

MOLEBIO LAB #12: Bacterial Culture Techniques – Part I

Introduction:

This lab introduces an introduction to plating and culturing *E. coli* on LB agar plates such that single cells can be isolated from one another. Each cell then reproduces to form a visible colony composed of genetically identical clones. Streaking cells to obtain individual colonies is usually the first step in genetic manipulations of microorganisms. Using cells derived from a single colony minimizes the chance of using a cell mass contaminated with a foreign microorganism. To demonstrate antibiotic resistance, the growth of wild-type *E. coli* and that of *E. coli* containing an ampicillin resistance gene are compared, using LB medium containing ampicillin. The resistant strain contains the plasmid pAMP, which produces an enzyme that destroys the ampicillin in the medium, thus, allowing these cells to grow.

Protocols involving bacterial growth, transformation, and plasmid isolation have been tested and optimized with *E. coli* strain MM294, derived in the laboratory of Matthew Meselson at Harvard University. MM294/pAMP has already been transformed with pAMP, an ampicillin resistance plasmid constructed at Cold Spring Harbor Laboratory. Other strains commonly used for molecular biological studies should give comparative results.

HANDLING AND DISPOSAL OF *E. coli* MM294

E. coli is a normal part of the bacterial fauna of the human gut. It is not considered pathogenic and is rarely associated with any illness in healthy individuals. MM294 and other commonly used lab strains, are ineffective in colonizing the human gut. Adherence to simple guidelines for handling and disposal makes work with *E. coli* a non-threatening experience.

1. To avoid contamination, always re-ignite inoculating loop or cell spreader on final time before placing it on the lab bench/countertop.
2. Keep nose and mouth away from tip end when pipetting suspension culture to avoid inhaling any aerosol that might be created.
3. Do not over-incubate plates. Because a large number of cells are inoculated, *E. coli* is generally the only organism that will appear on plates incubated from 15-20 hours. However, with longer incubation, contaminating bacteria and slower-growing fungi can arise. If plates cannot be observed following initial incubation, refrigerate them to retard growth of contaminants.
4. Collect for treatment bacterial cultures *and* tubes, pipettes, and micropipettor tips that have come into contact with the cultures. Disinfect these materials as soon as possible after use. Contaminants, often odorous and sometimes potentially pathogenic, are readily cultured over a period of several days at room temperature.
 - Disinfect by using a 10% bleach solution. Immerse contaminated pipettes, tips, and tubes (opened) directly into sink or tub containing bleach solution. Plates should be placed, with lids open, in sink or tub, and flooded with bleach solution. Allow materials to stand in bleach for 15 minutes or more. Then drain excess bleach solution, seal materials in plastic bag, and dispose in accordance with local regulations.
5. Wipe down lab bench/countertop with soapy water, 10% bleach solution, or disinfectant at the end of the lab.
6. Always WASH YOUR HANDS before leaving the lab.

Procedure:

We will prepare LB and LB/amp plates for this protocol. This will most likely involve coming in after school to pour the plates after using the autoclave. Plan to stay after school!

PREPARING LB AGAR PLATES

1. Weigh out:
 - a. 2.5 g tryptone (an assortment of peptides from digested casein – cheese – protein)
 - b. 1.25 g of yeast extract
 - c. 2.5 g of NaCl (m.w. = 58.44)
 - d. 3.75 of agar

(We might use a prepared mixture of these instead...)
2. Add all ingredients to a clean 500 mL flask (or beaker) that has been rinsed with distilled water.
3. Add 250 milliliters of deionized or distilled water.
4. Add 0.125 mL (which is 125 μ L) of 4 N NaOH.
5. Stir to dissolve dry ingredients. Any undissolved materials will dissolve during autoclaving.
6. Cover flask mouth with aluminum foil/cotton, and autoclave solution of 15 minutes at 121 $^{\circ}$ C.
FOLLOW DIRECTIONS ON THE USE OF THE AUTOCLAVE. IMPROPER USE CAN CAUSE SEVERE BURNS OR WORSE!
7. After the sterilization run in the autoclave, let solution to cool down so that the flask(s) can be held in bare hands (55-60 $^{\circ}$ C). If solution cools too long and the agar begins to solidify, remelt by briefly heating it in a microwave for a few minutes.
8. While agar is cooling, wipe down the lab bench with ethanol to sterilize working area.
9. Continue using sterilized lab bench. Mark *culture plate bottoms* with the date and description of the media (e.g., LB or LB/amp). If using presterilized polystyrene plates, carefully cut the end of plastic sleeves, and save the sleeves for storing the poured plates. Keeping the culture plates closed, spread them out on the lab bench.
10. When the agar flask is cool enough to hold, lift lid of culture plate only enough to pour solution. *Do not place lid on lab bench.* Quickly pour in agar to just cover plate bottom (~20 – 25 mL). Tilt the plate to spread the agar, and immediately replace lid.
11. To remove any bubbles in the surface of the poured agar, touch plate surface with the flame from the Bunsen burner while agar is still liquid. Let agar solidify undisturbed for 5 – 10 minutes.
12. Incubate plates *lidside down* for several hours at 37 $^{\circ}$ C (overnight recommended). This dries the agar, limiting condensation when plates are stored under refrigeration. It also allows the ready detection of any contaminated plates.
13. Stack plates and mark once down the side of the stack with a blue marker – representing LB plates. We will use other colors for other plate preparations. (Add a red mark down as well for LB/amp)
14. Store at 4 $^{\circ}$ C; plates should last for 3 months.

PREPARING LB AGAR + ANTIBIOTIC (amp) PLATES

15. Follow the above steps for LB agar plates through step 8.
16. When agar flask is cool enough to hold, sterilely add 2.5 mL of 10 mg/mL ampicillin. Ampicillin (and many other antibiotics in that class) is destroyed at temperatures above 60 $^{\circ}$ C; therefore, *it is essential to cool agar before adding antibiotic.* Swirl flask(s) to mix. Resume with step 9.

PLATE-STREAKING TECHNIQUE

As with sterile pipetting, plan out manipulations before beginning to streak plates. Organize the lab bench to allow plenty of room and work quickly.

17. Use a permanent marker to label the *bottom* of each plate with your name (or group/class ID #) and the date (of inoculation). Each plate will have been previously marked to indicate whether it is plain LB agar or LB agar + ampicillin, along with the date of preparation.
18. Take two LB plates. Mark one plate –pAMP for cells without plasmid and the other plate +pAMP for cells with a plasmid.
19. Take two LB/amp plates. Mark one plate –pAMP for cells without plasmid and the other plate +pAMP for cells with a plasmid.
20. Hold the inoculating loop like a pencil, and sterilize the loop in the Bunsen burner flame until it glows red hot. Then continue to pass the lower shaft through the flame.
21. Cool for 5 seconds. *To avoid contamination, do not place inoculating loop on lab bench.*
22. Use one of the following techniques to scrap up *E. coli*.
If working from a culture plate:
 - a. Remove the lid from the *E. coli* culture plate with your free hand. *Do not place lid on the lab bench!* Hold the lid face down just above the culture plate to help prevent contaminants from falling on the plate or lid.
 - b. Stab inoculating loop into a clear area of the agar several times to cool.
 - c. Use the loop tip to scrap up a visible cell mass from a colony. Do not gouge agar. Replace culture plate lid and proceed to step 23.
23. Select LB –pAMP plate and lift lid only enough to perform streaking as directed below and also shown in **Figure 1 on the next page**. *Do not place top on lab bench.*
 - *Streak 1:* Glide inoculating loop tip back and forth across the agar surface to make a streak across the top of the plate. Avoid gouging agar. Replace lid of plate between streaks.
 - *Streak 2:* Reflame inoculating loop and *cool* by stabbing it into the agar away from the first (primary) streak. Draw loop tip through the end of the primary streak and, without lifting loop, make a zigzag streak across one quarter of the agar surface. *Replace plate lid.*
 - *Streak 3:* Reflame loop and *cool* in the agar as described above. Draw loop tip through the end of the secondary streak, and make another zigzag streak in the adjacent quarter of the plate *without touching the previous streak.*
 - *Streak 4:* Reflame loop and *cool* in the agar as described above. Draw tip through the end of the tertiary streak, and make a final zigzag streak in remaining quarter of plate.

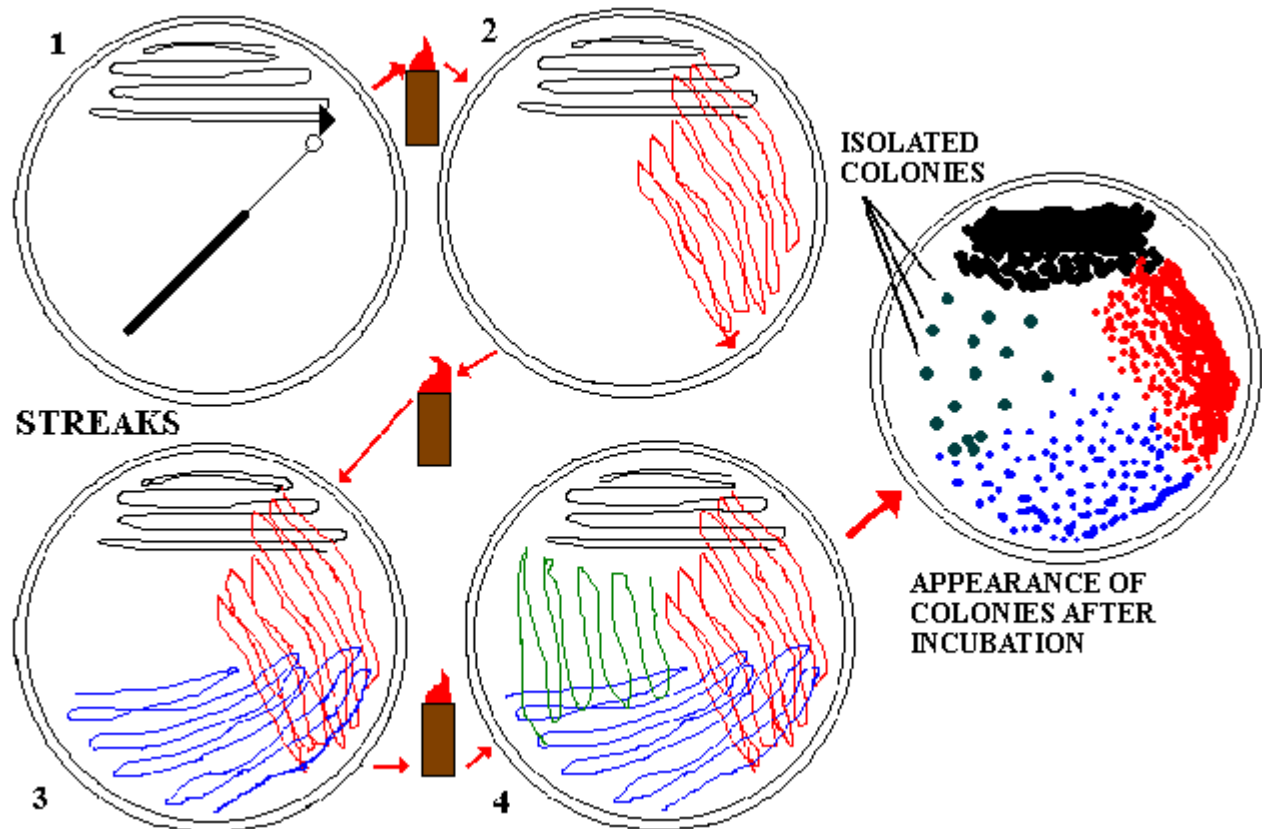


Figure 1: Streaking technique to isolate individual colonies.¹

24. Repeat steps 20 – 23 to streak *E. coli* onto LB/amp –pAMP plate.
25. Repeat steps 20 – 23 to streak *E. coli* /pAMP onto LB +pAMP plate.
26. Repeat steps 20 – 23 to streak *E. coli* /pAMP onto LB/amp +pAMP plate.
27. Reflame the loop, and allow it to cool, before placing it on lab bench. **Make it a habit to always flame the loop one last time!**
28. Place plates upside down in a 37 °C incubator and incubate for 15 – 20 hours. (Plates are inverted to prevent condensation that might collect on the lids from falling back on the agar and causing colonies to run together.)
29. Take time for a responsible cleanup.
30. Optimal growth of well-formed, individual colonies is achieved in 15 – 20 hours. At this point, colonies should range in diameter from 0.5 mm to 3.0 mm. Wrap with parafilm to retard drying and store at 4 °C.

Micklos, David A. Freyer, Greg A. *DNA Science*. 2nd edition. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press, 2003. 321-330. Print.

¹ <http://www.slic2.wsu.edu:82/hurlbert/micro101/pages/101lab3.html>

Name: _____

Name: _____

Observe plates and use the matrix below to record which plates have bacterial *growth* and which have *no growth*. On plates with growth, distinct, individual colonies should be observed within one of the streaks.

On the LB/amp plate, growth must be observed in the secondary streak to count as antibiotic-resistant growth. In a heavy inoculum, nonresistant cells in the primary streak may be isolated from the antibiotic on a bed of other nonresistant cells.

	Transformed cells +pAMP	Wild-type cells -pAMP
LB/amp	(experiment)	(negative control)
LB	(positive control)	(positive control)

On the LB/amp +pAMP plate, tiny "satellite" colonies may be observed radiating from the edges of large well-established colonies. These satellite colonies are NOT ampicillin-resistant, but grow in an "antibiotic shadow," where ampicillin in the media has been broken down by the large resistant colony. Satellite colonies are generally a sign of antibiotic weakened by not cooling the medium enough before adding the antibiotic, long-term storage of more than 30 days, or overincubation.

1. Describe the appearance of a single *E. coli* colony. Why can the cells of this colony be considered genetically homogeneous?

2. Upcoming laboratories use cultures of *E. coli* cells derived from a single colony. Why is it important to use this type of culture in genetic experiments?
