JOHNS HOPKINS BLOOMBERG SCHOOL of PUBLIC HEALTH | Center for Health Security

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

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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.

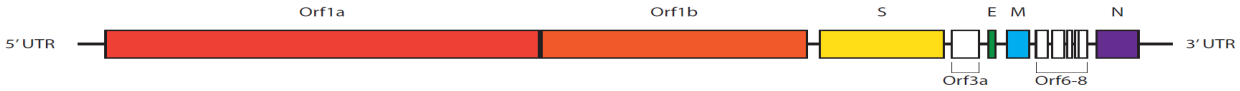

Figure 1. SARS-CoV-2 genome (NC_004718.3). Orf, open reading frame; S, spike gene; E, envelope gene; M, membrane gene; N, nucleocapsid gene. Gene size drawn to scale.

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.

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.

GATATCGGTAATTATACAGTTTCCTGTTCACCTTTTACAATTAATTGCCAGGAACCTAAATTGGGTAGTCTTGTAGTGCGTTGTTCGT
TCTATGAAGACTTTTTAGAGTATCATGACGTTCGTGTTGTTTTAGATTTCATCTAAACGAACAAACTAAAATGTCTGATAATG**GACCC** US, N1
CAAAATCAGCGAAATGCACCCCGCATTA**CGTTTGGTGGACCCTCAGATTCAACTGGCAGTAACCAGAATGGAGAACGCAGTGGGG**C
GCGATCAAAACAACGTCGGCCCCAAGGTTTACCCAATAATACTGCGTCTTGGTTCACCGCTCTCACTCAACATGGCAAGGAAGACCTT
AAATTCCCTCGAGGACAAGGCGTTCCAATTAACACCAATAGCAGTCCAGATGACCAAATTGGCTACTACCGAAGAGCTACCAGACGA
ATTCGTGGTGGTGACGGTAAAATGAAAGATCTCAGTCCAAGATGGTATTTCTACTACCTAGGAACTGGGCCAGAAGCTGGACTTCC
CTATGGTGCTAACAAAGACGGCATCATATGGGTTGCAACTGAGGGAGCCTTGAATACACCAAAAGATCACATTGGCACCCGCAATCC
TGCTAACAATGCTGCAATCGTGCTACAACTTCCTCAAGGAACAACATTGCCAAAAGGCTTCTACGCAGAAGGGAGCAGAGGCGGCA
GTCAAGCCTCTTCTCGTTCCTCATCACGTAGTCGCAACAGTTCAAGAAATTCAACTCCAGGCAGCAGTA**GGGGAACTTCTCCTGCTA**
GAATGGCTGGCAATGGCGGTGATGCTGCTCTTGCTTTGCTGCTGCTTGACAGATTGAAC**CAGCTTGAGAGCAAAATGTCTG**GTAAA
GGCCAACAACAACAAGGCCAAACTGTCACTAAGAAATCTGCTGCTGAGGCTTCTAAGAAGCCTCGGCAAAAACGTACTGCCACTAAA
GCATACAATGTAACACAAGCTTTTGGCAGACGTGGTCCAGAACAAACCCAAGGA**AATTTTGGGGACCAGGAACTAATCAGACAAGG**
AACTGATTACAAACATTGGCCGCAAATTGCACAATTTGCCCCCAGCGCTTCAGCG**TTCTTCGGAATGTCGCGC**ATTGG**CATGGAAG** US, N2
TCACACCTTCGGGAACGTG**GTTGACCTACACAGGTGC**CATCAAATTGGATGACAAAGATCCAAATTTCAAAGATCAAGTCATTTTGC
TGAATAAGCATATTGACGCATACAAAACATTCCCACCAACAGAGCCTAAAAAGGACAAAAAGAAGAAGGCTGA

Developed in	Institution	F Primer 5' – 3'	R Primer, 5' – 3' *(reverse compliment of below sequences highlighted in map)*	Amplicon Size
United States	US CDC	N1: GACCCCAAAATCAGCGAAAT N2: TTACAAACATTGGCCGCAAA	N1: TCTGGTTACTGCCAGTTGAATCTG N2: GCGCGACATTCCGAAGAA	N1: 71 bp N2: 67 bp
Thailand	National Institute of Health	CGTTTGGTGGACCCTCAGAT	CCCCACTGCGTTCTCCATT	57 bp
China	China CDC	GGGGAACTTCTCCTGCTAGAAT	CAGACATTTTGCTCTCAAGCTG	99 bp
Hong Kong	University of Hong Kong, Li Ka Shing School of Medicine	TAATCAGACAAGGAACTGATTA	CGAAGGTGTGACTTCCATG	110 bp
Japan	National Institute of Infectious Diseases	AATTTTGGGGACCAGGAAC	TGGCAGCTGTGTAGGTCAAC	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.

Master Table of national RT-PCR primers, probes, and protocol specifications (where available). Source: https://www.who.int/docs/default-source/coronaviruse/whoinhouseassays.pdf?sfvrsn=de3a76aa_2

Country	Primer Target(s)	Primers (sequence) *Forward 5'-3'*	Primers (sequence) *Reverse 5'-3'*	Primers (sequence) *Probe 5'-3'*	Amplicon Size *(base pairs)*	Sensitivity	Specificity	Concentration/ Volume of Reagents	Does the protocol recommend specific kits?
USA (CDC)	N1	GACCCCAAAATCAGCGAAAT	TCTGGTTACTGCCAGTTGAATCTG	FAM-ACCCCGCATTACGTTTGGTGGACC-BHQ1	71 bp	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.	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.	20 µM primers, 5 µM probe; 15 µL total volume	For the RT-qPCR, they recommend TaqPath™ 1-Step RT-qPCR Master Mix. For extraction, they recommend bioMérieux NucliSens® systems, QIAamp® Viral RNA Mini Kit, QIAamp® MinElute Virus Spin Kit or RNeasy® Mini Kit (QIAGEN), EZ1 DSP Virus Kit (QIAGEN), Roche MagNA Pure Compact RNA Isolation Kit, Roche MagNA Pure Compact Nucleic Acid Isolation Kit, and Roche MagNA Pure 96 DNA and Viral NA Small Volume Kit, and Invitrogen ChargeSwitch®, Total RNA Cell Kit.
	N2	TTACAAACATTGGCCGCAAA	GCGCGACATTCCGAAGAA	FAM-ACAATTTGCCCCCAGCGCTTCAG-BHQ1	67 bp				
	N3 (removed from diagnostic panel 3/15/20)	GGGAGCCTTGAATACACCAAAA	TGTAGCACGATTGCAGCATTG	FAM-AYCACATTGGCACCCGCAATCCTG-BHQ1	72 bp				
China (CDC)	Orf1ab	CCCTGTGGGTTTTACACTTAA	ACGATTGTGCATCAGCTGA	FAM-CCGTCTGCGGTATGTGGAAAGGTTATGG-BHQ1	—	Not stated	Not stated	Not stated	Not stated
	N	GGGGAACTTCTCCTGCTAGAAT	CAGACATTTTGCTCTCAAGCTG	FAM-TTGCTGCTGCTTGACAGATT-TAMRA	—	Not stated	Not stated	Not stated	Not stated
	nCoV_IP2	ATGAGCTTAGTCCTGTTG	CTCCCTTTGTTGTGTTGT	Hex-AGATGTCTTGTGCTGCCGGTA-BHQ1	108 bp	95% hit rate for approx. 100 copies of RNA	No cross-reactivity with other respiratory viruses	Final concentration of 0.4 µM of each	RNA extraction via NucleoSpin Dx Virus (Macherey Nagel 740895.50)

France (Institut Pasteur)	nCoV_IP4	GGTAACTGGTATGATTTCG	CTGGTCAAGGTTAATATAGG	FAM-TCATACAAACCACGCCAGG-BHQ1	107 bp	genome equivalent (LOD) for 1E7 RNA copies of transcript, it's ~21 cylces, for 1E4 it's ~30 cycles	(influenza A, H3N2, etc)	primer and 0.2 µM of probe	Invitrogen SuperscriptTM III Platinum® One-Step qRT-PCR system (ref: 11732-088)
	E gene/E_Sarbeco	ACAGGTACGTTAATAGTTAATAGCGT	ATATTGCAGCAGTACGCACACA	FAM-ACACTAGCCATCCTTACTGCGCTTCG-BHQ1	125 bp				
Japan (National Institute of Infectious Disease)	N	AAATTTTGGGGACCAGGAAC	TGGCAGCTGTGTAGGTCAAC	FAM-ATGTCGCGCATTGGCATGGA-BHQ	—	Average Cq value of specimen was 36.7 and 35.0 for the positive control (500 copies of RNA transcript)	Not stated	1 ul of 20 xprimer and probe mix in a 20 ul reaction with 5 ul of RNA. F primer at 500 nM, R primer at 700 nM, probe at 200 nM.	RNA extracted using QIAamp virual RNA mini kit (Qiagen). Reverse transcription via Super Script IV Reverse Transcriptase (Thermo). RT-PCR via QuantiTect Probe RT-PCR Kit (Qiagen).
Germany (Charité)	RdRP	GTGARATGGTCATGTGTGGCGG	CARATGTTAAASACACTATTAGCATA	P1: FAM-CCAGGTGGWACRTCATCMGGTGATGC-BBQ, P2: FAM-CAGGTGGAACCTCATCAGGAGATGC-BBQ	—	LOD: 3.8 RNA copies/rxn, 95% hit rate; 95% CI: 2.7-7.6 RNA copies/reaction	No nonspecific reactivity with water samples detected. No reactivity with other human respiratory viruses and bacteria (used RNA from patient samples).	RdRP: F-600 nM/reaction, R-800 nM/rxn, P-100 nM each/rxn,	RNA extracted using MagNA Pure 96 system (Roche), RT-PCR via Superscript III one step RT-PCR ystem with Platinum Taq Polymerase (Invitrogen).
	E gene	ACAGGTACGTTAATAGTTAATAGCGT	ATATTGCAGCAGTACGCACACA	FAM-ACACTAGCCATCCTTACTGCGCTTCG-BBQ	—	LOD: 5.2 RNA copies/reaction, at 95% hit rate; CI: 3.7-9.6 RNA copies/reaction		E gene: F-400 nM/rxn, R-400 nM/rxn, P-200 nM/rxn	
Hong Kong	Orf1b-nsp14	TGGGGYTTTACRGGTAACCT	AACRCGCTTAACAAAGCACTC	FAM-TAGTTGTGATGCWATCATGACTAG-TAMRA	132 bp	Not stated	No reactivity with respiratory cultured viruses and clinical samples.	10 µM primers, 10 µM probes	QIAamp Viral RNA Mini Kit (QIAGEN, Cat#52906) or equivalent and TaqMan Fast Virus Master mix.
	N	TAATCAGACAAGGAACTGATTA	CGAAGGTGTGACTTCCATG	FAM-GCAAATTGTGCAATTTGCGG-TAMRA	110 bp	Not stated			
Thailand	N	CGTTTGGTGGACCCTCAGAT	CCCCACTGCGTTCTCCATT	FAM-CAACTGGCAGTAACCABQH1	—	Positive control detected at less than 38 cycles.	Not stated	40 µM primers, 10 µM probe	Macherey-Nagel Nucleospin RNA virus (Cat. No 740956) and Invitrogen superscriptTM III Platinum One-Step Quantitative (Cat No. 11732-020 or 11732-088)

