Handbook for COVID-19 testing in Research Institutions

Introduction

(Adapted from WHO Interim guidance for laboratory testing: released 2nd March 2020)

Several viruses can lead to respiratory illness – the novel coronavirus pandemic is one such disease. Initially tentatively named 2019 novel coronavirus (2019-nCoV), the virus has now been named SARS-CoV-2 by the International Committee of Taxonomy of Viruses (ICTV) (2). This virus can cause the disease named coronavirus disease 2019 (COVID-19). WHO refers to the virus as COVID-19 virus in its current documentation and this document will also refer to the virus as COVID-19.

The purpose of this document is to provide interim guidance to laboratories and stakeholders involved in COVID-19 testing of patient samples. Adoption of these best practices are left up to the discretion of the laboratory supervisor.

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Acknowledgements

This handbook would not have been possible without the researchers named below putting together this excellent protocol, within a short span of time, which will help not just the established research labs, but labs across India where mentoring is absent and testing maybe needed to put into place rapidly, in exigencies.

The following group will, we hope also serve as an informal advisory for labs looking for setting up testing labs for COVID-19

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Part 1:

Am I eligible to do COVID-19 testing in my facility?

To be eligible for COVID testing you:

1.1 Have to be a government laboratory operational under the Department of Biotechnology (DBT), Department of science and Technology (DST), Council of Scientific & lndustrial Research (CSIR), Department of Atomic Energy (DAE), Ministry of Human Resource Development (MHRD) and Indian Council for Agricultural Research (ICAR)

1.2 Have a BSL-2 level laboratory facility with a molecular biology setup for human pathogen diagnosis.

1.3 Have a functioning and calibrated Biosafety cabinet Type 2A in the laboratory.

1.4 Have cold centrifuge for RNA extraction.

1.5 Have a functioning and calibrated real-time PCR (RT PCR) machine.

1.6 Have staff with hands-on understanding of laboratory biosafety and biosecurity, trained for handling respiratory samples for viral diagnosis, RNA extraction and RT PCR. Staff should also be versed with the theoretical underpinnings of each step of the process and its' implications.

1.7 A robust institutional policy on biomedical waste management of human origin.

1.8 Well defined arrangement for segregation and discarding of biomedical waste.

1.9 The laboratory should have a system to notify regulatory/concerned authorities regarding samples.

All points are mandatory. All laboratories with necessary facilities and personnel should engage in COVID testing.

Additional Information:

https://icmr.nic.in/sites/default/files/upload_documents/Protocol_for_approving_COVID19_testing_Lab_v2.pdf

Part 2:

What equipment/consumables do I need to start testing?

Sr. No	Equipment Checklist	Yes/No
1	2 BSL2 Hood (preferably 2, but 1 BSL2 + 1 Laminar hood at the minimum)	
2	Vortex mixer	
3	Sterile, RNase-free Micropipettes (2 or 10 µL, 200 µL and 1000 µL)	
4	Microcentrifuge (with rotor for 1.5 ml and 2 ml tubes)	
5	Benchtop minicentrifuge (optional)	
6	Calibrated Real time PCR machine with relevant detection channels	
7	Refrigerated microcentrifuge	
8	-80ºC (with free space for sample storage)	
9	-20 ºC	
10	4 ºC	

Sr. No	Reagents Checklist	Yes/No
1	RNASe Zap (Invitrogen Cat no: AM9782)	
2	Ethanol (absolute grade, high purity, For e.g., Sigma Molecular Biology E7023)	
3	70% ethanol in sterile water for disinfection	

4	1% Hypochlorite (Made from reagent grade Sodium hypochlorite; e.g., Sigma/ Merck 105614)	
5	Qiagen Viral RNA Mini kit. Catalog # 52904 (or equivalent)	
6a	ICMR approved RT PCR kit	
	or	
6b.1	Primer: probe mix (PP mix)	
6b.2	Molecular grade water, nuclease-free	
6b.3	One step RT-PCR Kit (like Superscript IIITM one step RT PCR kit)	

Sr. No	**Consumables Checklist**	**Yes/No**
1	1.5 ml and 2ml microcentrifuge tubes (screw cap, NOT flip tops)	
2	Racks for 1.5 mL and 2ml microcentrifuge tubes	
3	Sterile, RNase-free pipet tips (with aerosal barrier/ filter tips highly recommended)	
4	Plates and seal for RT PCR	
5	Beakers for waste collection	
6	Biohazard bags	
7	Waste bins (for PPE disposals)	

Sr. No	PPE Checklist	Yes/No
1	Gloves	
2	Surgical mask - for RT PCR set up	
3	N95 mask - for RNA isolation and sample handling	
4	Goggles	
5	Body Covers	
6	Shoe Covers	
7	Head Covers	

(Print and write Yes/No)

Part 3

What training does my personnel need before handling samples?

This is a guidance for staffing testing laboratories:

3.1 Be aware of ICMR guidelines on **eligibility for samples to be tested**. Do not process samples which fall outside these criteria. All staff need to be trained on sample receipt.

3.2 Ideally, all personnel involved in sample collection, receipt and analysis should be **tested for COVID-19** beforehand. Only those who test negative should be involved in testing. If resource limited, personnel should be made aware of the risks of working with COVID-19. For e.g., individuals with chronic respiratory issues, high blood pressure or diabetes are more likely to experience severe symptoms if infected with COVID-19.

3.3 Ideally, **staff trained to work in BSL2/3 facilities** should perform the RNA isolation from samples. Anyone new should be first trained on using **PPE** properly before handling samples. A trained observer should be around to monitor the wearing of PPE. Maintain a **log book** of everyone handling human samples.

3.4 All staff should be taken through **one pilot experiment** with complete PPE protocol (with synthetic positive control, if possible) with one non-infectious sample.

3.5 If possible, personnel should be engaged in **4 hour shifts**. In case 4 hour shifts are not feasible, shift time per worker should not exceed 8 hours. Different people should be involved in sample receipt, RNA isolation, RTPCR and final interpretation of the results. Experiments should not be performed by a single person; a trained observer/ or two people at the least should be in the room to ensure all protocols are correctly followed.

3.6 Staff picking up waste should be trained to appropriately **handle the waste** and provided appropriate PPE.

3.7 Assay principle (to be known by all staff):

Here, COVID 19 is detected based on the presence of viral RNA patient samples. It is a qualitative assay. Detection uses a process called Real Time Polymerase Chain Reaction (RT-PCR).

Two key steps in this process are:

a) RNA extraction/isolation from patient samples
b) amplification and detection of viral RNA using RT-PCR.

Manual as well as kit based assays rely on the differential solubility of RNA in specific organic solutions, to isolate it from patient samples.

In a one-step RT-PCR process, the reaction mix contains following key ingredients:

- Reverse transcriptase that converts RNA to complementary DNA (cDNA).
- Primers that specifically bind to COVID-19
- Probes that specifically bind to COVID-19. Each probe has a fluorophore at the 5' end and a quencher at the 3' end.
- Engineered *Taq* polymerase , dNTPs, buffer for the PCR.

In a positive sample, primers bind to viral cDNA and *Taq* polymerase makes copies of the that stretch of cDNA. The probes bind to copies of the amplified product. During extension, the *Taq* polymerase cleaves the fluorophore from the probe. Thus, more the viral cDNA, more is the free fluorophore, which results in increased fluorescence that is detected by the machine. Probes have different fluorophore, quencher pairs.

In a negative sample, there are no viral cDNA amplicons. Thus, the probe cannot bind a sequence and the fluorophore on the probe remains quenched i.e., there is little to no fluorescence to detect.

The number of cycles at which the detected fluorescence signal exceeds background levels (a default value and the reason why calibration of the instrument is important) is called the threshold cycle (Ct). Lower Ct values imply high levels of target RNA in the patient sample. Conversely, high Ct values imply low levels of target RNA in the patient sample.

There are 3 genes currently being tested for COVID-19: RdRp, E and N. A sequential positive RT-PCR result with any two is currently accepted as a sample positive for COVID-19.

Additional learning:

https://www.fda.gov/media/134922/download

http://dbtindia.gov.in/sites/default/files/OM_Interim_Guidance_COVID.pdf

Part 4:

Which is the correct kit for testing COVID-19?

Please check ICMR website for guidance on approved RT-PCR kits for diagnostic purposes. US FDA EUA/CE IVD approved kits can be used directly after due approval from DCGI and intimation to ICMR.

4.1 ICMR approved kits:

Below non- US FDA EUA/CE IVD real-time RT-PCR kits have been approved for use by ICMR as of 10th April 2020. The list of approved kits is regularly updated by ICMR, which can be accessed here.

Name of Company	Name of the Kit	Concordance among true negative (%)	Concordance among true positive (%)
1. Altona Diagnostics	RealStar SARS-CoV-2 RT-PCR kit 1.0	100	100
2. MY LAB	Patho Detect	100	100
3. KILPEST (BLACKBIO)	TRUPCR	100	100
4. Seegene	Allplex 2019-nCoV assay	100	100
5. SD Biosensor	nCoV Real-Time Detection kit	100	100
6. Huwel Lifesciences	Quantiplus CoV detection kit ver2.0	NA	NA

4.2 Main points of consideration while choosing a RT-PCR kit:

1. Check for approval - ICMR or US FDA EUA/CE IVD
2. Filters for detection of probes. Most kits use FAM, Cy5 and JOE
3. Some kits only provide PCR primers and not reaction master mix for PCR.
4. Some kits are only compatible with proprietary analytics software.
5. Check for true negative and true positive concordance as per ICMR validation results- both rates should be 100%
6. Check for batch numbers of the respective kits as tested and approved by ICMR.

Additional references:

https://www.fda.gov/media/134922/download

Part 5

How do I set up my laboratory for COVID-19 testing?

Ideally the laboratory should be divided into 4 sections:

5.1 Demarcated area for donning on PPE

5.2 Area for sample handling and RNA isolation

5.3 Area for RT PCR

5.4 Demarcated area for doffing of PPE

5.5 Identify trained staff. Guideline: lab in-charge for co-ordination (1); technologists for performing the testing (2-6); data entry operator (1); housekeeping (2). Except housekeeping staff, all personnel should be proficient in all aspects of testing. Housekeeping staff to be educated and tested on proficiency in biological spills management and decontamination protocols.

5.6 Train relevant staff with at least one pilot simulation from sample receipt through to RT-PCR.

5.7 Maintain a list of emergency contacts in case there is need of troubleshooting.

For more info on setting up a BSL2 lab:

https://www.who.int/malaria/areas/diagnosis/molecular-testing-dos-donts/en/

Part 6

What are the procedures for sample collection, receipt and storage?

Checklist (print and cut for future reference)

For Specimen Collection and Transport – Accept sample only after ticking below items

- ☐ Written Consent
- ☐ Test Requisition Form
- ☐ 1 tube of Viral Transport Media (VTM) per sample
- ☐ 1 Dacron swab (synthetic with flexible shaft) per sample
- ☐ Plastic bags for VTM tube transport
- ☐ Ice box/dry ice for transport for transport of multiple samples in plastic bags

Do NOT process / accept the sample if

- VTM tube contents have leaked

6.1 SAMPLE RECEIPT AND STORAGE

PLEASE FOLLOW ALL STEPS OUTLINED IN PARTS 7 AND 8 REGARDING USE OF PPE AND PREPARATION OF WORKPLACE BEFORE PROCEEDING WITH SAMPLE RECEIPT AND PROCESSING

1. Samples will be transported in collection tubes with VTM on ice.

2. Samples will be accompanied by a Test Requisition Form with relevant details.

3. Once received, the sample box has to be directly taken to the BSL-2 facility for further testing.

4. Wipe the external sample container box with 70% ethanol for disinfection, before removing any samples.

5. In the biosafety hood (Minimal requirement for Biosafety cabinet is Class II Type A2), keep the sample collection tubes on ice for thawing or in racks (for thawing at room temperature).

6. At this stage if samples need to be stored, the nasopharyngeal swab sample in the VTM tube is placed in the refrigerator at 4 - 8 °C until processing. Samples received during off-duty hours are kept in the refrigerator at 4 - 8 °C.

7. At the start of sample processing, the patient identification details on the VTM tube are double-checked against the Test Requisition Form.

8. The closed tube is briefly vortexed to bring the contents of the swab into the medium.

9. The tube is then opened and the swab is squeezed along the sides of the tube to express the medium.

10. The swab is then discarded into a discard bin containing 2% Lyzol or 5% freshly-prepared sodium hypochlorite.

11. The VTM contents are aliquoted into pre-labelled screw cap cryovials (150-200 uL per vial). 4 aliquots, sealed with parafilm, are recommended for storage at 4ºC. The remaining VTM is stored at -80°C and are further used for preparation of External Quality Controls (EQC).

12. For Endotracheal aspirates and Bronchoalveolar lavages – the contents of the tube are vortexed and aliquoted into screw cap cryovials similar to nasopharyngeal swab.

13. All tubes should be labelled correctly and legibly with a permanent marker. For e.g., virus_year_site_serialnumber (choose the serial number carefully). nCOV_20_KAR_0001

6.2 TROUBLESHOOTING

Do NOT process / accept the sample if

- VTM tube contents have leaked
- The Test Requisition Form is soiled with leaked contents
- At least two unique patient identifiers missing on sample tube and Test Requisition Form
- Samples not transported in ice pack.

Part 7

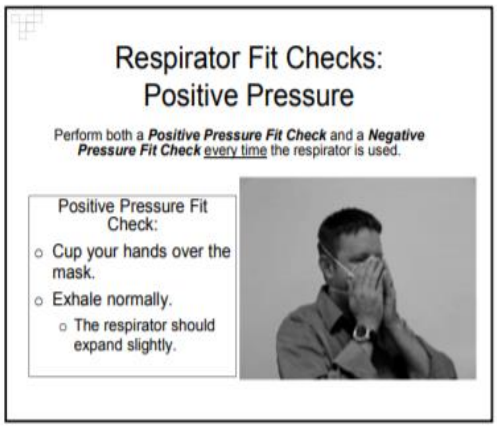
Personal Protective Equipment (PPE) Guidelines

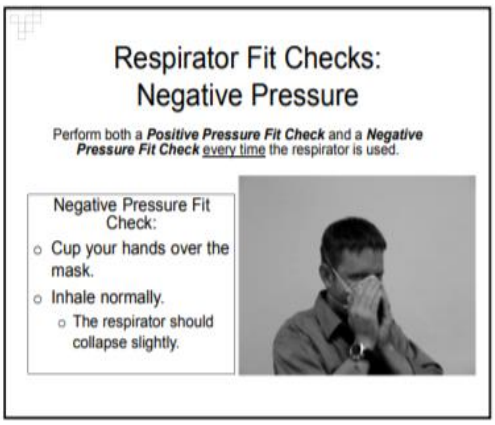
Key points

- All personnel at the testing centre must receive comprehensive training and demonstrate competency in using PPE.
- Allow personnel at least 30min to don and doff the gear. It should be done slowly and mindfully. It gets hot inside the gear, so deodorant is useful!
- An onsite manager must be present at all times.
- Preferably, a trained observer must supervise each step of every PPE donning and doffing.
- Individuals unable to comply with procedures should not carry out the testing procedures.
- **PPE designated area:** Sample aliquot room and DNA/RNA isolation rooms are PPE designated areas. In the rest of the rooms, mask and goggles along with lab coat will be sufficient.

7.1. Donning of PPE

1. Leave all personal belongings including gadgets, watches, rings etc, outside. Tie long hair back. Facial hair – beards, mustache etc – should be shaved.
2. Wash your hands with soap and water (recommended) or wipe with 70% alcohol before entering the facility.
3. Once you have entered the facility, wear assigned lab coat and shoe cover.
4. Enter the PPE donning area. Unpack PPE kit (surgeon gown, N-95 mask, Head cover, goggles, shoe cover and a pair of gloves). If gloves are powdered, do not use them for molecular biology work; use purple nitrile instead.
5. Follow the order to wear: gown→gloves→N95→goggles→disinfect hands. A printable visual procedure is included in the next page.
6. Ensure N95 is fit properly before putting on goggles. If air leaks between the face and face seal of the respirator when performing either fit check, readjust the nose clip, reposition the respirator and performs both pressure fit checks again.





7. Adjust PPE and make yourself comfortable**. After starting your work, DO NOT touch PPE**.
8. Before starting your work, wear another pair of gloves on the existing one**. It is very important to wear double gloves while handling pathogens.**

7.2 During Testing:

1. PPE must remain in place and be worn correctly for the entire duration.
2. PPE should not be adjusted
3. In the event of a significant splash, the personnel should immediately move to the doffing area to remove PPE.
4. Exception: visibly contaminated outer gloves can be changed while in the testing area and testing can continue. Contaminated outer gloves can be disposed of in the biohazard yellow bag.

7.3 Doffing

1. Discard outer pair of gloves.
2. Call an observer. Sometimes an assistant is required. Remove PPE in this order googles→gown→mask→gloves.
3. Ensure each time is discarded in the appropriate waste bin. PPE MUST NOT be recycled.
4. Once you exit PPE doffing area, wash hands thoroughly.
5. If there is showering onsite, change attire before going home. If not, get home as safely as possible, remove all clothes and shower. Clothes can be washed in regular wash cycle. Try not to keep the clothes around for too long.

7.4 Training on Correct Use of PPE

- The following elements are essential for PPE training:
    - How to safely don, adjust, use, and doff the specific PPE that the lab personnel will use;
    - How to safely conduct routine testing ;
    - Limitations of the PPE (e.g., duration of use, degree of protection);
    - What to do in the case of an equipment failure or detection of a breach in PPE;
    - How to maintain PPE and appropriately dispose of it after use; and
    - The possible physiologic strain associated with using PPE, and how to recognize and report early signs and symptoms, such as fatigue.
- Training must be interactive and should allow personnel to practice
- Institutes should ensure personnel understand training content and can correctly perform the required tasks.
- Regular refresher training is essential to maintaining these skills.

7.5 Use of a Trained Observer

- A trained observer can recite the steps to don and doff PPE.
- The observer should NOT provide physical assistance to lab personnel during donning & doffing, unless it is an emergency.
- The observer should be able to troubleshoot and understand the exposure management plan in the event of an unintentional break in procedure.
- Where a trained observer is not available, a senior lab member is to read out the steps to personnel donning and doffing PPE.

7.4. Designating Areas for PPE Donning and Doffing

- Ensure a designated area for donning and doffing PPE. It should be distinct from the testing area. Ideally, there is one-way flow from the donning area to the testing care area to the doffing area.

- Confirm that area is large enough to allow freedom of movement for safe doffing as well as space for a waste receptacle, a new glove supply, and alcohol-based hand rub (ABHR) used during the doffing process.
- Post signage to highlight key aspects of PPE donning and doffing, including
    - Designating clean areas vs. contaminated areas
    - Reminder to wait for a trained observer
    - Listing each step of the doffing/donning procedure
    - Reinforcing the need for slow and deliberate procedure.

7.6 Designate the following areas with appropriate signage

1. PPE Storage and Donning Area

- A clean area outside the testing room (e.g., a nearby vacant testing room, a marked area in the hallway outside the testing room) where clean PPE is stored.
- Do not store potentially contaminated equipment (e.g., PAPR components that have not been cleaned and disinfected), used PPE, or waste removed from the testing's room in the clean area.
- If waste must pass through this area, it must be properly contained.

2. PPE Doffing Area

- Designate an area near the testing's room (e.g., anteroom or adjacent vacant testing room that is separate from the clean area) where lab personnel leaving the testing room can stand to doff and discard their PPE.
- Alternatively, some steps of the PPE removal process may be performed in a clearly designated area of the testing's room near the door, provided these steps can be seen and supervised by a trained observer (e.g., through a window and provided that the lab personnel doffing PPE can hear the instructions of the trained observer).
- Do not use this designated area within the testing room for any other purpose.
- Stock gloves in a clean section of the PPE removal area accessible to the lab personnel while doffing.

- In the PPE removal area, provide supplies to disinfect PPE and perform hand hygiene and space to remove PPE, including an easily cleaned and disinfected seat where lab personnel can remove boot or shoe covers.
- If space allows, designate stations around the perimeter of the doffing room where each piece of PPE will be removed, moving from more contaminated to less contaminated areas of the room as PPE is doffed.
- Provide leak-proof disposable infectious waste containers for discarding used PPE.
- Frequently clean and disinfect the PPE removal area, including after each doffing procedure has been completed. One way such cleaning may be achieved is by having another lab personnel who has just donned their full PPE clean the doffing area, moving from cleaner to dirtier areas within the doffing area, before entering the testing's room.
- Facilities should consider making showers available for use for the comfort of lab personnels after doffing PPE at the end of their shift; the heat from wearing PPE is likely to cause significant perspiration.

7.6 What activities require what kind of PPE

If you have physically separate areas for sample processing, RNA isolation and RT-PCR, this guideline is effective. IF there is no physical barrier, full PPE is best.

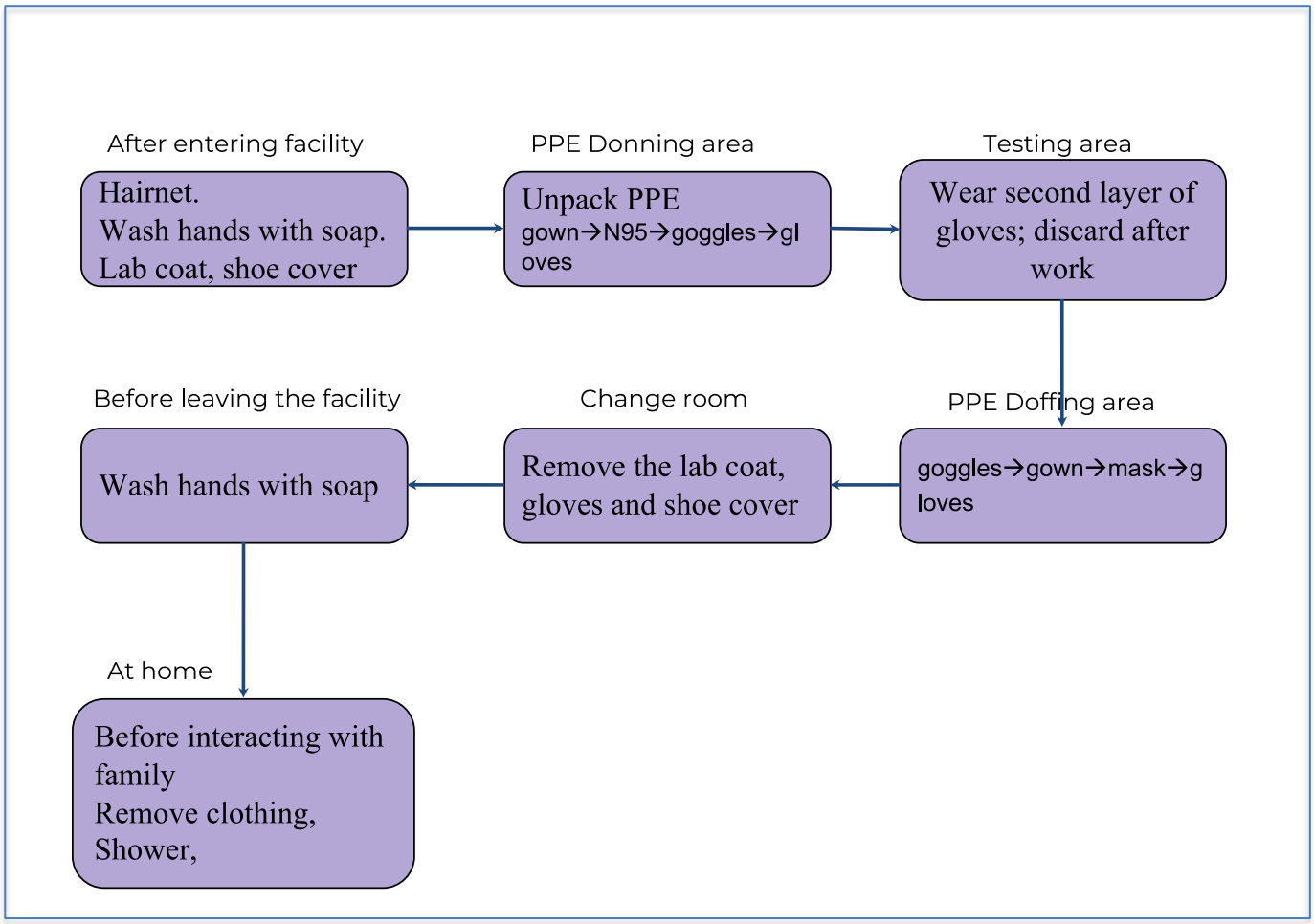
Sample storage	Full PPE
RNA isolation	Full PPE
RT-PCR	Lab coat, gloves, surgical mask may be sufficient.

7.7 References:

1. https://www.cdc.gov/vhf/ebola/clinicians/cleaning/waste-management.html

2. https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment_data/file/877658/Quick_guide_to_donning_doffing_standard_PPE_health_and_social_care_poster__.pdf
3. https://www.bu.edu/ehs/files/2014/08/N95-Respirators-Training.pdf
4. https://www.who.int/csr/resources/publications/biosafety/Biosafety7.pdf?ua=1

FLOW CHART FOR PPE



Sample storage	Full PPE
RNA isolation	Full PPE
RT-PCR	Lab coat, gloves, surgical mask may be sufficient.

CDC has downloadable images for donning and doffing PPE available at:

https://www.cdc.gov/hai/pdfs/ppe/PPE-Sequence.pdf

Print these images and stick in appropriate areas of the laboratory

Part 8

How do I set up the testing area?

Checklist (print and cut for future reference)

Material

- **Required pipettes**
- **Filter Pipette Tips**
- **70% alcohol**
- **Waste bins/biohazard bags**
- **Tissue rolls**
- **Eppendorf rack**
- **Waste bucket with 1% sodium hypochlorite**
- **Markers**

Protocol for preparation:

8.1: Unpacking sample (Mandatorily to be done in a BSL2 or BSL3 hood)

1. Keep 1% sodium hypochlorite ready for wiping the sample box.
2. Keep tubes labelled for aliquoting sample.

8.2: RNA isolation: (Mandatorily to be done in a BSL2 or BSL3 hood)

1. Clean hood before introduction of new samples.
2. Turn on UV in the hood for at least 30 minutes before use.
3. All pipettes to be used should be cleaned thoroughly with 1% sodium hypochlorite, water and then 70% ethanol.

4. Wipe down hood with 70% ethanol before starting the experiment.
5. Place all necessary items - pipettes, tips, 70% alcohol, tissue roll, eppendorf rack and RNA isolation reagents in the hood before introducing samples to be tested.
6. Mark all tubes before hand and keep sample interaction to the minimum.
7. Create worksheets with protocol flowchart and calculations ready.

8.3: RT PCR set up (Preferably to be done in a separate BSL2 hood; if another BSL2 hood is not available, a normal laminar hood may be used)

1. Clean hood before introduction of new samples.
2. Turn on UV in the hood for at least 30 minutes before use.
3. All pipettes to be used should be cleaned thoroughly with 1% sodium hypochlorite, water and then 70% ethanol.
4. Wipe down hood with 70% ethanol before starting the experiment.
5. Place all necessary items - pipettes, tips, 70% alcohol, tissue roll, eppendorf rack and RT PCR reagents in the hood before introducing samples to be tested.
6. Mark all tubes before hand and keep sample interaction to the minimum.
7. Create worksheets with protocol flowchart and calculations ready.

8.4 Routine decontamination of lab area

1. Virus aerosols are notorious for sticking around at several surfaces. It is important therefore to have a fixed decontamination schedule, preferably once every week with includes wiping down all surfaces including door knobs. Maintain a sheet of all surfaces. Proceed to wipe down each systematically. Procedure: wipe with 4% hypochlorite, wipe with alcohol, spray DNA away® and RNA zap® . For hood, turn on UV on for 30 minutes before and after each batch of samples are processed.
2. The best practice is to do a RT-PCR with the wipe swabs to ensure that these surfaces still test negative as amplicon contamination could be a major issue during testing.

8.5: Troubleshooting:

1. What if I run out of ethanol?

   70% isopropanol can be used as a substitute for 70% ethanol. If neither is available, do not accept any samples for processing.

2. If I don't have filter tips - would normal tips be okay to use?

   No. Use only filter tips for processing samples.

8.6 WHO guideline for laboratory biosafety guidance.

https://apps.who.int/iris/rest/bitstreams/1272450/retrieve

Part 9

How to proceed with RNA isolation?

This section deals with extraction of viral RNA using the Qiagen Viral RNA Mini kit. Catalog # 52904 (50); 52906 (250). Protocols have been adapted from the Qiagen handbook and manuals (see references). *If purchasing a commercial kit, check if it includes RNA isolation reagents; if yes, follow vendor protocol.*

Two methods are detailed:

9.1 Manual

9.2 Automatic, using QIAcube

9.1 Manual Extraction:

Kit Contents

- Spin columns
- Collection tubes (2mL)
- AVL Buffer
- AW1 Buffer
- AW2 Buffer
- AVE Buffer
- Carrier RNA

Required equipment and consumables

- BSL2 Hood
- Vortex mixer *(preferably inside the hood)*
- Micropipettes (Sterile, RNase free, 20 µL, 200 µL & 1000 µL)
- Pipet tips (Sterile, RNase free, filter tips)
- 1.5 ml microfuge tubes (Sterile, RNase free, screw cap)
- 2ml microcentrifuge tubes (Sterile, RNase free, screw cap)
- Racks for 1.5 mL and 2ml microcentrifuge tubes
- RNASe Zap (Invitrogen cat no: AM9782)
- Ethanol (high purity, For e.g., Sigma Molecular Biology E7023)
- 70% ethanol for disinfection
- Microcentrifuge (with rotor for 1.5 ml and 2 ml tubes)
- 1% Hypochlorite (reagent grade Sodium hypochlorite; e.g., Sigma/ Merck 105614)
- PPE: Gloves, mask, goggles
- Benchtop minicentrifuge *(optional) (preferably inside the hood)*

** Note: if AVL/AW1 with infectious material spills then use detergent and water to mop up and then 1% Sod. hypochlorite. Do not add bleach right away*

Checklist (print and cut for future reference)

Before starting the isolation, do the following…

1. **Carrier RNA- AVE mix**: Add 310µL of buffer AVE to 310µg carrier RNA. Dissolve thoroughly. Vortex and spin down. Aliquot this into microfuge tubes and store at -20◦C. Do not freeze thaw more than 3 times.
2. **AW1 buffer:** Add ethanol to the bottle: 44mL (50 nos kit); 130mL (250 nos kit). Store at RT.
3. **AW2 buffer**: Add ethanol to the bottle: 30mL (50 nos kit); 160mL (250 nos kit). Store at RT.
4. **AVL buffer:** Ensure it does not have crystals. If a precipitate is observed, place in water bath of up to 80◦C, until the precipitate dissolves.
5. **AVE buffer**: Bring to RT

9.1.1 RNA Extraction. (All steps to be performed at room temperature (RT))

1. Turn on UV in the hood for at least 20mins before starting.
2. Clean all surfaces of the hood, pipettes and tip boxes with RNAse ZAP, 1% hypochlorite, water and finally with 70% isopropanol. Ensure sufficient tips are in place for processing samples and you have access to enough 2mL and 1.5mL (not in the kit/ ~3 per sample) microfuge tubes. Ensure you have a disposal box set up, with a red bag (hazardous), lined with a small layer of 1% hypochlorite.
3. Withdraw samples from storage. Bring to RT. Count the number of samples to be processed. Calculate how much carrier RNA + AVL buffer (AVL*) is required. Table below lists volumes to prepare. This buffer has to be prepared fresh for each round of RNA isolation.

# of samples	AVL (mL)	AVE+Carrier RNA (µL)	# of samples	AVL (mL)	AVE+Carrier RNA (µL)
1	0.56	5.6	**13**	7.28	72.8
2	1.12	11.2	**14**	7.84	78.4
3	1.68	16.8	**15**	8.4	84
4	2.24	22.4	**16**	8.96	89.6
5	2.8	28	**17**	9.52	95.2
6	3.36	33.6	**18**	10.08	100.8
7	3.92	39.2	**19**	10.64	106.4
8	4.48	44.8	**20**	11.2	112
9	5.04	50.4	**21**	11.76	117.6
10	5.6	56	**22**	12.32	123.2
11	6.16	61.6	**23**	12.88	128.8
12	6.72	67.2	**24**	13.44	134.4

4. Remove aliquot of Carrier RNA-AVE mix from -20. Bring to RT. Vortex, spin down. *IMP* If working in BSL3 and having no access to vortex, invert the tube 10-15 times to mix contents.
5. In a fresh tube of appropriate size, dispense the AVL buffer. Add in Carrier RNA-AVE. Vortex. Keep at RT. This is **AVL*.**
6. Label 1.5mL microfuge tubes. Add 560µL of **AVL*** into each tube.
7. Add 140µL of VTM sample into respective tubes. Use a fresh tip for each sample. Close tube after dispensing each sample. Vortex briefly to mix well and spin down by pulse centrifuging at 8000 rpm/10s.

8. Incubate at RT for 10mins. *(This step enables viral lysis. Longer incubation times have no effect on the yield or quality of the purified RNA.)*
9. Add 560µl ethanol to the tube. Vortex briefly and spin down by pulse centrifuging at 8000 rpm/ 10s.
10. Carefully apply 630µl of above solution to the QIAamp spin column with 2ml collection tube without wetting the rim. Close the cap, and centrifuge at 6000 x g (8000 rpm) for 1min, RT. *Close each spin column as soon as the contents dispensed to avoid cross-contamination during centrifugation.* Ensure caps are labelled.
11. Place the QIAamp spin column into a new 2ml collection tube, and discard the tube containing the filtrate.
12. Carefully open the QIAamp spin column, and transfer the remaining liquid (~630µL) from step # 8. Discard the empty sample lysis tube. Repeat centrifugation at 8000 rpm for 1 min.
13. Place the QIAamp spin column in a clean 2 ml collection tube (provided), and discard the tube containing the filtrate.
14. Carefully open the QIAamp Mini column and add 500µl Buffer AW1. Close the cap, and centrifuge at 6000 x g (8000 rpm) for 1 min, RT.
15. Place the QIAamp spin column in a clean 2 ml collection tube (provided), and discard the tube containing the filtrate.
16. Carefully open the QIAamp Mini column and add 500 µl Buffer AW2. Close the cap and centrifuge at full speed (20,000 x g; 14,000 rpm) for 3 min, at RT.
17. Place the QIAamp Mini column in a new screw cap 1.5ml microfuge tube (not provided) and discard the old collection tube with the filtrate. Centrifuge at full speed (20,000 x g; 14,000 rpm) for 1min.
18. Open the QIAamp mini column inside the laminar flow hood and switch off the air and keep for 2 minutes to air dry.
19. Place the QIAamp Mini column in a new 1.5ml microfuge tube (not provided) and add 60µl **Buffer AVE** equilibrated to room temperature.
20. Close the cap, and incubate at room temperature for 2 min.
21. Centrifuge at 6000 x g (8000 rpm) for 1 min.

22. Label the tube with ID number of the sample, date of extraction and the test to be done.
23. Place the eluted RNA on ice. If not immediately proceeding for qRT-PCR, samples may be stored at -80^{o}C.
24. Clean all surfaces of the hood, pipettes and tip boxes with RNAse ZAP, 1% hypochlorite, water and finally with 70% isopropanol. Remove the discard box and place it in hazardous waste.
25. UV should be ON for 20 min before the next batch of the RNA isolation.

9.2 AUTOMATED RNA isolation using QIACUBE

This protocol has been adapted from the following Qiagen resources listed in the appendix. It is advisable to read the protocol and manual for the machine before starting with this procedure.

9.2.1 Setting up QIAcube:

1. Turn on UV in the hood for at least 20mins before starting.
2. Wipe down all work surfaces and all parts of the machine with 70% ethanol. If need be, use mild detergent. DO NOT use 1% hypochlorite for disinfection. DO NOT spray ethanol (wipe down instead) onto the machine unless parts are disassembled and lined up far from electronics.
3. Withdraw samples from storage. Bring to RT.
4. Prepare the samples and reagents to be used in the protocol. Refer to the section below for sample and reagent preparation
5. Close the instrument door.
6. Switch on the QIAcube at the power switch.
7. The beeper sounds, and the startup screen appears.
8. The instrument automatically performs initialization tests.
9. Select the appropriate application from the main menu by pressing “Virus”.
10. Select the correct kit name by pressing “⇑” or “⇓” to scroll through the list until the required protocol QIAamp Viral RNA MinElute spin is highlighted, then press “Select”.

11. Select the sample material "bodily fluids" by pressing "⇑" or "⇓" to scroll through the list until the required sample material is highlighted, and then press "Select".

12. Select the protocol by pressing "⇑" or "⇓" to scroll through the list until the required protocol QIAAMP Viral RNA MinElute spin is highlighted, then press "Select".

13. To start the protocol run, press "Start" and proceed to Open the worktable door.

14. Follow the instructions in the protocol sheet and displayed in the touchscreen for loading the work table with samples, reagents, and labware

15. You will be instructed to

    A. Empty the waste drawer.

    B. Place prefilled tip racks onto the worktable.

    C. Fill the reagent bottles and place the reagent bottle rack onto the work table. Be sure to remove the lids from the reagent bottles before placing on the worktable.

    D. Load opened accessory-buffer tubes required by the protocol in positions A, B, or C.

    E. Place the loaded rotor adapters into the centrifuge buckets.

    F. Load the shaker rack with samples, and place onto the shaker. Be sure to use the correct sample tubes and the correct shaker adapter, as described in the user manual (see pages 5-16).

16. Close the QIAcube door.

17. After you have set up the worktable, the QIAcube performs a load check, which consists of the following tasks:

    - The instrument is initialized.
    - The shaker is checked to make sure it is correctly loaded.

18. The number of rotor adapters in the centrifuge is checked to make sure that it corresponds to the number of samples in the shaker and that the rotor is correctly loaded.

19. The reagent bottle rack is checked to make sure that it is correctly loaded and there is sufficient buffer in the reagent bottles. *Note: The volume of accessory buffers in positions A, B, or C on the worktable is not checked. Be sure to fill the microcentrifuge tubes with the exact volume of buffer required.*

20. The filter-tips are checked to make sure that the correct type has been loaded and that there are sufficient filter-tips for the protocol run.

21. If the load check is unsuccessful, an error message will be displayed in the touchscreen. Resolve the error so that the protocol run can continue (see Section 7.2.1 of user manual). Note: When resolving a load check error, only move or change the affected samples or consumables.

22. End of the protocol run: When the protocol run has finished, a message is displayed in the touchscreen confirming that the samples have been processed.

23. Follow the instructions in the touchscreen for work table cleanup.

    1. Remove the microcentrifuge tubes containing purified nucleic acids or proteins from the rotor adapters.

    2. Discard sample tubes, used rotor adapters, and reagents according to your local safety regulations.

    3. Replace the lids of the reagent bottles and close tightly. Store the bottles according to the instructions in the relevant kit handbook.

    4. Empty the waste drawer.

    5. Run another protocol, or switch off the QIAcube.

24. Stopping a protocol: You can stop a QIAGEN protocol if there is an emergency by pressing “Cancel”. To confirm that you want to stop the protocol run, press “OK”. To cancel the stop protocol command, press “Cancel”.

    Note: If a protocol run is stopped, the run cannot be restarted; the samples must be processed manually

Internal features of the QIAcube

Internal view of the QIAcube.

1. Centrifuge lid
2. Centrifuge
3. Shaker
4. Reagent bottle rack
5. Tip sensor
6. Microcentrifuge tube slots
7. Tip racks
8. Disposal slots for tips and columns
9. Robotic arm

9.2.2. Setting up the Work table:

1. Loading Tips: Start with **positions # 7 & 8**. Tip racks. There are two slots for tip racks. For the Qiagen Mini Viral kit, only 1000uL tips are required. Fill the tips rack appropriately using the notch provided, the rack for 1000 µl filter-tips (light grey colored) has a notch at the left rear side as shown in (Figure below). Use only filter tips compatible with QIAcube. Qiagen: Cat No./ID: 990352 (1000uL). See below.

2. Loading Buffer AVE and Carrier RNA in Microcentrifuge tube slots (**position # 6**): a. Count the number of samples to be processed. Calculate how much carrier RNA is required. Refer to table below.

Number of samples	**Buffer AVE(µL)** **Position B**	**Diluted carrier RNA(µL)** **Position C**
2	256	125 (28 carrier RNA + 97 Buffer AVE)
3	364	150 (33.6 carrier RNA + 116.4 Buffer AVE)
4	472	175 (39.2 carrier RNA + 135.8 Buffer AVE)
5	580	200 (44.8 carrier RNA + 155.2 Buffer AVE)
6	688	225 (50.4 carrier RNA + 174.6 Buffer AVE)
7	796	250 (56 carrier RNA + 194 Buffer AVE)
8	904	275 (61.6 carrier RNA + 213.4 Buffer AVE)
9	1012	300 (67.2 carrier RNA + 232.8 Buffer AVE)
10	1120	325 (72.8 carrier RNA + 252.2 Buffer AVE)
12	1336	375 (84 carrier RNA + 291 Buffer AVE)

3. Remove aliquot of Carrier RNA from -20. Allow to come to RT. Vortex, spin down.

4. In a 2mL screw cap tube without a skirted base (Imp), dispense the appropriate amount of carrier RNA and buffer AVE. Place it in microcentrifuge tube slot Position C.

5. In a 2mL screw cap tube without a skirted base, dispense the appropriate amount of Buffer AVE and place it in microcentrifuge tube slot Position B.

6. Loading Reagent bottle rack: **Position # 4.**

   a. Attach the rack labeling strip by sliding it onto the flange at each side of the rack. The labeling strips fit onto the rack only in the correct orientation.

   b. The reagent bottle rack must only be used with the labeling strips attached. Carefully fill each bottle with a buffer, and place into the appropriate position in the reagent bottle rack indicated by the rack labeling strip. (Figure below).

   c. Pour carefully to ensure that the buffer does not foam or contain large air bubbles. Reagent volumes are checked during the load check at the start of the protocol run.

Reagent Bottle Rack

Rack labeling strip	QIAamp Viral RNA

Position	Reagent
1	Buffer AVL
2	100% ethanol
3	Buffer AW1
4	Buffer AW2
5	–
6	–

Loading the reagent bottle rack.

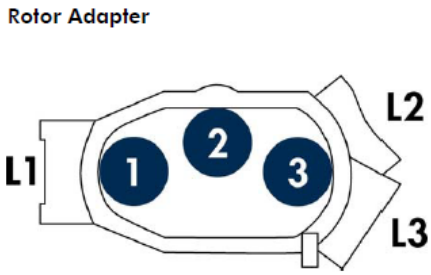
7. Loading Rotor Adaptors: **Position #2**

a. Rotor adapters need to be fitted into centrifuge buckets.

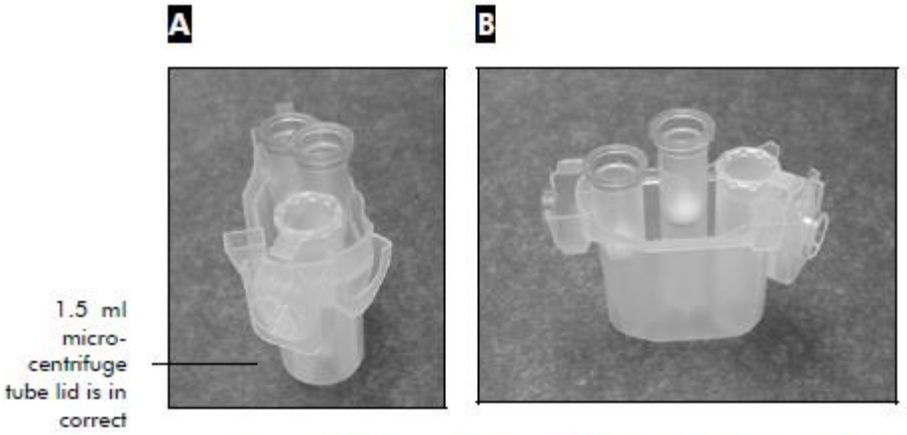
b. Place 1.5 ml microcentrifuge tubes (supplied with the rotor adapters) and QIAGEN spin columns into the appropriate positions in each rotor adapter as described in Figure below.

c. Ensure the 1.5 ml microcentrifuge tubes (comes with the bulk kit) and spin columns have been pushed firmly into the appropriate position.

d.



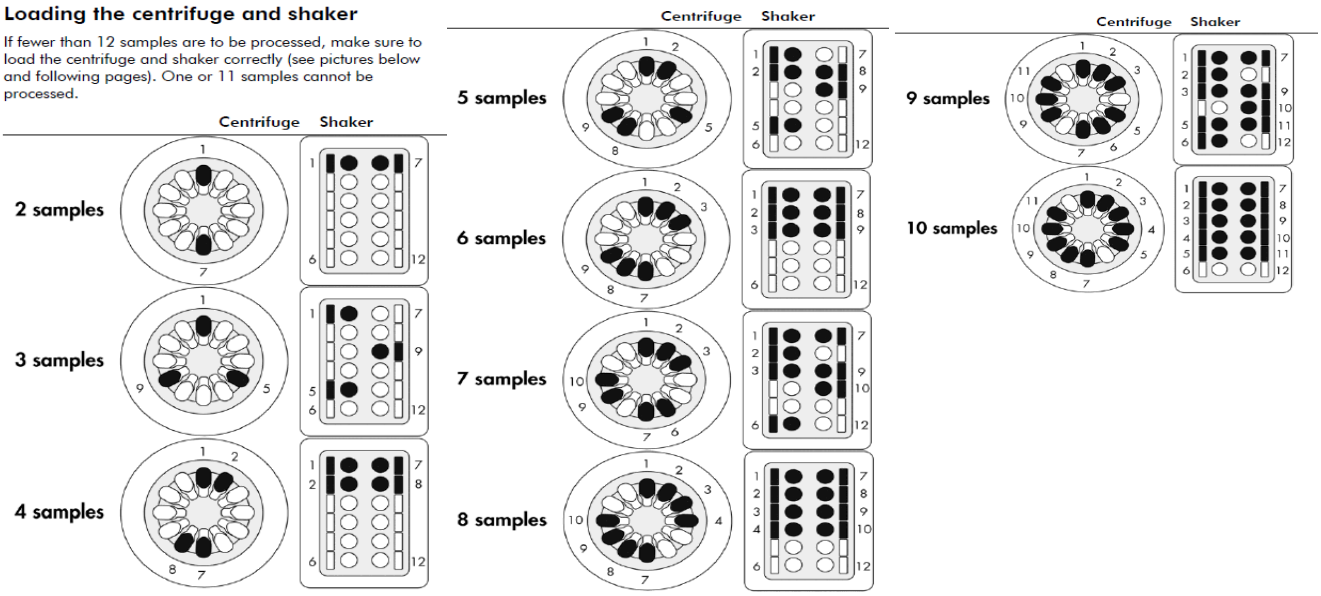
Position	Labware	Lid position
1	QIAamp Mini spin column	L1
2	–	–
3	1.5 ml collection tube†	L3



Correctly loaded rotor adapter. A Rotor adapter is correctly loaded and the 1.5 ml microcentrifuge tube lid is in the correct position; B Correctly loaded rotor adapter seen from the side.

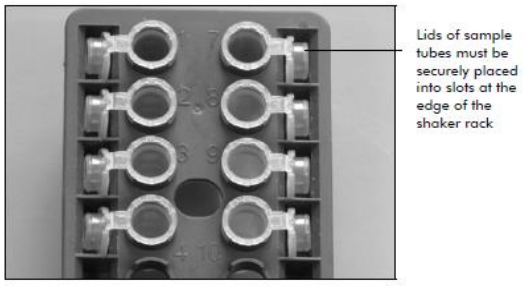
8. Place the loaded rotor adapters into the centrifuge buckets when instructed to do so by the software. For ease of use and high process safety, the rotor adapters only fit into the centrifuge buckets in one orientation.

Note: If processing fewer than 12 samples, make sure to load the centrifuge rotor symmetrically, you would typically for a centrifuge. I.e., it has to be balanced. Refer below.



9. Loading Sample tubes on Shaker:

    a. 140 uL of VTM sample is aliquoted to 1.5 ml microfuge tube (under BSL2 Cabinet). Sample tubes are set on the shaker as shown below.



Loading the shaker rack with samples tubes that have attached lids.

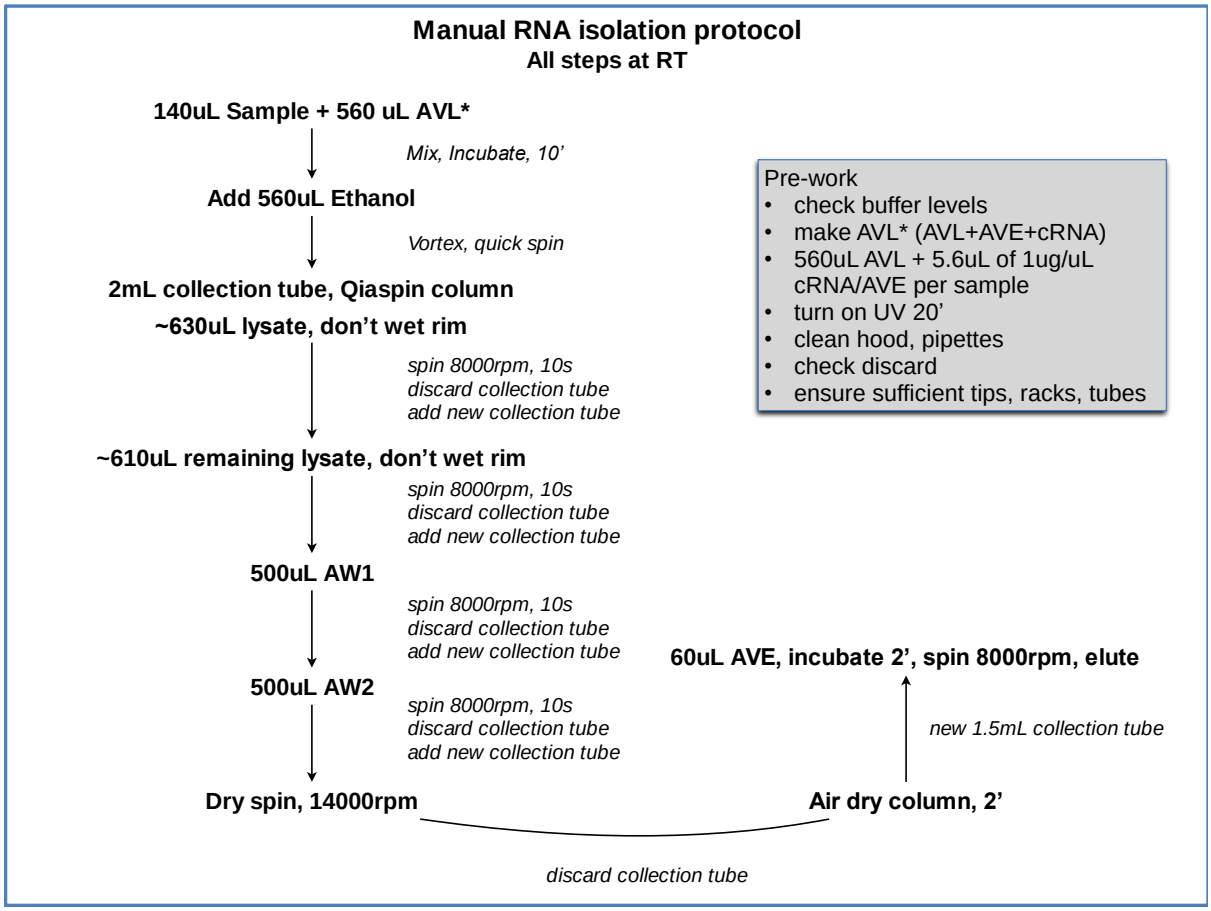
Note: If processing fewer than 12 samples, make sure that the shaker is correctly loaded. Refer above.

9.3 References:

1. QIAamp Viral RNA mini handbook https://www.qiagen.com/in/resources/resourcedetail?id=c80685c0-4103-49ea-aa72-8989420e3018&lang=en
2. Qiacube Specific Protocols(all): https://www.qiagen.com/in/qiacube/standard/search/
3. Qiacube viral RNA protocol sheet : https://www.qiagen.com/in/qiacube/standard/search/?ApplicationTypeID=2&ProductLineID=1000199&MaterialTypeID=0
4. QIAcube user manual https://www.qiagen.com/kr/resources/download.aspx?id=f7d77c6e-0479-4b2b-a2e0-5ca747114e34&lang=en

FLOW CHART

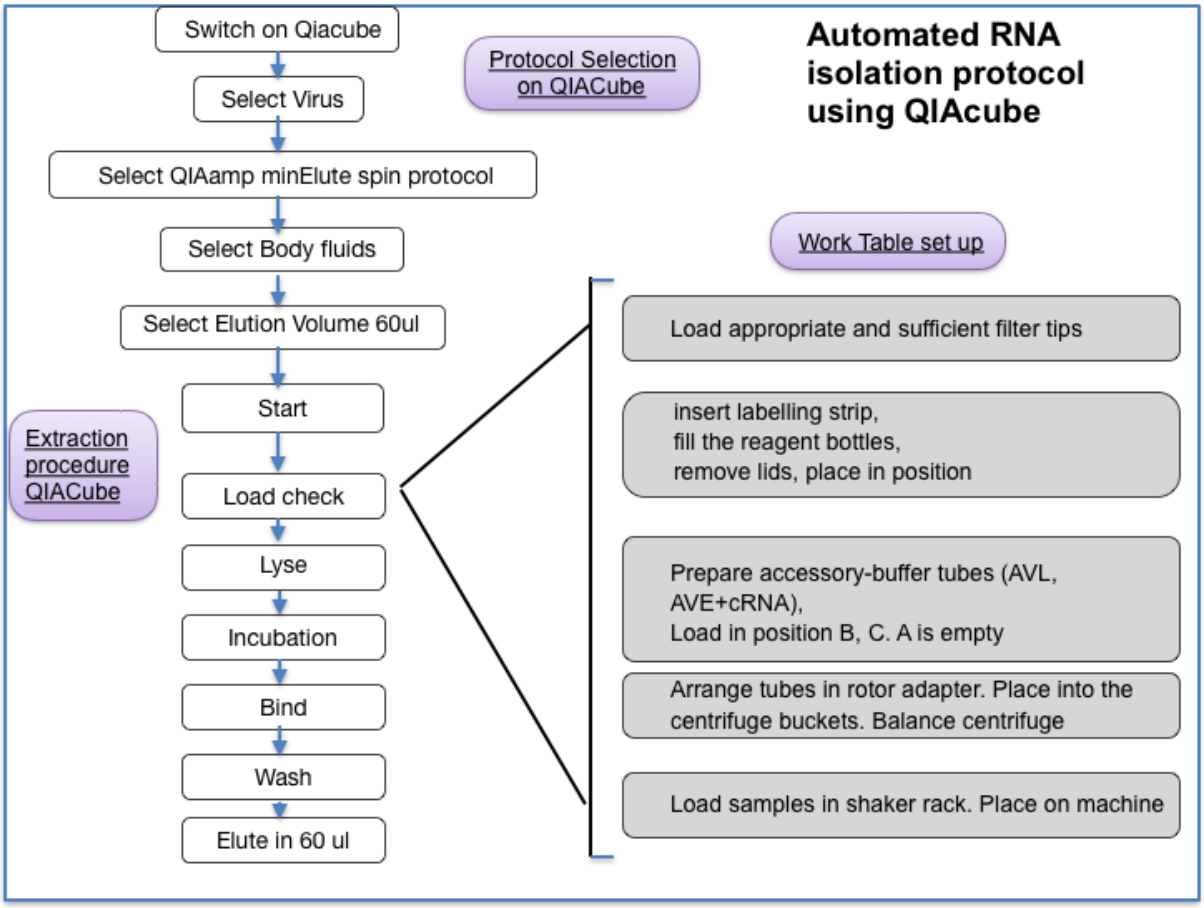
MANUAL RNA ISOLATION using Qaigen kit



Spin step: If there is no access to a vortex, invert the tube 10-15 times to mix contents thoroughly.

FLOW CHART

AUTOMATED RNA ISOLATION using QIAcube



Part 10

How to proceed with RT PCR?

Checklist (print and cut for future reference)

Equipment

- Calibrated micropipette set (2 or 10 µl, 200 µl and 1000 µl)
- Refrigerated microcentrifuge
- Calibrated Real Time PCR machine
- Real time PCR machine with detection channels for probe.

Reagents/consumables

- Primer: probe mix (PP mix)
- Molecular grade water, nuclease-free
- One step RT-PCR Kit (like Superscript IIITM one step RT PCR kit)
- 96-well plate for RT-PCR with covering seal recommended by the manufacturer
- aerosol resistant barrier tips for respective pipettes
- 0.5ml Eppendorf vials
- 1% sodium hypochlorite
- Dedicated racks, trays for transport and bench

If buying commercial kit, follow vendor instructions.

10.1 Reaction Master Mix and Plate Set Up

Note: Plate set-up configuration can vary with the number of specimens and workday organization. NTCs (no template controls) and nCoVPCs (positive control) must be included in each run.

1. PCR reactions are to be set up in a clean hood different from the hood in which RNA extraction was carried out.
2. Place rRT-PCR buffer, enzyme, and primer/probes on ice or cold-block. Keep cold during preparation and use.
3. Mix buffer, enzyme, and primer/probes by inversion 5 times. Centrifuge reagents and primers/probes for 5 seconds to collect contents at the bottom of the tube, and then place the tube in a cold rack.
4. Label one 1.5 mL microcentrifuge tube for each primer/probe set. Determine the number of reactions (N) to set up per assay. It is necessary to make excess reaction mix for the NTC, nCoVPC, HSC (if included in the RT-PCR run), and RP reactions and for pipetting error.

Use the following guide to determine N:

• If number of samples (n) including controls equals 1 through 14, then N = n + 1

• If number of samples (n) including controls is 15 or greater, then N = n + 2

For each primer/probe set (Table below), calculate the amount of each reagent to be added for each reaction mixture (N = # of reactions).

An example using Thermo Fisher Thermo Fischer /Invitrogen SuperScriptIII

E assay:

MasterMix:	25µl single rxn, µl
H_2O (RNAse free)	2.6
2x Reaction mix*	12.5
$MgSO_4$(50mM)	0.4
BSA (1 mg/ml)**	1
Fwd primer (10 µM)	1
Rev primer (10 µM)	1
Probe (10 µM)	0.5
SSIII/Taq EnzymeMix*	1
	20
Template RNA	5

Cycler:	
55°C 10'	
94°C 3'	
94°C 15"	45x
58°C 30"	
40°C 30"	
' = minutes; " = seconds	

OneStep RT-PCR System with Platinum Taq DNA Polymerase is given below:

5. Dispense reagents into each respective labeled 1.5 mL micro centrifuge tube. After addition of the reagents, mix reaction mixtures by pipetting up and down. Do not vortex. Centrifuge for 5 seconds to collect contents at the bottom of the tube, and then place the tube in a cold rack.

6. Set up reactions in a 96-well cooler rack. Dispense 15 µL of each master mix into the appropriate wells. Prior to moving to the nucleic acid handling area, prepare the No Template Control (NTC) reactions for column #1 in the assay preparation area.

RdRp- and N assay:

MasterMix:	25µl single rxn, µl
H_2O (RNAse free)	1.1
2x Reaction mix*	12.5
$MgSO_4$(50mM)	0.4
BSA (1 mg/ml)**	1
Fwd primer (10 µM)	1.5
Rev primer (10 µM)	2
Probe (10 µM)	0.5
SSIII/Taq EnzymeMix*	1
	20
Template RNA	5

Cycler:	
55°C 10'	
94°C 3'	
94°C 15"	45x
58°C 30"	
40°C 30"	
' = minutes; " = seconds	

7. Pipette 5 µL of nuclease-free water into the NTC sample wells (Figure below, column 1). Securely cap NTC wells before proceeding. Cover the entire reaction plate and move the reaction plate to the specimen nucleic acid handling area.

10.2 Nucleic Acid Template Addition

1. Gently vortex nucleic acid sample tubes for approximately 5 seconds. Centrifuge for 5 seconds to collect contents at the bottom of the tube. After centrifugation, place extracted nucleic acid sample tubes in the cold rack.
2. Samples should be added to columns 2-11 (column 1 and 12 are for controls) to the specific assay that is being tested as given in Figure below. Carefully pipette 5.0 µL of the first sample into all the wells labeled for that sample (i.e. Sample "S1" down column #2). Keep other sample wells covered during addition. Change tips after each addition.
3. Securely cap the column to which the sample has been added to prevent cross contamination and to ensure sample tracking.
4. Change gloves often and when necessary to avoid contamination.
5. Repeat steps #2 and #3 for the remaining samples.
6. Cover the entire reaction plate and move the reaction plate to the positive template control handling area.

10.3 Assay Control Addition

1. Pipette 5 µL of nCoVPC RNA to the sample wells of column 12 (see figure below).
2. Securely cap wells after addition of the control RNA.
3. Briefly centrifuge reaction tube strips for 10-15 seconds. After centrifugation return to cold rack. NOTE: If using 96-well plates, centrifuge plates for 30 seconds at 500 x g, 4°C.

Example of Sample and Control Set-up:

	1	2	3	4	5	6	7	8	9	10	11	12
A	NTC	S1	S2	S 3	S 4	S 5	S 6	S 7	S 8	S 9	S 10	Positive Control
B	NTC	S1	S2	S 3	S 4	S 5	S 6	S 7	S 8	S 9	S 10	Positive Control
C	NTC	S1	S2	S 3	S 4	S 5	S 6	S 7	S 8	S 9	S 10	Positive Control
D												
E												
F												
G												
H												

List of Primer and Probe used in the reaction:

Gene	Id	Sequence	Concentration
RdRp Gene	RdRP_SARSr-F2	GTGARATGGTCATGTGTGGCGG	use 600 nM per reaction
	RdRP_SARSr-R1	CARATGTTAAASACACTATTAGCATA	use 800 nM per reaction
	RdRP_SARSr-P2	FAM-CAGGTGGAACCTCATCAGGAGATGC-BBQ	Specific for Wuhan-CoV, will not detect SARSCoV use 100 nM per reaction and mix with P1
	RdRP_SARSr-P1	FAMCCAGGTGGWACRTCATCMGGTGATGCBBQ	Pan Sarbeco-Probe, will detect Wuhan virus, SARS-CoV and bat-SARS-related CoVs use 100 nM per reaction and mix with P2
E Gene	E_Sarbeco_F1	ACAGGTACGTTAATAGTTAATAGCGT	use 400 nM per reaction
	E_Sarbeco_R2	ATATTGCAGCAGTACGCACACA	use 400 nM per reaction
	E_Sarbeco_P1	FAM-ACACTAGCCATCCTTACTGCGCTTCG-BBQ	use 200 nM per reaction
N Gene	N_Sarbeco_F1	CACATTGGCACCCGCAATC	use 600 nM per reaction
	N_Sarbeco_R1	GAGGAACGAGAAGAGGCTTG	use 800 nM per reaction
	N_Sarbeco_P1	FAM-ACTTCCTCAAGGAACAACATTGCCA-BBQ	use 200 nM per reaction

10.4 After PCR set up

Disinfect your hands again with 70% alcohol and remove the glove. Discard into biohazard waste bin. Wash your hands with soap and water.

- ✔ Hands should be thoroughly lathered with soap, using friction, for at least 10 s, rinsed in clean water and dried using a clean paper or cloth towel (if available, warm-air hand-dryers may be used).

10.5 References:

(Adapted and modified from)

1. CDC. Coronavirus Disease 2019 (COVID-19) [Internet]. Centers for Disease Control and Prevention. 2020 [cited 2020 Apr 5]. Available from: https://www.cdc.gov/coronavirus/2019-ncov/lab/index.html

2. Corman V, Bleicker T, Brünink S, Drosten C, Landt O, Koopmans M, et al. Diagnostic detection of Wuhan coronavirus 2019 by real-time RT- PCR. :12.

3. https://www.fda.gov/media/134922/download

Part 11

How to interpret and report results?

11.1 CONTROLS

- **No Template Control (NTC)**

The NTC consists of using nuclease-free water instead of RNA. The NTC reactions for all primer and probe sets should not exhibit fluorescence growth curves. If any of the NTC reactions exhibit a growth curve that crosses the cycle threshold, sample contamination may have occurred. Invalidate the run and repeat the assay.

- **2019-nCoV Positive Control (nCoVPC)**

The nCoVPC consists of in vitro transcribed RNA. The nCoVPC should yield a positive result (Ct value < 36.00) with the following primer and probe sets: E, N, RdRp and RP.

- **Human RNase P (RP)**

All clinical samples should exhibit fluorescence growth curves in the RNase P reaction that cross the threshold line within 36.00 cycles (< 36.00 Ct), thus indicating the presence of the human RNase P gene.

Expected Performance of Controls Included in the CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel

Control	External Control Name	Used to Monitor	First Line Assay	Confirmatory Assay	RP	Expected Ct Values
Positive	nCoVPC	Substantial reagent failure including primer and probe integrity	+	+	+	< 36.00 Ct

Negative	NTC	Reagent and/or environmental contamination	-	-	-	None detected
Extraction	HSC	Failure in lysis and extraction procedure, potential contamination during extraction	-	-	+	< 36.00 Ct

If any of the above controls do not exhibit the expected performance as described, the data is invalid, and the samples are to be re-tested.

11.2 TEST SAMPLES

- **Assay E**

This is the first line screening assay. When all controls exhibit the expected performance, a specimen is considered negative if assay E cycle threshold growth curves DO NOT cross the threshold line within 36.00 cycles (< 36.00 Ct) AND the RNase P growth curve DOES cross the threshold line within 36.00 cycles (< 36.00 Ct). If the Ct value for E assay is <36 it is considered a positive and proceed to confirmatory assay.

- **Confirmatory Assay RdRp and N gene assay**

This is the confirmatory assay. If the RNase P growth curve DOES cross the threshold line within 36.00 cycles (< 36.00 Ct) and Ct values for both RdRp and N assays are <36 it is considered a positive test and proceed to report to ICMR-NIV and sender.

Checklist for data interpretation:

CONTROLS				
	Assay Name	Gene-	Ct Value	Report
Negative Control	NTC		<40.00	Invalid test
			>40.00	Proceed to check Positive control
Positive Control	nCoVPC	Rp	>36.00	Invalid test
		Assay E	>36.00	
		Rp	<36.00	Confirmed Negative test. Proceed to generate report for negative test
		Assay E	>36.00	
		Rp	<36.00	Proceed to check test sample
		Assay E	<36.00	
TEST SAMPLES				
First line assay	Assay E	Rp	>36.00	invalid test
		E gene		
		Rp	< 36.00	Positive. Proceed to confirmatory test
		E gene	<36.00	
Confirmatory Assay	Assay RdRp and N	Rp	>36.00	invalid test
		RdRp		
		N		
		Rp	<36.00	Postive for nCOVID-19. Proceed to generate report for positive test
		RdRp	<36.00	
		N	<36.00	

11.3 Reporting Results to ICMR:

1. Before any laboratory starts testing, they must ensure immediate/real-time reporting of the rest results along with the contract details to the ICMR HQ database accessible at http://cvstatus.icmr.org.in Login credentials for each lab for uploading the data will be provided by ICMR.
2. All reports will be delivered confidentially to the treating physician and the relevant authorities in the currently prescribed format

11.4 References:

(Adapted and modified from)

1. CDC. Coronavirus Disease 2019 (COVID-19) [Internet]. Centers for Disease Control and Prevention. 2020 [cited 2020 Apr 5]. Available from: https://www.cdc.gov/coronavirus/2019-ncov/lab/index.html

2. https://www.fda.gov/media/134922/download

3.https://icmr.nic.in/sites/default/files/whats_new/Notification_ICMR_Guidelines_Private_Laboratories.pdf

Part 12

Best practices for waste disposal

12.1 Waste Disposal Protocol

1. Keep separate color coded bins/ bags/ containers and maintain proper segregation of waste as Bio-Medical Waste Management (BMWM) Rules 2016, as amended and Central Pollution Control Board (CPCB) guidelines for implementation of BMWM Rules.

   Areas with designated waste disposal area:

   a. Sample collection room'
   b. PPE donning area
   c. BSL2 facility (RNA isolation/Pre PCR room)
   d. PCR/POST PCR processing room
   e. PPE doffing area
   f. Change rooms

2. As precaution double layered bags (using 2 bags) should be used for collection of waste from Sample collection room, BSL2 facility and PPE doffing area so as to ensure adequate strength and no-leaks;
3. Collect and store biomedical waste separately prior to handing it over the same Common Bio-medical Waste Treatment and Disposal Facilities (CBWTF). Use a dedicated collection bin labelled as "Covid-19 to store COVID-19 waste and kept separately in a temporary storage room prior to handing over to authorized staff of CBWTF. Biomedical waste collected from these rooms should go directly into CBWTF collection van.
4. In addition to mandatory labelling bags/containers used for collecting biomedical waste from sample collection rooms, BSL2 facility and PPE doffing area should be labelled as "COVID-19 waste". This marking would enable CBWTFs to identify the waste easily for priority treatment and disposal immediately upon the receipt.
5. General waste not having contamination should be disposed as solid waste as per SWM rules, 2016;

Safe Disposal of the COVID-19 waste along with the consumables used for the diagnostic purposes

The COVID-19 blood samples, spillage (biological samples and reagents) the laboratory consumables used for the diagnostic research (including disposable syringe, needles, and used cotton-swabs) and the outer disposable clothing used by the laboratory personnel need to be effectively disposed in accordance with the rules for safety disposal. The following guidelines need to be followed for safe disposal:

1. All waste generated from suspected or confirmed patients shall be disposed of as medical waste.
2. Put the medical waste into a double-layer medical waste bag, seal the bag with cable ties in a gooseneck fashion and spray the bag with 1% hypochlorite containing disinfectant.
3. Put sharp objects into a special plastic box, seal the box and spray the box with 1% bleach-containing disinfectant.
4. Put the bagged waste into a medical waste transfer box, attach a special infection label, fully enclose the box and transfer it.
5. Transfer the waste to a temporary storage point for medical waste along a specified route at a fixed time point and store the waste separately at a fixed location.
6. The medical waste shall be collected and disposed of by an approved medical waste disposal provider.
7. All the wet waste should be discarded into 5% hypochlorite solutions. This should be incubated at least for 20 minutes.
8. Note: Exception: All the wet waste from RNA isolation (manual/automated Qiacube) using Qiagen viral RNA mini kit should be treated with detergent and water and then treated with 1% Sodium hypochlorite solution (Refer Qiagen manual)
9. All the dry wastes should be discarded into biohazard bag and should be autoclaved before disposing off

10. Any spillage should be decontaminated by 1% bleach followed by 70% ethanol wiping. They should also be reported to the facility manager and biosafety officer.
11. The bio-medical wastes should be finally labeled as "COVID-19 Waste" before handing them over to the CBWTF (Common Biomedical Waste Treatment Facility) collection van.
12. All effluents from the lab should go to an effluent treatment plant.
13. Certification for waste generation and safe disposal from the Govt. Pollution Control Board is a must.

12.2 Decontamination/ Disinfection Protocol

1. Disinfection of the Laboratory Floor and walls

Visible pollutants shall be completely removed before disinfection and handled in accordance with disposal procedures of blood and bodily fluid spills. Disinfect the floor and walls with 1% bleach through floor mopping, spraying or wiping. Make sure that disinfection is conducted for at least 30 minutes. Carry out disinfection three times a day and repeat the procedure at any time when there is contamination.

2. Disinfection of Object Surfaces in the Laboratory

The surface of laboratory work-benches, machines and instruments used for the diagnostic procedures (such as pipette-mans) and manuals need to be disinfected routinely. Wipe the surfaces of objects with 1% bleach or wipes with effective chlorine; wait for 30 minutes and then rinse with clean water. Perform disinfection procedure three times a day (repeat at any time when contamination is suspected). Wipe cleaner regions first, then more contaminated regions: first wipe the object surfaces that are not frequently touched, and then wipe the object surfaces that are frequently touched. (Once an object surface is wiped clean, replace the used wipe with a new one).

3. Spillage Disinfection

Option 1: Absorb the spilled fluids for 30 minutes with a clean absorbent towel (containing peroxyacetic acid that can absorb up to 1 L of liquid per towel) and then clean the contaminated area after removing the pollutants.

Option 2: Completely cover the spill with disinfectant powder or bleach powder containing water-absorbing ingredients or completely cover it with disposable water-absorbing materials and then pour a sufficient amount of 10% bleach

chlorine-containing disinfectant onto the water-absorbing material (or cover with a dry towel which will be subjected to high-level disinfection). Leave for at least 30 minutes before carefully removing the spill. After removing the spills, disinfect the surfaces of the polluted environment or objects. The containers that hold the contaminants can be soaked and disinfected with 5% active chlorine-containing disinfectant for 30 minutes and then cleaned. The collected pollutants should be disposed of as medical waste (in double-layer biohazard bags).

4. Air Disinfection

Air circulating within the diagnostic labs needs to be sterilized appropriately. Plasma air sterilizers can be used and continuously run for air disinfection in an environment with human activity. If there are no plasma air sterilizers, use ultraviolet lamps for 1 hour each time. Perform this operation three times a day.

5. Precautions to be considered for Occupational exposure to COVID-19

The laboratory personnel handling COVID-19 samples are highly prone to occupational exposure and hence need to take the following important considerations to prevent infection during the handling of COVID-19 samples:

a. **Skin exposure**: The skin (or wounded skin) can be directly contaminated by exposure to visible bodily fluids/secretions obtained from the patient. In such conditions, remove the contaminants with clean tissues or gauze, then apply 0.5% iodophor or 75% alcohol to the skin and let the solution sit for at least 3 minutes for disinfection. Then, thoroughly flush with running water.

b. **Mucous membrane exposure**: Mucous membranes, such as the eyes and respiratory tract are directly contaminated by visible bodily fluids/secretions obtained from the patient. In such conditions, flush with plenty of normal saline or 0.05% iodophor for disinfection.

c. **Sharp object injury**: Piercing of the body by sharp objects that were directly exposed to visible bodily fluids/secretions obtained from the patient. Squeeze blood out from proximal end to distal end and flush the wound with running water. Then, disinfect with 75% alcohol or 0.5% iodophor.

d. **Direct exposure of respiratory tract**: Falling off of a mask, exposing the mouth or nose to a confirmed patient (1 meter away) who is not wearing a mask. Immediately leave isolation area. Gargle with plenty of normal saline or 0.05% iodophor. Dip a cotton swab into 75% alcohol, and wipe in a circular motion the nasal cavity gently.

Evacuate to the designated isolation room. Report this matter of occupational exposure to the relevant departments. Isolate and observe these personnel with occupational exposure other than intact skin exposure for 14 days. In case of symptoms, report to the relevant departments in a timely manner.

12.3 References:

2. Guidelines for handling treatment and disposal of waste during treatment diagnosis and quarantine of COVID-19 patients. https://www.tnpcb.gov.in/pdf_2020/Guideline_COVID_19_waste.pdf
3. Interim Laboratory Biosafety Guidelines for Handling and Processing Specimens Associated with Coronavirus Disease 2019 (COVID-19) https://www.cdc.gov/coronavirus/2019-ncov/lab/lab-biosafety-guidelines.html
4. Biomedical waste management rules 2016 https://dhr.gov.in/sites/default/files/Biomedical_Waste_Management_Rules_2016.pdf

Bio-Medical Waste Management Rules

Biomedical wastes categories and their segregation, collection, treatment, processing and disposal options

Category	Type of Waste	Type of Bag or Container to be used	Treatment and Disposal options
Yellow 	a) Human Anatomical Waste b) Animal Anatomical Waste c) Soiled Waste d) Expired or Discarded Medicines e) Chemical Waste f) Discarded linen, mattresses, beddings contaminated with blood or body fluid, routine mask and gown	Yellow coloured non-chlorinated plastic bags or containers	Incineration or Plasma Pyrolysis or deep burial* The discarded medicines shall be either sent back to manufacturer or disposed by incineration
	g) Micro, Bio-t and other clinical lab waste	Autoclave safe plastic bags or containers	
	h) Chemical liquid Waste	Separate collection system leading to effluent treatment system	After resource recovery, the chemical liquid waste shall be pre-treated before mixing with other wastewater
Red 	Contaminated Waste (Recyclable)	Red coloured nonchlorinated plastic bags or containers	Autoclaving or micro-waving/ hydroclaving followed by shredding or mutilation or combination of sterilization and shredding. Treated waste to be sent to recyclers. Plastic waste should not be sent to landfill sites.
White (Translucent) 	Waste sharps including Metals	Puncture proof, Leak proof, tamper proof containers	Autoclaving or Dry Heat Sterilization followed by shredding or mutilation or encapsulation in metal container or cement concret
Blue 	Glassware and Metallic Body Implants	Puncture proof and leak proof boxes or containers with blue coloured marking	Disinfection or through autoclaving or microwaving or hydroclaving and then sent for recycling

Source: Bio-Medical Waste Management Rules, 2016 and amended rules 2018