CHAPTER XIV
METHODS FOR STUDYING THE GENOME

1- PCR (Polymerase chain reaction)

. 2- RFLPs (restriction fragment length polymorphism).

3. DNA fingerprinting.

4.DNA sequencing.

2. Restriction Fragment Length Polymorphisms (RFLPs) as genetic marker:

That is the variations found within a species in the length of the DNA fragments generated from a particular DNA region by a specific restriction enzyme.

The RFPLs can be used as a genetic marker for study of many genetic traits.

For many genetic disorders the defective alleles have not been associated with any other gene that can serve as a marker.

Consequently, the location as well as linkage relationships of such a defective gene remain unknown.

Now methodology from molecular biology and recombinant DNA procedure is enabling to overcome this barrier.

In sickle-cell anemia a gene mutation can alter a specific restriction site within a gene.

Such a mutation, which alters a restriction site, can occur anywhere along the DNA.

These alterations of the restriction sites are inherited as codominant alleles and used as markers to follow the inheritance of genetic disorders from generation to another in an affected family.

If two individuals vary in a particular DNA sequence at a given site, a specific restriction enzyme will produce fragments of different sizes when it cuts the DNA from two sources.

PATTERN GENERATED DEPENDS MAINLY ON

- Differences in DNAs of selected strains
- Restriction enzymes used
- DNA probe employed for southern hybridization
- Point and frameshift mutations
- Differences in alleles for a particular sequence

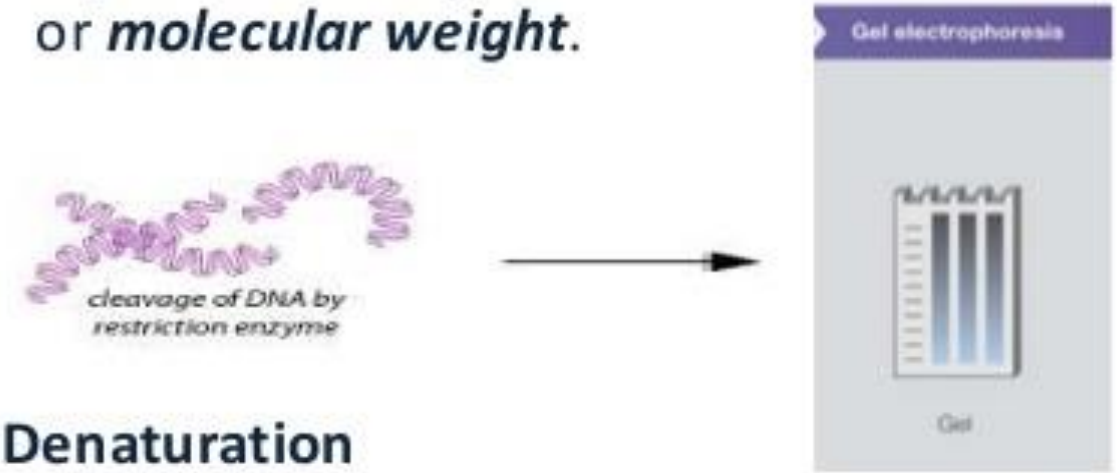
- **Step II: Restriction digest**

  The DNA in each sample is digested with the same restriction enzyme(s).

  The enzyme RE has specific restriction site on the DNA, so it cut DNA into fragments. Different size of fragments are generated along with the specific desired fragments.

- **Step III: Gel electrophoresis**

  The digested fragment are run in *polyacrylamide gel electrophoresis* or *Agarose gel electrophoresis* to separate the fragments on the basis of ***length*** or ***size*** or ***molecular weight***.



- **Step IV: Denaturation**

  The gel is placed in sodium hydroxide (NaOH) solution for denaturation so that single stranded DNA are formed.

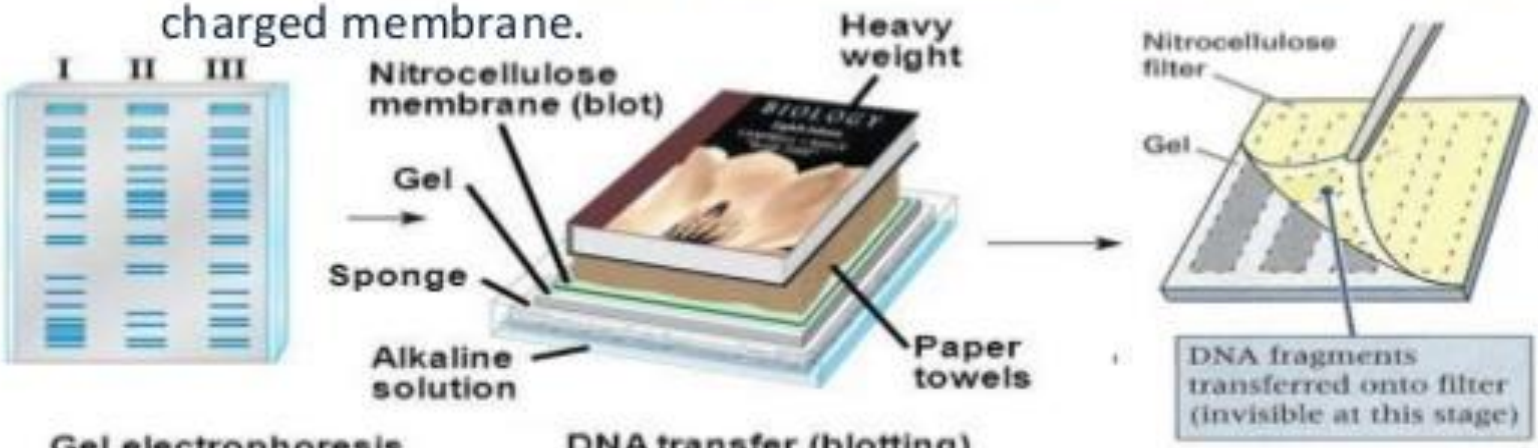
- **Step V: Blotting**

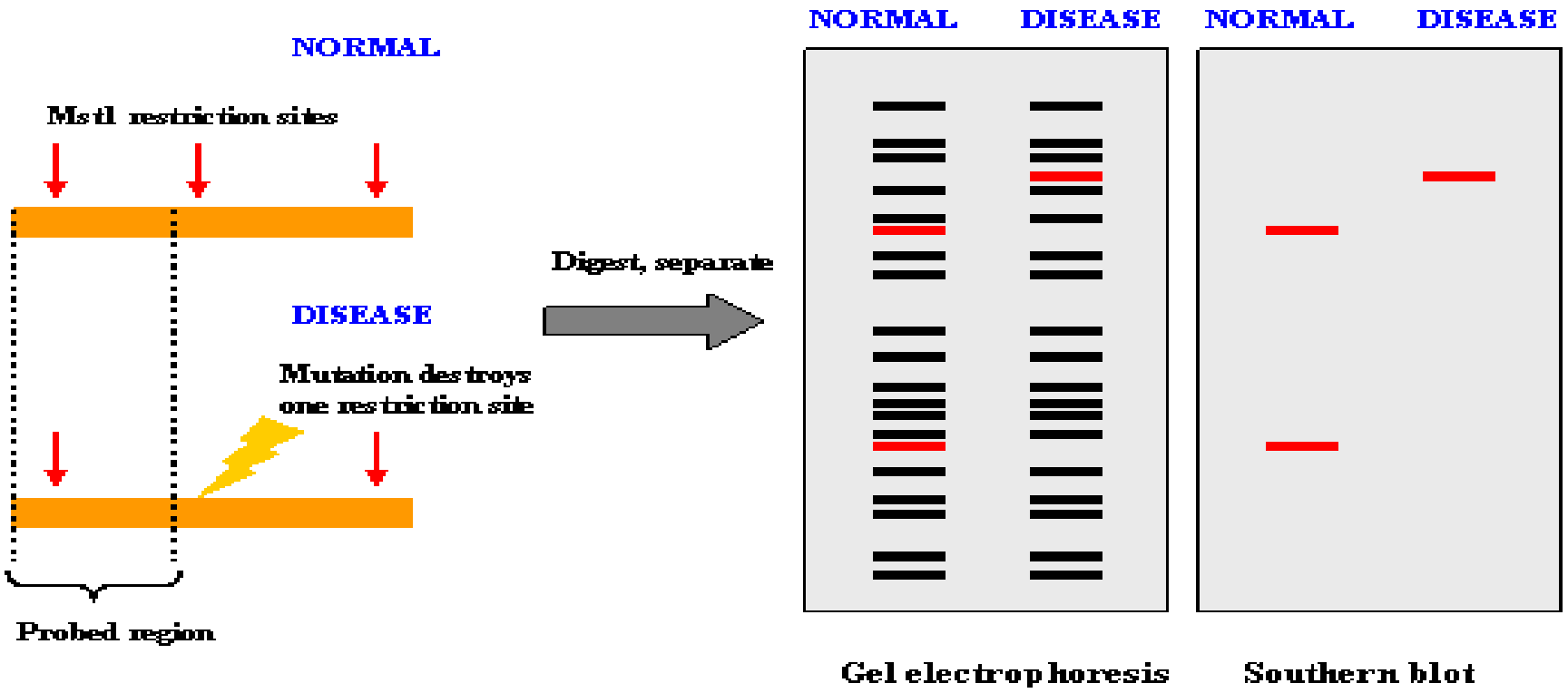
  The single stranded DNA obtained are transferred into charge membrane i.e. Nitrocellulose paper by the process called capillary blotting or electro-blotting.

- **Step VI: Baking and blocking**

  The nitrocellulose paper transferred with DNA is fixed by autoclaving.

  Then the membrane is blocked by using bovine serum albumin or casein to prevent binding of labeled probe nonspecifically to the charged membrane.




NORMAL
MstI restriction sites
DISEASE
Mutation destroys one restriction site
Probed region
Digest, separate
NORMAL
DISEASE
NORMAL
DISEASE
Gel electrophoresis
Southern blot

5. DNA fingerprinting:

A type of nucleotide variations, discovered in the mid 1980s, depends on variability in the length of repetitive DNA sequence clusters..

This and polymorphism in DNA led to the development of methods for identifying individuals and establishing degrees of genetic relatedness between individuals.

One of these techniques is called *DNA fingerprinting*.

5.a. Minisatellites and variable number tandem repeats (VNTRs)

One of the most useful forms of restriction fragment length polymorphism (RFLP) arises from variations in the number of tandemly repeated DNA sequences present between two restriction enzyme sites.

These sequences are minisatellites, or clusters of nucleotides from 2-100 nucleotides in length.

For examples, the base sequence GGGAAGGGAAGGGAAGGGAA, is composed of 4 tandem repeats of 5' nucleotide sequence GGAAG. Clusters of such sequences are widely dispersed in the human genome.

Typically, each repeat contains between 14-100 nucleotides and the number of repeats at each locus ranges from 2 to more than 100.

These loci are known as *variable number tandem repeats (VNTRs)*. The number of repeats at a given locus is a variable and each variation is a VNTR allele. Many loci have dozens of alleles each; as a result heterozygosity is common.

A pattern of bands is produced when the VNTR sequences are cut with restriction enzymes and visualized by *southern blotting*.

This pattern is one example of what is known as a ***DNA fingerprint.***

These patterns are equivalent of fingerprints because the pattern of bands is always the same for a given individual. The pattern varies from individual to individual.

DNA fingerprint analysis can be performed on very small sample of material (less than 60 μl of blood).

Analysis can also be performed using samples that are quite old (VNTR analysis has been performed on Egyptian mummies or 2400 years old). This increasing usefulness in legal cases.

5.b. Forensic application of fingerprints:

Since 1980, DNA fingerprints have been used as evidence in criminal trials in United States.

The method has also been used in a wide range of other applications including

1-Immigration cases

2-Disputes involving pure-breed dogs

3- paternity cases

4-Animal conservation studies

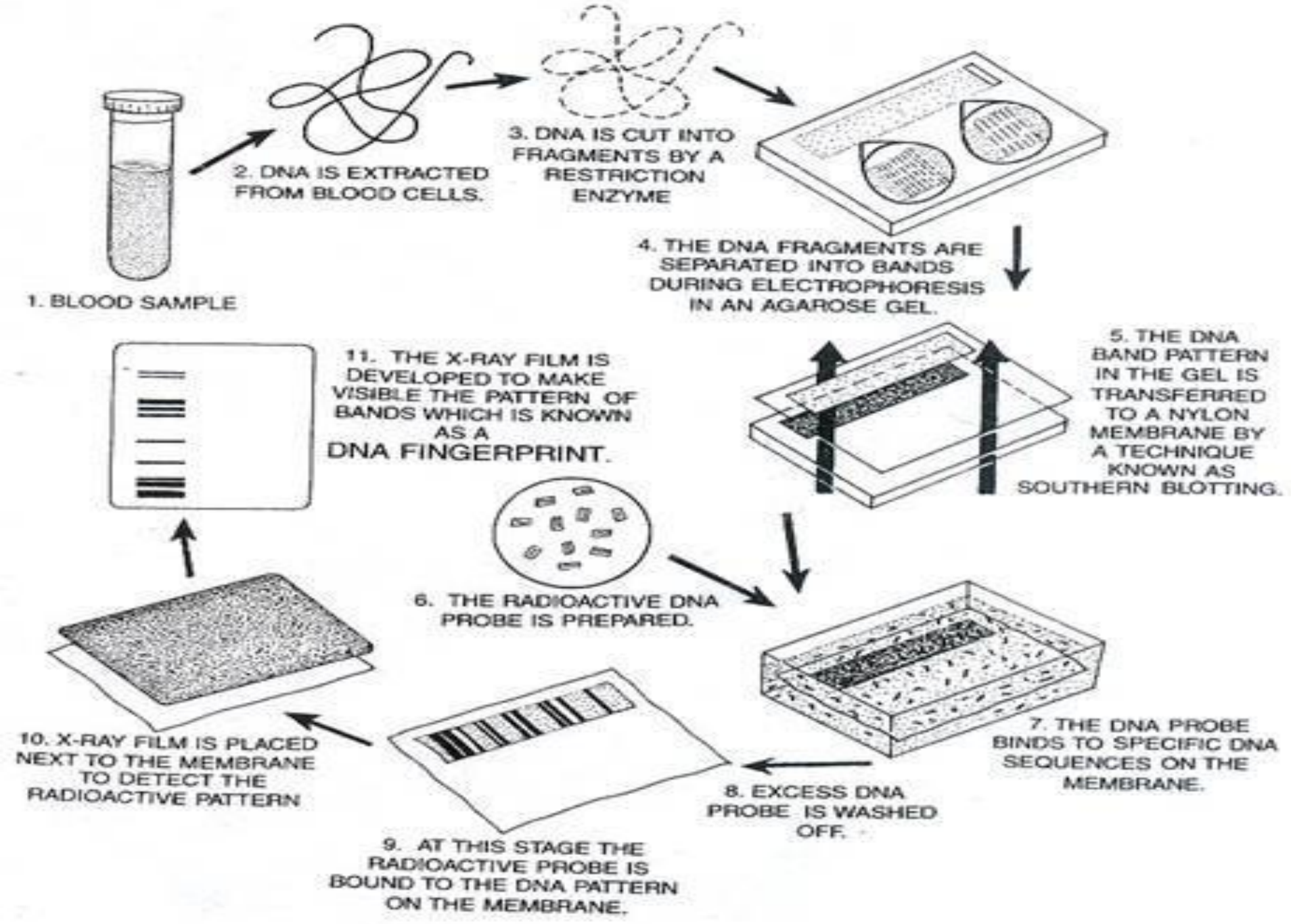
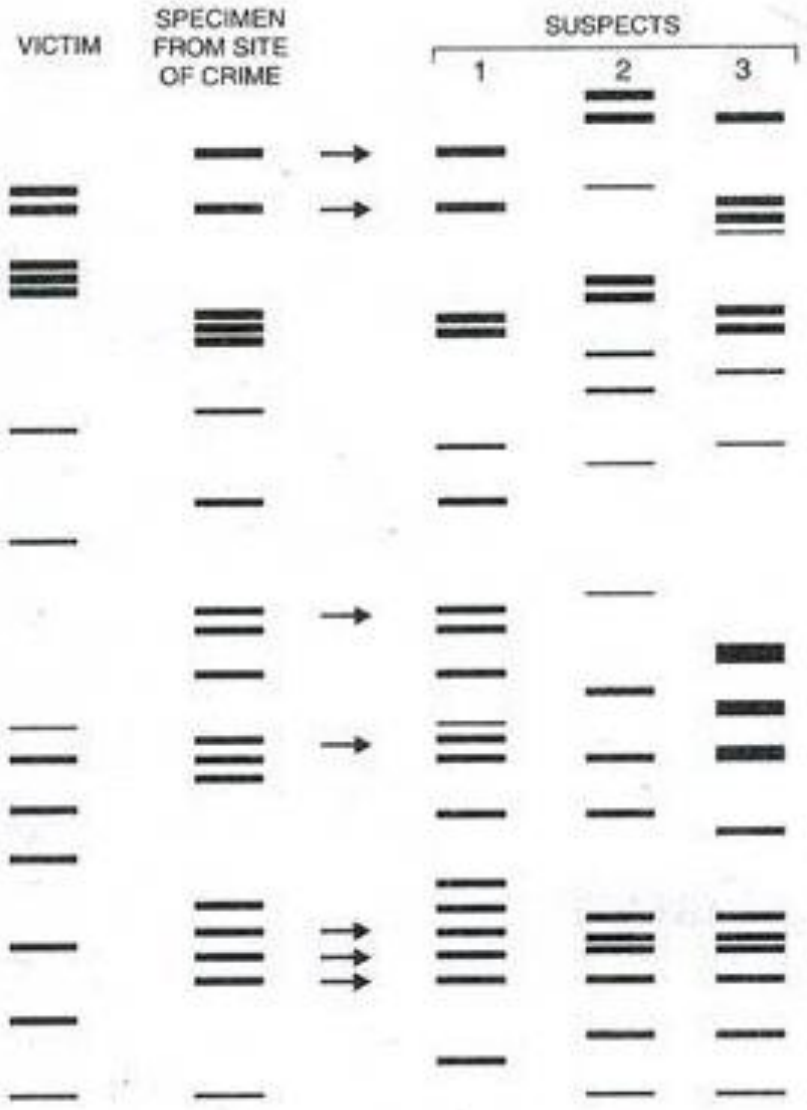
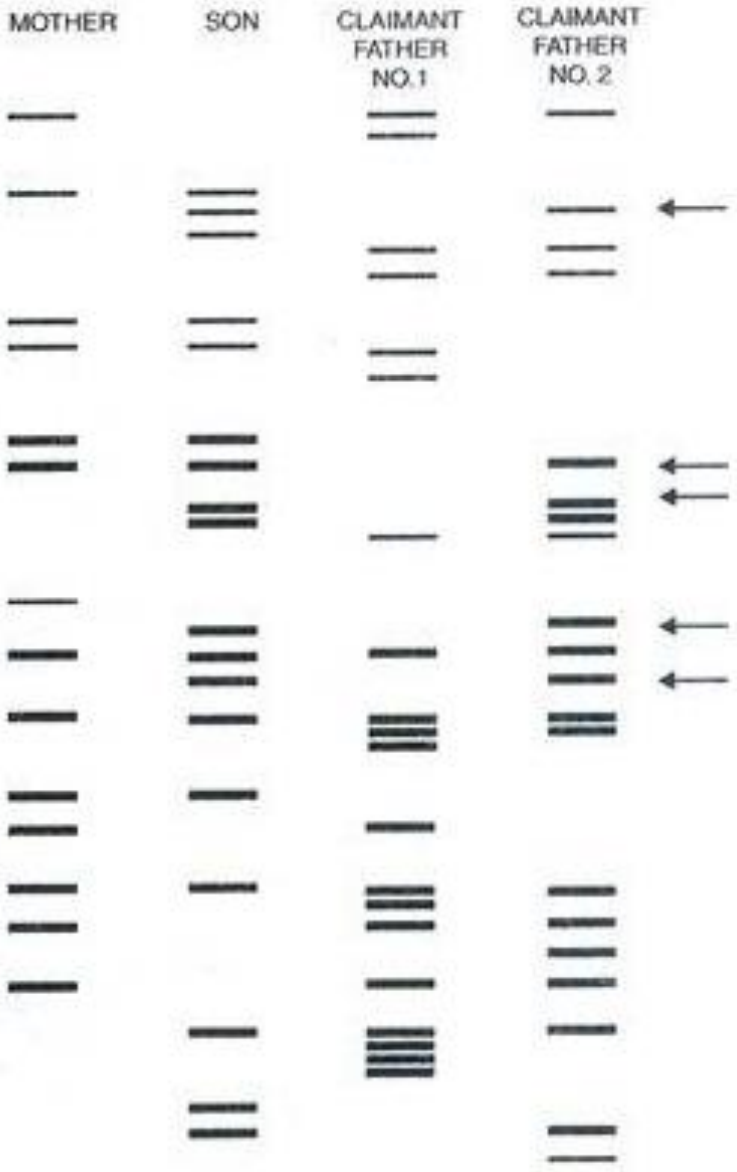


Fig. 6.40. The DNA Fingerprinting Process.



Identification of a criminal through DNA finger printing. Suspect number one is real culprit. as its VNTR bands are matching with specimen from site of crime.

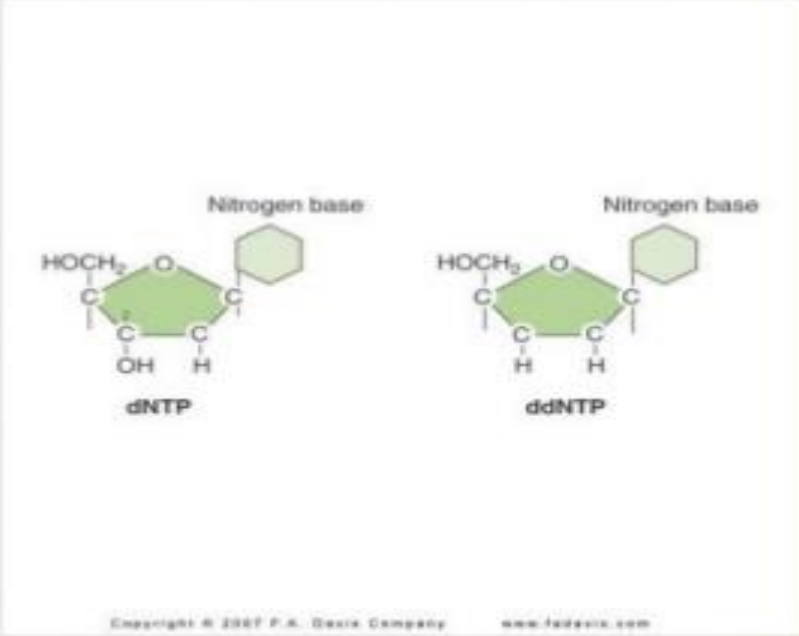


DNA fingerprints of mother under consideration, the child and two claimant fathers. Claimant father No. 2 proved to be the real/biological father.

4- DNAsequencing

REQUIREMENTS

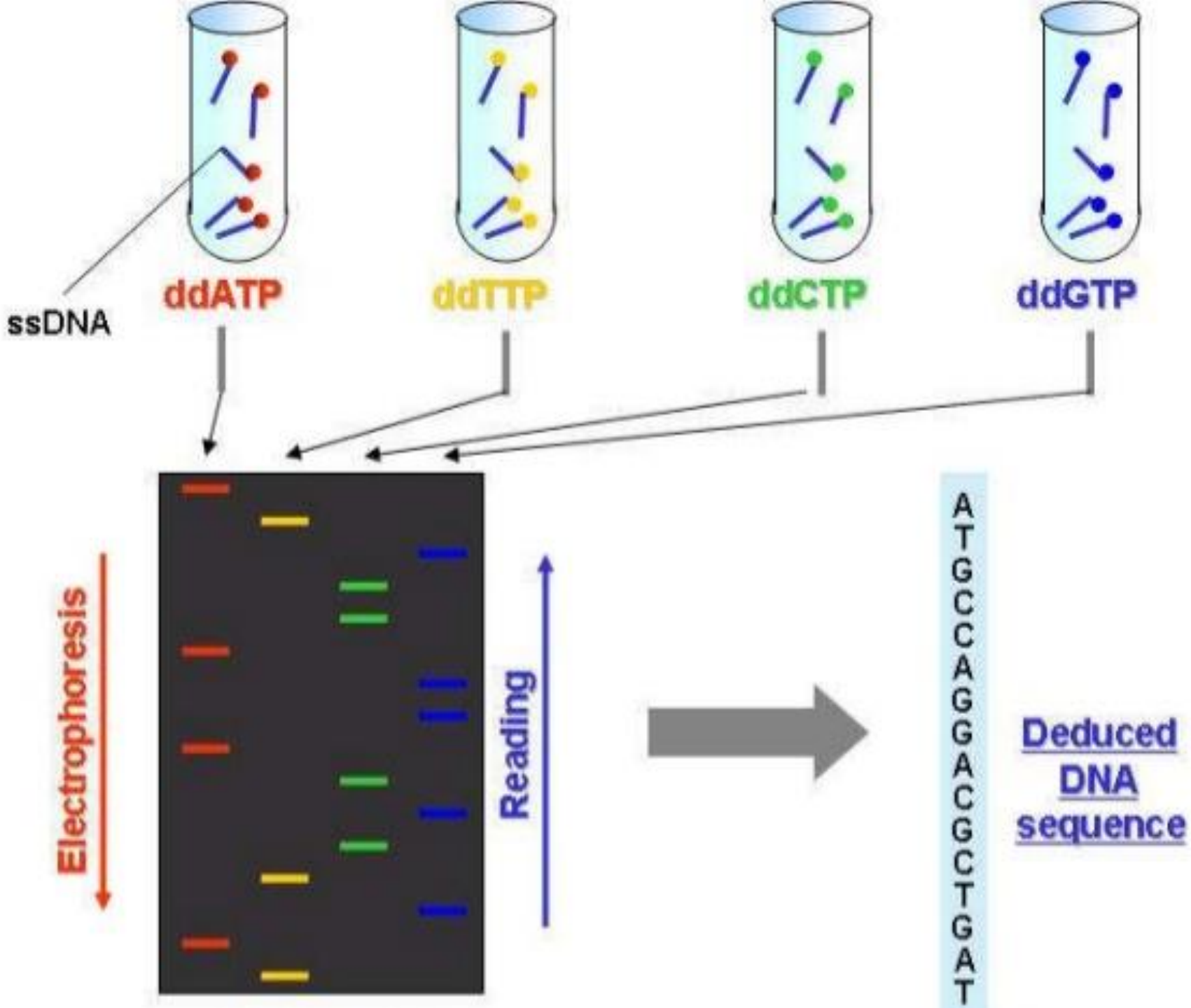
- Single Stranded template
- Primer
- DNA polymerase
- Di-Deoxynucleotide
- ✓ The 3'-OH group necessary for formation of the phosphodiester bond is missing in ddNTPs)
- ✓ Every nucleotide have its specific ddNTP form i.e., ddATP, ddGTP etc



PROCEDURE

- Steps:
  1. Denaturation
  2. Primer attachment and extension of bases
  3. Termination
  4. Gel electrophoresis

- The DNA template is treated with heat so that it becomes single stranded
- A short, single-stranded primer which is radioactively labelled is added to the end of the DNA template
- Add template DNA and primer in 4 Tubes.
- Now add ddNTPs In tubes in the way that single tube contain one type of ddNTP.
- Extension is start and band formed of various sizes.
- The fragments of DNA are separated by electrophoresis
- Overlap these sequences to find out sequence of Target DNA.


ssDNA
ddATP
ddTTP
ddCTP
ddGTP
Electrophoresis
Reading
ATGCCAGGACGCTGAT
Deduced DNA sequence

CHAPTER XV

INHERITED DISEASES OF BIOCHEMICAL ORIGIN

All simply inherited disorders and disease must have a biochemical origin.

However, until now, biochemical explanations have become possible for only a small proportion of inherited diseases

Gene mutation is the origin of all inherited disorders whose biochemical bases have been discovered.

The simplest alteration in polypeptide structure that can result from such a mutation is the substitution of only one amino acid for another at ant site along the polypeptide chain.

On the other hand, a mutation may lead to the complete failure of the cell to synthesize the polypeptide itself.

Whether the mutant polypeptide is unable to carry out its specific function properly, or if it is absent altogether, the end result is the same: there is a deficiency of the functional polypeptide.

Consequently, the physiological process in which the polypeptide is required becomes impaired.

I. Inborn Errors of Metabolism

A mutation in a gene specifying a polypeptide which acts as enzyme or as a part of an enzyme may result in a deficiency of that enzyme.

Consequently, a blockage occurs in the metabolic pathway at the point where the enzyme is required.

This way leads to faulty metabolism, accumulation of harmful metabolites etc. The diseases caused by such mutations are called ***inborn errors of metabolism.***

In general, enzymes are required in relatively small quantities. Accordingly, the deficiency in an enzyme which is controlled by an autosomal gene will not show in the heterozygote, as the normal allele will produce enough of the enzyme.

However, although no defect may be observed on the hetrozygous individual, yet the amount of the specific enzyme involved is less than in the normal homozygote.

Accordingly, it is possible in a number of such diseases to detect heterozygote carriers "heterozygote detection" by measuring (whenever possible) the particular enzyme activity in blood or other cell, which is expected to be only 50% of the normal level.

Now it is clear that:

Two alleles at one locus can show more than one type of gene action (e.g. complete and incomplete dominance) depending on the characteristics under consideration.

The following serves as example of inborn errors of metabolism in human being and in domestic animals.

a. Inherited Errors in the Metabolism of Phenylalanine:

The table presented below shows six different inborn diseases caused by seven autosomal recessive mutations leading to blockage in the metabolic pathway of phenylalanine at various points.

These include:

Normal pathway	Affected individual	Disorders
- Phenylalanine → Tyrosine	pp	Phenylketonuria Mental disorders
- Tyrosine → p-hydroxyphenyl pyruvic acid	tt	Tyrosinosis
- p-hydroxyphenyl pyruvic acid → 2, 5 dihydroxy phenylpyruvic acid	t't'	Tyrosinemia
- 2, 5 dihydroxy phenylpyruvic acid → homogentisic acid	hh	Alkaptonuria
Tyrosine → Thyroxine	cc	Cretinism
Tyrosine → DOPA (3, 4 dihydroxy phenyl alanine)	aa	Albinism
DOPA → melanin	a'a'	Albinism

b. Congenital Hemolytic Anaemia due to Pyruvate Kinase Deficiency:

This disease of dogs (Basengi & Beagle breeds) is due to a deficiency of the enzyme pyruvate kinase (PK) which is needed for anaerobic glycolysis (conversion of glycogen to glucose).

This leads to decreased glucose utilization and other deficiencies, mainly a decreased level of adenosine triphosphate (ATP).

Since ATP is major source of enzyme for erythrocytes, its decrease leads to a decreased life span of red blood cells and hence congenital Hemolytic anaemia.

Other symptoms include those associated with lack of enzyme, like weakness, excessive sleeping, etc. (this is a good example of pleiotropy, i.e. more than one effect of a single gene).

Heterozygous dogs (Dd) do not exhibit any abnormal external symptoms. However, they can be detected by measuring their PK activity which is approximately 50% of the normal value.

The reduced PK value is the result of the activity of the single D gene (which produces normal active PK), while the defective d gene produces very little or no active PK.

Thus the level of PK activity is directly proportional to the number of doses of the normal gene.

The ability to identify heterozygotes or carriers (heterozygote detection) is of major importance for the control of genetic diseases.

c. Inherited Lysosomal Storage Disease:

A variety of diseases result from defects in lysosomal catabolism. Lysosome are small membrane-bound organelles present in the cytoplasm.

Their function is the breakdown (catabolism) of different complex molecules (fats, carbohydrate, protein and nucleic acid) into simpler form (simple lipids, monosaccharides and amino acids).

For carrying out these functions, lysosomes contain a great variety of enzymes which act in a step by step manner.

If a particular enzyme is absent or inactive, then the step-wise degradation is stopped.

This results in building up (or storage) in the lysosome or elsewhere of the material that should have been broken down by the enzyme.

In other words, an inborn error of lysosomal catabolism produces a ***lysosomal storage disease.***

Among the inherited types of this disease two examples are given: Tay-sack's disease in humans and mannosidosis in cattle.

c.1. Tay-Sack's Disease:

infants homozygous for the autosomal recessive gene hex A (hex A hex A) do not produce the normal enzyme N-acetyl hexosaminidase A.

This enzyme is essential for purine metabolism. As a result of this absence the unmetabolized ganglioside lipid called GM2 accumulated in brain cells, leading to degradation of the brain, severe mental retardation called "*amaurotic idiocy*" beside other physical signs.

The symptoms appear soon after birth and end in death by three years of age.

c.2. Mannosidosis:

caused by an autosomal recessive gene which has been discovered in Angus cattle.

The recessive homozygote lacks the enzyme α-mannosidase which is required for metabolizing saccharides containing mannose.

As a result, these substances accumulate and cause a lysosomal storage disease that ends in death within the first year of life.

Many other inherited lysosomal storage diseases have been described in human and in animals

The following is a list of some inherited storage diseases in domestic animals

d. Porphyria (Pink Tooth):

This inherited error of metabolism is associated with a deficient in the synthesis of heme (the iron-containing group present in hemoglobin).

Inherited porphyria has been reported in humans and in cattle, cat and pigs.

It is caused by a recessive mutation in an autosomal gene which is responsible for manufacturing the enzyme uroperphyrinogen III cosynthetase.

In the recessive homozygote the deficiency of the enzyme leads to the lack of heme and the accumulation in the body of the intermediates of heme metabolism.

The lack of heme leads to hemolytic anemia, while the accumulation of uroporphyrinogen I (one of the intermediate porphyrinogens) leads to a characteristic red staining of teeth, bone and urine (hence the name pink tooth).

Also porphyrinogens are readily oxidized to porphyrin (hence the name porphyria), which absorbs visible light and induces photosensitivity.

The heterozygous carriers of the mutant gene have an enzyme activity intermediate between that of normal homozygotes and diseased individuals.

e. Dermatosparaxis:

This is a heritable disorder of connective tissue also due to an error of metabolism.

This causes the skin to be easily extendible and very fragile. The affected animals suffer severe laceration as a result of the lightest scratch, that in normal animals would cause only slight wounds.

The disease in sheep and cattle caused by a recessive mutation in an autosomal gene which specifies the enzyme procollagen peptidase.

In homozygous recessive individuals normal collagen (which is composed of cylindrical fibrils) cannot be formed and instead abnormal procollagen forms into flattened twisted ribbons.

This makes the skin very weak and becomes easily torn.

Inherited bleeding disorders other than hemophilia have an autosomal codominant or incompletely dominant mode of inheritance.

Most of these conditions were described in dogs and cats, and the deficient factors were coagulation factors I, II, VII, VIII (pseudohemophilia) X, XI and XII. In these diseases and the partial deficiency of the factor involved can be detected biochemically.

3. Inherited Haemoglobin Disorders

Mutations in genes specifying hemoglobin chains have been identified in human and in animals.

None of these variant hemoglobins has been found to cause any defect in hemoglobin function in animals.

In human beings, however, defective hemoglobins have been identified as the cause of hemoglobin disorders.

The most famous of these is sickle cell anemia which is inherited in a codominant pattern.