General Sterilization Techniques updated: Aug 2014

It is vital with sterile tissue culture that you work conscientiously and that you carefully follow procedures to ensure the cleanliness of the culture. Contamination can set work back days or even weeks. Here are some guidelines for sterile technique that will aid in keeping cultures contamination free.

The Laminar flow hood: ***Everything you want to keep contamination free must only be opened in the hood!!*** The Laminar flow hood should be running for at least 30 minutes and sterilized with 70% ethanol prior to use. The hood blows HEPA filtered air from the back, helping to keep external contamination out. This airflow is not very strong and how you move, place samples, or place objects affects the airflow. Make sure that everything that must remain sterile is receiving an unobstructed flow of air around it. The closer your sterile object is to the back of the hood, the safer it is. Be very considerate with what you bring into the hood and sterilize everything possible with 70% ethanol. When you are done using the hood, remove any unnecessary objects and spray the hood with 70% ethanol.
Remember: Your samples are being protect by a slight breeze of sterile air, act accordingly!

Utensils: Any metal object being used should be dipped in 200 proof (100%) ethanol and flame sterilized; this includes tweezers, scissors, and spatulas. Every part should be sterilized prior to use in the hood, including the parts you handle, and they should be sterilized after every time they touch anything EVER. The 200 proof ethanol used should be free of debris (floating bits of agar and moss). It is disposed of in a labeled Hazardous Waste jug in the hood in the molecular lab and new ethanol is housed under the hood.

Plates: Packages of plates that need to be kept sterile should only be opened and closed in the hood and follow the above described hood procedure. Try not to breathe onto plates. When taking lids off plates or when putting lids back on plates try to avoid obstructing the air flowing over and around the plate as much as possible. Rotate lids or plates toward the airflow whenever removing or placing lids on plates. If you must work with a plate that has contamination, make it the last plate you work with. Opening contaminated plates contaminates the hood.

Pipette tips: Pipette tips are autoclaved and must remain sterile. The box of tips should only be opened in the hood. Once a pipette tip has touched anything unsterile, such as the hemacytometer or the metal surface of the hood OR has been taken out of the hood, consider that tip no longer sterile, and don't allow it to come in contact with items that must remain sterile. If the tip box is opened outside of the hood consider the sterility of the contents compromised and re-autoclave.

1.5ml tubes: Theses tubes are largely used for diluting spore solutions. They are autoclaved in a metal container. This metal container should only be opened and closed in the hood following above described hood procedures to maintain sterile tubes. If the tube container is opened outside of the hood consider the sterility of the contents compromised and re-autoclave.

Water and other liquids: Water bottles have aluminum foil on the lid to limit exposure of the lip of the lid to contaminants while outside the hood. When a water bottle is first opened the aluminum must be removed to indicate the bottle's use and it must only be opened in the hood. The excess water should be disposed of after you are done using it. Any bottle of water that you

did not open should be considered non-sterile, don't use bottles without aluminum foil even if they are already in the hood. The empty bottles should be cleaned in the molecular lab and put on the drying rack to be used again. Any other liquids that must remain sterile follow the same procedure unless otherwise indicated.

Sporophyte Sterilization

When using field-collected moss sporophytes they must be surface sterilized prior to use. This procedure should be done in the Laminar flow hood and follow sterile techniques.

1. Prepare a small volume of 20% bleach solution, approximately 10-20ml (depending on the size of the beaker used), and put in a small beaker

2. Check existing 200 proof ethanol for sterilizing forceps– replace if not clean.

3. Light Bunsen burner for flame sterilization of forceps

4. Using a micropipette, add 1ml of autoclaved dH_2O to three 1.5ml tubes. *There will be three tubes per sporophyte.*

5. Label the last tube with sample name (DUR, CT, etc. this depends on where the population is from) and number. *E.g. 20-1, 20-2, 20-3, etc. In this example the 20 indicates the clump/sample of moss the sporophyte came from and the second number is used to simply number the sporophytes from that sample. Depending on how the sporophyte was picked from the sample it may already have the second number or you may have to number it yourself. This number is very important as it now designates an individual sporophyte these numbers once used should never be repeated.*

6. Using ethanol washed and flame sterilized tweezers, transfer **one** sporophyte into 20% bleach solution and soak for 30-40 seconds.

7. Using your tweezers, transfer the sporophyte to the first of the three tubes of autoclaved dH2O to rinse it. *Be exceptionally careful bleach is not carried over in between the tines of your forceps*

8. Repeat in the second tube of autoclaved dH2O

9. Ethanol wash and flame your tweezers, and transfer the sporophyte to third tube of autoclaved dH2O

10. Crush the capsule completely with edge of the tweezers

11. Place crushed sporophyte in fridge overnight before counting spores (this step is really important for freeing up the spores from the capsule tissue before counting)

Spore Counts

These spore counts are used to calculate the amount of spore solution you will need to make germination and dilution plates. The spore count data will be input in an excel file.

1. Rinse and dry hemacytometer slide and cover slip using dH2O. *Do not dry laser-etched surface with anything other than microscope lens paper. No Kimwipes!*

2. Place coverslip onto hemacytometer

3. Vortex sporophyte/spore suspension

4. Using a micropipette, inject 10μl of spore suspension into the grove in the hemacytometer slide making sure the suspension is drawn up under the coverslip. *Keep your sample tube in the hood when preforming this step, contaminating your stock spore solution at this step will contaminate all following steps.*

5. Repeat with the other groove. *Make sure to use a new pipette tip!*

6. Count spores in the 9 large grid squares on both sides of the hemacytometer. *The average number of spores present is calculated by averaging across all 18 grid squares and multiplying by a standard constant (10,000) to obtain the number of spores per 1ml of spore suspension. Each of the 18 grid cells contains 1/10,000 of a milliliter.*

Germination Data aka 9-spots

This data is extremely important to us. It is tedious but getting accurate counts should not be taken lightly.

To make 9-spots:

1. Use the excel file to find the amount of autoclaved dH_2O and spore solution needed
2. In the Laminar hood, micropipette the necessary amount of autoclaved dH_2O into an autoclaved 1.5ml tube
3. Vortex the sporophyte/spore suspension
4. Micropipette the necessary amount of spore solution into the 1.5ml tube with autoclaved dH_2O. *The total volume should be 100µl*
5. Label a Petri dish containing BCDA agar on the bottom with the sporophyte number, your initials, and the date. *Spell out the date so it does not get confused with other numbers e.g. April 4, not 4-4 (4-4 looks exactly like a sample/sporophyte label!)*
6. Set the Petri dish on top of the 9-spot template
7. Vortex the tube with the diluted spore solution
8. Micropipette 10µl of the spore solution onto each of the 9 spots shown on the template
9. Lid your Petri dish and allow time for the spots to dry
10. Tape the Petri dish and put in the growth chamber for 3 days

To count 9-spots:

1. Use a microscope with the darkfield light setting to count the germinated and ungerminated spores in each spot. *It is easier to see the spores if the lid of the Petri dish is removed but his does not have to be done in the Laminar hood. IF YOU CANT SEE THE UNGERMINATED SPORES AND GERMINATED SPORES CHANGE THE LIGHT SETTING*
2. Input the count data into the excel sheet
3. Dispose of the Petri dish in the appropriate waste bin

Dilution Plates and 16-spots plates

These plates are used to grow large enough amounts of tissue for DNA extraction. All procedures should be done in the Laminar hood.

To make dilution plates:

1. Use the excel file to find the amount of autoclaved dH_2O and spore solution needed

2. In the Laminar hood, micropipette the necessary amount of autoclaved dH_2O into an autoclaved 1.5ml tube

3. Vortex the sporophyte/spore suspension

4. Micropipette the necessary amount of spore solution into the 1.5ml tube with autoclaved dH_2O. *The total volume should be 200µl*

5. Label a Petri dish containing BCDA agar on the bottom with the sporophyte number, your initials, and the date. *Spell out the date so it does not get confused with other numbers e.g. April 4, not 4-4*

6. Vortex the tube with the diluted spore solution

7. Micropipette all of the diluted spore solution onto the Petri dish, lid and tape it

8. Using your hand, rapidly move the dish in a circular motion while it is on the table to distribute the spore solution across the agar and put in the growth chamber for 3 days

To pick dilution plates:

1. Using both hands **Carefully** put a microscope in the Laminar hood. *Remember to not let it restrict airflow to your plate.*

2. Light Bunsen burner for flame sterilization of tweezers

3. Label a Petri dish containing BCDA agar on the bottom with the sporophyte number, your initials, and the date. *Spell out the date so it does not get confused with other numbers e.g. April 4, not 4-4*

4. Set the Petri dish on top of the 16-spot template

5. Using ethanol washed and flame-sterilized tweezers, pick a single germinated spore onto each of the 16 spots. *It is very important that you pick up only ONE germinated spore!! We only want a single individual in each of these spots*

6. Repeat until you have 16 germinated spores on the plate, tape plate, and place in growth chamber.

7. In 2-3 days go back and look at your single spore picks to ensure there is only one spore present.