Development and validation of a multi-locus DNA metabarcoding method to identify endangered species in complex samples_SOP Version 2

Alfred J. Arulandhu,Martijn Staats,Rico Hagelaar,Marleen M. Voorhuijzen,Theo W. Prins,Ingrid Scholtens,Tamara Peelen and Esther Kok

Abstract

Background: DNA metabarcoding provides great potential for species identification in complex samples such as food supplements and traditional medicines. Such a method would aid CITES (the Convention on International Trade in Endangered Species of Wild Fauna and Flora) enforcement officers to combat wildlife crime by preventing illegal trade of endangered plant and animal species. The objective of this research was to develop a multi-locus DNA metabarcoding method for forensic wildlife species identification and to evaluate the applicability and reproducibility of this approach across different laboratories.

Results: A DNA metabarcoding method was developed that makes use of 12 DNA barcode markers that have demonstrated universal applicability across a wide range of plant and animal taxa, and that facilitate the identification of species in samples containing degraded DNA. The DNA metabarcoding method was developed based on Illumina MiSeq amplicon sequencing of well-defined experimental mixtures, for which a bioinformatics pipeline with user-friendly web interface was developed. The performance of the DNA metabarcoding method was assessed in an international validation trial by 16 laboratories, in which the method was found to be highly reproducible and sensitive enough to identify species present in a mixture at 1% dry weight content.

Conclusion: The advanced multi-locus DNA metabarcoding method assessed in this study provides reliable and detailed data on the composition of complex food products, including information on the presence of CITES-listed species. The method can provide improved resolution for species identification, while verifying species with multiple DNA barcodes contributes to an enhanced quality assurance.

Citation: Alfred J. Arulandhu,Martijn Staats,Rico Hagelaar,Marleen M. Voorhuijzen,Theo W. Prins,Ingrid Scholtens,Tamara Peelen and Esther Kok Development and validation of a multi-locus DNA metabarcoding method to identify endangered species in complex samples_SOP. **protocols.io**

dx.doi.org/10.17504/protocols.io.jwzcpf6

Published: 21 Sep 2017

Guidelines

This DNA metabarcoding method has been designed for the qualitative identification of multiple species in complex samples. DNA metabarcoding combines DNA barcoding with next-generation sequencing (NGS), and makes use of universal PCR primers to mass-amplify and sequence one or more taxonomically informative DNA barcodes. In this SOP, the following animal DNA barcodes are used: COI-2, cytochrome b (cyt *b*), 16S ribosomal RNA (16S). The following plant DNA barcodes are used: maturase K (*matK*), ribulose 1-5 biphosphate carboxylase large subunit (*rbcL*), the chloroplast *trnL* (UAA) intron, and the Internal Transcribed Spacer 2 (ITS2) region. Full-length barcodes and mini-barcodes with reduced sequence lengths are used for 16S, COI-2, cyt *b*, *rbcL*, ITS2 and *trnL* to accommodate the identification of species in degraded DNA samples. All amplicons are amplified in singleplex PCR.

Samples

The participating laboratories are provided with ten microcentrifuge tubes each containing 250 mg of powdered tissue stored in a cool box containing dry ice. The samples are labeled sample S1 to S10. In addition, two tubes containing positive control DNA (*Bos taurus* and *Lactuca sativa*) are shipped. The positive controls are labeled as 150 µl of 10 ng/µl Bos taurus and 150 µl of 10 ng/µl *Lactuca sativa.*

All samples should be stored frozen at -20°C until processed. Samples can be stored frozen indefinitely.

Required equipment, chemicals, reagents, consumables, and materials

Equipment

— 96-well thermocycler where the temperature range can be set between 8°C and 95°C, and the temperature accuracy is at least ±0.25°C (e.g. BIO-RAD CFX PCR Thermal Cycler).

— Spectrophotometer (regular or NanoDrop)

— Thermo-Shaker for 1.5 ml and 2 ml tubes capable of mixing at ~1000 rpm and heating at 65ºC (Mandatory).

— Centrifuge for 1,5-2 ml tubes capable of spinning at ~18,000xg.

— Mini-centrifuge for PCR tube strips capable of spinning at 10,000xg.

— Vortex machine for 1.5 ml and 2 ml tubes.

— Electrophoresis system to discriminate PCR amplification fragments between ~180 bp and ~920 bp (gel or microchip electrophoresis system)

— Electrophoresis gel imaging UV machine

— Full set of semi-automatic pipettes

— Analytical scales (max. capacity 100 g, readability 0.001 g)

— Fridge (or 4°C storage facility)

— Freezer (or -20°C storage facility)

— 1.5 ml and PCR tube racks

— Cold Block or ice

Chemicals and Reagents

— Nuclease-free molecular grade water (ddH2O)

— 150 µl RNase A (QIAGEN (Cat No./ID: 19101), solution 100 mg/ml molecular biological quality or equivalent)

— 500 µl Proteinase K (Fermentas (Cat No./ID: EO0491), solution 20 mg/ml, molecular biological quality or equivalent)

— 50 ml Chloroform

— 20 ml Ice-cold ethanol (96%)

— 20 ml 70% ethanol

— 1 ml 100 bp DNA Ladder (e.g. Invitrogen; for gel electrophoresis, if required).

— If using gel electrophoresis system (instead of chip electrophoresis system): the necessary chemicals and reagents required for the chip electrophoresis system to be used.

o 20 g Agarose

o 40 µl Ethidium bromide

o 1L TBE running buffer for gel electrophoresis

 54 g Tris

 27.5 g Boric acid

 20 ml 0.5M EDTA, pH 8

o 5 ml 10X loading dye

Consumables and Materials

— Centrifuge tubes (100-150 tubes 1.5 ml and 50 tubes 2 ml)

— 50 PCR tubes strips (8 tube strips, single attached domed cap, e.g. Bioplastics EU 8-tube cat no. C78201).

— Electrophoresis system, tray and comb used to discriminate PCR amplification fragments between ~180 bp and ~920 bp.

— If using gel electrophoresis system (instead of chip electrophoresis system): the necessary chemicals and reagents required for the chip electrophoresis system to be used.

— Micropipette filter tips (10 µl, 30 µl, 200 µl and 1000 µl tips)

— Small plastic spatula

— Disposable powder- free gloves

— Clean lab coat

— Cold Block or ice

— Aluminium foil

Chemicals and Reagents

All the provided chemicals and reagents are 2.5 times the amount required for the analysis.

— 17.5 ml of CTAB extraction buffer

— 25 ml of CTAB precipitation buffer

— 9 ml of 1.2M NaCl

— 6 tubes of HotStarTaq Master Mix and 3 tubes of water (Qiagen).

— Oligonucleotide primers (high purity), 20 µl (10 µM) of each primer.

— **Primer labelling information**:

16S-F COI-2-F CytB-F 16S-mini-F COI-mini-F CytB-mini-F Matk-F rbcL-F trnL(UAA)-F ITS2-F rbcL-mini-F trnL(P6loop)-F
16S-R COI-2-R CytB-R 16S-mini-R COI-mini-R CytB-mini-R Matk-R rbcL-R trnL(UAA)-R ITS2-R rbcL-mini-R trnL(P6loop)-R

CTAB DNA isolation

The protocol described is the CTAB DNA isolation procedure according to Murray and Thomson (1980).

Required reagents and chemicals

— Molecular grade water (ddH2O)

— 17.5 ml CTAB extraction buffer

— 150 µl RNase A (Qiagen, solution 100 mg/ml, molecular biological quality or equivalent)

— 500 µl Proteinase K (Fermentas, solution 20 mg/ml, molecular biological quality or equivalent)

— 50 ml Chloroform

— 25 ml CTAB precipitation buffer

— 9 ml NaCl

— 20 ml Ice-cold ethanol (96%) (e.g. stored at -20ºC)

— 20 ml 70% ethanol

PCR

Table 3: 96-well PCR plate setup for Plate 1 and Plate 2 for the 12 barcode markers and 10 samples.

Plate 1	Animal barcode markers						Plant barcode markers					
	16S	COI-2	CytB	16S-mini	COI-mini	CytB-mini	matK	rbcL	trnL(UAA)	ITS2	rbcL-mini	trnL(P6 loop)
	1	2	3	4	5	6	7	8	9	10	11	12
A	S1	S1	S1	S1	S1	S1	S1	S1	S1	S1	S1	S1
B	S2	S2	S2	S2	S2	S2	S2	S2	S2	S2	S2	S2
C	S3	S3	S3	S3	S3	S3	S3	S3	S3	S3	S3	S3
D	S4	S4	S4	S4	S4	S4	S4	S4	S4	S4	S4	S4
E	S5	S5	S5	S5	S5	S5	S5	S5	S5	S5	S5	S5
F	S6	S6	S6	S6	S6	S6	S6	S6	S6	S6	S6	S6
G	PA	PA	PA	PA	PA	PA	PP	PP	PP	PP	PP	PP
H	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC

Plate 2	Animal barcode markers						Plant barcode markers					
	16S	COI-2	CytB	16S-mini	COI-mini	CytB-mini	matK	rbcL	trnL(UAA)	ITS2	rbcL-mini	trnL(P6 loop)
	1	2	3	4	5	6	7	8	9	10	11	12
A	S7	S7	S7	S7	S7	S7	S7	S7	S7	S7	S7	S7
B	S8	S8	S8	S8	S8	S8	S8	S8	S8	S8	S8	S8
C	S9	S9	S9	S9	S9	S9	S9	S9	S9	S9	S9	S9
D	S10	S10	S10	S10	S10	S10	S10	S10	S10	S10	S10	S10
E	PA	PA	PA	PA	PA	PA	PP	PP	PP	PP	PP	PP
F	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC
G												
H												

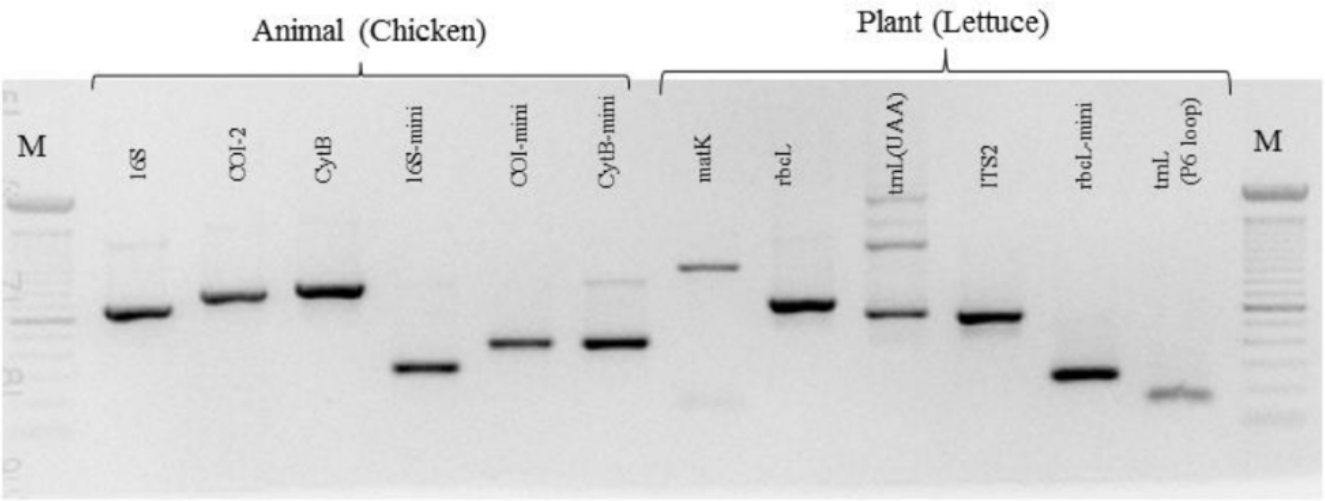

Figure 1: Animal and plant positive control image

Illumina MiSeq sequencing (BaseClear)

— The purified PCR products together with the (digital) gel images will need to be sent to RIKILT, clearly stating your name and affiliation, and referencing the "DECATHLON WP5 DNA metabarcoding" project. The DNA should be shipped on cool packs (blue ice) to:

RIKILT – Wageningen UR

Attn: Martijn Staats/Alfred Arulandhu

Akkermaalsbos 2

Building 123

6708 WB Wageningen

The Netherlands

— Please use (DHL/FedEx/TNT) express delivery service within your country for shipping the DNA to RIKILT.

— RIKILT will collect all the purified and pooled PCR products of all partners and send them to BaseClear B.V. for Illumina MiSeq sequencing.

Bioinformatics data analysis

Procedure

- The raw Illumina MiSeq data will be processed by RIKILT using the default settings of the CITESspeciesDetect pipeline. Details of the CITESspeciesDetect pipeline are available upon

request. **Note**: a user-friendly and platform-independent interface to the CITESspeciesDetect pipeline is currently under development, and is currently not available to the end-user.

- RIKILT will send the bioinformatics output to each participating partner for interpretation of results. The output will consist of the following files:
- The BLAST results file containing the 20 best BLAST results (i.e. bit-score, e-value, accession numbers etc.) for each Operational Taxonomic Unit [OTU]. Note that the results of the CytB and CytB mini-barcodes have, for technical reasons, been combined and presented both under "CytB". Also, the results of the COI-2 and COI-mini barcodes have been combined and presented both under "COI-2".

The BLAST output file is a tab-separated file that can be opened using spreadsheet software (e.g. Excel) or text editor software (e.g. notepad++ available from https://notepad-plus-plus.org/). The file contains the BLAST results for the 12 barcode markers separated by brackets. For each DNA barcode marker the results file contains the following headers:

— qseqid OTU (query) sequence identifier with the associated number of NGS reads supporting the size of the OTU.

— qlen OTU (query) sequence length.

— qcovs Percentage OTU (query) coverage.

— pident Percentage of identical matches.

— bitscore Bit score.

— evalue Expect value.

— sacc Subject accession.

— staxids Unique subject taxonomy identifier.

— species Subject scientific name.

— synonym Subject synonym name.

— genus Subject genus name

— family Subject family name.

— order Subject order name.

— Listed under CITES taxonomic listing (species/family/order).

— Category CITES appendix (I/II/III).

The BLAST tables are sorted based on bit score (bitscore) and it lists the most similar subject sequence(s) (sacc) on top. The BLAST output file often contains the concatenated hits for multiple OTUs (qseqid). For each DNA barcode marker these are named for example OTU_1;size=3863, OTU_2;size=1500, indicating that BLAST hits were found for two OTUs (OTU_1 and OTU_2), each of

which are used in species identification. The size indicates the number of NGS reads that support that OTU cluster.

Interpret the BLAST output and fill in tables 6 and 7 (Annex 1) using the following guidelines:

- To minimize the chance of erroneous species identifications, it should be verified that at least three hits with highest (near identical) bit scores have the same species designation.
- If multiple (three or more) hits are obtained with highest bitscores, but with different assigned species, the OTU fragment is assumed to lack the discriminatory power at the species level. In such cases, the OTU would be identified at the genus-level.
- If multiple (three or more) hits are obtained with highest bitscores, but with different assigned genera, the OTU fragment lacks the discriminatory power at the genus level. In such cases, the OTU would be identified at the family-level.
- If multiple (three or more) hits are obtained with highest bitscores, but with different assigned families, the OTU fragment lacks the discriminatory power at the family level. In such cases, the OTU would be identified at the order-level.
- In case less than three hits (top hits and other) are reported, the OTU is identified at the family-level.

Considerations when interpreting the (endangered) species identification:

- The completeness of the reference database is of importance. The current underrepresentation of DNA barcodes from species protected under CITES may critically hamper their identification. The identification of CITES species (and other species) will improve as DNA barcoding campaigns continue, such as the Barcode of Wildlife Project (www.barcodeofwildlife.org).
- The NCBI GenBank is known to contain erroneous taxonomic names and identifications. While it remains difficult to specify clear guidelines on how to recognise them, we recommend not to consider taxa with clearly deviant family or order assignments in BLAST results with otherwise unambiguous family listings. Also, GenBank entries without informative taxonomic information, such as scientific names containing “environmental sample”, should be ignored.
- The CITESspeciesDetect pipeline uses a 98% sequence similarity threshold to distinguish species based on DNA barcodes. This cut-off was chosen based on studies that found this divergence threshold to be sufficient to keep the majority of plants and animals apart using the standard *matK*, *rbcL*, cytB and *COI* DNA barcoding markers (add CITATION). The plant mini-barcode markers of *rbcL*, *trnL* (P6 loop) and ITS2 do not provide species-level resolution at the 98% level in all cases, but may nonetheless provide valuable taxonomic information at the genus-or higher level in degraded DNA samples.
- The OTU abundance as indicated by the size in the OTU query sequence identifier provides information about the number of Illumina reads supporting the OTU. Higher relative read numbers provide better support for the presence of an OTU compared to OTUs with lower read numbers. The OTU abundance should, however, not be interpreted in a quantitative manner (i.e. as a measure of species abundance).
- The quality of the identification depends on the length of the DNA barcode sequence used for identification. Smaller fragments have shown to lack the discriminatory power to distinguish

between species in a genus or higher taxon. For this reason, the pipeline automatically discards identifications obtained from OTUs shorter than 200 nt, except for the ITS2, *rbcL*, and *trnL* (P6 loop) mini-barcode markers for which OTUs need to be longer than 150 nt, 140 nt and 10 nt, respectively.

- Species in diverse groups such as Cycadaceae, Orchidaceae, Cactaceae, Euphorbia sp., and primates can have very similar DNA barcode sequences. For this reason, it is often an entire genus or family that is listed by CITES, rather than individual plant or animal species. The presence of CITES-listed species can be confirmed when three or more top BLAST hits (see the above guidelines) are reported with taxa listed by CITES at the species or higher level (genus/family/order).
- CITES appendices contain multiple exceptions for certain taxa, e.g. based on their geographic location, domestication status or the enforcement of trade quota. The pipeline is unable to take these exceptions into account as they are not made available in a structured format. Therefore, the user is advised to revert to the speciesplus.net website for additional information on these details.

Table 5: Example BLAST results file for DNA barcode matK. In this example, the results are shown for OTU_1 that has an OTU cluster size of 3863, and a sequence length of 264 bp (qlen). OTU_1 is assigned to multiple subject sequences (sacc) that are assigned to different genera (i.e. Thelocactus, Ferocactus) with identical Bit scores (bitscore). OTU_1 is therefore assigned to the family Cactaceae, which is listed by CITES under appendix II.

##### Results for: matK #####														
qseqid	qlen	qcovs	pident	bitscore	evalue	sacc	staxids	species	synonym	genus	family	order	Listed under	Category
OTU_1;size=3863	264	100	98.11	460	4.00E-126	HQ620892	1038490	Thelocactus leucacanthus		Thelocactus	Cactaceae	Caryophyllales	Cactaceae spp.	II
OTU_1;size=3863	264	100	98.11	460	4.00E-126	HQ620891	867532	Thelocactus bicolor		Thelocactus	Cactaceae	Caryophyllales	Cactaceae spp.	II
OTU_1;size=3863	264	100	98.11	460	4.00E-126	HQ620874	867048	Ferocactus emoryi		Ferocactus	Cactaceae	Caryophyllales	Cactaceae spp.	II
OTU_1;size=3863	264	100	98.11	460	4.00E-126	FN997491	130126	Ferocactus robustus		Ferocactus	Cactaceae	Caryophyllales	Cactaceae spp.	II
OTU_1;size=3863	264	100	98.11	460	4.00E-126	FN997414	867062	Ferocactus viridescens		Ferocactus	Cactaceae	Caryophyllales	Cactaceae spp.	II
OTU_1;size=3863	264	100	98.11	460	4.00E-126	FN997382	867053	Ferocactus johnstonianus		Ferocactus	Cactaceae	Caryophyllales	Cactaceae spp.	II
OTU_1;size=3863	264	100	98.11	460	4.00E-126	FN997376	867049	Ferocactus fordii		Ferocactus	Cactaceae	Caryophyllales	Cactaceae spp.	II
OTU_1;size=3863	264	100	98.11	460	4.00E-126	FN997335	867061	Ferocactus townsendianus		Ferocactus	Cactaceae	Caryophyllales	Cactaceae spp.	II
OTU_1;size=3863	264	100	98.11	460	4.00E-126	FN997299	867056	Ferocactus peninsulae		Ferocactus	Cactaceae	Caryophyllales	Cactaceae spp.	II
OTU_1;size=3863	264	100	98.11	460	4.00E-126	FN997205	867048	Ferocactus emoryi		Ferocactus	Cactaceae	Caryophyllales	Cactaceae spp.	II
OTU_1;size=3863	264	100	98.11	460	4.00E-126	FN997173	130178	Thelocactus conothelos		Thelocactus	Cactaceae	Caryophyllales	Cactaceae spp.	II
OTU_1;size=3863	264	100	98.11	460	4.00E-126	FN997171	867534	Thelocactus lausseri		Thelocactus	Cactaceae	Caryophyllales	Cactaceae spp.	II
OTU_1;size=3863	264	100	98.11	460	4.00E-126	FN997136	867050	Ferocactus gracilis		Ferocactus	Cactaceae	Caryophyllales	Cactaceae spp.	II
OTU_1;size=3863	264	100	98.11	460	4.00E-126	FN997135	867048	Ferocactus emoryi		Ferocactus	Cactaceae	Caryophyllales	Cactaceae spp.	II
OTU_1;size=3863	264	100	98.11	460	4.00E-126	FN997105	867532	Thelocactus bicolor		Thelocactus	Cactaceae	Caryophyllales	Cactaceae spp.	II
OTU_1;size=3863	264	100	98.11	460	4.00E-126	FN997047	867059	Ferocactus santa-maria	Ferocactus santamaria	Ferocactus	Cactaceae	Caryophyllales	Cactaceae spp.	II
OTU_1;size=3863	264	100	98.11	460	4.00E-126	FN997043	867058	Ferocactus pottsii		Ferocactus	Cactaceae	Caryophyllales	Cactaceae spp.	II
OTU_1;size=3863	264	100	98.11	460	4.00E-126	FN997019	867060	Ferocactus schwarzii		Ferocactus	Cactaceae	Caryophyllales	Cactaceae spp.	II
OTU_1;size=3863	264	100	98.11	460	4.00E-126	HM041787	130179	Thelocactus hastifer		Thelocactus	Cactaceae	Caryophyllales	Cactaceae spp.	II
OTU_1;size=3863	264	100	98.11	460	4.00E-126	HM041774	867515	Stenocactus coptonogonus		Stenocactus	Cactaceae	Caryophyllales	Cactaceae spp.	II

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Before start

Prepare the following solutions for CTAB DNA isolation:

- 50 ml Chloroform
- 20 ml Ice-cold ethanol (96%) (e.g. stored at -20ºC)
- 20 ml 70% ethanol

Protocol

CTAB DNA isolation

Step 1.
Perform the DNA isolation for all the 11 samples simultaneously, including the DNA isolation of the negative control.

Step 2.

Weigh 100 +/-10 mg per sample in a 2 ml tube.

AMOUNT
100 mg Additional info: Sample

Step 3.

Add 300 µl nuclease-free molecular grade water.

AMOUNT
300 µl Additional info: Nuclease-free molecular grade water

Step 4.

Add 700 µl CTAB extraction buffer and vortex at maximum speed (1000 rpm) for 10 seconds.

AMOUNT
700 µl Additional info: CTAB extraction buffer

Step 5.

Add 5 µl RNase (100 mg/µl) solution and gently shake the tube.

AMOUNT
5 µl Additional info: RNase (100 mg/µl) solution

Step 6.

Incubate at 65 °C for 15 minutes in order to achieve efficient cell disruption in a Thermo-Shaker at maximum speed (1000 rpm).

Step 7.

Add 20 µl of proteinase K solution (20 mg/ml) and shake the vial again.

AMOUNT
20 µl Additional info: Proteinase K solution

Step 8.

Subsequently incubate at 65 °C for 1 h, Thermo-Shaker (1000 rpm).

Step 9.

Centrifuge the sample at 18,000 x g for 10 minutes.

Step 10.

Fill a 1.5 ml Eppendorf tube with 500 µl of chloroform

AMOUNT
500 µl Additional info: Chloroform

Step 11.

Transfer the supernatant to the previously prepared 1.5 ml Eppendorf tube containing 500 µl of chloroform and shake the vial by hand for 30 seconds.

Step 12.

Centrifuge at 18,000 x g for 10 min. The two phases are clearly separated.

Step 13.

Transfer upper layer to a new 1.5 ml tube (700 µl) and add the equal volume of (700 µl) chloroform and shake for 30 seconds.

AMOUNT
700 µl Additional info: Chloroform

NOTES
Alfred J Arulandhu 16 Sep 2017

The supernatant to be transferred shall be transparent, and any elements of the blurry interface will result in unsuccessful DNA extraction and/or strong PCR inhibition later on.

Step 14.

Centrifuge for 10 minutes at 18,000 x g.

Step 15.

Transfer the upper layer to a new 2 ml tube.

NOTES

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Based on previous experience ~500-600 µl (depending upon the sample) of upper layer can be transferred to the new tube.

Step 16.

Add 2 volumes of CTAB precipitation buffer and mix by pipetting up and down.

NOTES
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To keep it even add 1200 µl of CTAB precipitation buffer for all the samples.

Step 17.
Incubate at room temperature for 1 h.

Step 18.

Centrifuge the sample for 10 min at 18,000 x g and remove the supernatant,

Step 19.

Add 350 µl of NaCl (1.2 M) to the precipitate DNA (pellet not visible sometime).

AMOUNT
350 µl Additional info: NaCl

Step 20.

Add 350 µl of chloroform to the tube containing precipitated DNA and NaCl, and vortex for 30 seconds.

AMOUNT
350 µl Additional info: Chloroform

Step 21.

Centrifuge the sample for 10 min at 18,000 x g

Step 22.

Transfer the upper layer to a new 1.5 ml tube.

Step 23.

Add 2 volumes of ice-cold ethanol (96%) mix the sample by gently inverting the tube several times.

NOTES
Alfred J Arulandhu 16 Sep 2017

Here, 700 µl of ice-cold ethanol needs to be added, to keep it even for all the samples.

Step 24.

The samples are now stored overnight at -20 °C.

Step 25.

On another day

Centrifuge for 30 minutes at 18,000 x g and remove the supernatant.

Step 26.

Add 500 µl of ethanol (70%) to wash the pellet.

AMOUNT
500 µl Additional info: Ethanol (70%)

Step 27.

Centrifuge for 5 minutes at 18,000 x g and remove the supernatant by pipetting.

NOTES
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Make sure that the supernatant is completely removed and also make sure not to displace the pellet.

Step 28.

Air-dry the pellet for 1 hr.

Step 29.

Add 100 µl of water and let the pellet dissolve overnight at 4 °C.

AMOUNT
100 µl Additional info: water

Step 30.

On another day

Vortex the dissolved DNA and short-spin (10 sec) using mini-centrifuge.

Step 31.

The concentration of dissolved DNA is measured using by spectrophotometry (e.g. NanoDrop ND1000) according to the manufacturer’s protocol.

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No DNA yield should be present in the DNA isolation negative control.

Step 32.

100 µl of DNA is required for the experiment, dilute the DNA to a final concentration of 10 ng/µl using nuclease-free molecular grade water.

Step 33.

Label (sample name, date) the 1.5 ml tubes containing the purified DNA and store the DNA at 4 °C.

PCR

Step 34.

Prepare for each DNA barcode marker primer pair (see the table below for primer information) a PCR mixture (12 in total) in a 1.5 ml Eppendorf tube using the provided chemicals and volumes listed under “Volumes (µl) X 13)” (Table 1).

Primer labelling information:

16S-F	**COI-2-F**	**CytB-F**	**16S-mini-F**	**COI-mini-F**	**CytB-mini-F**	**Matk-F**	**rbcL-F**	**trnL(UAA)-F**	**ITS2-F**	**rbcL-mini-F**	**trnL(P6loop)-F**
16S-R	**COI-2-R**	**CytB-R**	**16S-mini-R**	**COI-mini-R**	**CytB-mini-R**	**Matk-R**	**rbcL-R**	**trnL(UAA)-R**	**ITS2-R**	**rbcL-mini-R**	**trnL(P6loop)-R**

Before making the 12 master mixes, thaw the primers, vortex and spin down using mini-centrifuge. For example, use the primers 16SF and 16SR for PCR amplification of the full-length 16S marker.

Table 1: PCR reaction mixture

Component	Stock concentration	Final concentration	Volume (µl)	Volume (µl) X 15
HotStarTaq Master Mix	2x	1x	12.5	187.5
Forward primer	10 µM	0.2 µM	0.5	7.5
Reverse primer	10 µM	0.2 µM	0.5	7.5
ddH_2O			6.5	97.5
Total volume			20	260

Note: the above mixture should be prepared 12 times, one for each DNA barcode marker.

Step 35.

Vortex the PCR mixture for 10 sec.

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Vortexing will cause the liquid to be trapped in the cap of the tube. Before proceeding to the next step, make sure that this liquid is returned to the rest of the mixture by a15-second spin-down in a mini-centrifuge.

Step 36.
Aliquot 20 µl of PCR mixture to each well according to the scheme in Table 3.

AMOUNT
20 µl Additional info: PCR mixture

Step 37.

Aliquot 5 µl of sample DNA template (10 ng/µl) to each well according to the pipetting scheme in Table 3. Add 5 µl of the positive control DNA according to the setup in Table 3.

Step 38.

Use the provided Bos taurus DNA (10 ng/µl) as positive control DNA for the animal barcode markers. Use the provided Lactuca sativa DNA (10 ng/µl) as positive control DNA for the plant barcode markers. Add 5 µl of ddH2O instead of DNA as negative control according to the setup in Table 3.

Step 39.

Place the PCR tube strips in a thermocycler block with plastic caps on top and close the lid of the thermocycler.

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The second prepared example plate 2 can be stored at 4°C until further use in case only one PCR machine is available.

Step 40.

Start the PCR program with the following extended thermal profile:

Table 2: PCR thermal profile

Thermal cycler steps	T (°C)	Time	Cycles
Hot start	95	15 min	
Denaturation	94	30 s	
Annealing	49.5	40 s	5 cycles
Extension	72	1 min	
Denaturation	94	30 s	
Annealing	54	40 s	35 cycles
Extension	72	1 min	
Final extension	72	10 min	
Hold	8	∞	

Step 41.

Examine the PCR thermocycler to ensure that the run has completed at the correct temperatures and that there is no error messages.

Step 42.

S – Sample, PA- Positive control animal, PP- Positive control plant and NC- Negative control after the PCR run has been successfully completed, spin down all the PCR tube strips for 15 seconds using a mini-centrifuge to make sure that any liquid that might be trapped under the cap is returned to the rest of the cocktail.

Step 43.

Gently remove the cap of the PCR tube strip.

Step 44.

Visualise 5 µl of the sample S1-S10, positive control and negative control reactions by 1% agarose gel electrophoresis using an appropriate staining method (e.g. ethidium bromide) or on a microchip electrophoresis system. The fragment of the amplicons in the samples and the positive control reaction is verified by comparison to a 100 bp DNA Ladder. The expected fragment sizes of the amplicons are specified in the table below.

Table 4: Fragment sizes of barcoding markers

16S	COI-2	CytB	16S-mini	COI-mini	CytB-mini	matK	rbcL	trnL (UAA)	ITS2	rbcL-mini	trnL(P6 loop)
650 bp	720 bp	800bp	300 bp	400 bp	400 bp	920 bp	650 bp	600 bp	550 bp	250 bp	180 bp

Step 45.
An example gel image is included in Figure 1. No DNA bands should be observed for the negative control reactions.

Step 46.

The amplified products in the PCR tubes can be stored at 4 °C before pooling.

Step 47.

On another day

Vortex all the PCR tubes for 5 sec and spin-down using a micro-centrifuge for 10 sec.

Step 48.

Transfer 8 µl of each PCR reaction mixture of sample S1 to a single 1.5 ml Eppendorf tube (row A, from A1 to A12, of Plate 1). In this way, all different barcoding markers of sample S1 are pooled.

Step 49.

Repeat this procedure for samples S2 to S10.

Step 50.

The pooled PCR products of samples S1 to S10 are purified using the QIAquick PCR purification kit according to the manufacturer's protocol QIAGEN (Annex 3). Ethanol is already added to the PE buffer, ready to use.

Step 51.

Columns are eluted with 30 µl of the Elution buffer provided by the manufacturer.

AMOUNT
30 µl Additional info: Elution buffer

Step 52.

Quantify the pooled and purified PCR products by spectrophotometry (e.g. Nanodrop ND1000) according to the manufacturer's instructions.

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If the concentration of the pooled and purified PCR products is less than 15 ng/µl, then repeat the QIAquick PCR purification step for these samples using higher volumes (> 10 µl) of the PCR reaction mixtures.

Step 53.

For each sample, prepare an aliquot of at least 12.5 µl and with a concentration of at least 15 ng/µl.

Step 54.

Label (sample name, date, and laboratory-number) the purified PCR products and store the amplicons at 4 °C.

Warnings

To prevent contamination:

- When performing the DNA isolation, make sure to have enough space between each tube.
- When adding the DNA to the PCR, make sure that all the PCR tube caps are closed except the one that is used at that moment.
- When pooling the samples for NGS analysis make sure that only the PCR tubes corresponding to the sample is opened.