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Detection of 2019 novel coronavirus (2019-nCoV) in suspected human cases by RT-PCR

This protocol is designed to detect 2019-nCoV in human clinical specimens. The two monoplex assays described here are reactive with coronaviruses under the subgenus *Sarbecovirus* that includes 2019-nCoV, SARS-CoV and bat SARS-like coronaviruses. The rationales for using this detection approach are: 1) the genetic diversity of 2019-nCoV in humans and animals is yet to be fully determined and 2) many laboratories lack positive controls for 2019-nCoV. ***Viral RNA extracted from SARS-CoV can be used a positive control in the assays below.*** As SARS was eliminated in humans, suspected cases that are positive in these RT-PCR assays should be considered to be infected by the 2019-nCoV. The N gene RT-PCR is recommended as a screening assay and the Orf1b assay as a confirmatory one. In the event of a positive PCR result, sequence analyses of the amplicons will further help to confirm the result and to distinguish between SARS-CoV and 2019-nCoV. An N gene positive/Orf1b negative result should be regarded as indeterminate and the case is recommended to be referred to a WHO reference lab for further testing.

These assays have been evaluated using a panel of controls and only the positive control (SARS-CoV RNA) is tested positive in these assays. NB. Synthetic oligonucleotide positive controls or equivalents for 2019-nCoV is not available at present but will be available shortly.

Suitable biosafety precautions should be taken for handling human clinical specimens suspected to be 2019-nCoV infections (https://www.who.int/health-topics/coronavirus/laboratory-diagnostics-for-novel-coronavirus).

Materials required

- QIAamp Viral RNA Mini Kit (QIAGEN, Cat#52906) or equivalent
- TaqMan Fast Virus Master mix (TheromFisher, Cat# 4444432)
- Ethanol (96–100%)
- MicroAmp Fast Optical 96-well reaction plate (TheromFisher, Cat# 4346907)
- MicroAmp optical adhesive film (TheromFisher, Cat# 4311971)
- Microcentrifuge (adjustable, up to 13 000 rpm)
- Adjustable pipettes (10, 20, 100, 200 µl)
- Sterile, RNase-free pipette tips with aerosol barrier
- Vortex

- Microcentrifuge tubes (0.5ml and 1.5 ml)
- Thermocycler (TheromFisher, ViiA™ 7 Real-Time PCR)
- Positive control (Available from HKU, e-mail: llmpoon@hkucc.hku.hk)
- Primer sets

Primer and probe sequences

Assay 1 (Target: ORF1b-nsp14)

Forward primer (HKU-ORF1b-nsp14F): 5'-TGGGGYTTTACRGGTAACCT-3'

Reverse primer (HKU- ORF1b-nsp14R): 5'-AACRCGCTTAACAAAGCACTC-3'

Probe (HKU-ORF1b-nsp141P): 5'-FAM-TAGTTGTGATGCWATCATGACTAG-TAMRA-3'

Assay 2 (Target: N)

Forward primer (HKU-NF): 5'-TAATCAGACAAGGAACTGATTA-3'

Reverse primer (HKU-NR): 5'-CGAAGGTGTGACTTCCATG-3'

Probe (HKU-NP): 5'-FAM-GCAAATTGTGCAATTTGCGG-TAMRA-3'

Procedures

1. Extract viral RNA from clinical specimens by using QIAamp viral RNA mini kit according to manufacturer's instructions.

2. Prepare master mixture for one-step monoplex RT-PCR as below:

Reagent	**Vol for a single rxn (µl)**
H_2O (RNase free)	8.5
4x Reaction mix*	5
Forward primer (10 µM)	1
Reverse primer (10 µM)	1
Probe (10 µM)	0.5
RNA sample	4
Final rxn volume	20

*Reaction mix from TaqMan Fast Virus Master mix

3. Set the follow RT-PCR conditions*:

Temperature (°C)	Time (minute:second)	No. of cycle
50 95	5:00 0:20	1
95 60	0:05 0:30	40

*Both monoplex assays can be conducted under the same conditions.

Evaluation:

Positive controls: The tests were evaluated using serially diluted RNA samples extracted from SARS-CoV infected cells. These assays are confirmed to have a wide dynamic range (2^{-4}-2000 $TCID_{50}$/reaction, an amplification plot is shown an example). Upper respiratory and sputum samples spiked with SARS-CoV are shown to be positive in the test.

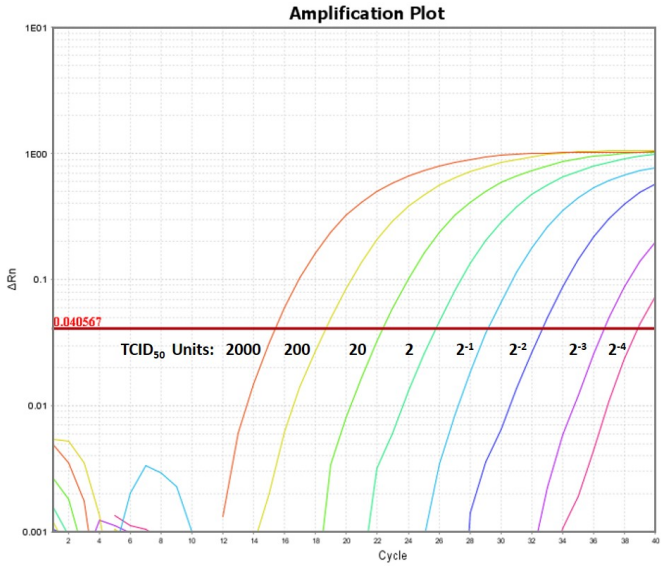


Figure. Amplification plot of the RT-PCR assay specific for N gene.
The viral titre ($TCID^{50}$) used in each reaction is shown as indicated.

Exclusivity: RNA extracted from respiratory cultured viruses and clinical samples (as described below) were included in the exclusivity panel. The assay yielded negative results against all of these preparations:

- RNA extracted from cultured viruses: human coronaviruses (229E, OC43 and MERS), camel coronavirus (HKU23), human influenza A viruses (H1N1, H3N2, H5N1 and H7N9 subtypes),

avian influenza (H1, H4, H6 and H9 subtypes), influenza B viruses (Yamagata and Victoria lineages), and adenovirus.

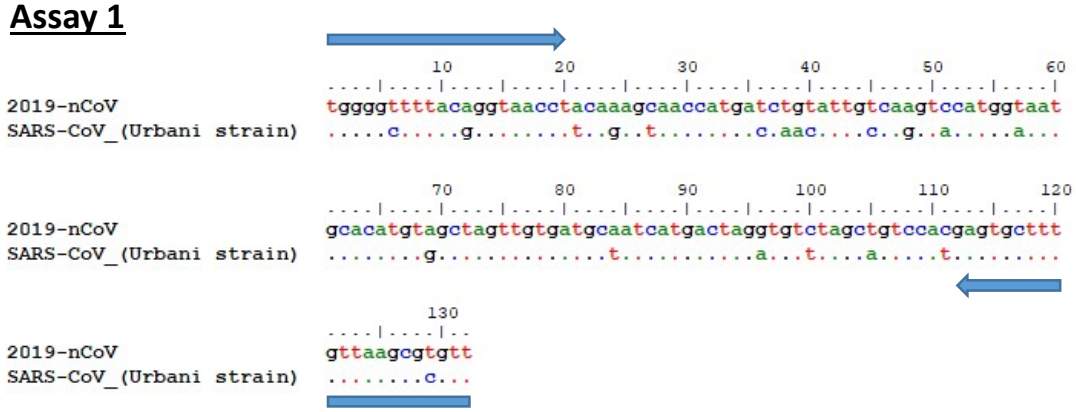
- RNA from retrospective human clinical specimens previously tested positive for other infections: coronavirus (229E, HKU1, NL63, OC43), influenza A viruses (H1N1 and H3N2 subtypes), influenza B viruses (Yamagata and Victoria lineages), adenovirus, enterovirus, human parainfluenza virus (PIV3), respiratory syncytial virus, human metapneumovirus, rhinovirus and human bocavirus.
- RNA from control human clinical specimens: Upper respiratory and sputum samples.

Remarks:

- The protocol is prepared by School of Public Health, The University of Hong Kong, Hong Kong (Leo Poon, Daniel Chu and Malik Peiris). For enquiry, please contact Leo Poon (llmpoon@hku.hk) or Malik Peiris (malik@hku.hk).
- Positive controls for the above assays may be available upon request.
- The amplicon sizes of Assay 1 and Assay 2 are 132 bp and 110 bp, respectively.
- A manual pan-coronavirus nested RT-PCR can detect a wide range of coronaviruses (J Virol. 85:12815-20). The identity of amplified DNA product can be confirmed by DNA sequencing.
- Primer-probe sets that are specific for 2019-nCoV are currently under evaluation. Please visit the nCoV laboratory website of WHO at https://www.who.int/health-topics/coronavirus/laboratory-diagnostics-for-novel-coronavirus.
- We encourage other labs to validate the described assay and share relevant finding with us.

Appendix 1: Sequence alignment of amplicons derived from 2019-nCoV and SARS-CoV

Assay 1



Assay 2