PROTOCOL

SOP wastewater sampling, transport, storage for SARS-CoV-2 RNA assays 13 May 2020
SOP wastewater SARS-CoV-2 RNA assays 23 May 2020

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Safety

Sewage sampling may expose you to sewage, which is generally contaminated with pathogenic micro-organisms. Adhere to your national Occupational Health and Safety regulations and guidelines. When applied properly, the standard protection is also adequately protecting you against SARS-CoV-2. At a minimum use latex gloves or similar when sampling and apply proper personal hygiene. Avoid spilling, absorb any spills and dispose as contaminated waste, disinfect surfaces that may have become contaminated by spills. Close containers and centrifuge tubes to avoid spills and aerosolization. Work in laminar flow cabinet

Chapter 1: SOP wastewater sampling, transport, storage for SARS-CoV-2 RNA assays

Scope: this protocol refers to the collection and storage of samples from 24h composite (auto) samplers that are installed at the inlet of the wastewater treatment or similar sample matrices. For surveillance of wastewater for SARS-CoV-2 RNA, 24h composite samples are required, because of the inherent variability in virus shedding and sewer flows. The composite sample should be taken at the inlet of the wastewater treatment plant, after the screening and grit removal steps and at a site that is well-mixed. The composite sample should be high frequency, and preferably flow-proportional composite and refrigerated during the sampling period. Volume or time composite sampling over 24 h is acceptable.

The composite sampler should adhere to requirements for composite sampling (e.g. https://www.epa.gov/sites/production/files/2017-07/documents/wastewater_sampling306_af.r4.pdf).

Prior to transfer of the sample from the composite sampler, the proper operation of the composite sampler over the last 24h and the maintenance status and calibration should be confirmed and documented in the sample notes.

Sample Collection Procedure

1. On the day of sampling, prepare the cooler with sufficient ice (packs).
2. Collect sufficient sample containers
3. Weigh each sample container and document the container net weight with a permanent marker on the container and the sample form.
4. Wear Personnel Protective Equipment (gloves + national OHS guidelines).
5. Wear Sample Protective Equipment: surgical face mask
6. Label the container(s) with sample ID, sampling location, date and time and also make a personal record.
7. Remove the tube cover and protect from contamination.
8. From the 24-hour composite, refrigerated wastewater sampler (from the plant inlet, post grit chamber) pour slowly approx. 100 ml into 4 individual sample containers.
9. Tightly close the container and place in the cooler on ice (packs).

Sample storage procedure: Following collection of the 4 x 100ml samples, start sample processing on 2 samples as quickly as possible upon receipt of the sample and freeze the remaining 2 samples for later analysis.

If you cannot process 2 samples on the day of sampling, store the samples for a maximum of 7 days in a fridge (i.e. +5 +/- 3°C) before starting the processing.

If samples cannot be analysed within 7 days of collection, freeze all 4 samples until analysis can be undertaken. Do not pasteurize.

Chapter 2 Quality assurance of the sampling, transport, storage and analysis of wastewater for SAR-CoV-2 RNA

Scope: these are recommendations for quality assurance of the sampling, transport, storage and analysis of wastewater for SAR-CoV-2 RNA

Sampling

- Confirm and document proper operation of the composite sampler over the last 24h
- Confirm the storage of the composite sample at 5 ± 3 °C
- Confirm and document the maintenance and calibration status of the composite sampler
- Label the sample container with a unique sample ID that cannot be erased
- Blanks: take samples of tap water

Transport and storage

- Confirm temperature of the samples during transport and storage has been 5 ± 3 °C
- Confirm time of transport and storage is ≤ 4 days
- Transport controls: the testlab can occasionally send samples that have been analysed for SARS-C0V-2 RNA for re-analysis to confirm stability during transport and storage

Sample processing

- Document all volumes in all steps of the processing to determine the equivalent wastewater volume that is analysed in the qRT-PCR assay
- Every assay: spike concentrates after centrifugation and ultrafiltration with 100-1000 gene copies of a coronavirus, such as Mouse Hepatitis Virus (alternatively spike replicate samples with inactivated SARS-CoV-2, such as ATCC® VR-1986HK™) to determine the recovery efficiency of the RNA extraction and qRT-PCR
- Every batch: include qRT-PCR blanks, positive controls (N-gene RNA, E-gene RNA, such as https://crm.jrc.ec.europa.eu/p/EURM-019 or https://www.european-virus-archive.com/detection-kit/2019-ncov-e-gene-stabilized-rna-positive-control-shipping-room-temperature), include blank samples
- Frequently: matrix spike; spike wastewater samples with 100-1000 gene copies of a coronavirus, such as Mouse Hepatitis Virus (alternatively spike replicate samples with inactivated SARS-CoV-2, such as ATCC® VR-1986HK™) to determine the recovery efficiency of the overall sample processing and qRT-PCR assays

Standard curve

- Every batch: confirm the concentration of DNA or RNA in the material obtained to generate standard curves for quantification of the dRT-PCR signal, using digital droplet PCR.

Chapter 3 Sample preparation

3.1 KWR protocol

This protocol describes how to concentrate and extract SARS-CoV-2 RNA from communal wastewater samples. The protocol has been designed and tested for samples of 50 ml of raw wastewater (or primary effluent) and 100 ml of secondary effluent (after biological treatment and sedimentation). The protocol has been designed and tested using the three targets on the nucleoprotein gene (N1. N2. N3) and one on the envelope gene (E).

1. Centrifuge the sample in the weighed 50 conical centrifuge tubes at 4654xg for 30 mins without brake. This step is to remove larger particles (debris, bacteria). A proportion of SARS-CoV-2 may be removed with the particles. This has not been tested.
2. Decant the supernatant in pre-weighed Centricon® Plus-70 centrifugal ultrafilters with a cut-off of 100 kDa (Millipore, Amsterdam, The Netherlands). Concentrate the virus through these ultrafilters by centrifugation at 1500xg for 15 minutes. Extract the concentrate (0.5-1.5 g) from the Centricon filters with a micropipette with filtertip. Top up to 1.5 ml with ultrapure DNAse/RNAse free distilled water.

3.2 Alternative simplified protocol for Sars-COV-2 extraction from wastewater via PEG-precipitation

This protocol is a modified version of the “biobot-protocoll” published in Wu et al. (2020)[6] prepared by Nina Lackner and Rudolf Markt, Universitaet Innsbruck (AT), Department of Microbiology; Katarzyna Slipko, Technische Universitaet Wien, Institute for Water Quality and Resources Management.

Precautions

- change gloves very frequently, work on ice or cold blocks, cool down all centrifuges to 4°C before use, also keep samples in the fridge whenever they are not needed
- use only filter tips! Preferably freshly opened box,
- use plastic not glass,
- avoid warming up (and freezing) of the samples at any time!
- keep bench times as short as possible!
- clean benches with bleach before, in the middle and after work,
- work with protective mask (for your protection and to not breathe and spread RNases into your samples!),
- be double careful with the movement of your hands:
  - do not touch the inner lid of the tubes,
  - do not hold tubes in your hands (to not warm them up),
  - do not move your hands above the tubes or tips box,
  - keep the trash bin as far as possible but in the reach of your hand,
  - take fresh tubes from the bag with sterile tweezers,
- avoid to use anything that was autoclaved (prefer to purchase sterile single use material)

Table 1 Materials

Material	Amount per sample
Pipette: 20 ml graduated glass pipettes (treated at 180°C for 150 min) with pipettors OR 25 ml sterile plastic pipettes	2x
1,000 µl pipette with sterile tips (preferable filter tips!)	
50 ml Falcon tubes (sterile)	2 (4)
Polyethylenglycol (PEG) 8000 (mw)	4 g per Falcon tube
Sodium chloride	0.9 g per Falcon Tube
Refrigerated (4°C) centrifuge with fixed angle rotor for 50 mL falcons (min 12,000 g)	
Vortexer (2,500 rpm)	
100 ml (200ml) sample stored at 4°C	
Balance	

Sample processing

1. Cool the centrifuge to 4 °C.
2. Use pipette (e.g. 25 ml sterile plastic with pipetboy) and transfer min. 45 ml of wastewater influent (more than 44 ml in any case) to a 50 ml falcon (F1). Balance out all the samples for centrifugation. Centrifuge at least 2 tubes per sample (better 4 –> ~200ml sample needed!).
3. Centrifuge the samples for 30 min at 4,500 g without brake to remove particulate biomass (Medema et al. 2020).
4. While waiting, put in 4 g PEG 8000 and 0.9 g sodium chloride into a fresh 50 ml Falcon tube (F2).
5. After centrifugation, **carefully** transfer 40 ml (2x20 ml) of supernatant with sterile pipette from F1 to F2. Be careful as the pellet is very instable. The ~ 5ml excess helps. If the pellet resuspends early, repeat the first centrifugation step (10 min 4,500 g without brake should be sufficient).
6. The PEG has to dissolve in the transferred supernatant (F2). Using a head-over-head shaker this may take up to 5 min at room temperature (RT). Alternatively, samples can be carefully inverted by hand. This may take about 15 min. Do not shake them heavily and avoid warming up in your hands!
7. Use RNase-free water and 1,000 µl pipette for balancing out samples for the next centrifugation step.
8. Before putting the Falcons into the centrifuge, mark the outer/upper side of the Falcon (the spot which is hit by the highest force) in order to indicate position of the pellet.
9. Centrifuge the samples for 99 min at 12,000 g and 4 °C without brake.
10. After centrifugation, decant the sample with the carefully via the opposing side of the pellet (= marked side up). The pellet usually sticks quite strongly to the tube, but in most cases you can't see it.
11. Return the Falcons to the refrigerated (4°C) centrifuge. Be sure, that the same (marked) side of the tube is facing upwards again.
12. Centrifuge the Falcons at 12,000 g for 5 minutes. You can set the brake to 3 (of 9). (You can use the time to prepare RNA extraction material.)
13. Carefully aspirate and discard the remaining liquid with a 1,000 µl pipette without touching the pellet. A transparent pellet should be visible.
14. Add 800-1000 µl lysis buffer or equivalent (e.g. trizol) and vortex the sample for 15 sec at 2,500 rpm, this is enough to fully dissolved the pellet. Be sure that you catch the entire pellet. Vortex also horizontally on the side that you expect to have the pellet

15. Centrifuge the sample again at 1,000 – 2,000 g for a few seconds (with brake) to collect all the lysis buffer droplets at the bottom of the falcon.
16. Transfer the entire solution to a micro reaction tube (Eppi) or directly on a purification column. (If Trizol was used to dissolve the pellet, the sample can be stored at 4°C; otherwise)
17. Proceed with RNA-Purification according to your protocol immediately.

Chapter 4 RNA extraction

4.1 KWR protocol

Extract the virus nucleci acids using the magnetic extraction reagents of the Biomerieux Nuclisens kit (Biomerieux, Amersfoort, the Netherlands) in combination with the semi-automated KingFisher mL (Thermo Scientific, Bleiswijk, The Netherlands) purification system[1,2]. One millilitre of every concentrated water sample was added to a Nuclisens tube containing 2 mL of Nuclisens lysis buffer and incubated for 10 min at 20 °C. A mixture containing 60 µL of magnetic silica and 940 µL Nuclisens buffer was added to the lysate and mixed briefly by vortexing and incubated for 10 min at 20 °C. The samples were centrifuged for 2 min at 1500 g to pellet the magnetic silica particles, the supernatant was removed, the silica particles were suspended in 500 µL wash buffer 1 and transferred to the first tube of a KingFisher ml magnetic particle processor (Thermo scientific, Breda, the Netherlands), and this device was used to automatically perform the washing steps. The magnetic silica particles were subsequently washed in wash buffer 1 (for 1 min with fast dual mixing), transferred to the second tube containing 500 µL wash buffer 2 (wash step for 1 min with fast dual mixing), transferred to the third tube containing 500 µL wash buffer 2 (wash step for 1 min with fast dual mixing), transferred to the fourth tube containing 800 µL wash buffer 3 (wash step for 10 s with slow mixing) and then suspensed in 100 µL elution buffer. The magnetic bead suspension is transferred to 1.5 mL eppendorf tubes and nucleic acids are released from the beads by incubating the magnetic bead suspension at 60 °C for 5 min. The magnetic beads are removed by incubating the tube on a magnetic separation stand (Promega, Leiden, the Netherlands) for 1 min and transferring the supernatant to a new tube. The resulting NA extract is 100 ul.

4.2 Alternative RNA extraction/purification approach if you do not have access to the Biomerieux Nuclisens kit

- If you have RNA purification established in your lab, proceed with your established SOP.
- If you never did RNA purification before: don’t be afraid – commercial kits are your friend!

Wastewater samples contain a lot of inhibitors that affect RT-PCR performance. Therefore, while choosing the RNA extraction kit, one needs to consider the type of the analyzed sample. As we do not want to state a specific brand, we only recommend using kits for stool or environmental (soil) samples (not for tissue, blood, bacterial cultures, or urine).
Ensure that your kit:

(i) contains an inhibitor removal step (for many manufacturers it is a step when after addition of a reagent the sample turns colloid white and needs to be incubated at 4 °C for a certain amount of time) and

(ii) removal of DNA (step when DNase I is applied).

RNA is highly degradable and each freezing-thawing cycle results in loss of the genetic material. Therefore, aliquot your extracts (min. 3 aliquots are recommended: the more the better) and store frozen at – 20 °C or – 80 °C.

RNA quantification: To check if your extraction was successful, you can use A260/A280 ratio fur purity (it should be around 2 for pure RNA if DNA removal step was applied). There are also fluorescent dyes for highly accurate measurements.

Chapter 5 Real-time RT-PCR

5.1 KWR protocol

Primers/probe sets that were published by US CDC[3] and a European study[4] have been tested successfully with this protocol. Four primer sets were selected (Table 1): the N1-N3 set from CDC that each target a different region of the nucleocapsid (N) gene and the set targeting the envelope protein (E) gene from Corman et al.[4], to include targets against two separate SARS-CoV-2 genes. The specificity of these primer/probe sets against other (respiratory) viruses, including human coronaviruses, had been confirmed by others.[4,5] Each individual qRT-PCR reaction contains:

- 5 µl of the total volume of 100 µl eluted RNA template (meaning that 5% of each sewage sample is analysed with each qRT-PCR),
- 4 µl of 5x Taqman Fast Virus 1-Step Master Mix (Applied Biosystems, Fisher Scientific, Landsmeer, The Netherlands),
- different concentrations of primers and probes (Table 1),
- 2 µl of 4 mg/ml BSA (Bovine Serum Albumin, Roche Diagnostics, Almere, The Netherlands)
- adjust the reaction volume to a final volume of 20 µl with ultrapure DNAse/RNAse free distilled water (Invitrogen, Fisher Scientific, Landsmeer, The Netherlands or equivalent).

Thermal cycling reactions were carried out at 50 °C for 5 minutes, followed by 45 cycles of 95 °C for 10 and 60 °C for 30 seconds on a CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories, Veenendaal, The Netherlands).

Reactions were considered positive if the cycle threshold was below 40 cycles. Quantification of N1, N2 and N3 specific assays was performed using four 10-fold dilutions (ranging from 1.0E+02 to 1.0E+05 copies per reaction) of the 2019-nCoV_N_Positive Control plasmid DNA from IDT (Leuven, Belgium) with specified concentrations as calibration suspension.

5.2 Generic alternative

Use your own in house methods; please document these in the reporting sheet.

Chapter 6 Quality control procedures

6.1 Matrix spike

In a subset of samples, add 100-1000 gene copy equivalents of Mouse Hepatitis Virus A59 to the wastewater sample before the first centrifugation step. Process and analyse the sample as normal sample and analyze with qRT-PCR using the primers/probes for MHV-A59. To calculate the recovery efficiency of the total method, divide the gene copies per ml detected by the gene copies per ml added and express the result as percentage.

Alternatively, replicate samples can be spiked with inactivated SARS-CoV-2 virus (such as ATCC® VR-1986HK™) in gene copy numbers that clearly exceed the expected SARS-CoV-2 gene copies present in the sample, and analyse with one or more of the qRT-PCR assays (this control has not been tested by KWR yet)

6.2 RT-PCR controls

To check the recovery efficiency RNA extraction for the qRT-PCR, add MHV-A59 RNA to the lysed sewage concentrates as an internal control (IC). Quantification of IC-RNA was done by performing qRT-PCR's on serial 10-fold dilutions of quantified synthetic RNA (IDT, Leuven, Belgium). The concentration of IC-RNA in the extracted sample-RNA was compared with the concentration in the added IC-RNA suspension to determine the recovery efficiency (%) of the IC-RNA by the RNA-extraction procedure and the possible presence of qRT-PCR inhibitors. Include negative controls by performing qRT-PCR reactions on RNA isolated from 1 ml of ultrapure DNAse/RNAse free distilled water (negative extraction control) and by performing qRT-PCR reactions directly on 5 µl ultrapure DNAse/RNAse free distilled water (negative qRT-PCR control).

Table 2. Primer-probe sets.

Assay	Target gene	Primer/Probe	Concentration	Sequence[a]	Ref
N1	Nucleo-capsid (N)	2019-nCoV_N1-F	200 nM	5'-GACCCCAAAATCAGCGAAAT-3'	3
		2019-nCoV_N1-R	200 nM	5'-TCTGGTTACTGCCAGTTGAATCTG-3'	3
		2019-nCoV_N1-P	200 nM	5'-FAM-ACCCCGCATTACGTTTGGTGGACC-ZEN/Iowa Black-3'	3
N2	Nucleo-capsid (N)	2019-nCoV_N2-F	200 nM	5'-TTACAAACATTGGCCGCAAA-3'	3
		2019-nCoV_N2-R	200 nM	5'-GCGCGACATTCCGAAGAA-3'	3
		2019-nCoV_N2-P	200 nM	5'-FAM-ACAATTTGCCCCCAGCGCTTCAG-ZEN/Iowa Black-3'	3
N3	Nucleo-capsid (N)	2019-nCoV_N3-F	200 nM	5'-GGGAGCCTTGAATACACCAAAA-3'	3
		2019-nCoV_N3-R	200 nM	5'-TGTAGCACGATTGCAGCATTG-3'	3
		2019-nCoV_N3-P	200 nM	5'-FAM-AYCACATTGGCACCCGCAATCCTG-ZEN/Iowa Black-3'	3
E	Envelope (E)	E_Sarbeco_F	400 nM	5'-ACAGGTACGTTAATAGTTAATAGCGT-3'	4
		E_Sarbeco_R	400 nM	5'-ATATTGCAGCAGTACGCACACA-3'	4
		E_Sarbeco_P1	200 nM	5'-FAM-ACACTAGCCATCCTTACTGCGCTTCG-ZEN/Iowa Black-3'	4

[a] *Y=C/T. FAM: 6-carboxyfluorescein; ZEN/Iowa Black: internal ZEN and Iowa Black double-quenched probe*

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