BASICS OF DNA CLONING

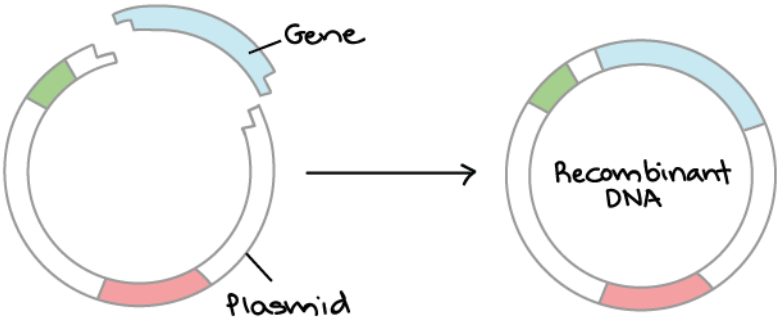
Introduction

The general term "cloning" means obtaining multiples copies of something, let it be individuals of an animal species, or molecules. DNA cloning is a Molecular Biology technique that allows us to get multiple copies of identical sequence-specific double-stranded DNA fragments, for various purposes.

When you hear the word "cloning," you may think of the cloning of whole organisms, such as Dolly the sheep. However, all it means to **clone** something is to make a genetically exact copy of it. In a molecular biology lab, what's most often cloned is a gene or other small piece of DNA.

Overview of DNA cloning

DNA cloning is the process of making multiple, identical copies of a particular piece of DNA. In a typical DNA cloning procedure, the gene or other DNA fragment of interest (perhaps a gene for a medically important human protein) is first inserted into a circular piece of DNA called a **plasmid**. The insertion is done using enzymes that "cut and paste" DNA, and it produces a molecule of **recombinant DNA**, or DNA assembled out of fragments from multiple sources.



Next, the recombinant plasmid is introduced into bacteria. Bacteria carrying the plasmid are selected and grown up. As they reproduce, they replicate the plasmid and pass it on to their offspring, making copies of the DNA it contains.

What is the point of making many copies of a DNA sequence in a plasmid? In some cases, we need lots of DNA copies to conduct experiments or build new plasmids. In other cases, the piece of DNA encodes a useful protein, and the bacteria are used as "factories" to make

the protein. For instance, the human insulin gene is expressed in *E. coli* bacteria to make insulin used by diabetics.

Steps of DNA cloning

DNA cloning is used for many purposes. As an example, let's see how DNA cloning can be used to synthesize a protein (such as human insulin) in bacteria. The basic steps are:

1. Cut open the plasmid and "paste" in the gene. This process relies on restriction enzymes (which cut DNA) and DNA ligase (which joins DNA).
2. Insert the plasmid into bacteria. Use antibiotic selection to identify the bacteria that took up the plasmid.
3. Grow up lots of plasmid-carrying bacteria and use them as "factories" to make the protein. Harvest the protein from the bacteria and purify it.

Let's take a closer look at each step.

1. Cutting and pasting DNA

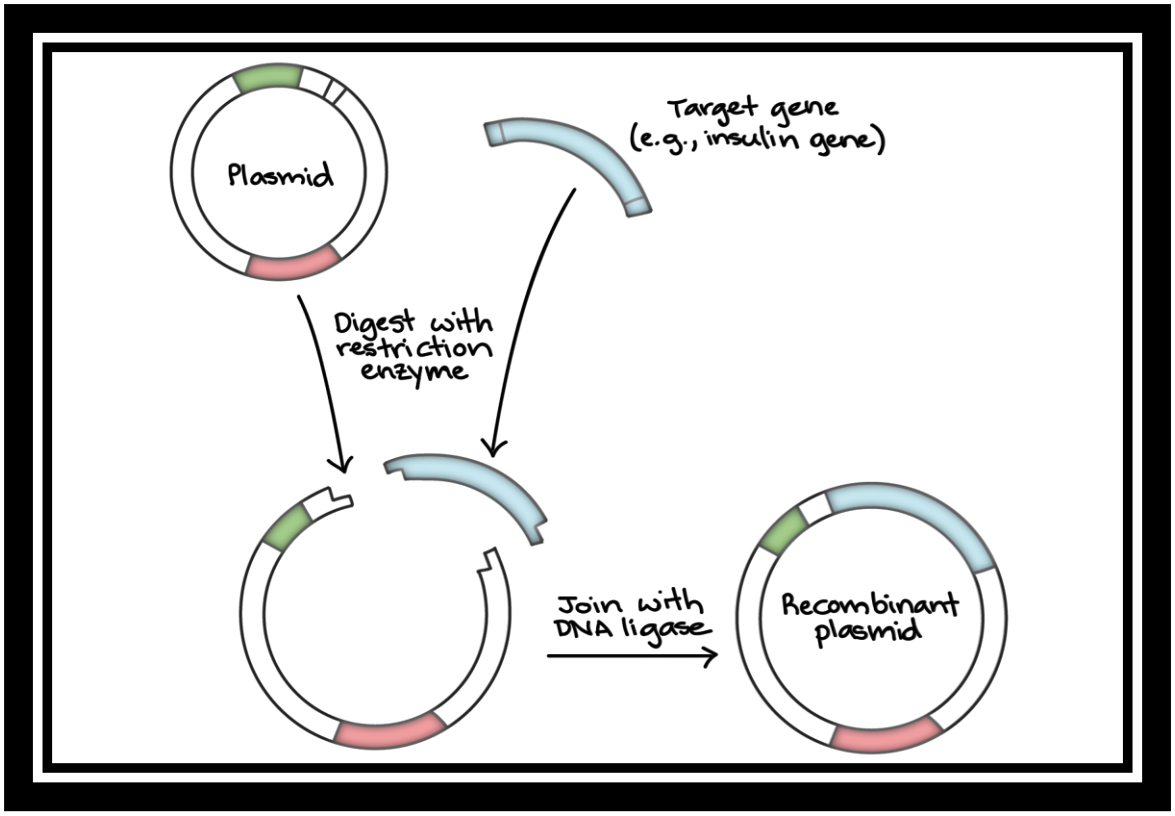
How can pieces of DNA from different sources be joined together? A common method uses two types of enzymes restriction enzyme & DNA ligase.

A **restriction enzyme** is a DNA-cutting enzyme that recognizes a specific target sequence and cuts DNA into two pieces at or near that site. Many restriction enzymes produce cut ends with short, single-stranded overhangs. If two molecules have matching overhangs, they can base-pair and stick together. However, they won't combine to form an unbroken DNA molecule until they are joined by **DNA ligase**, which seals gaps in the DNA backbone.

Our goal in cloning is to insert a target gene (e.g., for human insulin) into a plasmid. Using a carefully chosen restriction enzyme, we digest:

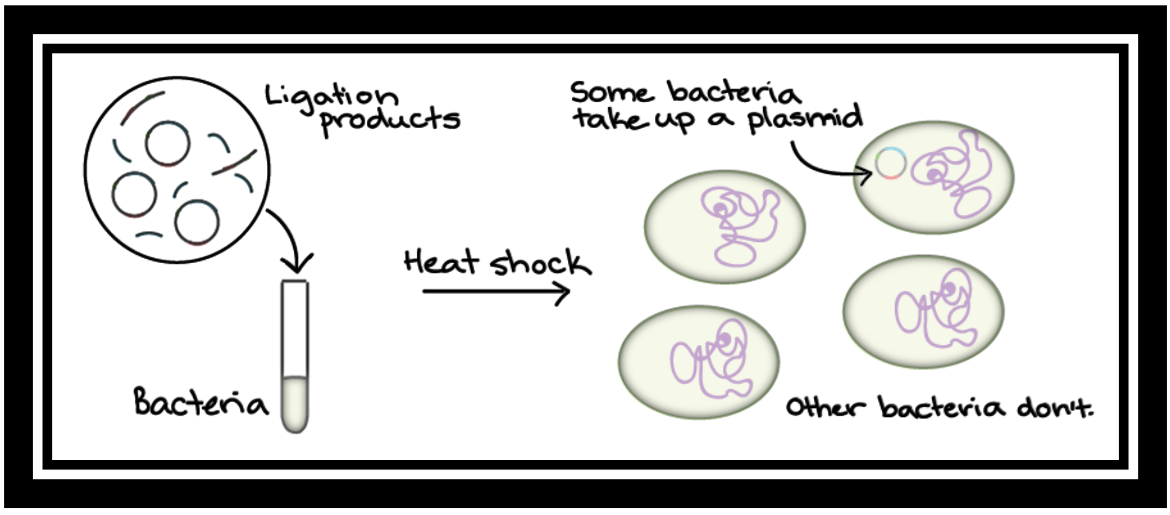
- The plasmid, which has a single cut site
- The target gene fragment, which has a cut site near each end

Then, we combine the fragments with DNA ligase, which links them to make a recombinant plasmid containing the gene.



2. Bacterial transformation and selection

Plasmids and other DNA can be introduced into bacteria, such as the harmless *E. coli* used in labs, in a process called **transformation**. During transformation , specially prepared bacterial cells are given a shock (such as high temperature) that encourages them to take up foreign DNA.



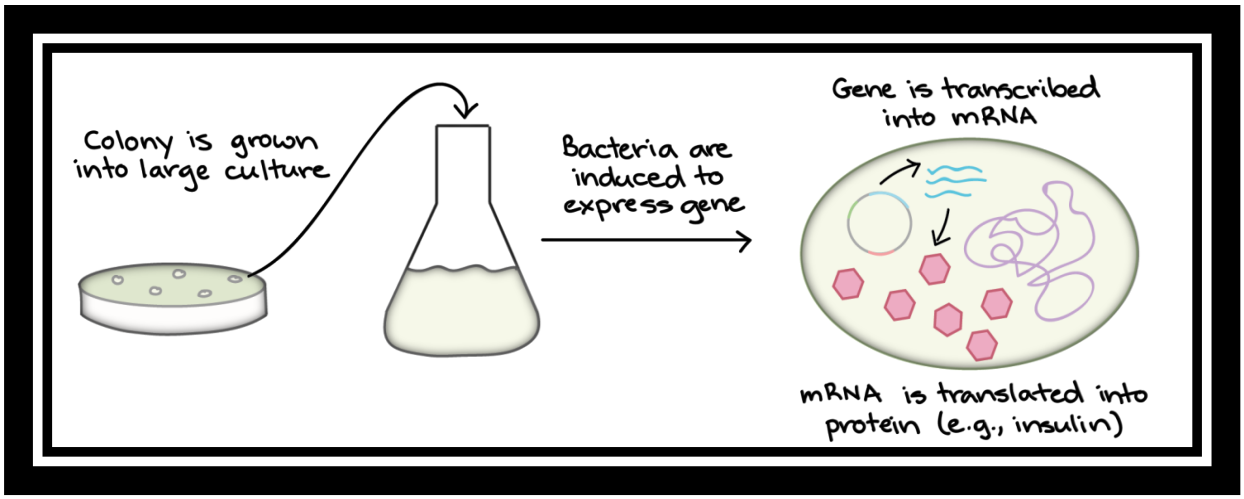
Not all colonies will necessarily contain the right plasmid. That's because, during a ligation, DNA fragments don't always get "pasted" in exactly the way we intend. Instead, we must

collect DNA from several colonies and see whether each one contain the right plasmid. Methods like resriction digestion enzyme andPCR are commonly used to check the plasmids.

3. Protein production

Once we have found a bacterial colony with the right plasmid, we can grow a large culture of plasmid-bearing bacteria. Then, we give the bacteria a chemical signal that instructs them to make the target protein.

The bacteria serve as miniature “factories," churning out large amounts of protein. For instance, if our plasmid contained the human insulin gene, the bacteria would start transcribing the gene and translating the mRNA to produce many molecules of human insulin protein.



Once the protein has been produced, the bacterial cells can be split open to release it. There are many other proteins and macromolecules floating around in bacteria besides the target protein (e.g., insulin). Because of this, the target protein must be **purified**, or separated from the other contents of the cells by biochemical techniques. The purified protein can be used for experiments or, in the case of insulin, administered to patients.

Uses of DNA cloning

DNA molecules built through cloning techniques are used for many purposes in molecular biology. A short list of examples includes:

- **Biopharmaceuticals.** DNA cloning can be used to make human proteins with biomedical applications, such as the insulin mentioned above. Other examples of recombinant proteins include human growth hormone, which is given to patients who are unable to synthesize the hormone, and tissue plasminogen activator (tPA), which is used to treat strokes and prevent blood clots. Recombinant proteins like these are often made in bacteria.

- **Gene therapy.** In some genetic disorders, patients lack the functional form of a particular gene. Gene therapy attempts to provide a normal copy of the gene to the cells of a patient's body. For example, DNA cloning was used to build plasmids containing a normal version of the gene that's nonfunctional in cystic fibrosis. When the plasmids were delivered to the lungs of cystic fibrosis patients, lung function deteriorated less quickly

- **Gene analysis.** In basic research labs, biologists often use DNA cloning to build artificial, recombinant versions of genes that help them understand how normal genes in an organism function.
  These are just a few examples of how DNA cloning is used in biology today. DNA cloning is a very common technique that is used in a huge variety of molecular biology applications.

Cloning using linkers and adaptors

An adapter, or a linker in genetic engineering is a short, chemically synthesized single-stranded or double stranded oligonucleotide that can be ligated to the ends of other DNA or RNA molecules. Double stranded adapters can be synthesized to have blunt ends to both terminals or to have sticky end at one end & blunt end to the other.

For instance, a double stranded DNA adapter can be used to link the ends of two other DNA molecules (i.e., ends that do not have sticky ends that is complementary protruding single strands by themselves). It may be used to add sticky ends to cDNAallowing it to be ligated into the plasmid much more efficiently .

Two adapters could base pair to each other to form dimers. A conversation adapter is used to join a DNA insert cut one restriction enzyme, say EcoR1, with a vector opened another enzyme, Bam HI. This adapter can be used to convert the cohesive end produced by Bam HI to one produced by EcoR1 or vice versa.

One of its applications is ligating cDNA into a plasmid or other vectors instead of using Terminal deoxynucleobletransferase enzyme to add poly A to the c DNA fragment.

CLONING INTO VARIOUS KINDS OF VECTORS

Introduction

A cloning vector is a small piece of DNA that can be stably maintained in an organism, and into which a foreign DNA fragment can be inserted for cloning purposes.The cloning vector may be DNA taken from a virus, the cell of a higher organism, or it may be the plasmid of a bacterium. The vector therefore contains features that allow for the convenient insertion or removal of a DNA fragment to or from the vector, for example by treating the vector and the foreign DNA with a restriction enzyme that cuts the DNA. DNA fragments thus generated contain either blunt ends or overhangs known as sticky ends, and vector DNA and foreign DNA with compatible ends can then be joined together by molecular ligation. After a DNA fragment has been cloned into a cloning vector, it may be further subcloned into another vector designed for more specific use.

There are many types of cloning vectors, but the most commonly used ones are genetically engineered plasmids. Cloning is generally first performed using *Escherichia coli* , and cloning vectors in *E. coli* include plasmids, bacteriophage (such as Lambda phage), cosmids, and bacterial artificial chromosome BACs). Some DNA, however, cannot be stably maintained in *E. coli*, for example very large DNA fragments, and other organisms such as yeast may be used. Cloning vectors in yeast include yeast artificial chromosome (YACs).

Features of cloning vectors

All commonly used cloning vectors in molecular biology have key features necessary for their function, such as a suitable cloning site and selectable marker. Others may have additional features specific to their use. For reason of ease and convenience, cloning is often performed using *E.coli*. Thus, the cloning vectors used often have elements necessary for their propagation and maintenance in *E. coli*, such as a functional origin of replication (ori). The ColE1 origin of replication is found in many plasmids. Some vectors also include elements that allow them to be maintained in another organism in addition to *E. coli*, and these vectors are called shuttle vector.

Types of cloning vectors

A large number of cloning vectors are available, and choosing the vector may depend a number of factors, such as the size of the insert, copy number and cloning method. Large insert may not be stably maintained in a general cloning vector, especially for those with a high copy number, therefore cloning large fragments may require more specialized cloning vector.

Plasmid

Plasmids are autonomously replicating circular extra-chromosomal DNA. They are the standard cloning vectors and the ones most commonly used. Most general plasmids may be used to clone DNA insert of up to 15 kb in size. One of the earliest commonly used cloning vectors is thepBR322 plasmid. Other cloning vectors include the pUC series of plasmids, and a large number of different cloning plasmid vectors are available. Many plasmids have high copy number, for example pUC19 which has a copy number of 500-700 copies per cell,[14] and high copy number is useful as it produces greater yield of recombinant plasmid for subsequent manipulation. However low-copy-number plasmids may be preferably used in certain circumstances, for example, when the protein from the cloned gene is toxic to the cells.

Some plasmids contain anM13 bacteriophage origin of replication and may be used to generate single-stranded DNA. These are called phagemid,and examples are the pBluescript series of cloning vectors.

Bacteriophage

The bacteriophages used for cloning are the lambda phage and M13 phage . There is an upper limit on the amount of DNA that can be packed into a phage (a maximum of 53 kb), therefore to allow foreign DNA to be inserted into phage DNA, phage cloning vectors may need to have some non-essential genes deleted, for example the genes for lysogeny . since using phage λ as a cloning vector involves only the lytic cycle. There are two kinds of λ phage vectors - insertion vector and replacement vector. Insertion vectors contain a unique cleavage site whereby foreign DNA with size of 5–11 kb may be inserted. In replacement vectors, the cleavage sites flank a region containing genes not essential for the lytic cycle, and this region may be deleted and replaced by the DNA insert in the cloning process, and a larger sized DNA of 8–24 kb may be inserted.

There is also a lower size limit for DNA that can be packed into a phage, and vector DNA that is too small cannot be properly packaged into the phage. This property can be used for selection - vector without insert may be too small, therefore only vectors with insert may be selected for propagation.

Cosmid

Cosmidsare plasmids that incorporate a segment of bacteriophage λ DNA that has the cohesive end site (*cos*) which contains elements required for packaging DNA into λ particles. It is normally used to clone large DNA fragments between 28 and 45 Kb.

Bacterial artificial chromosome

Insert size of up to 350 kb can be cloned in bacterial artificial chromosome (BAC). BACs are maintained in *E. coli* with a copy number of only 1 per cell. BACs are based on F piasmid, another artificial chromosome called the PAC is based on the P1 phage.

Yeast artificial chromosome

Yeast artificial chromosome are used as vectors to clone DNA fragments of more than 1 mega base (1Mb=1000kb) in size. They are useful in cloning larger DNA fragments as required in mapping genomes such as in human genome project. It contains a telomeric sequence, an autonomously replicating sequence(features required to replicate linear chromosomes in yeast cells). These vectors also contain suitable restriction sites to clone foreign DNA as well as genes to be used as selectable markers.

Human artificial chromosome

Human artificial chromosome may be potentially useful as a gene transfer vectors for gene delivery into human cells, and a tool for expression studies and determining human chromosome function. It can carry very large DNA fragment (there is no upper limit on size for practical purposes), therefore it does not have the problem of limited cloning capacity of other vectors, and it also avoids possible insertional mutagenesis caused by integration into host chromosomes by viral vector.

Animal and plant viral vectors Viruses that infect plant and animal cells have also been manipulated to introduce foreign genes into plant and animal cells. The natural ability of viruses to adsorb to cells , introduce their DNA and replicate have made them ideal vehicles to transfer foreign DNA into eukaryotic cells in culture. A vector based on Simian virus 40 (SV40) was used in first cloning experiment involving mammalian cells. A number of vectors based on other type of viruses like Adenoviruses and Papilloma virus have been used to clone genes in mammals. At present , retroviral vectors are popular for cloning genes in mammalian cells. In case of plants like Cauliflower mosaic virus , Tobacco mosaic virus and Gemini viruses have been used with limited success.

SELECTION OF SCREENING OF CLONES

SELECTION

After the introduction of recombinant DNA into the host cells which received rDNA molecule- screening(or) selection.

The vector or foreign DNA present in the recombinant cells expresses certain characters or traits, while non-recombinants do not express the traits.

Following the methods for screening or selection of recombinant clones.

DIRECT SELECTION OF RECOMBINANTS

The simplest example of direct selection occurs when the desired gene specifies resistance to an antibiotic. As an example will consider an experiment to clone the gene for kanamcycin resistance from plasmid R6-5.

This plasmid carries genes for resistances to four antibiotics: kanamycin, chloramphenicol, streptomycin and sulphonamide. The kanamycin resistance gene lies within one of the 13 EcoRI fragments.

To clone this gene EcoR1 fragments of R6-5 would be inserted into the EcoR1 site of a vector such as pBR322. The ligated mix will comprise many copies 13 different recombinant DNA molecules, one set of which carries the gene for kanamycin resistance.

Insertional inactivation cannot be used to select recombinants when the EcoR1 site of pBR322 is used .this is because this site does not lie in either ampicillin or the tetracycline resistance of this plasmid.

But this is immaterial for cloning the kanamycin resistance gene because in this case the cloned gene can be used as the selectable marker .Transformants are plated onto kanamycin agar, on which the only cells able to survive and produce colonies are those recombinants that the cloned kanamycin resistance gene.

INSERTIONAL INACTIVATION SELECTION METHOD

- Insertional inactivation is the inactivation of a gene upon insertion of another gene in its place within its coding sequence
- This helps in selection of recombinant colonies in rDNA technology.

Blue-White Screening & Protocols for Colony Selection

Identification of Recombinant Bacteria

Blue-white screening is a rapid and efficient technique for the identification of recombinant bacteria. It relies on the activity of β-galactosidase, an enzyme occurring in *E. coli*, which cleaves lactose into glucose and galactose.

Disrupting the LacZ Gene

The presence of lactose in the surrounding environment triggers the lacZ operon in *E. coli*. The operon activity results in the production of β-galactoisdase enzyme that metabolizes the lactose. Most plasmid vectors carry a short segment of lacZ gene that contains coding information for the first 146 amino acids of β-galactosisdase. The host *E. coli* strains used are competent cells containing lacZ M15 deletion mutation. When the plasmid vector is taken up by such cells, due to α-complementation process, a functional β-galatosidase enzyme is produced.

The plasmid vectors used in cloning are manipulated in such a way that this α-complementation process serves as a marker for recombination. A multiple cloning site (MCS) is present within the lacZ sequence in the plasmid vector. This sequence can be nicked by restriction enzymes to insert the foreign DNA. When a plasmid vector containing foreign DNA is taken up by the host *E. coli*, the α-complementation does not occur, therefore, a functional β-galactosidase enzyme is not produced. If the foreign DNA is not inserted into the vector or if it is inserted at a location other than MCS, the lacZ gene in the plasmid vector complements the lacZ deletion mutation in the host *E. coli* producing a functional enzyme.

How Does Blue White Screening Work?

For screening the clones containing recombinant DNA, a chromogenic substrate known as X-gal is added to the agar plate. If β-galactosidase is produced, X-gal is hydrolyzed to form 5-bromo-4-chloro-indoxyl, which spontaneously dimerizes to produce an insoluble blue pigment called 5,5'-dibromo-4,4'-dichloro-indigo. The colonies formed by non-recombinant cells, therefore appear blue in color while the recombinant ones appear white. The desired recombinant colonies can be easily picked and cultured.

Isopropyl β-D-1-thiogalactopyranoside (IPTG) is used along with X-gal for blue-white screening. IPTG is a non-metabolizableanalog of galactose that induces the expression of lacZ gene. It should be noted that IPTG is not a substrate for β-galactosidase but only an inducer. For visual screening purposes, chromogenic substrate like X-gal is required.

Steps inBlue White Screening

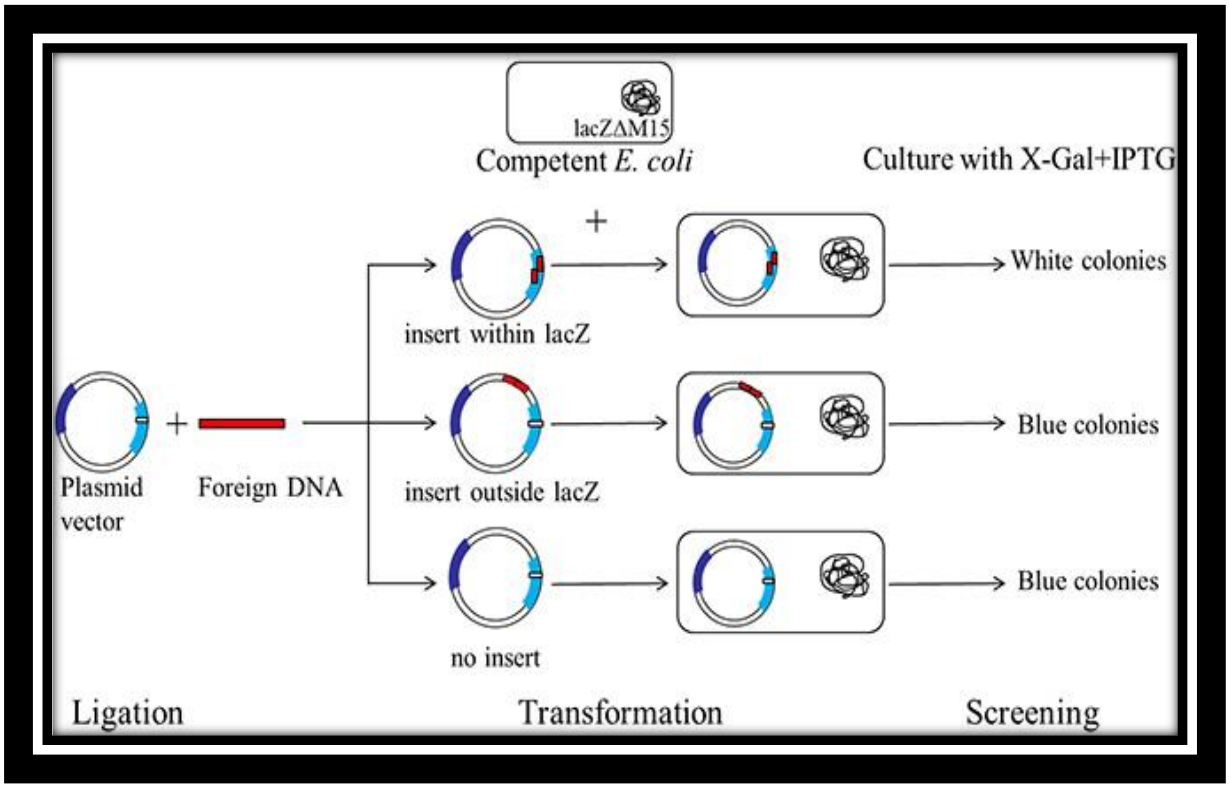


Plate showing Blue and White colour colonies

- Bacterial colonies contain recombinant vector – resistant to ampicillin & unable to hydralyze x –gal (White colonies)
- Bacterial clones contain vector without the new gene- Blue colonies

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