transcript possibly goes in favour of mutation in noncoding regions (introns) of the gene, like at splice junction of an intron–exon border.

## DNA FINGERPRINTING TECHNOLOGY

A British geneticist Dr. Alec Jeffreys pioneered DNA fingerprinting technology in 1985. It has now become a routine method for crime investigations in all parts of the world. It is used for identification of the criminals. Using DNA from the suspect and from the site of crime, if the two match perfectly one can conclusively say that the suspect under consideration is the criminal. However, there are two prime questions before an ordinary person accepts the statement. The first, how reliable is the technology and secondly, how fool proof are its conclusions. To answer these questions one can recapitulate some well known facts.

People differ from each other only at about 1% nucleotide locations. In human genome there are over 3 billion nucleotides. Even 1% of 3.3 billion amounts to 33 million such locations. Considering that at each of these locations, there are four alternative nucleotides that may occur. Further one knows that there are two DNA strands that make up DNA of a person. Making all the permutations and combinations one can safely assert that the complete DNA sequencing of any two individuals on the earth cannot be identical unless they are identical twins.

**Hypervariable regions of the genome.** In our genome there are hundreds of specific regions. At each of these, the nucleotide arrangement differs from individual to individual. Literally thousands of possible types could be observed. With this technology one can reliably type individuals at several such regions either sequentially or simultaneously. Combining these observations one gets almost an error-free DNA profile to identify an individual.

Introduction of DNA typing in courts rests upon similar genetic principles. It has high level of discrimination among individuals and can be performed with many source materials, e.g. bones, skin, hair follicles, saliva, epithelial swabs, etc. Therefore it is superior to traditional blood typing. Stability of DNA molecules allows genetic typing even from fossilized or decomposed materials. This has helped in establishing identity from fragmented material from the war victims, exhumed bodies or even from mummified bodies. Identification of members of Czar family through DNA typing of excavated bodies from the burial ground is a glaring example of this technology.

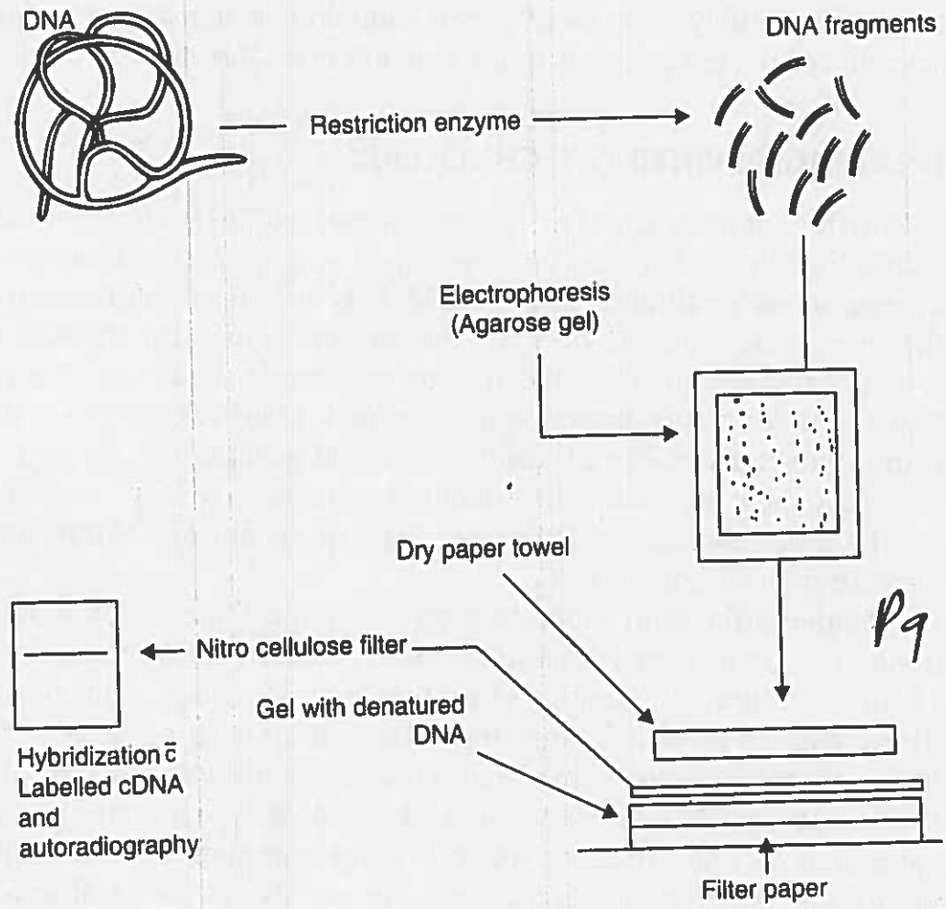


**Fig. 3.13.** The Southern blot technique.

4. Denatured DNA (single stranded) is then transferred on nitrocellulose filter by blotting.
5. Now to identify and localize a particular fragment on the filter, a radioactive labelled DNA probe $p^{32}$ is used.
6. Probe is allowed to hybridize with DNA fragments in 'Southern blot' and subsequently autoradiographed (Fig. 3.13).

## NORTHERN BLOTTING

In this technique, mRNA is isolated and run on an electrophoretic gel and is transferred to a filter. This is called Northern blotting. Hybridization of the Northern blot with a radiolabelled probe allows determination of the size as well as the quantity of the mRNA transcript. Some single gene disorders in which no mutation has been identified in exons (coding sequences), an alteration in the size of mRNA

2. A small quantity of template DNA (5–10 ng) is also sufficient.
3. A highly purified DNA sample is not essential.
4. Number of samples can be used e.g. peripheral blood, bone chips, single sperm, hair follicle and even paraffin embedded tissues.

### Problems of PCR

**False positive reaction:** Any contamination in the sample can be amplified giving false positive signal. This could be from the previous reaction or from the exogenous source.

**False negative reaction:** This may be due to very low yield or the absence of the specific product. The conditions of PCR amplification may be altered to overcome this. If essential, even the sequence of the primers can be changed.

### Applications of PCR

It can be used in:

1. Diagnosis
2. Therapeutics
3. Criminology, etc.

### Amplification Refractory Mutation System (ARMS) PCR

It is a special type of PCR in which the 3' end base of the primer is mutant specific. The sequence of the primer is selected in such a way that its 3' end base matches with the mutant base under question. Thus two primers, a mutant and a normal are used.

## SOUTHERN BLOT TECHNIQUE

It is used in DNA analysis. The technique was evolved in 1975 by Edwin Southern at Edinburgh. The steps involved in this technique are as follows (Fig. 3.13):

1. DNA is cleaved by restriction enzymes.
2. DNA fragments are separated by agarose gel electrophoresis. Small fragments move faster than the large ones.
3. DNA is then denaturated with alkali. This makes the DNA single stranded.

temperature depends upon how farther the primers are placed along the DNA molecule.

These steps 1, 2 and 3 make up one cycle in PCR reaction. At the end of this, the original DNA molecule under consideration is quantitatively doubled. The thermal cycler is programmed to repeat the cycle 25–30 times. With each cycle the desired DNA (between two primers) is doubled. Therefore it is possible to selectively amplify any given DNA several million times in few hours.

## Analysis of PCR product

This is one of the most important steps. Sometimes the primer/s bind to nonspecific locations if the annealing temperature is lowered, which favours annealing of mismatched bases.

Analysis is accomplished as under:

**Estimation of size:** The PCR product is subjected to the agarose gel electrophoresis and its size is estimated by running known DNA size markers. The newly synthesized DNA should be of the size between the extreme ends of primers. Mostly it is sufficient to conclude between the positive and negative results.

**Nested PCR:** The PCR product can be reamplified using a new primer pair that is located within the ends of the first primer pair. This PCR product is shorter than the first PCR product.

**Hybridization:** A short oligonucleotide may be synthesized which is complementary to one of the DNA strands of the PCR product. Such an oligonucleotide is radiolabelled and the hybridization signal can then be obtained on autoradiography.

**Restriction enzyme mapping:** The PCR product may be subjected to restriction digestion in order to confirm the identity of the product. This also gives us information regarding RE site gained or lost due to mutation/s.

**Cloning and sequencing:** Alternatively the PCR product can be cloned in an appropriate vector and its authenticity can be verified by nucleotide sequencing. The sequencing of PCR product can be done even without cloning.

## Advantages of PCR

1. The technique is rapid as well as sensitive.

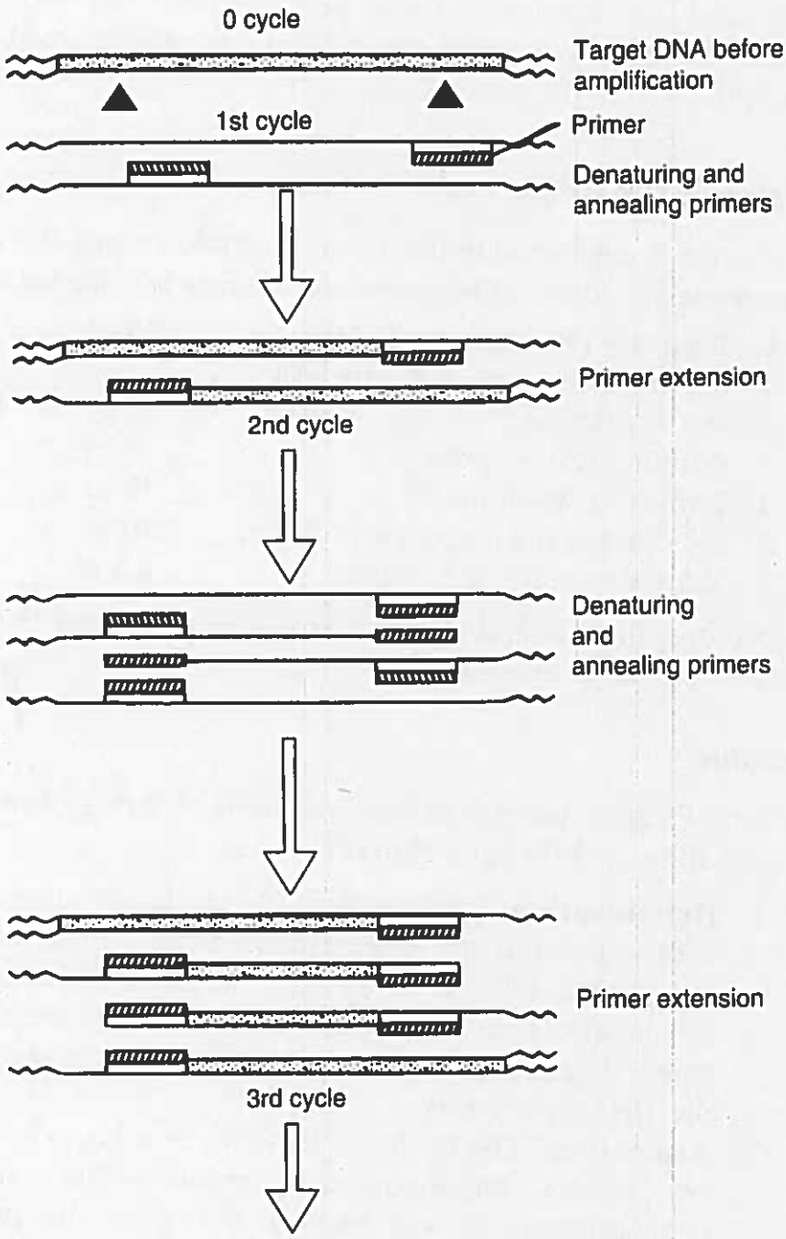


**Fig. 3.12.** Schematic representation of polymerase chain reaction (PCR).

one at a time. The nucleotide sequence of new DNA is decided by the sequence of template DNA. Since the primers are designed to extend in opposite directions, the intervening DNA (between the two primers) is synthesized.

Usually this step is carried out at 72°C because most of the thermostable DNA polymerases commercially available have an optimum activity at this temperature. The incubation time at this

The reaction tube (containing test DNA, primer pair, dNTPs, Mg, buffer and thermostable DNA polymerase (Taq polymerase) enzyme) is put in a thermal cycler which is programmed to carry out amplification.

## The standard reaction

The reaction is conducted in the 0.5 ml Eppendorf tubes, the following components are added and the reaction volume is made up to 100 µl.

1. Template (100 ng–1 µg DNA) ... 10 µl
2. Buffer (containing KCl, Tris-HCl, $MgCl_2$ gelatine) ... 10 µl
3. Primer 1 (20–50 pmol) ... 10 µl
4. Primer 2 (20–50 pmol) ... 10 µl
5. dNTP mix (200 µm of each dNTP) ... 10 µl
6. Taq polymerase (2.5 units) ... 0.5 µl

Two drops of mineral oil are then added to overlay the reaction mixture to avoid evaporation.

### *Procedure*

The amplification procedure involves three steps: (1) denaturation, (2) annealing, and (3) extension (Fig. 3.12).

1. **Denaturation:** Since the test DNA is double stranded, it has to be converted into single stranded one. This is achieved by heating the DNA at 92–95°C for about 30–60 seconds. This breaks the hydrogen bonds between the complementary bases, thus separating the two strands. This does not damage the DNA in any way.
2. **Annealing:** The cycler is then set to a lower temperature i.e. 55–65°C for about 30–60 seconds. Since the primer concentration is very high in the tube, the primer will anneal with A = T and C = G base pairing, to the test DNA at the specified locations (as per primer). The high concentration of the primers prevents reannealing of the original (test) DNA strands. The annealing temperature is critical and depends upon the composition of the DNA sequence and the length of primers.
3. **Primer extension (DNA synthesis):** In this step the annealed primers are extended. The DNA chain, as learnt earlier, grows in 5'–3' direction. The nucleotides are added

### *DNA probe*

The process of making recombinant DNA involves the insertion of a large number of random restriction fragments into plasmids. This obviously makes it important to be able to recognize a particular gene. It is possible, if mRNA of the desired type is available. With the help of an enzyme, reverse transcriptase, a complementary DNA (cDNA), can be transcribed from a known RNA. The enzyme, reverse transcriptase, is obtained from RNA tumour viruses. The final product of the transcription is a radioactive cDNA molecule also referred to as DNA probe. The probe hybridizes to the specific gene or its mRNA under suitable conditions.

## POLYMERASE CHAIN REACTION

The PCR was discovered by Kary Mullis and developed by Saiki and others in 1985. It has revolutionized both the diagnostic as well as therapeutic ability of the doctors. It has become a powerful tool in the field of molecular biology. Before we turn to PCR let us acquire little background knowledge of DNA synthesis.

The DNA synthesis requires a template (to be copied), a primer which is extended as a newly synthesized DNA, dNTPs, Mg and an enzyme—DNA polymerase. The DNA is synthesized by polymerase action, i.e. by adding one nucleotide at a time and the chain extends.

**Primer:** It is a short stretch of nucleotides having complementary sequences. It attaches to a long template molecule at specific region. The 3' end of the primer gets extended by addition of an appropriate nucleotides, i.e. T (thymine) if the template has A (adenine), G (guanine) if the template has C (cytosine). The 5' end of the primer remains fixed. The new DNA is synthesized only in 5'–3' direction.

**PCR:** Polymerase chain reaction is an in vitro method of synthesis of nucleic acids, wherein, a specific DNA segment is amplified rapidly without concomitant replication of the rest of the DNA molecule. Thus in PCR a limited region of a DNA molecule is amplified. Basic requirement for this assay is nucleotide sequence information at two ends of the part to be amplified. Then a pair of oligonucleotide primers (about 20–30 nucleotide long) are then synthesized. One of them is complementary to upper/one strand of DNA at one end and the other primer is complementary to the other end of the lower/other strand of the DNA to be amplified.

into the host cell by exposing the host (bacterial) cell to calcium salts. This turns the cell permeable to plasmid.

4. The host cells containing vectors are put in a culture medium to produce clones, i.e. obtain multiple copies of foreign DNA fragments incorporated in recombinant vector.
5. Selection of clones possessing proper DNA fragment.

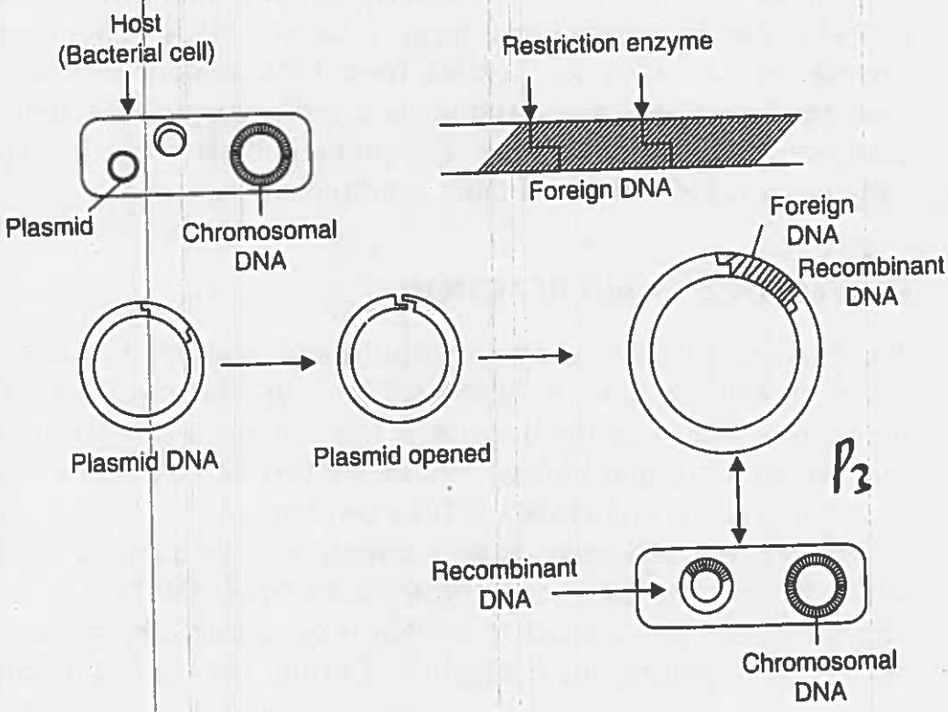


**Fig. 3.11.** Constructing recombinant plasmid and transformation of host cell.

## *Applications of recombinant DNA technology*

1. It gives us a rational approach to the understanding of molecular basis of numerous diseases, e.g. Sickle cell disease, familial hypercholesterolaemia, thalassaemias, cystic fibrosis and Huntington's chorea, etc.
2. Using this technology, human proteins can be produced for therapeutic purposes, e.g. insulin and growth hormone.
3. Production of proteins for vaccination, e.g. hepatitis B.
4. Proteins can be produced for diagnostic tests, e.g. AIDS test.
5. Gene therapy for Sickle cell disease or thalassaemias or other diseases.

from *E. coli* known as pBR 322 is 4362 bp (base pair) in length. Plasmids have an advantage as a vector in that they have a limited number of unique restriction sites and can also carry resistance to particular antibiotics. This character is used to identify recombinant clones.

### *Phages (Bacteriophages)*

These are viruses that infect bacteria and multiply within bacteria. Subsequently they cause lysis of a bacterial cell. This releases a phage progeny which then infects another bacteria. Phages have an advantage over plasmids that much larger fragments of DNA can be cloned in them. Plasmids can be used as vectors up to 8 kb (1 kb = 1000 bp). Phages can be useful for fragments up to 15 kb and for still larger fragments of 35–45 kb, cosmids are used.

### *Cosmids*

These are plasmids in which the maximum DNA has been removed to permit largest possible insert for cloning but still have DNA essential for in vitro packaging into phage particle. In short, they are phage-plasmid hybrids capable of carrying relatively large DNA inserts.

### *Yeast artificial chromosomes (YACs)*

YACs consist of plasmid possessing DNA sequences essential for (a) centromere formation, (b) telomere formation and other DNA sequences known as autonomous replication sequences. YACs can incorporate DNA fragments up to 1000 kb (kilobase) in size.

The choice of vector used in cloning depends on number of factors such as the restriction enzyme used and the size of DNA fragment to be inserted.

### *Recombinant DNA procedure*

It involves five steps (Fig. 3.11):

1. DNA is cut into fragments by restriction endonuclease enzyme.
2. Incorporation of these fragments in a suitable vector using DNA ligase.
3. Transformation of host organism, e.g. *E. coli* by recombinant vector, i.e. the recombinant vector (plasmid) is reinserted

3. **Cryopreservation facility:** Samples of 'synthetic seeds', embryos, gametes can be preserved by this technique. This is possible either by immersion in liquid nitrogen (–196°C) or by keeping in its vapour phase around –150°C. This is however according to the protocols using computerized freezing-thawing rates and also chemical cryoprotectants.
4. **Clonal repository:** Germplasm collections of vegetatively propagated crops like ginger, turmeric, sweet potato and banana are being maintained.

India is one of the bio-diversity rich countries. The plant wealth in India and in Indian Gene Bank can be imagined that it held 132,619 dehydrated, sealed seed samples till 1994. Accessions still under multiplication and characterization in NBPGR network recorded that time were 120,226, and more than that 188,263 were still under multiplication and upgrading at Indian NBPGR system.



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## Recombinant DNA

Among recent advances in the field of genetics, recombinant DNA occupies a prominent position.

P1

### *Definition*

It is an artificially synthesized DNA which is constructed by insertion of foreign DNA into DNA of an appropriate organism so that foreign DNA is replicated along with the host DNA.

### *Restriction enzyme*

Restriction endonucleases are enzymes that can cleave DNA at specific sites. They were discovered by Hamilton Smith and his associates in 1970. Today more than 200 of them are known.

### *Vectors*

They are used to carry foreign DNA fragments. They are as follows: plasmids, phages, cosmids and yeast artificial chromosomes (YACs).

### *Plasmids*

Plasmid is a circular extrachromosomal element in bacteria. It can replicate independently. Plasmids vary in size. One of the plasmids