**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.