Vericheck ddPCR™ Empty-Full Capsid Kit User Guide

Catalog #	Description
17010072	**Vericheck ddPCR Empty-Full Capsid AAV5 Kit**
17010082	**Vericheck ddPCR Empty-Full Capsid AAV9 Kit**
17010081	**Vericheck ddPCR Empty-Full Capsid AAV2 Kit**
17009844	**Vericheck ddPCR Empty-Full Capsid AAV8 Kit**

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Introduction

Adeno-associated virus (AAV) vectors are used in cell and gene therapy as vehicles to deliver therapeutic genes to patients. Producing AAV vectors is a complex process that requires rigorous testing and verification to meet robust safety standards and ensure high efficacy. One critical test in this process is the assessment of the empty-to-full capsid ratio, which is essential for preventing potential immunotoxicity. AAV vectors consist of a capsid filled with genome cargo. During manufacturing, empty capsids lacking the intended genome cargo may be produced. The Vericheck ddPCR Empty-Full Capsid Kit can be used to simultaneously quantitate AAV genomes and capsids to produce a highly precise measurement of the percentage of filled capsids. The kit is compatible with both purified and unpurified samples.

Protocol Overview

The protocol is executed in four distinct stages, shown in Figure 1.

1. **Sample prep:** AAV samples, a positive control, and a negative control are treated with a nuclease to remove unencapsulated DNA. Samples are diluted within dynamic range.
2. **Binding:** AAV samples, positive control, and negative control are bound with antibodies.
3. **Ligation:** A new template is created by a ligation reaction, which is then stopped by transferring the reaction to new wells containing Stop Solution.
4. **Droplet Digital™ PCR (ddPCR):** Each stopped ligation reaction is tested in three ddPCR wells.

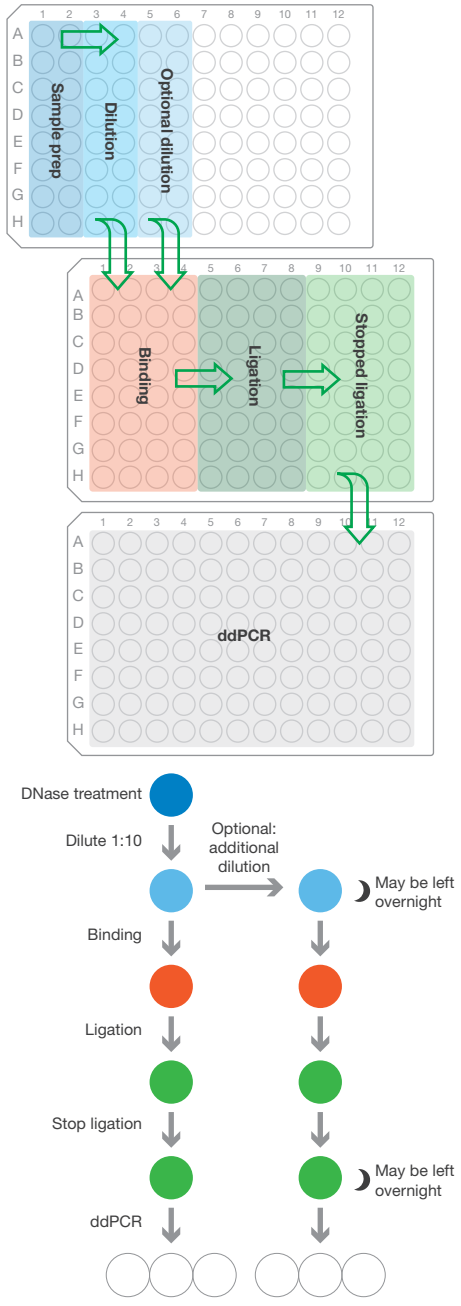


Fig. 1. Protocol schematic.

Kit Contents

The kit is provided in two boxes, the contents of which are shown in Tables 1 and 2. There are four subsets of kit reagents corresponding to each stage of the protocol. There are sufficient reagents for 18 sample prep reactions, 32 binding reactions, 32 ligation reactions, and 96 ddPCR reactions. Store both boxes at –20°C.

Table 1. Reagent kit contents.

Reagent	1x Volume	Kit Volume	Stage
DNase	2 µl	50 µl	Sample prep
10x DNase Buffer	2 µl	50 µl	Sample prep
Dilution Buffer	60 µl	1.5 ml	Sample prep
20x Ligase	1 µl	40 µl	Ligation
10x Ligase Buffer	2 µl	80 µl	Ligation
10x Splint	2 µl	80 µl	Ligation
Stop Solution	18 µl	700 µl	Ligation
ddPCR Supermix for Probes (No dUTP)	11 µl	700 µl × 2	ddPCR
20x ddPCR Capsid Detection Assay (FAM)	1.1 µl	120 µl	ddPCR
20x ddPCR ITR2 Assay (HEX)	1.1 µl	120 µl	ddPCR

Table 2. Serotype-specific kit contents.

Reagent	1x Volume	Kit Volume	Stage
AAV Positive Control*	2 µl	30 µl	Sample prep
AAV Antibody 1	2.5 µl	90 µl	Binding
AAV Antibody 2	2.5 µl	90 µl	Binding

* Biological source material. Treat as potentially infectious. The positive control contained in this kit is a replication-incompetent AAV that is not known to cause illness in healthy adults. Standard laboratory precautions, including engineering controls and proper personal protective equipment (PPE) to prevent contact, are recommended.

Required Equipment, Reagents, and Consumables

Table 3. Additional required materials.

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 Heat 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)

A. Sample Preparation

Note: AAV samples must be DNase-treated prior to the binding workflow. This kit includes DNase I, but other nucleases may also be suitable.

Note: Alternative nucleases should be validated before usage. When using alternative nucleases, dilute the samples 1:10 in AAV dilution buffer after digestion and proceed to the binding protocol. Heat inactivation of nucleases is not recommended.

1. The dynamic range of the test is ≈ $1 \times 10^9 – 1 \times 10^{12}$, with 1×10^{11} optimal for both genome copies (GC)/ml and viral particles (VP)/ml. More concentrated samples may be treated with DNase and diluted further in step 9.
2. Thaw, briefly vortex, and spin down AAV sample(s), positive control, and sample prep reagents
3. Assemble reaction (Table 4).

Table 4. Sample prep reaction recipe.

Component	Volume for 1 Reaction, µl	Volume for 16x + Overage, µl
10x DNase Buffer	2	35.2
DNase	2	35.2
Nuclease-free H_2O	14	246.4
Total	**18**	316.8

4. Aliquot 18 µl of reaction mix into the wells of a 96-well plate or PCR strip tubes.
5. Add 2 µl of each AAV or positive control sample to each well. To a negative control well, add 2 µl of Dilution Buffer.
6. Seal plate or cap tubes, pulse mix, and centrifuge briefly.
7. Incubate in a thermal cycler for 30 min at 37°C, then cool to 4°C.
8. Dilute DNase-digested AAV samples and controls 1:10 by adding 2 µl sample prep reaction to 18 µl Dilution Buffer.
9. Optional: To expand the dynamic range of the test, perform additional 1:10 serial dilutions in Dilution Buffer, resulting in the ranges specified in Table 5. Sufficient reagents are included to test two dilutions per sample.

   Note: It is not necessary to perform additional dilutions of the negative or positive controls.

Note: DNase-digested AAV can be stored at –20°C for at least 1 month.

Table 5. Dilution guidance. For unknown samples, select two dilutions.

Dilution	Dynamic Range	
1:10	$1 \times 10^9 – 1 \times 10^{12}$	Required in step 8
1:100	$1 \times 10^{10} – 1 \times 10^{13}$	Optional
1:1,000	$1 \times 10^{11} – 1 \times 10^{14}$	Optional
1:10,000	$1 \times 10^{12} – 1 \times 10^{15}$	Optional

B. Binding

1. Bring DNase-digested samples, controls, and antibody probes to room temperature. Pulse vortex tubes to mix and centrifuge briefly.
2. Assemble binding reaction (Table 6).

Table 6. Binding reaction mix recipe.

Component	Volume for 1 Well, µl	32x + Overage, µl
Antibody 1	2.5	88
Antibody 2	2.5	88
Dilution Buffer	3	105.6
Total	**8**	**281.6**

3. For each sample to be tested, dispense 8 µl of binding reaction mix per well in a 96-well plate.
4. Add 2 µl DNA-digested and diluted AAV sample or negative control from section A to each reaction.
5. Heat seal plate using the PX1 PCR Plate Sealer at 180°C for 5 sec. Allow the foil seal to briefly cool before performing the next step.
6. Vortex the plate briefly and centrifuge at 1,150 rcf for 1 min.
7. Incubate in a C1000 Touch Thermal Cycler with 96-Deep Well Reaction Module according to the conditions in Table 7 per serotype.

Table 7. Incubation conditions by AAV serotype.

AAV2	60 min at 30°C, then cool to 4°C
AAV5	60 min at 37°C, then cool to 4°C
AAV8	60 min at 30°C, then cool to 4°C
AAV9	60 min at 37°C, then cool to 4°C

C. Ligation

1. Bring ligation reagents to room temperature. Pulse vortex to mix and centrifuge briefly.
2. Assemble ligation reaction (Table 8).

Table 8. Ligation reaction mix recipe.

Component	Volume for 1x Reaction, µl	Volume for 32x Reaction + Overage, µl
10x Ligase Buffer	2	70.4
20x Ligase	1	35.2
10x Splint	2	70.4
Nuclease-free H_2O	11	387.2
Total	**16**	**563.2**

3. Remove and discard heat seal and dispense 16 µl ligation reaction mix into a clean well in the plate for each binding reaction.
4. Add 4 µl of binding reaction to ligation mix wells.
5. Heat seal plate using PX1 PCR Plate Sealer at 180°C for 5 sec.

   Note: Allow the foil seal to briefly cool before next step.

6. Vortex plate briefly and centrifuge at 1,150 rcf for 1 min.
7. Incubate in a C1000 Touch Thermal Cycler with 96-Deep Well Reaction Module for 10 min at 37°C, then cool to 4°C.
8. Remove heat seal and add 18 µl of Stop Solution to a different clean well in the plate for each ligation reaction.
9. Transfer 2 µl of ligation reaction to wells containing Stop Solution to stop the ligation reaction.

   Note: The ligation reaction should be transferred promptly to Stop Solution after incubation.

10. Heat seal plate using PX1 PCR Plate Sealer at 180°C for 5 sec. Allow the foil seal to briefly cool before proceeding to the next step.
11. Vortex plate briefly and centrifuge at 1,150 rcf for 1 min.

Note: Stopped ligation reactions may be stored at 4°C overnight before testing in ddPCR reaction.

D. Droplet Digital PCR

1. Bring ddPCR reagents to room temperature. Pulse vortex to mix and centrifuge briefly.
2. Assemble ddPCR mix for three replicate wells per ligation reaction (Table 9).

Table 9. ddPCR master mix recipe.

Component	Volume for 1 Well, µl	96x + Overage, µl
ddPCR Supermix for Probes (No dUTP)	11	1,161
20x ddPCR Capsid Detection Assay (FAM)	1.1	116
20x ddPCR ITR2 Assay (HEX)	1.1	116
Nuclease-free H_2O	4.4	465
Total	**17.6**	**1,858**

3. Dispense 17.6 µl ddPCR master mix in each well of a new 96-well plate.
4. Add 4.4 µl of stopped ligation reaction.

   Note: AAV may settle out of solution if left sitting for an extended period of time. Mix samples well.

5. If necessary, add 22 µl of 1x ddPCR Buffer Control for Probes (#1863052) to empty wells of the plate. A 1:1 mixture of supermix and water can be used if no buffer control is available.
   a. **For the ■ QX200 Droplet Generator and ■ AutoDG Instruments**, droplet generation occurs in columns. Add 22 µl 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.
   b. **For the ■ QX ONE ddPCR System**, droplet generation occurs in sets of 2 columns (for example, droplets for columns 1 and 2 are generated simultaneously). If necessary, add 22 µl of buffer control to any unused wells in each set of columns from which droplets will be generated.
6. Heat seal plate using PX1 PCR Plate Sealer at 180°C for 5 sec. Allow the foil seal to briefly cool before next step.
7. Vortex briefly and centrifuge at 1,150 rcf for 1 min.
8. Droplet generation steps differ slightly depending on which system is used. Transfer the reaction mix from the tubes or plate prepared in step 7 to the appropriate Droplet Generation Cartridge or ddPCR Plate and generate droplets as follows for the system in use:

- **For manual droplet generation (■ QX200 ddPCR System)**, load 20 µl of each reaction mix into the sample wells of a DG8 Cartridge. Then load 70 µl of Droplet Generation Oil for Probes into the oil wells. For detailed instructions, refer to the QX200 Droplet Generator Instruction Manual (10031907).
- **For automated droplet generation (■ QX200 or ■ QX600 AutoDG ddPCR Systems)**, place the sealed plate in the Automated Droplet Generator and follow instructions in the Automated Droplet Generator Instruction Manual (10043138).
- **For the ■ QX ONE ddPCR System**, load 20 µl of each reaction mix into the wells of a GCR96 Cartridge. Follow subsequent instructions for heat sealing, centrifuging, and loading the plate as specified in the QX ONE Droplet Digital PCR System and QX ONE Software Instrument Guide (10000116512). Use the appropriate thermal cycling conditions, as specified in Table 10.

  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:** Unknown or Pos Ctrl or Neg Ctrl
  - **Supermix:** ddPCR Supermix for Probes (No dUTP)
  - **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. 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 Droplet Generator**, after droplet generation, carefully transfer each column of the droplet emulsion into a clean ddPCR 96-Well Plate using a multichannel pipette. Seal the plate using the PX1 plate sealer at 180°C for 5 sec. Proceed to thermal cycling with conditions specified in Table 10.
- **For the ■ Automated Droplet Generator**, 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 with conditions specified in Table 10.
- **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 10. Cycling conditions.*

Cycling Step		Temp, °C	Time	Number of Cycles	Ramp Rate
Hold (QX ONE ddPCR System only)		25	3 min	1	2°C/sec
Initial denaturation		95	10 min	1	
Denaturation		94	30 sec	40	
Annealing/extension		55	1 min		
Enzyme deactivation		98	10 min	1	
Hold	QX200 and QX600 ddPCR Systems	4	10 min	1	
	QX ONE ddPCR System	25	1 min	1	

* For the C1000 Touch and PTC Tempo Thermal Cyclers. 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:

- **For the ■ QX200 Droplet Reader:**
  The Empty-Full Capsid Kit is compatible with QX Manager Software v. 1.2 or higher (Standard, Regulatory, and Premium editions). Refer to the QX Manager Software Standard Edition (10000107223), Regulatory Edition (10000107224), or Premium Edition (1000153878) User Guides for detailed information on the QX200 instrument and plate setup.
- **For the ■ QX600 Droplet Reader:**
  The Empty-Full Capsid Kit is compatible with QX Manager Software, Standard Edition, v. 2.0 or higher and QX Manager Software, Premium Edition, v. 2.1 or higher. Refer to the QX Manager Software Standard Edition (10000107223) or Premium Edition (1000153878) User Guides for detailed information on the QX600 instrument and plate setup.
- **For both the ■ QX200 and ■ QX600 Droplet Readers:**
  Place the sealed 96-well plate in the Droplet Reader

  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:** Unknown or Pos Ctrl or Neg Ctrl
  - **Supermix:** ddPCR Supermix for Probes (No dUTP)
  - **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. Press Start Run. See Figure 2 for an example.
- **For the ■ QX ONE ddPCR System:** The Empty-Full Capsid Kit is compatible with QX ONE Software v. 1.2 or higher (Standard and Regulatory Editions). Data acquisition is integrated into and sequentially performed by the system itself. Hence, no additional equipment or sample handling is required for this step.

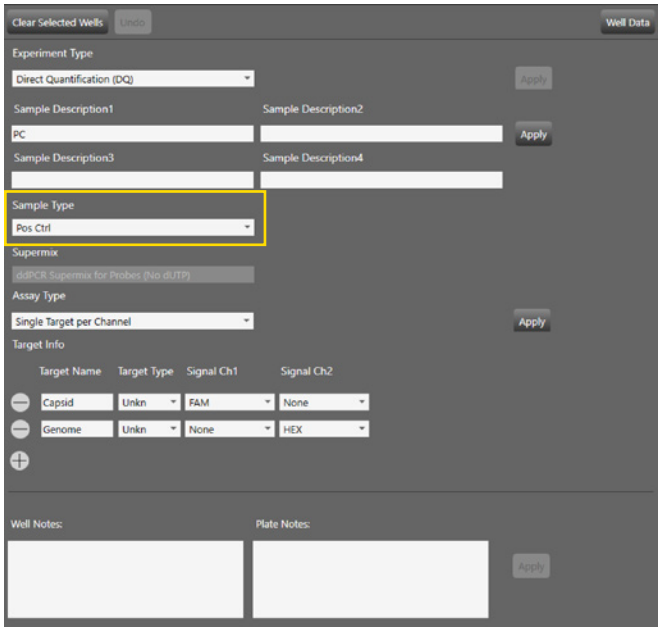


Fig. 2. Example of plate setup in the Plate Editor screen. Positive control wells should have the sample type Pos Ctrl (■).

Data Analysis

The Vericheck ddPCR Empty-Full Capsid Kit is compatible with QX Manager Software and QX ONE Software v. 1.2 or higher (all editions). All compatible editions of software offer 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 user guide for the software in use for detailed instructions about data analysis.

1. Ensure that the positive control wells for the assay are marked as having 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 to mark the sample type for the selected wells. See Figure 2 for details.
2. Navigate to the 2-D Amplitude tab. Select all wells, including control wells.
3. Click Auto, 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 crosshair tool.

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

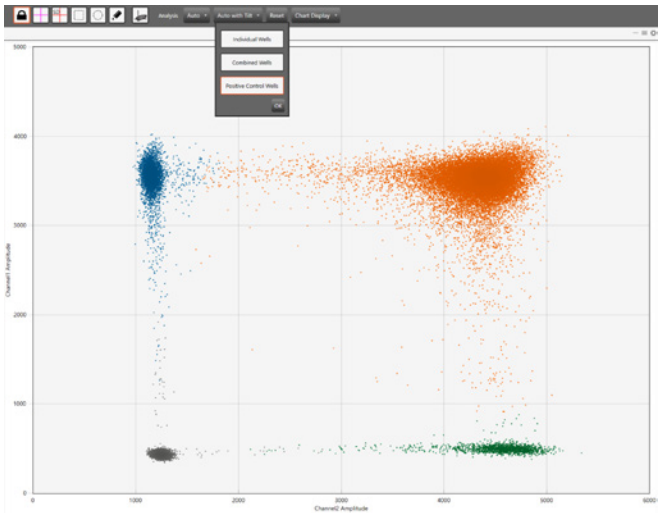
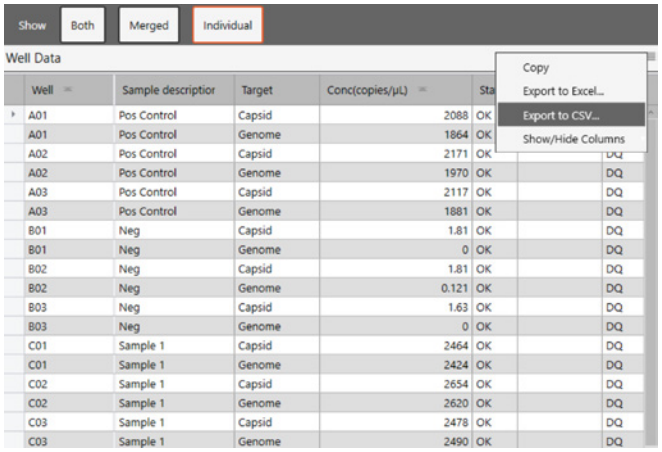


Fig. 3. Autothresholding using Positive Control Wells. On the 2-D Amplitude tab, select all wells and choose Positive Control Wells from the Auto dropdown menu.

5. To export data, navigate to the Data Table tab.
6. Select Individual.
7. Click the Table Menu icon on the far right and click Export to CSV from the dropdown menu (Figure 4).



Well	Sample description	Target	Conc(copies/µL)	Sta		
A01	Pos Control	Capsid	2088	OK		DQ
A01	Pos Control	Genome	1864	OK		DQ
A02	Pos Control	Capsid	2171	OK		DQ
A02	Pos Control	Genome	1970	OK		DQ
A03	Pos Control	Capsid	2117	OK		DQ
A03	Pos Control	Genome	1881	OK		DQ
B01	Neg	Capsid	1.81	OK		DQ
B01	Neg	Genome	0	OK		DQ
B02	Neg	Capsid	1.81	OK		DQ
B02	Neg	Genome	0.121	OK		DQ
B03	Neg	Capsid	1.63	OK		DQ
B03	Neg	Genome	0	OK		DQ
C01	Sample 1	Capsid	2464	OK		DQ
C01	Sample 1	Genome	2424	OK		DQ
C02	Sample 1	Capsid	2654	OK		DQ
C02	Sample 1	Genome	2620	OK		DQ
C03	Sample 1	Capsid	2478	OK		DQ
C03	Sample 1	Genome	2490	OK		DQ

Fig. 4. Exporting data for analysis. On the Data Table tab, select Individual and choose Export to CSV... from the dropdown menu.

Data Interpretation

Use the equations in Table 11 to calculate genome titer, percent full, and capsid titer.

Table 11. Manual calculations.

Metric	Formula
Genome concentration	= HEXConcentration
$FAM_{adjusted}$	$= FAMConcentration_{Unknown} - FAMConcentration_{NegCtrl}$
Efficiency	$= \frac{Linkage}{HEXConcentration}$
Percent full	$= \frac{Linkage}{FAM_{adjusted}}$
Capsid concentration	$= \frac{FAM_{adjusted}}{Efficiency}$
Original genome titer	= genome concentration × 125,000 × extra dilutions
Original capsid titer	= capsid concentration × 125,000 × extra dilutions

The Vericheck ddPCR Empty-Full Capsid Kit works by simultaneously measuring capsids and genomes in the same droplet. Genome concentration is simply the HEX concentration. As the capsids are lysed within droplets, it is not necessary to account for two copies of ITR. Both copies will always be inside the same droplet.

The capsid signal in the FAM channel must be calibrated, as it is not 100% efficient and has a small background signal. To begin, subtract the background signal from a negative control from all unknown samples. Next, use the genome signal to calibrate the capsid signal with two assumptions: first, that PCR is 100% efficient, but the capsid assay may not be; second, that if a sample has been treated with a nuclease, then all viral genomes will be located within capsids. Droplets that have a genome signal in HEX but no capsid signal in FAM therefore represent capsids that exist but were not directly detected. The efficiency of the capsid labeling reaction is therefore the concentration of genomes colocalized with capsids (linkage) divided by the genome concentration. Capsid concentration is then calculated by correcting the FAM signal for efficiency.

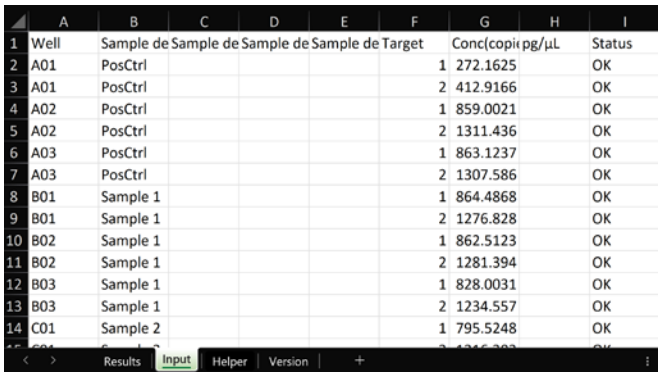
The percent of full capsids is the ratio of linkage to adjusted FAM concentration, which is mathematically identical to the ratio of genome to capsid concentration. To ensure precision, both FAM and HEX concentrations must be <8,000 copies/µl, and the FAM concentration in test samples should be at least 3x higher than the negative control.

Additionally, the genome and capsid titer of the original sample can be back-calculated. When performed as described above, the original sample has been diluted 1:125,000. That is, 1:10 in the DNase digestion reaction, 1:10 dilution afterward, 1:5 in the binding reaction, 1:5 in the ligation reaction, 1:10 in stopping the ligation reaction, and 1:5 in the ddPCR reaction. If additional dilutions were performed, incorporate those changes into your calculations.

Data Interpretation Using the Vericheck ddPCR Empty-Full Capsid Analysis Worksheet

The Vericheck ddPCR Empty-Full Capsid (EFC) Analysis Workbook is available to automatically calculate genome titer, capsid titer, and percent full metrics. Negative control wells must be specified by Sample Type in QX Manager before exporting data for the workbook to function.

1. Export to .csv, as shown in Figure 4.
2. Open .csv file with exported data and the Vericheck ddPCR EFC Analysis Workbook.
3. Select all (Ctrl+A) data in the .csv file and copy and paste to cell A1 of the Input tab of the Workbook (Figure 5).
4. Calculations are performed on the Helper tab of the Workbook. Results for Genome Titer, Capsid Titer, and Percent Full will automatically populate in the Results tab of the Workbook.



	A	B	C	D	E	F	G	H	I
1	Well	Sample de	Sample de	Sample de	Sample de	Target	Conc(copi	pg/µL	Status
2	A01	PosCtrl				1	272.1625		OK
3	A01	PosCtrl				2	412.9166		OK
4	A02	PosCtrl				1	859.0021		OK
5	A02	PosCtrl				2	1311.436		OK
6	A03	PosCtrl				1	863.1237		OK
7	A03	PosCtrl				2	1307.586		OK
8	B01	Sample 1				1	864.4868		OK
9	B01	Sample 1				2	1276.828		OK
10	B02	Sample 1				1	862.5123		OK
11	B02	Sample 1				2	1281.394		OK
12	B03	Sample 1				1	828.0031		OK
13	B03	Sample 1				2	1234.557		OK
14	C01	Sample 2				1	795.5248		OK

Results | Input | Helper | Version

Fig. 5. Importing data into the Vericheck ddPCR EFC Analysis Workbook. Copy data from exported .csv file and paste to cell A1 of Input tab in Workbook.

Appendix A. Alternative Genome Reference Targets

The Empty-Full Capsid Kit uses AAV2 ITR as a reference target. If desired, a user-provided assay may be used instead. To do so, simply replace the ITR2 assay in the protocol above. Alternative assays should use a HEX or VIC probe. Primers should be designed to have a T_m of approximately 55°C, with the T_m of the HEX probe 3–10°C higher than that of the primers. Each primer should be used at 900 nM final concentration, and probes should be used at 250 nM final concentration. Manual thresholding may be required, as the included positive control will likely not be detected by an alternative assay. No modifications to the data analysis calculations are required.

Appendix B. Troubleshooting

This section lists some common failure modes with their phenotypes, descriptions, and suggested resolution. 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 or Low Total Droplet Counts

Problem: Wells have droplet counts <10,000.

Resolution: Exclude wells with low droplet counts from analysis. For the QX200 or QX600 Systems, repeat ddPCR analysis and allow the plate to hold at the final 4°C step for at least 30 min prior to droplet reading to increase droplet numbers. If the problem persists, contact Technical Support.

PCR Inhibition

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

Resolution: If a concentration cannot be calculated, exclude the well from analysis. Ensure sample was properly DNase-treated prior to antibody binding, as excessive residual DNA may inhibit ddPCR reactions.

Mirroring

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

Resolution: Exclude the well from analysis or repeat ddPCR analysis, preferably with a different lot of droplet generation consumables.

Droplet Shredding

Problem: Shredded droplets appear on the diagonal through the negative droplet cluster.

Resolution: Exclude well from analysis or repeat ddPCR analysis.

Incorrect Autothresholding

Problem: Droplets are incorrectly thresholded using autothresholding.

Resolution: Ensure that the wells were thresholded using the positive control-based autothresholding. 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.

Inconsistent Concentration Results

Problem: Concentration estimates are inconsistent between technical replicates. This is likely due to insufficient mixing at one or more steps in the protocol.

Resolution: Repeat Droplet Digital PCR. When creating technical replicates, thoroughly mix the reaction mixture (master mix, sample, and assays) by pipetting the reaction mixture up and down ten times, using 90% volume strokes. Alternatively, pulse vortex the reaction mixture for 15 sec followed by spinning down the sample. Do not assemble or mix reaction mixtures in the DG8 Cartridge. For accurate results from the ddPCR Empty-Full Capsid Kits, reactions at all steps must be thoroughly mixed before proceeding.

Wrong Serotype Kit Used

Problem: Low or no estimated concentration in the FAM channel of sample wells while Positive Control shows expected clusters.

Resolution: The ddPCR Empty-Full Capsid Kits are serotype specific. Presence of signal from the ITR2 Assay (HEX) but not from the Capsid Detection Assay (FAM) indicates an AAV sample not recognized by the kit antibody probes. Ensure that samples tested correspond to the correct AAV serotype of the kit used.

Low FAM Signal

Problem: High genome concentration detected, but very low capsid assay concentration.

Resolution: Ensure sufficient binding time for antibodies (≥1 hour). Ensure that nucleases are deactivated by dilution before addition of antibodies. Avoid heat inactivation of all reactions containing AAV.

No Linkage

Problem: No linkage detected, but high concentrations in FAM and HEX. Percent full and capsid titer cannot be calculated.

Resolution: Check thresholding. Check thermocycling conditions. Ensure sample has not been heated or lysed after antibody binding.

No Negative Droplets

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

Resolution: Exclude well from analysis. Further dilute DNase-treated sample with provided AAV Dilution Buffer and test again.

High ITR2 (HEX) Signal in Negative Control

Problem: Presence of >10 copies/µl in ITR2 (HEX) Channel. Negative control should be free of AAV particles and not include any ITR2 DNA.

Resolution: Ensure correct sample was selected as Negative Control. Make sure that good laboratory practices for PCR are being followed to prevent sample contamination of the Negative Control, including wiping down area and equipment with 5–10% bleach, preparing samples in a template-free environment, and not reusing ddPCR consumables.

High Capsid (FAM) Signal in Negative Control

Problem: Negative Control Capsid concentration (FAM) exceeds 50 copies/µl with no/few ITR2 positive droplets.

Resolution: Exclude plate data and repeat protocol. Ensure ligation reaction is diluted in provided Stop Solution promptly after completing incubation.

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

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