



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

Created: May 23, 2017; Last modified Nov 07, 2018; Version: 2.0

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 gene² (underlined primer sites)

AAGCTCCGCGTCTTTACCAAGGAAATAACATCACTGTAACCTGCCTATGCAAACGGCGACCATGCCGTCACAGTTAAGGACGCCA
AATTCATTGTGGGGCCAATGTCTTCAGCCTGGACACCTTTCGACAACAAAATTGTGGTGTACAAAGGTGACGCTCTATAATATGGA
CTACCCGCCCTTTGGCGCAGGAAGACCAGGACAATTTGG | CAGACCACGCTACGGCGTGCTACTCTGCGGAGAGTGCAGTCTGCG
ATAGTGGCCAGGAGGACTGGGTTAACAAAGGCAAACCAACGCCCCACGCGGCCCTAG | AAGGACTAGAGGTTAGAGGAGACCAT
GTCCATGTCACCCACGGTCATCATCTGGCATGTCTTGGGTTCTATCCTGCTGTCTCTACAGCATCATTCAGGCACAGAACGCC
| AAGGACTAGAGGTTAGAGGAGACCCCCCAACACAAAACAGCATATTGACGCTGGGAAAGACCAGAGATCCTGCTGTCTCTGC
AACATCAAATCCAGGCACAGAGCGCC | AAATACACATACCAAAAACAAAGTGGTGAAGGTTCTCAGACCAGCTGAAGGAGGGAAAAAC
AGTCATGGACATCATCTCAAGA CAAGACCAGAGAGGGAGTGGG | ATGTATGTGAGTGTGCTGATGCCACGAAATGGTACCAGGAGA
TAATTCGGCAAAGTTAAGAGATTCACACAGGCATTATATGATGGCTTGTCTCAGATGAGAA GTTAAAAATGTTGCGTTGTTGATGC |
ATGTAACACCTCTACAATGATG CCGACAAGATTGTATTCAAAGTCAATAATCAGGTGGTCTCTTTGAAGCCTGAGATTATCGTG
GATCAAATATGAGTACAAGTACCCCTGC | GCATAGCTGTAGGAAGGACTCTCAAGGCATTTTTCTCTGGTGGCTTACTGACCCCTTT
AGGGAACGAAGCCCTGGGGATACTGTCTTGAAGTGGATGCTT | GCAACGCGGATTCAGTTCTCTTACATAATCGCCCC
GAGCTCGCTTATCGTTTAAAGCAGCTCTGCGCTACTATGGGTCCCGTGTAGAGGC | TGATGATGCCGTCGTGTGCTACAA CAGTAA
CTATGCGGCTCAAGGTTTAGTAGCTAGCATTAAGAATTTAAGGCAGTTCTGTATATCAAGATAATGTGTTTCATGTCTGAGGCA
AAATGTTGGACTGAGACTGACCTTACTAAA GGACCTCACGAATTTTGCTACA





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 ₂ O | 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 |

| Total time: 6 min | | |
|-------------------|-------|---------|
| 95 °C | 2 min | 1 cycle |
| 4 °C | 2 min | |

Run RT-1 program in Axygen TC-1 →

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

| Total time: 1:12 hrs | | |
|----------------------|--------|---------|
| 38 °C | 60 min | 1 cycle |
| 95 °C | 5 min | |
| 4 °C | 5 min | |

Run RT-2 program in Axygen TC-1 →

Polymerase Chain Reaction (PCR)

| | | 1x |
|---------------------|--------|-----------|
| dH ₂ O | 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 |

| Total time: 2:10 hrs | | |
|----------------------|--------|-----------|
| 95 °C | 3 min | 40 cycles |
| 94 °C | 15 sec | |
| 60 °C ³ | 60 sec | |
| 95 °C | 15 sec | |
| 60 °C | 20 sec | |
| Ramp ³ | 60 min | |
| 95 °C | 15 sec | |

Run generic program in Applied Biosystems 7500 →

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 ₂ O | 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 |

↓

| | | |
|--|--|--|
| Run Affymetrix program in Applied 7500 | | |
|--|--|--|

→

| | | |
|----------------------|--------|-----------|
| Total time: 2:00 hrs | | |
| 50 °C | 10 min | 40 cycles |
| 95 °C | 10 min | |
| 95 °C | 15 sec | |
| 58 °C | 30 sec | |
| 95 °C | 15 sec | |
| 60 °C ³ | 20 sec | |
| Ramp ³ | 60 min | |
| 95 °C | 15 sec | |

Note 3: Data acquisition.

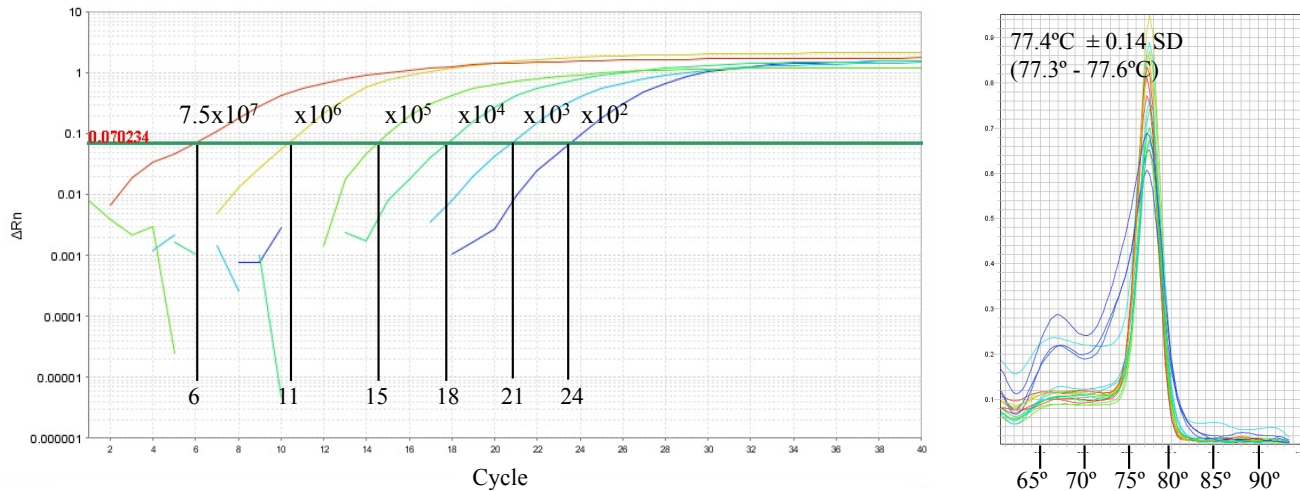
Titration curve preparation for quantitative analysis of viral titres

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 7.51×10^7 cp/μL, working dilution of AG prepared at 7.51×10^6 cp/μL).

| | AG stock | dH ₂ O vol | Ct mean | |
|-----------------|-------------------------|-----------------------|---------|---------------|
| 1 st | 7.5×10^7 cp/μL | 10 μL | 90 μL | 5.74 (±0.55) |
| 2 nd | 7.5×10^6 cp/μL | 10 μL | 90 μL | 10.69 (±0.31) |
| 3 rd | 7.5×10^5 cp/μL | 10 μL | 90 μL | 14.65 (±0.59) |
| 4 th | 7.5×10^4 cp/μL | 10 μL | 90 μL | 17.72 (±0.15) |
| 5 th | 7.5×10^3 cp/μL | 10 μL | 90 μL | 20.57 (±0.76) |
| 6 th | 7.5×10^2 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 7.51×10^7 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 T_m : $77.43^\circ\text{C} \pm 0.14 \text{ SD}$ (77.29 to 77.57°C)
 Limit of detection (LODet): $7.51 \times 10^2 \text{ cp}/\mu\text{L}$
 Limit of discrimination (LODis): $7.51 \times 10^2 \text{ cp}/\mu\text{L}$

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 7.5×10^2 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

- 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.
- 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.

