Standard Genetic Methods – Sanger Sequencing

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 and are responsible for understanding any hazards associated with those chemicals (SDS).
Wear gloves when working with bleach and work in a well-ventilated area.

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

I. DNA EXTRACTIONS

- **WEAR CLEAN GLOVES**
- **WORK CAREFULLY USING ASEPTIC 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 (10%) before beginning. First wipe down the surface with tap water before applying diluted bleach as a precaution against a negative reaction with any residual guanidine hydrochloride.

*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

*When finished, always make separate aliquots (20-30uL) of DNA for downstream processing – store these aliquots in the pre-PCR fridge (4ºC) and the stock DNA in the freezer (-20ºC)

II. DNA QUANTIFICATION

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

1) Follow the instructions on the Nanodrop for quantifying double-stranded DNA.
2) In your lab notebook, note the concentration (ng/uL) and the 260/280 ratio for each sample.

III. POLYMERASE CHAIN REACTION

- **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 mix BSA or Taq.**
- **Only UV crosslink reagents that DO NOT contain any DNA sequence or enzyme: 10X PCR Buffer, MgCl2, pure H2O**

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 (e.g., date first opened).

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 UV-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. Make pooled master mix of all the reagents without the DNA using DNA-free PCR 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) Record the thermocycler used and the start time for the program protocol.

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

IV. GEL ELECTROPHORESIS

- **WEAR CLEAN GLOVES**
- **WORK CAREFULLY USING AESEPTIC TECHNIQUE**
- **DO NOT 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.**

*Once PCR is complete, unless samples are to sequenced via metabarcoding (if so, see Metabarcoding SOP), then use non-filter pipette tips for all post-PCR steps to reduce costs.

*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) Assembly 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 (a.k.a. smiling).

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 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. 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 ~50min 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 PURIFICATION WITH EXO-SAP

- **WEAR CLEAN GLOVES**
- **WORK CAREFULLY USING AESEPTIC TECHNIQUE**
- **DO NOT USE AEROSOL PIPETTE TIPS**
- **Concentrated EXO-SAP IT should be stored in the freezer. Diluted EXO-SAP IT must be stored in the refrigerator!**
- **Mix all samples via vortex, then do quick spin down on centrifuge to get liquid off the interior lid.**

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

2) Ensure that you are using already diluted (1:10) EXO-SAP IT --- if not dilute some.

    a. To make this dilution, mix 5uL of EXO-SAP IT (from the freezer) with 45uL of PCR grade water (from the hood) in a new, clean 1.5 mL centrifuge. Label this tube 1:10 diluted EXO-SAP IT and the date it was made and store in the fridge (4ºC pre-PCR).

3) For every sample, mix 1uL of diluted EXO-SAP IT for up to 10uL of PCR product in the newly labeled strip tubes. (You can use less PCR product if for some reason you don't have that much.)

4) Run on thermocycler with EXO-SAP IT protocol

    a. 30-40 minutes at 37ºC, 10-15 minutes at 80ºC, 12ºC forever.

    b. Record the thermocycler used and the start time for the program protocol.

VI. CYCLE-SEQUENCING

- **WEAR CLEAN GLOVES**
- **WORK CAREFULLY USING AESEPTIC TECHNIQUE**
- **DO NOT USE AEROSOL PIPETTE TIPS**
- **Mix all samples via vortex, then do quick spin down on centrifuge to get liquid off the interior lid.**

- **Make sure primers are diluted to 10 uM**

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

2) Remember to create two master mixes – we sequence in both directions, so you need one forward master mix (with only the forward primer) and a reverse master mix (with only the reverse primer).

   1X reaction using the v3.1 Big Dye Terminator kit:

   a. 1.5 uL of 10X sequencing buffer (stored in 4C fridge)
   b. 0.7 uL of Big Dye
   c. 0.3 uL of 10 uM primer (*one primer per master mix)
   d. 6.5 uL of PCR water (UV cross-linked before adding to master mix)

3) Leave the BigDye in a cold block or on ice while making the Master Mix.

4) Make sure to set-up this reaction in a post-PCR area.

5) Once set-up is complete, run the SEQ program on the thermocycler. Record the thermocycler used and the start time for the program protocol.

VII. SEQUENCE CLEAN-UP WITH SEPHADEX

- **WEAR CLEAN GLOVES**
- **WORK CAREFULLY USING AESEPTIC TECHNIQUE**
- **DO NOT USE AEROSOL PIPETTE TIPS**
- **NOTE: Sephadex is a very slippery powder that is also very expensive, so we keep it in a plastic container in order to avoid spillage. Change gloves once done handling the Sephadex as your gloves will be slippery.**

This step removes all remaining primers and dideoxy nucleotides from the cycle sequencing products, which can cause problems during sequencing and make for some messy looking chromatograms.

1) Sephadex must first be hydrated before it can be used. Also, we re-use the Millipore plates (which are very expensive) for Sephadexing. Make sure to rinse out any used Millipore plate with DI water before adding Sephadex and hydrating.

   a. If hydrating overnight, place plate with wet paper towel in a plastic bag and put it in the fridge.
   b. Can clean used Millipore plates by filling wells with DI water and then centrifuge. Make sure plates are balanced before starting the centrifuge.

2) Instructions for volume to add, centrifuge programs, etc. are located in the container with the Sephadex. Follow these instructions carefully, taking special note of the requirement for an appropriate balance at each step.

3) Once the final step in complete, cover with a foil lid and place in the freezer until time to take to MSC.

VIII. STANDARD RECIPIES AND STOCKS

All stocks made with fresh aliquots of PCR-grade water.

Primer Hydration:

- **WEAR CLEAN GLOVES**
- **WORK CAREFULLY USING ASEPTIC TECHNIQUE**
- **ONLY USE AEROSOL PIPETTE TIPS**
- **WORK UNDER THE CLEAN BENCHES (RIVENDALE)**
- **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.**

When primers are ordered from a company, they arrive in a dry state (referred to as "dried down") and must be reconstituted before use to 100uM.

To create 100uM primer stocks, you must add the appropriate amount of pure water to each tube. The volume of water will differ for each primer.

> Reminder: PCR_grade water is purchased from Qiagen (DNAse and RNAse free) and kept in the Marine Disease Ecology Lab (Mathias 1051).

Remove 50-100 μL aliquots for individual use. Do not freeze-thaw the stock each time as degradation will eventually occur. A conventional 25 μL PCR generally uses 0.5 μL of each primer**.**

1) Before beginning, label the top of each stock tube with the primer name and "100uM"

2) Using a personal centrifuge, centrifuge all the tubes for ~5seconds. This will ensure that all of the primer is located at the bottom of the tubes.

3) Water used for hydration MUST be clean!!! UV crosslink the pure water specifically set aside for primer hydration (in sterile hood of Rivendale). You MUST use filter tips and a new tip for each tube containing primer.

   a. NOTE: For the UV crosslinker to be fully effective, plastics and reagents should be opened before being placed into the machine. Screw caps should be placed with the inside of the cap facing up. Close all tubes and reagents immediately upon removal from the machine.

4) On the individual tubes, the nmol amount is listed. You must add 10x the amount of water as the nmol listed to create a 100uM stock.

   a. Example: if a primer is listed as being 18.8nmol, then you add 188uL of water to create 100uM stock

5) Once the water is added, vortex at maximum speed for ~5 seconds to homogenize the solution

6) Aliquot 20-30uL into a 0.5mL tube, which should be UV crosslinked before filling and labeled with the primer name and 100uM solution.

7) Store working primer aliquots at 4ºC and primer stocks at -20ºC.

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

Primer Aliquoting:

For PCR, we are using 100uM primers. For sequencing, we are using 10uM primers.

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- **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) To aliquot primers, get a new 0.5mL tube and put it in the UV crosslinker.

   a. NOTE: For the UV crosslinker to be fully effective, plastics and reagents should be opened before being placed in to the machine. Screw caps should be placed with the inside of the cap facing up. Close all tubes and reagents immediately upon removal from the machine.

   b. DO NOT put the primers in the crosslinker – they will be destroyed!

2) In the sterile hood in Rivendale, thaw the primer stock solution. The tube must be completely thawed before an aliquot is taken.

3) Vortex the thawed solution and then quickly spin down the in the personal centrifuge to remove liquid from the sides and inside the cap.

4) Using filter tips, pipette 20-30uL of primer solution into the clean 0.5mL tube. Label the top of the tube with the name of the primer and "100uM."

5) Return the stock to the -20ºC pre-PCR freezer. Store the primer aliquot at 4ºC.

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