**Polymerase Chain Reaction (PCR):**

**Introduction:**

 The molecular technique called PCR is *in vitro* amplification of a specific segment of DNA using a thermostable enzyme. Although it is a fairly new technique, invented in 1985 by Cary Mullis, it is widely used in hundreds of labs all over the world. The PCR process makes millions of copies of DNA in just a few hours. It is a replication reaction, which uses reagents very similar to what is needed for DNA replication inside a cell. Each strand serves as a template for synthesis of its complementary strand.

 PCR has many applications. 1) It is a form of direct cloning of DNA (without the need for bacteria). This is convenient when there is little DNA to work with. 2) PCR can produce a DNA fingerprinting pattern for forensics purposes, such as identifying blood at a crime scene. 3) PCR can be used in prenatal diagnosis of genetic diseases. 4) It can be used for evolutionary analyses, to look at genetic relationships among or within taxa (genera, species, populations). 5) PCR can detect allelic sequence variation and chromosomal rearrangements. 6) It is involved in the DNA sequencing process. 7) It is the newest technique in detecting viral or bacterial infection within a host.

 The requirements for PCR are as follows: dsDNA, dNTP nucleotides (dATP, dGTP, dCTP, dTTP), magnesium ion (Mg++), a forward primer and a reverse primer, *Taq* polymerase, and buffer. *Taq* polymerase is an enzyme extracted from the bacterium, *Thermus aquaticus*. This bacterium lives in hot springs, thus it can withstand high temperatures. *Taq* polymerase is thermostable at close to boiling temperatures. During PCR, the sample is heated to 90°C (for 30 sec) to denature the DNA (separate the 2 strands). Then the sample is cooled down to 50°C (for 1 min) to allow the 2 primers to anneal to each DNA strand. Finally, the sample is heated to 72°C (for 2 min), which is the optimal temperature for *Taq* polymerase to add on nucleotides after each primer. This cycle of denaturing, annealing and extending is repeated 30-40 times. For each cycle, the targeted gene is doubled in number.

**1** --> 2 --> 4 --> 8 --> 16 --> 32 --> 64 --> 128 --> 256 --> 512 --> 1024 --> 2048 --> 4096 -->

 8192 --> 16,384 --> 32,768 --> 65,536 --> 131,072 --> 262,144 --> 524,288 --> **1,048,576**

As shown above, after 20 cycles, over a million copies of a gene from one molecule of DNA is generated. This logarithmic process is called amplification.

**Polymerase Chain Reaction (PCR)**



**Materials:**

micropipettors & tips microfuge thermocycler

microtubes (1.5 ml & 0.5 ml) vortexer PCR kit of reagents

waste beaker kimwipes tube of mineral oil

dsH2O gloves microtube rack & marker

**Procedure:**

1) **Put on gloves**. Place the lid of the 0.5 ml tube container up-side-down on the lab bench. Pour some of the tubes slowly on top of the lid. With gloves on and without touching the lid, place 4 tubes in the blue rack. They are for 2 samples, a positive control and a negative control. Pour the extra tubes back in the container and close the lid. (This technique is used to avoid any contamination of the tubes). Label the top of the tubes with the permanent marker. Write +C and -C for the controls, and number each sample.

2) Spin down the PCR kit reagents, DNA samples, and +C. Place all tubes in the gray microfuge. Make sure they are balanced. Every tube should have another tube opposite it in the microfuge. If there is an odd number of tubes to spin, then make a balance tube by adding some water to an empty tube using a micropipettor. Put pressure on the lid with one hand and press the green button to start spinning. Press the red stop button after ~10 sec.

3) Below is a recipe of what your cocktail mix will contain. To determine the volumes of each item, multiply the volume in the cocktail by the number of tubes you have in your experiment, plus one more for pipette error (i.e. if you have 4 samples, multiply each number by 5). Fill in the table below. This is a recipe for a 25 μl PCR reaction.

Note\*\*\*\*This recipe is for DNA concentrations ranging from 10-150 ng/μl. If your DNA concentration is out of this range, then adjust the amount of dsH2O and DNA accordingly to reach a 25 μl volume reaction (DNA + dsH2O = 13.8 μl).

|  |  |
| --- | --- |
|  **1X Cocktail** | **?X Volume Master Mix** |
|  2.5 μl buffer  |  |
|  1.5 μl MgCl2 |  |
|  0.5 μl dGTP |  |
|  0.5 μl dATP |  |
|  0.5 μl dTTP |  |
|  0.5 μl dCTP |  |
|  2.5 μl Osp2' forward primer |  |
|  2.5 μl OspB3 reverse primer |  |
|  10.8 μl dsH2O |  |
|  0.2 μl *Taq* polymerase |  |

4) Set your micropipettors to the proper settings. You will have to reset them as you make your cocktail.

5) **In a 1.5 ml tube**: add all of the items in order of the recipe (table), **using a new sterile pipette tip every time**. Avoid any contamination!!! Then ask the instructor for the *Taq* polymerase and add to your tube. This is called your master mix.

**Note\*\*\*\*** The rest of the procedure needs to be accomplished as quickly as possible (the enzyme is more active at room temp than at 4°C).

6) Vortex your master mix very gently using a slight touch, then spin briefly using a balance tube in the microfuge.

7) Open all PCR tubes. Aliquot **22 μl** of the **master mix** to each tube, and close each cap as you fill them. (Use the same pipette tip, since the tubes are sterile and you are using the same mix. This saves time).

8) Open each tube (except the -C), one at a time, and add **3 μl** of each appropriate **DNA** sample, including the positive control. For this, you have to change your pipette tip every time!!!

9) Make sure the rotor inside the microfuge holds 0.5 ml tubes. If not, then change the rotor. The rotor pops right out as you lift it up, and the replacement rotor will click into place as you push it down. Spin all PCR tubes briefly in the microfuge.

10) Place tubes in a row, in the microtube rack. Open all tubes and, using a **yellow pipette tip**, add 2 small drops of oil to each, by holding the micropipettor vertically above the tubes without touching them. Since you are not touching the tubes, you will use the same yellow tip like an assembly line.

11) Place tubes together in the thermocycler in a row angled slightly to aid in fitting. Avoid the holes in the corners.

**A Typical Temperature Profile:**

**40 cycles** of:

 93°C / 30 sec (DNA denaturation)

 50°C / 1 min (primer annealing)

 72°C / 2 min (DNA extension)

then 4°C final holding temp

 The amplification process takes 3-4 hours.

# GEL ELECTROPHORESIS

**Introduction:**

The size of a DNA fragment can be estimated by gel electrophoresis. This technique separates fragments by charge, size (molecular weight) and shape. First, an agarose gel is made with slots (wells) in it. The DNA sample is dispensed in the well, a buffer solution is placed in the apparatus, and an electric current is run through the gel. DNA molecules are negatively charged due to the phosphates in its backbone, and when placed in an electric field starting at the negative (black) electrode, it will migrate towards the positive (red) electrode. The gel is a complex molecular network containing narrow passages. Smaller DNA molecules pass through more easily (less friction) and migrate faster through the gel than larger size fragments. Linear molecules also migrate faster through a gel, compared to globular forms.

 Since the DNA fragments generated in this experiment are all negatively charged and linear in shape, the only variable to observe during electrophoresis is size. To determine the size of a molecule, a standard or positive control is run concurrently in the gel. The standard consists of fragments of DNA of known size. A positive control contains the same size fragment (in our case 662 bp) as potential positive samples.

 **Gel Electrophoresis**

 

red

black

 DNA fragments are viewed under ultra violet light, and a brightly colored band indicates the presence of DNA. Ethidium bromide (EtBr) is used in the gel to view the DNA. The gel is stained with EtBr after the electrophoresis run, or is placed in the gel prior to gel solidification.EtBr is an intercalating agent that fits into the middle of the DNA molecule, causing it to bulge. UV light fluoresces the bulging areas of the DNA, which gives it a bright appearance.

**EtBr is a mutagen and possible carcinogen. Therefore, it is extremely important that you only handle the compound with gloves. If EtBr is accidentally spilled, immediately ask for assistance to clean it up.**

**Materials:**

micropipettors vortexer microwave

pipette tips gloves (3 sizes) rubber hothands

waste beaker microfuge weigh balance

microtube rack agarose

loading dye chloroform TAE buffer

125 ml bottle / funnel power supply electrophoresis equip.

**Procedure:**

 **I) Prepare a 1% agarose gel**

 **II) Prepare the gel for loading**

 **III) Prepare the samples for loading**

 **IV) Perform gel electrophoresis**

 **V) View PCR products under ultraviolet light**

**I. Prepare 1% Agarose Gel:** (with gloves)

1) Place the funnel in a 125 ml bottle and place on the balance. Press **tare**.

2) Pour 0.5 g of agarose down the funnel.

3) Add 50 ml 1X TAE buffer to the funnel. Try to get all residual agarose from the sides of the funnel into the bottle. [TAE = Tris acetate EDTA]

4) Close cap and shake to make sure no agarose is stuck to bottom of the bottle.

5) Loosen cap and microwave for **1 min** only. If solution starts to boil, stop the microwave immediately to avoid spillage. Clean microwave tray if this occurs.

6) Swirl gently using a hothand and microwave for 10 seconds more.

7) While waiting for the gel to cool, place the gel mold (that has side gaskets) in the middle of the electrophoresis chamber so that the gaskets are tightly fitted to the sides to avoid leakage.

8) If the agarose bottle can be held without discomfort, then continue. (Do not wait too long. Otherwise, the gel will start to solidify). With gloves, get ethidium bromide from instructor and, using a sterile white tip, add **1 μl EtBr** (be careful).

9) Swirl until you do not see the red EtBr.

10) Pour into mold and place 2 rows of combs, on top and in the middle.

11) Rinse out bottle immediately before the excess agarose solidifies.

**II.** **Prepare Gel for Loading:** (with gloves)

1) After gel has solidified (takes about 15-20 min), pull gel mold up and out of chamber.

2) Place gel into electrophoresis apparatus, with the top row at the end with the black (negative) electrode.

3) Add 1X TAE to both sides of the chamber until there is a small amount above the gel (touch top of gel with finger to judge). Do not over pour.

4) Take out combs carefully, by lifting straight up. The buffer on top of the gel helps the combs to slip out without tearing the gel.

**III.** **Prepare Samples to load in Gel:** (with gloves)

1) Spin samples briefly and place tubes in rack. Open tubes.

2) Using the same white pipette tip, add **4 μl loading dye** to each cap.

3) Using the same blue pipette tip, add **250 μl chloroform** to each tube. Close each cap as soon as you add the chloroform (it vaporizes quickly).

4) Pick up 2 tubes at a time, shake quickly up and down, and place in microfuge; Do the same for all of the tubes.

5) Spin briefly. The chloroform dissolves the oil. The PCR mix is now the blue bubble floating at the top.

6) Load ~**25 μl** of each sample into each well. Each group has its own row to load. Make sure you draw a picture indicating which wells contain which samples.

**IV.** **Gel Electrophoresis:**

1) Slide the lid on top. Make sure you do not spill any buffer. If you do, then use a kimwipe to dry it up. You do not want to get electrocuted when it is turned on!

2) Place the black electrode into the black hole of the power supply. Place the red electrode into the red hole. Turn on power supply. Set the electric current to 80-100 volts. Press the *runner* button to start.

3) In 15 minutes, check to see how far the dye has migrated in the gel. If it is midway between wells (or from the end for the second set of wells), then turn the power off.

**V.** **Viewing of PCR Products under U.V. Light:** (with gloves)

1) Carefully slide the lid off. Try to avoid spilling the buffer.

2) Grab both sides of the gel that have the gaskets and lift. Holding these sides will avoid the gel from sliding off. Tilt to pour off any extra buffer.

3) Place your gel inside the photodocumentation system using the U.V. box.

4) If possible, capture an image of the gel using the photodocumentation system. Save your image on a zip disk.

5) Print out your gel image using the photodoc system.

6) Lift the Plexiglas lid of the transilluminator and place the gel on top. Close the lid over your gel and put on goggles. Cut out the PCR bands that were positive (except for the positive control). Use a razor blade and gently cut down on all four sides of each band. Using the razor blade, flip the band on its side and trim off the bottom, where there is no sign of DNA. Place the band in a 1.5 ml tube. Label the tube with your initials and the sample number. You will now prepare each sample for cycle sequencing.