Author: Institute Pasteur, Paris

Technique: Real Time RT-PCR

Methodology: The protocol is designed in order to detect SARS-CoV-2 with two *RdRp* gene targets (IP2 and IP4) in a multiplex reaction. A singleplex confirmatory assay analysis of *E* gene is also performed.

Material:

- Kit Extraction NucleoSpin Dx Virus (Macherey Nagel; 740895.50)
- SuperScript™ III Platinum® One-Step Quantitative RT-PCR System (Invitrogen; 1732-020)
- LightCycler 480 (96)
- Positive control – *in vitro* transcribed RNA derived from strain BetaCoV_Wuhan_WIV04_2019 (EPI_ILS_402124) (available upon request; grippe@pasteur.fr)

- Primers and Probes sequences

RdRp gene / *nCoV_IP2* (108 bp)

nCoV_IP2-12669Fw: 5'-ATGAGCTTAGTCCTGTTG-3'

nCoV_IP2-12759Rv: 5'-CTCCCTTTGTTGTGTTGT-3'

nCoV_IP2-12696bProbe(+): 5'-HEX-AGATGTCTTGTGCTGCCGGTA-BHQ-1-3'

RdRp gene / *nCoV_IP4* (107 bp)

nCoV_IP4-14059Fw: 5'-GGTAACTGGTATGATTTCG-3'

nCoV_IP4-14146Rv: 5'-CTGGTCAAGGTTAATATAGG-3'

nCoV_IP4-14084Probe(+): 5'-FAM-TCATACAAACCACGCCAGG-BHQ-1-3'

E gene / *E_Sarbeco* (125 bp)

E_Sarbeco_F1: 5'-ACAGGTACGTTAATAGTTAATAGCGT-3'

E_Sarbeco_R2: 5'-ATATTGCAGCAGTACGCACACA-3'

E_Sarbeco_P1: 5'-FAM-ACACTAGCCATCCTTACTGCGCTTCG-BBQ-1-3'

Protocol:

1. Viral RNA extraction, performed according to NucleoSpin Dx Virus kit manufacturer's instructions. RNA extracted from 100 µL of original sample, is eluted in 100 µL of elution buffer.

2. Preparation of real-time RT-PCR master mix as presented below. While the analysis of *E* gene should be performed in a simplex mix, the primer sets of *nCoV_IP2* and *nCoV_IP4* can be multiplexed. Negative and positive controls should be performed.
   [Other kits can be used to perform RT-PCR. For the matter, the optimized concentrations of the reagents are described below for each reaction mixture]

Simplex Mix		
Reagent	**Volume (µL) per reaction**	**Final Concentration**
H_2O PPI	3.60	
Reaction Mix 2x	12.50	3 mM Mg
$MgSO_4$ (50mM)	0.40	0.8 mM Mg
SuperScript III/Platinum Taq Mix	1.00	
Primer FW (10 µM)	1.00	0.4 µM
Primer RV (10 µM)	1.00	0.4 µM
Probe (10 µM)	0.50	0.2 µM
Total reaction mix	20	
Template RNA, add	5	
Total volume	25	

Multiplex Mix		
Reagent	**Volume (µL) per reaction**	**Final Concentration**
H_2O PPI	1.30	
Reaction Mix 2x	12.50	3 mM Mg
$MgSO_4$ (50mM)	0.40	0.8 mM Mg
SuperScript III/Platinum Taq Mix	1.00	
Primer FW (10 µM)	1.00	0.4 µM
Primer RV (10 µM)	1.00	0.4 µM
Primer FW (10 µM)	1.00	0.4 µM
Primer RV (10 µM)	1.00	0.4 µM
Probe (10 µM)	0.4	0.16 µM
Probe (10 µM)	0.4	0.16 µM
Total reaction mix	20	
Template RNA, add	5	
Total volume	25	

3. Set the follow RT-PCR conditions:

Temperature (°C)	Time	No of Cycles
55 95	20 min 3 min	1
95 58	15 sec 30 sec	50
40	30 sec	1

Results Evaluation: Ct values for the reactions are presented below. The values may vary from instrument to instrument by up to 2 cycles, while he interval between two dilutions steps is constant (ΔCt).

Ct values for the performed reactions			
	Multiplex		**Singleplex**
RNA copies of transcript	**nCoV_IP2**	**nCoV_IP4**	**E_Sarbeco**
1,00E+07	21.67	21.97	24.72
1,00E+06	24.97	25.12	28.19
1,00E+05	28.00	27.88	30.96
1,00E+04	31.84	30.51	33.33