RECOMBINANT DNA TECHNOLOGY

Gene Cloning/ Molecular Cloning/ Genetic Engineering

- Recombinant DNA technology is a technology that allows DNA to be produced through artificial means.
- It is a set of methods used to locate, analyse, alter, study and recombine DNA Sequences to combine genetic material from different organisms
- Recombinant DNA is the one in which nucleotide sequences from two different sources are combined in the laboratory to produce a new combination of genes (Hybrid DNA/ Chimeric DNA)
- Recombinant DNA technology is one of the recent advances in biotechnology, which was developed by two scientists named Herbert W. Boyer and Stanley N. Cohen in 1973

PRINCIPLE

- The DNA molecule (foreign/ insert/ target/ clone DNA) is inserted into another DNA molecule called VECTOR
- The recombinant vector is then introduced into a host cell where it replicates itself
- The recombinant gene is then produced

STEPS IN VOLVED IN RDT

- Identification and Isolation of DNA (gene of interest/ foreign DNA)
- Cutting DNA (mechanically/restriction enzymes)
- Separation and Visualization of DNA (gel electrophoresis)
- Joining DNA into a suitable vector (ligation)
- Amplifying DNA

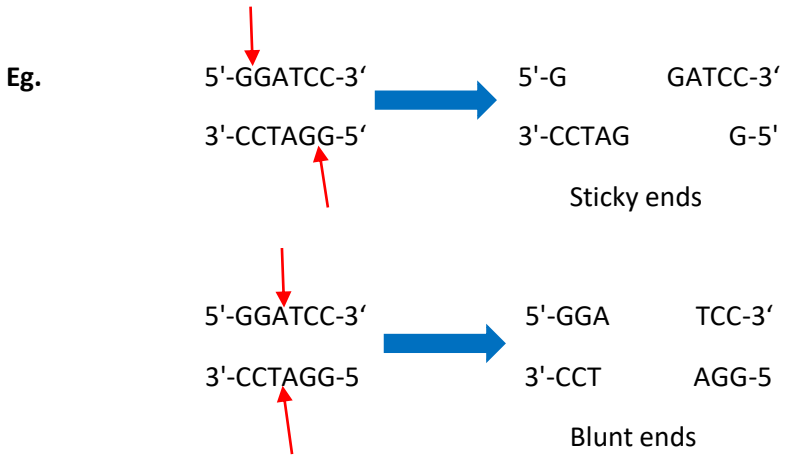
- Cloning – introduction of vector into a suitable organism/host cell (Transformation)
- Identification of clones/selection of recombinant cells with gene of interest (markers)
- Sequencing individual fragment, multiplication or expression of gene of interest

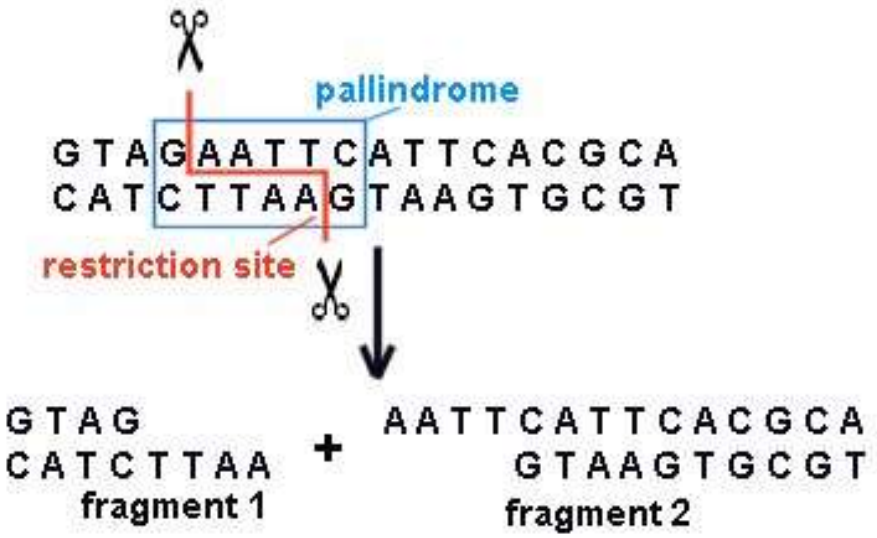
DNA EXTRACTION

- Cells are lysed using **lysozyme/EDTA** and **detergent** that disrupts the cell wall and plasma membrane respectively
- Cell contents are treated with **protease/phenol** to destroy protein and **ribonuclease/RNAase** to destroy RNA
- Cell debris is pelleted in a centrifuge. The supernatant containing the DNA is transferred to a clean tube
- The DNA is precipitated with **ethanol**. It forms viscous strands in the solution

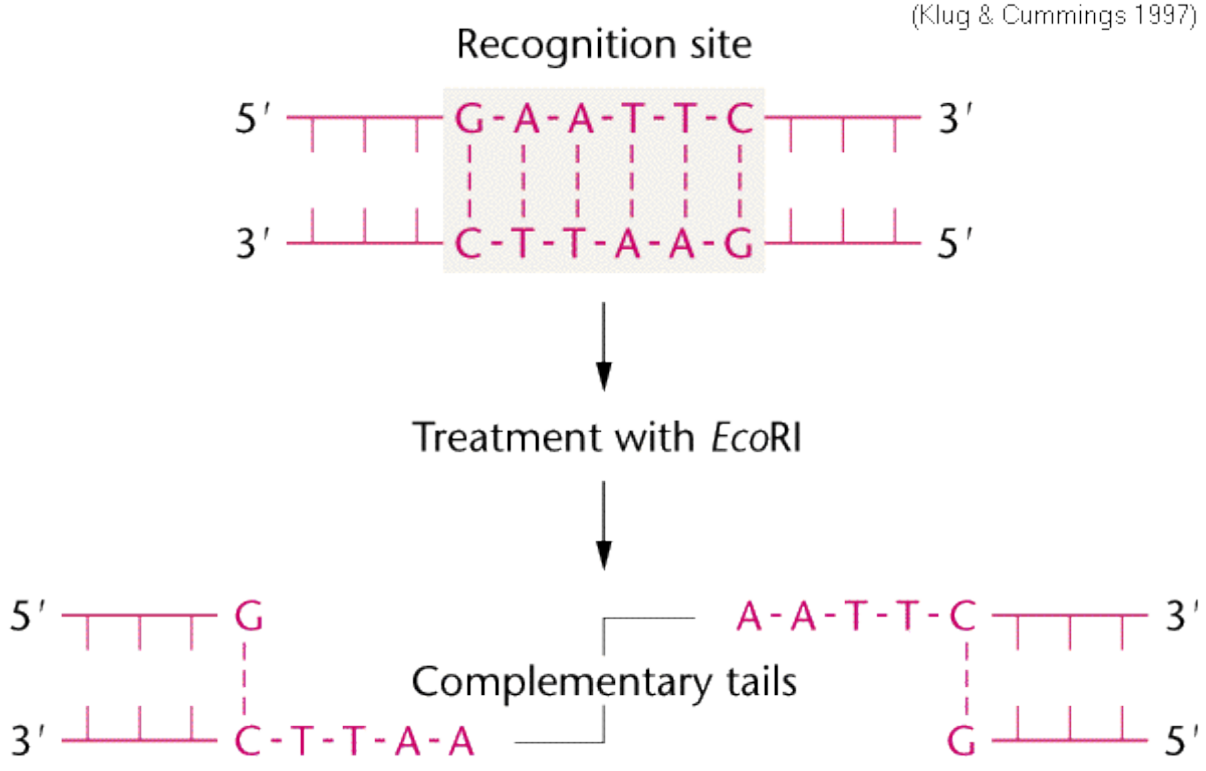
DNA CUTTING (DIGESTION)

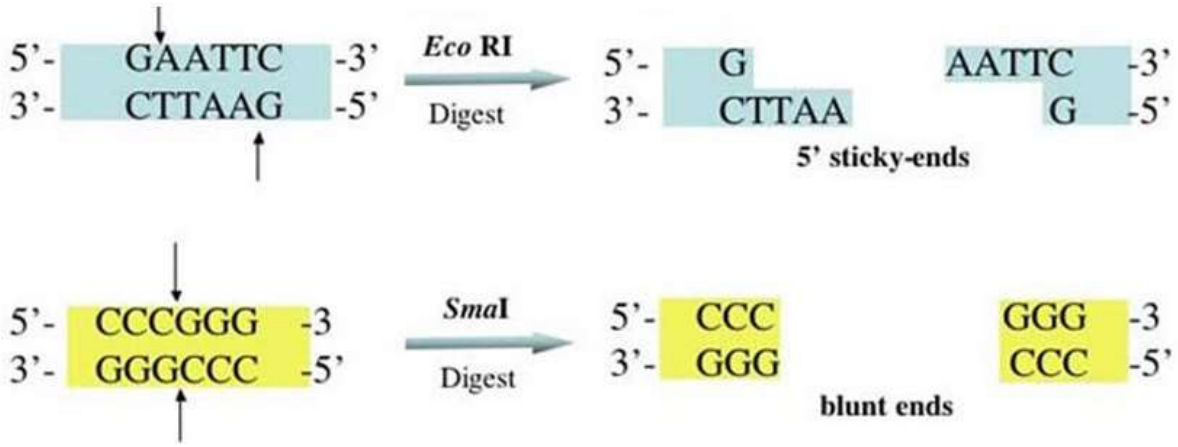
- DNA can be cut into large fragments by **mechanical shearing**
- **Restriction enzymes** are used as molecular scissors. These are special class of sequence-specific enzymes found in bacteria
- They cleaves DNA only at specific nucleotide sequence
- Restriction enzymes recognize the DNA sequences that are palindrome
- They make staggered cuts with complementary base sequences for easy circularization
- A **palindromic sequence** is a sequence made up of nucleic acids within double helix of DNA and/or RNA that is the same when read from 5' to 3' on one strand and 3' to 5' on the other, complementary, strand.





(Klug & Cummings 1997)





Eco RI stands for ***Escherichia coli*** strain RI

Sma stands for ***Serratia marcescens*** strain I

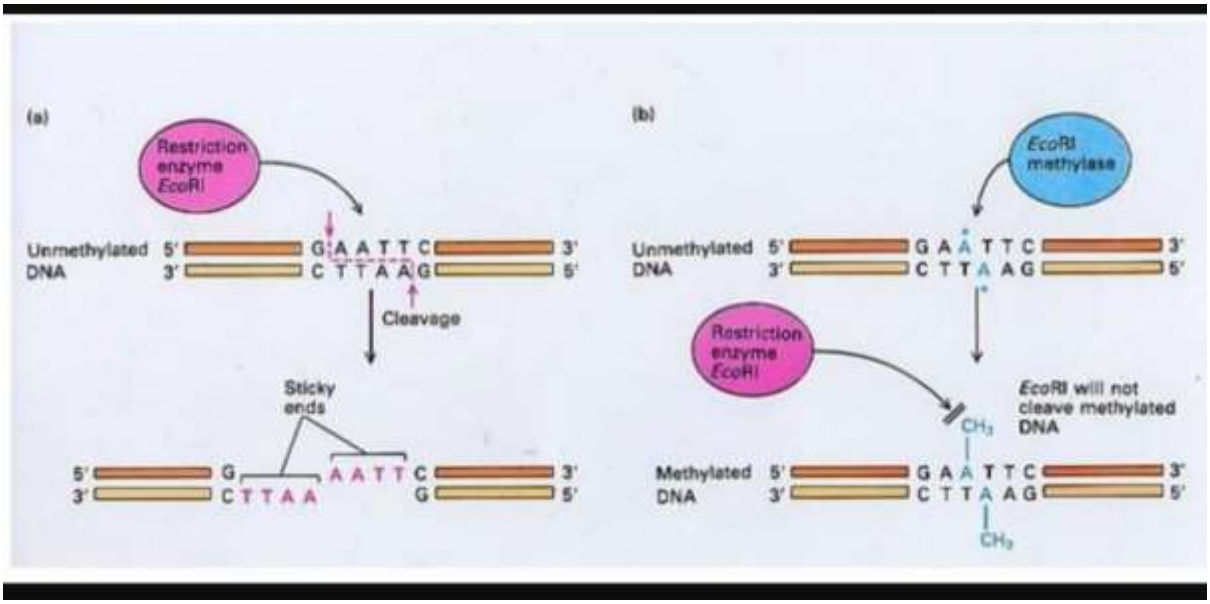
Genetic engineering became possible with the discovery of mainly two types of enzymes:

- the <u>cutting enzymes</u> called **restriction endonucleases** (molecular knives, molecular scissors or molecular scalpels)

  Restriction endonucleases or restriction enzymes, recognize unique base sequences in a DNA strand and cleave the strand of the molecule at a place within or, at some distance from the recognition site

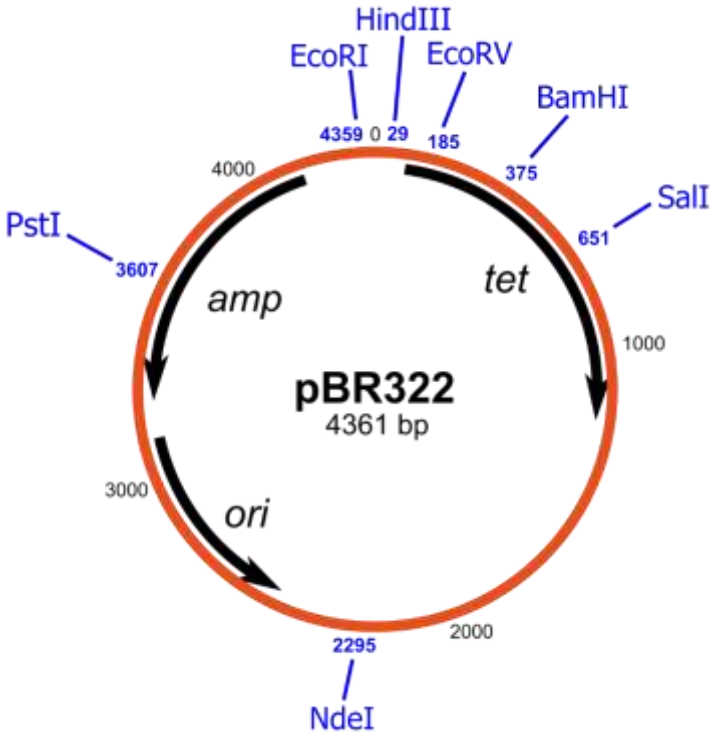
- the <u>joining enzymes</u> called **ligases**

  Ligase is the enzyme that joins a 5′ end of a DNA with a 3′ end of the same or of another strand

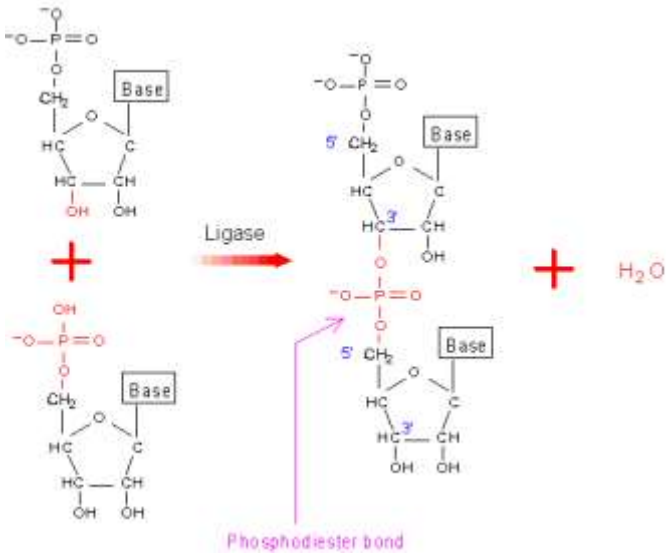


JOINING DNA TO A VECTOR (Ligation)

- A **vector** is a DNA molecule that has the ability to replicate autonomously in a host cell and into which the gene of interest (foreign DNA) is integrated eg. Plasmids, cosmids, phagemids, YACs, BACs, etc.
- DNA Ligase (ligation – to seal the nicks that remain in the recombinant DNA molecule)
- It joins by forming phosphodiester bonds between the nucleotide ends of the vector and the DNA fragment to be inserted
- Ligation efficiency depends on the types of ends : Blunt or Sticky ends



PLASMID



AMPLIFYING THE RECOMBINANT DNA

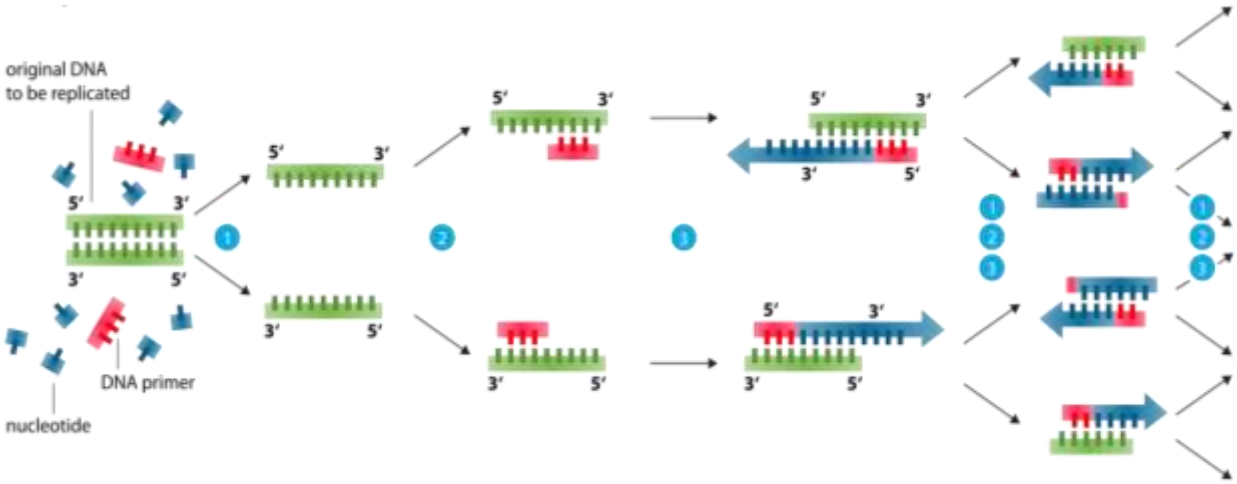
By transferring the recombinant DNA into a bacterial host strain

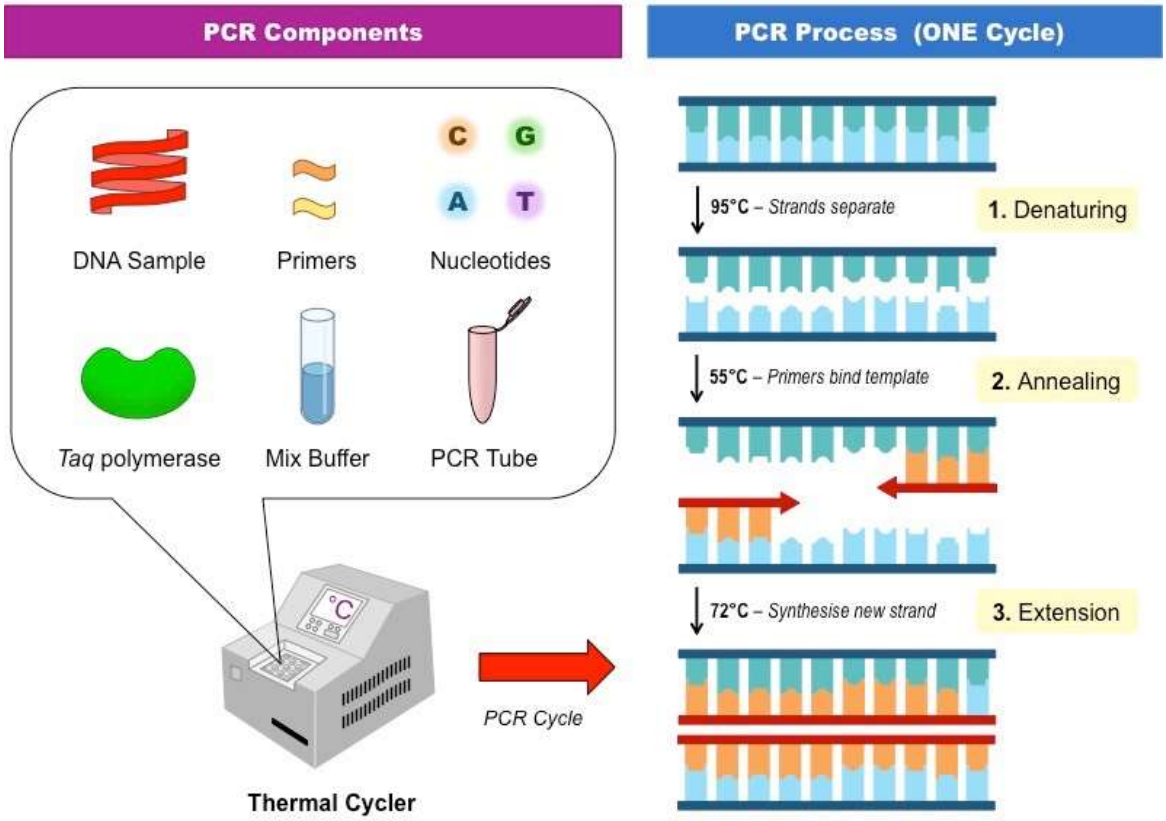
- Cells treated with $CaCl_2$
- DNA (recombinant) is added
- Cells are heat shocked at 42°C
- Once in a cell, the recombinant DNA will be replicated
- The replicated recombinant molecules go to both daughter cells which themselves will divide later.
- DNA is amplified
- PCR is very popular for Amplification of DNA nowadays.

PCR (POLYMERASE CHAIN REACTION)

Thermal Cycler (PCR Machine)

STEPS INVOLVED IN PCR





- ***Denaturation*: The reaction mixture is heated to 95°C [94-98°C] for a short time period (about 15–30 sec) to denature the target DNA into single strands that can act as templates for DNA synthesis.**
- ***Annealing:* The mixture is rapidly cooled to a well defined temperature which allows the two primers to bind to the sequences on each of the two strands flanking the target DNA.**

  Primers are short, single-stranded sequences of nucleic acid (i.e., oligonucleotides usually 20 to 30 nucleotides long) selected to specifically hybridize (anneal) to a particular target nucleic acid sequence, (like probes).

  The primers bind only to the desired DNA sequences (usually at around 55°C [50-68°C]).

  One primer binds to each strand. The two parental strands do not re-anneal with each other because the primers are in large excess over parental DNA.
- ***Extension:* The temperature of the mixture is raised to about 72°C [70-74°C] and kept at this temperature for a pre-set period of time to allow DNA polymerase to elongate each primer by copying the single-stranded templates.**

  Annealing of primers to target sequences allows the DNA polymerase to add nucleotides to the 3' terminus of each primer and extend complementary sequence to the target template.

  ***Taq polymerase* is the enzyme commonly used for primer extension, which occurs at 72°C. This enzyme is used because of its ability to function efficiently at elevated temperatures and to withstand the denaturing temperature of 94°C through several cycles.**

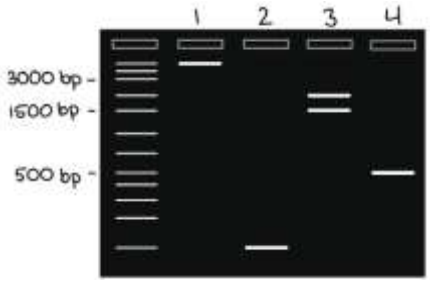
- **The amplified DNA is then introduced into the host cells where it multiplies (self-replicates) and also transforms the host DNA by combining with the host genome to produces the desired effects. This taking up of the new genetic material is transformation.**
- **The cells containing recombinant sequences are the selected and the clones with desired inserts ore screened**

TOOLS OF RECOMBINANT DNA TECHNOLOGY

- **Target DNA**
- **Host DNA**
- **Enzymes (Restriction enzymes, DNA ligases, Polymerases, reverse transcriptase, etc.)**
- **Vectors (plasmid, bacteriophage, cosmid, yeast artificial chromosomes, BACs, etc.,)**
- **Probes**
- **Linkers and adapters**
- **Instruments**

TECHNIQUES USED IN RECOMINANT DNA TECHNOLOGY

- **Extraction procedures**
- **Centrifugation**
- **Gel Electrophoresis – A technique used to separate DNA fragments (or other macromolecules, such as RNA and proteins) based on their size and charge.**

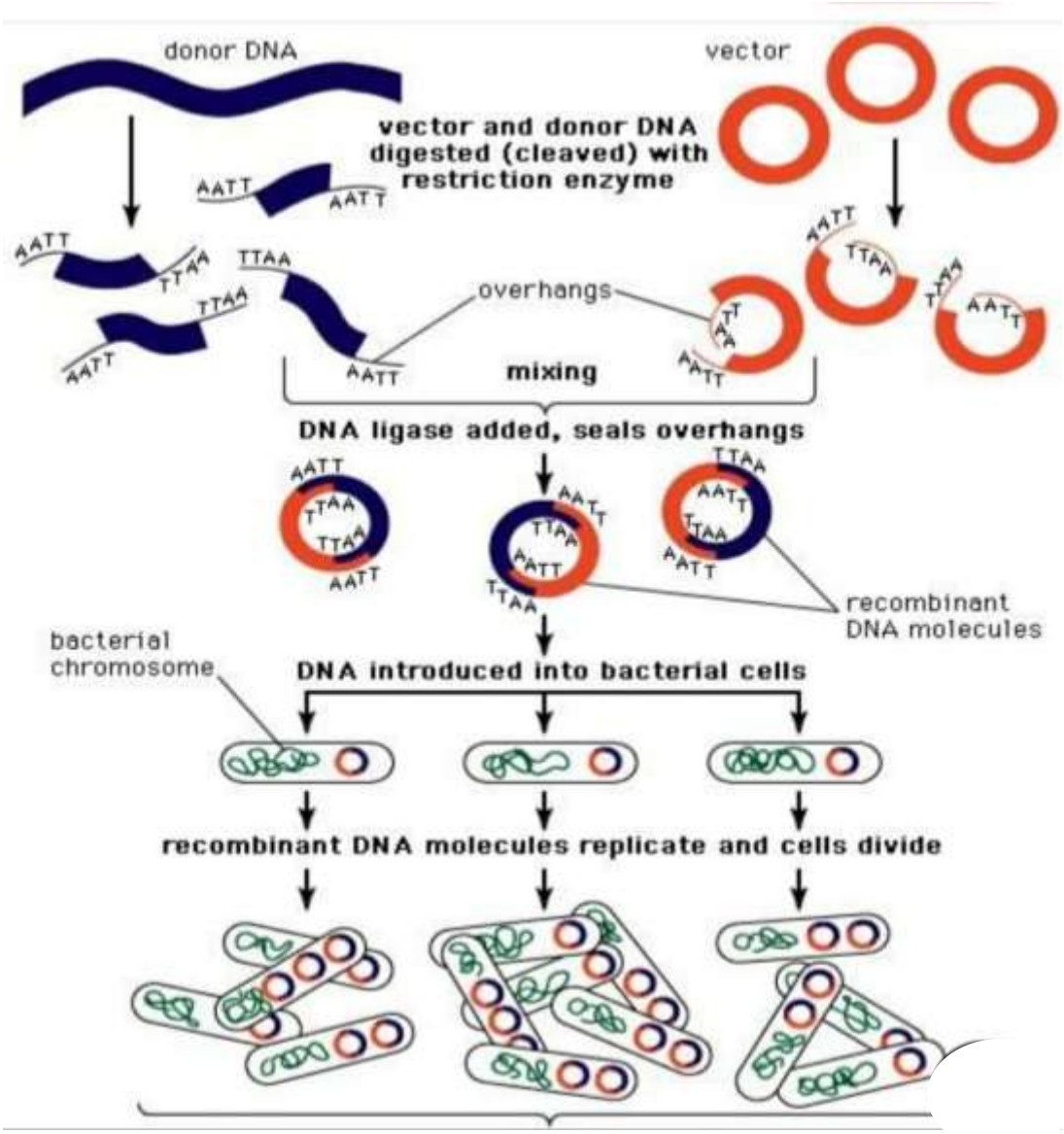


- **Restriction digestion**
- **DNA Amplification (PCR)**
- **Cloning**
- **Gene transfer methods**
- **Nucleic Acid Hybridization**
- **Blotting**

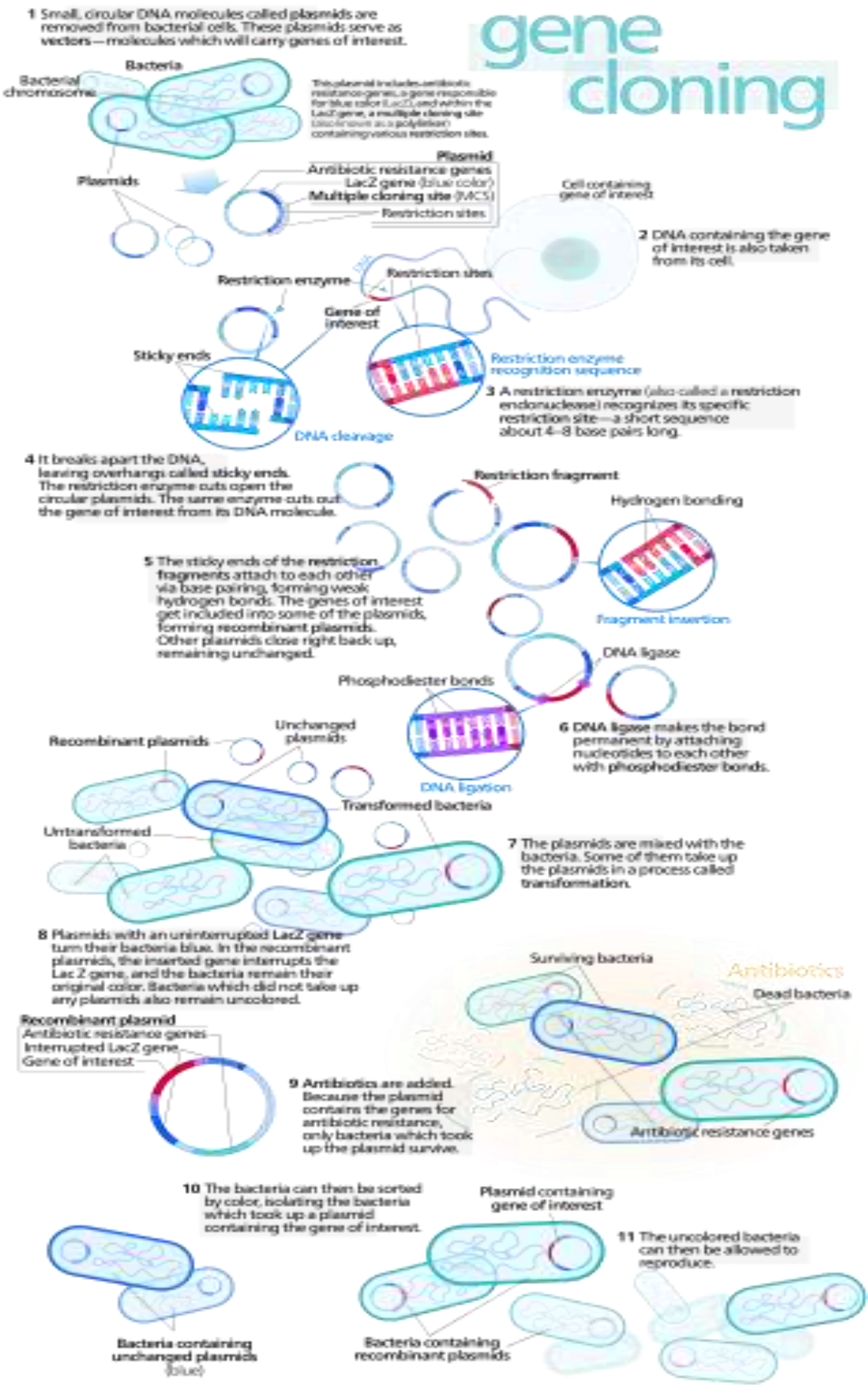
Overall Steps of Gene Cloning/Molecular cloning/Genetic engineering/Recombinant DNA technology

- Choice of host organism and cloning vector
- Preparation of vector DNA
- Preparation of DNA to be cloned
- Creation of recombinant DNA with DNA ligase
- Introduction of recombinant DNA into host organism
- Selection of organisms containing vector sequences
- Screening for clones with desired DNA inserts and biological properties

DIAGRAMMATIC REPRESENTATION OF THE PROCEDURE OF PREPARING RECOMINANT DNA



2012, Encyclopaedia Britannica. Inc


gene cloning
1 Small, circular DNA molecules called plasmids are removed from bacterial cells. These plasmids serve as vectors—molecules which will carry genes of interest.
Bacteria
Bacterial chromosome
Plasmids
Plasmid
Antibiotic resistance genes
LacZ gene (blue color)
Multiple cloning site (MCS)
Restriction sites
Cell containing gene of interest
2 DNA containing the gene of interest is also taken from its cell.
Restriction enzyme
Restriction sites
Gene of interest
Sticky ends
Restriction enzyme recognition sequence
DNA cleavage
3 A restriction enzyme (also called a restriction endonuclease) recognizes its specific restriction site—a short sequence about 4–8 base pairs long.
4 It breaks apart the DNA, leaving overhangs called sticky ends. The restriction enzyme cuts open the circular plasmids. The same enzyme cuts out the gene of interest from its DNA molecule.
Restriction fragment
Hydrogen bonding
5 The sticky ends of the restriction fragments attach to each other via base pairing, forming weak hydrogen bonds. The genes of interest get included into some of the plasmids, forming recombinant plasmids. Other plasmids close right back up, remaining unchanged.
Fragment insertion
DNA ligase
Phosphodiester bonds
6 DNA ligase makes the bond permanent by attaching nucleotides to each other with phosphodiester bonds.
DNA ligation
Recombinant plasmids
Unchanged plasmids
Transformed bacteria
Untransformed bacteria
7 The plasmids are mixed with the bacteria. Some of them take up the plasmids in a process called transformation.
8 Plasmids with an uninterrupted LacZ gene turn their bacteria blue. In the recombinant plasmids, the inserted gene interrupts the Lac Z gene, and the bacteria remain their original color. Bacteria which did not take up any plasmids also remain uncolored.
Surviving bacteria
Antibiotics
Dead bacteria
Recombinant plasmid
Antibiotic resistance genes
Interrupted LacZ gene
Gene of interest
9 Antibiotics are added. Because the plasmid contains the genes for antibiotic resistance, only bacteria which took up the plasmid survive.
Antibiotic resistance genes
10 The bacteria can then be sorted by color, isolating the bacteria which took up a plasmid containing the gene of interest.
Plasmid containing gene of interest
11 The uncolored bacteria can then be allowed to reproduce.
Bacteria containing unchanged plasmids (blue)
Bacteria containing recombinant plasmids

Further Reading:

- Gene Cloning – T.A. Brown
- Principles of gene manipulation and genomics – Primrose
- Biotechnology – B.D. Singh
- Elements of Biotechnology – P.K. Gupta

Source of Above Figures:

- Gene Cloning – T.A. Brown
- Principles of gene manipulation and genomics – Primrose
- Wikipedia – Molecular cloning