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Phenotypic Characterization of Auxotroph Mutants Obtained by Transposon Mutagenesis

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The experiments presented in this workshop are part of a semester-long inquiry-based molecular biology laboratory course offered to fourth year biological science undergraduate students. The experiments presented here can however be easily adapted to second or third year level laboratories. In the lab course, *E. coli* transposon mutants were created using a suicide plasposon (a plasmid containing a transposon). The transposon mutants were then screened for auxotroph mutants and the auxotrophs obtained were further characterized phenotypically and genotypically. This workshop focuses on the isolation and phenotypic characterization of auxotroph mutants. During the workshop, the participants will be introduced to the use of a plasposon, pRL27 (containing the Tn5-RL27 transposon), to create random transposon mutation. Participants will then screen for auxotroph mutants, and identify and confirm the phenotype of the mutants using plating techniques. Analysis of the results and ability to adapt the experiment for the screening of other types of mutants will be discussed.

Keywords: plasposon pRL27, auxotroph mutants; transposon, phenotypic characterization

Introduction

The experiments presented in this workshop are part of a semester-long laboratory course in molecular biology. The course, offered to fourth year undergraduate students in Biological Sciences specialized in Biotechnology, is an intensive lab course with 6 h of labs per week (3 h lab sessions over two consecutive days). Part of the course (such as the experiments presented in this workshop) can easily be adapted to second- or third-year level laboratories. The course is designed as a mini research project. This format exposes students to the process of "doing" research and helps them develop critical thinking and originality. The course also focuses on the theoretical background behind key molecular biology techniques, reinforcing the students' ability to properly perform the experiments and interpret the results obtained, while enhancing their "trouble-shooting" skills. Other learning outcomes include maintaining a laboratory notebook, performing oral presentations, and writing a scientific manuscript on their research.

The course starts with the creation of transposon mutants in a wild type strain of *Escherichia coli, E. coli* R10. The transposon mutants are screened for auxotrophs (mutants that are unable to synthesize an essential nutrient, such as amino acids, nucleic acids or vitamins). The nature of the auxotroph mutants is phenotypically characterized using a plate assay and selected auxotrophs are further characterized using genetic analysis including Southern blot, cloning and sequencing of the mutated genes, identification of the mutated genes using bioinformatics, and finally cloning of the wild type genes using PCR in an attempt to complement the mutations.

The experiments presented in this workshop focus on the first part of the course, namely the creation of the transposon mutants and the screening and phenotypic characterization of the auxotroph mutants. These experiments can be completed within five, 2-3h laboratory sessions.

Transposon and Transposon Mutagenesis

Transposons are mobile genetic elements that have been found in organisms from bacteria to man (Babakhani, and Oloomi, 2018; Muñoz-López and García-Pérez, 2010). The characteristic of these elements is that they are capable of transposition, meaning that they can insert themselves into other DNA molecules or in different locations of the same DNA molecule. Transposition does not require base sequence homology with the DNA host in order to insert itself. In molecular biology, transposons are used to mutate genes (insertion mutation) in order to study their function. Transposons that are the most useful for this purpose are the ones that insert themselves randomly in the

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host genome and insert only once, so that the new observed phenotype can be attributed to mutation into a single gene.

In its simplest form, a transposable element is defined by the presence of inverted repeat sequences (identical sequences reading in opposite directions also called mosaic elements, ME; see Figure 2 in the student's section) flanking a DNA fragment containing the *tnp* gene. The *tnp* gene codes for the transposase enzyme. This enzyme is responsible for the transposition of the transposon. In "cut and paste" type of transposons, the transposase:

- recognizes and binds to the inverted repeat sequences and excises the transposon out of its original location;
- cuts the host double-stranded DNA
- inserts the transposon into the host genome.

It was observed that the transposon itself was not required to carry the *tnp* gene; the transposase enzyme provided *in trans* was indeed able to induce transposition. This discovery led to the creation of novel transposition systems in which the transposase enzyme was either provided *in trans* as a protein or have the *tnp* gene cloned in a suicide plasmid (plasmid unable to replicate in the recipient cells). In both cases, the lack of transposase enzyme being produced in the recipient cells, eliminated the risk of a transposon being able to jump from one place to another on the host genome and therefore ensured stability of transposition insertion. The pRL27 plasposon (a plasmid containing a transposon; Larsen *et al*., 2002) used here, is an example of this type of novel transposition system.

Characteristic of the pRL27 Plasposon

The pRL27 plasposon (see Figure 1), created from the transposon Tn5, is made of two distinct portions: the transposon portion (in red) and the vector portion (in blue).

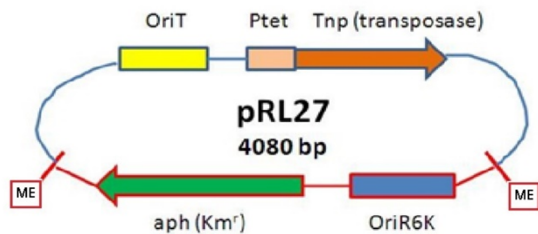


Figure 1. Genetic map of pRL27 plasposon

(a) The transposon portion of pRL27 (called Tn5-RL27) is flanked by the inverted repeats (ME; red tick marks) of the Tn5 transposon. Inside the transposon, the *aph* gene provides resistance to the antibiotic kanamycin (for selection), and a conditional origin of replication (OriR6K) allows for the plasposon to replicate in cells (such as *E. coli* BW20767) that produce the π protein, encoded by the *pir* gene. When these plasposons are introduced into non-producing π hosts (such as the wild type *E. coli* R10 strain), they are unable to replicate and therefore behave as suicide plasmid.

(b) The vector portion of pRL27 contains the RP4 origin of transfer (OriT) and a *tnp* gene, coding for the transposase enzyme, under the control of a *tetA* promoter. The OriT allows the plasposon to be transferred to other cells via conjugation as long as the donor cell (such as *E. coli* BW20767) possesses the RP4 transfer *tra* genes. The *tnp* gene in pRL27 codes for a mutated hyperactive version of the Tn5 transposase enzyme with higher than normal transposition rates. It is also constitutively expressed under the control of the *tetA* promoter. Because the *tnp* gene is located on the vector portion of the plasposon (outside of the transposon), the plasposon and therefore the *tnp* gene will be lost shortly after conjugation into the host cell as the plasposon (suicide plasmid) cannot replicate in cells that do not produce the π protein. Therefore when transposition occurs in the host DNA (such as *E. coli* R10), the resulting transposon insertion will be very stable due to the lack of transposase enzymes in the host cell.

Summary of Laboratory Activities and Tips

For the lab experiments presented here, it is best if students already have some basic lab skills such as knowledge on how to use micropipettes, how to perform dilutions and sterile techniques. If these skills are new to the students, you may want to dedicate a lab session teaching these skills before starting the activities.

Safety: All the experiments presented in this workshop should be performed using sterile techniques. Sterile techniques involve working closely to a live flame as well as flaming tools and flaming alcohol when sterilizing the spreader. Make sure each student knows how to work safely with a live flame. Sterile techniques should be taught or reviewed in the first lab session.

In lab 1, the students will learn about sterile techniques and perform the conjugation experiment.

- The students start by learning/reviewing sterile techniques including the transfer and inoculation of media, as well as spreading and streaking plates.

- For the conjugation experiment, the students receive overnight cultures of the donor and recipient cells grown in antibiotic containing media. The donor cells (BW20767/pRL27; Larsen *et al.*, 2002; Metcalf *et al.* 1996) are grown in a growth media containing kanamycin (Km) to ensure that only cells containing the plasposon are growing. The recipient cells (*E. coli* R10) are gown in ampicillin (Amp) media as the cells

are naturally resistant to this antibiotic. The cells are first "washed" with saline solution to remove the antibiotics and are conjugated by spotting the two cell types on top of each other on a LB plate. The two cell types are also spotted on their own as controls. The only difficulty in this part of the experiment is to make sure the spots are deposited carefully onto the plate to avoid splashes that would lead to contamination of the controls.

In lab 2, the students start by preparing plates for the selection of the transposon mutants. They also resuspend their mating and control spots, perform serial dilutions (Appendix A) and plate on selective plates.

- The plates prepared include LB media supplemented with Km and Amp (LB KmAmp) as well as plates with Amp alone (LB Amp). The first set of plates select for the *E. coli* R10 transposon mutants (*E. coli* R10::Tn5-RL27). The Amp plates are used to determine the number of R10 recipient cells.

- While the plates are drying, the students resuspend the mating and control spots into saline solution and perform serial dilutions before spreading the diluted spots on the selection plates. Reviewing how to perform dilutions and how to accurately measure volumes with a micro-pipettor is critical for the success of this part of the experiment.

In lab 3, the students count the number of colonies on their plates, record their data and calculate the transposition efficiency (# of mutated cells / total number of recipient cells). Then they patch well isolated single colonies from the LB KmAmp plates (the one containing the transposon mutants) on both M9-glucose and LB KmAmp plates in this order.

- When the students count their plates, make sure that they only count the dilutions that give them "countable numbers" of colonies. Countable numbers are between 20 and 200 colonies per plate. Below 20, the number of colonies is considered to be too low to be accurate. Above 200, they are too many colonies to count accurately. Be careful, the students tend to have a hard time understanding that they are plating different dilutions of a bacterial culture in order to find the ONE dilution that gives them countable numbers; they usually try to count colonies from all the plates!

- The screening for auxotroph mutants uses the fact that these mutants are unable to grow on minimal media such as M9-glucose as they are unable to synthesize an essential nutrient but can grow on complex media such as LB as this media provides all the nutrients. Therefore, when patching single and well-isolated colonies on M9-glucose followed by LB KmAmp plates, the colonies that can grow on LB media but cannot grow on M9-glucose are likely to be auxotroph mutants. We typically have each student patch 150-200 colonies following a 50 squares grid templates of the size of a petri dish (see Appendix B); the instructor and TA usually join the patching party so enough mutants can be screened! Note that students can pick-up colonies for patching from any LB KmAmp plates (not just the countable one), as long as the colonies are well isolated. Ideally, you want the class to screen > 2,500 colonies. Out of those, we usually end up with ~20 potential auxotroph mutants. Note that the patching of colonies is a fairly repetitive work during which students are instructed not to talk to avoid contaminating their plates. So we turn on the music to make the task less monotonous….. ♫♫♫

In lab 4, the patches of the potential auxotroph mutants obtained in lab 3 are resuspended in saline solution and spotted on M9-glucose, the 11 auxanography plates and LB KmAmp plates in this order, to identify the type of auxotroph mutants the students have isolated. This assay is based on the fact that the auxotroph mutants can grow on minimal media as long as the media is supplemented with the nutrient they cannot synthesize.

- The auxanography screening system was developed by Davis *et al*. (1980) in which each M9-glucose plate is supplemented with 5 amino acids, nucleic acids and/or vitamins (see Table 1 in the Student Outline section). The combination of nutrients added in the plate is not random; it follows the biosynthesis pathways of those nutrients. Two plates will have one nutrient in common so growth on two plates will identify the nutrient the auxotroph cannot produce. Please notice that there are exceptions where some mutants will only grow on one plate or will grow on more than 2 plates. Also, the nutrients on plate 11 only are present in this plate.

- In addition to plating on the auxanography plates, the auxotrophs are plated again on M9-glucose and LB KmAmp plates to confirm that they are indeed auxotroph transposon mutants (no growth on M9-glucose but growth on LB KmAmp). We typically also spot *E. coli* R10 wild type strain as a control which will show growth on M9-glucose and on the auxanography plates but not on LB KmAmp.

During lab 4 all potential auxotroph mutants are also streak-purified on LB KmAmp in order to

obtain single colonies.

The day before lab 5, a single colony of each auxotroph is inoculated into LB Km broth and grown overnight so that cultures are available for the labs. We typically only inoculate the strains whose auxotrophy can be determined from the results of the auxanography plating performed in lab # 4. We encourage the students to come and perform this activity but if they are unable to come, we will perform this task for them.

In lab 5, the students confirm the nature of the auxotroph mutants by plating them on M9-glucose supplemented with the amino acid, nucleic acid or vitamin they believe the auxotroph cannot synthesize.

- To do so, the nutrient stock solution is simply spread onto a M9-glucose plate at a concentration of 5 mL/L. Considering that a Petri dish contains ~25 mL of agar media, the students are asked to figure out how much stock solution (0.125 mL) they need to spread onto the plate.

- Once the plate is dried, the auxotroph is streaked onto the plate and the plate is allowed to grow for 24-48h. The presence of single colonies on the supplemented plate is confirmation that the auxotroph was unable to synthesize the nutrient added to the plate. The students record the entire class data (for all the characterized auxotroph mutants) as results for the experiment.

Student Assignments and Evaluations

To prepare for each experiment, students have to maintain a laboratory notebook. Before each lab, they have to write the title of the experiment, some background information and the materials and methods of the experiment performed that day. During the lab, the students can update their procedure (if different from what they have written before coming to the lab) and record the raw data of their experiment. In between the lab sessions, they need to perform the analysis of the results obtained and conclude their experiment.

Students also need to read and understand the lab manual of the experiment they will perform in order to do well on the 10-minute closed-book quiz given at the beginning of each lab (quiz questions sample can be found in Appendix C). Finally, students are required to write a manuscript-style lab report for the whole experiment.

Student Outline

Objectives:

- Use pRL27 to create random insertion mutations into the genome of the wild type *Escherichia coli* R10 strain.
- Screen for auxotroph mutants
- Perform phenotypic characterization of the mutants
- Confirm the phenotypic characterization of the mutants

Introduction

In this laboratory you will perform a mini-research project, which involves the creation of transposon mutants in a bacterial strain and phenotypically characterizing some of the mutants obtained. The project starts by mutating the genomic DNA of *Escherichia coli* R10 using transposon mutagenesis. The transposon mutants are first screened for auxotroph mutants. The type of auxotrophy is then phenotypically characterized and confirmed using plating assays.

Prototroph Versus Auxotroph

Prototrophs are organisms that are able to synthesize all the essential cellular compounds they need from simple inorganic substances, water and a source of energy such as glucose. In the laboratory, this environment can be re-created in what we call a minimal media such as M9. As long as a carbon source (i.e. glucose) is provided, these organisms are able to grow on this media. Some mutations in prototroph organisms render them unable to synthesize specific essential compounds (such as amino acids, nucleic acids, vitamins). Such mutants are called auxotrophs because they have to obtain the essential compound they can no longer synthesize from their environment in order to grow. In nature, these organisms would very likely die. In the lab, these organisms cannot grow on M9-glucose media anymore but they can grow on M9-glucose supplemented with the compound they cannot synthesize and they can also grow on complex media, such as LB, because complex media contain vitamins, amino acids, organic and inorganic compounds. For example, bacteria with a mutation in one of the genes involved in the pathway for synthesis of arginine is referred to as an arginine auxotroph because it cannot synthesize arginine anymore. An arginine auxotroph cannot grow on M9-glucose media, except if the minimal media is supplemented with arginine. This organism is able to grow on LB because this is a complex media that contains all nutrients.

Mutagenesis

Mutagenesis is a process whereby the genetic information of an organism is modified. This can occur spontaneously or due to exposure to mutagens. Greater understanding of the different processes leading to mutations has prompted researchers to design laboratory procedures that are able to induce mutation in organisms for a variety of reasons, such as:

- Determining the function of a gene and the relationship between structure and function of a protein (using site-specific mutation of a gene)
- Identifying the location of transcriptional units
- Identifying genes involved in a physiological process so that their identity, relative size, number, and genes organization can be studied
- Creating strains with desired properties, such as the ability to overproduce a desired metabolite or enzyme

The basic process for creating mutations involves the alteration of a cell’s DNA followed by the screening and selection for the desired phenotype. For example, if you were studying the histidine biosynthetic pathway in a microorganism, you might first mutagenize the DNA of the cells and then screen for mutants that can no longer grow in the absence of histidine (histidine auxotroph). Four general treatments can be used to mutagenize organisms: ionizing radiation, chemical mutagens, site-directed mutagenesis and transposons.

Transposons and Transposon Mutagenesis

Transposons are mobile genetic elements that have been found in organisms from bacteria to man. The characteristic of these elements is that they are capable of transposition; meaning that they can insert themselves into other DNA molecules or in different locations of the same DNA molecule. Transposons that are the most useful for gene analysis via insertion mutation, are the ones that can insert themselves randomly into the host DNA sequence and that only transpose once per genome. Some transposons do not require base sequence homology with the DNA

host for transposition to occur and can therefore randomly insert themselves into the host genome. This allows for the isolation of many different mutants, which is a great way to elucidate genes involved in a given pathway, for example. Single insertion of the transposon into a host genome is also important to ensure that the new phenotype observed is due to mutation into a single gene.

In its simplest form, a transposable element is defined by the presence of inverted repeat sequences (identical sequences reading in opposite directions; also called mosaic elements, ME; see Figure 2), flanking a DNA fragment containing the *tnp* gene. The *tnp* gene codes for a protein called a *transposase* enzyme. This enzyme is responsible for the transposition of the transposon. In a "cut and paste" type of transposon, the transposase recognizes and binds to the inverted repeat sequences of the transposon, excises the transposon from its original DNA location, cuts the host double-stranded DNA and inserts the transposon into the host genome. Note that the transposase enzyme produces sticky ends (short stretches of single stranded DNA) when cutting the double-stranded DNA of the host so that, after the ligation of the transposon into the host DNA, the sticky ends are filled following the Watson-Crick base pairing. This creates bases duplication at each end of the transposon leading to the formation of identical **direct repeats** at the site of insertion. The number of base pairs duplicated is transposon-specific and ranges from 2 to 13 (See Figure 2).

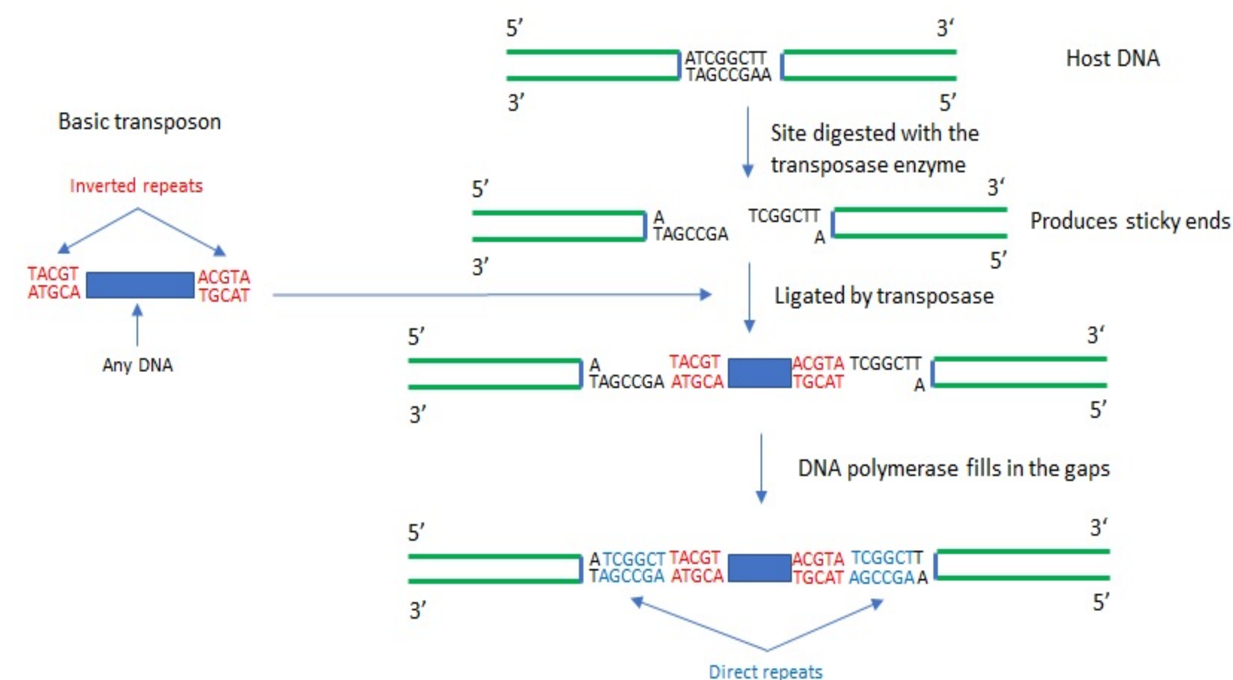


Figure 2. Structure of a basic transposon on the left. Inverted repeats also called mosaic ends are shown in red. Insertion of a transposon into a target DNA and creation of direct repeat sequences (in blue) at the site of transposon insertion is shown on the right side of the figure.

Transposition Using Tn5-derivatives

The Tn*5* transposon has been extensively used in molecular biology as it can transpose into a large variety of gram-negative bacteria, and provide random and unique insertion into the host genome. Numerous modified Tn*5* derivatives carrying different selectable markers, mutated transposase enzyme with increased transposition efficiency, and Tn*5* containing plasmids called "plasposons" have been developed over the years. Tn5 plasposons are plasmids that contain a modified transposon as well as an origin of transfer (*oriT*). The origin of transfer allows the plasposon to be transferred from a donor strain encoding the transfer functions (*tra* genes) to recipient bacteria via conjugation.

It was observed that transposons that have lost or have a non-functional transposase enzyme can still be transposed if the transposase enzyme is provided "*in trans*". The only requirement for recognition of the transposon by the transposase enzyme is the presence of compatible inverted repeats. In other words, it is possible to create

transposons by simply surrounding ANY piece of DNA of interest with inverted repeat sequences recognized by a given transposase enzyme. These transposons are only able to transpose when the transposase enzyme is provided *in trans*. This means that these transposon insertions are very stable as they cannot be transposed unless the transposase enzyme is purposely provided to the cell. In this lab, we will use the plasposon called pRL27, which derives from Tn5, to create random mutations in the *E. coli* R10 strain.

Characteristic of the pRL27 Plasposon

The pRL27 plasposon (see Figure 3; Larsen *et al.* 2002) is made of two distinct portions: the transposon portion (in red) and the vector portion (in blue).

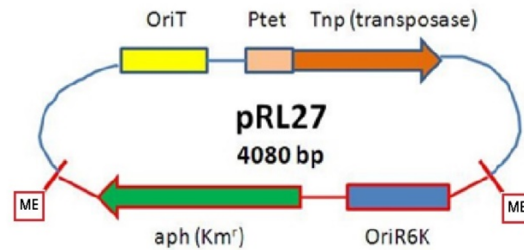


Figure 3. Genetic map of pRL27 plasposon

a) The transposon portion of pRL27, Tn5-RL27, outlined in red, is delimited by the Tn5 inverted repeats (ME; red tick marks). The DNA contained within the transposon's mosaic ends code for the *aph* gene and a conditional origin of replication (OriR6K).

- The gene *aph* codes for a protein that provides resistance to the antibiotic kanamycin. Kanamycin resistance is used to select cells containing the transposon (selection marker).

- The conditional origin of replication OriR6K has two purposes:
  - Plasmids containing the OriR6K origin of replication can only replicate in cells that produce the π protein, encoded by the *pir* gene. Therefore, when these plasmids are conjugated into non-π producing hosts, the plasmids, unable to replicate, behave like suicide plasmids. Kanamycin-resistance can only be acquired by the recipient hosts if the transposon inserts itself into the host genome.

  - The presence of an origin of replication in the transposon can also be used to recover the genomic DNA in which the transposon is inserted in a process called **rescue cloning**. In rescue cloning, the host DNA adjacent to the transposon can be cloned, along with the transposon, and be maintained as a plasmid when transferred into permissive *pir* producing *E. coli* hosts. The DNA flaking the transposon can then be sequenced to identify the gene mutated by the transposon insertion.

b) The vector portion of pRL27 contains the RP4 origin of transfer (OriT) and a transposase enzyme under the control of a Tet promoter.

- An *E. coli* strain expressing the RP4 transfer functions (encoded by the *tra* genes) can transfer plasmids containing the RP4-OriT to another bacterial strain via conjugation.

- The *tnp* gene in pRL27 codes for a mutated version of the Tn5 transposase enzyme. This enzyme is a hyperactive version of the transposase enzyme allowing higher than normal rate of transposition. The gene is also constitutively expressed under the control of the *tetA* promoter. Because the *tnp* gene is located on the vector portion of the plasposon (outside the transposon), the plasposon and therefore the *tnp* gene will be lost shortly after conjugation in the host cell as the plasposon cannot replicate (suicide plasmid) in cells that do not produce the π protein. Therefore, when transposition occurs in the host DNA, the resulting transposon insertion is very stable due to the lack of transposase enzyme in the host cell.

Procedures

Safety First!

The labs presented below require the use of sterile techniques to make sure you do not contaminate your cultures. Sterile techniques imply working close to a live flame as well as using the flame to sterilize tools. You will learn/review how to perform sterile techniques in the first lab. Please follow the guidelines below when working with live flame:

- Wear lab coat (preferably made of cotton or polyester) and safety glasses at all times when working by the flame.
- Restrain loose clothing, such as large lab coat sleeves, and avoid wearing hanging jewelries.
- Long hair must be tied back.
- Be aware of the flame at all times and make sure the person next to you is aware that you have the flame on. Be careful hot flames are blue and may be difficult to see.
- Turn off the flame as soon as you are done with it and if you have to move away from your bench; do not leave an open flame unattended, even for a short period of time.
- Make sure the area next to the flame is clutter-free and move papers and any flammable objects away from the flame. If there are shelves above your working area, make sure they are also paper-free.

Lab # 1: Conjugation

The first part of the lab is dedicated to the review of sterile techniques. This will prevent the contamination of stock solutions and of your own experiment. The sterile techniques taught in this lab include how to transfer media, how to inoculate media with bacterial cultures as well as streaking and spreading plates.

Bacterial conjugation allows for the transfer of genetic material between two bacteria using a tube-like connection called a pilus. In order to perform a conjugation, one of the bacteria, the *donor*, must contain conjugative or mobilizable genetic elements located either on a conjugative plasmid (also called fertility plasmid) or on the genome itself. The conjugative elements are characterized by the presence of *tra* genes that codes for proteins involved in the formation of pili as well as proteins required for the transfer of genetic information from the donor cell to the recipient. The exact role of the pilus is still unclear; it may act as a tube through which the DNA passes from donor to recipient or, its primary function may be to create contact between the two cells in order to bring the two cell walls close together, which allows for the transfer of the DNA. The transferred DNA, usually a plasmid, must contain a locus called the origin of transfer, *OriT,* compatible with the Tra proteins present in the donor strain. During conjugation, a single strand of the plasmid is transferred from the donor into the recipient. The single strand is created by a nick at the *oriT*. The transferred, or *T-strand*, is unwound from the double helix of the plasmid and transferred into the recipient bacterium in a 5' to 3' direction. The remaining strand is replicated, either independent of conjugative action or in concert with conjugation. Conjugative replication is similar to the rolling circle replication of lambda phage. The complementary strand of the T-strand is also synthesized in the recipient cell to re-create the double-stranded plasmid.

In our experiment, *E. coli* BW20767 (Metcalf *et al.,* 1994) act as the donor cells. They carry the transfer function (the RP4- *tra* genes) on their chromosome. The pRL27 plasposon, containing the RP4-O*riT* locus, is conjugated into wild type *E. coli* R10 strain for the purpose of creating transposon mutants in R10 (see Figure 4).

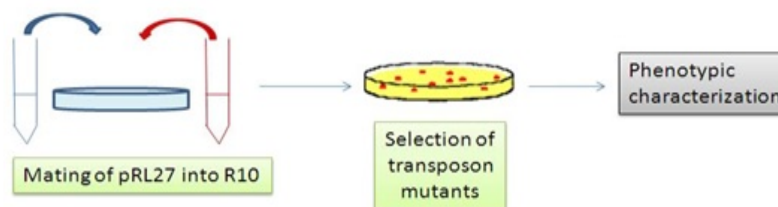


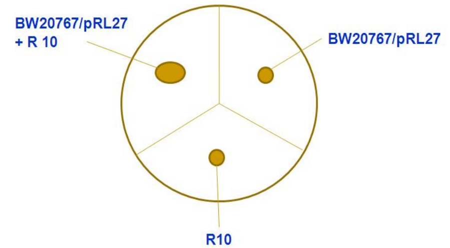
Figure 4. Schematic of the experimental procedure

1. Overnight culture of *E. coli* BW20767 containing the plasposon pRL27 (*E. coli* BW20767/pRL27) and *E. coli* R10 strains were prepared by inoculating a single colony into LB Km^{25} broth and LB Amp^{50} broth, respectively. The cultures were grown overnight under aeration at 37°C.
   Note that the antibiotic concentration mentioned in superscript is expressed in µg/mL.

2. Working under sterile conditions, transfer 1 mL of each overnight cultures into two labeled sterile microtubes and centrifuge the tubes for 1 minute at maximum speed. Pour the supernatant in a waste beaker; quick spin the tubes for 10 seconds and withdraw the remaining liquid with a micro-pipettor without touching the pellet.

3. Wash each pellet with 1 mL of NaCl solution. To do so, resuspend the pellets in 1 mL of 0.85% NaCl, centrifuge and discard all the supernatant as performed in step 2. Resuspend each pellet in 120 µL of 0.85% NaCl.

4. Take a LB agar plate and split it into three portions by drawing lines on the bottom portion of the plate (the one containing the agar); see side figure.



   a. In one of the portions, mix the two strains together by spotting 50 µL of each strain on top of each other on the LB agar plate. **Do not spread the spots. Avoid splashing** so you do not contaminate the controls that will be spotted in the other portions of the plate.

   b. In the two other portions, spot 50 µL of each strain on their own (will serve as controls). Do not disturb the plate until the spots are fully dried. Incubate the plate overnight at 37°C.

   During incubation, the plates should always be placed agar side up to prevent the dripping of condensation on your spots.

Lab # 2: Isolation of E. coli R10 Transposon Mutants

After mating the donor strain, *E. coli* BW20767/pRL27 (BW), and the recipient strain, *E. coli* R10 (R10), we expect that the conjugation of the pRL27 plasposon into the *E. coli* R10 has occurred and we also hope that the transposon (Tn5-RL27) on the pRL27 plasposon has transposed itself onto the recipient genome to create *E. coli* R10::Tn5-RL27 mutants. In order to successfully complete this experiment, continue working using sterile techniques and make sure you review how to perform serial dilutions and know how to pipet accurately with a micropipette.

1. Pipet 1 mL of 0.85% NaCl into three microtubes, labeled M (for mating), BW and R10 for the controls. Using sterile toothpicks, gently scrape the mating spot as well as the two control spots and resuspend the cells in their corresponding saline-containing tubes (M, BW and R10). Mix well by vortexing for 10 seconds.

2. Perform 10X serial dilutions of the solution in the M tube up to a dilution of 10^{-8} using 0.85% NaCl as solvent (see Appendix A for information on how to perform 10X serial dilutions).

   Draw in your lab notebook how you will prepare your serial dilution, in a way similar to what is shown in the Appendix.

3. Spread 100 µL of the 10^{-2} (1:100), 10^{-3} (1:1000) and 10^{-4} (1:10000) diluted M solutions (3 plates for each dilution) onto LB $Km^{75}Amp^{50}$ plates. Also spread 100 µL of each of the undiluted controls, BW and R10 (one plate each) on the same media (LB $Km^{75}Amp^{50}$).

4. Spread 100 µL of the 10^{-6}, 10^{-7} and 10^{-8} diluted M solutions (3 plates for each dilution) onto $LBAmp^{100}$. Store the remaining dilutions in the fridge in case you want/need to spread more later on.

   You may have to plate additional mating solution to obtain enough simple colonies for the screening of mutants (in lab 3). So, keep your dilutions in the fridge.

5. Incubate the plates overnight at 37°C. The grown plates can be stored in the fridge until the next lab session.

Lab # 3: Isolation of E. coli R10 Auxotroph Mutants

In this lab, you will start by recording the number of colonies on the countable plates obtained from lab # 2. From this, you will determine the number of cells/mL, average the triplicate values and calculate the transposition efficiency. The transposition efficiency relates the number of transposon mutants obtained to the total number of recipient cells present in the mating and is a measure on how efficient the transposition system is.

The rest of the lab is dedicated to screening *E. coli* R10::Tn5-RL27 colonies for auxotroph mutants by patching sequentially well isolated *E. coli* 10::Tn5-RL27 colonies from the LB KmAmp plates (from lab # 2) onto M9/glucose and LB KmAmp plates, in this order. Colonies that are able to grow on LB KmAmp plates but do not grow on M9-glucose plates are likely to be auxotroph mutants.

1. Part I: Record the number of colonies on countable plates ONLY. Countable plates are the dilution that gives between 20 to 200 colonies per plate. Do not count plates that have less than 20 colonies as this number will not be statistically significant. Do not count plates that have more than 200 colonies as colonies will be too close to each other to count accurately. Calculate the number of colonies per mL, taking into consideration the dilution factor on the plates you counted. Also note that you have plated 100 µL of cells, so to calculate the number of cells per mL, don't forget to multiply your counts by 10!
   Then calculate the average number of cells/mL from both plates and determine the transposition efficiency (T), using the following formula:

$$T = \frac{Number\ of\ transposon\ mutants\ (in\,\frac{cell}{mL})}{Number\ of\ recipient\ cells\ (in\,\frac{cell}{mL})}$$

   From which plates can you determine the number of transposon mutants?
   From which plates can you determine the number of recipient cells?

2. Part II: In order to screen for auxotroph mutants, label the bottom of a M9/glucose and a LB $Km^{75}Amp^{50}$ plates with the name of the strain, the date and your initials. Place a tick mark at the top of the plate with a permanent marker and label both plates # 1.

3. Place each of the plates on a 50 square grid template, making sure to align the top of the grid with the tick mark on the plate. The plates should be placed with the agar portion on the grid.

4. Using a sterile toothpick, touch a **single** well isolated *E. coli* R10::Tn5-RL27 colony from a LB $Km^{75}Amp^{50}$ plate (from the Lab 2 experiment) and patch the colony at position 1 of the grid on the M9/glucose plate and then at position 1 on the LB $Km^{75}Amp^{50}$ plate (in this order and using the same toothpick). Discard the toothpick in an empty Petri dish.

5. Using another sterile toothpick, touch another single colony from the LB $Km^{75}Amp^{50}$ plate and patch on position 2 of the grid, on both M9/glucose and LB $Km^{75}Amp^{5}$ plates, in this order. Discard the toothpick in the Petri dish.

6. Repeat until you have patched the 50 spots of the grid.

7. Patch additional plates as described in steps 2 to 6, making sure you label the second set of M9-glucose/ LB $Km^{75}Amp^{50}$ plates # 2; the third set # 3, etc.

8. On one of the plates, use one spot on the grid to patch an appropriate control.

   Which control should you patch?

9. Incubate the plates upside down overnight at 37°C. Place the plates in the fridge after 48 h incubation.

10. Next lab: Record the presence or absence of growth on both M9/glucose and LB $Km^{75}Amp^{50}$ plates for each spot. Mutants that can grow on LB $Km^{75}Amp^{50}$ but not on M9/glucose are potential auxotroph mutants.

Lab # 4: Phenotypic Characterization of the Potential Auxotroph Mutants

The phenotype of the potential *E. coli* R10::Tn27 auxotroph mutants can be determined by plating the mutants on the 11 auxanography plates. Determining the phenotype of auxotrophic mutants can be rather time consuming if each mutant has to be tested on M9-glucose plates supplemented with every possible amino acids, nucleic acids, and vitamins. Davis *et al.* (1980) designed an auxanography plate system that allows for the characterization of auxotrophic mutants using only 11 agar plates. Each plate is a M9-glucose media supplemented with 5 different amino acids/nucleic acids/vitamins. A given amino acid/nucleic acid/vitamin is present on two different plates. So, if an auxotrophic mutant only grows on two plates (i.e. 3 and 6) that both contain cysteine, the mutant is likely a cysteine auxotroph. The composition of the plates is as followed:

Table 1. Composition of the auxanography plates.

Plate	1	2	3	4	5
6	adenosine	guanine	cysteine	methionine	thiamine
7	histidine	leucine	isoleucine	lysine	valine
8	phenylalanine	tyrosine	tryptophan	threonine	proline
9	glutamine	asparagine	uracil	aspartic acid	arginine
10	thymine	serine	glutamic acid	DAP*	glycine
11	pyridoxine, nicotinic acid, biotin, pantothenate, alanine				

*DAP: diaminopimelic acid

Note that the plate composition was not designed at random but rather take into consideration the commonality between the pathways involved in the synthesis of the different amino acids, nucleic acids and vitamins.

Some exceptions to the two plates characterization system:

- Some purine mutants grow on either adenosine or guanine; so, they will grow on plates 1, 2, and 6.
- Some purine mutants require adenosine + thiamine; they will only grow on plate 6.
- *pyrA* mutants require uracil + arginine; they will grow only on plate 9.
- Mutants in the branched pathway can lead to a double auxotrophy requiring both isoleucine + valine and will grow only on plate 7.
- Mutants with early blocks in the aromatic pathway will only grow on plate 8.
- Early blocks in the lysine pathway grows only on plate 4.
- Plate 11 is a catch-all of mostly vitamins.

1- Record results from Lab 3: Record the presence or absence of growth on both M9/glucose and LB $Km^{75}Amp^{50}$ plates for each patch in each given M9/LB set. Make sure you align the tick marks. Mutants that can grow on LB $Km^{75}Amp^{50}$ but not on M9-glucose are potential auxotroph mutants. Circle these potential mutants on the LB $Km^{75}Amp^{50}$ plates and give those plates to your instructor.

   - Your lab instructor will number all the auxotroph mutants obtained by the class (from 1 to …) and will distribute a certain number of potential mutants to each group of students to study further.

2- Prepare a microtube, for each auxotroph mutants you received from your lab instructor, with 0.5 mL of 0.85% NaCl. Label the tubes with the mutant number assigned by your instructor.

3- With a sterile toothpick, gently scrape some of the patch of the potential auxotroph mutant from the LB $Km^{75}Amp^{50}$ plate and resuspend the bacteria in the saline containing tube with corresponding number. Vortex to create a homologous solution.

Do not scrap the whole streak. Also make sure you do not touch the other streaks when collecting your bacteria.

4- With a marker, split each the following plates (one M9-glucose, the 11 auxanography plates (1 to 11), and one LB $Km^{75}Amp^{50}$) into sections according to the number of mutants you have to test. Spot 5 µL of each resuspended bacteria in the same section on each of the plates and in the order mentioned. **Let the spots dry** before moving the plates.

5- Streak-purify each potential auxotrophs onto a new LB $Km^{75}Amp^{50}$ plate in order to obtain well isolated single colonies of the potential auxotroph mutants. Make sure you label the plate with the mutant number.

6- Incubate the plates up-side-down at 37ºC for up to 48 h. Store the plates in the fridge.

Between Lab #4 and lab #5 (Done for you):

1- The day before the lab, inoculate a single colony of each of the auxotroph mutants into 3 mL of LB Km^{25}. Let the cultures grow overnight at 37ºC under agitation.

a. The single colonies are coming from the streak-purified plates plated in Lab # 4
b. Only inoculate the strains for which the auxotrophic nature could be determined form the results of the auxanography plating performed in lab # 4.

Lab # 5: Confirming the nature of the auxotroph mutants

1- Record the growth results obtained from the spotting done in lab 4, and refer to the Auxanography Table (Table 1) to identify the mutants' growth requirement (the phenotype of the mutant).

The auxanography plates results should allow you to identify the phenotype of 90% of your auxotrophs. Please note that not all auxotroph mutants can be identified by this method as not all vitamins are present in the plates. Mutations of unknown nature are also sometimes isolated. Note that some of those mutants may be deficient in glucose utilization and will not be able to grow on any of the M9-glucose plates.

To confirm the results of the auxanography plating, the auxotrophs must be plated on minimal media containing the compound they cannot synthesize. To do this:

2- Spread the compound you believe your auxotroph cannot synthesize on a M9-glucose plate. The concentrated solutions of each compound were prepared so that an amount of 5 mL/L can be spread on the M9-glucose plate. Let the plate dry for 15 min.

Note: A M9-glucose plate contains ~ 25mL of media. How much concentrated solution of the compound should you spread on the M9-glucose plate?

3- Transfer 0.5 mL of each overnight auxotroph culture to a microtube properly labeled. Centrifuge for 1 min at 8,000 rpm.

4- Discard supernatant in a waste beaker. Quick spin and remove the droplet of liquid with a micropipette.

5- Resuspend pellet in 0.5 mL of 0.85% NaCl. Centrifuge 1 min at 8,000 rpm. Discard supernatant in a waste beaker.

This step is necessary to remove the nutrients from the LB media before plating the cells on the minimal media.

6- Resuspend pellet in 0.5 mL of 0.85% NaCl.

7- Streak your auxotroph onto the supplemented plate using a loop. Let the plate grow for 24-48 h at 37°C. Record your results. The phenotype of the auxotroph mutants is confirmed if single colonies are able to grow on the supplemented media.

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Materials

This lab is designed for a class of twenty students, working individually or in groups of two. Students or pairs of students are provided at the beginning of the semester with a box of general materials including a set of micropipettes (P1000, P200, P20, P2) and corresponding tip boxes, and racks for test tubes and microtubes, inoculating loop, a Bunsen burner with flint, sterile toothpicks in a glass petri dish, and 50 mL of sterile 0.85% NaCl. Materials are refilled as needed. Some other general equipment such as micro-centrifuges and vortexes are accessible on each bench. The spreader kit, made of a beaker containing 70% ethanol and a glass hockey stick is stored in the flammable cabinet when not in use. The lab should also be equipped with (or students should have access to) a growth incubator set at 37ºC. We use a rotating wheel in the incubator to provide aeration for the growth of liquid cultures (up to 10 mL). This speeds up the growth of the cultures. Bunsen burner and flint are used during every lab session in order to work under sterile techniques.

Media, Strains and Growth Conditions

The media used in the experiments include the complex media LB and the minimal media M9-glucose (Miller, 1972; Sambrook *et al.*, 1989). LB media may be supplemented with antibiotics. Heat-resistant solutions are autoclaved at 120ºC for 20 min. Heat-sensitive solutions are filter-sterilized through a 0.2 µm filter membrane attached to a syringe.

The bacterial strains used in these labs are *E. coli* BW20767/pRL27 (Metcalf *et al*., 1996; Larsen *et al.*, 2002) and *E. coli* R10 (can be ordered from Salmonella Genetic Stock Center, University of Calgary). The strains are stored at -80ºC in 15% glycerol containing media. The bacteria are streaked from the stock and grown on LB plates with antibiotics at 37ºC a day before the lab sessions. After inoculation, plates are placed up-side-down on a shelf of the incubator and are typically grown for 24-48 h at 37ºC. Liquid cultures are placed on the wheel for aeration and are grown for 24 h at 37ºC.

LB (Luria Broth, Lysogeny Broth)

To prepare 1 L of LB broth, weigh 10 g tryptone, 5 g yeast extract and 10 g sodium chloride. Add 800 mL ddH_2O and mix by stirring and then bring the volume to 1 L. To prepare 1 L of LB agar add 15g agar to the above solution. Autoclave to sterilize. You can also buy LB broth Powder from Sigma (product # 1016290500) or other companies.

M9 Minimal Media

For 1 liter of agar media prepare:

- 500 mL of 2X M9 by dissolving 5.8 g of Sodium phosphate $NaHPO_4$), 3 g of potassium phosphate (KH_2PO_4), 0.5 g of Sodium chloride (NaCl) and 1 g of Ammonium chloride (NH_4Cl) in ddH_2O. The solution is autoclaved.
- 480 mL of 2X agar by adding 15 g agar to ddH_2O and autoclaving the solution.

Once the bottles are cooled to 55°C, mix the 2X agar and 2X M9 solutions and add the following filter sterilized ingredients: 1 mL of 1 M Magnesium sulfate ($MgSO_4.7H_2O$), 1 mL of 0.1 M Calcium chloride ($CaCl_2.2H_2O$), 20 mL of 20% (v/w) glucose (for more information about how to prepare these solutions refer to Appendix D).

Antibiotic Stock Solution Preparation

The antibiotic powders for ampicillin (Amp) and kanamycin (Km) are purchased from Sigma/Aldrich. The stock solutions of the antibiotics are prepared at concentrations of 10 mg/mL for Amp and 5 mg/mL for Km (for more information on how to prepare these solutions, refer to Appendix D). The solutions are then filter-sterilized and stored in a -20ºC freezer.

When preparing antibiotics containing media, the number written in superscript (LB Km^{25}) indicate the concentration of the antibiotic expressed in µg/mL (so Km^{25} means 25 µg in 1 mL of LB media). When adding antibiotics to agar containing media, make sure the media has cooled down to 55 ºC before adding the antibiotics. Typically, the concentration of antibiotics in liquid media is ½ the concentration of the same antibiotics in agar media.

Equipment and Media for Each Lab

Lab 1

- 10 mL of overnight culture of *E. coli* BW20767/ pRL27 grown in LB Km^{25} broth (1 mL per group)
- 10 mL of overnight culture of *E. coli* R10 grown in LB Amp^{50} broth (1 ml per group)
- 10 LB agar plates (1 per group)

A few days before the lab, the *E. coli* BW20767/pRL27 and *E. coli* R10 strains are streaked form the -80ºC stock on LB plates containing Km^{50} and Amp^{100}, respectively and are grown overnight. To prepare the overnight liquid cultures of the *E. coli* strains, a single, well-isolated colony from each plate is inoculated into 10 mL of LB Km^{25} for BW or LB Amp^{50} for R10 using sterile techniques. The cultures are grown overnight at 37ºC under aeration and dispensed into 1 mL aliquots before the lab.

Lab 2
- 110 LB $Km^{75}Amp^{50}$ plates (11 plates per group)
- 90 LB Amp^{100} plates (9 plates per group)
- 10 bottles of 300 mL LB agar media
- Antibiotic stock solution; Km (5 mg/mL); Amp (10 mg/ML)
- Sterile toothpicks
- Spreader kit

The LB agar media can be prepared from scratch and autoclaved 2 h before the lab. Or you can prepare several bottles of LB agar media in advance, autoclave the media and store the bottles on a shelf. Two hours before the lab, place the number of bottles needed in the autoclave to melt the agar. The bottles with melted agar media are placed in a 55ºC water bath for 30 min to cool it down before supplementing them with antibiotics. Note that 12 plates are usually poured from 300 mL bottles of media. Once the plates are poured, they are placed in the biosafety cabinet to dry with lids removed to prevent the accumulation of condensation. The plates should be dried for ~ 45 min before using them. Left over plates containing antibiotics can be stored in the fridge for up to two weeks.

Lab 3
- *E. coli* R10 to be used as a control; you can use single colonies from a plate or in liquid culture
- *E. coli* R10::Tn5-RL27 (transposon mutants from LB $Km^{75}Amp^{50}$ plates obtained in Lab 2)
- ~60 M9-glucose plates (~3 per student)
- ~60 LB $Km^{75}Amp^{50}$ plates (~3 per student)
- Sterile toothpicks
- 40 x 50 squares grid template, Appendix B (2 per person)

The goal here is to screen as many colonies as possible. The minimum number of mutants screened should be 2,500. The numbers above are for the screening of 3,000 *E. coli* R10::Tn5-RL27 mutants. The more mutants screened the more auxotrophs can be isolated.

Lab 4
- *E. coli* R10 to be used as a control; can be single colonies from a plate or in liquid culture
- *E. coli* R10::Tn5-RL27 potential auxotrophs from Lab 3
- 10 M9-glucose plates (1 per group)
- 10 of each auxanography plates (11 plates per group)
- ~30 LB $Km^{75}Amp^{50}$ plates (1 per group for the auxanography plating and extras to streak-purify the potential auxotroph mutants)

The auxanography plates are made by supplementing M9-glucose agar with 5 amino acids, nucleic acids or vitamins based on the plate number indicated in Table 1. The catalog number, the concentration of the supplement's stocks solutions, the solvent in which to prepare the solutions and the sterilization technique are reported in Table 2 in Appendix E. The supplements stock solutions are added, under sterile conditions, to melted M9-glucose agar cooled to 55ºC just before the plates are poured. All stock solutions are such that 5 mL should be added to 1 L of medium for normal supplementation level. All supplements were purchased from Sigma-Aldrich.

Between Lab 4 and Lab 5
- *E. coli* R10::Tn5-RL27 auxotrophs that were phenotypically characterized in Lab 4
- LB Km^{25} media: 3 mL per auxotroph mutant isolated
- Inoculating loop

Use single and well isolated colony of each mutant to inoculate the growth media. The cultures are grown overnight at 37ºC under agitation.

Lab 5
- M9-glucose plates (depends on the number of auxotrophs tested)
- Supplement stock solutions (depends on the phenotype of the auxotroph mutants obtained in Lab 4)
- Spreader kit
- Inoculating loop

We usually divide the number of auxotrophs isolated by the number of groups and multiply by two so that each group test an equal number of auxotrophs and that each auxotroph is tested independently by two groups to ensure accuracy. The number of M9-glucose plates needed also depends on whether you have the students streak two mutants of same phenotype on the same plate or streak each mutant on their own plate.

The supplements are added at a concentration of 5 mL/L. So, considering that a plate contains ~25 mL of media, the students will have to calculate and spread 125 µL of the supplement stock on the plates. If a mutant requires more than one supplement, spread the first supplement, let the plate dry, and spread the second supplement on top.

Disposal

All the liquid waste, plates, toothpicks, tips and gloves are disposed into proper biohazard containers and are autoclaved (waste cycle; 30 min at 121ºC) before disposal. Benches are cleaned before and after each lab by wiping them with the Decon™ Conflikt™ Detergent and Disinfectant (from Fisher Scientific cat # 04-355-34). Alternatively, ethanol 70% can also be used to wipe the benches.

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About the Authors

Dr. Sylvie Bardin obtained her PhD. from McMaster University, Ontario, Canada for her dissertation on plant-microbe symbiosis entitled “Phosphate uptake in *Rhizobium meliloti.*” She continued studying plant-microbe interactions in the context of biocontrol of *Pythium* damping-off plant diseases during her post-doc at University College Cork, Ireland and as a plant pathologist at the Lethbridge Research Centre, Agriculture and Agri-Food Canada, Alberta. Sylvie Bardin is currently an Associate Teaching Professor at Ontario Tech University, focussing on lecturing, designing and coordinating laboratories for first year general biology, second year cell biology, third year advanced microbiology and fourth year laboratory methods in molecular biology courses.

Zahra Mortaji, MSc, obtained a BSc in Agricultural engineering from Isfahan University of Technology and her first MSc in Agricultural biotechnology from University of Tehran. She obtained her second MSc in Applied Bioscience from Ontario Tech University, Canada. Zahra is currently a biology lab tech in Faculty of Science at Ontario Tech University. She has taught and coordinated several science laboratory courses including first year biology, cell biology, microbiology, lab methods, and physiology. She is particularly enthusiastic about microbiology and biotechnology projects.

Appendix A

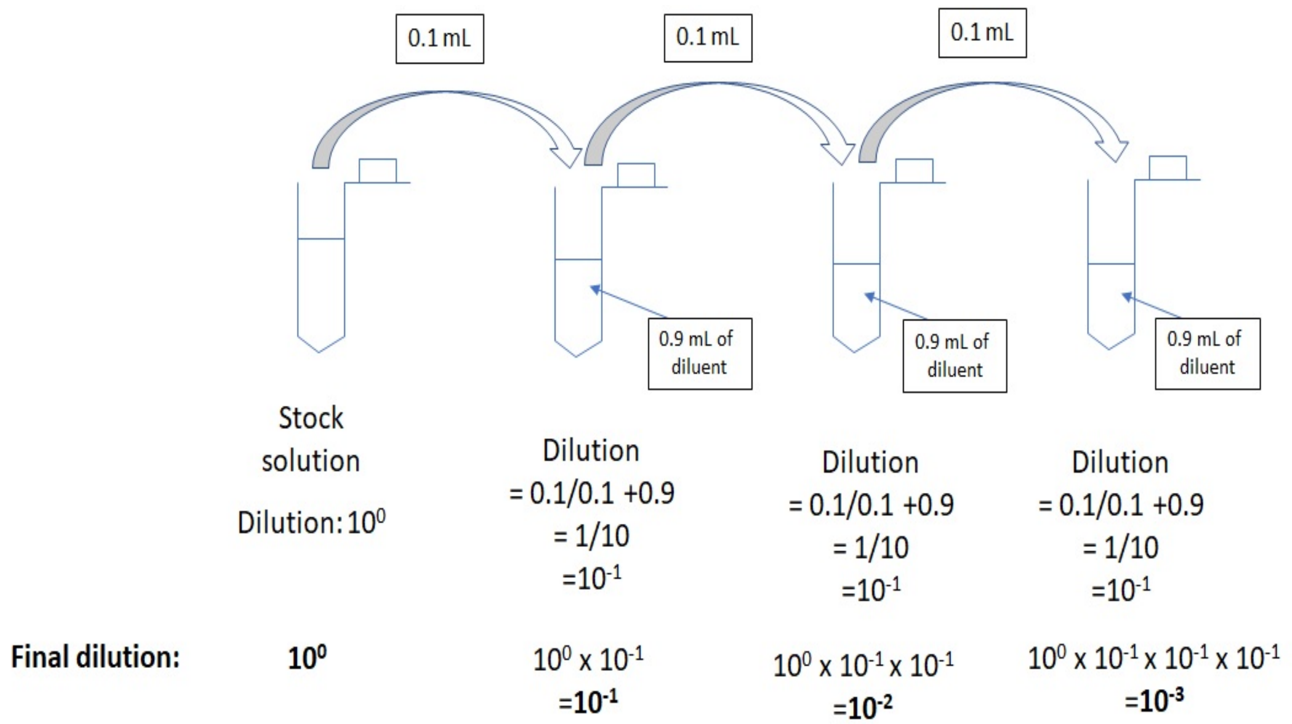

Figure 5. Performing serial dilutions.

Appendix B

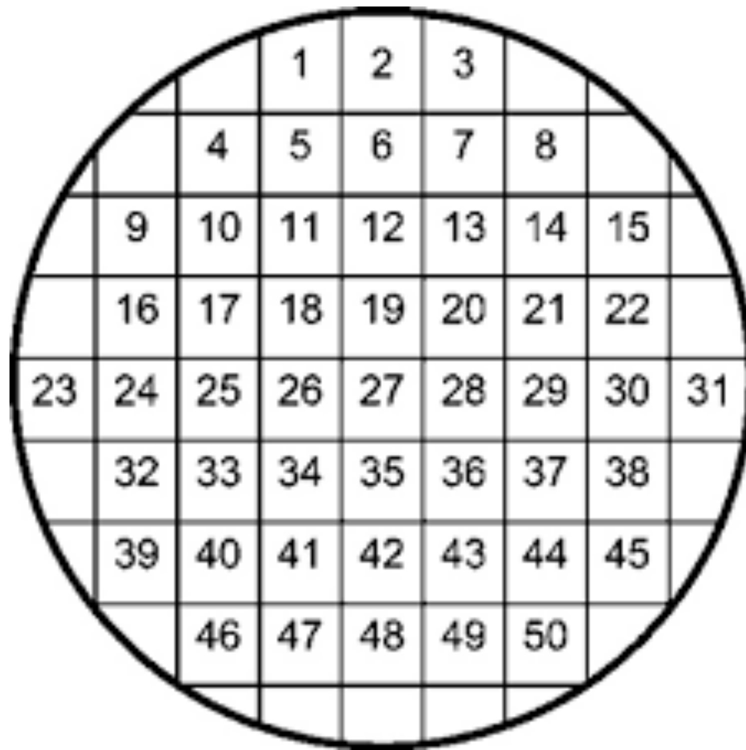

Figure 6. The 50 squares grid template.

Appendix C

Sample Questions for the Quizzes:

Introduction
1. What is needed for transposition of a DNA fragment to occur?
2. What are mosaic ends and what is their role/importance?
3. What are direct repeats and how are they created?
4. What are the functions of a transposase enzyme?
5. What are the characteristics of the pRL27 plasposon?

Lab 1:
1. Which *E. coli* cells are the donor cells and which one are the recipient cells during the mating experiment?
2. Why are you washing the cell pellets with saline in step 3?

Lab 2:
1. What are you selecting for, when plating the mating on LB $Km^{75}Amp^{50}$?
2. What are you selecting for, when plating the mating on LB Amp^{100}?
3. What do you expect to see when plating the controls onto LB $Km^{75}Amp^{50}$?

Lab 3:
1. What is an auxotroph?
2. You patch *E. coli R10*::Tn5-RL27 mutants onto M9/glucose and LB $Km^{75}Amp^{50}$ plates. You obtain four types of results (see table below). How can you explain these phenotypes?

Strains	**Growth on M9/glucose**	**Growth on** LB/ Amp^{50} Km^{75}
a	-	+
b	-	-
c	+	-
d	+	+

Lab 4:
1. You obtained growth on auxanography plates 3 and 10; no growth on M9-glucose and growth on LB $Km^{75}Amp^{50}$. What type of mutant do you have? Briefly explain.
2. You obtained growth on auxanography plates 1, 2, 3, 7 and 9; no growth on M9-glucose and growth on LB $Km^{75}Amp^{50}$. What type of mutant do you have? Briefly explain.
3. You obtained no growth on any of the M9-glucose plates (M9-glucose and the 11 auxanography plates), but there was growth on LB $Km^{75}Amp^{50}$. What type of mutant do you have? Briefly explain.

Lab 5:
1. If the volume of media in a Petri dish is 30mL, how much of the nutrient stock solution do you have to spread in order to end up with 8mg/mL of nutrient on the plate? Show your calculations
2. What would happen if you skipped strep 5? Briefly explain.

Appendix D

Solutions Preparation:

- **Magnesium sulfate** ($MgSO_4.7H_2O$). To prepare a 1 M solution: Dissolve 24.65 g of $MgSO_4.7H_2O$ in a final volume of 100 mL of ddH_2O. Filter-sterilize through a 0.2 µm filter. Store the solution at room temperature.

- **Calcium chloride** ($CaCl_2.2H_2O$). To prepare 0.1 M solution: Dissolve 1.47 g of $CaCl_2.2H_2O$ in a final volume of 100 mL of ddH_2O. Filter-sterilize through a 0.2 µm filter. Store the solution at room temperature.

- **Glucose**. To prepare 20 % (w/v) glucose solution: dissolve 20 g of glucose in a final volume of 100 mL of ddH_2O. Filter-sterilize through a 0.2 µm filter. Store the solution at room temperature.

- **Ampicillin** (Sodium salt' Sigma/Aldrich cat# A9518) stock solution 10 mg/ml: dissolve 100 mg of ampicillin in 10 ml ddH_2O. Filter-sterilize through a 0.2 µm filter membrane using a 10 ml syringe. Store the solution in a -20°C freezer.

- **Kanamycin** (Sulfate salt, Sigma/Aldrich cat# 60615) stock solution 5 mg/ml: 50 mg of kanamycin dissolve in 10 ml ddH_2O. Filter-sterilize through a 0.2 µm filter membrane using a 10 ml syringe. Store the solution in a -20°C freezer.

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Appendix E

Table 2. Preparation of the supplement stock solution for the auxanography plates (adapted from Davis et al., 1980).

Nutrient	Plate Conc (mM)	Stock sol (%)	Sterilization[2]	Solvent[3]	Product number[4]
Adenosine	0.5	2.67	Filter	1 N NaOH	A4036
L-Alanine	0.47	0.84	Autoclave		A7469
L-Arginine monohydrochloride	0.6	2.53	Autoclave		A5131
L-Asparagine	0.32	0.84	Filter	1 M HCl	A0884
Aspartic acid potassium salt	0.3	1	Filter	1 M HCl	A6558
Biotin	0.1	0.49	Filter	1 N NaOH	B4639
L-Cysteine	0.3	0.73	Filter		C7352
2,6-Diaminopimelic Acid (DAP)	0.1	0.38	Autoclave		D1377
L-Glutamic acid	5	14.8	Filter	1 M HCl	G1251
L-Glutamine	5	14.6	Filter	1 M HCl	G8540
Glycine	0.13	0.2	Autoclave		G7126
Guanine	0.3	0.91	Filter	1 M HCl	G6779
L-Histidine	0.1	0.31	Filter	0.5 M HCl	H8000
L-Isoleucine	0.3	0.79	Autoclave		I7403
L-Leucine	0.3	0.79	Autoclave		L8912
L-Lysine monohydrochloride	0.3	1.1	Autoclave		L8662
L-Methionine	0.3	0.9	Autoclave		M5308
Nicotinic acid	0.1	0.25	Autoclave		N0761
D-Pantothenic acid hemicalcium[1]	0.1	0.48	Autoclave		P5155
L-Phenylalanine	0.3	0.99	Autoclave	0.01 M HCL	P5482
L-Proline	2	4.6	Autoclave		P5607
Pyridoxine HCl[1]	0.1	0.41	Autoclave		P8666
L-Serine	4	8.4	Autoclave		S4311
Thiamine hydrochloride[1]	0.05	0.337	Autoclave		T1270
L-Threonine	0.3	0.71	Autoclave		T8441
Thymine	0.32	0.81	Autoclave	1 N NaOH	T0895
L-Tryptophan[1]	0.1	0.41	Filter		T8941
L-Tyrosine	0.1	0.36	Filter	0.1 N NaOH	T8566
Uracil	0.1	0.224	Autoclave		U1128
L-Valine	0.3	0.7	Autoclave		V4638

1 These nutrients are light-sensitive and should be kept in a dark bottle.
2 All solutions are autoclaved unless stated otherwise.
3 Deionized water is used as solvent unless stated otherwise.
4 Product numbers are from Sigma-Aldrich Company.

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