Protocols for High-Volume DNA Barcode Analysis

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DNA Working Group

Consortium for the Barcode of Life

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Introduction

This draft report describes protocols and equipment that will enable single laboratories to achieve production rates of 50K sequences per year. However, most suggestions are also relevant to labs with lower production goals as they seek simply to minimize the costs of analysis and speed its execution.

Most steps of the analysis (specimen to PCR product) can be carried out in facilities with modest infrastructure ($20K). However, the establishment of a sequencing facility capable of analyzing 50-100K samples per year is much more expensive (circa $1M). As a result, it may often be appropriate to funnel PCR products from 'satellite' laboratories to a central sequencing facility.

Although commercial kits are available for varied stages of the analytical chain (e.g. DNA extraction, PCR amplification, product detection), 'home-made' reagents can lower costs dramatically. We describe both approaches in many cases.

1. Specimen Collection/Preservation

Whenever possible, specimens should be killed in a DNA-friendly fashion (freezing, cyanide, immersion in ethanol), avoiding even brief exposure to killing/preservation agents such as ethyl acetate or formalin that damage DNA. DNA in dried specimens ordinarily remains in good condition for at least a year, but degradation becomes increasingly problematic as time passes. DNA in frozen specimens (especially those held in cryogenic conditions) remains stable indefinitely, but DNA in ethanol-preserved material often degrades due to acidification. As a result, barcode analysis should follow collection as quickly as possible.

2. Tissue Sampling/Handling

All specimen samples should be handled on a clean working surface and all instruments should be acid or flame sterilized between each sample. A Bunsen burner flame is convenient for sterilization; small propane tanks are ideal for settings where 'gas' is not on-line.

In any laboratory that seeks high production rates, it is critical to carry out all stages of barcode analysis in 96-well plates. Care must be taken when loading these plates with samples to avoid cross-contamination between wells. This risk can be reduced by covering each plate with strip caps and opening just one row at a time. Soaking dry insect legs in ethanol for a few minutes before extraction is also helpful as it prevents specimen 'flying' due to static electricity.

3. Genomic DNA Isolation/Purification

3.1. FAST DNA EXTRACTION- LOW COST

CHELEX 100 (DRYRELEASE)

Fresh or frozen specimens are ordinarily an easy target for barcode analysis, allowing the use of rapid Chelex resin protocols (Walsh et al. 1991) for DNA isolation. Chelex extraction (Jaulnac et al. 1998) can be combined with proteinase K treatment to create a simple, cheap and efficient 96-well protocol for DNA extraction. This protocol has been used successfully with arthropods, fish, birds (including feathers) and mammals (including skin and hairs). It requires only a small amount of tissue (1-3 mm^3), making even a single insect leg sufficient for several DNA extractions.

Some Chelex protocols involve grinding tissue in liquid nitrogen (Gregory & Rinderer 2004), an approach that is not readily compatible with 96-well format extractions. By combining Chelex extraction with proteinase K treatment, the need for tissue disruption is eliminated. Tissue samples usually dissolve completely after overnight incubation, while chitinous parts remain intact, but DNA is released. Therefore, the **Chelex/Proteinase K combination can be used for non-destructive DNA extraction from small invertebrates** (e.g. collembolans, rotifers). In this case, the entire specimen is placed in the solution and removed at the end of the procedure.

Chelex-based extraction is not suitable for samples with high levels of PCR inhibitors (e.g. haemoglobin) or for samples where DNA is degraded. A second disadvantage of this method lies in the fact that the extracted DNA is relatively impure and is, hence, often unstable for more than a few weeks.

Two commercial kits provide very fast options for DNA extraction (Sigma-Aldrich Extract-N-Amp* PCR* Kit, Genereleaser), but are more costly and are not currently available in a 96-well format.

3.2 Silica or Silica Membrane DNA Extraction

Various silica and silica-membrane based protocols produce relatively pure DNA. These approaches are also more effective in extracting DNA, a factor that makes them particularly useful for studies on specimens with degraded DNA. These approaches rely on DNA binding to silica in the presence of a high concentration of chaotropic salt (Boom et al. 1990; Hoss and Paabo 1993). This class of methods provides an alternate non-destructive approach for extracting DNA that involves soaking samples in guanidinum-thiocyanate (GuSCN) with subsequent sorption of DNA to silica (Rohland et al. 2004).

We have tested four commercially available systems that employ silica-binding.

a) Sigma-Aldrich GenElute™ Mammalian Genomic DNA Miniprep Kit

b) QIAGEN DNeasy tissue kit (DNeasy 96 tissue kit)

c) Promega Wizard® SV 96 Genomic DNA Purification System

d) Macherey-Nagel Nucleospin® 96 tissue

The GenElute kit is sensitive, but it is not available in a 96-well format and is relatively slow to use. The other three kits are available in 96-well formats. A multi-channel pipettor is required to effectively perform 96-well DNA extractions with any of these kits. For those that are considering very high volume production, most of these kits can be automated on robotic liquid handling stations.

3.3 Magnetic Bead-Based DNA Extraction

Dynal Biotech's Dynabeads® DNA DIRECT™ and Dynal MPC® -auto 96 Magnet Station

This system might be effectively applied in cases where a robotic liquid handling device is available.

4. Genomic DNA Quantitation

It is not usually necessary to quantify genomic DNA extracts because even a few copies of the target gene are sufficient for PCR amplification. However, the quantity of extracted DNA can be determined with a plate reader.

5. PCR Amplification of Barcode Region

5.1 PRIMERS

Primer design is critical and minor adjustments can have large impacts on barcode recovery. The first phase of any study on a new group should involve a serious effort to identify optimal primers. Whenever we have done this, we have gained very high success in barcode recovery. For example, the 2 primer sets that we routinely employ for lepidopterans recover the barcode region from more than 99% of species and our 2 primer sets for fishes have about 97% success.

DEGENERATE PRIMERS, MODIFIED BASES (E.G. INOSINE)

Single bp mismatches at the 3'-end of a primer usually prevent PCR amplification (Simsek & Adnan 2000). This problem can often be solved by the use of degenerate or inosine containing primers (Batzer et al. 1991; Shultz & Regier 2000; Candrian at al. 1991; Christopherson et al. 1997). Sorenson et al. (1999) suggest that primers with appropriate degenerate sites are also less likely to preferentially amplify nuclear pseudogenes because they accommodate usual differences between nuclear and mtDNA sequences (e.g. 3rd positions changes in the mtDNA copy).

Primers with 2-4 degenerate positions will often rescue barcodes from 'recalcitrant' specimens. Early results with primers containing inosine show that they are also effective in amplifying difficult samples.

5.2 REACTION MINIMIZATION, REACTION MIXES AND PCR ENHANCERS

Although Chelex-based DNA extracts sometimes resist amplification because of the presence of inhibitors, this can usually be overcome by incorporating amplification facilitators such as bovine serum albumin (BSA), betaine or DMSO (Al-Soud & Rådström 2000) in the PCR mix. Betaine exerts its effect by stabilizing AT base pairs while destabilizing GC base pairings, resulting in a net specific destabilization of GC-rich regions (Rees et al. 1993, Henke et al. 1997). Most commercial 'PCR-enhancing' buffers contain betaine (Frackman et al. 1998). The addition of amplification enhancers also improves the specificity of PCR and allows the amplification of GC-rich templates.

To reduce costs we have lowered reaction volumes; we regularly employ 10 µl (versus standard 25-50 µl). In order to accurately dispense such small volumes, it is useful to make up a larger volume master mix. This can be dispensed into 96-well plates and stored frozen till use, but freezing is not possible without a cryoprotectant. Such pre-mixing of PCR reagents speeds an otherwise time-consuming step and aids quality assurance.

Trehalose is widely used as a cryoprotectant (Franks 1990; Spiess et al. 2004). It also acts as a potent PCR enhancer by both lowering the DNA melting temperature and stabilizing Taq polymerase (Spiess et al. 2004). Because of these properties, trehalose is ideal to stabilize frozen PCR mixes and to overcome the effect of inhibitors that may be present in Chelex extracts, resulting in improved PCR success. Aliquoted 'ready to use' PCR mixes can be stored at -20^{o}C for 3 months and do not degrade even after several freeze-thaw cycles. Thus, we regularly fill large numbers of 96-well PCR plates with 10 µl of standardized mix and hold them frozen until use.

5.3 THERMAL CYCLERS

The latest generation of thermal cyclers (e.g. Eppendorf MasterCycler) have faster thermal ramping that allows PCR amplifications to be completed more quickly (2 vs 3 hours). There are also capillary cyclers that enable the completion of amplification in 20 minutes, but they are not 96-well compatible.

5.4 ALTERNATE POLYMERASES

There is a growing diversity of polymerases or polymerase cocktails that have varied effects. Some enable PCR to be executed much more quickly; others aid the amplification of damaged templates or permit high fidelity replication.

a) **Fast Taqs**: e.g. Z Taq (Takara). This enzyme allows PCR completion in 20 minutes using a standard cycler.

b) **DNA Repair**: e.g. Restorase and Restorase II (Sigma). By repairing DNA damage, these mixes aid the recovery of barcodes from specimens with degraded DNA.

c) **High-fidelity/specificity**: e.g. DeepVent Taq (NEB), Diamond Taq (Bioline). These enzymes are of limited utility in barcode analysis as PCR artifacts are generally unimportant because a large population of molecules is available for amplification.

5.5 FIELD –FRIENDLY PCR

Dried PCR reagents will undoubtedly be a convenient solution for barcode acquisition in 'field' settings because they are stable at room temperature. The resultant PCR products can be air or vacuum dried before transportation to a DNA sequencing facility.

Dried reagents e.g. Amersham PuReTaq Ready-To-Go™ PCR Beads

6. PCR Product Check/Quantitation

For projects that are examining compliant specimens, it is possible to proceed directly from the barcode PCR to a sequencing reaction. However, it is often critical to screen PCR reactions for product when working with older specimens or with a new taxonomic group. This has traditionally been a laborious task involving gel casting and the loading of individual reaction products into the gel. We have explored two options to reduce this time – **microfluidic technology** and **pre-cast agarose gels**.

6.1 MICROFLUIDIC TECHNOLOGY

This approach involves microfluidic devices that are able to sip a small volume of the PCR product from each well in a plate and determine its size and concentration. Both existing devices have several drawbacks- they are expensive, have high operating costs, and are relatively slow.

CALIPER LS 90

This instrument provides sizing and quantification of PCR products. However, it requires considerable operator attention as the 96-well plate has to be changed every hour. Moreover, this system is not compatible with PCR additives (e.g. trehalose) because the microfluidic channels clog.

AGILENT 5100 AUTOMATED LAB-ON-A-CHIP PLATFORM (ALP)

This instrument requires less operator attention, allowing the unattended analysis of up to 10 plates (96 or 384 well format) (Pike et al. 2004). Our preliminary results show that this system is less sensitive to debris and PCR additives than the Caliper LS 90. However, its capital and operating costs are very high, while its sensitivity is lower than agarose gel screening methods.

6.2 PRE-CAST AGAROSE GELS

Several manufacturers make pre-cast agarose gels that are designed to analyze 96-well plates. We have considerable experience with the E-gels by Invitrogen, but similar products from Bio-Rad or Amersham Bisciences are worth investigating, especially if they have a lower cost.

E-GEL 96® SYSTEM

The E-gel 96 system is user-friendly and has a low capital cost ($0.5K). It involves the use of pre-cast agarose gels that are run in a bufferless electrophoresis system. Results are obtained in 6-15 minutes. The system is sensitive and compatible with 8-, 12- or 96-tip robotic liquid handling systems. E-gel software provides a simple way to incorporate results into spreadsheets that track specimen analysis. The cost per sample is about $0.30, which is lower than microfluidic systems, but about 10X the cost of 'home-made' agarose gels.

7. PCR Product Normalization & Hit Picking

At present, we sequence all wells on a plate if at least 80 of the samples have PCR amplified. When success is lower, we use spreadsheets with incorporated E-gel images to aid hit picking and normalization of successful PCR reactions. This is a difficult and time consuming task; this is one point in the analytical chain where a robotic system could substantially aid production.

8. PCR Product Cleanup

PCR products are often 'cleaned-up' to remove un-incorporated nucleotides and residual primers. If this step is omitted, it leads to degradation in the sequencing results for the first 50 or so bp. Such degradation is of little concern when the PCR product is slated for bi-direction sequencing. However, when the PCR product is sequenced in just a single direction, there are varied kits to support cleanup as well as traditional ethanol precipitation.

a) Milllipore MultiScreen® Filter Technology

b) QIAGEN QIAquick Robo96 PCR Clean-up Kit

c) Promega's Wizard® MagneSil™ PCR Clean-Up System

9. Sequencing Setup

9.1 DECREASING CONCENTRATION OF BIGDYE AND LOWER REACTION VOLUMES

To reduce costs, we use 10 µl sequencing reactions containing just 0.25 µl of BigDye (1/16 concentration). Because BigDye is so expensive, lowering its usage is a critical step in minimizing costs.

We dispense our sequencing reaction cocktail into either 96-well plates or into larger volume tubes (to provide the opportunity to use different sequencing primers) and then freeze it for up to 3 months. Trehalose is added to ensure stability of the enzyme during freeze-thaw cycles.

We find that 10 pmoles of the sequencing primer is optimal for analyzing PCR products without clean up. Primers can be reduced to 3 pmoles if the PCR product has been cleaned up.

9.2 SEQUENCING CHEMISTRY

We employ **ABI BigDye™ v. 3.1. Cycle Sequencing Kit** because it provides a robust sequencing chemistry. It generally produces long reads (circa 750 bp), even on GC-rich templates.

Amersham Biosciences DYEnamic™ ET Terminator Cycle Sequencing Kits are fully compatible with ABI instruments (mobility files can be downloaded from the Amersham web site) and appear to perform well. This chemistry option deserves more serious study as it is lower in cost.

The **CounterTrace II™ system** from **Nucleics** is also compatible with ABI sequencers and BigDye™ v1.1 and 3.1 chemistries. CounterTrace II typically leads to an increase in read length (15-20% in high throughput laboratories) and allows greater dilution of Big Dye.

10. Sequencing Reaction Cleanup

SEPHADEX

There are many protocols available to cleanup sequencing reactions. Many high-volume genomics facilities use either ethanol precipitation or magnetic bead protocols. At present, we use a Sephadex column based approach which is available in a 96-well format. However, other options like **SPRI (Solid Phase Reversible Immobilisation)**-based dye terminator removal system e.g. **AgenCourt Cleanseq** or **ethanol precipitation** might be employed.

11. Sequence Analysis

Capillary sequencers have now largely displaced slab gel instruments, but ABI PRISM® 377 and 373 sequencers provide a low-cost sequencing solution for labs that seek to analyze no more than 50 templates a day. However, for higher production goals and greater automation, a multi-capillary instrument is critical. Applied Biosystems has long dominated the sequencer marketplace and they produce several highly reliable instruments with varied production capacities.

LOW-THROUGHPUT LABS

ABI PRISM® 3100 Avant Genetic Analyzer – 4 capillaries, syringe system of polymer delivery (upgradeable to **3130** with automated system of polymer delivery).

MID-THROUGHPUT LABS

ABI PRISM® 3100 Genetic Analyzer – 16 capillaries, syringe system of polymer delivery (upgradeable to **3130xl** with automated system of polymer delivery).

HIGH-THROUGHPUT LABS

Applied Biosystems 3730 or 3730XL DNA Analyzer – 48-96 capillaries, automated polymer delivery.

OTHER OPTIONS

Amersham Biosciences MegaBACE™ 500 and MegaBACE™ 1000. Both systems accept a variable number of capillary arrays with up to 48 capillaries in the MegaBACE™ 500 and twice that many in the MegaBACE™ 1000.

Amersham Biosciences MegaBACE 4000 – This system has 384 capillaries, the highest capacity of any sequencer.

Beckman-Coulter CEQ 8000/8800 – This instrument has not proven effective for barcode analysis.

12. Sequence Editing

Whenever possible, barcode products should be sequenced bidirectionally if they are destined for inclusion in the barcode reference library. Bidirectional sequencing aids the generation of full length barcode sequences by avoiding problems in signal deterioration that often occur near the end of a read. It has also allowed the creation of specialized software that generates a consensus sequence from the 2 reads and determines a quality score (e.g. PHRED) for each position. However, there remains a need for manual sequence editing to fully extract information from sequence records.

Two widely used DNA analysis software packages are effective for the analysis of barcode sequences.

1) Sequencher™ (Gene Codes Corporation). Sequencher was first introduced in 1991 and remains very popular. It is easy to learn and can be served over TCP/IP, AppleTalk, or IPX, a feature that makes it popular in large research centers and universities.

2) SeqScape® (Applied Biosystems) is another powerful tool for sequence analysis. As indels are rare in COI, sequences are easily assembled against a reference sequence. This program includes internal base callers (including KB basecaller), automatic alignment and trimming against a reference sequence. It also allows the easy import of sequences into a project, and can create different layers for varied regions of interest. It requires a powerful computer for normal operation.

DNA Star (Lasergene®) provides another option and it couples software for sequence analysis with the capacity for primer design, DNA map drawing, etc. The latest release of this package is Sequencher compatible.

13. Sequence Alignment

We employ the Barcode of Life database to organize sequence records and keep them in alignment. This software also allows results from different projects to be merged into a 'virtual project' enabling sequence comparisons and tree generation for specimens from different projects.

As COI sequences rarely have indels or deletions, they can be easily aligned in any DNA alignment or editing software. We usually align against a reference sequence at the editing stage. When the generated sequence is shorter, all missing bases are replaced with 'N's to stabilize the length of submitted sequences.

14. Acknowledgements

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15. Appendix

An Appendix that provides detailed protocols, sources for key reagents, and more detailed time/cost estimates for various analytical steps will be available for distribution at the joint meeting of the DNA and Database Working Groups on February 9th.

16. References

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TABLE 1: Costs in obtaining a bi-directional barcode sequence from fresh or archival specimens. The cost estimate for archival specimens is based on PCR amplification and contig assembly of two 350 bp amplicons.

	Fresh/Frozen	Archival
DNA Extraction	$0.34	$2.00
PCR Amplification	$0.24	$0.48
PCR Product Check	$0.35	$0.70
Cycle Sequencing	$1.04	$2.08
Sequencing Clean up	$0.32	$0.64
Sequence	$0.40	$0.80
Subtotal:	**$2.69**	**$6.70**
Staff time	$2.00	$5.00
Service contracts	$0.50	$1.00
Total:	**$5.19**	**$12.70**

Note: If barcode analysis is only being used for species identification, the PCR products can be sequenced in a single direction, reducing the cost to approximately $3.60 for fresh samples and to $8.80 for archival specimens. Bi-directional analysis is critical for sequences that will be used as 'barcode references'.

Robotic instruments will produce cost savings through reduced staff time, but will increase capital costs and service contracts.

TABLE 2: Detailed cost analysis for barcode assembly

Reagents and Disposables	Description	Catalogue #	Price	Units	Units/Sample	Price/Sample	Price/96
1) Genomic DNA Isolation/Purification							
1A							
Microplates	Eppendorf twin.tec 96-well microplates	E951020427	147.59	25	0.01	0.06	5.90
Extraction Kit	Sigma GenElute Mammalian Genomic DNA Kit	G1N350-KT	607.69	350	1	1.74	166.68
Tips (1-200 µl)	ProGene (Ultident) Tips - boxed; 1-200 µl	24-TR222-CR	31.95	10 x 96	6	0.20	19.20
						2.00	**191.78**
1B							
DryRelease reagents	BioRad Chelex 100	142-1253	104.00	50 g	0.006 g	0.01	1.25
	Invitrogen Proteinase K	25530-015	128.05	100 mg (5 ml of 20 mg/ml)	10 µl	0.26	25.70
	Sigma Sodium Azide	S2002-2KG	377.20	2 kg	0.0001	<<0.01	<0.01
Microplates	Eppendorf twin.tec 96-well microplates	E951020427	147.59	25	0.01	0.06	5.90
Tips (1-200 µl)	ProGene (Ultident) Tips - boxed; 1-200 µl	24-TR222-CR	31.95	10 x 96	0.08 (8 tips/96)	<<0.01	0.26
						0.34	**33.11**
2) PCR Amplification							
Taq & buffer	New England Biolabs	M0267L	288.00	2000	0.3125 u/rxn	0.045	4.50
dNTPS	New England Biolabs	N0447L	384.00	4 ml	0.0625 µl/rxn	0.006	0.60
Primers	Invitrogen Custom Oligos	N/A	0.50/base (~15.00/primer)	5000 µl of 10 µM	0.25 µl	0.007	0.70
Trehalose	Sigma Trehalose	T9531	159.3	100 g	0.0006	<<0.01	0.10
Tips	Corning (Fisher) filter tips - boxed; 0.1-10 µl	CS004807	82.70	10 X 96	1	0.09	8.27
Microplates	Eppendorf twin.tec 96-well microplates	E951020427	147.59	25	0.01	0.06	5.90
Strip caps	ABGene (Ultident) 8-Strip flat PCR caps	SP-0030	24.95	120	0.125	0.03	2.50
						0.24	**22.57**
3) PCR Product Check							
Pre-cast gels	Invitrogen E-gel 96 2%	G7008-02	242.05	8	N/A	0.32	30.26
Tips (1-200 µl)	ProGene (Ultident) Tips - boxed; 1-200 µl	24-TR222-CR	31.95	10 x 96	1	0.03	3.20
						0.35	**33.46**
4) Cycle Sequencing							
Primer	Invitrogen Custom Oligos	N/A	0.50/base (~15.00/primer)	5000 µl of 10 µM	1 µl	0.028	2.80
BigDye	ABI BigDye Terminator v3.1 Ready Reaction Mix (comes with 5X buffer)	4337456	10 584.00	1000 rxns (8000 µl)	0.25 µl	0.33	31.68
Trehalose	Sigma Trehalose	T9531	159.3	100 g	0.005 g	<0.01	0.80
Tips (1-200 µl)	Corning (Fisher) filter tips - boxed; 0.1-10 µl	CS004807	82.70	10 X 96	1	0.09	8.27
Microplates	Eppendorf twin.tec 96-well microplates	E951020427	147.59	25	0.01	0.06	5.90
						0.52	**49.45**
5) Sequencing Cleanup							
Sephadex	Sigma-Aldrich Sephadex G-50	G5080	1108.60	500 g	3.4 g/96	0.08	7.54
Plates	Millipore Multiscreen HV Plate	MAHVN4550	793.92	50	1 (reuse 2X)	0.08	7.93
						0.16	**15.47**
6)Sequencing							
Microplates	Barcoded Plate	4306737	109.88	20	1 (reuse 5X)	0.01	1.09
Polymer	POP 7	4335612	445.17	25 ml/150 runs*	3.4 µl	0.06	5.94
Buffer	10X Running Buffer	4335613	255.79	12 ml/10 runs*	N/A	0.01	1.23
Capillary	Capillary (50cm)	4331250	4480.67	800 runs*	N/A	0.06	5.60
Formamide	Formamide	4300320	38.42	1 ml/run*	0.01	0.01	1.54
Tips (1-200 µl)	ProGene (Ultident) Tips - boxed; 1-200 µl	24-TR222-CR	31.95	10 x 96	1	0.03	3.20
Tips (1-300 µl)	ProGene (Ultident) Tips - boxed; 1-300 µl	24-T350CRS	39.95	10x96	0.08 (8 tips/96)	<<0.01	0.33
Label	Barcode label			1		<<0.01	0.10
						0.20	**19.03**

* 1 run = 48 samples on ABI 3730 sequencer
Note: for bi-directional sequencing, double the cost/sample in 'Cycle-Sequencing', 'Sequencing Cleanup' and 'Sequencing'

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BARCODES OF LIFE

DNA BARCODING ANIMAL LIFE USING COI

DETAILED LABORATORY PROTOCOLS:

DNA EXTRACTION (RECENT) 1.1

GENERAL PRACTICES

- Clean the bench top with alcohol **before** and **after** setting up extractions.
- Always use clean, acid or flame sterilized forceps between specimens.
- For acid sterilization 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.
- For flame sterilization soak forceps in 95-100% ethanol and ignite on propane burner for 1-2 seconds. Repeat if necessary.
- Use small amounts of tissue from single animals (eg. single legs for Lepidoptera, whole animals for daphniids).

FOR RECENT SPECIMENS

1) Use a simple Chelex/Proteinase K extraction (**DryRelease**).
2) Aliquot 100 µl of Working Solution in each well of a 96-well plate.
3) Put a tiny amount of tissue (e.g. 1-2 mm of insect leg or 1-2 mm^3 of ethanol preserved tissue) into each well of a 96-well Eppendorf plate containing 100 µl of premixed DryRelease. To prevent cross contamination work with one row at a time. Close lids. Don't shake to make sure that the tissue fragment remains in the solution.
4) Incubate for 12-24 hours at 55ºC.
5) FOR ANIMAL TISSUE only – tape over the lids and shake on Vortex.
6) Centrifuge the plate at 1000 rpm for 5 minutes. Incubate samples in a
7) thermocycler at 95ºC 20 min to denature the Proteinase K enzyme.
8) Store extractions at –20ºC.
9) Before PCR set up, centrifuge the plate at 1000 rpm for 5 minutes.
10) Use 1-2 µl of DNA sample for PCR. Make sure that Chelex granules do not go into the PCR reaction.

CHELEX REAGENTS (DRYRELEASE)

Extraction Buffer (100 ml)	
Chelex-100 BIO-RAD	5-6 g
1% Sodium Azide	10 ml
1 Tris-HCl pH 8.3	1 ml
ultrapure H_2O	to 100 mls
Store at +4ºC in 10 ml aliquots	
Note: ***Sodium Azide is toxic (see MSD!)***	

Proteinase K (20 mg/ml)	
Proteinase K	100 mg
ultrapure H_2O	5 ml
Store in 0.1-1 ml aliquots at –20ºC	
Do not freeze-thaw more than 1 time.	
Store unused aliquot for 1-3 days at +4°C.	

Working Chelex Solution

Add 10 µl of Proteinase K to 100 µl of Chelex solution. To set up a plate – add 1 ml of Proteinase K to 10 ml of Chelex solution.

Different final extraction volume 40-110 µl could be used for different samples.

Aliquot Working Chelex Solution using multichannel or regular pipette (use wide-bored tips), mix solution while aliquoting to make sure that the resin is equally dispersed between wells (tubes).

DNA EXTRACTION (RECENT AND ARCHIVAL) 1.2

FOR RECENT AND ARCHIVAL SPECIMENS

Currently we use the Genomic DNA NucleoSpin® 96 Tissue Kit (Machery-Nagel) with modifications as follows:

1) Note: Use filter-plugged tips to avoid contamination of samples and reagents. Dilute Proteinase K with Proteinase Buffer and Buffer B5 with 95% ethanol prior to first use, as described in the instruction manual. Although elution Buffer BE is provided with the kit for Step #15 (the final elution of DNA from surface of columns), we use ddH_20 instead.
2) Add a tiny amount of tissue (e.g. 2-4 mm of insect leg or 1-3 mm^3 of ethanol preserved tissue) to each well of a Round-well Block supplied with the kit.
3) Prepare a working solution of Proteinase K by combining 180 µl of Buffer T1 with 25 µl of Proteinase K for each sample. Transfer 200 µl of the working solution into each well of the Round-well Block.
4) Seal wells with Cap Strips provided and shake vigorously for 10 - 15 seconds to mix. Centrifuge at 1500 g for 15 seconds to collect samples at the bottom of the wells.
5) Incubate at 56ºC (dry-bath) for a minimum of 6 hours or overnight to allow digestion. Tape down Cap Strips to prevent them from occasionally popping off.
6) After digestion, centrifuge at 1500 g for 15 seconds to remove any condensate from the Cap Strips.
7) Premix ethanol and Buffer BQ1: For each sample, add 200 µl of 95% ethanol and 200 µl of binding Buffer BQ1 to adjust DNA binding conditions. Shake vigorously for 10 - 15 seconds and centrifuge at 1500 g for 10 seconds to remove any sample form Cap Strips.
8) Remove Cap Strips and transfer lysate (about 600 µl) from the wells of the MN Round-well Block into the wells of the NucleoSpin® Tissue Binding Plate placed on top of an MN Square-well Block. Seal plate with Self-adhering PE foil supplied with kit.
9) Centrifuge at 5600 - 6000 g for 10 minutes to bind DNA to silica membrane.
10) First wash step: Add 500 µl of Buffer BW to each well of the NucleoSpin® Tissue Binding Plate. Use a new Self-adhering PE foil to seal the plate and centrifuge at 5600 - 6000 for 2 minutes.
11) To accommodate the volume of flowthrough, replace the current MN Square-well Block with a new, sterile MN Square-well Block underneath the NucleoSpin® Tissue Binding Plate.
12) Second wash step: Add 700 µl of Buffer B5 to each well of the NucleoSpin® Tissue Binding Plate. Use a new Self-adhering PE foil to seal the plate then centrifuge at 5600 - 6000 for 4 minutes.
13) Remove the Self-adhering PE foil and place the NucleoSpin® Tissue Binding Plate on an open rack with MN Tube Strips. Incubate at 56℃ for 30 minutes to evaporate residual ethanol.
14) Dispense 30 - 50 µl ddH_20 directly onto the membrane in each well of the NucleoSpin® Tissue Binding Plate and incubate at room temperature for 1 minute.
15) To elute highly pure DNA, centrifuge 5600 - 6000 g for 2 minutes and again at 5600 - 6000 g for 2 minutes after rotating NucleoSpin® Tissue Binding Plate and MN Tube Strips 180°. Remove NucleoSpin® Tissue Binding Plate and transfer eluted DNA to a 96 PCR plate and seal. Keep DNA at 4ºC for temporary storage or at -20ºC for long-term storage.
16) Use 1-2 µl of the DNA sample for PCR.

GENERAL PRACTICES

- The basic recipe for a Polymerase Chain Reaction (PCR) is given in the table at the right. The amount of DNA extract 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.
- **Addition of trehalose facilitates PCR and makes possible freezing of aliquoted master-mixes.** Aliquots in tubes can be stored at -20 ^{o}C for 1-3 months (1-3 freeze-thaw cycles don't affect performance).The content of a tube should be mixed by pipetting before use. Another option – aliquot mixes directly into 96-well plates, cover plates with PCR film, store at -20^{o}C for 1 month.
- 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 sterile 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 individual reagents are given on the following page.

PCR REACTION MIX

Volumes for One Reaction		
	25 µl	**12.5 µl**
10% trehalose	12.5	6.25
H_2O+DNA	8	4
10X PCR Buffer	2.5	1.25
50 mM $MgCl_2$ (2.5 mM)	1.25	0.625
10 mM dNTP	0.125	0.0625
10 µM Primer 1	0.25	0.125
10 µM Primer 2	0.25	0.125
Taq polymerase	0.125	0.0625

MULTIPLE SAMPLES

When doing multiple reactions with the same primer set, make a master mix that contains everything except DNA template. It is OK to round up volumes of dNTPs, primers and Taq to the nearest 0.5 µl.
If you plan to fill several 96-well plates include extra volume to allow for pipetting mistakes and dead volume in the digital multichannel pipettor (e.g. for making 10 plates with 12.5 µl reactions each, include about 40 extra reactions)

Volumes for 96-well plate		
	25 µl(x100)	**12.5 µl(x104)**
DNA (e.g. 2 µl)	-	-
10% trehalose	1250	650
H_2O	600	208
10X PCR Buffer	250	130
50 mM $MgCl_2$	125	65
10 mM dNTP	12.5	6.5
10 µM Primer 1	25	13
10 µM Primer 2	25	13
Taq polymerase	12.5	6.5

Aliquot 1/8 of total mix volume in 8-tube PCR strip (if making more than one plate – to disposable container) and dispense desired volume (23 µl for 25 µl reactions or 10.5 µl for 12.5 µl reactions) in 96-well plate and then add 2 µl of DNA extract.

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 g
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

10% trehalose

D-(+)-Trehalose dihydrate	5 g
ultrapure H_2O	to 50 mls

store frozen in 1-2 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 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) for 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 **Eppendorf mastercycler ep*gradient*** thermocycler is shown at the right.
- Step 1 is an initial 94ºC soak to completely denature the original DNA template.
- 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 holds the samples until they are removed.

Eppendorf PCR Program for COI

Step	Action	Time
1	94°C	1 min
2	94°C	40 sec
3	45°C	40 sec
4	72°C	1 min
5	GOTO step 2	5 more times
6	94ºC	40 sec
7	51ºC	40 sec
8	72ºC	1 min
9	GO TO step 6	35 more times
10	72°C	5 min
11	4°C	0:0:0

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**
0.7	800-12000
1.0	500-10000
1.2	400-7000

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.

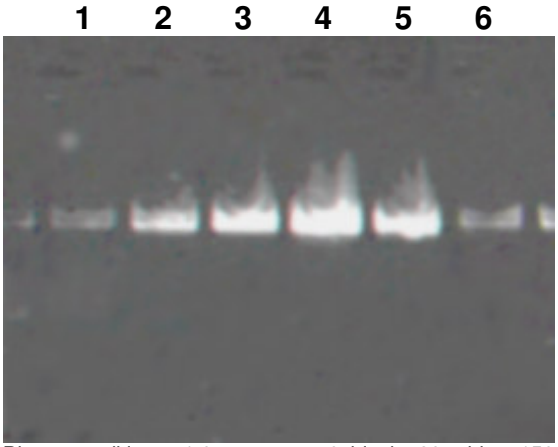


Photo conditions: 1.0 exposure, 0 black, 90 white, 150 gamma.

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).

INVITROGEN E-GEL® 96 SYSTEM AND SOFTWARE

We now employ pre-cast agarose gels from Invitrogen. This system is bufferless, so exposure to Ethidium Bromide is minimized. However, gloves should be worn when handling and loading the gel.

Loading and Running E-gel® 96 Gels:

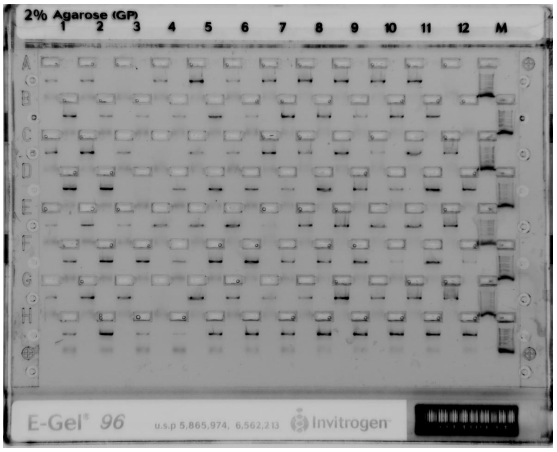
The recommended program for E-gel® 96 gel is EG and the run time is 6-12 min.
Plug the Mother E-Base into an electrical outlet. Press and release the pwg/prg (power/program) button on the base to select program EG.

- Remove gel from the package and remove plastic comb from the gel.
- Slide gel into the two electrode connections on the Mother or Daughter E-Base™.
- Load 15 µl of ddH_20 or diluted Gel Dye into wells with 8- or 12-multichannel pipettor.
- Load appropriate DNA markers in the marker wells
- Load 4-5 µl of sample.
- To begin electrophoresis, press and release the pwd/prg button on the E-Base™. The red light changes to green.
- At the end of run (signaled with a flashing red light and rapid beeping), press and release the pwr/prg button to stop the beeping.
- Remove gel cassette from the base and capture a digital image of a gel on UV transilluminator equipped with digital camera.
- Analyze the image and align or arrange lanes in the image using the E-Editor™ 2.0 software available at www.invitrogen.com/egels.
- Incorporate E-gel image into lab spreadsheet for estimation of concentration and hit picking.

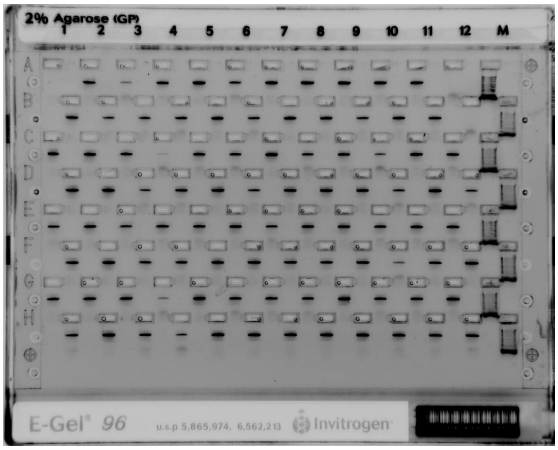
TYPICAL E-GEL IMAGES (NEGATIVE) OF COI AMPLIFICATION – DNA EXTRACTED WITH CHELEX

The dark band indicate product; the clear slots are the loading wells.

Regular PCR master mix without trehalose (A12, B12 – negative controls; M – size marker)



PCR master mix with 5% trehalose (A12, B12 – negative controls, M – size marker)



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).

SEQUENCING REACTION 5.1

DYE TERMINATOR SEQUENCING OF COI FOR THE 3730 DNA ANALYZER

- 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 or 1-2 µl (depending on intensity of the band) for directly sequenced PCR products.

Reagent	COI (~700 bp)
Dye terminator mix **v3.1**	0.25 µl
5 X Sequencing Buffer	1.875 µl
10% trehalose	5 µl
10 µM Primer	1 µl
H_2O	0.875 µl
DNA	1[up to 2] µl
Final Volume	10 µl

Note: 5X Sequencing buffer is: 400 mM Tris-HCl pH 9 + 10 mM $MgCl_2$ or 5X ABI sequencing buffer.

- 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	2 min
2	96°C	30 sec
3	55°C	15 sec
4	60°C	4 min
5	GOTO step 2	29 more times
6	4°C	00:00:00

SEQUENCING REACTION CLEAN-UP 6.1

SEPHADEX CLEAN-UP

- Measure Dry Sephadex with the Black column loader into the Sephadex Plate.
- Hydrate the wells with 300 µl of Autoclaved Distilled Water.
- Let the Sephadex swell overnight in the fridge OR for 3 to 4 hours at room temperature before use.
- Put Sephadex plate together with Centrifuge Alignment frame and "balance" plate – hold together with at least 2 rubber bands.
- Make sure the two sets weigh the same (adjust weigh by using different rubber bands.
- Centrifuge at 750 rcf for 3 minutes – this is to drain the water from the wells.
- Add the entire DNA from the Sequencing Reaction to the center of Sephadex columns.
- Add 10 µl of Formamide to each well of the autoclaved 3730 96 well plate.
- To elute DNA attach 3730 96 well plate to the bottom of the Sephadex plate – secure them with tape and with rubber bands.
- Make sure the sets weigh the same (adjust weight by using different rubber bands).
- Centrifuge at 750 rcf for 3 minutes.
- Cover the top of plate with Septa.
- Place 3730 96 well plate into the black plate bases and attach the white plate retainer.
- Stack assembled plate in the 3730 – don't forget your barcode and plate record.