Severe Acute Respiratory Syndrome Coronavirus (SARS-CoV) detection & quantitation by qRT-PCR (SYBR green).

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This protocol describes the real-time polymerase chain reaction with reverse transcription (qRT-PCR) approach developed for the detection and quantitation of viral titres using a one-step SYBR-Green modality (but easily TaqMan adaptable). CoV are a group IV ((+)ssRNA) species of the Nidovirales order, Coronaviridae family, Coronavirinae subfamily and Betacoronavirus genus having a twin species (SARS-CoV). SARS-CoV is distinct from MERS CoV and distinct from the common-cold CoV and known endemic human HCoV-OC43 and HCoV-HKU1. The 29.7 kb CoV genome is non-segmented and contains 5′-methylated caps and 3′-polyadenylated tails encoding for replicase, structural proteins like spike glycoprotein (S), envelope protein (E), membrane protein (M) and nucleocapsid protein (N) which are essential for virus‑cell-receptor binding and virion assembly, and immunomodulatory effects. The partially overlapping 5′-terminal ORF (ORF1a/b) encodes the large replicase polyprotein 1a (pp1a) and pp1ab which are cleaved by proteases to produce non-structural proteins, RNA-dependent RNA polymerase (RdRp) and helicase (Hel), involved in the transcription and replication of CoVs.

Oligonucleotide primers

Name	Sequence[1]	bp	%GC	Tm	Hair	HomD	HetD	Amplicon
SARS-F	5´- TGA-TGA-TGC-CgT-YGT-GTG-CTA-YAA -3´	23	47.8	58.8	0.01	-8.63	-6.69	168
SARS-R	5´- TGT-GAG-CAA-AAT-TCG-TGA-GGT-CC -3´	23	47.8	57.7	0.15	-5.36		

Note 1: Reverse oligonucleotide primer sequences given in this table are the reverse-complement of sequence present in alignments and as they should be ordered for synthesis; Hairpin (Hair), homodimer (homD) and heterodimer (HetD) ΔQ's given in kcal/mol.

Oligonucleotide primer map in artificial gene[2] (underlined primer sites)

AAGCTCCGCGTCCTTTACCAAGGAAATAACATCACTGTAACTGCCTATGCAAACGGCGACCATGCCGTCACAGTTAAGGACGCCA
AATTCATTGTGGGGCCAATGTCTTCAGCCTGGACACCTTTCGACAACAAAATTGTGGTGTACAAAGGTGACGTCTATAATATGGA
CTACCCGCCCTTTGGCGCAGGAAGACCAGGACAATTTGG|CAGACCACGCTACGGCGTGCTACTCTGCGGAGAGTGCAGTCTGCG
ATAGTGCCCCAGGAGGACTGGGTTAACAAAGGCAAACCAACGCCCCACGCGGCCCTAG|AAGGACTAGAGGTTAGAGGAGACCAT
GTCCATGTCACCCACGGTCATCATCCTGGCATGTCTTGGGTTCTATCCTGCTGTCTCTACAGCATCATTCCAGGCACAGAACGCC
|AAGGACTAGAGGTTAGAGGAGACCCCCCCAACACAAAAACAGCATATTGACGCTGGGAAAGACCAGAGATCCTGCTGTCTCTGC
AACATCAATCCAGGCACAGAGCGCC|AAATACACATACCAAAACAAAGTGGTGAAGGTTCTCAGACCAGCTGAAGGAGGGAAAAC
AGTCATGGACATCATCTCAAGACAAGACCAGAGAGGGAGTGGA|ATGTATGTGAGTGCTGATGCCACGAAATGGTCACCAGGAGA
TAATTCGGCAAAGTTTAAGAGATTCACACAGGCATTATATGATGGCTTGTCAGATGAGAAGTTAAAATGTTGCGTTGTTGATGC|
ATGTAACACCTCTACAATGGATGCCGACAAGATTGTATTCAAAGTCAATAATCAGGTGGTCTCTTTGAAGCCTGAGATTATCGTG
GATCAATATGAGTACAAGTACCCTGC|GCATAGCTGTAGGAAGGACTCTCAAGGCATTTTTCTCCTGGTCGCTTACTGACCCTTT
AGGGAACGAAGCCCCTGGGGGATACTGTCTTGAAAAGTGGATGCTT|GCAACGCGCGATTCAGTTCCTCTTCACATAATCGCCCC
GAGCTCGCTTATCGTTTAAGCAGCTCTGCGCTACTATGGGTCCCGTGTAGAGGC|TGATGATGCCGTCGTGTGCTACAACAGTAA
CTATGCGGCTCAAGGTTTAGTAGCTAGCATTAAGAACTTTAAGGCAGTTCTGTATTATCAAGATAATGTGTTCATGTCTGAGGCA
AAATGTTGGACTGAGACTGACCTTACTAAAGGACCTCACGAATTTTGCTCACA

Note 2: Primer sites for different pathogen sequences shown in red, TaqMan probe sites shown in blue, SARS-CoV specific primer binding sites shown in grey highlight. SARS-CoV artificial gene sequence derived from ORF 1 ab gene sequence as suggested by Balboni 2012.

Two-step (separate RT and PCR) components and conditions

Dual (separate RT and PCR) uses Vivantis M-MULV (Cat. No.: ME2305) RT Enzyme and Biorad iTaq (Cat. No.: 1725150) qPCR Master Mix.

First strand synthesis (RT)

		1x
dH_2O	cf	2.6 µL
10 µM Forward oligo	1.125 µM	2.25 µL
10 µM Reverse oligo	1.125 µM	2.25 µL
10 mM dNTPs 10 mM	250 µM	0.5 µL
RNA	-	10 µL
		vf: 17.6 µl

↓

Run RT-1 program in Axygen TC-1 →

Total time: 6 min		
95ºC	2 min	1 cycle
4 ºC	2 min	

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RT Buffer	1x cf	2 µL
RT Enzyme 250 IU/µl	5 IU/µL	0.4 µL
		vf: 20 µl

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Run RT-2 program in Axygen TC-1 →

Total time: 1:12 hrs		
38 ºC	60 min	1 cycle
95 ºC	5 min	
4 ºC	5 min	

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Polymerase Chain Reaction (PCR)

		1x
dH_2O	cf	3.7 µL
Master mix	1 x	5 µL
10 µM Forward oligo	150 nM	0.15 µL
10 µM Reverse oligo	150 nM	0.15 µL
Template	10 ng	1 µL
		vf: 10 µl

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Run generic program in Applied Biosystems 7500 →

Total time: 2:10 hrs		
95 ºC	3 min	
94 ºC	15 sec	40 cycles
60 ºC[3]	60 sec	
95 ºC	15 sec	
60 ºC	20 sec	
Ramp[3]	60 min	
95 ºC	15 sec	

Note 3: Data acquisition.

One-step qRT-PCR components and conditions

The One-Step qRT-PCR procedure uses VeriQuest SYBR Green One-Step qRT-PCR Mix (USB Affymetrix Cat. No.: 75700).

qRT-PCR		1x
dH_2O	cf	3.1 µL
qRT-PCR Master mix	1 x	5 µL
RT Enzyme mix	1 x	0.1 µL
10 µM Forward oligo	400 nM	0.4 µL
10 µM Reverse oligo	400 nM	0.4 µL
Template	< 1 µg	1 µL
		vf: 10 µl

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Run Affymetrix program in Applied 7500 ➔

Total time: 2:00 hrs		
50 ºC	10 min	
95 ºC	10 min	
95 ºC	15 sec	40 cycles
58 ºC	30 sec	
95 ºC	15 sec	
60 ºC[3]	20 sec	
Ramp[3]	60 min	
95 ºC	15 sec	

Note 3: Data acquisition.

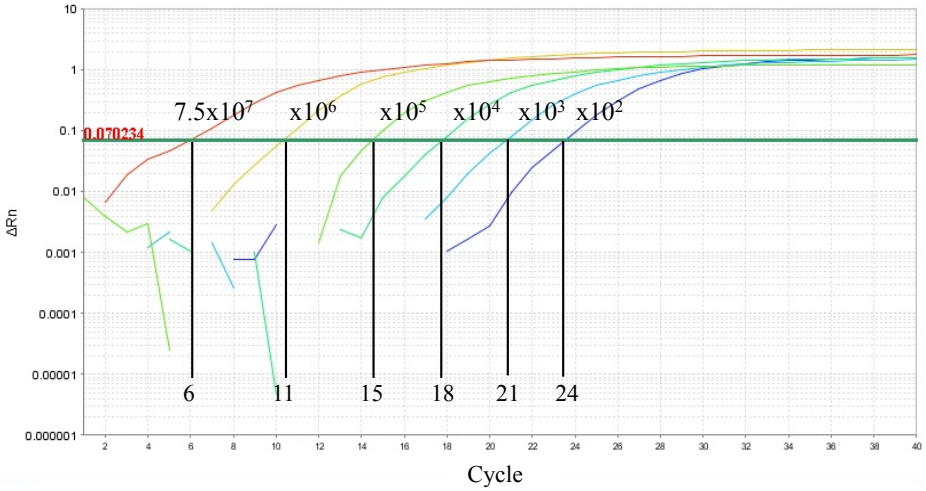
Titration curve preparation for quantitative analysis of viral titres

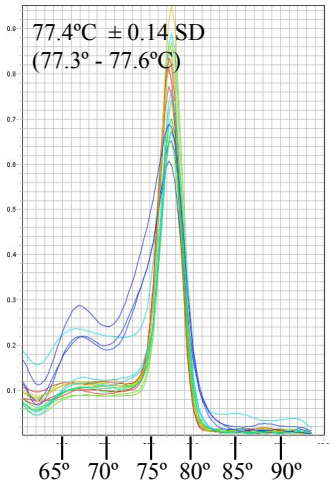
Using Artificial Gene (AG) sequence stock having all viral references (CHIKV, WNV, DENV1-3, DENV2-4, ZIKV, SNV, RABV, TCRV, MERS and SARS) prepare the following log titres (stock currently at $7.51x10^7$ cp/µL, working dilution of AG prepared at $7.51x10^6$ cp/µL).

		AG stock	dH_20 vol	Ct mean
1st	$7.5x10^7$ cp/µL	10 µL	90 µL	5.74 (±0.55)
2nd	$7.5x10^6$ cp/µL	10 µL	90 µL	10.69 (±0.31)
3rd	$7.5x10^5$ cp/µL	10 µL	90 µL	14.65 (±0.59)
4th	$7.5x10^4$ cp/µL	10 µL	90 µL	17.72 (±0.15)
5th	$7.5x10^3$ cp/µL	10 µL	90 µL	20.57 (±0.76)
6th	$7.5x10^2$ cp/µL	10 µL	90 µL	23.61 (±0.55)

Add 90 µL to each of the 6 PCR 0.2 mL tubes. Take 10 µL of initial working stock (at $7.51x10^7$ cp/µL) and dispense into 1st PCR tube, wash tip 30 times, cap, vortex for 10 seconds and spin down for 10 seconds. Retrieve 10 µL from volumetric centre of PCR tube and dispense into 2nd PCR tube repeating exactly the same procedure for further dilutions.

Performance summary





Standard curve: ***m* = -3.488, Y = 34.246, R^2 = 0.983**
Target amplicon Tm: **77.43ºC ± 0.14 SD (77.29 to 77.57 ºC)**
Limit of detection (LODet): **$7.51x10^2$ cp/µL**
Limit of discrimination (LODis): **$7.51x10^2$ cp/µL**

Interpretation

For samples to be considered as positive, their Ct should not be prior to that of the last standard curve's Ct (i.e., < cycle 24, corresponding to the $7.5x10^2$ LODet titre. In addition, the melting curve of the corresponding sample should be higher than surrounding unspecific melting peaks and preferably clean (without accompanying shoulders or unspecific melting peaks).

References

1. Balboni, A., Gallina, L., Palladini, A., Prosperi, S., & Battilani, M. (2012). A Real-Time PCR Assay for Bat SARS-Like Coronavirus Detection and Its Application to Italian Greater Horseshoe Bat Faecal Sample Surveys. The Scientific World Journal, 2012, 1-8.

2. Zumla A, Chan JF, Azhar EI, Hui DS, Yuen KY. Coronaviruses - drug discovery and therapeutic options. Nat Rev Drug Discov. 2016 May;15(5):327-47. doi: 10.1038/nrd.2015.37. Epub 2016 Feb 12. Review. PubMed PMID: 26868298.

Revision history

1.0 Original document.

2.0 Changes to document format only.