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Technique: Real Time RT-PCR

Methodology: The protocol is designed in order to perform three consecutive assays for the identification of infection by 2019-nCoV. The first line screening assay targets the *E* gene, and the confirmatory assay targets the *RdRp* gene (the second is performed if the result is positive for the first, and will detect 2019-nCoV, SARS-CoV and bat-SARS-related CoVs). A third assay is performed (discrimatory assay) if the result of the second is positive, which is specific for 2019-nCoV.

Material:

- MagNA Pure 96 system (Roche) (for clinical samples)
- Viral RNA mini kit (Qiagen) (for cell cultures supernatants)
- SuperScript™ III One-Step RT-PCR System with Platinum™ Taq DNA Polymerase (ThermoFisher)
- $MgSO_4$ (50 mM) (Sigma)
- Non-acetylated bovine serum albumin (Roche)

- Primers and Probes sequences
  (W is A/T; R is G/A; M is A/C; FAM, 6-carboxyfluorescein; BBQ, blackberry quencher)

  E gene:

  E_Sarbeco_F1: 5'-ACAGGTACGTTAATAGTTAATAGCGT-3'

  E_Sarbeco_R2: 5'-ATATTGCAGCAGTACGCACACA-3'

  E_Sarbeco_P1: 5'-FAM-ACACTAGCCATCCTTACTGCGCTTCG-BBQ-3'

  RdRP gene:

  RdRP_SARSr-F2: 5'-GTGARATGGTCATGTGTGGCGG-3'

  RdRP_SARSr-R1: 5'-CARATGTTAAASACACTATTAGCATA-3'

  RdRP_SARSr-P1: 5'-FAM-CCAGGTGGWACRTCATCMGGTGATGC-BBQ-3'

RdRP_SARSr-P2: 5'-FAM-CAGGTGGAACCTCATCAGGAGATGC-BBQ-3'

- Positive controls (available from Charité, Berlin, via EVAg; https://www.european-virus-archive.com/)

Protocol:

1. Viral RNA extraction, performed according to manufacturer's kit instructions

2. Preparation of RT-PCR master mix as below. Each assay is performed in order, depending if the prior result is positive (If assay No 1 is positive, continue to assay No 2; If assay No 2 is positive, continue to assay No 3).
   [Other Real-Time RT-PCR reagents can be used. In this case, oligonucleotides final concentrations should be taken in consideration).

Volumes (µL) per reaction for each assay			
Reagent	**Assay No 1** (E assay) First line screening	**Assay No 2** (RdRp assay) Confirmatory	**Assay No 3** (RdRp assay) Discrimatory)
H_2O (RNAse free)	2.6	0.6	1.1
2x Reaction Mix*	12.5	12.5	12.5
$MgSO_4$ (50mM)**	0.4	0.4	0.4
BSA (1 mg/ml)***	1	1	1
SSIII/Taq EnzymeMix*	1	1	1
Primer E_Sarbeco_F1 (10µM stock solution)	1	-	-
Primer E_Sarbeco_R2 (10µM stock solution)	1	-	-
Probe E_Sarbeco_P1 (10µM stock solution)	0.5	-	-
Primer RdRP_SARSr-F2 (10µM stock solution)	-	1.5	1.5
Primer RdRP_SARSr-R1 (10µM stock solution)	-	2	2
Probe RdRP_SARSr-P1 (10µM stock solution)	-	0.5	-
Probe RdRP_SARSr-P2 (10µM stock solution)	-	0.5	0.5
Total reaction mix	20	20	20
Template RNA, add	5	5	5
Total volume	25	25	25

* Thermo Fischer/Invitrogen: SuperScriptIII OneStep RT-PCR System with Platinum® Taq DNA Polymerase

** $MgSO_4$ (50 mM) [Sigma]. Not provided with the OneStep RT-PCR kit

*** non-acetylated bovine serum albumin [Roche].

3. Set the follow RT-PCR conditions (Both monoplex assays can be conducted under the same conditions):

Temperature (°C)	Time	No of Cycles
55 94	10 min 3 min	1
94 58	15 sec 30 sec	45