TECHNIQUES IN MOLECULAR BIOLOGY – LAB EXPERIMENT 1: PIPETTING AND STERILE CULTURE

Mastery of micropipetting and sterile technique is essential for reliable success in molecular biology. Take these exercises and our discussions about pipetting seriously.

I. Micropipetting Exercise The purpose of this exercise is to conduct the 3 volumes x 10 Weighing pipette calibration protocol using a standard table-top balance. Chose one of three pipettes at your bench for the experiment.

1. Set pipette to 10%, then 50%, and then 100% of the pipette range.
2. Pipette 10 samples of distilled water into a weigh boat on an analytical balance
3. Tare the balance before each pipetting
4. Record the weight in the table below in mg
5. Prewet the pipette tip before starting measurements.
6. Use the same pipette tip for all measurements.
7. Record the weights in Table 1, 2, and 3 respectively
8. ***Recreate the table in your ELN***

Table1. Weight Recordings of 10 Consecutive 10% pipette range with _____ Pipette.

Sample	1	2	3	4	5	6	7	8	9	10
Weight (mg)										
Volume (µl)										
Volume Setting (µl)										
Mean Weight (mg)					Standard Deviation (Precision)					
Mean Volume (µl)					Standard Error (Accuracy)					
Pass Accuracy					Pass Precision					

Table 2. Weight Recordings of 10 Consecutive 50% pipette range with _____ Pipette.

Sample	1	2	3	4	5	6	7	8	9	10
Weight (mg)										
Volume (µl)										
Volume Setting (µl)										
Mean Weight (mg)					Standard Deviation (Precision)					
Mean Volume (µl)					Standard Error (Accuracy)					
Pass Accuracy					Pass Precision					

Table 3. Weight Recordings of 10 Consecutive 100% pipette range with _____ Pipette.

Sample	1	2	3	4	5	6	7	8	9	10
Weight (mg)										
Volume (µl)										
Volume Setting (µl)										
Mean Weight (mg)					Standard Deviation (Precision)					
Mean Volume (µl)					Standard Error (Accuracy)					
Pass Accuracy					Pass Precision					

Use this table to determine your final calculations shown above. A copy of just the table for your ELN will be found on the class webpage. Enter the table into your ELN.

RAININ PIPET-LITE, PIPET-PLUS, AND EDP3

MODEL VOLUME	VOLUME SET µL	ACCURACY %	ACCURACY µL(±)	PRECISION %	PRECISION µL(≤)
2	0.2	12.0	0.024	6.0	0.012
	1	2.7	0.027	1.3	0.013
	2	1.5	0.030	0.7	0.014
10	1	2.5	0.025	1.2	0.012
	5	1.5	0.075	0.6	0.03
	10	1.0	0.1	0.4	0.04
20	2	7.5	0.15	2.0	0.04
	10	1.5	0.15	0.5	0.05
	20	1.0	0.2	0.3	0.06
100	10	3.5	0.35	1.0	0.1
	50	0.8	0.4	0.24	0.12
	100	0.8	0.8	0.15	0.15
200	20	2.5	0.5	1.0	0.2
	100	0.8	0.8	0.25	0.25
	200	0.8	1.6	0.15	0.3
1000	100	3.0	3	0.6	0.6
	500	0.8	4	0.2	1.0
	1000	0.8	8	0.15	1.5

II. Practice Sterile Use of a 10 ml Serological Pipet

Use the following protocol to test your ability to transfer sterile LB media (NO antibiotic) into a 5 ml sterile round bottom tube.

1. Loosen caps on any tube or media container about to be used (sterile culture tube, container of sterile LB broth) so they can be easily lifted off.

2. Open the end of the pipet sleeve and attach the pipet bulb. Squeeze the bulb so that it is prepared to suck. Remove the sleeve from the pipet.

3. Lift the lid from the LB broth container (*do not set down*), insert the pipet and withdraw 5 ml of broth into the pipet.

4. Replace the LB broth container lid loosely.

5. Remove the lid from the culture tube (*do not set down*), and transfer the LB broth to the tube. Replace the lid, with the lid on the 'fingers.'

6. Place the tube in a rack in the 37° C *shaking* incubator to test its sterility (you may do this also with your LB broth container). The broth should be clear tomorrow if sterility was maintained.

7. YOU MUST CHECK your culture tomorrow, take an image and upload in the ELN and dispose of the tube and LB as directed. Forgetting to do so will result in a 5-point deduction.

III. Isolation of Individual Colonies by Streaking

Good bacteriological technique dictates that any culture is begun with a single well-isolated bacterial colony, which represents a clone – the progeny of a single bacterium. This way one is reasonably certain that the culture grown contains a single, isogenic entity (all the same genotype), and not more than one kind of bacteria or other microbe. This exercise teaches you how to make a bacterial culture plate that has such isolated bacterial colonies.

This exercise also demonstrates the phenomenon of antibiotic resistance by comparing the growth of two different strains of bacteria – one with an ampicillin resistance gene on a plasmid, and one without – on culture plates with or without the antibiotic ampicillin.

In general, an unmarked plate (for this lab) is considered to contain LB agar without antibiotic. Plates with antibiotic (or other components) are typically striped on the side with a color code indicating additions to the plate. It is important that the media portion of the plate is marked and not only the lid. Otherwise you should mark the plate on the bottom.

Ideally culture plates are removed from the refrigerator where they are stored long-term and allowed to warm to room temperature before use (incubating at 37°C). Too rapid warming (transfer directly from 4°C to 37°C) can cause bubbles to appear in or under the plate making it difficult to see colonies on the plate. If the plate is still cool, condensation may need to be wiped off immediately prior to writing with a marker.

Note that plates should be stored with the lid down/media side up (unless wet) – this is **bacteriologically 'right side up'** – this way any airborne contaminants are more likely to land on the lid than on the media in the plate.

1. Use a marker to label the *bottom* of each agar plate (the portion with the LB agar medium) with the name of the bacterial strain, your initials and date, and (as needed) the type of bacterial plate (LB or LB/Amp). See the illustration below ("Anatomy of a Bacterial Streak Plate"). Write small and near the edges of a plate.

You will streak a total of 3 plates (2 LB Agar only, 2 LB/Amp, and 2 LB/Kan) with two starter bacteria (DH5 or BL21 without plasmid and the same strain with pQE60 wgMDH). One bacterial strain vs each type of plate.

In your ELN create a table of your strains and the expected level of growth for each plate/strain.

	Culture plate type		
Bacterial Strain	LB	LB / Amp	LB / Kan
DH5/BL21			
pQE60 wgMDH + DH5/BL21			

2. Light your Bunsen burner.

3. Hold the inoculating loop in the flame until it is red hot, tilted to sterilize the maximal part of the wire. Make sure the entire wire portion up to the handle has at least been passed through the flame.

4. Cool the loop in an unused part of the bacterial plate (under the writing is a good spot). Return the plate to its lid on the bench.

5. With the lid resting on the bench, lift a culture plate (media part with colonies) and scrape the sterile loop through several isolated colonies. Close the plate, and open your clean plate. Smear the bacteria in a single large streak back and forth at the top of the plate (near the 'loop cooling' region – see illustration).

6. Flame the loop again and cool.

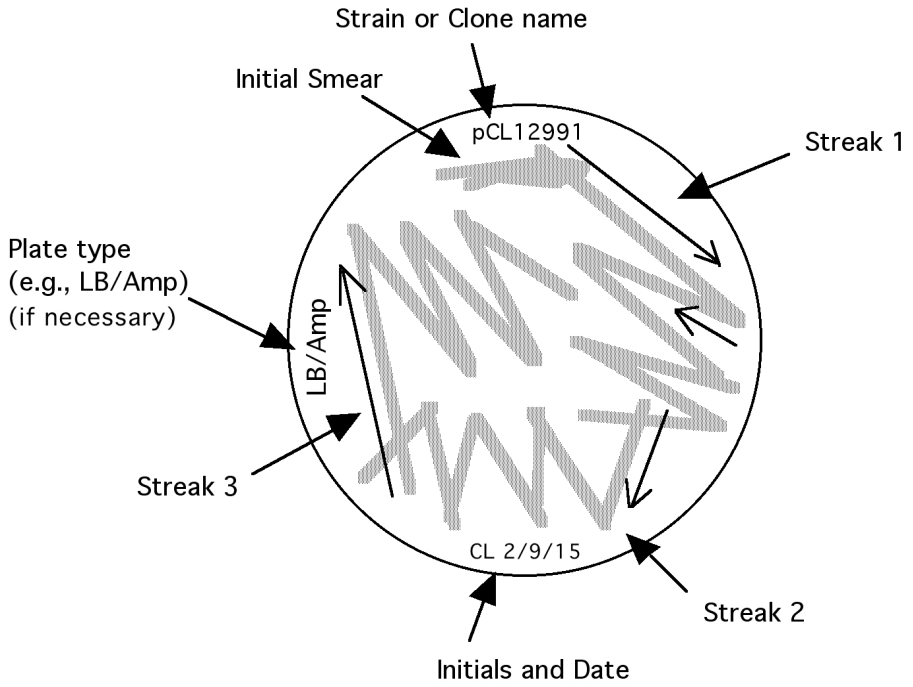
7. Pass the loop ONCE through the initial smear region, and continue into a clean region of the plate. Slide the loop on the surface back and forth several times (actually 2 or 3 times more than the illustration indicates). Use about 1/3 – 1/4 of the plate area.

Note that if you tilt the plate to reflect the ambient light just right you should be able to make out on the surface of the plate where you have previously streaked with your loop.

8. Flame the loop again, cool, and pass once through the *end* of the first streak, and again streak back and forth through about 1/3 – 1/4 of the plate area.

9. Repeat one more time to make a 3rd streak.

Figure: Anatomy of a Bacterial Streak Plate



Notes: Each new streak begins in the final pass of the previous streak. (The number of back & forth spreading is actually much more than illustrated.) When labeling the plate, write small, near the edges, on the media-containing part of the plate (not the lid). Write the plate type if there is any potential for confusion.

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10. Repeat this for the remaining 3 plates. Then place all your plates (lid down) in the 37°C incubator to grow overnight.

Next Day (parafilm, scissors needed)

11. Remove plates from the incubator. Observe and record the growth on the plates (recreate the chart you made above AND using your phone record an image of each plate). If you do not have well isolated colonies for either of the plates we are keeping, repeat the streaking process and grow again.

IV. 5 ml Culture Growth Curve *Using the bacteria harboring the pQE60 you will generate a simple growth curve to monitor the time it takes for cells to reach stationary phase and begin to dye. To do this you will isolate one of your colonies from the appropriate plate in experiment III above. Then observe the OD 600 to determine the relative cell growth in your culture. Start the experiment in the morning (8-9 am) take at least two readings before 6 pm and then again the next morning for several hours. A total of 5-8 readings should give your group a reasonable growth curve.*

1. Using the bacteria harboring your bacterial streak plate from the refrigerator and remove the parafilm.

2. Prepared a culture tube with 5 ml LB broth with Amp. Light the burner and flame the loop. Cool the loop in a clean part of the plate.
   - Determine the OD 600 of your culture after using the "common" LB broth blank. Be certain the blank doesn't have growth or it will not be a proper blank. Use your own plastic cuvette. Transfer ~500 ul of culture into the cuvette. After recording the reading, discard the media and wash the cuvette.
   - Create a table in your ELN for this and all following readings.

3. Pick most or all of one large isolated colony with a uniform appearance. (If you do not have a plate with any isolated colonies, arrange to use someone else's plate.) Without setting the cap down, place the colony into the broth, and agitate the loop to dislodge the bacteria into the LB broth. Re-flame the loop.

3. Replace the cap on the 'fingers' to allow air to enter the tube. Do not close or the culture will grow poorly.

4. Place your culture tube into the rack in the 37° shaking incubator in the autoclave room. Add your tube to the tube rack in the shaker at the bottom of the incubator. Ensure the shaker is on. Be sure the door to the incubator is completely closed.

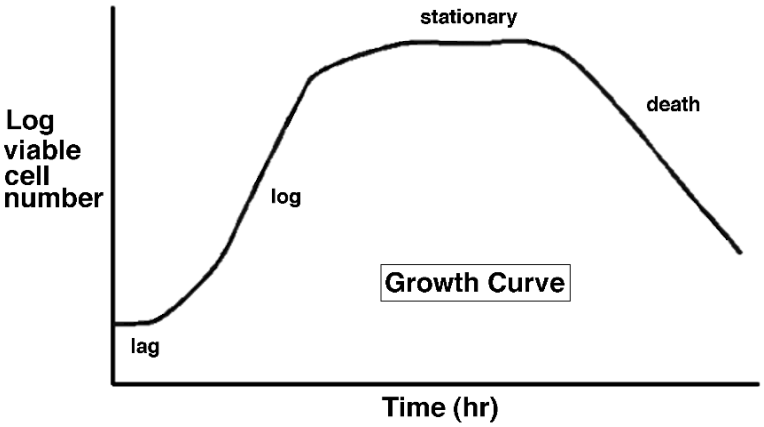
5. Take a few moments for proper cleanup: Return equipment to where you found it. Re-parafilm your bacterial plate and return to the refrigerator. Wash your hands before you leave.

Note the time you started your overnight culture in your lab book. Create a table of times and readings for each. Take an image of the tube once you can see a "cloudy" growth. This will help you judge the density and timing of growth for this and other laboratories.

6. Design and plan with your group the times to collect your results. Carefully review the comments at the beginning of this section to create your plan. Include your plan with appropriate tables in your ELN.

Why do shorter cultures? See the bacterial growth curve at right – after the cells run out of nutrients and accumulate waste products, entering 'stationary phase' in which no further cell division occurs, eventually cells begin to die. The actual course of the growth curve depends on the bacterial strain and the precise growth conditions.

Figure modified from image found at http://pathmicro.med.sc.edu/fox/growth_c.jpg



Create a growth curve graph in your ELN. Share your results with at least two other groups to see if the growth curves are similar.

Discuss how long you might leave a culture if you want, early, late log culture or stationary culture of bacteria without cell death.