PCR of Tape Measure Protein (TMP) gene

Introduction to PCR

The polymerase chain reaction (almost always simply referred to as PCR) was developed by Kary Mullis and colleagues in 1983. Mullis envisioned a method to replicate specific pieces of DNA in such a way that the target DNA was amplified. Then, as now, a cycle of PCR included:

- Denaturation. The template DNA is heated to 95˚C to denature the strand. (Remember that DNA polymerase synthesizes a new strand of DNA as it moves along a *single stranded* template.)
- Annealing. The reaction temperature is dropped to 50–60˚C to allow two primers to anneal to the template DNA. Primers are designed to bind at one end or the other of the target DNA sequence.
- Extension. The reaction temperature is raised to 72˚C and DNA polymerase uses free dNTPs (deoxynucleoside triphosphates) to synthesize a new strand complementary to the template DNA.
- And the steps are repeated for 30–40 cycles. At the end, the number of copies of the target sequence is several orders of magnitude greater than at the beginning of the reaction.

Bacterial and algal mats surrounding thermal pool at Yellowstone National Park.

Initially, PCR required that DNA polymerase be added to the reaction after each denaturation step because heating the reaction to the temperature required to denature DNA also permanently denatured the DNA polymerase. However, DNA polymerases were discovered in organisms that live the thermal pools at Yellowstone National Park — since the bacteria lived in >65˚C water, their enzymes had to function at those temperatures. A DNA polymerase isolated from the bacteria *Thermus aquaticus* (called *Taq* polymerase) made PCR the common tool we know today (although other heat stable DNA polymerases have since been discovered).

The other important development in the history of PCR was the invention of the thermal cycler. One cycle of PCR requires the reaction be at three different temperatures for different lengths of time. Initially, the cycling was done manually, lifting the reaction tubes from one water bath to the next for hours (remember that PCR involves 30–40 cycles). In the late 1980s, an instrument was developed that automated the temperature cycles. Thermal cyclers have a metal block that holds sample tubes. The temperature of the block can be raised and lowered very rapidly, and PCR reaction tubes have very thin walls to facilitate rapid temperature cycling. PCR can now be done in only a few hours by simply putting reaction tubes in a thermal cycler and pressing a few command buttons. The results are analyzed using gel electrophoresis.

Using PCR to assign subcluster with the tape measure protein gene

Genomic similarity has long been a core principle used to establish evolutionary relationships under the phylogenetic species concept across the tree of life. Mycobacteriophages are no different in this regard; those that have similar genomes are more closely related than those with less similar genomes. Phages are grouped together in units called clusters and subclusters based on shared genomic features. Members of a subcluster share more similarities than members of a cluster—you can think of it loosely as species vs. genus.

Traditionally, mycobacteriophages have been assigned to clusters and subclusters through whole genome comparison which requires the phage's genome to be sequenced first. More recently, Smith *et al.* (2013) developed a rapid method for assigning phage subclusters without any sequencing. They developed a PCR system that screens the tape measure protein (TMP) gene with a series of primers to determine subcluster. The tape measure protein (TMP) gene is an excellent choice for this experiment since all phages have it, yet it varies greatly between subclusters. Each primer pair used in the experiment anneals to regions of the tape measure protein gene from a specific subcluster. For example, if you have an A4 phage, the A4 primers will anneal to the complimentary regions of DNA and you will see a 588 base pair fragment when you analyze the PCR products with gel electrophoresis. You should not see a fragment (or at least not one of the expected size) in the reactions that contained other primers.

Primer list

Sample Number	Subcluster	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')	Expected product size (in bp)
1	A1	CYGCYGGTAACTTCGGCTCG	CTGGGCYAGCGTCTTCTGC	704
2	A2	SCAGGGYCTGATCAACGGC	AGGAACTGCTTSCCAGTCGC	597
3	A3	CSTTCAACTTCAACTTCG	AAGATGAACTGCTCRCC	512
4	A4	GGTCACGCCGCTKATCTCC	CCGCCGAGTTCCTTCAGC	588
5	A5 mix	GATCATCCCGTTCACCGTGG, SASCTCGAAGCCAAGATCCC	CRGAGCCGAACGACGGCAGG, CRGAGCCGAACGACGGCAGG	248, 849
6	A6	ACATCGCAARCGCCATCG	TTGATGCCKCCGAGGAAGC	829
7	B1	AAAGGTGATCGTGCCCATCG	GAACCTCGTGAACAGGTCGG	493
8	B3	CGGAACAARAAGAAGGGCGG	AKGGGCAYACCGCCGACGCC	205
9	E	CCAGTCGTCGCAGAACATCC	CTGYGCGACRTTGCGGAGG	736
10	F1	TGTCGGGGTATGAGGGTGC	GRCCCTGCTTCACCCCACC	303
11	F2	CCCCCCTGCCACTGTTCC	TTGWAKCCCCGCTTGAACC	873
12	G	GGCGTCGTCTGGGGATGG	GAGATTGCCGAGCCGATGC	431
13	K	GGCGTGGGWGTCGATACAGC	GMCCCAGACGATTTGCGTGC	298
14	N	GCGATCCCGYATGTCRACGC	CGATGACGTCGTTGCGKGCC	430

Smith, *et al.*: **Phage cluster relationships identified through single gene analysis**. *BMC Genomics* 2013 **14**:410.

Today you will prepare a lysate from a dilution of your phage solution (HVL), then probe the DNA with primers that will amplify sections of DNA from tail protein genes. From the results of this experiment, you may be able to determine the cluster and subcluster to which your phage belongs.

To prepare the lysate, you will heat your phage to 95˚C, which will break apart the phage heads, making the genomic DNA accessible. That DNA will be the template for the PCR. The PCR reaction tubes have been prepared for you, so you will just have to add your DNA to each tube. There are 14 primer pairs (a pair is a forward and a reverse primer for the same target DNA). In addition, you will run a positive control (DNA lysate from Watson with a primer for Watson's subcluster) and a negative control (adding water in place of template DNA with Watson's primers). The reaction components are:

- template DNA
- primers
- dNTPS*, a nucleotide mixture (dATP, dTTP, dCTP, and dGTP)
- *Taq* DNA polymerase*
- reaction buffer*

* The dNTPs, *Taq* DNA polymerase, and PCR buffer are combined in a PCR "**mastermix**"

You will have two 8-well strips of PCR tubes. Labeling will be *very* important, so follow the directions carefully.

In the next lab session, you will analyze your results on a 2% agarose gel.

Materials per person

Item	Quantity
screw top microcentrifuge tube	1
sterile distilled water (shared)	1 ml
PCR tube rack	1
PCR strips with mastermix	2
Watson lysate (shared)	10 µl

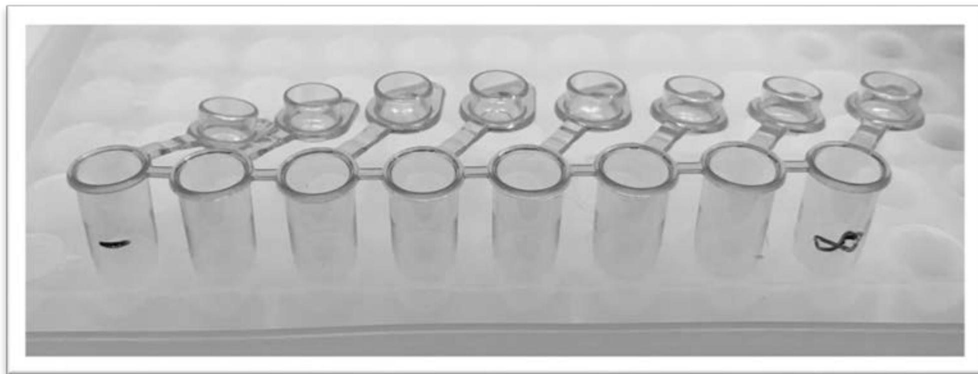
Procedure 1: Preparing template DNA

1. Remove your HVL bottle from the refrigerator and swirl well to mix.
2. Obtain a screw top microcentrifuge tube. Make a 1:100 dilution of your phage solution by adding 5 µl of HVL to 495 µl sterile distilled water in the screw top tube.
3. Heat to 95˚C for 10 min in a heat block.

Procedure 2: Setting up PCR reactions

1. Obtain a rack with 2 PCR tube strips containing mastermix and primers. Remember that the two strips have different contents. (See table below.)
    a. The first 8 primers are in blue tubes (tubes 1–8)
    b. The next 6 primers and the primers for the controls are in pink tubes (tubes 9–16)

   To get the correct orientation of the tube strips, place them so that the caps are slanting to the right. When you do that, tube 1 will be the first tube on the left. The TAs will label the first strip with 1 and 8, and the second strip with 9 and 16.



2. Label the back side of the strips with your initials. (Write on the tubes, not the lids.) Be careful that you do not rub off the writing. Check the labeling when you turn the strips in to your TA.
3. Vortex your heated lysate to mix completely. Using a new pipette tip for each sample, add 5 µl of your lysate to tubes 1–14.
4. Get a tube of Watson lysate and add 5 µl to tube 15 for the positive control. Return the Watson lysate to the front of the room, as other students will be sharing the tube.
5. Add 5 µl of sterile distilled water to tube 16 for the positive control.

6. Cap all the tubes. Double check that they are all tightly closed, as the samples will evaporate during PCR if they are not.
7. Take the samples to your TA. Check that the labeling is still clear and legible.
8. The thermal cycler will be programmed as follows:

Purpose	**Temperature**	**Time**	**Number of cycles**
Initial denaturation	95˚C	5 min	1
PCR	95˚C	30 sec	31
	55˚C	30 sec	
	72˚C	45 sec	
Final extension	72˚C	5 min	1
Hold	4˚C	∞	

9. After PCR, the samples will be stored at –20˚C until the next lab period, when you will analyze them on a 2% agarose gel.

Samples

Strip	**PCR Tube**	**Subcluster primer**	**Expected product size (in bp)**
Strip 1 (**BLUE**)	1	A1	704
	2	A2	597
	3	A3	512
	4	A4	588
	5	A5 mix	248, 849
	6	A6	829
	7	B1	493
	8	B3	205
Strip 2 (**PINK**)	9	E	736
	10	F1	303
	11	F2	873
	12	G	431
	13	K	298
	14	N	430
	15	A3	512
	16	A3	–