Example Concentration Protocols for Wastewater Surveillance for SARS-CoV-2

Used to prepare and concentrate wastewater samples prior to nucleic acid purification and quantification of SARS-CoV-2

IDEXX

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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. 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”[1].

1. When using BRSV as the exogenous Matrix Recovery Control: idexx.com/BRSV.
When using PMMoV as the endogenous Matrix Recovery Control: idexx.com/PMMoV.

General Considerations

The protocols in this document have been validated to provide repeatable quantification of SARS-CoV-2 in untreated wastewater when used in conjunction with the IDEXX Water DNA/RNA Magnetic Bead Kit and Water SARS-CoV-2 RT-PCR Test. They are provided as examples that may be modified to meet different testing objectives. Other concentration methods may also be used with the IDEXX Water DNA/RNA Magnetic Bead Kit and Water SARS-CoV-2 RT-PCR Test.

Two different options for wastewater concentration are provided, each based on a different approach:

- Option 1 uses precipitation with polyethlene glycol (PEG) and centrifugation to concentrate SARS-CoV-2 in the sample. This method does not require special consumable devices but does require a refrigerated centrifuge and rotor capable of achieving 12,000 RCF. The PEG method requires more total time than the ultrafiltration approach but provides a consistent output volume and produces a sample with low risk of inhibition at the PCR step.

- Option 2 uses a centrifugal ultrafiltration device to concentrate SARS-CoV-2. This method requires a lower maximum RCF of 4,700 and is compatible with a broader range of centrifuge equipment compared to the PEG approach. However, it does require special consumable devices that experienced supply chain issues during the COVID-19 pandemic. The method requires less total time and, in many cases, can achieve a higher throughput compared to the PEG approach. The method is more affected than the PEG approach by sample-to-sample differences and can produce different concentrate volumes for different samples.

Warnings and Precautions

- 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 specimens are handled.
- Dispose of waste in compliance with the local, state, and federal regulations.

Wastewater Sample Collection

- Wastewater samples should be collected in an appropriate container to provide sufficient sample volume for testing.
- Refer to the Water Research Foundation and the US Centers for Disease Control and Prevention for more information on collecting untreated wastewater for quantification of SARS-CoV-2:
    - Water Research Foundation: Best Practices for Collection and Storage of Wastewater Samples to Support Wastewater Surveillance of the COVID-19 Signal in Sewersheds.[2]
    - US Centers for Disease Control and Prevention: Developing a Wastewater Surveillance Sampling Strategy.[3]
- Keep samples cold but unfrozen during transportation of the sample to the laboratory for processing. Wastewater samples can be stored at 2–8°C for up to 72 hours after collection.

Pasteurization (Optional)

Procedural Notes

- The objective of this procedure is to achieve a sample temperature of 60°C for 60 minutes.
- It is recommended to verify correct exposure by monitoring the temperature of a representative sample or water blank with a thermometer placed directly in the sample inside the covered container. The monitored sample should be examined under similar conditions to those used during routine testing, including: container type, sample fill volume, initial sample temperature, and the number of samples to be treated simultaneously.
- A variety of different water bath configurations can be used. The best option will depend on the volume and number of samples processed. In general, larger water baths, active recirculation, and equipment with microprocessor thermal control will provide the most rapid and consistent performance. It is also recommended that the water bath fill level be above the liquid fill level in the sample container, and that sample containers are spaced sufficiently apart for effective water circulation.
- The recommended time and temperatures below are expected to be sufficient in most cases and were developed using up to ten 1L samples in a large recirculating water bath. A shorter time may be sufficient for smaller volumes and quantities of wastewater.

Materials

- Water bath
- Alcohol, UV light, or suitable alternative

Procedure

1. Decontaminate exterior surface of container. Surface disinfection may be performed, for example, with 70% isopropanol and/or exposure to short-wave (UV-C) light using appropriate contact or exposure times, respectively.
2. Incubate sample in a 60±1°C water bath for 1.5 hours, mixing once during incubation. The water level must cover the container sufficiently for the container and sample volume used to ensure that the target temperature is reached.
3. Cool sample to 2–8°C before proceeding with concentration. Pasteurized samples may be stored overnight at 2–8°C.

2. https://www.waterrf.org/sites/default/files/file/2020-06/COVID-19_FieldSampleCollectionForm.pdf
3. https://www.cdc.gov/healthywater/surveillance/wastewater-surveillance/developing-a-wastewater-surveillancesampling-strategy.html

Option 1: Wastewater Concentration using PEG Precipitation

Procedural Notes

- Larger or smaller volumes of wastewater can be analyzed by modifying the basic procedure with appropriate centrifugation equipment, including centrifuge, rotor, tubes or bottles, and other required accessories. Appropriate fill volumes must be used to ensure the sample remains contained during centrifugation.
- Where possible, maintain samples at cold temperatures near 4°C throughput the procedure. It is recommended to use refrigerated centrifuge equipment and keep rotors, buckets, and other processing equipment cold where practical. It is recommended to minimize handling time and avoid delays while working with tubes outside of the centrifuge to minimize warming of the sample and centrifuge rotor.
- PEG and NaCl may be electrostatically attracted to plastic centrifuge tubes. Adherence of the powders to the tube rim must be avoided to prevent interference with the cap seal.
- After PEG and NaCl dissolution, liquid adhering to the inner cap surface may unexpectedly be transferred to outside of the tube when the cap is removed. To prevent this, the following technique is recommended when opening the tubes: let tube sit undisturbed for 1 minute to allow excess fluid to drain from the cap; loosen cap 1/2 turn; pause for one second; continue to loosen cap until threads are disengaged; then carefully lift cap straight up off tube.
- Flash spins may be performed by bringing the tubes momentarily up to approximately 2,000 RCF (±1000) then quickly stopping the rotor with strong braking force.
- If using BRSV as an exogenous matrix control, it is recommended to follow the procedure described in the *IDEXX Water Matrix and Fecal Control Kit* product insert.

Materials

- Sterile 50 mL centrifuge tubes rated for a relative centrifugal force (RCF) of at least 12,000 x RCF (maximum)
- Refrigerated centrifuge, with rotors and needed accessories for centrifugation at 12,000 x RCF (maximum RCF)
- Polyethylene glycol (PEG), average molecular weight 8000, molecular biology grade or equivalent
- NaCl, molecular biology grade or equivalent
- Nuclease free water, molecular grade
- Nuclease-free, aerosol-resistant pipette tips
- Microcentrifuge tubes (DNase/RNase free)
- Pipettes, including micropipettes and serological pipettes, as needed.
- Container for liquid waste (size dependent on volume and number of samples being processed)

Procedure

1. Mix sample well, then add 35±1 mL to each of three (3) empty 50 mL centrifuge tubes.
2. Centrifuge all three tubes at 4700 RCF for 30 minutes at 4±1°C. A swinging bucket rotor is recommended to provide a stable bacterial pellet. Use little or no braking force to prevent the bacterial pellet from being disturbed.
3. Add 3.5±0.1 g PEG 8000 and 0.788±0.01 g NaCl into three (3) empty 50 mL centrifuge tubes. Note: complete this step in advance or while step 2 is in process.
4. Promptly and gently, remove centrifuge buckets from centrifuge. Carefully decant supernatant, smoothly and in one consistent motion, from each tube into a 50 mL tube containing PEG and NaCl to prevent the pellet from being disturbed.
5. Mix the tubes containing PEG and NaCl at ambient temperature until completely dissolved.

6. Open tubes carefully (see recommended technique in the Concentration Procedural Notes above) and decant liquid into a new 50 mL centrifuge tube. This step prevents leaks during centrifugation due to powder interference with the cap seal.
7. Mark a location near the bottom of the tube and orient to the outside of the rotor during centrifugation. This will aid the resuspension of viral pellets that are not clearly visible
8. Centrifuge at 12,000 RCF for 120 minutes at 4±1°C. Use little or no braking force to prevent the viral pellet from being disturbed.
9. Promptly and gently, remove rotor from centrifuge. Carefully decant and discard most of the supernatant from each tube. Decant smoothly in one consistent motion to prevent the pellet from being disturbed. It is not necessary to remove all liquid as the remainder will be removed in the next steps.
10. Centrifuge at 12,000 RCF for 5 minutes at 4±1°C. Use little or no braking force to prevent the viral pellet from being disturbed.
11. Promptly and gently, remove rotor from centrifuge. Use a pipette to carefully remove and discard the remaining supernatant from each tube. Do not contact or disturb pellet with pipette tip.
12. Use a pipette to transfer 0.4 mL nuclease-free water to one of the tubes containing a viral pellet.
13. Resuspend the viral pellet by repeatedly pipetting to rinse the inside surface of the tube around the expected location of the pellet. Rinse a wide area surrounding the expected pellet location to ensure all precipitated virus is recovered. Some of the resuspension liquid will adhere to the sides of the tube. This liquid will be recovered in the next step.
14. Flash spin the tube at 2,000 RCF (+/-1000) to collect all the liquid at the bottom of the tube.
15. Pipette up and down several times to homogenize the concentrate, then transfer the entire volume to the second tube containing a viral pellet. Repeat steps 13 and 14 to resuspend the pellet and collect all the liquid.
16. Pipette up and down several times to homogenize the concentrate, then transfer the entire volume to the third tube containing a viral pellet. Repeat steps 13 and 14 to resuspend the pellet and collect all the liquid.
17. Transfer the recovered concentrate to a RNase free microtube. The volume should be approximately 0.4 mL
18. Proceed with extraction immediately, or store concentrate overnight at –25 to –15°C.

Option 2: Wastewater Concentration Using Ultrafiltration

Procedural Notes

- Refer to the Centricon Plus-70 manufacturer's product insert for complete handling and usage instructions, and specific terminology used to refer to different parts of the device.
- The procedure below was validated using devices with a 10 kDa molecular-weight cutoff (MWCO). Other products with different MWCO were not evaluated.
- Where possible, maintain samples at cold temperatures near 4°C throughout the procedure. It is recommended to use refrigerated centrifuge equipment and keep rotors, buckets, and other processing equipment cold where practical. It is recommended to minimize handling time and avoid delays while working outside of the centrifuge to minimize warming of the sample.
- The volume of concentrate obtained may vary from sample-to-sample, or from device-to-device. To obtain accurate measurements of recovery efficiency, for example when using the *IDEXX Matrix and Fecal Control Kit*, it is recommended to measure the volume of concentrate obtained for each sample as indicated in step 8 and use the specific volume obtained for each sample when calculating recovery efficiency.
- Under ideal circumstances, the Centricon device will provide a concentrate volume of approximately 0.35 mL, however, processing of some wastewater samples will result in larger volumes, for example, up to 2 mL. If required, for example to increase assay sensitivity, the duration of centrifugation during step 5 can be increased to reduce the

concentrate volume. As a starting point, it is recommended to test an additional 5 to 15 minutes of centrifugation.

- In limited testing, optional pre-rinsing of the filter units performed in step 4.A was found to provide an approximately two-fold increase in concentration for SARS-CoV-2 and the BRSV and PMMoV matrix recovery controls. A recommended process for this optional step is therefore provided if needed, for example, to potentially increase assay sensitivity. The effectiveness of this approach on a wide variety of samples has not been evaluated.
- Centricon devices are indicated by the manufacturer to hold a maximum of 70 mL. However, it is recommended that a slightly smaller volume be used to reduce the possibility of sample overflow and cross-contamination during handling. The procedure below uses a 66 mL volume to reduce this risk.
- Sample volumes larger than 66mL can be processed by refilling the device and repeating the centrifugation step. For example, a total volume of 100 mL could be processed by successive filtration of two 50 mL volumes. If using this approach, it is recommended to keep the unconcentrated sample cold until all processing is complete, and to mix the sample thoroughly before refilling the filtration device. For best performance, it may be necessary to extend centrifugation times for the second filtration step as described above.
- If using BRSV as an exogenous matrix control, it is recommended to follow the procedure described in the *IDEXX Water Matrix and Fecal Control* Kit product insert.

Materials

- Ultrafiltration device; for example, Centricon Plus-70, 10 kDa MWCO (Millipore #UFC701008)
- Sterile 50 mL centrifuge tubes rated for at least 4,700 relative centrifugal force (RCF)
- Refrigerated centrifuge, with rotors and accessories for centrifugation at 4,700 RCF (maximum)
- Nuclease-free, aerosol-resistant pipette tips
- Microcentrifuge tubes (DNase/RNase free)
- Pipettes, including micropipettes and serological pipettes, as needed
- Nuclease free water, molecular grade (optional)
- Container for liquid waste (size dependent on volume and number of samples being processed)

Procedure

1. Mix wastewater sample well, then add 33 mL of sample into each of two (2) empty 50 mL centrifuge tubes.
2. Centrifuge all sample tubes at 4,700 RCF for 15 minutes at 4±1°C. A swinging bucket rotor is recommended to provide a stable bacterial pellet. Use little or no braking force to prevent the bacterial pellet from being disturbed.
3. Being careful not to disturb the pellet, promptly and gently remove the centrifuge tubes from the centrifuge.
4. **A.** If performing *optional* procedure to pre-rinse the Centricon device (see note above):
   i) Being careful not to disturb the pellet, decant supernatant smoothly and in one consistent motion, from each tube into a new centrifuge tube (or an equivalent clean container). Keep transferred sample cold until step 4.A.v.
   ii) Remove Cap from Centricon device and add at least 20 mL of PCR grade water to the Sample Filter Cup. Replace Cap and centrifuge at 3,500 RCF for 5 minutes. Strong braking force can be used.
   iii) Remove Sample Cup from the Filtrate Collection Cup. Pour excess water from both the Filtrate Collection Cup and the Sample Cup into a waste container.
   iv) Place Sample Cup upside-down into a Concentrate Collection Cup. Flash spin, for example at 1,000 RCF for 1 minute, to remove residual water and discard into a waste container.
   v) Place the Sample Cup back into the Filtrate Collection Cup and pour all of the

sample supernatant from step 4.A.i into the Sample Cup of one Centricon device. The total volume transferred will be approximately 66 mL.

B. If not pre-rinsing the Centricon device: Being careful not to disturb the pellet, decant supernatant smoothly and in one consistent motion, from each tube into the Sample Cup of one Centricon device. The total volume transferred will be approximately 66 mL.

5. Replace Cap on Centricon device, and centrifuge at 3,500 RCF for 30 minutes at 4±1°C.
6. Remove the Sample Cup from the Filtrate Collection Cup. Discard flow through into a waste container.
7. Carefully place the Sample Cup upside-down into a Concentration Collection Cup. Flash spin the device, for example at 1,000 RCF for 1 minute.
8. Carefully remove the Sample Cup from the Concentration Collection Cup. Using a pipette, measure and record the volume of concentrate recovered from device.
9. Transfer the recovered concentrate to a RNase free microtube.
10. Proceed with extraction immediately, or store concentrate overnight at –25°C to –15°C.

Concentration Method Verification

IDEXX recommends a verification procedure be performed to ensure the full method, including concentration, extraction, and PCR are working correctly. This procedure can be performed when first adopting the method and afterwards, when needed to meet laboratory quality standards. Contact IDEXX Technical Support for more information.

Next Steps

Extraction or nucleic acid purification can be performed with the Water DNA/RNA Magnetic Bead Kit (98-0014719-00, WCOV2MAG). Reference the Water DNA/RNA Magnetic Be information and detailed instructions for use. Other extraction or lysis methods may also be used once validated by the laboratory.

For Technical Support, please call:

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