

NCXT Tissue Culture Lab Protocol

(Adapted from Lauffenburger lab procedures)

This protocol describes tissue culture techniques and regulations at NCXT. These guidelines **must** be followed- failure to do so can result in damage to your and the experiments of others. Repeated failures to follow the guidelines can result in loss of privileges.

Dealing with Biohazards:

Although most cell lines are reasonably safe to work with, be aware that you are working with or around potentially dangerous materials (you should have already taken Blood Borne Pathogen training since we use human cell lines and blood samples in our facilities). To protect everyone's safety, follow these guidelines:

- 1) Label **EVERYTHING**. Minimal labeling includes your initials, the date, and the cell line nomenclature. For cell passages, it is usually best to include passage # and split ratio for your own information.
- 2) For long term experiments update the plate with the last date you changed media, etc. In general, cells that haven't been observed in several days should be disposed of to minimize contamination risks.
- 3) Anything that has touched cells should be placed in bio-waste container, not general waste. Media/serum that has not been used on cells can be disposed of in the sink.

Aseptic Technique:

Contamination by microorganisms is a major problem in tissue culture. Bacteria, mycoplasma, yeast, and fungal spores may be introduced via the operator, the atmosphere, work surfaces, solutions and many other sources. Proper aseptic technique seeks to eliminate these contaminants. It is important to establish and maintain aseptic techniques particularly given the importance of cell culture to everyone's experiments in the lab. Contamination can be minor and confined to one or two cultures, can spread among several cultures and infect a whole experiment, or can be widespread and wipe out your entire stock. Catastrophes can be minimized if 1) cultures are checked carefully under a microscope, 2) cultures are maintained without antibiotics for at least part of the time to reveal cryptic contaminations, 3) reagents are checked for sterility, 4) bottles of media are not shared with other people, and 5) the standard of sterile technique is kept high at all times. All materials that will come into direct contact with the culture must be sterile. Aseptic technique is a combination of procedures designed to reduce the probability of infection. Most cell culture work is done in a laminar flow hood.

General Rules:

1. Wear gloves – but note that these are to protect you, and are not inherently clean!

2. Keep the work surface clean (clean with 70% ethanol and keep the work area clear enough to allow work without reaching over an item and preventing inadvertent brushing of a sterile tip against another object.)
3. Use sterile reagents and media and work to keep them that way (do not reuse tips, clean the outside of reagent bottles with 70% ethanol, autoclave or sterile filter as appropriate)
4. Keep bottles, flasks and tubes covered as much as possible.
5. Remove liquid from containers at an angle.
6. Clean with 70% ethanol often (surfaces and gloves).
7. Remember that it is the inside of containers (autoclaved bottles, centrifuge tubes, etc) that is sterile.. treat the outside as dirty and clean with EtOH.

The major advantage of working in a laminar flow hood is that the working environment is protected from contamination by a constant flow of filtered air. A vertical flow hood is where air blows down from the top of the hood onto the work surface. Follow these guidelines when using the laminar flow hood. Keep this direction of flow in mind when setting up your work area.

Start-up in the hood

- 1) The hood must be operating correctly to be used- be sure UV light is OFF, turn on regular light if needed. Sash should be opened to upper tape mark (approximately 8 inches of clearance). Hood blowers are not to be turned off- if they were turned off for any reason, allow to run for 15-20 minutes to clean the air in the hood.
- 2) Remember that everything that goes INTO the hood must be cleaned with 70% EtOH. This includes your hands. Spray them down before starting.
- 3) Swab down the work surface liberally with 70% EtOH. Wipe with a Kimwipe, starting from the back of the hood and moving forward. Wipe off anything you intend to use in the hood (pipettor, vacuum line/valve, Pasteur pipet box, etc).
- 4) Bring stuff into the hood that you intend to use – pipets, tips, etc. Each of these items needs to be cleaned with 70% EtOH. In addition, if you plan to use tips or microcentrifuge tubes, be sure to keep an autoclaved stock that is kept for hood use only.
- 5) Dry media bottles thoroughly if they have been taken out of the water bath (this water is a great source of contamination). Swab with 70% EtOH, especially at the neck and bottom before placing in hood.
- 6) Ironically, the greatest source for contamination comes from the cell culture flask itself. If you notice that you have dripped media onto a flask, either replace the lid or carefully wipe down with 70% EtOH before returning to the incubator.

Working in the Hood

- 1) As stated above, **everything** that goes into the hood must be wiped with 70% EtOH before putting it onto the bench in the hood. This means your hands, new boxes of tips, new beakers of tubes, the exterior of bottle-top filter units, etc. **Everything**.
- 2) Keep the air intake (the vent in the bottom of sash, underneath your elbows) clear of **all** items at all times. If this vent is blocked, it allows room air to enter the hood,

which is great source of contamination! Also, keep items from directly blocking the vent in the back of the hood.

- 3) Bring only the items you need for a particular procedure into the hood to prevent cluttering your working space. Having a clear working space will significantly reduce the chance of contamination! Ensure easy access to items in the hood and maintain plenty of clear space in the center of the hood to work in.
- 4) This is a vertical laminar flow hood, which means that air flows straight down from the top of the hood. Do not work directly over any open vessels, or contaminants from your hands could be blown into your vessel. Always work at an angle, off to one side.
- 5) Watch what you are pipetting! The replaceable filters in the air pipettors are expensive. Ensure that you don't suck fluid up inside them. Especially watch the 1 mL pipettes...they fill really fast!
- 6) If you spill anything in the hood, clean up immediately to prevent cross-contamination and damage to working surface. Stop what you are doing and wipe up the spill – salts in particular can corrode the metal if left. Wipe the area with 70% EtOH before returning to work.
- 7) Styrofoam is not recommended in the hood – it often flakes and is difficult to keep out of the vents. Use plastic racks instead (if you need more racks, be sure to clean with EtOH before putting into the hood).

Incubators and Microscope

These are shared equipment, so they present a great method to spread contamination!

- 1) Before using the microscope, spray a Kimwipe with 70% EtOH and wipe down the stage. Do this also when you are done to prevent media spills from spreading between plates.
- 2) If you need counter space to set plates down on by the microscope, clean area with 70% EtOH.
- 3) The incubators are not technically sterile- however, every effort must be made to maintain their cleanliness to prevent contamination from spreading. If media has spilled in the incubator, clean the spill with EtOH. Spills into the water bath **MUST** be immediately taken care of- talk to either the lab manager or the tissue culture czars.
- 4) Put plates carefully into incubator- be sure not to bump other people's plates, minimize stacking as much as possible, and keep your own plates set up to allow you easy access to what you need next.

When You Are Finished

- 1) Trash must be disposed of properly- sharps/pipettes in the sharps waste bins, non-sharps that have contacted cells must be bleached and disposed of in autoclave bins, and all other non-sharp/non-biohazard waste goes in regular trash.
- 2) Remove all of your media/solution bottles (tightly capped), tubes, etc from the hood.
- 3) Be sure the pipet-aid is returned to its rack to turn off pump. Close the vacuum line.
- 4) Restock Pasteur pipettes if empty with a new container of autoclaved pipettes– remember to clean the container with EtOH before putting in hood.
- 5) Wipe down the bench with 70% EtOH.

- 6) Check serological pipettes and restock if necessary.
- 7) All waste media and/or cell cultures should be bleached. Add bleach to a final concentration of 10% and let stand for 20 minutes. Then discard down the sink and put plates, etc into biohazard bins. **ALL BIOHAZARD WASTE MUST BE PROPERLY DISPOSED OF BEFORE YOU LEAVE FOR THE DAY!**

Detailed Aseptic Technique

There are tons of ways to contaminate your cells, and this list is not exhaustive but includes many general guidelines. Don't worry- this will quickly become second nature..

- 1) Solids (tips, etc) are autoclaved by the lab helpers. The indicator stripes on the autoclave tape should turn black if an object has been properly autoclaved.
- 2) Sterile filter liquids in the tissue culture hood into an autoclaved bottle.
- 3) Parafilm may be used to cover the outside of small media bottles before warming in the water bath (to help prevent water seepage into your media). When removing, clean with EtOH first, then remove the parafilm and EtOH treat as normal.
- 4) Bottles should always be tightly capped when outside the hood (*i.e.*, they should have been tightly capped the last time they were *in* the hood).
- 5) Never pour from one sterile container to another. Pouring will generate a liquid path to introduce infection from the outside to the inside. Always pipette or use filters when transferring from one bottle to another.
- 6) Do not fill a dish/flask so full or swirl it such that the medium spills over the edge. This will introduce a path of infection via liquid and may cause cross-contamination.
- 7) Avoid working too closely to the front, or the non-sterile area, of the hood. Keep working area at the center or towards the back. Keep the objects needed for the current procedure within reach; keep the others in the back.
- 8) Avoid working above an open bottle or dish in vertical laminar flow. Always work around them unless they are capped or covered.
- 9) Avoid leaving bottles, dishes, and flasks open when they are not in use. If the cap must be laid down, place it face-up/face-down towards the back of the hood where there is less traffic and less chance of being touched or crossed over. Correct cap placement has been debated. Having a cap facing up can potentially introduce airborne particles and drive non-sterile lid liquid onto the interior face of the cap, where contaminations can fall into the bottle upon recapping. If face-down placement is preferred, then make sure to swab the area specifically and thoroughly before the cap is placed down there. Conversely, if hood surface sterility cannot be *absolutely* guaranteed due to high traffic or cluttering, then face-up is a better option. The best placement, however, is to place the cap on its side and towards the back of the hood. This way the interior is not in contact with the air flow or with the work surface. However, this is not possible with dishes. Therefore, exercise good judgment in light of individual operating style and the hood setup.
- 10) Withdraw a pipettes from wrappers in the center of the working area, attaching directly to pipet-aid. Ensure that the tip does not come into contact

with anything (including the outside of the wrapper). Dispose of wrapper immediately.

- 11) Avoid contact between the tip of the pipette and the mouth of the bottle. The mouth and neck of the bottle (both inside and out) present a potential source of contamination.
- 12) When working with Pasteur pipettes, do not remove pipettes directly from the tube with fingers. Shake the tube gently to cause the pipettes to slide out slightly, and then withdraw a pipette with fingers without touching the other pipettes and the tube interior.
- 13) Handle the pipette with a steady hand. Avoid large motions and do not let the tip touch anything non-sterile. Keep the tip away from the front and far above the objects in the hood.
- 14) To keep the hood from being cluttered, do not leave any trash in the hood. Immediately discard uncontaminated wrappers in the regular trash. Put all pipette tips and biologically contaminated sharps in the sharps biohazard waste container. Put all biologically contaminated tissue culture plates, flasks, and other non-sharps in the non-sharps biohazard waste container (after bleaching). However, an effort to minimize entry/exit from the hood should be made to minimize disturbances in the laminar flow at the entrance, which may create the potential to waft in contaminants.
- 15) New cell lines are a potential danger to the lab stocks- they should be kept in the designated incubator until you have ascertained that they are free of bacteria, yeast, fungi, and mycoplasma contamination. After you have done that, move cells to your incubator space the next time you passage rather than moving the old plates.