Protocol for POOL Testing of COVID-19

2077

Provincial Government
Ministry of Social Development
Province 1 Biratnagar,
We are grateful to Mr. Jivan Ghimire, honorable minister, Ministry of Social Development Province 1 for giving us this opportunity to prepare this document. His strong leadership and untiring efforts in the control of COVID 19 is highly appreciable. His interest and initiation of this collaborative activity among the three laboratories designated for testing SARS-COV-2 in the Province 1 is praiseworthy. It is expected that personnel of these three laboratories will work together by sharing knowledge, technical expertise and resources within the rules and regulation of institute/facility they are associated with. We would like to acknowledge Dr Ratna Baral, Dr Abhilasha Sharma, Dr Shraddha Swakoti, Dr Lok Bahadur Shrestha, Dr Rinku Sah, Mr Ganesh Prasad Sah and entire molecular team of BPKIHS for their valuable contribution in the document. We would like to express our thankfulness to Prof Dr Gyanendra Giri, Vice Chancellor BPKIHS and Dr Sangita Kaushal Mishra, Medical Superintendent KZH for their support to accomplish this task.

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## Abbreviation

<table>
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<th>Description</th>
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<tr>
<td>BPKIHS</td>
<td>B. P. Koirala Institute of Health Sciences</td>
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<tr>
<td>KZH</td>
<td>Koshi Zonal Hospital</td>
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<tr>
<td>PPPL</td>
<td>Provincial Public Health Laboratory</td>
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<tr>
<td>BSL2</td>
<td>Bio Safety Level 2</td>
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<tr>
<td>PPE</td>
<td>Personnel protective equipment</td>
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<tr>
<td>RT-PCR</td>
<td>Real Time PCR</td>
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<tr>
<td>WHO</td>
<td>World Health Organization</td>
</tr>
<tr>
<td>CICT</td>
<td>Case Investigation and Contact Tracing</td>
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<tr>
<td>RDT</td>
<td>Rapid Diagnostic Test</td>
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<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>ICMR</td>
<td>Indian Council of Medical Research</td>
</tr>
<tr>
<td>SOP</td>
<td>Standard Operating Procedure</td>
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<tr>
<td>AHU</td>
<td>Air Handling Unit</td>
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<tr>
<td>UV</td>
<td>Ultra Violet</td>
</tr>
<tr>
<td>VTM</td>
<td>Viral Transport Medium</td>
</tr>
<tr>
<td>EDCD</td>
<td>Epidemiology and Disease Control Division</td>
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<tr>
<td>OT</td>
<td>Operation Theatre</td>
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<tr>
<td>HCW</td>
<td>Health Care Worker</td>
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Background

Province one has 14 districts, and 137 municipalities that include 1 metropolitan city (Biratnagar), 2 sub-metropolitan cities (Itahari and Dharan), 49 Municipalities (Nagarpalika) and 88 Rural Municipalities (Gaupalika). There are around 4.5 million people in the Province, with a population density of 175.6 per square kilometer. Of the total population, 52.6% are female and 47.4% are male. The Province covers an area of 25,905 sq km comprising of the Himalayan region in the north, Hilly region in the middle, and the Terai region in the southern part between the altitudes of 70m to 8848m. The lowest point of the Province is also the lowest point of the country, Kechanalakwal of Jhapa district; at 70m and the highest point of the Province is Mount Everest, the highest mountain of the world. The major religion of this province is Hinduism (63.6%), Kirat Mundhum (17.1%), Buddhism (9.2%), Islam (3.6%), Christianity (1.7%), Prakrti (1.3%) and other or not religious (0.4%).

COVID 19 is a respiratory illness caused by Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2). The outbreak was first identified in Wuhan, Hubei Province of China on December 31, 2019. The Virus causes symptoms like fever, cough, and shortness of breath. WHO had declared Public Health Emergency of International Concern on 30 January 2020 and later declared global Pandemic on 11 March 2020. There have been more than 4 million cases worldwide and more than 3 hundred thousand deaths worldwide by mid-May 2020. USA, Italy, Spain, France has one of the highest numbers of cases worldwide with cases increasing at a high rate in Russia, the UK, and Brazil.

In Nepal, the first case was reported on 23 January 2020, a 32-year old Nepali man returning from Wuhan. After the first case was reported, the government of Nepal took immediate action and tightened its border security both with India and China. Health desks were installed at Tribhuvan International Airport. To avoid further cases in the country and to slow down the rate of transmission, the country went into lockdown on 23 March 2020.

Province 1 has also been under Lockdown since March 23, 2020. Province one had seen its first COVID-19 case on April 17, 2020 in Udyapur district among Indian nationals. PCR Testing for COVID-19 was started in 27th March 2020 in BPKIHS which was the first COVID testing lab outside
Kathmandu. It was soon followed by Koshi Hospital, and Province Public Health Laboratory in collaboration with Veterinary Hospital Laboratory. Labs across all the districts are being equipped with trained personnel and equipment for COVID-19 Lab testing. Currently, 200 Hospital beds are arranged for COVID-19 but the capacity can be increased up to 1000 hospital beds. Till May 16, 2020, there have been a total of 36 confirmed cases of COVID-19 in the Province 1 and all of the cases are under medical supervision. Contact tracing is going on for all of the cases. Case Investigation and Contact Tracing (CICT) guidelines has been developed and finalized, CICT teams have been formed in all the districts with proper training and orientation. There has been a total of 2756 PCR testing and 9185 RDT testing for COVID-19 carried out along with ongoing contract tracing.

On one hand number of COVID-19 cases is on the rise all over Nepal, making the need of immediate expansion of testing pertinent. On the other hand, there is pressing issue of optimal utilization of resources for testing. PCR detects the virus even at early stage of infection, thus is crucial from the timely management and prevention of spread perspectives. And number of samples with positivity is still low. In such situation, testing large number of population with the existing resources is a challenge.

“Pool testing RT-PCR” is one of the practical approaches to address this challenge. These tests “pool” more than two samples, preferably 5 so that use of resources and chance of false negative by dilution effect is balanced. If “pool” is negative, all samples in the pool are negative, whereas if “pool” is positive, then all samples in the pool need to be tested individually, a process known as “pool deconvolution”.

Pool PCR technique is set to be useful in the area where prevalence of COVID-19 is less than 2%. According to government of Nepal’s guidelines, pool is applicable only for samples from individuals in quarantine. However, even in area with positivity between 2-5%, it has been recommended for community survey or surveillance among asymptomatic individuals. (ICMR, advisory on feasibility of using pooled samples in molecular testing of COVID-19, 13.04.2020).
Objective of the committee:

To prepare protocol of RT-PCR for the detection of SARS-COV-2 using pooled samples collected from Nasopharyngeal and throat swabs.

Each method described in this protocol here is based on the “experiment on pool testing” performed in Department of Microbiology, B P Koirala Institute of Health Sciences (BPKIHS). This protocol has been finalized after extensive discussion among all the members in ZOOM meeting, email exchanges and WHATSAPP discussion and incorporation of their scientific inputs. Other labs are requested to adapt this protocol to prepare their specific operating procedures according to their facilities and resources.

Situation in which Pool testing is recommended

- Individual in quarantine (if positivity for COVID-19 is <2%)
- Asymptomatic individuals (if positivity for COVID-19 is between 2-5%)
- Community survey (if positivity for COVID-19 is between 2-5%)

Situation in which pool testing is not recommended

- Clinical suspicion of COVID-19, Severe Acute Respiratory Illness (SARI) and confirmation of virus after recovery
- Individual with known contact with confirmed cases
- Health care provider in direct contact with COVID-19 cases
- Population with more than 5% positivity for COVID-19
COVID-19 Testing Facilities at BPKIHS/KZH/PPHL

A. Laboratory Workflow:
   
   a. Specimen Handling: SOP (SOP 1)
   b. Duty Roster of Specimen receipt (Prepare Departmental/KZH/PPHL duty roster)
   c. If laboratory negative pressure installed along with BSL II then the strength of the
      lab can be recognized as BSL II+. The laboratory with BSL II may skip steps
      (indicated in italics) which are specific for use and operation of negative pressure.

C.1. RNA Extraction Room

   i. **Switch on the first knob to open AHU Unit.**
   ii. **Switch on the second knob to open Exhaust unit.**
   iii. **Switch on the third knob to open the central Air-condition**
   iv. Enter the room
   v. Switch on the Biosafety cabinet, UV light on for 30 minutes and internal
      air-condition of the both rooms. Keep the temperature of 21°C in the air-
      condition
   vi. Clean the floor, slab and benchtop with 1% Sodium Hypochlorite solution
   vii. Clean the wall of the room with 1% Sodium Hypochlorite solution (Do Not
        Use on Metallic surface)
   viii. **Clean the wall with by 70% Ethanol**
   ix. Leave the room undisturbed for 1 hour in order to maintain the flow of the
      room
   x. **Switch on the flow of the cabinet**
   xi. Wipe the surface of the cabinet, equipment and pipettes with 1% Sodium
       Hypochlorite
   xii. Wipe the surface of the cabinet, equipment and pipettes by 70% Ethanol
   xiii. **Switch on the light of the cabinet**
   xiv. Check all the consumables and reagents in the room
d. Preparation of the tubes:
   i. Prepare the tubes in separate room preferably in laminar flow.
   ii. Switch on the air flow of the laminar flow.
   iii. Leave the flow for 10 minutes
   iv. Wear the latex gloves
   v. Clean the surface of the flow with 1% Sodium Hypochlorite and then with
      70% Ethanol
   vi. Clean the rack with 70% Ethanol
   vii. Change the gloves and wear the Nitrile gloves
   viii. Label the tubes for RNA elution with the preprinted label
   ix. Label of the RNA Extraction column with marker pen on the top of the spin
       column
   x. Place the extra collection column in the rack (If sufficient collection tubes
      are not provided then remove the cap of the 2.0 ML Micro centrifuge tube
      and use them as the collection tubes)
   xi. Label the sample storage screw capped cryo-vial (these samples need to
       be stored at -80°C)

e. Donning of PPE for the RNA Extraction (SOP 5)
   Required PPE: Cover all/Gown, Gloves (double), N95 Mask, head cover, shoe cover
   or boots, goggles, face shield

f. Sample preparation for RNA Extraction
   i. Switch on the flow of the Biosafety cabinet type II
   ii. Switch on the UV light and wait for 10 minutes
   iii. Clean the surface of Safety cabinet with 1% Sodium Hypochlorite solution
   iv. Wipe the surface in the cabinet with 70% Ethanol
   v. Switch on the light in the cabinet
   vi. Place all the samples inside the safety cabinet
   vii. Check the original label and place the unique sample ID given by PCR lab
       in the sample vial.
viii. Keep the samples in increasing order of the sample ID
ix. Prepare a document to indicate the samples ID, Pool ID, name of the individual, date of collection, address of the individual etc.
x. Transfer the properly arranged sample rack to the 4°C refrigerator
xi. Wipe the surface of the cabinet with 1% Sodium hypochlorite solution
xii. Wipe the surface of the cabinet with 70% Ethanol
xiii. Switch off the light in the cabinet
xiv. Switch off the flow of the cabinet
xv. Switch on the UV light of the cabinet for 10 minutes
xvi. Check the status of Autoclave (switch on to confirm the temperature, pressure, water level, electricity supply etc.)

g. RNA Extraction
i. Enter the RNA extraction room with all component of PPE worn carrying the rack containing all the preparation of consumables into the identified room for RNA Extraction
ii. Collect the samples kept in refrigerator dedicated for sample storage for short time
iii. Place the sample rack inside the Biosafety safety cabinet II

h. Sample handling:
i. Transfer 2ml of each sample in the previously labeled respective sterile storage vial
ii. Close the lid of the sample collection vial.
iii. Discard the sample collection vial along with swab in the autoclavable bags
iv. Pipette out the required volume of the sample from storage vial to the 1.5 ml DNase and RNase free Microfuge tube for RNA Extraction or make a pool of 5 samples into 1.5 ml sterile DNase and RNase free Microfuge tubes. Volume to be transferred for RNA extraction depends upon the available kit for RNA extraction
v. Change the gloves (Use only Nitrile gloves)
i. RNA Extraction from specimen (This procedure depends upon the availability of the kit)

j. Cleaning of the lab (SOP 3)

k. Doffing of PPE (SOP 5)

l. Disposal of waste (SOP 4)

m. Negative pressure handling after the work
   i. Leave it in operation for one hour after the work
   ii. Switch off the AHU, Exhaust and Air-condition knob
   iii. Switch off all the light

n. Transfer of RNA
   i. Collect the RNA containing box and vial containing samples for storage
   ii. Place them immediately in the box containing icepacks

o. Transfer the box to the room for template addition. Handover the RNA and samples to the identified person (Prepare delegation log) in room for template addition. (in Biosafety cabinet type II)

p. Pre-PCR Preparation
   i. Wear the lab coat
   ii. Put on the Nitrile gloves
   iii. Switch on the UV light of the hood for 10 minutes
   iv. Switch on the flow of the hood
   v. Never switch on the light (you work with Probe)
   vi. Wipe the hood surface and other equipment with RNase ZAP
   vii. Wipe all the hood surface and other equipment with 70% Ethanol
   viii. Clean the cooling rack with 70% Ethanol
   ix. Prepare the required tubes for PCR reaction and keep them in the cooling rack
   x. Label the tubes with sample ID, positive control (P) and No template control (NTC)
   xi. Prepare the master mix (Follow the manufacturer instruction)
xii. Aliquot the master mix in each tube
xiii. Remove the cooling rack from the hood
xiv. Wipe the hood surface and equipment with 70% Ethanol
xv. Switch off the flow of the hood
xvi. Switch on the UV light for 10 minutes
xvii. Change the lab coat
xviii. Transfer the tubes immediately to template addition room
q. Donning of the PPE to work for Template addition (SOP 5)
r. Template addition room
   i. Switch on the Biosafety Cabinet Type II
   ii. Switch on the UV light for 10 minutes
   iii. Switch on the flow of the cabinet
   iv. Wipe the surface/equipment with 1% Sodium hypochlorite solution
   v. Wipe the surface/equipment with 70% Ethanol
   vi. Add RNA template in each tube, pipette up and down of the sample before adding the template into the tube containing master mix
   vii. Close the tube firmly
   viii. Remove the cooling rack from the cabinet and store immediately in refrigerator
   ix. Wipe the cabinet surface/equipment with 1% sodium hypochlorite
   x. Wipe the surface/equipment with 70% Ethanol
   xi. Close the flow of the cabinet
   xii. Switch on the UV light for 10 minutes
   xiii. Transfer the tubes to other room where centrifuge is located
s. Doffing of PPE: (SOP 5)
t. Addition of positive and no template control: Add required amount of positive and No template control
u. Centrifuge all the tubes including positive and no template control: Spin down for 10 seconds (if bubbles seen, remove the bubbles with centrifugation; especially in
RGQ machine, bubbles should be completely removed from the bottom of the tube.

v. Sample loading in the machine
   i. Place the positive control in position 1, no template control in position 2 then place other samples in the rotor of the machine
   ii. Fit the rotor in the machine
   iii. Close the machine
   iv. Run the programme in the computer (Note that machine and computer should be connected)
   v. Connect all the electrical wire to the dedicated power supply (in order to prevent the loss of electricity during the procedure)

w. Read the results, interpret, dispatch the reports and keep the records securely with back up.

x. Dispose the waste in adherence to (SOP 4)

γ. Requirement of the laboratory consumables and stock maintenance (List of the items in Annex)
SOP 1: Specimen Collection, Packaging and Transport for diagnosis of SARS COV-2

1. Scope and application

Diagnosis of COVID-19 is established by RT-PCR on the nasopharyngeal (NP)/or oropharyngeal(OP)/throat swab obtained from suspected case of COVID-19. After collection, the sample is packaged in triple layer and kept in ice-pack box and transported to SARS-CoV2 laboratory.

2. Responsibilities

<table>
<thead>
<tr>
<th>Responsible personnel</th>
<th>Activities</th>
</tr>
</thead>
<tbody>
<tr>
<td>Doctor / Health care provider on duty</td>
<td>Collection of NP/OP or throat swab</td>
</tr>
<tr>
<td></td>
<td>Transport to Microbiology lab, BPKIHS, Pathology lab KZH, PPHL</td>
</tr>
</tbody>
</table>

3. Procedures

3.1.1 Materials and samples

3.1.2 Materials

- Viral transport medium
- Collection swab plastic with Dacron or nylon tip
- PPE (full set: N95 mask, goggles, Coverall/gown, disposable gloves, disposable shoe cover or boot)
- Torch
- Hand sanitizer
- Zip lock medium or large size
- Absorbent materials: Cotton or tissue paper
- Leak proof secondary container: zip-lock pouch, cryo box, 50ml centrifuge tube, or a plastic container
- Hard frozen gel/ice packs
- Paraffin, scissors, cello tape
- Ice Box
- Requisition form or Patient record form of EDCD/NPHL/PPHL/BPKIHS wherever applicable
- Waste basket for infectious waste

3.1.3 Procedure

- Take decision on the indication of collection of the NP and OP swab per the case definition given by Ministry of Health, Nepal.
- Consider all specimens as potential hazardous and infectious
- Fill up the requisition form completely and legibly
- Label the VTM with patient’s ID
- Don PPE (Refer to SOP 5 on Donning and Doffing of PPE).

Procedure to collect nasopharyngeal swab

- Ask the patient to take off mask and blow his/her nose into a tissue to clear excess secretions from the nasal passages.
- Tilt the patient’s head back (70°) slightly, so that the nasal passages become more accessible.
- Ask the patient to close eyes to lessen the mild discomfort of the procedure.
- Gently insert the swab along the nasal septum, just above the floor of the nasal passage, to the nasopharynx, until resistance is felt.
- Leave the swab in place for several seconds to absorb secretions and then slowly remove the swab while rotating it.
- Place the swab in the VTM and break the shaft.
Procedure to collect Oropharyngeal swab

- Ask the subject to open his or her mouth wide open.
- Depress the tongue.
- Swab the posterior pharynx behind the tonsils with another swab stick.
- Avoid the tongue, teeth and gums.
- Place the swabs immediately into the VTM in which NP swab was kept breaking the swab stick.
- Close the VTM tube.
- Seal the neck of VTM with Parafilm.
- Dispose the waste in the basket for infectious waste.

Packaging

1.1 Cover the VTM with the absorbent materials (cotton wools/tissue papers) and keep inside the centrifuge tube or Ziploc bag. Lock the bag tightly.

1.2 Put this in a container with hard surface (secondary receptacle) (a type of plastic bottle depending on the availability, if not available another larger Zip lock bag can be used). Many primary receptacles can be kept inside one secondary receptacle.

1.3 Keep the whole 3 layered items in ice box, with ice pack maintaining temperature 2-8°C

1.4 Outer shipping package (box) protects its contents inside from outside influences such as physical damage and water while in transit.

1.5 Keep the specimen data forms, letters and other types of information that identify or describe the specimen in a transparent folder or zip lock bag and paste it securely on the outer side of ice box or keep inside the box.

1.6 Doff PPE (Refer to SOP on Donning and Doffing of PPE).
1.7 Dispose PPE into the waste basket for final disposal as infectious waste.

Transport

- Transport the box as UN3373 label which indicates "Biological substance category B" maintaining cold chain 2-8°C.

Receipt at BPKIHS/KZH/PPHL lab

- Contact duty doctor of Microbiology/hospital/lab before dispatching and during transport of the sample
- Receive the Sample Box (usually ice box or vaccine carrier) in a separate room or area designated for the purpose
- Disinfect the box with 1.0% Sodium Hypochlorite wipe.
- Open the box, check if triple layer packaging and cold chain is maintained.
- Open the outer layer
- Verify the label in the VTM with that in the form
- Criteria for rejection
  - Leaking specimen
  - Cold chain not maintained
  - Not labeled or information in the label not matching with that in the form
- Take the accepted sample to SARS CoV2 lab
- Store the sample till assayed at 2-8 °C for up to ≤ 72 hours. If testing is anticipated after 72 hours, store at −80 °C

4. Reference

SOP 2: Sample receipt and storage for SARS COV-2

1. Scope and application

This SOP is applicable for receiving and storage of sample for SARS –COV-2 in PCR laboratory, Department of Microbiology/Koshi Zonal Hospital/PPHL.

2. Responsibilities

<table>
<thead>
<tr>
<th>Responsible personnel</th>
<th>Activities</th>
</tr>
</thead>
<tbody>
<tr>
<td>Laboratory personnel delegated by respective lab/institution for the specific activities (in accordance with interim guidelines by NPHL Nepal)</td>
<td>Receiving and storing the samples sent for SARS –COV 2 PCR testing.</td>
</tr>
</tbody>
</table>

3. Procedures

3.1.1 Precautions

- Handle all the samples as potential biohazard materials.

3.2 Safety condition:

- Follow all safety guidelines for disposal of waste.
- Wear protective head cover, Gown (OT linen), plastic apron, N95 mask and disposable gloves, goggles.
- Wash hands thoroughly before and after the completion of tasks.

3.1.3 Materials required

- Appropriate PPE
- Sodium hypochlorite 1%
- Cotton roll
- 70% ethanol
• Tissue paper
• Hand sanitizer
• Discarding bucket
• Marker pen

3.1.4 Procedure

1. Receive the ice box containing the VTM and patients swab sample at designated area/room along with the patient's information sheet.
2. Disinfect the outer part of the box with 1% sodium hypochlorite wipe.
3. Open the box carefully and clean the inner part too.
4. Check the condition of the ice packs inside.
5. Take out the sample and check for proper triple packaging.
6. Remove the first pack and check for patient's details in VTM inside the zip lock bag.
7. Check whether the patient's details in the VTM and information sheet match or not.
8. Write the details of patient in the register and write your lab's ID number/sample number in register as well as the zip-lock bag.
9. Carry the sample to the biosafety cabinet identified for the purpose
10. Remove the zip lock bag and take out the VTM.
11. Wipe the screw cap and the sides of VTM with 1% sodium hypochlorite.
12. Write down the given lab ID on the VTM.
13. Store the VTM at 2-8°C in refrigerator till RNA extraction (within 72 hours)
14. Return the ice box to the carrying person after cleaning the inner and outer side of the box.
15. Wipe the work area with 1% sodium hypochlorite.
16. Dispose all the wastes as infectious waste.

3.1.5 Criteria for Rejection
- Leaking specimen
- Cold chain not maintained
- Not labeled or information in the label not matching with that in the form
SOP 3: Cleaning and Disinfection of PCR Lab

1. Scope and Application

Environmental cleaning is part of standard precautions. COVID-19 virus can potentially survive in the environment for several hours/days. Therefore, proper cleaning and disinfection of all areas-surfaces and rooms of PCR laboratory is crucial. This activity should be performed routinely and after each batch of work.

2. Responsibilities:

<table>
<thead>
<tr>
<th>Responsible personnel</th>
<th>Activities</th>
</tr>
</thead>
<tbody>
<tr>
<td>Designated Cleaning Staff</td>
<td>Cleaning and Disinfecting lab</td>
</tr>
</tbody>
</table>

3. Procedure

3.1 Materials Required

- Bucket of appropriate size or containers for disinfectants
- Mops Separate for BSL lab and other areas
- Cottons swab
- 70% Ethanol
- 1% Sodium Hypochlorite
- Detergent
- 0.1% Sodium Hypochlorite

3.2 Cleaning Procedure

- Clean and disinfect the laboratory daily before starting the test and after completion of test (frequency being at least twice a day)
➢ Wear water resistant gowns, goggles, head cover, N95 mask, heavy duty gloves, Boots

➢ Prepare 1% Sodium Hypochlorite solution fresh in a small bucket

➢ Wipe with detergent and water with separate mop daily or if visibly soiled before disinfecting with Sodium hypochlorite

➢ Disinfect slabs with 1% sodium hypochlorite

➢ Mop floor and wipe the wall with 1% sodium hypochlorite

➢ Wipe all frequently touched areas (door handles, Knobs, switches, chairs, metal cabinets, switches button, computer, keyboards etc.) with 70% Ethanol

➢ Give the contact time of at least 10 minutes

➢ Progress from the least soiled areas to the most soiled areas from high surfaces to low surfaces during mopping/cleaning

➢ When cleaning the floor, begin farthest from the door and move towards the door (in to out).

➢ Do not “DOUBLE – DIP” mops (dip the mop only once in the cleaning solution, as chance of recontamination is increased dipping multiple times re-contaminates it)

➢ Never shake Mops: Minimize turbulence to prevent the dispersion of dust.

➢ After completing the work, clean the mops first with detergent and water then with 0.1% sodium hypochlorite

➢ Clean the buckets with detergent and water

➢ Dry in sunlight

➢ Change the mop head when heavily soiled or at the end of the day

➢ Collect waste, handle plastic bags from the top (do not compress with hands)

➢ Clean hands on leaving room

➢ Go for shower after completion of cleaning
Note:

Apply sodium Hypochlorite or 70% ethanol to the surfaces with wipe.

Do not use spray; as coverage is uncertain and spraying may promote aerosols.

3.3. Spill management

Required materials:

- Scoop and scraper
- Absorbent material (tissue paper/cotton/gauze)
- Gloves
- Water impermeable gowns
- N95 Masks
- Goggles
- Infectious waste bags and ties
- Detergent
- 1% Sodium Hypochlorite
- Instruction chart

Note: Buckets and mops of spills should be different from the regular mops and buckets and should be cleaned separately

Protocol:

1. Immediately attend spillage area
2. Mark the spill area
3. Put wet floor signage
4. Wear appropriate PPE (N95 Mask, water impermeable gowns, goggles, gloves)
5. Confine the spill with absorbent materials
6. Remove the absorbent cloths and discard as infectious waste

7. Pour the 1% sodium hypochlorite solution over the spilled area

8. Leave for 10 minutes (contact time)

9. Rinse the area with clean water to remove the disinfectant residue

4. References:

   Best Practices for Environmental Cleaning in health care facilities in Resource – limited Settings. CDC, November, 2019

SOP 4: Disposal of wastes generated during PCR work

Materials Required

- Containers color coded
  o Red: Infectious plastics generated during the PCR work and during receipt of samples
  o A small plastic container: for disposing the used micro tips
  o Blue: Non-infectious materials such as papers
  o Black: Non-infectious plastics
- Autoclavable Plastics Bags and ties
- Autoclave

Placement of Buckets, Autoclave

1. Keep small red bucket lined with autoclavable bag inside the BSL cabinet
2. Keep big bucket with autoclavable bag inside in the BSL room/Negative pressure room near the cabinet
3. Keep autoclavable container in the BSL cabinet for the purpose of discarding the micropipette tips (optional)
4. Keep autoclavable bag in the duffing room
5. Place autoclave for infectious waste disposal close to donning area

Waste management

Discard any swabs /tissues/ VTM/used gloves/ or any waste generated inside BSL cabinet in the small red container lined with biohazard bag placed inside

- Discard used Micropipette tips in the container provided for the purpose
- Discard the doffed PPE in autoclavable biohazard bag.
- Autoclave all the waste generated inside PCR room

Decontamination and cleaning of Reusable coverall

- Dip them in 0.1% Sodium hypochlorite for 20 min
- Flush the liquid Sodium hypochlorite
- Clean with water and detergent
- Dry in sunlight
- **Boot** to be disinfected with 0.1% sodium hypochlorite
- Then clean with water and detergent.
- Keep for drying

**Management of Waste inside the Autoclave**

- After autoclave is completed, let it cool, then take out the bag and transfer it into red container for recycling or incineration.
- Maintain the log for autoclave and its transfer outward
- Bag with non-infectious wastes (plastic and non-plastic) are discarded in the blue and black waste container
- Reusable linens (gowns and caps) will go to laundry.

**Local/institutional regulation should be followed for segregation and disposal of waste**

**Management Gloves used in areas other than BSL room**

- Discard in a separate plastic container and keep it in the designated place finally to be disposed as infectious waste

**Laundry**

- Keep all used linens (OT gowns, trousers, cap) and other linens in the double layer plastic bag with label as COVID lab
- Spray 0.1% Sodium hypochlorite outside the bags
- Transfer it to the laundry section
Appendix

Preparation of disinfectants

<table>
<thead>
<tr>
<th>Disinfectant Concentrations</th>
<th>Dilution protocol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Preparation of 1% from 4% sodium hypochlorite (for 1000ml) [Hypochlorite 1%]</td>
<td>Take 250 ml of 4% sodium hypochlorite solution to 750 ml of distilled water</td>
</tr>
<tr>
<td>Hypochlorite 0.5%</td>
<td>1Lt of 1% Sodium hypochlorite + 1Lt water</td>
</tr>
<tr>
<td>Hypochlorite 0.1%</td>
<td>100ml of 1% Sodium hypochlorite + 900 ml of water</td>
</tr>
<tr>
<td>70% Ethanol</td>
<td>70ml of 95% Ethanol and enough water to bring the volume to 100 ml</td>
</tr>
</tbody>
</table>
SOP 5: Donning and Doffing of Personal Protective Equipment (PPE)

1. Scope and application

This SOP is applicable for the health care providers (HCP) involved in direct patient care, cleaning of environment or equipment or any procedure that expose to potential contact with SARS-COV 2.

2. Storage of PPE

All PPE should be stored in a manner which ensures that it will not be exposed to dust or other contaminants before use. HCP should be aware of the location of all PPE.

3. Hand Hygiene

- Apply standard precautions when using all PPE
- Perform hand hygiene before putting on and after removing PPE.

Note: The donning and doffing process is guided and supervised by a trained observer, who confirms visually that all PPE has been donned and doffed successfully.

I. Materials required

- Soap and water
- Hand sanitizer
- Surgical scrub and dedicated washable footwear
- Gloves
- Coverall/Gown
- N95 mask
- Face shield
- Shoe cover

II. Procedure

A. Donning of PPE

1. Engage Trained Observer
2. Remove Personal Clothing Change into surgical scrubs (or disposable garments) and dedicated washable (plastic or rubber) footwear
3. Perform Hand Hygiene
4. Inspect PPE Before Donning
5. First put on shoe Covers
6. Perform hand Hygiene
7. Wear the Inner Gloves
8. Put on Gown or Coverall
9. Then wear the Outer Gloves
10. Put on N95 Respirator and do the fit checking and testing
11. Put on the Full face-shield
12. Wear the Outer Apron (if used)
13. Verify: After completing the donning process, the trained observer should verify

B. Doffing of PPE

1. Engage Trained Observer
2. Inspect the PPE to assess for visible contamination, cuts, or tears before starting to remove
3. Remove outer Gloves
4. Disinfect and Remove Outer Gloves
5. Inspect and Disinfect Inner Gloves
6. Remove Face Shield
7. Disinfect Inner Gloves
8. Remove Gown or Coverall
9. Disinfect Inner Gloves
10. Remove Boot Covers
11. Disinfect Washable Shoes
12. Disinfect Inner Gloves
13. Remove Respirator
14. Disinfect and Remove Inner Gloves
15. Perform Hand Hygiene
16. Inspect: Both the trained observer and the healthcare worker perform a final inspection.

17. Leave the PPE removal area wearing dedicated washable footwear and surgical scrubs or disposable garments, proceeding directly to showering area where these are removed.
SOP 6: Use of pooled samples (Pool Testing) for detection of SARS-CoV2 by RT-PCR

1. Scope and application

Number of COVID-19 cases in Nepal is rising. Therefore, it is very important to increase the number of tests. Positivity rate is still low meaning result of most of the PCR is negative. The technical advancement has introduced high throughput PCR so that multiple samples can be pooled and tested into one tube. Such methods should be efficient to detect the genetic materials present in the samples. These would also save the time and cost of the reagents. However, their implementation should be identified together with the prevalence of the diseases, observing positivity of PCR < 2% from the existing data or recent literature also suggests that it can also be performed in selected situation even if the PCR positivity is between 2 to 5% of the population.

This SOP is applicable for the RT–PCR testing for detection of SARS-CoV2 RNA by pooling of clinical samples i.e. nasopharyngeal and oropharyngeal swabs collected in VTM. Five VTM samples are aseptically mixed in separate tube and detection of SARS-CoV2 RNA is done by PCR complying with specific standard operating procedures.

2. Responsibilities

<table>
<thead>
<tr>
<th>Responsible personnel</th>
<th>Activities</th>
</tr>
</thead>
<tbody>
<tr>
<td>Laboratory personnel delegated by the respective lab/institution for the specific activities (in accordance to the interim guidelines by NPHL, Nepal)</td>
<td>To extract the RNA from nasopharyngeal swab in VTM by making a pool from 5 samples</td>
</tr>
<tr>
<td></td>
<td>RT-PCR</td>
</tr>
<tr>
<td></td>
<td>Technical validation, Reporting</td>
</tr>
</tbody>
</table>
3. Laboratory Workflow

3.1 Specimen Handling/Receipt

- Always use the calibrated micropipette.
- Put the label in tube with great care.
- Equilibrate all reagents at room temperature before use.
- Before using any reagents, spin down, vortex and spin down.

3.2 Safety

- Put on gloves during handling samples.
- Always clean the working surface before and after work with 1% sodium hypochlorite (wait for 3 minutes) followed by disinfecting with 70% ethanol.
- Always use the Biological Safety Cabinet Level -2 when processing the samples and PCR cabinet to prepare the reagents.
- Always done PPE (Personal Protection Equipment) before starting extraction.

3.3 Storage

- Use fresh samples in VTM for pooling.
- In case of delay, store the VTM fluid at 4-8°C for less than 72 hours
- For long term storage, preserve the VTM fluid at -80°C
- After pooling, keep each VTM at 4-8°C until the report
- In case of positive result of pool, test individually
- Store the positive samples at -80°C
- Discard the negative VTM

3.4 Materials and samples

3.4.1 Materials

A. Plastic wares
   i. Micro-centrifuge tube 1.5ml
ii. VTM with swab
iii. Micropipette tips (1000µl, 200µl, 10µl)

B. Equipment and tools
i. Balance
ii. Vortex
iii. Centrifuge
iv. Micropipette

3.5 Procedures

1. Take a 1.5ml micro centrifuge tube and stick the label (e.g. BPK/COV/PL00001) and record the sample codes for the pooling in register (e.g. BPK/COV/00001, BPK/COV/00002, BPK/COV/00003, BPK/COV/00004, BPK/COV/00005 etc.).
2. Follow the SOP for use Biosafety cabinet
3. Transfer the samples to Biosafety cabinet and transfer 30 to 60 µL (depending upon the type of extraction protocol used in the lab; as different company kit recommends different starting volume of VTM to test) of each VTM samples to the pre-labelled (with pool number) 1.5ml micro-centrifuge tube.
4. Briefly centrifuge the pooled samples and pulse vortex for 10 sec.
5. Again, centrifuge to remove the VTM at wall of tube.
6. Go for RNA extraction [Follow the RNA extraction Protocol]
7. Proceed the real time PCR protocol [Follow the PCR protocol for SARS CoV2 diagnosis]
8. If PCR result is negative for the pool, report the results as Negative for all samples individually
9. In case of Positive PCR result in a pool, repeat RNA extraction of samples individually and re-do the PCR separately to detect the PCR positive samples [See Figure 1].
40 uL* (Roche, but Qiagen use 28uL VTM each; depend on the extraction kit)

→ POOL

→ Brief Centrifuge-Vortex-Centrifuge

→ RNA extraction & PCR

branch 1: PCR negative
  → Report all samples Negative

branch 2: PCR positive
  → Re-do RNA extraction & PCR Individual and Report the Result separately

Figure 1: Pool testing results with high ct (BPK/COV/340) and low ct (BPK/COV/344) of two different samples in Pools with dilution and un-dilution of samples.
Figure 2: Amplification plot of pool samples with high ct and low ct values.

Quality control

- Extraction control is added during the RNA extraction. The addition of extraction control and its co-amplification by PCR indicate no inhibition in reaction;
- Positive and no template controls are added in each batch of PCR to confirm the validity

References


• Advisory on feasibility of using pooled samples of molecular testing of COVID 19. Indian Council of Medical Research, Dept of Health Research, 13.04.2020
SOP 7: Viral RNA Extraction from Nasopharyngeal and Throat swab using Qiagen RNA extraction Kit

1. Scope and application

This SOP is applicable for the viral RNA extraction from the naso-pharyngeal swab samples collected in Viral Transport Medium (VTM). QiaAmp Viral RNA Mini kit (Qiagen, cat #52904, #52906) protocol is used for the extraction of RNA for the real-time PCR analysis. The protocol is adapted for the diagnosis of RNA viruses (e.g. SARS CoV2).

2.1 Responsibilities

<table>
<thead>
<tr>
<th>Responsible personnel</th>
<th>Activities</th>
</tr>
</thead>
<tbody>
<tr>
<td>Laboratory personnel delegated by the respective lab/institution for the specific activities (in accordance to interim guidelines by NPHL, Nepal)</td>
<td>To extract the RNA from nasopharyngeal swab in VTM</td>
</tr>
<tr>
<td></td>
<td>Technical validation of procedure</td>
</tr>
<tr>
<td></td>
<td>Reports</td>
</tr>
</tbody>
</table>

2.2 Precautions

- Do not use the chemicals/reagents beyond expiry date.
- Label the tube with great care.
- Equilibrate all reagents at room temperature before use.
- Before using any reagents, spin down, mix and spin down.

2.3 Safety

- Avoid the reagent from direct contact with skin.
- Put on gloves during handling samples.
- Always disinfect the working surface before and after work by 1% sodium hypochlorite (wait for 3 minutes) followed by wiping with 70% ethanol.
- Always use the laminar flow for reagent preparation and biological Safety Cabinet Level -2 for processing of samples
- Work on the Negative pressure environment (if available)
- Always done PPE before starting extraction.

2.4 Storage

Always preserve the RNA at -80°C.

3. Materials and samples

3.1 Materials

i. Reagents

   i. Reagents provided in QIAamp viral RNA mini kit (250 extraction, cat nr. 52906, Qiagen)

   a. Preparation of Carrier RNA
      - Add 310 µl Buffer AVE to the tube containing 310 µg lyophilized carrier RNA to obtain a solution of 1 µg/µl.
      - Dissolve the carrier RNA thoroughly, divide 50 µl aliquots
      - Store it at -20°C.
      - Do not freeze–thaw the aliquots of carrier RNA more than 3 times.

   b. AVL lysis buffer

   c. AW1 wash buffer; delivered is concentrated, and add 130 ml of ethanol (100%) to make working solution

   d. AW2 wash buffer; delivered is concentrated, and add 160 ml of ethanol (100%) to make working solution

   e. AVE elution buffer

   ii. Reagents not provided in kit: Ethanol (100%)

   iii. Extraction control (EC): it is provided with COVID diagnosis Kit and aliquot in 130µl, store at -80°C.

C. Equipment and tools

   i. Balance

   ii. Vortex

   iii. Centrifuge
iv. Heating Block
v. Micropipette

3.2 Samples

VTM fluids with Nasopharyngeal Swab, oropharyngeal swab

4. Procedures

i. Switch on UV light in the laminar air flow for 30 minutes

ii. Switch on the flow.

iii. Take a 1.5ml sterile microfuge tube, label with pool number and extraction date.

This tube will be used to collect and store the 60 μl RNA at the end of the procedure.

iv. Identify 5 samples appropriate to form "a pool"

v. Take an aliquot of (a) Carrier RNA (b) extraction control out of -20°C and let it thaw

vi. Add 560 μl AVL buffer + 5.6 μl carrier RNA as following table into the labelled 1.5ml tube (Do not vortex, mix by inversion):

Table 1. Volumes of Buffer AVL and carrier RNA–Buffer AVE mix required for the QIAamp Viral RNA Mini procedure

<table>
<thead>
<tr>
<th>No. Samples</th>
<th>Buffer AVL (μl)</th>
<th>Carrier-RNA-AVE (μl)</th>
<th>No. Samples</th>
<th>Buffer AVL (μl)</th>
<th>Carrier-RNA-AVE (μl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.56</td>
<td>5.6</td>
<td>13</td>
<td>7.28</td>
<td>72.8</td>
</tr>
<tr>
<td>2</td>
<td>1.12</td>
<td>11.2</td>
<td>14</td>
<td>7.84</td>
<td>78.4</td>
</tr>
<tr>
<td>3</td>
<td>1.68</td>
<td>16.8</td>
<td>15</td>
<td>8.4</td>
<td>84</td>
</tr>
<tr>
<td>4</td>
<td>2.24</td>
<td>22.4</td>
<td>16</td>
<td>8.96</td>
<td>89.6</td>
</tr>
<tr>
<td>5</td>
<td>2.8</td>
<td>28</td>
<td>17</td>
<td>9.52</td>
<td>95.2</td>
</tr>
<tr>
<td>6</td>
<td>3.36</td>
<td>33.6</td>
<td>18</td>
<td>10.08</td>
<td>100.8</td>
</tr>
<tr>
<td>7</td>
<td>3.92</td>
<td>39.2</td>
<td>19</td>
<td>10.64</td>
<td>106.4</td>
</tr>
<tr>
<td>8</td>
<td>4.48</td>
<td>44.8</td>
<td>20</td>
<td>11.2</td>
<td>112</td>
</tr>
<tr>
<td>9</td>
<td>5.04</td>
<td>50.4</td>
<td>21</td>
<td>11.76</td>
<td>117.6</td>
</tr>
<tr>
<td>10</td>
<td>5.6</td>
<td>56</td>
<td>22</td>
<td>12.32</td>
<td>123.2</td>
</tr>
<tr>
<td>11</td>
<td>6.16</td>
<td>61.6</td>
<td>23</td>
<td>12.88</td>
<td>128.8</td>
</tr>
<tr>
<td>12</td>
<td>6.72</td>
<td>67.2</td>
<td>24</td>
<td>13.44</td>
<td>134.4</td>
</tr>
</tbody>
</table>

vii. In order to make 140 μl of VTM fluid in a 1.5ml sterile tubes labelled with pooled number, take out 28 μl sample from each of 5 VTM identified as a pool
viii. Repeat this transfer for other VTMs too.

**Figure-1**

<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>AVL buffer:</td>
<td>560 µl</td>
<td>560 µl</td>
<td>560 µl</td>
<td>560 µl</td>
</tr>
<tr>
<td>Carrier RNA:</td>
<td>5.6 µl</td>
<td>5.6 µl</td>
<td>5.6 µl</td>
<td>5.6 µl</td>
</tr>
<tr>
<td>VTM:</td>
<td>140 µl</td>
<td>140 µl</td>
<td>140 µl</td>
<td>140 µl</td>
</tr>
<tr>
<td>Extraction Control:</td>
<td>10 µl</td>
<td>10 µl</td>
<td>10 µl</td>
<td>10 µl</td>
</tr>
</tbody>
</table>

ix. Add 10 µl extraction control (EC, 1:100 dilution) in each tube and pulse vortex for 15 seconds.

x. Incubate at room temperature for 10 min for viral particle lysis.

xi. Spin down to remove droplets from the tube lid.

xii. Add 560 µl of ethanol (100%), pulse-vortex for 15 sec. Mix by gentle vortexing for 15 seconds.

xiii. Spin briefly to collect liquid from the lid.

xiv. Carefully apply 630 µl of the solution from step xiii to the QIAamp Mini column (in a 2 ml collection tube) without wetting the rim. Close the cap, and centrifuge at 6000 g (8000 rpm) for 1 min.

xv. Place the QIAamp Mini column into a clean collection tube, and discard the tube containing the filtrate.

xvi. Carefully open the QIAamp Mini column, and add remaining 630 µl of lysate (step xiii) to the column and centrifuge at 6000 g (8000 rpm) for 1 min.

xvii. Place the column in a new collection tube and discard the tube with filtrate.

xviii. Carefully open the QIAamp Mini column, and add 500 µl of Buffer AW1. Close the cap, and centrifuge at 6000 g (8000 rpm) for 1 min.

xix. Place the QIAamp Mini column in a clean collection tube, and discard the tube containing the filtrate.

xx. Add 500 µl of Buffer AW2. Close the cap and centrifuge at full speed (20,000 g; 14,000 rpm Maximum) for 3 min.
xxi. Place the QIAamp Mini column in a clean 1.5 ml micro-centrifuge tube (prepared in step iii). Discard the old collection tube containing the filtrate.

xxii. Add 30 μl of AVE buffer equilibrated to room temperature. Close the cap, and incubate at room temperature for 1 min. Centrifuge at 6000 x g (8000 rpm) for 1 min.

xxiii. Again, add 30 μl of AVE buffer incubate for 1 min at room temperature and centrifuge at 6000 g (8000 rpm) for 1 min.

xxiv. Finally, tube containing 60 μl of RNA must be stored as soon as possible at-80°C.

5. Quality control

Extraction control is added during the RNA extraction. The addition of extraction control and its co-amplification by PCR indicate no inhibition in reaction.

6. References


SOP 8: Viral RNA Extraction from Nasopharyngeal and Throat swab using Roche RNA extraction Kit

1. Scope and application

This SOP is applicable for the viral RNA extraction from the naso-pharyngeal swab samples collected in Viral Transport Medium (VTM). High Pure Viral RNA kit (Roche, 11 858 882 001) protocol is used for the extraction of RNA for the real-time PCR analysis. The protocol is adapted for the diagnosis of RNA viruses (e.g. SARS CoV2).

2. Responsibilities

<table>
<thead>
<tr>
<th>Responsible personnel</th>
<th>Activities</th>
</tr>
</thead>
<tbody>
<tr>
<td>Laboratory personnel delegated by the respective lab/institution for the specific activities (in accordance to guidelines by NPHL, Nepal)</td>
<td>To extract the RNA from nasopharyngeal swab in VTM</td>
</tr>
<tr>
<td></td>
<td>Technical validation of procedure</td>
</tr>
<tr>
<td></td>
<td>Reports</td>
</tr>
</tbody>
</table>

3.1 Precautions

- Do not use the chemicals/reagents beyond expiry date.
- Label in tube with great care.
- Equilibrate all reagents at room temperature before use.
- Before using any reagents, spin down, mix and spin down.

3.2 Safety

- Avoid the reagent from direct contact with skin.
- Put on gloves during handling samples.
- Always clean the working surface area before and after work by 1% sodium hypochlorite (wait for 3 minutes) followed by cleaning with 70% ethanol.
- Always use the laminar flow for the reagent preparation and Biological Safety Cabinet Level -2 for sample processing.
- Work in the Negative pressure environment (if available)
- Always done PPE before starting extraction.

3.2 Storage

Always preserve the RNA at -80°C.

3.4 Materials and samples

3.4.1 Materials

A. Reagents

i. Reagents provided in High Pure Viral RNA kit (100 extraction, cat nr. 11 858 882 001, Roche)

<table>
<thead>
<tr>
<th>Vial / Bottle</th>
<th>Cap</th>
<th>Label</th>
<th>Function / Description</th>
<th>Content</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Green</td>
<td>Binding Buffer</td>
<td>Contains 4.5 M guanidine-HCl, 50 mM Tris-HCl, 30% TritonX-100(w/v), pH 6.6 (+25°C)</td>
<td>2 x 25ml</td>
</tr>
<tr>
<td>2</td>
<td>Poly A</td>
<td>For binding of RNA</td>
<td>2mg poly(A) carrier RNA (lyophilizate)</td>
<td></td>
</tr>
<tr>
<td>3a</td>
<td>black</td>
<td>Inhibitor Removal Buffer</td>
<td>Contains 5 M guanidine-HCl, 20 mM Tris-HCl, pH 6.6 (+25°C) (final concentration after addition of ethanol)</td>
<td>33ml, add 20ml absolute ethanol</td>
</tr>
<tr>
<td>3</td>
<td>blue</td>
<td>Wash Buffer</td>
<td>20 mM NaCl, 2 mM Tris-HCl, pH 7.5 (+25°C) (final concentrations after addition of ethanol)</td>
<td>2 x 10ml, add 40ml absolute ethanol to each vial</td>
</tr>
<tr>
<td>4</td>
<td>colorless</td>
<td>Elution Buffer</td>
<td>Water, PCR grade</td>
<td>30ml</td>
</tr>
<tr>
<td>5</td>
<td>High Pure Filter Tubes</td>
<td>For use of up to 700µl sample volume.</td>
<td>2 bags with 50 polypropylene tubes with two layers of glass fiber fleece</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Collection Tubes</td>
<td></td>
<td>8 bags with 50 polypropylene tubes (2 ml).</td>
<td></td>
</tr>
</tbody>
</table>
## Working Solution

<table>
<thead>
<tr>
<th>Content</th>
<th>Reconstitution/Preparation</th>
<th>Storage and Stability</th>
<th>For use in...</th>
</tr>
</thead>
<tbody>
<tr>
<td>Poly (A) carrier (Vial 2)</td>
<td>Dissolve poly(A) carrier RNA (vial 2) in 0.4ml Elution Buffer (vial 4). Prepare aliquots of 50 μl for running 8 x 12 purifications. Prepare aliquots of 100 μl for running 4 x 25 purifications. For 12 purifications, thaw one vial with 50 μl poly(A) carrier RNA and mix thoroughly with 5ml Binding Buffer (vial 1). For 25 purifications, thaw one vial of 100 μl poly(A) carrier RNA and mix thoroughly with 10 ml Binding Buffer (vial 1). <strong>Prepare always fresh before use! Do not store!</strong></td>
<td>• Store at -15°C to -25°C. • Stable for 12 months</td>
<td>For the preparation of working solution Protocol Step 1</td>
</tr>
<tr>
<td>Inhibitor Removal Buffer (Vial 3a; black cap)</td>
<td>Add 20 ml absolute ethanol to Inhibitor Removal Buffer. <strong>Label and date bottle accordingly after adding ethanol.</strong></td>
<td>• Store at +15°C to +25°C. • Stable through the expiration date printed on kit label.</td>
<td>Protocol Step 5: To remove PCR inhibitors Protocol Step 6 and 7: Removal of residual impurities</td>
</tr>
<tr>
<td>Wash Buffer (Vial 3; blue cap)</td>
<td>Add 40ml absolute ethanol to Wash Buffer. <strong>Label and date bottle accordingly after adding ethanol.</strong></td>
<td>• Store at +15°C to +25°C. • Stable through expiration Date printed on kit label.</td>
<td>Protocol Step 6 and 7: Removal of residual impurities</td>
</tr>
</tbody>
</table>

**ii. Reagents not provided in the extraction kit**

a. Ethanol (100%)

b. Extraction control (EC): it is provided with COVID diagnosis Kit and aliquot in 130μl, store at -80°C.

**B. Equipment and tools**

a. Balance

b. Vortex

c. Centrifuge

d. Heating Block

e. Micropipette

**3.4.2 Samples**

- VTM fluids with Nasopharyngeal Swab, oropharyngeal swab
4. Procedures

i. Switch on UV light in the laminar air flow for 30 minutes

ii. Switch on the flow.

iii. Take a 1.5ml sterile microfuge tube, label with pool number and extraction date. This tube will be used to collect and store the 60 µl RNA at the end of the procedure.

iv. Identify 5 samples appropriate to form “a pool”

v. Identify 5 samples to form “each pool”

vi. Take an aliquot of (a) Carrier RNA and (b) Extraction Control (working dilution 1/100) out of the -20°C and let it thaw to room temperature.

vii. Prepare the working solution with carrier RNA as per the table 1. Adapt the table 1 to Prepare the solution as the number of pools which is going to be tested in the lot. For example, for 1 pool, make the 400 µl working solution + 4 µl carrier RNA as following table (Do not vortex, mix by inversion):

<table>
<thead>
<tr>
<th>No. Samples</th>
<th>Working solution (ml)</th>
<th>Carrier-RNA (µl)</th>
<th>No. Samples</th>
<th>Working solution (ml)</th>
<th>Carrier-RNA (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.4</td>
<td>4</td>
<td>14</td>
<td>5.6</td>
<td>56</td>
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<tr>
<td>2</td>
<td>0.8</td>
<td>8</td>
<td>15</td>
<td>6.0</td>
<td>60</td>
</tr>
<tr>
<td>3</td>
<td>1.2</td>
<td>12</td>
<td>16</td>
<td>6.4</td>
<td>64</td>
</tr>
<tr>
<td>4</td>
<td>1.6</td>
<td>16</td>
<td>17</td>
<td>6.8</td>
<td>68</td>
</tr>
<tr>
<td>5</td>
<td>2.0</td>
<td>20</td>
<td>18</td>
<td>7.2</td>
<td>72</td>
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<td>6</td>
<td>2.4</td>
<td>24</td>
<td>19</td>
<td>7.6</td>
<td>76</td>
</tr>
<tr>
<td>7</td>
<td>2.8</td>
<td>28</td>
<td>20</td>
<td>8.0</td>
<td>80</td>
</tr>
<tr>
<td>8</td>
<td>3.2</td>
<td>32</td>
<td>21</td>
<td>8.4</td>
<td>84</td>
</tr>
<tr>
<td>9</td>
<td>3.6</td>
<td>36</td>
<td>22</td>
<td>8.8</td>
<td>88</td>
</tr>
<tr>
<td>10</td>
<td>4.0</td>
<td>40</td>
<td>23</td>
<td>9.2</td>
<td>92</td>
</tr>
<tr>
<td>11</td>
<td>4.4</td>
<td>44</td>
<td>24</td>
<td>9.6</td>
<td>96</td>
</tr>
<tr>
<td>12</td>
<td>4.8</td>
<td>48</td>
<td>25</td>
<td>10.0</td>
<td>100</td>
</tr>
<tr>
<td>13</td>
<td>5.2</td>
<td>52</td>
<td>26</td>
<td>10.4</td>
<td>104</td>
</tr>
</tbody>
</table>

xxv. In order to make 200 µl of VTM fluid in a 1.5ml sterile tubes labelled with pooled number, take out 40 µl sample from each of 5 VTM identified as a pool.
xxvi. Repeat this transfer for other VTMs too.

viii. Label all the tubes clearly.

ix. Add 400 μl Working solution (Carrier RNA supplemented Binding buffer), see also figure 1 into the tube containing 200 μl of VTM fluid.

x. Add 10 μl extraction control (EC, 1:100 dilution) in each tube and pulse vortex for 15 sec.

xi. Incubate at room temperature for 5 min for viral particle lysis.

xii. Spin down to remove droplets from the tube lid.

xiii. Insert one High Pure filter tube in one collection tube.

xiv. Transfer 610 μl of the solution from step xii to the filter tube. Close the cap, and centrifuge at 8,000 g (9,500 rpm) for 15 sec.

xv. Place the High Pure Filter tube into a clean collection tube, and discard the tube containing the filtrate.

xvi. Carefully open the High Pure Filter tube and add 500 μl of Inhibitor Removal Buffer. Close the cap, and centrifuge at 8,000 g (9,500 rpm) for 1 min.

xvii. Place the High Pure Filter tube in a clean collection tube, and discard the tube containing the filtrate.

xviii. Add 450 μl of Inhibitor Removal Buffer. Close the cap and centrifuge at 8,000 g; (9,500 rpm) for 1 min.

xix. Place the High Pure Filter tube in a clean collection tube, and discard the tube containing the filtrate.

xx. Add 450 μl Wash Buffer to the upper reservoir of the filter tube.

xxi. Centrifuge 8,000 g or 9,500 rpm for 1 min.

xxii. Leave the filter tube-collection tube assembly in the centrifuge and spin it for 10 sec at maximum speed at 13,000 g to remove any residual Wash Buffer.

xxiii. Place the High Pure Filter tube in a clean 1.5 ml micro-centrifuge tube (prepared in step iii). Discard the old collection tube containing the filtrate.
xxiv. Add 30 μl of Elution buffer equilibrated to room temperature. Close the cap, and incubate at room temperature for 1 min. Centrifuge at 8,000 x g (9,500 rpm) for 1 min.

xxv. Again, add 30 μl of Elution buffer, incubate for 1 min at room temperature and centrifuge at 8,000 g (9,500 rpm) for 1 min.

xxvi. Finally, tube containing 60 μl of RNA and must be stored as soon as possible at -80°C.

5. Quality control

Extraction control is added during the RNA extraction. The addition of extraction control and its co-amplification by PCR indicate no inhibition in reaction.

6. References


SOP 9: Diagnosis of SARS CoV2 (COVID) using E-gene (screening) specific real time PCR (TIB-MOLBIOL)

1. Scope and application

The purpose of this standard operating procedure is to describe a method for detection of E gene amplification of SARS-CoV2 virus in RNA extract from nasopharyngeal and oropharyngeal swabs by polymerase chain reaction (PCR). This SOP is applicable for screening of SARS-CoV2 virus by using the Rotor Gene Q (RGQ) machine and primer/probes from TIB-MOLBIOL, Catalogue number 400776-96 (Annex).

2. Responsibilities

<table>
<thead>
<tr>
<th>Responsible personnel</th>
<th>Activities</th>
</tr>
</thead>
<tbody>
<tr>
<td>Laboratory personnel delegated by the respective lab/institution for the specific activities (in accordance to guidelines by NPHL, Nepal)</td>
<td>To perform the real time PCR Report and technical validation of PCR</td>
</tr>
</tbody>
</table>

3. Procedures

3.1 Precautions

- Do not lift up the RGQ lid when in operation.
- Probes are highly light sensitive and always cover them aluminum foil.

3.2 Safety

- Put on nitrile gloves during handling reagents.
- Always wipe the working surface with 1% Sodium hypochlorite followed by 70% Ethanol, before and after work.
- Done on PPE before loading the RNA template.
3.3 Materials
   A. Reagents
      i. Supplied with TIB-MOLBIOL [cat nr. 40-0776-96]

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Description</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Reagent primer, probe (lyophilized)</td>
<td>Yellow cap</td>
<td>1 tube</td>
</tr>
<tr>
<td>Positive control COVID-19 E gene (lyophilized)</td>
<td>Black cap</td>
<td>1 tube</td>
</tr>
<tr>
<td>Extraction control (lyophilized)</td>
<td>White cap</td>
<td>1 tube</td>
</tr>
</tbody>
</table>

a) Preparation of Primer-probe
   - Briefly centrifuge (8000 rpm for 1 minute) the yellow cap containing primer probe tube
   - Add 50 μl of PCR grade water
   - Leave it for 5 minutes at room temperature
   - Gently vortex
   - Spin down
   - Make aliquot of 10 μl in different tubes
   - Store at -20°C.

b) Preparation of Positive control
   - Briefly centrifuge (8000 rpm for 1 minute) the black cap containing positive control tube
   - Add 160 μl of RNase/DNase free 10mM Tris buffer, pH 8
   - Mix by pipetting up and down 10 times.
   - Make aliquot of 20 μl in different tubes
   - Store at -20°C.

c) Preparation of Extraction control
   - Briefly centrifuge (8000 rpm for 1 minute) the white cap containing extraction control tube
   - Add 1,200 μl of RNase/DNase free 10mM Tris buffer, pH 8
   - Mix by pipetting up and down 10 times
- Make aliquot of 250 μl in different tubes
- Store at -20°C.

ii. Reagents required but not supplied with kit
   a. Nucleic extraction kit
   b. RT-Buffer
   c. RT-PCR Enzyme

B. Equipment and tools
   i. Centrifuge
   ii. Micropipette (1000 μL, 200 μL, 10 μL)
   iii. Rotor Gene Q machine (Qiagen)

C. Plastic wares
   a. PCR tubes, flat cap 0.2 ml
   b. Tube racks for 0.2ml and 1.5ml tubes.
   c. 1.5 ml micro centrifuge tube
   d. Filter tips (1000 μL, 200 μL, 10 μL)

4. Procedures

A. RNA extraction from VTM swab SOP number 7 or 8

B. Pre-PCR Work

3. Prepare the paper worksheet file for the real time PCR containing detail information about PCR ID, date of experiment, list of samples and master mix table
4. For calculation of the master mix, always include 10% extra reactions to take account of pipetting errors
5. Prepare the master mix table
6. Switch on the UV light of PCR cabinet for 10 minute
7. Switch on the flow of PCR cabinet
8. Disinfect the PCR cabinet, racks and pipettes with DNA exit plus (or1% Sodium hypochlorite solution)
9. Wipe the same surface and items with 70% Ethanol
10. Take out the master mix reagents from the freezer (-20°C). Take tubes that have already been used first (marked with “•”), and take an additional tube if not enough for the experiment.

11. Bring all the reagents at room temperature for complete thawing

12. Take out 0.1ml/0.2ml PCR tubes (number of tubes depend on the samples to be tested + Positive control + No template control) with the help of sterile forceps from the pack of the tubes and keep them in cooling rack.

13. Label all the tubes with sample ID, positive control (P) and No template control (N)

14. Take a 1.5ml PCR grade micro centrifuge tube and label it "MM" on top

15. Note batch number/lot number of each reagent in paper worksheet

16. Mix thawed reagent thoroughly by pipetting up and down followed by short spin down

17. Add the reagents of the master mix in the order indicated in the worksheet as per the table below

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>μl per reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water (PCR grade)</td>
<td></td>
</tr>
<tr>
<td>Reagent mix (primer, probe)</td>
<td></td>
</tr>
<tr>
<td>2X RT-PCR Buffer</td>
<td></td>
</tr>
<tr>
<td>25X-RT-PCR Enzyme Mix</td>
<td></td>
</tr>
<tr>
<td>RNA Template</td>
<td></td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>20</strong></td>
</tr>
</tbody>
</table>

18. Mix and spin down

19. Put all reagents from the mastermix back at -20°C as soon as possible. If you use any new stock reagent, mark it on the lid with “•”.

20. Aliquot the required volume of mastermix to each reaction tube and include positive and no template control

21. Add PCR water in No Template Control at PRE-PCR.
22. Transfer the tubes with master mix in cooling rack to template addition room (BSL II)

23. Disinfect the working surface of BSL II cabinet and pipettes with RNase Zap (or 1% sodium hypochlorite). Then, wipe with 70% ethanol.

24. Add template (RNA extract) to the master-mix.

25. Add positive control to the tube labelled with "P" in a separate room.

26. Spin down all the tubes.

27. Disinfect the pipette and work surface with 1% sodium hypochlorite solution and 70% ethanol wipe.

28. Store the RNA at -80°C.

C. Cycler room procedures

33. Take the worksheet and PCR tubes to the cycler room.

34. Insert the tubes into the 36/72 well rotor, put Positive control in position 1 and No Template control (NTC) in position 2, rest of samples in respective wells using the stand.

35. Attach the locking ring.
36. Check tube volumes. If not equal, note this on the worksheet.
37. Insert the rotor in the RotorGeneQ.
38. Close the lid.

39. Doff PPE
40. Discard as infectious waste.
41. Turn on the RotorGeneQ.
42. Turn on the PC and connect it to the RotorGeneQ using the COM serial port.
43. Check the OTV calibration date. If performed less than 6 months ago, circle ‘OK’ on the work sheet. If longer ago, circle ‘NOT OK’.
44. Start the software by double click “Rotor-Gene Q Series Software” at desktop.
45. Check the thermocyclic condition
46. Follow the wizard to start the run.

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature °C</th>
<th>Time</th>
<th>Cycler repeats</th>
<th>Fluorescence detection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hold</td>
<td>55 5</td>
<td>5 min</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>95 9</td>
<td>5 min</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Cycling</td>
<td>95 9</td>
<td>10 sec</td>
<td>45</td>
<td>FAM/Green, Cy5/Red</td>
</tr>
<tr>
<td></td>
<td>58 5</td>
<td>30 sec</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

47. Edit the sample list from the main menu on the right, and save.
48. After run is completed, save it again.
49. Close the RotorGene program.
50. Put on gloves.
51. Check the volumes of the tubes. Note on the sample sheet which volumes deviated from the rest. If the volume from a particular test tube deviates, interpret its result with caution! It is best to repeat the respective sample when negative.

52. Discard the samples, close and turn off the Rotor Gene Q.

E. Post-run procedure

52. Transfer the "rex" file from the RGQ computer to the analysis computer.

53. Double click the file to open the rex file.

54. Click the analysis tab a to show Cycling A Green in menu.

55. Select the linear scale, choose threshold and observe the Ct values.
56. Place the computer cursor over the graph and right click, new window appear.

57. Click on “Edit Chart in TeeChart office” and new window appear.
58. Enlarge the picture by dragging bottom right corner of the picture.

59. Click view in menu bar and select “Legend”.

60. Right click to the Legend appear in screen and select bottom. Now Legend will appear at bottom of picture.

61. Double click in Sample label 1, new window appear “Editing DBChart1”.
62. Now click “Point” and go to Style, click drop down button and select the square. The line in graph will appear with square annotation.

63. Again go to second sample and select circle, the line will appear with circle annotation, and do the same for required line to be visualized.

64. Then click “Close”.

65. Go to “File” in menu bar, and select “Export” and click on as JPEG, give the EXPT number and the Channel name (Green, Yellow...)

66. Close the TeeChart Office and go to RGQ window.

67. Place the cursor in window “Quant. Results” then right click:
68. Click on "Export to Excel", save in the folder called "Ct", the excel file will contain the Ct values of the respective samples.

69. Store in the worksheet folder, always save experiment number and receiving channel.

70. This is Multiplex PCR, so we need to do same by clicking "Analysis" button and select the channel from RED channels.

71. The threshold cutoff values for the each channels are fixed as following table:

72. Copy all the content from the Excel file (GREEN and RED filter) and paste in the INPUT sheet of Report EXCEL file prepared for each experiment!!! Careful with the sample name each time!!!
4.1 Interpretation of result

First check the amplification of positive control and negative control, if they are valid then proceed for amplification of samples.

<table>
<thead>
<tr>
<th>GREEN Channel 530 Sample</th>
<th>Red Channel 660 Control Reaction</th>
<th>GREEN Channel 530 NTC control</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>No amplification</td>
<td>Detectable</td>
<td>Negative</td>
<td>Not detectable</td>
</tr>
<tr>
<td>Amplification Cp &lt;36</td>
<td>Not relevant</td>
<td>Negative</td>
<td>SARbecoV Positive</td>
</tr>
<tr>
<td>No amplification</td>
<td>Not detectable</td>
<td>Not relevant</td>
<td>PCR failure Repeat</td>
</tr>
<tr>
<td>Amplification signal</td>
<td>Not relevant</td>
<td>Positive</td>
<td>Contamination Repeat</td>
</tr>
</tbody>
</table>

4.2 Quality control

Each batch of PCR should contain positive and no template in each PCR run.

5. References:

3.3 TIB-MOLBIOL, Germany handbook. Synthese labor GmbH, Eresburgstr. 22-23, D-12103 Berlin, Germany.

SOP 10: Diagnosis of SARS-Cov2 (COVID) using RdRP (Wuhan specific) specific real time PCR (TIB-MOLBIOL)

1. Scope and application

The purpose of this standard operating procedure is to describe method for detection of RdRP (wuhan specific) gene amplification of SARS-CoV2 virus in RNA extract from nasopharyngeal and oropharyngeal swabs by polymerase chain reaction (PCR). This SOP is applicable for screening of SARS-CoV2 virus by using the Rotor Gene Q (RGQ) machine and primer/probes from TIB-MOLBIOL, Catalogue number 53-0777-96 (Annex).

2. Responsibilities

<table>
<thead>
<tr>
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<th>Activities</th>
</tr>
</thead>
<tbody>
<tr>
<td>Laboratory personnel delegated by the respective lab/institution for the specific activities (in accordance to guidelines by NPHL, Nepal)</td>
<td>To perform the real time PCR Report and technical validation of PCR</td>
</tr>
</tbody>
</table>

3.1 Precautions

- Do not lift up the RGQ lid when in operation.
- Probes are highly light sensitive and always cover them aluminum foil.

3.2 Safety

- Put on nitrile gloves during handling reagents.
- Always wipe the working surface with 1% Sodium hypochlorite followed by 70% Ethanol, before and after work.
- Done on PPE before loading the RNA template.

3.3 Materials

A. Reagents
   i. Supplied with TIB-MOLBIOL [cat nr. 40-0776-96]
Table 1 Reagents supplied with TIB-MOLBIOL Kit

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Description</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Reagent primer, probe (lyophilized)</td>
<td>Yellow cap</td>
<td>1 tube</td>
</tr>
<tr>
<td>Positive control COVID-19 E gene (lyophilized)</td>
<td>Black cap</td>
<td>1 tube</td>
</tr>
</tbody>
</table>

a) Preparation of Primer-probe

- Briefly centrifuge (8000 rpm for 1 minute) the yellow cap containing primer probe tube
- Add 50 µl of PCR grade water
- Leave it for 5 minutes at room temperature
- Gently vortex
- Spin down
- Make aliquot of 10 µl in different tubes
- Store at -20°C.

b) Preparation of Positive control

- Briefly centrifuge (8000 rpm for 1 minute) the black cap containing positive control tube
- Add 160 µl of RNase/DNase free 10mM Tris buffer, pH 8
- Mix by pipetting up and down 10 times.
- Make aliquot of 20 µl in different tubes
- Store at -20°C.

ii. Reagents required but not supplied with kit

   a. Nucleic extraction kit
   b. RT-Buffer
   c. RT-PCR Enzyme

B. Equipment and tools

   i. Centrifuge
   ii. Micropipette (1000 µL, 200 µL, 10 µL)
iii. Rotor Gene Q machine (Qiagen)

C. Plastic wares
   a. PCR tubes, flat cap 0.2 ml
   b. Tube racks for 0.2ml and 1.5ml tubes.
   c. 1.5 ml micro centrifuge tube
   d. Filter tips (1000 µL, 200 µL, 10 µL)

4. Procedures
A. RNA extraction from VTM swab SOP number 7 or 8
B. Pre-PCR Work
   1. Prepare the paper worksheet file for the real time PCR containing detail information about PCR ID, date of experiment, list of samples and master mix table
   2. For calculation of the master mix, always include 10% extra reactions to take account of pipetting errors
   3. Prepare the master mix table
   4. Switch on the UV light of PCR cabinet for 10 minute
   5. Switch on the flow of PCR cabinet
   6. Disinfect the PCR cabinet, racks and pipettes with DNA exit plus (or 1% Sodium hypochlorite solution)
   7. Wipe the same surface and items with 70% Ethanol
   8. Take out the master mix reagents from the freezer (-20°C). Take tubes that have already been used first (marked with “X”), and take an additional tube if not enough for the experiment.
   9. Bring all the reagents at room temperature for complete thawing
   10. Take out 0.1ml/0.2ml PCR tubes (number of tubes depend on the samples to be tested + Positive control + No template control) with the help of sterile forceps from the pack of the tubes and keep them in cooling rack.
   11. Label all the tubes with sample ID, positive control (P) and No template control (N)
   12. Take a 1.5ml PCR grade micro centrifuge tube and label it "MM" on top
   13. Note batch number/lot number of each reagent in paper worksheet
14. Mix thawed reagent thoroughly by pipetting up and down followed by short spin down.

15. Add the reagents of the master mix in the order indicated in the worksheet as per the table below.

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>µL per reaction</th>
</tr>
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<tbody>
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</tr>
<tr>
<td>Total</td>
<td>20</td>
</tr>
</tbody>
</table>

16. Mix and spin down.

17. Put all reagents from the mastermix back at -20°C as soon as possible. If you use any new stock reagent, mark it on the lid with “•”.

18. Aliquot the required volume of mastermix to each reaction tube and include positive and no template control.

19. Add PCR water in No Template Control at PRE-PCR.

20. Transfer the tubes with master mix in cooling rack to template addition room (BSL II).

21. Disinfect the working surface of BSL II cabinet and pipettes with RNase Zap (or 1% sodium hypochlorite). Then, wipe with 70% ethanol.

22. Add template (RNA extract) to the master-mix.

23. Add positive control to the tube labelled with "P" in a separate room.

24. Spin down all the tubes.

25. Disinfect the pipette and work surface with 1% sodium hypochlorite solution and 70% ethanol wipe.

26. Store the RNA at -80°C.

C. Cycler room procedures
33. Take the worksheet and PCR tubes to the cycler room.
34. Insert the tubes into the 36/72 well rotor, put Positive control in position 1 and No Template control (NTC) in position 2, rest of samples in respective wells using the stand.

35. Attach the locking ring.

36. Check tube volumes. If not equal, note this on the worksheet.
37. Insert the rotor in the RotorGeneQ.
38. Close the lid.
39. Doff PPE
40. Discard as infectious waste.
41. Turn on the RotorGeneQ.
42. Turn on the PC and connect it to the RotorGeneQ using the COM serial port.
43. Check the OTV calibration date. If performed less than 6 months ago, circle ‘OK’ on the work sheet. If longer ago, circle ‘NOT OK’.
44. Start the software by double click “Rotor-Gene Q Series Software” at desktop.
45. Check the thermocyclic condition
46. Follow the wizard to start the run.

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature °C</th>
<th>Time</th>
<th>Cycler repeats</th>
<th>Fluorescence detection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hold</td>
<td>55</td>
<td>5 min</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>95</td>
<td>5 min</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Cycling</td>
<td>95</td>
<td>10 sec</td>
<td>45</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>58</td>
<td>30 sec</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

47. Edit the sample list from the main menu on the right, and save.
48. After run is completed, save it again.
49. Close the RotorGene program.
50. Put on gloves.
51. Check the volumes of the tubes. Note on the sample sheet which volumes deviated from the rest. If the volume from a particular test tube deviates, interpret its result with caution! It is best to repeat the respective sample when negative.
52. Discard the samples, close and turn off the Rotor Gene Q.

F. Post-run procedure

73. Transfer the "rex" file from the RGQ computer to the analysis computer.
74. Double click the file to open the rex file.
75. Click the analysis tab a to show Cycling A Green in menu.

76. Select the linear scale, choose threshold and observe the Ct values.
77. Place the computer cursor over the graph and right click, new window appear.

78. Click on “Edit Chart in TeeChart office” and new window appear.

79. Enlarge the picture by dragging bottom right corner of the picture.

80. Click view in menu bar and select “Legend”.

81. Right click to the Legend appear in screen and select bottom. Now Legend will appear at bottom of picture.
82. Double click in Sample label 1, new window appear “Editing DBChart1”.

83. Now click “Point” and go to Style, click drop down button and select the square. The line in graph will appear with square annotation.

84. Again go to second sample and select circle, the line will appear with circle annotation, and do the same for required line to be visualized.

85. Then click “Close”.

86. Go to “File” in menu bar, and select “Export” and click on as JPEG, give the EXPT number and the Channel name (Green, Yellow...)
87. Close the TeeChart Office and go to RGQ window.

88. Place the cursor in window “Quant. Results” then right click:

89. Click on “Export to Excel”, save in the folder called “Ct”, the excel file will contain the Ct values of the respective samples.
90. Store in the worksheet folder, always save experiment number and receiving channel.

91. This is Multiplex PCR, so we need to do same by clicking "Analysis" button and select the channel from RED channels.

92. The threshold cutoff values for the each channels are fixed as following table:

93. Copy all the content from the Excel file (GREEN and RED filter) and paste in the INPUT sheet of Report EXCEL file prepared for each experiment!!! Careful with the sample name each time!!!

4.1 Interpretation of result

First check the amplification of positive control and negative control, if they are valid then proceed for amplification of samples.

Table 2 Interpretation of Result

<table>
<thead>
<tr>
<th>GREEN Channel530 Sample</th>
<th>Red Channel 660 Control Reaction</th>
<th>GREEN Channel530 NTC control</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>No amplification</td>
<td>Detectable</td>
<td>Negative</td>
<td>Not detectable</td>
</tr>
<tr>
<td>Amplification Cp &lt;39</td>
<td>Not relevant</td>
<td>Negative</td>
<td>WH-CoV Positive</td>
</tr>
<tr>
<td>No amplification</td>
<td>Not detectable</td>
<td>Not relevant</td>
<td>PCR failure Repeat</td>
</tr>
<tr>
<td>Amplification signal</td>
<td>Not relevant</td>
<td>Positive</td>
<td>Contamination Repeat</td>
</tr>
</tbody>
</table>

4.2 Quality control

Each batch of PCR should contain positive and no template in each PCR run.

5. References:


SOP 11: Testing of human RNase P gene in swab specimen

1. Scope and application

The purpose of this standard operating procedure is to describe method for detection of human housekeeping RNase P gene in RNA extract from nasopharyngeal and oropharyngeal swabs by polymerase chain reaction (PCR). This SOP will be applied to check the quality of the sample by real time PCR.

2. Responsibilities

<table>
<thead>
<tr>
<th>Responsible personnel</th>
<th>Activities</th>
</tr>
</thead>
<tbody>
<tr>
<td>Laboratory personnel delegated by the respective lab/institution for the specific activities (in accordance to guidelines by NPHL, Nepal)</td>
<td>To perform the real time PCR</td>
</tr>
<tr>
<td></td>
<td>Report and technical validation of PCR</td>
</tr>
</tbody>
</table>

3.1 Precautions

- Do not lift up the RGQ lid when in operation.
- Probes are highly light sensitive and always cover them aluminum foil.

3.2 Safety

- Put on nitrile gloves during handling reagents.
  - Always wipe the working surface with 1% Sodium hypochlorite followed by 70% Ethanol, before and after work.
- Done on PPE before loading the RNA template.

3.3 Materials

  A. Reagents
a) Primer-probe

<table>
<thead>
<tr>
<th>Primer/probe (Concentration)</th>
<th>Sequence (5' to 3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 RNaseP F (10μm)</td>
<td>AGATTGGGACCTGCGAGCG</td>
</tr>
<tr>
<td>2 RNaseP R (10μm)</td>
<td>GAGCGGCTGTCCAAAGT</td>
</tr>
<tr>
<td>3 RNaseP P (5μm)</td>
<td>TTCTGACCTGAGGCTCTGC</td>
</tr>
</tbody>
</table>

Aliquot 50 μl and store at -20°C.

i. Reagents required but not supplied with kit
   a. Nucleic extraction kit
   b. One step RT-PCR mastermix

B. Equipment and tools
   i. Centrifuge
   ii. Micropipette (1000 μL, 200 μL, 10 μL )
   iii. Rotor Gene Q machine (Qiagen)

C. Plasticwares
   a. PCR tubes, flat cap 0.2 ml
   b. Tube racks for 0.2ml and 1.5ml tubes.
   c. 1.5 ml centrifuge tube
   d. epi-dual filter tips (1000 μL, 200 μL, 10 μL)

4. Procedures
   A. RNA extraction from VTM swab (SOP 7 and 8)
   B. Pre PCR, cycle run procedure and Post run procedures are the same indicated in SOP 9 and 10 except the cycling parameters.
   C. Prepare the master mix as per the table (volume can be adjusted as per leaflet/protocol )
Table 1 Mastermix preparation for the PCR

<table>
<thead>
<tr>
<th>RXN components</th>
<th>Volumes/ tube (μl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water (pcr grade)</td>
<td></td>
</tr>
<tr>
<td>RNaseP F (10 μm)</td>
<td></td>
</tr>
<tr>
<td>RNaseP R (10 μm)</td>
<td></td>
</tr>
<tr>
<td>RNaseP P (5 μm)</td>
<td></td>
</tr>
<tr>
<td>RT PCR MIX</td>
<td></td>
</tr>
<tr>
<td>Reverse Transcriptase</td>
<td></td>
</tr>
<tr>
<td>RNA Template</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>20</td>
</tr>
</tbody>
</table>

Thermocycling condition

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature °C</th>
<th>Time</th>
<th>Cycler repeats</th>
<th>Fluorescence detection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hold</td>
<td>50</td>
<td>10 min</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>95</td>
<td>2 min</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td>Cycling</td>
<td>95</td>
<td>15 sec</td>
<td>50</td>
<td>FAM/Green</td>
</tr>
<tr>
<td></td>
<td>58</td>
<td>30 sec</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

4.1 Interpretation of result

First check the amplification of positive control and negative control, if they are valid then proceed for amplification of samples.

4.2 Quality control

Each batch of PCR should contain positive and no template in each PCR run.

5. References:


2. CDC protocol of real-time RTPCR for influenza A(H1N1), 28 April 2009.
SOP 12: SARS CoV2 RT-PCR Reporting and Data management

1. Scope and application

This SOP is applicable for the reporting the real-time PCR result of Nasopharyngeal and Throat swab. This includes the PCR results entry and reporting format in MS-Access database. As the method of data entry may differ in these three laboratories. Each laboratory is requested to adapt this SOP as applicable to their settings.

2. Responsibilities

<table>
<thead>
<tr>
<th>Responsible Personnel</th>
<th>Activities</th>
</tr>
</thead>
<tbody>
<tr>
<td>Laboratory personnel delegated by the respective lab/institution for the specific</td>
<td>Laboratory work for PCR analysis</td>
</tr>
<tr>
<td>activities (in accordance to interim guidelines by NPHL, Nepal)</td>
<td>Technical validation of procedure</td>
</tr>
<tr>
<td></td>
<td>Reports</td>
</tr>
<tr>
<td></td>
<td>Maintenance of records</td>
</tr>
</tbody>
</table>

3.1 Precautions

- Carefully check the label of sample before entering into the database.

3.2 Safety

- Not applicable.

3.3 MS Access Database: Prepare your own database to include all the information indicated in the sample collection form designed by EDCD/NPHL/PPHL/BPKIHS

3.3.1 Laboratory analysis

Labelling of samples,
RNA Extraction,
RT-PCR analysis,
3.3.2 Database (example from BPKIHS database)

i. Open MS-Access database "COVID-19_BPKIHS_Database.accdb".

ii. Double click the table "COVID_BPKIHS" in the navigation window at left side:

![Snapshot of MS Access database. Navigation panel at left side corner of the database.](image)

3.4 Record entry

i. First enter the demographic information filled in request form with sample ID e.g. "BPK/COV/001".

ii. In field "Status of Lab analysis", three status could be chosen: (a) Progressing= demographic filled but PCR is not done, (b) Completed= PCR done and report is final, (c) Pending= PCR done but need to repeat again for further confirmation, either IC not amplified or suspicious positive results. Select the PCR status of each sample.

iii. Write the date of PCR performed for each sample in field "Date of SARS CoV2 PCR".
iv. Fill the PCR result in field "RESULT SARS CoV2 Result" either "Positive" or "Negative".

v. Enter the reporting date when the pcr result is final in field "Date of Reporting" in bikram sambat e.g. "2077/01/05" and also the time in field "Time of Reporting" e.g. 16:00 hours.

vi. Save the database and close the table "COVID_BPKIHS".

vii. Double Click "COVID_BPKIHS Query" in navigation panel and insert the required date of PCR in criteria "2077/01/05" and also select Status of Lab analysis... as "Completed".

viii. Click "Run", save and close the table.

ix. Open "Date wise PCR REPORT" in navigation panel and go to file → print → PDF printer.

x. Select the Download folder for PDF document.

xi. Check the content of sample number, sample ID.

xii. Upload the database every day in google drive of "mlab.bpkiihs@gmail.com", in folder name "Research Project/COVID-19/Database".

Prepare the distinct mechanism for dissemination of report to concerned authority. Preferably, dispatch the report by single portal. Maintain the confidentiality. Maintain the database securely with reliable backup

5. References

- Microsoft Access 2013. URL: products.office.com/microsoft-office-2013
SOP 13: Quality Assurance (QA)/Quality control (QC) SARS CoV2 Real-Time PCR (RT-PCR) testing

1. Purpose and scope

This document is prepared for Quality Assurance/Quality Control practices for sample collection, sample management, RNA extraction, PCR testing, reporting of results and data management for COVID-19 RT PCR tests at the following laboratories of Province 1, Nepal
1. Department of Microbiology, BPKIHS, Dharan
2. Koshi Zonal Hospital, Biratnagar, Biratnagar
3. Province Public Health Laboratory, Province 1, Biratnagar

2. Laboratory quality assurance

This section provides guidance on various aspects of PCR testing for SARS-CoV2 divided as:
1. Pre-analytical phase
2. Analytical phase
3. Post-analytical phase

2.1 Pre-analytical phase

- Laboratory should have sufficient staff designated to work in COVID 19 laboratory as per the interim guideline by NPHL, Nepal.
- In charge of the laboratory must assure that:
  - Each staff is well oriented in respective activities they are delegated for, including infection prevention and control and practice of safety measures.
  - Lab has delegation log of each staff with clear mention of alternative provision in case of leave/absence of concerned staff.
  - All activities such as analytical procedures, verification of the results, infection control measures and waste disposal are being followed as per respective SOPs.
  - Results are disseminated, recorded and archived in adherence to SOP.
Attire
- Dedicated laboratory coats and powder-free Nitrile gloves for each personnel
- Personnel protective equipment (PPE): Surgical mask, N95 masks, coverall, shoe cover, head cover, face shield, goggles, boot, linen gowns appropriate for activities
- Appropriateness of its use (number and type) depending upon the exposure risk. Disposable PPE to be autoclaved and sent for final disposal.
- Reusable items to be reused after disinfection, cleaning and washed in laundry depending upon the nature of items and recommended procedure.

Facility design and workflow
- The high sensitivity of PCR techniques requires that demanding assay conditions be followed.
- Facility and operation to be designed to prevent contamination of reactions with amplified products from previous assays and cross contamination between samples.
- Allocate physically separate space/area/room for each activity listed below,
  - Sample receipt, verification and preparation
  - Reagent preparation
  - RNA extraction
  - Master Mix preparation
  - Template addition
  - Amplification and analysis
  - Donning and doffing of PPE
- Do not share materials, supplies, or equipment among the rooms
- Prepare and follow SOP for each activity mentioned above

Sample acceptance procedure
As the sample used is nasopharyngeal and oropharyngeal swab assess its condition at the time of receipt. Ascertain the following:
- Sample is in good condition with its cold chain maintenance and intact triple layer
- All required information is recorded in the form designed for the purpose.
- Label on the VTM matches with that in record form
- Give unique identifier to the sample, mark it and use for logging, and tracking.

**Equipment**
- Use equipment suitable for the methods. Follow SOP for use of each.
- Calibrate the instruments and equipment periodically.
- Maintain the log of each equipment to document its functioning, schedule for maintenance, and any event of its malfunction.
- **Power supplies**
  - Check electrical cables and cable connections for breaks, fraying, corrosion, or looseness, regularly and replace if needed.

**Biological Safety Cabinet and PCR work stations**
- Get Biological Safety Cabinet II and PCR work station checked and certified annually and get it documented.
- Use and perform maintenance of Safety cabinet in adherence to the SOP.
- Use UV light, 70% ethanol, 1% Sodium hypochlorite for cleaning and disinfection

**Thermal cyclers**
- Great care should be taken to ensure that they are well maintained.
- Follow manufacturer instruction to test and to maintain.

**Centrifuges**
- Use separate refrigerated centrifuges for pre- and during the procedure.
- Follow manufacturer instructions for calibration and use.
- Balance the centrifuge before use to increase bearing life and minimize vibrations that can unsettle concentrates.

**Pipettes**
- Preferably use adjustable micropipettes.
  - Get it calibrated regularly.
  - For cleaning and disinfection: Clean with RNase ZAP RNase Decontamination solution (AM9782, Thermofischer Scientific) and then 70% Ethanol.

**Temperature-dependent equipment**
- Refrigerators: 1°C - 5°C
• Standard laboratory freezers: -20°C ± 5°C
• Ultra-low freezers: -70°C ± 10°C
• Monitor temperatures of equipment twice a day
• Keep separate refrigerators and freezers for samples, reagents, and final amplification products

2.2 Analytical phase

Analytical procedures and assessment
• Follow the standard operating procedure for each analytical technique used (SOP for each procedures)

Internal quality control procedures
• Use Positive control, no template control and extraction control (preferably use RNase P gene for extraction control) in each reaction.

PCR positive control
• Confirm the amplification of positive control in each batch of PCR
• Compare the ct value of positive control included in different reactions

Sources of RNase contamination:
RNase from several sources can contaminate RNA extracted from biological samples
• Body fluids such as perspiration
• Tips & tubes (always use tips and tubes that have been tested and certified RNase-free)
• Water and buffers
• Laboratory surfaces
• Endogenous RNases
• RNA samples
• RNA storage
• Chemical nucleases
• Enzymes
Preventing RNase contamination

Some basic precautions need to be taken when working with RNA. These include

- Wearing gloves throughout experiments and changing them after touching skin, door knobs, and common surfaces
- Having a dedicated set of pipettes that are used solely for RNA work
- Using tips and tubes that are tested and guaranteed to be RNase-free
- Using RNase-free chemicals and reagents

2.3 Post analytical phase

Data recording, record keeping, Report dissemination

- Verify the data correctness and completeness.
- Back up the electronically maintained data on a regular basis and stored in a separate location from the original data.

Equipment and consumables items

- Keep log of each equipment calibration and maintenance in appropriate logbooks.
- Establish a schedule of equipment calibration and maintenance with the procurement of each new piece of equipment and a paste a copy in the laboratory near the equipment.
- Maintain logbooks for all consumables and reagents, kits and document all pertinent information needed to identify possible sources of contamination, including the following:
  Product name, manufacturer, product number, formulations (reagents), sequence (primers and probes), receipt or preparation analysts' name and initials, storage location and location of components, location of the commercially available kit specifications and procedure, concentration of original primer stocks and working solutions, storage buffer, number of units (enzyme) and associated buffer (enzyme).
- Locate the reagent logbooks centrally and readily accessible.
3. Reference

https://www.google.com/search?client=safari&rls=en&q=The+Real-Time+PCR+Core+Facility+Quality+Assurance/Quality+Control+Guidance&ie=UTF-8&oe=UTF-8
SOP 14: Sample Storage and Archiving

1. Scope and application

This SOP is applicable for long term storage of the sample at -80°C.

2. Responsibilities

<table>
<thead>
<tr>
<th>Responsible Personnel</th>
<th>Activities</th>
</tr>
</thead>
<tbody>
<tr>
<td>Laboratory personnel delegated by the respective lab/institution for the specific activities (in accordance to interim guidelines by NPHL, Nepal)</td>
<td>Sample storage and archiving</td>
</tr>
<tr>
<td></td>
<td>Maintenance of records</td>
</tr>
</tbody>
</table>

3. Methods

a. Store all positive sample at -80°C in Pre-defined position of cryo box.
b. Maintain the register/MS Access Database to track the storage sample.
c. Discard the negative sample after reporting.
d. Record the temperature of -80°C deep freeze twice a day.
<table>
<thead>
<tr>
<th>SN</th>
<th>Name of reagents (for 5000 samples)</th>
<th>Manufacturer</th>
<th>Catlog Number</th>
<th>for 5000 Reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Sarbecot gene (primer, probe) with FAM dye *</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>SarbecoldRSP (primer, probe) with HEX dye *</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Sarbeco N gene (primer, probe) with CAL RED 610 *</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Human RNaseA ( primer, probe) with Quasar Red 670 *</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Qiaqon Virus Mini Kit</td>
<td>Qiagen</td>
<td>52906</td>
<td>250 x 20 boxes</td>
</tr>
<tr>
<td>6</td>
<td>One Step Multiplex RT master mix (RT Enzyme, buffer, PCR mix)</td>
<td>Roche/Invitrogen/Thermofischer</td>
<td>06 75 155 001</td>
<td>2 x 5000 kits</td>
</tr>
<tr>
<td>7</td>
<td>Strip Tube and Caps, 0.1ml (1000 reactions)</td>
<td>Qiagen</td>
<td>981103</td>
<td>10000 tubes</td>
</tr>
<tr>
<td>8</td>
<td>Safe-lock tube 1.5ml, 1000 pieces</td>
<td>Eppendorf/Tarson/Abdos</td>
<td>108543</td>
<td>12000 tubes</td>
</tr>
<tr>
<td>9</td>
<td>Molecular Grade Ethanol 500ml</td>
<td>Merck</td>
<td></td>
<td>1 liter</td>
</tr>
<tr>
<td>10</td>
<td>Dualfilter Tips 0.1-10µl, 96 Tips/Box</td>
<td>Eppendorf/Tarson/Abdos</td>
<td></td>
<td>30000 tips</td>
</tr>
<tr>
<td>11</td>
<td>Dualfilter Tips 2-20µl, 96 Tips/Box</td>
<td>Eppendorf/Tarson/Abdos</td>
<td></td>
<td>30000 tips</td>
</tr>
<tr>
<td>12</td>
<td>Dualfilter Tips 20-250µL, 96 Tips/Box</td>
<td>Eppendorf/Tarson/Abdos</td>
<td></td>
<td>20000 tips</td>
</tr>
<tr>
<td>13</td>
<td>Dualfilter Tips 50-1000µL, 96 Tips/Box</td>
<td>Eppendorf/Tarson/Abdos</td>
<td></td>
<td>50000 tips</td>
</tr>
<tr>
<td>14</td>
<td>Cardboard cryo-box 81 holes 13.4 x 13.4 x 7 cm</td>
<td>Himedia</td>
<td>CG280</td>
<td>150 boxes</td>
</tr>
<tr>
<td>15</td>
<td>Rease ZAP Rease Decontamination Solution</td>
<td>Thermofischer</td>
<td>AM9782</td>
<td>5 liter</td>
</tr>
<tr>
<td>16</td>
<td>Tissue paper (dust free, 100gram quality)</td>
<td></td>
<td></td>
<td>500 Roll</td>
</tr>
<tr>
<td>17</td>
<td>Biodegradable Autoclave bags for biomedical waste collection (5 liter, red color)</td>
<td></td>
<td></td>
<td>200 PCS</td>
</tr>
<tr>
<td>18</td>
<td>Transparent polythene bags 3 legs capacity</td>
<td></td>
<td></td>
<td>50 Kg</td>
</tr>
<tr>
<td>19</td>
<td>Biodegradable Autoclave bags for biomedical waste collection (large size, red color 44x48)</td>
<td></td>
<td></td>
<td>200Kg</td>
</tr>
<tr>
<td>20</td>
<td>Liquid soap</td>
<td></td>
<td></td>
<td>10 Liter</td>
</tr>
<tr>
<td>21</td>
<td>NITRLE GLOVES (Medium size), 100 Pairs</td>
<td></td>
<td></td>
<td>250 Box</td>
</tr>
<tr>
<td>22</td>
<td>Nitrite Gloves (Large size), 100 Pairs</td>
<td></td>
<td></td>
<td>250 Box</td>
</tr>
<tr>
<td>23</td>
<td>Sodium Hypochlorite solution</td>
<td></td>
<td></td>
<td>50 Liter</td>
</tr>
<tr>
<td>24</td>
<td>Ziplock bag large size</td>
<td></td>
<td></td>
<td>250 packet</td>
</tr>
<tr>
<td>25</td>
<td>Latex gloves medium size, 100 pairs</td>
<td></td>
<td></td>
<td>100 box</td>
</tr>
<tr>
<td>26</td>
<td>Latex gloves Large size, 100 pairs</td>
<td></td>
<td></td>
<td>50 Box</td>
</tr>
<tr>
<td>27</td>
<td>Ziplock bag Medium size</td>
<td></td>
<td></td>
<td>50 Box</td>
</tr>
<tr>
<td>28</td>
<td>Specimen Bag with Biohazard sign</td>
<td></td>
<td></td>
<td>5000 PCS</td>
</tr>
<tr>
<td>29</td>
<td>Complete Set of PPE (Cover all/Gowns, N95 Masks, Face shield, Goggles, Shoecover, reasuable boot, Head cover)</td>
<td></td>
<td></td>
<td>100 Sets</td>
</tr>
<tr>
<td>30</td>
<td>Spirit (70% Alcohol)</td>
<td></td>
<td></td>
<td>30 liter</td>
</tr>
</tbody>
</table>

* Primers probes information of each gene and required to test 5000 samples
Taking into consideration of Pandemic COVID-19, molecular diagnostic is being challenged in low resource countries like Nepal. Testing individual molecular markers would take time. Likewise, including only one molecular marker would also demand further confirmation by other molecular markers. These confirmation steps would increase the cost of testing, prolong the reporting time frame and pose threat for further laboratory contamination due to several manipulations of clinical samples in the lab. Therefore, to look for multiple markers would enhance the efficiency and widen the scope of testing in one step real time PCR. So far, E, RdRP (Wuhan specific) and N genes are recommended to detect COVID-19 so multiplex PCR with different emission spectrum of fluorophores (Like HEX, FAM, CalRed, Quasar 670 etc...) can be tagged in different probes together with the extraction control (most probably RNase P) to monitor the process control and sample quality.

**Test E, RdRP and N gene together with RNAse P in multiplex PCR**

(In case of process control of RNA extraction, a few samples can be spiked with known extraction control and a few samples can be tested without extraction control if RNAse P is planned to detect.)

<table>
<thead>
<tr>
<th>Name1</th>
<th>Sequence (5 to 3)</th>
<th>Fluorochrome dye</th>
<th>Synthetic Yield in a tubes upto</th>
<th>Required number of tubes</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>N GENE</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>COV2 N1-F</td>
<td>GACCCCAAAATCAGCGAAAT</td>
<td>CAL RED680</td>
<td>100 micromole</td>
<td>20</td>
</tr>
<tr>
<td>COV2 N1-R</td>
<td>TCTGGTTACTGCAAGTGTGGATCTG</td>
<td></td>
<td>100 micromole</td>
<td>20</td>
</tr>
<tr>
<td>COV2 N1-P</td>
<td>ACCCCCGATTTGCTTGGGACGC</td>
<td></td>
<td>50 micromoles</td>
<td>10</td>
</tr>
<tr>
<td><strong>HUMAN RNAse P</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HUM RNAse P-F</td>
<td>AGATTGGGACCTCGGAGGCC</td>
<td>CAL RED680</td>
<td>100 micromole</td>
<td>20</td>
</tr>
<tr>
<td>HUM RNAse P-R</td>
<td>GACCGCGTTCGCTTCCAACAGT</td>
<td></td>
<td>100 micromole</td>
<td>20</td>
</tr>
<tr>
<td>HUM RNAse P-P</td>
<td>TCTGGACCTGAAGGCTCTGCGGC</td>
<td>QUASAR RED670</td>
<td>50 micromoles</td>
<td>10</td>
</tr>
<tr>
<td><strong>RdRP GENE</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RdRP SARS1-F</td>
<td>GTGARATGGTCCATGTGGGCGG</td>
<td>HEX</td>
<td>100 micromole</td>
<td>20</td>
</tr>
<tr>
<td>RdRP SARS-R1</td>
<td>CARATGTAAACACACCATTTAGCAT</td>
<td></td>
<td>100 micromole</td>
<td>20</td>
</tr>
<tr>
<td>RdRP SARS-P2</td>
<td>CAGGTGGAACCTCACTCAGGAGATGC</td>
<td></td>
<td>50 micromoles</td>
<td>10</td>
</tr>
<tr>
<td><strong>E GENE</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E-Sarbeco-F1</td>
<td>ACACGGTTAACCTTAATGTTAATAGCGT</td>
<td>HEX</td>
<td>100 micromole</td>
<td>20</td>
</tr>
<tr>
<td>E-Sarbeco-R2</td>
<td>ATATGGCAACAGTACGGCAACA</td>
<td></td>
<td>100 micromole</td>
<td>20</td>
</tr>
<tr>
<td>E-Sarbeco-P1</td>
<td>ACACCTGCCATCTTTACTGCGCTCG</td>
<td>FAM</td>
<td>50 micromoles</td>
<td>10</td>
</tr>
</tbody>
</table>
Interim Guideline for the establishment and operationalization of molecular laboratory for COVID 19 testing in Nepal

Physical Infrastructure:

The laboratory should have at least 3 rooms with physical barriers, with unidirectional workflow of humans and materials to be maintained.

Equipment:

All the equipment in the laboratory needs to be calibrated/validated for its performance periodically by an agency mandated for quality validation. As most of the equipment require uninterrupted power supply, the lab should have dedicated feeder line for electricity supply or linked to automated electric power generator or UPS. A 10KVA UPS would satisfy the need for the required equipment.

- Functional Biosafety cabinet type 2A/2B-1
- Laminar Flow hood/PCR cabinet-1
- Real time PCR machine-1
  - with UPS for minimum two hour uninterrupted power supply (10KVA capacity)
  - Air conditioned room to maintain the room temperature
- Autoclave-1
- Deep freezer (-20°C)-1
- Deep freezer (-80°C): Optional for storage of samples.
- With compartments for reagents and samples (better to have separate deep freezers for samples and reagent storage)

- Refrigerator with temperature range of 2-8°C

- Microfuge/Refrigerated centrifuge with adjustable rotors for PCR tubes

- Vortex Mixer 2

- Spinner-2 (for strip as well as Eppendorf)

- Automatic Pipette with stands:
  - Fixed volume: 1000μl - 1; 500μl - 1, 200μl - 1, 10μl - 2
  - Adjustable: 0.5 to 10 μl - 2 nos; 2 to 20 μl - 2 nos; 10-100 μl - 2; 20 to 200 μl - 2 nos; 100 to 1000 μl - 2 nos

**Consumables:**

- Personnel Protective Equipment (disposable gowns, masks, gloves, eye cover and face shield, shoe covers etc.) - as required

- Pipette tips with filters & compatible with pipette size - as per requirement, minimum 1000 each

- Sample and PCR tubes: Eppendorf tubes 1.8 ml capacity: minimum 1000 pcs. Cryovials with capacity of 2 ml as per requirement, minimum 1000

- Steel container to store Eppendorf tubes: 1

- Cryobox with 81/100 slots for storage of reagents, samples, products - 10 (can be more depending upon sample size)

- Real time PCR compatible PCR strip (with cap) - as per requirement

- PCR cooler - 2

- Icebox - 2
- Gel packs – as required
- Dust bin -6 pieces (minimum2 colour coded)
- Biohazard bag as per requirement
- Discard Jars -3
- Aluminium foil as per requirement
- Reagents required for PCR

Human Resource

- Masters level Medical Microbiologist (preferable)/Masters in life science - 1; Some experience in microbiological molecular work will be desirable
- Supporting laboratory staffs with Bachelor/Certificate level in Laboratory sciences - 2

Personnel working in a PCR laboratory should undergo training in the methodology that covers PCR theory and practice. The course work should also include biosafety in a PCR laboratory as well as quality issues and troubleshooting PCR-related problems. Hands-on training should be completed for each technique under the supervision of experienced personnel. The time required for training will vary depending on the trainee and technique. Personnel should demonstrate that they can successfully perform the method by testing confirmed positive and negative control samples before being allowed to analyse diagnostic samples.
प्रेस विज्ञप्ति

समाजवाद विषय वर्तमान मानवीय विज्ञान संस्था द्वारा कार्यकर्ता शासन के साथ-साथ हैं भारत के राज्य में।

भारत के नया विशेष वित्त पापटज्ज्वाला की रूपरेखा प्रकाशित हुई। इसका केन्द्र भारत के सामाजिक और राजनीतिक मूलभूत स्तर पर बनाया गया है।

प्रेस विज्ञप्ति के अन्तर्गत, केंद्रीय सरकार एवं राज्य सरकारों के अंतर्गत व्यवस्थापन के अंतर्गत विभिन्न विषयों पर वित्त परिषद् की रूपरेखा जारी की गई है।

बारेमा ने सामाजिक विज्ञान अनुसन्धान संस्था से मदद के रूप में राज्य सरकारों को भेजा।

विदेशी मंत्री के अंतर्गत, सरकारों के अंतर्गत वित्त परिषद् की रूपरेखा जारी की गई है।

केंद्रीय सरकार के अंतर्गत विदेशी मंत्री के अंतर्गत सरकारों के अंतर्गत वित्त परिषद् की रूपरेखा जारी की गई है।

निम्न लिखित पत्र नामक नीति पर विचार करने के लिए हस्ताक्षर किया गया है।

निम्न लिखित पत्र २०२० वर्ष के लिए हस्ताक्षर किया गया है।
प्रेदेश सरकार
सामाजिक-विकास मन्त्रालय

प्रमुख सचिव:

विषय: मनोमय सम्पर्कमा।

प.पा. बुधुमा चनाल,
बि.पी.कोईराला स्वास्थ्य विद्यालय प्रतिनिधि, धरार।

प्रस्तुत विषयमा यस मन्त्रालयको मिति २०७६/०२/०१ मार्गदेश निर्देशन सँग मध्ये लाई कोनल-१९ परिषद गर्न सम्बन्धमा परिवर्तन (Pool testing) का लागि मार्गदेश (Protocol) निर्धारण गरेका भएका हुने सत्ताको जिम्मेवारी तथा सरकारले व्यक्ति अनुरोध गर्दै। सँग उक्त सम्बन्धमा सामन्तलका महत्त्वाकर्षण रहन्ने रुपमा रुपरेत।

मनोमय हुन भएकोमा भएकाँ हुन।

पुर्व: उक्त मार्गदेसन ३ दिन विच मन्त्रालयमा पेश गर्दै।

संपर्कलिपि:

१. संपर्कको संख्याको संख्या: प.पा. बुधुमा चनाल,
बि.पी.कोईराला स्वास्थ्य विद्यालय प्रतिनिधि, धरार।
२. सरोवर: डा.सुरेश मेहता, जनस्वास्थ्य प्रशासन, सामाजिक विकास मन्त्रालय, प्रदेश नं ६।
३. सरोवर: डा.मनोज कापौल, विद्रोह विज्ञान कलेज तथा टीमिङ अस्पताल विदर्भ गायमार।
४. सरोवर: डा.कार्मना शर्मा, कौटी अस्पताल, विदर्भ गायमार।
५. सरोवर: डा. राक्षिक बुझाई, निर्देश, प्रेदेश जनस्वास्थ्य प्रशासन, प्रदेश नं ६।
६. सरोवर: डा. जेवल राई, बि.पी. कोईराला स्वास्थ्य विद्यालय प्रतिनिधि, धरार।
७. सरोवर: उ.भ. नगराल भुट्रवाई, बि.पी. कोईराला स्वास्थ्य विद्यालय प्रतिनिधि, धरार।

रक्तमूलक पदक
मुख्य सचिव

नाम: न. न. मुजफ्फर आली

रक्तमूलक पदक
मुख्य सचिव

नाम: न. न. मुजफ्फर आली

नम्बर: ००७-२३३५०९ | ईमेल: mndprovi@gmail.com |