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Metabarcoding Fecal Swabs or Stomach Contents for Fish and Crustaceans using 2-PCR protocol and Illumina MiSeq V.3

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Protocol status: Working
We use this protocol and it's working

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Abstract

This protocol describes a method to metabarcode a 170bp region of the mitochondrial16S 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&hvrand=12953200023550024012&hvpone=&hvptwo=&hvqmt=&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
B0BZWXZMZ2	SKU
https://www.amazon.com/Magnetic-Rack-Purification-centrifuge-Tubes/dp/B0BZWXZMZ2/ref=asc_df_B0BZWXZMZ2/?tag=hyprod-20&linkCode=df0&hvadid=652498086131&hvpos=&hvnetw=g&hvrand=6716034042841103246&hvpone=&hvptwo=&hvqmt=&hvdev=c&hvdvcmdl=&hvlocint=&hvlocphy=9	LINK

Protocol materials

- Buffer EB **Qiagen Catalog #**19086 — In 2 steps
- MiFish-F **Integrated DNA Technologies, Inc. (IDT) Catalog #**custom — In 2 steps
- MiFish-R **Integrated DNA Technologies, Inc. (IDT) Catalog #**custom — In 2 steps
- Takara High Fidelity PCR EcoDry Premix **Takara Bio Inc. Catalog #**639280 — Step 5.1
- Crustacean16S-F **Integrated DNA Technologies, Inc. (IDT) Catalog #**custom — In 2 steps
- Crustacean16S-R **Integrated DNA Technologies, Inc. (IDT) Catalog #**custom — In 2 steps
- Agencourt AMPure XP **Beckman Coulter Catalog #**A63880 — Step 16.1
- 2x Kapa HiFi Hotstart Readymix **Kapa Biosystems Catalog #**KK2602 — Step 22
- Nuclease-free water **Integrated DNA Technologies, Inc. (IDT) Catalog #**11-05-01-14 — In 2 steps

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):
TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGNNNNGTCGGTAAAACTCGTGCCAGC

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

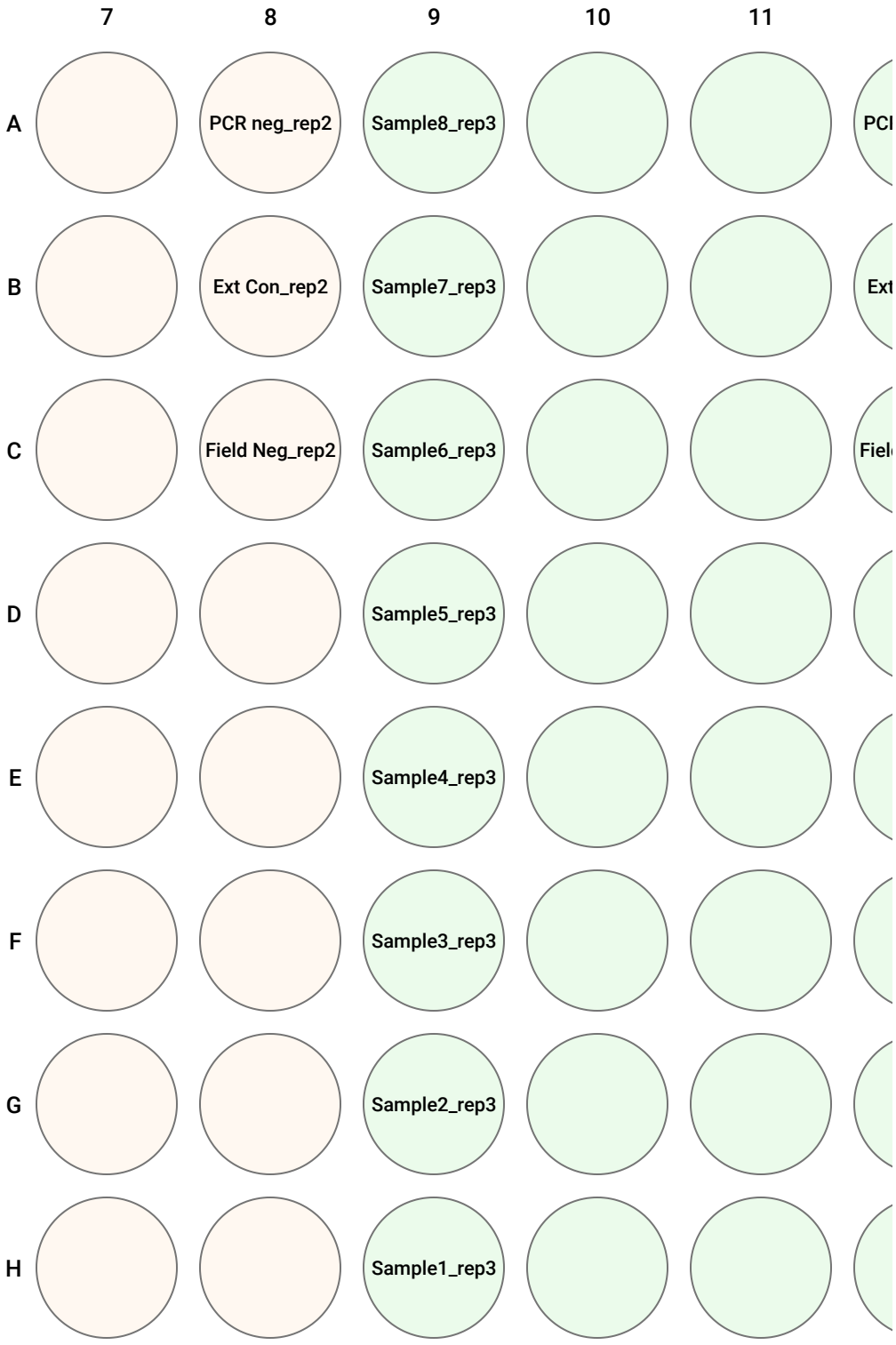
We got ours from **https://www.idtdna.com/** as 4 nmole Ultramer DNA Oligos, with standard desalting.

2 Briefly centrifuge primer tubes, then reconstitute primers to [M] 100 micromolar (µM) stock solutions by adding 40 µL (number of nano moles of primer *10) 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.



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 Make your MiFish Mastermix:

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

0.7 µL [M] 5 micromolar (µM)

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

0.7 µL [M] 5 micromolar (µM)

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

22.6 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.

5.1 Add 24 µL of your MiFish metabarcoding mastermix to each well of

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

5.2 Add 1 µL DNA extracted from stomach contents or fecal swabs.

5.3 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.

5.4 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 [M] 5 micromolar (µM)

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

2 µL [M] 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 4m

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

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 multitip 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 ¾ 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. Check that the initial PCR worked before continuing on with the library-building procedure.

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 µL 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&hvrand=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.

Tip: If you do get beads in your pipette tip, just put the liquid and beads back into the well and wait until the solution clears before trying again.

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 until all rows are cleaned.

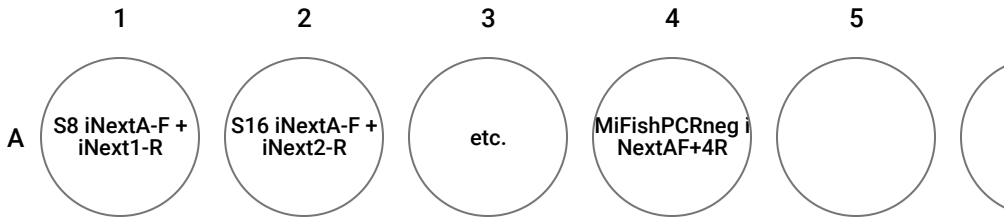
Note

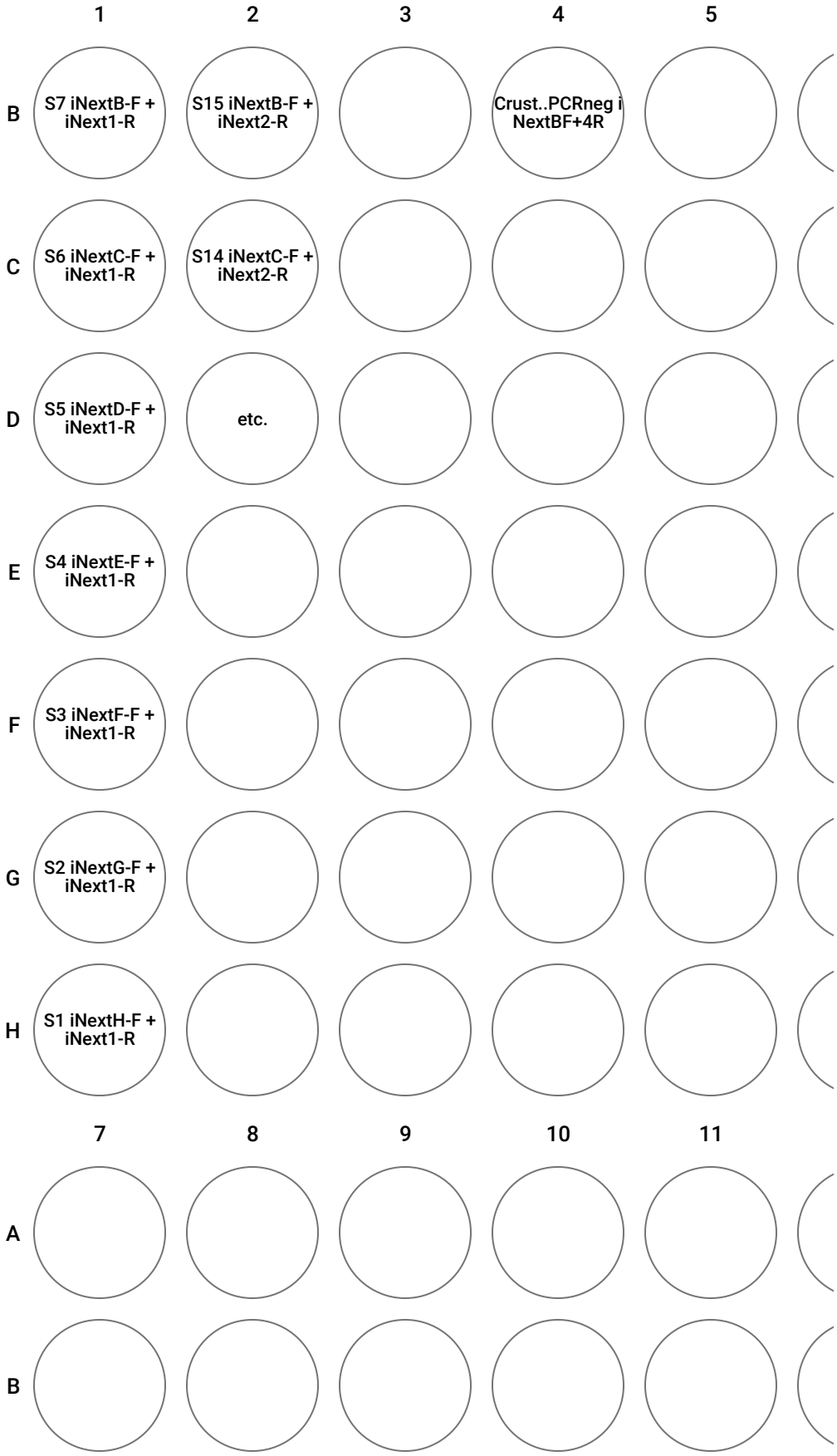
Safe stopping point. Samples can be stored at 4C after this step.

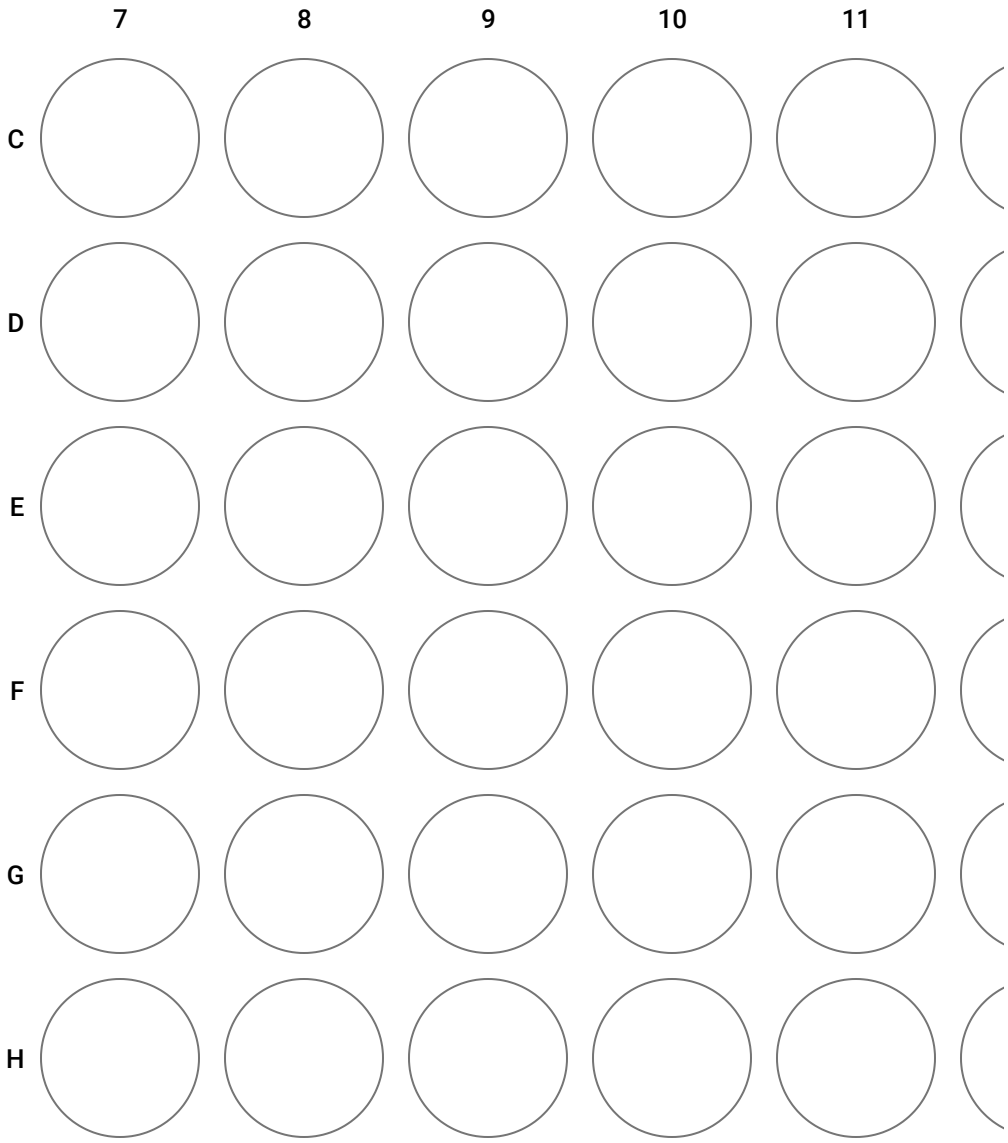
Prepare Indexing PCR

19 Work in a pre-PCR area, preferably a cleaned and UV-sterilized hood to prepare your indexing reactions before going into the post-PCR area to add the PCR1 products.

20 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
B
S7 iNextB-F + iNext1-R
S15 iNextB-F + iNext2-R
Crust..PCRneg i NextBF+4R
C
S6 iNextC-F + iNext1-R
S14 iNextC-F + iNext2-R
D
S5 iNextD-F + iNext1-R
etc.
E
S4 iNextE-F + iNext1-R
F
S3 iNextF-F + iNext1-R
G
S2 iNextG-F + iNext1-R
H
S1 iNextH-F + iNext1-R
7
8
9
10
11
A
B



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 **for information and spreadsheet to help color-balancing the indexes.**

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

22 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.

23 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.

24 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

25 95 °C 00:03:00 4m 50s

8 cycles of:

98 °C 00:00:20

65 °C 00:00:15

72 °C 00:00:15

final extension of:

72 °C 00:01:00

then hold 4 °C

Gel to check Indexing PCR Products

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

Combine and Clean all indexed samples from each plate

27 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.

28 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

29 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

10m

30 Make enough fresh 80% EtOH to have 2x the total volume of the beads+library pool plus a bit extra.

31 Place 1.5mL tube into a magnetic rack

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

B0BZWXZMZ2

https://www.amazon.com/Magnetic-Rack-Purification-centrifuge-Tubes/dp/B0BZWXZMZ2/re
20&linkCode=df0&hvadid=652498086131&hvpos=&hvnetw=g&hvrand=67160340428411032

and incubate Room temperature for 00:05:00

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

1m

33 Repeat the ethanol wash a second time go to step #34 , 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).

34 Resuspend beads with 100 µL Buffer EB **Qiagen Catalog #**19086 by pipette mixing thoroughly. Incubate Room temperature 00:10:00 10m

35 Place 1.5 mL tube back on magnet rack and wait until liquid is clear, approximately 00:02:00 2m

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

37 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.

38 Quantify with Qubit Broad range and visualize in a gel, then send an aliquot of the cleaned, pooled libraries for sequencing. Check with the sequencing core you're working with for their minimum concentration and volume and try to exceed it by a decent (10-50%) margin if possible.

Optional Quantification and Visualization

39 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 the sequencing core's minimum concentration. If it's below that concentration, go to step #29 and elute in 30 µL instead of 100uL in step 34.

Send for Illumina MiSeq Sequencing 28m

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

Tip: We have sequenced up to 1.75 plates of diet samples per MiSeq run, and still gotten sufficient coverage.

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