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Version 4

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

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Coronavirus Method De...



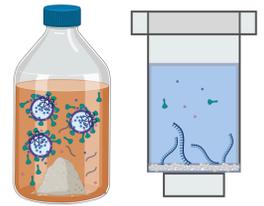
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Abstract

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.

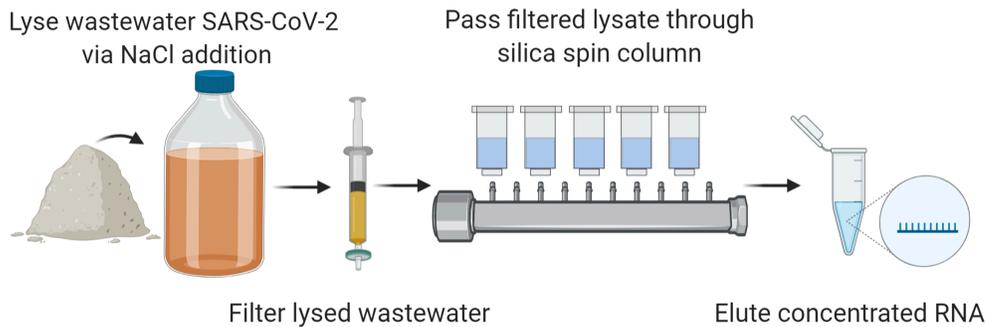


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Materials

MATERIALS

Tris

EDTA

Sodium Chloride **Catalog #PubChem CID: 5234**

Microcentrifuge

Ethanol

Zymo III-P column **Zymo Research Catalog #C1040-5**

EZ-Vac Vacuum Manifold **Zymo Research Catalog #S7000**

Durapore® Membrane Filter 5.0 µm **Merck MilliporeSigma (Sigma-Aldrich) Catalog #SVLP04700**

Magnetic Funnel 300mL 47mm **Pall Catalog #4242**

Bovilis Coronavirus Calf Vaccine **Merck Animal Health Catalog #16445**

Swinnex Filter Holder **Merck MilliporeSigma (Sigma-Aldrich) Catalog #SX0004700**

ZymoPURE Elution Buffer **Zymo Research Catalog #D4200-7-30**

STEP MATERIALS

ZymoPURE Elution Buffer **Zymo Research Catalog #D4200-7-30**

TE buffer

Bovilis Coronavirus Calf Vaccine **Merck Animal Health Catalog #16445**

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 Tris

 ZymoPURE Elution Buffer **Zymo Research Catalog #D4200-7-30**

 Microcentrifuge

 TE buffer

 Ethanol

 Zymo III-P column **Zymo Research Catalog #C1040-5**

 EDTA

 Sodium Chloride **Catalog #PubChem CID: 5234**

Troubleshooting

Safety warnings

-  **Safety Precautions:** Follow all institutional guidance. All steps should be carried out with BSL2 precautions in a certified Class II biosafety cabinet, PPE (gloves, lab coat, and safety glasses) to be worn during all steps. Hands should be washed before and after the experiment.

Before start

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:

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

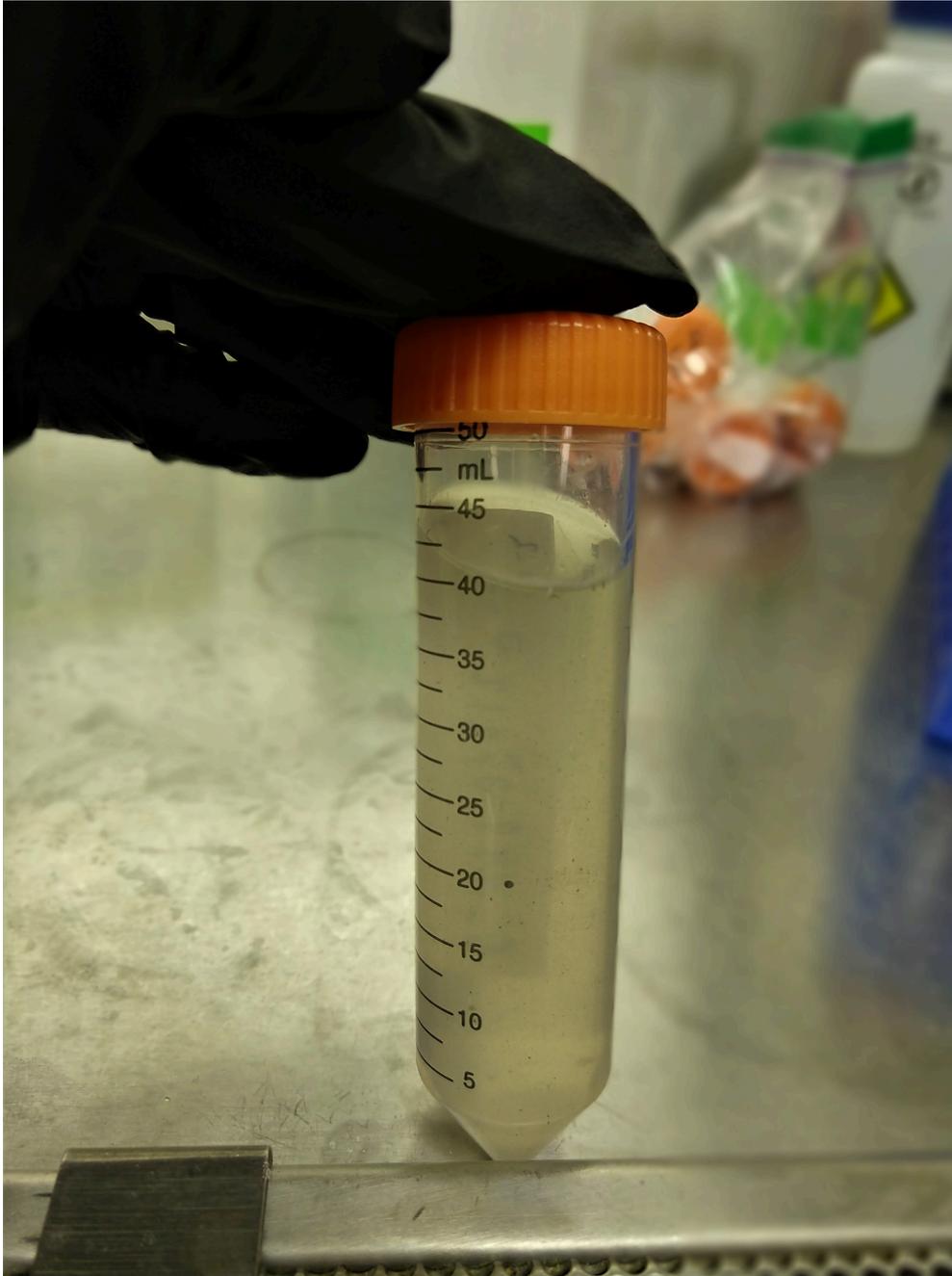
Add 10 mL of 1 Molarity (M) pH 7.2 TRIS

Agitate to fully mix buffer solution

- 1.3 Prepare two tubes containing lysis salts (one for the sample and one as a matched negative control) by adding 9.35 g of sodium chloride to each sterile 50mL tubes. Make $\text{pH } 7.2$ TE buffer (1 Molarity (M) TRIS, $100 \text{ millimolar (mM)}$ EDTA). Add $400 \mu\text{L}$ 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 $4 \text{ }^\circ\text{C}$ 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 process control. Agitate sample to fully mix bCoV or other spiked-in controls with the wastewater sample.

 Bovilis Coronavirus Calf Vaccine **Merck Animal Health Catalog #16445**

Note

Other spike-in 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. 30m

4 (RECOMMENDED) Heat inactivate sample at  70 °C for  00:45:00 . Our unpublished analyses have shown that this step may improve some RNA species' enrichment and detection. 45m

5 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.

⊗ Durapore® Membrane Filter 5.0 μm Merck MilliporeSigma (Sigma-Aldrich) Catalog #SVLP04700

⊗ Swinnex Filter Holder Merck MilliporeSigma (Sigma-Aldrich) Catalog #SX0004700

⊗ Magnetic Funnel 300mL 47mm Pall Catalog #4242



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 IM1 70 % volume to the 40 mL of filtrate.



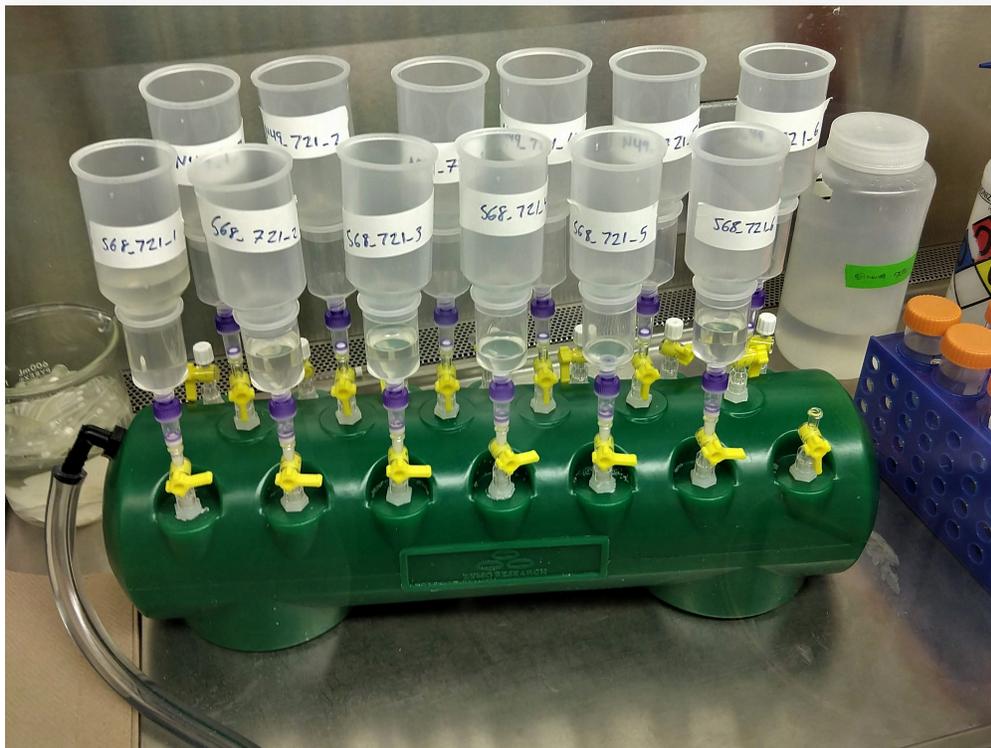
Filtered sample before ethanol addition. Filtrate should be semi-clear.

- 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.

 EZ-Vac Vacuum Manifold [Zymo Research Catalog #S7000](#)

 Zymo III-P column [Zymo Research Catalog #C1040-5](#)

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, 20°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 μ L of ZymoPURE elution buffer or  200 μ L  8 TE buffer per RNA sample to  50 °C in a heat block, waterbath or incubator.

 ZymoPURE Elution Buffer **Zymo Research Catalog #D4200-7-30**

 TE buffer

12.1 Add  200 μ L of pre-warmed elution buffer to each silica spin column. Incubate the elution buffer and column + collection tube assembly at room temperature for  00:01:00 .

1m

12.2 Spin at  10000 x g, 20°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.

Waste disposal

- 14 **Liquid waste:** Per sample, this protocol generates 90 mL of liquid waste containing 37.5% ethanol, 9.35 g NaCl, and trace amounts of Tris and EDTA. This should be treated as a hazardous chemical waste containing ethanol and should NOT be poured down the drain (see campus or local municipal guidelines).

Biohazard waste: All consumables and solid waste (e.g. filters, gloves, tubes) used in this protocol should be treated as biohazardous waste.

Any excess wastewater samples should be treated with bleach to final concentration of 10% v/v before removal from the biosafety cabinet for drain disposal.

Troubleshooting

- 15 In our hands, about 1% of Zymo III-P columns clog when passing the wastewater lysate through the columns.

Wastewater that remains after the column clogs can slowly be pushed through the column using a luer lock syringe, but note that this impacts the speed at which wastewater passes through the column, and thus possibly the yield. Use caution when analyzing RNA from clogged columns.