Introduction

Coronavirus belongs to the family of Coronaviridae, in the order of Nidovirales. It consists of a positive-sense single-stranded RNA, usually appears spherical with a size of 80-120nm and with crown-like spikes on the surface. This large family of the virus is commonly circulating among vertebrates, such as camels, cats, and bats. Novel coronavirus (COVID-19) has been identified as a new strain of coronavirus. Novel Coronavirus (SARS-CoV-2) is a single-stranded positive RNA (+ssRNA) virus that translates four structural proteins (S, M, E, and N) which are essential for virion assembly and infection to the host.[1] Spike on the surface (S-protein) are responsible for host Angiotensin-Converting Enzyme 2 (ACE2) receptor attachment and serine protease TMPRSS2 priming. Viral pathogenesis is carried out by E-protein. Viral replication is enhanced by N-protein with the help of RDRP (non-structural proteins).[2] Analyses of viral-specific genes by reverse transcription-polymerase chain reaction (RT-PCR) are suitable diagnostic modalities for qualitative analysis with high sensitivity and specificity.

Intended use

COVID-19 TRIPLEX RT-PCR DIRECT is a real-time RT-PCR test intended for the qualitative detection of SARS-CoV-2 Directly from the upper respiratory swab, nasopharyngeal or Oropharyngeal swabs, anterior nasal swabs, mid-turbinate nasal swabs, nasal or nasopharyngeal aspirates, nasal washes, and bronchoalveolar lavage samples collected in Normal saline or Preservative media, {Viral Transport Media (VTM) or Universal Transport Media (UTM)}, pH 7.2-7.4 to provide the molecular diagnostic basis for infected patients[3]. This test is also for the qualitative detection of RNA extracted from upper respiratory swab, nasopharyngeal or Oropharyngeal swabs, anterior nasal swabs, mid-turbinate nasal swabs, nasal or nasopharyngeal aspirates, nasal washes, and bronchoalveolar lavage samples collect from individuals suspected of COVID-19 by the healthcare provider.

The assay is intended for use by qualified and trained clinical laboratory personnel specifically instructed and trained in the techniques of real-time PCR.

The test results of this kit are for clinical reference only and should not be used as the only standard for clinical diagnosis. It is recommended to conduct a comprehensive analysis by combining the test results with patients' symptoms and other laboratory tests.

Principle

COVID-19 TRIPLEX RT-PCR DIRECT test includes primer and probe mix that adopts the Triple target gene design, which detects the specific conserved sequence encoding the Nucleocapsid (N) gene, Envelop (E) gene, and RNA Dependent RNA Polymerase (RdRp). The amplification of the RNA template can be monitored by the increasing fluorescence signal detected by a real-time PCR instrument. The primer probe set specific to detect RNase P (RP), is also included to serve as an endogenous internal reference. The result of internal control provides the accuracy of sampling and RNA availability, to avoid false-negative results

Equipment and Consumables Required (But Not Provided)

Biosafety cabinet, Preservative media & Swabs, RT-PCR instrument, Vortex mixer, Microcentrifuge and microcentrifuge tubes, Micropipettes, and aerosol barrier pipette tips, Gloves & Personal Protective Equipment (PPE).

Controls to be used & provided with the COVID-19 RT-PCR DIRECT

1. A No template control (Nuclease free water) is needed to verify the possibility of sample contamination on the assay run and is used on every assay run.
2. A positive control (COVID-19_P & RNase P) is needed to verify that the assay run is performing as intended and, is used on every assay run at a given concentration.
3. An Internal control (RNase P) targeting the human RNase P gene is needed to verify that nucleic acid is present in every sample and is used for every sample processed to ensure that samples resulting as negative contain nucleic acid for testing.

Components	Volume (100 Tests)	Volume (500 Tests)	Volume (1000 Tests)
RT Mix	150 µl	750 µl	1.5 mL
Master Mix	1.0 mL	5.0 mL	10.0 mL
Primers & probes set	350 µl	1.75 mL	3.5 mL
Positive control	100 µl	200 µl	300 µl
Nuclease Free Water	100 µl	200 µl	300 mL

Storage and Shelf life

All components of the COVID-19 TRIPLEX RT-PCR DIRECT diagnostic kit must be stored at -20°C ±5°C with protection from light. Reagents are stable for 12 months when stored at the recommended conditions. Repeated freezing and thawing may lead to inaccurate results. Leftover reagents should be stored at 4°C for no longer than 1 week. Do not use the kit after the expiry date or if the pack is damaged.

Instrument Compatibility

COVID-19 TRIPLEX RT-PCR DIRECT kit is compatible with real-time PCR instruments with FAM, HEX/VIC, Texas Red and Cy5 channels.

Sample Collection and Storage

Sample Type: Upper respiratory swab, nasopharyngeal or oropharyngeal swabs, anterior nasal swabs, mid-turbinate nasal swabs, nasopharyngeal aspirates, nasal washes, and bronchoalveolar lavage specimen collected in normal saline or virus preservative buffer (VTM or UTM), pH 7.2-7.4.

Sample Collection: Collect the sample following the conventional sample collection method.

Specimen collection should avoid possible contamination according to regulatory guidelines. Refer to Interim Guidelines for Collecting, Handling, and Testing Clinical Specimens from Patients Under Investigation (PUIs) for 2019 Novel Coronavirus (2019-nCoV).[4]

Sample Storage & Transportation: The sample to be tested can be processed immediately after collection or it may be stored at 2-8°C for up to 24 hours before testing. Sample to be tested can be processed immediately, or stored at 2-8°C for up to 24 hours, -20 ± 5°C for 3 months or -70°C for long term. The specimen should be shipped in low-temperature conditions using refrigerant packs or dryice.

Sample processing

50 µl of the specimen collected in normal saline or virus preservative buffer (VTM or UTM), pH 7.2-7.4 is subjected to heat treatment at 70°C for 15 minutes in water/dry bath. 5 µl of this heated specimen can be Directly used as template in reaction mix.
Alternatively, RNA extraction can be carried out following the manufacturer's instruction using RNA extraction kit. Extracted RNA can be used Directly for PCR detection. Otherwise, keep RNA sample at -70°C if not in use. Avoid repeated thawing and freezing. RNA should be used as a template in the RT-PCR reaction.

Assay Setup

1. In the reagent set-up room, clean the biosafety cabinet, place RT mix, Master Mix, and Primer/Probes on iceor cold-block.

2. Determine the number of reactions (N) to set above assay. It is necessary to make excess reaction mix for the negative & positive controls and compensating pipetting error.

3. The recommended sample volume used in the reaction is 05 µL. Prepare the reaction mix by combining Master Mix, RT Mix and Primers & Probe set as described below:

1 x Volume Required*	
Component	**Volume**
Master Mix	10.0 µL
RT Mix	1.5 µL
Primers & Probe set	3.5 µL
Total Volume	15.0 µL

*Multiply the numbers according to the number of tests.

4. Aliquot 15 µL of the above reaction mix into the PCR plate or tubes of the chosen PCR platform. Aliquot into wells according to the number of samples to be tested, include one well for the positive control and one well for the negative control.

5. Transfer the reaction mix to Sample Processing Area. Add 5.0 µL of the processed specimen or RNA and controls to the appropriate wells/tubes. After adding the samples, cover the lid immediately. Spin down briefly using a centrifuge to remove air bubbles. Transfer the mixture to the amplification area.

Selection of Fluorescence channel

Gene	**Dye**	**Color**
N Gene	FAM	Green
RDRP gene	HEX/VIC	Yellow
E gene	Texas Red	Orange
RNase P	Cy5	Red

Thermal Cycling

Enter the amplification program. Recommended as below:

Step	**Cycles**	**Temperature**	**Time**
cDNA Synthesis	1	50°C	30 min
Initial Denaturation	1	95°C	2 min
PCR cycling	45	95°C	15 sec
		60°C	40 sec

6. Place the tubes on the sample holder in the instrument. Set up the test panel according to the positions of positive control, negative control, and RNA samples. Save the file after settings and run the reaction.

7. **Result Interpretation:** (Please refer to the user manual of instrument for setting, the following analysis uses ABI series instruments as an example)

   After the reaction is completed, the results are automatically saved and the amplification curves of the detected target DNA and the internalcontrol are analyzed individually.

8. According to the analysis, the amplification plot will adjust the Start value, End value, and Threshold value of the Baseline (Users can adjust the values according to the actual situation. Start value can be set within 3~15, End value can be set within 5~20; Users can adjust the amplification curve of negative control to make it linearor below the threshold line). Click "Analyze" to perform the analysis and the parameters should meet the following requirements mentioned in "Quality Control". Lastly, record the qualitative results in the Plate window.

Quality Control

All test controls should be examined before the interpretation of patient results. If the controls are not valid, the patient results cannot be interpreted.

COVID-19 TRIPLEX RT-PCR DIRECT – Positive, Negative and Internal Controls

The expected results generated from each control and acceptance criteria are as follows:

- No Template Control (NTC): No data or no sigmoid amplification should be observed in FAM, HEX/VIC Texas Red and Cy5 channels before Ct value 36. If amplification is observed with any channel in the no template control (NTC) reactions, sample contamination may have occurred and repeat testing is recommended.
- Positive Control – FAM, HEX, and Texas Red channels, Ct ≤ 36 and Cy5 channel, Ct ≤ 36.
- Internal Control (RNase P) - Internal Control (Cy5) channels, Ct ≤ 36.

If controls do not perform as described above, the run is considered invalid and the specimen must be reanalyzed, and the test should be repeated from the sample processingstep.

Quality Control				
Control	N gene	RDRP gene	E gene	Internal control-RNaseP
No Template Control	No Ct or Ct>36	No Ct or Ct>36	No Ct or Ct>36	No Ct or Ct>36
Positive Control	Ct≤36	Ct≤36	Ct≤36	Ct≤36

Target	Ct value	Result
N gene	Ct≤36	SARS-CoV-2 N positive
RDRP gene	Ct≤36	SARS-CoV-2 RDRP positive
E gene	Ct≤36	SARS-CoV-2 E positive
Internal Control – RNase P	Ct≤36	Internal Control

Analysis of Results

a) First to analyze the amplification curve of internal control Cy5 channel. If Ct ≤ 36, it indicates that the detection is valid, and users can continue the subsequent analysis:
   1) If a typical S-type amplification curve is detected in the FAM or HEX/VIC or Texas red channel, with Ct ≤ 36, it indicates that the COVID-19 virus is positive.
   2) If FAM, HEX/VIC and Texas Red channels do not detect a typical S-type amplification curve (No Ct) or Ct > 36, it indicates that the COVID-19 virus is negative.
   3) If anyone of FAM or HEX/VIC or Texas Red channels detects a typical S-type (sigmoidal) amplification curve with Ct ≥ 36 and ≤40, the sample shall be considered as suspected. The user should repeat the experiment. If upon repetition, the Ct value appears in the same Ct range, the sample shall be considered presumptive positive.

b) If the internal control Cy5 channel failed to detect Ct or Ct >36, it indicates that the concentration of the tested sample is too low or there is an inhibitory of RT-PCR reaction from the interfering substance. Users have to repeat the experiment.

c) If there is no typical S-shape amplification curve or Ct >36 or No Ct detected for N, RDRP, E, and RNase P, it indicates that the specimen concentration is too low, or there are interfering substances that inhibit the reaction. If upon retest, the result is invalid again, another fresh sampleshould be collected and tested.

Cut-off for all targets

Assessment of clinical specimen test results should be performed after the positive, negative (no template) and internal controls have been examined and determined to be valid and acceptable. If the RNase P assay does not produce a positive result for human clinical specimens, interpret as follows:

(i) If N, E, and RDRP genes are positive even in the absence of a positive RNase P, the result should be considered valid. Some samples may fail to exhibit RNase P growth curves due to low cell numbers in the original clinical sample. A negative RNase P signal does not preclude the presence of SARS CoV-2 virus RNA in a clinical specimen.

(ii) If all the 3 genes along with RNase P are negative for the specimen, theresult should be considered invalid for the specimen. If the residual specimen is available, repeat the sample processing procedure and repeat the test. If all markers remain negative after re-test, report the results as invalid and a new specimen should be collected if possible.

N Gene	RDRP gene	E Gene	RNase P Gene	Result	Report	Actions (Specimen from clinical sites)
-	-	-	+	SARS-CoV-2 not detected	SARS-CoV-2 Negative	Report results to sender
+	+	+	±	SARS-CoV-2 Detected	SARS-CoV-2 Positive	Report results to sender and appropriate public health authorities
If any two of the three targets is positive			±	SARS-CoV-2 Detected	SARS-CoV-2 Positive	Report results to sender and appropriate public health authorities
If any one of the three targets is positive			±	SARS-CoV-2 Inconclusive	Inconclusive	The sample is repeated, If the second repeat result remains inconclusive, the collection of new specimen is recommended, if clinically indicated
-	-	-	-	Invalid	Invalid	The sample is repeated, if a second failureoccurs, report to sender as invalid; recommend recollection of sample.

Warnings and Precautions

1. This product is only used for *in vitro detection*. Please read this manual carefully before use.

2. Laboratory personnel should be trained and familiar with the operating procedures and precautions of the instrument before the experiment. Quality control should be performed foreach experiment.

3. Laboratory management should be strictly following the regulations of PCR gene amplification laboratories. Laboratory personnel must be professionally trained and the experimental process should be strictly divided into sections. All consumables should be used only once after sterilization. Instruments and equipment should be assigned to each stage of the experiment and cannot be used alternatively. All samples should be regarded as potentially infectious materials. Laboratory workers should wear appropriate personal protective equipment (PPE) which includes disposable gloves, laboratory coat, or grown. Gloves should be changedregularly to avoid cross-contamination between samples.

4. Clinical laboratories involving manipulation of potentially infected specimens should be performed in a certified Class II Biological Safety Cabinet (BSC) in a BSL-2 facility. Diagnostic tests should follow standard laboratory practices, including Standard Precautions, when handling potential patient specimens. For laboratory waste, follow standard procedures associated with other respiratory pathogens.

Catalog No.	Description
TRT010-100	100 Test COVID-19 TRIPLEX RT-PCR DIRECT
RT010-500	500 Test COVID-19 TRIPLEX RT-PCR DIRECT
RT010-1000	1000 Test COVID-19 TRIPLEX RT-PCR DIRECT

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References

1. Satarker, S., & Nampoothiri, M. (2020). Structural Proteins in Severe Acute Respiratory Syndrome Coronavirus-2. Archives of medical research, 51(6), 482–491.
2. Cong, Y., Ulasli, M., Schepers, H., Mauthe, M., V'kovski,P., Kriegenburg, F., Thiel, V., de Haan, C., & Reggiori,
F. (2020). Nucleocapsid Protein Recruitment to Replication-Transcription Complexes Plays a Crucial Role in Coronaviral Life Cycle. Journal of virology, 94(4), e01925-19.
3. Smyrlaki, I., Ekman, M., Lentini, A. et al. (2020). Massive and rapid COVID-19 testing is feasible by extraction-free SARS-CoV-2 RT-PCR. Nat Commun 11, 4812.
4. https://www.fda.gov/media/135659/download.

Index of Symbols

IVD In Vitro Diagnostics only
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