Edvo-Kit # 300

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Blue/White Cloning of a DNA Fragment and Assay of β-Galactosidase

Experiment Objective:

The objective of this experiment is to clone a DNA fragment in the pUC-linker and select colonies that have DNA inserts based on color selection. The experiment is divided into three modules which focus on (1) Ligation, (2) Transformation and selection, (3) Growth of transformants and β-galactosidase assay.

See page 3 for storage instructions.

Version 300.230509

SAMPLE LITERATURE
Please refer to included weblink for correct version

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

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Experiment Components

		Storage	Check (√)
REAGENTS FOR DNA LIGATION			
L1	DNA Vector Linearized with EcoRI and DNA Fragments	-20°C Freezer	❑
L2	Control Superhelical Plasmid	-20°C Freezer	❑
L3	T4 DNA Ligase/ATP Reaction Tubes		
	(5 lyophilized T4 pellets in small screw-capped tubes)	-20°C Freezer	❑
L4	TE Buffer, Sterile	-20°C Freezer	❑
REAGENTS FOR TRANSFORMATION			
TR1	Ampicillin	-20°C Freezer	❑
TR2	IPTG	-20°C Freezer	❑
TR3	X-Gal	-20°C Freezer	❑
TR4	$CaCl_2$	-20°C Freezer	❑
TR5	Sterile Water	-20°C Freezer	❑
REAGENTS AND CELLS FOR TRANSFORMATION			
•	BactoBeads™ JM109	-20°C Freezer	❑
•	ReadyPour™ Agar (sterile)	Room Temp.	❑
•	Recovery Broth (sterile)	Room Temp.	❑
REAGENTS β-GALACTOSIDASE ASSAY			
A1	Bottle LB Growth Medium	Room Temp.	❑
A3	Sodium Phosphate Buffer	Room Temp.	❑
A5	Stop Buffer (Na_2CO_3)	Room Temp.	❑
REAGENTS β-GALACTOSIDASE ASSAY			
A2	Lysozyme	-20°C Freezer	❑
A4	ONPG	-20°C Freezer	❑

Experiment # 300 is designed for 5 groups.

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

DISPOSABLE SUPPLIES

- Microtest tubes (0.5ml)
- 1.5 mL microtest (microcentrifuge) tubes
- Transfer pipets (sterile)
- 10 mL pipets (sterile)
- Petri plates (sterile, 60x15 mm)
- Inoculation loops (sterile)
- Toothpicks

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Requirements *(not included with this kit)*

- Two water baths (37°C and 42°C)
- Microcentrifuge
- Table top clinical centrifuge or floor model centrifuge
- 37°C incubation oven
- Shaking incubator or shaking waterbath
- Automatic micropipets and sterile pipet tips
- Pipet pumps
- Balance
- Microwave or hot plate
- Spectrophotometer (Spectronic 20 or equivalent)
- Autoclave (optional)
- 10 - 125 mL sterile flasks with caps
- 80 - 13x100 mm test tubes
- 80 additional 1.5 mL microtest tubes
- Distilled or deionized water
- Ice

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Blue/White Cloning of a DNA Fragment and Assay of β-Galactosidase

The majority of specialized recombinant DNA molecules used in biotechnology have been constructed by subcloning procedures. Recombinant molecules are vectors designed to meet specific needs in molecular biology research. For example, some vectors have high copy numbers and will produce large amounts of subcloned DNA inserts. Others have been designed to facilitate *in-vitro* transcription and super-expression of proteins *in-vivo*.

Subcloning involves the ligation of a previously cloned and purified DNA molecule into a vector. The resulting recombinant molecule is then introduced into the appropriate host cell where the cloned gene is expressed.

This experiment involves three experimental modules. They are: 1) the ligation of a DNA fragment in a plasmid vector; 2) Introduction of the recombinant DNA into *E. coli* cells by transformation; and 3) selection of ampicillin resistant transformants; selection and growth of Lac^+ and Lac^- colonies; assay of these colonies for β-galactosidase activity. As an optional activity, the recombinant plasmids may be extracted from cells, digested with restriction enzymes, and analyzed by agarose gel electrophoresis (materials not provided).

PLASMID VECTOR

pUC8 is a 2700 base pair plasmid that possesses a single recognition site for *Eco* RI endonuclease, which is located in a M13 mp derived polylinker in the Lac Z fragment. The polylinker region is approximately 30 base pairs in length and contains several unique restriction enzyme sites to facilitate the ligation of DNA in the vector.

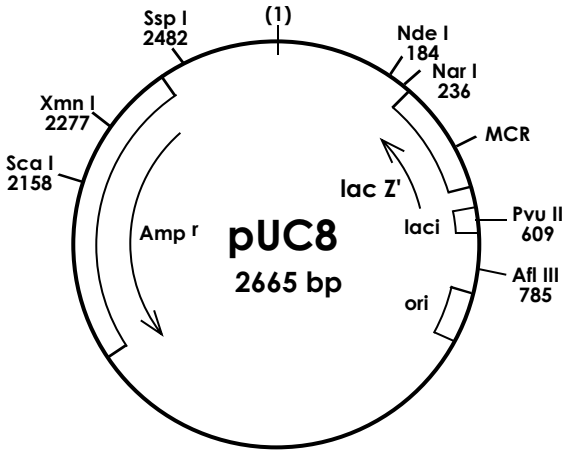

Figure 1

pUC8 is present in multiple copies in host *E. coli* cells. The plasmid has been modified by genetic engineering to contain part of the lac Z gene which codes for β-galactosidase, an enzyme involved in galactoside metabolism.

An operon contains structural genes which carry information for the synthesis of proteins such as enzymes, and regulatory genes that control the expression of structural genes. The lac operon consists of structural and regulatory genes. The lac Z gene is a structural gene required for galactoside metabolism. The pUC8 plasmid carries the alpha fragment of the lac Z gene. The alpha fragment is the amino-terminus of the protein and is not functional by itself. The alpha fragment is denoted by lac Z'.

Typically, the *E. coli* host strain used for transformation is a mutant strain that has a deletion of the alpha fragment of lac Z. The *E. coli* chromosome contains the omega fragment, which is the carboxy-terminus of the protein. The omega fragment is also non-functional. When the alpha and omega fragments are expressed, they interact, which results in a functional β-galactosidase protein. This interaction is called alpha complementation. Alpha complementation was discovered by Ullman, Jacob, and Monod in 1967.

The lac operon is highly regulated by repressors and inducers. Repressors are constitutively produced at low levels by a bacterial cell and keep the operon "turned off". When inducers are present, such as isopropyl-β-D-thiogalactopyranoside (IPTG), the repressor protein binds to the inducer instead of the regulatory site in the DNA molecule. Transcription of the structural genes can then occur, followed by translation into a functional protein molecule.

The substrates for the β-galactosidase enzyme are galactosides, such as lactose. Lactose is hydrolyzed into galactose and glucose. Artificial galactosides, such as 5-Bromo-4-Chloro-3-Indolyl-beta-D-galactoside (X-Gal), are also substrates for β-galactosidase. When hydrolyzed, X-Gal will release a blue precipitate, hence pUC8-transformed *E. coli* colonies will be

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Blue/White Cloning of a DNA Fragment and Assay of β-Galactosidase, continued

blue. Likewise, ONPG (orthonitrophenalgalactopyranoside) can be used as a colorimetric indicator for β-galactosidase activity. When hydrolyzed, it forms a yellow soluble product which can be quantified with a spectrophotometer.

The pUC8 plasmid has a Multiple Cloning Region (MCR) which is inserted into the lac Z' gene in a way that does not interfere with the lac Z function. The MCR or "polylinker" region is approximately 30 bases long and has several unique restriction enzyme sites which makes it versatile for molecular cloning. Foreign DNA can be inserted into the MCR, which interrupts the lac Z' gene and prevent the formation of a functional β-galactosidase protein. Such recombinants will appear as white colonies on selection plates.

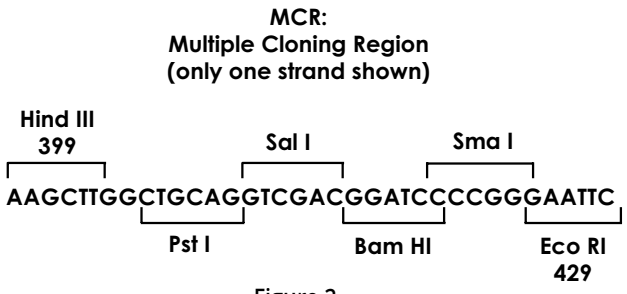

Figure 2

The plasmid also contains an ampicillin resistance gene which codes for β-lactamase. For this experiment, the plasmid has been linearized with *Eco* RI endonuclease to produce compatible termini for the subcloning experiment.

LIGATION

The ligation of the fragments to the linearized vector will be accomplished by the addition of T4 DNA ligase to a reaction mixture of the cleaved vector and DNA fragments (see Figure 3). The enzyme catalyzes the formation of phosphodiester bonds by the condensation of a 5' phosphate and 3' hydroxyl groups of adjacent nucleotides occurring at nicks or between cohesive or blunt termini of DNA. The DNA ligase is purified from T4 phage infected *E. coli*. It requires magnesium and ATP. Each phosphodiester bond formation results in the hydrolysis of ATP to AMP plus pyrophosphate. The catalytic efficiency of the enzyme is optimal at 37˚C. However, ligation of DNA fragments having cohesive termini is usually done at temperatures of 4˚C to 22˚C. Lower temperatures allow for annealing between complementary ends of DNA which is a prerequisite for the ligation of cohesive termini.

In the simplest case, ligation of a vector and the insert DNA result in a circular recombinant plasmid. Ligation of the DNA fragments would occur between the guanine 3' hydroxyl group and the adenine 5' phosphate in the *Eco* RI termini. However, the actual stoichiometry of the vector and insert joined in the ligation reaction is a complex function based on the lengths and

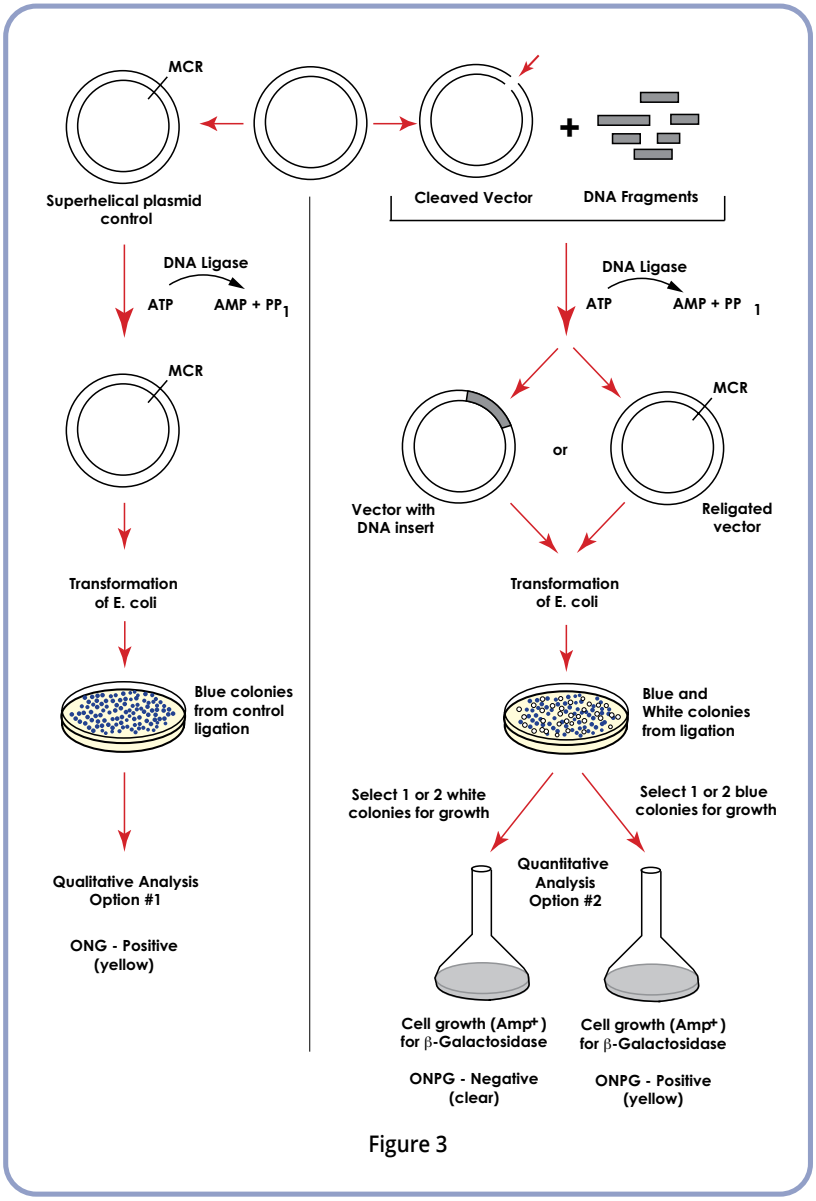

Figure 3

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Blue/White Cloning of a DNA Fragment and Assay of β-Galactosidase, continued

relative concentrations of the two DNA species. The concentration of enzyme and the ionic strength also have an effect. Due to the complimentarity of the *Eco* RI termini, the vector can undergo reclosure without an insert. At higher concentrations it can form concatamers, i.e., larger linear arrays consisting of repeating units of full length vector. Circularization and concatamer formation can also occur with the insert fragment. Alternative combinations and orientations between the vector and insert can also be envisioned. These multiple forms of DNA appear as complex banding patterns observed during electrophoresis of ligation reaction products.

Transformation of competent *E. coli* cells is very inefficient with linear DNA molecules. Therefore, production of circular molecules should be optimized. Furthermore, large recombinant molecules containing multiple arrays of vector and insert will not replicate efficiently and can complicate analysis. Linearized plasmid vectors are sometimes treated with alkaline phosphatase. This phosphomonoesterase removes the 5' phosphates at DNA termini, yielding a 5' hydroxyl group plus inorganic phosphate. Since ligase requires a 5' phosphate for phosphodiester bond formation, vector reclosure and concatamers are eliminated. In this case, the ligation of the insert in the DNA vector will produce nicks at the annealed junctions since only two instead of four phosphodiester bonds can be formed. The nicks are repaired in the transformed host. Concatamers of the insert can be reduced by lowering the concentration of the insert DNA. Increased yields of circular recombinant molecules can be obtained by adjusting the total DNA concentration and the molar ratio of vector to insert.

When the vector and insert contain the same cohesive termini, the orientation of the subcloned insert can vary between individual bacterial colonies that came from the same transformation experiment. This is due to the symmetrical nature of the termini and, statistically, one would expect to find a 50:50 occurrence of the two insert orientations if many colonies are analyzed. A single insert in the recombinant plasmid can be in either one of two directions relative to a fixed point in the vector.

TRANSFORMATION

Competent cells were prepared from cultures of *E. coli* JM109. This strain does not have any natural antibiotic resistance or plasmids and lacks restriction enzymes. In addition, the strain does not produce the RecA protein which reduces the possibility of intracellular recombination events. All these features make *E. coli* JM109 an excellent host for cloning and subcloning experiments.

Transformation with the ligation reaction products performs several functions. Transformation acts as a purification step since it separates the complex mixture of ligation reaction products into individual bacterial colonies or eliminates some of them entirely. Linear vector and very large concatamers are not taken up well by competent *E. coli*. Supercoiled and relaxed circular DNA have the highest transformation efficiencies. Only small amounts of DNA, typically less than 10 nanograms, is required for transformation. In fact, transformation is inhibited by amounts of DNA exceeding 100 nanograms. Only 1 in 10,000 cells successfully incorporate the exogenous DNA. The uptake of two different molecules of DNA by the same cell during transformation occurs at a low frequency.

Transformation efficiency is defined by the number of transformants obtained per microgram of DNA. For example, if 10 nanograms of DNA was used for transformation in 1 mL of cells and one tenth (0.1 mL) was plated and produced 100 colonies on a selective agar medium, this would equate to1000 transformants per mL. Keeping in mind that each colony grew from one transformed cell, the efficiency would be 1000/0.01µg = 1 x 10^5. Transformation efficiencies of 10^5 to 10^6 are sufficient for most subcloning experiments. When the cloning of single copy genes from genomic DNA is done, the required efficiencies are 10^7 to 10^8.

SELECTION FOR BLUE/WHITE COLONIES

Screening can often be tedious and time-consuming. Plasmid vectors usually contain antibiotic resistance genes that are used for the positive selection of bacteria containing the recombinant plasmid that contains the cloned DNA.

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Blue/White Cloning of a DNA Fragment and Assay of β-Galactosidase, continued

The aim of this experiment is to obtain two types of transformed bacterial colonies: blue and white in the presence of X-Gal and IPTG. The blue colonies contain "self" religated plasmids that do not have DNA inserts interrupting the lac Z gene. White colonies consist of bacteria that carry plasmids that have DNA insert fragments that interrupt the lac Z gene. The selection will be done on ampicillin containing medium.

β-galactosidase will be assayed from Lac^+ transformants (blue colonies that produce the active enzyme). A4-orthonitrophen-algalac-topyranoside (ONPG) will be used to assay for β-galactosidase. Upon catalysis, this substrate will form a yellow color. Lac^- (white colonies) will not hydrolyze ONPG and no yellow color will be observed.

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Experiment Overview

EXPERIMENT OBJECTIVE

The objective of this experiment is to clone a DNA fragment in the pUC-linker and select colonies that have DNA inserts based on color selection. The experiment is divided into three modules which focus on (1) Ligation, (2) Transformation and selection, (3) Growth of transformants and β-galactosidase assay.

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.

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.

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Laboratory Safety

Wear gloves and safety goggles

1. Gloves and goggles should be worn routinely as good laboratory practice.
2. Exercise extreme caution when working with equipment which is used in conjunction with the heating and/or melting of reagents.
3. DO NOT MOUTH PIPET REAGENTS - USE PIPET PUMPS OR BULBS.
4. The *E. coli* bacteria used in this experiment is not considered pathogenic. Although it is rarely associated with any illness in healthy individuals, it is good practice to follow simple safety guidelines in handling and disposal of materials contaminated with bacteria.
5. Properly dispose materials after completing the experiment:

   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.

6. Wear gloves, and at the end of the experiment, wash hands thoroughly with soap and water.

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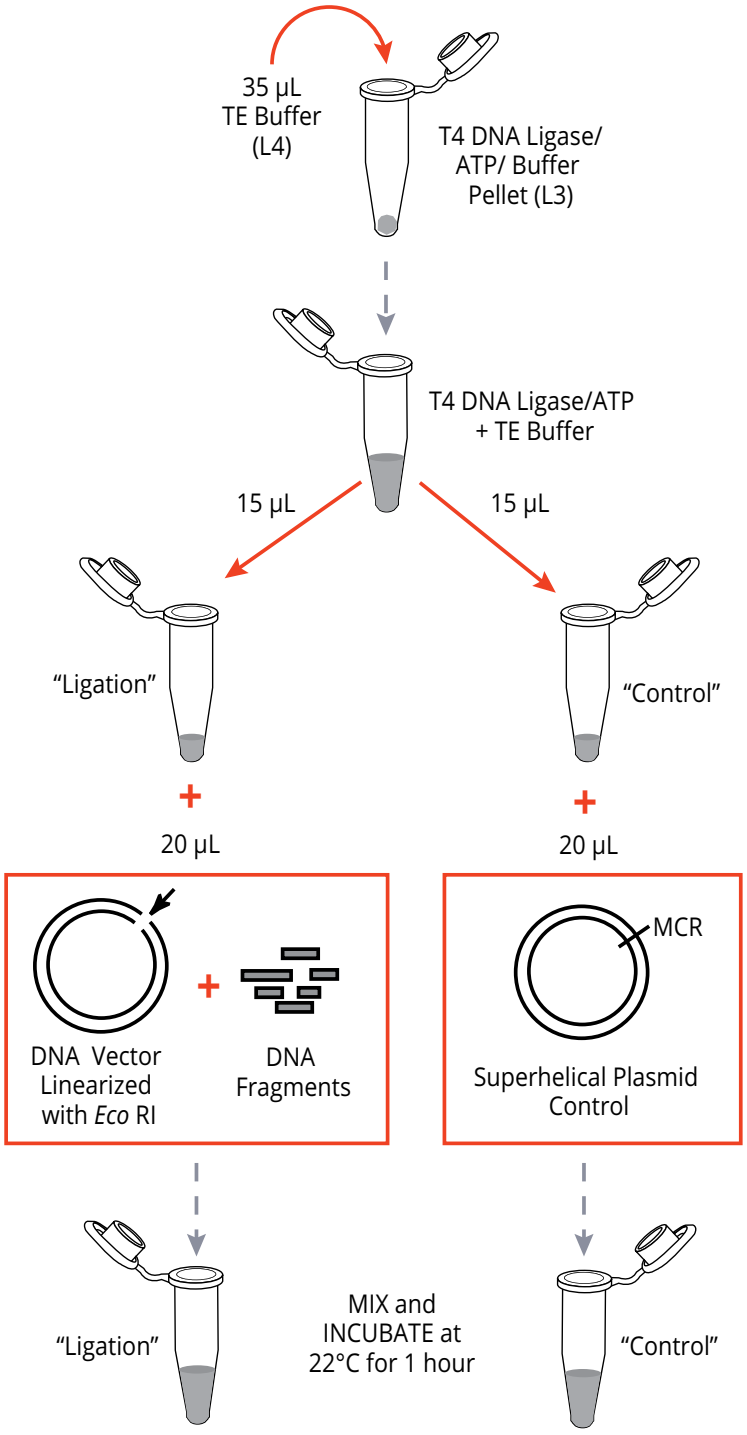
MODULE I OVERVIEW



Continue with the experiment, or freeze the Ligation and Control tubes until needed for Transformation in Module II.

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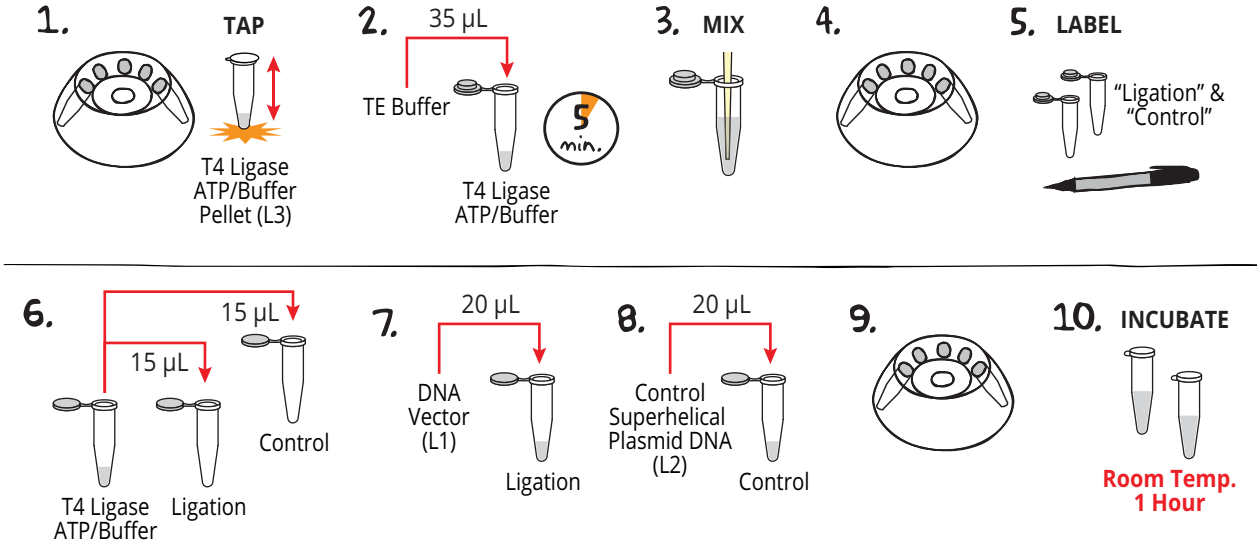
Module I: Ligation of a DNA Insert in the MCR of pUC8 Vector



1. **VORTEX** the tube containing the T4 Ligase/ATP/Buffer pellet (L3). **TAP** it on the lab bench to collect the pellet in the bottom of the tube.
2. **ADD** 35 µL sterile TE Buffer (L4) to the T4 Ligase/ATP Reaction Tube (L3). Allow it to **HYDRATE** for five minutes.
3. Carefully **STIR** the mixture with a pipet tip and gently pipet the solution up and down to **MIX** the buffer and ligase.
4. Briefly **PULSE** the tube in a microcentrifuge to collect all of the solution at the bottom of the tube.
5. **LABEL** and initial two 1.5 mL microtest tubes "Ligation" and "Control".
6. **ALIQUOT** 15 µL of the hydrated T4 Ligase/ATP/Reaction Buffer to the tubes "Ligation" and "Control".
7. To the tube "Ligation" **ADD** 20 µL DNA Vector Linearized with *Eco* RI and DNA Fragments (L1). **MIX** by vortexing or tapping briefly.
8. To the tube "Control", **ADD** 20 µL Control Superhelical Plasmid DNA (L2). **MIX** by vortexing or tapping briefly.
9. **PULSE** the tubes "Ligation" and "Control" in a microcentrifuge to collect all of the sample in the bottom of the tubes.
10. **INCUBATE** at room temperature (approximately 22°C) for 1 hour. **MIX** the tubes periodically by tapping or vortexing at 10 or 15 minute intervals.

OPTIONAL STOPPING POINT

Continue with the experiment, or freeze the Ligation and Control tubes until needed for Transformation in Module II.

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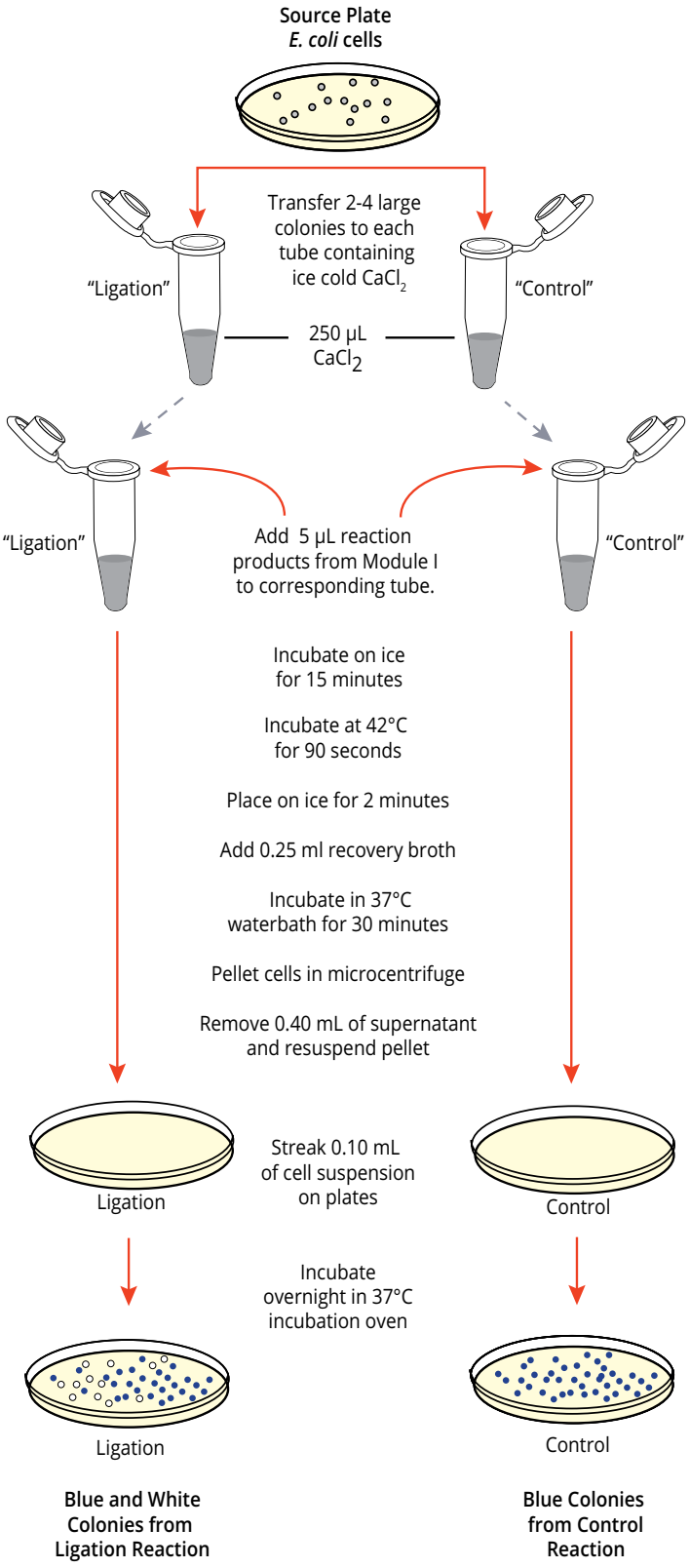
MODULE II OVERVIEW



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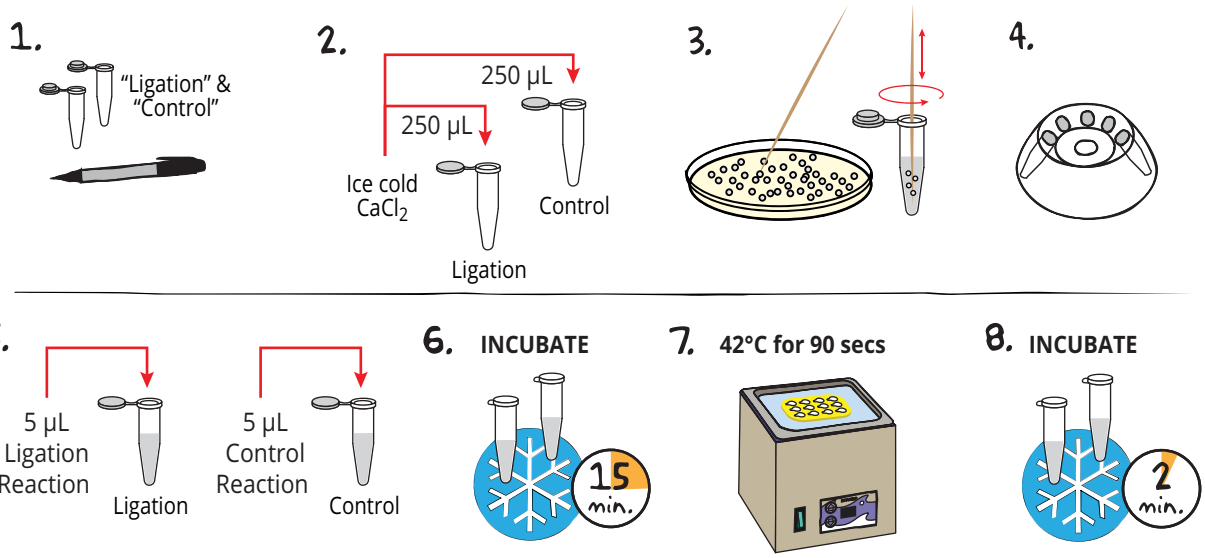
Module II: Transformation and Selection



SETTING UP THE TRANSFORMATION AND CONTROL EXPERIMENT

1. **LABEL** one microcentrifuge tube "ligation" (this will be the transformation tube with ligation DNA). **LABEL** a second microcentrifuge tube "control " (this will be the experimental control with superhelical plasmid DNA).

2. Using a sterile 1 mL pipet, **ADD** 250 µL (0.25 mL) of ice cold $CaCl_2$ solution to each tube.

3. **PICK** colonies from the source plate of *E. coli* cells. To each of the test tubes labeled "ligation" and "control":

   - Use a sterile toothpick to **TRANSFER** 2 colonies (2-4 mm) from the source plate to the test tubes.
   - Between your fingers, **TWIST** the toothpick vigorously and up and down in the $CaCl_2$ solution to dislodge and emulsify the cells.

4. **SUSPEND** the cells in both tubes by tapping or vortexing.

5. **ADD** reaction products from Module I:

   - To the tube labeled "ligation" **ADD** 5 µL of the ligation reaction.
   - To the tube labeled "control" **ADD** 5 µL of the control reaction.

6. **INCUBATE** the two tubes on ice for 15 minutes.

7. Place both tubes in a waterbath at 42°C for 90 seconds for the heat shock step. This facilitates the entry of DNA in bacterial cells.

8. Return both tubes immediately to the ice bucket and incubate for two minutes.

NOTES FOR STEP 3:

Avoid scraping up agar when transferring the cells from the source plate to the tubes with calcium chloride solution. It is important that the cells are resuspended in the calcium chloride solution and are not left on the toothpick or on the wall of the tube.

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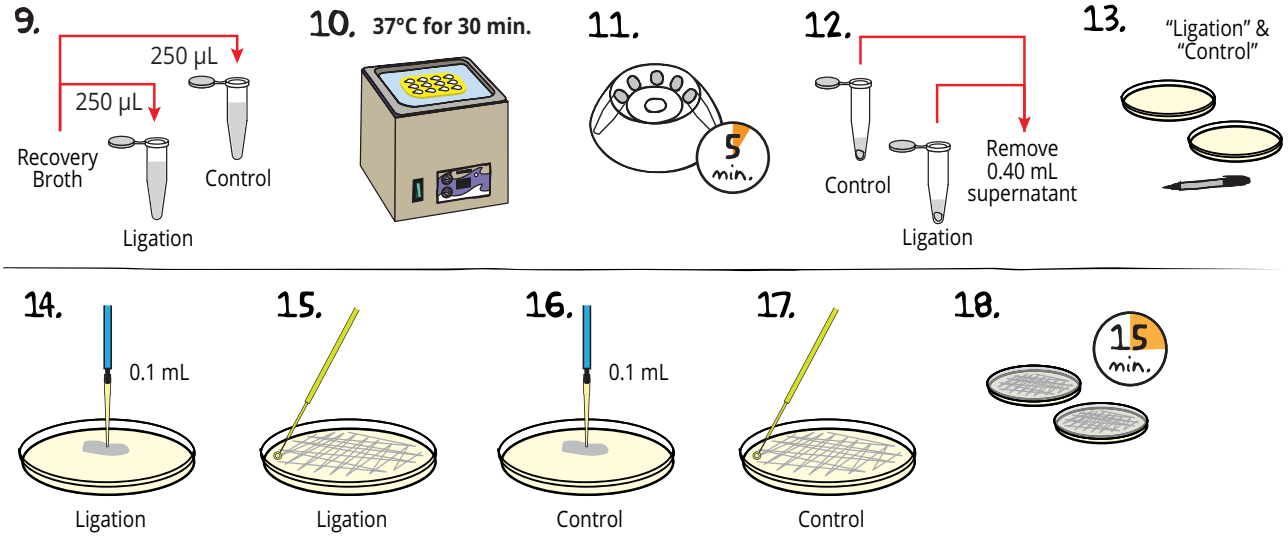
Module II: Transformation and Selection, continued



9. Using a sterile pipet, add 250 µL (0.25 mL) of Recovery Broth to each tube and mix. The Recovery Broth does not contain antibiotic.

10. Incubate the cells for 30 minutes in a 37°C waterbath for a recovery period. This allows the cells to recover and begin to express the antibiotic resistance genes.

11. After the recovery period, remove the tubes from the waterbath and place them in a microcentrifuge and spin for 5 minutes to pellet the cells.

12. Remove and discard 0.40 mL of supernatant and resuspend pellet in remaining liquid.

PLATING THE CELLS

13. Obtain two agar plates ("Ligation" and "Control") and label them with your initials or lab group number.

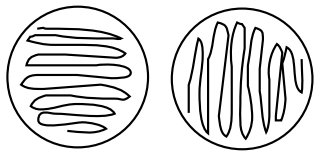
14. Pipet 0.1 mL of the recovered transformed cells in the tube labeled "Ligation" tube to the center of the agar plate labeled "Ligation".

15. Using a sterile loop, spread the cells evenly and thoroughly over the entire surface. Turn the plate 90° and thoroughly spread again.

16. With a fresh pipet, transfer 0.1 mL of recovered cells in the tube labeled "Control" to the middle of the agar plate labeled "Control".

17. Using a fresh loop, spread the cells over the entire surface of the plate as described.

18. Cover both plates and allow the liquid to be absorbed (approximately 15-20 minutes).



QUICK REFERENCE:

DNA and competent cells are combined in a suspension. After the cells have incubated with the DNA, growth medium (recovery broth) is added. Bacterial cells continue to grow through the recovery process, during which time the cell wall is repaired. Cells recover and begin to express the antibiotic resistance gene.

IMPORTANT NOTES:

To avoid contamination when plating, do not set the lid down on the lab bench - lift the lid of the plate only enough to allow spreading. Be careful to avoid gouging the loop into the agar.

If the cells have not been absorbed into the medium, it is best to incubate the plates upright. The plates are inverted to prevent condensation on the lid, which could drip onto the culture and may interfere with experimental results.

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Module II: Transformation and Selection, continued

PREPARING PLATES FOR INCUBATION

19. Stack your group's set of plates on top of one another and tape them together.

20. Put your initials or group number on the taped set of plates.

21. Place the set of plates in a safe place where they will not be disturbed. The plates should be left in the upright position to allow the cell suspension to be absorbed by the agar for 15 - 20 minutes.

22. Place the plates in the inverted position (agar side on top) in a 37°C incubation oven for overnight incubation (15-20 hours).

VIEWING PLATES AFTER INCUBATION

23. Proceed to analyzing your results.

24. After analyzing your results, save the plates to pick up colonies for inoculating liquid bacterial cultures. For other materials used in Module II, properly dispose contaminated materials.

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Module II: Transformation and Selection, continued

DETERMINATION OF TRANSFORMATION EFFICIENCY

Transformation efficiency is a quantitative determination of how many cells were transformed per 1 µg of plasmid DNA. In essence, it is an indicator of how well the transformation experiment worked.

You will calculate the transformation efficiency from the data you collect from your experiment.

1. Estimate the number of transformants (both white and blue colonies) on both plates. A convenient method to keep track of counted colonies is to mark the colony with a marking pen on the outside of the plate.

2. Calculate the transformation efficiencies for total transformants and for colonies that contain vectors with inserts (white colonies).

   The final recovery volume of the cells was 0.50 mL. Because the cells were centrifuged, the volume plated is 0.10 mL. The quantity of DNA used was approximately 25 ng.

   Determine the transformation efficiency using the formula:

$$\frac{\text{Number of transformants}}{\text{µg of DNA}} \times \frac{\text{final vol at recovery (mL)}}{\text{vol plated (mL)}} = \text{Number of transformants per µg}$$

EXAMPLE: Assume you observed 40 colonies:

$$\frac{\text{40 transformants}}{\text{0.025 µg}} \times \frac{\text{0.5 mL}}{\text{0.1 mL}} = \text{8000 } (8 \times 10^3) \text{ transformants per µg}$$

QUICK REFERENCE FOR EXPT. 300:

25 ng of DNA is used.

The final volume at recovery is 0.50 mL.

The volume plated is 0.10 mL.

OPTIONAL STOPPING POINT

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

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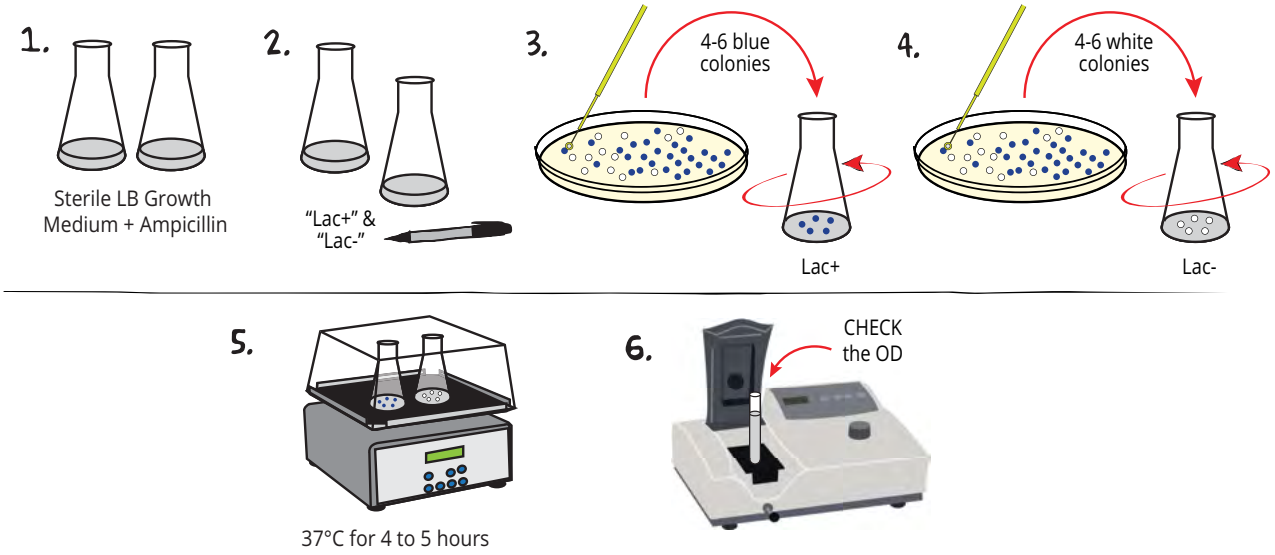
Module III: Assay for β-galactosidase in Blue and White Colonies



Grow Lac$^+$ and Lac$^-$ Cultures for Assay

Four to five hours before the lab, inoculate Lac$^+$ (blue colonies) and Lac$^-$ (white colonies) bacterial culture medium. Alternatively, follow instructions from your instructor.

1. **OBTAIN** 2 flasks (125 mL) that contain 25 mL each of sterile LB Growth Medium + Ampicillin.
2. **LABEL** one flask Lac$^+$ and the other Lac$^-$.
3. With a sterile inoculating loop, **PICK** several (4 to 6) individual blue transformant colonies and **INOCULATE** the flask labeled Lac$^+$. **SWIRL** the flask to suspend bacteria. ***NOTE: Shake loop in broth to allow bacteria to come off the loop and enter the broth.***
4. With a sterile inoculating loop, **PICK** several (4 to 6) individual white transformant colonies and **INOCULATE** the flask labeled Lac$^-$. **SWIRL** the flask to suspend bacteria. ***NOTE: Shake loop in broth to allow bacteria to come off the loop and enter the broth.***
5. **INCUBATE** cultures with shaking at 37°C for 4 to 5 hours.
6. **CHECK** the optical density (OD) at 600 nm. ***NOTE: It should be 0.5 to 0.7 by placing 3 mL in a 13 mm x 100 mm glass tube or 1 mL in a cuvette and placing in a blanked spectrophotometer.***

For cell growth: Use leftover LB + AMP as a blank for OD_{600} absorbance readings.

For β-Galactosidase Assay: Use Distilled H_2O as a blank for OD_{420} and OD_{600} absorbance readings.

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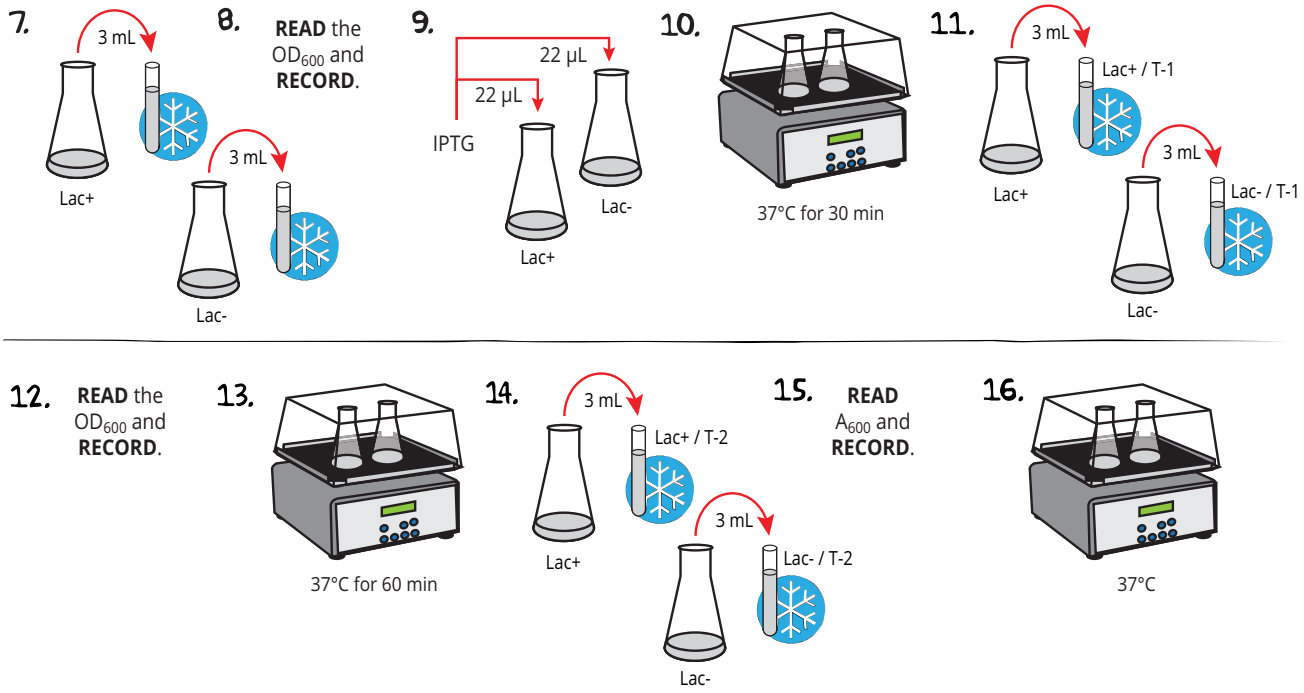
Module III: Assay for β-galactosidase in Blue and White Colonies, continued



Induction OF β-Galactosidase and Sampling

7. **REMOVE** 3 mL from each of the flasks. **RETAIN** these samples as the zero timepoint for the β-galactosidase assay. **LABEL** the tubes Lac^+/ T-0 and Lac^-/ T-0. **PLACE** them on ice for the assay.

8. **READ** the OD_{600} and **RECORD**.

9. To each of the remaining 22 mL of culture, **ADD** 22 µL IPTG as the inducer of β-galactosidase activity.

10. **RETURN** the cultures to the 37°C shaking incubator for 30 minutes.

11. After 30 minutes, **REMOVE** 3 mL from each culture and **PLACE** in a 13 x 100 mm tube. **LABEL** the tubes Lac^+/ T-1 and Lac^-/ T-1. **PLACE** them on ice.

12. **READ** the OD_{600} and **RECORD**.

13. **RETURN** the remaining cultures (19 mL) to the 37°C shaking incubator.

OPTIONAL STEPS 14-16:

14. To obtain better results, after an additional 60 minutes, **REMOVE** 3 mL from each culture and **PLACE** in a 13 x 100 mm tube. **LABEL** the tubes Lac^+/ T-2 and Lac^-/ T-2. **PLACE** them on ice.

15. **READ** A_{600} and **RECORD**.

16. **RETURN** cultures (16 mL) in flask to the 37°C shaking incubator.

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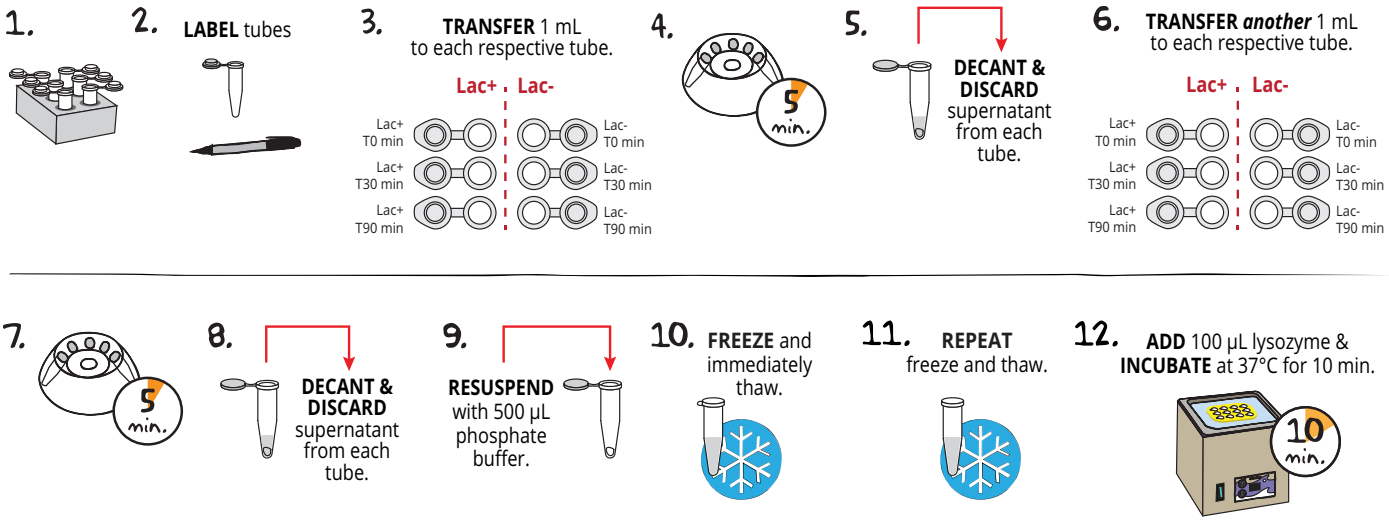
Module III: Assay for β-galactosidase in Blue and White Colonies, continued



β-GALACTOSIDASE ASSAY

1. **SET UP** the assay tubes by placing six (6) 1.5 mL microcentrifuge tubes in a rack.

2. **LABEL** the 6 tubes:

Lac^+ / T0 min	Lac^- / T0 min
Lac^+ / T30 min	Lac^- / T30 min
Lac^+ / T90 min	Lac^- / T90 min

3. **TRANSFER** 1 mL of the cultures on ice from each sample into each respective assay tube.

4. **SPIN** the cells for 5 minutes in a microcentrifuge tube to pellet the cells.

5. **DECANT** and **DISCARD** the supernatant.

6. **TRANSFER** another 1 mL of the cultures on ice into each respective assay tube.

7. **SPIN** the cells again for 5 minutes to pellet the cells.

8. **DECANT** and **DISCARD** the supernatant and **SAVE** each pellet.

9. **RESUSPEND** the pellets in 500 µL phosphate buffer (A3).

10. **FREEZE** the suspension until solid and immediately thaw. ***NOTE: Cells can be frozen quickly in dry ice or by spreading tubes out (lay flat) in a -20°C freezer. Cells can be thawed at room temperature or by a brief incubation in the 42°C waterbath (just long enough to thaw).***

11. **REPEAT** the freezing and thawing a second time.

12. **ADD** 100 µL of lysozyme to each tube and **INCUBATE** at 37°C for 10 minutes.

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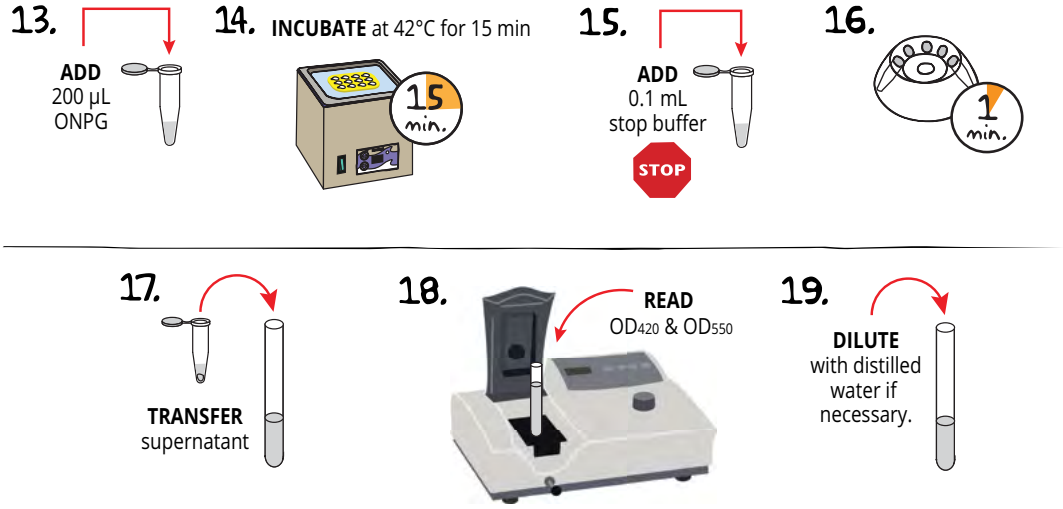
Module III: Assay for β-galactosidase in Blue and White Colonies, continued



13. **ADD** 200 µL of ONPG to each assay tube.

14. **INCUBATE** the assay tubes for 15 minutes in a 42°C waterbath.

15. **ADD** 0.1 mL of stop buffer (Na_2CO_3) to stop the reactions.

16. **CENTRIFUGE** the tubes in a microcentrifuge for 1 minute to pellet the cells.

17. For each tube, **TRANSFER** the clear supernatant to a clean tube or cuvette and **LABEL** them appropriately.

18. **USE** distilled water as a blank. **READ** OD_{420} and OD_{550}.

19. If reading is higher than 0.8, **DILUTE** with distilled water and **RECORD** the dilution factor.

20. **DETERMINE** the units of enzyme activity. Units are defined as Miller units based on the equation which follows.

$$\text{Miller Units} = \frac{1000 \times [\, OD_{420} - 1.75 \times OD_{550}\,]}{T \times V \times OD_{600}}$$

where:
- OD_{420} and OD_{550} are read from the ONPG reaction
- OD_{600} is read from the cell culture optical density
- T is the time in minutes of the ONPG reaction
- V is the volume of the cell culture used in the ONPG reaction in mLs

21. **DISINFECT** all liquids, medium, plates, and plasticware that has been in contact with bacterial cells by soaking them in 10% bleach overnight or sterilize by autoclaving.

The reading at 420 nm is the combined absorbance from O-nitrophenol and light scattering by particle materials such as cell debris. Absorbance at 550 nm corrects for light scattering with no contribution from the O-nitrophenol reaction. Light scattering at 420 nm is equal to (-1.75 x OD_{550}).

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Study Questions

Answer the following study questions in your laboratory notebook or on a separate worksheet.

1. Why does this cloning experiment yield both blue and white colonies?
2. Do all the white and blue colonies contain a plasmid?
3. Will the lysozyme used to lyse cells denature β-galactosidase?
4. Which restriction enzyme is best suited for cloning in pUC8?

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Instructor's Guide

IMPORTANT READ ME!

Transformation experiments contain antibiotics which are used for the selection of transformed bacteria. Students who have allergies to antibiotics such as penicilllin, ampicillin, kanamycin or tetracycine should not participate in this experiment.

ORGANIZING AND IMPLEMENTING THE EXPERIMENT

Class size, length of laboratory sessions, and availability of equipment are factors which must be considered in the planning and the implementation of this experiment with your students.

Prior to commencing this experiment, carefully check that you have all the necessary experiment components and required equipment. Check the lists of Components and Requirements on pages 3 and 4 to ensure that you have a complete inventory to perform the experiment.

The guidelines that are presented in this manual are based on five laboratory groups. The following are implementation guidelines, which can be adapted to fit your specific set of circumstances. If you do not find the answers to your questions in this section, a variety of resources are available at the EDVOTEK web site. In addition, Technical Service is available from 8:00 am to 5:00 pm, Eastern time zone. Call 1-800-EDVOTEK for help from our knowledgeable technical staff.

This experiment has three modules:

I. Ligation of a DNA Insert in the Multiple Cloning Region (MCR) for pUC8 Vector
II. Transformation and Selection
III. Picking and Growth of Lac^+ and Lac^- Transformants

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Notes to the Instructor

NATIONAL CONTENT AND SKILL STANDARDS

By performing this experiment, students will develop skills necessary to do scientific inquiry, learn new techniques using several types of biotechnology equipment, and will learn standard procedures used in transformation. Analysis of the experiments will provide students the means to transform an abstract concept into a concrete explanation.

APPROXIMATE TIME REQUIREMENTS

1. After setting up the ligation, Module I requires a 1 hour incubation. The experiment can be temporarily stopped after the completion of Module I and later resumed. Experimental results will not be compromised if instructions are followed as noted under the heading "Optional Stopping Point" at the end of the module.
2. Module II includes a 30 minute incubation in a 37°C waterbath. There is also an overnight incubation of plates at 37°C before students can perform Module II and before proceeding to Module III.
3. Module III requires a 4-5 hour culture incubation to allow for growth of transformant colonies. This requires that the instructor or student(s) prepare the cultures for incubation (at 37°C with shaking) prior to induction of β-Glactosidase and sampling, followed by the enzyme assay.

Preparation For:	What to do:	When:	Time Required:
Module I	Prepare reagents for ligation	Before the lab period.	15 min.
Module II	Prepare LB agar plates	2-7 days before use	1 hour
	Prepare *E. coli* 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	Preparation of β-Galactosidase assay reagents	The day before (store frozen) or the morning of the activity.	20 min.

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

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Pre-Lab Preparations - Module I

LIGATION OF DNA FRAGMENTS IN pUC8

FOR MODULE I
Each Group should receive:

- 1 Tube with 25 µL of L1, DNA vector linearized with Eco RI and DNA fragments
- 1 Tube with 25 µL of L2, Control Superhelical Plasmid DNA
- 1 Tube with 50 µL of L4, TE Buffer

Enough reagents are provided to perform 5 ligation reactions. You may aliquot the reagents for each lab group as described in step 2.

Alternatively, students can share the stock tubes in a central location.

NOTE: Sharing the tubes increases the risk of a spill or contamination.

1. Shortly before the lab begins, **THAW** and place on ice:

   L1 DNA vector linearized with *Eco* RI and DNA fragments
   L2 Control Superhelical Plasmid DNA

2. For each lab group, **TRANSFER** the following volumes into separate, **ice cold** 0.5 mL microtest tubes that are appropriately labeled.

   - 25 µL of L1 DNA vector linearized with *Eco* RI and DNA fragments
   - 25 µL of L2 Control Superhelical Plasmid DNA
   - 50 µL of L4 TE Buffer

3. **KEEP** all of the tubes on ice.

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Pre-Lab Preparations - Module II

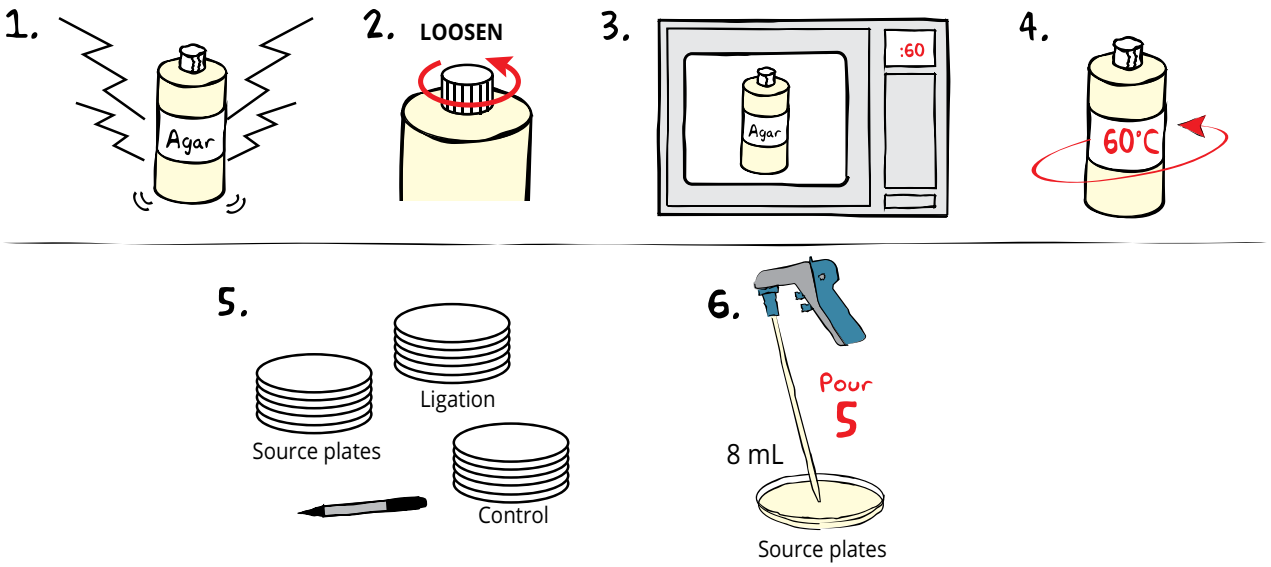
POURING LB-AGAR PLATES

For optimal results, prepare plates two days prior to plating and set aside the plates inverted at room temperature. If they are poured more than two days before use, they should be stored inverted in the refrigerator. Remove the plates from the refrigerator and store inverted for two days at room temperature before use.

Preparing the Reagents:

- **THAW** the X-Gal solution (TR3) and Sterile Water (TR5).
- **ADD** 0.75 mL (750 µL) Sterile Water (TR5) to the tube containing Ampicillin (TR1). **VORTEX** or **SHAKE** vigorously to dissolve the powder and **PLACE** on ice.
- **ADD** 0.70 mL (700 µL) Sterile Water (TR5) to the tube containing IPTG (TR2). **VORTEX** or **SHAKE** vigorously to dissolve the powder and **PLACE** on ice.

Heating the Agar and Pouring the Plates:



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 **REMOVE** 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 ReadyPour medium is cooling, **LABEL** a total of 15 petri plates. **LABEL** these plates on their bottom halves:
   - 5 plates: Source plates
   - 5 plates: Ligation
   - 5 plates: Control
6. Once the ReadyPour™ has cooled to 60°C, **POUR** the 5 source plates 8 mL each (See Quick Reference: Pouring Agar Plates).

CONTINUED

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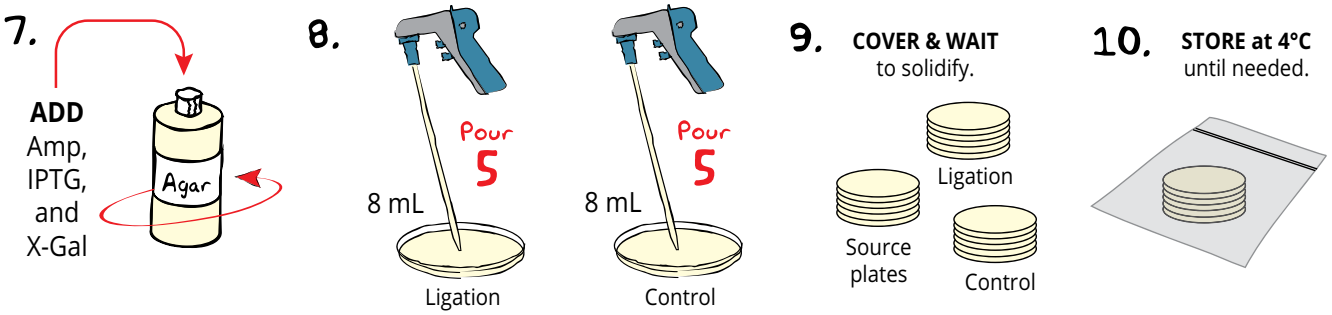
Pre-Lab Preparations - Module II



7. **ADD** 0.30 mL of ampicillin (TR1), 0.30 mL of IPTG (TR2), and all of the X-Gal (TR3) to the medium with sterile pipets. ***ONLY ADD REAGENTS TO COOLED AGAR.*** **SWIRL** the medium to mix. **RETURN** the remaining ampicillin and IPTG to the freezer for Module III.
8. **POUR** the remaining plates, 8 mL each. (See Quick Reference: Pouring Agar Plates).
9. **COVER** and **WAIT** for the LB-agar plates to solidify. For optimal results, leave plates at room temperature overnight.
10. **STORE** plates at 4°C until needed. Plates should be inverted and placed in a sealable plastic bag to ensure that they do not dry out.

NOTE:
Add ampicillin, IPTG, and X-Gal to medium which has been **cooled**. Hot medium will cause rapid decomposition of ampicillin.

NOTE: If plates are prepared more than two days before use, they should be left on the bench overnight to solidify and dry. The following day, store inverted plates 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 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.

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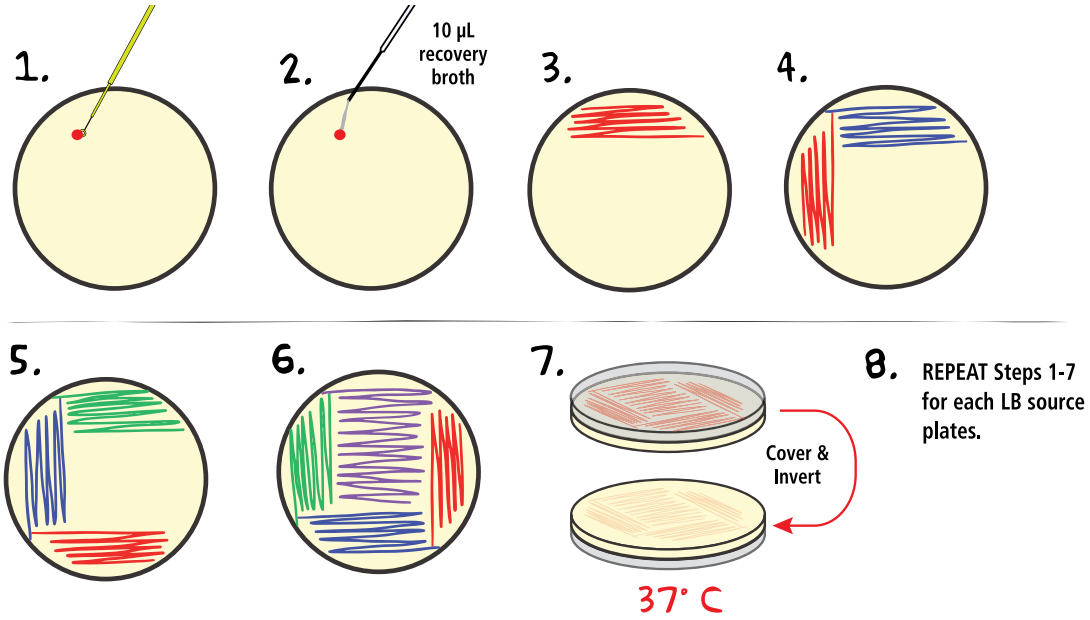
Pre-Lab Preparations - Module II

PREPARATION OF *E.coli* SOURCE PLATES

For best results, the *E. coli* source plates should be streaked 18-22 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.



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. **DISSOLVE** the bead by adding 10 µL of recovery broth.
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. **ROTATE** the plate 90°. STREAK the loop through primary streak once, then zig-zag across a clean part of the agar several times to create a secondary streak.
5. **ROTATE** the plate. **STREAK** the loop through the secondary streak once and then across a clean part of the agar several times.
6. **ROTATE** the plate once more. **STREAK** the loop through the third streak and then zig-zag across the remaining clean agar. This should produce isolated colonies.
7. **COVER** the plate and **INCUBATE INVERTED** at 37°C for 18-22 hours. If you do not have an incubator, colonies will form at room temp. in approximately 24 - 48 hours, although transformation efficiency will decrease.
8. **REPEAT** the above steps for each of the LB source plates using a new loop for each plate.

NOTE: If growth on plates is heavy (i.e. lawn of colonies), instruct students to transfer a loopful of cells into the $CaCl_2$ solution.

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Pre-Lab Preparations - Module II

OTHER PREPARATIONS FOR TRANSFORMATION EXPERIMENT

- **Day of the Lab**

1. **DISPENSE** 1 mL of $CaCl_2$ (TR4) into microcentrifuge tubes labeled "$CaCl_2$" for each of the groups and place on ice.
2. **ALLOW** ample time for the equilibration of water baths and incubation ovens.
3. **ASSEMBLE** reagents and materials for 5 lab groups.

FOR MODULE II
Each Group should receive:

- 1 Ligation plate
- 1 Control plate
- 1 *E. coli* source plate
- 1 Ligation reaction from Module I
- 1 Ligation control from Module I
- 1 Tube of 1 mL $CaCl_2$

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Pre-Lab Preparations - Module III

MODULE III - Assay for β-Galactosidase in Blue and White Colonies

1. **THAW** the Ampicillin (TR1). **PREPARE** growth medium by adding 0.4 mL of ampicillin to LB growth medium (A1).
2. **OBTAIN** 10 sterile (autoclaved) 125 mL flasks and aliquot 25 mL of growth medium + ampicillin into each.
3. **ARRANGE** for students to inoculate assay cultures 4 to 5 hours before the lab.

   Alternatively, the instructor may inoculate the cultures. Cultures may be grown to early exponential phase (OD_{540} = 0.3 to 0.5) and can be stored on ice for up to 4 hours.
4. **ADD** all of the Sodium phosphate buffer (A3) to 27 mL of distilled water. **ALIQUOT** 5.5 mL for each group into covered tubes.
5. **ALIQUOT** 1.5 mL of stop buffer (A5) into covered tubes for each group.
6. **DISSOLVE** the ONPG (A4-orthonitrophenalgalactopyranoside) in 20 mL distilled water (the ONPG may be difficult to get into solution). **ALIQUOT** 3 mL into covered tubes and store on ice. The final concentration is 4 mg/ml of ONPG.
7. **THAW** the IPTG (TR2) and aliquot 60 µL into 5 microtest tubes.
8. **DISSOLVE** the lysozyme (A2) in 10 mL distilled water and dispense 1.5 mL into 5 tubes labeled "lysozyme". Store on ice.
9. **PREPARE** a 42°C waterbath for the latter part of Module III.

Qualitative β-galactosidase Reaction Analysis

Students can also use the plates from the transformation experiment. Pick an equal number of blue and white colonies (15-20 colonies each) and place them in two microcentrifuge tubes. Suspend in 500 µL of phosphate buffer and follow the protocol outlined on page 20 starting with step 5 in the β-galactosidase assay.

FOR MODULE III
Each Group should receive:

- 2 flasks containing 25 mL of growth medium + ampicillin
- Sterile loops
- 60 µL diluted IPTG (TR2)
- 5 mL tubes
- Six 1.5 mL microcentrifuge tubes
- 5.5 mL diluted phosphate buffer (A3)
- 1.5 mL diluted stop buffer (A5)
- 3 mL diluted ONPG (4 mg/mL)
- 1.5 mL diluted lysozyme (A2)

- Access to 37°C shaking incubator
- Access to 37°C & 42°C waterbaths
- Access to microcentrifuge
- Cuvettes + access to spectrophotometer (OD420, OD550, OD600)

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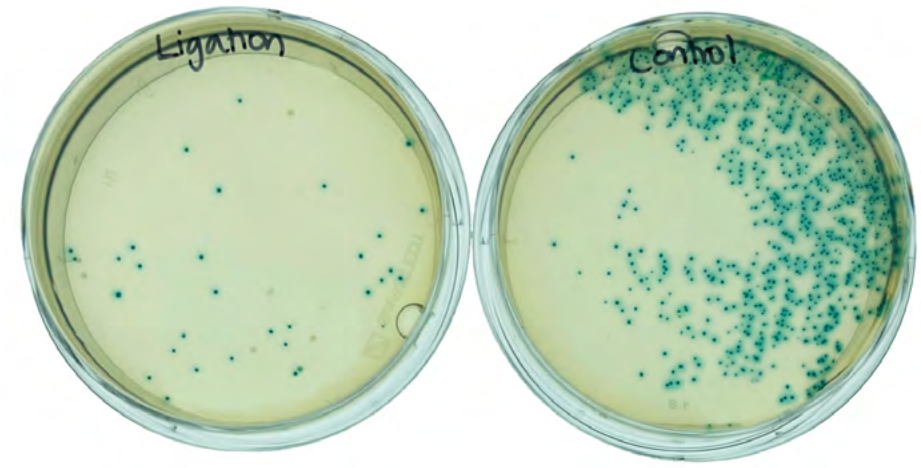
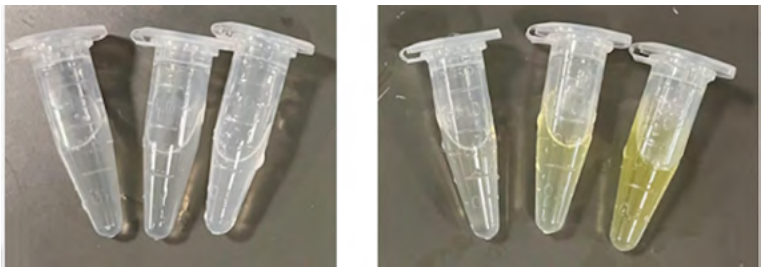
Experiment Results and Analysis

MODULE II RESULTS



MODULE III RESULTS



β-gal Assay: Lac- samples on left, Lac+ samples on right.

OD600			
	T0	T1	T2
Lac+	.446	.492	.667
Lac-	.796	.990	1.406

OD550			
	T0	T1	T2
Lac+	.09	.111	.146
Lac-	.128	.314	.384

OD420			
	T0	T1	T2
Lac+	.230	.720	1.435
Lac-	.205	.492	.575

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

Appendix A
Troubleshooting Guides

TRANSFORMATION TROUBLESHOOTING GUIDE

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 18-22 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.
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 (18-22 hours).
Colonies appeared smeary on transformation plate	Plates containing transformants were inverted too soon	Allow cells to fully absorb 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 individual colonies seen on source plates	Cells were not properly quadrant streaked.	Have students transfer a small loopful of bacteria to the $CaCl_2$.
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 and are properly calibrated.
	Incorrect host cells used for transformation	Confirm that correct bacterial strain was used for transformation
	Cells were not properly heat shocked	Ensure that temp. was 42°C & heat shock step took place for exactly 45 seconds.
	Incorrect antibiotics	Be certain that the correct antibiotic was used.
	Cells not well resuspended in $CaCl_2$	Completely resuspend the cells in the $CaCl_2$, leaving no cell clumps (vortex or pipet up and down to fully resuspend cells). Cell suspension should be cloudy.
Low transformation efficiency	Not enough cells used for transformation	Pick more colonies from source plate (5 colonies @ 1-1.5 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 up and down 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 celll suspension on ice 10-15 min. (should not exceed 30 min. total). This increases the transformation efficiency.
	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 45 seconds.
	Antibiotics were degraded prior to pouring plates	Make sure ReadyPour is cooled to 60°C before adding antibiotic.
	Incorrect concentration of antibiotics	Ensure that the correct concentration of antibiotic was used in plates.

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