Laboratory Manual

Molecular diagnosis of SARS-CoV-2 from clinical specimens

17 September 2020

IEDCR
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Disclaimer:

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More than 12.8 million COVID-19 cases have been reported across 188 countries and territories, resulting in more than 945,000 deaths till mid-September 2020. Bangladesh has also been experiencing the pandemic wave of COVID-19 and as of 17 Sep 2020, there were more than 344,000 confirmed COVID-19 cases in Bangladesh, including 4859 related deaths. To combat the COVID-19 situation, it was a tough job for the Government of Bangladesh to ensure credible and high-quality COVID-19 diagnosis all over the country with minimum resources and skills. The most reliable way to diagnose COVID-19 is real time PCR technology. Government of Bangladesh (GoB) has extended the testing facilities to several district level hospitals of Bangladesh but is lacking high skilled manpower. Until now, a total of 95 laboratories all over the country are testing COVID-19 by using Real-Time RT-PCR and it is very important to establish a generalized and uniform testing platform for the diagnosis of COVID-19. The objective of this manual/SOP is to establish a common testing strategy in all COVID-19 laboratories by implementing same protocol across the labs. This manual can also be used as a training manual. I believe that the manual/SOP for COVID-19 diagnosis will help in generating quality data and outcomes that could strengthen the country wide testing capacity for COVID-19 in Bangladesh.

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1. Severe Acute Respiratory Syndrome-Coronavirus-2 (SARS-CoV-2): an overview

Coronaviruses are a group of related viruses that cause diseases in mammals and birds. In humans, coronaviruses cause respiratory tract infections that can be mild, such as some cases of the common cold and others that can be lethal, such as SARS-CoV and MERS-CoV.

In December 2019, a novel coronavirus was detected in three patients with acute respiratory illness from Wuhan, Hubei Province, China. The virus has been named “SARS-CoV-2” and the disease it causes has been named “Coronavirus Disease 2019” (abbreviated “COVID-19”). On January 30, 2020, the International Health Regulations Emergency Committee of the World Health Organization (WHO) declared the outbreak a “public health emergency of international concern”. As of April 30, 2020, most of the countries and territories around the world have reported a total of 3.3 million confirmed cases of the coronavirus COVID-19 and a death toll of 234K. On March 11, the WHO declared COVID-19 a pandemic as the number of infected countries grows.

The SARS-CoV-2 virus is a beta-coronavirus, like MERS-CoV and SARS-CoV. All 3 of these viruses have their origins in bats. The respiratory tract is the major route of entry which makes it more contagious. Details about how the disease is spread are still being determined. The World Health Organization and Centers for Disease Control state that it is primarily spread during close contact and by respiratory droplets produced when people cough or sneeze; with close contact being within 1 to 2 meters (3 to 6 feet). It may also spread when one touches a contaminated surface and then touches their eyes, nose, or mouth.

2. Diagnosis of SARS-CoV-2

Polymerase chain reaction (PCR) is regarded as a gold standard test for the molecular diagnosis of viral and bacterial infections with high sensitivity and specificity. The respiratory specimens such as – nasopharyngeal, oropharyngeal, throat swab, sputum are more relevant for the early stage detection of the respiratory virus. Real-time reverse transcriptase-PCR (RT-PCR) detection is currently favored for the detection of coronavirus because of its advantages as a specific, and simple quantitative assay. Moreover, real-time RT-PCR is more sensitive for the diagnosis in early infections. Therefore, the real-time RT-PCR assay still is the preferred method to be applied for the detection of all kinds of coronaviruses including SARS-CoV-2. One recent study from Wuhan, which evaluated the performance of a fluorescence-based RT-PCR kit distributed by the Chinese Center for Disease Control and Prevention, suggests that nasopharyngeal swabs/ throat swabs offer greater consistency than sputum samples.

The real-time PCR technique is one of the emerging techniques that has become the method of choice for quantification of DNA and RNA levels in clinical specimens. Compared to conventional PCR, the real-time PCR technique makes use of the 5’-3’ exonuclease activity of this same Taq polymerase to cleave a dual-labeled fluorescent probe, giving rise to fluorescent emission (Fig. 1). Therefore, next to the presence of two primers in the PCR reaction, real-time PCR makes use
of an additional oligonucleotide primer that is dually labeled with two fluorophores. Importantly, this oligonucleotide primer, also called hydrolysis or TaqMan probe, anneals to the target internally of the two primers and emits a fluorescent signal only upon cleavage. In its free, intact form no fluorescent emission can be measured because fluorescent emission of the reporter dye is absorbed by the quenching dye. Quantification of the initial amount of target DNA is based on the increase in fluorescent emission, measured in every cycle of the PCR reaction. Therefore, PCR amplification and data acquisition (by measuring the increase in fluorescent emission as the PCR reaction proceeds) are performed in one single step, such that no post-PCR processing is required. In summary, the prospects of real-time PCR in molecular diagnostics are:

1. Amplification and detection are performed in one single step, which significantly reduces hands-on-time as well as contamination.
2. Reproducibility of the method is extremely high.
3. The use of the technique has been greatly facilitated for high throughput application such as infectious diseases surveillance.
4. Relative quantification of providing an idea of viral load.

![TaqMan probe chemistry mechanism](https://en.wikipedia.org/wiki/TaqMan)
3. Bio-safety

All procedures must be performed based on risk assessment and only by personnel with demonstrated capability in strict observance to any relevant protocols at all times. Initial processing (before inactivation) of all specimens should take place in a validated biological safety cabinet (BSC) or primary containment device. Non-propagative diagnostic laboratory steps (e.g. specimen processing and nucleic acid extraction) should be conducted at facilities and procedures equivalent to BSL-2 and propagative work (e.g. virus culture, isolation or neutralization assays) at a containment laboratory with inward directional airflow (BSL-3). Appropriate disinfectants with proven activity against enveloped viruses should be used, e.g. 0.5% hypochlorite (bleach) and 70% alcohol. For surface cleaning bleach exposure should be for 1 min and for liquid waste it should be for 20 minutes. Human coronaviruses in general are known to persist on inanimate surfaces such as metal, glass or plastic for up to 9 days.

Personal protective equipment:

1. Gloves (for molecular biology works better to use powder less gloves), one-time use
2. Safety gown (apron), Shoe cover, Head cover, Goggles, reuse
3. Masks (N95 for specimen processing), one-time use
4. Toe covered shoe

If disposable PPE are available, then after work discard all. If disposables are not available, it is recommended to wash after each day work. For decontamination, autoclave or clean with 0.5% hypochlorite solution the clothes for 20 min and then wash.
4. Organization of workspace

Workspace should be organized to ensure that the flow of work occurs in one direction (Fig. 2), from clean areas (mastermix preparation) to dirty areas (sample processing). Have separate designated rooms, or at minimum physically separate areas, for:

1. Sample processing, Nucleic acid extraction, and Template addition
2. Mastermix preparation
3. PCR amplification and handling of amplified products

![Diagram of workspace organization](image)

**Figure 2:** Unidirectional management of working area. R represents refrigerator.

All staff should follow a unidirectional workflow. In special situations if something otherwise is required then personnel must take care to thoroughly wash hands, change gloves, use the designated lab coat and not introduce any equipment they will want to take out of the room again, such as lab books. If any emergency equipment is needed to be moved from one area to another it should be cleaned with 0.5% hypochlorite solution and then with distilled water.
Equipment

Each room/area needs a separate set of clearly labeled items:
1. Pipettes
2. Filter tips
3. Tube racks
4. Vortexes
5. Centrifuges (if relevant)
6. Pens/Markers
7. Lab reagents
8. Lab coats, shoe cover, gloves, goggles
9. Freezer (-20°C)
10. Refrigerator (4°C)
11. Water bath (temperature up to 99.9°C)
12. Biosafety cabinet (class II) for specimen processing
13. PCR hood (for master mix preparation)
14. PCR hood for template addition (if RNA is extracted separately)

These items must remain in their designated locations. Reagents and equipment should not be moved from a dirty area to a clean area. All PPE including lab coat, mask, shoe cover, gloves and goggles should be left when leaving from any designated area.

5. Good Laboratory Practices

a) Avoiding contamination

Molecular detection methods can produce a large volume of nucleic acid through the amplification of trace quantities found in samples. While this is beneficial for enabling sensitive detection, it also introduces the possibility of contamination through the spreading of amplicon aerosols in the laboratory environment. When conducting experiments, measures can be undertaken to avoid the contamination of reagents, laboratory equipment and bench space, as such contamination may generate false-positive (or false-negative) results.

Handling reagents
1. **Spin down** (brief centrifuge) reagent tubes before opening to avoid the generation of aerosols.
2. **Aliquot reagents** to avoid multiple freeze-thaws and the contamination of master stocks (if comes in large volume).
3. Clearly **label and date all reagents** and reaction tubes and maintain logs of reagent lot and batch numbers used in all experiments.
4. Pipette all reagents and samples using **filter tips and** ensure tips are compatible with pipette.
b) Cleaning bench and equipment after and before work

After use, bench spaces should be cleaned with 0.5% hypochlorite, followed by sterile water (to remove residual bleach) and 70% ethanol. After all work are done, turn on the UV lights of safety cabinets (if available for at least one hour or overnight). If manufacturer instructions permit autoclave the pipettes. Otherwise always clean with 0.5% bleach followed by sterile water and 70% alcohol before and after use. Bench spaces MUST be cleaned again before resuming next laboratory work.

c) General molecular biology advice

1. Use powder-free gloves to avoid assay inhibition.
2. Correct pipetting technique is paramount to reducing contamination.
3. Centrifuge tubes before opening, and open them carefully to avoid splashing.
4. Close tubes immediately after use to avoid the introduction of contaminants.
5. It is recommended to set up the mastermix on ice or a cold block.
6. Protect reagents containing fluorescent probes from light in order to avoid degradation.

d) Waste disposal

1. Collect biological waste in biohazard bags. Decontaminate by autoclaving or using 0.5% bleach for 20 minutes first and then dispose by incineration.
2. If autoclave or incinerator is not available, collect all contaminated materials in sufficient volume of 1% hypochlorite, leave for overnight, collect all decontaminated solid waste in biohazard bag and transfer to a remote incinerator at intervals.

6. Specimen handling

A specimen received by the laboratory must be accompanied by sufficient information to identify what it is, when and where it was taken or prepared.

a) Storage and transportation

Specimens to be tested can be immediately processed, and specimens to be tested within 24 hours can be stored at 4°C. Specimens that cannot be tested within 24 hours should be stored at -70°C or below (in the absence of -70°C, specimens to be tested can be stored at -20°C for 10 days and nucleic acid can be stored at -20± 5°C for 15 days). Repeated freezing-thawing should be avoided. The specimens should be transported with ice or sealed foam box with ice.
The following supplies should be ensured for sample transportation.

1. Specimen collection tube with viral transport media and swab (flocked)
2. Cool box with ice pack (4°C to 8°C) for shorter transportation time, i.e. less than 24 hours
3. Dry shipper/liquid nitrogen tank for longer transportation (up to two weeks)
4. Specimen rack (to hold tubes)

b) Specimen reception

Following measures should be taken during specimen reception:

1. Receive the paper document separately.
2. The “cool box” should enter lab through pass box.
3. Check the temperature of the cool box (a thermometer is a must). It should be between 2-8°C.
4. Specimens should be in safe racks inside the cool box.
5. After receiving, clean the surface of the cool box with 70% alcohol.
6. Then clean the inside of the cool box with 70% alcohol.
7. The specimen rack should be placed in a container full of 0.5% hypochlorite solution for 20 minutes.
8. Return the cool box to the specimen reception area.

Check the tubes with specimens for any leakage and presence of swab stick. Any discrepancy should be noted and reported. Leaking specimens should be rejected.

c) Documentation

(i) Specimen log
All specimens must be documented properly. From hospital or any specimen delivery center all specimens should accompany with a “specimen delivery sheet” (appendix 1). Laboratory staff should check all specimens for proper labeling, proper transport condition (principally the maintenance of temperature) and sign of any leakage. If everything matches as expected laboratory staff can accept the specimen for testing. After receiving the specimen document in laboratory specimen log (appendix 2). It is recommended to enter all information in a laboratory information management database (MS Excel or other preferred software).

(ii) Experiment plan
Before running any PCR determine the number or specimens to be tested. Never run any experiment without controls (negative control and positive control). Maintain experiment log sheet (appendix 3) during every run. After the run is complete preserve the experiment log as future reference.
7. SARS-CoV-2 Testing Procedure

***BEFORE TESTING PLEASE MAKE SURE WHAT KITS HAVE BEEN PROVIDED. THE METHOD WILL VARY FROM KIT TO KIT. STRICTLY FOLLOW THE MANUFACTURER INSTRUCTIONS.

a) Sample Processing and heat inactivation (restricted to section I workspace)

Mix samples thoroughly with shaking (use the Vortex Mixer) inside the biosafety cabinet, then inactivate the sample at 56°C for 30 minutes or 65°C for 10 minutes in a metal bath or water bath before experiment.

*Note: This step can be avoided if not instructed by the manufacturer of the kit.*

b) RNA Extraction (restricted to section 1 workspace)

**OPTION 1: Spin Column-based Nucleic Acid Extraction**

If the diagnostic kit requires separate nucleic acid extraction, use spin column-based extraction kit and follow the manufacturers' protocol. See Appendix-4 for an example.

**OPTION 2: Release buffer-based One tube method**

If the diagnostic kit does not require separate nucleic acid extraction and provides RNA release reagent.

1. Inside the Biosafety cabinet II, take required numbers of 1.5 ml tubes and label with the sample id.
2. Pipette 10 μl RNA release buffer in each tube.
3. Add 10μl specimen into the labeled 1.5 ml tube containing release buffer.
4. Mix by vortex followed by short spin. If vortex/centrifuge is not available mix by pipetting.

   **DO NOT MIX RNA RELEASE REAGENT WITH SPECIMEN INTO A 8-STRIP PCR TUBE OR FULL PLATE.**

5. Stand the samples for 10 minutes under room temperature for lysis.
Real time RT-PCR:

(i) Materials:

Equipment:
1. Biosafety cabinet
2. Laminar airflow cabinet
3. Vortex
4. Pipettes
5. Tube compatible centrifuge for specimen (1.5 ml tube)
6. Real Time PCR instrument with computer

Consumables:
1. PCR strip and cap (compatible with Real Time PCR instrument) or PCR plate
2. PCR tube rack
3. 10 µL/100µL/200µL pipettes compatible tips
4. Timer (Stop watch)

Reagents:
1. SARS-CoV-2 specific Real Time PCR kits

Prepare experiment plan and print three copies for different areas (master mix preparation area, specimen processing area and laboratory documentation)

(ii) Master mix preparation (restricted to section 3 workspace)

The protocol used here is based on most common Real Time PCR kits used only for example. During clinical sample testing follow the provided PCR diagnostic manual before use.

1. Properly clean the top of the area with 0.5% hypochlorite solution. Wipe the relevant pipettes dedicated for master mix with 0.5% hypochlorite solution.
2. Take out each component (e.g., PCR mix, Enzyme mix, Controls, nuclease free water) from the diagnostic kit and thaw. Spin briefly each of them respectively for later use.
3. According to the quantity of test specimens including negative control and positive control, pipette appropriate quantity components in proportion and fully mix them into a 1.5 ml tube considering the total volume (DO NOT VORTEX). Spin shortly for 10-15 seconds (example table 1).

Table1: Components of Master mix (the amount and composition may vary with different kits)

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount in µL</th>
<th>For 8 samples</th>
<th>For 96 samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR Mix buffer</td>
<td>a</td>
<td>aX8</td>
<td>aX96</td>
</tr>
<tr>
<td>PCR-enzyme mix</td>
<td>b</td>
<td>bX8</td>
<td>bX96</td>
</tr>
<tr>
<td>Total</td>
<td>a+b</td>
<td>(a+b)X8</td>
<td>(a+b)X96</td>
</tr>
</tbody>
</table>
Notes: In a set of 8 specimens there should be 1 negative control and 1 positive control. Therefore, total of 6 clinical specimens can be tested.
In 96 well plate there should be 1 negative and 1 positive control. Therefore, 94 samples can be tested in one run.
In some experiment, distilled water is added as an extraction control to monitor any possible carry over and contamination during extraction.

4. Transfer a+b μL (e.g. Table 1) into each 8-strip PCR tubes or full plate and transfer the master mix/plate to the template addition area.
5. Always keep the master mix on ice or cold block (4°C to 8°C).

(iii) Template addition *(Restricted to section 2 workspace)*

1. Bring the lysed/extracted RNA in tubes to the template addition area and spin for 10-15 seconds.
2. Carefully take c μL RNA (as specified in the kit manual) and add to the wells with master mix according to the plate design.
3. Dispose of and use a new tip after each sample addition.
4. Positive control should be added very carefully at the end.
5. Cover the plate with caps/plate sealer and centrifuge at 2,000 rpm for 10-15 seconds.

Note: If centrifuge is not available, carefully tap the plate or spin the plate manually to avoid droplet on the wall of the wells.

(iv) PCR amplification *(restricted to section 2 workspace)*

*(Refer to user manual of each instrument for the settings. Here is as example- the kits contain two targets (ORF1ab and N genes) for SARS-CoV-2 to be detected by FAM and ROX channels respectively and internal control to be detected by HEX channel)*

1. Turn on the Real Time PCR instrument and computer (depending on the instrument for starting sequence consult with the manufacturer or local vendor)
2. After login open the software (instrument specific)
3. Open the PCR run file template (recommended to prepare once and save as template). During every run enter the specimen ID, save the file using “save as” option. Detail of template preparation should be available on instrument specific manual.
4. Save each run file into a designated folder.
5. Set up Positive Control, Negative Control and unknown specimen in the corresponding sequence and input sample information.
6. Select PCR test channel:
   a) Select dye channels (for example FAM, ROX, Cy5, Cy3 etc) to detect the RNA of SARS-CoV-2 virus (for example ORF1ab, RdRp, N, and E genes).
   b) Select the dye channel specific for internal control (for example Cy5, TAMRA etc.).

*Note: The dyes and targets can be different for different kits.*
7. Recommend Cycle parameter
   (this is a standard method but ALWAYS follow the kit provided manual):

<table>
<thead>
<tr>
<th>Steps</th>
<th>Temperature</th>
<th>Time</th>
<th>Cycle</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Reverse Transcription</td>
<td>50 °C</td>
<td>30 minutes</td>
<td>1</td>
</tr>
<tr>
<td>2 cDNA pre-denaturation</td>
<td>95 °C</td>
<td>1 minutes</td>
<td>1</td>
</tr>
<tr>
<td>3 Denaturation</td>
<td>95 °C</td>
<td>15 seconds</td>
<td></td>
</tr>
<tr>
<td>4 Annealing, extension and</td>
<td>60 °C</td>
<td>30 seconds</td>
<td>45</td>
</tr>
<tr>
<td>fluorescence collection</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 Instrument cooling</td>
<td>25 °C</td>
<td>10 seconds</td>
<td></td>
</tr>
</tbody>
</table>

8. Save volume to (a+b+c) µL (Table 1).

9. For data management purpose all PCR data files should use a common name format. Recommended naming convention could be YYYYMMDD_CoV_InitialofStaff_RunNumber. For example, if the PCR is run on 14 April 2020 by a staff named “Ishrat Jahan” and it is the run number 20 for CoV the name could be 20200414_CoV_IJ_20.

10. Place PCR reaction tubes into the specimen wells of the PCR machine.

11. Run the PCR program.

12. Complete setting, save the file, run reaction program.

13. After the run remove the PCR strips. NEVER open the cap, DISCARD the tubes directly to biohazard bag.

(v) Result Analysis
   (refer to the user manual of each instrument for the settings. Cut-off Ct value used here as an example)

Results are saved automatically when reactions are completed. The amplification curves of detection targets and internal targets are analyzed respectively.

1. Adjust threshold values of baseline of the graph according to the analysis result (Users can adjust the values according to the actual situation. Adjust the amplification curve of negative control to be flat or below threshold).

2. Click “Analyze” to implement the analysis and make sure each parameter satisfies the requirements given in “Quality control”

3. Quality control-
   a) SARS-CoV-2-pcr-negative control: The target(s) dye channels and internal control channel should have no Ct value (or follow the kit recommendation).
   b) SARS-CoV-2-pcr-positive control: The target(s) dye channels (for example FAM, ROX, Cy3) and internal control (for example Cy5, TAMRA) channels should have S-shaped curve with good Ct values (see the kit recommendation).
   c) The above requirements should be met in the same experiment at the same time. Otherwise, this experiment is invalid and needs to be repeated.
Notes: If following the above algorithm ultimate interpretation (positive/negative) cannot be reached, record the specific test as “indeterminate”. For indeterminate cases either repeat the experiment or consult with experts.

4. After each run, export the Real Time PCR data to readable format (e.g., Microsoft Excel). The exported file name should be EXACTLY the same as original run file. It is recommended to print the data including specimen id, Ct value and interpreted results (positive, negative, indeterminate or invalid).

(vi) Interpretation of test results

Invalid: The internal control must detect a typical S-shaped amplification curve. If internal control does not detect a typical curve, the specific sample test is invalid (see the kit recommendation for any exceptions). Recollect the sample for repeat testing.

Positive: According to WHO, at least two different targets on the SARS-CoV-2 virus genome (of which at least one target is specific for SARS-CoV-2 virus) as well as internal Control channel, must detect typical S-shaped amplification curves (WHO/COVID-19/laboratory/2020.5), report the SARS-CoV-2 is positive (Fig. 3).

If only one target produces S-shaped curve, repeat the test or confirm it by sequencing partial or whole genome of the virus. However, interpretation of test results may vary with different kits and should be decided based on manufacturer’s recommendation.

Figure 3: Positive specimen scenario
**Negative**: If gene targets (for example ORF1ab, RdRp, N, and E) do not detect a typical S-shaped amplification curve (NoCt), but the Internal Control has typical amplification curve, the SARS-CoV-2, is negative (Fig. 4).

![Amplification Graph](image)

**Figure 4**: Negative sample scenario

8. **Reading material:**

6. [https://www.who.int/news-room/q-a-detail/q-a-coronaviruses](https://www.who.int/news-room/q-a-detail/q-a-coronaviruses)
9. [https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7090637/](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7090637/)
12. [https://www.nature.com/articles/d41587-020-00002-2](https://www.nature.com/articles/d41587-020-00002-2)
Appendix 1: Specimen delivery form (Can vary from organization to organization)

NAME OF ORGANIZATION

Date and time of delivery to the lab:

Total no of specimen:

Cool box temperature:

<table>
<thead>
<tr>
<th>ID</th>
<th>PATIENT ID</th>
<th>COLLECTION DATE</th>
<th>AGE</th>
<th>SEX</th>
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<tbody>
<tr>
<td>1</td>
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Specimens sent by:  
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Signature: ________________  Signature: ________________

Date: ________________  Date: ________________
Appendix 2: Laboratory specimen management log (typical register book)

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Appendix 3: Experiment plan log

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Appendix-4. RNA extraction using spin column-based kit (vary with kit to kit and used here as example)

Before starting
1. This SOP describes general procedure for spin column-based RNA extraction procedure.
2. The SOP can vary from manufacturer to manufacturer.
3. Read and strictly follow the manufacturer’s protocol.
4. All centrifugation steps are carried out at room temperature (15–25°C).
5. Equilibrate samples to room temperature (15–25°C).
6. Equilibrate Buffer AVE to room temperature for elution.

Preparation of reagents

Addition of carrier RNA to lysis buffer
1. Add 310 µl carrier RNA solvent to the tube containing 310 µg lyophilized carrier RNA to obtain a solution of 1 µg/µl. Dissolve the carrier RNA thoroughly, divide it into conveniently sized aliquots, and store it at –30 to –15°C. Do not freeze–thaw the aliquots of carrier RNA more than 3 times.
2. Check lysis buffer for precipitate, and if necessary, incubate at 80°C until the precipitate is dissolved. Calculate the volume of lysis buffer-carrier RNA mix needed per batch of samples.

Preparation of wash buffer 1 and wash buffer 2
Add 25 ml ethanol (96–100%) to wash buffer 1 and 30 mL ethanol (96–100%) to wash buffer 2.

Procedure:
1. Pipet 560 µl of prepared lysis buffer containing carrier RNA into a 1.5 ml microcentrifuge tube. If the sample volume is larger than 140 µl, increase the amount of lysis buffer–carrier RNA proportionally (e.g., a 280 µl sample will require 1120 µl lysis buffer-carrier RNA) and use a larger tube.
2. Add 140 µl swab sample to the lysis buffer–carrier RNA in the microcentrifuge tube. Mix by pulse-vortexing for 15 s. To ensure efficient lysis, it is essential that the sample is mixed thoroughly with Buffer AVL to yield a homogeneous solution. Frozen samples that have only been thawed once can also be used.
3. Incubate at room temperature (15–25°C) for 10 min.

   Note: Viral particle lysis is complete after lysis for 10 min at room temperature. Longer incubation times have no effect on the yield or quality of the purified RNA. Potentially infectious agents and RNases are inactivated in lysis buffer.
4. Briefly spin the tube for 10-20 seconds to remove drops from the inside of the lid.
5. Add 560 µl of ethanol (96–100%) to the sample, and mix by vortexing for 15 s. After mixing, briefly spin the tube to remove drops from inside the lid.

   Note: Only ethanol should be used since other alcohols may result in reduced RNA yield and purity. Do not use denatured alcohol, which contains other substances such as
methanol or methylethylketone. If the sample volume is greater than 140 µl, increase the amount of ethanol proportionally (e.g., a 280 µl sample will require 1120 µl of ethanol). To ensure efficient binding, it is essential that the sample is mixed thoroughly with the ethanol to yield a homogeneous solution.

6. Carefully apply 630 µl of the solution from step 5 to the spin column (in a 2 ml collection tube) without wetting the rim. Close the cap, and centrifuge at 6000 x g (8000 rpm) for 1 min. Place the spin column into a clean 2 ml collection tube, and discard the tube containing the filtrate. Close each spin column to avoid cross-contamination during centrifugation. Centrifugation is performed at 6000 x g (8000 rpm) to limit microcentrifuge noise. Centrifugation at full speed will not affect the yield or purity of the viral RNA. If the solution has not completely passed through the membrane, centrifuge again at a higher speed until all of the solution has passed through.

7. Carefully open the spin column, and repeat the above step. If the sample volume was greater than 140 µl, repeat this step until all of the lysate has been loaded onto the spin column.

8. Carefully open the spin column and add 500 µl of wash buffer 1. Close the cap, and centrifuge at 6000 x g (8000 rpm) for 1 min. Place the spin column in a clean 2 ml collection tube (provided), and discard the tube containing the filtrate. It is not necessary to increase the volume of wash buffer 1 even if the original sample volume was larger than 140 µl.

9. Carefully open the spin column and add 500 µl of wash buffer 2. Close the cap and centrifuge at full speed (20,000 x g; 14,000 rpm) for 3 min. **Note**: Residual wash buffer 2 in the eluate may cause problems in downstream applications. Some centrifuge rotors may vibrate upon deceleration, resulting in flow-through, containing wash buffer 2 contacting the spin column. Removing the spin column and collection tube from the rotor may also cause flow-through to come into contact with the spin column.

10. Place the spin column in a new 2 ml collection tube (not provided), and discard the old collection tube with the filtrate. Centrifuge at full speed for 1 min.

11. Place the spin column in a clean 1.5 ml microcentrifuge tube (not provided). Discard the old collection tube containing the filtrate. Carefully open the spin column and add 60 µl of elution 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.

12. A single elution with 60 µl of elution buffer is sufficient to elute at least 90% of the viral RNA from the spin column. Performing a double elution using 2 x 40 µl of elution buffer will increase yield by up to 10%. Elution with volumes of less than 30 µl will lead to reduced yields and will not increase the final concentration of RNA in the eluate. Viral RNA is stable for up to one year when stored at −30 to −15°C or −70°C.

13. After finishing the work, wipe out the BSC II cabinet with 0.5% bleach followed by distilled water and then with 70% alcohol and allow to run for 10 minutes before turning it off.

14. Discard gloves and masks in the biohazard bag in the covered trashcan. Wash hands thoroughly with soap and water in the washbasin before leaving the lab. Remove lab coats and shoes in the changing room.