SYLLABUS

MOLECULAR BIOLOGY AND ANIMAL BIOTECHNOLOGY

17BCP211

MOLECULAR BIOLOGY

1. Isolation of DNA from liver
2. Isolation of RNA and estimation -UV method
3. Estimation of DNA by Diphenylamine method
4. Estimation of RNA by Orcinol method
5. Reverse-Transcription-PCR
6. Agarose gel electrophoresis of DNA
7. Preparation of competent E coli -transformation (demonstration)
8. Determination of Molecular weight of polypeptides by SDS PAGE(group) and Western Blotting (Demonstration)

ANIMAL TISSUE CULTURE (Demonstration)

9. Preparation and Sterilization of media
10. Cell lines and maintenance-Trypsinisation, Passaging, Staging and cell viability determination

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Ex No.1 ISOLATION OF DNA

Aim

The aim is to isolate the DNA from the given animal tissue sample.

Principle

Extraction of DNA basically consists of four major steps:

- Preparation of a cell extract
- Purification of DNA from cell extract
- Concentration of DNA samples
- Measurement of purity and DNA concentration

Reagents

1. Lysis buffer (50 mM Tris-HCl (pH 8.0), 10 mM EDTA, 0.5% sodium lauryl sarcosiante, 0.5 µg/ml proteinase K).
2. Ribonuclease A (10 ml, 0.5 µg/ml)
3. Phenol (Molecular Biology Grade)
4. Chloroform
5. Isoamyl Alcohol
6. Sodium Acetate (0.5 M)
7. Ethanol

Procedure

1. To extract DNA from cells of interest, cells are lysed with 100 –200 µl of lysis buffer [50 mM Tris-HCl (pH 8.0), containing 10 mM EDTA, 0.5% sodium lauryl sarcosinate and 0.5 µg/ml proteinase K].
2. Incubate for 1 h at 50ºC.
3. Add Ribonuclease A (10 µl, 0.5 mg/ml) and incubate for an additional 1 hr at 50ºC.
4. Add 1ml of phenol, shake well for 5 to 10 min and then centrifuge at 3000 rpm for 5 min at 4ºC.
5. Transfer the supernatant to a new microcentrifuge tube with 500 µl of phenol and 500 µl of chloroform / isoamyl alcohol (24:1), shake it well for 5 to 10 min and centrifuge at 3000 rpm for 5 min at 4ºC.
6. After centrifugation, transfer the supernatant to a new tube and add 25 – 50 µl of 3 M sodium acetate (pH 5.2) and 1 ml of ethanol, shake gently till the DNA to precipitate.
7. Then place it under -8^{0}C for 20 minutes and centrifuge at 12000 rpm for 20 min to recover the DNA.
8. Rinse the pellet with 1 ml of 70% ethanol and spin for 10min.
9. Discard the supernatant and air dry the pellet at room temperature.
10. Dissolve the DNA in 0.5 – 1.0 ml of Millipore water to determine the concentration and purity of DNA by absorbance at 260/280 nm in a UV-spectrophotometer.

Result

The purity of DNA obtained is _____.

Ex No.2 ISOLATION OF TOTAL RNA

Aim

To isolate RNA from the given tissue sample.

Principle

Single step guanidium acid phenol method emphasizes on the ability of guanidium isothiocyanate (GITC) to lyse cells, denature protein and inactivate intracellular ribonuclease rapidly. The presence of β mercaptoethanol in the mixture increases the solubilization properties of the GITC extraction buffer. Acid phenol extraction (pH< 5.0) selectivity keeps cellular DNA in the organic phase and help in extraction of proteins and lipids. The addition of chloroform further removes lipids and produce two distinct phases containing the DNA, proteins and lipids and an aqueous phase containing the RNA.

Reagents required

1. TRIR kit has the following components:
   Phenol, guanidium isothiocyanate, urea, detergents, buffering agents and stabilizers.
2. Chloroform (molecular biology grade)
3. Isopropanol (molecular biology grade)
4. 75% ethanol (molecular biology grade)

Procedure

1. Add 1 ml of TRIR to the homogenised tissue sample and swirl gently for 15 min and then keep at 4°C for 5 min to permit complete dissociation of nucleoprotein complexes.
2. To this, add 0.2 ml chloroform, shake vigorously for 15 sec and place on ice at 4°C for 5 min.
3. The lysate is to be then centrifuged at 12,000 x g for 15 min at 4°C to yield lower organic phase containing DNA and proteins and upper aqueous phase containing RNA.
4. The volume of the aqueous phase will be approximately 40-50% of the total volume of the lysate.
5. The aqueous phase to be carefully transferred to a fresh eppendorf micro centrifuge tube without disturbing the interphase. Equal volume of isopropanol to be added, mixed and kept at 4°C for 10 min. It is to be again centrifuged at 12,000 x g for 15 min at 4°C to precipitate the RNA.
6. Discard the supernatant and wash the pellet twice with 75% ethanol and air dried. Dissolve the RNA pellet in 50µl of sterile milliQ water and place in a water bath at 60°C for 10 min to ensure maximum solubility of RNA.

7. Vortex gently the RNA sample and quantify before storing at -80°C.

Quantification of RNA

Diluted RNA sample to be quantified spectrophotometrically by measuring the absorbance (A) at 260 nm. An absorbance of 1OD is equivalent to RNA concentration of 40 μg/ml. Therefore, the yield can be calculated by multiplying the absorbance at 260 nm with dilution factor and 40 μg. The purity of RNA preparations were assessed by determining the ratio of absorbance of sample at 260 nm and 280 nm.

Result

The purity of RNA obtained is _____.

Ex No.3 ESTIMATION OF DNA BY DIPHENLYAMINE METHOD

AIM

To estimate the amount of DNA present in the given sample.

PRINCIPLE

When DNA is treated with diphenylamine under acid conditions, the compound is formed with a sharp adsorption maximum at 595nm. This reaction is given by 2-deoxypentoses in general and is not specific for DNA (adenine and guanine). In acidsolution ,the straight chain from of a deoxypentose is converted to highly reactive α-hydroxylevulinic acid and which reacts with diphenylamine to give a blue coloured complex.

MATERIALS REQUIRED :

DNA STANDARD SOLUTION :

0.010mg of DNA as weighed accurately and make up to 50ml with 0.03 sodium hydroxide.

DIPHENYLAMINE REAGENT:

Dissolve 3g of diphenylamine in 292.2ml of glacial acetic acid .Add slowly 15ml of concentrated sulphuric acid .Stir well and store for future use.

PROCEDURE:

Pipette out different aliquots of standard DNA solution in the range of 0.1,0.2,0.3,0.4,0.5ml with (0.1ml interval) .

Make content of each tube to 1ml using distilled water.

To the aliquots add 5ml of diphenylamine reagent .

Close the test tubes with aluminium foil and keep it firm by rubber bands .

Heat on a boiling water bath for 10min, cool and read the OD value at 595nm . Read the test and µµstandards against water blank .

OBSERVATION:

Blue colour formation is observed.

RESULT:

The amount of DNA present in the given sample is µg/ml .

S.NO	VOLOF STANDARD SOLUTION (ml)	VOLUME OF WATER (ml)	CONC. OF DNA IN WORKING SAMPLE (µg)	VOLUME OF DIPHENY - LAMINE (ml)	INCUBATION	OPTI -CAL DENS -ITY (nm)
BLANK	0.0	1	-	5	INCUBATION FOR 10MIN IN BOILING WATER BATH	
S1	0.1	0.9	20	5		
S2	0.2	0.8	40	5		
S3	0.3	0.7	60	5		
S4	0.4	0.6	80	5ml		
S5	0.5	0.5	100	5ml		

TEST				5ml		

Ex No.4 ESTIMATION OF RNA BY ORCINOL METHOD

AIM:

To estimate the amount of RNA isolated from the liver using colorimetric method.

PRINCIPLE:

This method relies on the conversion of pentose sugar, ribose, present in RNA into furfural in the presence of hot acid. Furfural then reacts with orcinol in the presence of ferric chloride to give a green color. The intensity of the color produced largely depends on the concentrations of RNA, ferric chloride and orcinol as well as on the duration of boiling, etc. The green color developed is read at 665nm.

MATERIALS AND METHOD:

1. **Orcinol reagent:**

Dissolve 300mg orcinol in 5ml ethanol and add 3.5ml of this to 100ml of 0.1% solution of Fecl3 in concentrated Hcl.

2. **0.1% Fecl3 in concentrated Hcl:**

Weigh 100mg of Fecl3 and dissolve in 100ml of concentrated Hcl.

3. **RNA stock solution:**

Weigh 30mg of RNA and dissolve in 100ml of TAE buffer (Tris- acetate- EDTA). Concentration- 300µg/ml.

PROCEDURE:

Set up a series of clean test tubes and pipette out 0.1,0.2,0.3,0.4 and0.5 to the tubes labelled S1 to S5. The volume in all the tubes weremade upto 1ml using distilled water. 1ml of distilled water is taken in a separate tube and labelled as blank. Prepare a similar set of tubes with unknown RNA sample and labelled as U1 and U2. Add 3ml of orcinol reagent to all the tubes mix well and heat in the boiling water bath for 15 minutes. After 15mins, the tubes are cooled and the intensity of the green colour is measured at 665nm.

Draw a standard graph using a absorbance(A 665) on Y axis and concentration on X axis. Draw a straight line using principles of least squares and calculated the line equation containing X and Y. Calculate the amount of RNA (X) by using the equation.

RESULT:

The amount of RNA present in the isolated liver sample is estimated to be µg/ml.

Ex No.5

REVERSE TRANSCRIPTASE-POLYMERASE CHAIN REACTION

Aim

The aim is to reverse transcriptase of the resultant mRNA into cDNA using RT-PCR technique

Principle

The reverse transcriptase-polymerase chain reaction (RT-PCR) involves the conversion of mRNA of gene of interest present in the total RNA into cDNA and then amplifies a specific region of the cDNA. This enzyme reverse transcriptase catalyzes the conversion of mRNA into cDNA. This enzyme is isolated from retroviruses such as murine moloney leukemia virus (MMLV) and avian myeloblastosis virus (AMV). Reverse transcriptase polymerase chain reaction were done using a single step kit in which the reverse transcription reaction and the amplification can be carried out in a single vial.

Materials required

- **One step RT-PCR kit Components**

1. **Enzyme mix** containing 20 mM TrisHCl, 100 mM KCl,1 mM dithiothreitol (DTT), 0.1mM EDTA, 0.5%(V/V) Stabilizer; pH 9.0.
2. **5x RT-PCR master mix** containing Tris HCl, KCl, $(NH_4)_2SO4$, 12.5 mM $MgCl_2$, DTT, pH 8.7.
3. **dNTP mix** containing 10 mM each of dATP, dCTP, dGTP and dTTP.
4. PCR-grade RNase free water.
5. **Oligonucleotide primers**

For amplification of the individual gene, a 18-21 base primer needs to be identified and synthesized (available commercially)

Procedure

To 1.5 µg of total RNA, 2 µl of dNTP mix, 10 µl of 5X buffer, 2 µl of enzyme mix, 2 µl of ß actin (6 µM) and 5 µl of ALP or collagen (6 µM) of each primers were added and made up to final volume of 50 µl using RNAse free water.

- Reverse transcription (RT): 50°C for 30 min
- Termination of RT: 94°C for 2 min

- Denaturation: 94°C for 90 sec
- Annealing: 55°C for 90 sec
- Extension: 72°C for 90 sec

} (30 cycles)

- Final extension: 72°C for 10 min
- Hold at 4°C

Result

cDNA is synthesized from mRNA by reverse transcription.

Ex No.6 AGAROSE GEL ELECTROPHORESIS

Aim

To identify the purified DNA molecules from the given sample using Agarose Gel Electrophoresis

Principle

The generated cDNA fragments were resolved in 2% agarose gel under an applied electric field. DNA molecules migrate towards the anode due to negatively charged phosphate along the backbone of DNA. The rate of migration of linear DNA is inversely proportional to its molecular weight. Thus, the larger molecules travel at a much lower speed when compared to smaller one.

Reagents required

1. **TBE buffer 1X: (Tris, Boric acid. EDTA) (pH 8.0):** 3.78 g tris, 1.925 g boric acid and 0.260 g EDTA were dissolved in 350 ml of autoclaved RNAse and DNAse free water and the pH was adjusted to 8.0.
2. **1% Ethidium bromide in RNAse and DNAse free water**
3. **2% Agarose in 1x TBE buffer**
4. **Gel loading dye**
   The gel loading dye (6X) was procured commercially in ready to use form.

Procedure

1. Add 1 g of agarose to 50 ml 1X TBE buffer (2%). Then melt it on a microwave oven, make up the volume to 50 ml with TBE buffer and add 10 µl of 1% EtBr, evenly mix and cool to 40°C.
2. Then pour the mixed solution into a sealed gel-casting platform and insert the comb after ensuring the absence of air bubbles.
3. Then allow the gel to get harden. Remove the comb after 15 min by taking care of not disturbing the sample wells. Immerse the platform in the electrophoresis buffer tank.
4. Mix the 5 µl of PCR products from each reaction tube with 1µl of 6X gel loading dye and load to each well. Load simultaneously of 100 bp molecular weight marker DNA in the first lane to identify the cDNA products.

5. Turn on the power supply and adjust the electricity to 60 mA. Run the gel for 2 h. And then, visualize the resolved cDNA fragments gel for gel documentation.
6. Normalize the band intensity of cDNA fragments of ALP and Collagen with the internal control ß actin and express in OD units relative to GAPDH.

Ex No.7 INDUCTION OF LAC OPERON

Aim

To prepare competent cell, transform plasmid DNA and to assess induction of Lac Operon.

Introduction

Bacterial transformation is a process which involves genetic alteration of bacteria by incorporation and stable expression of a foreign genetic material from the environment or surrounding medium. Since DNA is a very hydrophobic molecule, it will not normally cross the bacterial cell membrane and hence bacterial cells needs to be made competent to ensure DNA uptake. Competence is the propensity of a bacterial cell to take up extracellular DNA from its environment. There are different methods of carrying out transformation, e.g. chemical transformation, electroporation, gene gun, liposome mediated transfer and microinjection. Chemical transformation includes the usage of Calcium chloride (CaCl2). This mode of transformation is easy to perform and requires minimum number of equipments.

Principle:

For the incorporation of plasmid into a cell, bacteria must first be made "competent". This process includes the treatment of cells with bivalent calcium ions in ice-cold condition. As a result small pores are formed on the cell membrane, which makes it permeable.

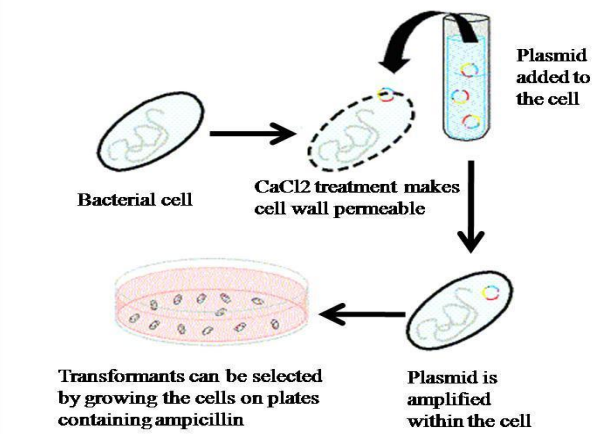

Fig 1: The process of bacterial transformation includes treatment of cells with CaCl2, which makes cells permeable, and plasmid DNA can enter the cell.

The plasmid DNA may adhere to the surface of the cell and uptake is mediated by a pulsed heat shock at 42^{O}C. A rapid chilling step on ice ensures the closure of the pores. These cells are allowed to propagate and selection of transformants can be done by growing the cells on a selective media which will allow only the plasmid containing cells to grow.

Plasmids are extrachromosomal DNA element capable of independent replication inside a suitable host. Plasmids encode a wide variety of genes, including those required for antimicrobial resistance. These genes act as selective markers when a transformation experiment is carried out.

The E. coli plasmid pUC19 encodes a gene that can be utilized as a selectable marker during a transformation experiment. pUC19 has ampicillin resistance marker that facilitate only transformed cells to grow on LB – Ampicillin plates. Transformants, thus having the ability to grow on ampicillin plates can be selected. This process of direct selection of recombinants is called insertional-inactivation. pUC19 also carries the N-terminal coding sequence for β-galactosidase of the lac operon. The E. coli host strain has a deletion at the amino terminal end of the LacZ gene, which codes for β-galactosidase. When pUC19 is transformed into the competent host cells, the truncated products from both complement each other leading to the production of enzymatically active β-galactosidase. This is referred as α-complementation. The tranformants turn blue on X-gal and IPTG containing plates due to the synthesis of β-galactosidase. X-gal is the chromogenic substrate of β-galactosidase and IPTG induce the expression of this β-galactosidase.

Step wise procedure:

1. The entire process needs to be carried out under sterile conditions.
2. **Preparation of 0.1M Calcium chloride (sterile):**
   To prepare 1000 ml of 11.1 gram of Calcium chloride was weighed and dissolved in 700 ml of sterile distilled water and made upto 1000 ml. This solution can be stored at 2-8^{O}C.
3. Pre-chill the tubes before competent cell preparation, 0.1M Calcium chloride solution and centrifuge tubes. Set the centrifuge at 4^{O}C and water bath at 42 ^{O}C.
4. 50 ng/µl of plasmid is used for transformation.
5. **Preparation of LB (Luria Bertani) broth (55 ml):** Dissolve 1.38 g of LB media in 55 ml of distilled water. The solution is to be made sterile by autoclaving.
6. **Preparation of LB (Luria Bertani) agar plates (20 ml):** 0.5 g of LB media and 0.3 g of agar are dissolved in 20 ml of sterile distilled water. The solution is to be made sterile by autoclaving.
7. **Preparation of Ampicillin:** Dissolve 30 mg of ampicillin powder in 600 µl of sterile double distilled water to prepare 50 mg/ml ampicillin solution.
8. **Preparation of LB (Luria Bertani) Agar plates containing Ampicillin, X-Gal and IPTG (100 ml):**
   Dissolve 2.5 g of LB media and 1.5 g of agar in 100 ml of distilled water. Sterilize by autoclaving and allow the media to cool down to 40-45 ^{O}C. Add 100 µl of ampicillin, 200 µl of X-Gal and of 100 µl IPTG to 100 ml of autoclaved LB agar media, mix well and pour on sterile petriplates.

9. Transformation to be carried out as soon as possible after the competent cells are prepared. Storage of competent cells decreases the transformation efficiency.
10. **Preparation of LB (Luria Bertani) agar plates (20 ml):** Dissolve 0.5 g of LB media and 0.3 g of agar in 20 ml of sterile distilled water. Sterilize by autoclaving and pour on sterile petriplate.
11. **Preparation of LB (Luria Bertani) Agar plates containing Ampicillin, X-Gal and IPTG (100 ml):**
    Dissolve 2.5 g of LB media and 1.5 g of agar in 100 ml of distilled water. Sterilize by autoclaving and allow the media to cool down to 40-45^{O}C. Add 100 µl of ampicillin, 200 µl of X-Gal and of 100 µl IPTG to 100 ml of autoclaved LB agar media, mix well and pour on sterile petriplates.

Procedure:

Day 1:

1. Open the bottle containing culture and reconstitute the pellet with 0.25 ml of LB broth.
2. Pick up a loopful of culture and streak onto LB agar plate and incubate overnight at 37^{0}C .

Day 2:

1. Inoculate a single colony from the revived plate in 1 ml LB broth and incubate overnight at 37^{0}C.

Day 3:

1. Take 50 ml of LB broth in a sterile flask. Transfer 1 ml of overnight grown culture into the flask containing 50 ml of LB broth and incubate at 37^{0}C in a shaker set at 3000 rpm for four hours.

A) Preparation of Competent Cells:

Note: Competent cells are to be prepared within 3 days of reviving the strain.

1. Transfer the above culture into a pre-cooled 50 ml polypropylene tube.
2. Incubate the culture at 4^{O}C for 10 minutes on ice.
3. Centrifuge at 5000 rpm for 10 minutes at 4^{O}C in a cooling centrifuge.
4. Decant the medium completely leaving no traces of medium.
5. The cell pellet is resuspended in 30 ml pre-chilled sterile 0.1 M Calcium chloride solution and incubate for 30 minutes on ice.
6. Centrifuge at 5000 rpm for 10 minutes at 4^{O}C.
7. Discard the calcium chloride solution.
8. The pellet is resuspended in 2 ml pre-chilled sterile 0.1M Calcium chloride solution.
9. This cell suspension contains competent cells and can be used for transformation.

B) Transformation of cells:

1. Take 200 µl of the above cell suspension in two 2.0 ml tubes and label control and transformed. Add 2 µl of plasmid DNA to the tube labeled as transformed and mix well.
2. Incubate the tubes at 4^{O}C for 30 minutes.
3. Transfer them to a pre-warmed water bath set at a temperature of 42^{O}C for 2 minutes to produce heat shock.

4. Rapidly transfer the tubes on ice-bath. Allow the cells to cool for 5 minutes.
5. Add 800 µl of LB Broth to both the tubes. Incubate the tubes for 1 hour at $37^{o}C$ to ensure the bacteria to recover and express the antibiotic resistance marker encoded by the plasmid.
6. Take four LB agar plates containing ampicillin, X-Gal, IPTG and label them as control, X, Y and Z. Plate 200 µl of culture from the „control" tube and plate it on the corresponding plate with a sterile spreader. Plate 50 µl, 100 µl and 200 µl of cell cultures from the transformed tube on the plates labeled as X, Y and Z.
7. Store at room temperature till the plates are dry.
8. Incubate the plates overnight at $37^{o}C$.

Observation and Result

After incubation observe the plates for the bacterial growth and count the number of visible colonies. Calculate the efficiency of transformation.

Record your observations as follows:

Sr. No.	Plate	Growth	Number of colonies	Transformation Efficiency
1.	Control plate			
2.	Transformed plate (X)			
3.	Transformed plate (Y)			
4.	Transformed plate (Z)			

Denote +ve when you observe bacterial growth, -ve when there is no growth

Calculation of transformation efficiency

Transformation efficiency is defined as the number of cells transformed per microgram of supercoiled plasmid DNA in a transformation reaction**.**

Transformation Efficiency = Number of colonies x 1000 ng/ Amount of DNA plated (ng) = /g.

Interpretation

On transformation of cells with pUC19 plasmid, antibiotic resistance is conferred on the host as this plasmid carries gene for ampicillin resistance. As a result, those cells that grow in presence of ampicillin are transformed cells. The transformed colonies are blue on X-Gal, IPTG plates due to α-complementation.

Ex No.8 **WESTERN BLOT ANALYSIS**

Procedure

Following electrophoresis, the gel is prepared for electroblotting using standard transfer or semi-dry blotting system. Before assembling the transfer system, soak blotting papers and blotting sponges in transfer buffer until saturated and wet the PVDF membrane with 100% methanol and then soak it in cold transfer buffer. It is important that gloves are worn while handling the membrane to prevent cross contaminations. Generally, a transfer sandwich is assembled, with the following layers in order from anode(+) to cathode(-).

1) +ve end
2) Sponge
3) Filter paper soaked in transfer buffer
4) Membrane
5) Gel
6) Filter paper soaked in transfer buffer
7) Sponge
8) -ve end

The transfer sandwich is then placed in the transfer apparatus filled with transfer buffer and subjected to an electric current 100 V for 3 h under cold condition. After the transfer, remove the sandwich from the transfer system and remove the membrane

Blocking non-specific binding sites

In a Western blot, it is important to block the unreacted sites on the membrane to reduce the amount of non-specific binding of proteins during subsequent steps in the assay. Typical blocking solutions include 10% non-fat dried milk in tris or phosphate buffered saline. Incubation in blocking solution for 30 min at 37oC is sufficient to block the membrane.

Antibody incubations

Primary antibody

The choice of primary antibody for a Western blot will depend on the antigen to be detected. Alternatively, a primary antibody may be made to recognize the antigen of interest. Both polyclonal and monoclonal antibodies work well for Western blotting. Polyclonal antibodies have high affinity for the antigen, but non-specific bands may appear. Monoclonal antibodies are valued for their specificity, purity and consistency that result in lower background. Antibodies for Western blotting are typically used as dilutions, ranging from 1/100 to 1/500,000. Antibody dilutions are typically made in the wash buffer containing a blocking agent. The presence of small amount of blocking agent and detergent in the antibody diluent often helps to minimize background.

Procedure

When the blocking is over, decant the blocking buffer from the blot and incubate the membrane with diluted primary antibody for 30 min at 37°C, or one hour at room temperature, or overnight at 4°C with gentle agitation. Consult the individual product datasheets for suggested dilution

ranges. Following incubation in primary antibody, the blot is washed in three times (10 min each) with T-TBS

Secondary antibody

A wide variety of labelled secondary antibodies can be used for western blot detection. The choice of secondary antibody depends upon the species of animal in which the primary antibody was raised (the host species). For example, if the primary antibody is a mouse monoclonal antibody, the secondary antibody must be an anti-mouse antibody obtained from a host other than the mouse.

Procedure

After washing the membrane to remove unbound primary antibody, the secondary antibody is incubated with the membrane for 45 min at room temperature in 1/5000 dilutions or 1/8000 dilutions. Following secondary antibody incubation, the membrane is washed in washing buffer to wash away the unbound secondary antibodies.

Protein detection

The method of detection is dependent upon the label that has been conjugated to the secondary antibody. The most common antibody label used in western blotting is an enzyme such as alkaline phosphatase or horseradish peroxidase (HRP), which can be detected visually through the conversion of a colorimetric substrate (chromogen) to a coloured precipitate at the site of antibody binding. Chemiluminescent substrates are commonly employed, which emit light upon conversion by enzyme. The light emitted at the site of substrate conversion can be captured on X-ray film. Bands corresponding to the detected protein of interest will appear as dark regions on the developed film. Band densities in different lanes can be compared providing information on relative abundance of the protein of interest. Chemiluminescence has an advantage of perhaps an order of magnitude greater sensitivity than the dye based methods. However, it requires a darkroom to perform and is more expensive in reagents.

Reagents for Western blotting

1) RIPA Buffer (150 mM NaCl, 50 mM Tris, 1 mM EDTA, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, pH 7.4)

For 500 ml of RIPA buffer:

	Tris base		3.029 g
NaCl		4.383 g	
	EDTA		186 mg
Na-deoxycholate		2.5 g	
SDS		500 mg	
NP-40		5 g	

Dissolve these chemicals in 350 ml water and adjust pH to 7.4 with HCl. Make up the volume to 500 ml with water. Store in refrigerator.

2) Running gel buffer (1.5 M Tris-HCl; pH 8.8)
For 250 ml solution, dissolve 45.375 g Tris in 200 ml water and adjust the pH with HCl. Make up the volume up to 250 ml with water. Store at room temperature.

3) Stacking gel buffer (0.5 M Tris-HCl; pH 6.8)
For 250 ml solution, dissolve 15 g Tris in 200 ml water, adjust pH with HCl and make up the volume up to 250 ml with water. Store at room temperature.

4) 10% SDS
For 100 ml solutions, take 10 g SDS in a measuring cylinder and make up the volume up to 100 ml with water and store at room temperature.

5) 10x SDS Electrophoresis buffer (25 mM Tris, 1.92 mM glycine, 1% SDS)
For 1 L of solution, 30 g Tris, 144 g glycine and 10 g SDS. Store at room temperature.

6) 1x SDS Electrophoresis buffer (25 mM Tris, 1.92 mM glycine, 1% SDS)
50 ml of 10x SDS electrophoresis buffer and 450 ml water.

7) 10x transfer buffer (25 mM Tris, 1.92 mM glycine)
Dissolve 30.3 g Tris (BioRad) and 144 g glycine (BioRad) in about 800 ml of water. Adjust volume to 1 L. Store at room temperature.

8) 1x transfer buffer (25 mM Tris, 1.92 mM Glycine, 20% methanol)
100 ml of 10x transfer buffer + 200 ml of methanol. Adjust the volume to 1 L with water. Prepare on the day of use and keep cold.

9) 6x sample buffer with reducing agents (12% SDS, 40% glycerol, 30% β-mercaptoethanol, 300 mM DTT, 120 mM EDTA. 1 mg/ml bromophenol blue, 375 mM Tris-Cl, pH 6.8)
For 10 ml: 1.2 g SDS, 4 ml glycerol, 3ml β-mercaptoethanol, 460 mg dithiothreitol, 446 mg EDTA, 454 mg Tris, 2 ml water, adjust pH to 6.8 with concentrated HCl then adjust the volume to 10 ml. Add 10 mg bromophenol blue. Store frozen as 500 µl aliquots.

10) TBS (20 mM Tris, 500 mM NaCl, pH 7.5)
Dissolve 4.84 g Tris and 58.48 g NaCl in about 1.5 L of water. Adjust pH to 7.5 with 1 N HCl and adjust volume to 2 L with water.

11) T-TBS (0.2% Tween-20 in TBS)
Add 1 ml of tween-20 to 500 ml of TBS. Rinse the pipette tip repeatedly with the solution being made until all the detergent is dissolved. Store in refrigerator.

12) Blocking solution (PBS with 10% glycerol, 10% non fat dry milk and 0.2
Tween-20) Prepare one day prior to use and discard unused portion. Add 50 ml of 10X PBS, 50 ml glycerol and 4.5 mg NaCl to a 500 ml graduated cylinder and about 400 ml water. Cover with parafilm and invert repeatedly until dissolved. Transfer to a 750 ml beaker and add
50 g carnation instant milk powder and stir until dissolved. Add 1 ml of Tween-20 (using a blue pipetteman tip with the tip cut off) to 500 ml of TBS. Rinse the pipet tip repeatedly with the solution being made until all the detergent is dissolved. Adjust the volume to 500 ml with water. Store in refrigerator.

How to do a Western Blot

A. **Preparation of gel**

1. Assemble the glass plates and spacers
2. Pour the running gel to about 1 cm below the wells of the comb
3. Seal with 1 ml water-saturated 1-butanol and leave the gel overnight to polymerize.
4. After polymerization, pour off the butanol and rinse with deionized water.
5. Pour the stacking gel (~5 ml) and insert the comb immediately.
6. When the stacking gel has polymerized, place in gel rig and immerse in buffer.

B. **Preparation of cell lysates**

1. Collect cells (confluent T-25) by trypsinization and spin.
2. Lyse the pellet with 100 µl RIPA buffer with protease inhibitors on ice for 10 min (For 500,000 cells, lyse with 20 µl).
3. Spin at 12,000 rpm (16,000 x g) in a microcentrifuge tube for 10 min at 4°C.
4. Transfer the supernatant to a new tube and discard the pellet.
5. Determine the protein concentration
6. Take x µl (= y µg protein) and mix with x µl of 2x sample buffer.
7. Boil for 5 min.
8. Cool at RT for 5 min.
9. Flash spin to bring down condensation prior to loading gel.

C. **Separation of Proteins in Gel**

1. After flash spinning the samples, load into the wells.
2. Be sure to use markers. We use 8 µl BioRad prestained MW markers
3. Run with constant current (100 V). Usual running time is about 45 min.

D. **Processing of the Membrane for Blotting**

1. Cut a piece of PVDF membrane (Millipore Immobion).
2. Wet for about 30 s in methanol at room temperature.
3. Remove methanol and immerse the membrane in 1x transfer buffer until ready to use.

E. **Transfer of Proteins to the Membrane**

1. Assemble "sandwich" for BioRad's Transblot.
2. Prewet the sponges, filter papers (slightly bigger than gel) in 1x blotting buffer.
Sponge - filter paper - gel - membrane - filter paper - sponge
3. Transfer for 3 h at 100 V at 4°C on a stir plate. Bigger proteins might take longer time to get transferred.
4. When finished, immerse membrane in blocking buffer overnight at room temperature.

F. **Antibodies and detection**

1. Incubate with primary antibody diluted in blocking buffer for the required time at required temperature.
2. Wash 3 x 10 min with 0.05% Tween 20 in TBS.
3. Incubate with secondary antibody diluted in blocking buffer for 45 min at room temperature.
4. Wash 3 x 10 min with 0.05% Tween 20 in TBS.

5. Detect with Amersham ECL kit.
6. Dry the membrane by dabbing on tissue paper. Place membrane on plastic film. Add 2 ml of detection mix. Incubate for 1 min. Tip off ECL and wrap in new plastic film.
7. Expose the wrapped membrane to X-ray film.
8. Develop the film.
9. Quantify the band intensity.

Expt No.9 Preparation of media for the animal cell culture.

PRINCIPLE

All the Animal cells can be grown in a liquid culture medium consisting of a mixture of vitamins, salts, glucose, amino acids and growth factors. Moreover, Calf serum is an easily available source of growth and attachment factors. Antibiotics are added to prevent the growth of bacteria. Under these conditions cells will grow at physiological pH (7.4) and at body temperature (37ºC) to form a monolayer on the culture vessels.

MATERIALS REQUIRED

Medium Adult bovine serum Membrane filter (Millipore 0.45µ) Sterilize

Double distilled water 1000 ml

1 litre measuring cylinder

100 ml measuring cylinder

1 litre filtration flask

Medium storage bottles

Other Glasswares

Method:

Sterilize the laminar air flow by UV irradiation for 45 minutes before using it.

1.Take 500ml of sterile double distilled water in a 1000 ml measuring cylinder.

2.Transfer the contents of the powdered medium into 1 litre measuring cylinder add 3.7 gms of $NaHCO_3$ in the absence of CO_2 incubator.

3.Mix thoroughly to dissolve the powdered medium, and add enicillin/streptomycin/gentamycin.
4.Fill the cylinder with1 litre double distilled water mix and transfer to sterile 2 litre flask and mix. Pinkish red color of the medium indicates normal pH range.

5.Assemble the filter sterilization set-up and carry out the filtration under negative pressure.

6.Prepare 400 ml of medium containing 10% Adult bovine serum using 100 ml measuring cylinder and store in a 500 ml sera lab bottle.

7.Transfer the remaining medium without serum into big glass bottles.

8.Store the medium in refrigerator, dispose the used membrane and immerse the used glassware in water for washing.

9.Different types of medium is used for various kind of Experiments.

10The components of different types of medium is given in the following tables

Passaging the Cells

1. Warm the trypsin-EDTA solution, balanced salt solution Dulbecco's Phosphate Buffered Saline without calcium or magnesium, ATCC® No. 30-2200], and complete growth medium to the appropriate temperature in water bath for the cell line. In most cases, this is the temperature used to grow the cells (usually 37°C). For some sensitive cells, the trypsin-EDTA solution may need to be used at room temperature or 4°C.

2. Aspirate the cell culture medium from the flask.

3. Rinse the cell monolayer with Dulbecco's PBS without calcium or magnesium and aspirate.

4. Add 2 mL to 3 mL of the trypsin-EDTA solution and incubate at the appropriate temperature. Check the progress of cell dissociation by microscopy. To avoid clumping, do not agitate the cells by hitting or shaking the flask while waiting for them to detach.

5. Once the cells appear to be detached (5 to 15 minutes for most cell lines; they will appear rounded and refractile under the microscope), add 6 to 8 mL of complete growth medium with a pipette to the cell suspension to inactivate the trypsin. Gently wash any remaining cells from the growth surface of the flask. Check the cells with the microscope to be sure that most (>95%) are single cells. If cell clusters are apparent, continue to disperse the cells with gentle pipetting.

6. Add 12 mL to 15 mL of fresh culture medium to a new flask and equilibrate this medium to the appropriate pH and temperature.

7. Count the cells in suspension and determine their viability or simply divide them according to a routine split ratio and dispense them into the medium of the newly prepared flask. Do not add a concentrated cell suspension to an empty culture vessel as this can result in uneven cell attachment and growth.

8. Place the flask back into the incubator. Examine the culture the following day to ensure the cells have reattached and are actively growing. Change the medium as needed; for most actively growing cultures two to three times per week is typical.

Expt No.10 **ASSESSMENT OF CELL VIABILITY**

MTT Assay

Principle

MTT assay depends on the reduction of a soluble yellow tetrazolium dye (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) into an insoluble blue purple product. Only living cells with active mitochondria can convert the pale yellow substrate (MTT) into dark blue insoluble crystals (MTT formazan).

Reagents

1. MTT: MTT was dissolved at a concentration of 0.5 mg/ml in serum-free medium
2. Phosphate buffered saline (PBS) (pH 7.4): NaCl (8 g), KCl (0.2 g), Na2HPO4 (1.44 g) and KH2PO4 (0.2 g) were dissolved in 800 ml of distilled water. The pH was adjusted to 7.4 using 1 N HCl. Then the buffer was made up to 1 litre with distilled water and sterilized by autoclaving.
3. Dimethyl sulfoxide (DMSO) (100 %)

Procedure

1. Cells were plated with FBS containing medium in 96-well plate at a concentration of 5 X 103 cells/well. After 24 h, cells were washed twice with FBS-free medium and starved for an hour at 34ºC. After starvation, the medium was replaced and cells were exposed to serum free medium containing 1 mM hydrogen peroxide.
2. After the treatment period, hydrogen peroxide containing medium was removed and the cells were washed with PBS. To the cells, 200 µl of MTT reagent was added and incubated at 37°C for 4 h.
3. After incubation, the MTT-containing medium was aspirated out and the cells were washed with PBS, then 100 µl of DMSO (100%) was added to dissolve the crystals in each well.
4. Then, the cells were kept for 30 min with constant shaking and the intensity of the colour developed was measured using an ELISA reader at 490 nm.
5. The colour developed was directly proportional to the number of viable (metabolically active) cells present in the culture. Results are expressed as % viable cells.

Possible viva questions

1. How might you optimize the purity of DNA?
2. Why must you handle DNA gently in the extraction procedure?
3. What are the importance of DNA isolation?
4. What are DNA? Mention its types?
5. What are the principles of DNA isolation?
6. Mention the uses of alcohol in isolation of DNA.
7. What is the need of cell lysis in DNA isolation?
8. What are the action of lysozyme in DNA isolation?
9. How acetate purify DNA during DNA isolation?
10. What are chemicals need for DNA isolation?
11. What is the end use of isolated DNA?
12. What is the importance of RNA?
13. How do you detect RNA in solution?
14. What are the difference between plasmid and chromosomal DNA?
15. What is single digestion and double digestion?
16. Mention properties of agarose.
17. What is electrophoresis?
18. What are the different types of electrophoresis?
19. What are the uses of ethidium bromide DNA separation?
20. What are the factors associated with nucleic acid mobility in electrophoresis?
21. What are the purposes of using buffer in electrophoresis unit?
22. What are the principles of DNA amplification?
23. Who invented PCR?
24. What is primer?
25. Explain the principle beyond lac operon induction.
26. Mention the principle of affinity chromatography in isolation of mRNA
27. What is blotting?
28. Western blotting is used to assess which macromolecule?
29. What are the methods available to transfer proteins to membrane?
30. What are the cautions required for preparing cell culture media?
31. How do you passage the cells?
32. What is trypsinization?
33. How do you assess viability of cells in culture?
34. What are the advantages and limitation of MTT assay?