GOODGENE COVID-19 QUADPLEX REAL-TIME RT-PCR ASSAY (GG COVID-19 Quadplex Real-Time RT-PCR)

For Emergency Use Only

Instructions for use
-Rotorgene 5 Plex machine

Catalog # GGCOV-Q-01/02/03/04
50/100/200/1,000 reactions

For In-vitro Diagnostic (IVD) Use
Rx Only

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Edition for:
Emergency Use in The United States

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Forword

The GoodGene Coronavirus-19 Nucleic Acid Detection Kit (GoodGene COVID-19 Real-Time Reverse Transcription Polymerase Chain Reaction Assay; GG COVID-19 Quadplex Real-Time RT-PCR, herein after described as "GG COVID-19 Quadplex Real-Time RT-PCR") is a Real-Time RT-PCR in vitro diagnostic test intended for the qualitative detection of nucleic acid (RNA) from the SARS-CoV-2 virus in human respiratory specimens. Results are for the identification of SARS-CoV-2 RNA which induces pandemic of COVID-19 globally. SARS-CoV-2 RNA is generally detectable in human oropharyngeal swab, nasopharyngeal swab, and sputum specimens during the acute phase of infection. Positive results are indicative of presence of SARS-CoV-2 RNA. However, clinical correlation with patient history and other diagnostic information is necessary to determine patient infection status. Negative results do not preclude SARS-CoV-2 infection and should not be used as the sole basis for patient management decisions.

GG COVID-19 Quadplex Real-Time RT-PCR uses TaqMan-based Real-Time RT-PCR technique to conduct in vitro reverse transcription of SARS-CoV-2 RNA, DNA amplification and fluorescence detection. The assay targets specific genomic regions of SARS-CoV-2: nucleocapsid (N) gene, RdRp and ORF1ab. The TaqMan probes for the three amplicons are labeled with FAM, Texas Red and Cy-5 fluorescent dyes respectively to generate target-specific signal. The assay amplifies an endogenous RNA internal control to monitor the processes from nucleic acid extraction to fluorescence detection. The IC probe is labeled with VIC fluorescent dye to differentiate its fluorescent signal from SARS-CoV-2 targets. GG COVID-19 Quadplex Real-Time RT-PCR showed good correlation with a pre-approved assay (>95% correlation in detection of SARS-COV-2) and LOD of 10 copies/ul in 2 commonly used Real-Time PCR machines.

GG COVID-19 Quadplex Real-Time RT-PCR is intended for use by qualified and trained clinical laboratory personnel specifically instructed and trained in the techniques of RT-Real-Time PCR and in vitro diagnostic procedures. Use of GG COVID-19 Quadplex Real-Time RT-PCR in the territory of United Sates of America will be limited to laboratories certified under the Clinical Laboratory Improvement Amendments of 1988 (CLIA), 42 U.S.C. to perform high complexity tests.

There are **3 types of GoodGene Coronavirus-19 Nucleic Acid Detection Kit** as summarized below.

	Name	Target genes to be analyzed	Strengths	Indications
1	Standard kit (Quadplex Real-Time RT-PCR assay)	IC and N, ORF1ab and RdRp of SARS-Cov-2	>99% sensitivity and specificity	Optimal for standard laboratory
2	Extended kit (Pentaplex Real-Time RT-PCR assay, 2 tube)	IC + N , ORF1ab and RdRp (2 probes) of SARS-Cov-2	>99% sensitivity and specificity. Can differen-tiate SARS-1 and MERS	Best for epidemiological study
3	Basic kit (Duplex/Triplex Real-Time RT-PCR assay)	IC and N or RdRP of SARS-Cov-2 or IC and N/RdRp of SARS-Cov-2	>95% sensitivity and specificity	Best for cost effective screening of COVID-19

The present kit is a Standard GoodGene Coronavirus-19 Nucleic Acid Detection Kit:
GG COVID-19 Quadplex Real-Time RT-PCR assay (Catalog GGCOV-Q-01/02/03).

NOTE) This manual is proprietary of GoodGene Inc., and intended only for customer use in connection with the product(s) described herein and for no other purpose. This document and its contents shall not be used or distributed for any other purpose without the prior written consent of GoodGene Inc. Follow the protocol included with the kit.

1. Product Name

Proprietary Name –

GoodGene COVID-19 Quadplex Reverse Transcription Real-Time Polymerase Chain Reaction Assay

GoodGene Coronavirus-19 Nucleic Acid Detection Kit (Standard)

Established Name –

GG COVID-19 Quadplex Real-Time RT-PCR

2. Intended Use

The GG COVID-19 Quadplex Real-Time RT-PCR is a Real-Time RT-PCR *in vitro* diagnostic test intended for the qualitative detection of nucleic acid from the SARS-CoV-2 virus in human respiratory specimens including oropharyngeal and nasopharyngeal swab, sputum and brochoalveolar lavage collected from individuals with signs and symptoms suspected of COVID-19. Testing in United States of America 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 SARS-CoV-2 RNA. SARS-CoV-2 RNA is generally detectable in human respiratory specimens during the acute phase of infection. Positive results are indicative of presence of SARS-CoV-2 RNA but do not rule out bacterial infection or co-infection with other viruses.; clinical correlation with patient history and other diagnostic information is necessary to determine patient infection status. Positive results do not rule out bacterial infection or co-infection with other viruses. 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 SARS-CoV-2 infection and should not be used as the sole basis for patient management decisions. Negative results must be combined with clinical observations, patient history, and epidemiological information.

The GG COVID-19 Quadplex Real-Time RT-PCR is intended for use by qualified and trained clinical laboratory personnel specifically instructed and trained in the techniques of Real-Time PCR and *in vitro* diagnostic procedures. The GoodGeneCoronavirus-19 Nucleic Acid Kit in United States of America is only for use under the Food and Drug Administration's Emergency Use Authorization.

3. Device Description and Test Principle

The GoodGene Coronavirus-19 Nucleic Acid Detection kit uses TaqMan- based real-time PCR technique to conduct in vitro reverse transcription of SARS-CoV-2 RNA, DNA amplification and fluorescence detection.
GG COVID-19 Quadplex Real-Time RT-PCR Assay includes primers and dual-labeled hydrolysis (Taqman®) probes to be used in the in vitro qualitative detection of COVID-19 virus RNA isolated from clinical specimens including nasopharyngeal, oropharyngeal, nasal swab, and sputum.
The assay targets specific genomic regions of SARS-CoV-2: nucleocapsid (N) gene, ORF1ab(Open reading frame 1ab), and RNA dependent RNApolymerase (RdRp). The

TaqMan probes for the 3 amplicons are labeled with fluorescent dyes to generate target-specific signal. For the N specific probe, the signal from the fluorescent dye (Texas Red) on the 5' end is quenched by BHQ-2 on its 3' end. For the Orf1ab specific probe, the signal from the fluorescent dye (Cy5) on the 5'end is quenched by BHQ-2 on its 3' end. For the RdRP specific probe, the signal from the fluorescent dye (FAM) on the 5' end is quenched by BHQ-1 on its 3' end.
The assay detects an internal control (IC, human beta-actin; a housekeeping gene) to monitor the processes from nucleic acid extraction to fluorescence detection, which is also separately provided as an endogenous in vitro transcript RNA to be run alongside the specimens. The IC probe is labeled with Hex (VIC) fluorescent dye on the 5'end and BHQ-1 and its 3' end to differentiate its fluorescent signal from SARS-CoV-2 targets.
RNA isolated and purified from upper and lower respiratory specimens is reverse transcribed to cDNA and subsequently amplified in one of the 2 commonly used real time RT-PCR machines and associated 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 in corresponding channels at a threshold of 0.05.

4. Components of Kit

1) Components Included with the Test

Standard kit (GoodGene COVID-19 Quadplex Nucleic Acid assay)

- Catalog number: GG-COV-Q-01 (50 tests/kit)
- Catalog number: GG-COV-Q-02 (100 tests/kit)
- Catalog number: GG-COV-Q-03 (200 tests/kit)
- Catalog number: GG-COV-Q-04 (1,000 tests/kit)
- Following is component of GG COVID-19 Quadplex REAL-TIME RT-PCR assay for each catalog number (Catalog number: GG-COV-Q-02, 100 tests/kit). The volume of each component varies with each of kit (GG-COV-Q-01, 50 tests/lit, GG-COV-Q-03, 200 tests/kit, GG-COV-Q-04, 1,000 tests/kit), which decreases or increases in proportion to number of testing to be done.

Table 1. Components manufactured by Good Gene, InC and supplied with the test

Components of GG COVID-19 Quadplex Real-Time RT-PCR kit (Unit; uL, Store at -20 °C)	Q-01	Q-02	Q-03	Q-04
Primer mix	200	400	800	4,000
Probe mix	200	400	800	4,000
Positive Control	30	60	120	600
Internal Control	10	20	40	200
One step reverse transcription (RT) PCR enzyme mix	25	50	100	500
RT-PCR buffer (5x)	625	1250	2,500	12,500

Hot Taq	25	50	50	50
RNase free water	300	600	600	600

Notes:

1) The reference materials and other components in the kit should be treated as potential sources of infection.

2) The use of this kit should be strictly in accordance with the nucleic acid amplification guidelines to operate in compliance with the requirements of the appropriate laboratories.

3) The components in different batches of the kit cannot be used interchangeably.

4) Note: Actual volume in kit allows for 10-20% more considering errors in pipetting, etc.

2) Components Required But Not Included with the Test

3)-1. RNA Extraction Options

For each of the kits listed below, GOOD GENE has confirmed that the external lysis buffer is effective for inactivation of COVID-19. For questions about using alternative nucleic acid extraction methods, please contact GoodGene at jung.moon.md@goodgene.co.kr.

Table 2. RNA extraction kit

Instrument/Manufacturer	Extraction Kit	Catalog No.
QIAGEN	QIAmp DSP Viral RNA Mini Kit	50 extractions (61904)
	QIAamp Viral RNA Mini Kit	50 extractions (52904) 250 extractions (52906)

3)-2. Equipment and Consumables Required (But Not Provided)

Real-Time PCR instrument and software as below will be used. (additional machines may contact manufacturer for questions):

1) Rotor-Gene Q 5-plex HRM (Qiagen, Hilden, Germany)
   Rotor-Gene Q Series Software 2.1.0

2) CFX96 Touch Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA)
   CFX ManagerTM Software V3.1; or CFX MaestroTM Software V1

Additional tools and Nuclease-free (RNase/DNase free) consumables may be required as listed below:

Instruments for automatic nucleic acid extraction and PCR setup may be indicated.

- 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
- Extraction systems (instruments):
- Molecular grade water, nuclease-free
- 70% ethanol or 0.5% hydroxyperoxide or sodium hypochlorite (0.1% and above, 1,000 ppm, 1 minute)
- DNAZapTM (Ambion, cat. #AM9890) or equivalent
- RNAse AwayTM (Fisher Scientific; cat. #21-236-21) or equivalent
- PPE: Disposable powder-free gloves, goggles or face barriers, N95/KF94 masks, and surgical gowns
- Aerosol barrier pipette tips
- 1.5 mL microcentrifuge tubes (DNase/RNase free)
- Rotorgene Q 5Plex
    - Tubes 0.2 ml; Strip Tubes 0.1 ml (4 tubes)
    - Rotor-Disc 72; Rotor-Disc 100(Up to 100 samples per run using a Rotor-Disc 100)
- CFX96:
    a. Hard-Shell® 96-Well PCR Plates, low profile, thin wall, skirted, white/white (Cat. No. HSP9655, Bio-Rad)
    b. Optical Flat 8-Cap Strips (Cat. No. TCS0803, Bio-Rad)

3) Controls included in the kit; instructions upon receiving kit

1) ***Control Material(s) to be Used:***

(1) COVID-19 Positive Control 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 3 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.
4) Use through the entire sample processing procedure, excluding the extraction.
5) Used to prove the functionality of the reaction mix for amplification of the pathogen target, detect improper assay setup, reagent failure including primer and probe degradation and rules out inhibition when used together with IC

(2) COVID-19 Internal Control 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 1 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.
4) Use through the entire sample processing procedure, excluding the extraction.
5) Used to prove the functionality of the reaction mix for amplification of the pathogen target, detect improper assay setup, reagent failure including primer and probe degradation and rules out inhibition when used together with PC

(3) No Template Control (Negative Control; NTC; NC)
1) Sterile, nuclease-free water of same quantity as the template on every run
2) Aliquot in small volumes
3) Used to check for contamination during PCR plate set-up
4) Use through the entire sample processing procedure, including the extraction.
5) Used to detect assay or extraction reagent contamination

(4) Negative extraction control (NEC) (not provided)
1) Clinical patient specimen that has been previously tested negative, prepared by extracting RNA of same quantity as the template on every run
2) Aliquot in small volumes
3) Used as the negative extraction control for the entire testing system to check adequacy of RNA extraction and to check for contamination during PCR plate set-up
4) Prepare at least 1 negative extraction control (NEC) each time RNA is extracted from a clinical specimen or sample. NEC is added to extraction system.
5) Used to detect cross-contamination during extraction, inefficient lysis of specimen, poor specimen collection, improper assay setup, extraction failure

3.Target Population, Collection, Storage and Shipment of Specimens

10.1. The target population:
PUI (Person under investigation);
Patients who meet CDC COVID-19 criteria /Individuals suspected of COVID-19 by their health care provider

Table 3. Specimen requirements

Specimen Type	Nasopharyngeal Swab, Nasal swab, or Oropharyngeal swab collected according to standard technique and immediately placed in 2-3 mL of transport media. *
Minimum Sample Volume	0.3 mL (300 µL)
Transport and Storage	Samples should be processed and tested with the GG COVID-19 Test as soon as possible.
	If storage is required, samples can be held:
	•At room temperature for up to 4 hours (15-25ºC)
	•Refrigerated for up to 2 days (2-8ºC)
	•Frozen (≤-15ºC or ≤-70ºC) for up to 30 days

10.2 Collection of specimens

Respiratory specimens include upper respiratory specimens (nasopharyngeal and oropharyngeal swabs) and lower respiratory specimens (sputum and bronchoalveolar lavage).

Refer to 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/lab/guidelines-clinical-specimens.html?CDC_AA_refVal=https%3A%2F%2Fwww.cdc.gov%2Fcoronavirus%2F2019-ncov%2Fguidelines-clinical-specimens.html

Use only synthetic fiber swabs with plastic shafts. Do not use calcium alginate swabs or swabs with wooden shafts, as they may contain substances that inactivate some viruses and inhibit PCR testing. Place swabs immediately into sterile tubes containing 3 ml of viral transport media. For initial testing, nasopharyngeal swab specimens are recommended. Collection of oropharyngeal swabs is a lower priority and is acceptable if other swabs are not available. 10.1

1) For nasopharyngeal swab (NP): Insert a swab into nostril parallel to the palate. Swab should reach depth equal to distance from nostrils to outer opening of the ear. Leave swab in place for several seconds to absorb secretions. Slowly remove swab while rotating it.

2) For Oropharyngeal swab (e.g., throat swab, OP): Swab the posterior pharynx, avoiding the tongue.

* Lower respiratory tract specimens

1)Bronchoalveolar lavage, tracheal aspirate

-Two to 3 mL should be collected in a sterile, leak-proof, screw-cap sputum collection cup or sterile, dry container.

-If clinically indicated (eg, if the patient is undergoing invasive mechanical ventilation), collection and testing of a lower respiratory tract aspirate or bronchoalveolar lavage sample should be performed.

2)Sputum

-The patient should rinse his or her mouth with water and then expectorate deep cough sputum directly into a sterile, leak-proof, screw-cap sputum collection cup or sterile, dry container.

-Only patients with a productive cough should undergo sputum collection.

10.3. Specimen Storage

Store specimens at 2-8°C for up to 72 hours after collection. If a delay in testing or shipping is expected, store specimens at -70°C or below.

10.4 Specimen Shipping

Specimens from PUI's must be packaged, shipped, and transporte according to the current edition of the International Air Transport Association (IATA) Dangerous Goods Regulation External Icon. Store specimens at 2-8°C and ship overnight to the lab on ice pack. If a specimen is frozen at -70°C ship overnight to the lab on dry ice. Additional useful and detailed information on packing, shipping, and transporting specimens can be found at Interim Laboratory Biosafety Guidelines for Handling and Processing Specimens Associated with Coronavirus Disease 2019 (COVID-19).

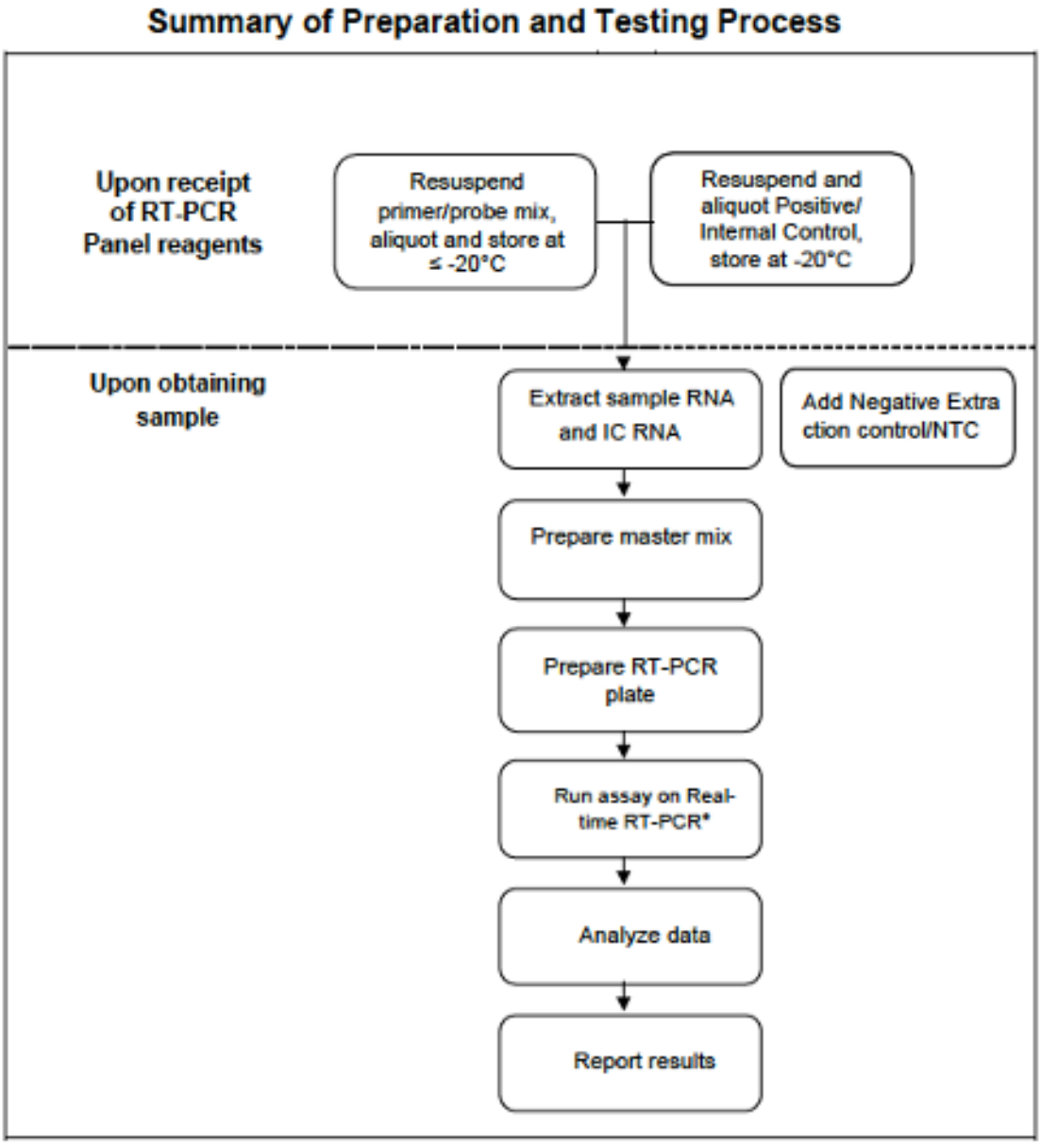
Note)

Refer to: Interim Guidelines for Collecting, Handling, and Testing Clinical Specimens from Persons for Coronavirus Disease 2019 (COVID-19) https://www.cdc.gov/coronavirus/2019-nCoV/guidelines-clinical- specimens.html

Refer to Interim Laboratory Biosafety Guidelines for Handling and Processing Specimens Associated with Coronavirus Disease 2019 (COVID-19) https://www.cdc.gov/coronavirus/2019-nCoV/lab-biosafety-guidelines.html

5. Assay procedure

Figure 1. Outline of Procedure



Step 1. Sample processing:

a. RNA should be collected from as fresh a specimen as possible to ensure suitable RNA quality and quantity.

b. The positive control, NEC (Negative extraction control), and no template (negative) control should be processed simultaneously alongside the specimen.
c. RNA should be extracted using the QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany), QIAamp DSP Viral RNA Mini Kit (Qiagen) or Qiagen EZ1Advanced XL Kit (Qiagen) according to the manufacturer's instructions.
d. Extracted RNA (>140 µL) should be eluted in a final volume of 20 µL.
e. Following extraction, the RNA should be used immediately and/or residual RNA stored at -20°C for later use with correct labeling.
f. When handling the positive control, avoid contamination of the specimen sample as failure to take proper precautions when handling the positive control could result in a false positive result.

#Sputum recommended processing protocol*:

1) proteinase K and DNase I treatment (PK-DNase): NucliSENS easyMAG protocol (bioMérieux SA, Marcy-l'Etoile, France).
   - 100 uL of proteinase K (1 mg proteinase K/1 mL proteinase K buffer; Promega, Madison, WI, USA) added to 100 µL of sample and incubated for 15 min at 55°C.
   - mix samples by vortexing every 5 min.
   - 20-µL of DNase I solution (1 U/µL) is added for every 100 µL of sample, followed by incubation at 37°C for 30 min
2) treatment with a phosphate buffer containing 1 g/L of protease K in an equal volume of sputum and vortexed for 1 min. The cell lysates are centrifuged and clarified supernatants transferred to sterile microtubes.

*Reference: Comparative Evaluation of Three Homogenization Methods for Isolating Middle East Respiratory Syndrome Coronavirus Nucleic Acids From Sputum Samples for Real-Time Reverse Transcription PCR
Heungsup Sung, et al. Ann Lab Med. 2016 Sep; 36(5): 457–462. Published online 2016 Jun 24. doi: 10.3343/alm.2016.36.5.457. PMCID: PMC4940489

Step 2. Reagent preparation (hereinafter is described based on Rotorgene Q 5-plex HRM RT-PCR machine)

g. Prepare all reagent mixture inside a biological safety cabinet in preparation area. To begin, take out the GG OneStep RT-PCR kit contents from freezer and thaw thoroughly at ambient temperature. Vortex and centrifuge briefly. The Enzyme Mix should be kept on ice or cold-block at all times.
h. Take out the GG COVID-19 Internal Control, GG COVID-19 Positive Control and GG COVID-19 Negative Control from freezer and completely thaw them at room temperature. Votex and centrifuge briefly. Take out the GG COVID-19 probes and primers solutions and place them on ice as well.
i. Calculate the number of reactions (N) that will be included in the test. Be sure to include the no template (negative) control (1 tube), negative extraction control (NEC), internal control (1 tube), positive control (1 tube), and each specimen. Prepare a volume of master mix as in the table below. It is recommended to prepare

110% of the calculated amount of PCR mix to account for pipetting carryovers. Mix by petting up and down a few times.

Table 4. Component list and amount of component per reaction

Component	Amount/reaction	Final concentration	Per kit (50T/-01 Cat)
Template (eluted RNA)	3.5 ul	100ng – 1ug/ul	N/A
F/R Primer Mix	4 ul	5 pmole/ul each	200 ul
Probe Mix	4 ul	2 pmole/ul	200 ul
RT-PCR Enzyme Mix	0.5 ul		25 ul
RT-PCR Buffer (2x)	12.5 ul	1x; 2.5mM Mg^{2+}	625 ul
dNTP Mix (10 mM)	0.5 ul	10 uM of each dNTP	25 ul

	Component	Volume / test	Volume for N samples and 4 controls	110% of volume (ul)
1	GG One Step RT-PCR Enzyme mixture	0.5 ul	(N+4) x 0.5 ul	(N + 4) x 0.5 x 1.1
2	Mixture of primers, probes, TE buffer	20.5 ul	(N+4) x 20.5 ul	(N + 4) x 20.5 x 1.1
3	GG One Step primers, probes, RT-PCR Enzyme, buffer, dNTP	21.5 ul	(N+4) x 21.5 ul	(N + 4) x 21.5 x 1.1

j. Prepare 72-well disks for real-time RT-PCR based on the estimated number of reactions (N) and prepare the PCR-Mix ingredients as described in above Table 4.
k. Pipette 21.5 μL of PCR-Mix into each well.
l. Cover and transfer the disk into sample processing area.
m. The remaining Reaction Mix and Enzyme Mix must be stored at -20°C immediately.

Step 3. Sample Addition:

n. Add 3.5 μL of the extracted sample RNA to the well pre-filled with reagent mix in the following order: no template (negative) control, Negative extraction control, patient specimen(s), and positive control.
o. Seal the disk and centrifuge at 2000 rpm for 10 seconds to avoid bubbles.
p. Place the plate into real-time RT-PCR system and record the exact location of controls and each specimen.

Step 4. Real-Time PCR (hereinafter is described based on use of Rotor-Gene Q 5-plex HRM, Qiagen, Germany)

q. 25 μL of reaction mixture containing 3.5 μL of the template RNA is tested
r. Set up and run the Rotor-Gene Q 5-plex HRM Real Time PCR instrument. Refer to its Reference Guideline for detailed instructions. Usually start by double clicking Rotor-GeneQSeries Software 2.1.0 and proceed to Set Up Experiment

Properties > Setup the Targets and Samples in Plate Setup > Setup Run Method, then click Run and Start.

s. When setup Experiment Properties, please check the following run settings and choose the correct settings.
   - Instrument: Rotor-Gene Q5-plex HRM (72-well)
   - Run type: Quantitation - Standard Curve
   - Run reagent: TaqMan reagents
   - Run mode: Standard

t. When setting up the Targets and Samples, create the following detectors with the quencher set as none. The passive reference must be set as None.

Table 5. Target and respective reader/quenchers

Target Name or Detector	Reader	Quencher
N	Texas Red	BHQ2
RdRP	FAM	BHQ1
Orf1ab	Cy5	BHQ2
IC (beta-actin)	HEX	BHQ1

u. Set up the plate layout by assigning a unique sample name to each well

v. Assign a Task to each well
   - Unknown: for patient samples
   - Standard for Positive Control
   - NTC for Negative Control

x. Set Run method as followings for PCR amplification and fluorescence detection. The sample volume is 25 ul.

Table 6. Real Time PCR setting

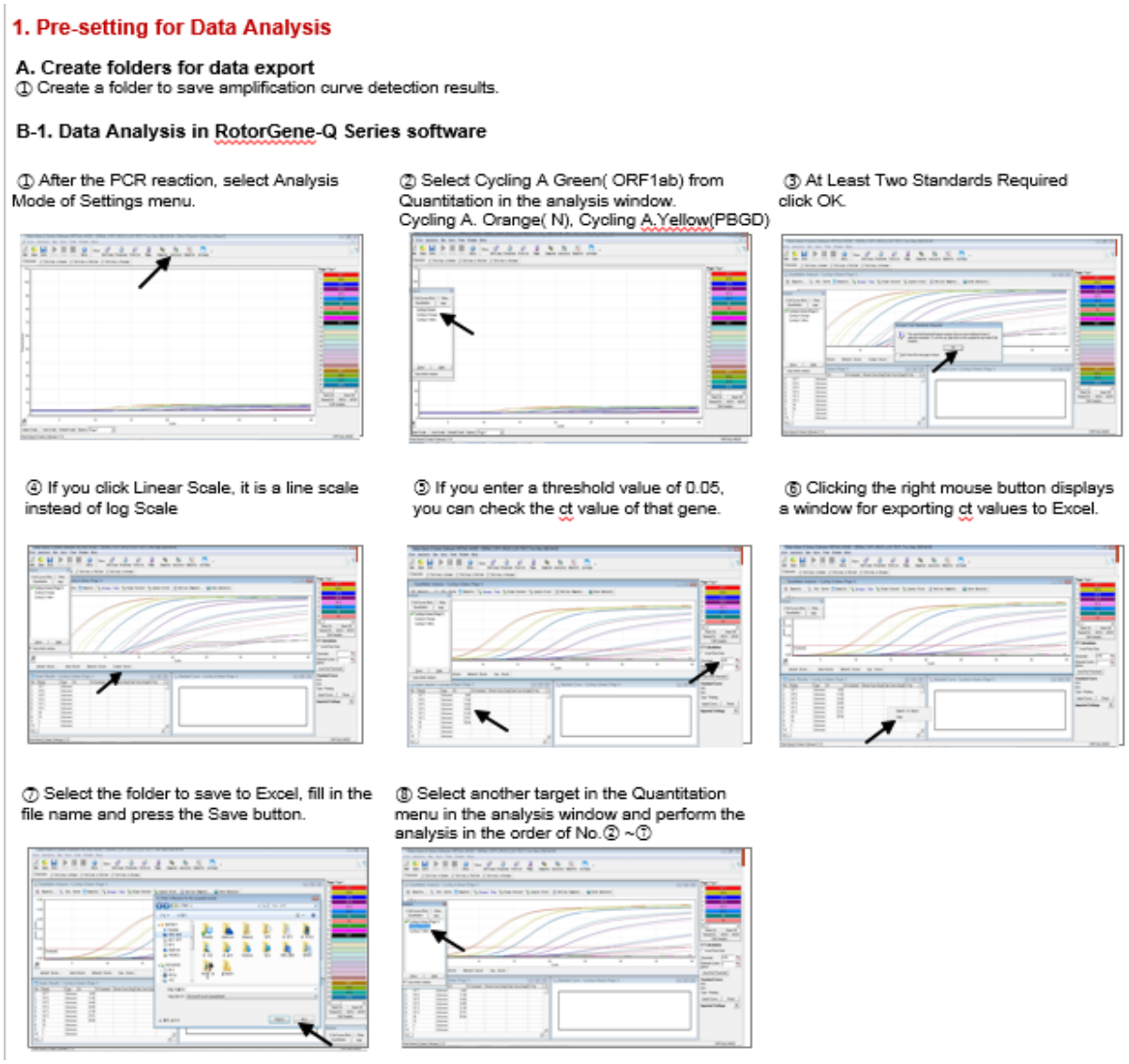
Step	Name	Temperature	Time	Number of cycle
1	Reverse transcription (RT)	42 °C	30 minutes	1
2	Initial denaturation	95 °C	10 minutes	1
3	PCR	95 °C	15 seconds	40 cycles
		56 °C*	40 seconds	
4	Final extension	40 °C	10 seconds	1

*Check fluorescence at the final 56 °C step

Step 5. After Real time PCR

When the run is complete, store and analyze the data according to the device manufacturer's instructions. Ct value of each target of each sample is checked and analysis should be performed for each target individually by using manual threshold value setting (threshold value, 0.05). Threshold values should be within the exponential phase of the fluorescence curve and adjusted above the background signal. The procedure you choose to set the threshold value should be used consistently.

Figure 2. Data Analysis (Rotorgene Q 5 Plex HRM RT-PCR Machine)



3) Control Material(s) to be Used: ; Read instructions upon receiving kit

(1) COVID-19 Positive Control :

1) This is a mixture of in vitro transcript RNA of N, ORF1ab and RdRp of SARS-Cov-2 (100 cp/ul)

2) Use through the entire sample processing procedure, excluding the extraction.

3) Used to prove the functionality of the reaction mix for amplification of the pathogen target, primer and probe integrity, and rules out inhibition when used together with IC

4) Precautions: This reagent should be handled with caution in a dedicated nucleic acid handling area to prevent possible contamination. Maintain on ice when thawed.

5) Aliquot 3 ul upon receival and store at ≤-20°C until use. Avoid repeated freeze-thaw cycles.

(2) COVID-19 Internal Control Material :

1) This contains in vitro transcript RNA of internal control gene (human beta-actin) (100 cp/ul)

2) Use through the entire sample processing procedure, excluding the extraction.

3) Used to prove the functionality of the reaction mix for amplification of the pathogen target and rules out inhibition when used together with IC
4) Precautions: This reagent should be handled with caution in a dedicated nucleic acid handling area to prevent possible contamination. Maintain on ice when thawed.
5) Aliquot 1 ul upon receival and store at ≤-20°C until use. Avoid repeated freeze-thaw cycles.

(3) No Template Control (Negative Control; NTC; NC) (not provided with Kit)
1) Sterile, nuclease-free water of same quantity as the template on every run
2) Used to check for contamination during PCR plate set-up
3) Use through the entire sample processing procedure, including the extraction.

(4) Negative extraction control (NEC) (not provided with Kit)
1) Clinical patient specimen that has been previously tested negative, prepared by extracting RNA of same quantity as the template on every run
2) Used as the negative extraction control for the entire testing system to check adequacy of RNA extraction and to check for contamination during PCR plate set-up
3) Prepare at least 1 negative extraction control (NEC) each time RNA is extracted from a clinical specimen or sample. NEC is added to extraction system.

H. INTERPRETATION OF RESULTS

1. First Step: Examination and Interpretation of Control Results:

The controls for the Real-Time Fluorescent RT-PCR Kit for Detecting COVID-19 are evaluated using the nucleic acid amplification curve and Ct values generated by the RT-PCR system software. The Ct cut-off values are determined using the receiver operator characteristic curves of the tested clinical samples. The no template (negative) control should provide Ct values at FAM, Texas-Red, Cy-5 and VIC/HEX channels of "0" or "no data available" and there should be no sigmoidal amplification curve. If any of the channels are positive, repeat from the RT-PCR step using residual extraction material. If repeat results are not as expected (positive for any of orf1ab/N/Beta-actin), re-extract and re-test (RT-PCR run) all samples.

The positive and internal control should provide an amplification curve in the FAM/Texas-Red/Cy-5 and HEX channels, respectively, that appear to be in a sigmoidal shape.

I. Experimental analysis found that the Ct values for positive COVID-19 samples should be no higher than 40 for orf1ab/N/RgdP (Rotorgene Q, Qiagen). Thus, the Ct value in the FAM, Texas-Red, Cy-5 channel for a valid positive control should be no higher than 40 (Ct value<40) and there should be a sigmoidal amplification curve. However, the positive control should be negative for Beta-actin (IC marker; Ct value<40). If positive results are obtained for Beta-actin target, the RT-PCR run is invalid. Repeat from the RT-PCR step using residual extraction material. If results are not as expected, re-extract and re-test (RT-PCR run) all samples.

II. Experimental analysis found that the Ct values for human samples should be no higher than 40 for beta-actin (Rotorgene Q, Qiagen). Thus, the Ct value in the hex channel for a valid positive control should be no higher than 40 (Ct value<40) and there should be a sigmoidal amplification curve. If negative results are obtained for Beta-actin target, the RT-PCR run is invalid. Repeat from the RT-PCR step using residual extraction material. If results are not as expected, re-extract and re-test (RT-PCR run) all samples.

III. The negative control (NTC; NC) should be negative for Orf1ab, N, and RgdP marker (Ct value>40) but positive for beta-actin (IC marker; Ct value<40). If positive results are obtained for either N, Orf1ab, or RgdP targets, the RT-PCR run is invalid. Repeat from the RT-PCR step using residual extraction material. If results are not as expected, (positive for any of orf1ab/N/RgdP), re-extract and re-test (RT-PCR run) all samples and use multiple NTCs during rRT-PCR run. Discard working reagent dilutions and remake from fresh stocks. Clean potential DNA contamination from bench surfaces and pipettes in the reagent setup and template addition work areas

IV. The negative extraction control (negative clinical specimen; endogenous internal control) should be negative for Orf1ab, N, and RgdP marker (Ct value>40) but positive for beta-actin (IC marker; Ct value<40). If positive results are obtained for N or Orf1ab targets, the extraction run and the RT-PCR run are invalid and entire process should be repeated using residual patient sample.

V. Do not go to the next step unless these controls are valid and acceptable.

Table 8. Interpretation of control results

Control Type	Control Name	Used to Monitor	Expected results and Ct Values			
			N	Orf1ab	RdRP	beta-actin
Negative	Negative control(NC;NTC)	Assay or extraction reagent contamination	Negative Ct ND	Negative Ct ND	Negative Ct ND	Negative Ct ND
Positive	Positive Template Control (PC)	Improper assay setup, reagent failure including primer and probe degradation for Orf/N/RdRP	Positive Ct < 40.0	Positive Ct < 40.0	Positive Ct < 40.0	Negative Ct ND
Internal	Internal control (IC)	Improper assay setup, reagent failure including primer and probe degradation for beta-actin	Negative Ct ND	Negative Ct ND	Negative Ct ND	Positive Ct < 40.0
Extraction control	Negative Extraction Control (NEC)	Cross-contamination during extraction, inefficient lysis of specimen, poor specimen collection, improper	Negative Ct ND	Negative Ct ND	Negative Ct ND	Positive Ct < 40.0

		assay setup, extraction failure				

4)-2. Second Step: Examination and Interpretation of Patient Specimen Results:
Assessment of clinical specimen test results should be performed after the positive , internal negative, and no template controls have been examined and determined to be valid and acceptable (Except in case #; described in **4)-1.-V.**). If the controls are not valid, the patient results cannot be interpreted. To be deemed valid, a test must satisfy all the no template control and positive control requirements noted above. If neither requirement is satisfied, or if only one requirement is satisfied, the test is invalid.

I. If the Ct value is less than or equal to 40, it is positive. If the Ct value is greater than 40 or "0" or "no data available" (no sigmoidal amplification curve), it is determined as negative.
II. Determine the result as shown in the Table 9. below.
III. In case of 2/3 positive (N and/or ORF1ab and/or RgdP), report as "COVID-19 presumptive positive".
IV. In case of 1/3 positive (N or ORF1ab or RgdP), run RT-PCR again, as there is a high possibility of COVID-19 positivity.
   a. (if positive in 2/3) : report as "COVID-19 presumptive positive"
   b. (if positive in 1/3, same gene) :
      - RdRP, N or different marker positive: report as "invalid" and ask for a new patient specimen
      - Orf1ab: report as "presumptive negative"
V. In case of beta-actin (internal control) negativity, proceed from RT-PCR again, and if results are not as expected, start from nucleic acid extraction again from leftover sample. If results are not as expected, ask for a new patient specimen and report as "invalid".
VI. Additional confirmatory testing may be conducted if "clinically indicated" (i.e., re-test or use an alternative method for detection of SARS-CoV-2).

Table 9. Interpretation of patient specimen results

Beta-actin	**+**	-	+	+/-	-
COVID-19 ORF1ab	**+**	2/3 positive	-	1/3 positive	+/-
COVID-19 N	**+**		-		+/-
COVID-19 RgdP	**+**		-		+/-
Result for SARS-Cov-2 RNA	**Positive**	Presumptive Positive*	Negative	Inconclusive	Invalid
Additional test	**Positive**	Not necessary	Not necessary	Restart from RT-PCR again, (refer to IV.)	Restart from nucleic acid extraction

NOTE: All positive test results generated using the GG COVID-19 Quadplex Real-Time RT-PCR by Good Gene, InC must be reported to CDC. Please refer to the Goodgene korea website (www.goodgenekorea.com). For questions regarding this policy, please contact the GG Helpdesk at goodgene@goodgene.co.kr.

Figure 5.A. Example of "Covid-19 Negative" Analysis of Result of Reverse Transcription Real Time PCR by Using GG COVID-19 Quadplex RT Real Time PCR Kit

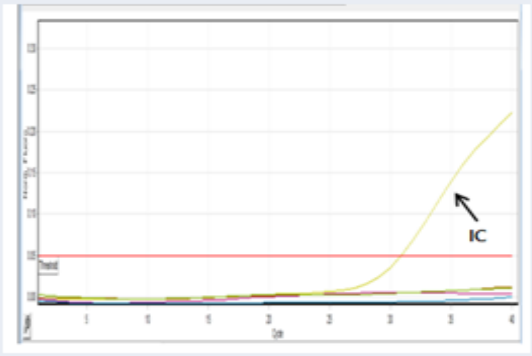
IC	Positive	Image
ORF1ab	Negative	
N	Negative	
RdRp	Negative	
Result	SARS-Cov-2 not detected	
Interpretation	COVID-19 Negative	

Figure 5.B. Example of "Covid-19 Positive"

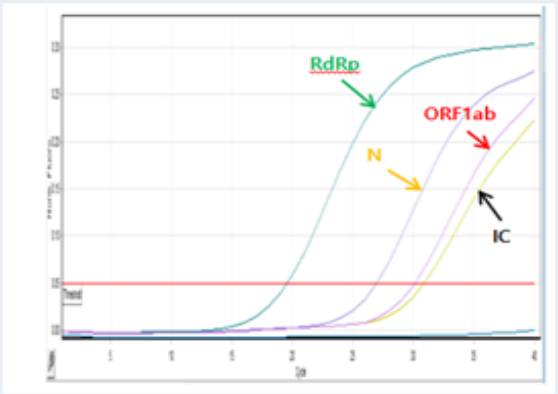
IC	Positive	Image
ORF1ab	All of 3 positive	
N		
RdRp		
Result	SARS-Cov-2 detected	
Interpretation	COVID-19 Positive*	

Figure 5.C. Example of "Covid-19 Presumptive Positive"

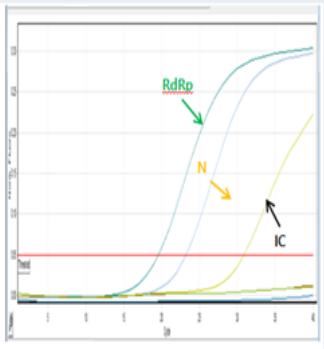
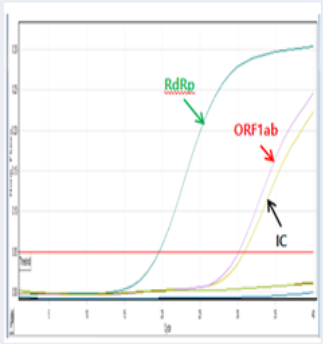
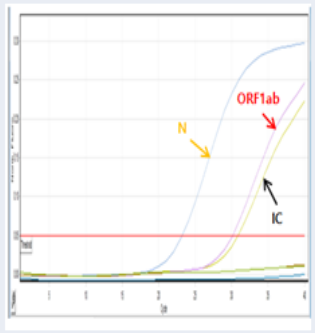
IC	Positive	Image 1	Image 2	Image 3
ORF1ab	Two out of 3 positive			
N				
RdRp				
Result	SARS-Cov-2 detected			
Interpretation	Presumptive Positive for COVId-19**			

Figure 5.D. Example of "Inconclusive Result"

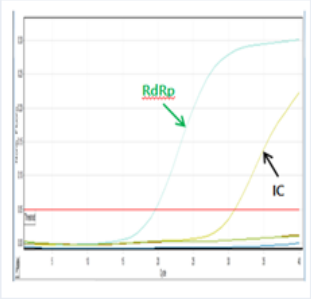
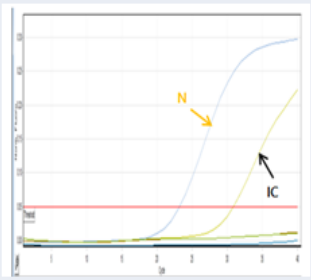
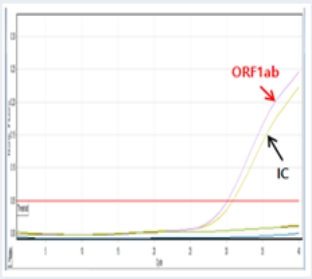
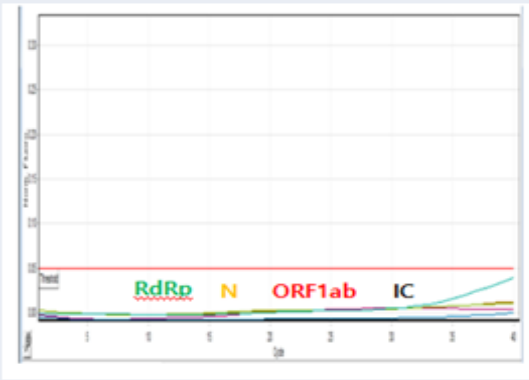
IC	Positive	Image 1	Image 2	Image 3
ORF1ab	One out of 3 positive			
N				
RdRp				
Result	Inconclusive			
Interpretation	Retest***			

Figure 5.E. Example of "Invalid Test, Retest Required"

IC	Negative	Image
ORF1ab	Positive or Negative	
N	Positive or Negative	
RdRp	Positive or Negative	
Result	Invalid Test	
Interpretation	Retest****	

I

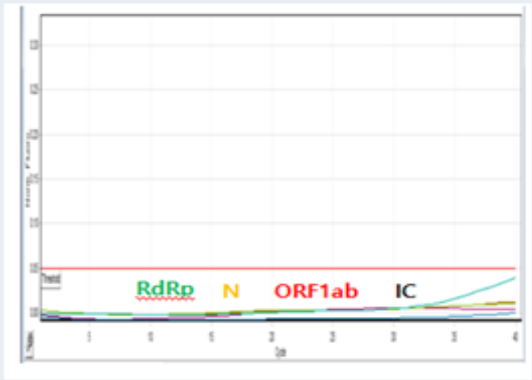
IC	Negative	Image
ORF1ab	Positive or Negative	
N	Positive or Negative	
RdRp	Positive or Negative	
Result	Invalid Test	
Interpretation	Retest****	

Table 10. Troubleshooting

Observation	Possible Cause	Solution
No signal from samples expected to be positive	RNA sample contains PCR inhibitors	• Use less starting sample as input for your RNA isolation procedure.
		• Increase the number or stringency of washes during RNA isolation.
		• Use less RNA sample in the qRT-PCR. Follow the guidelines below.
Low signal from samples expected to be positive	RNA sample contains low level of PCR inhibitors	Samples containing minimal amounts of inhibitors may yield successful qRT-PCR reactions if less RNA sample (and therefore less inhibitor) is added to the reaction. For example: • Reduce the sample volume to 1 to 2 µL, then add Nuclease-free Water to bring the reaction to the proper volume. -Or- • Dilute the RNA sample 1:10 using the solution used to elute the nucleic acid at the end of the nucleic acid isolation procedure, then use the diluted RNA in the qRT-PCR reaction. -Or- • Dilute the RNA sample 1:10 using 10 mM Tris-HCl pH 8, 0.1 mM EDTA, then use the diluted RNA in the qRT-PCR reaction.
Signal detected in no template control (NTC)	PCR contamination	• Repeat the qRT-PCR reaction with fresh reagents and decontaminated pipettors. • Set up and run the qRT-PCR reaction in an area that is isolated from areas used for nucleic acid isolation and PCR product analysis.

6. Storage & Handing Requirements

1. Store all reagents at -25 to -15°C.
2. Use the reagents within 6 months of manufacturing date.
3. Use the reagents within 30 days once kit is opened.
4. Avoid excessive freeze/thaw cycles for reagents.

7. Limitations of The Procedure and Warnings and Precautions

- For in vitro diagnostic use (IVD).
- For emergency use only. For Rx 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 COVID-19 https://www.cdc.gov/coronavirus/COVID-19/lab-biosafety-guidelines.html.
- Specimen processing should be performed in accordance with national biological safety regulations.
- If infection with COVID-19 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.
- The procedures in this handbook must be followed as described. Any deviations may result in assay failure or cause erroneous results.
- Good laboratory practice is required to ensure the performance of the kit, with care required to prevent contamination of the kit components. Components should be monitored for contamination and any components thought to have become contaminated should be discarded as standard laboratory waste in a sealed pouch or zip-lock plastic bag.
- As with any molecular test, mutations within the target regions of the GG COVID-19 Quadplex Real-Time PCR assay could affect primer and/or probe binding resulting in failure to detect the presence of virus.
- False negative results may be caused by:
    - Unsuitable collection, handling and/or storage of samples.
    - Sample outside of viraemic phase.
    - Failure to follow procedures in this handbook.
    - Use of unauthorized extraction kit or PCR platform.
- False positive results may be caused by:
    - Unsuitable handling of samples containing high concentration of COVID-19 viral RNA or positive control template.
    - Unsuitable handling of amplified product All results should be interpreted by a health care professional in the context of patient medical history and clinical symptoms.
- This test cannot rule out diseases caused by other pathogens.

- A negative result for any PCR test does not conclusively rule out the possibility of infection.
- Always include a negative control (NTC), and the appropriate positive control (GG Positive Control) in each amplification and detection run. All clinical samples should be tested for Pbgd gene (IC) to control for specimen quality and extraction.