Tissue Collection Protocol for Genetic Research

K.E. Leyse, A.J. Lind, W.K. Savage, H.B. Shaffer, and M.R. Stephens*
University of California, Davis
June 2003

*Corresponding author contact information:
Amy Lind
Section of Evolution and Ecology - Storer Hall
One Shields Avenue
University of California, Davis 95616
ajlind@ucdavis.edu
(530)-752-1112, (530)-759-1702

This document provides recommended standards of field and laboratory methods for collection of tissue to be used in genetics research. It was developed from the collective experience of the authors as well as existing literature on the subject. It does not address the equally important issue of sampling design (numbers of individuals, distribution and numbers of localities, etc.). Sampling designs and analysis strategies should be clearly defined prior to field collecting to reduce impacts to animal populations.

GENERAL INFORMATION

Planning Ahead

Be sure you have thought through your sample collection logistics ahead of time. It is important to visit sites known to have infected amphibians last, if possible, to reduce the likelihood of inadvertently spreading disease between sites.

Prepare ahead of time. Gather all of the necessary collection and disinfection equipment, so that you don't have to resort to lower standards of collection once you are in the field.

Be aware of permit restrictions (State, Federal, National Park, etc.), particularly the localities and number of individuals per locality that you are allowed to collect. Use discretion when collecting, particularly for sensitive/declining species.

Consider the ultimate use(s) of the tissue as that may determine field collecting procedures. If the tissue is for genetic work only, preservation in ethanol or immediate freezing is appropriate. However, if work is to be done on disease (e.g. histology studies), freezing will make samples unusable, and storage in lower % ethanol is recommended. Contact the appropriate researcher to determine the proper collection protocol when collecting for disease study or other purposes.

Field Collecting

Collecting whole larvae is preferable if they are available and abundant. This insures there will be enough tissue for genetic work as well as providing voucher specimens for localities and opportunities for assessment of disease status.

Toe samples are typically taken for juvenile/adult frogs and either toe or tail samples can be taken for large larval or juvenile/adult salamanders.

Prepare several microtubes before going into the field by partially filling them with 95% ethanol (may use ≥70% ethanol, if necessary).

Store all chemicals (e.g. ethanol, euthanasia solutions, hydrogen peroxide, etc.) in a cool, dark place to maintain highest quality. Avoid prolonged exposure to heat and light. For example, when traveling by car in the summer, place chemicals in a cooler. If backpacking, pack chemicals in the middle of the pack rather than on the top.

Start with clean implements when collecting tissues. Clean scissors or scalpels with hydrogen peroxide and dry them with a clean paper towel or Kimwipe prior to going into the field. Clean plastic holding jars/bottles/tupperware with between localities to avoid disease transmission.

Remember that ethanol is a DNA preservative and hydrogen peroxide breaks down DNA, so be sure to use these chemicals appropriately.

Reducing Transmission of Disease

Cleaning of all field equipment between field sites is strongly recommended to prevent disease transmission, but is not addressed in detail here. Main issues to consider are:

* Minimizing cross-contamination of localities.
* Minimizing contamination among individuals at a locality.

For more information, see:

New South Wales (NSW) National Parks and Wildlife Service. 2001. "Hygiene protocol for the control of disease in frogs" on the web at:
http://www.npws.nsw.gov.au/wildlife/licence/hyprfrog.pdf

Speare et. al. 1998. "How to reduce the risks of you transmitting an infectious agent between frogs and between sites" on the web at:
http://www.jcu.edu.au/school/phtm/PHTM/frogs/prevent.htm

TOE AND TAIL SAMPLES

When taking toe clips, cut slowly (but firmly), avoiding cutting at the tip of the scissors because toes have a tendency to jettison, and a cleaner cut is obtained further inward on the scissor blades. Use sharp scissors (medical/scientific grade) to make a clean cut (Figure 1).

Cut at least a 3-4 mm clip of toe or tail, but 5-10 mm is better. With tail clips, include some muscle tissue as well as tail fin. If the animal is small (e.g. a juvenile frog), two toe clips should be done, one on a front foot and one on a rear foot. Avoid clipping toes that are important for reproductive or other functions for the study species (e.g. thumbs of most male frogs and outer toes on species that dig burrows).

Use tweezers or forceps to place samples from each individual into one microtube (one individual per tube) and top off the tube with 95% ethanol. Store them in a cooler, refrigerator or freezer for a minimum of the first 24 hours after collection. If possible, samples should be stored at –20 ^{o}C, but otherwise a refrigerator will suffice for the short term.

After each toe/tail is clipped, clean scissors, tweezers/forceps, and all other implements with hydrogen peroxide. This is best accomplished by pouring hydrogen peroxide into a sturdy cup and soaking implements for 10 seconds or so between individuals. Make sure to change the peroxide frequently, as it loses its potency with repeated uses. Rinse implements well with water and wipe equipment dry with a paper towel or Kimwipe before processing the next individual. Because hydrogen peroxide breaks down DNA, it is important that there is no peroxide on the scissors or forceps. Be aware that sometimes tissue clings to cutting implements. Material from one individual must not be contaminated with tissues from another individual (or with blood or skin of the investigator).

WHOLE LARVAE OR SMALL METAMORPHIC AMPHIBIANS

Animals should be euthanized in a large jar filled with a liter or so of either chlorotone or benzocane solutions, prior to storage in ethanol. This can either be done in the field or in the lab/office setting depending on conditions. If live animals are transported to the lab/office for processing, put them in large plastic bottles or *Ziploc* bags in a cooler to reduce stress. Provide water for larvae and moisture (wet paper towel) for metamorphs.

Preparing chlorotone solution - prepare a saturated solution of chlorotone by incrementally dissolving the powdered chemical in a liter of water until particles will no longer dissolve.

Preparing benzocane solution – dissolve 1g of ethyl p-amino benzoate (benzocaine) in 5 ml of ETOH and then mix that into 1 liter of water in a larger jar. This is 3x the concentration used for anesthetizing amphibians and should work for euthanization.

For either chlorotone or benzocane, the same solution can be saved in the jar and used multiple times so it will not need to be disposed of in the field. Following the field season, these solutions should be disposed of properly in the laboratory.

Euthanizing - Place animals to be euthanized in the jar and leave them there for a duration of at least 5 minutes <u>after</u> they have stopped moving.

Rinse the animals in clean water, place one individual in each microtube, and top off the tube with at 95% ethanol. If tadpoles are too large for the microtube, their gut may be removed using

a scalpel prior to placing them in the tube. The optimal ratio of ethanol:tissue is 10:1; don't go below 2:1. Store tubes in a cooler, refrigerator or freezer for a minimum of the first 24 hours after collection. If possible, samples should be stored at -20 ^{o}C, but otherwise a refrigerator will suffice for the short term.

ROAD KILLS

Road killed amphibians and reptiles can be used for genetic samples if decomposition has not set in. Collect the entire animal and store it in *Ziploc* bag or other plastic container in a standard freezer. These specimens can either be shipped whole on dry ice or be "tissued" and the resulting samples sent to the interested genetics lab. Tissuing requires dissection of the animal to remove muscle and/or organ material. This tissue is then placed in microtubes and labeled as was described for toe and tail clipping methods described above.

DATA COLLECTION AND TEMPORARY STORAGE OF SAMPLES

Give each individual (toe clip, tail tip, or whole larvae) a field number that uniquely identifies that individual (preferably with field site name/number and an individual sample number – e.g. ltb-a-23). Write that field number on the outside of the plastic microtube and cap, usually before the tissue is added. Extra fine tip *Sharpie* markers work best for labeling vials. Optionally, an additional label (marked with pencil or India ink; eg. *Micron* archival ink pens) can be placed inside the vial. Note that *Sharpie*'s may not be ethanol-proof, so be sure to dry the microtubes thoroughly prior to marking them and avoid spilling ethanol on the tubes once they are marked. For each collection site, put all individual microtubes in a *Ziplock* bag with some locality label inside the bag and store in cooler, refrigerator, or freezer.

<u>Create a spreadsheet/database with at least the following information</u>:
- Collection date
- Species
- Type of tissue – toe or tail clip or whole larvae/metamorph
- Field site name, code, and/or number
- Individual sample number
- Locality information, including lat/longs or UTM's from a map or GPS system as well as a narrative description (e.g. 4 miles north along Forest Service Rd. 2N34, from Calif. highway 88, at south end of Tadpole Lake, El Dorado National Forest, Alpine Co., California).
- Name, affiliation, and contact information for field collectors.
- Gender, if identifiable.
- Notes on unusual deformities, possible infections, or unusual behavior (eg. adult appears to have sloughing skin, is unresponsive to being touched - doesn't blink or try to turn away).

See Figure 2. for an example spreadsheet.

VOUCHERING

Vouchering is documenting the species' identity and locality for the genetics lab (or museum) that will ultimately house the specimen. There are three situations that require somewhat different approaches: road kills or otherwise salvaged animals, collection of whole live animals, and clipping and releasing of animals.

For road kills or other-wise salvaged animals, the entire specimen should be saved and sent as a voucher. This means freezing or fixing the specimen in ethanol (less desirable, but it works) or in formalin (much better, but requires more chemical training/permits), or taking a sample of tissue and storing it in 95% ethanol, freezing the rest of the specimen, and sending both the tissue and frozen specimen.

For live animals that are collected whole and euthanized, if they are small enough they can be put directly into a microtube and covered with ethanol. They can also be euthanzied and frozen whole or placed in ethanol in a larger container and shipped either on dry ice (frozen specimens) or in a sealed plastic jar (ethanol specimens).

For live animals that are being clipped and released, the standard is to identify them in the field, clip, and release, resulting in no animal voucher. The most reasonable way to voucher these animals is with photographs that include both dorsal and ventral views and some sort of size reference (ruler or other another commonly know object like a coin). A rule of thumb is that the harder an animal is to identify, the more careful the vouchering should be.

SHIPPING

Whenever shipping of tissue samples is planned, be sure to contact the genetics lab (or museum) prior to shipping so that someone will be prepared to receive the samples. In the case of frozen tissue and shipping on dry ice, shipping should be overnight/next day only to ensure that tissue remains frozen.

Shipping early in the week is strongly advised, so that samples do not sit in a warehouse or mailroom over the weekend.

US Postal Service *(General Info: 800-275-8777)*
Check with your local post office for requirements prior to shipping. U.S. Postal Regulations prohibit the shipping of ethanol through the mail, but see U.S. D.O.T. 49 for details.

Federal Express *(General Info: 800-463-3339; Hazardous Materials: 901-344-3000)*

Ethanol and Other Chemicals
Small quantities of a chemical (e.g. ethanol) can be shipped via air express service if packaging conforms to certain standards and a statement is made on the outside of the package. The following packaging is required:
- inner packaging (e.g. microtubes) must have less than 1 oz =30ml of chemical per container.

- total package weight cannot exceed 64 lbs.
- inner packaging must be taped or wired closed.
- the package must contain cushioning material.
- the package must be able to withstand a 6 ft. drop with no damage to the contents.

The following statement must then appear on the outside of the package: "This package conforms to the conditions and limitations specified in 49CFR173.4." Also check the box(s) on the FedEx form that say: "yes, dangerous goods", "shipper's declaration not required".

Dry Ice
FedEx requires two forms be completed for shipping on dry ice. On the FedEx air bill (shipping label) the "dry ice section" must be filled out. In addition, a "dry ice" label must filled out and placed on the outside of the box. These labels, which cover all " U.S. D.O.T. Class IX Miscellaneous Hazards", can be obtained at a FedEx shipping center or can be ordered with a valid FedEx account number.

United Parcel Service *(General Info: 800-742-5877; Hazardous Materials Support Center: 800-554-9964)*
Refer to the Material Safety Data Sheet (MSDS) for chemical in question and check the transportation or D.O.T. section. If this section says that the chemical can be considered non-hazardous for shipping than it can be packaged and sent via regular UPS.

EQUIPMENT

Chemicals and Chemical Storage
- 95% ethanol* (may use ≥70%, if necessary) DO NOT SUBSTITUTE ANY OTHER TYPE OF ALCOHOL!
- chlorotone or benzocane solution
- hydrogen peroxide (drug store grade – 3%), replenish at least every 6 months, as it degrades once the bottle is opened.
- filtered water
- various plastic jars/bottles with leak-proof lids - for transporting chemicals into the field.

* Ethanol is highly flammable; do not place near an open flame. High vapor concentrations or consumption can cause narcotic effects, and methylated ethanol poses serious health risks when ingested. Do not breathe vapors or consume.

Capture/Holding Equipment
- Dip nets or D-nets
- Leak-proof plastic jars/bottles or tupperware – one for each locality (pond or stream) visited in a day.
- *Ziploc* bags – small and large.

Toe/Tail Clipping Implements and Tissue Storage
- sharp (medical or scientific grade) scissors
- scalpel
- tweezers or forceps
- 2 ml microtubes, screw cap with o-ring – one for each individual sampled.
- eye dropper or squeeze bottle - for filling microtubes with ethanol.
- small dip-net (clean, no field use) - for removing larvae from euthanasia jar.
- small tupperware container - for rinsing larvae in water after euthanasia.

Miscellaneous
- Fine point *Sharpie* markers
- *Micron* archival ink pens
- Pencils
- Small linen labels to put inside microtubes.

Figure 1. Example of toe clipping technique.

	A	B	C	D	E
1	ORIG ID	DATE	SPCODE	LOC	LOCATION
2	14824	5/5/1992	RABO	1	In the Eel River. Rich.…
3	14825	5/5/1992	RABO	1	In the Eel River. Rich.…
4	14826	5/5/1992	RABO	1	In the Eel River. Rich.…

F	G	H	I	J	K
LAT - dd	LONG -dd	COUNTY	STATE	SOURCE	TISSINFO
38.999	-123.690	Humboldt	California	Shaffer Cat	Tad, frozen whole
38.999	-123.690	Humboldt	California	Shaffer Cat	Tad, frozen whole
38.999	-123.690	Humboldt	California	Shaffer Cat	Tad, frozen whole

Figure 2. Example spreadsheet for locality and tissue sample tracking.

REFERENCES

NSW National Parks and Wildlife Service. 2001. Hygiene protocol for the control of disease in frogs. Information Circular Number 6. NSW NPWS, Hurstville NSW, Australia.

Speare, R., L. Berger, and H. Hines. 1998. How to reduce the risks of you transmitting an infectious agent between frogs and between sites. School of Public Health and Tropical Medicine, James Cook Universtiy, Townsville, and Department of Environment, Moggill, Queensland, Australia.