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# DNA metabarcoding protocol for siphonophore gut contents V.2

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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.

DOI

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

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**protocols.io**
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Corrected the barcode names and start-end positions on the 18S gene to reflect the hypervariable regions as reference. Corrected mistyped annealing time values in the PCR thermocycler programs.

protocol ,

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59815

MATERIALS

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

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## DNA Extractionion 4h

4h

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

-Digestion at **56 °C 1-2h**

-Elution using 2 rounds of incubation and 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

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| A | B | C | D | E | F | G | H | I | J | K | L | M | N |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Original Name | Type (F/R) | Barcode Region | 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 | V5-V7S | TGACGGAAGGGCACCACCAG | 20 | 62.7 | 64.6 | 63.2 | 63 | 0.1 | None | 152 | 1187 | 1207 |
| 152R | R | V5-V7S | TCCACCAACTAAGAACGGCC | 20 | 60 | 60 | 55 | None | None | None | 152 | 1319 | 1339 |
| 166F | F | V3 | AACGGCTACCACATCCAAGG | 20 | 60 | 60 | 55 | None | None | None | 146 | 420 | 440 |
| 166R | R | V3 | CACCAGACTTGCCCTCCAAT | 20 | 60 | 60 | 55 | 31.9 | None | None | 146 | 546 | 566 |
| 272F | F | V5-V7L | AAACGATGCCGACTAGCGAT | 20 | 59.9 | 59.9 | 50 | 44.6 | 7.9 | 6.7 | 272 | 1067 | 1087 |
| 272R | R | V5-V7L | TCCACCAACTAAGAACGGCC | 20 | 60 | 60 | 55 | None | None | 6.7 | 272 | 1319 | 1339 |
| 179F | F | V7 | GGCCGTTCTTAGTTGGTGGA | 20 | 60 | 60 | 55 | None | None | 11 | 170 | 1319 | 1339 |
| 179R | R | V7 | TGCGGCCCAGAACATCTAAG | 20 | 60.1 | 60.1 | 55 | None | None | 11 | 170 | 1469 | 1489 |
| 261F | F | V7p+V8 | AACAGGTCTGTGATGCCCTT | 20 | 59.2 | 59.2 | 50 | 44.1 | 4.2 | 14 | 215 | 1472 | 1492 |
| 261R | R | V7p+V8 | TGTGTACAAAGGGCAGGGAC | 20 | 59.9 | 59.9 | 55 | None | None | 14 | 215 | 1667 | 1687 |
| 134F | F | V9 | CTTTGTACACACCGCCCGTC | 20 | 61.6 | 61.6 | 60 | None | None | None | 115 | 1675 | 1695 |
| 134R | R | V9 | CCTTGTTACGACTTTTACTTCCTCT | 25 | 58.8 | 58.8 | 40 | None | None | None | 115 | 1765 | 1790 |

T1. Primer sequences and properties. Positions based on the 18S Gene of *Lymnaea diaphana* (GenBank JF909497.1).

4 1m 45s

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

-Separate and label two PCR plates,
one for V9 (annealing at **48 °C** for **00:00:45** per cycle)
one for V3, V5-V7S, V5-V7L, V7, and V7p+V8 (all annealing at **54 °C** for **00:01:00** x30 cycles ). 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.



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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 .

| A | B |
|---|---|
| **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 10m

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.

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 30m

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.

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 1m

Seal the plates ( **00:00:06** under the plate press) with cellophane cover.
Vortex plates.
**1000 rpm, 00:00:10 , Benchtop manual plate centrifuge** and spin it down.

10 1h 45m

Insert plates (V9) and (V3, V5-V7S, V5-V7L, V7, V7p+V8) in two separate thermocyclers. Run PCRs following the programs specified in T5A and T5B respectively.



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SimpliAmp Thermal Cycler
PCR
**Applied Biosystems** **A24811**
Any standard PCR thermocycler will suffice

| A | B | C | D | E | F |
|---|---|---|---|---|---|
| **Temperature (C)** | 95 | 95 | 48 | 72 | 72 |
| **Time** | 2m | 30s | 45s | 1m | 5m |
| **Cycles** | | x30 | x30 | x30 | |

T5A. PCR program for barcode V9.

| A | B | C | D | E | F |
|---|---|---|---|---|---|
| **Temperature (C)** | 95 | 95 | 54 | 72 | 72 |
| **Time** | 2m | 30s | 1m | 1m | 5m |
| **Cycles** | | x30 | x30 | x30 | |

T5B. PCR program for barcodes V3, V5-V7S, V5-V7L, V7, and V7p+V8.

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 ordered by amplicon size: V9:V5-V7S, V3:V7, V7p+V8:V5-V7L 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 for barcodes V3 to V9.

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

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



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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.

| A | B | C | D | E | F |
|---|---|---|---|---|---|
| **V9** | **V5-V7S** | **V3** | **V7** | **V7p+V8** | **V5-V7L** |
| 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 V9:V5-V7S:V3, and one combining V7:V7p+V8:V5-V7L.

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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