V.3 - Direct wastewater RNA capture and purification via the "Sewage, Salt, Silica and SARS-CoV-2 (4S)" method V.3

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Coronavirus Method Development Community

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Version created by Oscar N Whitney
ABSTRACT

This protocol describes the procedure of the “4S” (Sewage, Salt, Silica and SARS-CoV-2) method for SARS-CoV-2 RNA extraction from wastewater. Offering a highly efficient, modular and economical alternative to existing wastewater RNA purification methods, this procedure lowers the barrier to entry for SARS-CoV-2 wastewater-based epidemiology. This procedure is intended to be carried out in a BSL2+ laboratory space, with precautions when handling raw wastewater samples.

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IMAGE ATTRIBUTION

Figures created with BioRender.com

GUIDELINES

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MATERIALS

- Tris Contributed by users
- EDTA Contributed by users
- Sodium Chloride Contributed by users Catalog #PubChem CID: 5234
- Microcentrifuge Contributed by users
- Ethanol Contributed by users
- Zymo III-P column Zymo Research Catalog #C1040-5
- EZ-Vac Vacuum Manifold Zymo Research Catalog #S7000
- Durapore® Membrane Filter 5.0 µm Millipore Sigma Catalog #SVLP04700
- Magnetic Funnel 300mL 47mm Pall Catalog #4242
- Bovilis Coronavirus Calf Vaccine Merck Animal Health Catalog #16445
- Swinnex Filter Holder Millipore Sigma Catalog #SX0004700
- ZymoPURE Elution Buffer Zymo Research Catalog #D4200-7-30

STEP MATERIALS

- ZymoPURE Elution Buffer Zymo Research Catalog #D4200-7-30
- TE buffer Contributed by users
- Bovilis Coronavirus Calf Vaccine Merck Animal Health Catalog #16445
- Durapore® Membrane Filter 5.0 µm Millipore Sigma Catalog #SVLP04700
- Swinnex Filter Holder Millipore Sigma Catalog #SX0004700
- Magnetic Funnel 300mL 47mm Pall Catalog #4242
- EZ-Vac Vacuum Manifold Zymo Research Catalog #S7000
- Zymo III-P column Zymo Research Catalog #C1040-5
PROTOCOL MATERIALS

- **Bovilis Coronavirus Calf Vaccine** Merck Animal Health Catalog #16445
  - Step 3
- **Durapore® Membrane Filter 5.0 µm** Merck MilliporeSigma (Sigma-Aldrich) Catalog #SVLP04700
  - Step 5
- **Swinnex Filter Holder** Merck MilliporeSigma (Sigma-Aldrich) Catalog #SX0004700
  - Step 5
- **Magnetic Funnel 300mL 47mm** Pall Catalog #4242
  - Step 5
- **EZ-Vac Vacuum Manifold** Zymo Research Catalog #S7000
  - Step 7
- **Zymo III-P column** Zymo Research Catalog #C1040-5
  - Step 7
- **ZymoPURE Elution Buffer** Zymo Research Catalog #D4200-7-30
  - Step 7
- **TE buffer** Contributed by users
  - Step 12

SAFETY WARNINGS

- Wastewater is intrinsically hazardous, so we advise handling wastewater samples in a biosafety cabinet in a BSL2+ laboratory space.
We developed this procedure to provide a highly efficient, economical, and rapid method for extraction of SARS-CoV-2 RNA from wastewater. Using this procedure at the University of California Berkeley, we have captured and quantified SARS-CoV-2 in the raw wastewater influent of six San Francisco Bay Area treatment plants, as well as at dozens of locations within Bay Area sewersheds. We have also used this method to detect Bacteroides, 18S rRNA, and pepper mild mottle virus (PMMoV) RNA in wastewater, which could serve as indicators of wastewater fecal concentration with which to normalize SARS-CoV-2 concentrations.

This procedure relies on vacuum column processing, which can be performed with a vacuum manifold and vacuum pump or central vacuum line. In our laboratory, this procedure yields concentrated and purified wastewater RNA in less than 3 hours.

In our laboratory, this purification method enables the detection of SARS-CoV-2 N and E gene RNA as well as PMMoV RNA via RT-qPCR probe-mediated detection. Depending on sample origin, we are able to recover an average of 35 ng RNA/mL of purified wastewater sample (min = 9.33 ng/mL, max = 95 ng/mL).

### Preparing RNA wash buffers

1. Prepare 1 L each of two wash buffers - Wash buffer #1 (4S-WB1) and #2 (4S-WB2), for later use during cleanup of RNA bound to silica columns.

   1.1 4S-WB1 composition:

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Original molarity/%</th>
<th>Final molarity/%</th>
<th>Volume per liter of buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>5 M</td>
<td>1.5 M</td>
<td>300 mL</td>
</tr>
<tr>
<td>Ethanol</td>
<td>100%</td>
<td>20%</td>
<td>200 mL</td>
</tr>
<tr>
<td>TRIS pH 7.2</td>
<td>1 M</td>
<td>10 mM</td>
<td>10 mL</td>
</tr>
<tr>
<td>Pure water (MilliQ or distilled)</td>
<td>NA</td>
<td>NA</td>
<td>490 mL</td>
</tr>
</tbody>
</table>

   Add 490 mL water to sterile bottle
   Add 300 mL of 5 Molarity (M) NaCl
   Add 200 mL of 100 % volume Ethanol
   Add 10 mL of 1 Molarity (M) TRIS

   Agitate to fully mix buffer solution

   1.2 4S-WB2 composition:
<table>
<thead>
<tr>
<th>Reagent</th>
<th>Original molarity/%</th>
<th>Final molarity/%</th>
<th>Volume per liter of buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>5 M</td>
<td>100 mM</td>
<td>20mL</td>
</tr>
<tr>
<td>Ethanol</td>
<td>100%</td>
<td>80%</td>
<td>800mL</td>
</tr>
<tr>
<td>TRIS pH 7.2</td>
<td>1 M</td>
<td>10 mM</td>
<td>10mL</td>
</tr>
<tr>
<td>Pure water (MilliQ or distilled)</td>
<td>NA</td>
<td>NA</td>
<td>170mL</td>
</tr>
</tbody>
</table>

Add **170 mL** water to sterile bottle
Add **20 mL** of **5 Molarity (M)** NaCl
Add **800 mL** of **100 % volume** Ethanol
Add **10 mL** of **1 Molarity (M)** TRIS
Agitate to fully mix buffer solution

1.3 Prepare two tubes containing lysis salts (one for the sample and one as a matched negative control) by adding **9.5 g** of sodium chloride to each sterile 50mL tubes.
Make **7.2 M** TE buffer (**1 Molarity (M)** TRIS, **100 millimolar (mM)** EDTA).
Add **400 µL** of TE buffer to each 50mL tube with salt. Gently shake.

Sample preparation, RNA preservation and particle lysis

2 Obtain a **40 mL** wastewater sample and pour directly into the pre-salted tube. Agitate sample until all NaCl dissolves in the wastewater. Maintain at **4 °C** during transport to the lab.
Raw wastewater containing NaCl, TRIS & EDTA. With the salt and the wastewater, the total volume in the tube will be about 44mL.
Note

Here, NaCl lyses lipid-protein envelopes, denatures proteins and disrupts RNA-protein interactions. EDTA inhibits the enzymatic degradation of RNA by RNases present in wastewater and TRIS provides optimal buffering conditions for nucleic acids.

2.1 Obtain 40 mL sterile 1x PBS and pour directly into the second pre-salted tube. Agitate sample until all NaCl dissolves in the PBS. Maintain at 4 °C during transport to the lab. Perform same steps with the PBS-only negative control as described below for the wastewater sample.

3 Resuspend dry bovine coronavirus stock (Bovilis Coronavirus Calf Vaccine) in 2 mL of PBS. Dilute this resuspended stock into PBS at a dilution of 1:10 (100 µL of stock into 900 µL PBS). Spike 50uL of diluted bCoV into the wastewater sample as a recovery efficiency control. Agitate sample to fully mix bCoV or other spiked-in controls with the wastewater sample.

Note

Other recovery controls can be used instead of bCoV, such as Phi6 bacteriophage. In addition, purified RNAs can be used to quantify the extraction efficiency of "free RNA".

3.1 Heat 200 µL of remaining bCoV spike-in aliquot at 75 °C for 00:30:00. Freeze for later quantification via RT-qPCR. This enables more accurate assessment of the bCoV spike-in concentration.

4 (OPTIONAL) Heat inactivate sample at 70 °C for 00:45:00. Our unpublished analyses have shown that this step may slightly improve some RNA species' enrichment and detection.
Filter the sample through a 5-um PVDF filter via syringe filtration or funnel top vacuum into a sterile 100mL tube.
Syringe filter setup: Wastewater is filtered through a 47-mm reusable filter membrane holder.
Wastewater filtering through a 5-um PVDF filter in a Pall filter holder.

Direct RNA extraction (RNA Binding, Washing, Eluting)

6. Add 40 mL of 70% volume ethanol to the 40 mL of filtrate.

Filtered sample before ethanol addition. Filtrate should be semi-clear.
6.1 Agitate sample to mix ethanol and wastewater lysate.

7 Attach Zymo III-P (or other) silica spin column to a vacuum manifold. Agitate the wastewater and lysate by inverting the tube five times, then pour into the spin column and vacuum the full 80 mL of wastewater lysate & ethanol through the spin column.

**Note**

Commercial silica spin columns vary in their silica membrane packing tightness, changing the flow rate of lysed wastewater. We advise the use of the Zymo III-P column to avoid column clogging issues, but columns such as the Qiagen RNeasy, QIAamp Mini Spin and Zymogen II-CR can act as substitutes, depending on vacuum strength and sample particulate content. Large-format "maxiprep" style columns are also able to purify wastewater RNA, but require a large volume RNA elution up to 20mL (Step 13) and a downstream precipitation-concentration step (Isopropanol precipitation, see companion protocol, Step 12).
Passing lysed & filtered samples through Zymo III-P columns for direct RNA capture.

8 Vacuum 5 mL wash buffer #1 (4S-WB1) through the silica spin column.

9 Vacuum 10 mL wash buffer #2 (4S-WB2) through the silica spin column.

**RNA elution**

10 Detach silica spin column from vacuum manifold, remove any attached reservoirs/funnels and place column into a 1.5-mL centrifugation-compatible flowthrough collection tube.

11 Centrifuge silica spin column in tube at 10000 x g, 4°C, 00:02:00 to remove any residual 4S-WB2 present in the column.

11.1 Discard the collection tube and place silica column into a new 1.5-mL centrifugation-compatible flowthrough collection tube.

12 Pre-warm 200 µL of ZymoPURE elution buffer or 200 µL TE buffer per RNA sample to 50 °C in a heat block, waterbath or incubator.
12.1 Add 200 µL of pre-warmed elution buffer to each silica spin column. Incubate the elution buffer and column + collection tube assembly in a heat block or incubator warmed to 50 °C for 00:10:00.

12.2 Spin at 10000 x g, 37°C, 00:05:00 to elute RNA from the column. The flowthrough present in the collection tube contains the purified RNA.

Storage

13 The eluted RNA is now ready for downstream analysis. Store RNA at 4 °C for same-day use or freeze at -80 °C for later use and storage.