

May 22, 2024 Version 2

Metabarcoding Fecal Swabs or Stomach Contents for Fish and Crustaceans using 2-PCR protocol and Illumina MiSeq V.2

DOI

dx.doi.org/10.17504/protocols.io.ewov1qxokgr2/v2



Eldridge Wisely¹

¹University of Arizona

EWisely



Eldridge Wisely

University of Arizona

OPEN  ACCESS



DOI: dx.doi.org/10.17504/protocols.io.ewov1qxokgr2/v2

Protocol Citation: Eldridge Wisely 2024. Metabarcoding Fecal Swabs or Stomach Contents for Fish and Crustaceans using 2-PCR protocol and Illumina MiSeq. **protocols.io** <https://dx.doi.org/10.17504/protocols.io.ewov1qxokgr2/v2> Version created by **Eldridge Wisely**.

License: This is an open access protocol distributed under the terms of the **[Creative Commons Attribution License](#)**, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited

Protocol status: Working

We use this protocol and it's working

Created: November 19, 2023

Last Modified: May 22, 2024

Protocol Integer ID: 100313



Abstract

This protocol describes a method to metabarcode a 170bp region of the mitochondrial 16S rRNA gene of crustaceans and a 163-185bp region of the mitochondrial 12S rRNA gene of fishes. These regions are subjected to PCR separately in multiple replicates and the resulting PCR products are pooled by sample and then indexed for sequencing on an Illumina MiSeq platform.

Image Attribution

Haley Capone

Guidelines

The PCR conditions described here are different from the PCR conditions described by Miya et al., and Berry et al. in their respective publications introducing the primers used here. This difference is due to the use of the Takara High Fidelity PCR EcoDry Premix in this protocol.

Materials

96-well PCR plates
Adhesive foil PCR plate covers

1.5mL tubes

Glenn et al. Adapterama I iNext indexing primers A-H and 1-12.

PCR machine

Equipment to run gels
optionally: equipment for fluorometric quantification

Equipment	
96-well Magnetic Rack Separator	NAME
Magnetic Rack Separator	TYPE
Sergi Lab Supplies	BRAND
B08134P9RT	SKU
https://www.amazon.com/Magnetic-Separator-Protein-Purification-Format/dp/B08134P9RT/ref=asc_df_B08134P9RT/?tag=&linkCode=df0&hvadid=416872221972&hvpos=&hvnetw=g&hvrnd=12953200023550024012&hvpone=&hvptwo=&hvmmt=&hvdev=c&hvdvcmdl=&hvlocint=&hvlocphy=903024	LINK

Equipment

Magnetic Rack for for 1.5 mL Tubes

NAME

Magnetic Rack for DNA, RNA Purification; for 1.5 mL centrifuge Tubes

TYPE

Sergi Lab Supplies

BRAND

B0BZWXZM22

SKU

https://www.amazon.com/Magnetic-Rack-Purification-centrifuge-Tubes/dp/B0BZWXZM22/ref=asc_df_B0BZWXZM22/?tag=hyprod-20&linkCode=df0&hvadid=652498086131&hvpos=&hvnetw=g&hvrand=6716034042841103246&hvpone=&hvptwo=&hvqmt=&hvdev=c&hvdvcmdl=&hvlocint=&hvlocphy=9

LI
N
K

Protocol materials

⊗ Buffer EB **Qiagen Catalog #19086** In 2 steps

⊗ Agencourt AMPure XP **Beckman Coulter Catalog #A63880** Step 16.1

⊗ MiFish-F **Integrated DNA Technologies, Inc. (IDT) Catalog #custom** Step 1

⊗ MiFish-R **Integrated DNA Technologies, Inc. (IDT) Catalog #custom** Step 1

⊗ Crustacean16S-F **Integrated DNA Technologies, Inc. (IDT) Catalog #custom** In 2 steps

⊗ Crustacean16S-R **Integrated DNA Technologies, Inc. (IDT) Catalog #custom** In 2 steps

⊗ 2x Kapa HiFi Hotstart Readymix **Kapa Biosystems Catalog #KK2602** Step 20


Before start


Work in a pre-PCR lab, as separated as possible from post-PCR products.


Clean work area with 10% bleach solution before beginning work for the day, then change gloves so that no bleach carryover to your samples or reactions occurs.




Prepare Primers







- 1 Order metabarcoding primers with diversity spacers and Illumina overhang sequences (Illumina, 2013):  **MiFish-F Integrated DNA Technologies, Inc. (IDT) Catalog #custom** (Miya et al., 2015):
TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGNNNGTCCGTAAACTCGTGCCAGC

 **MiFish-R Integrated DNA Technologies, Inc. (IDT) Catalog #custom** (Miya et al., 2015):
GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGNNNNCATAGTGGGGTATCTAATCCCAGTTTG

 **Crustacean16S-F Integrated DNA Technologies, Inc. (IDT) Catalog #custom** Berry et al., 2017): TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGNNNGGGACGATAAGACCCTATA

 **Crustacean16S-R Integrated DNA Technologies, Inc. (IDT) Catalog #custom** (Berry et al., 2017):
GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGNNNATTACGCTGTTATCCCTAAAG

We got ours from <https://www.idtdna.com/> as custom oligos at 25nm scale, with standard desalting.

- 2 Reconstitute primers to [M] 100 micromolar (μM) stock solutions by adding  40 μL of  **Nuclease-free water Integrated DNA Technologies, Inc. (IDT) Catalog #11-05-01-14**
- 3 Make [M] 5 micromolar (μM) working solutions of each primer by adding  95 μL of  **Nuclease-free water Integrated DNA Technologies, Inc. (IDT) Catalog #11-05-01-14** and  5 μL of primer stock solution for each  100 μL of primer that you intend to use within the next week or so.

Create Plate Map



- 4 Determine which sample will go into each well. This should be the same for each primer set and each replicate. Include at least one extraction control (you can combine aliquots of the extraction controls from each round of DNA extraction into one tube, and use that as your single extraction control), and include a PCR negative control for each plate of PCR. See example below of 21 samples, a field negative sample, a combined extraction control, and a PCR negative.

	1	2	3	4	5
A	Sample8_rep1	Sample16_rep1	Sample24_rep1	PCR neg_rep1	Sample8_rep2
B	Sample7_rep1	Sample15_rep1	Sample23_rep1	Ext Con_rep1	Sample7_rep2
C	Sample6_rep1	Sample14_rep1	Sample22_rep1	Field Neg_rep1	Sample6_rep2
D	Sample5_rep1	Sample13_rep1	Sample21_rep1	Sample29_rep1	Sample5_rep2
E	Sample4_rep1	Sample12_rep1	Sample20_rep1	Sample28_rep1	Sample4_rep2
F	Sample3_rep1	Sample11_rep1	Sample19_rep1	Sample27_rep1	Sample3_rep2
G	Sample2_rep1	Sample10_rep1	Sample18_rep1	Sample26_rep1	Sample2_rep2
H	Sample1_rep1	Sample9_rep1	Sample17_rep1	Sample25_rep1	Sample1_rep2




	7	8	9	10	11	
A		PCR neg_rep2	Sample8_rep3			PCR
B		Ext Con_rep2	Sample7_rep3			Ext
C		Field Neg_rep2	Sample6_rep3			Field
D			Sample5_rep3			
E			Sample4_rep3			
F			Sample3_rep3			
G			Sample2_rep3			
H			Sample1_rep3			

- 4.1 Do not mix sample types between invasively sampled methods (fecal swabs, or stomach contents) and non-invasively sampled methods (eDNA from water or sediment) in the same PCR procedure. And don't plan to sequence both types in the same sequencing run with the



combinatorial indexing scheme used here. The potential for contamination of the lower quantity eDNA samples by the higher quantity fDNA samples is too high.

MiFish Takara PCR Recipe

- 5 Add  24 μL of your MiFish metabarcoding mastermix to each well of  Takara High Fidelity PCR EcoDry Premix **Takara Bio Inc. Catalog #639280**
- 5.1 Add  1 μL DNA extracted from stomach contents or fecal swabs.
- 5.2 Mix and stir together with pipette tip, swirling to make sure the liquid is in the bottom, and bringing any bubbles to the surface of each reaction.
- 5.3 Cap each row of reaction tightly before beginning any other PCR reaction in the same room.


MiFish Takara PCR Conditions

- 6  95 °C for  00:01:00 3m 30s
35 cycles of:
 95 °C for  00:00:30
 66 °C for  00:01:00
followed by:
 68 °C for  00:01:00
Hold at  4 °C

Crustacean_16S Takara PCR Recipe

- 7 Make your Crustacean_16S Mastermix:

For each **PCR replicate of each sample** you intend to process (+10% overage), mix:

 2 μL  5 micromolar (μM)

 Crustacean16S-F **Integrated DNA Technologies, Inc. (IDT) Catalog #custom**

 2 μL  5 micromolar (μM)

 Crustacean16S-R **Integrated DNA Technologies, Inc. (IDT) Catalog #custom**



20 µL

 Nuclease-free water **Integrated DNA Technologies, Inc. (IDT) Catalog #11-05-01-14**

For a full plate of 96 reactions, multiply 105.6*the per-sample volumes in the recipe to make the mastermix.

8 Add 24 µL of your Crustacean_16S metabarcoding mastermix to each well of

 Takara High Fidelity PCR EcoDry Premix **Takara Bio Inc. Catalog #639280**

8.1 Add 1 µL DNA extract

8.2 Mix and stir together with pipette tip, swirling to make sure the liquid is in the bottom, and bringing any bubbles to the surface of each reaction.

8.3 Cap each row of reaction tightly before beginning any other PCR reaction in the same room.

Crustacean_16S Takara PCR Conditions

4m

9 95 °C for 00:01:00

35 cycles of:

95 °C for 00:00:30

50 °C for 00:01:00

68 °C for 00:00:30

followed by:

68 °C for 00:01:00


then hold at 4 °C

4m

Combine PCR Products of Biological Samples by Sample and Negatives by Primer



10 Briefly vortex to mix and lightly centrifuge PCR products to get any bubbles from the bottom and any droplets off of the lids.
8-strip tubes can be put into a plate holder and spun down as a plate if this option is available.



- 11 Get a new sterile 96-well plate out of its packaging and immediately cover with foil (a 15-minute treatment under a UV light is helpful to sterilize before covering with foil).
- 11.1 For biological samples (not PCR negative controls): combine  10 μL of each of the 6 PCR products **by sample** into the new sterilized plate.

Peel back one row of foil at a time and using a multitiip pipette, open only one row of PCR products at a time to combine. Cap the resulting combined row of the new plate with a strip-cap and close the PCR products of the previous row before opening a new row.

This should result in 60uL of an equal volume of MiFish and Crustacean-16S PCR products, each from the same original sample.

- 11.2 For negative controls: combine  20 μL of each of the 3 negative PCR controls **by primer**. You should have one MiFish combined negative and one Crustacean-16S negative, each with a total of  60 μL




Visualize PCR Products

- 12 Make a 1.7% to 2% agarose gel and run a representative sample of reactions on it to make sure the PCRs worked, producing bands in the 250-300bp range. Use a ladder than allows you to distinguish small bands between 100-500bp. Check some PCR negatives to see that they don't have bands. Be very careful opening the PCR plate wells at this point to avoid cross-contamination.
- 12.1 Run the gel at 100V until the dye band has traveled $\frac{3}{4}$ of the length of the gel, then visualize.
- 12.2 Any DNA smaller than 250 bp will be excess primer and oligos and will need to be cleaned with SPRI beads. If any sample in the gel has excess primer or primer-dimer fragments, the whole experiment will need to include a bead cleanup step next.




Prepare EtOH for bead cleanup, and bring beads to room temperature

12m 30s

- 13 Get AmpureXP beads out of the refrigerator, and bring to room temp, swirl to mix occasionally, or use a rocking platform.
- 14 Make fresh 80% EtOH so that you will have at least  200 μL of EtOH per well of the combined plate.



- 15 Get 2 sterile DNAase/RNase free 96-well PCR plates out of their packaging and immediately cover with adhesive foil or if possible, UV clean the plates for  00:15:00 , then immediately cover with adhesive foil.

15m

One plate will be for the bead-cleanup steps, and the other will be for the final, cleaned reactions.


Perform a 1.5x bead cleanup with Ampure XP beads.


12m 30s

- 16 In the bead-cleanup plate, do the following steps for one 8-sample row of the plate at a time, pulling back the foil cover for each row after the previous one has been completed.


- 16.1 Add 1.5x the sample volume of Ampure XP beads.

5m

In this case you have 60uL of combined PCR products per well, so you will add  90 μ L of room temperature, well-mixed

 Agencourt AMPure XP **Beckman Coulter Catalog #A63880** to each well, and pipette mix very thoroughly, by stirring and pipetting up and down ten times.

Incubate  00:05:00 at room temperature.

- 17 After the  00:05:00 incubation, place 96-well plate on a

7m

Equipment


96-well Magnetic Rack Separator

Magnetic Rack Separator
















Sergi Lab Supplies

B08134P9RT

<https://www.amazon.com/Magnetic-Separator-Protein-Purification-Format/dp/B08134P9RT/tag=&linkCode=df0&hvadid=416872221972&hvpos=&hvnetw=g&hvrnd=1295320002355002>

for  00:02:00 or until liquid is clear.



- 18 remove and discard liquid from the row, being careful not to touch the beads with the pipette or to let the beads dry for more than 30 seconds.
- 18.1 Add  100 μL of fresh 80%EtOH to each well of beads, without disturbing the beads or removing the plate from the magnet. Incubate at  Room temperature for  00:00:30 30s
- 18.2 Remove the EtOH, then immediately add another  100 μL of 80% EtOH to the wells, incubate for  00:00:30  Room temperature . 30s
- 18.3 Remove ALL EtOH, and let the row of beads dry just enough to lose some shine but not enough to start cracking. This should be approximately  00:00:30 to  00:01:00 . 1m 30s
- 18.4 Remove the plate with new row of cleaned beads from the magnetic plate, and add  30 μL of  Buffer EB **Qiagen Catalog #19086** to each well of beads, pipette mixing each well thoroughly. Incubate  00:05:00 at  Room temperature 5m
- 18.5 Place back on the magnetic rack for  00:01:00 until liquid is clear again. 1m
- 18.6 Roll back the foil on the final cleaned reactions plate for the appropriate row. Remove  28 μL clear eluate from the bead-cleanup plate, and place in the appropriate wells of the final cleaned reactions plate. Immediately cover this cleaned PCR product with 8-strip caps.
- 18.7 uncover the next row of samples for cleaning and  go to step #16.1 until all rows are cleaned. II

Note

Safe stopping point. Samples can be stored at 4C after this step.



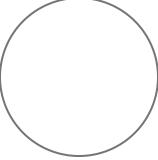
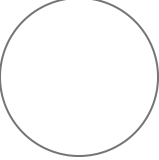
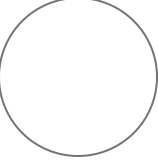
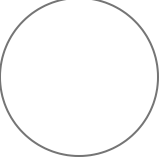
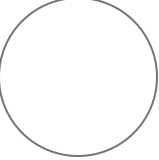

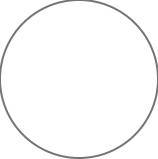
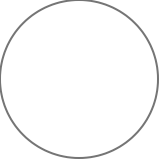
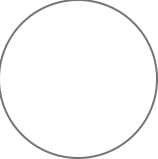
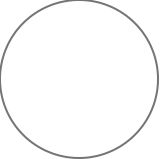
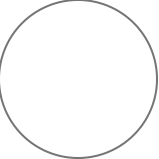

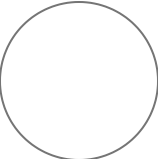
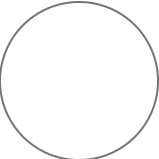
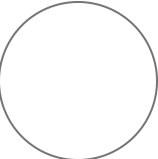
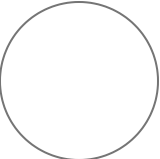
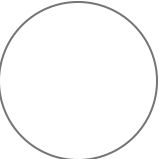

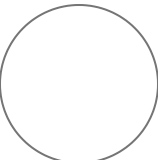
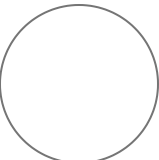
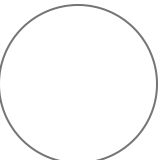
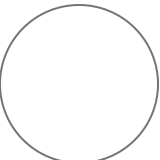
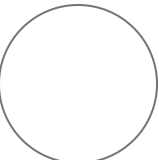

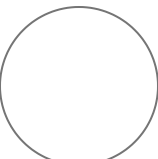
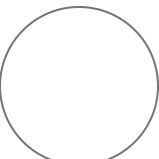
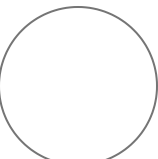
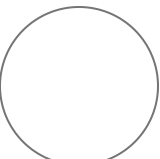
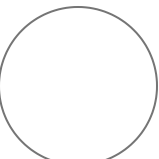

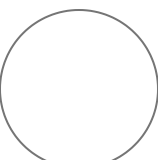
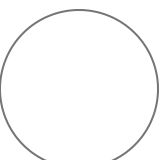
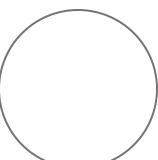
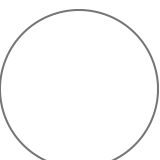
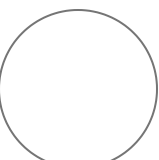

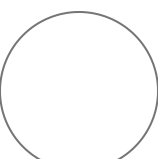
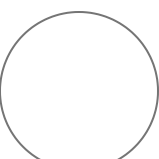
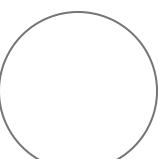
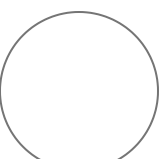
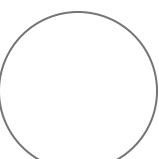

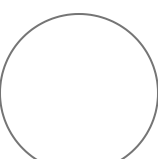
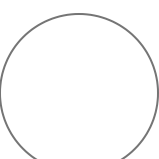
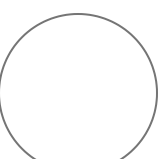
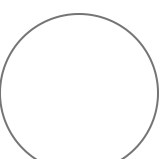
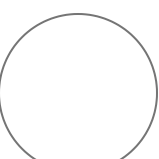

Prepare Indexing PCR

- 19 Create an indexing plate map and **make sure your chosen indexes (iNext indexes) are color balanced if you aren't doing full 96-well plates at one time.**



	1	2	3	4	5	
A	S8 iNextA-F + iNext1-R	S16 iNextA-F + iNext2-R	etc.	MiFishPCRneg i NextAF+4R	Crust..PCRneg i NextAF+5R	
B	S7 iNextB-F + iNext1-R	S15 iNextB-F + iNext2-R				
C	S6 iNextC-F + iNext1-R	S14 iNextC-F + iNext2-R				
D		etc.				
E						
F						
G						
H						



	7	8	9	10	11	
A						
B						
C						
D						
E						
F						
G						
H						

See: Glenn, Travis C., Roger A. Nilsen, Troy J. Kieran, Jon G. Sanders, Natalia J. Bayona-Vásquez, John W. Finger, Todd W. Pierson, et al. 2019. "Adapterama I: Universal Stubs and Primers for 384 Unique Dual-Indexed or 147,456 Combinatorially-Indexed Illumina Libraries (iTru & iNext)." *PeerJ* 2019 (10). <https://doi.org/10.7717/peerj.7755>. Supplemental file S10



Prepare working solutions of [M] 5 micromolar (μM) of each indexing primer you intend to use.

20 Indexing PCR Mastermix Recipe:

6 μL 2x Kapa HiFi Hotstart Readymix **Kapa Biosystems Catalog #KK2602**

2.1 μL

Nuclease-free water **Integrated DNA Technologies, Inc. (IDT) Catalog #11-05-01-14** per sample.

Multiply by number of wells *10% as explained above, to create master mix.

21 In a new, clean 96-well plate (UV before use if possible and prepare in a pre-PCR space):

Add 8.1 μL Indexing Mastermix to each well that will be used and add 0.7 μL of the [M] 5 micromolar (μM) iNext forward indexed primer for each horizontal row of the plate (8 letters), and 0.7 μL [M] 5 micromolar (μM) of the iNext reverse indexed primer for each vertical column of the plate (12 numbers) according to the indexing plate map.

22 Take the prepared indexing reactions to the post-PCR space to add the cleaned PCR product.

In the post-PCR area, add 2.5uL of cleaned PCR 1 product to their associated wells from the indexing plate map.

Indexing PCR Conditions

23 95 °C 00:03:00

4m 35s

8 cycles of:

98 °C 00:00:20

65 °C 00:00:15

72 °C 00:01:00

then hold 4 °C

Gel to check Indexing PCR Products

24 Visualize PCR products in a 1.7-2% gel. Bands should be around 350-400bp.



Combine and Clean all indexed samples from each plate

- 25 Combine 10uL of up to 70 indexed samples (library) into a single 1.5mL (or 1.7mL) tube. If there are more than 70 samples, you will need another tube.
- 26 Multiply the volume of the pooled libraries in each tube by 0.9 to get the volume of Ampure XP beads needed to clean up the reactions.

For 70 uL of combined libraries you will need 63uL of beads for a total of 133uL of beads+library pool.

Perform a 0.9x bead cleanup with Ampure XP beads

28m

- 27 In the 1.5mL tube of pooled libraries, add 0.9x volume of Ampure XP beads and pipette mix well. incubate Room temperature for 00:10:00
- 28 Make enough fresh 80% EtOH to have 2x the total volume of the beads+library pool plus a bit extra.
- 29 Place 1.5mL tube into a magnetic rack

10m

5m

Equipment

Magnetic Rack for for 1.5 mL Tubes

Magnetic Rack for DNA, RNA Purification; for 1.5 mL centrifuge Tubes

Sergi Lab Supplies

B0BZWXZM22

https://www.amazon.com/Magnetic-Rack-Purification-centrifuge-Tubes/dp/B0BZWXZM22/ref=sr_1_1?crid=1383333333&pf_rd_p=652498086131&pf_rd_r=67160340428411032&linkCode=df0&hvadid=652498086131&hvpos=&hvnetw=g&hvrand=67160340428411032





and incubate Room temperature for 00:05:00

- 30 Discard liquid and add an equal or greater volume of 80% EtOH. Incubate Room temperature for 00:01:00

1m




31 Repeat the ethanol wash a second time [↩ go to step #30](#) , then after the second 80% EtOH wash, remove all EtOH and dry the beads slightly (just until no longer wet-looking but not cracking either).

32 Resuspend beads with  100 μ L  Buffer EB **Qiagen Catalog #19086** by pipette mixing thoroughly. Incubate  Room temperature  00:10:00

10m

33 Place 1.5 mL tube back on magnet rack and wait until liquid is clear, approximately

 00:02:00


2m

34 remove 100 μ L of the clear eluate from the tube with beads while on the magnet and place in a new 1.5mL tube.

35 If you had more than one 1.5mL tube, combine equal volumes of the resulting cleaned pooled libraries (the clear eluates) into a new tube.

36 Quantify with Qubit Broad range and visualize in a gel, then send an aliquot of at least 4nM 100 μ L for sequencing on a lane of MiSeq.

Optional Quantification and Visualization

37 If you have a Qubit available, quantify with the Broad Range chemistry so that you know that the aliquot you send for sequencing is at least 4nM concentration. If it's below that concentration, [↩ go to step #27](#) and elute in  30 μ L instead of 100 μ L in step 33.

Send for Illumina MiSeq Sequencing

28m

38 Send 100 μ L of cleaned, pooled libraries for sequencing on the MiSeq platform. (This combinatorial indexing strategy is not compatible with NovaSeq).



Protocol references

- Berry, Tina E., Sylvia K. Osterrieder, Dáithí C. Murray, Megan L. Coghlan, Anthony J. Richardson, Alicia K. Grealy, Michael Stat, Lars Bejder, and Michael Bunce. 2017. "DNA Metabarcoding for Diet Analysis and Biodiversity: A Case Study Using the Endangered Australian Sea Lion (*Neophoca Cinerea*)." *Ecology and Evolution* 7 (14): 5435–53. <https://doi.org/10.1002/ece3.3123>.
- Miya, M., Y. Sato, T. Fukunaga, T. Sado, J. Y. Poulsen, K. Sato, T. Minamoto, et al. 2015. "MiFish, a Set of Universal PCR Primers for Metabarcoding Environmental DNA from Fishes: Detection of More than 230 Subtropical Marine Species." *Royal Society Open Science* 2 (7): 150088. <https://doi.org/10.1098/rsos.150088>.
- Glenn, Travis C., Roger A. Nilsen, Troy J. Kieran, Jon G. Sanders, Natalia J. Bayona-Vásquez, John W. Finger, Todd W. Pierson, et al. 2019. "Adapterama I: Universal Stubs and Primers for 384 Unique Dual-Indexed or 147,456 Combinatorially-Indexed Illumina Libraries (iTru & iNext)." *PeerJ* 2019 (10). <https://doi.org/10.7717/peerj.7755>.
- 16S Metagenomic Sequencing Library Preparation." 2013. Illumina. https://support.illumina.com/content/dam/illumina-support/documents/documentation/chemistry_documentation/16s/16s-metagenomic-library-prep-guide-15044223-b.pdf