Unit 13

Methods in Biology

Unit Map

13.A Molecular biology and recombinant DNA methods

13.A.1 Isolation and purification of RNA and DNA

Isolation and purification of nucleic acids are very important for any molecular biology experiment. It is the starting material for all types of molecular biology experiments like restriction digestion, cloning, PCR, DNA-libraries and sequencing. By manipulating this extracted DNA or RNA and new DNA with different characteristics can be obtained. Genetic engineering process relies on DNA, RNA and proteins only. The basic process of extracting DNA involves the release of DNA from the cells, purification of the DNA to be used in the experiments. The pH of all solutions is maintained at pH-8.0 throughout the extraction procedure.

The isolation and purification of DNA mainly include four steps

i. Cell lysis
ii. Enzymatic treatment
iii. Purification of DNA/RNA
iv. Quantification of DNA or RNA

i. Cells lysis Cell lysis is needed in order to release their components from the cells. The nature of the treatment will vary widely according to the cell type. For cell lysis we need a culture of bacteria or yeast cells of extracting DNA which is homogenized so that individual cells can be lysed. The cell walls present in bacteria which are needed to be lysed to release the cell content is carried out by using lysozyme. The other material which can be used is EDTA and SDS detergent. EDTA acts as chelating agent, and chelates divalent cations which act as co-factor for enzymes. SDS an anionic detergents break the di-sulphide binds and helps the membrane proteins to degrade. In case of plants the cell walls are degraded either by enzymatic treatment like use of pectinase, cellulose etc. The alternate method of breaking the cell wall of plants and fungi is by using liquid N_2 (liquid Nitrogen) and crushing/homogenizing the cells in mortar-pestle. Further cell lysis is carried out by treatment with mild anionic detergent CTAB (Cetyl Trimethyl Amino Bromine). β- mercaptoethanol can be used to break the thio bonds.

ii. Enzymatic treatment Removal of RNA from a mixture of nucleic acids is easily achieved by treatment with RNase which is a very heat stable enzyme. Protein contamination can be removed by digestion with a proteolytic enzyme such as proteinase K.

iii. Purification of nucleic acids

- **Phenol chloroform extraction** To get pure DNA solution we need to remove proteins and other impurities. This is achieved by extraction with phenol or a mixture of phenol and chloroform. Phenol and chloroform are immiscible with water. When the mixture is vigorously agitated, the proteins will be denatured; lipids and carbohydrates solubilize in the organic solvent and get precipitated at the interphase, when centrifugal at high-speed (15000 rpm). The aqueous phase containing the soluble DNA for the top layer and the solvent forms the bottom phase.
- **Alcohol precipitation** After phenol extraction the sample which is obtained by above extraction will be free from protein impurities and will be much diluted so to concentrate the sample alcohol precipitation is done by precipitating nucleic acids using chilled ethanol or isopropanol. A small vol (1/10) of 3 M Sodium Acetate solutions is added before precipitation step. The acetate ions help form the network and thus enhance precipitation. On addition of alcohol a precipitate will appear which can then be collected at the bottom of the test tube by centrifugation.
- **Centrifugation** As mentioned in above paragraph centrifugation is used in DNA purification for separation of cellular debris from solution and for recovering precipitated nucleic acids. Other method which can be used is cesium gradient centrifugation for separation of plasmid DNA from bacterial genomic DNA, or of RNA from DNA. Sucrose gradient can also be used for size selection of large DNA fragments when constructing genomic library.
- **Plasmid Extraction** Plasmids are extracted by alkali denaturation method in which, bacterial cells with the desired plasmid are lysed under alkaline conditions and the crude lysate is purified using centrifugation. Three buffer solutions are mainly used suspension buffer, neutralization buffer and lysis buffer. Suspension buffer contains glucose, EDTA and Tris HCl buffer. Glucose maintains osmotic pressure and EDTA chelates divalent ions like Mg^{2+}, Ca^{2+} which prevents DNases from damaging the plasmid and also helps in destabilizing the cell wall. Lysis buffer contains NaOH and SDS. NaOH helps to break cell wall and disrupts hydrogen bonding between DNA bases converting dsDNA including genomic DNA and plasmid DNA into ssDNA. SDS solublizes the phospholipids and protein component of bacterial cell membrane leading to lysis and release of cell content to obtain plasma. Neutralization buffer contains potassium acetate, glacial acetic acid and water. Potassium acetate decreases alkalinity of mixture. The potassium acetate also precipitates the SDS from solution, along with the cellular debris. The *E. coli* chromosomal DNA, a

partially renatured tangle at this step, is also trapped in the precipitate. The plasmid DNA remains in solution. Acetic acid neutralizes the pH, allowing the DNA strands to renature.

- **Column purification** Mainly two types of chromatography can be done for purification of nucleic acids like size exclusion chromatography and affinity chromatography. In size exclusion chromatography sample mix is passed through a matrix of small porous beads. Smaller molecules such as salts and some nucleotides will enter the beads whereas larger molecules such as longer nucleic acid will pass right through the column. In affinity chromatography the macromolecule will bind to resins of column. The resins could be an anionic resins or oligo-dT sequences which specifically bind to the poly-A tail of eukaryotic mRNA molecules. In both chromatography cases undesirable molecules can be washed from the column and elution of nucleic acid bound to column can be eluted out by changing pH conditions or other.
- Quantitation by spectrophotometer: Both quality and quantity of DNA

13.A.2 Different separation methods

Chromatography It is an analytical process for the separation of mixture which involves passing of a sample (analyte solute) in the mobile phase; often in a stream of solvent, through the stationary phase. Some form of material that will provide resistance by virtue of chemical interaction between components of sample and the material.
Chromatography is defined as a separation technique based on partition or distribution of a sample (solute) between a mobile phase and a stationary phase.

Stationary phase May be a solid, gel, liquid or a solid / liquid mixture immobilized.
Mobile Phase May be liquid / gaseous for which is passed over the stationary phase after the mixture of analytes to be separated has been applied to the stationary phase.
The basis of all the chromatography in the distribution or Kd (Patton cost) and describes the way in and the compound distributes between two immiscible phase.
For such two phase A and B K_d is

$$\text{Conc. in phase A} \xrightarrow{K\alpha^2} \text{Conc. in phase B}$$

It is widely used because of following advantages

- Very small quantities of substances can be analyzed quantitatively and qualitatively. Equipment is very simple.
- Actual operation is fairly simple and no special skill is required.
- The results are remarkable reproducible.

The basic technique

- **Stationary Phase** absorbent.
- **Mobile Phase** Gas/liquid moves the sample through a region containing solid/liquid.
- The molecular species separate as they differ in their distribution between two phases and it depends on driving force and retarding force.

➢ **HPLC High Performance Length Chromatography**
It is a chromatographic technique that can separate a mixture of compounds and identify, quantify and purify the individual components of mixture.

- **Principle** Organic solvents are used as the mobile phase. The analyte is first dissolved in a solvent and then force it through a chromatographic column under high pressure. In the column, mixture is resolved into its components. The stationary phase is defined as the immobile packing material in the column.
  As the analyte is forced under high pressure, it spends less time in the column, leading to narrower phase and hence better selectivity and better sensitivity. Often, a gradient, over time in the solvent composition passing through the column is used to separate analyte mixtures.
- **Stationary Phase** Three forms are available, based on a rigid solid as opposed to gel, structure as the material need to withstand high pressures generated in the column. All forms are spherical particles of a uniform size to minimize spare for diffusion and hence brand broadening to occur.
  Three forms are
  i. **Microporous** ramify through the particles and are generally 5-10 nm in diameter.
  ii. **Pellicular** In which porous particles are coated onto an inert solid concentration such as a glass bead of bout 40mm in diameter.
  iii. **Bonded** In which stationary phase is chemically bonded onto on inert support (Silica).

- **Mobile Phase** The choice of mobile phase depends on the separation being carried out.
  Some common properties are
  - **Purity** Very high purity solvents with no particulate matter. Suspended particles tend to plug the column and chemical impurities produce sporous peak in detector. To purify laser grade solvent multiple filter is used.
  - **Low Viscosity** High viscous solution are avoided as they require longer time to pass through the column which results in peak broadening, power resolution and higher pressure to force solvent through the column.
  - **Chemical Inertness** Mobile phase must not react with any sample.
  - **Degassing** It should be degassed to avoid air bubbles in column or detector and partially block the column and is done by warming, storing, vacuum ultrasonication.

➢ **Ion Exchange chromatography (IEC)**

- **Principle** It relies on the attraction between opposite charged particles. Many biological materials like amino acids and proteins have ionisable groups that may carry a net positive or net negative charge. IEC is frequently chosen for separation and purification of proteins, peptides, nucleic acids, polynucleotide and other charged molecules mainly because of high resolving power and high capacity.
  Two types of Ion exchanges
  i. **Cation** Passes negatively charged groups and attracts positively charged cation. It is also called acidic ion exchanges because their negative charges result from ionisation of acidic groups.
  ii. **Anion** has positively charge groups and attracts negatively charged anions. It is also called as basic ion exchangers as negative charges results from association of protons with basic groups.
  Ion exchange much consists of five main steps
  i. **Diffusion of ion to the exchanger surface** This occurs very quickly in homogenous solutions.
  ii. **Diffusion of ion through the matrix structure of the exchanger to the exchanger site** It is dependent on degree of cross linkage of exchanger and concentration of soln. This process is thought to be a feature that controls the rate of whole ion exchange process.
  iii. **Exchange of ions at the exchange site** It occurs instantaneously and is an equilibrium process.
  Cation exchanger
  RSO_3^- --- $Na^+ + N^+ H_3 R^1 \leftrightarrow$ RSO_3^--- $N^+ H_3 R^1 + Na^+$
  Bound ion Exchanged ion

  Anion Exchanger
  $(R)_4 N^+$ ---- $Cl^- + {}^-OOCR' \leftrightarrow (R)_4 N^+$ ---- $^-OOCR' + Cl^-$

  The more highly charged the ionized molecules to be exchanged, lighter it binds to the exchanger and the less readily it is displayed by other ions.
  iv. **Diffusion of the exchanged ion through the exchanger to the surface.**
  v. **Selective adsorption by the eluent and diffusion of the molecule into external eluent** Selective desorption of the bound ion is achieved by changes in pH and/or ionic concentration or by affinity elution.

- **Material** Matrices used include polystyrene, cellulose and agarose. Final ionic groups include SO_3^- and $-N^+R_3$. Ion exchangers are stable upto 60^0C and separation is carried out within these temps because very high temp decreases viscosity of mobile phase and thereby increasing efficiency of separation.
- **Applications** Ion exchange chromatography is prominently used as preparatory chromatography to isolate a desired compound from mixture. Hence the applications are meant to obtain pure compounds.
  - For deionisation and softening of water.
  - Purification of solution to keep them ion free.
  - In biochemistry for separation of drugs and metabolites from blood, urine etc.
  - Separation of organic mixture of acidic and basic compounds.
  - For extraction of enzymes from tissues.
- **Uses**
  Ion exchange chromatography has many uses including
  - Separation of proteins from foods, for example, to investigate the effects of individual food components on health this type of analysis is used in nutrition research.
  - Separation of high value proteins from substances.

- **Limitations**
  - Costly equipment and more expensive chemicals
  - Turbidity should be below 10ppm.

➢ **Affinity chromatography**

It does not rely on difference in physical properties of molecules to be separated. It was originally developed for purification of enzymes, but exceeded for nucleic acids, nucleotides, immunoglobins, and membrane receptors and even to whole cells and cell fragments. This technique requires that the material to be isolated is capable of binding reversibly to a specific ligand that is attached to an insoluble matrix.

$$M + L \underset{K_{-1}}{\overset{K_1}{\leftrightarrow}} ML$$

Macromolecule Ligand complex

When a complex mixture containing the specific compound to be purified is added to immobilize ligand, generally contained in a conventional chromatography column, only that compound will bind to the ligand. All other compounds can then be washed away and the compound is subsequently recovered from the ligand.

It requires preliminary knowledge of the structure and biological specificity of the compound to be purified so that separation conditions can be planned.

In case of enzyme a ligand can be S, IRI, RI or allosteric activator.

Matrices used

Cross linked dextrans, agarose, polyacrylamide, silica, porous glass, cellulose, and polystyrene.

Applications

- Many enzymes and other protein including receptor proteins and Igs have been purified by this technique.
- It can be used for nucleic acids also. m-RNA is isolated by selective hybrid poly (u)- sepharose 48 by exploiting its Poly A tail.
- Single stranded DNA can be used for isolating complementary DNA or RNA. It is performed by using SS DNA on nitro cellulose membrane.
- It is used for separation of mixture of cells into homogenous populations.

Uses

- It is specific and high degree of purity is obtained.
- The absorbed material is separated from proteolytic enzyme and can be stabilized by ligand binding at active site.
- Adsorbent can be regenerated several times.

Limitation

- High cost
- Difficulties associated with scale up and high labour intensity

➢ **Paper Chromatography**

Principle The principle involved is partition chromatography where in the substances are distributed or partitioned between to liquid phases. One phase is the water which is held in pores of filter paper used and other phase is that of mobile phase which moves over the paper. The compounds in the mixture get separated due to differences in their affinity towards water (in stationary phase) and mobile phase solvents during the movement of mobile phase under the capillary action of pores in the paper.

The principle can also be adsorption chromatography between solid and liquid phases, where in the stationary phase is the solid surface of paper and the liquid phase is of mobile phase. But most of the applications of paper chromatography work on the principle of partition chromatography i.e. partitioned between to liquid phases.

Material used

Chromatography paper (stationary phase) Coffee filter or blotting paper or paper towel may also work. Different papers have different properties and will separate substances more or less successfully. Chromatography chamber. 1 liter glass jar

- Paper clip.
- Skewer (or pencil).