Bacterial Culture Techniques

LABORATORY 2 CONTAINS MOST OF THE CULTURE TECHNIQUES used throughout the course. We suggest that Part A (Isolation of Individual Colonies) be done in sequence between Laboratory 1 and Laboratory 3. Part B (Overnight Suspension Culture) need be done only in conjunction with plasmid purification in Laboratories 8 and 12. Part C (Mid-log Suspension Culture) is done in preparation for making competent cells by the standard calcium chloride procedure in Laboratory 10.

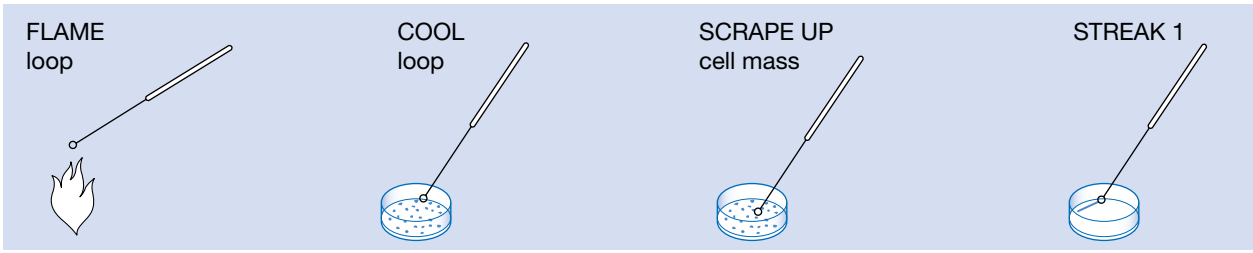
- Part A provides a technique for streaking *E. coli* cells onto LB agar plates such that single cells are isolated from one another. Each cell then reproduces to form a visible colony composed of genetically identical clones. Streaking cells to obtain individual colonies is usually the first step in 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 an *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.
- Part B provides a protocol for growing small-scale suspension cultures of *E. coli* that reach stationary phase with overnight incubation. Overnight cultures are used for purification of plasmid DNA and for inoculating mid-log cultures. When growing *E. coli* strains that contain a plasmid, it is best to maintain selection for antibiotic resistance by growing in LB broth containing the appropriate antibiotic. Strains containing an ampicillin resistance gene (such as pAMP) should be grown in LB broth plus ampicillin.
- Part C provides a protocol for preparing a mid-log culture of *E. coli*. Cells in mid-log growth can generally be rendered more competent to uptake plasmid DNA than can cells at stationary phase. Mid-log cells are used in the classic transformation protocol described in Laboratory 10. 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 (reculture) 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

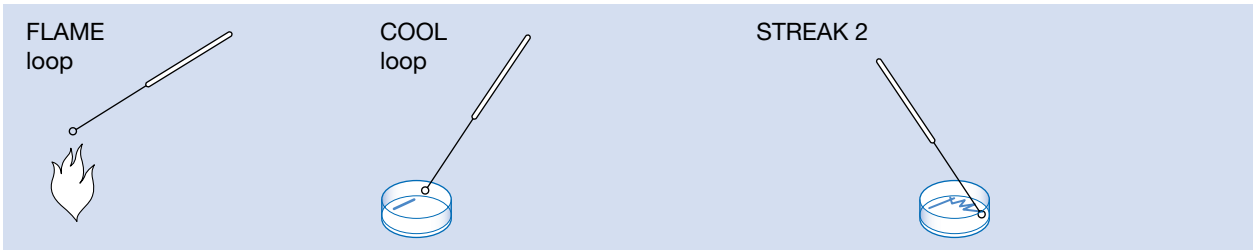
lag phase, the cells enter the log-growth phase. As a general rule, 1 volume of overnight culture (the *inoculum*) is added to 100 volumes of fresh LB broth in an Erlenmeyer flask. To provide good aeration for bacterial growth, the flask volume should be at least four times the total culture volume.

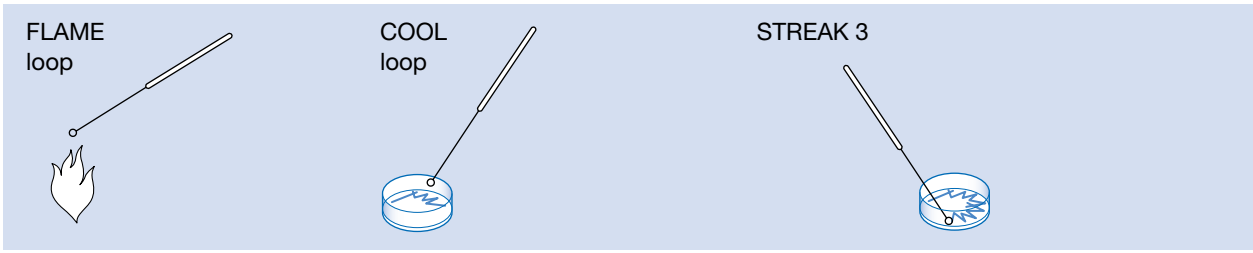
Equipment and materials for this laboratory are available from the Carolina Biological Supply Company (see Appendix 1).

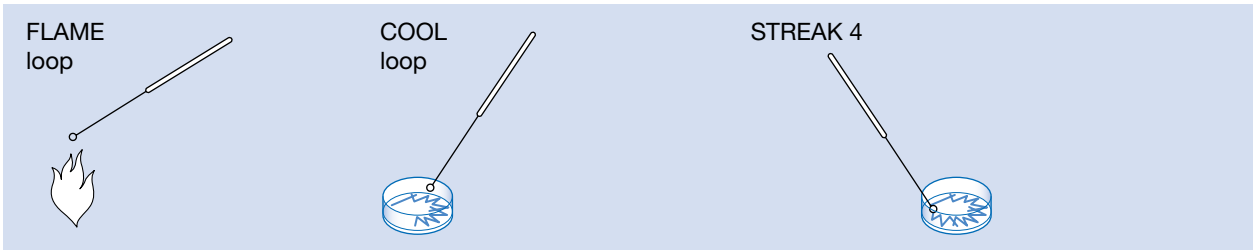
PART A

Isolation of Individual Colonies









PRELAB NOTES

E. coli Strains

All 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 been transformed with pAMP, an amplicillin resistance plasmid constructed at Cold Spring Harbor Laboratory. Other strains commonly used for molecular biological studies should give comparable results. However, growth properties of other *E. coli* strains in suspension culture may differ significantly. For example, the time needed to reach mid-log phase and the cell number represented by specific optical densities differ from strain to strain.

Nutrient Agar

We prefer LB (Luria-Bertani) agar, but almost any rich nutrient agar can be used for plating cells. Presterilized, ready-to-pour agar is a great convenience. It needs only to be melted in a microwave oven or boiling water bath, cooled to approximately 60°C, and poured onto sterile culture plates.

> **CAUTION**
> To prevent boiling over, the container should be no more than half full. Loosen the cap to prevent the bottle from exploding.

Ampicillin

Plasmids containing ampicillin resistance are most commonly used for cloning DNA sequences in *E. coli*. Ampicillin is very stable in agar plates, thresholds for selection are relatively broad, and contaminants are infrequent. Despite its stability, ampicillin, like most antibiotics, is inactivated by prolonged heating. Therefore, it is important to allow the agar solution to cool until the container can be held comfortably in the hand (~60°C) before adding antibiotic. Use the sodium salt, which is very soluble in water, instead of the free acid form, which is difficult to dissolve.

Responsible Handling and Disposal of *E. coli*

A commensal organism of *Homo sapiens, 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. Furthermore, K-12 *E. coli* strains, including MM294 and all 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 nonthreatening experience.

1. To avoid contamination, always reflame inoculating loop or cell spreader one final time before placing it on the lab bench.
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 for 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 bacteria-contaminated materials in one of two ways:
   a. Autoclave materials for 15 minutes at 121°C. Tape three to four culture plates together and loosen tube caps before autoclaving. Collect contaminated materials in a "bio bag" or heavy-gauge trash bag; seal bag before autoclaving. Dispose of autoclaved materials in accordance with local regulations.

      or

   b. Treat with solution containing 5000 parts per million (ppm) available chlorine (10% bleach solution). Immerse contaminated pipettes, tips, and tubes (open) 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 solution 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 with soapy water, 10% bleach solution, or disinfectant (such as Lysol) at the end of lab.
6. Wash hands before leaving lab.

PRELAB PREPARATION

Before performing this Prelab Preparation, please refer to the cautions indicated on the Laboratory Materials list.

1. About 1 day to 1 week before class, MM294 must be streaked onto several LB agar plates and MM294/pAMP must be streaked onto several LB+ampicillin (LB/amp) plates. Following overnight incubation at 37°C, wrap the plates in Parafilm or plastic wrap to prevent drying and store at 4°C (in a refrigerator) until they are needed. Alternately, streak directly from stab or slant cultures.
2. Prepare for each experiment:

   2 LB agar plates
   2 LB/amp plates
3. Make sure that the plate type is clearly marked on the bottom of the plate (not on the lid).
4. Prewarm incubator to 37°C.

MATERIALS

CULTURE AND PLATES	SUPPLIES AND EQUIPMENT
LB agar (LB) plates (2)	"Bio bag" or heavy-duty trash bag
LB+ampicillin▼ (LB/amp) plates (2)	Bleach (10%)▼ or disinfectant
MM294 culture	Bunsen burner
MM294/pAMP culture	Incubator (37°C)
	Inoculating loop
	Permanent marker

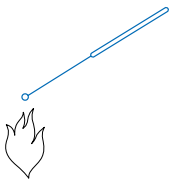
▼ See Appendix 4 for Cautions list.

METHODS

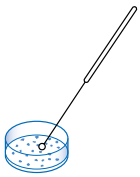
Plate-streaking Technique (15 minutes)

As with sterile pipetting, plan out manipulations before beginning to streak plates. Organize the lab bench to allow plenty of room and work quickly. If working from a stab or slant culture, loosen the cap before starting.

1. Use a permanent marker to label the *bottom* of each agar plate with your name and the date. Each plate will have been previously marked to indicate whether it is plain LB agar (LB) or LB agar+ampicillin (LB/amp).
2. Select the two LB plates. Mark one plate –pAMP for cells without plasmid and the other plate +pAMP for cells with plasmid.
3. Select the two LB/amp plates. Mark one plate –pAMP for cells without plasmid and the other plate +pAMP for cells with plasmid.



4. 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 lower half of shaft through flame.
5. Cool for 5 seconds. *To avoid contamination, do not place inoculating loop on lab bench.*
6. Use one of the techniques below to scrape up *E. coli*.



If working from culture plate:

a. Remove the lid from the *E. coli* culture plate with free hand. *Do not place lid on 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 scrape up a visible cell mass from a colony. Do not gouge agar. Replace culture plate lid, and proceed to Step 7.

or

If working from stab culture:

a. Grasp the bottom of the *E. coli* culture vial between thumb and two fingers of free hand. Remove vial cap using little finger of same hand that holds inoculating loop. *Avoid touching rim of cap.*

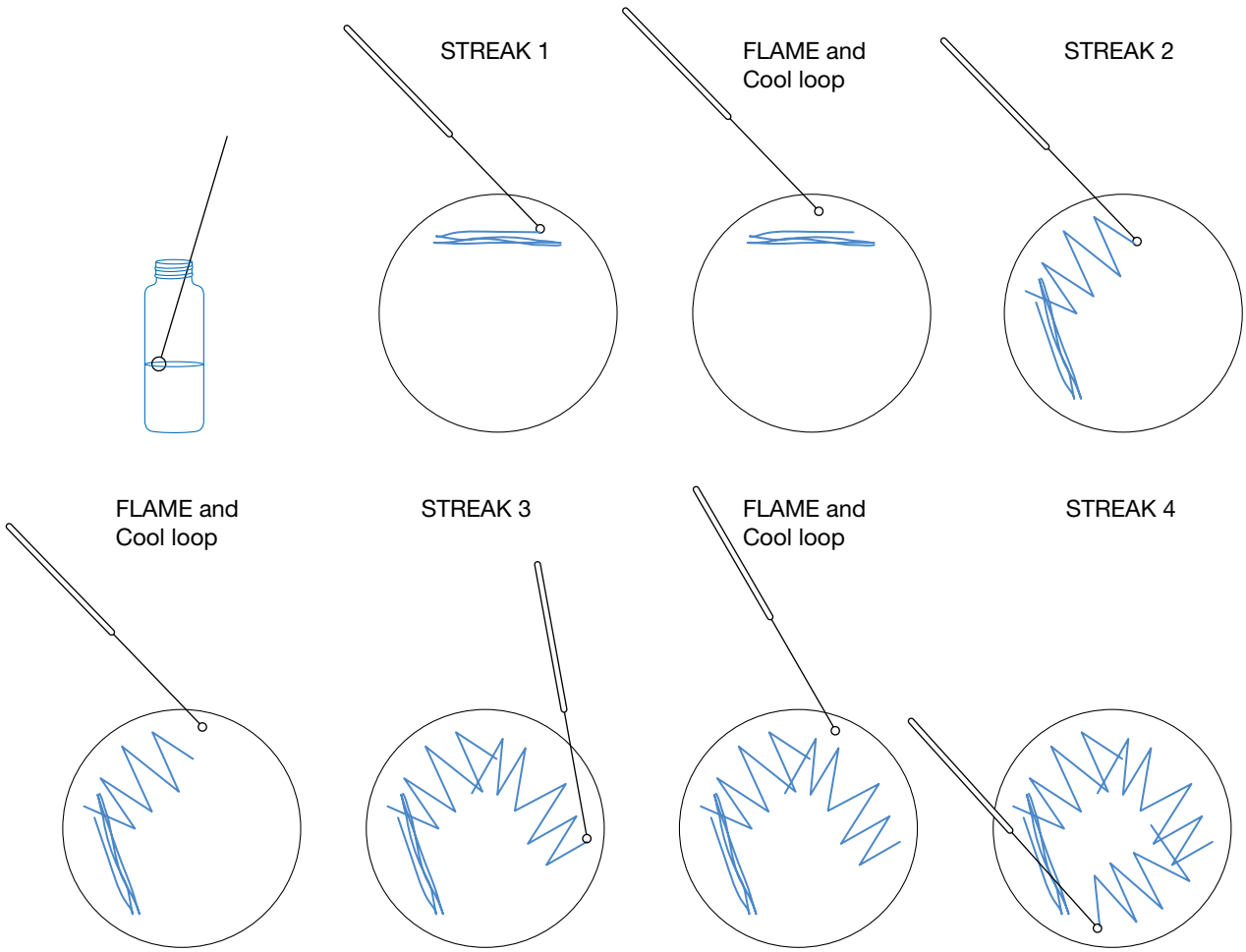
b. Quickly pass mouth of vial several times through burner flame.

c. Stab inoculating loop into side of agar several times to cool.

d. Scrape the loop several times across area of culture where bacterial growth is apparent. Remove loop, flame vial mouth, and replace cap. Proceed to Step 7.

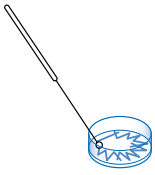
7. Select LB –pAMP plate and lift lid only enough to perform streaking as shown below. *Do not place top on lab bench.*

- *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.

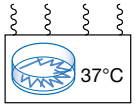


Streaking to Isolate Individual Colonies (Step 7)

- *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 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* it as above. Draw tip through the end of the tertiary streak, and make a final zigzag streak in remaining quarter of plate.



8. Repeat Steps 4–7 to streak *E. coli* onto LB/amp –pAMP plate.
9. Repeat Steps 4–7 to streak *E. coli*/pAMP onto LB +pAMP plate.
10. Repeat Steps 4–7 to streak *E. coli*/pAMP onto LB/amp +pAMP plate.
11. Reflame the loop, and allow it to cool, before placing it on the lab bench. Make it a habit to always flame loop one last time.



12. 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.)

If cells are to be used for colony transformation, continue incubating plates at room temperature for 1 day. During this time, colonies grow large and become sticky, making them easier to pick up with an inoculating loop.

13. 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 mm.
14. Take time for responsible cleanup.
    a. Segregate bacterial cultures for proper disposal.
    b. Wipe down lab bench with soapy water, 10% bleach solution, or disinfectant (such as Lysol) at end of lab.
    c. Wash hands before leaving lab.

RESULTS AND DISCUSSION

This laboratory demonstrates a method to streak bacteria to single colonies. It also introduces antibiotics and plasmid-borne resistance to antibiotics—topics that will be important in several laboratories that follow.

There are two classes of antibiotics: *bacteriostats,* which prevent cell growth, and *bacteriocides*, which kill cells outright. Two antibiotics are used in this course. Ampicillin is a bacteriostatic agent (inhibits the growth of bacteria) and kanamycin is a bacteriocide (kills bacteria). Ampicillin, a derivative of penicillin, blocks synthesis of the peptidoglycan layer (sometimes referred to as the "cell wall") that lies between the *E. coli* inner and outer cell membrane. Thus, it does not affect existing cells with intact cell walls, but kills dividing cells as they synthesize new peptidoglycan. Kanamycin (introduced in later laboratories) is a member of the aminoglycoside family of antibiotics, which block protein synthesis by covalently modifying the bacterial ribosome. Thus, kanamycin quickly kills both dividing and quiescent cells.

The ampicillin resistance gene carried by the plasmid pAMP produces a protein, β-lactamase, that disables the ampicillin molecule. β-lactamase cleaves a specific bond in the β-lactam ring, a four-membered ring in the ampicillin mol-

ecule that is essential to its antibiotic action. β-lactamase not only disables ampicillin within the bacterial cell, but because it leaks through the cell envelope, it also disables ampicillin in the surrounding medium. The enzyme kanamycin phosphotransferase prevents kanamycin from interacting with the ribosome.

Antibiotic-resistant Growth

If the plates cannot be observed on the day after streaking, store them at 4°C to arrest *E. coli* growth and to slow the growth of any contaminating microbes. Wrap in Parafilm or plastic wrap to retard drying.

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.

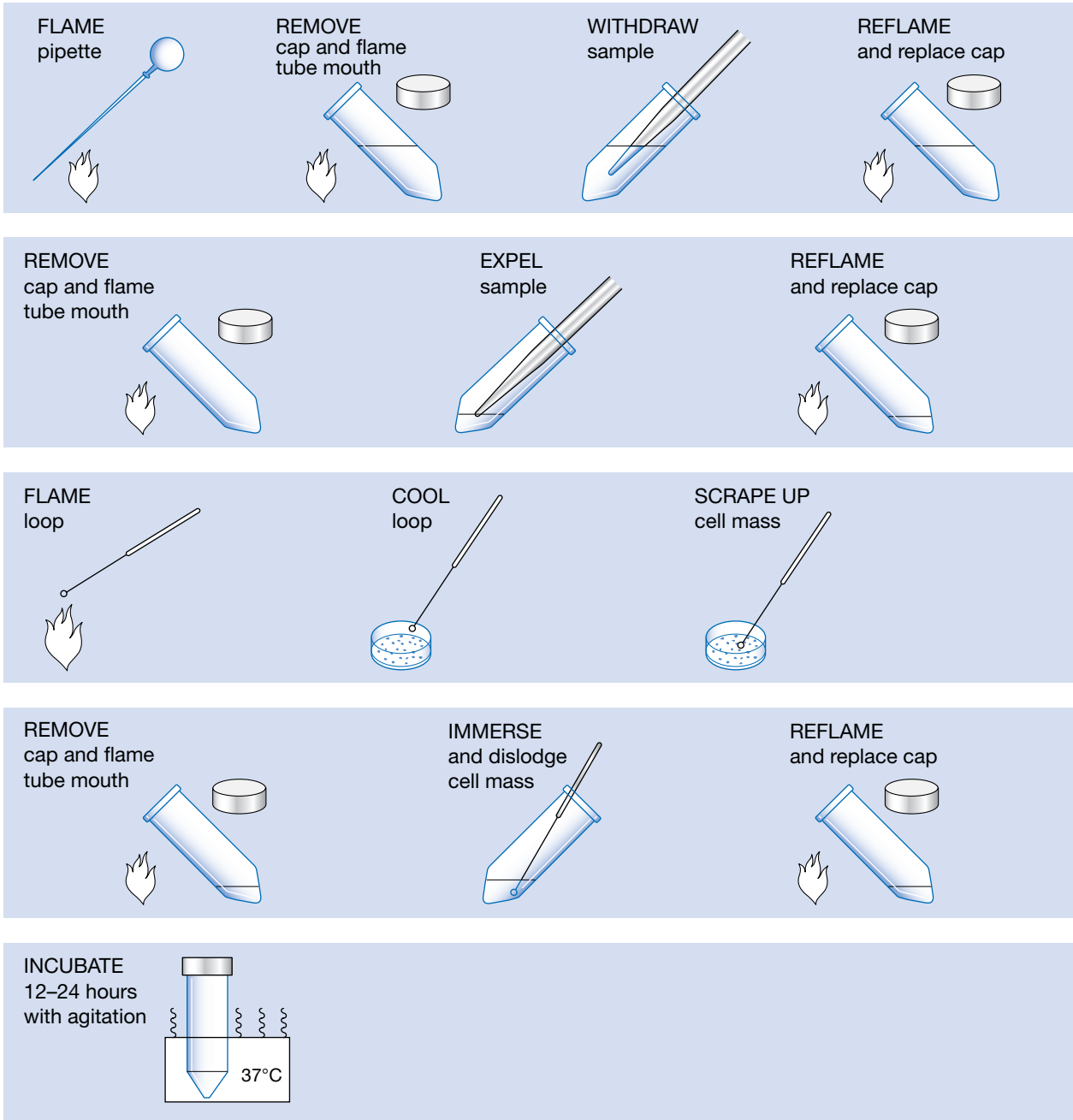
	Transformed cells +pAMP	Wild-type cells –pAMP
LB/amp	experiment	negative control
LB	positive control	positive control

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. Were results as expected? Explain possible causes for variations from expected results.
2. In Step 7:
   a. What is the reason for the zigzag streaking pattern?
   b. Why is the inoculating loop resterilized between each new streak?
   c. Why should a new streak intersect only the end of the previous one only at a single point?
3. Describe the appearance of a single *E. coli* colony. Why can it be considered genetically homogeneous?
4. Upcoming laboratories use cultures of *E. coli* cells derived from a single colony or from several discrete parental colonies isolated as described in this experiment. Why is it important to use this type of culture in genetic experiments?
5. *E. coli* strains containing the plasmid pAMP are resistant to ampicillin. Describe how this plasmid functions to bring about resistance.
6. A major medical problem is the ever-increasing number of bacterial strains that are resistant to specific antibiotics. Antibiotic resistance is carried on circular DNA molecules, called plasmids, which are generated separately from the cell's chromosome. With this in mind, suggest a mechanism through which new antibiotic-resistant strains of bacteria arise.

PART B

Overnight Suspension Culture



PRELAB NOTES

Review Prelab Notes in Laboratory 2A, Isolation of Individual Colonies.

Although it is always best to grow any overnight culture with shaking, it is not absolutely essential when growing cells for purifying plasmid or for inoculating a larger culture. For these purposes, suspensions can be incubated, without shaking, in a rack within a 37°C incubator. However, the cultures will need to incubate for 1 day or more to obtain an adequate number of cells.

A 50-ml conical tube is preferable for growing overnight cultures. It provides greater surface area for aeration than does a 15-ml culture tube, although a 15-ml tube can be also be used. In overnight cultures for plasmid preparations, it is best, but not essential, to maintain antibiotic selection of the transformed strain by growing in medium containing the appropriate antibiotic. It is prudent to inoculate a "back up" overnight culture, in case the first was not inoculated properly.

PRELAB PREPARATION

Before performing this Prelab Preparation, please refer to the cautions indicated on the Laboratory Materials list.

1. For plasmid purification: Make sure a freshly streaked plate (less than 1 week old) of a transformed or plasmid-bearing *E. coli* strain is available.
2. For mid-log culture: Make sure a freshly streaked plate of wild-type MM294 or other *E. coli* strain is available. It is convenient to make the overnight culture exactly the size of the inoculum needed to start mid-log culture: 1-ml overnight per 100-ml mid-log culture. The entire overnight culture is then simply poured into a flask of fresh, sterile LB broth.
3. Prewarm shaking water bath or incubator to 37°C.

MATERIALS

CULTURE AND MEDIA

E. coli plate
LB broth with appropriate antibiotic

SUPPLIES AND EQUIPMENT

Bleach (10%)▼ or disinfectant
Bunsen burner
Conical tube (50-ml), sterile
Inoculating loop
Permanent marker
Pipette (10-ml standard)
Pipette aid or bulb
Shaking water bath (37°C) (or 37°C dry shaker or dry shaker + 37°C incubator)

▼ See Appendix 4 for Cautions list.

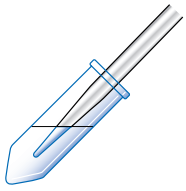
METHODS

Prepare Overnight Culture (10 minutes)

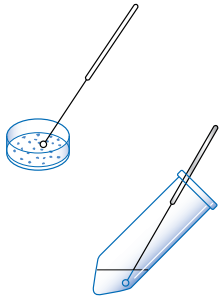
Review Sterile Use of 10-ml Standard Pipette, section III in Laboratory 1, Measurements, Micropipetting, and Sterile Techniques. Think sterile! A pipette should be considered contaminated whenever the tip end comes into contact with anything in the environment—lab bench, hands, or clothing. When contamination is suspected, discard pipette and start with a fresh one. Plan out steps to perform, organize lab bench, and work quickly.

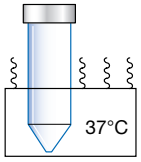
If working as a team, one partner should handle the pipette and the other should handle the tubes and caps.

Pipette flaming can be eliminated if individually wrapped pipettes are used.



Loop flaming is eliminated if an individually wrapped, sterile plastic loop is used.





1. Label a sterile 50-ml tube with your name and the date. The large tube provides a greater surface area for good aeration of culture.
2. Use a 10-ml pipette to sterilely transfer 5 ml of LB broth into the tube.
   a. Attach pipette aid or bulb to pipette. Briefly flame pipette cylinder.
   b. Remove cap of LB bottle using little finger of hand holding pipette bulb. Flame mouth of LB bottle.
   c. Withdraw 5 ml of LB. Reflame mouth of bottle, and replace cap.
   d. Remove cap of sterile 50-ml culture tube. Briefly flame mouth of culture tube.
   e. Expel sample into tube. Briefly reflame mouth of tube, and replace cap.
3. Locate well-defined colony 1–4 mm in diameter on a freshly streaked plate.
4. Sterilize inoculating loop in the Bunsen burner flame until it glows red hot. Then, continue to pass lower half of its handle through the flame.
5. Cool loop tip by stabbing it several times into agar near the edge of plate.
6. Use loop to scrape up a visible cell mass from selected colony.
7. Sterilely transfer colony into culture tube:
   a. Remove cap of the culture tube using little finger of hand holding loop.
   b. Briefly flame mouth of culture tube.
   c. Immerse loop tip in broth, and agitate to dislodge cell mass.
   d. Briefly reflame mouth of culture tube, and replace cap.
8. Reflame loop before placing it on lab bench.
9. Loosely replace cap to allow air to flow into culture. Affix a loop of tape over the cap to prevent it from becoming dislodged during shaking.
10. Incubate for 12–24 hours at 37°C, preferably with continuous agitation. Shaking is not essential for a culture to be used for plasmid purification. The culture can be incubated at 37°C, without shaking, for 1 or more days.
11. Take time for 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 or disinfectant.
    c. Wipe down lab bench with soapy water, 10% bleach solution, or disinfectant (such as Lysol).
    d. Wash hands before leaving lab.

RESULTS AND DISCUSSION

E. coli has simple nutritional requirements and grows slowly on "minimal" medium containing (1) an energy source such as glucose, (2) salts such as NaCl and $MgCl_2$, (3) the vitamin biotin, and (4) the vitamin thiamine (B1). *E. coli* sythesizes all necessary vitamins and amino acids from these precursors and grows rapidly in a complete medium, such as LB. Yeast extract and hydrolized milk protein (casein) provide a ready supply of vitamins and amino acids.

A liquid bacterial culture goes through a series of growth phases. For approximately 30–60 minutes following inoculation, there is a *lag phase* during which there is limited cell growth. The bacteria begin dividing rapidly during *log phase,* when the cell number doubles every 20–25 minutes. As nutrients in the media are depleted, the cells nearly stop dividing and the culture enters *stationary phase*. During *death phase,* waste products accumulate and cells begin to die.

Optimum growth in liquid culture is achieved with continuous agitation, which aerates the cells, facilitates the exchange of nutrients, and flushes away waste products of metabolism. It can be safely assumed that a culture in complete medium has reached stationary phase following overnight incubation with continuous shaking.

A stationary-phase culture will look very cloudy and turbid. Discard any overnight culture where vigorous growth is not evident. Expect less growth in cultures incubated for 1–2 days *without continuous shaking.* To gauge growth, shake the tube to suspend cells that have settled at the bottom of the tube.

1. Why is 37°C the optimum temperature for *E. coli* growth?
2. Give two reasons why it is ideal to provide continuous shaking for a suspension culture.
3. What growth phase is reached by a suspension of *E. coli* following overnight shaking at 37°C?
4. Approximately how many *E. coli* cells are in a 5-ml suspension culture at stationary phase?

FOR FURTHER RESEARCH

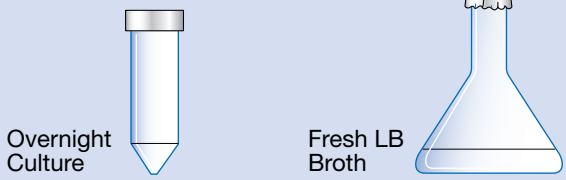
The number of cells in an overnight culture can be determined with a simple experiment.

1. Set up five tubes, marked -2, -4, -6, -8, -10, with 1 ml of sterile water or LB in each of them.
2. Use a micropipettor with a sterile tip to add 10 µl of overnight culture to the first tube (labeled "-2"). Mix the tube thoroughly and use a fresh tip to transfer 10 µl of diluted cells in the -2 tube to the -4 tube. Mix thoroughly and transfer 10 µl from the -4 tube to the -6 tube, to the -8 tube, and finally to the -10 tube.
3. Use a sterile tip to transfer 100 µl of diluted cells from the -10 tube onto an LB plate and spread the cells across the plate. See instructions in Laboratory 5 for spreading bacteria onto plates.

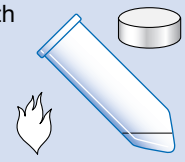
4. Repeat with fresh plates for the -8 and -6 tubes. Incubate the plates overnight and count colonies. Each colony represents a single original cell.
5. Based on the number of colonies/original cells counted and the dilution factor for each tube, calculate the concentration of the cells in the original culture. For example, if there were 15 colonies on the plate spread from the -8 tube, then there were 150 bacteria in the -8 tube (you removed 100 µl out of 1 ml, which is a tenth of the total, so 15 colonies x 10 = 150 bacteria in the -8 tube). The -8 tube was a 10^8-fold dilution of the original culture, so 150 bacteria x 10^8 = 150 x 10^8 or 1.5 x 10^{10} bacteria per milliliter in the original overnight culture.

PART C

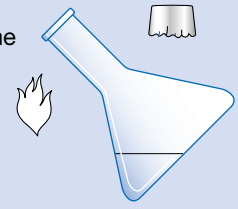
Mid-log Suspension Culture



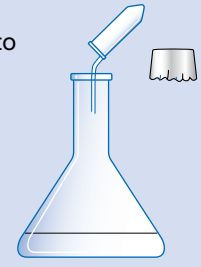
REMOVE
cap and flame
tube mouth



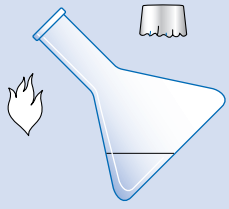
REMOVE
foil cap and flame
flask mouth



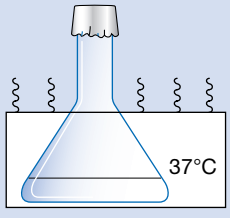
POUR
overnight into
flask



REFLAME
flask mouth and
replace foil cap



INCUBATE
1 3/4–2 hours
with agitation



PRELAB NOTES

Competent Cell Yield

In Laboratory 10A—Classic Procedure for Preparing Competent Cells—each experiment will require 20 ml of mid-log cells, which yields 2 ml of competent cells. If competent cells are being prepared in large quantity for group use, remember that the ratio of mid-log cells to competent cells is 10 to 1. A 100-ml mid-log culture will yield 10 ml of competent cells, sufficient for 50–200 µl transformations.

Sterile Technique

Scrupulous sterile technique must be used when preparing overnight and mid-log cultures. No antibiotic is used, and any contaminant will multiply as cells are repeatedly manipulated and/or stored for future use.

Aeration of Culture

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. An economical alternative to a shaking water bath or temperature-controlled dry shaker is to place a small platform shaker inside a 37°C incubator. For adequate surface-to-volume ratio for the exchange of air, cells should be grown in an Erlenmeyer flask with a volume of LB broth not exceeding one third of the total volume of the flask.

Timing of Culture

Inoculate a mid-log culture 2–4 hours before Laboratory 10 begins. Using the protocol below, *E. coli* strain MM294 reaches mid-log phase after 1.75–2.0 hours of incubation. Cells can be used immediately or held on ice for up to 2 hours before beginning Laboratory 10.

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. A culture begun by inoculating into LB broth prewarmed to 37°C will reach mid-log phase more quickly than one begun at room temperature. Different strains of *E. coli* display different growth properties. Strain MM294 may exhibit different growth properties in a nutrient broth other than LB broth.

PRELAB PREPARATION

Before performing this Prelab Preparation, please refer to the cautions indicated on the Laboratory Materials list.

1. The day before performing this protocol, start a culture of MM294 or other *E. coli* strain to be transformed with plasmid DNA according to instructions in Laboratory 2B, Overnight Suspension Culture.

2. It is convenient to make the overnight culture exactly the size of the inoculum needed to start a mid-log culture, for example, 1 ml of overnight culture for 100 ml of mid-log culture. Then, simply pour the entire overnight culture into a flask of fresh LB broth. Pipetting is not necessary.
3. Sterile flasks of LB broth can be prepared weeks ahead of time. Add 100 ml of LB broth to a 500-ml Erlenmeyer flask and cap with aluminum foil as a microbe barrier. Autoclave for 15 minutes at 121°C (250°F). Cool and store at room temperature, until ready for use.

MATERIALS

CULTURES AND MEDIA

LB broth (sterile)
MM294 overnight culture

SUPPLIES AND EQUIPMENT

Bleach (10%)▼ or disinfectant
Bunsen burner
Erlenmeyer flask (500-ml), sterile
Pipettes (10-ml), sterile (optional)
Shaking water bath (37°C) (or 37°C dry shaker or dry shaker + 37°C incubator)
Spectrophotometer (optional)

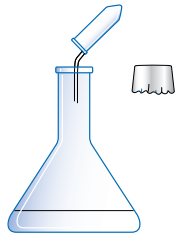
▼ See Appendix 4 for Cautions list.

METHODS

Prepare Culture

(2 hours, including incubation)

Cells will reach mid-log phase more quickly if overnight culture is inoculated into LB prewarmed to 37°C. Time estimate in Step 4 is based on inoculation of room-temperature LB.

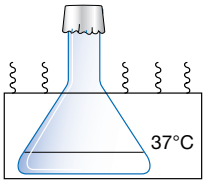


1. Sterilely transfer 1 ml of overnight culture into 100 ml of LB broth at *room temperature.*
2. *If using a 1-ml overnight culture:*
   a. Remove cap from overnight culture tube, and flame mouth. *Do not place cap on lab bench.*
   b. Remove foil cap from flask, and flame mouth. Do not place cap on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

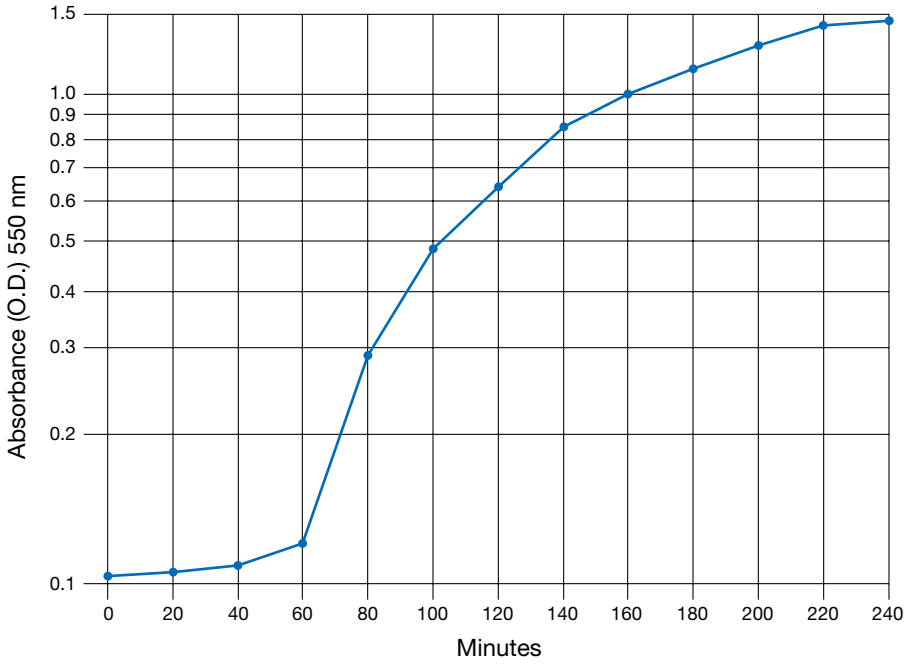
e. Expel overnight sample into flask. Reflame mouth of flask, and replace foil cap.



3. Incubate at 37°C with continuous shaking.

4. *If a spectrophotometer is available:* Approximately 1 hour after inoculation, sterilely withdraw a 1-ml sample of the culture, and measure absorbance (optical density at 550 nm). Repeat procedure at approximately 20-minute intervals. An MM294 culture should be grown to an OD_{550} of 0.3–0.5.

   If spectrophotometer is not available: It can 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 below, an MM294 culture reaches an OD_{550} of 0.3–0.5 in 1 hour, 30 minutes. However, less ideal conditions often result in slower growth.



Growth Curve for *E. coli* Strain MM294

5. Store mid-log culture on ice until ready to begin Laboratory 10. This arrests cell growth. Cells can be stored on ice for up to 2 hours prior to use.

6. Take time for responsible cleanup.

   a. Segregate for proper disposal bacterial cultures *and* tubes and pipettes that have come into contact with the cultures.

   b. Disinfect overnight culture, mid-log culture, tubes, and pipettes with 10% bleach or disinfectant.

   c. Wipe down lab bench with soapy water, 10% bleach solution, or disinfectant (such as Lysol).

   d. Wash hands before leaving lab.

RESULTS AND DISCUSSION

1. What variables influence the length of time for an *E. coli* culture to reach mid-log phase?
2. What are the disadvantages of beginning a mid-log culture from a colony scraped off a plate, as opposed to an inoculum of overnight culture?

FOR FURTHER RESEARCH

This experiment can be started in the morning by one experimenter and continued by others throughout the day, until late afternoon.

1. Start a 500-ml *E. coli* culture as described in the above protocol. Determine the optical density of samples sterilely withdrawn at 20-minute intervals, from time zero for as many hours as possible. Plot a graph of time *versus* OD_{550}.
   a. What is the slope of the curve at a point that corresponds to an OD_{550} of 0.3?
   b. Describe the growth of the culture at this point.
2. Perform the following experiment to correlate the optical density of culture with actual number of viable *E. coli* cells. Observe sterile technique.
   a. Inoculate 500 ml of LB with 5 ml of *E. coli* overnight culture. Swirl to mix.
   b. Immediately remove a 10-ml aliquot (time = 0) of the culture, and place on ice to arrest growth. Then incubate the remaining culture at 37°C with vigorous shaking.
   c. Remove additional aliquots from shaking culture every 20 minutes for a total of 4 hours. Hold each aliquot on ice until ready to perform Steps d–f.
   d. Determine the OD_{550} of each aliquot.
   e. Make a 10^2 dilution by mixing 10 µl of the aliquot with 990 µl of fresh LB broth. Prepare three serial dilutions of each aliquot for plating in Step f:

      10^4 = 10 µl of 10^2 culture + 990 µl of LB
      10^5 = 100 µl of 10^4 culture + 900 µl of LB
      10^6 = 100 µl of 10^5 culture + 900 µl of LB

   f. Spread 100 µl of each dilution onto an LB agar plate, for a total of three plates for each time point (aliquot). *Label each plate bottom with time point and dilution.* Invert plates, and incubate for 15–20 hours at 37°C.
   g. For each time point, select a dilution plate that has between 30 and 300 colonies. Multiply the number of colonies by the appropriate dilution factor to give cell number per milliliter in the original aliquot.
   h. Plot two graphs:

      time (*x* axis) *versus* OD_{550} and cell number (*y* axis)
      cell number (*x* axis) *versus* OD_{550} (*y* axis)

   i. An OD_{550} 0.3–0.4 corresponds to what number of cells?

j. What is the average cell number at each of the following points:

lag phase
first third of log phase (early log)
second third of log phase (mid log)
final third of log phase (late log)
stationary phase

k. Do OD_{550} measurements distinguish between living and dead cells?