Amplification and Sequencing of Amplicons

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Amplicon sequencing targets a specific region of DNA which is known to be variable. Using specific primers, we are able to amplify this target region and increase the amount of DNA from it. By carefully choosing a target region which is conserved among our taxa of interest (e.g., bacteria, symbiodiniaceae), we can use these sequences to analyze the community composition.

Materials:

5x Phusion polymerase buffer
10 uM (2.5 uM each) dNTPs
2 uM forward primer (diluted from stock)*
2 uM reverse primer (diluted from stock)*
Phusion DNA polymerase
MilliQ water

* Use primers corresponding to the region you want to amplify. For symbionts, this is the ITS2/SYM_VAR primers. For bacteria, the 16S V3/V4 primers.

1. Before starting, quantify the DNA concentration of your samples. Initially, this can be done with nanodrop, as we will PicoGreen all samples prior to sequencing.
2. Dilute all samples to 10 ng/uL. To do this, use the equation C1*V1 = C2*V2. For small numbers of samples you can do this by hand in your lab notebook. However, when processing more than ~20 samples, I strongly recommend you do this in Excel, print it, and take it to the lab with you.

Sample ID	Starting Conc (C1)	Input Volume (V1)	Desired Conc (C2)	Final Volume (V2)	H2O Volume
Coral 1	131.9 ng/uL	5 uL	10 ng/uL	65.95 uL	60.95 uL

3. Based on the number of samples you have, make your PCR master mix. Be sure to include excess master mix for pipetting error and a negative control.

   (# Samples + 1 negative control) x 1.1 = # Error adjusted samples

   Note: Add the reagents in the order listed here. This will help you pipette smaller volumes and ensure we use the cheapest reagents first in case there is a mistake

Reagent	Amount/Sample
MilliQ (or nuclease free) water	8.4 uL
5x Phusion polymerase **buffer**	4 uL
2 uM forward primer	1 uL
2 uM reverse primer	1 uL
10 uM dNTPs	0.4 uL
Phusion DNA polymerase	0.2 uL
Total Volume	**15 uL**

4. After making your master mix, mix by flicking it (the polymerase is fragile, so you do not want to vortex). Spin it down in the tabletop centrifuge. Pipette 15 uL in each sample well + 15 uL in the well for your negative control.
5. Add **5 uL of DNA @ 10 ng/uL** to each sample well.
6. Add **5 uL of MilliQ water** to the negative control well.
7. After preparing your sample wells and negative control, make one "no primer" (NP) control. This is to ensure that we aren't getting nonspecific amplification in our PCR/haven't contaminated our samples. To make the NP control, add the following **directly into an empty well**.

Reagent	Amount
MilliQ (or nuclease free) water	10.4 uL
5x Phusion polymerase **buffer**	4 uL
10 uM dNTPs	0.4 uL
Phusion DNA polymerase	0.2 uL
Total Volume	**15 uL**

\+ **5uL DNA @ 10ng/uL from any 1 sample**

8. Mix wells by flicking and spin down. If you are preparing an entire plate, use the plate vortex **on its lowest setting (1-2)**, then spin down using the plate centrifuge in the gel room. *Note: the plate centrifuge is incredibly sensitive to imbalances, so you will need to make another plate containing water of exactly the same weight to use it.*

9. After you have spun down your samples, run them in the thermocycler using the following program (currently in the “CARLYNEW” folder, called “SYM_AMP”). Be sure to check the program to make sure it follows the correct time and number of cycles - sometimes the programs get edited by other users.

Time	Temp	Cycles
1 min	98 C	
30s	95 C	
30s	56 C	x20
30s	72 C	
5 min	72 C	

10. Set a timer to know when the PCR finishes.
11. While the PCR runs, cast a 1.5% gel to view your product.

    Gel Recipe:

1x TE	35 mL
Agarose	0.5 g
Gel Red	2 uL

- Mix the 1xTE and agarose together in a beaker. Microwave for 45s. Swirl. Microwave for 15s.
- Cool the mixture by swirling in water for appx. 15s.
- Add 2 uL gel red. Swirl.
- Pour into the casting rig. Don’t forget the combs!
- Let set in the dark for 20-30 minutes until firm to the touch.

12. After the PCR finishes, visualize your product on a gel to ensure successful and even amplification. Leave your sample at room temperature until you have viewed the gel! If processing an entire plate, randomly pick 12-24 samples to view on the gel.
    a. Run the gel at 95V for 25-30 minutes.
    b. Load 2 uL each sample + appx. 1 uL loading dye
    c. Add 2 uL 2log ladder to the first and last well of each row
    d. Photograph gel when finished.
13. If your samples look good, congrats! You can proceed to the barcoding step (not yet written)

14. If your samples only have faint bands, add another 3 cycles of the PCR program. Visualize on a gel.
15. If your samples have no bands, add another 5 cycles. Visualize on a gel.

Never exceed 40 PCR cycles!!!