

Edvo-Kit #

301

Edvo-Kit #301

Construction and Cloning of a Recombinant DNA

Experiment Objective:

In this experiment, students will assemble and analyze DNA molecules *in vitro* using several recombinant DNA techniques, including gene cloning, plasmid extraction, and restriction enzyme analysis.

See page 3 for storage instructions.

Version 301.220315

PROTOCOL HAS BEEN UPDATED!
Please review before beginning experiment!

EDVOTEK®

Table of Contents

	Page
Experiment Components	3
Experiment Requirements	4
Background Information	5
Experiment Procedures	
Experiment Overview and Laboratory Safety	11
Module I: Ligation of the Plasmid Vector to the kan ^R Gene Fragment	13
Module II: Transformation of the Recombinant DNA into <i>E.coli</i>	18
Module III: Culturing of kan ^R Transformants	22
Module IV: Extraction of Recombinant Plasmid DNA	24
Module V: Restriction Enzyme Analysis	27
Study Questions	30
Instructor's Guidelines	
Overview of Pre-lab Preparations	31
Pre-Lab Preparations	
Module I Preparations	32
Module II Preparations	34
Module III Preparations	37
Module IV Preparations	38
Module V Preparations	39
Experiment Results and Analysis	40
Study Questions and Answers	44
Appendices	45
A EDVOTEK® Plasmid Map	46
B EDVOTEK® Troubleshooting Guide	47
C Bulk Preparation of Agarose Gels	51
D Data Analysis Using a Standard Curve	52

Safety Data Sheets can be found on our website: www.edvotek.com/safety-data-sheets

EDVOTEK, The Biotechnology Education Company, InstaStain, and Dryzymes are registered trademarks of EDVOTEK, Inc. ReadyPour, BactoBeads, and Ultra-Spec Agarose are trademarks of EDVOTEK, Inc. SYBR is a registered trademark of Thermo Fisher Scientific.



1.800.EDVOTEK • Fax 202.370.1501 • info@edvotek.com • www.edvotek.com

Duplication of any part of this document is permitted for non-profit educational purposes only. Copyright © 2022 EDVOTEK, Inc., all rights reserved. Version 301.220315

Experiment Components

MODULE I - DNA LIGATION

	Storage	Check (✓)
A Ultra-pure Water	-20 °C Freezer	<input type="checkbox"/>
B DNA Fragments for Ligation (Linear vector and kan ^R gene)	-20 °C Freezer	<input type="checkbox"/>
C T4 DNA Ligase in Reaction Tube	-20 °C Freezer	<input type="checkbox"/>

Experiment #301 is designed for 5 groups of 2-4 students.

MODULE II - TRANSFORMATION

D Kanamycin Sulfate	-20 °C Freezer	<input type="checkbox"/>
E1 Supercoiled Control DNA Sample 1	-20 °C Freezer	<input type="checkbox"/>
E2 Supercoiled Control DNA Sample 2	-20 °C Freezer	<input type="checkbox"/>
E3 Supercoiled Control DNA Sample 3	-20 °C Freezer	<input type="checkbox"/>
F JM109 BactoBeads™	-20 °C Freezer	<input type="checkbox"/>
G 0.05 M CaCl ₂	-20 °C Freezer	<input type="checkbox"/>
• ReadyPour™ LB Agar	Room Temperature	<input type="checkbox"/>
• Recovery Broth	Room Temperature	<input type="checkbox"/>

IMPORTANT NOTE:
Storage conditions for some components have changed. Please carefully review storage conditions after receiving the kit.

MODULE III - GROWTH OF kan^R TRANSFORMANTS

• Growth Medium	Room Temperature	<input type="checkbox"/>
-----------------	------------------	--------------------------

NOTE:
Some components will be used in multiple modules.

Three new control plasmids are included. See page 37 for more information.

MODULE IV - PLASMID EXTRACTION

H Tris-EDTA-Glucose Buffer (TEG)	4 °C Refrigerator	<input type="checkbox"/>
I 2M NaOH	4 °C Refrigerator	<input type="checkbox"/>
J 10% SDS	4 °C Refrigerator	<input type="checkbox"/>
K RNase (DNase-free)	4 °C Refrigerator	<input type="checkbox"/>
L Tris-EDTA buffer	4 °C Refrigerator	<input type="checkbox"/>
M Acidified Potassium Acetate	4 °C Refrigerator	<input type="checkbox"/>

NEW
Troubleshooting Guides see pages 47-50.

MODULE V - RESTRICTION ENZYME ANALYSIS

N Ultra-pure Water for Restriction Digests	-20 °C Freezer	<input type="checkbox"/>
O 10X Restriction Enzyme Reaction Buffer	-20 °C Freezer	<input type="checkbox"/>
P Dryzymes® Rehydration Buffer	-20 °C Freezer	<input type="checkbox"/>
Q DNA Standard Marker	-20 °C Freezer	<input type="checkbox"/>
R Supercoiled Plasmid Vector Standard	-20 °C Freezer	<input type="checkbox"/>
S EcoRI Dryzymes®	-20 °C Freezer	<input type="checkbox"/>
T PvuII Dryzymes®	-20 °C Freezer	<input type="checkbox"/>
U XhoI Dryzymes®	-20 °C Freezer	<input type="checkbox"/>

All experiment components are intended for educational research only. They are not to be used for diagnostic or drug purposes, nor administered to or consumed by humans or animals.

Reagents and Supplies *(included with this kit)*

Store all components below at room temperature.

- SYBR® Safe DNA Stain
- 10x Gel loading solution
- 50x Electrophoresis buffer
- UltraSpec-Agarose™
- Screw cap tubes (sterile)
- Microtest tubes (0.5 mL)
- 1 mL pipets (sterile)
- Petri plates (sterile, 60 x 15 mm)
- 1.5 mL microcentrifuge tubes
- Sterile inoculating loops/needles
- 10 mL pipets (sterile)
- Sterile 50 mL culture tubes
- Petri Plates (sterile, 100 x 15 mm)

Requirements *(not included with this kit)*

- Horizontal gel electrophoresis apparatus
- DC power supply
- Waterbath, 37 °C and 42 °C (EDVOTEK [Cat. # 539](#) highly recommended)
- UV transilluminator or blue light visualization system ([Cat. #557](#) recommended)
- UV safety goggles
- Microcentrifuge (maximum speed should be 10,000Xg or greater)
- Table top, clinical centrifuge or floor model centrifuge
- 37 °C incubation oven
- Shaking incubator or shaking waterbath
- Automatic micropipets (5-50 µL, 20-200 µL 100-1000 µL) with sterile pipet tips
- Pipet pump
- Balance
- Microwave or hot plate
- Hot gloves
- Disposable laboratory gloves
- 95-100% ethanol and isopropyl alcohol
- Distilled or deionized water
- Ice buckets and ice
- Bleach solution or laboratory disinfectant



Background Information

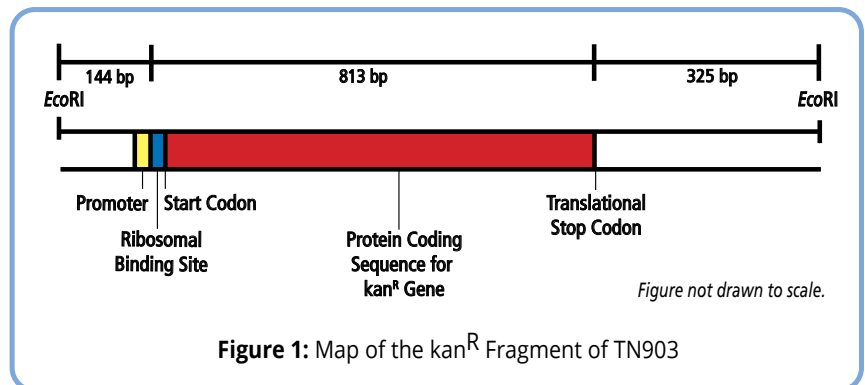
CLONING AND CONSTRUCTION OF RECOMBINANT DNA

After James Watson and Francis Crick published the structure of the double helix, scientists around the world raced to unlock the secrets encoded in our DNA. However, they found it difficult to study the properties of an individual gene because a single chromosome can contain hundreds of genes. As such, gene cloning techniques were developed to isolate, combine, and reproduce specific DNA sequences. First, using an enzyme called a restriction endonuclease, the DNA could be cut into smaller pieces. Then, a second enzyme called DNA ligase was used to ligate, or link, the DNA fragment with a bacterial plasmid. Finally, the hybrid DNA molecules were forced into bacteria, where they can be reproduced to create millions of copies of the starting DNA sequence. The development of recombinant DNA technology launched the era of biotechnology by making DNA mapping, sequencing and various genome-wide studies possible.

In this exploration, students will assemble and analyze recombinant DNA molecules using the same gene cloning techniques used in research laboratories. First, students will ligate the Kanamycin resistance (kan^R) gene into a plasmid vector. The modified plasmid is transformed into *E. coli*, which is then plated on media containing Kanamycin, and allowed to grow overnight. Because the newly-constructed plasmid contains the kan^R gene, only successfully transformed cells should grow into colonies. However, the plasmid's presence in the cells does not tell us anything about the orientation or the number of Kanamycin inserts present within the vector. To do this, the plasmid within a single transformant is grown, purified, and analyzed using restriction digestion and agarose gel electrophoresis.

CREATING RECOMBINANT DNA

Kanamycin is an aminoglycoside antibiotic that binds to the 30S subunit of the prokaryotic ribosome, preventing the correct translation of the messenger RNA. The Kanamycin resistance enzyme, an aminoglycoside phosphotransferase, inactivates the antibiotic through covalent modification. Once modified, the drug can no longer bind to the ribosome. Molecular biologists identified the gene for Kanamycin resistance as part of the *E. coli* TN903 transposon. The 1282 base pair fragment of the transposon used for this experiment includes the following features (summarized in Figure 1):



1. **Promoter:** a DNA sequence located just before (or “upstream” of) the coding sequence of a gene. Promoter sequences recruit RNA polymerase to the start of the gene sequence where it can begin transcription.
2. **Ribosomal Binding Sequences:** A short sequence that recruits ribosomes to a given mRNA.
3. **Start codon** – A sequence (ATG) that signals the start of translation.
4. **The protein coding sequence for the kan^R gene.** This 813-base pair DNA sequence will be transcribed into the mRNA that will be translated into a 30-kD protein consisting of 271 amino acid residues.
5. **Stop codon** – A specific sequence (TAG, TAA, TGA) that signals the end of translation. The stop codon is located 325 base pairs upstream of the nearest *EcoRI* site (see Figure 1).

In this experiment, we will be ligating the kan^R gene to a small, circular piece of bacterial DNA known as a plasmid. In nature, plasmids allow bacteria to exchange beneficial genes. In the laboratory, specialized bacterial plasmids called vectors are used to introduce DNA from different sources into bacteria. The vector used for this experiment, a 3000-base pair plasmid derived from the pUC series of vectors, contains the following three features that make it useful for our experiment (Figure 2):

1. **Origin of Replication:** a DNA sequence that allows bacteria to copy the plasmid.
2. **Multiple Cloning Site (or MCS):** a short DNA sequence that contains many unique restriction enzyme sites and allows scientists to insert specific genes into the plasmid.
3. **Selectable marker:** a gene that codes for resistance to a specific antibiotic (in pUC plasmids, ampicillin). When using selective media, only cells containing the marker should grow into colonies. This allows researchers to identify transformed cells.

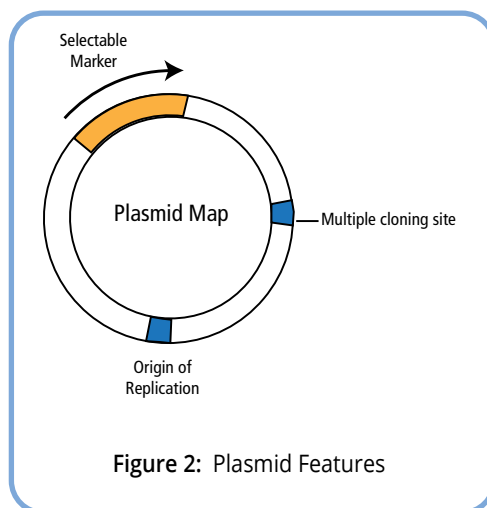


Figure 2: Plasmid Features

To link the kan^R gene into this vector, both pieces of DNA are cut with an enzyme known as a restriction endonuclease. These endonucleases (also known as restriction enzymes) act like molecular scissors, cutting double-stranded DNA at specific sequences. Many restriction enzymes recognize and cut palindromic stretches of DNA, generally 4-8 base pairs in length. Digestion by a restriction enzyme generates DNA fragments with one of two types of DNA ends -- "blunt" or "sticky" (Figure 3). To illustrate this, first consider the recognition site and cleavage pattern of *HaeIII*. This enzyme cuts both DNA strands at the same position, between the G and the neighboring C, which generates fragments without an overhang. These so-called "blunt" ends can be joined with any other blunt end.

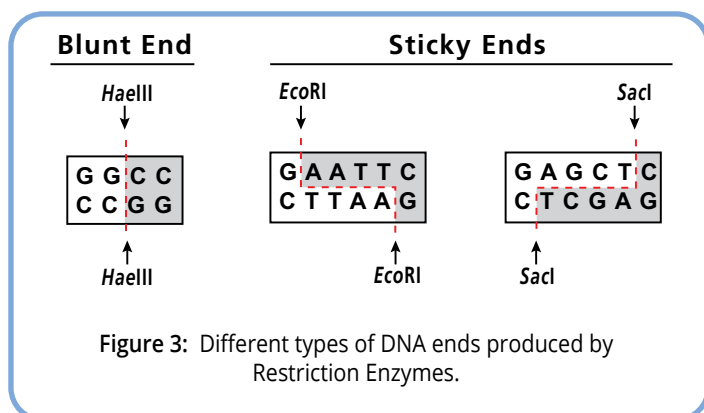


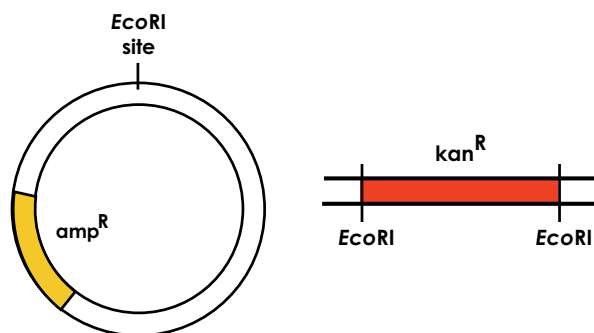
Figure 3: Different types of DNA ends produced by Restriction Enzymes.

In contrast to *HaeIII*, *EcoRI* cleaves between the G and neighboring A, as indicated by the arrows in the right side of the figure. It is important to note that the positions of the cleavage are staggered, so the resulting fragments project short overhangs of single-stranded DNA with complementary sequences. Such overhangs are referred to as "sticky" ends because the single-strands can interact with—or stick to—other overhangs with a complementary sequence. Digestion of the same piece of DNA using different enzymes can produce sticky ends of different lengths and strand orientation (5' vs. 3').

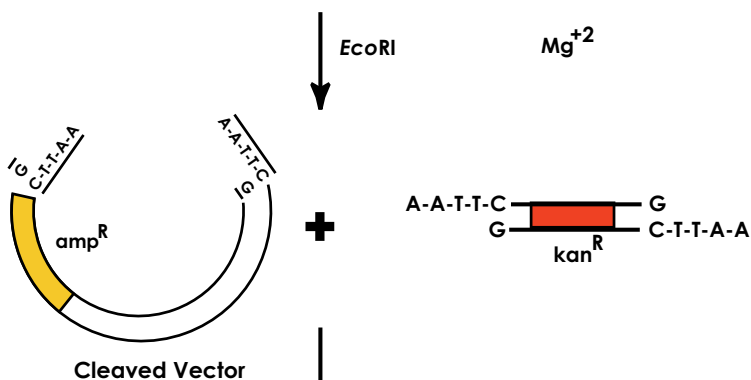
To create compatible sticky ends for our ligation, both pieces of DNA (the pUC vector and the kan^R insert) are cut with the same restriction enzyme – *EcoRI*. This enzyme cuts once within the multiple cloning site of our vector, creating a linear piece of DNA with sticky *EcoRI* overhangs at each end (Figure 4A). *EcoRI* overhangs are also created at each end of the kan^R fragment when digested with the enzyme.

After the DNA fragments are cut, they are linked together *in vitro* (Figure 4B). First, the unpaired nucleotides at the ends of the DNA fragments base-pair to one another, creating a circular DNA molecule. Then, DNA ligase catalyzes the formation of a phosphodiester bond between the 5' phosphate at the end of one fragment and 3' hydroxyl group at the end of the other fragment (Figure 4C). ATP is added to the reaction to supply the required energy for bond formation (Figure 4D). Although DNA ligase is most active at 37 °C, ligation of DNA fragments with cohesive termini is usually performed between 4 °C and 22 °C. Lower temperatures allow for a more stable interaction between the sticky ends.

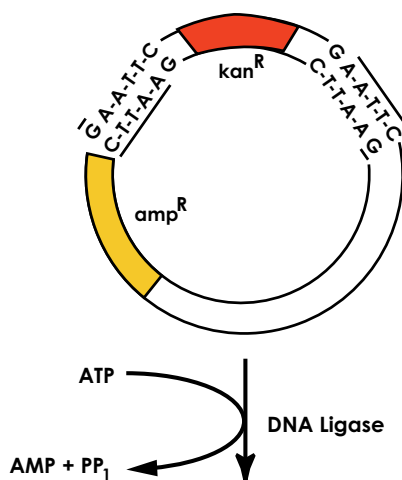
A. Restriction Digest of Plasmid and Insert



B. Base-pairing between plasmid and insert



C. Ligation



D. Recombinant DNA molecule

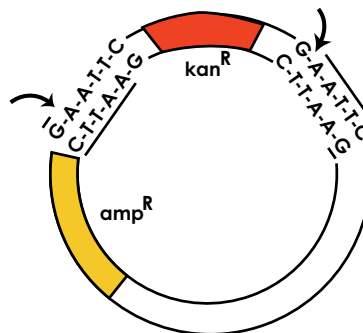


Figure 4: Creation of a Recombinant DNA Molecule.

Once the two pieces of DNA are ligated together, we have created a recombinant DNA molecule. Ideally, a single kan^R insert is ligated to the vector in the correct orientation, creating a 4282-base pair plasmid. In practice, the ligation is not limited to one insert per vector -- many combinations and orientations between vector and insert can be created using the two pieces of DNA (summarized in Figure 5). Because the vector is cut with one enzyme, the ends of the plasmid can self-ligate. Furthermore, a vector molecule can link to another vector instead of the insert, forming a long linear chain of plasmid DNA called a concatemer. The kan^R fragment may also circularize or concatemerize; however, since this sequence does not contain an origin of replication, it cannot reproduce in bacteria.

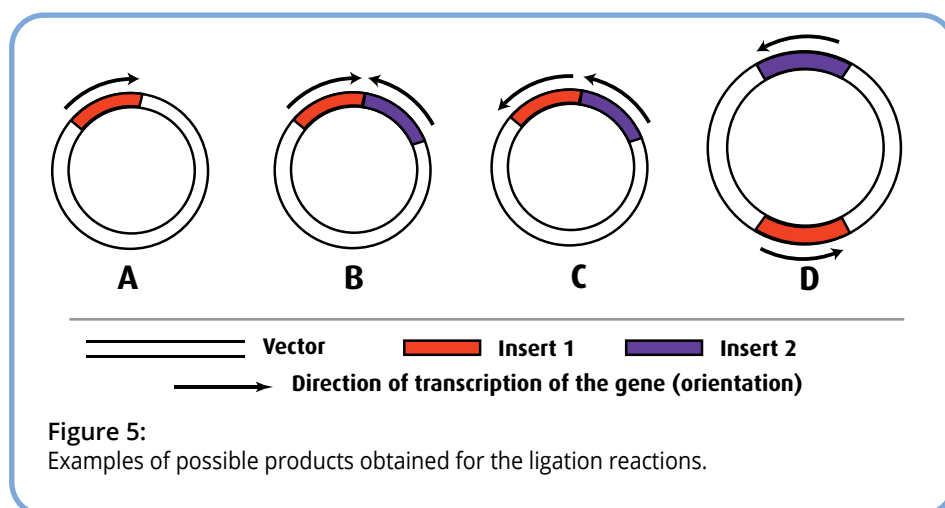


Figure 5:
Examples of possible products obtained for the ligation reactions.

When cloning, the ligation reaction can be optimized multiple ways to produce the maximum quantity of the correct plasmid. For example, we can prevent the vector from ligating to itself by choosing two different restriction enzymes that produce different overhangs at either end of the vector and the insert. This common practice prevents the vector from annealing to itself; in addition, the insert can only go into the vector in one direction. Additionally, linearized plasmid vectors can be treated with alkaline phosphatase, an enzyme removes the 5' phosphates from the cut ends of the DNA. This eliminates problems associated with vector reclosure and concatemers, because ligase requires a 5' phosphate for bond formation. The nicks in the sugar-phosphate backbone after the ligation of DNA insert to vector are repaired in the transformed host. Finally, increased yields of the correct recombinant molecules can be obtained by adjusting the molar ratio of vector to insert.

TRANSFORMATION

In the laboratory, scientists can induce cells – even bacteria like *E.coli* that are not naturally able to be transformed – to take up plasmid DNA. To do this, the cells are treated with specific chemicals that make them competent, or able to take up DNA from the environment. Next, DNA is added to the cells mixture, and the suspension is “heat shocked” — or moved quickly between widely different temperatures. It is believed that a combination of chemical ions and the rapid change in temperature alters the permeability of the cell wall and membrane, allowing DNA molecules to enter the cell. Once the plasmid is transformed into the bacteria, the cells are then allowed to grow and multiply overnight, producing millions of copies of the plasmid. For this experiment, we will be transforming our recombinant DNA into a strain of *E.coli* that does not have any natural antibiotic resistance, plasmids, or restriction enzymes. These characteristics make the bacteria an excellent host for cloning and subcloning experiments.

In practice, transformation is a highly inefficient process -- only one in every 10,000 cells successfully incorporates the plasmid DNA. Furthermore, transformation efficiency is even lower with linear DNA molecules or very large concatemers. However, because many cells are used in a transformation experiment (about 1×10^9 cells), only a few cells must be transformed to achieve a positive outcome. If bacteria are transformed with a plasmid containing a selectable marker and plated on both selective and nonselective agar medium,

$$\frac{\text{Number of transformants}}{\mu\text{g of DNA}} \times \frac{\text{final vol. at recovery (ml)}}{\text{vol. plated (ml)}} = \text{Number of transformants per } \mu\text{g}$$

Specific example:

$$\frac{100 \text{ transformants}}{0.01 \mu\text{g}} \times \frac{1 \text{ mL}}{0.1 \text{ mL}} = \frac{100,000}{(1 \times 10^5)} \text{ transformants per } \mu\text{g}$$

Figure 6:
Bacterial Transformation Efficiency Calculation

we will observe very different results. Nonselective agar plates will allow both transformed and untransformed bacteria to grow, forming a bacterial “lawn”. In contrast, only transformed cells expressing the marker will grow on the selective agar plate, resulting in isolated colonies.

Because each colony on the selective agar plate originates from a single transformed cell, we can calculate the transformation efficiency, or the number of cells transformed per microgram (μg) of plasmid DNA (outlined in Figure 6). For example, if 10 nanograms ($0.01 \mu\text{g}$) of plasmid were used to transform one milliliter (mL) of cells, and plating 100 microliters (μL) of this mixture gives rise to 100 colonies, then there must have been 1,000 transformed bacteria in the one mL mixture. Dividing 1,000 transformants by $0.01 \mu\text{g}$ DNA means that the transformation efficiency would be 1×10^5 cells transformed per μg plasmid DNA. In general, transformation efficiency ranges from 1×10^5 to 1×10^8 cells transformed per μg plasmid.

SELECTION AND PURIFICATION OF RECOMBINANT PLASMIDS

In most cases, the vector's antibiotic resistance gene is used to select for bacteria that contain the recombinant DNA. The plasmid DNA is then screened to determine whether the insert is present. In this experiment, we can perform a simple and rapid selection for the insert by plating the transformants on nutrient agar plates containing Kanamycin. Only cells that contain the insert will grow. However, we must still analyze our recombinant DNA to verify the results of the ligation reaction. For example, the insert may be present in the reverse orientation, or multiple inserts may be present (Figure 5). To determine the nature of the recombinant plasmid, the DNA is digested with three restriction enzymes, used alone or in combination. The various digestions will create different patterns depending on the presence and the orientation of the insert.

Before we analyze the recombinant DNA, we must isolate plasmid from the *E. coli* cells. First, a single colony from the transformation plate is used to inoculate liquid media. Because bacteria will often eliminate their plasmids, the liquid media contains Kanamycin to maintain this selective pressure. The bacteria are then allowed to grow and multiply in the media overnight, producing millions of copies of the plasmid. Finally, the plasmid is purified from the bacteria using the alkaline-lysis method and analyzed.

The alkaline-lysis plasmid extraction technique is a simple and reliable method to purify the recombinant DNA from the bacterial cells. First, the cells are harvested from the liquid culture by centrifugation, creating a pellet of cells at the bottom of the tube. The cell pellet is then suspended in a solution that contains the detergent sodium dodecyl sulfate (or SDS). SDS disrupts the cell membrane, releasing the DNA from the bacteria in a process called lysis. The high pH of the solution irreversibly denatures the bacteria's chromosomal DNA and aids in protein denaturation and RNA degradation. In addition, the nuclease RNase is added to the cell lysate to degrade any residual RNA in the sample. In contrast, supercoiled plasmid is not affected because its phosphate backbone remains unbroken.

Once the effects of lysis are complete, an acidic potassium acetate solution is added to the lysate to neutralize the pH and precipitate SDS and membrane/protein complexes. Furthermore, most of the chromosomal DNA will also precipitate because it is associated with the cell membrane. This leaves a solution that primarily contains the supercoiled plasmid. If high-quality plasmid is required for sequencing, the residual protein can be further removed from the DNA by organic solvents such as phenol and chloroform.

In this experiment, isopropyl alcohol is used to purify and concentrate DNA from cellular extracts. When the alcohol is added to the solution, electrostatic interactions between the water molecules and the sugar-phosphate backbone of the plasmid are disrupted, forcing the DNA out of solution as a sticky white precipitate. The precipitated plasmid is concentrated at the bottom of a micro-test tube by high-speed centrifugation. Once the supernatant is removed, the DNA pellet is dried before being resuspended in Tris-ETDA buffer for restriction digestion analysis.

RESTRICTION ENZYME ANALYSIS OF RECOMBINANT DNA

To verify the presence of the kan^R insert, the recombinant plasmid is digested with *EcoRI*. Remember, this is the site that was used to clone the insert into the plasmid. Digestion of the plasmid with this enzyme will generate two fragments of 3000 and 1282-base pairs that represent the vector and the insert, respectively. This digestion does not confirm the orientation of the insert nor does it identify the presence of multiple inserts because each insert will produce the same 1282-base pair product (Figure 5, A-C).

The presence of multiple inserts can be confirmed by cleaving the plasmid with *PvuII* endonuclease. The only *PvuII* recognition site in the recombinant plasmid is found in the plasmid vector, about 128 base pairs downstream (in the 3' direction) from the vector's *EcoRI* site in the polylinker. Consequently, when the DNA is digested with this enzyme, the plasmid will be linearized. The size of the linearized plasmid can be used to estimate how many inserts are present in the recombinant DNA molecule. For example, if two adjacent inserts were ligated to the vector, the linearized plasmid would be 5564 base pairs in length ($1282 + 1282 + 3000$).

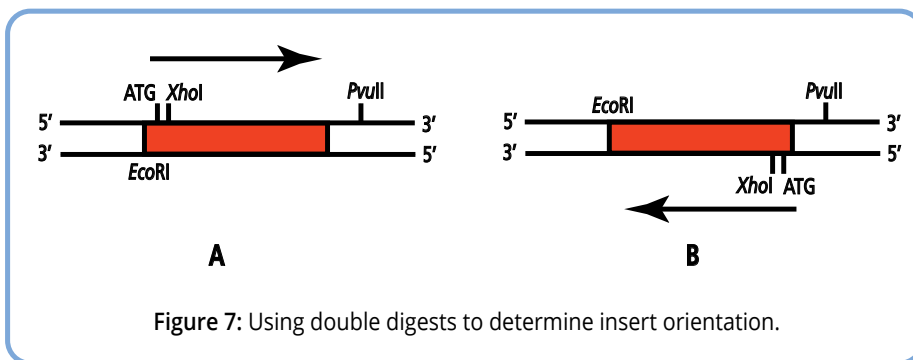


Figure 7: Using double digests to determine insert orientation.

The single *PvuII* site in the vector can be used as a fixed reference point to determine the orientation of the kan^R insert. The insert possesses a single *XhoI* recognition site located ~177 base pairs from the 5' end of the DNA. Because the relationship between the *XhoI* site and the *PvuII* site changes depending on the orientation of the kan^R gene within the plasmid, a “double digest” of the recombinant DNA with these enzymes will produce a fragment with a distinguishing length, which can be used to determine the orientation of the insert (Figure 7). For example, a digest of the plasmid in Figure 7A with *XhoI* and *PvuII* will yield 1233 and 3049-base pair fragments after the double digest. When the insert is in the opposite orientation, a double digest will yield 305 and 3977-base pair fragments.

Statistically, one would expect to find a 50:50 occurrence of the two insert orientations if many colonies were analyzed. It should be noted that the orientation of the kan^R insert does not have any large effects on its expression because the fragment has its own promoter. However, when the vector supplies the promoter required for expression, one of the insert orientations can abolish expression of the subcloned gene.

If multiple inserts are present in a single circular vector, the determination of orientation becomes more complex (Figure 5B). Assume the *PvuII* site in the vector is 128 base pairs to the right (3') of *EcoRI* restriction site (going in the clockwise direction). A *PvuII* - *XhoI* double digest of the recombinant in Figure 5A would yield three fragments having lengths of 305, 1282 and 3977 base pairs.

ANALYSIS OF RESTRICTION DIGESTS

In order to view the results of a restriction digest, students will utilize agarose gel electrophoresis. At first glance, an agarose gel appears to be a solid at room temperature. On the molecular level, the gel contains small channels through which the DNA can pass. A mixture of DNA molecules is added into depressions (or “wells”) within a gel, and then an electrical current is passed through the gel. Because the sugar-phosphate backbone of DNA has a strong negative charge, the current drives the DNA through the gel towards the positive electrode. Small DNA fragments move through these holes easily, but large DNA fragments have a more difficult time squeezing through channels. Therefore, molecules with dissimilar sizes and/or shapes travel at different speeds, become separated, and form discrete “bands” within the gel. After the current is stopped, the bands can be visualized using a stain that sticks to DNA.

Each student group will observe different banding patterns after electrophoresis of ligation reaction products due to the different plasmids that are built by the experiment. However, because plasmids can exist in different conformations, several unexpected bands may be present in the undigested plasmid DNA samples. First, the supercoiled plasmid DNA is tightly wound in a compact secondary structure. This allows the DNA to be efficiently packaged within the cell. This DNA will appear smaller than its molecular weight when analyzed by electrophoresis. In contrast, if the DNA backbone is nicked or cut during purification, the plasmid will lose its compact structure and run at the appropriate size. This will create two distinct bands when analyzed by electrophoresis. Large chains of interlocked plasmids called catenanes are also present in the sample. The catenanes, being the largest of the DNA isoforms, will appear much larger than the linear DNA. All of these isoforms are digested by restriction enzymes, so they will produce the same patterns. After restriction digest, all of these isoforms will produce the same series of DNA fragments when analyzed by electrophoresis.

Experiment Overview

EXPERIMENT OBJECTIVE

In this experiment, students will assemble and analyze DNA molecules *in vitro* using several recombinant DNA techniques, including gene cloning, plasmid extraction, and restriction enzyme analysis.

LABORATORY NOTEBOOKS

Scientists document everything that happens during an experiment, including experimental conditions, thoughts and observations while conducting the experiment, and, of course, any data collected. Today, you'll be documenting your experiment in a laboratory notebook or on a separate worksheet.

Before Starting the Experiment:

- Carefully read the introduction and the protocol. Use this information to form a hypothesis for this experiment.
- Predict the results of your experiment.

During the Experiment:

- Record your observations in your lab notebook.

After the Experiment:

- Interpret the results – does your data support or contradict your hypothesis?
- If you repeated this experiment, what would you change? Revise your hypothesis to reflect this change.

BRIEF DESCRIPTION OF EXPERIMENT

In this experiment, you will create a recombinant plasmid by covalently linking (ligating) a kanamycin resistance gene and a pUC vector. You will then use gel electrophoresis to confirm this ligation. Next, you will prepare a strain of competent *E. coli* cells to take up exogenous DNA including the new kan^R plasmid. Transformed bacteria will be selected for by growing the cells on kanamycin plates. Surviving colonies will then be cultivated overnight in a kanamycin growth media. Finally, you will verify the presence of the kan^R insert(s) in your bacteria culture and determine the insert(s) orientation by isolating the plasmids, digesting them with multiple restriction enzyme combinations, and analyzing the resulting fragments using gel electrophoresis.

The following are the four modules to this experiment:

- Module I Ligation of the Plasmid Vector to kan^R Gene Fragment
- Module II Transformation of the Recombinant DNA into *E. coli*
- Module III Culturing of kan^R Transformants
- Module IV Extraction of Recombinant Plasmid DNA
- Module V Restriction Enzyme Analysis

Laboratory Safety

IMPORTANT READ ME!

Transformation experiments contain antibiotics to select for transformed bacteria. Students who have allergies to antibiotics such as penicillin, ampicillin, kanamycin or tetracycline should not participate in this experiment.

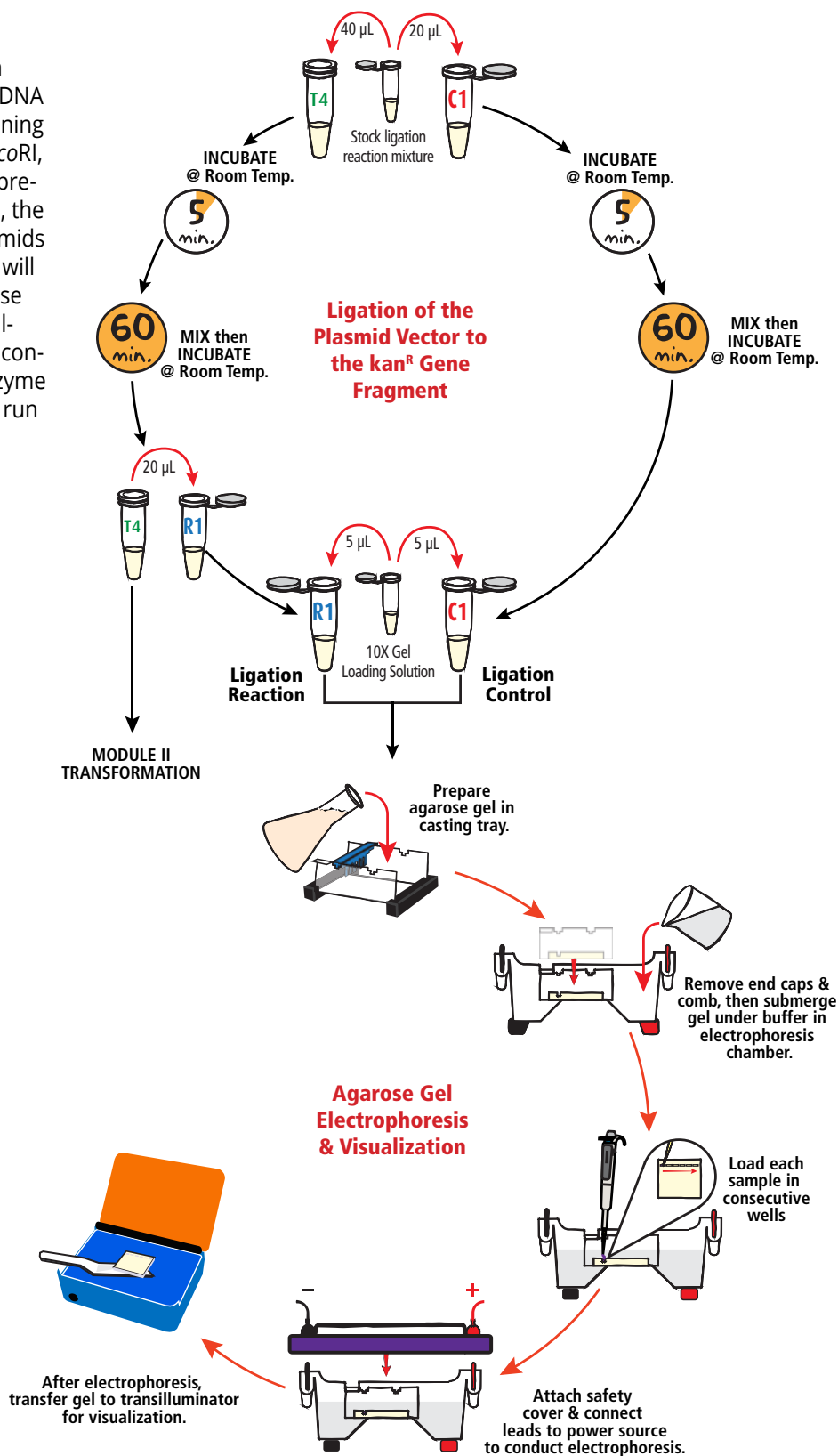
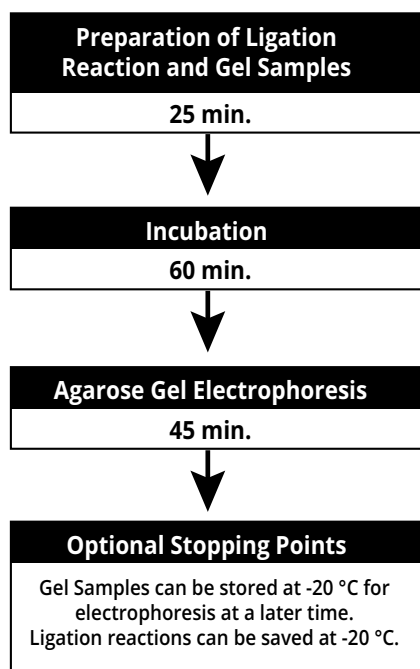
1. Wear gloves and goggles while working in the laboratory.
2. Exercise extreme caution when working in the laboratory - you will be heating and working with high voltages, which could be dangerous if performed incorrectly.
3. DO NOT MOUTH PIPET REAGENTS - USE PIPET PUMPS OR BULBS.
4. The *E. coli* bacteria used in this experiment is not considered pathogenic. Regardless, it is important to follow simple safety guidelines in handling and disposal of materials contaminated with bacteria.
 - A. Wipe down the lab bench with a 10% bleach solution or a laboratory disinfectant.
 - B. All materials, including petri plates, pipets, transfer pipets, loops and tubes, that come in contact with bacteria should be disinfected before disposal in the garbage. Disinfect materials as soon as possible after use in one of the following ways:
 - Autoclave at 121 °C for 20 minutes.
Tape several petri plates together and close tube caps before disposal. Collect all contaminated materials in an autoclavable, disposable bag. Seal the bag and place it in a metal tray to prevent any possibility of liquid medium or agar from spilling into the sterilizer chamber.
 - Soak in 10% bleach solution.
Immerse petri plates, open tubes and other contaminated materials into a tub containing a 10% bleach solution. Soak the materials overnight and then discard. Wear gloves and goggles when working with bleach.
5. Always wash hands thoroughly with soap and water after working in the laboratory.
6. If you are unsure of something, ASK YOUR INSTRUCTOR!



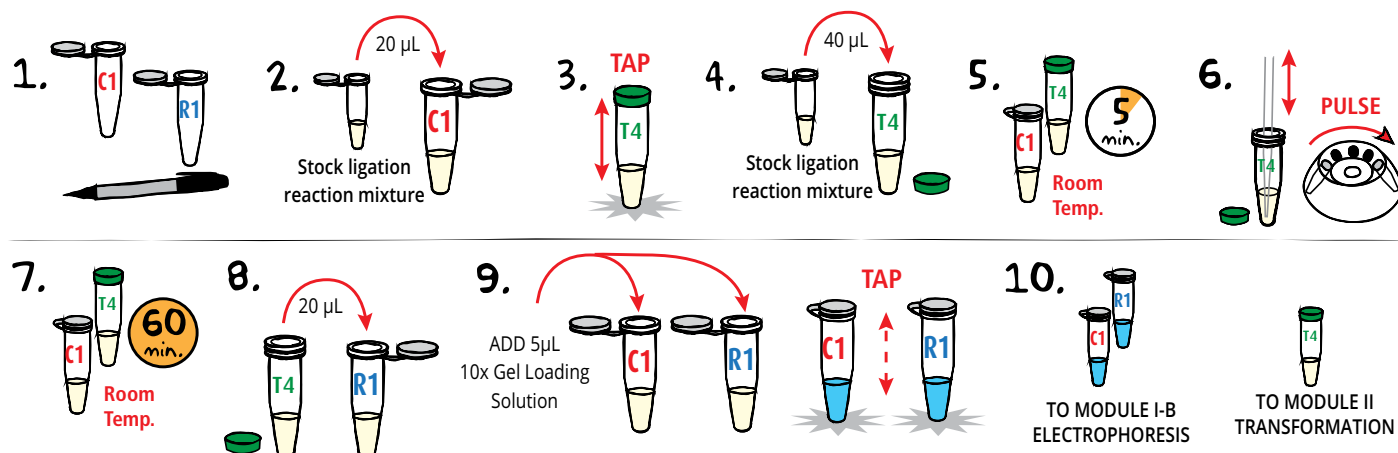
Module I Overview

In Module I, you will perform a ligation reaction by combining the enzyme T4 DNA Ligase (T4) with a stock solution containing water, pUC vectors predigested with *Eco*RI, and Kanamycin resistance genes also predigested with *Eco*RI. During incubation, the sticky ends of many of the precut plasmids and genes will link and the T4 enzyme will catalyze the formation of bonds at these sites resulting in new recombinant molecules. You will also prepare a control containing the vector and gene but no enzyme (C1). A portion of both samples will be run on an agarose gel to confirm ligation.

TIMING REQUIREMENTS:



Module I-A: Ligation of the Plasmid Vector to the kan^R Gene Fragment



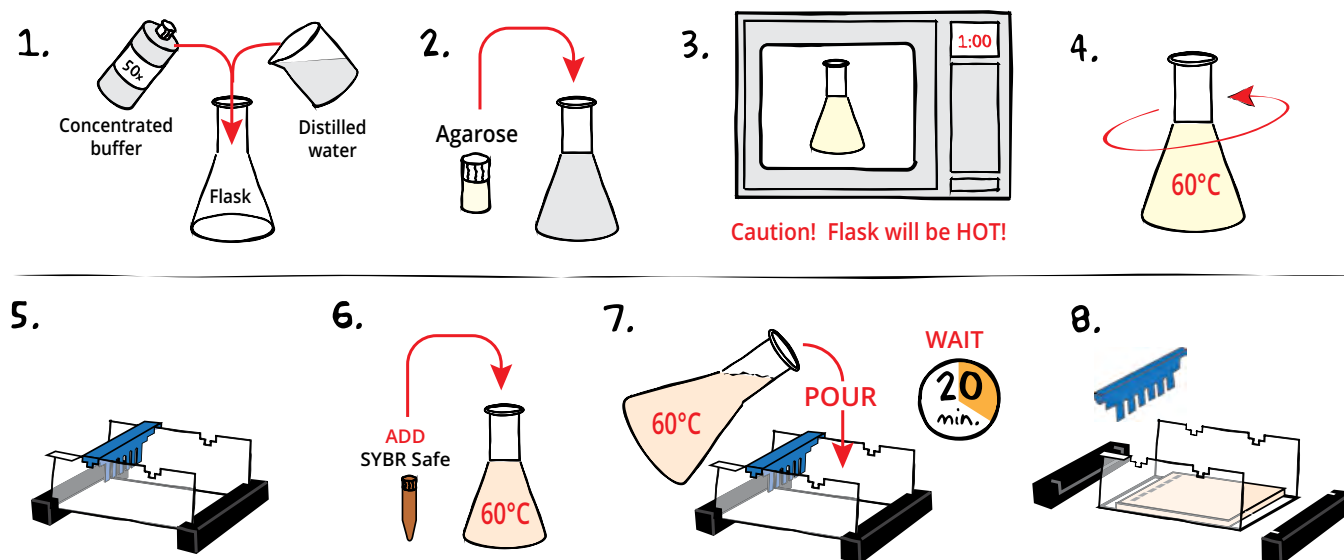
- LABEL** two 1.5 mL microcentrifuge tubes "C1" and "R1" and with your student group.
 - "C1" will contain the Ligation Control gel sample, without any T4 ligase.
 - "R1" will contain the Ligation Reaction gel sample, with T4 ligase.
- Using a fresh pipet tip, **TRANSFER** 20 µL of the Stock Ligation Reaction mixture (provided by your instructor) to the Ligation Control Tube "C1".
- Gently **TAP** the tube containing the T4 DNA Ligase on the tabletop or **CENTRIFUGE** it to collect the enzyme at the bottom of the tube.
- Using a fresh pipet tip, **ADD** the 40 µL of Stock Ligation Reaction mixture to the T4 DNA Ligase Tube.
- INCUBATE** both samples at room temperature for 5 minutes.
- Using a fresh pipet tip, carefully **MIX** the stock ligation reaction mixtures with the T4 DNA Ligase by pipetting the solution up and down. The solution may appear cloudy after mixing. Briefly **PULSE** in a microcentrifuge to collect the ligation reaction at the bottom of the tube.
- INCUBATE** the samples at room temperature (~ 22 °C) for 1 hour. During the incubation, periodically **MIX** the sample by tapping or vortexing the tube.
- Using a fresh tip, **TRANSFER** 20 µL of sample from the T4 DNA Ligase Tube to Tube R1.
- ADD** 5 µL of 10x Gel Loading Solution to Tubes C1 and R1. **DO NOT** add 10x Gel Loading Solution to the T4 Ligase tube. **MIX** by tapping or briefly vortexing.
- PROCEED** to electrophoresis with the samples in Tubes C1 and R1. The sample in the T4 DNA Ligase Tube will be used in the transformation in Module II.



OPTIONAL STOPPING POINT:

The ligation control and the ligation reaction gel samples (Tubes "C1" and "R1") can be stored at -20 °C for electrophoresis at a later time. The ligation reaction in the T4 DNA Ligase Tube should be stored at -20 °C until needed for Transformation in Module II.

Module I-B: Agarose Gel Electrophoresis



CASTING THE AGAROSE GEL

1. **DILUTE** concentrated (50X) buffer with distilled water to create 1X buffer (see Table A).
2. **MIX** agarose powder with 1X buffer in a 250 mL flask (see Table A).
3. **DISSOLVE** agarose powder by boiling the solution. **MICROWAVE** the solution on high for 1 minute. Carefully **REMOVE** the flask from the microwave and **MIX** by swirling the flask. Continue to **HEAT** the solution in 15-second bursts until the agarose is completely dissolved (the solution should be clear like water).
4. **COOL** agarose to 60°C with careful swirling to promote even dissipation of heat.
5. While agarose is cooling, **SEAL** the ends of the gel-casting tray with the rubber end caps. **PLACE** the well template (comb) in the appropriate notch.
6. Before casting the gel, **ADD diluted SYBR® Safe** to the molten agarose and swirl to mix (see Table A).
7. **POUR** the cooled agarose solution into the prepared gel-casting tray. The gel should thoroughly solidify within 20 minutes. The gel will stiffen and become less transparent as it solidifies.
8. **REMOVE** end caps and comb. Take particular care when removing the comb to prevent damage to the wells.



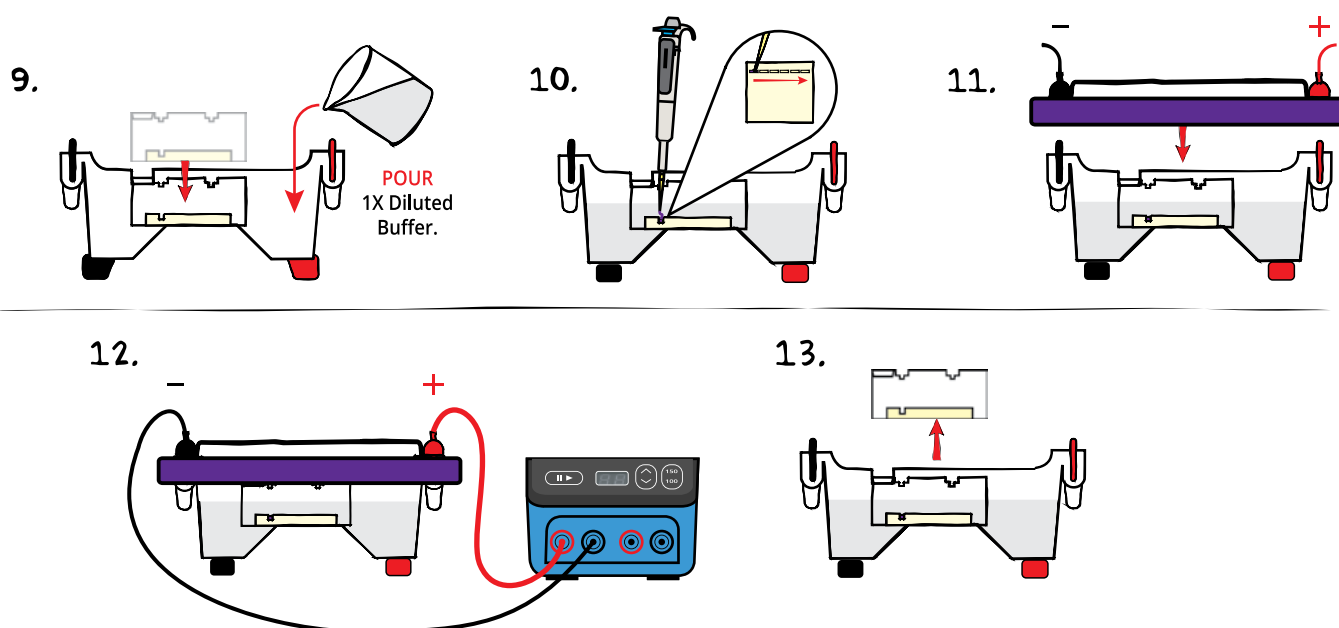
Table
A

Individual 0.8% UltraSpec-Agarose™ Gel with SYBR® Safe Stain

Size of Gel Casting tray	Concentrated Buffer (50x)	+ Distilled Water	+ Amt of Agarose	= TOTAL Volume	Diluted SYBR® (Step 6)
7 x 7 cm	0.6 mL	29.4 mL	0.24 g	30 mL	30 µL
10 x 7 cm*	0.9 mL	44.1 mL	0.36 g	45 mL	45 µL
14 x 7 cm	1.2 mL	58.8 mL	0.48 g	60 mL	60 µL

* Recommended gel volume for the EDGE™ Integrated Electrophoresis System.

Module I-B: Agarose Gel Electrophoresis, continued



RUNNING THE GEL

9. **PLACE** gel (on the tray*) into electrophoresis chamber. **COVER** the gel with 1X electrophoresis buffer (See Table B for recommended volumes). The gel should be completely submerged.
10. **LOAD** the entire sample (25 μ L) into the well in the order indicated by Table 1.
11. **PLACE** safety cover. **CHECK** that the gel is properly oriented. Remember, the DNA samples will migrate toward the positive (red) electrode.
12. **CONNECT** leads to the **power source** and **PERFORM** electrophoresis (See Table C for time and voltage guidelines).
13. After electrophoresis is complete, **REMOVE** the gel and casting tray from the electrophoresis chamber.

REMINDER:

Before loading the samples, make sure the gel is properly oriented in the apparatus chamber.

TABLE 1: Gel Loading

Lane 1	DNA Standard Marker
2	Ligation Control (C)
3	Ligation Reaction (R)

Table B 1x Electrophoresis Buffer (Chamber Buffer)

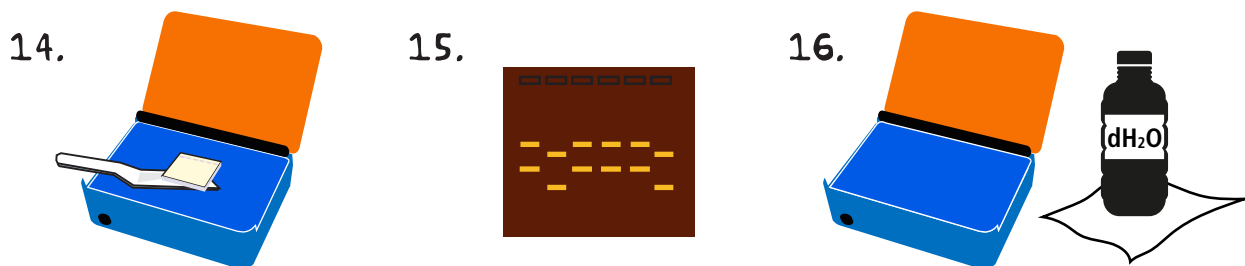
EDVOTEK Model #	Total Volume Required	50x Conc. Buffer	Dilution + Distilled Water
EDGE™	150 mL	3 mL	147 mL
M12	400 mL	8 mL	392 mL
M36	1000 mL	20 mL	980 mL

Table C Time and Voltage Guidelines (0.8% Agarose Gel)

Volts	Electrophoresis Model	
	EDGE™	M12 & M36
	Min/Max (minutes)	Min/Max (minutes)
150	10/20	20/35
125	N/A	30/45
100	15/25	40/60

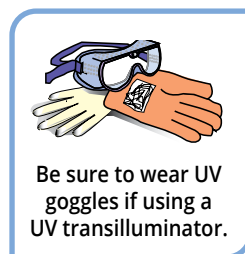
*Gels that have previously been removed from their trays should be “anchored” back to the tray with a few drops of molten agarose before placing into the electrophoresis chamber. This will prevent the gels from sliding around in the trays and the chambers.

Module I-B: Agarose Gel Electrophoresis, continued

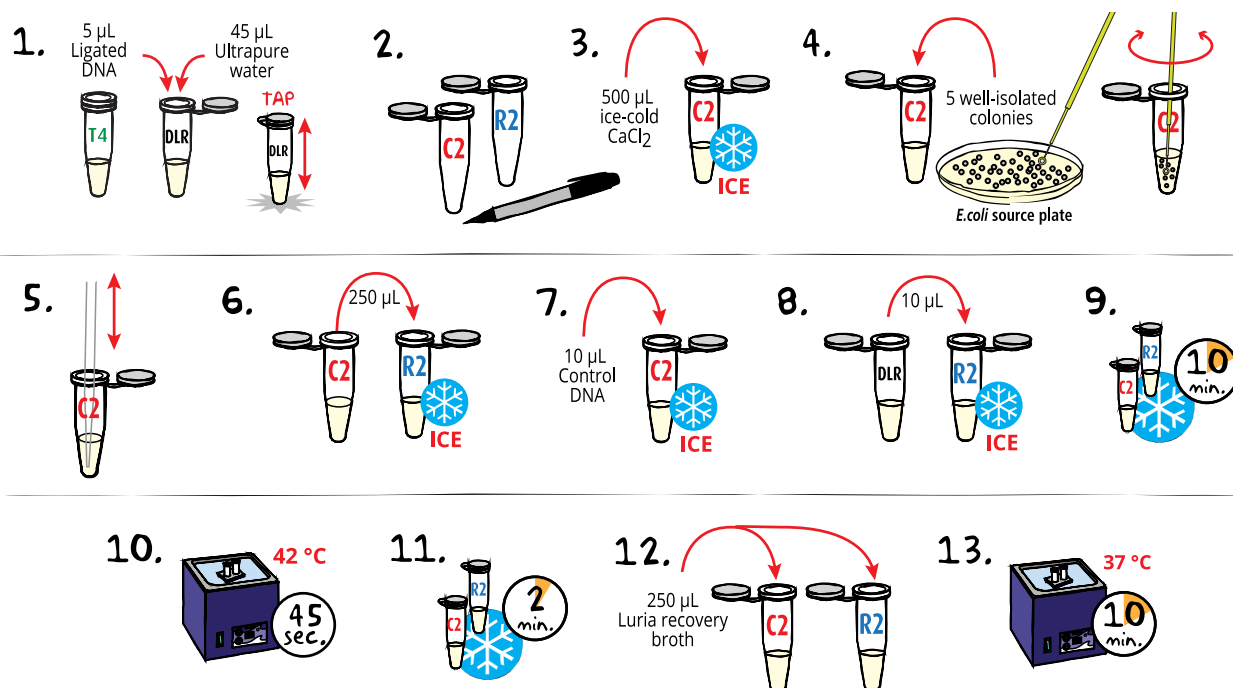


VISUALIZING THE SYBR® GEL

14. **SLIDE** gel off the casting tray onto the viewing surface of the transilluminator.
15. **TURN** the unit on. DNA should appear as bright green bands on a dark background. **PHOTOGRAPH** results.
16. **REMOVE** and **DISPOSE** of the gel and **CLEAN** the transilluminator surfaces with distilled water.



Module II: Transformation of the Recombinant DNA into *E.coli*

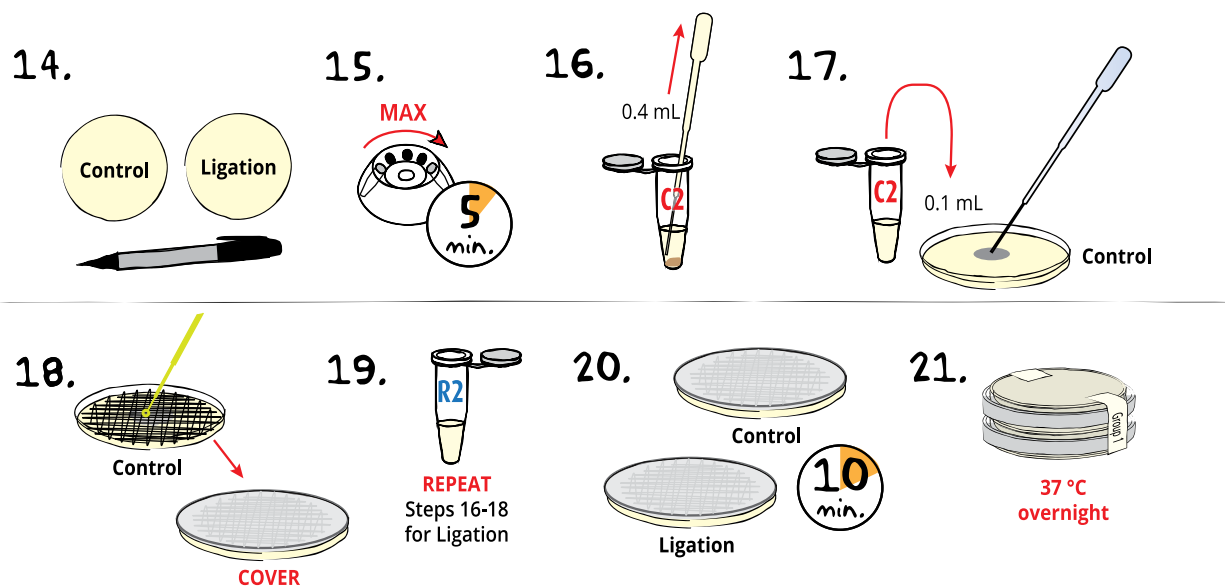


- DILUTE** the ligation reaction by mixing 5 µL of DNA from the T4 DNA Ligase tube (Module I) with 45 µL of Ultra-pure water in a fresh microcentrifuge tube. **LABEL** this tube "DLR" (Diluted Ligation Reaction). **MIX** by tapping or briefly vortexing.
- LABEL** one microcentrifuge tube with "C2" for Control and a second microcentrifuge tube with "R2" for Ligation Reaction.
- TRANSFER** 500 µL ice-cold CaCl_2 solution into the "C2" tube (Control) using a sterile one mL pipet. **PLACE** tube on ice.
- Using a sterile inoculation loop, **TRANSFER** 5 well-isolated colonies from the *E.coli* source plate to the "C2" tube. **TWIST** the loop between your fingers to free the cells. Check the tube to confirm that the bacteria have come off of the loop.
- RESUSPEND** the bacterial cells in the CaCl_2 solution by pipetting up and down until no clumps of cells are visible and the cell suspension looks cloudy.
- TRANSFER** 250 µL of the cell suspension to the "R2" tube. **PLACE** tubes on ice.
- ADD** 10 µL of Control DNA to the "C2" tube. **PLACE** tubes on ice.
- ADD** 10 µL of the diluted ligation reaction DNA (DLR) to the "R2" tube (Ligation). **PLACE** tubes on ice.
- GENTLY MIX** both samples by flicking the tubes. **INCUBATE** the tubes on ice for 10 minutes.
- PLACE** the transformation tubes in a waterbath at 42 °C for 45 seconds. This heat shock step facilitates the entry of DNA into *E.coli*.
- Immediately **RETURN** the tubes to the ice bucket and **INCUBATE** for two minutes.
- TRANSFER** 250 µL of Luria Recovery Broth to the tubes using a sterile 1 mL pipet. Gently **MIX** by flicking the tube.
- INCUBATE** the cells for 10 minutes in a 37 °C waterbath. This recovery period allows cells to repair their cell walls and to express the antibiotic resistance gene.

STEP 5 REMINDER:
For best results, make sure that the cells are completely resuspended!

IMPORTANT:
For best results, ensure that the CaCl_2 is ice cold and that the cells are rapidly moved from ice to 42 °C and then immediately back to ice. Follow all times exactly.

continued

Module II: Transformation of the Recombinant DNA into *E.coli*, continued

14. While the cells are recovering, **LABEL** the bottom of two agar plates with "Control" and "Ligation".
15. After the recovery period, **REMOVE** the tubes from the waterbath. **CENTRIFUGE** the cells for 5 minutes at full speed (10,000 x g).
16. Using a pipet, **REMOVE** and **DISCARD** 0.4 mL of the supernatant from the "C2" tube. **RESUSPEND** the cell pellet in the remaining 0.1 mL of liquid.
17. Using a fresh pipet tip, **TRANSFER** all of the recovered cells (0.1 mL) from the "C2" tube to the middle of the corresponding plate.
18. **SPREAD** the cells over the entire plate with a sterile inoculating loop. Make sure the cells have been spread over the entire surface of the plate. **COVER** the plates.
19. **REPEAT** steps 16 thru 18 with the ligation cells from the "R2" tube. Be sure to use a fresh pipet tip and loop for this sample.
20. **WAIT** 10 minutes for the cell suspension to be absorbed by the agar. **DO NOT** invert the plates until the cell suspension has been completely absorbed into the medium.
21. **STACK** the plates on top of one another and **TAPE** them together. **LABEL** the tape with your initials or group number. **PLACE** the plates in the inverted position (agar side on top) in a 37 °C bacterial incubation oven for overnight incubation (16-18 hours).
22. **OBSERVE** the ligation and control plates. **ANALYZE** the results by calculating the transformation efficiency.

NOTE for Step 20:

It may take longer for the cell suspension to be absorbed by the agar. If liquid remains on the plate, leave the plates upright for another 10 minutes.

**OPTIONAL STOPPING POINT:**

The plates may be wrapped and stored in the refrigerator for up to one week.

Module II: Transformation of the Recombinant DNA into *E.coli*, continued

DETERMINATION OF TRANSFORMATION EFFICIENCY

Transformation efficiency is a quantitative determination of the number of cells transformed per 1 µg of plasmid DNA. In essence, it is an indicator of the success of the transformation experiment. The transformation efficiency is calculated using the data collected from your experiment.

1. Count the number of colonies on the control and ligation plate. A convenient method to keep track of counted colonies is to mark each colony with a lab marking pen on the outside of the plate.
2. Determine the transformation efficiency using the following formula:

$$\frac{\text{Number of transformants}}{\mu\text{g of DNA}} \times \frac{\text{final vol. at recovery (ml)}}{\text{vol. plated (ml)}} = \frac{\text{Number of transformants}}{\text{per } \mu\text{g}}$$

For example, imagine that you transformed cells with 0.125 µg of DNA. The final volume at recovery was 1.05 mL, but only 0.25 mL of the cells were spread on the agar plate before overnight incubation. The next day, you observed 40 colonies on the plate. The transformation efficiency calculation would look like this:

$$\frac{40 \text{ transformants}}{0.125 \mu\text{g}} \times \frac{1.05 \text{ mL}}{0.25 \text{ mL}} = \frac{1344 \text{ (} 1.3 \times 10^3 \text{) transformants}}{\text{per } \mu\text{g}}$$

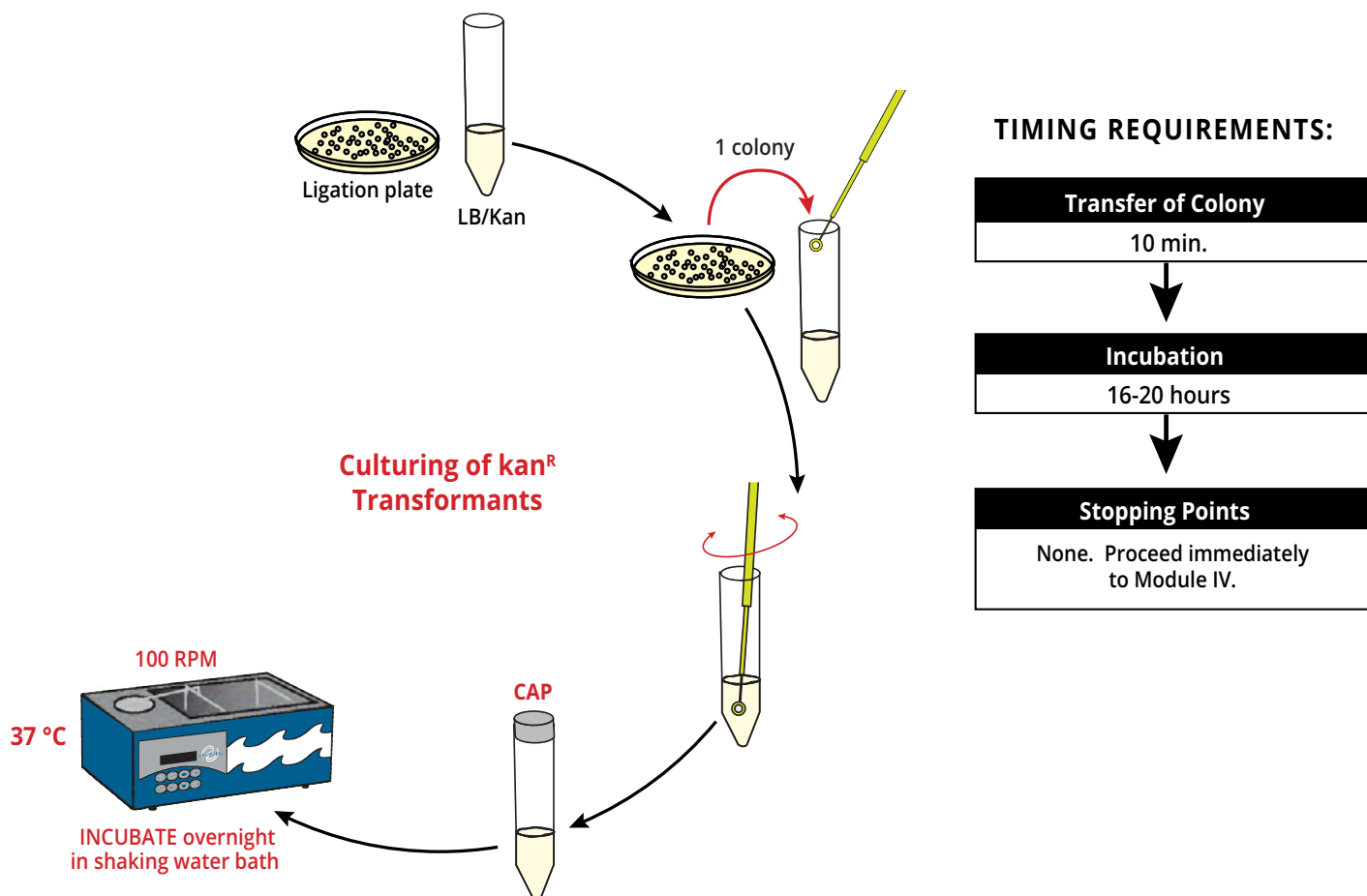
Although we plated 0.1 mL cells, this volume equals the entire final recovery volume (0.5 mL, Module II step 13) because the cells were concentrated by centrifugation before plating. Approximately 25 ng of DNA were used in the transformation.

QUICK REFERENCE for Expt. 301:

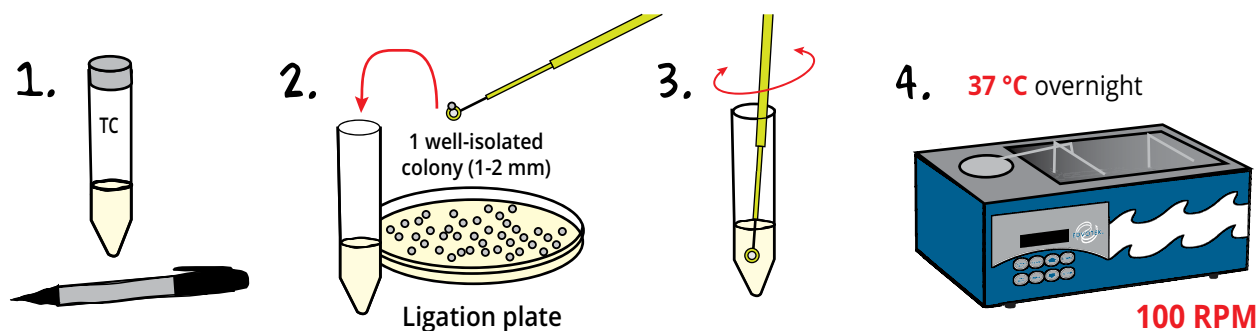
25 ng of DNA is used.
The final volume at recovery is 0.50 mL.
The entire volume of cells is plated.

Module III Overview

In Module III, you will select a single well-isolated colony of transformed bacteria and grow it overnight in order to create a large cell culture for use in Module IV. Kanamycin is included in this mixture because bacteria will often eliminate plasmids that are not under some type of selective pressure.



Module III: Culturing of kan^R Transformants



IMPORTANT! BE SURE TO PICK COLONIES FROM THE "LIGATION" PLATE FOR GROWTH AND ANALYSIS.

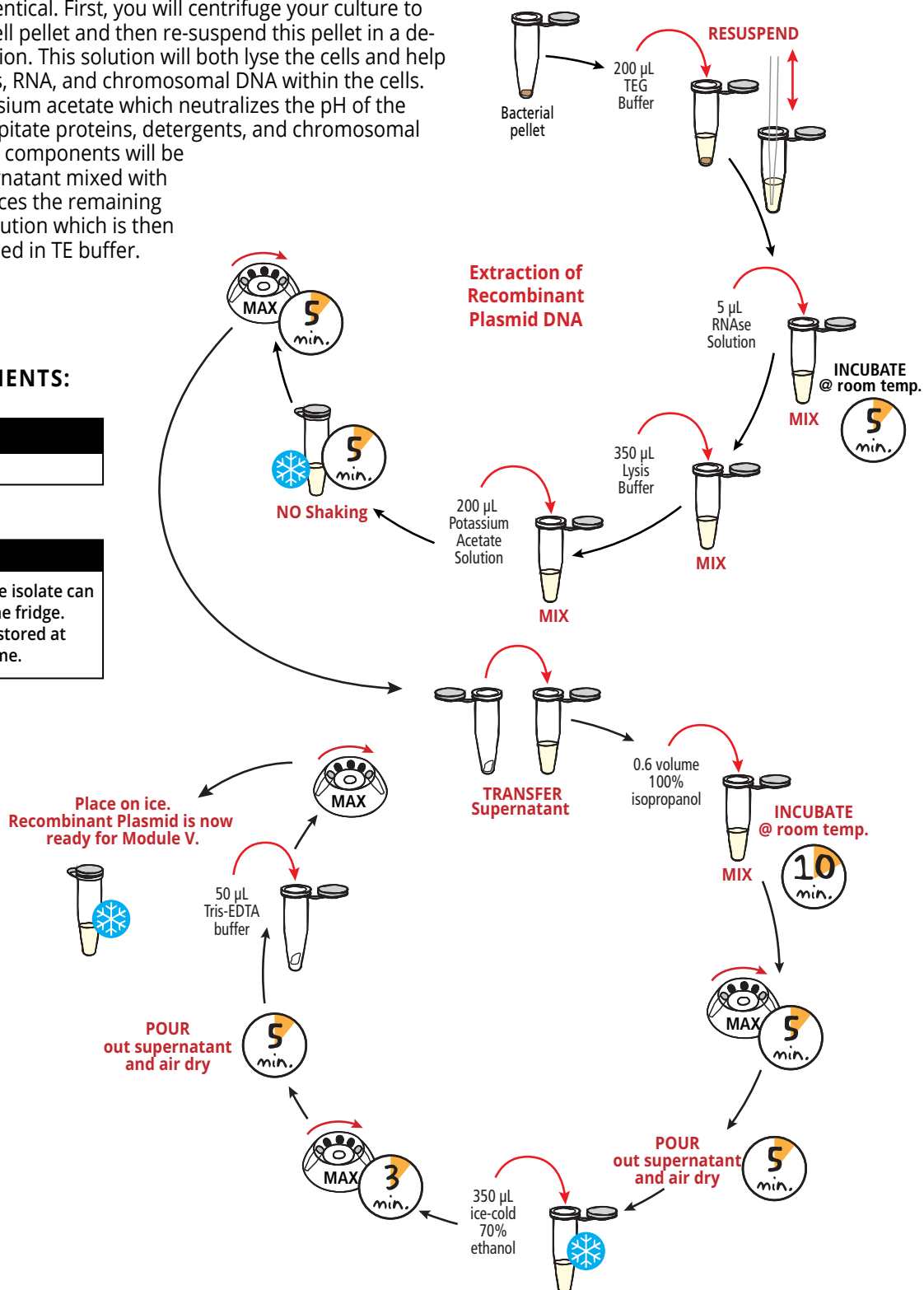
- 1. PICK UP** a culture tube containing liquid medium with Kanamycin from your instructor. **LABEL** the tube with your initials or lab group number.
- Using a sterile inoculating loop, **PICK** a single, well-isolated colony from the agar plate labeled "Ligation". For best results, choose a colony 1-2 mm in diameter.
- INOCULATE** the medium by **SHAKING** and **TWISTING** the loop in the broth. This transfers the bacteria from the loop to the broth. Tightly **CAP** the tube.
- PLACE** the tubes in a 37 °C shaking incubator or shaking waterbath set to 100 RPM (if available) overnight for 16-20 hours for best results. Otherwise, a standard waterbath or incubator can be used.

Module IV Overview

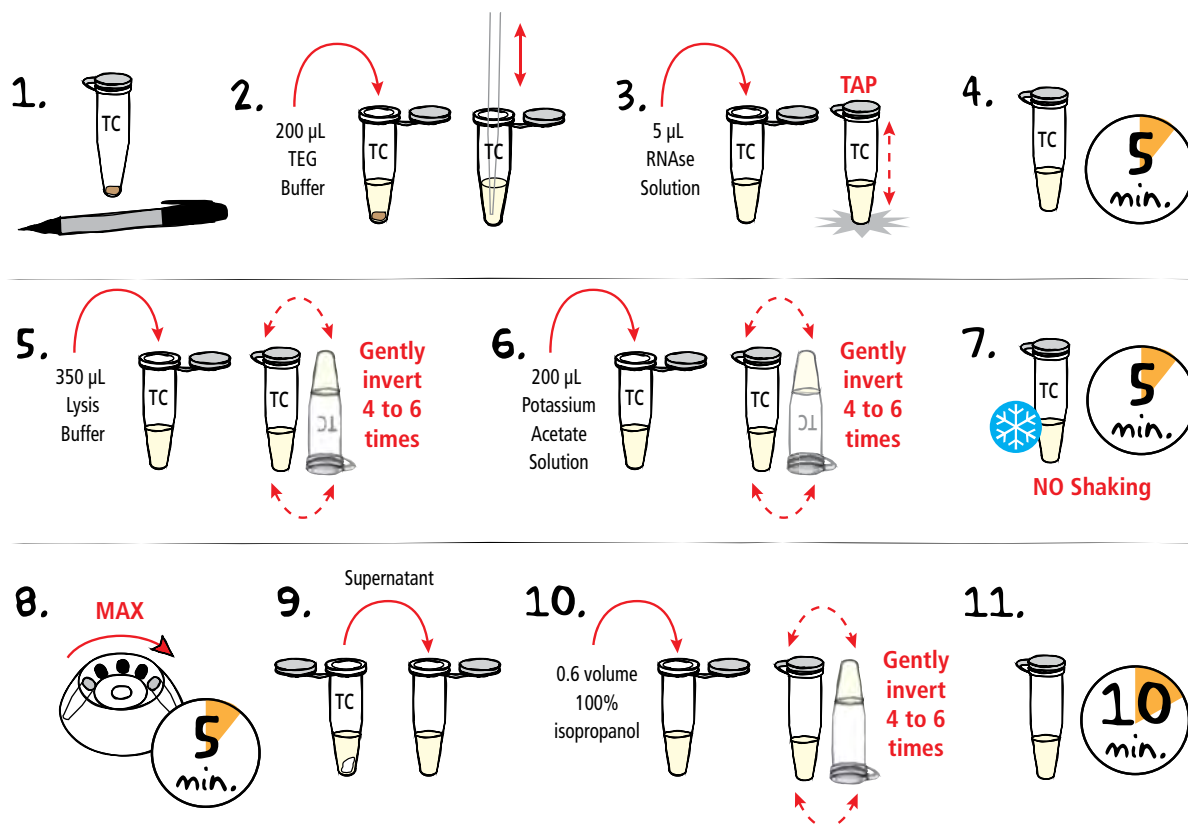
In Module IV, you will isolate the plasmids from the culture you created in Module III. If a single colony was selected in Module III, all plasmids should be genetically identical. First, you will centrifuge your culture to create a concentrated cell pellet and then re-suspend this pellet in a detergent and RNase solution. This solution will both lyse the cells and help to denature the proteins, RNA, and chromosomal DNA within the cells. Next, you will add potassium acetate which neutralizes the pH of the solution and helps precipitate proteins, detergents, and chromosomal DNA. These precipitated components will be discarded and the supernatant mixed with isopropanol. Alcohol forces the remaining (plasmid) DNA out of solution which is then pelleted and re-suspended in TE buffer.

TIMING REQUIREMENTS:

Extraction
60 min.
Stopping Points
After adding isopropanol, the isolate can be stored overnight in the fridge.
Final extractions can be stored at -20 °C for a later time.



Module IV: Extraction of Recombinant Plasmid DNA



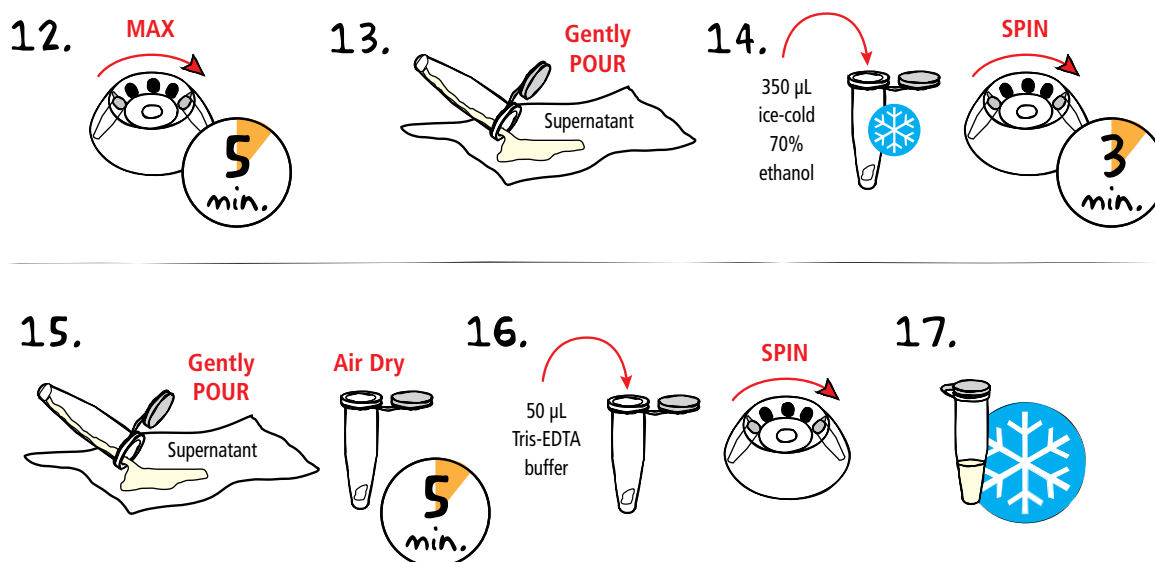
1. **LABEL** a microcentrifuge tube of pelleted *E.coli* cells with your initials or group number on it.
2. **ADD** 200 µL of TEG Buffer to the bacterial pellet. **RESUSPEND** the pellet by pipetting up and down.
3. **ADD** 5 µL of RNase solution to the cell suspension and **MIX** by tapping the tube.
4. **INCUBATE** the cell suspension for 5 minutes at room temperature.
5. **ADD** 350 µL of freshly prepared Lysis Buffer to the cell suspension. **CAP** the tube and **MIX** well by inverting gently 4 to 6 times. To avoid damaging the plasmid DNA, **DO NOT** vortex the sample.
6. **ADD** 200 µL of Potassium Acetate Solution to the suspension. **CAP** the tube and **MIX** thoroughly by inverting the tube 4 to 6 times. A white precipitate should form.
7. **INCUBATE** the sample in ice for 5 minutes. **DO NOT** shake the tube during this incubation.
8. **CENTRIFUGE** the sample for 5 minutes at full speed.
9. Carefully **TRANSFER** the supernatant into a clean 1.5 mL microcentrifuge tube. Avoid transferring the white cellular debris with the supernatant. **DISCARD** the tube containing the pellet.
10. **ADD** 0.6 volume of 100% isopropanol to the supernatant (i.e. 0.6 mL isopropanol for 1 mL of supernatant). **MIX** gently by inverting the tube 4 to 6 times.
11. **INCUBATE** the sample for 10 minutes at room temperature.



OPTIONAL STOPPING POINT:

The samples can be stored in the freezer overnight in lieu of step 11. Thaw completely before continuing with step 12.

Module IV: Extraction of Recombinant Plasmid DNA, continued



12. **INSERT** the tubes in the rotor so that the hinges are facing towards the outside edge. **CENTRIFUGE** the sample for 5 minutes at full speed. The precipitated plasmid DNA will form a small, white pellet at the bottom of the hinge side of tube after centrifugation.
13. **REMOVE** and **DISCARD** the supernatant. To prevent pellet loss, we recommend gently pouring the supernatant onto a paper towel.
14. **WASH** the DNA pellet by adding 350 μ L of ice-cold 70% ethanol to the tube. **CENTRIFUGE** at full speed for 3 minutes.
15. **REMOVE** the supernatant and air dry the pellet for 5-10 minutes to remove the residual ethanol.
16. **RESUSPEND** the pellet in 50 μ L of 1x Tris-EDTA buffer. **CAP** the tube and **MIX** by shaking and vortexing. Briefly centrifuge to get all the contents to the bottom of the tube.
17. **PLACE** sample on ice. **PROCEED** to Module V: Restriction Digest.

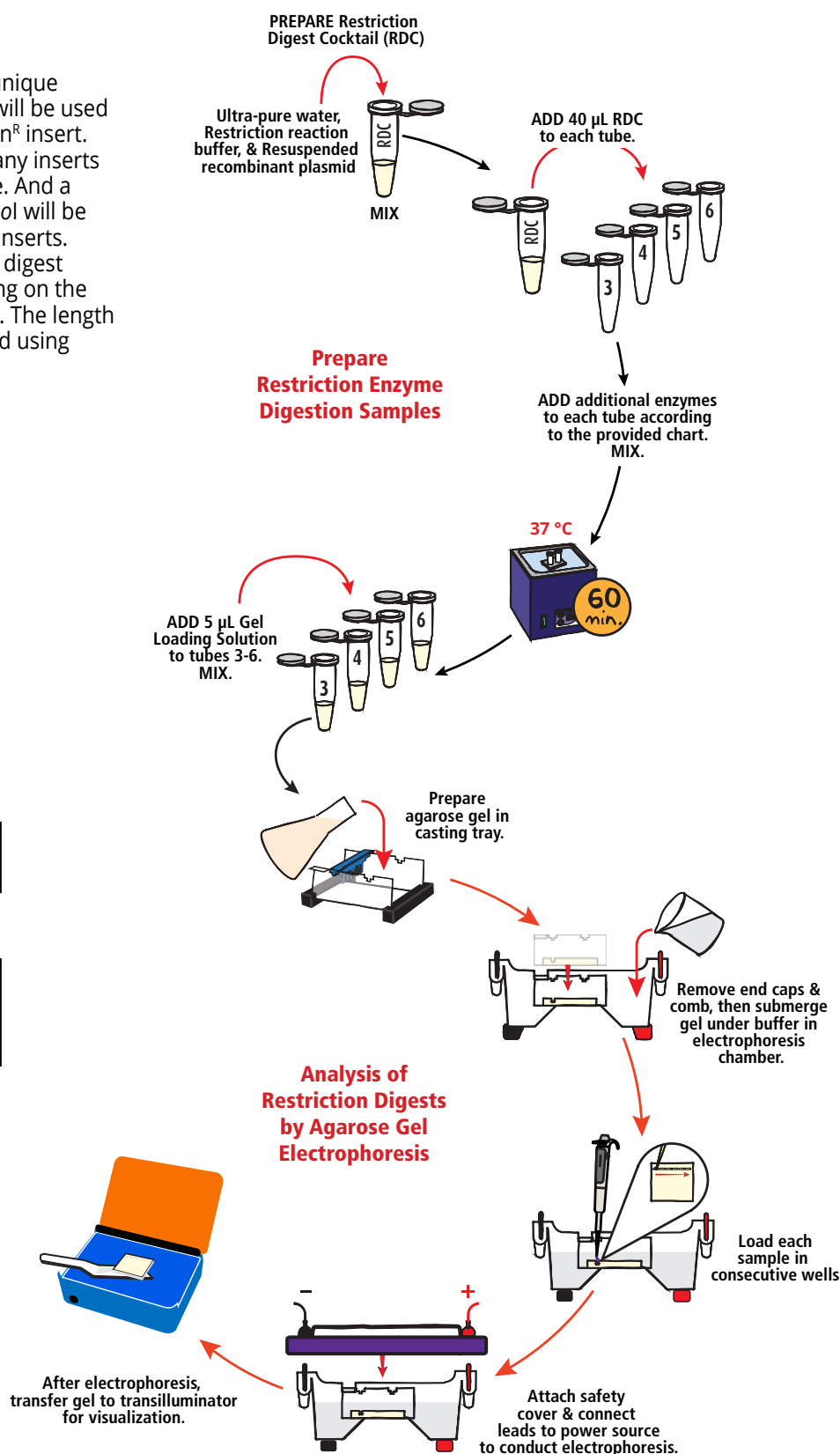
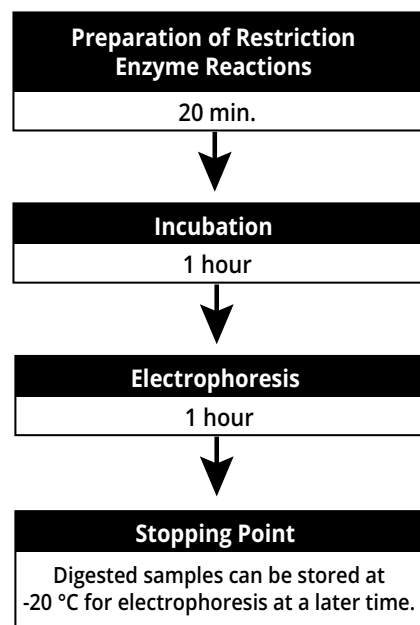
**OPTIONAL STOPPING POINT:**

The purified plasmid can be stored in the freezer for restriction digestion at a later time.

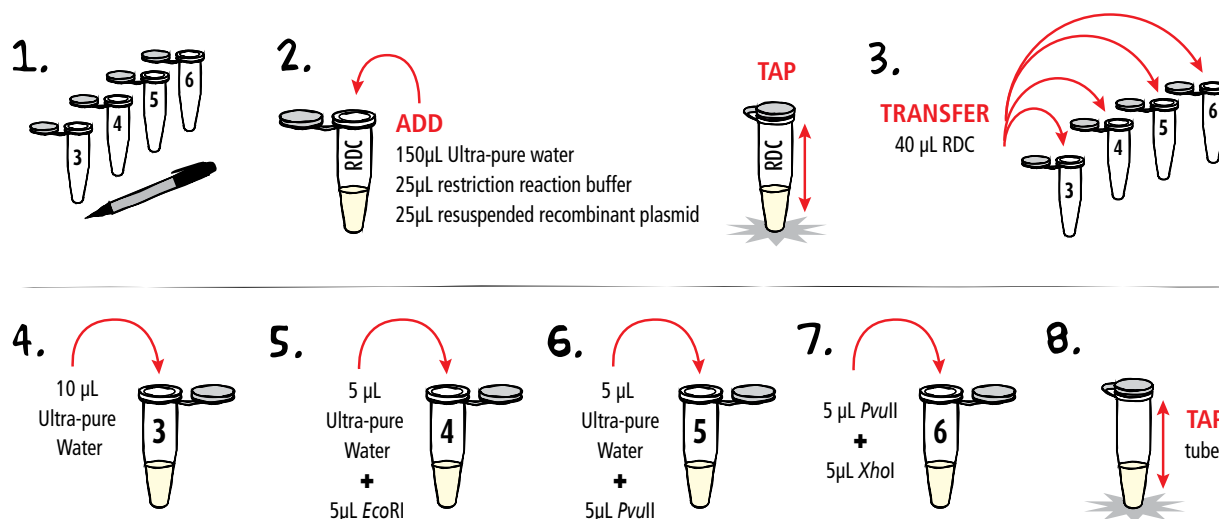
Module V Overview

In Module V, you will perform three unique restriction enzyme digestions. *EcoRI* will be used again to verify the presence of the kan^R insert. *PvuII* will be used to estimate how many inserts are in the new recombinant molecule. And a double digestion of both *PvuII* and *XhoI* will be used to reveal the orientation of the inserts. Each reaction will result in restriction digest fragments of distinct length depending on the unique characteristics of the insert(s). The length of these fragments will be determined using electrophoresis.

TIMING REQUIREMENTS:



Module V-A: Restriction Enzyme Analysis



IMPORTANT:

To prevent contamination, be sure to use a fresh pipet tip before going into the enzyme, DNA, and buffer stocks. Keep the enzymes on ice when not in use.

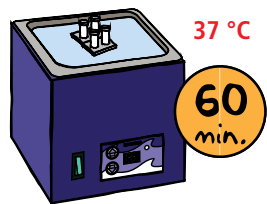
continued

Summary of Restriction Enzyme Digestion Reactions

Reaction Tube	Reaction Cocktail (µL)	Ultra-pure Water (µL)	<i>EcoRI</i> Enzyme (µL)	<i>PvuII</i> Enzyme (µL)	<i>XhoI</i> Enzyme (µL)	Final Reaction Volume (µL)
3	40	10	-	-	-	50
4	40	5	5	-	-	50
5	40	5	-	5	-	50
6	40	-	-	5	5	50

Module V-A: Restriction Enzyme Analysis, continued

9.



10.



9. **INCUBATE** the samples at 37 °C for 1 hour.
10. After the incubation, **ADD** 5 µL of 10x gel loading solution to each reaction tube. **CAP** the tubes and **MIX** by tapping the tube or vortexing vigorously.
11. **PROCEED** to electrophoresis with the samples in tubes 3, 4, 5, and 6.



OPTIONAL STOPPING POINT:

The restriction digest samples can be stored at -20 °C for electrophoresis at a later time.

Module V-B: Analysis of Restriction Digests by Electrophoresis

Each group will require one 0.8% gel with 6 sample wells for electrophoresis. Refer to Module I-B for detailed instructions about casting agarose gels and performing electrophoresis experiments.

Load 25 µL of each DNA sample into the wells in the following order:

TABLE 2: Gel Loading	
Lane 1	DNA Standard Marker
2	Supercoiled Plasmid Vector Standard
3	Restriction Digest Control
4	<i>EcoRI</i> Digest
5	<i>PvuII</i> Digest
6	<i>PvuII/XhoI</i> Double Digest

Study Questions

1. Did you observe a discreet band of DNA after the electrophoresis of the ligation reaction products? Explain. Did you observe any bands that migrated faster than the 1282 base pair kan^R fragment? If so, how could these DNA forms have been generated? (Hint: DNA does not always circularize as a relaxed molecule).
2. Which of the following pairs could be ligated together? (All termini are cohesive and complementary.)
 - a. 5'-dephosphorylated linear insert DNA + linear vector
 - b. Supercoiled vector + linear insert DNA
 - c. 5'-dephosphorylated linear vector + linear 5'-dephosphorylated insert DNA
 - d. Linear 5'-dephosphorylated vector + linear insert DNA
 - e. Nicked vector + linear insert DNA

In general, which of the above possibilities would be the best approach in a subcloning experiment like the one you have done? Why?

3. Assume the transformants produced with the ligated DNA were also plated on ampicillin medium. Would you expect to see a significant difference in the number of colonies compared to the Kanamycin plates? Why? (Hint: the linear vector was not dephosphorylated before the ligation). Why would it be unwise to pick a transformant from an ampicillin plate if you were trying to isolate the recombinant DNA? If you had, is there a step in this series of experiments that would have prevented the propagation of the incorrect plasmid?
4. Did the electrophoretic pattern of your uncut recombinant plasmid contain many forms of DNA like your ligation reaction? Explain.
5. Did your recombinant plasmid have more than one insert? What was the orientation of the insert(s)? Make a rough map of your recombinant plasmid.
6. Can the size of a supercoiled plasmid be calculated by comparison to linear DNA fragments of known size that have been run in parallel?
7. A kan^R transformant was found to contain the supercoiled pUC vector without an insert in addition to the expected supercoiled recombinant plasmid. How can this be explained?

Instructor's Guide

OVERVIEW OF INSTRUCTORS PRELAB PREPARATION

In this experiment, students will assemble and analyze recombinant DNA molecules using authentic gene cloning techniques. While we have optimized this experiment to work in the classroom laboratory, student results may vary. To address this, we have included three pre-ligated control plasmids (Components E1, E2, and E3) which can be used in lieu of the ligated DNA. Please refer to the instructors guide for guidelines in using the new control plasmids.

Preparation For:	What to do:	When:	Time Required:
Module I (1 hour)	Prepare reagents for ligation	Before the lab period.	15 min.
	Prepare diluted TAE buffer & SYBR® Safe Stain for electrophoresis	Any time before the class period.	10 min.
	Prepare molten agarose and pour gels	One day to 30 minutes before performing the experiment.	30 min.
Module II (2 hours)	Prepare LB agar plates	2-7 days before use	1 hour
	Prepare <i>E. coli</i> source plates	The day before the experiment	20 min. to streak plates; 16-18 hours to incubate plates
	Dispense control plasmid, CaCl ₂ , and recovery broth	One day to 30 minutes before performing lab period.	30 min.
	Equilibrate waterbaths at 37°C and 42°C, incubator at 37°C	One to two hours before the experiment.	10 min.
Module III (30 minutes)	Preparation of Kanamycin medium	The day of the lab experiment.	5 min.
Module VI (1.5 hours)	Harvesting of cells	The day AFTER Module III is performed.	30-45 min.
	Prepare and dispense lysis reagents	The day of the lab experiment.	30 min.
Module V (1.5 hours)	Prepare and aliquot restriction enzymes	30 minutes before use.	30 min.
	Equilibrate waterbath to 37°C	One to two hours before the experiment.	5 min.
	Prepare diluted TAE buffer for electrophoresis	Any time before the class period.	10 min.
	Prepare molten agarose and pour gels	One day to 30 minutes before performing the experiment.	30 min.

Red = Prepare immediately before module. Yellow = Prepare shortly before module. Green = Flexible / prepare up to a week before the module.

MODULE I-A: Pre-Lab Preparations

LIGATION OF THE PLASMID VECTOR TO THE kan^R GENE FRAGMENT

This kit provides enough reagents to perform 5 ligation reactions. We recommend finishing this module in one class period. If necessary, the samples can be stored in the freezer directly following the ligation (step 7). The samples should be completely thawed before proceeding to step 8.

We recommend aliquoting the reagents into sets for each lab group as described below. Alternatively, the students can share the stock tubes placed in a central location. Note that sharing the tubes increases the risk of a spill or contamination.

FOR MODULE I-A Each Group Will Need:

- 65 μL Ligation Reaction Mixture
- 1 Tube of TR DNA Ligase (C)
- 10 μL 10x Gel Loading Solution
- 2 1.5 Microcentrifuge Tubes

1. Shortly before the lab begins, thaw the Ultra-pure Water (A) and DNA fragments for ligation (B) and place on ice.
2. Prepare DNA for ligation:
 - a. Label five 0.5 mL microcentrifuge tubes with LRM for Ligation Reaction Mixture. Place the tubes on ice.
 - b. Add 300 μL of Ultra-pure Water (A) to the tube containing the DNA fragments for ligation (B). Mix well by pipetting up and down. Save the remaining water for use in Module II.
 - c. Aliquot 65 μL for each group. Keep the tubes on ice until use.
3. Each group requires one T4 DNA Ligase Reaction Tube (C). Label this tube "T4" before distributing.
4. Each group requires 10 μL of 10x Gel Loading Solution.

MODULE I-B: Pre-Lab Preparations

AGAROSE GEL ELECTROPHORESIS

This experiment requires a 0.8% agarose gel per student group. You can choose whether to prepare the gels in advance or have the students prepare their own. Allow approximately 30-40 minutes for this procedure.

Prepare SYBR® Safe Stain

1. Prepare 1x Electrophoresis Buffer by combining 20 μ L of 50X Concentrated Buffer with 980 μ L of distilled water.
2. Add 600 μ L of the 1X buffer from step 1 to the tube of SYBR® Safe and mix by tapping the tube several times. The diluted SYBR® Safe Stain is now ready to be used during agarose gel preparation.

Individual Gel Preparation

Each student group can be responsible for casting their own individual gel prior to conducting the experiment. See Part 1 in the Student's Experimental Procedure. Students will need 50x concentrated buffer, distilled water, agarose powder, and diluted SYBR® Safe Stain.

Batch Gel Preparation

To save time, a larger quantity of agarose solution can be prepared for sharing by the class. See Appendix C.

FOR MODULE I-B Each Group Will Need:

- 50x concentrated buffer
- Distilled Water
- UltraSpec-Agarose™ Powder
- Diluted SYBR® Safe Stain
- DNA Standard Marker

Preparing Gels in Advance

Gels may be prepared ahead and stored for later use. Solidified gels can be stored in the refrigerator for up to 2 weeks. Place 1-2 mL of electrophoresis buffer in a sealable bag with the gels to prevent them from drying out. Excessive buffer will cause SYBR® Safe to diffuse out of the gels.

Do not freeze gels at -20 °C as freezing will destroy the gels.

Gels that have been removed from their trays for storage should be “anchored” back to the tray with a few drops of molten agarose before being placed into the tray. This will prevent the gels from sliding around in the trays and the chambers.

Preparing the DNA Standard Marker

The DNA Standard Marker for this experiment are provided in bulk in screw-top microcentrifuge tubes. Students can share the sample tubes, or they can be pre-aliquoted into individual tubes (not included).

Visualizing SYBR® Safe-Stained Gels

SYBR® Safe is a DNA stain that fluoresces when bound to double-stranded DNA, allowing us to visualize our samples. This DNA stain is compatible with both UV and blue-light transilluminators. For best results, we recommend the TruBlu™ 2 Blue Light Transilluminator (Cat #557).

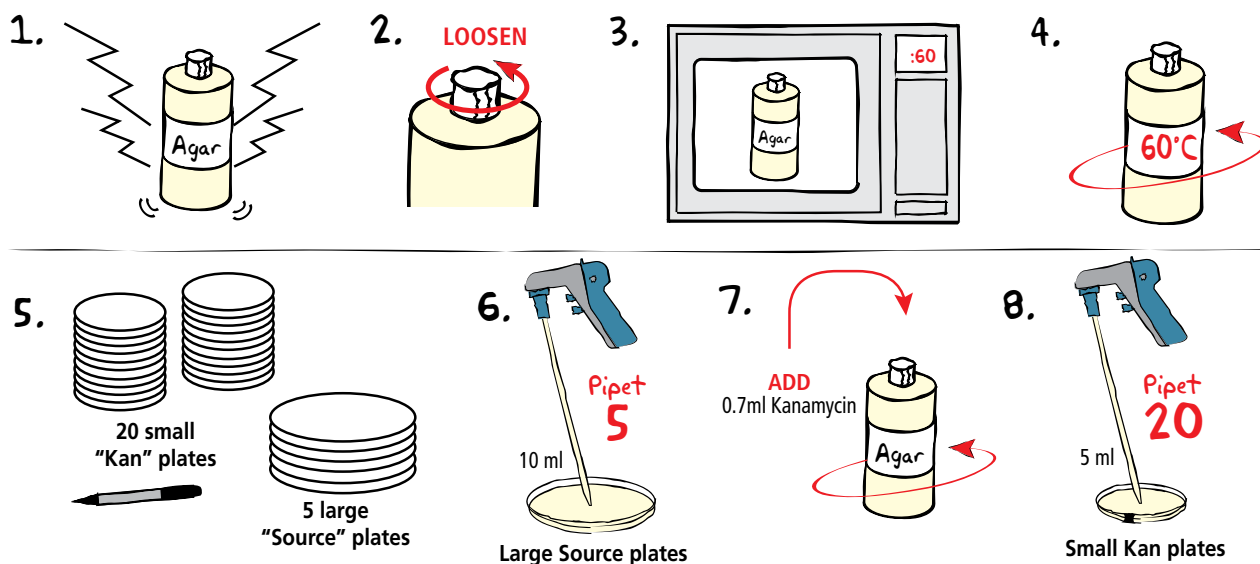
MODULE II: Pre-Lab Preparations

PREPARATION OF THE LB-AGAR PLATES

One bottle of Ready Pour Luria Broth Agar will make 5 large LB source plates and 20 LB-kan plates. Ten plates will be used for the transformation. The additional plates can be used to transform extra control plasmids if necessary.



Wear hot gloves and goggles during all steps involving heating.



1. **BREAK** solid ReadyPour™ LB Agar into small chunks by vigorously squeezing and shaking the plastic bottle.
2. **LOOSEN**, but DO NOT REMOVE, the cap on the ReadyPour™ Agar bottle. This allows the steam to vent during heating. CAUTION: Failure to loosen the cap prior to heating may cause the bottle to break or explode.
3. **MICROWAVE** the ReadyPour™ Agar on high for 60 seconds to melt the agar. Carefully **RE-MOVE** the bottle from the microwave and **MIX** by swirling the bottle. Continue to **HEAT** the solution in 30-second intervals until the agar is completely dissolved (the amber-colored solution should be clear and free of small particles).
4. **COOL** the ReadyPour™ Agar to 60 °C with careful swirling to promote even dissipation of heat.
5. While the medium is cooling, **LABEL** the bottom of the large petri plates "Source" and the bottom of the small petri plates "kan".
6. **PIPET** 10 mL of the cooled ReadyPour™ Agar into each of the five large "Source" plates using a 10 mL pipet and pipet pump.
7. **ADD** 0.7 mL of Kanamycin (D) to the remaining Ready-Pour Agar. **RECAP** the bottle and **SWIRL** to mix the media. **ONLY ADD KANAMYCIN TO COOLED AGAR.** Kanamycin degrades at high temperatures. **RETURN** the remaining Kanamycin to freezer.
8. Using a fresh 10 mL pipet, **PIPET** 5 mL of the LB-kan medium into the 20 small plates.

NOTE for Step 3:

Use extra care and make sure the agar does not boil out of the bottle. Pay close attention and stop the heating if it starts to bubble up.

REMINDER:

Only add reagents to cooled agar (60 °C)!

MODULE II: Pre-Lab Preparations

9. **COVER** and **WAIT** at least twenty minutes for the LB-agar plates to solidify. For optimal results, leave plates at room temperature overnight.
10. **STORE** plates at room temperature for no more than two days, then in the refrigerator for up to two weeks. Plates should be inverted and placed in a sealable plastic bag to ensure that they do not dry out.

***NOTE:** If plates are prepared more than two days before use, they should be stored inverted in a plastic bag in the refrigerator (4 °C). Remove the plates from the refrigerator and warm in a 37 °C incubator for 30 minutes before use.*

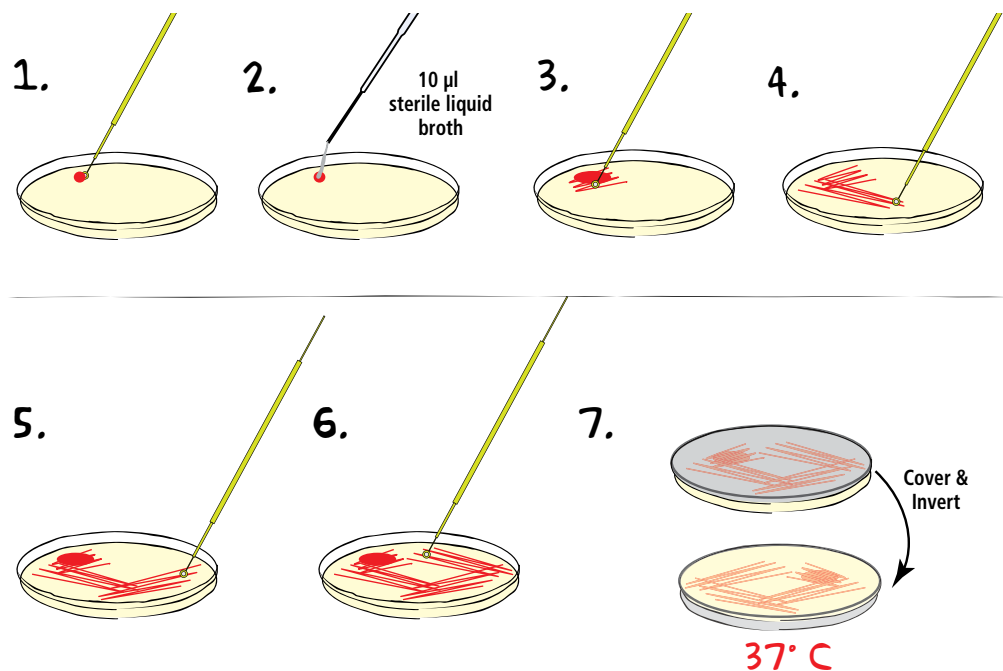
QUICK REFERENCE: POURING LB AGAR PLATES

- Use a sterile 10 mL pipet with a pipet pump to transfer the designated volume of medium to each petri plate. Pipet carefully to avoid forming bubbles.
- Rock the petri plate back and forth to obtain full coverage.
- If the molten medium contains bubbles, they can be removed by passing a flame across the surface of the medium.
- Cover the petri plate and allow the medium to solidify.

MODULE II: Pre-Lab Preparations

PREPARATION OF *E.coli* SOURCE PLATES

For best results, the *E.coli* source plates should be streaked 16-20 hours before the experiment is performed. Preparing the source plates more than 24 hours before the laboratory may compromise the success of the transformation experiment. If you do not have an incubator, colonies will form at room temperature in approximately 24 - 48 hours.



1. **REMOVE** a single BactoBead™ from the vial using a sterile inoculating loop. Using aseptic technique, **TRANSFER** the bead to the edge of a large petri plate (LB source plate) and replace lid. **CAP** the vial immediately after using to limit exposure to moisture in the air.
2. Instantly **DISSOLVE** the bead by adding 10 µL of sterile liquid broth or sterile water.
3. **STREAK** the loop back and forth through the dissolved BactoBead™ to make a primary streak at the top of the plate. Try not to gouge the loop into the medium.
4. **STREAK** the loop through primary streak to a clean part of the agar several times to create a secondary streak.
5. **ROTATE** the plate. **STREAK** the loop through the secondary streak to a clean part of the agar several times.
6. **ROTATE** the plate once more. **STREAK** the loop through the third streak to a clean part of the agar. This should produce isolated colonies.
7. **COVER** the plate and **INCUBATE INVERTED** at 37 °C for 16 to 20 hours. If you do not have an incubator, colonies will form at room temperature in approximately 24 - 48 hours.
8. **REPEAT** the above steps for each of the LB source plates.

NOTE: If growth on plates is heavy (i.e. lawn of colonies), instruct students to transfer a loopful of cells into the CaCl₂ solution.

MODULE II: Pre-Lab Preparations

PREPARATION OF CONTROL DNA FOR TRANSFORMATION

This kit includes three control plasmids. Each control plasmid represents a different combination of insert and vector. If the ligation experiment is not successful, the bacteria transformed with the control plasmids can be used for Modules III, IV, and V. Aliquots of plasmid DNA can be prepared the day before the lab and stored at 4 °C.

- Place the tubes of Control DNA for Transformation (E1, E2, and E3) on ice to thaw.
 - E1 represents a single insert in the 5'-3' orientation
 - E2 represents a single insert in the 3'-5' orientation
 - E3 represents a double insert in the 3'-5' orientation
- Assign each group one of the three control plasmids for transformation.
- Label five 0.5 mL microcentrifuge tubes with "Control" and the group name/number.
- Before dispensing, tap the tubes of control plasmids until all the samples are at the tapered bottom of the tubes. Using an automatic micropipet, dispense 12 μ L of the control plasmid into the appropriately labeled tube. NOTE: Students will use 10 μ L for the transformation experiment.
- Cap the tubes and place them on ice.
- Return the plasmid DNA to the freezer. This DNA can be restriction digested in Module V if the plasmid preparation in Module IV is unsuccessful.

NOTE:

New controls may be used in Modules II and III. Read carefully before performing experiment.

PREPARATION FOR TRANSFORMATION (Day of the Lab)

- Equilibrate waterbaths at 37 °C and 42 °C; incubator at 37 °C.
- Dispense 1 mL of CaCl_2 into microcentrifuge tubes for each of the 5 groups and place on ice. Label tubes CaCl_2 . Temperature of the CaCl_2 is critical. We recommend storing the CaCl_2 in the refrigerator the night before the experiment to ensure that it is at the correct temperature for transformation.
- Dispense 1.5 mL of Luria Broth Medium ("Recovery broth") into tubes for each of the 5 groups and keep at room temperature. (Alternatively, the Recovery Broth bottle can be placed at a classroom pipetting station for students to share)
- Dispense 50 μ L ultra-pure water (A) into 0.5 mL microcentrifuge tube for each of the groups. Label tubes H_2O .

FOR MODULE II, Each Group Requires:

- Ligation Mixture from Module I
- One microcentrifuge tube containing 12 μ L control DNA (E1, E2, or E3)
- One microcentrifuge tube containing 1 mL 0.05M CaCl_2
- One microcentrifuge tube containing 50 μ L ultra-pure water
- One microcentrifuge tube containing 1.5 mL recovery broth
- One *E.coli* source plate
- Two LB-Kan plates
- Two 1.5 mL microcentrifuge tubes
- Two sterile inoculating loops
- Two sterile 1 mL pipets
- Toothpicks
- Ice and ice bucket

MODULE III: Pre-Lab Preparations

CULTURING OF kan^R TRANSFORMANTS

If ligation was unsuccessful, students can pick a single colony off of the transformation control plate for Modules III, IV, and IV (Growth, Purification, and Restriction Digest).

NOTE: Kanamycin Medium should be prepared on the day of the Module III laboratory.

- Thaw the Kanamycin (D).
- Withdraw 0.25 mL of the Kanamycin with a sterile 1 mL pipet.
- Add 0.25 mL of Kanamycin to the growth medium. Cap and swirl to mix.
- Aseptically transfer 10 mL of the medium to each of 5 sterile culture tubes (sterile 50 mL conical tubes). Close the tubes. (NOTE: The culture tubes have a substantially larger volume than 10 mL to allow for adequate aeration during growth.)

MODULE IV: Pre-Lab Preparations

EXTRACTION OF RECOMBINANT PLASMID DNA

Harvesting the Transformed Cells from Liquid Culture:

Cells must be harvested after the 16-20 hour overnight incubation period. At this point, sterility is no longer important.

If your centrifuge has a rotor that can accommodate the 50 mL conical tubes:

1. Harvest the cells by centrifuging the entire culture at 4000 RPM for 5 minutes.
2. Pour off most of the medium, leaving about 500 μ L at the bottom of the tube.
3. Resuspend the pellet in the remaining media and transfer the cells into a fresh 1.5 mL microcentrifuge tube. Make sure to label this tube with the appropriate student group name or number.
4. Centrifuge the tubes again at 4000 RPM for 5 minutes.
5. Pour off the supernatant. The pellet should be the size of a match head.
6. Drain off residual medium by leaving the tubes inverted on paper towels for a few minutes. Take care not to let the cell pellet slip out of the tube.
7. If Module IV (Plasmid Extraction) will be performed on the same day as the cell harvest (steps 1-6), put the cells on ice or in the refrigerator until use. If Module IV will be performed at a later date, freeze the cells until they are required.

FOR MODULE IV, Each Group Requires:

- One 1.5 mL microcentrifuge tube containing a cell pellet
- One clean 1.5 mL snap-top microcentrifuge tube
- One microcentrifuge tube containing 500 μ L Cell Lysis Solution
- One microcentrifuge tube containing 250 μ L Potassium Acetate Neutralization Buffer
- One microcentrifuge tube containing 250 μ L TEG
- One microcentrifuge tube containing 100 μ L 1x TE Solution
- One microcentrifuge tube containing 10 μ L RNase Solution
- Isopropanol - 95-100%
- Ethanol - 70%

If your centrifuge DOES NOT have a rotor that can accommodate the 50 mL conical tubes:

1. Label five 1.5 mL microcentrifuge tubes with each student group name or number.
2. Transfer 1.5 mL of the bacterial culture to the appropriately labeled microcentrifuge tube.
3. Harvest the cells by centrifuging the culture at 4000 RPM for 5 minutes.
4. Pour off the entire volume of medium, leaving the pellet at the bottom of the tube.
5. Transfer another 1.5 mL of the bacterial culture into the appropriate microcentrifuge tube. Centrifuge the tubes again at 4000 RPM for 5 minutes.
6. Repeat steps 4 and 5 until the bacterial pellet is the size of a match head.
7. Drain off residual medium by leaving the tubes inverted on paper towels for a few minutes. Take care not to let the cell pellet slip out of the tube.
8. If Module IV (Plasmid Extraction) will be performed on the same day as the cell harvest (steps 1-7), put the cells on ice or in the refrigerator until use. If Module IV will be performed at a later date, freeze the cells until they are required.

PREPARATION OF LYSIS REAGENTS

The reagents can be aliquoted for each group of students. We recommend the aliquoting the reagents into sets for each lab group as described below. Alternatively, the students can share the stock tubes placed in a central location. Note that sharing the tubes increases the risk of a spill or contamination. Set up each pipetting station with a designated 1 mL pipet for the reagent.

1. Prepare the Cell Lysis Solution. (NOTE: This solution should be prepared fresh and kept at room temperature.) The SDS will precipitate out of the lysis solution if it is stored on ice or in the refrigerator.
 - a. In a small beaker, add all of the Sodium Hydroxide solution (I) to 8 mL of distilled water. Mix.
 - b. Add all of the SDS solution (J) to the beaker. Mix.
 - c. Label this beaker "Cell Lysis Solution". Cover the beaker and keep at room temperature until use.
 - d. If desired, label five 1.5 mL microcentrifuge tubes "Lysis Solution". Dispense 500 μ L of Cell Lysis Solution into each tube.
2. Label five 1.5 mL microcentrifuge tubes "Tris." Dispense 100 μ L of 1X Tris-EDTA buffer (L) into each tube.
3. Label five 1.5 mL microcentrifuge tubes "KOAc." Dispense 250 μ L of Potassium Acetate Solution (M) into each tube.
4. Label five 1.5 mL microcentrifuge tubes "TEG." Dispense 250 μ L of Tris-EDTA-Glucose buffer (H) into each tube.
5. Label five 0.5 mL microcentrifuge tubes "RNase." Dispense 10 μ L of RNase Solution (K) into each tube.
6. Each student group will require 95-100% Isopropanol and 70% Ethanol. These reagents can be dispensed into 1.5 mL aliquots if desired. Cool the 70% Ethanol by placing it on ice 30-60 minutes before use.

MODULE V: Pre-Lab Preparations

RESTRICTION ENZYME ANALYSIS

This kit contains enough restriction enzyme to perform five sets of restriction digests. If the student DNA extractions (Module IV) were successful, the isolated plasmid DNA will be used for restriction digest in Module V. However, if the students DNA extractions were unsuccessful, or if you have fewer than five groups, the control plasmid DNA (Components E1, E2, E3) can be used for the restriction digest.

NOTE:

New controls may be used in Module V. Read carefully before performing experiment.

1. Place the tubes of Control DNA for Transformation (E1, E2, and E3) on DNA on ice to thaw. Once thawed, tap the tubes of control plasmids until all the samples are at the tapered bottom of the tubes.
2. Using an automatic micropipet, dispense 30 μ L of the control plasmid into an appropriately labeled tube.

NOTE: Students will use 25 μ L for the restriction digest experiment.

3. Return control DNA to freezer.

Day of the Lab: Prepare restriction digest within 30 minutes of reconstituting Dryzymes®

1. Equilibrate a 37 °C waterbath.
2. Thaw the Restriction Enzyme Reaction Buffer (O), Dryzymes® Rehydration Buffer (P), and Ultra-Pure Water (N). Place on ice.
3. Rehydrate the Dryzymes® in the rehydration buffer.
 - a. Make sure the solid material is at the bottom of the Dryzymes® tubes. If not, centrifuge the tubes in a microcentrifuge at full speed for 20 seconds or tap the tube on the lab bench.
 - b. Add 100 μ L Rehydration Buffer (P) to the solid at the bottom of each tube containing Dryzymes®.
 - c. Allow the samples to hydrate for 1 minute.
 - d. Mix the samples vigorously by flicking the tubes with your finger or by vortexing for 30 seconds until the solid appears to be completely dissolve.
 - e. Centrifuge for 20 seconds or tap the tubes on the lab bench. At this point the Dryzymes® should be gathered at the bottom of tubes and should have no undissolved particulate matter. If not completely dissolved, repeat mixing or vortexing.

NOTE: At this point, the enzymes can no longer be stored. They must be used as soon as possible. Keep the tubes on ice until use.

4. Aliquot the following reagents into labeled 0.5 mL microcentrifuge tubes. Store on ice until use.
 - a. 200 μ L Ultra-Pure Water (N)
 - b. 40 μ L Restriction Enzyme Buffer (O)
 - c. 7 μ L Diluted *Eco*RI
 - d. 14 μ L Diluted *Pvu*II
 - e. 7 μ L Diluted *Xho*I

AGAROSE GEL ELECTROPHORESIS

Each group will require one 0.8% gel with 6 sample wells for electrophoresis. See Module I-B or Appendix C for detailed preparation information.

1. Aliquot 25 μ L of DNA Standard Marker (Q) and 25 μ L of Supercoiled Plasmid Vector Standard (R) into 0.5 μ L microcentrifuge tubes.
2. Metric rulers and semi-log graph paper (semi-log) will be needed if the students are calculating the restriction fragment sizes by creating a linear standard curve (Appendix C). However, the students should be able to estimate the sizes of the DNA fragments by visual inspection of the gel or a photograph of the gel.

For Module V, Each Group Requires:

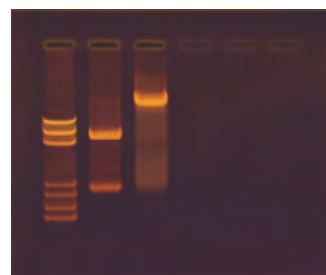
- One purified DNA sample (from Module IV) OR control plasmid DNA
- Five clean 1.5 mL microcentrifuge tubes
- One microcentrifuge tube containing 200 μ L UltraPure water
- One microcentrifuge tube containing 40 μ L Restriction Enzyme Dilution Buffer
- One microcentrifuge tube containing 7 μ L diluted *Eco*RI
- One microcentrifuge tube containing 7 μ L diluted *Xho*I
- One microcentrifuge tube containing 14 μ L diluted *Pvu*II

Experiment Results and Analysis

MODULE 1 RESULTS:

The gel from Module 1 will have 3 lanes: the DNA standard ladder, the ligation control sample, and the ligation reaction sample. The ligation control sample will show 2 bands, the unligated vector and insert at 3,000 and 1,300 bp respectively. The ligation reaction sample should result in just one large band if the ligation reaction was successful. The purpose of this is to visualize the ligated vector and insert from Module 1. Depending on how many inserts were ligated in the vector during the reaction the size of the band will vary. It will be at least 4,300 bp as that is the combined molecular weight of the vector and insert.

Lane	Sample	Result	Molecular Weight (bp)
1	DNA Standard Marker	-----	6751, 3652, 2827, 1568, 1118, 825, 630
2	Ligation Control Sample	Two bands— unligated vector and insert	3000, 1300
3	Ligation Reaction Sample	One large band	Depends on ligation products. May appear larger than 10,000 bp



Experiment Results and Analysis

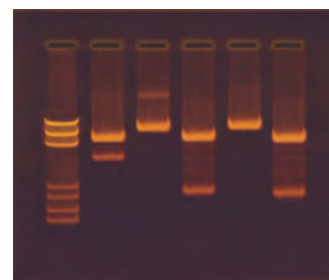
MODULE V RESULTS:

The Module V results will help to determine the orientation of the inserts in the plasmid you extracted in Module IV. Plasmid DNA will be digested with 3 different restriction enzymes that will allow for the creation of a plasmid map. *EcoRI* is used to verify the presence of the *kan^R* insert, *PvuII* is used to determine how many inserts there are, and *XhoI* is used together with *PvuII* as a double digest to determine the orientation of the insert(s). Orientation and gene inserts can be visualized in Figure 5 on page 8.

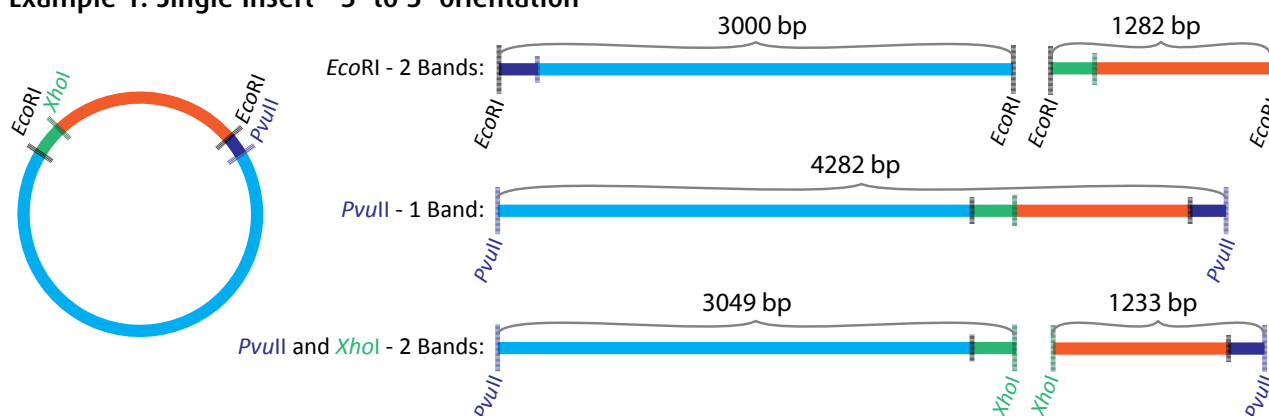
Example 1 (Component E1): Single Insert 5'-3' Orientation

In this example, the gel results indicate a single insert in the 5'-3' direction. Undigested DNA will generally show multiple bands, indicating a mix of linear, nicked, and supercoiled plasmid. The *EcoRI* digest (lane 4) reveals two bands: the 3000 bp plasmid backbone and 1282 bp *kan^R* gene insert. The *PvuII* digest (lane 5) reveals a single band of 4282 bp. Finally, the *PvuII/XhoI* double digest (lane 6) indicates that the *kan^R* gene is in the 5'-3' orientation due to the presence of the 1,233 bp band which is composed of the *kan^R* gene and the *PvuII* site.

Lane	Sample	Result	Molecular Weight (bp)
1	DNA Standard Marker	-----	6751, 3652, 2827, 1568, 1118, 825, 630
2	Supercoiled Plasmid Vector Standard	Several bands, may vary from student to student	Linear DNA is 3000 bp. Supercoiled plasmid appears smaller. Nicked plasmid appears larger.
3	Restriction Digest Control	Several bands, may vary from student to student	Linear DNA is 4282 bp. Supercoiled plasmid appears smaller. Nicked plasmid appears larger.
4	<i>EcoRI</i> Digest	Two bands	3000, 1282
5	<i>PvuII</i> Digest	One band	4282
6	<i>PvuII/XhoI</i> Double Digest	Two bands	3049, 1233



Example 1: Single Insert - 5' to 3' orientation



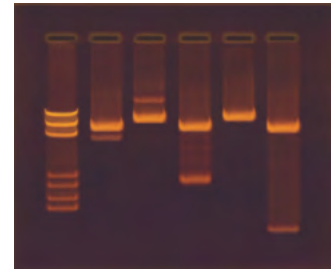
Experiment Results and Analysis

MODULE V RESULTS:

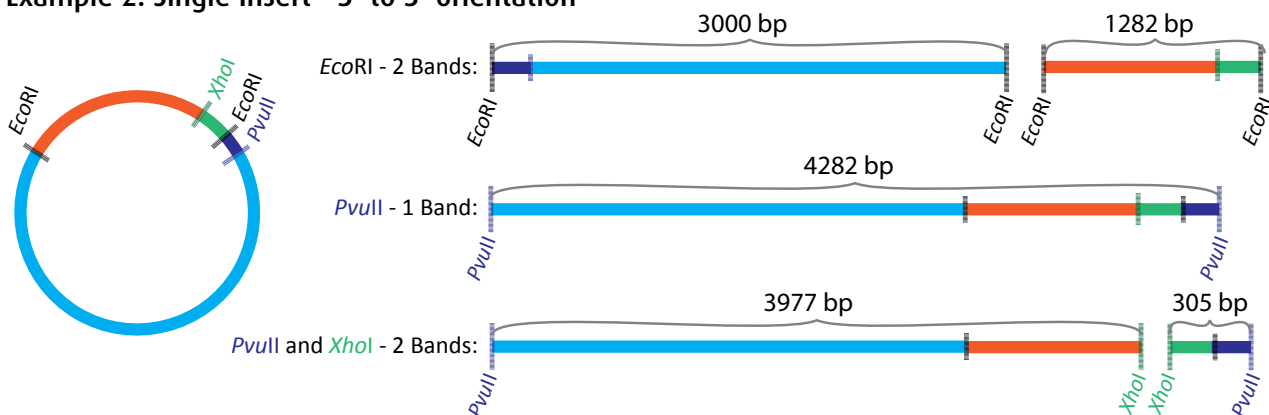
Example 2 (Component E2): Single 3'-5' Orientation

Based on the explanation from Example 1, we can see that the insert is present due to the 2 bands in the *EcoRI* digest lane (lane 4) and that this gel only has one insert because of the single band in the *PvuII* digest lane (lane 5). Once again, the orientation of the insert can be determined from the *PvuII/XhoI* double digest (lane 6). In this example, the presence of 3,977 bp and 305 bp bands indicates that the insert is in the 3'-5' orientation.

Lane	Sample	Result	Molecular Weight (bp)
1	DNA Standard Marker	-----	6751, 3652, 2827, 1568, 1118, 825, 630
2	Supercoiled Plasmid Vector Standard	Several bands, may vary from student to student	Linear DNA is 3000 bp. Supercoiled plasmid appears smaller. Nicked plasmid appears larger.
3	Restriction Digest Control	Several bands, may vary from student to student	Linear DNA is 4282 bp. Supercoiled plasmid appears smaller. Nicked plasmid appears larger.
4	<i>EcoRI</i> Digest	Two bands	3000, 1282
5	<i>PvuII</i> Digest	One band	4282
6	<i>PvuII/XhoI</i> Double Digest	Two bands	3977, 305



Example 2: Single Insert - 3' to 5' orientation



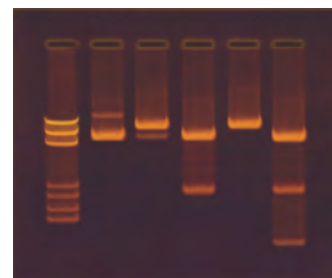
Experiment Results and Analysis

MODULE V RESULTS, CONTINUED:

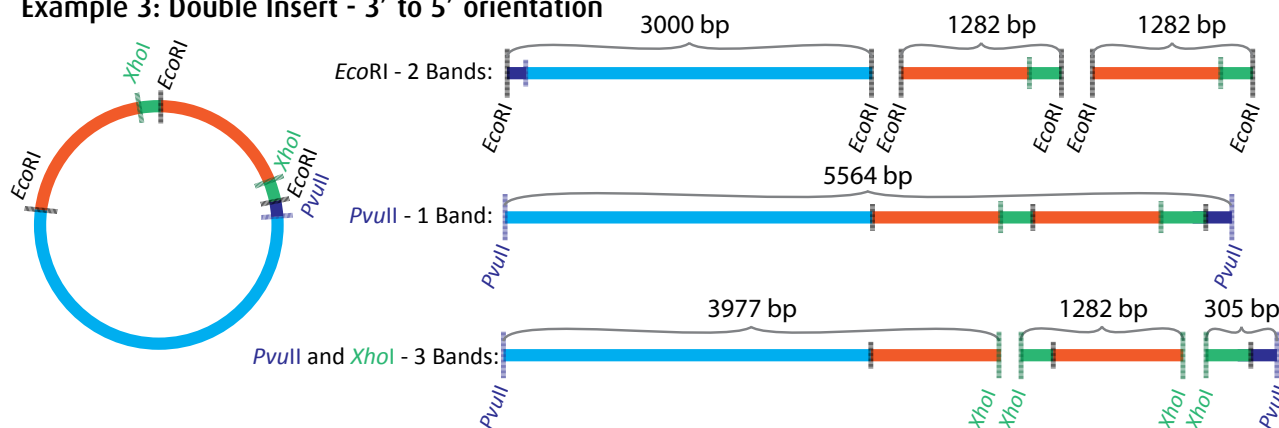
Example 3 (Component E3): Double Insert 3'-5', 3'-5' Orientation

Although there are 2 inserts of the *kan^R* gene, there are only 2 bands visible in the *EcoRI* digest lane because the 2 inserts are the same length of 1,282 bp. Since they're the same length they will run identically on the agarose gel and present as one band. The *PvuII* digest (lane 5) once again shows a single band, although in this example it is 5,564 bp. Finally, the *PvuII/XhoI* double digest (lane 6) shows three bands of 3,977 bp, 1,282 bp, and 305 bp, indicating double inserts, both in the 3'-5' direction.

Lane	Sample	Result	Molecular Weight (bp)
1	DNA Standard Marker	-----	6751, 3652, 2827, 1568, 1118, 825, 630
2	Supercoiled Plasmid Vector Standard	Several bands, may vary from student to student	Linear DNA is 3000 bp. Supercoiled plasmid appears smaller. Nicked plasmid appears larger.
3	Restriction Digest Control	Several bands, may vary from student to student	Linear DNA is 5564 bp. Supercoiled plasmid appears smaller. Nicked plasmid appears larger.
4	<i>EcoRI</i> Digest	Two bands	3000, 1282
5	<i>PvuII</i> Digest	One band	5564
6	<i>PvuII/XhoI</i> Double Digest	Three bands	3977, 1282, 305



Example 3: Double Insert - 3' to 5' orientation



**Please refer to the kit
insert for the Answers to
Study Questions**

Appendices

- A EDVOTEK® Plasmid Map
- B EDVOTEK® Troubleshooting Guide
- C Bulk Preparation of Agarose Gels
- D Data Analysis Using a Standard Curve

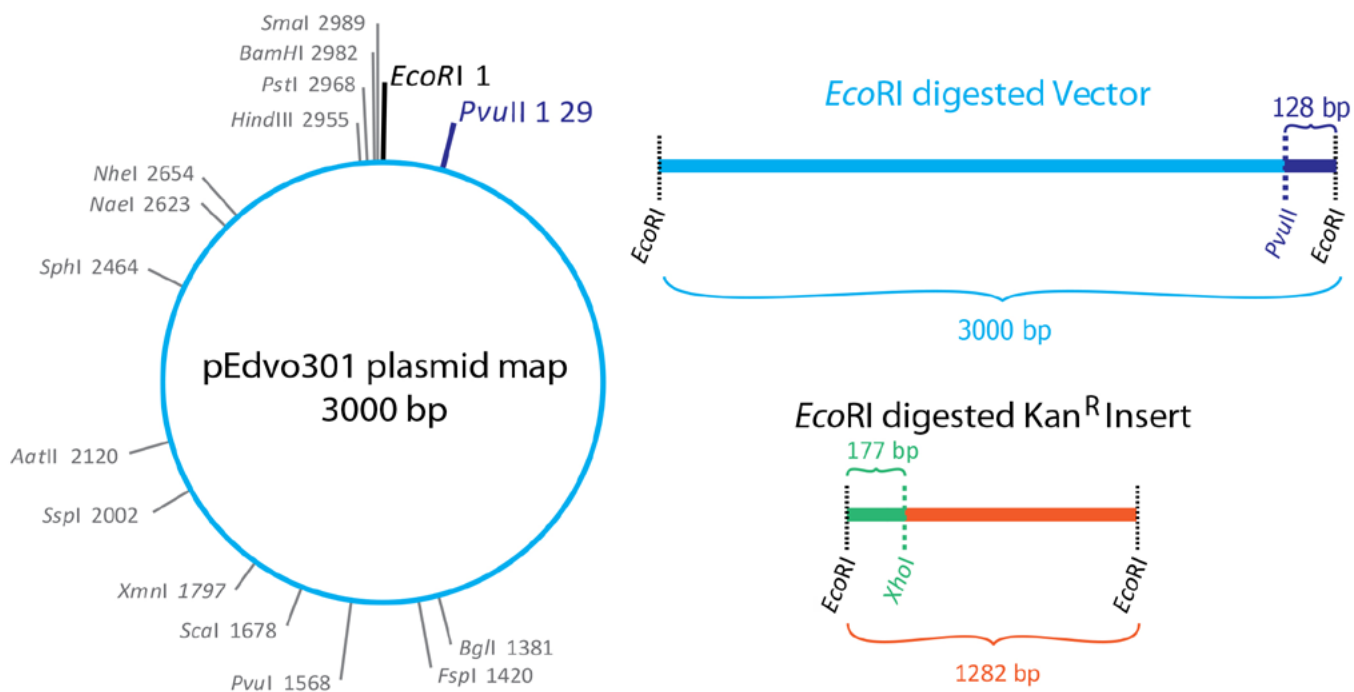
Safety Data Sheets:

Now available for your convenient download on www.edvotek.com/safety-data-sheets

Appendix A

EDVOTEK® Plasmid Map

pEdvo301 Plasmid and Kan^R Insert



Appendix B

EDVOTEK® Troubleshooting Guides

LIGATION AND TRANSFORMATION

PROBLEM:	CAUSE:	ANSWER:
Poor cell growth on source plate	Incubation time too short	Continue to incubate source plate at 37°C for a total of 16-20 hours.
	Antibiotic added to source plate	When pouring plates, be sure to add antibiotics & additives at the correct step.
	Incorrect incubation temperature	Use a thermometer to check incubator temperature. Adjust temp. to 37°C if necessary.
	Agar plate too dry	If plates are prepared more than two days in advance, they should be wrapped in plastic and stored in the refrigerator.
Satellite colonies seen on transformation plate	Incorrect concentration of antibiotics in plates	Ensure the correct concentration of antibiotic was added to plates - Make sure ReadyPour is cooled to 60° C before adding antibiotic.
	Antibiotic is degraded	Make sure ReadyPour is cooled to 60° C before adding antibiotic.
	Plates were incubated too long	Incubate the plates overnight at 37°C (16-18 hours).
Colonies appeared smeary on transformation plate	Plates containing transformants were inverted too soon	Allow cell suspension to fully absorbed into the medium before inverting plates.
	Experimental plates too moist	After pouring plates, allow them dry overnight at room temp. Alternatively, warm plates at 37°C for 30 min. before plating cells
No colonies seen on transformation plates	Plasmid DNA not added to transformation mix	Ensure plasmid DNA was added to transformation tube. Make sure that pipets are used properly. If using micropipets, make sure students practice using pipets
	Incorrect host cells used for transformation	Confirm that correct bacterial strain was used for transformation
	Cells were not properly resuspended in CaCl ₂	Be sure to completely resuspend the cells by vortexing or pipetting up and down.
	Cells were not properly heat shocked	Ensure that temp. was 42°C & heat shock step took place for no more than 45 seconds.
	Incorrect antibiotics	Be certain that the correct antibiotic was used.
	Too many/too few colonies picked from source plate	Pick five colonies from the source plate.
	The source plates are too old.	Important that the source cells grow no longer than 20 hours.
	CaCl ₂ not cold enough	Pre-chill CaCl ₂ before adding the bacterial cells.

continued...

Appendix B

EDVOTEK® Troubleshooting Guides

LIGATION AND TRANSFORMATION, CONTINUED

PROBLEM:	CAUSE:	ANSWER:
Colonies present on control plate but not on ligation plate	Ligation was not successful	Be sure the T4 DNA ligase is completely mixed with the ligation reaction mixture. For best results, incubate the ligation samples at 16° C for 30 minutes.
	DNA was not properly diluted before transformation	Make sure to mix the Diluted Ligation Reaction thoroughly before transformation.
Low transformation efficiency	Incorrect number of cells used for transformation	Carefully pick more colonies from source plate (5 colonies @ 1-2 mm width per 500µl CaCl ₂)
	Source plates were incubated for more than 20 hours	Important that source cells grow no longer than 20 hrs. Refrigerate plates after 20 hrs if necessary. Do not use source plates that have been incubated longer than 24 hours, refrigerated or not).
	Experimental plates too old	Prepare transformation plate and use shortly after preparation
	Cells not well resuspended in CaCl ₂	Completely resuspend the cells in the CaCl ₂ , leaving no cell clumps (vortex or pipet vigorously to fully resuspend cells). Cell suspension should be cloudy.
	CaCl ₂ solution not cold enough	Pre-chill CaCl ₂ before adding cells to the CaCl ₂
	Cell solution not cold enough	Extend incubation of cells in CaCl ₂ + DNA on ice (extra 10-15 min. but not more than 30 min). This allows more DNA to adhere to outside of bacterial cell).
	Too much or too little plasmid DNA added to cell suspension	Ensure that correct volume of plasmid was added to the transformation tube. If using micropipets, make sure students practice using pipets.
	Cells were not properly heat shocked	Ensure that temperature was 42°C and that heat shock step took place for no more than 90 seconds.
	Antibiotics were degraded prior to pouring plates	Make sure ReadyPour is cooled to 60°C before adding antibiotic.
	Incorrect concentration of antibiotics in plates	Ensure that the correct concentration of antibiotic was used

Appendix B

EDVOTEK® Troubleshooting Guides

PLASMID PREPARATION

PROBLEM:	CAUSE:	ANSWER:
Cells did not grow in liquid LB/Kan media	Colony used to inoculate media did not contain the plasmid. ("Satellite" colony protected by secreted antibiotic resistance)	Be sure to pick isolated colonies 1-2 mm in diameter.
There is a precipitate in my Lysis Buffer.	The Lysis Buffer was placed on ice, and the SDS precipitated out	Gently warm the Lysis Buffer to dissolve the SDS. Keep the solution at room temperature before use.
No/small DNA pellet after extraction.	Not enough cells used for lysis.	Be sure the bacterial cell pellet is the size of a match head before moving forward. Make sure cell pellet is completely resuspended in the TEG buffer before adding the Lysis Buffer.
	Cells did not lyse.	Lysis solutions did not mix well. Make sure to thoroughly but gently mix the sample after each buffer addition.
	Plasmid DNA was sheared.	Lysis solutions were mixed too vigorously. Be sure to mix the solutions gently by inverting the tube 4-6 times. The cells should not be incubated in the lysis solution for more than five minutes.
	Pellet was aspirated during wash.	Try gently pouring the supernatant out of the tube instead of using a pipet.
Pellet is hard to resuspend	Pellet was dried for too long.	Dry the pellet for 5-10 minutes
	Some of the white precipitate was left behind in the supernatant.	Make sure to avoid the white pellet when transferring the supernatant to a fresh tube.

Appendix B

EDVOTEK® Troubleshooting Guides

RESTRICTION DIGEST AND ELECTROPHORESIS

PROBLEM:	CAUSE:	ANSWER:
The plasmid DNA did not digest	The restriction enzymes were not active.	Be sure that the restriction enzymes were diluted in the correct buffer (P).
		For optimal activity, prepare the enzymes within 30 minutes of use.
There is only a small amount of SYBR® Safe in my tube.	SYBR® Safe is a concentrate that is diluted before use.	Centrifuge or tap the tube to move the contents to the bottom of the tube. Dilute the SYBR® Safe Stain before use as outlined on page 33..
There are bands on my gels that can't be explained by the restriction digests.	Some bands may represent partially digested DNA.	The sample was not digested at the right temperature.
		The sample was not digested for the appropriate amount of time.
I don't see well-defined bands after electrophoresis, only a smear.	Genomic DNA contamination.	Samples processed too vigorously during plasmid extraction.
The ladder and student samples are not visible on the gel.	The gel was not prepared properly.	Ensure that the electrophoresis buffer was correctly diluted.
		Make sure that the agarose solution is completely clear of "clumps" and glassy granules before pouring gels.
	The gel was not stained properly.	Ensure that diluted SYBR® Safe was added to the gel.
The ladder and control samples are visible on gel, but some student samples are not present.	Malfunctioning electrophoresis unit or power source.	Contact the manufacturer of the electrophoresis unit or power source.
	Student DNA sample was not concentrated enough.	Poor DNA extraction. Extract new DNA.
	Student DNA sample was degraded.	If DNA is not used right after extraction, store sample at -20°C.
Some students have more/less plasmid DNA than others.	Wrong volumes of DNA and enzyme added to restriction digest.	Practice using pipettes.
		There is an inherent variability in the extraction process. Students with low yields can restriction digest supercoiled plasmid DNA (components E1,E2,E3) in lieu of their plasmid.
DNA bands were not resolved.	The tracking dye should migrate at least 4 cm from the wells to ensure adequate separation.	Be sure to run the gel at least 4 cm before staining and visualizing the DNA.

Appendix C

Bulk Preparation of Agarose Gels

To save time, the electrophoresis buffer and agarose gel solution can be prepared in larger quantities for sharing by the class. Unused diluted buffer can be used at a later time and solidified agarose gel solution can be remelted.

Bulk Electrophoresis Buffer

Quantity (bulk) preparation for 3 liters of 1x electrophoresis buffer is outlined in Table D.

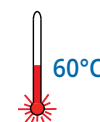
Table D Bulk Preparation of Electrophoresis Buffer			
50x Conc. Buffer	+	Distilled Water	Total Volume Required
60 mL		2940 mL	3000 mL (3 L)

Batch Agarose Gels (0.8%)

For quantity (batch) preparation of 0.8% agarose gels, see Table E.

1. Use a 1 L flask or bottle to prepare the diluted gel buffer.
2. Pour 4.8 grams of UltraSpec-Agarose™ into the prepared buffer. Swirl to disperse clumps.
3. With a marking pen, indicate the level of solution volume on the outside of the flask.
4. Heat the agarose solution as outlined previously for individual gel preparation. The heating time will require adjustment due to the larger total volume of gel buffer solution.
5. Cool the agarose solution to 60 °C with swirling to promote even dissipation of heat. If evaporation has occurred, add distilled water to bring the solution up to the original volume as marked on the flask in step 3.

Table E Batch Prep of 0.8% UltraSpec-Agarose™					
Amount of Agarose	+	Concentrated Buffer (50x)	+	Distilled Water	Total Volume
4.8 g		12 mL		588 mL	600 mL



6. Add the entire tube of **diluted SYBR® Safe** stain (see page 33) to the cooled agarose and mix well.

7. Dispense the required volume of cooled agarose solution for casting each gel. Measure 30 mL for a 7 x 7 cm tray, 45 mL for a 10 x 7 cm tray, and 60 mL for a 14 x 7 cm tray. **NOTE: For this experiment, 7 x 7 cm gels are recommended.**
8. Allow the gel to completely solidify. It will become firm and cool to the touch after approximately 20 minutes. Then proceed with preparing the gel for electrophoresis.

NOTE:

The UltraSpec-Agarose™ kit component is usually labeled with the amount it contains. Please read the label carefully. If the amount of agarose is not specified or if the bottle's plastic seal has been broken, weigh the agarose to ensure you are using the correct amount.

Appendix D

Data Analysis Using a Standard Curve

Agarose gel electrophoresis separates biomolecules into discrete bands, each comprising molecules of the same size. How can these results be used to determine the lengths of different fragments? Remember, as the length of a biomolecule increases, the distance to which the molecule can migrate decreases because large molecules cannot pass through the channels in the gel with ease. Therefore, the migration rate is inversely proportional to the length of the molecules—more specifically, to the \log_{10} of molecule's length. To illustrate this, we ran a sample that contains bands of known lengths called a "standard". We will measure the distance that each of these bands traveled to create a graph, known as a "standard curve", which can then be used to extrapolate the size of unknown molecule(s).

1. MEASURE AND RECORD MIGRATION DISTANCES

Measure the distance traveled by each DNA Standard Marker from the lower edge of the sample well to the lower end of each band. Record the distance in centimeters (to the nearest millimeter) in your notebook. Repeat this for each DNA fragment in the standard.

Measure and record the migration distances of each of the fragments in the unknown samples in the same way you measured the standard bands.

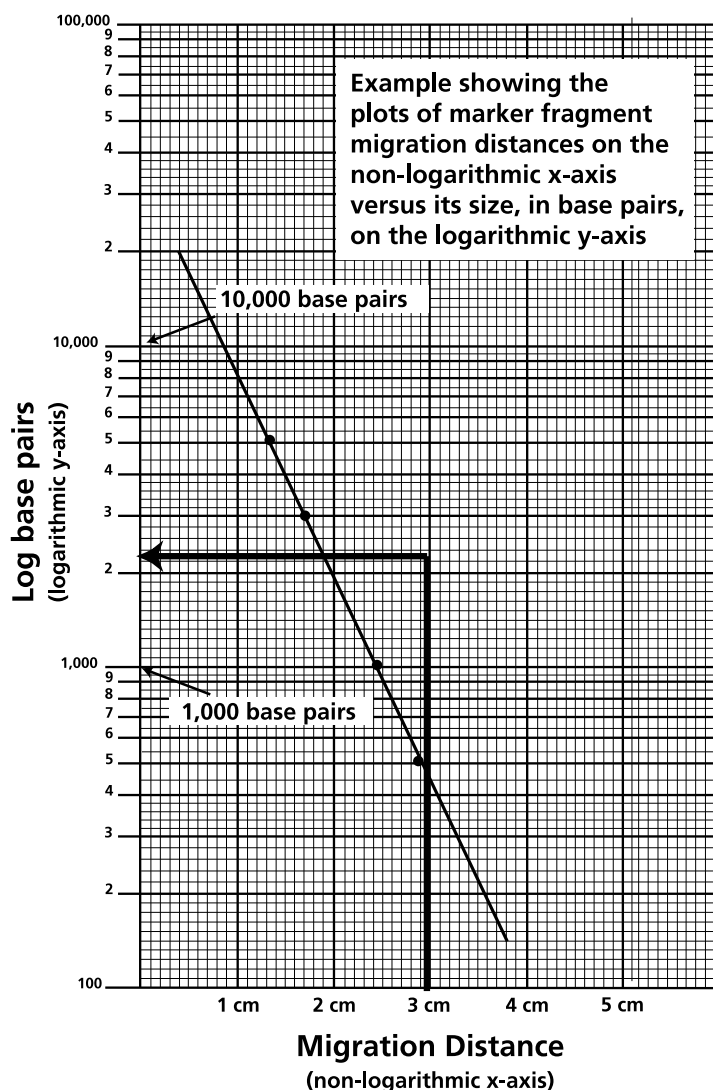
2. GENERATE A STANDARD CURVE

Because migration rate is inversely proportional to the \log_{10} of band length, plotting the data as a semi-log plot will produce a straight line and allow us to analyze an exponential range of fragment sizes. You will notice that the vertical axis of the semi-log plot appears atypical at first; the distance between numbers shrinks as the axis progresses from 1 to 9. This is because the axis represents a logarithmic scale. The first cycle on the y-axis corresponds to lengths from 100-1,000 base pairs, the second cycle measures 1,000-10,000 base pairs, and so on. To create a standard curve on the semi-log paper, plot the distance each Standard DNA fragment migrated on the x-axis (in mm) versus its size on the y-axis (in base pairs). Be sure to label the axes!



Figure 8: Measure distance migrated from the lower edge of the well to the lower edge of each band.

Figure 9: Semilog graph example



Appendix D: Data Analysis Using a Standard Curve, continued

After all the points have been plotted, use a ruler or a straight edge to draw the best straight line possible through the points. The line should have approximately equal numbers of points scattered on each side of the line. It is okay if the line runs through some points (see Figure 9 for an example).

3. DETERMINE THE LENGTH OF EACH UNKNOWN FRAGMENT

- Locate the migration distance of the unknown fragment on the x-axis of your semi-log graph. Draw a vertical line extending from that point until it intersects the line of your standard curve.
- From the point of intersection, draw a second line, this time horizontally, toward the y-axis. The value at which this line intersects the y-axis represents the approximate size of the fragment in base pairs (refer to Figure 9 for an example). Make note of this in your lab notebook.
- Repeat for each fragment in your unknown sample.

Includes EDVOTEK's All-NEW DNA Standard Marker

- Better separation
- Easier band measurements
- No unused bands

NEW DNA Standard ladder sizes:
6751, 3652, 2827, 1568, 1118, 825, 630



