MICROBIOLOGY LAB STANDARD OPERATING PROCEDURES

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Standard Microbiological Practices

1. Eating, drinking, smoking, handling contact lenses, and applying cosmetics are not permitted in the lab. Food for human consumption is never stored the lab.
2. A biohazard sign must be posted on the entrance to the laboratory when pathogenic agents are in use. Information to be posted includes:
    i. Agent(s) in use
    ii. Biohazard symbol
    iii. Biosafety Level 2
    iv. Investigator's name
    v. Telephone number
    vi. Personal Protective Equipment
    vii. Procedures required for exiting the laboratory
3. Persons wash their hands upon entering the lab, after they finish working in the lab, after removing gloves, and before leaving the laboratory.
4. Work surfaces are decontaminated prior to beginning any work in these rooms, on completion of work or at the end of the day with 10% bleach or a disinfectant solution. Any spill or splash of viable material should be decontaminated with 25% bleach solution.
5. All procedures are performed carefully to minimize the creation of splashes or aerosols. Any procedure that would potentially create aerosols will be performed within the biosafety cabinet.
6. Mouth pipetting is prohibited; mechanical pipetting devices are used.
7. A limited number of needles and syringes are used for reconstituting reagents. After use, these materials are placed in a puncture-proof red Sharps container. Do not recap needles.
8. All cultures, swabs, and waste containers are decontaminated before disposal by autoclaving.
9. Wear eye protection in the laboratory always. Prescription glasses do not qualify as laboratory eye protection when worn alone. Contact lens should not be worn in the lab.
10. Never sniff any chemicals.
11. Do not wear laboratory coats outside the laboratory area.
12. Review all chemical safety information mentioned in the Safety Data Sheets (SDS), prior to use or transport of any chemical.
13. Know the location and proper use of emergency equipment including gas lock off button, fire blanket, fire alarm, eyewash, and safety showers.
14. Be aware and follow the appropriate emergency protocols: including evacuation routes, spill clean-up procedures and proper waste disposal.

15. BSL2 agents are handled in the Microbiology prep room. Biohazard signs are posted on the doors. When appropriate BSL2 practices will be followed.

16. **Removal of media, equipment, or bacterial cultures from the laboratory is strictly prohibited.**

Regulations

Access, Training and Responsibilities

1. Access is granted through special permission and use of Broward College key cards. It is limited to individuals involved directly in media prep, clean up, lab prep, lab work and research.

2. The lab and prep room doors will be closed when a BSL2 agent is in use.

3. All staff and work study students are required to read, understand, and follow these regulations before working in the microbiology lab and prep areas.

4. All staff and students working in the lab and prep areas will receive training from trained lab technicians concerning the proper use of the equipment.

5. The lab's PI/Lab manager/Lab technician will train staff and work study students on aseptic techniques appropriate for handling pathogenic agents. This will include the potential hazards associated with the work involved, the necessary precautions to prevent exposures, and the exposure evaluation procedures.

6. Personnel will receive annual updates or additional training as necessary for procedural or policy changes.

7. Personnel are advised of special hazards and are required to read and follow instructions on practices and procedures.

8. Any staff or work study student found in violation of the regulations may have their access the microbiology prep and lab rooms terminated.

9. The supervising PI is responsible for seeing to the enforcement and resolution of regulations of work study student/staff actions, including correction of damages, violations and reproductions of experiments.

Accident Reporting

In case of an emergency call 911. However, in the case of a non-emergency situation immediately call, Broward College Campus Safety, to report all accidents or incidents and complete the appropriate forms.

Personal Protective Equipment (PPE)

Follow proper personal hygiene practices and use appropriate PPE:

a. Wear protective lab coat
b. Wear closed toe, sturdy shoes and full-length garments
c. Inspect all protective equipment prior to use and discard defective equipment
d. Use hearing protection when and if appropriate.

e. Wear appropriate hand protection based on the chemicals and/or tasks. (Note: Nitrile gloves are provided in the labs.)
f. Use appropriate eye and face protection (e.g. safety glasses, goggles).

Housekeeping

a. Keep walkways and path of egress clear.
b. Clearly label all stations and containers.
c. Safety Data Sheets (SDS) are located on the countertop.
d. Ensure all eye wash stations, emergency showers, fire extinguishers, and exits are always unobstructed and accessible.
e. First aid kits are mounted and located near the single eyewash unit.
f. Fire extinguishers are mounted on the wall by the exit door in the labs.
g. The emergency shower and eyewash unit is located by an exit door in the labs.
h. An additional eyewash station is mounted on the sink and located by the second exit door.
i. Store hazardous chemical in appropriate cabinets.
j. Use proper methods of transporting chemicals within the facility.
k. Immediately cover spilled cultures or broken culture tubes with paper towels. Then saturate them with disinfectant solution. After 15-20 minutes, dispose of the towels and broken items as indicated by the waste disposal protocols.
l. Bench tops are impervious to water and are resistant to moderate heat and the organic solvents, acids, alkalis, and bleach used to decontaminate the work surfaces.
m. Spaces between benches, cabinets, and equipment should be accessible for cleaning.
n. Chairs used in laboratory work should be made of or covered with a non-porous material that can be easily decontaminated.

Disinfectants

A wide variety of disinfectants are commercially available. Disinfection of blood or other potentially infectious material, as defined in the OSHA Bloodborne Pathogen Standard, requires the use of EPA-registered disinfectant: alcohol is not EPA-registered and should not be used in these cases.

The lab manager is responsible for assessing the risk level of microbes used in the prep or lab areas and determining the most appropriate disinfectant protocol for routine decontamination of laboratory surfaces and equipment.

Consideration should be given to the specific microbes used in a lab, the concentrations of microbes handled by people staff and students in the lab, and the various kinds of surfaces to be decontaminated.

Commonly used disinfectants for microbiology labs include 1:10 dilutions of bleach (approximately 0.5% sodium hypochlorite), 70% ethanol, and 70% isopropanol. Many others are available; efficacy and cost are considerations.

Squirt/Spray bottles stamped with ethanol or sodium hypochlorite are available. Instructors, lab technicians, and student assistants should be familiar with the proper concentrations utilized for each disinfectant and follow the manufacturer's instructions for proper application techniques and required **contact times.**

Sodium hypochlorite is readily available and inexpensive. Commercial bleach products are typically 5-6% aqueous solutions of sodium hypochlorite. Sodium hypochlorite is used to decontaminate surfaces; in waste containers for used pipettes, tips and swabs; and to clean up spills.

Bleach is corrosive to metals and should be used sparingly on stainless steel. Metal surfaces that have been treated with bleach should be "rinsed" with 70% ethanol or water **after a 20-minute contact time.**

Routine benchtop disinfection

Freshly prepared 1: 10 (10%) dilutions of commercially available bleach are suitable for general use to disinfect tabletops and work areas. Spray the 1:10 dilution of bleach solution on the benchtop, wipe the entire surface, and allow to air dry. Mix 100 ml bleach with 900 ml DI-water: the solution should be prepared fresh daily.

Disinfecting a Spill

- A 1:10 solution of bleach should be used to clean up spills and in discard containers for used pipettes, tips and swabs: for larger spills (>10ml, use undiluted to 1:4 diluted bleach). Following a spill, everyone in the lab should be made aware that there is a spill and the area evacuated.
- Allow aerosols to settle for at least 60 minutes.
- If splashed, remove PPE and don clean PPE (Lab coat, 2 pairs of gloves, eye protection, face mask, shoe covers (if spill is on floor)).
- Cover the spill with paper towels and pour disinfectant slowly around and over the spill, starting from the outside and working in.
- Saturate the area with bleach and allow it to remain undisturbed for at least 20 minutes.
- Clean up paper towels and place in the biohazard bag to be autoclaved/disposed: if the spill included any items that could cut or abrade your skin (glass, needles, and pipet tips) use tongs to pick up the paper towels.
- Spray the area again with bleach solution and allow to air dry
- Remove PPE and dispose in biohazard bag. Wash hands with soap and water.
- Sodium hypochlorite solutions should be mixed fresh daily.

    - Alcohols (ethanol and isopropanol) should not be used to clean up spills because of their volatility which preclude an appropriate contact time.

    - Alcohols are highly flammable and should not be used near an open flame. Alcohols are effective for routine decontamination of stainless-steel surfaces, such as those in biosafety cabinets, **but not spills.**

    - Alcohols can be used to remove residual bleach from metals to minimize corrosion. **However, alcohols are not effective against bacterial spores.**

    - Incubators, chemical fume hoods, and biological safety cabinets should be **thoroughly disinfected monthly.**

Hygiene

a. It's important to wash hands and wear gloves whenever the aseptic technique will be employed in order to protect the organism from contamination and to protect the technician from infection.

b. To properly wash hands, hands should be washed under moderately warm water with soap for a minimum of thirty (30) seconds vigorously. Special attention should be paid to washing the wrist during hand washing as the wrist(s) are generally not protected by normal latex and nitrile gloves.

c. Make sure hands are completely dry, free from any moisture prior to putting on gloves. Latex and nitrile gloves are both significantly more difficult to put on if the hands are moist.

d. Used gloves must be disinfected prior to disposal in the regular trash. If contaminated with bacteria these gloves must be disposed of in the biohazard bag.

e. Gloves should be taken off carefully to prevent contamination of the skin during removal. Use two fingers from the opposite hand to flip the glove inside out to prevent skin and environment exposure to the potential contaminants on the glove.

Follow Proper Waste Disposal Protocols

Sharps are devices or objects capable of cutting or piercing. Examples include scalpels, blades, and some broken glass items such as transfer pipettes, capillary tubes, and microscope slides. This specifically excludes most broken laboratory and non-laboratory glassware and bottles which should be collected in broken glass containers. While hypodermic syringes/needles are classified as sharps, they must be disposed in a separate sharps container and not mixed with other sharp's waste.

Biohazardous Sharps

• Biohazardous sharps must be collected in a red sharps container that is labeled, "Biohazardous Sharps Waste," sealed and disposed of in a Biohazard box for removal by Stericycle.

When Disposing of Sharps

- Do not dispose of sharps containers in the general waste.
- Do not throw needles/syringes/slides down the drains because they may then be washed out to other areas.
- Do not throw needles/syringes down toilets.

Biohazard Waste Disposal

Disposal of Materials and Decontamination

a. Laboratory equipment and work surfaces should be decontaminated with 10% bleach or disinfectant, before and after work with infectious materials is finished. Evident spills, splashes, or other contamination by infectious materials should be decontaminated with 25% bleach by first placing paper towels over the spill to prevent aerosolizing the contents of the spill.

b. Spills and accidents that result in evident exposures to infectious materials are immediately reported to the laboratory manager. Medical evaluation, surveillance, and treatment are provided as appropriate and written records are maintained.

c. Broken glassware that does not contain live cultures should be swept up with the broom and dustpan and discarded in the broken glass disposal box.

d. Broken glassware that contains live cultures should be saturated with bleach/disinfectant solution. After 20 minutes, the debris should be "swept" up into an autoclave bin and autoclaved. After being autoclaved, the glassware can go into the glass disposal box and the paper towels can go into the regular trash.

e. Biohazard waste generated during the regular functioning of the laboratory and prep rooms are regularly autoclaved.
   I. Markings and tapes are removed from the tubes then placed in an autoclave safe test tube rack.
   II. Place a strip of autoclave indicator tape on a test tube (Note: loosen screw caps)
   III. Place items on a metal cart until there are sufficient items to fill the autoclave tray.
   IV. For waste agar plates that have been inoculated and disposed of in biohazard bags. Seal and place a strip of autoclave indicator tape on the bag then place all the waste in the autoclave metal bin.
   V. Record waste materials to be autoclaved in the autoclave biohazard folder.

Aseptic Technique

Describes the skill set needed to successfully transfer bacteria without contamination by unwanted bacterial, viral, or fungal species, while maintaining sterility of the source culture, media to be inoculated, self, others, and the work environment.

In order to ensure proper observance of aseptic technique and ensure safe handling of microbial organisms, special attention must be given to hand washing, glove use, work area sanitization, Bunsen burner and flame use, as well as loop/pipet/swab handling.

Special Practices and Considerations

Access to the laboratory is limited or restricted by the laboratory manager when work with infectious agents is in progress. Persons who are at increased risk of acquiring infection – e.g., those who are immunocompromised or immunosuppressed – or for whom infection may have serious consequences, should consult with their physician to determine the appropriate level of participation in the lab.

Work Area Sanitization

Any surface that the organism could potentially contaminate while handled should be sanitized with a standard disinfectant that is anti-bacterial, fungicidal, and viricidal. If possible, inoculations should be performed under a laminar flow hood. One way to maintain aseptic conditions of a given workspace is to use a Bunsen burner/butane can or other device that provides an open flame that produces a blue flame.

Bunsen Burner & Flame

A properly lit burner should produce two types of flame:

(1) An inner cone or series of smaller cones depending on the type of burner being used, and an outer cone that should be slightly lighter than the inner cone.

(2) The inner-cone's color is an indication of the flame's temperature. The inner cone is the location where the flame's temperature is at its highest, which makes this the most effective place to sterilize loops and other inoculation tools.

A striker and/or match can be used to light a Bunsen burner, but never a butane cigarette lighter as a cigarette lighter can rupture, explode, or ignite from within causing serious injury.

Once lit the Bunsen burner can be adjusted in three (3) ways:

1. By the lab bench's gas valve adjustment knob, this knob manipulates the amount of gas supplied to the burner directly from the institutions source. This serves as the first line of defense to preventing a fire or unwanted ignition and should be the first valve to be closed in the event of an accident or emergency. This knob functions much like a coarse adjustment, as the amount of gas allowed to pass can't be toggled with much sensitivity.
2. At the base of the burner is a gear-like knob the can be used to adjust the amount of gas allowed to pass into the burner itself. This knob is useful because unlike the knob on the lab bench this knob can be used with much more sensitivity, comparable to the fine adjustment on a microscope.
3. The burner itself can turn about the base regulating the amount of oxygen allowed to feed the flame, the amount of oxygen alters the color of the flame and the flame's temperature.

Loop, Pipet, & Swab Handling

When using any inoculation tool, it is important to maintain complete control of the inoculation tool. The inoculation tool should only touch the inoculum and the tube or plate to be inoculated.

Loop

Sterilization of the loop when in use is the most important step to keep in mind. Proper sterilization of the loop requires that it be heated red hot from loop to handle. In addition to proper sterilization, one should know that the loop can be flipped around to make the loop into a needle. Any portion of the loop that encounters any surrounding objects that do not include the interior of the inoculum or the interior of the tube/plate to be inoculated should be considered contaminated, even if the area is considered sterile.

Pipet

When inoculating using a pipet one must be sure that the pipet is sterile. Care should always be taken when using the thin tipped transfer pipets as they have an increased tendency to produce bubbles. The rupture of these bubbles can disperse culture to the surrounding work area, gloves, tubes, or any other objects in the nearby workspace causing contamination. Successful inoculation of most cultures only requires two (2) or three (3) drops of inoculum.

When opening a sterile transfer pipet or swab, the packaging should be opened just enough for the handle or bulb of the pipet/swab to be accessed. Opening the pipet/swab in this way makes it possible to re-insert the pipet/swab into the packaging for disposal; this method also protects the workspace from possible contamination as well. Any transfer pipet that encounters any surrounding objects that do not include the interior of the inoculum or the interior of the tube/plate to be inoculated should be considered contaminated, even if the area is considered sterile.

Swab

The tip of the swab is to be considered contaminated if at any point it touches anything other than the interior of the inoculum or the interior of the tube/plate to be inoculated after it has been removed from its packaging. The swab is also to be removed and returned to its packaging after use. The packaging of sterile swabs and

pipets are not waterproof and if they happen to get wet prior to use should be assumed contaminated even if the packaging is untampered.

Media Preparations

In order to prepare media, the following materials should be gathered:

1. Hot plate
2. Spatula
3. Graduated cylinder
4. Distilled water
5. Weighing boat
6. Balance
7. Magnetic stirrer
8. Dehydrated media

When preparing media a few notes should be observed…

1. Magnetic stirrers should never be dropped into an Erlenmeyer flask. Instead, magnetic stirrers should be inserted into Erlenmeyer flask by tilting the flask to the side at approximately a forty-five (45°) angle and then gently sliding the magnetic stirrer carefully into the flask on the side wall of the flask (Magnetic stirrers dropped directly into Erlenmeyer flask can shatter the bottom of the flask).

2. It is also important to put the ingredients into the flask in the proper order, as to assist the media in dissolving evenly, preventing the media from burning, helping to maintain the accuracy of all the previously measured ingredients, and to ensure the media will be of the same quality each time it is prepared.

3. Once all the ingredients are in the flask it is important to be conscious of the temperature of the hot plate. Most media require that you bring them to a boil, however there are a few, which must not be boiled (Skim milk agar, gelatin, and litmus milk are examples of media that are damaged by extreme heat). Generally, media is best if heated between two-hundred degrees Celsius (200°C) and two-hundred fifty (250°C) degrees Celsius until it boils. It should also be noted that certain types of media transform from opaque to transparent once completely dissolved via boiling.

Caution: When making media the technician must take care for overheating the media as this leads to overflow of very hot liquid which can cause injury.

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4. How the media will be dispensed prior to autoclaving when making media for pouring plates should be considered. For example, if pouring the plates by hand it is best to transfer the media into five-hundred milliliter (500ml) bottles that are easier to handle.

5. After the media has been autoclaved, the media should be poured as close to fifty-five (55°C) degrees Celsius as possible. If needed autoclaved media bottles/flasks can be placed in a water bath set between sixty (60°C) degrees Celsius and seventy (70°C) degrees Celsius for a minimum of twenty (20 mins) minutes in order to bring the media to temperature. Bringing the media to temperature prevents the poured media from developing condensation and prevents the stacked plates from melting and warping that sometime can cause plates to adhere to one another.

6. When pouring plates be sure that the poured media covers the bottom of the plate and pour gently as to prevent the formation of bubbles in the liquid agar prior to solidification.

7. Label all media at every stage during its processing.

8. If needed any media that has been autoclaved and not poured or dispensed can be refrigerated to be poured or dispensed later. Keep in mind that media handled in this way has a greater chance of being contaminated, and any refrigerated media must be re-autoclaved to ensure it is sterile prior to pouring and/or dispensing and should be dated prior to being placed in the refrigerator.

Media Recipes

Brain Heart Infusion Broth

- Brain Heart Infusion (9.8g)
- Peptic Digest of Animal Tissue (10g)
- Sodium Chloride (5g)
- Glucose (2g)
- Disodium Hydrogen Phosphate (2.5g)
- Calf Brains Infusion (7.7g)

Brain Heart Infusion Agar

- Brain Heart Infusion (8g)
- Peptic Digest of Animal Tissue (5g)
- Pancreatic Digest of Casein (16g)
- Sodium Chloride (5g)
- Glucose (2g)
- Disodium Hydrogen Phosphate (2.5g)
- Agar (13.5g)

Eosin Methylene Blue (EMB)

- Pancreatic Digest of Gelatin (10g)
- Sucrose (5g)
- Lactose (5g)
- Dipotassium Phosphate (2g)
- Agar (13.5g)
- Eosin Y (.4g)
- Methylene Blue (.065g)

Fluid Thioglycollate Medium

- Pancreatic Digest of Casein (15g)
- Yeast Extract (5g)
- Dextrose (5g)
- Sodium Chloride (2.5g)
- L-Cystine (.5g)
- Sodium Thioglycollate (.5g)
- Agar (.75g)
- Resazurin (.001g)

Litmus Milk

- Skim Milk (100g)
- Azolitmin (.5g)
- Sodium Sulfite (.5g)

MacConkey Agar

- Pancreatic Digest of Gelatin (17g)
- Peptone (3g)
- Lactose (10g)
- Bile Salts (1.5g)
- NaCl (5g)
- Neutral Red (.03g)
- Crystal Violet (1mg)

Mannitol Salt Agar

- Sodium Chloride (75g)
- Proteose Peptone (10g)
- Mannitol (10g)
- Beef Extract (1g)
- Phenol Red (.025g)
- Agar (15g)

Milk Agar

- Pancreatic Digest of Casein (5g)
- Yeast Extract (2.5g)
- Glucose (1g)
- Agar (15g)
- Non-Fat Dry Milk (50g)

Minimal Salt

- Disodium Phosphate Anhydrous (33.9g)
- Monopotassium Phosphate (15g)
- Sodium Chloride (2.5g)
- Ammonium Chloride (5g)

MR-VP Broth

- Pancreatic Digest of Casein (3.5g)
- Peptic Digest of Animal Tissue (3.5g)
- Dextrose (5g)
- Potassium Phosphate (5g)

Mueller-Hinton Agar

- Beef Infusion (2g)
- Acid Digest of Casein (17.5g)
- Starch (Soluble) (1.5g)
- Agar (17g)

Nitrate Broth

Pancreatic Digest of Casein (20g)
Disodium Phosphate (2g)
Dextrose (1g)
Potassium Nitrate (1g)
Agar (1g)

Nutrient Agar

Beef Extract (3g)
Peptone (5g)
Agar (15g)

Nutrient Broth

Beef Extract (3g)
Peptone (5g)

Nutrient Broth w/Glycerol

Beef Extract (3g)
Peptone (5g)
Glycerol (30ml)

Nutrient Gelatin

Beef Extract (3g)
Peptone (5g)
Gelatin (120g)

Phenyl ethyl Alcohol Agar

Casein Enzymatic Hydrolysate (15g)
Soy Peptone (5g)
NaCl (5g)
Phenyl ethyl Alcohol (2.5g)
Agar (15g)

Phenol Red Broth(s)

Pancreatic Digest of Casein (3.5g)
Peptic Digest of Animal Tissue (3.5g)
Dextrose (5g)
Potassium Phosphate (5g)
Dextrose/Sucrose/Lactose/Mannitol (5g)

*Sugars are not to be mixed, only one sugar is to be used per batch of media.

Sabouraud Dextrose Agar

Peptic Digest of Animal Tissue (5g)
Pancreatic Digest of Casein (5g)
Dextrose (40g)
Agar (15g)

S.I.M.

Tryptone (20g)
Peptone (6.1g)
Ferrous Ammonium Sulfate (.2g)
Sodium thiosulfate (.2g)
Agar (3.5g)

Simmons Citrate Agar

Ammonium Dihydrogen Phosphate (1g)
Dipotassium Phosphate (10g)
Sodium Chloride (5g)
Sodium Citrate (2g)
Magnesium Sulfate (.2g)
Bromothymol Blue (.08g)
Agar (15g)

Snyder Test Agar

Proteose Peptone (10g)
Pancreatic Digest of Casein (10g)
Dextrose (20g)
Sodium Chloride (5g)
Agar (20g)
Bromocresol Green (.02g)

Starch Agar

Beef Extract (1g)
Peptone (5g)
Sodium Chloride (5g)
Yeast Extract (2g)
Agar (15g)
Soluble Starch (10g)

Triple Sugar Iron Agar

Pancreatic Digest of Casein (10g)
Peptic Digest of Animal Tissue (10g)
Sodium Chloride (5g)
Lactose (10g)
Sucrose (10g)
Dextrose (1g)
Ferric Ammonium Citrate (.2g)
Phenol Red (.025g)
Agar (13g)

Tryptic Soy Broth

Tryptone (17g)

Tryptic Soy Agar

Tryptone (17g)
Soytone (3g)
Dextrose (2.5g)
NaCl (5g)
Potassium Phosphate, Dibasic (2.5g)
Agar (15g)
Dextrose (2.5g)
NaCl (5g)
Potassium Phosphate Dibasic (2.5g)
Soytone (3g)

Urea

Peptone (1g)
Dextrose (1g)
Sodium Chloride (5g)
Potassium Phosphate, Monobasic (2g)
Urea (20g)
Agar (15g)
Phenol Red (.012g)

Inoculation Techniques

Stab Technique

The stab inoculation technique is used to introduce bacteria into the media as opposed to onto the media as with other solid media inoculations. T.S.I., S.I.M., and Nutrient Gelatin are all examples of media that require stab inoculation technique. The stab technique often exposes certain properties about organisms such as motility, gas, production, and hydrolysis of various molecules.

a. Stab inoculations are best done from solid inoculum.

b. In T.S.I. the stab is used to demonstrate gas production that can be seen when gas produced by the organism, with this in it is best to insert the inoculation needle to the very bottom of the T.S.I. butt.

c. S.I.M. media does not require that the media be inoculated with a very deep stab, instead S.I.M. deeps require that it be inoculated with extreme care. Motility is one of the properties that S.I.M. detects, for this reason it is imperative that the stab be done neatly with steady hands. A messy stab inoculation can sometimes be mistake for bacterial motility after incubation.

d. Nutrient Gelatin does not require a stab inoculation, however, is recommended by most text because it is most effective at demonstrating the positive result. Alternatively, a few drops of the inoculum will also demonstrate a positive result, but may require additional incubation time (A positive result for Nutrient Gelatin is demonstrated by the liquefying of the media).

Drops (Liquid Inoculation)

Liquid inoculum requires a sterile transfer pipet, swab or a micropipette with sterile tip(s), although on rare occasions a loop can also be used to manipulate liquid culture when needed in small volumes. In most cases, it is to the advantage of the technician to use liquid culture in conjunction with sterile transfer pipets as this combination increases inoculation speed and efficiency.

- Normally 2 to 3 drops of healthy/cloudy inoculum is enough to successfully culture most organisms, if the appropriate media is used.

- When inoculating slants, be sure to tilt the slant and slowly whirl it after inoculation to make sure that the entire surface of the slant is moistened by the inoculum so that the entire surface of the slant is covered with growth after incubation.

Lawn

When the entire surface of the plate is covered by uninterrupted bacterial growth it's referred to as a lawn. Liquid drops of bacterial growth are the most effective way to produce a lawn. Using a swab, three (3) to four (4) drops of dense inoculum can be swabbed in several overlapping directions to produce a lawn. Solid culture can be used in the same way; however, a seamless lawn is less likely using solid culture inoculum.

Streak

Streak techniques are by far the most useful inoculation techniques, because they demonstrate the widest variety of bacterial properties.

Linear Streak

This can be done with a swab, or loop depending on the nature of the inoculum, and the bacterial property to be observed. Any biochemical test on a plate that is inoculated in order to demonstrate biochemical hydrolysis or the lack thereof should be inoculated in this way.

4-Way Streak

The four-way (4) streak is important because this streak technique is the cornerstone of isolating organisms, which makes this technique an integral part of the quality control process. Is best if performed with a loop, however it is sometimes beneficial to use a sterile swab to inoculate the first quadrant as it tends to hold more bacteria than loops and needles.

Broad Streak

The broad streak is one of the most important streak techniques if not the most important technique with respect to the technicians work flow. Mostly, because it serves as an intermediate storage of all the organisms except for organism #10 *Clostridium sporogenes*. The broad streak is best if performed using a sterile swab, and can be accomplished using a liquid or solid culture as long as the inoculum is dense and healthy. Remember that if the broad plates are to be used for storage the life of the plates can be extended by keeping them refrigerated, and their purity secured by being wrapped when not in use. The broad streak consists of a series of linear streaks that overlap maximizing the surface area that the plate provides.

Spread

The spread technique is seldom used by technicians. It has no practical use for a technician in the line of quality control or general bacterial culturing of organisms. When coupled with a serial dilution the spread technique can produce isolated colonies, however the four way (4-Way) streak is far more efficient at producing isolated colonies. Although the spread plate technique has little use to a technician, the mid-term does call for a serial dilution to be performed using a mixed culture and plated for both the review and the exam.

Reconstitution

Reconstitution is the act of adding water to something that was dried. In terms of bacteria reconstitution means to reanimate an organism by adding a protein-rich media (such as nutrient broth, tryptic soy broth, or BHI broth). Reanimating an organism with one of the media not only rehydrates the dried organism but the

nutrients help the organism begin to sub-divide upon incubation. It is recommended that 5ml to 6ml of media be used to rehydrate dried bacteria.

There are two types of reconstitution that any prospective micro technician will need to be familiar with, one comes in a cap-less glass vial and the other in a rubber stoppered vial. Each of the two requires different methods to aseptically access their contents.

Cap-less Glass Vial Instructions

1. Flame the pointed tip of the glass vial over direct flame for approximately ten (10 secs.) seconds.

2. Using a distilled water dropper bottle, squirt several drops over the pointed glass tip before it cools (The glass should shatter).

3. Using a sterile pair of forceps break open the glass vial being careful (wearing goggles) to protect your eyes, as glass can potentially shatter and injury someone.

4. Once the glass vial is open, aseptically transfer one of the three non-specific media (such as nutrient broth, tryptic soy broth, or BHI broth) to the dehydrated culture to rehydrate the organism. It is best if one (1ml) to three (3ml) milliliters is used to rehydrate an organism, then the rehydrated organism should be incubated for a minimum of twenty-four (24 hrs.)

Rubber Stoppered Vial Instructions

Note: *It is important that a technician be extremely careful when reconstituting rubber stoppered vials because of the many pieces that must be manipulated in the process of opening the vial, there is the potential for contamination.*

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1. Remove the metal rim that caps off the rubber stopper. Be careful when removing the metal rim because it tends to tear through both latex and nitrile gloves. If the metal rim does tear through the gloves it is strongly recommended that the vial be thoroughly sterilized via and open flame after the rubber stopper is removed.

2. Once the rubber stopper is removed, aseptically transfer one of the three non-specific media (such as nutrient broth, tryptic soy broth, or BHI broth) to the dehydrated culture to rehydrate the organism. It is best if one (1ml) to three (3ml) milliliters is used to rehydrate an organism, then the rehydrated organism should be incubated for a minimum of twenty-four (24 hrs.)

Storage of Organisms

There are (3) three ways we keep and maintain bacterial organisms here at Broward College:

(1) Parafilm broad streaked plates

(2) Freeze nutrient broth with three percent (3%) glycerol tubes

(3) Refrigerate nutrient broth with three (3%) percent glycerol tubes. Each one of the methods mentioned has optimal particularities that need to be observed in order to ensure the purity and viability of the bacteria being cultured.

Parafilm Broad Streaked Plates

Broad streaked plates are wrapped for several reasons

a. Parafilm extends the life of the plate by trapping water in the enclosed plate and significantly reducing evaporation of the water within the plate's media.

b. Parafilm prevents the organism from being exposed to environmental contamination sources such as airborne spores both bacterial and fungal.

c. Parafilm also prevents pests from having access to potentially contaminate the broad plate(s) as well, sometimes ants fruit flies have been known to enter plates that aren't wrapped with parafilm.

d. In addition, wrapping plates with parafilm protects the organism and media within from potential contaminants.

e. The broad streak uses a large portion of the plate's surface area which helps the technician because the plate can be used on multiple occasions as long as aseptic technique is exhausted.

f. The broad streak is also useful in that since it doesn't cover the entire surface of the plate it allows for the edge of the organism's growth to be seen. Often contamination can be seen on the edge of growth where differences in morphology can be observed. The life of broad streaked plates varies based on how much media was initially poured into the plate. The plates are good until the media dries out.

Note: With time plates will lose moisture due to evaporation, and once the plate is completely dry it will no longer support any bacteria.

If the broad streaked plates are only accessed using strict aseptic technique and they are wrapped with parafilm properly after each use, they can last four (4) to six (6) months depending on the organism. It is recommended that they be tested and sub-cultured every three (3) months or every semester.

Freeze Nutrient Broths with 3% Glycerol Screw Capped Tubes

Nutrient Broths fortified with 3% Glycerol screw capped tubes are used to freeze sub-cultured organisms for long term use. Nutrient Broth is the media of choice because it is the most versatile and supports the growth of all but one organism on the organism list.

Glycerol is added because it helps to protect the bacterial cell membrane from puncture as the water within the media crystallizes and produces sharp pointed ice cycles. The screw cap helps secure the organisms purity for the long-term storage for which it is intended. A typical frozen viable for up to three (3) years.

Refrigerate Nutrient Broth with 3% Glycerol Screw Capped Tubes

Once thawed the frozen Nutrient Broth with three (3%) Glycerol Tubes can be used repeatedly as stock organisms if the organism is exposed to new media and subsequently incubated after each use.

Note: Incubation times and temperature vary depending on organism and media used.

Autoclave Validation

Requirements for autoclave validation vary by state. We have no state requirements re this practice in Florida. However, this practice is important in ensuring the autoclave is functioning reliably (sterilizing material). According to literature sterilization units can be evaluated for effectiveness with spores of *Bacillus stearothermophilus* at least once each 40 hours of operation or each week, whichever is less frequent.

Autoclaves are used to sterilize and decontaminate biological waste. The key components are:

Appropriate use of the autoclave to decontaminate biological waste

- Minimal parameters are 121°C at 15 psi for 15 min.
- Time may need to be increased for larger loads and larger volumes of fluid.
- Items should be loaded in a manner that ensures that steam can penetrate packages and test tubes.

Recordkeeping – There should be a log or notebook adjacent to the autoclave to indicate

- Date
- Time
- User name and contact number
- Type of load (liquids, hard goods, etc.)
- Items autoclaved (media, waste, pipettes, etc.) and amount
- The temperature, pressure and duration of the treatment.

Ideally, any autoclave paper tape is kept in the draw directly across from the autoclave unit. The waste log to verify autoclave parameters are also located just opposite the autoclave. Logs are maintained for at least 3 years.

Performance verification – threefold

1. If the autoclave has paper tape to record performance, this should be checked prior to opening the door to be sure all temperature, pressure, and/or time parameters were met.
2. Autoclave indicator tape should be clearly visible on each item placed in the autoclave (one per rack of tubes, one per beaker, one on a bag of used plates, etc.). Some biological waste bags have integrated heat sensitive indicators, e.g., Fisherbrand™ Orange Autoclave Bags with Sterilization Indicator.

3. The person in charge of the autoclave operation or a designated safety officer should conduct a monthly performance verification using the biological thermophilic spore former *B. stearothermophilus* (ATCC 7953).

4. There are several different verification methods that employ this organism. This testing should be documented monthly and readily available for inspection.

5. Waste shall not be considered sterilized if the tape or equivalent indicator fails to indicate that a temperature of at least 250 °Fahrenheit (121 °Celsius) was reached and a pressure of at least 15 psi was maintained during the process.

Annual Calibration and Maintenance

- This service is performed by an outside maintenance person, familiar with the operation of autoclaves.

Lab Classification

Biosafety Level 2 (BSL-2) facility, containment devices, administrative controls, and practices and procedures that constitute BSL-2 are designed to maximize safe working conditions for laboratory personnel working with agents of moderate risk to personnel and the environment.

BSL2 practices, equipment, facility design and construction are applicable to clinical, diagnostic, teaching, and other laboratories in which work is done with the broad spectrum of indigenous moderate-risk agents that are present in the community and associated with human disease of varying severity.

With good microbiological techniques, these agents can be used safely in activities conducted on the open bench, provided the potential for producing splashes or aerosols is low. Hepatitis B virus, H1V, the salmonellae, and Toxoplasma spp. are representative of microorganisms assigned to this containment level.

Extreme caution should be taken with contaminated needles or sharp instruments. Even though organisms routinely manipulated at Biosafety Level 2 are not known to be transmissible by the aerosol route, procedures with aerosol or high splash potential that may increase the risk of such personnel exposure must be conducted in primary containment equipment, or in devices such as a biological safety cabinet (BSC) or safety centrifuge cups.

Personal protective equipment (PPE) should be used as appropriate, such as splash shields, face protection, gowns, and gloves. Secondary barriers such as hand washing sinks and waste decontamination facilities must be available to reduce potential environmental contamination.

CDC Summary of Recommended Biosafety Levels for Infectious Agents

Biosafety Level	Agent Characteristics	Practices	Safety Equipment	Facilities (secondary barriers)
BSL-2	Associated with human disease, hazard from percutaneous injury, ingestion, mucous membrane exposure	Standard microbiological Practices Limited access Biohazard warning signs Sharps precautions Biosafety manual defining any needed waste decontamination or medical surveillance policies.	Class I or II biosafety cabinets (BSCs) or other containment devices used for all agents that cause splashes or aerosols of infectious materials Laboratory coats and gloves Face protection as needed	Open bench top sink Autoclave

Biological Safety Cabinets (BSCs)

Requirements

In microbiology teaching laboratories that handle organisms at BSL2, most standard pipetting and plating protocols, if done properly, do not generate aerosols such that a BSC is necessary for student use. In these cases, PPE, including eye-protection, and proper handling of materials are sufficient.

Whenever procedures have a potential for creating infectious aerosols, properly maintained BSCs inspected and certified annually), other appropriate PPE, or other physical containment devices must be used. These procedures are standard laboratory procedures and include some pipetting, centrifuging, grinding, blending, shaking, mixing, sonicating, and opening containers of infectious materials.

Additionally, the BSC is required when using high concentrations or large volumes of infectious agents or when opening sealed containers of organisms in a BSL2 lab that become depressurized upon opening and can result in the release of concentrated stock culture.

Certification

Biosafety cabinet certification is crucial to maintaining primary containment to keep the lab and personnel as safe as possible.

Class I and II biological safety cabinets are tested and certified when they are installed in a lab, any time they are moved to a new location, or following any maintenance procedure. The BSC should be certified annually.

This requires a knowledgeable NSF-certified technician and is not something lab personnel are able to perform. There should be a sticker prominently displayed on the front of the BSC that tells when the BSC was last certified and when it is due for recertification. Users should be in the habit of checking the certification each time they use the hood.

If you need assistance with cabinet re-certification contact Occupational and Environmental Health and Safety (OEHS) at (801-581-6590).

Class II BSCs are not fume hoods and must **NOT** be used with volatile chemicals, unless they are a Type B2 cabinet that is vented to the outside. The BSC is designed to contain biological specimens.

Class II BSCs have an inward airflow that will protect the user, a HEPA-filtered downward laminar airflow to protect the specimen, and a HEPA-filtered exhaust that protects the lab.

Care and Use

Personnel education is critical. The function of the BSC can be compromised by inappropriate actions or an unsuitable location within the lab. Inward flow of air may be disrupted by movement of others in the room, improper placement of materials within the hood, opening and closing doors to the lab, movement of arms into and out of the BSC, and sideways motion of hands in the cabinet.

BSCs should be surface decontaminated with an appropriate disinfectant prior to and at the end of any work performed in the cabinet: 70% ethanol is commonly used but is not appropriate after handling blood or other potentially infectious material as defined in the OSHA Bloodborne Pathogen Standard, in which case an EPA-registered disinfectant must be used.

The entire cabinet should be thoroughly cleaned at least once a month. This includes removal and disinfection of the bottom pan as well as thoroughly cleaning the front and back of the sash and all interior surfaces of the BSC.

References

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