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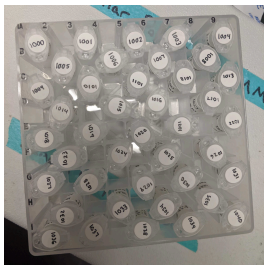
Environmental DNA (eDNA) COI PCR Amplification and Gel Electrophoresis Protocol



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Abstract

This is a protocol for amplifying DNA extracts via PCR using broad COI primers (Leray et al., 2013) and confirming successful amplification via gel electrophoresis.

This protocol originates with environmental DNA samples collected onto 0.22 µm capped Sterivex filters, e.g. through this sampling and filtration protocol:

Protocol

NAME

Coastal Environmental DNA Sampling & Gravity Filtration Protocol

CREATED BY

Meghan M. Shea

Preview

Samples are then extracted, e.g. through this extracted protocol, before proceeding through the current amplification protocol:

Protocol

NAME

DNA Extraction Protocol from Sterivex Filters

CREATED BY

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Preview

Acknowledgements & Attributions:

The PCR protocol is adapted from Curd et al. (2019; see Appendix 6) and the **detailed first PCR protocol** developed by **CALeDNA** (by Teia Schweizer, Emily Curd, and Rachel Meyer).

The gel electrophoresis protocol is adapted from an Agarose Gel Electrophoresis & Gel Visualization Protocol from the Boehm Lab (by Blythe Layton and edited by Wiley Jennings, Eily Andruszkiewicz Allan, and Winnie Zambrana), a 16S rRNA gene V4 Amplicon Sequencing Protocol from the Nelson Lab (updated by Lauren Kennedy based on the **Schloss Lab V4 Protocol**) and the **detailed Gel Electrophoresis protocol** developed by **CALeDNA** (by Teia Schweizer, Emily Curd, and Rachel Meyer).

We are grateful to the authors and editors of all the protocols above for creating and maintaining such helpful resources.

Attachments

XLSX Sample PCR Calculati... 11KB

PDF IDT jgHCO2198 Primer... 538KB

PDF IDT mlCOIintF Primer... 528KB

Image Attribution

Meghan M. Shea

Materials

General Laboratory Equipment:

	Equipment	Specific Model Used
	PCR Hood	CBS Scientific Co. (P-036-202) PCR Hood
	Vortex	VWR Mini Vortexer
	Mini Centrifuge	Onilab (D1008) Mini Centrifuge
	PCR & microcentrifuge tube holders	Various
	1000 µL pipette with sterile tips	Various
	200 µL pipette with sterile tips	Various
	20 µL pipette with sterile tips	Various
	10 µL pipette with sterile tips	Various
	10% bleach solution in spray bottle	NA
	>70% ethanol solution in spray bottle	NA
	RNase Away solution in spray bottle	NA
	UV Light Source	BioRad Universal Hood II Gel Doc System
	Gel Box with casting tray	Various
	Power supply for gel box	Various

Note

It's best to have designated pipettes for working with PCR products.

PCR Supplies:

	Material	Amount Needed	Source	Link	Approx. Cost
	Qiagen Multiplex PCR Kit	12.5 µL/rxn	Qiagen (206143)	https://www.qiagen.com/us/products/discovery-and-translational-research/pcr-qpcr-dpcr/pcr-enzymes-and-kits/end-point-pcr/qiagen-multiplex-pcr-kit	315.87/kit
	2 µL mlCOIintF forward primer (2 µM) and 2 µL jgHCO2198 reverse primer (2 µM)	2.5 µL of each/rxn	Integrated DNA Technologies	https://www.idtdna.com/pages/products/custom-dna-rna/dna-oligos	$52.1/one order
	8-well tube strips	1/every 8 rxns	Fisher Scientific (AB20005)	https://www.fishersci.com/shop/products/easystrip-plus-tube-strip-ultra-clear-caps/AB2005	$295.37/250 strips
	1.5/2 mL LoBind Eppendorf tubes (either size works; prefer 1.5 mL for storage)	Several	USA Scientific (4043-1021/4043-1048)	https://www.usascientific.com/dna-lobind-microcentrifuge-tubes/p/DNA-LB-Micro-Tubes	$38.95/250 tubes

Note

IDT spec sheets for primers are attached to this protocol. Primers were ordered with the overhang specified by **Georgia Genomics and Bioinformatics Core**, where samples are ultimately sequenced. Core primer sequences used are:

mlCOIintF: GGWACWGGWTGAACWGTWTAYCCYCC
jgHCO2198: TAIACYTCIGGRTGICCRAARAAYCA

Gel Electrophoresis Supplies:

	Material	Amount Needed	Source	Link	Approx. Cost
	Agarose	1.9 g/gel	Variable	NA	Unknown
	1X TAE buffer (0.04 M Tris-acetate, 0.002 M EDTA)	125 ml/gel	Variable	NA	Unknown
	GelRed Nucleic Acid Gel Stain 10,000X	12.5 µl/gel	Biotium	https://biotium.com/product/gelred-nucleic-acid-gel-stain/	Unknown
	6X gel loading dye	Variable	Variable	NA	Unknown
	100 base pair DNA ladder	10 µl/gel	Variable	NA	Unknown

Note

Working stock of 1X TAE buffer made from 980 mL DI water + 20 mL 50x TAE

Troubleshooting

Set-Up

1 Wipe down 10 µL pipette with 70% ethanol and RNase Away. UV for 10 minutes on each side

2 Wipe down a staging area near the PCR hood with 10% bleach, 70% ethanol, and RNase Away

3 Wipe down ice bin with 10% bleach, 70% ethanol, and RNase away, fill with ice, and remove DNA samples, PCR-grade water, Qiagen Multiplex Mix, and primers from the freezer to thaw. Place bin in staging area near PCR hood

4 Clean inside of PCR hood (including tube racks, etc.) with 10% bleach, 70% ethanol, and RNase Away

5 Wipe down PCR hood pipettes with 70% ethanol and RNase Away

6 Clean any items that need to go into the PCR hood and can be UVed with 10% bleach, 70% ethanol, and RNase Away and then place into PCR hood, including:
- Lab marker
- Bag of 8-well tube strips
- Bag of microcentrifuge tubes
- Strip tube holder
- Any new tip boxes (make sure you have enough in the hood for the rest of the protocol)

7 With PCR hood cover in place, run UV light for 10 minutes

8 While PCR hood is UVing, wipe down a vortex, centrifuge, work bench, and box of 10 µL pipette tips with 10% bleach, 70% ethanol, and RNase Away

9 Once UV timer on PCR hood is done, remove the cover and turn on the regular light

10 Once reagents have thawed, gently vortex and wipe down the tubes with RNase Away before bringing into the hood

11 Place a Kimwipe dampened with RNase Away just outside the hood to clean tubes you bring in and out of the hood

12 Set up your diagram of how you're organizing your samples and calculate the total amount of PCR reagents needed for all samples, e.g. using the attached Sample PCR Calculation Spreadsheet

PCR Preparation

13 Change gloves before beginning to work in the hood

14 Label tube strips with relevant information, including:
- Tube number
- Initials
- Date

15 Add primers and PCR-grade water to microcentrifuge tube(s) according to your combined reagent recipe (see attached Sample PCR Calculation Spreadsheet), remove from hood, vortex vigorously, wipe with RNase Away, and return to hood

16 Add Qiagen Multiplex Mix to microcentrifuge tube(s) according to your master mix recipe, remove from hood, vortex VERY gently, wipe with RNase Away, and return to hood

Note

Taq Polymerase does not like to be heavily vortexed

17 Using a pipette, aliquot 24 µL of master mix to each strip tube

Note

The amount, 24 µL, should be the final volume per reaction minus the amount of DNA template. You can reuse your tips unless you think you contaminated the tips.

Adding Template DNA

18 Remove the aliquoted master mix and put on bench

19 Vortex each DNA sample and pipette 1 µL into the appropriate tube strip. Mix the DNA and master mix solution by pipetting up and down

Thermocycling

20 Turn on thermocycler and select your program.

Primers:	CO1			
Step	**# of Cycles**		**Temperature**	**Time**
Activation	1x		95°	15 min
Touchdown	13x	*Denaturation*	94°	30 sec
		Annealing	69.5° *(-1.5°/cycle)*	30 sec
		Extension	72°	60 sec
Amplification	35x	*Denaturation*	94°	30 sec
		Annealing	50°	30 sec
		Extension	72°	60 sec
Final Extension and hold	1x		72°	10 min
			10°	infinity

PCR cycling adapted from Curd et al. 2019

Note

Even once you've programmed your thermocycler settings, always check the program before using.

21 Place your samples in to the thermocycler, and double check that all tubes are fully sealed

22 The thermocycler will take between 2 and 4 hours to complete the program. While the thermocycler is running, prepare for gel electrophoresis (below)

23 When the thermocycler has finished running, remove your tubes and turn off thermocycler

24 Spin your PCR tubes down using a centrifuge or microcentrifuge specialized for plates or tubes and label. Make sure the labels are clear.

Note

If you will use your PCR products within 1 week, it is safe to store it at 4^{O}C. If it will be more than 1 week, store your PCR products in a -20 ^{O}C freezer to avoid DNA degradation.

Gel Electrophoresis Preparation

25 Weigh out 1.9 g of agarose and measure out 125 ml of 1X TAE for a 1.5% gel

26 Heat agarose in TAE in microwave until the agar is completely dissolved. You will know the agarose is completely dissolved when there are no specks floating in the TAE.

Note

Be careful of boil-over as it occurs quickly and unexpectedly. Recommend heating for ~30 seconds at a time and swirling in between.

Note

Use a paper towel or hot-pad to hold the beaker as it will be very hot.

27 Allow the solution to cool to just above room temperature. Placing the flask on a few folded paper towels will prevent the agarose on the bottom from solidifying too soon.

28 Once cool, add 12.5 ul of GelRed per 125 mL of gel (want it to be 1X final concentration). Gently swirl until GelRed is dissipated evenly.

29 Fit the casting tray in the box

Note

Make sure the casting tray is oriented so that the solution does not run off. Also make sure that the rubber gasket is seated properly so that the casting tray does not leak.

30 Pour the agarose into the casting tray and place the combs in the tray. Use the “fatter” side of the combs to make it easier to pipet into.

31 Allow the gel to solidify

Note

Gel is cool when it is a uniform milky white color. If the center looks clear still, it needs more time to solidify. It usually needs to cool for ~15-20 minutes.

32 Remove the tray and put in running position with the combs closer to the negative (black) electrode

33 Fill the box with TAE buffer until it just covers the gel

34 Carefully remove the combs

Note

At this point, you can store the gel for later if needed. Fill a shallow container (e.g,. Tupperware) with enough 1X TAE to cover the gel. Carefully slide the gel out of the casting tray into the TAE. Cover with tin foil and store at 4°C.

35 Decide the subset of samples to visualize via gel electrophoresis to confirm successful amplification

Gel Electrophoresis

36 Load 5 µl of DNA ladder (at concentrations specified by the manufacturer) into the wells on either end of the row of samples

37 Dot 2 µl of loading dye (one dot per PCR product) onto a piece of parafilm

Note

If you have a large number of samples it can be easier to do this in a 96-well plate using a 12-channel pipetter.

38 Mix 4 µl of PCR product with the dye by pipetting up and down

39 Load the entire ~6 µl sample into a well, ejecting the sample slowly and carefully to avoid spillage or bubbles

Note

If there are any air bubbles in the wells, remove them with a pipet tip before loading sample into the well.

Note

Insert only the tip of the pipet tip into the well so you don't pierce the bottom of the gel.

40 Run gel at 110 volts for 60 minutes

Note

These parameters can be adjusted for various applications, i.e. longer running times at lower voltages for better separation of many products and/or long amplicons (>1 kb). Similarly, shorter running times and higher voltages (up to 120V) can be used if you're in a big hurry, but it's not ideal and can result in smearing.

41 Visualize and photograph gel using UV light box, GelDoc, etc

42 If visualizing with GelDoc, use the following steps:

42.1 Use a paper towel to open the drawer of the GelDoc.

42.2 Remove the tray from the gel box and carefully slide the gel onto the glass surface of the drawer

42.3 Remove one glove, close the drawer and open the main door of the GelDoc. Open the QuantityOne software and select File → GelDoc XR.

42.4 Turn on the white lamp by pressing the Epi White button and click "Live/Focus"

42.5 Use your gloved hand to center the gel

Note

You can put a KimWipe along the edge of the gel if it is sliding too much on the glass.

42.6 Close the door and turn on the UV lamp (Trans UV button)

42.7 Take a picture by clicking either “Auto Expose” or “Manual Acquire”, then “Freeze”. Adjust the exposure length as necessary until you are satisfied with the image.

Note

The brightness of the bands will decrease the longer they are exposed to UV, so keep the lamp on for as little time as possible.

42.8 Click “Save,” then “Print.”

Note

The software has a number of really useful features (via “Analyze”) for processing and analyzing the image, which is really helpful if you are doing DNA fingerprinting, multiplex PCR, etc. These can be explored at http://www.bio-rad.com/webroot/web/pdf/lsr/literature/4000126-14A.pdf

42.9 Dispose of the gel properly and clean the glass surface of the GelDoc with a paper towel.

Protocol references

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