

## Production of RFP+ or GFP+ Bacteria – Upstream Process Protocol Days 1-5

### Materials for Lessons 2-5.1/5.2:

1. **Lysogeny Broth (LB):** The media used to grow liquid cultures of lab strains of E. coli.
2. **Lysogeny Broth + Ampicillin (LB/Amp):** Ampicillin is the antibiotic used to select bacteria that contain a plasmid that contains the gene encoding GFP or RFP as well as a gene encoding a protein that confers resistance to ampicillin.
3. **A bacterial plate with RFP+ or GFP+ bacterial colonies:** You will pick a colony from this plate to inoculate your team's small liquid bacterial culture.
4. **Sterile loops:** Used for picking individual bacterial colonies from the plate.
5. **14mL snap-cap Falcon test tubes:** The first small scale bacterial culture will be grown in these tubes.
6. **Arabinose liquid stock (50X):** This will be added to LB/Amp media to induce bacterial production of GFP or RFP.
7. **125mL sterile glass baffled flask with lid:** The scaled up bacterial culture will be grown in this container.
8. **Five x 10 cm<sup>2</sup> LB + Ampicillin Agar Plates:** These will be used to determine your final bacterial culture count.
9. **Sterile 50mL plastic test tubes**
10. **Sterile plastic transfer pipette:** Used to move volumes of liquid larger than 1mL
11. **Sterile plastic spreaders:** Used for spreading bacterial cultures on agar plates.
12. **Sterile plastic inoculation loops:** Used for inoculating bacterial colonies into liquid media.
13. **Plastic cuvettes:** The correct container for bacterial cultures that will be read in the spectrophotometer.
14. **Micropipettes and tips:** For measuring small volumes of reagents or bacterial cultures
15. **Microfuge tubes of various sizes:** For containing small volumes of reagents or bacterial cultures
16. **Microfuge tube rack:** To hold microfuge tubes.
17. **Sharpie markers:** For correct labeling of samples.
18. **Colony Forming Unit (cfu) Calculation Record:** This form is a record of your team's bacterial yield calculation.
19. **Upstream Process Batch Record Form:** When properly filled out, this form is a record of your team's entire upstream process.

### Equipment:

20. **Bacterial Plate Incubator:** The equipment used to warm bacterial cultures on agar plates for optimal growth.
21. **Shaker/Incubator:** The equipment used to shake and warm bacterial cultures for optimal growth in liquid culture.
22. **Spectrophotometer:** The equipment used to measure the optical density of bacterial cultures – for plotting bacterial growth curves.

### Protocol: Day 1 – (Lesson 2) – Inoculation of Liquid Culture

This procedure will be carried out by the Upstream Process Technician. **NOTE:** The Upstream Process Technician should work with their team members to carry out this procedure.

Use aseptic technique at all times!!

**You are STRONGLY ENCOURAGED to check off each step below as you complete it.**

1. Using aseptic technique, prepare a 14mL Falcon tube containing 5mL LB + Ampicillin (LB/Amp) media. You will receive a bottle of the LB/Amp media from your teacher. **Put a tape label on this bottle with your team name. This will be your team's bottle of LB/Amp throughout all of the Upstream Process labs.**
2. **The Upstream Process Technician will work with all team members to carry out this procedure.**
3. The Upstream Process Technician will use a sterile transfer pipette to move 5mL of sterile LB/Amp media into the 14mL Falcon tube. If your class has Pipet-Aids and sterile pipettes, those can be used instead of transfer pipettes.
4. Label the tube with your team name, date, and contents (Example: RFP+ bacteria in LB/Amp).
5. Open the package of sterile bacterial loops. Make sure you open the end at the non-circular (handle) portion of the loops.
6. Pull a single loop out of the package taking care to not touch the circular part with anything.
7. Using aseptic technique, remove the parafilm from around your bacterial plate containing the RFP+ or GFP+ colonies and open it. You might want to have another team member open the plate for you. Use the 'clamshell' method to open the plate.
8. Touch the circular part of the loop to a single RFP+ or GFP+ colony. Pick up as much of the colony as you can.
9. Close the bacterial plate and re-parafilm it. Store it upside down in the refrigerator.
10. Carefully open the 14mL Falcon tube containing LB/Amp media. Again, you might want a team member to do this for you.
11. Dip the circular end of the bacterial loop into the media and swirl it a few times.
12. Remove the loop and place it in the biohazard waste.
13. Cap the tube to the first 'stop' – so the cap still moves on the tube but will not come off.
14. Your Instructor will properly program and check the shaker/incubator.

15. Place the tube into the shaker/incubator that has been properly checked and programmed by your instructor.
16. Shake the tube overnight at 200rpm, 37°C. When removed from the shaker/incubator, the tube can be stored in the refrigerator until the next class period.
17. Fill out the appropriate parts of the 'Upstream Process Batch Record'.
18. Properly dispose of all waste following the guidelines in the Aseptic Technique slidedeck.

### **Protocol: Day 2 – Lesson 3 – Bacterial Culture Scale-Up**

Bacterial scale up will be carried out by the Upstream Process Technician. The Process Engineers will check and ready the spectrophotometer for use. The QC Technician will take bacterial culture samples at time points and measure OD600.

**NOTE: All team members will assist the Process Engineer, the Upstream Process Technician, and the QC Technician as needed.**

**NOTE: Not all class periods are long enough for bacterial growth OD600 readings to be taken as they are described in this protocol. Your instructor may modify the protocol as needed..**

Before starting the protocol, turn on the spectrophotometer to give it time to warm up for the recommended 30 minutes. Your teacher may want to turn it on before the beginning of class.

**You are STRONGLY ENCOURAGED to check off each step below as you complete it.**

19. Carefully open the sterile baffled flask by pulling the metal cap off and setting it on your sterilized lab bench.
20. The Upstream Process Technician will add 50mL of LB/Amp to a sterile 125mL glass, baffled flask.  
**Note:** This can be done using a Pipet-Aid and sterile 10mL pipettes. If you do not have Pipet-Aids, it can be done by carefully pouring sterile 50mL LB/Amp media into a sterile 50mL tube and then carefully pouring it into the sterile 125mL baffled flask.
21. Place a tape label on the flask with your team name, date, and contents.
22. Using a p1000 carefully pipette up 500uL of your small liquid overnight culture. If this culture was stored in the refrigerator, be sure to swirl the tube to homogeneously mix the contents. Some of the bacterial will have settled out during storage.
23. Add the 500uL of your small liquid overnight culture to the 50mL of LB/Amp in the baffled flask.
24. Cap the flask and swirl gently to mix.

25. The Process Engineers from each team work together to properly blank the spectrophotometer using the 'Spectrophotometer Check SOP'. **Remember:** The spectrophotometer must warm up for 30 minutes before being used.  
**PLEASE NOTE:** Teachers who have other types of spectrophotometers such as: 96 well plate readers, Vernier SpectroVis Plus etc, may choose to use their own equipment. Creation of an SOP for how to use alternate spectrophotometric equipment is up to the teacher.
26. The QC Technician will remove 2mL of bacterial culture and place it into a cuvette.
27. Read the cuvette at OD600. Record the data in the Upstream Process Batch Record. This reading will be your 0 time point reading.
28. Discard the bacterial solution from the cuvette into your 10% bleach beaker. Set the cuvette aside to use again.
29. Place the flask in the shaker/incubator and shake at 250rpm, 37°C.
30. After 15 minutes, remove the flask from the shaker/incubator.
31. Remove 2mL of culture and place it in the cuvette.
32. Take another reading at OD600.
33. Discard the bacterial solution from the cuvette into your 10% bleach beaker. Set the cuvette aside to use again.
34. Record your 15min time point reading in the Upstream Process Batch Record.
35. Place the flask back in the shaker/incubator and shake for another 15 min.
36. Repeat steps 24-30 at 30 min, 45 min, 60 min and 75 min time points.
37. When finished, the Process Engineer cleans the cuvettes according to the 'Spectrophotometer Check SOP'.
38. The flask will shake overnight at 250rpm, 37°C in the shaker/incubator. After the overnight incubation in the shaker/incubator, the flask can be stored in the refrigerator until the next class period.
39. Make sure you have filled out all necessary parts of the Upstream Process Batch Record.
40. Properly dispose of all waste following the guidelines in the Aseptic Technique slidedeck.

### **Protocol: Day 3 – Lesson 4 – Induction of RFP or GFP Production**

This will be carried out by the Upstream Process Technician.

**NOTE: All team members will assist the Upstream Process Technician as needed.**

**You are STRONGLY ENCOURAGED to check off each step below as you complete it.**

41. Determine how much volume remains in the liquid culture in your baffled flask.  
You started with 50mL. At various intervals you removed 2mL aliquots to read OD600. The number of readings you took x 2mL = how much volume you have lost. Subtract this amount from 50 to determine how many mL remain in your flask.
42. Calculate how much of the 50X arabinose solution to add to your liquid culture to bring it to a 1X solution.  
This can be done using the equation  $(C1)(V1) = (C2)(V2)$   
Example calculation:  
 $C1 = 1X$  – the concentration you want the arabinose to be at in your flask  
 $V1 = 32\text{mL}$  remaining in your flask  
 $C2 = 50X$  - the concentration the arabinose stock is at  
 $V2 =$  How much of the 50X arabinose you need to add to your 32mL culture to bring it to 1X  
 $(1)(32\text{mL}) = (50)(Y\text{ mL})$   
 $32 = 50(Y\text{mL})$   
 $Y\text{mL} = 32/50 = 0.64\text{mL}$  or 640uL
43. Using a micropipette, add the appropriate amount of the 50X arabinose solution to your liquid culture.
44. Put your flask back in the shaker/incubator and let it shake at 250rpm, 37°C until the next day.  
At this point the flask can be stored in the refrigerator until the next class period.
45. Properly dispose of all waste following the guidelines in the Aseptic Technique slide deck.
46. Make sure you have filled out all necessary parts of the Upstream Process Batch Record.

**Protocol: Day 4 – Lesson 5.1 – Bacterial Harvest and Limiting Dilution Plating**

These tasks should be done by the QC Technician.

**NOTE: All team members will assist the QC Technician as needed.**

**You are STRONGLY ENCOURAGED to check off each step below as you complete it.**

47. Remove your flask from the shaker/incubator. Take note of the color. Are the bacteria producing RFP or GFP? How can you tell?

48. Harvest the bacteria by carefully pouring the liquid culture into a sterile 50mL tube.
49. Now you will carry out a limiting dilution plating experiment so you will be able to determine your total bacterial yield.
50. Label 5 bacterial plates with your team name and date. Be sure to put your label on the side of the plate that contains the agar. Be sure to put your label around the edge of the plate.
51. Place the 5 LB/Amp agar plates in the bacterial incubator to warm up. Make sure your plates are upside down.
52. Prepare 6 microfuge tubes and label them as follows:
  - a. 1:10
  - b. 1:100
  - c.  $1:10^3$
  - d.  $1:10^4$
  - e.  $1:10^5$
  - f.  $1:10^6$

53. Arrange the microfuge tubes in your microfuge tube rack in the order they are shown in Fig 1.

54. Add 90uL of LB+Amp to each tube.

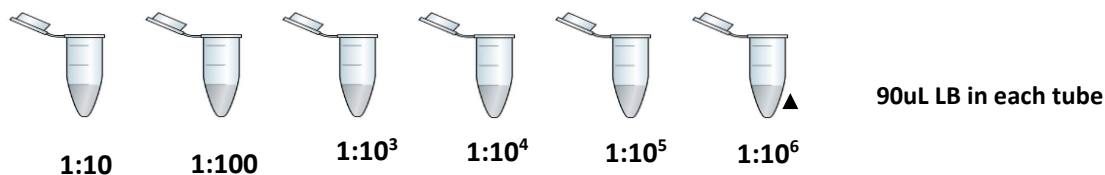


Fig. 1

55. Take 10uL of your harvested culture and add it to the 90uL of LB/Amp in your tube labeled 1:10. Cap the tube and mix by inverting 3-4 times. This is a 10-fold dilution of your harvested culture. (See Fig. 2)
56. Take 10uL of your 1:10 dilution and add it to the 90uL of LB/Amp in your tube labeled 1:100. Cap the tube and mix by inverting 3-4 times. This is a 100-fold dilution of your harvested culture.
57. Take 10uL of your 1:100 dilution and add it to the 90uL of LB/Amp in your tube labeled  $1:10^3$ . Cap the tube and mix by inverting 3-4 times. This is a 1,000-fold (or  $10^3$ -fold) dilution of your harvested culture.
58. Continue making dilutions to create your  $10^4$ -,  $10^5$ -, and  $10^6$ -fold dilutions.

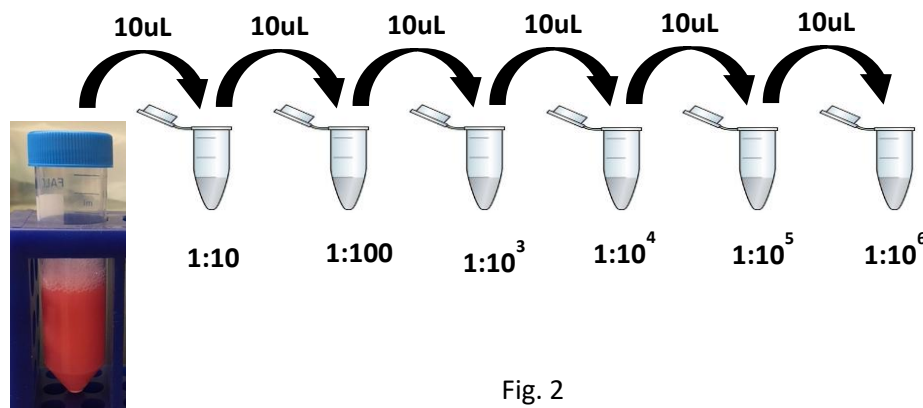


Fig. 2

### Your induced culture

59. Remove your LB/Amp agar plates from the bacterial incubator.
60. Have a team member open a package of sterile bacterial spreaders. Be sure they open the package where the non-spreader end of the spreaders is.
61. Have another team member remove a spreader being careful not to touch it to anything.
62. Using a p200 micropipette set to 50uL, remove 50uL of liquid from the 1:100 solution.
63. Have a team member open a plate. Add the 50uL of liquid drop-wise to the plate and spread it with a sterile spreader.  
NOTE: You will not plate your 1:10 dilution. It results in way too many colonies to count.
64. Add '1:100' to the plate label.
65. Repeat steps 55-58 for each of the rest of the bacterial dilutions.  
NOTE: You can use the same spreader for all plates IF you start with the lowest dilution first and IF you do not touch the spreader to anything but the plates.
66. Place the plates upside down in the bacterial incubator to grow overnight at 37 °C.  
After incubation overnight in the incubator, the plates can be stored in the refrigerator until the next class period. Be sure to store them upside down in the refrigerator so that moisture condensation does not drip onto your bacterial colony growth and smear them.
67. Store your harvested bacterial culture in the refrigerator.
68. Properly dispose of all waste following the guidelines in the Aseptic Technique slidedeck.
69. Make sure you have filled out all necessary parts of the Upstream Process Batch Record.

### **Protocol: Day 5 – Lesson 5.2 – Calculation of Bacterial Yield**

The calculation will be carried out as a team, by all team members. The QA Technician checks the Colony Forming Unit Calculation Record and the Upstream Process Batch Record.

**You are STRONGLY ENCOURAGED to check off each step below as you complete it.**

70. Remove your plates from the bacterial incubator.
71. Examine each plate to see how many colonies have grown.
72. If there is so much bacterial growth that you cannot see individual colonies, this is called a 'bacterial lawn'. Any plates with a bacterial lawn should be recorded as: 'lawn'. Plates with too many colonies to count should be recorded as 'too many to count'. Find any plates that have a number of individual bacterial colonies that you are able to count. Count the number of colonies on one of those plates and record the numbers in the appropriate boxes on the Colony Forming Unity Calculation Record.
73. Use the Colony Forming Unit Calculation Record document to calculate the total number of bacteria/mL (cfu/mL) of the culture of bacteria that you harvested. Use a plate that has a countable (but not too low) number of colonies. A count of 20-30 bacterial colonies is ideal.
74. The QA Technician reviews the Colony Forming Unit Calculation Record. If it is filled out properly, the QA Technician signs and dates it.
75. Record your count of cfu/mL on your Upstream Process Batch Record.
76. The QA Technician reviews the Upstream Process Batch Record.
77. If the record is filled out properly and completely, the QA Technician signs and dates it.
78. If the record is not filled out properly, the QA Technician deducts a point for every error made.

### **Protocol: Day 5 or 6**

#### **Final Documentation Check**

79. The QA Technician files the following documents in the group file:
  - a. Colony Forming Unit Calculation Record
  - b. Upstream Process Batch Record