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Title of resource

GN_10: PCR Amplification of the DNA Barcode Segment of the mitochondrial DNA Cytochrome Oxidase Subunit I (COI) gene

Authored by

When using this protocol, the following should be referenced:

Harrup, L.E (2018). The Pirbright Institute DNA Barcoding Protocols Version 3.

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Description

This method details the protocol and guidance notes for the amplification of the DNA Barcode (Hebert *et al.* 2003) segment of the mitochondrial DNA (mtDNA) cytochrome oxidase subunit I (COI) gene from genomic DNA (gDNA) or total nucleic acid extractions. Protocol from the Gnatwork Bangladesh workshop, September 3-6th 2018.

Intended use

Scientific research use and training purposes.

Restrictions on use

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Resource history

Updated version of: Harrup, L.E. (2014). The Pirbright Institute *Culicoides* DNA Barcoding Protocols, Version 2, available at www.ibvnet.com

GN_10: PCR Amplification of the DNA Barcode Segment of the mitochondrial DNA Cytochrome Oxidase Subunit I (COI) gene

Citation: Harrup, L.E (2018). The Pirbright Institute DNA Barcoding Protocols Version 3

A. Introduction

This method details the protocol and guidance notes for the amplification of the DNA Barcode (Hebert *et al.* 2003) segment of the mitochondrial DNA (mtDNA) cytochrome oxidase subunit I (COI) gene from genomic DNA (gDNA) or total nucleic acid extractions.

B. Materials required

Equipment

- PCR laminar flow cabinet (or microbiological safety cabinet Class II) (ideally two cabinets, to allow separation of mastermix preparation and template addition activities)
- Thermal cycler
- Fridge (+4°C)
- Freezer (-20°C)
- Reaction tube spinner, plate spinner, or centrifuge with plate rotor (appropriate to the plasticware to be used)
- Vortex
- Pipettes (0.1-2.0 µl, 1-20 µl, 20-200 µl, 100-1000 µl)
- Multichannel pipette (5-50 µl) (optional)
- Disposable pipette tips containing hydrophobic filters (2 µl, 20 µl, 200 µl, 1000 µl)
- Thin-wall reaction tubes as individual tubes, 8 or 12 tube strips or PCR plates plates (of an appropriate size (e.g. 200 µl) and type for the brand and model of thermal cycler to be used (see the thermal cycler's manufacturers guidance for further information on compatible plasticware)
- For primers: 1.5 ml screw cap or flip-top (e.g. Eppendorf® Safe Lock Tubes #0030 123.328) microcentrifuge tubes
- For mastermix preparation: 1.5 ml flip-top microcentrifuge tube or 15 ml centrifuge tubes (dependent upon volume of mastermix required to be prepared)
- PCR adhesive plate sealers (*only if using 96-well PCR plates)
- Solvent resistant marker pen
- Blue roll
- Label printer (*optional)
- Microcentrifuge tube labels
- Solvent resistant cryopen
- 96 well PCR tube rack
- Microcentrifuge tube rack
- Low volume reagent reservoir (or equivalent)
- Benchtop cooler for microcentrifuge tubes or ice bucket & ice (optional)

Reagents

- DNAZap™ (AM9890, ThermoFisher Scientific, UK) (or equivalent)
- Nuclease free water (NFW)
- 1 M Tris-HCL pH 8.0 (e.g. ThermoFisher Scientific, #15568025)
- Forward & reverse primers (see Table 1 & 2)
- Selected Mastermix Components (see below)

Platinum™ Taq Mastermix

- 10 mM dNTP Mix (e.g. D7295 Sigma, UK; 18427-088: ThermoFisher Scientific, UK)
- Platinum™ Taq DNA Polymerase (kit contains Platinum™ Taq polymerase, 10x reaction buffer and 50 mM Magnesium Chloride ($MgCL_2$) (10966018 120rxs; 10966026 300rxn; 10966034 600 reactions: ThermoFisher Scientific, UK)
- Bovine serum albumin (BSA) (20mg/ml molecular grade ultrapure non-acetylated) (e.g. 10711454001: Sigma Aldrich)

TopTaq Mastermix

- TopTaq mastermix kit (Qiagen: 200403)

C. Method

C.1 Review Table 1 and select the appropriate primer set, mastermix and thermal profile for the target taxa.

C.2 Ultraviolet (UV) sterilise the PCR laminar flow hood (or microbiological safety cabinet (MSC)) which is designated for mastermix preparation and if required also clean the working area, pipettes etc. with DNAZap™ (or equivalent). Once cleaned complete the following steps within the PCR laminar flow hood (or MSC).

C.3 Prepare a working stock of 10 mM Tris-HCL pH 8.0 (e.g. for 50 ml add 500 µl 1M Tris-HSL pH 8.0 to 49.5 ml nuclease free water), label with the name and concentration of the solution, your initials and the date made [store at 4°C when not in use].

C.4 Resuspend lyophilised primers to 100 µM with 10 mM Tris-HCL pH 8.0 using the volume indicated by the supplier (10 µl 10 mM Tris-HCL pH 8.0 per 1 nmole of primer e.g. 36.4 nmole primer add 364 µl 10 mM Tris-HCL pH 8.0 to each lyophilised primer and vortex briefly). Store at 4°C for approximately 20 minutes to allow primers to fully resuspend.

C.5 Make a working stock of each primer at the concentration required by the mastermix by briefly vortexing resuspended 100 µM primers, then make an appropriate dilution in a second appropriately labelled 1.5 ml microcentrifuge tube [store both re-suspended 100 µM primer and working stock 10 µM primer at -20°C when not in use]. For example:

- For 100 µl of 10 µM working primer stock add 10 µl re-suspended 100 µM primer to 90 µl 10 mM Tris HCL pH 8.0).

C.6 Appropriately label individual reaction tubes, tube strips or PCR plates so that tubes can be linked to each specimen/samples' unique identifier code (ensure plasticware is appropriate for the thermal cycler to be used).

C.7 Thaw if needed, then vortex briefly forward and reverse primers and other mastermix components. The mastermix components used in this protocol do not need to be prepared over ice.

C.8 In a microcentrifuge or centrifuge tube as appropriate to the volume require, make sufficient of the selected mastermix for the number of wells required for samples and positive and negative controls (volume per reaction x number of reactions required +10% (to account for wastage during pipetting, use of reagent reservoirs etc.)).

C.9 Vortex the mastermix and aliquot 20 µl into each well. For large numbers of wells, distribute the mastermix into a low volume reagent reservoir, eight-well tube strip or other appropriate plasticware and use a multichannel pipette to distribute the mastermix.

C.10 If separate mastermix preparation and template addition areas/hood are not available proceed directly to step C.11, if separate areas are available seal tubes/plates containing mastermix and transfer the plate/tubes containing mastermix to the designated template addition area then proceed to step C.11.

C.11 Thaw if needed, then briefly gently mix/vortex DNA extractions (DNA template) (caution excessive vortexing can increase DNA fragment shearing decreasing the quality of the DNA extraction).

C.12 Using a new pipette tip per well to prevent cross-contamination transfer 5 µl of each DNA template into the corresponding labelled 200 µl reaction tube and if using individual tubes or strips seal securely after each template addition to reduce the potential for cross-contamination.

C.13 Using a new pipette tip per well transfer 5 µl of the nominated positive control sample into the designated positive control well(s), if using individual tubes or strips seal securely after each template addition to reduce the potential for cross-contamination.

C.14 Using a new pipette tip per well transfer 5 µl of nuclease free water into the designated negative control well(s). If using individual tubs or strips of tubes seal securely after the addition of nuclease free water.

C.15 If using PCR plates seal the plate securely with PCR adhesive film/foil. Or if using individual reaction tubes or tube strips ensure they are all securely sealed.

C.16 Briefly vortex tubes/plate.

C.17 Spin reaction tubes/plate for approximately 10 seconds in the reaction tube spinner / centrifuge to concentrate reaction mix at the base of the wells.

C.18 Place reaction tubes in the thermal cycler and carry out PCR amplification using the thermal profile appropriate to the primers used see Table 1.

C.19 UV Sterilise the PCR Laminar flow hood(s)/MSCs and other working areas used and if required also clean working area, pipettes etc. with DNAZap™ (or equivalent).

C.20 When the thermal cycle is finished, remove reaction tubes from the thermal cycler and store PCR product at 4°C if to be used with 24 hours or store at -20°C until required for downstream analysis.

Table 1 PCR Primer sets for different taxonomic groups

Primer Set Name	PCR Primers		Sequencing Primers		Amplicon size (base pairs-bp) excluding primers	Mastermix & Thermal Profile
	Forward	Reverse	Forward	Reverse		
COIpanCUL	COIpanCULF1_t1	COIpanCULR1_t1	M13F(-21)	M13R(-27)	658 bp	Table 3 or 4
Lep/Fol Cocktail	LCO1490_t1 + LepF1_t1	HCO2198_t1 + LepR1_t1	M13F(-21)	M13R(-27)	658 bp	Table 5 or 6

Table 2 Primer Sequences (_t1 suffix indicates primer has M13 tail, M13F(-21) sequence shown in italics, M13R(-27) shown underlined)

Oligo Name	Sequence (5'-3')	Size (bp)	Reference
Sequencing			
M13F(-21)	TGTAAAACGACGGCCAGT	17	Messing, 1983
M13R(-27)	CAGGAAACAGCTATGAC	17	Messing, 1983
mtDNA COI			
COIpanCULF1_t1	TGTAAAACGACGGCCAGTTChACwAAyCAyAAArwTATTGG	41	Harrup, 2018
COIpanCULR1_t1	CAGGAAACAGCTATGACTAwACyTCdGGrTGICCrAArAATCA	43	Harrup, 2018
LCO1490_t1	TGTAAAACGACGGCCAGTGGTCAACAAATCATAAAGATATTGG	43	Folmer *et al.*, 1994
HCO2198_t1	CAGGAAACAGCTATGACTAAACTTCAGGGTGACCAAAAAATCA	43	Folmer *et al.*, 1994
LepF1_t1	TGTAAAACGACGGCCAGTATTCAACCAATCATAAAGATATTGG	43	Herbert *et al.*, 2004
LepR1_t1	CAGGAAACAGCTATGACTAAACTTCTGGATGTCCAAAAAATCA	43	Herbert *et al.*, 2004

Table 3 COIpanCUL Platinum™ Taq mastermix and thermal profile

Component	Initial Concentration	Final Concentration	Volume Per Reaction in µl
Nuclease Free Water			14.9
PCR Buffer	10x	1x	2.5
$MgCL_2$	50 mM	1.5 mM	0.75
dNTPs	10 mM	0.2 mM each	0.5
Bovine Serum Albumin	20 mg/ml	0. 2 mg/ml	0.25
Platinum™ *Taq* DNA Polymerase		1 U/rxn	0.1
Forward Primer			
COIpanCULF1_t1	10 µM	0.2 µM	0.5
Reverse Primer			
COIpanCULR1_t1	10 µM	0.2 µM	0.5

Initial denaturation	2 minutes	94˚C
3-step Cycling (40 Cycles)		
Denaturation	30 seconds	94˚C
Annealing	40 seconds	50˚C
Extension	1 minute	72˚C
Final Extension	10 minutes	72°C
Soak	∞	4˚C

Table 4 COIpanCUL TopTaq mastermix and thermal profile

Component	Initial Concentration	Final Concentration	Volume Per Reaction in µl
Nuclease Free Water			4.0
TopTaq Mastermix	2x	1x	12.5
CoralLoad Concentrate	10x	1x	2.5
Forward Primer			
COIpanCULF1_t1	10 µM	0.2 µM	0.5
Reverse Primer			
COIpanCULR1_t1	10 µM	0.2 µM	0.5

Initial denaturation	3 minutes	94˚C
3-step Cycling (40 Cycles)		
Denaturation	30 seconds	94˚C
Annealing	40 seconds	50˚C
Extension	1 minute	72˚C
Final Extension	10 minutes	72°C
Soak	∞	4˚C

Table 5 Lep/Fol Cocktail Platinum™ Taq mastermix and thermal profile

Component	Initial Concentration	Final Concentration	Volume Per Reaction in µl
Nuclease Free Water (NFW)			13.9
PCR Buffer	10x	1x	2.5
$MgCL_2$	50 mM	1.5 mM	0.75
dNTPs	10 mM	0.2 mM each	0.5
Bovine Serum Albumin	20 mg/ml	0.2 mg/ml	0.25
Platinum™ *Taq* DNA Polymerase		1 U/rxn	0.1
Forward Primers			
LCO1490_t1	10 µM	0.2 µM	0.5
LepF1_ t1	10 µM	0.2 µM	0.5
Reverse Primers			
HCO2198_t1	10 µM	0.2 µM	0.5
LepR1_t1	10 µM	0.2 µM	0.5

Initial denaturation	2 minutes	94˚C
3-step Cycling (40 Cycles)		
Denaturation	30 seconds	94˚C
Annealing	40 seconds	46˚C
Extension	1 minute	72˚C
Final Extension	10 minutes	72°C
Hold	∞	4˚C

Table 6 Lep/Fol Cocktail TopTaq mastermix and thermal profile

Component	Initial Concentration	Final Concentration	Volume Per Reaction in µl
Nuclease Free Water (NFW)			3.0
TopTaq Mastermix	2x	1x	12.5
CoralLoad Concentrate	10x	1x	2.5
Forward Primers			
LCO1490_t1	10 µM	0.2 µM	0.5
LepF1_ t1	10 µM	0.2 µM	0.5
Reverse Primers			
HCO2198_t1	10 µM	0.2 µM	0.5
LepR1_t1	10 µM	0.2 µM	0.5

Initial denaturation	3 minutes	94˚C
3-step Cycling (40 Cycles)		
Denaturation	30 seconds	94˚C
Annealing	40 seconds	46˚C
Extension	1 minute	72˚C
Final Extension	10 minutes	72°C
Hold	∞	4˚C

D. Results

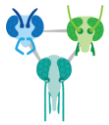
D.1 Where appropriate associated experiment results should be recorded in the relevant laboratory notebook.

D.2 Confirm successful amplification of the DNA Barcode segment of the COI gene via agarose gel electrophoresis on a 2% (*w/v*) agarose gel. If using the 2% 96-well E-Gel™ run the gel for 8 minutes on program EG.

D.3 Successful amplification of the DNA Barcode segment of the COI gene using this protocol is indicated by the presence of a band at the appropriate size for the target amplicon (see Table 1) plus the primer size (see Table 2), results are recorded as the presence or absence of a band at the correct size. No band should be present in the negative control lane(s).

E. Tips and troubleshooting

- Addition of between 1 ng to 1 µg of gDNA template per reaction is optimal, hence depending on the DNA yield of the DNA extractions the volume of DNA template added to each reaction may need to be increased or decreased as required, increasing or decreasing the volume of NFW to ensure the total volume including template is constant at 25 µl to maintain the concentration of other reagents in the mastermix.
- If after the PCR process the samples have appeared to condense, the caps or sealing film may not have been attached correctly or the heated lid may not have been set to be on during the thermal cycle. Correct and repeat as required.
- Platinum™ Taq DNA polymerase (ThermoFisher Scientific) is a recombinant Taq DNA polymerase complexed with an antibody that inhibits polymerase activity at ambient temperature. It is therefore stable for room temperature reaction setup hence reaction setup



over ice is not required, however it should be stored at -20°C when not in use. The polymerase is activated in a temperature-dependent manner (at 94°C) during the start of the PCR thermal cycle, once the antibody is dissociated, the Taq DNA polymerase regains its full activity.

- The TopTaq Mastermix Kit (Qiagen) is a ready to use PCR mastermix, containing PCR buffer, $MgCL_2$ and dNTPs reducing pipetting steps during mastermix setup. The TopTaq master mix may be stored at 4°C, reducing the requirement to thaw reagents prior to use and reactions do not need to be prepared over ice. The TopTaq Mastermix Kit also includes CoralLoad Concentrate a gel loading reagent with two gel-tracking dyes which can be included directly in the PCR mastermix reaction mix, reducing pipetting steps prior to agarose gel electrophoresis.
- This protocol may be adapted for use with a variety of other mastermixes and Taq DNA polymerases from other suppliers. Care must be taken, however, when selecting alternative reagents that cost-savings do not negatively influence the quality of resulting data. We have found that Qiagen TopTaq™ mastermix offers a good compromise between, cost-saving, ease of use and efficient and accurate amplification. The alternative Platinum™ Taq DNA Polymerase together with the inclusion of BSA is preferable with more difficult/low yield DNA templates.
- Bovine serum albumin (BSA) when used as an additive in PCR acts to enhance enzyme activity e.g. Taq and increases PCR yields from low purity templates. As an additive BSA is also helpful in reducing the loss of enzyme through non-selective adsorption to reaction tube walls, pipette tips and other plasticware.
- D-(+)-Trehalose may also be added to the Platinum™ Taq mastermixes listed in Table 3 and 5 at a final concentration of 5% as a PCR enhancer (reduce the volume of nuclease free water included in the reaction to ensure the final reaction volume remains at 25 µl). The addition of D-(+)-Trehalose when used with Invitrogen Platinum™ Taq DNA Polymerase also allows mastermixes to be aliquoted out into PCR plates and frozen ready for use. Ready-made frozen plates of this type should be used within 3 months. D-(+)-Trehalose can reduce the optimal annealing temperature of the primers a gradient PCR should be utilised to check that the inclusion of D-(+)-Trehalose in the reaction mastermix has not decreased the efficiency of the reaction due to changes in the optimal primer annealing temperature.
- The primer pairs listed in this protocol have been found to perform best with a relatively low annealing temperature producing a high abundance of the target PCR product with minimal non-specific amplification. However if non-specific products are observed when PCR products are visualised following agarose gel electrophoresis, try reducing the number of cycles (between 30-40 cycles is suitable for most DNA templates) and/or increasing the annealing temperature.
- The Platinum™ Taq and TopTaq mastermix recipes given in this protocol can be adjusted to include alternative primer pairs (see the literature for relevant primers details). Optimal concentration and annealing temperatures of new primer pairs should be confirmed using positive control samples prior to being utilised with field samples. If alternative primer concentrations are required, the volume of nuclease free water included in the reaction should be adjusted as required to ensure the final reaction volume remains at 25 µl.
- Platinum™ Taq DNA polymerase is also available as a 2x mastermix (13000013 colourless 200 reactions; 13001013 with tracking dyes 200 reactions) which can be substituted at 12.5 µl per reaction for the Platinum™ Taq DNA polymerase, 10x PCR Buffer, 50 mM $MgCl_2$ and the 10 mM dNTP components of the mastermix listed in Table 3 and 5, adjust the volume of NFW to ensure total reaction volume including mastermix, BSA, forward and reverse primers and DNA template remains at 25 µl.