
	<h1>ICMR-National Institute of Virology (ICMR-NIV), Pune</h1>	
<h2>Standard Operating Procedure For Detection of 2019 novel coronavirus (2019-nCoV) in suspected human cases by rRT-PCR : confirmation assay</h2>		

Purpose: This protocol is designed to detect 2019-nCoV in human clinical specimens

Introduction: The purpose of this document is to provide interim guidance to laboratories and stakeholders involved in laboratory testing of patients who meet the definition of suspected case of pneumonia associated with a novel coronavirus identified in Wuhan, China. (<https://www.who.int/health-topics/coronavirus/laboratorydiagnostics-for-novel-coronavirus>)

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.

Coronaviruses under the subgenus Sarbecovirus that includes 2019-nCoV, SARS-CoV and bat SARS-like coronaviruses were used to generate a non-redundant alignment. Confirmatory assays designed based on their matching to the Wuhan virus as per inspection of the sequence alignment. Suspected human sample should be first tested for E gene assay and then confirmatory assay by RdRp and N gene assay.

- **Confirmatory assay: RdRp, ORF gene assay**

Reference:



<https://www.who.int/health-topics/coronavirus/laboratory-diagnostics-for-novel-coronavirus>

Requirements:

a. Instruments:

1. Real Time PCR machines (Make : ABI, Rm. Real time PCR room)
 - Model:7500 Fast: Serial no: 275012996
 - Model:7500 Fast Dx: Serial no: 275030301
 - Model:7500: Serial no: 275006294
 - Model: 7500 Fast Dx: Serial no: 275005234
 - Model:7500 Step one Plus: Serial no: 27200433
2. Biosafety cabinet (Make: Micro FITT, Model: MFI BIO4X2, Serial no: 14476, Rm no: Reagent preparation room)

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b. Pipettes

1. Rm. Reagent preparation room

For reagent dilutions

0.5-10 µl (Make: BIOHiT, Serial no: 6519410)

20-200 µl (Make: Thermo, Serial no: CH17505)

100-1000 µl (Make: Thermo, Serial no: CH28611)

For master mix preparation(Make: Thermo)

0.5-10 µl (Serial no:V44877)

2-20µl (Serial no: V42740)

20-200 µl (Serial no: U75613)

100-1000 µl (Serial no: CH01229)

2.Rm. RNA addition room:

5-100 µl multichannel (Make: BIOHiT, Serial no. 6545582)

2-20 µl (Make: Thermo, Serial no. V17267)

3. Rm. Real Time PCR room (Positive control addition)

2-20 µl (Make: Thermo, Serial no.V90525)

c. Small equipments

Vortex V1 plus: (Make: BIOSAN, Serial no: 15975, Location: Rm no: Reagent preparation room),

Minispin : (Make: TAESON, Serial no: 1775, Location: Rm no: Reagent preparation room, Hood: (Make: Serial no. V-14971, Rm: Real time PCR room)

Miniplate spinner: (Make: Labnet, Serial no. V-15725, Rm: Real Time PCR room)

d. Plastic ware: MicroAmp Fast reaction tubes (8 tubes/strip) , 96 Thin wall PCR plates, 96



Thin wall PCR plates 0.1 ml, 1.7ml Eppendorf tubes, stand, micro tips, 96 well cooler

e. Consumables: Disposable powder free gloves, Lab coats, aerosol barrier tips (20ul, 200ul and 1000ul), Laboratory marking pen, tissue paper rolls

f. Reagents:

1. Invitrogen SuperScript™III Platinum® One-Step Quantitative Kit (Cat. No.11732088)
2. AgPath-ID™ One-Step RT-PCR
3. QIAamp Viral RNA Mini Kit (QIAGEN, Cat#52906) or equivalent RNA extraction Kit
4. Nuclease Free Water
5. Ethanol (96–100%)

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Primers and Probes

Assay/ Use	Oligonucleotide ID	Sequence (5'-3')
RdRp	RdRP_SARSr-F2	GTGARATGGTCATGTGTGGCGG
	RdRP_SARSr-R1	CARATGTTAAASACACTATTAGCATA
	RdRP_SARSr-P2 Specific for Wuhan-CoV	FAM-CAGGTGGAACCTCATCAGGAGATGC- QSY
HKU ORF gene	HKU-ORF1b-nsp14F	TGGGGYTTTACRGGTAACCT'
	HKU-ORF1b-nsp14 R	AACRCGCTTAACAAAGCACTC
	HKU-ORF1b-nsp14 P	FAM-TAGTTGTGATGCWATCATGACTAG- QSY

FAM, 6-carboxyfluorescein; QSY Quencher (select quencher none in plate set up)

Documentation:

- Clinical sample register
- RNA extraction Laboratory book
- Real time PCR Laboratory book
- Result record book

Procedure/Protocol:

1. Perform RNA extraction of clinical samples according to “RNA extraction- QIAmp viral RNA Mini Kit” protocol in RNA extraction area.
2. Perform real time PCR reactions as shown in table for RdRp, ORF and N gene assays Determine the number of reactions (N) to set up per assay. In addition, include Negative control, Positive control and MOCK (human source cell line) in the test.
3. Prepare excess reaction cocktail to account for pipetting error.

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

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4. In the **clean reagent preparation room** prepare the Master Mix:
Calculate the amount of each reagent to be added for each Primer /probe set reaction master mix. The calculations are as follows:

Component	Volume for RdRp gene	Volume for ORF
H ₂ O (RNase free)	5 µl	5 µl
2x Reaction mix	12.5 µl	12.5 µl
PP Mix	1.5 µl	1.5 µl
AgPath One-Step RT-PCR *	1 µl	1 µl
Template RNA	5 µl	5 µl
Total	25 µl	25 µl

*** Invitrogen SuperScrip III Platinum One-Step Quantitative Kit, use 0.5ul and adjust the water volume to 5.5µl**

5. Mix reaction mixtures by pipetting up and down. Do not vortex.
6. Centrifuge for 5-10 sec to collect contents at bottom of the tube, and then place the tube in cold rack.
7. Set up reaction strip tubes or plates in 96-well cooler rack.
8. Dispense 20µl of each master mix into each well as per the plate set up.
9. Before moving the plate to the nucleic acid handling area. Pipette 5ul of the nuclease free water into NTC wells. Cap NTC wells.
10. **In the nucleic acid extraction room**, add 5ul of each sample and 5ul of Mock extraction control into respective wells as per the set up.
11. Cap the column or cover the plate with tissue paper to which the samples and mock control has been added.

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12. Finally, pipette 5ul of positive viral template control into all VTC 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 reaction tubes.
13. For real time PCR set up follow the instructions given by the Real-time PCR system manual for plate set up. **Save your plate setup!**
14. The reaction volume is 25ul. Program the run method as follows:

Reverse Transcription	55°C for 30 min
Taq inhibitor inactivation	95°C for 3 min
PCR amplification (45 Cycles)	95°C for 15 Sec 58°C for 30 sec* (data collection)



Fluorescence data should be collected during the 58⁰C incubation step.

15. After completion of the run, save the run and analyze the collected data.

Interpretation/examination:

1. The NTC reactions for primer / probe sets **should not exhibit** fluorescence growth curves that cross the threshold line. If a false positive occurs with one or more of the primer and probe NTC reactions, sample contamination may have occurred. Invalidate the run and repeat the assay with stricter adherence to the procedure guidelines.
2. The MOCK should NOT exhibit fluorescence growth curves for primer/probe sets for 2019-nCoV RdRp, ORF and N gene. Only in RP target, MOCK should show fluorescence growth curve. If any 2019-nCoV RdRp, ORF and N gene specific primer/probes exhibit a growth curve that crosses the threshold line, interpret as follows:
 - a. Contamination of RNA extraction reagents may have occurred. Invalidate the run and confirm reagent integrity of RNA extraction reagents prior to further testing.

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- b. Cross contamination of samples occurred during RNA extraction procedures or assay setup. Invalidate the run and repeat the assay with stricter adherence to procedure guidelines.
3. PTC reactions should produce a positive result with the 2019-nCoV RdRp, and ORF and N gene reactions between 20 and 30 cycles. If expected positive reactivity is not achieved, invalidate the run and repeat the assay with stricter adherence to procedure guidelines. Do not use PTC reagents that do not generate expected result.
4. When all controls meet stated requirements, a specimen is considered confirmed positive for 2019-nCoV reaction growth curves cross the threshold line within 35 cycles for E gene, and both RdRp, ORF Or either RdRp or ORF

Limitations

1. Analysts should be trained and familiar with testing procedures and interpretation of results prior to performing the assay.
2. A false negative result may occur if inadequate numbers of organisms are present in the specimen due to improper collection, transport or handling.
3. A false negative result may occur if an excess of DNA/RNA template is present in the reaction.

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