Comparison of National RT-PCR Primers, Probes, and Protocols for SARS-CoV-2 Diagnostics

April 13, 2020

In this fact sheet, we provide a detailed comparison of several RT-PCR tests developed by various countries. Our review is limited to those listed in the World Health Organization (WHO) resource of in-house-developed molecular assays (accessible here) that have been used as the national test of choice in many countries and in regional reference laboratories. Depending on the test protocol, various primers, probes, concentrations of primers and probes, recommended extraction methods, and RT-PCR kits are used.

Primers are small fragments of single-stranded DNA designed to bind specifically to a single region in the genome to allow for precise amplification of the diagnostic target area. Probes bind to the target area and give off a fluorescent signal as amplification of that area increases. This fluorescent signal is then read by the quantitative real-time PCR machine where the reaction is occurring to give a diagnostic read-out. Molecular scientists use many different tools to design the most appropriate primers to create functional diagnostic targets. While there are many different protocols for reverse transcriptase polymerase chain reaction (RT-PCR) diagnostic testing for SARS-CoV-2, the causative agent of COVID-19 disease, they are not all designed to target the same segments of the genome (see Figure 1). Many nationally used RT-PCR protocols target the nucleocapsid phosphoprotein (N protein) of SARS-CoV-2. This is because, among other reasons, it is a highly conserved region in coronaviruses and likely to deliver consistent results as the pandemic progresses.

The N region genome map in Figure 2 below includes primers from 5 institutions that had at least 1 target within the N protein. As such, this is not an exhaustive look at all primers in use for RT-PCR diagnostics of COVID-19; however, it does illustrate the variability in the primer selection process between different institutions, even when creating primers for the same genomic region of the same virus.

Figure 1. SARS-CoV-2 genome (NC_004718.3). Orf, open reading frame; S, spike gene; E, envelope gene; M, membrane gene; N, nucleocapsid gene. Gene size drawn to scale.

How to read the N region genome map:

- Different colors represent primer sets from different protocols. In the case of the United States, there are 2 separate primer pairs, all in the same color.
- Primer pairs include a forward primer and a reverse primer in order to read both strands of DNA. Within the same protocol, the first color-blocked sequence is the forward primer, the intervening uncolored sequence is the area being amplified during PCR, and the second corresponding color-blocked sequence is the reverse primer.
- Note: Although SARS-CoV-2 is an RNA virus, during the RT-PCR process it is reverse-transcribed into DNA for increased stability and ease of use.
- Mixed colors denote areas of primer overlap between national protocols. For example, the forward primer from Thailand's protocol overlaps slightly with the reverse N1 primer from the United States protocol.

How to interpret sensitivity and specificity measures in the RT-PCR Master Table

In each set of primers described, sensitivity and specificity are included. Sensitivity and specificity measures were not available for all protocols.

Sensitivity refers to the level at which the protocol can recognize the presence of SARS-CoV-2 RNA. The higher the sensitivity, the less viral RNA is required for a positive test result. Tests with high sensitivity have a lower chance of missing a true positive (ie, declaring a false negative), an important factor when testing positive or negative can affect treatment and hospitalization decisions. Primer sets and probes can be tested for their sensitivity using a range of fixed concentrations of in vitro transcribed SARS-CoV2- viral RNA transcripts (positive controls). The limit of detection (LOD) is the lowest possible concentration of SARS-CoV-2 that can be detected under the experimental conditions in at least 95% of all reactions. Most tests aim for an LOD of 10 RNA copies per reaction. A test with a low LOD would be considered more sensitive. Having a low LOD means that the test can detect low-level infections.
Specificity refers to whether the test recognizes only SARS-CoV-2 RNA and not other closely related pathogens or human RNA transcripts. Tests with high specificity have a lower chance of declaring a false positive; rather, tests with high specificity have a greater ability to rule out true negatives from further clinical suspicion. Primer and probe specificity can be increased by ensuring they do not share great sequence similarity with other viral RNA sequences. Choosing primers and probes that recognize highly specific genome domains of SARS-CoV-2, such as the N domain, can help to increase specificity. Most laboratories have also assessed test specificity by checking for cross-reactivity with a panel of respiratory viruses, such as influenza A (H1N1), influenza A (H1N3), influenza B, rhinovirus, and other human coronaviruses. Ideally, cross-reactivity should be tested on RNA derived from patient samples. To be deemed adequately specific, the test should not read positive for any virus other than SARS-CoV-2.

The master table is not intended to make claims on which test is better or more reliable than others; more research would be required to answer those questions. Still, it is important to be able to understand what parameters the developing institutions used when validating their protocols.
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Note: The beginning and ending nucleotides represented on this page do not represent the actual boundaries of the N protein but were rather arbitrary points chosen for ease of visibility of the highlighted SARS-CoV-2 primers. The full sequence of the N protein can be accessed here.

GATATCGGTAATTATACAGTTTTCTGTTCACCTTTTACAATTAATGGCAGAACCTAATAATTGGGTAGTCTTTGAGTGCTCGTGTTCTGT
TCTATAGGACCTTTTTAGAGTATCATAGCAGTCGTGTTTGTGTTTGTGTTTACTTACTCAAAGAATTGCGTGTGATTGCG
US, N1
CAAAATCAGGGAAAATTGCAACCCCGCATTAAGTGGTTGGACCTCAAGTTCAACTGGCAGTAAACCAGAATGGGACAGCTGGG
GACCTAATAACCTGCTACATCAACTGCCAAACCTTCAAGGAAACACTGCTGCAAAGGGCTTCTACGCAGAAGGGAGCACAGACGA
ATTCGTTGTTGGAGGTCAGAATTCGATGGTACATGCAAATTGCTAGTACACCAGAAGATCGTACAGACGACAGAATTCGTTGTT
CTATGGTGCTAAGAAAGACGGCATCATATGGTGGTCACTTGAATACACCAAAAGATCACATTGGCAGCCAGAATCC
TGCATACATGCACTGTCACTACACACTTCCTAAAGGAAACACTGCTGCAAAGGGCTTCTACGCAGAAGGGAGCACAGACGA
AATTTTGAGGACCGAGAACAAATGTCTGTAAAAGGCGGCAACAACAAGGCCAAACTGTCACTAAGAAATGCTGCTGAGGCTTCTACC
GCCACAAATGTAACACAAGCTTTTGGCAGACGTGGTCCAGAACAAACCCAAGGAAATTTTGAGGACCGAGAACAAATGTCTGT
GGCCAACAACAACAGGCGCAACTGCTACTAAGAAATCTGCTGCTGAGGCTTCTACCAGCAGGCGCAAACGTACTGGCCACTAA
GCAATAATGGAACCTTGGTTACTGCCAGTTGAATCTG
N1: 71 bp
N2: 67 bp
Thailand National Institute of Health CGTTTGGTGGACCCTCAAGAT
57 bp
Hong Kong University of Hong Kong, Li Ka Shing School of Medicine TATACGACAAAGGAAACTGATTA
110 bp
Japan National Institute of Infectious Diseases AATTTTGGGGACCAGGAAC
155 bp
Primers source: https://www.who.int/docs/default-source/coronaviruse/whoinhouseassays.pdf?sfvrsn=de3a76aa_2

Figure 2. N region genome map and abridged table display the location of national primers and their true distance from each other in the SARS-CoV-2 genome.
<table>
<thead>
<tr>
<th>Country</th>
<th>Primer Target(s)</th>
<th>Primers (sequence) Forward 5'-3’</th>
<th>Primers (sequence) Reverse 5'-3’</th>
<th>Primers (sequence) Probe 5'-3’</th>
<th>Amplicon Size (base pairs)</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>Concentration/Volume of Reagents</th>
<th>Does the protocol recommend specific kits?</th>
</tr>
</thead>
<tbody>
<tr>
<td>USA (CDC)</td>
<td>N1</td>
<td>GACC.CCAAAATTCAGCGAAAT</td>
<td>TCTGGTTACTGCAACGT GAAATCTG</td>
<td>FAM-ACCCCGCAATTACCTGGTG GACC-BHQ1</td>
<td>71 bp</td>
<td>Limit of detection (LOD): 10^0.5 RNA copies/ul for Qiagen EZ1 Advanced XL Kit and 10 RNA copies/ul for Qiagen DSP Viral Mini Kit. 100% primer alignment with all available 2019-nCov sequences, 1 mismatch with the N1 F primer with 1 deposited sequence, but impact will be negligible, primers should still bind.</td>
<td>Probe showed high sequence homology with SARS coronavirus and Bat Sars-like coronavirus. F and R primer showed no sequence homology with SARS coronavirus or Bat Sars-like coronavirus. F primer showed high sequence homology to Bat SARS-like coronavirus. R primer and probe showed no significant homology with human genome, other coronaviruses, or human microflora.</td>
<td>20 μM primers, 5 μM probe; 15 μL total volume</td>
<td>For the RT-qPCR, they recommend TaqPath™ 1-Step RT-qPCR Master Mix. For extraction, they recommend bioMérieux NucleSpin® systems, QIAamp® Viral RNA Mini Kit, QIAamp® MinElute Virus Spin Kit or RNaseasy® Mini Kit (QIAGEN), EZ1 DSP Virus Kit (QIAGEN), Roche MagNA Pure Compact RNA Isolation Kit, Roche MagNA Pure Compact Nucleic Acid Isolation Kit, and Invitrogen ChargeSwitch®, Total RNA Cell Kit.</td>
</tr>
<tr>
<td></td>
<td>N2</td>
<td>TTACAAACATTGCGCAAA</td>
<td>GCGGACACATTGCGAAGAA</td>
<td>FAM-AACATTTGCCCCACGGCTT CAG-BHQ1</td>
<td>67 bp</td>
<td>Not stated</td>
<td>Not stated</td>
<td>Not stated</td>
<td>Not stated</td>
</tr>
<tr>
<td></td>
<td>N3</td>
<td>GGGAGCCTGAATACGACAAA</td>
<td>TGTAGACGATGTCAGATTTG</td>
<td>FAM-AAYCACATGGGCACCAGGAAT CCTG-BHQ1</td>
<td>72 bp</td>
<td>Not stated</td>
<td>Not stated</td>
<td>Not stated</td>
<td>Not stated</td>
</tr>
<tr>
<td></td>
<td>Orflab</td>
<td>CCGTGTGGTTTACACGCTGTAT</td>
<td>ACAGTTGCTGCTACGCTGA</td>
<td>FAM-CGCTTGCGGTATGGAA AGGTATGG-BHQ1</td>
<td>—</td>
<td>Not stated</td>
<td>Not stated</td>
<td>Not stated</td>
<td>Not stated</td>
</tr>
<tr>
<td></td>
<td>N</td>
<td>GGGGAACTGTCTTCTGCTGTAAT</td>
<td>CACAGATTGCTCTCAAAGCTG</td>
<td>FAM-TCGCTGCTGCTTGACAGATT TAMRA</td>
<td>—</td>
<td>Not stated</td>
<td>Not stated</td>
<td>Not stated</td>
<td>Not stated</td>
</tr>
<tr>
<td></td>
<td>nCoV_IP2</td>
<td>ATGACCTTAGCTCCTGGTG</td>
<td>CTCACCTCCCTGTGTGGTG T</td>
<td>Hex-AGATGTCTGTGGTGCGG TA-BHQ1</td>
<td>108 bp</td>
<td>95% hit rate for approx. 100 copies of RNA No cross-reactivity with other respiratory viruses</td>
<td>Final concentration of 0.4 μM of each</td>
<td>DNA extraction via NucleoSpin Dx Virus (Macherey Nagel 740895.50)</td>
<td></td>
</tr>
<tr>
<td>Country</td>
<td>RT-PCR Primers and Probes</td>
<td>Protocol Details</td>
<td>LOD</td>
<td>Positive Control</td>
<td></td>
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<tr>
<td>France (Institut Pasteur)</td>
<td>nCoV_IP4</td>
<td>nCoV_IP4 GGTAACTGGTATGATTTCG CTGGTCAAGGTTAATATAGG</td>
<td>107 bp</td>
<td>(Influenza A, H3N2, etc) primer and 0.2 μM of probe</td>
<td>Invitrogen Superscript™ III Platinum® One-Step qRT-PCR system (ref: 11732-088)</td>
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<tr>
<td>France (Institut Pasteur)</td>
<td>E gene/E_Sa rbeco</td>
<td>nCoV_IP4 GGTAACTGGTATGATTTCG CTGGTCAAGGTTAATATAGG</td>
<td>125 bp</td>
<td>Average Cq value of specimen was 36.7 and 35.0 for the positive control (500 copies of RNA transcript)</td>
<td>Not stated</td>
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<tr>
<td>Japan (National Institute of Infectious Disease)</td>
<td>nCoV_IP4</td>
<td>nCoV_IP4 GGTAACTGGTATGATTTCG CTGGTCAAGGTTAATATAGG</td>
<td>107 bp</td>
<td>LOD: 3.8 RNA copies/reaction, 95% hit rate; 95% CI: 2.7-7.6 RNA copies/reaction</td>
<td>Not stated</td>
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<tr>
<td>Germany (Charité)</td>
<td>nCoV_IP4</td>
<td>nCoV_IP4 GGTAACTGGTATGATTTCG CTGGTCAAGGTTAATATAGG</td>
<td>107 bp</td>
<td>LOD: 5.2 RNA copies/reaction, at 95% hit rate; Cl: 3.7-9.6 RNA copies/reaction</td>
<td>Not stated</td>
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<tr>
<td>Hong Kong</td>
<td>nCoV_IP4</td>
<td>nCoV_IP4 GGTAACTGGTATGATTTCG CTGGTCAAGGTTAATATAGG</td>
<td>107 bp</td>
<td>No reactivity with other human respiratory viruses and bacteria (used RNA from patient samples.)</td>
<td>Not stated</td>
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</tr>
<tr>
<td>Thailand</td>
<td>nCoV_IP4</td>
<td>nCoV_IP4 GGTAACTGGTATGATTTCG CTGGTCAAGGTTAATATAGG</td>
<td>107 bp</td>
<td>No reactivity with respiratory cultured viruses and clinical samples.</td>
<td>Not stated</td>
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