TECHNICAL MANUAL

GoTaq® Enviro Wastewater Flu A, Flu B, SC2 System

Instructions for Use of Product
AM2170

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TM738

Promega

GoTaq® Enviro Wastewater Flu A, Flu B, SC2 System

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1. Description

The GoTaq® Enviro Wastewater Influenza A (Flu A), Influenza B (Flu B), and SARS-CoV-2 (SC2) Target System is a four-dye four-target hydrolysis probe-based assay designed to quantify RNA levels of Flu A, Flu B and SC2 from test samples such as wastewater in a single-step RT-qPCR amplification. The system includes primer/probe sets that target Flu A, Flu B, and the SC2 3´ untranslated region (UTR) of the nucleocapsid (N) gene (1) and detect Pepper Mild Mottle Virus (PMMoV), an RNA virus commonly found in wastewater, as a control (2). The GoTaq® Enviro Master Mix 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.

GoTaq® Enviro Wastewater Flu A, Flu B, SC2 System includes:

Target Genes: A primer/probe set is supplied as 20X Primer/Probe mixture for detecting the targets in the following fluorescent channels. You can setup this system as a "four-dye" assay:

Target Gene	Fluorophore
Influenza A (Flu A)	FAM™
Influenza B (Flu B)	Yakima Yellow® (VIC®/HEX™ channel)
SARS-CoV-2 (SC2)	Texas Red®-XN (ROX™ channel)
PMMoV	Quasar® 670 (Cy®5 channel)

DNA Polymerase and Reverse Transcriptase: GoTaq® Enviro Master Mix contains thermostable DNA polymerase and 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 or feces.

RNA Quantitation Standards: GoTaq® Enviro Wastewater Flu A, Flu B, SC2 System contains four in vitro transcribed RNA fragments: includes Flu A, Flu B and SC2 RNA at 4×10^6 copies/µl; and PMMoV RNA at 4×10^6 copies/µl. These RNA fragments serve as 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 Flu A, Flu B, SC2 System	**200 reactions**	**AM2170**

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

- 2 × 100µl FluA/FluB/SC2/PMMoV Primer/Probe Mix, 20X
- 2 × 1ml GoTaq® Enviro Master Mix, 2X
- 1 × 100µl GoScript™ Enzyme Mix
- 2 × 1.25ml Nuclease-Free Water
- 1 × 100µl FluA/FluB/SC2 RNA Quant Standard
- 1 × 100µl PMMoV RNA, 4 × 10^6 copies/µl

Storage Conditions: Store all components of the GoTaq® Enviro Wastewater Flu A, Flu B, SC2 System at –30°C to –10°C. Limit freeze-thaws to five cycles or fewer. Store the 20X Primer/Probe Mix protected from light.

3. General Considerations

The GoTaq® Enviro Wastewater Flu A, Flu B, SC2 System is very sensitive; take precautions to minimize contamination. We recommend storing the reagents separately from RNA and total nucleic acid (TNA) 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 triplicates.

! 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™, Yakima Yellow® (HEX™), Texas Red®-XN (ROX™) and Quasar® 670 (Cy®5) dyes

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3.A. System Usage

The GoTaq® Enviro Wastewater Flu A, Flu B, SC2 System is designed to detect Influenza A, Influenza B and SARS-CoV-2 genetic signals from wastewater 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 may be achieved using the following Promega kits:

- Wizard® Enviro Total Nucleic Acid Kit (Cat.# A2991)
- Maxwell® RSC Enviro Total Nucleic Acid Kit (Cat.# AS1831)

Alternative viral concentration and nucleic acid extraction methods can also be used.

4. GoTaq® Enviro Wastewater Flu A, Flu B, SC2 System Protocol

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

4.A. Preparing Standard Curve Dilutions for Flu A, Flu B, SC2 and PMMoV RNA

1. Thaw the FluA/FluB/SC2 RNA Quant Standard and PMMoV RNA, 4×10^6 copies/µl. Place reagents and standards on ice after thawing to avoid long exposure to ambient temperature.
2. Add 4µl of FluA/FluB/SC2 RNA and 40µl of PMMoV RNA to 56µl of Nuclease-Free Water, resulting in a final concentration of 1.6×10^5 FluA/FluB/SC2 RNA copies/µl of and 1.6×10^6 PMMoV RNA 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 (FluA/FluB/SC2 RNA 1.6×10^5–16 copies/µl and PMMoV RNA 1.6×10^6–160 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. Concentration of Flu A, Flu B, SC2 and PMMoV RNA Standards in Standard Curve. Copy number of each RNA standard dilution shown in Figure 1.

Tube (Figure 1)	FluA/FluB/SC2 RNA Quant Standard (copies/µl)	FluA/FluB/SC2 RNA Quant Standard Copies/Well (20µl reaction)	PMMoV RNA (copies/µl)	PMMoV RNA Copies/Well (20µl reaction)
A	1.6×10^5	8×10^5	1.6×10^6	8×10^6
B	1.6×10^4	8×10^4	1.6×10^5	8×10^5
C	1.6×10^3	8×10^3	1.6×10^4	8×10^4
D	1.6×10^2	8×10^2	1.6×10^3	8×10^3
E	1.6×10^1	8×10^1	1.6×10^2	8×10^2

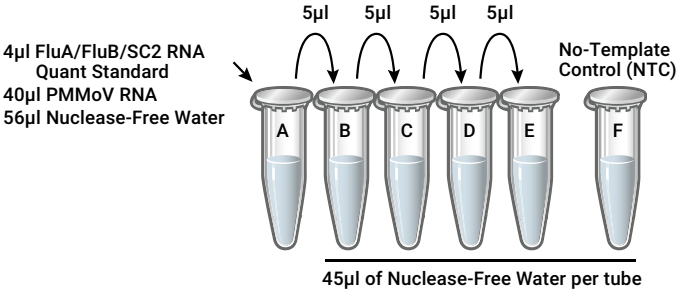

Figure 1. Dilution scheme for the combined Flu A, Flu B, SC2 and PMMoV RNA standards.

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

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

1. Vigorously vortex the GoTaq® Enviro Master Mix for 30–60 seconds to ensure homogeneity. Centrifuge briefly to collect contents at the bottom of the tube.
2. Determine the number of reaction wells needed. Include wells for the quantification standards and negative control reactions. Add one or two reactions to this number to compensate for pipetting error. While this approach consumes a small additional 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 a 20µl Final Reaction Volume.

RT-qPCR Amplification Mix	Volume per Reaction (X)	Number of Reactions (n)	Final Volume (X × n)
GoTaq® Enviro Master Mix (2X)	10.0µl		
GoScript™ Enzyme Mix (50X)	0.4µl		
FluA/FluB/SC2/PMMoV 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 a 96-well qPCR plate or qPCR strip tubes.
5. Add 5µl of extracted nucleic acid, standards or Nuclease-Free Water for NTC. The final reaction volume should be 20µl.
6. Seal the 96-well plate or strip tubes and then vortex to mix.
7. Centrifuge the 96-well plate or strip tubes at approximately 300 × *g* for 1 minute to ensure all liquid is collected at the bottom of the wells. Protect reaction mix from extended light exposure and elevated temperatures before cycling. The samples are now ready for thermal cycling.

   Note: Immediately start thermal cycling for best assay performance.

5. Thermal Cycling

The PCR cycling parameters and instrument settings shown in Table 3 are provided as guidelines and can be modified as necessary for optimal results.

Table 3. Recommended 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	58	60 seconds	
Extension	72	15 seconds	

Collect data from the following fluorescence channels at the end of each 72°C extension step. We do not recommend performing >40 PCR cycles because it can generate nonspecific amplification.

Table 4. Fluorescent Channels and Targets for the GoTaq® Enviro Wastewater Flu A, Flu B, SC2 System.

Fluorophores	Target
FAM™	Flu A
Yakima Yellow®/HEX™, VIC®	Flu B
Texas Red®-XN/ROX™	SC2
Quasar® 670/Cy®5	PMMoV

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

5. Thermal Cycling (continued)

	Flu A, Flu B, SC2 and PMMoV RNA Standards (copies), and NTC			Purified samples								
	1	2	3	4	5	6	7	8	9	10	11	12
A	8×10^5	8×10^5	8×10^5	Sample 3	Sample 3	Sample 3	Sample 11	Sample 11	Sample 11	Sample 19	Sample 19	Sample 19
B	8×10^4	8×10^4	8×10^4	Sample 4	Sample 4	Sample 4	Sample 12	Sample 12	Sample 12	Sample 20	Sample 20	Sample 20
C	8×10^3	8×10^3	8×10^3	Sample 5	Sample 5	Sample 5	Sample 13	Sample 13	Sample 13	Sample 21	Sample 21	Sample 21
D	8×10^2	8×10^2	8×10^2	Sample 6	Sample 6	Sample 6	Sample 14	Sample 14	Sample 14	Sample 22	Sample 22	Sample 22
E	8×10^1	8×10^1	8×10^1	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	Sample 1	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 GoTaq® Enviro Wastewater Flu A, Flu B, SC2 System. Note: PMMoV Standards are 10X higher concentration than listed in Figure 2 with a range of 8×10^6–800 copies.

6. Data Analysis and Interpretation

6.A. Evaluate qPCR Assay Standard Curves (FAM, HEX, ROX and Cy5)

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 $x = \text{Log}_{10}$ 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 ($\text{Log}_{10}(1) = 0$).

In general, the standard curve for each PCR target has an average slope (m) in the range of −3.1 to −3.9, which corresponds to a qPCR efficiency of 80–110%, and an r^2 value >0.970. We recommend monitoring y-intercept values for any significant changes from run to run.

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

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

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

6.C. 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 should not produce amplification curves. Sample contamination is indicated if FAM™, HEX™, ROX™ or Cy®5 reaction channels exhibit fluorescence curve with C_q value indicating copy number greater than the limit of quantification (LoQ).

6.D. 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 40 copies nucleic acid per reaction for the Flu A/Flu B/SC2 targets and 400 copies nucleic acid per reaction for the PMMoV target.

Limit of quantification (LoQ) is the lowest amount of analyte in a sample that can be quantitatively determined with a coefficient of variation of less than 25%. The assay LoQ is 80 copies per reaction for Flu A/Flu B/SC2 and 800 copies per reaction for PMMoV. If Flu A, Flu B, SC2, and PMMoV amplification signals appear after the LoQ signal, the quantitative target amounts in the sample cannot be determined with certainty. Any amplification signals that appear outside of the quantification standards range should be disregarded as false positives.

6.E. Calculating Viral Nucleic Acid

The following formula can be applied to quantitate the amount of Flu A/Flu B/SC2 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.

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

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6.F. Normalization with PMMoV

Quantitation of PMMoV genome copies can be performed using the same approach as for Flu A/Flu B/SC2 using the PMMoV RNA standard.

Changes in Flu A/Flu B/SC2 levels can be analyzed relative to the PMMoV levels by using this formula:

$$\text{Relative Flu A/Flu B/SC2 signal} = \frac{\text{Flu A/Flu B/SC2 signal (copies/L)}}{\text{PMMoV signal (copies/L)}}$$

7. Specificity Testing

Wastewater TNA isolates contain abundant nucleic acid originating from various bacterial and viral species. The GoTaq® Enviro Wastewater Flu A, Flu B, SC2 System was carefully designed to amplify only the designated Flu A, Flu B and SC2 genomic targets (Table 5).

Table 5. Respiratory Pathogens Tested with GoTaq® Enviro Wastewater Flu A, Flu B, SC2 System.

Pathogen	Test Results
Salmonella enterica	Negative
Campylobacter jejuni UA466	Negative
Legionella pneumophila	Negative
Pseudomonas aeruginosa 41501	Negative
Candida albicans MYA-2876D-5	Negative
Human coronavirus HKU1	Negative
Human coronavirus 229E	Negative
Betacoronavirus 1 OC43	Negative
Human coronavirus NL63	Negative
Human respiratory syncytial virus 18537	Negative

8. Appendix

8.A. References

1. Shu, B. *et al*. (2021) Multiplex real-time reverse transcription PCR for influenza A virus, influenza B virus, and severe acute respiratory syndrome coronavirus 2. *Emerg. Infect. Dis.* **27**, 1821–30.
2. 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.

8.B. Troubleshooting

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

Symptoms	Causes and Comments
Low yield of RT-qPCR product	RNA degradation. Always use nuclease-free, commercially autoclaved reaction tubes, sterile aerosol resistant pipette tips and gloves. Ensure that reagents, tubes and tips are kept RNase-free by using sterile technique.
	Reaction not mixed well. Mix the reaction by vortexing the 96-well plate or strip tubes and centrifuge at approximately 300 × *g* for 1 minute to ensure all liquid is collected at the bottom of the wells. Ensure that all wells were adequately sealed to prevent evaporation during thermal cycling.
	Wrong thermal cycling strip tubes or 96-well plates were used. Make sure to use thin-walled reaction tubes or 96-well plates as recommended by instrument manufacturers for optimal heat transfer during PCR.

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8.C. 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 Nucleic Acid 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