USFWS RECOMMENDED DNA SAMPLING METHODS FOR BAT SPECIES IDENTIFICATION

The information herein provides researchers with options and methods for collecting DNA samples from live bats for which visual species identification may be unreliable and a more definitive method is necessary. These recommendations are intended to mitigate impacts to individual bats, especially federally listed *Myotis septentrionalis*, and to ensure proper sample collection so that handling facilitates receive viable DNA samples for laboratory analysis.

Situations that may warrant the need for species confirmation via DNA include in-cave surveys and presence/probable absence mist-net surveys where an individual bat cannot be positively identified using visual inspection and morphometrics alone. Ear length measurements, combined with other physical traits such as tragus shape, are sometimes enough to confidently assign a species identification, yet biologists have encountered scenarios where physical characteristics overlap among species and a positive identification could not be confidently assigned in the field via visual methods.

The decision of whether DNA collection is necessary is at the discretion of the respective Service Field Office. DNA species confirmation is unlikely to be necessary for all captured Myotids, nor for those species with which they may be mistaken (e.g., *M. evotis with M. septentrionalis*), but rather will be based on expected range overlap as well as previous knowledge of the survey area and species occurrence records. If needed, however, DNA sampling should be included in study plans submitted to the local Service Field Office for review and coordination prior to field work.[1] Please note that collecting DNA samples via wing swabbing, buccal swabbing, and/or wing punching can only be completed by individuals whose Section 10(a)(1)(A) Recovery Permits authorize those activities. Guano collection should be added to a Permit only if the bat is being captured solely for the purpose of guano collection or is being held longer than the permitted 30-minute processing time allowed to collect guano.

The Service recommends the use of four DNA collection methods for bat species identification: wing swabs, buccal swabs, fecal collection, and 2 or 3mm wing biopsy punches[1]. When properly collected, efficacy for species identification from wing and buccal swabs as well as fecal samples (particularly if feces are fresh) may reach approximately 95%, while wing biopsy punches provide a slightly higher efficacy of approximately 98%[2]. Given those comparable and favorable percentages, the Service prioritizes the use of less invasive DNA sampling methods over those that may cause additional stress or harm to bats. Please note that these methods are reliable for collecting DNA toward species identification but may not be effective toward other types of genetic analyses.

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[1] Hair samples are another potential method to acquire DNA from bats; however, because we currently do not have a measure of efficacy for this method, and purposeful and painful pulling of numerous hair strands to obtain follicles is required, this method is not recommended nor described herein.

[2] Current as of September 2024, information provided by NAU Lab.

The Service recommends the use of the following methods, in order of highest to lowest preference:

1. Fecal collection – it has low risk of injury or additional stress if bats opportunistically defecate while being processed or if fresh feces are collected from below a known roost. However, we recognize that for mist net or harp trap surveys, fecal collection from captured bats may not be possible due to the short, allowed holding times and surveyors may need to use alternative methods outlined below.
2. Wing swabbing is the second preferred method – it provides both adequate DNA for analysis and is considered safe for the animals.
3. Buccal swabs are less preferable - this method presents potential for stress or injury as the swab is inserted and moved around within the bats' mouth.
4. Wing biopsy punches may be stressful and leave a hole and requires healing of skin tissue. Wing membranes contain elastin bundles and an array of embedded muscles which could be damaged by collection of wing punches. Additionally, biopsy holes have the potential for tearing the membrane or becoming infected. Therefore, this method is acceptable but is the least preferred. Further, the risk to bats via this method is increased if biopsies are taken in the early Spring, when bats' energy reserves are depleted, or in the Fall, when skin membranes may not have time to fully heal prior the bats entering torpor.

Approximate costs, materials, methods, and protocols of the four recommended DNA sampling methods are provided herein. The USFWS does not endorse any product or business and is currently aware of two laboratories that routinely process DNA samples from bats: Northern Arizona University's Species from Feces Laboratory (NAU Lab; https://in.nau.edu/bat-ecology-genetics/sff/) and Pisces Molecular (https://piscesmolecular.com/). Other laboratories may provide similar services with acceptable results. However, if choosing a different laboratory service, determine that entity's preferred sampling collection, preservation, documentation, and shipping protocols in advance of field work. Please compare procedures with those outlined below to evaluate whether sample quality will lead to similar results.

For questions or more information on this document, please send an email to the USFWS Guidelines Team at bat_survey_guidance@fws.gov.

LAB COSTS & PROCESSING TIME ESTIMATES

- Cost estimates[3] for DNA analysis at the NAU Lab
  - For individual species identification: $150/sample
  - Kits with tubes of RNAlater[4], available at cost from NAU: $3 each
  - Processing time: results anticipated 2-3 weeks after receipt of samples

3 https://in.nau.edu/bat-ecology-genetics/frequently-asked-questions/

4 RNAlater is a DNA stabilizer that performs well under field conditions and is safe for air transport. Obtaining a sterile RNAlater sampling kit in advance of a field outing is recommended. If not feasible or available, consider purchasing 1.5 – 2 mL microcentrifuge tubes to have on hand when sampling. Samples collected in these tubes must be frozen immediately (dry ice only) and delivered within the next two days to avoid DNA degradation. Do not collect guano in a resealable plastic bag because this makes sub-sampling difficult and may increase risk of cross-contamination.

- Cost estimates[5] for DNA analysis at Pisces Molecular
  - For individual species identification (CO1 sequencing): 142$/sample
  - Individual swab, tissue or fecal sample kits (50 samples): $57 each plus shipping
  - Processing time: results anticipated 2-3 weeks after receipt of samples. Rush and priority processing is available for extra cost.

RECOMMENDATIONS AND MATERIALS NEEDED FOR SAMPLING METHODS

DATASHEETS

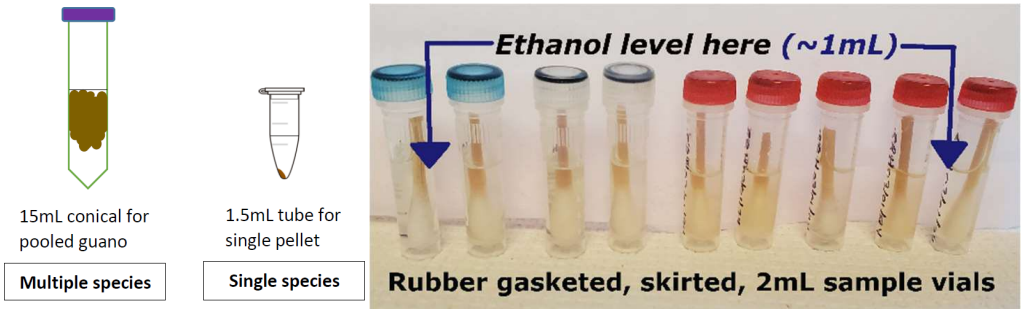
Contact Labs or check websites for up-to-date Data Sheets/Submission Forms, as they are required for submission with DNA samples.

SAMPLING VIALS AND SOLUTION

A variety of sterile vials and solution to preserve DNA are acceptable to Labs for storing collected DNA. Use 1.5 to 2 mL tubes or larger 15mL conical tubes if more space is needed (Figure 1). Use 500 µL RNAlater solution, and parafilm to seal tube (NAU preference), or if using 70% ethanol as preservative (Pisces accepts both RNAlater and ethanol), use skirted screwcap or rubber gasket vials. If using dry vials without preservative, ensure vials have lids to secure contents.

Please note that RNAlater preserves DNA and RNA and is more forgiving of field conditions such as warm temperatures or extended time before samples can be frozen than 70% or higher histological grade ethanol. RNALater preserves RNA and DNA very well for approximately 1 week at ambient temperatures and for one month at -4 Celsius. Therefore, especially in cases when extended hold time is necessary, the Service recommends using RNAlater for important samples where DNA preservation is a high priority.

Figure 1. Examples of the types of vials that can be used for storing DNA.



DNA COLLECTION AND PRESERVATION METHODS

Always adhere to white-nose-syndrome protocols when handling bats. Use fresh disposable nitrile gloves over leather gloves while handling bats and sterilized equipment to collect DNA from each bat (consider layering multiple sterile gloves for quick removal between bat/DNA

5 Current as of November 2024, information provided by Pisces Molecular and valid through Spring 2025. (to obtain updated and more accurate quote of costs, contact Pisces Molecular (https://piscesmolecular.com/discuss/).

specimens). Extreme care should be taken to avoid cross-contamination between samples or other sources of bat DNA for any sample method described below. Note that UV light exposure and mold are common causes of DNA degradation in field sample collections; caution must be taken in handling and preservation of samples. Bleach (hypochlorite) used to decontaminate equipment is also known to degrade DNA quickly, thus items must be thoroughly rinsed before reuse as even parts per billion of remaining hypochlorite can destroy DNA samples over the course of a few days/weeks as the sample moves from field to lab.

We recommend having the following items on hand for any method used:

- Powder-free nitrile gloves (wear over leather gloves if handling live bats)
- 70% ethanol - use as solution for immersion (and subsequent flaming) for tool sterilization
- Lighter (used with 70% ethanol to sterilize equipment such as wing punchers)
- Box or rack to hold sample vials
- Permanent, alcohol-resistant marker to label sample vials
- Resealable plastic bags (quart or gallon, depending on # of samples) or freezer boxes to organize sample vials

WING SWAB COLLECTION

For wing swabs, use lab grade/sterile swabs, generally same type recommended for detection of *Pseudogymnoascus destructans* (*Pd*), Rayon-flocked MedWire swabs or ordinary Puritan cotton swabs. Bulk packed swabs are less expensive but individually wrapped swabs are likely easier to keep uncontaminated in field conditions.

Open swab packet ensuring nothing touches the swab head. Moisten swab tip with sterile water. Swab wing by holding stem and gently running swab tip over the wing for 6 passes along the forearm while simultaneously rotating the swab. For example, video, see: https://www.usgs.gov/media/videos/collecting-a-skin-swab-white-nose-syndrome-surveillance

If using 1.5 or 2mL tubes (with O-rings) with RNAlater solution, immediately after swabbing place swab head in tube containing 500 µL of RNAlater solution. Press the plunger at top of swab stick, which ejects the swab head, or break off the swab stick by leveraging against the tube. Place lid on tube. Invert the tube several times to submerge all contents with RNAlater solution and to test its seal for shipment. Please note you should leave the tip submerged in the RNAlater solution. If swabs are stored dry (not in solution) they would have to be frozen after collection and sent to Lab on dry ice.

If using sterile, rubber-gasket vials with ethanol as preservative, break off excess swab stick by leveraging against tube or clip off with sterile scissors or other cutting tool. Add ~1 mL of ethanol - enough to cover entire sample, but not reach brim of vial. Ensure lid is secure. If using sterile vials without preservative, break off excess swab stick by leveraging against tube or clip off with sterile scissors or other cutting tool.

Follow Lab instructions for storage, transport, and shipment, which may differ based on preservative used. Be sure to understand shipping requirements prior to sample collection, as

Labs may require very specific storage (refrigeration, ice packs, dry ice, etc.) and shipment within 48 hours of sample collection.

BUCCAL SWAB COLLECTION

For buccal swabs, use lab grade/sterile swabs, generally same type recommended for detection of *Pseudogymnoascus destructans* (*Pd*), Rayon-flocked MedWire swabs. Researchers have found that larger swabs are difficult to use and do not fit well inside a bat's mouth. Swabs with diameter of approximately 0.14mm work best (for example product such as the Puritan HydraFlock Sterile Ultrafine Flock, see https://www.fishersci.com/shop/products/hydraflock-sterile-revised-ultrafine-flock-swab/22029693).

Open swab packet ensuring nothing touches the swab head. Insert swab inside the bat's mouth and swab for 1 minute. Rub it on the tongue and roof of mouth; bats will usually bite and chew the swab. Apply some pressure with the swab inside the mouth on whatever it is making contact with (cheeks, tongue, etc.) as you are trying to grab cells; take care not to apply too much pressure which could injure the bat.

If using tubes with RNAlater solution, immediately after swabbing place swab head in tube containing 500 µL of RNAlater solution. Break the excess swab stick by leveraging against the tube, or if there is a plunger at top of swab stick, press it to eject the swab head. Place lid on tubeInvert the conical several times to submerge all contents with RNAlater solution and to test its seal for shipment. Please note you should leave the tip submerged in the RNAlater solution.

If using sterile, rubber-gasket vials with ethanol as preservative, break off excess swab stick by leveraging against tube or clip off with sterile scissors or other cutting tool. Add ~1 mL of ethanol - enough to cover entire sample, but not reach brim of vial. Ensure lid is secure. If using sterile vials without preservative, break off excess swab stick by leveraging against tube or clip off with sterile scissors or other cutting tool.

Follow Lab instructions for storage, transport, and shipment, which may differ based on preservative used. Be sure to understand shipping requirements prior to sample collection, as Labs may require very specific storage (refrigeration, ice packs, dry ice, etc.) and shipment within 48 hours of sample collection.

FECAL SAMPLE COLLECTION

For successful species identification from fecal samples, it is critical that the fecal pellet(s) come from a single animal and that they are not contaminated by touching pellet(s) from another animal. Collecting a stray single pellet from a collection of pellets or guano beneath a roost where multiple species may occur is likely to have poor odds of success.

For fecal sample collection, follow instructions provided in NAU's Standard Operating Procedure for Fecal Collection for Genetics, last updated January 2021, and available here:

https://in.nau.edu/wp-content/uploads/sites/51/2021/02/Fecal-Collection-SOP_NAU-Bat-Team_January-2021.pdf

Be sure to understand shipping requirements prior to sample collection, as Labs may require very specific storage (refrigeration, ice packs, dry ice, etc.) and shipment within 48 hours of sample collection.

WING BIOPSY PUNCH COLLECTION

Membranes take approximately two weeks to heal after one 2 mm punch is taken, which provides sufficient DNA for analysis. Note: larger samples take longer to heal and are unnecessary for adequate DNA content, thus are not recommended - a single 2 mm punch is adequate unless otherwise specified by the Lab.

Wing (propatagium) biopsy punches should generally be taken midsummer. Discuss with the Field Office when taking biopsies is appropriate. The Service prefers researchers and surveyors to avoid taking wing punches during spring staging when bats are in weakened state from hibernation, fall swarming when the wound needs time to heal before hibernation, from pregnant females - particularly late in the pregnancy stage, when additional multiple actions like application of radiotelemetry devices and tags are also planned, when captured individuals show signs of stress (e.g., lethargy).

Do not punch the uropatagium (tail membrane), as this area is highly vascularized and far more likely to bleed when a biopsy is taken. To avoid punching venation in propatagium and to prevent bleeding, place small/dim light beneath transparent, sterilized container. Stretch bat wing over container to illuminate wing membrane. Place biopsy tool on portion of wing closer to the body, avoiding the membrane among the outer fingers. Press straight down firmly and quickly to obtain tissue sample. Use thin sterile tweezers to pick up tissue or remove it from biopsy tool and place in sample vial.

If possible (e.g., rare/few punches needed) use a new sterile biopsy tool for each punch. If not feasible to use a new tool each time (e.g., high number of punches needed) biopsy tools may be sterilized after each use. We do not recommend using the same tool more than ~10 times, as the tip dulls with each application and will not be able to cut tissue well, causing partial cuts that require re-attempts and taking more tissue than necessary. If reusing a biopsy tool, ensure no wing tissue is left in tool tip, dip biopsy tool in ethanol then burn the tip with lighter for a few seconds and rub clean with alcohol wipe.

Follow Lab instructions for storage, transport, and shipment, which may differ based on preservative used. Be sure to understand shipping requirements prior to sample collection, as Labs may require very specific storage (cold packs, dry ice, etc.) and shipment within 2 days of sample collection.

DOCUMENTATION

Labs will require data forms and that all samples are labelled to their standards. Typically, requested information includes species ID (if known), sex (if known), a unique number or code

for each conical and/or tube, date of collection, etc. Label the sample collected by writing sample ID directly on vial (do not affix labels or tape). Use resealable plastic bags to separate samples as appropriate by site or species. Contact the Lab for their data form. For example, NAU requires the following datasheet "Client Data Sheet" available on their website (https://in.nau.edu/bat-ecology-genetics/frequently-asked-questions/).

SHIPPING

Labs have requirements on how to store samples prior to shipping and for transport. Always contact Lab before shipping samples for Lab specific information and to make sure the Lab is ready to receive the samples. Typically, the Lab will require two copies of their preferred filled datasheets, an electronic copy, emailed to lab and a hard copy sent with shipped samples. They will also require that you pack samples inside resealable plastic bags or sealed containers to mitigate contamination in case of leaks.

If shipping RNAlater vials or vials without preservatives to NAU Lab:

- Samples in RNAlater may be packed with either ice packs or dry ice.
- Samples in sterile vials (not in RNAlater) must be shipped on dry ice within 2 days of collection to reduce DNA degradation.
- Ship via FedEx using overnight or 2nd day shipping to Dr. Faith Walker, Northern Arizona University, Applied Research and Development Bldg 56, PMI 2nd Floor, 1395 S. Knoles Dr., Flagstaff, AZ 86011-4073, USA

If shipping to Pisces Molecular:

- Ship 2 ml tubes in cryoboxes (plastic or cardboard) or in an open 80-well long rack plastic wrapped to keep samples upright.
- For overnight shipping, do not use FedEx First Overnight; drop-off occurs before Pisces opens. Instead use Priority overnight for frozen samples and Standard Overnight for all other samples.