Hildebrand BSL3 Biosafety Manual

University of California, Berkeley
Hildebrand Hall room 403

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Introduction to the Hildebrand Biosafety Level 3 (BSL3) Facility

This manual describes the general procedures for working with Biosafety Level 3 pathogens (specifically *M. tuberculosis*) in the Hildebrand BSL3 facility. Accompanying this manual is a separate document describing all approved protocols used in this BSL3 facility.

Working with BSL3 pathogens requires diligence from the laboratory worker to maintain safe laboratory conditions. This includes extensive knowledge of both the pathogen and the procedures, proper training and certification, and rigorous adherence to safety practices. Failure to meet any of these expectations will result in removal of BSL3 access privileges.

Mycobacterium tuberculosis is the major pathogen used in the Hildebrand BSL3 Facility and every user must be knowledgeable of the specific risks and precautions associated with *M. tuberculosis* work. Any pathogen other than *M. tuberculosis* used in the Facility must first be approved by the UC Berkeley Committee on Laboratory and Environmental Biosafety (CLEB) and the EH&S Biosafety Officer (BSO). Additional training will be involved for BSL3 pathogens other than *M. tuberculosis*.

The user is responsible for being well versed in the experimental design and execution of each protocol to be done in the BSL3. Also, all procedures must be approved and added to the **Approved Protocols** manual prior to being used in the BSL3 facility.

Training of users will take place prior to any work in the BSL3. Each user is required to take biosafety training through EH&S as well as BSL3-specific training. After the user has displayed adequate competency in safely handling BSL3 pathogens, the user will be tested under mock BSL3 conditions and evaluated for knowledge of procedures and technique. A short evaluation period is also required before the user can work in the BSL3 unsupervised.

Finally, adherence to safety practices is paramount to all BSL3 work. An unsafe worker jeopardizes the health and safety of all workers in the BSL3. Any reports of unsafe working conditions will be critically evaluated and appropriate measures will be taken, including retraining/certification, reduced privileges, or banning from the facility.

Contact Information

Working Hours:

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Emergency Contacts:

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Requirements for beginning work in the BSL3 Facility

In order to work in the BSL3 facility, all potential users must complete the following requirements.

- Enroll in the **medical surveillance** program
- Be fitted for a **respirator**
- Complete **EH&S general biosafety** training course
- Complete the **BSL3 safety** training
- Demonstrate **BSL3 safety techniques**
- Be evaluated by a BSL3 user during a short **trial period**
- Be listed on the BSL3 BUA

1. Medical surveillance

The first step towards working in the BSL3 facility is to enroll in the TB surveillance program. All personnel working in the BSL3 must be tested regularly for exposure to *M. tuberculosis*. TB surveillance consists of a Tuberculin skin test (PPD test) or chest X-ray. Both of these tests are administered by the **Tang Occupational Health Clinic** (www.uhs.berkeley.edu).

- Contact the **Facility Director** to add you to the list of personnel needing TB surveillance
- Prepare an IOC form to pay for the test (check price with Clinic Nurse)
- Bring a copy of any prior PPD tests or chest X-rays
- If you have not been PPD tested – you must obtain a 2 step test (get tested twice)
- If you are PPD+ (including those vaccinated with BCG- Bacille Calmette-Guerin) you are required to have a basal chest X-ray unless you have record of one from the last 12 months
- Give the result of either the PPD test or chest X-ray to the **Facility Director**

All personnel working in the BSL3 are required to be tested every 6 months. The Facility Director will keep track of TB surveillance scheduling and contact personnel when they are due for their tests. However, it is the responsibility of the user to go to the **Tang Occupational Health Clinic**, have the test administered and deliver the results to the **Facility Director**. Failure to do this will result in barring the user from the BSL3 facility until the tests are completed.

TB surveillance is required every 6 months:

- PPD– individuals are required to have a PPD skin test
    - In the event of conversion to PPD+, the user must immediately contact the **Facility Director**, who will then notify the **Biosafety Officer** and the **Principal Investigator**
- PPD+ individuals must complete a semi-annual health survey
    - All symptoms of illness that may be related to infectious organisms being used in the lab must be brought to the attention of the **Facility Director**, who will then notify the **Biosafety Officer** and the **Principal Investigator**

PPD screening consists of subcutaneous injection of tuberculin, which **must be read 48-72 hours later**

PPD screening times:

Monday.....10 to 11:30 AM
Tuesday .....2 to 4 PM
Thursday, Friday (PPD reading and symptom reviews only) .2 to 4 PM

2. Respirator fit testing

Respirators must be worn at all times in the BSL3 facility. Proper fit of the respirator is critical for function and must be tested by EH&S.

- Contact **Sara Souza** (sarasouza@berkeley.edu) and **Roy Waller** (r_waller@berkeley.edu) for respirator fit testing
- Complete the respirator medical clearance form prior to being tested and submit to Tang Center for review

All users must be fit tested every year and are responsible for staying up to date. Failure to complete the required fit testing will result in the user being barred from entering the BSL3 facility.

3. EH&S biosafety training

UC Berkeley EH&S (www.ehs.berkeley.edu) requires two training classes for work with pathogens in the BSL3 facility.

- Complete the Introduction to Biosafety class – Enroll for "Introduction to Biosafety" at the UC Berkeley Learning Center via the Blu portal at

http://blu.berkeley.edu. Once authenticated, search for "Biosafety" in the search box. Select and register for your date of choice.

3b. BSL3 safety training

The BSL3 safety training is the most intensive and specific training required to work in the BSL3 facility. All aspects of the BSL3 safety training are outlined in this manual. **The applicant is expected to have thoroughly read this manual before training begins.**

To begin BSL3 training, an active BSL3 user will be chosen by the **Facility Director** to introduce the applicant to generalized BSL3 procedures and provide specific technical training based on the submitted protocols. All training must include identification and control of the hazards with which personnel will be working. Furthermore, regardless of the techniques required for the proposed protocol, the training program must include performing mock procedures that cover the following topics:

- Adherence to general lab safety procedures, such as wearing double gloves and sterilizing gloved hands before removing them from the biosafety cabinet.
- Setting up, cleaning out, and properly using the biosafety cabinets.
- Bringing materials into and out of the cabinet.
- Culturing and manipulating *M. tuberculosis* safely, with emphasis on the importance of avoiding aerosol generation during all operations.
- Performing the essential procedures required of most protocols, such as centrifugation, plating, and incubating.
- Disposing of waste.
- Running the autoclave
- Bringing materials out of the BSL3
- Following emergency procedures, especially what to do in the event of a spill. Particular emphasis must be placed on the reporting of spills and accidents, no matter how minor the incident may seem.

At least two training sessions must be performed, with additional sessions at the discretion of the either the trainer or applicant. The applicant will be responsible for completing the **Documentation of Training Form**.

4. BSL3 safety testing

Following the training period, the applicant must demonstrate to the **Facility Director** proper BSL3 safety practices. The test will require the applicant to

demonstrate their ability to follow all the approved biosafety practices outlined in this manual. Typically, the applicant will have to complete a mock procedure that covers general *M. tuberculosis* culturing and manipulation. If the applicant fails to show competence in properly handling *M. tuberculosis*, more training will be required specifically focusing on problems that arise during testing and the applicant will have to be tested again by the Facility Director.

After satisfactorily demonstrating biosafety techniques, the Facility Director will approve the applicant to work with live bacteria.

5. Supervised trial period

Given the complexities and risks associated with BSL3 work, any new user must undergo a supervised trial period. For the first 2 sessions of work in the BSL3, an existing BSL3 user must be present to help the new user if needed and observed the user's technique. The new user will be responsible for documenting the date, time and who supervised these initial sessions and giving this information to the **Facility Director**.

After the supervised trial period, the new user will confer with the **Facility Director** to discuss any additional problems or suggestions and be able to work independently in the BSL3.

Personnel not working directly with *M. tuberculosis*

Physical Plant personnel, vendors and repair person, visitors, and inspectors who need to access the BSL3 lab for short periods of time must sign the **Acknowledgement of Risk and Agreement to Follow Guideline** form prior to entering the facility and complete the EHS BSL3 PP-CS training for the NAF and Hildebrand facilities which is given annually or more frequently as needed. Personnel will be given brief training by the **Facility Director** and **Biosafety Officer** or qualified designate on the rules of the facility and correct use of personal protective equipment. Personnel must contact the **Biosafety Officer** and **Facility Director** prior to entry and receive clearance.

Personnel wishing to use the BSL3 facility to work on pathogens other than *M. tuberculosis* must first get approval from **CLEB**, **EH&S**, the **Facility Director**, and the **PI**. Such personnel are required to fully follow the above guidelines for beginning work in the BSL3. Procedural testing will focus on a protocol that is appropriate for the work being done and will be done by the **Facility Director**.

Description of *Mycobacterium tuberculosis*

1. Background

Tuberculosis has existed for centuries, and used to be called "consumption". It is a serious disease that usually attacks the lungs, but can also affect other parts of the body, including the brain, the lymph nodes, and bones. Although most TB infections are curable with antibiotics, it continues to be a major health problem and kills as many as three million people worldwide every year. However, multiple-drug resistant TB is increasing world-wide, complicating treatment.

2. How TB is Spread

Mycobacterium tuberculosis (Mtb) is an airborne pathogen. It is usually spread through the air from person to person, when someone with infectious TB disease coughs or sneezes. In a laboratory setting, mechanical aerosol generation can also lead to the release of particles which can cause infection if inhaled. Likewise, direct inoculation via injection into the bloodstream can also cause infection.

3. Tuberculosis Infection and TB Disease

Most people who are exposed to *Mycobacterium tuberculosis* do not develop TB disease. In some cases, the person's immune system is able to kill the *Mtb*. When this doesn't happen, the bacteria can remain alive but inactive in the body, and this is called TB infection. A person with TB infection has no symptoms, is not sick, and poses no risk of spreading the bacteria.

TB infection may develop into TB disease if the infected person's immune system cannot stop the TB bacteria from growing. The risk of developing TB disease is highest in the first two years after someone is infected. About 10% of infected adults will develop TB disease at some point in their lives.

4. Risk Factors for Developing TB Disease

Anyone with a weakened immune system is at greater risk of developing TB disease. Conditions that weaken the immune system include HIV infection, end stage kidney disease, diabetes, cancer, organ transplants, silicosis, abuse of alcohol or drugs, pregnancy, and low body weight.

Babies, pre-school children, and the elderly are also at greater risk because their immune systems are weaker than those of healthy adults.

Also, certain population groups have an increased risk of TB infection. These groups include people born in or traveling to countries where TB is widespread, homeless people, and people who live in long-term care or correctional facilities.

People who work with any of these groups (such as health care workers) are also at greater risk.

5. The Symptoms of TB Disease

The symptoms of TB disease in the lungs can include a bad cough that lasts longer than two weeks, pain in the chest, coughing up blood or phlegm, weakness or feeling very tired, weight loss, lack of appetite, chills, fever, and night sweats.

A PPD skin test can determine whether you have TB infection. If you do, it is important to protect your immune system from becoming weak. Treating TB infection with antibiotics will help your immune system fight the TB bacteria and prevent the development of TB disease.

The BCG vaccine is still administered to many children around the world (not in the US), despite its dubious effectiveness for preventing TB in adults. BCG vaccination leads to reactivity against PPD and thus can lead to false-positive skin tests. Persons known to be vaccinated with BCG should not take the skin test and should be given a chest X-ray to rule out active disease.

You may also need additional tests or chest x-rays to determine whether you have TB disease. Anyone with TB disease must take antibiotics for at least six months to kill all of the TB bacteria.

People who do not finish a full course of antibiotic treatment are at greater risk for developing a strain of TB that is drug resistant and harder to cure. They also pose a greater risk of spreading TB to others.

Entering and exiting the BSL3 facility

1. Using personal protective equipment (PPE)

Designated personal protective equipment (PPE) must be worn by every person entering the BSL3. All PPE will be provided and will be stocked in either the ante-room or entry vestibule. Change into all PPE in the ante-room before entering the BSL3 facility. Required PPE includes:

- Respirator (N95 or PAPR)
- Eye protection (goggles or PAPR)
- Double gloves (thick inner pair, standard outer pair – preferably latex)
- Shoe covers
- Disposable, back closure lab gown

Put on Shoe covers first, then put on and tie the gown in the back. Place inner gloves over the cuffs of the gown and tape them to the gown to prevent gaps. Put on the second pair of gloves over the first. Form respirator to the face and put on eye protection.

All personnel must be certified physically able to wear a respirator and must be fit-tested by EH&S to ensure proper fit. One must be retested annually or if significant changes to the fit area occur, such as a sizable weight change or allowing growth of any facial hair. It is the job of all BSL3 users to detect compliance failures and report them to the **Facility Director**. Anyone not in compliance with the surveillance plan will be required to be retested. The Facility Director will advise users when their fit test has expired.

PAPR devices will be available as an extra safeguard for performing potentially aerosol-generating procedures.

2. Entering the BSL3 barrier

Before opening the door from the Ante-room to the BSL3, complete the following checklist:

- Be sure you are wearing proper PPE
- Note - Name, Date, Time, and Laboratory affiliation in log book
- Confirm negative pressure in the BSL3

    - Proper air balance is indicated by a green light on the meter next to the door
    - If there is a problem with the air balance, i.e. if the indicator light is either red or yellow, do not open the door. Call the **Facility Director** immediately and do not let others enter the laboratory.
- Only open the inner door to the BSL3 facility if the outer door of the ante-room is closed.

3. Exiting the BSL3 facility

Before exiting the BSL3 facility:

- Remove the outer layer of gloves and place in biohazard waste container inside the BSL3
- Look through the glass into the ante-room to be sure the door from the ante-room to outside is not open
- Enter the ante-room
- Remove shoe coverings and place in the biohazard container
- Remove and dispose of the gown, masks and latex gloves in the biohazard container
- Rinse off protective eye ware with water and return to shelf
- If using a PAPR, disconnect the hood and plug in the HEPA unit
- Note time of departure on the sign in sheet
- Wash hands thoroughly with antibacterial soap

Running the autoclave

All trash must be autoclaved before it can be removed from the BSL3 facility. All PPE must also be autoclaved before being picked up by EH&S. Each user is responsible for autoclaving trash and must be familiar with autoclave function.

The autoclave can only be set from the "clean" side – in the autoclave room. Once the autoclave is properly set, the cycle can be started from the BSL3 side remotely.

The autoclave must also be tested each month using the spores of *Bacillus stearothermophilus* to assess proper sterilization. Autoclave monthly test records are maintained for regulatory purposes and test records are kept next to the autoclave.

Always use an autoclave tray. Report any problems immediately to the **Facility Director**.

1. Autoclaving trash

- Check that the Jacket is on (to turn on - press the **Jacket** button)
    - Jacket pressure should read 22 psi
- Trash should be run in the "Dry" cycle
    - Press – **1** (cycle selection)
    - Press – **2** (Dry)
- Check the cycle you are running
    - Temperature = 121°C (250°F)
    - Time = 30 minutes
- Press – **9** (exit) until you reach the main menu (3x)
- Press the **Run** button
- Press **Enter** 2 times to begin cycle

If the cycle is set correctly, the autoclave can be started by pressing the **Remote start** button on the BSL3 side of the autoclave **twice**.

2. Autoclaving plates

Plates are the only trash that should contain live *M. tuberculosis* and requires a longer cycle. Follow the above directions except:

- **Set cycle time = 60 minutes**

Bags of plates must be completely contained within a tray to prevent molten agar from blocking drain.

3. Autoclave problems

Before removing autoclaved trash for pickup by EH&S, check the screen to be sure that the cycle completed successfully.

If the cycle did not complete do not open autoclave!

Reset the autoclave and run the cycle again following the above instructions. If the autoclave fails, notify the **Facility Director** immediately. Post signs on the autoclave instructing users not to open the autoclave.

Working with *M. tuberculosis* in the Biosafety Cabinet (BSC)

Direct manipulation of *M. tuberculosis* is only allowed inside a prepared Biosafety Cabinet (BSC). All *M. tuberculosis* cultures outside of the BSC must be in secondary containment unless specified otherwise.

1. Preparing the BSC

Once *M. tuberculosis* is introduced into the BSC, all equipment, materials and hands must be disinfected prior to removal from the BSC. Therefore, proper preparation of the BSC is essential to safely handling *M. tuberculosis*.

- Check the airflow indicator on the front panel of the BSC - negative pressure indicates that the unit is working properly and is safe to use
- Place a red biohazard bag in polypropylene container
- Bring in an open container of disinfectant (Vesphene IIse diluted 1:40 with water) with a soaked disposable blue towel
- Place materials and equipment that will be used including pipetters, pipets, pipet tips, sterile culture flasks, centrifugation tubes, media, etc., inside the BSC

Prior to the introduction of *M. tuberculosis*, materials may be taken in and out of the BSC without further precaution.

2. Working with *M. tuberculosis* in the BSC

Once sealed containers of *M. tuberculosis* have been placed inside the BSC nothing is to be removed without being wiped with Vesphene. Hands must be wiped thoroughly with Vesphene and removed from the BSC immediately. The outer layer of gloves should be discarded whenever there is the possibility of contamination with *M. tuberculosis*. All disposable materials that come into contact with *M. tuberculosis* are sterilized with Vesphene before being placed into the biohazard bag. Liquid waste is to be disinfected by addition of equal volumes of Vesphene in tightly sealed containers and placed into the biohazard bag.

3. Disinfection and removal of objects from the BSC

Objects are not to be removed from the BSC if there are open containers of *M. tuberculosis*. Objects to be removed will be wiped thoroughly with Vesphene and can be carried directly out, if gloves have been wiped with Vesphene. Alternatively, an area at the edge of the cabinet can be wiped with Vesphene, and materials to be removed from the BSC may be wiped thoroughly and placed on this sterilized area and then removed immediately. Vesphene is the only allowable disinfectant; ethanol is never used except to remove the residue from Vesphene. Reagents and materials that are to be reused will be clearly labeled with users initials and "**open only in BSC**" then tightly sealed, outer surfaces disinfected, and removed as above.

4. Disinfection of the BSC

At the end of a procedure, all objects should be removed from the BSC and the entire BSC should be disinfected so it will be clean for the next user.

- Remove objects from the BSC as described above
- Seal the biohazard trash bag with a rubber band
- Wipe all exposed surfaces of the bag with Vesphene
- Removed sealed trash bag from the BSC and place in large biohazard bag adjacent to BSC
- Sterilize the cabinet with disinfectant in a "wave of cleaning"
    - All objects that are to remain in the BSC are placed on one side, the other side of the cabinet is sterilized by wiping all surfaces with a Vesphene-soaked cloth. Equipment is then wiped and placed on the sterile side and the "wave" continues to the other side.
- Remove the container of Vesphene
- Clean the BSC with a solution of 70% ethanol to remove the residue of Vesphene
- Turn off the light - leave the blower ON (for long BSC shutdown - close sash, turn off blower and turn on UV light)

Clean up the area around the BSC and the benches and place any trash in the large Biohazard bins. If trash bags are full, seal the double bag with tape and autoclave. Turn off all equipment and inspect the BSL3 for hazards.

General techniques

- General microbiological aseptic technique is required in the BSC. If tube caps are placed on the surface of the BSC, it should be cap-up. Disposable loops or spreaders are to be used.

- Stable test tube racks and plastic boxes for culture flasks will be used to prevent a spill inside the BSC. All tubes and flasks will be properly sealed prior to removal from the BSC.

- Nothing may be removed from the BSC without every surface wiped with Vesphene.

- After use, pipettes will be rinsed (up and down pipetting once) with Vesphene, and discarded in the trash bag in the BSC.

- All cultures and surfaces of trash must be disinfected with Vesphene prior to placement into the trash bag. The only exception to this rule is agar plate disposal (see below).

- User will limit the number of items in the BSC during use, since clutter interferes with the airflow in the BSC.

1. Handling of liquid cultures

Special care must be taken when handling liquid cultures to avoid spilling of any liquid inside or outside the BSC. Also avoid generating aerosols, even inside the BSC. All manipulations of cultures must be done in the BSC.

- Supernatants will be poured or pipetted into a deep container with Vesphene.

- All pipetting is done slowly with a pipette-aid and care is taken to minimize expulsion of air into cultures.

- Liquid culture is grown in 3 types of containers.
    - Inkwell bottles or screw-cap centrifuge tubes are used to grow up small colonies into larger liquid medium. Inkwell bottles or centrifuge tubes containing growing cultures are placed within a large Tupperware container in the incubator.

- Larger roller bottles are used to grow up frozen stocks of liquid culture. Only thick-walled Corning "vacuum filter" roller bottles may be used. Roller bottles are kept in the roller bottle incubator. Before opening the roller bottle incubator, users should check the interior of the incubator for cracked bottles and leaks.
- Tissue-culture flasks with plug seal caps can also be used for standing cultures. If necessary, flasks can be capped with plug seal caps containing a 0.2 μm filter to allow gas exchange. Take special precaution to prevent any liquid from getting on these caps.

- Special care should be used when carrying roller bottles and tissue culture flasks from the incubator to the BSC.
  - If simply moving from the incubator to the adjacent BSC, carefully move only one culture at a time holding with both hands.
  - If you need to move cultures somewhere else in the BSL3, then a secondary container (such as an autoclave tray) should be used to house the roller bottles or flasks in transit.

2. Plating and handling of plates

All plating takes place in the BSC. Plates never leave the BSC without being in secondary containment. Plates can be wrapped in parafilm and a second layer aluminum foil or be placed in specialized canisters.

To photograph plates:

- Seal each plate with parafilm (unless previously sealed)
- Wipe each plate with Vesphene and place in a clean container
- Wipe out the container and carry to camera station
- Photograph each plate individually and leave the rest inside the secondary container
- Wipe the camera station with Vesphene
- Return plates to the BSC

Plate disposal is the **only** exception to the "no live TB in the trash" rule. The reason for this is that adding Vesphene to agar plates is extremely messy (which causes a hazard) and TB colonies are so clumpy that penetration of a chemical disinfectant is likely to be incomplete.

- Plates containing TB are put inside small biohazard bag inside the BSC
- The bag is sealed with a rubber band and wiped thoroughly with Vesphene

- Place this bag inside another bag, rubber banded shut, and wipe out of the BSC
- The double-bagged plates are autoclaved immediately for 60 minutes
- If the autoclave is busy, then the bag is left inside an operating BSC until it can be sterilized and a note is left on the BSC for other users

3. Pipetting

Pipetting can generate aerosols. Only pipettes and pipet tips with aerosol barriers are allowed in the BSL3. Aerosols are minimized by avoiding blowing out the contents of a pipette completely and by preventing the formation of bubbles. To avoid aerosol generation, whenever pipetting TB into a container, pipet onto the side wall of the container, and be careful not to eject any air after all of the liquid has been ejected. Similarly, avoid accidental intake of air when drawing liquid into a pipet or pipet tip. After pipetting, clean the inside and outside of the pipet by drawing Vesphene into the pipet and then allowing it to drain back into the pot of Vesphene. After the pipet has been rinsed, it can be disposed of in the waste bag.

For micropipet tips, following ejection of liquid, do not release the pipetman piston allowing air to be drawn into the pipet tip. Instead, holding the pipetman depressed, immerse the pipet tip in Vesphene and draw Vesphene into the tip. The tip can then be disposed of into the waste bag.

4. Freezing Cultures

TB cultures can be frozen in their 7H9 growth media.

- Prepare 2ml cryovial tubes by labeling them on both the side and cap of each tube and then place them into a rack
- Pipet 1ml of culture into each tube
- Deliver liquid along the side of the tube to avoid splashing and avoid producing bubbles
- Tighten the caps on all of the tubes
- Completely wash off an empty rack with Vesphene in the BSC
- Individually wipe down each cryovial and place them into the clean, empty rack
- Remove the entire rack from the BSC
- Outside the cabinet, transfer each tube into a clearly labeled freezer box and place the box into a metal tower in the freezer

5. Heat killing *M. tuberculosis*

Heat killing of *M. tuberculosis* can be accomplished by heating at 95°-100° C for 15 min or 80° C for one hour. Samples must be in screw-cap tubes.

- Bring a heating block or metal pot filled with water into the BSC and bring to appropriate temperature
- Do NOT plug anything into the outlets inside the hood. It will cause a short. Use outlets on the walls
- Place *M. tuberculosis* sample to be killed into the block or boiling water
- After the appropriate time, turn off heat source and remove sample
- Wipe tube with Vesphene prior to removal from BSC

6. Killing with chemical reagents

Killing *M. tuberculosis* cells with chemical reagent occurs inside the BSC in a sealed tube. At least an equal volume of reagent is used to sterilize liquid cultures. All of the following reagents may be used to kill *M. tuberculosis* and will sterilize a culture by one minute after addition:

- 2.5% Vesphene
- Chloroform:methanol (1:1)
- 100% Formalin
- 4% Formaldehyde or paraformaldehyde

After addition of reagent, samples in sealed tubes are inverted to mix contents. Cover slips must be entirely submerged. After sample is killed, tube is wiped with Vesphene prior to removal from BSC and again prior to removal from BSL3.

7. Transferring live *M. tuberculosis* between NWAF and BSL3

Transfer live cultures in screw-capped shipping containers with O-rings containing absorbent material.

- Disinfect outside of culture tube
- Place in shipping container
- Disinfect outside of shipping container with Vesphene
- Remove from BSC
- Disinfect again with Vesphene before removing container from BSL3 facility

- Label with Biohazard sticker and contents
- Transfer immediately to BSL3 (or NWAF)
- Only open secondary container within BSC in BSL3

8. Tissue culture

Infected cell lines must be handled as if they were regular liquid TB cultures. Therefore, minimizing aerosols and avoiding spills is of utmost importance.

- Use flasks equipped with 0.2 μm filters when possible.
- Multiwell plates may be used if necessary. Always use breathable film sealed to the top of the plate to avoid spills.
- All plates and flasks must be clearly labeled with user's name and infectious agent.
- To image tissue culture dishes, a porous film is sealed to the top of the plate. Be sure to fully seal the film to the plate to prevent spilling. The sealed dish is then wiped with Vesphene and placed in a clean Tupperware container. The container is carefully wiped out of the BSC and taken to the microscope. The dish can be taken out of the container and imaged. Only one dish should be out of the container at any time.
- Aspirate liquid from tissue culture dishes using the aspirating pipet attached to a sidearm flask containing Vesphene. Be sure the filter is attached between the flask and the vacuum port. After aspirating, suck Vesphene into the aspirator and disassemble the aspirator and wipe out each piece.

9. Waste

- **No waste can leave the BSL3 facility without being autoclaved**
- All waste must be double bagged, marked with autoclave tape, and placed in covered plastic bins for autoclaving.
- Each worker is responsible for correctly bagging their own waste
- All personnel are responsible for autoclaving waste when the bin gets full. The **Facility Director** will ensure proper and safe disposal of waste
- Autoclaved waste must be removed from the clean side of the autoclave and disposed of in the red bins when the cycle is complete
- All users must be familiar with the proper use of the autoclave. Failure to use the autoclave correctly can result in malfunction. The **Facility Director** is to be notified immediately in the case of autoclave malfunction.

- Autoclaved waste is taken to room 230 Hildebrand Hall for disposal (entry code 889889).

Using equipment in the BSL3

The most important aspects of working with equipment in the BSL3 are to maintain secondary containment of pathogens and avoid generating aerosols. Specific concerns and protocols for common BSL3 laboratory equipment are listed below. Do not bring in new equipment unless authorized by the **Facility Director**. Failure to follow these guidelines for safe equipment usage will result in additional training or banning of the user from the BSL3.

1. Vortexer

Vortexing generates aerosols. All vortexing must be done inside the BSC and should be avoided if possible. The Vortexer is kept in a sealable container. Do not open this container outside the BSC.

- Bring Vortexer container into the BSC
- Take Vortexer out of container and plug into wall outlets (NOT BSC outlets)
- Check Vortexer settings – vortex at the lowest setting possible
- **Only use Screw-cap tubes** for vortexing
- Wipe surface of sealed tube with Vesphene
- Vortex for the minimal amount of time possible
- When complete, wipe Vortexer with a damp Vesphene rag
- Place Vortexer back into the container
- Seal container and wipe out of the BSC

2. Table top centrifuge

Centrifugation can generate aerosols. Only screw-cap tubes can be centrifuged.

- Bring aerosol-tight centrifugation buckets into the BSC
- Wipe sealed tubes with Vesphene
- Place tubes in centrifugation buckets and seal bucket – check for O-ring
- **Be sure centrifuge is balanced**
- Wipe out buckets from BSC
- Centrifuge – **do not exceed 3500 rpm**
- Place buckets in BSC and open carefully
- Disassemble buckets and wipe out of BSC
- Rinse and dry buckets

3. Microcentrifuge

Centrifugation can generate aerosols. Only screw-cap tubes can be centrifuged.

- Bring aerosol-tight microcentrifuge rotor into the BSC
- Wipe sealed tubes with Vesphene
- Place tubes in microcentrifuge rotor and seal rotor
- **Be sure centrifuge is balanced**
- Wipe out rotor from BSC
- Attach rotor to microcentrifuge using the tool next to the microcentrifuge
- Centrifuge – **do not exceed 14000 rpm**
- Disassemble microcentrifuge and place rotor in BSC – open carefully
- Thoroughly clean the rotor with Vesphene and wipe out of BSC
- Rinse and dry rotor

4. Bath sonicator

Sonication generates aerosols. Only screw-cap tubes can be sonicated.

- Fill the bath with Vesphene
- Wipe out sealed tubes
- Place sealed tubes into the bath
- Turn on bath and sonicate
- When complete, immediately return tubes to BSC
- Turn off bath and empty sonicator

5. Electroporator

- Set Electroporator to desired settings
- Bring only the small portable electroporation unit into the BSC
- Place electroporation cuvette into electroporator (check for bubbles)
- Place moist Vesphene rag over electroporator
- Wipe out hands and begin electroporation
- **If electroporator arcs – treat as a spill inside the hood**

After electroporation, thoroughly clean electroporation unit and remove from BSC

6. FastPrep bead beater

Bead beating generates aerosols. Only use O-ring, Bio101 screw-cap tubes. Only use the FastPrep bead beater in the BSC.

- Move FastPrep into the BSC
- Plug into wall outlet (NOT BSC outlet)
- Insert tubes (O-ring, Bio101 screw-cap tube) into FastPrep
- Bead beat
- After bead beating, remove tubes and wipe entire FastPrep with Vesphene
- Remove from BSC

7. OD reader

All samples should be killed with Formalin prior to removal from BSC for OD reading. Formalin quickly kills *M. tb* without lysing the cells.

- Add 0.75 mL formalin to each cuvette
- Add 0.75 mL culture to each cuvette
- Carefully seal tubes with cuvette lids
- Mix and wipe tubes out of the BSC
- Wipe each cuvette with 70% ethanol and dry
- After reading the OD, place samples back in the waste bin in the BSC

Using radioactive materials

The use of radiation in the BSL3 combines two unique safety hazards and special precautions and training are necessary. Only users specifically trained in **Radioactive Material (RAM) Use** by the Facility Director are allowed to use radiation and all radioactive work must be approved prior to the experiment. Only **^{35}S** and **^{14}C** are currently allowed in the BSL3. Users are responsible for monitoring areas for contamination and reporting any radioactive contamination immediately to the Facility Director. The BSL2 support lab is available for further experiments using radioactivity and waste disposal. **No radioactive materials can be stored in the BSL3**.

1. RAM training

Radiation safety training by **EH&S** and the **Facility Director** is required to use radioactive materials (RAM) in the BSL3. Current BSL3 users who wish to conduct experiments in the BSL3 with RAM must be certified by EH&S for RAM usage. Users that are not certified by EH&S (i.e. not on an existing RUA) must first complete the required EH&S radioactive material safety training (http://ehs.berkeley.edu/radsafety/training.html). Following EH&S training, notify the Facility Director to be added to the BSL3 RUA #8906. Once added to the RUA, the Facility Director will give specific training on handling RAM in the BSL3. This training will cover:

- Experimental design with special precautions for working in the BSL3
- Obtaining RAM under RUA #8906
- General procedures for RAM work
- RAM waste disposal of TB contaminated materials
- Steps to control radiological contamination

The Facility Director will assist the user during the first experiment to ensure proper understanding of the rules and guidelines.

2. Ordering or transferring RAM to the BSL3

All RAM used in the BSL3 must be listed on the BSL3 RUA #8906. This is done through the EH&S "Radiation Safety Information System" (RSIS) (https://was.ehs.berkeley.edu/rsis/item/Item.jsp). Users will be instructed on how to use the system during EH&S radiation safety training. RAM can be

either purchased under the BSL3 RUA or an aliquot can be transferred to the RUA for each experiment. The steps to order/transfer RAM are detailed below:

- Inform the Facility Director exactly what material you wish to order/transfer (include isotope, chemical form, vendor, and activity)
- On the EH&S RSIS website:
    - Select "radioactive material request" tab in inventory
    - Fill in required information
    - Click "add to request"
    - You will receive an authorization number by email.
- Once approved, order the materials, including both the RUA number (8906) and the authorization number (All RAM must be purchased by your lab, the BSL3 does not provide RAM)
- **NO RADIOACTIVE MATERIALS CAN BE STORED IN THE BSL3**
- RAM can be stored in approved and marked locations in the BSL2 rm 407
- After the RAM have arrived, check the RSIS to ensure the correct item and amount is in the inventory and label the item with the 4 digit RSIS number
    - Log on to the inventory, find your chemical (it will be entered into the inventory by EH&S upon receipt), and change its location to "top shelf of deli case in Rm 407 (your initials)"
    - Transfer your material to the Rm 407 and use a permanent marker to write your name somewhere on the outer packaging.
    - If you plan to dilute your stock, you need to "create a new stock vial". Transfer all of the radioactivity from the original stock to the "new vial" and fill in the required information (including the location, as above, with your initials). The new stock will be assigned a new material number in the inventory. It is your responsibility to write this new number on a piece of tape and place it on the vial so that we can identify it in the future. Do not cover up the old material number so we have a record of it.

3. Using RAM in the BSL3

All procedures must be approved by the Facility Director prior to conducting a radioactive experiment. This is necessary to ensure proper handling of the radiological substance and proper killing of all bacterially contaminated materials. As with all experiments using RAM, try to keep waste and radiation as low as reasonably allowable.

Before starting a radioactive experiment

- Discuss experimental design with Facility Director
- Make sure the RAM is listed on the RSIS system under RUA #8906
- Create an experiment in the RSIS system (see RSIS user manual)

Set up the BSC for radioactive work

- Set up hood for normal work (biohazard bag, Vesphene pot, and rags)
- Place protective bench paper inside the BSC (secure with tape if necessary)
- Fill "RAM Waste" sealable bucket with enough Vesphene to kill all contaminated materials and place in hood on bench paper
- Bring in two "radioactive" rags for cleaning up RAM spills ***Be careful to segregate RAM contaminated rags and Vesphene***
- Bring in the Geiger Meter (GM) (check protective bag for tears)

General procedures

- **Do not use Serological Pipets** with RAM – they will not fit in the buckets and cannot be safely decontaminated!
- Conduct all RAM work over protective bench paper in case of spills
- Clearly label all RAM-contaminated cultures with the date, initials, RAM sticker, and isotope
- Any potentially contaminated waste must go into the "RAM Waste" bucket
- Check for contamination with the GM during all procedures
- If any non-disposable equipment is contaminated with radiation, clean thoroughly with "radioactive" Vesphene rag and dispose of rag in RAM Waste bucket

Cleaning up

- Check all equipment for contamination before wiping out with Vesphene (e.g. centrifuge rotors, racks, pipetmen, etc.)
- Add Vesphene to any roller bottles, seal tightly, and wipe out of hood
- Place all radioactive waste in the "RAM Waste", including bench paper
- Tightly seal the "RAM Waste" bucket and wipe out of the hood
- Survey the entire inside of the hood with the GM
- Wipe out the GM
- Disinfect BSC like normal

4. Monitoring

Users are responsible for monitoring **all** areas where radioactive work was performed (including BSC, bench, centrifuge, incubators, etc.). Slowly survey all work areas using the Geiger Meter (do not remove from protective bag). If contamination is detected, thoroughly clean the affected area with a damp Vesphene rag and dispose of rag in RAM Waste. Continue cleaning contaminated areas until no radiation is detected by the GM. Report any contamination to the Facility Director immediately for further monitoring.

5. RAM waste disposal

NO RAM WASTE IN THE BSL3 – NO RAM WASTE DOWN THE BSL3 SINK! All solid RAM waste is only disposed of in the BSL2 rm 407 and all liquid RAM waste down the BSL2 sink. Tb-contaminated radioactive waste will be disposed of by the Facility Director.

- Wipe out RAM Waste from the BSL3 into the anteroom
- Carefully take all RAM Waste (bucket, roller bottles) to the BSL2 rm 407 using the marked autoclave tray in the BSL2
- Leave RAM waste and the marked autoclave tray on the floor by the RAM area of the BSL2
- Check the cart or tray for contamination with the GM in rm 407 and clean any detected radiation
- Immediately notify the Facility Director (by e-mail or phone) to dispose of the waste (include the name of the experiment in the RSIS, isotope, and amount or radiation – the Facility Director will delete the experiment from the RSIS and record the waste in the system)

Emergency response

For ALL emergencies – call the **Emergency Response Hotline (642-9090).** They will contact the necessary personnel.

1. Biohazard spills inside the BSC

- Immediately disinfect the area: Place dry towels on spill to absorb liquid. Soak these towels with Vesphene, working from the outside in towards the center of spill area.
- Clean all affected areas within the BSC with Vesphene.
- Leave UV light on in BSC after disinfecting, as usual. The BSC blower is never turned off during a spill.

A spill inside the BSC must be cleaned up immediately, but does not require any further notification

2. Biohazard spill outside the BSC

DO NOT ATTEMPT TO CLEAN THE SPILL!

- Hold breath, back away from spill
- Tell everyone in the BSL3 to evacuate the facility immediately
- If gown or shoe covers are contaminated, douse with Vesphene and remove them at the exit and leave on floor inside the BSL3
- If clothing or gloves are contaminated, douse with Vesphene but keep them on
- Once inside ante-room, remove protective equipment and any potentially contaminated clothing and place in a bag for autoclaving
- Wash hands, face

All spills outside the BSC, regardless of size, must be reported immediately. Call the **College of Chemistry Emergency Response Hotline (642-9090)**, the **Facility Director** and the **Biosafety Officer**. If it is after normal working hours or the weekend, call the **College of Chemistry Emergency Response Hotline (642-9090)**.

Post signs so that others will not enter the contaminated area.

3. Decontamination of spill outside the BSC

Decontamination plan is established by the **Biosafety Officer**. No one can re-enter the facility without approval from the Biosafety Officer or other EH&S official. EH&S will make the determination for re-entry and has an emergency responder who will coordinate entry.

- After one hour, dress in protective clothing--rubber gloves, boots, disposable jumpsuit, head covering, and PAPR
- Pour Vesphene solution around spill and cover the area with paper towels soaked in disinfectant solution
- Let stand 30 minutes.
- Use paper towels to wipe up spill, working towards the center of the spill
- Swab area around spill, including floor, walls, counters, using paper towels soaked in Vesphene
- Dispose of all contaminated material used in cleanup in autoclave bags, and autoclave immediately

The Biosafety Officer will determine if gaseous decontamination is necessary.

After a spill, medical surveillance may be required for potentially affected workers.

4. Equipment failure

- Centrifuge rotor malfunction – ruptured centrifuge bowl
    - Treat as a spill outside the BSC and follow procedures outlined above
- BSC malfunction – red warning light and/or alarm signifies insufficient air flow
    - Cover and contain all infectious material, turn off vacuum, and close sash
    - Terminate work, notify all other workers to leave the room
    - Leave room, notify others not to enter by posting a sign
    - Notify the **Facility Director** and **Biosafety Officer** so that decontamination procedures can begin

5. Fire alarm

If there is not an immediate threat to your safety:

- Cover and contain all infectious material
- Close BSC sash
- Leave the BSL3 following normal procedures being careful to check for fire or smoke at doors
- Evacuate building using stairs
- Contact the **College of Chemistry Emergency Response Hotline (642-9090) or 911** to report a fire in the BSL3

If the main exit is blocked by fire:

- Go to emergency exit
- Remove PPE at the door
- Step onto the roof wait for fire department

If sprinklers are tripped:

- Cover and contain all infectious material
- Close BSC sash
- Leave the BSL3 following normal procedures being careful to check for fire or smoke at doors

6. Personal injury

- Needle sticks or cuts involving potential *M. tuberculosis* exposure
    - Allow wound to bleed – express the wound
    - Wash area with Vesphene
    - Wash affected area with soap and water
    - Call the **Emergency Response Hotline (642-9090)**
- Occupational injury such as exposure to hazardous chemicals
    - Report immediately to the **Facility Director** and **Biosafety Officer**
    - Seek medical attention

7. Room Pressure Failure

- If pressure sensors at the doors begin to alarm
    - Cover and contain all infectious material
    - Close BSC sash
    - Leave the BSL3 following normal procedures
    - Call the **Emergency Response Hotline (642-9090)**

BSL3 SAFETY TRAINING (ACTIONS BY INSTRUCTOR)

Introduction

- ☐ Verify completion of EH&S training, medical surveillance, respirator fit testing
- ☐ Reminder that Documentation of Training form must be completed
- ☐ Any questions about information in Safety Manual, Training Protocol

Administrative

- ☐ Ordering and restocking
- ☐ General lab contacts: director, other personnel, their responsibilities

Lab layout

- ☐ General principles of air handling and isolation
- ☐ General principles sterilization and autoclaving
- ☐ Designation of BSL2/3 areas
- ☐ Door alarms and indicators
- ☐ Autoclave operation (from outside)
- ☐ Emergency procedures and location of emergency contact information

Entry procedure

- ☐ Suiting up and signing in
- ☐ Use of PPE (needs certification from EH&S?)

Inside the BSL3

Lab layout

- ☐ Hoods, calendar, instrumentation and storage
- ☐ Autoclave operation (from inside) and waste handling policies
- ☐ Supply restocking policies

TRAINING DEMONSTRATION

General principles of sterilization

- ☐ Wipe-in/wipe-out, double gloves
- ☐ Bringing materials into and out of cabinet
- ☐ Culturing and manipulating *M. tuberculosis* safely
- ☐ Minimization of aerosols: use of equipment inside hood

Demonstration of procedures

- ☐ Biosafety cabinet set-up: use of two buckets and rag
- ☐ Bringing instruments/supplies into/out of hood.
- ☐ Transporting cultures
- ☐ Bringing cultures into/out of hood.
- ☐ Opening/closing culture containers
- ☐ Pipetting with pipettman
- ☐ Pipetting with pipets

- ☐ Taking an OD
- ☐ Centrifugation
- ☐ Plating (also parafilm/foil for incubation; autoclaving)
- ☐ Disposing of waste
- ☐ Cleaning hood – wave of cleaning
- ☐ Emergency procedures – handling spills and accidents

SAFETY TESTING (ACTIONS BY TRAINEE)

- ☐ Explain lab layout and general air handling principles.
- ☐ Identify door alarms and function.
- ☐ Locate emergency contact information, explain emergency procedures.
- ☐ Operate autoclave from outside.
- ☐ Prepare for lab entry.
- ☐ Prepare biosafety cabinet for *M. tuberculosis* work.
- ☐ Demonstrate experimental procedures
    - ☐ Inoculate a 50-mL liquid culture in a roller bottle from a 1-mL frozen stock using a 5-mL pipet. Put culture into incubator, bring back into BSC.
    - ☐ From this culture, take an OD600 measurement
    - ☐ Centrifuge the culture (2x 25mL) at full speed for 5 min
    - ☐ Resuspend one cell pellet using a P1000 pipet
    - ☐ Use resuspension inoculate a 10-mL culture in a tissue flask
    - ☐ Resuspend the other cell pellet using a 5-mL pipet
    - ☐ Inoculate a 20-mL culture in a shaker flask and put into incubator.
    - ☐ Plate 100 µL of culture on a solid agar plate; parafilm, foil, and bring out of BSC
- ☐ Disinfect the biosafety cabinet.
- ☐ Operate autoclave from inside.
- ☐ Restock item from stockroom.
- ☐ Exit the BSL3.

BSL3 Completed Training Documentation Form

- Submit completed form to the Director.
- Completed form must be on file before you are permitted to do unsupervised BSL3 work.

User Name: _____

Lab and Department Affiliation: _____

Status (circle one)**:** Grad student Postdoc Visiting scholar

EH&S Biosafety Training .....Date completed: _____

Initial Medical Surveillance.....Date completed: _____

If 2-stage test, provide both dates. Give clinic documentation to the Director.

Respirator Fit Test .....Date completed: _____

BSL3 Training .....Date completed: _____

Supervised by: _____

Signature of director: _____

BSL3 Testing.....Date completed: _____

Supervised by: _____

Signature of director: _____

BSL3 Supervised Trial Session 1 of 2.....Date completed: _____

Supervised by: _____

Circle one: Director Certified user

Signature of supervisor: _____

BSL3 Supervised Trial Session 2 of 2.....Date completed: _____

Supervised by: _____

Circle one: Director Certified user

Signature of supervisor: _____

I certify that I have completed all of the above training for work in the Hildebrand BSL3 Laboratory. I have read the Safety Manual and understand that I am responsible for all the information therein. I am also responsible for discussing and clearing with the Director any new procedures that I plan to perform in the BSL3.

Signature: _____ Date: _____

Name (print): _____

E-mail address: _____

Phone number: _____

Emergency contact name: _____

Emergency contact phone number: _____

"Exit" protocol completed and USER INACTIVE AS OF (date): _____

ADDITIONAL TRAINING

EH&S Radioactive Safety Training .....Date completed: _____

BSL3 Radiation Safety Training .....Date completed: _____

Supervised by: _____

Signature of director: _____

BSL3 Radiation Supervised Trial Session .....Date completed: _____

Supervised by: _____

Circle one: Director Certified user

Signature of supervisor: _____

BSL3 Exit Protocol

In the BSL3 lab

- Check and dispose of cultures in:
    - Standing incubators
    - Shaking incubator
    - Roller bottle incubator
- Autoclave all plates (60 min)
- Clean out and dispose of items in:
    - Refrigerator
    - -20 freezer
    - -80 freezer
- Clean out cabinet, removing /discarding personal items
- Archive any strains in -80
    - Inform lab members and facility director of location and number of boxes and their contents

In the BSL2 lab

- Check and dispose of cultures in:
    - Standing incubators
    - Shaking incubator
- Autoclave all plates (60 min)
- Clean out and dispose of items in:
    - Refrigerator
    - -20 freezer
    - -80 freezer
    - Radioactive material should be properly removed from the BSL3 and disposed of according to the radioactive guidelines (see Biosafety Manual)

- Radioactive samples in the -20 freeze and deli case should be catalogued with lab members
    - Remaining stock vials should be cataloged with lab members and the facility director

Inform the Facility Director of your leaving date

Director will remove your name from:

- BUA
- RUA
- BSL3 listserve
- Medical surveillance log