Concentration of viruses from sewage using HA filters V.2

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GUIDELINES
Each sewage samples is processed in triplicate: two technical duplicates and one archive.

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MATERIALS TEXT

Bovine coronavirus solution prepared according to Bovine coronavirus **Zoetis Catalog #CALF-GUARD** Cellulose esters filters 0.8 µm 47 mm diameter **Emd Millipore Catalog #AAWP04700** Step 6.2 RNeasy PowerMicrobiome Kit **Qiagen Catalog #26000-50** Step 6.5 ZR BashingBead Lysis Tubes (2mm) **Zymo Research Catalog #S6003-50** Step 6.5

Gast DOA-P704-AA
High-Capacity Vacuum Pump, with Gauge, Regulator, and Relief
Cole-Parmer **EW-07061-40**

Magnetic Filter Funnel
300 mL, 47 mm
Pall **4242**

Pipette controller
25 ml serological pipette

SAFETY WARNINGS

The filtration has to be performed in a biosafety cabinet to avoid any contact with raw sewage.

BEFORE STARTING

Before starting the filtration:

1. Clean the working area and all equipments: wipe down with 10% bleach and let stand for 10 min, then rinse with water and dry. Next, wipe down using 70% ethanol and let dry.
2. Verify that the carboy to receive filtration waste is not full or near to be full.
3. Keep forceps sterile by placing them in a small beaker containing 70% ethanol.
4. Prepare an ice bucket.
5. Thaw the bovine coronavirus (BCoV) recovery control on ice. Do not thaw the same aliquot more than twice.
6. Add $MgCl_2$ stock solution in 50-mL tubes for a final concentration of 25 mM.
7. Warm Solution PM1 (provided in RNeasy PowerMicrobiome kit) at 55°C for at least 10 min prior to dissolve precipitates. Shake to mix before transferring 650 µL into each 2-mL bead beating tubes (3 tubes needed per sample).

When work is completed, remove equipments and supplies from the cabinet. Wipe the work area with 10% bleach, let stand for 10 min, rinse with water, then with 70% ethanol. Finally, turn on the UV lamp for 15 minutes. Empty the carboy.

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Filtration of the sewage samples (in the biosafety cabinet)

1 Take the sample out from the refrigerator.

2 Homogenize the sample thoroughly, avoiding foaming.

3 Use a pipette controller, transfer 25 mL of the sample into a 50-mL tube (where $MgCl_2$ has been previously added [M]**25 Milimolar (mM) final concentration**). Repeat for each replicate. Save the rest of sample at 4ºC for archive or until decontamination and disposal.

4 Spike in each replicate 5 µL of Bovine Coronavirus recovery control (~100,000 cp/uL), prepared as Bovine coronavirus solution prepared according to http://dx.doi.org/10.17504/protocols.io.bpg8mjzw

5 Put the 50-mL tubes on ice until further processing.

6 Filter the samples in the 50-mL tubes:

6.1 Rinse the clean filtration funnel with sterile deionized water.

Magnetic Filter Funnel
300 mL, 47 mm
Pall **4242**

Make sure some water is still on the porous plate.

6.2 ⚠

Place the HA filter in the filtration funnel.
Cellulose esters filters 0.8 µm 47 mm diameter **Emd Millipore Catalog #AAWP04700** filter

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Make sure the filter gets wet due to the residual water on the porous plate.

6.3 Homogenize the sample in the 50-mL tube. Avoid as much as possible the liquid to touch the lid.

6.4 Filter the content in the 50-mL tube through a 0.80 µm 47mm cellulose ester filter at low vacuum pressure (~50 mmHg VAC).

Gast DOA-P704-AA
High-Capacity Vacuum Pump, with Gauge, Regulator, and Relief
Cole-Parmer EW-07061-40

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6.5

As soon as the filtration is complete, transfer the filter to a 2-mL ZR BashingBead Lysis Tubes containing 650 µL of PM1 buffer solution (previously added).

RNeasy PowerMicrobiome Kit **Qiagen Catalog #26000-50**

It is important not to “over-dry” the filter on the filtration system.

Fold the filter as shown in Figure 1. Do not crease the filters with the forceps.

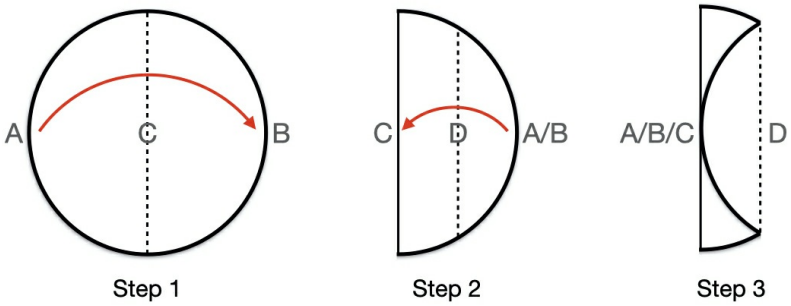

Figure 1 Filter folding procedure. Step 1: fold the filter in half. Step 2: repeat that step. Step 3: transfer to a 2-mL tube.

ZR BashingBead Lysis Tubes (2mm) **Zymo Research Catalog #S6003-50**

6.6 Put the 2-mL tubes on ice until further processing.

6.7 Repeat Steps 6 for the other replicates.

7 Store filters at -20ºC until all samples are processed

8

Store filters at -80ºC for a minimum of 2h or overnight prior to the nucleic acid extraction.
Archive filters at -80ºC.

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Nucleic acid extraction

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Nucleic acid extraction - RNeasy PowerMicrobiome Kit (Qiagen)
by McLellan Lab

PREVIEW RUN

9.1 For **HA filter** extractions, Add 6.5 µL of beta-Mercaptoethanol to each of the 2-mL tubes.

For **direct extraction** of raw sewage, homogenize the sewage sample (avoid foaming) and take 150 µL into a 2-mL tube (provided in the RNeasy PowerMicrobiome kit). Add 4 µL of beta-Mercaptoethanol and 400 µL of Solution PM1. Also add 5 µL of 1:10 diluted BCoV solution.

Add the beta-Mercaptoethanol while the HA sample still frozen. Let thaw at room temperature.

9.2

If you extract from **HA filters**, place the 2-mL tubes in the bead beater.
Skip step 2 if you do **direct extraction**.

Mini-Beadbeater-16
high-energy cell disrupter
BioSpec **607**
1 speed

9.2.1 Bead beat for **00:02:30** 2m

Start the bead beating when the beads start to be loose in the tubes.

9.2.2 Cooldown the samples on ice for **00:05:00** . 5m

9.2.3 Repeat Steps 9.1 and 9.2 once .

9.3 Centrifuge at maximum speed for 1 min at room temperature. **150000 rpm, Room temperature , 00:01:00**

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9.4 To extract from **HA filters**, transfer 450 µL of supernatant to a Collection Tube (provided in the RNeasy PowerMicrobiome kit). Transfer all supernatant for **direct extraction**.

9.5 To extract from **HA filters**, add 150 µL of Solution IRS and vortex briefly to mix. For **direct extraction**, add 100 uL of Solution IRS. Incubate at 2–8°C for 5 min.

9.6 Centrifuge at maximum speed for 1 min **150000 rpm, Room temperature , 00:01:00** . Avoiding the pellet and transfer the supernatant to a new Collection Tube.

9.7 Add 650 µL each of Solution PM3 and Solution PM4. Vortex briefly to mix.

9.8 Load 650 µL of the mixture into an MB Spin Column.

9.9 Centrifuge at max speed for 1 min **150000 rpm, Room temperature , 00:01:00** . Discard the flow-through and repeat until all the mixture has been loaded onto the MB Spin Column.

9.10 Shake to mix Solution PM5 and add 650 µL to the MB Spin Column.

9.11 Centrifuge at max speed for 1 min **150000 rpm, Room temperature , 00:01:00** .

9.12 Discard flow-through. Add 600 µL of Solution PM4.

9.13 Centrifuge at max speed for 1 min **150000 rpm, Room temperature , 00:01:00** .

9.14 Discard flow-through and centrifuge filter at max speed for an additional 2 min **150000 rpm, Room temperature , 00:02:00** .

9.15 Place the MB Spin Column in a clean 2-ml Collection Tube (provided in the RNeasy PowerMicrobiome kit).

9.16 Add 60 µL of RNase-Free Water (warmed to 55°C) to the center of the MB Spin Column membrane. Incubate at room

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temperature for at least 1 min **00:01:00** .

9.17 Centrifuge at max speed for 1 min **150000 rpm, Room temperature , 00:01:00** . Discard the MB Spin Column.

The DNA/RNA is now ready for downstream applications. RNA extract may be stored in RNase-free water at -80ºC for 1 year.

Quantification

10

Quantification by Droplet Digital PCR (ddPCR)
by McLellan Lab

PREVIEW RUN

10.1 When all reagents are thawed on ice, vortex Supermix, Reverse transcriptase and DTT throughly for 30 seconds. Vortex to mix primers and probes stocks.

10.2 Prepare the reaction matrix (for one well, beside sample RNA) according to the table below. Prepare Use a low-binding tube of appropriate volume to mix all the components according to the reaction numbers. Always include extra wells when setting up reaction to avoid potential volume shortage caused by pipetting.

Component	Volume per reaction, uL	Final concentration
Supermix	5.5	1x
Reverse transcriptase	2.2	20 U/uL
300 mM DTT	1.1	15 mM
Primer mix (forwad + reverse)	1.1	900 nM
Probe	1.1	250 nM
RNase-free water*	5.5	/
Total	16.5	/

* Note: Water volume can be replaced accordingly by another assay (e.g., duplex assay), or another RNA template (e.g., inhibition test).

10.3

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