3.1. General Materials

Glassware, plastics and heat stable solutions were sterilized by autoclaving; sterile deionised Milli-Q water (18 MΩ, Milli-Pore), sterile tubes, tips and solutions were used for DNA and protein handling. Heat labile solutions were filter sterilized using 0.2μm Acrodisc PF filters (Gelman Science).

3.2. Chemicals and Reagents

Laboratory reagents and chemicals were purchased from Himedia, Sigma and the Ranbaxi Chemicals Company unless otherwise stated. Sodium periodate and Carbomyl fuscin were purchased from HiMedia (Mumbai, India). Oseltamivir purchased from (Sigma, Chennai). Isopropanol and ethanol were purchased from Medox Pvt. Ltd. sodium dodecyl sulfate (SDS, 99%) and tetraethyl orthosilicate (TEOS, 98%) were purchased from Sigma-Aldrich Corporation (St. Louis, MO, USA). [Hydroxy(polyethyleneoxy)propyl-]triethoxysilane (PEG-silane, MW 575-750 g/mol, 8-12 EO, 50% in ethanol) was obtained from Gelest (Morrisville, PA). Ammonium nitrate (99.9%) and ammonium hydroxide (NH₄OH, 28-30 wt % as NH₃) were obtained from HiMedia (Mumbai, India). Absolute anhydrous ethanol and 95% ethanol were purchased from Changshu Yangyuan (Changshu, China). Calcium- and magnesium-free Dulbecco’s phosphate-buffered saline (PBS) was obtained from Invitrogen (Grand Island, NY). The deionized (D.I.) water was generated using a Millipore Milli-Q Biocel system (Billerica, MA). Primary antibody was purchased from Santa Cruz Biotechnology (sc-1724, Santa Cruz, CA).

3.3. Cell Culture and Culture Conditions

MDCK cells were cultured in Dulbecco’s Modified Eagles medium (DMEM) supplemented with 2mM L-glutamine and Earle’s BSS adjusted to contain 1.5g/L Na
bicarbonate, 0.1 mM nonessential amino acids, and 1.0 mM of Sodium pyruvate. The cultures were maintained in t-25 flask with the growth condition maintained at 37°C and 5% CO₂ in a air jacketed CO₂ incubator. After the cells attained 70–80% confluency following trypsinization, they were seeded in 96 well plates or 60-mm petriplates of tissue culture grade for experiments.

3.4. Cell Revival

Cells were taken out from −80°C or liquid nitrogen and were made active again. The cells were revived with DMEM containing 20% FBS. The following procedure should be performed in aseptic condition.

- The cells were collected from liquid nitrogen container and transfer immediately to water bath at 37°C.
- When 50% of the contents are thawed and wipe outside of vial with 70% alcohol.
- Add 0.2ml of the fresh 10% MEM to the vial drop by drop while gently mixing the contents thoroughly.
- Transfer cell suspension to tissue culture flask (75cm²) containing a fresh growth medium drop by drop with gentle mixing.
- Label the flask with cell name, passage number, Date of freezing, Date of revival, medium used.
- Incubate bottle at 37°C. Do not disturb.
- End of the day observe bottles, if cells are adherent carefully pipette out the medium (to get rid of Dimethyl Sulfoxide) DMSO and add fresh growth medium. If cells have not adhered, leave it overnight and change the medium the following morning.
- Incubate the bottle until confluent monolayer is formed.
3.5. Subculture of Adherent Cell Lines

Subculturing step gives media change to cells a day after revival of cells. Subculturing avoids possibility of medium in the flask turning acidic or alkaline. DMEM containing 10% FBS need only be used after subculturing.

- When the cells attained about 80% confluency, the culture medium was removed.
- Wash the cell monolayer with PBS. All serum must be removed before the addition of trypsin.
- Add trypsin/EDTA (1 ml/25cm² of surface area) to the washed cell monolayer. Rotate flask to cover the entire surface with trypsin. Return the flask to the incubator and incubate for 2–10 minutes or until cells are detached.
- Examine the cells using an inverted microscope to ensure that the cells are detached and floating. Gently tap the sides of the flask or dish to release adherent cells. Resuspend the cells in a small volume of fresh serum-containing medium to inactive the trypsin.
- Fresh medium containing 10% serum was added and resuspended gently using a Micropipette.
- Cells were counted using hemocytometer
- A fixed number of cells were seeded into either 96-well plates or Petriplates and stored for further experiments.
- Incubate as appropriate for the cell line.

3.6. Cell Seeding

- This step involves the transfer of cells onto 12-well plates (for flow cytometry) and 60-mm plates (for Western blotting).
The culture flasks containing the cells were taken out from the incubator.

The medium within each flask was discarded and the flasks were washed with PBS–EDTA.

Then a little amount of Trypsin was added into each of the flasks. The flasks were then kept for incubation for 1 min, after which they are taken out and observed under a microscope.

When all the cells were rounded and detached from the surface, a little amount of 10% DMEM was added to each flask and the detached cells were resuspended using a pipette.

Then a small amount of cells were transferred into 60-mm or 12-well plates according to the requirement. The amount added can be varied depending on the number of cells required. Then media was added onto the plates as well as the culture flasks.

After observing under a microscope, the cells were kept back in the incubator.

3.7. Cryopreservation of Cells

Cell freezing is very important to maintain a stock of the cell under culture; this is mostly done when cells in the culture flasks become confluent. If the cells become over confluent, then the media might become acidic, this is indicated by the color of the media changing from light pink to lemon yellow.

The culture flasks were taken out from the incubator and the media was discarded.

Then, it was washed with PBS–EDTA.

This was followed by the addition of trypsin to dislodge the cells from the surface. After trypsinisation, the flasks were kept undisturbed in the incubator for 1 min to speed up the rate of detachment of the cells.
The flasks were observed under the microscope to ensure that all the cells have
dislodged from the surface. The shape of the detached cells was round.

Approximately, 1 ml of 10% DMEM was added into the culture flasks and it was
resuspended using a pipette.

The contents of the flasks were transferred into cryovials or freezing vials, which
were also labeled.

The cryovials were then centrifuged at 5000 rpm for 5 min.

The supernatant was discarded and the pellet was dissolved in the freezing medium.

Place cryovials on ice for one hour before transferring to the -80°C freezer overnight.

For most cell cultures, the appropriate rate of cooling is between -1°C and -3°C per
minutes.

Transfer the vials to liquid nitrogen for long-term storage.

Table 3.1: Cell lines used in the present investigation and their sources

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Origin</th>
<th>Morphology</th>
<th>Identification code</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDCK</td>
<td>Dog Kidney Cells</td>
<td>Epithelial</td>
<td>ATCC CCL-34</td>
</tr>
<tr>
<td>Vero</td>
<td>Green Monkey Kidney Cells</td>
<td>Epithelial</td>
<td>ATCC CCL-81</td>
</tr>
<tr>
<td>A549</td>
<td>Human large cell lung carcinoma</td>
<td>Epithelial</td>
<td>ATCC CCL-185</td>
</tr>
</tbody>
</table>
Table 3.2: Equipments and Consumables for Isolation of Influenza viruses

<table>
<thead>
<tr>
<th>Equipment</th>
<th>Consumables</th>
</tr>
</thead>
<tbody>
<tr>
<td>Laminar BSL II Cabinet</td>
<td>T25 flask of MDCK cell line</td>
</tr>
<tr>
<td>Inverted Microscope</td>
<td>MEM, TPCK trypsin vials (0.2ml per vials),</td>
</tr>
<tr>
<td>-80°C deep freezer No 2</td>
<td>Nystatin vials (0.2ml per vial), Gentamycin vial,</td>
</tr>
<tr>
<td>Table Top Cooler</td>
<td>1ml, 5ml and 10ml pipette,</td>
</tr>
<tr>
<td>Refrigerator No-6.</td>
<td>mask, gloves, Spirit swab, discarding bowl</td>
</tr>
</tbody>
</table>

3.8. Isolation of Influenza viruses from throat and nasal swabs specimen

3.8.1. Procedure

- Microscopically examine 48-72hrs T25 flask of MDCK cell line for uniform monolayer formation and make sure that the cells are healthy, confluent and free from microbial contamination.

- Remove MEM and Gentamycin from fridge and TPCK trypsin vials and Nystatin vials from –20°C freezer.

- Switch on the laminar UV for half an hour before starting the work. After 30min switch off laminar UV and switch on laminar flow, light and exhaust, let the flow run for 10 minutes before starting the work.

- Prepare K cards and enter details of sample on K cards and K register. Mark T25 flask (one flask per sample plus one flask for control) with K number, sample number and date of inoculation.

- Wash hands, Wear lab coat and mask before entering the cubicle. Put on the gloves.
- Remove the throat and nasal swab specimens from –70°C freezer, thaw at room temperature under running water and place on wet ice. Enter use of vial in storage card.

- Wipe media bottle and vials with spirit swab.

- Prepare virus growth medium by adding TPCK trypsin (one 0.2 ml vial to 50ml medium), Nystatin (one 0.2 ml vial to 50ml medium) and Gentamycin (0.05ml to 50ml medium) in sterile 100ml bottle.

- Discard growth medium from T25 flask with monolayer MDCK culture, add 2.0ml of virus growth medium, rinse cell sheet, decant and add 3.0ml virus growth medium. Repeat this procedure for all T25 flasks to be used.

- Arrange T25 flask and corresponding specimens to be inoculated in corresponding order.

- Wipe the mouth of the sample vial with spirit. Decant virus growth medium from flask with matching sample number.

- Using 1ml/5ml sterile pipette aspirate complete content of specimen vial and add to the T25 flask gently, directly on the cell sheet. Discard pipette and tightly cork the flask.

- Before inoculating the next specimen, wipe gloves thoroughly with spirit and proceed. If wetting of the gloves with the specimen is suspected, discard and wear fresh gloves. Proceed in this manner till all the specimens are inoculated.

- Mock inoculate control bottle with transport medium.

- Allow the inoculum to adsorb for 30-60 minutes at 37°C

- With intermittent shaking.
Add 4 ml of virus growth media to each flask at the end of adsorption. Wipe gloves with spirit between handling of two flasks.

- Incubate the inoculated flasks at 37°C in incubator.
- Wipe the worktable with spirit swab and switch on the UV for half an hour.
- Observe the flask daily for the following:

**3.8.2. Toxicity:** Cell cultures show rapid degeneration within 1 or 2 days of inoculation, this may be due to non-specific toxicity of the specimen. Culture should be subjected to HA test before discarding. In case sample shows HA activity then subjected to second passage.

- Contamination: If cell cultures medium shows high turbidity or fungal culture, culture should be discarded and second aliquot of samples should be Millipore filtered before inoculation.
- Cytopathic effect: Cells become progressively granular, swollen and round. Amount of floating cells increase significantly. The CPE is graded from 1+ to 4+, depending on the percentage of cell sheet affected. (1+ = 25%, 2+ = 50%, 3+ = 75% and 4+ = 100%). At 3+/4+ CPE transfer flask to 4°C refrigerator and perform a HA test.
- Pass the supernatant from the entire flask to fresh T25 flask for second passage on 7th day.
- Passage 1 and 2 are performed separately. The procedure is the same as for passage 1.
- Harvest supernatant from all flask into sterile 10ml vials and perform a HA test of supernatant from all flasks. If the HA titer is >/= 1:8 remove 1.0ml and subject supernatant to HI test (SOP#006). To remaining supernatant add 0.5ml/10ml of 10% BSA in DW. Make 1.0ml aliquots and store at –80°C. Make storage cards and make entry in isolation data sheet.
Perform HA on all flask showing no CPE on day 7 of passage 2 and report negative if no titer is observed.

3.8.3. Safety conditions:

- All safety guidelines to be followed for disposal of waste
- Biosafety guidelines are adhered to.
- Viral cross-contamination: Utmost care should be taken to avoid viral cross contamination of cultures during inoculation and passage collection. Do not decant the supernatant from inoculated flasks. It should be removed only with a pipette. Take care to avoid aerosols created by vigorous pipetting. Perform Passage 1 & 2 in different sittings. Passage 2 of those found positive in Passage 1 are inoculated after the negatives.
- Spilled droplets should be immediately cleaned with disinfectant. Any spillage of infective material should be brought to the notice of the lab head.

3.9. HA & HAI test for identification of Influenza viruses

Table. 3.3. Equipments and Consumables for HA & HAI test

<table>
<thead>
<tr>
<th>Equipment</th>
<th>Consumables</th>
</tr>
</thead>
<tbody>
<tr>
<td>Laminar Flow</td>
<td>Guinea pig, 5ml syringe with 22 or 23 gauge needle, 15 ml centrifuge tube, 50ml conical flask, Alsever’s solution, PBS pH 7.2, WHO influenza reagent Kit, U/V bottomed Microtitre plate Ice pack</td>
</tr>
<tr>
<td>Vortex mixer</td>
<td>2ml Pipettes, U/V bottomed Microtitre plate</td>
</tr>
<tr>
<td>Micro pipette-5-50 and 20-200μl.</td>
<td>U/V bottomed Microtitre plate</td>
</tr>
<tr>
<td>Centrifuge maintained at room temperature</td>
<td>U/V bottomed Microtitre plate</td>
</tr>
<tr>
<td>Refrigerator No-6</td>
<td>WHO influenza reagent Kit, Sterile distilled water</td>
</tr>
<tr>
<td>-20 deep freezer No.2.</td>
<td>Phosphate buffered saline pH 7.2, Physiological saline 0.85% NaCl, Micropipettes, tips</td>
</tr>
</tbody>
</table>

HA: Haemagglutination

HAI: Haemagglutination inhibition

RBC: Red blood cell
3.10. Collection of blood and Preparation of RBC suspension

3.10.1. Collection of blood

Draw 0.5ml of Alsever’s solution in 5ml syringe fitted with a 22 or 23 gauge needle. Locate the point on the chest of the G.pig for cardiac puncture. Insert the needle of the syringe containing Alsever’s solution through the identified point into the heart take care to avoid puncture of the heart. Collect 5 ml of blood. Transfer the blood to a conical flask containing 12.5ml of Alsever’s solution. The suspension is mixed and stored at 4°C.

Washing of Guineapig Erythrocytes

1. Take a 50ml beaker and filter the RBC suspension through cotton gauze.
2. Transfer the filtered RBC suspension to a labeled 15ml centrifuge tube. Centrifuge at 1200rpm for 5min.
3. Remove and discard the supernatant fluid and the buffy layer of white blood corpuscles using a 10ml pipette, without disturbing the packed erythrocytes.
4. Add 10ml of PBS solution to the packed RBCs and swirl the tube gently to mix.
   Centrifuge at 1200rpm for 5min.
5. Repeat step # 3 and 4 thrice.
6. For final wash centrifuge at 1200rpm for 10min.
7. Now the cell suspension is ready for use in the HA/HAI tests. Store the packed cells in refrigerator.

3.10.2. Preparation of 0.75% RBC’s

1. Take a 15ml centrifuge tube and add 4.5ml of PBS with a 5ml pipette.
2. Using a micropipette dispense 462μl of PBS to make up the volume to 4.962ml.
3. To this add 38μl of packed RBCs with a micropipette to get a cell suspension of 0.75% concentration.

3.11. BIOSAFETY CABINET

- Daily before each use:
  - Switch on the ultraviolet light for 15 minutes after fully closing the view screen.
  - Switch on the blower of the cabinet after switching off the UV lamp. Run the airflow for 15 min before starting the work.
  - Switch on the light and open the view screen to start work.
  - Do not overcrowd the cabinet and never obstruct the front opening. Organize the work area so that sterile reagents and samples do not come into contact with each other (pots for liquid waste to the left and sterile media to the right with samples handled centrally).
  - Clean and decontaminate the inner surfaces of the cabinet after every working session with 1% Hypochlorite.
  - Switch on the u-v lamp after work for 15 minutes after fully closing the view screen.

**Every month**

- Check the sterility once a month by plate exposure method. Place Nutrient agar and blood agar plates in the anterior area, central and rear surface of the bench. Expose for half an hour, close and incubate at 37°C. Record observation for 48 hrs.

**Every 6 months**

- The service engineer Air particulate systems to inspect and validate the cabinet. DOP and airflow velocity to be tested. Record in the maintenance register.
Caution:

- Never leave the ultraviolet lamp on while personnel are working in the room.
- Do not use disinfectants such as bleach or iodine as they may corrode or stain the surface.
- Never operate the cabinet while the view screen alarm indicator is on.
- Never use the cabinet to store supplies or laboratory equipment.

Virus, cells and reagents

Influenza A/H1N1 (2009) and H3N2 viral strains were obtained from King Institute of Preventive Medicine & Research, Virology Department, Chennai. Virus was propagated in Madin-Darby canine kidney (MDCK) cells.

Preparation of Culture Media

Millipore double distilled water was collected in sterile 2L bottles. Millipore double distilled water was sterilized by autoclaving at 15lbs pressure, 121°C for 20min. Required volume of sterilized millipore double distilled water was transferred in 2L flask depending on the media to be prepared (growth media 10%, 5% or maintenance media). Add the MEM powder, mix well and sterilize at 15lbs pressure, 121°C. Allow cooling to room temperature and adding the ingredients in quantities as indicated in the protocol depending on the concentration of FCS.

MEM Preparation

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>10% Media</th>
<th>5% Media</th>
<th>2% Media</th>
</tr>
</thead>
<tbody>
<tr>
<td>MEM (SIGMA)</td>
<td>851 ml</td>
<td>901 ml</td>
<td>931 ml</td>
</tr>
<tr>
<td>P &amp; S (100Iu of penicillin)</td>
<td>1 ml</td>
<td>1 ml</td>
<td>1 ml</td>
</tr>
<tr>
<td>100µg of streptomycin)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Kanamycin (20mcg/ml) 1 ml 1 ml 1 ml
Fungizone (20mcg/ml) 1 ml 1 ml 1 ml
3% L-Glutamine 10ml 10ml 10ml
Foetal Bovine Serum 100ml 50ml 20ml
7.5% Sodium bicarbonate 30ml 30ml 30ml
Hepes buffer (IM) 15ml 15ml 15ml
(adjusted to pH 7.2)
Phenol red (0.4%) 1 ml 1 ml 1 ml
Total volume 1000ml 1000ml 1000ml

Preparation of Ingredients

1. **Penicillin and Streptomycin**: (Concentration 100IU of penicillin and 100µg of streptomycin) were dissolved with 20ml sterile distilled water. The solution was aliquot 2ml in 10 polypropylene vials store at -20°C.

2. **Kanamycin Acid Sulphate**: (Concentration 20µg/ml) 5gm of kanamycin acid sulphate was dissolved in 50ml millipore distilled water. Take 10ml from this concentration bulk and add to 40ml millipore distilled water mix well distribute in 1ml aliquots in vials. The solution was stored at -20°C until further use.

3. **Fungizone: Amphotericin B – 50µg** (Concentration 20µg/ml) The 20ml vial kept at -20°C. Thaw it to room temperature distribute in 1ml aliquots in vials.

4. **3% L-Glutamine**: 6gm of L-Glutamine was dissolved in 200ml of sterile millipore distilled water and mix well. Filter through Whatmann filter paper No.1 and distribute in 5ml aliquots in vials store at -20°C.
5. **7.5% Sodium-bi-carbonate solution**: 22.5 gm of Sodium Bicarbonate was dissolved in 300 ml of sterile millipore double distilled water. Filter through Whatmann filter paper No.1. The solution was distributed in 10 X 30 ml bottles and autoclave at 10 lbs for 10 minutes store at 4°C.

6. **Fetal Bovine Serum 500ml**: Thaw a bottle of FCS to room temperature. If floating particles are seen, filter through Seitz filter. Distribute in 100ml, 50ml and 20ml quantities in sterile bottles. Store in –20°C freezer.

7. **Preparation of Trypsin, PBS, Versene, Glucose (TPVG) – 1000ml**

   - **PBS** - 840 ml
   - 2% Trypsin - 50ml
   - 0.2% EDTA - 100ml
   - 10% Glucose - 5ml
   - P&S - 5ml

   All the ingredients were mixed well and adjust the pH to 7.4 by 0.1NHCl or 0.1N NaOH. Store at -20°C.

**PBS: 1000ml**

   - NaCl - 8gm
   - KCl - 0.2gm
   - Na₂HPO₄ - 2.88gm
   - KH₂PO₄ - 0.2gm
   - pH - 7.4

All the ingredients were dissolved in 1000ml of sterile millipore distilled water. The solution was filtered through Whatmann filter paper No.1 and autoclave at 15lbs for 15min.
Trypsin: 100ml

2gm of trypsin was dissolved in 100ml sterile millipore distilled water with magnetic stirrer for 1/2hrs. The trypsin solution was filtered through Whatmann filter paper No.1 and store at -20°C.

2% EDTA (Versene)

200mg of EDTA was dissolved in 100ml of sterile millipore distilled water and then autoclave at 15lbs/15min.

10% Glucose - 10ml

1gm of glucose was dissolved in 10ml of sterile millipore distilled water and filter through Whatman filter paper and autoclave at 10lbs/15min.

Freezing Media: 10% FCS with 10% DMSO

Ingredients:
MEM - 80ml
FBS - 10ml
DMSO - 10ml

Preparation of PBS (Phosphate Buffered Saline) pH 7.3

NaCl - 41.62 g
KCl - 1.04 g
Na₂HPO₄ - 5.21 g
KH₂PO₄ - 1.51 g

Add 350 ml distilled water to the beaker. When all the solid material has dissolved, adjust the pH to 7.3 with 1N NaOH solution. Pour solution into 500 ml graduated cylinder. Add distilled water to 500 ml mark. Pour solution into 5L beaker and add 4.5L H₂O.
General Materials & Methods

**Lysis Buffer**

- NP 40 - 1%
- Glycerol - 10%
- NaCl - 137 mM
- Tris–HCl (pH 7.4) - 20 mM
- Aprotinin - 1 μg/ml
- Leupeptin - 1 μg/ml
- NaF - 20 mM
- Sodium pyrophosphate - 1 mM
- Sodium orthovandate - 1 mM
- PMSF - 5 mM
- Triton-X 100 - 1%

**3.12. Chemicals Preparation for Western blotting Analysis**

**Bradford's Reagent**

Coomassie Blue G250 dissolved 100mg in 50ml of 90% ethanol; add 100ml of 85.7% phosphoric acid. Make the volume to 100ml with deionised water. Filter through Whatmann No. 1 filter paper.

**Sample Buffer (1x)**

- 10% SDS - 2 ml
- Glycerol - 1 ml
- 1 M Tris (pH 6.8) - 0.6 ml
- Bromo Phenol Blue - 0.005%
2-Mercapto ethanol - 1%

Water - 2.8 ml

**Lower Tris (1.5 M)**

Tris: 18.17g dissolved in 70ml distilled water.

Adjust to pH 8.8 using HCl. Add 2 ml 20% SDS and makeup the volume to 100 ml.

**Upper Tris (0.5 M)**

Tris: 6.06g dissolved in 70ml distilled water. Adjust to pH 6.78 using HCl. Add 2 ml 20% SDS and makeup volume up to 100ml.

**10% Separating Gel Composition: (20 ml)**

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distilled water</td>
<td>6.8 ml</td>
</tr>
<tr>
<td>30% Aeryl amide</td>
<td>5 ml</td>
</tr>
<tr>
<td>1 M Tris (pH 8.8)</td>
<td>3.8 ml</td>
</tr>
<tr>
<td>10% SDS</td>
<td>150 µl</td>
</tr>
<tr>
<td>10% APS</td>
<td>150 µl</td>
</tr>
<tr>
<td>TEMED</td>
<td>6 µl</td>
</tr>
</tbody>
</table>

**Stacking Gel Composition: (6 ml)**

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distilled water</td>
<td>4.1 ml</td>
</tr>
<tr>
<td>30% Aeryl amide</td>
<td>1 ml</td>
</tr>
<tr>
<td>1 M Tris (pH 6.8)</td>
<td>0.75 ml</td>
</tr>
<tr>
<td>10% SDS</td>
<td>60 µl</td>
</tr>
<tr>
<td>10% APS</td>
<td>60 µl</td>
</tr>
<tr>
<td>TEMED</td>
<td>6 µl</td>
</tr>
</tbody>
</table>
30% Acrylamide (100 ml)

- Acrylamide: 29.2 g
- Bis-Acrylamide: 800 mg

Electrode Buffer (8x)

- Tris: 12 g
- Glycine: 57.6 g

Volume makeup to 500 ml using water.

Electrode Buffer (1x)

- 8x Electrode buffer: 50 ml
- Distilled water: 350 ml
- 10% SDS: 4 ml

Transfer Buffer (Towbin’s Buffer)

- Tris: 3 g
- Glycine: 14.4 g
- Methanol: 200 ml

Volume makeup to 1 L using distilled water.

Tris Buffer Saline (TBS) (10x)

- Tris: 24.2 g
- NaCl: 80 g

Volume makeup to 1 L using distilled water and the pH was adjusted to 7.6.

TBS-Tween 20 (TBS-T)

- 10x TBS 50 ml

Volume makeup to 500 ml using distilled water, then, 1–2 ml of Tween 20 was added.
Ponceau’s Dye (stored at room temperature)

- Ponceau’s powder - 2 g
- Trichloroacetic acid (TCA) - 30 g
- Sulfosalicylic acid - 30 g
- Distilled water - 100 ml

Blocking Solution

- Non Fat Milk Powder - 1.5 g
- TBS-T - 30 ml

Developing solution (DAB Staining Solution) (10 ml)

- DAB (3,3’-di amino benzidene) (Sigma) - 6 mg
- 50 mM Tris (pH 7.6) - 10 ml
- Hydrogen peroxide - 20 µl

Instruments

- -20°C Freezer (Siemens)
- Centrifuge—(Sigma 3K30)
- CO₂ incubator—(Thermo Forma Series 11 Water Jacketed CO2 incubator)
- Laminar air flow—(Microfilm India Laminar flow ultra clean air unit)
- Ultra low temperature freezer (~80°C) (New Brunswick Scientific)
- AGE- apparatus
- SDS-PAGE- apparatus
- Western blotting apparatus (Bio-Rad).

3.13. TEM Analysis

- 4% Paraformaldehyde
2.5% Glutaraldehyde

4% Osmium tetroxide

0.5% Uranyl acetate

**Preparation of a Paraformaldehyde Fixative Solution**

For 10 ml of a 4% paraformaldehyde stock solution:

- Weigh out 0.4 g of paraformaldehyde, transfer powder into 15 ml conical tube.
- Add 8 ml of ddH$_2$O
- Add 5 µl of 1 M NaOH
- Heat to 70$^\circ$C (cap on), mix frequently until complete solubilization (~10 min at 70$^\circ$C; the solution should become clear)
- Put on ice and allow the solution to cool down to ~ room temperature
- Adjust volume to 9 ml with ddH$_2$O
- Carefully pH to pH 7.2-7.4.
- Add 1 ml of PBS 10X and mix.
- Filter solution using a 0.2 µm 25 mm nylon syringe filter.
- Prepare a 2% paraformaldehyde solution by diluting 4% paraformaldehyde solution in PBS.
- Use the fixative at room temperature

**Preparation of a Glutaraldehyde Fixative Solution**

For 50 ml of a 3% glutaraldehyde:

- 0.2 M phosphate solution
- 12 ml 25% glutaraldehyde
- 38 ml distilled water
4% stock Aqueous Osmium Tetroxide

- Take a 0.5 g osmium ampoule and score the neck with a glass cutter
- Break it using an ampoule snapper and immediately place both pieces into a Pyrex glass bottle containing 12.5mls of distilled water.
- Close tightly and leave overnight or until the osmium is in solution. If required urgently it can be dissolved rapidly in a sonicator.
- Stored at room temperature wrapped in aluminum foil or dark bottle. If stored properly, it is good for about 3 months. Do not use if it changes to a darker yellow.

3.14. Sodium Dodecyl Sulphate- Polyacrylamide Gel Electrophoresis of Protein - SDS-PAGE

Electrophoresis is used to separate complex mixture of proteins, to investigate subunit composition, to verify homogeneity of protein sample, and to purify proteins for use in further applications. SDS-PAGE of protein is carried out in the presence of SDS (CH₃(CH₂)₁₀CH₂OSO₃Na⁺), an anionic detergent that readily binds and dissociates oligomeric proteins in the presence of reducing agent, 2-mercaptoethanol into their subunits. Analysis and comparison of proteins in a larger number of samples is easily made on polyacrylamide gel slabs containing SDS under denaturing conditions.

Principle

A molecule with a net charge will move in an electric field. This phenomenon, termed electrophoresis offers a powerful means of separating protein and other macromolecules. The velocity of migration (V) of a protein in an electric field depends on the electric field strength (E), the net charge on the protein (Z) and the frictional coefficient (f).

\[ V = EZ/f \]
In SDS-PAGE, the migration rate of a protein is determined by the gel pore size and the protein charge, size and shape. Under the denature condition, SDS binds to the uncoiled molecules and also confers a negative charge on all of the proteins so that in an electric field they will migrate solely as a function of their molecular weights. This is articulated as the following expression.

\[ R_f = A - B \log_{10} MW \]

Where, \( R_f \) = Relative mobility  
MW = Molecular weight  
A & B = Constant for a particular experimental system.

Thus the size of the polypeptide chain of a given protein can be determined by comparing the electrophoretic mobility in SDS-PAGE with the mobility of marker protein of known molecular weights.

**Materials Required**

Acrylamide, Bis acrylamide, Tris-buffer, SDS, TEMED, Glycine, Ammonium per sulphate (APS), 2-mercaptoethanol, Glycerol, Bromophenol blue and distilled water. Gel plate, spacers, clamps, electrophoresis apparatus, power pack and power card.

**Reagents**

**Solution A**

Acrylamide - 29.02g  
Bis acrylamide - 0.80g

(Final volume is to be made up to 100 ml with distilled water. Filter, sterilize and store the solution in brown bottle at 4°C)

**Solution B**

*Lower gel Buffer (pH 8.8)*
0.75M Tris base

For 100ml preparation, 9.1g Tris base dissolved in 95ml of distilled water and adjust the pH with 30% HCl. Then made up to final volume 100ml with distilled water and store at 4°C.

**Upper Gel Buffer (pH 6.8)**

0.5M Tris base

For 10ml preparation 605mg of Tris base dissolved in 9ml of distilled water and adjust the pH with 0.1M HCl. Then made up to final volume 10ml with distilled water and store at 4°C.

**Solution C**

10% Sodium dodecyl sulphate (SDS) - 1g of SDS in 10 ml of distilled water.

**Solution D**

10% Ammonium per sulphate (APS) -100mg in 1ml distilled water; make fresh whenever necessary

**Solution E**

TEMED – Store at 4°C

**Running Buffer**

SDS - 1g
Glycine - 14.4g
Tris acetate - 3g H₂O to 1000ml

**SDS gel – Loading Buffer (10ml)**

Upper Tris - 1.25ml
Glycerol - 1.00ml
B-mercaptoethanol - 0.50ml
SDS - 150mg
General Materials & Methods

**Bromophenol blue** - pinch

**Distilled water** - 7.25ml

Store at 4°C.

**Staining Solution**

- Coomassie blue (R- 250) - 1g
- Methanol - 250ml
- Glacial acetic acid - 20ml

It is made up to 500 ml with the help of distilled water.

**Destaining Solution**

- Methanol - 150ml
- Glacial acetic acid - 35ml

**Sample Preparation**

Amount of protein sample required for loading in the SDS –PAGE is determined by the following adopting Bradford method.

\[
\text{Total volume of well (µl) } \times \text{ Std OD } \times \text{ Volume of sample (µ) } \\
\text{-----------------------------------------------} \\
\text{Std concentration (µg) } \times \text{ OD of the sample}
\]

- The protein samples are mixed with equal volumes of gel loading buffer.
- Heat sample in a microfuge tubes for 5min at 95°C in a float in a water bath.
- Allow to cool in room temperature.

**Casting the Gel**

- Fix spacers on both sides of the gel plate with the help of the clamps
- Seal the bottom with either agar or with another spacer
- prepare resolving gel and pour it in between two plates 2/3 of its height
 Add ethanol as gel overlay without disturbing the surface of the gel

 Once the gel is polymerized, decant the gel overlay by tilting the gel; wash gel surface gently with distilled water

 Prepare stacking gel, pour over the resolving gel and insert the Teflon comb immediately to form wells

 When the stacking gel is polymerized, remove the comb

 The glass plate with polymerized gel is then fixed to the electrophoresis tank

**Gel Electrophoresis**

 Assemble the electrophoresis equipment according to the instruction of the manufacturer

 Gently load the sample on to the wells; and then slowly fill up the top chamber with buffer without much agitation.

 Add buffer to the bottom reservoirs equally and then connect the tank to the power supply and turn the power on.

 Run the gel until the Bromophenol/tracking dye reaches the bottom of the resolving gel

**3.15. Coomassie Blue Staining**

**Procedure**

Once the gel front has reached the base of the gel, the gel is removed and the stacking gel is cut off from the resolving gel. The marker lane is indicated by a cut made at the end of the gel. This is then transferred on to a trough containing enough amount of staining solution so as to immerse the gel completely. The trough is placed on a shaker for 4-5hrs. The stain is removed and saved for further use.
**Staining**

Coomassie blue R250 - 0.025g

- Methanol - 63ml

- Acetic acid - 8.3ml made up to 100ml with distilled water.

**Destaining Solution**

The gel is transferred into a trough containing enough of the destaining solution and left for 2-3 hrs. The bands developed are then observed under illumination and subsequently photographed. Destaining solution contains 30ml methanol, 10ml acetic acid and 60ml of distilled water.

**3.16. Polymerase Chain Reaction-PCR**

**Introduction**

The polymerase chain reaction (PCR) is an extremely powerful method that allows one to amplify a selected DNA sequence in a genome million fold or even more after the invention of thermal cycler by Harry Mullis in 1986. PCR can be used to clone a given DNA sequence under *in vitro* condition without using living cells. This PCR technique can be applied only when the nucleotide sequence of at least one short DNA segment on each side of the gene of interest is known. The primers used in PCR are forward and reverse primers which are priming the respective 5’ and 3’ ends of the template sequence. This fabulous technique gained popularity after the identification of a thermo stable DNA polymerase enzyme from a bacterial species known as *Thermus aquaticus*. Later that peculiar enzyme was named as Taq polymerase and also commercialized as vent polymerase. The PCR procedure involves three major steps (i). The genomic DNA containing the sequence to be amplified is denatured by heating (ii). The denatured DNA is annealed with the constructed
specific oligonucleotide primers. (iii). Taq- polymerase is used to replicate the DNA segment between the sites complementary to the oligonucleotide primers.

Table 3.4. Primers used in this study

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer</th>
<th>Reverse primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>M. Gene</td>
<td>5'-AGTGAGCGGACTGCAGCGT-3'</td>
<td>5'-TAGCYTTAGCYGTRGTGCTGCG-3'</td>
</tr>
<tr>
<td>GAPDH</td>
<td>5'-CCCACACCATCTCCAGGAGC-3'</td>
<td>5'-CCAGTGAGCCTCCCTTCAGC-3'</td>
</tr>
<tr>
<td>NP Gene</td>
<td>5'-TGC TGG ATT CTC GTT CGG TC-3'</td>
<td>5'-CCT TTA TGA CAA AGA AGA AAT AAG GCG</td>
</tr>
<tr>
<td>β-actin</td>
<td>5'-TCA CCC GAG TCC ATC ACG AT</td>
<td>5'-GAA GTA CCC CAT TGA GCA CGG</td>
</tr>
</tbody>
</table>

**Principle**

An *in vitro* amplification of specific DNA using thermos table DNA polymerase enzyme and a minimum of 20-25 bp containing primers specific to the template DNA. During this processes, new DNA strands are produced from the reaction mixture containing appropriate ratio of dNTPs (A, T, G & C).

Based on three-temperature cycles new DNA is synthesized:

(a) Temperature at 70 - 85°C separates two strands of target DNA.

(b) Temperature at 40 - 60°C anneals two complementary primers to the ends of separated single strands of target DNA.

(c) Temperature at 72°C allows thermostable Taq polymerase to use single stranded target DNA and primers to synthesise new strands.

**Materials Required**

Template DNA

Taq DNA polymerase
Reagents

- 10 X Taq DNA polymerase buffer
- 25 mM MgCl₂
- DNA template & Primers
- PCR machine

Procedure

- Add 38 µL of sterile mile Q water (or) autoclaved double distilled water into a 0.2
  /0.5 µl microfuge tube.
- Add 5µL of 10 X polymerase assay buffer with magnesium chloride.
- Add 3 µL of 2.5 µl dwtps mix solution.
- Add 1 µL of template DNA (200 µg /µL).
- Add 1 µL of each of forward and reverse primers (250 ng /µL).
- Add 1 to 2 units of *Taq* DNA polymerase (3 units /µL).
- Mix the solution gently.
- Layer the reaction mix with 50µL of mineral oil to avoid any evaporation.
- Carry out the amplification using following reaction conditions for atleast 30 cycles.
- Initial temperature 94°C – 1 min.
  
  Denaturation → 94°C for 30 sec.
  
  Annealing → 48°C for 30 sec. 30 cycles.
  
  Extension → 72°C for 1 min.
  
  Final extension → 72°C for 2 min.
After the reaction is over, take out the reaction mixture and run 10 μL of aqueous layer in 1% agarose gel for 1 to 2 hrs at 100 volt.

Stain with ethidium bromide and visualise under UV transilluminator.

3.17. Statistical Analysis

All measurements were made in triplicate and all values were expressed as the mean ± standard error of the mean. The results were expressed as means±SD. Statistical significances were determined using the Student’s t-test, and p-values of <0.05 were considered statistically significant.

Instrumentation

3.18. X-RAY DIFFRACTION (XRD) ANALYSIS

Aim

X-ray scattering techniques are a family of non-destructive analytical techniques which reveal information about the crystallographic structure, chemical composition, and physical properties of materials and thin films. These techniques are based on observing the scattered intensity of an X-ray beam hitting a sample as a function of incident and scattered angle, polarization, and wavelength or energy.

Principle

When a monochromatic X-ray impinges upon the atoms in a crystal lattice, each atom act as a source of scattering the radiation of same wavelength. The crystal acts as a sense of parallel reflecting planes. The intensity of the reflected beam at a certain angle will be maximum, when the path difference between two reflected waves from two different planes is an integral multiple of wavelength.
**Diffraction and the Bragg Equation**

- Diffraction of an X-ray beam striking a crystal occurs because the λ of the X-ray beam is similar to the spacing of atoms in minerals [1 – 10 Å]. When an X-ray beam encounters the regular 3-D arrangement of atoms on a crystal most of the X-ray will destructively interface with each other and cancel each other out, but in some specific directions they constructively interface and reinforce one another.

- W.L. Bragg showed the diffracted X-rays act as if they were reflected from a family of planes within crystals. Bragg’s planes are the rows of atoms that make up the crystal structure. These reflections were shown to only occur under certain conditions which satisfy the equation:

\[
n\lambda = 2dsin\theta
\]

**Figure 3.1. Diffraction of X-ray from set of planes**

Where, ‘n’ is an integer [1, 2, 3 … n], λ is the wavelength, ‘d’ is the distance between atomic planes and ‘θ’ is the angle of incidence and the atomic planes. 2dsinθ is the path length difference between two incident X-ray beams. Figure show the diffraction of X-Ray from set of planes.
Determination of Structural parameters

1. Interplanar spacing ($d_{hkl}$)

From the XRD profiles, the inter planar spacing $d_{hkl}$ has been calculated using the Bragg’s relation,

$$d_{hkl} = \frac{n \lambda}{2 \sin \theta}$$

2. Crystalline size ($D$)

The crystalline size ($D$) was calculated using the Scherrer’s formula, from the Full Width at Half Maximum (FWHM).

$$D = \frac{K \lambda}{\beta \cos \theta}$$

Where, the constant ‘$K$’ is the shape factor = 0.94, ‘$\lambda$’ the wavelength of X-Rays (1.5406 for Cu $K\alpha$), ‘$\theta$’ the Bragg’s angle and ‘$\beta$’ the FWHM. The XRD pattern recorded in the 2theta angle range 20-90. The grain size of nanoparticles were calculated from X-ray diffraction pattern using equation.

Observation

The observations of spectrum reveal information about the crystallographic structure, chemical composition, and physical properties of materials

3.19. TRANSMISSION ELECTRON MICROSCOPE (TEM) ANALYSIS

Aim

Electron beam can have a very high resolving power, which is used in imaging technology. There are three types of electron microscopes, namely the transmission electron microscope (TEM), scanning electron microscope (SEM), and scanning tunneling microscope (STM). In this section, the principle of TEM was described.
Working principle

TEM works like a slide projector. A projector shines a beam of light which transmits through the slide. The patterns painted on the slide only allow certain parts of the light beam to pass through. Thus the transmitted beam replicates the patterns on the slide, forming an enlarged image of the slide when falling on the screen.

TEMs work the same way except that they shine a beam of electrons (like the light in a slide projector) through the specimen (like the slide). However, in TEM, the transmission of electron beam is highly dependent on the properties of material being examined. Such properties include density, composition, etc. For example, porous material will allow more electrons to pass through while dense material will allow less. As a result, a specimen with a non-uniform density can be examined by this technique. Whatever part is transmitted is projected onto a phosphor screen for the user to see.

Figure 3.2. The schematic outline of a TEM

Figure shows a schematic outline of a TEM. A TEM contains four parts: electron source, electromagnetic lens system, sample holder, and imaging system.
**Electron source**

The electron source consists of a cathode and an anode. The cathode is a tungsten filament which emits electrons when being heated. A negative cap confines the electrons into a loosely focused beam. The beam is then accelerated towards the specimen by the positive anode. Electrons at the rim of the beam will fall onto the anode while the others at the center will pass through the small hole of the anode. The electron source works like a cathode ray tube.

**Electromagnetic lens system**

After leaving the electron source, the electron beam is tightly focused using electromagnetic lens and metal apertures. The system only allows electrons within a small energy range to pass through, so the electrons in the electron beam will have a well-defined energy.

1. Magnetic Lens: Circular electro-magnets capable of generating a precise circular magnetic field. The field acts like an optical lens to focus the electrons.
2. Aperture: A thin disk with a small (2-100 micrometers) circular through-hole. It is used to restrict the electron beam and filter out unwanted electrons before hitting the specimen.

**Sample holder**

The sample holder is a platform equipped with mechanical arm for holding the specimen and controlling its position.

**Observations**

The imaging system consists of another electromagnetic lens system and a screen. The electromagnetic lens system contains two lens systems, one for refocusing the electrons after they pass through the specimen, and the other for enlarging the image and projecting it.
onto the screen. The screen has a phosphorescent plate which glows when being hit by electrons. Image forms in a way similar to photography. (Eg: SEM image of silver nanoparticles).

3.20. FOURIER TRANSFER INFRARED SPECTROSCOPY (FT-IR) ANALYSIS

**Aim**

To find out the functional group of synthesized nanomaterials using FTIR analysis.

**Working principle**

The application of traditional infrared spectroscopy for low concentration measurements, such as ambient and air measurements is limited by several factors. First is the significant presence of water vapour, CO$_2$ and methane, which strongly absorb in many regions of the infrared (IR) spectrum. Consequently, the spectral regions that can easily be used to search for pollutants are limited to 760-1300 cm$^{-1}$, 2000-2230 cm$^{-1}$, and 2390-3000 cm$^{-1}$. Another problem is that the sensitivity is not enough to detect very small concentrations in the sub-ppm level. Finally, spectral analysis was difficult since subtraction of background spectra had to be carried out manually.

The development of Fourier Transform Infra Red spectroscopy (FTIR) in the early 1970s provided a quantum leap in infrared analytical capabilities for monitoring trace pollutants in ambient air. This technique offered a number of advantages over conventional infrared systems, including sensitivity, speed and improved data processing.

**Basic components of FTIR**

- Infra Red Source
- Interferometer
- Sample
- Detector
- Signal and Data Processing
The basic components of an FTIR are shown in Figure the infrared source emits a broad band of different wavelength of infrared radiation. The IR source used in the Temet GASMET FTIR CR-series is a SiC ceramic at a temperature of 1550 K. The IR radiation goes through an interferometer that modulates the infrared radiation. The interferometer performs an optical inverse Fourier transform on the entering IR radiation. The modulated IR beam passes through the gas sample where it is absorbed to various extents at different wavelengths by the various molecules present. Finally the intensity of the IR beam is detected by a detector, which is a liquid-nitrogen cooled MCT (Mercury-Cadmium-Telluride) detector in the case of the Temet GASMET FTIR CR-series.

**Observations**

The detected signal is digitized and Fourier transformed by the computer to get the IR spectrum of the sample and functional groups.