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1. PURPOSE

To provide instructions for performing real-time reverse transcription polymerase chain reaction (rRT-PCR) for the detection of SARS-CoV-2 target genes in the RNA isolated from the clinical specimens.

2. INTRODUCTION

This SOP describes rRT-PCR method to detect SARS-CoV-2 from the clinical specimen. rRT-PCR, considered as the gold standard for the diagnosis of SARS-CoV-2 infection, is highly sensitive and, has very low limit of detection (1,2). The procedure is carried out after viral RNA has been extracted from ICMR approved specimen types, in a BSL 2 equivalent facility. Key steps in the procedure need to be performed in physically separated areas in the laboratory. Adherence to good laboratory practices is critical for quality test results.

3. PRINCIPLE

SARS-CoV-2 is detected based on the presence of viral RNA in patient specimens. RNA isolated from the specimen is converted to complementary DNA (cDNA). cDNA is then used to amplify viral target genes by PCR. Real time detection of amplified target during PCR is achieved by using fluorescent probes in the PCR reaction and detection of fluorescence signals by the instrument. Presence or absence of fluorescence signal/s specific to the viral target gene/s is the indicator of the presence of the virus in the specimen.

In a one-step rRT-PCR process, where reverse transcription and PCR reaction occur in the same PCR tube, the reaction mix contains following key ingredients:

- Reverse transcriptase that converts RNA to cDNA.
- Primers and probes that specifically bind to SARS-CoV-2 target gene of interest.
- Each probe has a fluorophore at the 5’ end and a quencher at the 3’ end. Fluorescence from the fluorophore is inhibited by the presence of quencher in the vicinity, therefore intact probe does not fluoresce (3).
- Taq polymerase, dNTPs, buffer for the PCR.

During PCR reaction, primers and probes bind to their specific target regions on cDNA template at a suitable annealing temperature. Taq DNA polymerase (Taq) binds to the 3’end of the primer and starts its polymerase activity. When Taq encounters the probe bound to the cDNA, it uses its exonuclease activity to degrade the bound probe and continues to synthesize the complementary DNA. When degraded, the fluorophore in the probe is released from the Quencher and emits Fluorescence. As amplification cycles progress more copies of the target DNA are generated to which free probes bind and subsequently get degraded by Taq DNA polymerase. Accumulation of free fluorophore in the reaction mix is detected by the real time PCR machine and is shown as gradual increase in fluorescence intensity. Therefore, presence of target in reaction tube will lead to detectable increase in fluorescence intensity.

In a sample with no SARS-CoV-2 specific cDNA, probes have no target to bind to and are unable to emit fluorescence, as they are inhibited by the quencher in the intact probe.

PCR primer-probe combinations can be designed in such a way that one can detect signal to multiple targets in one reaction. While designing such combinations one needs to make sure that the instrument specification matches with fluorophore excitation & emission wavelengths.

Ct Value: The number of cycles at which the detected fluorescence signal exceeds background levels is called the threshold cycle (Ct). Lower Ct value implies high levels of target RNA in the patient sample. Conversely, high Ct value implies low levels of target RNA in the patient sample (4).As a rule of thumb, Ct values above 38 are not considered valid.

4. CROSS REFERENCES

4.1 SOP#008 (RNA extraction)

4.2 Verification data for RT-PCR kit to be used

5. PERSONNEL QUALIFICATIONS & RESPONSIBILITIES

The lab personnel performing this procedure must have:

- Knowledge of the principle of the procedure being used
- Expertise in micro pipetting skills
- Knowledge of good laboratory practices
- Understanding of organization of workflow in a COVID - PCR lab
- Knowledge of contamination control methods
- Understanding of the importance of laboratory results for patient management

6. RESPONSIBILITIES

- It is the responsibility of the lab personnel to correctly understand and perform this procedure.
- All users of this procedure who do not understand it or are unable to carry it out as described are responsible for seeking advice from their supervisor.

7. EQUIPMENT & MATERIALS

7.1 Equipment & Tools:

7.1.1 Real time PCR machine

7.1.2 Biosafety cabinet (level II)

7.1.3 PCR hood or Laminar flow hood

7.1.4 Vortex mixer

7.1.5 Calibrated micropipettes for:

7.1.5.1 Master Mix preparation (10 µL, 200 µL and 1000 µL)

7.1.5.2 RNA addition (10 µL,)

7.1.5.3 Positive control addition (10 µL,)

7.1.6 Refrigerated microcentrifuge (with rotor for 1.5 ml centrifuge tube)

7.1.7 -70/ -80°C, -20°C freezers

7.1.8 4°C Refrigerators

7.1.9 Benchtop minifuge

7.1.10 96 well plate spinner

7.1.11 Tube racks
7.1.12 PCR tube rack

7.2 Consumables:

7.2.1 PCR 96 well plates
7.2.2 Plate covers
7.2.3 Plate sealers
7.2.4 PCR tubes
7.2.5 Aluminum foil
7.2.6 Cool racks
7.2.7 Nuclease free tips
7.2.8 Tube racks
7.2.9 Markers
7.2.10 Absorbent liners
7.2.11 Paper towels

7.3 Reagents & Disinfectants

7.3.1 RNAse Zap
7.3.2 CoviPath Covid -19 RT- PCR Kit that contains

- Covipath COVID 19 Assay Multiplex (A50780)
- Covipath 1- step Multiplex Master Mix (No ROX)
- Covipath COVID-19 Control
- **Should be stored at the temperature recommended by the manufacturer and always protected from light. The reagents should be aliquoted to avoid repeated freeze thaw.**

7.3.3 Nuclease free Water
7.3.4 Reagent grade Ethanol and Isopropanol
7.3.5 Absolute alcohol
7.3.6 Sodium Hypochlorite
7.3.7 Biohazard bags Red &Yellow

7.4 Personal Protecting Equipment (PPE)

7.4.1 Lab gowns
7.4.2 Surgical/medical masks/
7.4.3 N95 Mask
7.4.4 Gloves
7.4.5 Shoe Covers
7.4.6 Hair Covers

8. PROCEDURE

This procedure consists of following main steps:

- Master mix preparation
- RNA addition

- Positive and Negative Control addition
- PCR machine set up
- Data collection and PCR plate discard

For uninterrupted workflow, review your checklist and ensure all the necessary items are available at the site where you are going to be working. Maintenance of unidirectional workflow is important. Do not enter in master mix preparation room after entering into the RNA extraction room.

Note: It is better to have designated personnel to work in master mix area so as to minimize contamination. All sections such as pre PCR, PCR and Post PCR area must have dedicated equipment and consumables.

8.1 Master mix preparation:

Important:

Master mix preparation should be carried out in a RNA/ DNA free area, (preferably in a PCR hood or laminar flow cabinet). PPE, tools and consumables used in this area should not be used elsewhere. Minimize RNase contamination by frequently changing gloves, using RNase free water, molecular biology grade tips and tubes.

PPE to be worn during this procedure: Lab coat, gloves, hair cap, shoe covers, surgical mask.

8.1.1 Before starting this step calculate the amount of reagents needed by using a master mix calculation work sheet. (Annexure 1)

8.1.2 Determine the number of reactions (N) to set up per assay. Add the reactions for following controls:

- No template control (NTC)
- Extraction negative control (ENC)
- Positive control/ viral template control (VTC)
- Sample processing control (SPC; If recommended by manufacturer)

8.1.3 Add extra number of reaction/s to account for pipetting error.

- If number of samples (n) including controls = 1 to 10, then N = n + 1
- If number of samples (n) including controls = more than 10, then N = n + 2

8.1.4 Carry out the calculations on Mastermix Calculation worksheet.

8.1.5 In addition, prepare a layout of your sample reactions tubes or 96 well plate/384 well plate.(Annexure 2)

8.1.6 Carry the worksheet, sample list and sample layout with you to the master mix preparation area.

Cleaning the Master mix preparation area

8.1.7 Prepare sufficient amount of 1% sodium hypochlorite solution and 70% ethanol for cleaning. Ensure availability of waste collection bags and bins.

8.1.8 Clean the work surface work area, micro pipettes, tip boxes with freshly prepared 1% sodium hypochlorite. After 15 minutes of contact time, wipe off with 70% ethanol. Turn on UV (if available) in the hood for at least 20 minutes before use.

8.1.9 Place an absorbent liner. Place all necessary items - pipettes, tips, 70% alcohol, tissue roll, tube rack, waste collection bag in the hood. To avoid RNase contamination, frequently change gloves.

8.1.10 Take out the master mix reagents from the freezer and allow them to thaw on a cooler rack. Keep them protected from light by covering with aluminum foil if required. Meanwhile, label the master mix tube and reaction tubes or plates.

8.1.11 Gently tap the reagent tubes on the sides to mix the salts and then spin down the reagent tubes in a mini spin for 5 to 10 sec (do not spin the reagents for too long).

8.1.12 Pipette the calculated amount of reagents into the master mix tubes as per the table below:-

Master mix contents	**96 well**		**384 well**	
	1X	**N reaction**	**1X**	**N reaction**
Multiplex Master Mix	6.25µl	6.25*N	5 µl	5*N
RT PCR assay multiplex	1.25 µl	1.25*N	1 µl	1*N
NFW	7.5 µl	7.5*N	4 µl	4*N
Total reaction volume	15 µl		10 µl	

8.1.13 Mix the reagents by gently pipetting up and down. Do not vortex. Spin the master mix tubes for few seconds to collect contents at bottom, and then place the tubes in cold rack.

8.1.14 Set up reaction strip tubes or plates in 96-well cooler rack. Dispense appropriate volume of master mix into each well as per the plate set up.

8.1.15 Before moving the plate to the RNA addition area, pipette appropriate volume of the nuclease free water into negative control tubes (NTC)/ wells. Cap NTC tubes/wells. Cover the plate with aluminum foil to protect it from light.

8.1.16 Clean the work surface area with 1% sodium hypochlorite and 70% alcohol as described above.

8.2 RNA addition (IN THE SAMPLE PREPARATION AREA)

8.2.1 A separate PCR hood or laminar flow hood should be used for RNA addition. If that is not available, wear the appropriate PPE (COVER ALL/Lab gown, N95 mask, head cover, goggles, outer shoe cover, and two pairs of gloves).

8.2.2 Clean the biosafety cabinet used for RNA preparation thoroughly with 1% sodium hypochlorite, giving a contact time of 15 minutes, followed by wiping with 70% alcohol.

8.2.3 Items such as micro-pipettes, tips, tube racks used here should not be used elsewhere and they should be cleaned with cleaning agent such as RNAse ZAP to get rid of RNases.

8.2.4 Take out the RNA samples from the freezer on cold rack and bring them inside the BSC. Let them thaw. Gently tap on the sides of the tubes to mix the content and vortex for approximately 5 seconds.

8.2.5 Place the tubes back in the cold rack.

8.2.6 Add 15 µl (as recommended in RT-PCR kit manual) of RNA to each tube and the same volume of mock extraction control into the respective wells as per the set up. **Change the tips after each addition.**

8.2.7 Cap the tubes/cover the plate with aluminum foil to which the samples and mock control has been added.

8.2.8 **Positive control addition:** Positive control should be added in a positive control addition area or if the separate area is not available, it should be added after addition of sample RNA. The dilution for the control is as below:-

Positive control preparation-

Dilution 1 - 95µl of TE buffer + 5ul of Covipath Covid19 control (mix & centrifuge).

Dilution 2 - Take 75ul of dilution buffer + 25ul of dilution 1 (Mix &Centrifuge).

10 µl of this diluted positive control (dilution 2) will be used as a template in RT-PCR

Note: - The volume of the RNA template and the positive control will vary depending on the PCR plate that will be used,

8.2.9 If 96 well plate is used, seal the plate with optical sealer. Centrifuge the plate / tubes for10 seconds. Make sure that bubbles are eliminated from the bottom of the reaction tubes.

8.2.10 The kit contents included the controls should be stored at -20°C immediately

8.2.11 Now the PCR plate is ready to be transported to the Amplification room. Place it in a disinfected cold rack and cover with clean aluminum foil. Remember not to apply any disinfectant on the PCR plate or PCR tubes.

8.2.12 Discard the solid waste in the appropriate waste bin outside the BSC. Clean the work surface area with 1% sodium hypochlorite, followed by 70% ethanol.

8.2.13 Exit the RNA extraction area with PCR plate on cold rack and remove the PPE.

8.3 PCR machine set up (Amplification Room)

8.3.1 Don the Lab coat and gloves and enter the Amplification room.

8.3.2 For real time PCR set up follow the instructions given by the Real-time PCR instrument manual for plate set up. Save your plate setup.

8.3.3 Program the run method as per instructions provided in the RT-PCR kit:

STAGE	TEMPERATURE	TIME	CYCLES
1	25°	2 MIN	1
2	53°	10 MIN	1
3	95°	2 MIN	1
4	95°	3 SEC	40
	60°	30 SEC (Data collection)	

Passive Reference Dye-None

After the protocol of amplification is done, remove PCR Plate from the thermal cycler and discard them in a sealable plastic bag for autoclave and decontamination.

8.3.4 Fluorescent Channels

Reporter/Quencher	Target
FAM/None	ORF1ab
VIC/None	N gene
JUN/None	RNaseP

Detection of the Positive control in fluorescence channel FAM and VIC.

Detection of the internal control (IC) in fluorescence channel JUN in NTC.

Note: Set the threshold above the maximum level of No template control curve (random noise curve), then analyze the results.

8.4 Data Collection.

8.4.1 After completion of the run, save the result file. Ensure a proper backup of the data.

8.4.2 Remove the PCR tubes/ plates, inspect the wells for any visible signs of reaction mix evaporation. Note down the details.

8.4.3 Discard the plates in the amplification room in a waste bin lined with red biohazard bag.

9. QUALITY CONTROL

In accordance with manufacturer instructions, following internal controls should be run with every rRT-PCR reaction batch

9.1 No template Control

9.2 Sample Extraction Control (Nulcease free water that is extracted along with the samples)

9.3 Positive Control that is provided in the kit

9.4 Known positive and negative samples

10. RESULT & INTERPRETATION

10.1. The negative control reactions for probe/primer sets should not exhibit fluorescence growth curves (FAM and VIC) that cross the threshold line.

10.2. If a false positive occurs with one or more of the primers and probe non template control (NTC) reactions, sample contamination may have occurred.

10.3. The positive control reactions for each probe/primer reactions should give following Ct values:

Channels	Positive control	Expected Ct values
FAM	ORF1ab	≤37
VIC	N gene	≤37
JUN	RNase P	≤35

10.4. All clinical samples should exhibit RNase P reaction curves that cross the threshold line at or before 35 cycles.

10.5. Failure to detect RNase P in any of the clinical samples may indicate:

(a) Improper extraction of nucleic acid from clinical materials resulting in loss of RNA

(b) Carryover of RT-PCR inhibitors from clinical specimens

(c) Improper assay set up and execution

11. Limitations:

- One 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.
- A false negative result may occur if an excess of template is present in the reaction due to competitive inhibition.
- A false negative result may occur if RNA is degraded or is contaminated with RNase
- Not achieving desired Ct value for PC/ inappropriate test results may occur if pipetting is not being done properly.
- Handling by trained staff and timely calibration of pipettes would minimize these issues.
- The impacts of vaccines, antiviral therapeutics, antibiotics, chemotherapeutic or Imuno suppressant drugs have not been evaluated. The Covipath™ COVID-19 RT-PCR Kit cannot rule out diseases caused by other bacterial or viral Pathogens.

12.WASTE MANAGEMENT, CONTAMINATION CONTROL AND OTHER SAFETY PRECAUTIONS

12.1 Waste management:

The waste should be collected in bins lined with red bags, removed periodically from each room and autoclaved.

12.1.1 Always properly clean work area after completion of tasks

12.1.2 Establish a regular (e.g. weekly) and thorough laboratory cleaning protocols (floors, doors, walls)

12.2 Contamination Control

12.2.1 Tools and instruments used for master mix preparation area should be labelled accordingly and should not be used elsewhere. Never take anything from this site to RNA extraction/addition area.

12.2.2 Add RNA to PCR tubes in a PCR or laminar flow hood. If these facilities are not available, clean and decontaminate the RNA extraction BSC and use it for sample addition.

12.2.3 Positive controls should be handled very carefully. Dedicate a separate area and PCR Hood for positive control addition.

12.2.4 Change gloves frequently and especially if they get contaminated with RNA sample

12.2.5 Clean micropipettes, racks, instruments and the work area with freshly diluted 1% bleach (20 min), followed by 70% alcohol after every use

12.2.6 Use area-dedicated spray flasks or beakers (separate beakers for surface cleaning and instruments)

12.2.7 Do not take anything from one dedicated area either to other area of the lab

13 REFERENCES:

13.1 SARS-CoV2 Testing: The Limit of Detection Matters. Arnaout R, Lee RA, Lee GR, et al. Preprint. bioRxiv. 2020;2020.06.02.131144. Published 2020 Jun 4. doi:10.1101/2020.06.02.131144. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7302192/

13.2 Diagnostic testing for SARS-CoV-2: interim guidance document WHO, 11 September 2020: https://apps.who.int/iris/handle/10665/334254

13.3 Detection of specific polymerase chain reaction product by utilizing the 5'----3' exonuclease activity of Thermus aquaticus DNA polymerase. Holland PM, Abramson RD, Watson R, Gelfand DH. Proc Natl Acad Sci U S A. 1991 Aug 15;88(16):7276-80. doi: 10.1073/pnas.88.16.7276. PMID: 1871133; PMCID: PMC52277. https://pubmed.ncbi.nlm.nih.gov/1871133/

13.4 Phases of Real-Time PCR: https://www.labce.com/spg1913034_phases_of_real_time_pcr.aspx

13.5 CoviPath™ COVID-19 RT-PCR Kit - USER GUIDE

SOP CHANGE HISTORY

New version # / date	Old version # / date	No. of changes	Description of changes	Source of change request

ANNEXURES

ANNEXURE 1: MASTER-MIX CALCULATION WORKSHEET (96 reaction well plate)

Components	Volume required per reaction	Volume required for N reaction	
Multiplex Master Mix	6.25µl	6.25*N	
RT PCR assay multiplex	1.25 µl	1.25*N	
NFW	7.5 µl	7.5*N	
Total reaction volume	15 µl		

Date:
Kit:

ANNEXURE-2: AMPLIFICATION WORKSHEET

Master mix prepared by:_____Template addition by:_____

PCR run by:_____ PCR Checked by: _____

RT-PCR batch log

PCR machine name:					Batch no.:.....					Date:		
Name of rt-PCR kit:.....					Lot no.:					Expiry:		
	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												
Remark :												
Results -												
No. of positive results -												
No. of negative results -												
No. of presumptive positive -												
No. of invalid/indeterminate results -									Signature:			