

## PCR

(See the end of the document for primer sequences)

For all PCR reactions I use the same cocktail:

For Ex-taq:

25 $\mu$ L total volume

DH2O	16.37	
Ex-taq-Buffer	2.5	
Ex-taq-dNTPs	2.5	
Primer1	1.25	at 10 $\mu$ M
Primer2	1.25	at 10 $\mu$ M
Ex-taq	0.13	
+1-2 $\mu$ L of your template		

Cut everything down by  $\frac{1}{2}$  (12.5 $\mu$ L total volume) or  $\frac{3}{4}$  (6.25 $\mu$ L total volume) if everything is working well.

### **The following PCR regions work for almost all cicadas:**

PCR protocol:

#### **COI (3' end) (~800bp)**

94°C 2 mins  
94°C 45 secs-----  
56°C 45 secs \*30  
72°C 1min 15secs-----  
72°C 5 mins

#### **COII (~750bp)**

94°C 2 mins  
94°C 45 secs-----  
53°C 45 secs \*30  
72°C 1min 15secs-----  
72°C 5 mins

#### **Barcoding (COI 5' end) (~700bp)**

94°C 2 mins  
94°C 45 secs-----  
45°C 45 secs \*30  
72°C 1 min-----  
72°C 5 mins

#### **EF1a start (EF001-R752) (usually ~750bp)**

94°C 2 mins  
94°C 45 secs-----  
53°C 45 secs \*30  
72°C 2mins 30secs-----  
72°C 5 mins

<b>EF1a middle (f650-DVR1) (Usually ~750bp)</b>	<b>EF1a end (DVF1-R114) (Usually ~750bp)</b>
94°C 2 mins	94°C 2 mins
94°C 45 secs-----	94°C 45 secs-----
58°C 45 secs *30	55°C 45 secs *30
72°C 2mins 30secs-----	72°C 2mins 30secs-----
72°C 5 mins	72°C 5 mins

**Calmodulin (~650bp) and short pieces of Period (~600bp each)**

94°C 2 mins  
 94°C 45 secs-----  
 60-50°C 45 secs \*10 touchdown, -1°C per cycle  
 72°C 1 min-----  
 94°C 45 secs-----  
 50°C 45 secs \*25  
 72°C 1min-----  
 72°C 5 mins

**Period total (<1000bp)**

94°C 2 mins  
 94°C 45 secs-----  
 60-50°C 45 secs \*10 touchdown, -1°C per cycle  
 72°C 2 mins 30 secs-----  
 94°C 45 secs-----  
 50°C 45 secs \*25  
 72°C 2mins 30 secs-----  
 72°C 5 mins

**PCR clean-up**

We use ExoSAP-IT unless we have to run samples out on a gel and cut out the bands, where we use the Nucleospin Extract II kit.

**ExoSAP-IT**

Mix up ExoSAP-IT 1:5 with water. Add 1µL of this to 2.5µL of your sample. Place on PCR machine for 15mins at 37°C, then 15mins 80°C. Your 1:5 Exosap can be stored in the freezer for at least a few months (even if it freezes it seems to be OK still). If you have a really strong PCR band you may want to use a bit more ExoSAP (we don't actually know how much you can dilute the ExoSAP and have it still work).

Note: Exosap also cleans up primer-dimer, so if you have weak amplification and badly designed primers that can dimerise, then sometimes you cannot read the first 30-odd bases of your sequence because it is also sequencing the primer-dimer over the top.

## **Nucleospin Extract II kit**

Follow the manufacturer's instructions. Hint, you can re-use the columns, just run room-temperature water thru them 1-2 times (or you can boil them but sometimes this makes the ring above the membrane fall out, which makes the column unuseable). Hint2- NT3 buffer is just 76% EtOH, so you can just make more if you run out. Hint 3 - Nucleospin elution buffer reacts oddly with Nanodrop machines. If you are using a Nanodrop to spec' your DNA strength use either water or Qiagen AE (from DNEasy kit) elution buffer.

## **Cycle sequencing**

I currently use a 5 $\mu$ L recipe which works really well with about 8ng of DNA template. ExoSAP PCR products for some reason tend to work a lot better than those from the Extract II kit, which are a lot more susceptible to using either too much or too little template.

### **5 $\mu$ L cycle sequencing recipe:**

1 $\mu$ L BigDye v1.1 or v3.1

0.5 $\mu$ L BigDye Buffer

0.75 $\mu$ L Primer at 1 $\mu$ M conc.

0.2-2.5 $\mu$ L template (about 8ng)

+water to 5 $\mu$ L (usually I just add 1.75 $\mu$ L of water per sample, you don't need to make it exactly to 5 $\mu$ L unless you are trying to use really weak template)

### **Cycling Protocol (same for all genes)**

96°C 2mins

96°C 30secs-----

50°C 20secs \*30

60°C 2mins-----

60°C 5mins

### **Cycle Sequencing clean-up**

Clean cycle sequencing products using Sephadex. We use 500 $\mu$ L Sephadex per sample (either Sephadex individual column or Sephadex plate-spinning column). Spin sephadex for 2mins at 2800rpm. Add sample, then spin for another 2 mins at 2800rpm.

## Cicada primer list:

Note: It would be a good idea to check the sequence written here against the sequence printed in the references in case I made a mistake!

### mtDNA

These primer sets work in most cicadas:

COI 3'	C1-J-2195 +TL2-N-3014	TTGATTTTTTGGTCATCCAGAAGT (Simon et al. 1994) TCCAATGCACTAATCTGCCATATTA (Simon et al. 1994)
COI barcoding (5')	C1-J-1490 +C1-N-2198	GGTCAACAAATCATAAAGATATTGG (Folmer et al. 1994) TAAACTTCAGGGTGACCAAAAATCA (Folmer et al. 1994)
COII	TL2-J-3034 or TL2-J-3033 + TK-N-3786 or+A8-N-3914	AATATGGCAGATTAGTGCA (Simon et al. 1994) AATATGGCAGATTAGTGC (Sueur et al 2007) GTTTAAGAGACCATTACTT (Sueur et al 2007) TCATATTATTGGTGATATTTGAGG (Simon et al. 1994)
12s	SR-J-14233 +SR-N-14588	AAGAGCAACGGGGCGATGTGT (Arensburger et al. 2004) AAACTAGGATTAGATACCCTATTAT (Arensburger et al. 2004)
16s	LR-J-12887 +LR-N-13398	CCGGTCTGAACTCAGATCACGT (Arensburger et al. 2004) CGCCTGTTTAACAAAACAT (Arensburger et al. 2004)

This A6-A8 primer set is designed specifically for the genus *Maoricicada* but works in some other cicadas

A6-A8	TK-J-3799 + A6-N-4570 or+ C3-N-4834	GGCTGAAAGTAAGTAATGGTCTCT (Buckley et al. 2001) AAGACTGAATTATACAAACGGCTA (Buckley et al. 2001) ATACCAGATACAAAACCTTAGAGC (Hill et al. in press)
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This A6A8 primer set works in *Kikihia* and some other cicadas

A6-A8	FOR	GTAATTAACAAATACTCTTAATGG (Marshall et al. 2008)
	REV	GATGTCCAGCAATTATATTAGCTG (Marshall et al. 2008)

Many other primers in the mtDNA insect primer kit amplify easily in cicadas. We have successfully amplified pieces as long as 4500bp with just Ex-taq.

### Nuclear genes

These EF1a genes amplify well in almost all cicadas, but can have large (>1kb) inserts in some of the introns in some species. There is also an intronless copy that amplifies in some species. Start, middle and end overlap.

EF1a start (usually the most conserved)

EF1-F001-cicada	TCTACAAATGTGGTGGTATC	(Arensburger et al. 2004)
+EF1-R752-cicada	TCAGTGTTGTCCATTTTGTT	(Arensburger et al. 2004)

EF1a middle+end (this sometimes works well, but often it is easier to split up this section into the two below, because of the possibility of several large inserts)

EF1-F650-cicada TGCTGCTGGTACTGGTGAAT (Arensburger et al. 2004)  
or EF1a-F650-mod TGCTGCGGGTACTGGTGAAT (Sueur et al. 2007)  
or EF1a-F650-ambig TGCTGCKGGTACTGGTGAAT unpublished  
+EF1-R114-cicada TTGATAGACTTGGGATTTTC (Arensburger et al. 2004)

EF1a middle EF1-F650-cicada TGCTGCTGGTACTGGTGAAT (Arensburger et al. 2004)  
or EF1a-F650-mod TGCTGCGGGTACTGGTGAAT (Sueur et al. 2007)  
or EF1a-F650-ambig TGCTGCKGGTACTGGTGAAT unpublished  
+EF1-N-1419 ACACCAGTTTCAACTCTGCC (Sueur et al. 2007)

EF1a end DV-EF-F1 CAGGAYGTATACAAATTGGTG (Marshall et al 2008)  
+EF1-R114-cicada TTGATAGACTTGGGATTTTC (Arensburger et al. 2004)

Alternative EF1a primers: (work in *Maoricicada*, have not tried on other cicadas)

EF1a-97-for ACGCCCTGGACATAGAGAT (Buckley et al 2006)  
+EF1a-189-rev CAACCTGAGATTGGCACAAA (Buckley et al 2006)

EF1a-236-for GAAGCCCTTGATGCCATTT (Buckley et al 2006)  
+EF1a-352-rev TGTGTAGCCATTTGAAATTTGG (Buckley et al 2006)

18s (Too slow for cicadas (there is only a couple bp divergence across all Cicadidae), but good sequence, both regions together give about 1800bp). I'm not sure where these come from.

18s1F TACCTGGTTGATCCTGCCAGTA  
+18s5R CTTGGCAAATGCTTTTCGC

18s5F GCGAAAGCATTGCAAGAA  
+18s9R GATCCTTCCGCAGGTTACCTA

These Calmodulin primers only amplify a short piece (~650bp) but work well in almost all cicadas.

Calmodulin Cal-60-for AACGAAGTAGATGCCGATGG (Buckley et al. 2006)  
+Cal-72-rev GTGTCCTTCATTTNCKTGCCATCAT (UBC insect primer kit)

These Period primers were designed specifically for *Maoricicada* and do not seem to work in other cicadas. We have some other primers for Period that were developed for *Kikihia*, which may be more general within cicadas. email me if you want them. This whole piece is <1000bp in *Maoricicada*. The two sections overlap and the whole piece can also be amplified.

Period Per-205-For CAGTACAACTGTTTCGATCTGC (Buckley et al. 2006)  
+Per-925-Rev CCATTCGAGGAATTGAGCAT (Buckley et al. 2006)

Per-491-For GTGCACACCTGAATCCATTG (Buckley et al. 2006)  
+Per-1201-Rev AATGCCGCCACCATTAATAC (Buckley et al. 2006)

### **Primer references:**

- Arensburger P, Buckley TR, Simon C, Moulds M, Holsinger KE. 2004. Biogeography and phylogeny of the New Zealand cicada genera (Hemiptera: Cicadidae) based on nuclear and mitochondrial DNA data. *Journal of Biogeography* 31: 557–569.
- Buckley TR, Cordeiro M, Marshall DC, Simon C. 2006. Differentiating between hypotheses of lineage sorting and introgression in New Zealand alpine cicadas (*Maoricicada* Dugdale). *Systematic Biology* 55:411–425.
- Buckley TR, Simon C, Chambers GK. 2001. Phylogeography of the New Zealand cicada *Maoricicada campbelli* based on mitochondrial DNA sequences: ancient clades associated with Cenozoic environmental change. *Evolution* 55:1395-1407.
- Folmer O, Black M, Hoeh W, Lutz R, Vrijenhoek R. 1994. DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. *Molecular Marine Biology and Biotechnology* 3:294-299.
- Hill KBR, Marshall DC, Simon C, Chambers GK. 2008 in press. Surviving glacial ages within the Biotic Gap: phylogeography of the New Zealand cicada *Maoricicada campbelli*. *Journal of Biogeography* in press.
- Marshall DC, Slon K, Cooley JR, Hill KBR, Simon C. 2008. Steady Plio-Pleistocene diversification and a 2-million-year sympatry threshold in a New Zealand cicada radiation. *Molecular Phylogenetics and Evolution* 48: 1054-1066.
- Simon C, Frati F, Crespi B, Liu H, Flook PK. 1994. Evolution, weighting, and phylogenetic utility of mitochondrial gene sequences and a compilation of conserved PCR primers. *Annals of the Entomological Society of America* 87:651-701.
- Sueur J, Vanderpool D, Simon C, Ouvrard D, Bourgoïn T. 2007. Molecular phylogeny of the genus *Tibicina* (Hemiptera, Cicadidae): rapid radiation and acoustic behaviour. *Biological Journal of the Linnean Society* 91: 611-626.