

Cloning a Fluorescent Gene

Laboratory Protocols Handout v1.10

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Lab 1: Pipettes and Pipetting

Purpose

In this lab you will acquaint yourself with an air displacement pipette, learning how to correctly use the device. You will also dispense all of the reagents you will be using into microcentrifuge tubes to make them easily accessible for future use. To get used to using the pipette, you will start out with a simple exercise of mixing colored acid and base solutions using an indicator called Phenol Red.

Materials

Equipment

- 2-20 μ L Air displacement pipette (recommended: one instrument per 2 students)
- Pipette tips
- 12 Microcentrifuge tubes for 4 students
- Plastic bag (1 bag per 4 students)
- Permanent Marker (preferably black)

Reagents

- Small beaker of water
- Phenol Red Solution (1 tube for each group of 4)
- Acid Solution (1 tube for each group of 4)
- Base Solution (1 tube for each group of 4)
- Assorted stock solutions of various reagents from *gen.otyp* on instructor's bench.

Methods

In this laboratory session you will be taking small amounts of reagents, called **aliquots**, for future experiments. The members of your lab area (usually 4 students organized into 2 pairs) will assemble all the reagents that you will need for the rest of the lab. **Every student should individually perform steps 3-5.**

1. Choose Your Partner and Group

- You and your partner will share an air displacement pipette. **Your group of 4 students will share** the reagent **aliquots** that you are going to create in today's lab.

2. Watch the Chapter 1 video and pay careful attention to the instructions in the video.

3. Practice changing the microliter volumes on the pipette.

- Change the volume of your pipette to 7.5 microliters (μ L).
- Change the volume of your pipette to 12.0 microliters.

4. Practice adding liquid to the microcentrifuge tube.

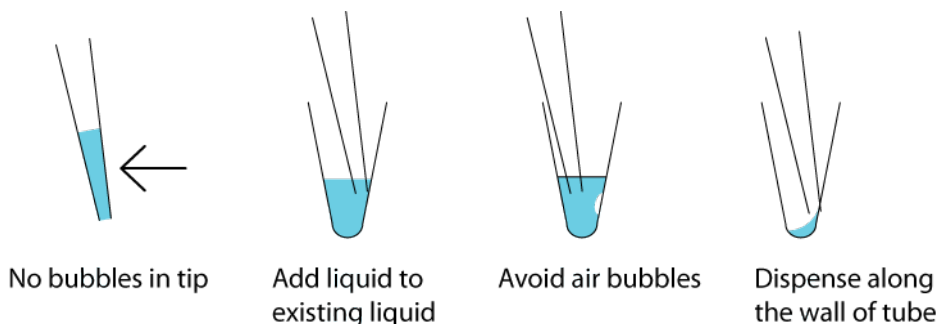


Figure 1.4. Pipetting Tips (no pun intended)

- Ready an empty microcentrifuge tube to practice mixing acids and bases. Every member of the group should practice on their own microcentrifuge tube.
- Set your pipette to 10.0 microliters.
- Draw in 10.0 microliters of yellow Phenol Red solution into a pipette tip. Observe the level of yellow solution in the pipette tip. There should be no bubbles in your tip.
- Expel the 10.0 microliters of Phenol Red into the bottom of the microcentrifuge tube. Observe the droplet on the bottom of the tube. It should be a yellow color. 
- Eject your tip and put a new one on your pipette. You are now ready to add liquid from a new solution.
- Add 5.0 μL of Base solution to the tube, pipetting up and down carefully to mix the liquids. It should now be a fuchsia color. 
- Change tips and add 10.0 μL of Acid solution to the tube. It should return to its original yellow color. Make sure to mix the solution until the entire solution is yellow. 
- Change tips and add 15.0 μL of Base solution to the tube, it should return to its fuchsia color. 

5. Practice removing liquid from the microcentrifuge tube.

- Set your pipette to 7.5 microliters.
- Touch the tip to the fuchsia liquid and draw in 7.5 microliters of liquid.
- Make sure you have not drawn in any air bubbles in the pipette tip.
- Eject the pipette tip. You are now trained to use an Air Displacement Pipette.

6. Assemble the reagents that you will use for your group of 4 students

- Number, initial, and label eight microcentrifuge tubes using a permanent marker according to the following table, and add the stated volumes from the stock solutions on the instructor's bench to each tube. Be sure to carefully add the volumes to the bottom of the labeled microcentrifuge tube, because you will need almost every microliter for your group's experiments. To add more than 20.0 μL to a tube, simply set the pipette to 20.0 μL and perform multiple transfers.

Label	Volume (μL)
Template	25.0
5x PCR Buf.	25.0
DNTPs	30.0
GFP For.	18.0
GFP Rev.	18.0
Vector	25.0
5x Lig Buf.	20.0
Water	40.0

Change tips for every different reagent!

- Divide the tubes among the different members of your group. One member is responsible for getting an aliquot of one reagent for the entire group. At the end of this step, your group of four should have 8 tubes total, each for a different reagent. Your group will share these reagents in Chapter 2 and Chapter 3.

7. Store your reagents

- Close the lids of each tube tightly
- Gently put all of the tubes into a sealable plastic bag (such as a Ziploc® bag)
- Label the bag with initials of group members, and give it to your instructor to be placed in the freezer until needed.

Clean-up

Make sure that each tube that you have dispensed your reagents in is clearly labeled with your group's initials and the name of the reagent. Place all of the tubes gently in the small plastic bag and label the

bag with your initials. The bag should be placed in the freezer until the next laboratory session, when some of the reagents will be used.

Conclusions

Now that you have some experience using air displacement pipettes and microcentrifuge tubes, you are capable of performing all the laboratory techniques required in the next two lab sessions. Though you may not understand the purposes of each of the reagents that you have parceled out, they will be revealed to you soon. Starting in the next laboratory session you will use your new skills to perform a polymerase chain reaction to amplify the green fluorescent protein gene.

Lab 2: Polymerase Chain Reaction

Purpose

We will use Polymerase Chain Reaction to amplify the GFP gene. Using a DNA Polymerase called “Taq” and two primers that complement the two ends of the GFP DNA, we will create a huge number of linear copies of GFP DNA. In the next lab this DNA will be inserted into an antibiotic resistant vector.

Materials

Equipment:

- 2-20 μ L Air displacement pipette (1 per 2 students)
- Pipette Tips
- 0.2mL (PCR) Tube
- Thermal Cycler (1 per class)

Reagents:

- Reagents from your group's bag
 - 5x PCR Buffer
 - DNTPs
 - GFP Forward Primer
 - GFP Reverse Primer
 - DNA Template
- Taq Polymerase

Methods

You will be mixing together the appropriate reagents in given amounts, just as you practiced in the first laboratory session. Use the table on the right below to reference how much of each reagent, adding each reagent in from top to bottom in the order listed. It is important to remember good pipetting skills to ensure that there is enough of each reagent for everyone in your group.

Each student should perform their own PCR reaction!

1. **Label** your PCR tube on the cap and the side with your initials using a dark permanent marker.
2. **Add the PCR reagents to the tube.** Wait for all reagents to thaw completely before pipetting.
 - a. Add 4.0 μ L of 5x PCR Buffer to the tube.
 - b. Add 5.0 μ L of DNTPs
 - c. Add 3.0 μ L of GFP Forward Primer
 - d. Add 3.0 μ L of GFP Reverse Primer
 - e. Add 4.0 μ L of DNA Template
 - f. Add 1.0 μ L of Taq Polymerase
 - g. Mix Tube gently.
3. Close the tube and bring it to the Thermal Cycler. Place the tube in an open tube slot. Make sure your tube is properly labeled. Once all the students in the class are finished putting

Reagent	Amount
5x PCR Buffer	4.0 μ L
DNTPs	5.0 μ L
GFP For.	3.0 μ L
GFP Rev.	3.0 μ L
DNA Template	4.0 μ L
Taq Polymerase	1.0 μ L
Total	20.0μL

together their reactions. The instructor should start the PCR machine on the "GFP_PCR" program (the details of this program are found in the CAGF Instructor's Manual).

Clean-up

PCR Tubes may remain in the Thermal Cycler overnight, but for longer periods, they should be stored in the refrigerator. Unused reagents may be thrown out unless your instructor is collecting them. Double check to make sure that your PCR Tube is labeled with your initials so that it does not become mixed up with other tubes.

Conclusions

By the end of the thermal cycling your PCR Tube should contain a large number of copies of short sequences of DNA that encode for only the GFP gene. During the next lab period you will be inserting this short DNA sequence into a larger piece of circular DNA that can then be put into bacteria for expression.

Lab 3: Ligation

Purpose

Today you will ligate your amplified DNA from the previous lab into a linearized vector that you placed into an aliquot on the first day. This procedure should generate circular plasmids containing GFP DNA.

Materials

Equipment:

- 2-20 μ L Air displacement pipette
- Pipette Tips
- 0.2mL (PCR) Tube

Reagents:

- Amplified GFP PCR product (from last lab)
- Reagents from your group's bag
 - Linearized Vector
 - 5x Ligase Buffer
- DNA Ligase Enzyme

Methods

1. **Label** your Microcentrifuge tube with your initials using a permanent marker.
2. Take out the 5x Ligase Buffer to **thaw**
3. **Add the Ligation Reagents to the tube**
 - a. Use a new pipette tip for each different reagent you use, discarding the old one in the trash.
 - b. Add 4.0 μ L of your PCR reaction to the bottom of the new 0.2mL tube
 - c. Add 6.0 μ L of Water to the tube
 - d. Add 4.0 μ L of Linearized Vector to your mix
 - e. Add 4.0 μ L of 5x Ligase buffer
 - f. Go to your instructor's lab table and add 2.0 μ L of DNA Ligase, which should be sitting on ice.
 - g. Shake the tube gently so that all the components are mixed well
4. **Let the reaction sit overnight** at room temperature
5. **Watch** the Chapter 4 video to get an idea of the techniques you will be using next lab.
6. The next morning, you or your instructor will put your reaction into the Thermal Cycler **and run the "Lig End" program**, which heats up your sample to denature your enzyme, ending the reaction and improving the efficiency of transformation.
7. Place your tube back in the plastic bag for your group after running the "Lig End" program. **Store the ligation reaction** in the freezer until next lab period.

Reagent	Quantity (μ L)
PCR Reaction	4.0
Water	6.0
Linearized Vector	4.0
5x Ligase Buffer	4.0
DNA Ligase	2.0
Total	20.0

Clean-up

After the reaction has been allowed to incubate overnight, be sure to put your ligation reaction in your group's bag and place the bag back in the freezer. Extra 5x Ligase buffer, Linearized Vector, and PCR product can either be stored for another experiment or thrown away.

Conclusions

Some of the pieces of linear DNA that you have mixed together in your reaction tube should have been joined together by DNA ligase into a circular plasmid. These circular pieces will remain mixed in with the un-ligated fragments until next lab session, when you will be putting the DNA into bacteria, and then only allowing the bacteria which have taken up the circular plasmid will grow.

Lab 4: Transformation

Purpose

Today you will perform heat shock transformation on chemically competent *E. coli* bacteria. Using a beaker of warm water you will force the bacteria to take in your ligated GFP-containing vector from the last lab.

Materials

Equipment

- 2-20 μ L Air displacement pipette
- Pipette tips
- 1 transfer pipette
- 1 hot plate, beaker with water, and thermometer OR 1 water bath
- 1 tube float (optional)
- Ice bucket (One per lab table recommended)
- 1 microcentrifuge tube per student

Equipment for Tomorrow

- 1 agar plate containing ampicillin and arabinose
- 1 bacteria spreader

Reagents

- Ligation Reaction from last session
- *E. coli* bacteria on ice
- Tube of SOCS broth

Methods

The laboratory today will be the most time-consuming of all the lab sessions, so in order to finish on time you must be organized, quick, and careful. You will be using your ligation reaction from last week to transform *E. coli* bacteria, recover those bacteria, and then plate them on an agar plate containing arabinose.

When handling live *E. coli* bacteria, be sure not to touch the bacteria with any part of your body, and quickly dispose of any pipette tip, spreader, or transfer pipette that touches the bacteria in a trash can lined with a plastic bag. These *E. coli* bacteria do not cause disease, but you still should not get them on yourself. If your classroom has them, wear gloves and goggles when handling bacteria.

1. *E. coli* bacteria should remain cold at all times, your instructor will remove the bacteria from the freezer and thaw them on ice.
2. Use your Air Displacement Pipette to add 40.0 μ L of bacteria from the lab stock tube to your microcentrifuge tube.
3. Let your bacteria sit on ice for 2-3 minutes to make sure they stay cold.
4. Thaw your ligation reaction from the last lab.
5. Use a pipette to add 10.0 μ L of the ligation reaction to the bacteria.
6. Cap and flick the tube a couple times to mix and then put the mixture with bacteria on ice.

7. Leave the tube on **ice for 20 minutes**, optimally the tube would be left on ice for 1 hour. During this time **re-watch the Chapter 4 Video**.
8. While the bacteria DNA mixture is on ice, heat a beaker of water to 42°C or prepare your water bath
9. Incubate your bacteria for 60 seconds in the 42° water. Don't let the bacteria spend much time at room temperature. Move them straight from the ice bucket into the water bath.
10. Remove the now-transformed bacteria from the water bath. Use the transfer pipette to add around 500µL of SOCS broth to the transformed bacteria. Place the bulb pipette on your bench to use again in ten minutes. Be sure not to mix your bulb pipette up with anyone else's pipette.
11. Tightly cap the microcentrifuge tube containing your transformed bacteria.
12. Holding the transformed bacteria in your hand to warm them to 37° C (body temperature) and shake the bacteria in an orbital motion for about a minute, then place the tube in the incubator at 37° C. You should come back **tomorrow morning** (at least 2 hours later) to plate the cells on the agar plate.
13. Write your name on the bottom of your agar plate in permanent marker.
14. Use the bulb pipette to add all of the bacteria in SOCS broth to the center of your agar plate and use the spreader to evenly distribute the liquid around the agar plate. Place the lid back on the plate.
15. **Let the plate sit** for about a minute to absorb some of the liquid, then place it face up in the 37° C incubator.
16. Tomorrow, after colonies have grown, the plates should to be moved from 37° C to the refrigerator to prevent overgrowth of bacteria. If you don't see any colonies, try leaving the plates in the incubator for another day (also double check that the incubator is at the appropriate temperature).

Clean-up

Make sure that all of the tubes containing *E. coli* bacteria are properly disposed of and that the ligation reaction is discarded. The microcentrifuge tube that contained your *E. coli* should be tightly capped before being thrown away. Be sure to put away the hot plate and beaker in addition to your pipette, as normal. Make sure the transfer pipette and spreader that you used are thrown away as well. Double check that your initials are on your agar plate and place it in the incubator.

Hand in your group's air displacement pipette to your instructor; you will not need it for the rest of the module.

Conclusions

Today you have inserted some circular plasmid DNA from a ligation reaction into *E. coli* bacteria. By plating the bacteria on ampicillin containing plates, only the bacteria that have the ampicillin resistance gene from the plasmid DNA will be able to grow. By tomorrow you should be able to see individual colonies started by a single bacterium on the plate, and hopefully some of them will express GFP!

Lab 5: Selection and Expression

Purpose

Today you will be visualizing the expression of Green Fluorescent Protein in bacterial colonies using a long-wave UV light. You will select one colony that expresses GFP (a successful transformant) and use bacteria from that colony to draw an elaborate picture of your own design.

Materials

- Agar plate with GFP expressing *E. coli* colonies from last lab.
- Long-wave UV lamp
- 1 Agar plate with ampicillin and arabinose
- 1 mL tube of LB broth
- Agar plate painting tools, which include but are not limited to
 - Pipette tip
 - Toothpick
 - Cotton swab

Methods

In today's session you will visualize the expression of GFP in your bacterial colonies using a UV light and then use a GFP-expressing clone to paint an entire picture in fluorescent bacteria which will grow up within a day.

When handling live *E. coli* bacteria, be sure not to touch the bacteria with any part of your body, and quickly dispose of any pipette tip, toothpick, or cotton swab that touches the bacteria in a trash can lined with a plastic bag. These *E. coli* bacteria do not cause disease, but you still should not get them on yourself. If your classroom has them, wear gloves and goggles when handling bacteria.

When using a Long-wave UV lamp, always keep the light pointed away from yourself and other people. If your classroom has them available, use goggles when handling the UV light. Long-wave UV light is less damaging to cells than the higher energy short-wave UV light, but exposure should be minimized nonetheless.

Before painting your fluorescent picture, you may want to check out Figure 6.3 in the next chapter, to see what other students have drawn with this same kit!

1. Retrieve your agar plate from last lab session; it should have a number of small round, whitish colonies growing on it.
2. Shine the long-wave UV lamp on the plate, and observe to see if you have any GFP-producing colonies, they should glow green when illuminated by the long-wave UV light.
3. On the bottom of the plate, use a permanent marker to circle one of the colonies that is expressing GFP. You will use this clone to paint your picture in bacteria.
4. Open the lid of your plate, and using a toothpick or pipette tip, gently touch the colony you are going to use, then mix the bacteria on the end of your tip or toothpick into the microcentrifuge tube of LB Broth.
5. Discard that tip or toothpick, close the lid on your tube of LB with bacteria in it, and replace the lid of your agar plate from last week.
6. Invert the tube containing LB and bacteria multiple times to ensure that they are well mixed.

7. Open the tube with LB and bacteria. Then touch the painting tool you are going to use to the LB in the tube. The microscopic bacteria in the LB will be lifted out on the end of the pipette tip, toothpick, cotton swab, or other instrument, allowing you to paint a detailed and beautiful picture onto the new agar plate!
8. Optional: if you have a detailed idea of the picture that you want to create, it may help to draw it out on paper first, and then place the paper underneath your agar plate while you are adding the bacteria in whatever design you choose.
9. When you are finished painting, be sure to correctly dispose of all of your painting tools and your bacteria in LB broth. Then invert your agar plate and place it in the 37 ° Celsius incubator.
10. The cells should be allowed to grow for anywhere between 12 and 24 hours, if the plates need to be kept for longer than this, they should be moved to the refrigerator after the 12 to 24 hours. An agar plate sealed with plastic wrap can last in the refrigerator for over a month.

Clean-up

Make sure that all agar plate painting tools are properly disposed of in the trash can. The microcentrifuge tube containing LB and the bacteria should be tightly capped and thrown away. Make sure that your agar plate has your initials before placing it in the incubator to grow.

Conclusions

Congratulations! You have successfully cloned the GFP gene from a template into a vector and then grown in bacteria. This technique is used by thousands of scientists to manipulate DNA for a broad range of experiments, and now you are among their ranks. If everything has worked perfectly to this point, you have done exceptionally well and worked carefully and accurately. Enjoy your fluorescent picture made of living organisms that will be ready to look at tomorrow.

You are also now ready to take the final assessment for the course, which will involve the application of many of the concepts you have learned during the course. Your instructor may distribute the final assessment today before you leave the laboratory.

Lab 6: Fluorescence

Purpose

Observe the designs made by you and your classmates using a UV lamp.

Materials

- Long-wave UV lamp
- Camera (optional)

Methods

Today you will simply be observing the fruits of your labors. Collect your agar plate from the incubator and use the UV light to illuminate your design! Compare your design with those of your classmates, and use a digital camera to take pictures.

If you email your pictures to us at data@otyp.es your art may be included in the next edition of the lab manual!

Clean-up

You may now throw away all of your agar plates and any extra tubes that are in your group's plastic bag in the freezer. Be sure to clean up your lab area with an ethanol-containing cleaning solution, like Windex®, which will kill any bacteria that got onto the lab bench.

Conclusions

This is the end of gen.otyp's *Cloning a Fluorescent Gene*. We hope that you have enjoyed the course and have learned some valuable skills to use in the laboratory. To those of you who are soon going to be in college, and may want to work in biology research laboratories, be sure to mention that you have experience doing PCR and cloning when applying for a job as a research assistant, it may help you secure the position! Best of luck to you all.