BARCODES OF LIFE

DNA BARCODING ANIMAL LIFE USING COI

LABORATORY PROTOCOLS:

DNA EXTRACTION
COI AMPLIFICATION
DNA SEQUENCING

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GENERAL PRACTICES

- Clean the bench top with alcohol **before** and **after** setting up extractions.
- Always clean forceps between specimens. Use a new pestle for each specimen. Dirty pestles can be re-used after soaking in 5N HCl and autoclaving.
- Directly before use, briefly soak forceps and pestles in 5N HCl for 30 sec.
- Rinse forceps and pestles in two separate ddH_2O washes to remove excessive HCl.
- Use small amounts of tissue from single animals (eg. single legs for Lepidoptera, whole animals for daphniids).

FOR SPECIMENS IN GOOD CONDITION

1) Use a simple Proteinase K digestion.
2) Aliquot 50-100 µl (dependent on amount of tissue) of Proteinase K buffer into 1.5 ml microfuge tubes.
3) Add tissue and grind with pestle.
4) Incubate for 24-48 hours at 55ºC.
5) Incubate samples in a 95ºC waterbath for 10 min to denature the Proteinase K enzyme.
6) Use 1-5 µl of DNA samples for PCR.

PROTEINASE K REAGENTS

Extraction Buffer (10 ml)	
1M Tris HCl pH8.3	100 µl
1 M KCl	500 µl
1% Tween 20	50 µl
1% nonidet p40 (NP40)	50 µl
20 mg/ml Pro K	100 µl
ddH_2O	9.2 ml
store frozen in 1 ml aliquots	

Proteinase K (20 mg/ml)	
Proteinase K	20 mg
ddH_2O	0.5 ml
Glycerol	0.5 ml
Store in 100 µl aliquots at –20ºC	

1% Tween 20	
Tween	100 µl
ddH_2O	9.9 ml
Store in 1 ml aliquots at –20ºC	

1% NP40	
NP40	100 µl
ddH_2O	9.9 ml
Store in 1 ml aliquots at –20ºC	

FOR OLD (SAMPLES >10 YEARS) OR DEGRADED SPECIMENS (POORLY PRESERVED)

We use the GenElute Mammalian Genomic DNA Miniprep Kit (Sigma Genosys) with modifications as follows:

Note: Use filter-plugged tips to avoid contamination of samples and reagents. Dilute Wash Solution with 95% ethanol prior to first use, as described in the instruction manual. Although an Elution Solution is provided with the kit for Step #12 (the final elution of DNA from surface of column), it contains EDTA. As EDTA is thought to inhibit PCR, we use ddH_2O instead.

1) Aliquot 180 µl of Lysis Solution T into a 1.5 ml microfuge tube.

2) Add 20 µl of Proteinase K.

3) Add tissue and grind with pestle.

4) Vortex sample. Incubate at 55ºC (dry-bath) for 2-4 hours to allow digestion.

5) While incubating, label 3 sets of regular 1.5 ml microfuge tubes (supplied by kit) and 1 red O-ring column microfuge tube for each specimen.

6) After digestion, add 200 µl of Lysis Solution C to the sample. Vortex WELL and incubate at 70ºC in a dry-bath for 10 minutes.

7) While incubating, add 500 µl of Column Preparation Solution to the red O-ring column microfuge tube and spin at 12000 g for 1 minute. Discard the flow-through. Replace red O-ring column into the same microfuge tube.

8) Following the 70ºC incubation, add 200 µl of 95% ethanol (molecular biology grade) to sample. Vortex well.

9) Transfer sample (about 600 µl) into the red O-ring column microfuge tube. Spin at 8000 g for 1 minute. Place red O-ring column into a new microfuge tube. Discard old tube.

10) Add 500 µl Wash Solution to red O-ring column micorfuge tube. Spin at 8000 g for 1 minute. Place red O-ring column into a new microfuge tube and discard old tube.

11) Add 500 µl Wash solution to red O-ring column microfuge tube. Spin at 16000 g for 3 minutes. Columns MUST be free of ethanol. If residual ethanol is present on surface of column, re-spin for an additional 1 minute. Place red O-ring column into the final microfuge tube and discard old tube.

12) Add 30 µl ddH_20 to red O-ring column and incubate at room temperature for 5 minutes.

13) Spin at 8000 g for 1 minute to elute DNA. Discard red O-ring column. Keep DNA at –20ºC for long-term storage.

14) Use 1-5 µl of the DNA sample for PCR. Start at 3 µl and if this doesn’t work, try 1 µl and 5 µl.

GENERAL PRACTICES

- The basic recipe for a Polymerase Chain Reaction (PCR) is given in the table at the right. The amount of DNA used will depend on the concentration of the sample. It is best to keep the volume of DNA template as low as possible to avoid adding enzyme inhibitors that may be present, and to avoid illegitimate amplification of excess DNA.
- The use of plugged tips is recommended for all PCR reagents to avoid contamination. Clean the bench top with alcohol before setting up reactions.
- Always use a clean tip when removing Taq polymerase and the other reagents from their tubes.
- Keep DNA templates (i.e. other PCR products) away from the PCR reagents while you are setting up the reaction mixes. Add DNA after all of the reagents have been returned to the freezer.
- Always include a sample without template as a negative control to check for contamination of the reagents. Include a positive control (a DNA sample that has amplified in the past) as well to test the effectiveness of the PCR reagents.
- The concentration of $MgCl_2$ will depend on the primers. Use the lowest concentration of $MgCl_2$ possible to give the cleanest product. The minimum concentration is 1.5 mM.
- Recipes for the individual reagents are given on the following page.

PCR REACTION MIX

Volumes for One Reaction (50 µl total)	
H_2O + DNA	41.5 µl
10X PCR Buffer	4.5 µl
50 mM $MgCl_2$ (2.5 mM)	2.5 µl
10 mM dNTP	0.25 µl
10 µM Primer 1 (5 pmol)	0.5 µl
10 µM Primer 2 (5 pmol)	0.5 µl
Taq polymerase	0.2 µl

MULTIPLE SAMPLES

When doing multiple reactions with the same primer set, make a master mix that does not contain the DNA templates.

Example: you want to amplify 2 µl of 20 DNA samples in a 50 µl reaction. Remember to include one reaction for the negative control and "one extra for the pot". It is OK to round up volumes of dNTPs, primers and Taq to the nearest 0.5 µl.

Volumes for 22 Reactions		
	1 Tube	22 Tubes
DNA	2 µl	----
H_2O	39.5 µl	869 µl
10X PCR Buffer	4.5 µl	99 µl
50 mM $MgCl_2$	2.5 µl	55 µl
10 mM dNTP	0.25 µl	5.5 µl
10 µM Primer 1	0.5 µl	11 µl
10 µM Primer 2	0.5 µl	11 µl
Taq polymerase	0.2 µl	4.5 µl

Aliquot 48 µl of the mix to each individual reaction tube and then add 2 µl of template.

PCR REAGENTS

Ultrapure H_2O
Store ultrapure H_2O frozen in 1 ml aliquots. Use a new, sterile filter-plugged tip to aliquot the water. Aliquot the entire bottle of water when it is first opened.

10X PCR Buffer

2 M Tris-HCl pH 8.3	5 ml
KCl	3.73 gm
ultrapure H_2O	to 100 mls

store frozen in 1 ml aliquots

10 mM dNTP Mix

100 mM dGTP	10 µl
100 mM dATP	10 µl
100 mM dTTP	10 µl
100 mM dCTP	10 µl
ultrapure H_2O	60 µl

store frozen in 100 µl aliquots

1 M $MgCl_2$

anhydrous $MgCl_2$	9.52
ultrapure H_2O	to 100 ml

use entire contents of a fresh bottle of $MgCl_2$, store frozen in 10-50 ml aliquots

50 mM $MgCl_2$

1 M $MgCl_2$	2 ml
ultrapure H_2O	39 ml

store frozen in 1 ml aliquots

PRIMERS

100 µM Stock
Dissolve desiccated primer in: number of nmol x 10 µl ultrapure H_2O

eg. dissolve 50 nmol primer in 500 µl H_2O

10 µM Working Stock
Dilute 100 µM primer stocks to 10 µM with ultrapure H_2O and store frozen in 100 µl aliquots

e.g. Add 10 µl of 100 µM primer stock to 90 µl ddH_2O

TIPS FOR PRIMER DESIGN

- Primers should be between 20-30 nt in length
- Avoid complementarity within and between primers
- The GC content should be approximately 50%
- Avoid mono- or dinucleotide repetition within primers
- The primer should end on a G or a C
- Primers within protein coding regions should end on the second (or first if necessary) position of a codon
- The melting temperatures of primer pairs should be within 5°C of one another
- To design COI primers for a particular taxonomic group, try aligning as many COI genes from closely related taxa as possible (try surfing GenBank) to the desired species group. Design primers that are situated in regions that are conserved across all taxa

PCR THERMOCYCLE PROGRAM

- A typical PCR program on the **MJ PTC100** thermocycler is shown at the right.
- Step 1 is an initial 94ºC soak to completely denature the original template, particularly if it is genomic DNA.
- Step 2 is the denaturing step.
- Step 3 is the annealing step whose temperature will depend on the sequence of the primers. For COI, it is ideal to begin annealing at a low temperature (45ºC) for a few initial cycles to allow the primers to bind to the template and then raise the temperature (51ºC) to avoid excessive non-specific binding of primers.
- Step 4 is the extension step whose time depends on the length of the product. Generally, extension steps should be at least 1 min/1000 bp.
- Step 5 repeats steps 2, 3, and 4 five more times.
- Steps 6, 7, 8, and 9 denature, anneal at 51ºC, and extend for 36 cycles.
- Step 10 is a soak at 72ºC that will allow the Taq polymerase to complete any unfinished products.
- Step 11 is a 4ºC soak that will last "forever" until someone cancels the program.

MJ PTC100 PCR Program for COI

Step	Action	Time
1	94°C	1 min
2	94°C	30 sec
3	45°C	1 min, 30 sec
4	72°C	1 min
5	GOTO step 2	5 more times
6	94ºC	30 sec
7	51ºC	1 min, 30 sec
8	72ºC	1 min
9	GO TO step 6	35 more times
10	72°C	5 min
11	4°C	00:00:00

AGAROSE GEL ELECTROPHORESIS

CAUTION: The gel will contain the mutagen Ethidium bromide. Gloves and a lab coat should be worn when handling and loading the gel. Take care not to contaminate countertops and sinks when handling the gel.

1) 1. Determine the total volume and concentration of gel required. For COI (~700 bp), use a 1% gel.

Recommended gel composition for resolution of linear DNA	
% Gel	**Optimum resolution in bp**
0.5	1000-30000
0.7	800-12000
1.0	500-10000
1.2	400-7000
1.5	200-3000
2.0	50-2000

2) Add the appropriate amount of agarose and 1X TBE buffer to a pyrex flask and swirl.

Example: 100 ml of 1.0% agarose

1X TBE	100 ml
Agarose	1.0 gm

3) Heat the agarose mixture in the microwave. Allow about 2 min for a 50-100 ml gel and 3-4 min for larger gels. Swirl the mixture and check to make sure that ALL of the agarose has melted. There should be no lumps or particles.

CAUTION: When you first remove the flask from the microwave, steam may escape explosively from the liquid, which may cause burns.

4) Allow the agarose to cool for several minutes and add 1 drop (2 ul) of Ethidium bromide (4 mg/ml) to it. Mix well.

5) Pour the agarose solution into a sealed gel tray and insert a comb at one end of the tray to form the sample wells.

6) After the gel has completely hardened, carefully remove the comb.

7) Pipette the samples into the wells, being careful not to puncture or tear their edges. Include the blue dye Bromophenol in at least one lane so that you can monitor the progress of the gel run. If necessary, include a size standard (for example, lambda DNA digested with the Hind III enzyme) mixed with the blue dye Bromophenol in at least one lane.

8) Place the gel into the electrophoresis chamber and carefully submerse it in 1X TBE running buffer. Do not pour the buffer directly onto the sample wells. There should be a 2-4 mm layer of buffer over the gel.

9) Connect the electrical leads to the electrophoresis chamber. Remember to ***run to red***. That is, connect the black lead to the end of the gel containing the sample wells. The DNA samples, which are negatively charged, should migrate towards the red (positive) lead.

10) Turn on the power supply and adjust the voltage level as follows:

Gel Size	**Maximum voltage**
50 ml	100
100 ml	100
$\geq$ 200 ml	125

11) Allow the bromophenol blue marker dye in the standard lane to migrate within 2-4 cm from the end of the gel.

12) TURN OFF THE POWER SUPPLY and disconnect the leads.

13) The gel is now ready to be photographed. Wear goggles to protect eyes from UV exposure.

SOLUTIONS FOR RUNNING AGAROSE GELS

10 X TBE	
Tris Base	108 gm
Boric acid	55 gm
Na_2EDTA	9.3 gm
H_2O	to 1 L
Adjust to pH 8.3	

Ethidium bromide
4 mg/ml

Agarose Gel Dye (Bromophenol Blue)	
0.5 M EDTA pH 8	200 µl
Bromophenol blue	2 mg
H_2O	to 5 mls

Size Standard: Lambda DNA digested with HindIII (or buy pre-made commercial)	
Lambda DNA (0.5 µl)	180 µl
10 X HindIII buffer	20 µl
HindIII	2-5 µl
Digest for 3 hours in a 37°C water bath. Ethanol precipitate and resuspend in 250 µl H_2O. Add 50 µl of digest to 1 ml Bromophenol blue dye. Final [DNA] is 18 µg/ml. Load 10-20 µl of size standard per well.	

CLEANING OF COI PCR PRODUCT

- Following PCR amplification, prepare a 1% TBE agarose gel.
- Load 10 µl of PCR products into wells and run the gel.
- NOTE: DO NOT add the blue dye bromophenol to PCR samples, as it will inhibit direct sequencing. Load the dye in a separate lane.

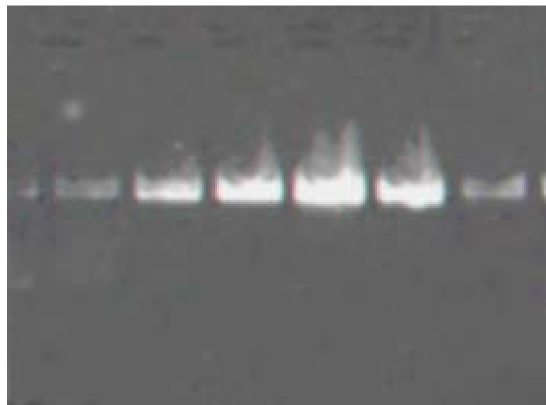

Photo conditions: 1.0 exposure, 0 black, 90 white, 150 gamma.

Figure: A gel image of COI PCR products. All lanes contain 10 µl of a 50 µl PCR reaction. All of these COI PCR products can be sequenced directly. For weak bands (lanes 1, 6), use 4-5 µl of COI PCR product in the sequencing reactions. For medium bands (lane 2), use 2-3 µl and for strong bands (lanes 3-5), use 1.5 µl.

- For samples showing clean, discrete PCR products (all lanes in above figure), proceed directly to sequencing (page 3.4).
- However, for samples with multiple bands or excessive smearing, excise the desired fragment from a gel under long wave (nm) ultraviolet light and purify using a kit (see page 3.3).

CLEANING OF COI PCR PRODUCT (CONT.)

We use the **QIAEX II Agarose Gel Extraction Kit (Qiagen)** to gel-purify PCR samples with modifications as follows:

1) Electrophorese the remaining PCR product (~40µl) on a subsequent gel.
2) Excise the desired band and place into a 1.5 ml microfuge tube.
3) Add 500 µl of Buffer QX1.
4) Vortex glassmilk suspension WELL (30 seconds) and add 7 µl to samples.
5) Incubate samples at 55ºC for 1 hour to melt agarose.
6) Spin samples at 13000 rpm for 1 minute.
7) Pour off supernatant. Add 500 µl of Buffer PE and resuspend the pellets by vortexing.
8) Spin samples at 13000 rpm for 1 minute.
9) Pour off supernatant. Add 500 µl of Buffer PE and resuspend the pellets again by vortexing.
10) Spin samples at 13000 rpm for 1 minute.
11) Pour off supernatant. Invert tubes and air-dry pellets for 15-20 minutes.
12) Add 8 µl of ddH_20, resuspend pellets and incubate at 55ºC for 10 minutes.
13) Spin samples at 13000 rpm for 1 minute. Pipet 7 µl of supernatant into clean microfuge tubes. Place aside.
14) Add 7 µl of ddH_20 to pellets, resuspend and incubate at 55ºC for an additional 10 minutes.
15) Spin samples at 13000 rpm for 1 minute. Pipet 6 µl of supernatant into the final microfuge tubes.
16) Electrophorese 3 µl of Qiaex cleaned products on a 1% TBE agarose gel to estimate its concentration.
17) Use 1-5 µl (dependent on COI band intensity) of purified PCR product in the sequencing reaction (next section).

DYE TERMINATOR SEQUENCING OF COI FOR THE ABI PRISM 377

- DNA must be very clean for good results.

- Set up a sequencing reaction according to the table below. Use **8 ng/100 bases** of DNA for Qiaex cleaned and direct PCR products.

Reagent	**COI (~700 bp)**
Dye terminator mix **v3.1**	2 µl
2.5 X Sequencing Buffer	1 µl
10 µM Primer	1 µl
DNA + H_2O	5 µl
Final Volume	10 µl

Note: 2.5X Sequencing buffer is: 200 mM Tris-HCl pH 9 + 5 mM $MgCl_2$.

- Run the sequencing reactions in a thermocycler under the conditions shown below. The annealing temperature can be varied according to the primer specificity but 55ºC works well for most COI sequencing reactions.

PCR Program for COI Sequencing		
Step	**Action**	**Time**
1	96°C	1 min
2	96°C	10 min
3	55°C	5 sec
4	60°C	4 min
5	GOTO step 2	29 more times
6	4°C	00:00:00

- Submit samples to an ABI sequencing facility.