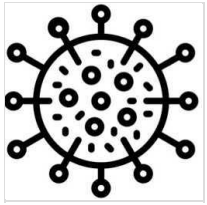

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Dec 05, 2021

High Throughput pre-analytical processing of wastewater settled solids for SARS-CoV-2 RNA analyses V.2

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Wastewater-based epidemiology working group

APHL Wastewater Surveillance Community of Practice

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This process instruction describes the steps for pre-analytical processing of primary settled solids from wastewater treatment plants for downstream nucleic acid purification and quantification. Previous research has demonstrated that enveloped viral particles, such as SARS-CoV-2, associate with the solids in wastewater. Therefore, concentrating the solids in the sample and removing the water concentrates the viral particles as well and increases the sensitivity of the assay.

Bovine coronavirus vaccine (BCoV) is spiked into samples before nucleic acid extraction and serves as a process control as well as an indicator of PCR inhibition.

The dry weight of the sample is determined to account for sample variability between different treatment plants and between samples collected on different days and allows for the final quantification to be normalized to the precise quantity of solids in the sample. A dry weight conversion factor to adjust for the final amount of solids in the quantified sample is determined by measuring the difference in mass between the “wet” dewatered solid and after the sample is dried at 110°C overnight.

For long term storage, up to 50mL of the original primary settled solid sample is stored at 4°C and small aliquot of the dewatered solids is stored at -80°C.

This process instruction applies to sample dewatering, BCoV control spike in, homogenization, dry weight measurement, aliquoting and storage.

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Equipment
●Rainin Single-Channel Pipettes L1000XLS+, L200XLS+, L20XLS+, L10XLS+, , L2XLS+
●SPEX™ SamplePrep 2010 Geno/Grinder™
●Sorvall LYNX 4000 Superspeed Centrifuge
●Mettler Toledo Excellence Plus Analytical Balance
●Zebra GX430t label printer

Reagents
●Zymo DNA/RNA Shield
●Nuclease Free Water

Consumables
●Rainin low retention, pre-sterilized, filter tips 1000 µL, 200 µL, 20 µL
●Rainin Wide Orifice, Low Retention, pre-sterilized, filter LTS tips 200 µL
●OPS Diagnostics 5/32” Grinding Balls
●Reagent Reservoirs
●VWR® Disposable Polypropylene Spatulas
●VWR® Disposable Aluminum Crinkle Dishes with Tabs
●Disposable Serological Pipets, Polystyrene, Sterile (Individually Wrapped) 2 mL (2 mL serological aspirating pipette)
●Disposable Serological Pipets, Polystyrene, Sterile, Plugged (Individually Wrapped) 5 mL (5 mL serological pipette)
●Disposable Serological Pipets, Polystyrene, Sterile, Plugged (Individually Wrapped) 10 mL (10 mL serological pipette)
●Disposable Serological Pipets, Polystyrene, Sterile, Plugged (Individually Wrapped) 25 mL (25 mL serological pipette)
●Falcon 50 mL conical tubes
●150mL bottle
●2.5” x 0.5” Cryogenic Labels for the Zebra GX430t label printer
●Large Kim Wipes

Samples and Test Materials
●Settled solid wastewater samples from wastewater treatment plants
●Attenuated bovine coronavirus vaccine (PBS Animal Health, Calf-Guard Cattle Vaccine)

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Preparation 5m

1
- Process fresh samples stored at **4 °C** if possible. If analyzing a frozen sample, thaw at **Room temperature** .
- Set centrifuge temperature to **4 °C** .
- Set oven temperature to **110 °C** .
- Remove an aliquot of BCoV from the freezer and thaw **On ice** .

Dewater Sludge Samples by Centrifugation

2 Label 50 mL conicals with the sample name.

3 Invert the sludge sample bottle a few times to mix.

4 In a biosafety cabinet, pour **50 mL** of well mixed primary sludge sample to corresponding labeled 50 mL conical. If the total volume of a sample is less than 50 mL, bring the volume up to 50 mL with nuclease free water.

5 Bring the rotor to a biosafety cabinet and arrange the conicals in the rotor in a manner to ensure that it is balanced.

6 Centrifuge samples at **24000 x g** for at least **00:30:00** at **4 °C** . Set the Centrifuge time to “Hold”. 30m

7 In a biosafety cabinet, aspirate supernatant into a liquid biohazard waste container.

BCoV Spike-in and Sample Homogenization

8 Apply a label to a new 50 mL conical for each sample and record the empty conical mass in grams.

With a spatula (either plastic or metal), scoop a small aliquot of dewatered pellet (~0.75 g) into

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9 the new, pre-weighed 50 mL conical. Record the mass in grams of the aliquot in the conical. Set the rest of the pellet aside for dry weight measurement.

It is important to note the wet mass of solids in this conical as it is essential in resuspending the solids in buffer to the right concentration and back calculating the concentration of the gene targets per dry mass of solids.

10 In a new 150 mL bottle, prepare a stock solution of DNA/RNA shield with BCoV spike-in by adding **1.5 µL** rehydrated BCoV vaccine per **1 mL** DNA/RNA shield. Mix well by inverting or gently shaking the bottle.

The concentration of BCoV target is approximately 500,000 copies / ml in this solution.

DNA/RNA Shield **Zymo Research Catalog #R1100-50**

Bovine coronavirus **Zoetis Catalog #CALF-GUARD**

11 Add DNA/RNA shield with BCoV spike-in to each conical containing solids so that the final concentration of dewatered solids resuspended in the BCoV-spiked DNA/RNA shield is ~ **75 mg/mL**

75 mg dewatered solids per ml BCoV-spiked DNA/RNA shield was chosen during an exercise where we titrated the solids in the mixture (spiked with gRNA of SARS-CoV-2) to determine the maximum mass per volume where we did not see inhibition of the SARS-CoV-2 assays in digital PCR. You may want to repeat this titration with your samples to ensure it is appropriate for alleviating inhibition.

11.1 Save the remaining DNA/RNA shield with BCoV spike-in to use as template in Extraction Positive Control during RNA Extraction.

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12 Add 5-10 grinding balls to each conical tube.

13 Place the conicals in the adapters on the stage of the Geno/Grinder and tightly secure the clamp.

Geno/Grinder
Automated Tissue Homogenizer and Cell Lyser
SPEX Sample Prep **1006-113806**

14 Shake the conicals in the Geno/Grinder for **00:02:00** at 1,000 rpm to homogenize the sample. 2m

15 Briefly centrifuge the conicals for **00:05:00** at **5250 x g** to pull down the bubbles generated during homogenization. Sample is ready for RNA extraction using the **Chemagic 360** (see accompanying protocol, extraction may also be done using Kingfisher or several manual Qiagen kits. See protocol for details). 5m

A modification of this step may provide greater sensitivity. The modification entails centrifuging at a higher speed of **24000 x g** to pellet the solids and then extracting the RNA from the resultant supernatant.

Dry weight measurement

16 Place aluminum weigh boats in oven to pre-heat to **110 °C** .

17 Weigh each pan and record the mass in grams of each empty pan.

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18 Using a spatula, scoop a generous amount of dewatered solid into the pan.

19 Record the mass in grams of each pan with the wet solids.

20 Place the dishes in the oven inside the biosafety cabinet and leave overnight to dry,

21 The next day or at least 6 hours later (**06:00:00** - **24:00:00** dry time), remove the pans from the oven and record the mass in grams. 1d 6h

The dry weight percent is calculated as:

(100)*(1 - (wet mass of solids - dry mass of solids) / (wet mass of solids))

This percentage is key for calculation of the concentration of the gene targets per mass dry weight.

22 Dispose of pans in the red biohazard waste bin.

Aliquot and Sample Storage

23 Apply a label for each sample to a 50 mL conical.

24 Pour up to **50 mL** of remaining primary settled solids sample and place at **4 °C** for storage until it is confirmed that results of the analyses pass quality control. Dewatered solids can be stored at -80 deg C. We recommend that you confirm the effects of storage on target quantification. We have noted that up to 7 d storage at 4 deg C has minimal affect on the RNA targets. However, freeze thaws may affect target quantification.

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25 Dispose of remaining sludge in 4 L Biohazard waste container prefilled with **400 mL** of bleach. Then dispose of the sample bottles as biohazard waste.

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