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# **PREvalence ddPCR SARS-CoV-2 Wastewater Quantification Kit**

## **User Guide**

### **Test for the Detection of SARS-CoV-2 Viral RNA in Wastewater Using Droplet Digital PCR**

Catalog #12015402

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## Section 1

# Introduction

Since its initial discovery in 2019, the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has become widespread. SARS-CoV-2 is a virus that can spread by respiratory droplets through air, fluids, and some surfaces. Monitoring its spread is becoming increasingly important. It can leave devastating impacts on those infected, and has led to an unprecedented global health crisis.

Testing wastewater for SARS-CoV-2 enables traceability and pre-emptive surveillance. This allows regions to determine viral loads within the sewage system and track the amount over time to predict viral outbreaks. This information can then be brought to the attention of health authorities for prompt implementation of safety guidelines.

The PREvalence ddPCR SARS-CoV-2 Wastewater Quantification Kit, based on the U.S. Centers for Disease Control and Prevention and World Health Organization RT-PCR primers for SARS-CoV-2 detection, allows for the detection of N2 and E genes in SARS-CoV-2. It also includes an assay for a murine coronavirus control, murine hepatitis virus (MHV), which is an enveloped, single-stranded RNA virus. MHV serves as a matrix recovery control, enabling estimation of the virus lost during sample processing. The test allows for all three targets to be detected in a single well using the kit.

## Section 2

# Droplet Digital PCR (ddPCR) Technology

Droplet Digital PCR is a quantitative, endpoint PCR that partitions a sample into approximately 20,000 individual droplets and allows for higher sensitivity and greater resistance to inhibition compared to other types of quantitative PCR (qPCR) methods. Each droplet is an individual PCR reaction that is counted as positive or negative after PCR amplification. The result is a quantitative description of the number of template copies in the total PCR reaction. This technology also yields high reproducibility and low variability.

## Section 3

# Kit Components

The PREvalence ddPCR SARS-CoV-2 Wastewater Quantification Kit contains sufficient reagents for 200 reactions (Table 1).

**Table 1. Kit components (catalog #12015402).**

Part Number	Kit Component	Quantity
16009200	ddPCR Triplex Assay (SARS-CoV-2 N2, E, and MHV)	2 x 140 µl
16008441	SARS-CoV-2 Standard	1 x 300 µl
10049218	300 mM DTT	2 x 1,000 µl
10049219	Supermix for one-step RT-ddPCR	2 x 200 µl
10049220	Reverse transcriptase for one-step RT-ddPCR	2 x 500 µl

## Section 4

# Shelf Life and Storage

Upon receipt, store the kit in a constant temperature freezer at  $-20^{\circ}\text{C}$ . Repeated freezing and thawing of the supermix is not recommended. Dithiothreitol (DTT) should be aliquoted to multiple tubes and stored at  $-20^{\circ}\text{C}$ . Reagents can be used until the expiration date indicated on the tube when stored properly.

## Section 5

# Materials Required but Not Supplied

This kit is designed for use with the QX200 AutoDG or QX200 Droplet Digital PCR System. It is also compatible with the QX ONE Droplet Digital PCR System. Materials and instruments required but not supplied are listed in Tables 2 and 3.

**Table 2. List of materials and instruments needed but not supplied with the PREvalence ddPCR SARS-CoV-2 Wastewater Quantification Kit for performing Droplet Digital PCR with the QX200 AutoDG ddPCR System.\***

Material	Vendor	Catalog #
Automated Droplet Generator	Bio-Rad	1864101
DG32 Automated Droplet Generator Cartridges	Bio-Rad	1864108
Pipet Tips for the AutoDG System	Bio-Rad	1864120
Pipet Tip Waste Bins for the AutoDG System	Bio-Rad	1864125
Automated Droplet Generation Oil for Probes	Bio-Rad	1864110
C1000 Touch Thermal Cycler with 96-Deep Well Reaction Module	Bio-Rad	1851197
ddPCR 96-Well Plates	Bio-Rad	12001925
PX1 PCR Plate Sealer	Bio-Rad	1814000
PCR Plate Heat Seal, foil, pierceable	Bio-Rad	1814040
QX200 Droplet Reader	Bio-Rad	1864003
ddPCR Droplet Reader Oil	Bio-Rad	1863004
QX Manager Software Standard Edition (v1.2 or later)	Bio-Rad	N/A
Vortexer	Any	N/A
Microcentrifuge	Any	N/A

\* Other materials required include standard molecular biology laboratory equipment, including pipets, pipet tips, 50 ml tubes, microcentrifuge tubes, tube racks, gloves, and other safety equipment. Additional reagents required but not supplied are listed in Table 4.

**Table 3. List of materials and instruments needed but not supplied with the PREvalence ddPCR SARS-CoV-2 Wastewater Quantification Kit for performing Droplet Digital PCR with the QX200 ddPCR System.\***

Material	Vendor	Catalog #
QX200 Droplet Generator	Bio-Rad	1864002
DG8 Cartridges for QX200 Droplet Generator	Bio-Rad	1864008
DG8 Gaskets for QX200 Droplet Generator	Bio-Rad	1863009
DG8 Cartridge Holder	Bio-Rad	1863051
Droplet Generation Oil for Probes	Bio-Rad	1863005
C1000 Touch Thermal Cycler with 96-Deep Well Reaction Module	Bio-Rad	1851197
ddPCR 96-Well Plates	Bio-Rad	12001925
PX1 PCR Plate Sealer	Bio-Rad	1814000
PCR Plate Heat Seal, foil, pierceable	Bio-Rad	1814040
QX200 Droplet Reader	Bio-Rad	1864003
ddPCR Droplet Reader Oil	Bio-Rad	1863004
QX Manager Software Standard Edition (v1.2 or later)	Bio-Rad	N/A
Vortexer	Any	N/A
Microcentrifuge	Any	N/A

\* Other materials required include standard molecular biology laboratory equipment, including pipets, pipet tips, 50 ml tubes, microcentrifuge tubes, tube racks, gloves, and other safety equipment. Additional reagents required but not supplied are listed in Table 4.

**Table 4. Additional reagents required for use of the PREvalence SARS-CoV-2 Wastewater Quantification Kit.**

Reagent	Vendor	Catalog #
Murine hepatitis virus*	American Type Culture Collection [ATCC] or ZeptoMetrix, LLC	VR-764 or NATMHV-ST
Nuclease-free phosphate buffered saline (PBS)	Any	N/A
Nuclease-free water	Any	N/A

\* Other sources of whole or inactivated MHV may be acceptable as long as they can be quantified with the PREvalence ddPCR SARS-CoV-2 Wastewater Quantification Kit.

Additional reagents available for use but not supplied are listed in Table 5.

**Table 5. Additional reagents for use with the PREvalence ddPCR SARS-CoV-2 Wastewater Quantification Kit (optional).**

Reagent*	Vendor	Catalog #
PREvalence ddPCR crAssphage Fecal Indicator Assay	Bio-Rad	12015961
PREvalence ddPCR PMMoV Fecal Indicator Assay	Bio-Rad	12015962

\* See Appendices A and B for more information about these assays.

## Section 6

# Precautions and Recommendations

This test should be performed only by adequately trained personnel. All wastewater samples should be handled as Biosafety Level 2 (BSL-2) with appropriate precautions taken and correct personal protective equipment (PPE) used. Samples should be handled in a biosafety cabinet by individuals trained for the appropriate equipment and samples. All waste should be placed in biohazard bags, sharps containers, or appropriate fluid waste containers, and then disposed of appropriately in accordance with local guidelines.

To avoid contamination, all surfaces, pipets, pipet tip boxes, and equipment that will interact with samples should be cleaned with 10% bleach followed by 70% ethanol before and after use.

The quality of results depends on strict compliance with good laboratory practice (see for example, the EN ISO7218 standard). Important considerations, especially regarding PCR, include:

- The laboratory equipment (pipets, tubes, etc.) must not circulate between workstations
- It is essential to use a positive control and no template control (NTC) for PCR runs
- Reagents should not be used after their expiration date
- Vortex reagents from the kit before use to ensure homogeneity. This is especially important for the supermix, which has a high viscosity. It is recommended to vortex this solution for 30 sec before use (after it has fully thawed)
- Periodically verify the accuracy and precision of pipets, as well as correct functioning of instruments
- Change gloves often, especially when changing environments or if you suspect that they are contaminated
- Clean workspaces before and after use by wiping them down with 10% bleach followed by 70% ethanol

While preparing the master mix, the reverse transcriptase enzyme should be kept on a cold block or on ice (4°C) the whole time it is outside of the -20°C freezer.

## Section 7 Protocol

It is strongly recommended to read the entire protocol before starting the test.

### 1. Preparation of the Coronavirus Controls Using Murine Hepatitis Virus

Live or inactivated MHV is used as part of the quality controls that monitor different steps of the wastewater analysis process. MHV is not included with this kit and recommended vendors are included in Table 4.

It is important to first quantify the MHV stock solution before use to be able to reliably determine the matrix recovery control concentration. Quantification is performed by extracting serial dilutions of the stock and quantifying them using the ddPCR Triplex Assay.

The MHV is used in the following ways:

- As the matrix recovery control (when spiked into samples prior to concentration) to evaluate the amount of virus lost during sample processing
- As part of the positive extraction control (when spiked into the SARS-CoV-2 Standard, included in the PREvalence ddPCR SARS-CoV-2 Kit)
- As part of the positive RT-ddPCR control (when the purified MHV RNA is spiked into the SARS-CoV-2 Standard, included in the PREvalence ddPCR SARS-CoV-2 Kit)

**Note:** Live or inactivated MHV is required for use as a valid matrix recovery control. Purified or unprotected MHV nucleic acid will yield an invalid matrix recovery control due to digestion by nucleases present in the sample.

### 2. Preparation of the Matrix Recovery Control

Following quantification of whole MHV with the ddPCR Triplex Assay, dilute MHV to 6,000 copies/ $\mu$ l. Create aliquots at this concentration for ease of use and to limit freeze-thaws. Add 10  $\mu$ l of this stock to each wastewater sample prior to sample processing.

### 3. Preparation of the Positive Extraction Control

To make a sample for the positive extraction control, MHV must be combined with the SARS-CoV-2 Standard included in the kit. The instructions provided in Table 6 assume an input sample volume into an RNA extraction of 200  $\mu$ l and an elution volume of 50  $\mu$ l to yield a control that contains 36 copies/ $\mu$ l of the MHV, N2, and E templates in the ddPCR reaction. If the input and output volumes of the RNA extraction are different, the volume of MHV and the SARS-CoV-2 Standard must be updated accordingly.

1. Prepare a 1:10 dilution of the 6,000 copies/μl MHV sample for a final concentration of 600 copies/μl.
2. Add the 600 copies/μl MHV solution to the SARS-CoV-2 Standard and PBS, as described in Table 6. Volumes can be scaled as needed.

**Table 6. Preparation of the positive extraction control formulation.**

Reagent	Volume, μl
SARS-CoV-2 Standard	30
MHV (600 copies/μl)	10
Nuclease-free PBS	260
<b>Total</b>	<b>300</b>

3. Perform the RNA extraction on the positive extraction control following the same extraction process as the rest of the wastewater samples.

#### 4. Preparation of the Positive Reverse Transcription ddPCR (RT-ddPCR) Control

To make the positive RT-ddPCR control, extracted MHV nucleic acid must be combined with the SARS-CoV-2 Standard, as described in section 7.1.

1. Extract the MHV stock to an RNA target concentration of 1,200 copies/μl.

**Note:** This can be achieved by diluting the 600 copies/μl MHV virus solution 1:1 with PBS, then extracting 200 μl of this solution using the Zymo Quick-RNA Viral Kit (Zymo Research, #R1034 or R1035) and a 50 μl elution volume.

2. Combine the extracted MHV nucleic acid with the SARS-CoV-2 Standard according to Table 7. This yields the positive RT-ddPCR control and may be used immediately or aliquoted and stored at –80°C for future use. Volumes can be scaled as needed.

**Table 7. Preparation of the positive RT-ddPCR control containing MHV nucleic acid.**

Reagent	Volume, μl
SARS-CoV-2 Standard	20
MHV nucleic acid (1,200 copies/μl)	10
Nuclease-free water	170
<b>Total</b>	<b>200</b>

#### 5. Sample Concentration

Samples should be stored and transported at 4°C for processing within 24 hours of collection. Samples may also be frozen for longer-term storage; it is the responsibility of the laboratory to demonstrate sample stability when frozen. Use a storage container with sufficient volume to allow for sample expansion upon freezing.

Process 40 ml of wastewater in a 50 ml conical tube. Wastewater samples should be spiked with 10 μl of the MHV dilution at 6,000 copies/μl prior to freezing or proceeding with sample concentration. MHV serves as a matrix recovery control, enabling quantification of viral recovery efficiency from sample collection to ddPCR analysis.

**Note:** MHV must be spiked into the sample and the RT-ddPCR positive control for correct operation of the PREvalence Wastewater Automated Analysis Application.

Proceed with the wastewater concentration step.

The PREvalence ddPCR SARS-CoV-2 Kit is compatible with a variety of wastewater sample concentration methods: Concentrating Pipette (Forés et al. 2021), polyethylene glycol (PEG) precipitation (Ahmed et al. 2020, Wu et al. 2020), centrifugal filtration (Forés et al. 2021), and silica membrane filtration (Whitney et al. 2021). For the convenience of the laboratory, the details for using the Concentrating Pipette method are provided.

## Concentrating Pipette (CP) Method

The Concentrating Pipette enables the rapid concentration of particles in wastewater. Materials needed but not supplied are listed in Table 8.

**Table 8. Materials needed (not supplied) for using the Concentrating Pipette method.**

Material	Vendor	Catalog #
Concentrating Pipette Select	InnovaPrep	CP Select
Ultrafiltration PS Hollow Fiber Tips	InnovaPrep	CC08003 or CC08004
Elution Fluid Can — Tris	InnovaPrep	HC08001
Storage Fluid	InnovaPrep	HC08558
Tween 20 (molecular biology grade)	Any	N/A
PBS (molecular biology grade)	Any	N/A

1. Set up the instrument according the manufacturer's instructions.
2. Set up the run protocol, COVID-WW, in the instrument before starting, as described in the [InnovaPrep Concentrating Pipette Select Wastewater Application Note, Revision B](#).
3. Concentrate the wastewater sample according to the application note protocol.
4. The eluted solution can be used immediately for RNA extraction or stored at  $-80^{\circ}\text{C}$  for future use.

## 6. RNA Extraction

### General Recommendations

All necessary safety precautions should be taken according to the laboratory guidelines. Precautions must also be taken to prevent cross-contamination of samples. If available, separate work areas should be used for:

- Nucleic acid extraction
- Reagent preparation (for example, preparation of RT-ddPCR master mix); **no** amplified reactions, target solutions, or clinical specimens should be brought into this area. After working in this area, laboratory coat and gloves should be changed before moving into the area where nucleic acid is added
- Nucleic acid addition
- Instruments (for example, thermal cyclers)

Proper aseptic technique should always be used when working with RNA. Hands and dust particles may carry bacteria and molds and are the most common sources of RNase contamination. Always wear powder-free latex, vinyl, or nitrile gloves while handling reagents, tubes, and RNA samples to prevent RNase contamination from skin or dust. Change gloves frequently and keep tubes closed. If debris is in the samples after the concentration step, avoid transferring that into the extraction step by separating the debris by centrifugation. Debris may cause filters to clog, causing the extraction to fail.



Clean working surfaces, pipets, etc. with 10% bleach or other solution that can destroy nucleic acids and RNases. To eliminate accelerated deterioration of any plastics and metals, wipe them down with 70% ethanol after using 10% bleach. Make sure all bleach is removed to eliminate possible chemical reactions between bleach and guanidine isothiocyanate, which is present in the extraction reagents.

Performance of the PREvalence ddPCR SARS-CoV-2 Kit is dependent upon the amount and quality of template RNA purified from wastewater samples.

The PREvalence ddPCR SARS-CoV-2 Wastewater Quantification Kit is compatible with a variety of commercially available viral RNA extraction kits. This kit has been qualified and optimized with the Zymo Quick-RNA Viral Kit. For this kit, follow the manufacturer's instructions while using 200 µl of concentrated wastewater sample and 50 µl of eluant. Extracted nucleic acid should be stored at 4°C if it is to be used within 4 hours, or at -70°C or lower if stored longer than 4 hours. This kit has also been validated for use with the IDEXX Water DNA/RNA Magnetic Bead Kit (IDEXX Laboratories, Inc., #WCOV2MAG) for sample extraction.

Positive and negative extraction controls should be processed with the wastewater samples. A positive extraction control should be prepared, as described in section 7.2. The negative extraction control should consist of nuclease-free water.

## 7. Droplet Digital PCR

### Preparation of One-Step RT-ddPCR Reactions

1. Clean all work surfaces and equipment with 10% bleach followed by 70% ethanol.
2. Retrieve the PREvalence ddPCR SARS-CoV-2 Wastewater Quantification Kit materials and let all reagents, except the one-step reverse transcriptase, thaw at room temperature. Also retrieve a cold block or ice to hold the reagents after they have thawed.

**Note:** The one-step reverse transcriptase must always be stored on ice or on a cold block while in use.

3. Label a clean microcentrifuge tube for preparing the master mix and place on the cold block or ice. Once the reagents have thawed, vortex and briefly centrifuge each to collect the contents at the bottom of the tubes.
4. **The one-step supermix is highly viscous and must be vortexed at maximum speed for at least 30 seconds prior to use. Failure to do so may cause poor results.**
5. At a minimum, run one positive RT-ddPCR control and one NTC with each plate to ensure validity of results.
6. Set up the master mix as described in Table 9.

**Table 9. Formulation of the master mix for 20 reactions.**

Reagent	1 Reaction, µl	1 Reaction with 20% Overage, µl*	20 Reactions with 20% Overage, µl*
One-step supermix	5	6	120
One-step reverse transcriptase	2	2.4	48
300 mM DTT	1	1.2	24
ddPCR Triplex Assay	1	1.2	24
Nuclease-free water	2	2.4	48
RNA sample**	9	10.8	216
<b>Total</b>	<b>20</b>	<b>24</b>	<b>480</b>

\* 20% overage is recommended to avoid losses in pipetting and transferring, particularly during droplet generation.

\*\* RNA sample will be added at a later step.

7. Vortex the master mix for 30 sec, and briefly centrifuge to collect the contents to the bottom of the tube.
8. Prepare a 96-well plate cold block or ice bucket and place a clean ddPCR 96-Well Plate onto it.
9. Add 12.1 µl of master mix to appropriate sample wells.
10. Seal the plate with a foil seal and move plate to template addition area. Ensure that the plate with master mix is kept on a 96-well plate cold block or on ice while working.
11. Add 9.9 µl of nuclease-free water to the NTC well.
12. Add 9.9 µl of sample to the appropriate sample wells.
13. Add 9.9 µl of the RT-ddPCR positive control solution to the positive control well.
14. Heat seal the sample plate for 5 sec at 180°C with a pierceable foil heat seal after all samples have been combined with master mix.
15. Vortex the plate for 30 sec at high speed.
16. Spin down the plate in a centrifuge at 1,150 rcf for 30 sec.
17. Ensure all wells have equal volume and that there are no bubbles at the bottom of the wells. If bubbles are present, centrifuge the plate again.

### Droplet Generation

There are two instruments available for droplet generation: QX200 Droplet Generator or Automated Droplet Generator (AutoDG). It is up to the laboratory to select a method based on sample throughput and availability of QX200 Droplet Generator or AutoDG. For protocols, troubleshooting guidance, and instructions for droplet generation, refer to the following specific instruction manuals:

- QX200 Droplet Generator Instruction Manual ([10031907](#))
- Automated Droplet Generator Instruction Manual ([D112916](#))

Following droplet generation, heat seal the plate with a foil seal. The plate sealer should be set to seal for 5 sec at 180°C. **Make sure that the red stripe on the foil seal is facing up before heat sealing.** Label the plate and take it directly to the thermal cycler.

### Thermal Cycling

Place the plate into the C1000 Touch Thermal Cycler with 96–Deep Well Reaction Module and ensure that it is properly seated in the heating element. Tighten the lid knob finger tight and do not overtighten.

**Note:** If you hear an audible click, the knob has been turned too far. Unscrew the knob one full turn, then tighten it one-half turn.

Set the protocol according to Table 10 on the thermal cycler.

**Table 10. Thermal cycling profile for the PREvalence ddPCR SARS-CoV-2 Wastewater Quantification Kit.\***

Step**	Description	Temperature, °C	Time	Cycles
1	Reverse transcription	50	60 min	1
2	Reverse transcriptase deactivation	95	10 min	1
3	Denaturation	94	30 sec	40
4	Annealing/extension	55	1 min	
5	Enzyme deactivation	98	10 min	1
6	Droplet stabilization	4	30 min	1
7	Hold	4	∞	1

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

\*\* Ramp rates should be set to 2°C/sec for each step.

This assay is also compatible with the S1000 Thermal Cycler with 96-Deep Well Reaction Module (Bio-Rad, #1852197). It is recommended to use the same parameters described in Table 10.

**Note:** Once the thermal cycling protocol is complete, the plate must remain at 4°C for at least 30 min to stabilize the droplets. The plate can be read after 30 min or held overnight at 4°C.

### Reading the Plate on the QX200 Droplet Reader

If not already installed, go to [bio-rad.com/QXSoftware](https://www.bio-rad.com/QXSoftware) to download and install the latest QX Manager Software Standard Edition.

For more information on setting up plate runs and operating the QX200 Droplet Reader, refer to the QX200 Droplet Reader and QX Manager Software Standard Edition User Guide ([10000107223](#)).

Using QX Manager Software Standard Edition, set up a plate run, being sure to use the following plate and well parameters:

- Experiment Type: **Direct Quantification (DQ)**
- Sample Type: **Unknown** for samples, **NTC** for NTC, and **Pos Ctrl** for the RT-ddPCR control
- Supermix: **One-Step RT-ddPCR Advanced Kit for Probes**
- Assay Type: **Probe Mix Triplex**
- Targets:
  - N2 (Signal Ch1: **FAM**; Signal Ch2: **None**)
  - E (Signal Ch1: **FAM**; Signal Ch2: **HEX**)
  - MHV (Signal Ch1: **None**; Signal Ch2: **HEX**)

**Note:** At least one positive control well must be designated as Pos Ctrl in the Sample Type section if using the PREvalence Wastewater Automated Analysis Tool. If none are selected, the tool will not be able to automatically threshold the plate.

Once the plate run has been defined, proceed with reading the plate.

## 8. Data Analysis

Data can be analyzed in QX Manager Software Standard Edition, Version 1.2 or later, directly after the plate finishes reading or later. The data can be analyzed manually, with the PREvalence Wastewater Automated Analysis Application, or through a combination of both methods.

**Note:** If not already installed, go to [bio-rad.com/QXSoftware](https://bio-rad.com/QXSoftware) to download and install the latest version of QX Manager Software Standard Edition.

### PREvalence Wastewater Automated Analysis Application

The PREvalence Wastewater Automated Analysis Application is a companion tool to QX Manager Software Standard Edition that automatically analyzes and exports results for PREvalence ddPCR SARS-CoV-2 Wastewater Quantification Kit data.

#### System Requirements and Installation

Minimum requirements:

- Windows 10 or later
- 8 GB of RAM or greater
- QX Manager Software Standard Edition, Version 1.2 or later

Go to [bio-rad.com/PREvalence](https://bio-rad.com/PREvalence) to download the PREvalence Wastewater Automated Analysis Application.

Once the installer for the PREvalence Wastewater Automated Analysis Application has been downloaded, simply double click the file to begin the installation process. After the installation process has completed, the application can be launched by double clicking the application's shortcut icon (Figure 1).



Fig. 1. PREvalence Wastewater Automated Analysis Application icon.

## Analyzing Data

1. Double click the application icon to launch the application. The interface in Figure 2 will be displayed.

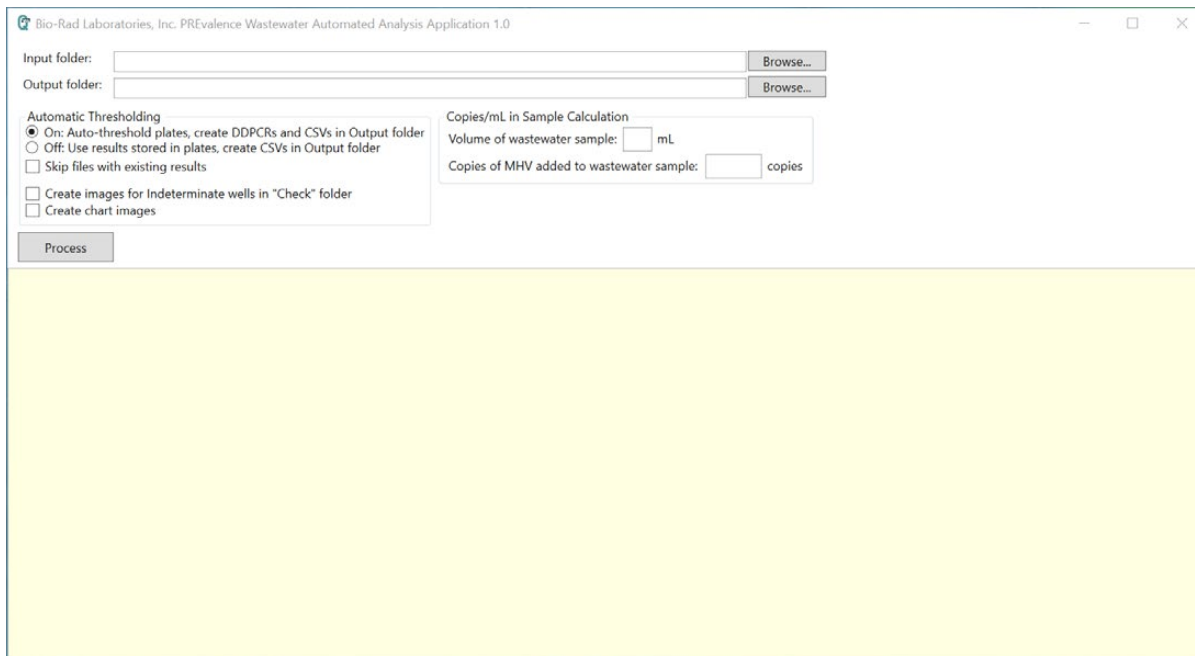


Fig. 2. Main interface for the PREvalence Wastewater Automated Analysis Application tool.

2. Specify an Input folder and an Output folder. The application will batch-process all ddPCR data files found in the Input folder provided. The Output folder will be used by the application to store the automatically clustered .ddpcr data files and .csv result export files generated by the application.

**Note:** Users who wish to use the same folder for both Input and Output must ensure that the **Skip files with existing results** option is selected. This will prevent duplicate analyses of already analyzed files.

- a. To create Input and Output folders, click the **Browse...** button directly to the left of the Input folder address bar.
- b. A window will pop up (Figure 3) where you can choose the location of the main folder that will contain the Input and Output folders.

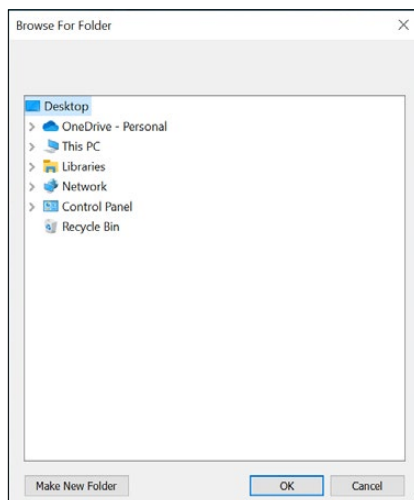


Fig. 3. Adding folders using the tool.

- c. Click **Desktop** to highlight it. Then click the **Make New Folder** button at the bottom of the pop-up window (Figure 4).

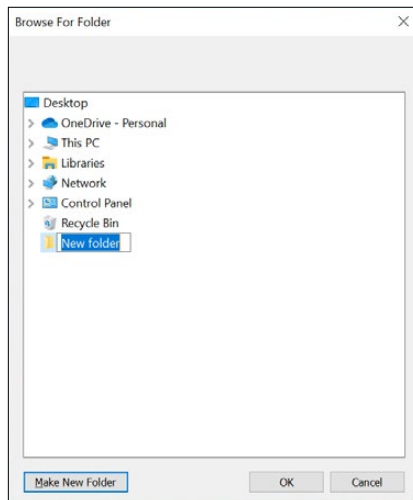


Fig. 4. Creating a folder that will hold the Input and Output Folders the tool uses.

- d. Right click the **New Folder**.
- e. Select **Rename** on the pop-up menu.
- f. Rename the folder Automated Analysis Data.
- g. Once complete, click anywhere else in the Browse for Folder window.
- h. Click the **Automated Analysis Data** folder from step 2f and click the **Make New Folder** button again. Name this new folder Input Folder. Click anywhere in the window once complete.
- i. Click the **Automated Analysis Data** folder and click the **Make New Folder** button. Name this new folder Output Folder. Click anywhere in the window once complete. All necessary folders for the tool have been created (Figure 5).

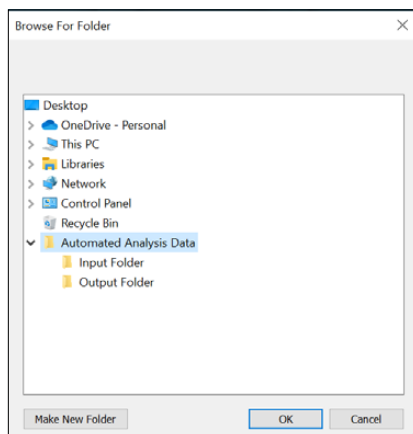


Fig. 5. Window with all folders needed.

**Note:** This can also be completed outside of the tool's interface in Windows File Explorer and the appropriate folders can later be selected in the tool.

- Now select the **Input Folder** created in step 2h and click **OK**. You will be returned to the tool's main interface (Figure 6).

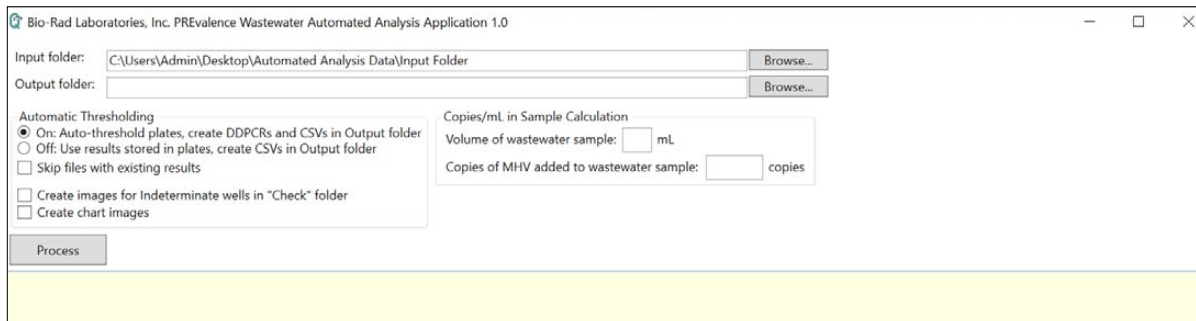


Fig. 6. Input folder specified with file path.

- Click the **Browse...** button directly to the right of the Output folder address bar, and locate the Output folder previously created. Select the folder and click **OK**. The Input and Output folders should now be specified (Figure 7).

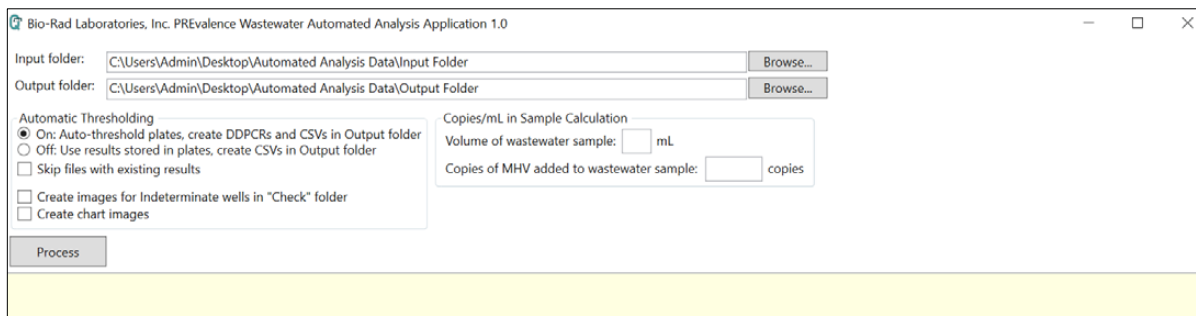


Fig. 7. Output folder specified with file path.

- Copy all the .ddpqr files (Figure 8) to be analyzed into the Input folder specified in step 3.

**Note:** The tool will save the file paths specified from the last time it was used and will also save the inputs into the Copies/mL in Sample Calculation fields for the next time it is opened again.

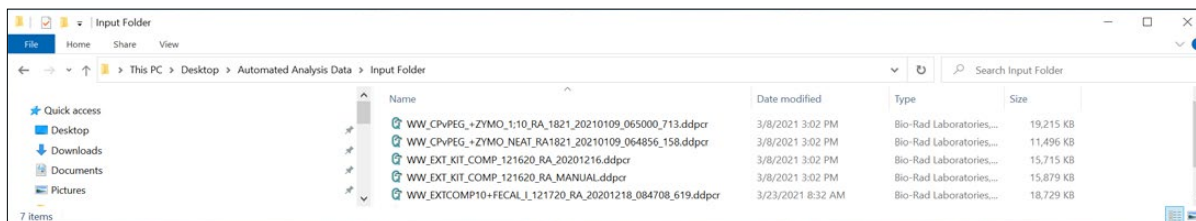


Fig. 8. Files to be analyzed are copied into the Input folder.

- Under the Automatic Thresholding section, ensure that the option **On: Auto-threshold plates, create DDPcRs and CSVs in Output folder** is selected (this is the default setting).

**Note:** The option **Off: Use results stored in plates, create CSVs in Output folder** can be selected if you wish to have result export .csv files created for data files that have already been analyzed or manually clustered.

- Click the **Process** button to begin the data file analysis.
- As the application proceeds to analyze the data files found in the Input folder, the user is able to track the progress in the Processing log section of the user interface (Figure 9).

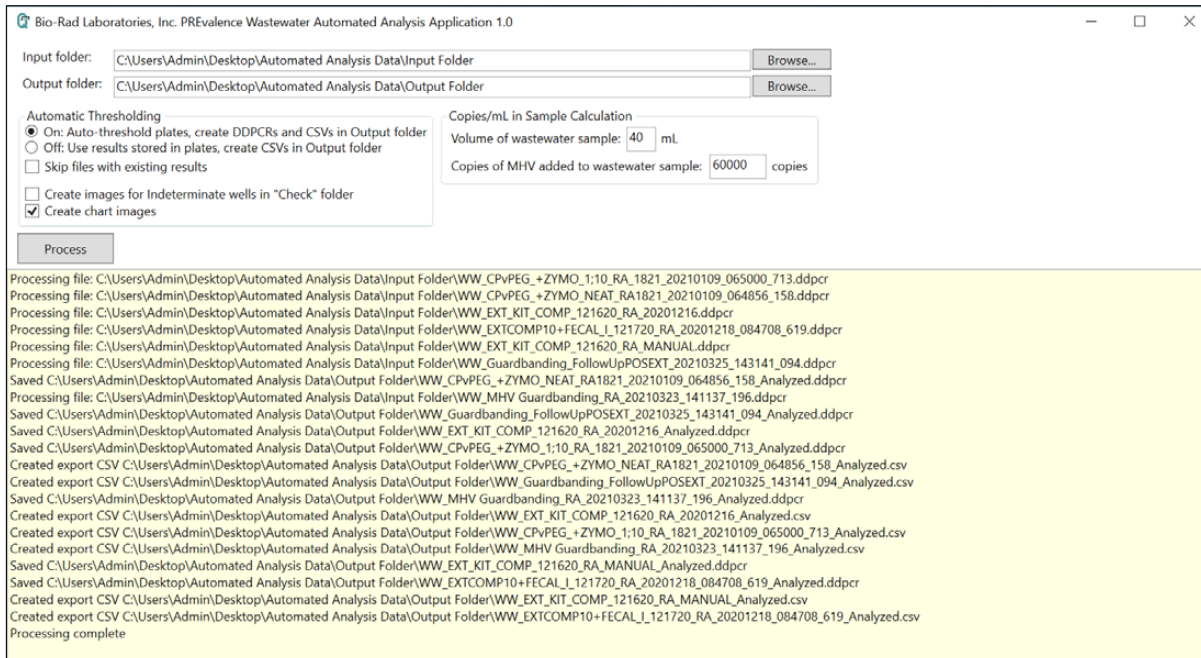


Fig. 9. Processing log.

- Once all data files have been analyzed and generated output files have been populated in the Output folder, the application will display the message Processing Complete. Autoanalyzed data files are identified by the word Analyzed appended to the output filenames.

**Note:** Once the tool has completed processing the files, it is critical to open and review the resulting analyzed .ddpccr files and check that all droplets were assigned to the correct cluster designation. If corrections are made to the .ddpccr clusters, you can have the tool create a new .csv export file with the corrected values by setting the Automatic Thresholding feature to **Off**.



## Application Options

### Indeterminate Wells

Selecting the option **Create images for Indeterminate wells** in the Check folder will generate 2D amplitude charts for any wells that are identified by the application as being Indeterminate. Wells are identified as indeterminate when no clusters can be determined by the application.

### Chart Images

Selecting the option **Create chart images** will generate 2D amplitude charts for the wells of each data file in the Input folder.

### Copies/ml in Sample Calculation

The application can calculate the concentration of assay targets (N2, E, and MHV) in Copies/ml in Sample based on the ddPCR copies/ $\mu$ l. To use this option, users must provide the following inputs:

- Volume of wastewater sample (in ml)
- Copies of MHV added to wastewater sample (in number of copies)

The calculated Copies/ml in Sample for each assay target can be found in the result export .csv file.

**Note:** Valid numeric data must be input into both fields for the application to perform the Copies/mL calculation (Figure 10).

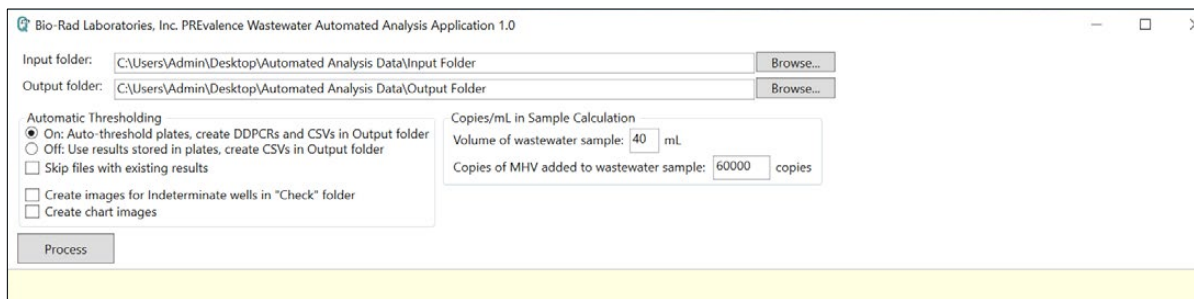


Fig. 10. Recommended configuration for the PREvalence Wastewater Automated Analysis Application.

## Manual Thresholding with QX Manager Software, Version 1.2 or Later

For Manual thresholding, follow these instructions:

1. Set the Assay Type for the plate to Triplex Probe Mix and make sure the targets are labeled/designated **N2** (FAM), **E** (FAM/HEX), and **MHV** (HEX).
2. In the 2D Amplitude tab, select all the wells to be analyzed.
  - a. Click the small gear icon to the right of the 2D plot. Under Options, select **Fixed**.
  - b. The 2D plot graph axes (minimum and maximum) can be adjusted to remove the peripheral white spaces (Figure 11).

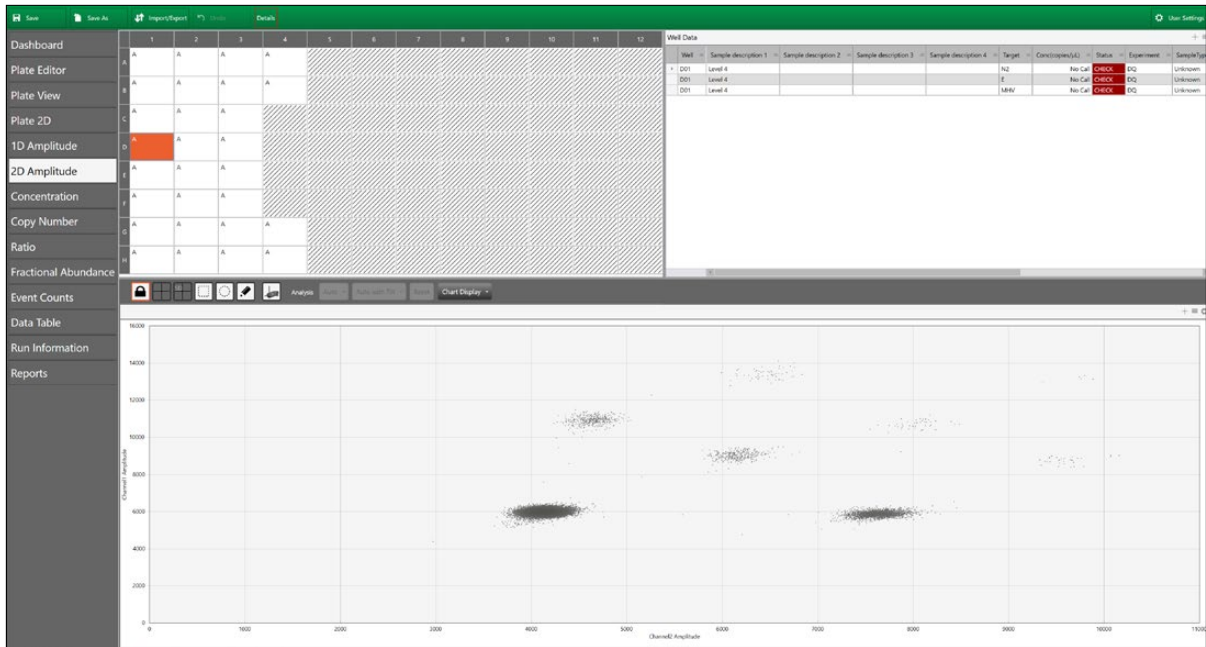


Fig. 11. 2D plot containing all possible clusters.

3. Select one of the threshold cluster modes. Preferred mode can be selected based on user preferences; the circular tool is easiest for most users. Keep **Graph View** at **Individual Wells**.
4. Using the mouse cursor, start by selecting the bottom left (triple-negative) cluster. The **Select to assign cluster** window pops up. Choose the bottom left, gray assignment (Figure 12).

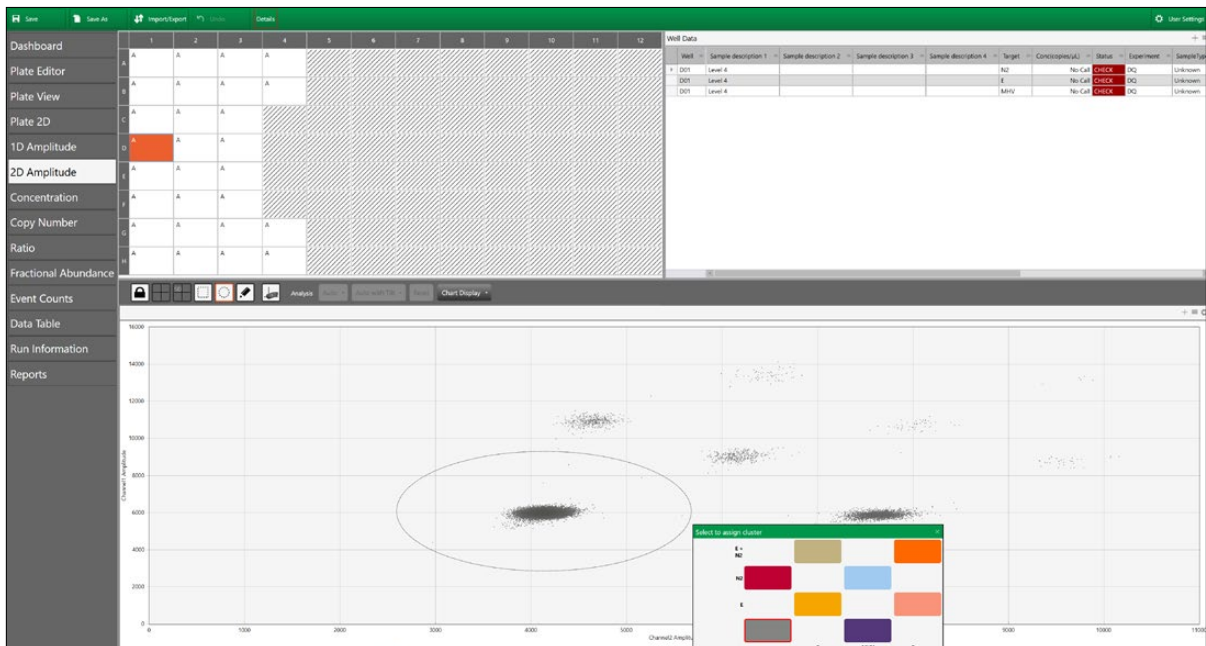


Fig. 12. Triple-negative (all negative) cluster.

5. Continue selecting and assigning cluster definitions until all clusters have been defined.
6. The cluster immediately to the right of the triple-negative cluster corresponds to **MHV** (single-positive, HEX channel) (Figure 13).

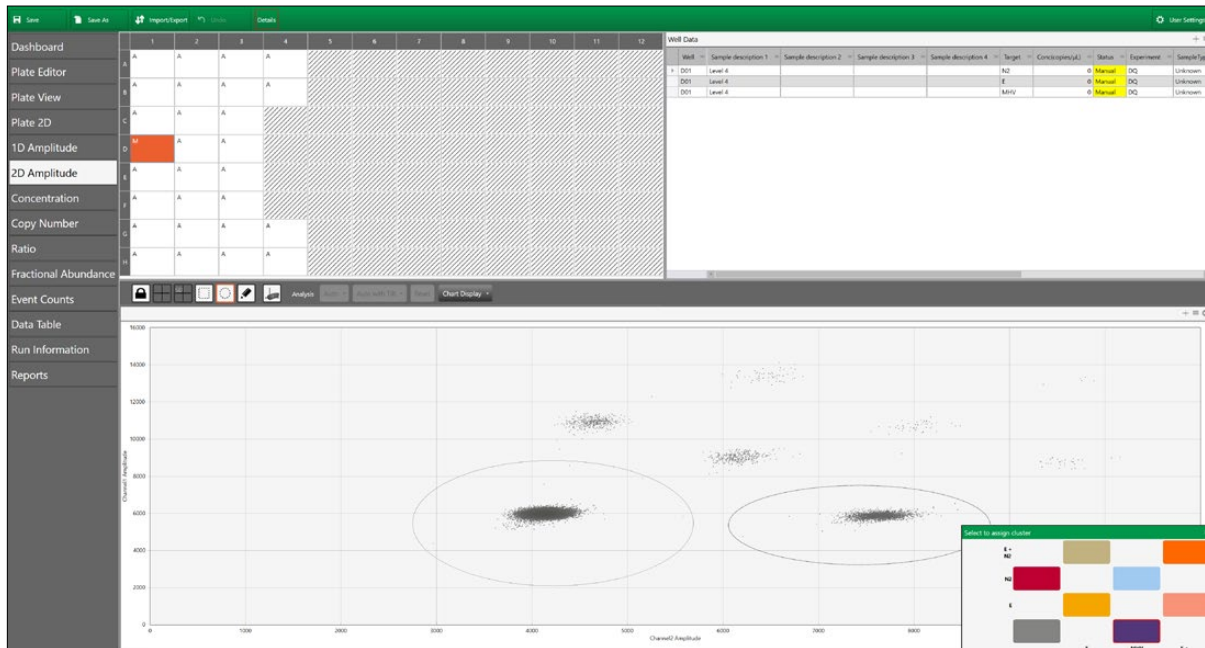


Fig. 13. MHV single-positive cluster.

7. The cluster immediately above the triple-negative corresponds to **N2** (single-positive, FAM channel) (Figure 14).

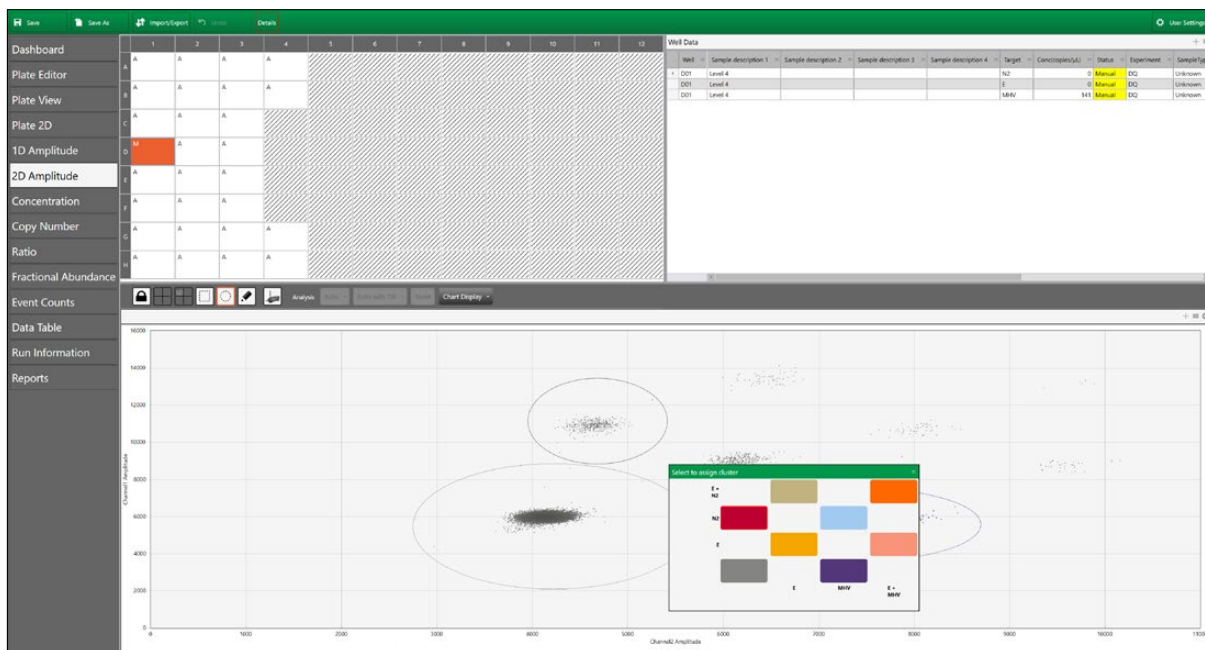


Fig. 14. N2 single-positive cluster.

- The cluster at a 45° angle from the triple-negative corresponds to **E** (double-positive, FAM and HEX channels) (Figure 15).

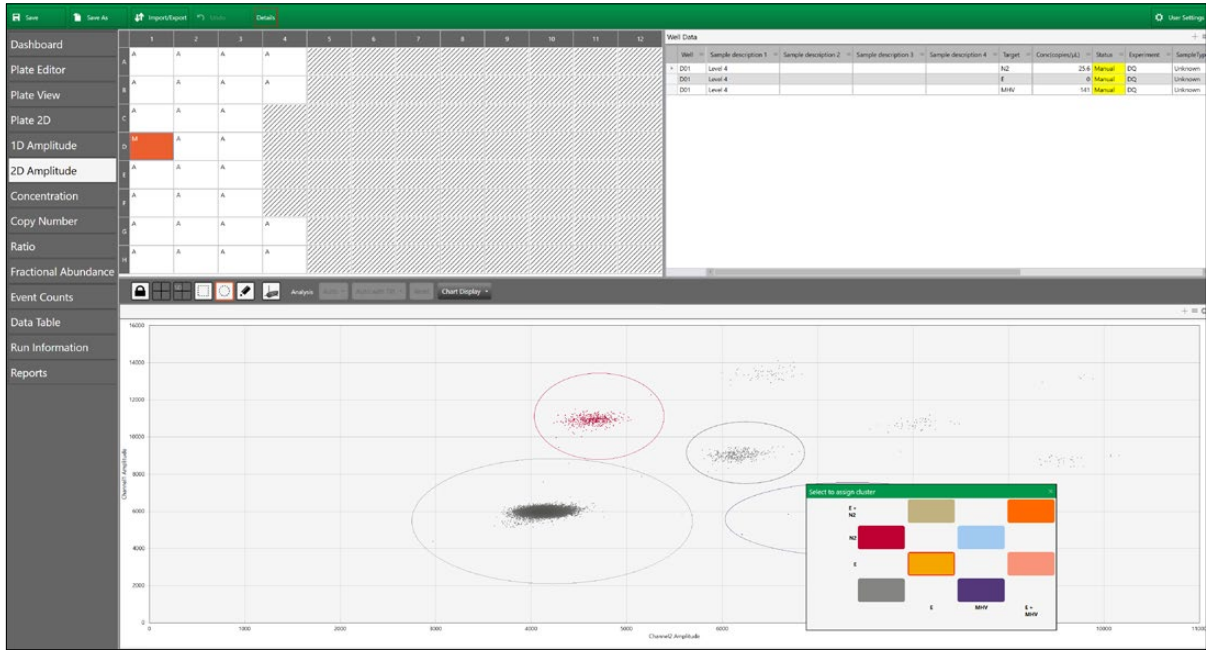


Fig. 15. E single-positive cluster.

- The cluster above the N2 and E single-positive clusters is a double-positive cluster corresponding to droplets that are both **N2** and **E** positive (Figure 16).

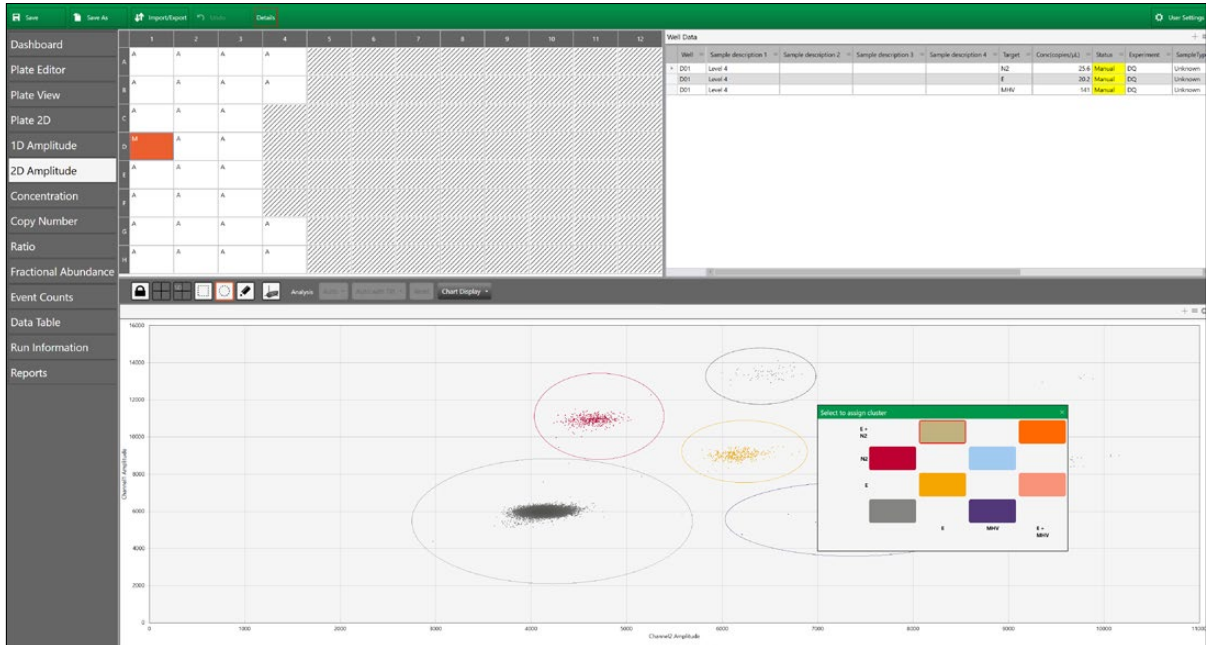


Fig. 16. N2 and E double-positive cluster.

- Above the MHV single-positive cluster, there is a double-positive cluster corresponding to droplets that are both **E** and **MHV** positive (Figure 17).

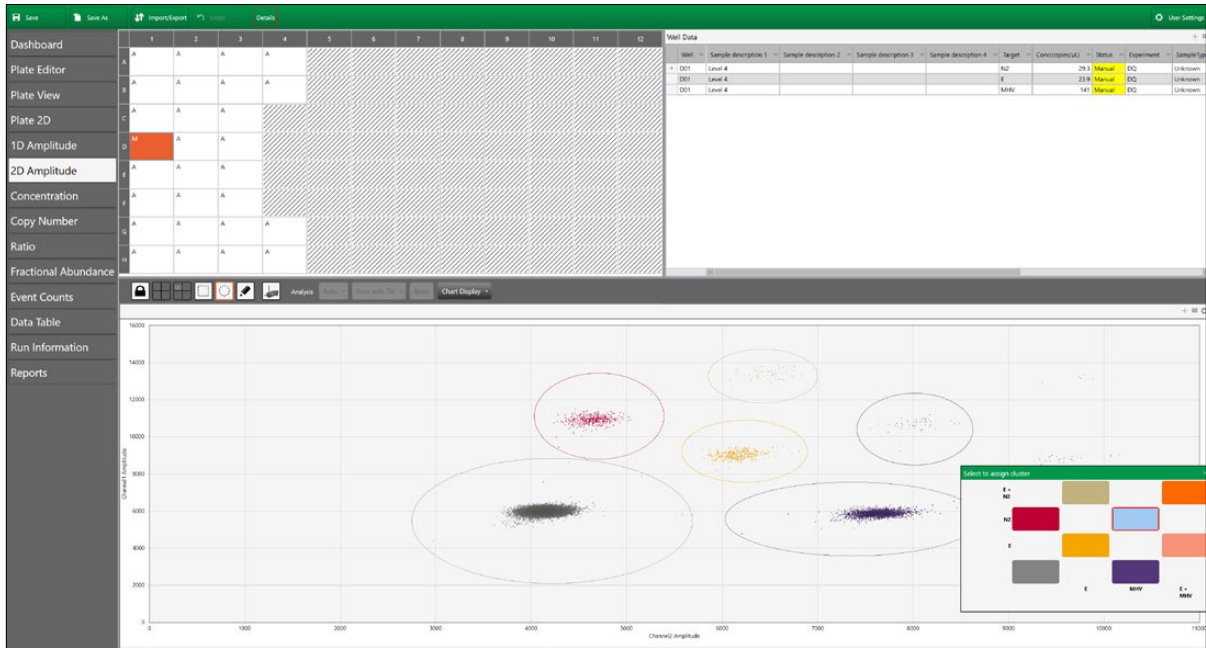


Fig. 17. E and MHV double-positive cluster.

- At a 45° angle from the E and MHV double-positive cluster is the **N2**, **E**, and **MHV** triple-positive cluster corresponding to droplets that are positive for all three targets (Figure 18).

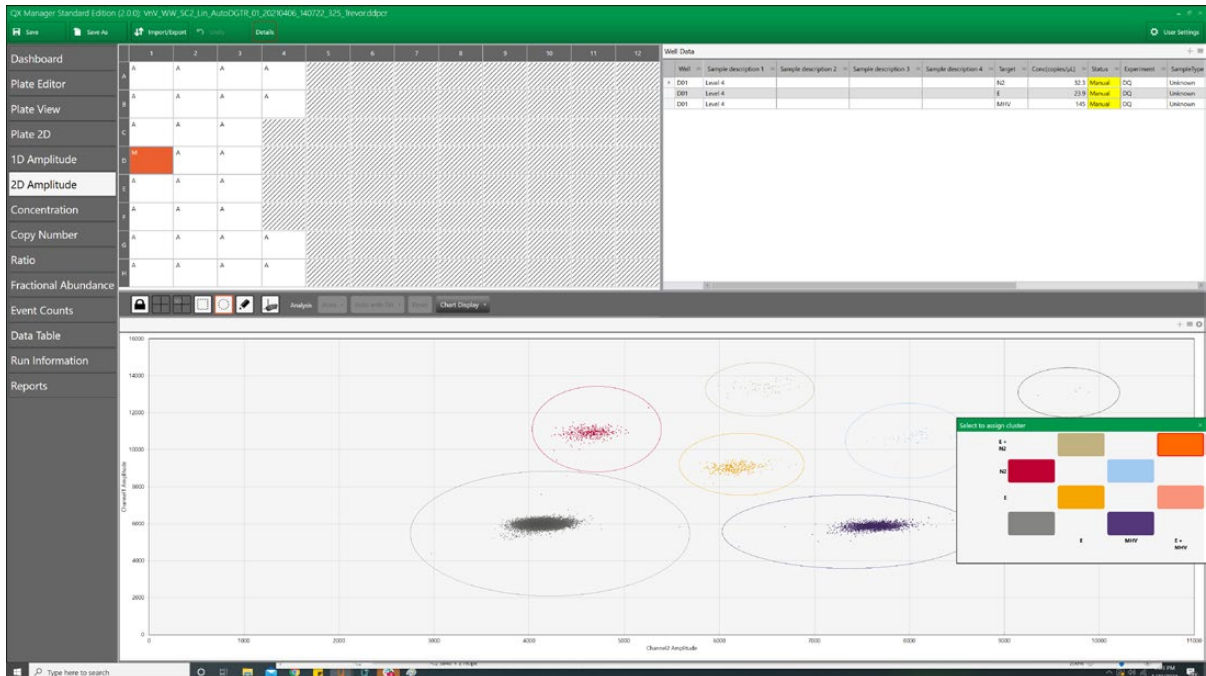


Fig. 18. N2, E, and MHV triple-positive cluster.

- Below the triple-positive cluster there is an E and MHV double-positive cluster corresponding to droplets that are both **E** and **MHV** positive (Figure 19).

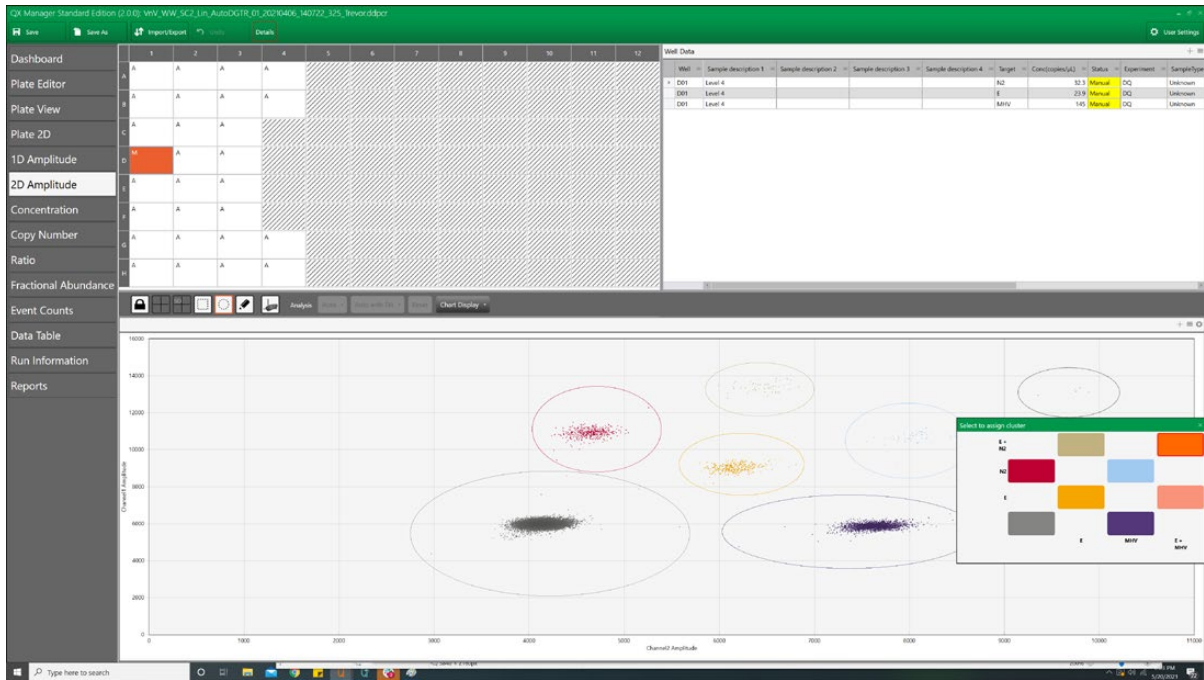


Fig. 19. E and MHV double-positive cluster.

- Cluster assignments are applied upon selection. See Figure 20 for the final configuration .

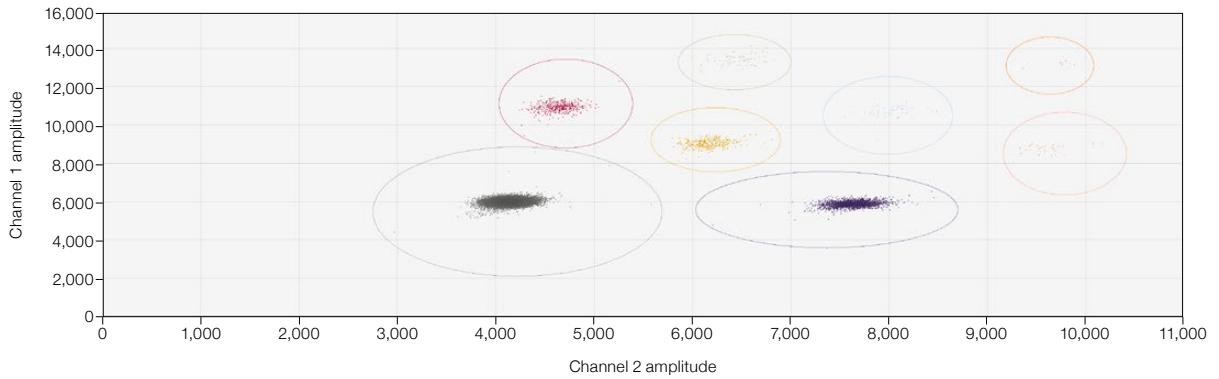


Fig. 20. 2D plot with all correctly designated clusters.

**Note:** After applying cluster designations on multiple wells at a time, it is essential to select each well and review cluster designations on an individual well basis to avoid errors and inaccuracies in clustering.

- The data can then be exported by selecting all wells, then selecting the **Data Table** tab and clicking any of the four horizontal bars toward the top left corner of the window (Figure 21). Then options of Export to Excel... or Export to CSV... are made available.

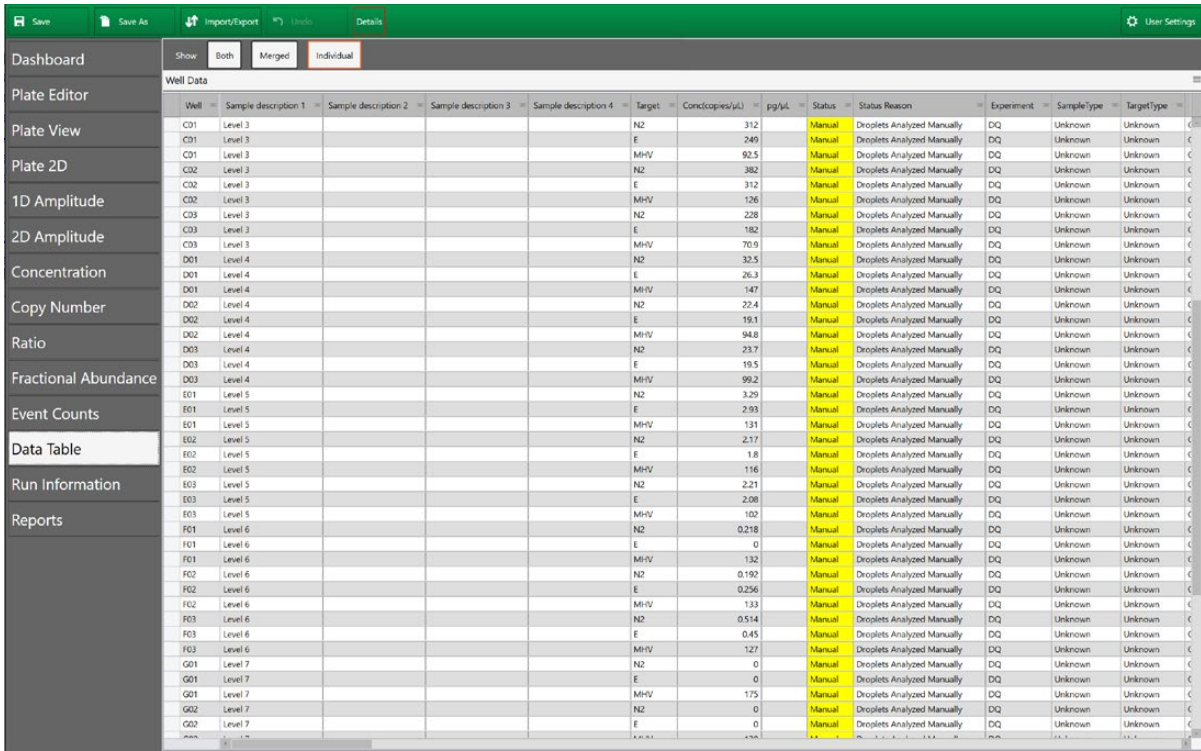


Fig. 21. Options in Data Table tab.

Refer to the QX200 Droplet Reader and QX Manager Software Standard Edition User Guide (10000107223) for troubleshooting any software errors.

## 9. Interpretation of Results

Results can be interpreted once data have been exported and clusters have been defined for all samples and controls.

All test controls should be examined prior to interpretation of results.

## Section 8 Test Performance and Validation

For the run to be valid, the controls must have the results summarized in Table 11.

Table 11. Interpretation of control results.

Control Material	N2 (FAM) Result	E (FAM/HEX) Result	MHV (HEX) Result	Interpretation	Process Result
(+) Extraction control	>5 copies/µl	>2 copies/µl	>10 copies/µl	Positive	Valid extraction
(-) Extraction control	≤0.1 copies/µl and ≤1 droplet	≤0.1 copies/µl and ≤1 droplet	≤0.1 copies/µl and ≤1 droplet	Negative	
(+) RT-ddPCR control	>5 copies/µl	>2 copies/µl	>30 copies/µl	Positive	Valid RT-ddPCR
NTC	≤0.1 copies/µl and ≤1 droplet	≤0.1 copies/µl and ≤1 droplet	≤0.1 copies/µl and ≤1 droplet	Negative	

**Note:** If the results look irregular, if a sample is missing the triple-negative cluster, or if clustering is difficult, consider diluting the extracted RNA eluate 1:10 and testing again.

## Quantification of Viral Loads in Wastewater Samples

The concentration of SARS-CoV-2 targets in wastewater samples may be calculated from the ddPCR reaction concentrations according to equations 1 and 2. These calculations consider the recovery efficiency of the concentration and extraction steps based on the MHV matrix control.

Equation 1 for calculation of the concentration of N2 gene in the initial wastewater sample:

$$\text{N2 in wastewater sample } \left( \frac{\text{copies}}{\text{ml}} \right) = \frac{\text{MHV added to wastewater sample (copies)}}{\text{Volume of wastewater sample (ml)}} \times \frac{\text{N2 in ddPCR reaction } \left( \frac{\text{copies}}{\mu\text{l}} \right)}{\text{MHV concentration in ddPCR reaction } \left( \frac{\text{copies}}{\mu\text{l}} \right)}$$

Equation 2 for calculation of the concentration of the E gene in the initial wastewater sample:

$$\text{E in wastewater sample } \left( \frac{\text{copies}}{\text{ml}} \right) = \frac{\text{MHV added to wastewater sample (copies)}}{\text{Volume of wastewater sample (ml)}} \times \frac{\text{E in ddPCR reaction } \left( \frac{\text{copies}}{\mu\text{l}} \right)}{\text{MHV concentration in ddPCR reaction } \left( \frac{\text{copies}}{\mu\text{l}} \right)}$$

For positive results, check with local and public health agencies to see what actions need to be taken.

## Section 9 References

Ahmed W et al. (2020). Comparison of virus concentration methods for the RT-qPCR-based recovery of murine hepatitis virus, a surrogate for SARS-CoV-2 from untreated wastewater. *Sci Total Environ* 739, 139960.

Forés E et al. (2021). Evaluation of two rapid ultrafiltration-based methods for SARS-CoV-2 concentration from wastewater. *Sci Total Environ* 768, 144786.

Whitney ON et al. (2021). Sewage, salt, silica, and SARS-CoV-2 (4S): An economical kit-free method for direct capture of SARS-CoV-2 RNA from wastewater. *Environ Sci Technol* 55, 4,880–4,888.

Wu F et al. (2020). SARS-CoV-2 titers in wastewater are higher than expected from clinically confirmed cases. *mSystems* 5, e00614–00620.

## Section 10 Appendix

### Appendix A. The crAssphage Fecal Indicator Assay

For more information, refer to the PREvalence ddPCR crAssphage Fecal Indicator Assay Product Insert (10000145551).

### Appendix B. The PMMoV Fecal Indicator Assay

For more information, refer to the PREvalence ddPCR PMMoV Fecal Indicator Assay Product Insert (10000145552).





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