Document available from http://mountainlakesresearch.com/protocols/

Collection and Analysis of Amphibian Skin Swabs for qPCR Analysis of Bd Load

Roland Knapp and Alexa Lindauer, Sierra Nevada Aquatic Research Laboratory – 05 November 2020
Email: info@mountainlakesresearch.com,
alexalindauer@gmail.com | alindauer@ucsb.edu

Purpose. The purpose of collecting skin swabs from amphibians is to provide information on *Batrachochytrium dendrobatidis* (Bd) infection intensity (i.e., Bd "load").

Sample Collection

Reduce swabbing-induced disease spread. To avoid moving Bd from one animal to another, catch one animal, conduct the swabbing and related data collection, and release the animal. Do not hold multiple animals together (e.g., in a net) prior to swabbing. Prior to capturing each animal, clean your net and hands (e.g., sweep the net vigorously through the water, scrub hands in water) to remove as much Bd from previously-captured frogs as possible.

Swabbing. To collect a skin swab sample, remove a sterile swab from its packaging and swipe the animal 30 times. It is essential that the swabbed animal be wet (not just damp) when swabbed as this allows the swab to pick up skin cells. Therefore, dip the animal in water (from the collection site) immediately before swabbing. This also serves to clean the animal of any detritus and other extraneous materials. Such contaminants can impair the PCR reaction and cause spurious results. If water is not available at the site, bring sufficient sterile water with you and rinse the animal using a squirt bottle.

> **Post-metamorphic life stages.** On post-metamorphic frogs and toads, swipe the left and right sides of the abdomen five times each, left and right thighs five times each, and foot webbing of the left and right rear feet five times each.

> **Tadpoles** For tadpoles of Gosner stage <42 (no front legs), make all 30 swipes on the mouthparts. For tadpoles of Gosner stage ≥42 (front legs present), swipe the mouthparts 10 times, left and right sides of the abdomen five times each, and left and right thighs five times each.

Drying swabs. After making 30 swipes, allow the swab to dry (at least 30 minutes on a dry, warm, breezy day; at least 1 hour on a cloudy day or if late in the day) before putting it into a swab vial. To dry swabs, push the swab stick (not the rayon swab tip) into the ground and through the loop of the tether that connects the vial to the cap. This will ensure that each swab is associated with the correct vial (and swab id). When swabs are dry, insert the swab ~3/4 of the way into a vial, break off the swab stick against the rim of the vial (using your finger or swab cap to ensure that the swab stays in the vial), and close vial cap securely. Swabs need to be completely dry before they are sealed into a vial; damp swabs will become moldy which can compromise PCR results. If swabs are being collected from multiple widely-separated locations, it is helpful to have a headband that allows swabs to be transported securely with their associated vial (contact us for details).

Sample organization. Swab vials submitted to our laboratory must be labeled with a pre-printed swab_id, as described below under "Materials". Use swab vials in order of their swab_id (see labeling information below), and place them in freezer tube boxes in numerical order. Because swabs are analyzed in numerical order, shipping swabs in numerical order makes lab processing more efficient. Efficient lab processing is essential for us to keep the costs of swab analysis low. If this protocol is not adhered to, costs for swab analysis will increase accordingly.

Transport. During swab collection and subsequent transport, keep collected swab samples cool. Hot temperatures will degrade DNA. When in the field, putting the samples into a resealable plastic bag and out of the sun will usually suffice. If working in hot conditions, putting the samples in a small cooler with icepacks may be necessary. **After arranging the shipment with us**, ship samples in a small cooler containing ice packs using FedEx or UPS overnight delivery. Do not ship samples on a Thursday or Friday. Doing so may result in the samples sitting in a hot location over the weekend. Also, at the time of shipment please provide us with the tracking number. Ship samples to:

Molecular Laboratory, Sierra Nevada Aquatic Research Laboratory
1016 Mount Morrison Road
Mammoth Lakes, CA 93546

When you ship your samples to us, please also email Alexa a spreadsheet cataloging your samples and their swab_ids. A piece of paper in the shipping cooler that notes the swab_id range(s) is helpful but not required.

Materials. Please use 1.5 mL round-bottom vials with a tethered cap. A round bottom is necessary for the DNA extraction process due to the configuration of the heat plates used. A tethered cap improves efficiency in the DNA extraction process and reduces the chance of sample mix-up (i.e. placing the wrong cap on the wrong vial during DNA extraction). All vials must be labeled with a swab_id that is unique both within and among all research groups and qPCR clients. Please contact Alexa Lindauer (alindauer@ucsb.edu) before printing swab_id labels to ensure the use of unique ids for every sample. If you are purchasing your own vial labels, be sure to buy labels with adhesive that can withstand -80 °C, the temperature at which your processed DNA extracts will be stored. For shipping, please use a cooler and freezer boxes to numerically order your samples, as explained above. Cardboard freezer boxes with 81-cell dividers are inexpensive and reusable. We are happy to provide vials, labels printed with swab_ids, freezer boxes, and foam shipping coolers at cost if given ample time to process this request (2+ weeks). If you will be collecting a relatively small number of swabs, this may be cheaper for you than purchasing supplies through your organization.

From left to right: carboard freezer box with 81-cell divider; foam shipping cooler; 1.5 mL round bottom tether cap tube.

Supplies List:

- Gloves (non-powdered, sterile, disposable – optional)
- Swabs (only use Medical Wire, MW113; available from Advantage Bundling)
- Swab vials (use Fisher Scientific 1.5 mL microcentrifuge tubes, cat.no. 02-707-353; do not be confused by the skirted tube in the photo) – must be labeled with pre-printed Tough Spot (e.x.: USA Scientific: 9185-1000).
- Sterile water (if no water is available on site)
- Squirt bottle (for sterile water)
- Swab headband (optional – contact alindauer@ucsb.edu for details)
- Resealable plastic bags (separate bags for sample vials and trash)
- 81-place tube box (for convenient storage of collected samples – optional. e.x.: Fisher Scientific 13-994-070)

qPCR

qPCR analysis. Skin swab samples will be analyzed using standard qPCR methods as described in Boyle et al. (2004). Unless requested otherwise, samples will be run singly instead of in triplicate (see Kriger et al. 2006, for details). Every precaution will be taken to ensure that samples are processed using established protocols and that assay results are accurate. However, we are not liable for lost samples or for results that are inaccurate for reasons that we were not aware of, including improper sample collection or problems encountered during the qPCR analysis. When testing is complete, results will be shared with the client via a Google Doc file, and clients are invoiced for the analyses conducted. Invoices can be paid using a credit card, and payment details are provided with the invoice. Current per-sample cost is $7.00 and $10.96 for UC-based and non-UC clients, respectively.

Post-analysis sample disposition. Following analysis, DNA extracts are either discarded or returned to the client. Prior to shipping samples, please contact us to discuss which of these options you would like us to use. Due to limited storage space in our ultracold freezer, long term storage of DNA extracts will likely only be possible for samples of particular importance (e.g., those from previously unsampled populations).

Interpreting qPCR data

Units for Bd load. The Bd qPCR assay targets a portion of the ITS1 gene for amplification. As such, results ("bd_load") are expressed as the number of copies of the ITS1 gene per swab. In mountain yellow-legged frogs, values >600,000 ITS1 copies are typically associated with severe chytridiomycosis and increased frog mortality.

You have likely seen other units reported, such as zoospore equivalents (ZE). Bd load units, such as zoospore equivalents versus ITS1 copies, depend on which qPCR standard is used. When standards for quantifying Bd load are created using zoospores (by extracting DNA from an estimated number of zoospores), the resulting Bd load data are typically expressed as "zoospore equivalents". A more modern qPCR standard uses a piece of synthetic DNA that is a copy of a portion of the Bd ITS1 gene region. In this case, the units for resulting Bd load data are "ITS1 copies". This is the approach we use in our

molecular diagnostics lab because this standard is less subject to degradation over time and produces more consistent results.

The threshold load above which severe chytridiomycosis is evident in MYL frogs is 10,000 zoospore equivalents (based on analyses in Vredenburg et al. 2010). However, different Bd strains have zoospores with different numbers of copies of the ITS gene (Longo et al. 2013), and as a result it generally isn't possible to express these load data as number of ITS gene copies. To address this problem within our Sierra Nevada system, we ran a few hundred swab samples collected from mountain yellow-legged frogs using both types of standards. The results indicated that Sierran Bd strains have approximately 60 ITS copies per zoospore. That allowed us to generalize the 10,000 ZE threshold to results produced by the new standard, i.e. 600,000 ITS copies. It remains unknown if this conversion holds for populations of mountain yellow-legged frogs outside of our primary study area, or for other amphibian species.

High Cq values. The threshold cycle or cycle quantification value (Cq) reflects the number of PCR cycles it took to detect a signal from your sample. A lower Cq value means more target Bd DNA extracted from your swab. Conversely, a higher Cq values means lower amounts of target Bd DNA from your sample. Samples with Cq values greater than 38 likely indicate a **very** small quantity of Bd DNA. For example, Hyatt et al. (2007) suggest that a qPCR reaction takes 38 or more cycles (Cq > 38) to detect DNA from the equivalent of 0.1 Bd zoospores. High Cq values may also be false positives from contamination (in the field or in the lab), although this is less likely. As such, these very small quantities of Bd DNA should be interpreted with caution.

References

Boyle, D. G., D. B. Boyle, V. Olsen, J. A. T. Morgan, and A. D. Hyatt. 2004. Rapid quantitative detection of chytridiomycosis (Batrachochytrium dendrobatidis) in amphibian samples using real-time Taqman PCR assay. Diseases of Aquatic Organisms 60:141–148.

Hyatt, A. D., D. G. Boyle, V. Olsen, L. Berger, D. Obendorf, A. Dalton, K. Kriger, M. Hero, H. Hines, R. Phillott, and R. Campbell. 2007. Diagnostic assays and sampling protocols for the detection of Batrachochytrium dendrobatidis. Diseases of aquatic organisms, 73:3:175-192.

Kriger, K. M., J.-M. Hero, and K. J. Ashton. 2006. Cost efficiency in the detection of chytridiomycosis using PCR assay. Diseases of Aquatic Organisms 71: 149–154.

Longo, A. V., D. Rodriguez, D. da Silva Leite, L. F. Toledo, C. M. Almeralla, P. A. Burrowes, and K. R. Zamudio. 2013. ITS1 copy number varies among Batrachochytrium dendrobatidis strains: implications for qPCR estimates of infection intensity from field-collected amphibian skin swabs. PLoS One, 8:3:e59499.

Vredenburg, V. T., R. A. Knapp, T. S. Tunstall, and C. J. Briggs. 2010. Dynamics of an emerging disease drive large-scale amphibian population extinctions. Proceedings of the National Academy of Sciences, 107:21:9689-9694.