

BASIC SKILLS IN MOLECULAR BIOLOGY

Introduction

In our first laboratory you will learn some basic skills of molecular biology, the proper care and use of micropipettors and the proper handling of microorganisms. These are essential skills that you will use to set up your first reaction - cutting DNA with restriction enzymes.

LEARNING GOALS:

1. Learn how to work safely in the Molecular lab.
2. Know how to correctly use micropipettors.
3. Understand how to safely manipulate bacteria.

Topic I: MICROPIPETTORS AND PIPETTING SKILLS

Use of the micropipettors is one of the most fundamental skills in molecular biology. Since we must often accurately measure very small quantities, the proper use of micropipettors is also one of the most important skills to learn.

1. Types of Micropipettors

Pipettors are made by many different manufacturers and thus all do not look the same. However, if you learn how to correctly use one type of pipettor it is usually easy to understand other pipettors since the same basic principles are used for setting many pipettor models. Figure 2.1 shows several common micropipettors. The pipettors used in our lab are the Rainin Pipetman® brand and a few similar models.

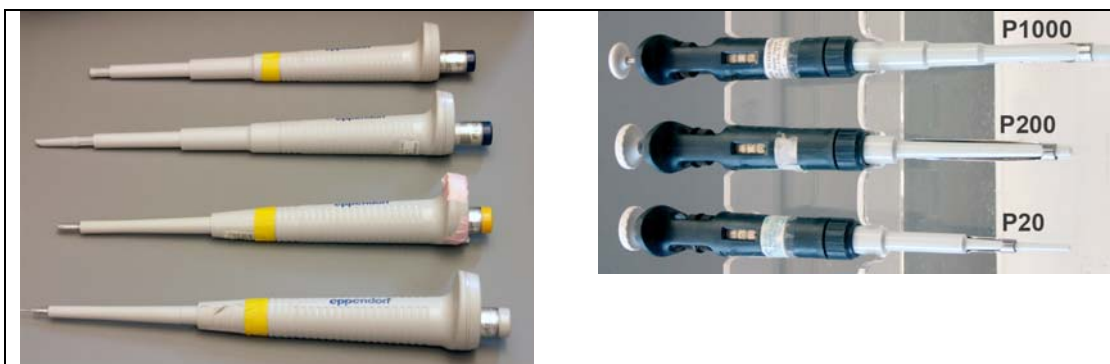


Figure 2.1 Common brands of micropipettors. Two brands of pipettors often found in research or clinical labs are the **(left)** Eppendorf® or **(right)** Rainin Pipetman® brands. (photos by S. Dellis)

The top of the plunger shows the pipettor size for the Pipetman models. Each pipettor has its own volume range and it is **CRITICAL** to use a pipettor only in its proper volume range. The “P-number” represents the maximum volume in uL that the pipettor can measure. Pipettors are more accurate in the *upper* part of their range. Thus, 20 uL should be measured with a P20 rather than with a P200. The three pipettor sizes used in our lab will measure from 1 uL - 1000 uL as shown in Figure 2.2, and Figure 2.3 identifies the parts of the pipettor.



Figure 2.2: The P1000, P200, and P20 models of Pipetman® (photo by S. Dellis)

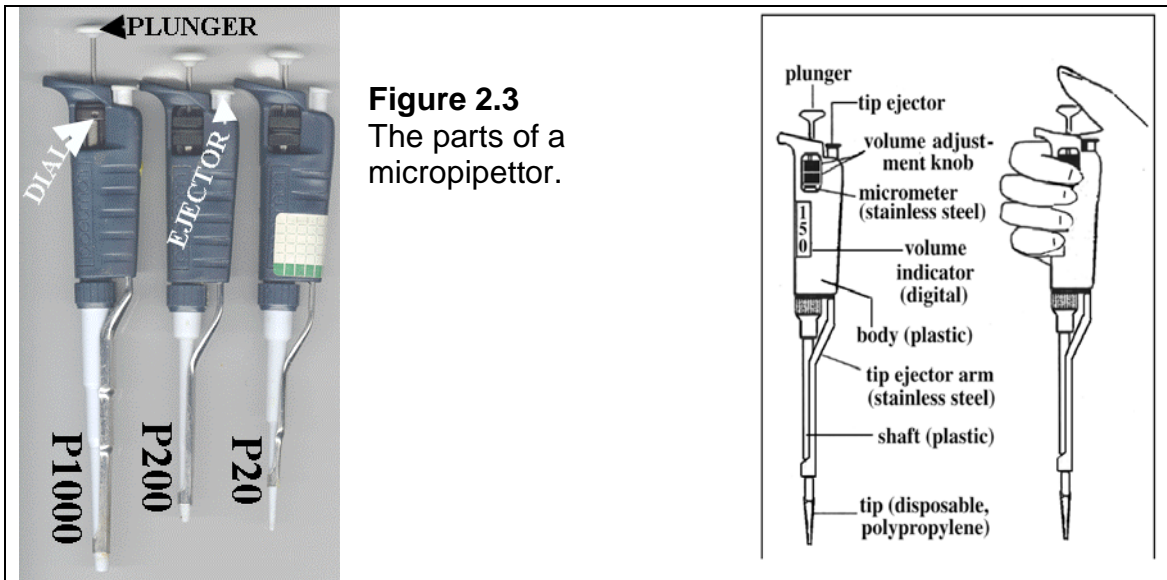


Figure 2.3
The parts of a micropipettor.

Left: the three sizes of Pipetman®. The large P1000 is easy to identify, however the P200 and P20 are often confused. Always *check the number* on the plunger to insure you have the correct pipettor.

http://a32.lehman.cuny.edu/molbio_course/pipettman.GIF

Right: the parts of a generic pipettor. Volume is set with the volume adjustment knob (or dial) and the volume is shown on the volume indicator. There are three numbers shown on the volume indicator but the units differ depending on the pipettor. The tip ejector button moves the ejector arm and releases the tip.

<http://sonhouse.hunter.cuny.edu/tech/TF-7.htm>

2. Correctly Adjusting the Pipettors

Figure 2.4 Adjustment range and volume indicators

Model	P20	P200	P1000
Volume range	1-20 uL	20-200 uL	200-1000 uL
Smallest measurement increment	0.02 uL	0.20 uL	2.0 uL

P-20	P-200	P-1000																		
<table border="1"> <tr><td>1</td><td>← tens</td></tr> <tr><td>7</td><td>← ones</td></tr> <tr><td>4</td><td>← tenths</td></tr> </table>	1	← tens	7	← ones	4	← tenths	<table border="1"> <tr><td>0</td><td>← hundreds</td></tr> <tr><td>5</td><td>← tens</td></tr> <tr><td>7</td><td>← ones</td></tr> </table>	0	← hundreds	5	← tens	7	← ones	<table border="1"> <tr><td>0</td><td>← thousands</td></tr> <tr><td>9</td><td>← hundreds</td></tr> <tr><td>7</td><td>← tens</td></tr> </table>	0	← thousands	9	← hundreds	7	← tens
1	← tens																			
7	← ones																			
4	← tenths																			
0	← hundreds																			
5	← tens																			
7	← ones																			
0	← thousands																			
9	← hundreds																			
7	← tens																			
= 17.4 ul	= 57 ul	= 970 ul																		

Figure 2.4 shows some sample pipettor settings. Using one of each of the pipettor sizes, answer the following questions:

1. Some of the Pipetman® models demarcate some of the measurement units with a **red** number to make it easier to determine if the pipettor is set properly. Which pipettor models (P20, P200, etc.) have these demarcations?

What units do they represent?

2. Draw the volume indicator for the following volumes.


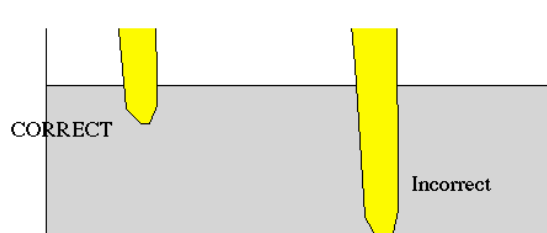
What pipettor size is used?

- A. 20.0 uL
- B. 350 uL
- C. 100.5 uL
- D. 2.76 uL
- E. 555 uL

3. Using the Pipettor

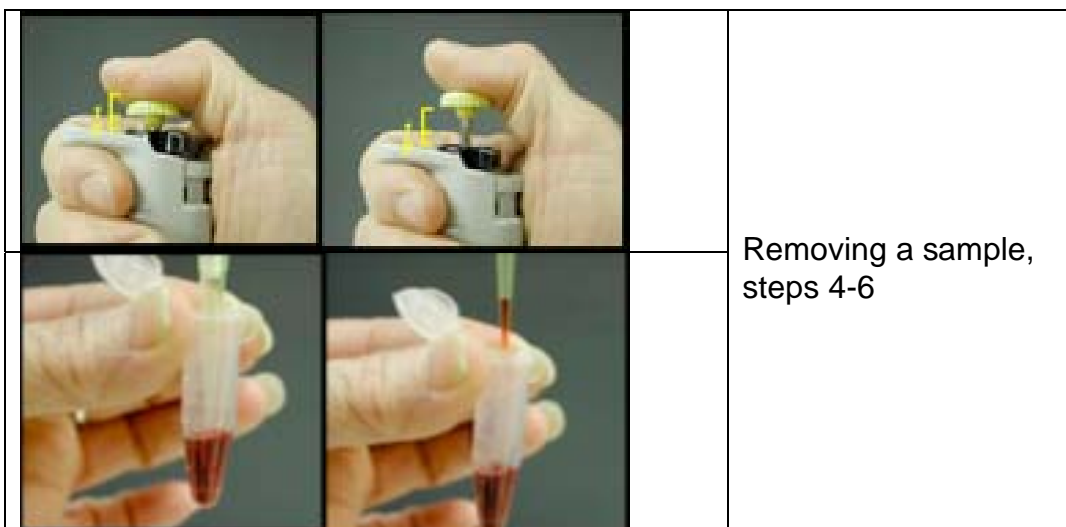
A) Drawing up a sample

1. Check the top of the pipettor plunger button to make sure that you have the pipettor that you need. We have three sizes. Refer to the ranges listed above.
2. Rotate the volume adjustment knob until the digital indicator reaches the desired volume.
3. Firmly place a disposable tip on the shaft of the pipettor. The P100 uses the large "blue tips". The P200 and the P20 both use the "yellow tips" (Figure 2.5, left).

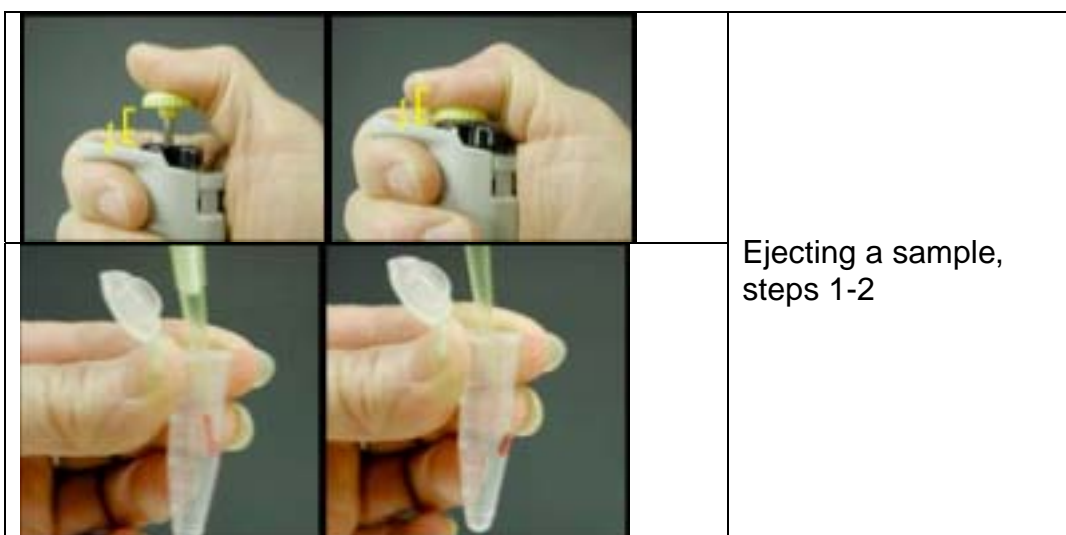
 <p style="text-align: center;">"yellow tips" "blue tips"</p> <p>(photo by S. Dellis)</p>	<p>Figure 2.5 Pipettor tips LEFT: the two common types of pipettor tips. RIGHT: the tip is immersed only a few millimeters into the solution.</p> 
<p style="text-align: center;">http://www.bio.davidson.edu/Courses/Bio111/Bio111LabMan/Preface%20D.html</p>	

4. Press down the plunger to the **first stop**. (You will be able to push past this point, but there is enough resistance to stop the movement.) This part of the stroke is the calibrated volume that you see on the digital micrometer.

5. Hold the pipettor vertically and immerse the disposable tip into the sample. Only place the tip in to a depth of several millimeters, do *not* immerse the entire tip! See Figure 2.5, right.
6. Slowly, with a nice even motion, return the plunger button to its original position. Do *not* allow the button to snap up.
7. Wait a couple of seconds to ensure that the full volume of the sample is drawn into the tip.
8. Withdraw the tip from the sample.



http://biog-101-104.bio.cornell.edu/biog101_104/tutorials/pipettor/emptying.html



http://biog-101-104.bio.cornell.edu/biog101_104/tutorials/pipettor/emptying.html

B) Dispensing the sample

1. Place the tip against the side wall of the receiving tube and push the plunger down to the **first stop**. Wait 2-3 seconds, and then depress the plunger to the **second stop** in order to expel any residual sample in the tip. It helps to keep the tip end against the dispensing container and use capillary action to drain the tip.

2. While the plunger is still pushed down, remove the pipettor from the tube and slowly return the plunger to its original position - no jerking movements.
3. Discard the disposable tip into Solid Waste by pushing the ejector button. (Be careful where you point.)

C) Important RULES for pipettor use

Pipettors are expensive (over \$250 apiece!) and must be sent out to repair. You must handle them with care.

1. Never rotate the volume adjustment knob past the upper or lower range of the pipettor.

2. Any type of liquid inside the barrel can ruin the inner workings of the pipettor.

Therefore:

Never use a pipettor without a tip in place.

Never lay the pipettor down on its side or invert it (upside-down) when it contains liquid, always hold it upright.

3. Never let the plunger snap back after withdrawing or ejecting fluid as this could damage the piston.

4. *Please ask* if you are not sure of which pipettor or setting to use. Your Instructor will gladly assist you.

5. When not in use, keep pipettors on the bench away from the edge. Before putting them away, adjust pipettors to their upper volume.

Topic II: BACTERIOLOGY FOR THE MOLECULAR BIOLOGIST

1. Bacteria

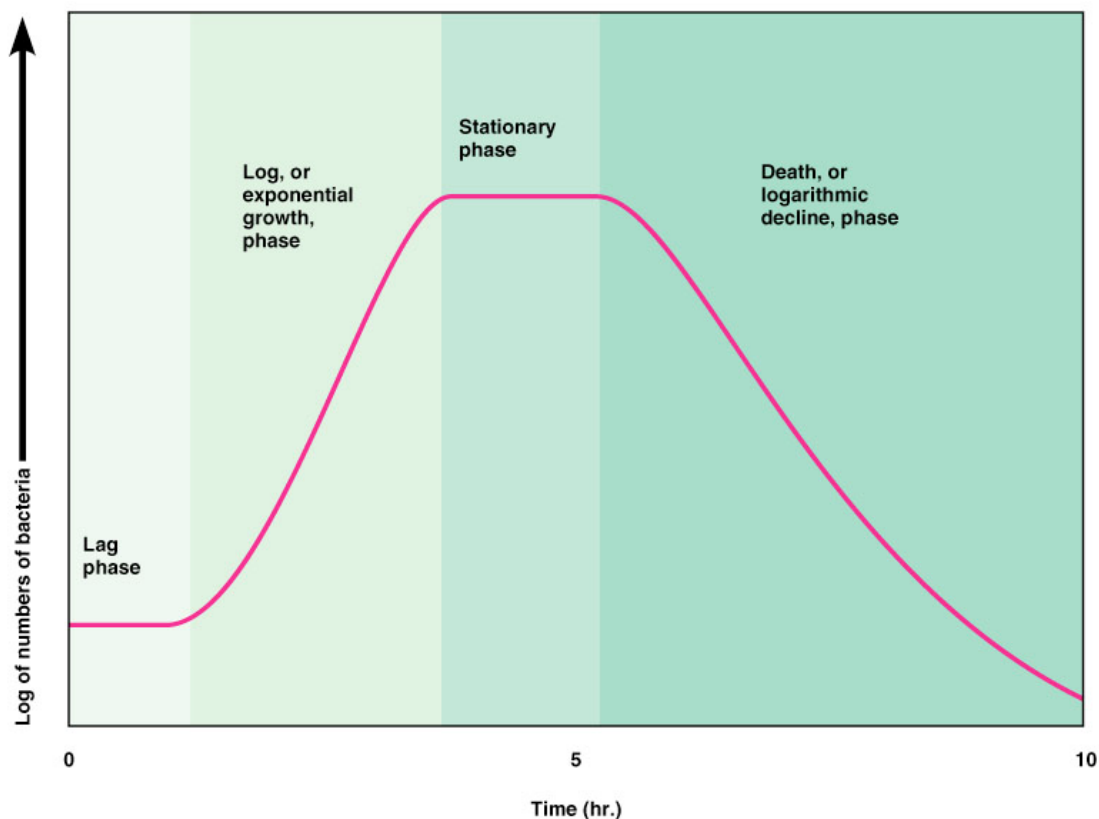
Bacteria are important tools to the Molecular Biologist. We use bacteria as little "factories" to make plasmid DNA or to express a particular protein we are studying. However, it is important to remember that bacteria are living organisms that do not always comply with our wishes. For example, if the protein you are trying to produce is harmful to the bacteria, you may find that it will only produce altered versions, or no protein at all.

The *Escherichia coli* (*E. coli*) bacterium is the most common in the Molecular Biology lab. There are many different strains of *E. coli*. Whenever a researcher introduces a change in the *E. coli* chromosome, she has created a new bacterial strain. It is important to know which strain you are working with and to understand how the mutations in the strain may affect your work.

Many molecular biology lab manuals and some of the biotechnology company catalogues have lists of the common *E. coli* strains used in molecular biology. The strain and its **genotype** are given. The genotype will list all mutations in that strain. If a gene is not listed, you can assume it is the **wild type**. (What strains are we using in this exercise?)

Bacteria are able to grow in liquid media or on solid media. There are many specialized types of bacterial growth media. One that is often used for *E. coli* is called Luria-Bertani broth, or LB. This is a rich medium that provides all the nutrients that the bacteria need to grow. If agar is added to liquid medium, it will solidify. LB agar petri plates provide a solid surface for bacterial growth. Thus, if you need a suspension of bacteria you can grow them in liquid media; however, for individual colonies, you need agar plates. Certain molecular biology procedures will require either solid or liquid media.

When bacteria are inoculated into a fresh tube of liquid medium, they begin to grow by binary fission. The bacterial **growth curve** has several distinct stages. See Figure 2.6 for a description of the bacterial growth curve. Different procedures may require a particular growth stage. For example, the bacteria must be in **exponential** (or log) phase when we make them competent to take up plasmid DNA. When we want to isolate plasmid DNA, we often let the bacteria grow overnight ("overnight culture") and by the next morning they are in **stationary** phase. You should know the stages of bacterial growth and which stage is used for a particular molecular biology procedure for the midterm exam.



Copyright © 2004 Pearson Education, Inc., publishing as Benjamin Cummings.

<http://diverge.hunter.cuny.edu/~weigang/Lecture-syllabus.html>

Figure 2.6 The Bacterial Growth Curve. When bacteria are first introduced to new media, there is an initial lag phase when there is very little new growth. However, soon there is rapid binary fission and the bacteria undergo log or

exponential growth. At some point, growth slows and bacteria enter stationary phase. Limiting amount of a nutrient or buildup of toxins may cause this. If the culture is left untouched, it will enter the final phase, the death phase.

The optimal temperature for *E. coli* growth is 37°C (Why? Where is *E. coli* normally found?). *E. coli* in liquid medium can be grown in a 37°C incubator, but we usually use the hot air shaker adjusted to 37°C. The shaking motion keeps the bacteria in suspension and exposed to nutrients. In rich media (liquid) at the optimal temperature, *E. coli* have a **doubling time** of about 25 minutes.

When we inoculate bacteria on petri plates, the petri plates are put in the 37°C incubator and the closed petri plates are always incubated upside down. The lid half rests on the incubator shelf and the agar half is on top. This is done to prevent condensation from dripping onto the colonies and spreading them around.

Bacteria are **clonal**. *An isolated colony on a petri plate represents a population of bacteria derived from a single cell, and thus is genetically identical.* This is very important to the Molecular Biologist. We must have confidence that all our DNA manipulations are performed on a genetically identical population.

Sterile technique is necessary when handling all bacteria. There are two important considerations to remember: (1) you must always protect yourself and your lab mates from being contaminated with bacteria. (2) You must also protect the bacterial strain that you are working with from contamination by other bacteria. All the supplies that you use for manipulating bacteria must be sterile, and after they are used they must be disposed of properly.

Key point

Bacteria can be manipulated on solid or on liquid media, and easily transferred from one to the other as needed.

- **Streaking a plate** - we can begin with either a liquid culture or a colony on a petri plate. A loop is used to spread the bacteria around the plate in a certain pattern, reducing the concentration of the **innoculum** along the way. The goal is to isolate individual colonies on the plate. See Figure 1.7 below for the pattern we will be using in lab. This procedure is often used to verify that the culture is not contaminated, or to pick one clonal colony for further analysis.
- **Spreading a plate** - this technique is more specific and is used to isolate individual colonies of bacteria after **transformation** with a plasmid (transformation will be covered later). As above, the goal is to obtain individual colonies that are clonal. Liquid culture is spread evenly onto the surface of a petri plate. Even though thousands of bacteria are spread on the plate, only the bacteria that now contain the plasmid will be able to grow and form a colony.

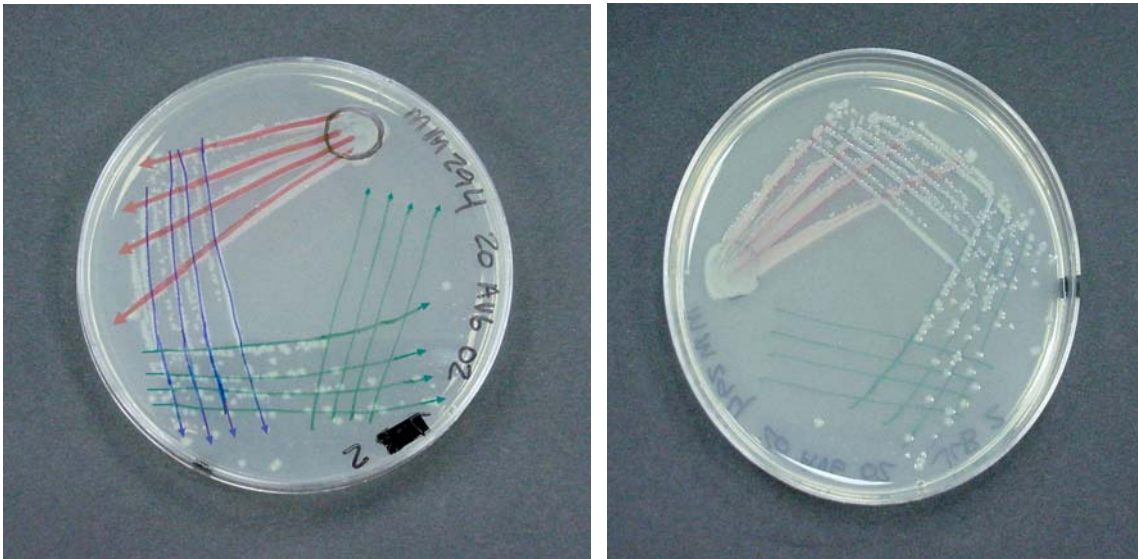


Figure 2.7 An example of a good streak plate.

#1 a loopful of bacteria is obtained from a resuspended liquid culture, and a dime to penny-sized circle is made on the plate (BLACK).

#2 a second loop is used to make four lines moving out from the circle (RED).

#3 a third loop is used to make four more lines that overlap the first four (BLUE).

#4 a fourth loop is used to make four more lines that overlap the second four (GREEN).

NOTE that the *same* loop is used to make the final four lines (GREEN).

Please see the virtual lab book for other examples:

<http://www.cofc.edu/~delliss/virtuallabbook/>

2. Plasmids

Plasmids are small circles of DNA that replicate in the cytoplasm of bacterial cells, and thus are **extrachromosomal** elements. Many different plasmids have been isolated from bacteria. Some of them carry genes that are useful to the bacterial host, such as resistance to heavy metals. Other naturally occurring plasmids have no selective advantage that we can detect. Molecular biology depends on plasmids for many common techniques such as gene cloning and protein expression. The naturally occurring plasmids have been drastically altered to serve as **cloning vectors**. The plasmid is called the vector, while the piece of DNA to be cloned is called the **insert**.

Plasmid cloning vectors have one or more genes that code for antibiotic resistance. These antibiotic resistance genes produce a product (often an enzyme) that enable a bacterium that contains the plasmid to survive exposure to the antibiotic. The antibiotic resistance is a **selectable marker** for bacteria that contain the plasmid. If a bacterium contains the plasmid, it survives exposure to the antibiotic; if it lacks the plasmid, it dies. Antibiotics can be added to liquid or

to solid media. There are many types of antibiotics in use for medicine or research. They have different modes of action, but all antibiotics target some fundamental difference between prokaryotic and eukaryotic cells.

Five Basic Mechanisms of Antibiotic Action Against Bacterial Cells:

1. Inhibition of Cell Wall Synthesis (most common mechanism)
Penicillin, Ampicillin, Vancomycin, Bacitracin
2. Inhibition of Protein Synthesis (Translation) (second largest class)
Kanamycin, Tetracycline, Chloramphenicol
3. Alteration of Cell Membranes
Polymyxin, Bacitracin
4. Inhibition of Nucleic Acid Synthesis
Metronidazole, Rifampin, Bacitracin
5. Antimetabolite Activity
Trimethoprim, Sulfonamides, Dapsone

<http://www.life.umd.edu/classroom/bsci424/Chemotherapy/AntibioticMechanisms.htm>

The antibiotics that we use in lab today are Ampicillin (Amp) and Kanamycin (Kan). Ampicillin affects cell wall biosynthesis of gram-negative bacteria (like *E. coli*) so newly dividing bacteria cannot form a cell wall and die. The resistance protein, β -lactamase, cleaves the ampicillin molecule so it is nonfunctional. Kanamycin affects the prokaryotic ribosome and stops protein synthesis. Since any living cell is undergoing some protein synthesis, Kanamycin is considered to be a "harsher" antibiotic than Ampicillin. The resistance protein is an enzyme that alters the structure of Kanamycin so it cannot bind to the ribosome.

Figure 2.8 shows the parts of a plasmid.

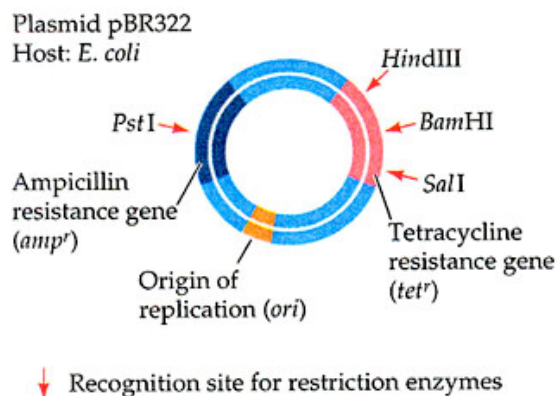


Figure 2.8 A typical plasmid. A plasmid must have an origin of replication, a selectable marker (such as a drug resistance gene), and restriction sites for cloning foreign DNA.

<http://www.blc.arizona.edu/INTERACTIVE/recombinant3.dna/Plasmids.html>

BRIEF GLOSSARY

Agar: Provides a solid matrix to support bacterial growth. Contains nutrient mixture of carbohydrates, amino acids, nucleotides, salts, and vitamins.

Antibiotic Selection: The plasmid used to move genes into bacteria also contains the gene for beta-lactamase that provides resistance to the antibiotic ampicillin. The beta-lactamase protein is produced and secreted by bacteria that contain the plasmid. The secreted beta-lactamase inactivates the ampicillin present in the LB/agar, which allows for bacterial growth. Only bacteria that contain the plasmids, and express beta-lactamase can survive on the plates that contain ampicillin. Only a very small percentage of the cells take up the plasmid DNA and are **transformed**. Nontransformed cells, cells that do not contain the plasmid, cannot grow on the ampicillin selection plates.

Colony: A clump of genetically identical bacterial cells growing on an agar plate. Because all the cells in a single colony are genetically identical they are called clones.

Culture Media: The liquid and solid media are referred to as LB (named after Luria-Bertani) broth and agar are made from an extract of yeast and an enzymatic digest of meat byproducts that provides a mixture of carbohydrates, amino acids, nucleotides, salts, and vitamins, all of which are nutrients for bacterial growth. Agar, which is from seaweed, polymerizes when heated to form a solid gel (very analogous to Jell-O), and functions to provide a solid support on which to culture the bacteria.

Plasmid: A circular DNA molecule, capable of autonomous replication, carrying one or more genes for antibiotic resistance proteins and a cloned foreign gene such as Green Fluorescent Protein (GFP).

Screening: The process of identifying wanted bacteria from a population of bacteria.

Selection: Manipulating the experimental conditions so only the desired bacteria are able to grow. For example, using petri plates with antibiotic in the agar to select a population of bacteria containing a drug-resistant plasmid and kill other bacteria.

Sterile Technique: Minimizing the possibility of outside bacterial contamination during an experiment through observance of cleanliness and using careful laboratory techniques.

Streaking: The process of passing an inoculating loop with bacteria on it across an agar plate.

Transformation: The process of introducing a plasmid into a bacterial cell. This process occurs in nature, but can be forced in the lab by treating the bacteria.

REFERENCES

Bloom, M., Freyer, G., Micklos, D. 1996. Laboratory DNA Science. Benjamin Cummings, CA

http://a32.lehman.cuny.edu/molbio_course/Basic_techniques.htm

<http://www.bio.davidson.edu/Courses/Bio111/Bio111LabMan/Preface%20D.html>

http://biog-101-104.bio.cornell.edu/BioG101_104/tutorials/pipettor.html

<http://diverge.hunter.cuny.edu/~weigang/Lecture-syllabus.html>

<http://sonhouse.hunter.cuny.edu/tech/TF-7.htm>

http://www.biology.arizona.edu/molecular_bio/problem_sets/Recombinant_DNA_Technology/05t.html

<http://www.blc.arizona.edu/INTERACTIVE/recombinant3.dna/Plasmids.html>

<http://www.life.umd.edu/classroom/bsci424/Chemotherapy/AntibioticMechanisms.htm>