Activity #1 - Lab Safety, Aseptic Technique, Inoculation

A. Laboratory Safety – adapted from the American Society of Microbiology website. 
(http://www.asm.org/images/asm_biosafety_guidelines-FINAL.pdf)

The Microbiology laboratory is an active and potentially hazardous place. You will regularly work with boiling liquids, hot instruments, caustic or toxic chemicals, stains and high concentrations of microorganisms – much higher than would normally be found in nature. Students in the lab must understand and observe the following rules:

1. Wash hands after entering and before exiting the laboratory and at any time contamination is suspected.

2. Tie long hair back, wear personal protective equipment (eye protection, coats, gloves, closed shoes; glasses may be preferred to contact lenses), and use such equipment in appropriate situations.

3. Disinfect lab benches and equipment prior to and at the conclusion of each lab session, using an appropriate disinfectant and allowing a suitable contact time.

4. Never eat, drink, apply cosmetics, handle contact lenses, or place objects (fingers, pencils, etc.) in the mouth or touch the face in the laboratory.

5. Store personal items (coats, backbacks) away from the workspace. Do not handle personal items (cell phones, pens) in the laboratory.

6. Minimize or contain the production of aerosols and understand the hazards associated with aerosols

7. Report all spills and broken glassware to the instructor and carry out instructions for cleanup.

8. Properly identify and dispose of different types of waste.

9. Conduct good lab practice procedures, including returning materials to proper locations, proper care and handling of equipment, and keeping the bench top clear of extraneous materials.

10. Report all injuries immediately to the instructor. Locate emergency equipment (eye wash stations, first aid kits, fire extinguishers, chemical safety showers, telephones, and emergency numbers)

11. Immune-compromised students (including those who are pregnant or may become pregnant) and students living with or caring for an immune-compromised individual should consult physicians to determine the appropriate level of participation in the laboratory.

By signing this laboratory safety agreement, I certify that I have read and understand the safety rules of the laboratory. __________________________________________
B. Aseptic technique

Good aseptic or sterile technique is of critical importance in many areas of biology, particularly in Microbiology. Aseptic technique has two purposes:

1. Preventing contamination of experimental materials with microbes from the environment, including from your body.
2. Preventing contamination of the environment, including your body, with microbes that are in use.

List a reason that each is important. What could be consequences of contamination?

To minimize the number of microbes in your work environment, always wash your hands with soap and water and disinfect your lab bench at the beginning of lab.

At first, many of the manipulations and methods of handling materials will seem awkward. Within a few weeks, however, these procedures will be second nature. We will begin by performing several different types of transfers with different tools. Your instructor will demonstrate each technique before you attempt it.

1. **Always begin work by disinfecting your work area.** Spray disinfectant on the benchtop and wipe off with paper towels.

2. **Transferring liquids using sterile glass pipettes** – frequently done to distribute sterile liquid growth medium into sterile culture tubes. Always think of ways to minimize the potential for contamination.
   a. Label 5 sterile 16x125 mm tubes with your initials and 1, 2, 3, 4 and 5; and 4 sterile 13x100 mm tubes with your initials and 6, 7, 8, and 9.
   b. Aseptically transfer 7 ml tryptic soy broth (TSB) from the flask into each of the 16x125 mm tubes.
   c. Transfer 2 mL TSB from tube 1 into tubes 6 & 7, and from tube 2 into tubes 8 & 9.

   • Loosen caps of source and recipient containers.
   • Keep tip and shaft of pipette in sterile environment while attaching pre-aspirated bulb or pipetting device.
   • After removing pipette from container, do not hold it with hand above pipette.
   • Remove cap of source container, pick up container and hold at an angle so that opening is less accessible from above.
   • Draw sample into pipette.
   • **Put source container down**, replace cap.
   • Remove cap of recipient container, pick up container and hold at an angle so that opening is less accessible from above.
   • Dispense sample into recipient container, replace cap.
   • If reusing (glass) pipette, pass it through flame before drawing more sample.
3. **Transferring liquids using a micropipettor** – frequently done when making a dilution series or adding supplements such as antibiotics to medium.

   a. Review use of micropipettors if necessary

   #### Use of Pipettors - Review and Reference

   Small volumes are measured out using micropipettors. A “set” consists of three pipettors that together can accurately measure and dispense volumes from 0.5 μL to 1000 μL (1 mL).

   - Volumes between 0.5 μL and 20 μL are measured using the smallest pipettor, the I-20. The yellow digit on the bottom of the setting display indicates tenths of a microliter, e.g. setting of “0 - 5 - 3” corresponds to 5.3 μL.
   - Some sets have I-100’s and some have I-200’s for the intermediate volumes between 20 μL and 100 or 200 μL. The 3 digits on the display show hundreds, tens and ones; e.g. “0 – 7 – 5” corresponds to 75 μL.
   - The largest pipettor, a I-1000, is used for volumes between 100 and 1000 μL. The yellow digit at the top of the setting display indicates thousands of μL; e.g. “0 – 8 – 0” corresponds to 800 μL (or 0.80 mL).

   Your lab instructor will demonstrate the use of the pipettors.

   1. Set dial to desired volume. Do not try to turn volume setting dial beyond the maximum volume for the pipettor. This is how students most frequently damage the pipettors.
   2. Attach a pipette tip. The small yellow or white tips are used on the I-20, I-100 and I-200’s; the large blue tips are used with the I-1000 pipettors. Be sure tip is attached securely to pipettor, liquid will drip out if seal is not tight.
   3. Press plunger down to 1st stop. Place tip into liquid
   4. Slowly release plunger so that liquid is drawn into pipette tip. Always hold pipettors with the shaft pointing down, especially when liquid is in the tip. If liquid does get into the shaft, please tell your instructor immediately, so that it can be cleaned out. Damage and corrosion can occur if salt solutions dry inside pipettor.
   5. Dispense liquid into desired tube by pushing down plunger to the second stop. Remove tip from liquid before releasing plunger, otherwise you will draw liquid back into tip.
   6. Eject used tips into coffee cans at your bench.

   b. Transfer 50 μL TSB from tube 1 into tubes 6 and 7.

   - This task is done essentially as described on the previous page, except:
     - Pipette tip box must be opened *immediately* before and closed *immediately* after removing every tip. Do not leave tip box open!!
     - One must avoid contacting wall of tube with shaft of pipettor.
     - Dispose of tip by ejecting into autoclave bag.
C. Inoculating microbes into/onto medium.

Before learning about the manipulation of cultures, one must first become acquainted with the tools of the microbiologist. **Inoculating loops** or **needles** are used to transfer microorganisms from solid or liquid media to other media. Since microorganisms are ubiquitous, the loops and needles are contaminated with microorganisms from the environment. Before needles or loops can be used, they must first be sterilized (ensuring that all microorganisms have been destroyed) by passing them through or into the hottest part of the flame of the Bunsen burner or electronic microincinerator. Heat the wire first and then slowly draw the loop into the flame. The wire portion must always be sterilized in addition to the loop. This type of sterilization is sterilization by **incineration**. (Exercise caution that hair or clothing do not pass near the open flame of the Bunsen burner.) The entire length of the wire should turn red hot in the flame. You must always sterilize wires before and after transferring cultures. This is done to ensure that organisms are not carried over from a previous transfer or contaminate your working environment.

Proper inoculation technique is critical in Microbiology. Often, having too many bacteria on the loop can cause problems with obtaining a proper dilution, seeing individual cells in a gram stain, and it uses up your culture faster. When inoculating, one should barely be able to see any bacteria on the loop. When inoculating **streak plates**, one usually seeks to obtain single colonies. To do this, single cells must be deposited onto a certain area of the agar-containing medium. That single cell will divide to form a pile of genetically identical cells called a **colony**. The key in streaking plates is proper dilution from one area of the plate to another. When streaking plates, the **first area inoculated by moving the loop back and forth many times** will contain tens of millions of bacteria at a high density in a small area. As these grow, they will run into each other to form a solid **lawn** of growth. After streaking this area, there are still many bacteria on the loop. Therefore, before streaking in the second area, the loop must be flamed, so that the only bacteria in the second area will be those picked up by dragging the loop through the first area. **After dragging the flamed loop through the first area, move the loop back and forth many times in the second area** to dilute the bacteria. DO NOT go back into the first area, or else you will pick up more bacteria, and the goal here is dilution. Repeat these steps to inoculate a third area with bacteria picked up by dragging through the second area.

When labeling plates (**always on the agar side**), it is important to include the name of the organism, the experimental conditions (if any), your initials (all 3) and the **date**.

![Image of inoculation technique]
You are also selecting your known and unknown organisms to work with for the semester. Each person should write his/her name on one sector of the known and unknown plates that have been provided for you. **Record the complete genus, species, strain, and culture collection designations on the data table at the end of this lab packet.** E.g. *Flavobacterium aquatile* LMG 4008T

- Your instructor may have obtained the known organism from an international culture collection. You will recognize such strains by strain prefixes such as:
  - ATCC = American Type Culture Collection
  - DSM = Deutsche Sammlung von Mikroorganismen und Zellkulturen (German)
  - KACC = Korean Agricultural Culture Collection
  - CCUG = Culture Collection of the University of Goteborg (Sweden)
  - LMG = Belgian Co-ordinated Collections of Micro-organisms (BCCM)

- The superscript “T” at the end of a strain designation signifies that the organism is a Type strain, which means that is the official representative of that particular species.

- The assignment of a genus and species to any strain that does end with a superscript “T” should always be considered tentative, unless its genome sequence has been determined and shown to have 95% Average Nucleotide Identity to the Type strain for the species.

- Any known strain designations that do not have one of the prefixes above were probably identified here at Lycoming by students.

- The known organisms have been recovered from permanent stocks stored at -80C. Their identities **should** be correct, unless there was an error in stock preparation.

- All organisms should have a Lycoming College Culture Collection number (LCCC). This allows the permanent cultures to be easily located in the boxes stored at -80C.

- **The unknown organisms were isolated from the Loyalsock Creek, near the bridge into Montoursville on 1/2/2016.** A sample of water and sediment was collected, spread onto R2A (R), Shieh (S), and tryptic soy agar (T) media, and incubated at room temperature for two days. Colonies were picked and patched onto two plates of the same type of medium they were picked from, and one plate was incubated at 37C and the other plate was incubated at room temp. Only organisms that **did not grow at 37C** were streaked from the room temp patches onto new plates for use as unknown organisms for the class. **Because these organisms do not grow at 37C, it is extremely unlikely that they will be pathogenic to humans.** They have a current strain designation based on the type of medium they were isolated on, and a number corresponding to the patch plates.
1. Inoculate tube 4 with known organism (K) & tube 5 with unknown organism (UK).
   a. Label the tubes with your initials (all 3), K or UK, date, and name of the organism for the known.
   b. Flame inoculating loop.
   c. Lift the top of stock culture plate with your left hand (if right-handed) enough that the inoculating loop will be able to get to the agar surface.
   d. Touch loop to the agar surface to cool the loop, then pick up bacteria from one colony – one should barely be able to see anything on loop.
   e. Replace lid on plate.
   f. Pick up tube to be inoculated, remove cap with pinky, insert inoculating loop into medium, shake it around, remove loop, replace cap. Flame loop.
   g. Vortex sample to disperse bacteria in tube.
   h. Place tubes 4 & 5 on the room temp shaker (research lab) at a 30-45° angle to maximize aeration. These tubes will be used for the wet mount on Thursday.

2. Inoculate agar slants from plate.
   a. Repeat steps 1a-1d as described above, using the same colonies.
   b. Smear bacterial inoculum on surface of slant, do not dig loop into agar.
   c. Label the tubes with your initials (all 3), K or U, date, and name of the organism for the known.

3. Inoculate plates from plate.
   a. Repeat steps 1a-1d as described above (using the same colonies).
   b. Streak bacteria in one section of the plate.
   c. Flame and cool the loop, then drag it through the last area streaked to pick up some bacteria and streak them in an adjacent area of the plate. Your unknown plate should be labeled with the designation UK-XXX where XXX are your initials.

4. Place all tubes (except 4 & 5) in the can, label the can, flask, and plates with your name, and place into the room temp incubator (Agar side up to keep plates from drying out).

5. Always conclude work by disinfecting your work area. Spray disinfectant on the benchtop and wipe off with paper towels.

6. After 2 days of incubation, remove materials from incubator. Describe your results below.
   a. For tubes: use clear (no growth), cloudy (growth).
   b. For plates, measure the largest single colonies with a ruler, use the following guide to describe the shape, elevation and texture of the colonies.
   c. If the streak plates show a good number of single colonies, and all have a similar appearance, you probably have a pure culture! Use a digital camera to document the appearance of your colonies.
D. Preparation of Long Term/Permanent Stock Cultures

The DNA replication process is not perfect. In *E.coli*, the initial error rate has been estimated in the 10⁻⁶/base range, and after proofreading, it is in the 10⁻⁹/base range. Given an average genome size of 3 x 10⁶ bases, one could expect that 1 cell in 300 will carry a new mutation. If a culture contains 10⁸ bacteria/mL, there will be many different mutants represented in this supposed “pure” culture. Choosing a single colony to start a new culture will result in a starting point corresponding to the particular mutations carried by the cell that gave rise to that single colony. Repeated subculturing of bacterial strains can result in selection for mutants that are well adapted for rapid growth on a particular type of medium in the laboratory (The Microbial Zoo), but may not accurately reflect the characteristics of this microbe in its natural environment. Therefore, it is important that we prepare frozen permanent stock cultures now, before there has been much opportunity for selection. If your organism has never been studied before (i.e. is novel), it will be particularly important to have it in its original state.

**Prepare permanents of unknown cultures** – not “formal” because we do not know the genus species classification of the unknown organism. We are preparing two copies so that one can go into each -80 C freezer.

1. Inoculate your unknown organism into a tube with R2A or TSB liquid medium and incubate in the 30C shaker overnight (this is tube 5 from the inoculation activity above)

2. Label one bead vial, and one empty vial with UK-XXX 2016 where XXX are your initials.

3. Add 1.0 mL of the liquid culture (tube 5) to the bead vial and shake the vials well (do not vortex) to mix the culture with the cryogenic fluid.

4. Use a 200 uL pipettor to transfer **ALL** of the liquid to the empty vial. This is EXTREMELY important to be able to remove individual beads from the frozen vial in the future.

5. Place the tubes into Styrofoam box with -80C lab armor beads.
<table>
<thead>
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<th>colony shape</th>
<th>colony elevation</th>
<th>colony texture</th>
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<td>Tube 9-</td>
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<th>colony shape</th>
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Activity #1 - Lab Safety, Aseptic Technique, Inoculation Results