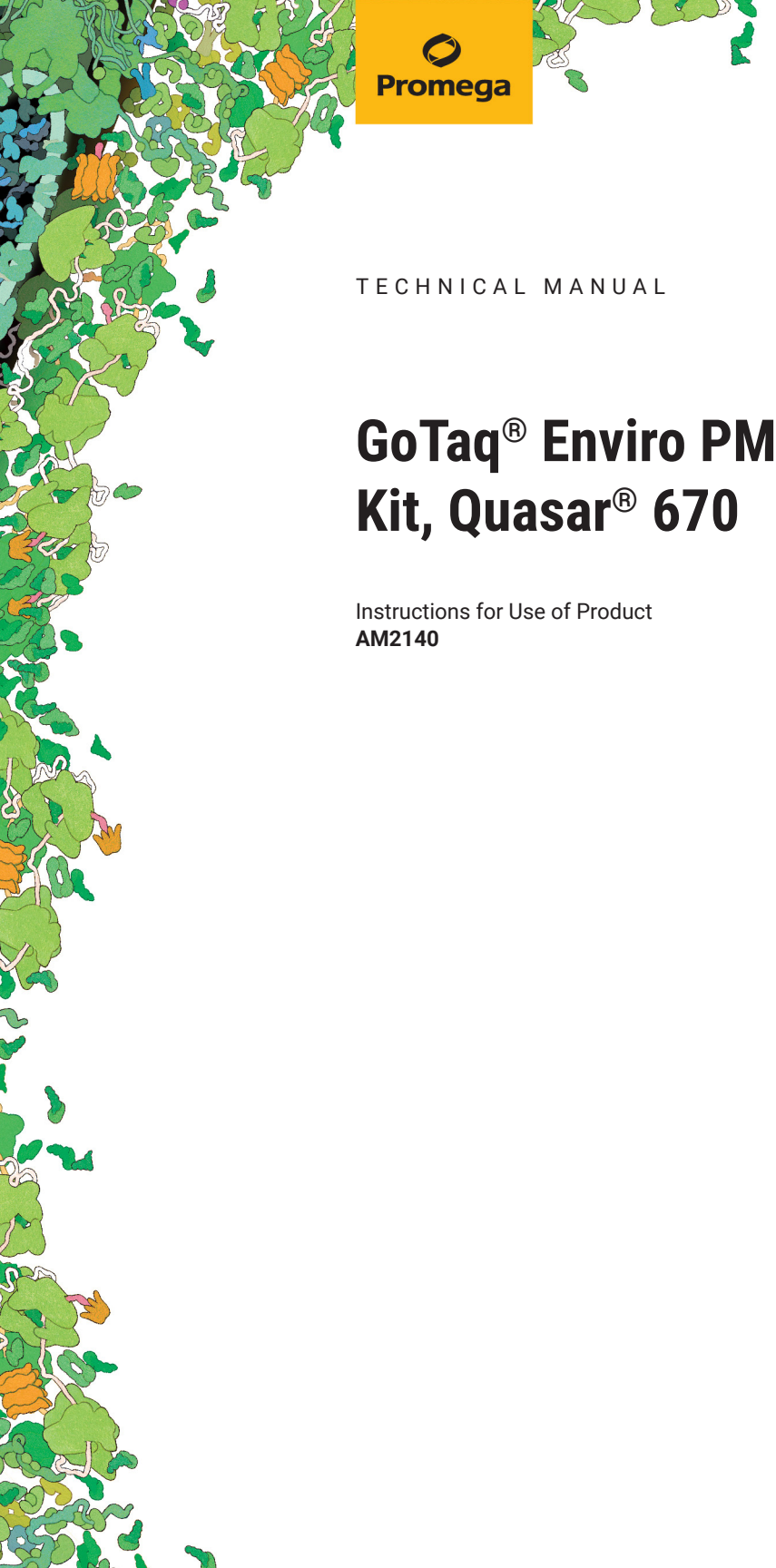

TECHNICAL MANUAL

GoTaq® Enviro PMMoV Quant Kit, Quasar® 670

Instructions for Use of Product
AM2140

Revised 4/24
TM667

GoTaq® Enviro PMMoV Quant Kit, Quasar® 670

All technical literature is available at: www.promega.com/protocols/
Visit the website to verify that you are using the most current version of this Technical Manual.
Email Promega Technical Services if you have questions on use of this system: techserv@promega.com

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1. Description

PMMoV (Pepper Mild Mottle Virus) is a single-stranded RNA virus infecting peppers. Due to the nature of the human diet, the virus and its genetic signature are present in human feces, acting as a human fecal indicator. PMMoV is widely distributed and commonly found in wastewater, surface water and other environmental water sources (1,2). The GoTaq® Enviro PMMoV Quant Kit, Quasar® 670 (Cat.# AM2140) can be used as a positive process control to normalize data for wastewater-based epidemiology.

The RT-qPCR master mix used in this kit, GoTaq® Enviro Master Mix, 2X, includes proprietary enzymes and formulations that tolerate reverse transcriptase (RT) and PCR inhibitors like humic acid that can be present in nucleic acid samples purified from wastewater.

The GoTaq® Enviro PMMoV Quant Kit, Quasar® 670, can be used for: identifying human fecal contamination in a sample, process control for sample preparation, RT-qPCR analysis of wastewater and other relevant environmental samples and normalizing other nucleic acid targets (e.g., SARS-CoV-2 RNA in wastewater).

The GoTaq® Enviro PMMoV Quant Kit, Quasar® 670, includes:

PMMoV Primer/Probe Mix, Quasar® 670: Contains primers and hydrolysis probe for amplifying and detecting PMMoV RNA. Quasar® 670 has spectral properties similar to Cy®5 spectral properties.

DNA Polymerase and Reverse Transcriptase: GoTaq® Enviro Master Mix, 2X, contains a thermostable DNA polymerase and the GoScript™ Enzyme Mix contains reverse transcriptase. These mixes are designed to tolerate a diverse range of DNA polymerase and reverse transcriptase inhibitors, including those found in wastewater.

Passive Reference Dye: CXR Reference Dye (carboxy-X-rhodamine) that has similar spectral properties to ROX™ dye.

PMMoV RNA, 4 × 10^6 copies/µl: An RNA encoding a fragment of the PMMoV genome serves as a stable quantitation standard and is supplied at 4 × 10^6 copies/µl for generating a standard curve.

Nuclease-Free Water: Can be used as a negative no-template control (NTC), for diluting the PMMoV RNA, 4 × 10^6 copies/µl, and for adjusting volumes of RT-qPCR reaction mixes during setup.

2. Product Components and Storage Conditions

PRODUCT	SIZE	CAT. #
GoTaq® Enviro PMMoV Quant Kit, Quasar® 670	**100 reactions**	**AM2140**

Not For Medical Diagnostic Use. Contains sufficient reagents for 100 reactions of 20µl each. Includes:

- 1 × 100µl PMMoV Primer/Probe Mix, Quasar® 670, 20X
- 1 × 1,000µl GoTaq® Enviro Master Mix, 2X
- 1 × 100µl GoScript™ Enzyme Mix
- 1 × 1.25ml Nuclease-Free Water
- 1 × 100µl PMMoV RNA, 4×10^6 copies/µl
- 1 × 100µl CXR Reference Dye, 30µM

Storage Conditions: Store all components of the GoTaq® Enviro PMMoV Quant Kit, Quasar® 670, at –30°C to –10°C. Limit the number of freeze-thaws cycles to five or less. The PMMoV Primer/Probe Mix, Quasar® 670, and CXR Reference Dye, 30µM, are light sensitive and must be stored in the dark.

3. General Considerations

GoTaq® Enviro PMMoV Quant Kit, Quasar® 670, is a sensitive detection system; take precautions to minimize contamination. We recommend storing the reagents separately from RNA samples. We also recommend using clean designated work areas and separate pipettes for pre- and post-amplification steps to minimize the potential for cross contamination between RNA samples and to prevent carryover of nucleic acid from one run to the next. Wear a lab coat and protective eyewear. Wear gloves and change them often. Prevent contamination by using aerosol-resistant pipette tips. Always include a no-template control (NTC) reaction to detect contamination. We recommend performing NTC reactions in triplicate.

Do not unseal reaction plates after amplification is complete. Unsealing the plates increases the risk of contaminating subsequent reactions with amplified product.

Materials to Be Supplied by User

- sterile aerosol-resistant barrier pipette tips
- pipettes dedicated to pre-amplification work
- 1.5ml tubes to prepare the reaction mixes
- 0.5ml low-bind tubes (e.g., Eppendorf Cat.# 022431005) to prepare the standard dilutions
- qPCR plates or strip tubes with caps
- qPCR thermocycler (FAM™, HEX™, Cy®5 channels; ROX™ channel if reference dye is required; see Section 7.A)

3.A. Kit Usage

The GoTaq® Enviro PMMoV Quant Kit, Quasar® 670, is designed to detect PMMoV RNA as a human fecal indicator from various samples, including but not limited to environmental samples, wastewater, drinking water, recreational water and irrigational/agricultural water. Upstream processing includes viral concentration and purification of nucleic acid. The purified nucleic acid can then be used in RT-qPCR.

Viral concentration and purification can be achieved using the following Promega kits:

- Wizard® Enviro TNA Kit (Cat.# A2991)
- Maxwell® RSC Enviro TNA Kit (Cat.# AS1831)

Alternative viral concentration methods can also be used, such as PEG 8000/NaCl precipitation, membrane filtration, centrifugal ultrafiltration, skimmed milk flocculation and others. Nucleic acid purification can be performed on the concentrated viral material using manual or automated systems.

4. GoTaq® Enviro PMMoV Quant Kit, Quasar® 670 Assay Setup

Note: To avoid contamination of samples with external sources of PCR templates, perform all steps with aerosol-resistant pipette tips.

4.A. Assembling the RT-qPCR Amplification Mix

We recommend preparing three replicates of each sample for RT-qPCR. Increasing the number of replicate samples increases the statistical power of your results. The final reaction volume is 20µl: Combine 15µl of reaction mix with 5µl of extracted nucleic acid, PMMoV RNA, 4×10^6 copies/µl, or NTC.

Vigorously vortex the GoTaq® Enviro Master Mix, 2X, for 30–60 seconds to ensure homogeneity before use. Briefly centrifuge to collect contents at the bottom of the tube. Once the reaction mix is assembled, vortex briefly to mix and centrifuge to collect contents at the bottom of the tube.

Determine the number of reaction wells needed. This should include reactions for quantitation standards and negative control reactions. Add 1 or 2 reactions to this number to compensate for pipetting error. While this approach consumes a small additional amount of each reagent, it ensures that there will be enough RT-qPCR amplification mix for all samples. It also ensures that each reaction contains the same RT-qPCR amplification mix.

Table 1. Reaction Mix Worksheet.

RT-qPCR Amplification Mix	Volume per Reaction (X)	Number of Reactions (n)	Final Volume (X × n)
GoTaq® Enviro Master Mix, 2X	10µl		
GoScript™ Enzyme Mix	0.4µl		
PMMoV Primer/Probe Mix, Quasar® 670, 20X	1µl		
CXR Reference Dye, 30µM* (optional)	0.02µl		
Nuclease-Free Water to a final volume of	**15µl**		

*See Section 7.A.

1. Pipet 15µl of RT-qPCR amplification mix into each well of the 96-well qPCR plates. Add 5µl of extracted nucleic acid, PMMoV RNA standard dilutions (Section 4.B) or Nuclease-Free Water as an NTC.

4.B. Preparing the Standard Curve Dilutions for PMMoV RNA

1. Thaw the PMMoV RNA, 4×10^6 copies/µl, avoiding long exposure to ambient temperatures. Dilute the PMMoV RNA, 4×10^6 copies/µl, 100-fold by adding 2µl to 198µl of Nuclease-Free Water to obtain a concentration of 4×10^4 copies/µl (Figure 1 and Table 2).
2. Perform subsequent serial tenfold dilutions in low-binding 0.5ml tubes (Figure 1). For example, add 5µl of PMMoV RNA to 45µl of Nuclease-Free Water to obtain the standard curve dilutions (4×10^4–4 copies/µl) shown in Figure 1 and Table 2.

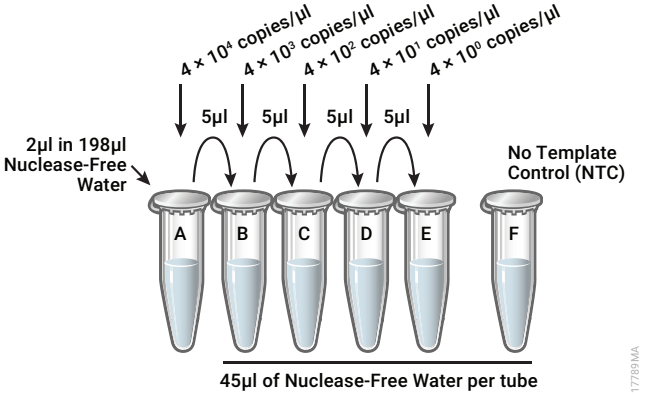

Figure 1. PMMoV RNA Quant Standard dilution scheme.

4.B. Preparing the Standard Curve Dilutions for PMMoV RNA (continued)

Table 2. RNA Standard Curve Dilutions.

Tube (Figure 1)	PMMoV RNA (copies/µl)	Copies/Well (5µl Sample/20µl Reaction)
A	4×10^4	2×10^5
B	4×10^3	2×10^4
C	4×10^2	2×10^3
D	40	2×10^2
E	4	20

3. Pipet 5µl of diluted PMMoV RNA from each tube into the corresponding wells containing master mix.
4. For the NTC, add 5µl of Nuclease-Free Water. The final PCR volume should be 20µl.
5. Seal and centrifuge the plate (300 × *g* for 1 minute) to ensure that all liquid is collected at the bottom of the wells.

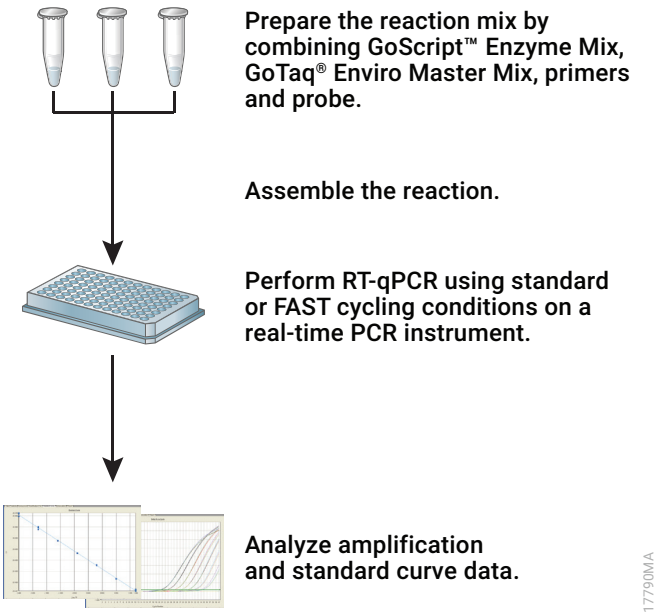

Figure 2 . An overview of the GoTaq® Enviro PMMoV Quant Kit, Quasar® 670, protocol.

5. Thermal Cycling

The PCR cycling program and instrument settings shown below are provided as guidelines and can be modified as necessary for optimal results.

Standard Cycling Conditions

Step	Temperature (°C)	Time	Number of Cycles
Reverse transcription	45	15 minutes	1
RT inactivation/GoTaq® activation	95	2 minutes	1
Denaturation	95	15 seconds	40
Annealing/extension	62	60 seconds	

FAST Cycling Conditions

Step	Temperature (°C)	Time	Number of Cycles
Reverse transcription	45	15 minutes	1
RT inactivation/GoTaq® activation	95	2 minutes	1
Denaturation	95	3 seconds	40
Annealing/extension	62	30 seconds	

Collect data from the following fluorescence channels at the end of each 62°C annealing/extension step. Performing >40 PCR cycles is **not** recommended as it may generate nonspecific amplification products. If multiplexing with other targets is desired, cycling conditions may need to be optimized.

Fluorophores	Target
Quasar® 670/Cy®5	PMMoV
optional: ROX/CXR	Reference Dye

Dispose of PCR plates in biohazard waste per your institution's guidelines. To avoid DNA contamination of your lab space and future samples, do not open the PCR plates after completing the amplification reaction.

6. Calculating Viral Nucleic Acid

The following formula can be applied to quantitate the amount of PMMoV nucleic acid in a sample:

$$\text{Viral genome (copies/liter)} = \frac{\text{Copies in RT-qPCR} \times 1{,}000}{\text{Volume of nucleic acid extract used in RT-qPCR (ml)*} \times \text{Concentration factor}}$$

*If 5µl of nucleic acid extract is used in RT-qPCR, the value in ml is 0.005.

$$\text{Concentration factor} = \frac{\text{Wastewater sample volume used (ml)}}{\text{Volume of nucleic acid extracted (ml)}}$$

6.A. Normalizing Targets to PMMoV

Quantitating other nucleic acid targets can be performed using the equations in Section 6 (above).

$$\text{Normalized Concentration} = \frac{\text{Target of interest (copies/liter)}}{\text{PMMoV (copies/liter)}}$$

7. Appendix

7.A. qPCR Instruments and Reference Dye Requirements

Instruments that do not require reference dye:

- Bio-Rad CFX96 Real-Time PCR Detection System
- Bio-Rad/MJ Research Chromo4™ Real-Time Detector
- Bio-Rad iCycler iQ® and iQ®5 Real-Time PCR Detection Systems
- Roche LightCycler® 480 Real-Time PCR System
- MyGo Pro IT-IS

Instruments that require low levels (30nM) of reference dye:

- Applied Biosystems® 7500 and 7500 FAST Real-Time PCR System
- Applied Biosystems® QuantStudio® Real Time PCR Systems
- Applied Biosystems® ViiA® 7 Real-Time PCR System
- Stratagene/Agilent Mx3000P® and Mx3005P® Real-Time PCR Systems
- Stratagene/Agilent Mx4000® Multiplex Quantitative PCR System

Instruments that require high levels (500nM) of reference dye:

- Applied Biosystems® StepOne™ and StepOnePlus™ Real-Time PCR Systems
- Applied Biosystems® 7300 and 7900HT Real-Time PCR System

7.B. Troubleshooting

For questions not addressed here, please contact your local Promega Branch Office or Distributor. Contact information available at: **www.promega.com**. Email: **techserv@promega.com**

Symptoms	Causes and Comments
Failure to detect qPCR signal	Improper nucleic acid extraction from samples, resulting in loss of RNA, RNA degradation or both.
	Inhibition of reverse transcriptase and/or DNA polymerase by inhibitors in the sample.
	Absence of sufficient nucleic acid due to poor collection or pasteurization of sample.
	Improper assay set up or execution. Reagent or equipment malfunction.
Low yield of RT-qPCR product	RNA degradation. Always use nuclease-free, commercially autoclaved reaction tubes, sterile aerosol resistant tips and gloves. Ensure that reagents, tubes and tips are kept RNase-free by using sterile technique.
	Isolate RNA in the presence of RNasin® Ribonuclease Inhibitor. Use RNasin® Ribonuclease Inhibitor to inhibit degradation of target during cDNA synthesis (20u/20µl reaction).
	Extension time was too brief for amplicon length. To minimize interactive effects of reverse transcriptase and thermophilic DNA polymerase, design the thermal cycling program with a longer extension time in each cycle. Begin with 1 minute per kilobase per cycle and increase to 2 minutes or more if necessary.
	Too few PCR cycles. To detect rare or difficult RNA targets by RT-PCR, increase the cycle number to 40 to maximize sensitivity.

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7.B. Troubleshooting (continued)

Symptoms	Causes and Comments
Low yield of RT-qPCR product (continued)	Wrong reaction tubes were used. Use thin-walled reaction tubes for optimal heat transfer during PCR. Use only sterile, nuclease-free commercially autoclaved tubes, strip tubes or plates for PCR. Autoclaving eliminates volatile contaminants that inhibit amplification.
	Reverse transcriptase effect on primer-dimer artifact synthesis in RT-PCR. Make sure to thoroughly heat-inactivate the reverse transcription reactions prior to use (6,7).

7.C. References

1. Kitajima, M. *et al.* (2018) Pepper mild mottle virus as a water quality indicator. *npj Clean Water* **1**, 19.
2. Rosario, K. *et al.* (2009) Pepper mild mottle virus as an indicator of fecal pollution. *Appl. Environ. Microbiol.* **5**, 7261–7.

7.D. Related Products

Amplification Systems and Reagents

Product	Size	Cat.#
GoTaq® Enviro Wastewater SARS-CoV-2 System, N1/N2/E*	200 reactions	AM2100
GoTaq® Enviro Wastewater SARS-CoV-2 System, N1*	200 reactions	AM2110
GoTaq® Enviro Wastewater SARS-CoV-2 System, N2*	200 reactions	AM2120
GoTaq® Enviro Wastewater SARS-CoV-2 System, E*	200 reactions	AM2130
SARS-CoV-2 (N+E) dsDNA Quant Standard	100µl	AM2060
PMMoV RNA Quant Standard	100µl	AM2070
SARS-CoV-2 (N+E) RNA Quant Standard	100µl	AM2050
GoTaq® Enviro qPCR System*	200 reactions	AM2000
	1,000 reactions	AM2001
GoTaq® Enviro RT-qPCR System*	200 reactions	AM2010
	1,000 reactions	AM2011
IPC qPCR Inhibition Control Assay, CAL Fluor® 560*	100 reactions	AM2030
IAC RT-qPCR Inhibition Control Assay, CAL Fluor® 560*	100 reactions	AM2040

*For Research Use Only. Not for use in diagnostic procedures.

Manual Nucleic Acid Purification Systems and Reagents

Product	Size	Cat.#
Wizard® Enviro TNA Kit	25 preps	A2991
Vac-Man® 96 Vacuum Manifold	1 each	A2291
Eluator™ Vacuum Elution Device	4 each	A1071
PEG 8000, Molecular Biology Grade	500g	V3011
Sodium Chloride, Molecular Biology Grade	1kg	H5273

Automated RNA Purification

Product	Size	Cat.#
Maxwell® RSC Enviro TNA Kit	48 preps	AS1831

8. Summary of Changes

The following changes were made to the 4/24 revision of this document:

1. Added Section 7.B, Troubleshooting.