Standard Operating Procedure No: MPX 6.7

Components of SOP: Molecular Biology: Mol Diagnostic RT.-PCR

Title	**Multiplex Real-Time PCR for detection of SARS-CoV-2 using TaqPath COVID-19 Combo Kit (Applied Biosystems).**
Document code	**SARS-CoV-2** -mol-multiplex RT PCR –diagnostic-MPX 6.7
Implementation Date	**07.04.2020**

1. **Introduction**:

The purpose of this document is to provide interim guidance to laboratories involved in laboratory testing of patients who meet the definition of suspected case of pneumonia associated with a novel coronavirus identified in Wuhan, China.

TaqPath™ COVID-19 Combo Kit contains the assays and controls for a real-time reverse transcription polymerase chain reaction (RT-PCR) test intended for the qualitative detection of nucleic acid from SARS-CoV-2 in nasopharyngeal swab, nasopharyngeal aspirate, and bronchoalveolar lavage (BAL) specimens from individuals suspected of COVID-19 by their healthcare provider. TaqPath™ COVID-19 Combo Kit is for use only under Emergency Use Authorization (EUA).

2. **SourceReference**:

https://assets.thermofisher.com/TFS-Assets/LSG/manuals/MAN0019181_TaqPath_COVID-19_IFU_EUA.pdf

3. **Testing criteria/Objective:**

Detection of SARS-CoV-2 in human clinical specimens using TaqPath COVID-19 Combo Kit (Applied Biosystems)

4. **Principle:**

The real time assay uses the TaqMan fluorogenic probe based chemistry that uses the 5′ nuclease activity of Taq DNA polymerase and enables the detection of a specific PCR product as it accumulates during PCR cycles.

COVID-19 Real Time PCR Assay Multiplex-Multiplexed assays that contain three primer/probe sets specific to different SARS-CoV-2 genomic regions and primers/probes for phage MS2 (Internal process control for nucleic acidextraction).

5. **Safety procedures:** According to Laboratory Safety Manual. (WHO,2011)
6. **Sample requirements:** 250µl of specimen or as per recommendedkit.
7. **Standard andcontrols:**

(1) Positive control (Supplied withKit)

(2) Water is used as no template control(NTC).

8. Scope anddefinition:

Highly specific and efficient detection of SARS-CoV-2 by Multiplex Real Time PCR.

9. Requirements:

Equipment	Consumables	Reagents and samples
Water bath, Bio Safety Cabinet clean laminar flow hood with micro-centrifuge with plate rotor and vortex, MiniSpin. Pipette set, Real Time PCR machine.	Mask, gloves, Lab Coats sterile filter tips, tissue paper, 0.2 ml, 0.5 ml and 1.5 ml micro centrifuge tubes, micro tips, 0.5-10 µl, 20-200 µl and 1000 µl tips. Real Time PCR Plates and sealers or tubes and strips	TaqPath COVID-19 Combo Kit (Applied Biosystems), Milli Q Water, Extracted viral Nucleic samples,

10. TestProcedure:

1. *Add 10 µL MS2 Phage Control to each sample well and to the Negative Control well during extraction and perform RNA extraction of clinical samples using your laboratory protocol. Extracted RNA will be the starting point for thereaction.
2. Prepare real time PCR worksheets (KGMU- VIRO-RTM-MPX-PCR-PP-6.7-copy attached at theend)
3. Perform multiplex real time PCR reaction as shown in table1 for corona ORF1ab gene, N gene, S gene and MS2 (Internal process control for nucleic acid extraction) in a single tube (as per manufacturers instruction).
4. Determine the number of reactions (N) to set up per assay. In addition, include Negative control & Positive control in thetest.
5. Prepare excess reaction cocktail to account for pipettingerror.
   If number of samples (n) including controls = 1 to 10, then N = n + 2
6. In the **clean reagent preparation room** prepare the MasterMix:

   Calculate the amount of each reagent to be added for each set reaction master mix.

Table 1: The calculations are as follows:

S.No.	Component	Volume for one reaction(N=1)	Volumefor(N=)
1.	TaqPath™ 1-Step Multiplex MasterMix(NoROX™)(4X)	6.25 µL	
2.	COVID-19 Real Time PCR Assay Multiplex	1.25 µL	
3.	Nuclease-free Water	12.50 µL	
	Total Reaction Mix volume	**20.0 µL**	

7. Mix reaction mixtures by pipetting up anddown.
8. Centrifugefor 5-10secto collect contents at bottomofthetube,andthenplacethetube incoldrack.

   Set up reaction strip tubes or plates in 96-well cooler rack.
9. Dispense 20µl of each master mix into each well as per the plate setup.
10. Before moving the plate to the nucleic acid handling area. Pipette 5ul of the nuclease free water into NTCwells.
11. **In the nucleic acid extraction room**, add 5ul of each sample and 5ul of extraction control into respective wells as per the setup.
12. Capthecolumn orcover theplate withtissue papertowhichthe samplesandcontrol has beenadded.
13. Finally, pipette 5 µl of positive viral template control (Positive Control) into wells in **positive control addition area.** Cap VTC wells/ or seal the plate with optical sealer. Centrifuge the plate for 10 seconds. Make sure that bubbles are eliminated from the bottom of the reactiontubes.
14. For real time PCR set up follow the instructions given by the Real-time PCR system manual for plate set up. **Save your platesetup!**
15. The reaction volume is 25µl.

Table 2: Program the run method as follows:

Step	Temperature	Time	Number of cycles
UNG incubation	25°C	2 minutes	1
Reverse transcription	53°C	10 minutes	1
Activation	95°C	2 minutes	1
Denaturation	95°C	3 seconds	45
Anneal/extension*	60°C	30 seconds	

*Fluorescence data should be collected during the 60ºC incubation step.

Table 3: Target Genes & Reporter dyes

Reporter dye	Detector
FAM	ORF1ab
VIC	N gene
ABY	S gene
JUN	MS2

16. After completion of the run, save the run and analyze the collecteddata.

11. Recording & reporting and Interpretation of theresults:

Interpretation of the results is performed by the Applied Biosystems COVID-19 Interpretive Software (Optional).

One Negative Control and one Positive Control are processed with each run.

Table 4: Result interpretation for patient samples

ORF1ab	N gene	S gene	MS2	Status	Result	Action
NEG	NEG	NEG	NEG	Invalid	NA	Repeat test. If the repeat result remains invalid, consider collecting a new specimen.
NEG	NEG	NEG	POS	Valid	SARS-CoV-2 Not Detected	Report results to healthcare provider. Consider testing for other viruses.
Only one SARS-CoV-2 target = POS			POS or NEG	Valid	SARS-CoV-2 Inconclusive[#]	Repeat test. If the repeat result remains inconclusive, additional confirmationtesting should be conducted if clinicallyindicated.
Two or more SARS-CoV-2 targets			POS or NEG	Valid	Positive SARS- CoV-2	Report results to healthcare provider and appropriate public health authorities.

[#] Samples with a result of SARS-CoV-2 Inconclusive shall be retested one time.

12. Quality control procedures:

For the results to be valid positive control must be positive; NTC must be negative. Check MS2 (if added during RNA Extraction) for all the samples. All the sample should have MS2 Positive. Otherwise, laboratory in charge must be informed and repeat testing is performed. Another experienced staff must countercheck all results.

13. Limitations

1. Analysts should be trained and familiar with testing procedures and interpretation of results prior to performing theassay.
2. A false negative result may occur if inadequate numbers of organisms are present in the specimen due to improper collection, transport orhandling.
3. This assay doesn't provide control over quality of samplecollected.

Note:

MS2 (Internal process control for nucleic acid extraction) testing can be ignored as it will not reflect the quality of sample collected. Hence, laboratories which have machine with no calibrated JUN dye filter or without JUN filter should not add MS2

control during extraction. MS2 control is for only for extraction procedure and if your machine doesn't support the JUN dye you can omitthis.

It is recommended that separate RNase P or any other human house-keeping gene for which primers & probe are available in your laboratory should be run parallel in a separate tube for RT PCR assay. This will check both the quality of sample collected and nucleic acid extractionprocedure.

Report: Communicate the result on daily basis to ICMR

Report Format

Sample ID	Patient State & place	Category of Patient	Sample received Date & time testing lab	Severity/ condition of patient	Result for SARS-CoV-2 virus

Worksheet for Multiplex Real-Time PCR for detection of SARS-CoV-2 using TaqPath COVID-19 Combo Kit (Applied Biosystems).
(One Step Reaction)

ReactionmixPreparation: ***Total Volume:*** *25µl (for one reaction)*

Corona ORF1ab Gene Probe:*Reporter-* ***FAM,*** ***Corona N Gene Probe:*** *Reporter-* ***VIC***

Corona S Gene Probe:*Reporter-* ***ABY*** ***MS2-IC Probe:*** *Reporter-****JUN***

S.No.	Item	Quantity(N=1)	Quantity(N=)	Done
1.	TaqPath™ 1-Step Multiplex Master Mix (No ROX™) (4X)	6.25 µL		
2.	COVID-19 Real Time PCR Assay Multiplex	1.25 µL		
3.	Nuclease-free Water	12.50 µL		
	Total Reaction Mix volume	**20.0 µL**		

Addition of Template

Template (TNA/RNA)	5 µl	

Sample details (sample ID):

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

Results for SARS-CoV-2:

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

Comments:

Done by: **Checked by:**