**U. S. Geological Survey**
**ALASKA SCIENCE CENTER**
**Biological Sciences Office**
**Molecular Ecology Laboratory**
**1011 E. Tudor Road, MS701**
**Anchorage, AK 99503**
**Phone: 907-786-3582; Fax: 907-786-3636**

**Sandra Talbot, Research Geneticist (Wildlife)**
**office: 907-786-3683**
**sandy_talbot@usgs.gov**

**John Pearce, Research Wildlife Biologist**
**office: 907-786-3893**
**john_pearce@usgs.gov**

**Kevin Sage, Geneticist**
**office: 907-786-3975**
**kevin_sage@usgs.gov**

**Judy Gust, Geneticist**
**office: 907-786-3949**
**judy_gust@usgs.gov**

This protocol was obtained from Enrique Lessa (now at Universidad de la República, Montevideo, Uruguay) and is used with some modifications in the ASC Molecular Ecology Laboratory for extraction of DNA from small amounts (no more than 20mg) of muscle or organ tissue samples, and from 1-10µl of nonmammalian vertebrate blood. It is also useful for extracting DNA from vascularized eggshell membranes, blood spots on filter paper, feathers, and buccal swabs. This protocol yields samples adequate for PCR amplification of microsatellite DNA as well as for mtDNA and nuclear DNA sequencing.

## DNA EXTRACTION FOR PCR *SANS* PHENOL/CHLOROFORM

This protocol is designed for DNA extraction from small amounts (no more than 20mg) of frozen tissues, which are likely to provide high molecular weight DNA that can be precipitated with alcohol. This procedure is also suitable for extraction from 1-10µl of blood from nonmammalian vertebrates (Medrano et al. 1990). For extracting DNA from whole mammalian blood, a Differential Lysis of the Red And White Blood Cells must precede this extraction. For obtaining DNA from museum specimens continue to use the phenol/chloroform extraction (MVZ protocol, June 1990, in MEL extraction notebook). Salt extractions work fine for some museum skins, but not all. If you're not sure how the skins were prepared, it is a good idea to use phenol-chloroform extractions on them. Also, DNA to be cut with restriction enzymes should be extracted via phenol/chloroform.

The protocol uses sodium chloride instead of phenol/chloroform for extracting proteins away from DNA. The salt extraction method is two steps shorter than the phenol procedure. Yields are about 30-60 µg total DNA from 10-20 mg frozen tissue, and amplification and sequencing for mitochondrial and nuclear segments is as good as with DNA extracted with organic solvents.

FOR BRAND NEW USERS, STUDENTS, AND NOVICES: (and old users who are having problems): before using this protocol, read through it several times to make sure you understand each of the steps pertinent to your extraction, tissue source, and species. If you have any

questions (or even if you don't), ask someone who routinely gets good yields using this protocol with your species group and tissue type.

Also, until you are able to get yields of > 250ng/mL on average using this procedure for avian blood, we recommend you don't process more than 16 – 24 samples at a time. This is non-negotiable (heads up, staff). It may *seem* efficient to process more samples at a time (and is, if you're experienced), but it is extremely easy to make mistakes using any extraction procedure; one way to make mistakes is to process too many samples at once, especially if you are just starting and are moving slowly through the steps. In the long run, it's more efficient to do the procedures slowly and carefully the first few times, running these few samples through the entire process from extraction to genotyping or other downstream use, to see how you are doing.

We understand that to err is human. We also believe that the wise person learns from the mistakes of others. Here I have listed the most common mistakes that we have either made ourselves, or seen others make, that result in poor yields using this (and most other similar) extraction procedures:

1. Mixing up the labels. Don't make this mistake. But, if you think you did, then start over. It's not worth the "saved" time, since it affects the quality of the data and it's likely we'll eventually find out anyway and you'll just have to start over (and we'll have to throw away results to that point).

2. Putting too much blood/tissue in the lysis buffer. Read the protocol. If you don't understand it, ask us. If you want to know what 10, 15, or 150 µL looks like, then measure out 10, 15 or 150µL using a pipettman, so you know what it looks like.

3. Lysing for too short a period of time. IF THERE IS UNDIGESTED TISSUE PRESENT, perform the procedure at step 5. Don't skip over this step to save time. You won't and again, you'll irritate the bosses. Alternatively, just let the sample lyse for a longer period of time. Despite what your Graduate Advisor says, it's okay to let the samples lyse for up to a week or so; in some cases (feathers), you won't get very high yields if you don't. Some folks think lysing for a long time degrades the DNA. This is likely true if you're doing whole genomic work, but we have had no difficulty genotyping, sexing and sequencing numerous loci (100 – 1500 kb) from samples lysed for long periods (up to a week). Check out the notebooks for proof.

4. Centrifuging for too short a period. Please centrifuge for the period indicated in the protein precipitation or alcohol precipitation steps. So, for the protein precipitation step, centrifuge for 30 minutes. For the ethanol or isopropanol step, centrifuge for 30 minutes. Of course, you can be creative and do side-by-side experiments to demonstrate that 30 minute spins yield lower or equal yields than 15 minute spins at either or both steps, but please make sure the information is 1) publishable and 2) written up and published. Alternatively, you can just take our word for it and save yourself some time.

5. Allowing the samples to sit too long prior to pouring off the 70 – 75% ETOH in step 9. This step is difficult to do quickly if you are processing too many samples, so, again, get good at this before you "cowboy" this step. Don't let it sit for > 5 minutes. The bosses don't let it sit for > 1 minute.

6. Hydrating DNA for too short a period of time (step 10). It takes awhile for the DNA to hydrate (sometimes up to several days at room temperature), and if there are high yields and too little TE buffer, all the DNA won't go into solution. This is easily detectable because when you are mixing the DNA, the pipette tip will clog with a "snot wad" (i.e. the glob of DNA). If this happens, add more TE and continue to hydrate. You can hurry the process a bit by placing in the 37°C incubator for an hour or two (at most), but don't forget it's there.

We are convinced that there are more mistakes than this, so don't feel cheated that we are asking you to avoid these. There will be more. If you are creative enough to make mistakes not included here, please feel free to add to the list.

## SUPPLIES

### A. Chemicals

- Lysis buffer (MVZ buffer)

| | |
|---|---|
| 50mM Tris-HCl, pH 8.0 | 5 ml 1M Tris-HCl, pH 8.0 |
| 50mM EDTA, pH 8.0 | 50ml 100mM EDTA, pH 8.0 |
| 1% sodium dodecyl sulfate (SDS) | 10ml 10% SDS |
| 100mM NaCl | 10ml 1M NaCl |
| 1% 2-mercaptoethanol* | 24ml NanoPure$H_20$ (np$H_20$) |

  Store at room temperature

  **add 2-mercaptoethanol (1% final concentration) to aliquot just before using; i.e., for 9.9ml lysis buffer, add 100µl 2-mercaptoethanol for about 18 tissue extractions*
- Proteinase K (20mg/ml) in np$H_20$
- Dithiothrietol (DTT, 100mg/ml) (<u>for feather/skin extractions</u>)
- RNase A (10mg/ml)
- 5M sodium chloride (NaCl)
- Ethanol (EtOH, 100%) or Isopropanol (IsOH) at -20°C
- 70% EtOH at -20°C
- 1X TE buffer, pH 8.0
- 1X STE buffer, pH 8.0
- glycogen (20mg/ml; Roche Cat. No. 901 393), <u>for feather/skin extractions</u>

### B. Equipment

- 1.5 ml autoclaved micro-centrifuge tubes (FLATOPS preferable). <u>Label tops of tubes</u>. For the Lysis step and <u>only</u> for the lysis step, it is best to use Eppie tubes with safe-locks.
- Pipetmen: 20 (P20), 200 (P200) and 1000 (P1000) microliter; yellow and blue autoclaved tips
- Racks for microtubes
- Microcentrifuge
- Vortex mixer
- Disposable gloves
- Should grinding of tissue be necessary, use P1000 blue tips that have been briefly melted and sealed at the tip with a flame.

## PROTOCOL

*NOTE: Carry out at least two extraction blanks for every extraction series you perform. These will provide for important controls in subsequent PCR experiments.*

1. <u>Muscle/Organ tissue</u>: Weigh out no more than 20mg of tissue in a 1.5mL microcentrifuge tube. Use clean, sterilized (alcohol-flamed) forceps and scissors or a scalpel to do this in order to prevent cross-contamination of tissues. Otherwise, interpretation of genetic data might be compromised.

   <u>Blood</u>: If extracting blood samples, **skip to step 3** and add 10 -- 15µL whole (nucleated) blood directly to the lysis buffer (up to 100µL if blood is in blood preservation buffer. DO

NOT USE OVER 150µL OF BLOOD STORED IN LONGMIRE BUFFER (Longmire et al. 1988), unless you let it sit in lysis for a week or so, or [more likely] start over at the beginning.). If you are extracting DNA from non-nucleated blood (e.g., mammals), use 200µL.

Feathers: Wet feathers using EtOH, to keep parts from flying around and driving you crazy. Strip off the vanes, dispose. Mince the calamus end of the feather with a sterile razor blade, or snip into tiny pieces using a pair of sterile scissors. The entire rachis of contour feathers can be minced, but for larger feathers such as goose primary feathers, just use the first ½ inch or so, including the calamus (that's where most of the DNA is hiding). Transfer to the microcentrifuge tube. **Skip to step 3.**

Nucleated Blood on Filter Paper: Cut a 5 $mm^2$ square containing the blood spots, using sterilized scissors (see Muscle/Organ tissue above). Place in microcentrifuge tube using sterilized forceps, and proceed as per a blood protocol (**skip to Step 3: also, see modification at Step 5 below**). We have had difficulty with blood on filter paper if the blood has been stored in SET or Queen's Buffer, but have seen no problem with whole blood on filter paper, or blood in ethanol (ETOH). Although we have no data to suggest that whole blood on filter paper becomes degraded, we recommend that whole blood transferred to filter paper be "fixed" using 95-100% ETOH, as soon as possible, to inactivate DNAses.

Buccal Swabs: We have used this extraction procedure to extract DNA from freshly-collected epithelial cells on buccal swabs, or swabs stored in Longmire Buffer. See Handel et al. (2006) for description of buccal swab collection from avian species. Roll the buccal swab around the inside perimeter of the microcentrifuge tube containing the MVZ lysis buffer (if you have stored the buccal swab in Longmire buffer, you can roll the swab around the perimeter in the tube with Longmire, then draw off about 100 µL of Longmire buffer, and transfer it to the lysis tube). The procedure then **skips to step 3**, following the procedure for blood, but with lysis for 1 – 3 days.

2. Wash tissue with 1 ml cold STE buffer three times, rapidly. Remove wash fluid with P1000.

3. Add 550µL of lysis buffer (don't forget the 2-mercaptoethanol) and, immediately afterwards, 5.5µL Proteinase K* (20mg/mL), and (for feathers only) 5µL of DTT to microtube. If necessary, grind with P1000 microtips melted at the tip. Finger vortex the mixture. Incubate in rocker at 55°C in dry air incubator until there is disintegration of the tissue (from 2 hours to 4 days; the longer, the better the yield. Ignore the folks that think this degrades the DNA, unless you're doing whole genomic sequencing).
   ***A cheaper alternative that works well is to add 5.5µL Protease E (20mg/ml) and incubate at 37°C; this is recommended for blood and tissue, but not feathers.**

4. One hour before the end of incubation, add 10.0 µL RNase A (10mg/mL, DNase free). This is generally considered an optional step in extraction protocols, but if you are working with tissue samples that were rapidly expressing at the time of collection, such as nestling bloods or blood quills, then definitely use RNase A.

5. Should there still be "serious" amounts of undigested tissue present, centrifuge samples at max speed for 10 minutes, and transfer supernatant (approximately 550µL) to a new microcentrifuge tube with a P1000 (for extraction of blood on filter paper, remove the paper at this step). **Otherwise, skip this step and:**

6. Add 350µl 5M sodium chloride (2M final NaCl; adjust the amount of NaCl if you added a lot of blood preservation buffer). Vortex for 15 seconds and centrifuge for 30 minutes at maximum speed (16,100 x g) in the microcentrifuge. Don't be impatient: spin for the full 30 minutes.

7. The original protocol calls for you to transfer 450µL of the supernatant (which contains the DNA) to a new tube. Add 900µL of cold, absolute ethanol (Alternatively, put 400µL into each of 2 tubes, add 800 µL EtOH to each, etc.)

   **However, you can also use 0.7 volumes of Isopropanol (IsOH), rather than 2 volumes of EtOH, for this step. Thus, if you transfer 850µL of the supernatant to a clean tube, add 595µL of IsOH, invert gently about 20 times, and incubate as above. This is the preferred method, since you get most of the DNA without having to use 2 tubes.**

   For feathers and buccal swabs, you can add up to--but no more than--1 µL of glycogen (20 mg/mL) at this step. Glycogen is used as a carrier for the precipitation of nucleic acids.

   Invert gently several times. You should see "ropes" of DNA come out of solution (but not always, so don't panic yet!). Incubate samples at –20°C for 2 hours to overnight.

8. Microcentrifuge for 30 minutes at max rpms (16,100 X g on most tabletops) to pellet the DNA. Pour off the supernatant. Invert the tubes on clean/sterile paper towel in order to drain as much of the fluid as possible. Be sure the pellet does not slide down the tube. This step can also be done by removing the supernatant with a Pipetman. (Optional: Remove the last remaining bit of fluid by touching the edge of the tube to a clean, untouched side of a Kimwipe)

9. Wash the pellet once with 500µl 70%-75% EtOH, microcentrifuge (6000 rpm, 5 minutes, or 1 minute, max rpms), and thoroughly remove the wash fluid (pour off, if you're brave, or use the "Kimwipe technique") each time. Do not let the samples sit in 70% EtOH for over 5 minutes or you'll end up losing your pellet. Then cover the open tubes with Kimwipes and incubate at 37°C for 30 minutes or until dry; typically overnight at room temperature works just as well.

10. Add 100 µL 1X TE, (less for feather and buccal swab samples (25-30µL) and incubate at 55 °C for 2 hours. If DNA has not completely suspended, continue incubation at 55°C and check tubes every 30 minutes. Finger vortex gently to suspend pellet. Quantify via spectrophotometry (see below) or fluorometry. Store DNA at 4°C or -20°C for long-term storage.

**DETERMINATION OF DNA CONCENTRATION VIA SPECTROPHOTOMETRY**

1. Add 10µL sample to 990µL 1X TE buffer (1:100 dilution). Take optical density readings in spectrophotometer at 260nm and 280nm. Calculate DNA concentration using the following equation: A260nm = 1 O.D. = 50µg/mL

2. For example, if a given sample with an O.D. at A260nm is 0.235:
   0.235 X dilution factor (100) 50µg/mL = 1175µg/mL.

3. Note: the ratio A260/A280 should be > 1.5. Do not take readings below 0.1 at 260nm wavelength. If necessary, add 10 µl increments from your DNA stock to the cuvette to reach O.D. above 0.1.

**References**

Handel, C. M., L. M. Pajot, S. L. Talbot and G. K. Sage. 2006. Use of buccal swabs for sampling DNA from nestling and adult birds. *Wildl. Soc. Bull*.: in press.

Longmire, J. L., A. K. Lewis, N. C. Brown, J. M. Buckingham, L. M. Clark, M. D. Jones, L. J. Meincke, J. Meyne, R. L. Ratliff, F. A. Ray, R. P. Wagner and R. K. Moyzis. 1988. Isolation and molecular characterization of a highly polymorphic centromeric tandem repeat in the family Falconidae. *Genomics* 2:14-24.

Medrano, J.F., E. Aasen, & L. Sharrow. 1990. DNA extraction from nucleated red-blood cells. *Biotechniques* 8: 43.