ABSTRACT

The following protocol describes the "4S" (Sewage, Salt, Silica and SARS-CoV-2) workflow applied to using dry silica powder as an RNA-binding matrix instead of silica spin columns. This offers an even more economical alternative, requiring only centrifugation to extract RNA from wastewater. This procedure is intended to be carried out in a BSL2+ laboratory space, with precautions when handling raw wastewater samples.

DOI

dx.doi.org/10.17504/protocols.io.biwfkfbn

PROTOCOL CITATION


KEYWORDS

SARS-CoV-2, COVID19, Wastewater-based epidemiology, Direct capture, RNA extraction
MATERIALS

**MATERIALS**

- Tris Contributed by users
- EDTA Contributed by users
- Sodium Chloride Contributed by users Catalog #PubChem CID: 5234
- Sodium acetate Merck Millipore Catalog #1.06268.1000 Step 12
- Centrifuge Contributed by users
- TE buffer Thermo Fisher Scientific
- Ethanol Contributed by users
- Isopropanol Merck Millipore Catalog #109634 Step 12
- Silicon dioxide ~99% 0.5-10 µm (approx. 80% between 1-5 µm) Millipore Sigma Catalog #SIGMA S5631 Step 1.3

**STEP MATERIALS**

- Durapore® Membrane Filter 5.0 µm Millipore Sigma Catalog #SVLP04700 Step 6
- Swinnex Filter Holder Millipore Sigma Catalog #SX0004700 Step 6
- Magnetic Funnel 300mL 47mm Pall Catalog #4242 Step 6
- Silicon dioxide ~99% 0.5-10 µm (approx. 80% between 1-5 µm) Millipore Sigma Catalog #SIGMA S5631 Step 1.3
- Bovilis Coronavirus Calf Vaccine Merck Animal Health Catalog #16445 Step 3
- Isopropanol Merck Millipore Catalog #109634 Step 12
- Sodium acetate Merck Millipore Catalog #1.06268.1000 Step 12

**SAFETY WARNINGS**

Wastewater is intrinsically hazardous, so we advise handling wastewater samples in a biosafety cabinet.

**BEFORE STARTING**

We developed this alternate procedure to allow the purification of wastewater RNA without access to a vacuum source or silica spin column. This companion method to "4S" enables highly efficient and extremely economical extraction of SARS-CoV-2 RNA from wastewater, but is more time and labor consuming. Using this procedure at the University of California Berkeley, we have captured and quantified SARS-CoV-2 and pepper mild mottle virus (PMMoV) present in a variety of San Francisco Bay Area raw wastewater influent samples and samples collected upstream of wastewater treatment plants. Results may vary depending on wastewater sample type and laboratory setting.

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**Citation:** Oscar N Whitney, Basem Al-Shayeb, Alex Crites-Cristoph, Mira Chaplin, Vinson Fan, Hannah Greenwald, Adrian Hinkle, Rose Kantor, Lauren Kennedy, Anna Maurer, Robert Tjian, Kara L. Nelson, UC Berkeley Wastewater-based epidemiology consortium (07/27/2020). Direct wastewater RNA extraction via the "Milk of Silica (MoS)" method - A companion method to "Sewage, Salt, Silica and SARS-CoV-2 (4S)", [https://dx.doi.org/10.17504/protocols.io.biwfkfbn](https://dx.doi.org/10.17504/protocols.io.biwfkfbn)

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This procedure relies on centrifugation. In our laboratory setting, this procedure yields pure Wastewater RNA in approximately 6 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 25.7 ng RNA/mL of purified wastewater sample (min = 13.1 ng/mL, max = 58.2 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 particles.

Prepare a "Milk of Silica" suspension of dry silica.

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) pH 7.2] 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) pH 7.2] TRIS
Agitate to fully mix buffer solution

1.3 Prepare a "Milk of Silica" silica suspension by resuspending 5 grams of silicon dioxide powder in 5 mL of pure water. Scale "Milk of Silica" suspension volume by number of wastewater samples (5mL/sample).

Silicon dioxide ~99% 0.5-10 µm (approx. 80% between 1-5 µm) Millipore

Sigma Catalog #SIGMA S5631
Sample preparation, RNA preservation and particle lysis

2. Obtain a 40 mL wastewater sample in a sterile sample collection tube. Maintain at 4 °C during transport to the lab.

Sodium chloride and TE buffer (Go to step 4) can be added to sample immediately after collection. Our unpublished analysis demonstrates that Sodium chloride & TE buffer preserve RNA present in wastewater.

3. Spike a known volume and titer of bovine coronavirus (bCoV) into the wastewater sample as a recovery efficiency control. Agitate sample to fully mix bCoV with the wastewater sample. Agitate sample to fully mix bCoV or other spiked-in controls with the wastewater sample.

Other recovery controls can be used instead of bCoV. Some candidates include Phi6 bacteriophage and coronavirus OC43. In addition, purified RNAs can be used to quantify the extraction efficiency of 'free RNA'.

Citation: Oscar N Whitney, Basem Al-Shayeb, Alex Crits-Cristoph, Mira Chaplin, Vinson Fan, Hannah Greenwald, Adrian Hinkle, Rose Kantor, Lauren Kennedy, Anna Maurer, Robert Tjian, Kara L. Nelson, UC Berkeley Wastewater-based epidemiology consortium (07/27/2020). Direct wastewater RNA extraction via the "Milk of Silica (MoS)" method - A companion method to "Sewage, Salt, Silica and SARS-CoV-2 (4S)". https://dx.doi.org/10.17504/protocols.io.biwfkfbn

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Add 9.5 g of sodium chloride to 40 mL wastewater sample.
Make pH 7.2 TE buffer (1 Molarity (M) TRIS, 100 Milimolar (mM) EDTA).
Add 400 µl of TE buffer to 40 mL wastewater sample.

Here, NaCl lysed 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.

4.1 Agitate sample until all NaCl dissolves in the wastewater. Vortex or shake sample for 00:00:30 to promote lysis.
5 (OPTIONAL) Heat inactivate sample at \( \textbf{70} \, ^\circ \text{C} \) for \( \textbf{00:30:00} \). Our unpublished analyses have shown that this step will not affect SARS-CoV-2 RNA enrichment and detection.

6 Filter the sample through a 5-um PVDF filter via syringe filtration or funnel top vacuum.

Syringe filter setup: Wastewater is filtered through a 47-mm reusable filter membrane holder.
Direct RNA extraction via addition of silica slurry (RNA Binding, Washing, Eluting)

7. Aliquot 40 mL filtrate into two 20 mL aliquots. Add 20 mL of 70% volume ethanol to each 20 mL sample filtrate aliquot.

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Filtered sample before ethanol addition. Filtrate should be semi-clear.

7.1 Agitate sample to mix ethanol and wastewater lysate.

8 Resuspend "Milk of Silica" suspension by inverting the slurry 10 times. Add 2.5 mL of the 1g/mL "Milk of Silica" slurry to each aliquot containing 40 mL of wastewater lysate with ethanol.

8.1 Invert tube with lysate & silica 10 times to mix. Incubate mixture at room temperature for 00:10:00.

In this step, the silica particles bind RNA present in the processed wastewater sample.
8.2 Centrifuge tubes containing silica & bound RNA at 4000 x g, 4°C, 00:05:00. The silica will form a firm pellet at the bottom of the tube. Remove the tubes from the centrifuge and decant and discard the supernatant.

Here, the silica & bound RNA is precipitated to the bottom of the tube, separating it from the wastewater matrix.

9 Add 20 mL of 4S Wash buffer #1 (4S-WB1) to each silica pellet. Agitate or vortex tubes until silica is resuspended and appears milky.

9.1 Merge the two aliquot containing 20 mL 4S-WB1 and silica suspension by pouring the silica suspension from one tube into the other.

9.2 Centrifuge tubes containing silica, bound RNA and 4S-WB1 at 4000 x g, 4°C, 00:05:00. The silica will form a firm pellet at the bottom of the tube. Remove the tubes from the centrifuge and decant and discard the supernatant.

10 Add 40 mL of 4S Wash buffer #2 (4S-WB2) to the silica pellet. Agitate or vortex tubes until silica is resuspended and appears milky.

10.1 Centrifuge tubes containing silica, bound RNA and 4S-WB2 at 4000 x g, 4°C, 00:05:00. The silica will form a firm pellet at the bottom of the tube. Remove the tubes from the centrifuge and decant and discard the supernatant.

10.2 Add 40 mL of 4S Wash buffer #2 (4S-WB2) to the silica pellet. Agitate or vortex tubes until silica is resuspended and appears milky.

10.3 Centrifuge tubes containing silica, bound RNA and 4S-WB2 at 4000 x g, 4°C, 00:05:00. The silica will form a firm pellet at the bottom of the tube. Remove the tubes from the centrifuge and decant and discard the supernatant.

10.4 Vacuum aspirate any excess 4S-WB2 or allow tubes to incubate at room temperature for 00:10:00 to evaporate excess 4S-WB2.

11 Resuspend silica & RNA pellet in 20 mL of pure water (DNase and RNase-free) pre-warmed to 37 °C. Vortex, agitate or pipette silica until fully resuspended. Allow silica & water suspension to incubate for 00:10:00.
Here, water elutes RNA from the silica particulate. The sample RNA is now present in the aqueous phase.

11.1 Centrifuge tubes containing silica & eluted RNA \(\times 4000\ x\ g, \ 37^\circ C, \ 00:05:00\). The silica will form a firm pellet at the bottom of the tube and the RNA will be present in the aqueous phase. Pipette or decant the aqueous supernatant into a sterile conical bottom centrifugation-compatible (4000xg) tube for further concentration.

This step separates the free, eluted RNA from the silica binding matrix, allowing downstream RNA concentration.

Concentration of eluted RNA (Isopropanol precipitation)

12 Add 20 mL of [\(100\ \%\) volume] Isopropanol and 4 mL of [3 Molarity (M) \(\text{pH}\ 5.2\)] sodium acetate to the eluted RNA. Invert tube 10 times to mix solution and incubate mixture at room temperature for 00:10:00.

- Isopropanol Merck
  - Millipore Catalog #109634
- Sodium acetate Merck
  - Millipore Catalog #1.06268.1000

Isopropanol and sodium acetate alongside centrifugation precipitate the eluted RNA from the 20mL aqueous matrix.

12.1 Centrifuge sample at \(\times 4000\ x\ g, \ 4^\circ C, \ 01:00:00\). A semi-translucent nucleic acid pellet will form at the bottom of the conical tube.

Depending on sample type and source, the pellet may be brown or grey, as shown in the image in step 12.2

12.2 Carefully decant and discard the excess isopropanol & water from the nucleic acid pellet.
Side view of pellet after removal of isopropanol, water and sodium acetate mixture
Wash pellet with ethanol by adding **40 mL 75 % volume** Ethanol to the nucleic acid pellet containing tube. Invert, vortex or agitate until the pellet loosens from the bottom of the tube and fully contacts the ethanol. Depending on sample type and origin, the pellet may fracture or remain intact during ethanol washing.

13.1 Re-precipitate nucleic acid pellet by centrifuging the sample at **4000 x g, 4°C, 00:30:00**. After centrifugation, the nucleic acid pellet becomes visible at the bottom of the conical tube.

13.2 Carefully decant and discard as much supernatant **75 % volume** Ethanol as possible from the nucleic acid pellet. Add **1 mL 70 % volume** Ethanol to the nucleic acid pellet.

13.3 Using a pipette, resuspend the pellet in the **1 mL 75 % volume** Ethanol. Transfer the pellet and ethanol mixture to a 1.5mL microcentrifuge tube.

To facilitate pellet transfer, use sterile scissors to cut the opening of 1mL pipette tips, allowing easier aspiration and transfer of the nucleic acid pellet.

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After pellet transfer, centrifuge the microcentrifuge tube at 5000 rpm, 4°C, 00:05:00. The nucleic acid pellet will form at the bottom and side of the microcentrifuge tube.

14.1 Carefully pipette-aspirate the supernatant [70 % volume] Ethanol from the nucleic acid pellet. Use pipette tips with a small opening to remove excess ethanol without aspirating the pellet.

14.2 Open the lid of the microcentrifuge tube and incubate the tube at 37 °C for 00:10:00. This allows excess ethanol to evaporate, yielding ethanol-free RNA.

14.3 Resuspend the nucleic acid pellet in 200 µl of pure water or TE buffer. Vortex or pipette-mix the resuspended RNA to facilitate resuspension. It is possible for residual silica particles to remain in the final eluted RNA. In this case, briefly centrifuge the resuspended RNA and transfer the silica-free supernatant to a new sterile 1.5mL microfuge tube.

Storage

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.

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