COVID-19 RT-PCR Kit User Manual v.1

For One-Step Real Time RT-PCR for Detection of COVID-19 Corona Virus

For use with BioRad CFX 96, CFX 384 systems

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GeneAid Biomed COVID-19 Real time RT-PCR Kit

1. INTENDED USE

GeneAid Biomed COVID-19 RT-PCR kit is for *in vitro* diagnostic test. It is a One-Step Real Time RT-PCR for detection of COVID-19 corona virus in human nasopharyngeal and oropharyngeal swab or sputum specimens.

2. PREFACE

The ongoing outbreak of the recently emerged novel coronavirus (COVID-19) poses a challenge for public health laboratories as virus isolates are unavailable while there is growing evidence that the outbreak is more widespread than initially thought, and international spread through travelers does already occur.

The Middle East respiratory syndrome (MERS) virus, another beta coronavirus, appears more distantly related. The closest RNA sequence similarity is to two bat coronaviruses, and it appears likely that bats are the primary source; whether COVID-19 virus is transmitted directly from bats or through some other mechanism (through an intermediate host) is unknown.

Pneumonia appears to be the most frequent manifestation of infection, characterized primarily by fever, cough, dyspnea, and bilateral infiltrates on chest imaging. Although many of the reported infections are not severe, approximately 20 percent of confirmed patients have had critical illness (including respiratory failure, septic shock, or other organ failure requiring intensive care). The overall case-fatality rate is uncertain but appears to be around 2 percent in China. Most of the fatal cases have occurred in patients with underlying medical comorbidities.

Asymptomatic infections and mild respiratory illnesses have also been described, but their frequency is unknown. In some patients, gastrointestinal symptoms (nausea and diarrhea) have been reported, but this is uncommon. In patients with COVID-19, the white blood cell count can vary. Leukopenia, leukocytosis, and lymphopenia have been reported, although lymphopenia appears most common. Elevated aminotransferase levels have also been described. On admission, many patients with pneumonia have normal serum procalcitonin levels; however, in those requiring intensive care unit (ICU) care, they are more likely to be elevated.

Full-genome sequencing and phylogenic analysis indicated that the coronavirus that causes COVID-19 is a beta coronavirus in the same subgenus as the severe acute respiratory syndrome (SARS) virus (as well as several bat coronaviruses), but in a different clade. The apparent structure of the receptor-binding gene region is very similar to that of the SARS coronavirus, and there is speculation that it will be shown to use the same receptor for cell entry.

Among the foremost priorities to facilitate public health interventions is reliable laboratory diagnosis. In acute respiratory infection, RT-PCR is routinely used to detect causative viruses from respiratory secretions. Here we demonstrated the feasibility of introducing robust detection technology based on real-time RT-PCR in public health laboratories during international health emergencies.

3. NAME AND FUNCTION

The Geneaid Biomed COVID-19 RT-PCR kit is a molecular diagnostic kit, developed by GeneAid Biomed, Taiwan. It is for the use of COVID-19 screening test in human nasopharyngeal and oropharyngeal swab or sputum specimens, and plays an important role in anti-viral drug efficacy evaluation. The COVID-19 primer and probe set is designed for the specific detection of COVID-19 targeting on ORF1b gene using Taqman probe real-time RT-PCR technology.

4. PRINCIPLE OF THE TEST

The kit is intended for the *in vitro* detection and quantification of COVID-19 RNA in human nasopharyngeal and oropharyngeal swab or sputum specimens utilizing 1-step Reverse Transcriptase-Polymerase Chain Reaction (1-step RT-PCR) and TaqMan probe technology. The COVID-19 Primers and Probes contain primer pairs and TaqMan probes specific for COVID-19 target/standard. The COVID-19 RNA signal is detected in the HEX channel. The human 5S RNA is used as the IC (HEX channel). In each run, the P (positive control) and N (negative control) are also included.

The COVID-19 kit P (positive control) is a non-infectious synthetic DNA molecule containing COVID-19 ORF1b gene sequence. The COVID-19 kit N (negative control) is the dd H_2O contain no template. The sample is considered as COVID-19 positive (+) if the unknown sample Ct is faster than that of the N.

5. REAGENTS PROVIDED IN THE KIT (50 TESTS/KIT)

Color code	Labeling and contents	Volume (ul)/test	Volume (µl) /50 tests	Quantity 50 tests	Storage
	COVID-19 Primers and Taqman probe mix	1.35	67.5	1	-20C
	IC Primers and Taqman probe mix	1.35	67.5		
	2x RT-PCR buffer	10	1000		
	Enzyme mix	0.35	35		
	PCR Grade Water	3.3	330		
	P (E7 copies/5 ul)	5	15		
	NTC (dd H_2O, 0 copy/5 ul)	5	15		
	C (unkn) provided by user				

P=Positive control

NTC=No template control

C=Clinical sample=Unknown sample

For each kit, 3 positives and 3 negatives are included.

6. STORAGE

The COVID-19 kit should be stored at -20°C. These kit components are stable until expiry date stated on the label. Repeated thawing and freezing should be avoided.

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7. ADDITIONALLY REQUIRED MATERIALS AND DEVICES

1. Latex gloves, powderless
2. Microcentrifuge
3. 1.5 ml RNase-free and DNase-free centrifuge tubes
4. 10 µl, 20 µl, 200 µl micropipettes and disposable RNase-free and DNase-free tips with aerosol barrier
5. Viral Nucleic Acid Extraction Kit II (GeneAid)
6. BioRad CFX96 or BioRad CFX384 systems with BioRad 96-well reaction plates with optical adhesive covers or optical strips and optical strip caps.

8. GENERAL PRECAUTIONS FOR USERS

1. This reagent kit is for *in vitro* diagnosis only.
2. This reagent kit must not be used after expiration date.
3. Do not pool reagents from different lots or from different bottles of the same lot.
4. Reagents must be protected form microbial contamination.
5. The P (positive control) is made of sequence from COVID-19 genome. For safety, it must be treated as infectious material.
6. Thaw all components thoroughly at room temperature, mix the components and centrifuge briefly before starting an assay.
7. Do not smoke, eat or drink in work areas, or pipet by mouth.
8. Latex gloves must be worn when handling reagents or specimens and must be changed before leaving the area. Wash hands thoroughly afterwards.
9. To avoid cross contamination between specimens and reagents, workflow in the laboratory must proceed in a uni-directional manner from the Pre-Amplification Area to Post-Amplification Area.
10. Thoroughly clean and disinfect all work surfaces with 5% sodium hypochlorite, followed up by drying with 70% ethanol.
11. Before disposal, all waste materials should be properly disinfected by autoclave for at least one hour at 121.1°C or incineration.
12. To performing color detection with HEX-labeled TaqMan probe, routinely color calibration must be done to ensure the best instrument performance.

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.

9. SAMPLE PREPARATION AND STORAGE

Specimen Handling and Storage

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

Sample Collection, Storage, and Transport

The kit is for use with nasopharyngeal and oropharyngeal swab or sputum specimens. The nasopharyngeal and oropharyngeal swabs are collected in Dacron or polyester flocked swabs. The samples are stored and transported at 4C. The testing should be conducted soon after collection. The nasopharyngeal and oropharyngeal swabs should be placed in the same tube to increase the viral load. The sputum sample is collected in sterile sputum container. The samples are stored and transported at 4C. The testing should be conducted soon after collection. It should be ensured that the sputum is from the lower respiratory tract.

Sample processing

Nasopharyngeal and oropharyngeal swab sample

The Nasopharyngeal and oropharyngeal swab can be immersed into the 400 ul of VB lysis buffer and then the eluted out lysis buffer can be subjected to the viral purification procedures as described below.

Sputum sample

Specimens are digested by the *N*-acetyl-L-cysteine-NaOH (NALC-NaOH) method. An equal volume of NALC-NaOH solution (2% NaOH, 1.45% Na-citrate, 0.5% NALC) is mixed with the specimen and incubated at room temperature for 20 min. The digested samples are mixed with VB lysis buffer (2x volumes) and then can be subjected to the viral purification procedures as described below.

COVID-19 RNA Purification

Viral Nucleic Acid Extraction Kit II Protocol (GeneAid)
important before use!

- Add absolute ethanol (see the bottle label for volume) to the AD Buffer prior to initial use
- Add absolute ethanol (see the bottle label for volume) to the Wash Buffer prior to initial use
- Additional requirements: absolute ethanol, microcentrifuge tubes (DNase and RNase-free), Phosphate-Buffered Saline Quick Protocol Binding Wash Elution DNA/RNA Virus Cell Lysis

Step 1. Lysis

• Transfer 200 µl sample to a 1.5 ml microcentrifuge tube. NOTE: If the sample is less than 200 µl, adjust the sample volume to 200 µl with PBS.
• Add 400 µl of VB Lysis Buffer to the sample then mix by vortex.
• Incubate at room temperature for 10 minutes.

Step 2. Nucleic Acid Binding

• Add 450 µl of AD Buffer (make sure ethanol was added) to the sample lysate.
• Shake the tube vigorously to mix.
• Place a VB Column in a 2 ml Collection Tube.
• Transfer 600 µl of the lysate mixture to the VB Column.
• Centrifuge at 14-16,000 x g for 1 minute.
•Discard the flow-through then place the VB Column back in the 2 ml Collection Tube
• Transfer the remaining mixture to the VB Column.
• Centrifuge at 14-16,000 x g for 1 minute.
• Discard the 2 ml Collection Tube containing the flow-through.
• Transfer the VB Column to a new 2 ml Collection Tube.

Step 3. Wash

• Add 400 µl of W1 Buffer to the VB Column then centrifuge at 14-16,000 x g for 30 seconds.
• Discard the flow-through then place the VB Column back in the 2 ml Collection Tube.
• Add 600 µl of Wash Buffer (make sure ethanol was added) to the VB Column.
• Centrifuge at 14-16,000 x g for 30 seconds.
• Discard the flow-through and place the VB Column back in the 2 ml Collection Tube.
• Centrifuge at 14-16,000 x g for 3 minutes to dry the column matrix.

Step 4. Nucleic Acid Elution

• Place the dried VB Column in a clean 1.5 ml microcentrifuge tube.
• Add **25 µl** of RNase-free Water to the CENTER of the VB Column matrix.
• Let stand for at least 3 minutes to ensure the RNase-free Water is absorbed by the matrix.
• Centrifuge at 14-16,000 x g for 1 minute to elute out the viral nucleic acid. Use 5 ul for each RT-PCR assay.

10. PREPARING THE RT-PCR

In every PCR run, please make sure that quantitative standards and one negative control (water, PCR grade) are included. All reagents need to be thawed thoroughly and centrifuged briefly before use. For the preparation of the PCR assay please use the following pipetting scheme.

1. Mix enough COVID-19 Primers/Taqman Probes mix, 2x RT-PCR buffer, and Enzymes Mix, and PCR Grade Water as the following table, and then centrifuge briefly. Dispense 15 µl of reagent mix into each of optical tubes (or plate).

2. Add 5 µl of P (positive control) to optical tubes as positive quantitation standards.
3. Add 5 µl of NTC to one optical tube as negative controls.
4. Add 5 µl of purified RNA samples to optical tubes.
5. Seal the tubes (plate) and centrifuge briefly. Run the reaction immediately.

Table. COVID-19 RT-PCR protocol

component	volume (ul)/each assay			
	P	N	C	IC
COVID-19 Primers/Taqman probe mix	1.35	1.35	1.35	
IC Primers/Taqman probe mix				1.35
2x RT-PCR buffer	10	10	10	10
Enzymes Mix	0.35	0.35	0.35	0.35
RNA Sample or P or NTC	5 (P)	5 (NTC, H_2O)	5 (C)	5 (C)
PCR Grade Water	3.3	3.3	3.3	3.3
Total volume (ul)	20	20	20	20

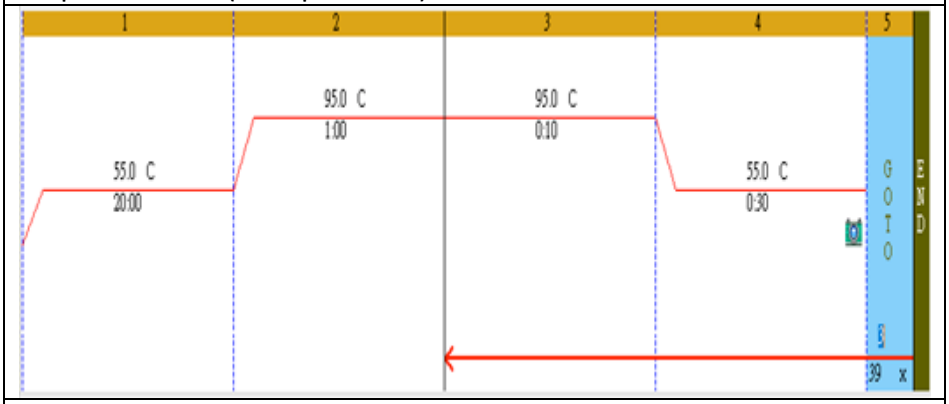
Table. COVID-19 RT-PCR program

Times and Temperatures			
Initial steps		40 cycles	
cDNA synthesis	Inactivate RT and activate HS Taq	Melt	Anneal, extend, and fluorescence collection
55℃ 20 min	95℃ 1 min	95℃ 10 sec	55℃ 30 sec

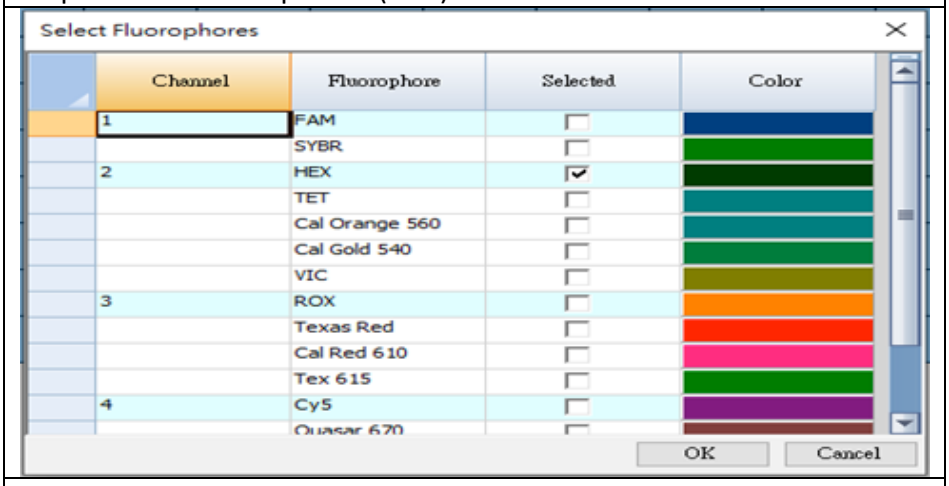
The program runs approximately for 1 hour and 20 minutes.

Schematic Programming Procedure:
Turn on instrument and select software
Select Fluorescence Detectors (Filter channel)
Set up Sample Plate with Tasks, Quantities, and Detectors
Check the Finished Plate Set-UP
Protocol Set up
After run finished with success, analyze result of target by Ct
Analyze Results of P, N, and C (unkn)
Report of Assay (COVID-19 positive or negative)

Step1: Protocol (1-step RT-PCR)



Step 2: Select fluorophores (HEX)



Step 3: Plate set (for 10 samples)

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B	NTC N	Unk C1	Unk C2	Unk C3	Unk C4	Unk C5	Unk C6	Unk C7	Unk C8	Unk C9	Unk C10	Pos P
C		Pos IC	Pos IC	Pos IC	Pos IC	Pos IC	Pos IC	Pos IC	Pos IC	Pos IC	Pos IC	
D												
E												
F												
G												
H												

Step 4: Start run

Step 5: Data analysis

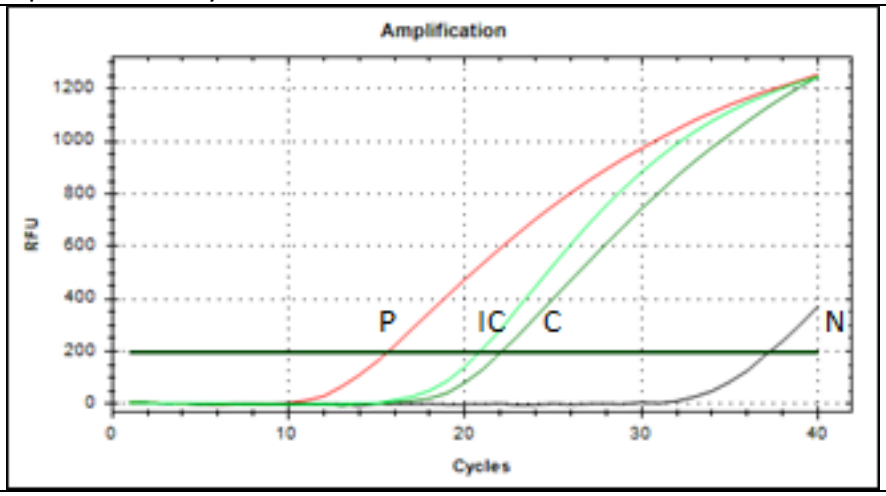
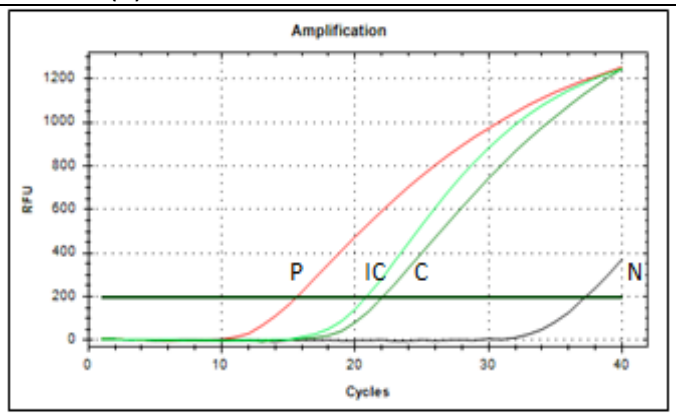
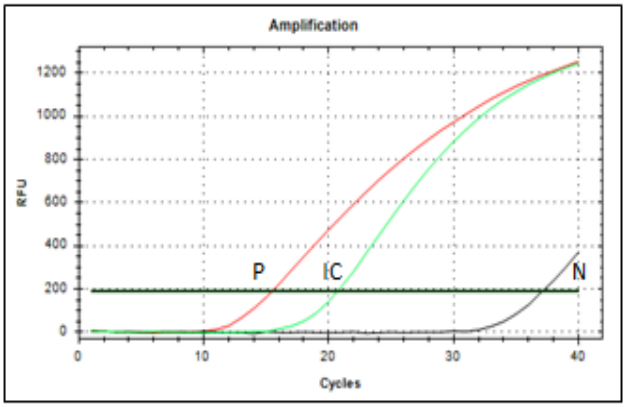
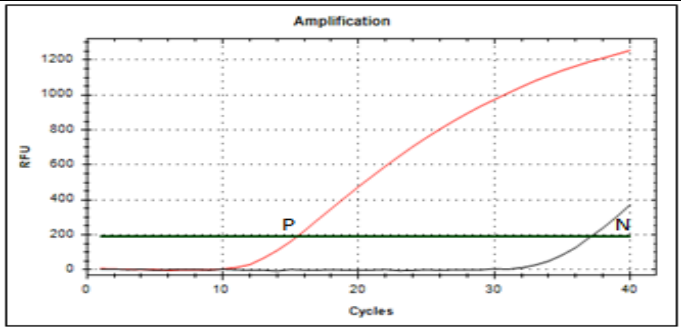
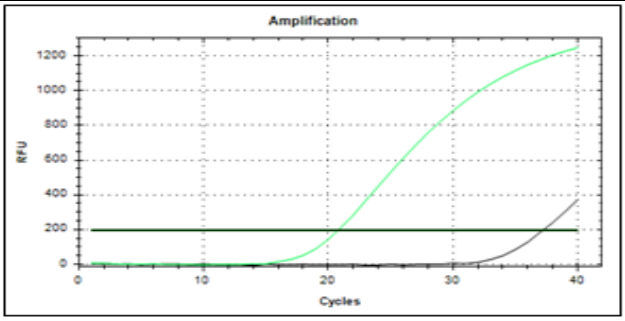


Fig. The 5 major steps are high-lighted; Set protocol, select fluorophore, Set plate, start run, and data analysis.

11. DATA ANALYSIS

Positive (+)	Negative (-)
	

Invalid results:

IC failure (purification problem)	P failure
	

P failure, IC failure

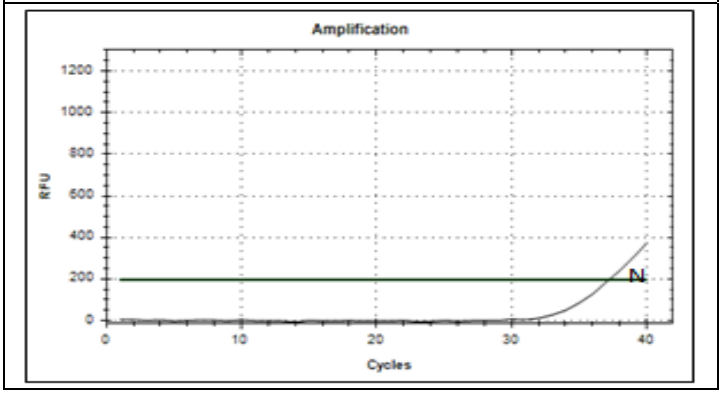


Fig. Data analysis. When finished, analyze the Ct from the HEX fluorescence channel. Adjust the baseline at RFU of 200. The P has a Ct of between 15-18. The N has a Ct <=36. When the C has a Ct <=36 it is considered as a positive (+). When the C has a Ct >=36, it is considered as a negative (-). (Top) COVID-19 positive (+). Red: P, Black: N, Green: C (unkn). (Bottom) COVID-19 negative (-). Red: P, Black: N, Green: C (unkn).

12. PERFORMANCE VALIDATION

Reproducibility

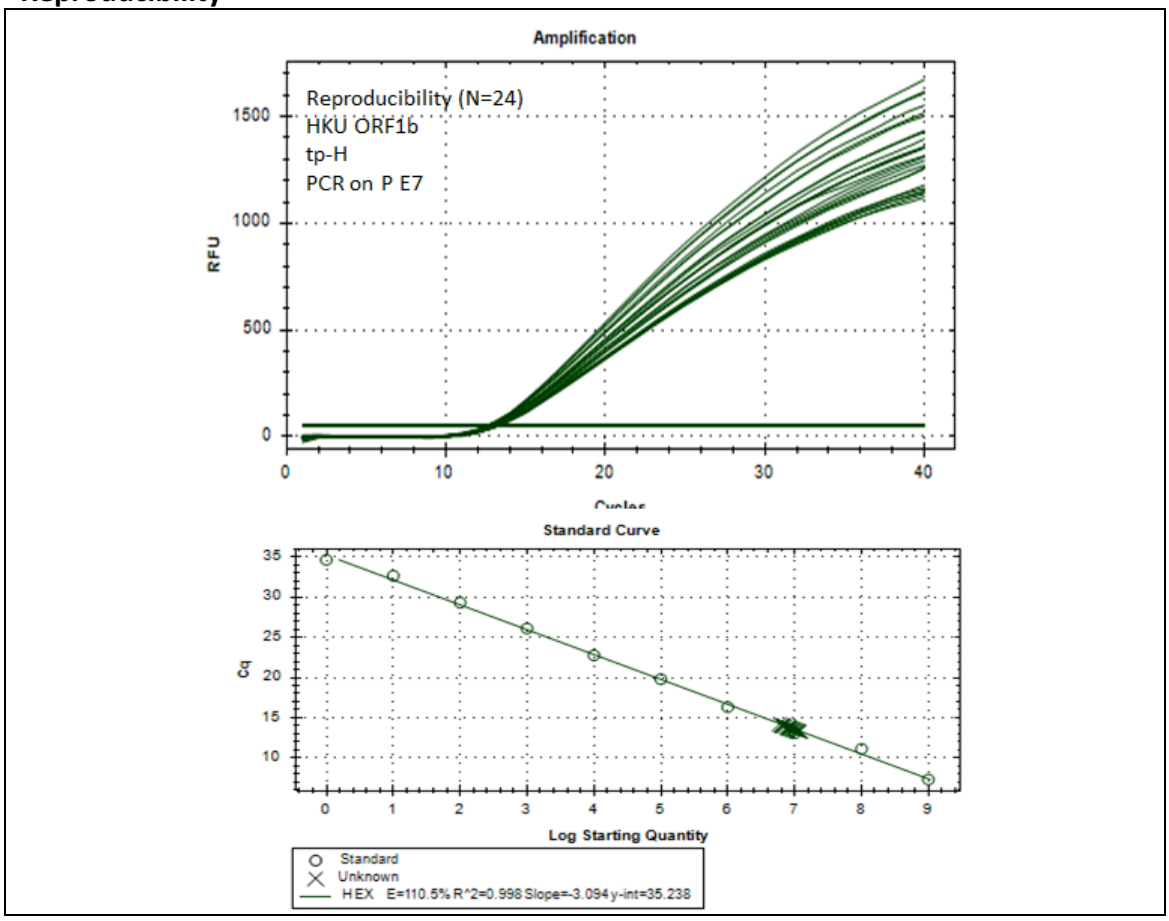


Fig. Reproducibility. When N=24 of P (E7 copies input) were run, the results show this assay is highly reproducible. The average Ct has a very narrow SD (2-3%).

Sensitivity and detection dynamic range

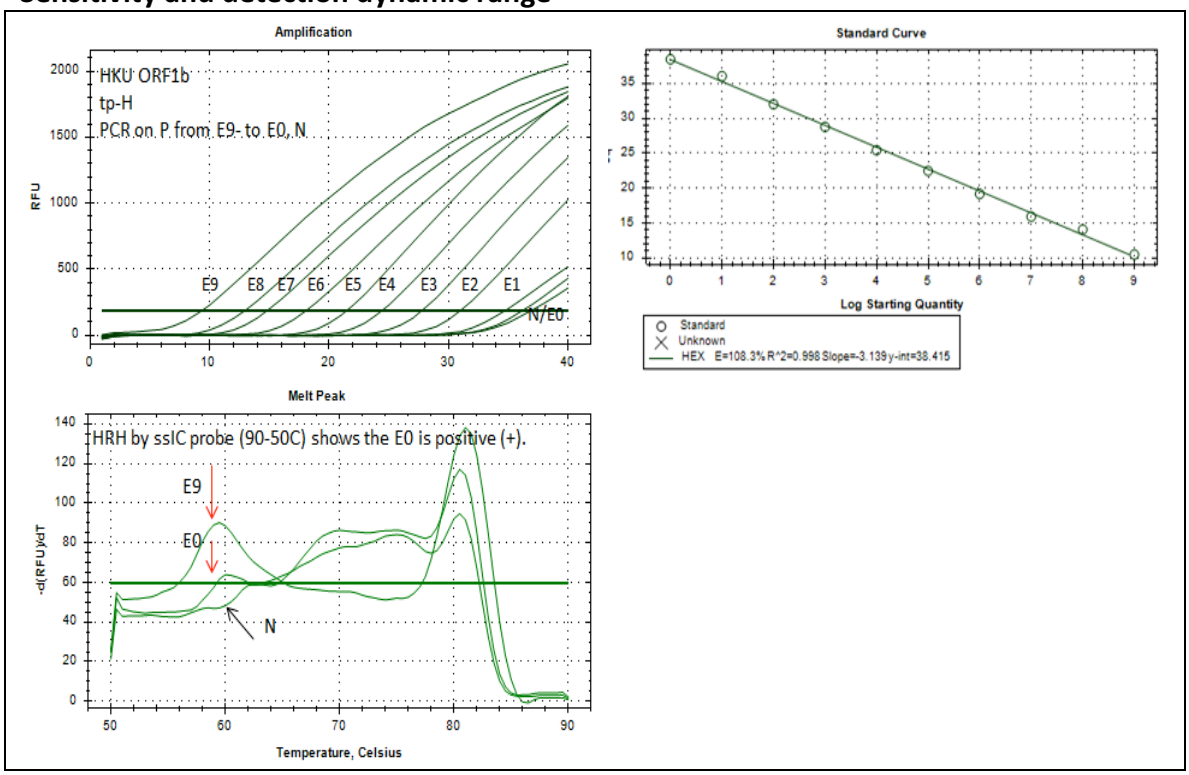


Fig. Sensitivity and detection dynamic range. The assay sensitivity is to E1 copies by real-time Taqman probe qRT-PCR assay. The E0 is also shown being positive by HRH using a probe. The real-time assay lineal (dynamic) range is E9-E1 copies covering of 9 logs range. If coupling to a HRH (high resolution melting) method (GeneAid Biomed proprietary technology), the sensitivity can go up to E0 copy.

13. SPECIFICATIONS

Analytical Sensitivity

The assay sensitivity is to E1 copies by real-time Taqman probe qRT-PCR assay. The real-time assay lineal (dynamic) range is E9-E1 copies covering of 9 logs range. If coupling to a HRH (high resolution melting) method (GeneAid Biomed proprietary technology), the sensitivity can go up to E0 copy (see above Fig.)

Specificity

This assay has been designed based on the published COVID-19 sequence in the ORF1b gene. With extensive NCBI data base blast analyses and some test, this combination of primer and probe sequence under this assay condition do not cross react with SARS CoV, MERS CoV, HBV, HCV, HCV, HTLV1, RSV, parainfluenza virus, human metapneumovirus, rhinovirus, enterovirus, and other respiratory pathogens such as respiratory bacteria and fungus.

14. REFERENCES

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