DNA Extraction from Whole Tissue

1. Set up three beakers, one containing diluted Micro, one containing ultrapure water, and one empty waste beaker. Fill a squirt bottle with ultrapure water.

2. Dip a pair of forceps and scissors into the Micro solution, then the water, then squirt water on the tools over the waste beaker. Dry tools with Kimwipe. This destroys any residual DNA and cleans contaminants off.

3. Arrange "n" number of labeled 1.5 mL microcentrifuge tubes, fill ¾ of the way with ultrapure water. "n" represents the number of samples to be extracted in this procedure.

4. Remove tissue sample from ethanol, cut approximately 25 mg of tissue into small pieces, and place pieces into corresponding microcentrifuge tube containing ultrapure water.

> **Note**: The tissue sample can be placed on a fresh Kimwipe during cutting. Dispose of Kimwipe immediately after cutting and replace for next sample.

5. Repeat step 3 each time a tissue sample is handled, and repeat step 4 until all tissue samples are completed.

6. Incubate samples in the fridge at 5°C for up to an hour. Allowing the ETOH preserved tissues soak in the water pulls the ETOH from the tissue.

> **Note**: During this time, it is advisable to prepare the final 1.5 mL microcentrifuge storage tubes. Using lab tape, write the following for each sample and wrap around tube:

Your initials X-DNA Genus & species Accession # Date(Extraction #, Sample #)

ELS X-DNA Cordylus cataphractus AMB 8375 04.01.08 5.2

Label the top of each tube using your initials, the extraction number, and sample number (ex. ELS 5.2)

7. Pipette out water from tubes and discard, being careful not to remove any bits of tissue.

> **Note:** Approximately 10 min before completion of step 7, turn on the 55°C hotblock. We use the incubator in 401.

8 Add 180µl of **ATL Buffer** to each sample.

9. Add 20µl of **Proteinase K**, vortex immediately.

10. Incubate samples at 55°C on the hotblock until tissue is completely lysed.

    Note: It is best to allow tissue to digest overnight, although the minimal time to allow for soft tissue (ex. liver) digestion is 1 hour. If possible, vortex occasionally.

11. **Next day or several hours later (when tissue is sufficiently digested)**, turn on the 70°C hotblock. Prepare labeled DNEasy spin columns for each sample.

12. Remove samples from hotblock and vortex at least 15 sec.

13. Add 200µl of **Buffer AL**, tip mix, then vortex immediately.

14. Incubate samples at 70°C for 10 minutes.

15. Remove samples from hotblock, add 200µl of **EtOH**, vortex.

16. Pipette samples into labeled DNEasy spin columns.

17. Centrifuge at 8000 RPM for 1 minute, discard collection tube and replace with fresh collection tube.

    Note: Remove DNeasy spin column carefully so that the column does not come into contact with the flow through. If does occur, replace the collection tube and re-spin.

18. Add 500µl of **AW1 Buffer**, centrifuge at 8000 RPM for 1 minute, discard collection tube and replace with fresh collection tube.

19. Add 500µl of **AW2 Buffer** centrifuge at max speed for 3 minutes discard collection tube and replace with the 1.5 mL microcentrifuge tube.

21. Add 200µl of **Buffer AE**, incubate 1 minute.

22. Centrifuge at 8000 RPM for 1 minute. **DO NOT DISCARD TUBE**.

24. Repeat steps 21 and 22.

23. Transfer flow-through to the 1.5 mL microcentrifuge final storage tube labeled with tape.

26. Spin down the 1.5 mL final storage tubes at max speed for 20 minutes.

    Note: This settles any residual pigment to the bottom that would otherwise negatively affect PCR reactions.

PCR Protocol

1. Remove extracted genomic samples to be used in the PCR from the freezer and let thaw.
2. Remove 5X gotaq, 10X(MgCl2), DNTPs, and Primers from freezer and allow them to thaw (**DO NOT** bring out Taq!)
3. Label individual PCR tubes, be sure to include initials, PCR #, and sample #

Note: For example, JRPCR 10.1, JRPCR 10.2, JRPCR 10.3, etc. This stands for JR PCR #10 sample ".n".

4. In a labeled 1.5 mL tube, create the "master mix" to be aliquotted into the individual PCR tubes.

Total amounts: dH_2O	= 7.5µl x (n + 1)
5X	= 2.0µl x (n + 1)
10X	= 1.0µl x (n + 1)
dNTPs	= 1.0µl x (n + 1)
Forward Primer	= 1.0µl x (n + 1)
Reverse Primer	= 1.0µl x (n + 1)
Taq	= 0.10µl (n + 1)

Note: "n" is the total number of samples for the PCR, it is best to add 1 to this number for calculations to make up for any calibration or pippetting errors and to insure that there is enough for a negative control.

Note: Make sure to add Taq last, store Taq in freezer until all other reagents are added, extract proper amount of Taq, add to the mix and immediately place Taq back in freezer.

5. Gently vortex the "master mix" for 2-3 sec, or tip mix thoroughly.
6. Add 13.6 µL of master mix to each PCR tube, making sure to pipette from the middle of the master mix liquid.

Note: Taq naturally settles to the bottom in the mix, pipetting from the top of the liquid will not allow enough Taq into the mixture, pipetting from the bottom will suck up most of the Taq and there will be little or none left for remaining samples.

7. Add 1.0 µL of pertinent extracted DNA to the PCR tubes, making sure to match genomic DNA extractions to the proper sample number.
8. Spin down tubes for 5 sec at ~(3,000 RPM).

Note: No liquid should be beaded up on the sides of PCR tubes nor should there be any bubbles present, it should all be collected at the bottom of the tubes to ensure a proper reaction will take place. Take care when removing PCR tubes from centrifuge and during transport to not splash mixture onto the sides of the tube.
9. Immediately load samples into PCR thermocycler and begin running program, or immediately store in freezer until a thermocycler is available.

Note: PCR reactions can be stored in freezer up to overnight if all machines are in use. When ready to run, simply remove tubes from freezer, put in thermocycler and begin program. The first stage will melt the reaction mixtures, so no pre-thawing is necessary.
10. Load the samples and begin the proper program on the thermocycler.

11. After PCR thermocycling program has been completed, remove tubes, centrifuge tubes for 5 sec at max speed, then store in fridge at 5°C.

Note: Condensation will be present on inside of tube walls. It is important to spin tubes down to collect this condensation at the bottom; otherwise it will evaporate if placed directly into fridge.

PCR DNA Check gel

1. Make 1.0% agarose gel

0.7 grams Agarose, 70mL 1x TAE buffer. Mix and microwave (make sure that the flask doesn't overflow) for 1.5 min until agarose is in solution. Cool by running the flask under cold water for 1 minute. Then add 2µl of Sybr safe (located in the little refridgeator) and pour into casting tray. Push any bubbles to the sides of the tray. Insert combs. Allow 20 minutes for gel to set up.

2. Load samples into wells (you can load the gel wet or dry).

3. Add 3 µl of your sample to each well skipping the first well.

4. The first well add 3 µl of DNA size ladder (also located in the refrigerator).
5. Run samples at 140V until dye runs ~8-10 minutes.
6. Take tray out of rig and take picture with UV camera
7. Make sure to clean up after yourself.

PCR Clean up using Millipore plates

1. Bring the total volume of the PCR reaction to 100 µl with diH20 (88.4 µl).
2. Transfer all 100 µl of diluted PCR reaction to a PCR 96 well Millipore plate.
3. Put the plate on the vacuum manifold for 7-10 minutes (8min is fine) until the wells are empty, making sure pressure gauge is at -22hg.
    a. Wells will appear shiny, so they will look slightly wet all the time.
4. Blot bottom of the plate on a paper towel to remove excess water.
    a. Empty collection plate.
5. Add 100 µl of diH2O to the plate and re-vacuum.
6. Resuspend DNA with 30 µl of diH20.
7. Place plate on shaker/ mixer for 15 minutes (or longer depending on the concentration of the DNA you want)..
8. Pipette product out of the wells and transfer to clean storages tubes (labeled with the PCR number and the sample number) or storage plate.

Sequencing Reactions

	1/8 rxn	1/16 rxn	1/32 rxn
diH20	**4.5 ul**	**6.4 ul**	**7.65 ul**
5x buffer	**1.5 ul**	**2.1 ul**	**2.1 ul**
Primer 10mM	**1.0 ul**	**1.0 ul**	**0.5 ul**
Big dye	**1.0 ul**	**0.50 ul**	**0.25 ul**
DNA (clean PCR)	**2.0 ul**	**2.0 ul**	**1.5 ul**
Total Vol.	**10.0 ul**	**12.0 ul**	**12.0 ul**

Sephadex Cleanup of sequencing reaction

1. Obtain Millipore multiscreen filter plates (96 well).
2. Pour Sephadex on plate loader and fill all holes (except for H6 and H12). Place left over Sephadex back into bottle.
3. Turn filter plate upside down and slide onto plate loader. Tap plate loader to get the Sephadex to fall into the wells.
4. Add 300ul dH2O to each well (make sure the wells are full).
5. Add a used v-bottom 96well plate to the bottom of filter plate making sure rows A-H are aligned on both plates.
6. Let plate stand the plate is running on the thermocycler.
7. Spin plate in centrifuge (using balance plate with water) for 3 minutes at 3700rpm.
8. Empty and rinse v-bottom 96 well plate and place back in cupboard.
9. Add a new (labeled) v-bottom 96 well plate to filter plate making sure rows A-H are aligned on both plates.
10. Add 15ul dH2O to sequencing reaction
11. Transfer all sequencing samples to filter plate
12. Spin plate for 3 minutes at 3700 rpm.
    a. Dump out used columns and rinse filter plate thoroughly with dH2O and let dry. Return balance plate to fridge.
13. Dry samples in Vacufuge at 60º for 45 minutes. Make sure to use balance plate.
14. Cover plate and mark off all unused wells.
15. Write DNA sequence submission number on plate and cover
16. Place plate in DNASC fridge.