ILLUMINA PCR AMPLIFICATION OF 16S REGION

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BACKGROUND

- This protocol is used to amplify the 16S region from DNA extractions in order to carry out gene sequencing. It uses primers and refers to the following paper from the Josh Neufeld Lab:
- Bartram et al. **Generation of multimillion-sequence 16S rRNA gene libraries from complex microbial communities by assembling paired-end illumine reads**. Appl Environ Microbiol.2011 Jun;77(11):3846-52

NOTES

- Keep Taq and primers cold at all times
- Once you are in the PCR hood, do not touch anything outside with your gloves on
- Aliquot all reagents for 2-3 times use in order to prevent any degradation or external DNA contamination
- Radiate MgCl2, buffer and BSA before use for about 10 minutes
- Do NOT radiate ddH20, primers, enzymes or template
- Use filter tips throughout the entire procedure
- Keep everything closed unless it is being used
- Always have a negative control (blank) and if possible, a positive control
- Be sure to use a different V3R barcodes for each sample because this is what differentiates the samples when they are sent for gene sequencing

EQUIPMENT

- Equipment:
  - PCR hood
  - Small tubes and lids for PCR reaction
- Reagents:
  - 10x buffer
  - 50mM MgCl2
  - 10mM dNTPs
  - 10mg/ml BSA
  - 1uM V3F_mod2 primer (5 pmoles)
  - 1uM V3R barcoded primers
  - Ultra pure distilled water (DNase/ RNase free)
  - Taq Polymerase
  - Loading Dye
  - RedSafe
  - 100bp DNA ladder

PROTOCOLS

Pre-work:

A. Make BSA in pure water, aliquot and irradiated on UV transilluminator for 30 minutes to get rid of any contaminating DNA.
B. Radiate MgCl2, buffer and BSA before use for about 10 minutes.

Assembling the reaction:

1. Calculate the amount of template and ddH20 needed for each reaction. The total reaction volume is 50ul. This includes: the master mix, ddH20, V3R barcode, and template.
    a. Master mix is 14.75ul
    b. V3R barcode is 5ul
    c. Calculate template to 30-50ng
    d. Fill the rest of the reaction up to 50ul using ddH20
2. Make master mix including the buffer, MgCl2, BSA, V3F, Taq and the dNTPs.

Reagent	x1	x (# of samples + 1)
Buffer	5ul	
MgCl2	1.5ul	
BSA	2ul	
V3F	5ul	
Taq	0.25ul	
dNTPs	1ul	
Total	**14.75ul**	

3. Add in the calculated amounts of water to each reaction.
4. Add 14.75ul of master mix to each reaction tube.
5. Add corresponding V3R barcodes.
6. Add template.
7. Split the 50ul reaction tube into three tubes with 16.7ul each for a reaction in triplicate.
8. Program to run the PCR on:
    a. 94°C for 2 minutes
    b. 94°C for 30 seconds
    c. 50°C for 30 seconds
    d. 72°C for 30 seconds (300bp product)
    } 30 cycles
    e. 72°C for 10 minutes
        - After this, the product can be stored at room temperature or be held in the PCR machine at 4°C

Visualizing the Products on a Gel

1. Prepare a 2% gel to run the samples on.
2. Combine the three tubes with 16.7ul of product each into one tube.
3. Add 5ul of 6x Loading Dye to each of your samples.
4. Use a 100bp ladder to run the gel. The product bands should appear around 300bp and the primer bands should appear around 80bp.
5. Run gel at 100V for 30-35 minutes (the small gels can run for 30 mins, and the larger gels can run for 35 minutes).
6. Visualize under the UV transilluminator light.
7. Under the UV light, carefully cut around the bands trying to avoid any excess gel that does not contain product.
8. These bands can be stored at -20°C until you are ready to purify and sequence them.

TROUBLESHOOTING PCR REACTIONS

What to do if your bands are not showing up:

A. Double the amount of Taq used to 0.5ul and alter the remaining calculations accordingly.
B. Use a new set of dNTPs or Taq (to see if any of the enzymes have been contaminated or degraded).
C. Use a new bottle of Ultra Pure distilled water and aliquot into 1ml tubes to prevent opening the entire bottle many times and allowing external DNA to enter.
D. Double the amount of template added (and if it still doesn't work, try doubling it again etc).

E. If your bands are not showing up, you should have a bright primer band (since nothing is being amplified, the primers should still be present in excess). And if you have a bright product band, it is likely that the primer band will be faint. This tells you that your reaction is working but the template concentration might be too small to be amplified.
F. If nothing is showing up, the entire PCR reaction needs to be optimized more.
G. You can also try heating up your template at 65°C before adding it to the reaction in order to ease along the PCR reaction.
H. If there is still no band after trying to trouble shoot, recheck the nucleic acid concentrations of your template using a BioAnalyzer since this is more precise.
I. Finally, it might be an issue with the purity of the template (from the initial extraction stage).