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 (Thermofisher, 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’-TGGGGYTTTACRGGAACCT-3’
Reverse primer (HKU- ORF1b-nsp14R): 5’-AACRCGCTTAACAAAGCAGCTC-3’
Probe (HKU-ORF1b-nsp141P): 5’-FAM-TAGTTGTGATGCWATCATGACTAG-TAMRA-3’

**Assay 2 (Target: N)**
Forward primer (HKU-NF): 5’-TAATCAGACAAAGGAACTGATTA-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:

<table>
<thead>
<tr>
<th><strong>Reagent</strong></th>
<th><strong>Vol for a single rxn (µl)</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>H2O (RNase free)</td>
<td>8.5</td>
</tr>
<tr>
<td>4x Reaction mix*</td>
<td>5</td>
</tr>
<tr>
<td>Forward primer (10 µM)</td>
<td>1</td>
</tr>
<tr>
<td>Reverse primer (10 µM)</td>
<td>1</td>
</tr>
<tr>
<td>Probe (10 µM)</td>
<td>0.5</td>
</tr>
<tr>
<td>RNA sample</td>
<td>4</td>
</tr>
<tr>
<td>Final rxn volume</td>
<td>20</td>
</tr>
</tbody>
</table>

*Reaction mix from TaqMan Fast Virus Master mix*
3. Set the follow RT-PCR conditions*:

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Time (minute:second)</th>
<th>No. of cycle</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>5:00</td>
<td>1</td>
</tr>
<tr>
<td>95</td>
<td>0:20</td>
<td></td>
</tr>
<tr>
<td>95</td>
<td>0:05</td>
<td>40</td>
</tr>
<tr>
<td>60</td>
<td>0:30</td>
<td></td>
</tr>
</tbody>
</table>

*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.

![Amplification Plot](image)

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

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2019-nCoV
SARS-CoV_(Urban strain)
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Assay 2

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2019-nCoV
SARS-CoV_(Urban strain)
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