

PCR Amplification of a Segment of Bacteriophage Lambda DNA

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Introduction

DNA replication is essential to life. It allows all individual cells, organisms and viruses to pass along their genetic material to the next generation. Through evolution, each living system has developed a way to replicate its DNA with a speed and accuracy that best ensures its survival and reproduction.

Inside any living cell, the process of replicating DNA requires the coordination of a number of different tasks. Like a construction crew building a house, each member of the team has a defined job. The DNA being replicated (the **template**) serves as the blueprint all the workers must follow. For this project, however, Martha the electrician, Randy the plumber, and Julio the brick layer are enzymes. The first worker on the scene is an enzyme (gyrase) that pulls the two strands of the double helix apart creating a small single-stranded region, or “bubble”. This allows enough room for the entry of another enzyme (helicase) whose job is to travel down the DNA molecule breaking the hydrogen bonds that hold the two strands together. The next crew member to arrive at the site is an enzyme (primase) that synthesizes small complementary strands of RNA, called **primers**. These primers bind (anneal) to the single strands of the DNA template within the replication bubble. The enzyme **DNA polymerase** then adds bases onto the ends of the primers until an exact copy of the template is constructed.

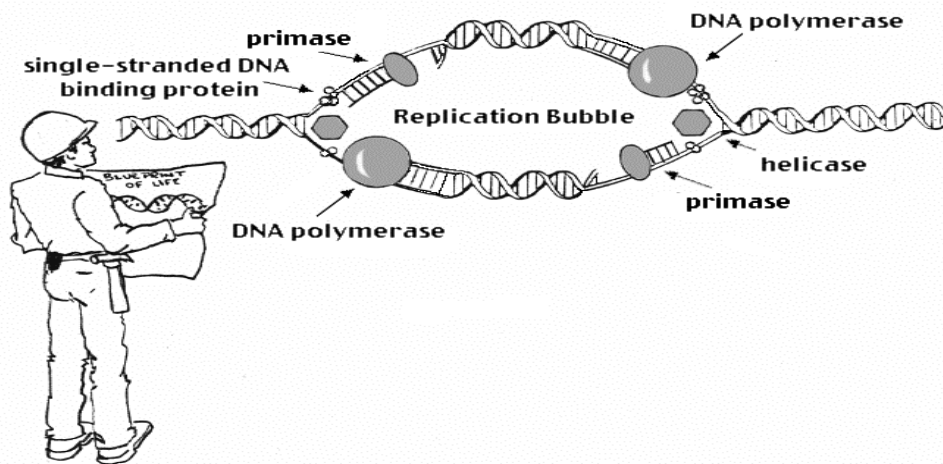


Figure 1. Within a cell, construction of a new DNA molecule requires a number of different enzymes.

In 1983, a biochemist at a small biotechnology company near San Francisco hit upon an idea for replicating DNA in a test tube. It didn't require an elaborate mix of enzymes. It didn't require fancy or expensive equipment. It could amplify just a small amount of DNA to over a million fold. It could be used to solve a vast number of biological problems from narrowing down the location of a particular gene within the 3 billion base pairs of the human genome to determining who left a spot of blood on a sidewalk at the scene of a murder. It was so simple, so elegant, so obvious that half the molecular biologists at the time could be heard cursing to themselves, "Now why didn't I think of that!" Its inventor, Kary Mullis, won a Nobel Prize. It has been the subject of multimillion dollar patent disputes. The technique is called PCR (for the polymerase chain reaction) and you will perform it in this laboratory exercise

To set up a reaction for amplifying a segment of DNA by PCR, you need to add several components into a reaction tube. These include the following:

Template DNA. This is the DNA molecule containing the segment of DNA you wish to amplify.

Primers. Small pieces of DNA (typically 18 to 25 bases long) that bind to complementary regions on opposite strands of the template. The primers serve as replication start points. Two primers are used to amplify one DNA segment.

DNA Polymerase. The enzyme used to replicate DNA. It adds bases onto the 3' ends of the annealed primers. For use in PCR, the DNA polymerase should be stable at high temperatures. You will use the enzyme AmpliTaq, a DNA polymerase isolated from bacteria that thrive in the hot springs of Yellowstone National Park.

Magnesium chloride. Provides magnesium ion (Mg^{2+}) to the reaction. Mg^{2+} is a cofactor for DNA polymerase. Without Mg^{2+} , DNA polymerase will not function.

dNTPs (deoxynucleoside triphosphates). The nucleotides added by the DNA polymerase enzyme onto the 3' ends of the annealed primers.

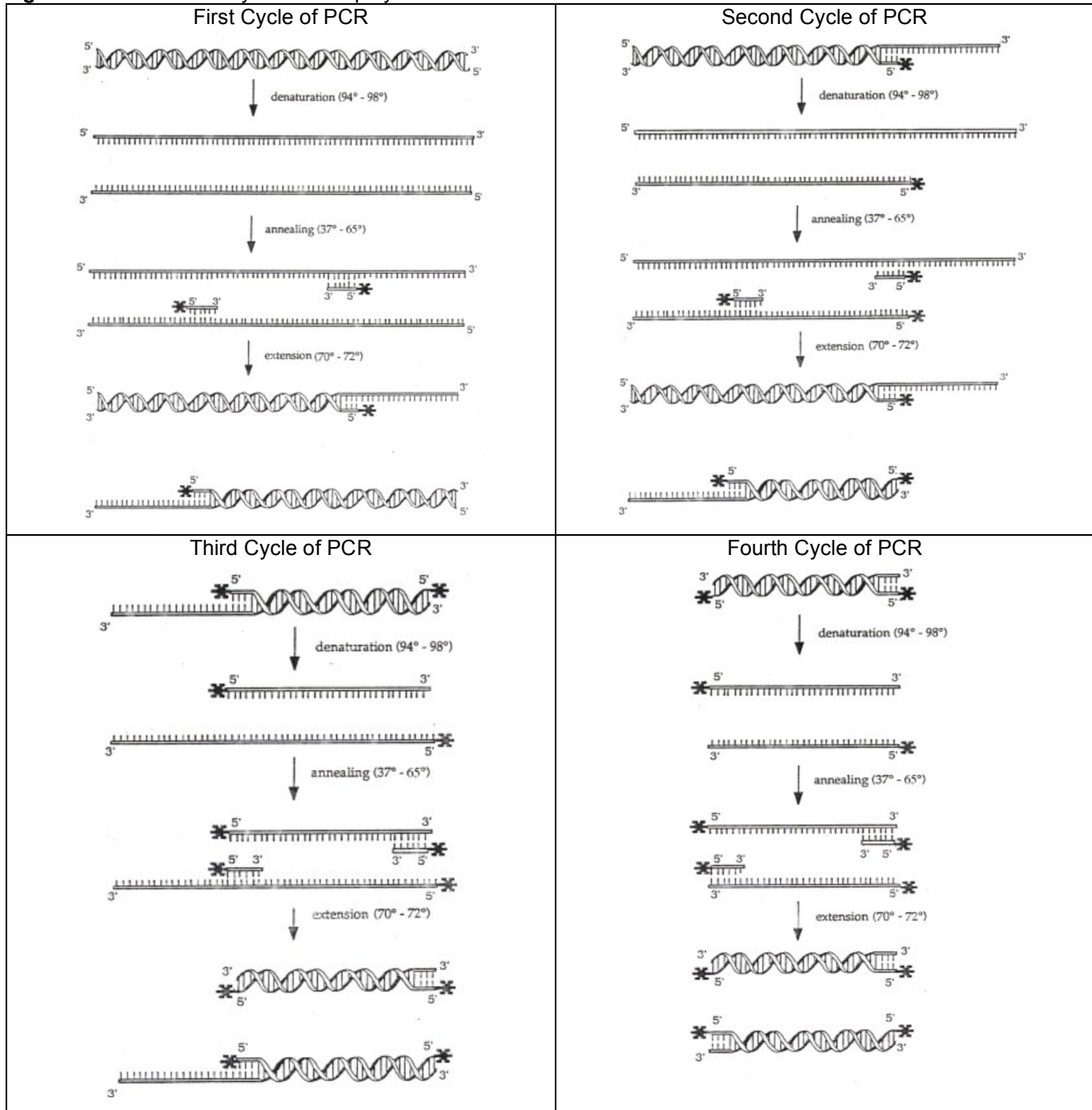
Buffer. Maintains the pH of the reaction at a level where the DNA polymerase is most active.

In PCR, the temperature of the reaction is cycled between three different levels. The first level is at a high temperature and is used to break the hydrogen bonds between the complementary bases that hold the two strands of the template together. This step is called **denaturation**. The reaction is then taken down to a lower temperature at which point the two PCR primers can anneal to the template strands. This step is called **annealing**. In a final step called **extension**, the reaction is brought up to an intermediate temperature at which the DNA polymerase adds nucleotides onto the ends of the annealed primers. The steps of denaturation, annealing, and extension make up one complete **cycle**. Making enough copies of DNA for analysis by gel electrophoresis typically requires from 25 to 40 cycles. Temperature cycling is performed in an instrument called a **thermal cycler**.

During the reaction, each new DNA fragment made in one PCR cycle can serve as a template in the next. This results in a doubling of the amount of amplified PCR product with each cycle. Mathematicians call this **exponential amplification** and describe the rate at which new products accumulate by the expression 2^n , where n is the number of cycles. If a PCR amplification is carried through 25 cycles, for example, the DNA would be amplified by 2^{25} , or about 33 million fold.

Illustration of the Polymerase Chain Reaction

Figure 2. The first four cycles of the polymerase chain reaction.



An excellent animated tutorial showing the steps of PCR is available at the DNA Learning Center web site. <http://www.dnalc.org/ddnalc/resources/pcr.html>

Another 3D PCR animation can be found at the DNAi website: <http://www.dnai.org/b/index.html>

Note: Once on this webpage, choose the "Techniques" box in the bottom. In the "Techniques" page, click on "amplifying" to view the 3D PCR animation.

Laboratory Exercise

In this laboratory exercise, a DNA segment of a bacteriophage called lambda will be amplified by PCR. A bacteriophage is a virus that infects bacteria. Lambda (λ), one of the most extensively studied bacteriophages, infects the common intestinal bacterium *Escherichia coli*. The lambda genome is 48,502 base pairs (bp) in length. You will amplify a 500 bp section of the virus' genome.

PCR is such a powerful technique because of its capacity to amplify very small amounts of DNA. This very quality can lead to a problem in those laboratories that amplify the same segment of template DNA over and over again. PCR product can find itself on laboratory equipment, on pipettes, in unused reaction tubes, on clothing, and in places you might never expect. For this reason, it is a good idea to also prepare a negative control, a reaction in which no template DNA is added, every time you set up a PCR experiment. If a PCR product shows up in your negative control reaction, then it is likely that you have a contamination problem. In this exercise, you will work with a partner. One of you will prepare a negative control reaction.

When the amplification reactions are complete, you will use gel electrophoresis and staining to detect the PCR product you have made. You will determine the length of the PCR product by running a DNA size marker in one of the adjacent gel lanes.

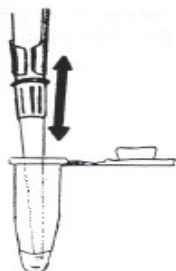
Careful laboratory technique is essential for successful PCR amplification and for minimizing contamination between samples. Therefore, when preparing your PCR amplifications, use the following laboratory techniques to help ensure your success.

Objectives:

1. The student will be able to identify the components necessary to perform PCR.
2. The student will be able prepare a PCR for the amplification of a segment of lambda DNA.
3. The student will be able to identify a PCR product on a stained agarose gel and to estimate its size.
4. The student will be able to identify characteristics of primer design that can lead to the formation of primer dimer.

IMPORTANT LABORATORY PRACTICES

- Add reagents to the bottom of the reaction tube, not to its side.
- Add each additional reagent directly into previously-added reagent.
- Do not pipette up and down, as this introduces error.** This should only be done only when resuspending the cell pellet and not to mix reagents.
- Make sure contents are all settled into the bottom of the tube and not on the side or cap of tube. A quick spin may be needed to bring contents down.



- Pipet slowly to prevent contaminating the pipette barrel.
- Change pipette tips between each delivery.
- Change the tip even if it is the same reagent being delivered between tubes. Change tip every time the pipette is used!



Keep reagents on ice.



Check the box next to each step as you complete it.



Setting up Lambda PCR Reactions

1. You will set up these reactions in pairs. Between you and your lab partner, you will set up one reaction using lambda template DNA and one negative (no template DNA) control reaction.
2. Obtain two PCR tubes and label the side of one with your initials/identification info and the symbol "I". On the side of the other tube, write your initials and "-C" (for negative control).
3. Add the following reagents in the order listed.
4. **Change pipette tips between deliveries!**
5. Add reagents directly to the bottom of each tube. Each time you add a new reagent, slowly pipet the reaction mixture up and down several times to ensure proper mixing.
6. Check off each reagent after you have added it to the appropriate reaction tube.
7. **Reaction set up table:**

Reagent	Lambda DNA reaction	Negative control reaction
Distilled water	26.0 μ L <input type="checkbox"/>	31.0 μ L <input type="checkbox"/>
10X PCR Gold Buffer	5.0 μ L <input type="checkbox"/>	5.0 μ L <input type="checkbox"/>
25 mM MgCl ₂	3.0 μ L <input type="checkbox"/>	3.0 μ L <input type="checkbox"/>
10 mM dNTP mix	4.0 μ L <input type="checkbox"/>	4.0 μ L <input type="checkbox"/>
Diluted AmpliTaq (1.25U/ μ L)	2.0 μ L <input type="checkbox"/>	2.0 μ L <input type="checkbox"/>
PC01 Forward Primer	2.5 μ L <input type="checkbox"/>	2.5 μ L <input type="checkbox"/>
PC02 Reverse Primer	2.5 μ L <input type="checkbox"/>	2.5 μ L <input type="checkbox"/>
Lambda ("I") DNA (0.1 ng/mL)	5.0 μ L <input type="checkbox"/>	0 μ L <input type="checkbox"/>
Final Volume:	50 μL	50 μL

8. After you finish preparing your reactions, place your tubes in the thermal cycler and record the spaces you fill on the grid provided by your teacher.

Lambda PCR Cycling Parameters

95°C / 2 minutes

94°C / 15 seconds

37°C / 15 seconds

72°C / 15 seconds

25 cycles

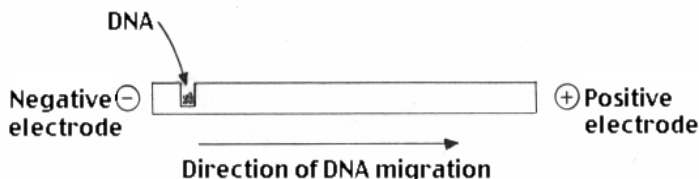
72°C / 7 minutes

15°C / HOLD

Agarose Gel Electrophoresis

Following the completion of the cycling program, you will need to determine whether or not your amplification was successful. This will be done by using a process called **gel electrophoresis**; a technique which separates DNA fragments according to their size. Gel electrophoresis utilizes electric current to separate molecules that have a charge. Since DNA is negatively charged, it will migrate in an electric field towards the positive electrode (Figure 3). When electrophoresed through a gel, shorter fragments of DNA will move at a faster rate than longer ones. The actual separation of DNA molecules of different sizes is achieved by forcing them, in an electric field, to travel through a porous gel material (agarose).

Figure 3. Side view of an agarose gel showing DNA loaded into a well and the direction of DNA fragment migration during electrophoresis.



Your instructor will prepare the gels made out of **agarose**. Agarose is extracted from seaweed and is manufactured as a dry powder. To prepare a gel, the agarose granules are placed in a solution of buffer and heated to boiling until they dissolve and the mixture becomes clear. The heated solution is allowed to cool slightly and is then poured into a casting tray mold having a plastic comb supported in an upright position within it. In about 15 minutes, the agarose solidifies into a gel having all the look and feel of coconut Jell-O. The solidified gel is placed in an electrophoresis chamber and buffer is added until the gel is completely submerged. The comb is pulled out to form the wells into which you will load your Lambda PCR product samples. The buffer chamber is then connected to an electrophoresis power supply.

You will add a small amount of **loading dye** solution to a sample of your reaction. Loading dye is a colored, viscous liquid containing a dye (making the liquid easier to see) and sucrose or glycerol (making the mixture denser than water so that it will drop to the bottom of the well). Once loading dye has been added, the sample is pipetted into the wells of the agarose gel. Either your instructor or an assigned student will load a **100 bp ladder** size marker into one of the gel lanes. The size marker contains fragments that differ in length by 100 base pairs. It will be used to help you determine the length of the PCR product you have made. Once all samples have been loaded onto the gel, the power supply is turned on to draw the DNA within the sample towards the positive electrode (Figure 3). Electrophoresis is continued until the dye is close to the bottom of the gel.

Calculations for Preparing 2% Agarose gel

You will need a 2% agarose gel solution, by mass, for electrophoresis of your PCR products.

Q: If your agarose gel casting trays holds 50 mL, then how much agarose and buffer would you need? *Note:* Definition of % in biology is usually grams (mass) / 100mL (volume). Therefore, for 2 % agarose solution, it will be 2 g /100 mL buffer.

Calculations:

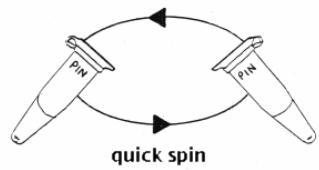
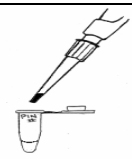
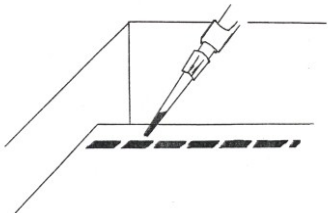
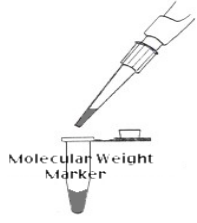
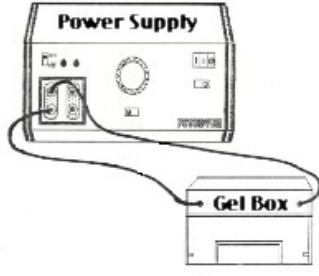

Step 1: Calculate mass of agarose needed for 50 mL total volume of agarose solution.

$$\frac{2 \text{ g}}{100 \text{ mL}} = \frac{X \text{ g}}{50 \text{ mL}} ; \quad X = 1 \text{ gram}$$

Step 2: Calculate amount of buffer needed to bring the agarose solution to 50 mL.

By standard definition, 1 gram of H₂O ≈ 1 mL of H₂O. The amount of buffer for the 2% agarose solution will be 49 mL (50 mL – 1 mL (1 gram of agarose)).

Electrophoresis of Amplified DNA

<p>1. Retrieve your PCR tube and place it in a balanced configuration in a microcentrifuge. Spin it briefly (~ 10 seconds) to bring the liquid to the bottom of the reaction tube.</p> <p>Note: Make sure the centrifuge adapters are in place before putting the tiny PCR tube into the centrifuge rotor.</p>	 <p style="text-align: center;">quick spin</p>
<p>2. Add 5 μl of loading dye to your PCR tube.</p>	
<p>3. Carefully load 15 to 20 μl of the DNA/loading dye mixture into a well in your gel. Make sure you keep track of what sample is being loaded into each well.</p> <p>Note: Avoid poking the pipette tip through the bottom of the gel or spilling sample over the sides of the well. Use a new tip for each sample.</p>	
<p>4. One student (or the instructor) should load 15-20 μl of 100 bp ladder (molecular weight marker) into one of the wells of each gel.</p>	 <p style="text-align: center;">Molecular Weight Marker</p>
<p>5. When all samples are loaded, attach the electrodes from the gel box to the power supply. Have your teacher check your connections and then electrophorese your samples at 150 Volts for 25 - 40 minutes.</p>	 <p style="text-align: center;">Power Supply</p> <p style="text-align: center;">Gel Box</p>
<p>6. After electrophoresis, the gels will be ready to stain and photograph.</p>	

Staining and Photographing Agarose Gels

Your teacher will stain your agarose gel and take a photograph for you so that you may analyze your PCR results. Gel staining is done as follows.

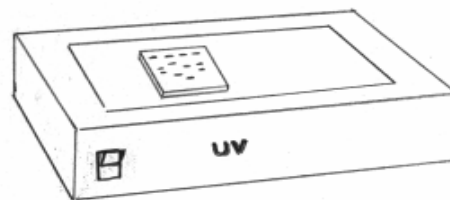
1. Place the agarose gel in a staining tray.
2. Pour enough ethidium bromide (0.5 μ g/mL) to cover the gel. Wait **20** minutes.
CAUTION: Ethidium bromide is a carcinogen. Always wear gloves and safety glasses when handling.
3. Pour the ethidium bromide solution back into its storage bottle. Pour enough water into the staining tray to cover the gel. Wait 5 minutes.
4. Pour the water out of the staining tray into a hazardous waste container and place the stained gel on a UV light box.
CAUTION: Ultraviolet light can damage your eyes and skin. Always wear protective clothing and UV safety glasses when using a UV light box.
5. Place the camera over the gel and take a photograph.

Figure 4. Ethidium bromide molecules stacked between DNA base pairs.



The PCR products run on your agarose gel are invisible to the naked eye. If you look at your gel in normal room light, you will not be able to see the amplified products of your reaction. In order to “see” them, we must stain the gel with a fluorescent dye called **ethidium bromide**. Molecules of ethidium bromide are flat and can nestle between adjacent base pairs of double stranded DNA (Figure 4). When this interaction occurs, they take on a more ordered and regular configuration causing them to fluoresce under ultraviolet light (UV). Exposing the gel to UV light after staining, allows you to see bright, pinkish-orange bands where there is DNA (Figure 5).

Figure 5. After staining an agarose gel with ethidium bromide, DNA bands are visible upon exposure to UV light.



Results

The stained gel of your results may look something like Figure 6 below.

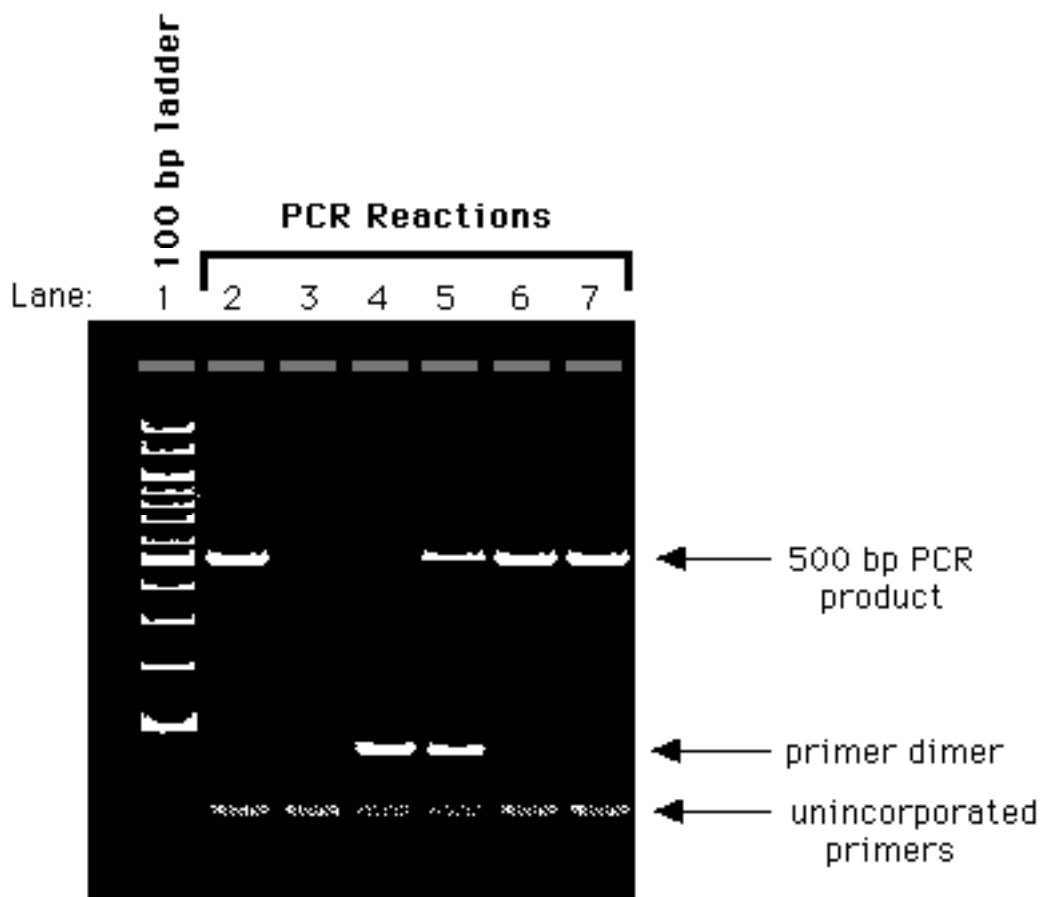


Figure 6. Representation of possible gel results obtained from this laboratory exercise. **Lane 1:** 100 base pair ladder size marker. Each band is different from the ones above and below it by 100 base pairs. To determine fragment lengths of the marker bands, start from the bottom of the gel (furthest from the wells) and count the bands upward. The smallest band is 100 base pairs in length, the next one up the gel is 200 base pairs in length, the next 300 bp, etc. The 500 and 1,000 bp fragments of the 100 bp ladder will be brighter than the others. **Lanes 2-7:** Samples showing the 500 bp desired PCR product, primer dimer, and unincorporated primers. The desired PCR product should run at a rate equivalent to the 500 bp band of the 100 bp ladder.

When the gel you have used to analyze your amplification reaction is viewed on a UV lightbox, it is possible that you might observe an artifact of PCR called **primer dimer**. It is a small but sometimes prominent band towards the bottom of the gel. It is caused by an interaction between the PCR primers.

In this experiment, one primer (designated PC01) has the following sequence:
5'-GATGAGTTCGTGTCCGTACAACCTGG-3'.

The base sequence of the other primer (PC02) is:
5'-GGTTATCGAAATCAGCCACAGCGCC-3'.

Notice that the two bases at the 3' end of the PC01 primer (GG) are complementary to the two bases at the 3' end of the PC02 primer (CC). During the low temperature steps of the PCR amplification (and while the reaction is

Review Questions:

1. What are the six reaction components needed to perform a PCR amplification? What is the purpose of each component?

Reagent	Purpose

2. What is a primer? How many primers do we use in this experiment and why?

3. Explain the three phases of the PCR reaction?

4. Your agarose gel for this experiment shows that you have primer dimer and yet you do not see the expected 500 base pair product. Even though the desired 500 base pair PCR product was not produced, because primer dimer was formed, the reaction did have some activity. For such a result, which of the PCR reagents must be working?

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