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Escherichia coli - GFP
CORE PRODUCTION SYSTEM

PRODUCTION

Upstream Processing

NSF NBC² Global Biomanufacturing Curriculum

Title: Batch Culture of *Escherichia coli* Recombinant for Jellyfish Green Fluorescent Protein SOP

Approvals:
Preparer:_____Deb Audino _____ Date _____18Apr07_____
Preparer:_____Kari Britt _____ Date _____18Apr07_____

1. **Purpose:**
   1.1. To produce a batch culture of bacterial host cells.
2. **Scope:**
   2.1. Applies to the production of green fluorescent protein from recombinant *E. coli* cells.
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. LB Broth manufacturer instructions
   4.2. LB Agar manufacturer instructions
   4.3. autoclave SOP
   4.4. shaking incubator SOP
   4.5. water bath SOP
   4.6. spectrophotometer SOP
   4.7. incubator SOP
   4.8. pH meter SOP
   4.9. Gram stain SOP
   4.10. microscope SOP
5. **Definitions:** N/A
6. **Precautions:**
   6.1. Recombinant *E. coli* is a BL2 microorganism. Use BL2 safety measures and discard waste in biohazard containers.
7. **Materials:**
   7.1. 1mL vials of *E.coli* recombinant for GFP (-86°C freezer)
   7.2. Luria-Bertani (LB) Broth premixed powder (room temp)
   7.3. Luria-Bertani (LB) Agar premixed powder (room temp)
   7.4. Arabinose (room temp)
   7.5. Ampicillin powder (4-8°C)
   7.6. 70% Isopropanol (room temp)
   7.7. deionized water
   7.8. small beaker
   7.9. 30cc syringe
   7.10. sterile syringe filter (0.2µm)
   7.11. sterile 50mL centrifuge tube
   7.12. 500 mL Erlenmeyer shake flask with cap
   7.13. 2L Erlenmeyer flask with cap
   7.14. petri dishes (100x15mm, approx. 55 per batch of LB agar)

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7.15. 125 mL glass bottle with cap
7.16. 25mL, 100mL, 250mL, 1000mL graduated cylinders
7.17. magnetic stir plate
7.18. magentic stir bars
7.19. autoclave tape
7.20. laboratory film such as Parafilm
7.21. sterile pipettes (25mL, 2mL) and pipet pumps
7.22. 1.5mL microfuge tubes
7.23. cell Spreader
7.24. spectrophotometer Cuvettes
7.25. balance
7.26. autoclave
7.27. 55°C water bath
7.28. 37°C radial shaking incubator
7.29. spectrophotometer
7.30. micropipettors and sterile pipette tips
7.31. micro-scale pH meter (pH7 and pH4 commercially prepared buffers)
7.32. microscope with 1000x magnification
7.33. Gram stain reagents

8. Procedure:

8.1. **Solution and Media Preparation**

8.1.1. **Culture Broth:** Luria-Bertani (LB) Broth, Ampicillin (0.09mg/mL), Arabinose (1.8mg/mL)

8.1.1.1. Gather the following items and place on a clean lab bench area:
500mL Erlenmeyer shake flask with cap
125mL glass bottle with cap
250mL graduated cylinder
magnetic stir bar and magnetic stir plate
autoclave tape

8.1.1.2. Weigh out approximately:

LB Broth premix	4g
arabinose	0.36g

8.1.1.3. Add LB broth premix and arabinose to a clean 500mL shake flask.
8.1.1.4. Measure about 200mL of deionized water using a 250mL graduated cylinder and add to the LB/ARA broth.
8.1.1.5. Stir to dissolve the ingredients using a magnetic stir bar and stir plate.
8.1.1.6. Measure approximately 100mL of the LB/ARA broth using the 250mL graduated cylinder and transfer to the 125mL glass bottle.
8.1.1.7. Remove the stir bar.
8.1.1.8. Place caps on 500mL shake flask and 125mL bottle loosely to allow air flow (but not so cap can fall off). Place a small piece of autoclave tape on each.
8.1.1.9. Label shake flask: LB/ARA, [date], [initials], GFP, [group #].
8.1.1.10. Label glass bottle: LB/ARA, [date], [initials], GFP, [group #].

8.1.2. Autoclave the broth per the following instructions.

8.1.2.1. Place the following items into the autoclave:

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500mL shake flask of LB/ARA medium
125mL glass bottle of LB/ARA broth only

8.1.2.2. Autoclave per SOP for 20 minutes at 121°C.
8.1.2.3. Remove items from the autoclave when pressure reaches <5 psi and <80°C.
8.1.2.4. Tighten cap on 125mL glass bottle and shake flask. Store at room temperature.

8.1.3. **Culture Agar:** Luria-Bertani (LB) Agar, Ampicillin (0.09mg/mL), Arabinose (1.8mg/mL).

8.1.3.1. Gather the following on a clean lab bench area:
Clean 2L Erlenmeyer flask with cap
Clean 1L graduated cylinder
Magnetic stir bar and magnetic stir plate

8.1.3.2. Weigh out approximately:
LB Agar (premix) 35g
Arabinose 1.8g

8.1.3.3. Add ingredients to a clean 2L flask.
8.1.3.4. Add about 1L of deionized water.
8.1.3.5. Stir to mix using a magnetic stir bar and stir plate.
Note: The agar may not dissolve completely until it is autoclaved.
8.1.3.6. Remove the stir bar.
8.1.3.7. Place cap on 2L Erlenmeyer flask loosely to allow air flow (but not so cap can fall off). Place a small piece of autoclave tape on flask and bottle caps.
8.1.3.8. Label flask: LB/ARA, [date], [initials].
8.1.3.9. Autoclave for 20 minutes at 121 °C.
8.1.3.10. Remove from autoclave when pressure reaches <5 psi and <80°C.
8.1.3.11. Cool to the touch in a 55°C water bath.

8.1.4. **Ampicillin Stock Solution** (6mg/mL)

8.1.4.1. Gather the following items on clean lab bench area:
Small beaker
25mL graduated cylinder
Sterile syringe filter (0.2µm) and 30cc syringe
Sterile 50mL centrifuge tube

8.1.4.2. Weigh out approximately 0.12g of ampicillin powder, place in small beaker.
8.1.4.3. Measure approx. 20mL deionized water using the 25mL graduated cylinder. Add to the ampicillin and swirl to dissolve.
8.1.4.4. Sterile filter solution using sterile syringe filter:
8.1.4.4.1. Draw up solution into 30cc syringe.
8.1.4.4.2. Attach sterile syringe filter (clear side of filter faces syringe).
8.1.4.4.3. Depress plunger and expel solution into sterile 50mL centrifuge tube.
8.1.4.5. Label tube: AMP 6mg/mL, [date], [initials], storage: -20°C, disposal: autoclave/then drain.

8.1.5. **Add Ampicillin**

8.1.5.1. Gather the following on a clean lab bench area:

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Ampicillin solution (6mg/mL)
Cooled media
2mL and 25mL pipets and pump

8.1.5.2. Aseptically add 1.5mL of sterile filtered ampicillin stock solution (6mg/mL) to shake flask containing 100mL culture medium (media must be cool to the touch!), swirl to mix.

8.1.5.3. Revise the label on the shake flask to reflect that Ampicillin has been added (LB/ARA/AMP).

8.1.5.4. Aseptically add 15mL of sterile filtered ampicillin stock solution (6mg/mL) to the 2L flask containing 1L agar medium, swirl to mix.

8.1.5.5. Revise the label on the 2L flask to reflect that Ampicillin has been added (LB/ARA/AMP).

8.1.6. **Pour Culture Plates**

8.1.6.1. Disinfect a lab bench by spraying with 70% isopropanol.

8.1.6.2. Spread 55 sterile plastic petri dishes on the disinfected lab bench (save plastic sleeve).

8.1.6.3. Label plates on the edge of the bottom plate (as small as possible) with description of media, LB/AMP/ARA, the date and operator initials.

8.1.6.4. Pour agar plates and allow agar to solidify undisturbed with covers on.

8.1.6.5. Turn over plates and incubate plates 24-48 hours, inverted at 37ºC.

8.1.6.6. Check for contamination, autoclave and discard any plates showing microbial growth.

8.1.6.7. Store proofed plates, stacked upside down in their plastic sleeve, at room temperature.

8.1.7. **Proof the LB Broth**

8.1.7.1. Place the shake flask into the flask holders in the shaking incubator.

8.1.7.2. Set shaking incubator to 37°C and 200 RPM, turn on just the temp switch.

8.1.7.3. Incubate for 24-48 hours.

8.1.7.4. Check for contamination. If contaminated, add bleach and dispose down the drain.

8.2. **Inoculation**

8.2.1. Set up Equipment and Supplies:

8.2.1.1. Set water bath (Belly Dancer) to 37°C.

8.2.1.2. Set shaking incubator to 37°C and 200 RPM, turn on just the temp switch.

8.2.1.3. Pre-warm shake flask containing 100mL LB/AMP/ARA medium at 37° C.

8.2.2. Thaw the 1mL Vial of Frozen *E.coli* cells.

8.2.2.1. Remove the E.coli vial from storage in the -86° C freezer.

8.2.2.2. Thaw contents rapidly by agitation in a 37°C water bath (Belly Dancer).

8.2.3. Inoculate thawed *E.coli* Cells into 100mL of *E.coli* Cell Culture Medium.

8.2.3.1. Wipe off the 1mL vial with 70% isopropanol.

8.2.3.2. Using a sterile 2mL pipet, aseptically transfer the entire contents of the 1mL vial of thawed *E.coli* cells into the previously prepared shake flask containing 100mL LB/AMP/ARA medium.

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8.2.3.3. Swirl to mix.
8.2.3.4. Immediately take a sample.

8.3. **Sampling**

8.3.1. **Set up equipment and supplies.**

8.3.1.1. Turn on spectrophotometer to read *E.coli* (550nm) per the spectrophotometer SOP.
8.3.1.2. Autozero the spectrophotometer with LB broth in the bottle per the spectrophotometer SOP.
8.3.1.3. Gather the following items on a clean lab bench:
micropipettors (100µL and 1000µL volumes) and sterile pipet tips
2mL pipets (>15) and pipet pump
bunsen burner
cell spreader
isopropanol in a beaker (~ 200mL)
15 spectrophotometer cuvettes
beaker containing sterile 1.5mL microfuge tubes
LB/ARA/AMP plates placed on clean lab bench away from working area

8.3.2. Aseptically withdraw 2 - 2.2mL of *E.coli* culture and place into cuvette. Cover cuvette with a small piece of laboratory film. Begin countdown to next sample.
8.3.3. Return suspension culture to the shaking incubator, making sure to turn on shaker and check temperature.
8.3.4. Take OD reading of sample at 550nm using the spectrophotometer.
Reminder: Invert the covered cuvette, before taking reading.
Reminder: If the initial sample OD reading is greater than 1.0, the sample should be diluted until it reads below 1.0 and then multiply by the dilution factor to obtain the absorbance value.
8.3.5. **Prepare serial dilutions for plating**
Note: Mix cells before each transfer.

8.3.5.1. For each time point label the bottom of 6 plates (as small as possible on the EDGE of the plates): [date], [product lot #], [time point], [dilution factor representing: **10^3**, **10^4**, **10^5**, **10^6** dilutions], [operator name].
8.3.5.2. Place 6 sterile microfuge tubes in a rack.
8.3.5.3. Label the tubes with the appropriate time point (t_{0min} or t_{60min} or t_{120min} etc).
8.3.5.4. Label each tube with a dilution factor (10, 10^2, 10^3, 10^4, 10^5, 10^6).
8.3.5.5. Add 900µL LB Broth from the bottle into EACH microfuge tube.
8.3.5.6. Add 100µL of sample from cuvette to the 10 dilution tube. Cap and mix well by inverting several times.
8.3.5.7. Change pipet tip and add 100µL of 10 dilution tube to the 10^2 tube. Cap and mix well by inverting several times.
8.3.5.8. Change pipet tip and add 100µL of 10^2 dilution tube to the 10^3 tube. Cap and mix well by inverting several times.
8.3.5.9. Change pipet tip and add 100µL of 10^3 dilution tube to the 10^4 tube. Cap and mix well by inverting several times.

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8.3.5.10. Change pipet tip and add 100 µL of 10^4 dilution tube to the 10^5 tube. Cap and mix well by inverting several times.
8.3.5.11. Change pipet tip and add 100 µL of 10^5 dilution tube to the 10^6 tube. Cap and mix well by inverting several times.
8.3.6. Spread 200µl of each dilution (10^3 – 10^6) onto an LB/AMP/ARA plate (a total of four plates for each time point sample). Allow fluid to absorb into the media before turning plate over.
8.3.7. Incubate plates overnight in 37°C incubator.
8.3.8. Obtain pH reading for *E.coli* sample using pH meter per the pH meter SOP.
8.3.9. Repeat all of the sampling steps approximately every 60 minutes.
8.3.10. Determine viable cell concentration using the spread plates after overnight incubation.
8.3.10.1. Count colonies on appropriate plate for each time point.
8.3.10.2. Select a plate from each time point that has between 30 and 300 colonies.
8.3.10.3. Divide the number of colonies counted by the volume of sample spread on the plate to get the concentration of cells/µL and multiply by 1000 to get the cells/mL.
8.3.10.4. Multiply the number of cells/mL by the appropriate dilution factor to get the cell number per milliliter (cells/mL) in the original sample.
8.3.11. Identify cells as Gram Positive or Gram Negative
8.3.11.1. Perform Gram stain on one colony from each time point per Gram stain SOP

8.4. **Evaluate production of GFP.**
8.4.1. Place one plate from each time point on the UV light and observe if the colonies fluoresce.

8.5. **Prepare Growth Curve**
8.5.1. Plot OD, Viable Cell Number, and pH vs. Time on one graph.
8.5.2. Attach Growth Curve to the Batch Record.

8.6. **Calculate Growth Rate (u) and Doubling Time (Td)**
8.6.1. $u = (\ln OD_2 - \ln OD_1)/T_2 - T_1$
8.6.2. $Td = \ln 2/u$
8.6.3. Attach calculations to the Batch Record

9. Attachments:
9.1. Data table

10. History:

Name	Date	Amendment
S. Wallman	1997	Initial Release
E. Raitt, Deb Audino	2005	Put into 2005 SOP format, removed addition of NaOH to broth and agar, increased number of plates made, increased shaking speed, removed use of ice bucket, increased volume of sample for spread plate
Deb Audino, Kari Britt	05Sep06	Moved preparation of ampicillin after the autoclave step. LB culture section: add ARA to shake flask before removing 100mL to bottle.

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Deb Audino	18 Apr07	Put effective date into new format. Separated inoculation and sampling sections.

TIME POINT (min)	OD (550nm)	pH	Colony Count on 10^3 plate	Colony Count on 10^4 plate	Colony Count on 10^5 plate	Colony Count on 10^6 plate	VIABLE CELL COUNT (cells/mL)	Gram (-) Gram (+)	GLOWING (YES/NO)
T_0									
T_{60}									
T_{120}									
T_{180}									
T_{240}									
T_{300}									
T_{360}									
T_{420}									
T_{480}									
T_{540}									
T_{600}									
T_{660}									

Title: Filter Integrity Testing Protocol

Approvals:
Preparer: __Kari Britt_____Date ____03Aug10_____
Reviewer: __Sonia Wallman_____Date____03Apr10_____

1. **Purpose:**
   1.1. Test the membrane integrity of used syringe filters.
2. **Scope:**
   2.1. Applies to previously used syringe filters to confirm sterility of filtered solutions.
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:** N/A
5. **Definitions:** N/A
6. **Precautions:** N/A
7. **Materials:**
   7.1. small beaker
   7.2. 250 mL deionized water
   7.3. 7-10 inch silicone tubing
   7.4. pressure gauge
   7.5. 30mL syringe
   7.6. 0.22µm or 0.45µm syringe filter being tested
8. **Procedure:**
   8.1. Gather the following; small beaker, 250mL of deionized water, 7- 10 inch silicone tubing, pressure gauge, a 30mL syringe, and the used syringe filter being tested.
   8.2. Take the 30mL syringe and fill it with deionized water.
   8.3. Attach the 30mL syringe to the filter.
   8.4. Push water through until the filter is completely wet.
   Note: If filter is not completely wet there will be no resistance for the pressure gauge to read.
   8.5. Detach the syringe from the filter.
   8.6. Refer to figure 1 for bubble point assembly and perform the following steps.
      8.6.1. Draw air into the 30mL syringe, and then attach the syringe to the syringe adaptor on the pressure gauge so that it cannot come apart.
      8.6.2. Take the used syringe filter and attach it to the filter adaptor on the pressure gauge. Make sure it is twisted in tightly enough to prevent air from escaping, but do not over tighten.
      Note: Over tightening can result in the plastic threads being damaged.
      8.6.3. Attach a 7- 10 inch piece of silicone tubing securely to the nozzle on the used syringe filter.
   8.7. Test the integrity of the syringe filter.
      8.7.1. Fill the small beaker with approximately 250mL of deionized water.

Title: Filter Integrity Testing Protocol

8.7.2. Place the end of the silicone tubing into the beaker so that it is submerged in the water.
8.7.3. Push the air from the syringe out, until the pressure gauge reaches 60psi for 0.22µm filter or 30psi for 0.45um filter.
8.7.4. When the designated pressure is reached on the pressure gauge a spurt of bubbles should emerge from tubing and into water.
8.7.5. If the pressure does not reach the designated psi level, then the filter is damaged.

9. Attachments:

9.1. Figure 1: Bubble Point Assembly
9.2. Figure 2: Pressure Gauge

10. History:

Name	Date	Amendment
Katrice Jalbert	25Mar06	Initial Release
Deb Audino	04Apr08	College name change
Kari Britt	03Aug10	Changed name of document from Bubble Point SOP to Filter Integrity Protocol. Also made formatting and grammar edits as needed throughout the document.

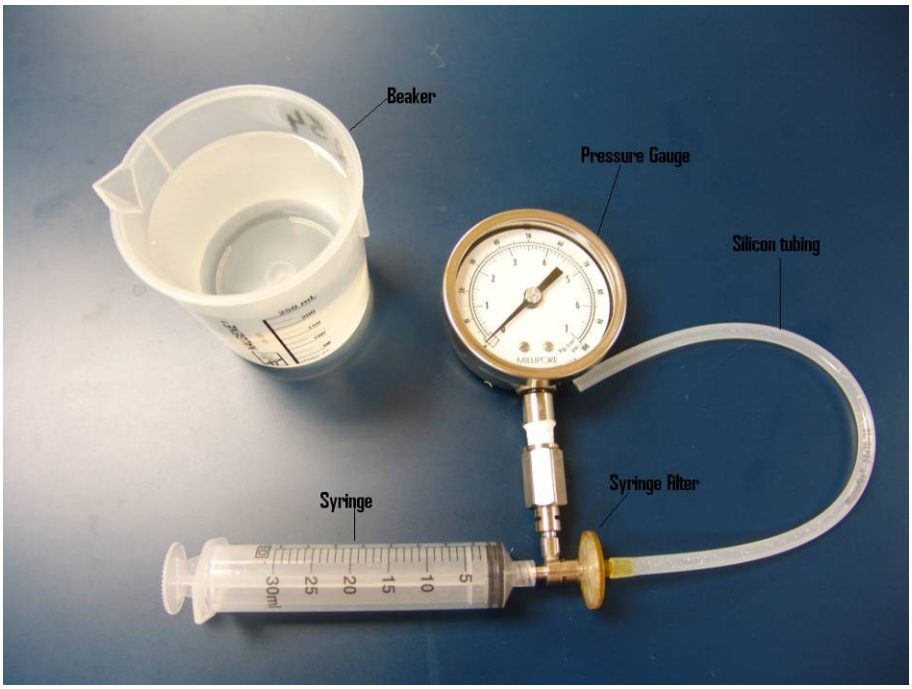

Figure 1: Bubble Point Assembly

Title: Filter Integrity Testing Protocol

Figure 2: Pressure Gauge

Title: Shimadzu UV-Visible Spectrophotometer SOP

Approvals:
Preparer: _____ Deb Audino _____ Date _____22Jul08_____
Reviewer:_____Bob O'Brien_____ Date _____22Jul08_____

1. **Purpose**:
   1.1. Operation of the Shimadzu UV-Visible Spectrophotometer.
2. **Scope:**
   2.1. This machine is used to measure how much light of a given wavelength is absorbed by a liquid sample.
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. Shimadzu UV-Visible Spectrophotometer manufacturer's instructions
5. **Definitions:**
   5.1. Cuvette: A small, transparent vessel. This is what a sample is put into for testing in the UV-Visible Spectrophotometer.
6. **Precautions:**
   6.1. N/A
7. **Materials:**
   7.1. sample to be tested
   7.2. cuvettes (visible or ultraviolet)
   7.3. lab tissue
   7.4. blank solution.
   7.5. 2mL pipettes
8. **Procedure:**
   8.1. Open panel door and make sure cuvette holders are empty, then close the panel door.
   8.2. Turn spectrophotometer "ON" by flipping the yellow switch on the side of the machine.
   8.3. The machine will automatically initialize and make a base line correction.
   8.4. Select **"8" CONDITION SET,** then press **ENTER**.
   8.5. Select **"5"** for **LAMP SELECT** to turn off UV light bulb, then press **ENTER.**
   8.6. Press the **FILE**, Key.
   8.7. Select one of the following numbers appropriate for the sample being tested.
      Select **"3"** for **E. COLI**
      Select **"7"** for **PICHIA**
      Select **"8"** for **CHO**
      Select **"10"** for **PROTEIN**
   8.8. Once you have made your selection press **ENTER.**
   8.9. It will then ask you for a **"Parameter Change Y/N**", Select **NO**, and press **ENTER**.

Title: Shimadzu UV-Visible Spectrophotometer SOP

8.10. Fill 2 of the same cuvettes each with about 2mL of blank solution. Hold the cuvette from the top to prevent tampering with the measurements, and wipe the sides with a lab tissue.
8.11. Open panel door and place the cuvettes with blank solution in the cuvette holders. **Make sure to use the appropriate orientation for the cuvettes you're using. Also make sure that the cuvettes used for the autozeroing are the same cuvette you use for the sample reading.** If using a standard cuvette, see fig 9.3, any orientation of the cuvette in the holder is acceptable, just make sure you wipe the cuvette's sides. If using a micro cuvetter, see figure 9.3, the microcuvette **MUST** be oriented in the holder so the the 1cm path length goes from left to right.
8.12. Press the **AUTO ZERO** key, then press **ENTER.**
8.13. When the the Auto Zero is complete, open the panel door and remove the **front** cuvette.
8.14. Do not replace cuvette in rear holder.
8.15. Using the same cuvette style, fill an empty cuvette with about 2-ml of the sample.
8.16. Clean the cuvette with a lab tissue.
8.17. Place in front cuvette holder, using the appropriate orientation and close the panel door.
8.18. Press **START** to take a reading.
8.19. Record the results or press **COPY** for a hard copy printout.
Note: If the initial sample OD reading is greater than 1.0, the sample should be diluted until it reads below 1.0 and then multiply by the dilution factor to obtain the absorbance value.
8.20. Open panel door and remove test sample from front cuvette holder.
8.21. To test additional samples: Place cuvettes in front holder and press start for a reading.
8.22. Record results, or press **COPY** for a hard copy printout.
8.23. Press **RETURN** to bring you back to step 8.9. Note: This will erase your old data.
8.24. Press **FILE** to return to the original screen.
8.25. Remove cuvettes remaining in holders.
8.26. Flip power switch located on the side, to turn off the machine.

9. Attachments:

9.1. Figure 1: Spectrophotometer
9.2. Figure 2: Spectrophotometer Control Panel
9.3. Figure 3: Micro and Standard Cuvettes

10. History:

Name	Date	Amendment
Sonia Wallman	1997	Initial release
Margaret Stiles,	2005	into 2005 SOP format
Stephen A. Derdau,	28Sep05	Amended section 8.13 regarding orientation of cuvette, and added attachments. Modified step 8.11 added the original step 8.11 to 8.11, "wipe with Kim wipe."
Deb Audino	04Apr08	Changed Kimwipe to lab tissue. College name change

Title: Shimadzu UV-Visible Spectrophotometer SOP

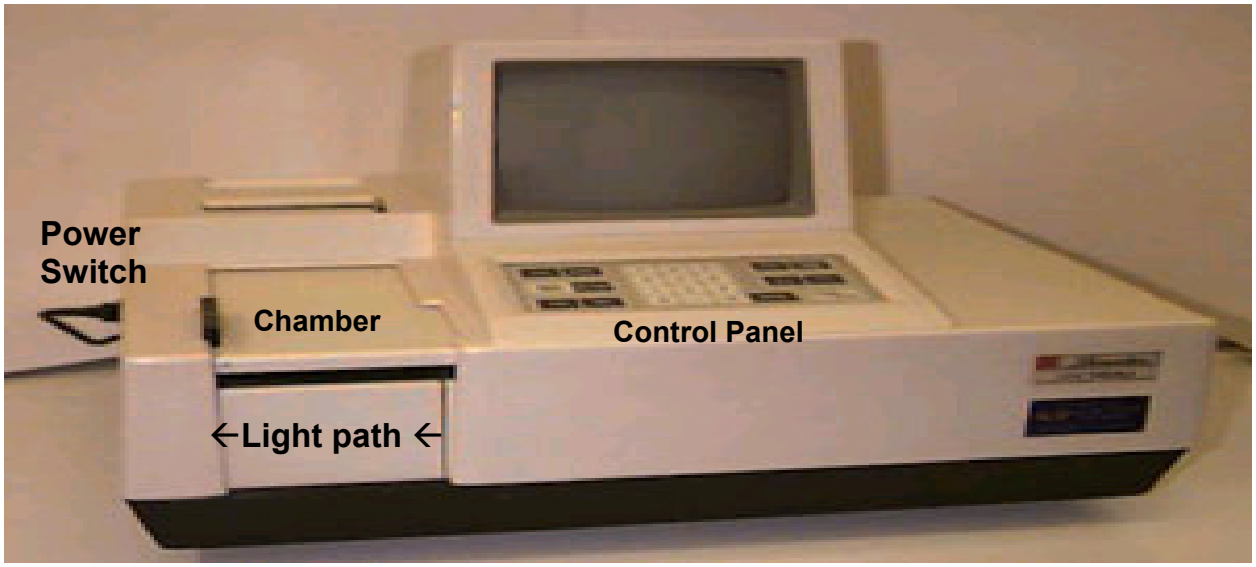

Figure 1: Spectrophotometer
Note: Light source runs from the right to the left through the sample in the cuvette chamber.

Figure 2: Spectrophotometer Control Panel

Title: Shimadzu UV-Visible Spectrophotometer SOP

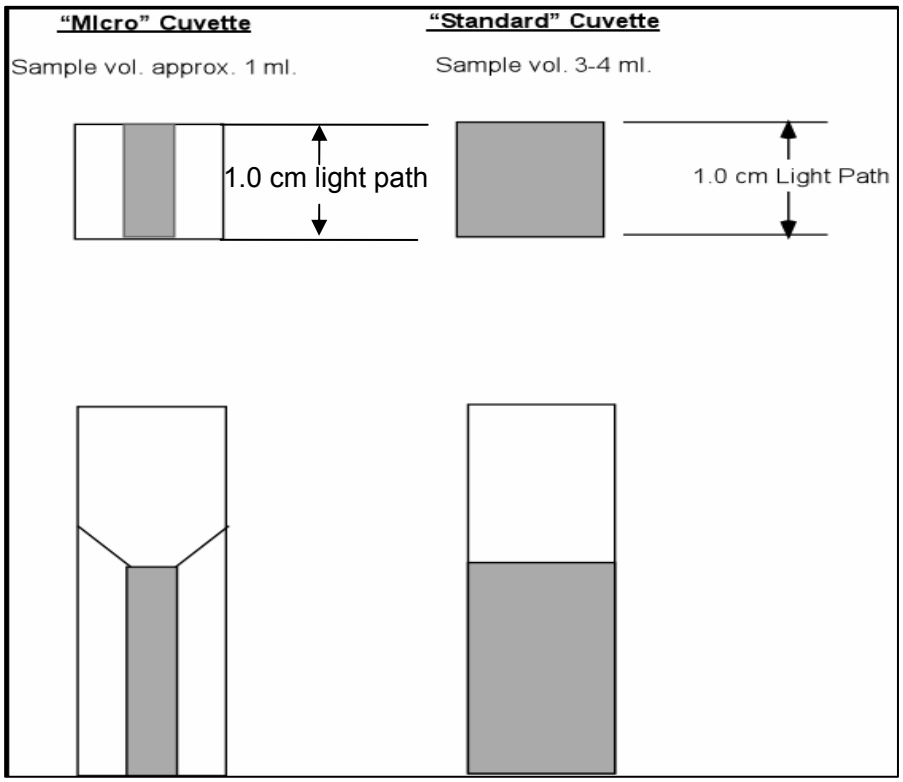

Figure 3: Micro and Standard Cuvettes
Gray Area = Sample
Image: http://bio.classes.ucsc.edu/bio100l/A%20SPECTROSCOPY/cuvettes.pdf

Title: Four Step Gram Stain SOP

Approvals:
Preparer:___Bob O'Brien_____ Date ____04Apr08_____
Reviewer:__ Deb Audino _____ Date ____04Apr08_____

1. Purpose:
1.1. To Gram stain samples.

2. Scope:
2.1. Applies to Gram staining samples using the 3-step method to detect the presence of Gram positive and Gram negative microorganisms.

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 Gram stain pack insert
4.2. microscope SOP

5. Definitions:
5.1. Gram positive microorganism: a microorganism that stains dark purple when treated with Gram staining solutions.
5.2. Gram negative microorganism: a microorganism that stains pink when treated with Gram staining solutions.

6. Precautions:
6.1. Gram Stain reagents are harmful. Wear gloves while performing this SOP.

7. Materials:
7.1. 4-step Gram stain kit
7.2. microscope slide
7.3. P20 pipet and tips
7.4. Bunsen burner
7.5. safety gas lighter with flint
7.6. tongs
7.7. inoculation loop
7.8. isopropanol
7.9. slide staining rack
7.10. timer
7.11. water
7.12. immersion oil
7.13. microscope with 1000X magnification
7.14. lab tissues
7.15. lab towels

8. Process:
Note: Refer to figures 1-6 as needed before performing this SOP and throughout the procedure as needed.
8.1. **Sample preparation**

Title: Four Step Gram Stain SOP

8.1.1. Label a glass microscope slide with pertinent information.
8.1.2. Prepare slide following directions for the appropriate sample source:
8.1.2.1. If sample is from a liquid culture, pipet 10µL of the culture onto the microscope slide.
8.1.2.1.1. Spread into a thin film with the pipet tip.
8.1.2.2. If sample is from a colony, pipet 10µL of water onto the slide.
8.1.2.2.1. Take a sample of the colony using a sterile loop.
8.1.2.2.2. Place the loop full of sample on the glass microscope slide, mix with water and spread into a thin film.
8.1.3. Gently heat fix the microbes to the slide.
Note: Do not overheat the slide. Excessive heating will cause atypical staining.

8.2. **Gram stain**
8.2.1. Place the slide on a slide rack to cool to room temperature before staining.
8.2.2. Cover the fixed sample on the slide with crystal violet stain and leave for approximately 1 minute.
8.2.3. Wash with a stream of water until the water runs clear.
8.2.4. Cover the fixed sample on the slide with iodine mordant and leave for approximately 1 minute.
8.2.5. Wash with a stream of water until the water runs clear.
8.2.6. Rinse with decolorizer.
8.2.7. Wash with a stream of water until the water runs clear.
8.2.8. Cover the fixed sample with safranin and leave for 30-60 seconds.
8.2.9. Wash with a stream of cold water until the water runs clear.
8.2.10. Air-dry or blot with lab tissue.
Note: Do not rub glass slide with the lab tissue.
8.2.11. View with the light microscope at 100x magnification (using oil).
8.2.12. Record whether cells are Gram positive (dark purple) or Gram negative (pink).
8.2.13. Discard the slide in the biohazard sharps container.

9. Attachments: N/A
9.1. Figure 1: Taking sample colony
9.2. Figure 2: Spreading sample colony thin film
9.3. Figure 3: Heat fix sample
9.4. Figure 4: Sample covered with crystal violet
9.5. Figure 5: Sample covered with iodine mordant
9.6. Figure 6: Sample covered with safranin

10. History:

Name	Date	Amendment
Bob O'Brien	12Jun07	Initial release
Deb Audino	04Apr08	College name change

Title: Four Step Gram Stain SOP

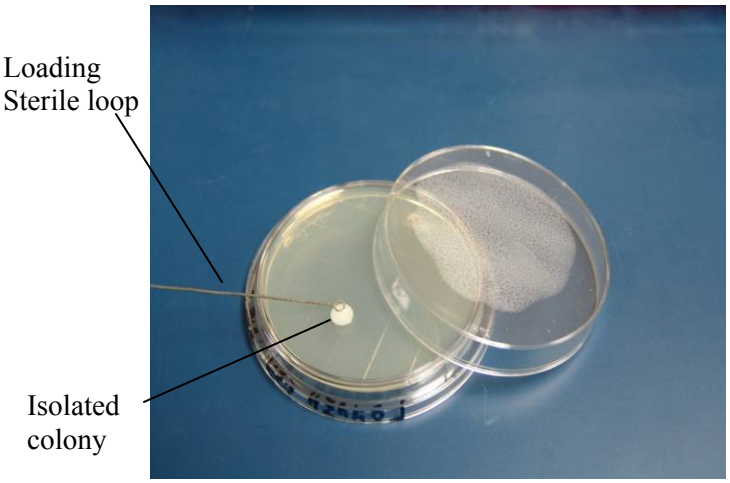

Figure 1: Taking sample colony

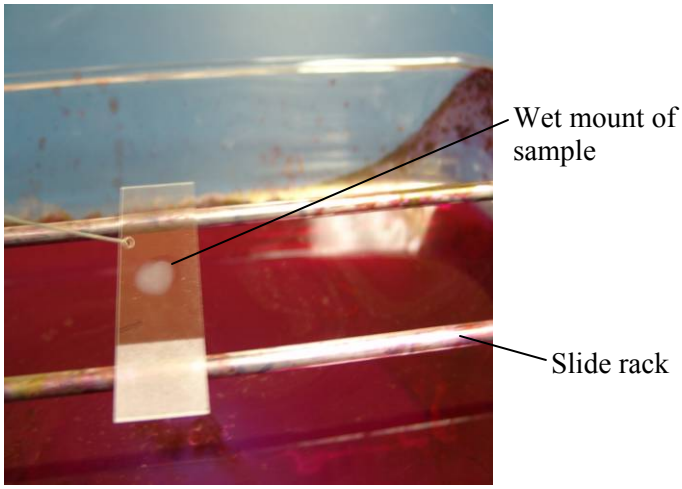

Figure 2: Spreading sample colony thin film

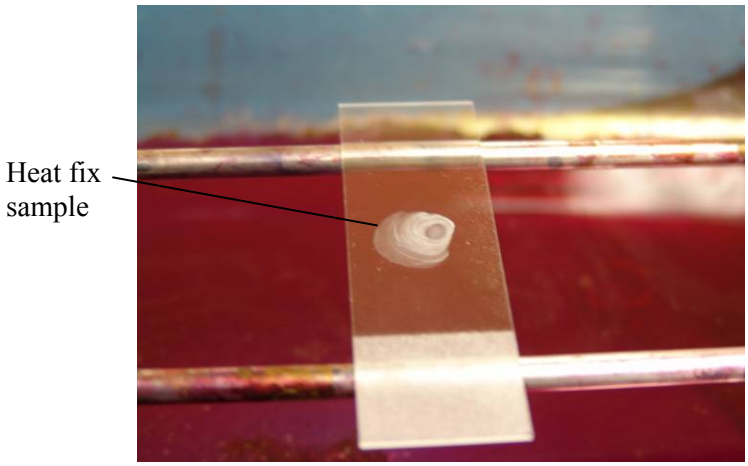

Figure 3: Heat fix sample

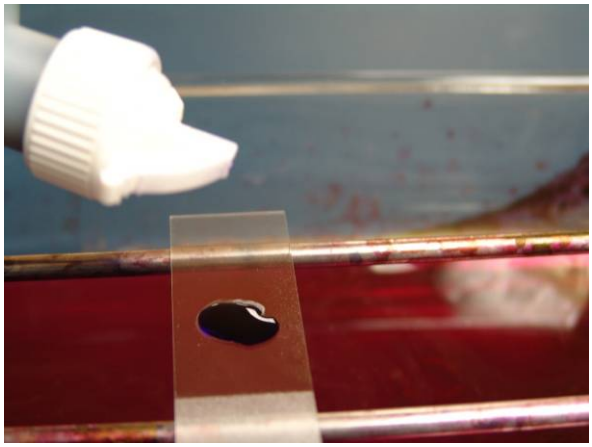

Figure 4: Sample covered with crystal violet

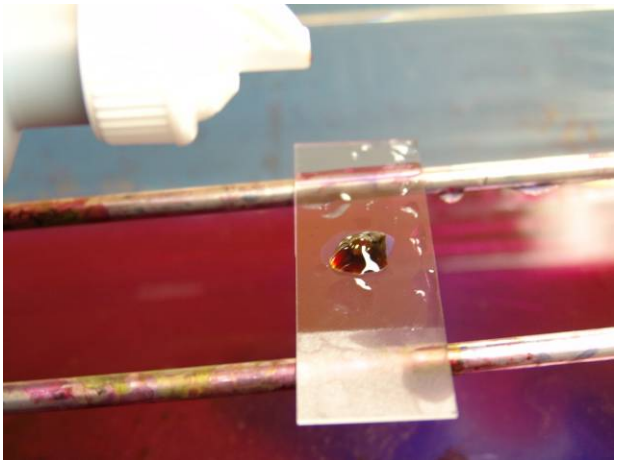

Figure 5: Sample covered with iodine mordant

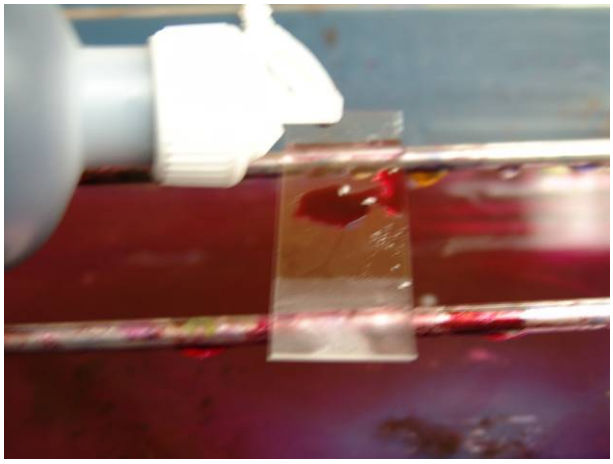

Figure 6: Sample Covered with safranin

Title: Leica DME Microscope

Approvals:
Preparer: _____Kari Britt_____ Date __03Aug10_____
Reviewer:_____Sonia Wallman_____ Date __03Aug10_____

1. **Purpose:** Operation of the Leica DME microscope.
2. **Scope:** Applies to the proper usage of the Leica DME microscope.
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. Leica DME instruction manual
   4.2. autoclave SOP
5. **Definitions:** N/A
6. **Precautions:**
   6.1. Use care when handling biological materials. Wear gloves at all times.
7. **Materials:**
   7.1. Leica microscope
   7.2. plastic protective cover
   7.3. power cord
   7.4. immersion oil
   7.5. specimen slide
   7.6. cover slips
   7.7. 70% IPA
   7.8. lab tissue
   7.9. lab towels
   7.10. sharps container
   7.11. biohazard container
   7.12. autoclave
8. **Procedure:**
   8.1. **Operation**
      8.1.1. Always use your microscope on a hard stable secure surface.
      8.1.2. Remove plastic protective dust cover from microscope.
      8.1.3. Verify that the power cord is plugged into an appropriate power source if necessary. Refer to Figure 1.
      8.1.4. Turn on illumination for the microscope by rotating the illumination power switch on the bottom left side of microscope by turning it towards the operator. Refer to Figure 1.
      8.1.5. Set the illumination control to the lowest setting.
      8.1.6. Fully open the aperture diaphragm of the condenser by rotating the ring to the right 40X. Refer to Figure 3.

Title: Leica DME Microscope

8.1.7. Using the substage condenser focusing knob, raise the condenser to the top of its excursion. Refer to Figure 3.
8.1.8. Verify that the 4X objective is in the working position by rotating the revolving nose piece, until the 4X objective is in the working position. Refer to Figure 2.
8.1.9. Utilize the X axis stage knob by turning it clockwise to bring the stage forward to allow the specimen to be placed on the stage. Refer to Figure 2.
8.1.10. Open the specimen slide clips. Refer to Figure 3.
8.1.11. Place specimen slide onto the stage in the specimen clips.
8.1.12. Verify that the specimen is properly placed in the specimen clips and that the clips are closed, holding the specimen in place.
8.1.13. Utilize the X axis stage knob to align the specimen under the objective and over the light path of the condenser. Refer to Figure 2.
8.1.14. Utilize the Y axis adjustment knob for the specimen clamp to bring the specimen under the objective and over the light path of the condenser. Refer to Figure 2.

8.2. **Focusing**

8.2.1. Adjust the interpupillary distance of the eyepiece.
8.2.2. Adjust the eye piece to allow the operator to look through the eyepieces. Refer to Figure 1.
8.2.3. Looking through the right eye piece with your right eye only, close the left eye, turn the course adjustment knob to its position to raise the stage to bring the specimen into focus. Refer to Figure 2.
8.2.4. Looking through the left eye piece with your left eye only and adjust the diopter adjustment ring to focus the specimen. Refer to Figure 1.
8.2.5. Using the fine adjustment knob to raise the stage to bring the specimen into focus. This should only require ± 1.5 rotations of focus adjustment. Refer to Figure 2.
8.2.6. The specimen slide can be scanned by utilizing the X axis adjustment knob in combination with the Y axis adjustment knob for specimen clamp to bring different parts of the specimen under the objective and into view.
8.2.7. Rotate the nose piece so that the 10X objective is in the working position.
8.2.8. Refocus by using the fine adjustment knob to raise the stage to bring the specimen into focus. This should only require ±1.5 rotations of focus adjustment.

8.3. **Immersion Oil**

8.3.1. Focus the object on the specimen slide with a lower power objective.
8.3.2. Rotate the revolving nose piece to the so that the 40X objective is out of the way. Note: Do not allow oil to touch any objective beside the 100X.
8.3.3. Place a single drop of immersion oil on the slide directly above where the light is shining through the specimen slide. (Since air bubbles in the oil will impair the image of the object, make sure the oil is free of bubbles.)
8.3.4. Rotate the revolving nose piece to allow the 100X objective to come into place Note: The lens should go into the drop of immersion oil and *not hit the specimen slide*.
8.3.5. Looking through the oculars, you may need to increase the amount of light by turning the condenser to 100X or it may be necessary to turning the illuminator knob.

Title: Leica DME Microscope

8.3.6. Your object on the specimen slide should still be in the field of vision, but slightly out of focus. Use the fine adjustment knob as necessary to clearly focus the object on the specimen slide.
8.3.7. Once you're in the immersion oil **do not go back to the 40X objective**. If you go back, the 40X objective will get oil on it, which will damage the lense.
8.3.8. When you have completed your observation of the specimen, rotate the revolving nose piece to allow the 4X objective to come into the working position.
8.3.9. Utilize the X axis stage knob by turning the knob in a clockwise direction to allow the removal of the specimen.
8.3.10. Remove the specimen slide from the microscope and dispose of the specimen slide in the appropriate waste container. (Microscope slides with microbial organisms shall be placed in biohazard waste container).
8.3.11. The 100X lens should be cleaned of the residual oil. This can be accomplished by spraying a lab tissue with 70% IPA and gently wiping the lens with the tissue. Then take a dry lab tissue, and, with very light pressure, wipe the 100X objective lens.
8.3.12. Dispose of lab tissue in the appropriate waste container. (Lab tissues that may have microbial organisms shall be placed in biohazard waste container).

8.4. **Cleaning and Storage**
8.4.1. After each usage clean the microscope.
8.4.2. Lightly spray a lab towel with 70% IPA and wipe the external surface of the microscope with the lab towel. Finish at the stage area.
8.4.3. Dispose of the lab towel in the biohazard waste container.
8.4.4. Allow any residual IPA to evaporate.
8.4.5. Rotate the revolving nose piece to allow the 4X objective to be placed in the working position.
8.4.6. Place the plastic protective cover over microscope.

8.5. **Transporting microscope**
8.5.1. Loosely wrap the electrical cord around the arm of the covered microscope. Keep the cord from coming into contact with the stage.
8.5.2. Pick up the microscope with one hand on the arm and the other hand placed under the base of the scope.
8.5.3. Place the microscope securely on a cart for transport.

9. Attachments
9.1. Figure 1: Leica DME microscope left side component view
9.2. Figure 2: Leica DME microscope right side component view
9.3. Figure 3: Leica DME microscope front component view
9.4. Equipment log sheet 4.4.3

10. History:

Name	Date	Amendment
Bob O'Brien	18May07	Initial Release
Bob O'Brien	04Apr08	College name change
Kari Britt	03Aug10	Made formatting and grammar edits as needed.

Title: Leica DME Microscope

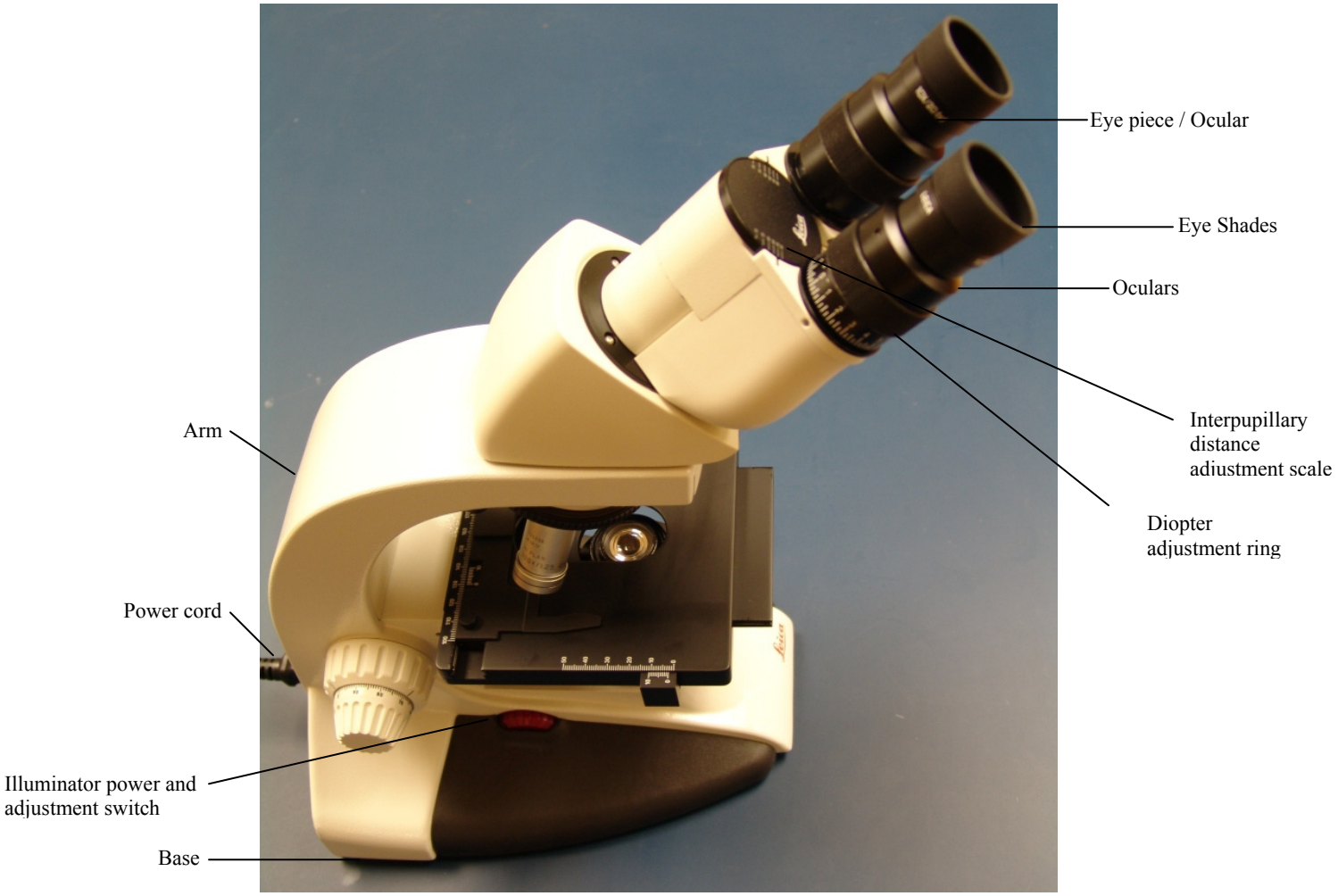

Figure 1: Leica DME microscope left side component view

Title: Leica DME Microscope

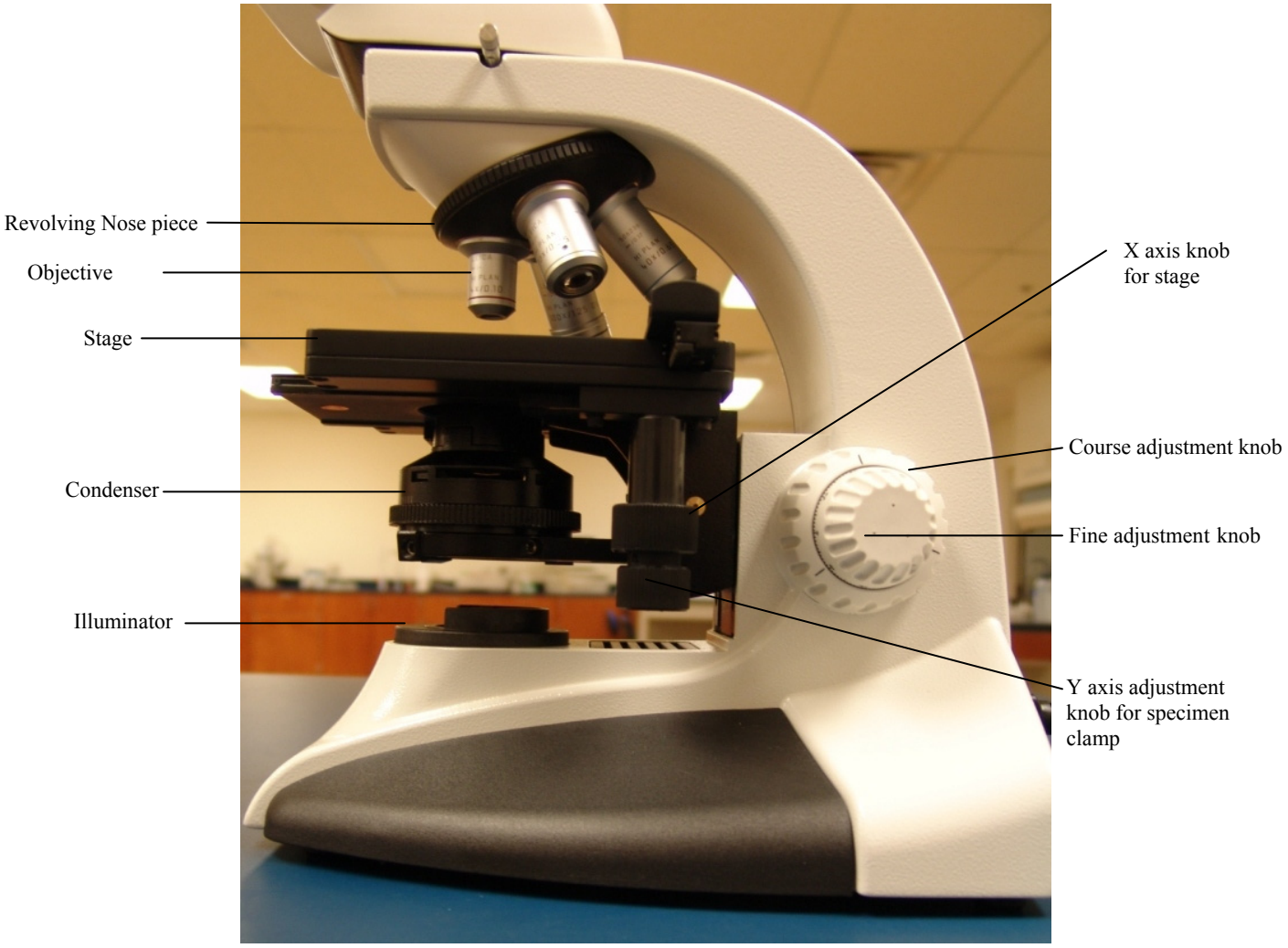

Figure 2: Leica DME microscope right side component view

Title: Leica DME Microscope

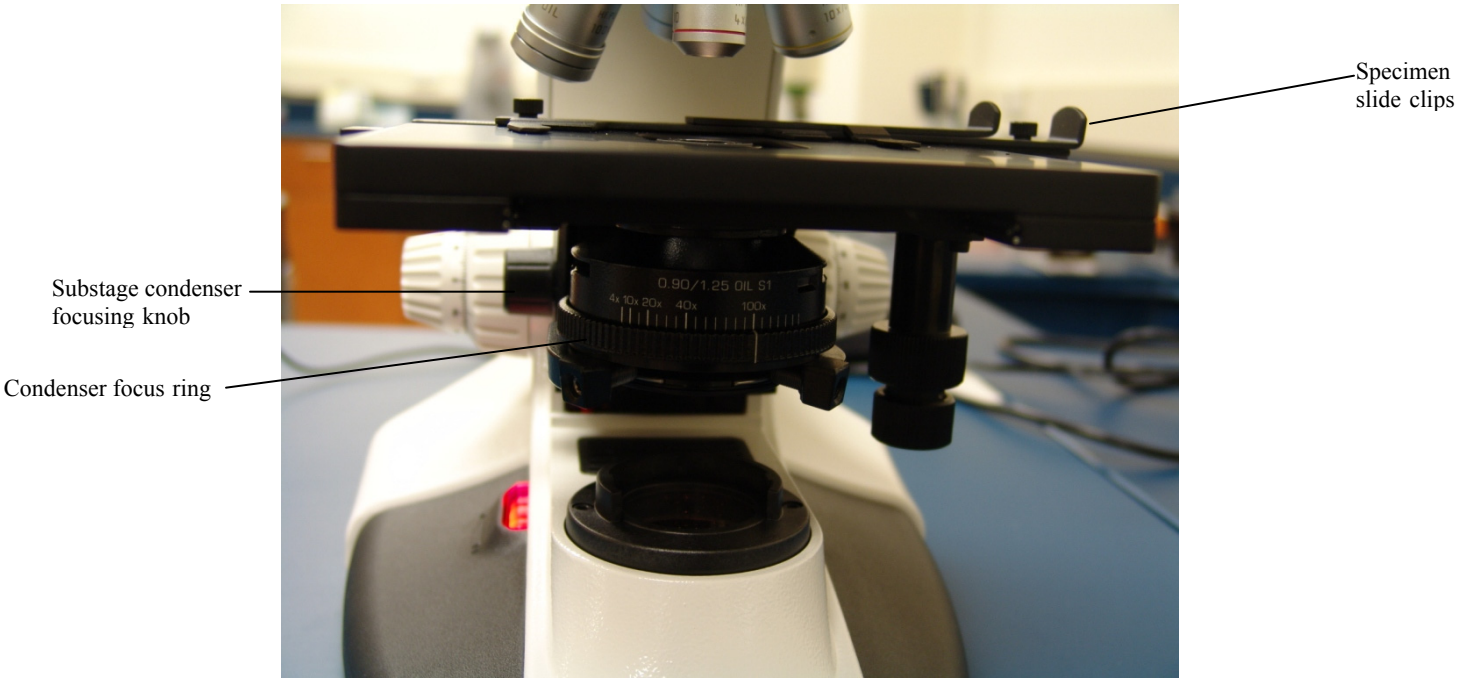

Figure 3: Leica DME microscope front component view

Batch Record: GFP Production from *Escherichia coli* Upstream Process

GFP Lot Number _____

Record Keeping Standards:

For each step in the batch record: the operator of the task will enter their initials (each operator has their own unique set of initials) and the date in the appropriate section(s) of the batch record. Another operator must initial and date in the appropriate section of the batch record to verify that the task was completed per SOP. No operator will verify their own work at any point. "If you didn't document it, you didn't do it!"

Batch records will be completed in blue or black ball point pen ONLY, and must be legible.

Any errors on a batch record will be crossed out with a single line through the error with the initials of the operator and the date. Corrections will be written in next to the crossed out error.

Use the following format to record dates: DDMMMYY. For July 10, 2006 use 10JUL06.

Use the 24 hour clock or "military time" to record time: 3:00pm would be written as 15:00.

Any and all deviations from a protocol or SOP, including abnormal results or retests performed, will be entered into the comments section at the end of each batch record. Be as detailed and specific as possible, include all steps taken before and/or after an abnormal reading, and provide an explanation for any deviations from a step.

Batch Record: GFP Production from *Escherichia coli* Upstream Process

GFP Lot Number _____

1. Prepare Media Broth Culture Broth: Luria-Bertani (LB) Broth, Ampicillin (0.09mg/mL), Arabinose (1.8mg/mL)		
Weigh out approximately 4g of LB Broth premix. Manufacturer:_____Catalog number: _____ Lot number: _____Expiration date:_____ Amount weighed:_____grams	Operator/Date	Verifier/Date
Weigh out approximately 0.36g of arabinose powder. Manufacturer:_____Catalog number: _____ Lot number: _____Expiration date:_____ Amount weighed:_____grams	Operator/Date	Verifier/Date
Add LB broth premix and arabinose to a clean 500mL shake flask. Flask I.D. number:_____	Operator/Date	Verifier/Date
Measure about 200mL of deionized water using a 250mL graduated cylinder and add it to the 500mL shake flask. Stir to dissolve. Volume of DI water measured:_____mL	Operator/Date	Verifier/Date
Measure approximately 100mL of the LB/ARA broth using the 250mL graduated cylinder and transfer it to the 125mL glass bottle. Volume of LB Broth transferred from shake flask to bottle:_____mL	Operator/Date	Verifier/Date
Autoclave per autoclave SOP for 20 minutes at 121°C. Autoclave I.D. #:_____ Time:_____minutes Temperature:_____ºC Pressure:_____ psi	Operator/Date	Verifier/Date
Comments:	Operator/Date	Verifier/Date

Batch Record: GFP Production from *Escherichia coli* Upstream Process

GFP Lot Number _____

2. Prepare Culture Agar Luria-Bertani (LB) Agar, Ampicillin (0.09mg/mL), Arabinose (1.8mg/mL)		
Weigh out approximately 35g of LB Agar (premix): Manufacturer:_____Catalog number: _____ Lot number: _____Expiration date:_____ Amount weighed:_____grams	Operator/Date	Verifier/Date
Weigh out approximately 1.8g of arabinose powder: Manufacturer:_____Catalog number: _____ Lot number: _____Expiration date:_____ Amount weighed:_____grams	Operator/Date	Verifier/Date
Measure approximately 1L of deionized water using a 1L graduated cylinder and add to the 2L Erlenmeyer flask. Stir to mix. Volume of DI water measured:_____L	Operator/Date	Verifier/Date
Autoclave per autoclave SOP for 20 minutes at 121°C. Autoclave I.D. #:_____ Time:_____minutes Temperature:_____ºC Pressure:_____ psi	Operator/Date	Verifier/Date
Remove from autoclave when pressure reaches <5psi and <80°C. **Cool** in a 55ºC water bath.	Operator/Date	Verifier/Date
Comments:	Operator/Date	Verifier/Date

Batch Record: GFP Production from *Escherichia coli* Upstream Process

GFP Lot Number _____

3. Prepare Ampicillin Solution and Add to Media Broth and Agar Ampicillin Stock solution: 6mg/mL		
Weigh out approximately 0.120g of ampicillin powder and place in small beaker. Manufacturer:_____Catalog number: _____ Lot number: _____Expiration date:_____ Amount weighed:_____grams	Operator/Date	Verifier/Date
Measure approximately 20mL deionized water with a 25mL graduated cylinder and add to the beaker containing the ampicillin. Swirl to dissolve. Volume of DI water measured:_____mL	Operator/Date	Verifier/Date
Sterilize filter solution using sterile 0.2 µm syringe filter as per the SOP: <u>Filter information:</u> Manufacturer:_____ Catalog number: _____ Lot number: _____	Operator/Date	Verifier/Date
Evaluate the integrity of the filter by performing the bubble point test per the Bubble Point Test SOP. Pressure needed to generate a steady flow of bubbles: _____psig Does the filter pass the test (pressure >45psig): YES/NO (circle one)	Operator/Date	Verifier/Date
Aseptically add approximately 1.5mL of sterile filtered ampicillin stock solution (6mg/mL) to shake flask containing 100mL culture medium (Media must be cool to the touch!), swirl to mix. Volume of Ampicillin added: _____mL	Operator/Date	Verifier/Date
Aseptically add 15mL of sterile filtered ampicillin stock solution (6mg/mL) to the 2L flask containing 1L agar medium, swirl to mix. Volume of Ampicillin added: _____mL	Operator/Date	Verifier/Date
Comments:	Operator/Date	Verifier/Date

Batch Record: GFP Production from *Escherichia coli* Upstream Process

GFP Lot Number _____

4. Pour Culture Plates/Proof LB Broth		
Pour agar plates and allow agar to solidify undisturbed with covers on as per SOP.	Operator/Date	Verifier/Date
Check for contamination. Autoclave and discard any plates showing microbial growth. Total number of plates:_____ Number of plates with contamination:_____	Operator/Date	Verifier/Date
Place the shake flask into the flask holders in the shaking incubator and incubate at 37 ºC and 200rpm. Incubator ID:_____ Temperature: _____ ºC Shaking Speed:_____ RPM Incubation Time: _____ hours	Operator/Date	Verifier/Date
Check for contamination. If contaminated, autoclave and dispose down the drain. Contamination: yes / no (circle one)	Operator/Date	Verifier/Date
Comments:	Operator/Date	Verifier/Date

Batch Record: GFP Production from *Escherichia coli* Upstream Process

GFP Lot Number _____

5. Inoculation		
Prewarm shake flask containing 100ml LB/AMP/ARA medium at 37 ºC.	Operator/Date	Verifier/Date
Remove the *E.coli* vial from storage in the -86 ºC freezer. Vial ID:_____	Operator/Date	Verifier/Date
Thaw contents rapidly by agitation in a 37 ºC water bath.	Operator/Date	Verifier/Date
Aseptically transfer the *E.coli* cells into the previously prepared shake flask per the SOP and immediately take a sample.	Operator/Date	Verifier/Date
Comments:	Operator/Date	Verifier/Date

Batch Record: GFP Production from *Escherichia coli* Upstream Process

GFP Lot Number _____

TIME POINT (min)	OD (550nm)	pH	Operator/Date	Verifier/Date
0				
60				
120				
180				
240				
300				
360				
420				
480				
540				
600				
660				
720				

Batch Record: GFP Production from *Escherichia coli* Upstream Process

GFP Lot Number _____

Time Point (min.)	Colony Count on 10^3 plate	Colony Count on 10^4 plate	Colony Count on 10^5 plate	Colony Count on 10^6 plate	Viable Cell Count (cells/ml)	Gram (-) Gram (+)	Glowing (Yes/No)	Operator/Date	Verifier/Date
0									
60									
120									
180									
240									
300									
360									
420									
480									
540									
600									
660									
720									

*** Attach growth curve and double time calculation.**

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.