Protocol: Real-time RT-PCR assays for the detection of SARS-CoV-2

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This protocol describes procedures for the detection of SARS-CoV-2 for two RdRp targets (IP2 and IP4).

Based on the first sequences of SARS-CoV-2 made available on the GISAID database on January 11, 2020, primers and probes (nCoV_IP2 and nCoV_IP4) were designed to target the RdRp gene spanning nt 12621-12727 and 14010-14116 (positions according SARS-CoV, NC_004718).

As a confirmatory assay, we used the E gene assay from the Charité protocol[1]

Material

Kits:

Kit Extraction NucleoSpin Dx Virus — Ref: Macherey Nagel 740895.50
SuperScript™ III Platinum® One-Step Quantitative RT-PCR System — Ref: Invitrogen 1732-020

Primers and probes

Name	Sequences (5'-3')	Length (bases)	PCR product size	Ref.
RdRp gene / nCoV_IP2				
nCoV_IP2-12669Fw	ATGAGCTTAGTCCTGTTG	17	108 bp	1
nCoV_IP2-12759Rv	CTCCCTTTGTTGTGTTGT	18		
nCoV_IP2-12696bProbe(+)	AGATGTCTTGTGCTGCCGGTA [5']Hex [3']BHQ-1	21		
RdRp gene / nCoV_IP4				
nCoV_IP4-14059Fw	GGTAACTGGTATGATTTCG	19	107 bp	1
nCoV_IP4-14146Rv	CTGGTCAAGGTTAATATAGG	20		
nCoV_IP4-14084Probe(+)	TCATACAAACCACGCCAGG [5']Fam [3']BHQ-1	19		
E gene / E_Sarbeco				
E_Sarbeco_F1	ACAGGTACGTTAATAGTTAATAGCGT	18	125 bp	2
E_Sarbeco_R2	ATATTGCAGCAGTACGCACACA	20		
E_Sarbeco_P1	ACACTAGCCATCCTTACTGCGCTTCG [5']Fam [3']BHQ-1	20		

1/ National Reference Center for Respiratory Viruses, Institut Pasteur, Paris.
2/ Corman et al. Eurosurveillance[1]

Primer sets **nCoV_IP2** and **nCoV_IP4** can be multiplexed. Both reaction mixtures are described below.

PCR amplification regions (positions according to SARS-CoV, NC_004718)

nCoV_IP2 / 12621-12727
nCoV_IP4 / 14010-14116
E gene / 26141-26253

NUCLEIC ACID EXTRACTION

RNA is extracted from specimens using the NucleoSpin Dx Virus (Macherey Nagel ref. 740895.50). RNA extracted from 100 µl of original sample, is eluted in 100 µl of elution buffer.

MIX PREPARATION FOR ALL SEPARATE PRIMER/PROBE COMBINATIONS

All primers and probes described below were validated under the following conditions.

RT-PCR Mix kit:

- Invitrogen Superscript™ III Platinum® One-Step qRT-PCR system (ref: 11732-088)

Real-time PCR equipment:

- LightCycler 480 (96)

Adjustments may be required for the use of other kits or other real-time PCR instruments. All Assays used the same conditions. Primer and probe sequences, as well as optimized concentrations are shown in table below. **A 25µl reaction was set up containing 5µl of RNA.**

Simplex Mix	**Vol (µl)**	**[final]**
H_2O PPI	3.60	
Reaction mix 2X	12.50	3 mM Mg
$MgSO_4$ (50mM)	0.40	0.8 mM Mg
Forward Primer (10µM)	1.00	0.4 µM
Reverse Primer (10µM)	1.00	0.4 µM
Probe (10µM)	0.50	0.2 µM
SuperscriptIII RT/Platinum Taq Mix	1.00	
Final Volume	**20.00**	

Multiplex Mix (nCoV_IP2&IP4)	**Vol (µl)**	**[final]**
H_2O PPI	1.3	
Reaction mix 2X	12.50	3 mM Mg
$MgSO_4$ (50mM)	0.40	0.8 mM Mg
Forward Primer (10µM)	1.00	0.4 µM
Reverse Primer (10µM)	1.00	0.4 µM
Forward Primer (10µM)	1.00	0.4 µM
Reverse Primer (10µM)	1.00	0.4 µM
Probe (10µM)	0.4	0.16 µM
Probe (10µM)	0.4	0.16 µM
SuperscriptIII RT/Platinum Taq Mix	1.00	
Final Volume	**20.00**	

CONTROLS

Each real-time RT-PCR assay includes in addition of unknown samples:

- Two negative samples bracketing unknown samples during RNA extraction (negative extraction controls)
- Positive controls (in duplicate); when using *in vitro* synthesized transcripts as controls include five quantification positive controls (in duplicate) including 10^5, 10^4 and 10^3 copies genome equivalent (ge) of *in vitro* synthesized RNA transcripts.
- One negative amplification control.

AMPLIFICATION CYCLES (LIGHTCYCLER SYSTEM)

Reverse transcription	55°C	20 min	x1	
Denaturation	95°C	3 min	x1	
Amplification	95°C	15 sec	x50	Acquisition
	58°C	30 sec		
Cooling	40°C	30 sec	x1	

SENSITIVITY

For the nCoV_IP and E_Sarbeco real-time RT-PCR

Sensitivity, in terms of 95% hit rate is about 100 copies of RNA genome equivalent per reaction (this amount of target sequences is always detected), the probability to detect lower amounts of virus decreases, but samples containing 10 copies could be detected with multiplex assay.

	Multiplex (Ct values)		Simplex (Ct values)
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

Ct values may vary from instrument to instrument by up to 2 cycles, while the interval between two dilutions steps is constant (ΔCt).

SPECIFICITY

Cross-reactivity with other respiratory viruses was tested with specimens known to be positive for a panel of respiratory viruses (influenza A(H1N1)pdm09, A(H3N2), B-Victoria, B-Yamagata; influenza C; RSV A, B; hBoV; hPIV; hMPV; HRV/enterovirus; adenovirus; hCoV (HKU1, OC43, 229E and NL63); MERS-CoV. None of the tested viruses showed reactivity with PCR2 and PCR4.

POSITIVE CONTROL FOR SARS-CoV-2 REAL-TIME RT-PCR

One specific control has been designated.

Positive control for real-time RT-PCR is an *in vitro* transcribed RNA derived from strain BetaCoV_Wuhan_WIV04_2019 (EPI_ISL_402124). The transcript contains the amplification regions of the ***RdRp*** *and* ***E gene*** as positive strand. Each microtube contains 10^{11} copies of target sequences diluted in yeast tRNA, and lyophilised.

Reconstitution of transcribed RNA

Add 100 µl of RNase/DNAse-free H2O to obtain a solution at a concentration of 10^9 copies/µl. Store at -80°C. Dilute to prepare a master bank at $2x10^6$ copies/µl. Store at -80°C.

From this prepare a working bank of reagent at $2x10^4$ copies/µl in order to avoid freeze/thaw cycles. Working tubes may be stored at -20°C for less than one week.

Positive controls are available upon request (grippe@pasteur.fr)

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Reference

1- Corman VM, Landt O, Kaiser M, et al. Detection of 2019 novel coronavirus (2019-nCoV) by real-time RT-PCR. Euro Surveill 2020;25.