To quickly develop SARS-CoV-2 testing capacity for Queensland, Public Health Virology (PHV) at FSS called upon 40 years of test horizon scanning, literature review, design, optimisation and validation expertise to create its real-time RT-PCR (RT-rPCR) tests. Our first test was developed using the initial SARS-CoV-2 genome sequence (https://virological.org/t/novel-2019-coronavirus-genome/319) before any other lab had published their tests. This test was designed and ordered on the day the initial genome sequence was released (Jan 11th, AEST). It targeted the nucleoprotein gene. This test aimed to accommodate both the possibility of ongoing mutational change and any virus variants which may exist but which had not yet arrived in Australia. A second RT-rPCR was made to help confirm positives later that month. It targeted the ORF1ab region. Test designs included a computer-based comparison of primer and probe sequences against all known human coronaviruses and other bat coronaviruses to understand, predict and where necessary, avoid interactions. The design process also predicted the thermodynamic properties, secondary structures and interactions of each primer or probe to select the most efficient primer and probe sequences.

Also, another region of the genome viral was targeted using sets of RT-PCR primers to generate a partial genome sequence to confirm that any positive results were specific to SARS-CoV-2 (called "the novel coronavirus" at the time) and not another virus. Early findings were put onto public sequence databases, e.g.
https://www.ncbi.nlm.nih.gov/nuccore/MT050414.1,
https://www.ncbi.nlm.nih.gov/nuccore/MT050415.1,
https://www.ncbi.nlm.nih.gov/nuccore/MT050416.1,
https://www.ncbi.nlm.nih.gov/nuccore/MT050417.1 .

The team continued to evaluate (optimise and compare) many other tests subsequently published by different laboratories from around the world (tests listed in https://www.who.int/publications/m/item/molecular-assays-to-diagnose-covid-19-summary-table-of-available-protocols) to ensure Queensland RT-rPCR testing was as effective as possible. The optimisation included testing each primer using five concentrations and each probe using thee concentrations with a synthetic RNA SARS-CoV-2 template. Each optimised test was applied to a panel of known positive sample extracts to compare the effectiv3ness of different tests. The best tests were taken forward to a validation. Validation examines the test's repeatability, reproducibility and performance. We challenge the best-performing tests using at least 50 SARS-CoV-2 previously positive sample extracts (sensitivity; looking for false negatives), which included testing SARS-CoV-2 RNA purified from cell-cultured material prepared from confirmed Queensland COVID-19 cases, at PHV (https://www.protocols.io/view/culture-of-the-severe-acute-respiratory-syndrome-c-bcduis6wv). Further, at least 100 samples from patients with related illnesses due to different pathogens, including human coronavirus 229E (specificity; looking for false positives) and influenza viruses were tested with the final tests.

PHV currently uses two tests as its frontline RT-rPCR tools - CCDC ORF (http://ivdc.chinacdc.cn/kyjz/202001/t20200121_211337.html) and US CDC N1 (https://www.cdc.gov/coronavirus/2019-ncov/lab/rt-pcr-panel-primer-probes.html).

Summary table of available protocols in this document

Institute	Gene targets
China CDC, China	ORF1ab and N
Institut Pasteur, Paris, France	Two targets in RdRP
US CDC, USA	Three targets in N gene
National Institute of Infectious Diseases, Japan	Pancorona and multiple targets, Spike protein
Charité, Germany	RdRP, E, N
HKU, Hong Kong SAR	ORF1b-nsp14, N
National Institute of Health, Thailand	N

***Disclaimer**: The order on the list is by country of the hosting institution and does not imply any preference of WHO. Neither the names of vendors or manufacturers included in the protocols are preferred/endorsed by WHO. The protocols have not yet been validated through a WHO process.*

***Assumptions:** Most procedures assume a basic familiarity with PCR/RT-PCR assays.*

***Safety Information**: Specimen processing should be performed in accordance with pertaining national biological safety regulations and following the recommended WHO guidelines on biosafety and biosecurity.*

***Protocol use limitations**: Optional clinical specimens for testing has not yet been validated.*

China CDC Primers and probes for detection 2019-nCoV (posted on 24 January 2020)

新型冠状病毒核酸检测引物和探针序列（Specific primers and probes for detection 2019 novel coronavirus)

来源：病毒病所 发布时间：2020-01-21

1.新型冠状病毒核酸测定（实时荧光RT-PCR方法）

推荐选用针对新型冠状病毒的开放读码框1ab（open reading frame,ORF1ab）、核壳蛋白（nucleoprotein，N）基因区域的引物和探针。

Target 1（ORF1ab）：

正向引物（F）：CCCTGTGGGTTTTACACTTAA

反向引物（R）：ACGATTGTGCATCAGCTGA

荧光探针（P）：5’-FAM-CCGTCTGCGGTATGTGGAAAGGTTATGG-BHQ1-3’

Target 2（N）：

正向引物（F）：GGGGAACTTCTCCTGCTAGAAT

反向引物（R）：CAGACATTTTGCTCTCAAGCTG

荧光探针（P）：5’-FAM-TTGCTGCTGCTTGACAGATT-TAMRA-3’

可以核酸提取和实时荧光RT-PCR反应体系参考相关厂家试剂盒说明。

2.结果判断

阴性:无Ct值或Ct为40。

阳性：Ct值<37，可报告为阳性。

可疑：Ct值在37-40之间，建议重复实验，若重做结果Ct值<40，扩增曲线有明显起峰，该样本判断为阳性,否则为阴性。

Protocol: Real-time RT-PCR assays for the detection of SARS-CoV-2

Institut Pasteur, Paris

This protocol describes procedures for the detection of SARS-CoV-2 for two RdRp targets (IP2 and IP4).

Based on the first sequences of SARS-CoV-2 made available on the GISAID database on January 11, 2020, primers and probes (nCoV_IP2 and nCoV_IP4) were designed to target the RdRp gene spanning nt 12621-12727 and 14010-14116 (positions according SARS-CoV, NC_004718).

As a confirmatory assay, we used the E gene assay from the Charité protocol[1]

Material

Kits:

Kit Extraction NucleoSpin Dx Virus — Ref: Macherey Nagel 740895.50
SuperScript™ III Platinum® One-Step Quantitative RT-PCR System — Ref: Invitrogen 1732-020

Primers and probes

Name	Sequences (5'-3')	Length (bases)	PCR product size	Ref.
RdRp gene / nCoV_IP2				
nCoV_IP2-12669Fw	ATGAGCTTAGTCCTGTTG	17	108 bp	1
nCoV_IP2-12759Rv	CTCCCTTTGTTGTGTTGT	18		
nCoV_IP2-12696bProbe(+)	AGATGTCTTGTGCTGCCGGTA [5']Hex [3']BHQ-1	21		
RdRp gene / nCoV_IP4				
nCoV_IP4-14059Fw	GGTAACTGGTATGATTTCG	19	107 bp	1
nCoV_IP4-14146Rv	CTGGTCAAGGTTAATATAGG	20		
nCoV_IP4-14084Probe(+)	TCATACAAACCACGCCAGG [5']Fam [3']BHQ-1	19		
E gene / E_Sarbeco				
E_Sarbeco_F1	ACAGGTACGTTAATAGTTAATAGCGT	18	125 bp	2
E_Sarbeco_R2	ATATTGCAGCAGTACGCACACA	20		
E_Sarbeco_P1	ACACTAGCCATCCTTACTGCGCTTCG [5']Fam [3']BHQ-1	20		

1/ National Reference Center for Respiratory Viruses, Institut Pasteur, Paris.
2/ Corman et al. Eurosurveillance[1]

Primer sets **nCoV_IP2** and **nCoV_IP4** can be multiplexed. Both reaction mixtures are described below.

PCR amplification regions (positions according to SARS-CoV, NC_004718)

nCoV_IP2 / 12621-12727
nCoV_IP4 / 14010-14116

E gene / 26141-26253

NUCLEIC ACID EXTRACTION

RNA is extracted from specimens using the NucleoSpin Dx Virus (Macherey Nagel ref. 740895.50). RNA extracted from 100 µl of original sample, is eluted in 100 µl of elution buffer.

MIX PREPARATION FOR ALL SEPARATE PRIMER/PROBE COMBINATIONS

All primers and probes described below were validated under the following conditions.

RT-PCR Mix kit:

- Invitrogen Superscript™ III Platinum® One-Step qRT-PCR system (ref: 11732-088)

Real-time PCR equipment:

- LightCycler 480 (96)

Adjustments may be required for the use of other kits or other real-time PCR instruments. All Assays used the same conditions. Primer and probe sequences, as well as optimized concentrations are shown in table below. **A 25µl reaction was set up containing 5µl of RNA.**

Simplex Mix	**Vol (µl)**	**[final]**
H_2O PPI	3.60	
Reaction mix 2X	12.50	3 mM Mg
$MgSO_4$ (50mM)	0.40	0.8 mM Mg
Forward Primer (10µM)	1.00	0.4 µM
Reverse Primer (10µM)	1.00	0.4 µM
Probe (10µM)	0.50	0.2 µM
SuperscriptIII RT/Platinum Taq Mix	1.00	
Final Volume	**20.00**	

Multiplex Mix (nCoV_IP2&IP4)	**Vol (µl)**	**[final]**
H_2O PPI	1.3	
Reaction mix 2X	12.50	3 mM Mg
$MgSO_4$ (50mM)	0.40	0.8 mM Mg
Forward Primer (10µM)	1.00	0.4 µM
Reverse Primer (10µM)	1.00	0.4 µM
Forward Primer (10µM)	1.00	0.4 µM
Reverse Primer (10µM)	1.00	0.4 µM
Probe (10µM)	0.4	0.16 µM
Probe (10µM)	0.4	0.16 µM
SuperscriptIII RT/Platinum Taq Mix	1.00	
Final Volume	**20.00**	

CONTROLS

Each real-time RT-PCR assay includes in addition of unknown samples:

- Two negative samples bracketing unknown samples during RNA extraction (negative extraction controls)
- Positive controls (in duplicate); when using *in vitro* synthesized transcripts as controls include five quantification positive controls (in duplicate) including 10^5, 10^4 and 10^3 copies genome equivalent (ge) of *in vitro* synthesized RNA transcripts.
- One negative amplification control.

AMPLIFICATION CYCLES (LIGHTCYCLER SYSTEM)

Reverse transcription	55°C	20 min	x1	
Denaturation	95°C	3 min	x1	
Amplification	95°C	15 sec	x50	Acquisition
	58°C	30 sec		
Cooling	40°C	30 sec	x1	

SENSITIVITY

For the nCoV_IP and E_Sarbeco real-time RT-PCR

Sensitivity, in terms of 95% hit rate is about 100 copies of RNA genome equivalent per reaction (this amount of target sequences is always detected), the probability to detect lower amounts of virus decreases, but samples containing 10 copies could be detected with multiplex assay.

	Multiplex (Ct values)		Simplex (Ct values)
RNA copies Of transcript	nCoV_IP2	nCoV_IP4	E_Sarbeco
1,00E+07	21,67	21,97	24,72
1,00E+06	24,97	25,12	28,19
1,00E+05	28,00	27,88	30,96
1,00E+04	31,84	30,51	33,33

Ct values may vary from instrument to instrument by up to 2 cycles, while the interval between two dilutions steps is constant (ΔCt).

SPECIFICITY

Cross-reactivity with other respiratory viruses was tested with specimens known to be positive for a panel of respiratory viruses (influenza A(H1N1)pdm09, A(H3N2), B-Victoria, B-Yamagata; influenza C; RSV A, B; hBoV; hPIV; hMPV; HRV/enterovirus; adenovirus; hCoV (HKU1, OC43, 229E and NL63); MERS-CoV. None of the tested viruses showed reactivity with PCR2 and PCR4.

POSITIVE CONTROL FOR SARS-CoV-2 REAL-TIME RT-PCR

One specific control has been designated.

Positive control for real-time RT-PCR is an *in vitro* transcribed RNA derived from strain BetaCoV_Wuhan_WIV04_2019 (EPI_ISL_402124). The transcript contains the amplification regions of the ***RdRp*** *and* ***E gene*** as positive strand. Each microtube contains 10^{11} copies of target sequences diluted in yeast tRNA, and lyophilised.

Reconstitution of transcribed RNA

Add 100 µl of RNase/DNAse-free H2O to obtain a solution at a concentration of 10^9 copies/µl. Store at -80°C. Dilute to prepare a master bank at $2x10^6$ copies/µl. Store at -80°C.

From this prepare a working bank of reagent at $2x10^4$ copies/µl in order to avoid freeze/thaw cycles. Working tubes may be stored at -20°C for less than one week.

Positive controls are available upon request (grippe@pasteur.fr)

Aknowledgements

We gratefully acknowledge the Authors, the Originating and Submitting Laboratories for their sequence and metadata shared through GISAID (EPI_ISL_402119; EPI_ISL_402121; EPI_ISL_402120; EPI_ISL_402123; EPI_ISL_402124; EPI_ISL_402125).

Reference

1- Corman VM, Landt O, Kaiser M, et al. Detection of 2019 novel coronavirus (2019-nCoV) by real-time RT-PCR. Euro Surveill 2020;25.

DEPARTMENT OF HEALTH & HUMAN SERVICES Public Health Service
Centers for Disease Control and Prevention (CDC)
Atlanta, GA 30333

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2019-Novel Coronavirus (2019-nCoV) Real-time rRT-PCR Panel Primers and Probes

Division of Viral Diseases

2019-Novel Coronavirus (2019-nCoV) Real-time rRT-PCR Panel Primers and Probes				
Name	**Description**	**Oligonucleotide Sequence (5'>3')**	**Label[1]**	**Working Conc.**
2019-nCoV_N1-F	2019-nCoV_N1 Forward Primer	5'-GAC CCC AAA ATC AGC GAA AT-3'	None	20 µM
2019-nCoV_N1-R	2019-nCoV_N1 Reverse Primer	5'-TCT GGT TAC TGC CAG TTG AAT CTG-3'	None	20 µM
2019-nCoV_N1-P	2019-nCoV_N1 Probe	5'-FAM-ACC CCG CAT TAC GTT TGG TGG ACC-BHQ1-3'	FAM, BHQ-1	5 µM
2019-nCoV_N2-F	2019-nCoV_N2 Forward Primer	5'-TTA CAA ACA TTG GCC GCA AA-3'	None	20 µM
2019-nCoV_N2-R	2019-nCoV_N2 Reverse Primer	5'-GCG CGA CAT TCC GAA GAA-3'	None	20 µM
2019-nCoV_N2-P	2019-nCoV_N2 Probe	5'-FAM-ACA ATT TGC CCC CAG CGC TTC AG-BHQ1-3'	FAM, BHQ-1	5 µM
2019-nCoV_N3-F	2019-nCoV_N3 Forward Primer	5'-GGG AGC CTT GAA TAC ACC AAA A-3'	None	20 µM
2019-nCoV_N3-R	2019-nCoV_N3 Reverse Primer	5'-TGT AGC ACG ATT GCA GCA TTG-3'	None	20 µM
2019-nCoV_N3-P	2019-nCoV_N3 Probe	5'-FAM-AYC ACA TTG GCA CCC GCA ATC CTG-BHQ1-3'	FAM, BHQ-1	5 µM
RP-F	RNAse P Forward Primer	5'-AGA TTT GGA CCT GCG AGC G-3'	None	20 µM
RP-R	RNAse P Reverse Primer	5'-GAG CGG CTG TCT CCA CAA GT-3'	None	20 µM
RP-P	RNAse P Probe	5'-FAM – TTC TGA CCT GAA GGC TCT GCG CG – BHQ-1-3'	FAM, BHQ-1	5 µM

[1]TaqMan® probes are labeled at the 5'-end with the reporter molecule 6-carboxyfluorescein (FAM) and with the quencher, Black Hole Quencher 1 (BHQ-1) (Biosearch Technologies, Inc., Novato, CA) at the 3'-end.

Note: Oligonucleotide sequences are subject to future changes as the 2019-Novel Coronavirus evolves.

CDC 2019-Novel Coronavirus (2019-nCoV) Real-Time RT-PCR Diagnostic Panel

For Emergency Use Only

Instructions for Use

Catalog # 2019-nCoVEUA-01
1000 reactions

For *In-vitro* Diagnostic (IVD) Use

Rx Only

Centers for Disease Control and Prevention
Division of Viral Diseases
1600 Clifton Rd NE
Atlanta GA 30329

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Intended Use

The CDC 2019-Novel Coronavirus (2019-nCoV) Real-Time RT-PCR Diagnostic Panel is a real-time RT-PCR test intended for the qualitative detection of nucleic acid from the 2019-nCoV in upper and lower respiratory specimens (such as nasopharyngeal or oropharyngeal swabs, sputum, lower respiratory tract aspirates, bronchoalveolar lavage, and nasopharyngeal wash/aspirate or nasal aspirate) collected from individuals who meet 2019-nCoV clinical and/or epidemiological criteria (for example, clinical signs and symptoms associated with 2019-nCoV infection, contact with a probable or confirmed 2019-nCoV case, history of travel to geographic locations where 2019-nCoV cases were detected, or other epidemiologic links for which 2019-nCoV testing may be indicated as part of a public health investigation). Testing in the United States is limited to laboratories certified under the Clinical Laboratory Improvement Amendments of 1988 (CLIA), 42 U.S.C. § 263a, to perform high complexity tests.

Results are for the identification of 2019-nCoV RNA. The 2019-nCoV RNA is generally detectable in upper and lower respiratory specimens during infection. Positive results are indicative of active infection with 2019-nCoV but do not rule out bacterial infection or co-infection with other viruses. The agent detected may not be the definite cause of disease. Laboratories within the United States and its territories are required to report all positive results to the appropriate public health authorities.

Negative results do not preclude 2019-nCoV infection and should not be used as the sole basis for treatment or other patient management decisions. Negative results must be combined with clinical observations, patient history, and epidemiological information.

Testing with the CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel is intended for use by trained laboratory personnel who are proficient in performing real-time RT-PCR assays. The CDC 2019-Novel Coronavirus (2019-nCoV) Real-Time RT-PCR Diagnostic Panel is only for use under a Food and Drug Administration's Emergency Use Authorization.

Summary and Explanation

An outbreak of pneumonia of unknown etiology in Wuhan City, Hubei Province, China was initially reported to WHO on December 31, 2019. Chinese authorities identified a novel coronavirus (2019-nCoV), which has resulted in thousands of confirmed human infections in multiple provinces throughout China and many countries including the United States. Cases of asymptomatic infection, mild illness, severe illness, and some deaths have been reported.

The CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel is a molecular *in vitro* diagnostic test that aids in the detection and diagnosis 2019-nCoV and is based on widely used nucleic acid amplification technology. The product contains oligonucleotide primers and dual-labeled hydrolysis probes (TaqMan®) and control material used in rRT-PCR for the *in vitro* qualitative detection of 2019-nCoV RNA in respiratory specimens.

The term "qualified laboratories" refers to laboratories in which all users, analysts, and any person reporting results from use of this device should be trained to perform and interpret the results from this procedure by a competent instructor prior to use.

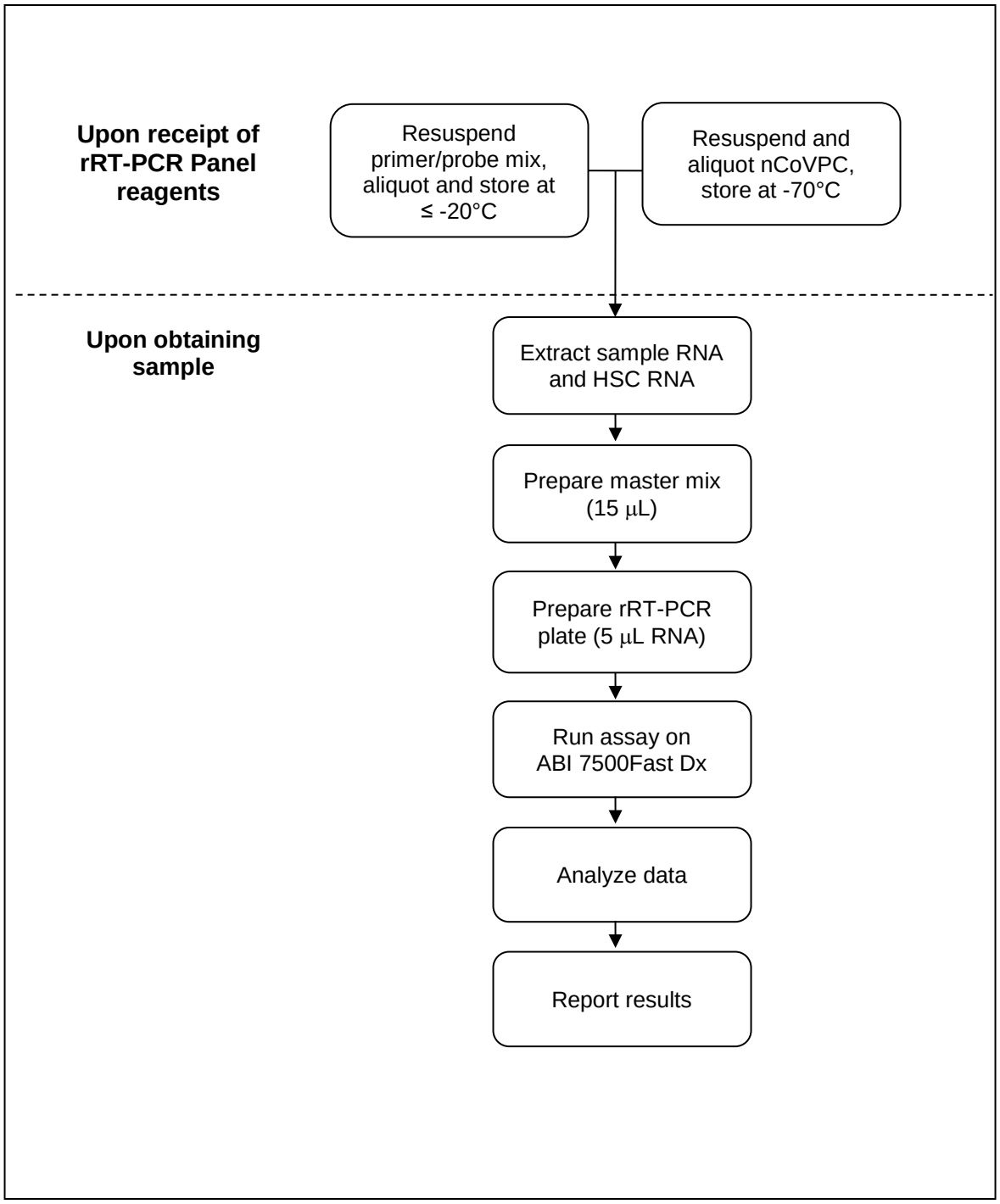
Principles of the Procedure

The oligonucleotide primers and probes for detection of 2019-nCoV were selected from regions of the virus nucleocapsid (N) gene. The panel is designed for specific detection of the 2019-nCoV (two primer/probe sets). An additional primer/probe set to detect the human RNase P gene (RP) in control samples and clinical specimens is also included in the panel.

RNA isolated and purified from upper and lower respiratory specimens is reverse transcribed to cDNA and subsequently amplified in the Applied Biosystems 7500 Fast Dx Real-Time PCR Instrument with SDS version 1.4 software. In the process, the probe anneals to a specific target sequence located between the forward and reverse primers. During the extension phase of the PCR cycle, the 5' nuclease activity of Taq polymerase degrades the probe, causing the reporter dye to separate from the quencher dye, generating a fluorescent signal. With each cycle, additional reporter dye molecules are cleaved from their respective probes, increasing the fluorescence intensity. Fluorescence intensity is monitored at each PCR cycle by Applied Biosystems 7500 Fast Dx Real-Time PCR System with SDS version 1.4 software.

Detection of viral RNA not only aids in the diagnosis of illness but also provides epidemiological and surveillance information.

Summary of Preparation and Testing Process



Materials Required (Provided)

Note: CDC will maintain on its website a list of commercially available lots of primer and probe sets and/or positive control materials that are acceptable alternatives to the CDC primer and probe set and/or positive control included in the Diagnostic Panel. Only material distributed through the CDC International Reagent Resource and specific lots of material posted to the CDC website are acceptable for use with this assay under CDC's Emergency Use Authorization.

This list of acceptable alternative lots of primer and probe materials and/or positive control materials will be available at:
https://www.cdc.gov/coronavirus/2019-nCoV/lab/index.html

Primers and Probes:

Catalog #2019-nCoVEUA-01 Diagnostic Panel Box #1:

Reagent Label	*Part #*	*Description*	*Quantity / Tube*	*Reactions / Tube*
2019-nCoV_N1	RV202001 RV202015	2019-nCoV_N1 Combined Primer/Probe Mix	22.5 nmol	1000
2019-nCoV_N2	RV202002 RV202016	2019-nCoV_N2 Combined Primer/Probe Mix	22.5 nmol	1000
RP	RV202004 RV202018	Human RNase P Forward Primer/Probe Mix	22.5 nmol	1000

Positive Control (either of the following products are acceptable)

Catalog #2019-nCoVEUA-01 Diagnostic Panel Box #2:

Reagent Label	*Part #*	*Description*	*Quantity*	*Notes*
nCoVPC	RV202005	2019-nCoV Positive Control (nCoVPC) For use as a positive control with the CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel procedure. The nCoVPC contains noninfectious positive control material supplied in a dried state and must be resuspended before use. nCoVPC consists of *in vitro* transcribed RNA. nCoVPC will yield a positive result with each assay in the 2019-nCoV Real-Time RT-PCR Diagnostic Panel including RP.	4 tubes	Provides (800) 5 µL test reactions

Catalog #VTC-04 CDC 2019-nCoV Positive Control (nCoVPC)

Reagent Label	*Part #*	*Description*	*Quantity*	*Notes*
nCoVPC	RV202005	2019-nCoV Positive Control (nCoVPC) For use as a positive control with the CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel procedure. The nCoVPC contains noninfectious positive control material supplied in a dried state and must be resuspended before use. nCoVPC consists of *in vitro* transcribed RNA. nCoVPC will yield a positive result with each assay in the 2019-nCoV Real-Time RT-PCR Diagnostic Panel including RP.	4 tubes	Provides (800) 5 µL test reactions

Materials Required (But Not Provided)

Human Specimen Control (HSC)

Description	Quantity	CDC Catalog No.
Manufactured by CDC. For use as an RNA extraction procedural control to demonstrate successful recovery of RNA as well as extraction reagent integrity. The HSC consists of noninfectious (beta-Propiolactone treated) cultured human cell material supplied as a liquid suspended in 0.01 M PBS at pH 7.2-7.4.	10 vials x 500uL	KT0189

Acceptable alternatives to HSC:

- Negative human specimen material: Laboratories may prepare a volume of human specimen material (e.g., human sera or pooled leftover negative respiratory specimens) to extract and run alongside clinical samples as an extraction control. This material should be prepared in sufficient volume to be used across multiple runs. Material should be tested prior to use as the extraction control to ensure it generates the expected results for the HSC listed in these instructions for use.
- Contrived human specimen material: Laboratories may prepare contrived human specimen materials by suspending any human cell line (e.g., A549, Hela or 293) in PBS. This material should be prepared in sufficient volume to be used across multiple runs. Material should be tested prior to use as the extraction control to ensure it generates the expected results for the HSC listed in these instructions for use.

CDC will maintain on its website a list of commercially alternative extraction controls, if applicable, that are acceptable for use with this assay under CDC's Emergency Use Authorization, at: https://www.cdc.gov/coronavirus/2019-nCoV/lab/index.html

rRT-PCR Enzyme Mastermix Options

Reagent	Quantity	Catalog No.
TaqPath™ 1-Step RT-qPCR Master Mix, CG (ThermoFisher)	1000 reactions	A15299
	2000 reactions	A15300

RNA Extraction Options

For each of the kits listed below, CDC has confirmed that the external lysis buffer is effective for inactivation of SARS-CoV-2.

Instrument/Manufacturer	Extraction Kit	Catalog No.
QIAGEN	[2]QIAmp DSP Viral RNA Mini Kit	50 extractions (61904)
	[2]QIAamp Viral RNA Mini Kit	50 extractions (52904) 250 extractions (52906)
QIAGEN EZ1 Advanced XL	[2]EZ1 DSP Virus Kit	48 extractions (62724) Buffer AVL (19073) EZ1 Advanced XL DSP Virus Card (9018703)
	[2]EZ1 Virus Mini Kit v2.0	48 extractions (955134) Buffer AVL (19073) EZ1 Advanced XL Virus Card v2.0 (9018708)
[1]Roche MagNA Pure LC	[2]Total Nucleic Acid Kit	192 extractions (03 038 505 001)
[1]Roche MagNA Pure Compact	[2]Nucleic Acid Isolation Kit I	32 extractions (03 730 964 001)
[1]Roche MagNA Pure 96	[2]DNA and Viral NA Small Volume Kit	576 extractions (06 543 588 001) External Lysis Buffer (06 374 913 001)
[1]QIAGEN QIAcube	[2]QIAmp DSP Viral RNA Mini Kit	50 extractions (61904)
	[2]QIAamp Viral RNA Mini Kit	50 extractions (52904) 250 extractions (52906)
[1, 3]bioMérieux NucliSENS® easyMAG® and [1, 3]bioMérieux EMAG® (Automated magnetic extraction reagents sold separately. Both instruments use the same reagents and disposables, with the exception of tips.)		EasyMAG® Magnetic Silica (280133) EasyMAG® Lysis Buffer (280134) EasyMAG® Lysis Buffer, 2 mL (200292) EasyMAG® Wash Buffers 1,2, and 3 (280130, 280131, 280132) EasyMAG® Disposables (280135) Biohit Pipette Tips (easyMAG® only) (280146) EMAG®1000µL Tips (418922)

[1]Equivalence and performance of these extraction platforms for extraction of viral RNA were demonstrated with the CDC Human Influenza Virus Real-Time RT-PCR Diagnostic Panel (K190302). Performance characteristics of these extraction platforms with 2019-nCoV (SARS CoV-2) have not been demonstrated.

[2] CDC has confirmed that the external lysis buffer used with this extraction method is effective for inactivation of SARS-CoV-2.

[3] CDC has compared the concentration of inactivating agent in the lysis buffer used with this extraction method and has determined the concentration to be within the range of concentrations found effective in inactivation of SARS-CoV-2.

Equipment and Consumables Required (But Not Provided)

- Vortex mixer
- Microcentrifuge
- Micropipettes (2 or 10 µL, 200 µL and 1000 µL)
- Multichannel micropipettes (5-50 µl)
- Racks for 1.5 mL microcentrifuge tubes
- 2 x 96-well -20°C cold blocks
- 7500 Fast Dx Real-Time PCR Systems with SDS 1.4 software (Applied Biosystems; catalog #4406985 or #4406984)
- Extraction systems (instruments): QIAGEN EZ1 Advanced XL
- Molecular grade water, nuclease-free
- 10% bleach (1:10 dilution of commercial 5.25-6.0% hypochlorite bleach)
- DNA*Zap*™ (Ambion, cat. #AM9890) or equivalent
- RNAse AwayTM (Fisher Scientific; cat. #21-236-21) or equivalent
- Disposable powder-free gloves and surgical gowns
- Aerosol barrier pipette tips
- 1.5 mL microcentrifuge tubes (DNase/RNase free)
- 0.2 mL PCR reaction plates (Applied Biosystems; catalog #4346906 or #4366932)
- MicroAmp Optical 8-cap Strips (Applied Biosystems; catalog #4323032)

Warnings and Precautions

- For *in vitro* diagnostic use (IVD).
- For emergency use only.
- Follow standard precautions. All patient specimens and positive controls should be considered potentially infectious and handled accordingly.
- Do not eat, drink, smoke, apply cosmetics or handle contact lenses in areas where reagents and human specimens are handled.
- Handle all specimens as if infectious using safe laboratory procedures. Refer to Interim Laboratory Biosafety Guidelines for Handling and Processing Specimens Associated with 2019-nCoV https://www.cdc.gov/coronavirus/2019-nCoV/lab-biosafety-guidelines.html.
- Specimen processing should be performed in accordance with national biological safety regulations.
- If infection with 2019-nCoV is suspected based on current clinical and epidemiological screening criteria recommended by public health authorities, specimens should be collected with appropriate infection control precautions.
- Performance characteristics have been determined with human upper respiratory specimens and lower respiratory tract specimens from human patients with signs and symptoms of respiratory infection.
- Perform all manipulations of live virus samples within a Class II (or higher) biological safety cabinet (BSC).
- Use personal protective equipment such as (but not limited to) gloves, eye protection, and lab coats when handling kit reagents while performing this assay and handling materials including samples, reagents, pipettes, and other equipment and reagents.

- Amplification technologies such as PCR are sensitive to accidental introduction of PCR product from previous amplifications reactions. Incorrect results could occur if either the clinical specimen or the real-time reagents used in the amplification step become contaminated by accidental introduction of amplification product (amplicon). Workflow in the laboratory should proceed in a unidirectional manner.
    - Maintain separate areas for assay setup and handling of nucleic acids.
    - Always check the expiration date prior to use. Do not use expired reagent. Do not substitute or mix reagent from different kit lots or from other manufacturers.
    - Change aerosol barrier pipette tips between all manual liquid transfers.
    - During preparation of samples, compliance with good laboratory techniques is essential to minimize the risk of cross-contamination between samples, and the inadvertent introduction of nucleases into samples during and after the extraction procedure. Proper aseptic technique should always be used when working with nucleic acids.
    - Maintain separate, dedicated equipment (e.g., pipettes, microcentrifuges) and supplies (e.g., microcentrifuge tubes, pipette tips) for assay setup and handling of extracted nucleic acids.
    - Wear a clean lab coat and powder-free disposable gloves (not previously worn) when setting up assays.
    - Change gloves between samples and whenever contamination is suspected.
    - Keep reagent and reaction tubes capped or covered as much as possible.
    - Primers, probes (including aliquots), and enzyme master mix must be thawed and maintained on cold block at all times during preparation and use.
    - Work surfaces, pipettes, and centrifuges should be cleaned and decontaminated with cleaning products such as 10% bleach, "DNA*Zap*™" or "RNase AWAY®" to minimize risk of nucleic acid contamination. Residual bleach should be removed using 70% ethanol.
- RNA should be maintained on cold block or on ice during preparation and use to ensure stability.
- Dispose of unused kit reagents and human specimens according to local, state, and federal regulations.

Reagent Storage, Handling, and Stability

- Store all dried primers and probes and the positive control, nCoVPC, at 2-8°C until re-hydrated for use. Store liquid HSC control materials at ≤ -20°C.
  Note: Storage information is for CDC primer and probe materials obtained through the International Reagent Resource. If using commercial primers and probes, please refer to the manufacturer's instructions for storage and handling.
- Always check the expiration date prior to use. Do not use expired reagents.
- Protect fluorogenic probes from light.
- Primers, probes (including aliquots), and enzyme master mix must be thawed and kept on a cold block at all times during preparation and use.
- Do not refreeze probes.
  Controls and aliquots of controls must be thawed and kept on ice at all times during preparation and use.

Specimen Collection, Handling, and Storage

Inadequate or inappropriate specimen collection, storage, and transport are likely to yield false test results. Training in specimen collection is highly recommended due to the importance of specimen quality. CLSI MM13-A may be referenced as an appropriate resource.

- Collecting the Specimen
  - Refer to Interim Guidelines for Collecting, Handling, and Testing Clinical Specimens from Patients Under Investigation (PUIs) for 2019 Novel Coronavirus (2019-nCoV) https://www.cdc.gov/coronavirus/2019-nCoV/guidelines-clinical-specimens.html
  - Follow specimen collection device manufacturer instructions for proper collection methods.
  - Swab specimens should be collected using only swabs with a synthetic tip, such as nylon or Dacron®, and an aluminum or plastic shaft. Calcium alginate swabs are unacceptable and cotton swabs with wooden shafts are not recommended. Place swabs immediately into sterile tubes containing 2-3 ml of viral transport media.
- Transporting Specimens
  - Specimens must be packaged, shipped, and transported according to the current edition of the International Air Transport Association (IATA) Dangerous Goods Regulation. Follow shipping regulations for UN 3373 Biological Substance, Category B when sending potential 2019-nCoV specimens. Store specimens at 2-8°C and ship overnight to CDC on ice pack. If a specimen is frozen at -70°C or lower, ship overnight to CDC on dry ice.
- Storing Specimens
  - Specimens can be stored at 2-8°C for up to 72 hours after collection.
  - If a delay in extraction is expected, store specimens at -70°C or lower.
  - Extracted nucleic acid should be stored at -70°C or lower.

Specimen Referral to CDC

For state and local public health laboratories:

- Ship all specimens overnight to CDC.
- Ship frozen specimens on dry ice and non-frozen specimens on cold packs.
- Refer to the International Air Transport Association (IATA - www.iata.org) for requirements for shipment of human or potentially infectious biological specimens. Follow shipping regulations for UN 3373 Biological Substance, Category B when sending potential 2019-nCoV specimens.
- Prior to shipping, notify CDC Division of Viral Diseases (see contact information below) that you are sending specimens.
- Send all samples to the following recipient:

Centers for Disease Control and Prevention
c/o STATT
Attention: Dr. Stephen Lindstrom (Unit 84)
1600 Clifton Rd., Atlanta, GA 30329-4027
Phone: (404) 639-3931

The emergency contact number for CDC Emergency Operations Center (EOC) is 770-488-7100.

All other laboratories that are CLIA certified and meet requirements to perform high complexity testing:

- Please notify your state and/or local public health laboratory for specimen referral and confirmatory testing guidance.

Reagent and Controls Preparation

NOTE: Storage information is for materials obtained through the CDC International Regent Resource. If using commercial products for testing, please refer to the manufacturer's instructions for storage, handling and preparation instructions.

Primer and Probe Preparation:

1) Upon receipt, store dried primers and probes at 2-8°C.
2) Precautions: These reagents should only be handled in a clean area and stored at appropriate temperatures (see below) in the dark. Freeze-thaw cycles should be avoided. Maintain cold when thawed.
3) Using aseptic technique, suspend dried reagents in 1.5 mL of nuclease-free water (50X working concentration) and allow to rehydrate for 15 min at room temperature in the dark.
4) Mix gently and aliquot primers/probe in 300 µL volumes into 5 pre-labeled tubes. Store a single aliquot of primers/probe at 2-8°C in the dark. Do not refreeze (stable for up to 4 months). Store remaining aliquots at ≤ -20°C in a non-frost-free freezer.

2019-nCoV Positive Control (nCoVPC) Preparation:

1) Precautions: This reagent should be handled with caution in a dedicated nucleic acid handling area to prevent possible contamination. Freeze-thaw cycles should be avoided. Maintain on ice when thawed.
2) Resuspend dried reagent in each tube in 1 mL of nuclease-free water to achieve the proper concentration. Make single use aliquots (approximately 30 µL) and store at ≤ -70°C.
3) Thaw a single aliquot of diluted positive control for each experiment and hold on ice until adding to plate. Discard any unused portion of the aliquot.

Human Specimen Control (HSC) (not provided)

1) Human Specimen Control (HSC) or one of the listed acceptable alternative extraction controls must be extracted and processed with each specimen extraction run.
2) Refer to the Human Specimen Control (HSC) package insert for instructions for use.

No Template Control (NTC) (not provided)

1) Sterile, nuclease-free water
2) Aliquot in small volumes
3) Used to check for contamination during specimen extraction and/or plate set-up

General Preparation

Equipment Preparation

Clean and decontaminate all work surfaces, pipettes, centrifuges, and other equipment prior to use. Decontamination agents should be used including 10% bleach, 70% ethanol, and *DNAzap*™ or *RNase AWAY*® to minimize the risk of nucleic acid contamination.

Nucleic Acid Extraction

Performance of the CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel is dependent upon the amount and quality of template RNA purified from human specimens. The following commercially available RNA extraction kits and procedures have been qualified and validated for recovery and purity of RNA for use with the panel:

Qiagen QIAamp® DSP Viral RNA Mini Kit or QIAamp® Viral RNA Mini Kit

Recommendation(s): Utilize 100 µL of sample and elute with 100 µL of buffer or utilize 140 µL of sample and elute with 140 µL of buffer.

Qiagen EZ1 Advanced XL

Kit: Qiagen EZ1 DSP Virus Kit and Buffer AVL (supplied separately) for offboard lysis
Card: EZ1 Advanced XL DSP Virus Card
Recommendation(s): Add 120 µL of sample to 280 µL of pre-aliquoted Buffer AVL (total input sample volume is 400 µL). Proceed with the extraction on the EZ1 Advanced XL. Elution volume is 120 µL.

Kit: Qiagen EZ1 Virus Mini Kit v2.0 and Buffer AVL (supplied separately) for offboard lysis
Card: EZ1 Advanced XL Virus Card v2.0
Recommendation(s): Add 120 µL of sample to 280 µL of pre-aliquoted Buffer AVL (total input sample volume is 400 µL). Proceed with the extraction on the EZ1 Advanced XL. Elution volume is 120 µL.

Equivalence and performance of the following extraction platforms were demonstrated with the CDC Human Influenza Virus Real-Time RT-PCR Diagnostic Panel (K190302) and based on those data are acceptable for use with the CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel.

QIAGEN QIAcube
Kit: QIAGEN QIAamp® DSP Viral RNA Mini Kit or QIAamp® Viral RNA Mini Kit
Recommendations: Utilize 140 µL of sample and elute with 100 µL of buffer.

Roche MagNA Pure LC
Kit: Roche MagNA Pure Total Nucleic Acid Kit
Protocol: Total NA External_lysis
Recommendation(s): Add 100 µL of sample to 300 µL of pre-aliquoted TNA isolation kit lysis buffer (total input sample volume is 400 µL). Elution volume is 100 µL.

Roche MagNA Pure Compact
Kit: Roche MagNA Pure Nucleic Acid Isolation Kit I
Protocol: Total_NA_Plasma100_400
Recommendation(s): Add 100 µL of sample to 300 µL of pre-aliquoted TNA isolation kit lysis buffer (total input sample volume is 400 µL). Elution volume is 100 µL.

Roche MagNA Pure 96
Kit: Roche MagNA Pure 96 DNA and Viral NA Small Volume Kit
Protocol: Viral NA Plasma Ext Lys SV Protocol
Recommendation(s): Add 100 µL of sample to 350 µL of pre-aliquoted External Lysis Buffer (supplied separately) (total input sample volume is 450 µL). Proceed with the extraction on the MagNA Pure 96. (**Note: Internal Control = None)**. Elution volume is 100 µL.

bioMérieux NucliSENS® easyMAG® Instrument
Protocol: General protocol (not for blood) using "Off-board Lysis" reagent settings.
Recommendation(s): Add 100 µL of sample to 1000 µL of pre-aliquoted easyMAG lysis buffer (total input sample volume is 1100 µL). Incubate for 10 minutes at room temperature. Elution volume is 100 µL.

bioMérieux EMAG® Instrument
Protocol: Custom protocol: **CDC Flu V1** using "Off-board Lysis" reagent settings.
Recommendation(s): Add 100 µL of samples to 2000 µL of pre-aliquoted easyMAG lysis buffer (total input sample volume is 2100 µL). Incubate for 10 minutes at room temperature. Elution volume is 100 µL. The custom protocol, **CDC Flu V1**, is programmed on the bioMérieux EMAG® instrument with the assistance of a bioMérieux service representative. Installation verification is documented at the time of installation. Laboratories are recommended to retain a record of the step-by-step verification of the bioMérieux custom protocol installation procedure.

Manufacturer's recommended procedures (except as noted in recommendations above) are to be followed for sample extraction. HSC must be included in each extraction batch.

Disclaimer: Names of vendors or manufacturers are provided as examples of suitable product sources. Inclusion does not imply endorsement by the Centers for Disease Control and Prevention.

Assay Set Up

Reaction Master Mix and Plate Set Up

Note: Plate set-up configuration can vary with the number of specimens and workday organization. NTCs and nCoVPCs must be included in each run.

1) In the reagent set-up room clean hood, place rRT-PCR buffer, enzyme, and primer/probes on ice or cold-block. Keep cold during preparation and use.
2) Thaw 4X Reaction Mix prior to use.
3) Mix buffer, enzyme, and primer/probes by inversion 5 times.
4) Centrifuge buffer and primers/probes for 5 seconds to collect contents at the bottom of the tube, and then place the tube in a cold rack.
5) Label one 1.5 mL microcentrifuge tube for each primer/probe set.
6) Determine the number of reactions (N) to set up per assay. It is necessary to make excess reaction mix for the NTC, nCoVPC, HSC (if included in the RT-PCR run), and RP reactions and for pipetting error. Use the following guide to determine N:
   - If number of samples (n) including controls equals 1 through 14, then N = n + 1
   - If number of samples (n) including controls is 15 or greater, then N = n + 2
7) For each primer/probe set, calculate the amount of each reagent to be added for each reaction mixture (N = # of reactions).

TaqPath™ 1-Step RT-qPCR Master Mix

Step #	Reagent	Vol. of Reagent Added per Reaction
1	Nuclease-free Water	N x 8.5 µL
2	Combined Primer/Probe Mix	N x 1.5 µL
3	TaqPath™ 1-Step RT-qPCR Master Mix (4x)	N x 5.0 µL
	Total Volume	**N x 15.0 µL**

8) Dispense reagents into each respective labeled 1.5 mL microcentrifuge tube. After addition of the reagents, mix reaction mixtures by pipetting up and down. ***Do not vortex***.
9) Centrifuge for 5 seconds to collect contents at the bottom of the tube, and then place the tube in a cold rack.
10) Set up reaction strip tubes or plates in a 96-well cooler rack.
11) Dispense 15 µL of each master mix into the appropriate wells going across the row as shown below (**Figure 1**):

Figure 1: Example of Reaction Master Mix Plate Set-Up

	1	2	3	4	5	6	7	8	9	10	11	12
A	N1	N1	N1	N1	N1	N1	N1	N1	N1	N1	N1	N1
B	N2	N2	N2	N2	N2	N2	N2	N2	N2	N2	N2	N2
C	RP	RP	RP	RP	RP	RP	RP	RP	RP	RP	RP	RP
D												
E												
F												
G												
H												

12) Prior to moving to the nucleic acid handling area, prepare the No Template Control (NTC) reactions for column #1 in the assay preparation area.
13) Pipette 5 µL of nuclease-free water into the NTC sample wells (**Figure 2**, column 1). Securely cap NTC wells before proceeding.
14) Cover the entire reaction plate and move the reaction plate to the specimen nucleic acid handling area.

Nucleic Acid Template Addition

1) Gently vortex nucleic acid sample tubes for approximately 5 seconds.
2) Centrifuge for 5 seconds to collect contents at the bottom of the tube.
3) After centrifugation, place extracted nucleic acid sample tubes in the cold rack.
4) Samples should be added to columns 2-11 (column 1 and 12 are for controls) to the specific assay that is being tested as illustrated in **Figure 2**. Carefully pipette 5.0 µL of the first sample into all the wells labeled for that sample (i.e. Sample "S1" down column #2). *Keep other sample wells covered during addition. Change tips after each addition.*
5) Securely cap the column to which the sample has been added to prevent cross contamination and to ensure sample tracking.
6) Change gloves often and when necessary to avoid contamination.
7) Repeat steps #4 and #5 for the remaining samples.

8) If necessary, add 5 µL of Human Specimen Control (HSC) extracted sample to the HSC wells (**Figure 2**, column 11). Securely cap wells after addition. NOTE: Per CLIA regulations, HSC must be tested at least once per day.
9) Cover the entire reaction plate and move the reaction plate to the positive template control handling area.

Assay Control Addition

1) Pipette 5 µL of nCoVPC RNA to the sample wells of column 12 (**Figure 2**). Securely cap wells after addition of the control RNA.

NOTE: *If using 8-tube strips, label the TAB of each strip to indicate sample position.* ***DO NOT LABEL THE TOPS OF THE REACTION TUBES!***

2) Briefly centrifuge reaction tube strips for 10-15 seconds. After centrifugation return to cold rack.

NOTE*: If using 96-well plates, centrifuge plates for 30 seconds at 500 x g, 4°C.*

Figure 2. 2019-nCoV rRT-PCR Diagnostic Panel: Example of Sample and Control Set-up

	1	2	3	4	5	6	7	8	9	10	11[a]	12
A	NTC	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	nCoV PC
B	NTC	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	nCoV PC
C	NTC	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	nCoV PC
D												
E												
F												
G												
H												

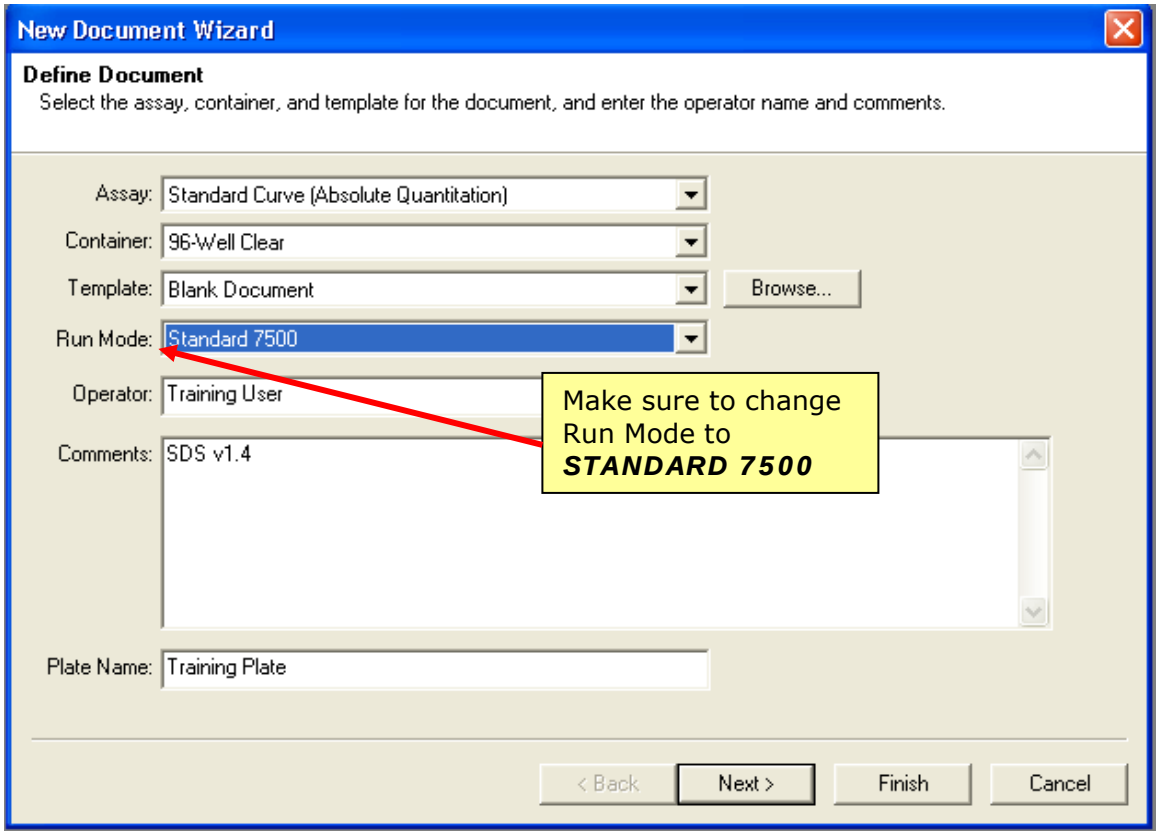
[a]Replace the sample in this column with extracted HSC if necessary

Create a Run Template on the Applied Biosystems 7500 Fast Dx Real-time PCR Instrument (Required if no template exists)

If the template already exists on your instrument, please proceed to the **RUNNING A TEST** section.

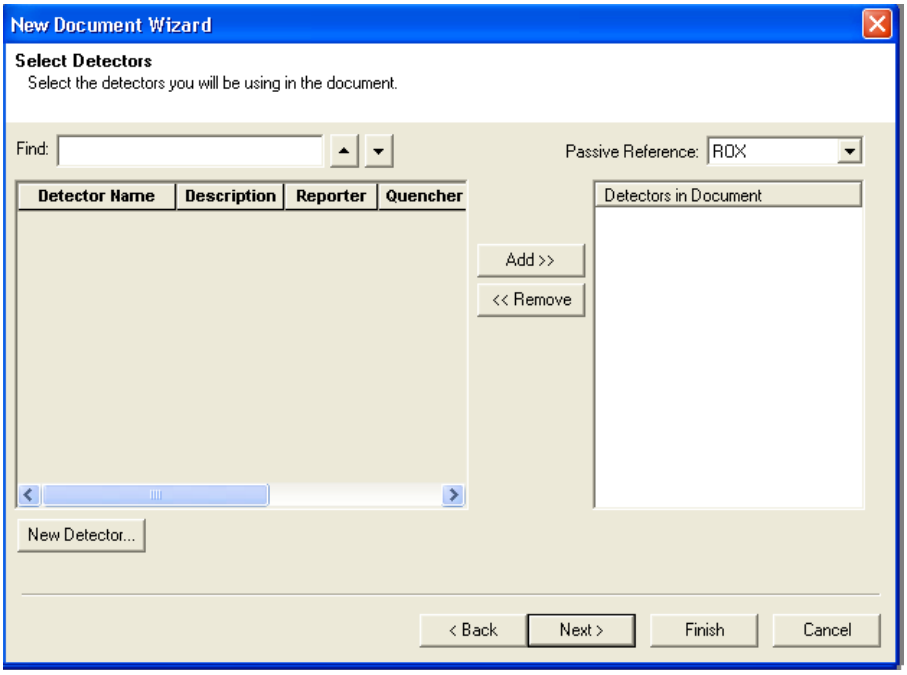
1) Launch the Applied Biosystems 7500 Fast Dx Real-time PCR Instrument by double clicking on the Applied Biosystems 7500 Fast Dx System icon on the desktop.
2) A new window should appear, select **Create New Document** from the menu.

Figure 3. New Document Wizard Window



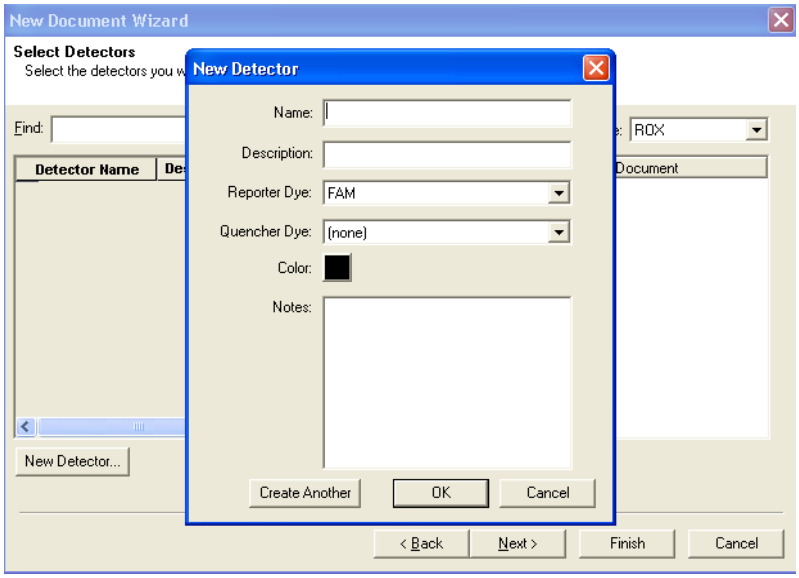
3) The **New Document Wizard** screen in **Figure 3** will appear. Select:
   a. Assay: **Standard Curve (Absolute Quantitation)**
   b. Container: **96-Well Clear**
   c. Template: **Blank Document**
   d. Run Mode: **Standard 7500**
   e. Operator: ***Your Name***
   f. Comments: **SDS v1.4**
   g. Plate Name: ***Your Choice***
4) After making selections click **Next** at the bottom of the window.

Figure 4. Creating New Detectors



5) After selecting next, the ***Select Detectors*** screen (**Figure 4**) will appear.
6) Click the **New Detector** button (see **Figure 4**).
7) The **New Detector** window will appear (**Figure 5**). A new detector will need to be defined for each primer and probe set. Creating these detectors will enable you to analyze each primer and probe set individually at the end of the reaction.

Figure 5. New Detector Window

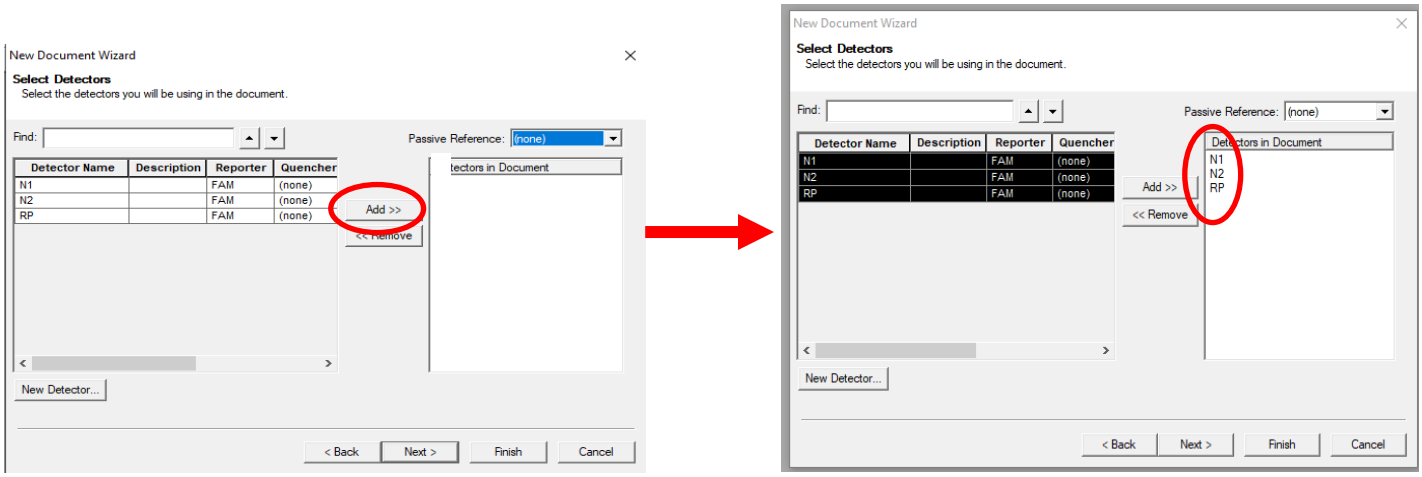


8) Start by creating the N1 Detector. Include the following:
   a. Name: **N1**
   b. Description: *leave blank*
   c. Reporter Dye: **FAM**
   d. Quencher Dye: **(none)**
   e. Color: *to change the color of the detector indicator do the following:*
      ⇒ Click on the color square to reveal the color chart
      ⇒ Select a color by clicking on one of the squares
      ⇒ After selecting a color click **OK** to return to the New Detector screen
   f. Click the **OK** button of the New Detector screen to return to the screen shown in **Figure 4**.
9) Repeat step 6-8 for each target in the panel.

Name	Reporter Dye	Quencher Dye
N1	FAM	(none)
N2	FAM	(none)
RP	FAM	(none)

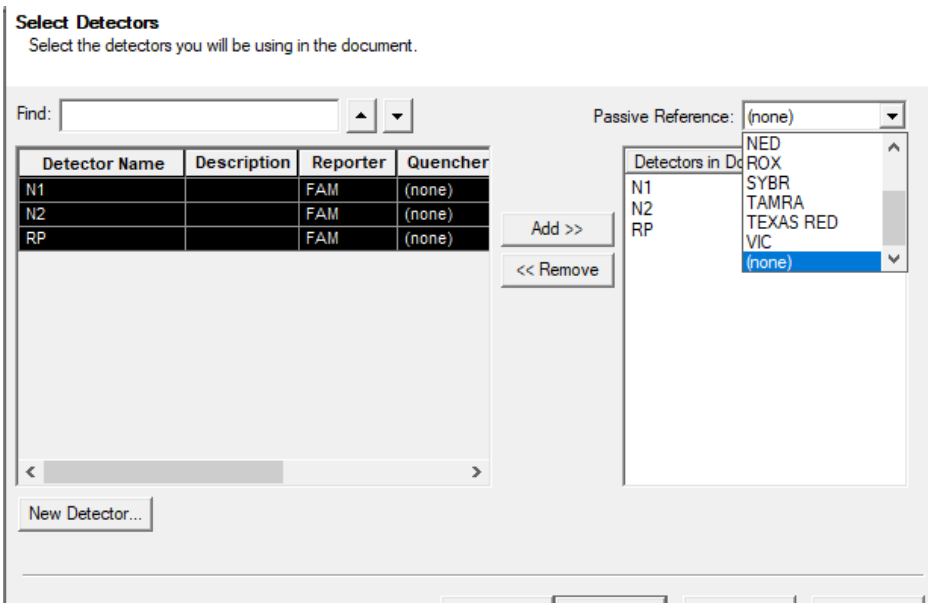
10) After each Detector is added, the **Detector Name**, **Description**, **Reporter** and **Quencher** fields will become populated in the **Select Detectors** screen (**Figure 6**).
11) Before proceeding, the newly created detectors must be added to the document. To add the new detectors to the document, click **ADD** (see **Figure 6**). Detector names will appear on the right-hand side of the **Select Detectors** window (**Figure 6**).

Figure 6. Adding New Detectors to Document



12) Once all detectors have been added, select **(none)** for **Passive Reference** at the top right-hand drop-down menu (**Figure 7**).

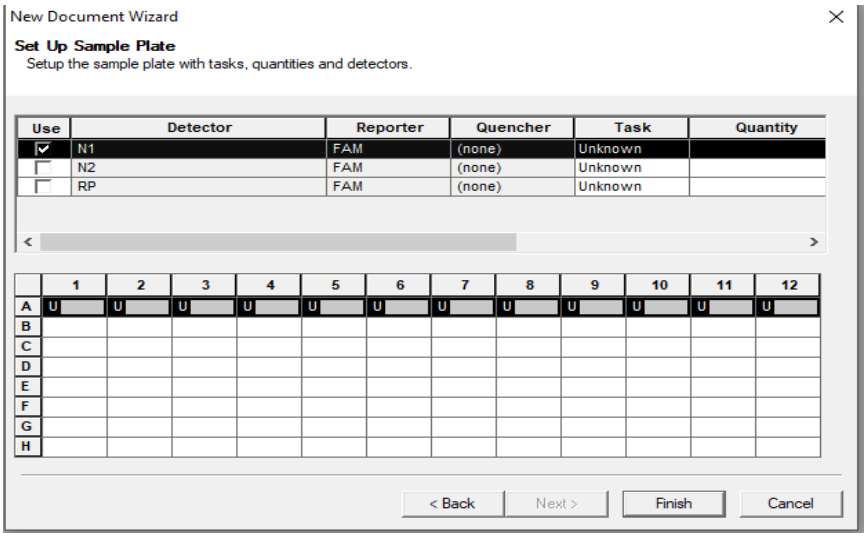
Figure 7. Select Passive Reference



Passive reference should be set to "(none)" as described above.

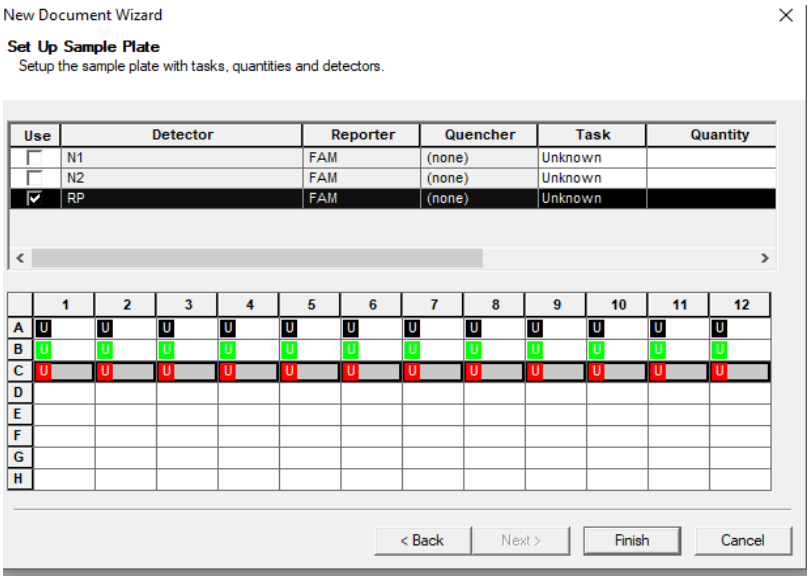
13) Click **Next** at the bottom of the **Select Detectors** window to proceed to the **Set Up Sample Plate** window (**Figure 8**).
14) In the **Set Up Sample Plate** window (**Figure 8**), use your mouse to select row A from the lower portion of the window, in the spreadsheet (see **Figure 8**).
15) In the top portion of the window, select detector **N1**. A check will appear next to the detector you have selected (**Figure 8**). You will also notice the row in the spreadsheet will be populated with a colored "U" icon to indicate which detector you've selected.
16) Repeat step 14-15 for each detector that will be used in the assay.

Figure 8. Sample Plate Set-up



17) Select **Finish** after detectors have been assigned to their respective rows. (**Figure 9**).

Figure 9. Finished Plate Set-up

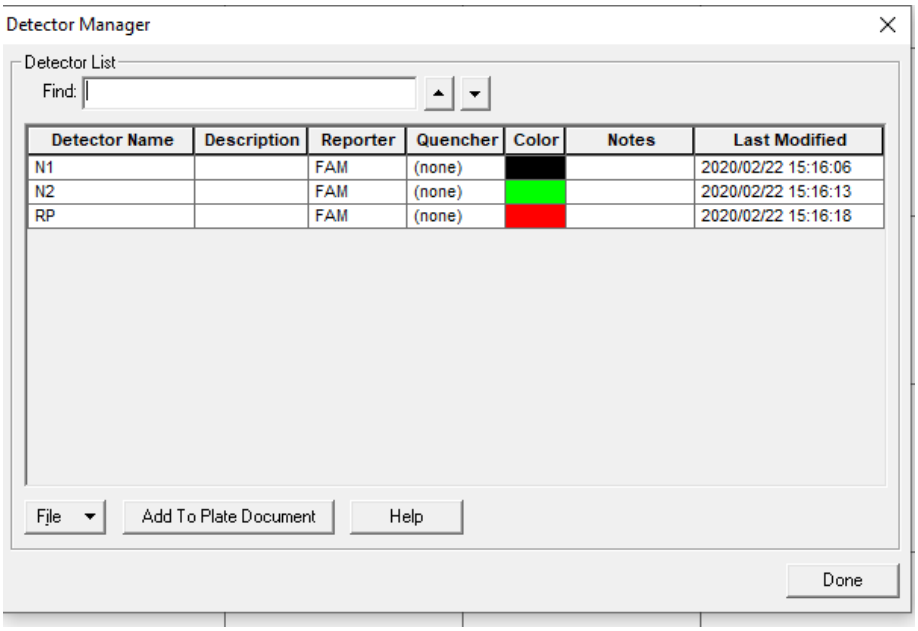


18) After clicking “Finish”, there will be a brief pause allowing the Applied Biosystems 7500 Fast Dx to initialize. This initialization is followed by a clicking noise. ***Note: The machine must be turned on for initialization.***
19) After initialization, the **Plate** tab of the Setup (**Figure 10)** will appear.
20) Each well of the plate should contain colored U icons that correspond with the detector labels that were previously chosen. To confirm detector assignments, select **Tools** from the file menu, then select **Detector Manager.**

Figure 10. Plate Set-up Window

21) The Detector Manager window will appear (**Figure 11**).

Figure 11. Detector Manager Window



22) Confirm all detectors are included and that each target has a **Reporter** set to **FAM** and the **Quencher** is set to **(none)**.
23) If all detectors are present, select **Done**. The detector information has been created and assigned to wells on the plate.

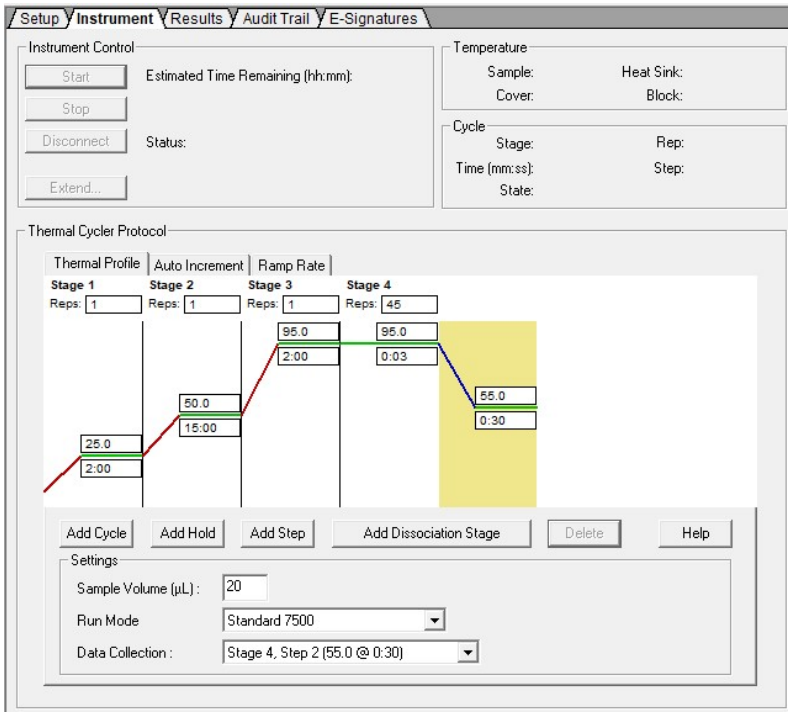
Defining the Instrument Settings

1) After detectors have been created and assigned, proceed to instrument set up.
2) Select the **Instrument** tab to define thermal cycling conditions.
3) Modify the thermal cycling conditions as follows (**Figure 12**):

TaqPath™ 1-Step RT-qPCR Master Mix, CG (ThermoFisher)

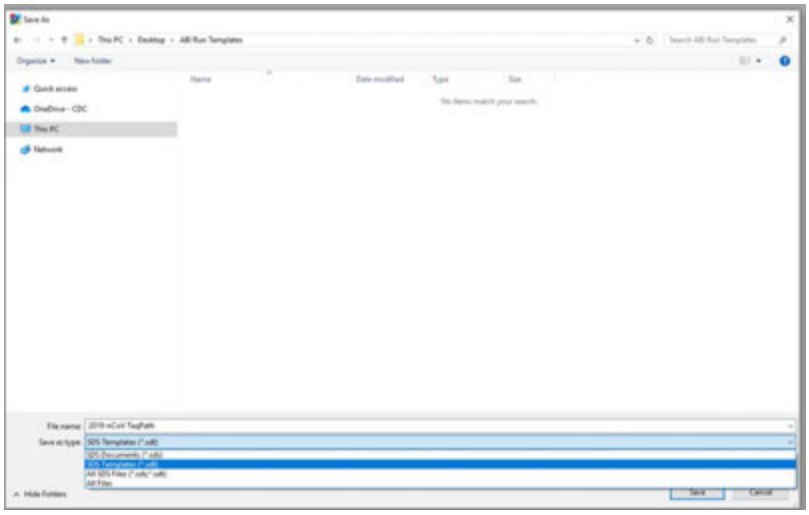
a. In Stage 1, Set to 2 min at **25°C**; **1 Rep**.
b. In Stage 2, Set to 15 min at **50°C**; **1 Rep**.
c. In Stage 3, Set to 2 min at **95°C, 1 Rep.**
d. In Stage 4, Step 1 set to **3 sec** at **95°C**.
e. In Stage 4, Step 2 set to **30 sec** at **55.0°C.**
f. In Stage 4, Reps should be set to **45.**
g. Under **Settings** (**Figure 12**), bottom left-hand box, change volume to 20 µL.
h. Under **Settings**, **Run Mode** selection should be **Standard 7500**.
i. Step 2 of Stage 4 should be highlighted in yellow to indicate data collection (see **Figure 12**).

Figure 12. Instrument Window



4) After making changes to the **Instrument** tab, the template file is ready to be saved. To save the template, select **File** from the top menu, then select **Save As**.
5) Save the template as **2019-nCoV TaqPath** as appropriate in the desktop folder labeled "***ABI Run Templates***" (*you must create this folder*). Save as type should be SDS Templates (*.sdt) (**Figure 13**).

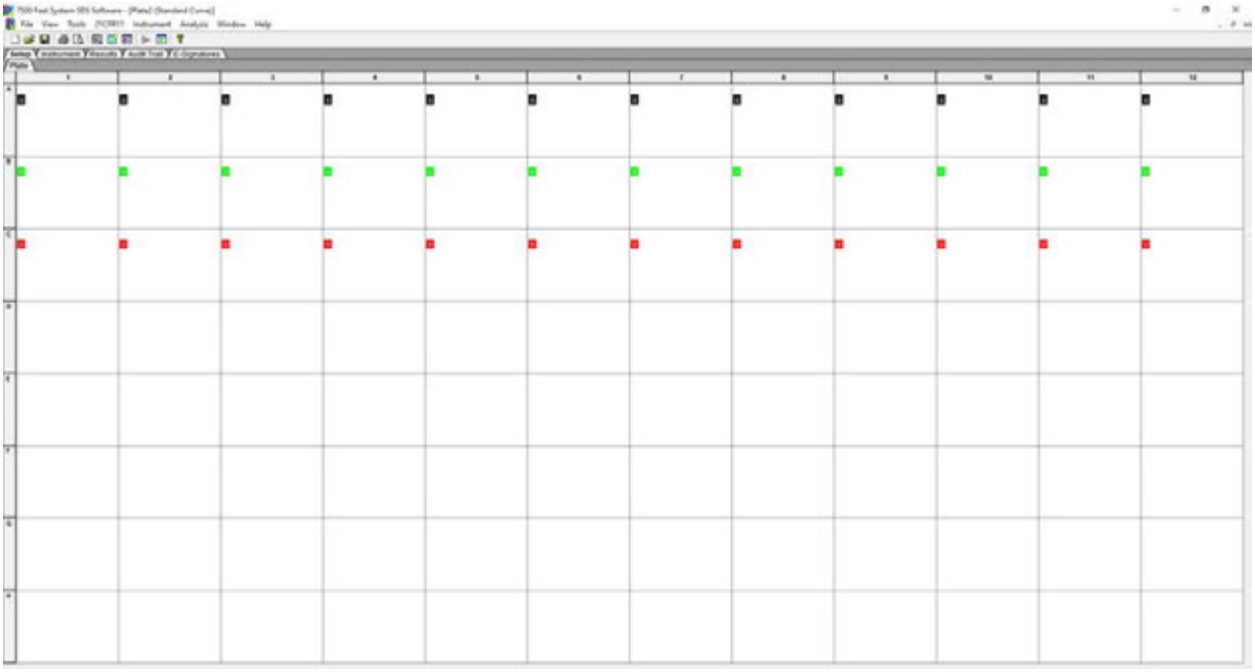
Figure 13. Saving Template



Running a Test

1) Turn on the ABI 7500 Fast Dx Real-Time PCR Instrument.
2) Launch the Applied Biosystems 7500 Fast Dx Real-time PCR System by double clicking on the 7500 Fast Dx System icon on the desktop.
3) A new window should appear, select **Open Existing Document** from the menu.
4) Navigate to select your ABI Run Template folder from the desktop.
5) Double click on the appropriate template file (**2019-nCoV TaqPath)**
6) There will be a brief pause allowing the Applied Biosystems 7500 Fast Dx Real-Time PCR Instrument to initialize. This initialization is followed by a clicking noise. ***Note: The machine must be turned on for initialization.***

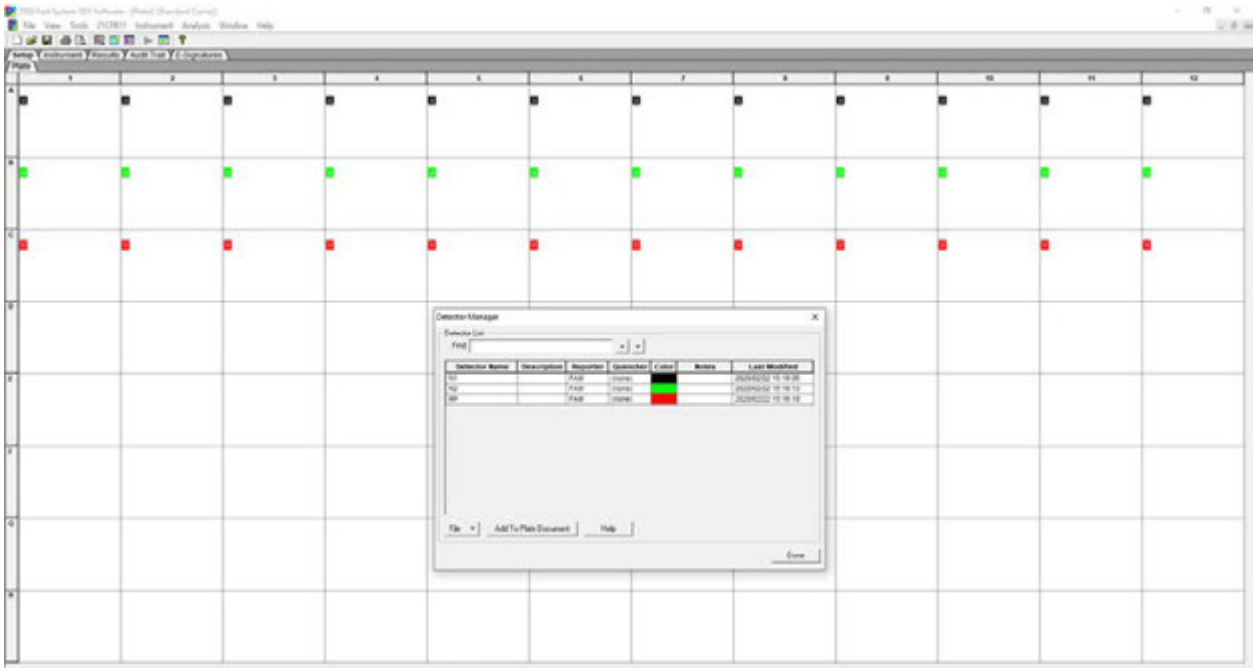
Figure 14. Plate Set-up Window



7) After the instrument initializes, a plate map will appear (**Figure 14**). The detectors and controls should already be labeled as they were assigned in the original template.

8) Click the **Well Inspector** icon  from the top menu.
9) Highlight specimen wells of interest on the plate map.
10) Type sample identifiers to **Sample Name** box in the **Well Inspector** window (**Figure 15**).

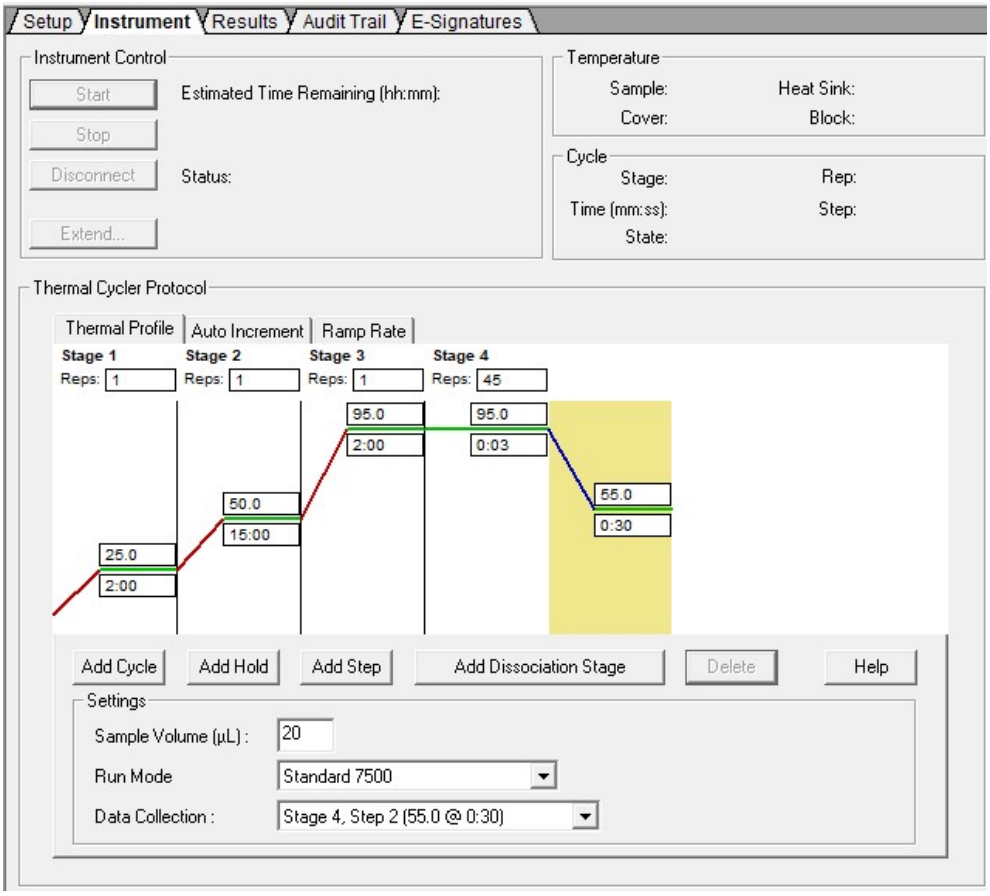
Figure 15. Labeling Wells



11) Repeat steps 9-10 until all sample identifiers are added to the plate setup.

12) Once all specimen and control identifiers are added click the **Close** button on the **Well Inspector** window to return to the **Plate** set up tab.
13) Click the **Instrument** tab at the upper left corner.
14) The reaction conditions, volumes, and type of 7500 reaction should already be loaded. (**Figure 16**).

Figure 16. Instrument Settings

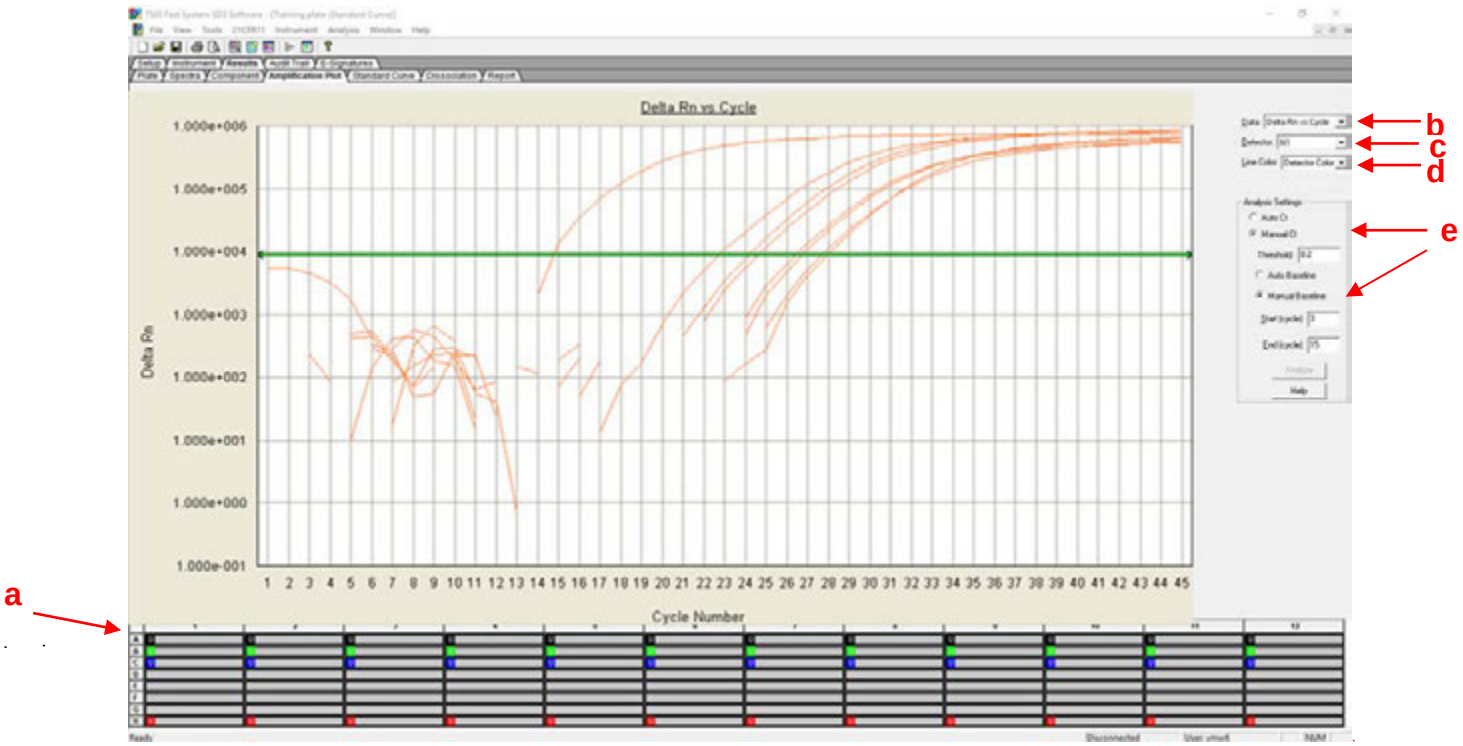


15) Ensure settings are correct (refer to the *Defining Instrument Settings*).
16) Before proceeding, the run file must be saved; from the main menu, select **File,** then **Save As**. Save in appropriate run folder designation.
17) Load the plate into the plate holder in the instrument. Ensure that the plate is properly aligned in the holder.
18) Once the run file is saved, click the **Start** button. *Note: The run should take approximately 1hr and 20 minutes to complete.*

Data Analysis

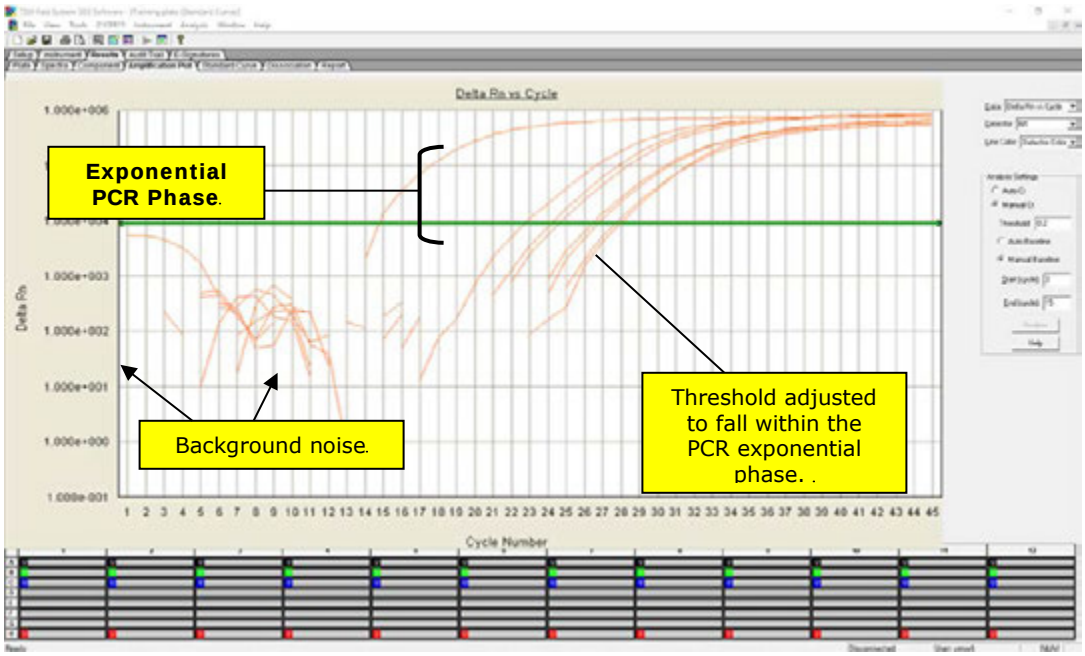
1) After the run has completed, select the **Results** tab at the upper left corner of the software.
2) Select the **Amplification Plot** tab to view the raw data (**Figure 17**).

Figure 17. Amplification Plot Window



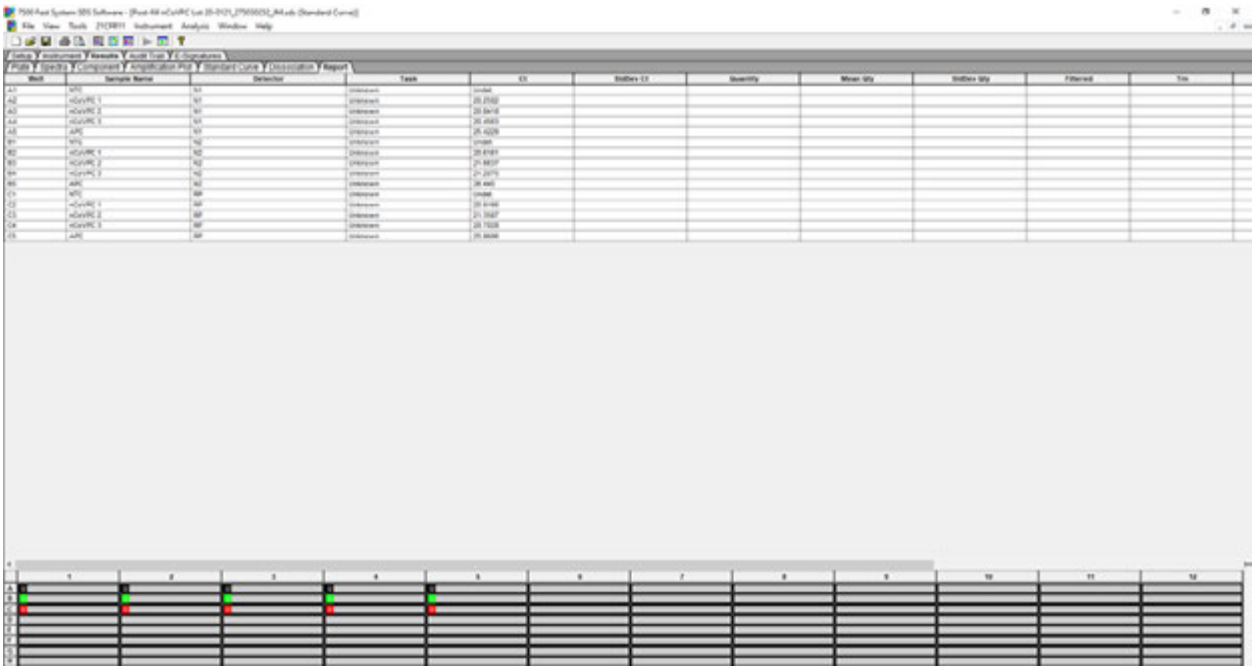
3) Start by highlighting all the samples from the run; to do this, click on the upper left-hand box **(a)** of the sample wells (**Figure 17**). All the growth curves should appear on the graph.
4) On the right-hand side of the window **(b)**, the **Data** drop down selection should be set to **Delta Rn vs. Cycle**.
5) Select **N1** from **(c)**, the **Detector** drop down menu, using the downward arrow.
    a. Please note that each detector is analyzed individually to reflect different performance profiles of each primer and probe set.
6) In the **Line Color** drop down **(d)**, **Detector Color** should be selected.
7) Under **Analysis Settings** select **Manual Ct (e)**.
    b. Do not change the **Manual Baseline** default numbers.
8) Using the mouse, click and drag the red threshold line until it lies within the exponential phase of the fluorescence curves and above any background signal (**Figure 18)**.

Figure 18. Amplification Plot



9) Click the **Analyze** button in the lower right corner of the window. The red threshold line will turn to green, indicating the data has been analyzed.
10) Repeat steps 5-9 to analyze results generated for each set of markers (N1, N2, RP).
11) Save analysis file by selecting **File** then **Save As** from the main menu.
12) After completing analysis for each of the markers, select the **Report** tab above the graph to display the Ct values (**Figure 19**). To filter report by sample name in ascending or descending order, simply click on **Sample Name** in the table.

Figure 19. Report



Interpretation of Results and Reporting

Extraction and Positive Control Results and Interpretation

No Template Control (NTC)

The NTC consists of using nuclease-free water in the rRT-PCR reactions instead of RNA. The NTC reactions for all primer and probe sets should not exhibit fluorescence growth curves that cross the threshold line. If any of the NTC reactions exhibit a growth curve that crosses the cycle threshold, sample contamination may have occurred. Invalidate the run and repeat the assay with strict adherence to the guidelines.

2019-nCoV Positive Control (nCoVPC)

The nCoVPC consists of in vitro transcribed RNA. The nCoVPC will yield a positive result with the following primer and probe sets: N1, N2 and RP.

Human Specimen Control (HSC) (Extraction Control)

When HSC is run with the CDC 2019-nCoV rRT-PCR Diagnostic Panel (see previous section on Assay Set Up), the HSC is used as an RNA extraction procedural control to demonstrate successful recovery of RNA as well as extraction reagent integrity. The HSC control consists of noninfectious cultured human cell (A549) material. Purified nucleic acid from the HSC should yield a positive result with the RP primer and probe set and negative results with all 2019-nCoV markers.

Expected Performance of Controls Included in the CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel

Control Type	External Control Name	Used to Monitor	2019 nCoV_N1	2019 nCoV_N2	RP	Expected Ct Values
Positive	nCoVPC	Substantial reagent failure including primer and probe integrity	+	+	+	< 40.00 Ct
Negative	NTC	Reagent and/or environmental contamination	-	-	-	None detected
Extraction	HSC	Failure in lysis and extraction procedure, potential contamination during extraction	-	-	+	< 40.00 Ct

If any of the above controls do not exhibit the expected performance as described, the assay may have been set up and/or executed improperly, or reagent or equipment malfunction could have occurred. Invalidate the run and re-test.

RNase P (Extraction Control)

- All clinical samples should exhibit fluorescence growth curves in the RNase P reaction that cross the threshold line within 40.00 cycles (< 40.00 Ct), thus indicating the presence of the human RNase P gene. Failure to detect RNase P in any clinical specimens may indicate:
  - Improper extraction of nucleic acid from clinical materials resulting in loss of RNA and/or RNA degradation.
  - Absence of sufficient human cellular material due to poor collection or loss of specimen integrity.
  - Improper assay set up and execution.
  - Reagent or equipment malfunction.
- If the RP assay does not produce a positive result for human clinical specimens, interpret as follows:
  - If the 2019-nCoV N1 and N2are positive even in the absence of a positive RP, the result should be considered valid. It is possible, that some samples may fail to exhibit RNase P growth curves due to low cell numbers in the original clinical sample. A negative RP signal does not preclude the presence of 2019-nCoV virus RNA in a clinical specimen.
  - If all 2019-nCoV markers AND RNase P are negative for the specimen, the result should be considered invalid for the specimen. If residual specimen is available, repeat the extraction procedure and repeat the test. If all markers remain negative after re-test, report the results as invalid and a new specimen should be collected if possible.

2019-nCoV Markers (N1 and N2)

- When all controls exhibit the expected performance, a specimen is considered negative if all 2019-nCoV marker (N1, N2) cycle threshold growth curves DO NOT cross the threshold line within 40.00 cycles (< 40.00 Ct) AND the RNase P growth curve DOES cross the threshold line within 40.00 cycles (< 40.00 Ct).
- When all controls exhibit the expected performance, a specimen is considered positive for 2019-nCoV if all 2019-nCoV marker (N1, N2) cycle threshold growth curves cross the threshold line within 40.00 cycles (< 40.00 Ct). The RNase P may or may not be positive as described above, but the 2019-nCoV result is still valid.
- When all controls exhibit the expected performance and the growth curves for the 2019-nCoV markers (N1, N2) AND the RNase P marker DO NOT cross the cycle threshold growth curve within 40.00 cycles (< 40.00 Ct), the result is invalid. The extracted RNA from the specimen should be re-tested. If residual RNA is not available, re-extract RNA from residual specimen and re-test. If the re-tested sample is negative for all markers and RNase P, the result is invalid and collection of a new specimen from the patient should be considered.
- When all controls exhibit the expected performance and the cycle threshold growth curve for any one marker (N1 or N2 but not both markers) crosses the threshold line within 40.00 cycles (< 40.00 Ct) the result is inconclusive. The extracted RNA should be retested. If residual RNA is not available, re-extract RNA from residual specimen and re-test. If the same result is obtained, the laboratory should coordinate transfer of the specimen to CDC for further analysis.
- If HSC is positive for N1 or N2, then contamination may have occurred during extraction or sample processing. Invalidate all results for specimens extracted alongside the HSC. Re-extract specimens and HSC and re-test.

2019-nCoV rRT-PCR Diagnostic Panel Results Interpretation Guide

The table below lists the expected results for the 2019-nCoV rRT-PCR Diagnostic Panel. If a laboratory obtains unexpected results for assay controls or if inconclusive or invalid results are obtained and cannot be resolved through the recommended re-testing, please contact CDC for consultation and possible specimen referral. See pages 10 and 40 for referral and contact information.

2019 nCoV_N1	2019 nCoV_N2	RP	Result Interpretation[a]	Report	Actions
+	+	±	2019-nCoV detected	Positive 2019-nCoV	Report results to CDC and sender.
If only one of the two targets is positive		±	Inconclusive Result	Inconclusive	Repeat testing of nucleic acid and/or re-extract and repeat rRT-PCR. If the repeated result remains inconclusive, contact your State Public Health Laboratory or CDC for instructions for transfer of the specimen or further guidance.
-	-	+	2019-nCoV not detected	Not Detected	Report results to sender. Consider testing for other respiratory viruses.[b]
-	-	-	Invalid Result	Invalid	Repeat extraction and rRT-PCR. If the repeated result remains invalid, consider collecting a new specimen from the patient.

[a]Laboratories should report their diagnostic result as appropriate and in compliance with their specific reporting system.

[b]Optimum specimen types and timing for peak viral levels during infections caused by 2019-nCoV have not been determined. Collection of multiple specimens from the same patient may be necessary to detect the virus. The possibility of a false negative result should especially be considered if the patient's recent exposures or clinical presentation suggest that 2019-nCoV infection is possible, and diagnostic tests for other causes of illness (e.g., other respiratory illness) are negative. If 2019-nCoV infection is still suspected, re-testing should be considered in consultation with public health authorities.

Quality Control

- Quality control requirements must be performed in conformance with local, state, and federal regulations or accreditation requirements and the user's laboratory's standard quality control procedures. For further guidance on appropriate quality control practices, refer to 42 CFR 493.1256.
- Quality control procedures are intended to monitor reagent and assay performance.
- Test all positive controls prior to running diagnostic samples with each new kit lot to ensure all reagents and kit components are working properly.
- Good laboratory practice (cGLP) recommends including a positive extraction control in each nucleic acid isolation batch.
- Although HSC is not included with the 2019-nCov rRT-PCR Diagnostic Panel, the HSC extraction control must proceed through nucleic acid isolation per batch of specimens to be tested.
- Always include a negative control (NTC), and the appropriate positive control (nCoVPC) in each amplification and detection run. All clinical samples should be tested for human RNAse P gene to control for specimen quality and extraction.

Limitations

- All users, analysts, and any person reporting diagnostic results should be trained to perform this procedure by a competent instructor. They should demonstrate their ability to perform the test and interpret the results prior to performing the assay independently.
- Performance of the CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel has only been established in upper and lower respiratory specimens (such as nasopharyngeal or oropharyngeal swabs, sputum, lower respiratory tract aspirates, bronchoalveolar lavage, and nasopharyngeal wash/aspirate or nasal aspirate).
- Negative results do not preclude 2019-nCoV infection and should not be used as the sole basis for treatment or other patient management decisions. Optimum specimen types and timing for peak viral levels during infections caused by 2019-nCoV have not been determined. Collection of multiple specimens (types and time points) from the same patient may be necessary to detect the virus.
- A false negative result may occur if a specimen is improperly collected, transported or handled. False negative results may also occur if amplification inhibitors are present in the specimen or if inadequate numbers of organisms are present in the specimen.
- Positive and negative predictive values are highly dependent on prevalence. False negative test results are more likely when prevalence of disease is high. False positive test results are more likely when prevalence is moderate to low.
- Do not use any reagent past the expiration date.
- If the virus mutates in the rRT-PCR target region, 2019-nCoV may not be detected or may be detected less predictably. Inhibitors or other types of interference may produce a false negative result. An interference study evaluating the effect of common cold medications was not performed.
- Test performance can be affected because the epidemiology and clinical spectrum of infection caused by 2019-nCoV is not fully known. For example, clinicians and laboratories may not know the optimum types of specimens to collect, and, during the course of infection, when these specimens are most likely to contain levels of viral RNA that can be readily detected.
- Detection of viral RNA may not indicate the presence of infectious virus or that 2019-nCoV is the causative agent for clinical symptoms.

- The performance of this test has not been established for monitoring treatment of 2019-nCoV infection.
- The performance of this test has not been established for screening of blood or blood products for the presence of 2019-nCoV.
- This test cannot rule out diseases caused by other bacterial or viral pathogens.

Conditions of Authorization for the Laboratory

The CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel Letter of Authorization, along with the authorized Fact Sheet for Healthcare Providers, the authorized Fact Sheet for Patients and authorized labeling are available on the FDA website:
https://www.fda.gov/MedicalDevices/Safety/EmergencySituations/ucm161496.htm
Use of the CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel must follow the procedures outlined in these manufacturer's Instructions for Use and the conditions of authorization outlined in the Letter of Authorization. Deviations from the procedures outlined are not permitted under the Emergency Use Authorization (EUA). To assist clinical laboratories running the CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel, the relevant Conditions of Authorization are listed verbatim below, and are required to be met by laboratories performing the EUA test.

- Authorized laboratories[1] will include with reports of the results of the CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel, all authorized Fact Sheets. Under exigent circumstances, other appropriate methods for disseminating these Fact Sheets may be used, which may include mass media.

- Authorized laboratories will perform the CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel as outlined in the CDC 2019-Novel Coronavirus (2019-nCoV) Real-Time RT-PCR Diagnostic Panel Instructions for Use. Deviations from the authorized procedures, including the authorized RT-PCR instruments, authorized extraction methods, authorized clinical specimen types, authorized control materials, authorized other ancillary reagents and authorized materials required to perform the CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel are not permitted. [2]

- Authorized laboratories that receive the CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel must notify the relevant public health authorities of their intent to run the test prior to initiating testing.

- Authorized laboratories will have a process in place for reporting test results to healthcare providers and relevant public health authorities, as appropriate.

- Authorized laboratories will collect information on the performance of the test and report to DMD/OHT7-OIR/OPEQ/CDRH (via email: CDRH-EUA-Reporting@fda.hhs.gov) and CDC

[1]Authorized Laboratories: For ease of reference, the Letter of Authorization refers to "laboratories certified under the Clinical Laboratory Improvement Amendments of 1988 (CLIA), 42 U.S.C. § 263a, to perform high complexity tests" as "authorized laboratories."
[2]If an authorized laboratory is interested in implementing changes to the CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel that are not in the scope (Section II) of this letter of authorization FDA recommends you discuss with FDA after considering the policy outlined in *Immediately in Effect Guidance for Clinical Laboratories and Food and Drug Administration Staff: Policy for Diagnostics Testing in Laboratories Certified to Perform High Complexity Testing under CLIA prior to Emergency Use Authorization for Coronavirus Disease-2019 during the Public Health Emergency* (https://www.fda.gov/media/135659/download).

(respvirus@cdc.gov) any suspected occurrence of false positive or false negative results and significant deviations from the established performance characteristics of the test of which they become aware.

- Authorized laboratories will report adverse events, including problems with test performance or results, to MedWatch by submitting the online FDA Form 3500 (https://www.accessdata.fda.gov/scripts/medwatch/index.cfm?action=reporting.home) or by calling 1-800-FDA-1088

- All laboratory personnel using the test must be appropriately trained in RT-PCR techniques and use appropriate laboratory and personal protective equipment when handling this kit and use the test in accordance with the authorized labeling.

- CDC, IRR, manufacturers and distributors of commercial materials identified as acceptable on the CDC website, and authorized laboratories will ensure that any records associated with this EUA are maintained until otherwise notified by FDA. Such records will be made available to FDA for inspection upon request.

Performance Characteristics

Analytical Performance:

Limit of Detection (LoD):

LoD studies determine the lowest detectable concentration of 2019-nCoV at which approximately 95% of all (true positive) replicates test positive. The LoD was determined by limiting dilution studies using characterized samples.

The analytical sensitivity of the rRT-PCR assays contained in the CDC 2019 Novel Coronavirus (2019-nCoV) Real-Time RT-PCR Diagnostic Panel were determined in Limit of Detection studies. Since no quantified virus isolates of the 2019-nCoV are currently available, assays designed for detection of the 2019-nCoV RNA were tested with characterized stocks of in vitro transcribed full length RNA (N gene; GenBank accession: MN908947.2) of known titer (RNA copies/µL) spiked into a diluent consisting of a suspension of human A549 cells and viral transport medium (VTM) to mimic clinical specimen. Samples were extracted using the QIAGEN EZ1 Advanced XL instrument and EZ1 DSP Virus Kit (Cat# 62724) and manually with the QIAGEN DSP Viral RNA Mini Kit (Cat# 61904). Real-Time RT-PCR assays were performed using the ThemoFisher Scientific TaqPath™ 1-Step RT-qPCR Master Mix, CG (Cat# A15299) on the Applied Biosystems™ 7500 Fast Dx Real-Time PCR Instrument according to the CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel instructions for use.

A preliminary LoD for each assay was determined testing triplicate samples of RNA purified using each extraction method. The approximate LoD was identified by extracting and testing 10-fold serial dilutions of characterized stocks of in vitro transcribed full-length RNA. A confirmation of the LoD was determined using 3-fold serial dilution RNA samples with 20 extracted replicates. The LoD was determined as the lowest concentration where ≥ 95% (19/20) of the replicates were positive.

Table 4. Limit of Detection Confirmation of the CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel with QIAGEN EZ1 DSP

Targets	2019-nCoV_N1			2019-nCoV_N2		
RNA Concentration[1]	$10^{0.5}$	$10^{0.0}$	$10^{-0.5}$	$10^{0.5}$	$10^{0.0}$	$10^{-0.5}$
Positives/Total	20/20	19/20	13/20	20/20	17/20	9/20
Mean Ct[2]	32.5	35.4	NA	35.8	NA	NA
Standard Deviation (Ct)	0.5	0.8	NA	1.3	NA	NA

[1] Concentration is presented in RNA copies/µL

[2] Mean Ct reported for dilutions that are ≥ 95% positive. Calculations only include positive results.

NA not applicable

Table 5. Limit of Detection Confirmation CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel with QIAGEN QIAmp DSP Viral RNA Mini Kit

Targets	2019-nCoV_N1			2019-nCoV_N2			
RNA Concentration[1]	$10^{0.5}$	$10^{0.0}$	$10^{-0.5}$	$10^{0.5}$	$10^{0.0}$	$10^{-0.5}$	$10^{-1.0}$
Positives/Total	20/20	20/20	6/20	20/20	20/20	20/20	8/20
Mean Ct[2]	32.0	32.8	NA	33.0	35.4	36.2	NA
Standard Deviation (Ct)	0.7	0.8	NA	1.4	0.9	1.9	NA

[1] Concentration is presented in RNA copies/µL

[2] Mean Ct reported for dilutions that are ≥ 95% positive. Calculations only include positive results.

NA not applicable

Table 6. Limit of Detection of the CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel

Virus	**Material**	**Limit of Detection (RNA copies/µL)**	
		QIAGEN EZ1 Advanced XL	**QIAGEN DSP Viral RNA Mini Kit**
2019 Novel Coronavirus	N Gene RNA Transcript	$10^{0.5}$	10^{0}

FDA Sensitivity Evaluation: The analytical sensitivity of the test will be further assessed by evaluating an FDA-recommended reference material using an FDA developed protocol if applicable and/or when available.

In Silico Analysis of Primer and Probe Sequences:

An alignment was performed with the oligonucleotide primer and probe sequences of the CDC 2019 nCoV Real-Time RT-PCR Diagnostic Panel with all publicly available nucleic acid sequences for 2019-nCoV in GenBank as of February 1, 2020 to demonstrate the predicted inclusivity of the CDC 2019 nCoV Real-Time RT-PCR Diagnostic panel. All the alignments show 100% identity of the CDC panel to the available 2019-nCoV sequences with the exception of one nucleotide mismatch with the N1 forward primer in one deposited sequence. The risk of a single mismatch resulting in a significant loss in reactivity, and false negative result, is

low due to the design of the primers and probes with melting temperatures > 60°C and run conditions of the assay with annealing temperature at 55°C to tolerate one to two mismatches.

Specificity/Exclusivity Testing: In Silico Analysis

BLASTn analysis queries of the 2019-nCoV rRT-PCR assays primers and probes were performed against public domain nucleotide sequences. The database search parameters were as follows: 1) The nucleotide collection consists of GenBank+EMBL+DDBJ+PDB+RefSeq sequences, but excludes EST, STS, GSS, WGS, TSA, patent sequences as well as phase 0, 1, and 2 HTGS sequences and sequences longer than 100Mb; 2) The database is non-redundant. Identical sequences have been merged into one entry, while preserving the accession, GI, title and taxonomy information for each entry; 3) Database was updated on 10/03/2019; 4) The search parameters automatically adjust for short input sequences and the expect threshold is 1000; 5) The match and mismatch scores are 1 and -3, respectively; 6) The penalty to create and extend a gap in an alignment is 5 and 2 respectively.

2019-nCoV_N1 Assay:
Probe sequence of 2019-nCoV rRT-PCR assay N1 showed high sequence homology with SARS coronavirus and Bat SARS-like coronavirus genome. However, forward and reverse primers showed no sequence homology with SARS coronavirus and Bat SARS-like coronavirus genome. Combining primers and probe, there is no significant homologies with human genome, other coronaviruses or human microflora that would predict potential false positive rRT-PCR results.

2019-nCoV_N2 Assay:
The forward primer sequence of 2019-nCoV rRT-PCR assay N2 showed high sequence homology to Bat SARS-like coronaviruses. The reverse primer and probe sequences showed no significant homology with human genome, other coronaviruses or human microflora. Combining primers and probe, there is no prediction of potential false positive rRT-PCR results.

In summary, the 2019-nCoV rRT-PCR assay N1 and N2, designed for the specific detection of 2019-nCoV, showed no significant combined homologies with human genome, other coronaviruses, or human microflora that would predict potential false positive rRT-PCR results.

In addition to the *in silico* analysis, several organisms were extracted and tested with the CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel to demonstrate analytical specificity and exclusivity. Studies were performed with nucleic acids extracted using the QIAGEN EZ1 Advanced XL instrument and EZ1 DSP Virus Kit. Nucleic acids were extracted from high titer preparations (typically ≥ 10^5 PFU/mL or ≥ 10^6 CFU/mL). Testing was performed using the ThemoFisher Scientific TaqPath™ 1-Step RT-qPCR Master Mix, CG on the Applied Biosystems™ 7500 Fast Dx Real-Time PCR instrument. The data demonstrate that the expected results are obtained for each organism when tested with the CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel.

Table 7. Specificity/Exclusivity of the CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel

Virus	Strain	Source	2019-nCoV_N1	2019-nCoV_N2	Final Result
Human coronavirus	229E	isolate	0/3	0/3	Neg.
Human coronavirus	OC43	isolate	0/3	0/3	Neg.
Human coronavirus	NL63	clinical specimen	0/3	0/3	Neg.
Human coronavirus	HKU1	clinical specimen	0/3	0/3	Neg.
MERS-coronavirus		isolate	0/3	0/3	Neg.
SARS-coronavirus		isolate	0/3	0/3	Neg.
bocavirus	-	clinical specimen	0/3	0/3	Neg.
Mycoplasma pneumoniae		isolate	0/3	0/3	Neg.
Streptococcus		isolate	0/3	0/3	Neg.
Influenza A(H1N1)		isolate	0/3	0/3	Neg.
Influenza A(H3N2)		isolate	0/3	0/3	Neg.
Influenza B		isolate	0/3	0/3	Neg.
Human adenovirus, type 1	Ad71	isolate	0/3	0/3	Neg.
Human metapneumovirus	-	isolate	0/3	0/3	Neg.
respiratory syncytial virus	Long A	isolate	0/3	0/3	Neg.
rhinovirus		isolate	0/3	0/3	Neg.
parainfluenza 1	C35	isolate	0/3	0/3	Neg.
parainfluenza 2	Greer	isolate	0/3	0/3	Neg.
parainfluenza 3	C-43	isolate	0/3	0/3	Neg.
parainfluenza 4	M-25	isolate	0/3	0/3	Neg.

Endogenous Interference Substances Studies:

The CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel uses conventional well-established nucleic acid extraction methods and based on our experience with CDC's other EUA assays, including the CDC Novel Coronavirus 2012 Real-time RT-PCR Assay for the presumptive detection of Middle East Respiratory Syndrome Coronavirus (MERS-CoV) and the CDC Human Influenza Virus Real-Time RT-PCR Diagnostic Panel-Influenza A/H7 (Eurasian Lineage) Assay for the presumptive detection of novel influenza A (H7N9) virus that are both intended for use with a number of respiratory specimens, we do not anticipate interference from common endogenous substances.

Specimen Stability and Fresh-frozen Testing:

To increase the likelihood of detecting infection, CDC recommends collection of lower respiratory and upper respiratory specimens for testing. If possible, additional specimen types (e.g., stool, urine) should be collected and should be stored initially until decision is made by CDC whether additional specimen sources should be tested. Specimens should be collected as soon as possible once a PUI is identified regardless of symptom onset. Maintain proper infection control when collecting specimens. Store specimens at 2-8°C and ship overnight to CDC on ice pack. Label each specimen container with the patient's ID number (e.g., medical record number), unique specimen ID (e.g., laboratory requisition number), specimen type (e.g., nasal swabs) and the date the sample was collected. Complete a CDC Form 50.34 for each specimen submitted.

Clinical Performance:

As of February 22, 2020, CDC has tested 2071 respiratory specimens from persons under investigation (PUI) in the U.S. using the CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel. Specimen types include bronchial fluid/wash, buccal swab, nasal wash/aspirate, nasopharyngeal swab, nasopharyngeal/throat swab, oral swab, sputum, oropharyngeal (throat) swab, swab (unspecified), and throat swab.

Table 8: Summary of CDC 2019-Novel Coronavirus (2019-nCoV) Real-Time RT-PCR Diagnostic Panel Data Generated by Testing Human Respiratory Specimens Collected from PUI Subjects in the U.S.

Specimen Type	2019 nCoV Negative	2019 nCoV Positive	Inconclusive	Invalid	Total
Bronchial fluid/wash	2	0	0	0	2
Buccal swab	5	1	0	0	6
Nasal wash/aspirate	6	0	0	0	6
Nasopharyngeal swab	927	23	0	0	950
Nasopharyngeal swab/throat swab	4	0	0	0	4
Oral swab	476	9	0	0	485
Pharyngeal (throat) swab	363	10	0	1	374
Sputum	165	5	0	0	170
Swab (unspecified)[1]	71	1	0	0	72
Tissue (lung)	2	0	0	0	2
Total	2021	49	0	1	2071

[1]Actual swab type information was missing from these upper respiratory tract specimens.

Two thousand twenty-one (2021) respiratory specimens of the 2071 respiratory specimens tested negative by the CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel. Forty-nine (49) of the 2071 respiratory specimens tested positive by the CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel. Only one specimen (oropharyngeal (throat) swab) was invalid. Of the 49 respiratory specimens that tested positive by the CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel, seventeen (17) were confirmed by genetic sequencing and/or virus culture (positive percent agreement = 17/17, 95% CI: 81.6%-100%)

During the early phase of the testing, a total of 117 respiratory specimens collected from 46 PUI subjects were also tested with two analytically validated real-time RT-PCR assays that target separate and independent regions of the nucleocapsid protein gene of the 2019-nCoV, N4 and N5 assays. The nucleocapsid protein gene targets for the N4 and N5 assays are different and independent from the nucleocapsid protein gene targets for the two RT-PCR assays included in the CDC 2019-nCoV Real-Time RT-

PCR Diagnostic Panel, N1 and N2. Any positive result from the N4 and/or the N5 assay was further investigated by genetic sequencing.

Performance of the CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel testing these 117 respiratory specimens was estimated against a composite comparator. A specimen was considered comparator negative if both the N4 and the N5 assays were negative. A specimen was considered comparator positive when the N4 and/or the N5 assay generated a positive result, and the comparator positive result(s) were further investigated and confirmed to be 2019-nCoV RNA positive by genetic sequencing.

Table 9: Percent Agreement of the CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel with the Composite Comparator

CDC 2019-nCoV Panel Result	**Composite Comparator Result**	
	Positive	Negative
Positive	13[1]	0
Inconclusive	0	0
Negative	0	104

[1]Composite comparator results were available for 13 of 49 CDC 2019-nCoV Panel positive specimens only.

Positive percent agreement = 13/13 = 100% (95% CI: 77.2% - 100%)
Negative percent agreement = 104/104 = 100% (95% CI: 96.4% - 100%)

Disposal

Dispose of hazardous or biologically contaminated materials according to the practices of your institution.

References

1. Ballew, H. C., *et al*. “Basic Laboratory Methods in Virology,” DHHS, Public Health Service 1975 (Revised 1981), Centers for Disease Control and Prevention, Atlanta, Georgia 30333.

2. Clinical Laboratory Standards Institute (CLSI), “Collection, Transport, Preparation and Storage of Specimens for Molecular Methods: Proposed Guideline,” MM13-A

3. Lieber, M., *et al*. "A Continuous Tumor Cell Line from a Human Lung Carcinoma with Properties of Type II Alveolar Epithelial Cells." *International Journal of Cancer* 1976, 17(1), 62-70.

Revision History

Revision #	Effective Date	Summary of Revisions
1	February 4, 2020	Original Instructions for Use
2	March 15, 2020	• Intended use update • Removal of N3 primer and probe set from Diagnostic Panel • Performance data update • Addition of alternative nucleic acid extraction platforms • Addition of acceptable alternatives to HSC and addition of QIAGEN RUO extraction reagents • Positive results no longer presumptive. No confirmation of positive results required

Contact Information, Ordering, and Product Support

For technical and product support, contact the CDC Division of Viral Diseases directly.

Send email to: respvirus@cdc.gov

Note: If your laboratory is using reagents sourced from someone other than the CDC International Reagent Resource, please refer to the manufacturer's instructions provided with the commercial materials.

DO NOT DISCARD: Important product-specific information

CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel

For use under EMERGENCY USE AUTHORIZATION (EUA) only.

Rx only

CATALOG: 2019-nCoV EUA-01

KIT LOT:

EXPIRATION DATE: YYYY-MM-DD (3 Years from DOM)

INTENDED USE

The CDC 2019-Novel Coronavirus (2019-nCoV) Real-Time RT-PCR Diagnostic Panel is a real-time RT-PCR test intended for the qualitative detection of nucleic acid from the 2019-nCoV in upper and lower respiratory specimens (such as nasopharyngeal or oropharyngeal swabs, sputum, lower respiratory tract aspirates, bronchoalveolar lavage, and nasopharyngeal wash/aspirate or nasal aspirate) collected from individuals who meet 2019-nCoV clinical and/or epidemiological criteria (for example, clinical signs and symptoms associated with 2019-nCoV infection, contact with a probable or confirmed 2019-nCoV case, history of travel to a geographic locations where 2019-nCoV cases were detected, or other epidemiologic links for which 2019-nCoV testing may be indicated as part of a public health investigation). Testing in the United States is limited to laboratories certified under the Clinical Laboratory Improvement Amendments of 1988 (CLIA), 42 U.S.C. § 263a, to perform high complexity tests.

Results are for the identification of 2019-nCoV RNA. The 2019-nCoV RNA is generally detectable in upper and lower respiratory specimens during infection. Positive results are indicative of active infection with 2019-nCoV but do not rule out bacterial infection or co-infection with other viruses. The agent detected may not be the definite cause of disease. Laboratories within the United States and its territories are required to report all positive results to the appropriate public health authorities.

Negative results do not preclude 2019-nCoV infection and should not be used as the sole basis for treatment or other patient management decisions. Negative results must be combined with clinical observations, patient history, and epidemiological information.

Testing with the CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel is intended for use by trained laboratory personnel who are proficient in performing real-time RT-PCR assays. The CDC 2019-Novel Coronavirus (2019-nCoV) Real-Time RT-PCR Diagnostic Panel is only for use under a Food and Drug Administration's Emergency Use Authorization .

PACKAGE CONTENTS

PACKAGING	COMPONENT	PART NUMBER	COMPONENT LOT NUMBER	VIALS PER KIT	QUANTITY /VIAL	STATE
Oligonucleotide Box	2019-nCoV_N1 Combined Primer/Probe Mix	RV202001		1	22.5 nmol	Dried
	2019-nCoV_N2 Combined Primer/Probe Mix	RV202002		1	22.5 nmol	Dried
	RP Combined Primer/Probe Mix	RV202004		1	22.5 nmol	Dried
Control Box	nCoVPC 2019-nCoV Positive Control (non-infectious)	RV202005		4	1×10^4 copies/µL	Dried

STORAGE INSTRUCTIONS

Upon receipt, store at 2-8°C. Refer to the CDC 2019-Novel Coronavirus (2019-nCoV) Real-Time RT-PCR Diagnostic Panel Instructions for Use before opening and preparing reagents for use.

PROCEDURE/INTERPRETATION/LIMITATIONS

Users should refer to the **CDC 2019-Novel Coronavirus (2019-nCoV) Real-Time RT-PCR Diagnostic Panel Instructions for Use** posted on the FDA website for all IVD products used under Emergency Use Authorization, http://www.fda.gov/MedicalDevices/Safety/EmergencySituations/ucm161496.htm.

IVD

PRECAUTIONS

This reagent should be handled in an approved BSL-2 handling area to avoid contamination of laboratory equipment and reagents that could cause false positive results. This product is non-infectious. However, this product should be handled in accordance with Good Laboratory Practices.

REAGENT COMPLAINTS/QUESTIONS

If you have a question/comment about this product, please contact the CDC Division of Viral Diseases/Respiratory Viruses Branch by email at respvirus@cdc.gov.

DISTRIBUTED BY

Manufactured by the Centers for Disease Control and Prevention, 1600 Clifton Road, Atlanta, Georgia, 30329, USA

IVD

*** *DO NOT DISCARD: Important product-specific information* ***

CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel – Verification Requirements

Please consult the following guidance from CMS regarding Emergency Use Authorized diagnostic tests: https://www.cms.gov/Medicare/Provider-Enrollment-and-Certification/SurveyCertificationGenInfo/Policy-and-Memos-to-States-and-Regions-Items/QSO18-19-CLIA

INTENDED USE

The CDC 2019-Novel Coronavirus (2019-nCoV) Real-Time RT-PCR Diagnostic Panel is a real-time RT-PCR test intended for the qualitative detection of nucleic acid from the 2019-nCoV in upper and lower respiratory specimens (such as nasopharyngeal or oropharyngeal swabs, sputum, lower respiratory tract aspirates, bronchoalveolar lavage, and nasopharyngeal wash/aspirate or nasal aspirate) collected from individuals who meet 2019-nCoV clinical and/or epidemiological criteria (for example, clinical signs and symptoms associated with 2019-nCoV infection, contact with a probable or confirmed 2019-nCoV case, history of travel to a geographic locations where 2019-nCoV cases were detected, or other epidemiologic links for which 2019-nCoV testing may be indicated as part of a public health investigation). Testing in the United States is limited to laboratories certified under the Clinical Laboratory Improvement Amendments of 1988 (CLIA), 42 U.S.C. § 263a, to perform high complexity tests.

Results are for the identification of 2019-nCoV RNA. The 2019-nCoV RNA is generally detectable in upper and lower respiratory specimens during infection. Positive results are indicative of active infection with 2019-nCoV but do not rule out bacterial infection or co-infection with other viruses. The agent detected may not be the definite cause of disease. Laboratories within the United States and its territories are required to report all positive results to the appropriate public health authorities.

Negative results do not preclude 2019-nCoV infection and should not be used as the sole basis for treatment or other patient management decisions. Negative results must be combined with clinical observations, patient history, and epidemiological information.

Testing with the CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel is intended for use by trained laboratory personnel who are proficient in performing real-time RT-PCR assays. The CDC 2019-Novel Coronavirus (2019-nCoV) Real-Time RT-PCR Diagnostic Panel is only for use under a Food and Drug Administration's Emergency Use Authorization.

REQUIRED MATERIALS

The 2019 novel coronavirus positive control (nCoVPC) is provided with the CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel and should be prepared according to the instructions for use. The nCoVPC consists of an RNA transcript of the 2019-nCoV N gene as well as human RNase P gene segment. nCoVPC will yield a positive result with the following primer and probe sets: 2019-nCoV_N1, 2019-nCoV_N2, and RP.

Approximately 2 mL of an upper respiratory specimen (e.g. nasopharyngeal swabs (NPS) in transport media) will be needed for testing. Specimens may be pooled if less than 2mL of one specimen is available.

Refer to CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel package insert (manufacturer instructions) for additional reagents, materials, and instructions.

PRECAUTIONS

This reagent should be handled in an approved BSL-2 handling area to avoid contamination of laboratory equipment and reagents that could cause false positive results. This product is an

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RNA transcript and is non-infectious. However, the nCoVPC should be handled in accordance with Good Laboratory Practices.

Store reagent at appropriate temperatures (see instructions for use) and hold on ice when thawed.

Please use standard precautions when handling respiratory specimens.

INSTRUCTIONS FOR PREPARING SAMPLES BEFORE EXTRACTION WITH THE QIAamp DSP VIRAL RNA MINI KIT OR THE QIAamp VIRAL RNA MINI KIT

- Refer to the 2019-nCoV Real-Time RT-PCR Diagnostic Panel instructions for use for reconstitution of the materials for use. RNA should be kept cold during preparation and use.
- Make a 1/10 dilution of nCoVPC by adding 5 µL of nCoVPC into 45 µL of nuclease-free water or 10 mM Tris
- Aliquot 560 µL of lysis buffer into each of nine tubes labeled 1-9.
- Add 140 µL of upper respiratory specimen (e.g. NPS in viral transport media) into each of the nine labeled tubes with lysis buffer
- To prepare samples at a moderate concentration, spike 14 µL of undiluted nCoVPC (rehydrated as described in the CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel instructions for use) into each tube labeled 1-3 containing lysis buffer and specimen
- To prepare samples at a low concentration, spike 14 µL of 1/10 dilution of nCoVPC into each tube labeled 4-6 containing lysis buffer and specimen
- To prepare negative samples, spike 14 µL of nuclease-free water into each tube labeled 7-9 containing lysis buffer and specimen
- Perform extractions of all nine samples according to the CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel instructions for use

INSTRUCTIONS FOR PREPARING SAMPLES BEFORE EXTRACTION WITH THE QIAGEN EZ1 ADVANCED XL

- Refer to the 2019-nCoV Real-Time RT-PCR Diagnostic Panel instructions for use for reconstitution of the materials for use. RNA should be kept cold during preparation and use.
- Make a 1/10 dilution of nCoVPC by adding 5 µL of nCoVPC into 45 µL of nuclease-free water or 10 mM Tris
- Aliquot 280 µL of lysis buffer into each of nine tubes labeled 1-9.
- Add 120 µL of upper respiratory specimen (e.g. NPS in viral transport media) into each of the nine labeled tubes with lysis buffer
- To prepare samples at a moderate concentration, spike 12 µL of undiluted nCoVPC (rehydrated as described in the CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel instructions for use) into each tube labeled 1-3 containing lysis buffer and specimen
- To prepare samples at a low concentration, spike 12 µL of 1/10 dilution of nCoVPC into each tube labeled 4-6 containing lysis buffer and specimen
- To prepare negative samples, spike 12 µL of nuclease-free water into each tube labeled 7-9 containing lysis buffer and specimen
- Perform extractions of all nine samples according to the CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel instructions for use

INSTRUCTIONS FOR PREPARING SAMPLES BEFORE EXTRACTION WITH THE ROCHE MagNA PURE TOTAL NUCLEIC ACID KIT OR THE ROCHE MagNA PURE NUCLEIC ACID ISOLATION KIT I

- Refer to the 2019-nCoV Real-Time RT-PCR Diagnostic Panel instructions for use for reconstitution of the materials for use. RNA should be kept cold during preparation and use.
- Make a 1/10 dilution of nCoVPC by adding 5 µL of nCoVPC into 45 µL of nuclease-free water or 10 mM Tris
- Aliquot 300 µL of lysis buffer into each of nine tubes labeled 1-9.
- Add 100 µL of upper respiratory specimen (e.g. NPS in viral transport media) into each of the nine labeled tubes with lysis buffer

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- To prepare samples at a moderate concentration, spike 12 µL of undiluted nCoVPC (rehydrated as described in the CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel instructions for use) into each tube labeled 1-3 containing lysis buffer and specimen
- To prepare samples at a low concentration, spike 12 µL of 1/10 dilution of nCoVPC into each tube labeled 4-6 containing lysis buffer and specimen
- To prepare negative samples, spike 12 µL of nuclease-free water into each tube labeled 7-9 containing lysis buffer and specimen
- Perform extractions of all nine samples according to the CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel instructions for use

INSTRUCTIONS FOR PREPARING SAMPLES BEFORE EXTRACTION WITH THE ROCHE MagNA PURE 96 DNA AND VIRAL NA SMALL VOLUME KIT

- Refer to the 2019-nCoV Real-Time RT-PCR Diagnostic Panel instructions for use for reconstitution of the materials for use. RNA should be kept cold during preparation and use.
- Make a 1/10 dilution of nCoVPC by adding 5 µL of nCoVPC into 45 µL of nuclease-free water or 10 mM Tris
- Aliquot 350 µL of lysis buffer into each of nine tubes labeled 1-9.
- Add 100 µL of upper respiratory specimen (e.g. NPS in viral transport media) into each of the nine labeled tubes with lysis buffer
- To prepare samples at a moderate concentration, spike 12 µL of undiluted nCoVPC (rehydrated as described in the CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel instructions for use) into each tube labeled 1-3 containing lysis buffer and specimen
- To prepare samples at a low concentration, spike 12 µL of 1/10 dilution of nCoVPC into each tube labeled 4-6 containing lysis buffer and specimen
- To prepare negative samples, spike 12 µL of nuclease-free water into each tube labeled 7-9 containing lysis buffer and specimen
- Perform extractions of all nine samples according to the CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel instructions for use

INSTRUCTIONS FOR PREPARING SAMPLES BEFORE EXTRACTION WITH THE BIOMÉRIEUX NucliSENS easyMAG OR THE BIOMÉRIEUX EMAG

- Refer to the 2019-nCoV Real-Time RT-PCR Diagnostic Panel instructions for use for reconstitution of the materials for use. RNA should be kept cold during preparation and use.
- Make a 1/10 dilution of nCoVPC by adding 5 µL of nCoVPC into 45 µL of nuclease-free water or 10 mM Tris
- Aliquot 1000 µL or 2000 µL of pre-aliquoted easyMAG lysis buffer into each of nine tubes labeled 1-9 for the easyMAG or eMAG, respectively.
- Add 100 µL of upper respiratory specimen (e.g. NPS in viral transport media) into each of the nine labeled tubes with lysis buffer
- To prepare samples at a moderate concentration, spike 12 µL of undiluted nCoVPC (rehydrated as described in the CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel instructions for use) into each tube labeled 1-3 containing lysis buffer and specimen
- To prepare samples at a low concentration, spike 12 µL of 1/10 dilution of nCoVPC into each tube labeled 4-6 containing lysis buffer and specimen
- To prepare negative samples, spike 12 µL of nuclease-free water into each tube labeled 7-9 containing lysis buffer and specimen
- Perform extractions of all nine samples according to the CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel instructions for use

PROCEDURE

Follow the CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel instructions for use for testing the 9 extracted samples at least once.

EXPECTED RESULTS

Moderate nCoVPC samples should be positive for 2019-nCoV.
Low nCoVPC samples should be positive for 2019-nCoV.
Negative upper respiratory samples should be negative for 2019-nCoV.

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≥ 90% of test results should be in agreement with the expected results. If test results are less than 90% in agreement with expected results, contact CDC at respvirus@cdc.gov.

QUESTIONS

Please send questions or comments by email to respvirus@cdc.gov.

DISTRIBUTION:

Distributed to qualified laboratories by Centers for Disease Control and Prevention, 1600 Clifton Road, Atlanta, GA, 30329 USA

Erratum: Nao, N., et al. Detection of second case of 2019-nCoV infection in Japan

The authors wish to make the following change to their report.

The reverse primer (NIID_2019-nCOV_N_R2) sequence should be replaced with TGGCAGCTGTGTA**G**GTCAAC. The corrected nucleotide is bold and underlined.

The authors apologize for any inconvenience this may cause.
The report will be updated and the corrected version of the report will be online.

Detection of second case of 2019-nCoV infection in Japan (corrected version)

[1]Naganori Nao, [1]Kazuya Shirato, [2]Harutaka Katano, [1]Shutoku Matsuyama, and [1]Makoto Takeda

[1]Laboratory of Acute Viral Respiratory Infections and Cytokines, Department of Virology III, National Institute of Infectious Diseases, 4-7-1 Gakuen, Musashimurayama, 208-0011 Tokyo, Japan: [2]Department of Pathology, National Institute of Infectious Diseases, 1-23-1 Toyama, Shinjuku, Tokyo 164-8640, Japan

Method & Results

1) Nested RT-PCR

Total RNA was extracted from pharyngeal swab using QIAamp viral RNA mini kit (Qiagen) following manufacture's instruction. First strand cDNA was synthesized using Super Script IV Reverse Transcriptase (Thermo) with random primer (Thermo) and oligodT primer (Thermo). PCR reaction was performed using Quick Taq HS Dyemix (TOYOBO, Japan) using two 2019-nCoV specific primers (Table 1). The PCR condition was as follows: 94°C for 1 min; 40 cycles of 94°C for 30 sec, 56°C for 30 sec, and 68°C for 1 min. After 1st PCR, nested PCR was performed using 2nd PCR primers and 1μL of 1st PCR product under the same condition as 1st PCR. The primer concentrations were 400nM for all. DDW was used as negative control. The amplicons were visualized by 2% agarose gel electrophoresis (Fig. 1). As the result, both primer sets detected desired size of bands. The rest of PCR products were purified with AMpure XP, and then direct sequencing analysis was performed using seq primers. The analyzed sequences showed 100% match with the sequence of WH-human1 (MN908947).

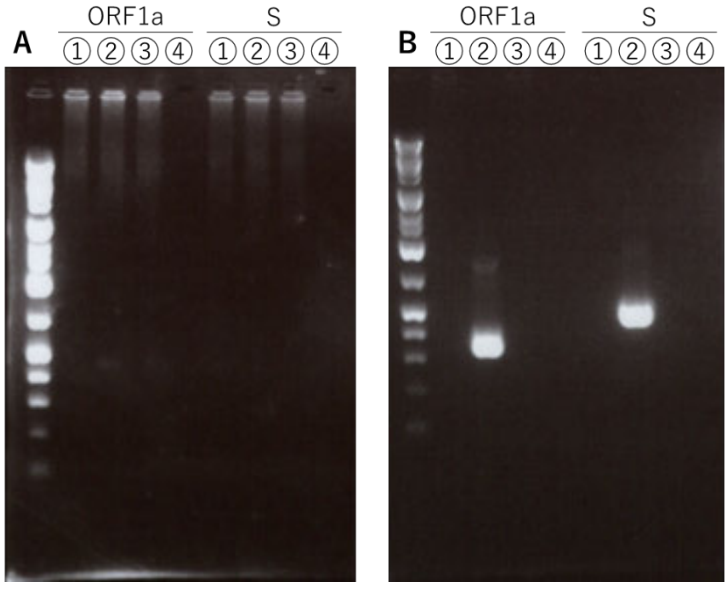


Figure 1. Three specimens were tested this time. Agarose gel electrophoresis of 1st (A) and 2nd (B) PCR reaction. DNA marker (Gene Ladder Wide 1, Nippon gene) was used as a reference for fragment size. Lane 1 and 3 were negative clinical samples, lane 2 was positive clinical sample, and lane 4 was negative control.

Table 1. Primer used for 2019-nCoV.

No.		Name	direction	sequence (5' to 3')	Expected size (bp)
ORF1a set					
1	1st	NIID_WH-1_F501	Sense	TTCGGATGCTCGAACTGCACC	413
2	1st	NIID_WH-1_R913	Antisense	CTTTACCAGCACGTGCTAGAAGG	
3	2nd	NIID_WH-1_F509	Sense	CTCGAACTGCACCTCATGG	346
4	2nd	NIID_WH-1_R854	Antisense	CAGAAGTTGTTATCGACATAGC	
5	Seq	NIID_WH-1_Seq_F519	Sense	ACCTCATGGTCATGTTATGG	
6	Seq	NIID_WH-1_Seq_R840	Antisense	GACATAGCGAGTGTATGCC	
S set					
7	1st	WuhanCoV-spk1-f	Sense	TTGGCAAAATTCAAGACTCACTTT	547
8	1st	WuhanCoV-spk2-r	Antisense	TGTGGTTCATAAAAATTCCTTTGTG	
9	2nd	NIID_WH-1_F24381	Sense	TCAAGACTCACTTTCTTCCAC	493
10	2nd	NIID_WH-1_R24873	Antisense	ATTTGAAACAAAGACACCTTCAC	
11	Seq	NIID_WH-1_Seq_F24383	Sense	AAGACTCACTTTCTTCCACAG	
12	Seq	NIID_WH-1_Seq_R24865	Antisense	CAAAGACACCTTCACGAGG	

2) Real-time RT-PCR

Real-time one step RT-PCR was performed using QuantiTect Probe RT-PCR Kit (Qiagen) on LightCycler96 system (Roche). A 20μL of reaction contains 10 μL of 2×Master Mix, 0.2 μL of RT mix, 3.8 μL of DDW, 1 μL of pre-diluted 20 ×primer and probe mix, and 5μL of extracted RNA. The PCR condition was as follows: 50°C for 30 min; and 95°C for 15 min; and 40 cycles of 95°C for 15 sec and 60°C for 1 min. The synthesized RNA, which contained artificial sequences that could distinguish the laboratory contamination, was used as positive control. DDW was used as negative control. The reaction was performed in duplicate, and both positives within 40 cycles were considered as positive. As the result, the average Cq value of specimen was 36.7, and that of positive control

(500 copies) was 35.0. Negative controls showed no signals. Therefore, the specimen could be considered as positive.

Table 2. Primers and probe sequence for real-time RT-PCR

Primer	Sequence (5' to 3')	Position (MN908947.1)	Concentration
NIID_2019-nCOV_N_F2	AAATTTTGGGGACCAGGAAC	29142-29161	500 nM
NIID_2019-nCOV_N_R2	TGGCAGCTGTGTAGGTCAAC	29299-29280	700 nM
NIID_2019-nCOV_N_P2	FAM-ATGTCGCGCATTGGCATGGA-BHQ	29239-29258	200 nM

Diagnostic detection of 2019-nCoV by real-time RT-PCR

-Protocol and preliminary evaluation as of Jan 17, 2020-

Victor Corman, Tobias Bleicker, Sebastian Brünink, Christian Drosten
Charité Virology, Berlin, Germany

Olfert Landt, Tib-Molbiol, Berlin, Germany

Marion Koopmans
Erasmus MC, Rotterdam, The Netherlands

Maria Zambon
Public Health England, London

Additional advice by Malik Peiris, University of Hong Kong

Users looking for a workflow protocol consult the last three pages of this document

Contact: christian.drosten@charite.de
https://virologie-ccm.charite.de/en/

Positive control material is available from Charité, Berlin, via EVAg (https://www.european-virus-archive.com/).

This is document Version 2.
Changes against Version 1 (Jan 13, 2019): Workflow protocols included, N gene assay removed, data for single probe versions of RdRp assay added; information on availability of controls updated.

We acknowledge the originators of sequences in GISAID (www.gisaid.org): National Institute for Viral Disease Control and Prevention, China, Institute of Pathogen Biology, Chinese Academy of Medical Sciences, Peking Union Medical College, China, and Wuhan Jinyintan Hospital Wuhan Institute of Virology, Chinese Academy of Sciences, China). We acknowledge Professor Yong-Zhen Zhang, Shanghai Public Health Clinical Center & School of Public Health, Fudan University, Shanghai, China for release of another sequence (MN908947).

We use the term “SARS-related Coronavirus” to include the SARS virus as well as the clade of betacoronaviruses known to be associated with (mainly) rhinolophid bats across the Palearctic. The latest taxonomy classifies these viruses in a subgenus termed *Sarbecovirus*.

Background
We used known SARS- and SARS-related coronaviruses (bat viruses from our own studies as well as literature sources) to generate a non-redundant alignment (excerpts shown in Annex). We designed candidate diagnostic RT-PCR assays before release of the first sequence of 2019-nCoV. Upon sequence release, the following assays were selected based on their matching to 2019-nCoV as per inspection of the sequence alignment and initial evaluation (Figures 1 and 2).

All assays can use SARS-CoV genomic RNA as positive control. Synthetic control RNA for 2019-nCoV E gene assay is available via EVAg. Synthetic control for 2019-nCoV RdRp is expected to be available via EVAg from Jan 21st onward.

First line screening assay: E gene assay
Confirmatory assay: RdRp gene assay

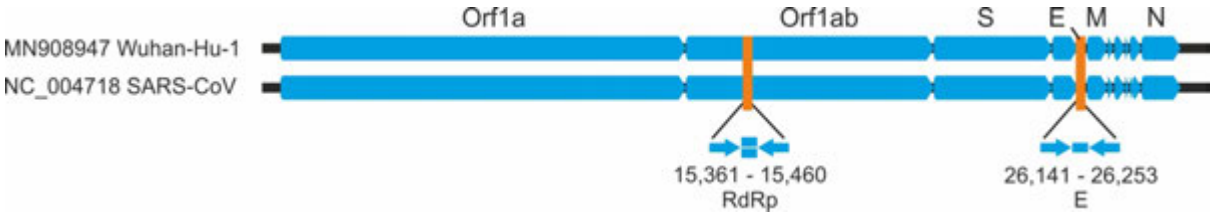


Figure 1 relative positions of amplicon targets on SARS-CoV an 2019-nCoV genome. ORF: open reading frame; RdRp: RNA-dependent RNA polymerase. Numbers below amplicon are genome positions according to SARS-CoV, NC_004718.

Materials and assay formulation

Clinical samples and CoV cell culture supernatants
Respiratory samples were obtained during 2019 from patients hospitalized at Charité medical center and tested by the NxTAG® Respiratory Pathogen Panel (Luminex) or in cases of MERS-CoV by the MERS-CoV upE assay as published before (1).

Cell culture supernatants from typed coronaviruses were available at our research and clinical laboratories. The typed avian influenza virus RNA (H5N1) was obtained from the German Society for Promotion of Quality Assurance in Medical Laboratories (INSTAND) proficiency testing panels. RNA was extracted from clinical samples by using the MagNA Pure 96 system (Roche) and from cell culture supernatants by the viral RNA mini kit (Qiagen).

Assay design
For oligonucleotide design and in-silico evaluation we downloaded all complete and partial (if >400 nucleotides) SARS-related virus sequences available at GenBank by January 1st, 2020. The list (n=729 entries) was manually checked and artificial sequences (lab-derived, synthetic etc.), as well as sequence duplicates removed, resulting in a final list of 375

sequences. These sequences were aligned and the alignment used for assay design. The alignment was later complemented by sequences released from the Wuhan cluster. All presently release sequences match the amplicons (Figure 2). An overview of oligonucleotide binding sites in all unique sequences of bat-associated SARS-related viruses is shown in the appendix.

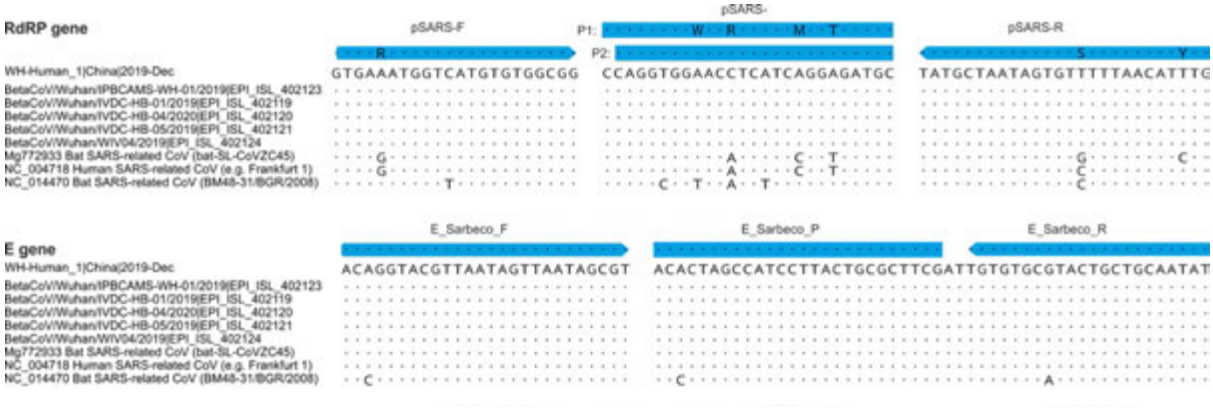


Figure 2 Partial alignments of oligonucleotide binding regions. Panels show six available sequences of 2019-nCoV, aligned to the corresponding partial sequences of SARS-CoV strain Frankfurt 1, which can be used as a positive control for all three RT-PCR assays. The alignment also contains the most closely-related bat virus (Bat SARS-related CoV isolate bat-SL-CoVZC45, GenBank Acc.No. MG772933.1) as well as the most distant member within the SARS-related bat CoV clade, detected in Bulgaria (GenBank Acc. No. NC_014470). Dots represent identical nucleotides compared to sequence Wuhan-Hu 1. Substitutions are specified. More comprehensive alignments in the Appendix.

Real-time reverse-transcription polymerase chain reaction

All assays used the same conditions. Primer and probe sequences, as well as optimized concentrations are shown in Table 1. A 25-µl reaction was set up containing 5 µl of RNA, 12.5 µl of 2 X reaction buffer provided with the Superscript III one step RT-PCR system with Platinum Taq Polymerase (Invitrogen; containing 0.4 mM of each deoxyribonucleotide triphosphates (dNTP) and 3.2 mM magnesium sulfate), 1 µl of reverse transcriptase/Taq mixture from the kit, 0.4 µl of a 50 mM magnesium sulfate solution (Invitrogen – not provided with the kit), and 1 µg of nonacetylated bovine serum albumin (Roche). All oligonucleotides were synthesised and provided by Tib-Molbiol, Berlin. Thermal cycling was performed at 55°C for 10 min for reverse transcription, followed by 95°C for 3 min and then 45 cycles of 95°C for 15 s, 58°C for 30 s.

Table 1. Primers and probes
Optimized concentrations are mol per liter of final reaction mix.
(e.g., 1.5 microliters of a 10 micromolar (uM) primer stock solution per 25 microliter (ul) total reaction volume yields a final concentration of 600 nanomol per liter (nM) as indicated in the table)
-note that standard, non-optimized reaction conditions as indicated by suppliers of one-step RT-PCR kits will generally yield sufficient sensitivity-

Assay/ Use	Oligonucleotide ID	Sequence (5'–3')	Comment
RdRP gene	RdRP_SARSr-F2	GTGARATGGTCATGTGTGGCGG	use 600 nM per reaction
	RdRP_SARSr-R1	CARATGTTAAASACACTATTAGCATA	use 800 nM per reaction
	RdRP_SARSr-P2	FAM-CAGGTGGAACCTCATCAGGAGATGC-BBQ	Specific for 2019-nCoV, will not detect SARS-CoV use 100 nM per reaction and mix with P1
	RdRP_SARSr-P1	FAM-CCAGGTGGWACRTCATCMGGTGATGC-BBQ	Pan Sarbeco-Probe, will detect 2019-nCoV, SARS-CoV and bat-SARS-related CoVs use 100 nM per reaction and mix with P2
E gene	E_Sarbeco_F1	ACAGGTACGTTAATAGTTAATAGCGT	use 400 nM per reaction
	E_Sarbeco_R2	ATATTGCAGCAGTACGCACACA	use 400 nM per reaction
	E_Sarbeco_P1	FAM-ACACTAGCCATCCTTACTGCGCTTCG-BBQ	use 200 nM per reaction

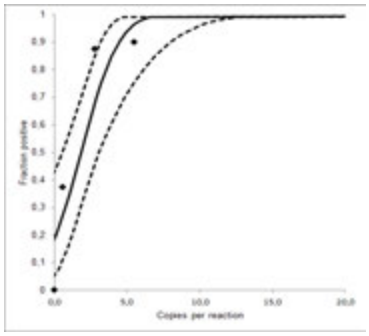
W is A/T; R is G/A; M is A/C ; FAM, 6-carboxyfluorescein; BBQ, blackberry quencher

Technical sensitivity testing

Preliminary assessment of analytical sensitivity for RdRp assay.

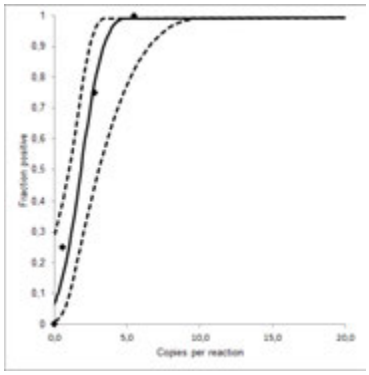
We tested purified cell culture supernatant containing SARS-CoV strain Frankfurt-1 virions grown on Vero cells, and quantified by real-time RT-PCR assay as described in Drosten et al. (2) using a specific *in-vitro* transcribed RNA quantification standard. The results are shown in Figure 3. All assays are highly sensitive.

A



A. First line assay: E gene
Technical limit of detection (LOD) = 5.2 RNA copies/reaction, at 95% hit rate; 95% CI: 3.7-9.6 RNA copies/reaction.

B



B. Confirmatory assay: RdRP gene
Technical LOD = 3.8 RNA copies/reaction, at 95% hit rate; 95% CI: 2.7-7.6 RNA copies/reaction.

Figure 3. A, E-gene assay, B, RdRp gene assay. X-axis shows input RNA copies per reaction. Y-axis shows positive results in all parallel reactions performed, squares are experimental data points resulting from replicate testing of given concentrations (x-axis) in parallels assays (8 replicate reactions per datum point). The inner line is a probit curve (dose-response rule). The outer dotted lines are 95% confidence intervals.

RdRp assay sensitivity with single probe application using the assay variant that only contains the 2019-nCoV specific probe.

SARS 2019-nCoV

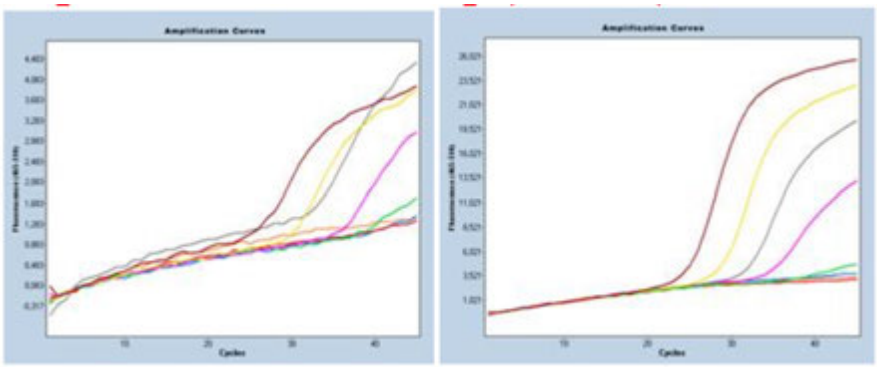


Figure 4. Preliminary experiment comparing single probe assay for SARS-CoV (probe RdRP_SARSr-P1, left panel) with single probe assay for 2019-nCoV (probe RdRP_SARSr-P2, right panel). Note that the fluorescent signal in these assays is suboptimal due to the use of PCR-generated targets.

Breadth of detection

To show that the assays will detect other bat-associated SARS-related viruses, we tested bat-derived fecal samples available from Drexler et al., (3) und Muth et al., (4) using the novel assays.

KC633203, Betacoronavirus BtCoV/Rhi_eur/BB98-98/BGR/2008
KC633204, Betacoronavirus BtCoV/Rhi_eur/BB98-92/BGR/2008
KC633201, Betacoronavirus BtCoV/Rhi_bla/BB98-22/BGR/2008
GU190221 Betacoronavirus Bat coronavirus BR98-19/BGR/2008
GU190222 Betacoronavirus Bat coronavirus BM98-01/BGR/2008
GU190223, Betacoronavirus Bat coronavirus BM98-13/BGR/2008

All samples were successfully tested positive by the E gene assay. Detection of these relatively distant members of the SARS-related CoV clade suggests that all Asian viruses are likely to be detected.

Specificity testing

1. Chemical stability
To exclude non-specific reactivity of oligonucleotides among each other, both assays were tested 40 times in parallel with water and no other nucleic acid except the provided oligonucleotides. In none of these reactions was any positive signal detected.

2. Cross-reactivity with other coronaviruses
Cell culture supernatants containing human coronaviruses (HCoV)-229E, -NL63, -OC43, and -HKU1 as well as MERS-CoV were tested in all three assays (Table 2). For the non-cultivable HCoV-HKU1, supernatant from human airway culture was used. Virus RNA concentration in all samples was determined by specific real-time RT-PCRs and in-vitro transcribed RNA standards designed for absolute viral load quantification.

Table 2. Cell-culture supernatants tested by all assays

Cell culture supernatants	Tested concentration	Result
Alphacoronaviruses		
Human coronavirus NL63	4x10^9 RNA copies/ml	No reactivity with any of three assays
Human coronavirus 229E	3x10^9 RNA copies/ml	No reactivity with any of three assays
Betacoronaviruses		
Betacoronavirus 1 (strain HCoV-OC43)	1x10^10 RNA copies/ml	No reactivity with any of three assays
Human coronavirus HKU1 (HCOV-HKU1)	1x10^5 RNA copies /ml	No reactivity with any of three assays
Middle East respiratory syndrome-related coronavirus (strain EMC/2012)	1x10^8 RNA copies/ml	No reactivity with any of three assays

3. Tests of human clinical samples previously tested to contain respiratory viruses

Both assays were applied on human clinical samples from our own diagnostic services, previously tested positive for the viruses listed in Table 3. All tests returned negative results.

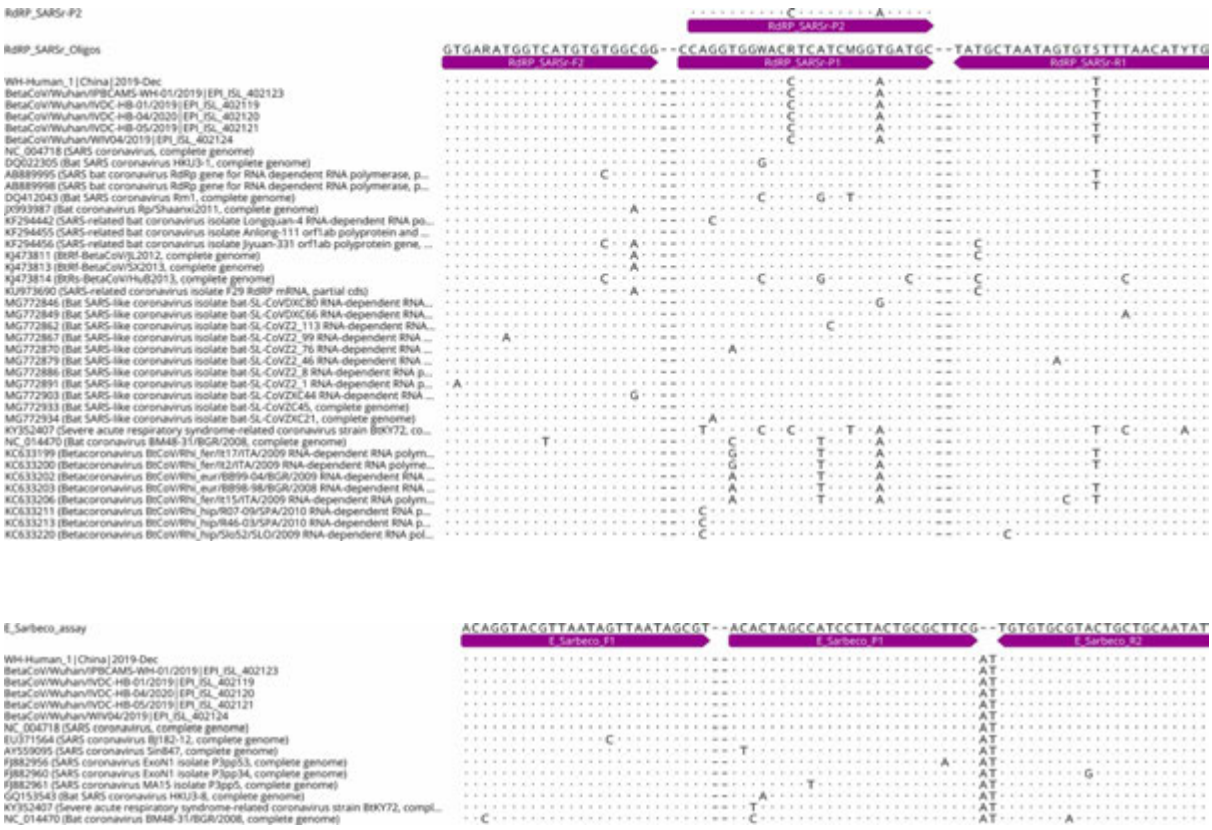
Table 3. Tests of known respiratory viruses and bacteria in clinical samples

Clinical samples with known viruses	**Number of samples tested**
HCoV-HKU1	2
HCoV-OC43	5
HCoV-NL63	5
HCoV-229E	5
MERS-CoV	5
Influenza A (H1N1/09)	6
Influenza A (H3N2)	5
Influenza A(H5N1)	1
Influenza B	3
Rhinovirus/Enterovirus	3
Respiratory syncytial virus (A/B)	6
Parainfluenza 1 virus	3
Parainfluenza 2 virus	3
Parainfluenza 3 virus	3
Parainfluenza A or -B virus	5
Human metapneumovirus	3
Adenovirus	3
Human Bocavirus	3
Legionella spp.	3
Mycoplasma spp.	3
Total clinical samples	75

References

1. Corman VM, Eckerle I, Bleicker T, Zaki A, Landt O, Eschbach-Bludau M, et al. Detection of a novel human coronavirus by real-time reverse-transcription polymerase chain reaction. Euro Surveill. 2012;17(39).
2. Drosten C, Gunther S, Preiser W, van der Werf S, Brodt HR, Becker S, et al. Identification of a novel coronavirus in patients with severe acute respiratory syndrome. N Engl J Med. 2003;348(20):1967-76.
3. Drexler JF, Gloza-Rausch F, Glende J, Corman VM, Muth D, Goettsche M, et al. Genomic characterization of severe acute respiratory syndrome-related coronavirus in European bats and classification of coronaviruses based on partial RNA-dependent RNA polymerase gene sequences. J Virol. 2010;84(21):11336-49.
4. Muth D, Corman VM, Roth H, Binger T, Dijkman R, Gottula LT, et al. Attenuation of replication by a 29 nucleotide deletion in SARS-coronavirus acquired during the early stages of human-to-human transmission. Sci Rep. 2018;8(1):15177.

Annex:



Annex figure. Non-redundant alignments of SARS-related CoVs focused on oligonucleotide binding sites of all assays (top to bottom: RdRp, E, N). Viruses not present in these alignments have been removed because their binding sites are 100% identical to one of the members of the alignment. ("--") means sequence gaps not covered by oligonucleotides. Note that these alignments contain only one sequence of 2019-nCoV while Figure 2 above contains all presently released sequences. We will fuse this into one figure.

Workflow Protocol

1. First line screening assay

E assay:

MasterMix:	**Per reaction**	
H_2O (RNAse free)	2.6 µl	
2x Reaction mix*	12.5 µl	
$MgSO_4$(50mM)	0.4 µl	
BSA (1 mg/ml)**	1 µl	
Primer E_Sarbeco_F1 (10 µM stock solution)	1 µl	ACAGGTACGTTAATAGTTAATAGCGT
Primer E_Sarbeco_R2 (10 µM stock solution)	1 µl	ATATTGCAGCAGTACGCACACA
Probe E_Sarbeco_P1 (10 µM stock solution)	0.5 µl	FAM-ACACTAGCCATCCTTACTGCGCTTCG-BBQ
SSIII/Taq EnzymeMix*	1 µl	
Total reaction mix	20 µl	
Template RNA, add	5 µl	
Total volume	25 µl	

* Thermo Fischer/Invitrogen: SuperScriptIII OneStep RT-PCR System with Platinum® Taq DNA Polymerase
** MgSO4 (50 mM) [Sigma], This component is not provided with the OneStep RT-PCR kit
*** non-acetylated [Roche].

Cycler:

55°C 10’
94°C 3’
94°C 15’’ | 45x
58°C 30’’ |

If assay No 1 is positive, continue to assay No 2.

2. Confirmatory assay

RdRp assay:

MasterMix:	**Per reaction**	
H_2O (RNAse free)	0.6 µl	
2x Reaction mix*	12.5 µl	
$MgSO_4$(50mM)	0.4 µl	
BSA (1 mg/ml)**	1 µl	
Primer RdRP_SARSr-F2 (10 µM stock solution)	1.5 µl	GTGARATGGTCATGTGTGGCGG
Primer RdRP_SARSr-R1 (10 µM stock solution)	2 µl	CARATGTTAAASACACTATTAGCATA
Probe RdRP_SARSr-P1 (10 µM stock solution)	0.5 µl	FAM-CCAGGTGGWACRTCATCMGGTGATGC-BBQ
Probe RdRP_SARSr-P2 (10 µM stock solution)	0.5 µl	FAM-CAGGTGGAACCTCATCAGGAGATGC-BBQ
SSIII/Taq EnzymeMix*	1 µl	
Total reaction mix	20 µl	
Template RNA, add	5 µl	
Total volume	25 µl	

* Thermo Fischer/Invitrogen: SuperScriptIII OneStep RT-PCR System with Platinum® Taq DNA Polymerase
** MgSO4 (50 mM) [Sigma], This component is not provided with the OneStep RT-PCR kit
*** non-acetylated [Roche].

Cycler:

55°C 10’
94°C 3’
94°C 15’’
58°C 30’’ | 45x

If assay No 2 is positive, continue to assay No 3.

3. Discriminatory assay

RdRp assay:

MasterMix:	**Per reaction**	
H_2O (RNAse free)	1.1 µl	
2x Reaction mix*	12.5 µl	
$MgSO_4$(50mM)	0.4 µl	
BSA (1 mg/ml)**	1 µl	
Primer RdRP_SARSr-F2 (10 µM stock solution)	1.5 µl	GTGARATGGTCATGTGTGGCGG
Primer RdRP_SARSr-R1 (10 µM stock solution)	2 µl	CARATGTTAAASACACTATTAGCATA
Probe RdRP_SARSr-P2 (10 µM stock solution)	0.5 µl	FAM-CAGGTGGAACCTCATCAGGAGATGC-BBQ
SSIII/Taq EnzymeMix*	1 µl	
Total reaction mix	20 µl	
Template RNA, add	5 µl	
Total volume	25 µl	

* Thermo Fischer/Invitrogen: SuperScriptIII OneStep RT-PCR System with Platinum® Taq DNA Polymerase
** MgSO4 (50 mM) [Sigma], This component is not provided with the OneStep RT-PCR kit
*** non-acetylated [Roche].

Cycler:

55°C 10’
94°C 3’
94°C 15’’ | 45x
58°C 30’’ |

Assay No 3 is specific for 2019-nCoV

Note: Other generic real-time RT-PCR reagents can be used for all assays. In this case, use oligonucleotides at concentrations indicated. If using Light Cycler instrument with glass capillaries, use Light Cycler-specific reagents or add BSA as indicated in the detailed documentation above.

HKU Med LKS Faculty of Medicine School of Public Health 香港大學公共衞生學院

Detection of 2019 novel coronavirus (2019-nCoV) in suspected human cases by RT-PCR

This protocol is designed to detect 2019-nCoV in human clinical specimens. The two monoplex assays described here are reactive with coronaviruses under the subgenus *Sarbecovirus* that includes 2019-nCoV, SARS-CoV and bat SARS-like coronaviruses. The rationales for using this detection approach are: 1) the genetic diversity of 2019-nCoV in humans and animals is yet to be fully determined and 2) many laboratories lack positive controls for 2019-nCoV. ***Viral RNA extracted from SARS-CoV can be used a positive control in the assays below.*** As SARS was eliminated in humans, suspected cases that are positive in these RT-PCR assays should be considered to be infected by the 2019-nCoV. The N gene RT-PCR is recommended as a screening assay and the Orf1b assay as a confirmatory one. In the event of a positive PCR result, sequence analyses of the amplicons will further help to confirm the result and to distinguish between SARS-CoV and 2019-nCoV. An N gene positive/Orf1b negative result should be regarded as indeterminate and the case is recommended to be referred to a WHO reference lab for further testing.

These assays have been evaluated using a panel of controls and only the positive control (SARS-CoV RNA) is tested positive in these assays. NB. Synthetic oligonucleotide positive controls or equivalents for 2019-nCoV is not available at present but will be available shortly.

Suitable biosafety precautions should be taken for handling human clinical specimens suspected to be 2019-nCoV infections (https://www.who.int/health-topics/coronavirus/laboratory-diagnostics-for-novel-coronavirus).

Materials required

- QIAamp Viral RNA Mini Kit (QIAGEN, Cat#52906) or equivalent
- TaqMan Fast Virus Master mix (TheromFisher, Cat# 4444432)
- Ethanol (96–100%)
- MicroAmp Fast Optical 96-well reaction plate (TheromFisher, Cat# 4346907)
- MicroAmp optical adhesive film (TheromFisher, Cat# 4311971)
- Microcentrifuge (adjustable, up to 13 000 rpm)
- Adjustable pipettes (10, 20, 100, 200 µl)
- Sterile, RNase-free pipette tips with aerosol barrier
- Vortex

- Microcentrifuge tubes (0.5ml and 1.5 ml)
- Thermocycler (TheromFisher, ViiA™ 7 Real-Time PCR)
- Positive control (Available from HKU, e-mail: llmpoon@hkucc.hku.hk)
- Primer sets

Primer and probe sequences

Assay 1 (Target: ORF1b-nsp14)

Forward primer (HKU-ORF1b-nsp14F): 5'-TGGGGYTTTACRGGTAACCT-3'

Reverse primer (HKU- ORF1b-nsp14R): 5'-AACRCGCTTAACAAAGCACTC-3'

Probe (HKU-ORF1b-nsp141P): 5'-FAM-TAGTTGTGATGCWATCATGACTAG-TAMRA-3'

Assay 2 (Target: N)

Forward primer (HKU-NF): 5'-TAATCAGACAAGGAACTGATTA-3'

Reverse primer (HKU-NR): 5'-CGAAGGTGTGACTTCCATG-3'

Probe (HKU-NP): 5'-FAM-GCAAATTGTGCAATTTGCGG-TAMRA-3'

Procedures

1. Extract viral RNA from clinical specimens by using QIAamp viral RNA mini kit according to manufacturer's instructions.

2. Prepare master mixture for one-step monoplex RT-PCR as below:

Reagent	**Vol for a single rxn (µl)**
H_2O (RNase free)	8.5
4x Reaction mix*	5
Forward primer (10 µM)	1
Reverse primer (10 µM)	1
Probe (10 µM)	0.5
RNA sample	4
Final rxn volume	20

*Reaction mix from TaqMan Fast Virus Master mix

3. Set the follow RT-PCR conditions*:

Temperature (°C)	Time (minute:second)	No. of cycle
50 95	5:00 0:20	1
95 60	0:05 0:30	40

*Both monoplex assays can be conducted under the same conditions.

Evaluation:
Positive controls: The tests were evaluated using serially diluted RNA samples extracted from SARS-CoV infected cells. These assays are confirmed to have a wide dynamic range (2^{-4}-2000 $TCID_{50}$/reaction, an amplification plot is shown an example). Upper respiratory and sputum samples spiked with SARS-CoV are shown to be positive in the test.

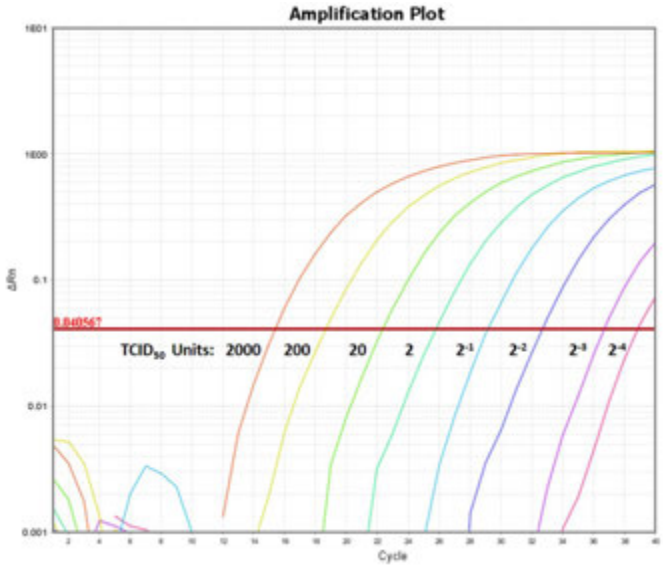


Figure. Amplification plot of the RT-PCR assay specific for N gene.
The viral titre ($TCID^{50}$) used in each reaction is shown as indicated.

Exclusivity: RNA extracted from respiratory cultured viruses and clinical samples (as described below) were included in the exclusivity panel. The assay yielded negative results against all of these preparations:

- RNA extracted from cultured viruses: human coronaviruses (229E, OC43 and MERS), camel coronavirus (HKU23), human influenza A viruses (H1N1, H3N2, H5N1 and H7N9 subtypes),

avian influenza (H1, H4, H6 and H9 subtypes), influenza B viruses (Yamagata and Victoria lineages), and adenovirus.

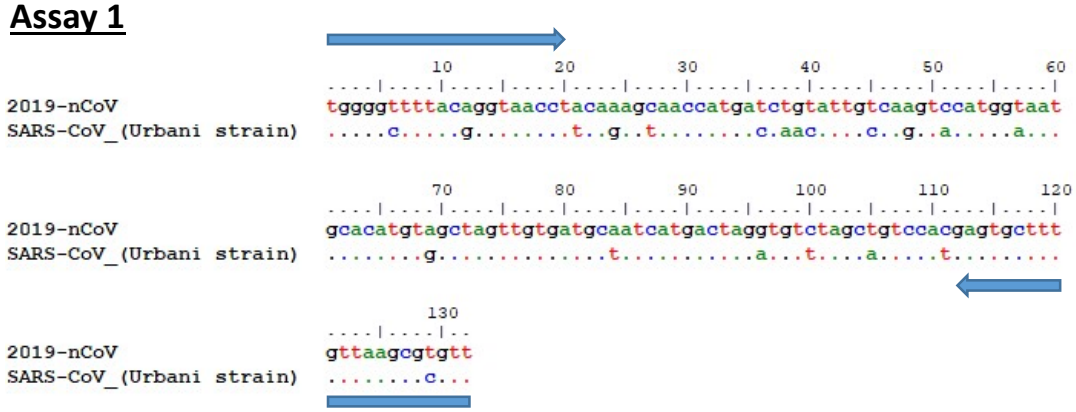
- RNA from retrospective human clinical specimens previously tested positive for other infections: coronavirus (229E, HKU1, NL63, OC43), influenza A viruses (H1N1 and H3N2 subtypes), influenza B viruses (Yamagata and Victoria lineages), adenovirus, enterovirus, human parainfluenza virus (PIV3), respiratory syncytial virus, human metapneumovirus, rhinovirus and human bocavirus.
- RNA from control human clinical specimens: Upper respiratory and sputum samples.

Remarks:

- The protocol is prepared by School of Public Health, The University of Hong Kong, Hong Kong (Leo Poon, Daniel Chu and Malik Peiris). For enquiry, please contact Leo Poon (llmpoon@hku.hk) or Malik Peiris (malik@hku.hk).
- Positive controls for the above assays may be available upon request.
- The amplicon sizes of Assay 1 and Assay 2 are 132 bp and 110 bp, respectively.
- A manual pan-coronavirus nested RT-PCR can detect a wide range of coronaviruses (J Virol. 85:12815-20). The identity of amplified DNA product can be confirmed by DNA sequencing.
- Primer-probe sets that are specific for 2019-nCoV are currently under evaluation. Please visit the nCoV laboratory website of WHO at https://www.who.int/health-topics/coronavirus/laboratory-diagnostics-for-novel-coronavirus.
- We encourage other labs to validate the described assay and share relevant finding with us.

Appendix 1: Sequence alignment of amplicons derived from 2019-nCoV and SARS-CoV

Assay 1



Assay 2

This protocol was kindly developed and provided by

Department of Medical Sciences, Ministry of Public Health, Thailand.

This document contains RT-PCR protocol for the detection of 2019-nCoV.

Diagnostic detection of Novel coronavirus 2019 by Real time RT-PCR

Materials & Methods

1.1 Macherey-Nagel Nucleospin RNA virus (Cat. No 740956)
1.2 Invitrogen superscript™ III Platinum One-Step Quantitative (Cat No. 11732-020 or 11732-088)

Primer	Sequence (5′ → 3′)	Working conc.
WH-NIC N-F	CGTTTGGTGGACCCTCAGAT	40 µM
WH-NIC N-R	CCCCACTGCGTTCTCCATT	40 µM
WH-NIC N-P	FAM-CAACTGGCAGTAACCA-BQH1	10 µM

Real-time RT-PCR Set-up Procedure

Place your samples on ice. Follow the procedure below to prepare the RT-PCR Master Mix.

a. Prepare the Master Mix as shown in the table below.
b. Pipette 20 µl of the Master Mix into each required reaction tubes/plate.
c. Add 5 µl isolated RNA or 5 µl the controls (Positive Control or Blank Control).
d. Make sure that every run including at least one Positive Control and one Blank Control.
e. Cap or seal the reaction tubes/plate and centrifuge using an appropriate centrifuge for 30 seconds at approximately 2,000 rpm.
f. Ensure that all liquid is at the bottom of the tubes/plate.
g. Perform the following protocol in the instrument.

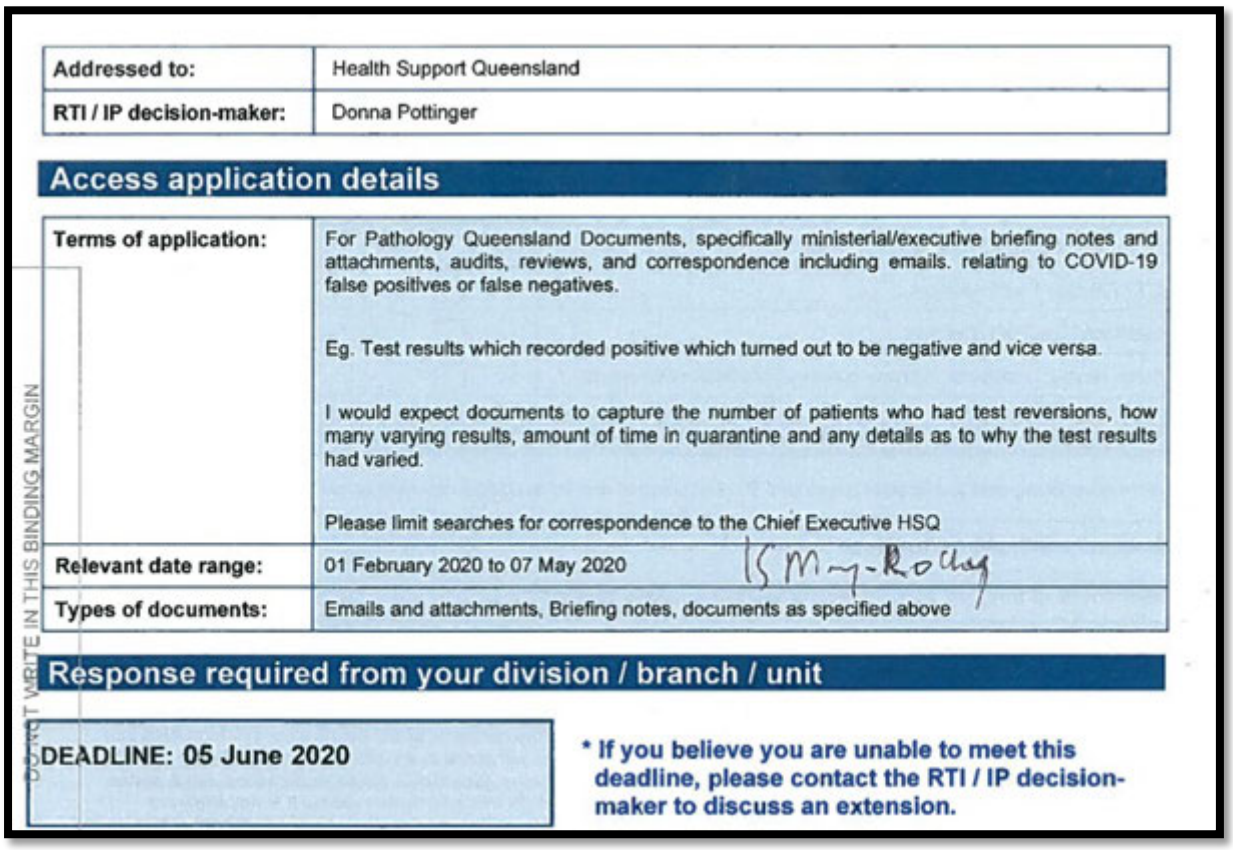
Reagents	microliter/r	Thermal cycler condition

	eaction			
Rnase free H_2O	5.5	**Round**	**Temp.**	**Time**
2x PCR Master Mix	12.5	1X	50 ^{0}C	30 min.
Forward primer (40 µM)	0.5	1X	95^{0}C	2 min.
Reverse primer (40 µM)	0.5	45X	95^{0}C	15sec.
Probe (10 µM)	0.5		55^{0}C**	30sec.**
Superscript –Tag Mix	0.5			
Total	20.0	** Fluorescence data (FAM) collect		
Add RNA	5			

Data Analysis and Interpretation

- Threshold setting : Above the maximum level of Blank Control
- Quality control : Prior to evaluating the specimen results, the Positive Control and Blank Control should be interpreted. Negative control should be undetected. Positive control should be detected with Ct ≤ 38
- The Positive Control and Blank Control should be included per PCR run.
- If the Positive Control and Blank Control do not meet the criteria, the entire run is invalid and results should not be reported. Repeat the entire process (specimen and control preparation, amplification and detection).
- Viral transport media or negative specimen can be used as a negative control.

PQ Response



Addressed to:	Health Support Queensland
RTI / IP decision-maker:	Donna Pottinger

Access application details

Terms of application:	For Pathology Queensland Documents, specifically ministerial/executive briefing notes and attachments, audits, reviews, and correspondence including emails. relating to COVID-19 false positives or false negatives. Eg. Test results which recorded positive which turned out to be negative and vice versa. I would expect documents to capture the number of patients who had test reversions, how many varying results, amount of time in quarantine and any details as to why the test results had varied. Please limit searches for correspondence to the Chief Executive HSQ
Relevant date range:	01 February 2020 to 07 May 2020
Types of documents:	Emails and attachments, Briefing notes, documents as specified above

DO NOT WRITE IN THIS BINDING MARGIN

Response required from your division / branch / unit

DEADLINE: 05 June 2020

*** If you believe you are unable to meet this deadline, please contact the RTI / IP decision-maker to discuss an extension.**

Introduction:

The first case in Australia was on Jan 25th, 2020 and first case in Queensland was detected by the Forensic and Scientific service lab on 29th Jan. Pathology Queensland began testing for COVID-19 on Feb 7th 2020. Since then Pathology Queensland has performed over 480 000 PCR COVID-19 tests in conjunction to the Pandemic response of Queensland Health.

The ability to detect cases of SARS-CoV-2 infection early is central to the strategies that can be employed in order to prevent transmission in Australia. A high rate of well-targeted testing is essential to provide confidence that cases will be detected as control measures are adjusted.

The Communicable Diseases Network Australia (CDNA) recommends that all people with fever or respiratory symptoms are tested for SARS-CoV-2. The rationale is that people with symptoms consistent with COVID-19 have a much higher probability of testing positive for SARS-CoV-2 than people without such symptoms and present a higher risk of transmission to others. Where there is a low prevalence of disease, testing people beyond those with COVID-19 symptoms, i.e. asymptomatic people, is likely to result in a higher rate of false positives.

Ideally, testing rates for COVID-19 should align with the prevalence of acute respiratory illness (ARI) in the community. Currently, levels of respiratory illness are low in Queensland due to physical distancing measures; however, can be expected to increase. The proportion of ARI attributed to COVID-19 in the coming months is yet to be determined. There is a need to optimise testing for groups and populations that have been under-represented to date and are at high risk for outbreaks.

The key means of optimising testing rely on: identifying individuals and groups at high risk of transmission and/or high risk of severe disease increasing community awareness of testing improved access to testing removing barriers in addition to access novel surveillance mechanisms.

Background to SARS-CoV-2 PCR Nucleic acid Testing in Australia

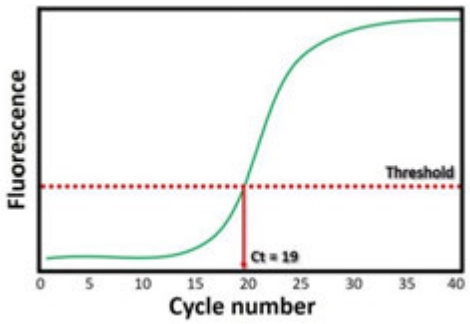
There is not one, uniformly used NAT within Australian diagnostic laboratories for the detection of SARS-CoV-2, but rather multiple different types of tests targeting one or more different regions of the SARS-CoV-2 genome. In addition, several laboratories may utilise multiple different supplemental assays in the event of an initial positive result.

Prior to the availability of TGA-approved commercial assays, "in-house" PCR assays were first developed and used by the public health laboratories within each jurisdiction. Subsequently, private and public hospital laboratories also developed testing capacity through the use of in-house and/or commercial assays.

Assay Performance and Sources of False Positive Results

All currently used diagnostic assays in Australia are known as real time PCR. The PCR reaction amplifies a highly specific region of the SARS-CoV-2 genome. SARS-CoV-2 amplicons are identified by fluorescent probes or melt curve analysis of intercalating dye-based non-probe based PCR assays. Each amplification reaction is known as a cycle. A usual PCR assay runs for 35-45 cycles. The cycle threshold (Ct) value of a reaction is the cycle number when the fluorescence of a PCR product can be detected above the background signal (Figure 1). Whilst extending the number of cycles may increase the sensitivity of the assay (or ability to detect very low levels of virus in the specimen) it may also increase the risk of false positivity due to non-specific amplification. This may be identified by assessing the Ct value (usually high), shape of the amplification curve and/or performing a melt curve analysis. It should be noted however that whilst the Ct value provides an indication of the amount of virus present in the specimen, it is not a standardised quantitative measure of viral load and for the same specimen may vary across different assays.

Figure 1: Typical amplification curve of a positive (detected) result



Commercial assays often have in-built analysis systems to interpret a result as detected (positive) or not detected (negative). To comply with TGA requirements, the laboratory must report the results according to the manufacture's recommendations.

For in-house assays, the parameters for determining a detected (positive) or not detected (negative) result have been set by the laboratory through the assay development and evaluation process, potentially allowing for greater scope for scientific interpretation and identification and reporting of equivocal/borderline/indeterminate results.

False positive test results may occur as a result of (1) the intrinsic performance characteristics of the test related mainly to test specificity or (2) pre-analytical, analytical or post analytical testing errors

Impact of testing on high versus low infection prevalence groups and rates of false positive results

With appropriate laboratory quality control, the analytical sensitivity (percentage of people with the infection that will have a positive test) and specificity (percentage of people without the infection that will have a negative test) of SARS-CoV-2 PCR assays are constant and pre-determined.

On the other hand, the positive predictive value (percentage of people with a positive test that will have the infection) and negative predictive value (percentage of people with a negative test do not have the infection) are variable, and dependent upon the pre-test likelihood of the person having infection.

For example, if a test with a sensitivity of 90% and specificity of 99% is used to test symptomatic passengers on a cruise ship where the likelihood of infection is 50%, the positive predictive value is 99% (i.e. for every 100 people with a positive test result, 1 person will have a false positive result and do not have infection).

However, using the *exact same test*, if a low risk asymptomatic population is tested where the likelihood of infection is 5 in 10,000 (i.e. 0.05%), the positive predictive value is 4.3% (i.e. for every 100 people with a positive test result, 95-96 people will have a false positive result and do not have infection).

These examples emphasise the importance of interpreting the test results in light of the pre-test likelihood of infection (risk factors and clinical features), even with a test that has very high specificity.

The scientific PCR testing performance of the assay does vary slightly between the instrument being used and the method used to test for the virus. A summary of all TGA approved testing methods for COVID-19 can be found at the TGA web site https://www.tga.gov.au/covid-19-test-kits-included-artg-legal-supply-australia. The post market review of COVID-19 test can be found at https://www.tga.gov.au/post-market-review-covid-19-point-care-tests

PCR is the most reliable testing method performed by the microbiology laboratory. The rate of false negatives and false positives is less than 1%. Note that there is no test in Pathology that is 100% correct 100% of the time.

Commercial Testing Method used by Pathology Queensland

Manufacturer: Roche Molecular Systems Inc (USA)
Name of test: cobas® SARS-CoV-2
Australian sponsor: Roche Diagnostics Australia Pty Limited
Date approved for supply: 20 Mar 2020

Laboratory
Nucleic Acid Test

Manufacturer: Cepheid (USA)
Name of test: Xpert® Xpress SARS-CoV-2
Australian sponsor: Cepheid Holdings Pty Ltd
Date approved for supply: 22 Mar 2020

Laboratory/Point-of-care test*
Nucleic Acid Test

Manufacturer: Hologic Inc (USA)
Name of test: Panther Fusion® SARS-CoV-2 Assay (Panther Fusion® System)
Australian sponsor: Hologic (Australia & New Zealand) Pty Ltd
Date approved for supply: 20 Mar 2020

Laboratory
Nucleic Acid Test

Taking into consideration the information provided above and between the period requested Pathology Queensland has had 8 patient swabs that have been classified as divergent or further clinical investigation and interpretation is required.

This is where we have had only single swab reported by the initial testing method as Detected, and the second confirmation test reported as a Not-Detected result plus 6 where there were no other specimens to assist with confirmation process.

These are listed below.

20407-13710	KN025355
20520-86539	BN097498
20885-24579	IP227984
20885-25031	SC0134191
20885-46079	SC0169325
20886-48594	TN927053
20887-18820	SC0113258
20935-41176	GC1043849

False positivity rate based on period Feb-1-2020 to 7-May-2020

- Based on Pathology Queensland requests (57036) the rate is 0.01 -0.02%
- Based on NCVPCR+CRTAQ requests i.e. 1089, rate = 0.6 – 1.2%

Australian TGA Web Site

https://www.tga.gov.au/covid-19-testing-australia-information-health-professionals

COVID-19 testing in Australia - information for health professionals

22 May 2020

Tests for COVID-19 aim to detect the causative virus, SARS-CoV-2, or an immune response to SARS-CoV-2.

The reliability of COVID-19 tests is uncertain due to the limited evidence base. Available evidence mainly comes from symptomatic patients, and their clinical role in detecting asymptomatic carriers is unclear.

COVID-19 test performance

COVID-19 is an emerging viral infectious disease. There is limited evidence available to assess the accuracy and clinical utility of available COVID-19 tests.

Due to the urgent nature of the COVID-19 pandemic, a number of SARS-CoV-2 tests have undergone an expedited assessment by the TGA to enable their legal supply in Australia. These expedited assessments are based on the limited clinical and performance data currently available. All SARS-CoV-2 tests currently approved for supply are required to provide updated evidence to support the ongoing safety and performance of the tests to the TGA.

How accurate are test results?

No test gives a 100% accurate result; tests need to be evaluated to determine their sensitivity and specificity, ideally by comparison with a “gold standard.” The lack of such a clear-cut “gold-standard” for covid-19 testing makes evaluation of test accuracy challenging.

Below are examples of the analytical sensitivity of the Commercial Assays used by Pathology Queensland as provided by the manufacturer.

Analytical sensitivity using cultured virus serially diluted to determine limit of detection - see below (TCID=tissue culture infectious dose)

As shown in Table 10, the concentration level with observed hit rates greater than or equal to 95% were 0.009 and 0.003 $TCID_{50}$/mL for SARS-CoV-2 (Target 1) and pan-Sarbecovirus (Target 2), respectively. As shown in Table 11, th Probit predicted 95% hit rates were 0.007 and 0.004 $TCID_{50}$/mL for SARS-CoV-2 (Target 1) and pan-Sarbecovirus (Target 2), respectively.

Clinical evaluation
"100 individual negative clinical samples and 50 contrived positive clinical samples"

Performance against the expected results are:
Positive Percent Agreement 50/50 = 100% (95% CI: 86.7% - 100%)
Negative Percent Agreement 100/100 = 100% (95% CI: 96.3% - 100%)

- **SARS-CoV-2 Assay (Panther Fusion® System)**

Panther Fusion SARS-CoV-2 Assay Performance

Analytical Sensitivity

The analytical sensitivity (limit of detection or LoD) of the Panther Fusion SARS-CoV-2 Assay was determined by testing serial dilutions of pooled negative clinical nasopharyngeal swab specimens spiked with inactivated cultured SARS-CoV-2 virus (USA-WA1/2020; BEI Resources, NR-52281). Ten replicates of each serial dilution were evaluated using two assay reagent lots across two Panther Fusion systems. The LoD was determined to be $1x10^{-2}$ $TCID_{50}$/mL and verified by testing an additional 20 replicates with one assay reagent lot.

Clinical Performance
The clinical performance of the Panther Fusion SARS-CoV-2 Assay was evaluated in comparison to a panel of contrived specimens. For the study, a panel of 178 remnant clinical nasopharyngeal specimens was tested using two Panther Fusion SARS-CoV-2 Assay reagent lots. All specimens were collected from US patients with signs and symptoms of respiratory infection. The panel consisted of 69 SARS-CoV-2 positive and 109 SARS-CoV-2 negative specimens. Of the 69 positive specimens, 45 were at concentrations 1-2x LoD and 24 were at concentrations 3-5x LoD using inactivated cultured SARS-CoV-2 virus (USA-WA1/2020; BEI Resources; NR-52281) as the target.

Table 4: Panther Fusion SARS-CoV-2 Performance Relative to Expected Results

		Contrived Specimen Expected Result	
		Positive	Negative
Panther Fusion SARS-CoV-2 Assay	Positive	69	0
	Negative	0	109

Positive Percent Agreement: 100% (94.7% – 100%)
Negative Percent Agreement: 100% (96.6% – 100%)
Overall Agreement: 100% (96.6% – 100%)

Pathology (■ xxxx) **xxx(xxx)**, xxx

CORRESPONDENCE

Contamination of SARS-CoV-2 RT-PCR probes at the oligonucleotide manufacturer

Sir,

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) was first identified in Wuhan, China, in December 2019 as the aetiological agent of Coronavirus disease 2019 (COVID-19).[1,2] Since then, the disease has spread rapidly worldwide and the World Health Organization (WHO) declared a pandemic on 11 March 2020.[3,4] At the beginning of the outbreak, rapid development and implementation of reliable detection methods became an immediate priority for clinical laboratories worldwide, and reverse transcription polymerase chain reaction (RT-PCR) methods, including those provided by the WHO,[5,6] have been implemented broadly. At the early stage of the outbreak, however, positive control material for RT-PCR assays (from positive patient samples, or viral culture) were not readily available. In such circumstances laboratories often turn to using synthetic controls (synDNA fragments or plasmids).[7,8] These synthetic controls have their advantages, particularly in that the controls can be acquired as readily as PCR primers and probes. Yet, depending on how they are designed, precautions must be taken when handling such controls as trace amounts of this material can potentially cause contamination in the same way as that caused by PCR products. Here we report contamination of a SARS-CoV-2 probe that our evidence suggests occurred at the oligonucleotide manufacturer, and was due to the manufacturer synthesising full length control oligonucleotides (spanning from the forward to reverse primers) in parallel with our probe orders.

Two commonly used assays, E-gene and RdRP, reported by Corman *et al.*,[5] were utilised by our laboratory in the early stages of the pandemic. To establish the assays, we were fortunate enough to have nucleotide stocks and positive control material provided to us from another local laboratory and the assays performed well in our hands. Needing new oligonucleotide stocks, we ordered primers and probes from supplier 'X' on 28 January 2020. The primers arrived on 31 January and were subjected to routine quality control checks. These included checking and recording oligonucleotide batch and reconstitution details, and master mix using new primers or probe was prepared and tested against previously checked reagents. The new primers passed quality control checks. The probes arrived on 11 February, however both were contaminated, providing positive results in the negative controls of both the E-gene and RdRP assays.

Suspecting that the probes had been contaminated by 'full length control oligonucleotides' (ordered by customers from elsewhere) we developed a series of alternative flanking primers for both assays (Fig. 1, Table 1). Each of these alternative flanking primers was designed to gradually 'step away' from the original target region. Full-length synthetic controls typically would only contain sequences from the original target region (i.e., not any additional sequences sitting outside of the original primer pair), and so this type of contamination can be identified by testing alternative primer sets targeting regions further away from the original target site. Therefore, for our reagents, if the contamination was from synthetic controls, the original primers would produce false positive results while the flanking primers would generate negative results when testing non-template controls (NTCs). The flanking primers as well as the original primers from supplier X were tested against the supplier X probes. The experiments were replicated using probes sourced from another supplier, supplier 'Y'. Two SARS-CoV-2 positive clinical samples and two NTCs were tested in each primer probe combination. This study was approved by the Children's Health Queensland Human Research Ethics Committee (HREC/LNR/19/QCHQ/49476).

All results are shown in Table 2. In brief, the known positive samples were positive by all oligonucleotide combinations. Notably, the NTCs were only positive in the supplier X probes using the original supplier X primers, and not in any other NTC, including the original supplier X primers with the supplier Y probes. Of concern were the cycle threshold (Ct) values for the supplier X probe NTCs for the RdRP assay which were very low at ~26 cycles (Table 2), indicating very high levels of contamination, whereas the NTC Ct values for the E assay were ~36 cycles. These results show the supplier X probes were contaminated with nucleic acid fragments consistent with the size, but not larger than, the expected PCR products for the E and RdRP assays.

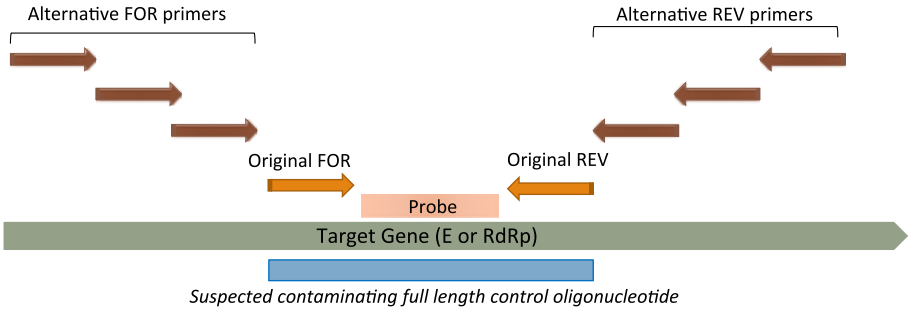


Fig. 1 Illustration of flanking primer designs for detection of contaminated probe. Note that six additional primers were designed for the E-gene but only four for the RdRP assay. FOR, forward primer; REV, reverse primer.

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Table 1 List of oligonucleotides used in this study

Name	Oligonucleotides	Nucleotide position[a]	Notes
Sarbeco_E-F1	5′ACAGGTACGTTAATAGTTAATAGCGT	26237−26262	E-Gene Original primer[5]
Sarbeco_E-R2	5′ATATTGCAGCAGTACGCACACA	26328−26349	E-Gene Original primer[5]
Sarbeco_E-probe	5′ACACTAGCCATCCTTACTGCGCTTCG	26300−26325	E-Gene Original probe[5]
Sarbeco_E-altF2	5′CTTATGTACTCATTCGTTTCGGAAGA	26210−26235	E-Gene Flanking primer
Sarbeco_E-altF3	5′GTAAGCACAAGCTGATGAGTACGA	26185−26208	E-Gene Flanking primer
Sarbeco_E-altF4	5′ GACGACGACTACTAGCGTGCCTT	26161−26183	E-Gene Flanking primer
Sarbeco_E-altR2	5′ GAAGGTTTTACAAGACTCACGTTAACA	26350−26376	E-Gene Flanking primer
Sarbeco_E-altR3	5′ GAAGAATTCAGATTTTTAACACGAGAGTAAA	26385−26415	E-Gene Flanking primer
Sarbeco_E-altR4	5′ GTTCGTTTAGACCAGAAGATCAGGAA	26421−26446	E-Gene Flanking primer
RdRP_SARSr-F2	5′ GTGARATGGTCATGTGTGGCGG	15399−15420	RdRP Gene Original primer[5]
RdRP_SARSr-R1	5′ CARATGTTAAASACACTATTAGCATA	15473−15498	RdRP Gene Original primer[5]
RdRP_SARSr-P2	5′ CAGGTGGAACCTCATCAGGAGATGC	15438−15462	RdRP Gene Original probe[5]
RdRP_SARSr-altF3	5′ GTTTCTATAGATTAGCTAATGAGTGTGCTCAA	15360−15391	RdRP Gene Flanking primer
RdRP_SARSr-altF4	5′ CTTGTTCTTGCTCGCAAACATACAA	15314−15338	RdRP Gene Flanking primer
RdRP_SARSr-altR2	5′ GCATTAACATTGGCCGTGACA	15505−15525	RdRP Gene Flanking primer
RdRP_SARSr-altR3	5′ TCGGCAATTTTGTTACCATCAGTAGATA	15531−15558	RdRP Gene Flanking primer

[a] Nucleotide position used reference genome Genbank ID: MN938384.

Table 2 E-gene and RdRP RT-PCR results with different oligonucleotide combinations

Assay and oligonucleotides	Notes	SARS-CoV-2 positive clinical sample 1 (Ct)	SARS-CoV-2 positive clinical sample 2 (Ct)	NTC 1 (Ct)	NTC 2 (Ct)
E-gene					
F1/R2 + probe Y	Original primer set with probe from supplier Y	26.37	20.42	ND	ND
F1/R2 + probe X	Original primer set with probe from supplier X	29.04	22.59	35.44	37.05
F1/altR2 + probe X	Alternative primer combinations with probe from supplier X	29.51	23.15	ND	ND
F1/altR3 + probe X		29.3	23.25	ND	ND
F1/altR4 + probe X		30.14	23.71	ND	ND
altF2/R2 + Probe X		28.85	22.94	ND	ND
altF2/altR2 + Probe X		29.49	23.5	ND	ND
altF2/altR3 + Probe X		30.44	23.93	ND	ND
altF2/altR4 + Probe X		29.86	23.76	ND	ND
altF3/R2 + Probe X		29.32	23.2	ND	ND
altF3/altR2 + Probe X		29.87	23.84	ND	ND
altF3/altR3 + Probe X		30.51	24.24	ND	ND
altF3/altR4 + Probe X		30.22	24.24	ND	ND
altF4/R2 + Probe X		29.9	23.8	ND	ND
altF4/altR2 + Probe X		30.85	24.65	ND	ND
altF4/altR3 + Probe X		30.48	24.29	ND	ND
altF4/altR4 + Probe X		30.75	24.72	ND	ND
RdRP					
F2/R1 + probe Y	Original primer set with probe from supplier Y	29.48	23.97	ND	ND
F2/R1 + probe X	Original primer set with probe from supplier X	26	24.31	26.24	26.11
F2/altR2 + probe X	Alternative primer combinations with probe from supplier X	ND	28.74	ND	ND
F2/altR3 + probe X		29.27	23.63	ND	ND
altF3/R1 + probe X		ND	26.14	ND	ND
altF3/altR2 + probe X		28.49	22.97	ND	ND
altF3/altR3 + probe X		29.06	23.46	ND	ND
altF4/R1 + probe X		31.29	25.72	ND	ND
altF4/altR2 + probe X		28.64	23.05	ND	ND
altF4/altR3 + probe X		29.31	23.36	ND	ND

alt, alternative; Ct, cycle threshold; F, forward primer; ND, not detected; R, reverse primer.

We have since contacted supplier X and they have now implemented new quality control measures to address this issue.

Overall, our study highlights the potential for contamination of oligonucleotide probes at the manufacturer and is due to customers ordering ‘full length control oligonucleotides’. This is alarming in the context of reagent shortages and delays associated with the pandemic, and would have left our laboratory in a precarious position had we not also ordered probes from supplier Y. We affirm that synthetic

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controls can be useful as positive control material for rare or emergent diseases but should be manufactured and used carefully. Oligonucleotide suppliers should consider how to better handle such requests.

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Laboratory testing for SARS-CoV-2: Information and FAQs

29 June 2020

The capacity to identify and isolate cases of COVID-19 is critical to limit the spread of the virus, protect vulnerable persons in the community and ensure healthcare systems maintain the capacity to deliver quality healthcare.

Laboratory testing for SARS-CoV-2 has evolved over the course of the pandemic and continues to advance as more is known about the virus and testing capability is enhanced.

Testing for SARS-CoV-2 should be done in conjunction with assessment and testing for other potential causes of the person's presentation, as deemed appropriate by the treating clinician and in line with local protocols.

What types of tests are available for SARS-CoV-2?

Testing can be broadly grouped into:

- SARS-CoV-2 specific testing (testing directly for the virus), done by nucleic acid testing AND
- Serology (testing for antibody response).

Nucleic acid testing (NAT)

NAT is performed by real time polymerase chain reaction (PCR). This method involves amplification of RNA of the SARS-CoV-2 virus. PCR is the appropriate test for diagnosis of acute COVID-19 infection.

PCR is most commonly performed on upper respiratory tract specimens. For best results from upper respiratory tract sampling, both deep nasal and throat swabs should be collected. Both sites can be sampled using the same swab.

PCR can also be performed on lower respiratory tract specimens including sputum, bronchoalveolar lavage and tracheal aspirate. Lower respiratory tract specimens contain higher viral loads in SARS-CoV-2, and therefore should be tested wherever appropriate.

PCR is occasionally performed on faecal and tissue specimens in special circumstances. Testing of faeces or tissue is only available by special request on the advice of a microbiologist, infectious diseases physician or public health physician.

Serology

Serology tests are performed on serum to look for antibodies that are produced by the person against SARS-CoV-2. They do not detect the virus itself.

A validated SARS-CoV-2 serology assay is now available at Queensland Health Forensic and Scientific Services (QHFSS). The assay detects both IgM and IgG antibodies in serum from patients who have been infected with the virus. Serology assays are currently being assessed by other laboratories in Queensland and will likely become available soon.

Serology is not recommended for diagnosis of acute infection. Acute and convalescent specimens collected 10–14 days apart can assist in diagnosis of COVID-19 in persons with negative PCR tests where there remains a high suspicion of COVID-19, or in persons suspected to have recovered from COVID-19 who did not undergo PCR at the time they were unwell.

Serology testing is currently limited in Queensland and must be prioritised for epidemiological investigations where the results will be used to provide information relating to current community transmission. Examples of this include:

- an active outbreak or cluster investigation, or
- investigation of potential upstream contacts of a confirmed case of COVID-19 where the case has no apparent epidemiological links (i.e. contact with other known cases or travel to an area where there is known community transmission of the virus).

Serology test requests via QHFSS must be discussed with the local public health unit and approved by the QHFSS microbiologist.

Point of care (POC) serology tests

Point of care (POC) serology tests, or finger-prick tests, are yet to be validated. The accuracy and clinical utility of these tests is unknown and therefore they are not endorsed for use by Queensland Health outside of the research setting.

Please refer to the following Chief Health Officer direction in relation to the use of POC serology tests in Queensland, effective from 23 April 2020, available at https://www.health.qld.gov.au/system-governance/legislation/cho-public-health-directions-under-expanded-public-health-act-powers/point-of-care-serology-tests

Is there a possibility of false negative or false positive PCR results?

There are no pathology tests that are completely accurate, however the accuracy of PCR assays used to detect SARS-CoV-2 in diagnostic laboratories in Queensland perform very well.

Factors that can influence the accuracy of PCR testing and correct classification of cases include:

- a variable presence of virus in different body sites at different phases of illness
- the quality of the sampling (how well the swab is taken)
- technical factors specific to the assay
- specimen handling and processing.

If the results of testing do not fit with the clinical and epidemiological context of the person being evaluated, this should be discussed with a microbiologist, infectious diseases physician or public health physician and consideration given to repeat testing.

Despite more than 250 000 tests for SARS-CoV-2 having been performed in Queensland to date, fewer than 10 have been identified as false positive reports. For further information on false positive PCR tests, refer to the "Public Health Laboratory Network Statement on Nucleic Acid Test False Positive Results for SARS-CoV-2", available at: https://www1.health.gov.au/internet/main/publishing.nsf/Content/2FCDB8DA4EB40BA9CA257BF000211F2A/$File/Nucleic-Acid-Test-False-Positive-Results-SARS-CoV-2-PHLN.pdf

What platforms are available for PCR?

There are a variety of platforms in use for PCR testing across different laboratories. These platforms are continually being evaluated and enhanced as new information on the virus and testing methods becomes available.

PCR is principally performed using high throughput instruments, processing specimens in large batches. These tests take approximately six hours to process once they are loaded onto the instrument in the laboratory.

In regional sites and some hospitals across Queensland, there are also GeneXpert instruments available. GeneXpert can process a small number of specimens with a quicker processing time than high throughput instruments.

The availability of testing kits for GeneXpert are currently very limited worldwide, and therefore their use in Queensland must be prioritised for investigation of suspected cases of COVID-19 where the confirmation of a positive or negative result would prompt:

- a rapid public health response, or
- a significant change to immediate clinical management.

The use of GeneXpert tests require approval from a clinical microbiologist, infectious diseases physician, public health physician or executive director of medical services (EDMS), depending on processes in place at your facility.

GeneXpert are sometimes referred to as 'point of care' PCR tests. Specimens being evaluated for SARS-CoV-2 using the GeneXpert must still be processed in a laboratory or clinic environment with adequate safety precautions in place to protect the operator of these tests from contracting COVID-19 from the specimens they are handling.

Can a serology test prove that a person is immune to COVID-19?

The presence of antibodies detected on serology testing demonstrates recent or past COVID-19 infection. They cannot be used to demonstrate immunity to the virus. Some people may never make antibodies to the virus, particularly if they are immunosuppressed.

It is currently not known how long antibodies remain in the body, and there is a possibility that they wane over time.

Serology results are not used to shorten a person's period of quarantine.

Can test results be fast-tracked?

Laboratory turn-around times are very good for SARS-CoV-2 testing and continue to improve. Laboratories in Queensland operate extended hours, seven days per week. Queensland currently tests around 5000 people per day.

If the testing processes were interrupted in order to fast-track one or more specimens, this would slow down the turn-around time for results for all the other specimens being processed on that run. Therefore, prioritisation of testing is not done.

Is there a shortage of SARS-CoV-2 tests?

There are worldwide shortages of some of the reagents used for SARS-CoV-2 testing. Diagnostic laboratories in Queensland have responded to these shortages by diversifying their testing platforms and by using innovative processes within the laboratory to ensure there is enough testing capacity to respond to the COVID-19 pandemic.

Testing should continue to be requested for persons who have symptoms of COVID-19 infection in accordance with state and national guidelines.

Should I test asymptomatic people for SARS-CoV-2?

Testing asymptomatic persons should only occur in specific circumstances, for example, under the guidance of your public health unit during investigation of an outbreak or prior to organ donation. Although asymptomatic and pre-symptomatic shedding of SARS-COV-2 is described, as the current prevalence of COVID-19 in Australia is so low, the predictive value of testing asymptomatic persons without epidemiological risk factors is significantly limited. Increased testing of asymptomatic persons in a low prevalence setting can lead to an increased proportion of false positive tests results.

Where can I find further information about SARS-CoV-2 testing?

- PHLN guidance on laboratory testing for SARS-CoV-2, https://www.health.gov.au/sites/default/files/documents/2020/05/phln-guidance-on-laboratory-testing-for-sars-cov-2-the-virus-that-causes-covid-19_1.pdf
- Queensland Health public health alerts, https://www.health.qld.gov.au/clinical-practice/guidelines-procedures/novel-coronavirus-qld-clinicians/public-health-alerts
- Coronavirus Disease 2019 (COVID-19) - CDNA National guidelines for public health units, https://www1.health.gov.au/internet/main/publishing.nsf/Content/cdna-song-novel-coronavirus.htm
- Australian Health Protection Principal Committee (AHPPC) coronavirus (COVID-19) statements on 14 May 2020, https://www.health.gov.au/news/australian-health-protection-principal-committee-ahppc-coronavirus-covid-19-statements-on-14-may-2020