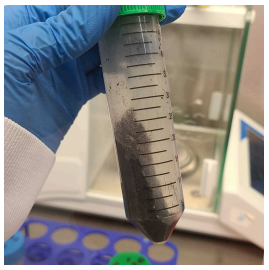
Aug 06, 2020

Extraction of RNA from Wastewater Primary Solids Using a Direct Extraction Method for Downstream SARS-CoV-2 RNA Quantification



In 1 collection

DOI
https://dx.doi.org/10.17504/protocols.io.bi6skhee

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DOI: https://dx.doi.org/10.17504/protocols.io.bi6skhee

Protocol Citation: Stephanie Loeb, Katy Graham, Marlene Wolfe, Krista Wigginton, Alexandria Boehm 2020. Extraction of RNA from Wastewater Primary Solids Using a Direct Extraction Method for Downstream SARS-CoV-2 RNA Quantification. **protocols.io https://dx.doi.org/10.17504/protocols.io.bi6skhee**

Protocol status: Working
We have used this protocol extensively with solids from a number of wastewater treatment plants.

Created: July 30, 2020

Last Modified: August 06, 2020

Protocol Integer ID: 39858

Keywords: wastewater, solids, SARS-CoV-2, influent, coronavirus, virus, rna from wastewater, extraction of rna, extract rna, solids samples from wastewater treatment plant, extraction procedure, direct extraction method for downstream sar, wastewater, biosafety concerns concentration, wastewater primary solid, extraction, rna, wastewater treatment plant, strict biosafety level, direct extraction method, raw sample, downstream sar, using standard laboratory safety guideline, standard laboratory safety guideline, settled solids sample, digital pcr protocal,

Disclaimer

Abstract

Overview

This SOP describes pre-analytical procedures to be followed for the isolation and identification of SARS-CoV-2 RNA in primary settled solids samples from wastewater treatment plants. This protocal should be paired with our digital PCR protocal.

The protocol follows the approximate workflow:

1. Separate and aliquot sample (~4 hrs)
2. Spike with BCoV (~1 hr)
3. Extract RNA (~ 12 hours total, may be split into 2-3 steps/days)
4. Inhibitor removal (~1 hr)

Biosafety Concerns

Concentration and extraction procedures that utilize raw samples must adhere to strict Biosafety Level 2+ procedures. These procedures should be performed in a dedicated room. Downstream products may be handled using standard laboratory safety guidelines.

This protocol was developed by Stephanie Loeb, Katy Graham, Marlene Wolfe, Krista Wigginton, and Alexandria Boehm at Stanford University and University of Michigan.

Materials

MATERIALS

- Phenol-chloroform-isoamyl alcohol 25:24:1 (PCI) **Invitrogen - Thermo Fisher Catalog #**15593049
- RNeasy® PowerSoil® Total RNA Kit **Qiagen Catalog #**12866-25
- OneStep PCR Inhibitor Removal Kit **Zymo Research Catalog #**D6030
- Bovilis Coronavirus Calf Vaccine **Merck Animal Health Catalog #**16445

STEP MATERIALS

- RNeasy® PowerSoil® Total RNA Kit **Qiagen Catalog #**12866-25
- Bovilis Coronavirus Calf Vaccine **Merck Animal Health Catalog #**16445
- UltraPure™ Phenol:Chloroform:Isoamyl Alcohol (25:24:1, v/v) **Thermo Fisher Catalog #**15593031
- OneStep PCR Inhibitor Removal Kit **Zymo Research Catalog #**D6030

Separate Solids and Spike with BCoV

Materials

- 100p pipet tips
- 50mL falcon tubes
- Percent solids materials (listed in part 2)
- PowerBead Tubes (from RNeasy PowerSoil Kit)
- 5 mL cryovials
- Bovine coronavirus (BCoV) stock
- 25mL serological pipettes
- Disposable polypropylene spatulas

Equipment

- Biosafety cabinet
- 100p pipet
- Centrifuge with high speed rotor
- Balance
- Tube rack
- Automatic serological pipettor

Percent Solids Measurements

Materials

- Aluminum weigh dish
- Disposable polypropylene spatulas
- Aluminum foil

Equipment

- Oven set at at 105°C
- Analytical balance
- Dehydrator (not essential)

RNA Extraction

Materials:

- Qiagen RNeasy PowerSoil Total RNA extraction kit
- LoBind tubes (for -80°C storage)
- Molecular grade mixture of 25 parts phenol, 24 parts chloroform, and one part isoamyl alcohol
- 1000p pipet tips (RNAse/DNase-free)
- 200p pipet tips (RNAse/DNase-free)
- 10p pipet tips (RNAse/DNase-free)
- Disposable polypropylene spatulas

Equipment:

- 1000p pipet
- 200p pipet
- Heat block (in BSC)
- 10p pipet
- Vortex with 15 mL falcon tube adapter (in BSC)
- Centrifuge

Inhibitor Removal

Materials

- Zymo OneStep PCR Inhibitor Removal Columns
- LoBind 1.5 mL tubes
- P100 pipet tips- molecular biology grade

Equipment

- Mini-centrifuge
- 100p pipet

Protocol materials

- RNeasy® PowerSoil® Total RNA Kit **Qiagen** **Catalog #**12866-25
- OneStep PCR Inhibitor Removal Kit **Zymo Research** **Catalog #**D6030
- Bovilis Coronavirus Calf Vaccine **Merck Animal Health** **Catalog #**16445
- UltraPure™ Phenol:Chloroform:Isoamyl Alcohol (25:24:1, v/v) **Thermo Fisher** **Catalog #**15593031
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Safety warnings

Concentration and extraction procedures that utilize raw samples must adhere to strict Biosafety Level 2+ procedures. These procedures should be performed in a dedicated room. Downstream products may be handled using standard laboratory safety guidelines.

Before Starting

30m

1 **Day before sample processing:**

- Thaw sample Overnight at 4 °C. Record the weight and volume of the sample.

Day of sample processing:

- Place aluminum dishes in oven 01:00:00 before use
- Set centrifuge temperature to 4 °C 00:30:00 before use

Separate Solids and Spike BCoV

5h

2 Load 40 mL samples of primary sludge at a time into the centrifuge rotor. Balance with equally weighted tubes filled with water.

3 Centrifuge at 14500 rpm, 4°C, 00:30:00 , (equivalent to 24,000 xg) .

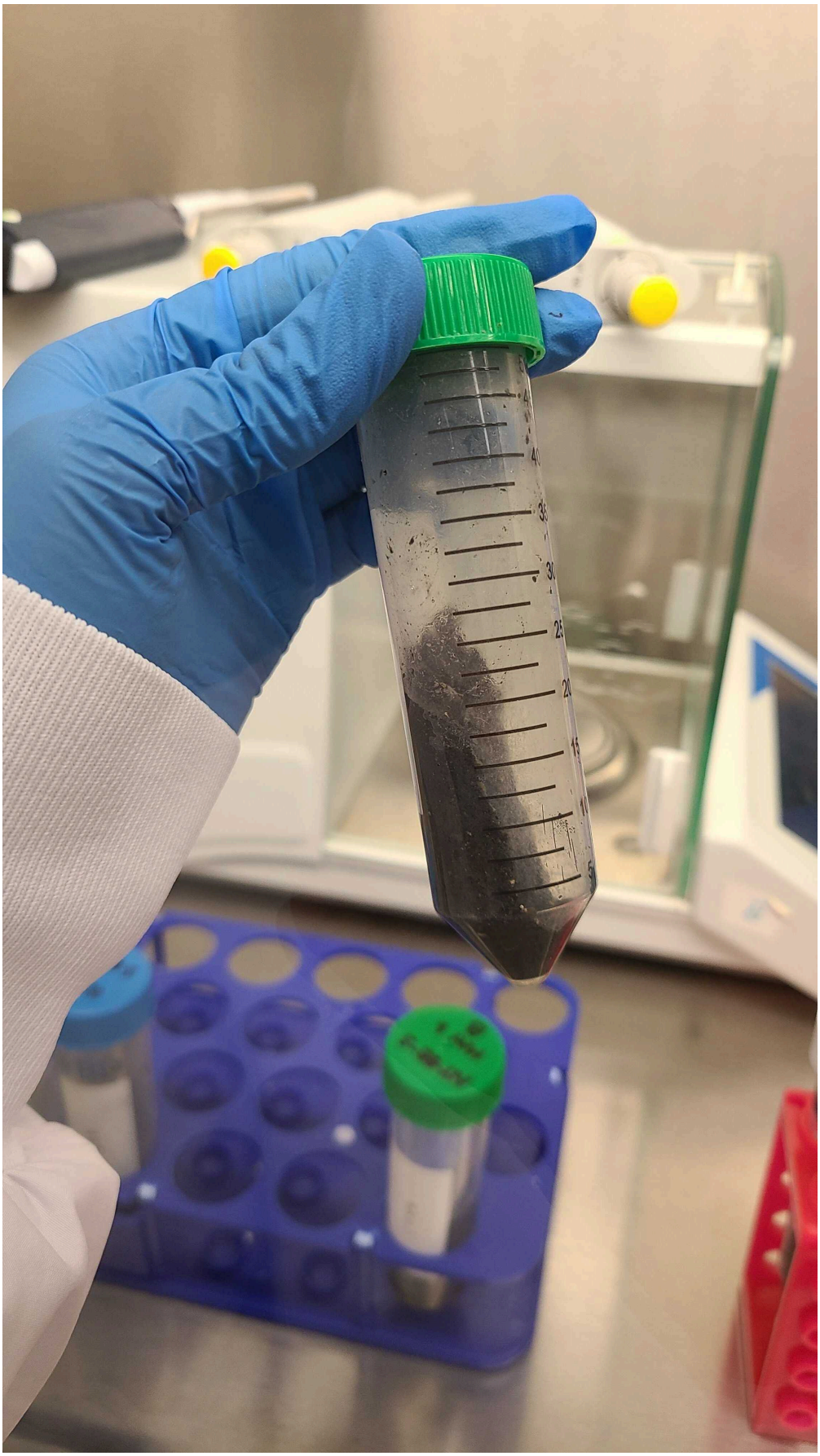
Safety information

Bring the centrifuge rotor to the BSC before loading or opening.

4 Decant the supernatant into an empty 50 mL falcon tube or larger container. Record the weight and volume of the remaining solids.

Safety information

Discard liquid as biohazard waste.



Separated solids with liquid removed

5 Transfer solids for percent solids measurement: Transfer a generous (~ 500 mg) aliquot of the solid fraction onto a pre-weighed aluminum weigh dish. The aluminum weigh dish should be used directly out of the oven and not allowed to rehydrate. Record the total weight of the weigh dish before covering with aluminum and transferring to the oven to measure dry weight following EPA method Method 106.3. (Page 62) **Link to EPA method. See page 62.**

Note

If there is less than 2 g of solids available, % solids may be performed with a smaller aliquot.

6 Aliquot solids as follows into tarred or pre-weighed tubes using a sterile spatula. Note exact mass aliquoted, with a target range from 2.0 g to 2.5 g .

6.1 One 2g aliquot can be place directly into a 15 mL PowerBead tubes from RNeasy PowerSoil Total RNA extraction kit to prepare for extraction. Depending on the number of replicates required, additional aliquots can also be placed in PowerBead tubes for extraction.

RNeasy® PowerSoil® Total RNA Kit **Qiagen Catalog #**12866-25

6.2 Additional 2g aliquots may be placed in as many 5mL cryovials as possible for storage at -80 °C .

7 Individually spike each sample that has been aliquoted in a 15mL PowerBead tube with 20 µL BCoV stock. Pipet the spike directly into the solids and mix well with a spatula to homogenize. Equilibrate the sample at 4°C for 1 hour.

Note

Vials of BCoV vaccine should be rehydrated with molecular grade water before use via needle and syringe, and the solution may be stored at 4°C. For each batch of BCoV, a known dilution in PBS should be processed and analyzed in order to determine the concentration spiked

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

8 Store samples in PowerBead tubes at -80 °C until extraction. Archive samples in cryovials at -80 °C.

Extract RNA

12h

9 **Prepare for extractions using the RNeasy PowerSoil Total RNA kit** to extract and isolate viral RNA into 100uL of eluent. This extract can be used for one step ddPCR or reverse transcribed for qPCR.

Before starting

- Prepare a sterile workspace
- Heat Solution IRS at 60 °C until any precipitate dissolves.
- Wear RNase-free gloves at all times and remove RNase from the work area.

Safety information

All sample processing must occur in the biosafety cabinet until the bead beating step is completed. After bead beating, cells have been completely lysed and the samples are considered non-infectious.

10 Remove samples from the freezer and place On ice. Samples do not need to be thawed before processing. Use Solution IRS while it is still warm.

11 *For processing samples already in PowerBead tube*

Add 2.5 mL of PowerBead Solution, 0.25 mL of Solution SR1 and 0.8 mL of Solution IRS directly to the tube containing the sample. Vortex the tube vigorously to suspend the solids.

Note

If the sample is not in a powerbead tube, perform step 11 in the sample tube, transferring the entire mixture to the PowerBead tube prior to step 12. If a large amount of residual solids remain in the tube after step 11, perform step 4 in two “washes” of 1.75 mL each phenol/chloroform/isoamyl alcohol. If a disposable spatula was used, the spatula can also be rinsed in this solution as well.

12 Add 3.5 mL of phenol/chlorogorm/isoamyl alcohol (pH 6.5–8.0, not included in kit) to the sample tube. Vortex vigorously.

UltraPure™ Phenol:Chloroform:Isoamyl Alcohol (25:24:1, v/v) **Thermo Fisher Catalog #**15593031

13 Cap and vortex the PowerBead Tube to mix until the biphasic layer disappears. Place the PowerBead Tube on a Vortex Adapter and vortex at maximum speed for 00:15:00 .

14 Remove the PowerBead Tube and centrifuge at 2500 x g for 00:10:00 at Room temperature . After centrifugation, two or three phases may be visible. Total nucleic acids are retained in the upper aqueous phase.

15 Transfer the upper aqueous phase to a clean 15 ml Collection Tube. Take care not to transfer material from the lower phases.

Safety information

After transferring the upper aqueous, the remaining fractions can be discarded as BSL2 waste.

16 Add 1.5 mL of Solution SR3 to the aqueous phase and vortex to mix. Incubate at 4 °C for 00:10:00 and then centrifuge at 2.500 x g for 00:10:00 at Room temperature .

17 Transfer the supernatant, without disturbing the pellet, to a new 15 ml Collection Tube.

18 Add 5 mL of Solution SR4 to the supernatant in the Collection Tube and invert or vortex to mix.
Incubate at -20 °C for 00:30:00 .

Note

Samples can also be incubated at -20 °C Overnight , to continue procedure on the next day.

19 Centrifuge at 2500 x g for 00:30:00 .

20 Decant the supernatant and invert the 15 ml Collection Tube on a paper towel for 00:05:00 .

Note

Check that the pellet seems to be firmly in the bottom of the tube - if so, you can carefully pour off the supernatant to decant.

21 Shake Solution SR5 to mix and add 1 mL to the 15 ml Collection Tube. Resuspend the pellet completely by repeatedly pipetting or vortexing.

Note

The pellet will be difficult to resuspend. Place the tube in a heat block or water bath at 45 °C for 00:10:00 , followed by vortexing and/or pipetting up and down. Repeat until the pellet is resuspended. When the pellet is fully re-suspended, the solution will appear as a relatively opaque homogenous cloudy yellow-white solution. Continue heating and vortexing until no visible precipitates remain. Failing to fully re-suspend the pellet will result in large downstream losses of nucleic acids.

22 Prepare one JetStar Mini Column from the RNeasy kit for each RNA isolation sample. Remove the cap of a 15 ml Collection Tube (provided) and place the JetStar Mini Column inside it. The column will hang in the Collection Tube.

23 Add 2 mL of Solution SR5 to the JetStar Mini Column. Allow it to completely gravity flow through the column and collect in the 15 ml Collection Tube.

Note

Do not allow the column to dry out before loading sample in the next step.

24 Add the RNA isolation sample from Step 21 onto the JetStar Mini Column and allow it to gravity flow through the column into the 15 ml Collection Tube.

Note

Expect the flow-rate through the column to be slow (and highly variable between samples) - reserve enough time accordingly. If possible, allow samples to flow through under gravity only. If time to flow exceeds 1 hr, add positive pressure to the column using a syringe plunger. Do not force the flow at a rate of >1 drop per second.

25 Add 1 mL of Solution SR5 to the JetStar Mini Column and allow it to completely gravity flow into the 15 ml Collection Tube.

Note

Apply positive pressure with syringe plunger to samples that are stalled. Do not force the flow at a rate of >1 drop per second.

26 Transfer the JetStar Mini Column to a new 15 ml Collection Tube. Shake Solution SR6 to mix and then add 1 mL to the JetStar Mini Column to elute the bound RNA. Allow Solution SR6 to gravity flow into the 15 ml Collection Tube.

Note

Apply positive pressure with syringe plunger to samples that are stalled. Do not force the flow at a rate of >1 drop per second.

27 Transfer the eluted RNA to a 2.2 ml Collection Tube. Add 1 mL of Solution SR4. Invert at least once to mix and incubate at 20 °C for a minimum of 00:10:00 .

> Note
>
> Samples can also be incubated at -20 °C Overnight , to split continue the procedure on the next day.

28 Centrifuge the 2.2 ml Collection Tube at 13000 x g for 00:15:00 to pellet the RNA.

29 Decant the supernatant and invert the 2.2 ml Collection Tube onto a paper towel for 00:30:00 to air dry the pellet.

30 Resuspend the RNA pellet in 100 µL of Solution SR7. Observe the pellet carefully to make sure it is fully re-suspended. The RNA is now ready for downstream applications.

> Note
>
> Observe the base of the tube while slowly rotating the sample - there should not appear to be any material adhered to the side or base of the tube, and the solution should flow smoothly.

Inhibitor Removal 1h

31 Use the Zymo OneStep PCR Inhibitor Removal Columns to remove inhibitors that were co-concentrated with the nucleic acids from the extracts to prevent downstream issues.

OneStep PCR Inhibitor Removal Kit **Zymo Research Catalog #**D6030

32 Place one column into one collection tube for each sample you need and add 600 µL of Prep solution to each column.

33 Centrifuge collection tubes in columns at 8000 x g for 00:03:00 .

34 Discard collection tube with flow through and load column into a labeled 1.5mL LoBind tube.

35 Add the full volume of RNA/DNA extract (100 µL) to each column.

36 Centrifuge LoBind tubes with column at 16000 x g for 00:03:00

37 This extract can be used for cDNA synthesis or as template for a one-step PCR reaction. Immediately perfom Nanodrop analysis, Qubit analysis, and/or reverse transcriptase if desired, or store eluent at -80 °C for later use.