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1. ISOLATION, PURIFICATION AND ANALYSIS OF DNA (GENOMIC AND PLASMID) AND RNA

Isolation of genomic DNA:

The knowledge of gene isolation was developed after gaining the concept of physical and chemical characteristics of described DNA fragments: their sizes, shapes, and conformation can aid in the selection of methods used to isolate and purify those segments.

Plants provide several special challenges for researchers interested in recombinant DNA research. No other class of living organisms has three separate genomes to analyze, a nuclear genome containing high molecular weight linear chromosomes, a circular mitochondrial genome, and a circular chloroplast genome. Intact, high molecular weight (>150 kbp) plant nuclear gDNA is used to construct genomic DNA libraries and to probe the plant genome for the presence of DNA markers, such as randomly amplified polymorphic DNAs (RAPDs), and restriction fragment length polymorphisms (RFLPs). Chloroplast cpDNA and mitochondrial mDNA, which are thought to incur fewer structural changes over time, are often used to study plant systematics. They are also used to study in vitro synthesis and assembly of organelle proteins.

DNA extraction from plant tissue can vary depending on the material used. Essentially any mechanical means of breaking down the cell wall and membranes to allow access to nuclear material, without its degradation is required. For this, usually an initial grinding stage with liquid nitrogen is employed to break down cell wall material and allow access to DNA while harmful cellular enzymes and chemicals remain inactivated. Once the tissue has been sufficiently ground, it can then be resuspended in a suitable buffer, such as CTAB. In order to purify DNA, insoluble particulates are removed through centrifugation while soluble proteins and other material are separated through mixing with chloroform and centrifugation. DNA must then be precipitated from the aqueous phase and washed thoroughly to remove contaminating salts. The purified DNA is then resuspended and stored in TE buffer or sterile distilled water. This method has been shown to give intact genomic DNA from plant tissue. To check the quality of the extracted DNA, a sample is run on an agarose gel, stained with ethidium bromide, and visualised under UV light.

Steps are:

1. Grind 200 mg of plant tissue to a fine paste in approximately 500 µl of CTAB buffer.

2. Transfer CTAB/plant extract mixture to a microfuge tube. Incubate the CTAB/plant extract mixture for about 15 min at 55^{o}C in a recirculating water bath.

3. After incubation, spin the CTAB/plant extract mixture at 12000 g for 5 min to spin down cell debris. Transfer the supernatant to clean microfuge tubes.

4. To each tube add 250µl of Chloroform : Iso Amyl Alcohol (24:1) and mix the solution by inversion. After mixing, spin the tubes at 13000 rpm for 1 min.

5. Transfer the upper aqueous phase only (contains the DNA) to a clean microfuge tube.

6. To each tube add 50 µl of 7.5 M Ammonium Acetate followed by 500 µl of ice cold absolute ethanol.

7. Invert the tubes slowly several times to precipitate the DNA. Generally the DNA can be seen to precipitate out of solution. Alternatively the tubes can be placed for 1 hr at -20^{o}C after the addition of ethanol to precipitate the DNA.

8. Following precipitation, the DNA can be pipetted off by slowly rotating/spinning a tip in the cold solution. The precipitated DNA sticks to the pipette and is visible as a clear thick precipitate. To wash the DNA, transfer the precipitate into a microfuge tube containing 500µl of ice cold 70 % ethanol and slowly invert the tube.

9. After the wash, spin the DNA into a pellet by centrifuging at 13000 rpm for 1 min. Remove all the supernatant and allow the DNA pellet to dry (approximately 15 min). Do not allow the DNA to over dry or it will be hard to re-dissolve.

10. Resuspend the DNA in sterile DNase free water (approximately 50-400µl H_2O; the amount of water needed to dissolve the DNA can vary, depending on how much is isolated). RNaseA (10µg/ml) can be added to the water prior to dissolving the DNA to remove any RNA in the preparation (10 µl RNase-A in 10ml H_2O).

11. After resuspension, the DNA is incubated at 65^{o}C for 20 min to destroy any DNases that may be present and store at 4^{o}C.

12. Agarose gel electrophoresis of the DNA will show the integrity of the DNA, while spectrophotometry will give an indication of the concentration and cleanliness.

ANALYSIS OF DNA

Gel electrophoresis is one of the most basic but important tool used by the molecular biologists in study of DNA or its fragments. Gel electrophoresis technique is used:

i. To study the purity and intactness of DNA;

ii. To separate and identify the fragments;

iii. To analyze and characterize recombinant DNA molecules.

iv. To determine molecular weight of DNA fragments by running standard markers in parallel.

Gel electrophoresis is:

(a) A simple and rapid technique.

(b) It can resolve mixtures of DNA fragments that cannot be separated adequately by other methods like density gradient centrifugation.

(c) Location of DNA in gel can be determined directly by the use of fluorescent, intercalating dye like Ethidium bromide. Photographs can be taken of such developed gel. Upon UV irradiation orange fluorescence results.

(d) As little as 0.05 μg DNA can be detected.

Principle: When a molecule like DNA is placed in an electrical field, it will migrate to the appropriate electrode at a velocity or electrophoretic mobility proportional to:

(a) The field strength,

(b) The net charge on the molecule.

Since a non-reactive, stable medium is used to reduce convectional motion, the electrophoretic mobility is inversely proportional to the functional coefficient of a molecule. (Functional coefficient is a function of the size and shape of the molecule and viscosity of the medium). Therefore, a mixture of different molecules can be resolved electrophoretically on the basis of:

(a) Size of the molecule, which for most biological macromolecules refers to mass or length;

(b) The shape or conformation of the molecule;

(c) The magnitude of net charge on the molecule.

So when placed at the same position in an electrical field, these molecules will resolve into bands and migrate at different rates to different positions in the medium.

Theory: Under the physiological conditions, the phosphate groups in phospho-sugar backbone of nucleic acids are ionized. Polynucleotide chains of DNA and RNA are called 'Polyanions' and they will migrate to positive electrode (anode) when placed in an electrical field. Due to repetitive nature of phosphate sugar backbone, double stranded nucleic acids have roughly the same net charge to mass ratio and will migrate to anode at equal velocities

Migration of a molecule is inversely proportional to its frictional coefficient while frictional coefficient, is dependent on size and shape of the molecule But DNA molecule can assume different conformations (shapes) and this fact complicates electrophoretic analysis of DNA For example, a plasmid DNA such as pBR-322 can exist as:

(i) Super coiled (Form I),

(ii) Relaxed circle (Form II), and

(iii)Linear (Form III).

Compact super-coiled (SC) forms of plasmids migrate considerably faster than open circles (OC) and linear plasmid (L) migrates just ahead of the open circle.

When DNA fragments are electrophoresed heavier fragments remain closer to the loading point, where as the smaller ones move faster. Contamination with RNA if present, will result in a broad smear running at low molecular weight. RNA can be removed by the addition of RNase to sample. Contamination with the protein results in partial inhibition of restriction enzyme activity if digestion of DNA is being done. Protein contaminants can be removed by phenol-chloroform extraction and subsequent ethanol precipitation. There is a linear relationship between log of mobility and get concentration over a certain range of fragment sizes. So gel concentration must be chosen to separate the molecules in DNA population more effectively. Gels of 0.8% agarose are suitable for separating linear DNA molecules (0.5 to 10 kb in size). If marker fragments of known molecular weight of DNA are run in parallel with sample fragments, the direct comparison of movement of markers and unknown fragments gives rough but faster idea about molecular weight of unknown fragments. The log molecular weight of known marker fragments can be plotted against mobility. The resulting calibration curve can be used to determine molecular weight of unknown DNA fragment.

There is linear relationship between sedimentation coefficient, and electrophoretic mobility and mobility is also linear with $\sqrt{T}$ (Resolution is better with higher value of T). Electrophoretic behavior of nucleic acid is very complex and many models are suggested which employ a repetition mechanism (primary, secondary, biased etc.) and explain migration behavior, nucleic acid compositional effects, nucleic acid-gel interactions, electric field effects.

Gel material: Agarose or polyacrylamide is generally used for DNA separation by gel electrophoresis. Agarose polyacrylamide composite gels are used for separating high molecular weight RNAs. 0.5 % agarose addition gives mechanical strength to polyacrylamide gel. The gel may be a cylinder or slab, 10 cm. in length and 0.5 cm. in thickness. Horizontal slab gels are preferred because of ease of operations, loading and better support. Polyacrylamide gels are more commonly used now for DNA sequencing experiments. Fragments differing by only few basepairs can be separated better by polyacrylamide gel.

The polyacrylamide gels used for protein fractionation have pores which are too small for passage of large DNA molecules.

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