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A report submitted

upon completion of summer project

on

Detection and Characterization of Membrane Proteins in *Drosophila melanogaster* using Western blotting

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CERTIFICATE OF COMPLETION

This is to certify that Ms. M. Adishree of NISER, has successfully completed the summer project for the academic year of 2019 in biology in the institution of NISER.

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ACKNOWLEDGEMENT

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Lastly, I would like to thank my friends and family for their endless love and support in the long run of my life.

AIM

To detect and characterize proteins in Drosophila melanogaster using Western Blotting.

THEORY

- Western blotting is a technique that is widely used in molecular biology to detect specific proteins in a sample of tissue extract.
- This technique is used in HIV tests or BSE Tests.
- The whole process of western blotting involves several steps which include the transfer of the protein from the gel to the blotting membrane and then treating the membrane with a primary antibody that will bind to the protein and washing the excessive antibody and then treating it with a secondary antibody which will then bind itself to the secondary antibody.
- One has to be very careful while doing this because the errors can mess up the whole blot.

REQUISITES

(I) For SDS-gel prep:

- 1x SDS dye
- 1M Tris (Mol. Mass = 121.14g)

1M Tris = 121.14g Tris in 1000mL	
⇒	for 1000mL = 121.14g
⇒	for 300mL = 36.342g **[1M TRIS STOCK]**

1.5M Tris (Mol. Mass = 121.14g)

1.5M = 181.71g Tris in 1000mL	
⇒	1000mL = 181.71g
⇒	300mL = 54.513g **[1.5M TRIS STOCK]**

(II) For Western blotting

- **Anode Buffer 1**: **0.3M Tris + 10% Methanol + NaOH (pH - 10.4)**

  1M Tris = 121.14g in 1000mL

  0.3M Tris = 36.342g in 1000mL

  For 200mL of 0.3M Tris we need 7.268g:

  i) Take 50mL H_2O

  ii) Weigh 7.268g Tris & add it to H_2O

  iii) Stir well with magnetic beads

  iv) Adjust pH to 10.4 with NaOH

  v) 10% Methanol added (20mL)

  vi) Make up the volume to 200mL

- **Anode Buffer 2**: **0.25M Tris + 10% Methanol + NaOH (pH - 10.4)**

  From 1M Tris stock,

  $M_1V_1 = M_2V_2$

  $1000mM \times V_1 = 25mM \times 200mL$

  $V_1 = 5mL$

  i) 5mL 1M Tris taken

  ii) Add to 100mL H_2O

  iii) Adjust the pH to 10.4

  iv) Add 10% Methanol

  v) Make up the volume to 200mL

- **Cathode Buffer**: **0.25M Tris + 10% Methanol + 0.4M Glycine**

i) From 1M Tris Stock, we take

1000mM × V1 = 25mM × 200mL

V1 = 5mL

ii) 1M 50mL Glycine (Mol. Mass = 75.07g)

for 1000mL = 75.07g glycine

for 50mL = 3.75g **[1M GLYCINE STOCK]**

From 1M Glycine Stock,

1000mM × V1 = 40mM × 200mL

V1 = 8mL 1M Glycine

iii) 5mL 1M Tris + 8mL 1M Glycine + 100mL H20

iv) pH adjusted to 9.4

v) Add 10% Methanol

vi) Make up the volume to 200mL

- **10× TGS STOCK**

  30g Tris + 144g Glycine + 10g SDS in 1000mL H2O (Stir overnight with magnetic stirrer)

  (pH 8.3)

  From 10x TGS stock,

  10x × V1 = 1x × 2000mL

  V1 = 200mL

- **1x TBST (Wash Buffer)** :

  10 × TBS (Stock) ⟶ 5mL + H_2O ⟶ 45mL + 0.05% Tween ⟶ 25µL

  = 50ml wash buffer

  (Note: Tween is highly viscous, thus a blunt cut tip is used)

- **Block Buffer**:

  1% BSA

  1× TBS (or PBS)

  0.05% Tween

1% BSA	TBS: 10x × V1 = 1x × 50	Tween
1g in 100mL or, 0.5g BSA in 50mL H_2O	V1 = 5mL or, 5mL in 45mL H2O	0.05% ⇒ 25µl tween in 50ml H_2O

Mix these and form a 50mL solution, to be used as block buffer.

PROCEDURE

1. SDS-gel preparation

- Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) is a method of separating molecules based on the difference of their molecular weight using a polyacrylamide gel matrix.
- SDS is used to denature the proteins and give them a uniform charge to mass ratio.
- Most SDS gels use a discontinuous Tris buffer system.
- The stacking gel is usually formulated at pH 6.8. At this pH, ionized chloride ions migrate rapidly, raising the pH behind them and creating a voltage gradient with a zone of low conductivity, which causes glycine to ionize and migrate behind the chloride front.
- Most peptides in the sample, which have a negative charge due to the bound SDS migrate between the chloride and glycine, forming a narrow band and thus becoming stacked.
- Once the stack reaches the resolving gel, which is at a pH 8.8, the increased ionization of the glycine causes it to accelerate and overtake the peptides.
- Additionally, the smaller pore size of the resolving gel starts to have a sieving effect, resulting in the separation of peptides by size.

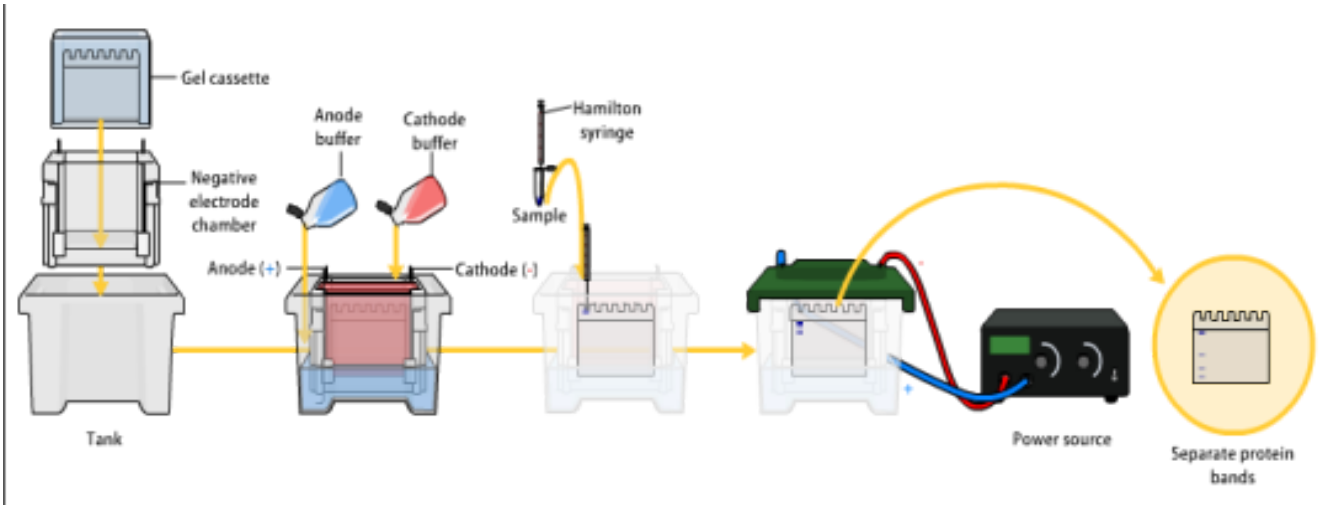


Figure 1: **SDS-PAGE apparatus**

(A) 10% 5mL Resolving Gel:

Solution Components	Volume (in mL)	Volume (in µL)
H2O	1.9	1900
1.5M Tris-HCl (pH – 8.8)	1.3	1300
30% Acrylamide mix	1.7	1700
10% SDS	0.05	50
10% APS	0.05	50
TEMED	0.005	5

(B) 5% 3mL Stacking Gel:

Solution Components	Volume (in mL)	Volume (in µL)
H2O	2.1	2100
1.5M Tris-HCl (pH – 6.8)	0.5	500
30% Acrylamide mix	0.38	380
10% SDS	0.03	30
10% APS	0.03	30
TEMED	0.003	3

Reagents used	Uses
Acrylamide	Sieving medium
Bisacrylamide	Cross-linker
TEMED	Catalyst
APS	Radical initiator
Isopropanol	Prevents oxygen exposure
Bromophenol blue	Dye

2. Sample Preparation

i. 10-15 anesthetized flies taken.
ii. Put them in 1.5 mL Eppendorf tubes.
iii. Sample kept in -80°C for 10-15 minutes.
iv. 100µL of 1x SDS dye added.
v. Flies crushed with micro-pestles and a homogenized sample is prepared.
vi. 100µL more 1x SDS dye added.

vii. Vortex them.
viii. Sample put in 98°C heat block and allowed to boil.
ix. Centrifuged at 10K rpm for about 15 min.
x. supernatant collected.
xi. 3µL sample loaded to the wells.
xii. Ladder loaded.
xiii. A gap of one well maintained after every two wells.
xiv. Gel run at 60-70V for 2.5 hours till the dye comes out.

The extraction is generally done after the resolving gel was set and the stacking gel was on the verge to solidify. Or else the sample can be stored at 40C but not for long.

The ladder –

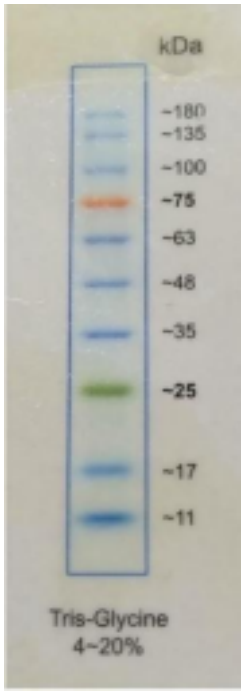


Figure 2: **Image of ladder used**

3. Preparing the membrane

pre-Requisites:

- Milli Q water
- Prepared gel

- Methanol
- Anode Buffer I
- Anode Buffer II
- Cathode Buffer
- Blotting Sheet
- Polyvinylidene difluoride (PVDF) membrane
- Scissor, Gloves and Container

Note: Do not touch the blotting paper with your bare hands, it'll leave marks on it and there will be noise in the final sheet.

(A) Membrane Activation:

i. Blotting sheet cut according to the dimensions of the gel (6 pieces per gel)

ii. PVD membrane cut according to the dimension of the gel.

iii. Membrane soaked in 100% methanol for 15 seconds.

iv. Membrane soaked in Milli Q water for 2 minutes.

(B) Membrane Equilibrium:

After 2 minutes exposure to milli Q, the membrane is soaked in Anode Buffer II for at least 5 minutes.

4. Semi-dry Transfer System

- The semi-dry transfer involves soaking up to six layers of gel, membrane, and filter paper in transfer buffer and sandwiching them between two horizontal plate electrodes.
- This is a technique to transfer the proteins from the gel to a nitrocellulose or PVDF (polyvinyl difluoride) membrane.
- It relies upon current and transferring buffer solutions (anode and cathode buffers) to drive the proteins to the membrane.

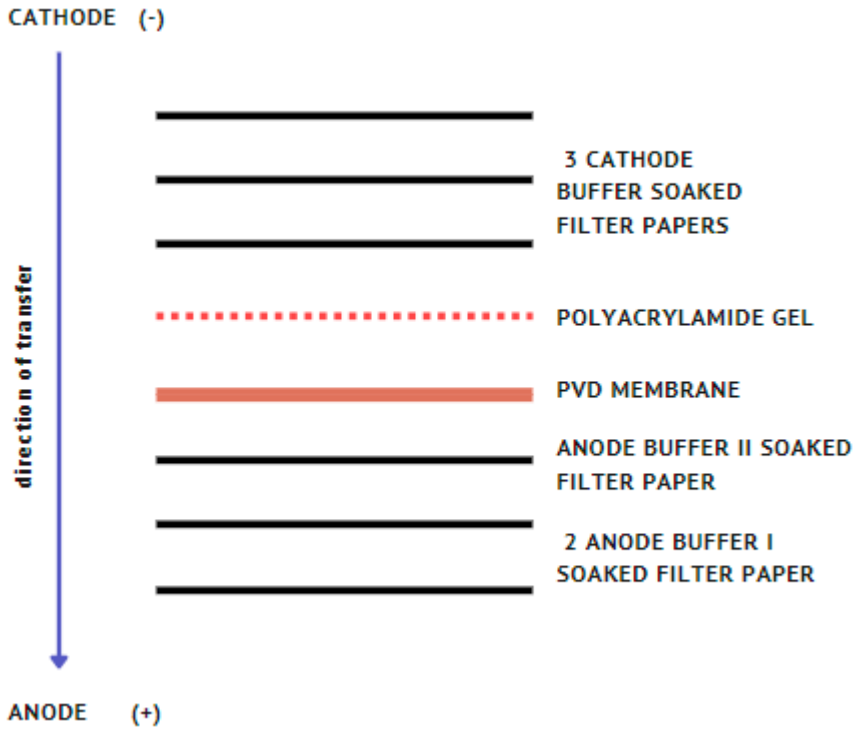


Figure 3: **An illustration of semi dry transfer system**

5. Dry the Blotted Membrane

i. The developed membrane is soaked in 100% methanol, for 15 seconds to dry out the H_2O.
ii. Membrane placed on a piece of filter paper and let methanol to evaporate (30 minutes)

6. Rapid Immuno Detection

- Based on the principle that an antibody specifically reacts with its corresponding antigen, several procedures have been developed in which antibodies or antigens labelled with appropriate indicators are used for the localization, detection or quantitation of substances of biological interest.

- Antibodies or antigens are labelled with enzymes either by covalent or by biospecific non-covalent linkages.
- During the detection process, the membrane is probed for the protein of interest with a modified antibody which is linked to a reporter enzyme; when exposed to an appropriate substrate, this enzyme drives a colourimetric reaction and produces a colour.
- The primary antibody is specific to the protein of interest and binds to it. Washing the membranes with buffer solutions helps minimizes background and removes unbound antibodies.
- The secondary antibody is specific to the primary antibody and binds to it.

i. As we are checking for different antibodies, the membrane is cut by the gap.
ii. Each piece of membrane is incubated in block buffer (previously made) + primary antibody for 3 hours.
iii. Antibody aliquot: 100µL stock antibody + 100µL glycerol
1:1 (50% diluted)
Primary antibody treatment: 50µL block buffer + 0.5 µL antibody
iv. Blot washed 3 times, 10 minutes each in 7-10mL of 1x TBST buffer (previously made)
v. Blot incubated in secondary antibody (509) for 60 minutes.
vi. 1% BSA in TBST (2mL): Secondary antibody (2µL) = 1000:1
vii. [Substrate (HRP conjugated antibody) added to the blot and incubated for an hour]
viii. Blot washed 3 times for 10 minutes each, in 7-10 mL 1x TBST buffer.

7. Visualising the blot by Chemiluminescent detection

- In the chemiluminescence reaction, horseradish peroxidase catalyzes the oxidation of luminol into a reagent which emits light when it decays.
- Since the oxidation of luminol is catalyzed by horseradish peroxidase, and the HRP is complexed with the protein of interest on the membrane, the amount and location of light that HRP catalyzes the emission of, is directly correlated with the location and amount of protein on the membrane.
    - Luminol: H_2O_2 = 1:1 {Mixed well and applied all over the blot}
    - Blot observed in ChemiDoc.

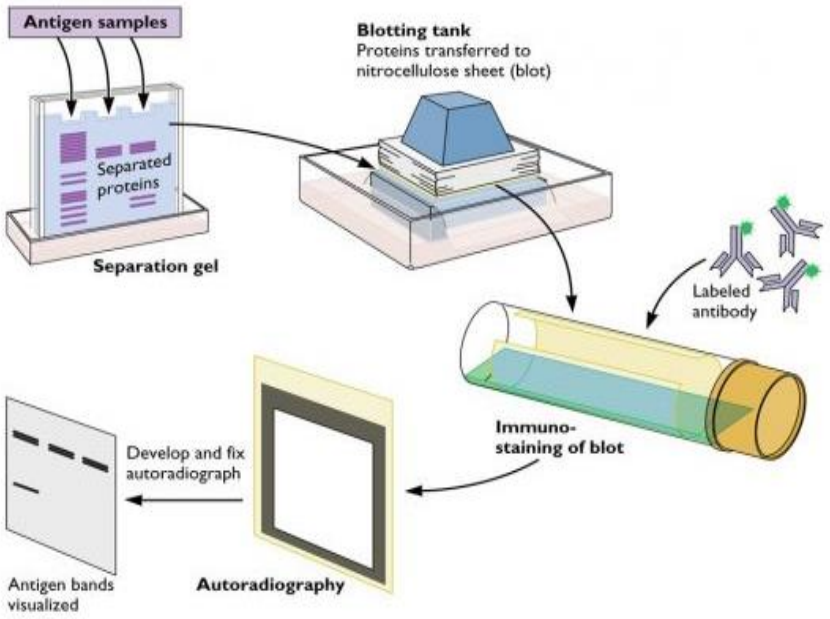


Figure 4: **Pictorial summary of Western blotting**

OBSERVATIONS

i. The molecular weights of all the proteins to be tested were checked from the site *flybase.org*.
ii. This site is specific to *Drosophila melanogaster*.
iii. The website has a search box entitled "jump to gene", where the gene symbol can be entered.
iv. The page that follows contains all the information about the gene.
v. Then in the decorated FASTA section on that page, the dropdown menu can be changed to *gene region, extended gene region, CDS, introns, exons, translations, transcripts, 3'UTR and 5'UTR*.
vi. As we required the protein weight, the translation option was chosen.
vii. The next page gives the FASTA sequence of all the protein isoforms of that gene.
viii. The desired isoforms FASTA sequence was selected and pasted in Expasy molecular weight calculator opened in the next tab.
ix. The result of the calculator was saved. Like this a file was made that contained the isoform no., name and molecular weight.

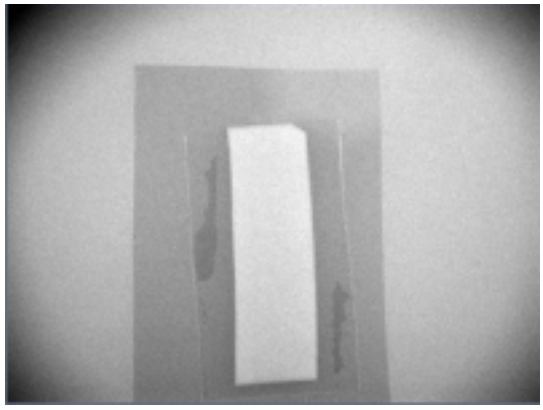
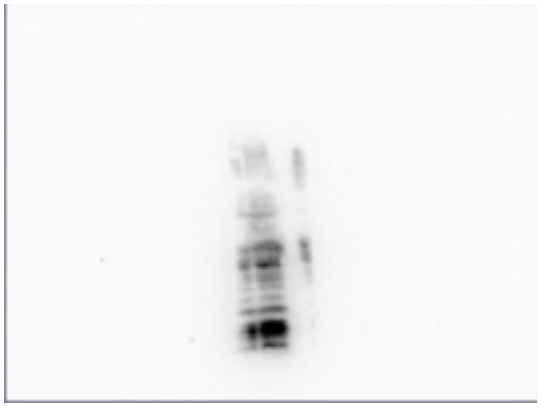
We observed the following blots for the respective antibodies –

Antibody	Name	Protein	Molecular weight
Ab 57	p1D9(anti-roh1)	rho1	21.7 kDa
Ab 62	anti-Spitz	spitz, lignad for sprouty	Predicted : 26 kDa
Ab 65	Rab7	RAb7/CG5915 protein	23.34 kDa
Ab 34a	Golgin84 12-1	Golgin-84	58.7 kDa
Ab 61	anti-Yan 8B12H9	yan, FGF Signalling	Predicted : 79 kDa
Ab 63	Cnx99A 6-2-1	Calnexin 99A	75.94 kDa

Ab57 (p1D9 or anti- rho1) –

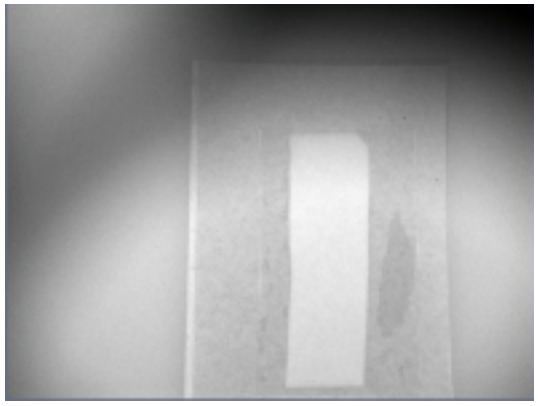
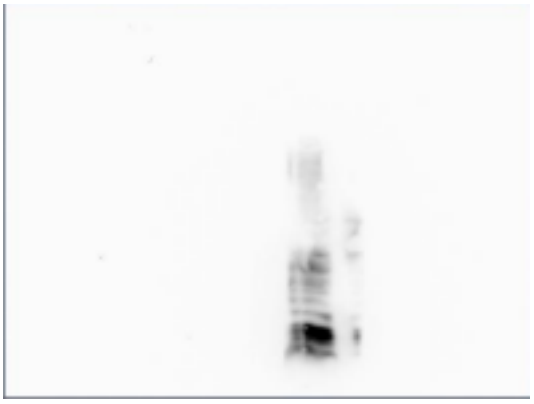
In this, we can see that the dark mark is in close proximity of the 25 line which confirms that the size of the protein is approximately equal to 21 kDa.

Ab62 (anti-spitz) –

The dark band is close to 25 kDa band and is close to 26kDa.

Ab65 (Rab7) –

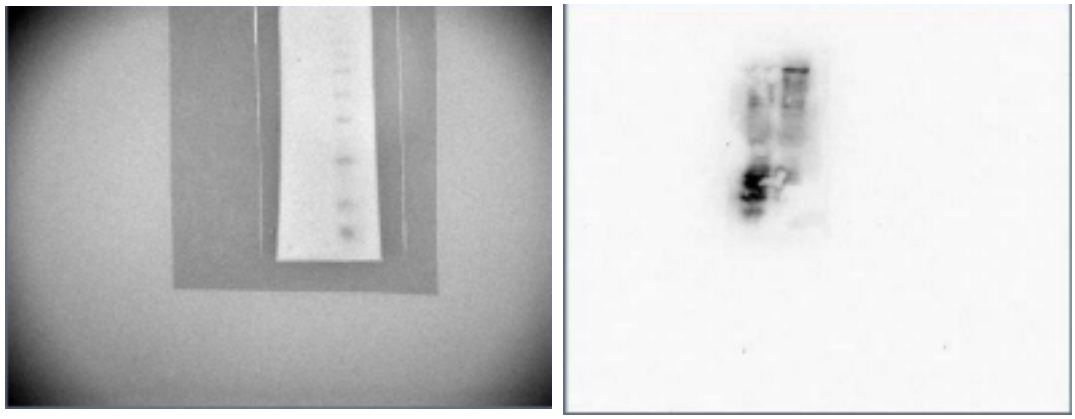



Again, the dark band is a little below the 25 kDa band and hence its size is close to 21 kDa

Ab34a –

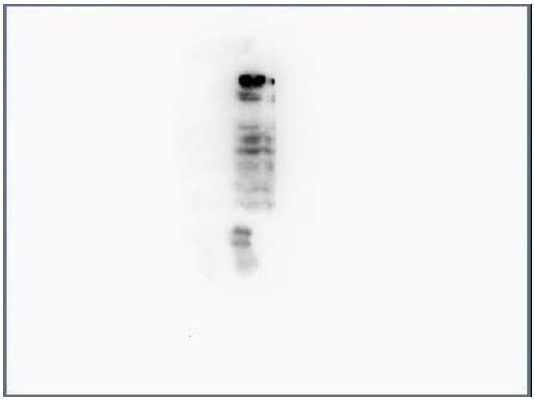
The dark band is at the last whereas the original size is 58.7 kDa. This might be due to some error in the gel running.

Ab61 -



The dark band is close to the top and hence its size is close to the theoretical size.

Ab63 -



The dark band is close to the top and hence its size is close to the theoretical size.

DISCUSSION

- Many unsepecific bands are seen. The reason for these unspecific bandings or smeared backgrounds can be an improper washing step or use of concentrated antibodies.
- In some cases the bands came at a different position other than the intended ones. This can be accounted for non-specific nature of the primary antibody.
- While in some cases bands were seen at other places along with the intended ones. This can be caused due to multispecific nature of the antibody.
- Most of the proteins were small. It can be considered that, due to improper transfer, bands were not detected. The transfer might have been done for alonger period as a result the proteins might have crossed the membrane and transferred into filter paper.
- To check if proper transfer has occurred or not, blots can also be stained.
- A second reason is lower specificity of antibody used.
- Even some larger proteins showed no signal. This can be caused due to washing off of antigen during PBST wash or lower concentration of antibody or less incubation time