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

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**Protocol status:** Working
**We use this protocol and it's working**

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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.

## Materials

STEP MATERIALS

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

### 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

## Protocol materials

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

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

**Safety information**

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 .

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5 Add 8 g / 100 mL PEG8000 and [M] 0.2 Mass Percent NaCl to each sample and shake to mix. The PEG8000 may clump together, so gentle vortexing may be applied.

**Note**

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.

Note

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 .

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.

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.

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.

Note

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 .

Note

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 .

> Note
>
> 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.

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.