M.Sc. IInd Semester

BIOCHEMISTRY PRACTICAL

1. Aim : Preparation of extract for enzyme assay (alkaline phosphatase).

Requirements :

Sample Tissue, Homogenizer, Tris Buffer (Ph 7.0-7.5), Distilled Water, Weighing Machine, Sucrose (0.25M), N-Butyl Alcohol.

Principle:

The initial procedure in the isolation of an protein, a protein complex, or a subcellular organelle is the preparation of an extract that contains the required component in a soluble form. The homogenization technique employed should, therefore, stress the cells sufficiently enough to cause the surface plasma membrane to rupture, thus releasing the cytosol; however, it should not cause extensive damage to the subcellular structures, organelles, and membrane vesicles.

The extraction of proteins from animal tissues is relatively straightforward, as animal cells are enclosed only by a surface plasma membrane (also referred to as the limiting membrane or cell envelope) that is only weakly held by the cytoskeleton. Animal tissues can be crudely divided into soft muscle (e.g., liver and kidney) or hard muscle (e.g., skeletal and cardiac). Reasonably gentle mechanical forces such as those produced by liquid shear may disrupt the soft tissues, whereas the hard tissues require strong mechanical shear forces provided by blenders and mincers. The homogenate produced by these disruptive methods is then centrifuged in order to remove the remaining cell debris.

Procedure :

1. A weighed amount of sample tissue in a while, with a known volume of buffer solution is taken.
2. Homogenizer causes vigorous mixing and turbulence as well as physical shearing of the sample into small pieces.
3. The resulting product of homogenization is semi solid in nature and is called the homogenate.
4. Each tissue sample is homogenised in 0.25M sucrose and 20% (w/v) n-Butyl alcohol at 4°C.
5. The resulting homogenate is incubated at 57°C for one hour.
6. Then it is kept overnight at 4°C and centrifuged at 100X g for 2 hours.
7. Decant the supernatant to minimise large particles on debris in the homogenate.

2. Aim : To estimate concentration of Protein in the tissue of Cytosolic Fraction by the Lowry's Method.

Materials Required : BSA stock solution, Analytical Reagent, Folin's Reagent, cuvette, spectrophotometer, test tube stand, test tubes, distilled water, pipette, and measuring cylinder.

Preparation of reagents

Reagent A :

- 2% Na_2CO_3 is mixed with 0.1N NaOH. (4gm of NaOH dissolved in the 100ml of Distilled Water)
- 4gm of Na_2CO_3 is dissolved in the 20ml of 1N NaOH and make the volume up to 200ml.

Reagent B :

- 0.5% $CuSO_4$ in 1% Sodium Potassium Tartarate ($C_4H_4O_6KNa \cdot 4H2O$)
- 50ml of each solution is made and mixed well in 1:1 ratio to make reagent B.

Reagent C :

- Reagent A and Reagent B are mixed in the ratio of 49:1.
- i.e. 98ml of Reagent A is mixed with 2ml of Reagent B to make Reagent C.

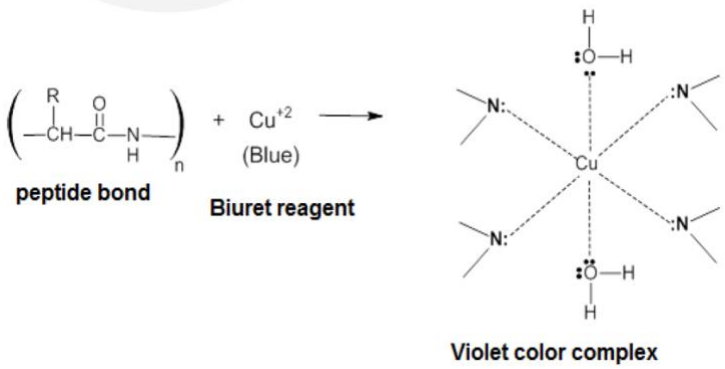
Reagent D :

- Folin is diluted to equal volumes with distilled water (1:1 ratio) to make reagent D

Bovine Serum Albumin (BSA) Standard :

- 20mg of BSA is weighed and mixed with 20ml of distilled water. (1mg/ml concentration)
- To make Standard BSA solution, again 16mg of BSA is weighed and mixed with 10ml of distilled water to make BSA Solution of 1.6mg/ml concentration.
- Concentration of 50µg/ml, 100µg/ml, 200µg/ml, 400µg/ml and 800µg/ml are prepared.

Principle : The –CO-NH- bond (peptide) in polypeptide chain reacts with copper sulphate in an alkaline medium to give a blue colored complex. In addition, tyrosine and tryptophan residues of protein cause reduction of the phosphomolybdate and phosphotungstate components of the Folin-Ciocalteau reagent to give bluish products which contribute towards enhancing the sensitivity of this method.

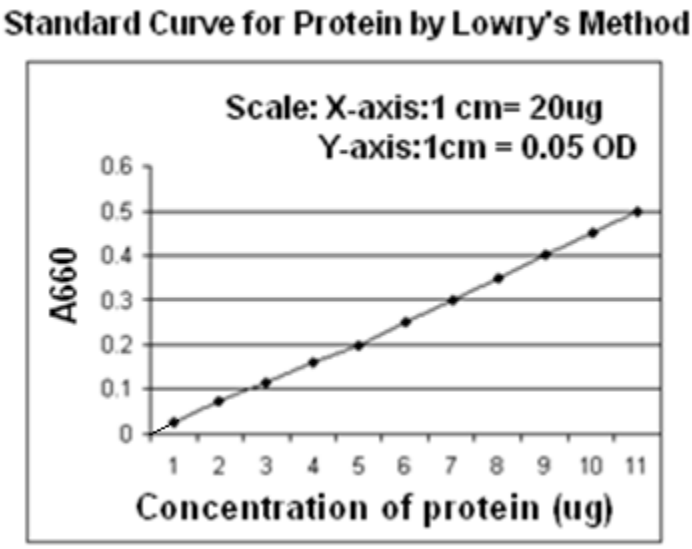


Procedure :

1. Pipette out 0.2, 0.4, 0.6, 0.8 and 1 ml of working standard in to the series of labelled test tubes.
2. Pipette out 1 mL of the sample in another test tube.
3. Make up the volume to 1 mL in all the test tubes. A tube with 1 mL of distilled water serves as the blank.
4. Now add 5 mL of reagent C to all the test tubes including the test tubes labelled 'blank' and 'unknown'.
5. Mix the contents of the tubes by vertexing / shaking the tubes and allow to stand for 10min.
6. Then add 0.5 mL of reagent D rapidly with immediate mixing well and incubate at room temperature in the dark for 30 min.
7. Now record the absorbance at 660 nm against blank.

Observations and Calculations :

Volume of standard BSA (ml)	Volume of distilled water (ml)	Concentration of Protein (µg)	Volume of reagent C (ml)		Volume of reagent D (ml)		Absorbance at 660nm
0.0	1.0	00	5	Incubate At Room Temp for 10 min	0.5	Incubate At Room Temp for 10 min	
0.2	0.8	40	5		0.5		
0.4	0.6	80	5		0.5		
0.6	0.4	120	5		0.5		
0.8	0.2	160	5		0.5		
1.0	0.0	200	5		0.5		
1.0 (Unknown)	0.0	Unknown	5		0.5		



Result :

The Protein concentration of the unknown given sample is _____ µg/ml.

Precautions:

- The concentration of the chemicals must be proportionate when making the reagent.
- The Folin reagent must not be exposed to the direct sunlight or light.

3. Aim : Estimation of the amino acids in the given sample (controlled and treated) by Ninhydrin Test.

Chemicals Required :

- Amino acid gradient (α-glutamine) 2µg/ml
- 8% w/v of Ninhydrin reagent
- 18gm of Ninhydrin dissolved in 100ml of Acetone
- 50% v/v ethanol (20ml) (10ml of ethanol + 10ml H_2O)
- Distilled water

Standard solution preparation :
10mg of α-glutamine is dissolved in 5ml of distilled water in volumetric flask. The concentration is 2mg/ml.

Principle:

Ninhydrin, which is originally yellow, reacts with amino acid and turns deep purple. It is this purple colour that is detected in this method. Ninhydrin will react with a free α-amino group, NH_2-C-COOH. This group is present in all amino acids, proteins or peptides. Whereas, the decarboxylation reaction will proceed for a free amino acid, it will not happen for peptides and proteins.

α-amino acids react with Ninhydrin involved in the development of colour is explained by the following steps :

1. α-amino acid + Ninhydrin → Reduced ninhydrin + α-amino acid + H_2O
2. α-amino acid + H_2O → α -keto acid +NH_3
3. α-keto acid + NH_3 → aldehyde + CO_2
4. α-amino acid + 2 ninhydrin → CO_2 + aldehyde + final complex (BLUE) + $3H_2O$

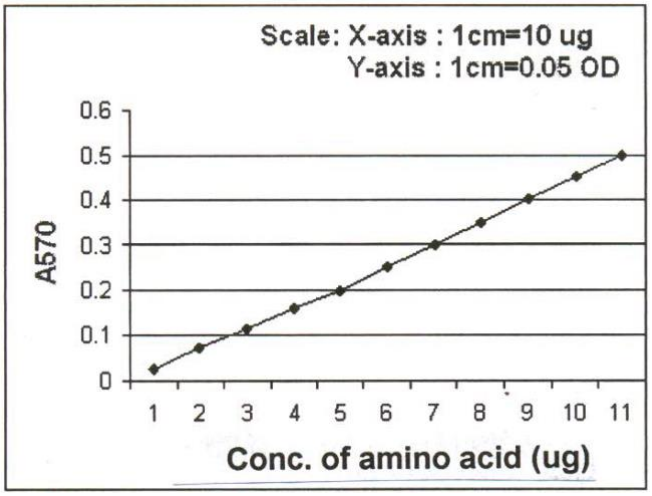
Procedure :

1. 0.20ml, 0.40ml, 0.80ml and 0.120ml of working solution is precipitated out in series of labelled test tube and add required volume of distilled water to make the volume 2ml.
2. 2ml of distilled water is added to test tube labelled blank.
3. 200µL of unknown sample (controlled and treated) is pipetted out and make up the volume to 2ml in the labelled test tube.
4. 500µg of Ninhydrin reagent is added to all the test tubes.
5. The contents of the test tubes are mixed by shake and mouth is sealed by aluminium foil.
6. All the tubes are placed in water bath for 15 minutes at approximately 60°C.
7. Then test tubes are to be cooled in the cold water and 500µL of ethanol is added to all the test tube and mixed well.
8. Absorbance is recorded at 570nm wavelength.

Observations and Calculations :

Volume of Standard Amino acid (ml)	Volume of distilled water (ml)	Concentration of Amino Acid (µg)	Volume of Ninhydrin (ml)		Volume of reagent D (ml)		Absorbance at 570nm
0.0	1.0	00	0.5	Incubate in Boiling Water bath for 15 min	0.5	Incubate At Room Temp for 10 min	
0.2	0.8	20	0.5		0.5		
0.4	0.6	40	0.5		0.5		
0.6	0.4	60	0.5		0.5		
0.8	0.2	80	0.5		0.5		
1.0	0.0	100	0.5		0.5		
1.0 (Unknown)	0.0	Unknown	0.5		0.5		

Standard Curve for amino acid by ninhydrin method



Result : The amino acid concentration of the unknown given sample is _____ µg/ml.

4. Aim : Study of the activity of the enzyme Glucose-6-Phosphate Dehydrogenase using the spectrophotometer.

Requirements :

Tris Chloride Buffer (pH 7.0)	100ml
$MgCl_2$	250µL
NADP	100µL
Glucose-6-Phosphate	250µL
Tissue Extract	50µL
Distilled Water	250µL

Principle :

Glucose-6-Phosphate dehydrogenase is the enzyme involved in the first step of pentose phosphate pathway and is responsible for the conversion of Glucose -6 phosphate to Phosphogluconolactone and produce NADPH. The concentration of NADPH is measured by spectrophotometrically to study the activity of enzyme. The interesting pattern of NADPH per minute is used to calculate the activity of the enzyme.

Procedure

- In a reaction mixture containing Tris-Chloride, $MgCl_2$, NADP and Glucose– 6 Phosphate placed in the Eppendorf tube and tissue extract is mixed with the reaction mixture.
- Followed by incubation at room temperature for 30 seconds to 1 minute.
- Now measure the per minute change of absorbance of NADPH at 340nm.

Observation and Calculation:

S. No.	Sample Reaction Time	A	B	C	Average	Change in OD
1						
2						
3						
4						
5						
6						
					Average Change in OD	

$$\text{Activity of G-6-P (unit/ml)} = \frac{\text{OD X Dilution factor mMol}}{6.22}$$

Result :

The activity of the enzyme Glucose-6-Phosphate Dehydrogenase is _____ unit/ml

5. Aim : Determination of Km and Vmax by Michaelis-Menten and Lineweaver-Burk Plot

Reagents:

- **Buffer:** 0.1M Sodium citrate buffer (pH 4.8).
- **Substrate solution:** 5 mM p-nitrophenyl phosphate (pNPP), dissolved in 0.1 M sodium citrate buffer (pH 4.8).
- 0.2N Sodium hydroxide solution

Principle :

The hyperbolic curve (initial velocity V0 vs. substrate concentration) is not entirely satisfactory for determination of Km and Vmax for an enzyme catalysed reaction. The Michaelis-Menten equation can be rearranged by taking reciprocals on both sides of equation to a form that is equivalent to the straight-line equation. By plotting 1/Vo on Y-axis (ordinate) and 1/[S] on X- axis (abscissa), a straight line relationship is observed which is known as Lineweaver- Burk plot, where the slope is Km/Vmax and the intercept on the Y- axis is I/Vmax. The X-intercept is -1/Km where Km is the Michaelis-Menten constant. In acid phosphatase catalyzed reaction using p-nitrophenyl phosphate as substrate, the inverse values of different concentrations of p- nitrophenol (i.e., 1/Vo) vs. the inverse values of pNPP concentrations (i.e. 1/[S]) are plotted to determine the Km and Vmax from the Lineweaver and Burk plot.

Procedure :

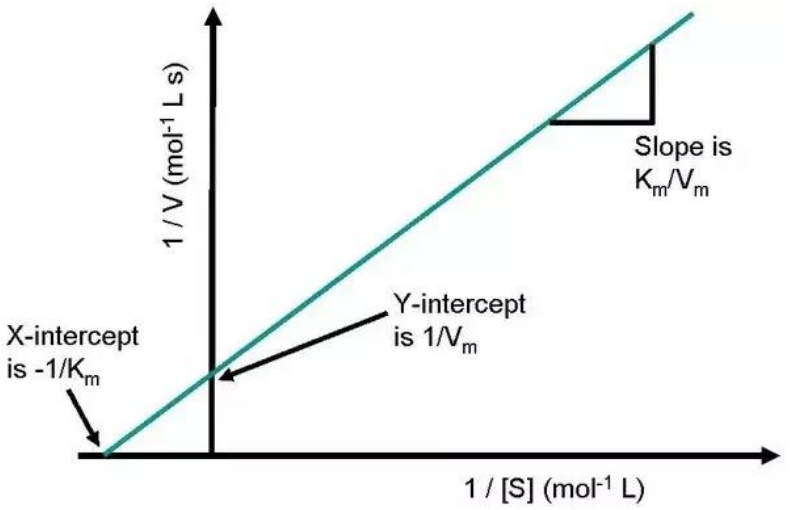
1. Take clean test tubes numbered from 1 to 9. Pipette out different volumes of 5 mM pNPP solution and the citrate buffer as follows (in duplicate). Prepare another set also and mark as Blank e.g. B1, B2, B3, B4 B5, B6, B7, B8, B9.

Reagent	**Tube**								
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
5 mM pNPP solution (mL)	0.025	0.05	0.10	0.15	0.20	0.25	0.3	0.6	0.9
0.1M Citrate buffer (pH 4.8) (mL)	0.875	0.85	0.80	0.75	0.70	0.65	0.6	0.3	-
	Mix properly and equilibrate at 37^{o} C for ~3 min								

2. Start the reaction by adding 0.1 mL of enzyme extract in each tube (assay tubes only). Note down the time of adding the enzyme extract in first tube.
3. Immediately mix properly and incubate at 37°C for 10 minutes.
4. Stop the reaction by adding 4.0 mL of 0.2N NaOH in each tube.
5. In blanks, add enzyme extract (0.1 mL in each tube) at the end after addition of 0.2N NaOH.
6. Record the absorbance of each tube (assay) against the corresponding blank (prepared for each pNPP concentration), at 410 nm using a spectrophotometer.

Observation and Calculations :

- Determine the amount of p-nitrophenol produced in each tube from the standard curve prepared as explained in experiment 1.
- Calculate the μ-moles of p-nitrophenol produced/min (termed as velocity, V0) for each concentration (mM) of pNPP, represented as [S].
- Plot V0 vs [S] and note the shape of curve.
- For the Lineweaver-Burk plot (double reciprocal plot), we have to find out the reciprocal of velocity (1/V0) and substrate concentration (1/[S]).
- Plot 1/ V0 on Y-axis and 1/[S] on X-axis, and determine the value of Km and Vmax of the acid phosphatase catalysed reaction.



Lineweaver-Burk plot (Double Reciprocal plot)

Precautions :

1. The substrate concentration should cover a wide range.
2. Pipetting of volumes should be very accurate.
3. Always wear gloves while working with p-nitrophenylphosphate and p-nitrophenol, avoid contact, inhalation etc.
4. Ensure equilibration at incubation temperature before adding the enzyme extract.

6. Aim : Isolation of the DNA

Chemicals Required:

Ethylene diamine tetra acetate (EDTA), NaOH, Tris-HCl, sucrose, MgCl2, Triton X100, Sodium dodecyl sulphate (SDS), NaCl, TE buffer or double distilled water, cold chloroform, cold ethanol.

Preparation of solutions

1) 0.5 M EDTA (pH 8.0) :

Add 146.1 g of anhydrous EDTA to 800 ml of distilled water. Adjust pH to 8.0 with NaOH (about 20 g). Make up the volume to 1 L with distilled water.

2) 1 M Tris-HCl (pH 7.6) :

Dissolve 121.1 g of Tris base in 800 ml of distilled water. Adjust pH with concentrated HCl (about 60 ml). Make up the volume to 1 L with distilled water.

3) Reagent A (Red Blood Cell Lysis Solution) :

Containing: 0.01M Tris-HCl (pH 7.4), 320 mM Sucrose, 5 mM MgCl2, and 1% Triton X100.

Add 10 ml of 1 M Tris to 109.54 g of sucrose, 0.47 g MgCl2 and 10 ml Triton X100 to 800 of distilled water. Adjust pH to 8.0; make up the volume to 1 L with distilled water.

4) Reagent B (Cell Lysis Solution)

Containing: 0.4 M Tris-HCl, 150 mM NaCl, 0.06 M EDTA, 1% SDS, pH 8.0.

Take 400 ml of 1 M Tris (pH 7.6), 120 ml of 0.5 M EDTA (pH 8.0), 8.75 g of NaCl, adjust pH to 8.0 with NaOH. Make up the volume to 1 L with distilled water. Autoclave at 15 p.s.i. for 15 min. After autoclaving the mixture, add 10 g of SDS.

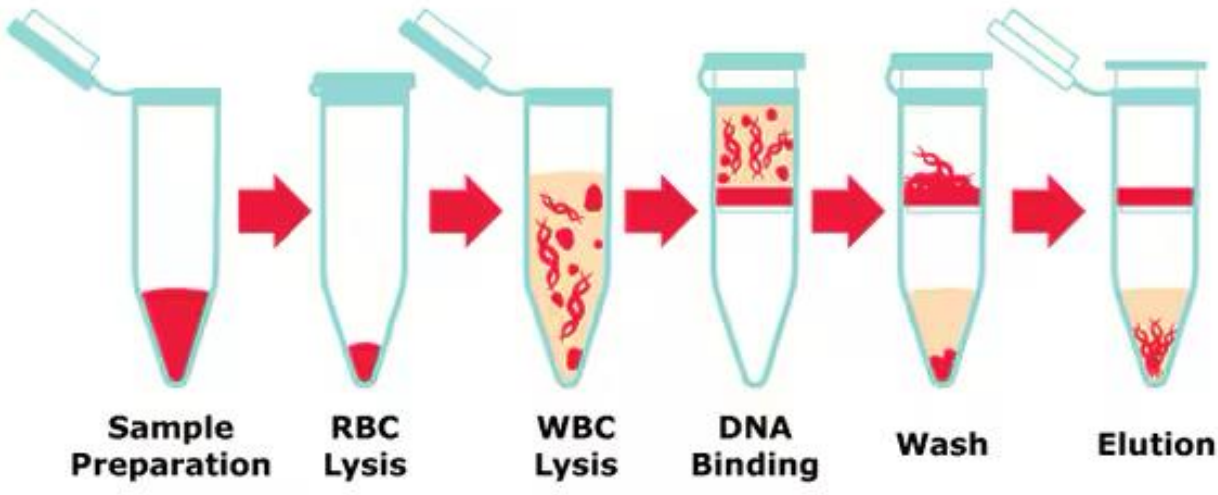
Principle:

Successful nucleic acid isolation protocols have been published for nearly all biological materials. They involve the physical and chemical processes of tissue homogenisation (to increase the number of cells or the surface area available for lysis), cell permeabilization, cell lysis (using hypotonic buffers), removal of nucleases, protein degradation, protein precipitation, solubilisation of nucleic acids and finally various washing steps. Cell permeabilization may be achieved with the help of non-ionic (non DNA-binding) detergents such as Triton.

Procedure :

1. Place 3 mL of whole blood in a 15-mL falcon tube (centrifuge tube).
2. Add 12 mL of reagent A.
3. Mix on a rolling or rotating blood mixer for 4 min at room temperature (to prevent leakage, close the lid tightly).

4. Centrifuge at 3000g for 5 min at room temperature.
5. Discard supernatant without disturbing cell pellet.
6. Remove remaining moisture by inverting the tube and blotting onto tissue paper.
7. Add 1 mL of reagent B and vortex briefly to re-suspend the cell pellet.
8. Add 250 µL of 5 M sodium chloride and mix by inverting tube several times.
9. Place tube in water bath for 15 to 20 min at 65 °C.
10. Add 2 mL of ice-cold chloroform.
11. Mix on shaker for 20 min.
12. Centrifuge at 2400g for 2 min.
13. Transfer upper phase into a clean falcon tube using a sterile pipette.
14. Add 2 to 3 ml of ice-cold ethanol and invert gently to allow DNA to precipitate (if a cloudy did not form, add more ethanol).
15. Using a clean Pasteur pipette spool the DNA onto the hooked end.
16. Transfer to a 1.5-mL Eppendorf tube and allow to air dry.
17. Re-suspend in 200 µL of TE buffer or sterilized water and label the tube.
18. As a final step in nucleic acid isolation, the characterization (concentration, purity, and integrity) of the extracted nucleic acid may need to be determined.



Results:

Cloudy precipitation can be seen by the naked eye, and it represent the isolated genomic DNA.

7. Aim : Isolation of the RNA

Materials Required:

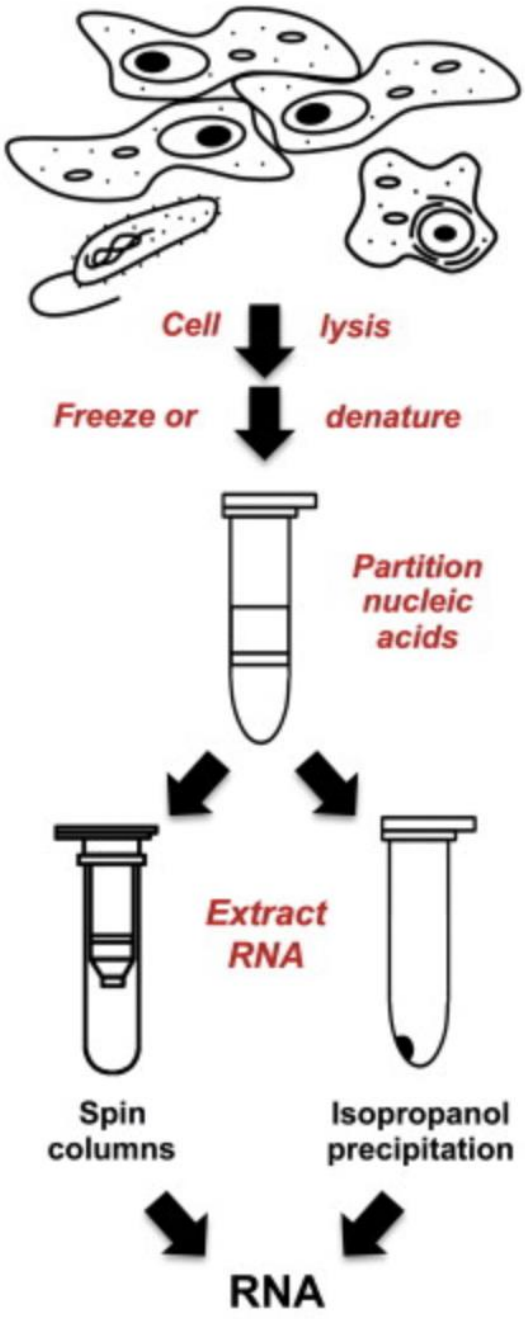
1. Dried Yeast.
2. Phenol solution: 900g/litre.
3. Potassium acetate: 200g/litre, pH 5.
4. Absolute ethanol.
5. Diethyl ether.
6. Glass wares, centrifuge, water bath *etc*.,

Principle:

Total yeast RNA is obtained by extracting a whole cell homogenate with phenol. The concentrated solution of phenol disrupts hydrogen bonding in the macromolecules, causing denaturation of protein. The turbid suspension is centrifuged and two phases appear; the lower phenol phase contains DNA, and the upper aqueous phase contains carbohydrates and RNA. Denaturated protein, which is present in both the phases, is removed by centrifugation. The RNA is then precipitated with alcohol.

Procedure:

1. Suspend 0.15 g of dried yeast in 0.6 ml of warm (37°C) water and incubate in an water bath for 15 min at 37°C.
   Then add 0.8 ml of concentrated phenol solution and stir the suspension mechanically for 30 min at room temperature, then centrifuge at 3000g for 15 min in cold (5°C) to break the emulsion.
2. Carefully collect the upper aqueous layer with a Pasteur pipette and centrifuge at 10000 g for 5 min in cooling centrifuge to sediment denatured protein.
3. After centrifugation collect supernatant into a fresh tube and add 1/10 volume of potassium acetate and two volumes of ethanol. Cool the solution in ice and leave to stand for one hr.
4. Collect the precipitate by centrifuging at 2000 g for 5 min at 5°C.
5. Wash the RNA with ethanol: water (3:1), ethanol and finally ether; air dry and preserve for future use.



Result:

RNA is isolated from yeast, which is observed as white fibrous material.

8. Aim: To study Reverse Transcriptase Polymerase Chain Reaction (RTPCR).

Requirements :

A typical RT-PCR assay requires nucleic acids, primers, a reverse transcriptase enzyme, RNA sample, DNA polymerase, and an RT-PCR buffer.

Principle :

RT-PCR uses RNA as starting material for in vitro nucleic acid amplification. The discovery of retroviral reverse transcriptase in the early 1970s ultimately made RT-PCR possible. Reverse transcriptase is an RNA-dependent DNA polymerase, catalyzing DNA synthesis using RNA as the template. The end product is known as complementary DNA (cDNA). cDNA is not subject to RNase degradation, making it more stable than RNA.

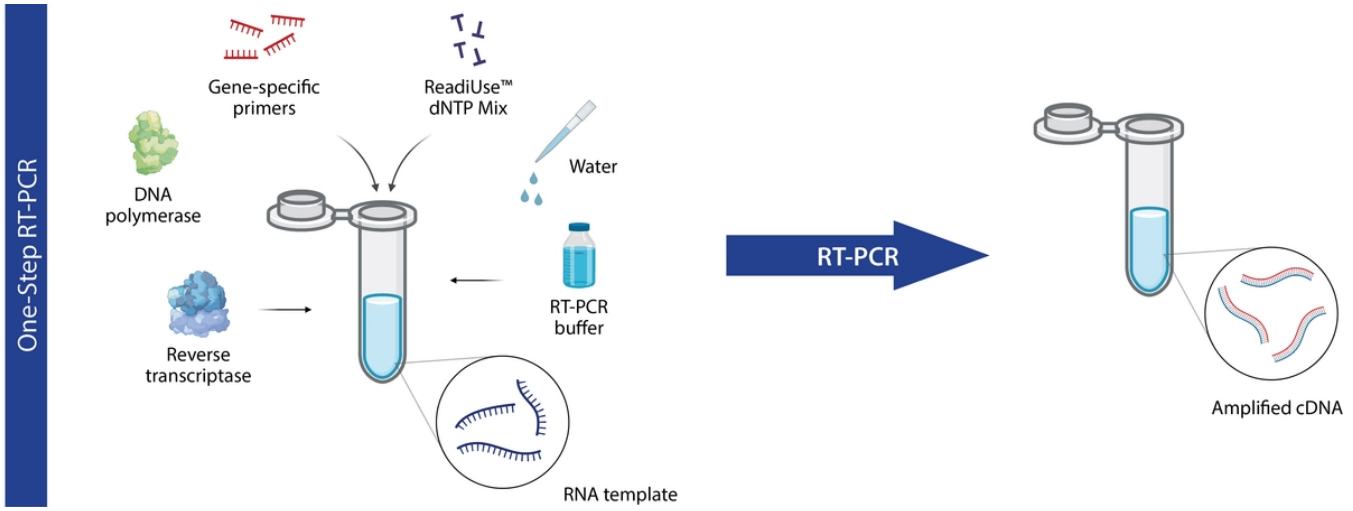
RT-PCR is a molecular diagnostic tool that works on the principle of converting the RNA template to a complementary DNA (cDNA) using the reverse transcriptase enzyme. This cDNA then undergoes exponential amplification using PCR to form multiple copies, which are then used for downstream analysis.

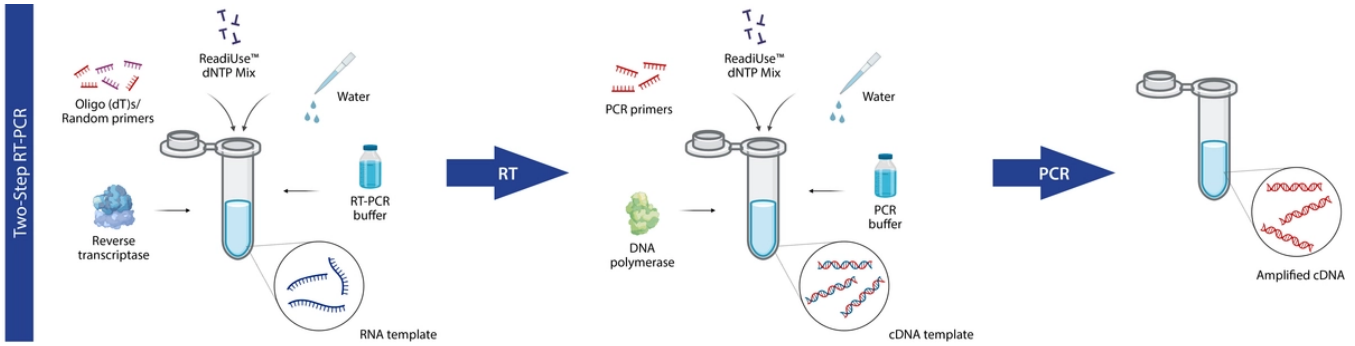
In a typical PCR, DNA is the template, and the enzyme used is Taq polymerase. However, in order to amplify RNA, reverse transcription needs to be carried out since RNA is not an efficient template for Taq polymerase. Hence, the modified version of PCR has an extra step of RNA being converted to DNA, then PCR is carried out.

RT-PCR can be performed in a one-step or a two-step assay. One-step assays combine reverse transcription and PCR in a single tube and buffer, using a reverse transcriptase along with a DNA polymerase. One-step RT-qPCR only utilizes sequence-specific primers. In two-step assays, the reverse transcription and PCR steps are performed in separate tubes, with different optimized buffers, reaction conditions, and priming strategies.

	ADVANTAGE	**DISADVANTAGE**
One-step	• Less experimental variation since both reactions take place in the same tube • Fewer pipetting steps reduces risk of contamination • Suitable for high throughput amplification/screening • Fast and highly reproducible	• Impossible to optimize the two reactions separately • Less sensitive than two-step because the reaction conditions are a compromise between the two combined reactions • Detection of fewer targets per sample

Two-step	• A stable cDNA pool is generated that can be stored for long periods of time and used for multiple reactions • The target and reference genes can be amplified from the same cDNA pool without multiplexing • Optimized reaction buffers and reaction conditions can be used for each individual reaction • Flexible priming options	• The use of several tubes and pipetting steps exposes the reaction to a greater risk of DNA contamination Time consuming • Requires more optimization than one-step





Procedure :

a. After RNA isolation is performed, the RNA is extracted.
b. An aliquot of extracted RNA sample is added to a reaction mixture. The reaction mixture consists of nucleotides, reverse transcriptase, and primers specific for the gene of interest.
c. Primers will anneal to the extracted RNA if the target is present.
d. Reverse transcriptase performs its function of synthesizing the complementary DNA (cDNA) strand.
e. The RNA/DNA hybrid now undergoes the steps of PCR:
    1. Denaturation: At 95°C the RNA/DNA strands denature.
    2. Annealing: Primers anneal to the newly formed cDNA
    3. Extension: Extending from the primer, the polymerase synthesizes a new DNA strand by replication.
f. Multiple cycles in the thermal cycler increase the number of copies of DNA as PCR products.

9. Aim: To study Western Blotting.

Reagents and materials:

1. Nitrocellulose membrane
2. Plastic staining box
3. Electroblotting apparatus
4. Whatmann No.1 filter paper
5. Transfer buffer (500 ml, pH 8.3)
   - Tris–HCl -25 mM
   - Glycine -192 mM
   - Methanol-20%
6. 10X Tris buffered saline (TBS) (100 ml, pH 7.6)
   - Tris -2.4 g
   - NaCl -8 g
   - They were dissolved in low amount of double distilled water, the pH was adjusted
7. And the total was made upto 100 ml with double distilled water.
8. Blocking solution (50mL)
   - 5% Non-dry fat milk powder - 0.25g
   - 1X TBS (pH 7.6) - 50mL
   - 0.1% Tween- 20 - 0.05mL
   - Washing buffer (100mL) (TBS)
   - 1X TBS (pH 7.6) -100 ml
   - 0.1% Tween - 20 - 0.1 ml
10. Preparation of primary antibodies
11. Preparation of secondary anti bodies
12. Colour indicator solution

0.05% 0f 3’3- diaminobenzidine tetra hydrochloride (DAB) substrate and 0.01% of H_2O_2 were dissolved in 1X PBS (pH 7.6). This chromogen substrate was prepared just prior to the treatment.

13. Ponceau S red solution (100mL)

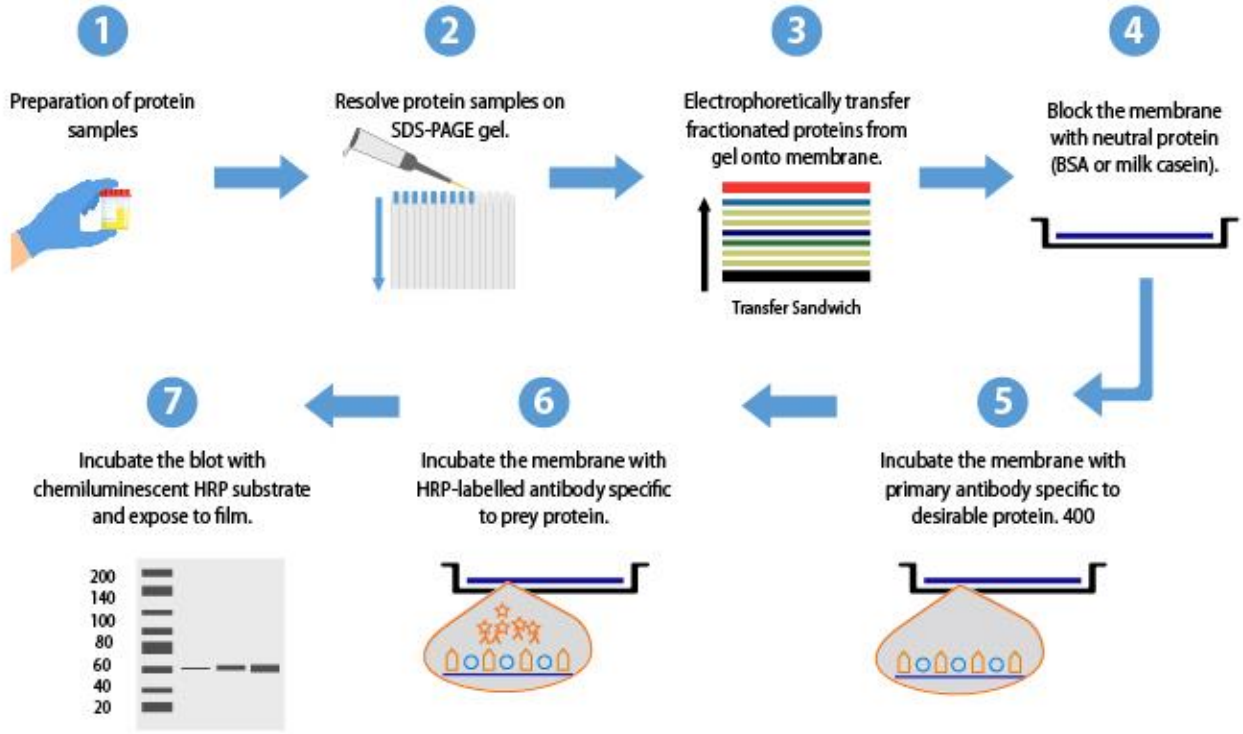
Ponceau S red - 0.5 g
Glacial acetic acid - 5%

Principle:

Western blotting (also known as protein blotting or immunoblotting) is a rapid and sensitive assay for detective and characterization of proteins. Western blotting technique exploits the inherent specificity by polyclonal or monoclonal antibodies.

It is an analytical method wherein a protein sample is electrophoresed on an SDS-PAGE and electro transferred onto nitrocellulose membrane. The transferred protein is detected using specific primary antibody and secondary enzyme labelled antibody and substrate. A protein sample is subjected to polyacrylamide gel electrophoresis. After this the gel is placed over a sheet of nitrocellulose and the protein in the gel is electrophoretically transferred to the nitrocellulose. The nitrocellulose is then soaked in blocking buffer (3% skimmed milk solution) to "block" the nonspecific binding of proteins. The nitrocellulose is then incubated with the specific antibody for the protein of interest. The nitrocellulose is then incubated with a second antibody, which is specific for the first antibody. For

example, if the first antibody was raised in mouse, the second antibody might be termed "goat anti-mouse immunoglobulin". What this means is that mouse immunoglobulins were used to elicit an antibody response in goats. The second antibody will typically have a covalently attached enzyme which, when provided with a chromogenic substrate, will cause a colour reaction. Thus the molecular weight and amount of the desired protein can be characterized from a complex mixture (e.g. crude cell extract) of other proteins by western blotting.



Procedure:

1. After SDS-PAGE, the gel was equilibrated in blotting buffer for 20 min at room temperature. While the gel was equilibrating, a piece of nitrocellulose membrane was cut into the same dimension as the gel it was wet slowly by sliding it at 45° angle into transfer buffer and was soaked for 20 min.

2. The pieces of Whatmann No.1 filter paper, four pads were also soaked in transfer buffer for 20 min.

3. Then, the pads, filter paper, nitrocellulose membrane and gel were assembled in the semi-dry blot apparatus in the following order:

- The two presoaked pads were placed at the bottom and a glass pipette was rolled over the surface of the pad to remove air bubbles. Then, the Whatmann No.1 filter paper was placed followed the nitrocellulose membrane. Carefully, the equilibrated gel was placed on top of the nitrocellulose membrane. The second Whatmann No.1

filter paper and followed it, the second set of pad were placed on top of the gel. (After each step care was taken to remove the bubbles). The transfer cell and plug was assembled and the gel transferred for 2h at 25V/130.

- After the transfer, protein were visualized by staining in ponceau S solution for 5 min, destained in the distilled water and the molecular marker was marked with in delible ink and destained for 10 min. The membrane was blocked in blocking buffer for 1h at room temperature. Then, the membrane was washed again with washing buffer and incubated with primary antibody overnight at 4oC. The next day, the membrane was washed again with washing buffer and incubated with HRP-conjugated secondary antibody for 2 h at room temperature. The membrane was washed and DAB solution was added and incubated at room temperature and watched for colour development, which is usually completed within 5–10 min. The membrane was rinsed with distilled water to stop the reaction of DAB. It was then placed on filter paper to air dry. Dilutions of the primary and secondary antibody were standardized after several trials. The specific protein was detected as a band in the nitrocellulose membrane.

Result :

The presence of specific protein or the presence of antigen or specific antibody was visualised as a bluish grey coloured band.

10. Aim: To study Northern Blotting.

Materials Required :

- Reagents for DNA isolation and purification
- Reagents for restriction digestion of DNA
- Reagents and buffers for agarose gel electrophoresis
- Apparatus for agarose gel electrophoresis
- Whatman filter papers
- Paper towels
- Positively charged nylon membrane
- Salmon sperm DNA
- Hybridization tube
- X-Ray films

Buffers Required:

10X TBE, used during gel electrophoresis:

- TRIS 1.3 M
- Boric acid 450 mM
- EDTA 25 mM

20X SSPE, used as a prehybridization and transfer buffer.

- NaCl 2.98 M
- EDTA 0.02 M
- Phosphate buffer (pH 7.4) 0.2M

Denaturing solution, used to denature dsDNA:

- NaCl 1.5 M
- NaOH 0.5 N
- Adjust the pH to ~13

Neutralizing solution, used to neutralize the gel after denaturing dsDNA:

- NaCl 1.5 M
- TRIS HCl 1 M
- Adjust the pH to 7.5

*Denhardt's solution, used during prehybridization to block non-specific DNA hybridizations:

- Bovine serum albumin 1%
- Ficoll 1%
- Polyvinylpyrrolidone 1%

Dissolve the components in water to a final volume of 50 mL, sterilize by filtration.

*2X Prehybridization solution, used to prepare the membrane for probe hybridization:

- 20X SSPE 30 mL
- 100X Denhardt's solution 10 mL
- 10% SDS 10 mL

- Water 50 mL

*Hybridization solution, used during the hybridization step.:
- 20X SSPE 30 mL
- 10% SDS 10 mL
- Water 60 mL

1X Probe buffer, used for the probe mix. (100 µL; make fresh):
- 1 M TRIS, pH 7.6 50 µL
- 2 M MgCl2 5 µL
- 0.5 M DTT 10 µL
- Water 35 µL

1X Probe mix (27 µL):
- Probe buffer 2.7 µL
- Oligonucleotide probe (0.2 µg/µL) 2 µL
- T4 phosphonucleotide kinase (PNK) 1 µL
- Water 11.3 µL
- 32P-ATP 10 µL

6X Low-stringency wash solution, used to remove low-homology hybridizations and reduce background noise. Prepare 600 mL of wash solution using the following reagents:
- 20X SSPE 180 mL
- 10% SDS 12 mL
- Water 408 mL

1X High-stringency wash solution, used to remove closely homologous hybridizations and further reduce background noise. Prepare 600 mL of wash solution using the following reagents:
- 20X SSPE 30 mL
- 10% SDS 12 mL
- Water 558 mL

Principle :

The principle of the northern blot is the same as all other blotting technique that is based on the transfer of biomolecules from one membrane to another. The RNA samples are separated on gels according to their size by gel electrophoresis. Since RNAs are single-stranded, these can form secondary structures by intermolecular base pairing. The electrophoretic separation of the RNA segments is thus performed under denaturing conditions. The separated RNA fragments are then transferred to a nylon membrane. Nitrocellulose membrane is not used as RNA doesn't bind effectively to the membrane. The transferred segments are immobilized onto the membrane by fixing agents. The RNA fragments on the membrane are detected by the addition of a labelled probe complementary to the RNA sequences present on the membrane. The hybridization forms the basis of the detection of RNA as the specificity of hybridization between the probe, and the RNA

allows the accurate identification of the segments. Northern blot utilizes size-dependent separation of RNA segments and thus can be used to determine the sizes of the transcripts.

Applications of Northern Blot

- The technique can be used for the identification and separation of RNA fragments collected from different biological sources.
- Northern blotting is used as a sensitive test for the detection of transcription of DNA fragments that are to be used as a probe in Southern Blotting.
- It also allows the detection and quantification of specific mRNAs from different tissues and different living organisms.
- Northern blotting is used as a tool for gene expression studies related to overexpression of cancer-causing genes, and gene expression during transplant rejects.
- Northern blotting has been used as a molecular tool for the diagnosis of diseases like Crohn's disease.
- The process is used as a method for the detection of viral microRNAs that play important roles in viral infection.

Procedure :

a. Separation of RNA on a denaturing gel

- The RNA gel solution is prepared by adding formaldehyde to the agarose solution.
- The cast is assembled, and the prepared denaturing gel is poured into the cast. As the gel begins to set, a comb with appropriate teeth is added to form wells.
- Once the gel is set, the comb is removed, and the gel is equilibrated with a running buffer for 30 minutes before running.
- 15 µg RNA sample is mixed with an equal volume of RNA loading buffer. Three µg of RNA markers are added in the same volume of RNA loading buffer.
- The samples are incubated at 65°C on a heating block for about 12-15 minutes.
- The samples are loaded to the equilibrated gel, and the first row of wells is filled with RNA markers.
- The gel is then run at 125V for about 3 hours.

b. Transfer of RNA from gel to the nylon membrane

- A nylon membrane is cut that is larger than the size of the denaturing gel, and a filter paper with the same size as the nylon membrane is also prepared.
- Once the electrophoresis process is complete, the RNA gel is removed from the tank and rinsed with water.
- An oblong sponge that is slightly larger than the gel is placed on a glass dish, and the dish is filled with SSC to a point so as to leave the soaked sponge about half-submerged in the buffer.
- A few pieces of Whatman 3mm papers are placed on top of the sponge and are wetted with SSC buffer.
- The gel is then placed on top of the filter paper and squeezed out to remove air bubbles by rolling a glass pipette over the surface.
- The nylon membrane prepared is wetted with distilled water on an RNase-free dish for about 5 minutes.

- The wetted membrane is placed on the surface of the gel while avoiding any air bubbles formation.
- The surface is further flooded with SSC, and a few more filter papers are placed on top of the membrane.
- A glass plate is placed on top of the structure in order to hold everything in place. The structure is left overnight to obtain an effective transfer.

c. Immobilization

- Once the transfer is complete, the gel is removed and rinsed with SSC, and allowed to dry.
- The membrane is placed between two pieces of filter paper and baked in a vacuum oven at 80°C for 2 hours.
- In some cases, the membrane can be wrapped in a UV transparent plastic wrap and irradiates for an appropriate time on a UV transilluminator.
- d. Hybridization
- The DNA or RNA probes to be used are to be labelled to a specific activity of >108 dpm/µg, and unincorporated nucleotides are to be removed.
- The membrane carrying the immobilized RNA is wetted with SSC.
- The membrane is placed in a hybridization tube with the RNA-side-up, and 1 ml of formaldehyde solution is added.
- The tube is placed in the hybridization oven and incubated at 42°C for 3 hours.
- If the probe used is double-stranded, it is denatured by heating in a water bath or incubator for 10 minutes at 100°C.
- The desired volume of the probe is pipette into the hybridization tube and further incubated at 42°C.
- The solution is poured off, and the membrane is washed with a wash solution. The membrane is then observed under autoradiography.

Result :

The RNA bands are observed under radiography in the form of bands. The distance of the bands from the markers can be used to determine the length and semi quantification of the RNA fragments.