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Protocol status: In development
 We are still developing and optimizing this protocol

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 44287

🌐 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.

MATERIALS

Bovine coronavirus solution prepared according to

Bovine coronavirus Zoetis Catalog #CALF-GUARD

Cellulose esters filters 0.8 µm 47 mm diameter Merck Millipore (EMD Millipore) Catalog #AAWP04700

RNeasy PowerMicrobiome Kit Qiagen Catalog #26000-50

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

Equipment	
Gast DOA-P704-AA	NAME
High-Capacity Vacuum Pump, with Gauge, Regulator, and Relief	TYPE
Cole-Parmer	BRAND
EW-07061-40	SKU
https://www.coleparmer.com/i/gast-doa-p704-aa-high-capacity-vacuum-pump-with-gauge-regulator-and-relief-1-1-cfm-25-5-hg-115-vac/0706140	LINK

Equipment	
Magnetic Filter Funnel	NAME
300 mL, 47 mm	TYPE
Pall	BRAND
4242	SKU
https://shop.pall.com/us/en/products/zid4242?CategoryName=ID29&CatalogID=Laboratory	LINK

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 START INSTRUCTIONS

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.

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 millimolar (mM) final concentration). Repeat for each replicate. Save the rest of sample at 4°C 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

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.

Equipment

Magnetic Filter Funnel

NAME

300 mL, 47 mm

TYPE

Pall

BRAND

4242

SKU

[https://shop.pall.com/us/en/products/zid4242?
CategoryName=ID29&CatalogID=Laboratory](https://shop.pall.com/us/en/products/zid4242?CategoryName=ID29&CatalogID=Laboratory)

LINK

Safety information

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 Merck Millipore (EMD Millipore) Catalog #AAWP04700

filter

Safety information

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).

Equipment

Gast DOA-P704-AA

NAME

High-Capacity Vacuum Pump, with Gauge, Regulator, and Relief

TYPE

Cole-Parmer

BRAND

EW-07061-40

SKU

<https://www.coleparmer.com/i/gast-doa-p704-aa-high-capacity-vacuum-pump-with-gauge-regulator-and-relief-1-1-cfm-25-5-hg-115-vac/0706140>

LINK

6.5

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

RNeasy PowerMicrobiome Kit Qiagen Catalog #26000-50

Safety information

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

Safety information

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 -20°C until all samples are processed

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

Archive filters at -80°C.

Nucleic acid extraction

9

Protocol

NAME

Nucleic acid extraction - RNeasy PowerMicrobiome Kit (Qiagen)

CREATED BY

McLellan Lab

PREVIEW

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.

Safety information

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**.

Equipment	
Mini-Beadbeater-16	NAME
high-energy cell disrupter	TYPE
BioSpec	BRAND
607	SKU
https://biospec.com/product/mini-beadbeater-16	LINK
1 speed	SPECIFICATIONS

9.2.1 Bead beat for 00:02:30 2m

Safety information

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

9.4 To extract from **HA filters**, transfer 450 μ L 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 µL 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 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.

Safety information

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

Quantification

10

Protocol

NAME

Quantification by Droplet Digital PCR (ddPCR)

CREATED BY

McLellan Lab

PREVIEW

10.1 When all reagents are thawed on ice, vortex Supermix, Reverse transcriptase and DTT thoroughly 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 (forward + 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