MOLEBIO LAB #15: Bacterial Culture Techniques – Part IV

Introduction:

This protocol is for preparing a mid-log culture of *E. coli*. Cells in mid-log growth can generally be rendered more competent to uptake plasmid DNA that can cells at stationary phase. Mid-log cells are used in the classic transformation protocol that we will try later in the year. The protocol begins with an overnight suspension culture of *E. coli*. Incubation with agitation has brought the culture to stationary phase and ensures a large number of healthy cells capable of further reproduction. The object is to subculture a small volume of the overnight culture in a large volume of fresh nutrient broth. This "re-sets" the culture to zero growth, where after a short lag phase, the cells enter the log-growth phase. As a general rule, 1 volume of overnight culture (the inoculums) is added to 100 volumes of fresh LB broth in an Erlenmeyer flask. To provide good aeration for bacterial growth, the flask volume should be at least four times the total culture volume.

A shaking incubator is necessary for growing *E. coli* for competent cells. Proper aeration and nutrient exchange are essential to achieve vigorous growth; only cells collected during the middle part of log (mid-log) phase will produce competent cells with a high transformation frequency.

Timing of the culture to reach mid-log phase is likely to be affected by any change in the protocol. For example, a culture inoculated with an overnight culture that was grown without shaking will take longer to reach mid-log phase. Different strains of *E. coli* display different growth properties. Different nutrient broths also will affect the growth of the culture.

Procedure:

STANDARD PREPARATION OF A MID-LOG SUSPENSION CULTURE

1. Sterilely transfer 1 volume of overnight culture into 100 volumes of LB broth at *room temperature*.
2. *If using a 1 mL overnight culture:*
    a. Remove cap from overnight culture tube, and flame mouth. *Do not place cap on lab bench*.
    b. Remove foil cap from flask, and flame mouth. *Do not place cap on lab bench.*
    c. Pour entire overnight culture into a flask. Reflame mouth of flask, and replace foil cap.

    If transferring only a portion of larger overnight culture:
    a. Flame pipette cylinder.
    b. Remove cap from overnight culture tube, and flame mouth of tube*. Do not place cap on lab bench.*
    c. Withdraw 1 mL of overnight suspension for every 100 mL of media prepared. Reflame mouth of overnight culture tube, and replace cap.
    d. Remove foil cap from flask, and flame mouth. *Do not place cap on lab bench.*
    e. Expel overnight sample into flask. Reflame mouth of flask, and replace foil cap.
3. Incubate at 37 °C with continuous shaking.
4. It can safely be safely assumed that an MM294 culture has reached OD_{550} 0.3 – 0.5 after 2 hours, 15 minutes of incubation with continuous shaking. Note that under ideal conditions, as represented in **Figure 1**, an MM294 culture reaches an OD_{550} 0.3 – 0.5 in 1 hour, 30 minutes. However, less ideal conditions often result in slower growth.

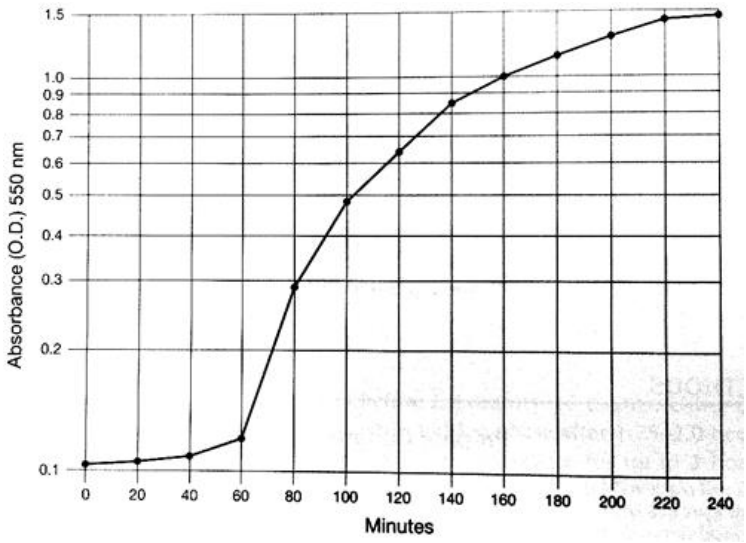

Figure 1: Growth curve of bacterial culture measured by absorbance at 550 nm.

5. This mid-log culture can be stored on ice until ready for use for up to two hours. During this time, cell growth is temporarily arrested.
6. Take time for a responsible cleanup.
    a. Segregate for proper disposal bacterial cultures *and* tubes, pipettes, and micropipettor tips that have come into contact with the cultures.
    b. Disinfect overnight culture and pipettes and tips with 10% bleach solution, or disinfectant.
    c. Wipe down lab bench with soapy water, 10% bleach solution, or disinfectant.
    d. Wash hands (as always) before leaving lab.
7. Answer questions 1 and 2.

FOR FURTHER RESEARCH (PART A)

8. Start a 250 mL *E.coli* culture as described in the above protocol. Determine the optical density of ~5 mL samples sterilely withdrawn directly into your cuvettes at 20-minute intervals, from time zero for as many hours as possible using a spectrophotometer. The blank used to calibrate the spectrophotometer at a wavelength of 550 nm will be LB broth that has NOT been inoculated. Using Excel (or any spreadsheet software), construct a data table with all of your measurements and plot a graph of OD_{550} *versus* time (no trendline).

FOR FURTHER, FURTHER RESEARCH (PART B)

Perform the following experiment to correlate the optical density of culture with actual number of viable *E. coli* cells. As always, observe sterile technique!

9. Inoculate 500 mL **(amount may be modified – depending on the number students in class)** of LB with 5 mL of *E. coli* overnight culture. Swirl to mix.
10. Immediately remove a 10 mL aliquot of the culture and place in a new 15 mL tube. Also remove one of the 'blanks' that is also located in the shaking water bath. Label both tubes with the correct time interval and your initials. This first sample will represent time = 0. Place the bacterial sample tube on ice to arrest growth. Place the blank tube into the 'blank' rack. Finally, incubate the remaining culture at 37 °C with vigorous shaking.

11. Remove additional 10 mL aliquots from the incubating culture (and one of the 'blanks' from the shaking water bath) every 20 minutes for a total of 4 hours. Place the blank in the 'blank rack' and place each aliquot on ice until ready to perform steps 12 – 15.
12. Determine the OD_{550} of each aliquot. (Don't forget to calibrate the spectrophotometer!)
13. Make a 10^2 dilution by mixing 10 μL of the aliquot with 990 μL of fresh LB broth. Prepare three additional serial dilutions of each aliquot for plating in step 14:
    a. 10^4 = 10 μL of 10^2 culture + 990 μL of LB
    b. 10^5 = 100 μL of 10^4 culture + 900 μL of LB
    c. 10^6 = 100 μL of 10^5 culture + 900 μL of LB
14. Spread 100 μL of the 10^4, 10^5, and 10^6 dilutions each onto a separate LB agar plate, for a total of three plates for each time point (aliquot). *Label each plate bottom with time point and dilution.* **There will be a lot of plates, so organization is crucial – LABEL CLEARLY!** Invert plates, and incubate for 15 – 20 hours at 37 °C.
15. For each time point, select a dilution plate that has between 30 and 300 colonies. Calculate the number of colonies for the original aliquot (as we did in Lab #8).
16. Using Excel, construct a data table and plot the following:
    - OD_{550} (y axis) *versus* time (x axis)
    - cell number (y axis) *versus* time (x axis)
    - OD_{550} (y axis) *versus* cell number (x axis)
17. Take time for a responsible cleanup.
    a. Segregate for proper disposal bacterial cultures *and* tubes, pipettes, and micropipettor tips that have come into contact with the cultures.
    b. Disinfect overnight culture and pipettes and tips with 10% bleach solution, or disinfectant.
    c. Wipe down lab bench with soapy water, 10% bleach solution, or disinfectant.
    d. Wash hands (as always) before leaving lab.
18. Answer the remaining questions.

Micklos, David A. Freyer, Greg A. *DNA Science*. 2nd edition. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press, 2003. 331-350, 393-394. Print.

1. What variables influence the length of time for an *E. coli* culture to reach mid-log phase?

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2. What are the disadvantages of beginning a mid-log culture from a colony <u>scraped off a plate</u>, as opposed to inoculums of overnight culture in liquid?

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3. Using your graph, an OD_{550} 0.3 – 0.4 corresponds to what number of cells?

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4. Indicate what is the average cell number at each of the following points (based on your graphs):

a) _____ lag phase
b) _____ first third of log phase (early log)
c) _____ second third of log phase (mid log)
d) _____ final third of log phase (late log)
e) _____ stationary phase