**DECON SARS-CoV-2 qPCR Protocol**

**Purpose**

To detect SARS-CoV-2 from clinical specimens through reverse transcriptase quantitative PCR (RT-qPCR).

**Materials & Equipment**

* QuantStudio 5
* Applied Biosystems 96 well plates & seals
* TaqMan Fast Virus 1-Step Master Mix
* N1 Forward Primer (20µM)
* N1 Reverse Primer (20µM)
* N1 Probe (5µM)
* Plasmid DNA control (P2)
* Synthetic RNA control (1 x 104 copies/µL)
* Molecular grade water
* RNase/DNase free tubes
* P1000 pipette & tips
* P200 pipette & tips
* P20 pipette & tips

**Preparation Steps**

1. Deep clean the PCR hood with RNase wipes and ethanol.
2. Remove N1 F and N1 R primers and probe from freezer and place within hood to thaw at room temperature.

**RT-qPCR Procedure:** *Protocol adapted from “CDC 2019-Novel Coronavirus (2019-nCoV) Real-Time RT-PCR Diagnostic Panel” and utilizing product information sheet from the TaqMan Fast Virus 1-Step Master Mix.*

1. Prepare the master mix solution immediately before preparing PCR plate.
   1. Remove a master mix (MM) aliquot from -20C. Store on ice until ready to use.
   2. Use the table below to prepare the appropriate amount of master mix for the number of samples.
   3. Carefully mix by pipetting and spin down to remove droplets from the lid. **Do not vortex.**
   4. Keep solution on ice until ready to prepare the PCR plate.

**Table 1:** Master Mix Solution Recipe

|  |  |  |  |
| --- | --- | --- | --- |
|  | **1X** | **96X** | **+10%** |
| N1 F Primer (20µM) | 0.5 µL | 48 µL | 52 µL |
| N1 R Primer (20µM) | 0.5 µL | 48 µL | 52 µL |
| N1 Probe (5µM) | 0.5 µL | 48 µL | 52 µL |
| TaqMan Fast Virus 1-Step Master Mix | 5 µL | 480 µL | 520 µL |
| Molecular Grade Water | 8.5 µL | 816 µL | 898 µL |
| Template | 5 µL | - | - |

1. Prepare the standard curve dilutions.
   1. Remove P2 from -80C and allow to thaw.
   2. Label five tubes the following: P3, P4, P5, P6, and P7.
   3. Add 45µL of molecular grade water to each tube.
   4. Conduct five 1:10 serial dilutions as follows. Mix by pipetting and change tips between tubes.

**Table 2:** Standard Curve Dilutions

|  |  |  |  |
| --- | --- | --- | --- |
| **Tube** | **Plasmid DNA** | **Molecular Grade Water** | **Copies/µL** |
| P2 | *Aliquots premade in -80C* | - | 20,000 copies/µL |
| P3 | 5µL from P2 | 45 | 2,000 copies/µL |
| P4 | 5µL from P3 | 45 | 200 copies/µL |
| P5 | 5µL from P4 | 45 | 20 copies/µL |
| P6 | 5µL from P5 | 45 | 2 copies/µL |
| P7 | 5µL from P6 | 45 | 0.2 copies/µL |

* 1. Keep dilutions on ice until ready to prepare the PCR plate.

1. Gather RNA samples and positive control for PCR.
   1. When conducting a qPCR in triplicate, a maximµM of 24 RNA samples can be run. The master mix calcuLation above can be adjusted if less samples are to be run.
   2. Remove samples and one tube of “104 RNA” positive control from -80C.
      1. Note: previously used 104 RNA may be stored at -20C for up to two additional PCR runs.
   3. Record samples to be tested and their order on the PCR plate. Mark top of sample tubes to indicate that they have been freeze/thawed and PCR tested.
   4. While samples thaw, continue to prepare PCR plate.
2. Prepare qPCR plate.
   1. Place the qPCR plate on an ice block while filling the plate.
   2. Pipette 15 µL of master mix solution into each well.
   3. Pipette 5 µL of each sample, controls and a non-template control (molecular grade water) into their designated well using the plate layout below.
      1. Utilize reverse pipetting technique to minimize the introduction of bubbles.
      2. Pipette up and down three times to mix, again being careful to not introduce bubbles.

**Table 3:** Plate Layout

|  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
| A | 1 | 1 | 1 | 9 | 9 | 9 | 17 | 17 | 17 | 104 RNA | 104 RNA | 104 RNA |
| B | 2 | 2 | 2 | 10 | 10 | 10 | 18 | 18 | 18 | P2 | P2 | P2 |
| C | 3 | 3 | 3 | 11 | 11 | 11 | 19 | 19 | 19 | P3 | P3 | P3 |
| D | 4 | 4 | 4 | 12 | 12 | 12 | 20 | 20 | 20 | P4 | P4 | P4 |
| E | 5 | 5 | 5 | 13 | 13 | 13 | 21 | 21 | 21 | P5 | P5 | P5 |
| F | 6 | 6 | 6 | 14 | 14 | 14 | 22 | 22 | 22 | P6 | P6 | P6 |
| G | 7 | 7 | 7 | 15 | 15 | 15 | 23 | 23 | 23 | P7 | P7 | P7 |
| H | 8 | 8 | 8 | 16 | 16 | 16 | 24 | 24 | 24 | NTC | NTC | NTC |

* 1. Once all samples are added carefully seal the plate using the optical adhesive cover.
  2. Spin down the plate for 1 minute at 2,000g to remove any bubbles.

1. Run qPCR on QuantStudio 5
   1. Open the “DECON” template for the qPCR run, which should be set up as follows.
   2. “TaqMan” Chemistry, “Fast” run mode, “20 µL” sample volume

**Table 4:** QS5 Settings

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
|  | **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 | 60C | 30 sec | 3 | 40 |

* 1. When run is complete dispose of plate and export data on USB drive.
  2. Continue to documentation procedure for data management and analysis.

**Documentation Procedure**

1. After starting the PCR run, search for the tested RNA samples in LabVantage.
2. For each sample set, generate a LabVantage excel report and copy the sample information into “LabVantage Info” sheet in the DECON qPCR excel template.
   1. Detailed instructions about the template are listed on the first excel sheet.
3. Click the “Testing Complete” button for each sample set in LabVantage. This ensures that all tested RNA is accounted for in the system.
4. When the PCR run is complete, copy the “Results” sheet from the raw QS5 data file to the “Results” sheet in the DECON qPCR excel template.
5. Check that the R2 value is > 0.98; if not the qPCR must be redone. Enter the slope and intercept of the regression into the indicated fields in order to calcuLate the ng/µL of RNA for each sample.
6. Copy the data from the “Final” sheet into the “DECON Masterlist”.
7. Upload the qPCR template, raw excel data, and raw experiment data into the appropriate BOX folders.

**Reagent Dilutions**

1. TaqMan Fast Virus 1-Step Master Mix
   1. Large vials of master mix should be thawed at room temperature.
      1. NEVER vortex the master mix solutions.
   2. Prepare several Eppendorf tubes.
   3. Once the master mix is thawed, carefully aliquot 550µL into each tube.
      1. Note: the master mix is extremely viscous and must be pipetted slowly.
   4. After aliquoting all of the available master mix, label each tube with “MM” and the date prepared.
   5. Store all master mix aliquots in their designated box at -20C (one tube is needed per PCR plate).
      1. After preparing a master mix solution with primers and probes, any unused master mix may be stored at -20C to be used for future experiments. These tubes should be marked to indicate that they have been thawed and refrozen.
2. IDT 2019-nCoV\_N\_Positive Control – 250µL stock (200,000 copies/µL)
   1. Conduct a 1:10 dilution to prepare the **P2 stock (20,000 copies/µL)**
      1. 100µL of stock plasmid solution into 900µL of molecular grade water.
   2. Thoroughly mix by pipetting and spin down to remove droplets from the lid.
   3. Aliquot 40µL into 25 Eppendorf tubes.
   4. Label each tube “P2” and the date the sample was prepared.
   5. Store all P2 aliquots at -80C until ready for use (one tube is needed per PCR plate).
3. Quantitative Synthetic SARS-CoV-2 RNA: ORF, E, N (ATCC® VR-3276SD™) – 100µL stock (1 x 105 – 1 x 106 copies/µL)
   1. Conduct a 1:100 dilution to prepare the 1 x 104 RNA standard positive control.
      1. 5µL of stock RNA into 495µL of molecular grade water
   2. Thoroughly mix by pipetting and spin down to remove droplets from the lid.
   3. Aliquot 50µL into 10 Eppendorf tubes.
   4. Label each tube “104 RNA” and the date the sample was prepared.
   5. Store all RNA aliquots at -80C until ready for use (one tube can be used for three PCR plates).
      1. Once a tube is used for a PCR, it may be stored at -20C. The tube lid should be marked for each freeze/thaw and can be disposed of after the third PCR is conducted.
4. nCOV\_N1 Forward Primer Aliquot, 50 nmol – 250µL stock (100µM)
   1. Conduct a 1:5 dilution to prepare the working forward primer stock (20µM).
      1. 200µL of forward primer stock into 800µL of molecular grade water.
   2. Thoroughly mix by pipetting and spin down to remove droplets from the lid.
   3. If desired, aliquot into multiple tubes.
   4. Label each tube “N1 F” and the date the sample was prepared.
   5. Store all N1 F primer aliquots at -20C until ready for use.
5. nCOV\_N1 Reverse Primer Aliquot, 50 nmol – 250µL stock (100µM)
   1. Conduct a 1:5 dilution to prepare the working reverse primer stock (20µM).
      1. 200µL of reverse primer stock into 800µL of molecular grade water.
   2. Thoroughly mix by pipetting and spin down to remove droplets from the lid.
   3. If desired, aliquot into multiple tubes.
   4. Label each tube “N1 R” and the date the sample was prepared.
   5. Store all N1 R primer aliquots at -20C until ready for use.
6. nCOV\_N1 Probe Aliquot, 25 nmol – 250µL stock (100µM)
   1. Conduct a 1:20 dilution to prepare the working probe stock (5µM).
      1. 50µL of probe stock into 950µL of molecular grade water.
   2. Thoroughly mix by pipetting and spin down to remove droplets from the lid.
   3. If desired, aliquot into multiple tubes.
   4. Label each tube “N1 P” and the date the sample was prepared.
   5. Store all N1 P aliquots at -20C until ready for use.