

# Biotechnology Explorer™

## Ligation and Transformation Module

### Instruction Manual

Catalog #166-5015EDU

[explorer.bio-rad.com](http://explorer.bio-rad.com)

This kit is shipped on blue ice. Open immediately upon arrival and store reagents bags at  $-20^{\circ}\text{C}$ .

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# Introduction

Cloning is the production of multiple exact copies of a piece of DNA, usually a gene, using molecular biology techniques. Cloning is frequently the first step of a research project, producing enough DNA for further study.

Using the Ligation and Transformation module students can subclone virtually any DNA fragment of interest that has been amplified using PCR. We recommend that the DNA fragment be approximately 200–2,000 base pairs (bp) in length for best results. Below is a typical workflow for cloning and sequencing a gene. The steps that the Ligation and Transformation module enable students to perform are in bold.

The Ligation and Transformation module is part of Bio-Rad's Cloning and Sequencing Explorer Series. The Cloning and Sequencing Explorer Series is a sequence of individual modules that have been designed to work in concert to give students the real world experience of a molecular biology research workflow. The additional modules of the Cloning and Sequencing Explorer Series can be purchased separately. Further information on the separate modules is available in the Biotechnology Explorer™ catalog or from [explorer.bio-rad.com](http://explorer.bio-rad.com).

- Amplify gene of interest using PCR<sup>1</sup>
- Purify PCR product<sup>2</sup>
- **Ligation of PCR product into pJet1.2 plasmid**
- **Transform ligated plasmid into bacteria**
- Culture bacteria and grow minipreps<sup>3</sup>
- Purify plasmid from minipreps<sup>4</sup>
- **Analyze plasmid by restriction digestion**
- Electrophoresis restriction digest reaction<sup>5</sup>
- Sequence plasmid and analyze<sup>6</sup>

<sup>1</sup> *GAPDH* PCR module (catalog #166-5010EDU) amplifies a fragment of the *GAPDH* gene from a preparation of plant genomic DNA.

<sup>2</sup> PCR Klean™ Spin module (catalog #732-6300EDU) purifies 25 PCR products.

<sup>3</sup> Microbial Culturing module (catalog #166-5020EDU) contains all required reagents for culturing bacteria for transformation using the Ligation and Transformation module.

<sup>4</sup> Aurum™ Plasmid Mini Purification module (catalog #732-6400EDU) contains reagents to purify plasmid DNA from 100 minipreps.

<sup>5</sup> Electrophoresis modules contain reagents to analyze plasmid restriction digests.

<sup>6</sup> Sequencing and Bioinformatics module (catalog #166-5025EDU) is designed to allow sequencing and bioinformatics analysis of plasmids generated using the Ligation and Transformation module.

Using the Ligation and Transformation module, students will clone a gene of interest. Prior to starting this laboratory activity, students must have already amplified a gene of interest using polymerase chain reaction (PCR) and subsequently purified the PCR product to remove excess primers, nucleotides, and DNA polymerase, which would otherwise interfere with subsequent experiments. Students can then use the Ligation and Transformation module to ligate the DNA fragment into the pJet1.2 blunted vector, which encodes *amp<sup>r</sup>*, an ampicillin-resistance gene. Following ligation, students will perform transformation to introduce the plasmid into living bacterial cells.

The pJet1.2 blunted vector enables positive selection of plasmids with the desired insert due to the disruption of *eco471R*, an otherwise lethal gene, that allows growth of successful transformants. Bacteria are then plated and incubated overnight at 37°C on the selective medium containing ampicillin and isopropyl β-D-1-thiogalactopyranoside (IPTG), which is added to increase expression of the *amp<sup>r</sup>* gene. Since transformed cells express an ampicillin-resistance gene, they will grow and divide, each forming a colony on the plate that is the product of a single transformation event.

The bacteria containing the cloned gene can be grown in liquid growth medium and the plasmid containing the insert can be purified from the bacteria. The pJet1.2 plasmid contains a Bgl II restriction enzyme recognition site on either side of the insertion site. Using the Bgl II enzyme students will analyze the cloned plasmid by restriction enzyme digestion and analyze their digests by agarose gel electrophoresis to confirm the presence of an insert and determine its size. The resulting fragment can then be compared to the size of the PCR fragment ligated into the plasmid. Finally, the DNA fragment can be then sequenced to determine the exact order of nucleotides in the DNA molecule.

### **What Skills Do Students Need to Perform this Laboratory Activity?**

This laboratory activity assumes that students and instructors have basic molecular biology and microbiology skills, such as proper pipeting techniques, pouring and streaking agar plates and performing agarose gel

electrophoresis. In addition, students must understand the principles of PCR and be able to perform PCR reactions. Bio-Rad's Biotechnology Explorer program has a full range of kits to help teach basic skills in individual laboratories.

### **What Is the Timeline for Completing the Ligation and Transformation Protocol?**

Before starting this activity, students must have already amplified a gene of interest using PCR. In addition, the PCR product should be purified to remove components of the amplification reaction that would otherwise interfere with the ligation step.

The amount of time it takes to complete the ligation and transformation protocols depends greatly on the level of your students and whether additional/optional techniques and analyses are performed in addition to the basic protocol. Steps using the Ligation and Transformation module are highlighted in bold. Additionally, there are a few incubation steps that add to the number of days it takes to complete the laboratory activity. A rough guide is provided on pages 4 and 5.

<b>When</b>	<b>Activity to Complete</b>	<b>Duration</b>
At least 1 day prior to starting the Ligation and Transformation module	Run a PCR reaction in thermal cycler to amplify a gene of interest	3–4 h
	Electrophorese the PCR products (optional)	1 h
	Purify PCR products	0.5 h
At least 3 days prior to the transformation step	Prepare LB and LB Amp IPTG agar plates	0.5 h
At least 2 days prior to the transformation step	Prepare LB broth	0.5 h
	Streak <i>E. coli</i> on a starter LB agar plate	5 min
	Grow <i>E. coli</i> starter plate at 37°C	16+ h
As late as possible the day before the transformation step	Inoculate starter culture	5 min
	Incubate starter culture at 37°C in a shaking water bath or incubator	8+ h
Day of ligation step	<b>Ligate PCR product</b>	1 h

**Note:** Bolded steps use reagents from the Ligation and Transformation module.

<b>When</b>	<b>Activity to Complete</b>	<b>Duration</b>
Immediately following ligation or during the next laboratory activity	<b>Transform <i>E. coli</i> with ligation mixture</b> and plate bacteria on LB Amp IPTG agar plates	1 h
	Incubate transformed bacteria at 37°C	16+ h
Next day after the transformation step	Analyze results	0.5 h
	Grow bacterial colony in LB Amp broth for miniprep	16+ h
Day after growing bacterial culture for miniprep	Perform miniprep plasmid purification to isolate plasmid carrying insert	1 h
Next laboratory activity	<b>Digest plasmid DNA with Bgl II restriction enzyme</b>	1 h
	Analyze digest by agarose gel electrophoresis	1 h
	Prepare the DNA insert for sequencing	0.5 h

**Note:** Bolded steps use reagents from the Ligation and Transformation module.

# Kit Inventory Checklist

This section lists equipment and reagents necessary to perform the ligation and transformation protocol in your classroom or teaching laboratory. Each kit contains sufficient materials for 12 student workstations, 12 ligation reactions, and 24 transformations. We recommend that students are teamed up – two to four students per workstation. Please use the checklist below to confirm inventory.

Kit Components	Number/Kit	(✓)
T4 DNA ligase, 10 $\mu$ l	1	<input type="checkbox"/>
Ligation reaction buffer (2x concentration), 100 $\mu$ l	1	<input type="checkbox"/>
Proofreading polymerase, 10 $\mu$ l	1	<input type="checkbox"/>
pJet1.2 blunted vector, 10 $\mu$ l	1	<input type="checkbox"/>
Sterile water, 1 ml	1	<input type="checkbox"/>
Bgl II restriction enzyme, 50 $\mu$ l	1	<input type="checkbox"/>
10x Bgl II reaction buffer, 1 ml	1	<input type="checkbox"/>
Isopropyl $\beta$ -D-1-thiogalactopyranoside (IPTG), 1 M, 0.1 ml	1	<input type="checkbox"/>
Transformation reagent A, 1.25 ml	4	<input type="checkbox"/>
Transformation reagent B, 1.25 ml	4	<input type="checkbox"/>
C-growth medium, 30 ml	1	<input type="checkbox"/>
Microcentrifuge tubes, clear, 1.5 ml	30	<input type="checkbox"/>
Microcentrifuge tubes, multicolor, 2.0 ml	120	<input type="checkbox"/>

Required Accessories	Number/Kit	(✓)
PCR product (previously amplified and purified by students)	1 per team	<input type="checkbox"/>
Microbial Culturing module (catalog #166-5020EDU)* containing the following:	1	<input type="checkbox"/>
• LB broth capsules (each for making 50 ml of LB broth)	12	<input type="checkbox"/>
• LB nutrient agar powder (to make 500 ml)	1 pouch	<input type="checkbox"/>
• Ampicillin	2 vials	<input type="checkbox"/>
• <i>E. coli</i> HB101 K-12, lyophilized bacteria	1 vial	<input type="checkbox"/>
• Culture tubes, sterile, 15 ml	75	<input type="checkbox"/>
• Petri dishes, sterile	40	<input type="checkbox"/>
• Sterile inoculating loops	80	<input type="checkbox"/>
Variable speed microcentrifuge (catalog #166-0602EDU)	1	<input type="checkbox"/>
Shaking water bath or shaking incubator (37°C)	1	<input type="checkbox"/>
Water bath (catalog #166-0504EDU), heating block, (catalog #166-0562EDU) or incubator (70°C)	1	<input type="checkbox"/>
Adjustable-volume micropipet		
0.5–10 µl (catalog #166-0505EDU)	12	<input type="checkbox"/>
20–200 µl (catalog #166-0507EDU)	12	<input type="checkbox"/>
100–1,000 µl (catalog #166-0508EDU)	12	<input type="checkbox"/>
Pipet Tips		
0.5–10 µl (catalog #223-9354EDU)	1 box	<input type="checkbox"/>
2–200 µl (catalog #223-9347EDU)	1 box	<input type="checkbox"/>
100–1,000 µl (catalog #223-9350EDU)	1 box	<input type="checkbox"/>
Ice bath	1	<input type="checkbox"/>
Parafilm sealing film	1	<input type="checkbox"/>
Marking pens	1	<input type="checkbox"/>

**\*Note:** Standard microbiological reagents may be used in place of the Microbial Culturing module (see Instructor's Advanced Prep section for requirements). Any *E. coli* strain commonly used for transformation (for example, DH5 $\alpha$ , DH10, JM107) may be used in place of *E. coli* HB101.

## **Optional Accessories**

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Vortex mixer (catalog #166-0610EDU)

Vacuum source

Agarose electrophoresis equipment

*GAPDH* PCR module (catalog #166-5010EDU)

PCR Kleen™ Spin Purification module (catalog #732-6300EDU)

pGLO™ Plasmid, 20 µg (catalog #166-0405EDU)

Aurum™ Plasmid Mini Purification Module (catalog #732-6400EDU)

Electrophoresis reagents:

Small Ethidium Bromide DNA Electrophoresis Reagent Pack (catalog #166-0451EDU)

Small Fast Blast™ DNA Electrophoresis Reagent Pack (catalog #166-0450EDU)

Sample Loading Dye, 5x, 1 ml (catalog #166-0401EDU)

EZ Load™ 500 bp Molecular Ruler (catalog #170-8354EDU)

Sequencing and Bioinformatics module (catalog #166-5025EDU)

## **Refills Available Separately**

Ligation module reagent refill (catalog #166-5016EDU)

Includes T4 DNA ligase, 2x ligation reaction buffer, proofreading polymerase, pJet1.2 blunted vector, sterile water

Bgl II reagent refill (catalog #166-5018EDU)

Includes Bgl II restriction enzyme and 10x Bgl II reaction buffer

Transformation module reagent refill (catalog #166-5017EDU)

Includes transformation reagent A, transformation reagent B, 1 M IPTG, C-growth medium

## Storage Instructions

The kit is shipped on blue ice. Open immediately upon arrival and store reagent bags immediately at  $-20^{\circ}\text{C}$ .

## Safety Issues

Eating, drinking, smoking, and applying cosmetics are not permitted in the work area. Wearing protective eyewear and gloves is strongly recommended.

Transformation reagent B contains dimethyl sulfoxide (DMSO, CAS #67-68-5), an organic solvent. Handle with care and follow standard laboratory practices, including wearing eye protection, gloves, and a laboratory coat to avoid contact with eyes, skin, and clothing. If the solution comes into contact with gloves, change the gloves. DMSO passes directly through latex gloves, readily penetrates skin, and may result in the absorption of toxic materials and allergens dissolved in the solvent. After handling, wash hands and any areas that came into contact with the solution thoroughly. Refer to MSDS for complete safety information.

Ampicillin may cause allergic reactions or irritation to the eyes, respiratory system, and skin. In case of contact with eyes, rinse immediately with plenty of water and seek medical advice. Wear suitable protective clothing. Ampicillin is a member of the penicillin family of antibiotics. Those with allergies to penicillin or any other member of the penicillin family of antibiotics should avoid contact with ampicillin.

The *E. coli* HB101 K-12 strain is not pathogenic. However, handling of *E. coli* HB101 K-12 requires the use of standard microbiological practices. These practices include, but are not limited to, the following: (1) work surfaces are decontaminated once a day after any spill of viable material; (2) all contaminated liquid or solid wastes are decontaminated before disposal; (3) persons should wash their hands: (a) after they handle materials involving organisms containing recombinant DNA molecules, and (b) before exiting the laboratory; (4) all procedures should be performed carefully to minimize the creation of aerosols and; (5) mechanical pipetting devices should be used—mouth pipetting is prohibited.

# **Background**

## **Cloning**

Cloning is the production of multiple exact copies of a piece of DNA, usually a gene, using molecular biology techniques. Cloning is frequently the first step used in a research project, producing enough DNA for further study. Once a gene or part of a gene has been amplified using PCR, the next step is to insert the DNA into a plasmid or cloning vector so that the DNA fragment can be propagated.

## **Plasmids as Cloning Vectors**

Many cloning vectors are derived from bacterial plasmids. Plasmids are circular extrachromosomal DNA molecules, usually around 2,000–100,000 base pairs (bp) long, although most plasmids used in cloning are 2,000–10,000 bp. Bacteria may naturally contain many copies of a single plasmid, or single copies of others. Plasmids are able to replicate independently of the host DNA and most plasmids carry at least one gene. Frequently these genes code for a factor or function that helps the bacteria survive. For example, resistance to the antibiotic ampicillin is conveyed by a plasmid carrying an ampicillin-resistance gene. Plasmids are capable of being transferred from one bacterium to another. These characteristics have resulted both in wonderful new uses for plasmids (such as their use in cloning, making many of the techniques of molecular biology possible) and in the emergence of dangerous pathogenic organisms (namely bacteria resistant to multiple antibiotics).

Plasmids thus already have many of the characteristics needed for use as cloning vectors, and other useful features have been added through genetic engineering. A wide variety of vectors are available commercially for various applications. A plasmid designed to clone a gene is different from a plasmid designed to express a cDNA (complementary DNA) in a mammalian cell line, which is different again from one designed to add a tag to a protein for easy purification. The primary characteristics of any good vector include:

- Self-replication — Plasmids have an origin of replication so they can reproduce independently within the host cell; since the origin of replication engineered into most cloning vectors is bacterial, the plasmid can be replicated by enzymes already present in the host bacteria
- Size — Most bacterial vectors are small, between 2,000–10,000 bp long (2–10 kilobases or kb), making them easy to manipulate
- Copy number — Each plasmid is found at specific levels in its host bacterial strain. A high copy number plasmid might have hundreds of copies in each bacterium, while a low copy number plasmid might have only one or two copies per cell. Cloning vectors derived from specific plasmids have the same copy number range as the original plasmid. Most commonly used cloning vectors are high copy number
- Multiple cloning site (MCS) — Vectors have been engineered to contain an MCS, a series of restriction sites, to simplify insertion of foreign DNA into the plasmid. An MCS may have 20 or more different enzyme sites, each site unique both in the MCS and in the plasmid. This means that for each restriction site included in the MCS, the corresponding restriction enzyme will cut the plasmid only at its single site in the MCS
- Selectable markers — Plasmids carry one or more resistance genes for antibiotics, so if the transformation is successful (that is, if the plasmid enters and replicates in the host cell), the host cell will grow in the presence of the antibiotic. Commonly used selectable markers are genes for resistance to ampicillin (*amp<sup>r</sup>*), tetracycline (*tet<sup>r</sup>*), kanamycin (*kan<sup>r</sup>*), streptomycin (*sm<sup>r</sup>*), and chloramphenicol (*cm<sup>r</sup>*)

- Screening — When bacteria are being transformed with a ligation reaction, not all of the religated vectors will necessarily contain the DNA fragment of interest. To produce visible indicators that cells contain an insert, vectors frequently contain reporter genes, which distinguish them from cells that do not have inserts. Two common reporter genes are beta-galactosidase ( $\beta$ -gal) and green fluorescent protein (GFP)

Some newer plasmid vectors use positive selection, in which the inserted DNA interrupts a gene that would otherwise be lethal to the bacteria. If foreign DNA is not successfully inserted into the MCS, the lethal gene is expressed and transformed cells die. If the foreign DNA is successfully inserted, the lethal gene is not expressed and the transformed bacteria survive and divide. Positive selection eliminates the need for reporter genes, as only cells transformed with vector containing an insert will survive

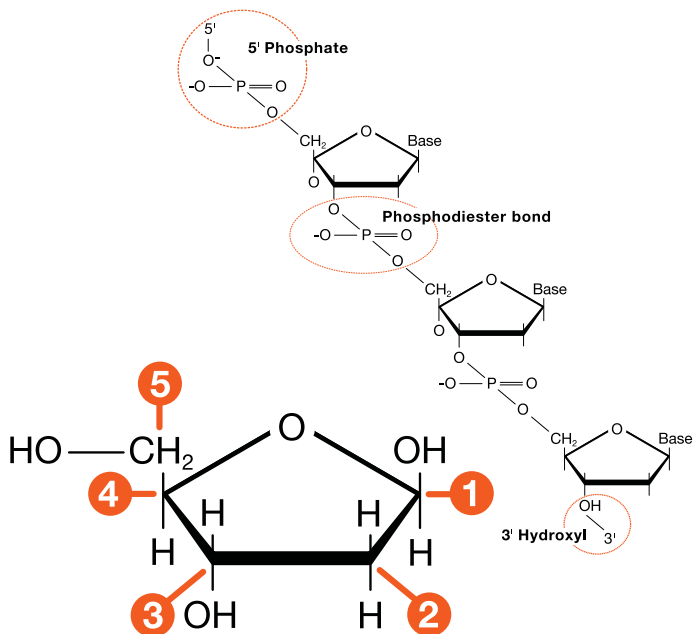
- Control mechanism — Most vectors have some control mechanism for transcription of the antibiotic resistance or other engineered gene. One of the best-known control mechanisms is the lac operon (an operon is a group of genes). When lactose (a sugar) is absent in the cell, the lac repressor protein binds to the lac operon, preventing transcription of the gene. When lactose is present in the cell, it binds to the lac repressor protein, causing the repressor protein to detach from the operon. With the repressor protein no longer bound to the operon, RNA polymerase can bind and the genes can be transcribed. In this system, lactose acts as an inducer. (A closely related compound, IPTG), is often used in the laboratory as an artificial inducer.) Genes from the lac operon have been engineered into many cloning vectors
- Size of insert — Plasmid vectors have limitations on the size of inserts that they can accept, usually less than the size of the vector. Other vectors have been developed for use if the target DNA is larger, for example, lambda phage (inserts up to 25 kb), cosmids (inserts up to 45 kb), bacterial artificial chromosomes (BACs; inserts from 100–300 kb), yeast artificial chromosomes (YACs; inserts from 100–3,000 kb), and bacteriophage P1 (inserts up to 125 kb)

## DNA Ligation

Ligation is the process of joining two pieces of linear DNA into a single piece through the use of an enzyme called DNA ligase. DNA ligase catalyzes the formation of a phosphodiester bond between the 3'-hydroxyl on one piece of DNA and the 5'-phosphate on a second piece of DNA.

The most commonly used DNA ligase is T4 DNA ligase (named because it originated in a bacteriophage named T4). There are several ways that the efficiency of DNA ligation can be optimized. First, like any enzyme, there are conditions that are optimal for ligase activity:

- T4 DNA ligase requires ATP and magnesium ions for activity
- The concentration of vector and insert DNA in solution must be high for efficient ligation
- The molar ratio of insert to vector DNA should be approximately equal, although the optimal ratio may not be 1:1

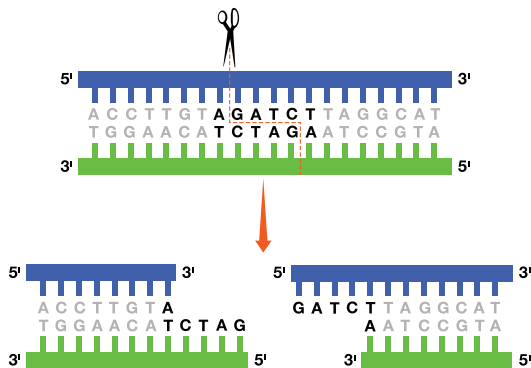


**Chemical structure of deoxyribose sugar and deoxyribose nucleic acid (DNA).**

Ligation is used to join vector DNA and insert DNA. There are two ways in which DNA can be ligated into a cloning vector, one using DNA with so-called sticky ends and the other using DNA with blunt ends. Unlike DNA with blunt ends, DNA with sticky ends has one or more unpaired bases at its ends that do not have complementary bases on the other strand of the double helix. When a DNA fragment is generated by *Taq* DNA polymerase by a process like PCR, it typically has sticky ends with a single adenosine (A). When a DNA fragment is generated by cutting a piece of DNA with a restriction enzyme (an enzyme that cuts both strands of double-stranded DNA), it may have either sticky ends or blunt ends, depending on the restriction enzyme.

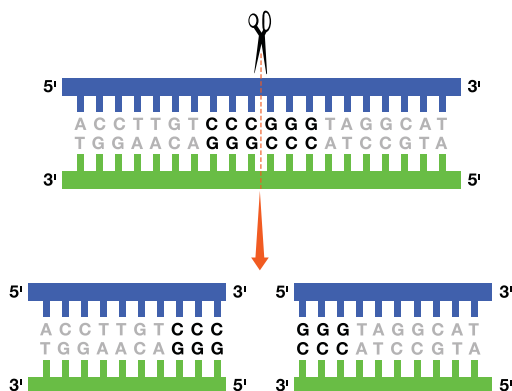
**DNA ligation with sticky ends** — To prepare a cloning vector for ligation with insert DNA, it is cut with a restriction enzyme within the MCS, opening it to receive the inserted DNA. If the insert has sticky ends, that is, overhangs on the end of the DNA strands, then the vector should be cut with the same enzyme, producing sticky ends that will be complementary to the ends of the insert DNA. For example, if the insert DNA has been prepared by cutting it at both ends with Bgl II, then the vector would also be cut with Bgl II. Having complementary sticky ends improves the efficiency of ligation, whereas mismatches in the sequences reduce efficiency.

Because the sticky ends on the vector and the insert are complementary, when they come into contact during the ligation reaction they will base-pair with each other using hydrogen bonds. (The base-paired sticky ends of the insert and vector are not stably associated, and they can dissociate prior to ligation.) While the insert and vector are associated, T4 DNA ligase forms a phosphodiester bond, covalently linking the two pieces of DNA. Actually, there are two ligations. The first ligation is intermolecular, between one end of the vector and one end of the insert, resulting in a linear DNA molecule. The second ligation is intramolecular, circularizing the molecule.



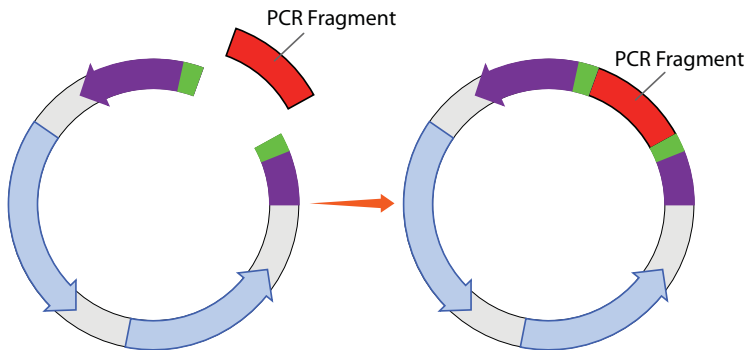
**Sticky end digestion.**

One advantage to sticky-end ligation is that it makes directional cloning possible. If it is desirable to have the insert in one orientation only (for instance, in the A  $\rightarrow$  B direction in the vector, but not in the B  $\rightarrow$  A direction), then the insert and vector can both be digested with two different restriction enzymes so that their ends are asymmetric. This is important if the DNA insert is a cDNA encoding a protein to be expressed in the transformed cell. When this is done, only the complementary ends will ligate and the insert will have a single orientation in the ligation products.



**Blunt end digestion.**

**DNA ligation with blunt ends** — Blunt-end ligation, in which both the inserted DNA and the vector have blunt ends, has an advantage compared to sticky-end ligation in that all DNA ends are compatible with all other ends. In other words, it is not necessary to cut the vector and insert with the same restriction enzymes to get complementary overhangs as for sticky-end ligation. Vectors used for blunt-end ligation have a blunt-ended ligation site in the MCS. They still have an MCS, as the restriction enzyme sites are very useful for subsequent manipulation of the inserted DNA.



**Ligation of PCR fragment into vector.**

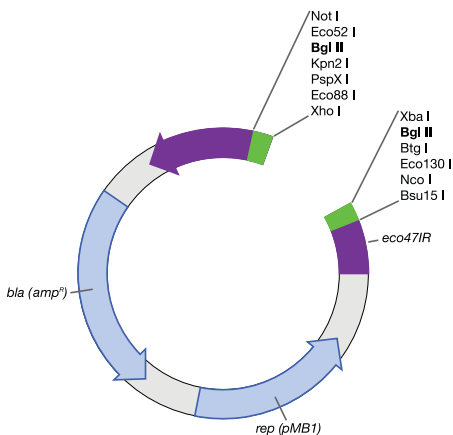
In PCR, *Taq* DNA polymerase adds a single nucleotide to the 3'-end of the PCR product, usually an A. Since this A overhang would prevent blunt-end ligation, it must be removed prior to ligation. Treating the PCR product with a proofreading DNA polymerase removes the 3'-A, leaving blunt ends ready for ligation. (Not all thermally stable DNA polymerases used in PCR leave an A overhang; some polymerases like *Pfu* DNA polymerase have proofreading ability.)

### **Features of the pJet1.2 blunted vector**

The pJet1.2 blunted vector has several features that make it a good choice for this laboratory activity:

- It is a vector designed for blunt-end cloning and is already linearized with blunt ends

- Its MCS has restriction enzyme sites that can be used for later manipulation of the DNA
- It is a high copy number plasmid
- It contains the  $\beta$ -lactamase gene, *amp<sup>r</sup>*, which confers resistance to ampicillin
- It contains the *eco47IR* gene, which allows positive selection of transformants. This gene codes for the *Eco47I* restriction enzyme, and when the enzyme is expressed, it is toxic to *E. coli*. When the *eco47IR* gene is disrupted by the insertion of DNA into the cloning site, the gene will no longer be expressed and the transformed cells will grow and divide on selective media
- It is 2,974 bp long and its sequence is readily available — the accession number for pJet1.2 is EF694056

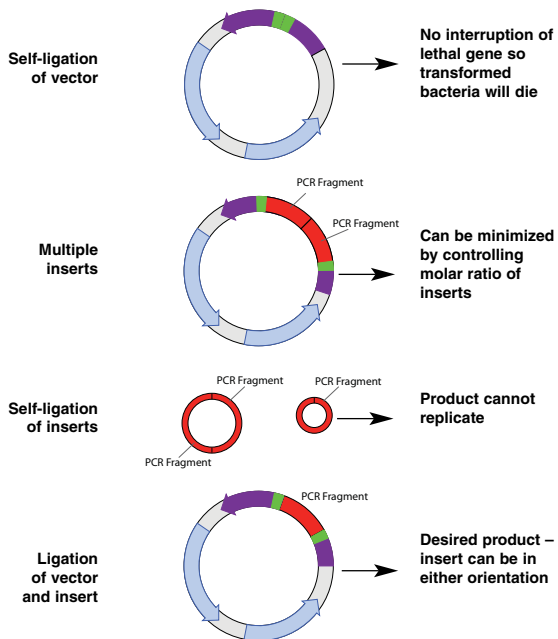


**pJet1.2 blunted vector.**

## Products of Ligation

Ligation is a very inefficient process; from millions of vectors and inserts, only 1–100 are expected to ligate together as desired and lead to growth of colonies from the desired transformants. There are several possible products from a ligation with a vector such as the pJet1.2 blunted vector:

- Self-ligation of the vector — A self-ligated vector, without any DNA inserted in the MCS, should have an intact lethal gene. The product of the lethal gene should kill any bacteria transformed by these vector-only constructs. (**Note:** The *eco47IR* gene is not 100% lethal, and so some bacteria may grow, resulting in tiny satellite colonies, which may be observed in no-insert ligation controls)
- Ligation of a vector with primer-dimers or other short DNA fragments from the PCR reaction — Even if these fragments are a small proportion of the total fragments available for ligation, small fragments ligate more easily than larger fragments
- Ligation of a vector with multiple inserts — Since the inserts all have the same blunt ends, they can ligate to each other and also ligate to the vector, giving a product with multiple inserts in a row. The number of these products can be reduced by controlling the molar ratio of insert to vector. If the ratio is high (that is, if there are many more insert molecules than vector), then it is more probable that inserts will ligate to each other rather than ligate to the vector. So the molar ratio can be an important factor in setting up ligation reactions
- Self-ligation of insert — It is possible for the insert molecules to self-ligate, forming closed circles. Since these molecules do not have any of the vector genes, they will not be able to replicate, so they are not of consequence
- Ligation of one insert into a vector — This is the desired result, and in ligation to a vector such as pJet1.2 blunted vector, there is no directionality in the cloning. In other words, the insert can ligate into the vector in either direction, because all of the ends are identical. So the products should be a 50:50 mix, with half of the inserts in one orientation and the other half in the reverse orientation

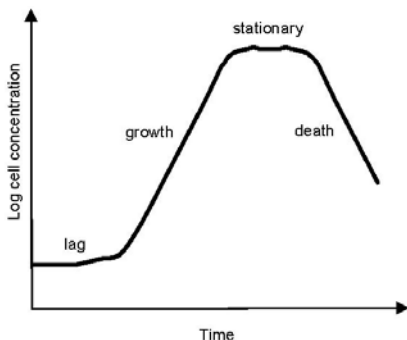


**Possible ligation products.**

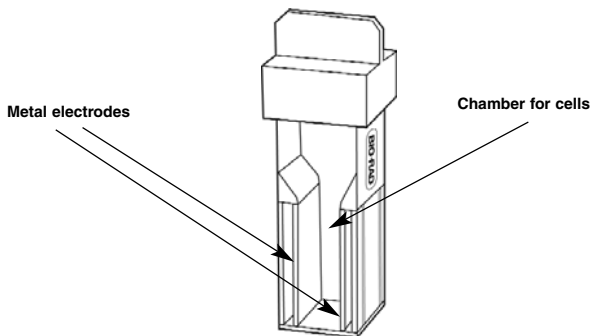
## Transformation

Once a gene or part of a gene has been amplified using PCR and ligated into a plasmid, the next step in cloning is transformation, introducing the plasmid into living bacterial cells so that it can be replicated. Heat shock transformation and electroporation are the two methods of bacterial transformation commonly used in the laboratory. Both methods require competent cells, bacterial cells that can take up DNA. Not all cells are naturally competent. For example some species, such as *Bacillus subtilis*, can be easily transformed, but for other species, such as *Escherichia coli*, only a small number of cells in a culture may be able to take up DNA. Competent cells may be prepared in the laboratory or purchased commercially.

- Heat shock is the most easily accomplished transformation method, as it does not require any equipment other than a water bath. Plasmid DNA and heat-shock competent cells in calcium chloride are mixed together and incubated on ice for several minutes. Although the mechanism is not fully understood, calcium chloride causes DNA to bind to the bacterial cell wall. The cells are then subjected to a brief heat shock resulting in the uptake of DNA into the bacteria. Traditionally bacteria are heat shocked by incubation at 42°C for 50 sec. However in this laboratory the bacteria are heat shocked by spreading the ice cold bacteria directly onto warm agar plates at 37°C. Cells intended for heat shock transformation must be in the exponential growth phase to be highly competent



- Electroporation is also commonly used for transformation, and its mechanism of enabling DNA uptake is somewhat better understood than heat-shock transformation. When bacterial cells are subjected to a brief electrical shock, small pores open in their cell wall, allowing DNA to enter the cells. For electroporation, electrocompetent bacteria and plasmid DNA are mixed and placed in a special type of cuvette, a square test tube with metal electrodes on two sides (see figure on page 22). The cuvette is placed in an instrument called an electroporator that delivers an electrical charge of specific strength and duration to the cells. The electricity travels through the cells between the two electrodes, which is why electrocompetent cells must be prepared in a solution of very low ionic strength. For electroporation to be successful, the cells themselves must carry the current across the gap between the electrodes. If there are many ions (like Na<sup>+</sup>) in the solution, the ions will carry the current instead of the cells, causing the cells to overheat and die



#### **Bio-Rad electroporation cuvette.**

There are ways to increase the number of competent cells in a bacterial culture. To prepare competent cells for heat shock transformation, the bacteria must be washed to remove the growth medium, then resuspended in an ice cold calcium chloride solution. For electroporation, the cells must be washed repeatedly in chilled buffer, and resuspended in a chilled sterile solution that has very low ionic strength. In both cases, the cells must be in solution and at a high concentration for transformation to be successful and the cells must be kept cold at all times prior to transformation. The cells are extremely fragile at this stage and the cold temperature keeps them inert. If they are warmed up in transformation solution, they will start to die. The transformation protocol also requires the cells to be cold; even though the exact process of transformation is still not fully understood, it is presumed that the cold hardens the cell membranes of the bacteria and the heat shock rapidly melts or breaks the membranes, which allows the DNA inside the cells.

Since bacteria have defense mechanisms that use restriction enzymes to degrade foreign DNA, only mutant strains that no longer have restriction activity can be used for transformation. Normal bacteria would degrade the plasmid DNA as soon as it enters the cell. Mutant strains for transformation are widely available, for example, the *E. coli* HB101 K-12 strain recommended for use in this activity.

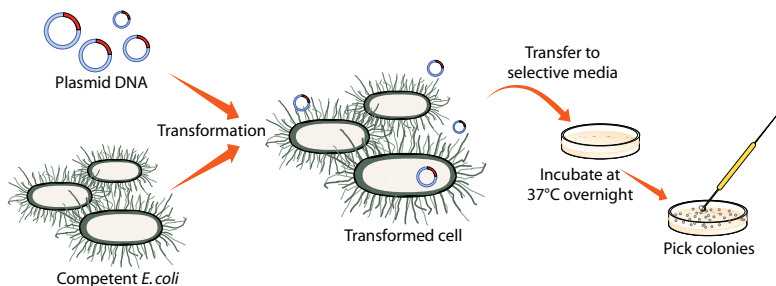
## **What Happens After Transformation**

After either transformation method, the cells are usually incubated in nutrient medium for up to 1 hour to allow them to recover from the stress

of the transformation (either the heat shock or the electrical pulse) and begin to express the genes on the plasmid (such as an antibiotic resistance gene), although this step may be omitted. The cells are plated on a selective medium for growth, usually agar plates containing nutrient medium and the antibiotic for which resistance is carried by the plasmid. For example, if the plasmid contains the *amp<sup>r</sup>* gene, providing resistance to ampicillin, the agar plates should also contain ampicillin. This means that only bacteria that have been successfully transformed and now carry the plasmid will be able to survive and divide on the ampicillin-containing plates. The plasmid will replicate in the bacterial cells (using the host cell's replication machinery) and, as the bacteria divide, the plasmids will be passed on to their offspring.

The plasmid that is used in this laboratory activity, pJet1.2 blunted vector, contains the *amp<sup>r</sup>* gene. IPTG is added to the selective medium to artificially increase expression of the *amp<sup>r</sup>* gene, which is under the control of the *lac* operon (normally regulated by lactose); this approach increases transformation efficiency with this plasmid.

Even though the efficiency of bacterial transformation can be optimized by using competent cells and by determining the best experimental conditions for transformation and selection, transformation is still an inefficient process, with only a small percentage of available DNA being taken into a small percentage of the competent bacteria. After the bacteria are plated on the selective medium, the antibiotic will prevent the untransformed cells from growing. Transformed cells, however, will grow and divide, each forming a colony on the plate that is the product of a single transformation event. In other words, all the cells in each colony are clones, hence the origin of the term cloning.



**Process of bacterial transformation.** Competent *E. coli* are transformed with plasmid DNA. Only a few bacteria take up the plasmid DNA. Bacteria are then plated on selective media and incubated overnight. Only bacteria that contain the plasmid will grow and form colonies. Bacteria colonies are then picked and grown for use in plasmid minipreps.

## Minipreps of Plasmid DNA

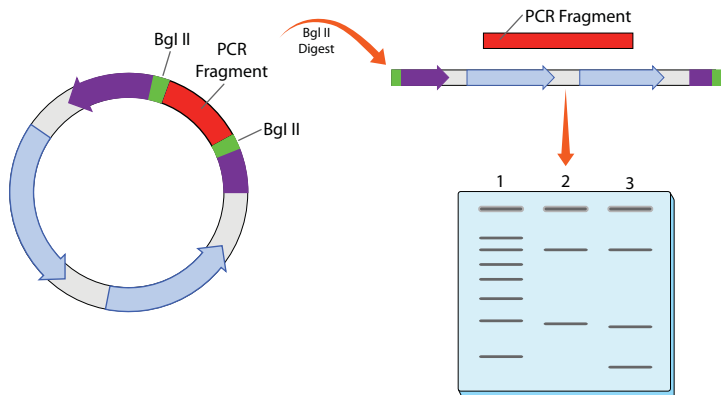
Once a plasmid has been introduced into competent bacterial cells and the cells have grown into colonies on a medium selective for cells containing the plasmid, the next step is to prepare a miniprep of the plasmid DNA in preparation for sequencing or further experiments. The three steps are: 1) growing cells in liquid culture; 2) purifying the plasmid DNA from the culture; and 3) performing a restriction digest on the purified DNA to determine whether the DNA insert in the plasmid is the expected size. To start the liquid culture, cells from an isolated bacterial colony are placed in selective medium (nutrient broth with an antibiotic to which the plasmid provides resistance). This placing of the cells into the medium is called inoculation. It is important to choose a single isolated colony from the plate, so that the liquid culture will contain cells that all have the same plasmid. If cells from more than one colony are used for inoculation, the miniprep may contain multiple plasmids and the mixed DNA will not be useful for sequencing or further experiments.

## Restriction digestion of plasmid DNA

Before proceeding with further experiments using the purified plasmid DNA, it is important to verify that the isolated plasmid contains the insert of interest using restriction digestion followed by gel electrophoresis.

Looking back at earlier steps in the experiment, a gene or portion of a gene was ligated into the plasmid vector. From previous work, the size of this insert should be known. By digesting a small portion of the miniprep DNA with a specific restriction enzyme such as Bgl II (see below), the insert should be cut out of the vector. Running the products of the restriction digestion on an agarose gel should give two DNA bands, one the size of the vector and the other the size of the inserted DNA.

If there are more than two bands in any digest, it may mean that the insert contains a Bgl II site. Does the size of the excised bands add up to the size of the original PCR fragment? Alternatively, two similarly sized fragments may indicate that a mixed culture was used to start the miniprep, instead of an isolated colony.



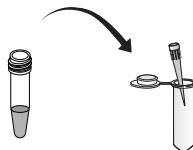
**Restriction enzyme digestion analysis of plasmid DNA.** Circular plasmid DNA purified from bacterial minipreps is isolated and digested with Bgl II, a restriction enzyme producing at least two linearized fragments — vector DNA and PCR fragment (lane 2). These fragments can be visualized using agarose gel electrophoresis. If the PCR fragment contains a Bgl II restriction site, three DNA bands may be observed (lane 3). A 500 bp molecular weight ruler is shown in lane 1.

## Ligation – Quick Guide

1. Label a microcentrifuge tube with your initials, plant name, and "ligation."
2. Briefly spin down the stock tubes of 2x reaction buffer and proof reading polymerase to collect the contents at the bottom of the tube.
3. Set up blunting reaction with the following reagents.

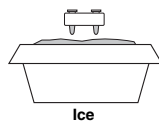
Reagent	Amount
2x ligation reaction buffer	5.0 $\mu$ l
Purified PCR product	1.0 $\mu$ l
Sterile water	2.5 $\mu$ l
Proofreading polymerase	0.5 $\mu$ l
<b>Total</b>	<b>9 <math>\mu</math>l</b>

4. Close the cap and mix well. Centrifuge briefly to collect the contents at the bottom of the tube.
5. Incubate the tube at 70°C for 5 min.



70°C water bath

6. Cool tube on ice for 2 min.



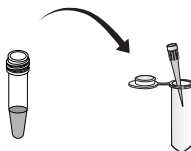
7. Once cool, centrifuge briefly to bring contents to the bottom of the tube and keep tube at room temperature.



8. Spin down stock tubes of pJet1.2 vector and the T4 DNA ligase to collect the contents at the bottom of the tube.

9. Set up a ligation reaction with the following reagents.

Reagent	Amount
Blunting reaction (already in microcentrifuge tube)	9.0 $\mu$ l
T4 DNA ligase	0.5 $\mu$ l
pJet1.2 blunted vector	0.5 $\mu$ l
<b>Total</b>	<b>10 <math>\mu</math>l</b>



10. Close the cap and mix well. Centrifuge briefly to collect the contents at the bottom of the tube.

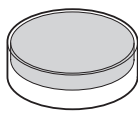


11. Incubate the tube at room temperature for 5–10 min.
12. Store the ligation at  $-20^{\circ}\text{C}$  or if proceeding directly to transformation step, store on ice.

# Transformation – Quick Guide

## Preparation for Competent Cells

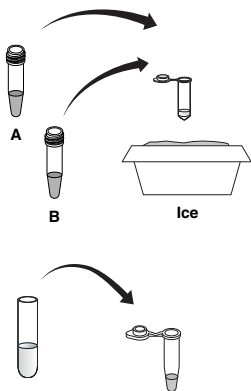
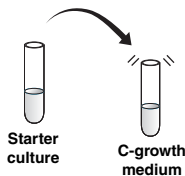
1. Label an LB Amp IPTG agar plate with your initials and place at 37°C.
2. If not already done, pipet 1.5 ml C-growth medium into a 15 ml culture tube. Label with your initials and warm to 37°C for at least 10 min. Also ensure your starter culture is shaking at 37°C.
3. Pipet 150 µl of fresh starter culture (inoculated yesterday) into the pre-warmed C-growth medium and place in shaking water bath at 37°C.
4. Label a 1.5 ml microcentrifuge tube with your initials and "competent cells."
5. Prepare transformation (TF) buffer by combining 250 µl of transformation reagent A and 250 µl of transformation reagent B into a tube labeled transformation buffer. Keep on ice.
6. After a 20–40 min incubation, transfer the actively growing culture in the C-growth medium to your competent cells microcentrifuge tube.



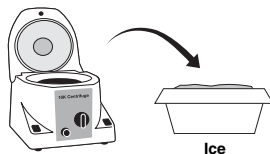
37°C incubator



37°C shaking  
water bath



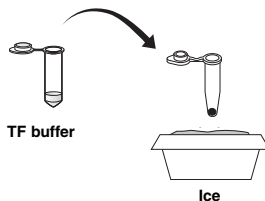
7. Centrifuge at top speed for one minute and immediately put tube on ice.



8. Use a 1000  $\mu$ l pipet or a vacuum source to remove culture supernatant avoiding the pellet. Keep the tube on ice.



9. Resuspend the bacterial pellet in 300  $\mu$ l of ice cold transformation buffer by very gently pipetting up and down in the solution above the pellet – do not touch the pellet.



10. Incubate the resuspended bacteria on ice for 5 min.

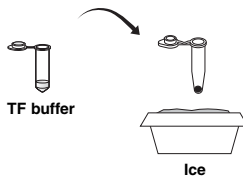
11. Centrifuge the bacteria at top speed for 1 min. Ensure the bacteria are on ice immediately prior to and immediately following centrifugation.



12. Using a 1000  $\mu$ l pipet or a vacuum source, remove the supernatant avoiding the bacterial pellet.



13. Very gently resuspend the bacterial pellet in 120  $\mu$ l of ice cold transformation buffer. Keep tube on ice.

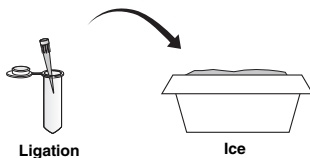


14. Incubate resuspended bacteria on ice for 5 min. The cells are now competent for transformation.

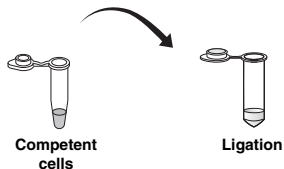
## Experimental Procedure for Transformation

15. Label one microcentrifuge tube with your initials and "transformation."

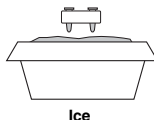
16. Pipet 5  $\mu$ l of your ligation reaction into your transformation microcentrifuge tube. Keep on ice.



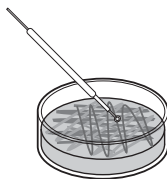
17. Using a fresh tip, very gently pipet the competent cells up and down two times then pipet 50  $\mu$ l of competent cells into your transformation tube. Very gently pipet up and down two times to mix and return to ice.



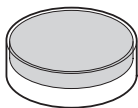
18. Incubate the transformation on ice for 10 min.



19. Retrieve the warm LP Amp IPTG agar plate from the 37°C incubator and pipet the entire volume of the transformation onto the labeled agar plate. Use an inoculation loop to very gently spread the bacteria around the plate — do not spread for more than 10 seconds.



20. Immediately place agar plate, upside down at 37°C and incubate overnight.



37°C incubator

21. The next day, analyze results or wrap plate in Parafilm and store at 4°C until required.

## Instructor's Advance Preparation

In the first part of this activity, students will insert (ligate) the purified PCR products into the pJet1.2 blunted vector. They will then transform competent bacteria with the ligation reaction mixture.

**Note:** Students can either proceed directly to transformation activity or store the ligation reaction at 4°C or –20°C until the next laboratory session.

**Note:** In order to complete the laboratory more efficiently, preparation for the transformation step can be initiated prior to performing the ligation reaction, allowing immediate transformation of competent cells with the products of the ligation reaction. Refer to **Tasks to Perform Prior to the Transformation Laboratory** on page 34 for details.

## Ligation Reaction

The pJet1.2 blunted vector is supplied ready to use, already opened, with blunt ends ready for ligation to PCR products. The pJet1.2 plasmid selects successful ligations through the disruption of an otherwise lethal gene, *eco47IR*, which enables positive selection of the recombinants. Before ligation, a 3'-A overhang must be removed from the PCR products by treating the PCR product with a proofreading DNA polymerase, leaving them with blunt ends ready to be ligated to the pJet1.2 blunted vector. This DNA polymerase is active at 70°C but not at lower temperatures, so it is not necessary to inactivate this enzyme after use. Once blunted, the PCR product is combined with the plasmid and T4 DNA ligase under conditions optimal for ligation. The ligation reaction is fast, complete in 5–10 min. Only a minimal increase in the number of transformants is gained by extending the ligation time beyond 10 min. Following ligation of a gene into a plasmid, students will perform transformation to introduce the plasmid into competent *E. coli* cells.

**Note:** Prior to starting this laboratory activity, students must have already performed a polymerase chain reaction (PCR) to amplify the DNA fragment they wish to clone and subsequently purify the PCR product to remove excess primers, nucleotides, and DNA polymerase, which would otherwise interfere with subsequent experiments.

**Note:** Components needed to carry out PCR reactions are not included in the Ligation and Transformation module. A kit that utilizes size exclusion chromatography, such as Bio-Rad's PCR Kleen™ Spin Purification module (catalog #732-6300EDU), can be used for purifying the PCR products. Once the purified PCR product is available, students may decide to electrophorese the PCR products to assess the quality and quantity of the sample. Electrophoresis of the purified sample is an optional activity and could be skipped.

## Tasks to Perform Prior to the Ligation Laboratory

1. Thaw the 2x ligation reaction buffer, proofreading polymerase, T4 DNA ligase, pJet1.2 blunted vector, purified PCR fragment, and sterile water and store on ice. Just before use, mix and centrifuge the reagents to collect contents at the bottom of the tubes. Do not aliquot the ligation reagents for students; the volumes required are too small. Have these reagents available at the common workstation for students. Also ensure students are familiar with methods to pipet small volumes.

## Transformation Laboratory

At this transformation step, students will transform bacteria with the ligation reaction. Following transformation, pJet1.2 enables positive selection of plasmids with the desired insert due to the disruption of an otherwise lethal gene, *eco47IR*, which allows growth of successful transformants. Bacterial transformation with ligation reactions is a very inefficient process (much more inefficient than transformation with plasmid DNA), so students should be encouraged to take special care during this protocol. There are many steps that if performed improperly can lead to reduced transformation efficiency or even no colonies. It is recommended that students perform a control transformation using 1 µl of 50–200 ng/µl of an ampicillin resistant control plasmid if available. (pGLO Plasmid (20 µg) (catalog #166-0405EDU) can be purchased separately and used as a control.) Each student team would require an additional LB Amp IPTG agar plate for their control transformation. **Note:** Each preparation of competent cells derives sufficient cells for two transformations.

The transformation protocol involves creating competent cells and immediately performing the transformation. Once made, the competent cells must be used immediately or discarded according to local regulations. They cannot be stored for later use. This transformation method permits

relatively high transformation efficiency ( $10^6$  transformants per  $\mu\text{g}$  DNA) without a requirement for a refrigerated centrifuge, commercial competent cells, or a  $-70^\circ\text{C}$  freezer to store the competent cells.

**Note:** Bio-Rad offers both chemically competent and electrocompetent cells for purchase should your teaching goals include electroporation or more traditional chemical transformation techniques. The commercially available cells require storage at  $-70^\circ\text{C}$  and have transformation efficiencies of  $10^9/\mu\text{g}$  DNA.

**Note:** The Ligation and Transformation module requires microbial culturing reagents, such as LB broth, LB agar, ampicillin, petri dishes, cell culture tubes, and inoculation loops, which are not included in this module. You may choose to purchase the Microbial Culturing module (catalog #166-5020EDU) for these components.

### Tasks to Perform Prior to the Transformation Laboratory

1. Prepare solid and liquid growth media at least 5 days prior to the transformation step. The Microbial Culturing module contains all reagents required for this activity, or alternatively the reagents may be prepared using standard protocols. (**Note:** Complete instructions for reagent preparation and streaking plates are available in the Microbial Culturing module instruction manual.) Each student team requires:  
1 LB agar plate for preparing starter colonies, 1–2 LB Amp IPTG agar plate with final concentrations of  $50\ \mu\text{g}/\text{ml}$  ampicillin and  $0.2\ \text{mM}$  IPTG for plating transformations (if performing a positive control transformation 2 plates will be required), 5 ml of LB broth for growing starter culture and 20 ml of LB Amp broth with final concentration of  $50\ \mu\text{g}/\text{ml}$  ampicillin for growing 4 minipreps. All reagents may be stored at  $4^\circ\text{C}$  for up to 1 month.
2. Inoculate growth media and culture cells
  - a. Streak an *E. coli* HB101 starter plate: At least **2 days** prior to the transformation, streak an *E. coli* culture appropriate for transformation on an LB agar plate using standard microbial techniques to allow formation of single colonies. If using the Microbial Culturing module, rehydrate the *E. coli* HB101 vial with  $250\ \mu\text{l}$  of sterile water and use  $10\ \mu\text{l}$  of the rehydrated bacteria to streak plates. Incubate plate at  $37^\circ\text{C}$  overnight. Once colonies have grown, wrap plate in Parafilm and store at  $4^\circ\text{C}$  for up to 2 weeks.

- b. Prepare starter culture: As late as possible the **day before** the transformation, inoculate a 2–5 ml LB culture with a starter colony from the *E. coli* starter plate. Incubate cultures in a shaking water bath or incubator **overnight** at 37°C and at least 200 rpm.

**Note:** It is important to use a fresh starter culture (<24 hours since inoculation) for the transformation, or transformation efficiency will be reduced.

**Note:** If a shaking water bath or incubator is not available, incubate starter cultures at 37°C 1 day prior to the transformation, manually shaking the vial containing the culture as frequently as possible to oxygenate the culture (a reduction in transformation efficiency may be observed).

3. Place 1 LB Amp IPTG agar plate per student team in 37°C incubator just before the transformation lab. **Note:** If performing a positive control transformation, a second plate per student team will be needed.
4. Pipet 1.5 ml of C-growth medium to one 15 ml culture tube per team and incubate at 37°C for at least 10 min prior to the transformation laboratory.

# Student Ligation Protocol

**Note:** Before use, the appropriate reagents must be defrosted, thoroughly mixed, and centrifuged to collect contents at the bottom of the tubes. Refer to **Tasks to Perform Prior to the Ligation Laboratory** on page 33 for details.

Listed below are materials and reagents required at the workstations prior to starting the ligation activity.

Instructor's (Common) Station	Quantity	(✓)
-------------------------------	----------	-----

Ice bucket containing stock tubes of:

- |                               |        |                          |
|-------------------------------|--------|--------------------------|
| o 2x ligation reaction buffer | 100 µl | <input type="checkbox"/> |
| o Proofreading polymerase     | 10 µl  | <input type="checkbox"/> |
| o T4 DNA ligase               | 10 µl  | <input type="checkbox"/> |
| o pJet1.2 blunted vector      | 10 µl  | <input type="checkbox"/> |
| o Sterile water               | 1 ml   | <input type="checkbox"/> |

Water bath, heating block, or incubator at 70°C	1	<input type="checkbox"/>
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Microcentrifuge (refrigerated, if available)	1	<input type="checkbox"/>
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Materials Required at Each Student Station	Quantity	(✓)
--	----------	-----

Purified PCR product	1–2 µl	<input type="checkbox"/>
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1.5 ml microcentrifuge tube	1	<input type="checkbox"/>
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10 µl adjustable-volume micropipet and tips	1	<input type="checkbox"/>
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Ice bucket	1	<input type="checkbox"/>
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Marking pen	1	<input type="checkbox"/>
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## Setting Up the Blunting Reaction

*This reaction removes the 3' nucleotide overhang left by the Taq DNA polymerase that would prevent blunt end ligation.*

1. Label a microcentrifuge tube with your initials, the name of your amplified gene, and "ligation."
2. Pulse spin the stock tubes containing the ligation reaction buffer and proofreading polymerase in a microcentrifuge for 10 sec to force contents to bottom of tubes prior to use.

**Note:** When pipetting very small volumes, take special care. When pulling up reagents, make sure only the soft stop of the pipet is used even though it may feel like a very small movement. Also, look at the end of the pipet tip and make sure that the correct volume of reagent is in the tip. After adding the reagent to the tube, be sure that the pipet tip is empty. Never reuse a pipet tip.

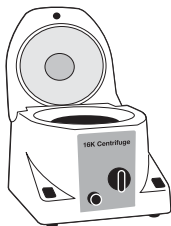
3. Set up a blunting reaction with the following reagents:

Reagent	Amount
2x ligation reaction buffer	5.0 $\mu$ l
Purified PCR product	1.0 $\mu$ l
Sterile water	2.5 $\mu$ l
Proofreading polymerase	0.5 $\mu$ l
<b>Total</b>	<b>9 <math>\mu</math>l</b>

**Note:** The amount of PCR product added to the reaction may be increased to 2  $\mu$ l if the PCR product is not very intense when analyzed on an agarose gel — for example if it is not as intense as the 1 kb band in the molecular weight marker. If the amount of PCR product is increased, remember to decrease the volume of sterile water to 1.5  $\mu$ l to compensate and keep the total volume of 9  $\mu$ l.

4. Close the cap and mix well. Centrifuge in a microcentrifuge for 10 sec to collect the contents at the bottom of the tube.

*This step is essential due to the very small volume used in this reaction.*



5. Place the tube at 70°C for 5 min.  
6. Place tube on ice to cool for 2 min.

*This recondenses water vapor to maintain reaction volume.*

7. Once cool, centrifuge the tube briefly to collect the contents at the bottom of the tube. Place tube at room temperature.

### Setting Up the Ligation Reaction

*This reaction inserts the PCR product into the pJet1.2 plasmid vector.*

8. Briefly centrifuge the stock tubes containing the pJet1.2 blunted vector and the T4 DNA ligase in a microcentrifuge to force the contents to bottom of tubes prior to using.  
9. Setup a ligation reaction with the following reagents:

Reagent	Amount
Blunting reaction (already in microcentrifuge tube)	9.0 $\mu$ l
T4 DNA ligase	0.5 $\mu$ l
pJet1.2 blunted vector	0.5 $\mu$ l
<b>Total</b>	<b>10 <math>\mu</math>l</b>

10. Close the cap and mix well. Centrifuge briefly in a microcentrifuge to collect the contents at the bottom of the tube.



11. Incubate tube at room temperature for 5–10 min.
12. Store the ligation reaction at  $-20^{\circ}\text{C}$ . If you are proceeding directly to transformation, pipet 5  $\mu\text{l}$  of the ligation reaction into a microcentrifuge tube labeled with your initials, the name of your gene, and "transformation," and store it on ice until needed for the transformation.

## Student Transformation Protocol

Listed are materials and reagents required at the workstations prior to beginning the transformation activity.

<b>Instructor's (Common) Workstation</b>	<b>Quantity</b>	<b>(✓)</b>
Water bath, heating block, or incubator (37°C)	1	<input type="checkbox"/>
Microcentrifuge (refrigerated, if available)	1	<input type="checkbox"/>

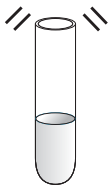
Each student team will require the following items to transform bacteria with one PCR product.

<b>Materials Required at Each Student Station</b>	<b>Quantity/Team</b>	<b>(✓)</b>
Ligation reaction from ligation laboratory (on ice)	5 µl	<input type="checkbox"/>
(Optional) Control plasmid 50–200 ng/µl (on ice)	1 µl	<input type="checkbox"/>
1.5 ml microcentrifuge tubes	4	<input type="checkbox"/>
15 ml culture tube containing 1.5 ml of C-growth medium	1	<input type="checkbox"/>
Transformation reagent A (on ice)	250 µl	<input type="checkbox"/>
Transformation reagent B (on ice)	250 µl	<input type="checkbox"/>
Sterile inoculating loops	2	<input type="checkbox"/>
Fresh LB Amp IPTG agar plate prewarmed to 37°C	1–2	<input type="checkbox"/>
Fresh starter culture plate of <i>E. coli</i> HB101	1	<input type="checkbox"/>
200 µl adjustable-volume micropipet and tips	1	<input type="checkbox"/>
1,000 µl adjustable-volume micropipet and tips	1	<input type="checkbox"/>
Marking pens	1	<input type="checkbox"/>
Parafilm sealing film	1 strip	<input type="checkbox"/>
(Optional) Vortex mixer	1	<input type="checkbox"/>
(Optional) Vacuum source	1	<input type="checkbox"/>

## Detailed Protocol for Transformation

### Preparation of Competent Cells

1. Approximately 20–40 min prior to starting the transformation, prepare competent cells by pipeting 150  $\mu$ l of fresh starter culture (inoculated one day prior) into the prewarmed C-growth medium and placing in a shaking 37°C water bath or incubator for 20–40 min.



**Note:** If a shaking waterbath is not available, manually shake the culture tubes every 5 min during the 20–40 min growth phase to oxygenate the culture.

**Note:** Your instructor may have already completed this step to save time.

2. Label 1–2 LB Amp IPTG agar plates with your initials. Also label one agar plate for your ligation (pJet + your insert name) and if you are performing a positive control transformation label the second agar plate for the positive control plasmid.

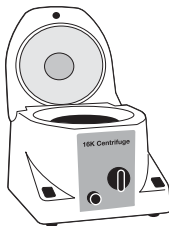
Place agar plates at 37°C.

3. If not already done, pipet 1.5 ml of C-growth medium to the 15 ml culture tube. Label tube with your initials and warm it at 37°C for at least 10 min.

Also ensure that the *E. coli* starter culture is at 37°C.

4. Label a microcentrifuge tube with your initials and "competent cells".
5. Prepare the transformation buffer by combining 250  $\mu$ l of transformation reagent A and 250  $\mu$ l of transformation reagent B into a microcentrifuge tube labeled "TF buffer" and mix thoroughly with a vortex mixer (if available). Keep on ice until use. (**Note:** This mixture must be used on the day of preparation.)

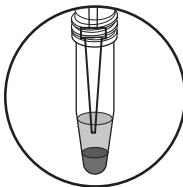
6. After bacteria have grown in C-growth medium for 20–40 min at 37°C with shaking, transfer the culture to your competent cells tube by decanting or pipetting it. It is better not to put the actively growing cell culture on ice at this step.
7. Centrifuge the culture in a microcentrifuge at top speed for 1 min. Make sure that the microcentrifuge is balanced and accommodate tubes of classmates to ensure economic use of the microcentrifuge. Immediately put the pelleted culture on ice.



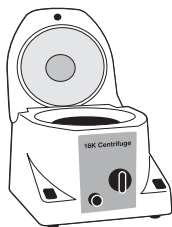
**Note:** After this step, it is very important to keep the bacteria on ice as much as possible during this procedure. Transformation efficiency will be severely compromised if the cells warm up.

**Note:** It is very important to treat the bacteria extremely gently during this procedure — the bacteria are very fragile and your transformation efficiency will be compromised unless you are very gentle.

8. Locate the pellet of bacteria at the bottom of the tube. Remove the culture supernatant, avoiding the pellet, using a 1,000  $\mu$ l pipet or a vacuum source. Keep the cells on ice.



9. Resuspend the bacterial pellet in 300  $\mu$ l of ice-cold transformation buffer by gently pipetting up and down in the solution above the pellet with a 1,000  $\mu$ l pipet, and gradually wear away the pellet from the bottom of the tube. Make sure that the bacteria are fully resuspended, with no clumps. Avoid removing the cells from the ice bucket for more than a few seconds.
10. Incubate the resuspended bacteria on ice for 5 min.
11. Centrifuge the bacteria in a microcentrifuge for 1 min. **Note:** Ensure that the bacteria are on ice immediately prior to and immediately following centrifugation. If the centrifuge is not close to the laboratory bench, take the entire ice bucket to the microcentrifuge so that the bacteria are only out of the ice bucket for 1 min. Use a refrigerated microcentrifuge, if available.



12. Remove the supernatant from the pellet using a 1,000  $\mu$ l pipet or vacuum source.
13. Resuspend the bacterial pellet in 120  $\mu$ l of ice-cold transformation buffer by gently pipetting up and down with a 200  $\mu$ l pipet. Be sure that bacteria are fully resuspended with no clumps. Avoid removing the cells from the ice bucket for more than a few seconds.
14. Incubate the resuspended bacteria on ice for 5 min.

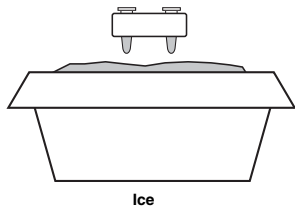
*The cells are now competent for transformation.*

### **Experimental Procedure for Transformation**

15. Label one microcentrifuge tube with your initials, insert name, and "TF" (referred to below as the "gene TF" tube).

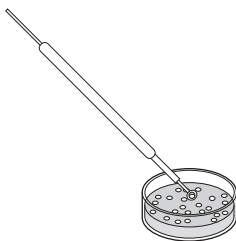
**Note:** If you are performing the ligation and transformation steps on the same day, use the microcentrifuge containing 5  $\mu$ l of the ligation mixture that was prepared and labeled at the end of the ligation step.

16. If not already done, pipet 5  $\mu$ l of the ligation reaction from the previous stage into the "gene TF" microcentrifuge tube. Store any remaining ligation reaction at 4°C or -20°C.
17. (Optional) If performing a positive control transformation, pipet 1  $\mu$ l of the positive control plasmid into an appropriately labeled microcentrifuge tube and keep on ice.
18. Using a fresh tip, pipet 50  $\mu$ l of competent bacteria directly into the ice-cold "gene TF" tube containing 5  $\mu$ l of your ligation and gently pipet up and down two times to mix.
19. (Optional) If performing a positive control transformation, pipet 50  $\mu$ l of competent bacteria directly into the ice-cold positive control plasmid tube and gently pipet up and down two times to mix.



20. Incubate the transformations for 10 min on ice.
21. Retrieve LB Amp IPTG agar plates from the 37°C incubator.
22. Pipet the entire volume of each transformation onto the corresponding labeled LB Amp IPTG agar plate, and using an inoculation loop or a sterile spreader, very gently spread the bacteria around the plate — remember that the bacteria are still very fragile! Once the plate is covered, stop spreading. Do not spread for more than 10 sec.

It is vital that the LB Amp IPTG agar plate be warm at this step to ensure sufficiently high transformation efficiency. Spreading the plate until it is dry will also reduce transformation efficiency.



23. Immediately place LB Amp IPTG agar plates upside down in the 37°C incubator and incubate them overnight.
24. The next day, analyze the results, or wrap the plates in Parafilm and place them at 4°C until required for inoculation of miniprep cultures (see Appendix A).

### **Analysis of Results of Ligation and Transformation**

Count the number of bacterial colonies that grew on the LB Amp IPTG agar plates.

**Note:** Occasionally, satellite colonies may grow using this ligation method. Count the large individual colonies, not the tiny colonies surrounding larger colonies.

<b>Transformation</b>	<b>Number of Colonies</b>
Ligation of gene of interest	
Control plasmid	

**Note:** If the number of colonies is very high and uncountable, enter "TNC" for "too numerous" to count in the results table.

## Appendix A

### Inoculating a Bacterial Colony for Plasmid Miniprep

Once the plasmid has been introduced into living bacterial cells and the cells have grown and divided on selective medium, the next step in the experiment is to prepare a miniprep of the plasmid DNA for sequencing or further experiments. The three steps are: 1) growing cells in liquid culture; 2) purifying the plasmid DNA from the culture; and 3) performing a restriction digest on the purified DNA to determine if the DNA insert in the plasmid is the expected size. To start the liquid culture, cells from an isolated bacterial colony are placed in selective medium (nutrient broth with antibiotic). It is important to choose an isolated single colony from the plate, so that the liquid culture will contain cells that all have the same plasmid. If cells from more than one colony are used for inoculation, the miniprep may contain multiple plasmids and the DNA may not be used for sequencing or further experiments.

Before proceeding to the miniprep protocol, grow transformed bacterial colonies in liquid cultures using the following instructions:

1. Prepare 25 ml of LB Amp broth (if not already prepared; refer to instructions in the Microbial Culturing module, catalog #166-5020EDU).
2. Label four 15 ml culture tubes with your initials and “pJet”, the name of your gene, and #1 through #4.
3. Using sterile technique, pipet 5 ml of LB Amp broth into each of the four 15 ml culture tubes.
4. One day prior to the next laboratory session, use a sterile loop or a sterile pipet tip to pick a single colony from the LB Amp IPTG agar plate containing the plated bacteria transformed with your ligation reaction. Inoculate an LB Amp culture tube with the colony. Repeat for a total of four miniprep cultures from four individual colonies.

**Note:** Occasionally satellite colonies may grow using this ligation method. Pick the large individual colonies, not the tiny colonies surrounding larger colonies. Be sure that a single colony is picked, or you may isolate multiple plasmids from your miniprep and these cannot be sequenced.

5. Place the miniprep cultures to grow in a shaking incubator or water bath at 37°C overnight.

**Note:** If no colonies grew on your team's agar plate from the pJet1.2 + gene ligation reaction, use colonies from another team's successful transformation. Relabel your 15 ml culture tubes accordingly.

6. Perform a miniprep using the Aurum™ Plasmid Mini Purification module (catalog #732-6400EDU).

## **Appendix B**

### **Restriction Digestion of Plasmid DNA with Bgl II Enzyme**

#### **Instructors Advanced Preparation**

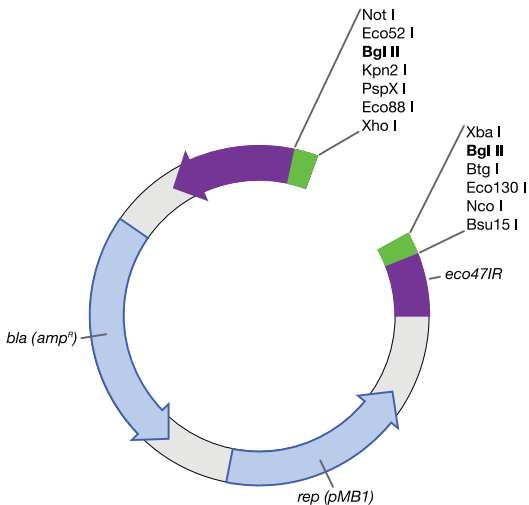
The Ligation and Transformation module contains Bgl II enzyme and reaction buffer to enable analysis of plasmids derived using the module and subsequently purified using a plasmid purification protocol. Electrophoresis reagents, including sample loading dye and a molecular weight ruler are also required for the analysis. The pJet1.2 plasmid vector is 2,974 bp in length, thus after restriction digestion of plasmids and subsequent electrophoresis a band of around 3 kb and a smaller band corresponding to the size of the PCR product insert is expected.

To digest and analyze four plasmids, each student team requires:

<b>Item</b>	<b>Qty</b>
Microcentrifuge tubes	4–8
Bgl II restriction enzyme	4 $\mu$ l
10x Bgl II reaction buffer	8 $\mu$ l
Sterile water	30 $\mu$ l
1% agarose gel	1
Electrophoresis running buffer (sufficient to fill electrophoresis chamber)	
Sample loading dye, 5x	50 $\mu$ l
Molecular weight ruler (10 $\mu$ l of EZ load molecular ruler per gel is recommended)	
2–20 $\mu$ l adjustable volume pipet and tips	1

#### **Background**

Once transformed bacteria are grown overnight in a liquid culture (see Appendix A), a miniprep is performed to purify the plasmid DNA from the bacteria. Before proceeding with further experiments using the purified plasmid DNA, it is important to verify that the isolated plasmid contains the insert of interest. During the ligation stage, a gene of interest was ligated into the pJet1.2 blunted vector, which contains a Bgl II restriction enzyme recognition site on either side of the insertion site. To determine the size of the fragment inserted, the purified plasmid can be analyzed by restriction enzyme digestion with Bgl II enzyme and subsequent agarose gel electrophoresis.



By digesting a small portion of the miniprep DNA with Bgl II enzyme (see instructions below), the insert should be cut out of the vector. Running the products of the restriction digestion on an agarose gel should give two DNA bands, one the size of the vector (2,974 bp) and the other the size of the inserted DNA (the original PCR product). If there are more than two bands in any digest, it may mean that the insert contains a Bgl II site. Does the size of the excised bands add up to the size of the original PCR fragment? Alternatively, two similarly sized fragments may indicate that a mixed culture was used to start the miniprep, instead of an isolated colony.

### Preparation for Restriction Digestion Analysis

1. Label one microcentrifuge tube with your initials and "Bgl II master mix."
2. The restriction digestion reactions will be performed on each miniprep in a final volume of 20  $\mu$ l with 10  $\mu$ l of plasmid DNA.
3. Prepare a master mix for Bgl II restriction enzyme digestion according to the following table, using stock reagents from the common workstation. Use a fresh tip for each reagent.

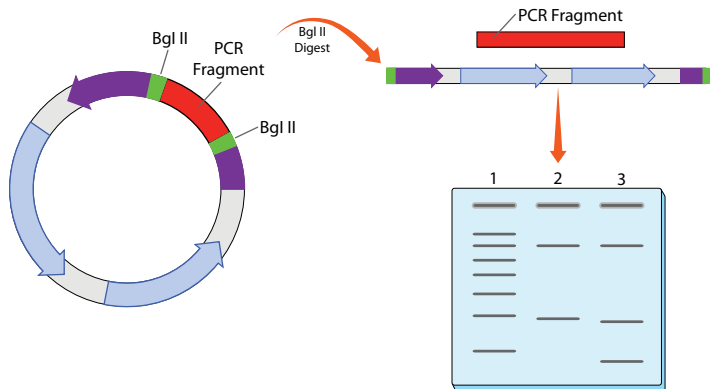
<b>Reagent</b>	<b>Volume for 1 Reaction</b>	<b>Volume for 5 Reactions</b>
10x Bgl II reaction buffer	2 $\mu$ l	10 $\mu$ l
Sterile water	7 $\mu$ l	35 $\mu$ l
Bgl II restriction enzyme	1 $\mu$ l	5 $\mu$ l
<b>Total</b>	<b>10 <math>\mu</math>l</b>	<b>50 <math>\mu</math>l</b>

## **Experimental Procedure for Restriction Digestion Analysis**

1. Label a microcentrifuge tube for each plasmid miniprep.
2. Prepare digestion reactions by combining 10  $\mu$ l of the Bgl II master mix and 10  $\mu$ l of each plasmid DNA in the appropriately labeled microcentrifuge tubes.
3. Mix tube components and spin briefly in a microcentrifuge to collect the contents at the bottom of the tube.
4. Incubate reactions at 37°C for 1 hr. If the reactions will not be analyzed by agarose gel electrophoresis immediately, store them at -20°C until analysis.

## **Preparation for Electrophoresis**

1. Place a 1% agarose gel in the electrophoresis chamber and add electrophoresis running buffer to just cover the gel.
2. Plan your gel electrophoresis experiment.
3. Add the appropriate amount of sample loading dye to the DNA samples and pipet up and down to mix.
4. Load 20  $\mu$ l of the restriction digestion reactions on the gel according to your plan and load an appropriate amount of molecular weight ruler in a separate lane.
5. (Optional) It is recommended that undigested DNA (at the same dilution as your restriction digests) also be run next to your digested samples.
6. Electrophorese samples at 100 V for 30 min and analyze results.



**Restriction enzyme digestion analysis of plasmid DNA.** Circular plasmid DNA purified from bacterial minipreps is isolated and digested with Bgl II, a restriction enzyme producing at least two linearized fragments — vector DNA and PCR fragment (lane 2). These fragments can be visualized using agarose gel electrophoresis. If the PCR fragment contains a Bgl II restriction site, three DNA bands may be observed (lane 3). A 500 bp molecular weight ruler is shown in lane 1.

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