Apprill Lab

Protocol for processing microbial biomass for 16S rRNA gene sequencing via Illumina MiSeq

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Getting started:

This protocol has a partner document called MiSeq_LibraryPrep_Template_2021 also in this drive folder. Several times I will reference that document – it's good to have it open and downloaded so you can adjust it for your samples and sequencing run. I will refer to the different sheets within the excel spreadsheet throughout (so don't change them without knowing what they are!)

Hot tips: Read over this protocol and take a look at the spreadsheet before hand

Reminder: NEVER PUT DNA OR PCR PRODUCT IN THE PCR HOOD. Pretty much do not put anything in the hood except what is designated below – we are trying to limit contamination

Overview of workflow:

1. **DNA Extraction**
2. **Quantify DNA with Qubit fluorometer**
3. **Screening PCR**
4. **Gel Electrophoresis – Visualizing PCR Products**
5. **Barcode PCR – Using 'barcode' primers, primers with unique identifiers**
6. **Purify PCR product – Gel or standard PCR purification**
7. **Diluting and Pooling – Dilute purified PCR products to equimolar concentrations and pool into a single tube to send off for sequencing**

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Protocol for preparing samples for MiSeq sequencing cont.

DNA Extractions:

Extract DNA from samples in accordance with the method that removes the most PCR inhibitors and yields high quality DNA from your sample type. Mostly we use commercially produced kits; the majority of our extractions are done using the Qiagen DNeasy PowerBiofilm Kit (Cat. No. 24000-50). We have protocols for using the PowerBiofilm kit with different sample types so have a look in Lab protocols for one tailored to your sample type/collection method:

- For swab samples, see the **Protocol for isolating nucleic acids from swabs with low biomass** (Appendix 1) or in the shared Apprill Drive: ApprillLab_Protocol for isolating nucleic acids from swabs with low biomass (e.g. low biomass collected on swabs, seawater collected on filters, and etc.).
- For seawater collected on 0.2 μM filters see **Protocol for isolating nucleic acids from filters** (Appendix 2) of this document or the shared Apprill Drive: ApprillLab_Protocol for isolating nucleic acids from filters

Quantify DNA – Qubit 2.0 Fluorometry

After DNA is extracted, it may be helpful to your project to check the concentration of your DNA. If you decide to do so, use the Qubit 2.0 Fluorometer. The Qubit is a fluorometric method for quantifying DNA and is generally accepted as the "better" method for quantifying DNA in preparation for sequencing. There are two types of Qubit assays:

1) Broad Range dsDNA assay for DNA extracted from samples that you would expect to have high yields (e.g. feces)
2) High Sensitivity dsDNA assay for DNA extracted from samples that will likely produce low yields (e.g. coral mucus or low volume samples of whale blow).

Protocol for quantifying DNA – Qubit 2.0 Fluorometry with the High Sensitivity dsDNA assay can be found in Appendix 3 or in the Apprill Drive: ApprillLab_Protocol for quantifying DNA_v2

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Protocol for preparing samples for MiSeq sequencing cont.

Screening PCR

1. After all DNA has been extracted from the samples, begin performing PCR screening tests on all samples. The purpose of this step is to group DNA samples that behave similarly during PCR (samples that require the same number of PCR cycles/ the same dilution manipulations) prior to tagging samples with unique bar-coded primers.
2. Use ScreenPCR Template: Don't forget to scroll all the way to the bottom of the sample input area for inputting the number of "extra" samples to use to make your master mix
3. Again, use only **one** set of bar-coded primers for this step (e.g.. only use B505 (forward primer) and SA705 (reverse primer) for all samples-saves time!! Please try to mix up which primers you choose for this because if everyone always uses 501A and 701, we will run out of aliquots for those two primers very quickly).
4. Be sure **to record the number of cycles & amount of DNA t**hat produces visible bands for all samples. This PCR screening can also be used to determine if certain DNA samples need to be diluted/ concentrated prior to amplification.
    - Try to aim for 30 cycles (less cycles is better) to produce a good quality, visible band. More difficult samples may require 35-40 cycles in tandem with dilutions (coral tissue and coral mucus) or an increase in the amount of DNA you add to the reaction. If you add more DNA to the reaction, you will have to adjust the amount of water that goes into the mastermix – see the table in the protocol for this adjustment.

5. The **Protocol for Screen PCR for MiSeq Library Preparation** can be found in Appendix 4 or in Apprill Drive: PCR screening for MiSeq_March2023_experienced user

Run a gel to visualize results of the screen PCR

Budget 2 hours. Run out PCR products ("amplicons") on an agarose gel to visualize the size and quality of PCR products. The **Protocol for visualizing PCR product using a 1% agarose gel** can be found in Appendix 5 of this protocol.

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Protocol for preparing samples for MiSeq sequencing cont.

Barcode PCR

Organize DNA to be used as PCR template into groups (samples that require the same number of PCR cycles, extracted from the same environmental material, DNA template from the same species . . . etc).

Once all the DNA samples have been screened and organized into similar groups, the barcode tagging process can begin!

The barcodes provide a unique ID for each sample – so we will be using the pair of forward and reverse primers (with the barcode attached) in order to genetically "tag" our samples. Pretty much everyone in our lab uses forward primer 515FY and reverse primer 806RB. We have:

- 10 forward primers (A501 – A505 and B501 – B505)
- 12 reverse primers (SA701 – SA712)

allowing for 120 unique combinations; thus, 120 is the total number of samples that could be used on one MiSeq pooled library. We use the primers at a concentration of 10 µM; aliquots are on the top shelf of the lab's -20°C freezer in sample storage boxes.

A well-planned barcode PCR is essential for success. To help you plan your PCR, there are templates in the MiSeq_Library_Prep document.

To start, you will need to decide on what size reaction you want to run: one 50µL reaction or two 25µL reactions, or one 25µL reaction. Some things to think about when deciding on reaction size:

- How much PCR product will you need? If you are doing gel extractions, you will want a 50µL reaction
- How fussy are the samples?
- Have you tried 50µL reactions in the past, and do they seem still yield low purified product?
- When you ask the PCR gods what to do, what do they say?

NOTE: Most often we run one 50µL reaction for barcoding PCRs.

Next you will need to assign unique barcode combinations to each sample you plan to sequence. Navigate to the BarcodePCR_50µLperWell and look at the plate map. When thinking about how assigning barcode combinations, please know it is a nice strategy to start with one unique Forward barcode for each row (there are 8 rows in a plate) and one unique Reverse barcode for each column (there are 12 columns in a 96-well plate) of a PCR plate. It is important to keep a

good record of which unique primer combinations have already been assigned- you do not want different samples to have the same primer combinations! Keep track by entering unique primer combinations next to the sample I.D. in the excel spreadsheet: Barcodes Assigned. You can then copy and paste the SAMPLE ID, FORWARD PRIMER, and REVERSE PRIMER into the columns for these in the BarcodePCR_50µLperWell or BarcodePCR_25µLperWell (depending on which size reaction you plan to run) but before you do so, you will want to make a copy of whichever template you will be using for each PCR you plan to run.

If you are new to barcoding, start with a smaller plate, either a quarter plate or half plate. Experienced users feel free to use 96-well plates. If performing duplicate 25µL PCR reactions per sample, 47 different samples can fit onto a plate (leaving room for 2 empty wells, a well for a positive and a well for a negative control).

The sample IDs + barcode combinations should automatically fill into the plate map if you are using the aforementioned templates.

Always make more mastermix than you need. To do so on the template, scroll all the way to the bottom to add extra samples

- If you are going to use a multichannel pipette and a reagent boat, err on the side of more
- Make sure the volume of water to be added to tubes is a whole number (adjust "extra" samples in orange until it's a whole number)

Protocol for assembling a barcode PCR (**Protocol for Barcode PCR for MiSeq Library Preparation**) can be found in Appendix 6 of this protocol and in the Apprill Drive: PCR Barcode for MiSeq_02March2023.pdf

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Protocol for preparing samples for MiSeq sequencing cont.

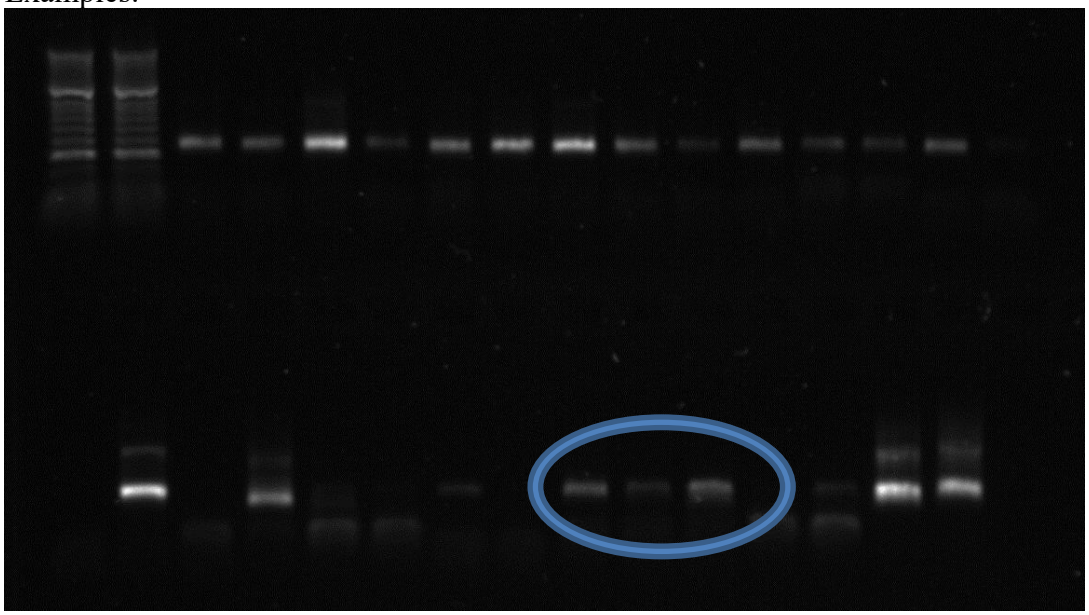
Run a gel to visualize results of the barcode PCR

1. If each sample was run in duplicate in the PCR, combine PCR products into one pool per sample. This can be done in the same well plate (recommended), another pre-sterilized well plate, or a pre-sterilized micro centrifuge tube.

2. Follow **Protocol for visualizing PCR product using a 1% agarose gel** (Appendix 5) to run samples out on a gel to check the quality of the bands.

3. PCR products can be used to generate the MiSeq pool if they do not contain multiple bands, have minimal primer dimers, and are not too bright/ concentrated when visualized on a gel (broad/bright bands is indicative of over-amplification and PCR bias that will affect the sequencing results).

Examples:



Most of the amplicons along the top row of the gel are suitable for generating the library (while they range in band brightness, they contain no double bands, minimal primer dimers, and are not "smeary" in appearance). Most of the amplicons along the bottom row of the gel contain double/bands and are smeared in appearance. The three middle amplicons along the bottom row (circles) are suitable for purification.

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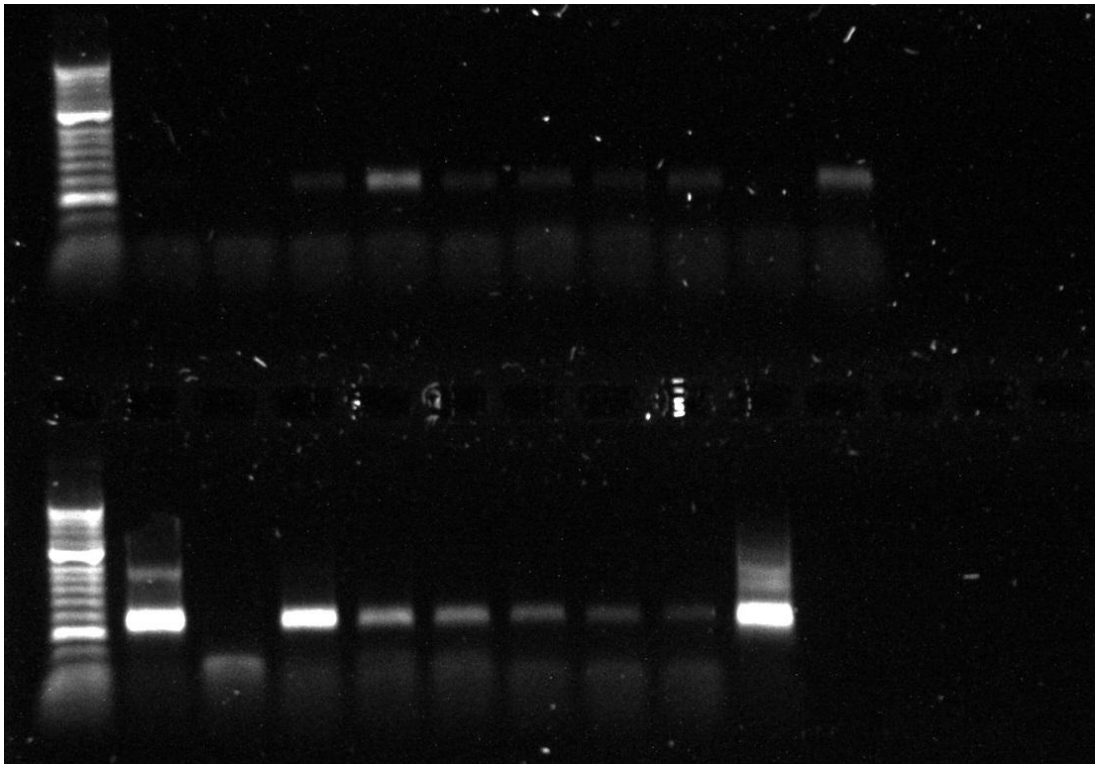
Protocol for preparing samples for MiSeq sequencing cont.



Another example of over-amplified/ double bands in bar-coded PCR products. The first, third, and last amplicons along the bottom are not suitable for generation of a MiSeq library because they contain multiple bands and are over-amplified. However, the amplicons in-between these samples are suitable to be used.

- **For over-amplified samples**: Conduct barcoded PCR reaction again using lower number of cycles to find sweet spot for each particular sample. May take a couple of tries . . . do not get discouraged!
- **For samples not amplified enough**: Increase the number of cycles, dilute template (if you think it's because of inhibitors)
- **For samples with multiple bands**: GEL EXTRACTIONS!

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Protocol for preparing samples for MiSeq sequencing cont.

Barcoded Amplicon Purification

1. For samples with a single band of the correct size: purify the acceptable bar-coded PCR amplicons using a PCR Purification Kit (one, nice bright band):

    - Qiagen MinElute Purification Kit or New England Biolabs Monarch PCR purifications kit. Follow the protocol directions
      -or-
    - PCR purification using the Qiagen Qiaquick 96-well PCR purification kit with vacuum manifold may be used (warning- evaporation/loss of final eluent will occur)

2. For samples with multiple bands: purify PCR products using a **Gel Extraction purification kit.** Follow **ApprillLab_Protocol for Gel Purification** (Appendix 7) or in Apprill Drive: ApprillLab_Protocol for Gel Purification

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Protocol for preparing samples for MiSeq sequencing cont.

Diluting Purified Amplicons prior to pooling:

1. **Assess concentrations of purified and bar-coded amplicons** using the dsDNA HS Qubit 2.0 Fluorometer assay. See Appendix 8 or Apprill Drive ApprillLab_Protocol for quantifying PCR Product_v2.pdf

2. **Perform dilutions on products.**

- See DiluteAndPool sheet in the MiSeqLibraryPrep_Template
- Aim: dilute to 1 ng/µL of PCR Product, and use 5µL of the purified product. We will just adjust the amount of water to add to dilute each product before adding it to the pool
    - We are using the equation $C_1V_1=C_2V_2$.
    - The calculations are done on the sheet, but this is what we do:
        - C1 = concentration of purified product after Qubit-ing
        - V1 = volume of purified product to put into dilution (5µL)
        - C2 = targeted concentration (1 ng/µL)
        - V2 = total volume of the dilution: SOLVE!

        So if we have 25.6 ng/µL of purified product, and we want to add 5µL into the dilution for a final concentration of 1ng/µL:

        (25.6*5)/1 = 128µL

        So we would add 128-5µL of water (123µL) for the dilution

    - Use the molecular grade sterilized water from the PCR hood but be sure to wear clean, fresh gloves before going into the hood to get water. WE DO NOT WANT PCR PRODUCT ANYWHERE NEAR THE HOOD.
    - Dilutions can be performed in either 96 well plates or individual centrifuge tubes
        - I make another template as above (see DiluteAndPool over by Column W) to include how much water to add to a new PCR plate
        - Add the water first, and then go to the PCR product area to add the purified product

3. OPTIONAL: Assess diluted PCR product concentrations using High Sensitivity DNA Qubit 2.0 Fluorometer assay. Adjust problem dilutions as necessary (either too dilute/too concentrated) and determine concentrations.

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Protocol for preparing samples for MiSeq sequencing cont.

Pooling bar-coded amplicons

1. Because all samples are now diluted to 1ng/µL, we are going to add 5µL of each to the pool (leading to a total of 5ng of DNA per sample in our pool)

2. Pipette 5µL of each sample into a 2mL cryovial.

3. Mix gently and then pipette approximately half of the pooled library into a second sterile cryovial so you can send one aliquot off for sequencing and keep one here as a backup in case anything happens to the first aliquot during shipping/sequencing.

Preparing Bar-coded, Purified, Diluted, and Pooled Samples for MiSeq Sequencing:

Directions for submission of pooled libraries to the University of Illinois, see: SubmittingToIllinois

1. in the Apprill lab Google Drive AND on the MiSeq_LibraryPrep_Template there are templates for preparing the spreadsheet for each pool: SampleSubmissionSheet_Example

- Information needed includes: sampleID (matches the one you are using in BarcodesAssigned), Species, library prep type, date of preparation, fragment size (450 bp), reverse (i7) name + sequence, forward (i5) name, and sequence
- Primer sequence information is in the MiSeq_LibraryPrep_Template on the PrimerIndexSequences sheet

Example of Submission spreadsheet:

Sample ID	Pooling Scheme	Species	Library Prep Type	Date Library Prepared	Concentration(nM)	Fragment Size(bp)	i7 Name	i7 Original Sequence(i7 Primer Orientation)	i7 Reverse Complementary	i5 Name	i5 Original Sequence(i5 Primer Orientation	i5 Reverse Complementary
Chile2017_1		whale	DNA_Amplicon	12/30/17	6	450	36RB_SA703	AGTAGCGT	ACGCTACT	FB_B504	TACGAGAC	GTCTCGTA
Chile2017_3		whale	DNA_Amplicon	12/30/17	6	450	36RB_SA706	CTACGCAG	CTGCGTAG	FB_A501	ATCGTACG	CGTACGAT
Chile2017_4		whale	DNA_Amplicon	12/30/17	6	450	36RB_SA702	ACTATGTC	GACATAGT	FB_B503	AGAGTCAC	GTGACTCT

2. Submit a WHOI Shipping request. Choose FedEx Priority overnight.

4. Ship samples in cooler box using ice packs that have been frozen at -80 C on Monday, Tuesday, or at the latest, Wednesday. Submit the submission spreadsheet Sample_SubmissionSheet_forsubmittingtoUIllnious

5. Update the Apprill Lab Record of Libraries in Apprill Drive Sequencing related folder

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Protocol for preparing samples for MiSeq sequencing cont.

General Hints/tips:

- Maintain organization (write/ record everything!). It is very easy to mislabel, confuse sample names, and use the same unique primer combinations on different samples within the same pool (which will be an issue when you get the results back).

- After you have barcoded and selected amplicons suitable for purification (and for the pool), record the PCR cycle number and date the amplicon was generated next to the sample name. Store the amplicons that will be used for library generation separately from the "junk/over-amplified" amplicons to ensure that you will purify and use the correct amplicons for generating your library pools.

- I find it useful to arrive early in the morning, set-up, and begin a PCR reaction. While waiting for that reaction to finish, I set up another PCR reaction and/or preload unique primer pairs into plates. After the first reaction has finished, I begin the next reaction. I pool and screen the samples using a gel, determine which products I will keep, organize and store accordingly (it is also helpful to have somebody make the gel, pool, and run the gel for you will you continue preparing PCR plates). I also find it helpful to run PCRs overnight (especially if they require 35-40 cycles).

- You may find it useful to preload primers into plates, cover them, and freeze them until needed (cuts down on PCR assembly time). Just be careful that you do not displace the 1µL primer droplets before you add the mastermix!

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Protocol for preparing samples for MiSeq sequencing cont.

Version history:

Laura Weber, Woods Hole Oceanographic Institution, 04/22/2014
Updated by Carolyn Miller, September 2020
Updated by Anya Brown, September 2021
Updated by Carolyn Miller, January 2024

Appendix 1.

DNA Extractions from swabs with low biomass

ApprillLab_Protocol for isolating nucleic acids from swabs with low biomass

Apprill Lab

Protocol for isolating nucleic acids from swabs with low biomass

Laboratory of Amy Apprill office 508 289 2649 aapprill@whoi.edu

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Reagents and tubes are from the DNeasy PowerBioFilm Kit (Qiagen, Inc., Germantown, MD)

Protocol adapted from manufacturers protocol for the MoBio PowerBiofilm DNA Isolation Kit

- Clean lab bench, centrifuge, tube racks, and incubator according to lab protocol
- Prepare reagents and equipment:
  - UV sterilize 1.8 or 2 mL microcentrifuge tubes for 15 minutes (one microcentrifuge tube for each swab)
  - Warm solution MBL 55°C for 5-10 min
  - Turn on incubator to 65°C
  - Put tube rack in 4°C
- Use filter tips

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1. Add **350 µL of solution MBL** to the cryovial containing the swab
2. Add **100 µL of solution FB** to the cryovial containing the swab
3. Gently agitate
4. Using flame-sterilized forceps, transfer swab with shaft down into a UV sterilized microcentrifuge tube
   Hint: having two pairs of forceps can be helpful for holding and guiding the swab into the centrifuge tube. Forceps with grips can also be helpful
5. Centrifuge 13,000 g for 1 min at room temp
6. Transfer the liquid (MBL + FB + sample) that came off of the swab to the bead tube for the sample (discard swab)
7. Also transfer the liquid (MBL + FB + sample) from the cryovial to the bead tube for the same sample.
   Hint: you can use the same pipette tip for both transfers.
8. Vortex briefly to mix
9. Incubate at 65°C for 5 minutes
10. Bead beat on vortex adapter for 15 minutes
11. Centrifuge 13,000 g for 1 min at room temp

12. Transfer supernatant to new 2 mL collection tube
13. Add **100 µL of solution IRS**
14. Vortex briefly to mix
15. Incubate at 4°C for 5 minutes
16. Centrifuge 13,000 g for 1 min at room temp
17. Warm solution MR for 5 minutes
18. Transfer supernatant to clean 2 mL collection tube, avoid pellet (expect ~ 375-500 µL)
19. Add **900 µL of solution MR**
20. Vortex briefly to mix
21. Filter supernatant:
    a. Load 650 µL of supernatant onto MB Spin Column (Spin Filter)
    b. Centrifuge 13,000 g for 1 min at room temp
    c. Discard flow thru
    d. Repeat until all supernatant has been loaded onto the spin filter
22. Place spin filter basket into clean 2 mL collection tube
23. Shake solution PW
24. Add **650 µL solution PW**
25. Centrifuge 13,000 g for 1 min at room temp
26. Discard flow through and centrifuge again at 13,000 g for 1 min at room temp
27. Add **650 µL ethanol**
28. Centrifuge 13,000 g for 1 min at room temp
29. Discard flow through
30. Centrifuge at 13,000 g for **2 min** at room temp
31. Place spin filter basket into clean 2 mL tube
32. Add **100 µL solution EB** to the center of the white membrane
33. Centrifuge 13,000 g for 1 min at room temp
34. Remove spin filter basket
35. Quantify DNA and store at -20°C

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Version history:

- 24 October 2019, Carolyn Miller: Created protocol by adapting from "ApprillLab_Protocol for isolating nucleic acids from samples of whale blow_v2"

Appendix 2.

DNA Extractions from filters

ApprillLab_Protocol for isolating nucleic acids from filters

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Protocol for isolating nucleic acids from filters

Laboratory of Amy Apprill office 508 289 2649 aapprill@whoi.edu

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Reagents and tubes are from the DNeasy PowerBioFilm Kit (Qiagen, Inc., Germantown, MD, USA, Catalog# 24000-50)

Protocol adapted from manufacturers protocol for the MoBio PowerBiofilm DNA Isolation Kit

- Clean lab bench, centrifuge, tube racks, and incubator according to lab protocol
- Prepare reagents and equipment:
  - Warm solution MBL 55°C for 5-10 min
  - Turn on incubator to 65°C
  - Put tube rack in 4°C

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1. Using flame-sterilized forceps, transfer filter into a bead tube
   Hint: having two pairs of forceps can be helpful for holding and guiding the swab into the centrifuge tube. Forceps with grips can also be helpful
2. Add **350 µL of solution MBL** to the bead tube
3. Add **100 µL of solution FB** to the bead tube
4. Vortex briefly to mix
5. Incubate at 65°C for 5 minutes
6. Bead beat on vortex adapter for 15 minutes
7. Centrifuge 13,000 g for 1 min at room temp
8. Transfer supernatant to new 2 mL collection tube
9. Add **100 µL of solution IRS**
10. Vortex briefly to mix
11. Incubate at 4°C for 5 minutes
12. Centrifuge 13,000 g for 1 min at room temp
13. Warm solution MR for 5 minutes

14. Transfer supernatant to clean 2 mL collection tube, avoid pellet (expect ~ 375-500 µL)
15. Add **900 µL of solution MR**
16. Vortex briefly to mix
17. Filter supernatant:
    a. Load 650 µL of supernatant onto MB Spin Column (Spin Filter)
    b. Centrifuge 13,000 g for 1 min at room temp
    c. Discard flow thru
    d. Repeat until all supernatant has been loaded onto the spin filter
18. Place spin filter basket into clean 2 mL collection tube
19. Shake solution PW
20. Add **650 µL solution PW**
21. Centrifuge 13,000 g for 1 min at room temp
22. Discard flow through and centrifuge again at 13,000 g for 1 min at room temp
23. Add **650 µL ethanol**
24. Centrifuge 13,000 g for 1 min at room temp
25. Discard flow through
26. Centrifuge at 13,000 g for **2 min** at room temp
27. Place spin filter basket into clean 2 mL tube
28. Add **100 µL solution EB** to the center of the white membrane
29. Centrifuge 13,000 g for 1 min at room temp
30. Remove spin filter basket
31. Quantify DNA and store at -20°C

Version history:

- 27 April 2021, Carolyn Miller: Created protocol by adapting from "ApprillLab_Protocol for isolating nucleic acids from swabs with low biomass"

Appendix 3.

Quantify DNA – Qubit 2.0 Fluorometry

ApprillLab_Protocol for quantifying DNA_v2

Protocol for quantifying DNA – Qubit 2.0 Fluorometry

Laboratory of Amy Apprill office 508 289 2649 aapprill@whoi.edu

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Equipment:	Qubit 2.0 fluorometer	(2nd drawer down of DNA bench)
	Qubit assay tubes	(2nd drawer down of DNA bench)
	15 mL conical tube – clean	(Shelf opposite DNA bench)

Reagents: Qubit dsDNA HS Assay Kit reagents:

- dsDNA HS Buffer (top drawer of DNA bench)
- dsDNA HS dye (top drawer of DNA bench)
- dsDNA Standards #1 & #2 (refrigerator top left)

Protocol adapted from manufacturers protocol

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1. Label tubes:
    a. one tube for each sample
    b. one tube for Standard #1
    c. one tube for Standard #2
2. Do calculations for preparing the working solution:
    a. Calculate total number of preparations (x).

        x = # of samples + 2 (for 2 standards) + 2 (extra just in case)

    b. Calculate the volume of dsDNA HS Buffer (V_b) needed:

        $V_b = x * 199\ \mu L$

    c. Calculate the volume of dsDNA HS Dye (V_d) needed:

        $V_d = x * 1\ \mu L$

3. Prepare working solution by combining HS Buffer (V_b) and HS Dye (V_d) in a clean 15 mL conical tube
4. Prepare standards:
    a. Aliquot 190 µL of the working solution into each standard tube
    b. Add 10 µL of each standard to their respective tubes
    c. Vortex 2 seconds and set aside for 2 minutes before using
5. Prepare samples:
    a. Aliquot 197 µL of working solution into each sample tube
    b. Add 3 µL of each sample's DNA to the respective tubes – Make sure to mix the DNA by flicking the tube before pipetting
    c. Vortex 2 seconds
    d. Set aside for 2 minutes before measuring

6. Plug in Qubit to turn it on
7. Select DNA
8. Select High Sensitivity (HS) dsDNA as the assay
9. Select 'Yes' for reading new standards
10. Final check of standards before measuring:
    a. Wipe tubes with the standards with a clean kim wipe
    b. make sure there are no bubbles (tap gently on the bench if there are)
    c. make sure there are no droplets on the side of the tube (tap gently on the bench if there are)
11. Read standards by following the prompts on the Qubit display
12. Final check of the samples before measuring:
    a. make sure there are no bubbles (tap gently on the bench if there are)
    b. make sure there are no droplets on the side of the tube (tap gently on the bench if there are)
    c. Wipe outside of tube with a clean tissue wipe
13. Measure samples:
    a. press "read new sample" on the Qubit display
    b. press 'Calculate stock concentration'
    c. make sure the units are set to ng/µL
    d. make sure the volume is set to 3 µL
    e. record the concentration in notebook
    f. repeat for all samples
14. Clean up:
    a. Assay tubes for both standards and samples can go into the autoclave waste
    b. Unplug Qubit and return to drawer
    c. Wipe down and tidy the bench

Ordering Information:
Qubit dsDNA HS Assay Kit (ThermoFisher Catalog#. Q32854)
Qubit assay tubes (ThermoFisher Catalog# Q32856)

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Version history:

- 30 April 2021, Carolyn Miller: Created protocol for Sam Bowman
- 02 March 2022, Carolyn Miller: updated with ordering information

Appendix 4.

Screen PCR

PCR screening for MiSeq_March2023_experienced user

Protocol for Screen PCR for MiSeq Library Preparation

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Equipment, reagents, primers, and consumables needed for this protocol are listed on page 3.

ALWAYS WEAR CLEAN GLOVES IN PCR HOOD

NEVER BRING DNA INTO PCR HOOD

1. Clean the PCR prep bench

2. Clean bench of PCR hood (and inside of front window if needed)

3. Label the following in the PCR hood:
   a. PCR plate, strips, or tubes with name, date, ctrls (+) & (-), and sample #s for strips/tubes)
   b. 2 mL microcentrifuge tube(s) for mastermix
   c. 2 mL microcentrifuge tube(s) for water – fill with DNA-free water

4. UV Sterilize the labeled items from step 3 for **15 MINUTES** (place very close to light):
   a. Be sure tubes are open and upright such that the light can reach inside
   b. If you will be using multi-channel pipette, add a reservoir to the UV sterilization

5. Thaw goTaq 5x flexi buffer and $MgCl_2$ (room temp is ok)

6. Vortex $MgCl_2$ & goTaq 5x flexi buffer well

7. Calculate volume of reagents for mastermix (see table 1 below)

8. Prepare first part of mastermix:
   a. Combine:
      i. H_20
      ii. goTaq 5x colorless flexi buffer
      iii. $MgCl_2$
   b. Vortex to mix
   c. Sterilize under UV light for **10 MINUTES** (open lid and place very close to light)

9. Thaw dNTPs, and primers (room temp is ok). Return goTaq 5x flexi buffer and $MgCl_2$ to freezer

10. Thaw DNA at 4°C. Remember a positive control.

11. Take goTaq DNA polymerase out of freezer and place in freezer cooler block made for 2 mL tubes.

12. Finish preparing mastermix:
    a. Add to first part of mastermix made above:
       i. Forward primer
       ii. Reverse primer
       iii. dNTPs
       iv. goTaq DNA polymerase
    b. Invert gently several times to mix

13. Aliquot mastermix into plate (24 µL/well for 1 µL DNA template or 23 µL/well for 2 µL template)

14. Aliquot H_20 into one well for negative control

15. Take PCR plate, strip(s), or tubes out of hood. If you have a plate, place it on the freezer cooler block made for 96-well plates. Use the freezer block that is labelled "PCR Prep" only!!
16. Turn on heat sealer
17. Prepare to, and add DNA to wells:
    a. Fetch thawed DNA from the refrigerator.
    b. Mix DNA samples (flick tube)
    c. Aliquot into wells or tubes
    d. Pipette up and down at least 5 times to mix DNA into mastermix.
    e. Add (+) control to one well
18. Heat seal PCR plate or close caps on tubes.
19. Centrifuge for about 15 seconds (3700 g)
20. Place plate in thermal cycler: MiSeq program. Edit to change the # repeats (1 < total # cycles you want). Change volume to that of your reaction. Verify program:
    a. Lid: 105°C
    b. Volume: 25 or 50 µL
        - 2 min 95°C
        - 20 sec 95°C
        - 15 sec 55°C
        - 5 min 72°C

        These steps repeat each cycle
        - # cycles
        - 10 min 72°C
21. Press run and then 'ok' to confirm the lid temp, volume, and thermocycler block
22. Store at 4°C short-term (<1 week) or -20°C long-term (>1 week)

Table 1. Mastermix recipes and summary of all components of screen PCR reactions.

volume DNA template you are planning to add to reaction:	For 25 µL screen reactions: 1 µL DNA	2 µL DNA
Mastermix component	volume per sample (µL)	
H_2O	14.75	13.75
buffer	5	5
MgCl2	2.5	2.5
forward primer - barcode 515FY	0.5	0.5
reverse primer - barcode 806RB	0.5	0.5
dNTPs	0.5	0.5
polymerase	0.25	0.25
Total volume matermix	24	23
DNA template	1	2
Total Volume of reaction	25	25

Protocol for Screen PCR for MiSeq Library Preparation

Laboratory of Amy Apprill office 508 289 2649 aapprill@whoi.edu

Equipment, reagents, primers, and consumables needed for PCR:

- Equipment:
  - Thermocycler
  - Centrifuge
  - Heater block for heat sealing
  - Pipettes (1000 µL, 200 µL, 20 µL, and 2 µL)

- Reagents:
  - GoTaq DNA polymerase (Promega Ref. M8295 for 1 pack, Ref. M8298 for large box)
  - GoTaq Flexi 5x Buffer – comes with DNA polymerase
  - GoTaq $MgCl_2$ – comes with DNA polymerase
  - Sterile, PCR-grade water (DNase-, RNase-free)
  - dNTPs 10 mM (Promega Cat. No. U1515)

- Primers
  - 515FY Forward Primers and 806RB Reverse Primers (10 µM)

- Consumables
  - PCR plates (96-well, 48-well, or 24-well), 0.2 mL PCR tubes, or PCR tube stripettes
  - 2 mL microcentrifuge tubes (Fisherbrand Cat. No. 05-408-129)
  - PCR hood
  - filter pipette tips (1000 µL, 200 µL, and 20 µL)
  - Heat seal (Eppendorf)

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Version History:

- originally written and revised by CAM over the years
- CAM: March 2023, added more detail, table, equipment etc list, added steps (e.g. when to turn on heat sealer, when to take out reagents).

Appendix 5.

Visualize results of a screen PCR

Protocol for visualizing PCR product using a 1% agarose gel

Apprill Lab

Protocol for visualizing PCR product using a 1% agarose gel

Laboratory of Amy Apprill office 508 289 2649 aapprill@whoi.edu

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1. Choose the appropriate size gel rig for the number of samples you want to visualize. Remember to leave one well at the beginning of each row for the ladder and two wells for your PCR controls. See **Table 1** of this protocol for details on how many wells each gel rig can hold.

   Hint for potentially difficult samples: you may want to run your positive control at the end of each row. If you will be running the extra-large gel, you may want to run the positive control in the middle and at the end of each row.

2. Make a 1% agarose gel. See recipes on page 3 of this protocol. Size will depend on the number of samples that need to be visualized.

   a. Weigh agarose on weigh paper, then pour into the clean flask
   b. Add 1X TBE buffer
   c. Add 10,000X SYBR Safe
   d. Mix together
   e. Heat flask in microwave until bubbles form. CAUTION: do not let it boil over. Repeat until fully dissolved – the mixture should be clear and pink

3. Let cool until it is warm (not hot) to touch for longer than 10 sec (about 55°C). You can run cool water along outside of flask while swirling to speed the cooling.

4. Pour the gel and let the gel solidify

5. When the gel is set (about 15-45 mins; will turn more translucent), fill the rig with freshly made 1X TBE buffer to the fill line and then take out the comb(s).

6. Pipette 1 µL loading dye per sample into a well of a 96-well plate or parafilm taped to the bench

7. For each sample, add 5 µL PCR product to the 1 µL of loading dye (pipetted in the previous step), and mix by pipette up and down a few times.
   Important: keep track of the order in which the samples are loaded!

Protocol for visualizing PCR product using a 1% agarose gel continued

8. When the PCR product from all samples has been mixed with the dye, then add each sample to a well (one sample per well). Remember to leave the first well of each row open for the ladder.

   Take care to not poke into/through the gel with the pipette tip when adding the sample to the well

9. Add the 3 µL DNA ladder to the first well of each row. If you haven't already, add the positive and negative controls from your PCR.

10. Attach the cords to begin running the gel. ALWAYS "RUN TO RED!", i.e. make sure the red, positive node is at the bottom of the rig (and gel). The phosphate backbone of DNA is negatively charged and when the electric current is added, it will move toward the positive (red) node. Hence, "run to red".

11. Run the gel for 40-60 min at 110 V and 60 mAmps

12. When the gel is finished, take it out of the rig and place on the INGENIUS UV gel viewer. Open GeneSys app on lab PC and go to "manual capture" to take a picture. Place gel on ethanol-cleaned deck, close door, and turn on UV light only for a short period to take a picture. UV light will slowly denature and degrade the DNA in your gel (think mutagen and cancer!) so only turn on when needed. Save picture as displayed.

13. Print out picture of gel and label the following on the picture:
    a. Date
    b. Sample type (seawater, coral, whale blow, etc…)
    c. Sample numbers
    d. Ladder size (50bp likely) and size of brightest ladder band
    e. amplicon target size
    f. number of PCR cycles
    g. Agarose gel %
    h. voltage and run time
    i. Any other important criteria you can think of

14. A successful PCR will have one single band at ~400bp for 515FY 806RB with Illumina adapter primers. There will be little amplification below the intended band size (measured relative to the 50bp DNA ladder), which are primer dimers.

    Please see images on page 4 for examples of good amplification and over-amplification

Protocol for visualizing PCR product using a 1% agarose gel continued

Table 1. Gel rig details

Gel Rig Size	# of wells / comb	# of combs	Total # wells / gel
Small	10	2	20
Medium	14	2	28
Large	20	4	80
Extra Large	34	3	102

Recipes for gels for PCR product visualization – 1% agarose

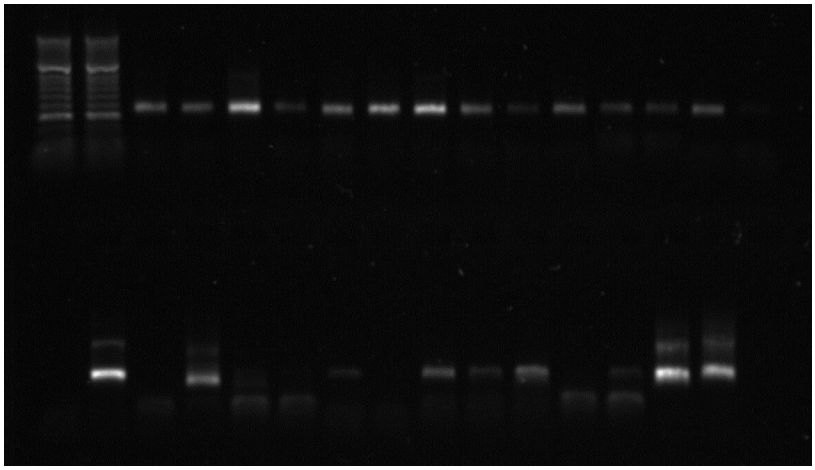
Small Gel – 1%
0.28 g agarose
28 mL 1x Buffer
2.8 µL 10,000x SybrSafe Dye

Medium Gel – 1%
0.5 g agarose
50 mL 1x Buffer
5.0 µL 10,000x SybrSafe Dye

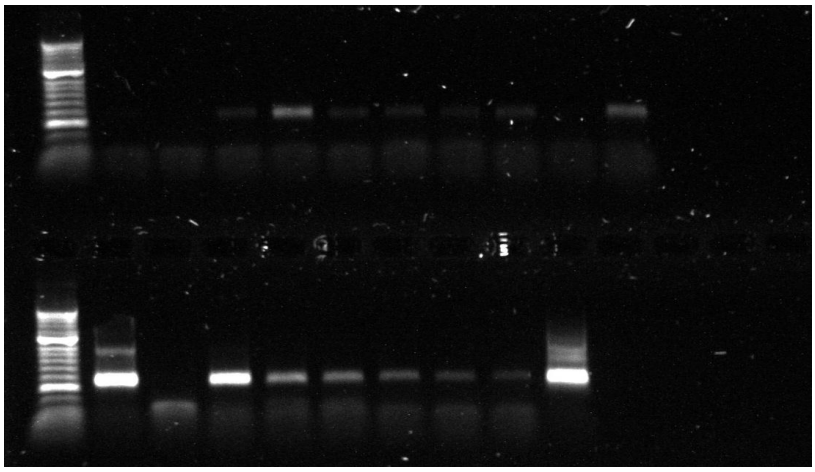
Large Gel – 1%
0.84 g agarose
84 mL 1x Buffer
8.4 µL 10,000x SybrSafe Dye

Largest Gel – 1%
1.0 g agarose
100 mL 1x Buffer
10 µL 10,000x SybrSafe Dye

Protocol for visualizing PCR product using a 1% agarose gel continued



Most of the amplicons along the top row of the gel are suitable for generating the library (while they range in band brightness, they contain no double bands, minimal primer dimers, and are not "smeary" in appearance). Most of the amplicons along the bottom row of the gel contain double/bands and are smeared in appearance. The three middle amplicons along the bottom row (circles) are suitable for purification.



Another example of over-amplified/ double bands in bar-coded PCR products. The first, third, and last amplicons along the bottom are not suitable for generation of a MiSeq library because they contain multiple bands and are over-amplified. However, the amplicons in-between these samples are suitable to be used.

- **For over-amplified samples**: Conduct PCR again using lower number of cycles to find sweet spot for each particular sample. May take a couple of tries – don't get discouraged!
- **For samples not amplified enough**: Increase the number of cycles of if you think there might be PCR inhibitors, dilute the template
- **For samples with multiple bands**: If they are not over amplified, you may proceed but you will likely have to purify with gel extractions

Protocol for visualizing PCR product using a 1% agarose gel continued

Equipment, Reagents, and consumables:

- Equipment:
  - Galileo gel rigs and INGENIUS UV-gel viewer
  - Microwave
  - Heater block for heat sealing
  - Pipettes (20 µL and 2 µL)
  - Hot gloves
  - Erlenmeyer flask

- Reagents:
  - 1X TBE Buffer (10X Tris-Borate-EDTA, Fisher BioReagents, 1L, BP1333-1)
  - Agarose
  - 10,000X SYBR Safe DNA gel stain (Invitrogen, Ref. S33102, 400 µL)
  - 5X Loading Dye (5X DNA Loading Buffer, blue; Bioline Cat. No. BIO-37045)
  - 50bp DNA Ladder (Bioline HyperLadder 50bp, 100 lanes)
  - PCR products from your PCR

- Consumables
  - White, opaque, bleached 96-well PCR plate from next to microwave
  - Filter pipette tips (20 µL)
  - Heat seal (Eppendorf)

Appendix 6.

Barcode PCR

PCR Barcode for MiSeq_02March2023.pdf

Protocol for Barcode PCR for MiSeq Library Preparation

Laboratory of Amy Apprill office 508 289 2649 aapprill@whoi.edu

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Equipment, reagents, primers, and consumables needed for this protocol are listed at end.

ALWAYS WEAR CLEAN GLOVES IN PCR HOOD

NEVER BRING DNA INTO PCR HOOD

1. Clean the PCR prep bench with the 10% bleach, then E-Pure water, followed by 70% alcohol

2. Clean bench of PCR hood (and inside of front window if needed) with the cleaning solution inside PCR hood.

3. Label the following in the PCR hood:
   a. PCR plate, strips, or tubes with name, date, ctrls (+) & (-), and sample #s for strips/tubes)
   b. Enough 2 mL microcentrifuge tube(s) for your volume of mastermix
   c. Enough 2 mL microcentrifuge tube(s) for water needed for mastermix – fill with DNA-free water

4. UV Sterilize the labeled items from step 3 as follows:
   a. Open tube(s) so light can sterilize inside the tubes
   b. Place the following very close to the UV bulb:
      i. PCR plate, strip(s), or tubes
      ii. open tube(s) of DNA-free water
      iii. tubes for mastermix
      iv. If you plan to use the multichannel pipette, put a reagent resevoir under the bulb as well
   c. UV sterilize for **15 MINUTES**

5. Thaw goTaq 5x flexi buffer and $MgCl_2$ (room temp is ok)

6. Vortex $MgCl_2$ & goTaq 5x flexi buffer well

7. Calculate volume of reagents for mastermix (see table 1 for recipe) based on the reaction volume you plan to run, the amount of DNA template you plan to use in that reaction, and the number of samples you plan to run plus some extra. Always make extra mastermix, especially if you plan to use the multichannel pipette and reservoir well. Also, adjust number of extras until the volume for the water is a whole number to make pipetting with the 1000 µL pipette easier and more precise.

8. Prepare first part of mastermix:
   a. Combine:
      i. H_20
      ii. goTaq 5x colorless flexi buffer
      iii. $MgCl_2$
   b. Vortex to mix, then tap to get any droplets off the lid and sides
   c. Sterilize under UV light for **10 MINUTES** (open lid and place very close to light)

9. Thaw dNTPs, and primers (room temp is ok). Return goTaq 5x flexi buffer and $MgCl_2$ to freezer

10. Thaw DNA at 4°C. Remember to also thaw a positive control.

11. Add barcode primers to **bottom** of each well such that each well has a unique combination of barcodes. **Carefully check pipette tip after aspirating and dispensing each primer!!**
    Be sure to change tips so as not to cross-contaminate your primers!!
    -For 25 µL reactions, add 0.5µL each of forward and reverse primers
    -For 50 µL reactions, add 1 µL each of forward and reverse primers

12. Set aside primer loaded PCR plate, strip(s), or tubes in PCR hood.

13. Take goTaq DNA polymerase out of freezer and place in freezer cooler block made for 2 mL tubes.
14. Finish preparing mastermix:
    a. Combine
        i. First part of mastermix made above
        ii. dNTPs
        iii. goTaq DNA polymerase
    b. Invert gently several times to mix
15. Aliquot mastermix into plate. The volume you will aliquot into each well will be determined by the size of reaction you planned and the volume of DNA template you plan to use (Table 1, total volume of mastermix).
16. For a negative control, aliquot H_20 into one well of your PCR plate or PCR tube. The volume you will aliquot will be the same as the volume of DNA template you plan to add.
17. Take PCR plate, strip(s), or tubes out of hood. If you have a plate, place it on the freezer cooler block made for 96-well plates. Use the freezer block that is labelled "PCR Prep" only!!
18. Turn on heat seal heater
19. Prepare to, and add DNA to wells:
    a. Fetch thawed DNA from the refrigerator
    b. Mix DNA samples (flick tube)
    c. Aliquot into wells or tubes
    d. Pipette up and down at least 5 times to mix DNA into mastermix.
    e. Add (+) control to one well
20. Heat seal PCR plate or close caps on tubes.
21. Centrifuge for about 15 seconds (3700 g)
22. Place plate in thermal cycler: MiSeq program. Edit to change the number of repeats (set to 1 less than total # cycles you want). Change volume to that of your reaction volume. Check to make sure the following settings are correct:
    a. Lid: 105°C
    b. Volume: 25 or 50 µL
        - 2 min 95°C
        - 20 sec 95°C
        - 15 sec 55°C
        - 5 min 72°C

        These steps repeat each cycle
        - # cycles
        - 10 min 72°C
23. Press run and then 'ok' to confirm the lid temp, volume, and thermocycler block
24. Store at 4°C short-term (<1 week) or -20°C long-term (>1 week)

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Table 1. Mastermix recipes and summary of all components of PCR reactions.

volume DNA template you are planning to add to the reaction:	For 50 µL reactions: 2 µL DNA	For 50 µL reactions: 4 µL DNA	For 25 µL reactions 1 µL DNA	For 25 µL reactions 2 µL DNA
Mastermix component	volume per sample (µL)			
H2O	29.5	27.5	14.75	13.75
buffer	10	10	5	5
MgCl2	5	5	2.5	2.5
dNTPs	1	1	0.5	0.5
polymerase	0.5	0.5	0.25	0.25
Total volume mastermix	46	44	23	22
forward primer - barcode 515FY	1	1	0.5	0.5
reverse primer - barcode 806RB	1	1	0.5	0.5
DNA template	2	4	1	2
Total volume of reaction (mastermix + primers+ DNA)	50	50	25	25

Equipment, reagents, primers, and consumables needed for PCR:

- Equipment:
  - Thermocycler
  - Centrifuge
  - Heater block for heat sealing
  - Pipettes (1000 µL, 200 µL, 20 µL, and 2 µL)

- Reagents:
  - GoTaq DNA polymerase (Promega Ref. M8295 for 1 pack, Ref. M8298 for large box)
  - GoTaq Flexi 5x Buffer – comes with DNA polymerase
  - GoTaq $MgCl_2$ – comes with DNA polymerase
  - Sterile, PCR-grade water (DNase-, RNase-free)
  - dNTPs 10 mM (Promega Cat. No. U1515)

- Primers
  - 515FY Forward Primers and 806RB Reverse Primers (10 µM)

- Consumables
  - PCR plates (96-well, 48-well, or 24-well), 0.2 mL PCR tubes, or PCR tube stripettes
  - 2 mL microcentrifuge tubes (Fisherbrand Cat. No. 05-408-129)
  - PCR hood
  - filter pipette tips (1000 µL, 200 µL, and 20 µL)
  - Heat seal (Eppendorf)

Protocol for Barcode PCR for MiSeq Library Preparation

Laboratory of Amy Apprill office 508 289 2649 aapprill@whoi.edu

Version History:

- originally written and revised by CAM over the years
- CAM: March 2023, added more detail, table, equipment etc list, added steps (e.g. when to turn on heat sealer, when to take out reagents).

Appendix 7.

Purification of barcoded amplicons via gel extraction

ApprillLab_Protocol for Gel Purification

Protocol for gel purification of PCR products

Laboratory of Amy Apprill office 508 289 2649 aapprill@whoi.edu

1. UV sterilize 2 mL microcentrifuge tubes for 15 minutes – one for each sample that you will be gel purifying.

2. Determine proper size gel and number of wells based on using 2 wells/sample with empty well on each side and remember to add one well of positive control and one well of ladder on the end of each end of the row and be sure to leave an empty well between these and your sample.

3. Make a 1.5% agarose gel of 1.5 times thickness. See recipes on next page. Size will depend on the number of samples that need to be purified.

    a. Weigh agarose on weigh paper, then pour into the clean flask
    b. Add 1X TBE buffer
    c. Add 10,000X SYBR Safe
    d. Mix together
    e. Heat flask in microwave until bubbles form. CAUTION: do not let it boil over. Repeat until fully dissolved – the mixture should be clear

4. Let cool until it is warm (not hot) to touch for longer than 10 sec (about 55°C). You can run cool water along outside of flask while swirling to help with the cooling.

5. Pour the gel and let the gel solidify

6. When the gel is set (about 15-45 mins; will turn more translucent), fill the rig with freshly made 1X TBE buffer to the fill line and then take out the comb(s).

7. Mix 15 uL product + 3 uL dye per sample, then add to a well. Do not poke through the gel with the pipette tip when adding to the well

8. Add your ladder (2 uL) and positive control (5 uL + 1 uL dye)

9. Attach the cords to begin running the gel. ALWAYS “RUN TO RED!” or make sure the red, positive node is at the bottom of the rig. The phosphate backbone of DNA is negatively charged and when the electric current is added, it will move toward the positive (red) node. Hence, “run to red”.

10. Run the gel for as long as possible so as to obtain good separation between the bands – typically about 1.5-2+ hours – at 110 V and 60 mAmps

11. Clean the gel viewer in the bottom drawer (not the UV box attached to the lab computer) with sterile alcohol wipes and let dry

12. When the gel is finished, take it out of the rig and place on the INGENIUS UV gel viewer. Open GeneSys app on lab PC and go to "manual capture" to take a picture. Place gel on ethanol-cleaned deck, close door, and turn on UV light only for a short period to take a picture. UV light will slowly denature and degrade the DNA in your gel (think mutagen and cancer!) so only turn on when needed. Save picture as displayed.

13. Transfer the gel to the gel viewer that was cleaned in the previous step

14. Set the tare on the balance for an empty pre-UVsterilized 2 mL microcentrifuge tube

15. Excise your bands: Choose correctly between the "junk" bands and the band with the appropriate number of base pairs ~380-393 bp for bar-coded Schloss primers) and combine the bands from the same sample into one 2 mL centrifuge tube.

    a. Excision Method 1:
        i. Use separate, clean gel extractor tools
        ii. Place opening over the two bands from the same sample
        iii. push extractor through the thickness of the gel
        iv. Pull extractor tool back up (should have the gel slice in it) and place over the opening over the labeled, sterilized and weighed 2 ml tube
        v. Press on bulb to release gel piece into the tube
        vi. Close tube and weigh

    b. Excision Method 2:
        i. Excise with a sterile single edge razor blade,
        ii. wiping with an alcohol wipe between samples
        iii. Put gel slice into the empty, sterile microcentrifuge tube.

16. Weigh the gel slice in the microcentrifuge tube (remember you have already set the tare on the balance so the reading of the weight should be the weight of your gel slice only) and record the weight in your notebook.

17. Purify the samples using either the Qiagen MinElute Gel Extraction Kit or the New England Biolabs Monarch Gel Extraction Kit. Follow the manufacturer's protocol for either except elute in 15 – 20 uL of elution buffer (depending on your needs and your anticipated concentrations.

NOTE: You can also pre-weigh all of your UV-sterilized 2mL microcentrifuge tubes

Recipes for gels for PCR product extractions (deeper wells) – 1.5% agarose of 1.5x thickness

Small Gel – 1.5%
0.63g agarose
42 mL 1x Buffer
4.2 uL 10,000x SybrSafe Dye

Add 400mL buffer to chamber

Medium Gel – 1.5%
1.125g agarose
75 mL 1x Buffer
7.5 uL 10,000x SybrSafe Dye

Add 600mL buffer to chamber

Large Gel – 1.5%
1.89g agarose
126 mL 1x Buffer
12.6 uL 10,000x SybrSafe Dye

Add 800mL buffer to chamber

Largest Gel – 1.5%
2.25g agarose
150 mL 1x Buffer
15 uL 10,000x SybrSafe Dye

Protocol for gel purification of PCR products continued

Equipment, Reagents, and consumables:

- Equipment:
  - Galileo gel rigs and INGENIUS UV-gel viewer
  - Microwave
  - Heater block for heat sealing
  - Pipettes (20 µL and 2 µL)
  - Hot gloves
  - Erlenmeyer flask

- Reagents:
  - 1X TBE Buffer (10X Tris-Borate-EDTA, Fisher BioReagents, 1L, BP1333-1)
  - Agarose
  - 10,000X SYBR Safe DNA gel stain (Invitrogen, Ref. S33102, 400 µL)
  - 5X Loading Dye (5X DNA Loading Buffer, blue; Bioline Cat. No. BIO-37045)
  - 50bp DNA Ladder (Bioline HyperLadder 50bp, 100 lanes)
  - PCR products from your PCR

- Consumables
  - White, opaque, bleached 96-well PCR plate from next to microwave
  - Filter pipette tips (20 µL)
  - Heat seal (Eppendorf)

Appendix 8.

Quantifying barcoded PCR product

ApprillLab_Protocol for quantifying PCR Product_v2

Protocol for quantifying PCR product – Qubit 2.0 Fluorometry

Laboratory of Amy Apprill office 508 289 2649 aapprill@whoi.edu

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Equipment:	Qubit 2.0 fluorometer	(2nd drawer down of DNA bench)
	Qubit assay tubes	(2nd drawer down of DNA bench)
	15 mL conical tube – clean	(Shelf opposite DNA bench)

Reagents: Qubit dsDNA HS Assay Kit reagents:

- dsDNA HS Buffer (top drawer of DNA bench)
- dsDNA HS dye (top drawer of DNA bench)
- dsDNA Standards #1 & #2 (refrigerator top left)

Protocol adapted from manufacturers protocol

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1. Label tubes:
    a. one tube for each sample
    b. one tube for Standard #1
    c. one tube for Standard #2
2. Do calculations for preparing the working solution:
    a. Calculate total number of preparations (x).

        x = # of samples + 2 (for 2 standards) + 2 (extra just in case)

    b. Calculate the volume of dsDNA HS Buffer (V_b) needed:

        $V_b = x * 199\ \mu L$

    c. Calculate the volume of dsDNA HS Dye (V_d) needed:

        $V_d = x * 1\ \mu L$

3. Prepare working solution by combining HS Buffer (V_b) and HS Dye (V_d) in a clean 15 mL conical tube
4. Prepare standards:
    a. Aliquot 190 µL of the working solution into each standard tube
    b. Add 10 µL of each standard to their respective tubes
    c. Vortex 2 seconds and set aside for 2 minutes before using
5. Prepare samples:
    a. Aliquot 199 µL of working solution into each sample tube
    b. Add 1 µL of each sample's PCR product to the respective tubes – Make sure to mix by flicking the tube before pipetting
    c. Vortex 2 seconds
    d. Set aside for 2 minutes before measuring

6. Plug in Qubit to turn it on
7. Select DNA
8. Select High Sensitivity (HS) dsDNA as the assay
9. Select 'Yes' for reading new standards
10. Final check of standards before measuring:
    a. Wipe tubes with the standards with a clean kim wipe
    b. make sure there are no bubbles (tap gently on the bench if there are)
    c. make sure there are no droplets on the side of the tube (tap gently on the bench if there are)
11. Read standards by following the prompts on the Qubit display
12. Final check of the samples before measuring:
    a. make sure there are no bubbles (tap gently on the bench if there are)
    b. make sure there are no droplets on the side of the tube (tap gently on the bench if there are)
    c. Wipe tubes with samples with a clean kim wipe
13. Measure samples:
    a. press "read new sample" on the Qubit display
    b. press 'Calculate stock concentration'
    c. make sure the units are set to ng/µL
    d. make sure the volume is set to 3 µL
    e. record the concentration in notebook
    f. repeat for all samples
14. Clean up:
    a. Assay tubes for both standards and samples can go into the autoclave waste
    b. Unplug Qubit and return to drawer
    c. Wipe down and tidy the bench

Ordering Information:
Qubit dsDNA HS Assay Kit (ThermoFisher Catalog#. Q32854)
Qubit assay tubes (ThermoFisher Catalog# Q32856)

Protocol for quantifying PCR product – Qubit 2.0 Fluorometry

Laboratory of Amy Apprill office 508 289 2649 aapprill@whoi.edu

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Version history:

- 30 April 2021, Carolyn Miller: Created protocol for Sam Bowman
- 14 September 2021, Carolyn Miller: modified quantifying DNA protocol for PCR product for using smaller volume (1 µL) of product
- 02 March 2023, Carolyn Miller: added ordering information