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DNA metabarcoding protocol for siphonophore gut contents

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ABSTRACT

Protocol for the SiphWeb DNA metabarcoding of siphonophore gut content.
Starting with DNA extractions from pooled frozen gastrozooids, this protocol takes the user through the steps of PCR amplification, PCR cleanup and pooling of amplicons from six complementary 18S barcodes that can be submitted into an Illumina MiSeq Lane.

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EXTERNAL LINK

https://docs.google.com/spreadsheets/d/1x71z9YLqxo9XszNcjAPB__p0cnHHYyMTEHNL4wb84j4/edit#gid=656913706

PROTOCOL CITATION

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MATERIALS TEXT

MATERIALS

GoTaq(R) Long PCR Master Mix, 10 Reactions **Promega Catalog #M4020**

DNA Extractionion 4h

1 Mainly identical to:
http://www.bea.ki.se/documents/EN-DNeasy%20handbook.pdf
With the following modifications: 4h

-Digestion at **56 °C 1-2h**

-Elution using 2 rounds of incubation adn centrifuge with **50 µl of AE buffer** , incubating **00:10:00** at **56 °C** , to a total of **100 µl**

-Label **2 mL** Eppendorf tubes to hold the extracted DNA. Include the extraction number, the siphonophore species, "GCDNA", the date of extraction, and your initials.
-Use Nanodrop to assess DNA yield (ng/µl) and 260:280 ratio.

NanoDrop™ 3300 Fluorospectrometer
Fluorospectrometer

NanoDrop™ 3300 **nd-3300**

Store eluted DNA in **-20 °C** freezer.

PCR 2h 52m

2 Based off Promega GoTaq protocol.

(Plan for a 96-well final plate [90 wells used for 13 templates, a positive, and a negative control, across 6 primer pairs (detailed in T1)], can be re-scaled as needed).
See reference planning spreadsheet here:
https://docs.google.com/spreadsheets/d/1x71z9YLqxo9XszNcjAPB__p0cnHHYyMTEHNL4wb84j4/edit#gid=656913706
Only select templates that have a DNA yield > 10ng/µl and a 260:280 > 1.7

3

Name	Type (F/R)	Primer sequence	Length (bp)	TM © min	TM © max	%GC	Hairpin TMC	Self Dimer TMC	Pair Dimer TMC	Insert size (bp)	Start position	End position
152F	F	TGACGGAAGGGCACCACCAG	20	62.7	64.6	63.2	63	0.1	None	152	3275	3301
152R	R	TCCACCAACTAAGAACGGCC	20	60	60	55	None	None	None	152	3425	3457
166F	F	AACGGCTACCACATCCAAGG	20	60	60	55	None	None	None	166	887	906
166R	R	CACCAGACTTGCCCTCCAAT	20	60	60	55	31.9	None	None	166	1152	1129
272F	F	AAACGATGCCGACTAGCGAT	20	59.9	59.9	50	44.6	7.9	6.7	272	3064	3085
272R	R	TCCACCAACTAAGAACGGCC	20	60	60	55	None	None	6.7	272	3457	3425
179F	F	GGCCGTTCTTAGTTGGTGGA	20	60	60	55	None	None	11	179	3425	3457
179R	R	TGCGGCCCAGAACATCTAAG	20	60.1	60.1	55	None	None	11	179	4139	4116
261F	F	AACAGGTCTGTGATGCCCTT	20	59.2	59.2	50	44.1	4.2	14	261	4081	4118
261R	R	TGTGTACAAAGGGCAGGGAC	20	59.9	59.9	55	None	None	14	261	4432	4397
134F	F	CTTTGTACACACCGCCCGTC	20	61.6	61.6	60	None	None	None	134	4,421	4443
134R	R	CCTTGTTACGACTTTTACTTCCTCT	25	58.8	58.8	40	None	None	None	134	4745	4779

T1. Primer sequences and properties.

4 5m
-Pull all reagents in T2 from the freezer and let them thaw.

-Separate and label two PCR plates,
one for 134 (annealing at **48 °C** , elongation **00:00:30**)

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one for 152, 166, 179, 261, and 272 (all annealing at **54 °C** , elongation **00:01:00**). Divide and label each pair of rows that will contain each primer pair mix.

5 10m

Reagent	Volume (µm)
Template (variable)	2
Forward primer (10µM)	0.5
Reverse primer (10µM)	0.5
MgCl2 (25mM)	2.5
dNTPs (10mM)	1
GoTaq	0.2
Buffer 5X (green)	2.5
BSA	1
H2O	14.8
TOTAL	25

T2. Reagent breakdown goal per well in the plate.

-Make master-mastermix for all primer pairs as in T3. Always vortex each reagent before pipetting, and vortex the mixture at the end.

Reagent	Volume (µm)
MgCl2 (25mM)	350
dNTPs (10mM)	140
GoTaq	28
Buffer 5X	350
BSA	140
H2O	2072
TOTAL	3080

T3. Master-mastermix volumes for 6 primer pair mastermixes downstream.

6 10m -Serve **440 µl** of this master-mastermix into 6 **2 mL** Eppendorf tubes. Lable the tubes with the name of each primer pair.

-Add **10 µl** of **10 Micromolar (µM)** Forward and **10 µl** of **10 Micromolar (µM)** Reverse primer into each primer-specific mastermix to end up with T4.

-Vortex .

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Reagent	Volume (µl)
Forward primer (10µM)	10
Reverse primer (10µM)	10
MgCl2 (25mM)	50
dNTPs (10mM)	20
GoTaq	4
Buffer 5X	50
BSA	20
H2O	296
TOTAL	460

T4. Volumes in primer pair mastermix.

7 In a set of 14 PCR tubes (can use two rows in either PCR plate too), serve an excess amount of template to seed each well with. I usually go for **20 µl** . Serve the templates in the same spatial arrangement as they will be seeded in each set of rows for each primer pair. 10m

Include the positive and negative controls. I tend to pick the elution AE buffer stock I used for extractions as negative control, and a previously successful template as positive control.

8 Using a single **200 µl** pipette tip on a multi-step pipette set to **23 µl** doses in 4 steps, seed each primer-specific pair of rows. Remember to get rid of the excess (before or after serving the steps, depending on the model of the pipette). Change tips in between primer-pair-specific mastermix. 30m

Using a multichannel 10-100µl, seed the **2 µl** of template in each well. Be careful to not accidentally aspire air at the bottom of the tubes/wells and end up with un-seeded wells. Always visually inspect the tips before serving into the wells. Change tips every time to avoid contamination.

9 Seal the plates (**00:00:06** under the plate press) with cellophane cover. 1m

Vortex plates.

1000 rpm, 00:00:10 , Benchtop manual plate centrifuge and spin it down.

10 Insert 134 and 152+166+179+261+272 plates in separate thermocyclers. Run PCRs following the programs specified in T5A and T5B respectively. 1h 45m

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SimpliAmp Thermal Cycler
PCR

Applied Biosystems **A24811**

Any standard PCR thermocycler will suffice

Temperature (C)	94	94	48	72	72
Time	2m	30s	30s	1m	5m
Cycles		x30	x30	x30	

T5A. PCR program for barcode 134.

Temperature (C)	94	94	54	72	72
Time	2m	30s	1m	1m	5m
Cycles		x30	x30	x30	

T5B. PCR program for barcodes 152, 166, 179, 261,and 272.

11 1m

Vortex plates.

1000 rpm, 00:00:10 , Benchtop manual plate centrifuge and spin it down.

Un-seal the plates (**00:00:06** under the plate press)

Gel Electrophoresis 1h 25m

12 30m

2 g of Agarose for every **100 mL** of TAE buffer.

TAE Buffer, 10X,
1000ml **Promega Catalog #V4271**

Agarose **Thermo Fisher Catalog #75510019**

For 90 wells, we will need 3 gels with 2 16-well combs each.
For 3 gels, we need to make **200 mL** of agar in one batch, and **100 mL** in another batch.
In the double batch, add **4 g** of agarose and **200 mL** of TAE buffer. Microwave for **00:03:00** . Stir and microwave for another **00:01:00** if solids are still not dissolved. When cool, add **4 µl** of SYBR-Safe.
In the single batch, add **2 g** of agarose and **100 mL** of TAE buffer. Microwave for **00:02:30** . Stir and microwave for another **00:01:00** if solids are still not dissolved. When cool, add **2 µl** of SYBR-Safe.

SYBR SAFE DNA stain **Life Technologies Catalog #S33102**

Pour gels and let them cool until solid.
Annotate distribution of samples on wells. I usually arrange a row per primer pair: 134+152, 166+179, 261+272 in 3 gels, and then the well sequence would go: Ladder, A1-B6 (letters vary with position in the PCR plate), -ve, +ve.

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13 Set up power source to 125V, 500mA, **00:45:00** 55m

Load **6 µl** of 100bp ladder on the left side of each row of wells (2 per gel).

Use adjustable-spacer multichannel pipette to load **5 µl** of PCR products onto the wells.

Well distance is **6.25 mm** , PCR plate distance is **9 mm** .

Run gels.

14 Place gels under UV light, take photograph, print. 2m

You should see a band over each well except the negative control. The bands should land at approximately the height (in bp) of the ladder corresponding to the name (expected amplicon length) of each primer pair.

PCR Cleanup 47m 6s

15 Put some nuclease-free water to warm up to **56 °C** . 10m

ExcelaPure™ 96-Well UF PCR Purification Plates (No Receivers) **Edge Bio Catalog #36181**

Add **55 µl** of water into each PCR well (except controls and failed reactions) using a multichannel 20-200µl and filter tips. Mix by pipetting up and down ~5 times. Transfer mix to ExcelaPure purification plate. Change tips every time.

16 **5000 rpm, 00:07:00** Centrifuge the purification plate with a collecting plate underneath. 7m

Make sure to balance the **plate centrifuge** with a couple of adequately weighted collecting plates.

17 Add **50 µl** of warm (**56 °C**) water to each well in the purification plate. Mix by pipetting up and down ~15 times with the multichannel pipette and filter tips. Change tips every time. 10m

18 Incubate at **56 °C** for **00:10:00** . 10m

Prepare a final clean product PCR plate: Label and annotate adequately to preserve sample location. I recommend drawing a line between each pair of rows, segregating primer pairs 134 to 272.

19 Mix the warm eluted clean products in the purification plates by pipetting up and down ~15 times with the multichannel pipette and filter tips. 10m
Transfer clean products to the final plate.
Change tips every time.

20 Seal the plates (**00:00:06** under the hot plate press) with cellophane cover. Store in **-20 °C** freezer. 6s

Amplicon isomolar poolingling 2h 50m

21 The goal here is to obtain an equal concentration of each barcode for each sample, so they can be pooled with equal representation for MiSeq Illumina sequencing. 40m

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First step is to quantify the DNA content in each cleaned PCR product well using Qubit HS.

Qubit 2.0 Fluorometer instrument Q33226
with Qubit RNA HS Assays

If you have the pre-mixed mastermix, just run the standards at **190 µl** of mastermix and **10 µl** of standard.
Vortex mix and incubate for 1min.
Prepare the assay tubes with **198 µl** MM and **2 µl** of template.
Vortex mix and incubate for 1min.
Pick the ng/µl units.

22 -Calculate amount of **template** to seed in each pooling well.
-Pool target concentrations are equal to the lowest concentration across barcodes for a sample excluding those that are <2ng/µl, which is the minimum required by YCGA for amplicon sequencing.

134	152	166	179	261	271
0.5	0.5	0.6	0.6	0.9	1

T6. Molar correction factors for each barcode based on the molecular weight of each expected amplicon length.

Template volume to add = (**50 µl** /(Qubit ng/µl value of original PCR product/Pool target concentration))*Molar_correction_factor
~ See T6 for Molar correction factors ~

23 -Calculate amount of **water** to add in each pooling well.
Water volume to add = (**50 µl** *Molar_correction_factor) - **Template volume to add**

24 Prepare a pooling PCR plate, labeled. 2h
Add the water and template quantities for each well. Change the pipette tip every time when handling PCR products.

25 Label Eppendorf **2 mL** tubes to hold to final amplicon pools. Include the extraction number, the genus of the siphonophore, "GC DNA pooled", and your initials. 10m
Using a multichannel pipette of 20-200µl, proceed to pool the amplicons by sample. If the volumes do not fit in a row on the pooling PCR plate, you can make 2 semi-pools, one combining 134+152+166, and one combining 179+261+272.

Be very careful on this step, since mistakes during pooling will result in having to re-do the whole protocol for the samples affected.

Using a single-channel 20-200µl pipette, transfer the pooled amplicons to the Eppendorf tubes. Vortex and close caps.
Store the amplicon pools in a **-20 °C** freezer.

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