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Molecular cloning & Cloning vectors

What is Molecular cloning?

In the broadest sense, cloning describes the method for creating exact genetic copies of cells, tissues, or animals. When applied to molecular biology, cloning refers to the creation of a DNA molecule with origins from multiple sources (i.e. recombinant DNA) to be replicated within host organisms. Through the cloning process, the host cell makes multiple copies of the DNA fragment, allowing scientists to use it in many types of molecular biology experiments.

Materials for cloning:

To start with, it is important to prepare some important materials for cloning:

1- DNA Fragment

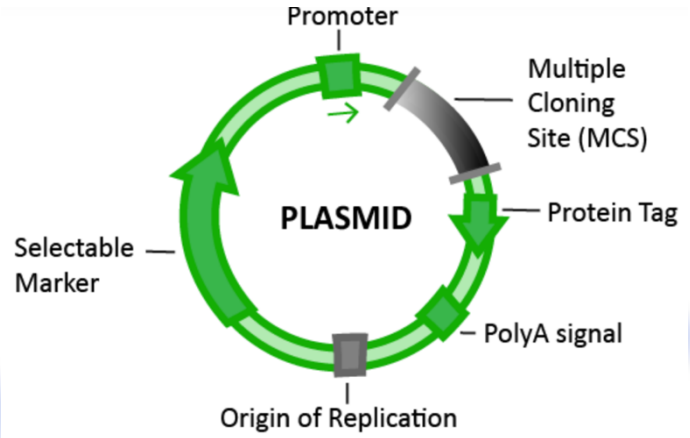
The source for a DNA fragment or "DNA insert" can be genomic DNA, complementary DNA, plasmid DNA, PCR product, or synthetic DNA. The DNA insert must contain particular sequences at the end of the fragments compatible with the prepared vector. You can add these particular sequences onto your DNA insert by PCR.

2- Vector

A vector is a DNA molecule which can carry a DNA insert to generate a recombinant DNA and replicate in a particular host. Examples of vectors are:

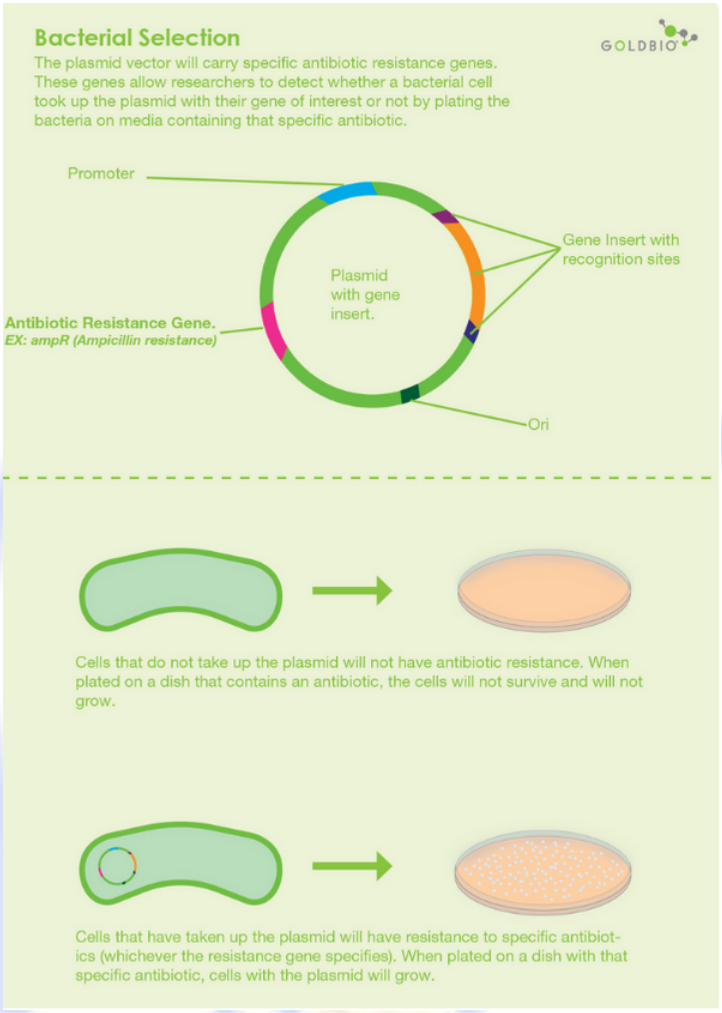
- **Cosmid**: a large DNA vector containing λ phage DNA sequence. It can carry large DNA fragment up to 45 kilobases into the host.
- **Artificial Chromosomes**: a large DNA vector which can perform the functions of a chromosome.

- **Plasmid**: a small extrachromosomal circular DNA which can replicate in a cell, commonly used in cloning.



Elements in a plasmid vector:

- **Origin of Replication (ORI)**: a required sequence or element on the plasmid for its replication inside the host.
- **Selectable Marker**: a required element for cloning to select a host, which carries the DNA construct. The only host cell growing in the growth medium containing a particular selective agent has the DNA construct with a selectable marker inside the cell. For an example, selectable markers in the DNA construct often contain antibiotic resistance genes. A transformed host has the antibiotic resistance gene on the DNA construct; therefore, it can grow in the medium with that particular antibiotic. On the other hand, the host cell without the DNA construct can't survive in the selective medium.
- **Multiple Cloning Site (MCS)**: an element on the plasmid fragment which contains restriction enzyme sites to allow DNA insertion. Compatible restriction enzymes cut on the MCS of plasmid and a DNA insert during preparation step of cloning.
- **Promoter Region**: a region which drives the protein expression of the cloned DNA.
- **Protein Tag**: a particular sequence which produces a protein with specific function, and it is usually attached to the recombinant protein. An example of a protein tag is luciferase or GFP, to monitor or quantify the protein.
- **Poly-adenylation signal**: an element containing poly-A which is important to produce a protein.



Competent Cells

After a DNA fragment is incorporated into the plasmid vector, the next cloning step is to perform a transformation step. In this transformation step, the recombinant DNA is introduced into the competent cell by chemical reaction or electroporation. Competent cells are cells which are temporarily permeable to extracellular DNA. The host organisms which are commonly used in the laboratories are *Escherichia coli* and *Saccharomyces cerevisiae.*

Selective Medium

It is a growth medium containing a selective agent to grow the transformed host. When you choose antibiotic selection for cloning, your growth medium must contain antibiotics. The most common antibiotics used for selection are Ampicillin, Kanamycin, and Chloramphenicol.

Scientists use molecular cloning for a variety of applications, including:

- Expressing proteins at high levels for medical or research purposes
- Mutating a gene to study the effects of the mutation on the cell
- Introducing a gene into an organism to give it a specific characteristic (ex: degrading chemicals in the environment, improving growth, etc.)

Before highlighting various molecular cloning techniques available to scientists today, we'll first look at the terminology used in molecular cloning and the general cloning process.

Common Terms in Molecular Cloning

- **Insert:** The piece of DNA that will be added to the cloning vector. This DNA is any DNA sequence of interest that the researcher wants to clone, amplify, and/or manipulate.
- **Cloning vector (plasmid):** A piece of DNA that can be maintained and replicated in an organism and often contains one or more antibiotic resistance genes and multiple cloning sites.
- **Construct:** An artificially-designed piece of DNA created using a cloning method and consisting of the insert and cloning vector
- **Clone (clonal population):** Collection of genetically identical cells (ex: bacterial colony) that have originated from a single ancestor (parent cell)
- **Antibiotic resistance gene:** Gene that allows bacteria to survive in the presence of a specific antibiotic
- **Multiple cloning site:** A short segment of DNA on the vector that contains multiple restriction sites, which help scientists introduce new genes and DNA fragments into the vector
- **Host cells:** Cells, commonly bacteria, used to replicate and maintain the cloned DNA

Overview of the Cloning Process

The cloning process generally involves joining insert DNA and a cloning vector together, inserting them into bacteria, identifying the bacteria that contain the correct construct, and verifying the sequence of the cloned DNA. Let's examine the steps in more detail:

Isolate and purify DNA fragments

The exact DNA fragment(s) you need depends on your cloning strategy (discussed below). These fragments originate from PCR reactions or other isolation methods. Once created, various enzymes, such as restriction enzymes or *Taq* polymerase, modify the fragment ends to be compatible with the vector DNA before ligation.

Prepare vector DNA

Vector DNA can be purchased from commercial vendors or prepared using PCR or restriction digests of existing vectors.

Ligate fragments together:

The ligation step uses enzymes (ex: DNA ligase) to join the insert DNA fragment(s) to the vector.

Transform into bacteria

This step introduces the ligation mixture into the bacterial host using either heat-shock or electroporation, two methods that allow DNA to cross the cell membrane. After this step, some bacteria will contain the correct clone, while others do not.

Identify correct clones

Scientists use various methods to help distinguish and/or identify the clones with the correct constructs. These methods involve growing the bacterial mixture in the presence of antibiotics so that only cells containing the vector backbone survive. Other methods use vectors with a gene that gives the bacteria a specific color (ex: blue-white screening). Inserting a DNA fragment within that gene inactivates it so scientists can visually distinguish clones where the vector contains the insert.

Isolate plasmids from individual clones

At this stage, bacterial colonies that likely contain the correct construct can be grown so scientists can harvest plasmid from them. After isolating the plasmid, it can be further analysed using restriction digests to check whether it produces DNA fragments of the expected size.

Verify plasmid sequence

To truly verify that the isolated construct contains the correct insert and that there are no errors, the constructs can be sequenced.

All cloning methods generally follow the above steps. The main difference between the methods occurs in how you prepare the insert and vector, the ligation step, and how to identify the correct clones.

Choosing a Molecular Cloning Method

With a plethora of cloning methods available, it can be difficult to decide which method to use. In designing a cloning experiment consider:

- **Number of inserts:** Some methods, such as restriction cloning, are straightforward when inserting one fragment into the vector. However, other methods like Gibson assembly are better suited for a larger number of inserts.
- **Size of insert** - Is the method suitable for the size of insert? For example, inserts that are particularly small or large may not work for specific methods.
- **Speed/complexity of method** - Does the reaction require one step or multiple steps? Some methods also require more careful primer design than others.
- **Flexibility** - Do you need to move your insert to other vectors? How easy is it to subclone into another vector?

- **Cost:** Different cloning methods have different costs associated with them. For example: do you need to purchase multiple enzymes, proprietary reagents, or vectors? Can you clone into a vector that you currently have in the lab?
- **Efficiency:** Do you have to screen many clones to find the right one or repeat your experiment?

The table below serves as a general guide to help you choose a cloning strategy for your experiment. However, please note each cloning experiment can behave differently due to the sequence of the insert or whether the gene expressed may be toxic to the cell. Therefore, there may be cases where the cloning method exceeds or doesn't meet the guidelines below.

	Restriction enzyme cloning	TOPO cloning	Gateway cloning	Gibson assembly	In-Fusion cloning	Golden Gate cloning
Number of inserts	1-3	1	1-4	1-15	1-5	1-20
Speed	Slow	Fast	Fast	Fast	Fast	Fast
Flexibility	Low	Low	High	High	High	High
Cost	Low	Medium	High	High	High	High