

## 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.*

## 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. 新型冠状病毒核酸检测（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) : CCCTGTGGGTTTACACTTAA

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) : CAGACATTTGCTCTCAAGCTG

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<sup>1</sup>

### 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.
<b>RdRp gene / nCoV_IP2</b>				
nCoV_IP2-12669Fw	ATGAGCTTAGTCCTGTTG	17		
nCoV_IP2-12759Rv	CTCCCTTGTGTTGTGTTGT	18		
nCoV_IP2-12696bProbe(+)	AGATGTCTTGCTGCCGGTA [5']Hex [3']BHQ-1	21		
<b>RdRp gene / nCoV_IP4</b>				
nCoV_IP4-14059Fw	GGTAACCTGGTATGATTTCG	19		
nCoV_IP4-14146Rv	CTGGTCAAGGTTAATATAGG	20		
nCoV_IP4-14084Probe(+)	TCATACAAACCACGCCAGG [5']Fam [3']BHQ-1	19		
<b>E gene / E_Sarboco</b>				
E_Sarboco_F1	ACAGGTACGTTAATAGTTAATAGCGT	18		
E_Sarboco_R2	ATATTGCAGCAGTACGCACACA	20		
E_Sarboco_P1	ACACTAGCCATCCTACTGCGCTTCG [5']Fam [3']BHQ-1	20		

1/ National Reference Center for Respiratory Viruses, Institut Pasteur, Paris.

2/ Corman et al. Eurosurveillance<sup>1</sup>

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 25 $\mu$ l reaction was set up containing 5 $\mu$ l of RNA.**

Simplex Mix	Vol ( $\mu$ l)	[final]
H <sub>2</sub> O PPI	3.60	
Reaction mix 2X	12.50	3 mM Mg
MgSO <sub>4</sub> (50mM)	0.40	0.8 mM Mg
Forward Primer (10 $\mu$ M)	1.00	0.4 $\mu$ M
Reverse Primer (10 $\mu$ M)	1.00	0.4 $\mu$ M
Probe (10 $\mu$ M)	0.50	0.2 $\mu$ M
SuperscriptIII RT/Platinum Taq Mix	1.00	
<b>Final Volume</b>	<b>20.00</b>	

Multiplex Mix (nCoV_IP2&IP4)	Vol ( $\mu$ l)	[final]
H <sub>2</sub> O PPI	1.3	
Reaction mix 2X	12.50	3 mM Mg
MgSO <sub>4</sub> (50mM)	0.40	0.8 mM Mg
Forward Primer (10 $\mu$ M)	1.00	0.4 $\mu$ M
Reverse Primer (10 $\mu$ M)	1.00	0.4 $\mu$ M
Forward Primer (10 $\mu$ M)	1.00	0.4 $\mu$ M
Reverse Primer (10 $\mu$ M)	1.00	0.4 $\mu$ M
Probe (10 $\mu$ M)	0.4	0.16 $\mu$ M
Probe (10 $\mu$ M)	0.4	0.16 $\mu$ M
SuperscriptIII RT/Platinum Taq Mix	1.00	
<b>Final Volume</b>	<b>20.00</b>	

## 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<sup>5</sup>, 10<sup>4</sup> and 10<sup>3</sup> 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\_Sarboco 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.

RNA copies Of transcript	Multiplex (Ct values)		Simplex (Ct values)  E_Sarboco
	nCoV_IP2	nCoV_IP4	
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 ( $\Delta Ct$ ).*

## 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  $\mu$ l of RNase/DNAse-free H<sub>2</sub>O to obtain a solution at a concentration of  $10^9$  copies/ $\mu$ l. Store at -80°C. Dilute to prepare a master bank at  $2 \times 10^6$  copies/ $\mu$ l. Store at -80°C.

From this prepare a working bank of reagent at  $2 \times 10^4$  copies/ $\mu$ l 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](mailto:grippe@pasteur.fr))

### Acknowledgements

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 <sup>1</sup>	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

<sup>1</sup>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



## Table of Contents

Intended Use .....	2
Summary and Explanation .....	2
Principles of the Procedure.....	3
Materials Required (Provided) .....	5
Materials Required (But Not Provided).....	6
Warnings and Precautions .....	8
Reagent Storage, Handling, and Stability .....	9
Specimen Collection, Handling, and Storage .....	10
Specimen Referral to CDC .....	11
Reagent and Controls Preparation .....	11
General Preparation.....	12
Nucleic Acid extraction .....	12
Assay Set Up.....	14
Create a Run Template on the Applied Biosystems 7500 Fast Dx Real-time PCR Instrument (Required if no template exists) .....	17
Defining the Instrument Settings.....	23
Running a Test.....	25
Interpretation of Results and Reporting.....	30
2019-nCoV rRT-PCR Diagnostic Panel Results Interpretation Guide.....	32
Quality Control .....	33
Limitations.....	33
Conditions of Authorization for the Laboratory.....	34
Performance Characteristics .....	35
Disposal.....	40
References .....	40
Contact Information, Ordering, and Product Support.....	41

## 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.

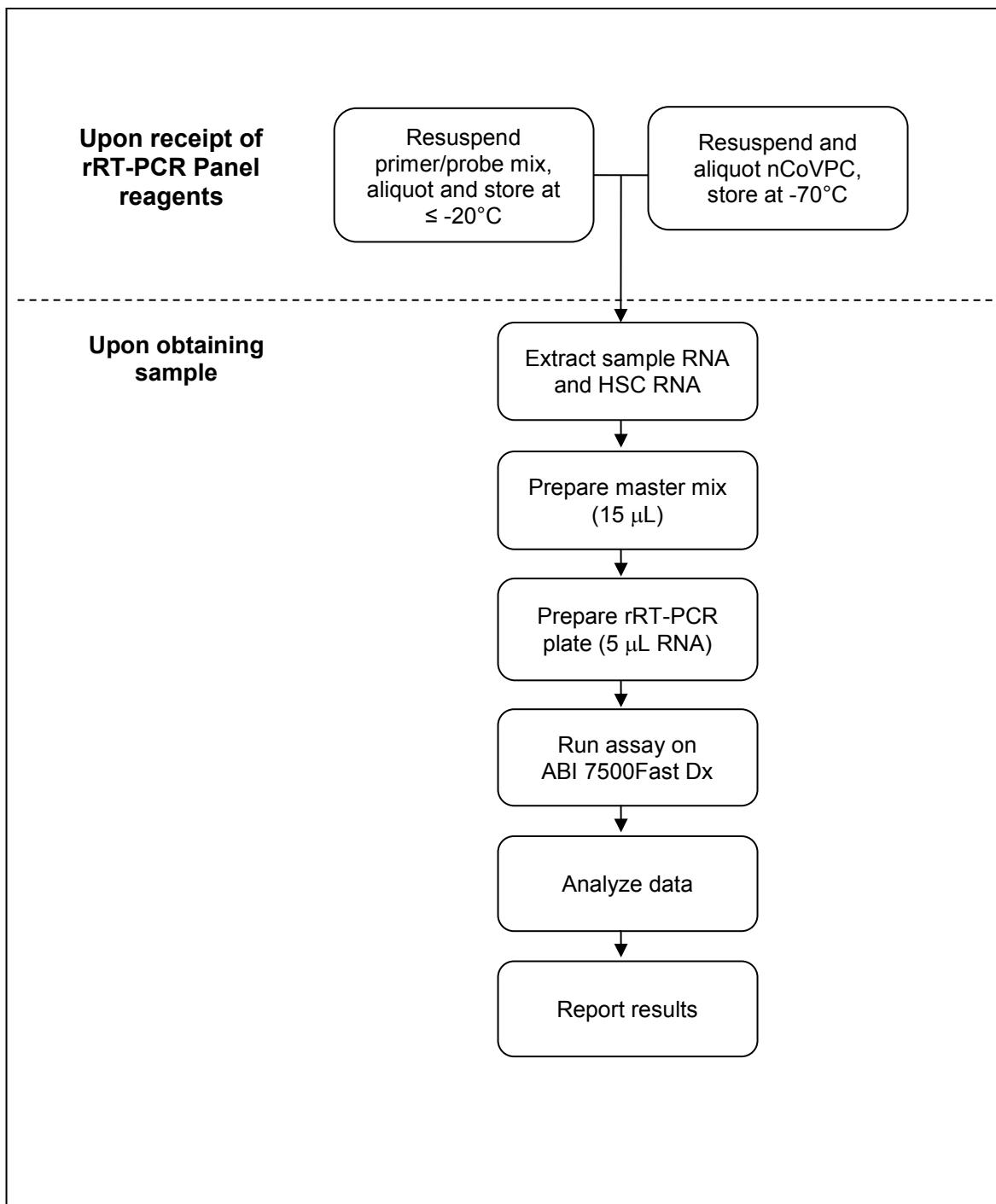
## Principles of the Procedure

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:**

<i>Reagent Label</i>	<i>Part #</i>	<i>Description</i>	<i>Quantity / Tube</i>	<i>Reactions / Tube</i>
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:**

<i>Reagent Label</i>	<i>Part #</i>	<i>Description</i>	<i>Quantity</i>	<i>Notes</i>
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 <i>in vitro</i> 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)**

<b>Reagent Label</b>	<b>Part #</b>	<b>Description</b>	<b>Quantity</b>	<b>Notes</b>
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 <i>in vitro</i> 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)**

<b>Description</b>	<b>Quantity</b>	<b>CDC Catalog No.</b>
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**

<b>Reagent</b>	<b>Quantity</b>	<b>Catalog No.</b>
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	<sup>2</sup> QIAamp DSP Viral RNA Mini Kit	50 extractions (61904)
	<sup>2</sup> QIAamp Viral RNA Mini Kit	50 extractions (52904) 250 extractions (52906)
QIAGEN EZ1 Advanced XL	<sup>2</sup> EZ1 DSP Virus Kit	48 extractions (62724) Buffer AVL (19073) EZ1 Advanced XL DSP Virus Card (9018703)
	<sup>2</sup> EZ1 Virus Mini Kit v2.0	48 extractions (955134) Buffer AVL (19073) EZ1 Advanced XL Virus Card v2.0 (9018708)
<sup>1</sup> Roche MagNA Pure LC	<sup>2</sup> Total Nucleic Acid Kit	192 extractions (03 038 505 001)
<sup>1</sup> Roche MagNA Pure Compact	<sup>2</sup> Nucleic Acid Isolation Kit I	32 extractions (03 730 964 001)
<sup>1</sup> Roche MagNA Pure 96	<sup>2</sup> DNA and Viral NA Small Volume Kit	576 extractions (06 543 588 001) External Lysis Buffer (06 374 913 001)
<sup>1</sup> QIAGEN QIAcube	<sup>2</sup> QIAamp DSP Viral RNA Mini Kit	50 extractions (61904)
	<sup>2</sup> QIAamp Viral RNA Mini Kit	50 extractions (52904) 250 extractions (52906)
<sup>1,3</sup> bioMérieux NucliSENS® easyMAG® and <sup>1,3</sup> 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)

<sup>1</sup>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.

<sup>2</sup> CDC has confirmed that the external lysis buffer used with this extraction method is effective for inactivation of SARS-CoV-2.

<sup>3</sup> 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)
- DNAZap™ (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, “DNAZap™” 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](http://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 ( $N = \# \text{ of reactions}$ ).

### **TaqPath™ 1-Step RT-qPCR Master Mix**

Step #	Reagent	Vol. of Reagent Added per Reaction
1	Nuclease-free Water	$N \times 8.5 \mu\text{L}$
2	Combined Primer/Probe Mix	$N \times 1.5 \mu\text{L}$
3	TaqPath™ 1-Step RT-qPCR Master Mix (4x)	$N \times 5.0 \mu\text{L}$
	<b>Total Volume</b>	<b><math>N \times 15.0 \mu\text{L}</math></b>

- 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											
B	N2											
C	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 <sup>a</sup>	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												

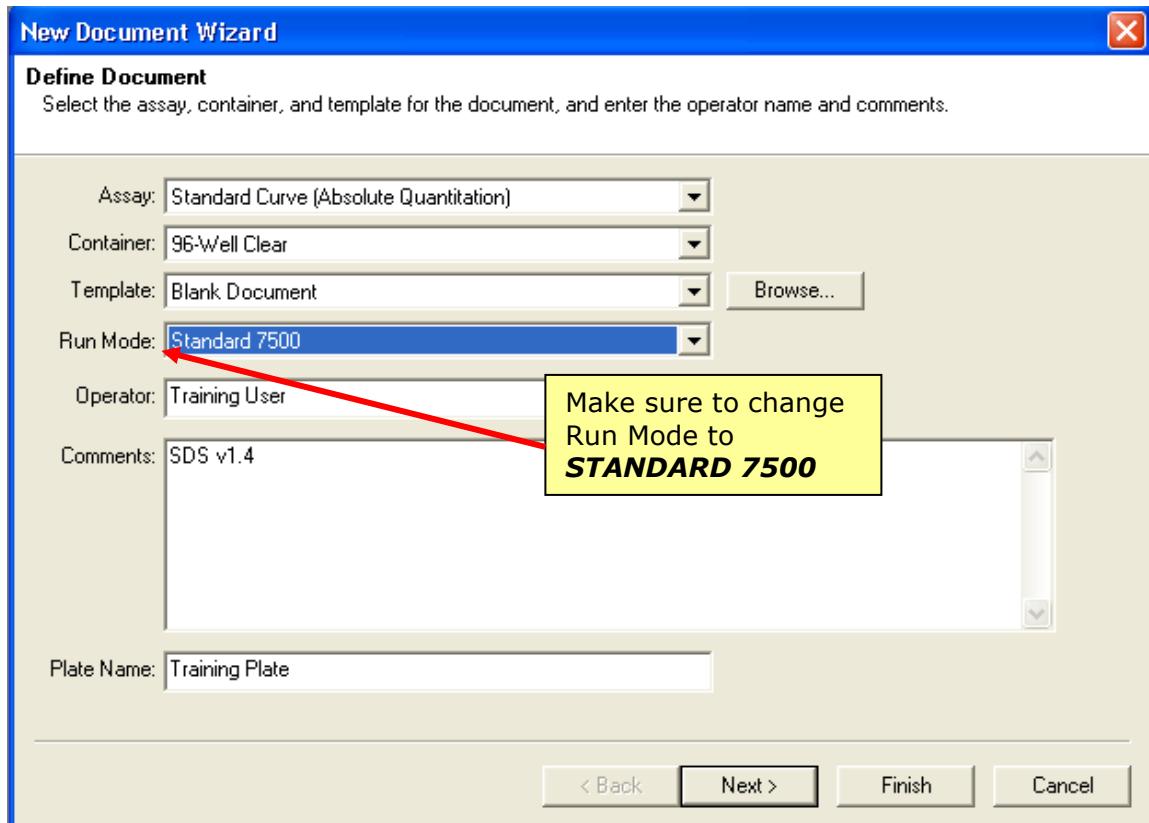
<sup>a</sup>Replace the sample in this column with extracted HSC if necessary

## 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.

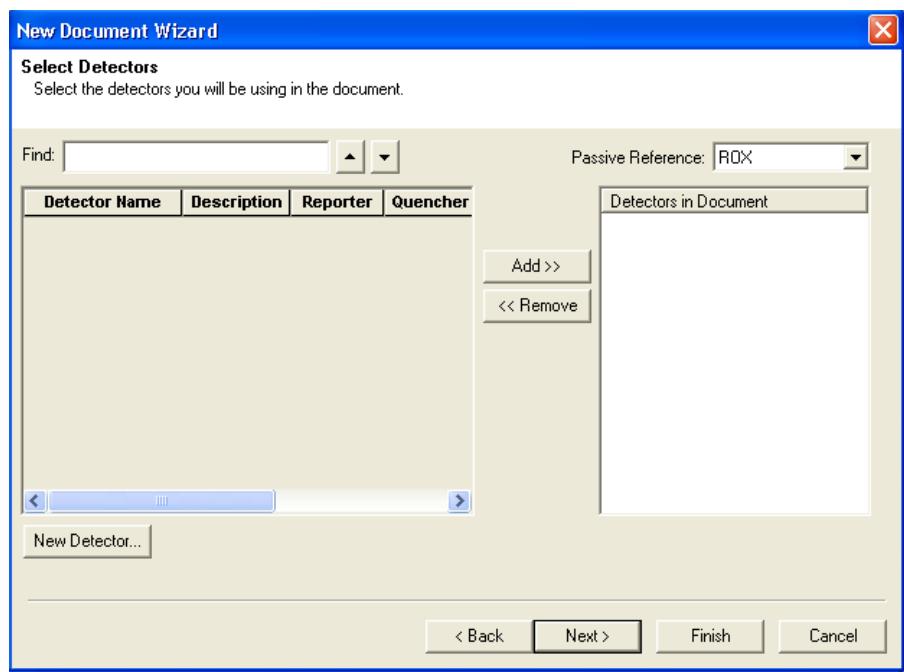
- 1) Launch the Applied Biosystems 7500 Fast Dx Real-time PCR Instrument by double clicking on the Applied Biosystems 7500 Fast Dx System icon on the desktop.
- 2) A new window should appear, select **Create New Document** from the menu.

**Figure 3. New Document Wizard Window**



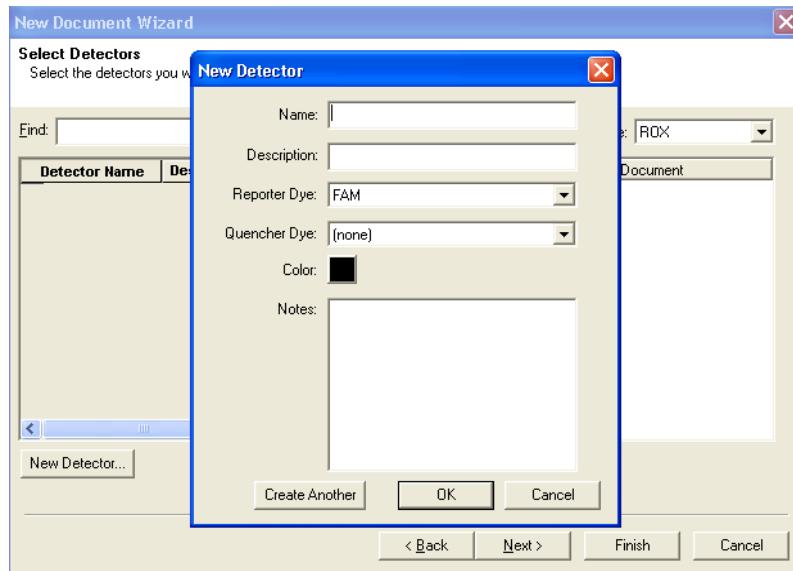
- 3) The **New Document Wizard** screen in **Figure 3** will appear. Select:
  - a. Assay: **Standard Curve (Absolute Quantitation)**
  - b. Container: **96-Well Clear**
  - c. Template: **Blank Document**
  - d. Run Mode: **Standard 7500**
  - e. Operator: **Your Name**
  - f. Comments: **SDS v1.4**
  - 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**

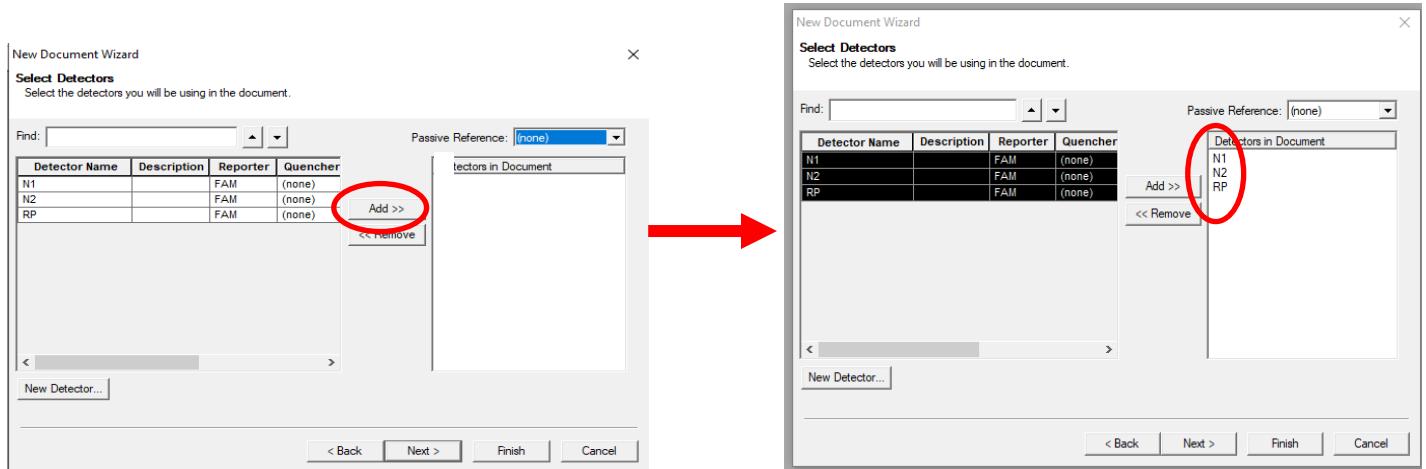


- 8) Start by creating the N1 Detector. Include the following:
  - a. Name: **N1**
  - b. Description: *leave blank*
  - c. Reporter Dye: **FAM**
  - d. Quencher Dye: **(none)**
  - e. Color: *to change the color of the detector indicator do the following:*
    - ⇒ Click on the color square to reveal 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)

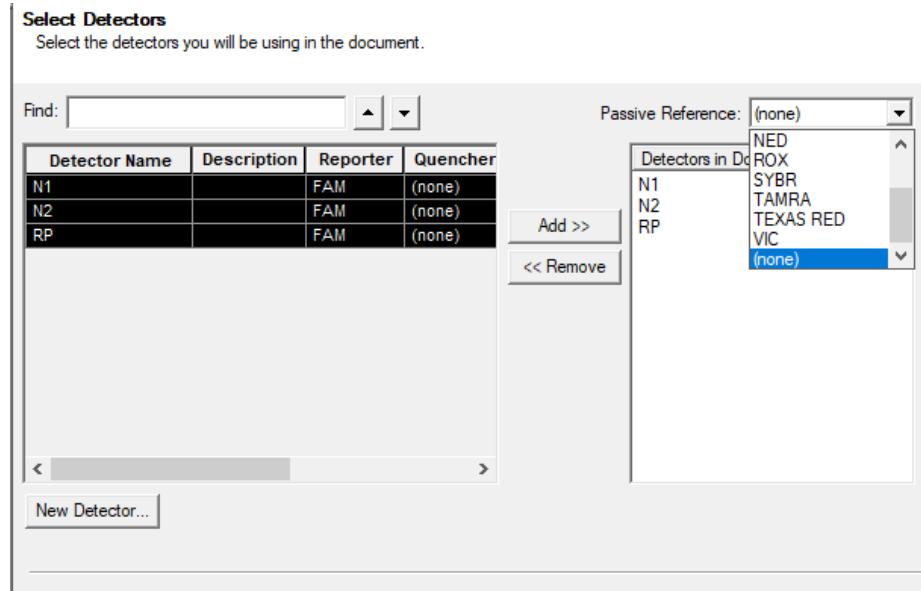
- 10) After each Detector is added, the **Detector Name**, **Description**, **Reporter** and **Quencher** fields will become populated in the **Select Detectors** screen (**Figure 6**).
- 11) Before proceeding, the newly created detectors must be added to the document. To add the new detectors to the 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**

The screenshot shows the 'Set Up Sample Plate' window. At the top, it says 'New Document Wizard' and 'Set Up Sample Plate'. Below that is a sub-instruction: 'Setup the sample plate with tasks, quantities and detectors.' The main area has two tables. The first table, titled 'Use', lists detectors N1, N2, and RP. N1 is checked and assigned FAM as the reporter. The second table is a 12x8 grid where rows are labeled A through H and columns are numbered 1 through 12. Row A is highlighted with a black border. The first column of the grid contains 'U' characters, indicating they are assigned to detector N1. The bottom of the window has buttons for '< Back', 'Next >', 'Finish', and 'Cancel'.

Use	Detector	Reporter	Quencher	Task	Quantity
<input checked="" type="checkbox"/>	N1	FAM	(none)	Unknown	
<input type="checkbox"/>	N2	FAM	(none)	Unknown	
<input type="checkbox"/>	RP	FAM	(none)	Unknown	

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

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

**Figure 9. Finished Plate Set-up**

This screenshot shows the same 'Set Up Sample Plate' window as Figure 8, but with the setup completed. The 'Use' table now shows detector RP checked and assigned FAM. The 12x8 grid shows colored 'U' characters: Row A has all 'U's. Rows B and C have some green 'U's. Row D has red 'U's. Rows E, F, G, and H are empty. The bottom buttons are '< Back', 'Next >', 'Finish', and 'Cancel'.

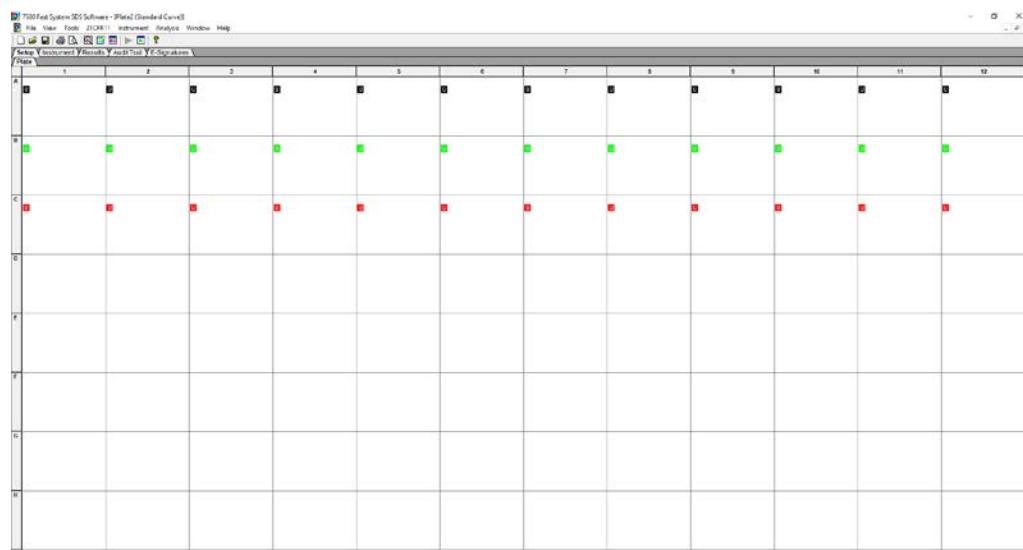
Use	Detector	Reporter	Quencher	Task	Quantity
<input type="checkbox"/>	N1	FAM	(none)	Unknown	
<input type="checkbox"/>	N2	FAM	(none)	Unknown	
<input checked="" type="checkbox"/>	RP	FAM	(none)	Unknown	

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

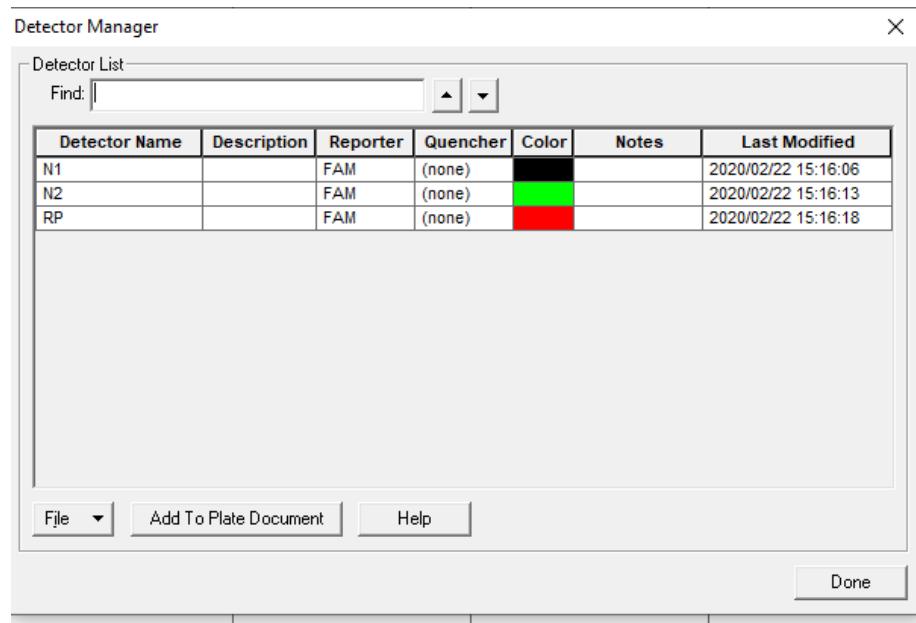
- 18) After clicking “Finish”, there will be a brief pause allowing the Applied Biosystems 7500 Fast Dx to initialize. This initialization is followed by a clicking noise. ***Note: The machine must be turned on for initialization.***
- 19) After initialization, the **Plate** tab of the Setup (**Figure 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.

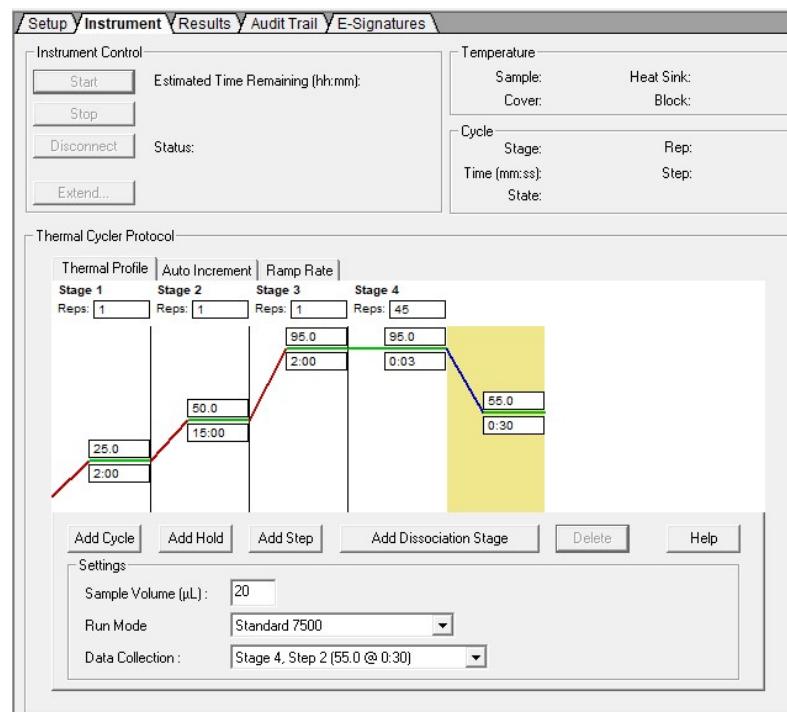
### Defining the Instrument Settings

- 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)**

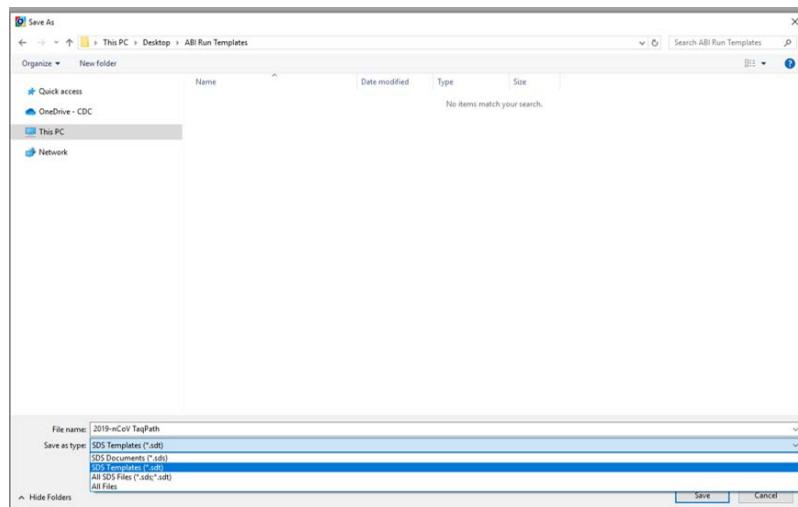
- a. In Stage 1, Set to 2 min at **25°C**; **1 Rep.**
- b. In Stage 2, Set to 15 min at **50°C**; **1 Rep.**
- c. In Stage 3, Set to 2 min at **95°C**, **1 Rep.**
- d. In Stage 4, Step 1 set to **3 sec** at **95°C**.
- e. In Stage 4, Step 2 set to **30 sec** at **55.0°C**.
- f. In Stage 4, Reps should 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**).

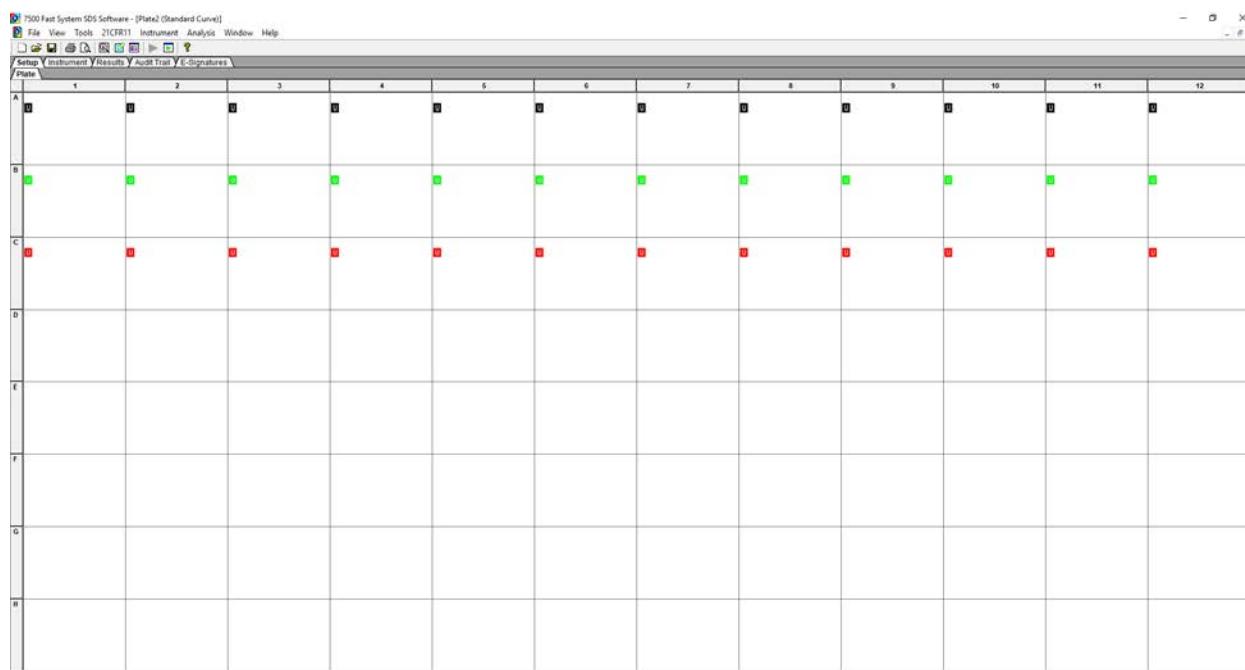
**Figure 13. Saving Template**



## 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.**

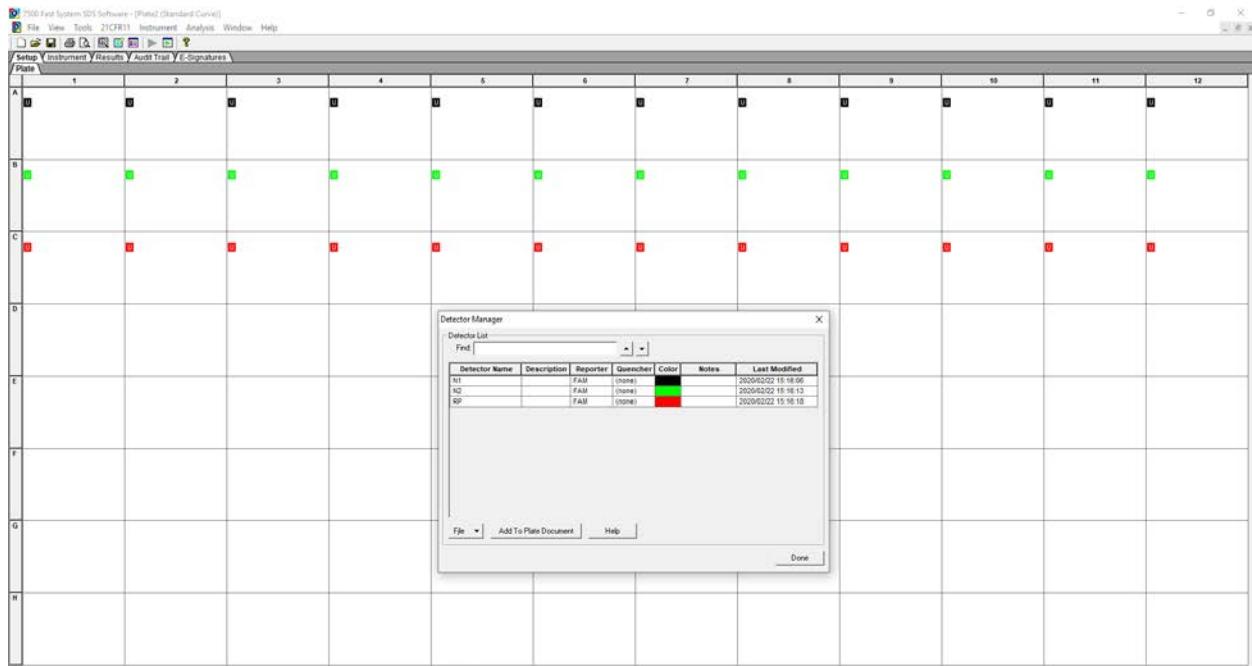
**Figure 14. Plate Set-up Window**



- 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**).

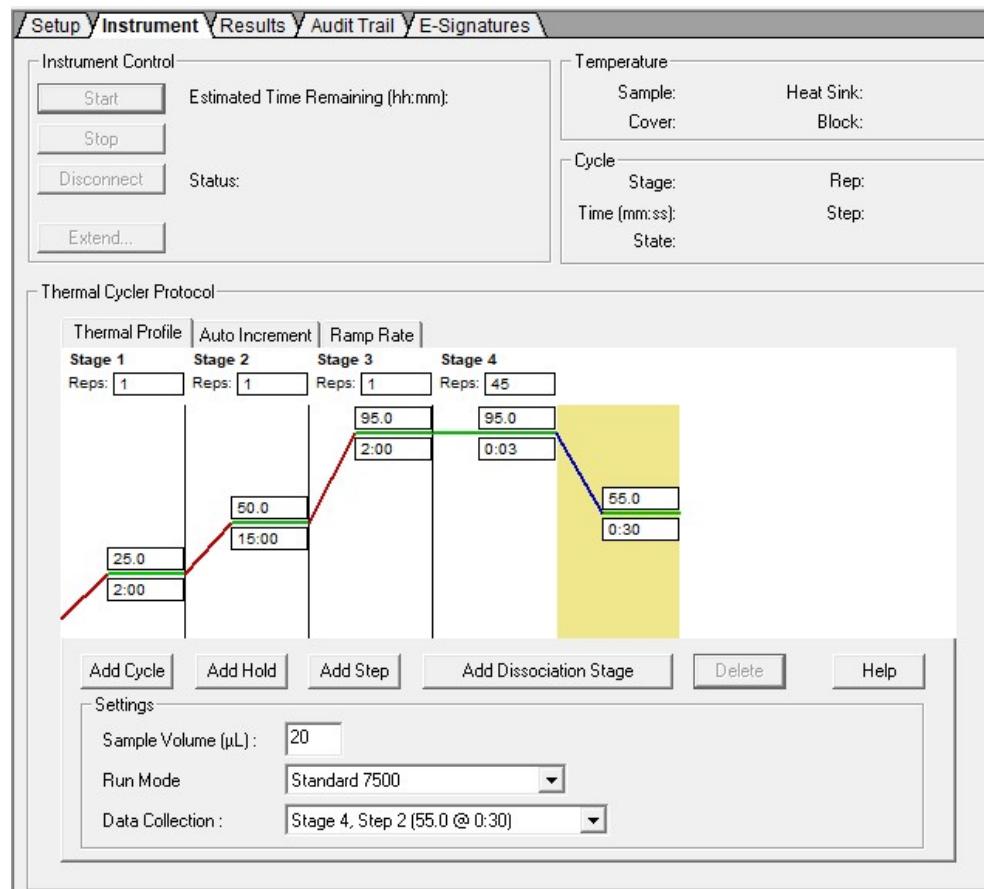
**Figure 15. Labeling Wells**



- 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**

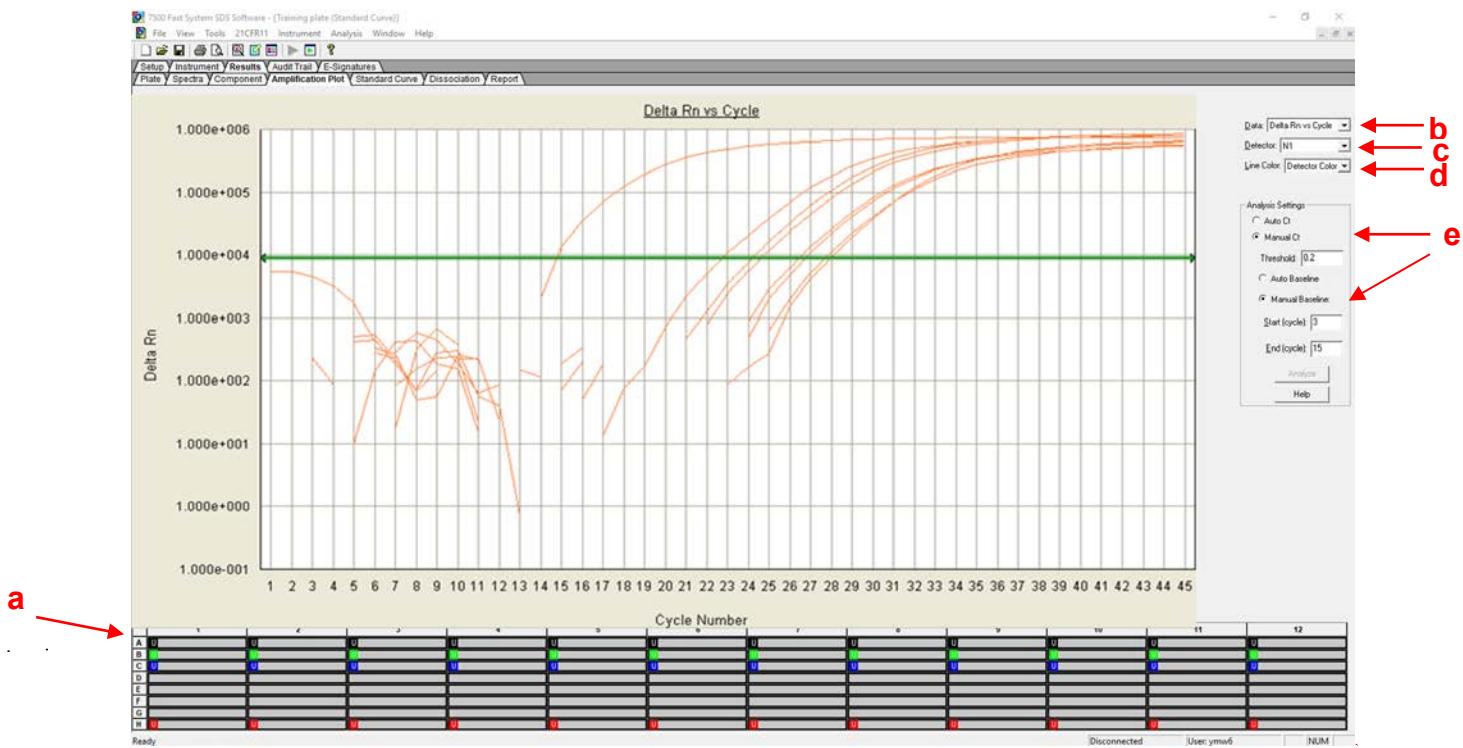


- 15) Ensure settings are correct (refer to the *Defining Instrument Settings*).
- 16) Before proceeding, the run file must be saved; from the main menu, select **File**, then **Save As**. Save in appropriate run folder designation.
- 17) Load the plate into the plate holder in the instrument. Ensure 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

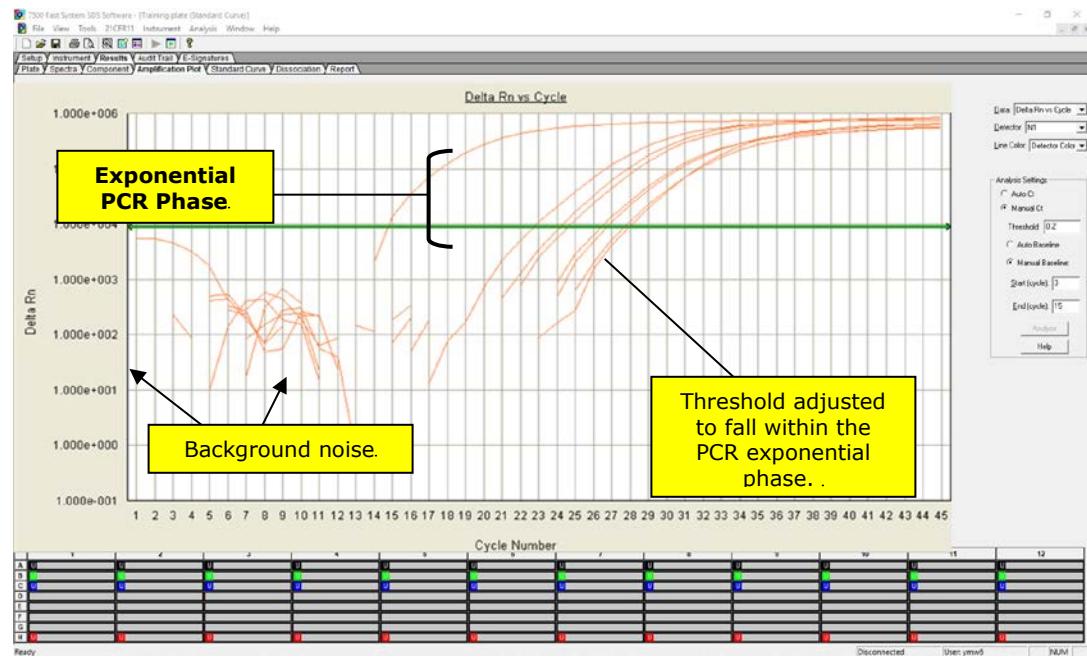
- 1) After the run has completed, select the **Results** tab at the upper left corner of the software.
- 2) Select the **Amplification Plot** tab to view the raw data (**Figure 17**).

**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**

Well	Sample Name	Detector	Task	Ct	StdDev Ct	Quantity	Mean Qty	StdDev Qty	Filtered	Tm
A1	NTC	N1	Unknown	Undet.						
A2	nGIVPC 1	N1	Unknown	20.2502						
A3	nGIVPC 2	N1	Unknown	20.4412						
A4	nGIVPC 3	N1	Unknown	20.4963						
A5	APC	N1	Unknown	25.4229						
B1	NTC	N2	Unknown	Undet.						
B2	nGIVPC 1	N2	Unknown	20.6161						
B3	nGIVPC 2	N2	Unknown	21.6637						
B4	nGIVPC 3	N2	Unknown	21.2075						
B5	APC	N2	Unknown	24.45						
C1	NTC	RP	Unknown	Undet.						
C2	nGIVPC 1	RP	Unknown	20.9166						
C3	nGIVPC 2	RP	Unknown	21.3587						
C4	nGIVPC 3	RP	Unknown	20.7028						
C5	APC	RP	Unknown	21.9999						

## 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 N2 are 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 <sup>a</sup>	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. <sup>b</sup>
-	-	-	Invalid Result	Invalid	Repeat extraction and rRT-PCR. If the repeated result remains invalid, consider collecting a new specimen from the patient.

<sup>a</sup>Laboratories should report their diagnostic result as appropriate and in compliance with their specific reporting system.

<sup>b</sup>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<sup>1</sup> 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.<sup>2</sup>
- 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

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<sup>1</sup>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."

<sup>2</sup>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](mailto: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/ $\mu$ L) 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 ThermoFisher 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  $\geq 95\%$  (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 <sup>1</sup>	10 <sup>0.5</sup>	10 <sup>0.0</sup>	10 <sup>-0.5</sup>	10 <sup>0.5</sup>	10 <sup>0.0</sup>	10 <sup>-0.5</sup>
Positives/Total	20/20	19/20	13/20	20/20	17/20	9/20
Mean Ct <sup>2</sup>	32.5	35.4	NA	35.8	NA	NA
Standard Deviation (Ct)	0.5	0.8	NA	1.3	NA	NA

<sup>1</sup> Concentration is presented in RNA copies/µL

<sup>2</sup> 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 QIAamp DSP Viral RNA Mini Kit**

Targets	2019-nCoV_N1			2019-nCoV_N2			
RNA Concentration <sup>1</sup>	10 <sup>0.5</sup>	10 <sup>0.0</sup>	10 <sup>-0.5</sup>	10 <sup>0.5</sup>	10 <sup>0.0</sup>	10 <sup>-0.5</sup>	10 <sup>-1.0</sup>
Positives/Total	20/20	20/20	6/20	20/20	20/20	20/20	8/20
Mean Ct <sup>2</sup>	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

<sup>1</sup> Concentration is presented in RNA copies/µL

<sup>2</sup> 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 <sup>0.5</sup>	10 <sup>0</sup>

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  $\geq 10^5$  PFU/mL or  $\geq 10^6$  CFU/mL). Testing was performed using the ThermoFisher 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.
<i>Mycoplasma pneumoniae</i>		isolate	0/3	0/3	Neg.
<i>Streptococcus</i>		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) <sup>1</sup>	71	1	0	0	72
Tissue (lung)	2	0	0	0	2
<b>Total</b>	<b>2021</b>	<b>49</b>	<b>0</b>	<b>1</b>	<b>2071</b>

<sup>1</sup>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 <sup>1</sup>	0
Inconclusive	0	0
Negative	0	104

<sup>1</sup>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

<b>Revision #</b>	<b>Effective Date</b>	<b>Summary of Revisions</b>
1	February 4, 2020	Original Instructions for Use
2	March 15, 2020	<ul style="list-style-type: none"><li>• Intended use update</li><li>• Removal of N3 primer and probe set from Diagnostic Panel</li><li>• Performance data update</li><li>• Addition of alternative nucleic acid extraction platforms</li><li>• Addition of acceptable alternatives to HSC and addition of QIAGEN RUO extraction reagents</li><li>• Positive results no longer presumptive. No confirmation of positive results required</li></ul>

## 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](mailto: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	$1 \times 10^4$ copies/ $\mu$ L	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**



2019-nCoV EUA-01

**CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel  
Product Information Sheet**

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](mailto:respvirus@cdc.gov).

**DISTRIBUTED BY**

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

IVD



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

**\*\*\* 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

**CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel – Verification Requirements****\*\*\* DO NOT DISCARD: Important product-specific information \*\*\***

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



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

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

- 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.



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

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

≥ 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](mailto:respvirus@cdc.gov).

### QUESTIONS

Please send questions or comments by email to [respvirus@cdc.gov](mailto: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**GGT**CAAC. 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)

<sup>1</sup>Naganori Nao, <sup>1</sup>Kazuya Shirato, <sup>2</sup>Harutaka Katano, <sup>1</sup>Shutoku Matsuyama, and <sup>1</sup>Makoto Takeda

<sup>1</sup>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; <sup>2</sup>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 manufacturer'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 1<sup>st</sup> PCR, nested PCR was performed using 2<sup>nd</sup> PCR primers and 1μL of 1<sup>st</sup> PCR product under the same condition as 1<sup>st</sup> 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).

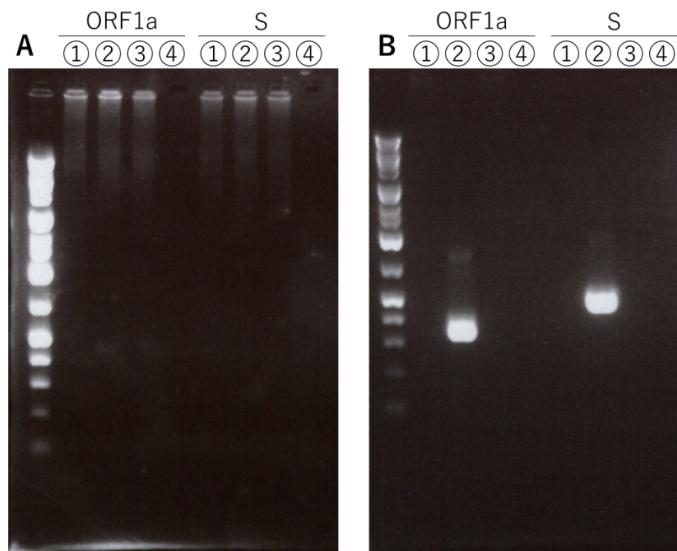


Figure 1. Three specimens were tested this time. Agarose gel electrophoresis of 1<sup>st</sup> (A) and 2<sup>nd</sup> (B) PCR reaction. DNA marker (Gene Ladder Wide 1, Nippon gene) was used as a reference for fragment size. Lane 1 and 3 were negative clinical samples, lane 2 was positive clinical sample,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

Table 1. Primer used for 2019-nCoV.

No.		Name	direction	sequence (5' to 3')	Expected size (bp)
<b>ORF1a set</b>					
1	1 <sup>st</sup>	NIID_WH-1_F501	Sense	TTCGGATGCTCGAACTGCACC	413
2	1 <sup>st</sup>	NIID_WH-1_R913	Antisense	CTTTACCAGCACGTGCTAGAAGG	
3	2 <sup>nd</sup>	NIID_WH-1_F509	Sense	CTCGAACTGCACCTCATGG	346
4	2 <sup>nd</sup>	NIID_WH-1_R854	Antisense	CAGAACGTTGTTATCGACATAGC	
5	Seq	NIID_WH-1_Seq_F519	Sense	ACCTCATGGTCATGTTATGG	
6	Seq	NIID_WH-1_Seq_R840	Antisense	GACATAGCGAGTGTATGCC	
<b>S set</b>					
7	1 <sup>st</sup>	WuhanCoV-spk1-f	Sense	TTGGCAAAATTCAAGACTCACTT	547
8	1 <sup>st</sup>	WuhanCoV-spk2-r	Antisense	TGTGGTTCATAAAATTCCCTTG	
9	2 <sup>nd</sup>	NIID_WH-1_F24381	Sense	TCAAGACTCACTTCTTCCAC	493
10	2 <sup>nd</sup>	NIID_WH-1_R24873	Antisense	ATTTGAAACAAAGACACCTTCAC	
11	Seq	NIID_WH-1_Seq_F24383	Sense	AAGACTCACTTCTCCACAG	
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	AAATTTGGGGACCAGGAAC	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](mailto: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](http://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*.

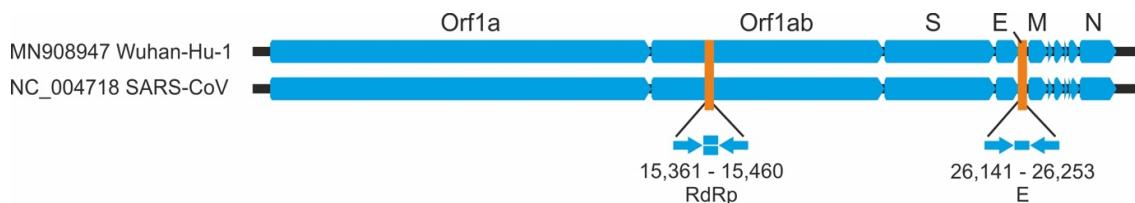
## Background

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

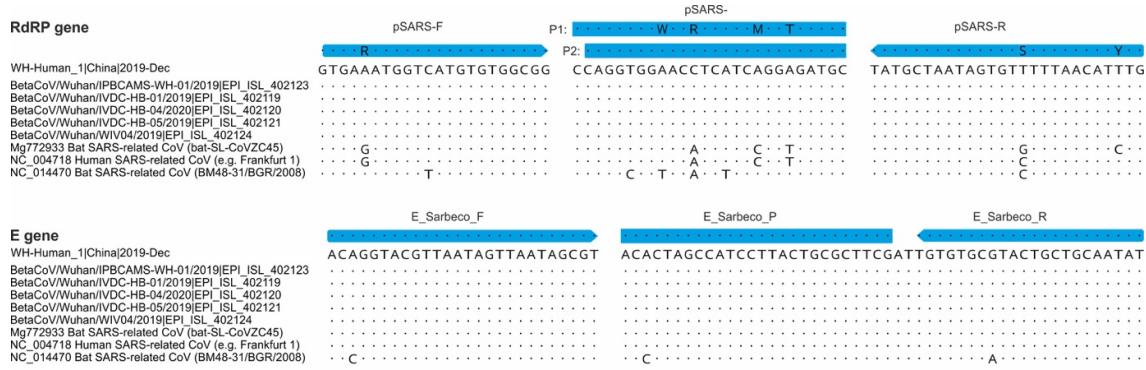
Respiratory samples were obtained during 2019 from patients hospitalized at Charité medical center and tested by the NxTAG® Respiratory Pathogen Panel (Luminex) or in cases of MERS-CoV by the MERS-CoV upE assay as published before (1).

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- $\mu$ l reaction was set up containing 5  $\mu$ l of RNA, 12.5  $\mu$ l 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  $\mu$ l of reverse transcriptase/Taq mixture from the kit, 0.4  $\mu$ l of a 50 mM magnesium sulfate solution (Invitrogen – not provided with the kit), and 1  $\mu$ g 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 ( $\mu$ M) primer stock solution per 25 microliter ( $\mu$ l) 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-

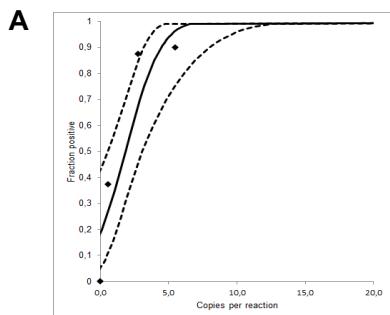
Assay/ Use	Oligonucleotide ID	Sequence (5'-3')	Comment
RdRP gene	RdRP_SARSr-F2	GTGARATGGTCATGTGTGGCGG	use 600 nM per reaction
	RdRP_SARSr-R1	CARATGTTAAASACACTATTAGCATA	use 800 nM per reaction
	RdRP_SARSr-P2	FAM-CAGGTGGAACCTCATCAGGAGATGC-BBQ	Specific for 2019-nCoV, will not detect SARS-CoV use 100 nM 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**

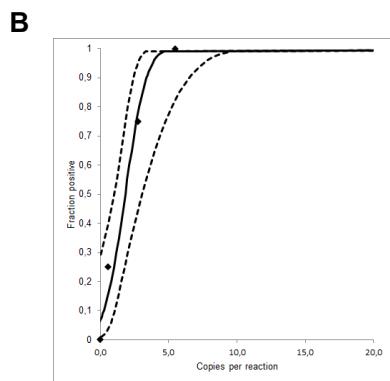
Preliminary assessment of analytical sensitivity for RdRp assay.

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. 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.

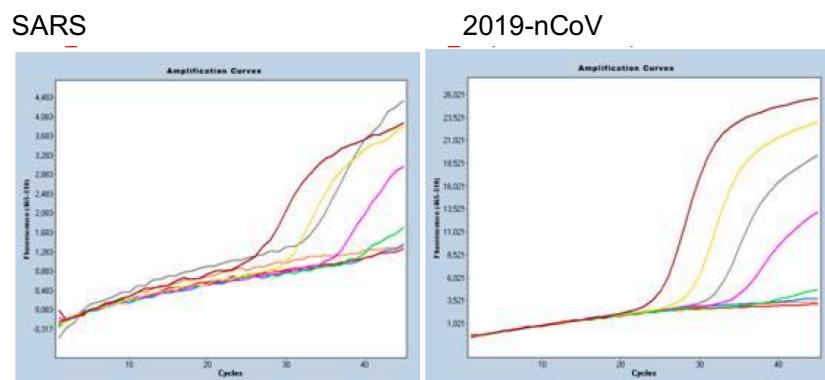


#### **B. Confirmatory assay: RdRP gene**

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.



**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**

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

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

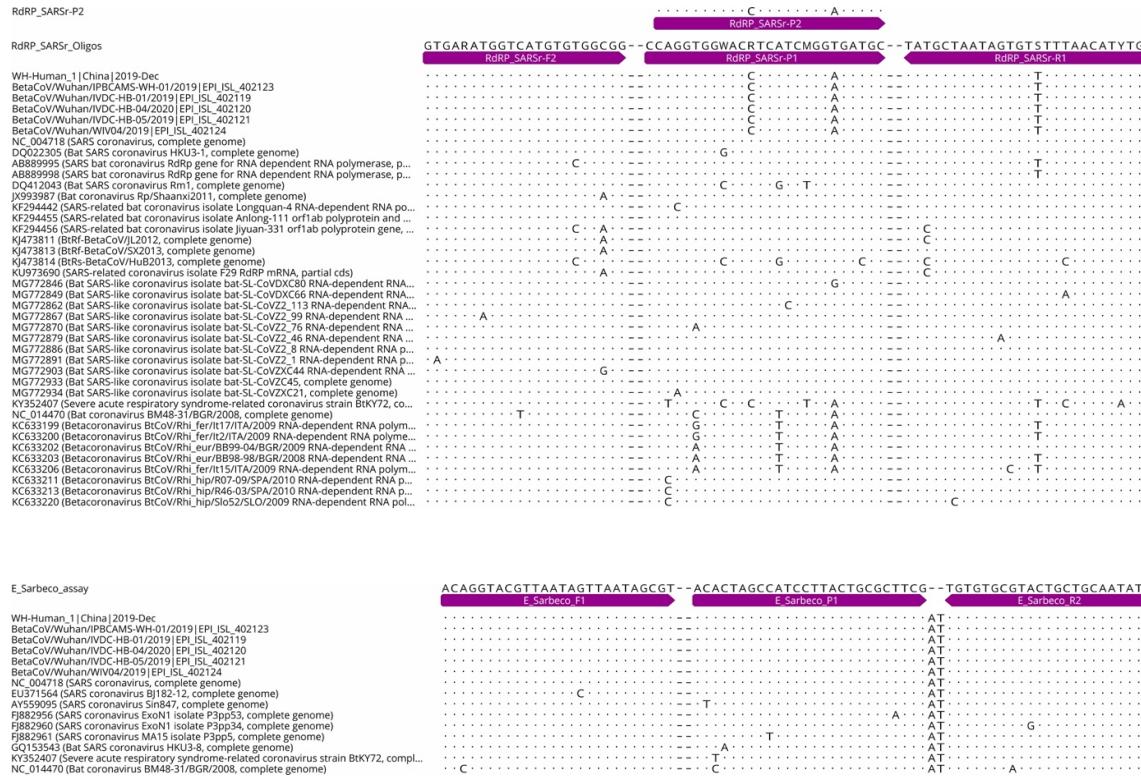
Both assays were applied on human clinical samples from our own diagnostic services, previously tested positive for the viruses listed in Table 3. All tests returned negative results.

**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
<b>Total clinical samples</b>	<b>75</b>

## 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:

<u>MasterMix:</u>	<u>Per reaction</u>	
H <sub>2</sub> O (RNase free)	2.6 µl	
2x Reaction mix*	12.5 µl	
MgSO <sub>4</sub> (50mM)	0.4 µl	
BSA (1 mg/ml)**	1 µl	
Primer E_Sarboco_F1 (10 µM stock solution)	1 µl	ACAGGTACGTTAACAGTTAATAGCGT
Primer E_Sarboco_R2 (10 µM stock solution)	1 µl	ATATTGCAGCAGTACGCACACA
Probe E_Sarboco_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

\*\* MgSO<sub>4</sub> (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 1 is positive, continue to assay No 2.

## 2. Confirmatory assay

### RdRp assay:

<u>MasterMix:</u>	<u>Per reaction</u>	
H <sub>2</sub> O (RNase free)	0.6 µl	
2x Reaction mix*	12.5 µl	
MgSO <sub>4</sub> (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

\*\* MgSO<sub>4</sub> (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. Discrimatory assay

#### RdRp assay:

<u>MasterMix:</u>	<u>Per reaction</u>	
H <sub>2</sub> O (RNase free)	1.1 µl	
2x Reaction mix*	12.5 µl	
MgSO <sub>4</sub> (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

\*\* MgSO<sub>4</sub> (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

**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**  
**香港大學公共衛生學院**

### **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 (ThermoFisher, 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'-TGGGGYTTACRGGTAAACCT-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'-TAATCAGACAAGGAAC TGATTA-3'

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

Probe (HKU-NP): 5'-FAM-GCAAATTGTGCAATTGCGG-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:

<b><u>Reagent</u></b>	<b><u>Vol for a single rxn (μl)</u></b>
H <sub>2</sub> O (RNase free)	8.5
4x Reaction mix*	5
Forward primer (10 μM)	1
Reverse primer (10 μM)	1
Probe (10 μM)	0.5
<u>RNA sample</u>	<u>4</u>
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	5:00	
95	0:20	1
95	0:05	
60	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<sub>50</sub>/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.

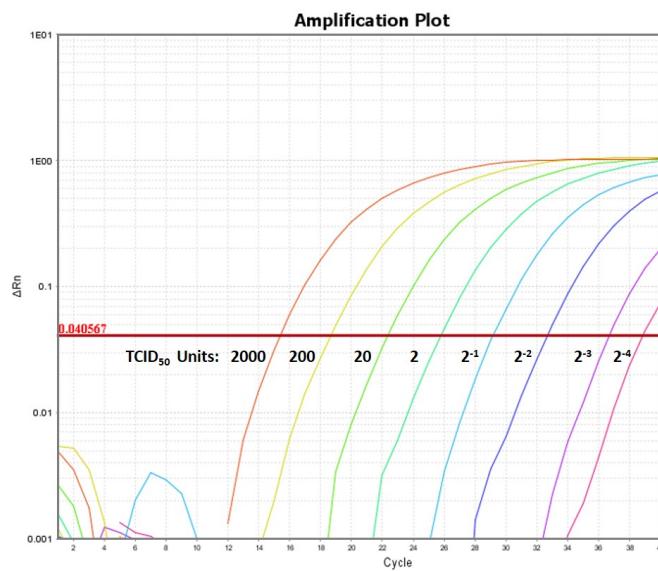


Figure. Amplification plot of the RT-PCR assay specific for N gene.  
The viral titre (TCID<sup>50</sup>) used in each reaction is shown as indicated.

*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 ([lmpoon@hku.hk](mailto:lmpoon@hku.hk)) or Malik Peiris ([malik@hku.hk](mailto: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)

<b>Primer</b>	<b>Sequence (5' → 3')</b>	<b>Working conc.</b>
WH-NIC N-F	CGTTGGTGGACCCTCAGAT	40 µM
WH-NIC N-R	CCCCACTGCGTTCTCCATT	40 µM
WH-NIC N-P	FAM-CAACTGGCAGTAACCA-BHQ1	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.

<b>Reagents</b>	<b>microliter/r</b>	<b>Thermal cycler condition</b>
-----------------	---------------------	---------------------------------

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