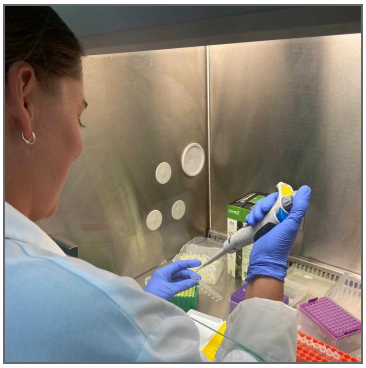

MAR 29, 2024



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dx.doi.org/10.17504/protocols.io.ewov1qxokgr2/v1

**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/v1



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

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

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University of Arizona

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.

**Created:** Nov 19, 2023

**Last Modified:** Mar 29, 2024

**PROTOCOL integer ID:** 91145

## 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

## 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

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

In 2 steps

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

In 2 steps

Agencourt AMPure XP **Beckman Coulter Catalog #**A63880 Step 14.1

2x Kapa HiFi Hotstart Readymix **Kapa Biosystems Catalog #**KK2602 Step 18

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

In 4 steps

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

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

In 2 steps

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

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

BEFORE START INSTRUCTIONS

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

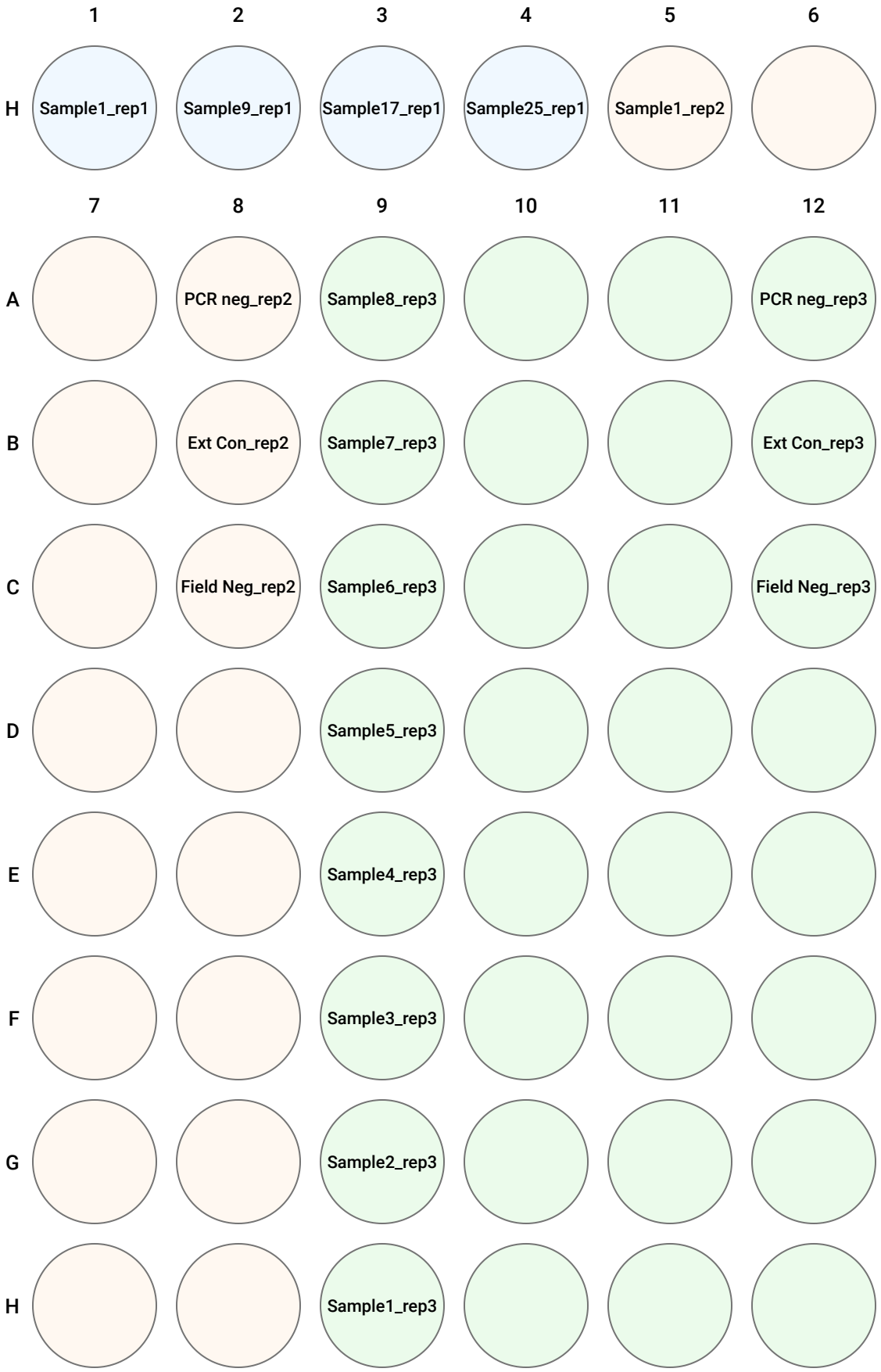
We got ours from https://www.idtdna.com/ as custom oligos at 25nm scale, with standard desalting.

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
6
H
Sample1_rep1
Sample9_rep1
Sample17_rep1
Sample25_rep1
Sample1_rep2
7
8
9
10
11
12
A
PCR neg_rep2
Sample8_rep3
PCR neg_rep3
B
Ext Con_rep2
Sample7_rep3
Ext Con_rep3
C
Field Neg_rep2
Sample6_rep3
Field Neg_rep3
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 3m 30s

95 °C for 00:01:00
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 4m

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

## Visualize PCR Products

10 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. 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.

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

12m 30s

11 Get AmpureXP beads out of the refrigerator, and bring to room temp, swirl to mix occasionally, or use a rocking platform.

12 Make fresh 80% EtOH so that you will have at least 200 µL of EtOH per well of the combined plate.

13 15m

Get 2 sterile DNAase/RNAse free 96-well PCR plates out of their packaging and immediately cover with adhesive foil.

UV clean the plates for 00:15:00

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

**14** 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.

**14.1** pipette mix 10 µL combined PCR product with 15 µL Agencourt AMPure XP **Beckman Coulter Catalog #**A63880 .

Incubate 00:05:00 at room temperature.

5m

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

7m

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 |

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

**16** 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.

**16.1** Add 100 µL of 80%EtOH to each well of beads. Incubate at Room temperature for 00:00:30 30s

**16.2** Remove the EtOH, then immediately add another 100 µL of 80% EtOH to the wells, incuba 30s for 00:00:30 Room temperature .

**16.3** Remove ALL EtOH, and let the row of beads dry just enough to lose some shine but not enou 1m 30s to start cracking. This should be approximately 00:00:30 to 00:01:00 .

**16.4** Remove the plate with cleaned beads from the magnetic plate, and add 30 µL of 5m Buffer EB **Qiagen Catalog #**19086 to each well of beads, pipette mixing each well thoroughly. Incubate 00:05:00 at Room temperature

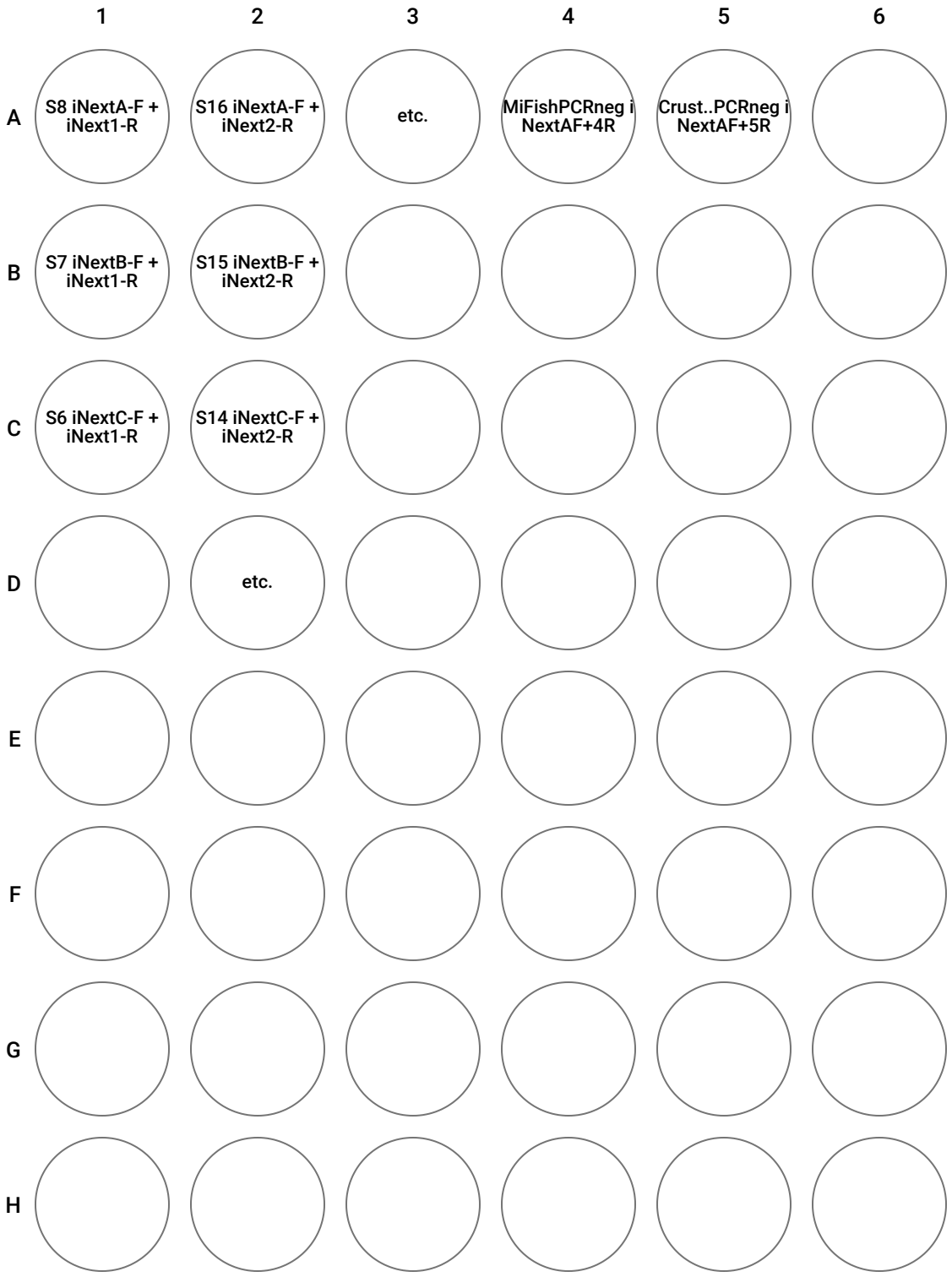
**16.5** Place back on the magnetic rack for 00:01:00 until liquid is clear again. 1m

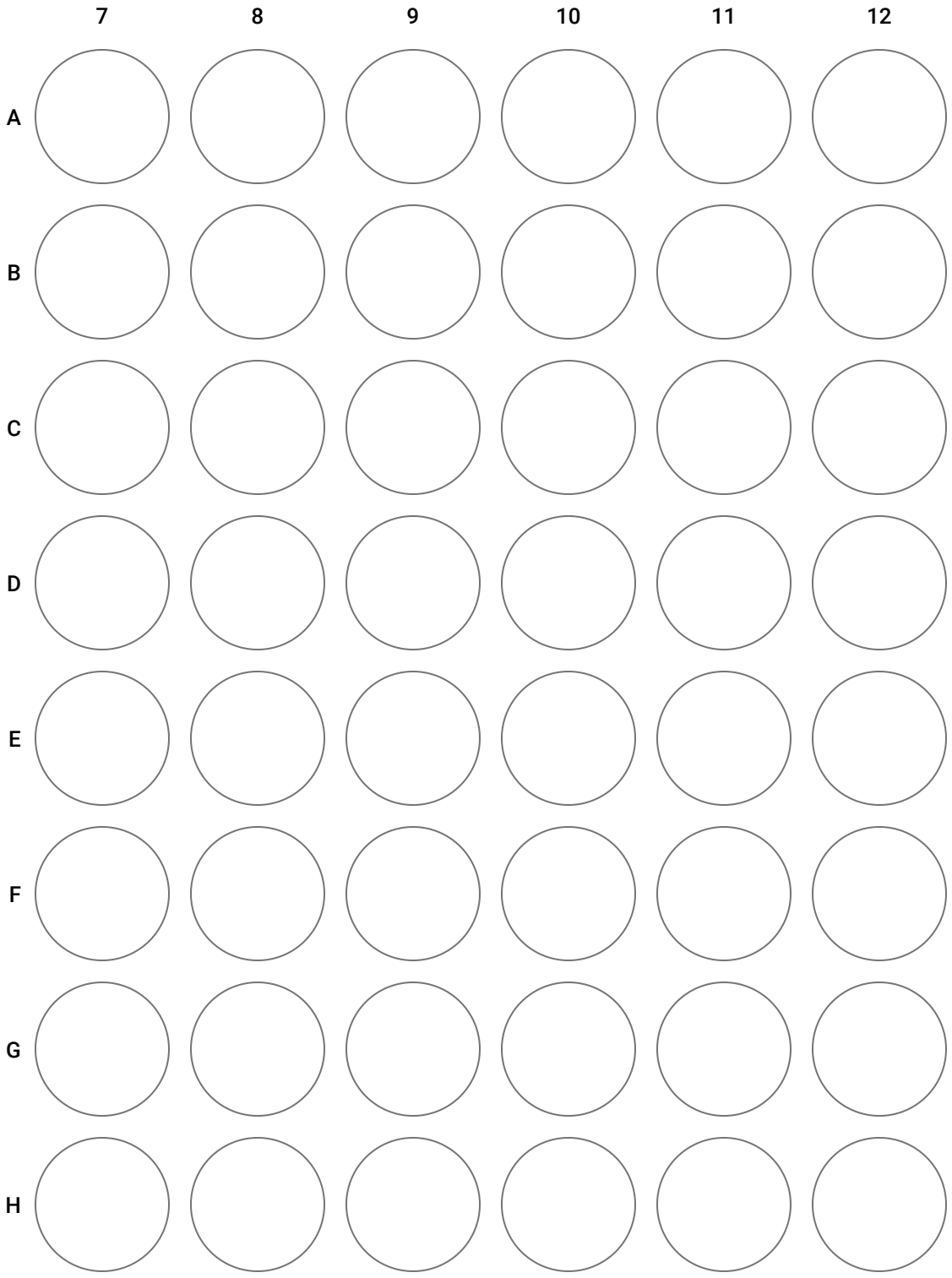
**16.6** Roll back the foil on the final cleaned reactions plate for the appropriate row. Remove the 30 µL clear eluate from the bead-cleanup plate, and place in the appropriate wells of the final cleaned reactions plate. Immediately cover this cleaned PCR product with either 8-strip caps.

**16.7** uncover the next row of samples for cleaning and go to step #14 until all rows are cleaned.

## Prepare Indexing PCR

17 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.






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.

18 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.

19 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.

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

20 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

21 4m 35s

95 °C 00:03:00

8 cycles of:

98 °C 00:00:20

65 °C 00:00:15

72 °C 00:01:00

then hold 4 °C

## Optional gel to check Indexing PCR

22 Optional: visualize PCR products in a 1.7-2% gel. Bands should be around 350-400bp.

## Combine and Clean all indexed samples from each plate

23 Combine 10uL of up to 70 indexed samples (library) into a single 1.5mL tube. If there are more than 70 samples, you will need another tube.

24 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.

## Perform a 0.9x bead cleanup with Ampure XP beads

28m

25 In the 1.5mL tube of pooled libraries, add 0.9x volume of Ampure XP beads and pipette mix well. incubate 10m
Room temperature for 00:10:00

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

27 Place 1.5mL tube into a magnetic rack 5m

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 |

and incubate Room temperature for 00:05:00

**28** Discard liquid and add an equal or greater volume of 80% EtOH. Incubate Room temperature for 00:01:00 1m

**29** Repeat the ethanol wash a second time go to step #28 , 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).

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

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

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

33 Quantify with Qubit Broad range and visualize in a gel, then send for sequencing on a lane of MiSeq.