Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) real-time RT-PCR ORF1ab 2020 (Wuhan-ORF1ab; 2019-nCoV-related test) V.3

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ABSTRACT

A real-time RT-PCR to specifically detect SARS-CoV-2 betacoronavirus also called nCoV-2019 or Wuhan seafood market pneumonia virus. Based on sequence MN908947 made available by Professor Yong-Zhen Zhang, Fudan University, Shanghai, China. The target region is within the ORF1ab sequence.

Notes

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

PROTOCOL CITATION

Judy A Northill, Ian M Mackay 2020. Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) real-time RT-PCR ORF1ab 2020 (Wuhan-ORF1ab; 2019-nCoV-related test). protocols.io

https://dx.doi.org/10.17504/protocols.io.bchvit66

KEYWORDS

CoV, coronavirus, Wuhan, Real-time, RT-PCR, PCR, virus, China, 2019-nCoV. ORF1ab, pneumonia, seafood market, WSMPV, sarbecovirus, SARS-CoV-2, COVID-19

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CREATED

Feb 13, 2020

LAST MODIFIED

Feb 14, 2020

PROTOCOL INTEGER ID

33045

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**

**Step Materials**

- SuperScript™ III Platinum™ One-Step qRT-PCR Kit
  
  Life Technologies Catalog #11732088

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**Mix**

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>WuhanORF1ab-F</td>
<td>AATCCACCTGCTCTACAAGATG</td>
<td>5455-5476</td>
</tr>
<tr>
<td>WuhanORF1ab-R</td>
<td>CATCACCTAACTCCTACTGTC</td>
<td>5566-5544</td>
</tr>
<tr>
<td>WuhanORF1ab-P</td>
<td>6FAM-AGCTTCACCAGGCCCTTGTCTCT-BHQ1</td>
<td>5505-5485</td>
</tr>
</tbody>
</table>

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

2. **Reagents**

- SuperScript™ III Platinum™ One-Step qRT-PCR Kit
  
  Life Technologies Catalog #11732088

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:**
   
   AAAATAATACGACTCTATAAGGTGAAGAGAATCCACAAGGAATTTGAAAGCTTCTACACCAGCCCTTGTCTACAGTGTCAG
   CAGGTCCTGTGAAAA

   **Primer control:**
   
   AAAATAATACGACTCTATAAGGAATCCACCTGCTCTACAGTGATGATCTGTCGACGGACCTCAGACAGTAGGTAG
   AGTTAGGATGAAAA

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 a 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.

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**Citation:** Judy A Northill, Ian M Mackay (02/14/2020). Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) real-time RT-PCR ORF1ab 2020 (Wuhan-ORF1ab; 2019-nCoV-related test). [https://dx.doi.org/10.17504/protocols.io.bchvit66](https://dx.doi.org/10.17504/protocols.io.bchvit66)

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<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>4.37</td>
<td></td>
</tr>
<tr>
<td>WuhanORF1ab-F (200uM)</td>
<td>0.07</td>
<td>700nM</td>
</tr>
<tr>
<td>WuhanORF1ab-R (200uM)</td>
<td>0.09</td>
<td>900nM</td>
</tr>
<tr>
<td>WuhanORF1ab-P (100uM)</td>
<td>0.03</td>
<td>150nM</td>
</tr>
<tr>
<td>2X 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 (25µM)*</td>
<td>0.04</td>
<td>50nM</td>
</tr>
<tr>
<td>TOTAL VOLUME</td>
<td>15</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>

*Fluorescence 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\textsubscript{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\textsubscript{T} value >40 cycles is considered a 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<sub>T</sub>) 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.