

The Polymerase Chain Reaction (PCR) Part 2

GOALS:

- Understand the basis and fundamentals of PCR.
- Design and implement an experiment using PCR.
- Analyze the PCR results with an agarose gel.

This laboratory is designed to provide you with the basics for generating a standard PCR from a DNA template. You will need to calculate the volumes needed to dilute your template DNA and to create your master mix prior to performing the experiment. The success of a PCR depends largely upon the stringency of primers binding to their target template DNA during the hybridization phase. This stringency is impacted by two major factors, (1) the temperature of annealing, and (2) the concentration of free Mg^{2+} ions. Reaction components, including template DNA, chelating agents, dNTPs, and proteins all affect the amount of free Mg^{2+} . The DNA polymerase we'll be using is inactive in the absence of Mg^{2+} , and has a greatly reduced fidelity that may increase the level of nonspecific amplification when too much Mg^{2+} is added. Another major factor involved with a successful PCR is quality and quantity of the template DNA. Reagents commonly used to isolate nucleic acids (e.g. salts, guanidine, proteases, organic solvents, SDS) may reduce the activity of DNA polymerase during the PCR. In addition, artifacts of the matrix the DNA was isolated from (e.g. blood, soil) may inhibit the reaction. In some instances, contamination issues can be resolved simply by diluting the template DNA (*dilution is the solution to pollution!*). The amount of template DNA required is dependent upon the complexity of the template DNA. For example, in a 4kb plasmid containing a 1 kb insert, 25% of the input DNA is the target of interest. Conversely, the same 1 kb gene in the human genome (3.3×10^9 bp) represents approximately 0.00003% of the total DNA. Therefore, ~825,000X more template DNA would be required in this case to maintain the same target dose (i.e., number of target copies).

For today's experiment, you will use your (or your partner's if yours did not work) sample of genomic DNA (gDNA) that was extracted from an environmental sample. Your goal is to (a) amplify the small subunit ribosomal RNA gene (SSU rDNA) from bacteria that were living in your sample and (b) to determine the best template concentration to maximize the yield of PCR product (i.e., does diluting the sample help remove contaminants?). If successful, your results will be used to answer significant questions regarding the bacterial community at your site. The source, concentration, and purity of your gDNA sample is different from others in the lab so do not expect similar results.

You will make a master mix (MM) to increase the comparability of each reaction and to reduce time, waste, and pipetting errors. Your first MM will contain all solutions except the template DNA, *Taq* polymerase and bovine serum albumin (BSA). You will add the template DNA after aliquoting MM #1 into the appropriate PCR tubes. After incubating this reaction at 95 °C for 2 minutes and letting it cool to 4 °C you will aliquot the second MM which contains *Taq* polymerase and BSA. Next, you will proceed to thermal cycling of 30 cycles of denaturation, annealing and extension at 94 °C for 1 min, 56 °C for 1.5 min, and 72 °C for 3 min, respectively. This is followed by an additional extension at 72 °C for 7 min, and a 4 °C hold. A 15 μ l aliquant of each amplification will be assayed for size and purity by electrophoresis on a 1% agarose mini-gel.

Stock Solutions:**10X PCR buffer:** 500 mM KCl, 100 mM Tris-HCl (pH 9.0 @ 25°C), 1.0% Triton X-100**[Mg²⁺]:** 25 mM**dNTPs:** 10 mM; 2.5 mM each**Forward Primer:** 50 μM**Reverse Primer:** 50 μM**High quality water:** q.s. to 50 μl**gDNA:** 10 ng/μl → plus 4 10-fold dilutions**50X BSA:** 10 mg/ml**Taq polymerase:** 5 units/μl

Table 1. Please complete the following table to solidify your understanding of the role of each reagent we are using for today's PCR experiment:

Reagent	Brief description as a PCR reagent
Template DNA	
1X PCR Reaction buffer	
2.5 mM MgCl ₂	
800 μM dNTPs (200 μM ea.)	
1 μM Forward Primer	
1 μM Reverse Primer	
Optimal H ₂ O	
0.2 mg/ml BSA	
5 units <i>Taq</i> DNA Polymerase	

You will be supplied with gDNA of known concentration. The array of 5 reactions will vary the gDNA amounts (50 ng, 5 ng, 500 pg, 50 pg, and 5 pg (note that these are simply serial 10-fold dilutions). For example, if your first tube contains 20 μl of a 10 ng/μl sample you will reserve 5 μl (50 ng) for PCR and use a portion of the remaining for diluting to your second tube. Your instructor will also provide you with instructions for running a positive and negative control to help troubleshoot potential problems. Fill in the remaining cells of the example table below and then construct a similar scheme in your notebook to create serial 10-fold dilutions for the template DNA in your experiment:

Example Table 2.

	Tube 1	Tube 2	Tube 3	Tube 4	Tube 5
C ₁	8 ng/μl	800 pg/μl	80 pg/μl	8 pg/μl	800 fg/μl
V ₁					
C ₂	800 pg/μl	80 pg/μl		800 fg/μl	
V ₂	20 μl	20 μl	20 μl	20 μl	
Volume for PCR	6.25 μl				
DNA mass for PCR	50 ng	5 ng	500 pg	50 pg	5 pg

Use the data given in this handout and what you determined to be the appropriate template DNA mixtures to fill out the following Master Mix table for your PCR. Be sure to add an extra sample ($n = 5 + 2 + 1 = 8$) when calculating volumes for your master mix to correct for potential loss of volumes during pipetting.

DNA AMPLIFICATION CHECKLIST

DATE _____

NAME _____

Template	H ₂ O	10X Buffer	MgCl ₂	dNTP's	#1 Primer	#2 Primer	Template DNA	BSA	TAQ Polymerase	Total Vol.

MM #1 (n = 8)

H₂O _____

Buffer _____

MgCl₂ _____

dNTP's _____

F-primer _____

R-primer _____

MM #2 (n=8)

BSA _____

Taq _____

Hot Start Temp/Time _____

Denaturation Temp/Time _____

Hybridization Temp/Time _____

Elongation Temp/Time _____

of Cycles _____

Notes:

**Before starting the experiment, have your instructor/TA check all data and please do not be weary of asking for assistance (this type of experimental setup can be confusing the first couple of times ☺).