

## DNA sequencing protocols BN Danforth, 6/7/2004

**Protocol for DNA extraction from small Hymenoptera** (Modified from Saghai-Marooof, et al. 1984. Ribosomal DNA spacer-length polymorphism in barley. Proc. Natl. Acad. Sci. USA 81:8014-8018, Steve Rissing and Ulrich Mueller).

### A.. Homogenization.

You will need: 2x CTAB, proteinase K (removed from freezer, thawed and thereafter kept on ice), disposable plastic pestels (autoclaved)<sup>1</sup>.

- (1) Place specimens individually into 1.5 ml. Eppendorf tubes on ice. Dip specimens in liquid nitrogen and place back into tube.
- (2) Shake the specimen to about the midpoint of the tube and insert a disposable plastic pestel crushing the specimen against the side of the tube. Grind the specimen until all the larger parts are mushed.
- (3) Add 350  $\mu$ l 2x CTAB to the tube and continue to grind the specimen. Add another 350  $\mu$ l 2x CTAB to wash off the pestel.
- (4) Add 10  $\mu$ l Proteinase K (10mg/ml stock) to the mixture.
- (5) Repeat for all specimens.
- (6) Incubate all the tubes at 55 °C for 1.5 to 2 hours. Gently stir the contents of the tube occasionally. Over incubation (i.e. 6 hours) clearly results in the degradation of DNA.

### C. Extraction and RNA digestion.

You will need: Phenol:Chloroform: Isoamylalcohol (25:24:1) [=PCI], Chloroform:Isoamylalcohol (24:1) [=CI], 10 mg/ml RNase (DNase free!).

- (1) Add 650  $\mu$ l CI to mixture and gently invert several times. This can be done in bulk by placing tubes in a covered box and inverting the box 15-20 times. **Do not vortex the tubes.** Spin in centrifuge at 13,000 RPM for 15 mins. Remove tubes and set a 20-200  $\mu$ l pipette to 180  $\mu$ l and carefully transfer the supernatant to another labelled tube without taking up the interface. You can remove almost all of the supernatant if you are careful. Use blunt pipette tips to avoid shearing the DNA.
- (2) Add 1  $\mu$ l RNase, and incubate for 30 mins at 37 °C to digest the RNA.
- (3) Add 650  $\mu$ l PCI to mixture and gently invert several times. Spin in centrifuge 15 mins. Transfer the supernatant to another labelled tube as before.
- (3) Add 650  $\mu$ l CI to mixture and gently invert several times. This step removes the phenol from the previous extraction. Spin in centrifuge 15 mins. Again transfer supernatant to another labelled tube.

### D. Precipitation

You will need: cold (-20) 100% EtOH, 3M Na-acetate, TE buffer.

- (1) The remaining aqueous phase of the extraction (with the DNA) should be about 400  $\mu$ l. Add 2.5 volumes (1000  $\mu$ l) cold 100% EtOH and 1/10 volume (40  $\mu$ l) 3M Na-

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<sup>1</sup> We get the disposable pestels from VWR Scientific, P.O. Box 1050, Rochester, NY, 14603; 716-247-0610 (cat. no. KT749520-0000)

acetate. This will give a final volume of about 1.5 ml. **Invert the tubes 10-20 times to mix the ethanol and Na-acetate.** Place the tubes in the -20 freezer overnight.

- (2) Spin down DNA 30 min. at 13,000 RPM at 4 C. Place tubes in ice and aspirate supernatant leaving pelleted DNA in the bottom of each tube. Do not disturb the pellet if possible.
- (3) Wash pellet in 1000  $\mu$ l cold 80% EtOH. [Make this up just before it is needed as the concentration will change if you make it up in advance.].
- (4) Spin again at 4 C for 20 minutes. Place tubes in ice. Remove all the supernatant without disturbing the pellet.
- (5) Spin for 3-5 min. in the vacuum centrifuge. The DNA pellets should not become totally dry. Ideally they will appear gelatinous.
- (6) Resuspend pellet in 50  $\mu$ l TE buffer.
- (7) Let tubes sit in the refrigerator overnight to allow the DNA to go into solution.
- (8) Measure concentration with Fluorometer or run a small aliquot (5-10  $\mu$ l) out on a 0.8% agarose gel to confirm that you have good, clean, high molecular weight DNA.

**2x CTAB extraction buffer:**

0.1 M Tris-HCl (pH 8.0)	25 ml 1M Tris-HCl (pH 8.0)
1.4 M NaCl	20.45 g NaCl
0.02 M EDTA	10 ml 0.5 M EDTA
2% CTAB	5 g CTAB
	0.5 ml 2-mercaptoethanol*
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	to 250 ml. w/ dH <sub>2</sub> O.

The CTAB will be hard to get into solution and may take several hours of stirring. It will go faster if you heat up the whole mixture slightly.

\*Add the mercaptoethanol last after buffer has come to room temp.

**Notes:**

A number of steps in this protocol are very important and result in substantial increases in yield.

- (1) Do not alter the volumes of original extraction buffer because you are working on a small bug rather than a big bug. The larger the volumes used the higher the yield no matter how much DNA you are able to remove. If you scale down the volumes you will only end up losing **more** DNA at each extraction.
- (2) The use of 2x CTAB results in a two-fold increase in DNA yield over 1x CTAB.
- (3) The initial CI extraction is very important, even though all other protocols usually call for a phenol or PCI step initially. The first CI step increases DNA yield by two-fold over any other possible combination of extraction steps.
- (4) A number of potential contaminants should be removed prior to extraction. If extracting females it may be necessary to dissect pollen from the crops to avoid contamination from plant DNA. Also, large hymenops may have enough eye pigments to really contaminate the extracted DNA. This will become apparent if the DNA pellets after ethanol precipitation are dark brown or black. To avoid this problem you can lop

the heads off prior to grinding.

The following DNA yields should serve as guidelines for success:

<i>Drosophila pseudobscura</i>	1 µg	
<i>Musca domestica</i>	2 µg	Blanchetot, 1991a
<i>Augochlorella striata</i>	5-12 µg	Mueller, 1992
<i>Perdita portalis</i>	1-3 µg	Danforth, pers. obs.
<i>Perdita texana</i>	3-5 µg	Danforth, pers. obs.
<i>Megachile rotundata</i>	2-8 µg	Blanchetot, 1992
<i>Apis mellifera</i> (workers)	2.4 µg	Blanchetot, 1991 b
<i>Apis mellifera</i> (workers)	5-7 µg	Danforth, pers obs.
<i>Apis mellifera</i> (queens)	8 µg	Blanchetot, 1991 b

### Protocol for PCR amplifications.

Since end primers work best on the automated sequencer it is best to amplify fragments of a size that can be completely sequenced from either end. Since the readable portion of the automated sequencing output is about 500 bp, We recommend amplifying fragments of from 600 to 800 bp in length and sequencing from both ends. You can use *Apis mellifera* as a positive control for most of our primers.

PCR reaction (per tube)  
11.9  $\mu$ l dH<sub>2</sub>O  
2.5  $\mu$ l 10x buffer  
2.5  $\mu$ l MgCl<sub>2</sub> (25 mM)  
5.0  $\mu$ l dNTPs (1mM each)  
1  $\mu$ l DNA<sup>1</sup>  
1  $\mu$ l primer + (~16: M)<sup>2</sup>  
1  $\mu$ l primer - (~16: M)  
0.1  $\mu$ l Taq (Promega @ 5 units/: l)  
  
25  $\mu$ l total

<sup>1</sup>For most DNA extractions I simply dilute the stock DNA solution (in 50  $\mu$ l) 1:10 with TE. Dilutions down to 1:100 seem to work equally well and I have gotten detectable PCR products with 1:1,000,000 (one in a million) dilutions of the original DNA stock solution.

<sup>2</sup>After synthesis of primers we dilute them in sterile HPLC water to 1  $\mu$ g/ $\mu$ l, aliquot 20-50  $\mu$ l into 0.5 ml Eppendorfs, and dry down, giving 20-50  $\mu$ g per tube. Primers are most stable when dry. We then redilute single tubes as needed with water to 75 ng/ $\mu$ l for a 15-mer, 100 ng/ $\mu$ l for a 20-mer, 125 ng/ $\mu$ l for a 25-mer, etc. Use 1  $\mu$ l of this working stock for each PCR reaction.

Make up a cocktail of all the ingredients you can combine at once (e.g., 10x buffer, MgCl, dNTPs, water and Taq) and aliquot the appropriate amount to each tube. Add the remaining ingredients (e.g., primers and template DNA) and overlay with one drop of mineral oil (Sigma M-5904)

We use the following thermal cycler conditions for amplifications:

1. 94 C, 45 sec. <---- initial denaturation
2. 94 C, 1 min.
3. 50-60 C, 1 min (anneal temp is dependent on the particular primer pair used. See Tables 1 and 2 for optimal annealing temps.)
4. 72 C, 1 to 1½ mins. (depending on the length of the product; expect 1 min. per 1000 bp).  
[35 cycles of steps 2,3,4]

5. 4 C, for holding the tubes after running the reactions.

Following PCR reaction, run 5-10  $\mu$ l out on a minigel along with the Boehringer Mannheim molecular weight marker X.

For sequencing we typically need around 70ul, so scale up the above reaction by three-fold and you will have enough DNA for gel purification.

**Gel purification of PCR products for sequencing** (Promega Wizard PCR preps DNA purification system).

The best sequencing results are obtained by using the same primers you used for the amplification for sequencing (=end primers). This assures a 100% match between PCR template and primer. We use two protocols for preparing PCR products for sequencing: (1) direct purification (which removes primer-dimers only) and (2) gel-purification (which allows you to cut out exactly which band you want to sequence). Some primer pairs produce multiple bands and when that is the case we recommend using gel-purification for the best results. For direct purification, follow the instructions in the Promega Wizard PCR preps DNA purification system manual. Follow the protocol below for gel purification using the Promega Wizard PCR preps DNA purification system (cat. no. A7170; \$1.50 per prep).

(1) Make a 1.2% agarose gel with a 1:1 ratio of standard agarose and low melting point agarose (FMC SeaKem LE. cat. no.50003 and NuSieve GTG. cat. no. 50082). For a large gel 1.96g of each agarose (3.92 g total)/327 mls 1x TBE. Add 15 ul Ethidium bromide to the gel after cooling but before pouring. After the gel hardens place the gel in the fridge for 30 minutes to get the gel really firm. Use the large-toothed combs (1.5 mm wide x 15 mm long; 12 teeth per comb) which allow you to load up to 100 ul per lane. [NOTE: It is important to use high quality agarose because contaminants in the agarose will really mess up the sequencing reactions, according to the ABI representative. FMC agarose works well]

(2) Transfer 70 ul from each PCR reaction into a 1.5ml Eppendorf. (If you started with a larger volume of PCR product you can use a vacuum centrifuge to reduce the volume to roughly 70 ul.) Add 15 ul dye before loading. Run the gels in the cold room (4 C) at low voltage (~55 volts for a large gel rig/16 hrs.) for best band resolution. The longer the run the better you will separate the fragments.

(3) Wash the gel briefly in H<sub>2</sub>O and then place on UV light table and, with the light setting on low, excise each band and place in the corresponding pre-labelled Eppendorf (we use the larger, 2.2ml, Eppendorf tubes). After a series of experiments, we have found the gel-slices weigh about 500 ug (equal to 500 ul in volume), so we just assume all the gel-slices weigh 500 ug and skip the weighing step

(4) Follow the Promega Wizard PCR preps DNA purification system protocol:

- i. Melt the gel slices at 70°C until completely melted.
- ii. Add 1 ml of Wizard PCR resin to the melted agarose; mix for 20 seconds (but do not vortex). Mix the resin well before use!
- iii. Attach a syringe barrel to the minicolumn and insert the minicolumn into the vacuum manifold. With the valve open, slowly add the 1400 ul of resin/agaose mix.
- iv. After all of the resin/agarose mix has passed through the column, wash the barrel with 2 mls of 80% isopropanol. Run the vacuum for about 20 seconds after all the isopropanol has passed through.
- v. Transfer the column to a new 1.5 ml Eppendorf and spin 2 mins. at 10,000 rpm to remove residual isopropanol.

- vi. Transfer the column to a new 1.5 ml Eppendorf and add 40 ul of sterile 10 mM Tris (pH 7.6). Do not use Tris-EDTA, as EDTA will interfere with sequencing reactions. Let the column sit for about 10-15 minutes and then spin for 1 min. at 10,000 rpm to elute the DNA.
- vii. Run out 5 ul of each sample on a 1.2% Synergel along with the Gibco Mass ladder to determine the concentration and purity of the resulting DNA. Each tube should give a nice clean, reasonably bright band.

### **Sequencing**

Once you have gel-purified the fragments and they are in 50 ul Tris, you need to calculate the concentration on the fluorometer or by running on a gel with a molecular weight and mass ladder. How you do the sequencing will determine what concentration you ultimately need. We use an ABI 3700 at Cornell and can sequence bands down to about 4 ng/ul. Ideally shoot for bands in the range of 10-20 ng/ul.

Appendix 1 -- **Useful recipes:**

1M Tris, pH 7.6

2.78 g Tris-base  
12.12 g Tris-HCl

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100 mls total (check pH to confirm)

1M Tris, pH8.0

5.30 g Tris-base  
8.88 g Tris-HCl

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100 mls total (check pH to confirm)

Tris-EDTA buffer, pH 7.6

10 mM Tris  
1 mM EDTA

5 mls 1M Tris, pH 7.6  
1 mls 0.5 M EDTA, pH 8

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500 mls

Making DNase free RNase (10 mg/ml)

RNase dilution buffer

10 mM Tris, pH 7.6  
15 mM NaCl

1 ml 1M Tris, pH 7.6  
1.5 ml 1M NaCl (5.84 g/100 mls)

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100 mls total

Add 10 mg RNase to 1 ml RNase dilution buffer (=1 ml total) and distribute 50  $\mu$ l to each of 20 tubes. Heat tubes in boiling water bath for 15 mins and let cool slowly to room temp before freezing at -20 C.

## Protocol for the Promega Wizard minipreps:

### 1. Gel purification

- ! Melt the gel slices at 70°C until completely melted (about 10 mins).
- ! Add 1 ml of Wizard PCR resin to the melted agarose; mix for 20 seconds (but do not vortex).
- Mix the resin well before use!**
- ! Attach a syringe barrel to the minicolumn and insert the minicolumns into the vacuum manifold. With the valve open and the vacuum off, add the 1400 ul of resin/agarose mix to each spin column.
- ! After all of the resin/agarose mix has been added to each spin column apply the vacuum (turn on the vacuum pump). When the last one goes through the spin column, turn off the vacuum.
- ! Add 2 mls of 80% isopropanol to each spin column. Apply vacuum again and monitor the spin columns as the alcohol gradually goes through. Once the alcohol has passed through each spin column shut the valve on that spin column. When all the spin columns have been washed with alcohol, turn off the vacuum pump.
- ! Transfer the column to a new 1.5 ml Eppendorf and spin 2 mins. at 10,000 rpm to remove residual isopropanol.
- ! Transfer the column to a new 1.5 ml Eppendorf and add 40 ul of sterile 10 mM Tris (pH 7.6). Do not use Tris-EDTA, as EDTA will interfere with sequencing reactions. Let the column sit for about 10-15 minutes and then spin for 1 min. at 10,000 rpm to elute the DNA.
- ! Run out 5 ul of each sample on a 1.2% Synergel along with the Gibco Mass ladder to determine the concentration and purity of the resulting DNA.

### 2. Direct PCR purification

- ! Add 100 ul of Direct Purification Buffer to each PCR reaction. Vortex briefly to mix.
- ! Add 1 ml of Wizard PCR resin to the tube and vortex briefly three times over a one minute period. **Mix the resin well before use!**
- ! Attach a syringe barrel to the minicolumn and insert the minicolumns into the vacuum manifold. With the valve open and the vacuum off, add the 1400 ul of resin/agarose mix to each spin column.
- ! After all of the resin/agarose mix has been added to each spin column apply the vacuum (turn on the vacuum pump). When the last one goes through the spin column, turn off the vacuum.
- ! Add 2 mls of 80% isopropanol to each spin column. Apply vacuum again and monitor the spin columns as the alcohol gradually goes through. Once the alcohol has passed through each spin column shut the valve on that spin column. When all the spin columns have been washed with alcohol, turn off the vacuum pump.
- ! Transfer the column to a new 1.5 ml Eppendorf and spin 2 mins. at 10,000 rpm to remove residual isopropanol.
- ! Transfer the column to a new 1.5 ml Eppendorf and add 40 ul of sterile 10 mM Tris (pH 7.6). Do not use Tris-EDTA, as EDTA will interfere with sequencing reactions. Let the column sit for about 10-15 minutes and then spin for 1 min. at 10,000 rpm to elute the DNA.
- ! Run out 5 ul of each sample on a 1.2% Synergel along with the Gibco Mass ladder to determine the concentration and purity of the resulting DNA.

Note: after using the vacuum pump, run it for approximately 5 minutes to dry out the inside of the pump.