DIVISION OF DEVELOPMENTAL BIOLOGY & TOXICOLOGY

SOPs followed in the lab

Dr. Balakrishnan Lab

Division of Developmental Biology and Toxicology

https://www.drbalakrishnanlabmsub.com/

Department of Zoology,

Faculty of Science,

The Maharaja Sayajirao University of Baroda, Vadodara-390002.

DNA Extraction

- Tissue is collected in lysis buffer and keep it 18-25°C for 1 hour.
- Homogenize the tissue in a unidirectional way in a precooled sterile mortar pestle.
- Add 1ml PCI to this and vortex till it mixes.
- Centrifuge at 8,000rpm for 10 minutes at 25°C.
- Collect the supernatant and add 1/10th volume of 3M sodium acetate.
- Vortex for mixing and add 3 times the volume of 100% ice cold ethanol.
- Vortex until mixed and keep it at -20°C for 5 minutes.
- Centrifuge at 10,000rpm for 10 minutes at 25°C.
- Discard the supernatant and wash the pellet with 1mL 70% ice-cold ethanol. Centrifuge at 8000rpm for 10 minutes at 25°C.
- Discard the supernatant and leave the pellet to dry.
- The pellet is dissolved in DEPC water (quantity of DEPC water is to be decided based on the pellet size).

RNA Extraction

- Tissue is collected in TRIzol (100 mg per 1mL).
- Homogenize the tissue in a unidirectional way in a precooled sterile mortar pestle.
- Collect the tissue sample in 1.5-2 ml Eppendorf tubes and centrifuge at 8,000g for 20 minutes at 4°C.
- Pipette out the supernatant in Eppendorf tubes containing 200µL of chloroform and gently shake the tubes for at least 15 minutes.
- Centrifuge the mixture at 12,000g for 15 minutes at 4°C. The organic and aqueous phases will separate with visible white interphase.
- Carefully dispense the upper layer into a microcentrifuge tube containing 500µL isopropanol. **Avoid contamination from the lower layers.**
- Gently mix and keep in -20°C deep freeze or on the ice at 4 degrees for at least 1 hour to allow precipitation.
- The tube is then centrifuged at 12,000g for 15 minutes at 4°C.

- The supernatant is discarded and the pellet is washed with 200µL 75% ethanol and centrifuged again for 5-10 minutes.
- After a total of two such washes, the tube is opened and left on ice for the pellet to dry.
- If the pellet is colored, give 2-3 ethanol washes.
- After excess ethanol had dried off, the pellet is dissolved in 30-50µL DEPC water depending upon pellet size.

Chemical Compositions

DEPC water: 0.1% in double distilled water, For 1000mL → 1mL DEPC in 1000mL double distilled water. Keep on magnetic stirrer for at least 6 hours.

RNA Quality Check

- RNA quality check is performed using 2% agarose gel electrophoresis.
- Add 3µL of RNA solution and 1 drop of tracking dye. Mix well.
- Add ethidium bromide (EtBr) depending upon the gel casting unit.
    - Small (15mL)- 2µL of EtBr
    - Medium (30mL)- 4µL of EtBr
    - Large (50mL)- 8µL of EtBr
- Three distinct (23s RNA, 18s RNA, 5s RNA) and sharp bands reflected good integrity of the RNA. Minimum 2 bands should be visible.

Chemical Compositions

Tracking Dye: Add a pinch of bromophenol blue in 1.5- 2ml glycelrol.

TBE (Tris/Borate/EDTA) buffer (1X in 1 Litre)		**250mL**
Tris	10.8g	27g
Borate	5.5g	13.75g
0.5M Na_2EDTA	4mL	10mL
DEPC water	996mL	240mL

RNA Quantification

- RNA is quantified using a fluorometric assay.
- Qubit assay: RNA solution is diluted at 1:5, 1:10, 1:40 depending on the band intensity on the Qubit 3.0 (Life Technologies, USA) using its fluorometric assay kit.
- The following formula is used to analyze the quantity of RNA.

qubit reading	dilution factor	µg/µL concentration	µL of RNA to be taken for 1µg	µL water to be added
Vary according to sample	10 (Vary according to sample)	qubit reading multiplied by the dilution factor	1/ (µg/µL concentration)	10- (µL of RNA to be taken for 1µg)

Calibration of Qubit 3.0 with Quantiflour RNA estimation kit (cat # E3310)

Step 1: Prepare 1X TE buffer from 20X TE buffer

e.g., For 1ml or 1X TE buffer: 50µL of 20X TE buffer + 950 µL of nuclease-free water

Step 2: Prepare Quantiflour RNA dye working solution

1µL of RNA dye + 400µL of 1X TE buffer

Step 3 (A): Preparing Blank (will be placed in Qubit as Standard #1)

In a 0.5mL tube (Total volume of the system should be 200µL):

100µL of RNA dye working solution (prepared in step 2) + 100µL of 1X TE buffer

Step 3 (B): Preparing standard #2

In a 0.5mL tube (Total volume of the system should be 200µL):

2µL of RNA standard (given in kit: 100µg/mL) + 98µL of 1X TE buffer + 100µL of RNA dye working solution

Note: Concentration before adding dye will be 2µg/µL and concentration after adding dye will be 1µg/µL.

Incubation: Incubate the standards and unknown samples at room temperature for 5min in the dark.

Unknown sample preparation

	RNA sample	1X TE buffer(µL)	RNA dye working solution(µL) Fixed volume
Sample	1	99	100

Note: Total volume of the assay should be 200µL. From which RNA should be 100µL and RNA dye working solution should be 100µL. So, make up the rest of the volume of your RNA sample to 100µL with 1X TE buffer as mentioned in the above table.

Setting up Qubit 3.0

If calibration is required: Home screen → Select oligo → ssDNA → Press Standards→ Read Std #1 → lnsert the blank (Std #1) → Read (will give you 0ng/mL) → Insert Std #2 (which is 1000ng/mL) → Read (will provide you with a plot with 1000ng/mL)

If you want to read samples directly: Home screen → Select oligo → ssDNA → Press Read Sample → Insert the sample vial → Read (will give you X ng/mL or µg/µL) → Repeat the same for other samples

cDNA Synthesis

- Complementary DNA is synthesized from the isolated RNA using High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, USA).
- The kit uses random hexamers/primers for reverse transcription of RNA into cDNA by reverse transcription.
- 1µg of total RNA is used from each sample for cDNA synthesis.

Components	Volume (µL)
10X RT buffer	2.0
10X RT random primers	2.0
25X dNTP mix (100mM)	0.8
MultiScribe Reverse Transcriptase	1.0
Nuclease-free water	4.2
RNA (diluted by DEPC water)	10

Total Volume	20

- The following program should be set on the thermal cycler:

Temperature (°C)	**Time (min)**
25	10
37	120
85	5
4	∞

- The cDNA prepared is collected and stored at -80°C till further use.

Real-time PCR

- For each reaction mix, following composition is prepared:

Components	**Volume (µL)**
2X SYBR Green master-mix	5.0
Forward primer (0.5µM)	0.5
Reverse primer (0.5µM)	0.5
cDNA template	1.0
Nuclease-free water	4.0

- The samples are taken in triplicates and the reactions run in 96-well plates (Genaxy, USA) sealed with clear sealing films.
- The following program is used for amplification:

Steps	**Temperature (ºC)**	**Time (Sec)**	
Initial denaturation	95	100	
Denaturation	95	10	35 Cycles
Annealing (acquisition)	58-62	30	
Extension	72	30	
Final extension	72	180	

Temperature (°C)	Time (Sec)
95	10
65	60
97	1

- Data analysis: Data is analysed using the LightCycler 96 software version 1.1.
- Cq values were obtained for each well; normalized Cq values were calculated by subtracting the Cq values of internal control gene or reference gene (18S rRNA) from those of the target gene.
- Mean of these normalized Cq values is plotted.

ΔCq = Cq of target gene − Cq of reference gene

- Mean of these normalized ΔCq values is plotted.

ΔΔCq = ΔCq of control − ΔCq of treated

- Fold change is calculated by $\mathbf{2^{-\Delta\Delta Cq}}$

Chemical Compositions

RNA loading dye (5x)	
EDTA 0.5µM	8µL
Formalin	72µL
Glycerol	200µL
Formamide	30µL
Bromophenol blue	25mg
TBE buffer	430µL
DEPC water	260µL
Stored at 8-12°C	

Ethidium Bromide: 25mM that is 7.885mg in 1mL deionized water. Stored at 8-12°C.

Primer Dilution

- Primers are stored at -20°C. Dilution is done in Laminar Air Flow.
- Add nuclease-free water to make primer 100µM stock. Volume of water is decided based on the primer datasheet.
- From this stock, 1:20 dilution is made.

  5µL of reconstituted primer+ 95µL sterile distilled water
- Working solution is kept at 4°C.

Protein Extraction

For tissue:

- Collect 50mg of tissue per mL of lysis buffer (1mL lysis buffer: 10% Protease inhibitor i.e., 10µL).
- Sample can be stored at constant -20°C for at least 4 months whereas at constant -80°C for at least 6 months until use.
- Homogenize with help of pre-cooled mortar pestle (in one direction only) at 4°C.
- Take solution in 1.5mL vial and centrifuge at 8000rpm for 20 minutes at 4°C.
- Collect the supernatant and discarded the rest.

For cells:

- 100µL lysis buffer for 10^6 cells.
- Add 100µL/200µL PBS to cells and detach the cells with the help of cell scraper.
- Add this solution to 100µL/ required volume of lysis buffer with 1X protease inhibitor (Lysis buffer should be mixed with protease inhibitor before adding cells mixed in lysis buffer)
- This whole method should be performed keeping vials on ice.
- Pipette the cells up and down once or twice in lysis buffer and allow it to stand on ice for 15 minutes.
- Centrifuge at 8000rpm for 20 minutes at 4°C.
- Collect the supernatant and discarded the rest.

Chemical Compositions

Lysis Buffer		
Components	**Molarity (mM)**	**Volume for 50mL**
Tris base (pH 7.5)	50	0.303g
NaCl	200	0.584g

$CaCl_2$	10	0.073g
Dissolve in 50mL autoclaved double distilled water and autoclave the solution		
Triton X-100	15	500µL
Protease inhibitor (Sigma, USA) is added freshly before use as per manufacturer's instructions		

Protein Quality Check

PAGE

- SDS-PAGE is performed at 100 volts and gel is taken for quality check.

Coomassie Staining

- Gel is processed for coomassie staining in order to check protein quality.
- Immerse gel in CBB stain for 1 hours.

Staining Solution:

Components	Concentration (%)	Volume (for 100mL)
Coomassie Brilliant Blue R250	0.5	0.5g
Methanol	40	40mL
Acetic acid	10	10mL
Distilled Water	50	50mL

- Remove and recollect stain.
- Immerse gel in de-staining solution overnight.

Destaining Solution:

Components	Concentration (%)	Volume (for 100mL)
Methanol	40	40mL
Glacial Acetic Acid	10	10mL
Distilled Water	50	50mL

- Observe the bands. If seen clearly without shear, the quality of sample is good.

Protein Quantification

Bradford reagent

- Protein is quantified using Bradford reagent.
- Sample (1µL) is added to the 200µL Bradford reagent in the well of 96-well plate.
- Assay to be done in triplicates to avoid technical variation in reading.
- Incubate in dark for 10 minutes.
- Take OD reading at 595nm wavelength in plate reader.
- The concentration is derived using the standard graph, which is prepared with the help of various concentrations of BSA.

	Distilled water	Sample	Bradford Reagent
Blank	10µL	-	200µL
Sample	9µL	1µL	200µL

Protein Sample for Loading

- Protein quantified using Bradford reagent will be made ready for loading.
- Volume for 2µg/µL, 4µg/µL, 8µg/µL, etc., can be prepared depending on the availability of the protein.

Volume for 2µg/µL	*100	Sample buffer (5x)	β-mercaptoethanol	Water	Total volume
Obtained on substituting mean OD upon slope obtained on standard bradford	Volume multiplied by 100 (if total volume is 100)	20mL	2mL	100-(Sum of the rest)	100

- Pierce the MCTs cap with a needle to avoid pressure buildup in the tube during heating.
- This mixture is heated at 80°C in water bath for 10 minutes.
- After cooling, the prepared samples were either used for PAGE and Western Blot or stored at -20°C for later use.

Sample buffer		50mL
Tris base	250mM	1.5143g
SDS	10%	5mL
Glycerol	50%	25mL
Bromophenol blue	0.1%	100µL
Distilled water	-	20mL
pH is adjusted to 6.5; At the time of use, β-mercaptoethanol is added at the final concentration of 100 mM; Stored at 8-12°C		

Bromophenol blue: 0.5g Bromophenol blue in 50mL distilled water.

Phosphate Buffer Solution (PBS)		
Components	**Concentration**	**Volume (for 100mL)**
NaCl	137mM	0.5g
KCl	2.7mM	40g
Disodium phosphate Na_2HPO_4	10mM	10g

Potassium phosphate KH_2PO_4	1.8mM	50g
Made in autoclaved double distilled water for cell culture. For other purposes, PBS can be made in RO water also.		

Sodium Dodecyl Sulphate - Polyacrylamide Gel Electrophoresis (SDS-PAGE)

- Clean PAGE unit equipment plates, spacers and combs with methanol.
- Assemble two plates with spacers, fit with clips, arrange them vertically.
- Sir and pour 1% agar to aluminum foil or use cello tape to seal the bottom of the plate assembly.
- Keep it aside and till it get solidified. Prepare stacking and resolving gel in the meantime.

4% Stacking Gel (3mL) {Above}		**12% Resolving Gel (5mL) {Below}***	
De-ionised Water	2.1mL	De-ionised Water	1.6mL
1M Tris Cl (pH 6.8)	380µL	1.5M Tris Cl (pH 8.8)	1.3mL
30% Gel stock	500µL	30% Gel stock	2mL
10% SDS	20µL	10% SDS	50µL
10% APS (freshly prepared)	20µL	10% APS (freshly prepared)	50µL
TEMED (added only before pouring the gel)	3µL	TEMED (added only before pouring the gel)	2µL

*(Depending upon the size of the protein, the percentage of the resolving gel varies)

- Pour the resolving gel as soon as TEMED is added to solution.
- Pour resolving gel solution in between the plates with the help of micropipette. Add layer of water immediately over it and allow the gel to solidify.
- When solidified remove water layer.
- Add TEMED to stacking gel and pour it over resolving till the edge of the plate.
- Immediately put comb slightly tilting to avoid air bubbles.
- Allow the gel to polymerize for 30-50 minutes.
- Samples are added with a loading dye tracking dye. 30µg protein for coomassie and 40µg protein for WB.
- Well's boundaries are to be marked using of marker for convenience.
- Arrange plates in apparatus. Pour tank buffer. Load the sample.

- Plug in a powerpack and run gel at 100 volts.

Chemical Compositions

Tank buffer (5X in 1 Litre)			
Tris Buffer	125mM	15.142g	Solution A
Glycine	1250mM	93.837g	
Distilled water	-	950mL	
SDS	10%	50mL	Solution B
Mix Solution A and Solution B			

10 % APS: Made in double distilled water. Light sensitive.

Semi dry blot buffer (1X in 100 mL)	
Components	**Volume (for 100mL)**
5X Tank buffer	20mL
Methanol	10mL
Distilled Water	70mL

30 % Gel stock: Acrylamide (29g) + Bis Acrylamide (1g) in 100mL distilled water.

BCIP-NBT Solution (50mL; pH 9.2)	
NBT in DMSO water	0.019g
BCIP in DMSO water	0.010g
Tris	0.06g
$MgCl_2$	0.6g

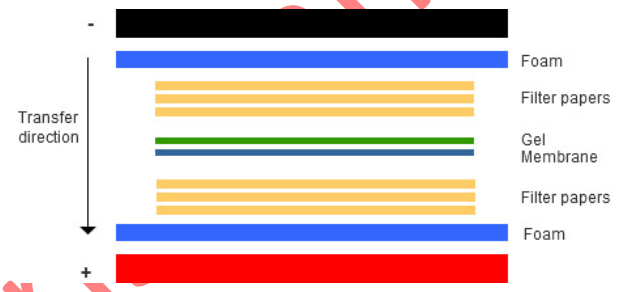
Western Blot

Charging of membrane

- 8-10 sheets of filter paper and PVDF membrane same size as the gel were cut
- Put PVDF membrane in absolute methanol 5-10 minutes for charging, then keep it in transfer buffer (Bjerrum-Schafer-Nielsen Buffer) for 20-30 minute. (Maximum 4 hours)
- Pre-soak filter papers in transfer buffer for 15 minutes.

Setting apparatus

- Put the PVDF membrane in between 4-4 filter papers in a tray and keep it soaked for 5 minutes.
- Make sandwich of gel, PVDF and filter papers



- Add transfer buffer on it, then roll the pipet on that in order to remove air bubbles.
- Close the apparatus and connect the circuit properly.

Protein size range	Transfer time (min)
Below 40 kDa	10
40 to 70 kDa	20
Above 70 kDa	25

- After transfer, stain membrane with Ponceau. Recollect the ponceau after use.
- Wash with distilled water till membrane background clears to show visible bands.

Probing

- Cut the PVDF membranes accordingly to the probe the particular molecular weight protein.
- Membrane can be stored in TBS for several hours in 4°C till next stage.
- Keep membrane in the blocking buffer for 1 hour. Discard the blocking buffer after one hour and incubate in primary antibody overnight at 4°C on rocker.
- On the next day, primary antibodies are recollected. The membrane is washed with TBST for 15 minutes 3 three times consecutively.
- Add biotinylated secondary antibody for 30-45 minutes. Recollect the antibody.
- Wash with TBST for 15 minutes 3 three times consecutively.
- Add streptavidin-ALP conjugate for 30 minutes. Recollect the conjugate.
- Wash with TBST for 15 minutes 3 three times consecutively.
- Add substrate and keep it for 20 minutes. Add water to stop reaction, once bands are visible.

Chemical Compositions

Bjerrum-Schafer-Nielsen Buffer (5X in 1 Litre, pH 9.2)		
Tris Buffer	48mM	5.814g
Glycine	39mM	2.927g
Distilled water	-	900mL
10 % Methanol	-	100mL

Blocking buffer: 5% milk powder dissolved in TBS.

TBS (10X in 100mL, pH 7.5)		
Tris	0.5M	6.05g
NaCl	1.5M	8.7g
Distilled water		100mL
TBS-T (1X in 100mL)		
TBS 1X	100mL	

TritonX	100µL

Protein Ladder preparation	
Ladder	1µL
BSA	1µL
Sample Buffer	2µL
β-mercaptoethanol	1µL
Sterile distilled water	5µL
Stored at 8-12°C	

Ponceau: 0.5% Ponceau in 1.5% acetic acid.

Cleaning Microscope

- Methanol can be used to clean lens on regular basis.
- Isopropyl alcohol can be used to clean stage.
- Xylene can be used to clean lens. But used rarely as it leaves spots on the lens.

Autoclave

- Add water in the cooker up to the level of the plate.
- Put the items to be autoclaved with correct packing. Plasticware should not touch the walls of the cooker.
- Tip boxes should be closed with cello tape and then wrapped in newspaper. Scissors, forceps, needles etc., should be wrapped in Aluminum foil. Glass bottles should be loosely closed.
- Put the cooker on gas stove and put whistle only when vapors come out.
- Switch off the gas 20 minutes after two whistle.

Laminar Air Flow

- Turn on switch.
- Turn on UV for 2-5 minutes.
- Turn on air flow.
- Turn on White light.
- Clean whole chamber with isopropyl alcohol before and after working.
- If burner required, switch on the gas line and start flame.
- Clean the chamber with iso-p after working also.
- Discard everything in the discard beaker and clean it.