Annexure III

Protocols

A. Dissection of avian embryonic heart (E: 5):

1. Collection of incubated chicken embryos
2. Isolation of the whole embryo
3. Localization of the whole heart
4. Dissection of the heart using required instruments
5. Hearts are harvested for epicardial explants

B. Dissection of avian Proepicardial organ (PEO) (E: 4):

1. Collection of incubated chicken embryos
2. Isolation of the whole embryo
3. Localization of the whole heart
4. Localization of the PEO near atrio-ventricular (AV) junction
5. Extraction of PEO for explants

C. Dissection of adult avian Aortic valve leaflets:

1. Collection of adult avian hearts
2. Dissection of the aorta
3. Aortic valvular leaflets are dissected out from the base region of the aorta of adult heart

Dissection Instruments:

- Forceps
- Scissors
- Dissection tray
- Petriplates
D. RNA Isolation:

**From tissue**

1. Dissection of the tissues from embryonic and adult heart
2. Tissues are placed in microcentrifuge tube adding 200μl Trizol for each set of tissues
3. Tissues are broken down by pipette up and down
4. 40μl Chloroform are added
5. Vortex for 10 secs
6. Samples in microcentrifuge tubes are put on ice for 2-3mins
7. Samples are centrifuged at 12000g 15mins (4°C)
8. Top aqueous layers are put in a new tube
9. 100μl Isopropanol is added to precipitate the RNA of samples
10. Samples are incubated for 10mins at room temperature at least 1hr at -80°C (or overnight at -20°C)
11. Samples are centrifuged at 12000g 10mins (4°C)
12. Supernatants are removed
13. RNA pellets are washed with 100 μl 75% EtOH
14. Samples are centrifuged at 7500g 5mins (4°C)
15. RNA pellets are air dried
16. RNA pellets are resuspended in water (Samples can be incubated at 65°C for 10 mins to help dissolve) [20-25μl for each tube]
17. Concentration of the RNA samples is taken
18. Store at -80°C

**From cell**

1. Cell media are removed by aspiration
2. Cell plates or chamber slides are washed with 1XPBS [1ml for 60mm plates and 500μl for chamber slides]
3. 200 μl of Trizol is added for plates and 100 μl of Trizol is added for each groove of the chamber slide
4. Kept for 5mins at room temperature (RT)
5. [4-18] steps of the previous protocol (RNA isolation from tissue) are followed
NB:

In step 3 depending upon the plate size the required amount of Trizol should be adjusted: 1ml for 100mm dish and 2ml for 250mm dish.

E. Measurements of RNA concentration:

1. Samples are 500 times diluted from the stocks
2. Optical density (ODs) are taken by absorbance at 280nm
3. Concentrations are calculated by RNA coefficient (40)

F. cDNA synthesis (each 20µl reaction):

1. Mini microcentrifuge tubes (200µl) are taken for cDNA synthesis
2. According to RNA concentration, amount of dH₂O is calculated
3. First, dH₂O is added to the tube
4. 4 µl of cDNA supermix (Buffer+dNTPs+ reverse transcriptase) is added to the tube
5. Required RNA is added to the tube
6. Reactions get ready for cDNA synthesis program
7. The program is followed according to manufacturer’s instructions:

<table>
<thead>
<tr>
<th>Step 1</th>
<th>Step 2</th>
<th>Step 3</th>
<th>Step 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>25°C</td>
<td>46°C</td>
<td>95°C</td>
<td>4°C</td>
</tr>
<tr>
<td>5mins</td>
<td>30mins</td>
<td>1min</td>
<td>∞</td>
</tr>
</tbody>
</table>

G. Primer Design:

1. FASTA sequences of each marker genes are collected from NCBI database
2. Sequences are given in IDT primer designing tool
3. Necessary parameters are changed there (GC content, primer length, base pair, Tm)
4. Primers are generated through the tool
5. Selected primers are used for reverse transcriptase and real time PCR
H. **Primer standardization:**

1. Gradient of annealing temperature is set according to $T_m$ (e.g. 50°-60°C) of every primers
2. Same PCR protocol is followed
3. Best and most intense band intensity among the given number of samples is selected for further PCR reaction for particular primers separately

I. **Reverse transcriptase PCR (20µl):**

1. Mini microcentrifuge tubes (200µl) are also taken for PCR reaction
2. The following calculation is used for each PCR reaction:

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>dH2O</td>
<td>15.4 µl</td>
</tr>
<tr>
<td>Buffer</td>
<td>2</td>
</tr>
<tr>
<td>dNTP</td>
<td>0.4 µl</td>
</tr>
<tr>
<td>Primer 1(Forward)</td>
<td>0.4 µl</td>
</tr>
<tr>
<td>Primer 2 (Reverse)</td>
<td>0.4 µl</td>
</tr>
<tr>
<td>Taq Polymerase</td>
<td>0.4 µl</td>
</tr>
<tr>
<td>cDNA Sample</td>
<td>1 µl</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>20 µl</strong></td>
</tr>
</tbody>
</table>

3. Prepared PCR reactions get ready for run and the program which is followed is mentioned below:

<table>
<thead>
<tr>
<th>Step 1</th>
<th>Step 2</th>
<th>Step 3</th>
<th>Step 4</th>
<th>Step 5</th>
<th>Step 6</th>
<th>Step 7</th>
</tr>
</thead>
<tbody>
<tr>
<td>94°C</td>
<td>94°C</td>
<td>Annealing Temperature</td>
<td>72°C</td>
<td>Go to step 2</td>
<td>72°C</td>
<td>4°C</td>
</tr>
</tbody>
</table>
### Agarose gel electrophoresis:

**MATERIALS NEEDED:**

1. Electrophoresis chamber
2. Power Supply
3. Gel casting trays
4. Sample combs
5. Electrophoresis buffer
6. Loading buffer/dye
7. Ethidium bromide
8. Magnetic stirrer
9. Transilluminator

### RECIPE OF TAE BUFFER

**50X TAE Electrophoresis Buffer of various volumes**

<table>
<thead>
<tr>
<th>REAGENTS</th>
<th>100ML</th>
<th>250ML</th>
<th>500ML</th>
<th>1000ML</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris base</td>
<td>24.2gm</td>
<td>60.5gm</td>
<td>121gm</td>
<td>242gm</td>
</tr>
<tr>
<td>Glacial Acetic Acid</td>
<td>5.71ml</td>
<td>14.27ml</td>
<td>28.55ml</td>
<td>57.1ml</td>
</tr>
<tr>
<td>0.5M EDTA(pH 8.0)</td>
<td>10ml</td>
<td>25ml</td>
<td>50ml</td>
<td>100ml</td>
</tr>
<tr>
<td>dH2O</td>
<td>Add to 100ml</td>
<td>Add to 250ml</td>
<td>Adjust to 500ml</td>
<td>Adjust to final volume 1000ml</td>
</tr>
</tbody>
</table>

**Preparation of TAE Buffer (for 1000ml)**
1. Dissolve Tris base in 750ml of H2O  
2. Dissolve by magnetic stirrer  
3. Add Glacial Acetic acid and EDTA  
4. Lastly, volume adjustment.

Procedure:

1. 2% Agarose gel slabs are used for PCR products  
2. Products are loaded for electrophoresis  
3. Detecting dye (bromophenol blue) is mixed with PCR products  
4. 100bp DNA ladder is used to compare the product size of the unknown samples  
5. After running, gels are imaged in transilluminator

K. Real Time PCR:

1. 96 well real time PCR plates are taken for real time PCR reaction  
2. SYBR green supermix (Buffer+dNTPs+MgCl₂+ Taq polymerase) is used for each 10µl reaction  
3. The following calculation is used for each PCR reaction:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>dH₂O</td>
<td>4.2 µl</td>
</tr>
<tr>
<td>Primer 1 (Forward)</td>
<td>0.4 µl</td>
</tr>
<tr>
<td>Primer 2 (Reverse)</td>
<td>0.4 µl</td>
</tr>
<tr>
<td>SYBR Mi</td>
<td>5 µl</td>
</tr>
<tr>
<td>cDNA Sample</td>
<td>1 µl</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>10µl</strong></td>
</tr>
</tbody>
</table>

4. Real time PCR run is performed using Bio-Rad CFX manager software  
5. Generated cq values are collected and further analysed statistically
L. Embryonic PEO explant culture:

1. 0.01% collagen solution is prepared on ice.
2. Chamber slides (1ml/groove) or 60mm plates (3ml/plate) are coated with collagen solution.
3. Left for at least 2 hours in CO\(_2\) incubator.
4. After isolating the whole avian embryos from embryonic egg obtained from local hatchery at E: 4 stage, these are taken it in a slide and dissected the PEO at room temperature on 1XPBS maintained at 37\(^\circ\)C.
5. Immediately after isolating the PEOs, these are placed in complete M199 media maintained at 37\(^\circ\)C.
6. From the collagen coated chamber slides or plates, excess collagen solution is removed and rinsed with 1XPBS (1ml/groove, 2ml/plate).
7. M199 complete media are added to chamber slides (1ml/groove) or 60mm plates (2-2.5ml/plate).
8. PEOs are re-suspended in M199 complete media on the collagen coated chamber slides or plates.
9. Complete media are further added if needed.

M. Embryonic epicardial (eEpi) explant culture:

1. 0.01% collagen solution is prepared on ice.
2. 60mm plates (3ml/plate) are coated with collagen solution.
3. Left for at least 2 hours in CO\(_2\) incubator.
4. After isolating the whole avian embryos from embryonic egg obtained from local hatchery at E: 5 stage, these are taken it in a slide and dissected the whole hearts at room temperature on 1XPBS maintained at 37\(^\circ\)C.
5. Immediately after isolating the PEOs, these are placed in complete M199 media maintained at 37\(^\circ\)C.
6. From the collagen coated plates, excess collagen solution is removed and rinsed with 1XPBS (1ml/groove, 2ml/plate).
7. M199 complete media are added to 60mm plates (2-2.5ml/plate).
8. Whole avian embryonic hearts are harvested in M199 complete media on the collagen coated plates.

9. Complete media are further added if needed.

**NB:**

- **0.01% Collagen solution:** (For 2ml)
  
  - dH$_2$O: 1.73ml
  - 10XPBS: 200µl
  - 1N NaOH: 1.75µl
  - Stock collagen (3mg/ml): 70µl

**N. Adult avian aortic valve interstitial cell (AVIC) culture:**

1. Collagenase solution is prepared (0.8mg/ml in 1X PBS) and kept it on 37°C water bath.
2. Chamber slides (1ml/groove) or 60mm plates (3ml/plate) are coated with collagen solution.
3. Left for at least 2hours in CO$_2$ incubator.
4. Whole adult hearts are collected and placed in chilled 1XPBS till dissection.
5. Aortic valvular cusps are dissected out from the adult heart on ice and transferred them next to chilled 1XPBS.
6. Till all the samples are dissected, the isolated cusps are transferred to complete M199 media maintained at 37°C water bath for a short time.
7. All the valve cusps are placed to the collagenase solution (2.5ml/cusps from 8-10 hearts) in conical flask and go for digestion in 37°C shaker water bath for 20mins.
8. M199+Pen-strep media (2ml) are added to the conical and taken the valve cusps out in a petri-plate.
9. Valve cusps are minced (25-30 time).
10. Those are taken in a falcon and go for centrifuge (800rpm for 5mins, 37°C).
11. Supernatant is saved in another 15ml falcon and placed it in CO$_2$ incubator.
12. Pellet is re-suspended in collagenase solution and carried out for next digestion in 37°C shaker water bath for 20mins.
13. M199+Pen-strep media (2ml) are added and transferred the content to falcon and are centrifuged (800rpm for 5mins, 37°C).

14. Again supernatant is saved in another 15ml falcon and placed it in CO2 incubator.

15. Previous (12-14) steps are repeated for (1-2) more times depending upon the pellet quantity.

16. From the collagen coated plates, excess collagen solution is removed and rinsed with 1XPBS (1ml/groove, 2ml/plate).

17. M199 complete media are added to 60mm plates (2-2.5ml/plate).

18. Total pooled supernatant is taken out from the CO2 incubator and centrifuged at 800rpm for 5mins, 37°C.

19. Supernatant is discarded and re-suspended the final desired VICs pellet in M199 complete media.

20. On the other hand, the last tissue pellet is re-suspended in M199 complete media and sieved through nylon mesh to get additional cell suspension.

21. Now, previous AVICs pellet and additional cell suspension are mixed and prepared final seeding solution in one new 15ml falcon.

22. Cell counting is performed using hemocytometer.

23. 0.6×10^6 cells/60 mm plates and its 1/4th number of cells are seeded in one groove of chamber slides.

24. Cell plates are kept and incubated for seeding and growth in CO2 incubator (37°C, 5% CO2).

O. Embryonic rat myoblast cell (Cell line H9C2) culture:

1. Stock H9C2 cell plates are obtained from Dr. Arun Bandopadhay’s lab (Indian Institute of Chemical Biology, IICB-Kolkata).

2. Cells are maintained in DMEM complete media following passage number.

3. Cells were seeded from stock plates according to the requirements for experimentation.

P. Phalloidin Staining:

1. Media are aspirated out from the cell plates.
2. Washed in 1XPBS (500 µl/ chamber slide, 1ml/60mm plate) [×2]
3. Cells are fixed in 1% paraformaldehyde (PFA)
4. Washed in 1X NP40 (500 µl/ chamber slide, 1ml/60mm plate) [×3-4, 5mins each, can be manipulated based on the condition of the cells]
5. Cells are kept in blocking for 1hr
6. Phalloidin stain is added (1:400 dilution) to the cell plates (200-300µl/groove of the chamber slides, 1-1.5ml /60mm plates)
7. Incubated for 1.5-2hrs
8. Cells are washed with 1X NP40 (500 µl/ chamber slide, 1ml/60mm plate) [×4, 5mins each]
9. 1-2 drops of DAPI with mounting media is added and kept for 3-4mins
10. Cells are mounted with coverslips
11. Kept at 4°C
12. Imaging

Q. Antibody Staining:

1. Cell media are aspirated out from the plates/ chamber slides
2. Plates are rinsed with 1XPBS [×2]
3. Cells are fixed with 1% PFA (3mins)
4. Cells are washed with 1XNP40 (10mins)
5. Again, cells are fixed with 4% PFA (10mins)
6. Cells are washed with 1XPBS [×3, 5mins each]
7. In this step, cells can be stored in 1XPBS for 1-3 weeks  
   or
8. Cells are rinsed with PBST (PBS+ 0.05% Tween20) solution
9. PBST is aspirated out and cells are kept in blocking adding 1NP40 (30mins-1hr)
10. Again, cells are rinsed with PBST solution
11. Primary antibody (1°) is applied (required dilution used)
12. Kept for overnight at 4°C with gentle shaking
13. Next day, cells are washed with 1XPBS [×3, 5mins each]
14. Cells are rinsed with PBST
15. Secondary antibody (2°) is applied (required dilution used)
16. Cells are washed with 1XPBS [×3, 5mins each]
17. 1-2 drops of DAPI with mounting media is added and kept for 3-4mins
18. Cells are mounted with coverslips
19. Kept at 4°C
20. Imaging

R. Alizarin Staining:

Qualitative assay

1. Cell media are aspirated out from the plates
2. Plates are rinsed with 1XPBS [×2]
3. Cells are fixed with 4% PFA (45mins-1hr) at room temperature (RT)
4. PFA is removed and cells are washed with dH₂O
5. 2% Alizarin Red S solution is prepared freshly
6. Alizarin solution is added to cell plates (2ml/ 60mm plates) and kept at RT with gentle shaking for 15-30mins
7. Now, excess alizarin solution is removed
8. Cells are washed with dH₂O [×2]
9. Now these plates are air dried
10. Imaging

Quantitative assay

1. Cell media are aspirated out from the plates
2. Plates are rinsed with 1XPBS [×2]
3. Cells are fixed with 4% PFA (45mins-1hr) at room temperature (RT)
4. PFA is removed and cells are washed with dH₂O
5. 40mM Alizarin Red S solution is prepared freshly
6. Alizarin solution is added to cell plates (2ml/ 60mm plates) and kept at RT with gentle shaking for 15-30mins
7. Now, excess alizarin solution is removed
8. Cells are washed with dH₂O [×2]
9. 800µl of 10% acetic acid is added to each cell plate and kept at RT for 30mins with gentle shaking
10. Cells are collected in acetic acid using cell scraper in microcentrifuge tube
11. Vortex for 30secs
12. Samples are heated at exactly 85°C for 10mins. [To avoid evaporation, 200-400µl of mineral oil is added on the top of the slurry]
13. Samples are incubated for 5mins on ice
14. Samples are now centrifuged at 20000g for 15mins
15. 500 µl of samples are transferred to new microcentrifuge tube
16. 100-200 µl of 10% ammonium hydroxide (NH₄PO₄) is added to the samples for acid neutralization
17. Samples are aliquotted in 96 well cell plate
18. Absorbances (ODs) are taken using microplate reader [405nm]
19. Statistical analyses are done

S. Coomassie Brilliant Blue staining:

1. 5% Coomassie Brilliant Blue solution is prepared using methanol solvent
2. Cell media are aspirated out
3. Washed with 1XPBS (1ml/60mm plate)
4. Coomassie solution is added to the cell plates (1-2ml/ 60mm plates, 500µl / groove of the slides)
5. Kept for 30-45mins at RT
6. Excess coomassie solution is removed and washed with tap water
7. Kept for air drying
8. Imaging

T. Flow cytometry:

1. Cell media are aspirated out
2. 1XPBS (1-2ml/60mm plate) is added to plates and made cell suspension using scraper
3. 1ml of cells are taken in a microcentrifuge tube
4. Samples are centrifuged at 3000rpm at 4°C for 7mins
5. Supernatant is removed and the pellet is re-suspended in 500µl of 1XPBS
6. Again, samples are centrifuged at 3000rpm at 4°C for 7mins
7. Supernatant is removed and the pellet is re-suspended in 500µl of cold binding buffer
8. Samples are centrifuged at 3000rpm at 4°C for 7mins
9. 450 µl of soup is taken
10. Blocking solution (0.5% BSA+2% FBS, dissolved in 1XPBS) is added for 45mins to 2hrs (200-300 µl for each tube)
11. Samples are centrifuged at 3000rpm at 4°C for 7mins
12. Again, 450 µl soup is taken in a tube
13. Primary antibody is added (1:50 or according to manufacturer’s instructions)
14. Kept at 4°C for 45mins
15. Samples are washed with cold binding buffer
16. Again, samples are centrifuged at 3000rpm at 4°C for 7mins
17. 500 µl of soup is taken out
18. Secondary antibody is added (1:100 or according to manufacturer’s instructions)
19. Kept at 4°C for 45mins
20. Samples are washed with cold binding buffer
21. Again, samples are centrifuged at 3000rpm at 4°C for 7mins
22. 1% PFA is added (1ml/tube) to the sample tubes
23. Covered with aluminium foil
24. Reading is taken in flow cytometer

U. Western Blot:

Reagents to be prepared:

1. Lysis buffer (RIPA)
2. Loading buffer (Laemmli buffer)
3. Running buffer (Tris/glycine/SDS)
4. Transfer buffer (Tris/glycine/Methanol)
5. Tris-buffered saline (TBS)
6. Tris-buffered saline with Tween20 (TBST) buffer
7. Blocking buffer

Procedure:

I. Protein extraction from the cells

1. Cell media are aspirated out from the plates
2. Washed with 1XPBS
3. Cells are scraped and made cell suspension and taken in 15ml falcon
4. Falcons are kept on ice for 5mins
5. Centrifuged at 16000g at 4°C for 20mins
6. Supernatant is removed and protein pellet is re-suspended RIPA buffer (protease+phosphatase added in 1:100 ratio)
7. Stored at -80°C for overnight (can be stored for 1-2days)
8. Next day, cell lysates are thawed down and froze down alternatively
9. Centrifuged at 16000g at 4°C for 20mins
10. Supernatants are collected in new tubes and stored at -80°C

II. Protein separation by gel electrophoresis

1. 10% SDS PAGE gel is prepared according molecular weight of used proteins
2. Equal amounts of required protein are loaded into the wells of a SDS PAGE gel along with molecular weight markers
3. Gel is run for 5mins at 50V
4. Voltage is increased up to 100-150V and run for 1-1.5hrs

III. Transfer of protein from gel to membrane

1. Gel is placed and kept in 1X Transfer buffer for 10mins
2. Transfer sandwich is assembled following the sequence (Mesh>filter paper>Gel>membrane>filter paper>mesh)
3. Transfer cassette is placed in a tank and kept at coldroom
IV. Antibody incubation

1. Bot is rinsed with TBST solution
2. Blot is stripped according to marker with molecular weight
3. Then blot is given for blocking applying blocking solution (10% non-fat dry milk in TBST) for 1hr at RT
4. Primary antibody is added to blots in 5% BSA solution
5. Blots are incubated for overnight at 4°C
6. Next day, blots are washed with TBST solution (×2, 4mins each)
7. Secondary antibody is added to blots in 10% non-fat dry milk solution
8. Blots are washed with TBST solution (×3, 4mins each)
9. Blots are placed TBS buffer and brought for developing
10. ECL substrate (chemiluminiscent) is applied to the blots
11. Blots are incubated for 5-6mins to complete the reaction
12. Imaging