Feb 20, 2022

RNA extraction from wastewater for detection of SARS-CoV-2

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David Thomson: Adapted and implemented the protocol
Alexander Corbishley: Developed the protocol
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dx.doi.org/10.17504/protocols.io.bzv5p686

2019-nCoV Wastewater Epidemiology
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bio_rdm

Citation: David Findlay, Julie Bolland, Brindusa Cerghizan, Kirsty Campbell, David Thomson, Alexander Corbishley, David Gally, Stephen Fitzgerald, Alison Tidswell, Sean McAteer, Livia C T Scorza RNA extraction from wastewater for detection of SARS-CoV-2ÃÂ https://dx.doi.org/10.17504/protocols.io.bzv5p686

As part of the global response to the 2019 novel Coronavirus (SARS-CoV-2) pandemic, it was determined that SARS-CoV-2 RNA was detectable in the faeces of both symptomatic and asymptomatic patients (1).

Further analysis demonstrated that a wastewater epidemiological (WWE) approach, similar to that used to track other viruses (i.e. Poliovirus), could be employed to monitor the spread of SARS-CoV-2. The presence of, or changes in concentration of viral RNA within the wastewater network can assist in monitoring the emergence of further viral peaks (2). Thus, monitoring the spread of Covid-19 using the WWE approach has been extensively explored in several countries (3).

This procedure, developed by the Scottish Environment Protection Agency (SEPA) based upon work of the Corbishley group at the Roslin Institute, University of Edinburgh, outlines the method for the concentration of viral RNA in wastewater, as well as in other types of environmental and potable water samples.

In this method, a known volume of sample is concentrated using a centrifugal filter to allow further extraction and detection of SARS-CoV-2 RNA.

For further analysis using RT-qPCR please search for "RT-qPCR for detection of SARS-CoV-2 in wastewater" by the same authors of this protocol on protocols.io

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DOI

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CREW (Scotland's Centre of Expertise for Waters)
Grant ID: CD2019_06 Tracking SARS-CoV-2 via municipal wastewater

Wastewater-based epidemiology, Infectious diseases, Public health, RNA extraction, RNA viruses, Covid-19, SARS-CoV-2

protocol ,

Nov 08, 2021

Feb 20, 2022

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PERFORMANCE AND LIMITATIONS

- RNA is highly susceptible to degradation by both temperature and enzymes known as RNases. Care must be taken to ensure that all reagents, equipment and working environment are free of RNase contamination and unless otherwise stated, all RNA work should be conducted “on ice” (RNase activity restricted at 4C).

- False negative result may occur if insufficient target is present due to improper sample transport or storage conditions.

- During this process substances which can inhibit enzyme activity and therefore interfere with the qPCR process can also be concentrated. Inhibition is monitored by the inclusion of an internal control added.

ANALYTICAL QUALITY CONTROL

- **Control: Samples are spiked with PRRS1* virus particles as a processing control**

Samples are spiked with a known quantity of non-target RNA (PRRS1) at the start of the process to demonstrate that the method has been successful at concentrating and recovering RNA particles. Successful recovery of this marker provides reassurance that any negative results are due to the absence of our target of interest in the sample and not because of any methodological failures.

*Porcine Reproductive and Respiratory Syndrome (PRRS) – An RNA virus in pigs that can used as a sample process control when testing samples for the presence of SARS-CoV-2 RNA.

- An **extraction Blank** shall be included as part of each analytical run.

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In addition to the facilities and equipment found in a general microbiology laboratory, the following are required:

- Microbiological Safety Cabinet Class II for sample processing
- Gravimetrically calibrated air displacement pipettors (50 µl to 1000 ml)
- Sterile, RNase-free, filtered pipette tips
- Amicon Centrifugal Filter Unit (15 ml; 10kDa) (Millipore)

NB Amicon centrifuge filters can accommodate 15ml (at a time) with a max speed of 4000 x g if using a swing bucket rotor or 12ml and 5000 x g if rotor is fixed.

- Centrifuge with 50ml tube compatible rotor and aerosol containment buckets
- Balance (0.001g)
- Sample Process Control – Inactivated PRRS virus
- QiAmp Viral RNA extraction kit (Qiagen)
- Micro-centrifuge
- Ethanol (96-100%)
- 0.2 and 2 ml low retention micro-centrifuge tubes
- RNase-free 15ml centrifuge tubes
- Cold blocks for 200 µl PCR tubes
- Ice maker and flaker

HEALTH AND SAFETY

It is not yet known whether samples which are positive for the presence of SARS-CoV-2 RNA are still viable and therefore present an infection risk. As such the precautionary principle should be applied with all analysis being conducted in a BSL level 2 laboratory. **Aerosol-generating procedures should be carried out in a Microbiological Safety Cabinet (MSC). Nitrile gloves must be worn at all times when carrying out this method.**

- All sample processing is to be carried out within a **Class II microbiological safety cabinet**.
- For all centrifugation steps, samples must be placed in centrifuge buckets or directly into a rotor and sealed prior to removal from the cabinet.
- All **spin steps to be carried out at 4 ºC** unless otherwise stated.
- **Samples should be processed as soon as possible after collection** but if this is not possible **may be stored at 4˚C for up to 72 hrs**. Upon receipt, samples are **aliquoted (2x 50ml) and stored at -80˚C**, to protect nucleic acid integrity, until ready for analysis.

Sample preparation 50m

1 Clean all surfaces with 0.5% Sodium Hypochlorite (10% bleach) before and after use. Rinse with water to remove any residue.

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2 If necessary defrost samples at **4 °C overnight**

3 Add a known quantity of Sample Process Control RNA (PRRS) to each sample (view guidelines for more information).

4 Remove solid matter from wastewater samples by centrifugation at **4669 x g, 4°C, 00:10:00** 10m

5 Carefully transfer the supernatant to a clean tube taking care not to disturb the pellet. Discard pellet.

RNA Concentration 50m

6 Transfer 15ml of the clarified supernatant from the previous step to a 15 ml Amicon/Centricon Filter (10kDa)

7 Concentrate samples ~ by centrifugation (**1500 x g, 4°C, 00:10:00**) 10m

8 Check the level of the concentrate in the filter device by transferring the concentrate to a pre-weighed tube and re-weighing the tube to determine the concentrate volume

9 If <250 uL proceed to RNA extraction
If >250 uL and < 1ml give tubes a further 10 minutes at 4000 x g.
If >1ml give tubes a further 15minutes at 4000 x g.

10 Remove concentrated sample, using a side to side pipettor motion to ensure maximum recovery, and transfer to a pre-weighed 2ml tube

11 Weight tube plus concentrate and record concentrate volume.
Samples should be processed immediately after concentration. If this is not possible then

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they must be stored, immediately at **-80 °C** until extraction can be carried out.

12 Extract viral RNA using **QA_Viral_RNA_Mini.pdf** according to next steps guidelines. Final elution in 60 µl AE buffer

Viral RNA Purification (Spin Protocol- QIAamp Viral RNA Mini Kit) 5m

13 Prepare the reagents supplied in the kit according to manufacturer's guidelines and equilibrate buffers and sample at room temperature before starting:

13.1 Carrier RNA preparation:
- Add 310 µl Buffer AVE to carrier RNA to obtain 1 µg/µl solution.
- Divide into 100 µl, single-use aliquots and store at -20°C. Do not freeze/thaw more than 3 times.
- 5.6 µl of carrier RNA-AVE is required per sample.
- For one sample, add 5.6 µl of carrier RNA-AVE to 0.56 ml of AVL buffer.
- For more than one sample, calculate the volume of Buffer AVL–carrier RNA mix needed per batch of samples (check Table 1 of QIAamp® Viral RNA Mini Handbook)

13.2 Resuspend buffer AW1 in ethanol (96-100%) – 130ml for 250 prep kit, 25ml for 50 prep kit.

13.3 Resuspend buffer AW2 in ethanol (96-100%) – 160ml for 250 prep kit, 30ml for 50 prep kit.

13.4 Before starting, ensure that buffer AVE and samples are equilibrated to **Room temperature**

***Add an extraction blank as part of each analytical run**.
*An **optional positive control** containing know quantities of SARS-CoV-2 and PRRS RNA can be included to determine extraction efficiency.

14 Transfer sample concentrate (to a maximum volume of 560µL) to a 1.5 ml tube, add 4x concentrate volume of AVL buffer containing carrier RNA and pulse-vortex for 15 seconds.

15 Incubate at room temperature for 10 minutes. **Room temperature**

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16 Add same volume of ethanol, as AVL, per sample and pulse-vortex to mix for 15 seconds.

17 Carefully transfer up to 700 µl to QIAamp mini column (in 2ml collection tube) without wetting the rim. Close cap and spin at **6000 x g, 00:01:00** 1m

18 Discarding flow-through after each spin, repeating the previous step until sample is fully processed.

19 Carefully add 500 µl buffer AW1 to the mini columns. Close cap and spin at **6000 x g, 00:01:00** . Discard the flow-through. 1m

20 Add 500 µl of buffer AW2 to the mini columns. Close cap and spin at maximum speed for **00:03:00** 3m

21 Transfer the mini-columns to a clean collection tube and spin at maximum speed for **00:01:00** . 1m

22 Transfer to a clean tube and add 60 µl (1 spin) or 40µl (2 spins) of AVE and incubate at room temperature for **00:01:00** 1m

23 Centrifuge at **6000 x g, 00:01:00** (or up to 13000 g) 1m
If performing a 2x 40 µl spin elution, repeat steps 22 and 23.

24 RNA is now ready for RT-qPCR.
NB Performing a single-spin elution should generate ~50µl of RNA in suspension whereas 2 spins should produce ~70 µl and may increase RNA yield by up to 10%.
Record final elution volume.

Determination of yield

25 "Yields of viral RNA isolated from biological samples are normally less than 1 µg and therefore difficult to determine photometrically. Keep in mind that the carrier RNA (5.6 µg per 140 µl sample) will account for most of the RNA present. Quantitative RT-PCR is

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recommended for determination of viral RNA yield" (from QIAamp® Viral RNA Mini Handbook)

26 Proceed with RT-qPCR for detection of SARS-CoV-2.

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