

Version 3 ▾

Oct 19, 2020

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

Oscar N Whitney¹, Basem Al-Shayeb², Alex Crits-Cristoph³, Mira Chaplin⁴, Vinson Fan¹, Hannah Greenwald⁴, Adrian Hinkle⁴, Rose Kantor⁴, Lauren Kennedy⁴, Anna Maurer¹, Robert Tjian⁵, Kara L. Nelson⁶, UC Berkeley Wastewater-based epidemiology consortium⁶

¹University of California, Berkeley, Tjian & Darzacq laboratory; ²University of California, Berkeley, Banfield & Doudna laboratory;

³University of California, Berkeley, Banfield laboratory; ⁴University of California, Berkeley, Nelson laboratory;

⁵University of California, Berkeley, HHMI; ⁶University of California, Berkeley

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

Coronavirus Method Development Community

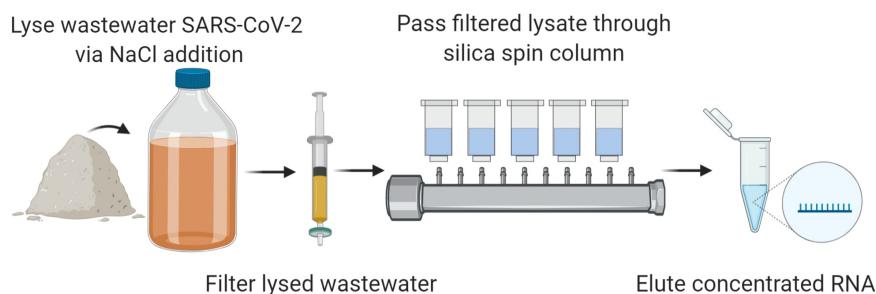


Oscar Whitney

University of California, Berkeley, Tjian & Darzacq Laborato...

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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Version created by Oscar Whitney



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
Durapore® Membrane Filter 5.0 µm	SVLP04700	Millipore Sigma
Swinnex Filter Holder	SX0004700	Millipore Sigma
Magnetic Funnel 300mL 47mm	4242	Pall
EZ-Vac Vacuum Manifold	S7000	Zymo Research
Zymo III-P column	C1040-5	Zymo Research

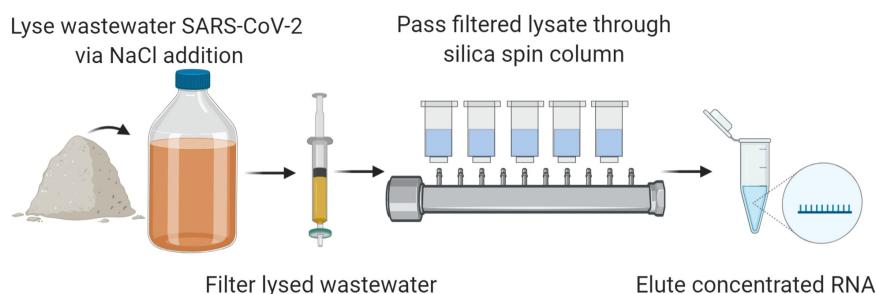
SAFETY WARNINGS

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

BSL2+ laboratory space.

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.



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 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 **[M]5 Molarity (M)** NaCl

Add **200 mL** of **[M]100 % volume** Ethanol

Add **10 mL** of **[M]1 Molarity (M)** **pH7.2** 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 **[M]5 Molarity (M)** NaCl

Add **800 mL** of **[M]100 % volume** Ethanol

Add **10 mL** of **[M]1 Molarity (M)** **pH7.2** TRIS

Agitate to fully mix buffer solution

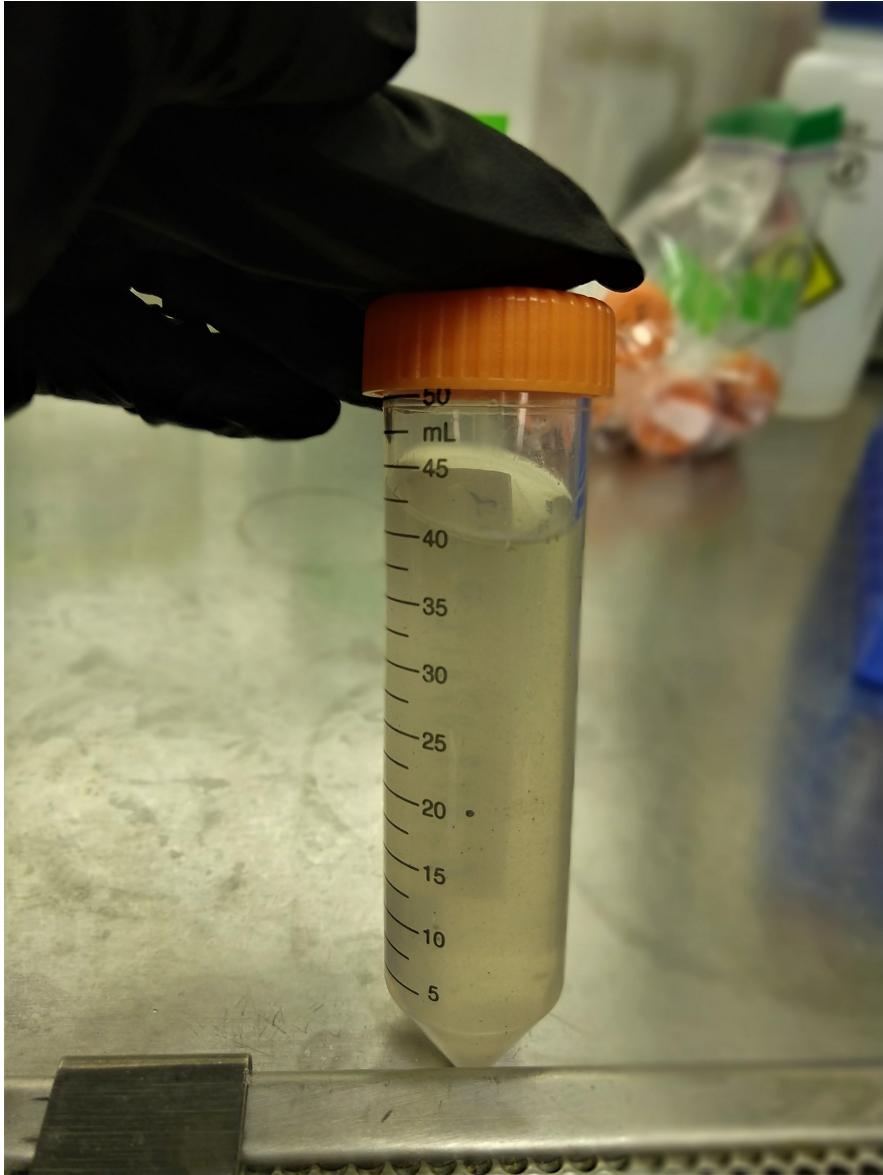
1.3 Prepare two tubes containing lysis salts (one for the sample and one as a matched negative control) by adding **9.5 g** of sodium chloride to each sterile 50mL tubes.

Make **pH7.2** TE buffer (**[M]1 Molarity (M)** TRIS, **[M]100 Milimolar (mM)** EDTA).

Add **400 µl** of TE buffer to each 50mL tube with salt. Gently shake.

Sample preparation, RNA preservation and particle lysis

- 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.



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 50µL 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.



Bovilis Coronavirus Calf Vaccine

by Merck Animal Health

Catalog #: 16445

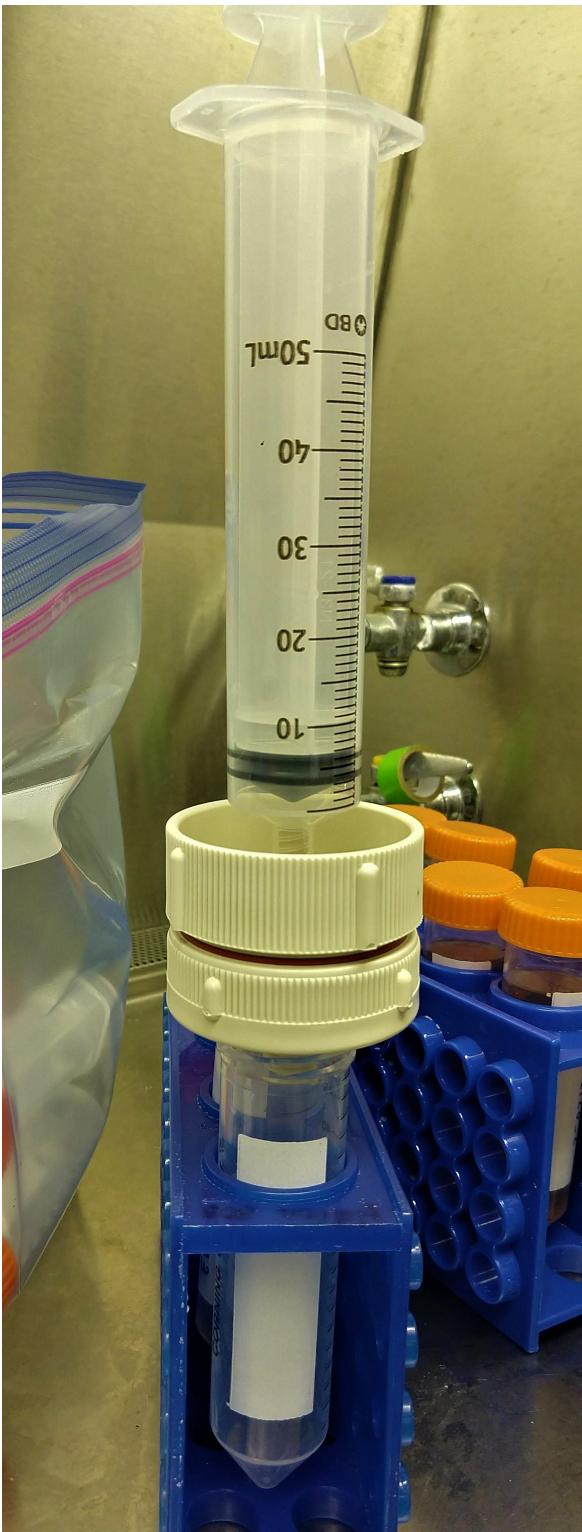


Other recovery controls can be used instead of bCoV, such as Phi6 bacteriophage. In addition, purified RNAs can be used to quantify the extraction efficiency of "free RNA".

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. 30m

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. 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 μ m

by Millipore Sigma

Catalog #: SVLP04700



Swinnex Filter Holder
by Millipore Sigma
Catalog #: SX0004700



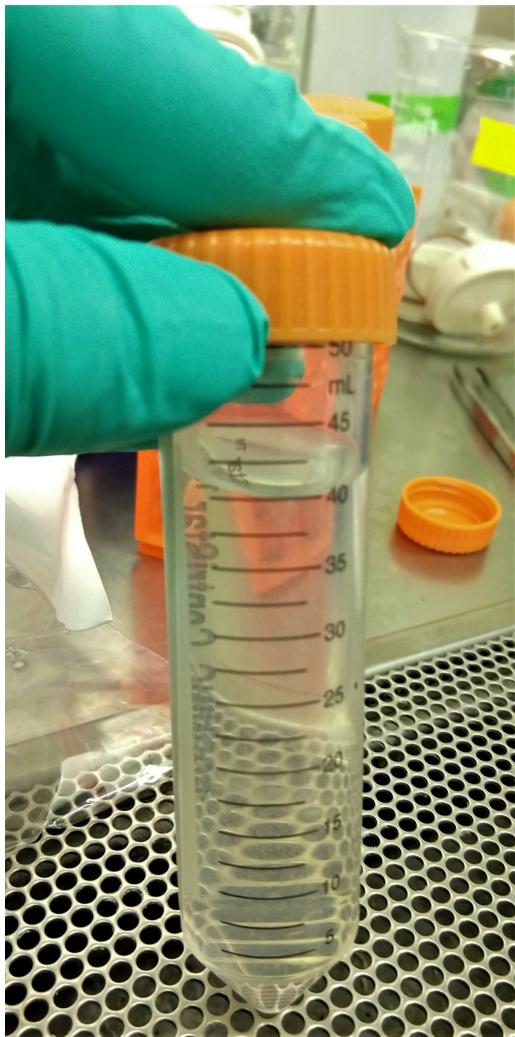
Magnetic Funnel 300mL 47mm
by Pall
Catalog #: 4242



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

Direct RNA extraction (RNA Binding, Washing, Eluting)

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



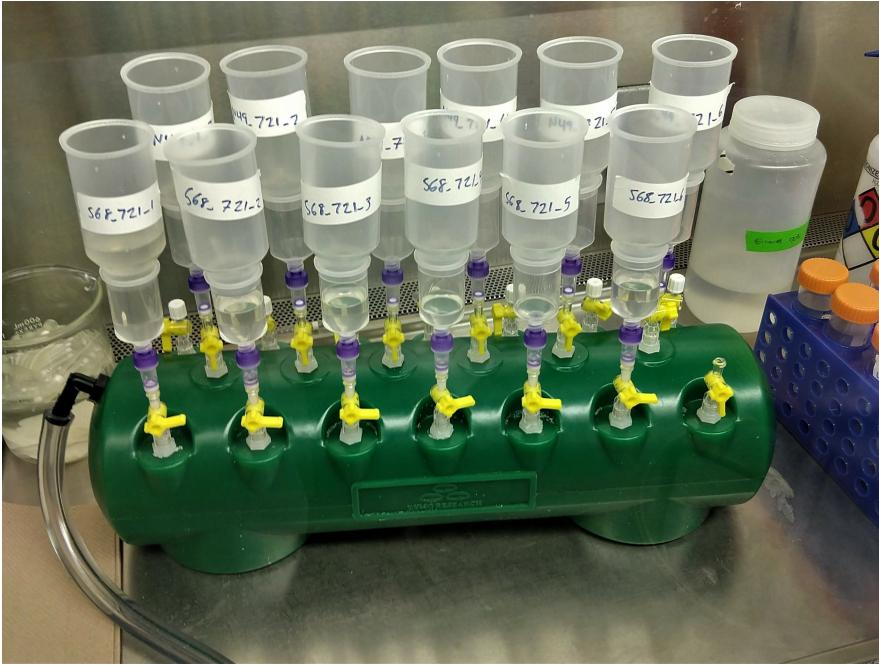
Filtered sample before ethanol addition. Filtrate should be semi-clear.

6.1 Agitate sample to mix ethanol and wastewater lysate.

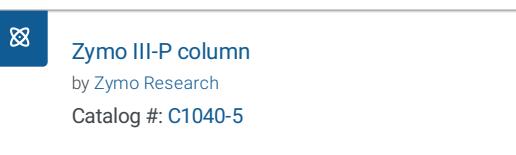
- 7 Attach Zymo III-P (or other) silica spin column to a vacuum manifold. Agitate the wastewater and lysate by inverting the tube five times, then pour into the spin column and vacuum the full **80 mL** of wastewater lysate & ethanol through the spin column.



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 **$\otimes 10000 \times g$, $4^\circ C$, 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 **$\square 200 \mu L$** of ZymoPURE elution buffer or **$\square 200 \mu L$** **pH 8** TE buffer per RNA sample to **$\delta 50^\circ C$** in a heat block, waterbath or incubator.



ZymoPURE Elution Buffer

by Zymo Research

Catalog #: D4200-7-30



TE buffer

12.1 Add **$\square 200 \mu L$** 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 **$\delta 50^\circ C$** for **$\otimes 00:10:00$** .

12.2 Spin at **$\otimes 10000 \times g$, $37^\circ C$, 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 **$\delta 4^\circ C$** for same-day use or freeze at **$\delta -80^\circ C$** for later use and storage.