

Production of RFP+ or GFP+ Bacteria – Upstream Process Protocol Days 4 & 5

Bacterial Harvest and Limiting Dilution Plating & Calculation of Bacterial Yield

Materials for Module 2, Lessons 4 and 5:

1. **Arabinose liquid stock (50X):** This will be added to LB/Amp media to induce bacterial production of GFP or RFP.
2. **125mL sterile glass baffled flask with lid containing your bacterial culture.**
3. **Five x 10 cm² LB + Ampicillin Agar Plates:** These will be used to determine your final bacterial culture count.
4. **Sterile 50mL plastic test tubes**
5. **Sterile plastic spreaders:** Used for spreading bacterial cultures on agar plates.
6. **Micropipettes and tips:** For measuring small volumes of reagents or bacterial cultures
7. **Microfuge tubes of various sizes:** For making serial dilutions
8. **Microfuge tube rack:** To hold microfuge tubes.
9. **Sharpie markers:** For correct labeling of samples.
10. **Colony Forming Unit (cfu) Calculation Record:** This form is a record of your team's bacterial yield calculation.
11. **Upstream Process Batch Record Form:** When properly filled out, this form is a record of your team's entire upstream process.

Equipment:

12. **Bacterial Plate Incubator:** The equipment used to warm bacterial cultures on agar plates for optimal growth.

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.

Use aseptic technique at all times!!

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

1. Remove your flask from the shaker/incubator. Take note of the color. Are the bacteria producing RFP or GFP? How can you tell?
2. Harvest the bacteria by carefully pouring the liquid culture into a sterile 50mL tube.
3. Now you will carry out a limiting dilution plating experiment so you will be able to determine your total bacterial yield.

4. 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.
5. Place the 5 LB/Amp agar plates in the bacterial incubator to warm up. Make sure your plates are upside down.
6. 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. $1:10^6$
7. Arrange the microfuge tubes in your microfuge tube rack in the order they are shown in Fig 1.
8. Add 90uL of LB+Amp to each tube.

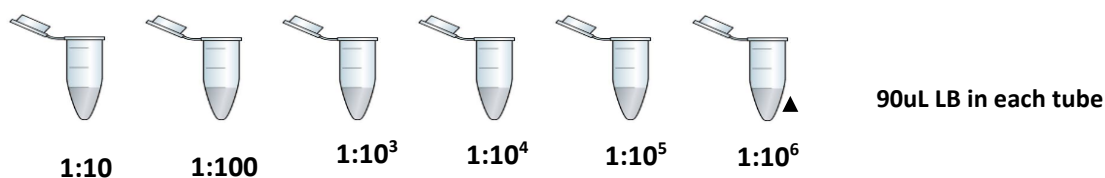


Fig. 1

9. 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)
10. 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.
11. 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.
12. Continue making dilutions to create your 10^4 -, 10^5 -, and 10^6 -fold dilutions.

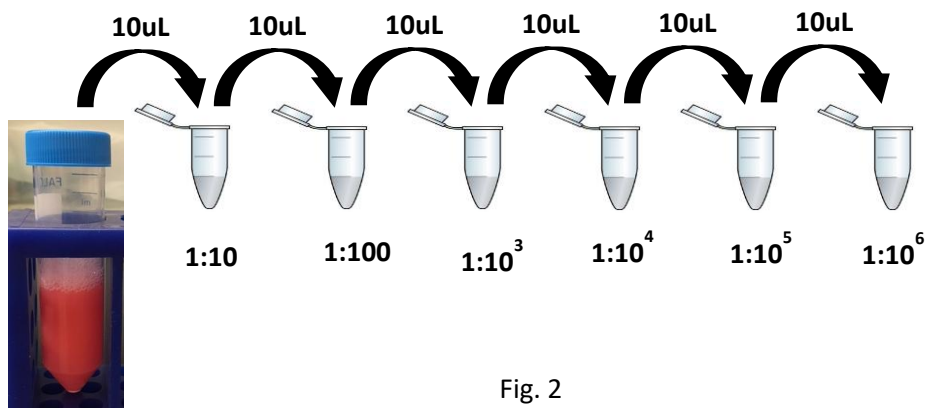


Fig. 2

13. Remove your LB/Amp agar plates from the bacterial incubator.
14. 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.
15. Have another team member remove a spreader being careful not to touch it to anything.
16. Using a p200 micropipette set to 50uL, remove 50uL of liquid from the 1:100 solution.
17. 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.
18. Add '1:100' to the plate label.
19. 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.
20. 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.
21. Store your harvested bacterial culture in the refrigerator.
22. Properly dispose of all waste following the guidelines in the Aseptic Technique slidedeck.
23. 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

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.

24. Remove your plates from the bacterial incubator.

25. Examine each plate to see how many colonies have grown.
26. 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 Unit Calculation Record.
27. 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.
28. The QA Technician reviews the Colony Forming Unit Calculation Record. If it is filled out properly, the QA Technician signs and dates it.
29. Record your count of cfu/mL on your Upstream Process Batch Record.
30. The QA Technician reviews the Upstream Process Batch Record.
31. If the record is filled out properly and completely, the QA Technician signs and dates it.
32. 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

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