

PREvalence ddPCR PMMoV Fecal Indicator Assay

Catalog #	Description
12015962	PREvalence ddPCR PMMoV Fecal Indicator Assay , 100 x 20 µl reactions

For research purposes only.

Description

The PREvalence ddPCR PMMoV Fecal Indicator Assay is designed to detect and quantify human fecal matter content in wastewater by Droplet Digital PCR (ddPCR). Normalizing human fecal matter content in wastewater samples can be an important step to interpret virus loads in wastewater, as the amount of human fecal matter in wastewater is impacted by changes in weather patterns or patterns of human activity. PMMoV is an RNA virus that infects pepper plants. It enters the wastewater stream through the dietary consumption of pepper plants and is highly associated with human fecal content.

The assay has been designed to pair with the PREvalence ddPCR SARS-CoV-2 Wastewater Quantification Kit (Bio-Rad Laboratories, Inc., catalog #12015402).

Storage and Stability

The ddPCR PMMoV Assay is stable through the expiration date printed on the label when stored at -20°C and protected from light. Repeated freezing and thawing of the kit is not recommended.

Kit Contents

Each kit includes 140 µl of 20x ddPCR PMMoV Assay, which is sufficient for 200 reactions. The 20x ddPCR PMMoV Assay includes reagents to detect PMMoV RNA in FAM.

Required Materials

This assay has been tested for use with the One-Step RT-ddPCR Advanced Kit for Probes (Bio-Rad, #1864021 or 1864022) using the QX200 AutoDG or QX200 Droplet Digital PCR System. Refer to the PREvalence ddPCR SARS-CoV-2 Wastewater Quantification Kit User Guide ([10000142158](#)) for a complete list of all other required materials and equipment.

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 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 ISO 7218 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 the kit reagents 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 should be kept on a cold block or on ice (4°C) the whole time it is outside of the -20°C freezer.

Protocol

Refer to the PREvalence ddPCR SARS-CoV-2 Wastewater Quantification Kit User Guide ([10000142158](#)) for sample concentration and RNA extraction protocols.

Preparation of One-Step Reverse Transcription ddPCR (RT-ddPCR) Reactions

- Clean all work surfaces and equipment with 10% bleach followed by 70% ethanol.
- Retrieve the PREvalence ddPCR PMMoV Fecal Indicator Assay and One-Step RT-ddPCR Advanced Kit for Probes. Let all reagents, except the one-step reverse transcriptase, thaw at room temperature for up to 15 minutes. Keep reagents in a cold block or on ice while working.

Note: The one-step reverse transcriptase must always be stored on ice or on a cold block while in use.
- 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.
- The one-step supermix is highly viscous and must be vortexed for at least 30 seconds prior to use. Failure to do so may cause poor results.**
- At a minimum, run one positive RT-ddPCR control and one NTC with each plate to ensure validity of results.
- Set up the master mix as described in Table 1.

Table 1. Formulation of the RT-ddPCR 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
PMMoV Fecal Indicator Assay	1	1.2	24
Nuclease-free water	2	2.4	48
RNA sample**	9	10.8	216
Total	20	24	480

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

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

- Vortex the master mix for 30 sec, and briefly centrifuge to collect the contents at the bottom of the tube.
- Prepare a 96-well plate cold block or ice bucket and place a clean ddPCR 96-Well Plate onto it.
- Add 12.1 µl of master mix to appropriate sample wells.

- Seal the plate with a foil seal and move plate to the area where template is added. Ensure that the plate with master mix is kept on a 96-well plate cold block or on ice while working.
- Add 9.9 µl of nuclease-free water to the NTC well.
- Add 9.9 µl of sample to the appropriate sample wells.

Note: Wastewater samples have a high abundance of PMMoV Assay template. Samples need to be diluted prior to reaction setup. Samples should be diluted from approximately 1:200 to 1:2,000 for the appropriate copies/µl in the ddPCR reaction.
- Add 9.9 µl of the RT-ddPCR positive control solution to the positive control well.
- 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.
- Vortex the plate for 30 sec at high speed.
- Spin down the plate in a centrifuge at 1,150 rcf for 30 sec.
- 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 2 on the thermal cycler.

Table 2. Thermal cycling profile for the PREvalence ddPCR PMMoV Fecal Indicator Assay.*

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 2.

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.

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.

Note: If not already installed, go to bio-rad.com/QXSoftware to download and install the latest version of QX Manager Software Standard Edition. For general instructions, refer to the QX200 Droplet Reader and QX Manager Software Standard Edition User Guide (10000107223).

1. On the Plate Editor Tab, ensure that the Assay Type is set to **Single Target per Channel** and make sure the Target Name is **PMMoV**, Signal Ch1 is **FAM**, and Signal Ch2 is **None**.
2. In the 1D Amplitude tab, select all the wells to be analyzed. Click the small gear icon to the right of the 1D plot for the FAM channel. Under Options, select **Fixed**.
3. Based on user preference, select one of the threshold cluster modes (**Threshold Multiple Wells** or **Threshold Single Wells**). Thresholded samples are shown in Figure 1, where blue indicates positive droplets for the PMMoV template, and gray indicates negative droplets.

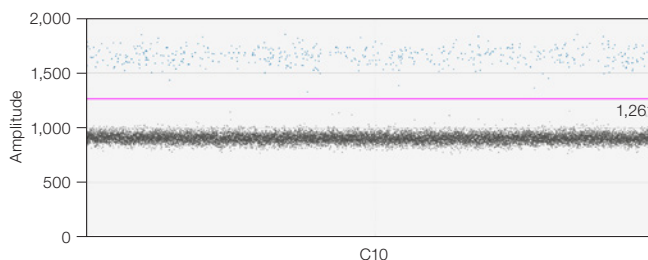


Fig. 1. 1D plot of a sample thresholded for the PMMoV template.

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.

Interpretation of Results

Test Controls

All test controls should be examined prior to interpretation of results. If the controls are not valid, the results should not be interpreted.

No Template Control

The NTC consists of nuclease-free water in the one-step RT-ddPCR reaction instead of RNA. The NTC reaction should not exhibit a positive signal in any channel (HEX or FAM). If a positive signal is observed, sample contamination may have occurred. Invalidate the run and repeat with the residual extracted nucleic acid with adherence to laboratory guidelines and PCR recommendations.

ddPCR Positive Control

The positive control contains template that is known to amplify. It may consist of extracted wastewater previously confirmed to have the PMMoV template, purified PMMoV nucleic acid, or synthetic DNA with the target assay sequence:

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TACATTAGGCGTAGATCCATTGGTGGCAGCAAAGGTAATGGTAGCTGTGGTTCAAATGAGAGT
GGTTTGACCTTAACGTTTGAGAGGCCTACCGAAGCAAATGTCGCACCTGCATTGCAACCGACA
ATTACATCAAAGGAGGAAGGTTTCGTTGAAGATTGTGTGTCGACAGCTAGGTGAGTCCCTCAA
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Normalization of Content in Wastewater Samples with the Fecal Indicator Assay

The fecal indicator assay can be used to normalize the concentration of targets, such as the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), in wastewater samples, especially when the human waste contributed to the sewershed can be expected to change over time (for example, weekday commuters, tourism, etc.).

To normalize the target, the ddPCR reaction concentration of the target may be divided by the ddPCR reaction concentration of the fecal indicator target multiplied by the sample dilution factor. This results in a dimensionless number:

$$\frac{\text{Target/}}{\text{PMMoV}} = \frac{\text{Target in ddPCR reaction} \left(\frac{\text{copies}}{\mu\text{l}} \right) \times \text{DF}_T}{\text{PMMoV in ddPCR reaction} \left(\frac{\text{copies}}{\mu\text{l}} \right) \times \text{DF}_{\text{PMMoV}}}$$

Where

DF_T = the dilution factor of the wastewater sample used in the ddPCR reaction for measuring the target of interest. For example, if the concentrated and extracted sample was diluted twofold before adding to the ddPCR reaction master mix, the DF_T is 2.

DF_{PMMoV} = the dilution factor of the wastewater sample used in the ddPCR reaction for measuring PMMoV. For example, if the concentrated and extracted sample was diluted 2,000-fold (1 μl of concentrated and extracted sample in a final volume of 2,000 μl) before adding to the ddPCR reaction master mix, the DF_{PMMoV} is 2,000.

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