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DNA Extraction Protocol for toe pad samples used in:

Harvey MG, Bravo GA, Claramunt S, Cuervo AM, Derryberry GE, Battilana J, Seeholzer GF, Shearer J, Faircloth BC, Edwards SV, Pérez-Emán J, Moyle RG, Sheldon FH, Aleixo A, Smith BT, Chesser RT, Silveira LF, Cracraft J, Brumfield RT, Derryberry EP. *In review*. Spatial dynamics of bird diversity in the Neotropics.

This protocol was designed by Andrés M. Cuervo (Instituto de Ciencias Naturales, Universidad Nacional de Colombia) and Brian T. Smith (American Museum of Natural History) based on available protocols (e.g. McCormack et al., 2015) with advice by Sabrina Taylor (Louisiana State University).

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If you use this protocol for samples obtained from **>50 year** old museum skins, then use the **spin columns from the QIAquick PCR purification kit** (purple lids) (http://www.qiagen.com/products/catalog/sample-technologies/dna-sample-technologies/dna-cleanup/qiaquick-pcr-purification-kit). Warning: **DO NOT** want to use QIAquick spin columns (purple lids) with fresh tissues samples or much more recent toe pads.
This protocol largely uses the DNeasy Blood and Tissue Kit reagents.

For samples obtained from **<50 year** old museum skins, use the **spin columns from the QIAamp DNA Micro kit** (yellow rims, stored in the freezer).
(http://www.qiagen.com/us/shop/sample-technologies/dna-sample-technologies/genomic-dna/qiaamp-dna-micro-kit/)

Work in a dedicated facility where no vertebrate PCRs are done (i.e., an environment free of modern bird DNA) and under a UV-hood.

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Recommendations for toe pad sampling:

1. Work on a white desktop (make layers of kimwipes if necessary and replace as needed).
2. Cut toe pad with a clean scalpel (or new razor blade) into a piece of curled foil. Store toe pads in a custom-made mini envelope or place directly in a microcentrifuge tube. Store toepad dry if necessary in a dedicated freezer.
3. Between each sample, change foil and scalpel blade and sterilize scalpel and forceps (in 10% bleach, rinse well, dry, UV sterilization)

Make sure you have a relatively large toepad sample. It better to make a big cut the first time then have to cut the skin twice. Total DNA yield is critical for the success.

DO NOT bring the toe pads to the standard lab or to the freezer with other bird DNA or reagents.

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Before you start the extraction:

Wipe down pipettes with DNAaway. Rinse racks and shaker in 10% bleach followed by diH20.
Put tubes, ddH20, pipettes, **filter** pipette tips, parafilm, razor blades, scalpels, gloves and racks in UV-hood
Wipe down countertops with 10% bleach (or DNAaway)
Make sure all reagents are ready and available (DTT, Carrier RNA, ethanol-mixed buffers AW1 and AW2, etc).

For DTT, make a stock solution at 1M (store in aliquots at -20˚C, avoid freeze/thaw cycles)

a. Molecular weight = 154.25g
b. 1.5425 g in 10 ml of ddH20

For Carrier RNA:

1. Place buffer AE in incubator.
2. Add 310 µl Buffer AE to the tube containing 310 µg lyophilized carrier RNA to obtain a solution of 1 µg/µl. Dissolve the carrier RNA thoroughly
3. Divide carrier RNA + buffer solution into conveniently sized aliquots, and store at –20°C. (Do not freeze–thaw the aliquots of carrier RNA more than 3 times.)
4. Calculate the volume of Buffer AL and dissolved carrier RNA needed per batch of samples by multiplying the number of samples to be **simultaneously** processed (1 ul dissolved carrier RNA to 200 uL buffer AL for tissues).
5. Gently mix Buffer AL and dissolved carrier RNA by inverting the tube 10 times. To avoid foaming, do not vortex. (Buffer AL containing carrier RNA is stable at room temperature (15–25°C) for up to 48 hours)

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Day 1: Toe pad digestion

<u>Toe pad wash</u>

1. Label microcentrifuge tube for each toepad (place toepad in)
2. Add 100 µl of absolute (100%) ethanol to each tube
3. Incubate at room temperature for 5 minutes
4. Remove ethanol carefully with pipette and discard

5. Add 200ul of buffer AE (or ddH20) to each tube
6. Incubate at room temperature for 5 minutes
7. Remove buffer AE (or ddH20) and discard.

Toe pad mince

1. Label new microcentrifuge tubes for each sample.
2. Add 180 ul buffer ATL to each tube and set aside
3. Remove toepad from washing tube (or envelope), and on a clean sheet of parafilm mince toe pad with clean razor blade.
4. Place all of toepad in labelled tube with buffer ATL. Discard parafilm.
5. Repeat for all samples.
6. Add 30-40 µl of ProK and 20 µl of DDT to each sample.
7. Close lid firmly, seal with parafilm. Limit the number of times you open and close tube before incubating.
8. Vortex well by pulsing, and place on (sterilized) rotator or shaker, in incubator at 56˚C overnight. Ensure all tissue is submerged before returning to rotator/incubator—you may need to spin down tubes. Prevent leaks. Don't do 180º rotations if tubes are not leak-proof. If possible, repeat vortex cycles every 1 or 2 hrs.

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Day 2: Finish extraction

Preparation steps:
- Prep Carrier RNA solution by adding buffer AE (see above). Optional: add Carrier RNA to buffer AL aliquots.
- Warm up ddH20 aliquot (or buffer AE) at 56ºC for the elutions step
- Warm up a heating block (or incubator) at 70ºC for step 1.
- Make new aliquots of fresh 100% EtOH and put them in the freezer (should be **cold** at -20 ºC)
- Gently mix samples by pulse-vortexing . Let it sit.
- Make sure the digested solutions are ready (clear solution with no hard toe-pad solids visible)
- Prep spin columns (label with number according to extraction worksheet in lab notebook)
- (Optional: prep centrifuge tubes as described in step 4).
-Make sure you have enough of new filtered pipette tips.

1) - Add 200 µl **Buffer AL** to the sample, mix well by pulse-vortexing for 15 s
   - Add 1 µl of Carrier RNA to each sample (optional: skip if Carrier RNA already in AL buffer)
   - Incubate at 70°C for 10 min.
   - Briefly centrifuge the 1.5 ml microcentrifuge tube to remove drops from inside the lid.

2) - Add 200 µl of fresh (and **cold**) absolute ethanol (100%) to the sample
   - Mix well by pulse vortexing for 15 s
   - Briefly centrifuge the 1.5 ml microcentrifuge tube to remove drops from inside the lid

- Incubate in 4°C fridge for one hour

3) - Make aliquots of enough **ddH20** for your samples (n x 300 ul) and heat to 56°
   - (optional: Instead of ddH20, make aliquots of AE buffer if you are not re-concentrating samples)

4) - Label 1.5 mL microcentrifuge tubes for permanent storage of the DNA extraction. Make sure they were in a UV-hood. Label properly on the cap (museum code and species name), and on the side of the tube (full species name, “toepad DNA”, and date). [skip if tubes already labeled]

5. - If there are still chunky bits in the sample mixture from step 2, spin the tubes down briefly.
   - Carefully transfer the sample mixture from step 2 (including the precipitate; but not chunky bits) to the spin column (QIAquick purple lids in a 2 ml collection tube for > 50 yr samples) **without wetting the rim.** For <50 year old samples use the Micro-kit spin columns from the freezer (yellow rim).
   - Close the cap. Incubate for 10 min at room temp.
   - Centrifuge at 8000 **rpm** for 1 min.
   - Place the spin column in a new clean 2 ml collection tube (provided), and discard the old collection tube containing the filtrate.

6. - Carefully open the spin column and add 500 µl Buffer **AW1** (check that Et-OH was added) without wetting the rim.
   - Close the cap, and centrifuge at 8000 **rpm** for 1 min.
   - Carefully remove the spin column from the collection tube containing the filtrate (do not wet the spin column)
   - Discard the collection tube with the filtrate
   - Put the spin column in a clean 2 ml collection tube (provided)

7. - Carefully open the spin column and add 500 µl Buffer **AW2** (check that Et-OH was added) without wetting the rim.
   - Close the cap, and centrifuge at full speed 14000 **rpm** for 3 minutes.
   - Carefully remove the spin column from the collection tube containing the filtrate (do not wet the spin column - the tube should be completely free of Et-OH)
   - Discard the collection tube with the filtrate, and place spin column in a new collection tube

8. - Re-spin sample: place the spin column (empty from previous step) in a new, empty collection tube and spin at full speed 14000 **rpm** for 3 mins (to remove residual Buffer AW2 and Et-OH.)

9. - Place the spin column in the labeled, clean 1.5 ml microcentrifuge tube.
   - Carefully open the spin column and **add 200 µl of warmed (56°C) ddH20.** *Make sure that the water covers the filter.* (Optional: use buffer AE instead if you are not going to re-concentrate samples).
   - Incubate at room temperature for 1 hour at least (up to 24 hrs)

- Centrifuge for 1 min at 8000 **rpm** (change settings in centrifuge from previous step)

10. - Make a second elution (as in the previous step) by placing the spin column in a separate, clean 1.5. mL tube. Put away the labeled tube with 100 µl of extract from 9 (in the fridge)
    - A**dd 50 µl of warmed (56°C) ddH20** to the spin column sitting on the new tube**.** *Make sure that the water covers the filter.*
    - Incubate at room temperature for at least 20 mins (up to 2 hrs).
    - Centrifuge for 1 min at 8000 **rpm**

11. - Make a third elution (by repeating the previous step 10): add another **50 µl of warmed (56°C) ddH20** to the spin column sitting on the same tube from #10 (which already contains 50 µl of extract). *Make sure that the water covers the filter.*
    - Incubate at room temperature for at least 20 mins (up to 2 hrs).
    - Centrifuge for 1 min at 8000 **rpm**

12. Transfer the resulting 100 µl from the last two elutions to the labeled, primary tube. For a total of 300 µl of extract. WARNING: High risk of cross-contamination. Do this with no rush.

13******. Speed-vac sample down to approximately 100 µl of DNA extraction (if extract is not in ddH20, but in buffer AE, then the DNA needs to be precipitated)

14. The 100 µl of DNA should be ready for DNA quantification in a Qubit or Picogreen method.

Store samples in dedicated freezer.

** Re-concentration of DNA samples by reducing extraction volume (from 300 to 100 µl). speed-vac centrifuge differs in temperature setting and vacuum pressure, make sure you have a handle at the one you will be using. Wipe down the bench areas beside the equipment you will be using with 10% bleach and then diH20 and leave/replace a large kimwipe on which you can set the rack that is holding your extractions. Use DNAaway (or a similar gentle decontaminator) for the internal part of the speed-vac centrifuge. Make a “ruler” centrifuge tubes with 100 µl of ddH20 and close the lid; use it as a yard-stick to compare the volume of the sample tubes. Place sample tubes with open lid carefully. Balance the centrifuge. It is OK to use a warm (40ºC) temp. Volume will seem to not be reducing at a really fast pace initially, but then around 120 µl or so (~after ca. 1 hr), the volume will start to reduce faster and faster. Monitor constantly the tubes (using your marked “yardstick” tube) to prevent extra evaporation. If samples are in buffer AE, you should consider precipitate the DNA.

Other general suggestions:

- leaving a set of gloves in the hood and using a new pair when you are working with the centrifuge/incubator/any other equipment in the lab OR just taking a new pair of gloves everytime you move back to working in the hood.

- Wipe down the bench areas beside the equipment you will be using with 10% bleach and then diH20 and leave/replace a large kimwipe on which you can set the rack that is holding your extractions and being used in the biosafety hood.
- After using/removing all supplies from the biosafety hood, wipe down with ethanol and run the UV light for 15 minutes (good practice for the next person who will use it).
- do NOT block/work over the vents near the edge of biosafety hood workbench – they are the source for air circulation in the hood.

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References

J. E. McCormack, W. L. E. Tsai, B. C. Faircloth, Sequence capture of ultraconserved elements from bird museum specimens. Molecular Ecology Resources. 16, 1189–1203 (2015).