

Standard Operating Protocol for *Mycobacterium tuberculosis*

**MFRC6042 and MFRC6041
6th floor BSL3 Containment Laboratories**

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1. Risk Assessment and Control of Biohazards.

Procedures to be performed under this standard operating protocol (SOP) involve the use of *Mycobacterium tuberculosis*. This organism has been designated a BSL3 agent by the Centers for Disease Control and Prevention and the National Institutes of Health. *M. tuberculosis* is primarily a pathogen of humans. The infectious dose for these organism is relatively low (<10 bacilli). However, productive infections are observed in <30% of individuals infected with *Mycobacterium* and those are primarily in immuno-compromised individuals. The primary mode of infection is via the aerosol route through the formation of droplet nuclei containing 1-3 organisms. *Mycobacterium* can also be acquired, although less frequently, by ingestion. While a vaccine (the live attenuated strain BCG) is available and administered at birth in most other regions of the world, it is not administered nor recommended in the United States. Alternatively, utilization of the purified protein derivative (PPD) skin test is suggested for detection of *Mycobacterium* infection. Several useful antibiotics are available and routinely prescribed to treat *Mycobacterium* infections including isoniazid, ethambutol, streptomycin, rifampin, and pyrazinamide. The *Mycobacterium* strains to be utilized in this laboratory have been previously tested and demonstrated to be sensitive to the antibiotics described above.

Adherence to the following SOP will be required for all investigators manipulating *Mycobacterium* strains. **While not documented here, all procedures to be performed in this facility will follow the recommended guidelines outlined in the Medical College of Wisconsin's Biosafety Level-3 Program Standard Operating Protocol. Failure to follow designated procedures will result in immediate termination of user privileges of the BSL3 lab.**

2. Entrance into MFRC6042 (BSL3 Anteroom) and MFRC6041 (BSL3 Lab).

All personnel entering MFRC6042 and MFRC6041 for tuberculosis research must receive permission from Dr. Zahrt (P.I.). All personnel must also receive authorization to use the BSL3 laboratory as outlined in the Biosafety Level-3 Program Standard Operating Protocol. Prior to entrance into the BSL3 laboratory, all personnel must sign the entrance log located outside room MFRC6041. The individual's name, time of entrance, and reason for entrance must be documented. This information will be retained as a permanent record of the MFRC6041 BSL3 lab usage. If the BSL3 lab is already in use, or will be used shortly by another investigator (as documented on the entrance log), the individual should contact that investigator to obtain permission to enter the facility. As the MFRC6041 BSL3 lab is multi-user, care must be taken to avoid entering the MFRC6042 anteroom when other personnel are present and donning their personal protective equipment. All personnel must access room MFRC6042 using the attached biometric hand reader and proximity card reader.

3. Donning of Personal Protective Equipment (PPE).

Prior to entrance into BSL3 lab MFRC6041, all individuals must change out of their street clothes and don the required personal protective equipment (PPE). PPE for tuberculosis research includes disposable scrub tops and pants, a standard back disposable water resistant gown, disposable sure grip shoe covers, disposable hair bonnet, 2 pairs of latex or nitrile gloves, and a fitted N95 filtering face piece respirator. All

lab personnel must be fitted for the respirator before they will be allowed entrance into the BSL3 lab. Individuals should contact Occupational Health at MCW to schedule an appointment for fitting. In donning clothing, one pair of latex or nitrile gloves should be secured underneath the water resistant gown and the remaining pair of latex or nitrile gloves over the knitted cuffs of the gown. It is recommended that personnel keep a pair of shoes in MFRC6042 that can be solely dedicated for utilization in the BSL3 lab. A supply of PPE will be maintained in MFRC6042. Extra boxes of latex or nitrile gloves, and a few additional ensembles of PPE, will also be available in MFRC6041 should they be required. If supply of any of the PPE items becomes low, personnel should contact Dr. Zahrt or the BSL3 lab director so that items can be replenished.

4. Obtainment and manipulation of *M. tuberculosis* within MFRC6041.

All work involving the manipulation of virulent *M. tuberculosis* must be performed in a class II laminar flow biosafety cabinet (BSC) located within MFRC6041. Organisms will be obtained from American Type Culture Collection (ATCC) and shipped to the MCW via a freight-prepaid carrier designated by ATCC. Dr. Zahrt may also routinely obtain cultures of *M. tuberculosis* from other investigators. These organisms will be shipped to the MCW in double sealed containers on dry ice via a freight pre-paid carrier. All cultures shipped to MCW will be stored in MFRC6041. All seed cultures or constructed strains of virulent *M. tuberculosis* or *M. bovis* will be stored in the -80°C freezer located in MFRC6041.

Cultures of *M. tuberculosis* will be grown on solid agar medium or in liquid medium within sealed incubators located within MFRC6041. Agar plates or broth medium used to grow organisms will be prepared outside MFRC6041 and transported into MFRC6041 as needed. Plates containing *M. tuberculosis* will be sealed in polypropylene bags before removal from the biosafety cabinet and transport into the incubator. Organisms in liquid culture will be grown in plastic, tightly-sealing screw-cap storage bottles (Corning). All manipulations involving infectious material will be performed using disposable reagents including plastic pipets, plastic spreaders, wood applicator sticks and wood toothpicks. All solid material coming into contact with infectious agents will be collected in a polypropylene biohazard bag which will be housed within the laminar flow hood. At no time will the biohazard bag be positioned over vent or blower openings within the biosafety hood. At the end of the experiment, the biohazard bag will be sealed and placed into a larger polypropylene biohazard bag for autoclaving. When liquid cultures are to be used for autoclaving, organisms will be transferred by pipeting into Corning conical screw-cap tubes or tightly sealing eppendorf tubes. Prior to removal from the biosafety cabinet, cultures will be placed in the appropriate centrifuge rotor and sealed with the corresponding aerosol-containment lid.

5. Experiments to be conducted in MFRC6041.

All experiments utilizing *M. tuberculosis* will be conducted to investigate the host-pathogen interactions of this organism. We will grow either liquid or solid medium cultures of *M. tuberculosis* for various experimental methodologies including DNA and RNA extraction, protein extraction, colony forming unit determinations, macrophage infection assays, and DNA transformations. **All assays to be conducted will be**

performed within the biosafety cabinet, or within tightly sealed tubes outside the biosafety cabinet. Detailed training on each of these procedures will be conducted by Dr. Zahrt and are documented in the MCW Biosafety Level 3 Program standard operating protocol.

Growth of *M. tuberculosis* cultures: *M. tuberculosis* will be routinely grown on solid Middlebrook 7H10 or 7H11 agar medium. Additionally, liquid cultures of *M. tuberculosis* will be grown in tightly sealing 15- or 50-ml conical tubes using Middlebrook 7H9 broth medium. Inoculated plate medium will be sealed in polypropylene bags within the biosafety cabinet to prevent desiccation during the 30 day incubation period. Prior to removal from the biosafety cabinet, conical tubes or sealed plate bags will be disinfected with Amphyl spray. All cultures will be grown in dry incubators located within the BSL3 laboratory.

DNA extraction: Cultures of *M. tuberculosis* will be processed to extract DNA for subsequent molecular assays. Cultures will be first concentrated in conical tubes by centrifugation using tightly sealing rotor-caps. Per normal operating procedures, the outside of conical tubes will be disinfected with Amphy, loaded into the centrifuge buckets with lids within the biosafety cabinet, and the centrifuge buckets disinfected with Amphyl prior to transport to the centrifuge. Once concentrated, sealed centrifuge buckets will be transported back to the biosafety cabinet. Bacteria will be suspended in 1.0 mls homogenization buffer and transferred to Sarsedt gasketed screw-cap centrifuge tubes for heat-inactivation (80°C in a heat block for 1 hour). Previous experiments using plate counts have demonstrated that this is sufficient to kill all viable organisms. Centrifuge tubes are then sprayed a final time with Amphyl and tubes removed for further processing outside the BSL3 laboratory. At the completion of centrifugation procedures, rotor buckets are disinfected with Amphyl and allowed to dry.

RNA extraction: Cultures of *M. tuberculosis* will also be processed to extract RNA for gene expression analysis (RT-PCR or Microarrays). Cultures will be first concentrated in conical tubes by centrifugation using tightly sealing rotor-caps as described above. Bacteria will be suspended in 0.5 mls RNA Later (Ambion) and incubated at 4°C overnight to inactivated RNAses prior to cell lysis. Cells will then be washed with 1X PBS to remove RNA later and immediately resuspended in RNazol B (Trizol) for lysis. This suspension is then transferred to Sarsedt gasketed screw-cap centrifuge tubes containing 300-400 microliters of silica beads and the tube subjected to bead-beating (3-30 sec pulses full power) using a Biospec Products bead-beater. Lysed cells are then centrifuged to remove cell debris and the resulting supernatant incubated with 0.2 volumes chloroform. The organic phase is separated by centrifugation and the upper aqueous phase removed. Genetic material is then precipitated by the addition of isopropanol. Previous experiments using plate counts have demonstrated that the Trizol/bead-beating treatment is sufficient to kill all viable organisms. Centrifuge tubes are then sprayed a final time with Amphyl and tubes removed for further enrichment of RNA outside the BSL3 laboratory.

Protein extraction: Cultures of *M. tuberculosis* will be processed to extract protein for 1D or 2D-SDS PAGE analyses. Cultures will be first concentrated in conical tubes by centrifugation using tightly sealing rotor-caps as described above. Bacteria will be suspended in homogenization buffer (0.3 M Tris, 0.1 M NaCl, 6 mM EDTA) and transferred to Sarsedt gasketed screw-cap centrifuge tubes containing a small amount of silica beads. The tube is subjected to bead-beating (3-30 sec pulses full power) using a

Biospec Products bead-beater. Lysed cells are then centrifuged to remove cell debris and the resulting supernatant is passed through a 0.45 micron filter. Previous experiments using plate counts have demonstrated that this treatment is sufficient to kill and/or remove all viable organisms from protein-containing samples. Centrifuge tubes are then sprayed a final time with Amphyl and tubes removed for further enrichment of protein outside the BSL3 laboratory. Alternatively, culture filtrate proteins (the secreted proteome of *M. tuberculosis*) will be isolated by centrifugation of *M. tuberculosis* cultures to pellet bacteria, and passage of the remaining media through a 0.45 micron filter. The outside of flasks containing this filtered media are then disinfected by Amphyl for subsequent removal from the BSL3 laboratory.

DNA transformations: High concentration cultures of *M. tuberculosis* will be prepared to introduce DNA by electroporation. Typically 200-ml cultures of the bacteria are grown, centrifuged as described above, and washed 4X in 10% glycerol. Following the final spin, cultures are resuspended in 2 mls of 10% glycerol and separated into 400 microliter aliquots within eppendorf tubes. This typically gives a concentration of $\sim 4\text{-}5 \times 10^{10}$ CFU/ml. DNA is then added to a given tube and the mixture incubated at 37°C for 5 minutes. The cell-DNA mixture is transferred to an electroporation cuvette and the top sealed. Electroporation of the mixture is conducted within the biosafety cabinet using standard settings, and the mixture transferred to a 15 ml conical tube containing 1 ml 7H9 medium for phenotypic expression at 37°C for 24 hours. The following day, the electroporated sample is then spread on agar medium and incubated for colony formation. Following electroporation, the cuvette holder is disinfected with Amphyl and transferred outside the biosafety cabinet.

Construction of *M. tuberculosis* mutants: A Mycobacterium-specific transducing phage will be utilized to generate marked and unmarked gene deletion mutations in strain H37Rv. Briefly, PCR fragments generated to 1.0-kb regions upstream and downstream of the gene of interest are directionally cloned adjacent to a hygromycin resistance cassette that is carried on a standard Mycobacterium cloning vector. This vector is recombined onto a Mycobacterium phasmid which is then used to generate Mycobacterium-specific transducing bacteriophage containing the disrupted gene of interest. Transduction of these bacteriophage into *M. tuberculosis* at the non-permissive temperature results in mutant derivatives that have undergone homologous recombination. Potential mutants will be screened by PCR and/or Southern blot.

Macrophage infection assays: The virulence of wild-type and mutant cultures of *M. tuberculosis* will be assessed by conducting macrophage infection assays *in vitro*. For these assays, mid-log phase cultures of *M. tuberculosis* are prepared, washed several times in 1X PBS as described above, and resuspended in tissue culture medium. An aliquot of this suspension is then transferred to a 50 ml conical tube containing sterile glass beads and vortexed to break up large clumps of organisms. The resulting suspension is subjected to a low-speed centrifugation step and subsequently allowed to rest at 4°C overnight to separate remaining clumps. The following day, 1.0 ml from the upper part of the suspension is transferred to a Sarsedt gasketed screw-cap centrifuge tube and subjected to heat-inactivation (80°C in a heat block for 1 hour). Previous experiments using plate counts have demonstrated that this is sufficient to kill all viable organisms. Centrifuge tubes are then sprayed a final time with Amphyl and tubes removed for counting organisms by microscopy outside the BSL3 laboratory using a fluorescent staining kit. Once bacterial numbers have been determined, an appropriate dilution of the viable *M. tuberculosis* suspension is prepared in the BSL3 lab allowing a

1:1 multiplicity of infection into macrophages. Macrophages are prepared outside the BSL3 laboratory and transported into the facility immediately prior to infection. Infections are allowed to proceed for 2 hours, and then infected macrophages washed several times in 1X PBS to remove extracellular organisms. Tissue culture medium is replaced, and at various times after infection macrophages are lysed with 1x PBS containing 0.05% Tween. Macrophage homogenates are then diluted and plated onto 7H10 agar medium to determine the number of *M. tuberculosis* colony forming units. Tissue culture plates are housed within larger, loosely sealing, Tupperware containers during times of incubation. The outside of this container is disinfected with Amphy prior to its removal from the biosafety cabinet for transport to the incubator.

Animal infection assays: Wild-type *M. tuberculosis* and constructed mutants will be tested for virulence in mice using the low-dose aerosol model of tuberculosis. Briefly, cultures of *M. tuberculosis* are prepared using standard techniques. A low dose inoculum corresponding to approximately 10^6 organisms per ml is then prepared and aliquoted into a 15-ml tightly sealing conical tube. This tube is then transferred into a DOT-approved shipping container and transported down to the ABSL3 laboratory in the TBRC for infection into animals. A 5-ml aliquot of this inoculum is then transferred within the biosafety cabinet located in the ABSL3 laboratory into the glass nebulizer unit of a GlasCol Inhalation Exposure System. This is a self-contained whole body aerosolization unit that provides reproducible animal exposure to droplet-borne contaminants and infectious agents. The unit is equipped with built in HEPA filters, an incinerator, and powerful ultraviolet lamps to decontaminate the exposure chamber. Following exposure, animals are removed from the infection basket using a large rubber-tipped forcep, and transferred back into their cages. At various timepoints after infection (typically 24 hrs out to 1 year), animals will be sacrificed and target organs of infection collected. Organs will be collected in 15-ml conical tubes, packed according to DOT-shipping regulations, and transported back to the BSL3 laboratory in MFRC6041. Within the biosafety cabinet of MFRC6041, organs will be homogenized using manual tissue grinders and homogenates plated to determine the number of colony forming units. At the completion of individual aerosolization studies, the nebulizer unit will be disinfected with a liquid Amphy solution, removed from the inhalation exposure system, and subsequently immersed in an Ampyl bath in a Nalgene pipet tray with removable cover. The tray and its contents will then be sterilized in the autoclave. The infection basket will also be double-bagged in large polypropylene autoclave bags and subjected to steam sterilization in the large autoclave. Finally, the inside of the infection chamber is disinfected using Amphy and the unit sealed until next use.

6. Disinfection of *M. tuberculosis* infected materials.

At the completion of all experiments, biohazard bags containing infectious waste or materials will be subjected to steam sterilization by autoclaving. The individual should initiate the pre-set autoclave program to run on the liquid cycle. Following sterilization, waste material will be recovered from the anteroom side of the autoclave (in room MFRC6042) and transported to the appropriate location for disposal. At no time will material contaminated with infectious agents be removed from MFRC6041 without first being sterilized or decontaminated (in the case of transport of infectious agents for animal infection studies-see section 7). As described in the Biosafety Level 3 Program SOP, all sterilization runs will be conducted with the appropriate monitoring materials and documented in the sterilization log book. All liquid waste and non-disposable solid

supplies (plastic spreaders, etc) are incubated for a minimum of 1 hr. in a 10% solution (final concentration) of bleach. At the completion of experiments, all work surfaces within the biosafety cabinet will be disinfected with a 10% bleach solution followed by a 70% solution of ethanol, or by spraying work surfaces with Amphyl spray.

7. Transport of *M. tuberculosis* materials outside MFRC6041.

At various times during the course of Dr. Zahrt's experiments, he or members of his laboratory may be required to transport infectious material containing live *M. tuberculosis* from MFRC6041 at MCW to the ABSL3 laboratories (room to be determined). Transportation of live organisms will be required for aerosolization experiments in mice. During these occasions, all cultures will be contained in Corning conical tubes that have been tightly sealed. Sealed Corning tubes containing viable *M. tuberculosis* cultures will then be packaged into a DOT-approved shipping container and tightly sealed. Prior to removal of conical tubes containing cultures from the biosafety cabinet in MFRC6041, all tubes will be thoroughly disinfected with the germicide Amphyl. Once disinfected, tubes will be removed from the biosafety cabinet and placed within a DOT-approved transport container. Once sealed, the outside of the DOT-approved transport container will also be sprayed with Amphyl disinfectant before removal of the entire container from the BSL3 lab. Stickers will be placed on the outside of all containers to designate them as biohazardous.

8. Exiting from MFRC6041 and MFRC6042.

All personnel exiting MFRC6041 will be required to remove both pairs of latex or nitrile gloves, the open back gown, the hair bonnet, and shoe covers prior to their entrance into the adjacent anteroom. A polypropylene biohazard bag will be used to collect lab clothing. It will be located adjacent to the door next to the steam sterilizer. When the biohazard bag becomes full, it is the responsibility of the investigator to include this material with other waste that is to be sterilized in the autoclave. Personnel will wash hands with antibacterial soap prior to exiting the BSL3 laboratory. The remaining safety garments including the N95 respirator will be removed once the investigator has exited to the anteroom in MFRC6042. A separate biohazard bag will also be located in MFRC6042. When the biohazard bag becomes full, it is the responsibility of the investigator to load this material into the autoclave on the anteroom side to sterilize materials. Sterilization of all materials will be performed and documented as previously described.

9. Strain storage and laboratory logbooks.

All strains will be permanently stored in the -80°C freezer located in BSL3 lab MFRC6041. Detailed listings of all strains including their genotype, date of deposition, and location position in the freezer will be documented in the strain logbook. Each investigator will document results from all procedures using the computer located in MFRC6041. This computer will be networked providing access to experimental details from outside the BSL lab.

10. Training procedures for laboratory personnel.

Dr. Zahrt will be responsible for directly training members of his laboratory in all aspects of the procedures utilizing *M. tuberculosis*. Prior to any member of his lab initiating experiments utilizing these agents, lab members will train in Dr. Zahrt's lab with the avirulent attenuated *M. bovis* strain BCG until BSL2 conditions, which behaves identical to *M. tuberculosis* in culture.

Dr. Zahrt and all members of his lab who will be involved in this project will be required to complete the BSL3 training program and participate in the Occupational Health and Safety Program offered at MCW. A copy of the training procedure for each researcher involved will be kept as a permanent record to verify training. Dr. Zahrt and all members of his lab obtain annual PPD skins, and have the TB Quantiferon test prior to their initiation of work with virulent *M. tuberculosis*. Results from this test will be kept as a permanent record to verify the absence of exposure. Those individuals previously vaccinated with the BCG strain will not be required to have the PPD skin test as they will test positive. In these instances, routine chest x-rays and blood draws for the Quantiferon test will be performed to confirm the absence of *Mycobacterium* infections. Personnel will also be fitted and tested annually for an N-95 respirator. This will be performed by the Department of Occupational Health and Safety.

11. Spill management and emergency procedures.

The procedures outlined in the MCW's Biosafety Level-3 Program SOP will be strictly followed in the event of a spill or other emergency procedure.

A. Biohazardous Spill in Open Space

Spill cleanup shall be conducted so that exposure potential is minimized. Actual spill cleanup procedures will depend on the contents of the spill. All spills, regardless of size, must be reported as soon as possible to the Principal Investigator, BSL-3 Laboratory Director and the Biological Safety Officer. After a spill, medical surveillance may be necessary for potentially exposed employees.

- a. **Major Spill:** Volume of spilled material greater than 10 ml or what a single blue absorbent under pad could cover. All exposed personnel **must** contact their supervisors and Occupational Health Services. During off hours, personnel may receive medical treatment from the FMLH Emergency Department.
 1. **The employee should hold their breath and exit the containment lab immediately.** Remove disposable scrubs, inner gloves and respirator.
 2. **Allow aerosols to settle and engineering control mechanisms to ventilate the lab.** The actual time depends on air exchange rate for the space. The BSL-3 containment laboratory exchanges the room air 19.7 times per hour. At least 30 minutes should elapse before attempting to enter the containment laboratory after a major spill.
 3. Dress in protective clothing for spill cleanup (spill suit, boots, gloves, PAPR-type (powered air purifying respirator), head cover) and enter the lab.
 4. Pick out any sharps with tongs or tweezers. Dispose of them in a sharps shelter containing disinfectant.
 5. Cover area with absorbent pads and paper towels. Pour more disinfectant around and on spill. Let stand 30 minutes.

6. Use paper towels to wipe up the spill by working from outside toward center of the spill.
 7. Swab areas around the spill, including floor, walls, and counters, using paper towels soaked in disinfectant.
 8. Autoclave all materials used to clean up spill, including personal protective equipment. Double-bag all material used for cleanup as well as disposable clothing. Autoclave immediately. Mop the floor or area with a 10% solution of bleach.
 9. Call the BSL-3 Laboratory Director and the BSO to report spill and potential exposure incident.
 10. Remain available to assist emergency personnel.
- b. **Minor Spill:** Volume of spilled material less than 10 ml or could be covered by blue absorbent under pad.
1. Cover spilled material with disinfectant-soaked blue absorbent under pad.
 2. Remove contaminated clothing in containment lab and bag for autoclaving. Leave respirator on until you are in the anteroom.
 3. Take care of any exposed personnel by washing exposed skin with disinfectant soap.
 4. Exit the containment lab. Allow aerosols to settle and the engineering controls to ventilate the lab.
 5. Dress in protective clothing for spill cleanup (spill suit, boots, gloves, PAPR-type (powered air purifying respirator), head cover).
 6. Pick out any sharps with tongs or tweezers. Dispose of them in a sharps shelter containing disinfectant.
 7. Cover area with paper towels. Pour more disinfectant around and on spill. Let stand 30 minutes.
 8. Use paper towels to wipe up the spill by working from outside toward center of the spill.
 9. Swab areas around the spill, including floor, walls, and counters, using paper towels soaked in disinfectant.
 10. Autoclave all materials used to clean up spill, including personal protective equipment.
 11. Contact the Principal Investigator, Biosafety Officer and BSL-3 Laboratory Director to discuss spill and potential exposure incident. Occupational Health Service may conduct any potential exposure follow-up with the BSO.

2. **Biohazardous Spill in Biosafety Cabinet**

- a. Leave cabinet on.
- b. Immediately disinfect the area: Place dry paper towels on the spill to absorb liquid. Soak these towels with disinfectant, working from outside towards the center of the spill. Carefully folding the paper and then discarding it can handle a spill on absorbent paper.
- c. Clean all areas affected within the biosafety cabinet with disinfectant.
- d. Contact the Biosafety Officer to determine if the cabinet needs to be decontaminated by an outside vendor.
- e. Leave UV light on in biosafety cabinet after disinfecting, as usual.