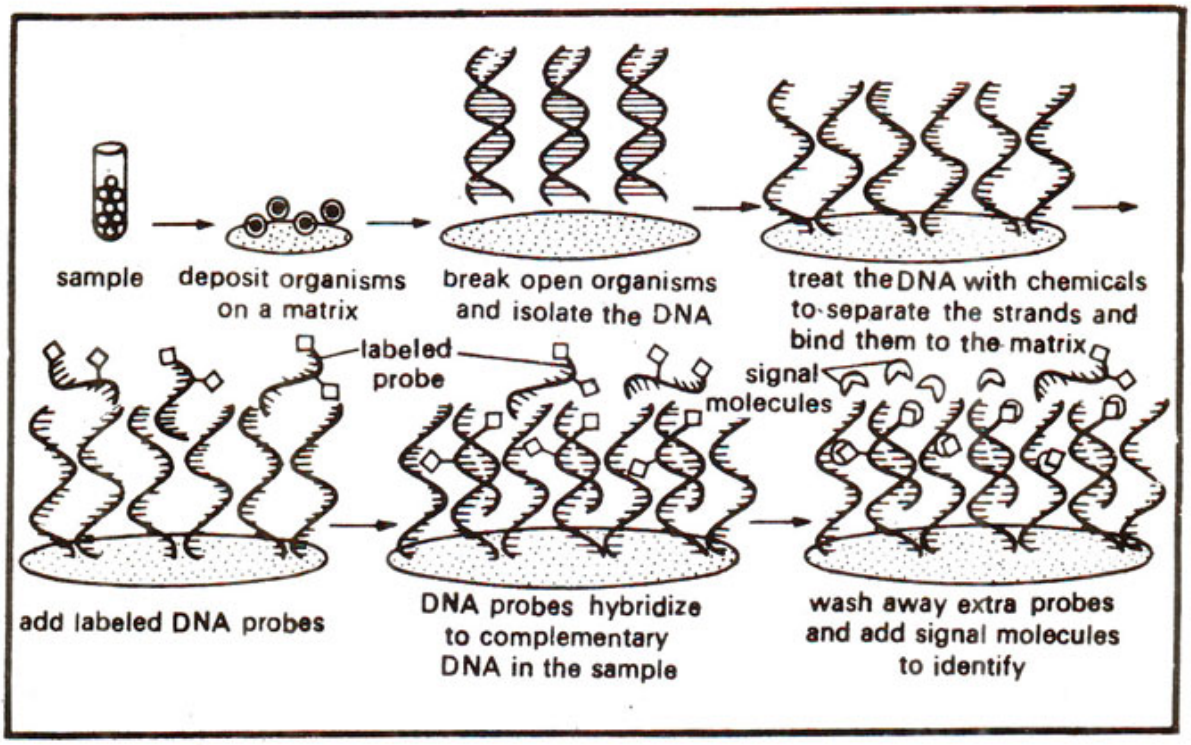
UNIT II- TECHNIQUES IN GENETIC ENGINEERING

PROBES- CONSTRUCTION AND LABELLING

Molecular probes are small DNA segments (genomic DNA, cDNA or synthetic oligonucleotides) or RNA segments (often synthesized on DNA template) that recognize complementary sequences in DNA or RNA molecules and thus allow identification and isolation of these specific DNA sequences from an organism. Antibodies are also occasionally use as probes to recognize specific protein sequences. These probes also frequently used for a variety of other purposes including diagnosis of infectious diseases, identification of food contaminants, variety of microbiological tests, forensic tests (e.g. fingerprinting of murderers or rapists), etc. Probes can also be used to identify strains of an organism e.g. varieties of a crop species (a plant breeder likes to have quick test to identify his variety to maintain his patent or breeder's right, so that a competitor may not use it in another name leading to infringement of rights).

DNA/RNA probe assays are faster and sensitive, so that many conventional diagnostic tests for viruses and bacteria involving culturing of the organism, are being fast replaced by antibody and DNA probe assays. While culture tests can take days or even months, molecular probe assays can be performed within few hours or minutes. Therefore, the use of DNA probes has become today's most sophisticated and sensitive technology for a variety of uses involving biological systems both in basic and applied studies including their commercial use.



Labelling of Gene Probes:

An essential feature of gene probe is that it can be visualized by some means. Therefore, labeling of probe is a must and is done by two methods, traditionally by using radioactive labels and by non-radioactive labels. Mostly used radioactive labels are phosphorous 32 (^{32}P), sulphur 35 (^{35}S) and tritium (^{3}H) which are detected by process of autoradiography.

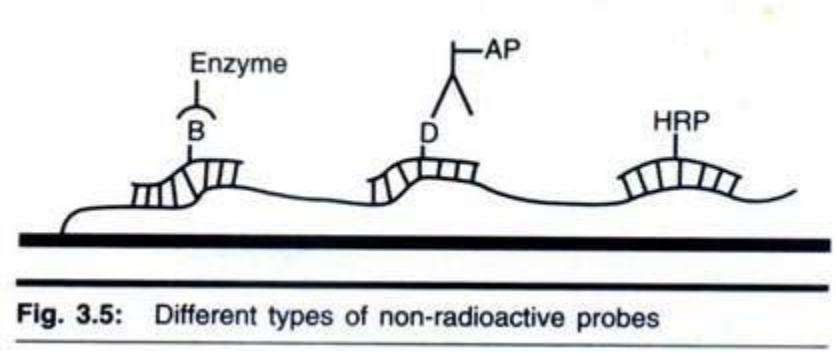


Fig. 3.5: Different types of non-radioactive probes

Non-radioactive probes, although less sensitive than radioactive probes but are safe to use. The labelling systems are termed either direct or indirect. Direct labelling allows an enzyme reporter such as alkaline phosphatase to couple directly to DNA. Indirect labelling method which is more popular involves binding of nucleotide that has label attached, e.g., Biotin, fluorescein and digoxigenin.

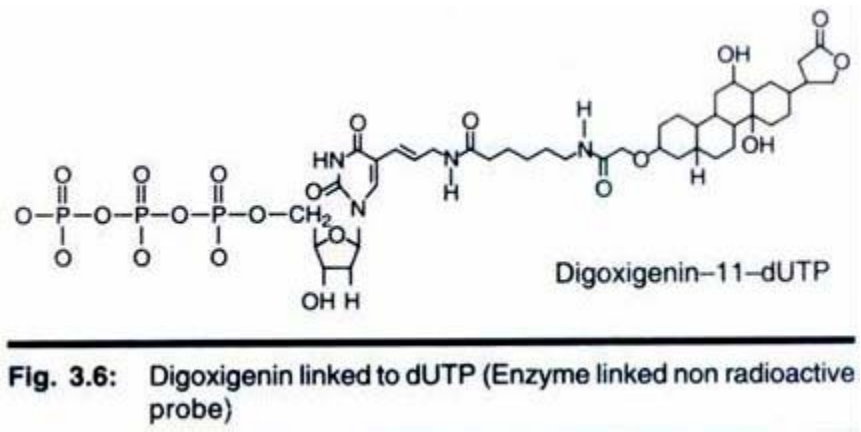


Fig. 3.6: Digoxigenin linked to dUTP (Enzyme linked non radioactive probe)

These molecules are covalently linked to nucleotides. Specific binding proteins may then be used as a bridge between nucleotide and reporter protein such as enzyme. For example biotin incorporated into DNA molecule is recognized with a very high affinity by Streptavidin. This may be in turn is coupled with enzyme alkaline phosphatase which is able to convert colourless compound p-nitro phenol phosphate (PNPP) into yellow colour compound p- nitro-phenol (PNP) and also offers a means of signal amplification.

Thus, rather than the detection system relying on autoradiography, which is necessary for radiolabels, a series of reaction resulting in colour, a light or a chemiluminescence are much

better, since autoradiography may take 1-3 days, where colour and chemiluminescence reaction takes a few minutes.

Table 3.1: Comparative chart of different gene probing methods

- random priming
  - — used for cloned DNA fragments
- T4 nucleotide kinase
  - — used primarily for synthetic oligonucleotides
- (nick translation)
  - — earlier method replaced by random priming
  - — quality control and reproducibility problems
- (terminal transferase)
  - — adds nucleotides to 3′ end
  - — used primarily for generation of homopolymer tails

End Labelling of DNA Molecules:

The simplest form of labelling is by 5′- or 3′-end labelling. 5′-end labelling involves a phosphate transfer or exchange reaction where the 5′-phosphate of the DNA to be used is replaced by ^{32}P. This is carried by two enzymes: the first, alkaline phosphatase; removing phosphate group followed by poly nucleotide kinase catalyzing the transfer of phosphate group (^{32}P) to 5′ end of DNA. The newly labelled probe is then purified, usually by chromatography through a sephadex column to remove any unincorporated radiolabel.

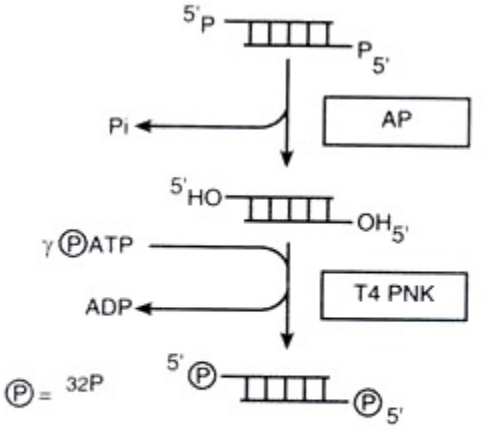


Fig. 3.7: End-labelling of a gene probe at 5′-end with alkaline phosphate and polynucleotide kinase

Using other end of DNA molecule, the 3′- end is slightly less complex. Here a new, labelled dNTP ([a^{32}-P]ATP or biotin-labelled dNTP) is added to the 3′-end of DNA by enzyme terminal

transferase. Although this is a simpler reaction, a potential problem exists because a new nucleotide is added to the existing sequence and so the complete sequence of the DNA is altered, which may affect its hybridization to its target sequence. End-labelling methods also suffer from the fact that only one label is added to DNA so such methods are of low activity in comparison to other who incorporate label throughout the length of sequence of DNA.

Random Primer Labelling:

The DNA to be labelled is first denatured and is then allowed to re-natured in presence of random sequences of hexamers or hexanucleotide. These hexamers will by chance bind to DNA sample wherever they encounter a complementary sequence and so the DNA will acquire hexanucleotide annealing to it.

Each of the hexamers can act as primer for synthesis of a fresh strand of DNA catalysed by DNA polymerase since it has free 3′- hydroxyl group. The Klenow fragment of DNA polymerase is used for random primer labelling since it lacks 5′ → 3′ exonuclease activity but still acts as 5′ → 3′ polymerase.

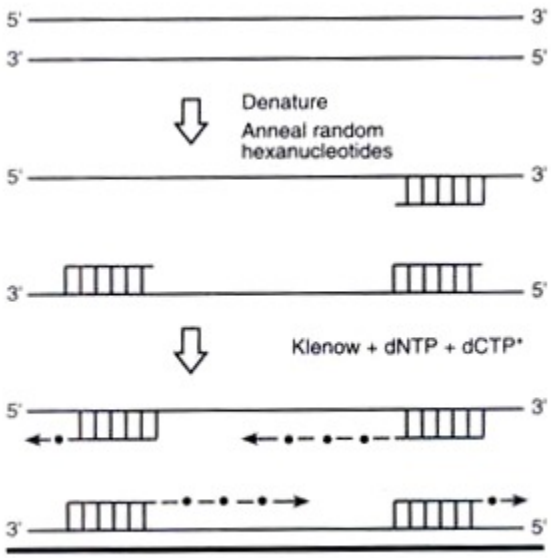


Fig. 3.8: Random primer gene probing

BLOTTING TECHNIQUE- SOUTHERN BLOTTING

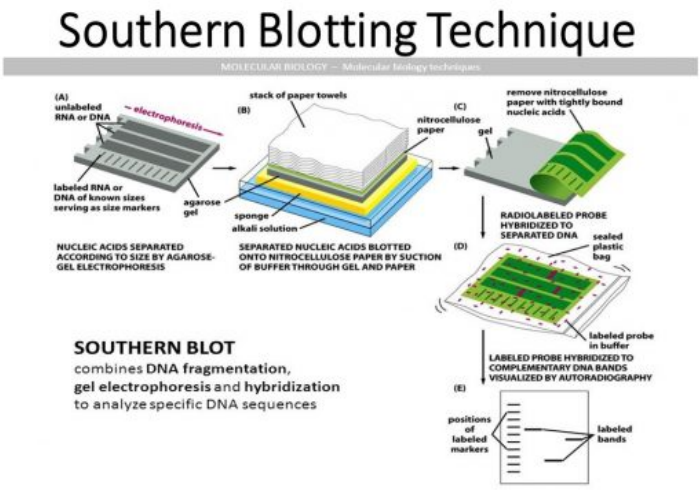
Southern Blotting

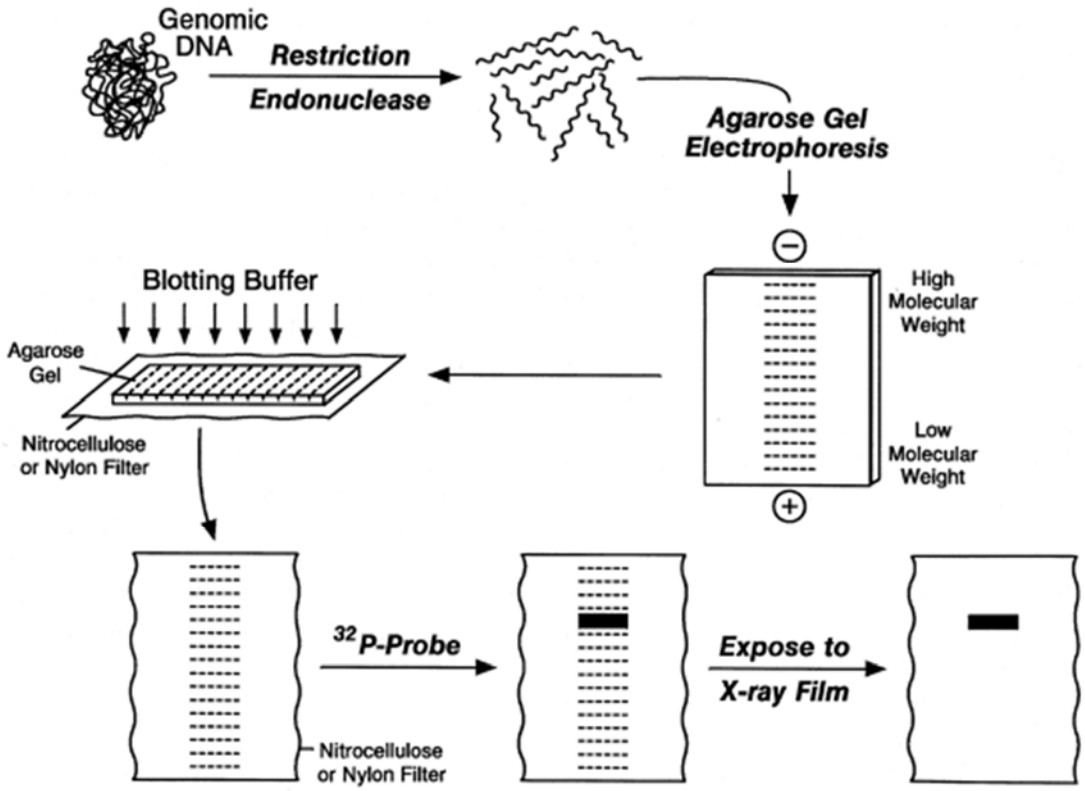
Principle:

- Southern blotting is an example of RFLP (restriction fragment length polymorphism). It was developed by Edward M. Southern (1975). Southern blotting is a hybridization technique for identification of particular size of **DNA** from the mixture of other similar molecules**.** This technique is based on the principle of separation of DNA fragments by gel electrophoresis and identified by labelled probe hybridization.
- Basically, the DNA fragments are separated on the basis of size and charge during electrophoresis. Separated DNA fragments after transferring on nylon membrane, the desired DNA is detected using specific DNA probe that is complementary to the desired DNA.
- A hybridization probe is a short (100-500bp), single stranded DNA. The probes are labeled with a marker so that they can be detected after hybridization.

Procedure/ Steps

1. Restriction digest: by RE enzyme and amplification by PCR
2. Gel electrophoresis: SDS gel electrophoresis
3. Denaturation: Treating with HCl and NaOH
4. Blotting
5. Baking and Blocking with casein in BSA
6. Hybridization using labelled probes
7. Visualization by autoradiogram





Step I: Restriction digest

- The DNA is fragmentized by using suitable restriction enzyme. RE cuts the DNA at specific site generating fragments
- The number of fragments of DNA obtained by restriction digest is amplified by PCR

Step II: Gel electrophoresis

- The desired DNA fragments is separated by gel electrophoresis

Step III: Denaturation

- The SDS gel after electrophoresis is then soaked in alkali (NaOH) or acid (HCl) to denature the double stranded DNA fragments.
- DNA strands get separated

Step IV: Blotting

- The separated strands of DNA is then transferred to positively charged membrane nylon membrane (Nitrocellulose paper) by the process of blotting.

Step V: Baking and blocking

- After the DNA of interest bound on the membrane, it is baked on autoclave to fix in the membrane.
- The membrane is then treated with casein or Bovine serum albumin (BSA) which saturates all the binding site of membrane

Step VI: Hybridization with labelled probes

- The DNA bound to membrane is then treated with labelled probe
- The labelled probe contains the complementary sequences to the gene of interest
- The probe bind with complementary DNA on the membrane since all other non-specific binding site on the membrane has been blocked by BSA or casein.

Step VII: Visualization by Autoradiogram

- The membrane bound DNA labelled with probe can be visualized under autoradiogram which give pattern of bands.

Applications of Southern blotting:

1. Southern blotting technique is used to detect DNA in given sample.
2. DNA finger printing is an example of southern blotting
3. Used for paternity testing, criminal identification, victim identification
4. To isolate and identify desire gene of interest.
5. Used in restriction fragment length polymorphism
6. To identify mutation or gene rearrangement in the sequence of DNA
7. Used in diagnosis of disease caused by genetic defects
8. Used to identify infectious agents

DNA SEQUENCING TECHNIQUE- SANGER AND COULSON METHOD

DNA sequencing is the process of determining the sequence of nucleotide bases (As, Ts, Cs, and Gs) in a piece of DNA. Today, with the right equipment and materials, sequencing a short piece of DNA is relatively straightforward.

Sequencing an entire genome (all of an organism's DNA) remains a complex task. It requires breaking the DNA of the genome into many smaller pieces, sequencing the pieces, and assembling the sequences into a single long "consensus." However, thanks to new methods that have been developed over the past two decades, genome sequencing is now much faster and less expensive than it was during the Human Genome Project^11start superscript, 1, end superscript.

Sanger sequencing: The chain termination method

Regions of DNA up to about 900900900 base pairs in length are routinely sequenced using a method called **Sanger sequencing** or the **chain termination method**. Sanger sequencing was developed by the British biochemist Fred Sanger and his colleagues in 1977.

In the Human Genome Project, Sanger sequencing was used to determine the sequences of many relatively small fragments of human DNA. (These fragments weren't necessarily 900900900 bp or less, but researchers were able to "walk" along each fragment using multiple rounds of Sanger sequencing.) The fragments were aligned based on overlapping portions to assemble the sequences of larger regions of DNA and, eventually, entire chromosomes.

Although genomes are now typically sequenced using other methods that are faster and less expensive, Sanger sequencing is still in wide use for the sequencing of individual pieces of DNA, such as fragments used in DNA cloning or generated through polymerase chain reaction (PCR).

Ingredients for Sanger sequencing

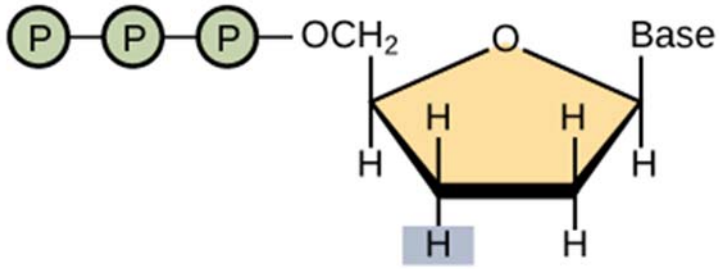
Sanger sequencing involves making many copies of a target DNA region. Its ingredients are similar to those needed for DNA replication in an organism, or for polymerase chain reaction (PCR), which copies DNA *in vitro*. They include:

- A DNA polymerase enzyme
- A **primer**, which is a short piece of single-stranded DNA that binds to the template DNA and acts as a "starter" for the polymerase
- The four DNA nucleotides (dATP, dTTP, dCTP, dGTP)

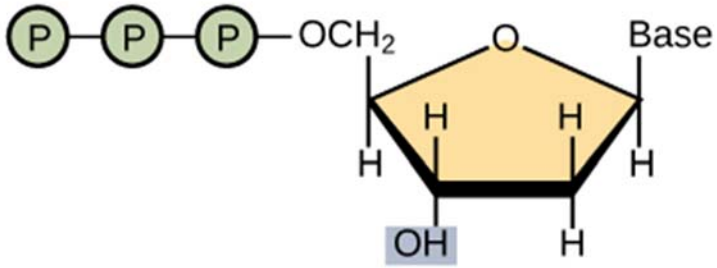
- The template DNA to be sequenced

However, a Sanger sequencing reaction also contains a unique ingredient:

- Dideoxy, or **chain-terminating**, versions of all four nucleotides (ddATP, ddTTP, ddCTP, ddGTP), each labeled with a different color of dye



Dideoxynucleotide (ddNTP)



Deoxynucleotide (dNTP)

Image credit: "Whole-genome sequencing: Figure 1," by OpenStax College, Biology (CC BY 4.0).

Dideoxy nucleotides are similar to regular, or deoxy, nucleotides, but with one key difference: they lack a hydroxyl group on the 3’ carbon of the sugar ring. In a regular nucleotide, the 3’ hydroxyl group acts as a “hook," allowing a new nucleotide to be added to an existing chain.

Once a dideoxy nucleotide has been added to the chain, there is no hydroxyl available and no further nucleotides can be added. The chain ends with the dideoxy nucleotide, which is marked with a particular color of dye depending on the base (A, T, C or G) that it carries.

Method of Sanger sequencing

The DNA sample to be sequenced is combined in a tube with primer, DNA polymerase, and DNA nucleotides (dATP, dTTP, dGTP, and dCTP). The four dye-labeled, chain-terminating dideoxy nucleotides are added as well, but in much smaller amounts than the ordinary nucleotides.

The mixture is first heated to denature the template DNA (separate the strands), then cooled so that the primer can bind to the single-stranded template. Once the primer has bound, the temperature is raised again, allowing DNA polymerase to synthesize new DNA starting from the primer. DNA polymerase will continue adding nucleotides to the chain until it happens to add a dideoxy nucleotide instead of a normal one. At that point, no further nucleotides can be added, so the strand will end with the dideoxy nucleotide.

This process is repeated in a number of cycles. By the time the cycling is complete, it's virtually guaranteed that a dideoxy nucleotide will have been incorporated at every single position of the target DNA in at least one reaction. That is, the tube will contain fragments of different lengths, ending at each of the nucleotide positions in the original DNA (see figure below). The ends of the fragments will be labeled with dyes that indicate their final nucleotide.

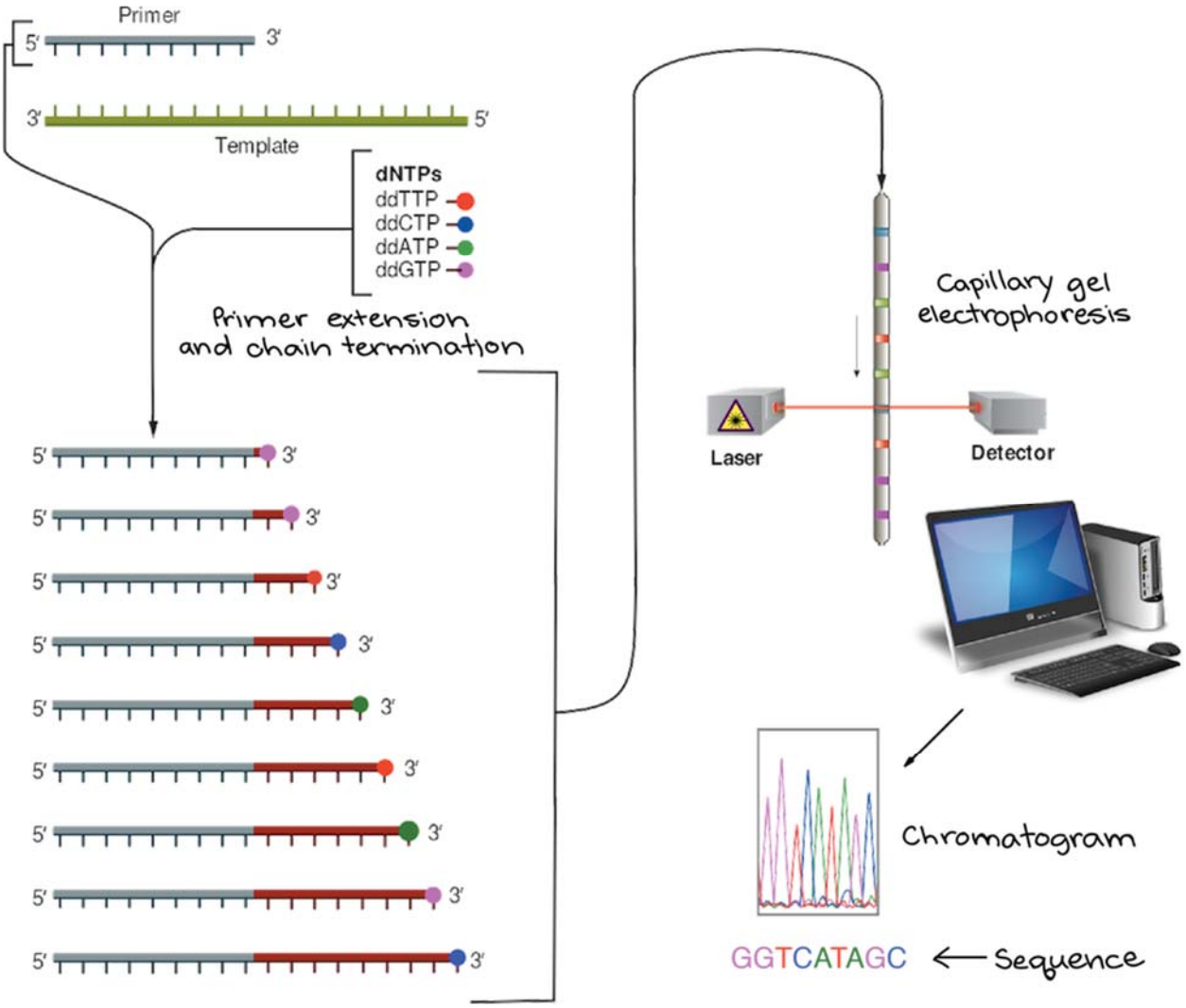


Image modified from "Sanger sequencing," by Estevezj (CC BY-SA 3.0). The modified image is licensed under a (CC BY-SA 3.0) license.

After the reaction is done, the fragments are run through a long, thin tube containing a gel matrix in a process called **capillary gel electrophoresis**. Short fragments move quickly through the pores of the gel, while long fragments move more slowly. As each fragment crosses the "finish line" at the end of the tube, it's illuminated by a laser, allowing the attached dye to be detected.

The smallest fragment (ending just one nucleotide after the primer) crosses the finish line first, followed by the next-smallest fragment (ending two nucleotides after the primer), and so forth. Thus, from the colors of dyes registered one after another on the detector, the sequence of the original piece of DNA can be built up one nucleotide at a time. The data recorded by the detector

consist of a series of peaks in fluorescence intensity, as shown in the **chromatogram** above. The DNA sequence is read from the peaks in the chromatogram.

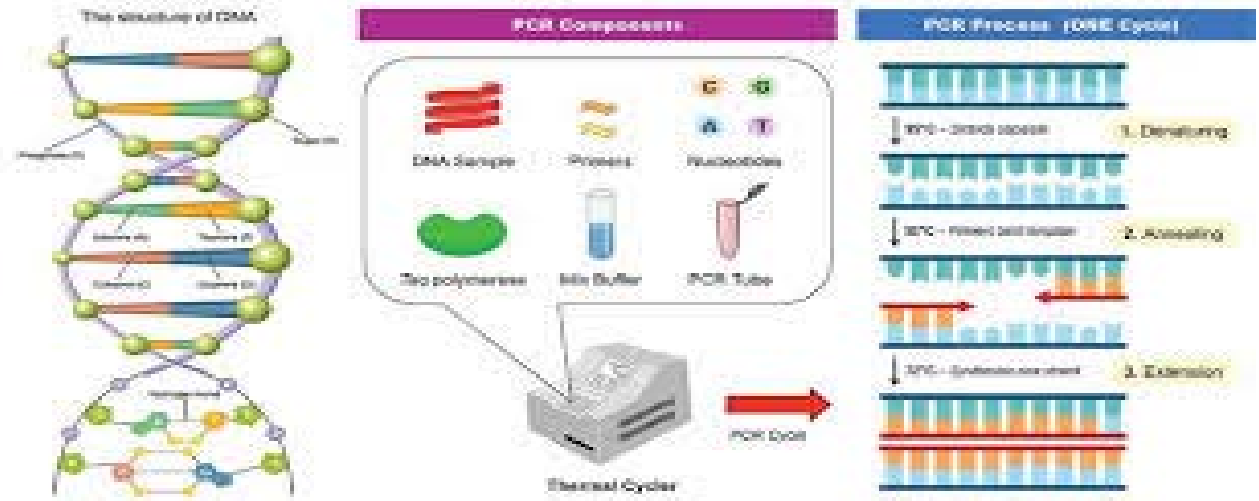
Uses and limitations

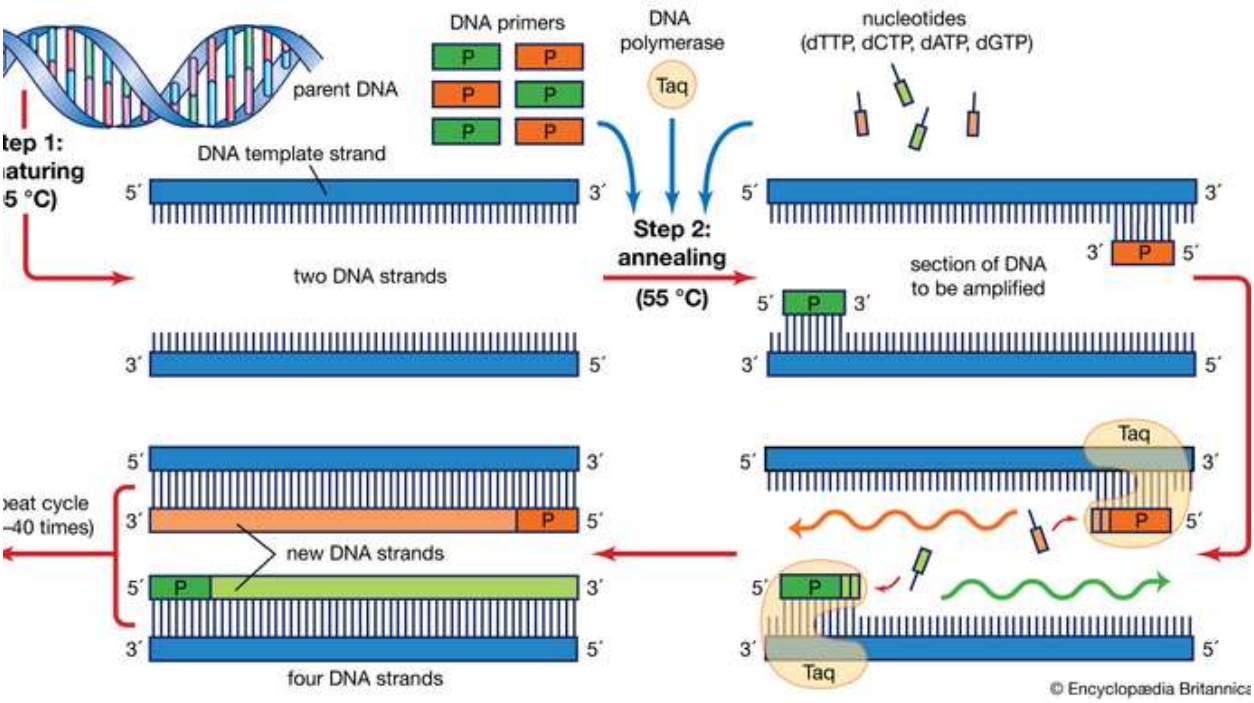
Sanger sequencing gives high-quality sequence for relatively long stretches of DNA (up to about 900900900 base pairs). It's typically used to sequence individual pieces of DNA, such as bacterial plasmids or DNA copied in PCR.

However, Sanger sequencing is expensive and inefficient for larger-scale projects, such as the sequencing of an entire genome or metagenome (the “collective genome” of a microbial community). For tasks such as these, new, large-scale sequencing techniques are faster and less expensive.

DNA AMPLIFICATION TECHNIQUE- PCR- TECHNIQUE AND APPLICATION

Polymerase chain reaction (PCR), a technique used to make numerous copies of a specific segment of DNA quickly and accurately. The polymerase chain reaction enables investigators to obtain the large quantities of DNA that are required for various experiments and procedures in molecular biology, forensic analysis, evolutionary biology, and medical diagnostics.





polymerase chain reaction

The three-step process of the polymerase chain reaction.

Encyclopædia Britannica, Inc.

PCR was developed in 1983 by Kary B. Mullis, an American biochemist who won the Nobel Prize for Chemistry in 1993 for his invention. Before the development of PCR, the methods used to amplify, or generate copies of, recombinant DNA fragments were time-consuming and labour-intensive. In contrast, a machine designed to carry out PCR reactions can complete many rounds of replication, producing billions of copies of a DNA fragment, in only a few hours.

The PCR technique is based on the natural processes a cell uses to replicate a new DNA strand. Only a few biological ingredients are needed for PCR. The integral component is the template DNA—i.e., the DNA that contains the region to be copied, such as a gene. As little as one DNA molecule can serve as a template. The only information needed for this fragment to be replicated is the sequence of two short regions of nucleotides (the subunits of DNA) at either end of the region of interest. These two short template sequences must be known so that two primers—short stretches of nucleotides that correspond to the template sequences—can be synthesized. The primers bind, or anneal, to the template at their complementary sites and serve as the starting point for copying. DNA synthesis at one primer is directed toward the other, resulting in replication of the desired intervening sequence. Also needed are free nucleotides used to build the new DNA strands and a DNA polymerase, an enzyme that does the building by sequentially adding on free nucleotides according to the instructions of the template.

PCR is a three-step process that is carried out in repeated cycles.

The initial step is the denaturation, or separation, of the two strands of the DNA molecule. This is accomplished by heating the starting material to temperatures of about 95 °C (203 °F). Each strand is a template on which a new strand is built.

In the second step the temperature is reduced to about 55 °C (131 °F) so that the primers can anneal to the template.

In the third step the temperature is raised to about 72 °C (162 °F), and the DNA polymerase begins adding nucleotides onto the ends of the annealed primers. At the end of the cycle, which lasts about five minutes, the temperature is raised and the process begins again. The number of copies doubles after each cycle. Usually 25 to 30 cycles produce a sufficient amount of DNA.

In the original PCR procedure, one problem was that the DNA polymerase had to be replenished after every cycle because it is not stable at the high temperatures needed for denaturation. This problem was solved in 1987 with the discovery of a heat-stable DNA polymerase called *Taq*, an enzyme isolated from the thermophilic bacterium *Thermus aquaticus*, which inhabits hot springs. *Taq* polymerase also led to the invention of the PCR machine.

Because DNA from a wide range of sources can be amplified, the technique has been applied to many fields. PCR is used to diagnose genetic disease and to detect low levels of viral infection. In forensic medicine it is used to analyze minute traces of blood and other tissues in order to identify the donor by his genetic "fingerprint." The technique has also been used to amplify DNA fragments found in preserved tissues, such as those of a 40,000-year-old frozen woolly mammoth or of a 7,500-year-old human found in a peat bog.