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

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1 Works for me dx.doi.org/10.17504/protocols.io.biwekfbe



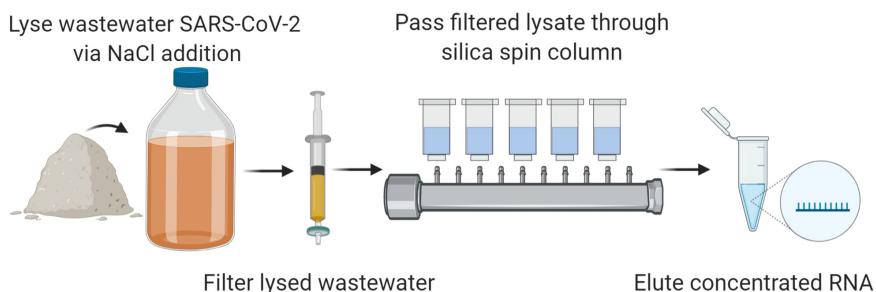
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ABSTRACT

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.



DOI

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KEYWORDS

SARS-CoV-2, Wastewater-based epidemiology, Direct capture, RNA extraction, COVID-19

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GUIDELINES

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MATERIALS

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

STEPS MATERIALS

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

SAFETY WARNINGS

Wastewater is intrinsically hazardous, so we advise handling wastewater samples in a biosafety cabinet in a BSL2+ laboratory space.

BEFORE STARTING

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 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.

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:

| Reagent | Original molarity/% | Final molarity/% | Volume per liter of buffer |
|----------------------------------|---------------------|------------------|----------------------------|
| NaCl | 5 M | 1.5 M | 300 mL |
| Ethanol | 100% | 20% | 200 mL |
| TRIS pH 7.2 | 1 M | 10 mM | 10 mL |
| Pure water (MilliQ or distilled) | NA | NA | 490 mL |

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:

| Reagent | Original molarity/% | Final molarity/% | Volume per liter of buffer |
|----------------------------------|---------------------|------------------|----------------------------|
| NaCl | 5 M | 100 mM | 20mL |
| Ethanol | 100% | 80% | 800mL |
| TRIS pH 7.2 | 1 M | 10 mM | 10mL |
| Pure water (MilliQ or distilled) | NA | NA | 170mL |

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

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 or other spiked-in controls with the wastewater sample.



Bovilis Coronavirus Calf Vaccine

by Merck Animal Health

Catalog #: 16445



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".

- 4 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.



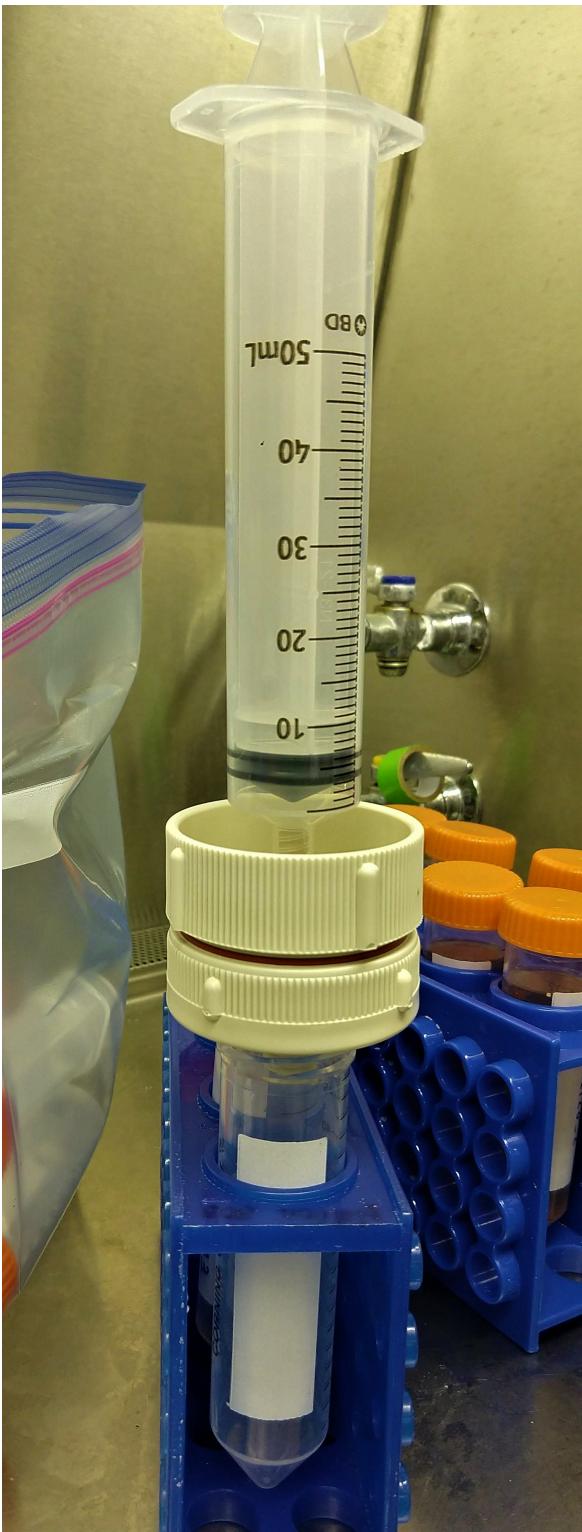
Here, NaCl lyses lipid-protein envelopes, denatures proteins and disrupts RNA-protein interactions. EDTA inhibits the enzymatic degradation of RNA by RNases present in wastewater and TRIS provides optimal buffering conditions for nucleic acids.

- 4.1 Agitate sample until all NaCl dissolves in the wastewater. Vortex or shake sample for **00:00:30** to promote lysis.



Raw wastewater containing NaCl, TRIS & EDTA.

- 5 (OPTIONAL) Heat inactivate sample at Δ **70 °C** for **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.



Durapore® Membrane Filter 5.0 μ m

by Millipore Sigma

Catalog #: SVLP04700



Swinnex Filter Holder
by Millipore Sigma
Catalog #: SX0004700



Magnetic Funnel 300mL 47mm
by Pall
Catalog #: 4242



Wastewater filtering through a 5-um PVDF filter in a Pall filter holder.

Direct RNA extraction (RNA Binding, Washing, Eluting)

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



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

7.1 Agitate sample to mix ethanol and wastewater lysate.

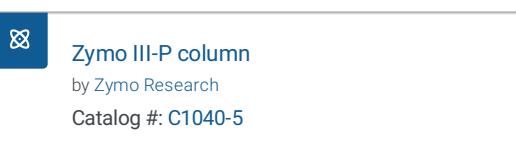
- 8 Attach Zymo III-P (or other) silica spin column to a vacuum manifold. Vacuum the full **80 mL** volume (both aliquots) of wastewater lysate & ethanol through the spin column.



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.



9 Vacuum **25 mL** wash buffer #1 (4S-WB1) through the silica spin column.

10 Vacuum **50 mL** wash buffer #2 (4S-WB2) through the silica spin column.

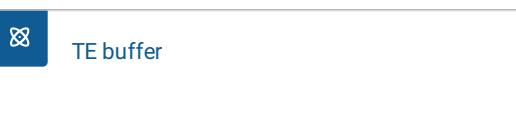
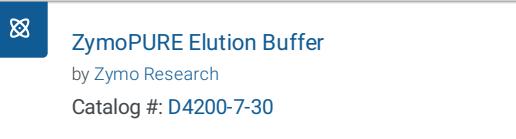
RNA elution

11 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.

12 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.

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

13 Pre-warm **200 µl** of ZymoPURE elution buffer or **200 µl pH 8** TE buffer per RNA sample to **50 °C** in a heat block, waterbath or incubator.



13.1 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**.

13.2 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

14 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.