Supplemental Materials

Note: The biosecurity practices listed here can be used for Institutional Biosafety Committee standard operating procedures, specifically for the United States.

Laboratory Biosecurity Practices for Working with *Batrachochytrium dendrobatidis and B. salamandrivorans*

Emergency Contact Information
Principal Investigator (PI) phone, office, email
Lab Manager phone, office, email
Environmental Health and Safety phone

Introduction
Batrachochytrium salamandrivorans (*Bsal*) and *B. dendrobatidis* (*Bd*) are emerging wildlife pathogens (Woodhams et al. 2018). They are not infectious to humans. In the U.S., these pathogens may be treated with Biosafety Level 2 working conditions (Meechan & Potts 2020) supplemented with security precautions established by the U.S. Department of Agriculture Animal and Plant Health Inspection Service (APHIS) Standard Safeguards to move pathogens (PPQ 526). *Batrachochytrium* species are not listed as pests of agriculture nor are they part of the APHIS national wildlife disease program. *Bsal* movement is currently indirectly managed in the U.S. by the listing of some salamanders as injurious by the Fish and Wildlife Agency under the Lacey Act, requiring permits to move salamanders internationally. Thus, it is up to the research community (Gray et al. 2015), informed in part by the North American *Bsal* Task Force, to adequately address the biosecurity risks associated with *Bsal* in laboratories, and the threats to wild populations.

Standard Safeguards adapted from APHIS include:

1. All microbes (i.e. *Bsal*) must be shipped in sturdy, escape-proof containers.
2. Upon receipt, all packing material media, substrate, soil and shipping containers shall be sterilized or destroyed immediately after removing pests.
3. Microbes shall be kept only within the laboratory or designated area.
4. No living microbes shall be removed from the confined area.
5. All microbes shall be destroyed at the completion of the intended use.
6. All necessary precautions must be taken to prevent escape of microbes. In the event of escape or detection of *Bsal* in the wild, we will follow guidance from A North American Strategic Plan to Control Invasions of the Lethal Salamander Pathogen *Batrachochytrium salamandrivorans*, and the *Bsal* Rapid Response Plan (www.salamanderfungus.org).
7. In addition, culture of *Bsal* follows standard protocols described in (Robinson et al. 2020) and experiments involving exposure of amphibians to pathogens follow these standard methods.

Personnel and Training
Provide names, title, and applicable training for all personnel involved and authorized to handle pathogens**.** Environmental Health and Safety trainings in Lab Safety and Biosafety are required for all PIs, as well as any faculty, students or staff involved in handling biological materials. Persons authorized to work on pathogens will be trained in the lab by reading this document and associated references, and training will be documented by **filling out and signing a blank "Pathogen Hazard**

Notification and Protocol Acknowledgement Form" that will be filed at [designated location]. In addition, all persons working in the laboratory must also be informed and sign the notification form.

Pathogen Acquisition, Storage, and Transport
Permitted shipments and field acquisitions of pathogens will be received at [designated location], and when not in use, will be stored under lock in [designated location]. *Batrachochytrium* must be shipped in sturdy, escape-proof containers according to best practices (Gray et al. 2017). Upon receipt, all packing material media, substrate, soil, and shipping containers shall be sterilized or destroyed immediately after removing cultures. Cultures will be restricted to designated areas including designated Biosafety Level 2 hoods, incubators, and temporary bench spaces that are appropriately marked. Spill-proof secondary containment will be used whenever cultures are transported. Elevators will not be used when transporting cultures. Gloves must always be worn when handling cultures. However, gloves should not be worn when transporting materials between labs or in hallways. All gloves and secondary containment should be treated as biohazardous waste or decontaminated after use.

Equipment Storage
Field equipment will never be stored in the laboratory, but stored in [designated area]. Returning field equipment will be decontaminated in the field following the below protocols. Laboratory equipment will be decontaminated by chemical disinfectants appropriate for *Batrachochytrium* as described below, or by autoclave disinfection in [autoclave location]. Analysis or use of permitted pathogens will take place in [designated location].

Supplemental Biosafety
Standard Biosafety Level 2 procedures (Meechan & Potts 2020) are expected to be followed to ensure containment of pathogens but are not reviewed in this document. Supplemental safeguards include foot protection in designated areas including disposable foot covers, designated footwear for use only in the designated lab space, or decontaminating foot baths. Floors will be regularly cleaned with a disinfectant.

Hazard Notification, Safeguards, and Awareness
Everyone working in the laboratory must be notified through this document that there are pathogens located in the laboratory. These pathogens could pose ecological harm and will be controlled by keeping the laboratory and freezer doors locked and the samples will be stored in designated and labeled locations such as the upright freezer and incubators in the laboratory. However, it is possible that during processing of the samples, that the samples may be located on benches and tables throughout the laboratory. Persons processing samples are required to adequately handle and label samples during processing, as listed in the protocols following; however, occupants/users of the laboratory should act as if any samples in the laboratory could be quarantined. If you should come in contact with any suspected pathogen samples, you should follow the protocols listed below, and in the case of environmental release, contact the Principal Investigator. On an annual basis, after reading this section and the following protocol section, you will need to sign the "Pathogen Hazard Notification and Protocol Acknowledgement Form" and that form will be held on file at [designated location].

Cleaning and Disinfection of Field Materials for *B. dendrobatidis* and *B. salamandrivorans* should follow published guidelines (Olson et al. 2021), briefly:

- Remove plant residue, mud, and other types of soil from boots, nets, etc.
- Rinse with water. Water from pond is sufficient. Make sure the materials in question are as clean as possible.
- Always disinfect materials as follows: Move a long distance away from any surface water (ponds, streams, etc.). Use a bucket or large container to disinfect your materials. Dispose the disinfectant as prescribed. It is preferable to use two or more sets of field materials in order to limit the use of chemical disinfectants.
- Wash your hands with a disinfectant.

Disinfectants Effective Against Pathogens

- *Batrachochytrium dendrobatidis*: Virkon S (1% solution, 1 min) is the preferred disinfectant. Other effective disinfectants are bleach (10% solution, 30 s), and Ethanol or spirit (85% alcohol content, 20 s) (Van Rooij et al. 2015)
- *Batrachochytrium salamandrivorans*: Ethanol (70% solution, 30 s), Bleach (4% solution, 30 s), and Virkon S (1% solution, 2 min) (Van Rooij et al. 2015)
- *Pseudogymnoascus destructans*: Submersible items should be placed in a hot water bath (>55 C) for at least 20 min. Chemical disinfectants for other items include ethanol (60% alcohol content) and isopropanol (60% solution). Autoclaved for 90 min. (Shelley et al. 2013, White-nose Syndrome Disease Management Working Group 2020).
- *Fusarium* sp.: Virkon S (1% solution, 2 min), Bleach (10%, 5 min) (Bennett et al. 2011)
- *Ophidiomyces ophiodiicola*: Virkon S (1% solution, 2 min), Bleach (10%, 2 min), Ethanol (70% solution, 2 min) (Rzadkowska et al. 2016)
- *Saprolegnia* sp.: Virkon S (1% solution, 5 min), Bleach (10%, 5 min) (Rahman & Choi 2018)
- Ranavirus: Bleach (4% solution, 1 min), Ethanol (70% alcohol content, 1 min), Virkon S (1% solution, 1 min), Nolvasan (0.75% solution, 1 min) (Bryan et al. 2009, Van Rooij et al. 2015)
- *Mucor hiemalis*: Virkon S (1% solution, 5 min), Bleach (10%, 5 min) (Unpublished data)

Clean all materials that may have come into contact with pathogens including pipettes, bench space, etc., after an experiment and at the end of the work day. **Always use fresh solutions. In some cases they can lose some of their disinfectant properties over time (e.g., ethanol evaporates).**

Disposal

All solid material (experimental plates and cultures, as well as all consumables such as tips and plasticware) that come in contact with pathogens will be placed in biohazard bags and autoclaved before disposal at 121°C (250°F) for at least 60 minutes. All liquids contaminated with pathogens will be disinfected with 10% bleach or with the addition of Virkon S powder (creating a solution roughly 1% or greater) for at least 5 minutes before being poured down the sink.

Emergency Protocols

Personal Contamination

1. Alert people in the spill area.
2. Remove contaminated articles.
3. Clean contaminated skin with 70% ethanol or ethanol-based hand sanitizer (at least 70% ethanol).
4. Vigorously wash exposed area with soap and water for at least 1 minute.
5. If eye exposure occurs, use eye wash per instructions.
6. Obtain medical attention as appropriate.
7. Report spill to Principal Investigator or Lab Manager

Surface Spill in the Lab Outside the Biosafety Cabinet

1. Clear the room of all personnel.
2. Call Environmental Health and Safety if you feel that you will need assistance [phone number].
3. Wait at least 30 minutes for aerosols to settle before reentry.
4. Wearing appropriate PPE for size and nature of the spill to include at a minimum: lab coat, gloves and safety glasses.
5. Place dry paper towels to establish a physical barrier between the spill and yourself. Then layer a second set of disinfectant-soaked towels over the spill.
6. Starting from the outside and working in, carefully soak the spill with disinfectant (Virkon (1%) or bleach (10%) being careful to minimize aerosolization.
7. Decontaminate all items within the spill area. Wait at least 20 minutes disinfectant contact time to allow for adequate inactivation.
8. Wipe equipment and reusable items with the disinfectant.
9. Wipe up spill and discard contaminated disposables in biohazardous waste stream.
10. If sharps are present, us a mechanical device such as a dust pan and brush to pick up the sharps and place in an approved sharps container.
11. If not wearing shoe covers, disinfect shoes in foot bath.

Spill Inside a Centrifuge

1. Clear area of personnel
2. Wait at least 30 minute for aerosols to settle before starting clean-up.
3. Wearing appropriate Personal Protective Equipment (PPE: lab coat, gloves and safety glasses.)
4. Wipe rotors and buckets with disinfectant (70% ethanol) then remove to nearest Biosafety cabinet for more extensive decontamination
5. Thoroughly disinfect inside of centrifuge with a minimum contact time of 20 minutes using a disinfectant that follows manufacturer's recommendations (bleach may corrode centrifuge) and is effective for spilled agent.
6. Dispose of contaminated materials in the biohazardous waste stream.

Spill Outside the Lab in Transit

1. To prevent or minimize a spill, all potentially biohazardous material is to be transported in a primary unbreakable, leak-proof, sealed primary container placed inside a secondary unbreakable, leak-proof, and break proof container. A biohazard symbol should be used on these containers.
2. Should a spill occur in a public area, do not attempt to clean up without the appropriate PPE.
3. Secure the area around the spill.
4. Call Environmental Health and Safety for assistance [phone number].
5. Stand by for further assistance if required.

Spill Inside the Biosafety Cabinet

1. Move the glass shield down and wait at least 5 minutes to allow the Biosafety cabinet to filter and clear aerosols.
2. Wearing appropriate PPE at a minimum lab coat, safety glasses and gloves. You may want to double glove in the event the outer pair becomes contaminated.
3. Move the glass shield back up and allow cabinet to equilibrate for an additional 5 minutes.
4. With the Biosafety cabinet operational, apply disinfectant (Virkon S [1%] noting that bleach [10%] may corrode that cabinet) for a minimum of 20 minute contact time directly on the spill and on all the potentially exposed surfaces of the cabinet.
5. Wipe up spill with disinfectant soaked towels or other appropriate absorbent material.
6. Wipe the walls, work surfaces, inside of sash and any potentially contaminated equipment with towels soaked in disinfectant (70% ethanol) before removing it from the Biosafety cabinet.
7. Lift exhaust grill and tray and wipe all surfaces with disinfectant (70% ethanol).
8. Discard contaminated disposable material using appropriate biohazardous waste disposal procedures.
9. Wipe down contaminated reusable items with disinfectant (70% ethanol) then place in autoclave bag or autoclave pans with lids for autoclaving.
10. Those items that are non-autoclavable should be wiped down with disinfectant (Virkon S [1%]) and kept wet for a minimum of 20 minutes before removal from Biosafety cabinet.
11. Remove protective clothing, when done and place in biohazard bag for disposal or autoclaving for reusable items.
12. Run the Biosafety cabinet for 10 minutes after clean-up before reusing.
13. All surfaces of the Biosafety cabinet that have been cleaned with virkon or bleach must be wiped afterwards with 70% ethanol.
14. WASH HANDS!

Autoclave Spore Test (Updated Jan. 26, 2016)

SUMMARY

This is an overview of how to conduct an autoclave spore test
using Getinge Assure Accufast Biological Indicator ampule/vial spore kits.

EQUIPMENT AND SUPPLIES

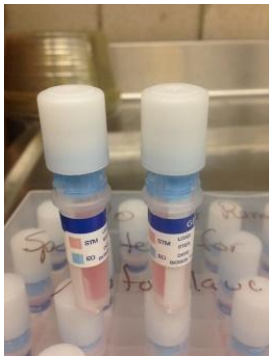
Autoclave, Getinge Indicator ampule/vials (61301606636), Heat Block, Camera, Log Book

SAMPLE PREPARATION AND STORAGE

Getinge Indicator ampules/vials are in the lab at [designated location].

PROCEDURE

1. Take out 2 ampules/vials from box located in the drawer in [designated location].



2. Record batch information in the **Log Book** located in the 3 ring binder on the shelf in [designated location].

3. Using an autoclave safe marker, cross out the STM (Steam) box on each vial. Write on one of the ampule/vial labels "Autoclave" and "Control" on the other ampule/vial label. **Write the date** on both vials.

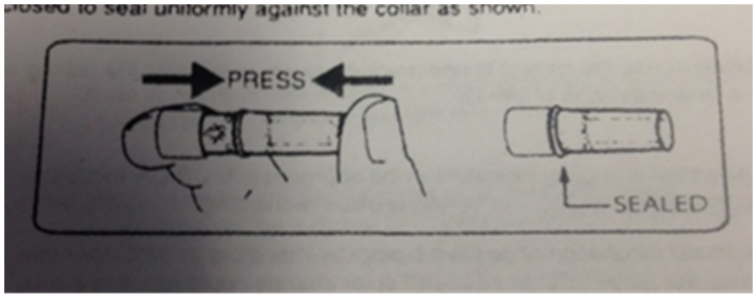
4. Prepare autoclave, following the "Autoclave SOP" in the "Autoclave Log and SOP" binder, using the following settings: **121°C (250°F) for 30 minutes at 15 lbs PSI**

5. Place the "autoclave" ampule/vial in autoclave and the "control" ampule/vial on the table top (control ampule/vial is not to be exposed to autoclave cycle). Run the Autoclave cycle.

6. Let the "autoclave" ampule/vial cool at least 10 minutes, until the ampule/vial cools to room temperature.

7. Examine the chemical integrator strip inside the vials. When the color changes from purple to green, it indicates correct exposure conditions of temperature, time, and steam. Biological spores should be killed under the same conditions.

8. Once the "autoclave" ampule/vial cools to room temperature, activate the media by breaking the ampules inside the vials.



9. Activate both ampules/vials by gently crushing the glass ampule inside of the vial using a pliers. Be careful not to tear open the plastic portion of the vial.

10. Turn on the heat block to **60°C, check with thermometer**.

11. Once the heat block temperature stabilizes, place the activated ampules/vials in a heat block slot for at least **10 hr incubation period** and record the starting time and temperature.

12. At 10 hrs, record the final temperature, color of the fluid and positive/negative growth, for each of the ampules/vials in the **Log Book.** Also take a photo, print photo, and tape into the **Log Book.**
    a. **Turn off Heat block**
    b. **Growth indications**
        i. Positive (Control) growth is indicated by a Yellow Color (control should turn yellow)
        ii. Negative growth is indicated by no color change (autoclave treatment should stay purple)
13. Keep both vials in a cardboard box in the drawer in [designated location].

CLEAN UP

Final disposal of both ampules/vials needs to be done by autoclaving using the following settings: **121°C (250°F) for 30 minutes at 15 lbs PSI**

SUPPLEMENTAL REFERENCES

Bennett RS, O'Neill W, Smith L, Hutmacher RB (2011) Activity of commercial detergents against conidia and chlamydospores of *Fusarium oxysporum* f. sp. *vasinfectum*. 15:8.

Bryan L, Baldwin C, Gray M, Miller D (2009) Efficacy of select disinfectants at inactivating Ranavirus. Dis Aquat Organ 84:89–94.

Gray M, Duffus A, Haman K, Harris R, Allender M, Thompson T, Christman M, Sacredote-Velat A, Sprague L, Williams J, Miller D (2017) Pathogen surveillance in herpetofaunal populations: guidance on study design, sample collection, biosecurity, and intervention strategies. Herpetol Rev 48:334.

Gray MJ, Lewis JP, Nanjappa P, Klocke B, Pasmans F, Martel A, Stephen C, Parra Olea G, Smith SA, Sacerdote-Velat A, Christman MR, Williams JM, Olson DH (2015) *Batrachochytrium salamandrivorans*: The North American response and a call for action. PLoS Pathog 11.

Meechan PJ, Potts J (2020) Biosafety in microbiological and biomedical laboratories. 6th edition. US Department of Health and Human Services, Public Health Service, Centers for Disease Control and Prevention, National Institutes of Health. https://www.cdc.gov/labs/BMBL.html

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Rahman HS, Choi T-J (2018) The efficacy of Virkon-S for the control of saprolegniasis in common carp, Cyprinus carpio L. PeerJ 6:e5706.

Robinson KA, Pereira KE, Bletz MC, Carter ED, Gray MJ, Piovia-Scott J, Romansic JM, Woodhams DC, Fritz-Laylin L (2020) Isolation and maintenance of *Batrachochytrium salamandrivorans* cultures. Dis Aquat Organ 140:1–11.

Rzadkowska M, Allender MC, O'Dell M, Maddox C (2016) Evaluation of common disinfectants effective against *Ophidiomyces ophiodiicola*, the causative agent of snake fungal disease. J Wildl Dis 52:759–762.

Van Rooij P, Martel A, Haesebrouck F, Pasmans F (2015) Amphibian chytridiomycosis: A review with focus on fungus-host interactions. Vet Res 46:1–22.

White-nose Syndrome Disease Management Working Group (2020) National White-Nose Syndrome Decontamination Protocol – October 2020. www.WhiteNoseSyndrome.org

Woodhams DC, Barnhart KL, Bletz MC, Campos AJ, Ganem SJ, Hertz A, LaBumbard BC, Nanjappa P, Tokash-Peters AG (2018) *Batrachochytrium*: biology and management of amphibian chytridiomycosis. eLS:1–18.

PATHOGEN HAZARD NOTIFICATION AND PROTOCOL ACKNOWLEDGEMENT FORM

I, _____ (Print Full Name) have read and understood the "**[Lab name] Biosecurity Practices for Working with *Batrachochytrium salamandrivorans*"** document, and had the opportunity to ask any questions. If working directly with *Batrachochytrium salamandrivorans*, I have also read the following references printed out in [designated location]:

(1) Pages 37-43 on Biosafety Level 2 protocols in Meechan & Potts (2020)
(2) Isolation and maintenance of *Batrachochytrium salamandrivorans* cultures by Robinson et al. (2020)
(3) Standard *Bsal* exposure methods (https://bsalproject.tennessee.edu/methodology/) by UT Knoxville Researchers, and
(4) Field decontamination protocols by Olsen et al. (2021)

and understand the ecological risks of working with *Batrachochytrium salamandrivorans*. I understand the biosecurity procedures that are put in place to reduce exposure and prevent release of pathogens into the environment. I also have asked any questions regarding the risks and protocols within the document. I also know where to find a copy of the "[Lab name] **Biosecurity Practices for Working with *Batrachochytrium salamandrivorans*"** document in case of an exposure/accident which also has information on who to contact if such an issue occurs. This form will be stored in a binder in the [Designated Location] for documentation, and renewed annually.

_____
Date

_____
Signature

_____
Date

_____
Witness