Standardized preservation, extraction and quantification techniques for detection of fecal SARS-CoV-2 RNA

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Abstract

COVID-19 patients shed SARS-CoV-2 RNA in stool, sometimes well after their respiratory infection has cleared. In our benchmarking study, we recommend a standardized protocol for the preservation, extraction and detection of viral RNA from stool. This protocol includes a preservative, viral RNA extraction steps, and PCR-based quantification methods to maximize yield and detection of SARS-CoV-2 RNA. Our protocol takes advantage of commercially available reagents and equipment to maximize ease of access and consistency across studies. Additionally, we apply an attenuated bovine coronavirus vaccine as a spike-in control, and synthetic RNA standards to improve standardization and reliability of the assay. While we recommend both ddPCR and RT-qPCR-based assays, we acknowledge that ddPCR may be prohibitively expensive due to the necessity of specialized equipment and reagents. This protocol was developed with a focus on SARS-CoV-2 RNA, but may apply to other coronaviruses as well. We estimate that this protocol takes between 6 to 8 hours total to quantify the viral RNA load in a fecal sample.

Introduction

Reagents

Zoetis Calf-Guard Bovine Rotavirus-Coronavirus Vaccine (Catalog # VLN 190/PCN 1931.20)

SARS-CoV-2 ATCC standard (Catalog # VR-3276SD)

QIAamp Viral RNA Mini Kit (Qiagen; Catalog # 52906)

TaqPath 1-Step RT-qPCR CG mastermix (Applied Biosystems, Catalog # A15300)

Nuclease-free water (Ambion, Catalog # AM9937)

Please find forward primer, reverse primer, and probe sequences in Supplementary Table 13 of our associated publication.

20x Custom ddPCR Assay Primer/Probe Mix (BioRad, Catalog # 10031277)

One-Step RT-ddPCR Advanced Kit for Probes (BioRad, Catalog # 1864021)

Equipment

Vortexer

Centrifuge

1.5 mL and 2 mL Eppendorf Tubes

qPCR Thermocycler (QuantStudio 12K Flex, Applied Biosystems)

ddPCR Thermocycler (BioRad T100)

Procedure

Preparation and Viral RNA Extraction

1. Prepare bovine coronavirus (BCoV) by resuspending one vial of lyophilized Zoetis Calf-Guard Bovine Rotavirus-Coronavirus Vaccine (Catalog # VLN 190/PCN 1931.20) in 3 mL of PBS to create an undiluted reagent as per manufacturer's instructions.

2. Prepare a standard curve by performing a five-point ten-fold dilution of the SARS-CoV-2 ATCC standard (Catalog # VR-3276SD) from 10^4 to 10^0 copies per µL of template.

3. Thaw the preserved stool sample and vortex well for 5 seconds before transferring 600 µL of into a 1.5 mL Eppendorf tube.

4. Add 12 µL of the prepared BCoV vaccine to each 600 µL sample and vortex for 3 – 5 seconds.

5. Centrifuge each sample at 10,000x *g* for 2 minutes to pellet solids. (If solids do not pellet, speed can be increased to 16,000x *g*).

6. Collect 200 µL of supernatant.

7. Extract viral RNA using the QIAamp Viral RNA Mini Kit (Qiagen; Catalog # 52906) according to manufacturer's instructions.

8. Store samples in a 96-well plate at -80°C.

Quantification of viral RNA by RT-qPCR

1. Prepare the master mix in the following proportions per sample to be assayed: 5 µL of TaqPath 1-Step RT-qPCR CG mastermix (Applied Biosystems, Catalog # A15300), 8.5 µL of nuclease-free water (Ambion, Catalog # AM9937), and 1.5 µL of primer/probe mix.

a. Be sure to account for assaying your samples, the standards, and the negative controls in duplicate.

b. We recommend increasing the final volume prepared by 10% to account for dead volume during reaction set up.

c. The primer/probe mix is composed of 200 nM each of forward primer, reverse primer and probe (Elim Biopharmaceuticals) with sequences summarized in Supplementary Table 13 of our associated publication. We designed the probes with 5' Fluorescein (FAM) and 3' 5-Carboxytetramethylrhodamine (TAMRA) dyes.

2. Add 15 µL of master mix per well for each sample to be assayed.

3. Add 5 µL of eluted sample RNA to each well for a total reaction volume of 20 µL.

a. Samples are mixed by pipetting.

4. Seal and spin plates down at 500x *g* for 2 minutes to remove droplets from the side and tops of wells.

5. Amplify the template using the following thermocycling program: 25°C for 2 min, 50°C for 15 min, 95°C for 2 min, 45 cycles of 95°C for 15 sec and 55°C for 30 sec with ramp speed of 1.6°C per second at each step.

Data Analysis of RT-qPCR Data

1. Calculate the concentration of RNA using a linear regression of the standard curve.

2. Pick the highest copy number from among the y-intercept of the line of best fit from the standard curve or negative controls to establish a limit of blank/detection threshold for each plate.

3. Treat samples with copy numbers that fall below this threshold as undetermined/undetected.

4. Average the copy numbers of replicate wells. If only one of the two wells yielded a detectable amount of viral RNA, treat the sample as having no detected viral RNA.

Quantification of viral RNA by ddPCR

1. Prepare the master mix in the following proportions per sample to be assayed: 5.5 µL Supermix, 2.2 µL reverse transcriptase, 1.1 µL of 300 nM Dithiothreitol (DTT), 1.1 µL of 20x Custom ddPCR Assay Primer/Probe Mix (BioRad, Catalog # 10031277) and 6.6 µL of nuclease-free water (Ambion, Catalog # AM9937). The Supermix, reverse transcriptase and DTT are from the One-Step RT-ddPCR Advanced Kit for Probes (BioRad, Catalog # 1864021).

2. Add 16.5 µL of master mix per well for each sample to be assayed.

3. Add 5.5 µL of eluted sample RNA to each well for a total reaction volume of 20 µL.

4. Seal and vortex the plate well for 30 seconds.

5. Follow manufacturer instructions to partition samples into droplets.

6. Amplify the template using the following thermocycling program: 50°C for 60 min, 95°C for 10 min, 40 cycles of 94°C for 30 sec and 55°C for 1 min, followed by 1 cycle of 98°C for 10 min and 4°C for 30 min with ramp speed of 1.6°C per second at each step.

7. Use a droplet reader to read 20 µL of amplified product, ensuring no bumping or rocking of the plate when transferring sample to droplet reader.

Thresholding ddPCR Data

1. Set the threshold between the mean positive and negative amplitudes of the negative and positive controls on each plate to minimize detected copies in the negative controls and reflect expected RNA concentration of positive controls.

2. Calculate the difference between the mean negative amplitude and threshold amplitude in the negative control reactions.

3. Add the difference obtained in step 2 to the mean negative amplitude for each sample, applying this as the new threshold.

4. Identify the highest copy number detected in the negative controls; treat samples with copy numbers that fall below this threshold as undetermined/undetected.

Troubleshooting

When thresholding ddPCR data, there may be too many positive droplets, saturating the signal. If this happens, dilute the sample in water and rerun.

Time Taken

3 hours for sample preparation and extraction

3 hours for RT-qPCR quantification

5 hours for ddPCR quantification

Anticipated Results

Quantification of SARS-CoV-2 viral load in a stool sample.

References

1. Natarajan, A., Han, A., et al. Standardized and optimized preservation, extraction and quantification techniques for detection of fecal SARS-CoV-2 RNA. Preprint at https://www.medrxiv.org/content/10.1101/2021.04.10.21255250v1

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