2021

SARS-CoV-2 VoCs RT-qPCR Multiplex Kit Instructions for Use (IFU)

Incorporating CDC Guidelines

200 REACTIONS

FOR *IN VITRO* DIAGNOSTIC (IVD) USE

Table of Contents

1. Intended Use

SARS-CoV-2 VoCs RT-qPCR Multiplex Kit is a real-time qPCR assay intended for the qualitative detection of nucleic acid from SARS-CoV-2 in respiratory specimens such as nasopharyngeal or oropharyngeal swabs and sputum from patients who exhibit clinical signs or symptoms related to COVID-19 or individuals who have been in close contact to persons or locations with positive SARS-CoV-2 cases.

Results are for the identification of SARS-CoV-2 RNA, which is generally detectable in upper and lower respiratory specimens during infection. Positive results are indicative of the presence of SARS-CoV-2 RNA and does not rule bacterial infection or co-infection with other viruses; other diagnostic information is necessary to determine the status of infection. 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 treatment or other patient management decisions. Negative results must be combined with clinical observations, patient history, and epidemiological information.

The SARS-CoV-2 VoCs RT-qPCR Multiplex Kit 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.

2. Specimen guidelines as recommended by CDC USA

Biosafety Precautions

Wear appropriate personal protective equipment (e.g. gowns, gloves, eye protection) when working with clinical specimens. Specimen processing should be performed in a certified class II biological safety cabinet following biosafety level 2 or higher guidelines. For more information, refer to:

- Interim Guidelines for Collecting, Handling, and Testing Clinical Specimens from Patients Under Investigation (PUIs) for 2019 Novel Coronavirus (SARS-CoV-2) https://.cdc.gov/coronavirus/SARS-CoV-2/guidelines-clinical-specimens.html
- Biosafety in Microbiological and Biomedical Laboratories 5th edition available at http://www.cdc.gov/biosafety/publications/.

Acceptable Specimens

- Respiratory specimens including: nasopharyngeal or oropharyngeal aspirates or washes, nasopharyngeal or oropharyngeal swabs, bronchoalveolar lavage, tracheal aspirates, and sputum.
    - Swab specimens should be collected only on swabs with a synthetic tip (such as polyester or Dacron®) with aluminum or plastic shafts. Swabs with calcium alginate or cotton tips with wooden shafts are not acceptable.
- Serum

Specimen Handling and Storage

- Specimens can be stored at 4°C for up to 72 hours after collection.
- If a delay in extraction is expected, store specimens at -70°C or lower.
- Extracted nucleic acids should be stored at -70°C or lower.

Specimen Rejection criteria:

- Specimens not kept at 2-4°C (≤ 4 days) or frozen at -70°C or below.
- Incomplete specimen labeling or documentation.
- Inappropriate specimen type.
- Insufficient specimen volume

3. Summary and Explanation

An outbreak of pneumonia of unknown etiology in Wuhan City, Hubei Province, China was initially reported to the World Health Organization (WHO) in late December 2019. Chinese authorities identified a novel coronavirus SARS-CoV-2 (previously SARS-CoV-2), which has since spread to more than 200 countries and territories, including the United States, affecting millions around the world. Cases have resulted in asymptomatic infection, mild to severe respiratory illness, and death. Patients can risk contracting SARS-CoV-2 through contact with a contaminated person or environment.

The SARS-CoV-2 VoCs Multiplex RT-qPCR Kit is an *in vitro* diagnostic assay that detects and discriminates wild-type (ancestral), Alpha, and Delta SARS-CoV-2 variants. The assay is based on widely used nucleic acid amplification technology. The product contains oligonucleotide primers, dual-labeled hydrolysis probes, and control material used in reverse transcriptase RT-PCR for the *in vitro* qualitative detection of SARS-CoV-2 RNA in lower and upper respiratory specimens.

4. Principles of the Procedure

The SARS-CoV-2 VoCs RT-qPCR Multiplex Kit is an *in vitro* diagnostic test based on real-time qPCR technology, developed for specific detection of SARS-CoV-2 viral RNA and two variants of concern. Real-Time PCR technology utilizes polymerase chain reaction (PCR) for the amplification of specific target sequences and target specific probes for the detection of the amplified RNA. The probes are labelled with fluorescent reporter and quencher dyes.

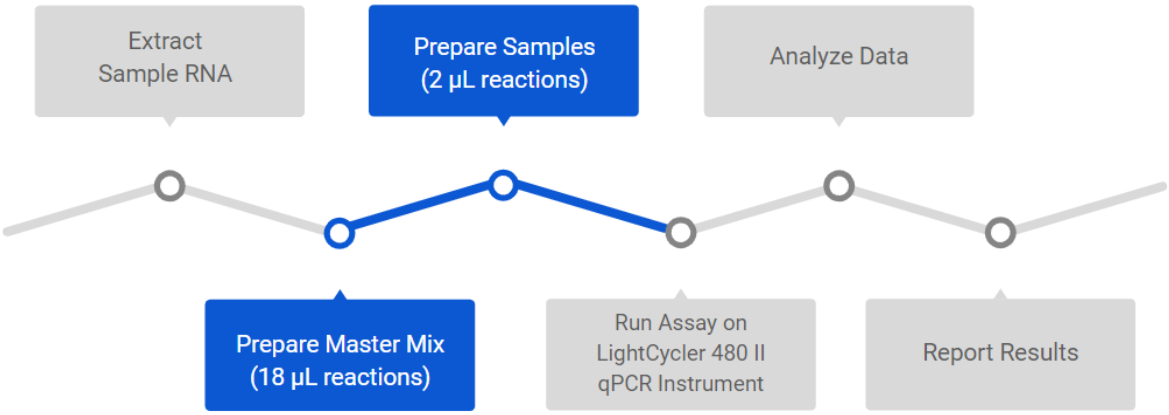
The oligonucleotide primers and probes were selected from regions of the virus, such as nucleocapsid (N) gene. An additional primer/probe set to detect the human RNase P gene (RP1) in control samples and clinical specimens is also included in the panel. The four probes are labelled with different fluorophores that excite and emit at different wavelengths for multiplex detection – The Delta variant probe contains HEX, probe contains FAM, the Alpha probe contains ROX, and RNase P probe contains Cy5.

RNA isolated and purified from respiratory specimens is reverse transcribed to cDNA and subsequently amplified using Real-Time RT-PCR machine. During PCR cycling, the probe anneals to a specific target sequence located between the forward and reverse primers. The probe is cleaved by the 5' nuclease activity of the Taq polymerase during the extension phase of the PCR cycle, causing the reporter dye to separate from the quencher dye, generating a fluorescent signal. With each cycle, additional reporter dye molecules are released from the probe, increasing the fluorescence intensity. Fluorescence intensity is monitored at each PCR cycle by the qPCR instrument.

New variants of SARS-CoV-2 have been circulating in many parts of the world. It is imperative that the molecular labs performing SARS-CoV-2 diagnostic tests be equipped with tools to rapidly and accurately detect and distinguish variants of concern. RT-qPCR is the primary method used to detect SARS-CoV-2. Development of a multiplex RT-qPCR test that can detect and distinguish SARS-CoV-2 variants will be a tremendous boost in understanding the prevalence of variants and help to adapt pandemic control policies.

The primary variant of concern driving the number of new infections world-wide is the Delta (B.617.2). Some parts of the world are still struggling with the Alpha variant (B.1.1.7) as well. The transmissibility of the Delta variant is higher than other variants, causing the pandemic to stretch and threatening the recovery. The Delta variant is also exhibiting partial resistance to some vaccines, which is a major concern for a post-pandemic world.

The Delta variant, also known as B.1.617.2 and which was first detected in India, has a unique alteration in the Spike gene (S). The Alpha variant, also known as B.1.1.7 and which was first detected in the United Kingdom, has a deletion of amino acids in the S gene as well. To detect and distinguish these variants of concern from wild-type SARS-CoV-2, we developed a multiplex kit that targets these regions of deletion. Primers and probe for the N gene will all variants and the wildtype, whereas primers and probe for human RP1 gene will serve as an internal control for successful sample collection.



5. Materials Included in Kit

The IQ-4 Multiplex Primer Mix was prepared as below:

Primer/Probe	Catalog #	Volume per 20 ul reaction	Volume for 200 reactions	Final concentration in 20 ul reaction
N F-primer (10 uM)	IQ4-CVD-P01	0.8 µL	160 µL	0.4 uM
N R-primer (10 uM)	IQ4-CVD-P02	0.8 µL	160 µL	0.4 uM
N-FAM Probe (5 uM)	IQ4-CVD-PR01	0.8 µL	160 µL	0.2 uM
RP1 F-primer (10 uM)	IQ4-CVD-P03	0.8 µL	160 µL	0.4 uM
RP1 R-primer (10 uM)	IQ4-CVD-P04	0.8 µL	160 µL	0.4 uM
RP1-Cy5 Probe (5 uM)	IQ4-CVD-PR02	0.8 µL	160 µL	0.2 uM
Alpha S gene F-primer (10 uM)	IQ4-CVD_AL-P01	0.8 µL	160 µL	0.4 uM
Alpha S gene R-primer (10 uM)	IQ4-CVD_AL-P02	0.8 µL	160 µL	0.4 uM
Alpha S gene-ROX probe (5 uM)	IQ4-CVD_AL-PR01	0.8 µL	160 µL	0.2 uM
Delta S gene F-primer (10 uM)	IQ4-CVD_DLT-P01	0.8 µL	160 µL	0.4 uM
Delta S gene R-primer (10 uM)	IQ4-CVD_DLT-P02	0.8 µL	160 µL	0.4 uM
Delta S gene-HEX probe (5 uM)	IQ4-CVD_DLT-PR01	0.8 µL	160 µL	0.2 uM
Final Volume		**9.6 µL**	**1920 µL**	

#	Reagent ID	Catalog #	Description	Storage	Volume for 200 reactions
1	**IQ4 Multiplex Primer Mix**	IQ4-CVD_DLT_AL-PM	Primers and probes for N and S gene of SARS-CoV-2 & human RP1 gene	-20°C	1920 µL
2	**Nuclease-Free Water**	IQ4-CVD-NFW	Molecular Biology grade Nuclease-free water	-20°C	680 µL
3	**One-Step RT-PCR Mix with UDG (4X)**	IQ4-CVD-PCR	Mixture of Reverse Transcriptase, DNA-dependent DNA Polymerase, UDG, dNTPs, and required buffer components for RT-PCR	-20°C	1000 µL

A Quick-RNA™ Viral Kit from Zymo Research can be included for the convenience of the user, at an additional cost. The Quick-RNA™ Viral Kit is a quick, purification system for viral RNA from plasma, serum, cell culture media, cellular suspensions, urine, blood, saliva and any other biological samples stored in DNA/RNA Shield™. DNA/RNA Shield™ ensures nucleic acid stability during sample storage/transport at ambient temperatures (4-25°C). The reagent effectively lyses cells and inactivates nucleases and infectious agents (virus). The kit also features a specialized buffer system that facilitates complete viral particle lysis for efficient RNA isolation from samples containing enteroviruses, rhinoviruses, coronaviruses, HIV, HCV, influenza A virus, flaviviruses, measles virus, parainfluenza virus, parvovirus (a ssDNA virus), etc. Viral RNA is bound to the column, washed and eluted. The isolated high-quality viral RNA is ready for all downstream applications such as Next-Gen sequencing, hybridization-based and RT/PCR detection.

Product Contents:

Quick-RNA™ Viral Kit (200 preps)	**Storage**	**Volume**
DNA/RNA Shield™ (2X)	Room Temp.	125 mL
Viral RNA Buffer		2 x 100 mL
Viral Wash Buffer		48 mL
DNase/Rnase-Free Water		10 mL
Zymo-Spin™ IC Columns		200
Collection Tubes		400
Instruction Manual		1

6. Equipment/Consumables Required – Not Provided

Disclaimer: Names of vendors or manufacturers are provided as examples of suitable product sources. Inclusion does not imply endorsement by ChemGenes Corporation.

RNA Extraction Kit	qPCR machine (LC 480 II)
Micropipettes (2 or 10 µL, 200 µL and 1000 µL)	qPCR machine (LC 480 II or other compatible machines)
Vortex Mixer	PCR plate cooler for 1.5 microcentrifuge tubes and 96-well 0.2 mL PCR reaction tubes
Sterile, nuclease-free 1.5 mL microcentrifuge tubes	0.2 mL PCR reaction tube strips with caps or 96-well qPCR plates with clear seals
Molecular-grade, nuclease-free water	Racks for 1.5 mL microcentrifuge tubes
Disposable powder-free gloves	RNA Extraction Kit
Surface decontaminants (RNaseZap; 10% bleach)	Ethanol, Absolute, Molecular Biology Grade

7. Nucleic Acid Extraction

- Performance of RT-PCR amplification-based assays depends on the amount and quality of sample template RNA. RNA extraction procedures should be qualified and validated for recovery and purity before testing specimens.
- Commercially available extraction procedures that have been shown to generate highly purified RNA when following manufacturer's recommended procedures for sample extraction include: bioMérieux NucliSens® systems, QIAamp® Viral RNA Mini Kit, QIAamp® MinElute Virus Spin Kit or RNeasy® Mini Kit (QIAGEN), EZ1 DSP Virus Kit (QIAGEN), Roche MagNA Pure SARS-CoV-2 RT-PCR Panel CDC/NCIRD/DVD Compact RNA Isolation Kit, Roche MagNA Pure Compact Nucleic Acid Isolation Kit, and Roche MagNA Pure 96 DNA and Viral NA Small Volume Kit, and Invitrogen ChargeSwitch® Total RNA Cell Kit.
- Retain residual specimen and nucleic extract and store immediately at -70℃
- Only thaw the number of specimen extracts that will be tested in a single day. Do not freeze/thaw extracts more than once before testing.

8. Quality Control

Due to the sensitivity of RT-PCR, these assays should be conducted using strict quality control and quality assurance procedures. Following these guidelines will help minimize the chance of false positive amplification.

General Considerations

- Personnel must be familiar with the protocol and instruments used.
- Maintain separate areas and dedicated equipment (e.g., pipettes, microcentrifuges) and supplies (e.g., microcentrifuge tubes, pipette tips, gowns and gloves) for assay reagent setup and handling of extracted nucleic acids.
- Work flow must always be from the clean area to the dirty area.
- Wear clean disposable gowns and new, previously unworn, powder-free gloves during assay reagent setup and handling of extracted nucleic acids. Change gloves whenever contamination is suspected.
- Store primer/probes and enzyme master mix at appropriate temperatures. Do not use reagents beyond their expiry dates.
- Keep reagent tubes and reactions capped as much as possible.
- Clean and decontaminate surfaces.
- Do not bring extracted nucleic acid or PCR products into the assay setup area.
- Use aerosol barrier (filter) pipette tips only.
- Use PCR plate strip caps only. Do not use PCR plate sealing film.

Assay Controls

- Assay controls should be run concurrently with all test samples.
- PTC - positive template control with an expected Ct value range
- NTC - negative template control added during RT-PCR reaction set-up
- RNASE P - all clinical samples should be tested for human Glyceraldehyde-3-phosphate dehydrogenase (RNASE P) gene to asses specimen quality
- Note: Keep running logs of PTC performance. After each RT-PCR run of clinical samples, the control Ct values should be recorded.

9. Protocol

Prior to starting the qPCR experiment:

- Decontaminate work surfaces and equipment with RNase Zap or 10% freshly prepared bleach.
- Thaw the provided primers and probes. Vortex and spin down contents thoroughly before use.
- Primer/probe mixes should be kept in amber tubes. Contact with light should be minimized.

I. Prepare the reaction:

Component	Volume per 20 µL reaction (µl)
IQ4 Multiplex Primer Mix	9.6
Nuclease-free Water	3.4
One-Step RT-PCR Mix with UDG (4X)	5
Total Volume (µl)	**18**

1. Place a 96-well plate or 8-tube PCR strips onto a PCR cold block.
2. Aliquot 18 µL of the corresponding master mix into separate wells of the plate/tubes.
3. Add 2 µL of each sample to be tested into the appropriate wells. *Note: triplicate reactions are recommended.*
4. Add 2 µL of nuclease-free water per replicate reaction for No Template Control (NTC).
5. Thoroughly seal/cap the sample plate/tubes.
6. Gently vortex the sample plate/tubes and centrifuge briefly to bring reaction to the bottom.

II. Instrument Set-up:

This set-up guide is specific to the Roche LightCycler 480 Instrument II.

Alternative qPCR instruments that can detect R6G (HEX), FAM, ROX, and Cy5 may also be used.

7. Create a new experiment in the Roche LightCycler® 480 Instrument II.
8. Set-up the following RT-PCR program in the '**Experiment**' tab:

Cycle Step	Temperature	Time	Cycles
Reverse Transcription	55 °C	10 min	1
Initial Denaturation	95 °C	60 seconds	1
Denaturation Extension*	95 °C 55 °C	10 seconds 30 seconds	40-45 *Analysis Mode: Quantification - Single

9. After inputting Experiment parameters, go to the '**Subset Editor**' tab and create a new subset by clicking on the +. Select the positions according to the sample plate/tubes to be run. Click '**Apply**' when finished.
10. Go to the '**Sample Editor**' tab.
11. Select '**Abs Quant**' for the workflow.
12. Click on the drop-down menu next to '**Subset**' and select the experiment subset.
13. Fill out the sample name and make sure '**Unknown**' is selected for '**Quantification Sample Type**.'
14. Make sure '**Negative Control**' is selected for the nuclease-free water reactions.
15. Create 'Replicates' according to the sample plate.

See the set-up below for an example of a six-sample run (S1 – S6) with the SARS-CoV-2 Positive Control (POS) and no-template control (NTC).

	1	2	3	4	5	6	7	8	9	10	11	12
A	POS	POS	POS	S1	S1	S1	S2	S2	S2	S3	S3	S3
B	S4	S4	S4	S5	S5	S5	S6	S6	S6	NTC	NTC	NTC
C												
D												
E												
F												
G												
H												

16. After all the experiment parameters are filled out, place the sample plate/tubes in the instrument according to the plate set-up and run the assay.

III. Analyzing the Data:

17. After the run is complete, go to the Analysis tab.
18. Select '**Abs Quant/2nd Derivative Max**' and the experiment subset. Click OK.
19. Click '**Calculate'** to perform absolute quantification analysis.
20. Export the Ct values by right-clicking the Results table and selecting '**Export Table**'.

10. Interpreting the Data

Gene	Expected Ct	Interpretation
RNase P	< 38	Failure to detect RNASE P may indicate: ● Improper assay set-up and/or execution ● Reagent or equipment malfunction Detection of RNASE P in Positive Control but failure to detect RNASE P in any clinical samples may indicate: ● Improper extraction of nucleic acid from clinical specimens ● Insufficient human cellular material due to poor collection or lost integrity. If the N gene is positive in the absence of positive RNASE P, the result should be considered valid.
N	< 38	The result is considered positive for SARS-CoV-2 if it has Ct values < 40 (RNASE P may not be positive for results to be valid). When all controls exhibit the expected performance, but the N markers and RNASE P do not cross the threshold line, the result is invalid. The extracted RNA from the specimen should be retested.
Alpha S gene	< 38	Failure to detect Alpha S gene but positive N indicates the sample contains Alpha RNA. If positive, the sample is wildtype in the region of detection and is a variant that is not detected specifically by this kit.
Delta S gene	< 38	Failure to detect Delta S gene but positive N indicates the sample contains Delta RNA. If positive, the sample is wildtype in the region of detection and is a variant that is not detected specifically by this kit.

SARS-CoV-2_N	SARS-CoV-2_Alpha S	SARS-CoV-2_Delta S	RNase P	Interpretation
+	+	+	±	SARS-CoV-2 detected
If two of the three targets are positive			±	SARS-CoV-2 Alpha or Delta detected
If only one of the three targets is positive			±	Inconclusive Result
-	-	-	+	SARS-CoV-2 not detected
-	-	-	-	Invalid Result

11. Assay Limitations

- Analysts should be trained and familiar with testing procedures and interpretation of results prior to performing the assay.
- A false negative result may occur if inadequate numbers of organisms are present in the specimen due to improper collection, transport or handling.
- RNA viruses in particular show substantial genetic variability. Although efforts were made to design RT-PCR assays to conserved regions of the viral genomes, variability resulting in mismatches between the primers and probes and the target sequences can result in diminished assay performance and possible false negative results.

12. Contact Information

Suggestions and questions concerning this procedure may be sent to:

Contact Person: Dr. Suresh Chandra Srivastava
Manufacturer: ChemGenes Corporation
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