Laboratory procedures for detection of Influenza virus in specimens from suspected human cases

Contents:

1. Intended use and Scope of the Document

The document is intended for the laboratories who perform or intend to perform laboratory diagnosis of influenza in humans. The purpose of this document is to provide guidance to the States in establishing new laboratories or for strengthening already existing laboratories for influenza testing. This document is not a regulatory but is an advisory document which is intended to address certain relevant issues and is based on existing knowledge (in terms of guidelines and standards) and experiences of the contributors.

The States are advised to form a technical committee comprising preferably of laboratory experts of the respective States to designate laboratories for Seasonal Influenza Testing.

2. Introduction

Influenza virus is an enveloped, segmented RNA virus which belongs to the family Orthomyxoviridae. There are three serotypes of influenza virus namely, Influenza virus A, B and C. Influenza type A is further classified into various subtypes on the basis of the surface glycoproteins Hemagglutinin (HA) and Neuraminidase (NA) e.g. H1N1, H3N2, H5N1 etc. The drastic changes in the antigenic structure (also called Antigenic shift) which is an infrequent occurrence gives rise to a completely new subtype eventually leading to development of a pandemic. On the other hand, mild antigenic changes which is an ongoing process (also called Antigenic drift) gives rise to frequent epidemics and regional outbreaks. In India, Influenza A (H1N1) is the predominating subtype which has been circulating for many years post the 2009 pandemic. Besides this, H3N2 and Influenza B are also found to occur but with a lesser prevalence.

Influenza viral infections, caused by different antigenic subtypes, can occur naturally in swine, horses, mink, seals and many domestic and wild avian species. Infections in humans and fowls are known to occur due to the interspecies transmission and reassortment of influenza A viruses. Many pandemics in the past like in 1918, 1957 and 1968, have occurred due to human influenza viruses which were found to be closely related to avian influenza viruses. The Influenza Type A (H1N1) which caused the worldwide pandemic in 2009 was also a reassortant of human, swine and avian subtypes.

Influenza virus causes an acute respiratory tract infection with the most common clinical manifestations being fever, headache, malaise, sore throat and cough. The wide spread nature of illness in forms of regional outbreaks, epidemics and pandemics and the high rates of mortality especially in the high risk groups is of concern.

3. Laboratory testing for influenza

Laboratory testing of samples from suspected cases of influenza should be done on the basis of clinical and epidemiological information. Influenza testing is recommended for the category C patients. (Summary of pre-requisites for testing for seasonal influenza: refer to Annexure A)

3.1. Specimen collection and handling

The validity of laboratory test is dependent on proper sample collection and handling which if done inappropriately can lead to incorrect diagnostic results. Human and animal samples should never be processed in the same laboratory. However, they could be processed in the same institution, if separate working rooms under stringent condition are assigned for animal and human specimens. The recommended procedures for collection, storage and transportation of human clinical samples for laboratory diagnosis of influenza are described in Guidelines for Collection, Storage and transportation of Human Clinical samples for Laboratory Diagnosis of Influenza.

3.2. Biosafety measures

3.2.1. Basic Biosafety Requirements

- BSL 2 or BSL 3 facility depending on the risk hazard (Refer to section 3.3.2) -
- PPE
    - gloves (latex)
    - laboratory coats or gowns(front closed full length apron)
    - head cover
    - protective eyewear
    - face protection (triple layered mask, N95 mask when handling novel or animal sample)
    - shoe cover
- puncture resistant autoclavable yellow coloured and red coloured biosafety bag with biosafety symbols
- biosafety spill kit
- Chemical disinfectants: sodium hypochlorite, 70% ethanol, quaternary ammonium compounds , detergents, iodophors, phenolic compounds

3.2.2. Biosafety practices:

3.2.2.1 *Preanalytic biosafety measures*

- The staff should be trained for the procedure.
- Staff should preferably be vaccinated yearly for Influenza
- Patient care and infection control policies and procedures should be followed in patient drawing areas.
- The laboratory should provide guidance on specimen collection that includes information on the required specimen containers and how to package and send specimens to the laboratory to minimize leakage. (section 3.1)
- SOP to deal with specimen receiving, leaking containers, and visible contamination of the outside of containers should be in place.

- Personal precautions such as hand washing, gloves and other required PPE should be available and worn by the lab personnel.
- Regular disinfection of lab surfaces with an intermediate level disinfectant each day of work should be followed.
- Staff shall refrain from touching eyes, nose, mouth and lips while in the laboratory.
- Storage of food items in the lab should be strictly prohibited
- staff should refrain from using cell phone and bringing personal items (purses, backpacks, books, magazines etc.) into the laboratory

3.2.2.2 *Analytic biosafety measures*

- Appropriate PPE (section 3.2.1) should be used by the lab personnel while performing the tests
- Lab personnel must be trained for containing any accidental spill. Spill kits should be readily available
- Aerosol generating procedures should be carried out in BSC.
- Biosafety cabinets should be used depending on the risk hazard(refer to section 3.3.2)
- Unidirectional work flow should be maintained within designated/ identified work area such as sample handling area, RNA extraction area, Clean reagent preparation area …etc.Tests should be performed only in designated area
- While performing RICTs (Rapid Immunochromatographic tests,) absorbent pads should be used

3.2.2.3 *Post analytic biosafety measures*

- Work surfaces and equipment should be decontaminated as soon as possible after specimens are processed as influenza viruses can survive on environmental surfaces for up to 2–8 hours
- PPE should be removed and discarded before exiting the lab – Annexure E
- Proper hand washing should be done. Steps of hand washing – Annexure D
- Safe segregation and disposal of biomedical waste as per Biomedical Waste Management (BMW) guidelines
- SOP for storage and inventory of infectious substances should be in place
- Trained personnel should be responsible for packing and shipping infectious substances for sending out of laboratory with appropriate triple layer packaging
- Shipping records should be regularly maintained and available

(Important considerations in design and practices of biosafety levels 2 and 3: Refer to Annexure B)

3.2.3 Occupational health

- All accidents or incidents should be documented and assessed with prompt action
- Regular medical evaluation should be performed on employees working in a BSL3 laboratories
- Lab personnel should be trained to identify any potential symptoms suggestive of influenza like fever and other symptoms such as cough, sore throat, runny or stuffy nose, body aches, headache, chills, and fatigue. Any influenza-like illness should be reported to your supervisor immediately.
- Appropriate immunization against influenza should be offered to all laboratory staff.
- Any issue that may affect the health of the lab personnel should be immediately dealt with
- Antiviral chemoprophylaxis is available and should be considered if required after accidental exposure.
- Lab personnel should regularly (annually) undergo BMW and biosafety training and competency assessment.

3.3. Laboratory Design

3.3.1 Biosafety levels

All laboratory facilities commensurate with each laboratory's function and the recommended containment level for the agents or materials being handled, based on risk assessment as per the WHO biosafety guidelines. The Laboratory levels for influenza testing should be either BSL2 or BSL3 depending on the risk hazard.

3.3.2 Recommended BSL facility of handling various Tests for influenza

- ✓ The risk assessment must take into consideration the potential for any laboratory activities to generate aerosols and the titre and volume of the virus to be worked with and thus be carried out in suitable containment.
- ✓ Laboratories performing serology or RT-PCR testing should handle potential seasonal influenza specimens using Standard Precautions (previously Universal Precautions, wear PPE [lab coat, face mask & gloves], avoid creating or contain aerosols) in BSL 2 facility.
- ✓ any procedures, either at BSL2 or BSL3, that are likely to give rise to aerosols of infectious material be carried out in a microbiological safety cabinet, or other suitable containment
- ✓ When centrifuging samples, use sealed centrifuge rotors or sample cups. Rotors and cups need to be loaded and unloaded in BSC.
- ✓ when handling newly recognised influenza viruses with poorly defined pathogenicity, The use of bio safety cabinets (i.e. Class II bio safety cabinets

when dealing with small quantities or low concentration of culture or other materials and Class III bio safety cabinets when using or manipulating large quantities or high concentration of cultures or other materials) should be considered.

BSL facility	Tests/ procedures that should be performed	Remarks
BSL-2 practices without Class II BSC	Rapid Immunochromatographic Diagnostic Tests (RIDTs) which are point of care tests may require just the dipping of swab tip into the test kit or pipetting specimens and reagents (not generating aerosols). Perform with splash protection :laboratory coat, gloves, eye protection, facemask (surgical, dental, medical procedure, isolation, or laser masks)	
BSL-2 practices within Class II BSC for	Performing diagnostic tests that don't involve propagation of viral agents in-vitro or In-vivo; RIDTs (more complex procedures (e.g., direct or indirect fluorescent antibody tests [DFA, IFA, culture*, molecular assays], which may involve procedures like vortexing. Nucleic acid extraction procedures involving untreated specimen	BSL2 is appropriate for laboratories that are not intentionally working with viruses of human pandemic potential and/or any Novel Influenza virus
BSL 3 facility	Attempt for Virus isolation/culture, should be in BSL3 Microneutralization In BSL 3	BSL3 is more appropriate for certain types of clinical samples, such as respiratory secretions from patients known or suspected of being infected with the avian strains, or with strains resistant to antiviral agents. Or newly recognised viruses with poorly defined pathogenicity
BSL 3 facility with BSL-3 work practices with	Highly pathogenic avian influenza (HPAI) A culture, (e.g. H5N1, with	

shower out facilities (BSL3+)	Specific BSL3+ conditions) which include controlled access double door entry with changing room and shower-out facilities. plus the use of negative-pressure, HEPA-filtered respirators or positive air-purifying respirators, and clothing change Therefore, respiratory virus cultures of patients suspected of having H5N1 infection must not be offered or performed in laboratories without BSL3+ facilities It is recommended that testing to be performed by PCR assays only	

*****(as per BMBL guidelines, Biosafety level-3 (BSL-3) practices are no longer required for viral isolation of seasonal human influenza isolates)

3.3.3 Organization of workspace and equipment (PCR testing)

- Workspace should be organized to ensure that the flow of work occurs in one direction, from clean areas to dirty areas
- Have separate designated rooms, or at minimum physically separate areas which are basically divided into the following 4 areas:

Rooms	**Clean area/rooms**	**Dirty area**		
	"No Template" laboratory reagent room	**Specimen processing laboratory**	**Nucleic acid loading area**	**PCR platforms/technology room**
Procedure performed	PCR reagents stored, mastermix preparation for cDNA and amplification	Specimens received, processed and stored.	Extracted nucleic acid added to master mixes	Depending on the molecular detection platforms used – this area can be divided into dedicated rooms /technology – depending on available space

Equipment	-Free of amplicon at all times -Movement control / dedicated staff for each area on a rotational basis - Dedicated equipment normally found in a no template room -20C Freezers, Fridge – reagent storage Dedicated pipettes (Colour coded) – filter tips - Dedicated vortex Cool pack for holding reagents and setting up PCR Dedicated microfuge, -laminar flow -Dedicated place to hang lab coats/ or disposable lab coats -All consumables necessary to perform work in area	-Dedicated equipment normally found in Specimen processing laboratory -Freezers and fridges for sample storage and extraction reagents storage. Dedicated -20◦C for reagent storage -80C for sample storage - Biosafety cabinets for initial sample preparation (all specimens regarded as infectious) -Centrifuges, microfuges -Dry heating blocks (with dedicated thermometers) -Dedicated pipettes (colour coded), filter tips, vortexes, timers -), -semi – automated extraction platforms, -fully automated extraction platforms -Storage space for tubes, pipette tips and other consumables	-Dedicated equipment normally found in a loading area -Freezer -20◦C and Fridge (positive controls and nucleic acid storage) -PCR workstations -Dedicated minifuge - Dedicated pipettes (Colour coded) – filter tips - Dedicated vortexer - Gloves -Labcoats.	-Viral load platforms -Real-timePCR platforms -Thermal cyclers -Line probe assays(ELISA based detection) GT Blot -Sequencing platform ◦ -Gel electrophoresis -Nothing from these areas should move back to the clean area! -Gloves and lab coats to be removed when leaving this area! -Dedicated pipettes, fridges, freezers, vortex, and centrifuges.

- In some settings, having 4 separate rooms is difficult. A possible but less desirable option is to do the mastermix preparation in a containment area, e.g. a laminar flow cabinet.
- Each room/area needs a separate set of clearly labelled pipettes, filter tips, tube racks, vortexes, centrifuges (if relevant), pens, generic lab reagents, lab coats and boxes of gloves that will remain at their respective workstations.
- Hands must be washed and gloves and lab coats changed when moving between the designated areas
- Reagents and equipment should not be moved from a dirty area to a clean area. Should an extreme case arise where a reagent or piece of equipment needs to be moved backwards, it must first be decontaminated with 10% sodium hypochlorite, followed by a wipe down with sterile water
- Preferable two persons conduct the test , If single person is doing the test ,he should first do the clean work and then move to Sample handling and RNA extraction area to avoid contamination

3.4 Various Laboratory tests for Influenza diagnosis

- **Molecular assays/Nucleic Acid Amplification Test (NAATs) – (Most recommended test is Real Time RTPCR)**
- **Virus isolation** – Performed only for research/vaccine production purposes
- **Other tests** available (but not currently recommended) are
    - Antigen detection tests
    - Antibody detection assays

✓ These assays may be used to detect (and initially identify) both seasonal and novel influenza A viruses in specimens from humans.

✓ The assays vary in the expertise and infrastructure required rapidity, cost, and sensitivity/specificity.

✓ Sensitivity and specificity of any test for influenza viruses in respiratory specimens might vary with the time from illness onset to specimen collection, respiratory source, the quality, handling and processing of the specimen and the time from specimen collection to testing.

✓ The post-test probability or predictive values (positive and negative predictive values) of an influenza virus test depend upon the prevalence of circulating seasonal influenza viruses in the patient population, and the specific test characteristics (sensitivity and specificity) compared to a “gold standard” comparison test (molecular assay or viral culture).

✓ As with any diagnostic test, results should be evaluated in the context of other clinical and epidemiologic information available to health care providers. Serological testing does not provide timely results to inform clinical management decisions.

- ✓ To maximize detection of influenza viruses, respiratory specimens should be collected as close to illness onset as possible (ideally <3-4 days after onset; molecular assays may detect influenza viral RNA in respiratory tract specimens for longer periods after illness onset than antigen detection assays).
- ✓ For quality assurance, only validated (by national institutes NIV, NCDC, NIB) or approved (by National or International bodies) tests should be used for diagnosis of Influenza

3.4.1 Description of Molecular assays/ Nucleic Acid Amplification Assays (NAATs)

NAATs have replaced culture techniques as the new gold standard in influenza diagnostics.

- ✓ Reverse transcriptase polymerase chain reaction (RT-PCR) :
  - -Real time reverse transcriptase PCR assay.
  - -Multiplex PCR systems-
  - - Conventional
- ✓ Other NAATs
- ✓ Rapid molecular assays

3.4.1.1 *PCR*

- PCR detects viral RNA present in clinical specimens (also in virus cultures). The targets could be a gene which is relatively conserved across all influenza A viruses (e.g. matrix gene) & Type B &subtype specific like the HA or NA genes
- Best upper respiratory tract specimens are nasopharyngeal swabs, washes or aspirates; other acceptable specimens are a nasal and/or throat swab

- ✓ In patients with lower respiratory tract disease, lower respiratory tract specimens (endotracheal aspirate or bronchoalveolar lavage fluid) should be collected and tested if influenza is clinically suspected and testing of upper respiratory tract specimens is negative. For critically ill patients with suspected influenza, even when testing by RT-PCR or other molecular assays is negative, consideration should be given to collecting additional multiple respiratory specimens, especially lower respiratory tract sample
- ✓ Antiviral treatment should be continued in such patients pending additional influenza testing.
- ✓ Respiratory specimens should ideally be collected as early as possible (ideally less than 4 days after illness onset when influenza viral shedding is highest) in persons without lower respiratory tract disease and tested as soon as possible. Some multiplex molecular assays are available that can detect influenza viral nucleic acids and distinguish influenza virus infection from other respiratory viruses. Only validated/evaluated kits should be used
- ✓ Advantages

- ✓ providing increased sensitivity and possibility of quantitation of the viral target gene
- ✓ very high sensitivity and specificity very high compared to other FDA-cleared assays that use different methods
- ✓ Some molecular assays are able to detect and discriminate between infections with influenza A and B viruses; other tests can identify specific seasonal influenza A virus subtypes [A(H1N1)pdm09, or A(H3N2)].
- ✓ Multiples PCR can detect other respiratory pathogens also and therefore be useful for management of severely immunosuppressed patients and for use in identifying the cause of an institutional outbreak of respiratory illness.
- ✓ *Use in Detecting Institutional Influenza Outbreaks and* support decisions to promptly implement prevention and control measures
- ✓ Can indirectly detect the presence of novel influenza by identifying unsubtypable Influenza A
- ✓ can detect influenza viral RNA (positive results) for a longer duration than other influenza tests (e.g., antigen detection – immunofluorescence or rapid influenza diagnostic tests)
- ✓ the interpretation of the result is less impacted by the level of influenza activity in the community

- <u>Disadvantages:</u>

  - ✓ RT-PCR and other molecular assays may not be available in all outpatient or emergency room settings. For hospitalized patients, these assays are not always available on-site.
  - ✓ Respiratory specimens may need to be sent to a state public health laboratory or commercial laboratory for RT-PCR. Therefore, although the test can yield results in 4-8 hours, the actual time to receive results may be substantially longer. This may lead to prolonged antiviral treatment as the treatment which is initiated immediately after collecting the sample may require modification after the results. Most FDA-cleared molecular assays are not approved to test lower respiratory tract specimens
  - ✓ RT-PCR and other molecular assays are generally more expensive than other influenza tests
  - ✓ Some molecular assays may not specifically identify all currently circulating influenza A virus subtypes. Depending on the test, a negative result for one influenza A virus subtype may not preclude infection with another influenza A virus subtype.

- ✓ the detection of influenza viral RNA or nucleic acids by these assays does not necessarily indicate detection of viable infectious virus or on-going influenza viral replication
- ✓ not all assays have been cleared by the FDA for diagnostic use

- Turn Around Time (45 min –several hours)
  - ✓ conventional RT-PCR assays is 6–8 hours
  - ✓ Real time RT-PCR methods is 3–4 hours

3.4.1.2 Rapid molecular assays (**only validated kits should be used**)

- Reported sensitivities of available rapid molecular assays range from 66-100%
- Rapid molecular assays and some commercially available molecular assays can produce results in a reasonable time period to inform clinical management (ranging from approximately 15-30 minutes to less than 1.5 hours).
- Isothermal nucleic acid amplification and has been reported to have high sensitivity and yields results in 15 minutes or less. Other platforms use RT-PCR and produce results in approximately 20-30 minutes
- The Infectious Diseases Society of America (IDSA) recommends use of rapid influenza molecular assays over rapid influenza diagnostic tests (RIDTs) for detection of influenza viruses in respiratory specimens of outpatients
- Some can detect and discriminate between Influenza A & B while some can even detect important subtypes like Influenza A (H1N1) and H3N2
- As with other molecular diagnostic tests, if treatment is clinically indicated, antiviral treatment should NOT be withheld from patients with suspected influenza while awaiting testing results during periods of peak influenza activity in the community when the likelihood of influenza is high.
- For point-of-care use.
- Some molecular assays are able to detect and discriminate between infections with influenza A and B viruses; other tests can also identify specific seasonal influenza A virus subtypes, for example A(H1N1)pdm09, or A(H3N2).
- FDA-cleared rapid molecular assays can provide results in 15-30 minutes, and some are CLIA-waived. Other molecular assays can provide results in 45-80 minutes or several hours, depending upon the assay.
- Some FDA-cleared multi-pathogen molecular assays are available that can detect influenza viruses and other respiratory pathogens.
- Some molecular assays come in cartridge based Fully automated, NAAT systems (e.g. CBNAAT).This system integrates and automates sample processing, nucleic acid amplification and detection of the target sequences in less than 2 hours

3.5. **Protocol of real time RTPCR for influenza detection (General procedure)**

3.5.1 Assay principle

- includes a panel of oligonucleotide primers and dual labelled probes to be used in real-time RT-PCR assays
- Qualitative detection and characterization of Influenza A(H1N1)pdm09 viruses in respiratory specimens and viral cultures.
- The InfA primer and probe set is designed for universal detection of all influenza type A viruses.
- Real time singleplex or duplex can be used

3.5.2 Safety Information

- Specimen processing should be performed in accordance with biological safety regulations.

3.5.3 Acceptable specimens – refer to Guidelines for Collection, Storage and transportation of Human Clinical samples for Laboratory Diagnosis of Influenza

Criteria for Rejection of samples:

- Specimen collected not within 7 days of post illness
- Specimens not kept at 2-4°C (≤4 days) or frozen at -70°C or below.
- Inappropriate specimens not listed above.
- No clinical data sheet available with sample
- Sample of Contacts case
- In case of leakage whole lot of samples should be rejected if not individually packed and transported

3.5.4 Materials

A) Reagents:

1. Molecular grade sterile distilled water (RNase and DNase free)
2. Forward and reverse primers (Sequences: Refer to Annexure G)
3. Dual-labelled fluorogenic oligonucleotide RT-PCR probes (TaqMan)
4. Nucleic acid extraction kits: Performance of RT-PCR amplification based assays depends on the amount and quality of sample template RNA. There certain commercially available extraction procedures for e.g. QIAamp® Viral RNA Mini Kit, or RNeasy® Mini Kit(QIAGEN), Roche MagNA Pure Compact RNA Isolation Kit, MagNA Pure LC RNA Isolation Kit II, and Roche MagNA Pure Total Nucleic Acid Kit, Ambion Mag Max total nucleic acid kit which have shown to generate highly purified RNA using manufacturer's recommended extraction procedures.

5. Controls used in PCR runs :

✓ Positive control (VTC/PTC)

-Contains target of interest

- identifies the amplification efficiency of the assay

✓ Negative control

a) No template control (NTC/Blank) : Validates the reagents integrity - Uses nuclease free water ◦

- Indicates if PCR reagents are contaminated ◦
- If positive – investigate, clean all pipettes, work areas and replace reagents, results cannot be used

b) Extraction negative control (Mock) : Validate extraction procedure

- Use cell line of human origin (Hep, HeLa etc.) as a source of RNP gene as Mock
- Always handle Mock after all the samples to be tested.
- The Mock should show amplification for RNP and not for any other target. If positive for any other target results cannot be used.
- This will ensure for cross contamination while handling the samples for RNA extraction.

- Internal control Human RNP control : Validate sample quality
  - All human sample should show amplification for Human RNP.
  - If the sample did not have RNP amplification, then repeat the procedure from RNA extraction.
  - Even after re extraction sample did not have RNP CT value it confirms that sample quality is poor
  - Monitor nucleic acid isolation procedure
  - Indicates possible inhibition of PCR reaction.
  - Control is run in same tube as sample

B) Supplies:

1. Laboratory marking pen
2. Cooler racks for 1.5 micro centrifuge tubes and PCR reaction tubes/wells *
3. Adjustable pipettes* and aerosol barrier tips
4. PCR reaction tube strips or plates*
5. Optical strip caps or optical seller
6. Sterile, nuclease free 1.5 ml micro centrifuge tubes
7. Disposable powder-free gloves

C) Equipment:

1. Micro centrifuge

2. Vortex

3. Real-time PCR detection system with thermocycler reaction block*

4. +/- Automated Extraction system

3.5.5 Procedure

A. Preparation:

1. Avoiding sample contamination (Good lab practices in PCR lab : Refer to Annexure C)

- Maintain separate areas for assay setup and handling of nucleic acids.
- Maintain separate, dedicated equipment (e.g., pipettes, micro centrifuges) and supplies (e.g., micro centrifuge tubes, pipette tips) for assay setup and handling of extracted nucleic acids.
- Wear a clean lab coat and powder-free disposable gloves (not previously worn) when setting up assays.
- Change gloves between samples and whenever you suspect they may be contaminated.
- Keep reagent and reaction tubes capped or covered as much as possible.

2. Equipment preparation (Good lab practices in PCR lab : Refer to Annexure C)

- Work surfaces, pipettes, and centrifuges should be cleaned and decontaminated with cleaning products such as 10% Sodium Hypochlorite or UV with ethanol or any commercially available nucleic acid destroying reagent to minimize risk of nucleic acid contamination.

3. Reagent preparation*

NOTE: Keep all reagents on cold rack during assay set up.

a) Primers and probes
   - Thaw frozen aliquots of primer and probes (Thawed aliquots of probes may be stored in the dark up to 3 months at 2-8°C. Do not re-freeze probes)*.
   - Vortex all primers and probes.
   - Briefly centrifuge all primers and probes and then place in cold rack.

b) Realtime RTPCR reagents
   - Place Master Mix and enzyme in cold rack
   - Thaw the Reaction Mix vial.

- Mix the Reaction Mix by inversion.
- Briefly centrifuge Reaction Mix and enzyme then place in cold rack

B. Tests for each RT-PCR run*

1. Each sample RNA extract is tested by separate primer/probe sets which may include InfA, H1 and other H/N targets, RNase P
2. The RNaseP primer and probe set targets the human RNase P gene and thus serves as an internal positive control for human nucleic acid.
3. No template controls (NTC) and positive template controls (VTC) for all primer/probe sets should be included in each run.
4. Mock Control provides a secondary negative control that validates the nucleic extraction procedure and reagent integrity.

C. Reaction setup*

1. Nucleic acid extraction
   - Pre extraction steps: liquefaction, centrifugation, external lysis.
   - Extraction
     - ✓ Manual extraction – vacuum/spin - column based
     - ✓ Semi – automated
     - ✓ Automated extraction Advantages:
       - More consistent results
       - Eliminates operator variability
       - Reduces hands on time
       - Reduces transcription errors
       - Increase in productivity
       - Higher throughput

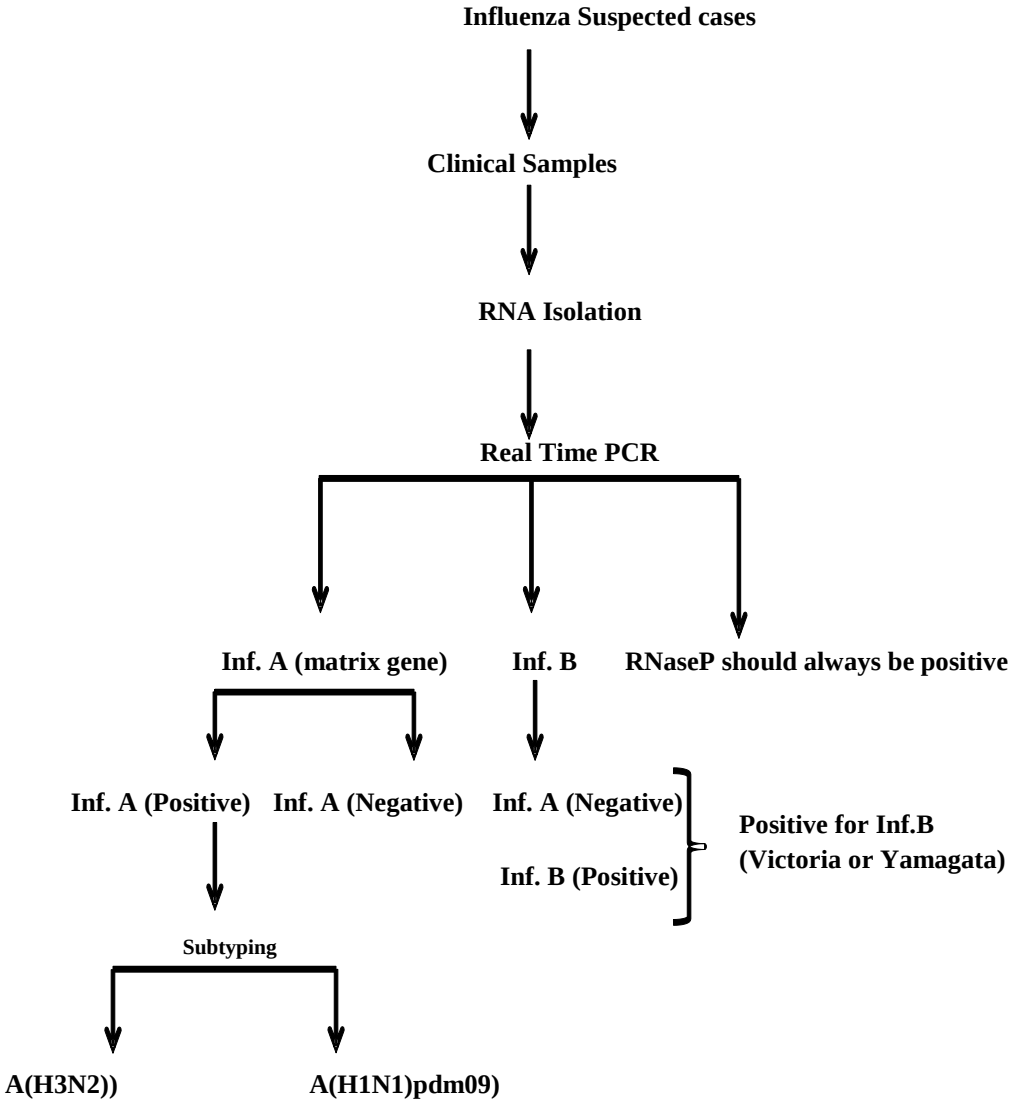
2. Reaction assay set up
   - Mixtures are made as a cocktail and dispensed into the reaction plate/tube.
   - Water and extracted nucleic acid or positive template controls are then added to the appropriate test reactions and controls.
   - Negative template controls (NTC) should be added first before any of the samples are added to check for contamination in the master mix.
   - Mock should be added after the samples have been added to check for cross-contamination during sample preparation or addition.
   - Positive template controls (PTC) should be added last after all samples and NTCs are sealed.

3. PCR run

    - Setting up of RT-PCR amplification conditions* in the PCR software
        - Select the reaction volume
        - Program the thermocycler (in terms of temp and time duration) for the following steps:
            - Reverse transcription
            - Taq inhibitor activation
            - PCR amplification (for specified cycles)
        - Add the sample details in the software
    - Load the reaction plate/tube format and proceed with the PCR run

*the requirements and procedure may vary depending on the type of reagent kit /thermocycler used. Kindly refer to the manufacturer's instructions

3.5.6 Test algorithm for RT PCR (most widely used test)



3.6. Test interpretation

3.6.1 Interpretation of Real time RT PCR Test:

- Proper interpretation of influenza testing results must consider a number of factors, including:
    - the predictive values of the test, test sensitivity and specificity compared to a "gold standard" test, prevalence of influenza in the patient population,
    - time from illness onset to specimen collection and

- whether the person may still have detectable influenza viral shedding, and
- source of the respiratory specimen (upper or lower respiratory tract

- False negative results due improper specimen collection and handling and when patient is no longer shedding detectable influenza virus, RT-PCR results may not always exclude a diagnosis of influenza.
- If clinical suspicion of influenza is high, antiviral treatment should continue in patients with severe illness or at high risk for complications while additional respiratory specimens are collected and further influenza testing is performed.
- False positive results, although rare, can occur (e.g., due to lab contamination or other factors)

3.6.2 *Validity of the test:*

Targets	**Inf A**	**Inf B**	**PdmH1N1**	**RNP**	**A(H3N2)**	**Type B Victoria**	**Type B Yamagata**
VTC/PTC	+ +	+ +	+ +	+ +	+ +	+ +	+ +
NTC	- -	- -	- -	- -	- -	- -	- -
MOCK	- -	- -	- -	- -	- -	- -	- -

- ✓ VTCs should be positive and within the expected Ct(24-30) value range for all primer/probe sets. **If VTCs are *negative*,**
  - Repeat testing samples for the targets for which positive control failed
  - If repeat testing generates negative VTC results, Change VTC
- ✓ If NTCs are *positive*,
  - Repeat testing samples for the targets for which negative control showed positive amplification
  - Clean all potential DNA contamination areas/ pipettes such as sample handling, clean reagent room, pipettes in the reagent setup and template addition work areas
  - Extract multiple times NTC (only water) and test the aliquots of reagent to ruled out possible contamination
  - Discard working reagents if the problem persist and remake from fresh aliquot
- ✓ Mock control should not exhibit fluorescence growth curves for primer/probe sets like InfA, H1 that cross the threshold line within 40 cycles. If any influenza specific primer/probes exhibit a growth curve that crosses the threshold line, interpret as follows:

- Contamination of RNA extraction reagents may have occurred. Invalidate the run and confirm reagent integrity of RNA extraction reagents prior to further testing.
- Cross contamination of samples occurred during RNA extraction procedures or assay setup. Invalidate the run and repeat the assay with stricter adherence to procedure guidelines

✓ RNP should be *positive* for each specimen, indicating that human cellular RNA/DNA is present in the sample and the extraction process was successful.

- RNP can be negative due to the following reasons:
  a. Improper extraction of nucleic acid from clinical materials resulting in loss of RNA or carry-over of RT-PCR inhibitors from clinical specimens
  b. Absence of sufficient human cellular material in sample to enable detection
  c. Improper assay set up and execution
  d. Reagent or equipment malfunction
- If RNP is *negative* for the specimen, and the virus-specific assays are negative,
  - Repeat sample extract for RNP and virus-specific assays
  - If virus-specific assay is positive, consider it a true positive (Note: Because inhibitors in the reaction could cause the RNP or other virus-specific targets to fail even though one of the targets amplified, it is advisable to repeat this sample for all negative targets for detection of potential co-infections.)
  - If RNP is negative after repeat extraction treat it as sample quality poor & report accordingly

- If all controls have performed properly, proceed to analyze each target.
- True positives should produce exponential curves with logarithmic, linear, and plateau phases if <u>the amplification curves cross the threshold line within 35 cycles</u>.

  (Note: Weak positives will produce high Ct values that are sometimes devoid of a plateau phase; however the exponential plot should be seen.)

3.6.3 *Real Time RT PCR Result interpretation*

Inf. A target gene	H3 target gene	Pdm H1 target gene	Result interpretation
Detected	Not detected	Detected	Inf. A Pdm H1N1 Positive
Detected	Detected	Not detected	Seas Inf. A (H3) Positive
Detected	Not detected	Not detected	Untypable Inf. A positive

*The influenza A(H1N1) pdm09 virus became a seasonal influenza virus and well established in humans. On this background testing of sample for Sw A target is not relevant.

Please communicate to Reference laboratory for further assistance if the sample is untypable for influenza

3.6.4 *Limitations:*

- Analysts should be trained and familiar with testing procedures and interpretation of results prior to performing the assay.
- A false negative result may occur if inadequate numbers of organisms are present in the specimen due to improper collection, transport or handling.
- A false negative result may occur if an excess of DNA/RNA template is present in the reaction.
- If inhibition of the RP control reaction is noted for a particular sample, extracted RNA can be tested at 2 or more dilutions (e.g., 1:10 and 1:100) to verify the result.

3.6.5 *Reporting of results*

- ✓ Standardized uniform reporting format (Refer to Annexure F)
- ✓ PC with internet facilities
- ✓ Fax machine

3.7 Other tests available

3.7.1 Virus Isolation

- Virus culture has been the gold standard for influenza diagnosis, however, is carried out only in reference laboratory.
- The virus can be isolated on cell lines or embryonated eggs
- Advantages
  - ✓ viruses are available for further antigenic and genetic characterization essential for surveillance and antigenic characterization of new seasonal influenza A and B virus strains that may need to be included in the next year's influenza vaccine
  - ✓ culture in appropriate cell lines can also detect other clinically important respiratory viruses
- Disadvantages
  - ✓ Important for public health purposes, but does not provide timely results to inform clinical management

- ✓ Positive influenza cultures may or may not exhibit cytopathic effects, thus a second step to specifically identify influenza viruses by immunofluorescence, haemagglutination – inhibition (HI) or RT-PCR is needed

- Turnaround Time
  - ✓ Shell vial culture: 1-3 days
  - ✓ Traditional tissue cell viral cultures : 3-10 days

3.7.2 Antigen detection tests (including rapid influenza diagnostic tests and immunofluorescence assays) – These tests are currently not preferred*

Viral antigen detection may be carried out by:

- Enzyme immunoassay (EIA) methods
- Immunofluorescence assays

3.7.2.1 A) *EIA based methods*

- Most RIDTs are immunoassays that utilize antibodies against the nucleoproteins of influenza A and B viruses to detect viral antigens
- Advantages
  - ✓ Simple and convenient to use.
  - ✓ can detect influenza viral antigens in 10-15 minutes
  - ✓ At present, directed at conserved viral antigens (e.g. virus nucleoprotein, matrix protein).
  - ✓ RIDTs are approved for specific kinds of respiratory specimens which vary by test, specific types of swabs and storage requirements
  - ✓ collecting specimens with appropriate methods within 24–72 hours after illness onset
- Specimens to be used with RIDTs should be collected as close as possible to the start of symptoms (e.g., less than 4 days after illness onset) except young children(few days) and immunosuppressed(weeks to months) who have longer periods of shedding
- Sensitivities of the RIDTs are low to moderate – approximately 50-70% while those with analyser devices have improved sensitivity of (75-80%) & specificity is approximately 90-95% when compared to viral culture & RT PCR i.e. False negative results are common with RIDTs especially during peak influenza activity in the community
- FDA has reclassified the RIDTs and published requirements for improved accuracy, including higher sensitivity i.e. the RIDTs must achieve80% sensitivity for detection of influenza A and influenza B viruses.(compared to RT PCR) & 90% sensitivity for detection of

Influenza A and 80% for influenza B (compared to Viral culture) ; 95% specificity for detection of influenza A and influenza B viruses.(compared to RT PCR & viral culture)

- Sensitivity is higher for influenza A virus detection in cases of human seasonal influenza but very low in cases of Avian influenza virus (H5N1) in humans.
- Some RIDTs are approved for point of care testing while some due to moderate complexity are required to be performed in a clinical laboratory
- Detection of influenza virus antigen does not necessarily indicate detection of viable infectious virus or on-going influenza viral replication.
- Variability in RIDT performance, especially at lower viral concentrations, negative RIDT test results might not exclude influenza virus infection in patients with signs and symptoms suggestive of influenza.
- Performance may vary depending on virus and its growth characteristics and affinity to antibodies used in RIDTs (as seen in analytic studies)
- Utility can be in outpatients (although Rapid molecular assays are recommended over RIDTs) and in outbreak situations
- Cannot be used for public health need i.e. surveillance purposes and for aiding in the selection of new influenza vaccine virus strains as influenza virus isolates are needed

3.7.2.1 B*) Immunofluorescence assays (**Not currently recommended for diagnosis***)*

- require use of a fluorescent microscope
- produce results in approximately 2-4 hours (assays with an analyzer device produce results in approximately 15 minutes)
- skilled technologists are needed
- moderate sensitivity and high specificity (superior to RIDTs)
- Both direct (DFA) and indirect fluorescent antibody (IFA) staining assays are available to detect influenza A and B viral antigens in respiratory tract specimens
- Subtyping or further identification of influenza A viruses is not possible by immunofluorescence assays.
- Most recently *digital immunoassay (DIA*) test platforms have been studied. These are lateral flow immunoassays with an instrument-based digital scanning facility of the test strip such that the sensitivity and specificity for detection of influenza virus antigens of Influenza A and B in patient's respiratory specimens is so much enhanced

that it has shown to approach that of viral cultures and / nucleic acid detection. They are also eliminate the need for an operator to visualize and interpret test results.

3.7.2.2 Test interpretation for RIDTs

- Proper interpretation of testing results, particularly negative testing results is very important.
- Interpretation of results must be in the context of the prevalence of influenza (level of influenza activity) in the patient population being tested as it affects the positive and negative predictive values of RIDTs considerably :
  - ✓ False-positive (and true-negative) influenza test results are more likely to occur when disease prevalence is low
  - ✓ False-negative (and true-positive) influenza test results are more likely to occur when disease prevalence is high, which is typically at the height of the influenza season
- If an important clinical decision is affected by the test result, the RIDT result should be confirmed by a molecular assay, such as reverse transcription polymerase chain reaction (RT-PCR).
- variability in RIDT performance, especially at lower viral concentrations, negative RIDT test results might not exclude influenza virus infection in patients with signs and symptoms suggestive of influenza
- Detection of influenza virus antigen does not necessarily indicate detection of viable infectious virus or on-going influenza viral replication.
- antiviral treatment, if indicated, should not be withheld from patients with suspected influenza because they have a negative RIDT test result

3.7.3 Antibody based tests (**not currently recommended for diagnosis***)

Serological tests available for the measurement of influenza A-specific antibody include:

  - ✓ haemagglutination inhibition test (HI)
  - ✓ enzyme immunoassay (EIA)
  - ✓ virus neutralization tests (VN)/ microneutralization (MN) assay
- Impractical for routine diagnostic testing of clinical cases as timely results for clinical decision making is not possible:
- Antibodies take up to several weeks to develop and become detectable in serum
- Requires paired acute and convalescent sera after 14 days
- Not generally recommended, except for research and public health investigations (e.g. for determination of extent of exposure in case contacts or at risk populations).

- Standard panels of reagents for H5N1 and other novel strains are not widely available
- Results among the laboratories performing these tests vary widely
- only available at a limited number of public health or research laboratories
- MN assay is technically difficult to perform
- VN and MN assays require the use of live virus, thus can only be used in laboratories with Biosafety Level 3 containment facilities
- Difficulties in determining endpoint in HI with horse red blood cells because of their small size
- Non-specific cross reactivity in all serological assays may occur due to the previous infections with human influenza viruses or other factors (serum adsorption is required)

Method	**Types Detected**	**Acceptable Specimens**	**Test Time**
Rapid Influenza Diagnostic Tests (antigen detection)	A and B	NPswab, aspirate or wash, nasal swab, aspirate or wash, throat swab	<15 min.
Rapid Molecular Assay [influenza viral RNA or nucleic acid detection]	A and B	NPswab, nasal swab	15-30 minutes
Immunofluorescence, Direct (DFA) or Indirect (IFA) Florescent Antibody Staining [antigen detection]	A and B	NP swab or wash, bronchial wash, nasal or endotracheal aspirate	1-4 hours
RT-PCR (singleplex and multiplex; real-time and other RNA-based) and other molecular assays [influenza viral RNA or nucleic acid detection]	A and B	NP swab, throat swab, NP or bronchial wash, nasal or endotracheal aspirate, sputum	Varies (1 to 8 hours, varies by the assay)

Rapid cell culture (shell vials; cell mixtures; yields live virus)	A and B	NP swab, throat swab, NPor bronchial wash, nasal or endotracheal aspirate, sputum; (specimens placed in VTM)	1-3 days
Viral tissue cell culture (conventional; yields live virus)	A and B	NP swab, throat swab, NP or bronchial wash, nasal or endotracheal aspirate, sputum (specimens placed in VTM)	3-10 days

*These recommendations of diagnostic tests are subject to change based on the updation in the current scientific knowledge.

3.8 Quality assurance.

- A quality laboratory must have:
  - ✓ quality policies and standards are in place
  - ✓ Standard operating procedures for all the processes should be made
  - ✓ Assay techniques and processes standardised
  - ✓ Validated methods, test validation with most recent virus strains including the appropriate use of positive and negative controls
  - ✓ Appropriate quality control(instruments and consumables)
  - ✓ Trained and competent staff
  - ✓ Proper Laboratory maintenance
  - ✓ Appropriate facilities
  - ✓ GLP for prevention of contamination

- Establishing a quality assurance plan is helpful, which can include:
  - ✓ Lists of reagent master stocks and working stocks
  - ✓ Rules for storing kits and reagents
  - ✓ Reporting of control results
  - ✓ Staff training programmes
  - ✓ Troubleshooting algorithms,
  - ✓ Remedial actions when needed.

ANNEXURE A

Summary of Important prerequisites for testing of specimens for Seasonal Influenza infection

General biosafety measures: Gloves (latex), shoe cover, head cover, goggles, triple layered mask, N95 mask, front closed full length apron, puncture resistant autoclavable bags (yellow and red) with biohazard sign, sodium hypochlorite solution

Lab Design: Separate dedicated areas for sample handling and PCR testing

Sample collection kit: Throat & nasal swab/ Nasopharyngeal swab with synthetic tip (Dacron or Polyester) and aluminium or plastic shaft. Sample collection vials or tubes (leak proof and autoclavable) containing 1-3ml viral transport media (with protein stabilizer) as primary container

Sample storage: Refrigerator (4-8C) for storage up to 48 hours, Deep freezer (-70C) for longer storage (back up sample for future testing should be kept at -70C)

Sample transport: Absorbent cotton, tissue paper, waste newspaper for wrapping primary container. Secondary container to hold primary container i.e. bigger tube or sealed plastic bag. Insulated ice box with icepack, sample proforma fastened onto the secondary container

Sample Handling: In BSL 3 Biosafety or BSL2+ facility with BSL 3 precautions with Class II BSC

Sample Testing: Real Time PCR test, by Real Time PCR machine using validated reagent accessories and protocol as per CDC/WHO guidelines/testing protocol and subsequent amendments published from time to time

Reporting of Results: Standardised uniform reporting proforma, PC with internet facilities, Fax machine

ANNEXURE B

Important points to consider in the design and practices of Biosafety levels 2 and 3

Points to consider in BSL2 lab

- Laboratory doors to be self-closing
- Laboratories to have a sink for handwashing that is located near the exit door
- Laboratories to be designed so that they can be easily cleaned and thoroughly disinfected (e.g. surfaces are smooth and free of cracks or crevices) and free of any carpets or rugs
- Materials used in laboratory fixtures and furniture designed for easy cleaning and disinfection, impervious to water, and resistant to solvents
- All chairs used for laboratory work constructed or covered with a nonporous material that can be easily cleaned and decontaminated with appropriate disinfectant
- Vacuum lines associated with biohazardous procedures protected with liquid disinfectant traps and assessed and replaced at a frequency appropriate to usage in the laboratory
- If laboratory windows are capable of being opened to the exterior, they should be fitted with screens
- All biological safety cabinets should be located away from doors and windows that can be opened, heavily travelled laboratory areas, fans, room air supply louvers, and other possible airflow disruptions
- Eyewash stations installed within each lab and maintained e.g. flushed and function checked weekly
- All laboratories should be supported by ventilation systems that provide an inward flow of air without recirculation to spaces outside of each laboratory
- If regulated medical waste is autoclaved on site, all required permits in place and have all autoclave units and cycles properly validated and verified on an ongoing basis with challenge testing using chemical and/or biological indicators

Points to consider in BSL3 lab:

- Laboratory access to be restricted
- Laboratory to be separated from areas that are open to unrestricted traffic flow within the building
- Access to the laboratory through an anteroom with two self-closing doors
- Laboratory to have a sink for handwashing which is hands-free or automatically operated and located near the exit door
- If segregated into different laboratories, a sink for handwashing in each zone as determined by risk assessment (i.e. points at which inner gloves might need to be removed and replaced).
- Laboratory (including floors, walls, and ceilings) designed so it can be easily cleaned and thoroughly disinfected (e.g. surfaces are smooth and free of cracks or crevices) and free of any carpets or rugs

- Floors should be slip resistant, impervious to liquids, and resistant to chemicals
- All seams of floors, walls, and ceilings are to be sealed to facilitate decontamination
- All laboratory windows are to be sealed
- All penetrations (e.g. spaces around doors and ventilation ducts) capable of being sealed to facilitate gaseous or vapour phase decontamination of the lab if that method of decontamination is appropriate based on risk assessment should be identified for sealing.
- Materials used in laboratory fixtures and furniture (including chairs) designed for easy cleaning and disinfection, impervious to water, and resistant to solvents
- Eyewash stations installed within the lab and maintained (e.g., flushed and function checked weekly)
- Biological safety cabinets are to be located away from doors, heavily travelled laboratory areas, room air supply louvers, and other possible airflow disruptions
- All vacuum lines protected with HEPA filters or their equivalent and assessed and replaced at a frequency appropriate to usage in the laboratory
- All equipment that may produce infectious aerosols should be contained in primary barrier devices that exhaust air through HEPA filtration or other equivalent technology before being discharged to the laboratory
- HEPA filters associated with primary barrier devices tested or replaced at least annually
- Single-pass air (i.e. 100% air supplied and exhausted without recirculation) to be provided
- Lab to have a ducted air ventilation system that provides sustained directional airflow by drawing air into the laboratory from “clean” areas toward “potentially contaminated” areas
- Air ventilation system to be designed and initially verified by a qualified individual such that under failure conditions, there is no reversal of air which originates within the BSL-3 that travels all of the way outside the containment boundary (note: the BSL-3 anteroom is considered to be within the containment boundary).
- A visual monitoring device to be provided at the laboratory entry which confirms directional airflow
- The lab should have local visual/audible alarms to notify personnel of airflow disruption
- The laboratory building exhaust air HEPA filtered or dispersed away from occupied areas and from building air intake locations
- Building HEPA filter housings have gas-tight isolation dampers and decontamination ports, or bag-in/bag-out capability with appropriate decontamination procedures
- The HEPA filter housing allow for leak testing of each filter and assembly
- The HEPA filters and housing certified at least annually
- A method (e.g. autoclaving, chemical disinfection, etc.) For decontaminating all BSL-3 laboratory waste available within the laboratory or facility
- There should be a protocol in place to assure that autoclaved waste is not removed from the laboratory until the successful run is verified with a biological indicator

- Any required permits to be in place for BSL-3 autoclaves and have all autoclave units and cycles been properly validated and verified on an ongoing basis with challenge testing using chemical and/or biological indicators
- The facility designed appropriately to accommodate required procedures, based on risk assessment, for decontaminating large pieces of equipment before removal from the laboratory
- All necessary facility enhancement (e.g. shower-out capabilities, HEPA filtration of laboratory exhaust air, access control devices, etc.) For environmental and personal protection been considered and integrated into the facility design based on risk assessment
- The BSL-3 facility design, operational parameters, and procedures verified by qualified individuals and documented prior to operation and at least annually thereafter for testing and performance of BSL-3 ventilation systems.

ANNEXURE C

GOOD LAB PRACTICES IN A PCR LAB

Contamination can be a PCR amplicon spill (post PCR) or a Target template (sample processing and extraction) which can contaminate reagents, equipment and bench spaces.

In order to prevent such contamination the following practices should be adopted:

Handling reagents

- Briefly centrifuge reagent tubes before opening to avoid the generation of aerosols.
- Aliquot reagents to avoid multiple freeze-thaws and the contamination of master stocks.
- Clearly label and date all reagent and reaction tubes and maintain logs of reagent lot and batch numbers used in all experiments.
- Pipette all reagents and samples using filter tips. Prior to purchase, it is advisable to confirm with the manufacturer that the filter tips fit the brand of pipette to be used.
- The 10% sodium hypochlorite solution must be made up fresh daily. When used for decontamination, a minimum contact time of 10 minutes should be adhered to.
- Alternatively, commercially available products that are validated as DNA-destroying surface decontaminants can be used if local safety recommendations do not allow the use of sodium hypochlorite or if sodium hypochlorite is not suitable for decontaminating the metallic parts of equipment.
- Ideally, staff should abide by the unidirectional work flow ethos and not go from dirty areas back to clean areas on the same day. However, there may be occasions when this is unavoidable. When such occasion arises, 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. Such control measures should be emphasized in staff training on molecular methods.
- After use, bench spaces should be cleaned with 10% sodium hypochlorite (followed by sterile water to remove residual bleach), 70% ethanol, or a validated commercially available DNA-destroying decontaminant. Ideally, ultra-violet (UV) lamps should be fitted to enable decontamination by irradiation. However, the use of UV lamps should be restricted to closed working areas, e.g. safety cabinets, in order to limit the laboratory staff's UV exposure. Please abide by manufacturer instructions for UV lamp care, ventilation and cleaning in order to ensure that lamps remain effective.
- If using 70% ethanol instead of sodium hypochlorite, irradiation with UV light will be needed to complete the decontamination.

- Do not clean the vortex and centrifuge with sodium hypochlorite; instead, wipe down with 70% ethanol and expose to UV light, or use a commercial DNA-destroying decontaminant. For spills, check with the manufacturer for further cleaning advice.
- If manufacturer instructions permit it, pipettes should be routinely sterilized by autoclave. If pipettes cannot be autoclaved, it should suffice to clean them with 10% sodium hypochlorite (followed by a thorough wipe down with sterile water) or with a commercial DNA-destroying decontaminant followed by UV exposure.
- Cleaning with high-percentage sodium hypochlorite may eventually damage pipette plastics and metals if done on a regular basis; check recommendations from the manufacturer first.
- All equipment needs to be calibrated regularly according to the manufacturer-recommended schedule. A designated person should be in charge of ensuring that the calibration schedule is adhered to, detailed logs are maintained, and service labels are clearly displayed on equipment.

Use and cleaning advice for the designated molecular space

1. Reagent aliquoting / mastermix preparation area

- This should be the cleanest of all spaces used for the preparation of molecular experiments and should ideally be a designated laminar flow cabinet equipped with a UV light.
- Samples, extracted nucleic acid and amplified PCR products must not be handled in this area.
- Amplification reagents should be kept in a freezer (or refrigerator, as per manufacturer recommendations) in the same designated space, ideally next to the laminar flow cabinet.
- Gloves should be changed each time upon entering the pre-PCR area or laminar flow cabinet.
- Laminar flow cabinet should be cleaned before and after use as follows: Wipe down all items in the cabinet, e.g. pipettes, tip boxes, vortex, centrifuge, tube racks, pens, etc. with 70% ethanol or a commercial DNA-destroying decontaminant, and allow to dry. In the case of a closed working area, e.g. a laminar flow cabinet, expose the hood to UV light for 30 minutes.

NOTE:

- Do not expose reagents to UV light; only move them into the cabinet once it is clean.
- If performing reverse transcription PCR, it may also be helpful to wipe down surfaces and equipment with a solution that breaks down RNases on contact. This may help to avoid false-negative results from enzyme degradation of RNA.
- After decontamination and before preparing the mastermix, gloves should be changed once more, and then the cabinet is ready to use.

2. Nucleic acid extraction/template addition area

- Nucleic acid must be extracted and handled in designated area only using a separate set of pipettes, filter tips, tube racks, fresh gloves, lab coats and other equipment.
- This area may also be used for the addition of template, controls and trend lines to the mastermix tubes or plates. To avoid contamination of the extracted nucleic acid samples that are being analysed, it is recommended to change gloves prior to handling positive controls or standards and to use a separate set of pipettes.
- PCR reagents and amplified products must not be pipetted in this area.
- Samples should be stored in designated fridges or freezers in the same area.
- The sample workspace should be cleaned in the same way as the mastermix space.

3. Area for Amplification and handling of the amplified product

- PCR reagents and extracted nucleic acid must not be handled in this area since the risk of contamination is high.
- This area should have a separate set of gloves, lab coats, plate and tube racks, pipettes, filter tips, bins and other equipment.
- Tubes must be centrifuged before opening.
- The sample workspace should be cleaned in the same way as the mastermix space.

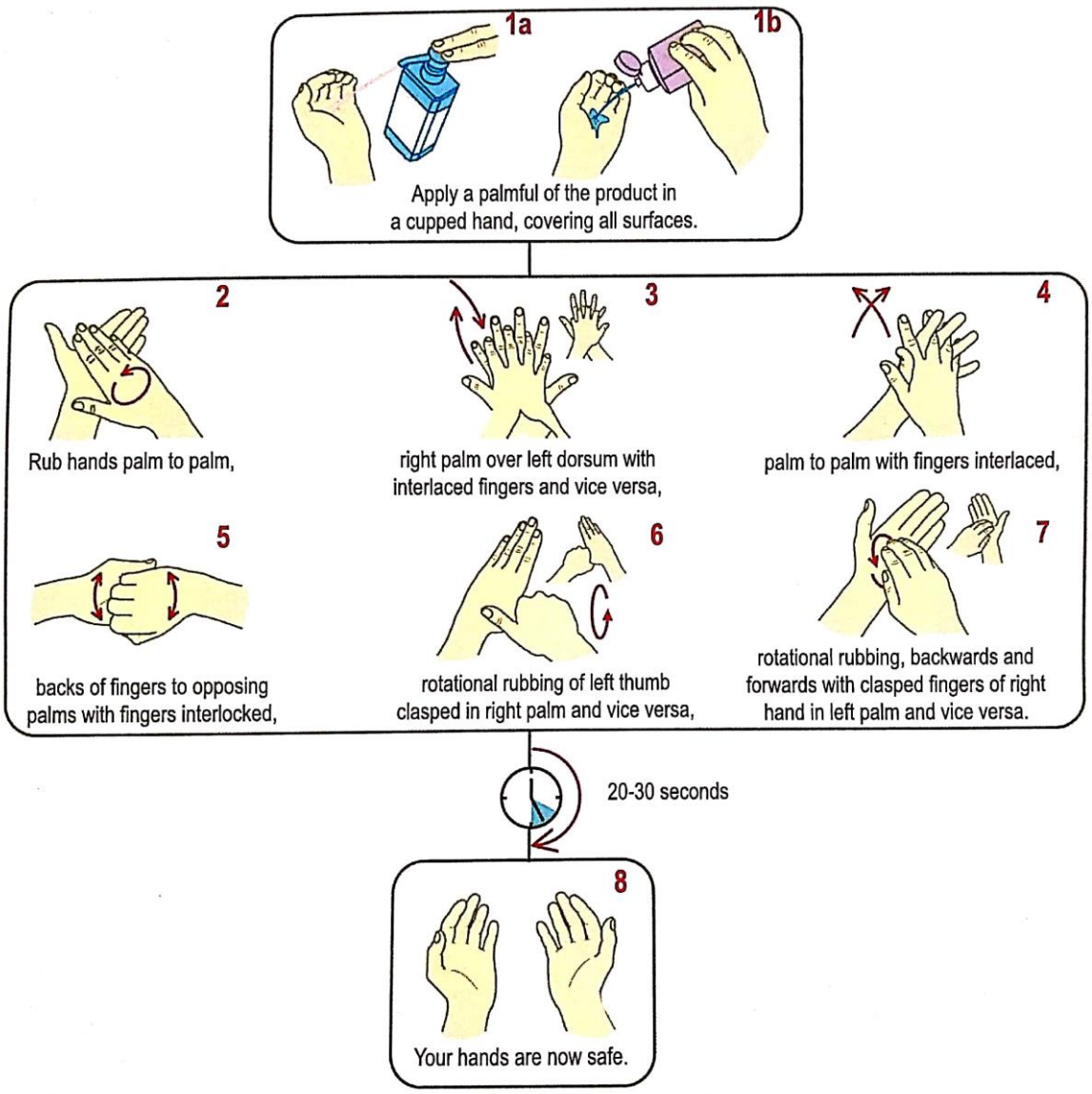
4. Area for Product analysis

- This area should have separate sets of gloves, lab coats, plate and tube racks, pipettes, filter tips, bins and other equipment.
- No other reagents can be brought into this area, excluding loading dye, molecular marker and agarose gel, and buffer components.
- The sample workspace should be cleaned in the same way as the mastermix space.
- Ideally, above mentioned first two areas should not be entered on the same day if work has already been performed in the above mentioned 3rd area.
- If this is completely unavoidable, ensure that hands are first washed thoroughly and that specific lab coats are worn in the rooms.
- Lab books and paperwork must not be taken into the above mentioned first two areas if they have been used in the above mentioned 3rd area; if necessary, take duplicate print-outs of protocols/sample IDs, etc.

Annexure D

Hand washing procedure

Using Alcohol based hand rub

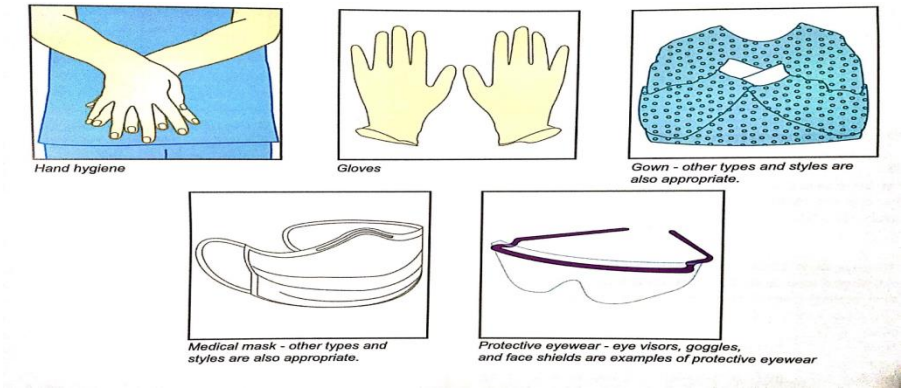


Using Soap and water

Annexure E

Donning and Doffing of PPE

Select appropriate PPE



***Personal Protective Equipment (PPE) used with Standard Precautions*:**

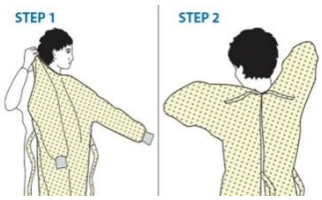
Scenario	Hand Hygiene	Gloves	Gown	Medical Mask	Eyewear
Always before and after patient contact, and after contaminated environment	✓				
If direct contact with blood and body fluids, secretions, excretions, mucous membranes, non-intact skin	✓	✓			
If there is risk of splashes onto health care worker's body	✓	✓	✓		
If there is a risk of splashes onto the body and face	✓	✓	✓	✓	✓

How to Don PPE

Step I

- ✓ Identify hazards & Assess risk. Gather the necessary PPE
- ✓ Plan where to put on and take off PPE (patients' room, doorway or anteroom)
- ✓ Take help of a mirror or colleague
- ✓ Deal with the waste

Step 2



- ✓ Put on a gown.
- ✓ Fully cover torso from neck to knees, arms to end of wrists, and wrap around the back
- ✓ Fasten in back of neck and waist(zip in case of a zipper at the back of the gown)

Step 3

- ✓ Put on a mask
- ✓ Secure the mask at the back of the neck and middle of the head
- ✓ Fit the flexible band on the nose bridge
- ✓ Snuggly fit the mask to face and below the chin
- ✓ Perform a respirator seal check

Step 4

- ✓ Put on eye protection e.g. Visor, face shields, goggles
- ✓ Caps (if need be) should be put on after eye protection

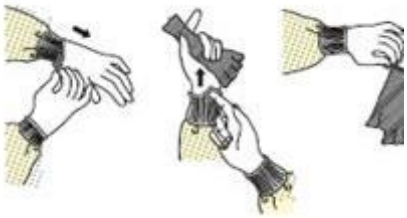
Step 5

- ✓ Put on gloves(over the cuff)

How to Doff the PPE

- Except for respirator, remove PPE at doorway or anteroom. Remove respirator after leaving patient room and closing door
- Avoid contamination of self, others & environment
- Remove the most heavily contaminated items first
- Perform hand hygiene in between the steps if hands become contaminated and immediately after removing PPE

Step1*



- ✓ Grasp outside of glove with opposite gloved hand and peel off,
- ✓ Hold removed glove in gloved hand
- ✓ Slide fingers of ungloved hand under remaining glove at wrist
- ✓ Peel glove off over the first gloves
- ✓ Discard gloves in waste container

Step 2

- ✓ Perform Hand Hygiene(Refer to Annexure D)

Step 3

- ✓ Remove cap(if worn)
- ✓ Remove eye protection from behind(handle by ear pieces or head band)
- ✓ Put eye protection in a separate container (designated receptacle) for reprocessing or waste container

Step 4*

- ✓ Remove gowns
- ✓ Unfasten ties
- ✓ Pull away from neck and shoulders, touching inside of gown only
- ✓ Turn gown inside out
- ✓ Fold or roll into a bundle and discard

Step 5

- ✓ Remove mask from behind
- ✓ Grasp bottom, then top ties or elastics and remove
- ✓ Discard in waste container

Step6

- ✓ Perform hand hygiene (refer to AnnexureD)

*[Note: Gowns and gloves can be removed in one step also as follows:

- Grasp the gown in front and pull away from the body, so that the ties break, touching outside of the gown only with gloved hands
- Fold the gown inside out into a bundle while removing. While removing the gown, peel off the gloves at the same time, only touching inside of the glove and gown with bare hands.
- Discard in waste container].

Perform a respirator seal check

Step 1

Cup the respirator in your hand with the nosepiece at your fingertips allowing the headbands to hang freely below your hand

Step2

Position the respirator under your chin with the nosepiece up

Step3

Pull the top strap over head resting it high at the back of your head. Pull thebottom strap over your head and position it around the neck below the ears

Step 4

Place fingers and both hands at the top of the metal nosepiece

Mould the nosepiece(using the two fingers of each hand) to the shape of your nose. Pinching the nosepiece using one hand may result in less effective respirator performance

Step 5

Cover the front of the respirator with both hands, being careful not to disturb the position of the respirator

Step5a) Positive seal check

Exhale sharply. A positive pressure inside the respirator means no leakage. If leakage is felt, adjust the position and /or tension straps. Retest the seal

Repeat the steps until the respirator is secured properly

Step5b) Negative seal check

Inhale deeply. If no leakage, negative pressure will make respirator cling to your face.

Leakage will result in loss of negative pressure in the respirator due to air entering through the gaps in the seal

Annexure F

Lab Reporting formats

A) Individual Patient Reporting format

Result of suspected case of Influenza (category C)

Name of the laboratory:

Contact details:

SNO	Lab. No./ Sample no.	Name of the Patient / CR NO.	Age / Sex	Date of sample collection	Address / Mob. No.	Name of Hospital	Type of sample	RESULT				
								Inf A	Inf. A Pdm. H1N1	Inf. A H3N2	Inf B	other

`

B) Line List for Reporting by the laboratories

SNO	Lab. No./ Sample no.	Name of the Patient / CR NO.	Age/ Sex	Date of sample collection	Address/ Mob. No.	Oseltamivir taken (YES/NO)	Symptoms					Comorbidities	Name of Hospital	Type of sample	RESULT				
							Fever	Cough	Sore throat	Nasal catarrh	Any other				Inf. A	Inf. A Pdm. H1N1	Inf. A H3N2	Inf B	other

Annexure G

Detection of Influenza Viruses
(Based on WHO/CDC protocol updated September 2016)

Note: primer/probe sets may undergo periodic modification

Primers and probes details	**Sequences (5' to 3')**	**Working Conc. pmole/ul**	**Target (gene)**	**Reference**
Influenza Detection				
InfA Forward	GAC CRA TCC TGT CAC CTC TGA C	10	M gene	CDC protocol
InfA Reverse	AGG GCA TTY TGG ACA AAK CGT CTA	10		
InfA Probe	**FAM-**TGC AGT CCT CGC TCA CTG GGC ACG- MGBNFQ	5		
InfB Forward	TCC TCA AYT CAC TCT TCG AGC G	10	NS gene	
InfB Reverse	CGG TGC TCT TGA CCA AAT TGG	10		
InfB Probe1	**NED or VIC -**CCA ATT CGA GCA GCT GAA ACT GCG GTG-MGBNFQ	5		
Pdm H1 Forward	GTG CTA TAA ACA CCA GCC TCC CATT	10	HA	
Pdm H1 Reverse	AGA **Y**GG GAC ATT CCT CAA TCC TG	10		
Pdm H1 Probe	**FAM-**ATA CAT CC**R** ATC ACA ATT GG**R** AAA TGT CCA AAMGBNFQ	5		
A/H3 Forward	AAG CAT TCC YAA TGA CAA ACC	10	HA	
A/H3 Reverse	ATT GCR CCR AAT ATG CCT CTA GT	10		
A/H3 Probe	**VIC-**CAG GAT CAC ATA TGG GSC CTG TCC CAG-MGBNFQ	5		
RnaseP Forward	AGA TTT GGA CCT GCG AGC G	10	RNasP	
RnaseP Reverse	GAG CGG CTG TCT CCA CAA GT	10		

RnaseP Probe	**VIC**-TTC TGA CCT GAA GGC TCT GCG CG- **MGBNFQ**	5		
Type B Lineage differentiation				
B HA BHA-188F	AGA CCA GAG GGA AAC TAT GCC C	10	Haemaglutinin	WHO
B HA BHA-270R	TCC GGA TGT AAC AGG TCT GAC TT	10		
Type B Victoria	**VIC**-CAGACCAAAATGCACGGGGAAHATACC MGBNFQ	5		
Type B Yamagata	**FAM**-CAGRCCAATGTGTGTGGGGAYCACACC MGBNFQ	5		

The fluorophore FAM,NED,VIC can be used to set duplex real time PCR while in Singleplex PCR all probe can be FAM labelled

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