

**Amphibian Monitoring Protocol for Klondike Gold Rush National Historical Park**  
**Standard Operating Procedure (SOP) No. 4**  
**Collecting Samples for *Batrachochytrium dendrobatidis* (BD) testing**  
**Version 1.0 (April 2009)**

**Revision History Log**

| Previous Version # | Revision Date | Author | Changes Made | Reasons for Change | New Version # |
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#### **4.1 Chytrid fungus in Klondike Gold Rush National Historical Park**

The fungus *Batrachochytrium dendrobatidis* (BD), also called chytrid fungus, is a disease agent responsible for chytridiomycosis in amphibians. The disease was originally described in 1998 from observations of dead, dying, and deformed frogs and is one of the main factors attributed to the loss of amphibian biodiversity worldwide (Adams 2007).

Although thought to have been spread throughout the world in just the last century, its extent and current distribution are unclear (Weldon 2004). Dispersal of the fungus is assumed to be through infected frogs, contaminated water, or an unknown host (Morgan 2007). Amphibians exposed to the fungus may die soon after their skin is infected and can quickly spread the fungus through an area, causing a rapid collapse of the entire population at the site. The chytrid fungus colonizes keratinized skin cells in individual adult amphibians. Often the fungus is concentrated in patches on the ventral surface near the groin and on the webbing between the toes of the hind legs (Poulter 2009), but few observable symptoms are exhibited in the individual until the final stages of infection and near death. Symptoms may include deformities and anomalies in tadpole mouthparts (Altig 2006).

Chytridiomycosis in amphibians can be diagnosed through several methods. Histological examination of skin tissue through a microscope is a widely used technique for detecting BD in skin scrapes and toe clips from amphibians (Weldon 2006). Recently-developed quantitative (real-time) polymerase chain reaction (PCR) assays provide a more sensitive and specific test to detect the presence of BD zoospores (Olsen 2004). Studies have shown that the PCR assay can be almost twice as likely to detect BD as histology and is sensitive to a single zoospore (Kriger 2006). However, presence of BD zoospores does not equate to a diagnosis of infection or chytridiomycosis (Smith 2007). Samples collected in KLGO are analyzed by Pisces Molecular LLC using PCR assays. Results from the data are used to monitor the presence of chytrid fungus in KLGO western toad populations.

Sampling for chytrid fungus in 2005 confirmed its presence in western toads (*Bufo boreas*) in Klondike Gold Rush National Historical Park (KLGO) (Adams 2007). Samples were collected again in 2006 and 2007 to better understand the presence and distribution of the fungus in KLGO western toad populations.

#### **4.2 Collection Objectives**

The objectives of collecting samples to monitor for the presence of *Batrachochytrium dendrobatidis* in KLGO are:

Monitor the presence and spread of *Batrachochytrium dendrobatidis* (BD) infection (chytridiomycosis) in KLGO western toad populations.

Detect changes in the distribution of chytrid fungus in infected breeding sites (core sites) in KLGO.

Contribute to chytrid studies that are studying the relationships between geographic distribution, climate gradients, population genetics, and human disturbance in relation to population declines of native amphibians in Southeast Alaska.

#### **4.3 Collection Frequency**

Sample collection and testing of western toads is conducted every other year, in odd-numbered years. Recaptured individuals that have PIT tags are opportunistically tested every year.

#### **4.4 Equipment Preparation**

Skin swab collection kits are ordered from Pisces Molecular LLC and consist of 2 ml vials of 70% ethanol and cotton swabs. Sampling kits are stored in the Resources office mini-fridge in the Mascot building if any are left from previous seasons.

Pisces Molecular LLC  
2200 Central Avenue, Suite F  
Boulder, CO 80301  
303-546-9033  
303-546-9400 fax  
jwood@pisces-molecular.com

#### **4.5 Collection Procedures**

The following field equipment is required for collecting samples:

Skin swab collection kits  
Disposable gloves, many pairs  
Plastic containers and bags, to hold individuals while sampling  
Labels or laboratory tape  
Alcohol-resistant permanent marking pen  
Plastic storage box, to transport samples

##### **4.5.1 Obtaining Skin Swab Samples**

Obtain the sample before doing other procedures with the animal (e.g. weighing, checking PIT tags, and measuring)

Using fresh gloves hold the animal in one hand. Gently but firmly swab, with the cotton swab, the ventral surface 25 times. For large animals, scrape the ventral surface 20 times and the feet and webbing 5 times. Place the swab, cotton side down, in a vial. Secure the lid and place in a rack or other container so that the tube remains upright.

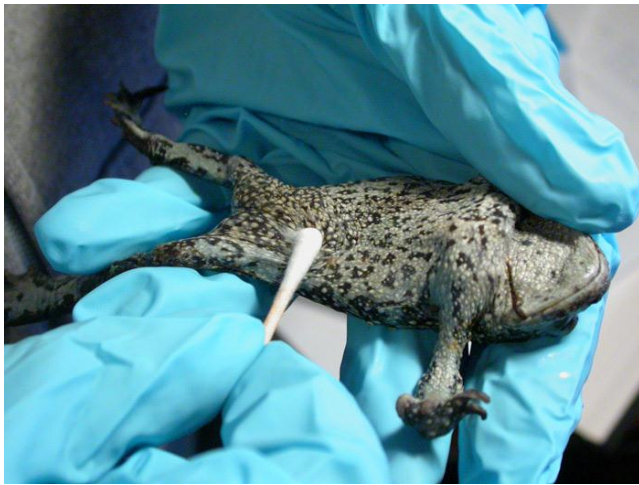


Figure 4.1. Swabbing the ventral surface of the amphibian

Label each sample vial with the site name (e.g. DY03), date (DDMMYY), 4-letter species code (e.g. BUBO for *Bufo boreas*), 1 letter describing lifestage ('A' for adult, 'J' for juvenile, 'M' for metamorph, 'L' for larva), followed by a number identifying the individual (A1 for adult number one). The full label for an adult western toad captured at Dyea site 3 on June 5, 2007 would read: DY0305JUN07A1.



Figure 2.2. Insert swab into tube with the sample at the bottom.

Use an alcohol-resistant permanent marking pen and store samples in plastic bags by groups to minimize damage to data interpretation if a sample should leak and cause contamination of other samples. Samples from the same geographic location should be grouped in the same bag. After collecting a sample, vials should remain upright to prevent leakage. Leakage from one tube with BD may get on other tubes and result in contamination of samples.



Figure 4.3. Example of label on tube.

Do not place sample information inside the tube. It can be difficult to extricate, may contaminate other samples through handling, and as paper may contain bleaching agents, may inhibit detection of the target DNA.

#### **Collecting Samples for Chytrid fungus testing**

1. Obtain the sample before doing other procedures with the animal
2. Using fresh gloves, hold the animal in one hand
3. Gently but firmly swab, with the cotton swab, the ventral surface 25 times
4. For large individuals, swab the ventral surface 20 times and the feet and webbing 5 times
5. Place the swab, cotton side down, in a vial
6. Secure the lid and place in a container so tube remains upright
7. Label each sample vial with the site number, date, and individual ID (eg. DY03Jul2J1)

Figure 4.4 Field instructions for collecting Chytrid fungus samples

#### **4.5.2 Obtaining Skin Tissue Samples**

Other skin tissues such as samples of ventral skin from dead animals may also be collected for PCR testing.

##### Equipment Requirements

Additional equipment is required to obtain other skin tissue samples:

Small, fine, metal scissors  
Alcohol wipes

##### **4.5.2.1 Procedures for obtaining other skin tissue samples**

Following hygiene procedures as for other sample collection, use fine scissors to obtain the tissue and place each sample in a 2ml vial containing 70% alcohol.

#### **4.6 Equipment cleaning and hygiene procedures (Livo 2005)**

Animals should be collected with clean, decontaminated equipment, individually handled with fresh disposable gloves, and placed in individual containers prior to obtaining the samples. Do not place multiple animals in the same container prior to sampling. In this situation, a single infected animal could infect others, and PCR tests could have inflated numbers of positive test results. See SOP No. 3: Hygiene Protocol for Control of Disease Transmission between Amphibian Study Sites.

Although using Isopropyl alcohol to clean equipment and hands will prevent the spread of live chytrid fungus between individuals, it will not prevent contamination of the lab samples with chytrid fungus DNA. Small, metal utensils, such as scissors, may be wiped with isopropyl alcohol and passed through a flame to destroy residual DNA. This procedure must be done between each individual.

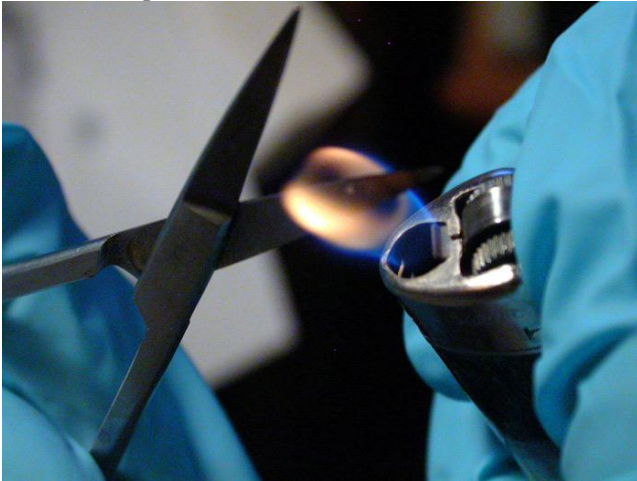


Figure 4.4 Passing scissor blades through flame to destroy residual DNA.

Equipment to be used on multiple individuals can be cleaned and bleached for reuse, but the equipment must be rinsed well to remove any residual bleach and be allowed to dry completely prior to reuse. Even parts per million of bleach in, on, or around a sample could possibly destroy all the chytrid fungus DNA in a sample over the course of a few weeks. Designate a plastic bag for the disposal of gloves and other materials (for example, alcohol wipes) to minimize the possibility of contamination.

Sample collection and preservation is only the first step in collecting accurate and useful data. Preventing contamination is the next step. Because of its extraordinary signal amplification, the lab test for BD is very sensitive to contamination. The BD assay has a demonstrated sensitivity of less than 0.1 zoospore. Therefore, all sample collection and subsequent handling procedures should be done to minimize contamination risks.

#### **4.7 Transporting samples for lab analysis**

Samples are shipped in a sturdy box or mailing tube, but never in a soft-sided envelope, and are returned to Pisces Molecular for analysis:

Pisces Molecular  
2200 Central Avenue  
Suite F  
Boulder, CO 80301

Another possibility for sample analysis is the lab at Cornell University's Department of Ecological and Evolutionary Biology. Arrangements need to be made with lab staff there before sending samples:

Angela Stevenson, or current lab manager  
Cornell University Dept.of Biology  
E145 Corson Hall  
Ithaca, NY 14853

## 1.8 References

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- Smith, K. G. (2007). "Use of quantitative PCR assay for amphibian chytrid detection: comment on Kriger et al. (2006a,b)." *Diseases of Aquatic Organisms* 73: 253-255.
- Weldon, C. and L. H. Du Preez (2006). "Quantitative measurement of *Batrachochytrium dendrobatidis* in amphibian skin." *Diseases of Aquatic Organisms* 72: 153-161.
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## Appendix A. Chytrid Sampling Field Reference Sheet

### Collecting Samples for Chytrid fungus testing

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