Centre for Environment Fisheries & Aquaculture Science

Cefas INTERNATIONAL CENTRES OF EXCELLENCE

Generic Protocol version 1.0

Quantification of SARS-CoV-2 in wastewater

Author: David I. Walker[1]

Contributors: James Lowther[1], Nick Evens[2], Jonathan Warren[2], Jonathan Porter[2], Kata Farkas[3], Davey Jones[3]

Contributor affiliations:

[1] Centre for Environment, Fisheries and Aquaculture Science, Weymouth, Dorset

[2] National Laboratory Service, Environment Agency, Exeter, Devon

[3] Environment Centre Wales, School of Environment, Natural Resources and Geography, Bangor University, Bangor, Gwynedd

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Introduction

Wastewater testing has been used as a tool for monitoring viral pathogens in human communities since at least the 1980s, when Israel starting its wastewater monitoring programme for poliovirus (Manor *et al.*, 1999). Since the demonstration by Medema *et al.* (2020) that SARS-CoV-2 can be detected in wastewater, there has been widespread interest in the use of wastewater monitoring to track COVID-19 outbreaks in communities. Wastewater monitoring from COVID-19 has since been shown by several laboratories to be an effective tool, and is it used by several governments (including the UK government), as part of their COVID-19 response strategy.

The quantification of SARS-CoV-2 RNA in wastewater by reverse transcriptase quantitative PCR (RT-qPCR) typically follows several steps including sample clarification, viral concentration, RNA extraction and RT-qPCR. Internationally the procedures for SARS-CoV-2 quantification from wastewater vary widely, with many laboratories using all or only a few of these steps. The use of different methods reflects the high degree of variation in sampling and testing environments. The choice of methods is also often a compromise between sample throughput and sensitivity.

The following protocol is based on the procedure that has been developed by the Environment Agency's National Laboratory Service and Bangor University for use in England in the UK government's Environmental Monitoring for Health Protection (EMHP programme). Since the start of the wastewater monitoring for SARS-CoV-2in the UK, the procedure has changed frequently as it has developed to meet the need of the UK government. Following initial trials using centrifuge-based ultrafiltration devices to concentrate SARS-CoV-2 from wastewater, it was found that filters often clogged and so a different method based on viral precipitation by polyethylene glycol (PEG) was adopted.

However, while the PEG method was proven to be efficient, it included an overnight incubation step, making the procedure too long for routine testing. Therefore, an alternate precipitation method using ammonium sulphate was developed. This method replaces the overnight incubation with a 1-hour incubation, reducing sample concentration times to 2-3 hours. This method has now been adopted for use in the EMHP programme.

This generic protocol was prepared by Cefas to allow laboratories to carry out SARS-CoV-2 quantification from wastewater samples in a way that is as similar to the EA's procedure, while still allowing some variation where necessary. It is intended that any laboratory that contributes data to the EMHP programme, should adopt this protocol. Users of this protocol would be expected to write their own standard operating procedures (SOP) that is specific to their own laboratory, using this protocol as a guide. Each laboratory should use their own internal control protocols to ensure that their SOP produces repeatable and reliable data.

Specific note about this version

This version of the protocol has been created to cover the procedures currently in place within the EMHP network. However, it is acknowledged that further work may be required for continued improvement of the method. Some elements for quality assurance are still missing from the procedure and are still under investigation at the time that this version was released.

Outstanding issues

The level of RT-qPCR inhibition in samples processed according to this protocol is not currently well understood. Initial studies by Cefas indicate that levels of inhibition may be variable. However, the exact nature of inhibition, its causes and its prevalence over time have not yet been studied. The process control (phi6) and the quality control cut-off for recovery require further investigation.

Variation in the process control stocks (both within and between stocks) may result in erroneous recovery results. No validated method for growth and quantification of phi6 currently exists. Additionally, the current 0.01% recovery requirement for recovery is very likely to be too lenient and may result in the generation of high levels of variation between samples.

Scope

This procedure describes the concentration and quantification of SARS-CoV-2 from wastewater as required by the Environmental Monitoring for Health Protection (EMHP) programme. It is based on the standard operating procedure developed by collaboration between Environment Agency National Laboratory Service and Bangor University.

Viral concentration is by ammonium sulphate precipitation followed by centrifugation. Viral RNA extraction is by lysis with guanidine isothiocyanate (GITC) and adsorption to silica. Extracted viral RNA is amplified and detected by quantitative RT-PCR (RT-qPCR). This protocol describes a method for quantification of virus RNA in the test sample and includes production of control materials.

Principle

Virus concentration

The wastewater samples are first clarified by centrifugation. A precipitant (ammonium sulphate) is then added before the sample is incubated for 1 hour at 4°C. This is followed by centrifugation to generate a viral pellet that is suitable for viral nucleic acid extraction. Details of addition of a spike process control (phi6) to the test samples are also described.

RNA extraction

It is necessary to extract RNA using a method that yields clean RNA preparations to reduce the effect of PCR inhibitors. In this protocol the chaotropic agent GITC is used to disrupt the viral capsid. RNA is then adsorbed to silica to assist purification through several washing stages. Purified viral RNA is released from the silica into a buffer prior to RT-qPCR. The use of the KingFisher™ Flex Purification System with BioMerieux's NucliSENS extraction reagents is described here. Other RNA extraction methods that use GITC followed by silica-based purification may also be applicable if they can be demonstrated to give equivalent results and are accepted by the reference laboratory.

Real-time reverse transcription polymerase chain reaction (RT-qPCR)

This protocol uses one-step RT-qPCR using hydrolysis probes. In one-step RT-qPCR, reverse transcription and PCR amplification are carried out consecutively in the same tube. Hydrolysis probe-based qPCR utilises a short DNA probe with a fluorescent label and a fluorescence quencher attached at opposite ends. The assay chemistry ensures that as the quantity of amplified product increases, the probe is broken down, and the fluorescent signal from the label increases proportionately. Fluorescence may be measured at each stage throughout the cycle. The first point in the PCR cycle at which amplification can be detected for any reaction is proportional to the quantity of template, therefore analysis of the fluorescence plots enables determination of the quantity of target sequence in the sample.

Safety precautions

The following procedures are for the analysis of wastewater (sewage). These samples are likely to contain high levels of pathogenic bacteria, viruses and parasites and should be treated as BSL-2 samples until sample lysis (step 18).

The procedures should be carried out in line with the laboratory's local health and safety requirements to ensure the safety of all people affected.

Equipment

For preparation of solutions/buffers

- Digital balance accurate to 0.01 g
- 1 l conical flask
- Magnetic stir bars
- Magnetic stirrer
- Freezer capable of temperatures <-15°C
- Autoclave capable of sterilising media at 121±2°C for 15 minutes (optional)
- Autoclavable bottles and tubes for storage of aliquots (size depends on aliquots sizes)

For preparation of control material

- Micropipettes and tips
- Incubator capable of maintaining a temperature of 25±1°C
- Freezer capable of temperatures <-70°C
- Bacterial culture loops
- Screwcap cryo-vials (1 ml recommended)
- Centrifuge tubes capable of holding 15 ml
- Centrifuge capable of spinning ≥15 ml tubes at 4,000 xg at 4±1°C
- Digital balance accurate to 0.1 g

For viral concentration

- Micropipettes and tips
- Biological safety cabinet
- Digital balance capable of weighing at least 150 g plus the weight of a centrifuge bottle to an accuracy of 0.5 g
- Centrifuges capable of spinning 200 ml samples at 10,000 xg at 4±1°C
- Centrifuge rotor with a bioseal lid to hold 200 ml samples
- Centrifuge bottles with a capacity of at least 200 ml
- Refrigerator capable of temperatures 3±2°C
- Orbital shaker (optional)
- Marker pens

For RNA extraction

- Micropipettes and tips
- Optional: liquid handling robot to automate pipetting
- Magnetic silica RNA extraction equipment
    - KingFisher™ Flex Purification System with 24-well head and associated plasticware if using the RNA extraction procedure described in this protocol.
- Vortex mixer
- Microcentrifuge tube spinner
- Microcentrifuge tubes

For RT-qPCR

- Micropipettes and tips
- Optional: liquid handling robot to automate pipetting
- Real-time PCR machine capable of supporting probe-based RT-qPCR

- Vortex mixer
- Microcentrifuge tube spinner
- Freezer capable of temperatures <-70°C
- Freezer capable of temperatures <-15°C
- Refrigerator capable of temperatures 3±2°C
- PCR cabinet with UV-C disinfection lamp

Reagents

Reagents used as purchased

For viral concentration

- Natural spring water (fit for human consumption according to Directive 2009/54/EC)
- Ammonium sulphate, reagent grade or equivalent
- Ringer ¼ strength solution:
    - Ringer ¼ strength solution (Oxoid BO0332)
      OR
    - Ringer ¼ strength solution tablets (Oxoid BR0052)
      OR
    - Sodium chloride, potassium chloride, calcium chloride hexahydrate and sodium biocarbonate, reagent grade or equivalent.

For RNA extraction

- Guanidine isothiocyanate (GITC) lysis buffer
    - NucliSENS lysis buffer. BioMerieux #280134 if using the RNA extraction procedure described in this protocol.
- RNA extraction reagents
    - Recommended: NucliSENS extraction buffers and magnetic silica beads. BioMerieux #280130, 280131, 280132, 280133
        - Alternative reagents may also be used if demonstrated to give equivalent results.
    - Thermo Scientific™ RNase AWAY™ Surface Decontaminant or a similar product to remove nucleases

For RT-qPCR

- Tris-EDTA (TE) buffer pH 8.0, molecular biology grade
- Triton™ X-100, molecular biology grade
- One-step RT-qPCR reagents
    - Recommended: Quantbio qScript™ XLT One Step RT-qPCR ToughMix® and Quantabio Ultra-Plex™ 1-Step ToughMix®
    - Alternative reagents may also be used if demonstrated to give equivalent results.

For preparation of phi6 process control

- Bacteriophage phi6 (DSM 21518)
- *Pseudomonas syringae* (DSM 21482)
- Tryptone Soya Broth (TSB):
    - Tryptone Soya Broth (Oxoid BO0369)
      OR

- Tryptone Soya Broth powder (Oxoid CM0876)
  OR
- Pancreatic digest of casein (Tryptone), papaic digest of soybean meal (Soytone), sodium chloride and dibasic potassium phosphate, reagent grade or equivalent.

- Glucose, reagent grade or equivalent.
- Tryptone Soya Agar (TSA):
  - Tryptone Soya Agar(Oxoid PO0163A)
    OR
  - powder (Oxoid CM0131)
    OR
  - Pancreatic digest of casein (Typtone), papaic digest of soybean meal (Soytone), sodium chloride and agar, reagent grade or equivalent.
- Glycerol, reagent grade or equivalent.

Prepared solutions/buffers

TEX buffer

For each 1000 ml of TEX buffer to be prepared, weigh 1±0.01 g of Triton X-100 and add to 1000±10 ml TE buffer. Mix thoroughly with a magnetic stir bar and stirrer before spitting into appropriately sized aliquots (e.g. 1 ml). Store at <-15°C.

Ringer's ¼ Strength Solution

Ringer's ¼ Strength Solution can be purchased premade and sterilised (Oxoid BO0332), made and sterilised from tablets (Oxoid BR0052) according to manufacturer instructions or made from constituent ingredients as follows:

Ingredient	Mass (g)
Sodium chloride	2.25
Potassium chloride	0.105
Calcium chloride hexahydrate	0.12
Sodium bicarbonate	0.05

Add all solid ingredients to 1000±10 ml deionised water in a conical flask with a magnetic stir bar. Place the flask on a magnetic stirrer and mix until all solids are completely dissolved. Separate into working aliquots and sterilise by autoclaving at 121±2°C for 15 minutes.

RT-qPCR PCR master mixes

RT-qPCR master mixes should be created to quantify the N gene of SARS-CoV-2 and the *phi-6S_1* gene of phi6 bacteriophage using the primers, probes and recipes listed in

Table 1, Table 2 and Table 3. Alternative master mixes may also be used if they follow the general requirements listed below and have been demonstrated to give equivalent results.

Table 1: A list of primers and probes used for RT-qPCR

Target	Name	Type	Sequence	Reference
SARS-CoV-2 N gene	2019-nCoV_N1-F	Forward primer	GAC CCC AAA ATC AGC GAA AT	(CDC, 2020)
SARS-CoV-2 N gene	2019-nCoV_N1-R	Reverse primer	TCT GGT TAC TGC CAG TTG AAT CTG	(CDC, 2020)
SARS-CoV-2 N gene	2019-nCoV_N1-P	Probe	ACC CCG CAT TAC GTT TGG TGG ACC	(CDC, 2020)
Phi6 *6S_1* gene	phi6_forward	Forward primer	TGG CGG CGG TCA AGA GC	(Gendron *et al.*, 2010)
Phi6 *6S_1* gene	phi6_reverse	Reverse primer	GGA TGA TTC TCC AGA AGC TGC TG	(Gendron *et al.*, 2010)
Phi6 *6S_1* gene	phi6_probe	Probe	CGG TCG TCG CAG GTC TGA CAC TCG C	(Gendron *et al.*, 2010)

Table 2: N1 RT-qPCR master mix recipe

Component	Volume per reaction	Final concentration
Quantbio qScript™ XLT One Step RT-qPCR ToughMix®	13 µl	1.3x
2019-nCoV_N1-F	-	400 nM
2019-nCoV_N1-R	-	400 nM
2019-nCoV_N1-P	-	100 nM
Template RNA	5 µl	-

Table 3: Bacteriophage phi6 RT-qPCR master mix recipe

Component	Volume per reaction	Final concentration
Quantabio Ultra-Plex™ 1-Step ToughMix®	6.5 µl	1.3x
phi6_forward	-	400 nM
phi6_reverse	-	400 nM
phi6_probe	-	100 nM
Template RNA	2 µl	-

General requirements for RT-qPCR master mixes

- Probes should be dual labelled with a 5' reporter and 3' quencher. Reporters should be chosen that are compatible with your RT-qPCR equipment.
- The original reporter/quencher combination used for 2019-nCoV_N1-P by CDC (CDC, 2020) was FAM/BHQ-1. If using a different combination, the quencher should be a compatible direct replacement of BHQ-1 (e.g. QSY, IQ4 or similar). Similarly, the original quencher for the phi6_probe was a BHQ (exact version unspecified) and so and replacement quencher for the probe should also be a compatible direct replacement for BHQ.
- Probes labelled with non-standard dyes and quenchers should be carefully tested and validated as different dyes and quenchers may affect the melting temperature of the probe.
- Multiplex RT-qPCR may be acceptable if laboratories can demonstrate that multiplex reactions work as well as the individual simplex reactions.

- If multiplex RT-qPCR will be used, then appropriate reporters and quenchers should be used which will not produce crosstalk between fluorescence channels. For example, commonly used dye pairs include FAM and VIC or FAM and HEX.
- RT-qPCR master mixes should be created using one step RT-qPCR reagents from reputable manufacturers and suppliers who can provide manufacturing quality control data for the products when requested.
- RT-qPCR master mixes must be optimised to consistently provide amplification of the standard curve with a slope between -3.1 and -3.6 (equivalent to an efficiency of between 90% and 110%) and $r^2 > 0.98$.

Control materials

Phi6 virus process control material

The *Pseudomonas* bacteriophage phi6 is used as a process control in this protocol. This control is added to samples near to the start of the procedure and the recovery in each sample is compared with a control. The phi6 recovery is used to indicate whether the process has been successful for individual samples. Phi6 recoveriess are used as quality assurance parameters only and not used to adjust test results.

Preparation of this control material requires bacterial culture facilities including incubator(s), culture consumables and media. The procedure for preparation of phi6 process control stocks is outlined in Appendix 1. Due to material sharing restrictions for phi6 and its bacterial host, all laboratories using this control must purchase their own stocks of phi6 and the host from the German Collection of Microorganisms and Cell Cultures (DSMZ).

Quantification standard

Quantification standards must consist of single stranded RNA (ssRNA) including primer and probe binding regions without mismatches. The amplicon produced by the ssRNA should be the same size and have a similar melt profile to the amplicon produced using the same primers with the reference SARS-CoV-2 N gene (Wuhan-Hu-1, NCBI NC_045512.2) or phi6 6S_1 gene (NCBI NC_003714.1). The target sequences are shown below:

N1 target sequence

GAC CCC AAA ATC AGC GAA ATG CAC CCC GCA TTA CGT TTG GTG GAC CCT CAG ATT CAA CTG GCA GTA ACC AGA

Bacteriophage phi6 target sequence

TGG CGG CGG TCA AGA GCA ACC CGG TCG TCG CAG GTC TGA CAC TCG CTC AGA TCG GAA GCA CCG GTT ATG ACG CCT ATC AGC AGC TTC TGG AGA ATC ATC C

Quantification standards may be commercially manufactured or made in-house. The production of ssRNA standards should be carried out in a quality control system to allow traceability of the production process and quality checks performed. Each new batch of ssRNA standards should not contain detectable levels of target DNA (e.g. cDNA or dsDNA). This can be demonstrated either by performing RT-qPCR in the usual manner, but without the RT step (therefore qPCR) or by preheating the RT-qPCR master mix to 95±1°C for 10 minutes prior to RT-qPCR. In either case, no target amplification should be seen.

All quantification standards used in the EMHP programme must be calibrated against the Reference Laboratory standard and their concentrations expressed as gene copies per µl (gc/µl). If new batches

of quantification standards need to be created, contact the Reference Laboratory lead as early as possible to arrange for this calibration to be carried out.

All ssRNA standards must also be checked quarterly against an equivalent dsDNA standard to ensure that no degradation of the ssRNA has occurred. This may be carried out by individual laboratories or by the Reference Laboratory.

Preparation of ssRNA standard dilutions

A tenfold dilution series should be created from the calibrated ssRNA standard stock. The dilution series should cover at least four concentrations of ssRNA within the range of 10^5-10^0 gc/µl. The dilutions should be carried out using TEX buffer as the diluent.

The ssRNA standard dilutions can be created daily for each set of RT-qPCRs individually, or created as a large batch, split into practical aliquots (e.g. 50 µl), and stored at ≤-70°C for up to 3 months.

Method

Preparation of daily phi6 process control working suspensions

1. On each day of testing, a new batch of phi6 process control working suspension should be made.
2. Remove an aliquot of phi6 stock from the freezer (prepared according to Appendix 1), thaw and mix thoroughly by vortexing.
3. Create a working suspension of phi6 by adding phi6 stock to Ringer's ¼ Strength Solution to a final volume of 50±0.5 ml and mixing thoroughly by inverting several times.

   Note: *The volumes of phi6 stock required will depend on the concentration of phi6 on the stock. The final concentration of phi6 in the working suspension needs to be high enough so that a 0.01% recovery can be quantified.*
   Note: *Larger volumes of phi6 process control working suspensions can also be made using the same proportions of phi6 stock to Ringer's ¼ Strength Solution if large numbers of samples are to be processed in a single day.*

Initial sample clarification

4. Mix each sample by inverting the sample bottle 10 times within a biological safety cabinet.
5. Measure approximately 200 ml of sample into a suitable centrifuge bottle.
6. Prepare at least two control centrifuge bottles with approximately 200 ml of spring water on each day of testing.
7. Centrifuge the samples and controls at 10,000 xg for 30 minutes at ambient temperature.
8. Place the centrifuge rotor into a biological safety cabinet and carefully remove the samples from the rotor being careful not to disturb the pelleted solids.

   Note: *If it is not feasible to remove the rotor from the centrifuge, then consideration should be made for how to deal with potential aerosolised wastewater in the event of a centrifuge bottle breakage.*

Virus concentration and lysis

9. On a digital balance, carefully decant 150±1 g of the clarified supernatant into clean bottles, being careful to minimise the amount of pelleted solids that are transferred.
10. To each 150 ml clarified sample and one of spring water control samples, add 1±0.01 ml of phi6 process control. Mix the samples well by shaking or inverting several times.
11. Pour the sample/phi6 mixtures in to centrifuge bottles containing 60±1 g of ammonium sulphate.

    Note: *The spring water control bottle with phi6 is the phi6 positive control, and the other spring water control is the total blank control.*
    Note: *The pellet in the centrifuge bottles can be disposed as biohazardous waste.*

12. Dissolve the ammonium sulphate by inverting the bottles several times, or by shaking on an orbital shaker at approximately 200 rpm until all of the ammonium sulphate has dissolved.
13. Incubate the samples at 3±2°C for at least 60 to 180 minutes.
14. Mark the outer sides of the bottle where the pellets will form during centrifugation.
15. Centrifuge the chilled samples for 30 minutes at 10,000 xg at 4±1°C with no braking.
16. Place the centrifuge rotor into a biological safety cabinet and carefully remove the bottles from the rotor being careful not to disturb the viral pellets, which may be invisible.

Note: *If it is not feasible to remove the rotor from the centrifuge, then consideration should be made for how to deal with potential aerosolised wastewater in the event of a centrifuge bottle breakage.*

17. Pour off the supernatant and dispose of this as biohazardous waste, keeping the pellet in the bottom of the bottle.
18. Add 2 ml of NucliSENS lysis buffer directly to the pellet.

Note: *If an alternative, validated RNA extraction method is to be used, then at this point an appropriate volume of GITC containing lysis buffer should be added to your sample according to that method.*

19. Resuspend the pellet in lysis buffer thoroughly by repeat pipetting. Be careful not to introduce air into the lysis buffer at this stage to avoid foaming.
20. If RNA extraction is not to be carried out immediately, pipette the lysis buffer and pellet mixture into clean tubes and store at 3±2°C for up to 48 hours before proceeding to RNA extraction 18.

RNA extraction

RNA extraction should be carried out using the KingFisher™ Flex Purification System described below or another RNA extraction system that has been demonstrated to give equivalent results and accepted by the reference laboratory.

KingFisher™ Flex Purification System method

21. Transfer the lysis buffer and pellet mixture to clean tubes or 24 well deep-well plates.
22. Incubate the lysis tubes or plates at room temperature for at least 10 minutes from when the pellets were resuspended.
23. To the same number of wells as samples + controls in a 24 well deep-well plate, add 50 µl of well-mixed NucliSENS magnetic silica beads.

Note: *Plates containing NucliSENS beads can be covered and stored at 3±2°C for up to 7 days before use.*

24. To the wells of two additional, clean 24 well deep-well plates, add 385 µl of NucliSENS wash buffer 1 to wells corresponding to those with magnetic silica beads prepared in step 23.
25. To the wells of two additional, clean 24 well deep-well plates, add 485 µl of NucliSENS wash buffer 2 to wells corresponding to those with magnetic silica beads prepared in step 23.
26. To the wells of one additional, clean 24 well deep-well plates, add 500 µl of NucliSENS wash buffer 3 to wells corresponding to those with magnetic silica beads prepared in step 23.
27. To the wells of one additional, clean 24 well deep-well plates, add 120 µl of NucliSENS wash buffer 3 to wells corresponding to those with magnetic silica beads prepared in step 23.

Note: *Plates containing NucliSENS wash buffers can be covered and stored at 3±2°C for up to 7 days before use.*

28. Transfer each sample lysate from step 22 to an individual plate well containing magnetic silica beads as prepared in step 23.
29. Load the SBPRO100 protocol on the KingFisher™ Flex Purification System.

Note: *The SBPRO100 protocol will be made available to laboratories by Environment Agency on request.*

30. Load the wash buffer and sample/magnetic silica bead plates onto the KingFisher™ Flex Purification System and press Start.
31. Following the 53-minute run, recover the elution plate and cover it.

    Note: *The elution plate the plate prepared at step 27, containing 120 µl of wash buffer 3 per well.*

32. If the RNA will not be used immediately for RT-qPCR following RNA purification, it should be stored at 3±2°C if it is intended to be used on the same day, <-15°C for use within 1 week or ≤-70°C for longer periods.
33. Decontaminate the KingFisher™ Flex Purification System to remove nucleases after each run using Thermo Scientific™ RNase AWAY™ Surface Decontaminant or a similar product.

RT-qPCR

The instructions below are for use with the EA's RT-qPCR master mixes for the N1 and phi6 assays. Alternative RT-qPCR master mixes and cycling conditions may also be used if they follow the general requirements listed in "General requirements for RT-qPCR master mixes" (page 11) and below and have been demonstrated to give equivalent results.

General requirements for RT-qPCR set-up

RT-qPCR should be carried out using the primers and probes outlined in "RT-qPCR PCR master mixes".

- Full sets of RT-qPCR controls and quantification standards should be used for every plate where sample RNA is assayed for that RT-qPCR target. For example, if a single RT-qPCR plate is to be used to analyse both SARS-CoV-2 and phi6 simultaneously, no template control (NTC) and ssRNA standard dilutions will be needed for both targets on the same plate.
- Prepare RT-qPCR master mixes immediately before starting procedure.
- Sample RNA and ssRNA quantification standards should be kept separate. It is preferable that they are added to the RT-qPCR plate in separate laboratories to avoid the potential for contamination of samples by ssRNA quantification standard. However, it is also possible to maintain separation of sample RNA and ssRNA quantification standards by using automated liquid handling systems. Laboratories must ensure that local quality procedures are maintained to reduce the possibility of cross contamination of this sort.

34. Make enough RT-qPCR master mix to test each sample (including total blank and phi6 positive controls), at least four ssRNA standard dilutions and TEX buffer NTC in duplicate for each assay (SARS-CoV-2 and phi6).

    Note: *Additional replicates can be used for the NTC and standard dilutions, but at least 2 replicates should be used for each reaction. Additional replicates of standard dilutions may increase the reliability of quantification and additional NTC reactions may be needed if background contamination of primer or probe stocks is suspected.*

35. Add the appropriate amount of RT-qPCR master mix to each well to be used in a PCR plate, leaving space for template RNA.
36. Add sample RNA, control template RNA or NTC to the appropriate wells. For the N1 assay the volume used is 5 µl for phi6, used and 2 µl.
37. Add ssRNA standard dilutions to the appropriate wells.

38. Cover RT-qPCR plates and carry out RT-qPCR in a real-time PCR machine capable of supporting probe-based chemistry using the following cycling conditions:
    - 20 minutes at 52°C
    - 10 minute at 96°C
    - 45 cycles of:
        - 15 seconds at 94°C
        - 1 minute at 60°C
39. Note the time when the RT-qPCR run finishes.

Analysis of results

Quality of amplification data

Analyse the amplification plots using the approach recommended by the manufacturer of the real-time PCR machine. The threshold should ideally be set so that it crosses the area where the amplification plots (logarithmic view) are parallel (the exponential phase).

Check all amplification plots to identify false positive results caused by high or uneven background signal (Figure 1)

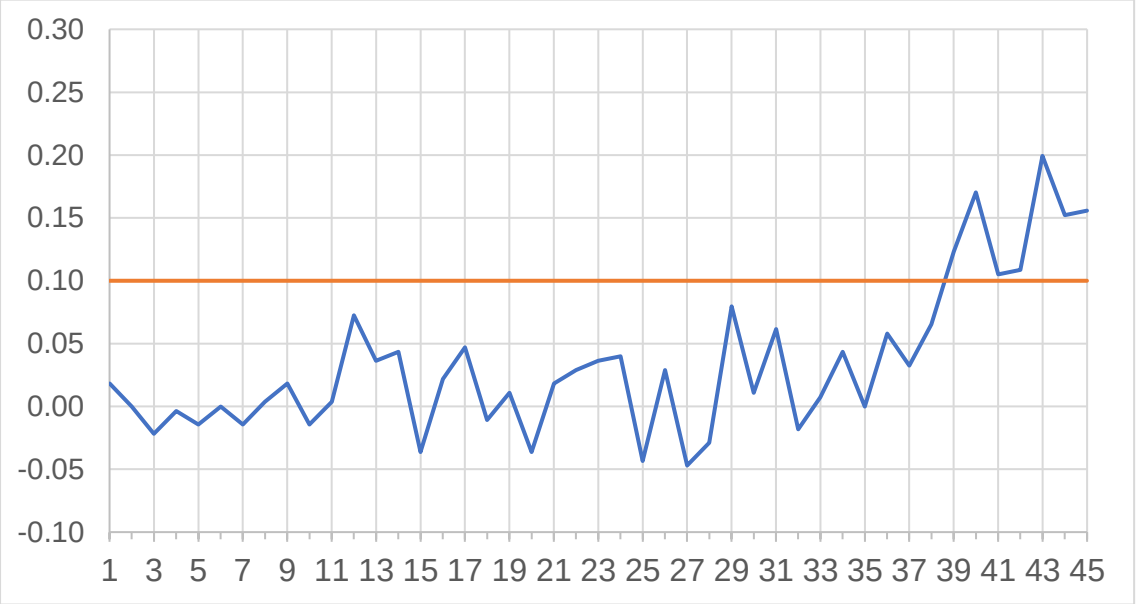

Figure 1: An example of a RT-qPCR amplification curve where a false positive was caused by uneven background signal.

Results for any wells affected in this way should be regarded as negative.

Check all amplification plots to identify true positive plots where the recorded quantification cycle (C_q) value is significantly distorted by high or uneven background signal. Approximate correct C_q values should be noted (in addition to the recorded value) for any wells affected in this way. Corrected C_q values should be used for all quantity calculations. Figure 2 shows a case where the recorded C_q value was 15.5, however it should be noted by the analyst that the correct figure should be e.g. 34.

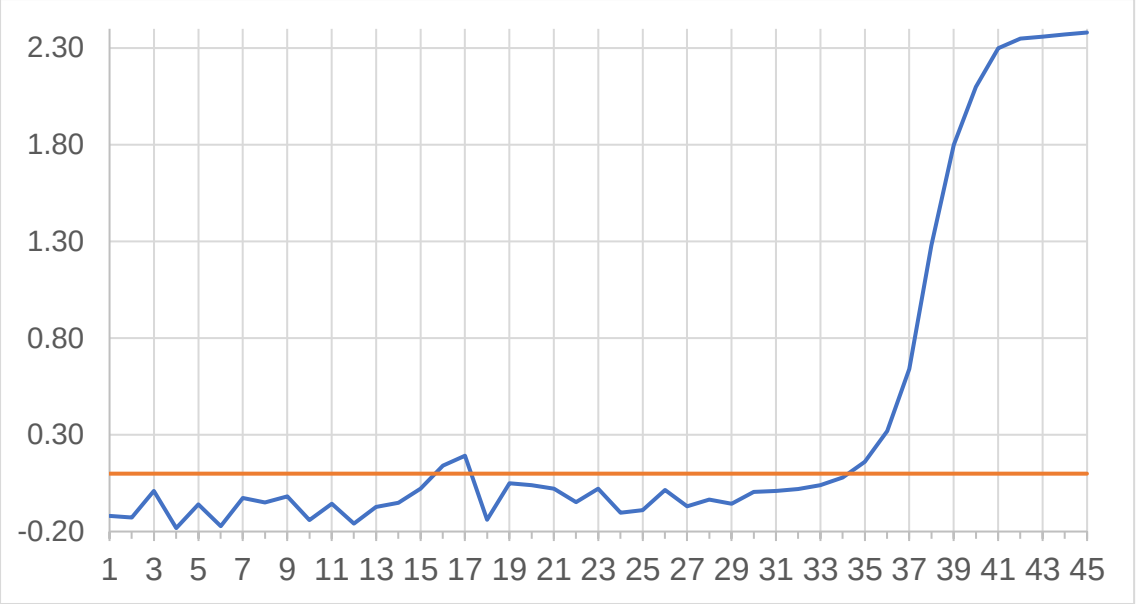

Figure 2: An example of a RT-qPCR amplification curve where uneven background signal caused an erroneously low C_q value to be reported.

Status of controls

No template controls (NTC)

Expected result: No amplification of N1, no amplification of phi6

Explanation: A positive result for the NTC indicates background contamination of the RT-qPCR.

Outcome of unexpected result: Repeat the entire RT-qPCR plate. The RT-qPCR set-up environment should be decontaminated. Also consider replacing PCR plasticware, TEX buffer, primer and probe working aliquots and RT-qPCR master mix. If contamination of NTC continues following multiple RT-qPCR repeats, investigate potential contamination sources and resolve as needed.

Total blank control

Expected result: No amplification of N1, no amplification of phi6

Explanation: A positive result for the total blank control indicates that there was contamination of the control at some point within the sample preparation process. This may have been due to contamination of the workstations where the samples were processed or by cross-contamination between samples.

Outcome of unexpected result: Repeat the RT-qPCR to confirm contamination. If no contamination is seen after repeating the RT-qPCR, then repeat RT-qPCRs for associated samples. If contamination of the total blank control is confirmed, all sample results associated with that control should be rejected.

Construction of standard curves

Calculate the r^2 (coefficient of determination), slope and intercept values for a plot of the C_q values of the ssRNA standard dilutions against $\log_{10}$ concentrations (copy numbers/µl).

Curves with r^2 <0.98 and the slope not between -3.6 and -3.1 (equivalent of 90% to 110% amplification efficiency) should not be used to calculate results.

If r^2 or slope fall outside these values, then up to two anomalous results may be removed from the standard curve and the r^2, slope and intercept values recalculated.

If r^2 or slope do not fall within these values after removal of anomalous results, then all RT-qPCR results should be rejected for that plate and the RT-qPCRs repeated for the associated sample RNAs.

Calculation of phi6 recovery

Estimate the recovery of the phi6 process control by comparing the C_q value for phi6 for each sample RNA reaction against the C_q value for the phi6 positive control as follows:

$$\text{Phi6 process control recovery } = 10^{(\Delta C_q/\text{m})} \times 100\%$$

where ΔC_q = C_q value (sample RNA) – C_q value (phi6 positive control virus RNA) and m = slope of the phi6 RNA standard curve.

For each sample, calculate the % recovery separately for each replicate then calculate an average (arithmetic mean) of all replicate recovery values to determine an overall recovery for the sample.

Where recovery is <0.01%, the results are not valid and the sample must be retested.

Calculation of gc/µl (G)

To calculate the gene copies per reaction (gc/µl), use the following equation:

$$G = 10^{(\Delta C_q/\text{m})}$$

where G =gc/µl, ΔC_q = C_q value (sample RNA) – standard curve intercept and m = slope of the ssRNA standard curve.

For example:

For a sample RT-qPCR run where the standard curve slope was -3.37 and the intercept was 35.6, for a sample RT-qPCR giving a result of C_q value of 30.5, the gc/µl would be calculated as:

$$\Delta C_q = 30.5 - 35.6$$

$$= -5.1$$

and so

$$\text{G} = 10^{(-5.1/-3.37)}$$

$$= 32.6\ gene\ copies/\mu l$$

RT-qPCR replicates with negative results should be given a concentration of 0 gc/µl.

Determining variability between replicates (ΔG)

Before using G to calculate gene copies per liter, determine whether the duplicate reactions fall within acceptable levels of variability i.e. if both replicate results are below limit of quantification (LOQ) or if for both replicates G differs from the mean of G by <50% of the mean of G.

LOQ and limit of detection (LOD) for the RT-qPCR must be calculated experimentally in your laboratory in line with guidance from the reference laboratory. In the absence of an experimentally determined LOQ, a nominal LOQ of 4 gc/µl should be used in the short-term. In the absence of an experimentally determined LOD, a nominal LOQ of 0.4 gc/µl should be used in the short-term. However, LOQ and LOD must be calculated as quickly as possible to ensure that results accurately reflect the limitations of your procedure.

If both replicates G differs from the mean of G by <50% of the mean of G, then the difference between replicate results in ≤3 times the lowest replicate. Therefore, this quality control parameter can be calculated using the following equation:

$$G_P = \frac{G_{max}}{G_{min}}$$

If G_P is ≤3, then the variability between replicates is acceptable. Examples of acceptable and unacceptable results are shown in Table 4.

Table 4: Examples of acceptable and unacceptable replicate results

Replicate 1 gc/µl	Replicate 2 gc/µl	RT-qPCR LOQ (gc/µl)	Acceptable variability?
150.0	300.0	4	Yes
150.0	450.0	4	Yes
150.0	600.0	4	No
1.0	3.8	4	Yes
1.0	4.0	4	No
1.0	4.2	4	No
4.0	6.0	4	Yes
3.0	10.0	4	No
150.0	300.0	10	Yes
150.0	450.0	10	Yes
150.0	600.0	10	No
1.0	3.8	10	Yes
1.0	4.0	10	Yes
1.0	4.2	10	Yes
4.0	6.0	10	No

Calculate gene copies per litre

Calculate the arithmetic mean average gc/µl for the duplicate RT-qPCR reactions (G_M).

The sample concentration in gene copies per litre (gc/l) should be calculated using the following equation:

$$G_L = 1000 \frac{V_e \, G_M}{V_S}$$

where G_L = sample concentration (gc/l), V_e = RNA elution volume (µl), V_S = volume of wastewater processed (ml).

For example:

Assuming an elution volume of 120 µl, where 150 ml of wastewater was processed. For a sample with duplicate G values of 32.6 gc/µl and 29.4 gc/µl, the sample concentration would be calculated as:

$$G_M = \frac{32.6 + 29.4}{2}$$

$$= 31\ gc/\mu l$$

and so

$$G_L = 1000\frac{120 \times 31}{150}$$

$$= 24{,}800\ gc/l$$

LOQ and LOD for the RT-qPCR must be calculated experimentally in your laboratory. In the absence of an experimentally determined LOQ, where a nominal LOQ of 4 gc/µl is applied, the LOQ for the whole process is 3,200 gc/l. In the absence of an experimentally determined LOD, where a nominal LOD of 0.4 gc/µl is applied, the LOD for the whole process is 160 gc/l. However, LOQ and LOD must be calculated as quickly as possible in line with guidance from the reference laboratory.

Summary of rejection and retesting criteria

- Positive result in NTC: Repeat RT-qPCR plate
- Positive result in RNA extraction negative control: Repeat RT-qPCR. Repeat RNA extractions if possible and if contamination is still present. Reject all sample results associated with RNA extraction negative control if is contamination still present.
- Positive result in total blank control: Repeat RT-qPCR. Reject all sample results associated with total blank control if is contamination still present.
- Quantification standard curve slope >-3.1 or <-3.7 after outliers removed: Repeat RT-qPCR plate
- Quantification standard curve r^2 <0.98 after outliers removed: Repeat RT-qPCR plate
- Recovery <0.01%. Repeat RNA extraction if possible. Reject sample result if recovery remains <0.01%.
- At least one RT-qPCR duplicate is >LOQ and ΔG is ≥50% of G_M: Repeat RT-qPCRs for that sample.

Reporting results

For all results obtained for the EMHP programme, results must be reported using the most up to date data template, which is available to testing laboratories from the programme data manager.

The following information from the RT-qPCR analyses must be reported for each sample in this template along with the rest of the sample information and data (e.g. sample metadata and inorganics data).

- RT-qPCR run end time and date
- Wastewater sample volume (150 ml)
- Name of the SARS-CoV-2 target (N1)
- Volume of template RNA added to RT-qPCR reactions
- Gene copies per µl of template RNA for each RT-qPCR replicate
    - For samples with no result, this should be left blank
- Mean gene copies per µl of template RNA

    - For samples with no result, this should be left blank
- C_q for each sample RT-qPCR reaction
    - Negative Cq values should be reported as 0.00
    - For samples with no result, this should be left blank
- SARS-CoV-2 concentration in sample (gc/l)
    - For samples with no result, this should be left blank
- Limit of detection (LOD) for SARS-CoV-2 (gc/l)
- Limit of quantification (LOQ) for SARS-CoV-2 (gc/l)

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Appendix 1 Preparation of phi6 process control material

Reagents required

Phi6 and *Pseudomonas* host

Phi6 and its bacterial host must be purchased from German Collection of Microorganisms and Cell Cultures (DSMZ):

- **phi6** DSM 21518
- ***Pseudomonas syringae*** DSM 21482

Tryptone soya broth (TSB)

Tryptone Soya Broth (TSB) can be purchased premade and sterilised (Oxoid BO0369), made and sterilised from powder (Oxoid CM0876) according to manufacturer instructions or made from constituent ingredients as follows:

Ingredient	Mass (g)
Pancreatic digest of casein (Typtone)	17.0
Papaic digest of soybean meal (Soytone)	3.0
Sodium chloride	5.0
Dibasic potassium phosphate	2.5
Glucose	2.5

- Add all solid ingredients to 1000±10 ml deionised water in a conical flask with a magnetic stir bar. Place the flask on a magnetic stirrer and mix until all solids are completely dissolved. Adjust to pH 7.3±0.2 1M NaOH or 1M HCl. Separate into working aliquots of 10±0.5 ml in centrifuge tubes and sterilise by autoclaving at 121±2°C for 15 minutes.
- Store at 3±2°C for up to 3 months.

Tryptone soya agar full strength (TSA)

Tryptone Soya Agar (TSA) can be purchased in pre-poured plates (Oxoid PO0163A), made and sterilised and poured from powder (Oxoid CM0131) according to manufacturer's instructions or made from constituent ingredients as follows:

Ingredient	Mass (g)
Pancreatic digest of casein (Typtone)	15.0
Papaic digest of soybean meal (Soytone)	5.0
Sodium chloride	5.0
Agar	15.0

- Add all solid ingredients to 1000±10 ml deionised water in a conical flask with a magnetic stir bar. Place the flask on a magnetic stirrer and mix until all solids are completely dissolved. Adjust to pH 7.3±0.2. Sterilise and melt by autoclaving at 121±2°C for 15 minutes. While still molten, pour approximately 20 ml of TSA1 into 90 mm Petri dishes and place lids on top of the dishes.
- Once set, store at 3±2°C for up to 3 months.

Ringer's ¼ Strength Solution

As described previously.

Calcium chloride solution

Dissolve 54.7 g calcium chloride hexahydrate in 500 ml deionised water. Autoclave at 121±2°C for 15 minutes and separate into aliquots of a practical working volume (e.g. 1 ml) under sterile working conditions.

Store at room temperature for up to 1 year.

Sterile glycerol

Separate glycerol into aliquots of a practical working volume (e.g. 10 ml) and autoclave at 121±2°C for 15 minutes.

Store at room temperature for up to 1 year.

Preparation of *Pseudomonas* host stock

Note: *The following procedures should be performed in a sterile environment to prevent contamination of cultures.*

1. Open a vial of freeze-dried *Pseudomonas* culture from DSMZ and rehydrate in 0.5 ml TSB.
2. Leave to rehydrate for 10 minutes and add to 8.5 ml of TSB.
3. Incubate, shaking at 25±1°C overnight (approximately 18 to 24 hours).
4. Check that the culture has become turbid overnight, indicating bacterial growth.
5. Add 1 ml of sterile glycerol and mix thoroughly by inverting several times.
6. Split into 150 µl aliquots in screwcap cryovials and store at ≤-70°C for up to 1 year.
7. New host stocks can be created by subculturing the host stock on TSA and using a single colony in place of the rehydrated *Pseudomonas* culture.
8. Completely new host stocks should be prepared annually using a new vial of freeze-dried culture.

Preparation of phi6 stock

Note: *The following procedures should be performed in a sterile environment to prevent contamination of cultures.*

9. Remove an aliquot of host stock from the freezer and allow it to thaw.
10. Mix gently by flicking the side of the vial.
11. Add 100 µl of host stock to 10 ml TSB in a sterile centrifuge tube and incubate, shaking at 25±1°C overnight (approximately 18 to 24 hours).
12. Check that the culture has become turbid overnight, indicating bacterial growth.

    Note: *An absence of turbidity indicates little or no bacterial growth. In this case, the culture process should be restarted, or the culture should be incubated for an additional 24 hours.*

13. Add 5 ml of TSB and 5 µl of calcium chloride solution to each of two centrifuge tubes.
14. Add 0.5 ml of overnight host culture to each centrifuge tube, incubate, shaking at 25±1°C overnight (approximately 18 to 24 hours).
15. Check that the culture has become turbid overnight, indicating bacterial growth.
16. Open and rehydrate a freeze-dried vial of phi6 and rehydrate in 500 µl Ringer's ¼ Strength Solution.
17. Add 100 µl of phi6 suspension to one of the host culture tubes, incubate both culture tubes, shaking at 25±1°C overnight (approximately 18 to 24 hours).
18. The culture broth with added phi6 should look clearer than the control broth following incubation.

Note: *if the culture broth with added phi6 does not look clearer than the control broth, phi6 growth may not have been efficient. In this case, either re-start the culture process, or incubate for a further 24 hours before continuing.*

19. Centrifuge the phi6 culture at 4,000 to 10,000 xg for 15 mins at 4±1°C and discard the control culture.
20. Add 0.8 g of sterile glycerol to two 15 ml tubes, and then fill each to 6 ml with the supernatant and invert to mix.
21. Split into 10 µl aliquots and store at <-70°C for up to one year.
22. New phi6 stocks should not be created by subculturing directly from broth cultures. Instead, the phi6 stock should be cultured by plaque assay according to Prussin *et al*. (2018) and individual plaques picked to be used as the starting phi6 material for step 16.
23. Completely new phi6 stocks should be created annually using a new vial of freeze-dried culture.