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

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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

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Author Comments:	As discussed previously, all four articles of this series would have to be filmed together, after concurrence of safety and security personnel.
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Editor(s)

Journal of Visualized Experiments

Dear Editor(s),

Please find attached our revised manuscript "***Safety Precautions and Operating Procedures in an (A)BSL-4 Laboratory: 2. General Practices***" by Mazur *et al.* for publication in the *Journal of Visualized Experiments*.

Thank you for your consideration.

Best Regards,

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biosafety; biosafety level 4; BSL4; BSL-4; Class II biosafety cabinet; Class II BSC; high containment; maximum containment; personal protective equipment; PPE

SHORT ABSTRACT:
Performing viral assays in a BSL-4 laboratory is more involved compared to work in a BSL-2 laboratory due to required additional safety precautions. Here, we present an overview of practices and procedures used inside a BSL-4 laboratory illustrating proper Class II biosafety cabinet usage, waste management/disposal, and sample removal.

LONG 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.

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

1.1 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.

1.2 Complete the BSL-4 entry procedure (outlined in detail in [7]).

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

2.1 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.

2.2 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.

2.3 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].

2.4 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.

2.5 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

3.1. Retrieve virus-containing samples and control virus from the storage location by hand and thaw materials in an incubator at 37 °C.

3.2. 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.

3.3. 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].

3.4. 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].

3.5. 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).

3.6. 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.

3.7. 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.

3.8. 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.

3.9. 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.

3.10. 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.

3.11. 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.

3.12. 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.

3.13. Rock the plates in a figure-8 motion to ensure proper dispersal of the sample over the cells every 15 minutes for 1 hour.

3.14. 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.

3.15. 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.

3.16. 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

4.1. Completely submerge all waste material in 5% disinfectant solution in the waste bucket for a contact time of at least 10 minutes. 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 minutes.

4.2. 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.

4.2.1. 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 minutes.

4.3. 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.

4.4. 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.

4.5. 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).

4.6. Pour the contents of the waste bucket into a strainer placed in the bottom of the sink.

4.7. 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.

4.9. Rinse out the waste bucket and place to dry on the rack next to the sink.

5. Autoclaving Waste

5.1. Remove the biohazard bag from the biohazard waste container and place in an autoclave tray on a cart.

5.2. 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.

5.3. Place a piece 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.

5.4. 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.

5.5. 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.

5.6. 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.

5.7. Place autoclave tray filled with waste and serological pipette tray onto the retractable autoclave platform and push it back into the autoclave.

5.8. Detach the autoclave loading/unloading cart from the autoclave and close the autoclave door.

5.9. Start the autoclave.

5.10. Do not leave the autoclave until the cycle has started. The operating screen of the autoclave will indicate time remaining for that run.

5.11 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

6.1. 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.

6.2. Carefully remove plaque assay plates from the incubator completed in step 3.1.2, and place into the Class II BSC by hand.

6.3. 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 minutes to inactivate the virus on the plates.

6.4. After inactivation, carefully remove the neutral buffered formalin/crystal violet mixture and place in a separate waste container to be neutralized prior to disposal.

6.5. Spray gloved hands with disinfectant and wipe the outside of the plates before passing them out of the Class II BSC as described above.

6.6. Proceed to the sink, rinse the plates to remove excess stain, and then place the plates on a cart to dry.

6.7. 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

7.1 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.

7.2 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.

7.3 Seal the second pouch and place the heat-sealed pouch into a dunk tank containing 5% disinfectant solution for at least 10 minutes to disinfect the outside of the heat-sealed pouch.

7.4 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.

7.5 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.

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.

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).

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).

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-minute 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.

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The authors have nothing to disclose.

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Figure 1

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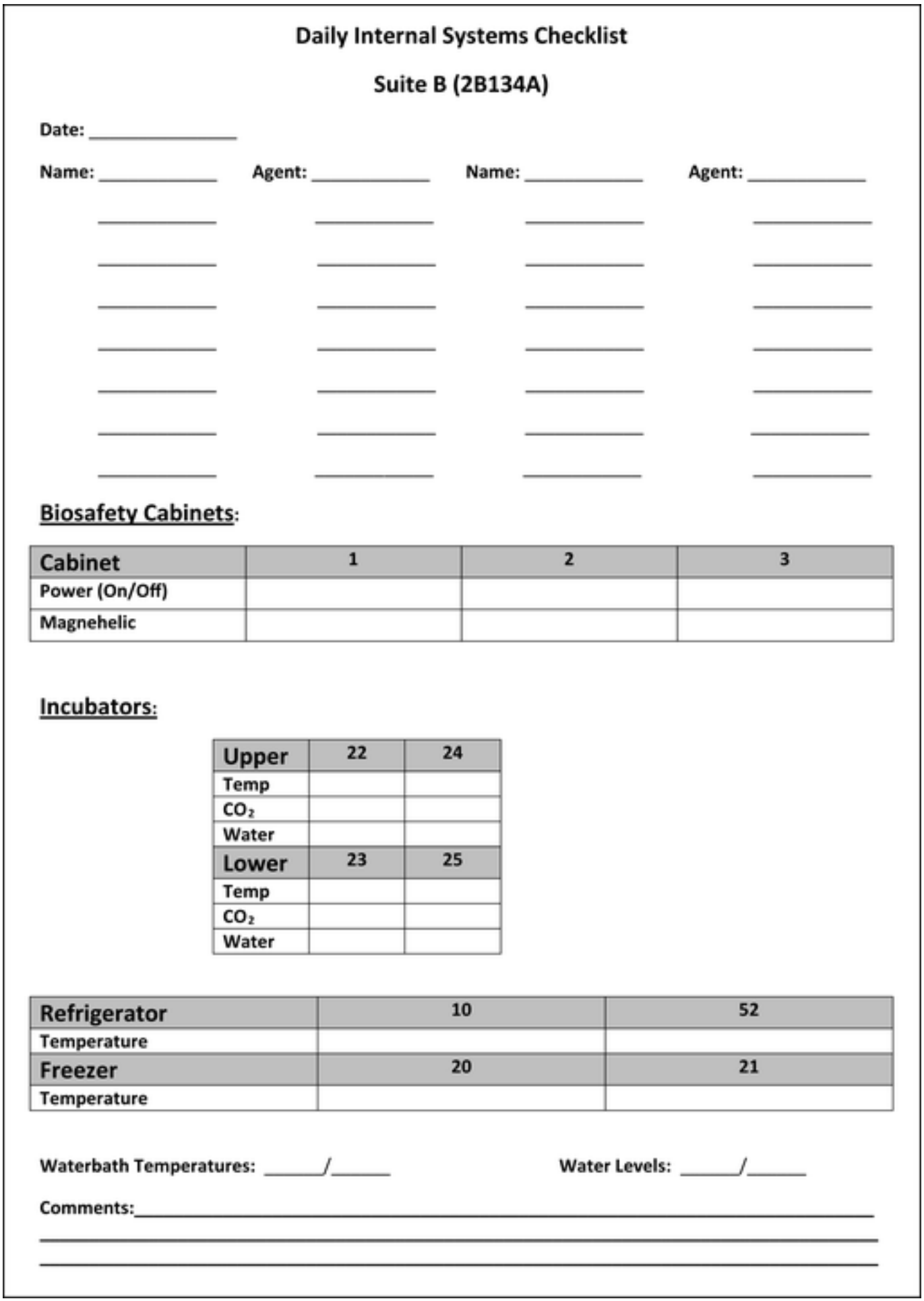

Daily Internal Systems Checklist

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 2
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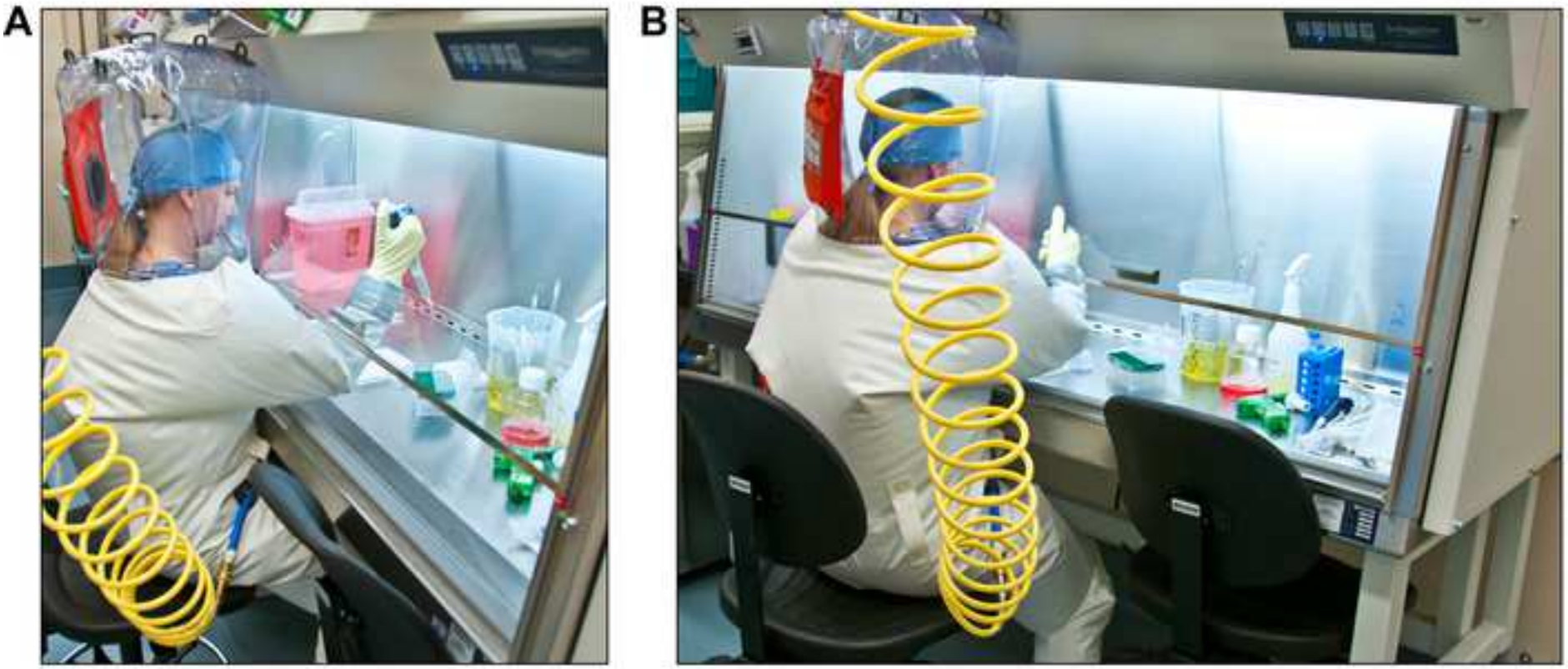

Figure 3

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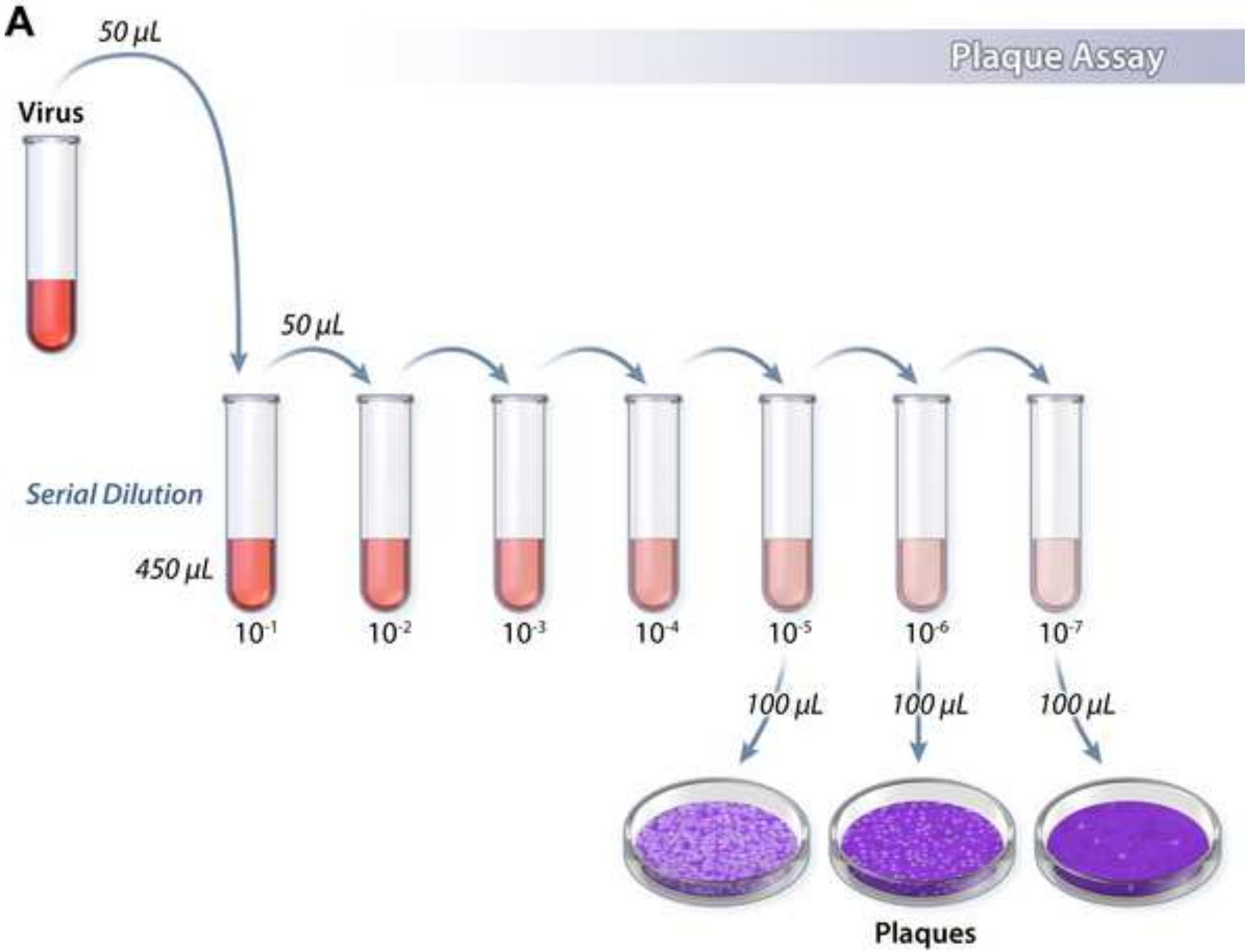

B

$$\frac{\text{number of plaque forming units (pfu)}}{d \times V\ (mL)}$$

d: dilution factor

V: volume of diluted virus added to the well

Excel Spreadsheet- Table of Materials/Equipment

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Name of Materials/Equipment	Company	Catalog Number
Micro-Chem Plus	National Chemical Laboratories	255
Ethanol	Fisher	BP2818500
2-ml 96-Deep Well Plates	Fisher	278743
10-ml Serological Pipette	Fisher	13-678-11E
25-ml Serological Pipette	Fisher	13-678-11
6-well plates	Fisher	140675
Crystal Violet	Sigma	HT90132-1L
10% Neutral Buffered Formalin	Fisher	22-050-105
Tragacanth	Fisher	50-702-2000
20-µl Pipette Tips	Fisher	21-402-550
200-µl Pipette Tips	Fisher	21-402-561
1000-µl Pipette Tips	Fisher	21-402-581
DMEM	Lonza	12-604Q
FBS	Sigma	F2442-500mL
Penicillin/Streptomycin	Lonza	17-602E
2X EMEM	Quality Biological	115-073-101
Pipettor	Drummond	4-000-101
1000-µl Pipette	Rainin	L-1000XLS+
200-µl Pipette	Rainin	L-200XLS+
12-Well, Multichannel 200-µl Pipettor	Rainin	L12-200XLS+
8-Well, Multichannel 1000-µl Pipettor	Rainin	LA8-1200XLS
Attest	Express Medical Supplies	MMM12192
Autoclave	Getinge	GEB 2404 AMB-2
Autoclave Bag	Fisher	01-828E
2000-ml Beaker	Fisher	02-591-10H
Autoclave Tray	Fisher	13-359-20B
Pipette Tray	Fisher	13-361-5
37°C Incubator	Fisher	WU-39321-00
Biohazard Can	Rubbermaid Commercial	FG614500 RED
Autoclave Tape	Fisher	15-903
Autoclave Rod	Made by IRF Facility	N/A
Light Box	Fisher	S11552
Heat Sealer	Fisher	NC9793612
Heat Seal Pouches	Fisher	01-812-25H
Biohazard Bag	Fisher	01-828E

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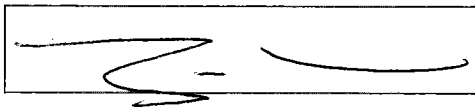
Signature:	 Date: 04/21/2015

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Editorial comments:
Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

We thank the editor for reminding us of the fact that there will be no copy-edit step. We proofread our manuscript and ensured there are no spelling or grammar errors. We also made some minor text changes to clarify meaning.

2. Typo: Line 218 – "any" instead of "and"

We thank the editor for catching this mistake, which has now been corrected.

3. Additional detail is required:
-3.6 – How is the sample mixed? Pipetting?

We agree with the editor that we should have been more specific. The sample is mixed by pipetting. We added this information to section 3.6.

-7.1 – Please provide a citation for inactivation methods.

We agree with the editor that we should have been more specific. Unfortunately, each institute uses slightly different protocols, all of which have to be approved by the CDC separately. To address the editor's concern, we qualified the statement by adding "approved by the internal biosafety office at the IRF" and added two general citations (1 and 19).

Reviewers' comments:

Reviewer #1:
Manuscript Summary:
The manuscript by Mazur et al describes safety precautions and operating procedures in an (A)BSL-4 lab. In general the article provides an overview of BSL-4 operations at the IRF-Frederick. The article is a high level overview and does not touch on the details of why certain safety precautions are performed which would be of greater value and cross the boundries of institutes making this review more universally applicable. This article would most likely be viewed by individuals outside of the BSL-4 community who are curious about work in a high containment lab. Individual institutes have slightly different operating procedures and this article would likely not benefit them.

We are delighted to see that our intentions came across. Indeed our article is targeted at individuals outside of the BSL-4 community, as outlined in the introduction.

Major Concerns:

Review document for spelling and grammatical errors. In several cases articles are missing from sentences.

We agree with the reviewer. We proofread our manuscript and ensured that there are no spelling or grammar errors. We also made some minor text changes to clarify meaning.

Line 118: typo "work in a biosafety…"
We thank the reviewer for catching this mistake, which has now been corrected.

Line 141: reference BMBL 5th edition
We agree with the reviewer and are now referencing the 5th ed. BMBL.

Line 143: What is meant by disseminated person-to-person? Perhaps this should be only transmitted since disseminated has multiple connotations?

We agree with the reviewer and deleted "disseminated or".

Lines 150-151: Filoviruses are not a good example of a high-consequence pathogen that has been poorly studies. On the contrary, this agent is one of the more well-studied agents in this category.

We agree with the reviewer. We deleted the paragraph pertaining to filoviruses.

Line 156: What is meant by "research in a BSL-4 lab is tightly controlled"? How is research controlled more in BSL-4 versus BSL-3 or 2? Select agent and dual use research rules are no different between BSL-3 and 4 in terms of acquiring appropriate approvals. This statement needs to be revised.

We agree with the reviewer that we should have been more specific. BSL-4 operations, rather than BSL-4 research, is 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 biosurety prerequisites. In addition, there are typically more physical security barriers in a BSL-4 facility versus a BSL-2 or BSL-3 facility. We have rephrased the paragraph for clarification.

Line 160: are risks avoided or mitigated?

We agree with the reviewer that the boundaries are fluid. We changed "avoided" to "avoided or mitigated" to cover the spectrum.

Lines 161-164: These examples seem to be exaggerated. Experienced staff typically have little to no difficulty doing fine manipulations in BSL-4 and certainly there is no difference when pipetting small versus larger volumes. These are all challenges that inexperienced staff might face as they are learning to maneuver in a containment environment. It does not represent the day-to-day norm.

We agree with the reviewer that pipetting smaller volumes are not a big challenge and therefore removed this sentence from the paragraph. All other examples in this paragraph are part of the IRF training program as they do pose challenges to new employees. We find them noteworthy as our article is targeted at potential collaborators/clients, who often are under the impression that they themselves could just walk into a BSL-4 laboratory and do the work without any specialized training.

Line 172: "Contaminated cell culture plates and other materials must be inactivated with chemical reagents such as neutral buffered formalin". This section of text seems to confuse two issues. Firstly there is the issue of surface decon of consumable plastics prior to autoclaving. The sentence listed here seems to refer to materials removed from containment for further analysis. In the case of the latter, two critical aspects are missing from the text. Firstly, it should state that materials are removed by institute-approved SOPs that specify chemical concentrations and contact time. The other aspect that bears mention is that materials may (and typically do) require safety testing, depending on the institute and method. This is a HUGE amount of time and effort and not needed for apathogenic viruses. Given all the recent issues at CDC, this should certainly be mentioned here. As written now, the text gives no mention to contact time, concentration of disinfectants, etc. This must be addressed.

We agree with the reviewer and added

> "At a minimum, these methods should meet CDC requirements."

to the procedure sections and

> "Quaternary ammonium disinfectant solution at the concentration listed is considered the gold standard for all US BSL-4 laboratories (Barr J, personal communication, 2015)."

Lines 205-209: There is no mention of contact time given here. If the purpose of this wipe down is for disinfection, then there should be a contact time. Why would cleaning be necessary prior to starting work if disinfection was performed at the end of the last work? Finally, it would be more appropriate to state a "disinfectant appropriate to the agent being utilized" since not all labs use MicroChem and MicroChem is not the disinfectant of choice for all agents.

Our procedures follow those outlined in the 5th edition of the BMBL (now cited). Wiping down surfaces/materials prior to starting work is an additional safety precaution for the (unlikely) case that a predecessor did not appropriately wipe down the work area after finishing work. We agree with the reviewer that MicroChem is not the only disinfectant – we therefore added "e.g.," in front of mentioning it and added or other disinfectant appropriate for the agent being used (section 2.2).

Line 217: what sort of disinfectant spray? What concentration? Perhaps keep generic to "disinfectant appropriate for the agent being used"

We agree with the reviewer and have changed the sentence to

> "In addition, ensure that a spray bottle with 5% dual quaternary ammonium disinfectant solution is available inside the Class II BSC to spray any items prior to removal and gloved hands during and after completion of the assay."

A generic comment is not possible due to the requirements of the journal.

Line 229: how can bottoms of plates be safely labeled without spilling? This would be best done outside of the BSL-4 before cells are seeded into plates. Typically folks mark the plates by drawing a line along the side from top to bottom of the plate if this is done in containment. This ensures proper lid matching and avoids lifting plates higher than necessary.

We agree with the reviewer and changed "bottom" to "body".

Line 253: typically SOPs for a plaque assay indicate removal of media. Leaving of "roughly" 500 ul is ambiguous.

We agree with the reviewer and deleted "roughly".

Line 257: inactivation is used to describe the process of treating consumables with chemical agents. Disinfection is more appropriate.

We agree with the reviewer and switched "inactivation of virus" to "decontamination of".

Line 264: are plates truly disinfected with gloved hands and not paper towel with disinfectant? What about secondary containment to transfer plates to the incubator?

We agree with the reviewer that we should have been clearer. Secondary containment is not required to transfer plates to the incubator. Gloved hands, coated in MicroChem, aren't really that different from a paper towel soaked in MicroChem and therefore using hands is a standard practice in several BLS-4 laboratories. However, a paper towel can also be used and may be a better portrayal of the process. We therefore updated the section to

> "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".

Lines 318-19: reword "one's name's initials"

We thank the reviewer for catching this mistake, which has now been corrected.

Line 424: "Using a clean-to-dirty approach" was not introduced or discussed previously to this.

We agree with the reviewer that we should have introduced this approach. The respective paragraphs have been modified to

> "3.3. 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"

and

> "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. Clean and contaminated materials and supplies should be segregated to limit the movement of contaminated items over clean items".

Line 427: proper waste management is essential for keeping more than the lab specialist safe. This underestimates the importance of following proper protocols.

We agree with the reviewer. We added "and the environment is not contaminated".

Figure 1: The text does not describe what the lab specialist should do if any values fall outside of a normal range. Presumably this is a stop work situation if the BSC fails. This should be addressed in the text.

We agree with the reviewer and added the following text:

> "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."

Figure 2 shows a lab specialist working at the BSC, however, there is an additional chair shown that restricts their movement. This seems to go against what the authors describe as properly setting up a BSC.

The BSCs in our laboratory are set up by default for pairwise work. Removing the chair would create a possible obstacle in the hallways/walkways. We therefore would like to keep these images.

What are the "Comments" listed on the last page?

We are as confused as the reviewer. Looks like these are unfortunate carry-overs from the Excel file pdf conversion. We will ensure these are not present in the revised manuscript.

Additional Comments to Authors:
None

Reviewer #2:
Manuscript Summary:
The manuscript „Safety Precautions and Operating Procedures in a BSL4 Laboratory: 2. General Practices" describes a general work flow under BSL4 conditions on a standard Plaque assay

protocol. The procedures are well and understandable described. The general principals appear clear and as the authors mentions there are differences between the BSL-4 laboratories based on equipment and management which require small modifications. However, clean and safe workflow under the class II BSC is indeed one of the most important factors to limit exposure risk to highly pathogenic pathogens.

Major Concerns:
No major concerns.

Minor Concerns:
No minor concerns.

Additional Comments to Authors:
N/A

We thank the reviewer for the positive assessment of our work.

Reviewer #3:
In this manuscript Mazur et al describe practices that can be followed in a full-suit BSL-4 laboratory using a plaque assay as an example procedure. This is of obvious interest to those who work in these laboratories, but there should also be significant interest from those who have never worked in these laboratories, simply because of curiosity. Further, understanding how to operate safely - which is exemplified by the procedures that are employed in maximum containment - can have significant benefit to those working at lower levels of containment. Overall, this is a useful manuscript that will have interest beyond those that work in maximum containment.

We thank the reviewer for the positive assessment of our work.

Major point:
While the manuscript is well written, it is written in a way that implies the authors' practices are the only way that work can be performed safely in a BSL-4 laboratory, which is inaccurate. For example, at the extreme, one can operate at BSL-4 using a cabinet or half suit line, which require very different practices. Even in a suit-lab, there are alternative ways to accomplish the goals in a safe and appropriate manner. This reviewer suggests that the final paragraph of the manuscript that states be moved to the beginning of the manuscript, and some of the text toned down to be consistent with this message.

We agree with the reviewer and moved the paragraph to the beginning as suggested.

Minor points:
* Line 249. Presumably the internal walls of the tip are decontaminated by exposure to the disinfectant solution - this should be clarified.

We agree with reviewer and added “decontamination of the inside of the pipette”.

* Comments section contains text that appears to be unnecessary

We are as confused as the reviewer. Looks like these are unfortunate carry-overs from the Excel file pdf conversion. We will ensure these are not present in the revised manuscript.

Reviewer #4:

Manuscript Summary:

The article describes performing a plaque assay at BSL-4 at the IRF and the methods used there to ensure laboratory biosafety. The article also describes the IRF's approach to waste management also linked to the plaque assay.

Major Concerns:

I feel the last paragraph, lines 434 to 442 are the most relevant of the article and should be emphasised more and mentioned in the abstract and main text. By their very nature most BSL-4 facilities have very specific design/layouts, equipment and ways of doing different procedures so I am not sure in the usefulness of this article for other BSL-4 researchers as each lab is unique. In addition, different countries outside of the US have their own national regulations and ways of working which should be acknowledged. I feel lines 179-181 hint at a more realistic use for the article in raising awareness of the difficulties surrounding BSL-4 work to potential collaborators and even funding bodies or the media. Without seeing the video, the text article seems a little light on substance relevant to a wide audience.

We agree with the reviewer that the "meat" of the article, in accordance with the journal scope, will be the video and not the accompanying text. To address the reviewer's concern regarding facility-specific SOPs, we have added a clarifying paragraph prior to the protocol section:

> "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."

Minor Concerns:

Minor points to consider clarifying.

Line 151 - Are these the right references. Should reviews of multiple agents be included? consider using date rather than "ongoing" for people reading the article in the future when the outbreak is over.

We agree with the reviewer. In light of reviewer 1's comments and the concerns of this reviewer, we have deleted the entire paragraph regarding filoviruses.

Line 174-175 - institute specific rule?

Many of the items listed are institute specific rules, and we have addressed this directly in the introduction.

Lines 179-181 - good point and worth expanding on.

We agree with the reviewer and moved this section into a new paragraph:

> "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."

Line 206-207 - give commercial name for disinfectant - presumably Microchem. Has the stated concentration and time been proven effective?

The reviewer is correct, it is MicroChem. However, the journal prohibits the use of brand names, which is why we left the statement as is. To address the other concern of the reviewer we added a statement on the approval of this method (see responses to reviewer 1).

Line 218 - word missing after "to spray and __"

We agree with the reviewer and deleted "and".

Section 3.1 and 3.12 and 6.2. How are materials moved round the lab(s)? Is virus carried by hand? How are plaque assay plates moved? Are boxes or sealable containers used?

Virus is carried from room to room in a sealed secondary container. Within a room it can be carried by hand to the hood. Plaque plates if moved from room to room would also be carried in a sealed container. Within the same room they are moved by hand, usually directly from the hood to the incubator. No container is used.

We added "by hand" at three different places. In addition, we added:

> "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".

Movement will also be demonstrated in the video.

Section 3.4/line 236 - is there a reference or diagram to back up this point?

We agree with the reviewer that this step needs to be demonstrated. This will be done in the video.

Section 3.6/line 243 - any suggestions on how to mix with minimal aerosol generation?

The reviewer raises an important point. We added "slowly and carefully".

Line 246 - Is last 50 ul of virus discarded into waste?

We agree with the reviewer that this should have been specified. We added "into waste container" to section 3.7.

Line 257 - In not into

We agree with the reviewer and fixed this mistake.

Line 271 - Readers may be unfamiliar with Tragacanth, consider explaining what it is and why used instead of agarose?

We agree with the reviewer and added the qualifying statement ", a semi-solid overlay that is easier to manipulate than agarose,".

Section 4. This section is confusing, particularly with regard to the tips. In 4.4/line 297 they are being pulled out (which does not seem safe) but in 4.6 it suggests using tweezers or a strainer to remove tips. Why do tips have to be separated into their own tray? Are BSCs fumigated after use?

We agree with the reviewer that we should have been clearer. Section 4 is now completely rewritten/re-organized.

Section 5.2 and 5.3 - Again, why are pipettes kept separate? Consider an image for some of these points as without the video they are a bit confusing>

We agree with the reviewer that we should have been more specified. We merged the two sections and updated the text to:

> "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)."

Section 5.5 - Great to use an internal control but please expand on how this is checked at the other end. An image of the rod and autoclave area would be helpful.

We agree with the reviewer. The video will illustrate the use of this control – we therefore feel an image is not needed. To address the reviewer's concern we added the following paragraph:

> "5.11 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 a new indicator will be assessed. If no growth is detected, remove the trash from the facility."

Section 6.3 - has this method of inactivation been proven?

Yes. We added two references.

Section 7 - This is quite important and safety critical. Please give some examples of what sort of things may need to be removed, and why and how

We agree with the reviewer and added the following sentence to the introduction:

> "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, we added a paragraph to section 7.1:

> "Follow one of two methods approved by the internal biosafety office at the IRF-Frederick: 10% neutral-buffered formalin (NBF) and Trizol LS (phenol, guanidine isothiocyanate, ammonium thiocyanate, sodium acetate, glycerol)[1,17]. Transfer samples to a new clean tube or plate outside of the BSC prior to packaging for removal from the BSL-4 laboratory."

Lines 434-442. Crux of the article.

> We agree with the reviewer.

Figure 1
Are there limits on the number of people allowed in the lab at any one time? Are there limits on how many viruses can be handled a day? Can one BSC be used for multiple viruses? Are there acceptable limitis or ranges that the cabinets, incubators, refrigerator and freezer etc must be at prior to work commencing?

The reviewer raises important questions. ~50 people can be in the laboratory at a given time but this is dependent on the available backup breathing air. There are no limits on the number of

viruses that can be handled in one day. Generally only one virus is handled in a single BSC at a time but this depends on the assay being performed. One single BSC can be used for many different viruses over its lifetime. The BSCs are certified annually and the functional criteria are determined at that point. Incubators, refrigerators and freezers all do have acceptance criteria to be deemed functional. However, the majority of these acceptance criteria is specific and fluid and is not captured directly on the checklist. Staff are informed when criteria change. Some of these items will be addressed during filming when the internal checklist is demonstrated/addressed. Because of the variability in the requirements, we will not be able to address them in the figure legend.

Comments "we dilute to 0.08%/1.6%" - Does this need to be included elsewhere?

We are as confused as the reviewer. Looks like these are unfortunate carry-overs from the Excel file pdf conversion. We will ensure these are not present in the revised manuscript.

Additional Comments to Authors:
N/A.