IDHAYA COLLEGE FOR WOMEN, KUMBAKONAM

DEPARTMENT OF MICROBIOLOGY

COURSE	**:**	**B.Sc., III MICROBIOLOGY**
SEMESTER	**:**	**VI**
TOPIC	**:**	**UNIT-V**
SUBJECT NAME	**:**	**RECOMBINANT DNA TECHNOLOGY**
SUB CODE	**:**	**16SMBEMB2**

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BLOTTING TECHNIQUES

INTRODUCTION

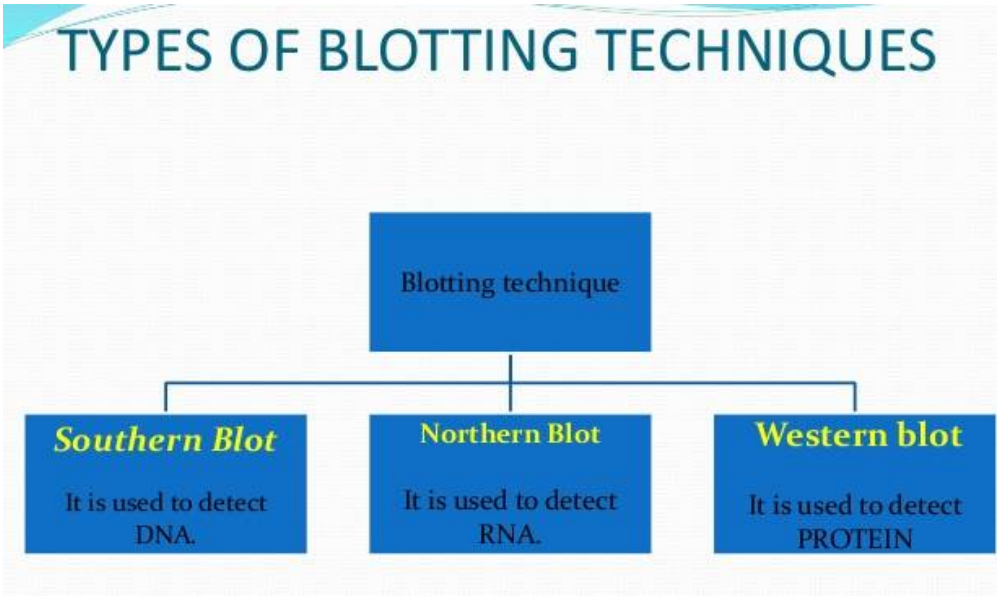
Blots are techniques for transferring DNA , RNA and proteins onto a carrier so they can be separated, and often follows the use of a gel electrophoresis. The Southern blot is used for transferring DNA, the Northern blot for RNA and the western blot for PROTEIN.

This is done after a gel electrophoresis, transferring the molecules from the gel onto the blotting membrane, and other times adding the samples directly onto the membrane.

After the blotting, the transferred proteins, DNA or RNA are then visualized by colorant staining (for example, silver staining of proteins), autoradiographic visualization of radiolabelled molecules (performed before the blot), or specific labelling of some proteins or nucleic acids.

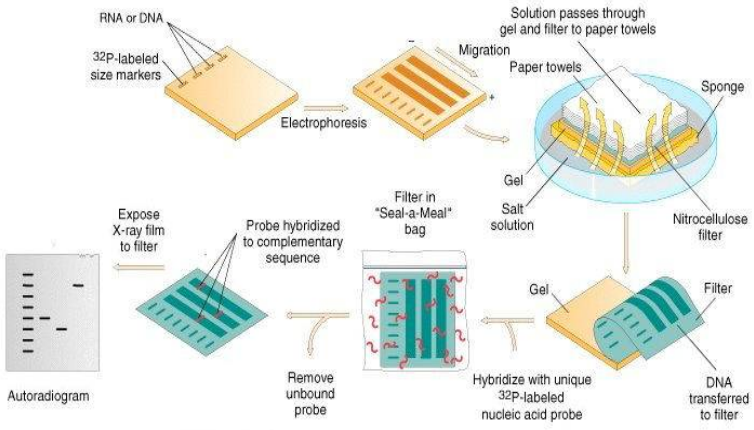
The latter is done with antibodies or hybridization probes that bind only to some molecules of the blot and have an enzyme joined to them.

After proper washing, this enzymatic activity (and so, the molecules we search in the blot) is visualized by incubation with proper reactive, rendering either a colored deposit on the blot or a chemiluminescent reaction which is registered by photographic film.



SOUTHERN BLOTTING

- A Southern blot is a method used in molecular biology for detection of a specific DNA sequence in DNA samples.
- Southern blotting combines transfer of electrophoresis-separated DNA fragments to a filter membrane and subsequent fragment detection by probe hybridization.
- Restriction endonucleases are used to cut high-molecular-weight DNA strands into smaller fragments.
- The DNA fragments are then electrophoresed on an agarose gel to separate them by size.
- If some of the DNA fragments are larger than 15 kb, then prior to blotting, the gel may be treated with an acid, such as dilute HCl.
- This depurinates the DNA fragments, breaking the DNA into smaller pieces, thereby allowing more efficient transfer from the gel to membrane.
- After the electrophoresis the separated DNA fragments are denaturated and transferred to a nitrocellulose (or nylon) membrane sheet by blotting.
- Southern blotting is designed to locate a particular sequence of DNA within a complex mixture.
- For example, Southern Blotting could be used to locate a particular gene within an entire genome.
- The amount of DNA needed for this technique is dependent on the size and specific activity of the probe.

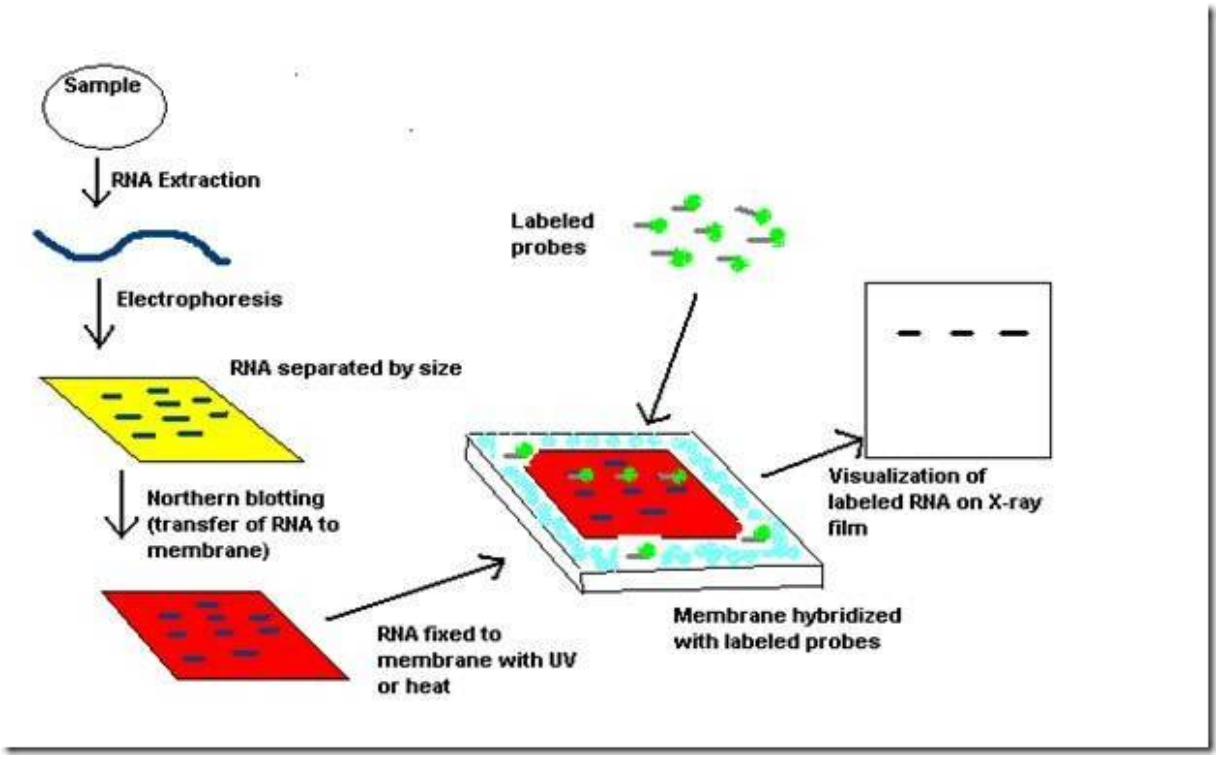


NORTHERN BLOTTING

The northern blot, or RNA blot, is a technique used in molecular biology research to study gene expression by detection of RNA (or isolated mRNA) in a sample.However, the entire process is commonly referred to as northern blotting.

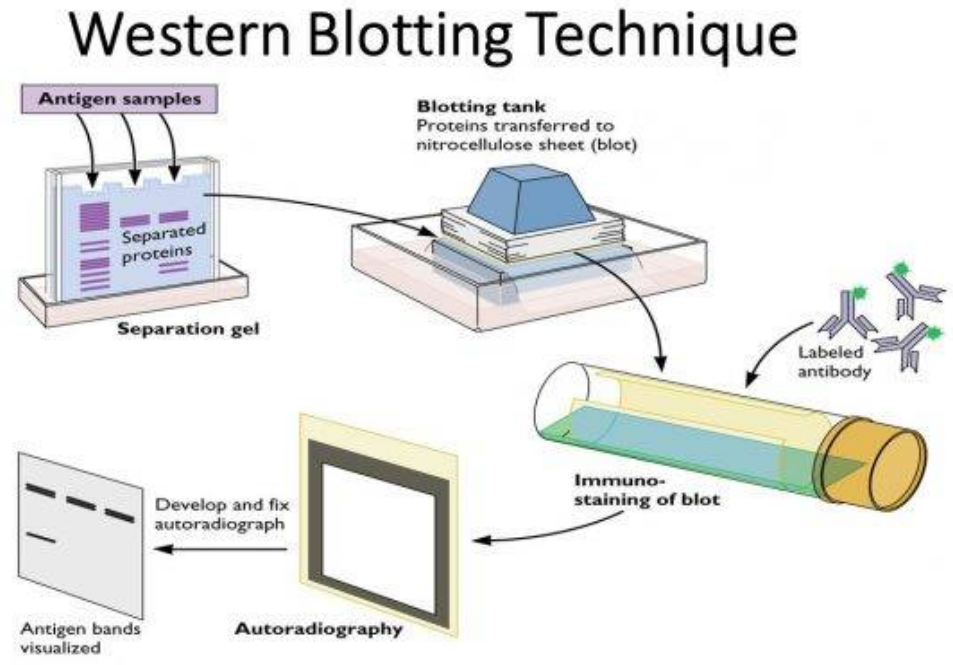
1. If the membrane was blotted from a glyoxal gel, soak the membrane in 20 mM Tris-HCl (pH 8.0) for 5 minutes to disassociate the glyoxal from the bound RNA.
2. Wet the membrane carrying the immobilized RNA in 6X SSC.
3. Place the membrane in a hybridization tube with the RNA-side facing in, and add ~1mL formamide prehybridization/hybridization solution per 10 cm^2. Alternatively one can use resealable bags. In which case, the membrane should be sealed into a bag after being wetted. Then a corner of the bag can be cut off to allow pipetting of the prehybridization/hybridization solution into the bag. The small corner can then be resealed.
4. Place the tube in a hybridization oven and incubate with rotation 15 minutes (3 hours if using a nitrocellulose membrane) at 42°C (DNA-probe) or 60°C (RNA-probe). If using resealable bags, it can be shaken or rocked slowly in a suitable incubator or water bath.
5. If the probe is dsDNA, denature by boiling for 5 minutes, and immediately place it on ice. Denatured probe should be added to the hybridization solution as soon as possible after denaturation.
6. Pipet the desired volume* of probe into the hybridization tube and continue to incubate with rotation overnight at 42°C (DNA-probe) or 60°C (RNA-probe). If using resealable bags the probe can be added by inserting a syringe into an uncut corner and injection of the probe. The bag must then be carefully be resealed to avoid leaking radioactivity. * The probe concentration in the hybridization solution should be 10 ng/mL if the specific activity is 10^8 dpm/µg or 2 ng/mL if the specific activity is 10^9 dpm/µg.
7. Pour off the hybridization solution into the radioactive waste, and replace with at least equal volume of 2X SSC/0.1% SDS. If using resealable bags, membrane can be transferred to a plastic dish for the following washes.
8. Incubate at room temperature for 5 minutes. Pour wash solution into radioactive waste, replace wash solution and repeat.

9. Low stringency wash: Replace wash solution with 0.2X SSC/0.1% SDS and incubate for 5 minutes with rotation at room temperature. Change wash solution and repeat.
10. Medium stringency wash (optional): Change wash solution with prewarmed (42°C) 0.2X SSC/0.1% SDS and incubate with rotation at 42°C for 15 minutes. Repeat once.
11. High stringency wash (optional): Change wash solution with prewarmed (68°C) 0.2X SSC/0.1% SDS and incubate with rotation at 68°C for 15 minutes. Repeat once.
12. Remove final wash and rinse membrane in 2X SSC at room temperature. Blot excess liquid and cover in UV-transparent (SaranWrap) plastic wrap. Do not allow membrane to dry out if it is to be stripped and reprobed.
13. Perform autoradiography making sure to mark the orientation of the membrane on the film.



WESTERN BLOTTTTING

- Western blot is often used in research to separate and identify proteins.
- In this technique a mixture of proteins is separated based on molecular weight, and thus by type, through gel electrophoresis.
- These results are then transferred to a membrane producing a band for each protein.
- Five steps are involved in western blotting procedure and detection assay, namely, transfer, blocking, primary antibody incubation, secondary antibody incubation and protein detection and western blotting analysis.
- The western blot is routinely used for verification of protein production after cloning. It is also used in medical diagnostics, e.g., in the HIV test or BSE-Test.
- The confirmatory HIV test employs a western blot to detect anti-HIV antibody in a human serum sample.



POLYMERASE CHAIN REACTION (PCR)

- Polymerase chain reaction, or PCR, is a technique to make many copies of a specific DNA region *in vitro* (in a test tube rather than an organism).
- PCR relies on a thermostable DNA polymerase, *Taq* polymerase, and requires DNA primers designed specifically for the DNA region of interest.
- In PCR, the reaction is repeatedly cycled through a series of temperature changes, which allow many copies of the target region to be produced.
- PCR has many research and practical applications. It is routinely used in DNA cloning, medical diagnostics, and forensic analysis of DNA.

What is PCR?

Polymerase chain reaction (PCR) is a common laboratory technique used to make many copies (millions or billions!) of a particular region of DNA. This DNA region can be anything the experimenter is interested in. For example, it might be a gene whose function a researcher wants to understand, or a genetic marker used by forensic scientists to match crime scene DNA with suspects.

Typically, the goal of PCR is to make enough of the target DNA region that it can be analyzed or used in some other way. For instance, DNA amplified by PCR may be sent for sequencing, visualized by gel electrophoresis, or cloned into a plasmid for further experiments.

PCR is used in many areas of biology and medicine, including molecular biology research, medical diagnostics, and even some branches of ecology.

Taq polymerase

Like DNA replication in an organism, PCR requires a DNA polymerase enzyme that makes new strands of DNA, using existing strands as templates. The DNA polymerase typically used in PCR

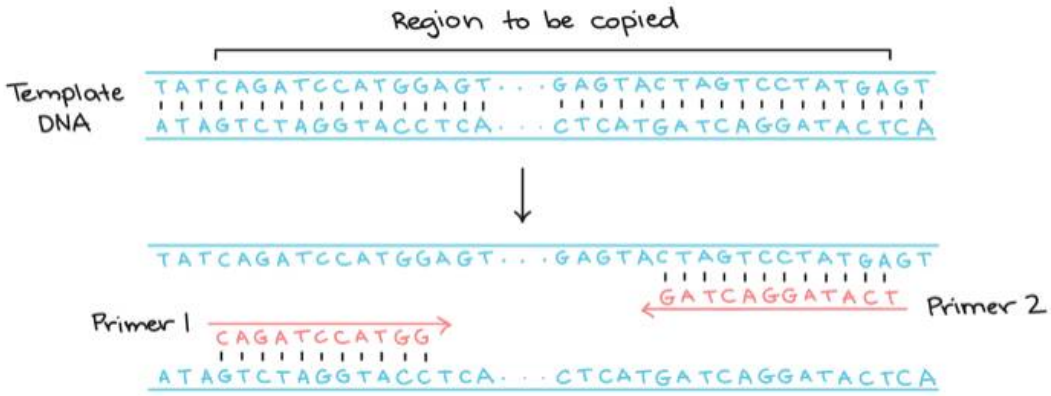
is called *Taq* polymerase, after the heat-tolerant bacterium from which it was isolated (*Thermus aquaticus*).

T. aquaticus lives in hot springs and hydrothermal vents. Its DNA polymerase is very heat-stable and is most active around 70°C70 °\text C70°C70, °, start text, C, end text (a temperature at which a human or *E. coli* DNA polymerase would be nonfunctional). This heat-stability makes Taq polymerase ideal for PCR. As we'll see, high temperature is used repeatedly in PCR to denature the template DNA, or separate its strands.

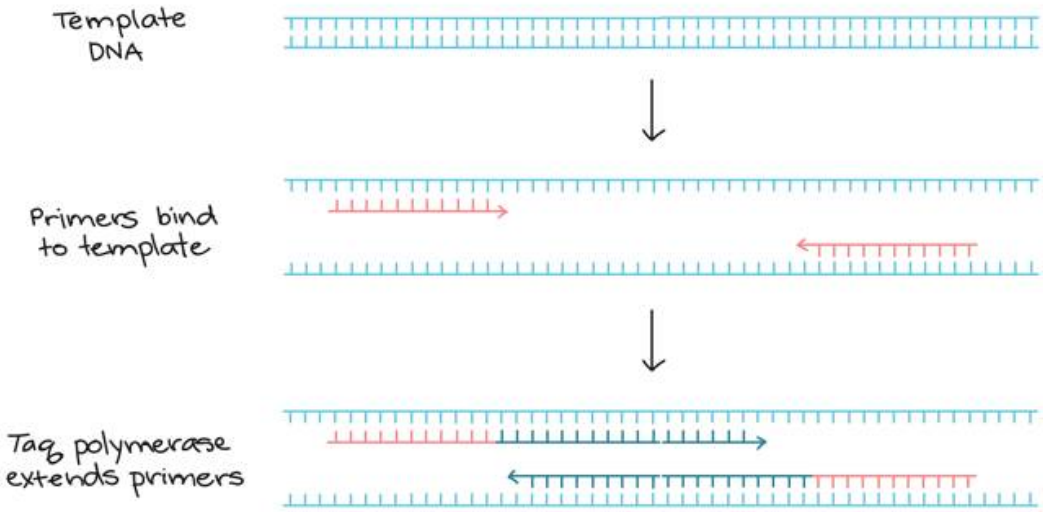
PCR primers

Like other DNA polymerases, *Taq* polymerase can only make DNA if it's given a primer, a short sequence of nucleotides that provides a starting point for DNA synthesis. In a PCR reaction, the experimenter determines the region of DNA that will be copied, or amplified, by the primers she or he chooses.

PCR primers are short pieces of single-stranded DNA, usually around 20202020 nucleotides in length. Two primers are used in each PCR reaction, and they are designed so that they flank the target region (region that should be copied). That is, they are given sequences that will make them bind to opposite strands of the template DNA, just at the edges of the region to be copied. The primers bind to the template by complementary base pairing.



When the primers are bound to the template, they can be extended by the polymerase, and the region that lies between them will get copied.

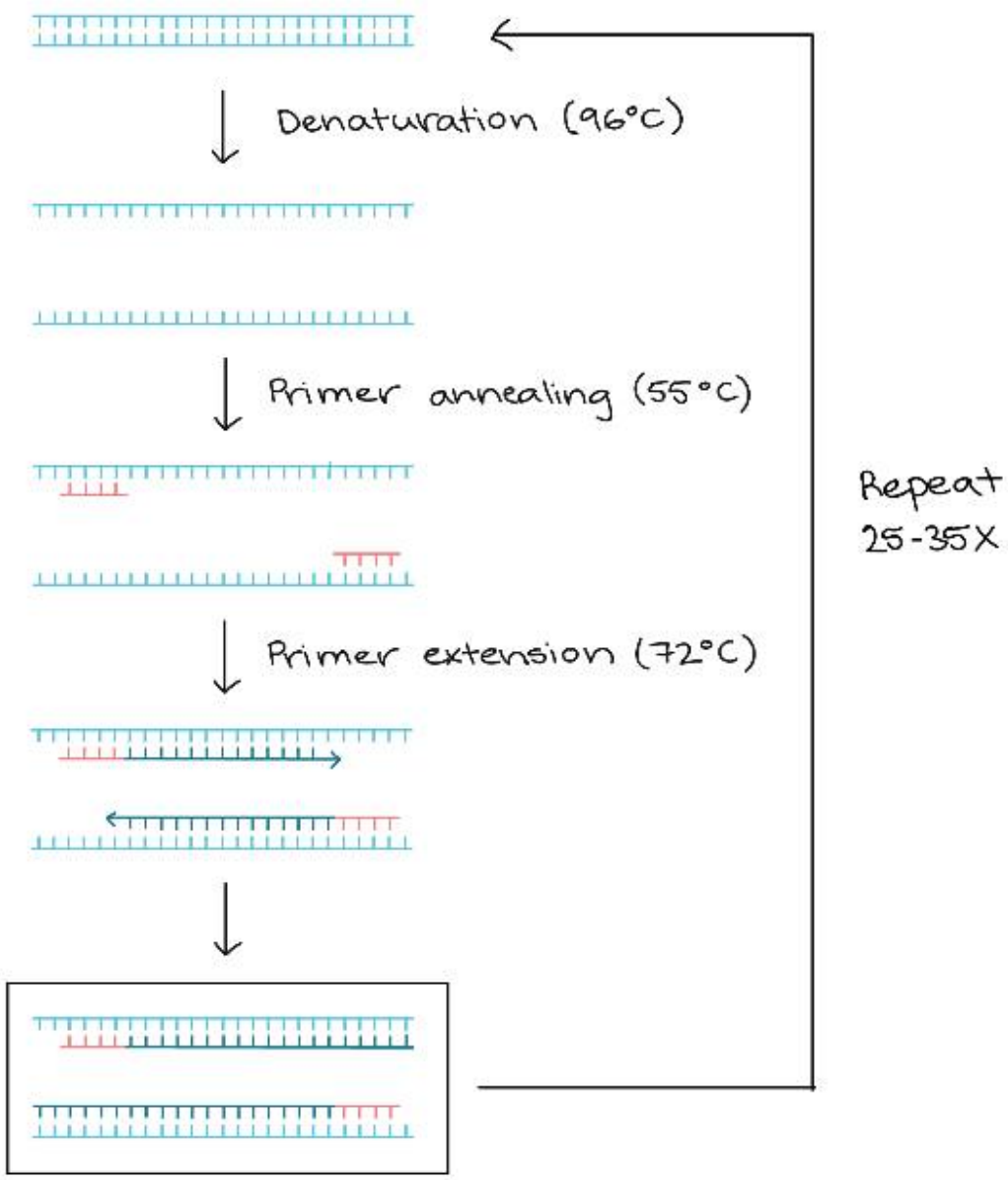


The steps of PCR

The key ingredients of a PCR reaction are *Taq* polymerase, primers, template DNA, and nucleotides (DNA building blocks). The ingredients are assembled in a tube, along with cofactors needed by the enzyme, and are put through repeated cycles of heating and cooling that allow DNA to be synthesized.

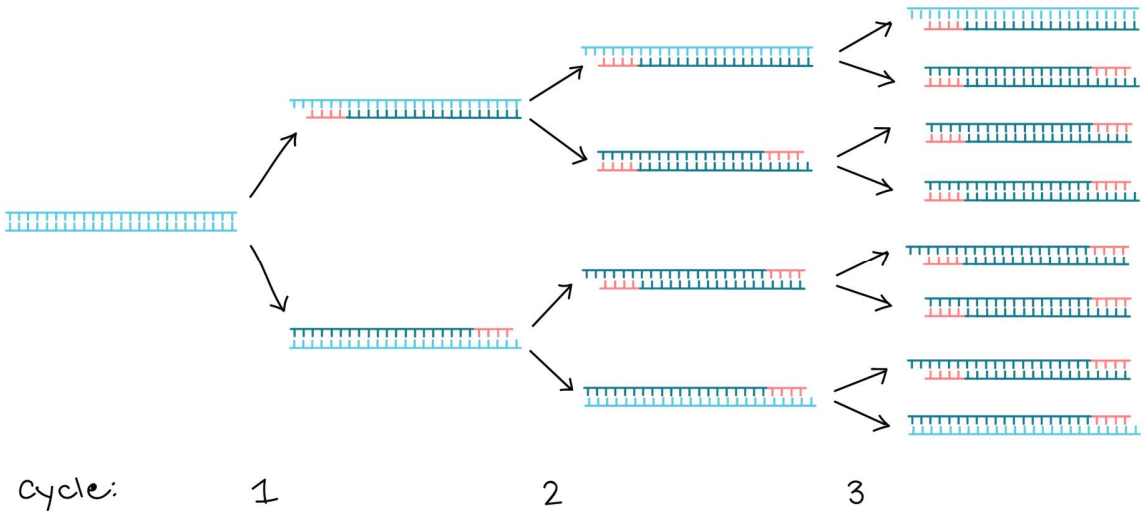
The basic steps are:

1. Denaturation (96°C96 °\text C96°C96, °, start text, C, end text): Heat the reaction strongly to separate, or denature, the DNA strands. This provides single-stranded template for the next step.
2. Annealing (55555555 - 65656565°C°\text C°C°, start text, C, end text): Cool the reaction so the primers can bind to their complementary sequences on the single-stranded template DNA.
3. Extension (72°C72 °\text C72°C72, °, start text, C, end text): Raise the reaction temperatures so *Taq* polymerase extends the primers, synthesizing new strands of DNA.



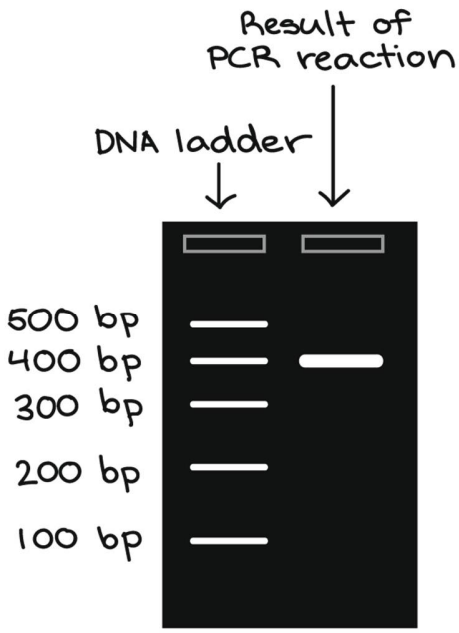
This cycle repeats 25252525 - 35353535 times in a typical PCR reaction, which generally takes 2222 - 4444 hours, depending on the length of the DNA region being copied. If the reaction is efficient (works well), the target region can go from just one or a few copies to billions.

That's because it's not just the original DNA that's used as a template each time. Instead, the new DNA that's made in one round can serve as a template in the next round of DNA synthesis. There are many copies of the primers and many molecules of *Taq* polymerase floating around in the reaction, so the number of DNA molecules can roughly double in each round of cycling. This pattern of exponential growth is shown in the image below.



The results of a PCR reaction are usually visualized (made visible) using gel electrophoresis. Gel electrophoresis is a technique in which fragments of DNA are pulled through a gel matrix by an electric current, and it separates DNA fragments according to size. A standard, or DNA ladder, is typically included so that the size of the fragments in the PCR sample can be determined.

DNA fragments of the same length form a "band" on the gel, which can be seen by eye if the gel is stained with a DNA-binding dye. For example, a PCR reaction producing a 400400400400 base pair (bp) fragment would look like this on a gel:



Left lane: DNA ladder with 100, 200, 300, 400, 500 bp bands.

Right lane: result of PCR reaction, a band at 400 bp.

A DNA band contains many, many copies of the target DNA region, not just one or a few copies. Because DNA is microscopic, lots of copies of it must be present before we can see it by eye. This is a big part of why PCR is an important tool: it produces enough copies of a DNA sequence that we can see or manipulate that region of DNA.

APPLICATIONS OF PCR

Using PCR, a DNA sequence can be amplified millions or billions of times, producing enough DNA copies to be analyzed using other techniques. For instance, the DNA may be visualized by gel electrophoresis, sent for sequencing, or digested with restriction enzymes and cloned into a plasmid.

PCR is used in many research labs, and it also has practical applications in forensics, genetic testing, and diagnostics. For instance, PCR is used to amplify genes associated with genetic disorders from the DNA of patients (or from fetal DNA, in the case of prenatal testing). PCR can also be used to test for a bacterium or DNA virus in a patient's body: if the pathogen is present, it may be possible to amplify regions of its DNA from a blood or tissue sample.

RANDOM AMPLIFIED POLYMORPHIC DNA (RAPD)

Description

RAPDs are DNA fragments amplified by PCR using short synthetic primers (generally 10 bp) of random sequence. These oligonucleotides serve as both forward and reverse primer, and are usually able to amplify fragments from 1-10 genomic sites simultaneously. Amplified fragments, usually within the 0.5-5 kb size range, are separated by agarose gel electrophoresis, and polymorphisms are detected, after ethidium bromide staining, as the presence or absence of bands of particular sizes. These polymorphisms are considered to be primarily due to variation in the primer annealing sites, but they can also be generated by length differences in the amplified sequence between primer annealing sites.

Strengths

The main advantage of RAPDs is that they are quick and easy to assay. Because PCR is involved, only low quantities of template DNA are required. Since random primers are commercially available, no sequence data for primer construction are needed. Moreover, RAPDs have a very high genomic abundance and are randomly distributed throughout the genome.

Weaknesses

The main drawback of RAPDs is their low reproducibility, and hence highly standardized experimental procedures are needed because of their sensitivity to the reaction conditions. RAPD analyses generally require purified, high molecular weight DNA, and precautions are needed to avoid contamination of DNA samples because short random primers are used that are able to amplify D NA fragments in a variety of organisms. Altogether, the inherent problems of reproducibility make RAPDs unsuitable markers for transference or comparison of results among research teams working in a similar species and subject. As for most other multilocus techniques, RAPD markers are not locus-specific, band profiles cannot be interpreted in terms of loci and alleles (dominance of markers), and similar sized fragments may not be homologous.

Applications

RAPDs have been used for many purposes, ranging from studies at the individual level (e.g. genetic identity) to studies involving closely related species. RAPDs have also been applied in gene mapping studies to fill gaps not covered by other markers. Variants of the RAPD technique include Arbitrarily Primed Polymerase Chain Reaction (AP-PCR) which uses longer arbitrary primers than RAPDs, and DNA Amplification Fingerprinting (DAF) that uses shorter, 5-8 bp primers to generate a larger number of fragments. Multiple Arbitrary Amplicon Profiling (MAAP) is the collective term for techniques using single arbitrary primers.

RESTRICTION FRAGMENT LENGTH POLYMORPHISM (RFLP)

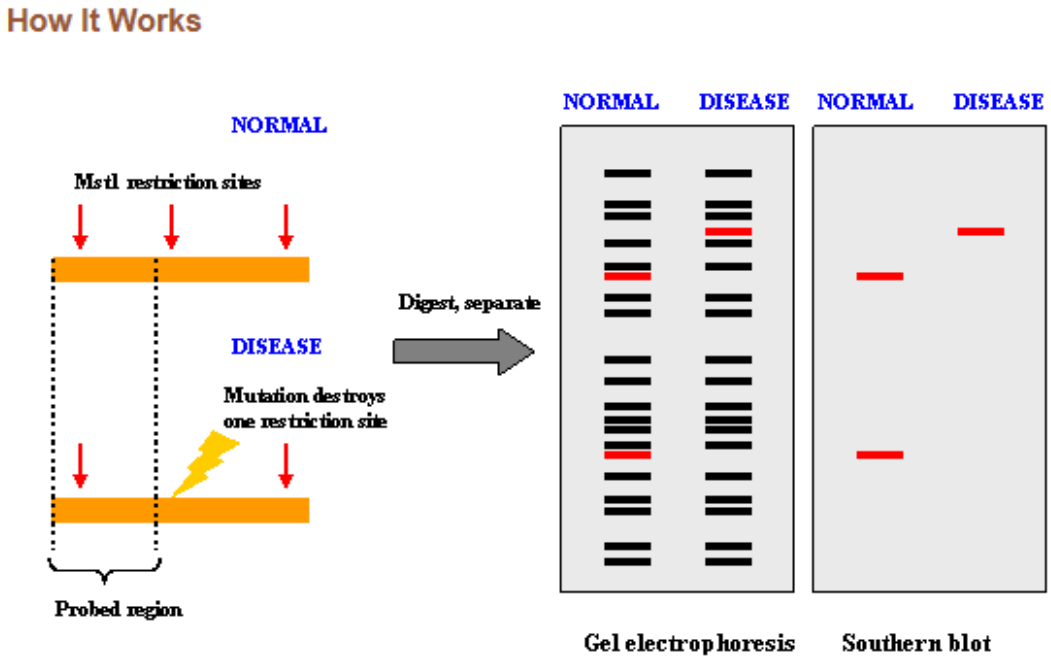
Restriction Fragment Length Polymorphism (RFLP)

is a difference in homologous DNA sequences that can be detected by the presence of fragments of different lengths after digestion of the DNA samples in question with specific restriction endonucleases. RFLP, as a molecular marker, is specific to a single clone/restriction enzyme combination.

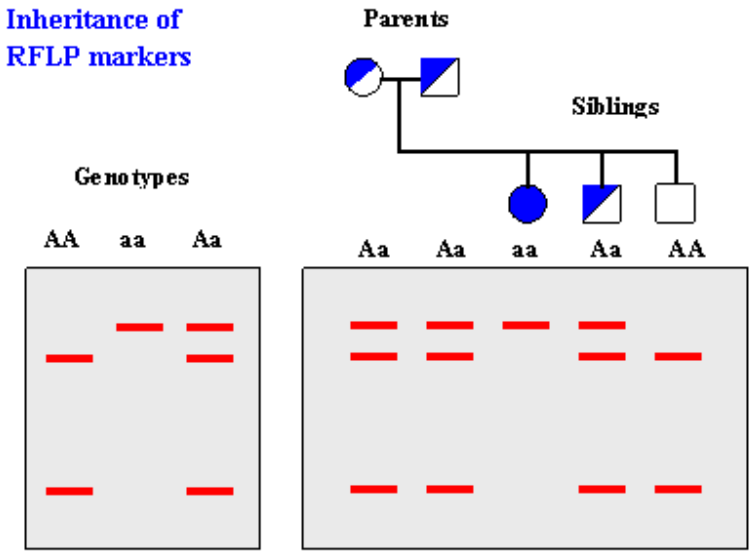
Most RFLP markers are co-dominant (both alleles in heterozygous sample will be detected) and highly locus-specific.

An RFLP probe is a labeled DNA sequence that hybridizes with one or more fragments of the digested DNA sample after they were separated by gel electrophoresis, thus revealing a unique blotting pattern characteristic to a specific genotype at a specific locus. Short, single- or low-copy genomic DNA or cDNA clones are typically used as RFLP probes.

The RFLP probes are frequently used in genome mapping and in variation analysis (genotyping, forensics, paternity tests, hereditary disease diagnostics, etc.).



Genotyping



DNA FINGERPRINTING

DNA profiling, DNA testing, DNA analysis, Genetic profile, DNA identification, genetic fingerprinting, and genetic analysis are some of the popular names used for DNA fingerprinting.

The present method is employed usually in criminal verification and crime scene investigation. However, it is also applicable to establishing a relationship between two persons and to know someone's identity. The testing method is practiced not only for humans but also for any organisms present on earth.

Since 1984, it has been a gold-standard method for personal verification. Because of the unique DNA pattern, two persons differ biologically. And this mechanism is the basis of the present method.

DNA is our blueprint, basis of life, encodes proteins, and regulates gene expression. It is made up of sugar, phosphate, and nitrogenous bases. DNAs are located on chromosomes. The whole set of DNA or chromosomes is known as the genome.

Interestingly, there are several regions in our genome that are unique and hypervariable.

Every person or organism has different patterns for those regions using which a DNA fingerprinting is performed

What is DNA fingerprinting?

The technique of DNA fingerprinting changed the era of identification, characterization, and classification of organisms.

Notably, approximately 99% of our DNA (deoxyribose nucleic acid) is similar. A 0.1% difference is sufficient to make someone so unique. We are using this 0.1% portion for DNA profiling which is often known as DNA fingerprinting.

There is a reason, why only DNA is used,

In all bodily parts, tissues, and cells (except germ cells) our DNA is the same. Even, after the death of the individual, it remains the same. It can remain stable even after 1000 years.

As we know, 97% of our genome is non-coding, repetitive, and junk, protein formation is regulated by only 3% portion, we know it as genes.

History:

Since the discovery of DNA in 1953 by *Watson* and *Crick*, genetic science has become more advanced and accurate. To know more on DNA and history of genetics, please read this article: DNA: Definition, Structure, Function, Evidence, and Types

DNA fingerprinting technique was originally developed by a British scientist *Alec Jeffreys* in 1984. However, Dr. *Lalji Singh* is known as a father of DNA fingerprinting in India.

Using the restriction digestion length polymorphism, Jeffrey created the first DNA profile. His method was actually a combination of RFLP and autoradiography.

Now h ere we have to understand some basics before going further. First, let’s understand what are the satellite regions of chromosomes.

Satellites DNA:

In Genetics, the satellites are repetitive DNA regions, located on telomeres and centromeres and abnormal repeats halt DNA replication. It clearly indicates that satellites help to do proper replication. Mutation in those sequences causes the end replication errors or problems.

Note one thing here, these satellite DNAs are non-coding.

The role of a non-coding region:

Gene exists in nature because it can encode various proteins. Through the collective efforts of replication, transcription and translation, DNA, mRNA, and proteins form, respectively. But the DNA other than genes aren't able to construct any protein, however, as per recent findings, non-coding DNA regulates gene expression.

Cell, tissue, and organs specific gene expression is regulated by the loosely packed, junk, and non-coding DNA. Read more: Gene regulation.

Steps:

1. Collection of a biological sample- blood, saliva, buccal swab, semen, or solid tissue.
2. DNA extraction
3. Restriction digestion or PCR amplification
4. Agarose gel electrophoresis, capillary electrophoresis or DNA sequencing
5. Interpreting results

Process of DNA fingerprinting:

Sample collection, DNA extraction, digestion or amplification and analysis results are major steps.

Step 1: Sample collection:

DNA can be obtained from any bodily sample or fluid. Buccal smear, saliva, blood, amniotic fluid, chorionic villi, skin, hair, body fluid, and other tissues are the major types of samples used.

In criminal cases, a buccal swab is taken usually. The buccal swab sample collection method is non-invasive and handy.

However, if not maintained properly, a buccal swab can easily be contaminated with bacteria. Further, the Buccal swab DNA yield is very less. A blood sample is a good replacement for a buccal swab sample. We can use a blood sample as well.

Step 2: DNA extraction

We have to first obtain DNA.

To perform any genetic applications, DNA extraction is one of the most significant steps. Good quality and quantity DNA increases the possibilities of getting good results.

You can use either of DNA extraction method enlisted below,

1. Phenol-chloroform DNA extraction method
2. CTAB DNA extraction method
3. Proteinase K DNA extraction method

Nevertheless, we strongly recommend using a ready to use DNA extraction kit for DNA fingerprinting.

The purity and quantity of DNA should be ~1.80 and 100ng, respectively to perform the DNA test. Purify the DNA using the DNA purification kit, if needed.

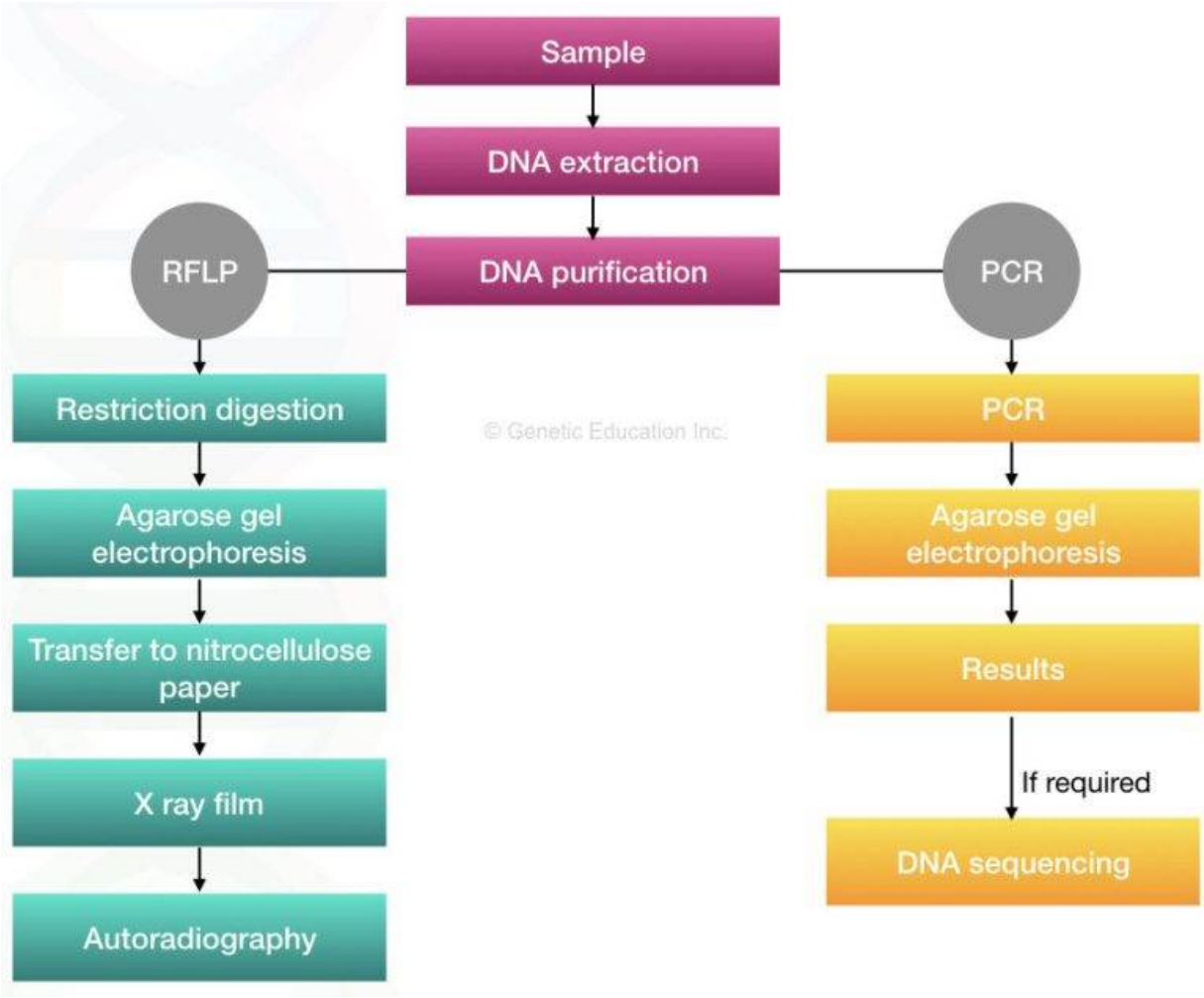
After that, quantify the DNA using the UV-Visible spectrophotometer. And perform one of the following method listed below,

DNA fingerprinting techniques:

Step 3: Restriction digestion, amplification or DNA sequencing

Three common methods are used:

1. RFLP based STR analysis
2. PCR based analysis
3. Real-time PCR analysis



APPLICATIONS OF RECOMBINANT DNA TECHNOLOGY

Recombinant DNA is widely used in biotechnology, medicine and research. Today, recombinant proteins and other products that result from the use of DNA technology are found in essentially every western pharmacy, physician or veterinarian office, medical testing laboratory, and biological research laboratory.

In addition, organisms that have been manipulated using recombinant DNA technology, as well as products derived from those organisms, have found their way into many farms,

supermarkets, home medicine cabinets, and even pet shops, such as those that sell GloFish and other genetically modified animals.

The most common application of recombinant DNA is in basic research, in which the technology is important to most current work in the biological and biomedical sciences. Recombinant DNA is used to identify, map and sequence genes, and to determine their function. rDNA probes are employed in analyzing gene expression within individual cells, and throughout the tissues of whole organisms. Recombinant proteins are widely used as reagents in laboratory experiments and to generate antibody probes for examining protein synthesis within cells and organisms.

Many additional practical applications of recombinant DNA are found in industry, food production, human and veterinary medicine, agriculture, and bioengineering.[4] Some specific examples are identified below.

Recombinant chymosin

Found in rennet, chymosin is an enzyme required to manufacture cheese. It was the first genetically engineered food additive used commercially. Traditionally, processors obtained chymosin from rennet, a preparation derived from the fourth stomach of milk-fed calves. Scientists engineered a non-pathogenic strain (K-12) of *E. coli* bacteria for large-scale laboratory production of the enzyme. This microbiologically produced recombinant enzyme, identical structurally to the calf derived enzyme, costs less and is produced in abundant quantities. Today about 60% of U.S. hard cheese is made with genetically engineered chymosin. In 1990, FDA granted chymosin "generally recognized as safe" (GRAS) status based on data showing that the enzyme was safe.

Recombinant human insulin

Almost completely replaced insulin obtained from animal sources (e.g. pigs and cattle) for the treatment of insulin-dependent diabetes. A variety of different recombinant insulin preparations are in widespread use.[15] Recombinant insulin is synthesized by inserting the human insulin gene into *E. coli*, or yeast (Saccharomyces cerevisiae)[16] which then produces insulin for human use.[17]

Recombinant human growth hormone (HGH, somatotropin)

Administered to patients whose pituitary glands generate insufficient quantities to support normal growth and development. Before recombinant HGH became available, HGH for therapeutic use was obtained from pituitary glands of cadavers. This unsafe practice led to some patients developing Creutzfeldt–Jakob disease. Recombinant HGH eliminated this problem, and is now used therapeutically. It has also been misused as a performance-enhancing drug by athletes and others.

Recombinant blood clotting factor VIII

A blood-clotting protein that is administered to patients with forms of the bleeding disorder hemophilia, who are unable to produce factor VIII in quantities sufficient to support normal blood coagulation.[20] Before the development of recombinant factor VIII, the protein was obtained by processing large quantities of human blood from multiple donors, which carried a very high risk of transmission of blood borne infectious diseases, for example HIV and hepatitis B.

Recombinant hepatitis B vaccine

Hepatitis B infection is controlled through the use of a recombinant hepatitis B vaccine, which contains a form of the hepatitis B virus surface antigen that is produced in yeast cells. The development of the recombinant subunit vaccine was an important and necessary development because hepatitis B virus, unlike other common viruses such as polio virus, cannot be grown in vitro. Vaccine information from Hepatitis B Foundation

Diagnosis of infection with HIV

Each of the three widely used methods for diagnosing HIV infection has been developed using recombinant DNA. The antibody test (ELISA or western blot) uses a recombinant HIV protein to test for the presence of antibodies that the body has produced in response to an HIV infection. The DNA test looks for the presence of HIV genetic material using reverse transcription polymerase chain reaction (RT-PCR). Development of the RT-PCR test was made possible by the molecular cloning and sequence analysis of HIV genomes. HIV testing page from US Centers for Disease Control (CDC)

Golden rice

A recombinant variety of rice that has been engineered to express the enzymes responsible for β-carotene biosynthesis. This variety of rice holds substantial promise for reducing the

incidence of vitamin A deficiency in the world's population. Golden rice is not currently in use, pending the resolution of regulatory and intellectual property issues.

Herbicide-resistant crops

Commercial varieties of important agricultural crops (including soy, maize/corn, sorghum, canola, alfalfa and cotton) have been developed that incorporate a recombinant gene that results in resistance to the herbicide glyphosate (trade name *Roundup*), and simplifies weed control by glyphosate application. These crops are in common commercial use in several countries.

Insect-resistant crops

Bacillus thuringeiensis is a bacterium that naturally produces a protein (Bt toxin) with insecticidal properties. The bacterium has been applied to crops as an insect-control strategy for many years, and this practice has been widely adopted in agriculture and gardening. Recently, plants have been developed that express a recombinant form of the bacterial protein, which may effectively control some insect predators. Environmental issues associated with the use of these transgenic crops have not been fully resolved.