NOT RECOMMENDED FOR SCREENING
The sensitivity of the assay has been found to be lower than expected and we no longer recommend it be used.
We do recommend the ORF1ab assay (Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) real-time RT-PCR ORF1ab 2020) or the E gene assay by Corman et al. (Protocol v2-1)

A real-time RT-PCR to designed to detect SARS-CoV-2 and other related sarbecoviruses. Based on sequence MN908947 made available by Professor Yong-Zhen Zhang, Fudan University, Shanghai, China.
The target region encodes the nucleocapsid (N).
Not tested on wild-type virus (as of 25Jan2020), it is expected to be capable of detecting Wuhan virus, bat-like SARS and SARS virus (members of the subgenus Sarbecovirus).
Limit of detection not yet determined.
A single 1 mismatch at probe-binding site identified with the BetaCoV/USA/CA1/2020|EPI_ISL_406034 variant of SARS-CoV-2 (as of 29JAN2020).
Probe is in the 3'-5' (reverse complement) direction.

Notes:
1. Assay is optimised (as of 24Jan2020).
2. This test has identified a clinical positive case of coronavirus disease (COVID-19)

DOI
dx.doi.org/10.17504/protocols.io.bchwit7e
GUIDELINES

- If using a different brand or model of real-time thermocycler, check the concentration of ROX is adequate.
- Method assumes the user is familiar with the thermocycler and software used to run the protocol.

MATERIALS TEXT

STEP MATERIALS

1 Oligonucleotides

<table>
<thead>
<tr>
<th>Oligo Name</th>
<th>Sequence 5'-3'</th>
<th>Location based on NC_045512*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wuhan-TM2020For</td>
<td>TCGTGCTACAACCTCTCAAG</td>
<td>28648-28668</td>
</tr>
<tr>
<td>Wuhan-TM2020Probe</td>
<td>6FAM-CCGCCTCTGCCTCCTGC-BHQ1</td>
<td>28714-28695</td>
</tr>
<tr>
<td>Wuhan-TM2020Rev</td>
<td>CTGCCWGGAGATTGAATTCTTG</td>
<td>28780-28759</td>
</tr>
</tbody>
</table>

*GenBank accession NC_045512 Wuhan seafood market pneumonia virus isolate Wuhan-Hu-1

2 Reagents

3 Synthetic controls

Synthetic controls are produced using the binary synthetic template oligonucleotide positive control for in-house diagnostic real-time RT-PCR method.
The oligonucleotide sequences required to make controls for this assay are:

**Probe control:**
AAAATAATACGACTCACTATAGGGTGAAGAGAATCCACAAGGAATTGAACCGCCTCTGCTCCCTTCTGCACAGTGTTCAGC
AGGTCCTGTTGAAAA

**Primer control:**
AAAATAATACGACTCACTATAGGGTCGTGCTACAACTTCCTCAAGATGATCTGGCACGGGACCCTCCAACAAGAAATTCAAC
TCCAGGCAAGAAAA

4 **Reaction Set-up**
- Assay has been designed to be used on both a Rotor-Gene 6000 / Rotor-Gene Q 5-plex using 100-place rotor discs and an ABI 7500 Fast real-time machine.
- Total reaction volume is 20µL.
- Prepare sufficient for number of reaction plus a 'dead volume' usually 2 extra. Adjust as necessary if using a robotic dispenser.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume (µl) X1</th>
<th>Final reaction concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nuclease free water</td>
<td>4.41</td>
<td></td>
</tr>
<tr>
<td>Wuhan-TM2020F (200uM)</td>
<td>0.05</td>
<td>500nM</td>
</tr>
<tr>
<td>Wuhan-TM2020R (200uM)</td>
<td>0.09</td>
<td>900nM</td>
</tr>
<tr>
<td>Wuhan-TM2020Probe (100uM)</td>
<td>0.01</td>
<td>50nM</td>
</tr>
<tr>
<td>2 X Reaction mix*</td>
<td>10</td>
<td>1X</td>
</tr>
<tr>
<td>Superscript III/Platinum Taq enzyme mix*</td>
<td>0.4</td>
<td></td>
</tr>
<tr>
<td>ROX reference dye (25uM)*</td>
<td>0.04</td>
<td>50nM</td>
</tr>
<tr>
<td><strong>TOTAL VOLUME</strong></td>
<td><strong>15</strong></td>
<td></td>
</tr>
</tbody>
</table>

*Superscript®III Platinum® One-Step qRT-PCR kit

Dispense 15µl to each reaction well.
Add 5µl of template, extracted RNA, controls or NTC (nuclease-free water).
Total reaction volume is 20µl.

5 **PCR amplification**

<table>
<thead>
<tr>
<th>1 cycle</th>
<th>40 cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>50°C 5min</td>
<td>95°C 3 seconds</td>
</tr>
<tr>
<td>95°C 2min</td>
<td>60°C 30 seconds*</td>
</tr>
</tbody>
</table>

*Florescence acquisition step

6 **Result Analysis**

The definition used for a satisfactory positive result from a real-time fluorogenic PCR should include each of the following:

1. A sigmoidal curve – the trace travels horizontally, curves upward, continues in an exponential rise and followed by a curve towards a horizontal plateau phase
2. A suitable level of fluorescence intensity as measured in comparison to a positive control (y-axis)
3. A defined threshold (C_T) value which the fluorescent curve has clearly exceeded (Fig.1 arrow) and which sits early in the log-linear phase and is <40 cycles
4. A flat or non-sigmoidal curve or a curve that crosses the threshold with a C_T value >40 cycles is considered a

Citation: Judy A Northill, Ian M Mackay (02/14/2020). Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) real-time RT-PCR N gene 2020 (Wuhan-N; 2019-nCoV-related test) -NOT RECOMMENDED. https://dx.doi.org/10.17504/protocols.io.bchwit7e

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negative result

5. NTCs should not produce a curve.

**Figure 1.** Examples of satisfactory sigmoidal amplification curve shape when considering an assay’s fluorescent signal output. The crossing point or threshold cycle (C\text{T}) is indicated (yellow arrow); it is the value at which fluorescence levels surpass a predefined (usually set during validation, or arbitrary) threshold level as shown in this normalized linear scale depiction. LP-log-linear phase of signal generated during the exponential part of the PCR amplification; TP—a slowing of the amplification and accompanying fluorescence signal marks the transition phase; PP—the plateau phase is reached when there is little or no increase in fluorescent signal despite continued cycling.