Lesson 8a: **TEACHER** Supplemental Prep Guidance.

Bend Question:
Why don't antibiotics work like they used to?

Lesson 8a: **Teacher Supplemental Prep Guidance, Procedures, Tips, and Safety Considerations**

E. coli Preparation

Depending on your budget and the number of teachers that need materials, there are two methods for preparation. Method #1 involves less time and allows for 3 preparations of bacteria for each vial of lyophilized *E. coli* (which should be enough to plate the bacteria for 3 classes). Method #2 involves more time, but will enable 10 classes to plate bacteria from only 1 vial of lyophilized *E. coli.*

Method #1 - link to demo video

1. 1 vial contains 30 mg of lyophilized *E. coli*.
2. Measure 30 mL of distilled water.
3. Uncap vial and use a sterile pipette to add some of the 30 mL of distilled water to the vial.
4. Cap vial and shake gently to hydrate and suspend the *E. coli*, then pour into small beaker.
5. Add more distilled water from the original 30 mL to the vial and rinse again and pour into small beaker. Repeat 1-2 more times to ensure the vial is rinsed of most of the bacteria, then dispose of the vial in a biohazard bag or a beaker of 10% bleach solution.
6. Add the rest of the distilled water (from the original 30mL) to the small beaker and mix thoroughly.
7. **This is your stock solution that contains 1mg *E. coli* / 1 mL water**
8. Label three small beakers "10mg *E. coli* / 40 mL water" and add 10 mL of your stock solution to each beaker.
9. Add 30 mL of distilled water to bring the volume up to 40 mL for the final mixture.

Method #2 (culturing *E.coli*)

1. Begin to warm 500 mL of distilled water in an Erlenmeyer flask on a hot plate.
2. As it warms, measure 10 g of Luria Broth Base and dissolve in the warming water.
3. Test with pH paper (should be 7.0) adjust pH if necessary using 1M NaOH.
4. Let the mixture reach boiling (without boiling over)
5. Move flask to the pressure cooker after covering the mouth of the flask with aluminum foil or a loose fitting cap.
6. Follow the directions on the pressure cooker or autoclave (Set to maximum pressure/temp for 20 minutes if using the pressure cooker method).
7. Let the broth cool to about body temperature or cooler.
8. Open the vial of E. coli and use a sterile pipette to transfer some broth to the vial.
9. Cap the vial and agitate to suspend the bacteria. Transfer the suspension to the large flask of broth.
10. Use 2-3 more rinses of broth to ensure the vial is rinsed of most of the bacteria, then dispose of the vial in a biohazard bag.
11. Flame the mouth of the flask and replace the foil cap.
12. Incubate at 34-37°C for 24 hrs (or in a warm room for 48 hrs). Swirl contents as often as possible (unless you are lucky enough to have access to a shaker).

50 mL of this culture can be divided into ten sterile flasks yield enough preparation for 10 classes. If other teachers are not ready on the same day as your classes, this culture may also be subcultured by transferring a portion to another 500 mL of sterile LB broth and growing overnight (If the culture grows for too many days, you get death and biofilm and a smelly room).

To prepare LB Agar plates:

250 mL of LB Agar - link to demo video

1. Begin to warm 250 mL of distilled water in an Erlenmeyer flask on a hot plate (bonus if you have one with a magnetic stirrer).
2. As it warms measure 5g of Luria Broth Base and dissolve in the warming water *(if you are using Luria Broth Agar base, add 8 g of that powder here and skip #5).*
3. Stir with glass rod (unless you have a magnetic stirring hotplate) until dissolved.
4. Test with pH paper (should be 7.0) adjust pH if necessary using 1M NaOH.
5. Measure 4g of Agar powder and add to the mixture. Continue to stir as it warms.
6. Let the mixture come to a boil stirring occasionally, (be watchful - it will boil over and make a huge mess without much warning).
7. Once boiling, the solution will become clear as all the agar dissolves.
8. Move the flask(s) to the pressure cooker after covering the mouth of the flask with aluminum foil. *Depending on how many plates I need to pour, I may have 4 of these 250 mL solutions going at once (that is how many safely fit in the pressure cooker). Double measurements for a 500 mL batch.*
9. Follow the directions on the pressure cooker or autoclave (for pressure cooker, set at maximum pressure/temp for 20 minutes).
10. Let the agar cool enough to touch it for 1-2 seconds without getting burned (around 15 min).

Pouring plates - link to demo video

1. Put on heat resistant glove(s) that have a good grip (wearing a heat glove on your pouring hand may only make it easier to lift the petri dish lids).
2. Prepare your work surface by cleaning the bench top with 70% ethanol or a 10% bleach solution.
3. Open the sterile packages of petri dishes and arrange them so you are ready to pour. Stacks of 4 or 5 dishes allow for quicker pouring.
4. Pour plates. Lift the cover on the bottom dish just far enough to pour the agar. Pour enough agar to cover the bottom of the plate, then replace the lid and repeat the process for the dish above it. Only touch the cover of the dish with your hand and be sure to not touch the dish with the flask. Move quickly and repeat the process until you have poured all prepared agar in the flask. *If agar begins to coagulate before you finish pouring all of your plates, reheat to boiling on hot plate. (*Optional: Flame mouth of flask before starting - use this technique if you are having issues with contamination in plates*).
5. Wait for agar to harden then flip over to cool (agar side on top so condensation doesn't accumulate on lid).

Note: A pressure cooker similar to this is large enough to sterilize up to four 250 mL flasks at one time and works well in the absence of an autoclave.

https://www.amazon.com/gp/product/B074CQYF9D/ref=oh_aui_detailpage_o01_s00?ie=UTF8&psc=1 Rubberized garden gloves from Costco provided both good grip and protection from heat while pouring: https://www.amazon.com/Gardena-Gardening-Gloves-Latex-Polyester/dp/B01MSAKJSA/ref=pd_lpo_vtph_86_tr_img_2?_encoding=UTF8&psc=1&refRID=SD47X8CT3R1T1VHA6YSM

Ampicillin preparation: link to demo video

Using this method will allow 3 separate teachers to have their own preparations of each of the necessary concentrations from a single vial of lyophilized ampicillin. If used in the same day, a single preparation can be used for several classes.

1. 1 vial contains 30 mg of lyophilized ampicillin.
2. Measure 30 mL of distilled water.
3. Uncap vial and place on a sterile surface. Use a sterile pipette to add some of the 30 mL of distilled water to the vial.
4. Return cap to vial and agitate to hydrate and suspend the lyophilized Ampicillin, then pour into small beaker labeled 1 mg/mL ampicillin.
5. Add more distilled water from the original 30 mL to the vial, rinse again, and pour into the 1 mg/mL ampicillin beaker. Repeat 1-2 more times.
6. Add the rest of the distilled water (from the original 30mL) to the beaker labeled 1 mg/mL ampicillin and mix.
7. ***This is your stock solution that contains 1mg/mL Ampicillin***.
8. To get the concentrations you need for the investigation:
    a. Label a small beaker 1 mg/10 mL – use a sterile graduated syringe or sterile pipette to add 1 mL of stock solution and then add 9 mL of distilled water
    b. Label a small beaker 3 mg/10 mL - use a sterile graduated syringe or sterile pipette to add 3 mL of stock solution to a small beaker and then add 7 mL of distilled water
    c. Label a small beaker 5 mg/10 mL - use a sterile graduated syringe or sterile pipette to add 5 mL of stock solution to a small beaker and then add 5 mL of distilled water

Note: you will have enough stock solution to make 3 beakers of each concentration

Once each concentration is made, use a hole punch to make enough filter paper disks for each group and let them soak directly in the ampicillin solution. A beaker of ethyl alcohol can be used to hold the forceps students will use to place their disks on their petri dishes. When students are ready to apply the disks to their plates, (after applying the bacteria to their dish) they come to each station, choose a disk from the beaker and place it on their plate (you could have them flame the forceps before taking a disk, if one of your goals is to highlight sterile technique).

Note: Make sure filter paper disks are not stuck together; if more than one disk is applied, a larger volume of antibiotic solution will be transferred to the dish which leads to a larger than expected zone of inhibition.

Teacher prep and general information

- Teachers should run through this activity before assigning it to students. This is a good way to catch potential difficulties, safety issues, and more.
- Students are determining how to conduct this experiment. You will want to supplement those directions with the safety protocols found in this document and any other directions you will need for your particular situation. Be sure to check students' directions to approve them before students proceed.
- If the Petri dishes were stored in a refrigerator, remove and place them on a clean, sterilized surface for at least several hours before use so they can reach room temperature.
- Make sure all materials are clearly labeled.
- Label a beaker that contains either 70% ethyl alcohol or a 10% bleach solution (prepared by you) and place in a safe location for students to use to dispose of all swabs as soon as they finish using them.
- Disinfect all lab surfaces with a 10% bleach solution before and after students in each class inoculate their Petri dishes. Caution students that a bleach solution will permanently remove color from clothing.
- The contaminated swabs should be left in the beaker containing the 10% bleach solution for at least 6 hours or overnight before disposing of permanently. Another alternative is to autoclave them in a heat-stable biohazard bag before disposal.
- Contaminated forceps and any other equipment students may use will also need to be disinfected in 70% ethyl alcohol or a 10% bleach solution.
- The incubator temperature should be between 34^0 C and 37^0 C. Monitor the incubator to be sure it is not too warm. This is especially important if your incubator does not maintain an even temperature throughout its interior. You don't want the agar to melt because that will make it difficult to view the bacteria and the colonies will likely blend together in the moisture. If you have not used your incubator in the past, you might consider placing a sample Petri dish with agar in it so you can monitor your incubator for 24 hours or more to see how the temperature affects the stability of the agar. If you do not have an incubator, place the Petri dishes in the warmest part of your room (but not in direct sunlight). Bacteria will take longer to grow at room temperature, so allow extra time. Be sure to store the dishes upside down (agar on top) during incubation period to ensure condensation does not affect results.
- Decide the size of your student groups.
- If you have students who have recently been ill or those with compromised immune systems, you should clear their participation in this lab with parents and your school's health clinic or nurse. You should also follow any other applicable procedures or requirements in place for your school.
- Lab stations should be free of clutter and only contain equipment and supplies that will be used today.
- Students should wear lab aprons, latex-free gloves, and chemical splash goggles (ANSI Z87.1).
- There should be no food or drink allowed in the laboratory.

Teacher-conducted demonstrations, safety review, location of all lab supplies, proper cleanup

- Go through all directions with students before they do this investigation. Good preparation is key to preventing mistakes and potential health risks.
- Do not allow students with allergies to penicillin to handle the antibiotic or the antibiotic disks.
- Be sure that students know they should immediately alert you if there is any sort of laboratory accident.
- Review with students by demonstrating all proper techniques they will be using in this investigation:
    - **Hand washing**
        - Students will need to wash their hands with an antiseptic soap and warm water during this investigation. If this is not available, they should use hand sanitizer.
        - They should put their goggles and lab aprons on prior to washing their hands.
    - **Labeling of Petri dishes**
        - The bottom of all Petri dishes should be pre-labeled with a permanent marker as you see fit. Students that are doing the labeling should wash their hands with antiseptic soap and warm water before and after handling Petri dishes. They should be labeled near the edge so as not to obscure future viewing of bacteria and the antibiotic disks. It is suggested that you give each group a letter designation. Then students could simply label the dish bottom with their group letter and the date. Be sure to have students also include the concentration of the antibiotic used in the Petri dish. For investigation 8b, each dish also needs to indicate if it is taken from the ZOI (zone of inhibition) or away from ZOI. The zip-lock bag the dish is stored in should be labeled with the same information.
    - **Proper opening of sterile cotton swab package**
        - Students should not open the package until they are ready to retrieve a sample. They will open the end that contains the handle for the swab (not the swab end). Demonstrate opening the package and removing the swab.
    - **Swabbing agar surface with bacterial samples**
        - Demonstrate how students are to get *E. coli* from the beaker of prepared sample to the surface of the agar using the pre-sterilized cotton swab.
            - One student in each group should be ready to lift the lid of the Petri dish at a 45^0 angle over the bottom of the dish as soon as the cotton swab is removed from the beaker of *E. coli* preparation.
            - A second student should use the swab to gently swipe the sample across the entire surface of the agar in a zigzag pattern, the dish should be carefully turned a quarter of the way, zig-zagged again, turned a quarter again, and zig-zagged one more time to ensure even transfer of bacteria (it is sometimes easier for the person who is swabbing the surface of the agar to hold the lid open as well - you may have your students practice on an empty dish first).
            - Then students should remove the swab and place in a beaker of disinfectant (either 70% ethanol or a 10% bleach solution). The lid should be immediately closed until they are ready to place the antibiotic disk on the agar.
            - You will have set up three stations - one for each antibiotic concentration. Each station will consist of a small beaker of 70% ethyl alcohol for the forceps to be disinfected between applications and a clearly labeled beaker with one of the ampicillin concentrations that has enough filter paper disks soaking for each group to place on their dish.
            - Students will bring their petri dish to the station with the concentration that matches the label on their dish. Ask students to double check that they are applying the disk to the petri dish labeled with the same concentration.
            - Students will take the forceps from the beaker of 70% ethyl alcohol, making sure there are no large drops of alcohol attached to the forceps, choose a disk from the appropriate concentration of antibiotic, gently place it on the properly labeled dish that has already been streaked with bacteria, and then close their dish and place it in an appropriately labeled zip-lock bag. They will repeat this procedure for all three

concentrations of antibiotic.

- **Disinfecting cotton swabs after use**
    - As soon as students are done using a cotton swab, they should place the swab in the 10% bleach solution.
- **Petri dishes**
    - Each dish will be placed in a ziplock bag, sealed, and labeled.
    - These should be placed upside down in the incubator. This position is preferable in the event that condensation forms on the lids.
- **Glove removal**
    - **https://www.youtube.com/watch?v=dyLEd9cng5U**
    - Gloves should be removed before students touch personal items such as notebooks, pencils, paper, etc.

- Review cleanup procedures that you want your students to follow..
- Remind students in the classroom of all safety rules in place that will be relevant to this laboratory exercise. Review the use of protective safety equipment—chemical splash goggles (ANSI Z87.1), latex-free gloves, laboratory aprons, eyewash station(s), and any other items you feel may be necessary in your situation—that will be used in this laboratory exercise.
- Students should be made aware of the location of all supplies to be used in this laboratory exercise.

Petri dish disposal

- After students are done with this investigation, carefully open a Petri dish.
- Using a pipet, saturate the agar with a 10% bleach solution. Repeat until all dishes have been saturated.
- Allow them to sit overnight, then seal them with masking tape and dispose of them in the trash.
- If you prefer, you may autoclave them in a heat-stable biohazard bag (without using bleach) and then dispose of them.

Disinfecting Goggles

- For more information about disinfecting goggles follow this link: http://www.nsta.org/safety/eyeprotection.aspx