DNA sequencing protocols
BN Danforth
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Note: for updated information on genes, primers and PCR conditions, see the information on our lab website: http://www.danforthlab.entomology.cornell.edu/resources.html

Protocol for DNA extraction from small Hymenoptera (Modified from Saghai-Maroof, 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).

(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 µl 2x CTAB to the tube and continue to grind the specimen. Add another 350 µl 2x CTAB to wash off the pestel.
(4) Add 10 µl Proteinase K (10mg/ml stock) to the mixture.
(5) Repeat for all specimens.
(6) Incubate all the tubes at 55 ^{o}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.
(7) Spin the tubes down at the end to remove condensation from tops of tubes.

B. Extraction and RNA digestion.

NOTE: We now skip the Rnase step.

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

(1) Add 650 µl 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 µl pipette to 180 µl 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 µl RNase, and incubate for 30 mins at 37 ^{o}C to digest the RNA.
(3) Add 650 µl 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 µl 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.

C. Precipitation

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

(1) The remaining aqueous phase of the extraction (with the DNA) should be about 400 µl. Add 2.5 volumes (1000 µl) cold 100% EtOH and 1/10 volume (40 µl) 3M Na-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 µl 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 µl TE buffer.
(7) Let tubes sit in the refrigerator overnight to allow the DNA to go into solution.
(8) Measure concentration with the Nanodrop or run a small aliquot (5-10 µl) 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*
	to 250 ml. w/ dH2O.

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.

Making Na-acetate: For 3M sodium acetate, mix 12.24 g Na-acetate in 30 mls distilled water. This can be made in a blue Falcon tube.

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 yeild no matter how much DNA you are able to remove. If you scale down the volumes you will only end up loosing **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:

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

Protocol for PCR amplifications [see sample PCR data sheet below].

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 800 bp, We recommend amplifying fragments of from 800 to 1200 bp in length and sequencing from both ends. You can use *Apis mellifera* as a positive control for most of our primers.

A PCR reaction form is printed below.

PCR reaction (per tube)
5.2 µl dH2O
3.0 µl 5x buffer
1.5 µl MgCl2 (25 mM)
3.0 µl dNTPs (1mM each)
1 µl DNA[1]
0.6 µl primer [2] + (~16 µM)
0.6 µl primer - (~16 µM)
0.075 µl Taq (Promega GoTaq [3] @ 5 units/µl)
_____

15 µl total [*we typically run 25 µl for sequencing*]

[1] For most DNA extractions I simply dilute the stock DNA solution (in 50 µl) 1:10 with water. 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.

[2] See Appendix 3 below for notes on primer mixing and concentrations. We generally assume the primers are around 16 µM in concentration.

[3] Promega Go-Taq Flexi DNA polymerase Promega M8291

Make up a cocktail of all the ingredients you can combine at once (e.g., 5x buffer, MgCl, dNTPs, primers, water and Taq) and aliquot the appropriate amount to each tube. Add the remaining ingredients (e.g., template DNA) and overlay with one drop of Dnase/RNase-free 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.

* Note: sometimes difficult-to-amplify reactions can be greatly improved by increasing the extension step by 30 sec.

Following PCR reaction, run 5-10 µl out on a 1.2% minigel along with the Bio-Rad Precision Molecular Mass Ruler (Bio-Rad cat. #: 1708207, 250mg, $101.00).

PCR product clean-up

There are a variety of methods for cleaning up the PCR products (i.e., removing the unincorporated dNTPs, primers, and PCR buffers). The simplest method is Exo-SAP followed by the Wizard miniprep protocols outlined below. For difficult to sequence fragments and for genes in which you need to size-select the bands, you will need to use gel purification. We start with the simplest protocol below.

EXO-SAP protocol

This protocol works well when you have bright, uniform PCR products of approximately the same length (e.g., 28S). You can use this in a 96-well format to purify a lot of samples very fast. Sequencing results appear to be comparable to PCR products purified with the Promega Wizard spin columns.

The following protocol will purify **8 µl** PCR product. Scale up for larger PCR-volume.

1. Transfer 8 µl of the PCR-product into new 0.2 ml PCR-tubes

2. Add 3-5 µl EXO-SAP cocktail (see below) to each sample. Use 3 µl for rather weak PCR-products and 5 µl for strong PCR products.

3. Put in Thermocycler using the following incubation program ("EXOSAP" in "CHRIS" directory)

30 min at 37 ° C
20 min at 80 ° C

4. The samples can now be used directly for sequencing. To estimate DNA concentration, use the gel estimations before the purification step and divide them by 1.6. Alternatively, because it is generally better to add too little DNA than too much DNA, just use the concentrations directly from the original PCR reactions.

Mix for 1 sample, **5 µl** EXO-SAP	Cocktail (for 100 rxts)	
DDH2O	3.6	360 µl
5x PCR buffer	1.0	100 µl
0.2 EXO (stock solution, 20u/ul)	0.2	20 µl
0.2 SAP (stock solution, 1u/ul)	0.2	20 µl
Total	5 µl	500 µl [add 5 µl/rxt]

Mix for 1 sample, **3 µl** EXO-SAP

DDH2O	2.0	200 µl
5x PCR buffer	0.6	60 µl
0.2 EXO (stock solution, 20u/ul)	0.2	20 µl
0.2 SAP (stock solution, 1u/ul)	0.2	20 µl
Total	3 µl	300 µl [add 3 µl/rxt]

Note: when you use the EXO-SAP protocol you are diluting your sample down slightly and that this should be taken into consideration when setting up the cycle sequencing reactions (see below).

Exonuclease I (E. coli): New England BioLabsTM Inc., M0293S

Shrimp Alkaline Phosphatase: USB Corporation, 70092Y

Two additional methods rely on the Promega Wizard PCR preps DNA purification system. For these methods you will need the following Promega products:

Wizard minicolumns	A7211	1 (250)	$178.20
Wizard PCR Preps DNA Purification Resin	A7181	1 (250 mls)	$186.30
Wizard PCR Preps Direct purification buffer	A7241	1 (25 mls)	$35.10

Direct purification of PCR products for sequencing (via Promega Wizard PCR preps DNA purification system).

This is a good protocol if the EXO-SAP protocol does not seem to be working. This method works well with single PCR products (i.e., a single, sharp band). However it is significantly more time-consuming than the EXO-SAP protocol and you cannot do it in a 96-well plate format.

1. Add 100 µl of Direct Purification Buffer to each PCR reaction. Vortex briefly to mix.
2. 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!**
3. Attach a syringe barrel to the minicolumn and insert the minicolums into the vacuum manifold. With the valve open and the vacuum off, add the 1400 µl of resin/agaose mix to each spin column.
4. 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.
5. 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, turm off the vacuum pump.
6. Transfer the column to a new 1.5 ml Eppendorf and spin 2 mins. at 10,000 rpm to

remove residual isopropanol.

7. Transfer the column to a new 1.5 ml Eppendorf and add 40 µl 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.
8. Run out 5 µl of each sample on a 1.2% Synergel along with the Bio-Rad Precision Molecular Mass Ruler (Bio-Rad cat. #: 1708207) to determine the concentration and purity of the resulting DNA.

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

This is the most time-consuming protocol, but is the only protocol that you can use when you have multiple PCR bands and you need to isolate one of them for sequencing (as with certain EF-1α primer pairs). This can also be a good protocol for PCR products that are difficult to sequence.

(1) Make a 1.2% agarose gel with "low melting point" agarose (NuSiece GTG. cat. no. 50082). For a large gel use 3.92 g agarose in 327 mls 1x TAE. Add 15 µl 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; 8 teeth per comb) which allow you to load up to 100 µl 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) Load from 15 to 25ul of your PCR reaction on the O/N, low-melt agaraose gel. Run the gels in the deli box fridge (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 water and then **gently** slide the gel directly onto the UV light table. 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 µl 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:

1. Melt the gel slices at 70°C until completely melted.
2. 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!
3. Attach a syringe barrel to the minicolumn and insert the minicolum into the vacuum manifold. With the valve open, slowly add the 1400 µl of resin/agaose mix.
4. After all of the resin/agarose mix has passed through the column, wash the barrel with 2 mls of 80% isoproponal. Run the vacuum for about 20 seconds after all the isopropanol has passed through.
5. Transfer the column to a new 1.5 ml Eppendorf and spin 2 mins. at 10,000 rpm to remove residual isopropanol.
6. Transfer the column to a new 1.5 ml Eppendorf and add 40 µl 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.

7. Run out 5 µl of each sample on a 1.2% Synergel along with the Bio-Rad Precision Molecular Mass Ruler (Bio-Rad cat. #: 1708207) to determine the concentration and purity of the resulting DNA. Each tube should give a nice clean, reasonably bright band.

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

Abbreviated protocols for the Promega Wizard minipreps:

A. 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 minicolums into the vacuum manifold. With the valve open and the vacuum off, add the 1400 µl of resin/agaose 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, turm 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 µl 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 µl of each sample on a 1.2% Synergel along with the Bio-Rad Precision Molecular Mass Ruler (Bio-Rad cat. #: 1708207) to determine the concentration and purity of the resulting DNA.

B. Direct PCR purification

- Add 100 µl 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 minicolums into the vacuum manifold. With the valve open and the vacuum off, add the 1400 µl of resin/agaose 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, turm 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 µl 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 µl of each sample on a 1.2% Synergel along with the Bio-Rad Precision Molecular Mass Ruler (Bio-Rad cat. #: 1708207) to determine the concentration and purity of the resulting DNA.

RTL Sequencing Protocol

Current RTL sequencing protocols are available on the BRC website:

http://www.biotech.cornell.edu/node/562

1. Assemble reaction in 96 or 384 well PCR Plate:

4.5 µl template/primer mix (containing 250 ng plasmid DNA or PCR product* of appropriate mass + pmoles primer)
0.5 µl BigDye (version 3.1)
0.5 µl 5M betaine (0.5 µl DMSO may be substituted)
2.5 µl 5x sequencing buffer
4 µl ddH2O

12 µl total volume/reaction

We typically use the following recipe (per reaction):

0.5 µl BigDye (version 3.1)
0.5 µl 5M betaine (0.5 µl DMSO may be substituted)
2.5 µl 5x sequencing buffer
0.13 µl primer**

3.6 µl total volume/reaction

Make a cocktail for each group of sequencing reactions.

We then use an Excel spreadsheet to calculate the relative amounts of water and DNA template to make up the remaining 8.4 µl volume. This excel spreadsheet is incredibly useful because you simply have to enter the fragment length (in bp), the concentration of the DNA (as estimated from the gel you ran after the PCR reactions) and the calculations are all made for you. This is also the file in which you can manage your sample names, which is important when you get to the final stage of entering sample names into the BRC website (see below).

* Amount of PCR product required for sequencing reaction: ng needed = (length of PCR product in bp)/5 For example: for 250 bp product, amount needed is 250/5 = 50 ng.
The Sequencing Handbook and ABI protocols contain calculations and recommendations for PCR products and large constructs plus additional information on purity considerations.

** same concentration as used for ordinary PCR reactions (= 2 picomoles)

2. Follow the standard ABI thermocycling program:

96 C 4 min

Followed by 35 cycles of:

96 C 10 sec
50 C 5 sec
60 C 3 min
4 C hold

Note: it is REALLY important to seal the microtitre plate well with sealing tape. You don't want these reactions to evaporate during the thermocycling program. Margarita Lopez recommends sealing the edges of the place with thin strips of metallic sealing tape.

3. To perform dye terminator removal by desalting, the BRC first adds 10 µl ddH2O to each 12 ul reaction before loading onto Edge Biosystems 96-well dye terminator removal columns (Edge part number 88415)*. We then follow the manufacturer's protocol. These plates are available from the BRC at a cost of $65 per plate. Reaction products are eluted in water into 96-well plates that fit the 3730xl instruments.

* This may not be necessary and some members of the lab don't add water to the cycle sequencing reactions.

Note: we use an excel spreadsheet to calculate the volume of template DNA, primer, and cocktail for each reaction. Our excel spreadsheet is available on most of the lab computers (*RTL_plate[template].xls*). We use a slightly different calculation for the amount of DNA to use. Instead of dividing the PCR product length by 5, we divide by 30. This seems to give good, clean DNA sequence data.

Edge Biosystems 96-well dye terminator removal protocol

For cleaning up the sequencing reactions, we use the Edge Biosystems 96-well dye terminator kit:

Performa® DTR V3 96-Well Short Plate Kit [10 plates]
Cat no. 89939
Price: $735.00

These are available from the BRC, Fisher Scientific, from Edge Biosystems:
http://www.edgebio.com/

Protocol:

1. Following the reaction, remove the flexible lid and add 10 µl of ddH2O* to each well (add more ddH2O if necessary to compensate for evaporation during the PCR reaction).

* This step is not recommended by some people.

2. Reaction cleanup is done using the Edge Biosystems Performa DTR V3 96-well Short Plates.

3. Remove the bottom and then the top adhesive tapes from the V3 96-well Short Plate (ensure that the plate remains horizontal to avoid losing any gel). Cover the plate with lid.

4. Stack the V3 96-well Short Plate on the top of a 96-well waste plate. Place the balancing weights and the stacked plates in the microplate centrifuge carriers.

5. Centrifuge for 3 minutes at 850 x g1 (~2212 RPM).

6. Transfer the reaction samples in a volume of 10 -15 µl to the **center** of each well in the V3 96-well Short Plate. Pipet slowly and do not touch the sides of the wells. Cover with lid.

7. Stack the V3 96-well Short Plate on the top of a Optical 96-well Reaction Plate. Place the balancing weights and the stacked plates in the centrifuge carriers.

8. Centrifuge for 5 minutes at 850 x g1 (~2212 RPM).

Full protocol is available from Edge Biosystems website:

http://www.edgebio.com/products/performa%C2%AE-dtr-v3-96-well-short-plate-kit-89939

Submitting RTL (= Ready to Load) sequences to the BRC:

The fastest and most cost-effective way to do sequencing is to submit your samples as "Ready to Load" (RTL) samples. These are samples in either a 96- or 384-well format. We always use the 96-well format. We do the sequencing reactions in the lab as well as the reaction clean-up. Basically, we are just using the sequencing lab to run the samples on the ABI 3730xl machine.

Submitting an RTL plate to the BRC is a two-step process. First, you will need to create and order and enter your sample names into the BRC website and then you will need to bring your samples over to the BRC for sequencing.

To use the BRC services you will need to be a registered user and have a username and password. You will also need a default account number for payment. Please talk to me about which account number to use for sequencing prior to submitting sequencing orders.

To start the process of submitting sequences, go to the BRC website:

BRC sample submission: http://www.biotech.cornell.edu/brc/genomics

Click on "Sample Submission" button

or go directly via:

BRC sample submission: https://cores.lifesciences.cornell.edu/userdev/placeorder.php

Enter your email address (=username) and your password.

Under "Facility" select "Ready to load sequencing"

Under "Service" select "Ready to load sequencing 96-well"

Then click "create new order"

Select a Cornell account number (this should already have been entered when you registered as a user)

On the next page you will be able to enter the 96 sample names. The easiest way to do this is to upload a text file (*.txt) that includes all the sample names in your 96-well plate. It is best to put some thought into this as the sample names will be the names associated with each ABI file that you will receive when the run is completed. I like to have these sample names include a brief, abbreviated name of the species (Hali = Halictus ligatus), the DNA extraction number (say, 1275), and the primer used for the sequencing (say, HaF2For1). The resulting sample would be "Hali1275HaF2For1" (which includes all the essential information you might need when editing, aligning, and analyzing your sequence results).

The easiest way to keep track of these taxonomic names, numbers, and primers is to create these names as part of the original calculations you made (in Excel) when you set up the original sequencing reactions. In the end I like to have a column in my excel file that has all the information needed to identify the species represented by each sequence, the DNA extraction number, and the primer.

Here is a sample of names and well identifiers from a typical RTL sequencing spreadsheet:

Hali203Bel	A1
Hali204Bel	B1
Hali205Bel	C1
Hali206Bel	D1
Hali207Bel	E1
Hali208Bel	F1
Hali209Bel	G1
Hali210Bel	H1
Hali211Bel	A2
Hali212Bel	B2
Hali213Bel	C2
Hali214Bel	D2
Hali215Bel	E2
Hali216Bel	F2
Hali217Bel	G2
Hali218Bel	H2
Hali219Bel	A3
Hali220Bel	B3
Hali221Bel	C3
Hali222Bel	D3
Hali223Bel	E3
Hali224Bel	F3
Hali225Bel	G3
Hali226Bel	H3
Hali227Mar	A4
Hali228Mar	B4
Hali229Mar	C4
Hali230Mar	D4
Hali231Mar	E4
Hali232Mar	F4
Hali233Mar	G4
Hali234Mar	H4
Hali235Mar	A5
Hali236Mar	B5
Hali237Mar	C5
Hali238Mar	D5
Hali239Mar	E5
Hali240Mar	F5
Hali241Mar	G5
Hali242Mar	H5
Hali243Mar	A6
Hali244Mar	B6
Hali245Mar	C6
Hali246Mar	D6
Hali247Mar	E6

Hali248Mar	F6
Hali249Mar	G6
Hali250Mar	H6
Hali251HaF2For1	A7
Hali252HaF2For1	B7
Hali253HaF2For1	C7
Hali254HaF2For1	D7
Hali255HaF2For1	E7
Hali256HaF2For1	F7
Hali257HaF2For1	G7
Hali258HaF2For1	H7
Hali259HaF2For1	A8
Hali260HaF2For1	B8
Hali261HaF2For1	C8
Hali262HaF2For1	D8
Hali263HaF2For1	E8
Hali264HaF2For1	F8
Hali265HaF2For1	G8
Hali266HaF2For1	H8
Hali267HaF2For1	A9
Hali268HaF2For1	B9
Hali269HaF2For1	C9
Hali270HaF2For1	D9
Hali271HaF2For1	E9
Hali272HaF2For1	F9
Hali273HaF2For1	G9
Hali274HaF2For1	H9
Hali275F2Rev1	A10
Hali276F2Rev1	B10
Hali277F2Rev1	C10
Hali278F2Rev1	D10
Hali279F2Rev1	E10
Hali280F2Rev1	F10
Hali281F2Rev1	G10
Hali282F2Rev1	H10
Hali283F2Rev1	A11
Hali284F2Rev1	B11
Hali285F2Rev1	C11
Hali286F2Rev1	D11
Hali287F2Rev1	E11
Hali288F2Rev1	F11
Hali289F2Rev1	G11
Hali290F2Rev1	H11
Hali291F2Rev1	A12
Hali292F2Rev1	B12
Hali293F2Rev1	C12
Hali294F2Rev1	D12
Hali295F2Rev1	E12
Hali296F2Rev1	F12
Hali297F2Rev1	G12
Hali298F2Rev1	H12

Note that the samples run from A1, B1, C1... F12, G12, H12. I.e., the samples are running from

the upper left corner of the plate **downward** and then consecutively across the plate from left to right.

You can simply cut from the Excel file to create a text file of the sequence names.

Upload text file with sample names. The final step is to “Proceed to verification”

The BRC will assign a number to your run. Write this number down and attach it to the microtitre place when you bring it over to the BRC. Drop the plate off with one of the staff in the BRC in Biotech.

You are done!

Appendix 1 -- **Useful recipes:**

1M Tris, pH 7.6

2.78 g Tris-base
12.12 g Tris-HCl

100 mls total (check pH to confirm)

1M Tris, pH8.0

5.30 g Tris-base
8.88 g Tris-HCl

100 mls total (check pH to confirm)

Tris-EDTA buffer, pH 7.6

10 mM Tris	5 mls 1M Tris, pH 7.6
1 mM EDTA	1 mls 0.5 M EDTA, pH 8
	500 mls

Making DNase free RNase (10 mg/ml)

RNase dilution buffer

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

Add 10 mg RNase to 1 ml RNase dilution buffer (=1 ml total) and distribute 50 µl 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.

Making Tris pH 8.0 (or 7.6):

Make up 1M Tris-base (60.5 g/500 ml)
Make up 1M Tris-Hcl (78.8 g/500 ml)

pH 8.0: 160 mls Tris-base + 200 mls Tris-HCL; adjust pH to 8.0
pH 7.6: 80 mls Tris-base + 200 mls Tris-HCL: adjust pH to 7.6

Making EDTA pH 8.0:

93.05 g/500 mls = 0.5 M

Add 10g NaOH pellets to adjust to pH 8.0

Autoclave both solutions

Appendix 2 -- Notes on mixing up dNTP's

Promega dNTPs (cat. no. U1330) come in a concentration of 100 mM each (100 µl per tube). To make up the working concentration of dNTPs (1 mM) dilute each dNTP down 1:100:

10 µl dATP
10 µl dTTP
10 µl dGTP
10 µl dCTP
960 µl H2O
_____
1000 µl total

The easiest way to do this is to make up 10ml of dNTP (in a 30 ml Falcon tube) and then aliquot 1000 µl dNTP to ten Eppendor tubes:

100 µl dATP
100 µl dTTP
100 µl dGTP
100 µl dCTP
9.6 ml H2O
_____
10 ml total

Aliquot 1ml [1000 µl]/Eppendorf tube, label and date the tubes, store in -20 C freezer.

Appendix 3 -- Primer dilutions and preparation:

Making up primers.

1. Add TE buffer (or water) to dried oligonucleotide at a concentration of 1ug/ul. Leave in fridge or on ice for one hour to fully dissolve primer.

2. Aliquot 20 µl of the stock solution into 1.5 ml. Eppendorf tubes. Label the top of the tube.

3. To make up a working solution, add distilled water. The amount of water added depends on the length of the primer. See table below.

Primer length	water (ul)	working conc. (ng/ul)
15	246.7	75
16	230.0	80
17	215.3	85
18	202.2	90
19	190.5	95
20	180.0	100
21	170.5	105
22	161.8	110
23	153.9	115
24	146.7	120
25	140.0	125
26	133.9	130
27	128.2	135
28	122.9	140
29	117.9	145

The final molar concentration = 20,000,000÷final volume÷molecular weight (mw). Final concentration should be roughly 16 uM.

When mixing up primers take particular care not to contaminate primer stock tubes.

4. Label primer stock tubes with a sticky label that indicates what volume of water to add to the 20 µl stock solution (see table above).

An alternative is to visit the IDT web site where there are links to tools for calculating primer concentrations and dilutions:

http://biotools.idtdna.com/gateway/

Appendix 4 – PCR reaction worksheet

PCR amplifications (w/ GoTaq)

PCR reaction (per tube)	Cocktail (tubes)
5.2 µl dH2O	_____
3.0 µl 5x buffer[1]	_____
1.5 µl $MgCl_2$ (25 mM)	_____
3.0 µl dNTPs (1mM each)	_____
1 µl DNA	_____
0.6 µl primer + (~16µM)	_____
0.6 µl primer - (~16µM)	_____
0.075 µl Taq (*Promega GoTaq*[2] @ 5 units/µl)	_____
15 µl total	_____ µl total

tube no.	specimen
1	_____
2	_____
3	_____
4	_____
5	_____
6	_____
7	_____
8	_____
9	_____
10	_____
11	_____
12	_____
13	_____
14	_____
15	_____
16	_____
17	_____
18	_____
19	_____
20	_____
21	_____
22	_____
23	_____
24	_____

Cycle conditions
_____
_____
_____

_____ cycles

Notes:
[1]Promega Taq now comes with two different 5x buffers: one with loading dye and one w/o.
[2] Promega *GoTaq Flexi* DNA polymerase (cat. no. M8291)