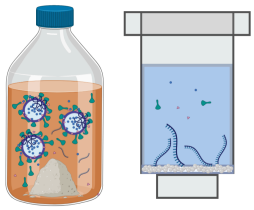
Aug 14, 2020 Version 2

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



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Oscar Whitney[1], Basem Al-Shayeb[2], Alex Crits-Cristoph[3], Mira Chaplin[4], Vinson Fan[1], Hannah Greenwald[4], Adrian Hinkle[4], Rose Kantor[4], Lauren Kennedy[4], Anna Maurer[1], Robert Tjian[5], Kara L. Nelson[6], UC Berkeley Wastewater-based epidemiology consortium[6]

[1]University of California, Berkeley, Tjian & Darzacq laboratory;
[2]University of California, Berkeley, Banfield & Doudna laboratory;
[3]University of California, Berkeley, Banfield laboratory; [4]University of California, Berkeley, Nelson laboratory;
[5]University of California, Berkeley, HHMI; [6]University of California, Berkeley

Coronavirus Method De...

Oscar N Whitney
University of California, Berkeley, Tjian & Darzacq Laborato...

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We use this protocol and it's working

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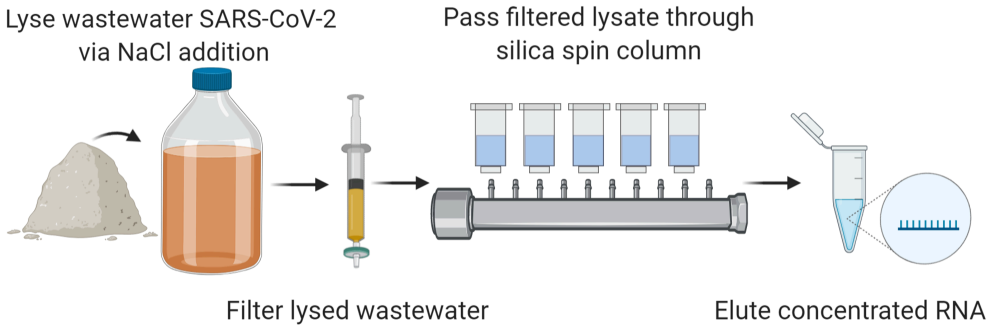
Abstract

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

- Durapore® Membrane Filter 5.0 µm **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**SVLP04700
- Swinnex Filter Holder **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**SX0004700
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- TE buffer
- Bovilis Coronavirus Calf Vaccine **Merck Animal Health Catalog #**16445

Protocol materials

- ZymoPURE Elution Buffer **Zymo Research Catalog #**D4200-7-30
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- Tris
- EDTA
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- EZ-Vac Vacuum Manifold **Zymo Research Catalog #**S7000
- Bovilis Coronavirus Calf Vaccine **Merck Animal Health Catalog #**16445
- TE buffer
- Microcentrifuge

Troubleshooting

Safety warnings

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

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 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 [M] 5 Molarity (M) NaCl

Add 200 mL of [M] 100 % volume Ethanol

Add 10 mL of [M] 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 [M] 5 Molarity (M) NaCl

Add 800 mL of [M] 100 % volume Ethanol

Add 10 mL of [M] 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.

Note

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 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.

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

Note

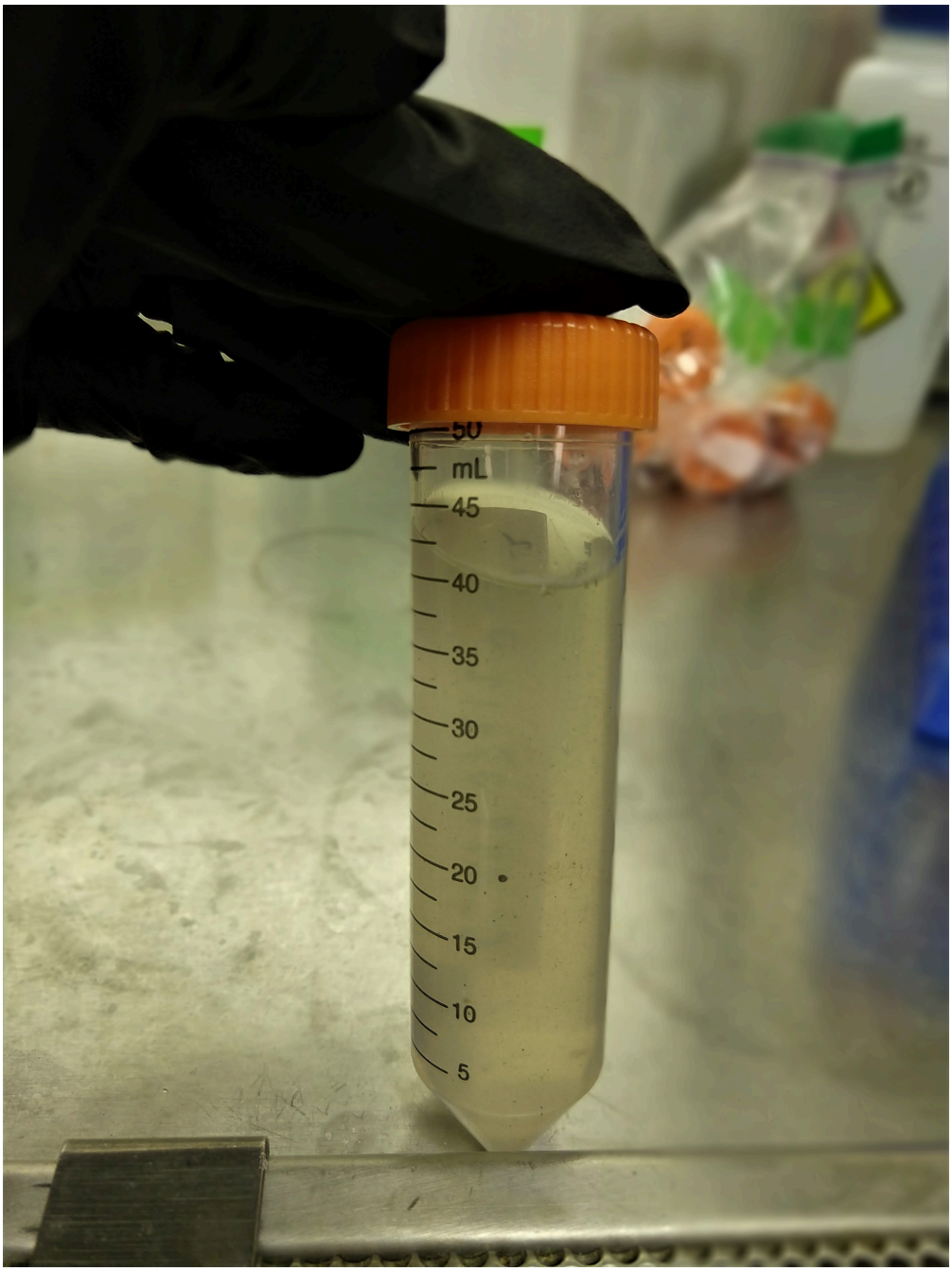
Other recovery controls can be used instead of bCoV. Some candidates include Phi6 bacteriophage and coronavirus OC43. In addition, purified RNAs can be used to quantify the extraction efficiency of "free RNA".

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

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.

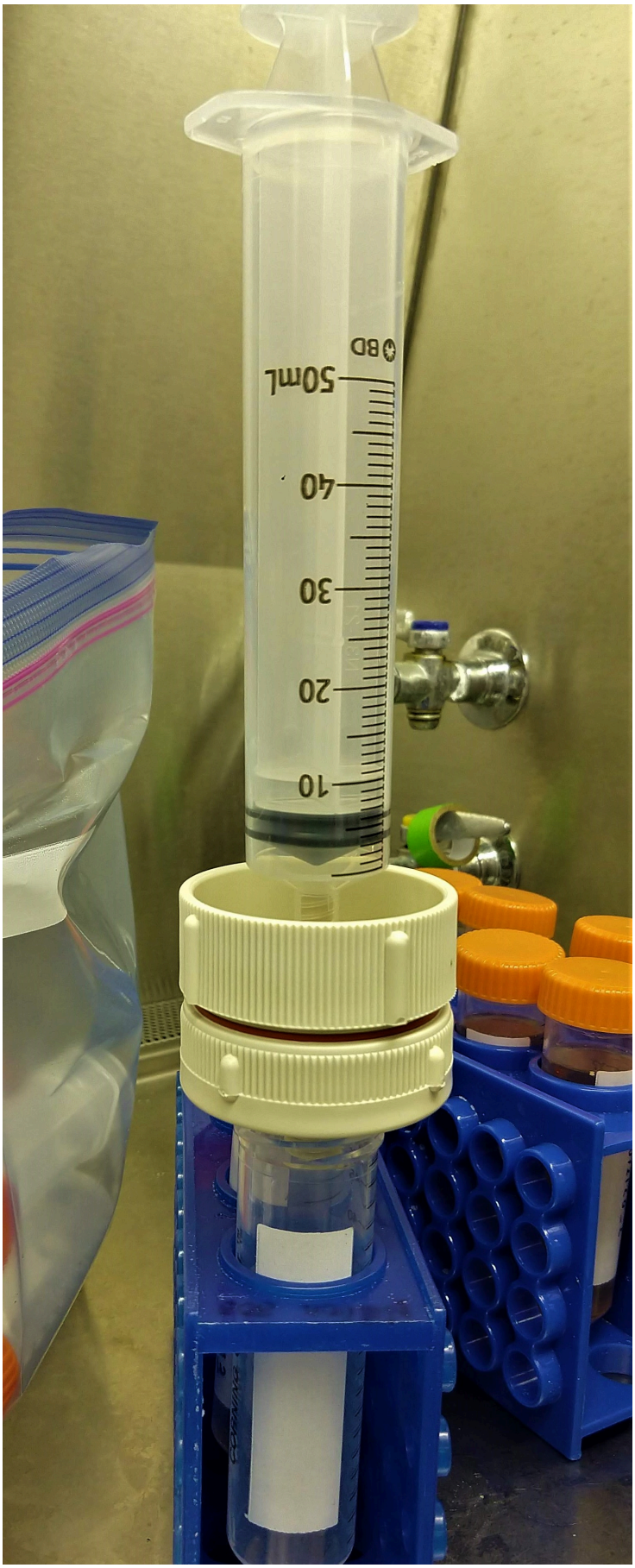
4.1 Agitate sample until all NaCl dissolves in the wastewater. Vortex or shake sample for 00:00:30 to promote lysis.

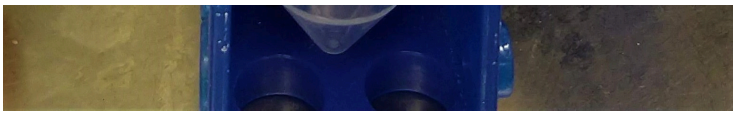


Raw wastewater containing NaCl, TRIS & EDTA.

5 (OPTIONAL) Heat inactivate sample at 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.


BD
50mL
40
30
20
10



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

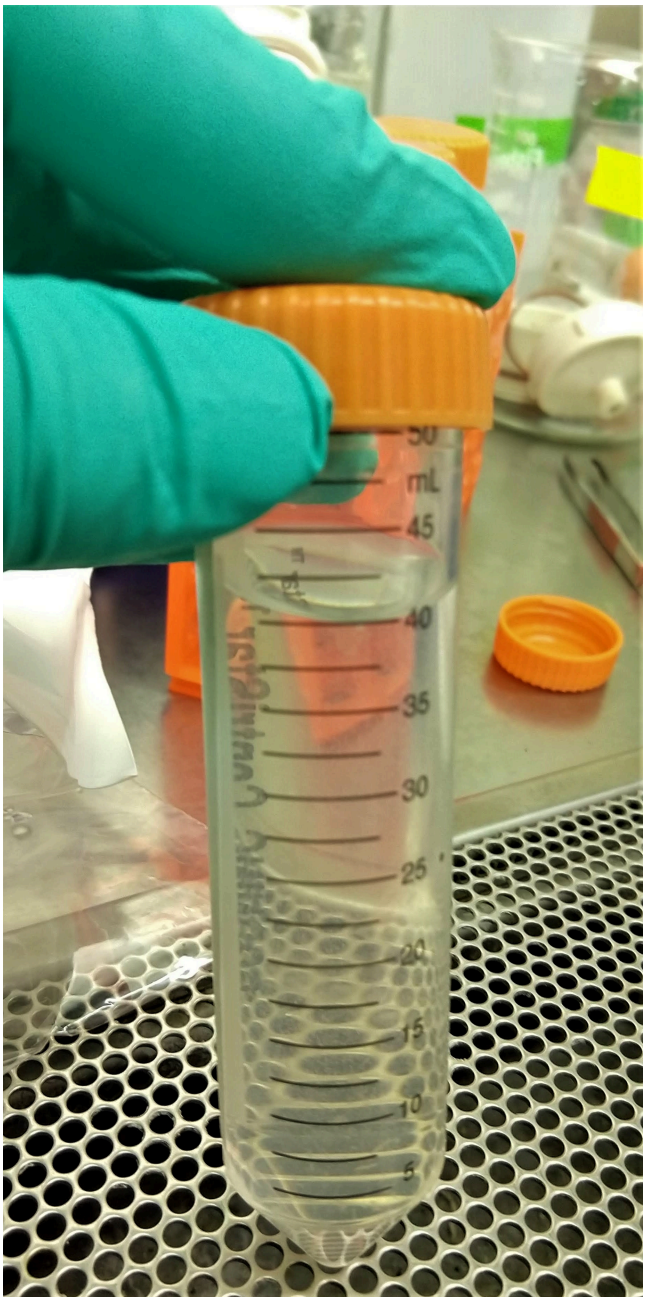
Swinnex Filter Holder **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**SX0004700

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)

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.



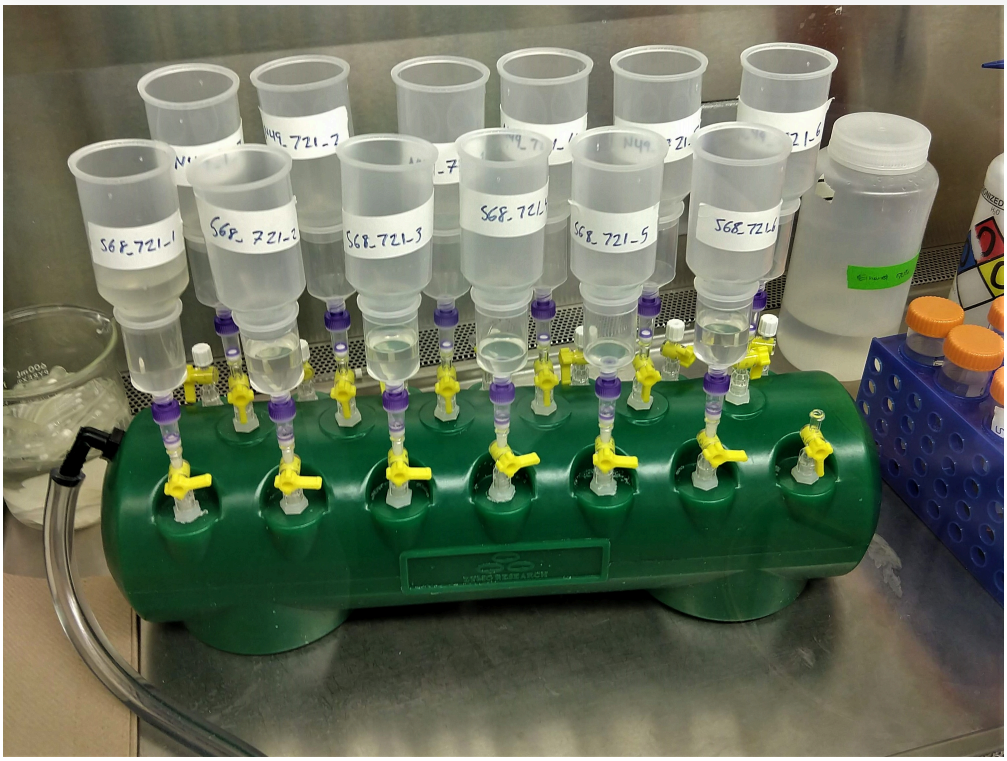
Filtered sample before ethanol addition. Filtrate should be semi-clear.

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.

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 dowstream 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

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

10 Vacuum 10 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.

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

TE buffer

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.