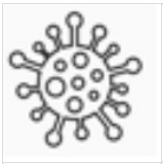

Sep 16, 2020

Concentration of viruses from wastewater influent using organic flocculation (PEG)

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1 Works for me dx.doi.org/10.17504/protocols.io.bi6jkhcn

Wastewater-based epidemiology working group

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ABSTRACT

This SOP describes pre-analytical procedures to be followed for the isolation and identification of SARS-CoV-2 RNA in influent wastewater samples from wastewater treatment plants. PEG precipitation is used to concentrate viruses in the samples. The concentrated pellet is recovered and resuspended to be utilized for the next steps of the protocol. Bovine coronavirus is used as a recovery control for the concentration step. The RNA extraction is acheived using a commercial kit and an inhibitor removal kit is used to remove inhibitors that were co-extracted with the RNA. The RNA from this protocol can be used in droplet digital RT-PCR assays. A protocol for the dd-RTPCR assays is available from our group.

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.

DOI

dx.doi.org/10.17504/protocols.io.bi6jkhcn

PROTOCOL CITATION

Katy Graham, Marlene Wolfe, Stephanie Loeb, Alexandria B Boehm, Krista Wigginton 2020. Concentration of viruses from wastewater influent using organic flocculation (PEG). **protocols.io** https://dx.doi.org/10.17504/protocols.io.bi6jkhcn

KEYWORDS

COVID-19, SARS-CoV-2, RNA, wastewater, Stanford, Michigan, influent, coronavirus

CREATED

Jul 30, 2020

LAST MODIFIED

Sep 16, 2020

PROTOCOL INTEGER ID

39851

STEPS MATERIALS

NAME	CATALOG #	VENDOR
Bovilis Coronavirus Calf Vaccine	16445	Merck Animal Health

MATERIALS TEXT

Materials for step 1 (Concentrate)

- 50 mL falcon tube
- Serological pipettes (25mL)
- Serological pipette
- Styrofoam bucket with ice
- PEG8000
- NaCl
- 1000 uL pipet tips
- 1000 uL pipet
- 1mL cryotube
- Centrifuge
- Eppendorf tubes
- Bovine coronavirus (BCoV) stock (~10^7 gc/mL when resuspended in 3 mL of water)
- Tube rack

Materials for step 2 (RNA extraction)

- Qiagen AllPrep PowerViral DNA/RNA extraction kit (#28000-50)
- b-mercaptoethanol (molecular biology grade)
- LoBind tubes (for -80°C storage)
- 1000p pipet
- 1000p pipet tips (RNAse/DNase-free)
- 200p pipet
- 200p pipet tips (RNAse/DNase-free)
- Heat block (in BSC)
- 10p pipet
- 10p pipet tips (RNAse/DNase-free)
- Vortex with the adapter (in BSC)

Materials for step 3 (inhibitor removal)

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

Concentrate: 3 hours on day 1 and 1 hour on day 2 for 8 samples 4h

1 Remove chosen samples from freezer storage, and thaw at 4°C. This should take about five hours for a 50 mL conical

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tube, and samples should not be placed at 4°C longer than 24 hrs prior to processing. Record the sample's volume.

Follow safe biosafety practices throughout. Samples should never be opened outside the biosafety cabinet (BSC). Bring the centrifuge rotor to the BSC before opening.

2 Load samples into centrifuge rotor, ensuring that samples are balanced. Centrifuge at **24000 x g, 4°C, 00:15:00** to remove suspended solids.

3 Remove rotor from centrifuge and open it in the BSC. Pipette off about 40mL of supernatant, avoiding the solid pellet, into a new 50mL falcon tube.

4 Individually spike each sample with **75 µl** BCoV stock. Pipet the spike directly into the samples. Gently vortex to mix and allow to equilibrate **On ice** for **00:30:00** .

Bovilis Coronavirus Calf Vaccine
by Merck Animal Health
Catalog #: 16445

5 Add **8 g** / **100 mL** PEG8000 and **0.2 Molarity (M)** NaCl to each sample and shake to mix. The PEG8000 may clump together, so gentle vortexing may be applied.

In order to determine the correct mass of PEG8000 and NaCl to add, calculate mass based on the sample volume.

6 Incubate sample at **4 °C** overnight.

If it is important to complete concentration in a single day incubation may be shortened to no less than 4 hours, however overnight incubation produces the best results.

7 Remove sample from 4°C the next morning. Load samples into centrifuge rotor, ensuring that samples are balanced. Centrifuge at **20000 x g, 4°C, 00:30:00** .

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8 Remove samples from the rotor, and remove and discard ~20mL of sample. Wash the sides of the tube with the remaining supernatant.

9 Centrifuge again at **20000 x g, 00:20:00** .

10 Remove samples from rotor and pipet off remaining supernatant. Pipet cautiously, as the pellet itself is invisible or may look like faint traces of dirt.

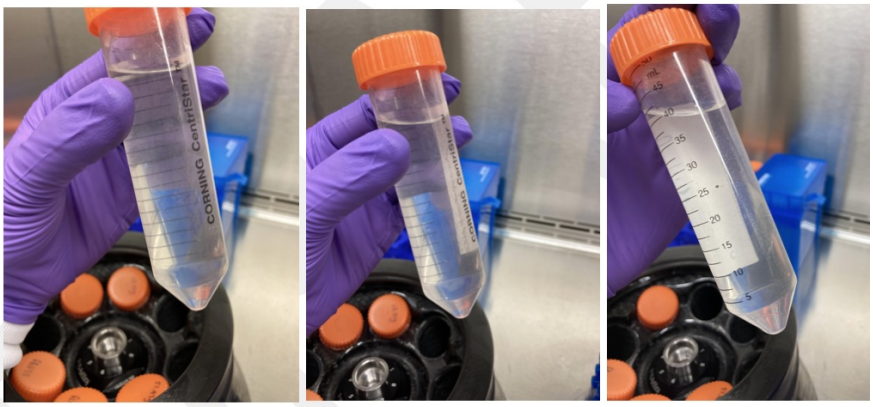

Figure 1: Various PEG pellets from different samples. The pellet is very faint and appears different from sample to sample.

11 Resuspend the pellet in a total volume of **200 µl** , using 1x PBS to supplement any liquid remaining in the tube.

12 Gently vortex to resuspend the pellet.

13 Transfer the resuspended pellet to a 1mL cryotube and store at **-80 °C** .

Extract RNA: 5 hours per 20 samples

14 RNA extraction is performed using the Qiagen AllPrep PowerViral Kit.

Before starting:

Prepare Solution PM1/β-ME: Warm Solution PM1 at **55 °C** for **00:10:00** prior to use to dissolve precipitates. Use Solution PM1 while still warm. Shake to mix before using.

Add β-ME to Solution PM1 to produce a mixture of Solution PM1/β-ME with a final β-ME concentration of 10 µl/ml (**6 µl** of β-ME with **594 µl** warm PM1 per sample). The mixture of Solution PM1/β-ME loses its effectiveness over time, so prepare a fresh batch each time you use the kit. You will need **600 µl** of the Solution PM1/β-ME mixture per prep.

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15 Pipet **200 µl** of viral concentrate into a PowerBead Tube, Glass 0.1 mm. Include one extraction blank per set of extractions.

16 Add **600 µl** of the Solution PM1/β-ME mixture to the PowerBead Tube.

17 Secure the bead tubes horizontally to a Vortex Adapter (cat. no. 13000-V1-24). The tube caps should be pointing toward the center of the adapter. Vortex at maximum speed for **00:10:00** in the BSC.

18 Centrifuge at **13000 x g, 00:01:00** at room temperature. Transfer the supernatant to a clean 2 ml Collection Tube.

Stick the pipet tip down into the beads to pipet out all of the supernatant- it is okay if you get some beads because they will be centrifuged into a pellet in the following steps.

19 Add **200 µl** of Solution IRS and vortex briefly to mix. Incubate at **4 °C** or on ice for **00:05:00** .

IRS is the inhibitor removal solution- this is a really important step!

20 Centrifuge at **13000 x g, 00:01:00** . Avoiding the pellet, transfer the supernatant to a clean 2.2 ml Collection Tube. Do not transfer more than **700 µl** .

If you have access to a QIAcube, this is where the QIAcube Connect protocol begins.

21 Add **600 µl** each of Solution PM3 and Solution PM4 to each tube. Vortex briefly to mix.

22 Load **625 µl** of supernatant into an MB Spin Column and centrifuge at **13000 x g, 00:01:00** . Discard the flow through and repeat until all the supernatant has been loaded onto the MB Spin Column.

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23 Shake to mix Solution PM5 and add **600 µl** to the MB Spin Column. Centrifuge at **13000 x g, 00:01:00** .

24 Discard flow-through. Add **600 µl** of Solution PM4. Centrifuge at **13000 x g, 00:01:00** .

25 Discard flow-through and Centrifuge at **13000 x g, 00:02:00** to remove residual buffer.

26 Place the MB Spin Column in a clean 2 ml Collection Tube.

27 Add **50 µl** of RNase-Free Water to the center of the MB Spin Column membrane. Incubate for **00:05:00** at room temperature. Pipet directly onto white membrane using a REACH pipet tip if needed.

28 Centrifuge at **13000 x g, 00:01:00** . Discard the MB Spin Column. The DNA/RNA is now ready for downstream applications. Proceed with inhibitor removal immediately before storing DNA/RNA for future use.

Inhibitor Removal: 1 hour per 24 samples

29 Place one column into one collection tube for each sample you need and add **600 µl** of Prep solution to each column.

30 Spin at **8000 x g, 00:03:00** ; discard collection tube with flow through.

31 Put column into a labeled 1.5mL LoBind tube.

32 Add ~ **50 µl** of RNA/DNA extract to each column.

33 Spin at **16000 x g, 00:03:00** .

34 Use cleaned extract for Nanodrop, Qubit, or RT, and store at **-80 °C** until analysis.

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