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Wastewater Concentration using the Electronegative Filtration method, Total Nucleic Acid Extraction and PCR inhibitor removal for recovery of viral pathogens V.1

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Wastewater Surveillanc...

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We use this protocol and it's working

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Abstract

This standard operating procedure (SOP) sets out the workflow for processing wastewater samples with the goal of recovering and detecting viral pathogens. The steps include virus concentration using the electronegative filtration (ENF) method, total nucleic acid extraction using MagMAX Wastewater Ultra Nucleic Acid Isolation Kit, and PCR Inhibitor removal using the Zymo OneStep PCR Inhibitor Removal Kit.

The raw wastewater/sewage samples are concentrated using the ENF method, which operates off the principle of adsorption-elution. The pH of the mixture is first adjusted, after which the sample is poured through a negatively charged membrane. Viruses are reversibly adsorbed to filters via charge attraction, after which they are eluted into a smaller volume. The resulting concentrate is then subjected to nucleic acid extraction and PCR inhibitor removal.

All procedures can be performed by suitably trained members of staff.

Safety information

Infectious agents – Wastewater contains pathogens, including viruses, bacteria and fungi. Appropriate safety precautions must be exercised when handling wastewater samples.

Materials

Reagents

- Hydrochloric acid 1M **Fisher Scientific UK** **Catalog #**10467640
- MagMAX Wasterwater Ultra Nucleic Acid Isolation Kit **Thermo Fisher** **Catalog #**A52606
- Molecular Biological Grade Water **Fisher Scientific UK** **Catalog #**15835408
- Ethanol, absolute 99.8% **Thermo Fisher** **Catalog #**10342652
- Zymo OneStep PCR Inhibitor Removal Kit **Zymo Research** **Catalog #**D6030

Equipment

EZ-Stream® vacuum pump

NAME

Merck

BRAND

EZSTREAM1

SKU

https://www.sigmaaldrich.com/GB/en/product/mm/ezstream1?utm_source=google&utm_medium=cpc&utm_campaign=15001183107&utm_content=127306761543&gad_source=1&gad_campaignid=15001183107&gclid=CjwKCAiAraXJBhBJEiwAjz7MZey-G5×6zzIStElKuApLrcoqbJ7VyRYhyZnSDl9cYXgX80

LINK

Equipment

EZ-Fit® Manifold Filtration Head

NAME

Merck

BRAND

EZFITMHTA3

SKU

https://www.sigmaaldrich.com/GB/en/product/mm/ezfitmhta3

LINK

Equipment

Silicone Tubing

NAME

Merck

BRAND

STREAMTUB

SKU

https://www.sigmaaldrich.com/GB/en/product/mm/streamtub

LINK

Equipment

Fisherbrand™ Microbead Sterilizer

NAME

Fisherbrand

BRAND

16391328

SKU

https://www.fishersci.co.uk/shop/products/microbead-sterilizer-9/16391328?ef_id=CjwKCAiAraXJBhBJEiwAjz7MZQhPF1lZN6R2Xf3k1yoqzyKDTKimSn09nYVAZA_NlFUldyU16kaMgRoCJoIQAvD_BwE:G:s&s_kwcid=AL!4428!3!763122804440!!!g!!&ppc_id=NonBrand_goog_21847340098_172478581

LINK

Equipment

Bochem™ 18/10 Stainless Steel Forceps, Blunt Tip

NAME

Bochem

BRAND

10169920

SKU

https://www.fishersci.co.uk/shop/products/18-10-stainless-steel-forceps/10169920

LINK

Equipment

Cole-Parmer BH-250D-4 Dri-Block Digital Block Heater; Quadruple Insert; 100Deg C; 230 VAC

NAME

Scientific Labs

BRAND

BLO1042

SKU

https://www.scientificlabs.co.uk/product/block-heaters/BLO1042

LINK

Equipment

Fisherbrand™ Lab Thermometer

NAME

Fisherbrand

BRAND

11597273

SKU

https://www.fishersci.co.uk/shop/products/liquid-filled-total-immersion-thermometer-1/11597273#?keyword=Thermometer

LINK

Equipment

Thermo Scientific™ HulaMixer™ Sample Mixer

NAME

Thermo Scientific™

BRAND

10548425

SKU

https://www.fishersci.co.uk/shop/products/hulamixer-sample-mixer/10548425#?keyword=15920D

LINK

Equipment

Fisherbrand™ Mini-Centrifuge 100-240V, 50/60Hz Universal Plug, Grey

NAME

Fisherbrand

BRAND

16617645

SKU

https://www.fishersci.co.uk/shop/products/fisherbrand-standard-mini-centrifuge/16617645

LINK

Equipment

DynaMag™-15 Magnet

NAME

DynaMag

BRAND

12301D

SKU

https://www.thermofisher.com/order/catalog/product/12301D

LINK

Equipment

Scientific Industries SI™ Vortex-Genie™ 2	NAME
SI™	BRAND
15557335	SKU
https://www.fishersci.co.uk/shop/products/vortex-genie-2-4/15557335#?keyword=vortex	LINK

Equipment

Eppendorf™ Centrifuge 5425/5425 R	NAME
Eppendorf™	BRAND
16845110	SKU
https://www.fishersci.co.uk/shop/products/centrifuge-5425-eu-ivd-8/16845110#?keyword=refrigerated%20centrifuge	LINK

- Pipettes
- KingFisher Duo System (Optional - if performing automated extractions)

Consumables

- 50mL conical centrifuge tubes, sterile, nuclease-free
- pH indicator strips (pH 0 -14)
- EZ-Fit filtration unit without absorbent pad 0.45 µm pore size (Sigma Aldrich, Catalog #: EFHAW10MS)
- Sterile, RNAse-free pipette tips
- 2.0 ml microcentrifuge tubes

- KingFisher 6-tip comb and 24 DW plate (Thermo Fisher Catalog #97003510. **KingFisher™ Plastics for 24 deep-well format 24 DW tip comb and plate | Buy Online**)
-

Preparation and setting up the ENF apparatus.

3m 30s

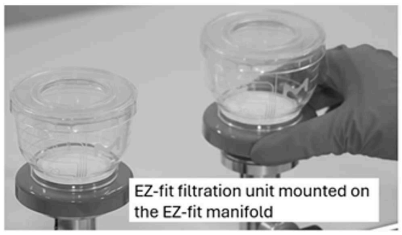
1 Turn on the glass bead sterilizer and allow sufficient time for it to reach the operating temperature before use.

2 Working inside the class II/A2 biological safety cabinet, connect the EZ-Fit manifold to the EZ-Stream vacuum pump (left or right side), then plug the pump into a power source.

3m

3 Securely mount the EZ-Fit filtration unit onto the EZ-Fit Manifold Base with HTA Manifold Head.

30s



EZ-fit filtration unit mounted on the EZ-fit manifold

Connected EZ-fit manifold to EZ-Stream pump with the EZ-fit filtration unit mounted on the manifold

Sample pH adjustment

3m 30s

4 Mix the 40 mL wastewater sample thoroughly by inverting several times.

30s

Safety information

Infectious Material - Handle sewage samples in a Class II/A2 biological safety cabinet.

5 Adjust the sample pH to 3.5 using hydrochloric acid, mixing thoroughly between titrations to ensure a homogeneous solution before proceeding.

3m

Sample Filtration and concentration.

13m 50s

6

3m

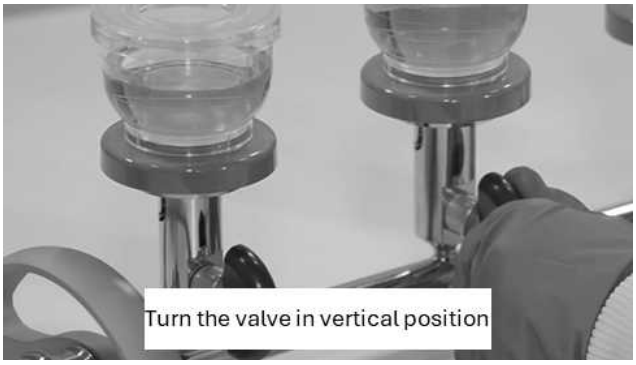
Note

Before adding the 40 mL wastewater sample, verify that the valve is in the horizontal (closed) position

Remove the lid on the EZ-fit filtration unit and carefully pour the 40 mL wastewater sample into the membrane filter.

Remove the top cover and pour the sample into the membrane filtration unit.

7 Place the lid back onto the EZ-fit filtration unit and turn the valve to the vertical position. 10s



8 Touch the button on top of the EZ-Stream vacuum pump to start filtration 10s

9 Allow the liquid to flow into the waste container until filtration is complete.

30s

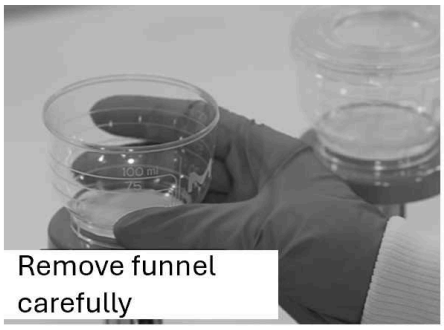
Note

Ensure the entire sample passes through the membrane filter.

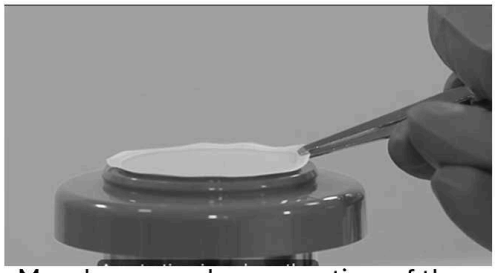
10 Remove the funnel carefully, ensuring the membrane remains on the lower portion of the funnel. 30s

Note

The protective rim minimizes cross-contamination during transfer to a clean 2 mL tube.



Remove funnel carefully



Membrane on lower portion of the funnel

11 Spray two forceps with 70% ethanol and sterilize them by briefly submerging in the glass bead sterilizer. 30s

12 Using both forceps, gently roll the membrane filter tightly ensuring that the filter is facing outwards and place it into a labelled clean 2 mL tube. 3m

Note

When concentrating multiple samples, after rolling each filter and placing it in a tube, repeat sterilization (step 11) for both forceps prior to moving onto the next sample.

13 Add 1 mL of lysis buffer from the MagMAX Wastewater Ultra Nucleic Acid Isolation Kit to the tube containing the rolled filter. 2m

14 Vortex vigorously for 30 seconds to 1 minute to mix thoroughly and then briefly centrifuge to collect the contents. 2m

15 Transfer the supernatant (lysate) to a clean 2.0 mL tube for immediate extraction. For automated extraction using the KingFisher system, transfer the lysate to the 24-deep-well plate (row A). 2m

Extraction using the MagMAX Wastewater Ultra Nucleic Acid Isolation Kit 2h 1m 50s

16 **Reagent preparation** 2m
Prepare 80% ethanol by diluting 100% (absolute) ethanol with nuclease-free water.
Prepare a minimum of 4 mL of 80% ethanol per sample.

17 **Bead Mix** 30s

> Note
>
> Prepare the bead mix on the day of use.

Vortex the Binding Beads vigorously to ensure complete resuspension.

17.1 Prepare the Binding Bead Mix by combining the required components below for the total number of samples plus an additional 10% excess. 3m

	Component	Volume per sample
	Binding Solution	2000 µl
	Binding Beads	100 µl
	Total volume	2100 µl

17.2 Mix the Binding Bead Mix well by inversion, then store at room temperature. 10s

18 **Automated extraction on KingFisher Duo**
Attached here is the KingFisher Duo Prime protocol for importing into your Instrument.
MagMAX_Wastewater_DUO24.bdz 3.5KB

Processing plate set up

Plate 1 containing the samples, tip comb, wash buffer and 80% ethanol

	Row ID	Plate row	Reagent	Volume per well
	Sample	A	Sample lysate + Proteinase K + Binding bead mix	4200 µL
	Tip Comb	B	Place a 6-tip comb in the plate	
	Wash 1	C	Wash buffer	4,000 µL
	Wash 2	D	80% Ethanol	4,000µL

Plate 2 containing the elution solution

	A	B	C	D
	Elution	A	Elution solution	100 µL
		B	Empty	
		C	Empty	
		D	Empty	

Note

To avoid evaporation and contamination, cover the prepared processing plates with paraffin film or MicroAmp Clear Adhesive Film until they are loaded into the instrument

18.1 Transfer 1 mL of lysate to row A of a new 24-deep-well sample plate. 2m

18.2 Add 160 µL of Proteinase K to each sample well. 2m

18.3 Invert the Binding Bead Mix several times to resuspend the beads, then add 2,100 µL of Binding Bead Mix to each sample in row A. 5m

Note

Keep the Binding Bead Mix well mixed throughout pipetting. Pipette slowly to ensure accurate volumes, and use fresh pipette tips for each addition. Do not reuse tips due to the high viscosity of the mix which will cause variations in the volumes added

18.4 Place a 6-tip comb into row B of the 24-deep-well plate. Label this as plate 1 10s

18.5 Add 4,000 µL of wash buffer to row C. 4m

18.6 Add 4,000 µL of 80% ethanol to row D. 4m

18.7 Add 100 µL of elution buffer into a separate 24 deep-well plate in position row A. Label this as plate 2. 1m

18.8 Select the MagMAX_Wastewater_DUO24 program on the KingFisher DUO

18.9 Start the run, then load the prepared plate 1 and 2 into position when prompted by the instrument

18.10 At the end of the run (~28 minutes), immediately remove the plate from the instrument and transfer the eluate to a new 1.5 mL tube. 28m

18.11 Keep the isolated nucleic acid on ice to be subjected to PCR inhibitor removal using the Zymo OneStep PCR Inhibitor Removal Kit or store at −80 °C until ready for processing.

Note

See section " Removing of PCR inhibitors using the Zymo OneStep PCR Inhibitor Removal Kit" for the procedure.

19 Manual Extraction

19.1 Transfer the 1mL lysate to clean 5 mL eppendorf tube 3m

19.2 Add 160 µL of Proteinase K to each sample. 2m

19.3 Invert the Binding Bead Mix several times to resuspend the beads, then add 2,100 µL of Binding Bead Mix to each sample. 5m

Note

Keep the Binding Bead Mix well mixed throughout pipetting. Pipette slowly to ensure accurate volumes, and use fresh pipette tips for each addition. Do not reuse tips due to the high viscosity of the mix which will cause variations in the volumes added

19.4 Transfer the sample tubes on a shaker or sample mixer and set to shake for 5 minutes at 900rpm. 5m

19.5 Incubate the sample at 65°C for 5 minutes. 5m

19.6 Place the sample tube on a magnetic stand for at least 5 minutes, or until all of the beads have collected. 5m

19.7 With the sample on the magnetic stand, carefully remove and discard the supernatant. 5m

Note

IMPORTANT! Avoid disturbing the beads.

19.8 Remove the sample from the magnetic stand, then add 4 mL of Wash Buffer to each sample. 2m

19.9 Close the tubes and then shake the sample at 800 rpm for 30 seconds on a shaker or sample mixer. 30s

19.10 Place the sample tube on the magnetic stand for 3 minutes, or until all of the beads have collected on the side of the tube. 3m

19.11 With the tube on the magnetic stand, carefully remove and discard the supernatant 3m

> Note
>
> IMPORTANT! Avoid disturbing the beads

19.12 Repeat step 19.8 through step 19.11, using 4 mL of 80% ethanol. 8m 30s

19.13 Briefly centrifuge and remove any residual solution with a small volume fine-tipped pipette, without disturbing the pellet. 3m

19.14 Air-dry the beads by leaving the tube open for 2 minutes to allow residual ethanol to evaporate.
Add 100 µL of Elution Solution to each sample. 2m

19.15 Add 100 µL of Elution Solution to each sample and vortex vigorously to fully resuspend the pellet. 3m

19.16 Incubate at 75°C for 5 minutes and the shake at 800 rpm for 5 minutes. 10m

19.17 Briefly centrifuge and place the sample tube on the magnetic stand for 3 minutes, or until all of the beads have collected to the side of the tube. 3m

19.18 With the sample tube on the magnetic stand, carefully transfer the eluates to a new clean 1.5 mL tube. 2m

19.19 Keep the isolated nucleic acid on ice to be subjected to PCR inhibitor removal using the Zymo OneStep PCR Inhibitor Removal Kit or store at −80 °C until ready for processing.

Removing of PCR inhibitors using the Zymo OneStep PCR Inhibitor Removal Kit 7m 30s

20 **Preparation of the column**

> Note
>
> Zymo-Spin™ III-HRC Columns need to be prepared before use

20.1 Insert column into a Collection Tube. 30s

> Note
>
> The matrix in the column may appear dehydrated, or powdery. This is normal

20.2 Open the cap, add 600 µl of Prep-Solution and centrifuge at 8,000 x g for 3 minutes. 3m

21 **Inhibitor Removal** 1m
Transfer the prepared column to a clean 1.5 ml microcentrifuge tube.

21.1 Add 100 µl total nucleic acid extract to the Zymo-Spin III-HRC Column and centrifuge at 16,000 x g for 3 minutes. 3m

21.2 The filtered total nucleic acid is now suitable for immediate PCR and downstream analysis use or can be store at −80 °C until ready for use.

Protocol references

Solana Narum, Thibault Stalder, Erik Coats, Eva Top 2022. Quantification of the SARS-CoV-2 using electronegative membrane filtration and dPCR. **protocols.io** https://dx.doi.org/10.17504/protocols.io.bp2l61xwrvqe/v2 Version created by **Solana Narum**