Tissue and Cell Culture User Guide

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Centre for
Heart Lung Innovation
UBC and St. Paul's Hospital

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HLI – Tissue and Cell Culture User Guide

December 6, 2019

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Chapter 1
General Outline

1. These tissue culture laboratories are a multi-user facility.

2. Research groups using the facility agree to abide by a number of general guidelines as a condition of use of the facility and these guidelines are intended to ensure:

- Safety of the users of the facility
- Good laboratory practice including sterile techniques, to ensure efficient and co-operative use of this research facility by all users.
- Appropriate and adequate storage of consumable materials for each group.

3. Designated users agree to share the cost of maintenance of the facility

4. The tissue culture administrative group consists of:
Dr. Delbert Dorscheid [Chair], Gurpreet Singhera [Cell Culture trainer/manager], Mary Zhang, Chung Cheung, Anthony Tam, Julia Kong, Teddy Chan, Jingchun Zhang, May Fouadi.

TC core advisory board has rights to fine the labs for being repetitive defaulters and failing to follow SOPs.

Please send the comments to Gurpreet.Singhera@hli.ubc.ca

A PDF of this guide can be found at http://home.hli.ubc.ca/services/cell_culture.html

Chapter 2
General Rules

The general principles of good laboratory practice, plus some additional principles apply to the tissue and cell culture facility:

1. **No eating, drinking or smoking in the laboratory**. Sign up for your TC experimental time.

2. **Yellow gowns shall be worn in the tissue culture laboratory**. Please keep a clean yellow gown on one of the pegs inside or outside the doors of tissue culture rooms. It is specifically for use in the tissue culture laboratory. Laboratory coats are notorious carriers of animal hair, dander etc. into the otherwise aseptic environment of the tissue culture laboratory.

3. **Materials that are known to be infected with viruses or other pathogens shall not be brought into the other cell culture rooms**.
See the "use of tissue and cell culture laboratory" (Chapter 5).

4. **Gloves shall be worn all the time when working in the cell culture facility including working in biosafety cabinets and when handling cultures from incubators and using microscope etc**.

If you are wearing gloves to protect yourself (rather than simply to ensure sterility of cultures) change them before touching things that will be touched by other workers. This particularly applies to incubators doorknobs and handles, the microscope and centrifuge. See "standard practices for cell culture" (Chapter 3).

5. **Sterile techniques and training in tissue culture techniques.**
Each research group is responsible for training its own new members in sterile techniques. The administrative group will assist each research group by offering a brief mandatory introductory course to the theory and general sterile techniques for each new staff/student, which include: general rules, standard practices for
cell culture, cleaning and waste disposal, use of tissue culture laboratories, use of equipment, including biosafety cabinets, incubators and inverted microscope and, in addition, procedures unique to the HLI tissue culture facilities.

6. **Clean up spills immediately!** Clean surfaces (especially in the biosafety cabinets and centrifuges) and sterilize them with alcohol after each use. Note that ethanol is a fixative and any spills containing proteins should be wiped up with water and/ or bleach prior to using ethanol for sterilization.

7. **You are responsible for ensuring that waste is disposed of appropriately** (including autoclaving it if necessary). See "waste disposal" (Chapter 4).

8. Each research group is responsible for their own supplies of chemicals, media and tissue culture consumables.

♦ There is limited storage space in the tissue culture laboratories, so supplies should, as much as possible, be stored outside these laboratories.

♦ Label your supplies clearly. Chemicals and media must be labeled appropriately with minimum of full name, date and contents. Outdated or unlabeled materials will be disposed.

♦ If you wish to borrow supplies, please ask the owner first and make a firm arrangement for the replacement of borrowed items.

9. If you are unsure of how to use a piece of equipment, please ask the tissue and cell culture administrative group members. Training is mandatory for each equipment prior to use. Failing to follow this rule will hold PI of the staff/student responsible for the repair cost of the damaged equipment.

10. **Bleach** mixed with water at a 1:9 ratio (i.e. 10 percent) is **potent** for about a day (it's more unstable in **it's** diluted form) – make fresh 10% bleach solution enough for one day use.

Chapter 3
Standard Practices for Cell Culture

1. Wash hands with antiseptic soap before tissue culture work.

2. Wear gloves at your own discretion, but particularly when handling tissues. Change gloves regularly, and be careful not to contaminate other members of the laboratory by touching communal surfaces or items with contaminated gloves.

3. Use biosafety cabinets for all cell and tissue manipulations (**see "Biosafety Cabinet user guide"** Chapter 6).

4. Only one cell line at a time should be handled in each BSC. If you wish to work beside another individual in the 6 foot "double hoods", ask whether the individual wants you to. The answer may be no, particularly if he or she is using hazardous chemicals or delicate cells.

5. Be careful not to create aerosols in handling liquids (see "sterile techniques", Chapter 7).

6. Decontaminate surfaces before and after each procedure (see "Cleaning and waste disposal", Chapter 4).
7. All cultures and supernatants should be handled as infectious materials by placing waste in 10% bleach for minimum 10 min.

8. All contaminated materials must be decontaminated appropriately before disposal (see Chapter 4).

9. Remove your gloves before leaving the room. Gloves are not to be worn outside the tissue culture room.

10. Wash your hands for 60 sec after your work. Gloves are not a substitute for hand washing.

Chapter 4
Cleaning and Waste Disposal

Cleaning:

1. Clean up spills immediately.
2. **Note that ethanol is a fixative and any spills containing proteins should be wiped up with water prior to using 70% ethanol for Sterilization**.
3. Clean surface (especially in the biosafety cabinets) and sterilize them with 70% alcohol after each use.
4. You are responsible for ensuring that waste generated by your experiments is disposed of quickly and appropriately (including autoclaving it if necessary).
5. The laboratories are cleaned on a regular basis (see "Clean tissue culture rooms and equipment", Chapter 11). This includes cleaning the floor, bench surfaces, water baths and biosafety cabinets, and dismantling and sterilizing shelves, walls and water tray in the incubators.

Waste Disposal:

1. All waste culture fluids should be handled as infectious materials:
To decontaminate: **Add 10% fresh bleach** (by adding nine parts water to one part laboratory bleach (sodium hypochlorite) and then **let sit for 10 min contact time before sink disposal**. Use plenty of water when disposing down the drain to dilute down bleach effect.
2. Flasks and used plastic ware and gloves: yellow bags.
3. Packaging and non-contaminated waste: black bags.
4. Sharps: biohazard sharps containers.

Chapter 5
Use of Tissue and Cell Culture Laboratories

1. Secondary Tissue and Cell Culture Rooms (Room M110, Room B50, Room B54):

These laboratories are designed for handling established continuous cell lines. If you bring cell lines into the laboratory, you should ensure that they are not contaminated. You should be aware that cell lines from less reputable sources might be contaminated with microorganisms or other cell lines.

- Follow the standard practices for cell culture and waste disposal (see Chapter 3 and Chapter 4).

2. Primary Tissue and Cell Culture Room (Room M203, M120, B53, ~~ASL 18020~~)

This laboratory is designed and equipped for handling animal and human primary cultures, including primary cell cultures, primary culture of isolated tissues, human biopsy materials and blood/fluids, etc. Individuals establishing primary lines or performing organ cultures should keep their cultures separate from established continuous cell lines. Use the designated incubator and culture hood in the primary culture room.

- Animal cells may harbor viruses or mycoplasma that can infect humans.
- Human cells and tissues may be infected with pathogens.
- All cultures and supernatants should be handled as though infectious.
- All unused animal/human tissues must be disposed of in a specified way. Animal tissues should be packed in a red anatomical waste bag appropriate to the size of the carcass and must be stored in -20 freezer until ready to and away through housekeeping special disposal.
- Follow the standard practices for cell cultures (see Chapter 3).

3. Viral Tissue and Cell Culture Room (Room MB03/Lab K and Room M120):

The laboratories are designed for handling cells or tissues that are infected or are going to be infected with viruses.

- Follow the standard practices for cell culture (see Chapter 3).
- "Special waste disposal": All materials including supernatants, cells, bottles, flasks, pipettes (standard glass or plastic pipettes), etc. should be appropriately decontaminated before disposal. Directions may be different for each room since different viruses are used.

- Individuals must obtain permission from Gurpreet Singhera for access to Room M120 or Mary Zhang for access to MB03. Refer to the Appendix under "SOP 3: Procedure for use of M120 viral tissue culture room" for M120 and "SOP2: CVB3 containment protocol" for detailed use of MB03.

Chapter 6
Use of Equipment

1. Biosafety Cabinets:

Most of the biosafety cabinets in the main laboratory are Class II type A biological safety cabinets. Both the inlet and outlet air are HEPA-filtered (HEPA is high efficiency particular air), so the air in the cabinet is sterile and air leaving the cabinet into the lab is filtered. Note that these are not "fume hoods"; volatile chemicals, such as dimethylsulphoxide (DMSO), introduced into the air in the cabinet will escape into the air in the laboratory. Do not use toxic or potentially toxic volatile chemicals in most of the hoods. The class II type A/B3 hood (Room 110, Main Culture Room, #1 Hood and Room 212) is capable of handling small quantities of toxic chemicals, provided that the external "receptacle" is activated. The biosafety cabinets in viral rooms (Room MR120 and MB03) are also vented outside. Please see respective SOPs in the Appendix.

Biosafety Cabinet (BSC) user guide

• Dedicated yellow gowns/ white lab coats should be used in TC labs. They SHOULD be left in same area after finishing your work. DO NOT bring lab coats from wet lab area to TC core as it can be a source of contamination.
• Gloves must be worn while working in BSC.
• Wear eye protection if there is a risk of splash (most likely outside the cabinet).
• Turn on the BSC for 10 min before use to purge non-sterile air.
• Wipe surface of the BSC with 70% ethanol (not absolute ethanol).
• Add equipment slowly to cabinet, wiping each item with ethanol. Don't block air grills. Wait 2 min before beginning experiments after setting up.
• Perform work. Don't move hands in and out of cabinet while flasks and media bottles are open.
• When finished, wipe all items with 70% ethanol as you remove them. Wipe the cabinet with distilled water first and then spray cabinet surface with 70% ethanol and let evaporate. (70% ethanol takes 10-20 min to be effective).
• Leave the BSC on for 10 min to purge airborne contamination.
• Turn off the BSC at the end of the day.
• Remove your gloves before leaving the room. Gloves are not to be worn outside the tissue culture room.
• Wash your hands for 60 sec. Gloves are not a substitute for hand washing.
• Walk slowly past biological safety cabinets to avoid creating drafts.

Waste Disposal:

• Flasks and used plastic ware and gloves: yellow bags.
• Packaging and non-contaminated waste: black bags.
• Sharps: biohazard sharps container.
• All waste culture fluids should be handled as infectious materials: add 10% bleach and then let sit minimum 10 min before sink disposal.

2. Incubators

The incubators are gassed with 95% air and 5% CO2.They must be humidified. Remember to check the water tray regularly and do not let it dry out. Do this only with distilled water **NOT milliQ water DE-IONIZED (DI**).

- **Distilled water is** free of contaminants and prevents mineral deposits in the **incubators** which **can** promote bacterial growth.
- The DI water, in its quest for ions, will aggressively attack the stainless steel in the incubator and water bath, pit the surface and allow corrosion to start. This will shorten the life of the unit.
- For the main tissue culture room on the first floor a distilled water outlet is available in the room. The temperature and concentration of CO2 should be checked on a regular basis.

3. Inverted Microscopy

The microscopes are capable of bright field and phase contrast microscopy. Manuals, spare lenses and attachments are kept in the drawers to the right of the scope. Please be careful in using them. If you wish to change the illumination mode, please obtain the assistance of someone who knows how the instrument works. Please keep the microscope covered when not in use.

4. Centrifuge

In main cell culture room, the instructions for the Beckman GS-6 centrifuge are posted above the instrument. In the primary tissue and cell culture room, there is a Beckman GPR centrifuge. Please balance tubes before running any of the centrifuges. Biohazard lids are available for the centrifuge buckets when centrifuging biohazardous materials (see respective MSDS provided in each facility). If you are unsure how to use the centrifuges, please ask your group cell culture supervisor (listed in Chapter 1).

Chapter 7
Sterile Techniques

Contamination by microorganisms remains a major problem in tissue culture cores. Bacteria, mycoplasma, yeast, and fungal spores may be introduced via the operator, the atmosphere, working surfaces, solutions, and many other sources. Proper sterile technique seeks to establish a strict code of practice and adherence to it, particularly if several people share the same working space. Catastrophes can be minimized if

1. Cultures are checked carefully by eye and by phase contrast microscope every time that they are handled;
2. Reagents are checked for sterility before use;
3. Bottles of media, etc., are not shared with other people or used for different cell lines; and
4. Standard of sterile technique is kept high at all times.

Elements of aseptic environment

• **Quiet Area:** Activities should be restricted in tissue culture laboratories. The tissue culture area should be kept clean and free of dust and should not contain equipment other than that connected with tissue culture. Non-sterile activities, such as sample processing, staining, or extractions, should be carried out elsewhere.
• **Working Surface:** one of the more frequent examples of bad technique is failure to keep the working surface clean and tidy. The following rules should be observed:
(a) Follow the "Biosafety Cabinet user guide" to do TC work;
(b) Start with a completely clean surface;
(c) Swab the surface liberally with 70% alcohol;
(d) Remove everything that is not required, and swab the surface down between the procedures;
(e) Arrange your work area so that you have easy access to all items;
(f) Mop up any spillage immediately and swab the area with 70% alcohol
(g) Remove everything when you have finished, and swab the work surface down again

• **Personal Hygiene:** Wash your hands before you start your experiment in the tissue culture laboratories. Gowns and caps are required under Good Manufacturing Practice (GMP) conditions [Food and Drug Administration, 1992]. If you have long hair, tie it back.

• **Reagents and Media:** Reagents and media obtained from commercial supplies will already have undergone strict quality control to ensure that they are sterile, but the outside surface of the bottle they come in is not. Some manufacturers supply bottles wrapped in polyethylene, which keeps them clean and allows them to be placed in a water bath to be warmed or thawed. The wrapping should be removed outside the hood. Unwrapped bottles should be swabbed in 70% alcohol.

Sterile Handling

• **Swabbing:** Swab down the work surface with 70% alcohol before and during work, particularly following any spillage, and swab it down again when you have finished. Swab bottles before using them for the first time each day, and also swab any flasks or boxes for the incubator.

• **Handling bottles and flasks:** When working on an open bench, you should not keep bottles and flasks open. When you are working in biosafety cabinet, bottles or flasks can be open and vertical, but don't let your hand or any other items come between an open vessel and the sterile pipette.

• **Pipetting:** Standard glass or disposable plastic pipettes (1ml, 2ml, 5 ml, 10 ml and 25 ml) are still the easiest way to manipulate liquids. It is necessary to insert a cotton plug into the top of a glass pipette before sterilization to keep the pipette sterile during use. The plug prevents contamination from the bulb or pipetting aid and reduces the risk of cross-contamination. A motorized pipetting aid is used for conventional graduated pipettes. Pipettors are particularly useful for small volumes (1 ml or less). Be sure to sterilize all of the pipettes and tips before use! Swab the motorized pipetting aid and pipettors with 70% alcohol before placing in the BSC.

• **Pouring:** Whenever possible, don't pour contents of sterile container into another, unless the bottle you are pouring from is to be used once only. The major risk in pouring lies in the generation of a bridge of liquid between the outside of the bottle and the inside, which may permit contamination to enter the bottle, so bottles or flasks that are stored or incubated after pouring are at a significantly higher risk of contamination.

Chapter 8
Contamination

Maintaining sterility is still one of the most difficult challenges to the newcomer to tissue culture. Part of the difficulty, of course, is due to awkwardness during early training, and experience will eventually cure the problem. However, in certain situations, even the most experienced worker will suffer from contamination. It cannot be overemphasized that general rules should be followed:

- Check living cultures regularly for contamination by using normal and phase-contrast microscopy and for mycoplasma by employing fluorescent staining of fixed preparations.
- Do not maintain all cell cultures routinely in antibiotics: grow at least one set of cultures of each cell line without antibiotics for a minimum of two weeks at a time, and preferably continuously, in order to allow cryptic contaminations to become overt.
- Do not attempt to decontaminate a culture, unless it is irreplaceable, and then do so only under strict quarantine.
- Do not share media or other solutions among cell lines or among operators, and check cell line characteristics periodically to guard against cross-contamination, especially when working with HeLa cells.

Sources of Contamination

There are several potential causes of contamination including:

- Failure in the sterilization procedures for glassware and pipettes
- Turbulence and airborne particulates (dust and spores) in the room;
- Poorly maintained incubators and refrigerators
- Faulty BSC;
- Contaminated cell lines or biopsies; and
- Poor sterile technique.

Types of Microbial Contamination

Bacteria, yeast, fungi, molds and mycoplasma all appear as contaminates in tissue culture and if protozoology is carried on in this same laboratory, some protozoa can infect cell lines.

Monitoring and handling Contamination

The following procedure is recommended to monitor for contamination:

- Check for contamination by eye and with a microscope at each handling of a culture.
- If it is suspected, clear the hood or bench of everything except your suspected culture and one can of Pasteur pipettes. Because of the potential risk to other cultures, this is best done after all your other culture work is finished. Check the culture with microscope again. If it is confirmed that the culture is contaminated, discard the pipettes, swab the hood or bench **with Conflict** (15 minutes contact time, followed by water cleaning and 70% ethanol), and do not use the hood or bench until the next day.

- Record the nature of the contamination. Send the culture medium and cells to the hospital laboratory for final identification (**may incur charges**) or you can use commercially available mycoplasma testing kits (Lonza).
- If the contamination is new and is not widespread, discard the culture, the bottle with the medium used to feed it, and any other bottles (e.g. trypsin) that have been used in conjunction with the culture. Discard all these into disinfectant (bleach for at least 10 min.).
- If the contamination is new and widespread, discard all media, stock solutions, trypsin, etc.
- All culture fluids, dishes, flasks and used plastic wares should be handled as infectious materials: add bleach and then let sit 10 min before disposal
- If you are unsure how to handle your cultures that are suspected to have contamination, please ask the tissue and cell culture administrative group members.

Visible Microbial Contamination

Characteristic features of microbial contamination are as follows:

- A sudden change in pH, usually a decrease with most bacterial infections, very little change with yeast until the contamination is heavy. This change in pH will be apparent by a change in the colour of the media (i.e., from red to yellow or pink) if a pH indicator such as phenol red has been included in the media.
- Cloudiness of the medium, sometimes with a slight film or scum on the surface or spots on the growth surface that dissipate when the flask is moved.
- Under low-power (~X 100) microscopy, spaces between cells will appear granular and may shimmer with bacterial contamination. Yeasts appear as separate round or ovoid particles that may bud off smaller particles. Fungi produce thin filamentous mycelia and, sometimes, denser clumps of spores. With toxic infections, some deterioration of the cells will be apparent.

• Under high-power microscopy (~X 400), it may be possible to resolve individual bacteria and distinguish between rods and cocci. At this magnification, the shimmering that is visible in some infections will be seen to be due to mobility of the bacteria.

Mycoplasma

Mycoplasma infection is one of the most serious contaminations in cell culture. Mycoplasma infections cannot be detected by the naked eye other than through signs of deterioration in the culture. The culture must be tested by fluorescent staining, PCR, ELISA assay, immunostaining, autoradiography, or microbiological assay. Fluorescent staining of DNA by Hoechst 33258 is the easiest and most reliable method and reveals mycoplasma infections as a fine particulate or filamentous staining over the cytoplasm at X500 magnification. The nuclei of the cultured cells are also brightly stained by this method and thereby act as a positive control for the staining procedure. Most other microbial contaminations will also show up with fluorescent staining, so low levels of contamination or particularly small organisms such as micrococci can be detected. It is important to appreciate the fact that mycoplasmas do not always reveal their presence by means of macroscopic alterations of cells or media. Many mycoplasma contaminations, particularly in continuous cell lines, grow slowly and do not destroy host cells. However, they can alter the metabolism of the culture in many different ways. Because mycoplasmas take up thymidine from the medium, infected cultures show abnormal labeling with [3H]-thymidine. Mycoplasma can alter cell behavior and metabolism in many other ways [Freshney, R.I. Culture of Animal Cells: a manual of basic technique (4th Edition) New York, Wiley- Liss (2000)], so there is an absolute requirement for routine, periodic assays to detect possible covert contamination of all cell cultures, particularly continuous cell lines.

Monitoring cultures for mycoplasmas:

♦ Chronic mycoplasma infection: a diminished rate of cell proliferation, reduced saturation density and agglutination during growth in suspension.

♦ Acute mycoplasma infection: total deterioration with perhaps a few resistant colonies, although these and any resulting cell lines are not necessarily free of contamination and may carry a chronic infection.

Performing mycoplasma testing (Hoechst 33258 staining):

The cultures are stained with Hoechst 33258, a fluorescent dye that binds specifically to DNA. Since mycoplasmas contain DNA, they can be detected readily by their characteristic particulate or filamentous pattern of fluorescence on the cell surface and, if the contamination is heavy, in surrounding areas. Monolayer cell cultures can be fixed and stained directly, but following centrifugation, the medium from cells growing in suspension will need to be added to an indicator cell (i.e. another monolayer) known to be free of mycoplasma, but also known to be good host for mycoplasma and to spread well in culture, with adequate cytoplasm to reveal any adherent mycoplasma.

Protocol: "Fluorescence Detection of Mycoplasma"

1. Seed indicator cells into Petri dishes without using antibiotics; seed enough to give 50-60% confluence after 4-5 days.
2. Add 1.5 ml of medium from the test culture.
3. Incubate 4-5 days until the cells reach 50-60% confluence.
4. Remove the medium and discard it.
5. Rinse the monolayer with *PBS-A, and discard the rinse.
6. Add fresh *PBS-A diluted 50:50 with ♦fixative, rinse the monolayer, and discard the rinse.
7. Add pure ♦fixative, rinse, and discard the rinse.
8. Add more ♦fixative (~0.5ml/cm2), and fix for 10 min.
9. Remove and discard the ♦fixative.
10. Dry the monolayer completely if it is to be stored. (Samples may be accumulated at this stage and stained later).
11. If you are proceeding directly with staining, wash off the ♦fixative with deionized water and discard the wash.
12. Add Hoechst 33258 in *PBS-A, and stain 10 min at room temperature (Hoechst 33258 concentration 50ng/ml).
13. Remove and discard the stain.
14. Rinse the monolayer with water and discard the rinse.
15. Mount a coverslip in a drop of mountant and blot off any surplus from the edges of the coverslip.
16. Examine the monolayer by Epifluorescence with a 330/380 excitation filter and an LP 440 nm barrier filter.

* PBS-A: Dulbecco's PBS without Ca2+ and Mg2+.

♦ Fixative: Freshly prepared acetic methanol (1:3, cold).

Analysis: Check for extra nuclear fluorescence. Mycoplasmas give pinpoints or filaments of fluorescence over the cytoplasm, and, sometimes, in intercellular spaces [Freshney, R.I. Culture of Animal Cells: a manual of basic technique (4th Edition) New York, Wiley-Liss Page 291-295 (2000)].

Chapter 9
Safety

1. Take the UBC chemical safety course and UBC bio-safety course.

2. If you intend to use radioisotopes in the tissue culture laboratory, you must take the UBC radioisotope handling and safety course first. The class II type B3 hood (Main culture room 101, hood #1) is capable of handling small quantities of toxic chemicals, provided that the external "receptacle" is activated. Please use class II type B3 hoods to handle radioisotope-related culturing.

3. Common chemical hazards in tissue culture.

CHEMICAL/AGENT	HAZARD
70% alcohol	Flammable
Dimethylsulphoxide (DMSO)	Penetrates skin, mucous membranes
Liquid Nitrogen (cell freezer)	Vials can explode when thawed, wear face shield
Ultraviolet light	Damage to skin and eyes
Biologically active	Follow provided MSDS sheets
Radioisotopes	Follow provided MSDS sheets

4. Accidents in the laboratory

Any accident/incident involving personal injury must be reported as soon as possible to lab supervisor, PI, HLI Operations director and safety officer. CARES report should be done within 48 hours from date of accident/incident. Talk to a person from your lab or contact MOM at 604-813- 0476 for emergency situation.

Chapter 10
Training in Tissue Culture Techniques

It is vital that new staff/student receives adequate training in techniques and procedures before being introduced into the sterile area. **Before they are allowed to work independently, they should be apprenticed to a skilled operator for a period; they should not learn from the last recruit to come in!** A brief introduction to the theory is helpful, but the major emphasis is on practical skills, so supervised operation is required for several weeks to three months**.** The training steps are as follows:

1. A mandatory brief introductory course on the theory and general sterile techniques plus procedures unique to the Centre for Heart Lung Innovation (HLI) facility offered by the tissue culture administrative group followed by a short examination must be successfully completed by each new staff/student before they are permitted to use any of the tissue culture facilities at the HLI Center. The course covers:

- ♦ General Rules (Chapter 1 & Chapter 2)
- ♦ Standard Practices for Cell Culture (Chapter 3)
- ♦ Cleaning and Waste Deposal (Chapter 4)
- ♦ Use of Tissue Culture Laboratories (Chapter 5)
- ♦ Use of Equipment, including BSC, Incubators and Inverted microscopes (Chapter 6)
- ♦ Safety (Chapter 9)

2. Each research group is responsible for supervising their new staff/student for a period (several weeks to three months), with emphasis on training the new staff/student's practical skills.

3. The examination has been designed to test the examinee's knowledge of general principles and techniques of good tissue and cell culture laboratory practice to ensure all of the rules and protocols will be followed properly.

4. **After passing the examination**, the staff/students are allowed to work under supervision within their research group to gain experience before working independently in the cell culture laboratories of the HLI Center.

Chapter 11
Clean Tissue Culture Rooms and Equipment

1. Each research group is responsible for regular cleaning and maintenance of the tissue culture rooms on a regular basis. Timetables for monthly rotations in the main tissue culture room are posted in this facility.

2. All of the tissue culture rooms and equipment should be cleaned on a regular basis. Keep a clean, clear space to work, and have on it only what you require at one time. There are standard procedures for cleaning the rooms and equipment:

Protocol 1. Clean Incubator every two months.
Protocol 2. Clean B**iosafety Cabinet** hood every four months.
Protocol 3. Clean Water Bath every week.
Protocol 4. Clean the open benches every week.
Please refer to details of each protocol in Appendix under SOP1
"Protocols for cleaning tissue culture rooms and equipment".

Chapter 12
Meetings and Administration

1. The regular meeting for tissue culture administrative group is held once a month in the Gourlay conference room.

2. The administrative group is responsible for the daily and regular work in cleaning and maintaining tissue culture rooms on a regular basis, including the following responsibilities:
• Oversee all culture facilities.
• Schedule and perform all maintenance.
• Standardize all procedures for each tissue and cell culture laboratory.
• Assist each group by offering a brief introduction to the theory and general sterile techniques for each new staff/student, which include: general rules, standard practices for cell culture, cleaning and waste disposal, use of tissue culture laboratories, use of equipment, including BSC, incubators and inverted microscopes, etc.
• Design and perform examinations to test the examinee's general principles and techniques of good tissue and cell culture laboratory practice and to make sure all the rules and protocols are followed properly.
• Provide clean yellow gowns for the culture rooms' daily needs. Take the dirty yellow gowns to the basement.
• Check the temperature and CO2 concentration of each incubator daily. Be sure the temperature is around 37˚C and CO2 concentration is around 5%.
• Measure the CO2 concentration of each incubator with the CO2 analyzer every week.
• Check the humidity tank of each incubator every week.
• Perform mycoplasma testing if contamination is suspected.
• Organize regular meetings for tissue culture room cleaning, safety and administration.

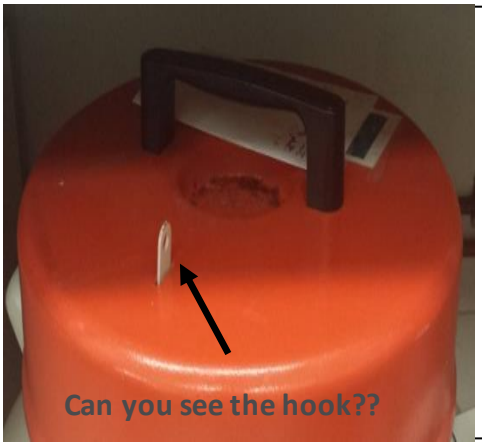
3. Each research group is responsible for regular cleaning and maintenance of the tissue culture rooms that they use (refer to Chapter 11 for posted timetable), including the following responsibilities:

- Clean the open benches every week (monthly rotation, see Appendix).
- Clean the water bath every week
- Clean incubators every two months (each group is responsible for cleaning their own incubators)
- Clean biosafety cabinet hood every four months (each group is responsible for cleaning one of the BSC)
- Check CO2 tank each day. Change CO2 tanks as needed AND ONLY if you are trained. Otherwise let TC supervisor know about it.
- Defrost the fridge and freezer (-20^{o} C) in the culture rooms as needed.

Chapter 13
Liquid Nitrogen handling

1. Proper PPE (Cryo apron, cryo gloves, face shield, full length pants and fully covered shoes) should be worn while handling with liquid nitrogen.
2. For cryo storage duers always make sure to put back retaining wire (holding the boxes in the rack). Failure to do so will cause boxes to fall out, leading to waste of time and money. TC core advisory group has rights to fine repetitive defaulters.
3. Make sure you see the hook protruding out after putting the lid back on the cry tank. Failure to do so will cause improer closure leading to quick evaporation of liquid nitrogen. **Consequences:** Lab can lose all of frozen stock in 24-48 hrs as liquid nitrogen will empty out bringing tank to room temperature, hence cell toxicity with DMSO.
4. For ergonomic purposes use step stool when taking out racks from the liquid nitrogen duer. This way less chances of injury due to heavy lifting.
5. Use a Styrofoam box to place the rack taken out of the cryo tank. This will help sudden temperature shock from the floor and will aslso save cracking of the floor.

   See figures below as examples



Appendix

SOP1: Protocols for cleaning tissue and cell culture rooms and equipment

Protocol 1. Cleaning Incubators

- Bleach the contaminated cultures right away.
- Remove **all non-contaminated cultures** to another CO2 incubator
- Remove all the shelves, the water tray, and any removable panels from the incubator.
- Spray the shelves and panels including all corners and crevices with Conflikt solution (ready to use) **for 15 mins contact time**, followed by rinsing with clean water in wash up room sink. Once dry, wrap in autolave sheets sitting on shelves in washup room and autoclave.
- Wipe the inside of the incubator door including glass door with Conflikt solution (ready to use) **for 15 mins contact time**
- Rinse/wipe off with distilled water followed by 70% ethanol.
- Return the autoclaved shelves and panels to the incubator.
- Replace the water tray and fill it with **distilled water (no MilliQ water) containing 2% Roccall**.
- Check the temperature and CO2 concentration. When both temperature and CO2 concentration have stabilized (overnight), return the cultures to the incubator.

Protocol 2. Cleaning Biosafety Cabinets (BSC)

- Remove all of the removable parts
- Wash these parts with Conflikt solution (ready to use) **for 15 mins contact time**, rinse/wipe off with distilled water and 70% ethanol.
- Wash the inside of the BSC (underneath and sides) with Conflikt solution (ready to use); try to reach all corners and crevices. Rinse them with distilled water.
- Returned the removable parts to the hood.

Protocol 3. Cleaning Water Bath

- Turn off the power and unplug. Empty the water bath.
- Wash water bath with Conflikt solution (ready to use) **for 15 mins contact time**, then rinse/wipe off them with distilled water followed by 70% ethanol.
- Place fresh **distilled water ONLY** (No Milli Q) into the water bath and fill it with water containing "Clean Bath Algicide" (15 drops).
- Turn on the power and make sure temperature comes back to 37 °C.

Protocol 4. Cleaning Open Benches

- Wash the open benches with Conflikt solution (ready to use) **for 15 mins contact time**. Let it sit for 10 mins, then wipe the open benches with distilled water.

SOP2: CVB3 containment protocol

Last Review: October 2018 by Dr. Mary Zhang

Background:

Everyone who is or will be working with CVB3 should know the risks involved so that they can make a sound, knowledgeable decision as to the capacity that they feel comfortable working with this virus.

Coxsackievirus B3 (CVB3) is a member of the enterovirus group within the Picornaviridae family. Enteroviruses cause a wide variety of diseases ranging from flu-like syndromes in adults to fatal pancreatitis and fatal myocarditis commonly in children. Enteroviruses multiply throughout the alimentary tract and are commonly transmitted via oraloral and/or fecal-oral mechanisms (1). The virus stocks that we propagate and utilize for infections are highly concentrated virulent isotypes of virus. By propagating such high titers of virus, we are circumventing traditional modes of infection and therefore, any oral or nasal contact of an aerosol can result in infection. Chronic coxsackieviral infections have been implicated in the etiology of dilated cardiomyopathy for which the only treatment is heroic.

Enteroviruses are stable at freezing temperatures for years, remain viable at refrigerated temperatures for weeks, and at room temperature for days. Enteroviruses are thermal labile and are rapidly destroyed at temperatures greater than 50oC, rapidly inactivated by exposure to ultraviolet light and usually by drying (2). Enteroviruses are resistant to all known antibiotics and chemotherapeutic agents. Alcohol (70%), 5% Lysol, 1% quaternary ammonium compounds, or similar disinfectants are not effective for viral inactivation (2). These viruses are insensitive to ether, deoxycholate, and various other detergents that destroy other viruses (2). **Treatment with 0.3% formaldehyde, 0.1 N HCl, or bleach at a level of 0.3-0.5 ppm causes rapid inactivation, but the presence of extraneous organic matter protects the virus from inactivation (2).**

All work performed with CVB3 or CVB3-infected animals which potentially generates virus-containing aerosol particles should be performed in a Class II type A biological safety cabinet for the protection of the worker and the environment. These particular safety cabinets have a HEPA filter that can trap virus associated with an aerosol or particle in addition to a carbon filter that can filter out small quantities of hazardous chemicals. Please be advised that the a portion of the biological safety cabinet air is filtered back into the room, therefore, copious quantities of hazardous chemical are not suited for use in these biological safety cabinets. Similarly, infected animals are isolated in micro-isolator cages in a horizontal flow animal containment rack within a risk level 2 animal containment room. All virally infected materials including infected animal tissues are inactivated by autoclaving and disposed of according to standard procedure. **No residual risk exists for those tissues which are fixed in 4% formaldehyde or glutaraldehyde, and paraffin-embedded in tissue blocks.**

General points regarding the CBV3 virus room MB03/Lab K:

1. All users planning experiments with virus must sign up for use of the biological safety cabinet. The current BSC is sized to accommodate a single user at one time. All users who have signed up will automatically have priority over someone who has not used the signup sheet on the virus room door. If others are waiting to conduct experiments, you may only book the BSC for one hour at a time
2. The virus room may only be used by authorized personnel who have been accredited by the virus room manager.
3. Lab K is a cell culture virus room only. No animals may enter this laboratory.
4. All personnel must don a surgical mask, yellow repellent gown, shoe covers, and bouffant cap in the anteroom before entering the virus room. Immediately upon entry into the containment facility, gloves must be worn. Full personal protection equipment (PPE) must be worn at all times whenever in the virus room.

5. Nitrile gloves are to be worn at all times inside the virus room. Handle everything wearing gloves only, including: the sink; spray and squirt bottles; the microscope and camera; the computer and telephone. A mask must be worn at all times, especially when using the telephone.
6. Authorization is required to bring any outside equipment into the virus room; all standard supplies remain as designated virus room equipment.
7. Food or drinks must be left in the lunch room and are not allowed in the virus room.
8. No lab books should be taken into the containment area. Protocols can be written on disposable paper. The telephone can be used to report data (if necessary) to the main laboratory and a networked computer workstation can be used to send digital photos, Excel spreadsheets and other data electronically.
9. It is the shared responsibility of all virus room users to clean the facility on a regular basis. The clean-up should follow a period of decontamination not less than one week when there has been no virus use.
10. There is a first aid kit located above the sink in the containment room. However in case of injury call 4777
11. It is the responsibility of the all users to ensure that supplies such as tips and pipettes are stocked, and general tidiness is maintained. It is the responsibility of designated users from each lab to ensure waste and gowns are autoclaved on a regular basis.
12. Garbage that has been autoclaved is 'clean', these autoclave bags can be placed in regular black garbage bags and taken to the main garbage pickup area located on the first floor autoclave/dishwasher room. Gowns can be removed from the autoclave bag, placed in the white cloth bag and left for removal. Any bags with biological waste (ie. animals, animal tissue, etc.) must be placed in a red biohazard bag, autoclaved, and collected in the refrigerator for subsequent incineration.
13. Any virus-containing samples (protein lysates, media supernatant etc.) which must be brought out of the containment room must be fully enclosed in a sealed screw cap container. The outside of the container must be completely wiped with a 10% bleach solution and the sample must be immediately stored in the designated freezer room. Transport containers entering and leaving the virus room are designated as such and/or are reserved only for this purpose; tube racks, tip boxes and the like are not to enter general lab supply circulation and should be labeled and separated.
14. Gloves are to be removed before exiting: do not touch the door handle with gloves. Do not wear gloves into the anteroom.

Handling of infected animals, tissue or cells:
All individuals handling infected animals, tissues, or cells must adhere to the following guidelines:

1. Clothing and protective laboratory wear.
   a. Individuals working with animals or tissues must change clothing and wear surgical greens.
   b. Clean masks, caps, shoe covers, gloves and gowns (protective laboratory wear) must be put on in the anti-room and worn at all times when in containment rooms in which infected animals are in a level 2 biosafety suite.
   c. After finishing procedures and cleanup of working area (i.e. equipment, benches, carts, etc), as described below, protective laboratory wear will be deposited in autoclave bags prior to leaving the virus room. This PPE is not reusable. Do not leave contaminated gowns, shoe covers or masks lying about the virus room and do not reuse any gowns, shoe covers or masks that have been left lying about the virus room; place them in the waste receptacles and don new PPE.
   d. All protective lab wear will be autoclaved after use. NOTE: Two separate containers with autoclave bags will be available for placement of disposable (i.e. masks, caps, shoe covers, gloves) and non-disposable (e.g. gowns) protective wear prior to autoclaving. These containers will be placed at the entrance of the isolation laboratory. If these become half full, please tape them up, and the designated lab workers will autoclave them. Do not overfill waste bags with gowns or garbage beyond half full.

2. Virus handling for cell culture:
a. The biological safety cabinet must be 'ON' and the exhaust fan (switch on the left wall) left on for at least 10-15 minutes before any work can proceed. The entire cabinet must be wiped once with 10% bleach to kill any residual virus, then with 70% ethanol to remove any chlorine salts that will corrode metal surfaces.
b. There should be a plastic waste container in the cabinet with 100% bleach. Although the initial bleach concentration is high, the solution will get diluted and any organic materials will cause some attenuation of the ability to kill the virus. All infected media, buffers, etc. should be added to the waste container. All infected pipette tips can either be rinsed in the bleach solution or placed in the container. Cell scrapers, 5 and 10 ml pipettes, Eppendorf tubes, tissue culture plates should be rinsed with the bleach solution and thrown into the autoclave bag.
c. Upon conclusion of the experiments, the waste container can be placed in the sink for disposal the following day. If there is bleached waste in the sink from the previous day, please throw it into the garbage. Any instruments that may be contaminated with virus should be wiped with 10% bleach and 70% ethanol. The cabinet must also be wiped with 10% bleach and 70% ethanol, and then shut off. Do not autoclave any garbage soaked with ethanol or bleach. Allow these to dry prior to autoclaving.

Note: Centrifuged tubes are often under pressure, please use caution when opening to reduce the production of virus-containing aerosol particles. Ensure that bleach is wiped dry before wiping with ethanol to eliminate the possibility of producing toxic chlorine gas. Please do not autoclave bleach as this will result in the production of toxic chlorine gas.

Comprehensive Autoclave SOP (Authorized users only)
1. Before entering Lab K, check that the autoclave has a jacket pressure of 22 psi and that the external autoclave door is sealed.
2. Garbage and gowns are to be autoclaved when the bag reaches half full.
Overfilled bags will not properly sterilize because the extra density will prevent steam from sufficiently heating the center of the bag.
3. Before loading the bags into the autoclave:
   a) Check that pipettes or other objects have not punctured the outside of the bag; if they have, then double-bag.
   b) Close the bag with tape, but do not seal the opening tightly; instead, leave a small space at the opening for steam to enter and air to escape.
4. Load the autoclave, ensuring that the plastic bags are not touching the inside walls or ceiling of the autoclave. The autoclave in Lab K can hold 2 half full bags.
5. Seal the interior autoclave door.
6. Run the sterilization program: cycle 1 for bagged waste, cycle 2 for yellow gowns. (1=40 min gravity @121, 5 min dry time). The cycles are pre-programmed. Press the cycle number once to check parameters, and again a second time to activate the cycle.
7. Important: garbage must be removed no later than 15-20 minutes after the cycle is complete, and taken to the main disposal area (the autoclave/dishwasher room on the 1st floor). Garbage may not be left in the autoclave overnight, or the bag will melt and make a mess. Garbage may not be left in the hallway outside Lab K.

References:
1. Hammond, G., 1988, Epidemiology, in: Coxsackieviruses: A general update (M. Bendinelli and H. Friedman, eds.), pp. 383-389, Plenum Press, New York and London.

2. Melnick, JL., 1990, Enteroviruses: Poliovirus, Coxsackieviruses, Echoviruses, and Newer Enteroviruses, in: Fields Virology, 2nd edition (B. Fields and D. Knipe, eds.), pp. 549-605, Raven Press, New York.

SOP3: Procedure for use of viral tissue culture room

Background

This room is designated for use with adenovirus, respiratory syncythial virus and parainfluenza.
Adenoviridae; non-enveloped, icosahedral virions, 70-90 nm diameter, doubled-stranded, linear DNA genome.

Pathogenicity**:** Varies in clinical manifestation and severity; symptoms include fever, rhinitis, pharyngitis, tonsillitis, cough and conjunctivitis; common cause of nonstreptococcal exudative pharyngitis among children under 3 years; more severe diseases include laryngitis, croup, bronchiolitis, or severe pneumonia; a syndrome of pharyngitis and conjunctivitis (pharyngoconjunctival fever) is associated with adenovirus infection.

Epidemiology**:** Worldwide; seasonal in temperate regions, with highest incidences in the fall, winter and early spring; in tropical areas, infections are common in the wet and colder weather; annual incidence is particularly high in children; adenovirus types 4 and 7 are common among military recruits (ARD). Mode of transmission**:** Directly by oral contact and droplet spread; indirectly by handkerchiefs, eating utensils and other articles freshly soiled with respiratory discharge of an infected person; outbreaks have been related to swimming pools; possible spread through the fecal-oral route.

Susceptibility to disinfectants**:** Susceptible to 1% sodium hypochlorite,
2% glutaraldehyde, 0.25% sodium dodecyl sulfate

Physical inactivation**:** Sensitive to heat >56°C; unusually stable to chemical or physical agents and adverse pH conditions
Survival outside host**:** Resistance to chemical and physical agents allows for prolonged survival outside of the body. Adenovirus type 3 survived up to 10 days on paper under ambient conditions; adenovirus type 2 survived from 3-8 weeks on environmental surfaces at room temperature

Containment requirements**:** Biosafety level 2 practices and containment facilities for all activities involving the virus and potentially infectious body fluids or tissues

Protective clothing**:** Laboratory coat; gloves when skin contact with infectious materials is unavoidable

Spills**:** Allow aerosols to settle; wearing protective clothing gently cover the spill with absorbent paper towel and apply 1% sodium hypochlorite starting at the perimeter and working towards the centre; allow sufficient contact time (30 min) before clean up

Disposal**:** Decontaminate all wastes before disposal; steam sterilization, incineration, chemical disinfection

Storage**:** In sealed containers that are appropriately labelled

Respiratory syncytial virus: Paramxyoviridae; pleomorphic, 150-300 nm diameter; enveloped virions; single stranded linear RNA; non-segmented negative sense genome; lack hemagglutinin and neuraminidase activities

Pathogenicity: Most common cause of common cold-like lower respiratory tract illness in infants and young children; causes common colds in adults, febrile bronchitis in infants and older children, pneumonia in infants, and bronchiolitis in very young babies; reinfection common and results in mild upper respiratory infection; can cause severe illness in the elderly and immunocompromised

Epidemiology: Worldwide; most common cause of viral pneumonia in children < 5 years; outbreaks peak in February and March in the northern hemisphere; tropical area peaks coincide with rainy seasons

Host range: Humans

Infectious dose: >100-640 infectious organisms when administered intranasally
Mode of transmission: Respiratory secretions; inhalation of large droplets, fomites; direct oral contact; indirectly by hands, handkerchiefs and eating utensils or other articles freshly soiled by respiratory discharges

Incubation period: 4 to 5 days

Communicability: Viral shedding may persist for several weeks after symptoms subside

Susceptibility to disinfectants: Susceptible to many disinfectants; 1% sodium hypochlorite, 70% ethanol, 2% glutaraldehyde, detergents

Physical inactivation: Sensitive to heating above 55° C, freezing and thawing; rapidly inactivated at pH < 5

Survival outside host: Virus contaminated nasal secretions remain viable up to 8 hours at room temperature on paper towels, cloths, rubber gloves and surfaces

Laboratory-acquired infections: One case was reported up to 1978; many cases probably occur but go unreported due to frequency of infection in the population and difficulty in tracing to laboratory cause

Sources/specimens: Nasopharyngeal swab, nasal washes, and secretions

Primary hazards: Droplet or aerosol exposure of mucous membranes

Special hazards: None

Containment requirements: Biosafety level 2 practices and containment facilities for activities involving infectious body tissues and fluids; cultures

Protective clothing: Laboratory coat; gloves when skin contact with infectious materials is unavoidable

Other precautions: None

Spills: Allow aerosols to settle; wearing protective clothing gently cover spill with absorbent paper towel and apply 1% sodium hypochlorite

starting at the perimeter and working towards the centre; allow sufficient contact time (30 min) before clean up

Disposal: Decontaminate all wastes before disposal; steam sterilization, chemical disinfection, incineration

Storage: In sealed containers that are appropriately labeled
Parainfluenza: for detail information refer to website links below.
Procedures: Follow the standard practices for cell culture (see Chapter 3).
"Special waste disposal": All materials including supernatants, cells, bottles, flasks, pipettes (standard glass or plastic pipettes), etc. should be appropriately decontaminated before disposal as respectively outlined above.

References

♦ Freshney, R.I. Culture of Animal Cells: a Manual of Basic Technique (4th Edition) New York, Wiley-Liss (2000)
♦ Freshney, R.I. Animal Cell Culture: A Practical Approach. Oxford University Press, New York (1992)
♦ Chen, T.R. In situ detection of mycoplasma contamination in cell culture by fluorescent Hoechst 33258 stain. Exp.Cell Res 104:255 (1997)

Adenovirus:
http://www.phac-aspc.gc.ca/msds-ftss/msds3e.html
RSV:
http://www.phac-aspc.gc.ca/msds-ftss/msds125e.html
Parainfluenza:
http://www.dhiusa.com/database_media/PI076v0503MSDS01-010000.pdf
http://www.chemicon.com/Product/ProductDataSheet.asp?ProductItem=3120

I have read the document and agree to follow the SOP. Failure to do so will result in penalties and could lose privileges.

PI Name	User Name	Signature	Date Signed

I have read the document and agree to follow the SOP. Failure to do so will result in penalties and could lose privileges.

PI Name	User Name	Signature	Date Signed