# ICMR-National Institute of Virology (ICMR-NIV), Pune

## Standard Operating Procedure For

**Detection of 2019 novel coronavirus (2019-nCoV) in suspected human cases by rRT-PCR : First Line Screening assay**

**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. Three assays based on their matching to the Wuhan virus as per inspection of the sequence alignment were designed

## First line screening assay: E gene assay

**Pl note along with novel corona real time PCR protocol, sample should be tested for Influenza detection**

**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)

**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%)

**Primers and Probes**

| Assay/ Use | Oligonucleotide ID | Sequence (5'–3') |
|---|---|---|
| E gene | E_Sarbeco_F1 | ACAGGTACGTTAATAGTTAATAGCGT |
| | E_Sarbeco_R2 | ATATTGCAGCAGTACGCACACA |
| | E_Sarbeco_P1 | FAM-ACACTAGCCATCCTTACTGCGCTTCG-BHQ |
| RNaseP gene (Internal Control) | RNaseP Forward | AGATTTGGACCTGCGAGCG |
| | RNaseP Reverse | GAGCGGCTGTCTCCACAAGT |
| | RNaseP Probe | FAM-TTCTGACCTGAAGGCTCTGCGCG-BHQ |

R is G/A; FAM, 6-carboxyfluorescein; BHQ, Black Hole Quencher

**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 E gene assays and RNasP housekeeping gene.
3. 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.
4. Prepare excess reaction cocktail to account for pipetting error.

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

5. 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 E gene | Volume for RNasP |
|---|---|---|
| $H_2O$ (RNAse free) | 5µl | 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**

6. Mix reaction mixtures by pipetting up and down. Do not vortex.
7. Centrifuge for 5-10 sec to collect contents at bottom of the tube, and then place the tube in cold rack.
8. 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 set up.
10. Before moving the plate to the nucleic acid handling area. Pipette 5ul of the nuclease free water into NTC wells. Cap NTC wells.
11. **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.
12. Cap the column or cover the plate with tissue paper to which the samples and mock control has been added.

13. Finally, pipette 5 µl of positive viral template control (E gene invitro transcribed RNA and for RNasP add pooled influenza 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.
14. 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!**
15. The reaction volume is 25 µl. 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<br>58°C for 30 sec* (data collection) |

Fluorescence data should be collected during the $58^0$C incubation step.

16. 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. All clinical samples should **exhibit RNase P reaction curves** that cross the threshold line at or before **35 cycles**, thus indicating the presence of sufficient RNA from human RNase P gene indicating the specimen is of acceptable quality.

However, it is possible that some samples may fail to give positive reactions due to low cell numbers in the original clinical sample.

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
b. RNA or carry-over of RT-PCR inhibitors from clinical specimens
c. Absence of sufficient human cellular material in sample to enable detection
d. Improper assay set up and execution
e. Reagent or equipment malfunction

3. The MOCK should NOT exhibit fluorescence growth curves for primer/probe sets for 2019-nCoV E gene. Only in RP target, MOCK should show fluorescence growth curve. If any 2019-nCoV E 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.
    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.
4. PTC reactions should produce a positive result with the 2019-nCoV E gene and RNaseP 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.
5. When all controls meet stated requirements, a specimen is considered presumptive positive for 2019-nCoV reaction growth curves cross the threshold line within 35 cycles.
6. Immediately send the sample to Reference laboratory i.e NIV Pune

**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. If inhibition of the RP control reaction is noted for a particular sample, extracted RNA can be tested at 2 or more dilutions (e.g., 1:10 and 1:100) to verify result.

**If the sample is positive, immediately send the sample to Reference laboratory i.e. ICMR –NIV Pune for Confirmatory testing. It is only after confirmatory test becomes positive, then the sample can be declared positive**

**Confirmatory assay Available at ICMR NIV**

- **ORF 1b**
- **RdRp gene assay**
- **E gene assay**
- **N gene assay**

**Report: Communicate the result on daily basis to ICMR NIV Pune**