

# RESTRICTION DIGESTS, GEL ELECTROPHORESIS, AND DNA LIGATION

## DNA Cloning

*“Every once in a while, the world experiences a revolution. Revolutions come in different forms, and in different fields. They also have different degrees of impact; some are localized, while others have regional and even worldwide impact. Some revolutions are political, while others are cultural, industrial, or religious in nature. In addition, others are scientific or technical in nature. Periodically, society can be transformed in a dramatic fashion by some ideological, philosophical, or technological innovation. The world of the twenty-first century is witnessing the unfolding of events some believe to be the biological revolution. Called **biotechnology**, this revolution began in the last twenty years and is currently impacting food, health, and the environment in very dramatic ways, the full extent of which is, as yet, unpredictable. Biotechnology is big business, and it promises to be even bigger with time.”*

*-George Acquah (Understanding Biotechnology 2004)*

The term **biotechnology** is often used to mean the manipulation of DNA. During this course we will manipulate DNA in a variety of ways. It is our hope that in doing so, you will come to appreciate that the tools and procedures we are using represent a basic skill set applicable to a wide range of fields including basic and applied research, pharmaceutical development, and medical diagnostics.

### LEARNING GOALS:

1. Be able to interpret and construct a cloning scheme.
2. Learn how to cut DNA with restriction enzymes.
3. Understand and perform gel electrophoresis.
4. Know how ligase enzyme connects the DNA pieces.

Modern tools for DNA manipulation include: enzymes that cut DNA at specific sites, enzymes that splice DNA strands together; techniques to visualize DNA; techniques to separate DNA fragments from one another; techniques to identify fragments of DNA with specific sequences; techniques that can amplify DNA

(which generate hundreds of thousands of copies of a specific gene fragment); and techniques to sequence DNA. We will use all of these tools this semester.

The availability of these modern tools for DNA manipulation allows biotechnologists to introduce genetic information from one organism into another. When a biologist causes a cell or organism to take up a gene from another organism, we say the cell or organism is **genetically modified**, or **genetically engineered**. The term **recombinant DNA** refers to DNA that contains sequences of DNA from different sources that were brought together using the tools of biotechnology.

The ability to produce genetically modified organisms is so powerful that the term *revolutionary* is often applied to it. Because DNA is the universal genetic material, biologists can integrate intact genes from one organism into another. For example, a human gene can be successfully integrated into bacterial DNA.

DNA contains coded information that can direct a cell into making a specific protein. When a protein is produced by a cell, we say that the DNA coding for that protein is **expressed**. Under the proper conditions, a genetically modified cell can express (i.e. produce) a protein coded for by an introduced gene. So why would anyone care to do this?

## 1. The Classic Recombinant DNA Story

Human insulin was among the first commercial products to be made by a genetically modified organism. Prior to the 1980s, all insulin to treat diabetes was purified from the pancreatic tissue of animals slaughtered for human consumption. Animal insulin is similar, but not identical, to human insulin; therefore, some diabetics developed allergies to the animal insulin. In addition, insulin derived from animals requires a large supply of material from slaughterhouses, which is not always convenient. In 1979, Herbert Boyer and Stanley Cohen developed methods to transfer the gene coding for human insulin into bacteria. This was a remarkable accomplishment; bacteria have absolutely no use for insulin and would never produce it without human intervention. Bacteria that contain the insulin gene can be grown in large quantities. In biotechnology, this large-scale cultivation of microorganisms is called **fermentation**. The resulting insulin can then be isolated and purified using protein separation techniques, thus providing an efficient, reliable production system for this medically important protein.

## 2. Modern Uses of Recombinant DNA

Biologists are currently making genetically modified plants, animals and human cells. This technology is having a tremendous impact on biological research. Genetically modified organisms are used daily to explore the role of genes, the mechanisms controlling gene expression, and other important questions. In terms of benefits to society, the uses of recombinant DNA are too numerous to count but include: more efficient plant crops, bacterial remediation to clean up the

environment, pharmaceuticals, and novel alternative fuels. New applications or recombinant DNA are continually being discovered.

Some steps are common to all recombinant DNA experiments that use plasmids:

1. The DNA of interest (**insert DNA**) that is to be put in the plasmid must be isolated.
2. A plasmid **vector** is obtained (usually commercially available).
3. The insert DNA and plasmid DNA are cut with **restriction enzymes** that make compatible ends (see Figure 3.2).
4. The insert DNA is joined by **ligation** into the plasmid (see Figure 3.2).
5. The recombinant DNA construct is transferred into, and maintained in, a host cell (typically *E. coli* bacteria) by the process of **transformation**. The vector replicates with the bacteria, producing identical copies or **clones** of the insert DNA.
6. The bacteria that have incorporated the foreign DNA are identified and isolated from untransformed cells.
7. The cloned DNA can be manipulated so that the protein product it encodes can be expressed by the host cell.

### **Topic I: RESTRICTION ENZYMES AND THEIR USE**

Restriction endonucleases (also called restriction enzymes) are some of the most powerful tools in molecular biology. Cutting DNA by using restriction enzymes is one of the most common molecular biology techniques and the availability of pure restriction enzymes was one of the first major advances in the new science of Molecular Biology. These enzymes occur naturally in bacteria and are used to protect the bacteria from invading foreign DNA such as bacterial viruses (bacteriophage).

Restriction enzymes **recognize specific sequences in DNA and then cleave the phosphodiester bonds between the nucleotides at that site**. Any time this sequence appears in DNA it will be cleaved by the enzyme, whether it is viral or frog or human DNA. This is why restriction enzymes are so critical to molecular biology; they are very specific, cutting only at their unique recognition sequence, but they are also general because they will cut any DNA having this sequence. They work by cutting the DNA at a specific nucleotide sequence, the **recognition sequence** of the enzyme. The result is the generation of DNA fragments (**restriction fragments**) from a DNA molecule having these **recognition sequences** or **cut sites**. Thus, digestion of a population of identical DNA molecules with a given enzyme will always result in identical restriction fragments. This is called an **enzyme digestion** and has made cloning and other DNA manipulations possible.

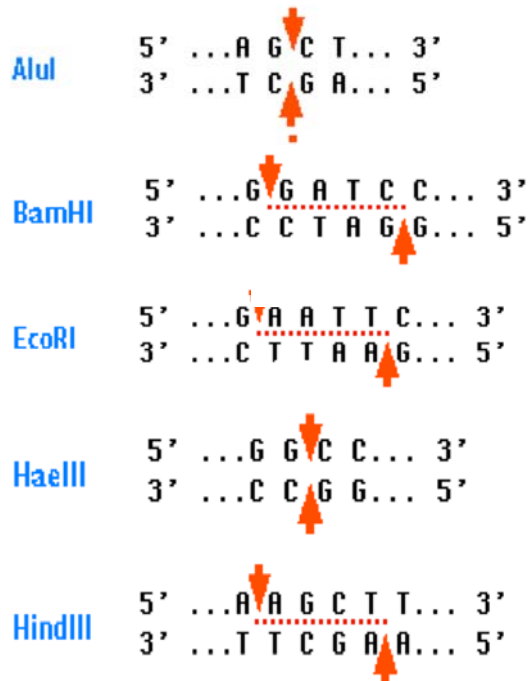
Restriction enzymes (abbreviated RE) usually recognize palindromic sequences in DNA (See Figure 3.1). For example, the widely used restriction enzyme

*Bam*HI recognizes the sequence GAATTC. This is considered a **palindrome** because *the complementary strand will have the identical sequence in the opposite direction*.

### 1. Blunt and Sticky Ends

A given enzyme will always cut between the same two nucleotides. As shown in Figure 3.1, some enzymes cut in the center of the recognition sequence, which results in the formation of “**blunt ends**” in DNA. Most restriction enzymes make cuts one or two bases away from the axis of symmetry, which results in the formation of single-stranded ends two or four base pairs long on the end of each fragment. Because these ends are complementary, they are referred to as “**cohesive**” or “**sticky ends**”. Since a given restriction enzyme produces identical ends in any DNA molecule, *restriction fragments from one source can be recombined with DNA from other sources if they are both cut with the same enzyme*. This characteristic allows the formation of **recombinant DNA molecules** and is the basis of most DNA cloning protocols. Typically, sticky ends are preferred for recombinant DNA work although blunt ends can be attached to other blunt ends when necessary (it is harder to join them together later).

Figure 3.1: Restriction enzyme recognition sites.



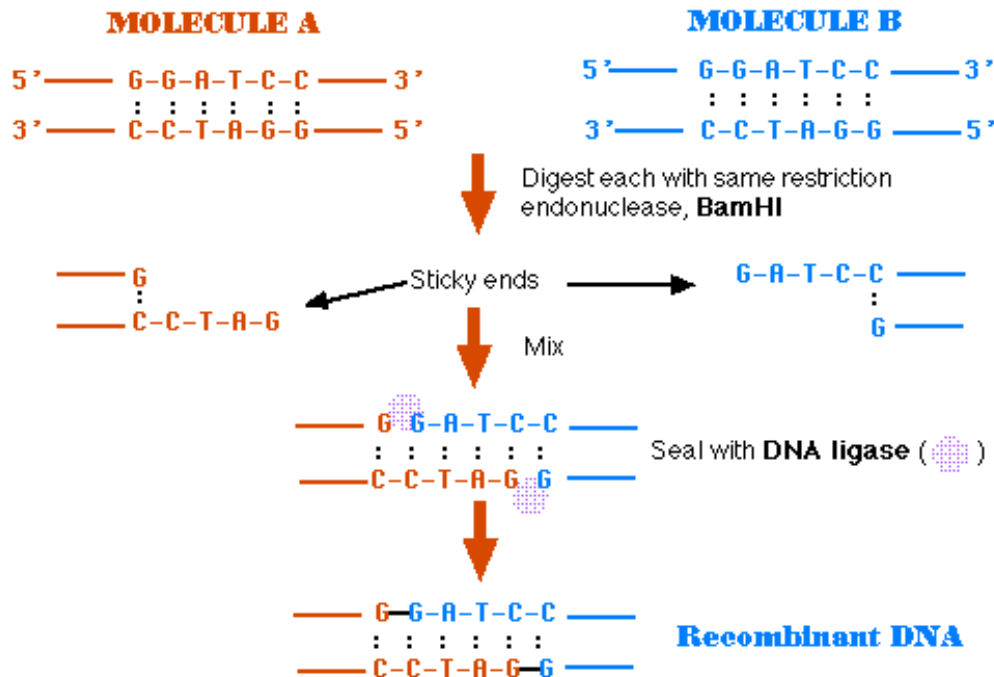
List the enzymes that leave “blunt ends” : \_\_\_\_\_

List the enzymes that leave “sticky ends” : \_\_\_\_\_

The top strand of the *Bgl*II palindrome is AGATCT. Draw the top and bottom strands below:

The general process of cutting and ligating (joining) DNA strands is shown in Figure 3.2. Two strands of DNA are cut with the same enzyme, leaving compatible sticky ends. These ends can then be joined with the enzyme **Ligase**, which *reforms the phosphodiester backbone*. A recombinant DNA molecule has been created.

Figure 3.2: Cutting DNA with RE and ligating the fragments together.



<http://users.rcn.com/jkimball.ma.ultranet/BiologyPages/R/RecombinantDNA.html>

## 2. Naming of Restriction Enzymes

Why do REs have such strange names? The name of the enzyme is taken from the first letter of the bacterial genus and the first two letters of the bacterial species. Thus the first three letters of the restriction enzyme name come from a formal genus and species designation. They must therefore be italicized or underlined, just like you would do for the bacterial species itself. The enzyme name may have additional numbers or letters and these may designate a particular strain, isolate, or plasmid. These numbers or letters should not be italicized or underlined. For example, *HaeIII* was isolated from *Haemophilus aegyptius*, and was the third RE isolated from this species. *EcoR1* comes from *Escherichia coli* **RY13** (this is the thirteenth **strain** of *E. coli* constructed by R.N. Yoshimori).

To date, hundreds of REs are commercially available, offering a wide variety of options for cutting DNA molecules at different sites. Restriction digestions are

done by adding the DNA to be digested and a restriction enzyme to a buffer that is optimal for that particular enzyme. Commercial enzyme manufacturers supply the appropriate buffer with each enzyme. Sometimes it is necessary to use two or more enzymes in the same digestion, called a “**double digest**”. This can usually be accomplished with a compromise buffer that provides sufficient activity from each enzyme.

### 3. Proper Use of Restriction Enzymes

Please remember these important rules when handling restriction enzymes.

1. RE must be kept on ice and returned to ice as soon as you are finished using them. Room temperature will cause them to lose activity.
2. Hold the tube by the center, do not hold the bottom of the tube with your \*warm\* hands.
3. Check the pipette tip to be sure that enzyme is there! We usually use 1uL of enzyme, and the enzyme solution is thick (stored in glycerol).

### 4. A Unit of Restriction Enzyme Activity

All enzymes are measured in units of activity but of course the activity varies from enzyme to enzyme, depending on its function. The **activity** of restriction enzymes is the *ability to cleave DNA*. The definition of **one Unit (U)** of restriction enzyme activity is given below. You should know, and understand, this definition.

The amount of restriction enzyme in microliters (uL) needed to completely digest (cleave) one microgram (ug) of substrate DNA in one hour at the optimal temperature of the enzyme in a 50-uL reaction volume.

Many enzymes are sold as 10 Units per uL of enzyme solution. Thus *1 uL of enzyme solution should cut 10 ug of DNA*. Normally, for the restriction digests that we perform in lab, 0.5 – 1.0 uL of enzyme is sufficient to cut the amount of DNA in the digest.

The number of units of activity will help you determine how much of the enzyme to use in a restriction digest. You need to use enough enzyme to cut the DNA to completion, but too great of an excess may be detrimental to the digest. Also, these enzymes are expensive and using more than we need wastes money.

Each enzyme has certain requirements for optimal activity, including the proper restriction enzyme buffer and incubation at the correct temperature. The enzyme’s supplier will provide buffer and instructions for optimal digest activity.

### 5. Setting up a Restriction Digest

Since the restriction digestion of DNA is one of the most common techniques of Molecular Biology, it is important to understand how digests are carried out. All restriction digests contain the following components:

- DNA (added third)
- Restriction enzyme (added last)

- Buffer (added second)
- Water (added first)

The question is, how do you determine the quantity of each of these components? Because restriction digests are done for different outcomes, there are no absolute hard and fast rules. However, there are general guidelines to follow whenever you set up a digest.

#### A. DNA

You must determine the amount of DNA to be cut, and the volume of DNA stock solution that is required. Suppose a restriction digest uses 4 uL of DNA. This DNA has a concentration of 0.1 ug/uL. So how many micrograms of DNA are being cut in this digest? \_\_\_\_\_

#### B. Restriction Enzyme

Remember the definition of a Unit (U) of restriction enzyme activity. You must determine how many Units are needed to cut the DNA. Then you have to decide what volume of enzyme will contain sufficient Units of activity to accomplish this task. Most of the enzymes that we use have a concentration of 10 U/uL. Knowing the definition for a Unit of activity, how many micrograms of DNA can be cut with one microliter of enzyme? \_\_\_\_\_ How many Units would be needed to cut the 4 ul of DNA (above)? \_\_\_\_\_ These values will be difficult to understand at first. However, these are the types of calculations that you need in order to determine how much enzyme to use and they will become easier with practice.

*There are two general guidelines for determining the amount of enzyme:*

- (1) Use at least the minimum number of Units necessary to cut the DNA. Most people use more Units than absolutely necessary. This speeds up the time needed and helps insure a complete digest.
- (2) Try to keep the total volume of restriction enzyme in the digest to 10% or less of the total digest volume. Thus if you have a 30 uL digest, don't use more than 3 uL total of enzyme.

Finally, always add the enzyme last. Even though enzyme listed it as the second component above, *the enzyme is always last* so it can be in the optimal reaction conditions and start to work immediately.

#### C. Buffer

Each enzyme has its optimal buffer. The first task is to make sure you use the right buffer. Then you must determine the volume of buffer to add to the digest. Restriction enzyme buffers are often sent as a 10X stock concentration. We always use a 1X working concentration in lab. This means that the stock is ten times more concentrated than the working solution. Therefore, the stock solution must be diluted 1:10 in the restriction digest. This is not as difficult as it sounds. It is simply a matter

of setting up a  $(C1)(V1) = (C2)(V2)$  proportion. If your final restriction digest volume is 30  $\mu$ L, then you use 3  $\mu$ L of the 10X stock buffer.

**D. Water**

Finally, water is added to whatever volume is not taken up by DNA, buffer, or enzyme. Often, when you actually set up a digest, the water is *added first* because it is the largest volume. Then it is easy to mix the buffer and the DNA into the water.

Here is a sample restriction digest:  
 Cleave 2  $\mu$ g of DNA with *Eco*RI in a 40  $\mu$ L reaction volume

	<b>STOCK</b>	<b>DIGEST</b>
DNA	0.5 $\mu$ g/ $\mu$ L	4 $\mu$ L
10x buffer	10x	4 $\mu$ L
<i>Eco</i> RI	8 Units/ $\mu$ L	0.5 $\mu$ L
water		31.5 $\mu$ L
<b>TOTAL</b>		<b>40 <math>\mu</math>L</b>

**Topic II: PLASMIDS**

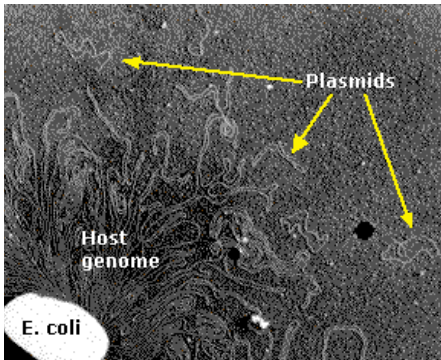


Fig. 3.3, left: Electron micrograph of an *E. coli* cell ruptured to release its DNA. The tangle is a portion of a single DNA molecule containing over 4.6 million base pairs encoding approximately 4,300 genes. The small circlets are plasmids. (Photo courtesy of Huntington Potter and David Dressler, Harvard Medical School).

Plasmids are molecules of DNA that are found in bacteria separate from the bacterial chromosome.

**1. Characteristics Of Plasmids**

They are small (a few thousand base pairs) and circular. Plasmids have a single origin of replication and they usually carry only one or a few genes.

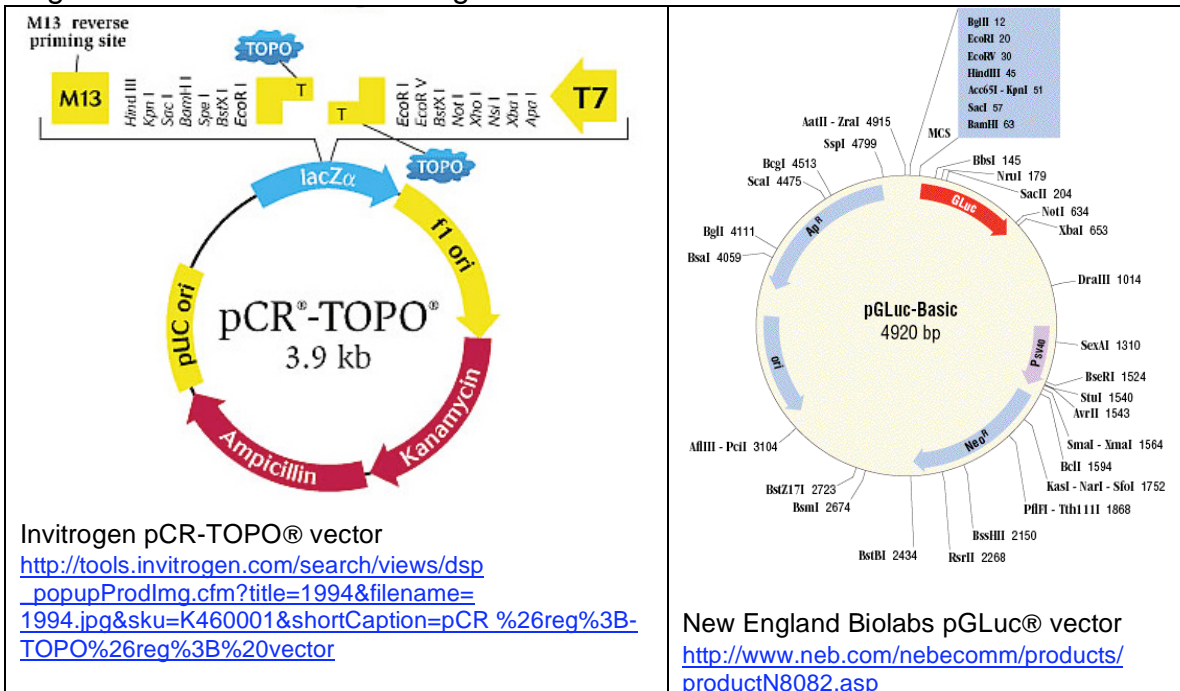
Plasmids are replicated by the same machinery that replicates the bacterial chromosome. Plasmids are present in the bacterial cell at a characteristic **copy number**. Some plasmids are copied at about the same rate as the chromosome, so a single cell is apt to have only a single copy of the plasmid. Other plasmids are copied at a high rate and a single cell may have 200 or more of them.

Plasmids used for cloning have a high **copy number**. Genes on plasmids with high copy number are usually expressed at high levels. In nature, these genes often encode proteins (e.g., enzymes) that protect the bacterium from one or more antibiotics or confer resistance to heavy metals. You should know the copy number of the plasmid you are working with. You may need to start with a greater number of cells to isolate enough of a low copy number plasmid to use in cloning. The majority of the vectors in use today are small plasmids, ranging from 3-5 kilobasepairs (kb), and they are high copy number.

Plasmids enter the bacterial cell with relative ease. This occurs in nature and may account for the rapid spread of antibiotic resistance in hospitals and elsewhere. Plasmids can be deliberately introduced into bacteria in the laboratory and **transforming** the cell with the incoming genes.

## 2. Plasmids As Cloning Vectors

Figure 3.4: Two modern cloning vectors.



The plasmids isolated from bacteria in nature are a far cry from the modern plasmid cloning vectors. Many unique features have been introduced into the naturally occurring plasmids to make cloning or gene expression easier for the researcher. A modern cloning vector is small, of high copy number, and contains a plasmid origin of replication (ORI). The vector may also have *other* origins for replication in mammalian cells. A **multiple cloning site (MCS)** or **polylinker** region is always present, and provides a cluster of useful restriction enzyme cleavage sites. A plasmid vector has one or more **selectable markers** so the researcher can keep track of the vector's presence in a cell. Some features help

the researcher **screen** for the presence of a vector with an insert (a positive clone). Other features may help with efficient expression of cloned genes or purification of the expressed gene product.

### Topic III: CLONING SCHEME

#### 1. Plasmid Vector

During this semester, we will use the cloning vector pGEM®-3Zf from Promega and insert a DNA fragment. This DNA fragment is about 500 bp, and is part of the human cardiac protein domain from Titin, called Ig 27. Let's examine this vector and the features it contains:

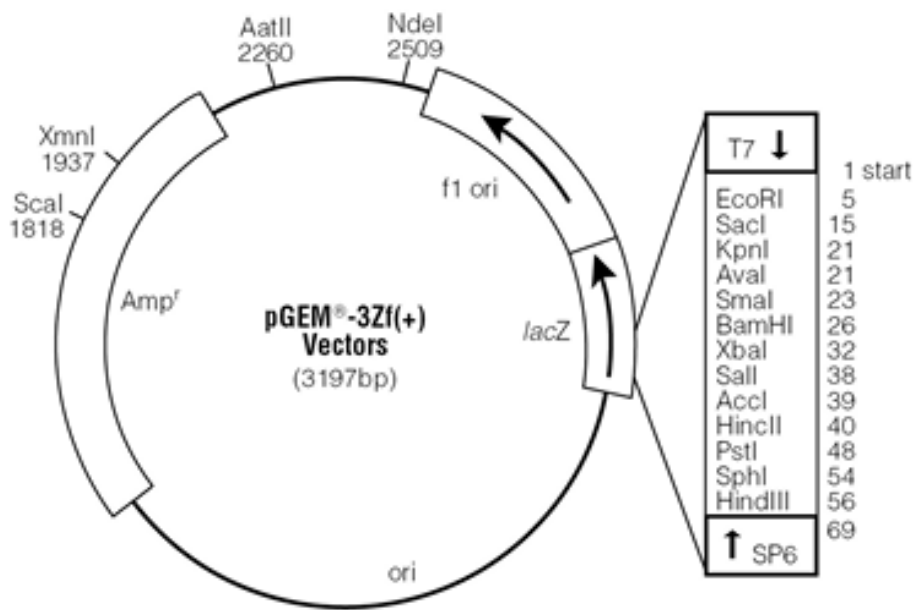


Figure 3.5. The plasmid pGEM®-3Zf from Promega Corporation.

[http://www.promega.com/catalog/catalogproducts.aspx?categoryname=productleaf\\_211#figure](http://www.promega.com/catalog/catalogproducts.aspx?categoryname=productleaf_211#figure)

#### Plasmid Map

The plasmid map shows the important features of the plasmid. The plasmid name and its total size are usually given in the center. Normally *only unique* (occurring only once) *restriction enzyme sites are given on a plasmid map*. Sites that occur more than once are not useful for cloning (Why?). The numbers next to the RE sites give the **nucleotide number (location)** of the RE site. For example, the numbering system of this plasmid starts at the T7 promoter (#1) and the *Bam*HI site is located at nucleotide #26.

Practice: determine the sizes of the two pieces that result when the plasmid is cleaved with *Hind*III and *Scal*: \_\_\_\_\_

### Amp<sup>R</sup> Gene

The Ampicillin resistance gene produces the enzyme  $\beta$ -lactamase, which inactivates the Ampicillin antibiotic in the medium. Thus we can **select** for bacteria that contain the pGEM plasmid because these bacteria are Ampicillin resistant.

### ORI

The pGEM®-3Zf(+/-)vectors contain the plasmid origin of replication (ORI) and the origin of replication of the filamentous phage f1. The phage f1 origin can be used to produce single-stranded DNA for sequencing of the cloned gene.

### Promoters

The plasmid contains phage T7 and SP6 RNA polymerase promoters. Bacteriophage SP6, T3 and T7 RNA polymerases are DNA-dependent RNA polymerases with strict specificity for their respective promoter sequences. The two promoters will allow strong expression of the cloned gene when the phage polymerase is supplied for transcription.

### MCS

Many unique RE sites are clustered in the multiple cloning site (MCS). In this plasmid the MCS is located within a fragment of the *lacZ* gene (this gene produces  $\beta$ -galactosidase). The fragment is called the  **$\alpha$ -peptide** coding region of  $\beta$ -galactosidase (Yanisch-Perron, C. *et al.*). When a cloned gene is inserted in the  $\alpha$ -peptide, this causes **insertional inactivation** of the  $\alpha$ -peptide.

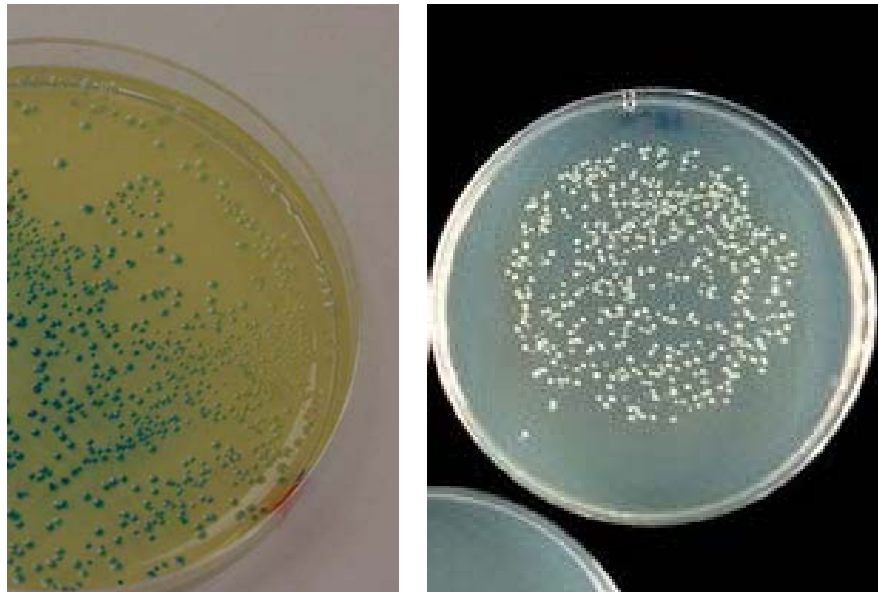


Figure 3.6. (Left) A petri plate spread with X-gal for the blue-white screen. (Right) An example of a normal spread plate of bacteria on a drug<sup>R</sup> plate. (right, photo by S. Dellis; left, photo from Wise and Paulson© <http://www.microbelibrary.org/ASMOOnly/details.asp?id=2320&Lang>)

### Blue-white Screening

Having the MCS located within the  $\alpha$ -peptide gives us a handy **screen** for plasmids that contain a DNA insert. A solution of “**X-gal**” (5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside) is spread on the petri plate. A plasmid that does **NOT** have DNA inserted into the  $\alpha$ -peptide will produce a **BLUE** color. However if the  $\alpha$ -peptide is inactivated by a DNA insert then the colony will be the normal **beige** of *E. coli*. *We want the boring beige colonies, because they will hopefully have our DNA insert.* Figure 3.6 (left) shows the blue colonies of the blue-white screen.

The molecular mechanism for blue/white screening is based on a genetic engineering of the *lac* operon in the *Escherichia coli* laboratory strain serving as a host cell, combined with subunit complementation from the cloning vector. The vector encodes the  $\alpha$  subunit of LacZ protein with an internal multiple cloning site (MCS), while the chromosome of the host strain encodes the remaining  $\Omega$  subunit to form a functional  $\beta$ -galactosidase enzyme.

The MCS can be cleaved by different restriction enzymes so that the foreign DNA can be inserted within the *lacZ $\alpha$*  gene, thus disrupting the production of functional  $\beta$ -galactosidase. The chemical required for this screen is X-gal, a colorless modified galactose sugar that is metabolized by  $\beta$ -galactosidase to form an insoluble product that is bright blue, and thus functions as an indicator.

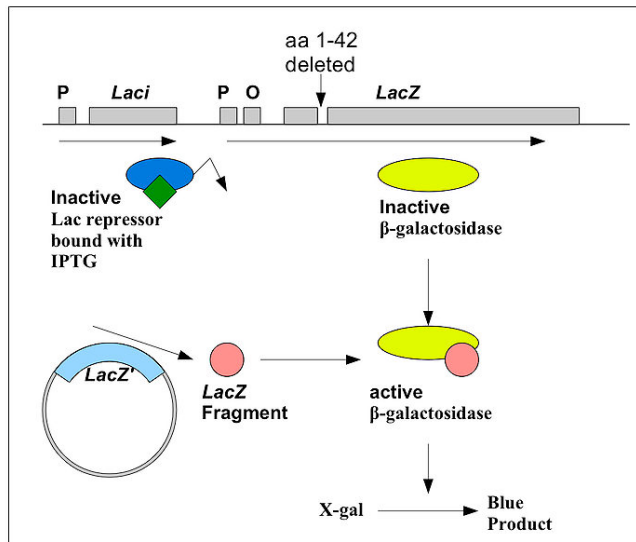


Figure 3.7, The blue-white screen.

[http://en.wikipedia.org/wiki/Blue\\_white\\_screen](http://en.wikipedia.org/wiki/Blue_white_screen)

**IMPORTANT POINT:** This plasmid lets us see the difference between a **selection** and a **screen**, two important genetics concepts. The presence of the Ampicillin resistance gene in the plasmid gives us a **selection** for all bacteria with a plasmid, because they will be resistant to the Ampicillin on the petri plate. By spreading the X-gal on the plate, we can **screen** for white colonies among the blue ones. These white colonies are our best chance of finding a plasmid with the DNA insert, but it is by no means a guarantee.

### 2. DNA Insert

We will be inserting a fragment of **Titin**. Titin, which consists of 34,350 amino acids, is the largest known protein. The molecular weight of the mature protein is

approximately 2,993,442.763 Da, Titin consists primarily of a linear array of two types of protein domains: type I (fibronectin type III domain) and type II (immunoglobulin domain). We are cloning a (roughly) 500 bp segment of the Immunoglobulin domain called **Ig27**.

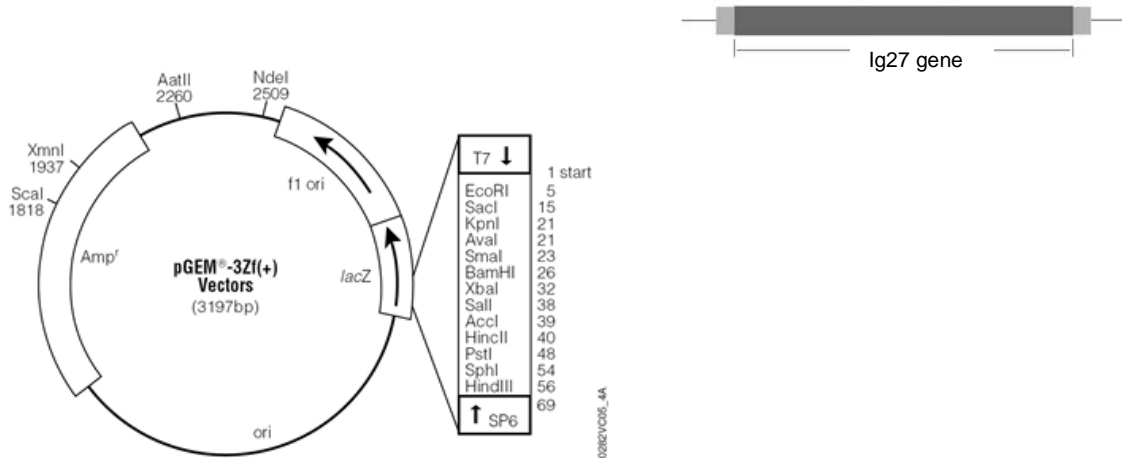
### 3. Cloning the DNA into the Plasmid

The Titin DNA is present in another plasmid. Your instructor has used Polymerase Chain Reaction to amplify the Ig27 gene so we can clone it into pGEM3zf.

*The vector will be cut with BamHI, while the insert will be cut with BglII. Return to page 4 and draw out the pallindromes for BamHI and BglII in the space below. Mark arrows to show where the enzyme cuts the top and bottom strands of the BamHI pallindrome. Examine the BglII restriction site. Where should the BglII enzyme cut so the BglII overhang will match the BamHI overhang? See Figure 3.2 for an example. Can you show the BamHI and BglII cut ends joining together? If so, draw it out below.*

On this page, draw the new construction showing the Ig27 gene inserted into pGEM. We will name the new construction plg. What is its size? Show the enzyme sites used to cut the vector and insert and also show these sites on plg.

Figure 3.8. The Ig27 gene will be inserted into the MCS of the pGEM plasmid. Ig27 is cut with *Bgl*II while pGEM3zf is cut with *Bam*HI.



**Topic IV: SEPARATING BY ELECTROPHORESIS**

In order to characterize DNA restriction fragments, verify that restriction digestions have worked, or to purify restriction fragments, it is necessary to separate the various sized fragments. An extremely common method to do this is called **gel electrophoresis**.

**1. Movement of DNA**

DNA is negatively charged (due to the phosphate residues on the DNA backbone) and as such can be separated in an electrical field. Digested DNA samples can be loaded into the wells of an agarose gel with the gel wells situated by the negative electrode. An electrical field applied across the gel causes the DNA fragments to migrate through the gel matrix toward the positive electrode. The gel matrix acts as a sieve through which smaller DNA fragments migrate faster than larger ones. The *migration* of DNA is inversely proportional to the log of its *size*. By loading appropriate DNA size standards in the gel, it is possible to determine the size of DNA restriction fragments after staining and photographing the gel. The concentration of agarose in the gel may be varied to allow resolution of differing sizes of DNA fragments (See Table below).

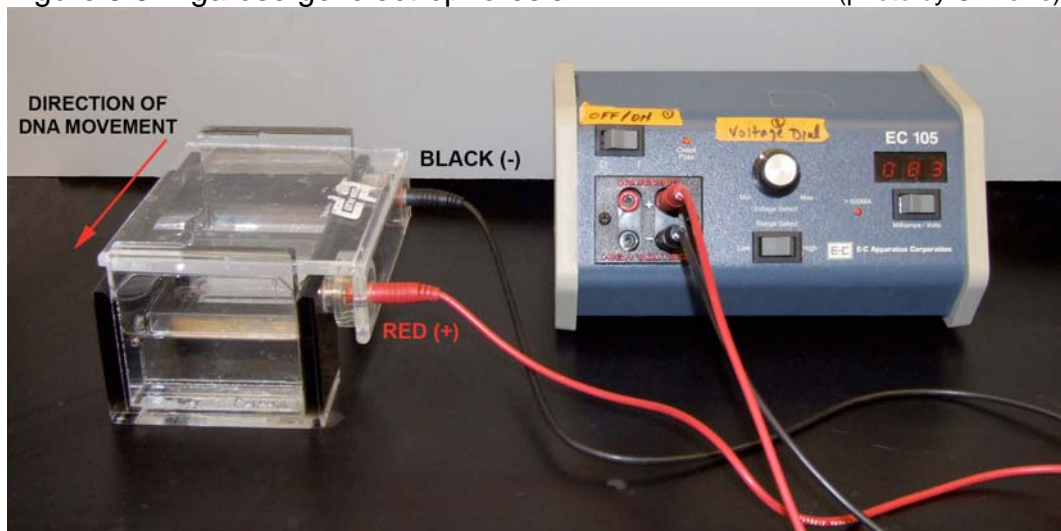
**Table 3.1: Separation of DNA in agarose gels.**

Percent Agarose in Gel	Size range of DNA molecules efficiently separated (kb)
0.6	1.0-20
0.8	0.6-12
1.0	0.4-8
2.0	0.3-6
1.5	0.2-4

Agarose Gel Electrophoresis units (called gel “*rigs*”) are varied in style but all have the same basic components (See diagrams below). A detailed explanation of gel rigs will be given in class.

Figure 3.5: Agarose gel electrophoresis.

(photo by S. Dellis)



## 2. Loading Dye and Ethidium Bromide

Two dyes are very important in gel electrophoresis. The first, called **loading** or **tracking dye**, has several uses at the start of the electrophoresis process.

First, the loading/tracking dye contains glycerol or sucrose to weigh down the DNA sample so it will sink to the bottom of the well. Second, the blue color comes from two (or sometimes three) different dyes. The first, **bromophenol blue**, is the “fast blue”. This dye runs with the same mobility as a small DNA fragment. The second, **xylene cyanol**, is the “slow blue” and runs with the same mobility as a large DNA fragment.

**IMPORTANT POINT:** *these dyes DO NOT interact with the DNA fragments.* They DO provide a guide for how far the gel has run, so we do not run the DNA off of the gel. **Figure 3.6** (right) shows these two dyes as they begin to separate during electrophoresis.

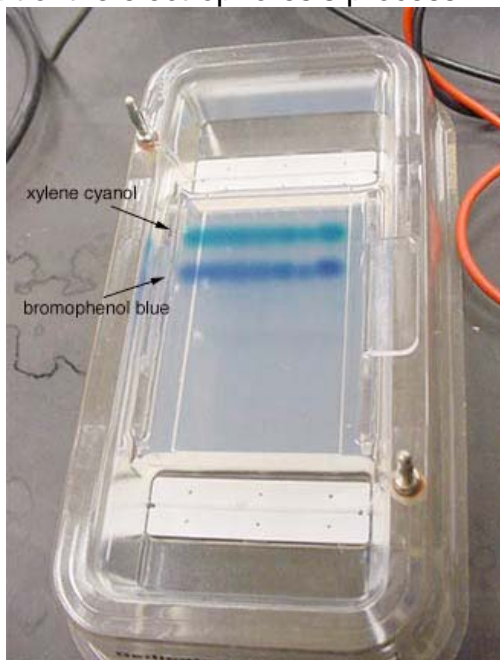


Figure 3.6: Separation of the tracking dyes during electrophoresis. (photo by S. Dellis)

The second dye is used after electrophoresis is complete. The gel is soaked in a solution of Ethidium Bromide. This planar (flat) molecule can get in between the DNA bases, or **intercalate** into the rungs of the helix. When the stained gel is put under UV light, *this is the first time we can actually see the bands of DNA.* This dye must be used with care as the molecule can intercalate into any DNA, including human DNA.

## 3. Electrophoresis Buffers

There are two commonly used buffers for agarose (or acrylamide) gel electrophoresis, TAE (Tris-Acetate-EDTA) and TBE (Tris-Borate-EDTA). The buffers provide the ions necessary to move the current during electrophoresis. These buffers are usually made in a concentrated stock solution, from 5X to 20X, and then diluted to 1X before use. It is critical that the agarose gel be made in the same buffer used to run the gel, or the results will be very poor.

The Table below shows that the tracking dyes (and also the DNA) have different mobilities in these two buffers. The mobility of the dyes in **TBE (in red)** is greater than that in TAE. For example, bromophenol blue in a 0.8% agarose gel moves with the same mobility as an 800-bp DNA fragment in TAE but with the same mobility as a 400-bp fragment in **TBE**. Thus you should be aware of the buffer used so you stop the gel before the DNA runs off.

**Table 3.2: Migration distances of gel tracking dyes.**

%TAE ( <b>TBE</b> ) agarose gel	xylene cyanol (light blue-green)	bromophenol blue (dark blue)	orange G (orange)
0.8	5000 bp ( <b>3000 bp</b> )	800 bp ( <b>400 bp</b> )	150 bp ( <b>&lt;100 bp</b> )
1.0	3000 bp ( <b>2000 bp</b> )	400 bp ( <b>250 bp</b> )	<100 bp ( <b>&lt;100 bp</b> )
1.5	1800 bp ( <b>1100 bp</b> )	250 bp ( <b>100 bp</b> )	<100 bp ( <b>&lt;100 bp</b> )
2.0	1000 bp ( <b>600 bp</b> )	200 bp ( <b>&lt;100 bp</b> )	<100 bp ( <b>&lt;100 bp</b> )
2.5	700 bp ( <b>400 bp</b> )	100 bp ( <b>&lt;50 bp</b> )	<50 bp ( <b>&lt;50 bp</b> )

<http://www1.qiagen.com/literature/handbooks/literature.aspx?id=1000252>

#### 4. Loading a Gel

	<p>Figure 3.7: Illustration of the three steps in loading a gel well. (photos by S. Dellis)</p>
<p>Place tip over well    Eject solution with even force    Remove tip then release plunger</p>	

Figure 3.8: Hints for loading a gel.

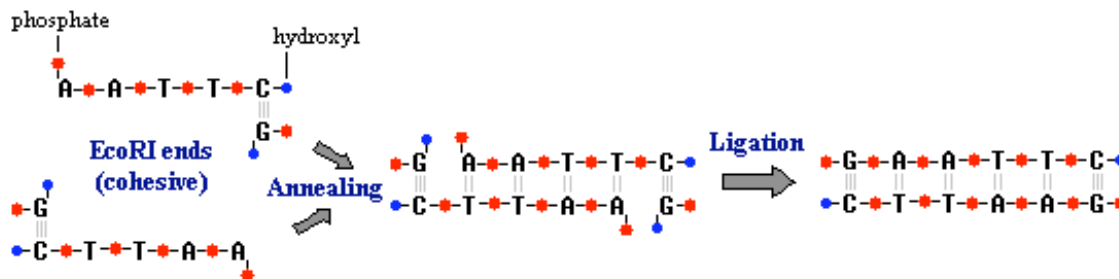
<p>DNA solution in tip. (photos by S. Dellis)</p>	<p>Tip in buffer and over the well.</p>

Figure 3.8 above shows how to load a gel. (Left) Be sure that there are no air bubbles in the DNA/loading dye solution. (Right) The pipette tip must go through the buffer layer and sit over the well – do not go down too far into the well or you can puncture the well. Also see the [Virtual Lab Book](#).

## Topic 5: LIGATION OF A GENE INTO A VECTOR

DNA ligation refers to the reaction that forms recombinant DNA molecules by covalently bonding together two restriction fragments with compatible ends. This reaction is one of the most important steps in any recombinant DNA project. The ligation reaction synthesizes an ester linkage between a 5'-phosphate (P) and a 3'-hydroxyl (OH) of two different DNA molecules or two ends of the same molecule. This reaction is catalyzed by the enzyme **DNA ligase**. This is the same enzyme that a cell uses to close nicks during DNA synthesis. The DNA ligase commonly used in molecular cloning is T4 DNA ligase, which is purified from an *E. coli* culture that is infected by the bacteriophage T4.

To carry out the ligation reaction, the DNA ligase requires the 5'-P and 3'-OH ends of DNA molecules to come together. With blunt-cut DNA fragments, this is a rare occurrence (although T4 ligase can ligate blunt-ended molecules under certain conditions). However, when DNA is digested with a restriction enzyme that generates sticky ends, compatible DNA ends can bond together. The few hydrogen bonds form a transient duplex as sticky ends are constantly annealing and separating. Ligase can covalently bind adjacent 5'-P and a 3'-OH with the energy source ATP, a  $Mg^{2+}$  cofactor, and proper incubation conditions (a reducing buffer at 4-37°C). See the example below:



[http://clickandlearn.org/Bio/Gr12Bio/U2\\_L5\\_Biotech.htm](http://clickandlearn.org/Bio/Gr12Bio/U2_L5_Biotech.htm)

When ligating restriction fragments into a cloning vector, the objective is to form recombinant molecules where a restriction fragment from the DNA being cloned (the “insert”) is ligated into the vector. Whether a recombinant molecule is produced is a matter of chance. If the ends of all molecules in the reaction (both the insert and the vector) have the same sticky ends, there are a number of possible products. First, the fragments of the original vector could ligate to produce an intact vector. Similarly, the insert molecules could ligate to themselves. Also, two ligated insert molecules could be ligated into the plasmid fragment. *The actual number of possible ligation products is almost limitless.*

**In most cloning experiments, the desired product is that a single vector ligates to both ends of a single insert to form a covalently closed circular recombinant molecule.** This situation creates the simplest conformation for

which to use the recombinant vector in additional DNA manipulations, DNA sequencing, or driving gene expression.

In the next part of this cloning experiment, you will set up ligation reactions with the restriction enzyme-digested DNA fragments. In general, ligations should have a several fold excess of insert DNA over vector DNA. The ligation you perform will likely result in several different types of molecules, and hopefully a few will contain a single copy of the gene in a covalently closed plasmid. Because we have the blue-white screen and a drug selection marker we should be able to identify clones containing our gene of interest.

Before setting up a ligation, we must **heat inactivate** the restriction enzymes in the reaction. Otherwise they will continue to cut the DNA we are trying to join.

#### IMPORTANT POINT:

Enzymes are the “tool kit” of the molecular biologist. You must put away the “saw” (restriction enzymes that cut DNA) before you use the “hammer and nails” (ligase to join DNA fragments). Always anticipate the next step in the process!

### BRIEF GLOSSARY

**Biotechnology** Applying biology in the real world by the specific manipulation of living organisms, especially at the genetic level, to produce potentially beneficial products.

**Cloning** When a population of cells is prepared by growth from a single cell, all the cells in the population will be genetically identical. Such a population is called clonal. The process of creating a clonal population is called “cloning”. Identical copies of a specific DNA sequence, or gene, can be accomplished following mitotic division of a transformed host cell.

**Genetic Engineering** The manipulation of an organism’s genetic material (DNA) by introducing or eliminating specific genes.

**Gene Regulation** Gene expression in all organisms is carefully regulated to allow for differing conditions and to prevent wasteful overproduction of unneeded proteins. The genes involved in the transport and breakdown of food are good examples of highly regulated genes. For example, the milk sugar lactose can be used as a source of energy and carbon by bacteria. The bacterial enzymes that are needed to break down or digest lactose for food are not expressed in the absence of lactose but are expressed when lactose is present in the environment.

**Induction** The *lac* operon controls expression of the lactose utilizing genes, and this operon has been genetically engineered to provide a screen for a DNA insert. Gene expression is induced when X-gal, an analog of lactose, is present in the medium and the protein  $\beta$ -galactosidase is then produced.

**Recombinant DNA Technology:** The process of cutting and recombining DNA fragments as a means to isolate genes or to alter their structure and function.

**Vector:** An autonomously replicating DNA molecule into which foreign DNA fragments are inserted and then propagated in a host cell (*i.e.* **plasmid**).

**Gel rig terminology**

**Casting tray:**

**Comb:**

**Wells:**

**Power Source:**

**Connection cords:**

**Voltage:**

**Preparation of gel**

**Agarose:**

**TAE Buffer:**

**Casting the gel:**

**Comb removal:**

**Loading the gel**

**Buffer:**

**Submergence:**

**Loading Dye/Tracking Dye:**

**Xylene cyanol and Bromophenol Blue:**

**Ladder (DNA Marker):**

**Red and Black Connector Cords:**

**Setting the Voltage:**

**Verification of Electrical Current:**

**Retrophoresis:**

"retrophoresis" is when you catch your mistake in time (before the samples run out the top of the gel), reverse the wires to "retro" your "phoresis" :(

**Visualization of migration**

**Gloves:**

**Eye Protection:**

**Ethidium bromide staining:**

**Intercalation:**

**Mutagens and Responsible Handling:**

**UV Transluminator:**

**Photograph Documentation:**

**Gel Disposal:**

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SUPPLEMENT: The buffers used by *Bam*HI and *Bgl*II:

*Bam*HI

Percent Activity in 4-CORE® Buffer System

A	B	C	D	E	MULTI-CORE™
75–100%*	75–100%	75–100%	50–75%	<b>100%</b>	<b>75–100%</b>

\*Buffer A is not recommended due to potential star activity.

*Bgl*II

Percent Activity in 4-CORE® Buffer System

A	B	C	D	MULTI-CORE™
25–50%	75–100%	75–100%	<b>100%</b>	<10%