Biochemical and Biophysical Techniques- Practical Manual

Course Code: CC-BTMP1001

2021

By

Dr. Diwakar Kumar Singh

Assistant Professor, Biotechnology

The Neotia University

Practical 1: Separation of nucleic acid by Agarose gel electrophoresis

Agarose is a polysaccharide extracted from seaweed and is used typically at concentrations 0.5 – 2% for electrophoresis of DNA and RNA. It forms a lattice with suitable pore size that allows the movement of nucleic acids to the positive electrode.

Materials and Reagents.

- Electrophoresis chamber with power supply, casting trays and combs
  An appropriate electrophoresis running buffer:
  1X TAE containing:
  0.04 M Tris-acetate (pH 7.6)
  0.001 M EDTA
  OR
  1XTBE containing:
  0.13 M Tris (pH 7.6)
  45 mM boric acid
  2.5 mM EDTA
- Loading buffer or prepared in 1X TAE or 1X TBE with the following:
  50% glycerol
  0.25% bromophenol blue
  0.25% xylene cynol
- Transilluminator

Procedure:

Preparation of agarose solution

- Measure the appropriate amount of agarose powder and add to 1X TAE buffer in a beaker or flask.
  Note: The amount of agarose will depend on the percentage of the gel required. The volume of agarose solution must be prepared according to the size of the gel tray used.
- Dissolve the agarose by heating on a magnetic hot plate. Alternatively, it can be heated in a microwave, swirling every minute till the agarose is completely dissolved.
- When the agarose is cooled to 50-60°C, add ethidium bromide (0.5 µg/mL final concentrations) to the solution. Another alternative is to immerse the gel in ethidium bromide solution after electrophoresis.
  IMPORTANT: Ethidium bromide is a carcinogenic agent. Always wear gloves and mask when handling ethidium bromide.
- While the agarose is cooling, prepare the gel tray for gel casting so that the agarose solution does not flow out before setting. This can be ensured by using tray dams, flexicaster or sealing with traditional laboratory tape. Place the comb in the grooves and place the tray in an even horizontal position.

- Pour the agarose solution slowly so that it is evenly distributed on the tray with no air bubbles trapped in the gel. Allow the gel to solidify at room temperature.
- Remove the combs and transfer the gel with the tray to the main tank and fill with 1X electrophoresis buffer until the gel is just covered with buffer.
- DNA sample is prepared with tracking dye.
- Load the samples carefully into the wells using pipettes. A suitable marker containing nucleic acid fragments of various sizes may also be loaded in same gel.
- Place the lid on the tank and connect to the power supply. The typical voltage to run the samples in agarose gels is 90 - 150 V.
- After electrophoresis, transfer the gel to UV transilluminator and acquire the image of the gel. The samples will appear as bright bands.

Practical 2: Separation of protein by poly acrylamide gel electrophoresis (PAGE).

Polyacrylamide gels are formed by the reaction of acrylamide and bis-acrylamide (N,N'-methylenebisacrylamide) that results in highly cross-linked gel matrix. Acrylamide gels can separate DNA fragments that differ by even 0.2% in length. While proteins must be denatured by SDS before separation on polyacrylamide.

Materials and reagents

- 30% polyacrylamide solution (filter through 0.45 mM filter and store in the dark at 4°C):
  29 g Acrylamide
  1 g Bis-acrylamide
  100 mL double-distilled water
- 10% Ammonium persulphate solution
- TEMED
- Electrophoresis buffer:
  5X gel loading buffer:
  80% glycerol 75%
  Bromophenol blue 0.25%
  Xylene cynol 0.25%
  1M Tris (pH 7.4) 10 mM
  5 M NaCl 10 mM
  0.5 M EDTA 10 mM
  10% SDS - 0.1%
- Geldoc System
- CBR stain/ Silver stain
- Vertical electrophoresis chamber with power supply, glass plates, spacers and combs
- Staining solution
- Destaining solution

Procedure

- Clean the glass plates and spacers of the gel casting unit with deionized water and ethanol.
- Assemble the plates with the spacers on a stable surface.
- Prepare gel solution using the following volumes depending on the percentage of gel required. (TEMED must be the last ingredient added)
- Pour the gel solution in the plates assembled with spacers. Insert the comb immediately ensuring no air bubbles are trapped in the gel or near the wells. Allow the gel to set for about 30-60 min at room temperature.
  The polymerized gel may be wrapped in Saran wrap and stored at 4°C for future use.
- When ready to perform electrotrophoresis, mount the plates with gel in the apparatus and fill the chambers with 1X electrophoresis buffer. It is recommended to perform a prerun for about 10 min at 5 V/cm.
- Prepare the samples by mixing 1 µL of sample with 1X-gel loading buffer. Load the samples carefully into the wells without introducing any air bubbles.
- Large gels may be run ~70 V for 8-12 h or 125-150 V for 3-5 h or until the dye front approaches the bottom of the gel. Ensure that there is no excessive heating. Alternatively, the samples may be run at a higher voltage in a cold room.
- Pry open the glass plates with a spatula, separate the upper glass plate. Stain the gel while still attached to the lower glass plate with suitable dyes for 10 -20 min.
- Soak the gel on the glass plate in distilled water for 5 min to remove it smoothly.
- Transfer the gel into staining solution for 30 minutes in shaking condition.
- Remove the stain and destain immediately with suitable solution.
- The samples will appear as bright bands can be captured in suitable instruments for further analysis.

Practical 3: Separation of amino acids by paper chromatography (Ascending).

Chromatography is used to separate mixtures of substances into their individual components. All forms of chromatography work on the same principle. They all have basic requirements of stationary phase (a solid or a liquid supported on a solid) and a mobile phase (a liquid or a gas). The mobile phase flows through the stationary phase and carries the components of the mixture with it. Different components travel at different rates based on their affinities toward stationary phase and mobile phase.In paper chromatography, the stationary phase is a very uniform adsorbent paper. The mobile phase is a suitable liquid solvent or mixture of solvents.

Material and Reagents:

- Glass beakers
- Whatmann filter paper
- Petridishes

- Measuring cylinder
- Developing chamber
- Capillary tubes etc.
- Chemicals: n-butanol Glacial acetic acid Distilled water (4:1:5) Amino acids (Tryptophan and threonine) Ninhydrin reagents.

Procedures:

Solvents system and its preparation methods

1. n-butanol and water are taken in 4:5 ratios in a conical flask and allow it to saturate for 24 hours.
2. By using separating funnel separate nbutanol and water.
3. The saturated n-butanol and Glacial acetic acid are taken in the ratio of 4:1 which can be used as a solvent system (or) mobile phase.
4. Ascending paper chromatography, the procedure for ascending paper chromatography method is quite simple as compared to other methods of chromatography.
5. The chromatography paper is cut into rectangular strips and marks a line on the paper with pencil at about 2 cm from the bottom.
6. With the help of capillary tube, the samples are applied at different points on the starting line
7. Now, place the chromatography paper in the developing chamber, which contains the mobile phase.
8. While placing the paper, it is important that the solvent level should not reach the starting line or the sample spots and paper shouldn't touch the walls of the developing chamber.
9. After sometime the solvent rises up the paper or the stationary phase by capillary action and dissolves the sample.
10. The components of the sample move along with the solvent in upward direction.
11. Check if the solvent has reached near the top level of chromatography paper.
12. The paper is removed when it reaches the top and marked the level with pencil.
13. This level (or) height is called the "solvent front". By using UV light, ninhydrin or iodine vapors examined the different spots of varied colors. Each spot represents a specific component of the sample.

Practical 4: Separation of amino acids by Thin layer chromatography.

Chromatography is a process used to separate a mixture of different substances back into their individual forms. For example, if you mixed red, yellow, green, and blue food coloring together chromatography could be used to separate them back into their individual colors. Thin Layer Chromatography (TLC) is used to separate solids from a liquid. The most common use is to separate amino acids from a liquid and each other. A spot of the sample is placed on a sheet of glass treated with an absorbent substance. The glass is then placed in a solvent that will travel up the absorbent surface and cause the solid to move out of the liquid with it. Different solids will move different distances on the sheet, but the distance will remain constant no matter how many

times chromatography is done. This distance is calculated into an amount called the Rf value, which can be used to determine the identity of the substance.

Material and Reagents:

- Chromatography plate
- Capillary tubes
- Amino acid solution A
- Amino acid solution B
- Chromatography solvent
- Hinge-topped vial
- Ruler UV black light
- Phenylalanine solution
- Graduated cylinder
- Paper towels
- Calculator

Procedure:

1. Obtain a TLC plate. Avoid touching the coated surface, since fingerprints can leave significant quantities of protein and therefore ruin them.
2. Draw a light pencil line about 1 cm from the bottom of the plate.
3. Evenly space 3 marks across the pencil line. These will be the places where you spot your amino acid samples on the plate. Label each mark at the bottom of the plate–P for the phenylalanine solution, A for amino acid solution A and B for amino acid solution B–so that you can identify them later.
4. Take your plate to the phenylalanine solution. Place the tip of the capillary tube into the phenylalanine solution and place the tip on the first mark labeled P. The spot should be small: more is not better! This step is very sensitive and large spots lead to incorrect results.
5. Allow the sheet to dry for 15-20 seconds and spot-mark P with the phenylalanine solution capillary tube again.
6. Spot a small sample of amino acid solution onto each mark with a capillary tube. Spots should be small: more is not better here! The method is very sensitive. Large spots lead to imprecise results.
7. Measure out 2 mL of chromatography solvent and CAREFULLY pour it into the bottom of the hinge-topped vial. Try to not allow the solvent to run down the sides of the vial.
8. Place the end of the TLC plate with the P, A, and B spots into the hinge-topped vial. MAKE SURE THAT THE LINE AND SPOTS DO NOT TOUCH THE SOLVENT!
9. Close the top on the vial to prevent the solvent from evaporating.
10. Watch the chromatography plate closely as the solvent moves up the strip. If the solvent reaches the very top before it is stopped, the experiment is invalid because an Rf value cannot be determined.

11. You will not see the amino acids moving on the plate since they are colorless. We will use a UV black light to see them.
12. Allow the solvent to move until it gets approximately 1 cm from the top of the plate. Remove the plate and place it flat on the paper towel to dry. If there is not enough time to allow the solvent to move that far, remove the plate regardless of the distance traveled since Rf values can still be calculated.
13. Make a mark in pencil on the strip where the solvent was stopped.
14. Pour any leftover solvent in the vial back into the chromatography solvent bottle.
15. Place the TLC plate under a black light source. The black light source should make any amino acids visible. Using the pencil, make a mark on the plate where the amino acids present in any of the samples stopped moving.
16. Measure the distance in millimeters from the start (pencil mark at the bottom of the strip) to the end of the solvent movement.
17. Measure the distance in millimeters from the starting point to where each of the amino acids stopped.
18. Calculate the Rf values for each amino acid and analyzed results

Practical 5: Separation of amino acids by paper chromatography (Descending).

NOTE: It will be the same as ascending chromatography but the only difference in mobility of solvent. It moves as opposite in ascending.