Biomanufacturing Module Two: Upstream Process

Please Note: These labs are designed to be flexible. All documents are provided as '.doc' or '.ppt' so that teachers can download them and modify them to suit their curriculum, classroom, student population. Please refer to the Module 2 syllabus for different ways to carry out the bacterial culture scale up and spectrophotometer readings.

The Upstream Process Protocol is prepared in two forms.

- The entire Protocol for Days 1-5 in one large document
- Separate Protocols for Day 1, Day 2, Day 3, Day 4+5

	Topic / Activity	Summary
Day 1	Introduction to aseptic technique - ● Practicing aseptic technique ● Testing the sterility of LB	On day one, students learn what aseptic technique is and perform an experiment to practice aseptic technique. Each student uses aseptic technique to test the sterility of the LB that will be used to grow RFP+ or GFP+ bacteria.
Day 2	Check Bacterial Plates Inoculate a small liquid culture with and RFP+ or GFP+ bacterial colony	On day two, student teams will check their bacterial plates to determine how well they performed aseptic technique. Using aseptic technique the Upstream biomanufacturing associates will inoculate a small liquid culture and grow it overnight.
Day 3	Scale up the bacterial culture to 50mL Take OD600 readings over a time course to plot a growth curve	On day three, Upstream Technicians scale up the bacterial culture to produce more RFP+ or GFP+ bacteria. Teams Process Engineers calibrate the spectrophotometer. The Upstream technician takes samples of the bacterial culture at designated time points. The QC Technician records the data. Student teams graph bacterial growth curves using their own data as well as a provided data set. The rest of the culture grows overnight.
Day 4	Induction of RFP or GFP production	On Day 4 Upstream Technicians add arabinose to the culture to induce protein production. The culture grows overnight again.
Day 5/6	Harvest of bacterial culture Bacterial plating to determine bacterial yield.	On Day 5 Upstream Technicians harvest the bacterial culture. The QC Technician carries out plating in order

	Documentation - batch record	to calculate bacterial yield. Students fill out the batch record. The QA Technician signs off on the upstream process batch record.

Biomanufacturing Kit Module 2 Syllabus

Please Note: These labs are designed to be flexible. All documents are provided as '.doc' or '.ppt' so that teachers can download them and modify them to suit their curriculum, classroom, student population etc

In particular, the bacterial culture scale up (Module 2, Lesson 3) can be done in a variety of ways.

- In a short class period, each team could take an OD600 reading of their 50mL culture at time = 0 minutes and then take another reading after the culture has grown for 24 hours.
- In a longer class period, each team could take several readings at 15 or 20 minute time points.
- A second class that occurs right after the first class could continue the readings on a culture started by teams in the first class.

In any case, there is also data provided that can be used by students to graph an entire growth curve.

Bacterial cultures, both liquid and on plates, can be stored in the refrigerator between classes. Plates should always be stored upside down (ie agar side up).

Upstream Process

1. Introduction to Aseptic Technique: Module 2, Lesson 1
    a. Aseptic Technique slide deck
    b. Aseptic Technique practice by all students: Testing the sterility of LB (SOP)

    Next class period:
    c. Examine plates
    d. Each team fills out the Aseptic Technique Check document
2. Bacterial Inoculation: Module 2, Lesson 2
    a. Review Upstream Process – Overview of Biomanufacturing slide deck
    b. Each team inoculates a small liquid culture in LB + ampicillin.
    c. Each team fills out the appropriate parts of the Upstream Process Batch Record document
    d. Recombinant Protein Production in Bacteria slide deck
3. Bacterial Culture Scale Up: Module 2, Lesson 3
    a. Each team scales up their culture to 50mL of LB + ampicillin.
    b. The Process Engineers prepare the spectrophotometer for use (SOP)
    c. Each team takes OD600 readings at various time points
    d. Each team records data in the appropriate part of the Upstream Process Batch Record

    e. Bacterial Growth Curve Graphing – teams graph their own data and/or a provided data set to generate a growth curve (this can be homework)
4. Induction of Recombinant Protein Production (RFP or GFP): Module 2, Lesson 4
    a. Induction of Protein Production slidedeck.
    b. Each team adds the appropriate amount of the sterile arabinose stock to their bacterial culture.
    c. Each team fills out the appropriate part of the Upstream Process Batch Record.
5. Bacterial Harvest and Determination of Bacterial Yield: Module 2, Lesson 5
    a. Each team harvests their bacterial culture
    b. Each team practices making serial dilutions (Serial Dilution Lab)
    c. Each team performs a bacterial plating experiment in order to determine their cfu/mL
    d. Each team fills out the appropriate part of the Upstream Process Batch Record.

    Next class period:
    e. Examine serial dilution plates
    f. Each team calculates their bacterial yield
    g. Each team fills out a Colony Forming Unit Calculation Record
    h. Each team completes their Upstream Process Batch Record.

Biomanufacturing Module 2

Lesson 1 – Bacterial Inoculation

Lesson objectives:

Students will understand:

- The importance of aseptic technique.
- How to perform aseptic technique.

Essential Question

- Why do you need to use aseptic technique in biomanufacturing?

Materials:

- Aseptic Technique slide deck
- Testing the Sterility of LB SOP
- Sterile bacterial spreaders (1 pack/team)
- LB media (1 microfuge tube of media/student)
- P200 Micropipette and tips
- Sharpie markers
- LB agar plates (1/student)
- Bacterial plate incubator
- Aseptic Technique Check Document

What Students Will Do

- Listen to/watch the Aseptic Technique slide deck
- Prepare themselves and their lab benches to perform aseptic technique
- Follow the Testing the Sterility of LB SOP as a method of practicing aseptic technique
- Each team fills out their Aseptic Technique Check Document

Teacher Preparation

- Prior to class make copies of
    - Testing the Sterility of LB SOP (one per team)
    - Aseptic Technique Check Document (one per team)
- Prior to class turn on the bacterial incubator to a temperature of 37 degrees Celsius.
- Prior to class remove the aliquots of LB media from the refrigerator and let them warm to room temperature.
- Prior to class remove the LB agar plates from the refrigerator and let them warm to room temperature.
- Provide each team
    - One package of sterile bacterial spreaders
    - One p200 micropipette
    - One box of p200 micropipette tips
    - Sharpie marker for labeling plates
    - One microfuge tube of LB per student
    - Five LB agar plates
    - Spray bottle of 70% ethanol

- Paper towels
- Team file folders

Organizer

Time	Activity	Materials
10 minutes	Present the Aseptic Technique slide deck	Slide deck
5 minutes	Members of all teams put on PPE	Lab coats, gloves, safety goggles
5 minutes	Teams sanitize and prepare their bench space	70% ethanol, paper towels, sterile LB media, sterile LB agar plates, micropipette, tips, sterile spreaders
20 minutes	Each team member practices aseptic technique by spreading sterile LB media on an LB agar plate.	Testing the Sterility of LB SOP, sterile LB media, sterile LB agar plates, micropipette, tips, sterile spreaders
5 minutes	Each member of the team labels their plate and places it in the bacterial incubator. The plates incubate overnight and can then be stored in the refrigerator until the next class period.	Sharpie markers Bacterial incubator
5 minutes – NEXT CLASS	Each student puts on gloves and removes their plate from the incubator	
10 minutes	Each student counts the colonies on their plate. Teams fill out the Aseptic Technique Check Document and file it	Aseptic Technique Check Document, Team File Folder

Procedure

Introduction to Aseptic Technique

1. Present the Aseptic Technique slide deck

Preparation of the staff and lab benches

2. Members of each team put on PPE
3. Each team sanitizes and organizes their bench space
4. Each team member follows the Testing the Sterility of LB SOP to plate sterile LB onto an LB agar plate.

5. Each team member puts their plate in the bacterial incubator to incubate at 37 degrees Celsius for 24 hours. After 24 hours in the incubator, plates can be stored in the refrigerator until the next class period.

Data Analysis – Next class period

6. Each team member puts on gloves and retrieves their plate from the incubator.
7. Each team member counts the colonies on their plate
8. Each team fills out their section of the Aseptic Technique Check document.
9. The completed Aseptic Technique Check document is filed in the team file.

Standard Operating Procedure for Performing a Sterility Test on Lysogeny Broth

SOP#: PPP-01
SOP Author: Pauline Fusco
Prepared: 03 March 2022,
Revision #2
Revised: 10 August 2022
Revisor: N. Jan Chalupny

1. Purpose

This procedure is designed to test the sterility of lysogeny broth (LB). If the broth will be used to grow a specific strain (type) of bacteria, the broth must be sterile. Otherwise, the resulting growth cannot be guaranteed to be the desired bacteria. Bacteria is everywhere, and LB is a media designed to support the growth of bacteria. The results of the sterility test will be recorded on the 'LB Sterility Results Report' document. A failed test would require the technician to trace all experiments that may have used that particular batch of LB. The LB was made previously, transferred into suitable flasks and bottles which are appropriately labeled, and autoclaved as a batch.

Each team member will perform this test using a sterile aliquot of LB that they have been provided. The test will involve a transfer of some of the LB liquid to an LB media plate followed by spreading of the liquid LB on the plate. The spread plate method is a technique to plate a liquid sample onto solid media in order to determine if any bacteria are growing in the liquid media. Bacterial growth on solid media in a plate is easy to see. If the liquid LB media is sterile, there will be no bacterial growth on the LB agar plate. The plates will be incubated for 24 hours at 37^{o}C in an incubator.

2. Scope

This Standard Operating Procedure is intended for students using the Shoreline Biotechnology Experience Biomanufacturing Kit.

3. Definitions

Media: A solution (liquid, gel, or some other type) used to grow bacteria.

Lysogeny broth (LB): A liquid growth medium, also called Luria broth, Lennox Broth, or Luria-Bertani medium. The standard recipe contains distilled water plus tryptone, yeast extract, and sodium chloride.

Batch record: Any reagent produced in a lab is identified by a batch record so it is traceable in the event of possible contamination or failed experiments. A batch record contains the recipe used to make the reagent, the concentrations of all the ingredients, the source of all the ingredients, the storage conditions and expected shelf life, who made it and when, and information relating to equipment or possible deviations and how they were corrected. This record is kept on file for future reference. The ID

of the batch record is also put on all containers of the reagent produced in that batch. Outside of your lab, you might see it as a lot number.

Sterility: The absence of bacteria or other living contaminants.

Bacteria: Small single-celled organisms found almost everywhere on earth.

Bacterial Strain: A genetic variant or subtype of a microorganism. The example in lab today is the DH5α strain of E. coli, which is one that is commonly seen in biotech labs because it has been bred to have specific mutations useful for inserting plasmids, otherwise known as subcloning or cloning. The DH5α strain is thus known as a good cloning vector. The DH5α strain is also non-pathogenic, meaning it will not make humans sick.

LB Agar Plate: LB with agar added. The agar allows the media to solidify in the plate. The result is a plate with a layer of a firm gel-like substance called LB agar. The LB agar plate is commonly used in biotech, but particularly for sterility tests.

4. References

Microbiology—004—Spread Plate Method. (n.d.). Microbiology Undergraduate Program. Retrieved April 11, 2022, from https://www.micro.iastate.edu/video/microbiology-004-spread-plate-method

Sezonov, G., Joseleau-Petit, D., & D'Ari, R. (2007). Escherichia coli Physiology in Luria-Bertani Broth. Journal of Bacteriology, 189(23), 8746–8749. https://doi.org/10.1128/JB.01368-07

Sterility Test. (n.d.). International Society of BioProcess Technology. Retrieved April 11, 2022 from https://www.isbiotech.org/about.html

SODIUM HYPOCHLORITE | CAMEO Chemicals | NOAA. (n.d.). Retrieved February 14, 2022, from https://cameochemicals.noaa.gov/chemical/4503

5. Materials, Reagents, and Equipment

5.1. Materials
- 5.1.1. A plastic or glass container around 500mL in volume for holding the 10% bleach solution.
- 5.1.2. Paper towels
- 5.1.3. Sharpie marker for labelling

5.2. Reagents
- 5.2.1. Lysogeny broth (LB) to be tested
- 5.2.2. LB Agar Plate
- 5.2.3. Bleach
- 5.2.4. 70% Ethanol spray (70% EtOH)
- 5.2.5. Dish soap spray (a spray bottle filled with a little dish soap added to water)

5.3. Equipment
- 5.3.1. Sterile Bacterial spreader
- 5.3.2. Micropipettor and tips
- 5.3.3. Shaker Incubator

6. Responsibilities of all team members

Lab personnel overseeing and performing the procedure are responsible for adhering to current lab safety procedures. Lab technicians must be trained to operate equipment and follow precautions listed below for handling potentially hazardous materials. Aseptic technique should be used. Bacterial waste should be disposed of in the proper manner.

7. Hazards and Safety Precautions

7.1. Warning: 10% bleach causes serious eye irritation, potential skin irritant.

7.2. Prevention: Wear Personal Protective Equipment (PPE), avoid contact, ingestion.

7.3. Response: Flush eyes with water for up to 30 minutes after exposure, wash hands after exposure.

8. Procedure

8.1. Pre-procedure preparation

8.1.1. Put on personal protective equipment

8.1.2. Make sure long hair is tied back.

8.1.3. Clear clutter off the lab bench.

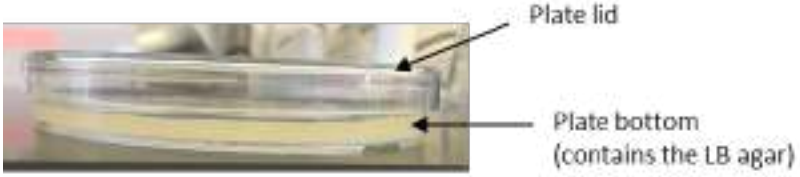
8.1.4. Organize your reagents, materials and equipment so they are close at hand.

8.1.5. Spray work surfaces with 70% ethanol (EtOH). Spread the EtOH evenly with a paper towel. Let surfaces dry.

8.1.6. Make sure the windows and doors of the room are closed.

8.2. LB Plating

8.2.1. Label a pre-warmed LB plate. The label should include the team member's initials, the date, and the purpose (LB test). Be sure to label the half of the plate containing the LB agar. Write the label on the edge of the plate (not across the middle). See **Figure 1** for an explanation of how to label your plate.





Turn the plate upsidedown.
Write the label on the bottom of the plate.
Write the label around the edge of the plate.

Figure 1: How to properly label a bacterial plate.

8.2.2. Using a p200 micropipette, remove 50uL of the LB from the container.
8.2.3. Carefully open your plate using the clam shell technique with the opening facing away from you. You may want a co-worker to do this for you.
8.2.4. Add the LB media to the surface of the LB Agar drop-wise.
8.2.5. Remove a sterile spreader from the package.
8.2.6. Carefully spread the LB over the surface of the plate.

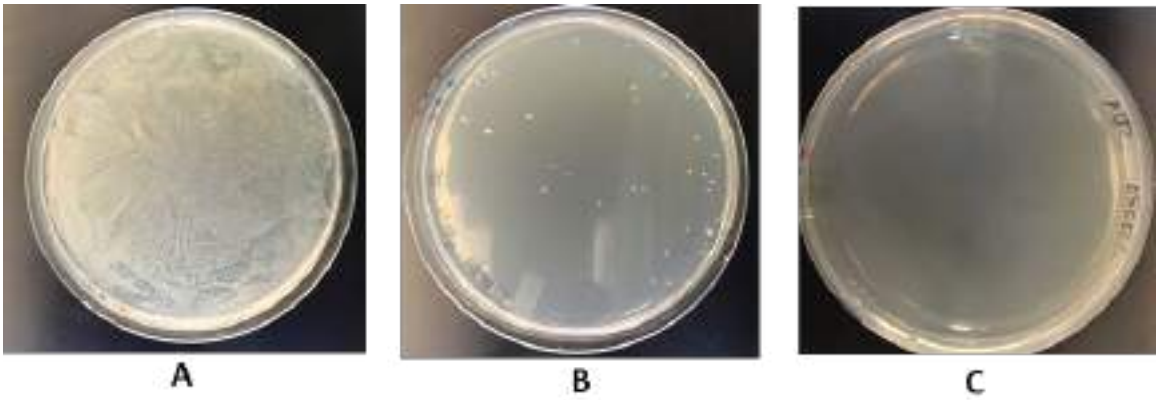


Figure 2: (A) Plate showing a lawn of bacteria. (B) Plate with individual bacterial colonies. (C) Plate with no bacterial growth.

8.3. Incubation

8.3.1. Incubate the plates in a 37 degree ˚C incubator for 24 hours. Make sure your plate is upside down in the incubator.
8.3.2. After the 24 hour incubation examine your plate and record your results on the LB Sterility Test Results Report. **Figure 2** shows examples of what plates might look like. If you have bacterial contamination, your plate may look like example A or B. If you have no contamination, your plate will look like example C.
8.3.3. Each team member should sign and date the Aseptic Technique Check Report
8.3.4. Give the Report to the QA Technician to review, sign, and date
8.3.5. The QA Technician files the LB Sterility Test Report in the team file.

8.4. Waste Disposal

8.4.1. Used bacterial spreaders should be disposed of in biohazard trash.
8.4.2. Remaining LB should be poured into a beaker containing 10% bleach. This beaker should be kept in the sink to minimize the risk of spills. This mixture should incubate at room temperature for 10 minutes and can then be poured down the sink.
8.4.3. After plates have been examined for colonies they should be disposed of in the biohazard trash.
8.4.4. All used micropipette tips should be disposed of in the biohazard waste.

Biomanufacturing Module 2

Lesson 2 – Bacterial Inoculation

Lesson objectives:

Students will understand:

- How to perform a bacterial inoculation.
- At what stage bacterial inoculation occurs during upstream processing.

Essential Question

- Why do you inoculate with a single bacterial colony?

Materials:

- Intro to Biomanufacturing slide showing upstream and downstream process steps
- Recombinant Protein Production in Bacteria slide deck
- Upstream Process Protocol Day 1 (Production of RFP+ or GFP+ Bacteria)
- Sterile inoculating loop (1/team)
- LB/Amp/Arabinose plate with single colonies of RFP+ or GFP+ E. coli
- LB + ampicillin (LB/Amp) media (1 bottle/team)
- 14mL Falcon snap cap tube (1/team)
- p1000 Micropipette and tips (1/team)
- Shaker/incubator
- Upstream Process Batch Record Document

What Students Will Do

- Review upstream process
- Listen to/watch the Recombinant Protein Production in Bacteria slide deck
- Inoculate a single colony of RFP+ or GFP+ bacteria into LB/Amp media
- Place the tube containing the inoculated bacteria in the shaker/incubator
- Each team fills out the appropriate parts of their Upstream Process Batch Record

Teacher Preparation

- Prior to class make copies of
    - Production of RFP+ or GFP+ Bacteria Protocol (one per team)
    - Upstream Process Batch Record Document (one per team)
- Prior to class turn on the bacterial shaker/incubator to a temperature of 37 degrees Celsius.
- Prior to class secure a tube rack onto the platform of the shaker/incubator.
- Prior to class remove the LB/Amp media from the refrigerator and let it warm to room temperature.
- Prior to class remove the LB/Amp/arabinose plate that has RFP+ or GFP+ colonies on it from the fridge. Place the plate in a central place so all teams can have access to it.
- Provide each team
    - One sterile inoculation loop
    - One p1000 micropipette and tips OR sterile transfer pipettes
    - One bottle of sterile LB/Amp media

- Sharpie marker for labeling tubes
- One 14mL Falcon snap cap tube
- Spray bottle of 70% ethanol
- Paper towels
- Team file folders

Organizer

Time	Activity	Materials
5 minutes	Review Upstream Process	Slide from Intro to Biomanufacturing slide deck
10 minutes	Present the Recombinant Protein Production in Bacteria slide deck	Slide deck
5 minutes	Members of all teams put on PPE	Lab coats, gloves, safety goggles
5 minutes	Teams sanitize and prepare their bench space	70% ethanol, paper towels, sterile LB/Amp media, Falcon snap cap tube, micropipette, tips, sterile inoculators, LB/Amp/arabinose plate with RFP+ or GFP+ colonies
15 minutes	Each team creates a bacterial inoculum	Upstream Process Protocol – Day 1, sterile LB/Amp media, Falcon snap cap tube, micropipette, tips, sterile inoculators
5 minutes	Each member of the team labels their plate and places it in the bacterial shaker/incubator	Sharpie markers Bacterial shaker/incubator
10 minutes	Teams fill out the appropriate portions of their Upstream Process Batch Record Document and file it	Upstream Process Batch Record Document, Team File Folder

Procedure

Introduction to Protein Production in Bacteria

1. Present the Introduction to Recombinant Protein Production in Bacteria slide deck

Preparation of the bacterial inoculum

2. Members of each team put on PPE

3. Each team sanitizes and organizes their bench space
4. Each team follows the appropriate section of the Upstream Process Protocol (Day 1) - Production of RFP+ or GFP+ Bacteria, to create a bacterial inoculum.
5. Each team puts their tube containing the inoculum in the shaker/incubator to shake at 37 degrees C for 24 hours. After 24 hours in the shaker/incubator, tubes can be stored in the refrigerator until the next class period.
6. Each team fills out the appropriate sections of the Upstream Process Batch Record and files it in their team file.

Module 2 – Shaker/Incubator Instructions

Using the Shaker/Incubator – Benchmark Incu Shaker Mini

1. Plug the unit in.
2. Turn the unit on. The on/off switch is on the back of the unit, on the right side (if you are facing the front of the unit.
3. The setting display will come on in the window.
4. The shaker/incubators are pre-set at:
    a. Temperature = 37 deg C
    b. Shaking speed = 220 rpm
    c. Timer = 36 hours
5. If you want to change settings, use the following instructions from the user's manual.



▶ **Basic View Screen**

ⓐ HEAT LED: Indicates if the heater is activated

ⓑ RUN LED: Indicates if the shaker is running.

ⓒ Time setting: Indicates the set or remaining time.
(To adjust, TOUCH the time value.)

ⓓ Temp setting: Displays the current temperature.
To set the desired temperature, TOUCH the temperature value.

ⓔ RPM setting: Displays the set or current RPM
To set the desired shaking speed, TOUCH the RPM value.

ⓕ Alarm ON/OFF: Alarm rings according to the condition
TOUCH to toggle the alarm on or off.

→ Alarm Alarm ON

→ Alarm Alarm OFF(mute)

ⓖ Menu : TOUCH to activate the main menu

ⓗ Shaker Start/Stop Button
TOUCH to Start or to stop the shaker.

Start Stop

ⓘShaker end: Indicates that time has expired and the shaker has stopped



▷ Temperature setting change

ⓐ TOUCH the temperature value, The numbers become red
ⓑ "SET MODE" is displayed (flashes)
ⓒ Input the desired value by pressing UP(▲)/DOWN(▼) buttons
NOTE: Press and hold the buttons to increase or decrease the value quickly.
ⓓ Apply: To store the newly set value TOUCH Apply
(Or wait 5 seconds for the value to store automatically)
ⓔ Exit: If you press Exit after changing any values, the values are not saved and the screen returns to the previous screen



▷ RPM setting change

ⓐ TOUCH the RPM value, The numbers become red
ⓑ "SET MODE" is displayed (flashes)
ⓒ Input the desired value by pressing UP(▲)/DOWN(▼) buttons
NOTE: Press and hold the buttons to increase or decrease the value quickly.
ⓓ Apply: To store the newly set value TOUCH Apply
(Or wait 5 seconds for the value to store automatically)
ⓔ Exit: If you press Exit after changing any values, the values are not saved and the screen returns to the previous screen



▷ Time setting change

ⓐ TOUCH the time value, The numbers become red
ⓑ "SET MODE" is displayed (flashes)
ⓒ Input the desired value by pressing UP(▲)/DOWN(▼) buttons
NOTE: Press and hold the buttons to increase or decrease the value quickly.
ⓓ Apply: To store the newly set value TOUCH
(Or wait 5 seconds for the value to store automatically)
ⓔ Exit: If you press Exit after changing any values, the values are not saved and the screen returns to the previous screen

NOTE: The time can not be adjusted during a run.
To change the time during a run,
you must TOUCH Stop .
Change the time value and press Apply and Start .

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^2 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 Incubato**r: 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^{o}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^{o}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^{o}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 = 32mL 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)(32mL) = (50)(Y mL)
    32 = 50(YmL)
    YmL = 32/50 = 0.64mL 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^{o}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.

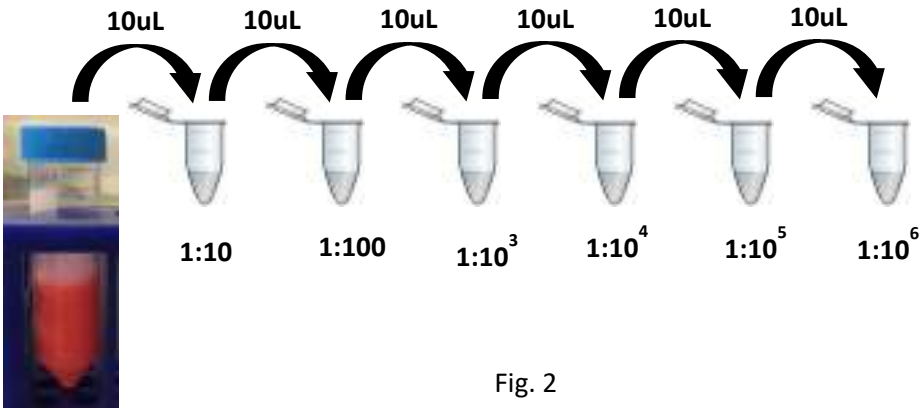


Fig. 2

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 ^{o}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

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^2 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 Incubato**r: 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^{o}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^{o}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^{o}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 = 32mL 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)(32mL) = (50)(Y mL)
    32 = 50(YmL)
    YmL = 32/50 = 0.64mL 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^{o}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.

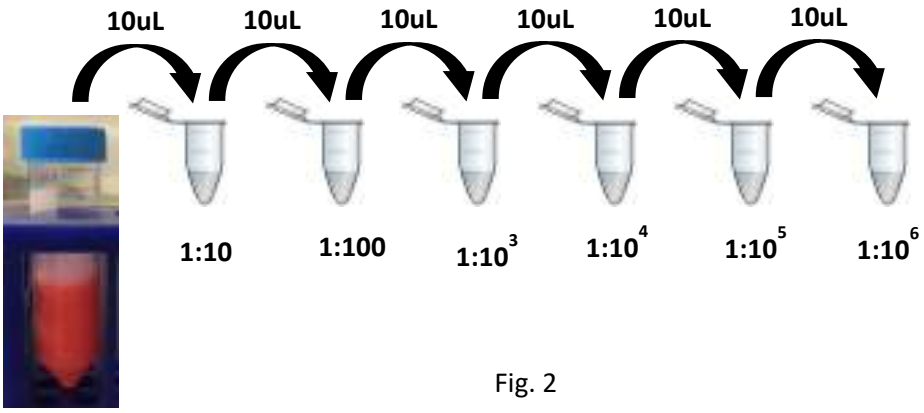


Fig. 2

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 ^{o}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

Biomanufacturing Module 2

Lesson 3 – Bacterial Culture Scale Up

NOTE: This lesson is best done during a class period that is longer than 50 minutes.

Lesson objectives:

Students will understand:

- How to scale up a liquid bacterial culture.
- Why the bacterial culture is scaled up.

Essential Question

- Why do you scale up the bacterial culture?

Materials:

- Review bacterial growth curve information in Recombinant Protein Production in Bacteria slide deck
- Upstream Process Protocol (Day 2)
- Spectrophotometer Check SOP
- Small liquid bacterial culture created in the last class period
- LB/Amp media (1 bottle/team)
- Sterile 50mL tube (1/team)
- Sterile 125mL baffled flask (1/team)
- Shaker/incubator
- Spectrophotometer
- Cuvettes (1/team and 1 for the Process Engineers to use for blanking the spectrophotometer)
- p1000 micropipette and tips (1/team)
- Upstream Process Batch Record Document
- Bacterial Growth Curve Graphing Protocol
- Bacterial Growth Curve Graphing Spreadsheet

What Students Will Do

- Review bacterial growth curve information in the Recombinant Protein Production in Bacteria slide deck
- Each team will scale up their liquid culture to 50mL
- Each team will label their flask
- Teams place the flask containing their scaled up culture in the shaker/incubator
- Process Engineers will ready the spectrophotometer using the Spectrophotometer Check SOP.

  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.
- Teams will take OD600 readings using the spectrophotometer at various time points
- Each team fills out the appropriate parts of their Upstream Process Batch Record

Teacher Preparation

- Prior to class get out the team folders which contain:
    - Upstream Process Protocol (one per team)
    - Upstream Process Batch Record Document (one per team)
    - Spectrophotometer Check SOP (one per team)
- Prior to class, turn on the bacterial shaker/incubator to a temperature of 37 degrees Celsius.
- Prior to class, turn on the spectrophotometer (needs to warm up for 30 min prior to use)
- Prior to class, secure 6 flask holders onto the platform of the shaker/incubator.
- Prior to class, remove the LB/Amp media from the refrigerator and let it warm to room temperature.
- Provide each team
    - Upstream Process - Production of RFP+ or GFP+ Bacteria Protocol
    - Spectrophotometer Check SOP
    - One sterile 50mL tube
    - One bottle of LB/Amp media
    - One p1000 micropipette
    - One box of p1000 micropipette tips
    - One 125mL sterile glass baffled flask
    - Sharpie marker and tape for labeling flasks
    - Spray bottle of 70% ethanol
    - Paper towels
    - Upstream Process Batch Record
    - Team file folders
    - A beaker half full of 10% bleach (in the sink)
    - Bacterial Growth Curve Graphing Protocol

Organizer

Time	**Activity**	**Materials**
10 minutes	Review bacterial growth curve information from the Recombinant Protein Production in Bacteria slide deck	Slide deck
5 minutes	Members of all teams put on PPE	Lab coats, gloves, safety goggles
5 minutes	Teams sanitize and prepare their bench space	70% ethanol, paper towels, sterile LB/Amp media, sterile 125mL baffled flask, micropipette, tips, small liquid bacterial culture started in previous class period

15 minutes	Each team takes their small liquid bacterial culture out of the shaker/incubator and uses it to make a scaled up 50mL culture	Upstream Process Protocol – Day 2, sterile LB/Amp media, sterile 125mL baffled flask, micropipette, tips, small liquid bacterial culture started in previous class period
10 minutes	Team Process Engineers prepare the spectrophotometer and blank it	Spectrophotometer, cuvettes, micropipette, tips, 10% bleach solution
60 minutes	Each team takes samples from their flask at specific times and measures the OD600 using the spectrophotometer	Spectrophotometer, cuvettes, micropipette, tips, 10% bleach solution
10 minutes	Teams fill out the appropriate portions of their Upstream Process Batch Record Document and file it	Upstream Process Batch Record Document, Team File Folder
30 minutes – homework or next class	Teams graph their own OD600 data and/or a provided data set to generate bacterial growth curves	Graphing a Bacterial Growth Curve Protocol, Bacterial Growth Curve Graphing Spreadsheet

Procedure

Bacterial Growth Curve Review

1. Review the slides covering bacterial growth curves in the Recombinant Protein Production in Bacteria slide deck

Scale up of the bacterial culture

2. Members of each team put on PPE
3. Each team sanitizes and organizes their bench space
4. Each team retrieves its small bacterial liquid culture from the incubator
5. Each team follows the appropriate section of the Production of RFP+ or GFP+ Bacteria Protocol to scale up their bacterial culture.
6. Each team puts their flask containing the scaled up culture in the shaker/incubator to shake at 37 degrees C for 24 hours.

Plotting a Bacterial Growth Curve

7. All Process Engineers work together using the Spectrophotometer Check SOP to prepare the spectrophotometer for use.
8. Each team takes OD600 readings with samples from their culture flask taken at assigned time points.

9. Each team records their OD600 readings in their Upstream Process Batch Record.
10. Each team plots their own data and/or a provided data set to create a bacterial growth curve graph.
11. Each team fills out the appropriate sections of the Upstream Process Batch Record and files it in their team file.

NOTE: You may want your students to test the difference in production of RFP (or GFP) between cells that are;

- induced at the time of scale-up vs.
- cells that are induced 24 hr after scale-up

If so, one team of students should induce expression of RFP in their 50mL bacterial culture at the time of scale up. Follow the Module 2, Lesson 4 protocol. The difference in expression under these two conditions can be seen in the 'RFP Induction Timeline Results' document (found in the Module 2, Lesson 4 section).

Production of RFP+ or GFP+ Bacteria – Upstream Process Protocol Day 2 Bacterial Culture Scale Up

Materials for Module 2, Lessons 3:

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. **125mL sterile glass baffled flask with lid**: The scaled up bacterial culture will be grown in this container.
4. **Sterile 50mL plastic test tubes**
5. **Plastic cuvettes**: The correct container for bacterial cultures that will be read in the spectrophotometer.
6. **Micropipettes and tips**: For measuring small volumes of reagents or bacterial cultures
7. **Microfuge tubes of various sizes:** For containing small volumes of reagents or bacterial cultures
8. **Microfuge tube rack**: To hold microfuge tubes.
9. **Sharpie markers**: For correct labeling of samples.
10. **Upstream Process Batch Record Form**: When properly filled out, this form is a record of your team's entire upstream process.

Equipment:

11. **Shaker/Incubator**: The equipment used to shake and warm bacterial cultures for optimal growth in liquid culture.
12. **Spectrophotometer**: The equipment used to measure the optical density of bacterial cultures – for plotting bacterial growth curves.

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.

Use aseptic Technique at all times!!

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

1. Carefully open the sterile baffled flask by pulling the metal cap off and setting it on your sterilized lab bench.

2. 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.

3. Place a tape label on the flask with your team name, date, and contents.

4. 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.

5. Add the 500uL of your small liquid overnight culture to the 50mL of LB/Amp in the baffled flask.

6. Cap the flask and swirl gently to mix.

7. 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.

8. The QC Technician will remove 2mL of bacterial culture and place it into a cuvette.

9. Read the cuvette at OD600. Record the data in the Upstream Process Batch Record. This reading will be your 0 time point reading.

10. Discard the bacterial solution from the cuvette into your 10% bleach beaker. Set the cuvette aside to use again.

11. Place the flask in the shaker/incubator and shake at 250rpm, 37^{o}C.

12. After 15 minutes, remove the flask from the shaker/incubator.

13. Remove 2mL of culture and place it in the cuvette.

14. Take another reading at OD600.

15. Discard the bacterial solution from the cuvette into your 10% bleach beaker. Set the cuvette aside to use again.

16. Record your 15min time point reading in the Upstream Process Batch Record.

17. Place the flask back in the shaker/incubator and shake for another 15 min.

18. Repeat steps 24-30 at 30 min, 45 min, 60 min and 75 min time points.

19. When finished, the Process Engineer cleans the cuvettes according to the 'Spectrophotometer Check SOP'.

20. The flask will shake overnight at 250rpm, 37^{o}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.

21. Make sure you have filled out all necessary parts of the Upstream Process Batch Record.

22. Properly dispose of all waste following the guidelines in the Aseptic Technique slidedeck.

Directions for Use of the Vis Spectrophotometer – Teacher Document

Please refer to Figure 1 for labeled pictures of the Vis Spectrophotometer.

1. Ensure the cuvette is clean. Wipe it free of smudges with a kimwipe.

2. After the spectrophotometer is warmed up, use the p1000 micropipette and tip to transfer **2 mL of lysogeny broth (LB)** into the cuvette. This cuvette is called the blank.

3. After the spectrophotometer is warmed up, use the p1000 micropipette and tip to transfer **2 mL of lysogeny broth (LB)** into the cuvette. This cuvette will be the blank.

4. Open the lid of the spectrophotometer. The spectrophotometer can hold four cuvettes at a time, if necessary. The cuvettes can be moved into the optical path by manually withdrawing or pushing in the knob on the front right of the spectrophotometer. Only one cuvette is needed to calibrate the spectrophotometer.

5. Place the cuvette into the cuvette holder in the slot that is closest to the front of the machine. Make sure the knob is pushed all the way in – this should put the front-most cuvette spot in the optical path. Make sure the blank cuvette is in the optical path. **Close the lid.**

6. Select the wavelength for bacterial cultures. For OD600 readings, select a wavelength of 600nm. Use the dial on the lower left of the top side of the spectrophotometer, and the dial display.

7. Select transmittance (%T) mode using the Mode button on the display panel on the upper top left of the spectrophotometer. The transmittance mode will be indicated by a light next to the label.

8. Tare by pressing the 100%T/abs button. The result should be 100% Transmittance of light through the LB. Transmittance and Absorbance are inversely related. So, the blank cuvette, which contains LB with no bacteria, should now have an Absorbance reading of zero.

9. Select the absorbance (Abs) using the Mode button on the display panel on the upper top left of the spectrophotometer. When the absorbance mode is selected you will see a light go on next to the 'Abs' label.

10. The readout should say zero. All subsequent readings will be based on the difference in absorbance between the sterile media of the blank and the media

containing the growing bacteria. This equates to measuring the amount of bacteria in a solution.

Please NOTE: These spectrophotometers were not made in the USA. A reading of 0,000 is a reading of 0.000. The comma translates to a decimal point.

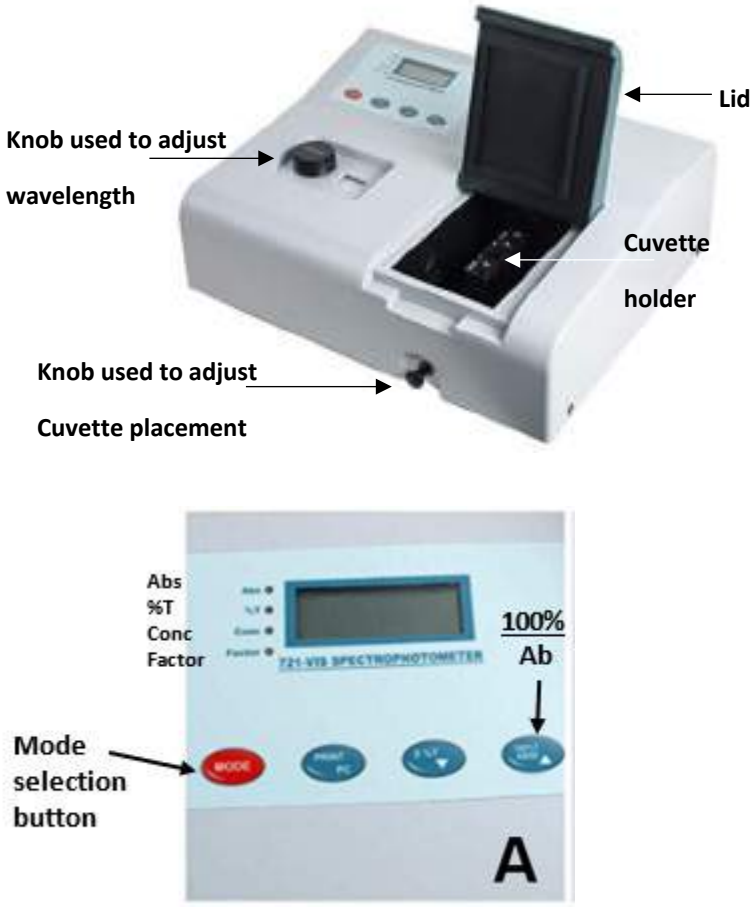


Fig 1

Standard Operating Procedure for Measuring Absorbance using the Vis Spectrophotometer

SOP#: PPP-02
SOP Author: Pauline Fusco
Prepared: 14 February 2022
Revision #2
Revised: 25 May 2022
Revisor: N. Jan Chalupny

1. Purpose

This procedure is for measuring absorbance of liquid bacterial culture samples using the Vis Spectrophotometer.

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.

2. Scope

This Standard Operating Procedure is intended for students using the Shoreline Biotechnology Experience Biomanufacturing Kit.

3. Definitions

Spectrophotometer: A spectrophotometer uses a light source, a lens, the sample in a cuvette, a slit, a concave mirror, and a diffraction grating to scatter the unabsorbed light onto a detection plate.

Vis: Short for wavelengths of visible light.

Cuvette: A container for use with the spectrophotometer, standardized to have a path distance of 1cm.

Transmittance: A perfectly clear object with nothing to absorb light—consider the clearest possible window—would have a transmittance of 100%. Transmittance and absorbance are inversely related.

Tare (or Blank): Using the liquid medium (typically lysogeny or LB broth) without inoculation as a baseline. By pressing abs0, the machine is told the absorbance of the liquid medium is considered to be zero, and all subsequent readings are relative to this. As a result, the absorbance of the liquid culture is assumed to be entirely due to bacterial growth.

Absorbance (A or Abs): The percent of the original light source not detected by the detection plate, presumed to have been absorbed by the molecules in the liquid sample in the cuvette. Absorbance of a material changes based on the wavelength of the light source.

Optical Density (OD): Considered a synonym for absorbance, commonly used in biology and biotechnology. The use of the word is being discouraged in favor of absorbance.

Wavelength: The distance between the crests of the light waves, in nanometers (nm). The wavelengths of visible light are 400 to 700nm. The wavelength used to measure bacterial growth is 600nm.

4. References

4.1. *2.1.5: Spectrophotometry*. (2013, October 2). Chemistry LibreTexts. https://chem.libretexts.org/Bookshelves/Physical_and_Theoretical_Chemistry_Textbook_Maps/Supplemental_Modules_(Physical_and_Theoretical_Chemistry)/Kinetics/02%3A_Reaction_Rates/2.01%3A_Experimental_Determination_of_Kinetics/2.1.05%3A_Spectrophotometry

4.2. *Diffraction Grating*. (n.d.). Retrieved February 14, 2022, from https://www.physics.smu.edu/~scalise/emmanual/diffraction/lab.html
4.3. *SODIUM HYPOCHLORITE | CAMEO Chemicals | NOAA*. (n.d.). Retrieved February 14, 2022, from https://cameochemicals.noaa.gov/chemical/4503

5. Materials, Reagents, and Equipment

5.1 Materials:

5.1.1 A plastic container for holding the 10% bleach solution.
5.1.2 A clean cuvette supplied
5.1.3 Roll of paper towels on hand
5.1.4 Micropipette p1000 plus tips
5.1.5 Box of KimWipes (delicate task wipes)
5.1.6 Sharpie marker

5.2 Reagents:

5.2.1 Sterile Lysogeny Broth (LB) for blank
5.2.2 Bacterial Culture to be measured

5.3 Equipment:

5.3.1 Vis Photospectrometer

6. Responsibilities of the Quality Control Technicion (QC Tech)

Lab personnel overseeing and performing the procedure are responsible for adhering to current lab safety procedures. Lab technicians must be trained to operate equipment and follow precautions listed below for handling potentially hazardous materials.

7. Hazards and Safety Precautions

7.1 Sodium Hypochlorite (bleach)

7.1.1 Warning: Causes serious eye irritation, potential skin irritant
7.1.2 Prevention: Wear Personal Protective Equipment (PPE), avoid contact, ingestion
7.1.3 Response: Flush eyes with water for up to 30 minutes after exposure, wash hands after exposure

8. Procedure

8.1 Startup and Calibration of Vis Spectrophotometer

Note: Refer to section 9 on page five for labeled images of the Vis Photospectrometer.

8.1.1 Turn on the Vis Spectrophotometer. It requires twenty minutes to warm up before use. The power button is on the back left-hand side of the machine.
8.1.2 Prepare 10% bleach solution as follows:

Mix, in a container that is about 500mL or larger, a 10% bleach solution. *Ex: If 10mL of bleach is used, add 90 mL of water*. Leave the container of 10% bleach solution in the sink.

8.1.3 Calibrate (tare) the spectrophotometer.

Note: Refer to section 9 on page five for labeled images of the Vis Photospectrometer.

8.1.3.1 Ensure the cuvette is clean. Wipe it free of smudges with a kimwipe.

8.1.3.2 After the spectrophotometer is warmed up, use the p1000 micropipette and tip to transfer **2 mL of lysogeny broth (LB)** into the cuvette. This cuvette is called the blank.

8.1.3.3 Open the lid of the spectrophotometer. The spectrophotometer can hold four cuvettes at a time, if necessary. The cuvettes can be moved into the optical path by manually withdrawing or pushing in the knob on the front right of the spectrophotometer. Only one cuvette is needed to calibrate the spectrophotometer.

8.1.3.4 Place the cuvette into the cuvette holder in the slot that is closest to the front of the machine. Make sure the knob is pushed all the way in – this should put the front-most cuvette spot in the optical path. Make sure the blank cuvette is in the optical path. **Close the lid.**

8.1.3.5 Select the wavelength for bacterial cultures. For OD600 readings, select a wavelength of 600nm. Use the dial on the lower left of the top side of the spectrophotometer, and the dial display.

8.1.3.6 Select transmittance (%T) mode using the Mode button on the display panel on the upper top left of the spectrophotometer. The transmittance mode will be indicated by a light next to the label.

8.1.3.7 Tare by pressing the 100%T/abs button . The result should be 100% Transmittance of light through the LB. Transmittance and Absorbance are inversely related. So, the blank cuvette, which contains LB with no bacteria, should now have an Absorbance reading of zero.

8.1.3.8 Select the absorbance (Abs) using the Mode button on the display panel on the upper top left of the spectrophotometer. When the absorbance mode is selected you will see a light go on next to the 'Abs' label.

8.1.3.9 The readout should say zero. All subsequent readings will be based on the difference in absorbance between the sterile media of the blank and the media containing the growing bacteria. This equates to measuring the amount of bacteria in a solution. **Please NOTE**: These spectrophotometers were not made in the USA. A reading of 0,000 is a reading of 0.000. The comma translates to a decimal point.

8.1.4 Remove the blank. Cap it with a small piece of plastic wrap or parafilm and set it aside in case reblanking is required. It is good practice to re-blank (other terms: recalibrate, tare) every fifteen to twenty minutes.

8.2 Collecting Absorbance Readings from Team Samples

8.2.1 Team QC Technicians will transfer 2 mL of the first sample to be recorded into a clean, empty cuvette.

8.2.2 Place the cuvette into the cuvette holder.

8.2.3 Repeat for up to four total samples. The cuvette holder can hold four samples at a time.

8.2.4 Move the cuvette of the first sample to be measured into the optical path. The cuvettes can be moved into the optical path by manually withdrawing or pushing in the knob on the front right of the spectrophotometer.

8.2.5 The spectrophotometer should already be set to absorbance. If not, press the mode button until the light next to *abs* is lit. Refer to

8.2.6 Record the reading.

8.2.7 Repeat as needed until all team cuvettes from each time point are measured.

8.2.8 Dispose of cuvette samples as follows:

8.2.8.1 Pour the contents of the cuvettes into the 10% bleach solution. The contents must sit for at least fifteen minutes from the time the last cuvette or culture is added to the bleach.

8.2.8.2 Reuse the cuvette for the next time point sample.

8.2.8.3 Soak the cuvettes in another container of 10% bleach solution for about a minute.

8.2.8.4 Drip dry for about a minute, then rinse with distilled water, wipe cuvette with Kimwipe, and set aside for reuse.

8.2.8.5 After fifteen minutes, the bleach solutions can be diluted with tap water until the container is full, then poured down the drain.

8.3 Clean Station and Cuvettes

8.3.1 Final Cuvette cleaning

8.3.1.1 Soak the cuvettes in another container of 10% bleach solution for 10 minutes.

8.3.1.2 Rinse and drip dry for about a minute.

8.3.1.3 Submerge cuvettes in a container of soapy wanter. Rinse once with tap water.

8.3.1.4 Rinse with distilled water twice.

8.3.1.5 Drip dry on paper towels, then put away. These cuvettes will be used again for measuring purified protein concentration.

8.3.2 Clean the lab bench by spraying with 70% ethanol (70% EtOH) solution, wiping evenly over all surfaces to be cleaned, then letting the solution dry on the surfaces. The time it takes the 70% EtOH to dry is approximately the time needed for the 70% EtOH to sanitize the surface. Do not spray the spectrophotometer.

8.3.3 Turn off the spectrophotometer.

9. Vis Photospectrometer



Figure 1: Vis Spectrophotometer

Major features starting from the upper left corner of machine:

(Upper Left) display + display indicators + control buttons;

(Lower Right) cavity with cuvette holder, knob for moving cuvettes in holder into the optical path;

(Lower Left) dial and readout for adjusting the wavelength.

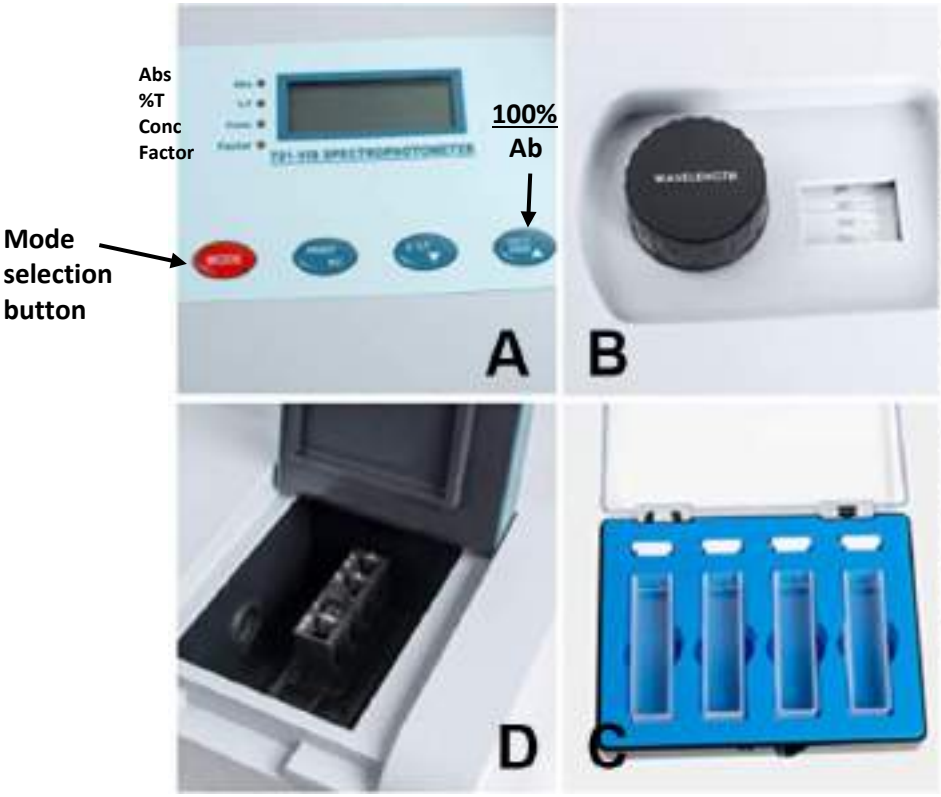
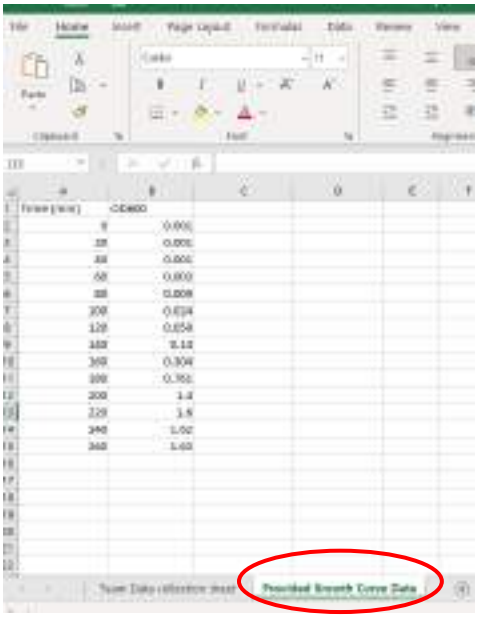


Figure 2: Features of Vis Spectrometer (clockwise from upper left): (A) 1. Digital display top middle. 2. Indicator lights, to left of display, showing which mode is currently selected. 3. Bottom from left to right: Mode, Print PC, 0% Transmittance (T), 100%T/Abs0/Blank. (B) Dial and indicator for adjusting wavelength. This is a polychromatic device. Remember OD or absorbance readings of bacteria cultures are taken at a wavelength of 600nm. (D) Cuvette holder capacity of (4) cuvettes. Round port visible in the left middle of the interior, and the counterpart–not visible in the image–is where the light at the selected wavelength is emitted and received. (C) Clean cuvettes are stored in this container. This may differ from how cuvettes are provided and stored in the lab.

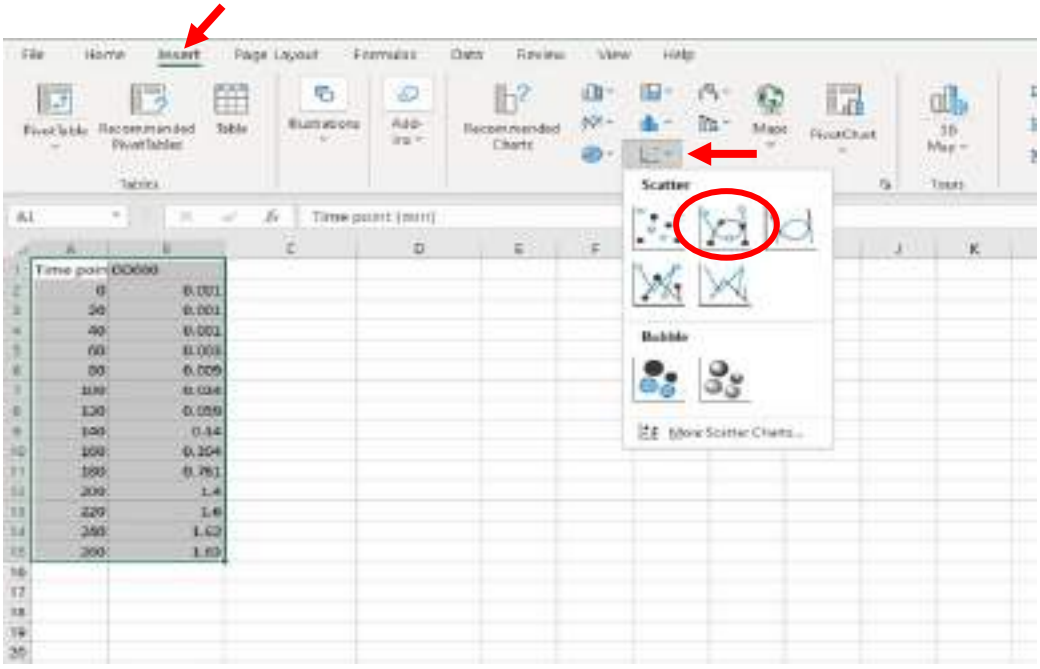
Graphing a Bacterial Growth Curve in Excel (PC)

You will be creating a bacterial growth curve using the Excel program, a commonly used spreadsheet program.

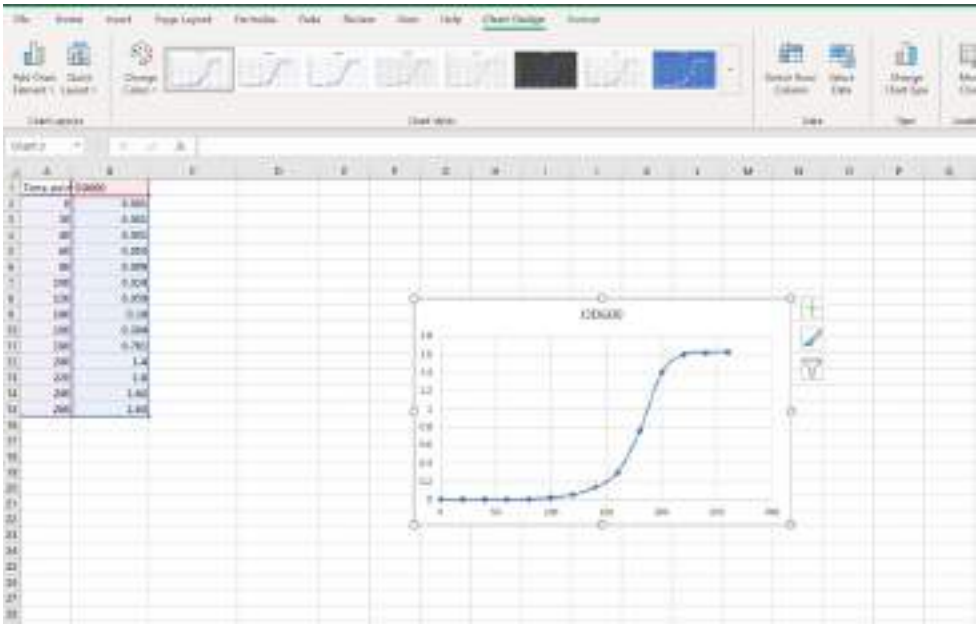
1. Open the 'Bacterial Growth Curve Spreadsheet'.
2. Click on the tab labeled 'Provided Growth Curve Data'. This will open a spreadsheet that already has bacterial growth data entered. You can see that column A contains time point data, in minutes. Column B contains OD600 readings taken at each time point.



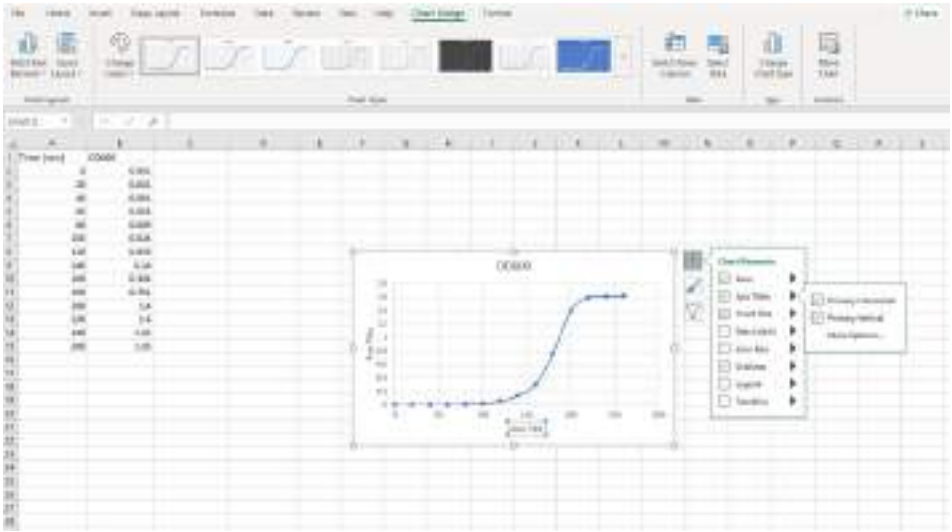
3. Select the entries in both column A and B. Select the 'Insert' tab at the top of the page (indicated by the slanted red arrow). Then select the line graph icon (indicated by the horizontal red arrow). When that window opens, select the type of graph shown in the figure below (red circle).



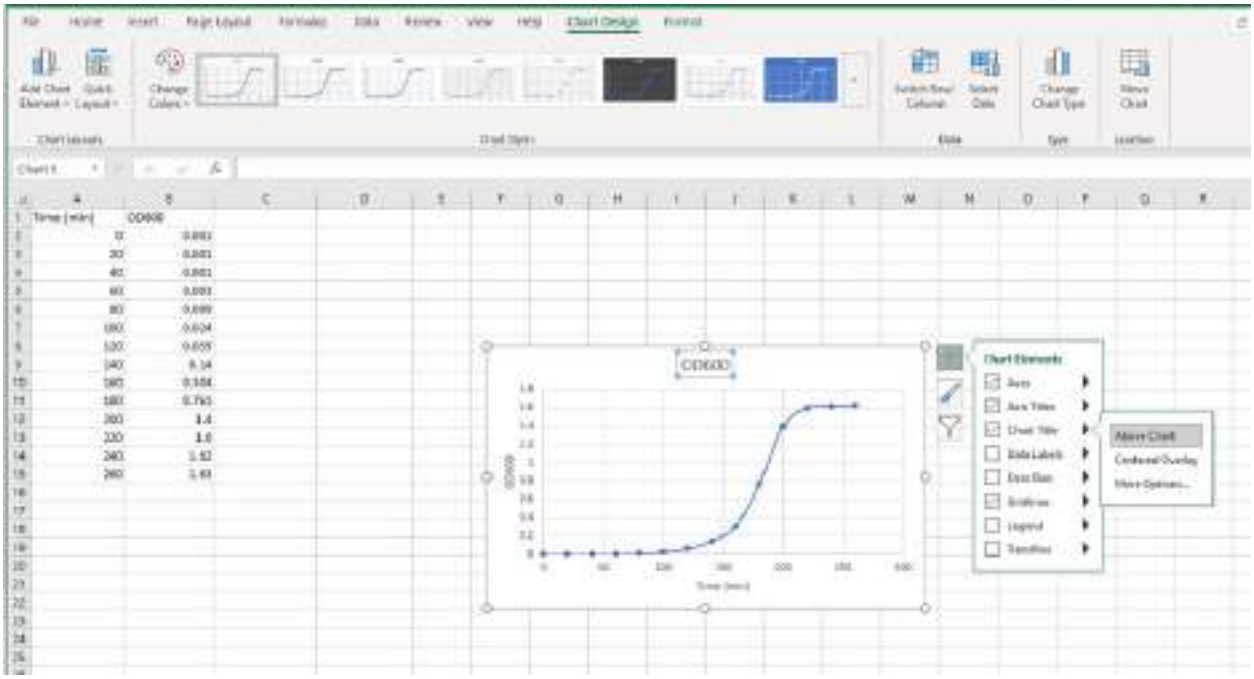
4. This will plot your data with the time points on the y-axis and the OD600 readings on the x-axis. The axes are not properly labeled yet and the graph title is incorrect.



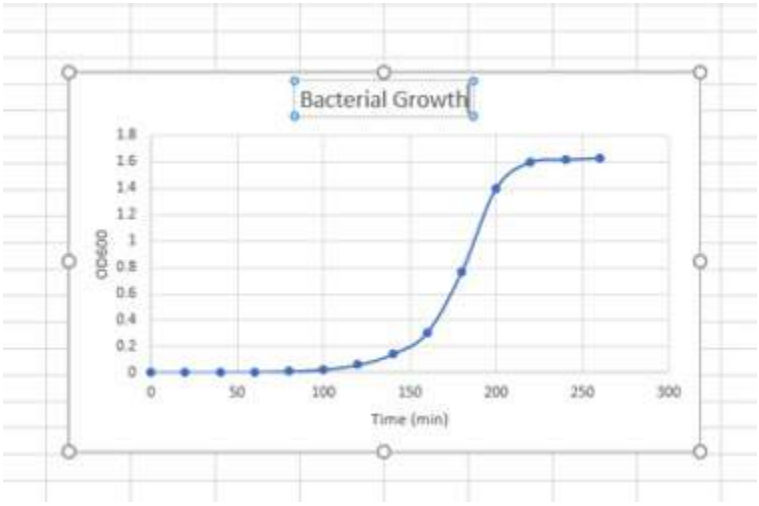
5. Click inside of the graph box to make the 3 icons appear to the right of the graph box. Click on the '+' icon. Make sure the 'Axis Titles' box is checked. Click on the arrow next to 'Axis Titles' and 'Primary Horizontal' and 'Primary Vertical' choices will appear. Make sure both of those boxes are checked. Now the axis labels will be activated and you can click on them and rename the axes.



6. Change the x-axis label to: Time (min). Change the y-axis label to: OD600. Now the graph should look like this. The chart title will still be incorrect. Click on the '+' icon again. Make sure the 'Chart Title' box is checked. Click on the arrow to the right of the 'Chart Title' box. This will open a window with choices for where the title appears. It also activates the chart title text. Click on the chart title text and change it to: Bacterial Growth.



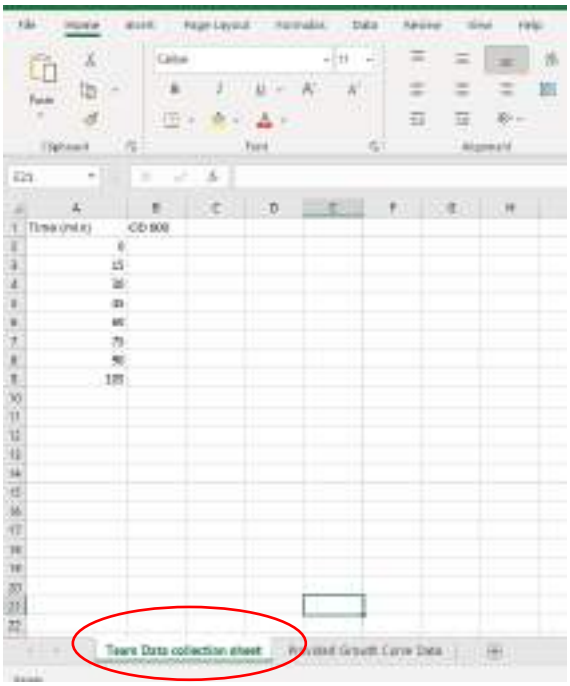
7. Now your graph should look like this.



What phases of the growth curve can you see?

_____

8. Next click on the tab labeled 'Team Data Collection Sheet' to open up a spreadsheet where you can input your own bacterial growth data. Change the time entries as needed. Input your OD600 readings. Create a graph using the protocol you have just used.



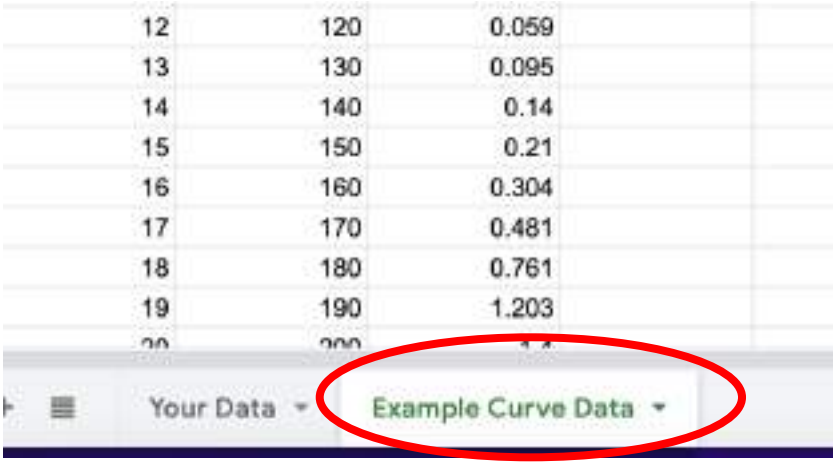
9. Which phases of the growth curve can you see in this graph?

Module 2, Lesson 3: Graphing a Bacterial Growth Curve

Graphing a Bacterial Growth Curve in Google Sheets

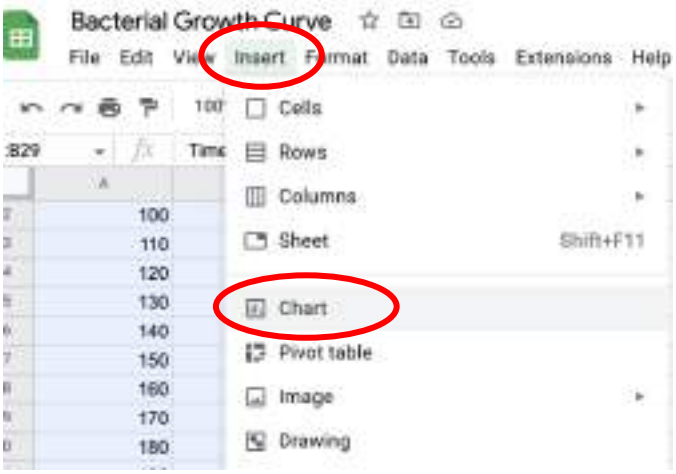
You will be creating a bacterial growth curve using google sheets, a commonly used spreadsheet program.

1. Open the 'Bacterial Growth Curve Spreadsheet' by clicking here.
2. Open the sheet at the bottom labeled example curve data.

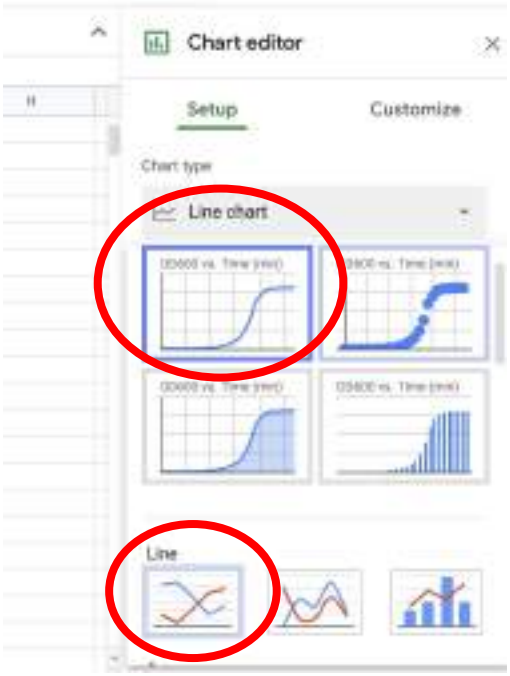


This will open a spreadsheet that already has bacterial growth data entered. You can see that column A contains time point data, in minutes. Column B contains OD600 readings taken at each point.

3. Select by clicking in the A1 box and holding and dragging until all of the boxes with data are highlighted.
4. Select insert at the top of the page. Once the dropdown opens select the chart.



5. This will auto generate a chart. Make sure that the chart that is selected is the line graph. →
6. Check to make sure the axes are labeled correctly.
    a. If not, click on each axis title and change it in the title text box.
7. Change the title of the graph by clicking on the title. Change the title to Bacterial Growth.

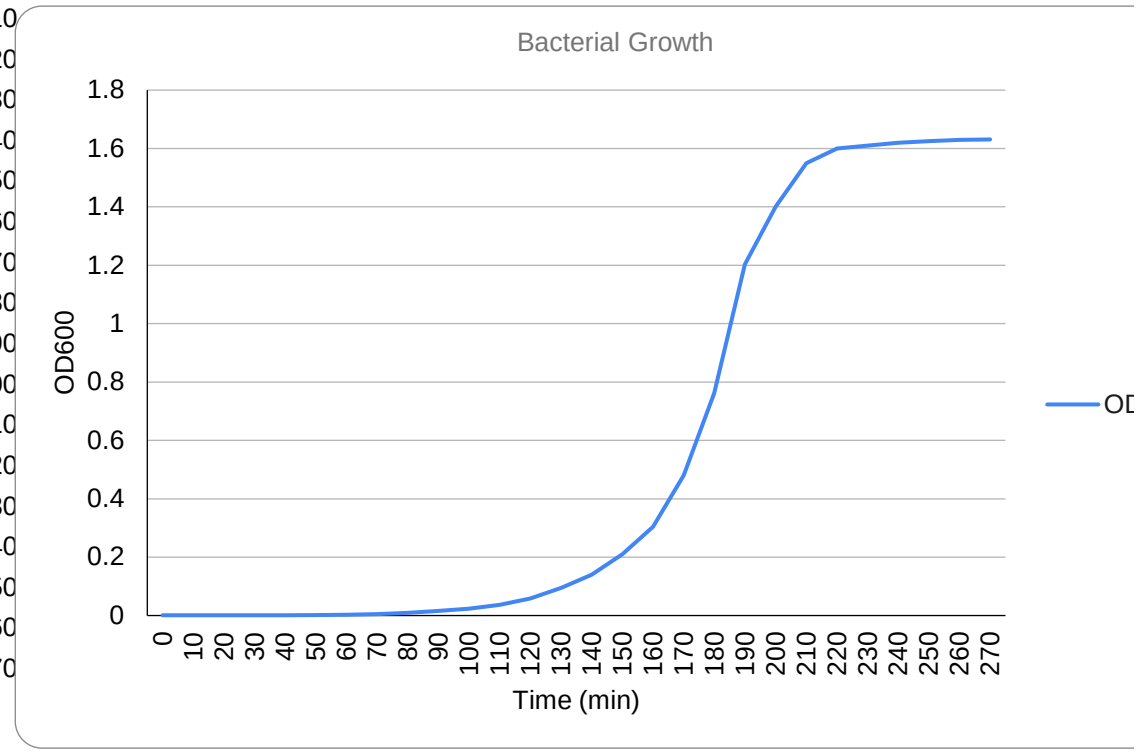


Time (min)	OD 600
0	
15	
30	
45	
60	
75	
90	
105	

Time (min)	OD600
0	0.001
20	0.001
40	0.001
60	0.003
80	0.009
100	0.024
120	0.059
140	0.14
160	0.304
180	0.761
200	1.4
220	1.6
240	1.62
260	1.63

Time (min)	OD600
0	
15	
30	
45	
60	
75	
90	
105	

Time (min)	OD600
0	0.001
10	0.001
20	0.001
30	0.001
40	0.001
50	0.002
60	0.003
70	0.005
80	0.009
90	0.016
100	0.024
110	
120	
130	
140	
150	
160	
170	
180	
190	
200	
210	
220	
230	
240	
250	
260	
270	



D600

Biomanufacturing Module 2

Lesson 4 – Inducing Production of the Recombinant Protein

Lesson objectives:

Students will understand:

- How protein production is induced.
- The benefits of inducing production of recombinant proteins.

Essential Question

- What are the benefits of inducing recombinant protein production at a specific time?

Materials:

- Induction of Recombinant Protein Production slide deck
- Upstream Process: Production of RFP+ or GFP+ Bacteria Protocol
- Scaled up liquid bacterial culture created in the last class period
- 50X arabinose stock solution
- Shaker/incubator
- Upstream Process Batch Record Document

What Students Will Do

- Students will listen to/watch the Induction of Recombinant Protein Production slide deck
- Teams will add arabinose to their liquid bacterial culture to induce production of RFP or GFP
- Teams place the flask containing their induced culture in the shaker/incubator
- Each team fills out the appropriate parts of their Upstream Process Batch Record

Teacher Preparation

- Prior to class, provide the team file folders containing:
    - Upstream Process: Production of RFP+ or GFP+ Bacteria Protocol (one per team)
    - Upstream Process Batch Record Document (one per team)
- Prior to class, turn on the bacterial shaker/incubator to a temperature of 37 degrees Celsius.
- Provide each team
    - Upstream Process: Production of RFP+ or GFP+ Bacteria Protocol
    - One p200 micropipette
    - One aliquot of 1mL 50X Arabinose stock solution
    - One box of p200 micropipette tips
    - Sharpie marker and tape for labeling flasks
    - Spray bottle of 70% ethanol
    - Paper towels
    - Upstream Process Batch Record
    - Team file folders

Organizer

Time	Activity	Materials
10 minutes	Watch/Listen to Induction of Recombinant Protein Production slide deck	Slide deck
5 minutes	Members of all teams put on PPE	Lab coats, gloves, safety goggles
5 minutes	Teams sanitize and prepare their bench space	70% ethanol, paper towels, sterile LB/Amp media, sterile 125mL baffled flask, micropipette, tips, small liquid bacterial culture started in previous class period
15 minutes	Each team takes their scaled up liquid bacterial culture out of the shaker/incubator and adds the appropriate amount of the arabinose stock to induce protein production	Upstream Process Protocol – Day 3, sterile 50X arabinose stock solution, micropipette, tips, scaled up bacterial culture started in previous class period
10 minutes	Teams fill out the appropriate portions of their Upstream Process Batch Record Document and file it	Upstream Process Batch Record Document, Team File Folder

Procedure

Induction of Recombinant Protein Production

1. Present the Induction of Recombinant Protein Production slide deck

Induce production of RFP or GFP in the bacterial culture

2. Members of each team put on PPE
3. Each team sanitizes and organizes their bench space
4. Each team retrieves its scaled up bacterial liquid culture from the incubator
5. Each team follows the Upstream Process Protocol – Day 3 to induce recombinant protein production in their culture.
6. Each team puts their flask containing the induced culture in the shaker/incubator to shake at 37 degrees C for 24 hours.
7. Each team fills out the appropriate sections of the Upstream Process Batch Record and files it in their team file.

Production of RFP+ or GFP+ Bacteria – Upstream Process Protocol Day 3 Induction of RFP or GFP Protein Production

Materials for Module 2, Lesson 4:

1. **Arabinose liquid stock (50X):** This will be added to the bacterial culture to induce bacterial production of GFP or RFP.
2. **125mL sterile glass baffled flask with lid that contains your bacterial culture.**
3. **Micropipettes and tips**: For measuring small volumes of reagents or bacterial cultures
4. **Microfuge tubes of various sizes:** For containing small volumes of reagents or bacterial cultures
5. **Microfuge tube rack**: To hold microfuge tubes.
6. **Sharpie markers**: For correct labeling of samples.
7. **Upstream Process Batch Record Form**: When properly filled out, this form is a record of your team's entire upstream process.

Equipment:

8. **Shaker/Incubator**: The equipment used to shake and warm bacterial cultures for optimal growth in liquid culture.

Protocol: Day 3 (Lesson 4) – Induction of RFP or GFP Production

This protocol is carried out by the Upstream Process Technician

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

Use aseptic technique at all times!!

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

1. 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.

2. 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 = 32mL 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)(32mL) = (50)(Y mL)
   32 = 50(YmL)
   YmL = 32/50 = 0.64mL or 640uL

3. Using a micropipette, add the appropriate amount of the 50X arabinose solution to your liquid culture.

4. Put your flask back in the shaker/incubator and let it shake at 250rpm, 37^{o}C until the next day. At this point the flask can be stored in the refrigerator until the next class period.

5. Properly dispose of all waste following the guidelines in the Aseptic Technique slidedeck.

6. Make sure you have filled out all necessary parts of the Upstream Process Batch Record.

Biomanufacturing Module 2

Lesson 5 – Bacterial Harvest and Determination of Bacterial Yield

Lesson objectives:

Students will understand:

- How to make serial dilutions
- How to use the spread plate technique to calculate bacterial yield

Essential Question

- How can we calculate the number of bacteria in a liquid culture?

Materials:

- Upstream Process Protocol – Days 4 & 5
- Serial Dilution Lab Protocol
- Colored water
- Microfuge tubes
- Induced liquid bacterial culture created in the last class period
- Sterile 50mL tube (1/team)
- Sterile LB/Amp media (1 bottle/team)
- LB/Amp plates (5/team)
- Sterile bacterial spreaders
- Bacterial plate incubator
- Upstream Process Batch Record Document

What Teams Will Do

- Remove their induced bacterial cultures from the shaker/incubator.
- Check the color of these cultures.
- Create 1:10 serial dilution set
- Plate their dilutions on LB/Amp agar
- Place their plates in the bacterial plate incubator
- Place the 50mL tube containing their harvested bacterial culture in the refrigerator.
- Each team fills out the appropriate parts of their Upstream Process Batch Record

Next Day

- Each team removes their plates from the incubator.
- Each team counts colonies and calculates bacterial yield using Colony Forming Unit (cfu) Calculation Record
- Each team fills out their Colony Forming Unit Calculation Record and files it

Teacher Preparation

- Prior to class make copies of
    - Colony Forming Unit (cfu) Calculation Record
- Provide each team
    - Production of RFP+ or GFP+ Bacteria Protocol
    - Serial Dilution Lab Protocol
    - One p200 micropipette

- One box of p200 micropipette tips
- Colored water
- 1.5mL microfuge tubes
- One bottle of LB/Amp media
- 5 LB/Amp agar plates
- Sharpie marker for counting colonies
- Sterile bacterial spreaders
- Spray bottle of 70% ethanol
- Paper towels
- Upstream Process Batch Record
- Colony Forming Unit (cfu) Calculation Record
- Team file folders

Organizer

Time	**Activity**	**Materials**
5 minutes	Members of all teams put on PPE	Lab coats, gloves, safety goggles
5 minutes	Teams sanitize and prepare their bench space	70% ethanol, paper towels, sterile LB/Amp media, sterile 125mL baffled flask, micropipette, tips, small liquid bacterial culture started in previous class period
5 minutes	Each team takes their induced liquid bacterial culture out of the shaker/incubator and decants it into a sterile 50mL tube.	Production of RFP+ or GFP+ Bacteria Protocol, sterile 50mL tube
20 minutes	Teams practice serial dilutions (if needed)	Serial Dilution Lab Protocol, 1.5mL microfuge tubes, colored water, plain water, p1000 micropipette and tips
30 minutes	Teams create serial dilutions from their bacterial harvest and plate them.	Upstream Process Protocol – Days 4 & 5, harvested bacterial culture, 1.5mL microfuge tubes, p200 micropipette and tips, LB/Amp plates, sterile spreaders
5minutes – NEXT CLASS PERIOD	Teams put on gloves and retrieve their plates from the incubator	
20 minutes	Teams count colonies on the most appropriate plate and calculate their bacterial yield	Colony Forming Unit (cfru) Calculation Record, pencils, scratch paper, sharpie markers

10 minutes	Teams fill out their Colony Forming Unit Calculation Record and file it	Colony Forming Unit Calculation Record Document
10 minutes	Teams fill out the appropriate portions of their Upstream Process Batch Record Document and file it	Upstream Process Batch Record Document, Team File Folder

Procedure

Bacterial Culture Harvest

1. Members of each team put on PPE
2. Each team sanitizes and organizes their bench space
3. Each team retrieves its induced bacterial liquid culture from the incubator
4. Each team examines the color of their induced bacterial culture.
5. Each team moves the bacterial culture to a 50mL tube.

Calculation of Bacterial Yield – Serial Dilution and Plating

6. Each team follows the appropriate section of the Upstream Process Protocol – Day 4 to make a serial dilution of their bacterial culture and plate each dilution on a separate LB/Amp plate.
7. Each team puts their plates in the bacterial plate incubator to incubate at 37 degrees C for 24 hours.

Calculation of Bacterial Yield – Serial Dilution and Plating

8. Each team retrieves their plates from the incubator.
9. Each team uses the instructions in the Colony Forming Unit Calculation Record document to calculate their bacterial yield.
10. Each team fills out their Colony Forming Unit Calculation Record and files it in their team file.
11. Each team fills out the appropriate sections of the Upstream Process Batch Record and files it in their team file.

Serial Dilution Lab

A serial dilution is the stepwise dilution of a substance in solution. Usually the dilution factor at each step is constant, resulting in a geometric progression of the concentration in a logarithmic fashion.

This lab is designed to introduce you to the process of 'serial dilution'. The word serial comes from the same root as the word 'series'. In this lab, you will be creating a series of solutions that are diluted 2-fold. Each solution will be 2-fold less concentrated than the solution it was diluted from. Or, to say it another way, each dilution will be half as concentrated as the one used to create it. The process of creating serial dilutions is used routinely in scientific assays in order to test reagents over a range of concentrations, to create a standard curve etc.

Procedure

1. Line up nine 1.5mL microfuge tubes in a tube rack.

2. Label the tubes from left to right: 1, 1/2 , 1/4 , 1/8 , 1/16 , 1/32 , 1/64 , 1/128 , 1/256.

3. Using a p1000 micropipette, place 1mL of a very dark green solution of food coloring and water (provided by the instructor) into tube #1.

4. Using a p1000 micropipette, place 0.5mL of water in each of the other tubes (See Fig 1.).

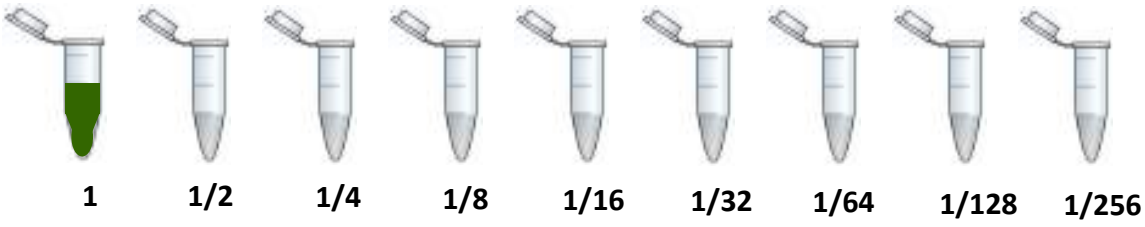


Fig. 1

5. To make your first serial dilution, use a p1000 micropipette to remove 0.5mL of the dark green solution in tube #1. Add the 0.5mL you have removed from tube #1 and mix it with the 0.5mL of water in the tube marked 1/2 . You now have 1mL in tube #1/2 and this solution is a slightly lighter green than the solution in tube #1 (See Fig. 2).

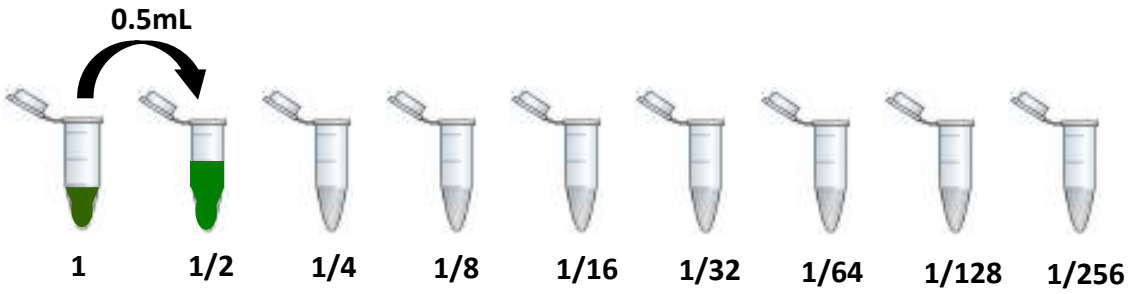


Fig. 2

6. To make your second serial dilution, use a p1000 micropipette to remove 0.5mL of the green solution in tube #1/2. Add the 0.5mL you have removed from tube #1/2 and mix it with the 0.5mL of water in the tube marked 1/4 . You now have 1mL in tube #1/4 and this solution is a slightly lighter green than the solution in tube #1/2 (See Fig. 3).

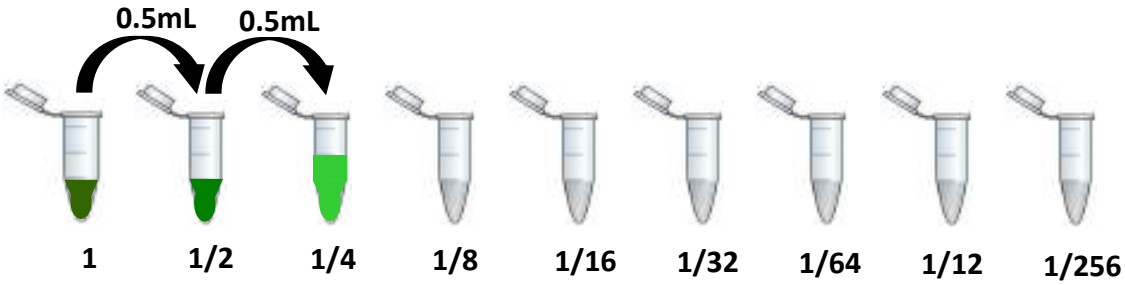


Fig. 3

7. Continue this procedure to create the rest of your serial dilutions.

8. Your final set of dilutions should look something like this in color (See Fig. 4).



Fig. 4

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 cm^2 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 Incubato**r: 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.

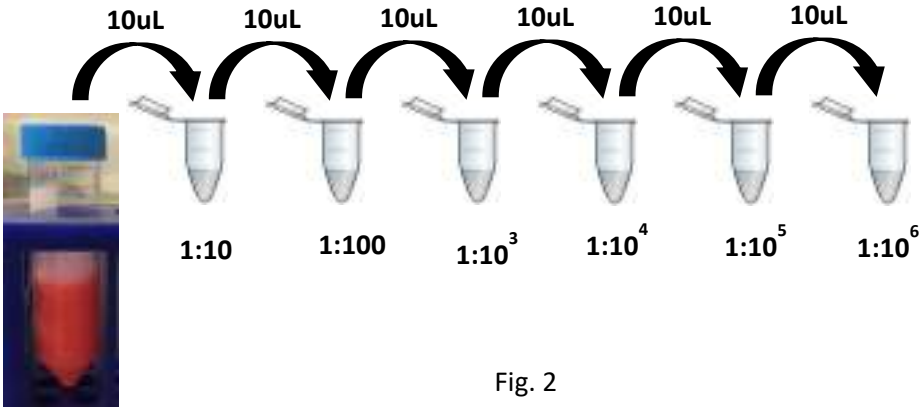


Fig. 2

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 ^{o}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 Unity 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

Team
Logo

Aseptic Technique Check Record

Team Name: _____

# colonies/plate	Name	Initials	Date Month Day Year Ex: 03242022

	Name	Initials	Date Month Day Year Ex: 03242022
QC Technician			
QA Technician			

Team Logo

Team Name:_____

Upstream Process Batch Record

1. Inoculate RFP+ or GFP+ bacterial into a small liquid culture of LB/Amp:

Name	Initials	Date Month Day Year Ex: 03242022

2. Culture scale up to 50 mL LB/Amp:

Time (minutes)	OD600 Readings
0	
15	
30	
45	
60	
75	
90	

Name	Initials	Date Month Day Year Ex: 03242022

Team Name:_____

3. Induce RFP/GFP expression by adding arabinose to a final concentration of 1X:

Name	Initials	Date Month Day Year Ex: 03242022

4. Determine bacterial concentration per mL via serial dilution /plating:

Number of bacteria (cfu/mL):

# of bacteria	Name	Initials	Date Month Day Year Ex: 03242022

Final Approval

	Name	Initials	Date Month Day Year Ex: 03242022
Upstream Technician:			
QA Technician:			

Team

Logo

Team Name:_____

Colony Forming Unit (Cfu) Calculation Record

1. Choose a plate that has separated, individual colonies.
    a. How many colonies are on this plate? _____ cfu
    b. What is the dilution for this plate? *(Example: 1:10, 1:100…)* _____
    c. What is the dilution factor for this plate? *(If plated 1:100, dilution factor is 100):* _____
    d. How many microliters were used to plate this specific plate? _____ ul
2. Calculate how many cfu/mL is in the dilution for this plate:
    a. General Equation: $\frac{\#\ cfu\ on\ dilution\ plate}{\#\ ul\ used\ to\ spread\ dilution\ plate}\ x\ \frac{1{,}000\ ul}{1\ mL}$ = cfu/mL of dilution plate
    b. Show your work using the equation in 2a.

3. Using the value of cfu/mL of the dilution plate (Result 2b), calculate the cfu/mL in the original culture (neat concentration)
    a. General Equation: (cfu/mL of dilution plate) x (dilution factor) = cfu/mL in original culture
    b. Show your work using the equation in 3a.

Position	Name	Initials	Date Month Day Year Ex: 03242022
Calculations: Upstream Process Technician			
Check: QC Technician			
Final Sign Off: QA Technician			

Extension Activities for Module 2

Build a Bioreactor

Using the supplies found in your 'Build a Bioreactor' kit, you will build a model bioreactor.

The plastic bottle contained in your kit will serve as the bioreactor itself. The lid is removable and will allow you to put components into the bioreactor.

The kit contains paper straws, colored card stock, fabric. You may use any supplies from your home as well. You may refer to the diagram at the end of these instructions to refresh your memory regarding the parts of a bioreactor.

Refer to the figure at the end of this document for reference.

Your model should have the following parts:

1. An **agitator** – comprised of an external power drive (motor) and at least one impeller. The agitator must enter the bioreactor, so that the impeller is inside of the bioreactor. You should be able to spin the impeller by spinning the external power drive (with your fingers).

2. A **sensor port** - for measuring pH, temperature and dissolved O_2. For the purposes of this model, this port does not need to actually enter the bioreactor. You need to represent the port on the outside of the bioreactor.

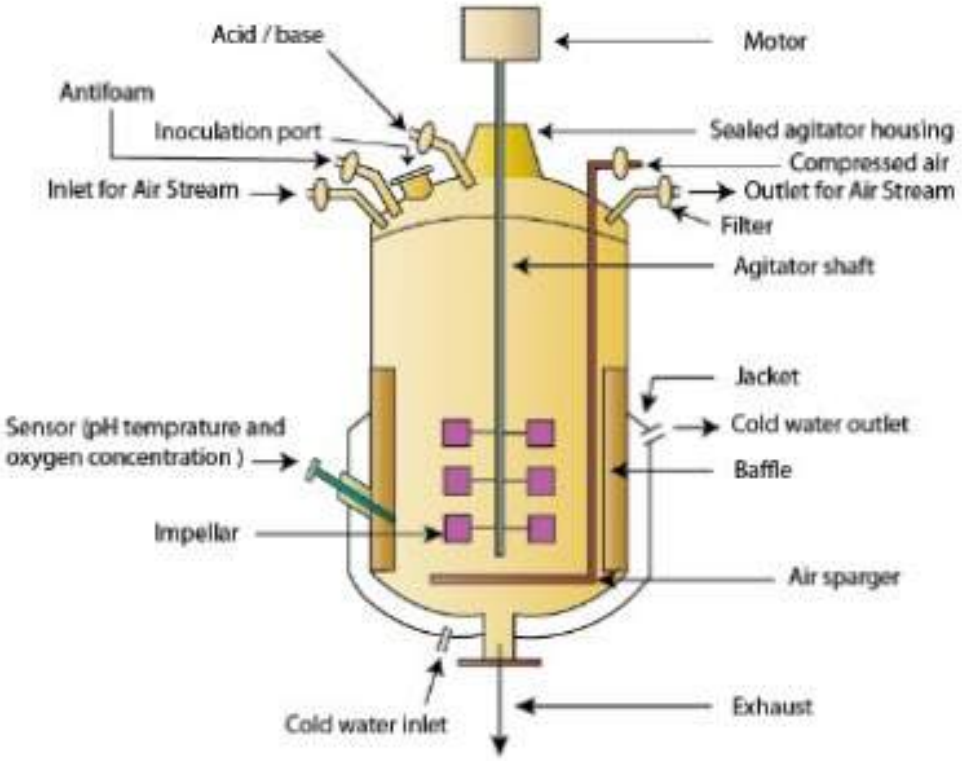
3. A **thermal jacket** – to keep the bioreactor at a constant temperature. The jacket should be removable.

4. An **air delivery system** – comprised of a compressed air inlet that connects to a sparger at the bottom of the bioreactor. The air delivery system should enter the bioreactor and the sparger should sit at the bottom of the bioreactor.

5. An **acid/base port** - for adjusting pH. For the purposes of this model, this port does not need to actually enter the bioreactor. You need to represent the port on the outside of the bioreactor.

6. An **anti-foam port** - for the addition of anti-foaming reagents. For the purposes of this model, this port does not need to actually enter the bioreactor. You need to represent the port on the outside of the bioreactor.

7. Take a photo of your bioreactor model. Drop the photo into ppt or some other type of file and label the required parts. Take multiple photos if needed to show all required parts of the model. In addition, take one photo with the thermal jacket on and one with it off.



Build a Bioreactor Kit

Contents:

1. 1 plastic flask with lid
2. 2 Paper straws
3. 4 Colored index cards
4. 3 Chenille stems
5. 1 piece of Felt (6” x 9”)

There is nothing in this kit that you need to return.

You provide:

1. Glue
2. Tape
3. Scissors

Build a Bioreactor Kit

Contents:

1. 1 plastic flask with lid
2. 2 Paper straws
3. 4 Colored index cards
4. 3 Chenille stems
5. 1 piece of Felt (6” x 9”)

There is nothing in this kit that you need to return.

You provide:

1. Glue
2. Tape
3. Scissors

Build a Bioreactor Kit

Contents:

1. 1 plastic flask with lid
2. 2 Paper straws
3. 4 Colored index cards
4. 3 Chenille stems
5. 1 piece of Felt (6” x 9”)

There is nothing in this kit that you need to return.

You provide:

1. Glue
2. Tape
3. Scissors

Module 2 Extension – Build a Bioreactor Kit

Calculation of Bacterial Growth

Calculate the Generation time G using the following equation: G = t/n

't' = time

'n' = the number of generations.

Please express your answer in minutes. Please show your work.

1. A culture of bacteria divides 10 times in 5 hours.

2. A culture of bacteria divides 100 times in 4 hours.

3. A culture of bacteria divides 4 times in 40 minutes.

4. A culture of bacteria divides 20 times in 2800 minutes.

Calculate the answers to the following questions using the following equation:

$$N(t) = N_0 \times 2^n$$

N = Number of bacteria

N(t) = Number of bacteria at time t

N_0 = Number of bacteria at time 0 – ie at the start

2 – refers to the doubling rate

n = the number of generations = Time period/generation time

Please show your work. Please put numbers of 4 digits or more into scientific notation. (Example: 1,560 = 1.56×10^3) Include only 2 digits to the right of the decimal place.

1. If you start with a population of 10 bacteria, and the Generation time is 30 min, how many bacteria will you have after 4 hours?

2. If you start with a population of 1 bacterium, how many generations does it take to get 120 bacteria?

3. If you start with a population of 1 bacterium, and the Generation time is 10 min, how many bacteria will you have after 10 hours?

Calculation of Bacterial Growth - KEY

Calculate the Generation time G using the following equation: G = t/n

't' = time

'n' = the number of generations.

Please express your answer in minutes. Please show your work.

1. A culture of bacteria divides 10 times in 5 hours.

   t = 5 hours*(60 min/1 hour) = 300 min.
   G = 300 min/10 = **30 min.**

2. A culture of bacteria divides 100 times in 4 hours.

   t = 4 hours*(60 min/1 hour) = 240 min.
   G = 240 min/100 = **2.40 min.**

3. A culture of bacteria divides 4 times in 40 minutes.

   t = 40 min.
   G = 40 min/4 = **10 min.**

4. A culture of bacteria divides 20 times in 2800 minutes.

   t = 2800 min.
   G = 2800 min/20 = **140 min.**

Calculate the answers to the following questions using the following equation:

$$N(t) = N_0 \times 2^n$$

N = Number of bacteria

N(t) = Number of bacteria at time t

N_0 = Number of bacteria at time 0 – ie at the start

2 – refers to the doubling rate

n = the number of generations = Time period/generation time

Please show your work.

Please put numbers of 4 digits or more into scientific notation. (Example: 1,560 = 1.56×10^3) Include only 2 digits to the right of the decimal place.

1. If you start with a population of 10 bacteria, and the Generation time is 30 min, how many bacteria will you have after 4 hours?

   N_o = 10 bacteria, G = 30 min., G = t/n n = t/G where t= 4 hr.*(60 min/1 hour) = 240 min.

   n = 240 min/30 min

   n = 8 generations or doublings

   $N_{(t)} = N_o \times 2^n$

   $N_{(t)} = 10 \times 2^8$

   $= 10 \times 256$

   $N_{(t)} = 2.56 \times 10^3$ bacteria

2. If you start with a population of 1 bacterium, how many generations does it take to get 120 bacteria?

   N_o = 1 bacterium, G = ?, $N_{(t)}$ = 120 bacteria

   $N_{(t)} = N_o \times 2^n$

   $120 = 1 \times 2^n$

$\ln 120 = \ln 2^n$

$\ln 120 = n * \ln 2$

$\ln 120 / \ln 2 = n$ **n = 6.91 or approximately 7 generations or doublings.**

3. If you start with a population of 1 bacterium, and the Generation time is 10 min, how many bacteria will you have after 10 hours?

N_o = 1 bacterium, G = 10 min., G = t/n n = t/G where t= 10 hr.*(60 min/1 hour) = 600 min.

n = 600 min/10 min

n = 60 generations or doublings

$N_{(t)} = N_o \times 2^n$

$N_{(t)} = 1 \times 2^{60}$

$= 1 \times (1.15 \times 10^{18})$

$N_{(t)} = 1.15 \times 10^{18}$ bacteria

Optimizing Cell Culture in Bioreactors – Cell Mixing

The purpose of this assignment is to increase your understanding of the purposes of the various parts of a bioreactor.

Watch the short video about the importance of cell mixing. Remember that it is the impeller which causes cell mixing in a bioreactor.

https://www.youtube.com/watch?v=mS_5x7cju7E

After watching the video, answer the following questions. Feel free to watch the video multiple times and/or pause it in specific places.

1. Why is it important to have dissolved oxygen in the cell culture medium?

2. Shear forces cause bubble break up. Why is bubble break up important? What does it do that is helpful for cell growth?

3. What can happen if shear forces are too strong in the medium?

4. Sometimes things need to be added to cell culture media - nutrients, a basic solution (to increase the pH). Why do you think it is important to achieve rapid homogeneity in the cell culture medium when things are added to the cell culture medium?

Optimizing Cell Culture in Bioreactors – Cell mixing - KEY

The purpose of this assignment is to increase your understanding of the purposes of the various parts of a bioreactor.

Watch the short video about the importance of cell mixing. Remember that it is the impeller which causes cell mixing in a bioreactor.

https://www.youtube.com/watch?v=mS_5x7cju7E (Links to an external site.)

After watching the video, answer the following questions. Feel free to watch the video multiple times and/or pause it in specific places.

1. Why is it important to have dissolved oxygen in the cell culture medium?

   Cells need oxygen to live. They need it to respire and metabolize.

2. Shear forces cause bubble break up. Why is bubble break up important? What does it do that is helpful for cell growth?

Bubble break up allows for the transfer of oxygen from the bubbles into the cell culture medium where it is accessible to cells.

3. What can happen if shear forces are too strong in the medium?

Strong shear forces are detrimental to cell health. Strong shear forces are physically detrimental to cell membranes etc.

4. Sometimes things need to be added to cell culture media - nutrients, a basic solution (to increase the pH). Why do you think it is important to achieve rapid homogeneity in the cell culture medium when things are added to the cell culture medium?

You don't want to have regions in the media that have too high or low concentration of nutrients or have too high or low pH levels – it's unhealthy for cells.

Optimizing Cell Culture in Bioreactors – pH

The purpose of this assignment is to increase your understanding of the purposes of the various parts of a bioreactor.

Watch the short video about the importance of pH. Remember that pH is measured on a scale of 1-14. Neutral pH is pH 7. As pH levels get lower, they are becoming more acidic. As pH levels get higher, they are becoming more basic.

https://www.youtube.com/watch?v=4gRK188sCKo (Links to an external site.)

After watching the video, answer the following questions. Feel free to watch the video multiple times and/or pause it in specific places.

1. Which factors impact the pH of cell culture medium?

2. What two substances do cells continually produce as they grow? How does an increasing level of each of those two substances affect the pH of the cell culture media?

3. What is the ideal pH range for most mammalian cells?

4. Name the various methods mentioned for stabilizing pH in cell culture.

Optimizing Cell Culture in Bioreactors – pH - KEY

The purpose of this assignment is to increase your understanding of the purposes of the various parts of a bioreactor.

Watch the short video about the importance of pH. Remember that pH is measured on a scale of 1-14. Neutral pH is pH 7. As pH levels get lower, they are becoming more acidic. As pH levels get higher, they are becoming more basic.

https://www.youtube.com/watch?v=4gRK188sCKo (Links to an external site.)

After watching the video, answer the following questions. Feel free to watch the video multiple times and/or pause it in specific places.

1. Which factors impact the pH of cell culture medium?

- Temperature
- Cell Growth
- Lactic Acid
- CO_2

2. What two substances do cells continually produce as they grow? How does an increasing level of each of those two substances affect the pH of the cell culture media?

Cells continually produce lactic acid (lactate) and CO_2. Production of both of these substances causes pH to drop, which has an adverse effect on cell growth.

3. What is the ideal pH range for most mammalian cells? 7.0 +/- 1.0

4. Name the various methods mentioned for stabilizing pH in cell culture.

    - Buffering components in the media
    - Addition of base solution
    - Sparging of nitrogen to strip CO_2 from the media

Optimizing Cell Culture in Bioreactors – Sparging

The purpose of this assignment is to increase your understanding of the purposes of the various parts of a bioreactor.

Watch the short video about the importance of sparging, the process of introducing air, usually oxygen (O_2), but sometimes also nitrogen (N_2), into cell culture media.

https://www.youtube.com/watch?v=7Pnrnjt6pyo (Links to an external site.)

After watching the video, answer the following questions. Feel free to watch the video multiple times and/or pause it in specific places.

1. What is more soluble in cell culture media, oxygen or glucose?

2. What two factors limit the amount of dissolved oxygen (dO_2) in cell culture media?

3. Is it better to have smaller bubbles in cell culture media or larger bubbles? Why?

4. Which size of bubbles rise most slowly in the media, small or large? How does a slower rise in the media help increase levels of dO_2 in the media?

Optimizing Cell Culture in Bioreactors – Sparging

The purpose of this assignment is to increase your understanding of the purposes of the various parts of a bioreactor.

Watch the short video about the importance of sparging, the process of introducing air, usually oxygen (O_2), but sometimes also nitrogen (N_2), into cell culture media.

https://www.youtube.com/watch?v=7Pnrnjt6pyo (Links to an external site.)

After watching the video, answer the following questions. Feel free to watch the video multiple times and/or pause it in specific places.

1. What is more soluble in cell culture media, oxygen or glucose?

Glucose is more soluble in cell culture media than oxygen.

2. What two factors limit the amount of dissolved oxygen (dO_2) in cell culture media?

I'm going for interfacial area and residence time. The video does mention: low oxygen solubility and rapid uptake of oxygen by cells, so some students gave those two answers which I accepted.

3. Is it better to have smaller bubbles in cell culture media or larger bubbles? Why?

It is better to have smaller bubbles in the cell culture media. Smaller bubbles have a larger surface area to volume ratio (interfacial area). A larger interfacial area allows oxygen to dissolve more easily and more quickly into the culture media.

4. Which size of bubbles rise most slowly in the media, small or large? How does a slower rise in the media help increase levels of dO_2 in the media?

Small bubbles rise more slowly. A slower rise translates into longer residency times for the bubbles. A longer residency time gives more time for oxygen to move from the bubbles into the cell culture media.

Optimizing Cell Culture in Bioreactors – Dissolved O_2 and CO_2

The purpose of this assignment is to increase your understanding of the purposes of the various parts of a bioreactor.

Watch the short video about the importance of dissolved oxygen (dO_2) and dissolved CO_2 (dCO_2).

https://www.youtube.com/watch?v=j-Lz35T_CJM (Links to an external site.)

After watching the video, answer the following questions. Feel free to watch the video multiple times and/or pause it a specific place.

1. How can reactive oxygen be harmful in biomanufacturing?

2. Dissolved CO_2 levels above 20% are detrimental to cell growth. Explain why this is true.

3. What methods can be used to measure dO_2 in a bioreactor?

4. What methods can be used to measure dCO_2 in a bioreactor?

Optimizing Cell Culture in Bioreactors – dO_2 and dCO_2 - KEY

The purpose of this assignment is to increase your understanding of the purposes of the various parts of a bioreactor.

Watch the short video about the importance of dissolved oxygen (dO_2) and dissolved CO_2 (dCO_2).

https://www.youtube.com/watch?v=j-Lz35T_CJM (Links to an external site.)

After watching the video, answer the following questions. Feel free to watch the video multiple times and/or pause it a specific places.

1. How can reactive oxygen be harmful in biomanufacturing?

Reactive oxygen species can chemically degrade the protein of interest.

2. Dissolved CO_2 levels above 20% are detrimental to cell growth. Explain why this is true.

Increases in CO_2 cause a decrease in pH. More acidic pH is not healthy for cells.

3. What methods can be used to measure dO_2 in a bioreactor?

dO_2 monitoring probe

optical sensors

4. What methods can be used to measure dCO_2 in a bioreactor?

dCO_2 monitoring probe

off gas analysis