

## Molecular Methods in Microbial Ecology

### Day 2: Set-up PCR

Next we are going to amplify the DNA collected from your columns using primers that are specific for two domains of life (Bacteria and Archaea) and three sets of primers specific for the functional genes involved in sulfate reduction (Dissimilatory Sulfite Reductase, *dsr*), methanogenesis (Methyl Coenzyme Reductase, *mcr*), and methane oxidation (Particulate Methane Monooxygenase, *pmo*). In addition, we will run a positive control (in which we add DNA that we know belongs to the target organism) and a negative control (in which we don't add any DNA just water and the PCR cocktail).

So in all we will have 7 reactions but you should make up enough master mix for 8. This accounts for small pipette errors. Use the worksheet below to work out how much of each reagent is needed. Since it is very important to keep primers and reagents as cold as possible, we will first create a "master mix" that contains sterile water, 5X PCR buffer, dNTPs, and the Taq polymerase. Your first step will be to calculate how much of each of these reagents that you need for your 8 reactions.

**PCR is extremely susceptible to contamination.** Wear your gloves at all times- they help protect your sample from you! In addition – you must be very very careful with pipettes and contamination. Be sure to change your tips so that you don't cross-contaminate anything. Please remember that the PCR reaction will amplify most all DNA present so even trace amounts of contaminant DNA in your master mix can be amplified and interfere with your results. This is why we run a negative control with just the master mix and no added DNA – if we do see DNA in the negative control it will be because you contaminated your mix so be careful with your reagents and with your pipette.

The master mix should contain the following amounts for each reaction.

Sterile H <sub>2</sub> O	26.8 $\mu$ l
5X PCR buffer	10 $\mu$ l
dNTPs (10 mM)	1 $\mu$ l
Taq polymerase (5 Units/ $\mu$ l)	0.2 $\mu$ l

Once you have made your master mix pipette 38  $\mu$ l into each of your 7 strip tubes.

Then add the following to each of the tubes. Be sure and mark which tube #1 is.

1. DNA template plus *dsr* specific primers dsr1F and dsr4R
2. DNA template plus *mcr* specific primers ME1 and ME2
3. DNA template plus *pmo* specific primers pa189F and pa682R
4. DNA template plus Bacteria specific primers 8F and 1492R
5. DNA template plus Archaea specific primers 21F and 958R
6. Positive control- *M. jannaschii* plus Archaea specific primers 21F and 958R
7. Negative control- no template plus Archaea specific primers 21F and 958R

You will be adding 2  $\mu$ l of each of template and 5  $\mu$ l of each of the primers above for a total volume of 50  $\mu$ l. Use the chart below to help you keep track of your additions. Use a new pipette for every addition.

Reagent	Volume ( $\mu$ l) per reaction	# of reactions	final volume
Sterile H <sub>2</sub> O	26.8		
5X PCR buffer	10		
dNTPs (10 mM)	1		
Taq polymerase (5 Units/ $\mu$ l)	0.2		

Tube	Master mix $\mu$ l	Target	Template	Vol $\mu$ l	F primer	Vol $\mu$ l	R primer	Vol $\mu$ l
1	38	Sulfate reducers	Column DNA	2	dsr1F	5	dsr4R	5
2	38	Methanogens	Column DNA	2	ME1	5	ME2	5
3	38	Methanotrophs	Column DNA	2	pa189F	5	pa682R	5
4	38	Bacteria	Column DNA	2	8F	5	1492R	5
5	38	Archaea	Column DNA	2	21F	5	958R	5
6	38	Archaea	+ control <i>M. jannaschii</i>	2	21F	5	958R	5
7	38	Nothing	- control (water)	2	21F	5	958R	5

Firmly close the tubes when you are done adding all reagents. Once they are closed, put your strip on ice. Make sure you have labeled the end of the strip cap with something so that you know what strip of tubes is yours. We'll use a thermal cycler in the Bay Paul Center Lab. I will run a gel and on Tuesday we will analyze the results.

Each primer set has a slightly different profile based on primer properties and optimizations carried out in our lab. Here is what we will use:

	<b>Bacteria</b>	<b>Archaea</b>	<b>dsr</b>	<b>mcr</b>	<b>pmo</b>
<b>Polymerase Activation</b>	94°C, 3 min	94°C, 3 min	96°C, 5 min	95°C, 5 min	95°C, 3 min
<b>Number of Cycles</b>	35	35	35	40	35
<b>DNA denaturation</b>	94°C, 40 s	94°C, 40 s	94°C, 1 m	95°C, 45 s	94°C, 35 s
<b>Primer annealing</b>	55°C, 1:30	55°C, 1:30	56°C, 1 m	50°C, 45 s	54°C, 1 m
<b>Extension creation</b>	72°C, 2 m	72°C, 2 m	72°C, 1 m	72°C, 1:30	72°C, 2 m
<b>Final Extension</b>	72 °C, 10 min	72 °C, 10 min	72 °C, 10 min	72 °C, 10 min	72 °C, 10 min