

Session 3

Cloning Overview & Polymerase Chain Reaction

Learning Objective:

In this lab exercise, you will become familiar with the steps of a polymerase chain reaction, the required reagents for a successful reaction, and the concept of making a “master mix.”

Introduction

Polymerase chain reaction (PCR) is perhaps the most important development in the field of molecular biology in the past 50 years. PCR allows the scientist or engineer to select part of a DNA sequence and ***amplify*** it ***in vitro***, rather than by growing up large quantities of cells containing that DNA. Usually only a small region, called the ***target sequence***, is amplified out of the starting DNA (the ***template***). Using PCR, many copies of the desired target sequence can be made, leaving only tiny amounts of the undesired portion of the template. PCR has many uses, some of which include: cloning, sequencing, mutagenesis, and quantifying DNA or RNA.

Background

Where We Are in the Cloning Process

Today we will be taking the second step in our cloning process: PCR amplification of the protein-generating-device sequence from our template DNA. At the end of this step, we will have a large quantity of the DNA to insert into our vector and complete the cloning process. PCR is explained below in more detail.

Reagents of the Polymerase Chain Reaction

PCR requires several reagents. First, you need the template (pattern) DNA which contains the target sequence you wish to amplify. The template can come from different sources of DNA including: previous clones, isolation from an organism, isolation from the environment, or chemical synthesis. The source of the template usually depends on the intended application and availability.

In order to build new DNA, you need to include the building blocks in the reaction mixture. DNA (deoxyribonucleic acid) is built out of ***deoxynucleoside triphosphates (dNTPs)***. These building blocks contain the nitrogenous base (A, T, C, or G), a deoxyribose sugar (the main component of the polymer backbone), and a triphosphate chain (which stores the energy required for the polymerization reaction and provides the link between the sugars). Since there are four bases used in the construction of DNA,

adenine (A), guanine (G), thymine (T), and cytosine (C), there are four dNTPs in the mixture (dATP, dGTP, dTTP, and dCTP).

The nucleotides must be assembled by a **DNA polymerase** enzyme. The DNA polymerase enzyme reads along a single strand of the template DNA and builds a brand-new complementary strand out of dNTPs, adding the complementary base at each point (pairing A/T and G/C). The polymerase enzyme requires a certain set of conditions to be operational. To achieve the appropriate concentrations of salt, metal ions, and pH, a **buffer** solution is added to the reaction mixture. The polymerase must also be able to tolerate the high temperatures used to separate double-stranded DNA for copying. Usually, scientists use a polymerase from a thermophilic bacterial species (*Thermophilus aquaticus*, aka **Taq**); this species grows at temperatures which would cook humans or *E. coli*.

The final component of a PCR reaction is the two **primers**, short (~20-base long) pieces of DNA. Recall that DNA has two strands and that each strand has a direction, with a beginning (**5' end**) and an end (**3' end**). The two strands are **anti-parallel**, running in opposite directions, so the 5' end of one strand is by the 3' end of the other strand. DNA polymerases copy in the 5'-to-3' direction, and need to be primed with a short piece of DNA (that is, it can only build a new strand by adding bases to an existing strand, and cannot start from nothing). The primers we add to a PCR reaction thus define what region of the template DNA will be copied, and the polymerase will not be able to copy anything outside that region. Thus, when we design primers, we select short pieces of the 5' ends of the target sequence. Because the primers dictate the target sequence, they must be designed for each PCR with the template in mind; you cannot PCR a sequence if you don't know anything about the template.

Steps in the Polymerase Chain Reaction

PCR is done in **cycles**. A reaction typically consists of 25-30 cycles. The perfect completion of a single cycle results in doubling the number of DNA copies from the beginning of the cycle. So, if you start with 1 piece of double-stranded DNA, after one cycle you will have 2 pieces, after two cycles you will have 4, after three cycles you will have 8, and so on; the number of DNA pieces grows **exponentially**.

There are three steps to a PCR cycle. The first step is called **denaturing**. In the denaturing step, the temperature is raised to about 95 degrees Celsius. This separates the double-stranded template DNA into two individual strands. The second step is called **annealing**. In the annealing step, the temperature is quickly cooled to about 55 C, and the primers attach to the individual strands of template DNA. The third step is called **elongating** or **extending**. The extending step is performed at about 72 C. In the extending step, the DNA polymerase copies the template strand by adding dNTPs, starting at the primer. The polymerase goes along until it reaches the end of the template strand runs out of DNA to copy. At the end of the extending step, the original double-stranded DNA has been turned into two double-stranded copies of DNA, each with a single strand of the original DNA. The process can now be repeated over and over again to make many more copies of the DNA.

Session 3: Pre-Laboratory Exercises

Name: _____

Date: _____

- 1) What is a dNTP?
- 2) Temperatures are very important in the PCR process.
 - a) Why do we heat the DNA in the first step?
 - b) What happens when the DNA is cooled back down in the second step?
 - c) What is the main factor deciding what the temperature should be in the third step?
- 3) What happens to the number of DNA molecules after each round of PCR?
- 4) Priming is a key part of the PCR process.
 - a) How many primers are needed for a PCR reaction?
 - b) Draw primers on the template strands shown below. Indicate the 5' and 3' ends. (x's show the ends of the target sequence.)

5' _____ x _____ x _____ 3'

3' _____ x _____ x _____ 5'

- c) Draw the rest of the strands that would be synthesized by the DNA polymerase during that cycle. Indicate the 5' and 3' ends.
- d) Below, re-draw one of the newly synthesized strands and show where a primer would bind to it in the next cycle. Indicate the 5' and 3' ends.

Laboratory Protocol

The exercise: You will be amplifying a protein generating device (containing pBad promoter, RBS, and fluorescent protein coding region) from a previously cloned template. You will be amplifying the gene with primers containing BioBrick restriction sites. The product will later be assembled with the vector you minipreped last week, in a **BioBrick assembly** process.

Materials: PCR tubes and caps
1.5 mL microcentrifuge tubes
Sterile Deionized Water
Template DNA
Forward Primer
Reverse Primer
dNTPs
Reaction Buffer
Taq DNA Polymerase

Equipment: Vortexer
Thermocycler

Protocol

1. To one of the PCR tubes, add 1 μL of the template DNA. To the other PCR tube add 1 μL of sterile water. Make sure to label the tubes indicating either template DNA or water, and your group color.
2. In a clean 1.5 mL tube, mix the following PCR reaction reagents IN THIS ORDER. Listed below are the amounts for one 50 μL reaction; calculate and prepare a mix for three reactions. Mix well by racking.

37.5 μL	Water
5 μL	10X Buffer
2.5 μL	Forward Primer
2.5 μL	Reverse Primer
1 μL	dNTPs
0.5 μL	Taq DNA Polymerase*

*Make sure to add polymerase last. Come to the instructor bench when you are ready to add it. You will need to use a P2 pipettor.

3. Add 49 μL of the master mix to each of the PCR tubes. Put the caps on the tubes and mix by pipetting up and down. Collect the liquid in the bottom of the tube by gently tapping the tube against the benchtop.

4. Put the PCR tubes into the thermocycler and record which position they are in.
5. Set the thermocycler to the following settings:
 - a. Initial Denaturing at 95° C for 2 minute
 - b. Denaturing at 93° C for 40 seconds
 - c. Annealing at 55° C 30 seconds
 - d. Elongation at 72° C for 1 minute
 - e. Final Elongation at 72° C for 10 minutes
 - f. Hold at 4° C

Cycle steps b-d for 30 cycles

Session 3: Post-Laboratory Exercises

Name: _____

Date: _____

- 1) What does the DNA you amplified today do? (Hint: look back to the Session 2 lab packet and/or do some searching on the internet)

2) PCR Calculations

Assuming you started with 1 double stranded piece of DNA...

- a. How many molecules are there after:
 - i. 5 cycles
 - ii. 10 cycles
 - iii. 30 cycles
 - iv. n cycles
- b. If the PCR process is only 50% efficient (i.e. only half of the strands are successfully copied), if you start with 100 copies of DNA, how many are present at the end of
 - i. 1 cycles
 - ii. 2 cycles
 - iii. n cycles
- c. If your target DNA sequence is 1,000 basepairs long, what is the maximum number of **double-stranded** DNA molecules that could be made starting with 50 μL of a 200 micromolar solution of dNTPs? (Hint: You will need to use Avogadro's number. Start by figuring out how many dNTPs you have in the solution.)

- 3) Where does “Taq” polymerase get its name? What is special about the organism it comes from that allows it to be used for PCR? What would happen if you tried to use a human or *E. coli* polymerase for PCR?

- 4) Based on the sequence below, design 10-base primers (please write in standard 5' to 3' notation) to amplify:

5' - ATGTGATGCC TAGCTTAGCT ACCTGTAAGA GGCATTACGT - 3'
3' - TACACTACGG ATCGAATCGA TGGACATTCT CCGTAATGCA - 5'

- a. The entire sequence
- b. The left half of the sequence
- c. The right half of the sequence

References & Additional Reading

Wikipedia:

Polymerase Chain Reaction http://en.wikipedia.org/wiki/Polymerase_chain_reaction

Deoxyribonucleoside Triphosphate <http://en.wikipedia.org/wiki/Nucleoside>

Deoxyribonucleic Acid <http://en.wikipedia.org/wiki/DNA>