Tissue Culture Orientation

1. Complete EH&S Biosafety training module.
   - General Biosafety Training
   - https://ehs.colorado.edu/training/general-biosafety-training/

2. Media Supplies Storage
   - Cold room
     - NO CARDBOARD or PAPER allowed in this room, plastic storage boxes only to prevent mold growth.
     - Media stocks: each project group is responsible for maintaining stocks of their own medium, it is not a common stock ordering item. Be sure to order medium again when one of the two boxes assigned to your project group is empty.
   - TC Aliquots
     - Aliquots of FBS (50mL), penicillin/streptomycin (6mL), and amphotericin B (fungizone)(1mL), and 10x trypsin (5mL in 50mL Falcon).
     - Aliquots are prepared by graduate students. If you use the last item in a rack, let your mentor and/or graduate students responsible for aliquoting know immediately so that they can be sure there are stocks available for everyone.

3. TC Room
   - Working stock of consumable supplies (dishes, flasks, PBS, pipettes, tips, conical tubes and epi tubes) are kept in the TC room for easy access and use. If you use the last of something, you must restock from the freezer room or general lab overstocks. If you use the last of any overstock, let your mentor know so that they can order more or talk with the lab member in charge of common stocks.
     - Freezer room: petri dishes, flasks, multi-well plates, conical tubes, serological pipettes, syringes, syringe filters, cryovials, bleach
     - Above water/bead bath: epi tubes, paper towels, aluminum foil, sterilization pouches
     - General lab storage: pipet tips, needles, gloves
   - Equipment in the tissue culture room is only for tissue culture use. Please keep these items in the tissue culture room and prevent them from wandering to your bench, fridge and freezer space or elsewhere in the lab.
     - This equipment includes but is not limited to: pipets, tube racks, and ethanol bottles.

4. Cell Freezers
   - Find a buddy - there must be 2 people present to go into a cell freezer to store or retrieve cells.

- Remove the entire stack from the liquid nitrogen and place on the ground *before* removing the safety bar – this ensures that no vials or boxes of cells fall into the liquid nitrogen causing problems for the entire lab.
    - REPLACE THE SAFETY BAR prior to replacing the stack into the liquid nitrogen.

5. CO2 tanks
    - Tanks are connected to a manifold mounted in the entry way of A391
    - Verify that 2 tanks in use + 2 full back-up tanks are connected at all times
    - Tank pressure: full tanks read 800-1000 psi. If you notice the pressure dropping, be sure to tell the person in charge to order new tanks.
    - Line pressure:
        - Never change the pressure valve!
        - Manifold is set to 15 psi. Do not exceed 15 psi or the incubators will be damaged.

6. Biohazard Waste
    - Anything that has come into contact with living cells, body fluids, viruses, clinical materials, and other microorganisms or any waste that involves the presence of organisms containing recombinant or synthetic nucleic acid molecules, or other organisms hazardous to human health.
    - Sharps
        - Sharps (needles, glass Pasteur pipettes, syringes *with or without* needles, razor blades) should be disposed of in the plastic sharps containers underneath each hood
        - If this is full, close the lid and place on the autoclave cart and tape closed with autoclave tape. Grab a new sharps container from the overstock room and place under TC hood.
    - Liquids
        - Liquids are aspirated into the vacuum flasks containing bleach. Check liquid level in aspirator flask *before and afte*r working in the TC hood.
        - When the flask is full, add more bleach and wait until liquid is clear before pouring down the drain. Add ~1" of bleach to the empty flask before hooking back-up to the vacuum.
        - To dispose of media and cell culture vessels outside of the hood, pour bleach in the vessel until medium is clear, swirl, and pour down the drain.
    - Plastic serological pipettes
        - Plastic pipettes are disposed of in the containers under the hoods. Only serological pipettes should go in these containers.
    - Tips
        - Plastic pipette tips are disposed of into the small biohazard bags in the hoods (*no conical tubes into these bags!*). When the bag is full, tape it shut, double bag to prevent punctures, and placed in the biohazard bag in the pedal containers.

- Other
    - Anything that does not fit into one of the above categories (e.g. culture dishes, epi tubes, multi-well plates, media bottles) that has been contaminated with biohazardous materials should be placed into the pedal containers. *No liquids may be left in any containers that go into this waste stream.*
- Solid Waste Disposal
    - Solid waste (serological pipettes and general biohazard waste) should be emptied when the containers are 90% full. Full biohazard waste bags should be twisted and taped with autoclave tape (in drawer under bead bath) and placed on the cart to be autoclaved and disposed.

7. Non-Biohazardous Waste
    - Recycling
        - Certain plastics can be recycled via boxes in the TC room – tearable plastic (such as the bags that serological pipets are packaged in) and the hard plastic portion of multi-well plate packaging. The paper portion of well plate packaging must be treated as trash.
    - Compost
        - Non-biohazardous paper towels may be composted through the green baskets throughout the lab. When full, lab members empty these baskets into the bathroom trash.

8. Sterile Technique
    - Spray 70% EtOH on work area, supplies and gloves
    - Gather all necessary supplies prior to starting work in order to minimize movement in and out of hood
    - Arrange items in hood to minimize clutter and airflow obstruction
    - Do not block air vents – interruption of airflow compromises hood sterility
    - Only open sterile bottles, tubes, supplies, etc. inside the TC hood
    - Do not pass anything over open bottles, tubes, plates, etc.
        - Generally, it is advised to loosen caps prior to starting so they can be removed and replaced with one hand while working.
    - Do not touch sterile tips or pipettes to surfaces.
        - When in doubt, change tips or pipettes!

9. Labeling
    - Anything used for more than a single use or over several days needs to be properly labeled. This includes, but is not limited to, petri dishes, T-flasks, and media/supplement bottles/tubes.
    - Proper labeling includes 1) initials (yours and mentor's if applicable), 2) date, and 3) contents. If something is found without a proper label it may be disposed of by another lab member.

10. Cell Culture
    - Maintenance, passage, and counting
    - Will depend on the specific cell lines with which you'll be working
    - Good technique will prevent most contamination, but it will still occur. Any contaminated cells or samples should be disposed of immediately and other lab members should be alerted so they can monitor for contamination.

11. Special notes for undergraduate tissue culture
    - Undergraduates should not aliquot common reagents (e.g. serum, trypsin, pen/strep). It is important that everyone have access to a consistent stock of tissue culture reagents. Graduate students and post-docs will take care to prepare these reagents in a consistent manner.
    - Undergraduates must be supervised by their mentor when accessing the cell dewars.

Tissue Culture Basics

Cells must be taken care of properly so they can stay happy and give you good, consistent results! This includes keeping them contamination-free, well fed, and in the proper atmospheric conditions. Typically, mammalian cells are kept at 37°C and 5% CO_2 in a humidified incubator, but some cells may be kept at other CO_2 levels.

The cells we culture are either primary cells (derived directly from the source, undergo senescence within a few passages) or cell lines (cells that have been transformed so they can keep dividing while maintaining the same phenotype). It is best to only use cell lines up the passage recommended, though, since repeated passaging can eventually lead to senescence or other phenotypic or genotypic changes.

Cells are typically cultured on rigid tissue culture dishes or plates, which are treated so the surface is hydrophilic, and cells can adhere to the surface through proteins deposited from the serum. Some cells, however, require coating of dishes with ECM proteins prior to cell seeding. Additionally, some cells grow in suspension and thus require untreated tissue culture dishes or plates.

<u>Feeding cells/Media</u>
Cells should typically be fed (media exchanged) every 2-3 days if they are in between passages. The media is different for each cell type, but typically they consist of: basal medium (contains essential amino acids, vitamins, glucose, ions, buffers, pH indicator coloring), serum (isolated from animal plasma and not so well-defined, but contains nutrients and growth factors required for cell growth), antibiotics/anti-fungals (penicillin/streptomycin and Fungizone), and L-glutamine (an essential amino acid that is less stable than others when stored above freezing and may require supplementation). Generally, a bottle of medium is good for about 1-2 months. Anything left at 4 degrees for longer than this timeline should be disposed of by treating with an appropriate amount of bleach.

Media color is often an indication of the culture status due to the inclusion of compounds such as phenol red: orange/yellow media indicates acid waste product buildup (you should feed cells before they get to this point!), which pink media indicates that the CO_2 level is low (check the incubator to make sure the CO_2 levels are OK).

<u>Passaging cells</u>
Cells need to be passaged, or subcultured into a more dilute fraction, every few days so they can continue dividing. The amount of time between passages (and the dilution of each passage) varies between cells types, but in general cells should be passaged before they reach confluence (100% density) to prevent senescence or other changes in cell behavior due to high cell density.

General protocol for passaging cells:

1) Heat media in the bead or water bath, trypsin-EDTA (0.25%) preferably at room temperature (since trypsin can self-digest and lose activity, especially at higher temperatures) for about 20-30 minutes.
2) Aspirate media, rinse cells with PBS (without calcium or magnesium) to wash out as much media as possible since serum contains protease inhibitors that can lower the activity of trypsin.
3) Aspirate PBS, add a small about of trypsin-EDTA so that it just covers the bottom of the dish/flask and place back in the incubator for 3-5 minutes (varies for cell type).
    a. Trypsin is a protease that cleaves proteins involved in cell-matrix and cell-cell bonds. It can be damaging to cells when they are exposed to too much or for too long so it's important to use as little trypsin as possible for as short an amount of time as possible.
    b. EDTA is a metal ion chelator that sequesters calcium required for integrin (cell-matrix) and cadherin (cell-cell) bonds.
4) When the cells are detached (you may need to rap the flask against the counter and verify that cells are detached via the microscope), add the desired amount of media for dilution into subsequent flasks and gently pipet a few times to break up cell clumps and distribute the cells evenly (while avoiding bubbles!). It is important to add enough media to inactivate the trypsin so it does not damage the cells (a 1:50 dilution is typical). Alternatively, you can suspend the cells in media, centrifuge down (1200rpm, 5 min), and resuspend in fresh media to remove nearly all the trypsin.

<u>Contamination</u>

We almost always make our media with antibiotics, so contamination with bacteria is not a common problem. However, if your cultures do become contaminated, you will generally be able to see cloudiness in your media within 18-24hr of contamination. **If you see contamination,** you should **dispose of your plate/flask immediately** by bleaching it and disposing of it in the biohazard waste. **Alert the others in your incubator** so they can check their cultures. **Alert your mentor and the TC Manager(s)** so that a systemic problem can be identified (e.g. a contaminated stock reagent).

<u>Freezing cells</u>

To store cells for later use, they can be kept in a solution of culture medium or serum + DMSO (a cryoprotectant) and stored in liquid nitrogen or a -80°C freezer indefinitely. The protocol varies slightly for each cell type, so it's best to refer to the protocol that usually arrives with a vial of new cells. Typically, though, cells are trypsinized, resuspended and centrifuged, and resuspended again in 90% serum + 10% DMSO, and vials contain 1mL of cell suspension at about 2-5 x 10^6 cells/mL. Place vials in an isopropanol-containing slow-freeze container (stored in cold room) and store at -80°C overnight, then transfer to liquid nitrogen for long-term storage. Return the slow-freeze container to the cold room.

Thawing cells

1) Remove from storage in liquid nitrogen or -80°C. Immerse immediately in 37°C water bath (do not immerse cap) or run under hot water for 1-2 minutes until the vial is completely thawed – quick thawing will minimize damage to cell membranes. Wipe vial with ethanol.
2) Slowly pipet vial contents into flask with pre-warmed media, and evenly distribute cells by rocking the flask/plate back in forth in multiple directions. Change media once cells have attached to remove residual DMSO which is toxic to cells. Alternatively, pipet vial contents into a conical tube with media, spin down in the centrifuge (1200 rpm, 5 min), aspirate the supernatant to remove the DMSO, and resuspend in fresh media.

For more detail, refer to *Culture of Animal Cells* by R. Ian Freshney, in the lab or available online through the CU library website.

Tissue Culture – Hands-On Training

Student: _____ Mentor: _____

Once completed, return your signed form to the Bio-side Coordinator or TC trainer.

TC Orientation Date: _____

EH&S Biosafety Training Date: _____

Exercise #1 – Sterile technique practice (to be completed in biosafety cabinet)

1. Pipet 30mLs of sterile PBS into a 50mL conical tube.
2. Pipet 8mLs from the 50mL conical tube into a 15mL conical tube.
3. Aspirate the PBS and repeat 1x more.
4. Pipet 15µL from the 50 mL conical tube into an epitube.
5. Pipet 150µL into an epitube.
6. Pipet 500µL into an epitube.
7. Aspirate all remaining PBS.
8. Dispose of Pasteur pipet, serological pipets, wrappers, and conical tubes.
9. Empty vacuum flask.

Date: _____ Student: _____ Mentor: _____

Date: _____ Student: _____ Mentor: _____

Exercise #2 – Cell culture and passage

1. Observe your mentor feeding cells.

Date: _____ Student: _____ Mentor: _____

2. Your mentor observes you feeding cells.

Date: _____ Student: _____ Mentor: _____

3. Observe your mentor passaging cells.

Date: _____ Student: _____ Mentor: _____

4. Your mentor observes you passaging cells.

Date: _____ Student: _____ Mentor: _____