

# Manual for the Detection of Pathogen 2019-nCoV Ver.2.6

February 17, 2020

Identify the novel coronavirus (2019-nCoV) by genetic testing, a 2-step RT-PCR method to specifically detect the two gene regions of 2019-nCoV, i.e., open reading frame 1a (ORF1a) and spike (S), or a real time one-step RT-PCR method using the TaqMan Probe.

## [How to operate]

### 1. Collection and storage of specimens

Please refer to “Manual of Collection/Transportation of Specimens Obtained from Patients Suspected of Having 2019-nCoV (Novel Coronavirus) Infection” (HP of the National Institute of Infectious Diseases).

For handling of sputum specimens, please refer to the appendix “Pretreatment Methods of Sputum Specimens.”

### 2. RNA extraction

A method using the widely used QIAamp Viral RNA Mini Kit is described; however, other viral RNA extraction kits can be used.

#### 2.1. Materials, devices, instruments and reagents

##### 1) Devices/instruments

Refrigerated centrifuge, high-speed refrigerated centrifuge for 1.5 mL Eppendorf tubes, desktop centrifuge for 1.5 mL Eppendorf tubes, vortex mixer, and tubes

##### 2) Reagents

QIAamp Viral RNA Mini Kit (QIAGEN, Cat.No.52904), ethanol, distilled water (deionized, sterile, autoclaved, DNase free, RNase free, Wako Pure Chemical Industries, Ltd., Cat No. 318-90105, etc. (hereinafter referred to as DDW), and positive control RNA

#### 2.2. Precautions for use

1) Specimens should be handled at biosafety level 2+. Handle specimens in a safety cabinet and wear personal protective equipment (PPE), i.e., disposable gown/cap/gloves/mask during operation. Perform centrifugation after opening the cover of the tube, and use a tube opener, etc. to prevent spreading of air from the tube as far as possible.

2) Close attention should be paid to prevent genetic contamination in laboratories and contamination of RNase. To prevent contamination, it is desirable to use physically separate places for preparation of reagents and for handling of samples such as the PCR product. If impossible, perform these operations in separate cabinets.

#### 2.3. RNA extraction with QIAamp Viral RNA Mini Kit

1) Preparation of reagents before use, etc.

(1) Let the sample return to room temperature (15-25°C).

(2) Preparation of 1 µg/µL of carrier RNA solution

Add 310 µL of buffer AVE to a tube containing 310 µg of carrier RNA (freeze-dried product) to prepare a 1 µg/µL solution. Carrier RNA solution should be stored at -20°C and to avoid repeated freezing-and-thawing cycles (up to three times), divide into appropriate amounts and store. If the Buffer AVL is precipitated, incubate it at 80°C, dissolve the precipitate, and then use it for preparation.

(3) Preparation of the mixture of Buffer AVL/carrier RNA

Prepare a mixture of Buffer AVL/carrier RNA to make 560 µL of Buffer AVL, 5.6 µL of carrier RNA solution per sample (for details, refer to the Handbook Table 1 enclosed with the kit). Since precipitate is generated when the mixture is stored at 2-8°C, incubate at 80°C immediately before use to dissolve it. This incubation should take less than 5 minutes. It is also convenient to dispense the mixture of Buffer AVL/carrier RNA into 560 µL portions each in advance and store them at -20°C.

(4) Preparation of buffer AW1, buffer AW2

Add 25 mL of 96-100% ethanol to Buffer AW1 (Kit Cat.No.51104).

Add 30 mL of 96-100% ethanol to Buffer AW2 (Kit Cat.No.51104).

## 2) Operating procedure

Perform all of the following procedures at room temperature.

(1) Put 560 µL of Buffer AVL/carrier RNA into a 1.5 mL tube.

(2) Operate the vortex mixer for 15 seconds to fully mix 140 µL of the specimen and buffer and leave it for 10 minutes at room temperature (15-25°C). Centrifuge with a desktop centrifuge for a few seconds to remove the liquid that adheres to the wall of the tube, etc. (spin-down)

(3) Add 560 µL of ethanol (96-100%) to a tube, operate the vortex mixer for 15 seconds, and then spin the tube down.

(4) Put 630 µL of the liquid in (3) in the QIAamp Spin Column (in a 2 mL collection tube), close the cover, and centrifuge at  $6,000 \times g$  (8,000 rpm) for 1 minute. Transfer the QIAamp spin column in a new 2 mL collection tube, put 630 µL of the remaining liquid in (3), and centrifuge it similarly to run the entire amount of liquid out (this operation is completed the second time).

(5) Open the QIAamp spin column and put 500 µL of buffer AW1 in. Close the cover and centrifuge at  $6,000 \times g$  (8,000 rpm) for 1 minute. Transfer the QIAamp spin column to a new 2 mL collection tube and dispose of the tube containing the filtrate.

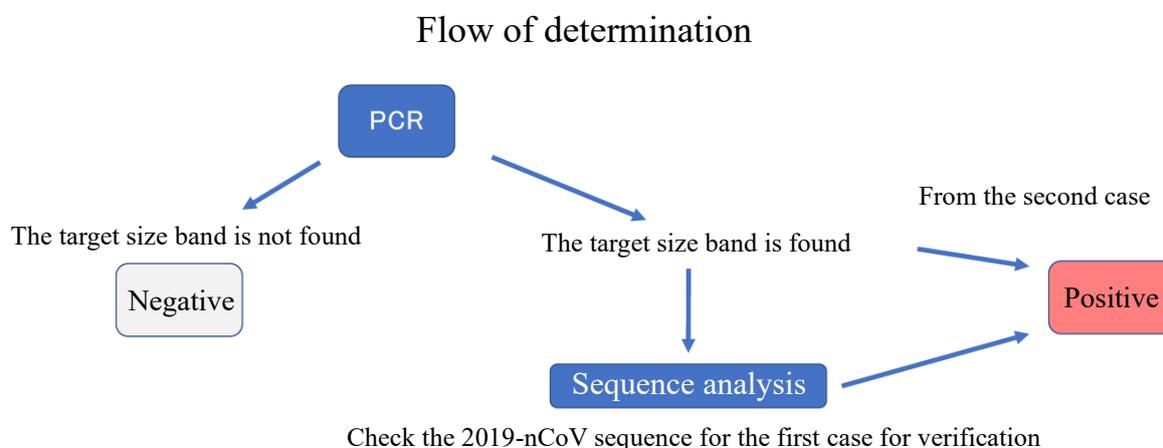
(6) Open the QIAamp spin column and put 500 µL of buffer AW2 in. Close the cover and centrifuge at  $20,000 \times g$  (14,000 rpm) for 3 minutes. Gently remove so that the spin column and filtrate, etc. do not make contact with each other. Perform (7) if they have made contact with each other.

(7) Transfer the QIAamp spin column to a new 2 mL collection tube and dispose of the tube containing the filtrate. Perform centrifugation at full speed ( $20,000 \times g$ ) for 1 minute.

(8) Transfer the QIAamp spin column to a new 1.5 mL tube with a cover and dispose of the tube containing the filtrate. Open the cover of the QIAamp spin column and put 60  $\mu$ L of the buffer AVE that has returned to room temperature; centrifuge at  $6,000 \times g$  (8,000 rpm) for 1 minute after it has been left for 1 minute with the cover closed, and collect the filtrate. It is desirable to store the extracted RNA at  $-80^{\circ}\text{C}$ .

### 3. Qualitative detection of 2019-nCoV by 2-step RT-PCR method

A diagrammatic overview of the test/determination of the result is shown below.



For example, the conditions for the reaction using SuperScript IV Reverse Transcriptase (Thermo) and Quick Taq HS Dymix (Toyobo) are shown. For the details, please refer to the manual enclosed with the kit. The protocol has also partly been changed in this manual. For the enzymes, etc. used for the reverse transcription and PCR reaction, similar products with use experience at each facility can be used. It is desirable to dispense all reagents, etc. on ice.

#### 3.1 Required instruments and reagents

##### 1) Instruments

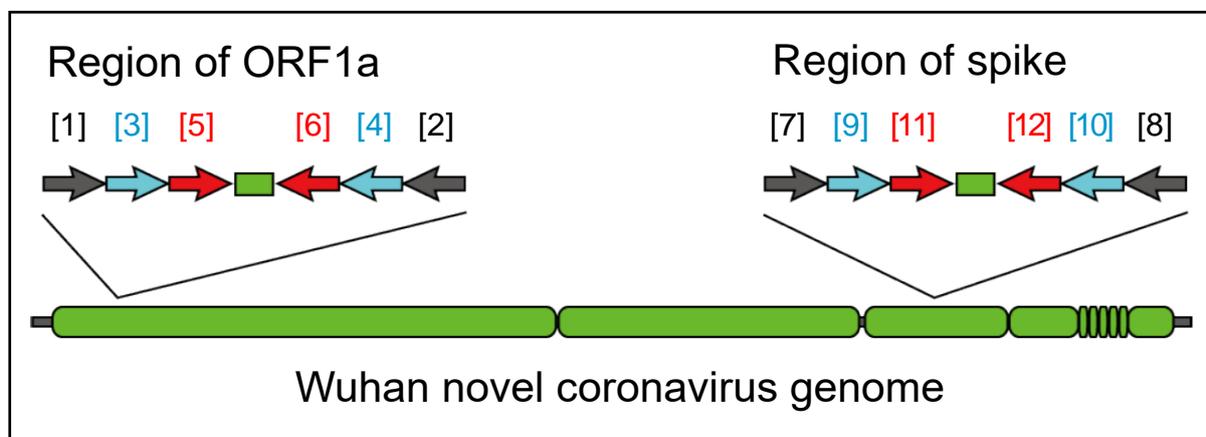
Thermal cycler, micropipette, tube, electrophoresis tank

##### 2) Reagents

SuperScript IV Reverse Transcriptase (RT) [Thermo, Cat.No. 18090010. 50, 200 or a similar product (e.g., PrimeScript RT reagent Kit, Takara RR037A)], [Quick Taq HS Dymix Toyobo DTM-101 or a similar product (e.g., PerfectShot Ex Taq, Takara RR005A)], 2019-nCoV-specific primer, oligo (dT) 12-18 primer (Thermo, 18418012 or a similar product), Random Hexamers (Thermo, N8080127, or a similar product. Not specified), Recombinant RNase Inhibitor (Takara-Bio, 2313A, or a similar product), PCR clean-up kit (Wizard SV Gel and PCR Clean-Up System, Promega A9281, or a similar product), DDW.

No.		Name	direction	sequence (5' to 3')	Expected size (bp)
ORF1a set					
[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	CAGAAGTTGTTATCGACATAGC	
[5]	Seq	NIID_WH-1_Seq_F519	Sense	ACCTCATGGTCATGTTATGG	
[6]	Seq	NIID_WH-1_Seq_R840	Antisense	GACATAGCGAGTGTATGCC	
S set					
[7]	1 <sup>st</sup>	WuhanCoV-spk1-f	Sense	TTGGCAAATTCAAGACTCACTTT	547
[8]	1 <sup>st</sup>	WuhanCoV-spk2-r	Antisense	TGTGGTTCATAAAAATTCTTTGTG	
[9]	2 <sup>nd</sup>	NIID_WH-1_F24381	Sense	TCAAGACTCACTTTCTTCCAC	493
[10]	2 <sup>nd</sup>	NIID_WH-1_R24873	Antisense	ATTTGAAACAAAGACACCTTCAC	
[11]	Seq	NIID_WH-1_Seq_F24383	Sense	AAGACTCACTTTCTTCCACAG	
[12]	Seq	NIID_WH-1_Seq_R24865	Antisense	CAAAGACACCTTCACGAGG	

### Diagrammatic primer position pattern



### 3.2 Synthesis of 1st strand cDNA

- 1) Positive controls dedicated to the ORF1a and the S set should be used. Add 990  $\mu\text{L}$  of DDW to 10  $\mu\text{L}$  of the positive control RNA diluted to  $10^5/\mu\text{L}$ .
- 2) Add an equivalent amount of DDW to 5  $\mu\text{L}$  of the positive control RNA in 1) to make 10  $\mu\text{L}$  and use it as follows (use 5,000 copies). If it seems difficult to detect 5,000 copies, investigate in advance and conduct for a detectable concentration.
- 3) Prepare the reaction solutions shown in the table using the extracted RNA solution and the positive control RNA, and put each in a 0.2 mL tube respectively.

Table 1 Preparation of the reaction solution of reverse transcription

5 × SSIV Buffer	5 μL
Random Hexamers	1 μL
Oligo(dT)12-18 Primer	1 μL
dNTP (2mM each)	5 μL
100 mM DTT	1 μL
Recombinant RNase Inhibitor	1 μL
SuperScript IV Reverse Transcriptase	1 μL
RNA	10 μL
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Total	25 μL

4) React according to the following program with a thermal cycler:

23°C	10 min
50°C	10 min
80°C	10 min

5) Add 35 μL of DDW to dilute after the reaction and use for the next PCR reaction.

### 3.3 1st PCR reaction

1) Adjust the reaction solutions as follows:

2 × Quick Taq HS DyeMix	25 μL
Forward primer (50 μM)	0.4 μL
Reverse primer (50 μM)	0.4 μL
DDW	19.2 μL
cDNA	5 μL
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Total	50 μL

[1] NIID\_WH-1\_F501 and [2] NIID\_WH-1\_R913 for the ORF1a set, and Use [7] WuhanCoV-spk1-f and [8] WuhanCoV-spk2-r for the S set.

2) Use 5 μL of DDW as the negative control.

3) Set the conditions of reaction as follows and conduct the PCR reaction:

94°C	1 min	} 40 cycles
94°C	30 sec	
56°C	30 sec	
68°C	1 min	
68°C	5 min	

### 3.4 2nd PCR reaction

1) Adjust the reaction solution as follows.

2 × Quick Taq HS DyeMix	25 μL
Forward primer (50 μM)	0.4 μL
Reverse primer (50 μM)	0.4 μL

DDW	23.2 $\mu$ L
1st PCR reaction solution	1 $\mu$ L
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Total	50 $\mu$ L

Use [3] NIID\_WH-1\_F509 and [4] NIID\_WH-1\_R854 for the ORF1a set, and [9] NIID\_WH-1\_F24381 and [10] NIID\_WH-1\_R24873 for the S set.

2) Use 1  $\mu$ L of DDW as negative control. One  $\mu$ L of the 1st PCR product of the negative control may be used. It has been confirmed that nonspecific amplification does not occur in either.

3) Set the conditions of the reaction as follows and conduct the PCR reaction:

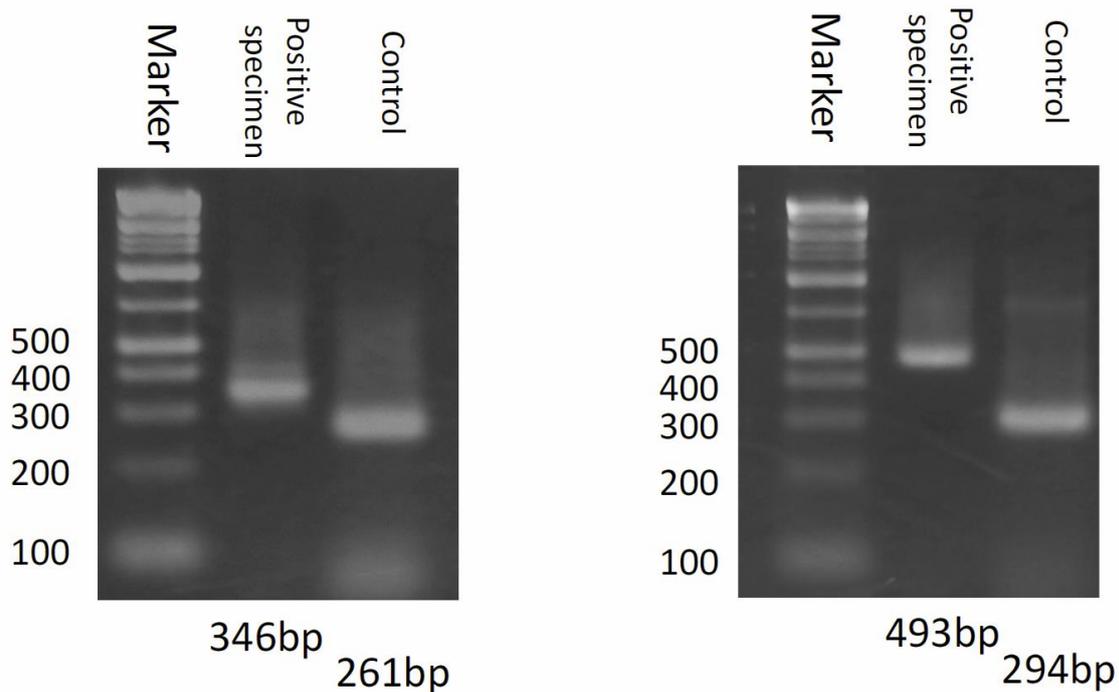
94°C	1 min	} 40 cycles
94°C	30 sec	
56°C	30 sec	
68°C	1 min	
68°C	5 min	

4) After the end of the reaction, 5  $\mu$ L of the amplification product is used for electrophoresis of the 1st PCR and 2nd PCR reaction solutions with 2% agarose gel (Agarose ME, Iwai Chemicals or an equivalent product) using TAE buffer (e.g., Nippon Gene); after staining with ethidium bromide (or a substitute), the presence or absence of bands is checked. Electrophoresis of the 1st PCR reaction solution may only be performed after the 1st PCR. Be sure to place a marker in the electrophoresis to be able to check the size of the band. It is recommended to use 2-4% agarose gel; however, other concentrations may also be used if the size of the band can be checked.

The sizes of amplification for positive control are as follows:

ORF1a set	1st PCR	292 bp
	2nd PCR	261 bp
S set	1st PCR	329 bp
	2nd PCR	294 bp

## Reference electrophoretogram of 2019-nCoV nested PCR



Electrophoresis of the PCR product of nested PCR for positive specimens and control show the following positional relationship.

5) A test is valid if a band of the target size is detected in a positive control but not in a negative control. A test is determined to be positive if a band of a size close to the target size is detected in the second PCR. Otherwise, the test is determined to be negative. Although the test is considered to be positive if a band is found in nested RT-PCR, it is recommended to conduct a sequence analysis for detection of the first case in each facility. Sequence analysis is unnecessary for the second and subsequent cases if a 2019-nCoV sequence is confirmed in the amplification product of the first case. The sensitivity of detection of this nested RT-PCR is estimated to be around 3 copies based on the number of copies in the clinical specimens for which the gene sequence analysis was successful.

### 3.5. Clean-up of PCR products (in sequence analysis)

Conduct sequence analysis after clean-up of the PCR product. An example of the use of Wizard SV Gel and PCR Clean-Up System is shown. Other systems of clean-up (e.g., Agencourt AMPure XP) can be used.

- 1) Mix the remaining PCR product (45  $\mu$ L) with the equivalent amount (45  $\mu$ L) of the membrane binding solution to that of the PCR product.
- 2) Insert the SV minicolumn into the collection tube, apply 1), culture for 1 minute at room temperature, and centrifuge at  $16,000 \times g$  for 1 minute.
- 3) Dispose of the waste liquid in the collection tube, return the SV minicolumn, apply 700  $\mu$ L of membrane wash solution with added ethanol, and centrifuge at  $16,000 \times g$  for 1 minute.

- 4) Dispose of the waste liquid in the collection tube, return the SV minicolumn, apply 500  $\mu$ L of membrane wash solution with added ethanol, and centrifuge at  $16,000 \times g$  for 5 minutes.
- 5) Dispose of the waste liquid in the collection tube, return to the SV minicolumn, and centrifuge at  $16,000 \times g$  for 1 minute.
- 6) Insert the SV minicolumn into a 1.5 mL tube, apply 30  $\mu$ L of TE buffer (or DDW), culture for 1 minute at room temperature, and centrifuge at  $16,000 \times g$  for 1 minute. Use the eluate for sequence analyses.

### 3.6. Sequence analyses (if conducted)

Use [5] NIID\_WH-1\_Seq\_F519 and [6] NIID\_WH-1\_Seq\_R840 for the ORF1a set, and [11] NIID\_WH-1\_Seq\_F24383 and [12] NIID\_WH-1\_Seq\_R24865 for the S set to conduct cycle sequence reactions. Conduct sequence analyses according to the specified method.

After sequences are obtained, conduct blast analysis, etc. and compare the results with the sequence of MN908947. If the results are almost consistent (approximately 95% or more), the test result is determined to be positive. If it is inconsistent, the test result is determined to be negative in case the human chromosome sequence is obviously different from that of the 2019-nCoV. If clear sequences cannot be obtained, for example, overlapped waves in sequence analysis, reconduct the sequence analysis from the cycle sequence considering maintenance condition of the sequencer. If clear sequences cannot be obtained in the second analysis, a certain nonspecific amplification is highly likely to exist, and the test result is determined to be negative.

- The test result is determined to be positive if a 2019-nCoV sequence is found either in the ORF1a set or the S set.

## 4. Detection of 2019-nCoV by the one-step RT-PCR method with the TaqMan Probe

### 4.1. Machinery and reagents

Use of the micropipettes (10, 20, 200, and 1,000  $\mu$ L), DDW, sterile microcentrifuge tubes (1.5 mL), 96-well real time PCR reaction plates, etc., as well as the 8-strip cap or plate seal, real time PCR device, primer, TaqMan Probe, QuantiTect<sup>®</sup> Probe RT-PCR Kit (QIAGEN Cat#204443) [AgPath-ID One-step RT-PCR Reagents (Thermo Cat# AM1005), TaqMan Fast Virus 1-Step Master Mix (Thermo Cat# 4,444,432) with LightCycler have been confirmed, and there are reports of operation with ABI instruments. These can be used with other agents if the detection sensitivity is assured.

### 4.2. Primer and probe for real time RT-PCR

N set

	Name	Sequence (5' to 3')	Position*	Concentration
(1)	N_Sarbeco_F1	CACATTGGCACCCGCAATC	28723-28741	600 nM

(2)	N_Sarbeco_R1	GAGGAACGAGAAGAGGCTTG	28850-28831	800 nM
(3)	N_Sarbeco_P1	FAM-ACTTCCTCAAGGAACAACATTGCCA-BHQ (or QSY**)	28770-28794	200 nM

Length of the amplification product 128 bp

Reference:

[https://www.who.int/docs/default-source/coronaviruse/wuhan-virus-assay-v1991527e5122341d99287a1b17c111902.pdf?sfvrsn=d381fc88\\_2](https://www.who.int/docs/default-source/coronaviruse/wuhan-virus-assay-v1991527e5122341d99287a1b17c111902.pdf?sfvrsn=d381fc88_2)

N set No.2 (N2 set)

	Name	Sequence (5' to 3')	Position*	Concentration
(4)	NIID_2019-nCOV_N_F2	AAATTTTGGGGACCAGGAAC	29142-29161	500 nM
(5)	NIID_2019-nCOV_N_R2	TGGCAGCTGTGTAGGTCAAC***	29299-29280	700 nM
(6)	NIID_2019-nCOV_N_P2	FAM-ATGTCGCGCATTGGCATGGA-BHQ (or QSY**)	29239-29258	200 nM

Length of the amplification product 158 bp

\*: The position is derived from the Wuhan seafood market pneumonia virus isolate Wuhan-Hu-1 MN908947.1.

\*\*: Operations have been checked with TAMRA.

\*\*\*: Although there is a mismatch from the sequence of Ver3, it has been confirmed that the sensitivity of detection of viral RNA is not affected.

[https://www.jstage.jst.go.jp/article/yoken/advpub/0/advpub\\_JJID.2020.061/\\_pdf](https://www.jstage.jst.go.jp/article/yoken/advpub/0/advpub_JJID.2020.061/_pdf)

#### 4.3. Real time one-step RT-PCR (TaqMan Probe method) reaction

The conditions of reaction for QuantiTect<sup>®</sup> Probe RT-PCR kit of QIAGEN K.K. are shown as an example. For the details, please refer to the manual enclosed in the kit. Dispense all reagents, etc. on ice.

#### Preparation and analysis of reaction plate

- 1) Use positive control RNA dedicated for the N set and N2 set, respectively. Add 10  $\mu$ L of positive control RNA diluted to  $10^5/\mu$ L to 90  $\mu$ L of DDW, sufficiently mix them, and spin down. Next, add 50  $\mu$ L of diluted positive control RNA to 450  $\mu$ L of DDW, mix well, and spin down ( $10^3/\mu$ L). For the positive RNA control, a determinable exogenous base sequence (sequence for the BamHI site and for checking H5 positive control) has been inserted for in case contamination has occurred in the laboratory, therefore be sure to use the specified control.
- 2) Make a 10-fold serial dilution of the positive control in 1) from  $5 \times 10^3/5 \mu$ L to  $5 \times 10^0/5 \mu$ L copies. For the dilution method, for example, add 50  $\mu$ L of control RNA to 450  $\mu$ L of DDW, mix well, and then make the next dilution. One point is enough if the detection

precision of the positive control has been confirmed.

3) Prepare the reaction solution shown in the table using the sample used for RNA extraction.

Table 1 Preparation of real time RT-PCR reaction solution

	N set	N2 set
2 × Master mix	10.0 μL	10.0 μL
Forward primer (10 μM)	1.2 μL	1.0 μL
Reverse primer (10 μM)	1.6 μL	1.4 μL
TaqMan probe (5 μM)	0.8 μL	0.8 μL
Quantitect RT mix	0.2 μL	0.2 μL
DDW	1.2 μL	1.6 μL
Template RNA	5 μL	5 μL
Total	20 μL	20 μL

Create mix by mixing the primer probe in advance. It can be kept at −30°C.

4) Put 15 μL of the reaction solution in each of the plate or 8-tube wells. Add 5 uL of the negative control to 2 wells each.

5) Add 5 μL of RNA to 2 wells each.

6) Add 5 μL of the positive control RNA to 2 wells each. (Add the solution of serial dilutions to wells in ascending order of concentration). To prevent contamination of the positive control, etc., measures such as sealing or covering with aluminum foil are recommended.

7) Set the conditions of the reaction as follows and start the reaction.

<Conditions of the reaction>

Since the optimum conditions of the reaction differ depending on the real time PCR device, the reagent and the reaction container, etc. that is used, be sure to check the sensitivity of detection, etc. in advance. The conditions of reaction in case QuantiTect® Probe RT-PCR Kit of QIAGEN K.K. is used as the reagent, and Applied Biosystems 7500 Fast Real-Time PCR System of Applied Biosystems or LightCycler 480 (or 480II, 96) of Roche Diagnostics K.K. is used as the real time PCR equipment are shown below.

Assay	Absolute Quantification (Standard Curve)
Run Mode	Standard 7500
Reporter	FAM
Quencher	None

When Applied Biosystems 7500 Fast Real-Time PCR System is used

The settings of the device are as follows

(Used in **Standard** mode)

50°C 30 min.

↓  
 95°C 15 min.  
 ↓  
 95°C 15 sec.  
 60°C 60 sec. (Data Collection) ] × 45 cycles

When LightCycler 480 II (480, 96) is used

	Analysis Mode	Cycle	Temperature (°C)	Time	Ramp Rate (°C/sec)	Acquisition Mode
RT	None	1	50*	30 min.	4.4	None
Denature	None	1	95	15 min.	4.4	None
PCR	Quantification	45	95	15 sec.	4.4	None
			60	60 sec.	2.2	Single
Cooling	None		40	30 sec.	4.4**	None

\*\* : The ramp rate of cooling can be the upper limit of the device.

8) A study is deemed to be valid when a rise in the amplification curve of the positive control is observed within 40 cycles and a rise in the amplification curve of the negative control is not observed. The results of previous analyses have suggested a higher sensitivity in the N2 set than the N set. The test result is deemed to be positive when a rise in the amplification curve is observed during reaction time in either or both of the 2 wells of the N2 set of specimens. Nonspecific amplification may be considered when only 1 well of the N set showed a positive test result in case the N2 set is negative, and the Cq value is high. Even in this case, a positive test result with the N2 set has been reported on re-analysis, although it is infrequent. Thus, in this case, re-test is recommended. The test result is determined to be negative when there is no rise in the amplification curve within the reaction time in any wells of the N set or the N2 set. It's to be noted that the test results may be determined to be positive even if a curve is not observed with an automatic determination method with the 2nd derivative method. Therefore, be sure to check the amplification curves for a rise in the curves in comparison with the control.

[Comparison between kits]

Moreover, when the kit of Roche Diagnostics K.K. (LightMix® Modular SARS and Wuhan CoV product number 518-499921 and 518-499914) is used for analysis, it is recommended to determine “SARS-like coronavirus positive” when both the E gene and the N gene are tested and both or either of them are determined to be positive. For sensitivity, these determination criteria are considered to be equivalent to the test method of this manual.

(Reference) Internal control and Master Mix: Modular EAV RNA Extract. Control (product number 518-

219963) of Roche Diagnostics K.K., LigthCycler Multiplex RNA Virus Master

Note 1: SARS-like coronavirus (SARS-CoV epidemic in 2003, and novel coronavirus 2019-nCoV this time)

Note 2: E gene and N gene of the kit of Roche Diagnostics K.K. cannot differentiate between the SARS-CoV epidemic of 2003 and this novel coronavirus 2019-nCoV. However, it is not considered problematic to determine positive test results with this study to be positive for the novel coronavirus 2019-nCoV with the current situation (not epidemic SARS-CoV).

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## Appendix

### Pretreatment Methods for Sputum Specimens Ver. 1

Since sputum is very viscous compared to nasal swabs, throat swabs, nasal discharge, and nasal lavage fluid, it is difficult to handle the specimens by pipette as such to test them.

It is possible to float viruses on the surface of sputum when sputum is suspended in PBS(-) etc.; however, it seems impossible to efficiently float viruses enclosed inside sputum. Adding a large amount of suspension such as PBS(-) dilutes the sputum itself and the concentration of floating viruses decreases, possibly resulting in low efficiency of the extraction of viral RNA and isolation of the viruses.

On the other hand, when sputum is dissolved, viruses enclosed inside the sputum are floated and efficient extraction of viral RNA and isolation of viruses can be expected. However, since contaminants such as genome contained in the sputum are also simultaneously eluted, it is important to perform DNase processing and dilution of the solution, etc. to prevent affecting the efficiency of viral RNA extraction.

This manual exemplifies the pretreatment methods for sputum specimens by sputum suspension using PBS(-), etc. and sputum dissolution using DTT for reference. Since there are also pretreatment methods such as the crushing method using a bead beater other than the indicated pretreatment methods, it is possible to consider the method at each facility.

#### 1. Pretreatment method for sputum specimens for viral gene tests

Use the supernatant of the DTT solution or PBS(-) suspension to extract RNA for viral gene tests. Since these solutions are very viscous and difficult to operate with a pipette as such, it is good to cut the tip of the pipette off.\*<sup>1</sup>

\*<sup>1</sup> To prevent contamination, cut the tip beforehand with a razor blade/cutter knife/scissors, etc. (which should be new or dedicated to prevent contamination) on aluminum foil in a clean environment such as the inside of a cabinet for preparation of the reagent, etc.

##### 1.1. Instruments and reagents required for sputum processing

Microcentrifuge, micropipette (e.g., 200  $\mu$ L, 1,000  $\mu$ L), sterile centrifuge tube (e.g., 15 mL, 50 mL), razor blade/cutter knife/scissors, etc., sterile microcentrifuge tube (e.g., 1.5 mL, 2.0 mL), PBS(-) (cell culture grade), dithiothreitol (DTT: molecular biology grades are recommended, Wako Cat# 044-29221, 29223), sterile distilled water, (e.g., disposable tweezers)

##### 1.2 Transportation and storage of sputum specimens

Perform pretreatment for sputum specimens collected in empty containers according to the

following methods 1.3.1 or 1.3.2. For sputum specimens collected on viral transport media, etc., the PBS(-) has to be read into the viral transport medium, etc., and pretreatment should be performed according to the following method in 1.3.2 (see the diagrammatic flow chart).

#### 1.3.1 When sputum specimens are dissolved in DTT

- 1) Use PBS(-) to create 10% w/v DTT solution (preparation in use<sup>\*2</sup>, hereinafter referred to as 10%DTT in PBS).
- 2) Add 10%DTT in PBS at the same volume as that of the sputum and suspend with a vortex mixer and inversion mixing. (If the sputum is divided into separate tubes in advance, it is good to divide it with a decanter or disposable tweezers, etc.)
- 3) Incubate the mixture for 15 minutes at room temperature. (The viscosity of the sputum decreases at this stage and it becomes easy to handle. A wide-tip pipette may be used without cutting the tip.)
- 4) Perform the DNase processing described in 2 above in advance.

<sup>\*2</sup> It is desirable to prepare the 10% DTT in PBS when it is to be used, and but it is also possible to divide it into small amounts and store it at 20°C for approximately 1 year. Avoid freezing and thawing.

#### 1.3.2 When sputum specimens are suspended in PBS(-)

- 1) Add PBS(-) at a volume of 1-3 times that of the sputum and suspend it with a vortex mixer and intense inversion mixing. (When sputum is divided into separate tubes in advance, it is good to divide it with a decanter or disposable tweezers, etc. Since the viscosity of sputum differs, change the volume as needed.)
- 2) Transfer the suspension (approximately 1 mL) to a 1.5 mL or 2 mL sterile microcentrifuge tube with a pipette of which the tip has been cut.
- 3) Centrifuge at 20,000 x g for 30 minutes at 4°C.
- 4) Extract RNA using the supernatant of suspension.

### 2 DNase processing before RNA extraction

When extracting RNA using the QIAamp Viral RNA Mini Kit, dissolve the components of sputum by adding the Buffer AVL. However, since the efficiency of the RNA extraction may be remarkably reduced by contaminants such as genome derived from sputum in the solution of the Buffer AVL, it is necessary to process the solution with DNase in advance if 10%DTT in PBS solution is supplied for RNA extraction.

#### 2.1 Instruments and reagents required for DNase processing

Micropipettes (10 µL, 200µL, and 1,000 µL), sterile microcentrifuge tubes (1.5 mL, 2.0 mL), syringe, needle, and RNase-free DNase Set (QIAGEN Cat# 79254)

## 2.2 DNase processing of sputum specimens

- 1) Dissolve the DNase I, RNase-Free enclosed with the RNase-Free DNase Set (QIAGEN Cat# 79254) using 550  $\mu\text{L}$  of the enclosed RNase-Free Water (the concentration of DNase after dissolution is 2.7 units/ $\mu\text{L}$ ). To prevent DNase (freeze-dried product) from being scattered and lost during dissolution, it is good to pierce a syringe with a needle into the rubber cover and not open the vial directly to add 550  $\mu\text{L}$  of the Water r, and dissolve DNase by inversion mixing (do not use a vortex mixer). To avoid the dissolved DNase I, RNase-Free from being frozen and thawed, divide it into some portions and store these at  $-20^{\circ}\text{C}$  (they can be stored for 9 months). When storing them at  $2-8^{\circ}\text{C}$ , use them up within 6 weeks.
  - 2) Add 1/10 of the Buffer RDD (RNase-Free DNase Set) and 1/100 of the DNase I, RNase-Free (RNase-Free DNase Set) to a part of the 10%DTT in PBS solution. (Example: Add 50  $\mu\text{L}$  of the Buffer RDD and 5  $\mu\text{L}$  of the DNase I, RNase-free to 445 $\mu\text{L}$  of the solution.)
  - 3) After 10 minutes of incubation at room temperature, extract RNA using the QIAamp Viral RNA Mini Kit, etc.
- \* This manual indicates a method of using the RNase-Free DNase Set (QIAGEN Cat# 79254) for DNase, but the products of other companies can also be used. However, when using the products of other companies, it should be examined in advance whether there are no problems with performing the reaction at room temperature, and whether RNA extraction is not affected.

Figure Flow chart of the pretreatment method of sputum specimens

