ABSTRACT

- 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).
- Tested on wild-type SARS-CoV-2 virus, it is expected to be capable of detecting SARS-CoV-2, bat-like SARS and SARS virus (members of the subgenus Sarbecovirus).
- Limit of detection not yet determined.
- The performance of the assay has not been tested with low viral load samples or samples from patients who are clinically well.
- The sensitivity of this assay was improved with the use of the SensiFast™ Probe Lo-ROX One-step kit.
- A single 1 mismatch at probe-binding site identified with the BetaCoV/USA/CA1/2020|EPI_ISL_406034 (GenBank MN994467.1) variant of SARS-CoV-2 (as of 23JUNE2020).
- Probe is in the 3’-5’ (reverse complement) direction.
- Reverse primers were replaced in March 2020.
- We also recommend the ORF1ab assay (Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) real-time RT-PCR ORF1ab 2020), US-CDC-N1 assay or the E gene assay by Corman et al. (Protocol v2-1)

Notes:

- Assay is optimised.
- This test has identified clinical positive cases of coronavirus disease (COVID-19)

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.
1 Oligonucleotides

<table>
<thead>
<tr>
<th>Oligo Name</th>
<th>Sequence 5'-3'</th>
<th>Location based on NC_045512.2*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wuhan-TM2020For</td>
<td>TCGTGCTACAACCTTCCCTCAAG</td>
<td>28743-28763</td>
</tr>
<tr>
<td>Wuhan-TM2020Probe</td>
<td>6FAM-CCGCCTCTGCTCCCTTGC-BHQ1</td>
<td>28809-28790</td>
</tr>
<tr>
<td>SARS2-28875R-G</td>
<td>CTGCCTGGAGTTGAATTTCTTG</td>
<td>28875-28854</td>
</tr>
<tr>
<td>SARS2-28875R-A</td>
<td>CTGCCTGGAGTTGAATTTCTTA</td>
<td>28875-28854</td>
</tr>
</tbody>
</table>

*GenBank accession NC_045512.2 Severe acute respiratory syndrome coronavirus 2 isolate Wuhan-Hu-1.

2 Reagents

SensiFAST™ Probe Lo-ROX One-Step Kit Bioline Catalog #BIO-78001

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:**
AAAATAATACGACTCACTATAGGGTGAAGAGAATCCACAAGGAATTGAACCGCCTCTGCTCCCTTCTGCACAGTGTTCAGCAGGTCCTGTTGAAAA

**Primer control:**
AAAATAATACGACTCACTATAGGGTCGTGCTACAACTTCCTCAAGATGATCTGGCACGGGACCCTCCAAYAAGAAATTCAACTCCAGGCAAGAAAA

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>4.2</td>
<td></td>
</tr>
<tr>
<td>Wuhan-TM2020F (200µM)</td>
<td>0.05</td>
<td>500nM</td>
</tr>
<tr>
<td>SARS2-28875R-G (200µM)</td>
<td>0.05</td>
<td>500nM</td>
</tr>
<tr>
<td>SARS2-28875R-A (200µM)</td>
<td>0.09</td>
<td>900nM</td>
</tr>
<tr>
<td>Wuhan-TM2020Probe (100µM)</td>
<td>0.01</td>
<td>50nM</td>
</tr>
<tr>
<td>2 X SensiFast Probe Lo-Rox One-Step mix*</td>
<td>10</td>
<td>1X</td>
</tr>
<tr>
<td>RiboSafe RNase Inhibitor*</td>
<td>0.4</td>
<td></td>
</tr>
<tr>
<td>Reverse transcriptase*</td>
<td>0.2</td>
<td></td>
</tr>
</tbody>
</table>
Dispense 15µl to each reaction well.
Add 5µl of template, extracted RNA, controls or NTC (nucléase-free water).
Total reaction volume is 20µl.

**Amplification**

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

**Result Analysis**

6 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\text{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\text{T} value >40 cycles is considered a negative result
5. NTCs should not produce a curve

![Figure 1](https://dx.doi.org/10.17504/protocols.io.bhpwj5pe)

**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.