The Plasmid Cloning Cycle

How to improve accuracy, increase efficiency and reduce errors

SnapGene

Introduction

Molecular cloning, the manipulation of DNA fragments to generate recombinant DNA that can be replicated within a host organism, is a technique that has revolutionized biological research and medicine. It was the discovery and isolation of various bacterial enzymes including restriction endonucleases in the 1950's and 1960's that propelled the beginnings of molecular cloning.[1] Further development of innovative technologies such as Polymerase Chain Reaction (PCR) have also contributed to advancements in molecular cloning techniques.[2]

Today, researchers have at their disposal a myriad of sophisticated, simple, and specialized cloning techniques that have many different applications including the study of gene and protein function and expression, clinical applications such as the discovery of therapeutic targets to treat disease and development of improved vaccines, and generation of transgenic organisms.

Cloning requires multiple experimental steps that can be summarized in the Plasmid Cloning Cycle as follows:

1. Manipulation of DNA (isolation of insert, cutting of the vector, and ligation)
2. Transformation of DNA into bacterial host
3. Selection and Screening for candidate recombinant molecules
4. Extraction of candidate recombinant molecules from bacteria
5. Analysis of candidate recombinant molecules

In traditional cloning, the insert is isolated using restriction enzymes that cleave DNA at specific sites from genomic DNA, a plasmid or a fragment. The same restriction enzymes are used to cut the vector, facilitating ligation of the insert and vector. The characteristics of the vector, insert, and host organism, as well as the downstream application must be carefully considered to generate a successful cloning strategy.

Since the birth of molecular cloning, many different and incredibly efficient cloning methods have been generated. Some of these techniques do not require restriction enzymes, can be performed in a few steps, and can be used to clone very large fragments of DNA. For example, instead of restriction enzymes, Gibson Assembly utilizes exonuclease, polymerase, and DNA ligase activities to join PCR-amplified fragments.

Along with advances in cloning techniques, tools, such as SnapGene's advanced software, have been developed to facilitate the cloning process. In this SnapGene Cloning Ebook, you will learn the basics of molecular cloning in Chapter 1. In Chapter 2, we explain the Plasmid Cloning Cycle and in Chapter 3 you can explore the array of tools SnapGene offers that will simplify the Plasmid Cloning Cycle, including documentation and annotation tools. In Chapter 3 you will also find how SnapGene helps create maps of your sequence's features, digitally manipulate DNA to visualize your cloning strategy, and facilitate analysis of your clone. Chapter 4 contains general cloning tips. In Chapter 5, we illustrate how SnapGene can facilitate key cloning techniques including Restriction Enzyme Cloning, Gateway Cloning, Gibson Assembly, In-Fusion Cloning, and TA Cloning.

1

Introduction to Molecular Cloning

Molecular cloning refers to the capture and isolation of a unique nucleic acid fragment (insert) so that it can be grown and propagated away from the genome it originated from. Once isolated, the fragment cannot be propagated until it has been transferred into a DNA cloning vector, which is a plasmid/virus that can transport the fragment into a host organism (cell) and assist in isolating or expressing the nucleic acid fragment.[3] The mixing of genetic material that occurs during cloning is described as "recombinant DNA."

The general molecular cloning process is illustrated below (Figure 1). The DNA cloning vector/plasmid (in yellow) and a genome (in blue) containing the DNA fragment of interest (in red) are cleaved with restriction endonucleases, resulting in isolation of the DNA fragment, which can then be introduced (ligated) to the DNA vector. The DNA vector containing the DNA fragment is called a "recombinant molecule" (or recombinant DNA) and can be transferred into a bacterial host that can produce many copies of recombinant DNA.

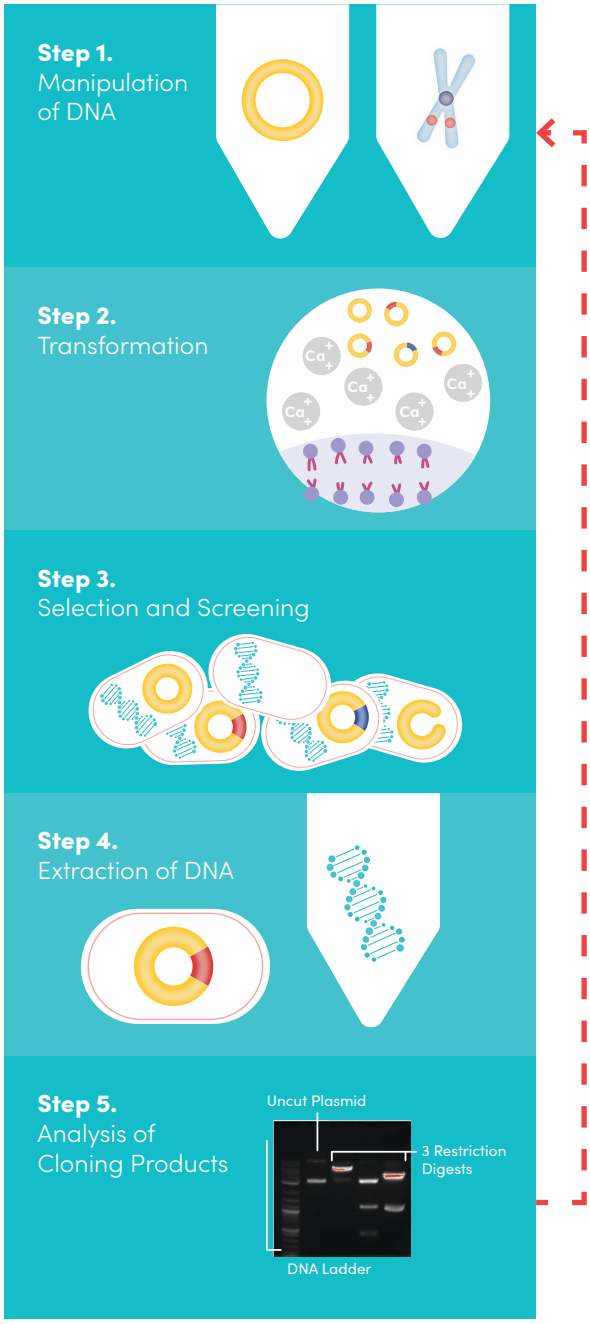


Figure 1. Molecular cloning process.

Features of a DNA Vector

The most common type of DNA vector is a bacterial plasmid, which is a small circular piece of DNA that grows in bacteria, independent from the bacterial chromosome. Plasmids have multiple features that facilitate the cloning process. These key features include enzyme restriction sites, an *E. coli* origin of replication, antibiotic resistance genes and a multi-cloning site (MCS). On a plasmid map, the name of the plasmid and the base pair length is indicated in the center of the plasmid.

Some of the most well-known plasmids are pBR322 and pUC19. We will use pBR322and pUC19 to illustrate the location and function of each of these key features.

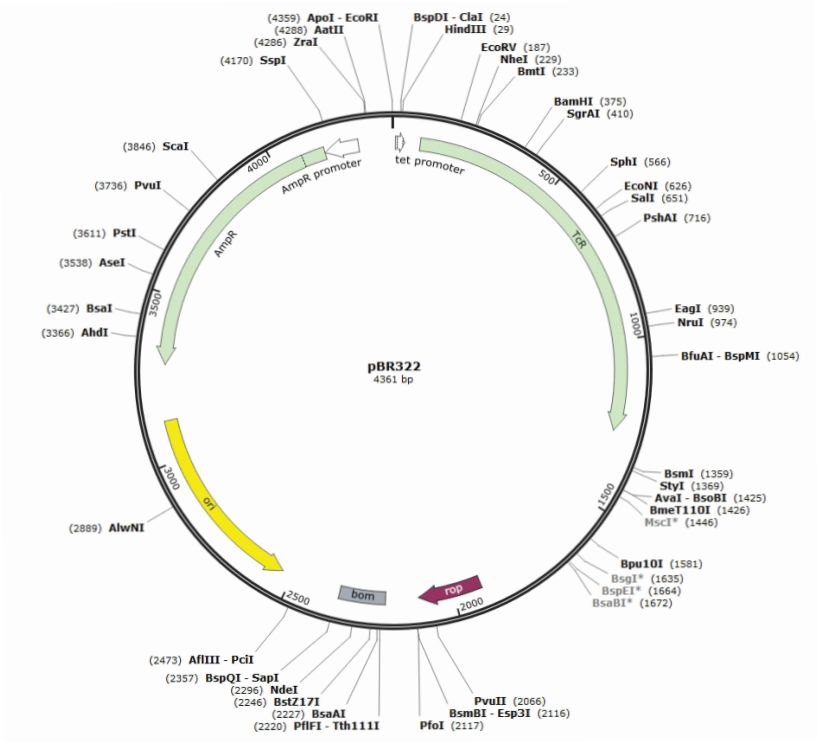


Figure 2. Map of E. coli plasmid pBR322.

Plasmids have multiple restriction sites (e.g., HindIII), which are indicated along the perimeter of the plasmid in the plasmid map. These restriction sites are recognized by restriction enzymes that cleave DNA at this site and are essential for specific cloning methods.

Another key feature is the "ori" or *E. coli* origin of replication (in yellow), which allows the plasmid to be replicated by *E. coli*'s DNA replication machinery, independently of the bacterial genome.[4]

Plasmids also have antibiotic resistance genes. pbR322, for example, has ampicillin and tetracycline resistance genes (green arrows), which also have associated promoters (white arrow). These resistance genes allow for selection of plasmids. Expression of the proteins encoded by either of the resistance genes protect the bacteria. The bacteria that do not contain this plasmid die when exposed to ampicillin or tetracycline. However, if the bacteria contain intact pBR322, they will survive. These resistance genes are called selectable markers. The type of selectable marker depends on the organism that the plasmid is intended to work with.

One important universal feature that has been added to plasmids is the MCS, which is a polylinker that contains several restriction sites that makes cloning easier by creating more flexibility in how DNA fragments can be inserted into a plasmid without disrupting essential elements of the plasmid. In pUC19 for example, the MCS is indicated in blue (Figure 3) and contains a cluster of restriction enzymes that you can use when cloning into this plasmid.[5] Note that the ampicillin resistance gene (AmpR) in green and the E. coli origin of replication (Ori) in yellow contain enzyme restriction sites. The use of these enzyme restriction sites may result in disruption of antibiotic resistance or replication, which could affect your cloning results. Thus, it is very important that the restriction sites that will be used in the cloning procedure be carefully selected.

SnapGene, a leading cloning tool preferred by scientists, has many functional features that aid in the cloning process. With SnapGene, the restriction sites that appear only once in a given sequence will be bolded as seen below (Figure 4) and there is an Enzyme Selection window where you can choose unique restriction sites, to ensure you are only cutting your plasmid at the site of interest.. You can learn about this tool here (link video).

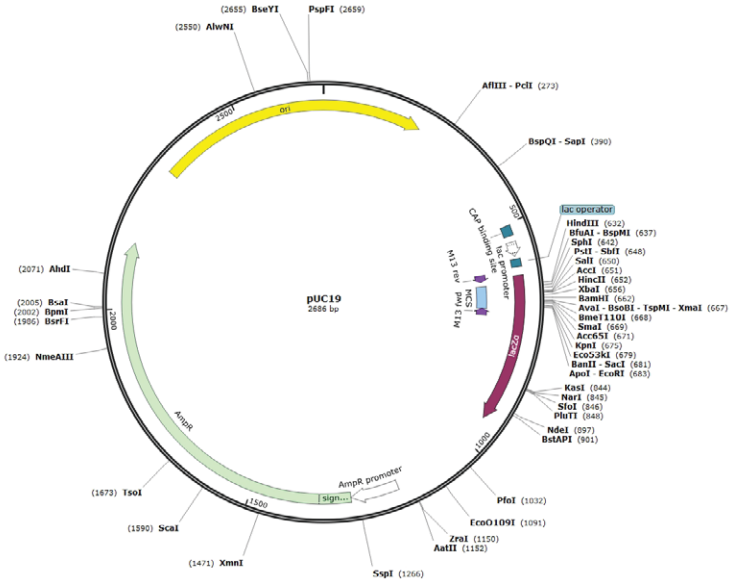


Figure 3. pUC19 plasmid.

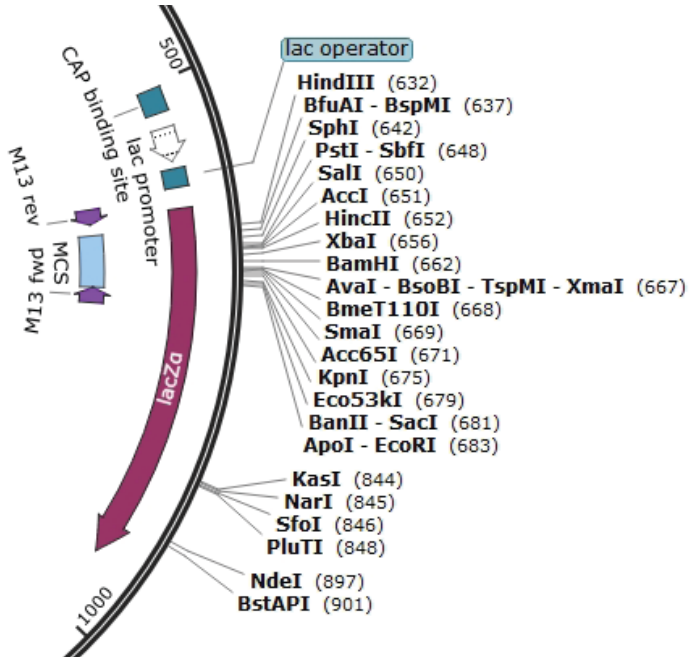


Figure 4. Enzyme restriction sites in pUC19.

Scientists use different model organisms for biological research. These organisms include mice, zebrafish, frogs, and yeast (Figure 5). Because the biology of these organisms varies vastly, specific and dedicated plasmids are needed to perform cloning and research in each unique organism. In other words, each organism requires vectors that contain features that allow their replication and gene expression in the model organism.

These organism specific functional elements, include: origins of replication, promoters and selectable markers. The sequences of commonly used vectors are available directly from SnapGene's website.

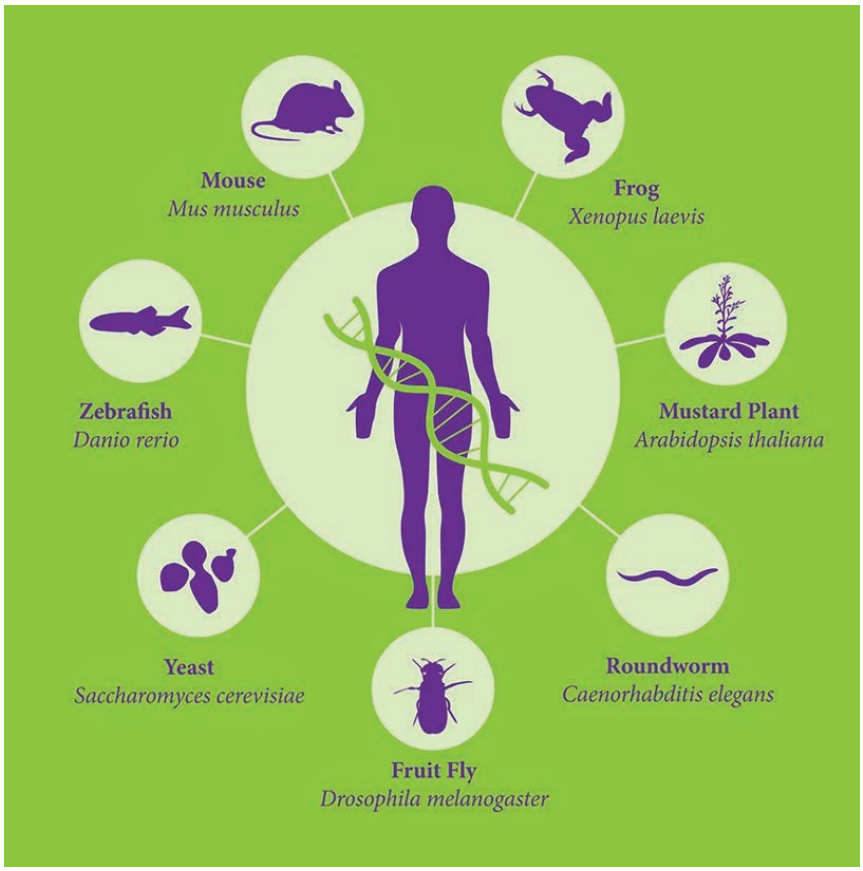


Figure 5. Organisms commonly used in biological research.

Visit **SnapGene.com** to learn more about cloning and ensure successful generation of recombinant DNA during your cloning experiments.

2

Introduction to the Plasmid Cloning Cycle

To successfully perform a cloning experiment, you must first understand how the different phases of cloning work. Here, we take you through the plasmid cloning cycle to illustrate the different phases of molecular cloning.

A series of steps must be performed successfully and in a specific order to clone a DNA fragment of interest into a cloning vector (plasmid). These steps are as follows:

1. Manipulation of DNA (isolation of insert, cutting of the vector, and ligation)
2. Transformation of DNA into bacterial host
3. Selection and Screening for candidate recombinant molecules
4. Extraction of candidate recombinant molecules from bacteria
5. Analysis of candidate recombinant molecules

Depending on the type of cloning experiment, you may need to perform this cycle one time. More complex cloning experiments may require that you perform this cycle of steps multiple times.

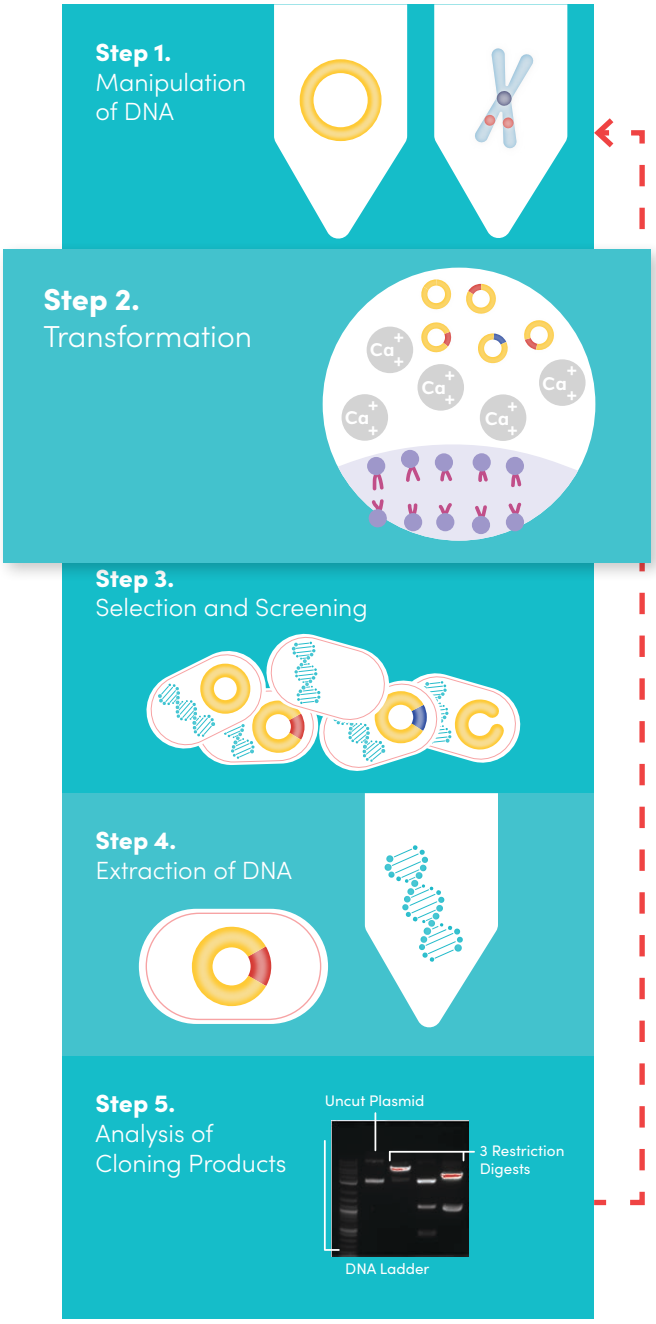


Figure 6. An overview of the plasmid cloning cycle.

Step 1: Manipulation of DNA

The first step is to manipulate DNA with the goal of generating a novel recombinant DNA molecule using specific starting materials (Figure 7). These starting materials include the **intact cloning vector/plasmid** (seen below in yellow), which is needed to help propagate the DNA of interest in a specific host, and the **DNA fragment of interest** (seen here in red), which is found in genomic DNA/Eukaryotic chromosome (in blue). The DNA material in red is the fragment that is to be isolated and cloned into the vector/plasmid.

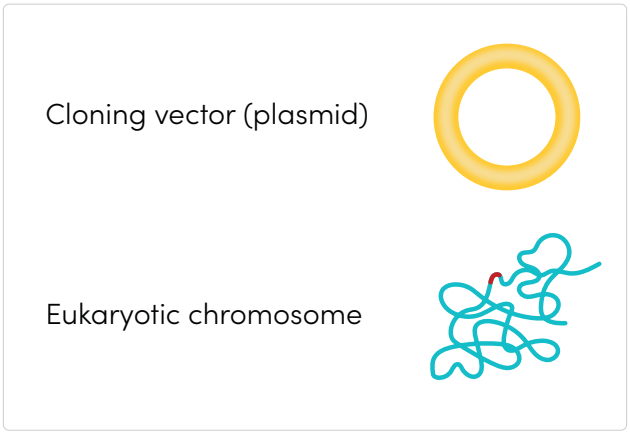


Figure 7. Starting material for the cloning procedure.

The classic approach to manipulate the plasmid and isolate the fragment of interest uses restriction enzymes. Restriction enzymes can be described as molecular scissors that cut DNA at specific sequences. It is also possible to manipulate DNA without restriction enzymes (this will be discussed later on).

A plasmid that is cut with restriction enzymes becomes linear. When the plasmid and DNA fragment are cut with the same restriction enzymes, they can be ligated back together using an enzyme called DNA ligase. The ligase "glues" fragments and the linearized plasmid together to generate a recombinant vector—a functional circular plasmid (Figure 8).

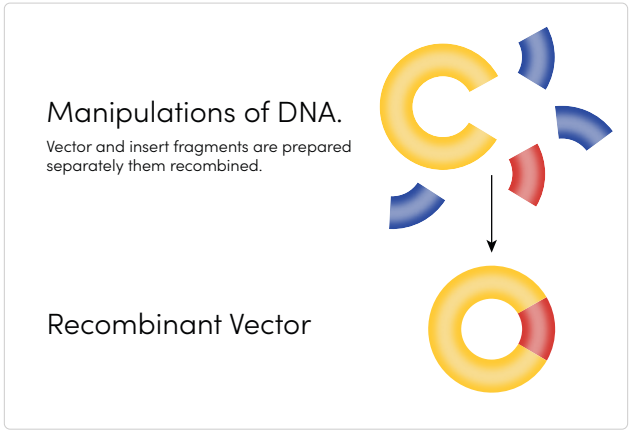


Figure 8. Manipulation of DNA. Genomic DNA and the plasmid are cut separately with the same restriction enzymes. Ligase is used to generate the recombinant vector.

Ligation does not guarantee that the DNA of interest has been ligated to the plasmid correctly. Analysis of your resulting clones is necessary to confirm that your cloning procedure has worked. This analysis will be covered in the Clone Verification section.

Once ligation occurs and recombinant DNA has been generated—and before clones can be analyzed—the recombinant DNA must be introduced into host cells.

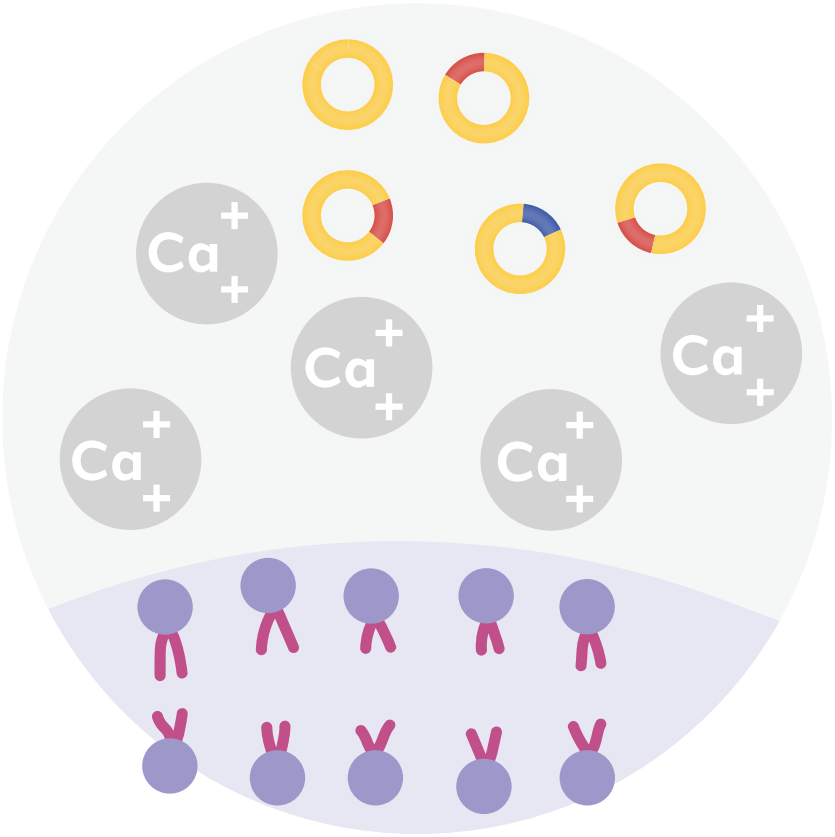


Figure 9. Chemically competent cells allow attachment of plasmid DNA to the cell membrane.

Step 2: Transformation

The DNA mixture can be introduced into bacterial cells through a process called transformation—a process that allows bacteria to make copies of the recombinant DNA.

Bacteria are very useful for cloning because they are able to absorb genetic material from their environment. Scientists have optimized this process to facilitate cloning experiments. The most common transformation technique used in laboratories today uses chemically competent cells, which are cells that have been treated with calcium chloride to allow attachment of plasmid DNA to the competent cell membrane (Figure 9).

Another technique uses electroporation, which involves exposing the cells to an electrical field (a shock), which increases the permeability of the cell membrane and allows plasmids to enter the cell. (Tylewicz).

With either technique, chemical transformation or electroporation, a very small proportion of cells are induced to take DNA from the surrounding environment. This is where the selectable marker on the DNA vector comes into play.

> Bacteria are very useful for cloning because they are able to absorb genetic material from their environment.

Step 3a: Selection (no cap required)

Once the plasmid has been introduced into the competent bacteria, the bacteria are allowed to rest and are then plated on agar plates so they can grow and propagate. In a process known as Selection, only the cells that received the circularized plasmid during transformation will grow (Figure 10).

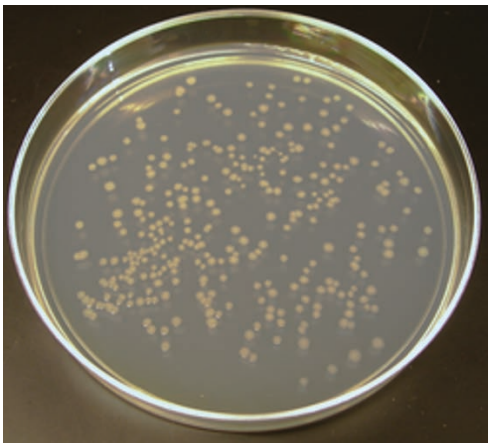


Figure 10. Bacterial colonies that are antibiotic resistant grow in an agar plate.

This selective growth is caused by the plasmids containing an antibiotic resistance gene that when expressed allows the bacteria to grow in media containing antibiotics (Figure 11).

Figure 11. Propagation of bacteria during Selection.

As mentioned previously, there are three types of plasmids that can result from cloning:

1. The vector can re-ligate back to itself (plasmid with no insert).
2. A blue fragment (not the DNA of interest) can ligate into the vector (plasmid with wrong insert).
3. A red fragment (DNA of interest) can ligate into the vector (plasmid with correct insert).

All of these plasmids will promote growth of bacteria in the presence of antibiotics. There may be several or just a few desired clones growing in the agar plates. Thus, the next step is to determine which clones contain the correct insert by performing a blue-white screen.

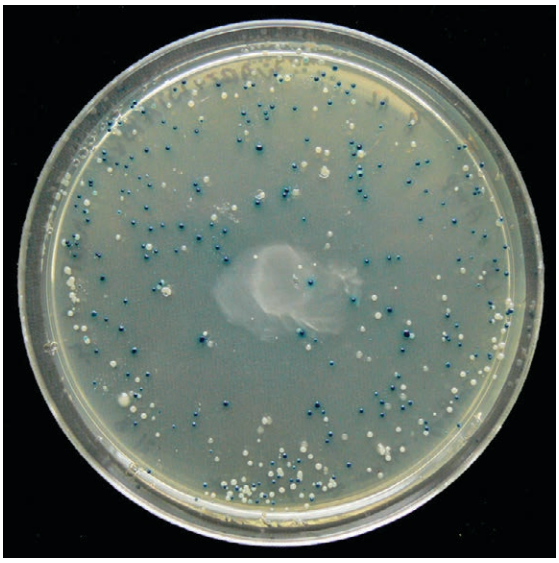


Figure 12. Blue and white colonies for a Blue-White screen.

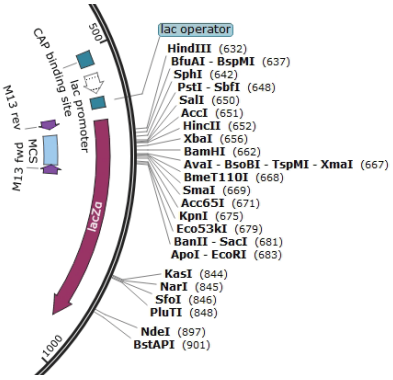


Figure 13. LacZα sequence in pUC19, indicated in purple.

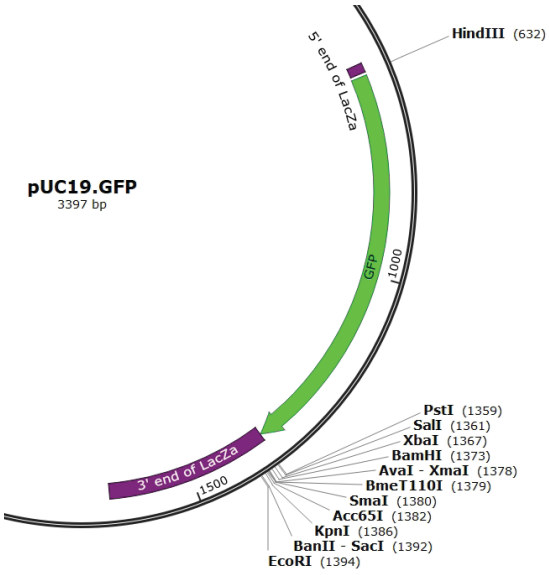


Figure 14. Cloning of the GFP sequence into the MCS disrupts LacZα.

Step 3b: Screening
Screening (no cap required)

Screening is distinct from Selection in that in Screening bacteria are examined for a specific phenotype. Blue-white screening is a very common screening technique, where the bacteria will appear blue or white depending on the presence of a plasmid. Other model systems rely on other phenotypes in their screening protocols.

Blue-white screening is possible due to the plasmid containing a specific functional feature—LacZα. When LacZα is disrupted by cloning into the MCS, then B-galactosidase cannot be produced and the bacteria cannot turn bright blue, the bacteria appear white (Figure 12). This is a very common screen and almost all plasmids carry the LacZα fragment. Depending on your cloning strategy, you may not need to perform this screen.

A white colony will appear when the insert has disrupted the MCS, and associated LacZ alpha fragment. There is no insert, colonies will remain blue (Figure 13).

For example, after insertion of the GFP-coding sequence (Figure 14) into pUC19, B-galactosidase cannot be produced and the bacteria will not turn bright blue.

Thus, when cloning GFP into pUC19, the starting materials are vector (pUC19) and the fragment of interest (GFP). We would be cloning a GFP fragment into the vector pUC19 using restriction enzymes to cut at restriction sites in the MCS of pUC19 and restriction sites in the insert (GFP). Once the fragments are cleaned up, they can be joined with ligase. The resulting DNA construct will be used to transform bacteria. Then, ampicillin selection and blue-white screening will be used to grow the bacteria and screen for colonies.

Step 4: Extraction from Bacteria

Once candidate clones are identified, small bacterial cultures are inoculated so that DNA can be extracted for analysis. DNA is extracted using a DNA miniprep, which is a rapid minipreparation of plasmid DNA. It is called a "mini" prep because it allows for isolation of small amounts of plasmid DNA—usually it yields around 30 micrograms of DNA. Currently, there are many DNA mini-prep kits available on the market that you can use. Once purified, DNA can be analyzed to determine whether the fragment of interest has been cloned as planned.

Step 5: Clone Verification

After isolation of the DNA, you must verify which clone (or clones) are the desired clone. There are multiple approaches to determine which clones are the correct ones.

One approach is sequencing, a method that is very accurate and yields sequences as shown below. A clone can be easily validated by aligning the sequence of the clone to a reference sequence, using SnapGene Tools.

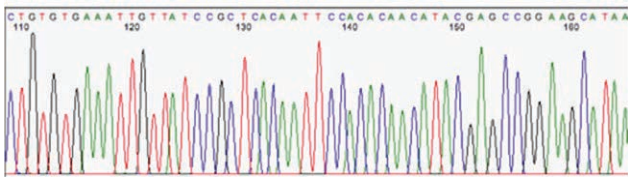


Figure 15. Sample sequence that is given once a clone is submitted for sequencing.

Restriction digest is another approach, and it requires the use of different restriction enzymes to cut the plasmid DNA at different sites. If your clone is the correct one, the resulting fragments will show that the insert is the correct and expected size (Figure 16). Another option is a diagnostic PCR reaction. It is important that you are 100 percent sure that your clone is the correct one before proceeding to the next step of your experiment.

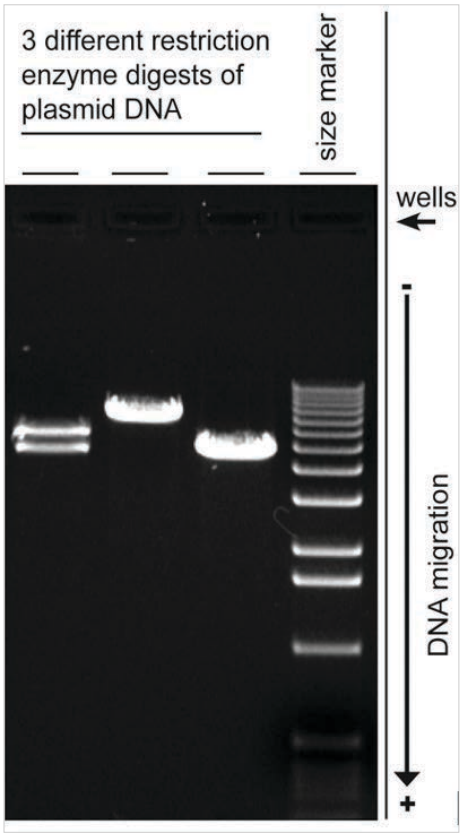


Figure 16. Restriction digests of plasmid DNA.

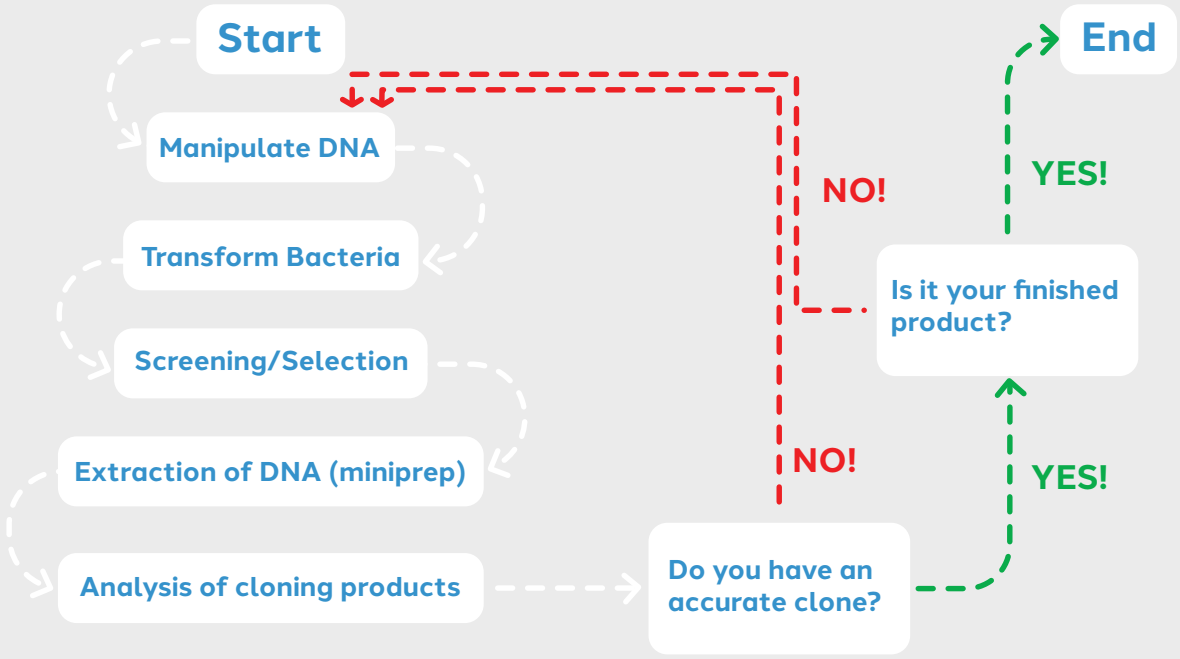
To summarize, the materials and steps required for cloning a fragment of interest (GFP) into a plasmid (pUC19) are the following:

Starting Material Vector Fragment of Interest	pUC19 GFP	SnapGene
1. Manipulation of DNA	Treatment with restriction enzymes and ligase	Lab bench SnapGene
2. Transformation	Transform bacteria with DNA mixture	Lab bench
3. Bacterial Growth	Ampicillin Selection Blue/white screening	Lab bench
4. Extraction of DNA	Standard miniprep procedure	Lab bench
5. Clone verification	Sequencing, PCR, digest	Lab bench SnapGene

Depending on your cloning objective, you may proceed through steps 1 through 5 just once before proceeding with other aspects of your experiment. Other situations may require that you "cycle" through the steps until your project is complete. Thus we can represent the steps of the Cloning Cycle in a flow chart.

The decision points come after analyzing candidate clones for completeness and accuracy.

1. The clone is not correct. Thus, you must start over at the manipulation of DNA-step. Troubleshooting may be needed.
2. The clone is correct but further modification is needed. Thus, you will manipulate the DNA again.
3. The clone is correct and is the desired finished product. In this case, you can proceed to other aspects of the experiment.



Visit **SnapGene.com** to learn more about cloning and ensure successful generation of recombinant DNA during your cloning experiments.

3

How SnapGene helps with the Plasmid Cloning Cycle

SnapGene has many different tools that will help with your cloning workflow. Here, we described how different SnapGene tools can be used throughout the Cloning Cycle (shown below).

In order to successfully create a molecular clone, you need to understand the features of the genetic material you are starting with. You also need a clear idea of how you will use those features in your completed construct. SnapGene Tools allow you to virtually assemble your final construct before proceeding to the bench. Visualizing your clone this way, before proceeding, allows to guarantee that you will be including all the elements that you need, and that they are positioned correctly.

> SnapGene has many different tools that will help with your cloning workflow.

Using SnapGene to Document and Annotate Sequences

In general, when starting a cloning experiment you must first document the sequence. In SnapGene, the functions for creating DNA files and documenting them are found in the **File Menu**. SnapGene allows you to **create a new sequence or import a sequence** from another format (Figure 17). This is especially helpful because you may receive sequences from different sources that use different formats.

Figure 17. Creating a new sequence in SnapGene.

Your sequence may come fully annotated or may need to be analyzed and annotated. In SnapGene, most of the annotating tools can be found in three menus: Enzymes, Features, and Primers (Figure 18).

It must be noted that prior to proceeding you must verify that you have received the correct plasmid. You must sequence at least part of the plasmid to determine whether it is the correct sequence. This is a very important step of cloning because it determines whether your experiment is successful or not. In SnapGene, you can easily align your cloned (original) sequence with one or more reference sequences. This function can be done using the Tools menu, where you can choose to align your sequence with other sequences, a copied sequence, or a sequence trace. You can see exactly how your original sequence matches to different reference sequences (Figure 19).

For more details about how to align sequences, check out this **SnapGene video tutorial**.

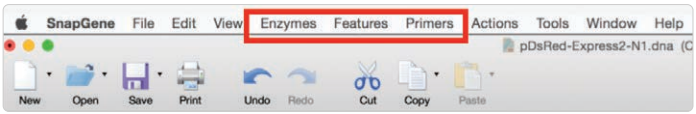


Figure 18. Annotating in SnapGene.

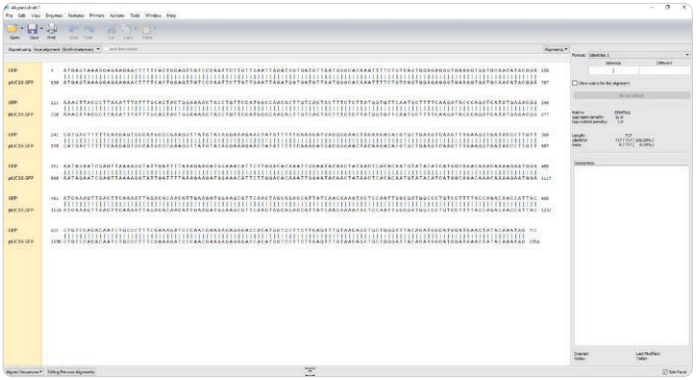


Figure 19. Aligning sequences in SnapGene for cloning verification.

To learn more about how to use Features in SnapGene watch this **video tutorial**.

Using SnapGene Actions for Your Cloning Strategy and Molecular Construct

The SnapGene Actions menu contains an array of tools that allow you to design and plan your cloning project in *silico*, allowing you to prepare for manipulation of DNA at the bench (Figure 20). This is a very useful tool because it allows you to simulate insertion of fragments into a desired plasmid and visualize the process from beginning to end. Throughout the cloning process, SnapGene allows you to see specific elements of the plasmid (e.g., restriction sites, MCS, antibiotic resistance genes).

Figure 20. Action menu in SnapGene.

The cloning techniques that SnapGene allows you to visualize include Restriction and Insertion Cloning, Gateway Cloning, Gibson and HiFi Assembly, and InFusion Cloning. These are discussed in more detail in Chapter 5.

Once you develop your cloning strategy and visualize the resulting construct using SnapGene, you can move to the bench and perform the necessary procedures: cutting with restriction enzymes, transformation, growing of bacterial cultures, and extraction of DNA.

With SnapGene, you can visualize the map, sequence, primers, and history of steps, which is very helpful when performing a cloning simulation

Using SnapGene Tools to Verify Your Clone

The functions of SnapGene 'Tools' are essential for clone verification. You may need to use a combination of Assemble or Align depending on the sequence you are analyzing. SnapGene's **Simulate Agarose Gel** can be used to predict your results when you are analyzing your clones with a diagnostic PCR reaction or a restriction digest.

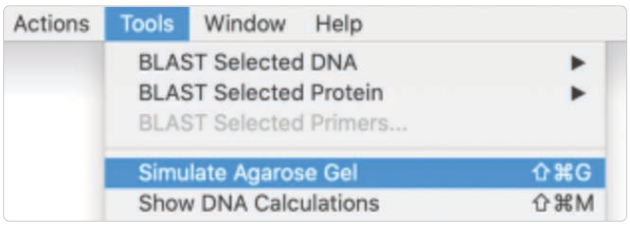


Figure 21. Simulate Agarose Gel function in Tools in SnapGene.

Under the Tools menu, you can access NCBI searches as well as local sequence assembly and alignment functions to help validate your molecular clone.

Thus, you can use SnapGene functions to facilitate your cloning project (Figure 22):

Starting Material Vector Fragment of Interest	Lab Bench	SnapGene
1. Manipulation of DNA	Various enzymes	Manipulate digitally in Actions
2. Transformation	Use of bacteria	n/a
3. Bacterial Growth	Use of selective media	n/a
4. Extraction of DNA	Minipreps	n/a
5. Clone verification	Sequence or Restriction digest	Analyze and compare in Tools and Agarose Gel Simulator

Figure 22. SnapGene functions useful for the Cloning Cycle.

1. The File menu allows you to create documents.
2. The Enzyme, Features, and Primers drop down menus allow you to add custom annotations to your sequences so that you can create a complete map of the features that are important in your sequences.
3. The Actions menu allows for digital manipulation of DNA.
4. The Tools menu including the Agarose Gel Simulator facilitates analysis of your clone.

Visit **SnapGene.com** to learn more about the different tools that will make your cloning possible.

4

General Tips for Cloning Success

Here, we present general tips that will help you achieve cloning success.

Tip 1. Respect Murphy's Law

If anything can go wrong, it will. However, by following the tips shared here you may avoid the many issues that may arise during cloning.

Tip 2. Understand the protocols and learn why each step is needed.

It is essential that when starting to use a cloning protocol, you understand what each step in the protocol accomplishes. As you become more familiar with the protocol and technique, you will learn which steps require more attention and precision, and which steps allow for more flexibility.

In some instances, you may increase efficiency by spending time choosing the best approach. For example, it is more efficient to clone a blunt-ended fragment into a blunt vector site, than into a site that has been blunted with Klenow or T4 DNA polymerase.

Tip 3. Prepare abundant and high-quality nucleic acids.

There is no substitute for good starting material with few contaminants. To obtain good-quality and abundant DNA, you must follow the DNA purification procedures carefully. Remember that it is easier to dilute something than to concentrate it. The quality of DNA is important. Thus, you must ensure that the DNA is clean.

Tip 4. Accurately quantitate your nucleic acids.

Ensure that you are quantifying small volumes of DNA accurately. Well-purified DNA has a characteristic curve over the measured UV spectrum. A sample of a classic UV spectrum observed on a NanoDrop instrument is shown on Figure 23. Absorbance at 260 nanometers is used to quantitate DNA. Quality of DNA is indicated by the 260/280 ratio. DNA with good purity will have a 260/280 ratio of 1.80-2.00. As seen below, the DNA is of good quality—which is also indicated by the shape of the curve.

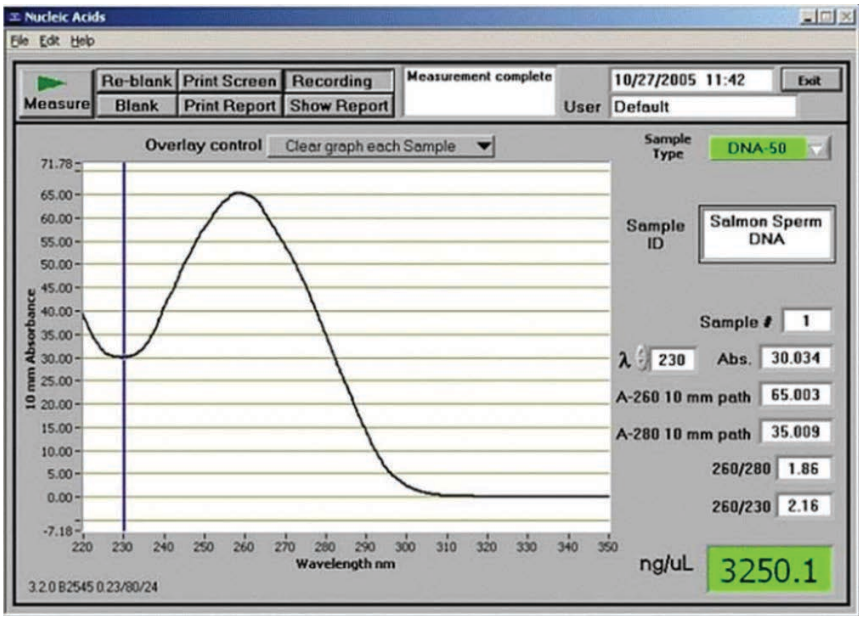


Figure 23. Sample quantification of DNA using a NanoDrop.

For more restriction cloning tips visit this SnapGene Resource.

A sample that has impurities will not have the peak shown above. Impurities can inhibit a downstream reaction. Impurities also interfere with accurate quantification of nucleic acids. It is also preferred to use samples that have less contaminants.

Tip 5. Carefully store and handle DNA

Freeze DNA to protect it from UV and oxidative damage. Avoid prolonged exposure to shortwave UV (UVB) when purifying DNA from agarose gel. Use the longer wavelength UV option (UVA). It would be best to use a non-UV lamp with the appropriate dye to avoid damaging DNA.

Tip 6. Understand molar ratios

Many protocols indicate the molar ratios needed. Thus, you must understand moral ratios. Specifically, you must understand the relationship between DNA mass and molar concentration. The mass of a DNA molecule is directly proportional to its length.

This example from SnapGene DNA calculations shows information about two different DNA molecules; pUC19 and M13/pUC Reverse (Figure 24). Full length pUC19 is 2,686 base pairs long and its molecular weight is 1.66 x 10^6 Daltons. The second fragment, M13/pUC Reverse, is a small oligonucleotide (primer) that is used for sequencing. Its molecular weight is 7,065.7 Daltons. As shown below, 1 nanomole of primer weighs 7.066 micrograms, whereas 1 nanomole of pUC19 weighs 1.659 milligrams. If these two fragments were mixed together based solely on their mass, we would have over 200x more moles of primer than of the plasmid, which may or may not be ideal. For most cloning experiments, insert to vector ratios will vary. Some protocols may call for 2:1 to 5:1 insert to vector ratio. The optimal molar ratio depends on the sizes of the fragments and the specific technique that is used.

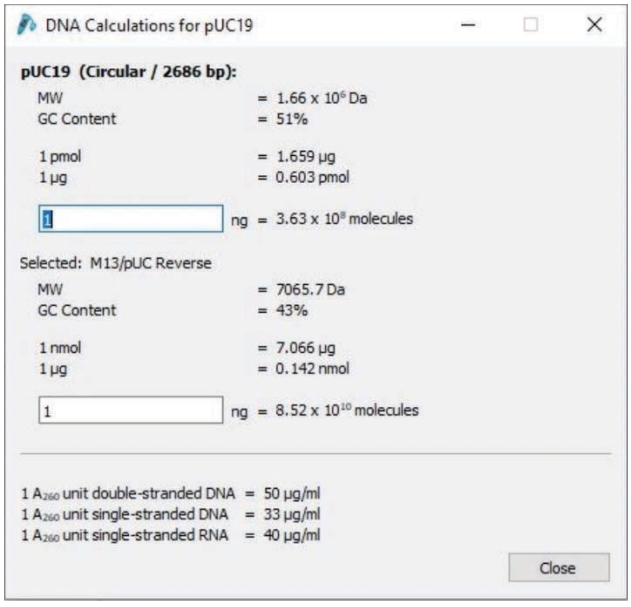


Figure 24. DNA calculation sample from SnapGene.

Tip 7. Learn to pipette as accurately as possible.

The less deviation you can introduce via pipetting the better. Accurate pipetting leads to accurate measurements and strong replicates.

Tip 8. Double check your work

For molecular biology experiments and cloning, it is important that you double check your calculations. In addition, learn what different volumes look like in your pipettors and Eppendorf tubes, and double check your labels.

Tip 9. Save your reaction intermediates

Saving reaction intermediates during cloning is beneficial when unexpected mishaps occur. Saving leftovers from reactions allows you to repeat steps if necessary. For example, you may have made a mistake when performing the transformation protocol. If you saved part of the ligation reaction, you can go back and repeat the transformation as needed.

Tip 10. Safely store and accurately document your finished products

Most reagents have optimal storage conditions. If these guidelines are not followed, then the materials will degrade, resulting failure of your experiment and wasted time and money.

Tip 11. Do not depend on kits

Kits are very useful, only when you understand what each step accomplishes. This is especially important when kits fail. Your understanding of the procedure will allow you to effectively troubleshoot the problem.

Tip 12. Simplify your cloning

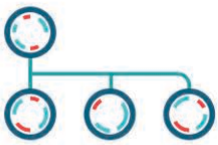
If possible, minimize the steps in your cloning protocol. As more steps are involved, there is a higher chance for error, loss of yield, mis-labeling, and mutation.

5

SnapGene's Key Cloning Techniques

Plan

Visualize



Document

SnapGene offers a wide range of user-friendly tools that support and facilitate simple and complex cloning projects. With SnapGene you can **plan** your cloning strategy, **visualize** the process, and **document** your work. SnapGene's tools prevent you from making errors and wasting time at the bench.

SnapGene helps streamline your cloning:

1. Specialized cloning tools ensure fast accurate construct design for all major molecular cloning techniques
2. Highly visual alignment of sequencing results to your simulated construct and simulation of agarose gel electrophoresis ensure accurate verification of your cloning results
3. Automatic documentation tools record your experimental procedures and create a graphical history
4. Data management tools allow storage, importing, sharing, and the organization of maps, files and sequences

Restriction Enzyme Cloning

Restriction enzyme cloning is a common technique that is used to prepare inserts and vectors for cloning using restriction enzymes. This method is used mainly when the insert and vector contain compatible restriction sites—the restriction sites must flank the region of interest and ideally be found within the MCS of the plasmid. In SnapGene, you can simulate cutting and ligation of plasmid and insert (Figure 25). In a few easy steps, you can import (or use copy/paste functions in SnapGene) sequences, cut them, and ligate them. You can then preview the final cloned plasmid, which will show the insert in red and the plasmid in black. You can see the step-by-step process here.

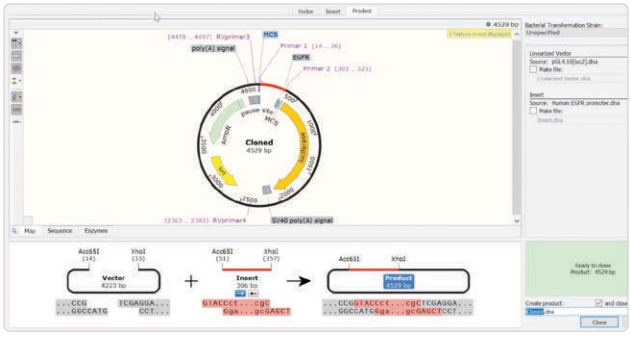


Figure 25. Simulation and visualization of restriction enzyme cloning in SnapGene.

Gateway Cloning

Gateway cloning is a highly efficient alternative to traditional restriction enzyme cloning and does not require restriction enzymes to insert DNA into a plasmid. Instead, an insert is moved into a vector through a two-step recombination process that takes advantage of integration and excision reactions using attachment sites *attL* and *attB*. The first step—a BP reaction—creates an entry clone containing the DNA insert flanked by two *attL* sites. The second step—an LR reaction—creates an expression clone containing the DNA insert flanked by two *attB* sites. Multisite Gateway cloning allows up to four fragments to be inserted simultaneously.

Below, you can see how SnapGene visualizes both reactions including the insert fragment and donor vector, which are needed for the BP reaction, as well as the resulting entry clone and the destination vector, which are needed for the LR reaction (Figures 26 and 27).

To learn more about SnapGene's Gateway Cloning simulation click **here**.

Figure 26. Creation of the entry clone using an insert sequence and a donor vector.

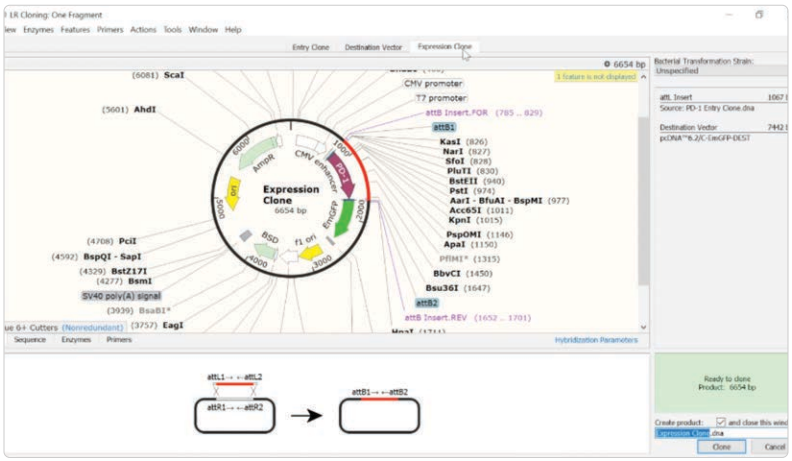


Figure 27. SnapGene snapshot of the expression clone, the product, through the LR reaction which uses the entry clone and a destination vector.

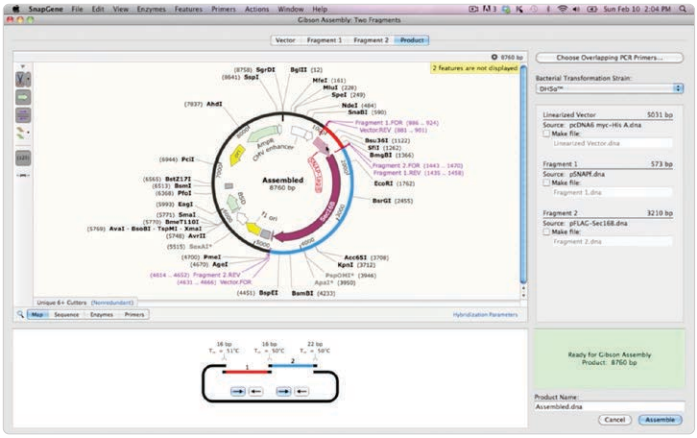


Figure 28. Product in SnapGene's simulation of Gibson Assembly performed with a vector and two fragments. Primers are indicated in purple. The fragments are shown in blue and red.

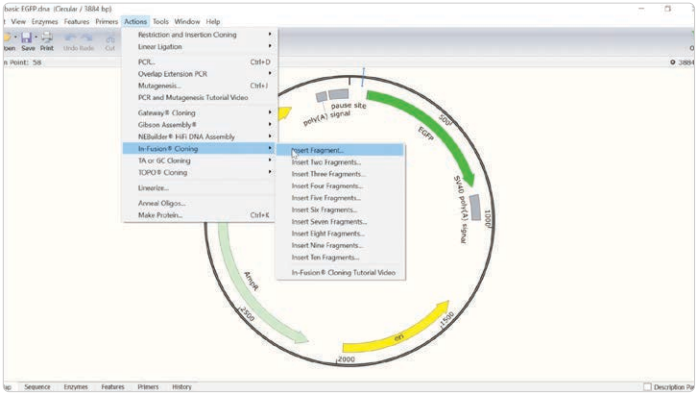


Figure 29. In SnapGene, In-Fusion cloning can be simulated using the Actions menu.

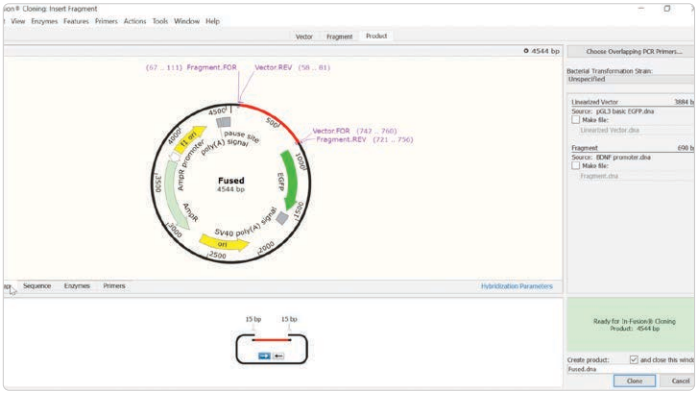


Figure 30. The product of In-Fusion Cloning simulation in SnapGene. The fragment is in red and the plasmid is in black. Primers are indicated in purple.

Gibson Assembly

Gibson Assembly is another efficient and easy-to-use cloning method that does not require restriction enzymes and allows insertion of multiple fragments into a plasmid in one reaction.

SnapGene simplifies your Gibson Assembly cloning procedure by letting you select and visualize the desired vector and fragments. SnapGene has automated primer design (Sidebar: After you design your primers on SnapGene, you can export them, which facilitates the primer ordering process) so that your fragments contain the overlaps needed for Gibson Assembly cloning (Figure 28). Below, Gibson Assembly was used to clone two fragments into a chosen vector.

See a detailed procedure for simulation of **Gibson Assembly in SnapGene**.

In-Fusion Cloning

In-Fusion cloning is a very efficient cloning method that does not require ligation. The insert and linearized vector are joined by the annealing of complementary ends (flanking homologous overlaps), which are generated via PCR, in a single step using the In-Fusion enzyme. It is important to note that the vector must be linearized through restriction digest or inverse PCR. In general, In-Fusion cloning is faster and has higher accuracy than Gibson Assembly.

In SnapGene, you can perform In-Fusion cloning by clicking on Actions In-Fusion Cloning Insert Fragment (Figures 29 and 30).

TA Cloning

TA cloning is a simple cloning technique that does not require restriction enzymes. Instead, these techniques utilize the addition of A overhangs to the 3' end of PCR products by DNA polymerase enabling ligation of these PCR products into vectors with complementary T overhangs.

In SnapGene, you can simulate TA cloning by clicking on Actions > TA or GC Cloning. Below you will see the drop down menu in SnapGene (Figure 31).

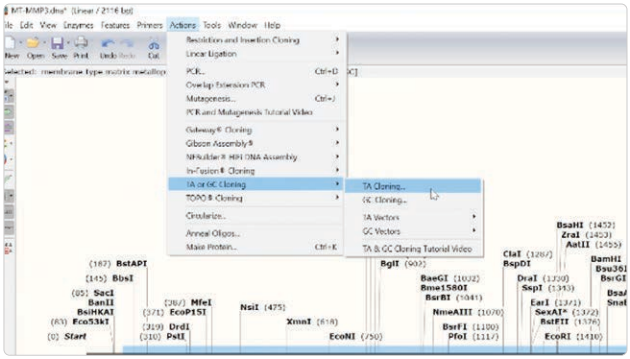


Figure 31. Snapshot of SnapGene's menu for TA and GC cloning simulation and visualization process.

As with other cloning techniques, you can select the insert and linearized vector files (Sidebar: With SnapGene choose a custom vector or a commercially available vector). Once selected, you will design the suitable PCR primers. In the Product tab, you will be able to see the insert and linearized vector with their overhang sequences, which are also indicated in the product (Figure 32). The vector is indicated in black and the insert is indicated in red.

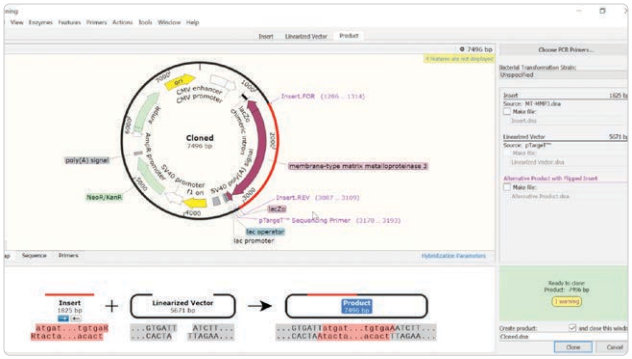


Figure 32. Snapshot of simulation of TA cloning in SnapGene. The resulting product is a plasmid with the indicated insert (in red).

To learn more about the detailed steps to use these cloning techniques in SnapGene check out these **SnapGene video tutorials**.

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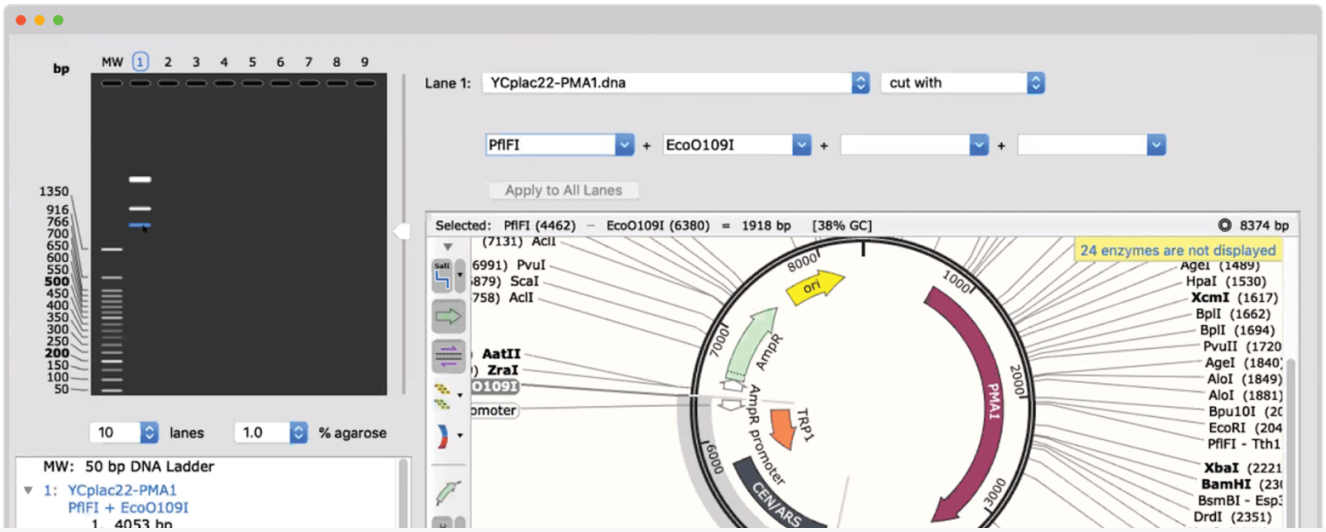

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