EXERCISE 9A - Lab Procedures

I. Treat *E. coli* cells so they become competent to take up plasmids

These 4 steps will be performed by your instructor. After the cells are competent, all student groups will obtain bacteria from this sample.

Instructor:

1. Locate the Eppendorf tube labeled *E. coli* and the vial of $CaCl_2$ in the ice bath.

2. Using a sterile transfer pipet, add about 0.5 mL of the $CaCl_2$ solution to the Eppendorf tube containing the *E. coli*. Mix by gently suctioning and expelling the solution into the Eppendorf tube with the pipet a few times.

3. Using the same pipet, transfer the *E. coli*/$CaCl_2$ mixture back to the vial containing the remainder of the $CaCl_2$ solution. Gently swirl or tap the vial to mix.

4. Place the vial containing the *E. coli* suspended in $CaCl_2$ in the ice bath and leave it there for at least 20 minutes. (Note: The suspension can remain in the ice bath as long as 12 hours.)

II. Transform the *E. coli* with the 3 unknown plasmid samples

In this part of the lab, you will attempt to transform the competent *E. coli* cells with 3 unknown plasmid samples. The unknown samples are in Eppendorf tubes labeled A, B, and C:

- One sample contains no DNA at all
- One sample contains normal pUC18 plasmids with functional lacZ and ampicillin resistance genes.
- One sample contains recombinant pUC18 plasmids that have a segment of λ DNA inserted at the EcoRI site. The λ DNA disrupts the lacZ gene, but the ampicillin resistance gene is unaffected.

1. Obtain three empty, sterile Eppendorf tubes and label them A, B, and C. Using tape, label a floating Eppendorf tube holder with your group name and "transformation." Place your A, B, and C Eppendorf tubes in it. You will be adding unknown plasmid samples to these tubes, but before you do, carefully read the three reminders below:

   ⇒ The 3 Eppendorf tubes that contain the unknown plasmid samples will be available at the instructor's desk or at the general supply area for your class. The small amounts of sample in each tube must supply all the groups in your class. Therefore, it is essential that you withdraw the proper amount and no more, so that there will be enough for everyone.

   ⇒ Be sure to use the micropipettor correctly. Remember, in Exercise 1 you saw that using it incorrectly could result in withdrawing as much as twice as much solution as you intended. Also, check to make sure you are using the correct micropipettor and that it is set to 10 μL.

   ⇒ It is essential not to cross-contaminate these samples. Use a new sterile tip *every time* you withdraw a sample. If one group is careless and mixes the different unknowns by not using a new tip each time, then every group to obtain sample afterwards will be attempting to perform this exercise with a contaminated sample. (Odds of obtaining decent results when using a contaminated sample are very poor.)

2. Using a sterile tip for the 2-20 µL micropipettor, withdraw 10 µL of the "A" plasmid unknown. Check to make sure you are measuring correctly; 10 µL is a very small amount, so the liquid should fill only about the last cm of a yellow tip

   Touch the tip of your micropipettor to the side of your sterile "A" tube near the bottom. After expelling the solution, you should see a small droplet on the side of the tube. Discard the tip and obtain a new sterile tip.

3. Transfer 10 µL of "B" plasmid unknown to your tube marked B. Replace the tip and transfer 10 µL of "C" plasmid unknown to your tube marked C.

4. Next you will add competent *E. coli* cells to your 3 unknown plasmid samples. Take your Eppendorf tubes to the ice bath containing the vial with *E. coli* in $CaCl_2$.

   The *E .coli* cells tend to settle to the bottom of the $CaCl_2$ solution, so gently swirl the vial each time before withdrawing the solution.

   Using a 100-1000 µL micropipettor with a sterile tip, add 100 µL of the *E. coli* suspension to your Eppendorf tube labeled A. Using a new sterile tip each time, add 100 µL of the *E. coli* suspension to your B and C tubes. Be sure to mix the plasmid DNA with the *E. coli* suspensions by tapping the tube. Dispose of your pipet tips immediately in a biohazard bag.

5. Place your *E. coli*/plasmid mixtures in an ice bath and leave them there for at least 30 minutes. It is okay for them to remain in the ice for up to 90 minutes. ***During this time, begin Part* III, *below.***

6. Remove the tubes from the ice and place them in a 42° C water bath for exactly 90 seconds. This is a heat shock step that favors the uptake of the plasmids by the *E. coli*. If a 42°C water bath is not available, place your tubes into a 37°C water bath for 5 minutes, instead.

7. Remove the tubes from the water bath and add 0.7 mL of nutrient broth to each tube, using a clean sterile micropipettor tip for each tube. Return the tubes to a 37° water bath for 30 minutes. ***During this time, pour your agarose electrophoresis gels as described in Part* IV.**

8. Obtain 3 Petri plates containing nutrient agar with Xgal and ampicillin, and label them. When labeling Petri plates, always label the bottom of the plate (the part that contains the agar), not the lid. Use small print, along the outer edge of the plate, so your writing does not interfere with viewing the contents of the plate later on. Label the three plates A, B, and C. In addition, write the following information on each plate:

   - "transformed *E. coli* cells"
   - your instructor's name
   - lab day and time
   - your group name
   - today's date

9. When the 30 minute incubation is complete, remove your "transformation" tubes from the 37° C water bath.

10. Using a micropipettor and sterile tip, transfer 0.25 mL of the contents of your Eppendorf tube A to the surface of the agar plate labeled A. Dispose of your pipet tip immediately in a biohazard bag.

11. With an inoculating loop, **gently** spread the liquid broth over the **surface** of the agar. Do not pierce or stab the surface of the agar. Dispose of your inoculating loop immediately in a biohazard bag.

12. Repeat steps 10 and 11 with your B and C samples, using a clean pipet tip and a new sterile inoculating loop each time.

13. Tape the plates closed and give them to your instructor for incubation. ***You are now ready to finish Part* IV, *beginning with step 3.***

III. Digest the plasmids in the 3 unknown samples with the restriction enzyme *EcoR*I

In this part of the lab, you will cut the plasmids in the 3 unknown samples with the restriction enzyme *EcoR*I. In the next part of the lab, you will use agarose gel electrophoresis to separate the resulting DNA fragments by size.

1. Label 3 empty, sterile Eppendorf tubes A, B, and C. Place the tubes in a floating Eppendorf tube holder labeled with your group name and the words "restriction digest."

2. Place 10 µL of *EcoR*I solution in the bottom of each of the 3 Eppendorf tubes. Since you are measuring the same solution into three empty, sterile tubes, you can use the same tip for all three transfers.

   Remember to use the micropipettor correctly and measure carefully, so there is enough *EcoR*I solution for all groups.

3. Take your 3 Eppendorf tubes to the area where the A, B, and C plasmid unknown stock solutions are located. With a new, sterile micropipettor tip, add 5µL of the "A" plasmid unknown to your A tube. Using a clean tip each time, add 5µL of the "B" plasmid unknown to your B tube and 5µL of the "C" plasmid unknown to your C tube.

4. Tap the tubes gently to mix the plasmids with the restriction enzyme and then spin them for a few seconds in the microcentrifuge to make sure all of the solution is at the bottom of the tube.

5. Place your 3 Eppendorf tubes in the 37° C water bath and leave them there for 60 minutes. ***During this time, return to Part* II, *step 6.***

IV. Separate the DNA fragments using agarose gel electrophoresis

1. Obtain a bottle of melted agarose from the water bath and pour the contents into the gel casting tray fitted with a 6-well comb as explained by your instructor.

2. Allow the gel to set until it has solidified (about 20 minutes.) ***While the gel sets, return to Part* II, *step 8.***

3. Plug in your power supply (which is not yet connected to the electrophoresis apparatus). Turn it on and set it to 100 volts. Then turn it off and unplug it.

4. After your gel is set, lower the 2 adjustable sides of the casting tray. Place your gel, in its casting tray, into the electrophoresis apparatus. Make sure that the wells of the gel are at the side where the cathode (black electrode) will connect.

5. Gently pour electrophoresis running buffer into the sides of the apparatus (not onto the gel itself) until the buffer is 2-3 mm above the gel. Ask your instructor to check your set-up before you proceed.

6. When the 60 minute incubation is complete (Part III, step 5), remove your 3 "restriction digest" tubes from the 37° C water bath. Add 5µL of Electrophoresis Sample Buffer to each tube. Locate the Eppendorf tube labeled "DNA markers", and spin all 4 tubes in the microcentrifuge for a few seconds.

7. Load 15 µL of sample into each well of your gel according to the plan below. To assign numbers to the lanes, view the gel with the wells oriented towards the top, then count from left to right.

   - Lane 1-- Unknown sample A + *EcoR*I
   - Lane 2-- DNA markers
   - Lane 3-- Unknown sample B + *EcoR*I
   - Lane 4-- DNA markers
   - Lane 5-- Unknown sample C + *EcoR*I
   - Lane 6-- leave empty

8. Place the lid on the electrophoresis apparatus and connect the power supply to it. Have your instructor check your set-up and then plug in the power supply and turn it on.

9. Allow the gel to run until the blue tracking dye is at the end of the gel. This should take about 30 minutes.

10. While the gel is running, use tape to label the bottom of a large Petri dish with your instructor's name, your lab day and time, your group name, and the date. Later, you will use this Petri dish to stain your gel.

11. When the blue tracking dye reaches the end of your gel, turn off the power supply and unplug it.

NEVER REMOVE THE COVER FROM THE ELECTROPHORESIS APPARATUS WHEN IT IS PLUGGED IN! SEVERE ELECTRICAL SHOCK COULD OCCUR!

After unplugging the apparatus, remove its cover. The buffer solution will be hot! Use a transfer pipet to withdraw and discard buffer until it is possible to remove the gel in its casting tray without burning yourself. Make sure you wear gloves when you remove the gel and casting tray from the electrophoresis apparatus.

12. Slide the gel out of the casting tray and into the labeled Petri dish. With the wells oriented towards the top of the gel, use a spatula to cut and discard a small triangular piece (approximately 5 mm on a side) from the lower right corner of the gel. When you analyze your gel, this will allow you to identify the lower right corner.

13. Pour enough stain into the Petri dish to cover the gel. Give the dish to your instructor who will store it in the refrigerator until the next lab period.

When you return to lab next week, you will examine and analyze your stained gel for bands of DNA, and check the Petri plates inoculated with* E. coli *for bacterial growth.

Clean up

Dispose of any materials that had contact with *E. coli* cells in a **biohazard bag**.

Be careful not to lose any parts of the gel apparatus, including the casting tray and the gel comb. Wash them in soapy water, rinse with tap water and dH_2O, and leave on absorbent toweling to dry.

Dispose of your excess solutions as directed by instructor.

Remove label tape and any marks made with a marking pen from all glassware. Wash and rinse all glassware, give it a final rinse with dH_2O, and leave it inverted at your work area in order to drain.

All disposable glassware goes into the special glass disposal receptacle.

All instruments should be turned off and unplugged.

Wipe off your workspace with a damp paper towel.

Make sure everything that you have used is clean, put away, or discarded. Leave your work area in the same order that you found it in.

Wash your hands and ask your instructor to check your work area before you leave.