	SKILLS CENTER **STANDARD OPERATING PROCEDURE**	**A BIOFIZZ** **PRODUCTON**
Polymerase Chain Reaction (PCR) #1 **Module Hours: 3.0**	**Effective Date: 11/12/2023** **PRQs** **Buffers and stock solution, Pipette calibration, DNA Agarose gel, Centrifugation**	**Revision # 1.0** **A. Siclair and Z. Hazlett** **Checked: M. Guzie and M. Stowell**

BACKGROUND

The polymerase chain reaction process (PCR), first invented by Kary Mullis in 1985, is still used today as the primary technique to amplify specific DNA sequences. At a basic level, the PCR process involves the design of primers accompanied by three stages of temperature cycling in the thermal cycler apparatus to generate copies of the DNA sequence.

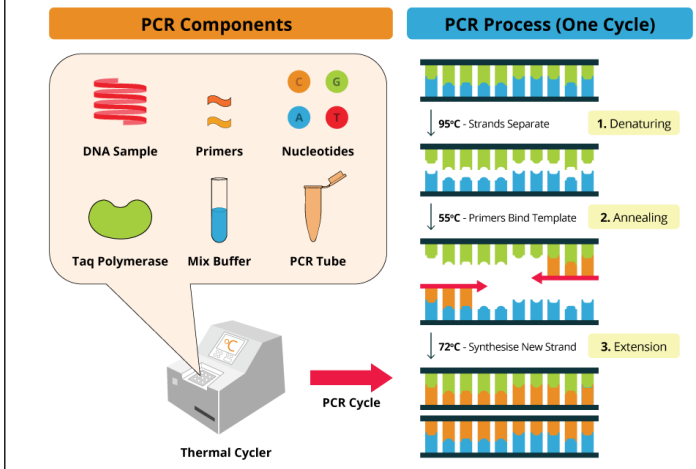

Figure 1: Basic components and process of a PCR cycle. (Boster Antibody and ELISA Experts)

- Denaturation:

The thermal cycler is heated to approximately 95℃, causing the bonds between the template DNA strands to break.

- Annealing:

The thermal cycler temperature is decreased between 55℃-65℃, allowing for the primers to bind to the template DNA in their designated locations. These primers serve as a signal to the DNA polymerase, determining what sequence section will be amplified.

- Elongation:

The thermal cycler temperature is slightly increased to around 72℃, where the heat-stable *Taq* polymerase adds free dNTPs to the template. This leads to the formation of a new DNA strand that is a replica of the sequence from the gene of interest.

Generating copious amounts of a region of interest is often a necessary first step to conduct other procedures, including the early stages of DNA sequencing. PCR has also been known for its role in pathogen detection, and in forensic DNA testing. This technique is used across the board in research and is a key skill to master in the lab.

1. PURPOSE

The purpose of this procedure is to understand the basic principles of PCR and to be able to conduct the process in the lab.

2. SCOPE

This procedure applies to qualified skills center users.

3. RESPONSIBILITY

3.1. It is the responsibility of the user to understand and perform the procedure described in this document.
3.2. It is the responsibility of the user performing the procedure to fully document any deviations from the written procedure.
3.3. It is the responsibility of the user to become trained on the procedure.

4. DEFINITIONS

4.1. Primer dimer – Two primers that have bound each other, meaning that there is complementarity between primers.
4.2. Hairpin loops – A primer looping on itself, meaning that there is complementarity within an individual primer.
4.3. Melting temperature – The temperature at which the DNA strands denature from one another.
4.4. Template DNA strand – The strand of DNA that contains the gene of interest that will be amplified.
4.5. Primers – Single stranded DNA sequences, between 17-28 base pairs long, that bind to the template DNA at the beginning and end of the desired amplification region.
4.6. *Taq* DNA polymerase – A DNA polymerase that is “heat stable”, meaning that it will retain its function during temperature increase.
4.7. dNTPs – free DNA nucleotides that can be utilized by the *Taq* polymerase.
4.8. Thermal cycler – Apparatus used to conduct the PCR process and change the temperature at specified times to ensure amplification.
4.9. DNA ladder – Used to assess the number of DNA base pairs that correlate with a specific band on the agarose gel.
4.10. DI water – deionized water, which is water that has had all impurities and ions removed. It is important that the water used in PCR is nuclease free to prevent degradation of the DNA.

5. MATERIALS/EQUIPMENT

5.1. Primer3Plus application or similar (only needed for PCR 2 module)
5.2. NCBI nucleotide or gene website
5.3. Thermal cycler
5.4. Template DNA: pML11 Plasmid
  5.4.1. Target fragment length 950bp (human Synaptophysin)
5.5. *Taq* DNA polymerase
5.6. Forward and reverse primers
  5.6.1. Forward primer (20 uM) : F001 (AAAACATATGCTGCTGCTGGCGGACATGGACG)
  5.6.2. Reverse primer (20 uM) : R005 (AAAACTCGAGCTACATCTGATTGGAGAAGGAGG)
5.7. Sterile (nuclease free) DI water
5.8. Enzyme reaction buffer
5.9. DNA loading dye
5.10. DNA ladder
5.11. 0.5 ml thin walled pcr tubes for each PCR reaction
5.12. P2, P20 and P200 pipettes
5.13. Sterile pipette tips for P2, P20 and P200 pipettes

6. RECIPES

6.1. Making a Stock Solution of Primers (Primers prepared for you in PCR 1 module; See materials above; If available, skip to 6.3)

- Primers are given in nanomoles, which must be converted to moles for the calculation. The desired result in this example is to have a 100 µM primer solution.

  (moles of primer) x (1L) = (100 x 10^{-6} moles/L) x (V_2)

- V_2 = amount of DI water needed to be added to the primers to get the desired concentration. This amount is in Liters after the original calculation, so must be converted to µL by multiplying the result by 10^6.

6.2. Making a Working Solution of Primers (Primers prepared for you in PCR 1 module; See materials above; If available, skip to 6.3)

- The working solution of primers is what is used as a component of the PCR reaction. To create this solution, the stock solution that was calculated in section 6.1 will be used. It is necessary to calculate the amount of stock solution used and the amount of water used to make the working solution.

- Utilize the $C_1V_1 = C_2V_2$ equation.

  (100 µM) x V1 = (10 µM) x (100 µL)
  V1 = 10 µL of stock solution
  100 µL – 10 uL of stock solution = 90 µL DI water

6.3. PCR Reaction Components (50 µl total; Promega, 2018)

10X DNA Polymerase buffer	= 5 µL
10 mM dNTP mix	= 1.5 µL
50 mM $MgSO_4$	= 1 µl
20 µM Primer 1 (Forward)	= 1 µL of working solution
20 µM Primer 2 (Reverse)	= 1 µL of working solution
Template DNA (pML11 plasmid)	= 1 µL
Taq DNA Polymerase	= 0.5 µL
DI, nuclease free water	= rest of the way up to **50 µl total volume**

7. PROCEDURE

The PCR procedure here will begin with determining PCR reaction cycle parameters, followed by using these parameters to execute your PCR experiment given DNA primers and template DNA.

7.1. Determine PCR Reaction Cycle Parameters

7.1.1. Determine the temperatures and times for each step of PCR. Recall the three primary steps of PCR: Denaturation, Annealing, and Extension (Figure 1). Temperatures and times of each of these steps vary based on the characteristics of the DNA polymerases, the types of PCR buffers, and the complexity of the template DNA (*REF*: https://tinyurl.com/2f8hctb9).

7.1.2. You will need to define each step of the below PCR cycle given your knowledge of the PCR conditions and characteristics of your reaction components. Please read carefully through the reference guidelines given in the reference here to determine your time and temperature conditions for each step: https://tinyurl.com/2f8hctb9.

PCR reaction (Complete and use for MMT)
Step 1 (*Initial Denaturation*): ____°C for ___ sec/min
Step 2 (Complete Cycle/All three steps):

Denaturation: ___ ^{o}C for ___ sec/min
Annealing: ___ ^{o}C for ___ sec/min
(will require you to determine the Tm of the primers; Show calculations if Tm determined manually)
Elongation: ___ ^{o}C for ___ sec/min (1 min per kb)
Number of cycles: Repeat 30 cycles
Step 3 (*Final Elongation*): 72 ^{o}C for 10 min
Step 4: Stop /Hold at 4-10^{o}C

7.2. CR Reaction

7.2.1. Put on gloves to prevent contamination during the procedure.

7.2.2. Prepare 0.5 ml sized thin wall pcr tubes. Labelled with “T” for Test and “NC” for negative control

7.2.3. Using the proper pipettes, and using new tips to avoid cross contamination, add the designated reaction components to each microcentrifuge tube. Keep all reaction components and reaction tubes on ice. The reaction components are denoted below (Promega, 2018) (See Recipes section for more details)

10X DNA Polymerase buffer	= 5 µL
10 mM dNTP mix	= 1.5 µL
50 mM $MgSO_4$	= 1 µl
20 µM Primer 1 (Forward)	= 1 µL of working solution
20 µM Primer 2 (Reverse)	= 1 µL of working solution
Template DNA (pML11 plasmid)	= 1 µL
TaqDNA Polymerase	= 0.5 µL
DI, nuclease free water	= rest of the way up to **50 µl total volume**

Note: The template DNA and the primers are the only components that might change from step to step. (EX: some controls might not have DNA, or might not have primers)

Negative Control: No DNA control (use equal volumes of sterile water instead of DNA to assess contamination of sample).

7.2.4. Spin the tubes briefly in a microfuge.

7.2.5. Place the microcentrifuge tubes in the thermal cycler. Set and run PCR reaction with the parameters you determined above. Feel free to review your cycle parameters with a proctor prior to running your experiment.
7.2.6. Remove the tubes from the Thermal cycler.
7.2.7. Analyze the PCR products on a 1.5% agarose gel (as described in the DNA agarose gel electrophoresis SOP).

8. TROUBLE SHOOTING

8.1. There are not any primer pairs with good “Any” or “End” scores: Go back to the start and use the default parameters in Primer3Plus instead of the input in section 7.2.4.
8.2. DNA bands show up in the negative control: This means that there was some other DNA contaminant in the sample, and that the bands shown might be amplifying a region that is not the intended gene of interest. The experiment needs to be repeated with a new DNA sample.
8.3. No bands show up in the positive control: This likely means that the DNA sample has degraded or was not properly put into the reaction mixture. The experiment needs to be repeated with a new DNA sample.
8.4. Multiple bands show up in a singular lane: This likely means that the primers are nonspecific and amplified multiple different regions of the DNA strand of differing base pair amounts. The primers need to be assessed to see if new primers need to be chosen.
8.5. Large, bright, thick bands occur at the bottom of the agarose gel past the DNA ladder: The primers are likely complementary to each other and are amplifying each other rather than the gene of interest.

9. REFERENCES

Boster Antibody and ELISA Experts. (n.d.) *PCR Principle*. Boster Antibody and ELISA Experts. https://www.bosterbio.com/protocol-and-troubleshooting/pcr-principle

New England BioLabs Inc. (2021). *PCR Protocol for Taq DNA Polymerase with Standard Taq Buffer (M0273).* New England BioLabs Inc. https://www.neb.com/protocols/0001/01/01/taq-dna-polymerase-with-standard-taq-buffer-m0273

Pilotte, N. (2013, Nov. 5) How does one calculate the required amount of primers for a PCR reaction? *Research Gate.* https://www.researchgate.net/post/How_does_one_calculate_the_required_amount_of_primers_required_for_PCR_reaction

Promega. (2018, April). *GoTaq® DNA Polymerase (M300) Protocol.* Promega.

https://www.promega.com/resources/protocols/product-information-sheets/g/gotaq-dna-polymerase-m300-protocol/

Skrenkova K., Song J.M., Kortus S., Kolcheva M., Netolicky J., Hemelikova K., Kaniakova M., Krausova B.H., Kucera T., Korabecny J., Suh Y.H. & Horak M. (n.d.) *Homo sapiens glutamate ionotropic receptor NMDA type subunit 1 (GRIN1), transcript variant GluN1-1a, mRNA.* NCBI Nucleotide. https://www.ncbi.nlm.nih.gov/nucleotide/NM_007327.4?report=genbank&log$=nuclalign&blast_rank=1&RID=776AD46801R

Vigers, A. (2020). *Lab 5 Primer design for PCR.* MCDB:3140. 2020S 3140 Lab 5Aand 5B[3683].pdf

10. MODULE MASTERY TASK

This task will test your conceptual knowledge of PCR and your abilities to correctly amplify your gene of interest and ensure that you are able to conduct PCR in an efficient and effective manner.

PCR Design and Execution

1. What is PCR and why is a useful technique?
2. Per the guidelines in 7.1.2, define the time and temperature conditions for each step of your PCR reaction. Provide a description below your cycle parameters justifying why you chose the time and temp for each step. In your description, please include the Tm you calculated for the given primers (with work shown if applicable) and how you used this Tm to determine the annealing temperature.
3. Describe three variables that might affect which PCR cycle parameters you choose.
4. Why is it important to have a negative control reaction? What happens if you have evidence of DNA amplification in a negative control reaction product?
5. When designing and testing your own primers to amplify a gene of choice (PCR 2 module), you will need to use a positive control. Describe what a positive control reaction might consist of if you were to design your own PCR reaction.

PCR Reaction and Calculations

1. What were the given nanomoles concentrations of each primer?
2. How much water did you calculate needs to be added to each primer to get the desired 100 µM solutions?

3. Document how much of each component you added to each reaction tube, including the control tubes.

Gel Electrophoresis

1. What reaction or control does each lane contain?
2. What time did you start running the gel?
3. What time did you stop running the gel?
4. Submit a well labelled picture of your resulting gel from the UV light camera.
5. Did your controls work? What evidence supports your answer?
6. Is there a band present in your experimental lanes? If so, what is the length of the amplified fragment in base pairs? (use the DNA ladder to establish this value)
7. Describe the resulting gel. Does there appear to be contamination in your samples? How can you tell?