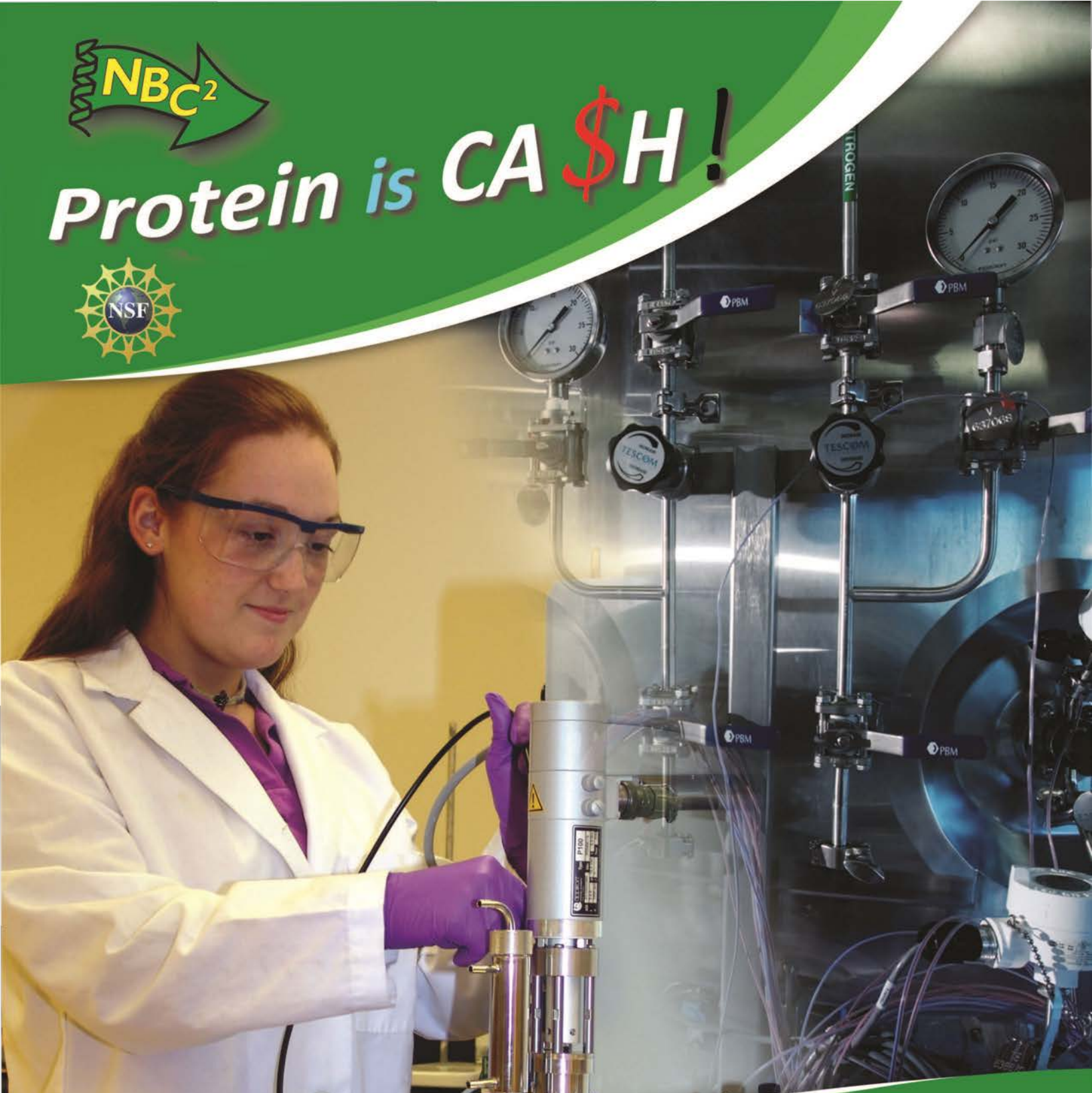
NBC²

Protein is CA $H!

NSF



Global Biomanufacturing Curriculum

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Protein is CA$H!

Revised August 2016

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Global Biomanufacturing Curriculum

DAY ONE

Metrology

Protein is CA$H!

Title: Gilson Pipetman® P-20 to P-1000 Operation and Maintenance

Approvals:
Preparer: Bob O'Brien Date: 02Jun08
Reviewer: Deb Audino Date: 02Jun08

1. **Purpose:**
   Operation of Gilson Pipetman® P-2 to P-1000

2. **Scope:**
   Applies to the operation, cleaning, and troubleshooting of the Gilson Pipetman®, designed to dispense precise volumes of liquid safely.

3. **Responsibilities:**
   3.1. It is the responsibility of the course instructor/lab assistant to ensure that this SOP is performed as directed and to update the procedure when necessary.
   3.2. It is the responsibility of the students/technicians to follow the SOP as described and to inform the instructor about any deviations or problems that may occur while performing the procedure.

4. **References:**
   4.1. Gilson Pipetman® P users guide
   4.2. Autoclave SOP

5. **Definitions:** N/A
6. **Precautions:**
   6.1. Volatile solutions: you should saturate the air-cushion of your pipette by aspirating and dispensing the solvent repeatedly before aspirating the sample.
   6.2. Acids or other corrosive liquids that emit vapors can damage pipettes. To avoid this, remove the tip holder and rinse the piston and O-ring and seal with distilled water.
   6.3. Temperature extremes can damage the pipetman. Do not pipette liquids having temperatures of above 70°C or below 4°C.

7. **Materials:**
   7.1. Pipetman®
   7.2. pipette tips
   7.3. beaker
   7.4. weigh boats
   7.5. distilled water
   7.6. deionized water (DI water)
   7.7. lab towels
   7.8. 70% isopropyl alcohol (IPA)
   7.9. autoclave

Title: Gilson Pipetman® P-20 to P-1000 Operation and Maintenance

8. Procedure:

8.1. Operation

8.1.1. The volume of liquid to be aspirated is set using the volume meter which is read top (most significant digit) to bottom (least significant digit).
8.1.2. Adjust the thumbwheel or push button to the desired volume. To obtain the maximum accuracy when setting the volume, set the volume 1/3 of a turn above the desired volume and then turn down to the desired volume
8.1.3. Double check that the set volume is correct while holding the volume meter at eye level.
8.1.4. Fit a tip into the tip holder, by using a slight twisting motion when pressing the Pipetman® tip holder into a pipette tip to ensure a firm and airtight seal.
8.1.5. Follow direction below to pre-rinse the tip by aspirating the first volume of liquid and then dispensing it back into the sample container or a waste container.
8.1.6. Aspirate by pressing the push button to the first stop (Figure 1). Make sure that you operate the pushbutton slowly and smoothly.
8.1.7. Hold the pipette vertically and immerse the tip into the liquid hold at a constant depth just below the surface of the liquid (Table 1).
8.1.8. Dispense by placing the tip against the inside wall of the recipient vessel at an angle of 10° to 40° and then pressing the pushbutton slowly and smoothly to the first stop. Wait for at least one second, then press the pushbutton slowly and smoothly to second stop to expel any residual liquid from the tip.
8.1.9. Keep pushbutton completely depressed while removing the pipette tip from the vessel. Draw up the tip along inside surface of the vessel.
8.1.10. Release the pushbutton slowly and smoothly.

8.2. Tip removal

8.2.1. Tip may now be ejected by pressing firmly on the tip ejector button into a waste container.
8.2.2. Tip changes are required only if aspirating a different liquid, sample or reagent or volume. Tips should also be changed if aseptic technique is compromised (e.g. if the tip touches the outside of a container).
8.2.3. When you are finished pipetting, re-set the volume of the Pipetman® to the maximum volume for proper storage.

Title: Gilson Pipetman® P-20 to P-1000 Operation and Maintenance

8.3. **Leak testing P20 – P200**

8.3.1. Fit a tip onto the Pipetman®.
8.3.2. Set the Pipetman® to the maximum volume given in the specification.
8.3.3. Using deionized water, pre-rinse the tip and then aspirate the set volume.
8.3.4. Hold the Pipetman® in a vertical position for approximately twenty seconds, while observing the end of the Pipetman® tip.
8.3.5. The water in pipette tip should remain constant.
8.3.6. If a droplet appears at the end of the tip there is a leak.
8.3.7. Verify that the tip is on tightly, and repeat the test.
8.3.8. If a droplet appears at the end of the tip there is a leak and the Pipetman® needs repair.

8.4. **Cleaning externally**

8.4.1. The Pipetman® is designed so that the parts that normally come in contact with liquid contaminants can easily be cleaned and decontaminated.
8.4.2. Wipe the outside of the entire Pipetman® with a lab towel dampened with a mild detergent solution.
8.4.3. Wipe the entire Pipetman® with a lab towel dampened with distilled water.
8.4.4. Remove the tip ejector.
8.4.5. Wipe the tip ejector with a lab towel dampened with a mild soap solution.
8.4.6. Wipe the tip ejector with a lab towel dampened with distilled water.
8.4.7. Refit the tip ejector and allow the pipette to dry.
8.4.8. Dispose all used lab towels in a biohazard waste container.

8.5. **Immersion decontamination**

8.5.1. The following components: tip ejector, tip holder, connecting nut and the metal and plastic components of the piston assembly can be removed and immersed in IPA for complete decontamination. See Figure 2 and disassemble instructions below.
8.5.2. Allow the components to dry prior to reassembly. See directions below for reassembly.

8.6. **Chemical decontamination**

8.6.1. Spray a lab towel with 70% IPA to dampen.
8.6.2. Wipe upper part of body with dampened lab towel.
8.6.3. Wipe tip holder and tip ejector with dampened lab towel.
8.6.4. Wipe entire Pipetman® with a lab towel dampened with DI water.
8.6.5. Leave Pipetman® to dry or wipe dry with lab towel.
8.6.6. Dispose all used lab towels in a biohazard waste container.

Title: Gilson Pipetman® P-20 to P-1000 Operation and Maintenance

8.7. **Trouble shooting**

8.7.1. See the trouble shooting table (Table 2).

8.8. **Disassemble the Pipetman®** (See Figure 2)

8.8.1. Remove tip ejector, Press the tip ejector button down and pull on the flanged upper part of the tip ejector with the other hand (moderate force may be required).

8.8.2. Remove the connecting nut by turning it counterclockwise direction by hand.

8.8.3. At this time carefully separate the lower components from the upper body assembly.

8.8.4. Remove the piston assembly and the o-ring and seal.

8.8.5. Clean the Pipetman® by following the decontamination instruction above.

8.8.6. Replace the necessary components as needed.

8.9. **Reassemble the Pipetman®**

8.9.1. Place fully assembled piston assembly into the tip holder.

8.9.2. Place connecting nut so that it can be pulled up over tip holder.

8.9.3. Holding the tip holder with connecting nut, line up and place the top of the piston assembly into the pipette upper body.

8.9.4. Tighten the connecting nut onto threads of upper pipette body.

8.9.5. Place tip ejector so it comes up over tip holder. Line up with tip ejector button rod in upper body.

8.9.6. Pull on the tip ejector flange with one hand with the other hand holding the upper body (moderate force may be required).

9. Attachments:

9.1. Figure 1: Pipetman®,

9.2. Figure 2: Pipetman® Component Breakout

9.3. Table 1: Immersion chart

9.4. Table 2: Troubleshooting guide.

10. History:

Name	Date	Amendment
Bob O'Brien	08162006	Initial release
Bob O'Brien	02Jun08	Re-worked photo. Added re-assembly. College name change

Title: Gilson Pipetman® P-20 to P-1000 Operation and Maintenance

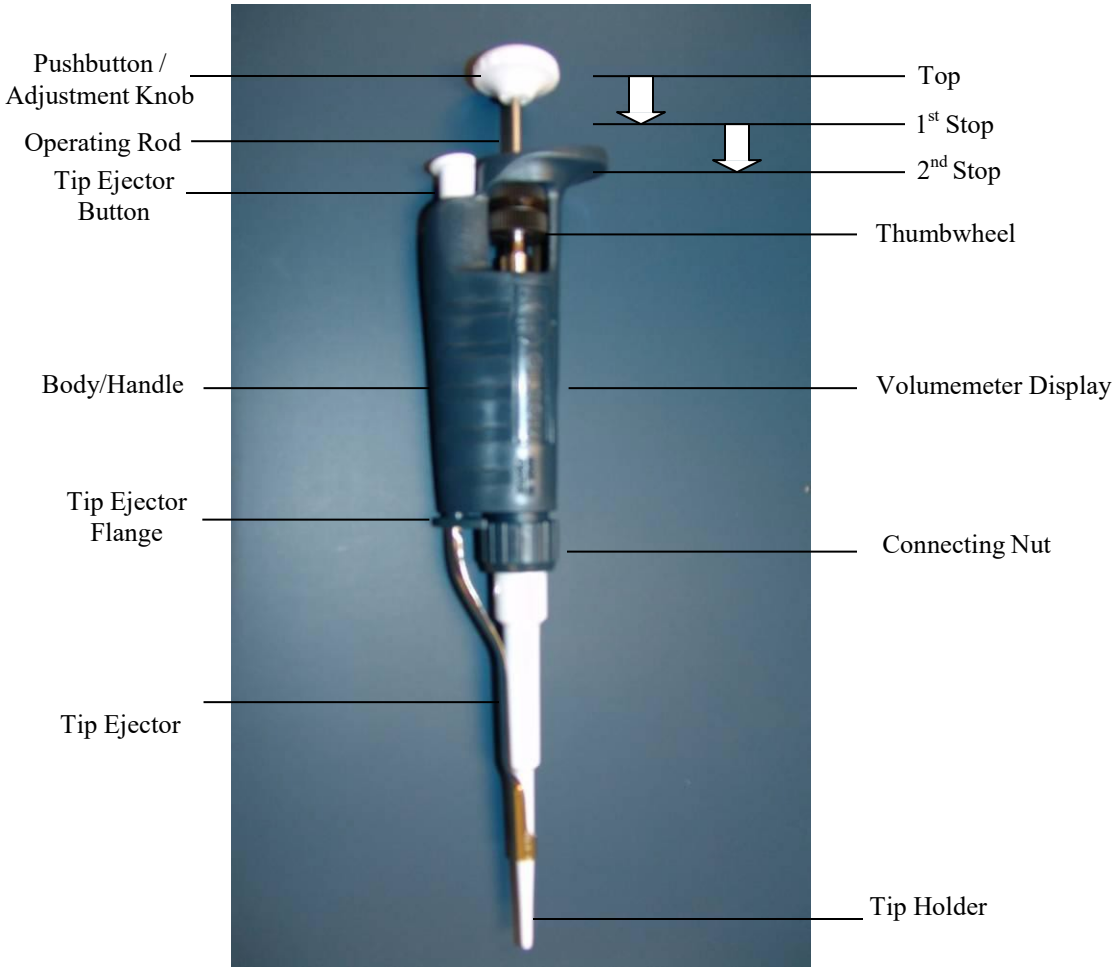


Figure 1: Pipetman®

Title: Gilson Pipetman® P-20 to P-1000 Operation and Maintenance

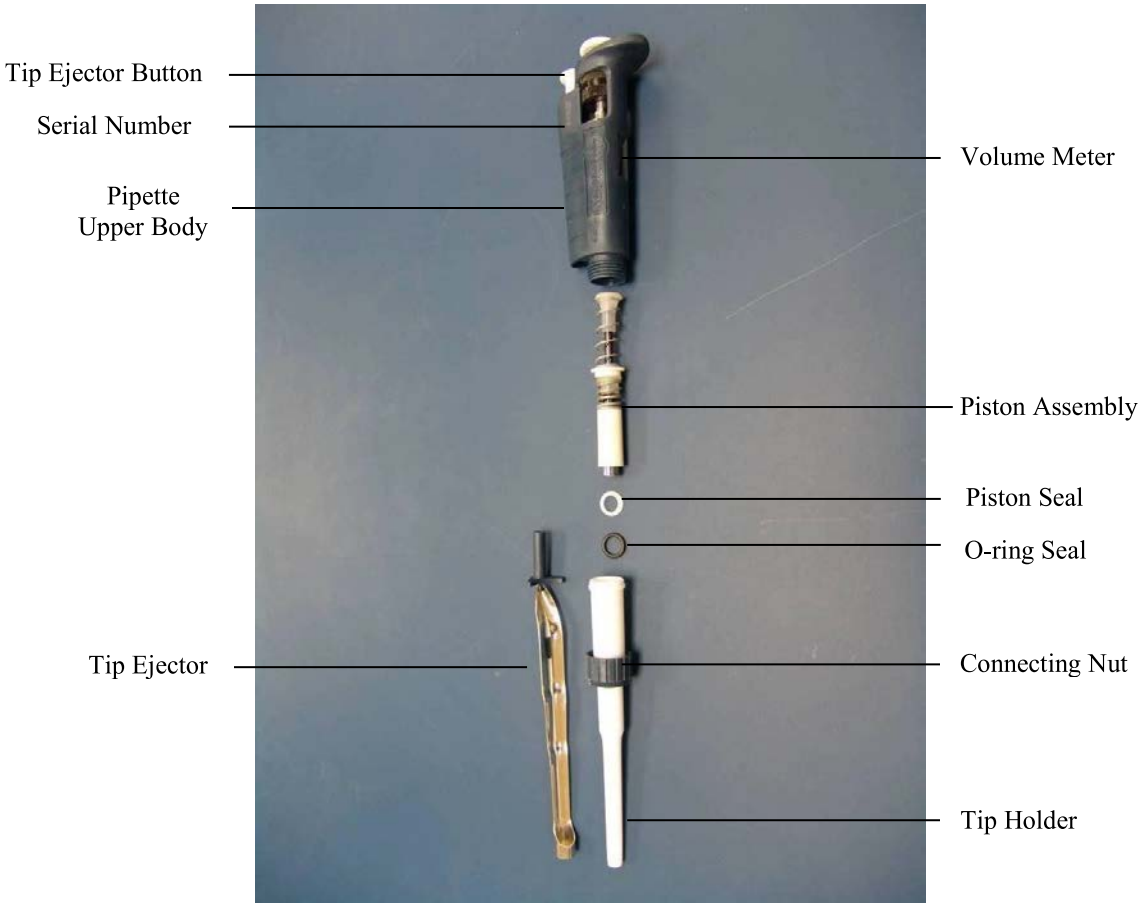


Figure 2: Pipetman® Component Breakout

Immersion Depth and Wait Time		
Model	**Immersion depth (mm)**	**Wait time (sec)**
P2	1mm	1
P10	1mm	1
P20	2mm to 3mm	1
P100	2mm to 4mm	1
P200	2mm to 4mm	1
P1000	2mm to 4mm	2 to 3

Table 1: Immersion chart

Title: Gilson Pipetman® P-20 to P-1000 Operation and Maintenance

Pipetman® Trouble Shooting		
Symptom	**Possible Cause**	**Action**
Pipette is leaking sample	O-ring or seal is worn	Replace both parts.
Pipette will not aspirate	O-ring or seal is worn Tip holder is loose Piston is damaged (chemically or mechanically) Damaged tip holder Connecting nut is loose	Replace both parts. Tighten connecting nut. Return pipette to supplier. Replace the tip holder. Tighten connecting nut.
Pipette is inaccurate	Improper assembly Unscrew tip holder Connecting nut is loose	See "Maintenance" Tighten connecting nut. Tighten connecting nut.
Pipette is not precise	Tip holder is loose Incorrect operator technique Worn O-ring or seal Piston is damaged (chemically or mechanically) Damaged tip holder	Tighten connecting nut. Operator training. Replace both parts. Tighten connecting nut. Return pipette to supplier. Replace tip holder.
Tips fall off or do not fit	Low quality tips Damaged tip holder Damaged tip ejector	Use better quality tips. Replace he tip holder. Replace the tip ejector.
Bent operating rod	Pipette damaged mechanical shock	Return pipette to supplier.
Operating rod has chemical damage	Chemically damaged rod	Return pipette to supplier.
Volume meter digits unclear	Pipette damaged	Return pipette to supplier.
Cannot set the maximum range	Pipette damaged, mechanical shock	Return pipette to supplier.
Volume meter thumbwheel is hitching	Pipette damaged, mechanical shock	Return pipette to supplier.
Tip ejector bent	Pipette damaged, mechanical shock	Replace ejector.
Tip ejector chemical damage	Chemical damage, damaged ejector	Replace ejector.

Table 2: Troubleshooting guide

Title: Pipette Performance Verification SOP

Approvals:

Preparer: Judith Fitzpatrick Date 13Oct09
Reviewer: Kari Britt Date 13Oct09

1. **Purpose:** To verify volume measurements for a single channel pipette.

2. **Scope:** Covers the cleaning, decontamination and verification of a single channel pipette.

3. **Responsibilities:**
   - **3.1.** It is the responsibility of the course instructor /lab assistant to ensure that this SOP is performed as directed and to update the procedure when necessary.
   - **3.2.** It is the responsibility of the students/technicians to follow the SOP as described and to inform the instructor about any deviations or problems that may occur while performing the procedure.

4. **References:**
   - **4.1.** Balance operation SOP
   - **4.2.** Balance calibration SOP
   - **4.3.** Autoclave SOP
   - **4.4.** Pipette Operation and Maintenance SOP

5. **Definitions:** N/A
6. **Precautions:** N/A

7. **Materials:**
   - **7.1.** Balance
   - **7.2.** 20g and 200g standard mass weights
   - **7.3.** 10 or 20mL bottle and cap for calibrating 20-50µL pipettes
   - **7.4.** 200mL bottle and cap for calibrating pipettes larger than 50µL
   - **7.5.** Beaker or suitable container
   - **7.6.** Pipette Performance Verification Form
   - **7.7.** Pipette Performance Verification Pass/Fail Form
   - **7.8.** Pipette tips
   - **7.9.** Pipette
   - **7.10.** 70% Isopropyl Alcohol (IPA)
   - **7.11.** Lab towels
   - **7.12.** Deionized (DI) water
   - **7.13.** Tweezers
   - **7.14.** Mild lab detergent
   - **7.15.** Thermometer
   - **7.16.** Calculator
   - **7.17.** Barometer

Title: Pipette Performance Verification SOP

8. Procedure:

8.1. Clean the pipette (Refer to figures 2 and 3)

Note: Most pipettes are designed so that the parts that normally come into contact with liquid contaminants can easily be cleaned and decontaminated.

8.1.1. Wipe entire pipette with a lab towel dampened with a mild detergent solution.
8.1.2. Wipe entire pipette with a lab towel dampened with deionized water.
8.1.3. Remove the tip ejector.
8.1.4. Wipe the tip ejector with a lab towel dampened with a mild soap solution.
8.1.5. Wipe the tip ejector with a lab towel dampened with deionized water.
8.1.6. Refit the tip ejector and allow the pipette to dry.
8.1.7. Dispose of lab towels in waste receptacle.

8.2. Chemical decontamination

8.2.1. Spray a lab towel with 70% IPA to dampen the lab towel.
8.2.2. Wipe upper part of body with dampened lab towel.
8.2.3. Wipe tip holder and tip ejector with dampened lab towel.
8.2.4. Wipe entire pipette with a lab towel dampened with deionized water.
8.2.5. Leave pipette to dry or wipe pipette dry with a lab towel.
8.2.6. Dispose lab towels in waste receptacle.

8.3. Verification

Note: To test the accuracy of the pipette you will pipette a set volume 10 times and then weigh the total volume dispensed. 1mL of DI water should weigh 1g and 1µL should weigh 1mg. You will then calculate error by subtracting the difference between your recorded weight and the expected weight and dividing this number by the expected weight. If the % error is ≤2% the pipette passes verification if it is greater than 2% the pipette fails. We will verify the pipette once at the maximum volume for the pipette, once at the ½ maximum volume, and once at the minimum volume. Altogether you will pipette 30 volumes and weigh 3 times for each pipette.

8.3.1. Record the necessary information on the Pipette Performance Verification Form.
8.3.2. Verify that the calibration label of the balance is within the dated calibration time period.
8.3.3. Verify that the balance is still in calibration by weighing 200g and 20g standard mass weights three times each. Refer to the balance operation SOP for proper operation of the balance.
Note: Standard mass weights can be placed directly on the balance weight pan. If the displayed value for the standard mass weight is greater than ±0.01g of the standard weight, then the scale needs to be calibrated. Refer to the balance calibration SOP for proper calibration of the balance.
8.3.4. Add DI water to bring a small sample bottle to approximately 1/3 full capacity.
8.3.5. Fill a small beaker with DI water.
8.3.6. Place the small sample bottle on the balance.

Title: Pipette Performance Verification SOP

8.3.7. Tare the balance and verify that 0.00 is being displayed.
8.3.8. Verify that the pipette is set to the maximum volume (e.g. the maximum volume for a P-200 pipette is 200µL).
8.3.9. On the Pipette Performance Verification Form, beside Selected Volume, enter the volume you will be pipetting, and the value of that volume times 10 (e.g. For a 200µL pipette you will record 200µL for the selected volume and 2000µL or 2mL for the selected volume times 10).
8.3.10. Convert the selected volume times 10 to expected mass using the following conversions: 1µL = 1mg and 1mL = 1g. Use the selected volume times 10 as the volume (e.g. for a 200µL pipette, 2000µL multiplied by 1mg/µL = 2000mg or 2g). Record the expected mass in the box beside Expected Mass.
8.3.11. Verify that the pipette is set to the maximum volume recommended by the manufacturer for the pipette.
8.3.12. Place pipette tip securely on the pipette.
8.3.13. Remove the sample bottle from the balance.
8.3.14. Aspirate DI water into pipette tip from the beaker and dispense it into the sample bottle. Refer to the pipette operation SOP for proper operation of the pipette.
8.3.15. Repeat the above step 9 times. Each time you dispense the selected volume mark the Pipette Performance Verification Form in the numbered box beside Dispense Repetitions.
8.3.16. Place the sample bottle on the balance and record the mass on the Pipette Performance Verification form next to Recorded Mass.
8.3.17. Tare the balance and verify that 0.00 is being displayed.
8.3.18. Set the volume of the pipette to half capacity (e.g. For a P-200 pipette, set it to 100µL) and verify the volume.
8.3.19. Repeat steps 8.3.9. through 8.3.16 with the pipette set to the half-capacity volume.
8.3.20. Tare the balance and verify that 0.00 is being displayed.
8.3.21. Set the volume of the pipette to the minimum capacity recommended by the manufacturer (e.g. For a 200µL pipette, the minimum capacity is usually 50µL.) Note: The minimum capacity for a 20µL pipette is usually 2µL. The minimum capacity for a 1000µL pipette is usually 200µL.
8.3.22. Repeat steps 8.3.9. through 8.3.16 with the pipette set to the minimum-capacity volume.
8.3.23. Calculate the % error (as directed in the note at the beginning of section 8.3) for each test (maximum, half-capacity, and minimum volumes) and record the results on the Verification form.
8.3.24. Verify that all fields of the Pipette Performance Verification Form have been filled out and fill out the Pipette Performance Verification Pass/Fail form according to the results of the tests.
8.3.25. This entire SOP can be repeated for pipettes of different capacities such as 20µL and 1000µL pipettes.

Title: Pipette Performance Verification SOP

9. Attachments:

9.1. Figure #1 photograph of the equipment on bench
9.2. Figure #2 photograph of the pipette stops
9.3. Figure #3 photograph of the breakdown of the pipette
9.4. Pipette Performance Verification Form
9.5. Pipette Performance Verification Pass/Fail Form

10. History:

Name	Date	Amendment
Bob O'Brien	10Jul07	Initial Release
Bob O'Brien	11May08	College name change, add photographs, reword to clarify
Judith Fitzpatrick Kari Britt	13Oct09	Added directions for using 3 different volumes for calibrating the same pipette. Also changed name from Pipette Calibration SOP to Pipette Performance Verification SOP.

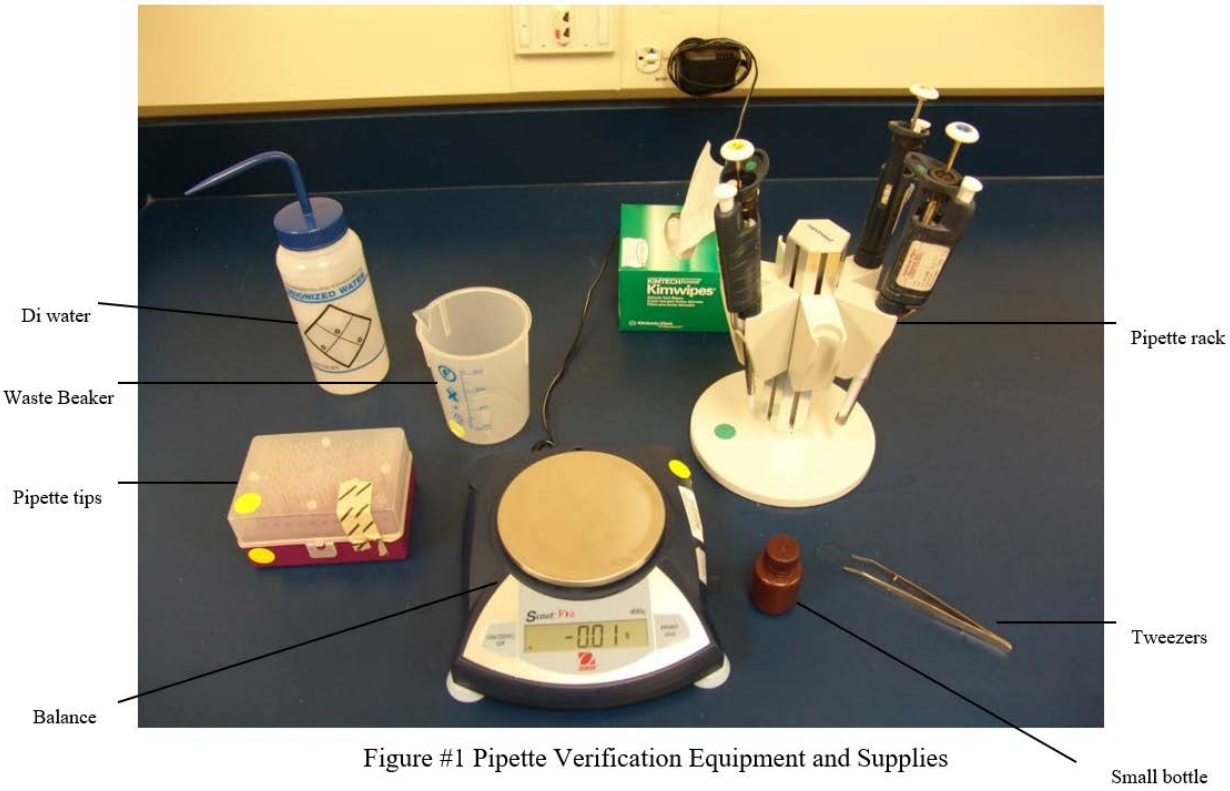


Figure #1 Pipette Verification Equipment and Supplies

Title: Pipette Performance Verification SOP

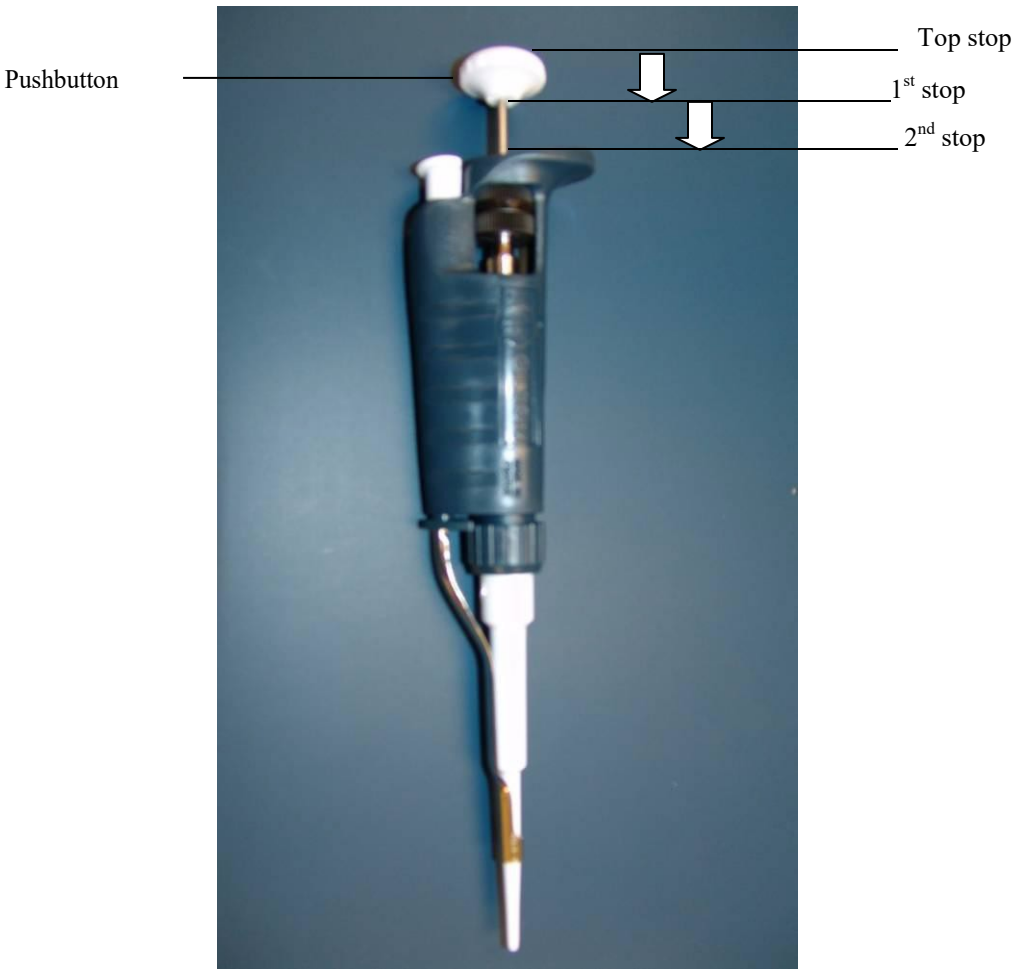


Figure #2 Gilson Pipetman ®

Title: Pipette Performance Verification SOP

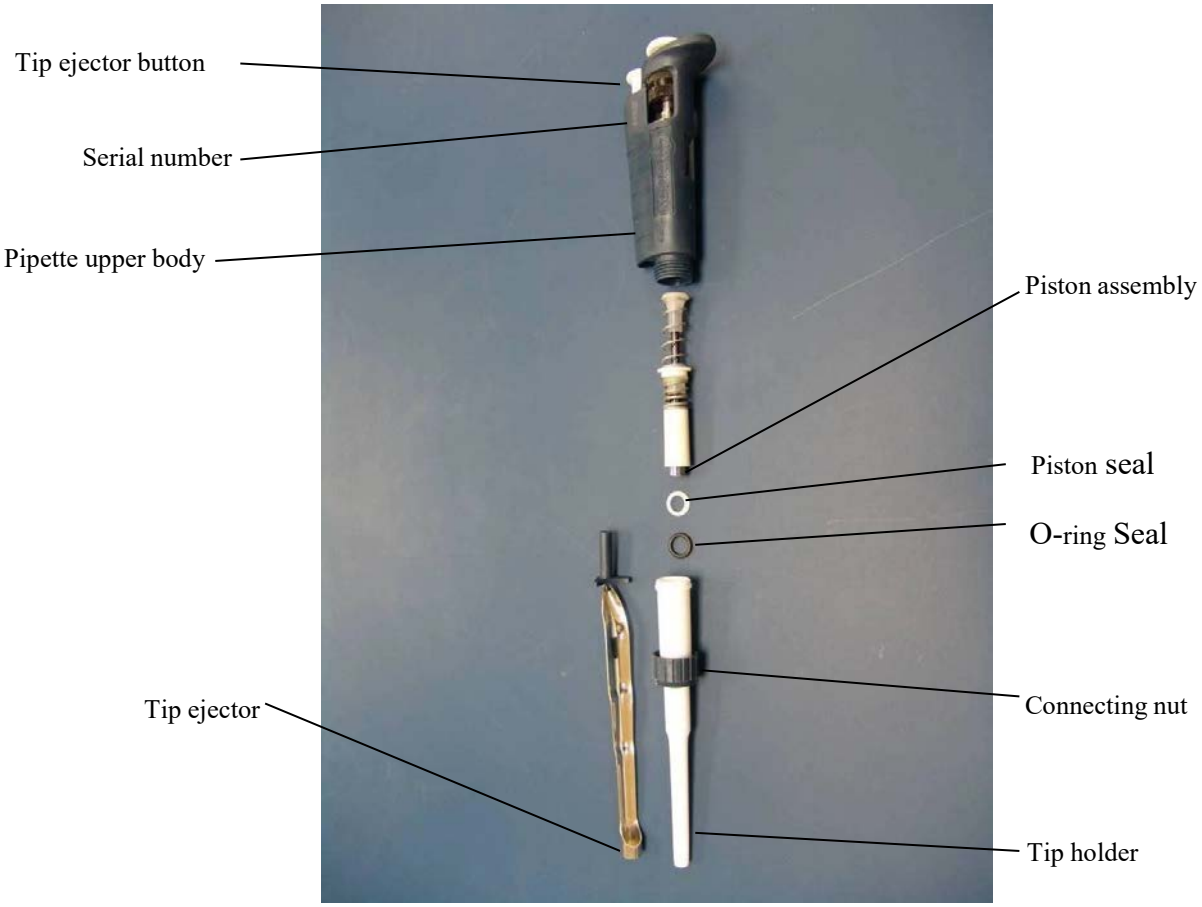


Figure #3 Gilson Pipetman® Component Breakout

Great Bay Community College
320 Corporate Drive
Portsmouth, NH 03801

Pipette Performance Verification Form

Customer Information:	**Equipment Information:**
Contact Name:_____	Name and Description:_____
Department Name: _____	Model:_____
Phone Number:_____	Serial Number:_ _____
	State and ID Number:

Identification											**Inspection completed**	
Model											Technician	
Serial #											Serial #	
Range											Date	
Number of Channels												
Test Conditions												
Balance Serial #											Balance model	
Sensitivity											Balance Calibration date	
Correction Factor												
Air Temperature												
Barometric Temperature												
Relative humidity												
Control data											**Test results**	
Calibration Date											% Error Test 1 (Maximum volume)	
Technician											% Error Test 2 (Half capacity volume)	
											% Error Test 3 (Minimum volume)	
											Pass or Fail	
Tests												
Test 1 (Maximum volume)												
Selected Volume											Expected Mass	
Selected Volume X 10											Recorded Mass	
Dispense Repetitions	1	2	3	4	5	6	7	8	9	10		
Test 2 (Half capacity volume)												
Selected Volume											Expected Mass	
Selected Volume X 10											Recorded Mass	
Dispense Repetitions	1	2	3	4	5	6	7	8	9	10		
Test 3 (Minimum volume)												
Selected Volume											Expected Mass	
Selected Volume X 10											Recorded Mass	
Dispense Repetitions	1	2	3	4	5	6	7	8	9	10		

Great Bay Community College
320 Corporate Drive
Portsmouth, NH 03801

Pipette Performance Verification Pass/Fail Form

Customer Information:
Contact Name: _____
Department Name: _____
Phone Number:_____

Equipment Information:
Name and Description: _____
Model: _____
Serial Number:_____
State and ID Number:_____

Verification: _____
_____
_____
_____

Technician_____
Date_____

Passed Verification:

Date of Verification_____

Technician_____

Verification Sticker:

☐Yes
☐No
☐Not Applicable

Verification Due Date_____

Failed Verification:

Reason for Failure_____
_____
_____
_____

Date Out of Service_____

Technician_____

Pipetman Challenge

MATERIALS:
blue (food dye) solution 2.0 ml
yellow (food dye) solution 2.0 ml
disposal beaker
P- 20µl pipetman, P-
200µl P-1000µl pipetman
pipet tips for both 200µL and 1000µL pipetman
microfuge tubes 2.0 mL
balance reading to the hundredth gram, 0.00 g

METHOD:
This activity includes the weighing of a microfuge tube before and after the Pipetman Challenge activity and requires some information on how to use a balance.
A common digital balance is needed to the precision of 0.00g. The balance you will be using today should have this precision.

1. Tare the balance.
    a. Press the balance on button.
    b. When the numbers appear, press the ZERO or TARE button and allow the balance to adjust to zero. The balance should read 0.00 then it is ready to be used.

View of the balance display:

0.00 grams

2. Place your empty 2.0 mL microfuge tube on the balance pan.
    a. Record the mass of the microfuge tube here: **mass =_____g**
    Record the pipetman's setting in the table below.
    b. Aspirate each desired volume of colored solution as indicated in the table using the 2.0 mL microfuge tube. Add all volumes into the same microfuge tube.

Pipetman Challenge

PIPETMAN CHALLENGE
TABLE

Add volume to the microfuge tube:	Use this color solution:	Record Pipetman used	Pipetman Setting
62µL	**Blue**		
18µL	**Yellow**		
0.975mL	**Blue**		
0.010mL	**Yellow**		
130µL	**Blue**		
75µL	**Yellow**		
0.020mL	**Blue**		
360µL	**Yellow**		
50µL	**Blue**		
0.300mL	**Yellow**		

Pipetman Challenge

RESULTS:

3. Determine the mass of the 2.0mL microfuge tube containing all of the colored solutions added from the activity above.

    a. The mass of the fluid in the microfuge tube =_____**g**

4. Determine the net mass of the combined solutions in the microfuge tube by weighing them and subtracting the mass of the tube itself. Record below.

    a. Total mass of tube + solutions =_____g

    b. Subtract the mass of the tube - _____g

    c. Net mass of the solutions =_____g

5. It is possible to find the actual volume in your microfuge tube by measuring the mass of all the colored solutions added to the tube and then solving the equation below to determine the actual volume:

$$\text{Density}^* = \frac{\text{Mass}}{\text{Volume}} \qquad D = \frac{\_\_\_\_g}{V\ mL}$$

***(density of solutions = 1g/1mL) hint: Solve for V**

    a. Actual Volume =_____mL

6 Calculate the total expected volume by adding the volumes listed on the pipetman in the first column of the table.

    a. Expected Volume = _____mL

7. Percent error can be calculated from the expected and actual volume values found are determined by using the following equation:

$$\frac{\text{Expected Volume - Actual Volume}}{\text{Expected Volume}} \times 100 = \%\ \text{Error}$$

8. The winner of the Pipetman Challenge is the student with the lowest percent error.

Maintenance/Instrumentation Technician

REF	Key Functions & Tasks (Maintenance/Instrumentation Technician)
1	**Work in compliance with EH&S.**
1.a	Wear appropriate personal protective equipment.
1.b	Participate in emergency drills and emergency response teams.
1.c	Identify unsafe conditions and take corrective action.
1.d	Appropriately and safely access production equipment.
1.e	Handle, label, and dispose of hazardous / biohazard materials.
1.f	Access and utilize Material Safety Data Sheets. (MSDS)
1.g	Comply with all permitting requirements. (Lock Out Tag Out, hot work, line breaking, CSE)
1.h	Carries out operations with attention to OSHA and EPA regulations, and any other applicable state and federal regulations.
1.i	Participate in all company safety training and audits as required.
1.j	Assists with waste treatment operations.
2	**Work in compliance with cGMPs.**
2.a	Follow SOPs for all operations.
2.b	Prepare required documentation for recording and notification of events and changes related to equipment, including: as-found/as-left data, maintenance logs, calibration certificates and instrument labels, deviations, OOT reports, and installation reports
2.c	Maintain equipment logbooks.
2.d	Control and receive parts and materials.
2.e	Maintain training documentation.
2.f	Maintain equipment and process utilities in a validated state.
2.g	Follow appropriate gowning procedures and behaviors for work in controlled/classified areas.
2.h	Ensure appropriate flow of personnel, equipment, and materials.
2.i	Follow change control procedures for process, equipment, and documentation.
2.j	Update status of equipment and materials.
2.k	Identify and report exception events and CAPA.
3	**Monitor, maintain, and repair process or laboratory equipment.**
3.a	Monitor, maintain and repair process vessels and fluid handling systems including pumps, pipes and valves.
3.b	Monitor, maintain and repair heat transfer equipment (heat exchangers, condensers, evaporators).
3.c	Monitor, maintain and repair process support equipment (autoclaves, ovens, dryers, washers).
3.d	Monitor, maintain and repair refrigeration equipment (freezers, lyophilizers).
3.e	Monitor, maintain and repair laboratory equipment (scales, hot plates, HPLC).
3.f	Monitor, maintain and repair fill/finish equipment including automated equipment (filling machines, cap/inspect machines, packaging).
3.g	Respond to alarms per procedure.
4	**Monitor, maintain, and repair plant utility systems.**
4.a	Monitor, maintain and repair utility systems including instrument air, process gases (CDA, nitrogen, argon, CO2).
4.b	Monitor, maintain and repair utility systems including steam, process water types (DI, WFI, USP) and cooling/heating fluids (glycol, liquid nitrogen).
4.c	Monitor, maintain and repair utility systems including HVAC, electrical, hydraulic.
4.d	Monitor, maintain and repair waste treatment systems (waste neutralization, inactivation, scrubbers).
4.e	Monitor, maintain and repair site safety systems (fire suppression, eye wash, safety showers, etc.).
4.f	Monitor, maintain and repair security system including controlled personnel access.
4.g	Monitor building management systems (BAS).
4.h	Respond to alarms per procedure.
4.I	Perform testing of plant utilities.
5	**Calibrate Control System Components.**

REF	Key Functions & Tasks (Maintenance/Instrumentation Technician)
5.a	Calibrate pressure, temperature, flow, weight, pH, DO, and other critical measurement devices and transmitters.
5.b	Calibrate control valves, actuators, positioners, and process switching devices.
5.c	Investigate, correct and document calibration failures (identify OOS results and report in CAPA).
5.d	Use calibration reference standards and other specialized equipment.
6	**Maintain control systems, equipment and instrumentation.**
6.a	Program and/or replace microprocessors, PC, PLC, and DCS controllers and devices.
6.b	Maintain and repair on-off actuators, diaphragms, electrical and pneumatic and hydraulic industrial control circuits and equipment.
7	**General Engineering Technology and Maintenance Skills**
7.a	Solve system/equipment problems using P&IDs, manuals, and other drawings as needed.
7.b	Participate in root cause analysis and verification, identification of solutions, and development of solution implementation work plans
7.c	Assist in installation, modification, commissioning, and validation of equipment.
7.d	Communicate with all applicable departments about scheduling, service needs and priorities, problems, status of equipment and repair jobs, and status of utility systems.
7.e	Maintain spare parts inventory.
7.f	Communicate with vendors and service suppliers to coordinate and schedule external calibration and maintenance activities.
7.g	Use Computerized Maintenance Management System (CMMS) to schedule and track work orders for corrective and preventive maintenance (PMs) (Maximo, Atlas, Blue Mountain).

DAY TWO

Transformation - Upstream Processing

Protein is CA$H!

Title: Bacterial Transformation and Upstream Processing

Approvals:
Preparer: Katherine Gorzyca Date:07Oct06
Reviewer: Mary Jane Kurtz Date:01Jun07

1. **Purpose:**
   To transform *Escherichia coli* and scale them up in cell culture in order to produce Green Fluorescent Protein (GFP).

2. **Scope:**
   2.1. To provide procedural instructions for the transformation of live *E. coli* with pGLO (a plasmid carrying the gene for Green Fluorescent Protein).
   2.2. To produce bacteria that can manufacture Green Fluorescent Protein.

3. **Responsibility:**
   3.1. It is the responsibility of the teacher/instructor/ lab assistant to ensure that these written instructions are performed as described.
   3.2. It is the responsibility of the teacher/instructor/lab assistant to complete the pre-lab preparation as set forth in the BIO-Rad Instruction Manual.
   3.3. It is the responsibility of the student to read and follow this SOP as described and to inform the instructor of any problems that he/she may encounter in doing so.

4. **References**:
   4.1. BIO-RAD Laboratories Instruction Manual: Biotechnology Explorer pGLO Bacterial Transformation Kit. (Cat. # 166-0003EDU)
   4.2. Burdin, David W. and Whitney, Donald B. Biotechnology: Proteins to PCR: A Course in Strategies and Laboratory Techniques, 1st ed. Birkhauser, 1995.
   4.3. Micklos, David A. and Freyer, Greg A. DNA Science: A First Course in Recombinant DNA Technology. Cold Spring Harbor Laboratory Press, 1990.
   4.4. Seidman, Lisa A. and Moore, Cynthia J. Basic Laboratory Methods for Biotechnology: Textbook and Laboratory Reference. Prentice Hall, 2000.

Title: Bacterial Transformation and Upstream Processing

5. Definitions:

5.1. **Transformation** – Process by which the properties of a cell are altered by the addition and expression of foreign DNA.

5.2. **Plasmid** – A small double stranded circular DNA molecule found in some bacterial cells. This DNA molecule is separate from chromosomal DNA, carries genes that enhance survival of the cell, can replicate on its own, and is often used as an expression vector.

5.3. **Antibiotic Resistance** – The ability of a microorganism to disable the action of an antibiotic or to prevent the transport of the antibiotic into the cell. This would allow the microorganism to grow in the presence of the antibiotic without being harmed.

5.4. **GFP** – Green Fluorescent Protein is naturally produced by a jellyfish (*Aequorea victoria*). This protein is responsible for the ability of the jellyfish to fluoresce or glow in the dark.

5.5. **pGLO** – This is an expression vector produced by Bio-Rad for this laboratory activity. It is a plasmid that contains both a gene for antibiotic resistance to ampicillin and the gene that codes for Green Fluorescent Protein. This expression vector also contains an important gene regulation system whereby the gene for GFP is "turned on" in the presence of the sugar arabinose and "turned off" when arabinose is absent.

5.6. **Transformation Efficiency** – This is a quantitative measure of the success of the transfer, uptake, and expression of the foreign DNA by the host cells.

6. Hazard Communication:

6.1. **Treatment of contaminated materials**:

6.1.1. All contaminated solutions and lab ware must be disinfected by autoclaving before disposal. If an autoclave is not available, alternate treatment with a 10% bleach solution for twenty minutes will also be effective.

6.2. **Harmful UV radiation**:

6.2.1. Ultraviolet radiation is harmful to the eyes and skin. The lamp used in this experiment is a long-wave UV lamp and is not as damaging as a short-wave lamp. Limited exposure and use of UV rated safety glasses or goggles are required.

Title: Bacterial Transformation and Upstream Processing

6.3. **Handling of live bacterial cultures**:

6.3.1. E.coli K-12 strain and its derivatives are laboratory strains and are not pathogenic or disease causing. They are unable to effectively colonize the human gut. But, as with all microbial exposure in the laboratory and standard microbiological safety practices must be followed. Standard Microbiological Practices include the following:

6.3.1.1. Work bench surfaces need to be decontaminated with a 10% bleach solution prior to setting up and after completing the lab.

6.3.1.2. Hands are to be washed on entering and before leaving the lab.

6.3.1.3. Specimens should be handled and mixed carefully to minimize the creation of aerosols.

6.3.1.4. No eating, drinking, smoking, or application of cosmetics are allowed in the lab.

6.3.1.5. Use of protective eyewear and gloves are required.

6.4. **Ampicillin**

6.4.1. Ampicillin is a member of the penicillin family of antibiotics. Avoid contact with ampicillin if you are allergic to penicillin or any other member of the penicillin family. Ampicillin may cause allergic reactions or irritations to the eyes, respiratory system, and skin. In case of contact with eyes, rinse immediately with plenty of water and seek medical advice.

7. Materials:

7.1. **BIO-RAD Transformation Kit Components – Reagents**

7.1.1. E.coli HB101 K-12, lyophilized
7.1.2. Plasmid (pGLO), lyophilized, 20 µg
7.1.3. Ampicillin, lyophilized, 30 mg
7.1.4. L(+) Arabinose, lyophilized, 600 mg
7.1.5. Transformation solution (50 mM $CaCl_2$, pH 6.1), sterile, 15ml
7.1.6. LB nutrient broth, sterile, 10 ml
7.1.7. LB nutrient agar powder, sterile (to make 500 ml)

7.2. **BIO-RAD Transformation Kit Components – Disposables**

7.2.1. pipets, sterile
7.2.2. inoculation loops, sterile, 10 µl
7.2.3. Petri dishes, 60 mm
7.2.4. microtubes, 2.0 ml and foam micro test tube holders

Title: Bacterial Transformation and Upstream Processing

7.3. **Accessories**
- 7.3.1. UV lamp – long wavelength
- 7.3.2. timer
- 7.3.3. microwave oven
- 7.3.4. 37ºC incubator
- 7.3.5. 42ºC water bath
- 7.3.6. glassware: 1L flask, 500 ml graduated cylinder
- 7.3.7. distilled water, 500 ml
- 7.3.8. crushed ice and containers (mini ice baths)
- 7.3.9. marker pen

8. Procedure:

8.1. **Assemble your work station**.
- 8.1.1. Collect the following materials from the common work station:
  - 8.1.1.1. *E.coli* starter plate
  - 8.1.1.2. 4 poured agar plates (1 LB, 2 LB/amp, 1 LB/amp/ara)
  - 8.1.1.3. 1 vial of Transformation Solution (TS)
  - 8.1.1.4. 1 vial of LB nutrient broth
  - 8.1.1.5. Sterile inoculation loops (1 pkg)
  - 8.1.1.6. 5 sterile pipets
  - 8.1.1.7. foam microtube test tube holder
  - 8.1.1.8. 2 sterile microtubes
  - 8.1.1.9. marker
  - 8.1.1.10. ice bath (foam cup with crushed ice)
  - 8.1.1.11. 20 ml cell culture tubes
  - 8.1.1.12. Parafilm
  - 8.1.1.13. pGLO plasmid DNA (shared between groups)

Title: Bacterial Transformation and Upstream Processing

8.2. **Inoculation of E.coli cultures**

8.2.1. There are four LB agar plates at your work station. One contains only LB agar (LB), two contain LB agar and ampicillin (LB/amp), and one contains LB agar, ampicillin, and arabinose (LB/amp/ara). With your marker pen, label the outside edge on the bottom (not the lid) of each of the plates as follows:

- "+pGLO LB/amp"
- "-pGLO LB/amp"
- "+pGLO LB/amp/ara"
- "-pGLO LB"

8.2.2. Label each plate with your group name

8.1.2.1. Obtain two capped sterile micro test tubes and place in test tube holder. Label one "+pGLO" and the other "-pGLO"; label both tubes with your group name

8.1.2.2. Take the vial labeled "transformation solution (TS)" and using a sterile pipet, transfer 250 µl of the solution to each of your labeled microtubes.

8.1.2.3. Place test tube holder with tubes into crushed ice bath. Make sure test tube is in contact with ice.

8.1.2.4. Use a sterile loop to pick up a 4 large or 8 small colonies of bacteria from the starter plate. Pick up the "+pGLO" tube and immerse the loop into the transformation solution at the bottom of the tube. Spin the loop between your index finger and thumb until the entire colony is dispersed in the transformation solution (with no floating chunks). Place the tube back in the tube rack in the ice.

8.1.2.5. Using a new sterile loop, repeat previous step for the "-pGLO" tube

8.3. Transformation of culture

8.3.1. Immerse a new sterile loop into the plasmid DNA stock tube and withdraw a loopful. NOTE: There should be a film of plasmid solution across the ring.

8.3.2. Mix the loopful into the cell suspension of the tube labeled "+pGLO". Alternatively, pipet 10 µL of the pGLO plasmid into the tube and mix

8.3.3. Do not add plasmid DNA to the tube labeled "-pGLO" (See Figure 1)

8.3.4. Incubate the tubes on ice for 10 minutes. Make sure to push the tubes all the way down in the rack so the bottom of the tubes sticks out and makes contact with the ice.

Title: Bacterial Transformation and Upstream Processing

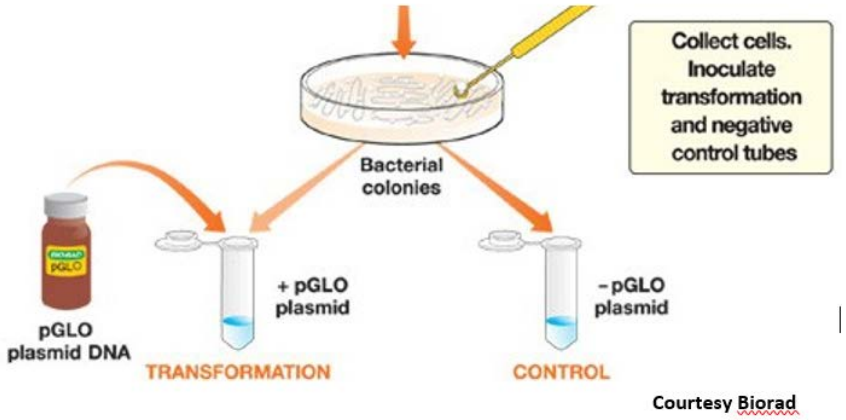


Figure 1 – Inoculation-Transformation of bacteria culture

8.4. Heat Shock Treatment

8.4.1. Using the foam rack as a holder, transfer both the +pGLO and –pGLO tubes from the ice bath to a heated water bath (42˚C) for exactly 50 seconds. Make sure the sample solutions are immersed in the warm water.

8.4.2. Return tubes to the ice bath and incubate on ice for 2 minutes

8.5. Gene Expression

8.5.1. Remove the tubes from the ice bath and place the rack on the bench.

8.5.2. Using a sterile pipet, add 250 µl of LB nutrient broth to the "+pGLO tube". Cap the tube. Using a new sterile pipet, add 250 µl of the LB broth to the "-pGLO" tube and cap the tube. Mix the solutions by tapping the bottom of the tubes with your finger.

8.5.3. Let the tubes stand at room temperature for 10 minutes.

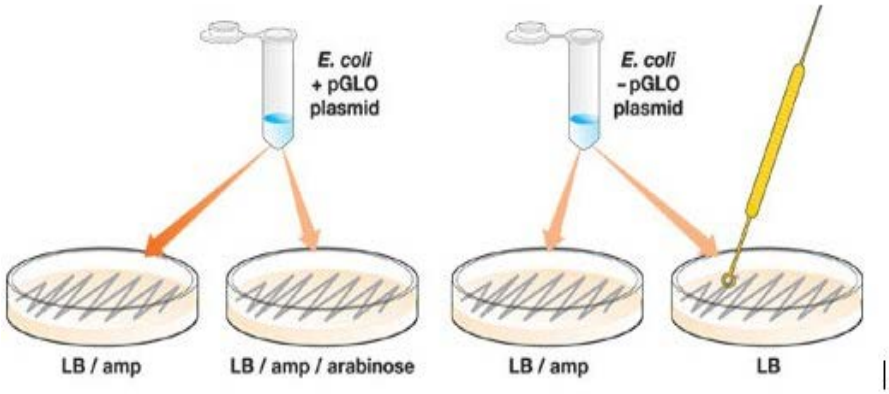
8.5.4. Mix the tubes by tapping on the bottom. Using a new sterile pipet for each transfer, place 100 µl of the "+pGLO" solution on each of the appropriately labeled LB/amp and LB/amp/ara plates. Again, using a new sterile pipet for each transfer, place 100µl of the "-pGLO" solution on the remaining LB and LB/amp plates.

Title: Bacterial Transformation and Upstream Processing

8.5.5. Using a new sterile loop for each plate, spread the suspension evenly on the surface of the agar, moving the flattened loop lightly back and forth across the surface. Do not press the loop into the agar. Cover the entire plate. Turn the plate 45° and repeat the streak. Cover each plate when complete. (See Figure 2)

8.5.6. Wait a few minutes for the suspension to be absorbed into the agar, and then stack the inoculated plates upside down. Parafilm and label the stack with your group name. Incubate the plates at 37°C for 24 hours. **Note**: If an incubator is not available, plates can be incubated at room temperature for a longer time. After incubation, observe plates under normal lighting. Make observations as to presence and amount of bacterial growth and color of colonies.

Figure 2



Courtesy BIO-RAD

Title: Bacterial Transformation and Upstream Processing

8.6. **Data Collection**:

8.6.1. After incubation, observe plates under normal lighting. Make observations as to presence and amount of bacterial growth and color of colonies.

8.6.2. Observe plates under UV light. (Refer to Hazard Communication)

8.7. Analysis of Results (See Figure 3)

When looking at your plates keep in mind that:

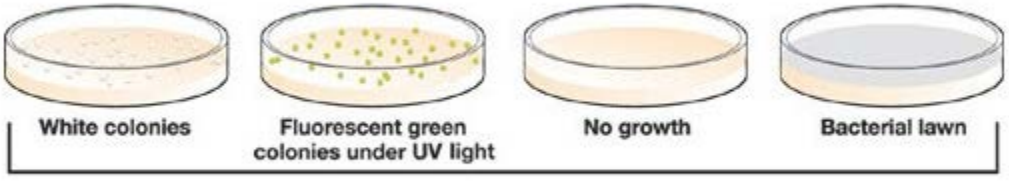
8.7.1. Each colony you see is the result of initial bacterial culture placed on the agar. Each cell grows and reproduces many times, doubling its number with each generation. After 24 hours of incubation, there are enough cells to detect visually as a colony. When you count the colonies, you are actually counting a mass of the originally plated cells growing together (in microbiology called colony forming units or CFU).

8.7.2. The "-pGLO" samples do not contain the pGLO plasmid and therefore do not have the gene for, or the ability, to produce green fluorescent protein. They also do not have the gene for ampicillin resistance. If the ampicillin is present in the culture media, they cannot survive.

8.7.3. The "+ pGLO" samples contain the pGLO plasmid. The E.coli cells exposed to the plasmid and heat shocked may or may not have taken up the plasmid. These cells may or may not express the genes for GFP or amp resistance. If transformation took place the cells will grow on LB/amp/arabinose media and will fluoresce under UV light.

8.7.4. Not all cells in the sample will be transformed. To quantify the success of this experiment in transforming E.coli with the pGLO plasmid, the instructor will explain how to calculate transformation efficiency.

Figure 3



Courtesy BIO-RAD

Title: Bacterial Transformation and Upstream Processing

8.8. **Upstream Processing (Cell Culture) of *E. coli* recombinant for GFP**

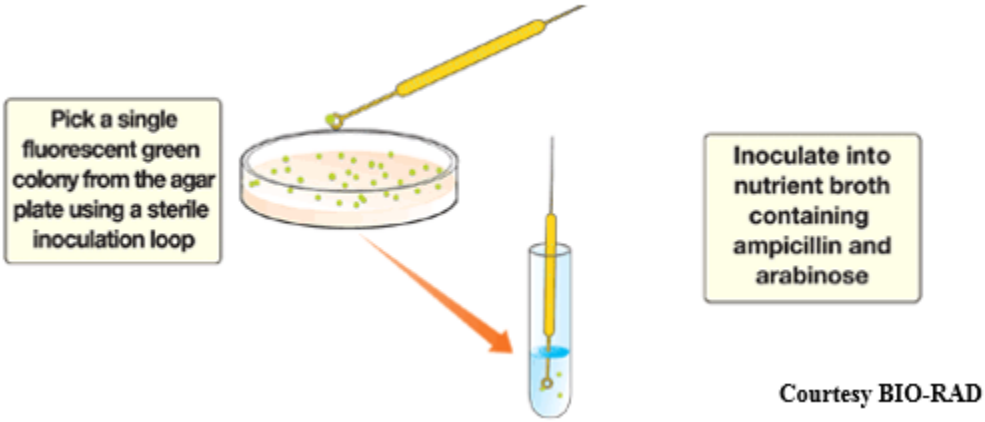
8.8.1. Nutrient cell culture broth has been prepared by the instructor by adding 60 mg arabinose and 100 µg of ampicillin to 10 ml of sterile nutrient broth.

8.8.2. Choose glowing colonies of pGLO transformed *E.coli* from the LB agar plate supplemented with L-arabinose and ampicillin (LB/amp/ara plate).

8.8.3. With a sterile loop obtain colonies of pGLO transformed *E. coli* cells and swirl them into the nutrient broth to form a homogenous mixture. See Figure 4 below.

8.8.4. Incubate cells for at least 24 hours at 37°C.

Figure 4



9. History

Name	Date	Amendment
Katherine Gorzyca	01Jun07	Initial release

Manufacturing Technician (Upstream)

REF	Key Functions & Tasks (Upstream Manufacturing Technician)
1	**Work in compliance with EH&S.**
1.a	Wear appropriate personal protective equipment.
1.b	Work in controlled environments.
1.c	Participate in emergency drills and emergency response teams.
1.d	Identify unsafe conditions and take corrective action.
1.e	Appropriately and safely access production equipment.
1.f	Handle, label, and dispose of hazardous / biohazard materials.
1.g	Access and utilize MSDS.
1.h	Perform permitting procedures.
1.i	Carries out operations with attention to OSHA and EPA regulations, and other applicable state and federal regulations.
1.j	Keeps work areas clean and safety equipment in order.
1.k	Participate in all company safety training and audits as required.
1.l	Assists with waste treatment operations.
2	**Work in compliance with cGMPs.**
2.a	Assists in environmental monitoring activities.
2.b	Follow SOPs for all operations.
2.c	Records process data and completes batch records as required.
2.d	Maintain equipment logbooks.
2.e	Control and receipt of raw materials.
2.f	Maintain training documentation.
2.g	Maintain equipment and processes in a validated state.
2.h	Working in controlled/classified areas (gowning, aseptic technique).
2.i	Ensure appropriate flow of personnel, equipment, and materials.
2.j	Change control for process, equipment, and documentation.
2.k	Label and apply status to equipment and materials.
2.l	Identify and report exception events and CAPA.
3	**Clean and maintain production areas.**
3.a	Housekeeping / pest control.
3.b	Sanitize and clean of controlled spaces.
3.c	Preparation of cleaning materials and solutions.
3.d	Assist in environmental monitoring for routine and changeover operations.
3.e	Document cleaning.
4	**Maintain effective communication.**
4.a	Deliver shift change update.
4.b	Communicate with coworkers and/or customers to ensure production or service meets requirements.
4.c	Suggest continuous improvements.
4.d	Coordinate with work teams / internal customers.
4.e	Maintain security and confidentiality.
4.f	Respond appropriately to internal auditors and external inspectors.
4.g	Assist in writing, reviewing, and commenting on technical documents.

REF	Key Functions & Tasks (Upstream Manufacturing Technician)
5	**Prepare process materials.**
5.a	Weigh, dispense, and label raw materials for use in production.
5.b	Dispense consumables and intermediates.
5.c	Control and reconcile inventory with enterprise control system (MRP, SAP, manual database).
5.d	Prepare and sterilize buffers and solutions.
5.e	Sample and test buffers and solutions.
5.f	Transfer buffers and solutions to use point.
5.g	Prepare filters for use.
5.h	Prepare, pasteurize / sterilize, and titrate media and feed solutions.
5.i	Manage chromatography resins.
6	**Prepare equipment.**
6.a	Clean CIP vessels, transfer lines, and filter trains .
6.b	Clean COP equipment (or sonicator).
6.c	Depyrogenate components and equipment.
6.d	Sterilize SIP vessels, transfer lines, and sampling ports.
6.e	Perform pressure test.
6.f	Prepare and assemble components and equipment.
6.g	Autoclave components and equipment.
6.h	Perform WFI flush of transfer lines.
6.i	Perform scheduled sanitizations of hoods.
6.j	Prepare and standardize probes and ancillary instruments.
6.k	Prepare, assembly, and integrity test filters.
6.l	Visually inspect equipment.
6.m	Maintain equipment logs and status tags.
6.n	Complete, review and approve equipment process records.
7	**Perform basic manufacturing operations.**
7.a	Perform processes following batch records, validation protocols, and/or SOPs.
7.b	Maintains and controls processes in an automated control environment.
7.c	Record process data.
7.d	Inspect materials at all stages of process to determine quality or condition.
7.e	Participate in the installation, modification, and upgrade of equipment.
7.f	Operate, monitor, and maintain equipment, tools, and workstation.
7.g	Recognize and respond appropriately to atypical events.
7.h	Participate in troubleshooting and root cause analysis of operations.
8	**Perform upstream manufacturing operations.**
8.a	Work in an aseptic environment (laminar flow hood / biosafety cabinet / cleanrooms).
8.b	Perform vial thaw from a working cell bank.
8.c	Perform cell culture expansion.
8.d	Monitor cell concentration by cell counting or measuring OD.
8.e	Inoculate seed reactor.
8.f	Transfer of seed culture to production reactors.
8.g	Monitor and control growth of cells in batch, fed-batch, and perfusion reactors.
8.h	Perform aseptic additions of media, solutions, and/or gases to reactors.
8.i	Perform CIP/SIP of bioreactors.
9	**Perform Sampling.**
9.a	Prepare sample port for aseptic sampling.
9.b	Obtain in-process samples according to batch records or sampling plans.
9.c	Label samples appropriately.
9.d	Record sample collection and distribution (storage and chain of custody).
9.e	Perform in-process chemical and/or microbiological tests.

DAY THREE

Downstream Processing

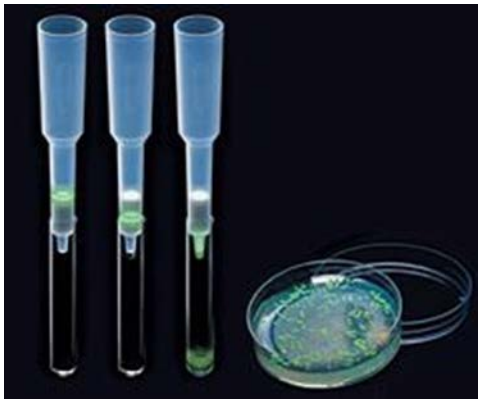
Protein is CA$H!

Title: Column Chromatography of Green Fluorescent Protein

Approvals:
Preparer: Mary Jane Kurtz Date: 24Apr14
Reviewer: Sonia Wallman Date: 24Apr14

1. Purpose:

1.1 The purpose of this SOP is to purify green fluorescent protein (GFP) from lysed *E. coli* cells using column chromatography.



Courtesy Bio-Rad

2. Scope:

2.1. This SOP will be executed whenever the purification of GFP is needed.
2.2. This SOP will be executed after the execution of the pGLO Bacterial Transformation and Upstream Processing SOP.

3. Responsibility:

3.1. It is the responsibility of the teacher/instructor/ lab assistant to ensure that these written instructions are performed as described.
3.2. It is the responsibility of the student to read and follow this SOP as described and to inform the instructor of any problems that he/she may encounter in doing so.

4. References:

4.1. Instructions from Biotechnology Explorer Green Fluorescent Protein (GFP) Purification Kit Instruction Manual (Bio-Rad catalog number 166-0005EDU
4.2. GFP Chromatography Kit. (Bio-Rad catalog number 166-0005EDU)

Title: Column Chromatography of Green Fluorescent Protein

5. Definitions:

5.1. **Hydrophobic Interaction Chromatography**: A process for separating non- charged molecules, called hydrophobic, from charged molecules, called ionic, by binding to non-charged media. This results in separating these two types of molecules by retaining one molecule while the other flows through the column.

5.2. **Elution:** The process of gently releasing molecules that are attached to the column media and moving them out of the column.

5.4 **Eluate:** The name given to the liquid which is travelling through the column during the elution of the column.

5.5 **Column Void Volume:** The open space around the solid media present in the prepared, poured column.

5.6 **Supernatant:** also called the lysate; upper layer of the cell fraction that is present after cell lysis and centrifugation. The lower portion, consisting of cellular particulate matter, forms a pellet.

6. Hazard Communication

6.1. Use Personal Protective Equipment (lab coat, gloves)

6.2. Wear eye protection

6.3. For disposal of biohazard waste use an autoclavable bag and follow proper sterilization techniques.

7. Materials

7.1. **General**

7.1.1. Lab coat, Safety glasses or goggles
7.1.2. Microfuge tubes
7.1.3. Pipettes
7.1.4. Microtube rack
7.1.5. Marker

7.2. **Part I – Crude Isolation of GFP from Lysed Cells**

7.2.1. E. coli GFP cell culture from Bacterial Transformation and Upstream Processing SOP
7.2.2. Incubator to grow E. coli transformed with GFP at 35°C
7.2.3. TE Buffer
7.2.4. Lysozyme in 1ml of TE Buffer
7.2.5. UV Lamp
7.2.6. Freezer (for -20°C storage)
7.2.7. Microfuge centrifuge
7.2.8. Vortex

Title: Column Chromatography of Green Fluorescent Protein

7.3. **Part II- Hydrophobic Interaction Chromatography**

- 7.3.1. 250 µl of bacterial cell lysate
- 7.3.2. Pre-packed Hydrophobic Interaction Chromatography Column (1 per group)
- 7.3.3. Waste tube or beaker
- 7.3.4. HIC Buffers:
  - 7.3.4.1. Binding buffer - 4.0M ammonium sulfate in TE Buffer pH 8.0
  - 7.3.4.2. Equilibration buffer -2.0M ammonium sulfate in TE Buffer pH 8.0
  - 7.3.4.3. Wash buffer -1.3M ammonium sulfate in TE Buffer pH 8.0
  - 7.3.4.4. Elution buffer - TE buffer pH 8.0
- 7.3.5. Test tube rack
- 7.3.6. Marker
- 7.3.7. 3 test tubes (5 ml volume)
- 7.3.8. disposable pipettes (3 ml volume)
- 7.3.9. Pipetman 1000 µl with tips
- 7.3.10. UV light
- 7.3.11. Microfuge tube

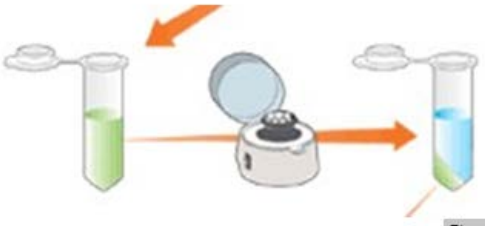
8. Procedure

8.1. **Part I - Crude Isolation of GFP from Lysed Cells**

- 8.1.1. Take 2 ml of pGLO transformed *E.coli* culture from the 20 ml culture tube and centrifuge in a microcentrifuge at 2,000 RPM for 20 minutes. The cell pellet should fluoresce when viewed by a UV lamp
- 8.1.2. While the culture is being centrifuged, rehydrate the lysozyme with 1ml of TE buffer. Rehydrated lyzozyme will be shared with other students.
- 8.1.3. Remove the cell culture from the centrifuge and pour off the E. coli supernatant into a 10% bleach solution. Keep the pellet, which is fluorescing, for the next step.
- 8.1.4. Add 750 µl of TE buffer to the pellet and resuspend it by pipetting the suspension up and down to make a lysate. Vortex gently if necessary.
- 8.1.5. Add 50 µL of the reconstituted lysozyme solution to the suspension. Mix the cell suspension gently and freeze at -20°C for at least 24 hours.
- 8.1.6. Remove the microfuge tube containing frozen cell lysate and thaw to hand warmth temperature.
- 8.1.7. Centrifuge for 10 minutes at maximum speed in the microcentrifuge
- 8.1.8. Observe the bacterial cell supernatant (lysate) with the UV light. It should fluoresce.
- 8.1.9. Remove the cell supernatant and place into a clean microfuge tube. This supernatant will be used for column chromatography and SDS- PAGE.

Title: Column Chromatography of Green Fluorescent Protein

Part I - Illustrated Overview



Courtesy Bio-Rad

8.2. **Part II- Hydrophobic Interaction Chromatography**

8.2.1. Remove caps from the top and bottom of the HIC column and then allow the fluid within the column to drain.

8.2.2. Add 2 ml of Equilibration buffer to the top of the column. Allow the column to drain into the waste beaker. Cap the bottom of the column.

8.2.3. Transfer 250µl of cell supernatant to a 1.0 ml microfuge tube and add 250 µl of binding buffer to it.

8.2.4. Label three 5 ml test tubes in sequence from 1-3.

8.2.5. Remove the cap from the bottom of the HIC column and place it in test tube number 1. Then add 300 µl of the cell lysate supernatant + binding buffer to the top of the HIC column. Allow the solution to drain completely into test tube 1.

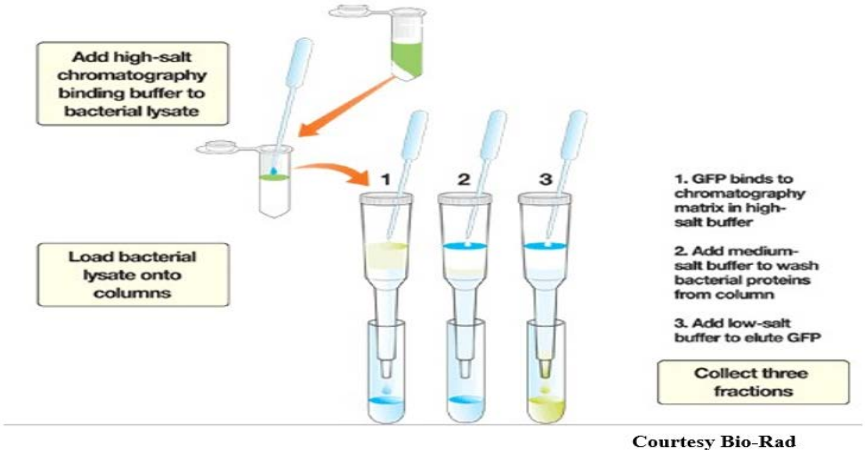
8.2.6. Remove the column from test tube 1 and place in test tube 2. Add 250µl of wash buffer. Allow the buffer to drain from the top of the column into test tube 2.

8.2.7. Remove the column from test tube 2 and place in test tube number 3. Add 1ml of Elution buffer to the column. Allow the buffer to drain completely into test tube 3. This tube should have pure GFP in it. It can be seen fluorescing using a long range UV lamp.

8.2.8. Note the GFP positive test tube(s) which glow with a UV lamp and make note. Save at least 50 µL of samples from test tubes 1-3 in a microfuge tubes for future analysis by SDS-PAGE.

Title: Column Chromatography of Green Fluorescent Protein

Part II- Illustrated Overview



Courtesy Bio-Rad

9. History:

Name	Date	Amendment
Mary Jane Kurtz	24April2014	Initial release

Manufacturing Technician (Downstream)

REF	Key Functions & Tasks (Downstream Manufacturing Technician)
1	**Work in compliance with EH&S.**
1.a	Wear appropriate personal protective equipment.
1.b	Work in controlled environments.
1.c	Participate in emergency drills and emergency response teams.
1.d	Identify unsafe conditions and take corrective action.
1.e	Appropriately and safely access production equipment.
1.f	Handle, label, and dispose of hazardous / biohazard materials.
1.g	Access and utilize MSDS.
1.h	Perform permitting procedures.
1.i	Carries out operations with attention to OSHA and EPA regulations, and other applicable state and federal regulations.
1.j	Keeps work areas clean and safety equipment in order.
1.k	Participate in all company safety training and audits as required.
1.l	Assists with waste treatment operations.
2	**Work in compliance with cGMPs.**
2.a	Assists in environmental monitoring activities.
2.b	Follow SOPs for all operations.
2.c	Records process data and completes batch records as required.
2.d	Maintain equipment logbooks.
2.e	Control and receipt of raw materials.
2.f	Maintain training documentation.
2.g	Maintain equipment and processes in a validated state.
2.h	Working in controlled/classified areas (gowning, aseptic technique).
2.i	Ensure appropriate flow of personnel, equipment, and materials.
2.j	Change control for process, equipment, and documentation.
2.k	Label and apply status to equipment and materials.
2.l	Identify and report exception events and CAPA.
3	**Clean and maintain production areas.**
3.a	Housekeeping / pest control.
3.b	Sanitize and clean of controlled spaces.
3.c	Preparation of cleaning materials and solutions.
3.d	Assist in environmental monitoring for routine and changeover operations.
3.e	Document cleaning.
4	**Maintain effective communication.**
4.a	Deliver shift change update.
4.b	Communicate with coworkers and/or customers to ensure production or service meets requirements.
4.c	Suggest continuous improvements.
4.d	Coordinate with work teams / internal customers.
4.e	Maintain security and confidentiality.
4.f	Respond appropriately to internal auditors and external inspectors.
4.g	Assist in writing, reviewing, and commenting on technical documents.

REF	Key Functions & Tasks (Downstream Manufacturing Technician)
5	**Prepare process materials.**
5.a	Weigh, dispense, and label raw materials for use in production.
5.b	Dispense consumables and intermediates.
5.c	Control and reconcile inventory with enterprise control system (MRP, SAP, manual database).
5.d	Prepare and sterilize buffers and solutions.
5.e	Sample and test buffers and solutions.
5.f	Transfer buffers and solutions to use point.
5.g	Prepare filters for use.
5.h	Prepare, pasteurize / sterilize, and titrate media and feed solutions.
5.i	Manage chromatography resins.
6	**Prepare equipment.**
6.a	Clean CIP vessels, transfer lines, and filter trains .
6.b	Clean COP equipment (or sonicator).
6.c	Depyrogenate components and equipment.
6.d	Sterilize SIP vessels, transfer lines, and sampling ports.
6.e	Perform pressure test.
6.f	Prepare and assemble components and equipment.
6.g	Autoclave components and equipment.
6.h	Perform WFI flush of transfer lines.
6.i	Perform scheduled sanitizations of hoods.
6.j	Prepare and standardize probes and ancillary instruments.
6.k	Prepare, assembly, and integrity test filters.
6.l	Visually inspect equipment.
6.m	Maintain equipment logs and status tags.
6.n	Complete, review and approve equipment process records.
7	**Perform basic manufacturing operations.**
7.a	Perform processes following batch records, validation protocols, and/or SOPs.
7.b	Maintains and controls processes in an automated control environment.
7.c	Record process data.
7.d	Inspect materials at all stages of process to determine quality or condition.
7.e	Participate in the installation, modification, and upgrade of equipment.
7.f	Operate, monitor, and maintain equipment, tools, and workstation.
7.g	Recognize and respond appropriately to atypical events.
7.h	Participate in troubleshooting and root cause analysis of operations.
8	**Perform downstream manufacturing operations.**
8.a	Receive material from upstream processing.
8.b	Separate cells from media using centrifugation or filtration (TFF or depth filtration).
8.c	Perform cell disruption techniques (mechanical or chemical).
8.d	Perform aqueous separations (liquid extraction, precipitation).
8.e	Perform chemical/enzymatic modifications to product.
8.f	Perform normal flow and tangential flow filtration (including microfiltration, ultrafiltration, or diafiltration).
8.g	Prepare chromatography columns (assembly, sanitization, resin packing, evaluation).
8.h	Perform chromatography steps (equilibrate, load, wash, elute, clean, store).
8.l	Perform viral clearance (removal/inactivation) steps.
8.j	Bulk fill purified product.
9	**Perform Sampling.**
9.a	Prepare sample port for aseptic sampling.
9.b	Obtain in-process samples according to batch records or sampling plans.
9.c	Label samples appropriately.
9.d	Record sample collection and distribution (storage and chain of custody)
9.e	Perform in-process chemical and/or microbiological tests.

DAY FOUR

Quality Control Biochemistry

Title: SDS-PAGE Electrophoresis of GFP

Approvals:
Preparer: Mary Jane Kurtz Date: 24Apr14
Reviewer: Sonia Wallman Date: 28Apr14

1. **Purpose:**
   To identify and characterize Green Fluorescent Protein by SDS-PAGE.

2. **Scope:**
   2.1. Applies to confirming the presence and purity of the green fluorescent protein (GFP) previously produced and purified.

3. **Responsibilities:**
   3.1. It is the responsibility of the course instructor/lab assistant to ensure that this SOP is performed as described and to update the procedure when necessary.
   3.2. It is the responsibility of the students/technicians to follow the SOP as described and to inform the instructor about any deviations or problems that may occur while performing the procedure.

4. **References:**
   4.1. Biotechnology Explorer Protein Electrophoresis of GFP pGLO Bacterial Transformation Kit Extension Application Note (BIO-RAD # 166-0013EDU)
   4.2. Gel box instruction instructions
   4.3. Gel Documentation Instrument SOP

5. **Definitions:** N/A

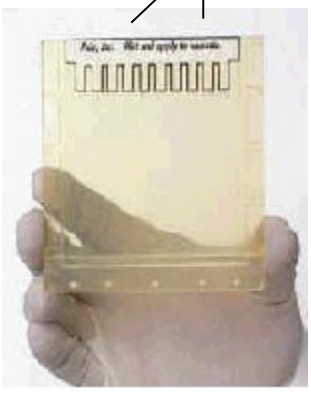
6. **Precautions:**
   6.1. Electrophoresis includes the use of high voltage of direct current. Use caution when operating the apparatus. Apparatus must be turned off and unplugged before the apparatus is disassembled.
   6.2. Acrylamide is a neurotoxin. Always wear protective gloves when handling the polyacrylamide gels.
   6.3. Fixative solution is acidic and flammable – keep away from sparks and flames. Dispose in Fixative Hazardous Waste bottle
   6.4. Coomassie Brilliant Blue stain is harmful. Handle carefully and dispose in hazardous waste bottle.

Title: SDS-PAGE Electrophoresis of GFP

7. Materials:

7.1 Vertical electrophoresis box (see example below)
7.2 Power Supply, 0-300 Volts
7.3 Pre-poured Polyacrylamide Gels prepared with 4-15% polyacrylamide in Tris-HCl, 10cm x 10cm
7.4 Pipette 20µl, 200µl
7.5 Pipette tips
7.6 Tris-Glycine Running Buffer, 0.05 M pH 8.8
7.7 2X Laemmli Sample Buffer with glycerol, bromophenol blue tracking dye and βME or DTT
7.8 Boiling water bath and microfuge holders
7.9 Kaleidoscope MW Protein standards
7.10 Staining trays
7.11 Coomassie Brilliant Blue Stain
7.12 Microfuge tubes
7.13 Markers
7.14 Kimwipes
7.15 Bio-Rad P6 Desalting Spin Columns (Optional)

Sample Wells



Assorted PAGE Gel Boxes

Title: SDS-PAGE Electrophoresis of GFP

8. Procedure:

8.1 Gather the samples and GFP positive fractions to test including:
- 8.1.1 Bacterial lysate supernatant
- 8.1.2 HIC column microfuge tube fractions 1,2,3
- 8.1.3 Kaleidoscope MW protein ladder, 10 µl
- 8.1.4 GFP standard diluted 1:5 in 1X PBS (2µL standard/10 µL), then suspended in equal volume of 2X Laemmli buffer

8.2 (Optional) Desalt HIC fractions using the P6 Spin Columns:
- 8.2.1 After re-suspending the gel in P6, snap off the tip of the column and place it in a 2.0 ml microfuge tube. Allow buffer to drain from column into tube.
- 8.2.2 Spin P6 Columns for 2 min in microcentrifuge to remove buffer and discard the buffer.
- 8.2.3 Place the column(s) into a clean 1.5 ml centrifuge tube(s), pipette 50µl sample from HIC microfuge tubes 1, 2, and 3 into separate spin columns
- 8.2.4 Spin columns 1 min and collect desalted HIC samples

8.3 Sample Preparation

8.3.1. Place 20 µl of samples from the following sources in labeled microfuge tubes
- Bacterial lysate supernatant (pre-column)
- HIC fraction 3 (post column); Fraction 2 could also be run as a sample

8.3.2 Place 20 µl of 2X Laemmli buffer into each of the microfuge tubes containing 20 µl samples

8.3.3 Place 15µl of diluted GFP standard into a microfuge tube, add 15 µl of 2X Laemmli sample buffer and mix

8.3.4 Heat all column samples at ~100°C (in a heated water bath or heating block) for 3 -5 minutes.

8.3.5 Place 10µL Kaleidoscope protein standard in a labeled microfuge tube. **Note: Do not add Laemmli buffer to or heat the Kaleidoscope protein standard**

Title: SDS-PAGE Electrophoresis of GFP

8.4 Assemble SDS-PAGE gel box according to manufacturer's instructions

8.4.1 After assembling the gel box, take the gel out of the package and **Make sure to remove green tape at the bottom of the gel!**

8.4.2 Place gel into the gel box per instructions

8.4.3 Rinse out each well with running buffer

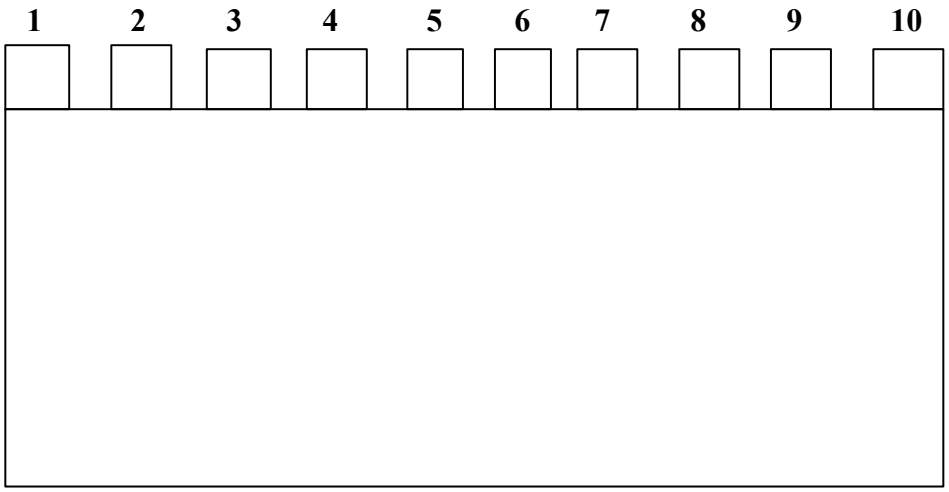
8.4.4 Place 20µl of each sample into assigned well of the gel as shown below in the diagram**. If possible, use the first two or three lanes to practice loading by loading 20 µL of sample buffer only to the wells.**

8.4.5 Add running buffer to the gel box according to manufacturer's instructions.

Recommended Lane Assignments

1	Practice lane – sample buffer	(20 µl for samples 2-9)
2	Practice lane- sample buffer	
3	Practice lane- sample buffer	
4	Bacterial Lysate Supernatant – Pre Column	
5	Chromatography Fraction 3 -Post Column	
6	Chromatography Sample	
7	Chromatography Sample	
8	Chromatography Sample	
9	GFP Standard	
10		
11	**Kaleidoscope MW Protein Std**	**(10 µl)**

Title: SDS-PAGE Electrophoresis of GFP



8.5 **Running SDS-PAGE Gel**

8.5.1 Wait for the instructor to assist you in turning on the Protean Tetra PAGE equipment. **DANGER: Care must be taken with high voltage.**

8.5.2 Run gels at 200 volts for 30 minutes. Do not allow running dye to go off the bottom of the gel. Turn off electricity and unplug electrophoresis equipment before opening to remove gels.

8.5.3 Remove gel from SDS-PAGE gel box and place in staining tray Wash with distilled water 2x for 1-2 minutes, then pour off all water.

Title: Title: SDS-PAGE Electrophoresis of GFP

8.6 **Visualizing the protein bands on SDS PAGE gel after Electrophoresis –** Staining and De-staining Gels

8.6.1 Pour Coomassie Brilliant Blue Stain over gel until it is completely covered with dye and stain them for 30 minutes while gently shaking

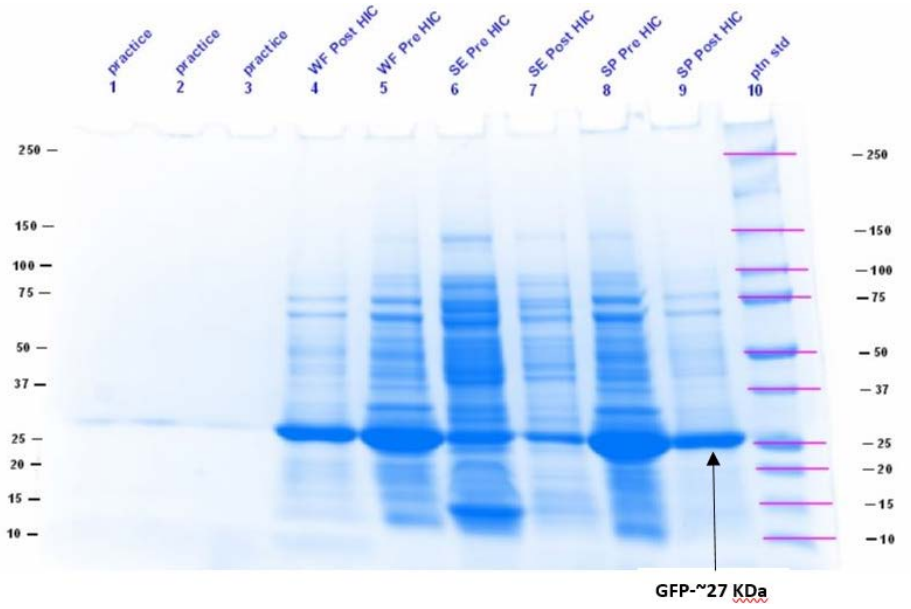
8.6.2 Pour off stain and wash 2x in DI water then Pour off DI water.

8.6.3 Destain with Coomassie destain solution changing the solution 2-3 times. Place pieces of twisted kimwipes in destain solution with gel. Watch for the bands to appear, which usually occurs within 1 hour.

8.6.4 Photo-document gel or draw the bands on paper as shown on gel

8.6.5 See Figure 1 below for image of SDS-PAGE gel of pre and post column fractions from HIC column

Figure 1- SDS-PAGE of GFP HIC pre and post column fractions



Title: SDS-PAGE Electrophoresis of GFP

8.7 Results and Analysis of Protein Bands Electrophoresed by SDS PAGE- Determining the molecular weight of unknown proteins

8.7.1 Running known proteins of known molecular weight which can be visualized on PAGE gels allows one to calculate the relative migration of the protein compared to the running dye front called the R_f value.

8.7.2 Figure 2 shows the standard stained proteins and their molecular weights as they appear on a 4-20% Tris-Glycine PAGE post electrophoresis and staining.

8.7.3 Rf value for each protein band is found by measuring how far the selected band moved compared to how far the running dye front moved from the beginning gel well.

Definition $R_f = \frac{\text{Distance from starting well to one protein band (cm)}}{\text{Distance from starting well to running dye front (cm)}}$

8.7.4 Table 1 contains the molecular weights in kiloDaltons and R_f values of the standard proteins used in this SDS PAGE activity

Figure 2
Standard Protein Bands on SDS PAGE

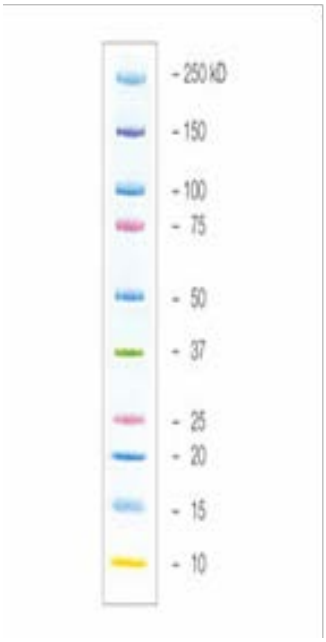


Table 1

Band Color	Molecular weight kD	R_f of band = Band to dye front in cm / gel top to dye front in cm
	y axis	x axis
blue	250	0.5/7.5 = 0.07
purple	150	1.2/7.5 = 0.16
blue	100	2.0/7.5 = 0.27
red	75	2.5/7.5 = 0.33
blue	50	3.4/7.5 = 0.45
green	37	4.2/7.5 = 0.56
red	25	5.1/7.5 = 0.68
blue	20	5.6/7.5 = 0.75
Lt blue	15	6.3/7.5 = 0.84
yellow	10	7.1/7.5 = 0.95

Title: SDS-PAGE Electrophoresis of GFP

8.8 Generate a protein standards graph using the semi-log graph shown below using the information in Table I.

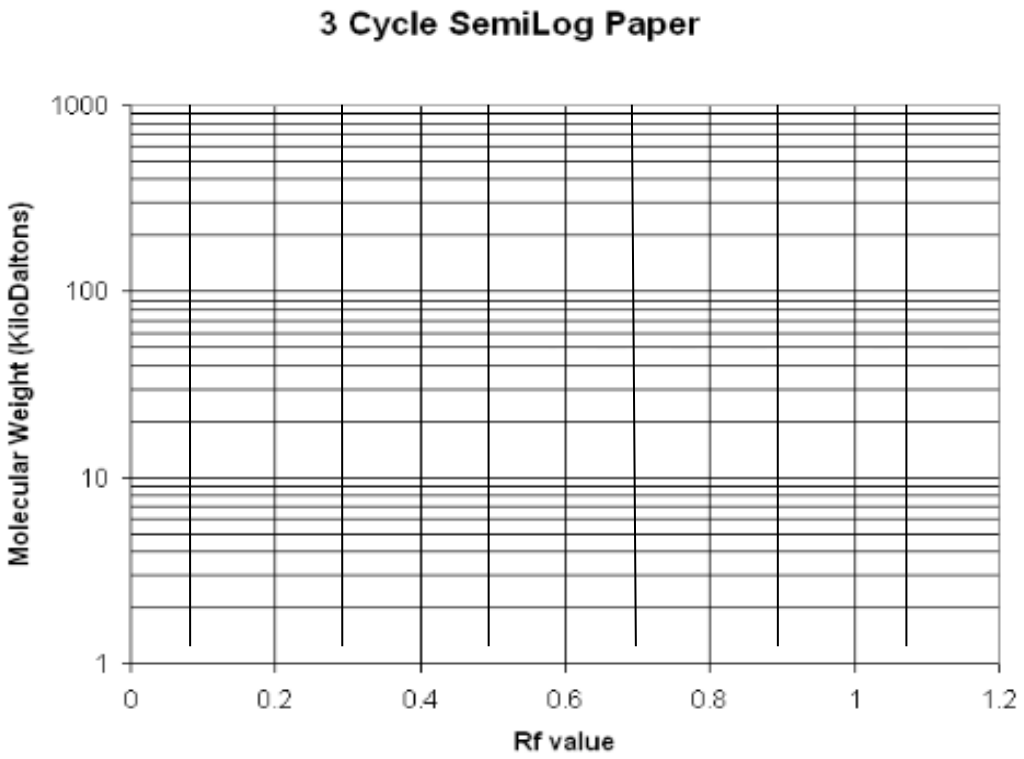
8.8.1 The protein standard has two values, a molecular weight in kilo Daltons found on the y axis and the Rf, found on the x axis. Find the point on a semi-log graph for the first standard protein, x and y determining one position on the graph.

8.8.2 Repeat the same process as in 8.8.1 for each known protein until all the proteins are plotted on the semi-log graph.

8.8.3 Draw a best fit straight line with a ruler, using the points on the graph.

Title: SDS-PAGE Electrophoresis of GFP

FIGURE 2 - Standard Curve of SDS PAGE proteins



8.9 Finding the molecular weight of an unknown protein using the graph of standard proteins created in 8.8.3.

8.9.1 Calculate the R_f of the unknown protein by determining the band distance migrated in cm of the unknown divided by the distance of the running dye in cm as shown in Figure 1.

8.9.2 Once the R_f is calculated for the unknown protein refer to the standard graph plotted as molecular weight vs R_f.

8.9.3 Find the R_f value on the x axis and draw a line vertically that will intersect with the line on the graph, the point that it crosses the line given for standard proteins.

8.9.4 Draw a line horizontally from this x axis to the line on the graph. the y axis for the point intersected on the y axis is its molecular weight.

Title: SDS-PAGE Electrophoresis of GFP

8.10 MW Determination of SDS PAGE pGLO transformed *E. Coli* lysate, and post column purification fractions

8.10.1 Determine which lane(s) on this PAGE gel shown below has a protein band that equals the Rf of GFP, (GFP, molecular weight 27.5 kD).

9. History

Name	Date	Amendment
Mary Jane Kurtz	28April2014	Initial release
C. Cooper	09 August 2016	Format changes; Figure 3- added new image of SDS-PAGE gel

Quality Control Technician (Chemistry)

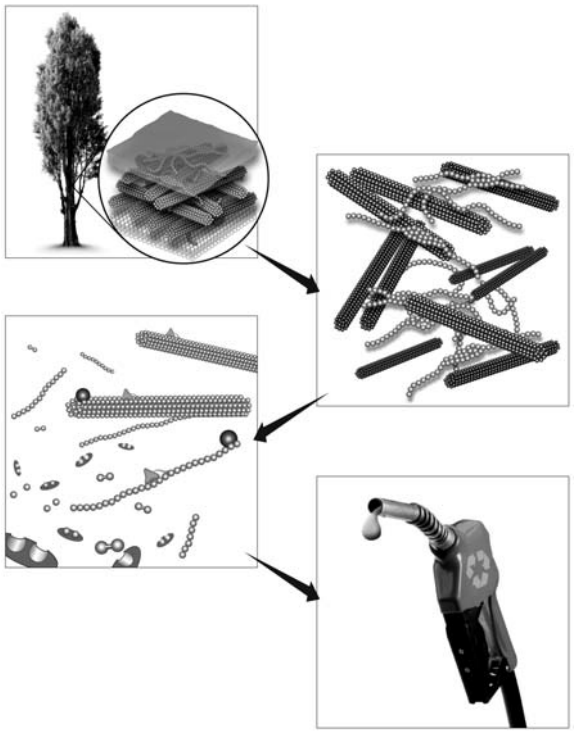
REF	Key Functions & Tasks (Quality Control Chemistry Technician)
1	**Work in compliance with EH&S.**
1.a	Wear appropriate personal protective equipment.
1.b	Work in controlled environments.
1.c	Participate in emergency drills and emergency response teams.
1.d	Use routine lab safety procedures, identify unsafe conditions, and take corrective action.
1.e	Appropriately and safely access production and laboratory equipment.
1.f	Handle, label, and dispose of hazardous / biohazard materials.
1.g	Access and utilize MSDS.
1.h	Perform permitting procedures.
1.i	Carries out operations with attention to OSHA and EPA regulations, and other applicable state and federal regulations.
1.j	Participate in all company safety training and audits as required.
1.k	Assists with waste treatment operations.
1.l	Dispose of wastes.
1.m	Follows SOPs, written test procedures, safety, regulatory requirements, and approved license requirements.
1.n	Use hoods where appropriate.
1.o	Dispose of sharps properly.
2	**Work in compliance with cGMPs.**
2.a	Follow SOPs for all operations.
2.b	Records laboratory data and completes lab records as required.
2.c	Maintain equipment and instrument logbooks.
2.d	Control and receipt of raw materials.
2.e	Maintain training documentation.
2.f	Maintain equipment and methods in a validated state.
2.g	Working in controlled/classified areas (gowning, aseptic technique).
2.h	Ensure appropriate flow of personnel, equipment, and materials.
2.i	Change control for process, equipment, and documentation.
2.j	Label and apply status to equipment and materials.
2.k	Identify and report exception events and CAPA.
2.l	Review data.
2.m	Participate in change control activities.
2.n	Archive documentation/data.
3	**Clean and maintain production areas.**
3.a	Housekeeping / pest control.
3.b	Sanitize and clean of controlled spaces.
3.c	Preparation of cleaning materials and solutions
3.d	Document cleaning.
4	**Maintain effective communication.**
4.a	Maintain security and confidentiality.
4.b	Respond appropriately to internal auditors and external inspectors.
4.c	Assist in writing, reviewing, and commenting on technical documents.
4.d	Consult appropriately with internal customers.
4.e	Report result to appropriate departments.
4.f	Interact with vendors.

REF	Key Functions & Tasks (Quality Control Chemistry Technician)
5	**Receive and/or collect samples.**
5.a	Receive/collect samples (stability, in-process, water, raw materials, final product, environment, validation) per batch records or plans.
5.b	Monitor controlled equipment.
5.c	Maintain equipment logs.
5.d	Label samples appropriately.
5.e	Record sample collection and distribution (storage and chain of custody).
6	**Laboratory Work.**
6.a	Prepare reagents.
6.b	Prepare samples.
6.c	Review testing procedures
6.d	Execute testing.
6.e	Perform mathematical and statistical calculations as appropriate.
6.f	Evaluate data with regard to specification.
6.g	Perform scheduled sanitizations of hoods.
6.h	Prepare and standardize probes and ancillary instruments.
6.i	Visually inspect equipment.
6.j	Maintain equipment logs and status tags.
6.k	Complete, review and approve equipment process records.
7	**Perform Chemical and biochemical testing.**
7.a	Perform chemical and biochemical testing for identity, potency, purity, consistency, and stability.
7.b	Perform chemical and biochemical testing for raw material, water, and other environmental and validation samples.
8	**Managing Information**
8.a	Support data trending activities.
8.b	Utilize Laboratory Information Management Systems (LIMS)
8.c	Records observations, generates reports, and maintains accurate records
9	**Provide Technical and/or Validation Support**
9.a	Troubleshoots basic technical issues and investigations of OOS results, instrument malfunctions, and methodology problems.
9.b	Assist in executing validation procedures
10	**Maintain laboratory systems and equipment**
10.a	Perform preventive maintenance (PM).
10.b	Standardize laboratory instrumentation.
10.c	Identify and assist in troubleshooting instrument and equipment failures.

DAY FIVE

Biofuels: A Crossover Industry

Protein is CA$H!



Biotechnology Explorer™

Biofuel Enzyme Kit

Catalog Number 166-5035EDU

explorer.bio-rad.com

Note: Kit contains temperature-sensitive reagents. Open immediately upon arrival and store components at 4°C as indicated.

Please visit explorer.bio-rad.com to access our selection of language translations for Biotechnology Explorer kit curricula.

BIO-RAD

Background for Instructors

The Biofuel Enzyme kit measures the enzymatic activity of cellobiase and identifies the optimal conditions for the enzyme. The reaction of cellobiase breaking down cellobiose is important in the process of making cellulosic ethanol, which is an efficient, more sustainable fuel to replace petroleum. The reaction is simple to set up and produces reliable, quantifiable data. The practical applications of this enzyme give students a real world experience that will make the abstract concepts involved in enzymatic reactions relevant.

General Information about Enzymes

Enzymes speed up the rate of chemical reactions. Since they do not chemically react with the substrate, they can work again and again to help convert reactants to products. Enzymes are generally proteins (some nucleic acid-based enzymes exist) with a specific3-D structure (tertiary structure). The active site is a cleft in the protein where the chemical reaction takes place. The charges and positions of the R groups (variable side chains) of the amino acids are critical for the activity of the enzyme. The properties of the active site are important because it is where the reactant(s) binds. The reactant in an enzyme-catalyzed reaction is called the substrate. The substrate fits into the active site because the amino acids facing the active site are attracted to the chemical groups on the substrate.

An enzyme speeds up the chemical reaction by positioning the substrate in such a way that the transition state of the reaction is stabilized. The enzyme reduces the energy needed to make the reaction occur (activation energy). Once the activation energy is lowered, the chemical reaction occurs at a much faster rate.

Changes in salinity and pH can affect the charges of the side chains in the active site, which can decrease the enzyme's effectiveness dramatically by influencing its affinity for its substrate. Temperature can also affect the speed of the reaction. Heat speeds up the movement of substrate and enzyme molecules in solution, which increases the number of collisions and therefore speeds up the reaction. However, at some point, the non-covalent attractions between the amino acids of the enzyme will begin to break, changing the shape of the enzyme. The point at which an enzyme changes shape (becomes denatured) will depend on the particular properties of that enzyme. Some enzymes can still efficiently convert substrate to product at temperatures close to boiling, whereas others are denatured at room temperature. Most enzymes, however, function best at moderate temperatures (20–40°C).

Optimal laboratory conditions for enzyme activity can be predicted by determining the conditions under which it operates in nature. For instance, the enzymes produced by bacteria living in hot springs will function best at a high temperature and the enzymes produced in a person's stomach work best at a very low pH.

The relative concentration of all molecules involved in the reaction affects the reaction rate as well. The higher the concentration of an enzyme, the faster the reaction will take place until there is excess enzyme. Similarly, increasing the concentration of a substrate will speed up the reaction until the point at which all the enzyme present is saturated with the substrate. Sometimes it helps to think of an analogy of workers (enzymes) producing a product from raw materials (substrate). If you increase the number of workers, the amount of product produced will increase until there are excess workers and not enough raw materials to work on. In the same way, increasing the amount of raw materials, while keeping the number of workers constant, will increase the rate of product production until you have given the workers excess raw materials. There is a maximum rate at which the product can be produced given a particular "concentration" of workers (enzyme) and raw

materials (substrate). In biochemical terms, this is called V_{max}. More details about enzyme kinetics can be found in Appendix A.

Students will use this kit to study the reaction rate of cellobiase, an enzyme involved in breaking down cellobiose to glucose. They will also analyze how temperature, pH, enzyme concentration, and substrate concentration affect the activity of cellobiase.

Organisms That Produce Cellulases

Cellulose, the structural polysaccharide found in the cell walls of plants, is a source of sugar to organisms that produce a family of enzymes known as cellulases. Cellulases catalyze the breakdown of cellulose to glucose. Humans and other animals do not produce cellulases. Many plant eating animals are hosts to other organisms that do possess these enzymes. For instance, termites have the protozoan *Trichonympha* living inside their gut. *Trichonympha* has a bacterium called Rs-D17 living inside it that produces cellulase enzymes that break down cellulose, the main component of wood (http://www.genomeweb.com/genome-termite-gut-bacteria-sequenced; http://www.sciencelinks.jp/content/view/826/258). Ruminants, such as cows, harbor a team of anaerobic microorganisms that digest the plants they eat. *Bacteroides succinogenes* is a common bacterium in the gut of cows that produces cellulases (http://sci.waikato.ac.nz/farm/content/microbiology.html). Many types of fungal decomposers derive much of their food from the cellulosic cell walls of plants. The filamentous fungus *Aspergillus niger* produces cellulases that it exudes from its hyphae to digest cellulose in its surroundings to use as a food source.

Cellulosic Ethanol: A Practical Application for Cellulases

The biofuel industry uses cellulases to convert the cellulose in plant cell walls to sugars, such as glucose. The sugar can then be converted to ethanol by microbial fermentation. This ethanol in turn can be used alone in certain engines or in combination with gasoline to power car, truck and airplane engines. To understand the process of cellulosic ethanol production in detail, a journey into the biochemical makeup of cell walls is helpful. A plant's biomass is mostly cell wall material. Plant cell walls are made up of a variety of polysaccharides and other compounds, but the primary component is cellulose. Cellulose is made up of a very long chain of glucose molecules. Each cellulose molecule is attracted to other cellulose molecules by the hydrogen bonds that form between their respective glucose molecules. These attractions form cellulose microfibrils made up of 60–80 individual strands of cellulose.

Plant cells can be alive or dead at maturity. Living plant cells, such as photosynthesizing mesophyll cells in leaves, have primary cell walls surrounding them. Primary cell walls are made up of cellulose microfibrils embedded in a matrix of other polysaccharides and protein. These cell walls are thick and relatively stretchy to allow for elongation. Cells with only primary cell walls are fairly soft like the majority of the cells found in a leaf.

Other plant cells are dead at maturity. They strengthen the plant and/or function to conduct water through the plant. These plant cells develop a second type of cell wall called the secondary cell wall before they die. Secondary cell walls are more rigid than primary cell walls. Plant tissues with secondary cell walls have water transport tissues such as xylem, the fibrous or hard tissues covering a coconut or walnut seed, and the stringy part of a celery stalk. These cell walls have additional molecules other than cellulose that contribute to their rigidity. Hemicellulose and lignin are found in high quantities in the secondary cell walls of woody or fibrous plant tissue. For cellulosic ethanol production, lignins must be removed because they inhibit enzymatic activity of cellulases. Hemicelluloses must be cleaved from the cellulose to allow enzymatic breakdown of the cellulose.

One way that ethanol can be produced from plant matter is by completing the following three tasks (Figure 2):

1. **Pretreatment**: Removal of non-cellulose biomolecules such as lignin
2. **Enzymatic hydrolysis**: Cellulases hydrolyze cellulose to produce six carbon sugars and enzymes can be added to hydrolyze hemicellulose to five carbon sugars
3. **Microbial fermentation**: Converts sugar products into ethanol

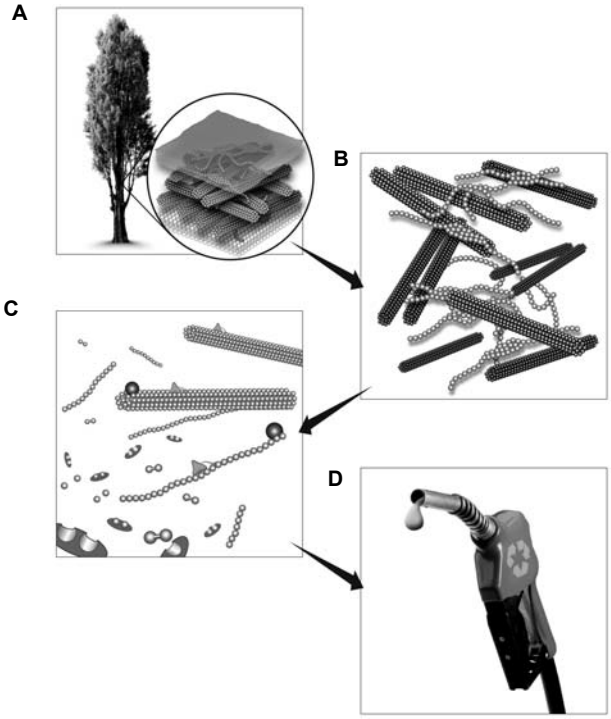
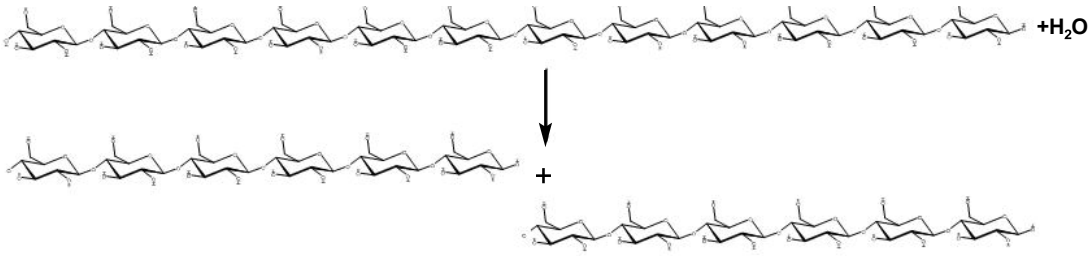


Fig. 2. The process of cellulosic ethanol production. A. Cellulose from sources such as poplar trees, switchgrasses, and corn stover is collected. **B**. The cellulose is isolated from these plant materials by heat treatment, physical crushing, acid or base treatment. Ideally, the cellulose is isolated from other compounds such as lignins that interfere with the breakdown of cellulose. **C**. The cellulose is enzymatically broken down to glucose . **D**. The glucose is fermented to ethanol and processed as fuel.

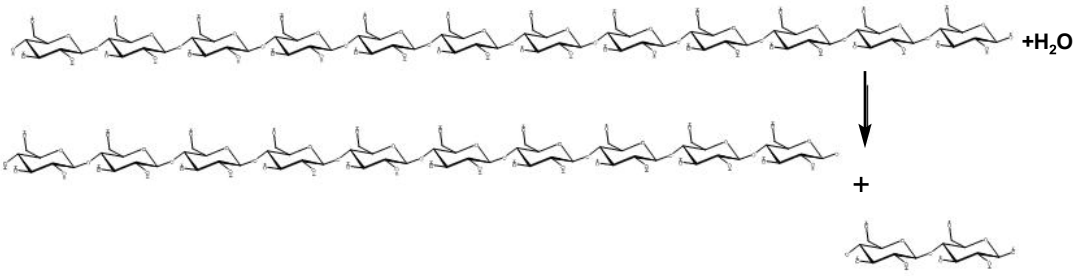
Types of Cellulases Needed to Break Down Plant Cell Walls into Glucose

The production of ethanol from plant material is a very complex procedure requiring multiple steps. Plant material is first processed mechanically, as well as with acids or enzymes and heat to remove lignin. Lignin is a highly complex, aromatic macromolecule found in high quantities in secondary cell walls of fibrous and woody plant tissue in close association with cellulose. Once the lignin is removed, the cellulose is more exposed and can be more readily broken down. Cellulose is broken down into glucose in three steps by three different types of enzymes.

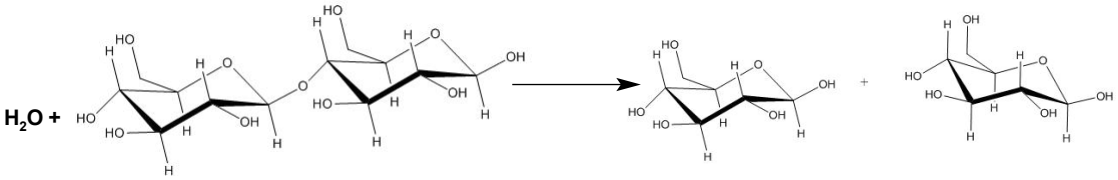
- Endocellulases — These enzymes break down the internal bonds of the long chains of glucose molecules that form cellulose.



- Exocellulases — These enzymes break the covalent linkages between the glucose units of cellulose that are on the end of the cellulose molecules, releasing cellobiose.



- Cellobiases (β-glucosidases) — These enzymes break down the cellobiose left behind as a result of the work of the first two enzymes.



Cellobiase Enzyme

Cellobiase, the enzyme provided in this kit, breaks down cellobiose, a disaccharide made up of two glucose molecules connected together by a 1,4 β–glucoside linkage (Figure 3). The breakdown of cellobiose by cellobiase is the final step in producing glucose from cellulose.

Glucose is the preferred source of sugar for microbial fermentation, an additional enzymatic reaction that produces ethanol.

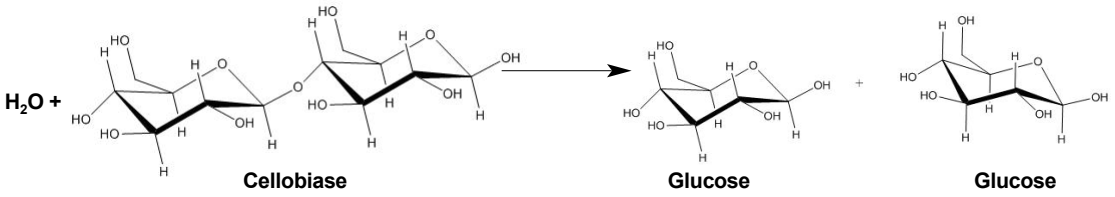


Fig. 3. Breakdown of cellobiose into two glucose molecules. Cellobiose is linked at carbon 1 and carbon 4 of two separate glucose units. The enzyme cellobiase can break this linkage resulting in two glucose molecules.

Detecting the Substrate Used in This Lab

Although cellobiose is the natural substrate of cellobiase, there is no simple method to quantitatively detect the product (glucose) or the disappearance of cellobiose. A simple colorimetric assay using an artificial substrate, *p*-nitrophenyl glucopyranoside, can be used to detect enzymatic activity of cellobiase. The substrate *p*-nitrophenyl glucopyranoside is composed of a beta glucose covalently linked to a molecule of nitrophenol (Figure 4). When the bond connecting these two molecules is cleaved with the help of cellobiase, the *p*-nitrophenol is released. To stop the activity of the enzyme and to create a colored product, the reaction mixture is added to a basic solution. When the *p*-nitrophenol is placed in a basic solution, the hydroxyl group on the nitrophenol loses an H^+ to the OH^- of the base, which changes the bonding within the phenolic ring, so that the molecule will absorb violet light (and reflect yellow light). This makes the solution yellow, which can be detected visually by comparing the deepness of the yellow color to a set of standards of known concentration of *p*-nitrophenol or by using a spectrophotometer to produce more accurate, quantitative results.

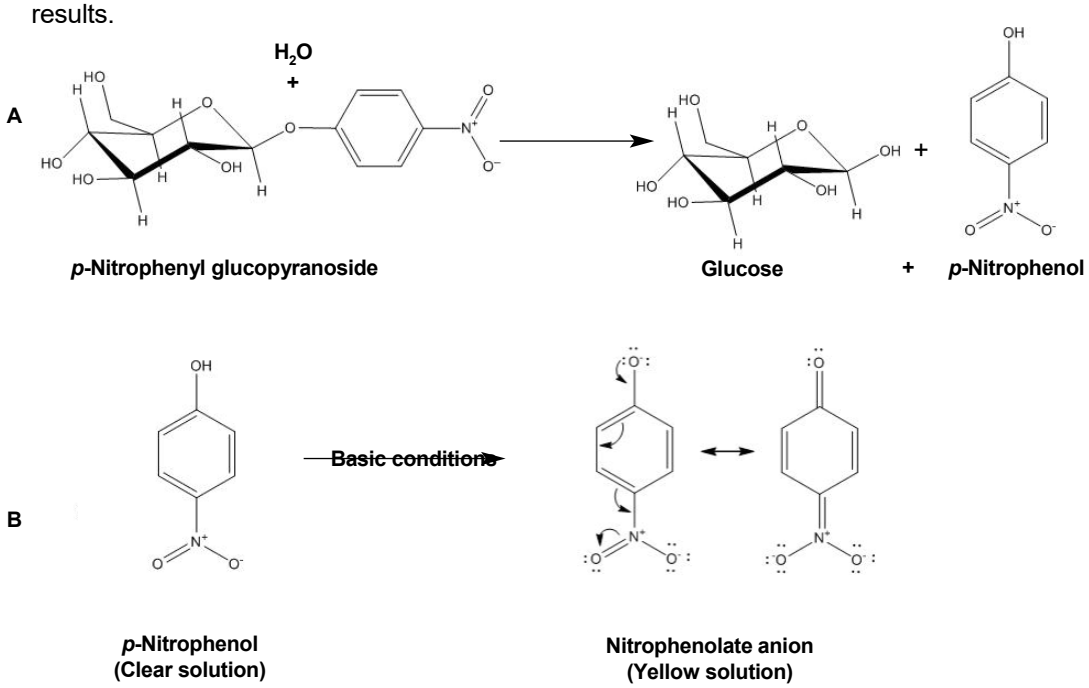


Fig. 4. Detecting glucose from the breakdown of cellobiose by cellobiase enzyme. A. An analog to cellobiose called *p*-nitrophenyl glucopyranoside is used to detect enzymatic activity and accumulation of glucose. Once cleaved by cellobiase, the *p*-nitrophenol is released, which can be detected by its yellow color in basic solutions. **B**. *p*-nitrophenol is colorless to slightly yellow at pH 5. However, under basic conditions, the hydrogen ion of the hydroxyl group (OH^- group) is removed, resulting in a negative charge due to an extra pair of electrons on the remaining oxygen group. This pair of electrons travels along the nitrophenolate anion, creating a resonance structure that produces the yellow color.

Activity 6: Test Ability of Mushroom Extracts to Increase Reaction Rate

Cellobiase that breaks down the 1,4 β-glucoside linkages in cellobiose is produced by many organisms. Fungi, such as molds, yeasts and mushrooms, produce this enzyme and can excrete it to digest cellobiose to produce glucose for energy usage. Many bacteria also contain cellulytic enzymes and cellobiase to break down plant cell walls. These bacteria can be found in the second stomach (rumen) of many hoofed animals such as cows and also in the gut of termites. Cellobiase can also be found in the seeds of bitter almonds where it is known as emulsin. Emulsin is actually thought to be a combination of cellobiase and other enzymes. In this activity, you will choose a potential source of cellobiase, extract proteins from this source, and take this extract and combine it with the substrate, *p*-nitrophenyl glucopyranoside, to determine if your extract has any enzymatic activity that allows it to break down the substrate.

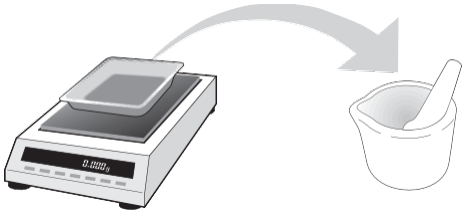
Student Workstation	**Quantity**	**(✔)**
Mushroom sample	1	❒
1.5 mM substrate	1	❒
Stop solution	1	❒
1x extraction buffer	1	❒
15 ml conical tube	1	❒
1.5 ml microcentrifuge tube	1	❒
DPTPs	4	❒
Cuvettes	6	❒
Marker	1	❒
Mortar and pestle	1	❒
Filter paper, cheese cloth, or strainer	1	❒
Beaker with deionized or distilled water to rinse DPTPs	1	❒
Stopwatch or timer	1	❒

Instructor's Workstation (Optional)	**Quantity**	**(✔)**
Spectrophotometer	1	❒

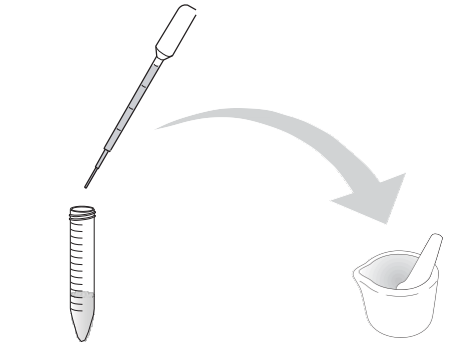
Protocol

1. Write down the name of your mushroom _____

2. Weigh out approximately 1 g of your mushroom and place it in a mortar._____g



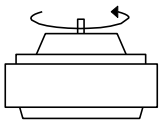
3. Add 2 ml of extraction buffer for every gram of mushroom into the mortar._____ml



Extraction buffer

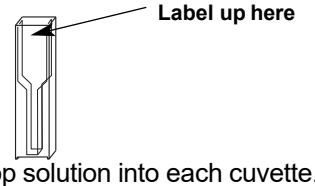
4. Using a pestle, grind your mushroom to produce a slurry.

5. Strain the solid particles out of your slurry using a piece of filter paper or cheese cloth into a 1.5 ml microcentrifuge tube. Alternatively, if you have a centrifuge, scoop the slurry into a 1.5 ml microcentrifuge tube and then pellet the solid particles by spinning at top speed for 2 minutes.

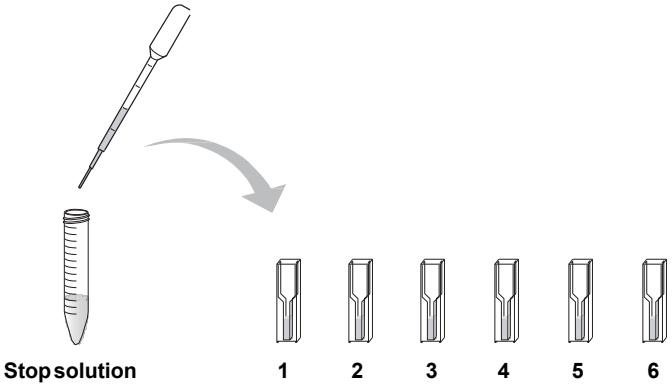


Note: You will need at least 250 µl of extract to perform the enzymatic reaction.

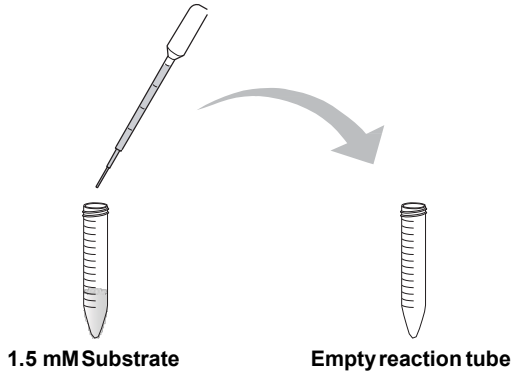
6. Label your cuvettes “1–6”. Only label on the upper part of the cuvette face.



7. Using a clean DPTP, pipet 500 µl of stop solution into each cuvette. Rinse out the DPTP thoroughly with water.

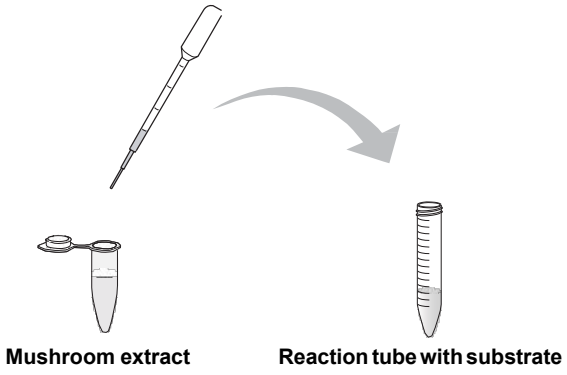


8. Label a 15 ml conical tube with the type of mushroom you are using. Using a clean DPTP, pipet 3 ml of substrate into the tube.

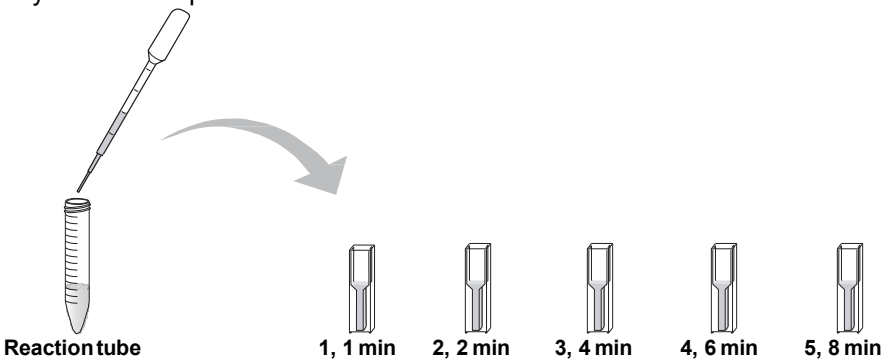


Please read and understand steps 10–11 fully before proceeding. These steps are time sensitive!

9. Using a clean DPTP, pipet 250 µl of your mushroom extract into the 15 ml conical tube containing 3 ml of substrate. **START YOUR TIMER**.

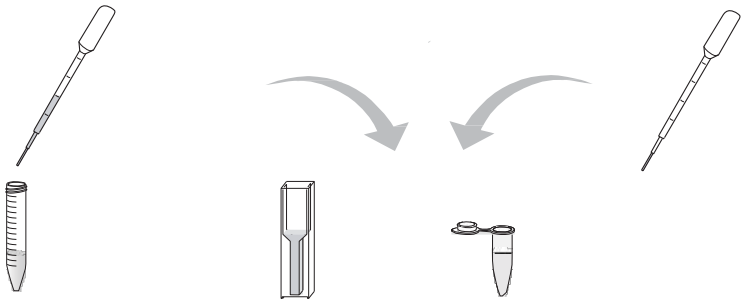


10. At the times indicated in the table below, remove 500 µl of mushroom extract/substrate mixture from the 15 ml conical tube, and add it to the appropriately labeled cuvette that already contains stop solution.



Time	Cuvette
1 min	1
2 min	2
4 min	3
6 min	4
8 min	5

11. Using a clean DPTP, add 500 µl of extraction buffer to cuvette #6. Clean the DPTP and then add one drop of enzyme extract. This will serve as the "blank" for this experiment.



12. Rinse out all DPTPs with copious amounts of water and save them for later activities. After you have finished your analysis, rinse out your reaction (conical) tubes and cuvettes with copious water and save them for later activities.

Note: Do not discard the unused stock solutions or cuvettes containing standards. They will be used for the next activity.

Quantitative Analysis of the Amount of Product Formed at Different Substrate Concentrations

1. Blank your spectrophotometer with the blank (cuvette #6) at 410 nm. Measure the absorbance values for your five cuvettes and record the absorbance values in column 2 of Table 17.

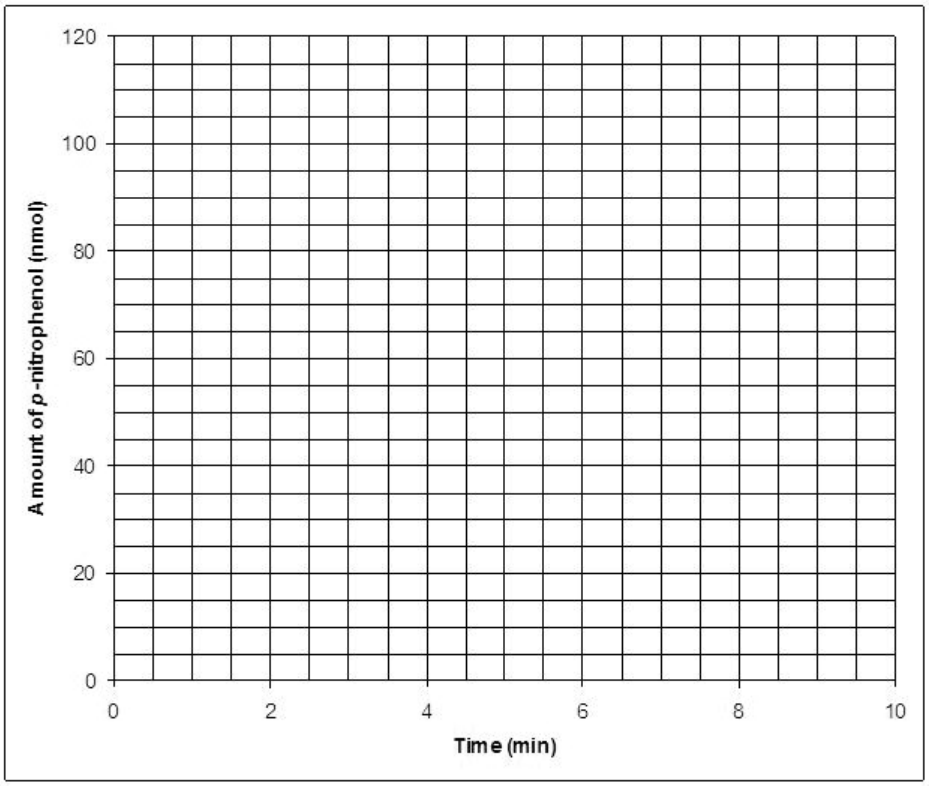
2. Using the protocols you learned in Activity 1, calculate the amount of *p*-nitrophenol formed in all of your samples and record it in column 3 of Table 17.

Table 17. Determination of *p*-nitrophenol produced by the mushroom extract breaking down the substrate based on a standard curve (similar to the one shown in Figure 8).

Cuvette	Absorbance at 410 nm	Amount of *p*-Nitrophenol Produced (nmol)
1		
2		
3		
4		
5		
6	0.00	

Analysis of Results

1. Plot the amount of product produced over time by your mushroom extract on the graph below.



2. Calculate the initial rate of reaction for mushroom extract

Initial rate of reaction =_____nmol/min

Activity 6 Analysis Questions

1. Did your mushroom extract break down the substrate (that is, produce any yellow product)?

2. Why did we use a blank for this experiment that was different from the one used in earlier experiments? **Hint**: What would be the effect on your absorbance readings if a mushroom naturally had some yellow color to it?

3. Compare the initial rate of reaction of your mushroom extract to the enzyme included in this kit. From what you have learned about the effect of pH, temperature, and enzyme concentration, can you explain some factors that might influence your enzyme extract's initial rate of reaction?

4. Scientists are constantly looking for sources of enzymes that can be used in industrial processes. If you were going to pick a source of cellobiase for ethanol production for biofuels, what type of organism might you look for as a source of this enzyme? **Hint**: The production of glucose to be converted to ethanol in biofuel production requires the reactions to occur at high temperatures and low pH.

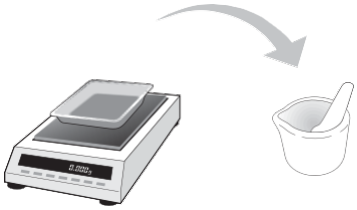
Quick Guide

Activity 6: Test the Ability of Mushroom Extracts to Increase the Reaction Rate

1. Write down the name of your mushroom

   _____

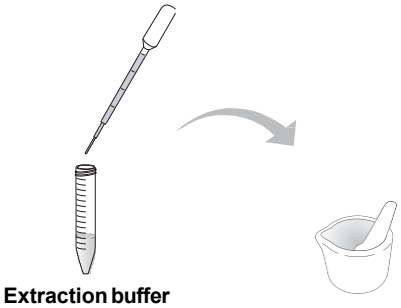
2. Weigh out approximately 1 gram of mushroom and put it into a mortar.



3. Add 2 ml of extraction buffer for every gram of mushroom into the mortar. To calculate the amount of extraction buffer you need, multiply the weight (in grams) of the mushroom by 2 and add that many milliliters.

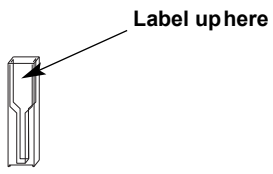
Weight of mushroom_____g x 2 =_____ml

4. Using a pestle, grind your mushroom to produce a slurry.

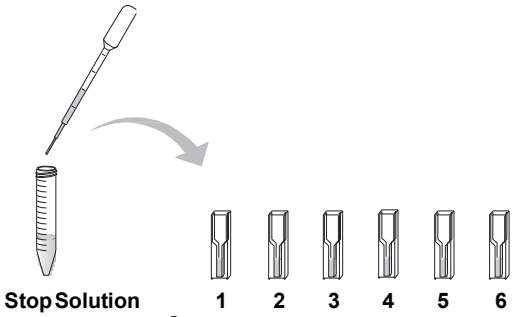


5. Strain the solid particles out of your slurry using a piece of filter paper or cheese cloth into a 1.5 ml microcentrifuge tube. Alternatively, if you have a centrifuge, scoop the slurry into a 1.5 ml microcentrifuge tube and then pellet the solid particles by spinning at top speed for 2 minutes. **Note**: You will need at least 250 µl of extract to perform the enzymatic reaction.

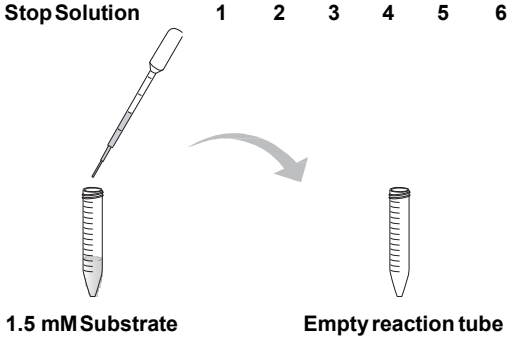
6. Label your cuvettes “1–6”. Only label on the upper part of the cuvette face.



7. Using a clean DPTP, pipet 500 µl of stop solution into each cuvette. Rinse out the DPTP thoroughly with water.

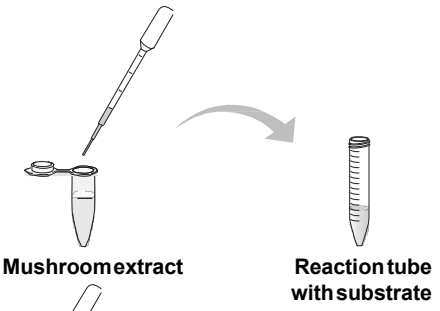


8. Label a 15 ml conical tube with the type of mushroom you are using and then using a clean DPTP, pipet 3 ml of substrate into the tube.

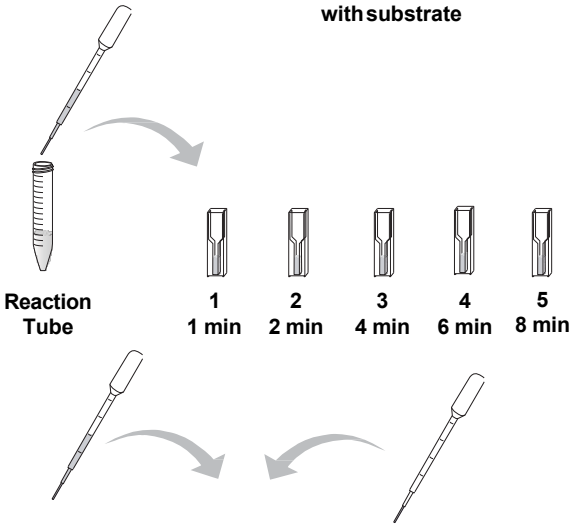


Please read and understand steps 9–10 fully before proceeding. These steps are time sensitive!

9. Using a clean DPTP, pipet 250 µl of your enzyme extract into your 15 ml conical tube of substrate. **START YOUR TIMER**.



10. At the times indicated, remove 500 µl of mushroom extract/substrate mixture from the reaction tube, and add it to the appropriately labeled cuvette that already contains the stop solution.



11. Using a clean DPTP, add 500 µl of extraction buffer to cuvette #6. Clean the DPTP and then add one drop of mushroom extract. This will serve as the “blank” for this experiment.

12. Proceed with the analysis of your samples. After you have finished your analysis, rinse out the reaction tubes, cuvettes, and DPTPs with copious water and save them for later activities.

Note: Do not discard unused stock solutions. They will be used for the next activity.

APPENDIX

Protein is CA$H!

The Drug Discovery, Development and Approval Process for Biopharmaceuticals (Biologics)

DISCOVERY DEVELOPMENT LAUNCH

Testing Phase	Discovery / Preclinical Testing
Test Population	Laboratory and animals studies
Purpose	Assess safety biological activity and formulations
Success Rate	5,000 compounds evaluated
Manufacturin Activities	Cell line construction, Cell banking
Years	6.5
Approximate Cost	$350M

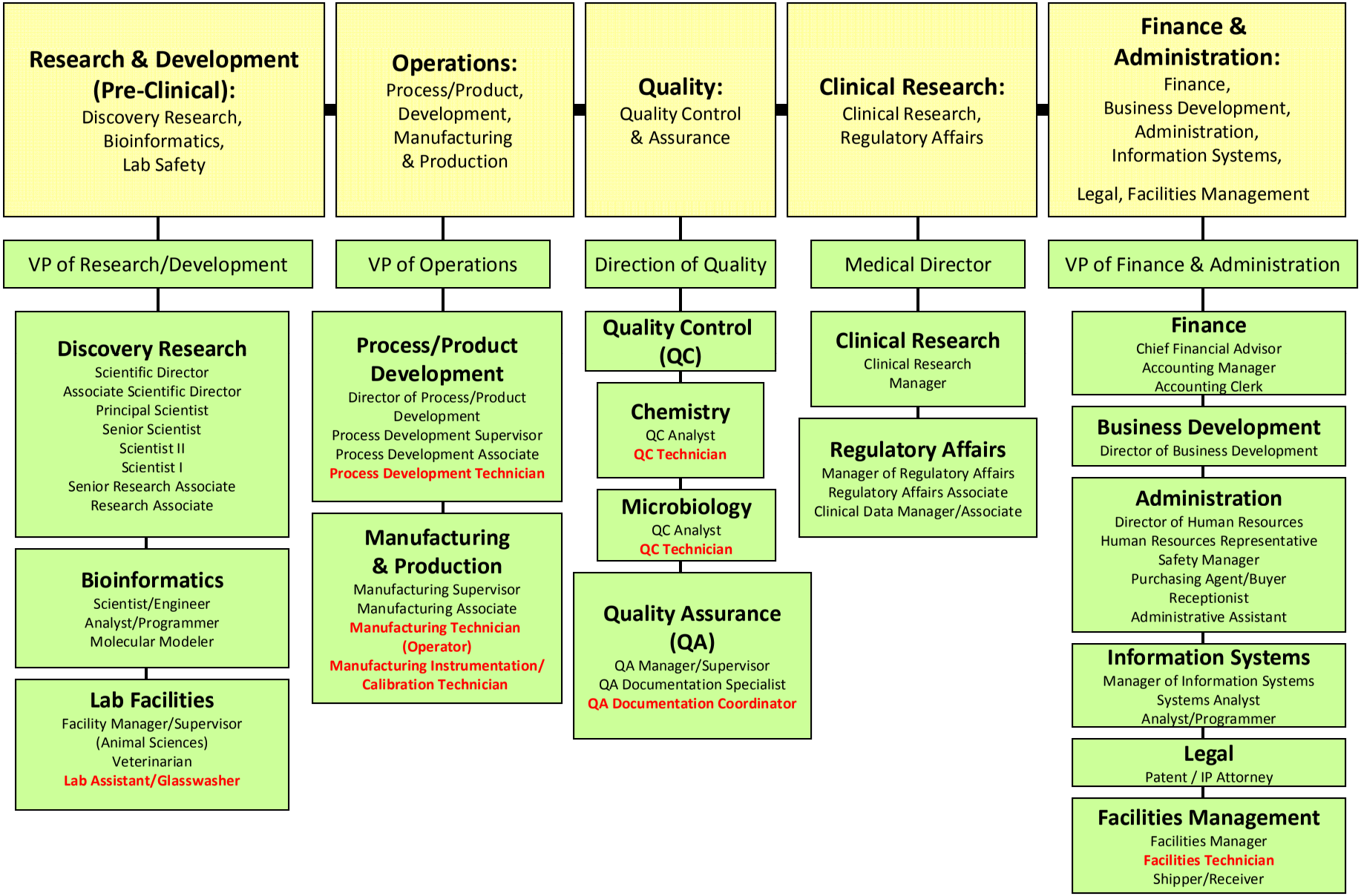
File IND at FDA

Clinical Trials		
Phase I	Phase II	Phase III
20 to 100 healthy volunteers	100 to 500 patient volunteers	1,000 to 5,000 patient volunteers
Determine safety and dosage	Evaluate effectiveness, look for side effects	Confirm effectiveness, monitor adverse reactions from long-term use
5 enter trials		
Process development, assay development, process optimization, scale-up, cGMP manufacture		
1.5	2	3.5
$70M	$100M	$200M

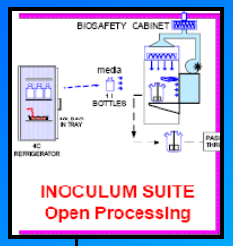
File NDA at FDA

File application	Phase IV
Review process / approval	Additional post-marketing testing required by FDA
1 approved	
Commercial manufacture	
1.5	=15
$80M	= $1B

Career Opportunities in Biotechnology/Biomanufacturing



Upstream/Downstream Manufacturing Overview



Working Cell Bank

Media Prep

Sub-Culture | Sub-Culture | Sub-Culture | Sub-Culture | Sub-Culture

Wave Bag

150L Bioreactor

Seed Bioreactors

750L Bioreactor

5,000L Bioreactor

Large Scale Bioreactor

26,000L Bioreactor

1 day

Centrifuge

Depth Filtration

Collection

Inoculum

24 days

Fermentation

31 days

Harvest/Recovery

Viral Inactivation

Harvest Collection Tank 1,500L

Filter

Chromatography Skid

Column

Eluate Hold Tank 8,000L

Anion Exchange Chromatography (QXL)

Chromatography Skid

Column

Eluate Hold Tank 20,000L

Hydrophobic Interaction Chromatography (HIC)

Purification

Filter

Chromatography Skid

Column

Protein A Chromatography

Eluate Hold Tank 6,000L

Viral Filtering

Post-viral Hold Vessel 3,000L

Filter

Chromatography Skid

Column

Anion Exchange Chromatography (QFF - Fast Flow)

Eluate Hold Tank 5,000L

Ultra Filtration Diafiltration

Bulk Fill

8 days

Bristol-Myers Squibb

Northeast Biomanufacturing Center and Collaborative

Supported by NSF DUE Award 1204974

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