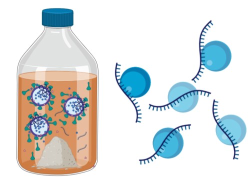
Jul 27, 2020

Direct wastewater RNA extraction via the "Milk of Silica (MoS)" method - A companion method to "Sewage, Salt, Silica and SARS-CoV-2 (4S)"



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Coronavirus Method Deve…

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Protocol status: Working
We use this protocol in our laboratory to routinely isolate RNA from wastewater samples

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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.

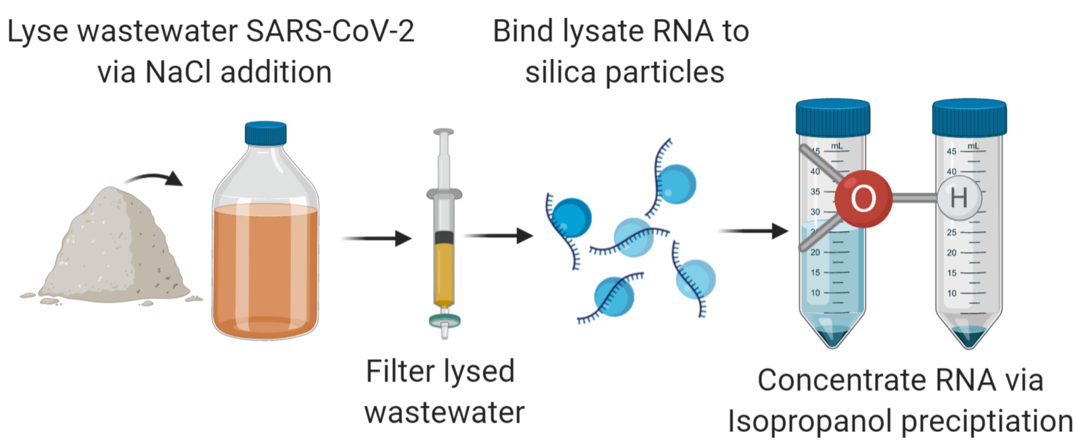


Image Attribution

Figures created with BioRender.com

Guidelines

DISCLAIMER – FOR INFORMATIONAL PURPOSES ONLY; USE AT YOUR OWN RISK

Materials

MATERIALS

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

STEP MATERIALS

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

Protocol materials

Ethanol Materials

Tris Materials

TE buffer **Thermo Fisher Scientific** Materials

Silicon dioxide ~99% 0.5-10 µm (approx. 80% between 1-5 µm) **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**SIGMA S5631

In Materials, Materials, Step 1.3

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

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

Materials, Step 6

Centrifuge Materials

Magnetic Funnel 300mL 47mm **Pall Catalog #**4242 Materials, Step 6

Bovilis Coronavirus Calf Vaccine **Merck Animal Health Catalog #**16445 Materials, Step 3

Sodium acetate **Merck Millipore (EMD Millipore) Catalog #**1.06268.1000 In Materials, Materials, Step 12

Swinnex Filter Holder **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**SX0004700 Materials, Step 6

Isopropanol **Merck Millipore (EMD Millipore) Catalog #**109634 In Materials, Materials, Step 12

EDTA Materials

Safety warnings

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

Before start

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.

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:

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

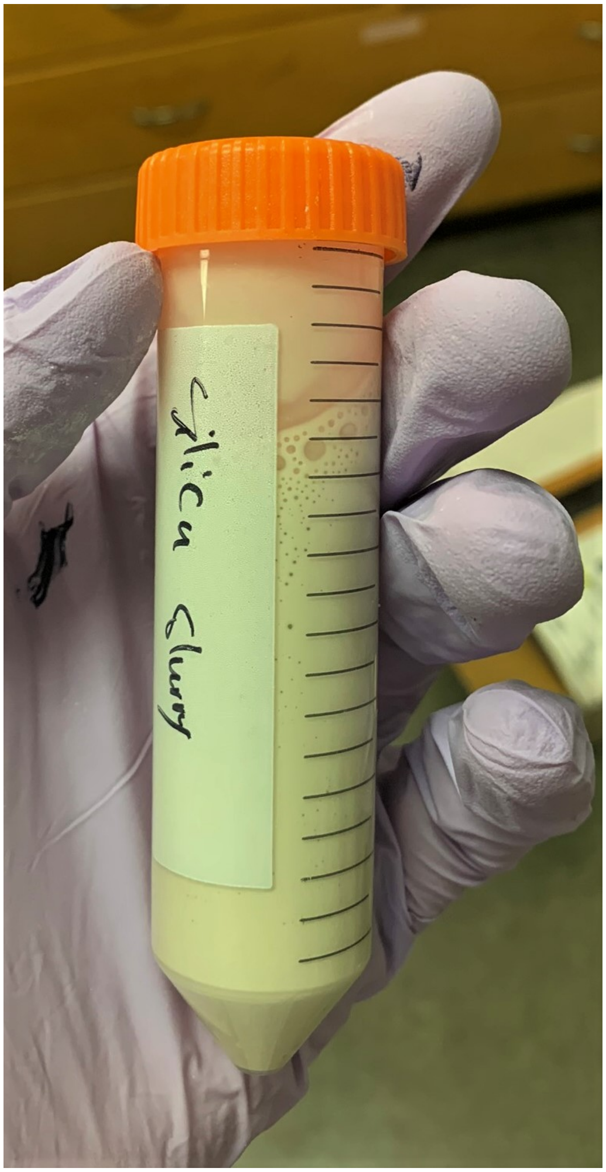
Add 800 mL of [M] 100 % volume Ethanol

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

Agitate to fully mix buffer solution

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) **Sigma Aldrich Catalog #**SIGMA S5631



"Milk of Silica" suspension (1g silicon dioxide/mL water)

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 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.

Bovilis Coronavirus Calf Vaccine **Sigma Aldrich 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.

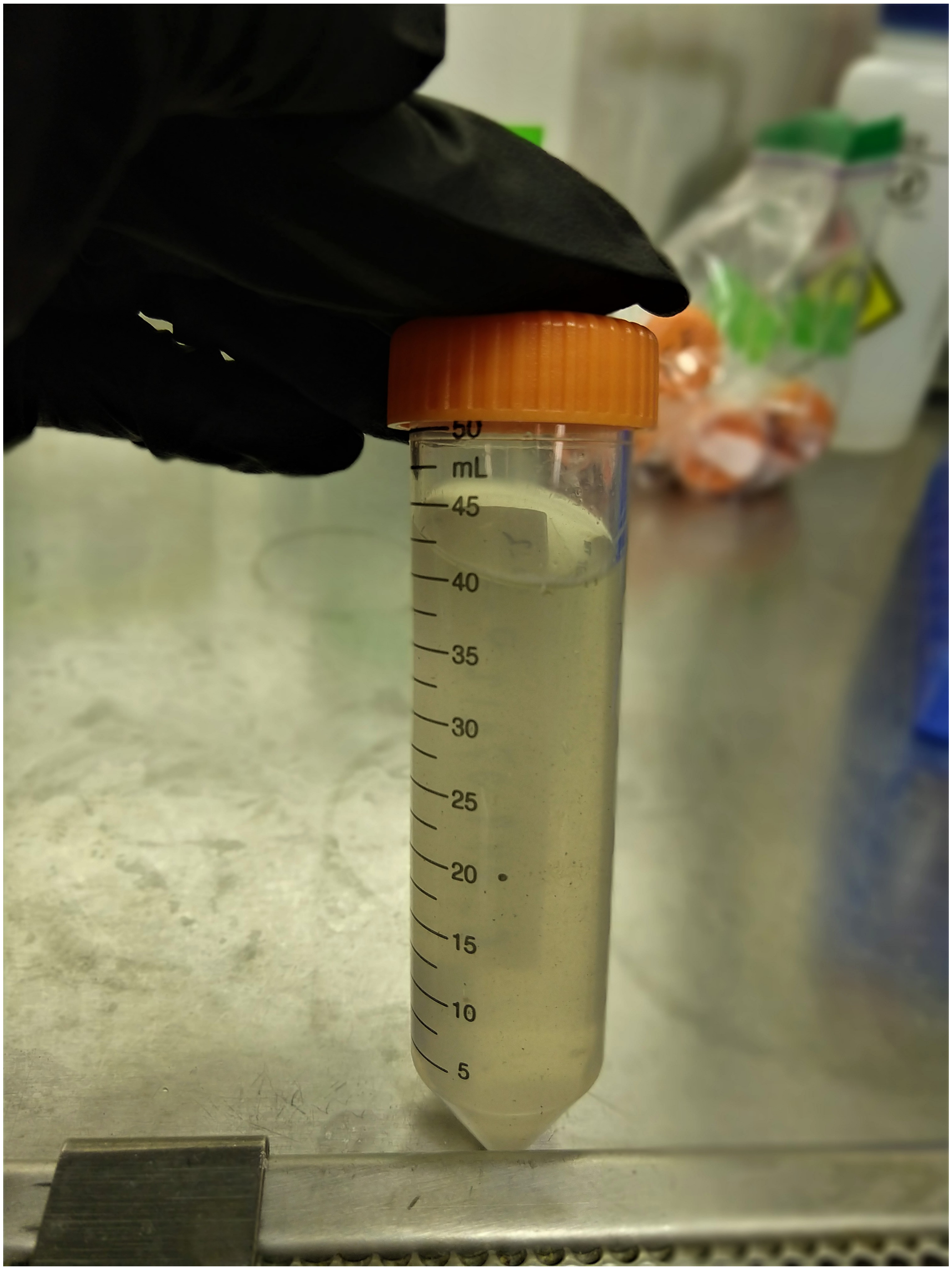
Make pH 7.2 TE buffer ([M] 1 Molarity (M) TRIS, [M] 100 millimolar (mM) EDTA).

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 **Sigma Aldrich** **Catalog #**SVLP04700

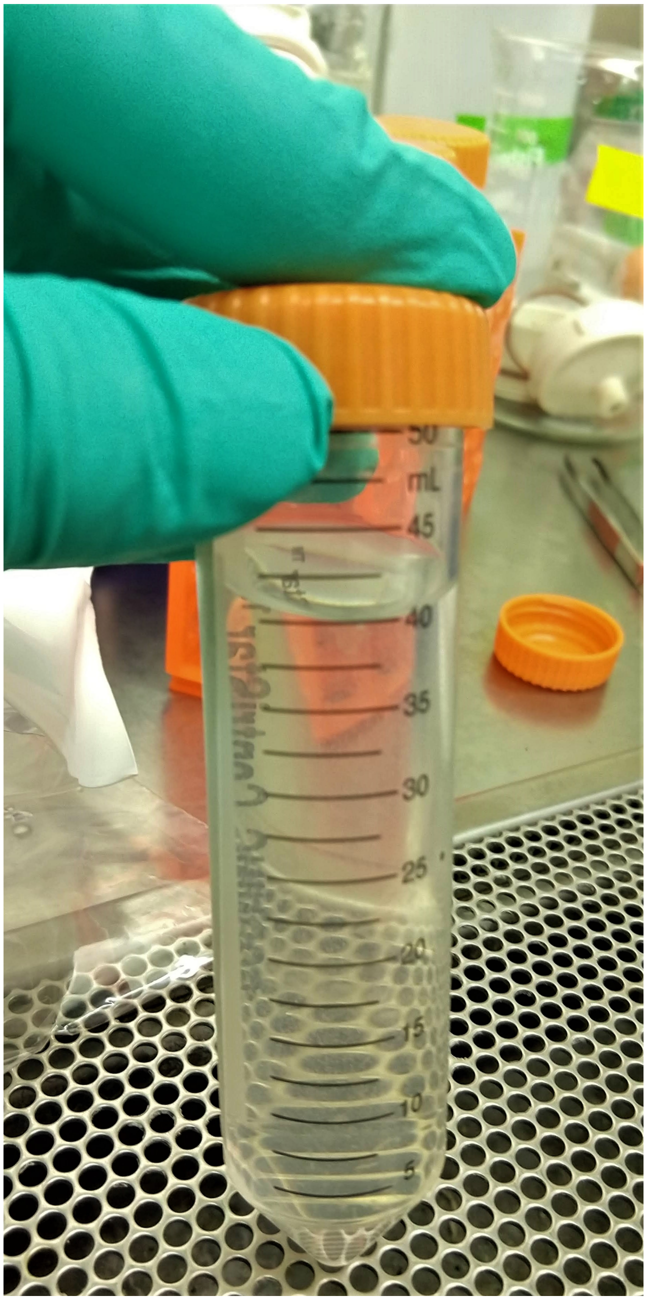
Swinnex Filter Holder **Sigma Aldrich** **Catalog #**SX0004700

Magnetic Funnel 300mL 47mm **Sigma Aldrich** **Catalog #**4242

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

Note

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.

Note

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 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 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 .

Note

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 4000 x g, 37°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.

Note

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 [M] 100 % volume Isopropanol and 4 mL of [M] 3 Molarity (M) 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 **Sigma Aldrich Catalog #**109634

Sodium acetate **Sigma Aldrich Catalog #**1.06268.1000

Note

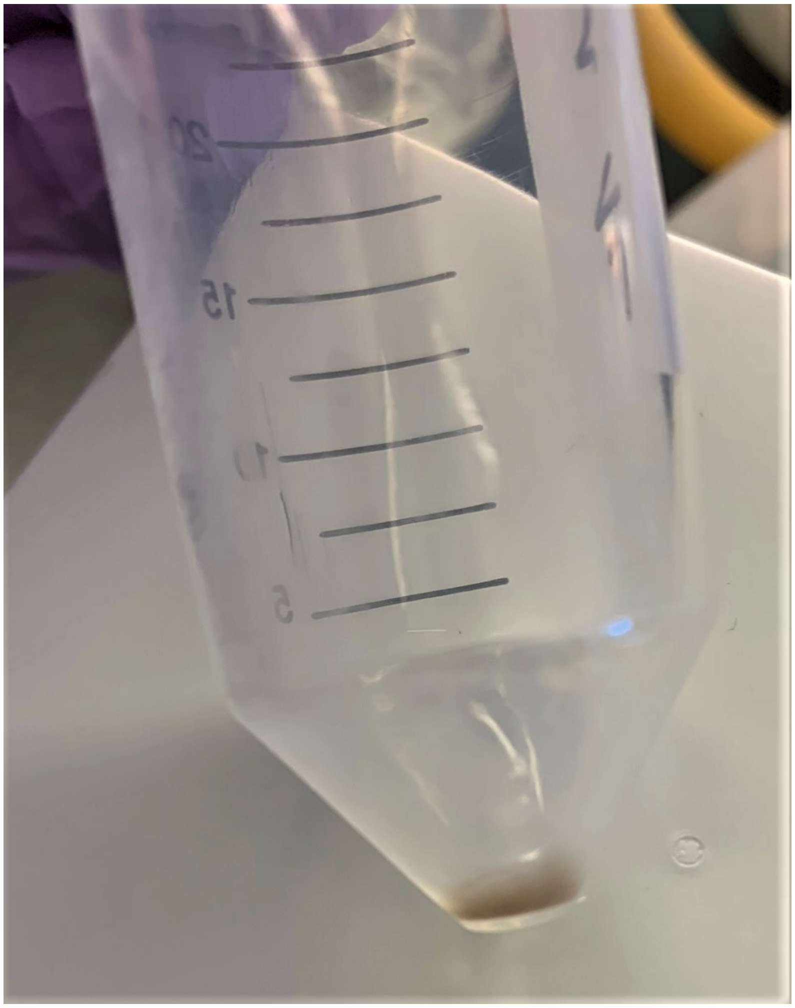
Isopropanol and sodium acetate alongisde centrifugation precipitate the eluted RNA from the 20mL aqueous matrix.

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

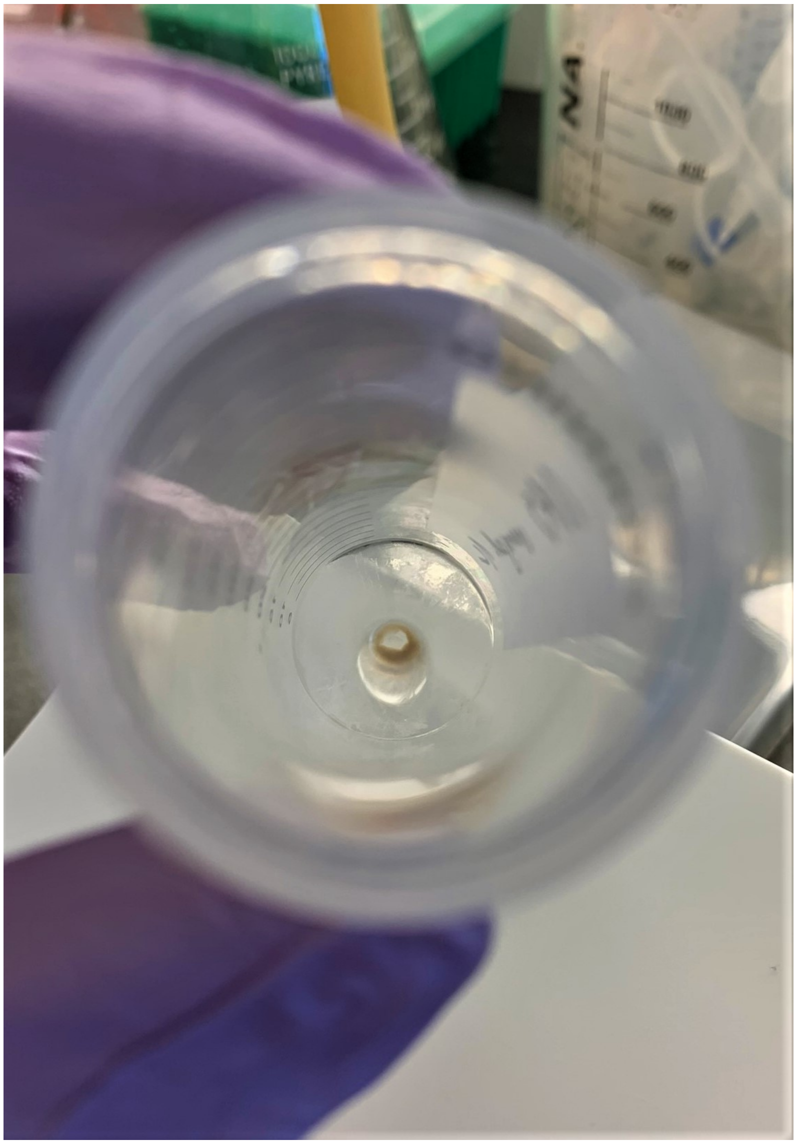
Note

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



Top view of pellet after removal of isopropanol, water and sodium acetate mixture

13 Wash pellet with ethanol by adding 40 mL [M] 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.

Note

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 [M] 75 % volume Ethanol as possible from the nucleic acid pellet. Add 1 mL of [M] 70 % volume Ethanol to the nucleic acid pellet.

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

Note

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

14 After pellet transfer, centruge 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 [M] 70 % volume Ethanol from the nucleic acid pellet.

Note

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 .

Note

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.

Note

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

15 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.