IEH Laboratories & Consulting Group

IEH SARS-CoV-2 RT-PCR Test Kit Instructions for Users

Validation information: FDA has not authorized this test. This test has been validated but FDA's independent review of this validation is pending

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Store at -20 °C upon arrival

(A) Intended Use

IEH SARS-CoV-2 RT-PCR Test is a real-time RT-PCR test intended for the qualitative detection of nucleic acid from the SARS-CoV-2 in in nasopharyngeal swabs, oropharyngeal (throat) swabs, anterior nasal swabs, mid-turbinate nasal swabs, nasal aspirates, nasal washed and bronchoalveolar lavage (BAL) fluid from patients suspected of COVID-19 by their healthcare provider. Testing is limited to those certified under the Clinical Laboratory Improvement Amendments of 1988 (CLIA), 42 U.S.C. §263a, to perform high complexity tests, or by similarly qualified non-U.S. laboratories.

Results are for the identification of SARS-CoV-2 RNA. SARS-CoV-2 RNA is generally detectable in respiratory specimens during the acute phase of infection. Positive results are indicative of SARS-CoV-2 RNA; clinical correlation with patient history and other diagnostic information is necessary to determine patient infection status. The agent detected may not be the definite cause of disease. 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 appropriate public health authorities.

Negative results do not preclude 2019-nCoV 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 IEH SARS-CoV-2 RT-PCR Test 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 IEH SARS-CoV-2 RT-PCR Test is only for use under the Food and Drug Administration's Emergency Use Authorization.

The assay has been validated in the following instruments:
- Stratagene MX-3005P (Software: Mx30005P v4. 10 Build 389, Schema 85)
- Biorad CFX96 (CFX Manager Software version 3.1 1517.0823)

The default RT-PCR software in each instrument will be used for data analysis.

(B) Principles of the Assay

IEH SARS-CoV-2 RT-PCR Test® is a hydrolysis probe-based (TaqMan) real-time reverse transcription Polymerase chain reaction test. This test contains two probes to detect two viral specific targets in the viral Nucleocapsid (N) gene. The TaqMan probes for the N gene, N1-P and N2-P are labeled with the FAM fluorescent dye at the 5' end. Each of the N1 and N2 amplifications are separately done. The assay uses human RNase P as an internal control to monitor the efficiency of human nucleic acid extraction, and uses a HEX dye labeled probe, RPP. N1 is multiplexed with the RPP probe. The fluorescence of each dye is quenched by the BHQ1 dye at the 3' end of the probe.

Purified viral RNA is reverse transcribed to cDNA by M-MuLV reverse transcriptase and subsequently amplified using Taq DNA Polymerase in a real time PCR machine. During the amplification process, the probe anneals to a specific target sequence located in the correctly amplified segment. During the extension phase of the PCR cycle, the 5' nuclease activity of Taq DNA Polymerase degrades the bound probe, causing the reporter dyes to separate from the quencher dye (BHQ1), generating a fluorescent signal. Fluorescence intensity is monitored at each PCR cycle by the instrument's fluorescent detector.

(C) Materials

Material required and provided

All enzymes, dNTPs, buffers, controls, and relevant primers/probes are supplied in the kit except for the no template control, which is nuclease free water (Table 1 and 2). Controls that will be provided with the test kit include a positive control for N1 and N2 targets. This is provided as a segment of the N gene RNA that is encapsulated in the MS2 bacteriophage capsid. MS2 encapsulated RNase P RNA is additionally provided as a negative extraction control.

Material storage

IEH SARS-CoV-2 RT-PCR Test should be stored at -15°C to -25°C in a manual defrost freezer.

Table 1: Kit components and storage specifications. 100 reactions per kit (product code PM-22) (protect from light).

Product Name	Product Description	Volume per vial	Storage condition
nCoV-1 N1/RPP Master Reagent. #B-1229	N1 primers/probe (FAM) and RNase P primers/probe (HEX), dNTPs, amplification buffer	2.4ml	-20°C
nCoV-2 N2 Master Reagent. #B-1230	N2 primers/probe (FAM), dNTPs, amplification buffer	2.4ml	-20°C
Mu-MLV reverse transcriptase; #M-1096	M-MuLV reverse transcriptase in storage buffer	20µl	-20°C
Taq DNA Polymerase, #T-1022	Hot Start Taq DNA Polymerase in storage buffer	20µl	-20°C
CoV-2 N gene Positive Control; #M-1097	MS2 phage encapsulated N1 and N2 RNA for positive control (1000 copies/ul)	500µl	-20°C
RNase P Extraction Control #M-1099	MS2 phage encapsulated Rnase P RNA for negative extraction control	500µl	-20°C

Validated kits for RNA extraction and specimen collection.
IEH Viral Nucleic Acid Extraction Kit #PM-25
IEH Viral Transport Media (VTM) #V-1008
IEH Viral Nucleic Acid Extraction Reagent Kit PM-23

Table 2: Primer and probe sequences used in the kit.

NAME	OLIGONUCLEOTIDE SEQUENCE (5'>3')
2019-nCoV_N1-F	5'-GAC CCC AAA ATC AGC GAA AT-3'
2019-nCoV_N1-R	5'-TCT GGT TAC TGC CAG TTG AAT CTG-3'
2019-nCoV_N1-P	5'-FAM-ACC CCG CAT TAC GTT TGG TGG ACC-BHQ1-3'
2019-nCoV_N2-F	5'-TTA CAA ACA TTG GCC GCA AA-3'
2019-nCoV_N2-R	5'-GCG CGA CAT TCC GAA GAA-3'
2019-nCoV_N2-P	5'-FAM-ACA ATT TGC CCC CAG CGC TTC AG-BHQ1-3'
RP-F	5'-AGA TTT GGA CCT GCG AGC G-3'
RP-R	5'-GAG CGG CTG TCT CCA CAA GT-3'
RPP	5'-HEX – TTC TGA CCT GAA GGC TCT GCG CG – BHQ-1-3'

Equipment and consumables required but not provided

- Recommended RNA extraction systems: see below (E)
- Vortex mixer
- Benchtop microcentrifuge
- Pipettes (10µL, 200µL and 1000µL) and multichannel pipettes (5-50µL and 1-10µL)
- A real time PCR system
- 10% bleach (1:10 dilution of commercial 5.25-6.0% hypochlorite bleach)
- Personal protective equipment (PPE): Gloves, lab coat, mask, sleeve protectors, goggles for eye protection
- Aerosol barrier pipette tips
- 1.5mL microcentrifuge tubes (DNase/RNase free)

- 0.2mL PCR reaction plates or Optical 8-cap Strips
- Class II (or higher) biological safety cabinet
- Nuclease free water

(D) Warnings and Precautions

- For in vitro diagnostic use (IVD).
- For emergency use only.
- Follow standard precautions. All patient specimens 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.
- Specimen processing should be performed in accordance with national biological safety regulations.
- If infection with SARS-CoV-2 is suspected based on current clinical and epidemiological screening criteria recommended by public health authorities, specimens should be collected with appropriate infection control precautions.
- Perform all manipulations of all clinical 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.
- Always check the expiration date prior to use. Do not use expired reagent. Do not substitute or mix reagent from different kit lots or from other manufacturers. Do not use damaged tubes.
- Change aerosol barrier pipette tips between all manual liquid transfers.
- Change gloves between samples and whenever contamination is suspected.
- Keep reagent and reaction tubes capped or covered as much as possible.
- Clinical RNA samples and the kit components must be always kept on ice or cold blocks during the procedure.
- Work surfaces and instruments should be cleaned and decontaminated with cleaning products such as 10% bleach to remove RNases and pathogens. Residual bleach should be removed using 70% ethanol.
- RNA extraction reagents and **IEH viral transport medium (IEH VTM)** contain guanidium salts that are irritants to skin, mucous membranes, and eye. Wear personal protective equipment (PPE) throughout the extraction procedure. Guanidium salts (isothiocyanate and chloride salts) should not be mixed with bleach. There is a potential risk of producing toxic gases (hydrogen cyanide, chlorine, chloramine etc.) if such mixing occur.
- Dispose unused kit reagents and human specimens according to local, state, and federal regulations.

(E) Specimen Collection, Processing and Archival

Upper respiratory tract samples should be collected using CDC recommended guidelines (https://www.cdc.gov/coronavirus/2019-nCoV/lab/guidelines-clinical-specimens.html), which include using polyester swabs with a plastic shaft, use of appropriate VTM and appropriate PPE. The preferred specimens are Nasopharyngeal specimens (NP), which should be collected from both nares. Alternatively, oropharyngeal (throat) swabs, anterior nasal swabs, mid-turbinate nasal swabs, or nasal aspirates can be used. A single swab can be used on multiple locations and be placed in a single vial. All collected swabs should be placed in a recommended viral transportation medium that has been validated for this test, which includes IEH VTM.

Poor specimen collection, transport and subsequent storage will likely produce false positives or false negatives. Refer to guidelines issued by relevant authorities in sample collection, transportation, and processing. Suspected SARS-CoV-2 patient specimens, cultures, or isolates should be transported as UN 3373 Biological Substance, Category B, in accordance with the current edition of the International Air

Transport Association (IATA) Dangerous Goods Regulations. If using the IEH VTM, viral RNA is stable up to two weeks and should be stored at -70C for long term storage.

Specimens from humans should always be considered infectious, not only with SARS-CoV-2 but with other pathogens. Follow standard precautions when handling clinical specimens, all of which may contain potentially infectious materials including highly infectious SARS-CoV-2. Standard precautions include hand hygiene and the use of personal protective equipment (PPE) such as laboratory coats, sleeve protectors, masks, gowns, gloves, and eye protection. Consider that the entire shipping package containing samples is infectious, therefore use decontamination using 2% bleach solution inside the shipping package including outside of vials.

Amplification technologies such as PCR are sensitive to accidental introduction of PCR product from previous amplifications reactions. Incorrect results could occur if either the clinical specimen or the real-time reagents used in the amplification step become contaminated by accidental introduction of amplification product (amplicon). Follow preventive measures during the entire assay.

Decontamination protocol: All work area should be decontaminated for potential pathogens and viral amplicons by using 10% bleach and subsequent wash with 70% isopropanol and UV irradiation.

Work area

To prevent amplicon contamination, three separate areas should be used for (i) sample preparation, (ii) RT-PCR setup and for (iii) amplification. Reagents, consumables, pipettors and instruments should not be shared among different prep areas. The experimental flow always should be from (i) to (iii).

(i) Sample preparation area: This area is only used for RNA extraction. Specimens should be handled in a certified BSL2 biosafety cabinet. All instruments, consumables, reagents, and handheld devices used in this area should remain dedicated. After each usage, all surfaces and instruments and lab coats should be decontaminated.

(ii) RT-PCR setup area: The RT-PCR reaction setup area should be separated from the sample prep area. Reaction setup should be done inside a lamina flow hood. After each usage, the surfaces and lab coats should be decontaminated. This area should be tested for amplicon contamination weekly or monthly.

(iii) Amplification area: This area is dedicated to amplification and should be located far away in a different room from the sample prep and reaction setup area. Any equipment including personal protective equipment belonging to this area should remain locally. A separate lab coat should be worn and cleaned daily. The RT-PCR machine should be handled with gloves. Completed reaction tubes should be placed in sealable bags and placed in the regular trash, not autoclavable trash, and disposed according to local regulations. Do not autoclave reaction tubes. Gloves should be discarded after each use within the amplification area. Surfaces of benchtops, equipment and hand-held devices should be decontaminated regularly using 2% bleach.

Viral RNA extraction

Samples can be stored at 4ºC for recommended periods (72 hours) in the IEH viral transport medium (IEH VTM). Long term storage should be at -70º C.

RNA extraction using chemical lysis and solid phase extraction:

The IEH SARS-CoV-2 RT-PCR Test kit has been tested with RNA isolated by kits developed by IEH based on well-established extraction methods (e.g., Boom method and column-based extraction methods). Two methods have been validated. **Please refer to their IFUs for detailed instructions.**

i) IEH Kingfisher RNA Extraction Protocol for automated extraction.
ii) IEH Viral Nucleic Acid Spin Column Extraction Kit.

Both methods uses reagents from the IEH Viral Nucleic Acid Extraction Reagent Kit (Table 3). This reagent kit contains buffers RV1 to RV3 which contain chaotropic salts and detergents for efficient viral lysis and denaturation of nucleases and protease. The preferred, automated method is used for large number of specimens.

Table 3. IEH Viral Nucleic Acid Extraction Reagent Kit (Kit Contents-1000 reactions)

Contents	**Volume**
Buffer RV1	400 mL
Buffer RV2 (as concentrate)[1]	440 ml
Buffer RV3 (as concentrate)[2]	220 ml
Buffer RV4 (elution buffer)	100 ml
Poly A Carrier RNA[3]	8 x 500 µl
Magnetic beads	10 mL

RNA Extraction steps (Refer to IFU IEH Kingfisher RNA Extraction Protocol for automated extraction SOP if more information is needed).

- **Prepare 1X concentrates of all reagents:** According to the set protocol.
- **Prepare wash plate:**
  - **WASH PLATE 1:** Dispense 400µl of 1X RV2 into all the wells in a deep well plate.
  - **WASH PLATE 2:** Dispense 400µl of 1X RV2 into all the wells in a deep well plate.
  - **WASH PLATE 3:** Dispense 400µl of 1X RV2 into all the wells in a deep well plate.
- **Prepare Elution Plate:** Dispense 100µl of RV4 into each well of elution plates.
- **Prepare Lysis Mix:** Calculate volume of the lysis mix components (see table 2 below) and add them to a reservoir.

Table 4. Lysis Mix

Contents	**96 Sample**
Buffer RV1	9 mL
Ethanol	18 mL
Poly A Carrier RNA	192 µL

- **Prepare Lysis Plate:** Dispense 312 µL of the lysis mix into each well of a deep well plate. Add the negative extraction control to G06 to G12 (MS2 encapsulated RNase P) and the positive N gene control to wells H06 and H12 (Table 5). Add 10µl of magnetic beads into each well.

Cover all control wells with a film.

Add specimens. Add 100 µL specimen samples into wells from C01 to F06 and from C7 to F12 wells.

- **Load Plates**:

- Load plates into appropriate plate holders in the Kingfisher96.
- ***Follow the protocol setup listed in the attached IFU for operating Kingfisher.***

- **Elution:** Elute the samples in 100 µL RV4 (nuclease free water) and store the RNA samples at -20°C for short term storage or -80°C for long-term storage.

Table 5: Control and specimen addition plate map (NTC: no template control; PCN: positive control; NEC: negative extraction control)

	1	2	3	4	5	6	7	8	9	10	11	12
A	NTC	S7	S15	S23	S31	S39	NTC	S7	S15	S23	S31	S39
B	NTC	S8	S16	S24	S32	S40	NTC	S8	S16	S24	S32	S40
C	S1	S9	S17	S25	S33	S41	S1	S9	S17	S25	S33	S41
D	S2	S10	S18	S26	S34	S42	S2	S10	S18	S26	S34	S42
E	S3	S11	S19	S27	S35	S43	S3	S11	S19	S27	S35	S43
F	S4	S12	S20	S28	S36	S44	S4	S12	S20	S28	S36	S44
G	S5	S13	S21	S29	S37	NEC	S5	S13	S21	S29	S37	NEC
H	S6	S14	S22	S30	S38	PCN	S6	S14	S22	S30	S38	PCN

(F) RT-PCR Procedure

Materials

- 1.25X nCoV-1 N1/RPP Master Reagent; #N-1009
- 1.25X nCoV-2 N2 Master Reagent; #N-1010
- M-MuLV reverse transcriptase; #M-1096
- Hot Start Taq DNA Polymerase; #M-1098
- CoV-2 N gene Positive Control; #M-1097
- RNase P Extraction Control; #M-1099
- Negative template control (NTC) (Nuclease free water; not provided)
- PCR tube and cap (or PCR plate and sealing film) (not provided)
- Clinical samples; purified.

Table 6. Plate map for dispensing master mixes.

	N1/RPP MASTER MIX						**N2 MASTER MIX**					
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A	N1/RPP	N1/RPP	N1/RPP	N1/RPP	N1/RPP	N1/RPP	N2	N2	N2	N2	N2	N2
B	N1/RPP	N1/RPP	N1/RPP	N1/RPP	N1/RPP	N1/RPP	N2	N2	N2	N2	N2	N2
C	N1/RPP	N1/RPP	N1/RPP	N1/RPP	N1/RPP	N1/RPP	N2	N2	N2	N2	N2	N2
D	N1/RPP	N1/RPP	N1/RPP	N1/RPP	N1/RPP	N1/RPP	N2	N2	N2	N2	N2	N2
E	N1/RPP	N1/RPP	N1/RPP	N1/RPP	N1/RPP	N1/RPP	N2	N2	N2	N2	N2	N2
F	N1/RPP	N1/RPP	N1/RPP	N1/RPP	N1/RPP	N1/RPP	N2	N2	N2	N2	N2	N2
G	N1/RPP	N1/RPP	N1/RPP	N1/RPP	N1/RPP	N1/RPP	N2	N2	N2	N2	N2	N2
H	N1/RPP	N1/RPP	N1/RPP	N1/RPP	N1/RPP	N1/RPP	N2	N2	N2	N2	N2	N2

Reaction setup

In the reaction setup area, setup 60 reactions for both N1 and N2 master mixes.

- Thaw all reagents at room temperature or at 30°C. Once thawed, store them in ice or cold blocks.
- Briefly vortex each vial for 5 seconds and then briefly centrifuge for 10 seconds.
- In a 1.5ml microfuge tube, make two master mixes, one for the N1 and the other for the N2 amplification. Add 1.2ml of 1.25X nCoV-1 N1 or N2 Master Reagent to each tube and then add

7.5µl of M-MuLV reverse transcriptase and 7.5µl of Hot Start Taq DNA Polymerase. Mix well and keep on ice.
- Using a multichannel pipettor, dispense 20µl of N1 and N2 master mix into a PCR plate as depicted in the 96 well plate map below (Table 6). If using 8 tube strips, use a similar scheme to include control samples.
- Add 5µl water to Negative template control (NTC) wells.
- Cover the whole plate and proceed with adding purified clinical specimen RNA.

Sample and positive control addition

- Add 5µl purified patient derived RNA samples. Make sure to cover the entire plate except the row to which the samples are being added (Table 5 and 6).
- Cover the plate with a RT-PCR compatible sealing film or if using strip tubes, use flat capped strip caps (do not write on top of the caps).
- Take the plate/strip tubes to the amplification area.

RT-PCR amplification

In the **amplification area**, turn on the RT-PCR machine. Program the instrument as depicted below.

Thermocycler program

- 50°C for 10min (reverse transcription).
- 95°C for 10min (RT enzyme inactivation).
- 95°C for 10sec, 60°C 30sec with 45 cycles (fluorescence acquisition during the annealing step).

Plate setup protocol.

- Use the template setup in Table 5 and 6 to define wells.
- Use the following table to define the probe labels.

TARGET NAME	FLUORESCENT REPORTER
N1	FAM
N2	FAM
RPP	HEX

- Before leaving the room, discard gloves and lab coat. At the end of the amplification, using PPE dedicated to the amplification area, remove the reaction plate and seal it in a sealable plastic bag and dispose according to local regulations. Do not autoclave.

(G) Results Interpretation

Results from all test controls should be examined prior to interpretation of patient results. If the control results are not valid, the patient results cannot be interpreted. Ct cutoff is used as part of the testing algorithm. Any positive samples, including weakly positive ones are considered SARS-CoV-2 RNA positive if the Ct value is less than 40 and all the controls are valid. Test result interpretation only involves default software in the RT PCR machine for Ct value determination.

Interpretation of SARS-CoV-2 RT-PCR test controls (Table 7)

(i) No template control (NTC): The NTC is nuclease free water used in place of sample nucleic acid. All NTC reactions must be negative, meaning no amplification curves would cross the PCR threshold (Ct is zero). If any of the N1, N2, or RP probe curve crosses the Ct, it is highly like that amplicon contamination occurred, or that the assay was setup improperly. ***The entire run is rejected and must be repeated.***

(ii) CoV-2 Positive Control N (PCN): Positive control samples are made of armored RNA and include both N1 and N2 targets in the same RNA segment. Both the N1 and N2 probes should give amplification curves with a Ct value less than 40. Any negative result (Ct >40 or no curve) indicates problems with the RNA extraction, integrity of the kit components, improper assay setup or RT-PCR reaction failure. ***The entire assay needs to be repeated.***

(iii) Negative extraction controls (NEC): RNase P armored RNA (NEC) is provided as a negative extraction control. A negative extraction control is needed to determine the efficiency of RNA extraction and to check for RT-PCR inhibitors and sample contamination. An invalid result with no RPP signal indicates problems with RNA extraction, RNA integrity and kit component integrity. This control is important for monitoring overall assay integrity because RP primers amplify both the DNA and RNA in clinical specimens whereas the RNase P armored RNA is devoid of DNA. If the RNase P negative extraction control is invalid, all negative results for SARS-CoV-2 within an assay should be redone by extracting new RNA or obtaining new patient specimens.

Table 7: Interpretation of SARS-CoV-2 RT-PCR test controls

CONTROL TYPE	PURPOSE	EXPECTED RESULTS (CT)			INTERPRETATION
		Probe N1	Probe N2	Probe RP	
Negative (NTC)	Amplicon contamination	Negative (Ct not detected)	Negative (Ct not detected)	Negative (Ct not detected)	Control results are valid
		Positive Ct value (<40) detected in any of the reactions			NTC results are invalid
Positive control wells N1 and N2 and RP	Kit integrity, assay setup and RT-PCR step	Positive (Ct <40)	Positive (Ct <40)	Negative (Ct not detected)	Control results are valid
		Negative	Negative		Positive control invalid
Negative Extraction control RNase P	Kit integrity, RNA extraction, assay setup, and RT-PCR step	Negative	Negative	Positive (Ct <40)	Negative extraction control valid
		Negative	Negative	Negative (Ct not detected)	Negative extraction control invalid

Examination and interpretation of patient specimen results (Table 8)

Assessment of clinical specimen test results should be performed after the positive and negative controls have been examined and determined to be valid and acceptable. If the controls are not valid, the patient results cannot be interpreted. The end user is not required to review fluorescent curves for weakly positive samples before final interpretation. If a weakly positive sample has a Ct value less than 40, the sample is considered positive for SARS-CoV-2.

Table 8: Interpretation of IEH SARS-CoV-2 RT-PCR Clinical Sample Results

N1 Signal	N2 Signal	RP Signal	Test Flag	Result Interpretation	Action
Negative	Negative	Positive	Valid	SARS-CoV-2 NOT detected	Report results as **negative** for SARS-CoV-2
		Negative	Invalid	Test invalid	Repeat test using new specimens
Either one or both N1 and N2 are positive		Positive	Valid	SARS-CoV-2 detected	Report results as **positive** for SARS-CoV-2
		Negative	Valid	SARS-CoV-2 detected	Report results as **positive** for SARS-CoV-2.

1. Internal extraction control for clinical samples (RNase P target) (Table 8)
All patient samples ideally should have positive results from the RP probe (RNase P target) with Ct values less than 40, indicating the presence of RNase P transcripts in samples. Absence of RNase P detection indicates either specimen degradation, improper sample collection, improper extraction of nucleic acid, or PCR inhibition in addition to equipment or master mix malfunction.

If the RP assay does not produce a positive result for human clinical specimens, interpret as follows:

(i) If N1 and N2 are positive in clinical samples: consider the results are valid. Some samples may have low human cell numbers but have high viral load. A negative RP signal for a clinical sample does not rule out SARS-CoV-2 in a clinical specimen.

(ii) If all N1 and N2 markers and RP signals are negative for the specimen, the result is invalid for the specimen. Repeat the assay using a fresh sample by extraction and repeating the test. Repeated negative results in all channels indicates either sample preparation or sample integrity problems. Run an RNA extraction control for the NTC and the PCN alone to rule out extraction issues and troubleshoot the RNA extraction protocol. If possible, get a new specimen from the patient.

2. SARS-CoV-2 markers (N1 and N2) in clinical specimens (Table 8)
Assuming that the positive controls for N1 and N2 probes are valid:

(i) If N1 and N2 signals are absent and the RP signal is positive (<40 Ct), the sample is negative for the viral RNA. Report as ***"Negative for SARS-CoV-2 RNA"***

(ii) If N1 and N2 signals are positive (Ct is <40) and the RP signal is positive (Ct <40), the sample is positive for SARS-CoV-2 RNA. Report as ***"Positive for SARS-CoV-2 RNA"***

(iii) If N1 and N2 signals are positive (Ct is <40) and the RP signal is negative (Ct >40), flag this result as ***"positive for SARS-CoV-2"***.

(iv) If one CoV-2 marker (N1 or N2, but not both markers) crosses the threshold line within 40.00 cycles (< 40 Ct) the result should be flagged as valid and ***"positive for SARS-CoV-2"***.

(H) Limitations

• Validation information: FDA has not authorized this test. This test has been validated according to the EUA guidance document, but FDA's independent review of this validation is pending.

• Users, analysts, and any person reporting diagnostic results should be trained to perform this procedure by a competent instructor. They should demonstrate their ability to perform the test and interpret the results prior to performing the assay independently.

• Performance of the IEH SARS-CoV-2 RT-PCR Test has only been established in upper respiratory specimens (such as nasopharyngeal, mid turbinate, anterior nares, or oropharyngeal swabs).

• 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. Optimum specimen types and timing for peak viral levels during infections caused by SARS-CoV-2 have not been determined. Collection of multiple specimens (types and time points) from the same patient may be necessary to detect the virus.

• A false negative result may occur if a specimen is improperly collected, transported, or handled. False negative results may also occur if amplification inhibitors are present in the specimen or if inadequate numbers of viral particles are present in the specimen.

• Do not use any reagent past the expiration date.

• If the virus mutates in the RT-PCR target region, SARS-CoV-2 may not be detected or may be detected less predictably.

(I) Additional instructions for laboratories

• Laboratories that receive the IEH SARS-CoV-2 RT-PCR Test must notify the relevant public health authorities of their intent to run the test prior to initiating testing.

• Laboratories will report fact sheets of IEH SARS-CoV-2 RT-PCR Test to health care personal and patients. Under exigent circumstances, other appropriate methods for disseminating these Fact Sheets may be used, which may include mass media.

• Laboratories will perform the test as outlined in the IEH SARS-CoV-2 RT-PCR Test Instructions for Use. Deviations from the authorized procedures, including the authorized RT-PCR instruments, authorized extraction methods, authorized clinical specimen types, authorized control materials, authorized other ancillary reagents and authorized materials required to perform the assay are not permitted.

• Laboratories will have a process in place for reporting test results to healthcare providers and relevant public health authorities, as appropriate.

• Laboratories should include the statement that this test has been validated but FDA's independent review of this validation is pending when reporting test results from the test to healthcare providers.

• Laboratories should collect information on any occurrence of false positive and false negative results and significant deviations from the established performance characteristics of this product to CDRH-14EUA-Reporting@fda.hhs.gov and to IEH laboratories at covid-19@iehinc.com.

(J) Performance Characteristics

1) Limit of detection studies to determine the assay sensitivity.

Limit of Detection (LoD) studies determine the lowest detectable concentration of SARS-CoV-2 at which approximately 95% of all (true positive) replicates would test positive.

(a) Limit of Detection determination for Stratagene MX-3005P - Using RNA directly in the RT-PCR:

The analytical sensitivity of the IEH RT-PCR assay was first determined by directly adding RNA into the RT-PCR reaction. A preliminary LoD for each primer was assayed using a 10-fold dilution of Twist synthetic RNA using the Stratagene MX-3005P instrument. This LoD was confirmed by using a 2-fold dilution series in 20 replicates. The LoD was determined as the lowest concentration where ≥ 95% (19/20) of the replicates would test positive. For N1 RT-PCR, the LoD was 2000 gene copies per ml. For N2 RT-PCR, the LoD was 2000 copies per ml (Table 9).

Table 9: LoD values for the N1 and N2 RT-PCR tests using Stratagene MX-3005P: RNA was added directly to PCR.

	SARS-COV-2 N1 RT-PCR			SARS-COV-2 N2 RT-PCR		
Genomic copy # per ml	$10^{0.8}$	**$10^{0.3}$**	$10^{0.1}$	$10^{0.8}$	**$10^{0.3}$**	$10^{0.1}$
Positives/Total	24/24	**24/24**	20/24	24/24	**23/24**	19/24
mean Ct	37.16	**38.55**	NA	37.64	**39.16**	NA

STD	0.49	**1.08**	NA	0.44	**1.03**	NA

(b) Limit of Detection determination for Stratagene MX-3005P, using IEH VTM based negative clinical matrix spiked with SARS-CoV-2 and extracted by the Kingfisher platform

Next, LoD studies were done using negative clinical matrix obtained from Molecular Epidemiology Inc, a WA State medical test site. (WA State CLIA #50D1077648 . WA State MTS # MTSC.FS.00005201). The negative clinical matrix is made of patients' nasopharyngeal swab specimens that has been transported using the IEH Viral Transportation Medium (2ml).

SARS-CoV-2 virus source: USA-WA1/2020 (BEI Resources/ATCC), heat inactivated, Catalog # NR-52286. Lot# 70033548. Concentration $1.16X10^{9}$ genome equivalents per ml.

Viral particles were diluted to 200 copies per µl in Hank buffered salt solution for matrix spiking. Virus was spiked at 5, 10, 15, 20 and 40 **Genomic Equivalent Copies per Reaction (GEC/Rx)**. Spiked clinical matrices were then purified using automated, magnetic bead based Kingfisher nucleic acid purification system. The LoD for Kingfisher based purification on this spiked negative clinical matrix was 2000 genome copies per ml for both N1 and N2 targets (Table 10)

Table 10: LoD values for the N1 and N2 RT-PCR tests performed in a Stratagene MX-3005P with viral genomic RNA extracted from IEH VTM using the Kingfisher platform.

	N1 Target			N2 Target		
Genome copies/ reaction	2.5 GEC/Rx	5 GEC/Rx	10GE/Rx	2.5 GEC/Rx	5 GEC/Rx	10GE/Rx
LoD #	0.25X	0.5X	1X	0.25X	0.5X	1X
Genome copies per ml	500	1000	2000	500	1000	2000
Positive/Total	20/24	20/24	24/24	19/24	18/24	24/24
Mean	35.20	34.37	35.26	37.29	37.01	34.55
Std Dev	1.14	1.35	0.73	1.02	1.07	0.70

(c) Limit of Detection determination for Stratagene MX-3005P, using alternative VTM based negative clinical matrix spiked with SARS-CoV-2 and extracted by the Kingfisher platform

To determine the LoD of specimens that are transported in non-IEH VTM, pooled negative clinical matrix obtained from the Washington State Public Health Laboratories (PHL) was used. These were from patients negative for Covid-19. Pooled matrix were spiked with the heat inactivated SARS-CoV-2 (BEI Catalog # NR-52286. Lot# 70033548) at 10, 20, 40 and 60 GEC/Reaction and extracted with the Kingfisher instrument and the IEH Viral Nucleic Acid Extraction Reagent Kit. 24 individual samples were extracted, and RT-PCR was done using the IEH kit. The LoD values were 2000GEC/ml for the N2 target and 4000 GEC/ml for the N1 target when alternative VTM was used for virus transport (Table 11).

Table 11. LoD values for the N1 and N2 RT-PCR tests using Stratagene MX-3005P. Negative clinical matrix obtained from WA State PHL was used to spike the SARS-CoV-2 virus. Kingfisher purification.

	N1 target: WA State PHL Panel				N2 target: WA State PHL Panel			
Genome copies/ reaction	5GEC/Rx	10GEC/Rx	20GEC/Rx	30GEC/Rx	5GEC/Rx	10GEC/Rx	20GEC/Rx	30GEC/Rx
LoD #	0.25X	0.5X	1X	1.5X	0.5X	1X	2X	3X

Genome copies per ml	1000	2000	4000	6000	1000	2000	4000	6000
Positive/Total	9/24	17/24	24/24	24/24	8/24	24/24	24/24	24/24
Mean	35.42	35.08	34.61	33.79	36.15	34.62	33.57	32.92
Std Dev	1.73	0.67	0.75	1.30	1.10	0.84	0.59	0.43

(d) Limit of Detection determination for Stratagene MX-3005P, using IEH VTM based negative clinical matrix spiked with SARS-CoV-2 and extracted using spin columns.

To determine the equivalent LoD of specimens purified using spin columns compared to that from Kingfisher based purification, IEH VTM based negative clinical matrix were spiked with the heat inactivated virus (BEI Catalog # NR-52286. Lot# 70033548) at 15, 20 and 40 GEC/Reaction. 24 individual samples were extracted, with specimen and elution volumes at 100µl, and RT-PCR was performed using the IEH kit. The bridging LoD were 3000GEC/ml for the N1 target and 4000 GEC/ml for the N2 target when the IEH VTM was used for virus transport (Table 12). These two values are within the required <3X LoD values of the Kingfisher purification.

Table 12. LoD values for the N1 and N2 RT-PCR tests performed in a Stratagene MX-3005P with viral genomic RNA extracted from IEH VTM using spin column purification.

	N1 Assay: Spin Columns			N2 Assay: Spin Columns		
Genome copies/ reaction	15GEC/Rx	20GEC/Rx	40GEC/Rx	15GEC/Rx	20GEC/Rx	40GEC/Rx
Genome copies/ ml	3000	4000	8000	3000	4000	8000
Positive/Total	24/24	24/24	24/24	22/24	23/24	24/24
Mean	36.24	35.54	34.21	34.76	34.45	33.78
Std Dev	1.028	0.889	0.471	0.547	0.741	0.598

(e) Claiming multiple instruments:

Limit of Detection determination for Biorad CFX96:
LoD was measured using protocols suggested by the FDA EUA document. According to the FDA protocol, confirmation of the LoD that was obtained for the Stratagene instrument was evaluated for the Biorad CFX96 using 4XLoD, 2XLoD, 1XLoD and 0.5X-fold LoD dilution series using Twist RNA series in eight replicates. Our results indicate that the LoD was 2000 copies per ml for the N1 RT-PCR and 4000 copies per ml for the N2 PCR (Table 13).

Table 13: Performance of Biorad CFX96 with the LoD values obtained from the Stratagene MX-3005P for the N1 and N2 targets.

	10 fold dilution for standard curve, N1 Target			Ct value determination using LoD dilution series for N1 target			
Twist RNA	**Ct value**	**Ct value**	**Well#**	**0.5XLOD,1 copy**	**1xLOD, 2 copies**	**2xLOD, 4 copies**	**4xLOD, 8 copies**
10^5	22.38	22.91	1	37.2	37.59	34.97	34.84

10^4	25.6	25.76	2	38.17	37.52	35.88	34.74
10^3	29.35	29.55	3	N/A	38.19	37.27	34.34
10^2	32.2	32.28	4	38.27	36.81	35.62	35.76
10^1	34.78	35.4	5	37.72	35.99	35.82	36.18
10^0	38.05	37.38	6	37.61	37.6	35.97	35.13
NTC	N/A	N/A	7	N/A	37.16	36.53	36.01
NTC	N/A	N/A	8	36.59	35.99	35.54	34.66

	10 fold dilution for standard curve, N2 Target			Ct value determination using LoD dilution series for N2 target			
Twist RNA	**Ct value**	**Ct value**	**Well #**	**0.5XLOD,1 copy**	**1xLOD, 2 copies**	**2xLOD, 4 copies**	**4xLOD, 8 copies**
10^5	24.01	23.92	1	40.35	N/A	38.05	39.07
10^4	28.01	27.49	2	44.97	42.27	40.16	40.9
10^3	31.38	31.17	3	N/A	41.23	39.33	38.43
10^2	34.4	34.11	4	N/A	N/A	38.95	41.4
10^1	38.74	38.62	5	42.66	41.77	37.51	37.48
10^0	40.72	41.69	6	44.24	42.66	39.49	38.98
NTC	N/A	N/A	7	39.93	40.46	39.72	40.96
NTC	N/A	N/A	8	43	38.15	40.78	37.56

2) Inclusivity

Primers and probes were analyzed using the NCBI Primer-BLAST tool, as well as a 'Virtual PCR' utility developed in-house at IEH. Probes were also verified by treating them as forward primers and pairing them with the matching reverse primer. Using both algorithms, primers listed in the table 2 matched with 100% sequence identity to all the sequenced SARS-CoV-2 genomes. N1 primer probe set did not have any mismatches. The 5' of the N2 probe had a one mismatch.

N1 amplicon consensus sequence from 8250 NCBI SARS2 sequences, with forward/reverse primers & probe:

```
GACCCCAAAATCAGCGAAATGCACCCCGCATTACGTTTGGTGGACCCTCAGATTCAACTGGCAGTAACCAGA
GACCCCAAAATCAGCGAAAT   ACCCCGCATTACGTTTGGTGGACC   CAGATTCAACTGGCAGTAACCAGA
********************  ************************  ************************
```

N2 amplicon consensus sequence from 8250 NCBI SARS2 sequences, with forward/reverse primers & probe:

```
TTACAAACATTGGCCGCAAATTGYWCAATTTGCCCCCAGCGCTTCAGCGTTCTTCGGAATGTCGCGC
TTACAAACATTGGCCGCAAA     ACAATTTGCCCCCAGCGCTTCAG   TTCTTCGGAATGTCGCGC
********************     **********************  ******************
```

This SNP, found 43 genomes, would not significantly affect the performance of the assay given their relative obscurity of the SNP and location within the probe. The exonuclease activity of Taq DNA Polymerase should not be affected by the displaced 5' base and the attached fluorophore.

3) Cross-reactivity (Analytical specificity)

Recommended List of Organisms to be analyzed *in silico* and by Wet Testing*

OTHER HIGH PRIORITY PATHOGENS FROM THE SAME GENETIC FAMILY	HIGH PRIORITY ORGANISMS LIKELY IN THE CIRCULATING AREA
Human coronavirus 229E	**Adenovirus (e.g. C1 Ad. 71)**
Human coronavirus OC43	**Human Metapneumovirus (hMPV)**
Human coronavirus HKU1	**Parainfluenza virus 1-4**
Human coronavirus NL63	**Influenza A & B**
SARS-coronavirus	**Enterovirus (e.g. EV68)**
MERS-coronavirus	**Respiratory syncytial virus**
	Rhinovirus
	Chlamydia pneumoniae
	Haemophilus influenzae
	Legionella pneumophila
	Mycobacterium tuberculosis
	Streptococcus pneumoniae
	Streptococcus pyogenes
	Bordetella pertussis
	Mycoplasma pneumoniae
	***Pneumocystis jirovecii* (PJP)**
	Pooled human nasal wash - *to represent diverse microbial flora in the human respiratory tract*
	Candida albicans
	Pseudomonas aeruginosa
	Staphylococcus epidermis
	Staphylococcus salivarius

i). Organisms analyzed by in silico for cross reactivity:

Primers and probes were analyzed using the NCBI Primer-BLAST tool as well as a 'Virtual PCR' utility developed in-house at IEH. Primer-BLAST was utilized by specifying each forward & reverse primer set individually to search for possible off-targets in the 'nr' (non-redundant) NCBI nucleotide database, not filtered by organism to search the entire database. ***Primer-BLAST showed no off-targets.*** The Virtual PCR ***utility also showed no off-targets***. This utility used the latest bacterial and viral genomes downloaded from NCBI, including all NCBI COVID-19 sequences and other Coronavirus variants (229E, OC43, HKU1, NL63, the original SARS, and MERS, among others). Probes were also verified by treating them as forward primers and pairing them with the matching reverse primer. Primers were not wet tested.

ii). Microbial Interference Studies:

FDA defines in silico cross-reactivity as greater than 80% homology between one of the primers/probes and any sequence present in the targeted microorganism. *In silico* cross reactivity studies demonstrated that primers used in the kit have no cross reactivity to any common pathogens except for close relatives of SARS-CoV-2. These close relatives such as Bat corona viruses, MERS-CoV and SARS-CoV-1 are currently not active as human pathogens. Therefore, interference studies are irrelevant.

4) Testing for endogenous interference substances

The test is based on RNA that has been purified using silica based purification methods. Although solid phase extraction can remove impurities that can modulate RT-PCR results, series of compounds that are commonly found in respiratory tract were tested to determine their modulatory effects on the IEH RT-PCR assay.

Negative respiratory matrix in IEH VTM were spiked at 1X, 2X and 4X LoD and extracted independently in triplicates using the Kingfisher method. RT-PCR was done to determine any inhibitory effects.

Summary of results:
Blood (in EDTA) at 10% inhibited the reaction (Virus GEC/Rx was 20). Blood at 4% did not inhibited reaction (Virus GEC/Rx was 10). None of the substances tested interfered with the RT-PCR assay at the given concentration when tested in triplicates (Table 14) except for Nasacort which had 2/3 results with 10 GEC/Rx.

Similar results were obtained when RNA were extracted with silica columns. No compounds inhibited at the given concentration when the virus was spiked at 10GEC/Rx.

Table 14. Results from triplicate RT-PCR assays to determine whether compounds found in the respiratory tract inhibit the assay.

Compound Name	N1 Ct	N2 Ct	N1 Ct	N2 Ct
Genomic copies/reaction	10GEC	20GEC	10GEC	20GEC
Mucin 1%	3/3	3/3	3/3	3/3
Blood 10%		0/3		1/3
Blood 4%	3/3		3/3	
Afrin 10%	3/3	3/3	3/3	3/3
Nasacort 10%	2/3	3/3	3/3	3/3
Flonase 10%	3/3	3/3	3/3	3/3
Zicam 10%	3/3	3/3	3/3	3/3
NyQuel 10%	3/3	3/3	3/3	3/3
Tobramycin 0.5%	3/3	3/3	3/3	3/3
oseltamivir 0.5%	3/3	3/3	3/3	3/3
Azithromycin 0.15%	3/3	3/3	3/3	3/3
Biotin 0.5%	3/3	3/3	3/3	3/3
Ampicillin 0.5%	3/3	3/3	3/3	3/3

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5) Clinical evaluation:

Clinical evaluation determines the sum performance of the extraction system, VTM and the RT-PCR assay in detecting positive and negative clinical specimens without any false positives or negatives. In this section, using two different clinical panels, the IEH-SARS-CoV-2 RT-PCR Kit has been validated for its clinical performance.

Clinical specimen sources:

Two sources have been used for the study solely based on the VTM used:

a) Clinical specimens obtained from Molecular Epidemiology Inc which are transported in the IEH VTM.
b) Clinical specimens obtained from the Washington State Public Health Laboratories which are transported in a VTM based VTMs that are either commercial or based on the CDC formulated VTM (SOP#: DSR-052-03).

Route of specimen collection: Nasopharyngeal.

RNA Extraction: The Kingfisher automated nucleic acid isolation system.

RT PCR kit used for the clinical performance validation: IEH SARS-Cov-2 RT-PCR Test

Alternative EUA approved test kits for comparisons: CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel:

The CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel was assembled using following components.

i). TaqPath 1-Step Multiplex Master Mix (No ROX) Catalog # A28523; LOT-2219558 (Thermofisher Scientific).

ii). CDC recommended IDT primer/probe sets from Integrated DNA Technologies: 2019-nCov CDC EUA Kit, 1000 rxn. Catalog# 10006770.

Study design:

- Positive and negative panels were obtained that have been previously characterized.
- RNA were extracted from both the positive and negative specimens using the Kingfisher system.
- RT-PCR was done again to confirm the positivity and negativity status of specimens using the IEH kit.
- Results were confirmed using the CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel.

Summary of the results:

40 positive and 40 negative nasopharyngeal specimens obtained from MEI Inc. were used for the study that has been transported in IEH viral transportation medium. These specimens were extracted for RNA, blinded, and tested. 30 positive and 30 negative nasopharyngeal specimens were additionally obtained from the Washington State Public Health Laboratories that has been transported in an alternative VTM that is either derived commercially or home made using the CDC VTM SOP. RNA was extracted using the Thermofisher Kingfisher automated platform. Results from the RT-PCR kit were confirmed by the CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel. The summary of the results is in Table 15 and 16. Regardless of the validation panel used, the observed positive percent agreement was 100% in detecting positive specimens between the two kits. Moreover, the observed negative percent agreement was 100% in detecting negative specimens between the two kits.

Table 15. Results from the clinical evaluation study using clinical specimens obtained from MEI that has been transported in IEH VTM.

		CDC 2019-nCoV Real-Time RT-PCR panel			
		SARS-CoV-2 RNA Detected	SARS-CoV-2 RNA **Not** Detected	Total Tested	% Agreement
IEH SARS-CoV-2 RT-PCR Test kit	SARS-CoV-2 Positive Specimens	40	0	40	100%
	SARS-CoV-2 Negative Specimens	0	40	40	100%
	Total Specimens	40	40	80	

Table 16. Results from the clinical evaluation study using clinical specimens obtained from WA State PHL that has been transported in alternative VTMs to IEH VTM.

		CDC 2019-NCoV Real-Time RT-PCR panel			
		SARS-CoV-2 RNA Detected	SARS-CoV-2 RNA **Not** Detected	Total Tested	% Agreement
IEH SARS-CoV-2 RT-PCR Test kit	SARS-CoV-2 Positive Specimens	30	0	30	100%
	SARS-CoV-2 Negative Specimens	0	30	30	100%
	Total Specimens	30	30	60	

(K) Technical Assistance

For technical assistance call IEH Laboratories & Consulting Group at (206) 522-5432, email covid-19@iehinc.com
For product assistance call Microbiologique Inc. (206) 522-5432, email: tech@microbiologique.com

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