Promega

APPLICATION NOTE

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Detection of SARS-CoV-2 in Municipal Wastewater Using the Azure Cielo™ 6 Real-Time PCR System and Promega GoTaq® Enviro Wastewater SARS-CoV-2 RT-qPCR System

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Kits:

- GoTaq® Enviro Wastewater SARS-CoV-2 System, N1 (Cat.# AM2110)
- Wastewater TNA purification system: Wizard® Enviro TNA Kit (Cat.# A2991) or Maxwell® RSC Enviro TNA Kit (Cat.# AS1831)

Materials Required:

- Azure Cielo™ 6 Real-Time PCR System (Cat.# AIQ060)
- Low-profile, white, semi-skirted 96-well plate (Azure, Cat.# AIQ322)
- Optical Sealing Film (Azure, Cat.# AIQ325)
- 0.5ml low-bind tubes (e.g., Eppendorf, Cat.# 022431005)

Abstract

Monitoring SARS-CoV-2 viral RNA in wastewater can be used to detect and predict COVID-19 disease outbreaks. Here, we describe a RT-qPCR protocol for detecting SARS-CoV-2 RNA from WW total nucleic acid (TNA). The Azure Cielo™ 6 Real-Time PCR System was used with the Promega GoTaq® Enviro Wastewater SARS-CoV-2 System to specifically detect and quantify viral RNA in TNA samples. Our results show that this method is a sensitive, robust and repeatable solution for SARS-CoV-2 wastewater-based epidemiology (WBE).

Introduction

Early in the COVID-19 pandemic, studies showed that SARS-CoV-2 viral RNA could be detected in wastewater (WW) containing the feces of infected individuals. Detection of SARS-CoV-2 in WW can indicate the presence or prevalence of COVID-19 within a community as well as the effectiveness of public health control measures. These efforts require quantitative and robust RT-qPCR methods for accurate acquisition and interpretation of WBE results (1).

To address the need for complete WBE detection workflows, we developed a multiplexed RT-qPCR assay (GoTaq® Enviro Wastewater SARS-CoV-2 System). This kit can accurately detect SARS-CoV-2 viral RNA from WW TNA samples purified using the Wizard® Enviro TNA Kit or Maxwell® RSC Enviro TNA Kit (2). The GoTaq® Enviro System pairs quantitative SARS-CoV-2 RNA detection with quantitation of PMMoV (a plant virus commonly found in feces), which is used as both a process control and normalization factor for SARS-CoV-2 WW signal. The kit utilizes SARS-CoV-2 and PMMoV RNA quantitative standards to construct RT-qPCR standard curves in the FAM and Cy5 channels, respectively. The standard curves are then used to quantify SARS-CoV-2 RNA in WW. Four different assay configurations are offered — all three N1/N2/E targets or each target (N1, N2 or E) separately to align with testing recommendations. In this application note, the Azure Cielo™ 6 Real-Time PCR System was used to amplify SARS-CoV-2 (N1) and PMMoV RNA standard curves to evaluate assay performance. These standard curves were used to quantify SARS-CoV-2 and PMMoV RNA concentrations in TNA samples purified from municipal WW collected in Dane County, WI, USA.

Methods

Total Nucleic Acid Purification from WW

1. Purify TNA from 40ml of municipal WW collected on four different dates using the Maxwell® RSC Enviro TNA Kit.

2. Follow the protocol outlined in the *Maxwell® RSC Enviro TNA Kit Technical Manual* #TM663, eluting TNA in 80µl of nuclease-free water.

Amplification and Quantitation of SARS-CoV-2 and PMMoV RNA from WW TNA Samples

1. Dilute the SARS-CoV-2 (N+E) RNA (4×10^6 copies/µl) and PMMoV RNA (4×10^6 copies/µl) 100-fold by adding 3µl of each RNA to 294µl of nuclease-free water, for a final concentration of 4×10^4 copies/µl.

2. Perform subsequent 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 (4×10^4–4×10^0 copies/µl; see Table 1)

Table 1. Standard curve dilutions for SARS-CoV-2 (N+E) and PMMoV RNAs.

Tube	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	4×10^1	2×10^2
E	4×10^0	20

3. Assemble RT-qPCR reaction mix for the desired number of reactions (n), as shown in Table 2. Add one or two reactions to this number to compensate for pipetting error.

4. Pipette 15µl of RT-qPCR reaction mix into wells of 96-well qPCR plates.

5. Add 5µl of purified WW nucleic acid, combined SARS-CoV-2 (N+E) RNA, PMMoV RNA standards, and no-template control (nuclease-free water). The final reaction volume should be 20µl.

Table 2. Reaction mix worksheet for 20µl reaction volume.

RT-qPCR Reaction Mix	Volume per Reaction (X)	Number of Reactions (n)	Number of Reactions (X × n)
GoTaq® Enviro Master Mix (2X)	10µl		
GoScript® RT (50X)	0.4µl		
Primer/Probe/IAC Mix (20X)	1µl		
Nuclease-Free Water	3.6µl		

6. Centrifuge the plate at approximately 300 × g for 1 minute to ensure all liquid is collected at the bottom of the plate wells.

7. Use the Azure Cielo™ 6 to run the RT-qPCR reaction using the cycling conditions and instrument settings shown in Tables 3 and 4.

Table 3. RT-qPCR cycling conditions. Collect data at the end of each 62° annealing/extension step.

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

Table 4. Fluorescence channels and corresponding RT-qPCR targets used in the GoTaq® Enviro WW protocol.

Fluorophores	Target
FAM	N1 (SARS-CoV-2)
HEX/JOE	Internal Amplification Control
Cy5	PMMoV

8. Analyze RT-qPCR data using the default settings in the Azure Cielo™ Manager software and detailed instructions in the *GoTaq® Enviro Wastewater SARS-CoV-2 System Technical Manual* #TM661.

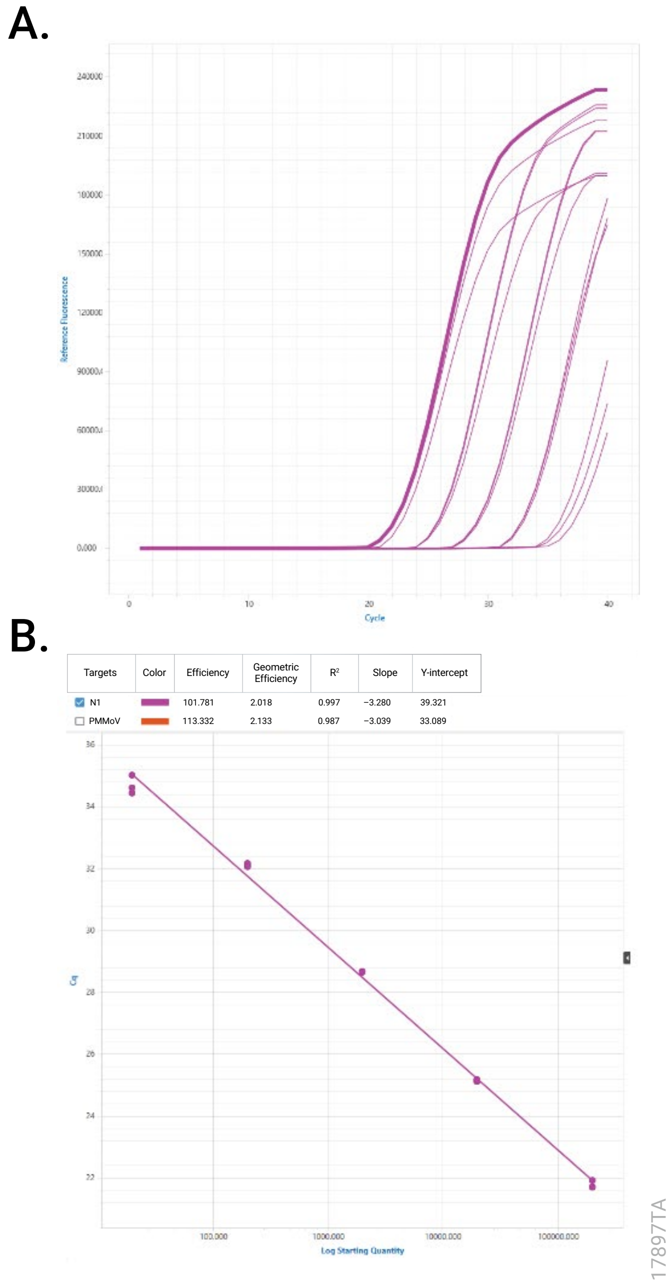

Figure 1. **SARS-CoV-2 N1 RT-qPCR standard curves**. Log-dilutions (20–200,000 copies/well) of N+E RNA quantitative standard were added to GoTaq® Enviro Wastewater SARS-CoV-2 System, N1 reaction mix and ran in an Azure Cielo™ 6 Real-Time PCR System and detected in the FAM channel. Graph traces are composed of three technical replicates at each dilution level. Standard curve derived from raw data shown in Panel A. Linearity (R^2) and efficiency measurements are listed in Panel B.

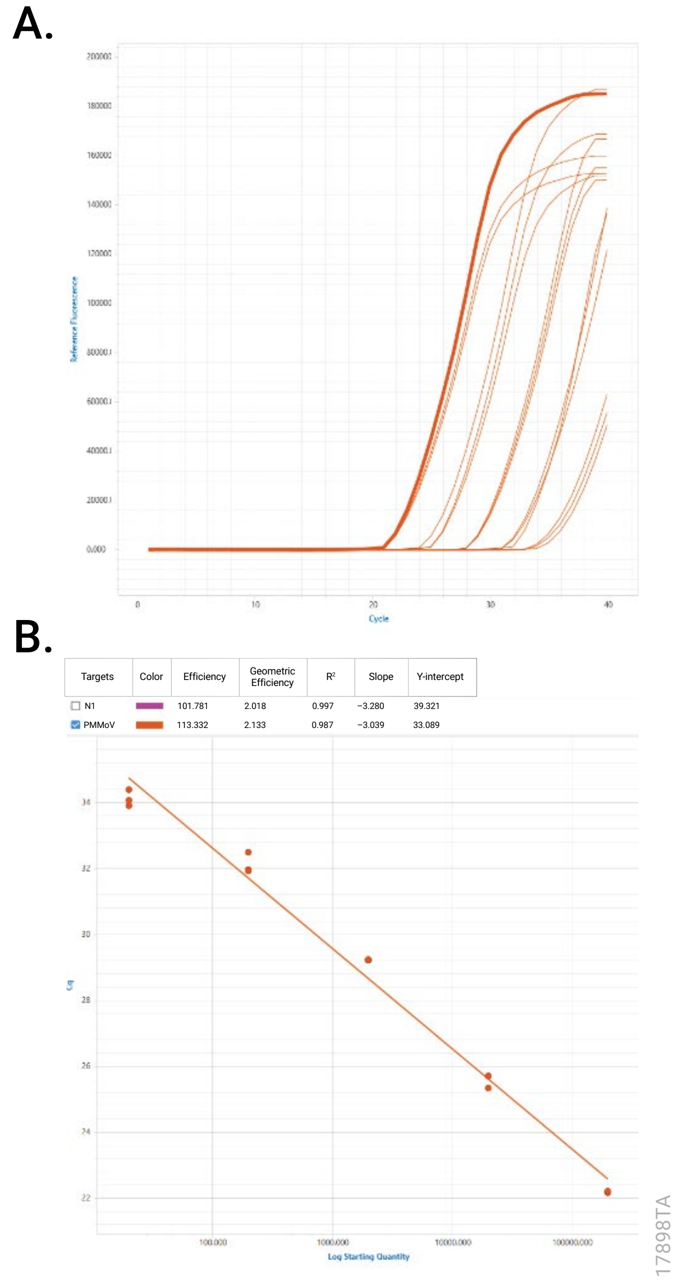

Figure 2. PMMoV RT-qPCR standard curves. Log-dilutions (20–200,000 copies/well) of PMMoV RNA quantitative standard were added to GoTaq® Enviro Wastewater SARS-CoV-2 System, N1 reaction mix and ran in an Azure Cielo™ 6 Real-Time PCR System and detected in the Cy5 channel. Graph traces are composed of three technical replicates at each dilution level. Standard curve derived from raw data shown in Panel A. Linearity (R^2) and efficiency measurements are listed in Panel B.

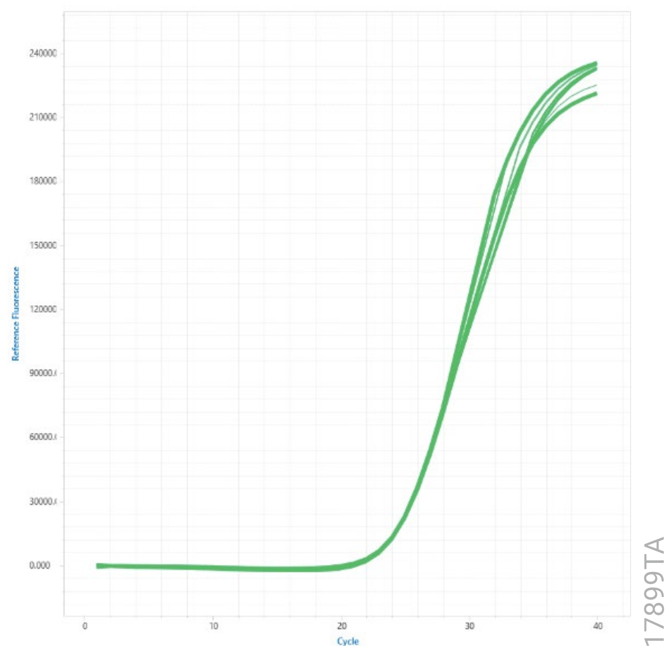

Figure 3. Internal Amplification Control (IAC) RT-qPCR inhibitor assessment. RT-qPCR reaction trace of IAC signal from both no-template control samples and municipal wastewater TNA samples. The IAC is a 285bp amplicon that is amplified from an endogenous RNA amplicon that is included in every reaction. RT-qPCR reactions were run on an Azure Cielo™ 6 Real-Time PCR System and IAC signal detected using the HEX channel.

Results

The Azure Cielo™ 6 successfully amplified SARS-CoV-2 (N+E) and PMMoV quantitative standard RNA over a 5-log range (20–200,000 copies, Figures 1A and 2A). Analysis of the standard curves (Figures 1B and 2B) demonstrate linearities (R^2>0.98) and efficiencies (86%–115%) within the acceptable range for accurate RT-qPCR detection. RT-qPCR inhibition was minimal, as no significant shift of endogenous IAC signal was observed (Figure 3). The GoTaq® Enviro Wastewater SARS-CoV-2 System, N1 also successfully amplified viral RNA from municipal wastewater using the Azure Cielo™ 6, allowing for comparison of WBE signal to clinical case counts (Figure 4).

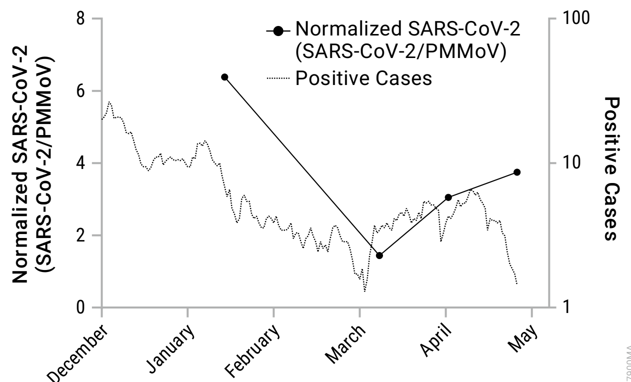

Figure 4. Comparison of the normalized SARS-CoV-2 RNA levels with the 7-day moving average of the new reported cases for a community in Dane County, WI.

Conclusion

A WBE workflow utilizing Promega purification and detection technology paired with the Azure Cielo™ 6 Real-Time PCR System provides sensitive and robust detection of SARS-CoV-2 RNA in municipal wastewater. Accurate quantitation of both SARS-CoV-2 and PMMoV-2 RNA in WW samples allows for comparisons of WBE trends in communities over time.

References

1. Kitajima, M. *et al.* (2020) SARS-CoV-2 in wastewater: State of the knowledge and research needs. *Sci Total Environ.* **739**, 139076.

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.* **795**, 148834.

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