IDEXX Water Matrix and Fecal Control Kit

English Version

Used with the IDEXX Water SARS-CoV-2 RT-PCR Test for quantification of BRSV Matrix Control and PMMoV Fecal Control in wastewater.

Table of Contents

Name and Intended Use....1

Overview of IDEXX Materials and Procedures Validated for Quantification of SARS-CoV-2 in Wastewater....1

Control Targets....2

Procedural Options....2

Verification....3

Materials and Storage....3

Reconstitution and Use of Kit Components....4

Warnings and Precautions....4

Procedure Option 1: Quantification of SARS-CoV-2 in Wastewater using BRSV as Matrix Recovery Control and PMMoV as Human Fecal Control....5

- Materials Not Provided....6
- 1.A. Sample processing....7-8
  - Addition of Matrix Recovery Control (BRSV) to each wastewater sample
  - Sample concentration
  - Internal Control
  - Nucleic-acid purification
- 1.B. RT-qPCR and Result Interpretation....9-13
  - Multiplex measurement of BRSV and PMMoV
  - Multiplex measurement of SARS-CoV-2 and Internal Control
- 1.C. Calculations....13-16
  - BRSV Matrix Recovery Efficiency
  - Quantification of SARS-CoV-2
  - Quantification of PMMoV

Procedure Option 2: Quantification of SARS-CoV-2 in Wastewater using PMMoV as endogenous Matrix Recovery Control and Human Fecal Control....17

- Materials Not Provided....18
- 2.A. Sample processing....19-20
  - Reserve unconcentrated portion of each wastewater sample
  - Sample concentration
  - Internal Control
  - Nucleic-acid purification
- 2.B. RT-qPCR and Result Interpretation....21-25
  - Measurement of PMMoV
  - Multiplex measurement of SARS-CoV-2 and Internal Control
- 2.C. Calculations....25-28
  - PMMoV Matrix Recovery Efficiency
  - Quantification of SARS-CoV-2
  - Quantification of PMMoV

English Version

IDEXX Water Matrix and Fecal Control Kit

Name and Intended Use

The IDEXX Water Matrix and Fecal Control Kit is for use with the IDEXX Water SARS-CoV-2 RT-PCR Test. This kit is used to provide information on both viral recovery during sample processing and the relative amount of human feces in the wastewater sample, thereby meeting current wastewater surveillance guidelines and enabling fecal normalization[1,2]. The materials and protocols provided in this kit have been validated for quantitative detection and measurement of Bovine Respiratory Syncytial Virus (BRSV) and Pepper Mild Mottle Virus (PMMoV) in wastewater. The kit provides primers and probes designed to detect BRSV and PMMoV in a multiplexed RT-qPCR reaction.

Overview of IDEXX Materials and Procedures Validated for Quantification of SARS-CoV-2 in Wastewater

IDEXX has validated an end-to-end protocol for detecting SARS-CoV-2 in wastewater and offers materials and/or procedures for each required step in the process. IDEXX has validated that these materials and procedures reliably quantify SARS-CoV-2 in untreated wastewater processed using PEG concentration. Validation data is available and demonstrates strong repeatability and sensitivity of the end-to-end protocol and materials. Please contact IDEXX Technical Support (contact information below) to request the validation report.

While IDEXX has validated the entire procedure, individual components can be used independently. For example, the IDEXX Water DNA/RNA Magnetic Bead Kit and SARS-CoV-2 RT-PCR Test can be used with a different concentration method than the example methods provided. Similarly, the IDEXX Water SARS-CoV-2 RT-PCR Test can be used with RNA purified using different extraction methods.

Because certain components of the test can be used independently, procedures for each component are detailed in separate documents. Laboratories that are interested in using the entire end-to-end and validated procedure for surveillance of SARS-CoV-2 in wastewater may do so by following the process outlined below, which is also summarized in a separate document, entitled “IDEXX Materials and Procedures Validated for Quantification of SARS-CoV-2 in Wastewater”[3].

1. U.S. Centers for Disease Control & Prevention. Wastewater Surveillance Testing Methods. https://www.cdc.gov/healthywater/surveillance/wastewater-surveillance/testing-methods.html
2. European Commission. Commission Recommendation on a common approach to establish a systematic surveillance of SARS-CoV-2 and its variants in wastewaters in the EU. Brussels, 17.3.2021, C(2021) 1925 Final. https://ec.europa.eu/environment/pdf/water/recommendation_covid19_monitoring_wastewaters.pdf
3. When using BRSV as the exogenous Matrix Recovery Control: idexx.com/BRSV. When using PMMoV as the endogenous Matrix Recovery Control: idexx.com/PMMoV.

Control Targets

Matrix Recovery Control: The efficiency of sample processing or concentration methods can vary, for example, due to changes in wastewater matrix properties or changes in laboratory methods. These differences can be measured using a surrogate target as a quality control to monitor the concentration process and provide information on the recovery of SARS-CoV-2 from a sample. Ideally the surrogate should have similar properties and structure to SARS-CoV-2 to provide similar behavior during processing. Two general approaches exist based on the detection of either an exogenous or endogenous surrogate viral target:

- Exogenous: A known amount of surrogate virus, which is not naturally present in the wastewater, is added to an unprocessed sample prior to concentration. This approach requires a suitable preparation of the surrogate virus for which the concentration is known or can be measured. IDEXX has tested different surrogate viruses and found that BRSV provides consistent and reliable results. Procedure Option 1 below provides methods for using a stock of BRSV virus to inoculate an unprocessed wastewater sample and measure the relative change in BRSV concentration after concentration. From these results the recovery efficiency (i.e. yield) can be calculated.

- Endogenous: An alternate approach is to measure a surrogate virus that is naturally found in wastewater. This approach does not require an exogenous virus preparation and may be preferable or required when a suitable source of BRSV is not available. PMMoV is consistently found in wastewater at high levels. Procedure Option 2 below provides methods for measuring the relative change in PMMoV, before and after wastewater concentration, to determine the relative change in viral concentration and calculate the recovery efficiency. This approach requires purification and analysis of two nucleic acid preparations for each wastewater sample.

Human Fecal Control: The amount of human fecal material in wastewater can vary due to multiple factors, including for example, dilution of wastewater due to rainfall, or changes in the number of people contributing to a wastewater system. One way to account for such differences is to measure the level of a specific target that is naturally present in human feces. IDEXX has tested different targets and found that PMMoV provides consistent and reliable results. Both procedural options below provide a method to detect and quantify PMMoV in a wastewater sample by RT-qPCR and determine its absolute concentration using a standard curve produced with an appropriate nucleic-acid reference material.

Procedural Options

The procedure for using and interpreting the above controls depends significantly on which approach to the Matrix Recovery Control is chosen. The procedures and materials required when using BRSV as the **exogenous** Matrix Recovery Control are materially different from the procedures and materials required when using PMMoV as the **endogenous** Matrix Recovery Control.

Given these differences, this document includes two complete and separate protocols, depending on which procedural option is chosen, as detailed in the table of contents above. Laboratories should **only follow one of these procedural options** and not both. Should a laboratory require assistance choosing between the two procedures, IDEXX Technical Support can provide additional information to help determine the most appropriate option for a particular laboratory.

Verification

The materials and procedures referenced in this document have been validated to provide recommended controls for wastewater surveillance. This validation was performed using general procedures for sample processing that are often used in the field of wastewater surveillance. These are described in a technical note available from IDEXX Technical Support, which also includes complete validation data.

A wide variety of accepted sample processing and extraction methods are used for wastewater surveillance, and significant variation of practices exists even within each method. It is therefore recommended that performance of this kit be verified using the specific processing method and typical wastewater samples to be examined by the laboratory, and using the recommendations and descriptions of expected results provided herein. Additional verification may be required if significant changes occur in the materials, processes, or samples used.

Furthermore, wastewater surveillance is a new field and new data and best practices frequently emerge. We encourage laboratories to contact IDEXX Technical Support for the latest information and best practices, and to understand how to best adapt the guidelines and recommendations in this document as necessary.

Materials and Storage

Identification/ general information	Cap color	Quantity	Storage		Freeze/thaw cycles
		100 tests	At receipt	After reconstitution	
Matrix and Fecal Control Mix (MFC), dried REF 61-56629-00 Contains primers and probes for the detection of BRSV and PMMoV on the FAM and HEX channels, respectively. Reconstitute to 1 mL in PCR Grade Water. Store the Matrix and Fecal Control Mix in the dark. The expiration date on the vial is valid for either the dry or reconstituted form.	Yellow	1 x 1.0 mL	–25 to 8°C	–25 to –15°C	≤6
RNA Master Mix (RNA MMx) REF 61-56622-00 Concentrated master mix that includes reverse transcriptase and hot-start polymerase. The RNA MMx is more viscous than most master mixes– see the RT-qPCR sections of the procedures below for handling recommendations. A reference dye (ROX) has been added for normalizing volume inaccuracies. Protect the RNA MMx from light.	Black	1 x 1.0 mL	–25 to –15°C (Long-term)	N/A	≤6
Positive Control, dried (PC), v2.0 REF 44-56624-00 The Positive Control provided in this kit contains synthetic nucleic acid targets for BRSV and PMMoV. This version of the PC, which can be identified by the part number and version 2 (“v2.0”) designation on the label, must be used as the PC with the Matrix and Fecal Control Kit. PC v2.0 also contains synthetic nucleic acid targets for SARS-CoV-2 N1 and the Water Internal Control, and therefore can also be used as the PC for the Water SARS-CoV-2 RT-PCR test kit. Reconstitute to 200 µL in PCR Grade Water. The expiration date on the vial is valid for either the dry or reconstituted form.	Blue	1 x 200 µL	–25 to 8°C	–25 to –15°C	≤6
PCR Grade Water REF 61-56624-00 PCR Grade Water has been qualified for reverse transcription-PCR (RT-PCR) use. It is used for the reconstitution of the Matrix and Fecal Control Mix and PC. It is also used as the PCR negative control for each test run. Do not transfer PCR Grade Water vials between PCR work areas. Separate vials of water are needed for each area to avoid contamination risk.	Clear	2 x 1.0 mL	–25 to 8°C	N/A	N/A

Note: See table at the end of the insert for a description of symbols used on the insert and labels.

Reconstitution and Use of Kit Components

Reconstitute the Matrix and Fecal Control Mix and Positive Control by pipetting PCR Grade Water to the volume indicated above and on the component label. Incubate at 18 to 26°C for at least 10 minutes; mix and microcentrifuge briefly prior to use. Once the Matrix and Fecal Control Mix and the Positive Control are reconstituted, aliquot as appropriate to enable use within the indicated freeze/thaw cycle limits, and store the solutions frozen as indicated in the Materials and Storage table. When handling frozen components, thaw at 18 to 26°C for approximately 15 to 30 minutes, mix gently and then microcentrifuge briefly to collect liquids at the bottom of the tube (2,000±1,000 RCF).

Warnings and Precautions

General

- Follow all local regulatory and safety guidelines for the handling of wastewater samples. In addition, follow local health authorities' recommended procedures for handling and processing of wastewater samples associated with SARS-CoV-2. One source of information is the U.S. Centers for Disease Control & Prevention (CDC) Interim Laboratory Biosafety Guidelines for Handling and Processing Specimens Associated with Coronavirus Disease 2019 (COVID-19). (https://www.cdc.gov/coronavirus/2019-ncov/lab/lab-biosafety-guidelines.html).
- Use personal protective equipment (PPE) consistent with current guidelines for the handling of potentially infectious samples.
- Do not eat, drink, smoke, apply cosmetics or handle contact lenses in areas where reagents and human samples are handled.
- Dispose of waste in compliance with the local, state, and federal regulations.

Extraction and PCR

- Reagents must be stored and handled as specified in these instructions for use.
- Do not use reagents past expiration date.
- Portions of the nucleic-acid extraction process and the entire RT-qPCR procedure must be performed under nuclease-free conditions.
- Wear powder-free gloves when working with the reagents and nucleic acids.
- Keep reagents and tubes capped or covered as much as possible.
- To avoid cross-contamination, use nuclease-free, aerosol-resistant pipette tips for all pipetting, and physically separate the workplaces for nucleic acid extraction/handling, PCR setup and PCR.
- Work surfaces, pipettes, and centrifuges should be cleaned and decontaminated with cleaning products such as 10% bleach, "DNAZap™" or "RNase AWAY®" to minimize risk of nucleic acid contamination. Residual bleach should be removed using 70% ethanol.

Procedure Option 1: Quantification of SARS-CoV-2 in Wastewater using BRSV as Matrix Recovery Control and PMMoV as Human Fecal Control

Refer to sections above for information on Materials and Storage, Reconstitution and Use of Kit Components, and applicable Warnings and Precautions.

Process Step	Procedure	Description	Protocol Document
1.A. Sample processing	1. Addition of Matrix Recovery Control (BRSV) to each wastewater sample	An exogenous virus is added to each wastewater sample for monitoring of subsequent processing steps.	IDEXX Water Matrix and Fecal Control Kit Product Insert (this document)
	2. Sample concentration	Concentration of viral particles and nucleic acids present in wastewater to improve limit of detection of the test.	Example Concentration Protocol for Wastewater Surveillance for SARS-CoV-2 by PEG Precipitation
	3. Internal Control	A synthetic nucleic acid is added during extraction to verify successful performance of nucleic acid purification and RT-qPCR steps.	IDEXX Water Internal Control Product Insert
	4. Nucleic-acid purification	Total nucleic acids are extracted and purified from the concentrated wastewater sample.	IDEXX Water DNA/RNA Magnetic Bead Kit Product Insert
1.B. RT-qPCR and Result Interpretation	1. Multiplex measurement of BRSV and PMMoV	Used to quantify the amount of BRSV and PMMoV RNA in the extracted sample via amplification of specific genetic sequences.	IDEXX Water Matrix and Fecal Control Kit Product Insert (this document)
	2. Multiplex measurement of SARS-CoV-2 and Internal Control	Used to quantify the amount of SARS-CoV-2 RNA in the extracted sample via amplification of specific genetic sequences. Detection of Internal Control in each sample determines result validity and possibility of PCR inhibition.	IDEXX Water SARS-CoV-2 RT-PCR Test Product Insert
1.C. Calculations	1. BRSV Matrix Recovery Efficiency	The proportion of virus recovered during sample processing, including concentration, is determined for process monitoring.	IDEXX Water Matrix and Fecal Control Kit Product Insert (this document)
	2. Quantification of SARS-CoV-2	The concentration of SARS-CoV-2 RNA is calculated using a standard curve produced from an appropriate quantitative reference material.	IDEXX Water SARS-CoV-2 RT-PCR Test Product Insert
	3. Quantification of PMMoV	The concentration of PMMoV RNA is calculated using a standard curve produced from an appropriate quantitative reference material.*	IDEXX Water Matrix and Fecal Control Kit Product Insert* (this document)

*A supplemental protocol is also available that provides information on preparing custom synthetic nucleic acid for use as PMMoV reference material.

Materials Not Provided

- **General**
  - Pipettes (5–1000 μL); dedicated pipettes for preparation of PCR Mix
  - DNase/RNase free aerosol-resistant pipette tips
  - DNase/RNase free microcentrifuge tubes
  - Microcentrifuge capable of reaching 1500 – 3000 x RCF (for low-speed "flash" spins after liquid mixing steps)
  - Vortex mixer
  - Additional materials for wastewater concentration, RNA extraction, and RT-qPCR as needed according to the respective protocols and product inserts (see table above).
  - Personal protective equipment (gloves, safety glasses, lab coat)
- **Section 1.A**
  - Stock of BRSV virus, (For example, a vaccine product containing BRSV as described in section 1.A.1, or another equivalent material. Contact IDEXX Technical Support for more information.)
  - Phosphate buffered saline (PBS)
  - DNase/RNase free molecular grade water
  - IDEXX Water DNA/RNA Magnetic Bead Kit (IDEXX part 98-0014719-00, or equivalent)
  - Optional: IDEXX Water Internal Control (IDEXX part 99-57010)
- **Section 1.B**
  - IDEXX Water SARS-CoV-2 RT-PCR Test Kit (IDEXX part 98-0014718-00)
  - PMMoV reference standard nucleic acid, (For example, a synthetic nucleic acid as described in section 1.B.1. Contact IDEXX Technical Support for more information on preparing a custom synthetic nucleic acid for use as PMMoV reference material)
  - TE pH 8.0. A low EDTA formulation containing 0.1 mM EDTA and 10 mM Tris is recommended.
  - Carrier RNA (tRNA or Poly A), (For example, Millipore Sigma part number: 10108626001 or equivalent)
  - 96- or 384-well format PCR plates and optical adhesive film/plate or suitable alternative.
  - Real-time PCR instrument: Performance of this kit has currently been validated on the Applied Biosystems QuantStudio 5 and Agilent AriaMx. This kit is also expected to be compatible with the following instruments, although these have not yet been evaluated by IDEXX: Applied Biosystems® 7500, Applied Biosystems® 7500 Fast Dx, Applied Biosystems® ViiA™ 7, Agilent Mx3000P™, Agilent Mx3005P™, Bio-Rad CFX96 Touch™, Bio Molecular Systems Mic qPCR Cycler, QIAGEN Rotor-Gene (72-Well Rotor only), Roche LightCycler® 480 or equivalent.
    Note: the Roche LC480 instrument requires additional calibration and software settings. Contact IDEXX Technical Support for guidance on use of this instrument.
  - Optional: Centrifuge with rotor and adapters for multi-well plates.

1.A.1. Sample processing: Addition of Matrix Recovery Control (BRSV) to each wastewater sample

Procedural Notes:

- The example procedure below uses a vaccine product as the source of BRSV virus. This approach provides a stable source material and simple handling steps. It is expected that other sources of BRSV may also be used. For example, the virus may be obtained from a culture collection. Modifications to the procedure may be needed depending on the source of virus used.
- This procedure has been validated for use with the example PEG concentration protocol provided by IDEXX, the IDEXX DNA/RNA Magnetic Bead kit, and the IDEXX Water SARS-CoV-2 RT-PCR Test. It is expected this procedure will be compatible with other concentration methods and kits, but this has not been evaluated by IDEXX.
- It is recommended to use consistent methods for preparation and inoculation of the control virus from run-to-run.
- The yield of BRSV may vary due to differences in the chemical, biological, and physical properties of different wastewaters. Viral recovery may be influenced by factors affecting BRSV that may be independent of the concentration method. For example, a fraction of the BRSV virus may degrade in wastewater.
- Rehydrated, undiluted BRSV vaccine stock may be stored at 4°C for at least 1 week with little or no change in detection.

Prepare virus stock:

1. Label a single-dose BRSV vaccine vial with the date of preparation.
2. Open vial using aseptic technique, and add 2 mL of PCR Grade Water to the lyophilized viral cake.
3. Incubate 5 minutes at room temperature to ensure complete dissolution, then mix gently.
4. Prepare a 10-fold dilution by combining 100 µL of resuspended BRSV stock solution with 900 µL of PBS in a microcentrifuge tube.

Inoculate wastewater samples:

5. Add 50 µL of the diluted virus stock per 40 mL of wastewater sample to be processed. For example, using the sample PEG concentration method provided by IDEXX, 43.8 µL of the diluted virus can be added to each tube containing 35 mL of wastewater, giving a total inoculation of 131.4 µL BRSV for the entire 105 mL wastewater sample.
6. Place the remainder of the undiluted BRSV stock solution at 4°C while the wastewater sample is being processed. RNA will be purified from a portion of this reserved material to provide the BRSV Recovery Control (section 1.A.4).

1.A.2. Sample processing: Sample Concentration

Concentrate the wastewater sample using a method that has been validated for SARS-CoV-2 in wastewater. IDEXX provides an example protocol for sample concentration: Example Concentration Protocol for wastewater surveillance for SARS-CoV-2 by PEG Precipitation. Other concentration methods can also be used (Please contact IDEXX Technical Support for more information).

1.A.3. Sample processing: Internal Control

It is recommended to use the IDEXX Water Internal Control during extraction (section 1.A.4) to provide verification that concentrated wastewater samples and the BRSV Recovery Control have been processed correctly and indicate the potential for PCR inhibition in extracted materials. To use the IDEXX Water Internal Control, follow the instructions in the IDEXX Water Internal Control and IDEXX Water DNA/RNA Magnetic Bead Kit product inserts.

1.A.4. Sample processing: Nucleic-acid purification

Procedural Notes

- Extraction of nucleic acids should be performed using a method validated for SARS-CoV-2 in wastewater. If using the IDEXX DNA/RNA Magnetic Bead Kit, follow the instructions in the product insert.
- The matrix recovery efficiency calculation provided below for BRSV (section 1.C.1) is based on the relative difference in results between a wastewater sample concentrate and the BRSV Recovery Control. For best results, both extractions should be performed at the same time, using the same batch of extraction reagents, and using the same volume of sample in each extraction. If using the IDEXX DNA/RNA Magnetic Bead Kit, the volume of sample processed is 200 μL.
- One nucleic acid preparation is required for each wastewater sample being analyzed; this material will contain SARS-CoV-2, BRSV, and PMMoV extracted from the sample and Internal Control (IC) from the reagents used during extraction.

Quality Controls

- Extraction Negative Control (PCR Grade Water): As described in the IDEXX Water DNA/RNA Magnetic Bead Kit and IDEXX Water SARS-CoV-2 RT-PCR product inserts, a “no template” (negative) control containing no nucleic acids should be extracted and tested with each set of wastewater samples to verify the absence of nucleic acid contamination in the extraction reagents and materials. One Extraction Negative Control must be included with each set of samples processed together using the same reagent preparation. The Extraction Negative Control should give a negative result for BRSV and PMMoV, in addition to SARS-CoV-2.
- BRSV Recovery Control: To calculate the relative recovery efficiency of BRSV, nucleic acids are extracted from a portion of the reserved, undiluted BRSV stock solution produced in section 1.A.1. This extraction provides the BRSV Recovery Control. Only one BRSV Recovery Control is required to analyze all samples inoculated with the same preparation of BRSV.
- Extraction Positive Control: A control material containing an RNA target should be extracted and tested with each batch of samples to demonstrate successful recovery of RNA during the extraction process. Detection of BRSV in the BRSV Recovery Control demonstrates recovery of single-stranded RNA from an encapsulated viral particle and this result can also be used as the Extraction Positive Control.
- Internal Control (IC): It is recommended to use the IDEXX Water Internal Control during extraction to verify that both the concentrated wastewater samples and BRSV Recovery Control have been processed correctly and indicate the potential for PCR inhibition for each sample. The Water Internal Control is detected in the multiplex SARS-CoV-2 RT-qPCR reaction (section 1.B.2), and interpretation of IC results are described in the Water SARS-CoV-2 RT-PCR Test insert. The IC is not detected in the RT-qPCR reaction provided in the Matrix and Fecal Control kit.

1.B.1. RT-qPCR and Result Interpretation: Multiplex measurement of BRSV and PMMoV

Procedural Notes:

- RT-qPCR for detection of BRSV and PMMoV is performed using the same general approach and recipe as described in the Water SARS-CoV-2 RT-PCR Test product insert.
- RT-qPCR reactions to detect BRSV and PMMoV can be incubated simultaneously in the same cycling program as reactions to detect SARS-CoV-2 and the Internal Control. For best results, first prepare RT-qPCR reactions using the Matrix and Fecal Control Mix, and then prepare additional reactions for detection of SARS-CoV-2 (section 1.B.2). This will prevent additional delays between preparation of the SARS-CoV-2 Mix and starting the thermocycling program, as required in the Water SARS-CoV-2 RT-PCR Test product insert.

Quality Controls

Controls that are provided with the Matrix and Fecal Control Kit are listed below:

- PCR Positive Control (PC): A positive template control is needed to confirm the PCR results are valid for detection of BRSV and PMMoV and should be included in each PCR run. The "v2.0" Positive Control provided in this kit contains synthetic targets for both BRSV and PMMoV and should test positive for each of these reactions on the FAM and HEX channels, respectively (see Materials and Storage).
- PCR Negative Control (PCR Grade Water): A "no template" (negative) control is needed to confirm the PCR results are valid for detection of BRSV and PMMoV and should be included in each PCR run. The negative control should test negative for BRSV on the FAM channel and negative for PMMoV on the HEX channel. PCR Grade Water provided in the kit can be used for the PCR Negative Control.

Additional controls that are recommended but not provided with the Matrix and Fecal Control Kit include:

- Extraction Negative Control (PCR Grade Water, see section 1.A.4)
- Extraction Positive Control (the BRSV Recovery Control can be used for this control; see section 1.A.4)
- BRSV Recovery Control (see section 1.A.4).
- PMMoV quantitative reference material: A suitable nucleic acid material should be prepared and tested in a dilution series to produce a standard curve (see section 1.C.3).

Note: Alternate materials may be used in place of the recommendations above. For more information, please refer to IDEXX Materials and Procedures Validated for Quantification of SARS-CoV-2 in Wastewater or contact IDEXX Technical Support.

PCR test procedure

Prepare the PCR Mix: Prepare sufficient volume to test the purified nucleic acids extracted from each wastewater sample, in addition to the control materials indicated above. For best results it is recommended to prepare a single mixture for all reactions that will be tested together.

PCR Mix	
Component	Volume per sample
Matrix and Fecal Control Mix (MFC)	10 µL
RNA Master Mix (RNA MMx)	10 µL
Total	20 µL

1. Mix the thawed RNA MMx by inversion or gentle vortex.
   Note: The RNA MMx is a viscous solution; always pipette it slowly.
2. To prepare the PCR Mix add 10 µL Matrix and Fecal Control Mix and 10 µL RNA MMx for each reaction.
   Note: first pipette the Matrix and Fecal Control Mix into the tube and then add the RNA MMx. Pipette up and down a few times to rinse the pipette tip containing MMx.
3. Gently vortex the solution to ensure the components are mixed well.
4. Load the PCR plate within 20 minutes or store at 2 to 8°C for up to 4 hours.
   Note: it is recommended to use a cold block if the plate loading time may exceed 20 minutes.

For each reaction, combine 20 µL of PCR Mix with 5 µL of each sample or control material. The final reaction volume is as follows:

PCR Reaction	
Component	Volume per sample
PCR Mix	20 µL
Sample	5 µL
Total	25 µL

5. Slowly pipette 20 µL of the PCR Mix into the required wells of the multi-well plate.
6. For each wastewater sample, add 5 µL of purified RNA to the appropriate well.
   The final reaction volume is 25 µL.
7. For each control reaction, add 5 µL to the appropriate well. The final reaction volume is 25 µL.
   Include reactions in each test run for each Quality Control material described above.
8. Cover the plate with adhesive film. Briefly spin, if necessary, to settle contents and remove air bubbles.

PCR Instrument setup

Instrument settings for detection of BRSV and PMMoV, including use of the FAM and HEX channels and thermocycling program, are identical to those used in the Water SARS-CoV-2 RT-PCR Test kit. Reporter and quencher settings for the Matrix and Fecal Control Mix are as follows:

Target	Reporter	Quencher
BRSV	FAM™	BHQ® (none)*
PMMoV	VIC™** (HEX™)	NFQ (none)*
Passive Reference	ROX™	N/A

***Note:** Some PCR systems do not require the quencher to be specified in instrument settings.

****Note:** The fluorescence characteristics of HEX and VIC are very similar and may be used interchangeably in instrument settings.

Cycling Program (used for all instruments)

Step	Temperature	Time	Cycles
Reverse transcription (RT)	50°C	15 min	1
Denaturation	95°C	1 min	1
Amplification*	95°C 60°C	15 sec 30 sec	45

***Note:** Ensure the instrument is set to record fluorescence following the 60°C amplification step.

Recommended Interpretation of Control Results and Batch Validity Criteria

The following control results for BRSV and PMMoV are expected for each batch of samples processed together during extraction and PCR. If expected batch control results are not observed, samples processed in the same batch should be retested or repeated as appropriate.

Control	BRSV		PMMoV	
	FAM Ct Value	FAM Result	HEX Ct Value	HEX Result
PCR Positive Control (v2.0)	< 38	Positive	< 38	Positive
PCR Negative Control	No signal	Negative	No Signal	Negative
Extraction Positive Control (BRSV)*	Any signal	Positive	No Signal	Negative
Extraction Negative Control	No signal	Negative	No Signal	Negative
BRSV Recovery Control**	Any signal	Positive	No Signal	Negative

***Note:** If the BRSV Recovery Control is used as the Extraction Positive Control, the Ct value obtained should be consistent with previous results obtained using the same BRSV source material and preparation method.

****Note:** For valid calculation of matrix recovery efficiency for each wastewater sample, the BRSV Recovery Control must test positive for BRSV. The Ct value observed will depend on the concentration of BRSV in the chosen source material and the methods used for sample spiking and processing, and thus a range of acceptable results are possible. For best results, the Ct value should be significantly above the limit of detection for the instrument and provide consistent results over time.

If the IDEXX Water Internal Control is used, the Extraction Positive Control, Extraction Negative Control, and BRSV Recovery Control should be analyzed for the Internal Control as described in the IDEXX Water SARS-CoV-2 RT-PCR Test product insert: all three materials should test positive for the Internal Control and there should be no significant evidence of inhibition.

Recommended Interpretation of Results for Wastewater Samples

Sample Validity: For best results, it is recommended that each wastewater sample is analyzed for validity using the IDEXX Water Internal Control as described in the IDEXX Water SARS-CoV-2 RT-PCR Test product insert (section 1.B.2). A valid Internal Control result demonstrates correct sample processing during extraction and indicates a low risk of RT-qPCR inhibition affecting BRSV and PMMoV detection. No detection, or very weak detection, of the Water Internal Control in the SARS-CoV-2 RT-PCR Test indicates a problem may have occurred during nucleic acid purification. If the Internal Control is not used, sample results are considered valid when analyzed on a plate demonstrating the expected batch control results as described above. In all cases, positive results should display a characteristic sigmoidal amplification curve.

The following table provides guidance for the interpretation of BRSV and PMMoV results for each wastewater sample, and information on expected results:

Result		Expected Result?	Recommended Interpretation
Channel	Signal Observed?		
FAM	Yes	Yes	BRSV is present and above the limit of detection, indicating successful sample processing. BRSV result may be used for calculation of recovery efficiency (section 1.C.1).[2]
	No	No[1]	BRSV recovery is below the limit of detection, indicating a problem may have occurred during sample concentration.[3]
HEX	Yes	Yes	PMMoV is present and above the limit of detection. PMMoV result may be used for quantification and fecal normalization (section 1.C.3).[4]
	No	No[1]	PMMoV recovery is below the limit of detection. If BRSV is also not detected, a problem likely occurred during sample processing. If BRSV is detected as expected, the wastewater sample may contain a very low fecal concentration, and fecal normalization may not be possible or reliable.[5]

[1] If unexpected results are observed, it is recommended to confirm the PCR result and simultaneously test for the presence of inhibitors by diluting the purified nucleic acid, for example using a five-fold dilution into PCR-grade water, and retest the dilution alongside the original undiluted sample in a new Matrix and Fecal Control reaction. A proportionally higher result from the diluted sample, after accounting for the dilution factor, indicates the presence of inhibitors. Results obtained with a diluted sample may be used for analysis if the signal is significantly above the limit of detection. If diluted nucleic acid does not show expected results, a new extraction is recommended. Alternatively if the Water Internal Control is used, sample extraction and the absence of inhibitors can be confirmed using the SARS-CoV-2 RT-qPCR Test as described in the Water SARS-CoV-2 RT-PCR Test product insert (see Sample Validity above).

[2] The concentration of BRSV used to inoculate the wastewater (section 1.A.1) should provide a strong Ct result that is significantly above the limit of detection and can be measured reliably after concentration. If high Ct values are observed close to the limit of detection, it is recommended to increase the concentration of BRSV in the wastewater to provide a stronger signal. Very high Ct values should be avoided due to the inherent variability of measurements close to the limit of detection.

[3] If sample extraction and PCR is confirmed to be correct (see note 1), it is recommended to repeat the concentration process.

[4] PMMoV is typically found at high levels in wastewater, and concentrated wastewater samples should show very strong positive results for PMMoV that are significantly above the limit of detection.

[5] A different sampling strategy or a concentration process providing an increased concentration factor may be necessary for PMMoV detection.

1.B.2. RT-qPCR and Result Interpretation: Multiplex measurement of SARS-CoV-2 and Internal Control

Follow the procedure in the Water SARS-CoV-2 RT-PCR Test product insert to prepare RT-qPCR reactions for detection and quantification of SARS-CoV-2 and the Internal Control in each wastewater sample. Ensure appropriate control materials are tested, including PCR positive and negative controls, and dilutions of a suitable SARS-CoV-2 reference material to produce a standard curve for quantification. Internal Control results obtained for each sample can be used to aid interpretation of results for BRSV and PMMoV (section 1.B.1).

1.C.1. Calculations: BRSV Matrix Recovery Efficiency

To determine the recovery of BRSV during the concentration process, the level of BRSV measured in the reserved, undiluted BRSV stock solution (section 1.A.1) is compared to the level of BRSV measured after wastewater concentration. Because the yield calculation depends only on the relative level of virus in each material, it is not necessary to determine the absolute concentration of BRSV and a standard curve is not required.

Procedural Notes:

- The calculations below assume that RNA was purified from the same volume of source material for both the wastewater sample and the reserved, undiluted BRSV stock solution. For example, when using the Water DNA/RNA Magnetic Bead Kit, RNA is purified from 200 μL of each source material. If different volumes are used the calculation below can be modified to account for the volume difference.
- When a group of wastewater samples is inoculated together using the same dilution of BRSV, recovery calculations for the entire group may be conducted using a single measurement for the undiluted BRSV stock solution, which will provide a single, common value for Ct_{stock}.

Calculations:

The yield of BRSV virus is calculated from the ratio of the observed change in BRSV concentration ($BRSV_{observed}$), which is measured experimentally, with the theoretical change in BRSV concentration ($BRSV_{theoretical}$), which is determined from the volumes used in the wastewater inoculation and concentration process.

The observed change in BRSV concentration is calculated as follows:

$$BRSV_{observed} = 2^{[Ct_{stock} - Ct_{concentrate}]}$$

where:

- Ct_{stock} = BRSV Ct value determined from the undiluted BRSV stock.
- $Ct_{concentrate}$ = BRSV Ct value determined from a concentrated wastewater sample.

The theoretical change in BRSV concentration is calculated as follows. An example calculation is given when the sample is inoculated with BRSV according to the procedure above (section 1.A.1) and concentrated according to the IDEXX-recommended PEG concentration protocol (section 1.A.2). If a different inoculation procedure or concentration method is used the calculation can be adjusted accordingly.

$$BRSV_{theoretical} = \left(\frac{BRSV\ stock\ volume}{BRSV\ dilution\ volume}\right) * \left(\frac{inoculated\ volume}{final\ concentrate\ volume}\right)$$

$$BRSV_{theoretical} = \left(\frac{0.1\ mL}{1.0\ mL}\right) * \left(\frac{0.131\ mL}{0.4\ mL}\right) = 0.0328$$

where:

- BRSV stock volume = the volume in milliliters of undiluted BRSV stock used to prepare the dilution for inoculation (0.1 mL)
- BRSV dilution volume = the final volume in milliliters of the prepared BRSV dilution (1.0 mL)
- inoculated volume = the volume in milliliters of diluted BRSV added to the wastewater (0.131 mL)
- final concentrate volume = the volume in milliliters of the final wastewater concentrate obtained after sample processing (0.4 mL)

To calculate the recovery of BRSV, the ratio of $BRSV_{observed}$ and $BRSV_{theoretical}$ is determined and expressed as a percentage:

$$BRSV\ Recovery\ \% = \left(\frac{BRSV_{observed}}{BRSV_{theoretical}}\right) \times 100$$

Expected Results

The level of BRSV recovery observed will depend on the choice of concentration method and characteristics of the wastewater sample. Repeat testing of the same sample, or of similar samples from the same source location, are expected to show similar levels of BRSV recovery. For example results obtained by IDEXX, please contact technical support for more information and to receive a copy of the IDEXX Technical Note.

It is recommended to track BRSV recovery levels over time to monitor and evaluate changes in process performance. Such changes may result, for example, from changes in wastewater composition or laboratory methods. For more information, please refer to guidance from the U.S. Centers for Disease Control & Prevention (CDC) on "Matrix recovery controls"[4].

1.C.2. Calculations: Quantification of SARS-CoV-2

Follow the procedure in the Water SARS-CoV-2 RT-PCR Test product insert to create a standard curve and determine the molecular concentration of SARS-CoV-2 in each wastewater sample.

1.C.3. Calculations: Quantification of PMMoV

Absolute quantification of PMMoV in wastewater can be performed using a standard curve produced with an appropriate nucleic-acid reference material. The standard curve is created by diluting the reference material in a series of known concentrations and then measuring a PMMoV Ct value by RT-qPCR for each concentration. The calculated relationship is used to convert a Ct value measured for an unknown sample into a concentration of RNA expressed as genome copy number per volume.

[4] https://www.cdc.gov/healthywater/surveillance/wastewater-surveillance/testing-methods.html

Procedural Notes

A variety of reference materials and dilution approaches may be used to produce a standard curve. For best results, the following is recommended:

- A standard curve should be determined within each RT-PCR run to avoid inaccuracies resulting from changes in procedures, reagents or equipment that may vary from run-to-run.
- Use consistent methods for preparation of dilutions and analysis of results.
- Verify results meet recommended quality acceptance criteria (see below).
- Different forms of the PMMoV target sequence, for example single-stranded RNA or double-stranded DNA, may be used as a reference standard. RNA is recommended to control for both the reverse transcription and polymerase amplification steps of the reaction. If DNA is used, it is recommended to use a linear molecule.
- The range of dilutions tested should encompass the levels of PMMoV observed in the wastewater samples being analyzed. PMMoV is typically found at high concentrations and it is suggested to test a dilution series starting at approximately 10^6 PMMoV copies per reaction.
- Dilutions providing PMMoV concentrations near the assay limit of detection may show inconsistent detection and may not be appropriate for inclusion in standard curve calculations.
- Serial ten-fold dilution levels are recommended. For higher accuracy, each dilution level may be tested in duplicate or triplicate.
- The following approaches are recommended to increase the stability of nucleic acid reference materials:
    - Prepare dilutions using TE pH 8.0 (10 mM Tris, 0.1 mM EDTA) containing carrier RNA (e.g. tRNA or Poly A). A low 0.1 mM concentration of EDTA is recommended. Carrier RNA can be used, for example, at a concentration of approximately 0.4 mg/mL.
    - Freeze and thaw nucleic-acid solutions as few times as possible. It is recommended to prepare single use aliquots. Store RNA solutions frozen, at or below -70°C.
    - Keep nucleic-acid solutions and associated materials cold during use.
- The presence of negative factors in wastewater samples that may affect quantification accuracy should be evaluated using either the Water Internal Control or through testing of a diluted nucleic acid sample (see section 1.B.1, Recommended Interpretation of Results for Wastewater Samples).
- Example procedures for preparing a custom, quantitative PMMoV reference material in a recommended dilution series are available; please contact IDEXX Technical Support for more information or to receive a copy of this protocol.

Expected Results

For the series of standard curve dilutions, a plot of the observed Ct value (y-axis) against the log of the known nucleic-acid copy number (x-axis) should show a linear relationship. The equation for this relationship is determined through linear regression. This equation can typically be produced by the analysis software provided with a real-time PCR system.

The quality of the resulting standard curve should be judged according to the following metrics:

- The difference in Ct value between each pair of dilutions separated by a 10-fold difference in nucleic-acid copy number should be approximately 3.32 cycles.
- The regression line should intersect the data points for all dilutions, and the calculated R^2 statistic should be >0.990. If replicates were tested for each dilution, there should be close agreement among the replicate Ct values.
- The slope of the regression line should be between -3.6 and -3.1.

Calculation of PMMoV Concentration in Wastewater

To calculate the number of copies of PMMoV virus detected in the PCR reaction, the following formula is used:

$$genome\ copy\ number\ = 10^{\frac{Ct-b}{m}}$$

where:

- Ct = threshold cycle value measured for the unknown sample
- b = y-intercept of the standard curve
- m = slope of the standard curve

To determine the concentration of PMMoV in an unknown sample, the genome copy number calculated above is expressed relative to the proportion of original wastewater sample volume that was tested in the PCR reaction. This can be determined using the following formula and the respective volumes used at each step in the sample concentration and purification process.

The example below shows the specific calculation performed when the sample has been processed using the IDEXX recommended procedure for PEG-based concentration and RNA purification using the IDEXX Water DNA/RNA Magnetic Bead Kit.

$$\begin{aligned} &virus\ genome\ copies\ per\ L \\ &= genome\ copy\ number * \left(\frac{RNA^{total}}{RNA^{PCR}}\right) * \left(\frac{concentrate^{total}}{concentrate^{extracted}}\right) * \left(\frac{1000\ \text{mL}}{wastewater}\right) \\ &= genome\ copy\ number * \left(\frac{0.1\ \text{mL}}{0.005\ \text{mL}}\right) * \left(\frac{0.4\ \text{mL}}{0.2\ \text{mL}}\right) * \left(\frac{1000\ \text{mL}}{105\ \text{mL}}\right) \\ &= genome\ copy\ number * 381 \end{aligned}$$

where:

- RNA^{total} = total volume of RNA eluted from magnetic-bead extraction (0.1 mL)
- RNA^{PCR} = volume of purified RNA tested in PCR (0.005 mL)
- $concentrate^{total}$ = total volume of wastewater concentrate (0.4 mL)
- $concentrate^{extracted}$ = volume of wastewater concentrate from which RNA was extracted (0.2 mL)
- $wastewater$ = volume of original wastewater sample processed with PEG procedure (105 mL)

Fecal Normalization of SARS-CoV-2 Results

The concentration of PMMoV in each sample may be used to normalize SARS-CoV-2 results from the same sample. For more information, please refer to guidance from the U.S. Centers for Disease Control & Prevention (CDC) on "Human fecal normalization"[5].

[5]https://www.cdc.gov/healthywater/surveillance/wastewater-surveillance/testing-methods.html

Procedure Option 2: Quantification of SARS-CoV-2 in Wastewater using PMMoV as endogenous Matrix Recovery Control and Human Fecal Control

Refer to sections above for information on Materials and Storage, Reconstitution and Use of Kit Components, and applicable Warnings and Precautions.

Process Step	Procedure	*Description*	Protocol Document
2.A. Sample processing	1. Reserve unconcentrated portion of each wastewater sample	A portion of each wastewater sample is processed without concentration to measure the initial concentration of PMMoV in each sample	IDEXX Water Matrix and Fecal Control Kit Product Insert (this document)
	2. Sample concentration	Concentration of viral particles and nucleic acids present in wastewater to improve limit of detection of the test.	Example Concentration Protocol for Wastewater Surveillance for SARS-CoV-2 by PEG Precipitation
	3. Internal Control	A synthetic nucleic acid is added during extraction to verify successful performance of nucleic acid purification and RT-qPCR steps.	IDEXX Water Internal Control Product Insert
	4. Nucleic-acid purification	Total nucleic acids are extracted and purified from both the concentrated and unconcentrated wastewater samples.	IDEXX Water DNA/RNA Magnetic Bead Kit Product Insert
2.B. RT-qPCR and Result Interpretation	1. Measurement of PMMoV	Used to quantify the amount of PMMoV RNA in the extracted sample via amplification of specific genetic sequences.	IDEXX Water Matrix and Fecal Control Kit Product Insert (this document)
	2. Multiplex measurement of SARS-CoV-2 and Internal Control	Used to quantify the amount of SARS-CoV-2 RNA in the extracted sample via amplification of specific genetic sequences. Detection of Internal Control in each sample determines result validity and possibility of PCR inhibition.	IDEXX Water SARS-CoV-2 RT-PCR Test Product Insert
2.C. Calculations	1. PMMoV Matrix Recovery Efficiency	The proportion of virus recovered during sample processing, including concentration, is determined for process monitoring.	IDEXX Water Matrix and Fecal Control Kit Product Insert (this document)
	2. Quantification of SARS-CoV-2	The concentration of SARS-CoV-2 RNA is calculated using a standard curve produced from an appropriate quantitative reference material.	IDEXX Water SARS-CoV-2 RT-PCR Test Product Insert
	3. Quantification of PMMoV	The concentration of PMMoV RNA is calculated using a standard curve produced from an appropriate quantitative reference material.*	IDEXX Water Matrix and Fecal Control Kit Product Insert* (this document)

*A supplemental protocol is also available that provides information on preparing custom synthetic nucleic acid for use as PMMoV reference material.

Materials Not Provided

General

- Pipettes (5–1000 μL); dedicated pipettes for preparation of PCR Mix
- DNase/RNase free aerosol-resistant pipette tips
- DNase/RNase free microcentrifuge tubes
- Microcentrifuge capable of reaching 1500 – 3000 x RCF (for low-speed "flash" spins after liquid mixing steps)
- Vortex mixer
- Additional materials for wastewater concentration, RNA extraction, and RT-qPCR as needed according to the respective protocols and product inserts (see table above).
- Personal protective equipment (gloves, safety glasses, lab coat)

Section 2.A

- IDEXX Water DNA/RNA Magnetic Bead Kit (IDEXX part 98-0014719-00, or equivalent)
- Optional: IDEXX Water Internal Control (IDEXX part 99-57010)
- Optional: Accuplex Verification Panel (Material Number: 0505-0168)

Section 2.B

- IDEXX Water SARS-CoV-2 RT-PCR Test Kit (IDEXX part 98-0014718-00)
- PMMoV reference standard nucleic acid, (For example, a synthetic nucleic acid as described in section 2.B.1. Contact IDEXX Technical Support for more information on preparing a custom synthetic nucleic acid for use as PMMoV reference material)
- TE pH 8.0. A low EDTA formulation containing 0.1 mM EDTA and 10 mM Tris is recommended.
- Carrier RNA (tRNA or Poly A), (For example, Millipore Sigma part number: 10108626001 or equivalent)
- 96- or 384-well format PCR plates and optical adhesive film/plate or suitable alternative.
- Real-time PCR instrument: Performance of this kit has currently been validated on the Applied Biosystems QuantStudio 5 and Agilent AriaMx. This kit is also expected to be compatible with the following instruments, although these have not yet been evaluated by IDEXX: (Applied Biosystems® 7500, Applied Biosystems® 7500 Fast Dx, Applied Biosystems® ViiA™ 7, Agilent Mx3000P™, Agilent Mx3005P™, Bio-Rad CFX96 Touch™, Bio Molecular Systems Mic qPCR Cycler, QIAGEN Rotor-Gene (72-Well Rotor only), Roche LightCycler® 480 or equivalent.
  Note: the Roche LC480 instrument requires additional calibration and software settings. Contact IDEXX Technical Support for guidance on use of this instrument.
- Optional: Centrifuge with rotor and adapters for multi-well plates.

2.A.1. Sample processing: Reserve unconcentrated portion of each wastewater sample

Procedural Notes:

- This procedure has been validated for use with the example PEG concentration protocol provided by IDEXX, the IDEXX Water DNA/RNA Magnetic Bead kit, and the IDEXX Water SARS-CoV-2 RT-PCR Test. It is expected this procedure will be compatible with other concentration methods and kits, but this has not been evaluated by IDEXX.
- The level of PMMoV detected can vary among different wastewaters.
- To obtain consistent results, it is recommended to make sure the unprocessed wastewater sample is homogenous before removing a subsample for RNA purification. This is important due to the relatively small volume of unprocessed wastewater that is examined compared to the equivalent wastewater volume tested after concentration. Consistent results can typically be obtained with raw wastewater using mixing techniques commonly employed prior to sample concentration (e.g. shaking). Depending on the size and distribution of solids in the sample, additional steps to ensure the sample is homogenous may be considered in some cases. The suitability of mixing techniques for a specific sample can be evaluated by comparing the consistency of PMMoV measured in different aliquots of the unprocessed sample.
- It is recommended to keep all wastewater samples at approximately 2 to 8°C to minimize any changes due to storage or handling prior to purification of nucleic acids (Section 2.A.4).

Procedure:

1. Thoroughly mix the wastewater sample to ensure it is homogenous (see additional notes above).
2. Remove a representative aliquot of each wastewater sample of approximately 1-5 mL to a fresh tube. Later in the procedure, RNA will be purified from these reserved aliquots to provide the PMMoV Recovery Control for each wastewater being analyzed (section 2.A.4).
3. Promptly store the reserved sample aliquots at 2 to 8 °C while another portion of each wastewater sample is concentrated (section 2.A.2).

2.A.2. Sample processing: Sample concentration

Concentrate the wastewater sample using a method that has been validated for SARS-CoV-2 in wastewater. IDEXX provides an example protocol for sample concentration: Example Concentration Protocol for wastewater surveillance for SARS-CoV-2 by PEG Precipitation. Other concentration methods can also be used (Please contact IDEXX Technical Support for more information).

2.A.3. Sample processing: Internal Control

It is recommended to use the IDEXX Water Internal Control during extraction (section 2.A.4) to provide verification that concentrated wastewater samples and PMMoV Recovery Controls have been processed correctly and indicate the potential for PCR inhibition in extracted materials. To use the IDEXX Water Internal Control, follow the instructions in the IDEXX Water Internal Control and IDEXX Water DNA/RNA Magnetic Bead Kit product inserts.

2.A.4. Sample processing: Nucleic-acid purification

Procedural Notes:

- Extraction of nucleic acids should be performed using a method validated for SARS-CoV-2 in wastewater. If using the IDEXX DNA/RNA Magnetic Bead Kit, follow the instructions in the product insert.
- The matrix recovery efficiency calculation provided below for PMMoV (section 2.C.1) is based on the relative difference in results between each wastewater sample concentrate and its respective PMMoV Recovery Control. For best results both extractions should be performed at the same time, using the same batch of extraction reagents, and using the same volume of sample in each extraction. If using the IDEXX DNA/RNA Magnetic Bead Kit, the volume of sample processed is 200 μL.
- Two nucleic acid preparations are required for each wastewater sample being analyzed. One extraction is performed using the wastewater concentrate (Section 2.A.3). A second extraction is performed using the respective, reserved sample aliquot (section 2.A.1).

Quality Controls

- Extraction Negative Control (PCR Grade Water): As described in the IDEXX Water DNA/RNA Magnetic Bead Kit and IDEXX Water SARS-CoV-2 RT-PCR product inserts, a "no template" (negative) control containing no nucleic acids should be extracted and tested with each set of wastewater samples to verify the absence of nucleic acid contamination in the extraction reagents and materials. One Extraction Negative Control must be included with each set of samples processed together using the same reagent preparation. The Extraction Negative Control should give a negative result for PMMoV, in addition to SARS-CoV-2.
- PMMoV Recovery Controls: To calculate the relative recovery efficiency of PMMoV, nucleic acids are extracted from a portion of each reserved, unprocessed wastewater sample aliquot (section 2.A.1). These extractions will provide a respective PMMoV Recovery Control for each sample.
- Extraction Positive Control: A control material containing an RNA target should be extracted and tested with each batch of samples to demonstrate successful recovery of RNA during the extraction process. Detection of PMMoV in the PMMoV Recovery Controls demonstrates recovery of single-stranded viral RNA and this result can also be used as the Extraction Positive Control. Because the concentration of PMMoV in wastewater can vary, use of PMMoV as the Extraction Positive Control may not be appropriate in all circumstances and some uncertainty will exist. It is recommended that PMMoV results for a representative wastewater sample, or group of wastewater samples, be evaluated over time to determine a range of results that are considered acceptable. Alternatively, the Accuplex Verification Panel (Material Number: 0505-0168) can be used as the Extraction Positive Control as described in the IDEXX Water DNA/RNA Magnetic Bead Kit and IDEXX Water SARS-CoV-2 RT-PCR product inserts.
- Internal Control (IC): It is recommended to use the IDEXX Water Internal Control during extraction to verify that both the concentrated wastewater samples and PMMoV Recovery Controls have been processed correctly and indicate the potential for PCR inhibition for each sample. The Water Internal Control is detected in the multiplex SARS-CoV-2 RT-qPCR reaction (section 2.B.2), and interpretation of IC results are described in the Water SARS-CoV-2 RT-PCR Test insert. The IC is not detected in the RT-qPCR reaction provided in the Matrix and Fecal Control kit.

2.B.1. RT-qPCR and Result Interpretation: Measurement of PMMoV

Procedural Notes:

- RT-qPCR for detection of PMMoV is performed using the same general approach and recipe as described in the Water SARS-CoV-2 RT-PCR Test product insert. In this procedure, only results from the HEX channel are used, and no signal should be observed for BRSV on the FAM channel.
- RT-qPCR reactions to detect PMMoV can be incubated simultaneously in the same cycling program as reactions to detect SARS-CoV-2 and the Internal Control. For best results, first prepare RT-qPCR reactions using the Matrix and Fecal Control Mix, and then prepare additional reactions for detection of SARS-CoV-2 (section 2.B.2). This will prevent additional delays between preparation of the SARS-CoV-2 Mix and starting the thermocycling program, as required in the Water SARS-CoV-2 RT-PCR Test product insert.

Quality Controls

Controls that are provided with the Matrix and Fecal Control Kit are listed below:

- PCR Positive Control (PC): A positive template control is needed to confirm the PCR results are valid for detection of PMMoV, and should be included in each PCR run. The "v2.0" Positive Control provided in this kit contains a synthetic target for PMMoV and should test positive for this reaction on the HEX channel (see Materials and Storage).
- PCR Negative Control (PCR Grade Water): A "no template" (negative) control is needed to confirm the PCR results are valid for detection of PMMoV and should be included in each PCR run. The negative control should test negative for PMMoV on the HEX channel. PCR Grade Water provided in the kit can be used for the PCR Negative Control.

Additional controls that are recommended but not provided with the Matrix and Fecal Control Kit include:

- Extraction Negative Control (PCR Grade Water, see section 2.A.4)
- Extraction Positive Control (one or more PMMoV Recovery Controls can be used for this control; see section 2.A.4)
- PMMoV Recovery Controls for each wastewater sample (see section 2.A.4)
- PMMoV quantitative reference material: A suitable nucleic acid material should be prepared and tested in a dilution series to produce a standard curve (see section 2.C.3).

Note: Alternate materials may be used in place of the recommendations above. For more information, please refer to IDEXX Materials and Procedures Validated for Quantification of SARS-CoV-2 in Wastewater or contact IDEXX Technical Support.

PCR test procedure

Prepare the PCR Mix: Prepare sufficient volume to test the purified nucleic acids extracted from each wastewater sample, in addition to the control materials indicated above. For best results it is recommended to prepare a single mixture for all reactions that will be tested together.

PCR Mix	
Component	Volume per sample
Matrix and Fecal Control Mix (MFC)	10 µL
RNA Master Mix (RNA MMx)	10 µL
Total	20 µL

1. Mix the thawed RNA MMx by inversion or gentle vortex.
   Note: The RNA MMx is a viscous solution; always pipette it slowly.
2. To prepare the PCR Mix add 10 µL Matrix and Fecal Control Mix and 10 µL RNA MMx for each reaction.
   Note: first pipette the Matrix and Fecal Control Mix into the tube and then add the RNA MMx. Pipette up and down a few times to rinse the pipette tip containing MMx.
3. Gently vortex the solution to ensure the components are mixed well.
4. Load the PCR plate within 20 minutes or store at 2 to 8°C for up to 4 hours.
   Note: it is recommended to use a cold block if the plate loading time may exceed 20 minutes.

For each reaction, combine 20 µL of PCR Mix with 5 µL of each sample or control material. The final reaction volume is as follows:

PCR Reaction	
Component	Volume per sample
PCR Mix	20 µL
Sample	5 µL
Total	25 µL

5. Slowly pipette 20 µL of the PCR Mix into the required wells of the multi-well plate.
6. For each wastewater sample, add 5 µL of purified RNA to the appropriate well. The final reaction volume is 25 µL.
7. For each control reaction, add 5 µL to the appropriate well. The final reaction volume is 25 µL. Include reactions in each test run for each Quality Control material described above.
8. Cover the plate. Briefly spin, if necessary, to settle contents and remove air bubbles.

PCR Instrument setup

Instrument settings for detection of PMMoV, including use of the HEX channel and thermocycling program, are identical to those used in the Water SARS-CoV-2 RT-PCR Test kit. Reporter and quencher settings for the Matrix and Fecal Control Mix are as follows:

Target	Reporter	Quencher
PMMoV	VIC™* (HEX™)	NFQ® (none)**
Passive Reference	ROX™	N/A

*__Note:__ The fluorescence characteristics of HEX and VIC are very similar and may be used interchangeably in instrument settings.

**__Note:__ Some RT-PCR systems do not require the quencher to be specified in instrument settings.

Cycling Program (used for all instruments)

Step	Temperature	Time	Cycles
Reverse transcription (RT)	50°C	15 min	1
Denaturation	95°C	1 min	1
Amplification*	95°C 60°C	15 sec 30 sec	45

*Ensure the instrument is set to record fluorescence following the 60°C amplification step.

Recommended Interpretation of Control Results and Batch Validity Criteria

The following control results for PMMoV are expected for each batch of samples processed together during extraction and PCR. If expected batch control results are not observed, samples processed in the same batch should be retested or repeated as appropriate.

Control	PMMoV	
	HEX Ct Value	HEX Result
PCR Positive Control (v2.0)	< 38	Positive
PCR Negative Control	No signal	Negative
Extraction Positive Control (PMMoV)*	Any signal	Positive
Extraction Negative Control	No signal	Negative
PMMoV Recovery Controls**	Any signal	Positive

*If a PMMoV Recovery Control is used as the Extraction Positive Control, the Ct value obtained for a representative PMMoV Recovery Control, or group of PMMoV Recovery Controls, may be evaluated. The Ct value observed for a PMMoV Recovery Control will vary depending on the fecal concentration in the sample and a range of results are possible. To be considered acceptable, the control result should be generally consistent with historical results obtained using the same wastewater source and preparation method and allow for typical variation in PMMoV levels (see 2.A.4 for additional information). For best results, Ct values for the PMMoV Recovery Controls should be significantly above the limit of detection for the instrument. Alternatively, if the Accuplex Verification Panel is used, the Extraction Positive Control is interpreted using detection of SARS-CoV-2 as described in the "Batch Validity Criteria" section of the Water SARS-CoV-2 RT-PCR Test product insert.

**For valid calculation of matrix recovery efficiency, the PMMoV Recovery Control for each sample must test positive for PMMoV. For best results, Ct values for the PMMoV Recovery Controls should be significantly above the limit of detection for the instrument.

If the IDEXX Water Internal Control is used during extraction, the Extraction Positive Control and Extraction Negative Control should be analyzed for the Internal Control as described in the IDEXX Water SARS-CoV-2 RT-PCR Test product insert. Both materials should test positive for the Internal Control and there should be no significant evidence of inhibition.

Recommended Interpretation of Results for Wastewater Samples

Sample Validity: For best results, it is recommended that each concentrated wastewater sample is analyzed for validity using the IDEXX Water Internal Control as described in the IDEXX Water SARS-CoV-2 RT-PCR Test product insert (section 2.B.2). A valid Internal Control result demonstrates correct sample processing during extraction and indicates a low risk of RT-qPCR inhibition affecting PMMoV detection. No detection, or very weak detection, of the Water Internal Control in the SARS-CoV-2 RT-PCR Test indicates a problem may have occurred during nucleic acid purification. If the Internal Control is not used, sample results are considered valid when analyzed on a plate demonstrating the expected batch control results as described above. In all cases, positive results should display a characteristic sigmoidal amplification curve.

The following table provides guidance for the interpretation of PMMoV results for each wastewater sample, and information on expected results:

Sample	Result		Expected Result?	Recommended Interpretation
	Channel	Signal Observed?		
PMMoV Recovery Control	HEX	Yes	Yes	PMMoV is present and above the limit of detection in the unconcentrated wastewater sample, indicating successful sample extraction and PCR.[2] PMMoV result may be used for calculation of recovery efficiency (section 2.C.1)
		No	No[1]	PMMoV is below the limit of detection in the unconcentrated sample, indicating a problem may have occurred during extraction or PCR.[3] Calculation of recovery efficiency is not possible without a Recovery Control measurement.
Concentrated Sample	HEX	Yes	Yes	PMMoV is present and above the limit of detection, indicating successful sample processing.[4] PMMoV result may be used for calculation of recovery efficiency (section 2.C.1), and quantification and fecal normalization (section 2.C.3).
		No	No[1]	PMMoV is below the limit of detection in the concentrated sample, indicating a problem may have occurred during sample concentration.[5]

1. If unexpected results are observed, it is recommended to confirm the PCR result and simultaneously test for the presence of inhibitors by diluting the purified nucleic acid, for example using a five-fold dilution into PCR-grade water, and retest the dilution alongside the original undiluted sample in a new Matrix and Fecal Control reaction. A proportionally higher result from the diluted sample, after accounting for the dilution factor, indicates the presence of inhibitors. Results obtained with a diluted sample may be used for analysis if the signal is significantly above the limit of detection. If diluted nucleic acid does not show expected results, a new extraction is recommended. Alternatively if the Water Internal Control is used, successful sample extraction and the absence of inhibitors can be confirmed using the SARS-CoV-2 RT-qPCR Test as described in the Water SARS-CoV-2 RT-PCR Test product insert (see Sample Validity above).
2. PMMoV is typically found at high levels in wastewater, and PMMoV Recovery Controls for each wastewater sample are expected to provide strong positive results for PMMoV that are significantly above the limit of detection. Result may also be used as the Extraction Positive Control if historical information is available for interpretation (section 2.A.4).
3. If sample extraction and PCR is confirmed to be correct (see note 1), the sample may contain very low fecal concentration and measurement of recovery efficiency and fecal normalization may not be possible or reliable. A different sampling strategy may be necessary for PMMoV detection.
4. Concentrated wastewater samples should show very strong positive results for PMMoV that are significantly above the limit of detection.
5. If PMMoV was detected in the corresponding Recovery Control, it is recommended to repeat the concentration process.

2.B.2. RT-qPCR and Result Interpretation: Multiplex measurement of SARS-CoV-2 and Internal Control

Follow the procedure in the Water SARS-CoV-2 RT-PCR Test product insert to prepare RT-qPCR reactions for detection and quantification of SARS-CoV-2 and the Internal Control in each wastewater sample.
Ensure appropriate control materials are tested, including positive and negative controls, and dilutions of a suitable SARS-CoV-2 reference material to produce a standard curve for quantification. Internal Control results obtained for each sample can be used to aid interpretation of results for PMMoV (section 2.B.1).

2.C.1. Calculations: PMMoV Matrix Recovery Efficiency

To determine the recovery of PMMoV during the concentration process, the level of PMMoV measured in a PMMoV Recovery Control for each sample is compared to the level of PMMoV measured after the respective wastewater sample has been concentrated. Because the yield calculation depends only on the relative level of virus in each material, it is not necessary to determine the absolute concentration of PMMoV and a standard curve is not required for this portion of the PMMoV analysis.

Procedural Notes:

The calculations below assume that RNA was purified from the same volume of source material for both the wastewater sample and the reserved wastewater aliquot. For example, when using the Water DNA/RNA Magnetic Bead Kit, RNA is purified from 200 μL of each source material. If different volumes are used the calculation can be modified to account for the volume difference.

Calculations:

The yield of PMMoV is calculated from the ratio of the observed change in PMMoV concentration ($PMMoV_{observed}$), which is measured experimentally, with the theoretical change in PMMoV concentration ($PMMoV_{theoretical}$), which is determined from the volumes in the wastewater concentration process.

The observed change in PMMoV concentration is calculated as follows:

$$PMMoV_{observed} = 2^{[Ct_{Recovery\ Control} - Ct_{concentrate}]}$$

where:

- $Ct_{Recovery\ Control}$ = PMMoV Ct value determined from the Recovery Control (unconcentrated wastewater) for the sample being analyzed.
- $Ct_{concentrate}$ = PMMoV Ct value determined from the concentrate prepared from the same wastewater sample.

The theoretical change in PMMoV concentration is calculated as follows. An example calculation is given for when the sample is concentrated according to the IDEXX-recommended PEG concentration protocol (section 2.A.2). If a different concentration method is used the calculation can be adjusted accordingly.

$$PMMoV_{theoretical} = \frac{initial\ sample\ volume}{final\ concentrate\ volume}$$

$$PMMoV_{theoretical} = \frac{105\ mL}{0.4\ mL} = 262.5$$

where:

- Initial sample volume = the volume in milliliters of wastewater sample that is concentrated (105 mL)
- final concentrate volume = the volume in milliliters of the final wastewater concentrate (0.4 mL)

To determine the recovery of PMMoV from the sample, the ratio of $PMMoV_{observed}$ and $PMMoV_{theoretical}$ is expressed as a percentage:

$$PMMoV\ Recovery\ \% = \left(\frac{PMMoV_{observed}}{PMMoV_{theoretical}}\right) \times 100$$

Expected Results

The level of PMMoV recovery observed will depend on the choice of concentration method and characteristics of the wastewater sample. Repeat testing of the same sample, or of similar samples from the same source location, is expected to show similar levels of PMMoV recovery. For example results obtained by IDEXX, please contact Technical Support for more information and to receive a copy of the IDEXX Technical Note.

It is recommended to track PMMoV recovery levels over time to monitor and evaluate changes in process performance. Such changes may result, for example, from changes in wastewater composition or laboratory methods. For more information, please refer to guidance from the U.S. Centers for Disease Control & Prevention (CDC) on "Matrix recovery controls"[6].

2.C.2. Calculations: Quantification of SARS-CoV-2

Follow the procedure in the Water SARS-CoV-2 RT-PCR Test product insert to create a standard curve and determine the molecular concentration of SARS-CoV-2 in each wastewater sample.

2.C.3. Calculations: Quantification of PMMoV

Absolute quantification of PMMoV in wastewater can be performed using a standard curve produced with an appropriate nucleic-acid reference material. The standard curve is created by diluting the reference material in a series of known concentrations and then measuring a PMMoV Ct value by RT-qPCR for each concentration. The calculated relationship is used to convert a Ct value measured for an unknown sample into a concentration of RNA expressed as genome copy number per volume.

[6] https://www.cdc.gov/healthywater/surveillance/wastewater-surveillance/testing-methods.html

Procedural Notes

A variety of reference materials and dilution approaches may be used to produce a standard curve. For best results, the following is recommended:

- A standard curve should be determined within each RT-PCR run to avoid inaccuracies resulting from changes in procedures, reagents or equipment that may vary from run-to-run.
- Use consistent methods for preparation of dilutions and analysis of results.
- Verify results meet recommended quality acceptance criteria (see below).
- Different forms of the PMMoV target sequence, for example single-stranded RNA or double-stranded DNA, may be used as a reference standard. RNA is recommended to control for both the reverse transcription and polymerase amplification steps of the reaction. If DNA is used, it is recommended to use a linear molecule.
- The range of dilutions tested should encompass the levels of PMMoV observed in the wastewater samples being analyzed. PMMoV is typically found at high concentrations and it is suggested to test a dilution series starting at approximately 10^6 PMMoV copies per reaction.
- Dilutions providing PMMoV concentrations near the assay limit of detection may show inconsistent detection and may not be appropriate for inclusion in standard curve calculations.
- Serial ten-fold dilution levels are recommended. For higher accuracy, each dilution level may be tested in duplicate or triplicate.
- The following approaches are recommended to increase the stability of nucleic acid reference materials:
    - Prepare dilutions using TE pH 8.0 (10 mM Tris, 0.1 mM EDTA) containing carrier RNA (e.g. tRNA or Poly A). A low 0.1 mM concentration of EDTA is recommended. Carrier RNA can be used, for example, at a concentration of approximately 0.4 mg/mL.
    - Freeze and thaw nucleic-acid solutions as few times as possible. It is recommended to prepare single use aliquots. Store RNA solutions frozen, at or below -70°C.
    - Keep nucleic-acid solutions and associated materials cold during use.
- The presence of negative factors in wastewater samples that may affect quantification accuracy should be evaluated using either the Water Internal Control or through testing of a diluted nucleic acid sample (see section 2.B.1, Recommended Interpretation of Results for Wastewater Samples).
- Example procedures for preparing a custom, quantitative PMMoV reference material in a recommended dilution series are available; please contact IDEXX Technical Support for more information or to receive a copy of this protocol.

Expected Results

For the series of standard curve dilutions, a plot of the observed Ct value (y-axis) against the log of the known nucleic-acid copy number (x-axis) should show a linear relationship. The equation for this relationship is determined through linear regression. This equation can typically be produced by the analysis software provided with a real-time PCR system.

The quality of the resulting standard curve should be judged according to the following metrics:

- The difference in Ct value between each pair of dilutions separated by a 10-fold difference in nucleic-acid copy number should be approximately 3.32 cycles.
- The regression line should intersect the data points for all dilutions, and the calculated R^2 statistic should be >0.990. If replicates were tested for each dilution, there should be close agreement among the replicate Ct values.
- The slope of the regression line should be between -3.6 and -3.1.

Calculation of PMMoV Concentration in Wastewater

To calculate the number of copies of PMMoV virus detected in the PCR reaction, the following formula is used:

$$genome\ copy\ number\ = 10^{\frac{Ct-b}{m}}$$

where:

- Ct = threshold cycle value measured for the unknown sample
- b = y-intercept of the standard curve
- m = slope of the standard curve

To determine the concentration of PMMoV in an unknown sample, the genome copy number calculated above is expressed relative to the proportion of original wastewater sample volume that was tested in the PCR reaction. This can be determined using the following formula and the respective volumes used at each step in the sample concentration and purification process.

The example below shows the specific calculation performed when the sample has been processed using the IDEXX recommended procedure for PEG-based concentration and RNA purification using the IDEXX Water DNA/RNA Magnetic Bead Kit.

$$virus\ genome\ copies\ per\ L$$

$$= genome\ copy\ number\ * \left(\frac{RNA^{total}}{RNA^{PCR}}\right) * \left(\frac{concentrate^{total}}{concentrate^{extracted}}\right) * \left(\frac{1000\ mL}{wastewater}\right)$$

$$= genome\ copy\ number\ * \left(\frac{0.1\ mL}{0.005\ mL}\right) * \left(\frac{0.4\ mL}{0.2\ mL}\right) * \left(\frac{1000\ mL}{105\ mL}\right)$$

$$= genome\ copy\ number\ * 381$$

where:

- RNA^{total} = total volume of RNA eluted from magnetic-bead extraction (0.1 mL)
- RNA^{PCR} = volume of purified RNA tested in PCR (0.005 mL)
- $concentrate^{total}$ = total volume of wastewater concentrate (0.4 mL)
- $concentrate^{extracted}$ = volume of wastewater concentrate from which RNA was extracted (0.2 mL)
- wastewater = volume of original wastewater sample processed with PEG procedure (105 mL)

Fecal Normalization of SARS-CoV-2 Results

The concentration of PMMoV in each sample may be used to normalize SARS-CoV-2 results from the same sample. For more information, please refer to guidance from the U.S. Centers for Disease Control & Prevention (CDC) on “Human fecal normalization”.[5]

[5] https://www.cdc.gov/healthywater/surveillance/wastewater-surveillance/testing-methods.htm

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