Video Article

Safety Precautions and Operating Procedures in an (A)BSL-4 Laboratory: 2. General Practices

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URL: https://www.jove.com/video/53600
DOI: doi:10.3791/53600

Keywords: Infection, Issue 116, biosafety, biosafety level 4, BSL4, BSL-4, Class II biosafety cabinet, Class II BSC, high containment, maximum containment, personal protective equipment, PPE, basic protocol

Date Published: 10/3/2016

Citation: Mazur, S., Holbrook, M.R., Burdette, T., Josleyn, N., Barr, J., Pusl, D., Bollinger, L., Coe, L., Jahrling, P.B., Lackemeyer, M.G., Wada, J., Kuhn, J.H., Janosko, K. Safety Precautions and Operating Procedures in an (A)BSL-4 Laboratory: 2. General Practices. *J. Vis. Exp.* (116), e53600, doi:10.3791/53600 (2016).

Abstract

Work in a biosafety level 4 (BSL-4) containment laboratory requires time and great attention to detail. The same work that is done in a BSL-2 laboratory with non-high-consequence pathogens will take significantly longer in a BSL-4 setting. This increased time requirement is due to a multitude of factors that are aimed at protecting the researcher from laboratory-acquired infections, the work environment from potential contamination and the local community from possible release of high-consequence pathogens. Inside the laboratory, movement is restricted due to air hoses attached to the mandatory full-body safety suits. In addition, disinfection of every item that is removed from Class II biosafety cabinets (BSCs) is required. Laboratory specialists must be trained in the practices of the BSL-4 laboratory and must show high proficiency in the skills they are performing. The focus of this article is to outline proper procedures and techniques to ensure laboratory biosafety and experimental accuracy using a standard viral plaque assay as an example procedure. In particular, proper techniques to work safely in a BSL-4 environment when performing an experiment will be visually emphasized. These techniques include: setting up a Class II BSC for experiments, proper cleaning of the Class II BSC when finished working, waste management and safe disposal of waste generated inside a BSL-4 laboratory, and the removal of inactivated samples from inside a BSL-4 laboratory to the BSL-2 laboratory.

Video Link

The video component of this article can be found at https://www.jove.com/video/53600/

Introduction

As safety of laboratory personnel handling high-consequence pathogens (no infection prophylaxes nor treatment options exist) is paramount, the US Department of Health and Human Services has established guidelines for facility construction and best practices for the safe conduct of work with pathogens in biomedical and clinical laboratories from a biosafety perspective[1]. Through legislation and regulation, many of the practices and procedures have become mandatory requirements that must be followed for work with these pathogens. In the US, pathogens that are easily transmitted from person to person, result in high case-fatality rates, and/or have the potential for major public health impact and bioterrorism, are categorized as National Institute of Health/National Institute of Allergy and Infectious Disease (NIH/NIAID) Priority A Pathogens and or Centers for Disease Control and Prevention (CDC) Bioterrorism Category A Agents[2]. In addition, high-consequence pathogens are classified as Tier 1 Select Agents if these pathogens are potential bioterrorism agents, have potential for mass casualties or devastating effects to the economy, critical infrastructure, or public confidence[3].

BSL-4 operations, including access to institutes with BSL-4 laboratories, are more highly controlled than BSL-2/3 operations. For instance, it is substantially more difficult to gain access to a BSL-4 laboratory compared to a BSL-2 or BSL-3 laboratory due to substantial suit training requirements, extensive mentorship requirements, and additional medical biosafety prerequisites. In addition, there are typically more physical security barriers in a BSL-4 facility versus a BSL-2 or BSL-3 facility[4-6]. As outlined in our first article on BSL-4 entry and exit procedures, laboratory staff undergo extensive training and psychological screening to qualify for entrance into the BSL-4 laboratory[7]. Within the BSL-4 laboratory, risk of infection and mistakes are avoided or mitigated by following established procedures. Research must proceed carefully and deliberately, with minimal multitasking or distractions. Bending over in positive pressure suits is difficult, and the face shield may restrict procedures such as microscopy. Bulky gloves impede the performance of fine motor tasks, such as handling small items or labeling tubes. To minimize time spent in BSL-4 laboratories, laboratory specialists should review work procedures to identify steps that can be done ahead in a BSL-2 laboratory and then transport these materials into the BSL-4 laboratory for completion of the task(s). When removing materials for further processing in BSL-2 laboratory, materials are fixed and removed from the BSL-4 laboratory in a sealed secondary container. Examples of samples that may need to be removed include: fixed plates or tubes of infected material that will be analyzed by enzyme-linked immunosorbent assay (ELISA), immunofluorescence assay (IFA), or polymerase chain reaction (PCR).

In addition to greater physical limitations imposed by personal protective equipment required in BSL-4 laboratories compared to those in BSL-2 laboratories, procedures for inactivation of high-consequence pathogens in cell culture plates and waste disposal are stricter than those needed for less pathogenic viruses studied in a BSL-2 laboratory. At a minimum, these methods should meet CDC requirement. For example, contaminated cell culture plates and other materials can be inactivated with chemical reagents, such as neutral-buffered formalin. Treated cell culture plates or tubes are to be placed into heat seal pouches containing formalin and removed from the laboratory via a dunk tank filled with a liquid disinfectant. Waste buckets filled with disinfectant solutions and spray disinfectants are used for temporarily receiving waste generated during the experiment and for disinfecting gloves, cleaning biosafety cabinet surfaces and instruments, respectively. Quaternary ammonium disinfectant solution at the concentration listed is considered the gold standard for all US BSL-4 laboratories (Barr J, personal communication, 2015). Solid waste from a waste bucket is autoclaved to eliminate potential for contamination.

In an effort to visually demonstrate the workflow and limitations of general BSL-4 procedures, we used a standard viral plaque assay as an example of a commonly used viral procedure. While the viral assay procedure is described in general, we stress the biosafety procedures used to ensure safety of laboratory personnel in this protocol. Please refer to previous classical plaque assay visualizations for additional background on the plaque assay technique[8,9].

The procedures presented here follow the BMBL specifications outlined by CDC[1]. However, the presented protocols are specific to the IRF-Frederick. Each BSL-4 facility has different standard operating procedures (SOPs) and methods of operation that impact the execution of experiments within the BSL-4 laboratory. Alternative procedures for waste stream management and execution of plaque assays may differ based on the management and operation of these laboratories. Nevertheless, a general understanding of the setup of a BSL-4 suit laboratory and procedures for performing work with Class II cabinets inside the BSL-4 environment will help scientists understand the constraints and safety implications when contemplating studies of high risk pathogens. Increased awareness of outside collaborators of the difficulties surrounding work in a BSL-4 laboratory can lead to adjusted expectations and greater ease in developing medical countermeasures in the research community.

Protocol

1. Laboratory Entry

1. Gather all supplies from a BSL-2 laboratory for the experiment (*e.g.*, cells, media, and consumables) prior to entry into the BSL-4 laboratory.
2. Complete the BSL-4 entry procedure (outlined in detail in reference[7]).

2. Preparation of a Class II Biosafety Cabinet in the BSL-4 Laboratory

1. Once inside the BSL-4 laboratory, ensure the daily internal checklist (**Figure 1**) has been completed. Complete the checklist if the checklist has not been previously filled out and indicate which viruses will be used. If the checklist has already been completed, add name and which virus will be used to the list.
2. Clean the Class II BSC by spraying down the entire inside of the BSC (including the sash) with 5% dual quaternary ammonium disinfectant solution (*e.g.*, n-alkyl dimethyl benzyl ammonium chloride, n-alkyl dimethyl ethyl benzyl ammonium chloride or other disinfectant appropriate for the agent being used) and wipe down with paper towels[10,11]. Spray 70% ethanol solution inside the cabinet and the sash to remove the tacky disinfectant solution.
3. If seated, set the chair in front of the Class II BSC at a comfortable height to ensure that the back of the cabinet can be reached and that the face of the laboratory specialist is situated above the front opening (**Figure 2**)[11].
4. Prepare a waste container for the biosafety cabinet. Ensure that the final concentration of dual quaternary ammonium disinfectant solution in the waste container is no less than 5%. Plan accordingly and make a 10% solution to which waste will be added that will dilute the disinfectant. In addition, place a spray bottle with 5% dual quaternary ammonium disinfectant solution inside the Class II BSC to spray any items prior to removal and gloved hands during and after completion of the assay.
5. Place the appropriate materials needed for the entire experiment in the Class II BSC as far back in the cabinet as possible to avoid repeated material introductions into the Class II BSC and disruption of the air flow[11].

3. Example: Plaque Assay

1. Retrieve virus-containing samples and control virus from the storage location by hand and thaw materials in an incubator at 37 °C.
2. Label the wells of the 6-well plates to be used according to the virus dilutions planned. Mark the lids and body of the plates to ensure successful matching if the lids and body are separated.
3. Bring materials from steps 3.1-3.2 into the Class II BSC. Place all items that have not or will not come in contact with virus on one side ("clean side") and waste on the other side ("dirty side"). If possible, keep "clean items" at least 30 cm apart from "dirty items" during aerosol-generating activities[11].
4. Work as close to the inside center of the Class II BSC as possible, as the inside center is designed to be the most effective position to protect oneself[11].
5. Remove 50 µl of virus sample and control virus and place into 450 µl of Dulbecco's modified Eagle's medium with 2% fetal bovine serum. Proceed with making serial dilutions as far out as needed (**Figure 3A**).
6. During the dilutions, mix the virus samples at least 5 times with a pipette tip slowly and carefully while trying to minimize air bubble generation in the samples. Change pipette tips after each addition to the next dilution well.
7. In the last dilution, after mixing the sample 5 times, discard 50 µl of virus sample into waste container to ensure equal volumes of dilutions.
8. When disposing of tips, rinse each tip with disinfectant solution from the waste bucket to decontaminate the inside and outside of the tip before expelling tip into the waste bucket.
9. Once the dilutions are made, set the dilution wells aside and begin aspirating media from the wells of cell culture plates, leaving 500 µl of media in each well of a 6-well plate.

10. When finished with the pipette tip, aspirate disinfectant solution from the waste bucket to the top of the pipette using a manual, adjustable volume, push-button pipettor, to ensure proper decontamination of the inside of the pipette. Leave the tip into the waste bucket until the end of the experiment.
11. Once the media have been removed from the plates, add 100 µl of the correct sample into the proper well in the pre-labeled plates in duplicate, changing tips each for each sample.
12. Upon completion of the plaque assay inoculations, spray off gloved hands with the disinfectant solution and use a paper towel soaked with disinfectant solution to wipe the outside of all plates before placing the plates back into the incubator by hand. Repeat this process until all plates are back into the incubator.
13. Rock the plates in a figure-8 motion to ensure proper dispersal of the sample over the cells every 15 min for 1 hr.
14. Upon completion of rocking plates, return plates into the Class II BSC along with a mixture of 2x Eagle's minimal essential medium and 1.6% tragacanth, a semi-solid overlay that is easier to manipulate than agarose, used for the overlay of the plaque assay.
15. Add 2 ml of the overlay mixture to each well in a 6-well plate and rock once again in a figure-8 motion for equal distribution of the overlay throughout the surface of the well. Repeat this process until every used well is overlaid with the overlay mixture.
16. Ensure that the tip of serological pipette does not touch any liquid in the wells to avoid cross contamination when performing the overlay procedure.

4. Waste Disposal and Cleaning of the Instruments and Biosafety Cabinet

1. Completely submerge all waste material in 5% disinfectant solution in the waste bucket for a contact time of at least 10 min. Disinfect pipette tips, serological pipettes, and other waste as described above. In addition, spray the waste bucket (inside and out) with 5% disinfectant solution let the solution remain in contact with the waste bucket for a contact time of 10 min.
2. While waiting for the contact time to elapse for the waste, soak a paper towel with 5% disinfectant solution, wipe down instruments such as micropipettes, and remove them from the BSC. Spray gloved hands with 5% disinfectant solution before bringing gloved hands out of the Class II BSC.
    1. After removing the micropipettes from the BSC, wipe them with a separate paper towel with 70% ethanol solution to avoid sticky buildup on the instruments. Spray any instruments that cannot be effectively wiped down with 5% disinfectant and leave in contact with the solution in the BSC for 10 min.
3. After sufficient contact time, remove all items from the BSC. Take items, including waste bucket containing waste materials, to the sink. Rinse items that can be reused to remove disinfectant residue. Return all items to their storage locations.
4. Clean the Class II BSC's work surface, cabinet sides and back, interior of glass, and sash[1] with 5% disinfectant solution followed by 70% ethanol solution.
5. Pull out and drain the serological pipettes from the waste bucket and place surface-disinfected serological pipettes into separate pipette trays for autoclaving (serological pipettes can present a sharps hazard and may tear through the trash bag. Place these pipettes in a hard-sided container before autoclaving).
6. Pour the contents of the waste bucket into a strainer placed in the bottom of the sink.
7. Bring a biohazard waste container that is lined with a red biohazard bag to the sink and dump the contents of the strainer into the red biohazard bag. Do not reach into the strainer to remove micropipette tips that may stick to the inside of the strainer. Hit the strainer along the inside of the waste container until all tips are removed, or use a pair of tweezers to remove tips from the strainer.
8. Rinse out the waste bucket and place to dry on the rack next to the sink.

5. Autoclaving Waste

1. Remove the biohazard bag from the biohazard waste container and place in an autoclave tray on a cart.
2. Leave biohazard bags open and place a piece of autoclave tape over the outside of the bag, connecting the sides of the autoclave tray to keep the bag secured in the tray.
3. Place two pieces of autoclave tape on the serological pipette tip tray and label it with one's initials and the date. Place the serological pipette tray on the cart with the autoclave tray.
4. Open the autoclave. Connect the provided autoclave loading/unloading cart to the autoclave and bring out the retractable autoclave platform to rest on the cart.
5. Remove the metal rod from the autoclave and open the top. Place a biological indicator vial containing spores of *Geobacillus stearothermophilus* into the metal rod to check that the autoclave sterilization cycle was completed successfully.
6. Place the metal rod into the center of the waste bag in the autoclave tray but ensure that the metal rod is still easily accessible.
7. Place autoclave tray filled with waste and serological pipette tray onto the retractable autoclave platform and push it back into the autoclave.
8. Detach the autoclave loading/unloading cart from the autoclave and close the autoclave door.
9. Start the autoclave.
10. Do not leave the autoclave until the cycle has started. The operating screen of the autoclave will indicate time remaining for that run.
11. After the autoclave run is completed, remove the biological indicator and evaluate for growth by heating in a specified incubator. If growth is detected on the biological indicator, re-run the trash in the autoclave, and assess anew indicator. If no growth is detected, remove the trash from the facility.

6. Example: Fixing and Staining of Plaque Assays

1. After the appropriate number of days (which is dependent on the virus used) has passed, return back into the laboratory and perform steps from sections 1 and 2 once again.
2. Carefully remove plaque assay plates from the incubator completed in step 3.1.2, and place into the Class II BSC by hand.
3. Pipette off all of the media and overlay material and dispense into disinfectant solution container and replace with a mixture of 10% neutral buffered formalin and 0.8% crystal violet[12,13]. Let the mixture remain on the plates for 30 min to inactivate the virus on the plates.

4. After inactivation, carefully remove the neutral buffered formalin/crystal violet mixture and place in a separate waste container to be neutralized prior to disposal.
5. Spray gloved hands with disinfectant and wipe the outside of the plates before passing them out of the Class II BSC as described above.
6. Proceed to the sink, rinse the plates to remove excess stain, and then place the plates on a cart to dry.
7. Once the plates are completely dry, use a light box to count the plaques. Record all the counts and calculate virus titers from the standard equation (**Figure 3B**).

7. Removing Samples from the BSL-4 Laboratory

1. Inactivate any samples that will be manipulated under BSL-2 laboratory conditions. Follow one of two methods approved by the internal biosafety office at the IRF-Frederick using 10% neutral-buffered formalin (NBF) or Trizol LS (phenol, guanidine isothiocyanate, ammonium thiocyanate, sodium acetate, glycerol)[1,14]. Transfer samples to a new clean tube or plate outside of the BSC prior to packaging for removal from the BSL-4 laboratory.
2. Heat-seal inactivated samples in tubes or plates in a heat-seal pouch containing sufficient 5% disinfectant or fixative solution to disinfect the inside of the bag and the outside of the sample tubes undergoing transfer out of the BSL-4 laboratory. Place this pouch into another pouch following same procedure.
3. Seal the second pouch and place the heat-sealed pouch into a dunk tank containing 5% disinfectant solution for at least 10 min to disinfect the outside of the heat-sealed pouch.
4. Fill out a dunk tank log book inside the lab by delineating the number and size of tubes, volume in tubes, agent used, inactivation method used, and room to where samples will be transferred.
5. Coordinate with a colleague on the outside of the BSL-4 laboratory to retrieve the pouch from the dunk tank and take samples to the BSL-2 laboratory.

Representative Results

Following proper procedures within the BSL-4 laboratory are critical for ensuring safe and effective completion of assays. By referring to the completed daily internal checklist (**Figure 1**), laboratory staff ensure that equipment is fully operational. Proper body positioning in the center of the BSC ensures that the experiment is performed under optimum air flow conditions (**Figure 2**). The virus sample is serially diluted to obtain plates that have 30-300 plaques per plate (**Figure 3A**) and to determine virus titer (**Figure 3B**). A number of factors affect formation of plaques, including virus tropism for host cell lines, inoculation technique, conditions for virus growth, appropriate dilution range, and overlay selection[8]. Waste generated in the BSC during the procedure is properly disinfected prior to removal from the BSC and again by autoclaving prior to leaving the BSL-4 environment. By following these procedures, no laboratory-acquired infections have been recorded during BSL-4 research at the IRF-Frederick.

Daily Internal Systems Checklist

Suite B (2B134A)

Date: _____

Name: _____ Agent: _____ Name: _____ Agent: _____

_____ _____ _____ _____

_____ _____ _____ _____

_____ _____ _____ _____

_____ _____ _____ _____

_____ _____ _____ _____

_____ _____ _____ _____

_____ _____ _____ _____

Biosafety Cabinets:

Cabinet	1	2	3
Power (On/Off)			
Magnehelic			

Incubators:

Upper	22	24
Temp		
CO_2		
Water		
Lower	**23**	**25**
Temp		
CO_2		
Water		

Refrigerator	10	52
Temperature		
Freezer	**20**	**21**
Temperature		

Waterbath Temperatures: _____/_____ Water Levels: _____/_____

Comments:_____

_____

_____

Figure 1: Sample daily internal systems checklist. Daily completion of this checklist ensures that laboratory staff has checked equipment within the laboratory (most importantly the BSC) prior to initiating work. If the BSC is found to be outside of the calibrated range, this BSC must not be used, and maintenance should be notified. All BSCs must be properly calibrated and functioning. Please click here to view a larger version of this figure.

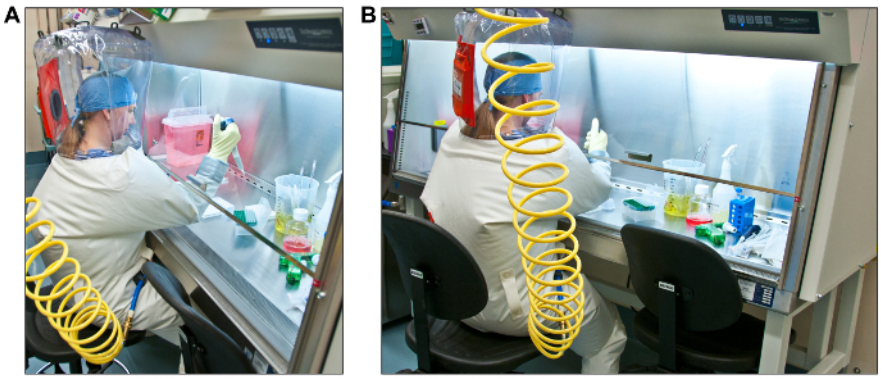

Figure 2: Back and side view of a laboratory specialist pipetting samples in a Class II biosafety cabinet. (**A**) Waste bucket containing yellow disinfectant solution and used pipettes are to the right of the well plates (**B**), and the disinfectant spray bottle is to the right of the waste bucket (**A**). Please click here to view a larger version of this figure.

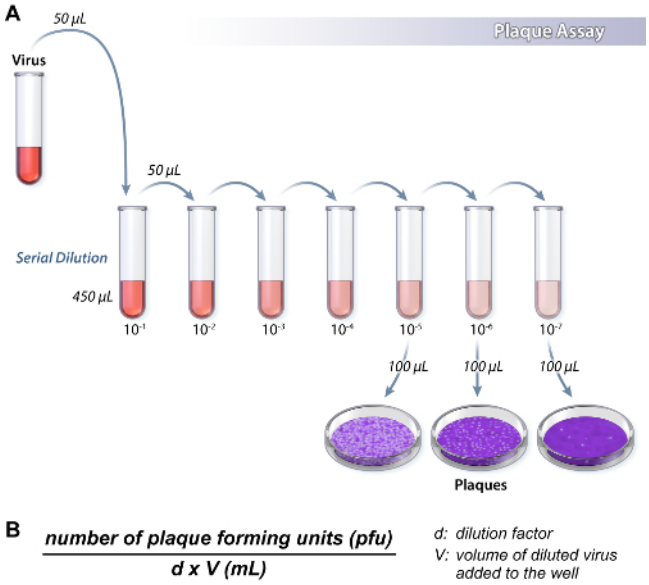

Figure 3: Calculation of viral titer of sample. Viral titer is expressed as plaque forming units (pfu) per ml. To calculate the viral titer, count the number of clearly defined plaques (pfu) and divide by the product of the dilution factor (d) times the volume of diluted virus added to the well (V). Please click here to view a larger version of this figure.

Discussion

Work in a BSL-4 laboratory requires considerable time and additional attention to detail. Any type of work in this environment requires well trained, thorough, and conscientious individuals. The standard viral plaque assay provides an accurate model of a common procedure for working with high-consequence pathogens in the BSL-4 laboratory, as the assay involves several major concepts in which laboratory workers must be trained.

The first major concept is the proper use and application of safe practices in Class II BSC, which functions as primary containment for high-consequence pathogens. Understanding of how a Class II BSC functions will dictate practices that greatly limit exposure risks to individuals. Work flow from a clean area ("clean side") to a contaminated area ("dirty side") across the work zone in the Class II BSC also helps to avoid cross contamination[11]. Clean and contaminated materials and supplies should be segregated to limit the movement of contaminated items over clean items.

The second major concept is physical and biological waste management. Proper steps in disposing both types of waste are essential in ensuring that the laboratory specialists stays safe and the environment is not contaminated. Steps during an experiment are designed to inactivate and destroy pathogens before samples are brought out of BSL-4 laboratory. Examples of such critical steps include: pipetting disinfectant into each tip, allowing at least a 10 min contact time of potentially contaminated materials with disinfectants, autoclaving waste, and validating sterility during autoclave cycles. These steps are designed to be redundant to ensure destruction of high-consequence pathogens.

Disclosures

The authors have nothing to disclose.

Acknowledgements

The content of this publication does not necessarily reflect the views or policies of the US Department of Health and Human Services (DHHS) or of the institutions and companies affiliated with the authors. This work was funded in part through Battelle Memorial Institute's prime contract with the US National Institute of Allergy and Infectious Diseases (NIAID) under Contract No. HHSN272200700016I. M.R.H., D.P., L.B., K.J. and J.W. performed this work as employees of Battelle Memorial Institute. Subcontractors to Battelle Memorial Institute who performed this work are: S.M. as an employee of MRI Global; M.G.L. as an employee of Lovelace Respiratory Research Institute, Inc.; and J.H.K. as an employee of Tunnell Government Services, Inc.

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