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Technique: Real Time RT-PCR

Methodology: The protocol is designed in order to study here different targets in *N* gene. A fourth target, namely *RNAse P* (RNP) gene is also analyzed to assess specimen quality.

Material:

- RNA extraction kit

[None is specified, but the following are accepted to generate highly purified RNA and the external lysis buffer is effective for inactivation of SARS-CoV-2 (for more details original protocol should be considered):

- QIAGEN: QIAmp DSP Viral RNA Mini Kit (61904); QIAamp Viral RNA Mini Kit (52904,52906)
- QIAGEN EZ1 Advanced XL: EZ1 DSP Virus Kit (62724), Buffer AVL (19073), EZ1 Advanced XL DSP Virus Card (9018703); EZ1 Virus Mini Kit v2.0 (955134), Buffer AVL (19073), EZ1 Advanced XL Virus Card v2.0 (9018708)
- Roche MagNA Pure LC: Total Nucleic Acid Kit (03038505001)
- Roche MagNA Pure Compact: Nucleic Acid Isolation Kit (03730964001)
- Roche MagNA Pure 96: DNA and Viral NA Small Volume Kit (06543588001), External Lysis Buffer (06 374 913 001)
- QIAGEN QIAcube: QIAmp DSP Viral RNA Mini Kit (61904); QIAmp Viral RNA Mini Kit (52904,52906)
- bioMérieux NucliSENS® easyMAG® and bioMérieux EMAG®: EasyMAG® Magnetic Silica (280133), EasyMAG® Lysis Buffer (280134), EasyMAG® Lysis Buffer, 2 mL (200292), EasyMAG® Wash Buffers 1,2, and 3 (280130, 280131, 280132), EasyMAG® Disposables (280135), Biohit Pipette Tips (easyMAG® only) (280146), EMAG®1000µL Tips (418922)]

- Positive Control
- TaqPath™ 1-Step RT-qPCR Master Mix, CG (ThermoFisher; cat # A15299)
- Primers-Probe sets
- Vortex mixer
- Microcentrifuge
- Micropipettes (2 or 10 µL, 200 µL and 1000 µL)
- Multichannel micropipettes (5-50 µl)
- Racks for 1.5 mL microcentrifuge tubes
- 2 x 96-well -20°C cold blocks
- 7500 Fast Dx Real-Time PCR Systems with SDS 1.4 software (Applied Biosystems; catalog #4406985 or #4406984)
- Extraction systems (instruments): QIAGEN EZ1 Advanced XL
- Molecular grade water, nuclease-free
- 10% bleach (1:10 dilution of commercial 5.25-6.0% hypochlorite bleach)
- DNAZapTM (Ambion, cat. #AM9890) or equivalent
- RNAse AwayTM (Fisher Scientific; cat. #21-236-21) or equivalent
- Disposable powder-free gloves and surgical gowns
- Aerosol barrier pipette tips
- 1.5 mL microcentrifuge tubes (DNase/RNase free)
- 0.2 mL PCR reaction plates (Applied Biosystems; catalog #4346906 or #4366932)

- MicroAmp Optical 8-cap Strips (Applied Biosystems; catalog #4323032)

- Primers and Probes sequences

Target N1

2019-nCoV_N1-F: 5'-GACCCCAAAATCAGCGAAAT-3'
2019-nCoV_N1-R: 5'-TCTGGTTACTGCCAGTTGAATCTG-3'
2019-nCoV_N1-P: 5'-FAM-ACCCCGCATTACGTTTGGTGGACC-BHQ1-3'

Target N2

2019-nCoV_N2-F: 5'-TTACAAACATTGGCCGCAAA-3'
2019-nCoV_N2-R: 5'-GCGCGACATTCCGAAGAA-3'
2019-nCoV_N2-P: 5'-FAM-ACAATTTGCCCCCAGCGCTTCAG-BHQ1-3'

Target RNAse P

RP-F: 5'-AGATTTGGACCTGCGAGCG-3'
RP-R: 5'-GAGCGGCTGTCTCCACAAGT-3'
RP-P: 5'-FAM-TTCTGACCTGAAGGCTCTGCGCG-BHQ-1-3'

Protocol:

1. Viral RNA extraction, performed according to the chosen kit manufacturer's instructions. An extra human specimen (HSC) extraction should be performed in order to provide a control of the extraction procedure.

2. Preparation of Primer FW, Primer RV and Probe Mix, for each target separately.

3. Preparation of one-step monoplex RT-PCR master mix as below for each of the combined Primers/Probe Mixes. Negative and extraction controls should be performed to the four Primers/Probe Mix, with water and HSC respectively. An extra positive control (nCoVPC) should be performed with an nCoV template.

Reagent	Volume (µL) per reaction
Nuclease-free Water	8.5
TaqPath™ 1-Step RT-qPCR Master Mix (4x)	5
Primer FW	1.5 (Mix of the components)
Primer RV	
Probe	
Total reaction mix	15
Template RNA, add	5
Total volume	20

4. Set the follow RT-PCR conditions (if not available, the creation of a run template on Applied Biosystems 7500 Fast Dx Real-Time PCR Instrument is detailed in the original protocol):

Temperature (°C)	Time	No of Cycles
25 50 95	2 min 15 min 2 min	1
95 55	3 sec 30 sec	45

Results Evaluation: Negative control should not exhibit fluorescence and nCoVPC should produce positive results with an expected Ct value of <40.00 Ct for each target. The results of *RNAse P* test should be positive for all clinical samples and HSC. HSC should be negative for 2019-nCoV specific primers (Targets N1 and N2).
Results interpretation should be performed as fallowing:

2019-nCoV rRT-PCR Diagnostic Panel Results Interpretation			
2019-nCoV_N1	**2019-nCoV_N2**	**RNAse P**	**Result Interpretation**
+	+	±	2019-nCoV detected
If only one of the two targets is positive		±	Inconclusive Result
-	-	+	2019-nCoV not detected
-	-	-	Invalid Result