Standard Genetic Methods – Metabarcoding

Standard Operating Procedures

** New users must be trained by a technician or post-doc! **

Whenever in the lab, all individuals must wear shoes that completely cover their feet.

Whenever working with any chemicals, reagents, or DNA, all individuals must wear clean nitrile gloves.

***If there are any questions or concerns, please do not hesitate to ask Ruth DiMaria or Katrina Lohan.**

Changes to protocol in 2019:

1) ExoSAPIT step (of replicate PCR pool) was removed. Suspected to interfere with library sequencing success.

2) Multiple rounds of AmPure Bead cleaning may be necessary to remove small, unwanted fragments. Appropriate bead ratio is dependent on the size of the DNA fragment amplified for the project. You can always re-clean your samples to remove unwanted fragments, but you cannot gain back DNA that you lost during cleaning!

3) The final library pool was bead cleaned prior to submission for sequencing. The same bead ratio that was successful in Step 2 above was then used to clean the final library pool.

I. DNA EXTRACTIONS

- **WEAR CLEAN GLOVES**
- **WORK CAREFULLY USING AESEPTIC TECHNIQUE**
- **USE AEROSOL PIPETTE TIPS**

*Follow all safety precautions outlined in instruction manuals for the appropriate kit

*Choose kit appropriate for sample type – animal tissue, culture, sediment, water

*Always wipe down areas with diluted bleach (e.g., 10%) before beginning. If in an extraction room, first wipe down the surface with tap water before applying diluted bleach as a precaution against a negative reaction with any residual guanadine hydrochloride.

*This protocol is extremely sensitive and highly prone to cross-contamination. Thus, it is imperative that tubes and bottles not be left open.

1. For reagents, remove the lid, pipette out any liquid needed, and place the lid back on top. The lid does not have to be secured in place until it is no longer needed, but it must be covered when not imminently in use.
2. For samples, only open one tube at a time. Do not touch the inside of the tubes with gloves. If you do so, CHANGE GLOVES immediately.

*Auto-crosslink all tubes (if not already sterile from packaging) before beginning

*All waste (solid or liquid) containing guanidine hydrochloride should be disposed of as hazardous waste in an appropriate container

*When finished, always make separate aliquots (20-30uL) of DNA for downstream processing – store these aliquots in the pre-PCR fridge and the stock DNA in the freezer (-20)

II. DNA QUANTIFICATION

- **WEAR CLEAN GLOVES**
- **WORK CAREFULLY USING AESEPTIC TECHNIQUE**
- **USE AEROSOL PIPETTE TIPS**
- **This protocol is extremely sensitive and highly prone to cross-contamination. Thus, it is imperative that tubes and bottles not be left open.**
- **For samples, only open one tube at a time. Do not touch the inside of the tubes with gloves. If you do so, CHANGE GLOVES immediately.**

1) Before starting, mix all samples via vortex, then do a quick spin down on centrifuge to get liquid off the interior lid.
2) Follow the instructions on the Nanodrop for quantifying double-stranded DNA.
3) In your lab notebook, note the concentration (ng/uL) and the 260/280 ratio for each sample.

III. POLYMERASE CHAIN REACTION (PCR)

Repeat 3x each for all samples that are to be included in the metabarcode library

- **WEAR CLEAN GLOVES**
- **WORK CAREFULLY USING AESEPTIC TECHNIQUE**
- **DON'T USE TOO MUCH TEMPLATE DNA**
- **DON'T USE PCR PRODUCTS IN PCR PREPARATION AREAS**
- ***ALWAYS, ALWAYS* INCLUDE WATER AND VERY DILUTE POSITIVE CONTROLS IN EVERY EXPERIMENT**
- **USE AEROSOL PIPETTE TIPS**

- **Mix all samples via vortex, then do quick spin down on centrifuge to get liquid off the interior lid.**
- **MIX THE FOLLOWING REAGENTS VIA VORTEX: Buffer, MgCl2, primers.**
- **DO NOT vortex BSA or Taq.**
- **Only UV crosslink reagents that DO NOT contain any DNA sequence or enzyme: 10X PCR Buffer, MgCl2, pure H2O**

***This protocol is extremely sensitive and highly prone to cross-contamination. Thus, it is imperative that tubes and bottles not be left open.**

a. **For reagents, remove the lid, pipette out any liquid needed, and place the lid back on top. The lid does not have to be secured in place until it is no longer needed, but it must be covered when not imminently in use.**
b. **For samples, only open one tube at a time. Do not touch the inside of the tubes with gloves. If you do so, CHANGE GLOVES immediately.**

1) Always put on a fresh pair of gloves before going anywhere near the PCR bench.

2) All users should use aliquoted reagents (dNTP's, primers, etc.)—that way if you contaminate your aliquots, you have not contaminated the concentrated stock. Note which reagents are currently in use with some mark on the top of the tube.

3) PCR water is purchased from Qiagen – it is DNAse and RNAse free. To avoid contamination, it is kept in the Marine Disease Ecology lab, not the Ecological Genomics Core. Make sure to auto-crosslink water aliquots before adding additional reagents to the mixture.

4) All PCR prep should be performed in the DNA extraction room (Mordor) or the hoods in Rivendale. First, make a pooled master mix of all the reagents without the DNA using DNA-free PCR-dedicated pipettes, then dispense to individual tubes, and finally add DNA to individual reactions, using different pipettes.

5) After PCR has been performed, none of the reaction products should go near the pre-PCR areas and never use PCR designated pipettors for post-PCR pipetting.

6) Observe proper operation of the pipettes on the PCR bench. If solution is sucked up into the pipette tip too fast, the pipette itself can become contaminated (it is for this reason that aerosol tips are incorporated).

7) **ALWAYS INCLUDE A NEGATIVE CONTROL AND A VERY DILUTE POSITIVE CONTROL IN EVERY EXPERIMENT**.

    a. The negative controls should be an indication of no contamination (use sterile water or additional PCR mixture instead of DNA).

b. The positive control should only use 1-5 ng of DNA.
c. Store the positive control aliquots and stock in separate containers from the DNA samples being processed.

8) The *Taq* polymerase should never be left out at room temperature. It should either stay in the freezer or in a freezer box.

9) Make sure to use the correct ingredients for the marker that is to be amplified and the number of samples to be processed.

10) General practice: record the thermocycler used and the start time for the program protocol.

IV. GEL ELECTROPHORESIS

Repeat for all amplifications (x3) for each sample

- **WEAR CLEAN GLOVES**
- **WORK CAREFULLY USING AESEPTIC TECHNIQUE**
- **USE AEROSOL PIPETTE TIPS**
- **ALWAYS USE A STANDARD DNA LADDER ON EVERY ROW OF THE GEL**
- **TO AVOID ELECTROCUTION, TURN OFF THE APPARATUS IF THERE IS ANY SPILLED LIQUID. DO NOT USE DAMAGED RIGS.**
- **For samples, only open one tube at a time. Do not touch the inside of the tubes with gloves. If you do so, CHANGE GLOVES immediately.**

*Continue to use aerosol tips only. At this point, the samples do not have unique bioinformatic barcodes that can be used to tell them apart, so it is imperative that precautions be taken to avoid any cross- or aerosolized- contamination.

*This step is done to confirm that the amplification protocol was successful with the presence of a band on the gel that is the correct size.

1) Assemble a gel rig that will accommodate the number of samples that need to be analyzed. One well per row will need to hold DNA ladder. For the X-Large rig (50-well combs), two wells per row should be used (first and last well) as samples on either ends of large gels can move at slightly different rates.

2) We re-use gels up to 3x. Check the fridge to see if a gel exists – if so, then microwave until the agarose is melted.

    a. If there is no gel in the fridge, mix the appropriate amount of agar (labeled MDE) and clean TBE buffer (in autoclave jars by the sink) to generate a 2% w/v agarose gel that will be large enough for all the samples required.
        a. Gel Density calculated as % = weight (g) / volume (mL)

b. We use TopVision Agarose Tablets. Follow the directions on the box for 2% gel.
c. See table below for calculations to mix 2% gel for different sized rigs and how much Gel Red to add.

Rig	TBE	Agarose	# Tablets	GelRed
X-SM	25 mL	0.5 g	1	1uL
SM	50 mL	1.0 g	2	1uL
MED	100 mL	2.0 g	4	2-3uL
X-LG	200 mL	4.0 g	8	3-5uL

*each tablet = 0.5 g; Pre-soak 4min in buffer before heating

b. Once the mixture is melted, DO NOT pour into the gel rig until the Erlenmeyer flask is cool enough to hold on the palm of your hand (~52ºC) otherwise you risk permanently warping the gel mold.
c. Add the appropriate amount of GelRed for the size gel being poured and gently swirl to mix before pouring. Easier to only add the amount of GelRed you need for a single run.
d. Use the bubble level to check that the gel rig setup is level before pouring the mixture into the mold.

3) Once poured, allow to cool to solid (~15 minutes).

4) Mix 3uL-5uL of PCR product (aerosol tips only) with 2uL of loading dye for each sample on a piece of parafilm. Make sure to use the same loading dye for all samples (purple and blue can run at different rates; purple dye comes with the DNA ladder kit and is limited quantity).

5) Once cooled, remove the comb, turn the rig so that DNA will move toward the positive electrode. Fill the rig with TBE buffer so that the agarose gel is completely submerged.

   a. Use a disposable dropper pipette to flush any air bubbles out of the wells before loading samples. Dropper can be reused.

6) Carefully, load all the samples and the ladder into the individual wells. You can use non-filter tips for this step. Do not stab through the gel. Do not pipette air bubbles, which will cause the DNA to come out of the well. Make sure there is one well available for the DNA ladder (at beginning or end of row). For the large gel rig (50 samples/comb), load DNA ladder in the first and last wells of each row.

7) Add 2-3uL DNA ladder (6X; 1:100 dilution) to each row of samples.

8) Put the cover on and turn on the electric current – run at 120 volts for ~35 minutes, depending on the size of the fragment (90 volts for ~50 minutes minimizes “smiling” bands). Check to make sure the loading dye has run far enough down the gel rig before turning off.

9) Turn off the electric current, then remove the lid.

10) Take a picture of the gel using the Multi-Doc IT. Save these pictures in the appropriate project folder within the MDE folder on the shared drive.

    a. Gel can either be saved and stored in 4ºC post-PCR fridge (we use up to 3x), or thrown away in general trash flow.

***TO AVOID UV DAMAGE TO EYES OR SKIN, DO NOT LOOK DIRECTLY AT UV LIGHT OR DISABLE ANY SAFETY SETTINGS ON THE MULTI-DOC IT.**

V. PCR POOLING

- **WEAR CLEAN GLOVES**
- **WORK CAREFULLY USING AESEPTIC TECHNIQUE**
- **USE AEROSOL PIPETTE TIPS**

1) Using gel images generated post-electrophoresis, compare the relative brightness of each band for the same replicate across all three replicates.

2) Based on the comparison across all three replicates, first write out the required volume to be pooled from each replicate.

    a. Band brightness generally has three levels: bright, faint, absent

    b. When all bands have the same brightness (regardless of the level): take 5uL from each replicate

        Ex. R1 = Bright (5uL), R2 = Bright (5uL), R3 = Bright (5uL)

        Ex. R1 = absent (5uL), R2 = absent (5uL), R3 = absent (5uL)

    c. When all bands are not equal brightness, then take 10uL from the fainter replicates and pool with 5uL from the brighter replicates

        Ex. R1 = faint (5uL), R2 = absent (10uL), R3 = absent (10uL)

3) Obtain, label, and cross-link new strip tubes.

4) Using new pipette tips with each replicate (aerosol only), pipette the replicates so that the appropriate volume from all three replicates is put into the same tube.

VII. INDEXING PCR

- **WEAR CLEAN GLOVES**
- **WORK CAREFULLY USING AESEPTIC TECHNIQUE**
- **DON'T USE TOO MUCH TEMPLATE DNA**
- **USE AEROSOL PIPETTE TIPS**
- **Mix all samples via vortex, then do quick spin down on centrifuge to get liquid off the interior lid.**

***This protocol is extremely sensitive and highly prone to cross-contamination. Thus, it is imperative that tubes and bottles not be left open.**

a. **For reagents, remove the lid, pipette out any liquid needed, and place the lid back on top. The lid does not have to be secured in place until it is no longer needed, but it must be covered when not imminently in use.**
b. **For samples, only open one tube at a time. Do not touch the inside of the tubes with gloves. If you do so, CHANGE GLOVES immediately.**

1) Prep work for indexing PCRs must be performed in the post-PCR space even though the bioinformatic barcodes have not been added to distinguish the individual samples. **Thus, use caution when mixing.**

2) Always put on a fresh pair of gloves.

3) Make sure each sample is indexed with a unique combination of i5 and i7 primers (dual-indexed). There is a template available to help with this step (ask Katrina or Ruth).

4) We use KAPA HiFi HotStart reagents for this protocol using the following combination of reagents:

    a. Use 1uL of pooled product as template in the indexing PCR.

    b. Indexing PCR done in 25uL total reaction volume using KAPA Ready Mix.

    c. 9.5uL water (volume to 25uL), 12.5uL KAPA ReadyMix (1x final concentration), 1uL of 10uM forward and reverse primers (indexing primers – 0.4uM final concentration), 0.5uL of template (pooled amplicons).

    NOTE: Due to high concentrations of unindexed primers in libraries, modifications moving forward include decreasing volume of template (from 1 to 0.5uL) and slightly increasing number of cycles (from 4 to 8)

95° 5 min
98° 20 sec
60° 45 sec } **x 8** **(# cycles varies depending on input)**
72° 45 sec
72° 5 min

d. The PCR parameters for indexing are still in flux and depend on how much material you have. Katrina greatly reduced them compared to what individuals do with genomic DNA.

e. PCR water is purchased from Qiagen – it is DNAse and RNAse free. To avoid contamination, it is kept in the Marine Disease Ecology lab, not the Ecological Genomics Core. Aliquots are made under the clean bench on the first floor and taken to the EGC. Make sure to auto-crosslink water aliquots before adding additional reagents to the mixture.

5) Use strip tubes to create a master mix for each i5 and i7 primer. The template to calculate reagent volumes for each master mix is included with the template used to assign unique a barcode combination to each sample.

6) Use a multi-channel pipette to dispense the master mixes into the appropriate reaction tube. Add 12uL of each i5 and i7 master mix to each reaction tube, following the template to track the combination of barcodes used for each sample (12uL i5 master mix + 12uL i7 master mix + 1uL template = 25uL total reaction).

7) Add DNA to individual reactions as the last step.

8) Observe proper operation of the pipettes. If solution is sucked up into the pipette tip too fast, the pipette itself can become contaminated (it is for this reason that aerosol tips are incorporated).

9) General practice, record the thermocycler used and the start time for the program protocol.

VIII. GEL ELECTROPHORESIS

Perform as described above (Part IV), with one addition – for each row in the gel, include both a ladder and a non-indexed control. This is to ensure that the pre- and post-indexed samples are different sizes (should be ~60bp different). *****1uL product used to load gel to check indexing**

IX. PURIFICATION WITH AMPURE BEADS

- **WEAR CLEAN GLOVES**
- **WORK CAREFULLY USING AESEPTIC TECHNIQUE**
- **USE AEROSOL PIPETTE TIPS**
- **Mix all samples via vortex, then do quick spin down on centrifuge to get liquid off the interior lid.**

***This protocol is extremely sensitive and highly prone to cross-contamination. Thus, it is imperative that tubes and bottles not be left open.**

***For reagents, remove the lid, pipette out any liquid needed, and place the lid back on top. The lid does not have to be secured in place until it is no longer needed, but it must be covered when not imminently in use.**

1) Obtain, label, and cross-link new strip tubes.

2) Follow the manufacturer's protocol for the AMPureXP beads. Remember that the ratio of beads used depends on the size of the DNA fragment that you are targeting for the project. You can always re-clean your samples to remove unwanted small fragments, but you cannot gain back DNA that you lost during cleaning. You may need to clean your samples multiple times.
    a. A 1.8X bead ratio will remove fragments 100bp and lower
    b. A 0.8X bead ratio will remove fragments 300bp and lower

3) Start with the manufacturer protocol using 1.8X – 1.5X bead ratio and 10uL PCR product.
    a. Use a clean trough and multi-channel to add the AMPureXP bead mix to the strip tubes. Only pour out into the trough as much as you need for the reactions you will be cleaning at the time.
    b. You may need to re-clean the samples (adjusting the bead ratio as necessary) if gel images show that multiple bands were not removed.

4) Aliquots of AMPureXP beads are stored in the pre-PCR fridge.

5) Make fresh 70% ethanol in a 50mL tube. Label the tube and parafilm the lid when not in use.

6) Magnet is in the MDE roller unit beneath the post-PCR workbench. Two magnet models are available: one to use with strip tubes and one to use with eppendorph tubes. **DO NOT USE MAGNET IF YOU HAVE A PACE MAKER!!!**

X. GEL ELECTROPHORESIS

Perform as described above (Part IV). This is to ensure that all small fragments have been removed with the AMPureXP bead clean-up. *****1uL cleaned product used to load gel**

You will need to re-clean any samples that have multiple gel bands as described above (Part IX). Adjust the bead ratio as necessary and re-run a gel to verify that unwanted small fragments have been removed before proceeding further.

XI. QUANTIFICATION WITH QUBIT

- **WEAR CLEAN GLOVES**
- **WORK CAREFULLY USING AESEPTIC TECHNIQUE**
- **USE AEROSOL PIPETTE TIPS**
- **Mix all samples via vortex, then do quick spin down on centrifuge to get liquid off the interior lid.**

1) Open only one tube at a time when conducting this protocol to avoid any cross-contamination

2) Follow the manufacturer's instructions for the Qubit High Sensitivity double-stranded DNA kit.

3) Record concentrations for each sample in nM or pM (preferably) or ng/uL if that is the only option. UPDATE 2019, ng/uL is preferred for pooling calculations.

XII. POOLING CALCULATIONS FOR FINAL LIBRARY

This step is all done on a computer within an excel spreadsheet – ask Katrina or Ruth for the template for these calculations.

Based on the concentrations from the Qubit for each sample, you need to correct for the fragment size, then determine the volume required for each sample to reach a final concentration of >10nM in at least 10uL for the entire library (though larger volumes and more concentrated are permitted).

- If the fragments in your library are the same size, you can pool based on equal concentrations.
- If the fragments in your library are different sizes, pool based on **equimolar** concentrations.

1) It is important to note that you should try to get the calculations so that you do not pipette less than 2uL per sample, as pipettes are less reliable with small volumes.

2) For negative controls, add 1uL for each. No calculations are necessary.

3) For samples whose Qubit reading was "Too Low," pool the entire sample volume.

XIII. CREATING FINAL LIBRARY POOL

- **WEAR CLEAN GLOVES**
- **WORK CAREFULLY USING AESEPTIC TECHNIQUE**
- **USE AEROSOL PIPETTE TIPS**
- **Mix all samples via vortex, then do quick spin down on centrifuge to get liquid off the interior lid.**

1) Label a single eppendorph tube with the name of the library, auto-crosslink this tube. The size tube needed will depend on the estimated final volume in the calculations from step XII (1.5mL and 2.0mL round-bottom tubes are available).

2) Using the calculations from step XII, pipette the corresponding volume for each sample into the tube.
    a. Since beads are not removed from product from step IX, use the strip tube magnet to separate beads before pipetting.

    b. Magnet is in the MDE roller unit beneath the post-PCR workbench. Two magnet models are available: one to use with strip tubes and one to use with eppendorph tubes. **DO NOT USE MAGNET IF YOU HAVE A PACE MAKER!!!**

3) If necessary once pooling is complete, reduce the final volume using a speed-vac (machine in RNA extraction room).

XV. PURIFICATION OF FINAL LIBRARY WITH AMPURE BEADS

- **WEAR CLEAN GLOVES**
- **WORK CAREFULLY USING AESEPTIC TECHNIQUE**
- **USE AEROSOL PIPETTE TIPS**
- **Mix all samples via vortex, then do quick spin down on centrifuge to get liquid off the interior lid.**

***For reagents, remove the lid, pipette out any liquid needed, and place the lid back on top. The lid does not have to be secured in place until it is no longer needed, but it must be covered when not imminently in use.**

We have had success cleaning the final library pool with AMPureXP beads (i.e., second bead-clean) prior to submission for sequencing.

1) Label a single 1.5mL tube with the name of the library (indicate this is the final library to submit for sequencing), crosslink this tube.

2) Follow the manufacturer's protocol for the AMPureXP beads. **Use the same bead ratio to clean the final library pool that was used during the initial cleaning** (Perform as described in Part IX above).

3) Bead clean a subset of the pooled library from step XIII.
    a. The subset volume you choose will depend on the initial pooled volume available from step XII. For example, for previous libraries with initial pool volume at 1000uL, a subset of 100uL-500uL was used to create the final library to submit for sequencing.
    b. Use an eppendorph tube and the tube magnet plate.
    c. If your estimated final molarity is low (<10nM) from the calculations in step XII, bead clean a larger volume and then elute the final library to a smaller volume, thereby concentrating the final library for submission (e.g., bead clean 500uL and elute to 50uL).

4) Make fresh 70% ethanol in a 50mL tube. Label the tube and parafilm the lid when not in use.

5) Magnet is in the MDE roller unit beneath the post-PCR workbench. Two magnet models are available: one to use with strip tubes and one to use with eppendorph tubes. **DO NOT USE MAGNET IF YOU HAVE A PACE MAKER!!!**

6) Qubit to check that the molarity of the final cleaned library is ≥4nM. For LAB MiSeq submissions using the v3 kits we need to prepare at least 10uL of the final library sample with final concentration ≥4nM. You can submit the final library at a higher concentration (it is easier for the sequencing techs to dilute a library than to concentrate).

7) Parafilm wrap the tube and freeze (-20ºC post-PCR freezer).

Notes on submitting your library for sequencing:

a. Before shipping for sequencing, the library should be completely frozen.
b. You will need to submit the required documentation to the sequencing core. Ask Ruth or Katrina for details.
c. Always ship via FedEx overnight, either on dry ice or surrounded by ice packs.
d. Before shipping, check that the recipient mail system does **not** irradiate packages.
e. Coordinate with Ruth if sending to LAB.
f. Copies of final submission details are stored on the shared drive: S:\MarineDiseaseEcology\Projects\ALL PROJECTS_LAB_MiSeq Submissions