

Non-lethal isolation of the fungal pathogen *Batrachochytrium dendrobatidis* from amphibians

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Bd Isolation from Biopsy Punch in the Field

Materials

- 1) TGhL agar with antibiotics
- 2) Sterile needles
- 3) Hand lens or magnifying glass (optional)
- 4) Sterile forceps
- 5) 70% ethanol
- 6) Lighter for flaming tools
- 7) Ziplock bags (2-gallon)
- 8) Parafilm
- 9) 1.5 or 3mm diameter biopsy punches and rigid, wipeable surfaces for performing punches

Tissue Collection

- 1) Place the frog on the rigid work surface and hold it securely while you spread the webbing of one hind foot
 - a. Choose the punch size that is appropriate for the size of the individual animal
 - b. Select a punch location that is in the middle of the webbing.

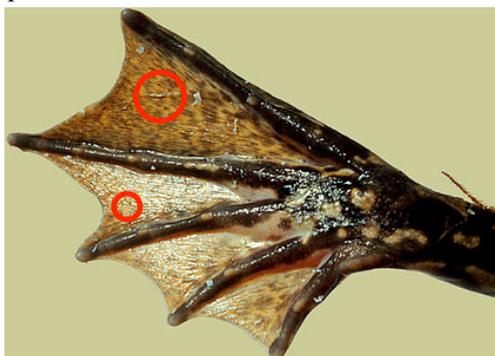


Figure 1: example punch locations

- 2) Position the punch on the webbing and press down firmly, then twist slightly to ensure a complete puncture
 - a. Remove the punch and pick up the skin piece using a sterile needle or forceps (make sure these are completely cool if flame was used for sterilization) and proceed with cleaning

Tissue Cleaning

- 1) Set up in a relatively cool, shaded spot
 - a. As much of the protocol as is practical should be performed inside the 2-gallon bag to minimize exposure of the skin pieces and tools to airborne microbes, especially fungal spores
 - b. Skin pieces and agar plates should be kept as cool as possible
- 2) Have one agar plate for cleaning and a separate (labeled) plate for holding the cleaned piece
 - a. TGhL agar: 1000 mL H₂O, 16 g Tryptone, 2 g Gelatin hydrolysate, 4 g Lactose, 10 g agar with antibiotics (300 mg/L Penicillin-Streptomycin)
- 3) Place the skin piece on a fresh section of the cleaning plate and use the needle to wipe the skin piece through the agar in order to clean off bacteria and fungus that are on the surface of the skin
 - a. Skin should be wiped at least three times on each side, and the needle should be wiped through the agar periodically to keep it clean
 - b. The same cleaning plate can be used for multiple skin pieces from different frogs as long as care is taken to use a separate section of the plate for each piece. Ensure you are not re-using sections of the cleaning plate to prevent accumulation of contaminants.
 - c. Be sure to keep the plates covered as much as possible to prevent exposure to environmental microbes

Tissue Culture

- 1) Once the skin is cleaned, place it on a fresh section of a holding plate with TGhL agar
 - a. The same holding plate can be used for multiple skin pieces from different frogs as long as care is taken to use a separate section of the plate for each piece
- 2) Once the holding plate is filled, seal it with parafilm and place in an insulated container with cold pack (if available)
- 3) To inhibit microbial growth, skin pieces should be kept cool at all times, but not frozen (ideal temperature is around 4°C)
 - a. If multiple days will be spent at a backcountry site, tissue samples should be collected as close as possible to the last day to keep the pieces as fresh as possible
 - b. For hike-in sites, chemical cold packs can be carried and activated on the day that toe punches are collected
 - c. If available, a soft foam cooler can be used to store the plates with the cold packs while hiking
- 4) Upon returning to the lab, incubate plates at 20–23°C
 - a. May need to “re-clean” pieces if there is extensive fungal overgrowth due to incomplete field cleaning
 - b. Bd growth may not occur for up to 4 weeks

LITERATURE CITED

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