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}									