Restriction Fragment Length Polymorphism (RFLP)

Restriction enzymes are site-specific DNAses that cleave a DNA molecule whenever the recognition sequence, which is usually a 4-6 base palindrome, is present. Because of the enzyme's sequence specificity, digestion of a particular DNA results in a reproducible array of fragments. RFLP, or length differences in homologous fragments between different DNA, are caused by changes in the primary sequence of the DNA.

These length differences can be the result of:

a. A point mutation resulting in the loss or gain of restriction enzyme cut site

b. An insertion or deletion of DNA between two restriction enzyme cut sites

c. A deletion which overlaps a restriction enzyme site

d. A DNA rearrangement, where one end of the rearranged segment resides between two restriction enzyme sites. When such differences occur, they can be detected by DNA hybridization and used as molecular markers in fingerprinting or genetic studies.

The flow diagram of RFLP analysis is shown in Fig. 9.5:

Isolate DNA to be Analysed

↓ Choose a DNA isolation procedure that works for the species being analysed. The main requirements are that the DNA be of high average molecular weight and that it be pure enough to be cleaved with restriction enzymes.

Digest DNA with Restriction Enzyme

↓ Restriction enzymes are site-specific nucleases that reproducibly cleave DNA. Over 100 are currently available with many different sequence specificities.

Perform Agarose Gel Electrophoresis

↓ Agarose is a long chain polysaccharide that forms a mesh of molecules when it is melted and allowed to solidify. During electrophoresis this mesh acts as a molecular sieve for the restriction fragments, which are separated by size with the smallest fragments moving fastest through the gel.

Transfer DNA from Gel to Membrane

Transfer of the DNA from the gel to a membrane in a form suitable for hybridization analysis is known as Southern transfer or blotting. This step consists of:

— denaturation of the DNA to single strands by treatment with alkali

— transfer of the DNA to a membrane by capillary action and permanently fixing the DNA to the membrane by UV treatment and/or drying.

↓ During this step water and DNA move together from the gel to the stack of dry paper towels. However, since the DNA can't pass through the membrane, at the end of the transfer process the membrane contains all of the DNA originally in the gel in the exact spatial orientation that it had in the gel.

Hybridize with Labeled Probe

↓ DNA hybridization takes advantage of the fact that DNA is normally a double stranded molecule with the two strands having complementary base sequences and held together by relatively weak hydrogen bonds between the base pairs. Since the DNA on the membrane is single stranded, the parts of the DNA molecules that are involved in interstrand base pairing are exposed. When the membrane is incubated with probe under conditions that promote the formation of hydrogen bonds between complementary base pairs, the probe will hybridize, wherever it finds a DNA sequence on the membrane that is complementary to it.

Wash Membrane to Remove Unhybridized Probe

↓ After hybridization there will be probes stuck nonspecifically to the membrane as well as weakly hybridized to DNA fragments where short segments of homology occurred by chance. Both of these events cause unwanted background and they can be removed by high temperature washes in a low salt solution. Conditions are chosen so that true probe : target hybrids are stable but the nonspecifically bound probes are not.

Autoradiography

↓ At this point, the membrane has tiny amounts of radioactivity bound to it. This can be detected by placing a piece of photographic film in contact with the membrane, a process called autoradiography. The film will be exposed at the regions that are close to radioactivity. This exposed sheet of film, with its variously placed bands, is the raw data of an RFLP experiment.

Reuse of Membrane

↓ Membranes can usually be reused up to ten times. Before reuse any previously hybridized probe should be removed by denaturing the probe : target DNA hybrid. After prehybridization, the membrane is then ready for another round of hybridization.

Fig. 9.5. Flow diagram of RFLP analysis

Advantages of RFLP:

a. Present everywhere,

b. Mendelian inheritance,

c. Co-dominant expression,

d. No pleiotropic effects,

e. Independent of the environment,

f. Present at each developmental stage,

g. Long stability of cDNA probes,

h. Different loci may be identified by one probe,

i. Heterologous genes may be used as probes,

j. Any number of probes can be produced,

k. Probes are producible for coding and silent sequences,

l. Probes show the variability of flanking sequences,

m. Several characters can be screened in the same sample.