## Spike-and-Recovery Procedures

### Materials/Equipment

* Pipettes
  + Gilson PG26567 (100-1000 µl)
  + Gilson P427242 (50-200 µl)
  + Denville P1590080 (2-20 µl)
* Thermo Scientific Sorvall Legend X1 Centrifuge with MicroClick 30 x 2 rotor (cat# 75-612-024)
* AirScience PCR work station (cat# Al-PCR-24)
* FisherBrand vortex (cat# 14-955-163); 500-2800 RPM, 50-60 Hertz
* PMT-DP
* Analyte: Heat-inactivated SARS-CoV-2 (ATCC VR-1986HK; 2019-nCoV/USA-WA1/2020 (4.2 x 105 copies/µl))
* DNase/RNase-free 1.5ml microcentrifuge tubes
* DNase/RNase-free 2ml microcentrifuge tubes
* P10, P100, P1000 pipettes/tips
* 10ml conical tubes
* Qiagen Viral RNA column-based extraction mini kit (cat# 52906)
* Ethanol (100%)
* Invitrogen RNase-free water

### Spiking Protocol

1. Products:
   1. If PMT-001, -002: thaw filtrate and create three 2ml aliquots and spin at room temperature for 20 min at 4,000 x g.
   2. If PMT-003: re-suspend the contents of one capsule into 5ml of LB and vortex briefly to homogenize.
2. Create seven 126µl aliquots of the resulting supernatant/resuspension from step 1 in 1.5ml microcentrifuge tubes and label #1-6. Create an eighth aliquot of 140µl of resulting supernatant and label #7.
3. Complete the serial dilutions outlined in the table below:

|  |  |  |  |
| --- | --- | --- | --- |
| **Tube** | **RNA (µL)** | **Fecal Filtrate/Capsule suspension (µL)** | **[RNA] (copies/mL)** |
| 1 | 14µL (from 4.2 10^5 copies/µl stock) | 126 | 4.2 x 107 |
| 2 | 14µL (from tube 1) | 126 | 4.2 x 106 |
| 3 | 14µL (from tube 2) | 126 | 4.2 x 105 |
| 4 | 14µL (from tube 3) | 126 | 4.2 x 104 |
| 5 | 14µL (from tube 4) | 126 | 4.2 x 103 |
| 6 | 14µL (from tube 5) | 126 | 4.2 x 102 |
| 7 | 14µL (from tube 6) | 126 | 4.2 x 101 |
| 8 | 14µL (from tube 7) | 126 | 4.2 x 100 |
| 9 | 0 (negative control) | 140 | 0 (negative control) |

1. Mix each tube well before continuing by pipetting gently up and down. Do not vortex. Avoid the creation of bubbles.
2. Place each tube within -80°C for ≥ 20min until frozen solid.
3. Continue to RNA extraction protocol.

### RNA Extraction (Recovery) Protocol

Before starting: Equilibrate samples to RT. Check that buffer AW1 and AW2 have been prepared. Add carrier RNA reconstituted in buffer AVE to buffer AVL.

|  |  |  |  |
| --- | --- | --- | --- |
| Buffer type | Buffer concentrate | Ethanol | Final volume |
| Buffer AW1 | 19ml | 25ml | 44ml |
| Buffer AW2 | 66ml | 160ml | 226ml |

Carrier RNA initial prep: Add 310 µl buffer AVE to the tube containing 310 µl lyophilized carrier RNA to obtain a solution of 1µg/µl. Dissolve the carrier RNA thoroughly, divide into conveniently sized aliquots and store at -20⁰C. DO NOT freeze-thaw the aliquots of carrier RNA > 3 times. [This step is done once when first using kit]

Carrier RNA subsequent prep: For the seven samples, done in triplicate in this experiment, combine 11.76 ml buffer AVL with 117.6 µl carrier RNA-AVE.

1. Pipet 560µl prepared buffer AVL containing carrier RNA into a 1.5ml microcentrifuge tube.
2. Add 140 µl of thawed sample to the buffer/carrier RNA containing tube. Mix by pulse-vortexing for 15s.
3. Incubate at RT for 10 min.
4. Briefly centrifuge the tube to remove drops from the inside of the lid.
5. Add 560µl ethanol (96-100%) to the sample and mix by pulse vortexing for 15s. After mixing, briefly centrifuge the tube to remove drops from the inside of the lid.
6. Carefully apply 630µl of the solution from step 5 to the QIAamp mini column (in a 2ml collection tube, provided) without wetting the rim. Close the cap, and centrifuge at 6,000 x g (8,000 rpm) for 1 min (can increase speed if necessary to pass membrane). Place the column into a clean 2ml collection tube and discard the tube containing the filtrate.
7. Open the column and repeat step 6.
8. Open the column, and add 500 µl buffer AW1. Close cap and centrifuge at 6,000 x g (8,000 rpm) for 1 min. Place the column into a new collection tube and discard the tube containing the filtrate.
9. Open the column and add 500µl buffer AW2. Close cap and centrifuge at full speed (20,000 x g; 14,000 rpm) for 3 min.
10. Place the column into a new collection tube and discard the old tube containing filtrate. Centrifuge at full speed for 1 min.
11. Place the column into a clean 1.5ml lo-bind microcentrifuge tube. Discard the collection tube containing the filtrate. Open the column and add 60μl RNase-free water. Close the cap and incubate at RT for 1 min.
12. Centrifuge at 6,000 x g (8,000 rpm) for 1 min.
13. Viral RNA is then stored at -80°C while waiting for downstream applications.

## RT-qPCR Procedures

### Materials/Equipment

* QS5 qPCR machine with corresponding plates
* 2019-nCoV RUO kit, 500 rxn (-20°C)(IDT #10006713)
  + N1 primer/probe contained within
* TaqMan Fast Virus 1-step Master Mix (cat # 4444434)
* Experimental RNA from spike-and-recovery steps
* Analytes:
  + Heat-inactivated SARS-CoV-2 (ATCC VR-1986HK; 2019-nCoV/USA-WA1/2020 (4.2 x 105 copies/µl))
  + SARS-CoV-2 RNA Standard (quantitative synthetic SARS-CoV-2 RNA: ORF, E, N (ATCC VR-3276SD) 105-106 copies/µl) pre-diluted to 104 copies/µl
  + IDT plasmid DNA (2019-nCoV\_N Positive Control(IDT 10006625))

### TaqMan Fast Virus 1-step Protocol

1. Deep clean PCR hood with RNase eliminating wipes, ethanol and ultraviolet light.
2. Collect reagents and place onto ice blocks, mixing and spinning down as needed. Do not vortex with the TaqMan master mix.
3. Create serial dilution of plasmid DNA according to the chart below:

|  |  |  |  |
| --- | --- | --- | --- |
| Tube | Plasmid DNA (µL) | Molecular Grade Water (µl) | [Plasmid DNA] (copies/mL) |
| P2 | 5µL (from 2 x 105 copies/mL stock) | 45 | 104 |
| P3 | 5µL (from tube 1) | 45 | 103 |
| P4 | 5µL (from tube 2) | 45 | 102 |
| P5 | 5µL (from tube 3) | 45 | 101 |
| P6 | 5µL (from tube 4) | 45 | 10-1 |

1. Mix each tube well before continuing by pipetting gently up and down ten times. Do not vortex. Avoid the creation of bubbles.
2. Create dilution of whole virus standard according to the chart below:

|  |  |  |  |
| --- | --- | --- | --- |
| **Tube** | **RNA (µL)** | **Molecular grade water (µL)** | **[RNA] (copies/mL)** |
| V1 | 14µL (from 4.2 10^5 copies/µl stock) | 126 | 4.2 x 107 |

1. Aliquot the diluted whole virus stock into 17.5µl volumes and store at -80°C until time of use.
2. Calculate how much mastermix needed, where n = number of reactions:

|  |  |
| --- | --- |
| Reagent | Vol. per reaction |
| Water | n x 8.5 µL |
| N1 | n x 1.5 µL |
| Taqman Fast Virus MM | n x 5 µL |
| Total Volume | n x 15 µL |

1. Create the mastermix in 1.5µL Eppendorf tubes; pipetting up and down to mix after each addition. Do not vortex Taqman or when the mastermix contains Taqman.
2. Fill 96-well plate according to the table and instructions below:

|  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
| A | FMT 2 - A | FMT 2 - A | FMT 2 - A | FMT 2 - B | FMT 2 - B | FMT 2 - B | FMT 2 - C | FMT 2 - C | FMT 2 - C | S3 | S3 | S3 |
| B | FMT 3 - A | FMT 3 - A | FMT 3 - A | FMT 3 - B | FMT 3 - B | FMT 3 - B | FMT 3 - C | FMT 3 - C | FMT 3 - C | P2 | P2 | P2 |
| C | FMT 4 - A | FMT 4 - A | FMT 4 - A | FMT 4 - B | FMT 4 - B | FMT 4 - B | FMT 4 - C | FMT 4 - C | FMT 4 - C | P3 | P3 | P3 |
| D | FMT 5 - A | FMT 5 - A | FMT 5 - A | FMT 5 - B | FMT 5 - B | FMT 5 - B | FMT 5 - C | FMT 5 - C | FMT 5 - C | P4 | P4 | P4 |
| E | FMT 6 - A | FMT 6 - A | FMT 6 - A | FMT 6 - B | FMT 6 - B | FMT 6 - B | FMT 6 - C | FMT 6 - C | FMT 6 - C | P5 | P5 | P5 |
| F | FMT 7 - A | FMT 7 - A | FMT 7 - A | FMT 7 - B | FMT 7 - B | FMT 7 - B | FMT 7 - C | FMT 7 - C | FMT 7 - C | P6 | P6 | P6 |
| G | FMT 8 - A | FMT 8 - A | FMT 8 - A | FMT 8 - B | FMT 8 - B | FMT 8 - B | FMT 8 - C | FMT 8 - C | FMT 8 - C | V1 | V1 | V1 |
| H | FMT 9 - A | FMT 9 - A | FMT 9 - A | FMT 9 - B | FMT 9 - B | FMT 9 - B | FMT 9 - C | FMT 9 - C | FMT 9 - C | NEG | NEG | NEG |
| P2-6 = Plasmid standard dilutions as described above.  V1 = Whole virus standard dilutions as described above.  S3 = Synthetic RNA standard | | | | | | | | | | | | | |

* 1. NEG = mastermix + molecular grade water
  2. Add 15 µL of the mastermix into all wells that will contain a sample or control.
  3. Add 5µl of each dilution or of each sample to the appropriate wells containing the mastermix; bringing the total volume to 20µL.
  4. Add 5µL of each standard to their appropriate wells.

1. Seal the plate with an optical adhesive cover, then mix by flicking wells and centrifuge.
2. On QS5 qPCR machine, create or select the following protocol (fast cycling mode from Taqman protocol, because reaction is < 30L)

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Step | Temp | Time | Stage | Cycles |
| Reverse Transcription | 50C | 5 min | 1 | 1 |
| RT inactivation/ initial denaturation | 95C | 20 sec | 2 | 1 |
| Denature | 95C | 3 sec | 3 | 40 |
| Anneal/Extend | 95C | 30 sec | 3 | 40 |

1. Select “Taqman” for Chemistry setting and “Fast” for Run Mode.
2. In method, create the correct steps and ensure volume is set to 25μL.
3. Load the plate into the machine and start the run, which should take ~50 minutes.
4. Export the data onto a USB drive (un-select “Open files after exporting” when exporting the csv)
5. Analyze using the standards to determine copy number.