	SKILLS CENTER **STANDARD OPERATING PROCEDURE**	**A BIOFIZZ** **PRODUCTON**
Polymerase Chain Reaction (PCR) **Module Hours: 4**	**Effective Date: 04/12/2021** **PRQs** **Buffers, stock solution, dilutions** **Pipette calibration** **DNA Agarose gels** **Centrifugation**	**Revision # 1.0** **A. Siclair** **Checked: M. Guzie**

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.

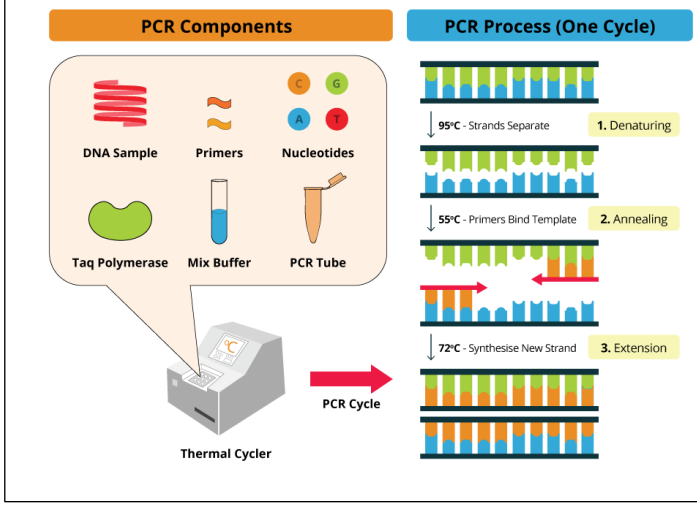

Figure 1: Basic components and process of a PCR cycle. (Boster Antibody and ELISA Experts)

- 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 in order 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
5.2. NCBI nucleotide or gene website
5.3. Thermal cycler
5.4. Template DNA
5.5. Control DNA
5.6. *Taq* DNA polymerase
5.7. Forward and reverse primers
5.8. Sterile DI water
5.9. Enzyme reaction buffer
5.10. DNA loading dye
5.11. DNA ladder
5.12. Microcentrifuge tubes for each PCR reaction
5.13. P20 and P200 pipettes
5.14. Sterile pipette tips for P20 and P200 pipettes

6. RECIPES

6.1. Making a Stock Solution of Primers

- Primers are given in nanomoles, which must be converted to moles for the calculation. The desired result 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

- 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 (Promega, 2018)

5X enzyme reaction buffer = 10 µL
10 M nucleotide mix = 1 µL
Primer 1 (Forward) = 1 µL of working solution
Primer 2 (Reverse) = 1 µL of working solution
Template DNA or cDNA = 1 µL
Taq. Polymerase = 0.25 µL
DI, nuclease free water = rest of the way up to **50 µl total volume**

7. PROCEDURE

The PCR procedure will begin with primer design, after a model organism and gene of interest have already been chosen.

7.1. Basic Rules for Primer Design

7.1.1. Primers should be between 17 and 28 nucleotides long. (Vigers, 2020)
7.1.2. The forward and reverse primers should not differ in length from each other by more than 3 base pairs. (Vigers, 2020)
7.1.3. The primers should amplify a region of your gene between 300 and 1000 base pairs long. (Vigers, 2020)
7.1.4. The melting temperature (Tm) of each primer should be between 52-62° C. (Vigers, 2020)
7.1.5. The forward and reverse primer Tms should not differ from each other by more than 5 degrees. (Vigers, 2020)
7.1.6. The GC content of the primer should be between 40-60%. (Vigers, 2020)
7.1.7. Avoid the primers being complementary to each other or to themselves. (Vigers, 2020)
7.1.8. Any and End scores below 4, indicating acceptable complementarity values. (Vigers, 2020)

7.2. Primer Design

7.2.1. Go to the Primer3Plus website: http://primer3plus.com/
7.2.2. Click Run Primer3Plus.
7.2.3. Select the General Settings tab. (Figure 2)
7.2.4. Change the Primer Size, Primer Tm, and Primer GC% parameters to match the values in Figure 2.

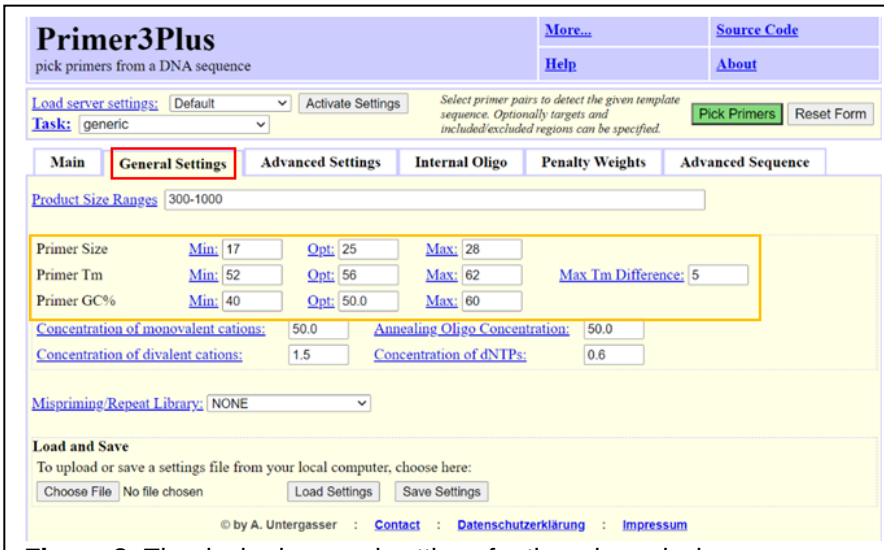

Figure 2: The desired general settings for the primer design process.

7.2.5. Go to the NCBI Nucleotide website and look up the gene of interest: https://www.ncbi.nlm.nih.gov/nucleotide/
7.2.6. Click on the CDS function to select only the coding sequence of the gene. (Figure 3)
7.2.7. Copy the coding sequence of the gene that is to be amplified and return to the Primer3Plus page. (Figure 3)
- The ascension number for the example used for this procedure: NM_007327.4

Figure 3: The CDS button to select the coding sequence of the example gene (left). A portion of the example coding sequence on the NCBI website (right).

7.2.8. Return to the Main tab of Primer3Plus. (Figure 4)

7.2.9. Paste the coding sequence into the Paste Template Sequence Below box. (Figure 4)

7.2.10. Click on the Pick Primers button. (Figure 4)

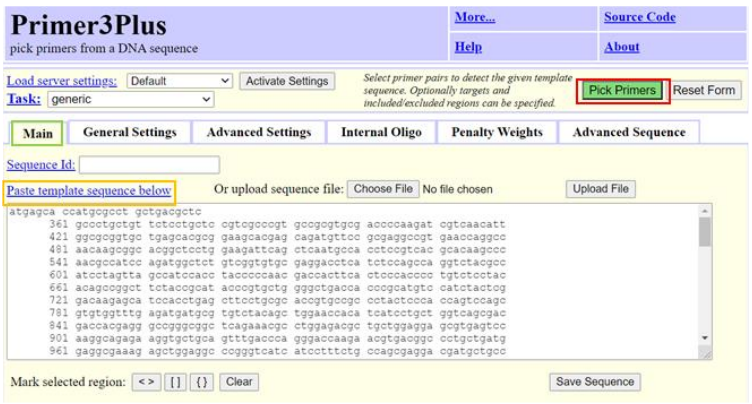

Figure 4: The main screen of Primer3Plus with the inserted coding sequence of the example gene.

7.2.11. A new window will pop up with different primer options for the given coding sequence. (Figure 5)

- The position of primer pair 1 will be highlighted in the coding sequence. (Vigers)

- Note: if the coding sequence used had number values in it as is given from some sites, you may receive a warning notification. It should not affect the given primer pairs. (Figure 5)

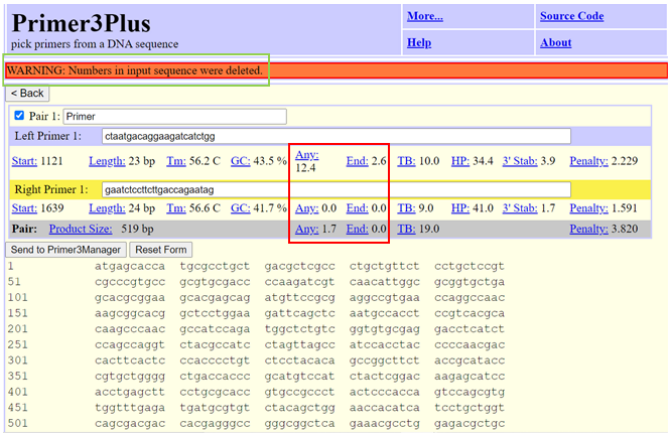

Figure 5: The resulting primer pair 1 from the example search coding sequence and parameters.

- Look for a primer pair that does not anneal to itself, meaning that it has Any and End scores below 4. (Figure 5) (Vigers)

7.2.12. Choose a primer pair based using the guidelines in section 7.1.

7.3. PCR Reaction

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

7.3.2. Label all of the microcentrifuge tubes, both on the top and sides.

7.3.3. Using the proper pipettes, and using new tips to avoid cross contamination, add the designated reaction 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)

5X enzyme reaction buffer = 10 µL
10 mM nucleotide mix = 1 µL
Primer 1 (Forward) = 1 µL
Primer 2 (Reverse) = 1 µL
Template DNA or cDNA = 1 µL
Taq. Polymerase = 0.25 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)

Controls:

1. Positive: primers with the control DNA. It is known that these primers amplify this DNA sequence.
2. Negative: no DNA control (use sterile water instead of DNA to assess contamination of sample).

7.3.4. Spin the tubes briefly in a microfuge.

7.3.5. Place the microcentrifuge tubes in the thermal cycler and carry out an initial denaturation at 98 °C for 3 minutes.

7.3.6. Cool the thermal cycler to annealing temp (around 80°C) and amplify the DNA for between 25 - 40 cycles.

Parameters for a typical cycle might be:

94°C	15 – 30 sec.	Denaturation
60°C	15 – 30 sec.	Annealing of primers
72°C	30 sec – 1min.	Extension of sequences

7.3.7. Conduct a final extension at 68 – 72°C for about 5 minutes

7.3.8. Remove the tubes from the Thermal cycler.

7.3.9. Hold the reaction products at 4 – 10°C and then 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

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10. MODULE MASTERY TASK

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

Part A.

Choosing a Gene for PCR

1. What is your model organism?
2. What is your gene of interest?
3. What is the coding sequence of your gene of interest?

Primer Design

1. What are the forward and reverse primers for your chosen primer pair?
2. What are the melting temperatures for each primer?
3. What is the GC content of your primers?
4. What are the Any and End scores for your primers?
5. What is the length of each primer in base pairs?
6. How long is the region of the gene of interest that your primers amplify?

Submit your Part A results to a Proctor for review. Once a proctor has signed off on Part A we will order your primers so you can complete Part B.

Part B.

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 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?