I. Restriction enzymes (REases)

In gene cloning, restriction endonuclease enzymes play an important role in cutting the desired gene as well as cleaving the vector:

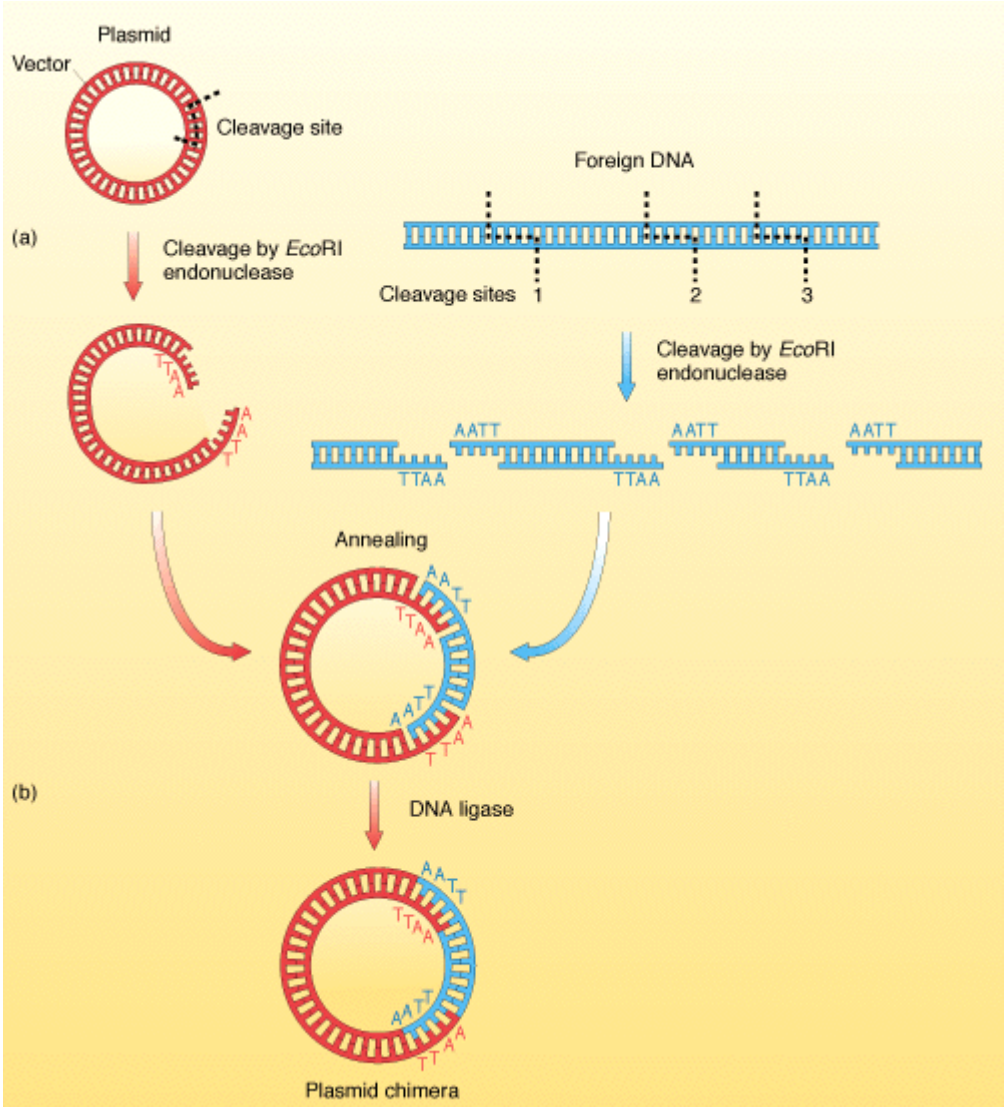
1. Cutting the gene:

The required DNA fragment from a large DNA molecule should be cleaved in a precise and repeatable manner for further genetic manipulations. This can be done using restriction enzymes or restriction endonucleases, which are naturally produced by many bacteria, and which cut DNA whenever a particular sequence of bases are recognized. They have a range of different properties, and recognize a wide variety of different sequences (some have longer recognition sequences and some have shorter ones). It is important that the restriction sites you want to clone into are unique so that you only cut the vector in one place. If the restriction enzyme cuts at several sites you might lose an important part of the vector.

2. Cutting the vectors:

The function of a vector DNA molecule is to carry a gene of interest to a second organism where it can express it and produce a gene specific product. During this technique the DNA to be cloned is integrated with the plasmid. Hence each vector molecule should be cleaved with same restriction site at a single position to open the circular form so that the new DNA fragment can be inserted at these complementary sites.

If foreign DNA is introduced into *E. coli* host, it may be attacked by restriction endonucleases active in a host cell. Because restriction phenomenon provides a natural defense against invasion by foreign DNA, so, it is usual to employ a K restriction deficient *E. coli* K12 strain as a host in transformation with newly created recombinant DNA molecules. This will eliminate the chance that the incoming sequence will be restricted.



Why bacteria produce REases?

Restriction endonuclease enzymes occur naturally in bacteria as a chemical weapon against the invading viruses.

Types:

There are four main groups of restriction endonucleases (REases) called Types I, II, III and IV, which differ primarily in structure, cleavage site, specificity, and cofactors.

Type I restriction systems consist of a single enzyme with 3 subunits that performs both modification (methylation) and restriction activities. These enzymes recognize specific DNA sequences, but cleave the DNA strand randomly, **at least 1,000 base pairs** (bp) away from the recognition site. Eg: HsdR is required for restriction, HsdM necessary for adding

methyl groups to host DNA (methyltransferase activity) and HsdS important for specificity of cut site recognition in addition to its methyltransferase activity.

Type III restriction systems have separate enzymes for restriction and methylation, but these enzymes share a common subunit. These enzymes recognize specific DNA sequences, but cleave DNA at random sequences approximately **twenty-five** bp from the recognition sequence.

Type IV enzymes target modified DNA, e.g. methylated, hydroxymethylated and glucosyl-hydroxymethylated DNA.

Neither type I, type III nor type IV restriction systems have found much application in recombinant DNA techniques.

Type II restriction enzymes; in contrast, are heavily used in recombinant DNA techniques (Restriction Mapping and Gene Cloning). Type II enzymes consist of single, separate proteins for restriction and modification. One enzyme recognizes and cuts DNA, the other enzyme recognizes and methylated the DNA. Type II restriction enzymes cleave the DNA sequence at the same site at which they recognize it.

Cleaving a single piece of DNA with multiple restriction enzymes creates a "DNA fingerprint." The pattern of fragments can be compared to similar DNA from another source treated with the same enzymes, to determine if the two are identical or different.

Type II restriction enzymes discovered to date collectively recognizes over 200 different DNA sequences. Type II restriction enzymes can cleave DNA into 2 types of cuts depending on whether they cut both strands at the center of the recognition sequence as follows:

– These enzymes cleave both DNA strands in the middle of a recognition sequence, generating blunt ends. These blunt ended fragments can be joined to any other DNA fragment with blunt ends, making these enzymes useful for certain types of DNA cloning experiments.

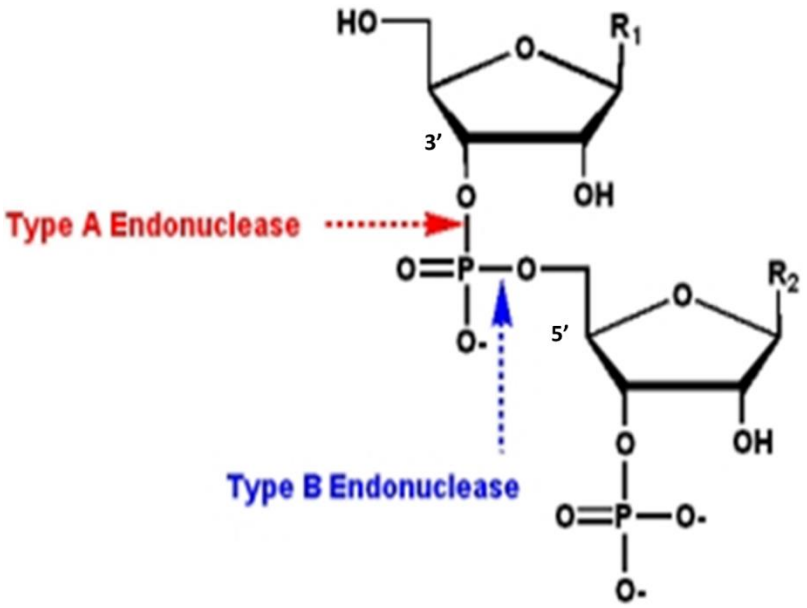
– Type II restriction enzymes can also cleave DNA to generate sticky (cohesive) ends and leave either a 3′ ("three prime") overhang or 5′ overhanging.

An overhang means that the restriction enzyme leaves a short single-stranded "tail" of DNA at the site where the DNA was cut. These 3′ overhanging ends can only join to another compatible 3′ overhanging end (that is, an end with the same sequence in the overhang). Finally, some type II enzymes can generate 5′ overhanging DNA ends, which can only be joined to a compatible 5′ end.

These overhanging ends are formed according to the way REases cleave on either side of the phosphodiester bond:

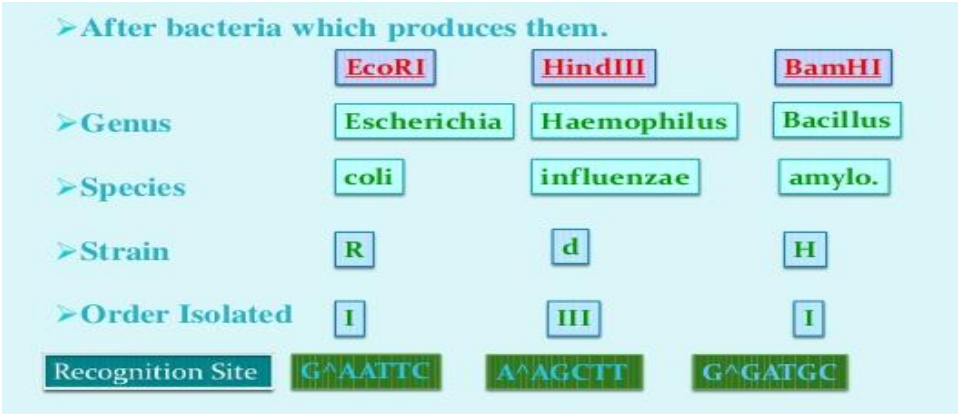
Type "A" nucleases cleave on the 3' side of the phosphodiester bond. Such a cleavage leaves the phosphate on the 5' side of the leaving polynucleotide.

Type "B" nucleases cleave on the 5' side of the phosphodiester bond. Such a cleavage leaves the phosphate remaining on the 3' end of the polynucleotide.



Nomenclature:

Since 1973, REases have been named based on an original suggestion by Smith and Nathans. They proposed that the enzyme names should begin with a three-letter acronym in which the first letter was the first letter of the genus from which the enzyme was isolated and the next two letters were the first two letters of the species name. The first three letters of the name were italicized. Extra letters or numbers could be added to indicate order of identification of the individual strains or serotypes.



Restriction Sites:

When a restriction endo-nuclease recognizes a particular sequence, it snips through the DNA molecule by catalyzing the hydrolysis (splitting of a chemical bond by addition of a water molecule) of the bond between adjacent nucleotides. To cut DNA, all restriction enzymes make two incisions, once through each sugar-phosphate backbone (i.e. each strand) of the DNA double helix.

The DNA sequences recognized by restriction enzymes are called palindromes. Palindromes are the base sequences that read the same on the two strands but in opposite directions. In addition, there is a point of symmetry within the palindrome.

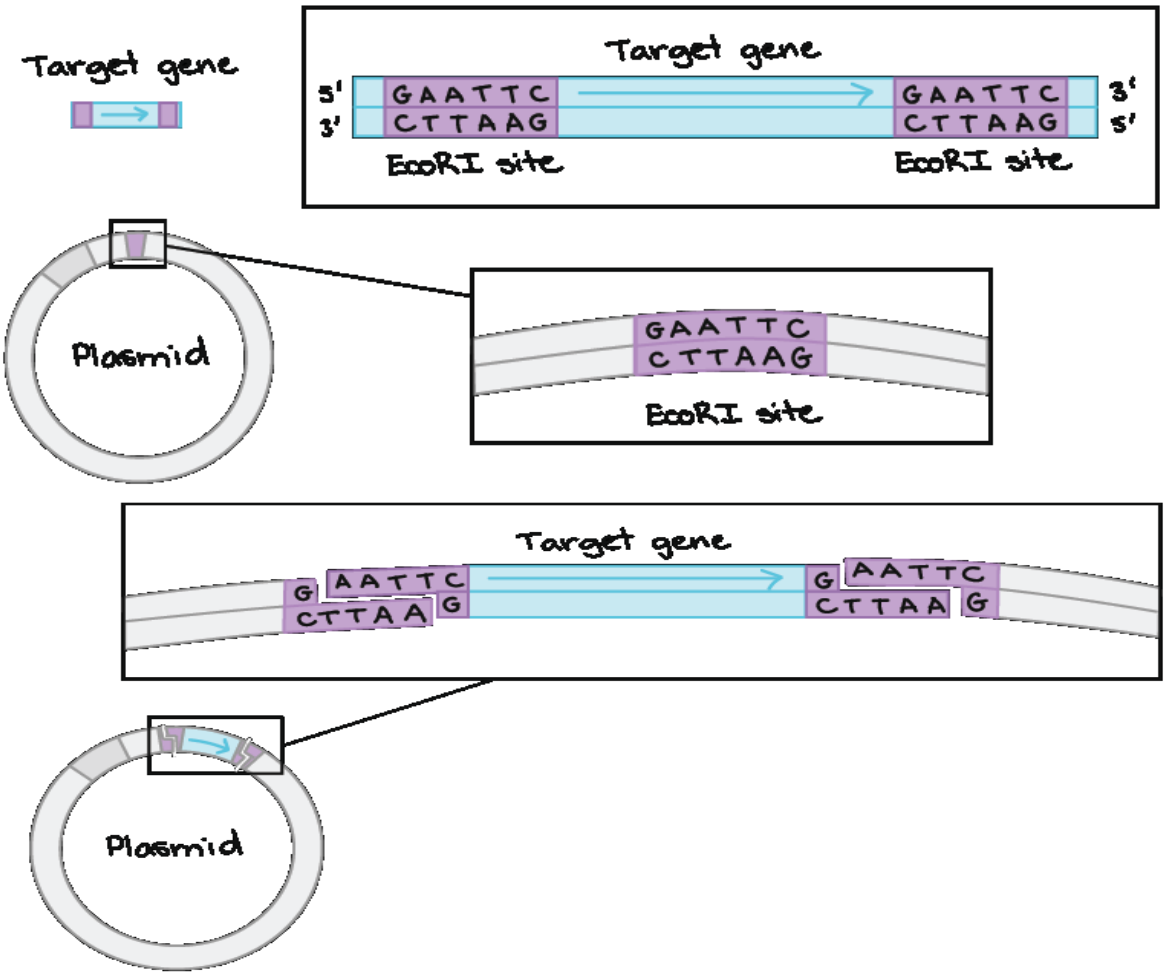
Eg if the sequence on one strand is GAATTC read in 5'→3′ direction, the sequence on the opposite strand is CTTAAG read in the 3'→5′ direction

The palindrome appears accordingly:

5′ GAATTC 3′

3′ CTTAAG 5′

So, there is a point of in the center between the AT/TA.



Types of DNA ends:

The value of restriction enzymes is that they make cuts in the DNA molecule around this point of symmetry. Some enzymes cut straight across the molecule at the symmetrical axis producing **blunt ends**.

Of more value, however, are the restriction enzymes that cut between the same two bases away from the point of symmetry on two strands, thus, producing a **staggered break**.

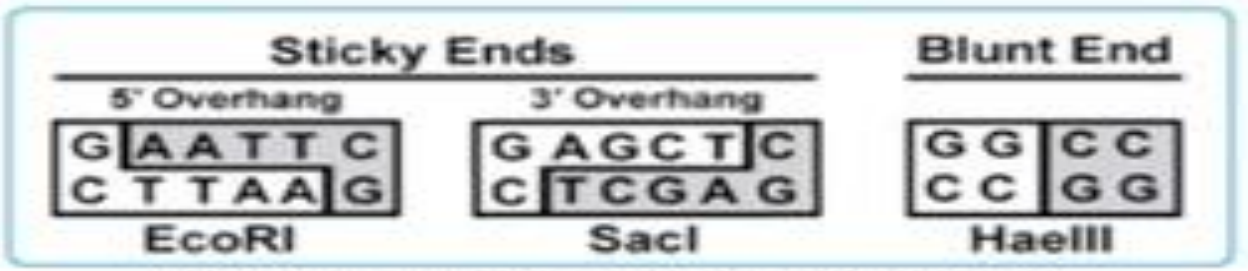


Figure 1 -- Different Types of DNA Ends Produced by Restriction Enzymes

TABLE 2. Some restriction enzymes, their origin and recognition sites

Enzyme	Organism	Recognition sequence (one strand mentioned)
*Eco*RI	*Escherichia coli* Ry13	5′G↓AATTC3′
*Bam*HI	*Bacillus amyloliquefaciens*	5′G↓GATCC3′
*Bgl*II	*Bacillus globigii*	5′A↓GATCT3′
*Hind*III	*Haemophilus influezae* R_d	5′A↓AGCTT3′
*Hin*fI	*Haemophilus influezae* R_f	5′G↓ANTC3′
*Sau*3A	*Staphylococcus aureus*	5′↓GATC3′
*Alu*I	*Arthobacter luteus*	5′AG↓CT3′
*Hae*III	*Haemophilus aegyptius*	5′GG↓CC3′
*Taq*I	*Thermus aquaticus*	5′T↓CGA3′
*Not*I	*Nocardia otitidis-caviarum*	5′GC↓GGCCGC3′
*Hpa*II	*Haemophylus parainfluenzae*	5′C↓CGG3′
*Pst*I	*Providentia stuartii*	5′CTGCAG3′
*Sma*I	*Serratia marcescens*	5′CCC↓GGG3′
*Xma*I	*Xanthomonas malvacearum*	5′C↓CCGGG3′

Table 13.1 Types of restriction endonucleases and their target sequences

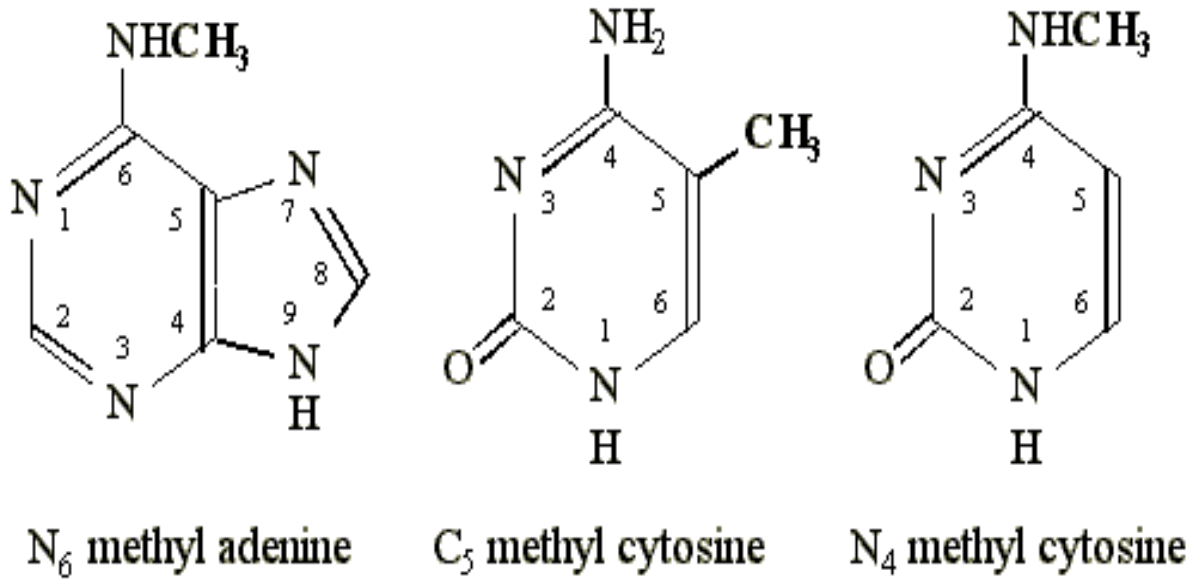
	Name of restriction systems	Target sequence	Type of DNA end
1.	Bam HI (*Bacillus amyloliquifaciens*)	G↓GATCC	5′ sticky end
2.	EcoRI (*Escherichia coli*)	G↓AATTC	5′ sticky end
3.	Hae III (*Haemophilus aegyticus*)	GG↓GC	Blunt end
4.	Sma I (*Serratia marcescence*)	CCC↓GGG	Blunt end
5.	Pst I (*Providentia stuartii*)	CTGCA↓G	3′ sticky end
6.	Hind III (*Haemophilus influenzae*)	A↓AGCTT	5′ sticky end
7.	Alu I (*Arthrobacter luteus*)	A↓GCT	5′ sticky end

Microorganisms	*Restriction enzymes*	*Cleavage sites*	*Cleavage products*
Bacillus amyloliquefaciens H	*Bam* HI	5-G↓GATCC-3 3-CCTAGG↑-5	5-G GATCC-3 3-CCTAG G-5
B. globigii	*Bgl* II	5-A↓GATCT-3 3-TCTAGA↑-5	5-A GATCT-3 3-TCTAG A-5
Escherchia coli RY13	*Eco* RI	5-G↓AATTC-3 3-CTTAAG↑-5	5-G AATTC-3 3-CTTAA G-5
Haemophilus influenzae Rd	*Hin* dIII	5-A↓AGCTT-3 3-TTCGAA↑-5	5-A AGCTT-3 3 -TTCGA A-5
H. parainfluenzae	*Hpa* I	5-GTT↓AAC-3 3-CAA↑TTG-5	5-GTT AAC-3 3-CAA TTG-5
Klebsiella pneumoniae OK 8	*Kpn* I	5-GGTAC↓C-5 3-C↑CATGG-3	5-GGTAC C-3 3-C CATGG-5
Streptomyces albus G	*Sal* I	5-G↓TCGAC-3 3-CAGCT↑G-5	5-G TCGAC-3 3-CAGCT G-5
Staphylococcus aureus 3AI	*Sau* 3AI	5-↓GATC-3 3-CTAG↑-5	5- GATC-3 3-CTAG 5

Host controlled restriction:

Certain strains of bacteria are immune to bacteriophages. This phenomenon is called host controlled restriction. This restriction is due to these restriction endonuclease enzymes (e.g., Eco RI) which could recognize and split specific loci in the foreign DNA. Thus these enzymes prevent or restrict the survival of foreign DNA in the host. This is analogous to an immune system.

All restriction sites in host chromosome of a bacterium are protected from its own restriction endonuclease enzyme due to a modification system. This system helps in preventing suicidal self-degradation. Such modification occurs by methylation of specific bases in the recognition sequence of the endonuclease. The enzymes involved in such modification are called methyltransferases. These enzymes methylate adenine (i.e., adds a methyl group to the base) in the N6 position and cytosine either in C5 or N4 position and produce 6 methyl adenine and 5 or 4 methyl cytosine, respectively.



N_6 methyl adenine C_5 methyl cytosine N_4 methyl cytosine

Unmodified foreign DNA entering the cell is degraded by the host restriction system. As both the enzymes, i.e., methyltransferases and endonucleases recognize the restriction site; they are together called as restriction and modification system.

Star Activity:

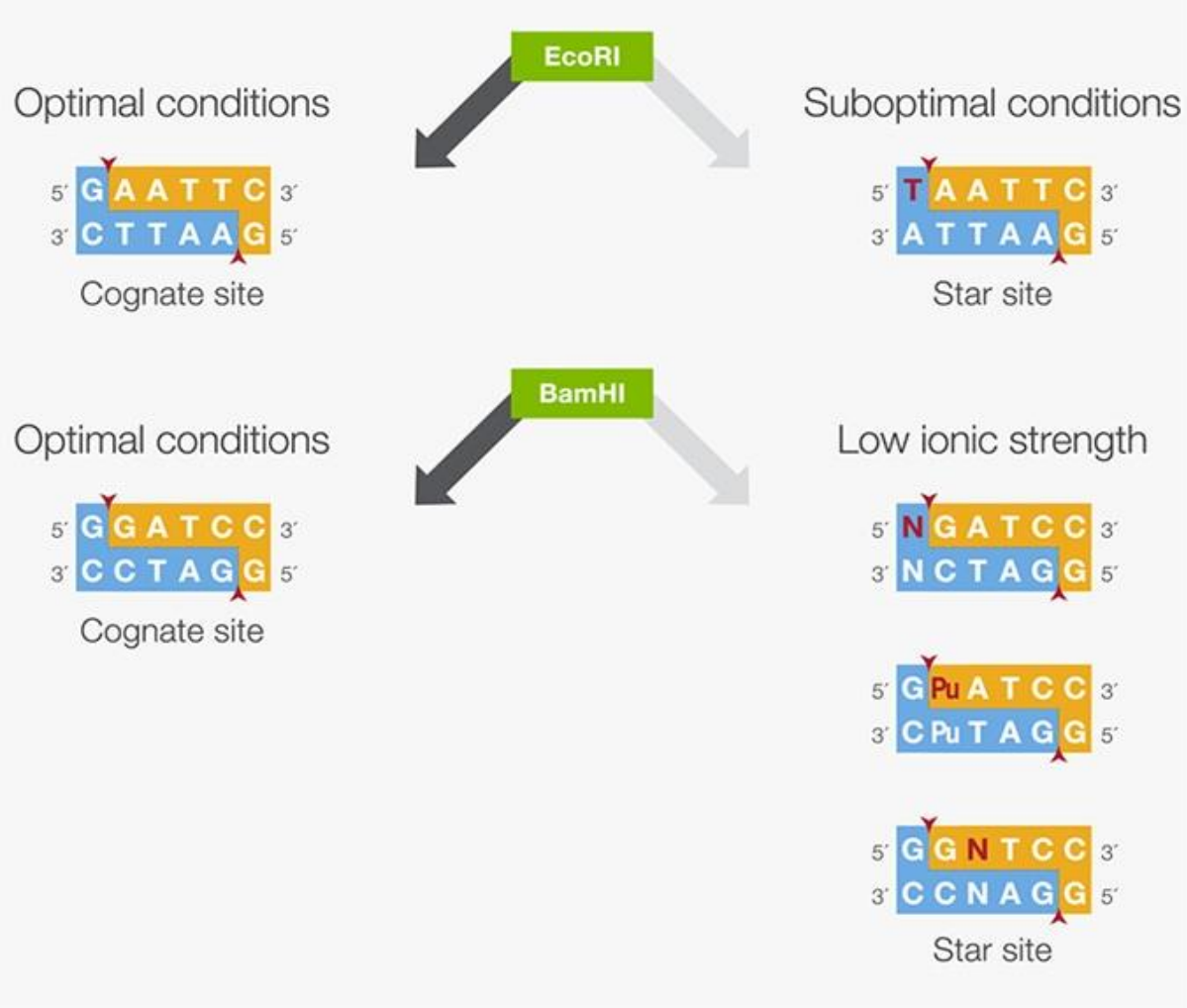
It is the relaxation or alteration of the specificity of restriction enzyme mediated cleavage of DNA under non-optimal conditions. Restriction enzyme may act on recognition sequences with minor differences from their main recognition sites which may produce a typical cleavage at non-specific recognition sites, or sometimes complete loss of specificity.

Differences which can lead to star include low ionic strength, high pH, high (> 5% v/v) glycerol concentrations and the presence of Mg^{2+} as well as the insufficient dilution of the enzyme solution can cause star activity; this problem most often arises during double or multiple digests.

For example, EcoRI recognizes and cleaves the site 5'-GAATTC-3', but its star activity may result in cleavage at 5'-TAATTC-3' and 5'-CAATTC-3'.

Similarly, BamHI may cut 5'-NGATCC-3', 5'-GPuATCC-3', and 5'-GGNTCC-3' in addition to its normal recognition sequence, 5'-GGATCC-3'.

* N in 5'-NGATCC-3'/ 5'-GGNTCC-3' means any nucleotide, Pu in 5'-GPuATCC-3'means purine.



Isoschizomers/ Neoschizomers/ Isocaudomer:

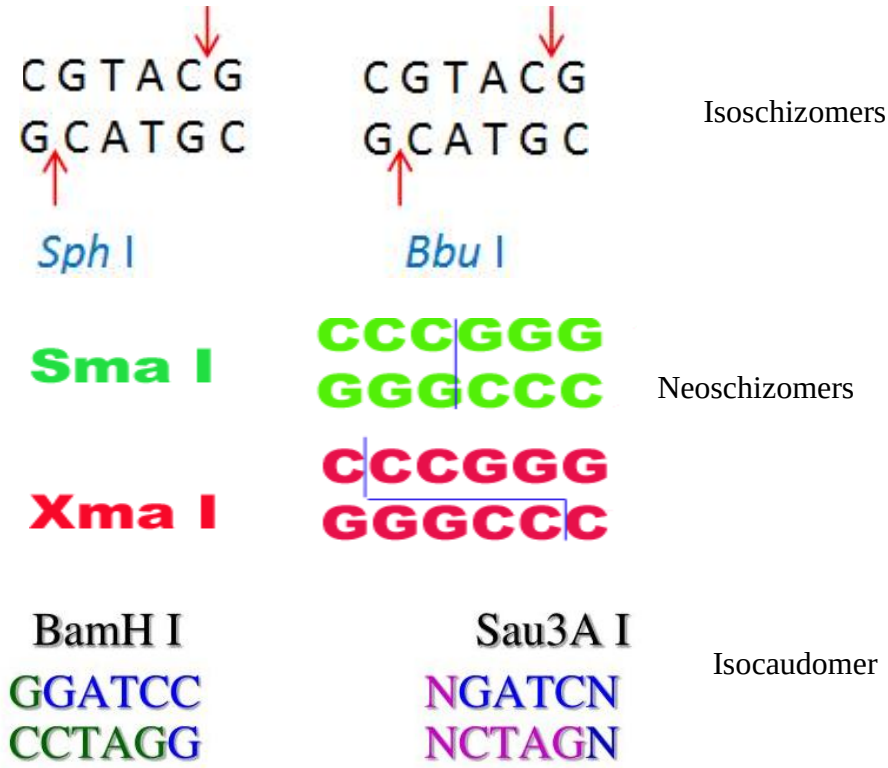
Isoschizomers are pairs of restriction enzymes , which are isolated from different bacteria, have the same recognition sequence and therefore may require different reaction conditions. For example, SphI (CGTAC/G) and BbuI (CGTAC/G) are isoschizomers of each other. All subsequently identified enzymes that recognize that sequence are isoschizomers.

In some cases, only one out of a pair of isoschizomers can recognize both the methylated as well as unmethylated forms of restriction sites. In contrast, the other restriction enzyme can recognize only the unmethylated form of the restriction site. This property of some isoschizomers allows identification of methylation state of the restriction site while isolating it from a bacterial strain.

For example, the restriction enzymes HpaII and MspI are isoschizomers, as they both recognize the sequence 5'-CCGG-3' when it is unmethylated. But when the second C of the sequence is methylated, only MspI can recognize it while, HpaII cannot.

An enzyme that recognizes the same sequence but cuts it differently is a **neoschizomer**. Neoschizomers are a specific type (subset) of isoschizomer. For example, SmaI (CCC/GGG) and XmaI (C/CCGGG) are neoschizomers of each other. Similarly, Kpn1 (GGTAC/C) and Acc651 (G/GTACC) are neoschizomers of each other.

An enzyme that recognizes a slightly different sequence, but produces the same ends is an **isocaudomer**. For example, BamH1 (G/GATCC) and Sau3AI (N/GATCN).



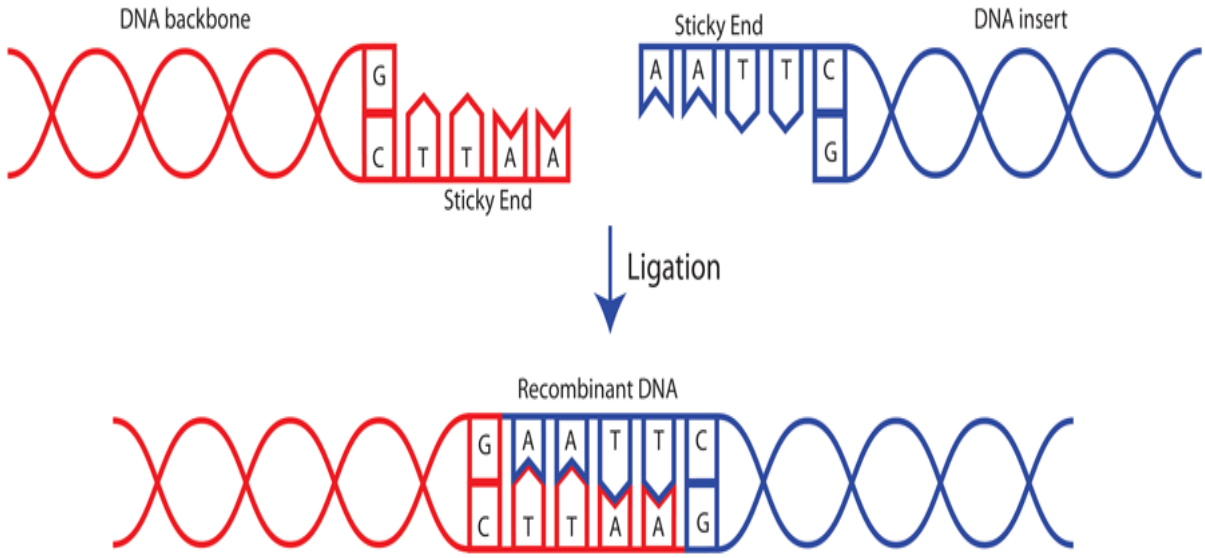
II. DNA Ligase

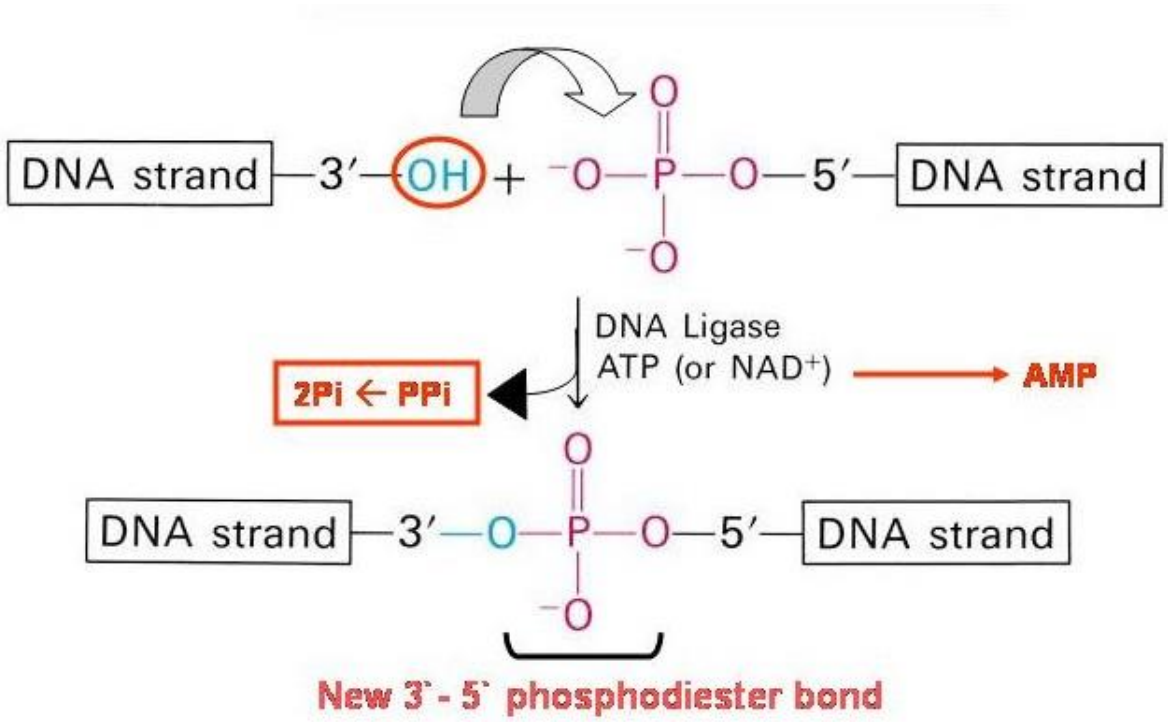
Having described a way of cutting DNA molecules up we now need to consider how to join them together in a new combination. The new molecule is called a recombinant.

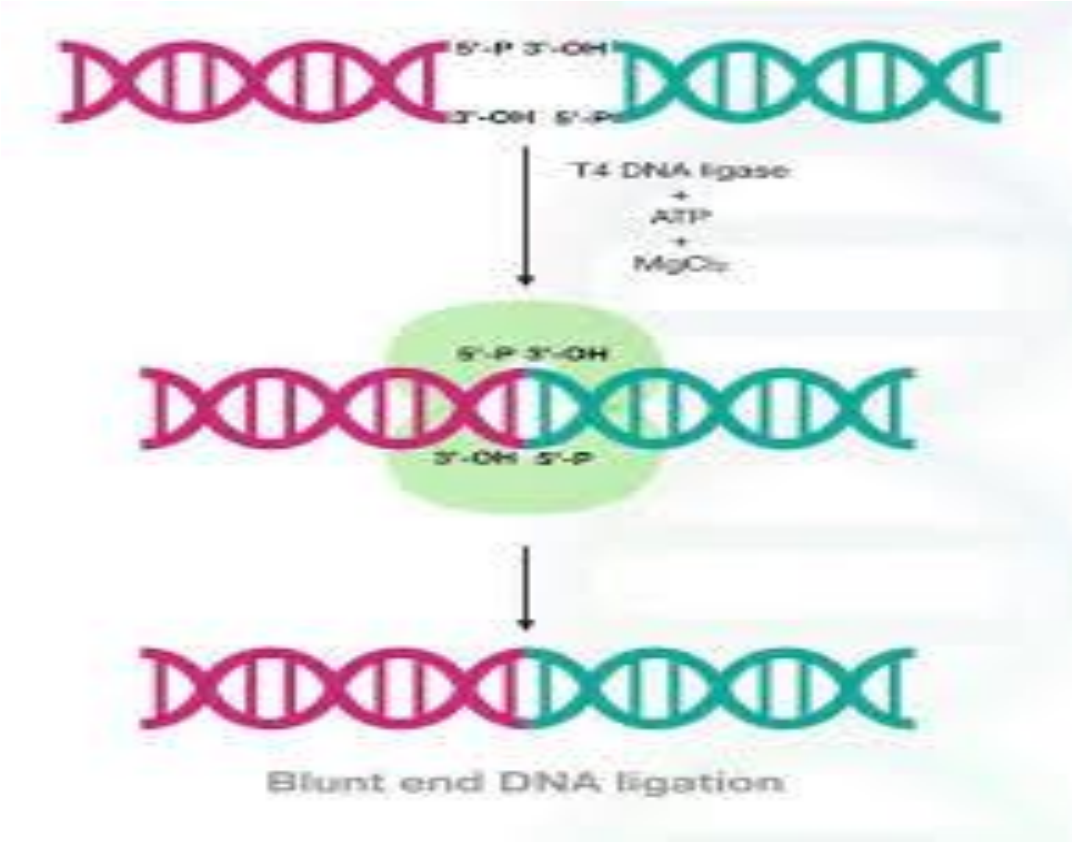
If the DNA has been cut up using a restriction enzyme like *Eco*RI, which produces sticky ends, then when two molecules with the same sticky ends come into contact, hydrogen bonding between the complementary bases will cause the molecules to stick together. This is in fact why these molecules are said to have sticky ends. This is not a very stable arrangement and the two molecules will soon drift apart again.

For gene cloning, you need to be able to covalently link the two molecules. The enzyme that is capable of doing this is called DNA ligase.

When two restriction fragments with sticky ends are transiently held together by hydrogen bonding there are in effect two single-stranded breaks in a double-stranded molecule; DNA ligase repairs these single-stranded breaks. DNA ligase catalyzes the formation of a covalent phosphodiester bond between the 5′ phosphate on one DNA strand and a 3′ hydroxyl on another. This process requires ATP as an energy source. The most commonly used DNA ligase is a protein produced by a T4 bacteriophage.







Blunt cut end: In case of the blunt cut end, the enzyme (e.g., Haelll, Smal) makes a simple double-stranded cut in the middle of the recognition sequence. Thus the blunt ends or flush ends are formed. The RE Hae III makes a cut in the 5′-GGCC-3′ target site The utility of generation of blunt end cuts during the joining of DNA fragments is that any pair of ends may be joined together irrespective of sequence. This is especially useful for those researchers who are interested to join two defined sequences without introducing any additional material between them.

Sticky cut end: Many restriction enzymes (e.g., Eco RI, Bam HI, and Hind III) make staggered, single-stranded cuts, producing short single-stranded projections at each end of the cleaved DNA, called sticky ends. Since the restriction sites are symmetrical, so that both strands have the same sequence when read in the 5′ to 3′ direction. Thus, such staggered cuts will generate identical single-stranded projections on the either site of the cut. These ends are not only identical, but complementary, and will base pair with each other; they are therefore, known as cohesive or sticky ends. Because of specificity of restriction enzymes, every copy of a given DNA molecule will give the same set of fragments when cleaved with a particular enzyme.

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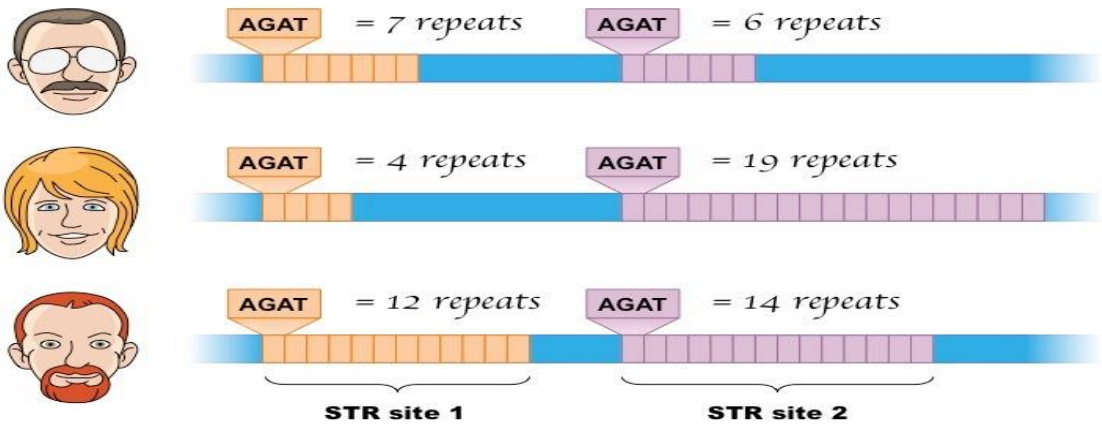
Applications of Gene cloning

Genetic engineering, molecular cloning, protein replication, and gene therapy are just some of the fields.

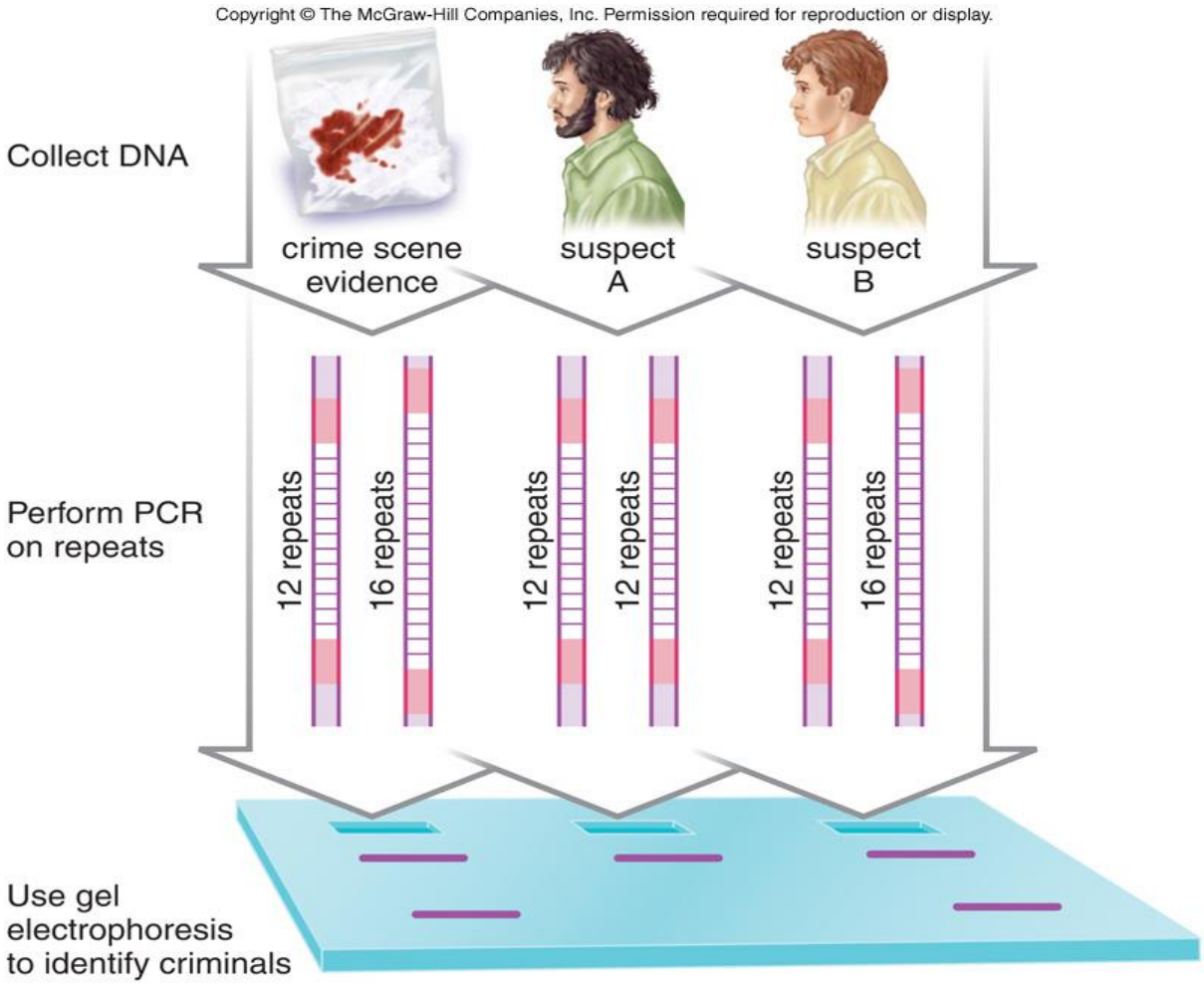
Gene cloning experiments can be useful in Forensics, Breeds, Foods, Medicals (diagnose and therapy) and Pharmaceuticals sciences.

Forensics:

One of the most high profile applications of DNA technology is in forensic science; you cannot read a newspaper article about a murder hunt, or watch a crime series on the television without hearing mention of DNA profiling. DNA profiling offers a way of looking at genetic loci which vary between individuals, and use these characteristics to identify individuals. The genetic loci used are regions of repetitive DNADNA profiling makes use of regions of the genome called microsatellite or short tandem repeat (STR) DNA. In these regions a short DNA sequence, of 5 bp or less, is repeated many times resulting in a repeat region of up to 150 bp. These STR loci are highly polymorphic with a wide variability in the number of repeats between individuals. Because of the problems encountered by DNA polymerase in copying highly repetitive DNA sequences these regions are copied incorrectly more often than non-repeat DNA. This slippage results in variation in the number of repeats within each microsatellite between individuals. DNA profiling uses these highly polymorphic STR loci in order to generate a pattern which can be used to identify individuals.

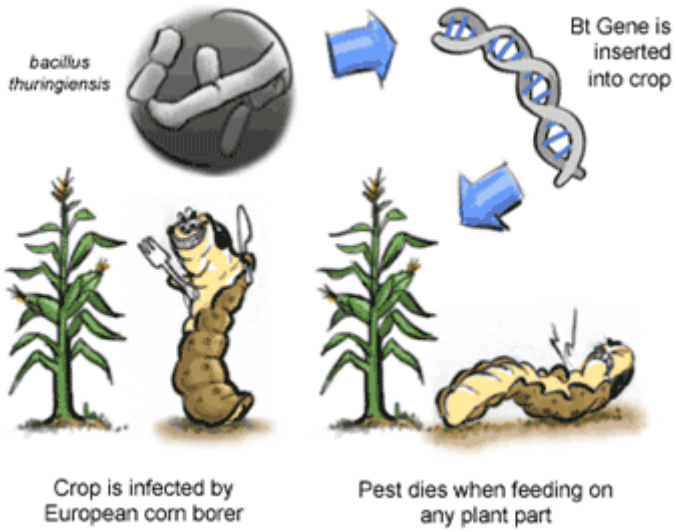


PCR has been key to the forensic analysis of variation between humans from the very beginning. It is amplification by PCR that makes it possible to analyze DNA found in small samples left inadvertently at a crime scene. Forensic samples are often blood, semen, hair, skin cells, plant or animal remains and without PCR it would usually be impossible to obtain sufficient DNA to perform tests; however in the case of DNA profiling PCR plays an even more integral role.



Breeds and Foods:

Researchers can use laboratory techniques to clone the resistance genes and introduce them into elite varieties of domestic crops to protect them against pathogens and pests such as rusts, powdery mildew and Hessian fly. By making crops more disease resistant (AgRenSeq*, speed cloning) it will help to improve yields and reduce the use of pesticides.



After years of detailed study and analysis, the Food and Drug Administration (FDA) has concluded that meat and milk from clones of cattle, and goats, and the offspring of clones from any species traditionally consumed as food, are as safe to eat as food from conventionally bred animals. This conclusion stems from an extensive study of animal cloning and related food safety, culminating in the release of three FDA documents in January 2008: a risk assessment, a risk management plan, and guidance for industry.

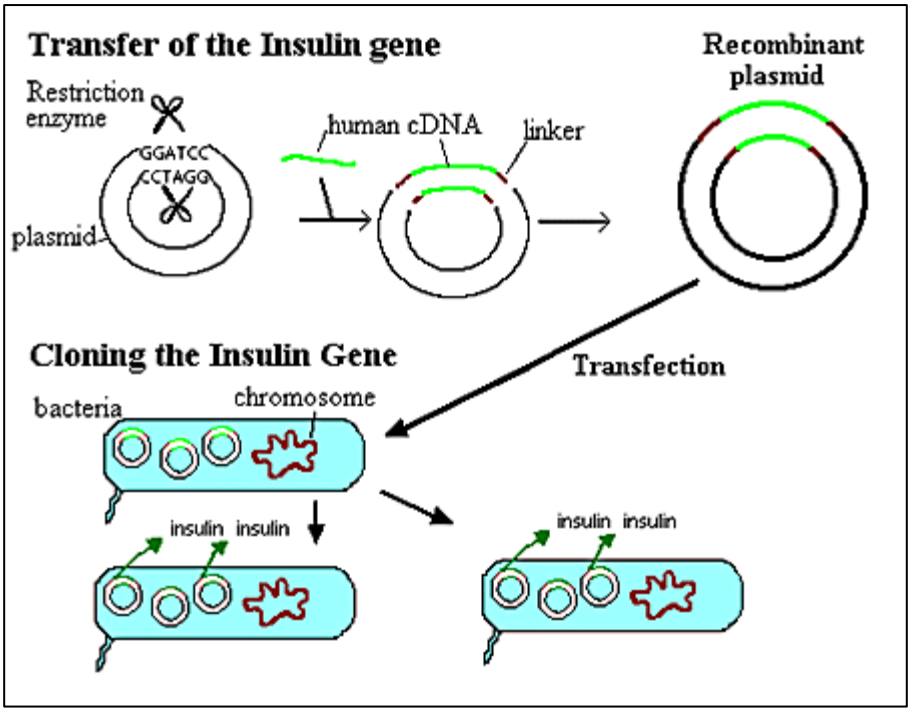
* AgRenSeq lets researchers search a library of resistance genes discovered in wild relatives of modern crops so they can rapidly identify sequences associated with disease fighting capability.

Medicals and Pharmaceuticals

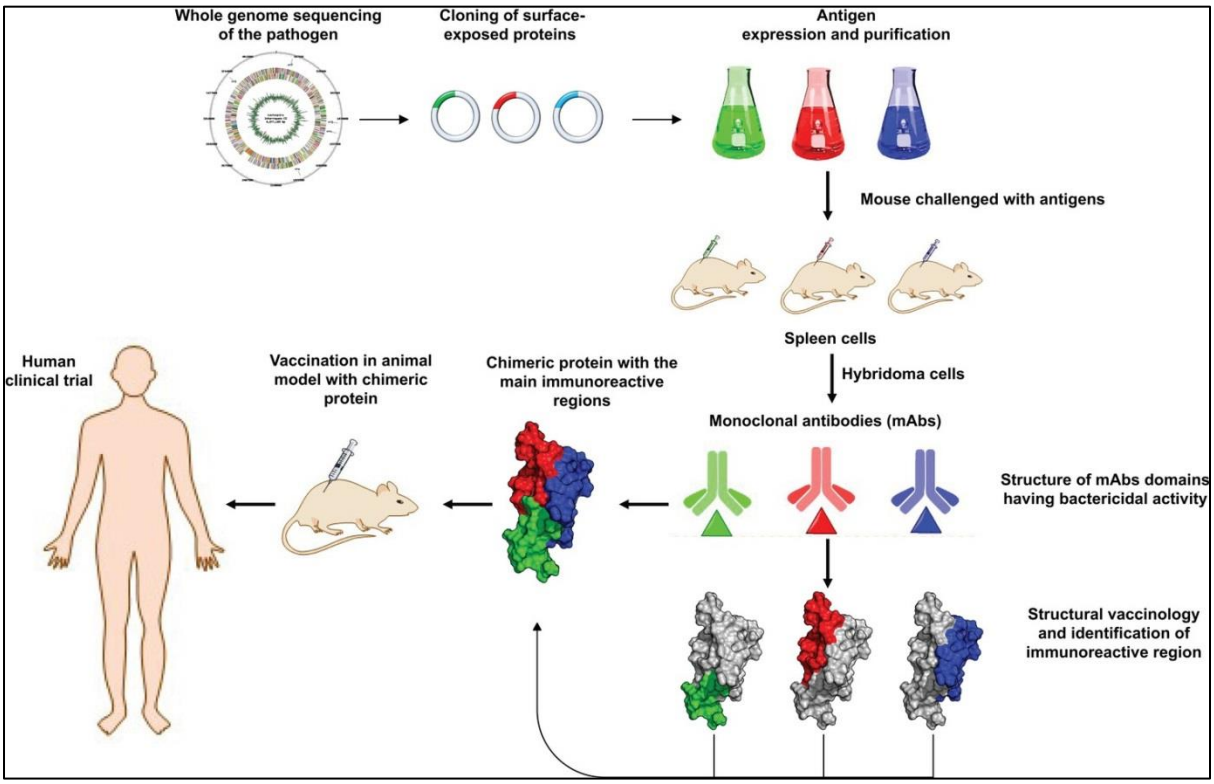
Molecular techniques are already routine in the diagnosis of genetic disease and the potential of the human genome project in allowing us to understand the functioning of the human body. As Plasmids are useful in cloning short segments of DNA, it can also be used to replicate proteins, such as the protein that codes for insulin, in large amounts. Additionally, plasmids are being investigated as a way to transfer genes into human cells as part of gene therapy. Cells may lack a specific protein if the patient has a hereditary disorder involving a gene mutation. Inserting a plasmid into DNA would allow cells to express a protein that they are lacking. Using transgenic animals to produce therapeutic products is the idea of "humanizing" animals as a donor of organs for use in xenotransplantation.

The use of cultured cells, whether bacteria or eukaryotic cells, offers a number of advantages. In many cases the only other possibility is to isolate the proteins from humans or animals. Proteins from humans carry the risk of transmitting disease, as in the case of HIV passed to hemophiliacs in factor VIII blood clotting factor. Proteins from other animals may not behave in exactly the same way as the human protein as in the case of insulin, one of the first pharmaceuticals to be produced by genetic engineering techniques.

Gene cloning has proved to be especially valuable in the production of safe and effective vaccines. Using this technology it is possible to clone specific proteins from a pathogen, for instance viral coat proteins from hepatitis B, produce these proteins in cultured cells and use them as a vaccine to elicit a protective immune response against the whole organism. These so-called subunit vaccines avoid many of the problems encountered with killed or attenuated vaccine preparations; the absence of whole organisms avoids any risk of causing an infection and also reduces side effects.



Many important pharmaceutical products are proteins and gene cloning is already used extensively in their production. Bacteria, yeast and tissue culture cells can be used in the production of proteins and pharmaceuticals. Introducing the idea of biopharming; of using transgenic plants or animals to produce pharmaceutical proteins: this technology is in its infancy, but it holds the promise of fields of plants or flocks of sheep cheaply and efficiently producing valuable pharmaceutical products. In most cases it is envisaged that the pharmaceutical product would be harvested and purified, although in the case of edible vaccines it is hoped that it will be possible to deliver the vaccine orally by eating the plant. The advantages of this approach are not only in the acceptability of this method of delivery to people who have an aversion to hypodermic needles, but also in being able to grow, harvest and store vaccines using low-tech farming techniques rather than expensive hightech pharmaceutical technology.


Whole genome sequencing of the pathogen
Cloning of surface-exposed proteins
Antigen expression and purification
Mouse challenged with antigens
Spleen cells
Hybridoma cells
Monoclonal antibodies (mAbs)
Structure of mAbs domains having bactericidal activity
Structural vaccinology and identification of immunoreactive region
Chimeric protein with the main immunoreactive regions
Vaccination in animal model with chimeric protein
Human clinical trial

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