TECHNICAL MANUAL

GoTaq® Enviro Wastewater SARS-CoV-2 Dual Target Systems

Instructions for Use of Products
AM2150 and AM2160

10/23
TM721

Promega

GoTaq® Enviro Wastewater SARS-CoV-2 Dual Target Systems

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1. Description

The GoTaq® Enviro Wastewater SARS-CoV-2 Dual Target Systems are four-dye, four-target hydrolysis probe-based reverse transcription quantitative polymerase chain reaction (RT-qPCR) assays that amplify SARS-CoV-2 genetic signatures. The systems include primer/probe sets prescribed by the United States Centers for Disease Control and Prevention (CDC) that target the SARS-CoV-2 nucleocapsid (N) gene fragments N1 and N2 (Cat.# AM2150), or N2 and envelope (E) gene (Cat.# AM2160). The systems are designed as a one-step RT-qPCR. For an internal process control and normalization, the systems include primers to detect Pepper Mild Mottle Virus (PMMoV), an RNA virus commonly found in wastewater (1), and an internal amplification control (IAC; 2). The GoTaq® Enviro Master Mix provided in these kits uses proprietary enzymes and formulations that tolerate reverse transcriptase and PCR inhibitors, such as humic acids, that can be present in nucleic acid samples purified from wastewater.

The GoTaq® Enviro Wastewater SARS-CoV-2 Dual Target Systems include:

Target Genes: Four primer/probe sets are supplied as 20X Primer/Probe mixture for detecting the targets in the following fluorescent channels:

Targets in Cat.# AM2150	Targets in Cat.# AM2160	Fluorophore
SARS-CoV-2 N1	SARS-CoV-2 E	FAM™
SARS-CoV-2 N2	SARS-CoV-2 N2	SUN™/(VIC®/HEX™ channel)
PMMoV	PMMoV	Quasar® 670/(Cy®5 channel)
IAC	IAC	Texas Red®/(ROX™ channel)

Controls: Two controls are included in this system. One detects PMMoV via the Quasar® 670 channel and the other is an internal amplification control (IAC) detected via the Texas Red® (ROX™ channel).

The 20X Primer/Probe/IAC Mixes contain two controls:

- **Process Control:** Detects the Pepper Mild Mottle Virus (PMMoV), an RNA virus commonly found in wastewater. The primers and Quasar® 670 dye-labeled probe amplify and detect a 68bp region of the PMMoV genome and are measured in the Cy®5 channel.
- **Internal Amplification Control (IAC):** The 20X Primer/Probe/IAC Mixes contain primers, probe and template for a 285bp product from an RNA template that is included in every amplification reaction. IAC performance is used to detect RT-qPCR inhibitors (both thermostable DNA polymerase and reverse transcriptase inhibitors) in the sample and are measured in the ROX™ dye channel.

DNA Polymerase and Reverse Transcriptase: The GoTaq® Enviro qPCR Master Mix contains 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.

RNA Quantitation Standards: GoTaq® Enviro Wastewater SARS-CoV-2 Dual Target Systems contain two in vitro transcribed RNA fragments: SARS-CoV-2 (N+E) RNA, 4×10^6 copies/µl, and PMMoV RNA, 4×10^6 copies/µl. These RNA fragments serve as stable quantitation standards that can be used to generate standard curves.

Nuclease-Free Water: Can be used as a negative no-template control (NTC), for diluting the quantitation standards, and for adjusting the setup volume for RT-qPCR amplification mixes.

2. Product Components and Storage Conditions

PRODUCT	SIZE	CAT. #
GoTaq® Enviro Wastewater SARS-CoV-2 N1/N2 System	**200 reactions**	**AM2150**

Not for Medical Diagnostic Use. The system contains sufficient reagents for 200 reactions at 20µl. Includes:

- 2 × 100µl N1/N2 & PMMoV Primer/Probe/IAC Mix, 20X
- 2 × 1ml GoTaq® Enviro Master Mix, 2X
- 1 × 100µl GoScript™ Enzyme Mix
- 2 × 1.25ml Nuclease-Free Water
- 1 × 100µl SARS-CoV-2 (N+E) RNA, 4 × 10^6 copies/µl
- 1 × 100µl PMMoV RNA, 4 × 10^6 copies/µl

PRODUCT	SIZE	CAT. #
GoTaq® Enviro Wastewater SARS-CoV-2 E/N2 System	**200 reactions**	**AM2160**

Not for Medical Diagnostic Use. The system contains sufficient reagents for 200 reactions at 20µl. Includes:

- 2 × 100µl E/N2 & PMMoV Primer/Probe/IAC Mix, 20X
- 2 × 1ml GoTaq® Enviro Master Mix, 2X
- 1 × 100µl GoScript™ Enzyme Mix
- 2 × 1.25ml Nuclease-Free Water
- 1 × 100µl SARS-CoV-2 (N+E) RNA, 4 × 10^6 copies/µl
- 1 × 100µl PMMoV RNA, 4 × 10^6 copies/µl

Storage Conditions: Store all components of the GoTaq® Enviro Wastewater SARS-CoV-2 Dual Target Systems at –30°C to –10°C. Limit freeze-thaws to five cycles or fewer. Store the 20X Primer/Probe/IPC Mix protected from light.

3. General Considerations

The GoTaq® Enviro Wastewater SARS-CoV-2 Dual Target Systems are very sensitive; take precautions to minimize contamination. We recommend storing the reagents separately from RNA and TNA (total nucleic acid) samples. We also recommend using clean designated work areas and separate pipettes for pre- and post-amplification steps to minimize the cross-contamination potential between RNA samples and prevent nucleic acid carryover from one reaction 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 products.

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 capable of detecting FAM™, SUN™ (HEX™/VIC®), Quasar® 670 (Cy®5) and Texas Red®-XN (ROX™) channels

3.A. System Usage

The GoTaq® Enviro Wastewater SARS-CoV2 Dual Target Systems are designed for detecting SARS-CoV-2 genetic signals from water and environmental samples that have been preprocessed. This preprocessing includes viral concentration and nucleic acid purification. The purified nucleic acid is then used for RT-qPCR.

Viral concentration and purification can be performed using following Promega kits:

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

Alternative viral concentration methods can also be used.

4. GoTaq® Enviro Wastewater SARS-CoV2 Dual Target Systems Protocol

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

4.A. Preparing Standard Curve Dilutions for SARS-CoV-2 (N+E) and PMMoV RNAs

1. Thaw the SARS-CoV-2 (N+E) RNA, 4×10^6 copies/µl, and PMMoV RNA, 4×10^6 copies/µl. To avoid long exposure to ambient temperature, place reagents and standards on ice after thawing.
2. Add 2µl of SARS-CoV-2 (N+E) RNA and 20µl of PMMoV RNA into 178µl of Nuclease-Free Water, resulting in a final concentration of 4×10^4 SARS-CoV-2 (N+E) copies/µl and 4×10^5 PMMoV copies/µl (Tube A in Table 1 and Figure 1).
3. Prepare serial tenfold dilutions in low-binding 0.5ml tubes. For example, combine 5µl of RNA with 45µl of Nuclease-Free Water to obtain the following standard curve dilutions: 4×10^4–4 SARS-CoV-2 copies/µl and 4×10^5–40 PMMoV copies/µl (see Table 1 and Figure 1). Vortex each dilution for 3–5 seconds prior to removing an aliquot for the next dilution. Change pipette tips between dilutions.

Table 1. Standard Curve of SARS-CoV-2 (N+E) and PMMoV RNAs Shown in Figure 1.

Tube (Figure 1)	SARS-CoV-2 (N+E) RNA (copies/µl)	SARS-CoV-2 RNA Copies/Well (20µl reaction)	PMMoV RNA (copies/µl)	PMMoV RNA Copies/Well (20µl reaction)
A	4×10^4	2×10^5	4×10^5	2×10^6
B	4×10^3	2×10^4	4×10^4	2×10^5
C	4×10^2	2×10^3	4×10^3	2×10^4
D	40	2×10^2	4×10^2	2×10^3
E	4	20	40	2×10^2

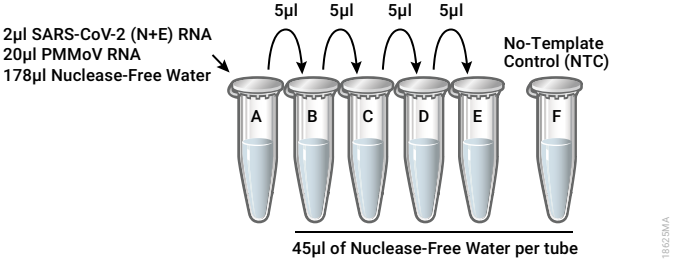

Figure 1. Dilution scheme for combined SARS-CoV-2 (N+E) and PMMoV RNA standards.

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4.B. Preparing the RT-qPCR Amplification Mix (20µl Reaction Volume)

We recommend preparing three RT-qPCR technical replicates for increased statistical power.

1. Vigorously vortex the GoTaq® Enviro Master Mix for 30–60 seconds to ensure homogeneity before use. Briefly centrifuge to collect contents at the bottom of the tube.
2. Determine the number of reaction wells needed. Include reactions for the quantification standards and negative control reactions. Add 1 or 2 reactions to this number to compensate for pipetting error. While this approach consumes a small amount of each reagent, it ensures that enough RT-qPCR amplification mix will be available for all samples. This also makes sure that each reaction contains the same RT-qPCR amplification mix.

   Table 2. Reaction Mixture Worksheet for 20µl Final Reaction Volume.

RT-qPCR Amplification Mix	Volume per reaction (X)	Number of reactions (n)	Final Volume (X × n)
GoTaq® Enviro-qPCR Master Mix (2X)	10µl		
GoScript™ Enzyme Mix (50X)	0.4µl		
Primer/Probe Mix, 20X	1.0µl		
Nuclease-Free Water	3.6µl		

3. Assemble the reaction mix by combining the GoTaq® Enviro Master Mix, GoScript™ Enzyme Mix, 20X Primer/Probe Mix and Nuclease-Free Water calculated in Step 2.
4. Pipette 15µl of RT-qPCR amplification mix into each well of the 96-well qPCR plate(s).
5. Add 5µl of extracted nucleic acid, standards or Nuclease-Free Water for NTC. The final reaction volume should be 20µl.
6. Seal and centrifuge the plate at approximately 300 × *g* for 1 minute to ensure all liquid is collected at the bottom of the plate wells. Protect the plate from extended light exposure and elevated temperatures before cycling. The samples are now ready for thermal cycling.

5. Thermal Cycling

The PCR cycling parameters and instrument settings shown here are provided as guidance and can be modified for optimal results.

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	

Collect data from the following fluorescence channels at the end of each 62°C annealing/extension step. **Note:** The annealing temperature may be optimized between 60–62°C. Certain variants of SARS-CoV2 amplify with greater efficiency at a lower annealing temperature using this assay.

Performing >40 PCR cycles is **not** recommended as it may generate nonspecific amplification.

Targets in Cat.# AM2150	Targets in Cat.# AM2160	Fluorophore
SARS-CoV-2 N1	SARS-CoV-2 E	FAM™
SARS-CoV-2 N2	SARS-CoV-2 N2	SUN™/(VIC®/HEX™ channel)
PMMoV	PMMoV	Quasar® 670/(Cy®5 channel)
IAC	IAC	Texas Red®/(ROX™ channel)

Dispose of PCR plates as biohazardous waste per your local institutional guidelines. To avoid DNA contamination of your lab space and subsequent samples, do not open the PCR plates after completing amplification and collecting data.

	SARS-CoV-2 (N+E) and PMMoV RNA Standards (copies), and NTC			Purified samples								
	1	2	3	4	5	6	7	8	9	10	11	12
A	2×10^5	2×10^5	2×10^5	Sample 3	Sample 3	Sample 3	Sample 11	Sample 11	Sample 11	Sample 19	Sample 19	Sample 19
B	2×10^4	2×10^4	2×10^4	Sample 4	Sample 4	Sample 4	Sample 12	Sample 12	Sample 12	Sample 20	Sample 20	Sample 20
C	2×10^3	2×10^3	2×10^3	Sample 5	Sample 5	Sample 5	Sample 13	Sample 13	Sample 13	Sample 21	Sample 21	Sample 21
D	2×10^2	2×10^2	2×10^2	Sample 6	Sample 6	Sample 6	Sample 14	Sample 14	Sample 14	Sample 22	Sample 22	Sample 22
E	20	20	20	Sample 7	Sample 7	Sample 7	Sample 15	Sample 15	Sample 15	Sample 23	Sample 23	Sample 23
F	NTC	NTC	NTC	Sample 8	Sample 8	Sample 8	Sample 16	Sample 16	Sample 16	Sample 24	Sample 24	Sample 24
G	Sample 1	Sample 1	Sample1	Sample 9	Sample 9	Sample 9	Sample 17	Sample 17	Sample 17	Sample 25	Sample 25	Sample 25
H	Sample 2	Sample 2	Sample 2	Sample 10	Sample 10	Sample 10	Sample 18	Sample 18	Sample 18	Sample 26	Sample 26	Sample 26

Figure 2. Example plate layout for the GoTaq® Enviro RT-qPCR System. Note: PMMoV Standards are 10X higher concentration than listed in Figure 2 with a range of 2×10^6–200 copies.

6. Data Analysis and Interpretation

6.A. Evaluate qPCR Assay Standard Curves (FAM and SUN/HEX/VIC/JOE [SARS-CoV-2]/Cy5 [PMMoV])

Common qPCR analysis software packages apply a linear regression to the standard dilution series data and calculate the best fit of the standard curve using y = mx + b, where x = Log_{10} concentration; y = C_q /C_t; m = slope. r^2 measures goodness of fit to the regressed line and m is a measure of efficiency, where m= −3.3 indicates 100% PCR efficiency (i.e., amplification product is doubled at each cycle). The y intercept (b in the equation) is the y value C_q at x = 0. For example, b corresponds to the C_q value for a sample with a concentration of 1 copy/reaction ($Log_{10}(1) = 0$). During development using the BioRad CFX96™ and Thermo Fisher Scientific QuantStudio™ 6 instruments, the standard curve for each PCR target had an average slope (m) in the range of −3.0 to −3.7, which corresponds to a qPCR efficiency of 86–115%. Generally, we observe y-intercept values in the range of 37–42. We recommend monitoring y-intercept values for any significant changes from run to run.

PMMoV amplification varies based on the sampling location and is typically detected at C_q = 15–30. Higher or lower values may occur.

6.B. Analyze Internal Amplification Control Signal (Texas Red/ROX)

The IAC can be used to evaluate overall performance of the SARS-CoV-2 RT-qPCR amplification reaction and to detect DNA polymerase and/or reverse transcriptase inhibition. The probe used is a dual-labeled probe (Texas Red/BHQ2). Depending on the qPCR instrument used, Texas Red®/ROX™ channels can be used to record the amplification signal. Depending on the qPCR instrument and analysis software used, the IAC C_t should fall in the range of 20–32 for the NTC reactions.

If the IAC C_t in a sample well is shifted significantly (C_t ≥2) compared to NTC well, PCR inhibitors are present in the experimental sample, and results should be considered qualitative and not quantitative. Repeat the purification or clean-up of nucleic acid if necessary. If a sample yields no detectable amplification for SARS-CoV-2 but exhibits IAC amplification (C_t = 20–32) and PMMoV amplification occurs, SARS-CoV-2 is not detectable with this system. If the IAC fails to amplify or the IAC C_t is shifted >3 C_t compared to NTC wells, no conclusions can be made about the absence of SARS-CoV-2 genetic material in a sample. Results can be considered invalid. See Table 3 in Section 6.F for examples.

IAC can fail to amplify if the assay is set up incorrectly.

6.C. Analyze PMMoV Process Control Signal (Cy5/Quasar 670)

Wastewater samples typically exhibit PMMoV fluorescence growth curves that cross the threshold at <40 cycles. PMMoV viral load varies based on the sampling location. It is typically detected at C_t = 15–30, higher or lower values may occur.

If a sample yields no detectable amplification for SARS-CoV-2 but exhibits IAC amplification (C_t = 20–32), and PMMoV amplification (C_t = 15–30), SARS-CoV-2 is not detectable with this system.

Failure to detect PMMoV in wastewater samples may indicate:

- improper nucleic acid extraction from samples, resulting in loss of RNA, RNA degradation or both
- inhibition of reverse transcriptase, DNA polymerase or both by inhibitors in the sample
- absence of sufficient nucleic acid due to poor collection or pasteurization of sample
- improper assay set up and/or execution
- reagent or equipment malfunction

If the PMMoV reaction (Cy®5 channel) is negative, IAC is positive and SARS-CoV-2 N1 or N2 or E are positive, the result can be considered valid because PMMoV negativity may reflect a low PMMoV viral load.

If all SARS-CoV-2 markers, PMMoV (process control) and internal amplification control (IAC) are negative for the specimen, the results are invalid. If residual sample is available, repeat the extraction procedure and retest. If all markers remain negative after retesting, report the results as invalid. A new specimen should be collected if possible.

6.D. No-Template Control

For a no-template control (NTC), use Nuclease-Free Water in the RT-qPCR instead of a nucleic acid-containing sample or RNA standards. NTC samples produce amplification curves for the IAC in the Texas Red® channel. Sample contamination is indicated if FAM™, HEX™ or Cy®5 reaction channels exhibit fluorescence curve with the C_q value indicating copy number greater than the limit of quantification (LoQ).

6.E. Limit of Detection and Limit of Quantification

Limit of detection (LoD) is the lowest amount of analyte in a sample that can be detected with 95% probability. The assay LoD is 8 copies of nucleic acid per reaction.

Limit of quantification (LoQ) is the lowest amount of analyte in a sample that can be quantitatively determined with less than 25% coefficient of variation. The assay LoQ was determined to be 20 copies per reaction for the GoTaq® Enviro Wastewater SARS-CoV-2 Dual Target Systems.

The LoQ of the assay is 20 copies per reaction for SARS-CoV-2 targets and 200 copies for PMMoV. If the SARS-CoV-2 or PMMoV amplification signal appears after the LoQ signal, the quantitative target amounts in the sample cannot be determined with certainty.

6.F. Interpreting Wastewater Signal Profiles

Table 3. Interpreting Wastewater Results.

SARS-CoV-2						
N1 (FAM)	N2 (SUN)	E (FAM)	NTC (FAM)	PMMoV Process Control (Cy5)	Internal Amplification Control (Texas Red)	Result
Any one or more is positive			–	+/–	+/–	SARS-CoV-2 detected
–	–	–	–	+	+	SARS-CoV-2 not detected
Any one or more is positive			+	+/–	+/–	Invalid (false positive)
–	–	–	–	+/–	–	Invalid (false negative)

6.G. Calculating Viral Nucleic Acid

The following formula can be applied to quantitate the amount of SARS-CoV-2 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 ml value is 0.005.

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

6.H. Normalization with PMMoV

Quantitation of PMMoV viral genome copies can be performed using the same approach as for SARS-CoV-2 using the PMMoV RNA Quant Standard.

Changes in SARS-CoV-2 levels can be analyzed relative to the PMMoV levels by using this formula:

$$\text{Relative SARS-CoV-2 signal} = \frac{\text{SARS-CoV-2 signal (copies/L)}}{\text{PMMoV signal (copies/L)}}$$

7. Specificity Testing

Wastewater TNA isolates contain abundant nucleic acid originating from various bacterial and viral species. The GoTaq® Enviro Wastewater SARS-CoV-2 Systems were carefully designed to amplify only the designated SARS-CoV-2 genomic target.

Table 4. Specificity of the GoTaq® Enviro Wastewater SARS-CoV-2 Systems When Tested Against Respiratory Pathogens.

Pathogen	N1	N2	E
SARS-CoV-2	+	+	+
OC43	–	–	–
229E	–	–	–
HKU1	–	–	–
NL63	–	–	–
Influenza A	–	–	–
RSV	–	–	–
E. coli	–	–	–
L. pneumophila	–	–	–
P. aeruginosa	–	–	–

8. Appendix

8.A. References

1. Symonds, E.M., Rosario, K. and Breitbart, M. (2019) Pepper mild mottle virus: Agricultural menace turned effective tool for microbial water quality monitoring and assessing (waste)water treatment technologies. *PLoS Pathog.* **15,** e1007639.
2. Mondal, S. *et al.* (2021) A direct capture method for purification and detection of viral nucleic acid enables epidemiological surveillance of SARS-CoV-2. *Sci. Total Environ.* **7,** 148834.

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8.B. Related Products

Amplification Systems and Accessories

Product	Size	Cat.#
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
GoScript™ Reverse Transcriptase	100 reactions	A5003
	500 reactions	A5004
RNasin® Plus RNase Inhibitor	2,500u	N2611
	10,000u	N2615
Set of dATP, dCTP, dGTP, dUTP	10µmol each	U1335
	40µmol each	U1245
RQ1 RNase-Free DNase	1,000u	M6101
$MgCl_2$	1.5ml	A3511
Nuclease-Free Water	50ml	P1193

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

Not For Medical Diagnostics Use.

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
Wizard® Enviro TNA Start-up Kit	110V	A3050
Wizard® Enviro TNA Start-up Kit	220V	A3060
Eluator™ Vacuum Elution Device	4 each	A1071
Vac-Man® Laboratory Vacuum Manifold	1 each	A7231
One-Way Luer-Lok ®Stop Cocks	10 each	A7261
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
Maxwell® RSC Enviro TNA Start-up Kit	110V	A3070
Maxwell® RSC Enviro TNA Start-up Kit	220V	A3070
Maxwell® RSC PureFood GMO and Authentication Kit	48 preps	AS1600