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RT-qPCR for detection of SARS-CoV-2 in wastewater V.2

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We use this protocol and it's working

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Abstract

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).

Viral RNA is extracted from a known volume of sewage treatment works influent (spiked with a know concentration of Porcine Reproductive and Respiratory Syndrome Virus, PRRSv, to act as an internal control) and amplified by Reverse Transcription Quantitative Polymerase Chain Reaction (RT-qPCR).

RT-qPCR is a process whereby RNA is first converted to complementary DNA (cDNA) after which it can undergo amplification and quantification via PCR. The amplification of a non-target sequence, in this case PRRSv, can also be carried out to act as an internal control.

This procedure, developed by Scottish Environment Protection Agency (SEPA) based on work in the Corbishley lab, Roslin Institute of the University of Edinburgh, outlines the method for amplification and quantification of the RNA extracted from crude sewage samples, but could equally apply to other RNA extracted from other types of environmental sample.

For viral RNA extraction from wastewater, please refer to "RNA extraction from wastewater for detection of SARS-CoV-2" by the same authors of this protocol on protocols.io (**dx.doi.org/10.17504/protocols.io.bzv5p686**).

For the data normalisation process to report the data as RNA copies per person, please see the protocol "Data normalisation of RT-qPCR data for detection of SARS-CoV-2 in wastewater" (**dx.doi.org/10.17504/protocols.io.b4eqqtdw**)

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1. Jones, D. L., Baluja, M. Q., Graham, D. W., Corbishley, A., McDonald, J. E., Malham, S. K., Hillary, L. S., Connor, T. R., Gaze , W. H., Moura , I. B., Wilcox, M. H., & Farkas , K. (2020).Shedding of SARS-CoV-2 in feces and urine and its potential role in person-to-person transmission and the environment-based spread of COVID-19.*Science of the Total Environment*.https://doi.org/10.1016/j.scitotenv.2020.1413644.

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3. Wastewater SARS Public Health Environmental Response (W-SPHERE).
https://sphere.waterpathogens.org/about

Guidelines

Performance and limitations

- Ribonucleic Acid (RNA) is a highly labile biomolecule that very susceptible to degradation by ubiquitous enzymes known as RNases. Care must be taken to ensure that all reagents, equipment and working environment are free of RNase contamination.

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

- RNA is a highly labile biomolecule that must be protected from near-ubiquitous degrading RNase enzymes temperature fluctuations. All RNA work should be conducted “on ice” (RNase activity restricted at 4C)

- During the concentration process substances which can inhibit enzyme activity and therefore interfere with the qPCR process can also be extracted. Inhibition is monitored by the inclusion of an internal control added. The addition of extra protein (i.e. BSA) can help to reduce inhibition.

- If target sequences are present in very low starting concentrations then a clear signal to noise ratio will be difficult to achieve.

In this protocol we used separated microbiological safety cabinets for mastermix preparation, positive template control and samples
This protocol describes an RT-qPCR using AriaMx or MxPro qPCR instruments (Agilent).

Interpretation guidelines

Once QC data has been checked raw data can be exported to XL where the number of gene copy equivalent per litre can be calculated. As is standard for laboratory tests of this nature, there are two numerical values which are important to understanding how much weight should be given to the value. These are known as the Limit of Detection (LoD) and the Limit of Quantification (LoQ). The limit of detection is the value at which the test has been determined to detect the virus material with certainty. The Limit of Quantification is the value, above which the test has been deemed to measure to virus material with a high degree of accuracy.

The Limit of Quantification was defined as the most dilute qPCR standard i.e. 5 copies per µl, equivalent to 1,500 genome equivalents per litre when 40 ml of wastewater was processed.

Values for reference:

- Limit of Detection (LoD) = 1,316 gc/L
- Limit of Quantification (LoQ) = 11,368 gc/L
- Detected but not quantifiable or "Positive (DNQ)" description is given to the sample where the average value of the replicates is between the LoD and the LoQ (i.e. between 1,316 and 11,386 gene copies per litre).

- "Weak Positive" will be given where two of three replicates return a positive signal but that signal is calculated as lower than the limit of detection (LoD).
- A sample is described as "Negative" where two replicates or two out of the three replicate tests return no response for the COVID markers.

Results are reported as follows:

If:

1. Replicate average > LoQ = Quantifiable positive result
Report average value as gene copies per liter – ex. “12345 gc/L"

2. Replicate average >LoD but <LoQ = Detected but not quantifiable
Report as “Positive (DNQ)”

3. Replicate average <LoD = Weak Positive
Report as “Weak Positive”

4. Two or more replicates produce No Cq (No Ct) result: Negative
Report as “Negative”

LoQ and LoD calculation - Methodology

LoD and LoQ values were calculated based on the methodology described in a JRC Technical Report "Verification of analytical methods for GMO testing when implementing interlaboratory validated methods" jrc109940_verification_of_analytical... ":

- “Procedure for Absolute LOQ (LOQabs): A dilution series of known amounts of copies per reaction is tested in at least 10 PCR replicates (e.g. 80, 60, 40, 20, 10, 5 copies and 1 copy per reaction). Then the RSDr is calculated for each dilution level. The LOQabs is estimated as the last dilution level where the RSDr* of the measurements is below 25 %.”
  *RSDr: Relative repeatability standard deviation

- “Procedure for Relative LOD (LODrel): a positive control material of low concentration can be measured in 10 PCR replicates and if all replicates are positive, this infers that the LODrel is below or equal to the positive control material level.”

Materials

Equipment

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

- Air displacement Pipettors (0.2µl to 1000µl)
- DNase/RNase free, filtered pipette tips
- 200-2000 µL, low retention tubes
- UV sterilisation cabinets
- 96 well plates and qPCR grade optically clear sealing caps
- Microcentrifuge
- Agilent (formerly Stratagene) MxPro or Aria Mx Real-Time PCR detection systems plus PC with relevant software
- Platefuge or Centrifuge with 96well plate rotor
- Cold blocks for 96 well plate/200 µl PCR tubes
- Ice maker and flaker

Reagents and Standards

- Molecular biology grade water – nuclease free
- RT-qPCR Master Mix – i.e. Luna One-Step RT-qPCR
- Bovine Serum Albumin (BSA) – working stock solution 1 µg/µl
- SARS-CoV-2 template for standard curve generation (we use EURM Reference Material ssRNA fragments of SARS-CoV-2 (EURM019))
- PRRSv template for standard curve generation (we use synthetic fragments of the ORF7 gene, purchased from IDT)

- SARS-CoV-2 assay primers & probes
- PRRSv assay primers and probes
- Primers_probes_list_SARS-CoV-2_...

Troubleshooting

Safety warnings

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).
- Only trained staff are permitted to perform RT-qPCR
- Before carrying out a lab procedure, staff must be familiar with all relevant COSHH assessments and lab's Health & Safety procedures.
- Due to the sensitivity of the assays, extra care must be paid to avoid sample contamination during analysis, and as such nitrile gloves must be worn at all times.

Before start

Samples should be processed as soon as possible after extraction. If previously frozen they must be analysed immediately after they are defrosted (on ice).

Sample and equipment setup

1 At start of day, clean all surfaces with RNase away or 10% v/v bleach solution.

2 Turn on UV lamps in the Master Mix preparation cabinet and Template addition cabinet for 20 minutes.

3 Prior to analysis; ensure there are sufficient cooler blocks and/or ice baths for each workstation.

4 Complete RT-qPCR Plate Worksheet and calculate the number of reactions (N) and volume of Master Mix required. See Appendix 1 for details.

Appendix_1_SARS-CoV-2_RT-qPCR...

5 Defrost sufficient RT-qPCR reagents and refrigerate until ready to use.
N.B. DO NOT REMOVE RT ENZYME FROM -20°C FREEZER UNTIL READY TO USE. MAINTAIN ON ICE AT ALL TIMES AND RETURN IMMEDIATELY AFTERWARDS.

6 Defrost positive control and any required samples and maintain on ice.

Standard curve preparation

7 For the standard curve preparation, use a 1:10 dilution series covering 5 dilution points (50000 to 5 copies per µl of SARS-CoV2 ssRNA and PRRSv DNA standards). See note below for standards details.

Note

STANDARDS

- To generate standard curves for quantification of SARS-CoV-2 we use EURM Reference Material ssRNA fragments of SARS-CoV-2 (EURM019)
- To generate standard curves for quantification of the Porcine reproductive and respiratory syndrome virus (PRRSv), we use synthetic fragments of the ORF7 gene, purchased from **IDT**

8 In positive template cabinet label 5 tubes and add 225 µl of RNase-free water to each and place on ice.

9 Transfer 25 µl from stock solution and add to labelled (5e+4) tube.

10 Mix and spin twice to ensure thorough homogenisation

11 Repeat step 9 and 10 until dilution series is complete. Store on ice until needed.

12 Go to "Standard curve analytical Control" steps for data interpretation (32 - 33)

Master Mix Preparation

13 DEFROST AND MAINTAIN REAGENTS ON ICE

	A	B	C
	Reagent	Volume (µl) per reaction (N)	Per sample (duplicate x 2.5)
	RT-qPCR reaction mix	10	25
	RT enzyme	1	2.5
	Primer/Probe mix	1.5	3.75
	BSA	2	5
	Nuclease-free water	0.5	1.25
	Total of master mix (without template)	15	37.5
	Template RNA	5	n/a

	A	B	C
	Total reaction volume	20	50

Table 1 Master Mix

The list containing primers and probes sequences and concentration can be found in the attached file below

Primers_probes_list_SARS-CoV-2_...

14 In the Master Mix cabinet, working on ice, combine the master mix reagents (excluding template). Invert x5 to mix.

15 Dispense 15 µl master mix to each well required of a 96 well PCR plate (on ice).

16 Add 5 µl of nuclease-free water to "no template control" (NTC) wells and cap.

17 Cover plate and transfer to sample addition cabinet

Plate setup

18 Add 5 µl of sample to appropriate wells and cap.

19 Cover and transfer to Template addition cabinet.

20 Add 5 µl of each positive control dilution series to appropriate wells and cap.

21 Spin plate down (1000 RCF for 1 minute) and transfer the plate to the qPCR instrument. Lower and lock the upper tray in place and close the door

RT-qPCR using AriaMx Real-Time PCR system

22 Select “New Experiment” and “Quantitative PCR (Fluorescence Probe)”.

23 In “Plate setup” select all wells and assign as per Plate Worksheet and choose relevant filters
N1 – FAM and ROX.
E gene (optional) – HEX and ROX
PRRS – Cy5 and ROX

24 In Thermal Profile tab set the following parameters:

	A	B	C
	Temperature	**Time**	**Cycles**
	55 ºC	10 min	1
	95 ºC	1 min	1
	95 ºC	10 seconds	40
	55 ºC	30 seconds	

RT-qPCR parameters

25 Save file with desired name

26 Select "Run experiment"

27 After Run Save file and copy to PC for detailed plate setup.

28 Open file using Aria software

29 Highlight all wells and assign ROX as reference dye.

30 Highlight sample wells and assign sample identifier (this can be imported from qPCR worksheet XL).

31 Highlight “Standard” wells and assign appropriate concentration values

Standard curve analytical quality control

32 Before results can be determined highlight all wells and select the following columns: Well, Well Name, Well Type, Ct (dRn) or Cq (ΔRn), Quantity (copies), RSq (dRn) or R^2 (ΔRn), Slope (dRn) and Efficiency (%).

33 Check the baseline threshold values (~0.03 FAM and ~0,1 Cy5).

33.1 Ensure that NTC wells are negative (No Cq).

33.2 Ensure that Standard Curve meets the following criteria:
R-Sq is >0.9,
Slope (y) is between -3.1 and -3.92 and
Efficiency is between 80 and 110%.

If any of these parameters are exceeded then analysis should be repeated.

Interpretation of raw data

34 Once QC data has been checked raw data can be exported to XL where the number of gene copy equivalent per litre can be calculated.

35 Results should be reported as follows:

1.Replicate mean >LoQ = Quantifiable Positive Result (ie 12345 gc/L)

2.Replicate mean >LoD but <LoQ = “Positive (DNQ)”

3.Replicate mean <LoD = “Weak Positive”

4.If both replicates produce No Cq result = “Negative”

LoQ= limit of quantification
LoD= limit of detection

Check "Guidelines & Warnings" section for more details on the LoQ and LoD values