AMERICAN MUSEUM OF NATURAL HISTORY

A Field Manual for the Collection, Storage, and Transportation of Biomaterials for Genetic Studies on Felids

George Amato, American Museum of Natural History
Salisa Rabinowitz, American Museum of Natural History
Luke Hunter, Panthera

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The following guidelines are intended for field biologists with previous wildlife experience who are working in association with Panthera. They have been developed by the Global Felid Genetics Project, a collaborative project between the American Museum of Natural History's Center for Conservation Genetics (CCG) and Panthera.

The main objectives of the Global Felid Genetics Project are: 1) to provide standardized methods for the collection of biomaterials from cats; 2) to provide assistance in the experimental design of genetic research projects; 3) to recommend the application of genetic techniques to questions of concern in the management and conservation of cats; 4) to assist in the interpretation of genetic results; and; 5) to provide recommendations, based on genetic findings, for the long-term management and conservation of wild cats.

Training sessions and consultation on biomaterial sampling, genetic analyses, and specific research issues related to genetic investigations of cats can be arranged through consultation with the CCG and Panthera staff (see Appendix I for contact information). Additionally, collection kits and materials are available from the CCG and Panthera; please contact us for materials if required.

I. QUICK-REFERENCE.

This manual provides details on all acceptable techniques for genetic sampling of felids. The 'Quick-Reference' section lists the minimum detail required for the techniques preferred by the AMNH. We encourage you to adopt the following techniques but please refer to the entire manual for much greater detail, and alternative methods.

1. We primarily use fecal samples for our genetic analyses. Recent studies have demonstrated that these samples are far superior to hair samples for DNA extraction, and provide sufficient material to avoid problems with allelic dropout or ghost alleles when genotyping. Additionally, CITES permits are not required for fecal samples. The best way to handle the samples is to air dry them and to store them individually in plastic bags. In humid conditions, storing the samples with silica beads (like the packages used in camera bags) will help reduce mold or fungal growth on the samples. Storing the samples dry is preferable to storing them in ethanol (ETOH) and reduces the cost and complexity of shipping.

2 If an animal is immobilized, there are two easy options:

a. 0.5 mls of whole blood should be placed in a cryo-tube with buffer (sample tubes with buffer can be obtained from the AMNH Center for Conservation Genetics).

b. A very small (2-3mm) disc of tissue can be cut from the ear and stored in a cryo-tube with 95% ethanol (ETOH)

Both types of samples can be stored at room temperature or in a refrigerator (**not** in a freezer).

3. Useful DNA can also be obtained from bones, teeth, dried skin/muscle, and other remains, including from carcases, prepared taxidermy specimens, hunting trophies etc. These should be stored dried in individual plastic bags. If fresh carcases are sampled, store tissue samples as in #2, above.

4. All samples should be individually labeled with the following information:

Scientific and/or common name; Collector and sample number; Locality (Country/ GPS lat & lon); Date; Individual ID (if known from monitoring/research effort); Sex and age (if known).

Example

Jaguar (*Panthera onca*); A. Rabinowitz #325; Belize (Latitude/Longitude); 23 May 2000; radio-collar M24; adult male

Write this information in indelible pen on the outside of tubes/bags. Write exactly the same information in pencil on a piece of paper and place this inside the tube/bag with the samples, guarding against the outside label wearing off.

II. INTRODUCTION[1]

The application of molecular techniques to the management of endangered animals has become an invaluable tool to the conservation biologist (Moritz 1994; Avise 1996; Smith and Wayne, 1996; Ashley 1999). Blood, tissue samples (liver, heart, kidney, spleen, muscle and brain) and intestinal epithelial cells recovered from feces are the preferred biomaterial for genetic studies. Deoxyribonucleic acid (DNA) extractions from tissue recover high molecular weight DNA and produce a high yield from a small portion of the sample. Although blood is a good source of high quality DNA, the ratio of mitochondrial to nuclear DNA in blood samples is extremely low when compared with that found in tissue. The best source for animal mtDNA is unfertilized eggs, heart, liver, kidneys, gonads, and brain. For vertebrates, muscle tissue produces the highest quality DNA, although liver usually produces the greatest yield (Dowling *et al*. 1994).

The acquisition of tissue is not always an option in the field, especially when dealing with highly endangered or easily-stressed animals. In recent years, technological advances have made it possible to extract DNA from a variety of sources, including feces and hair. DNA from fecal samples is retrieved from the sloughed intestinal epithelial cells found in the sample, and is the preferred source of collecting DNA by non-invasive means. In hair, DNA is extracted from the follicle of a single hair.

In recent years, several publications have reported on the extraction of DNA from material such as museum skins and fossils, some dating back several thousands of years (Poinar 1999). Wayne *et al.* (1999) have broadly defined ancient DNA (aDNA) as DNA isolated from plant or animal remains; typically however aDNA refers to the DNA extracted from museum materials and fossils. DNA extracted from non-traditional sources, such as hair and fecal samples, and aDNA encounter similar obstacles in the laboratory. The quality and quantity of DNA recovered from these sources are both low when compared to that recovered from tissue. Fragment sizes amplified from non-traditional sources are typically between 100-500 base pairs in comparison to the high molecular weight DNA (1000's of bases) recovered from a tissue sample (Pääbo 1990). Also, great care should be exercised when using PCR technology with DNA in very small quantities. Ideally, the optimal gene region should be determined and species-specific primers should be designed prior to amplification of the aDNA. In addition, problems with contamination and inhibitors are very real concerns when dealing with aDNA (Pääbo 1989; Handt *et al*. 1994). Studies have shown that when working with small amounts of DNA nuclear insertions and high rates of genotyping errors are definite concerns in the laboratory (Gagneux *et al.* 1997; Greenwood and Pääbo 1999). Reproducibility of results obtained using aDNA is also a concern, therefore it is suggested that multiple trials be completed in order to check for consistency in the results (Gerloff *et al*. 1995; Gagneux *et al.* 1997). Inhibitors to PCR have also been identified as a potential reason for the lack of amplification when using aDNA (Handt *et al*. 1994).

In conclusion, fecal, tissue or blood samples (in that order) are the preferred biomaterial for our genetic studies. The safety of the biologist and the welfare of the animal assume first priority when obtaining biomaterials for genetic studies, therefore the field biologist and the geneticist together must weigh the advantages and disadvantages of each method before making the decision of which biomaterials to collect (Taberlet et al. 1999).

[1] Refer to Appendix III for a glossary of commonly used genetic terms

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III. BIOMATERIAL SAMPLING FOR GENETIC STUDIES[1]

The highest priority when obtaining biomaterials for genetic studies is the safety of the biologist and the welfare of the animal. In order to ensure that the samples and data collected will adequately address the questions asked, it is strongly recommended that the field biologist consult with a geneticist prior to the collection of biomaterials in the field. The field biologist and the geneticist together must weigh the advantages and disadvantages of each method before making the decision of which biomaterials to collect. Another important consideration in the collection of biomaterials is that the proper sampling techniques are employed by the field biologist. It is imperative that all samples are collected, stored and transported properly to ensure that suitable biomaterials will be available for genetic and diagnostic testing.

Samples may be collected from immobilized animals (e.g. blood and tissue), from the field (e.g., feces and hair) and from carcasses (e.g., tissue samples). Fecal and tissue samples are the preferred biomaterial for genetic studies. In cases where these samples cannot be taken, DNA may be recovered from hair. It must be remembered however that DNA recovered from hair samples is of lower quality and quantity than DNA extracted from tissue and further complications, including contamination and inhibitors, are encountered.

In the event that samples are also being collected for health analyses, protocols in the Field Health Manual should be reviewed (Deem and Karesh 2000)

It is recommended that the field biologist ensure that shipping and permitting applications are in order prior to shipping (see Permits and Laws for Transporting Samples Internationally below).

A. SAMPLE COLLECTION, STORAGE, AND TRANSPORT

It is recommended that laboratory gloves and masks be worn whenever examining animals or collecting samples in the field. A basic equipment list for sample collection is listed in Appendix III.

It is recommended that buffer solutions be mixed in advance and stored under the appropriate conditions (Appendix IV).

Labeling

All biomaterials must have the following standard set of information accompanying them:

- Scientific and/or common name; Collector and number; Locality; Date; Individual ID (if available); Sex and age (if known).

[1] References for Sampling Procedures are located in Appendix VI.

Example

Jaguar (*Panthera onca*); A. Rabinowitz #325; Belize (Latitude/Longitude); 23 May 2000; tag#24; adult male

To ensure that the identification number and the sample are not separated, the sample information should be written in two separate locations. This is critical even when using permanent markers since although designated "permanent", occasionally information written in permanent ink marker may be erased, especially when using ethanol.

Field samples should be accompanied with a standard datasheet describing collection location, date, descriptive notes on the sample, and any other sign associated with the specimen. It cannot be emphasized enough that extensive field notes should be taken at the time of sample collection and be kept in a field journal. These notes should include a detailed description of the habitat where the sample was found and how it was collected. The condition of the animal should be noted in a field journal if information is available. In addition, if possible, a photograph of the animal should be taken.

The following labeling procedures are recommended:
All information should be written in permanent ink marker (e.g., Sharpie®) on the outside of the tube or container.

For samples placed in a FALCON® tube (15 ml or 50 ml) containing a buffer solution or silica beads, a piece of paper with the information written in pencil should also be placed in the buffer with the sample.

For samples placed in a NUNC® tube (1.8 ml) containing a buffer solution, an identification number should be written in permanent ink on the lid of the tube and on the side.

Samples wrapped in foil should have an identification number written on the foil in permanent ink marker and they should be placed in a separate Ziploc® baggie with an identification number written in permanent ink marker on the outside of the baggie and a piece of paper with the information written in pencil placed inside the baggie.

For samples placed in any other type of container, e.g. film canister or envelope, there should be identification information inside and outside the container.

B. ANIMAL IN HAND (ALIVE OR POST-MORTEM)
Tissue Samples
Collection
Laboratory gloves and a clean and sterilized knife, scalpel, livestock-ear notcher, or other blade is recommended for tissue collection.

For an immobilized animal, an ear notch (~ 2-3mm^3) sample should be collected.

For post-mortem samples, at least 1 cm^3 of incised sample from the liver; kidney; spleen; skeletal muscle; heart; and/or brain should be collected.

Storage
The following storage options are listed to accommodate the range of available equipment and facilities at the collection site. In all cases, Option 1 is the preferred and recommended storage protocol.

Option 1. Place each individual animal's tissue sample in a separate container with 70-99% ethanol. We recommend using the greatest concentration available. Store at 4°C, if possible.

Option 2. Place each individual animal's tissue sample in a separate container containing tissue storage buffer consisting of: 0.1M Tris, pH 8.0 wHCl; 0.1M EDTA•Na_2; 0.01M NaCl; 0.5% w/vol. and SDS with a final pH of 7.5-8.0. Ratio should be 1 part tissue: 5 parts buffer. Keep at room temperature away from heat and direct light.

Option 3. Place each individual animal's tissue sample in a cryotube or in a separate piece of foil and store at –20°C.

NOTE: Samples from the same animal may be stored together in the same tube.

Transport
Please ensure that tubes or containers are labeled according to the directions outlined above.

Please ensure that all samples are securely sealed. Parafilm® may be used to seal collection tubes.

Samples in a storage buffer or ethanol may be shipped at room temperature, however samples wrapped in foil must be kept frozen at all times.

Ensure shipping and permitting applications are in order before sending any samples.

Blood

Collection

It is strongly recommended that blood samples be collected under the supervision and/or training of a qualified veterinarian during immobilization. Collection protocols are described in detail in the Jaguar Field Health Manual (Deem and Karesh, 2000).

Ideally a total of 5 ml of whole blood per individual cat should be collected. If blood is being collected for health analyses, a sub-sample of this may be used for genetic analyses.

NOTE: Blood should not be collected from a cat carcass for the purposes of genetic analyses. In this case, other tissue samples (e.g. skeletal muscle, liver, kidney, heart) are the preferred samples.

Storage

The following storage options are listed to accommodate the range of available equipment and facilities at the collection site. In all cases, Option 1 is the preferred and recommended storage protocol.

Option 1. Add 100 microliters of blood to each of two (2) blood tubes containing 500 microliters of tissue storage buffer consisting of: 0.1M Tris, pH 8.0 wHCl; 0.1M EDTA•Na_2; 0.01M NaCl; 0.5% w/vol. SDS with a final pH of 7.5-8.0. Ratio for blood-to-blood storage buffer should be 1:5 for mammals*. Keep at room temperature away from heat and direct light. Samples collected in a tissue storage buffer may be maintained for several months under these conditions.

*In general, it is most important to ensure that the tissue storage buffer volume exceeds the blood volume.

Option 2. Draw 3-5 ml of blood for two (2) heparinized (green-top vacutainer) or EDTA (purple-top vacutainer) tubes. May be kept refrigerated for a maximum of 3 days, but these samples should be shipped immediately.

Transport

Please ensure that blood samples are labeled according to the directions outlined above.

Samples collected in a tissue storage buffer may be shipped at room temperature.

Samples collected in heparin or EDTA tubes must be shipped immediately. They **must** be kept cold, but not frozen.

Please ensure that all samples are securely sealed. Parafilm® may be used to seal blood tubes.

Ensure shipping and permitting applications are in order before sending any samples.

C. NON-INVASIVE SAMPLING

Fecal Samples

Collection

Great care should be exercised in the collection of fecal samples to ensure that contamination is minimized. It is recommended that the biologist: 1) wear laboratory gloves at all times during the collection: 2) sterilize instruments between sample collection, either by washing with ethanol or flaming and cooling; and, 3) use new, clean containers for the storage of each fecal sample.

Storage

The following storage options are listed to accommodate the range of available equipment and facilities at the collection site. In all cases, Option 1 is the preferred and recommended storage protocol.

Option 1. Air-dry away from direct sunlight and place individual fecal sample in a plastic baggie, paper bag, or FALCON® tube with silica bead packets at a ratio of 4:1 (beads:sample by weight) or.

Option 2. Place individual fecal sample in a container with 95-100% ethanol.

Label fecal samples following directions outlined above in Labeling.

Transport

Please ensure that fecal sample containers are labeled according to the directions outlined above.

Please ensure that all samples are securely sealed.

Ensure shipping and permitting applications are in order before sending any samples.

Hair Samples

Collection

Great care should be exercised in the collection of hair samples to ensure that contamination is minimized. It is recommended that the biologist wear laboratory gloves at all times during the collection and use new, clean containers for the storage of each hair sample.

Hair samples should be plucked using forceps or fingers. It is important to collect the root or hair follicle in this pluck. It is recommended that each sample consist of 3-10

hairs minimum per individual. It is also recommended that several samples/individual be collected.

Storage
It is recommended that each individual hair sample be placed in a sealed and labeled envelope. Baggies and other containers that trap humidity should not be used.

Label hair samples following directions outlined above in Labeling.

Transport
Please ensure that envelopes are labeled according to the directions outlined above.

Samples may be shipped at room temperature. Please ensure that all samples are securely sealed.

Ensure shipping and permitting applications are in order before sending any samples.

Skeletal Material
Hard tissue samples can include dried skin, bone, or teeth.
Storage
Place individual hard tissue sample in a sealed envelope or other container that will not trap moisture and cause mold to develop on the sample.

Label tissue samples following directions outlined above in Labeling.

Transport
Please ensure that containers used to store skeletal material are labeled according to the directions outlined above.

Samples may be shipped at room temperature. Please ensure that all samples are securely sealed.

Ensure shipping and permitting applications are in order before sending any samples.

D. PERMITS AND LAWS FOR TRANSPORTING SAMPLES INTERNATIONALLY

All diagnostic and genetic testing will by performed by staff of the Wild Cat Genetics Project at the AMNH's Center for Conservation Genetics. The ability of our personnel to complete these tests successfully is contingent on the exportation of samples from various countries within the cat's range and their importation into the US. Cats are listed on CITES Appendix I, therefore a CITES import and export permit are required

for transporting tissue samples into the US. CITES permits are not required from fecal samples, which is a significant advantage over other types of samples.

Permits must be issued by both the importing and exporting countries. In most instances, an export permit will not be issued until a valid import permit is presented to the office issuing the export. The U.S. Fish and Wildlife Service's Office of Management Authority is the Governmental agency which currently issues CITES import permits. In general, written proof of permission to work in the host country is required when applying for an import permit. For more information on permit requirements, you can visit http://international.fws.gov/permits/permits.html.

IV. LITERATURE CITED

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V. APPENDICES

Appendix I. Contact information

George Amato
Center for Conservation Genetics
American Museum of Natural History
Central Park West at 79th Street
New York, NY 10024
gamato@amnh.org
http://congen.amnh.org

Salisa Rabinowitz
Center for Conservation Genetics
American Museum of Natural History
Central Park West at 79th Street
New York, NY 10024
srabinowitz@amnh.org
http://congen.amnh.org

Appendix II. Glossary of commonly used genetic terms

Allele – one of a series of alternative forms of a given gene

Deoxyribonucleic acid – DNA - the primary genetic material of a cell: a polymer of the nucleotides (adenine, guanine, cytosine, and thymine) typically containing two polynucleotide chains in the form of a double helix.

Genetic distance – a measure of the number of allelic substitutions per locus that have occurred during the separate evolution of two populations or species.

Microsatellites[1]-class of DNA markers that consist of sequences containing a variable number of short (2-5 nucleotides) tandem repeats inherited in a single locus, codominant Mendelian manner (e.g. GT_6 or GTGTGTGTGTGT). Because of their high mutation rate, these markers tend to have a large number of alleles and high heterozygosity. Their highly polymorphic nature and small size have made them the ideal genetic system for population based studies.

Mitochondrial DNA[2] – mtDNA - the mitochondrial genome consists of a circular DNA duplex with 5-10 copies per organelle. Mitochondrial DNA has proven to be a useful tool in the discrimination of closely related taxa. Its unique properties include maternal inheritance, rapid rate of evolution, and lack of recombination.

Parsimony principle - the principle that the simplest sufficient hypothesis is to be preferred, even if others are possible. Also called Occam's razor.

Polymerase chain reaction – PCR - a technique for copying the complementary strands of a target DNA molecule simultaneously for a series of cycles until the desired amount is obtained.

Primers – a short strand of nucleic acid which provides the starting point required for the initiation of DNA replication by elongation.

Sequencing—the determination of the order of nucleotide residues of a DNA molecule or fragment

Taq *DNA polymerase* – a DNA polymerase synthesized by the thermophilic bacterium *Thermus aquaticus*. This enzyme, which is stable up to 95°C, is used in the polymerase chain reaction.

Excerpts from:

[1]Bruford, M. W., D. J. Cheesman, T. Coote, H. A. A. Green, S. A. Haines, C. O'Ryan, and T. R. Willimas. 1996. Microsatellites and their application to conservation genetics. *In* Molecular Genetic Approaches in Conservation (T. B. Smith and R. K. Wayne, eds.), pp. 278-297. Oxford University Press, New York, New York.

[2]Brown, W. M. 1983. Evolution of mitochondrial DNA, *In* Evolution of genes and proteins (M. Nei and R. K. Koehn, eds.), pp. 62-88. Sinauer, Sunderland, Massachusetts.

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Appendix III. Suggested supplies needed in the field to collect biomaterials for genetic studies and contacts for obtaining supplies

Collecting tubes containing tissue storage buffers-

Duct tape	Laboratory Gloves
Sharpie® pen	Laboratory face mask
Pencil	Foil
Scalpel and blades	Envelopes or paper bags
Forceps	Paper towels
Kim-Wipes®	1.8 mL Nunc® tubes
15 mL Falcon® graduated tubes	Parafilm®
50 mL Falcon® graduated tubes	Ethanol (96% vol)
Silica bead packets	Filter paper
Container to store used scapel blades	Plastic Ziploc® Baggies
Journal or Rite-in-Rain notebooks	

CONTACT INFORMATION FOR SCIENTIFIC SUPPLY COMPANIES IN U.S.

Fisher Catalog Products (Phone: 1-800-766-7000)	*Catalog#*
Kim-Wipes®	06-666A
1.8 mL Nunc® tubes	12-565-171N
15 mL Falcon® graduated tubes	05-527-90
50 mL Falcon® graduated tubes	14-432-23
Parafilm®	13-374-16

Sigma Catalog Products (Phone: 1-800-325-3010)	*Catalog#*
Silica bead packets	S8394
Ethanol (96% vol)	24106

NOTE: See Appendix IV for tissue storage buffer recipe

Appendix IV. Recipes for the Preparation of Tissue Storage Buffer

Tissue storage buffer (1:5 tissue:buffer): 0.1M Tris, pH 8.0 wHCl; 0.1M EDTA•Na_2; 0.01M NaCl; 0.5% w/vol. SDS (final pH 7.5-8.0)

To make 1 liter of the tissue storage buffer:
0.1 M Tris – 12.11 grams/ L
0.1 M EDTA•Na_2 – 37.22 grams/ L
0.01 M NaCl – 0.5844 grams / L
0.5% weight/volume SDS – 5 grams/ L

Final pH 7.5-8.0 with HCl

Store at room temperature, away from heat and direct light

Chemicals needed:

Name	Molecular weight (grams)	Sigma Catalog#
Trizma® base Tris Tris[hydroxymethyl]aminomethane	121.14	T6791
EDTA•Na_2 ethylenediaminetetraacetic acid•disodium salt	372.2	E1644
NaCl sodium chloride	58.44	S7653
SDS sodium dodecyl sulfate lauryl sulfate	n.a.	L4509

Appendix V. Standard datasheet for field specimens (from Rabinowitz 1993).

COLLECTED OR EXAMINED SPECIMENS

Name of Collector: ______________________

Date: ______________________

Locality: ______________________

Species of Specimen: ______________________

Sex of Specimen: ______________________

Measurements: ______________________

Weight: ______________________

Habitat: ______________________

If female, state of mammary glands (wet or dry) ______________________

Number of embryos in female ______________________

If male, state of testes ______________________

Stomach contents ______________________

PARASITE SAMPLES

Collector: ______________________

Date of Collection: ______________________

Locality: ______________________

Type of parasite collected: ______________________

Species of host: ______________________

Sex of host: ______________________

Part of host's body where collected: ______________________

If not found on a host, where it was found: ______________________

Appendix VI. References for sampling protocols.

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