The Polymerase Chain Reaction (PCR)

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 are given a sample of genomic DNA (gDNA) that was extracted from a community of microorganisms. 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 ^{o}C for 2 minutes and letting it cool to 4 ^{o}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^{o}C for 1 min, 56^{o}C for 1.5 min, and 72^{o}C for 3 min, respectively. This is followed by an additional extension at 72^{o}C for 7 min, and a 4^{o}C hold. A 15 µl 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
[Mg^{2+}]: 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_2O	
0.2 mg/ml BSA	
5 units *Taq* 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 ul 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	H2O	10X Buffer	$MgCl_2$	dNTP's	#1 Primer	#2 Primer	Template DNA	BSA	TAQ Polymerase	Total Vol.

MM #1 (n = 8)
H2O _____
Buffer _____
$MgCl_2$ _____
DNTP's _____
FPrim _____
Rprim _____

MM #2 (n=8)
BSA _____
Taq _____

Hot Start Temp/Time _____ # of Cycles _____
Denaturation Temp/Time _____
Hybridization Temp/Time _____
Elongation Temp/Time _____

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 ☺).