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# Concentration of viruses from sewage using HA filters V.4

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*In Development*

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ABSTRACT

**Concentration of viruses from sewage using HA filters**

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GUIDELINES

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

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

ZR BashingBead Lysis Tubes (2mm) **Zymo Research Catalog #S6003-50** Step 6.5

Maxwell(R) HT Environmental TNA Kit custom **Promega Catalog #AX9190** 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.

DISCLAIMER:



BEFORE STARTING

Before starting the filtration:

1. Clean the working area and all equipment: 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.



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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. Add 400 µL of CTAB in each 2-mL bead beating tubes (2 tubes needed per sample).

When work is completed, remove equipment 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 Milimolar (mM) final concentration** ). Repeat for each replicate. Save the rest of the 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.



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6.2 ⚠

Place the HA filter in the filtration funnel.

Cellulose esters filters 0.8 µm 47 mm diameter **Emd Millipore Catalog #AAWP04700** filter

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).




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Gast DOA-P704-AA
High-Capacity Vacuum Pump, with Gauge, Regulator, and Relief

Cole-Parmer EW-07061-40

6.5 ⚠

As soon as the filtration is complete, transfer the filter to a 2-mL ZR BashingBead Lysis Tubes containing 400 µL of CTAB buffer solution (previously added).

Maxwell(R) HT Environmental TNA Kit custom **Promega Catalog #AX9190** Transfer

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.

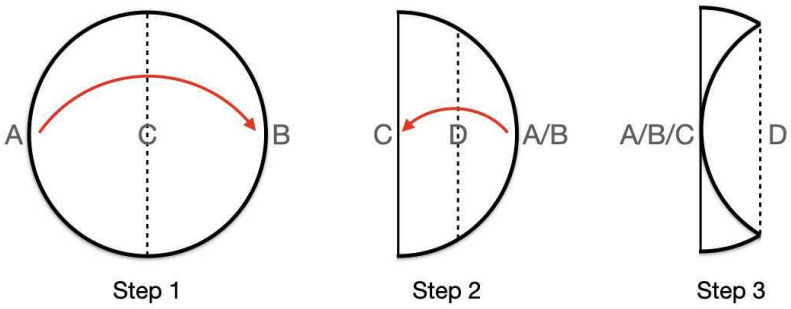


Figure 1 Filter folding procedure. Step 1: fold the filter in half. Step 2: repeat that step. Step 3: Transfer to a 2-mL tube.

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.



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

8

Store filters at -80ºC for a minimum of 1h or overnight prior to the nucleic acid extraction.
Archive filters at -80ºC.

Nucleic acid extraction

9

Total nucleic acid extraction - Maxwell(R) HT Environmental TNA Kit, custom (Promega)
**by McLellan Lab**

PREVIEW RUN

9.1 5m

For **HA filter** extraction, let the sample thaw on ice and go to **step 2**.

For **BCoV/BRSV** extraction (in duplicate), add 5 µL of BCoV/BRSV solution to the 2-mL tube containing 250 µL CTAB. Vortex for 15 seconds (speed 7 out of 10) and flash freeze the tube. Go to step 4.

For **Direct extraction**, add 150 µL of wastewater to the 2-mL tube containing 100 µL CTAB. Vortex for 15 seconds (speed 7 out of 10) and flash freeze the tube. Go to step 4.

9.2

For **HA filter** extraction, place the 2-mL tubes in the bead beater.

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

9.2.1 2m 30s

Bead beat for **00:02:30**

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

9.2.2 5m

Cooldown the samples on ice for **00:05:00** .



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9.2.3 Repeat Steps 9.1 and 9.2 once .

7m 30s

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

1m

9.4 For **HA filter** extraction, transfer 125-250 µL of supernatant to the **Lysis/Bind plate**.
For **BCoV/BRSV/Direct extraction**, transfer all supernatant to the **Lysis/Bind plate**.

10m

The default volume transferred is 250 µL. However, for WWTPs with "dirty" influents, we only transfer 125 µL.

9.5 Start the protocol Promega_Maxwell_HT_RNA_Wastewater_V1.bdz on the KingFisher Flex **01:14:00**

1h 14m

Kingfisher Flex
Automated Extraction System
**ThermoFisher** **5400630**

9.6 Transfer the purified sample from the **Elution plate** to the **microcentrifuge tubes**.

10m

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

9.7 Quantification by Droplet Digital PCR (ddPCR)

dx.doi.org/10.17504/protocols.io.bpg6mjze

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



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to mix primers and probes stocks.

10.2 

Plate Set up: see sub-steps below.

This step requires to keep the mixture cold/on ice.

10.2.1 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 other ingredients, such as another assay (e.g., duplex assay) or another RNA template (e.g., inhibition test).

1. Keep all reagents on ice during the process as well as the matrix.

2. Always prepare a total of 8* (N columns) wells for droplet generation, or use ddPCR Buffer Control for Probes (#1863052) to fill empty wells on the last cartridge.

3. Make sure sticky reagents are added the correct volumes and not left in the tips, i.e., Supermix, RT.

10.2.2 Place a 96-well PCR plate onto a low temp PCR rack, or on ice. Disperse 16.5 µL of reaction matrix into each well of a 96-well PCR plate. For runs with multiple columns, calculate (the matrix total volume/8) and evenly distribute the matrix into an 8-well PCR strip. Then use an appropriate multichannel pipette (e.g., 2- 20 µL range) to add 16.5 µL of matrix into each well, column by column.



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1. Keep the PCR plate cold/on ice during the process.

2. Lower the pipetting speed to avoid liquid leftover to the inner side of the tips.

10.2.3 Gently vortex at half speed to mix the RNA sample. Make sure no liquid is attached on the lid. Add 5.5 µL of sample RNA into each well containing 16.5 µL of reaction matrix, making the total volume of each reaction 22 µL.

1. Keep the PCR plate and RNA samples cold/on ice throughout the process.

2. Do not over vortex the RNA samples.

3. Pipetting robot may be used to add RNA samples to the matrix.

10.2.4 Seal the PCR plate. Centrifuge down gently at 1000 rpm for 30s. Take out the plate and vortex on a 96-well plate mixer at 1600 rpm for 30s. Centrifuge again at 1000 rpm for 30s to settle down the plate.

10.3 

Droplet generation handling.

It is NOT required to keep the plate cold/on ice during this process.

10.3.1 Prepare materials/reagents on the working bench top, e.g., cartridges, gaskets, ddPCR 96-well plate, droplet generation oil, foil cover. Label the cartridges with corresponding column numbers (e.g., 1 to 12 for a full plate).

10.3.2 Remove the sealing on the PCR plate. Place the cartridge in the cartridge holder. Align well the PCR plate and the cartridge on the bench.
Use an appropriate multichannel pipette (e.g., 2- 20 µL) for liquid transfer. Adjust the pipette at 20 µL and make sure the tips are well positioned. Gently mix the liquid by aspirating up to 2/3 of the tip height and then releasing to a lower level of the tip height, i.e., not to the end of the tip, to avoid creating bubbles. Repeat this mixing step 10 times. In the last movement, slowly aspirate to the full volume of 20 µL.

10.3.3 Transfer the 20 µL reaction matrix to the middle column of the cartridge. Position the tip end to the ridge in well (where the well wall connects to the bottom) at 15º angle. Avoid creating bubbles when releasing liquid from tips into the cartridge; this can be realized by only pressing the plunger to position 2 and not position 3 before pull the tips out from the cartridge. Make sure the cartridge wells are in the same direction as on the PCR plate.

10.3.4 Fill in 70 µl of Droplet Generation Oil into bottom wells of the cartridge and cover the cartridge with a red gasket. Loop outer holes of red gasket around hooks on left and right sides of cartridge holder. Place the gasket equipped cartridge into the droplet generator.



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You can start preparing the next column of droplet generation while waiting for the previous column to be done.

10.3.5 When droplet generation is done, take out the cartridge from the droplet generator and remove the red gasket. Using a Rainin multichannel with recommended Rainin tips, in a leaning position, count to 5 to aspirate all the liquid (i.e., 40 µl) from the droplets column, and press against side of wells of the corresponding column in the ddPCR 96-well plate (i.e., not the previous PCR plate), count to 5 to expel the droplets into the wells.

1. Avoid multiple times of liquid transfer.

2. Eye ball the ddPCR plate when droplet generation and transferring are done.

10.4 Turn on the PX1 PCR plate sealer and let heat to 180ºC. Correctly place the plate support block, the ddPCR plate, foil cover (i.e., red line up) and the metal holder. Seal the plate at 180ºC for 5vs and remove the plate immediately from the sealer.

Sealed plate should have indentations around wells. Always check the sealing before loading the plate on the PCR thermal cycler.

10.5 Load the plate on to a PCR thermocycler. Our lab's assay conditions are shown as below. We use Eppendorf Mastercycler Pro and the ramp speed is set to 50% for RT-ddPCR.

| Assay | Step 1 | Step 2 | Step 3 (40 cycles) | Step 4 (40 cycles) | Step 5 | Step 6 |
|---|---|---|---|---|---|---|
| N1/N2, BCoV, HepG | 50ºC 60 min | 95ºC 10 min | 94ºC 30s | 55ºC 1 min | 98ºC 10 min | 4ºC 30 mins and hold |
| PMMoV | 50ºC 60 min | 95ºC 10 min | 94ºC 30s | 60ºC 1 min | 98ºC 10 min | 4ºC 30 mins and hold |

10.6 List of assay primers and probes.



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| A | B | C | D |
|---|---|---|---|
| CDC N1 | GACCCCAAAATCAGCGAAAT | FAM-ACCCCGCATTACGTTTGGTGGACC- BHQ1 | TCTGGTTACTGCCAGTTGAATCTG |
| CDC N2 | TTACAAACATTGGCCGCAAA | HEX-ACAATTTGCCCCCAGCGCTTCAG-IowaBHQ | GCGCGACATTCCGAAGAA |
| BCoV | CTGGAAGTTGGTGGAGTT | FAM - CCTTCATATCTATACACATCAAGTTGTT-BHQ1 | ATTATCGGCCTAACATACATC |
| HepG | CGGCCAAAAGGTGGTGGATG | HEX-AGGTCCCTCTGGCGCTTGTGGCGAG- BHQ1 | CGACGAGCCTGACGTCGGG |
| PMMoV | GAGTGGTTTGACCTTAACGTTGA | FAM-CCTACCGAAGCAAATG- BHQ1 | TTGTCGGTTGCAATG CAA GT |



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