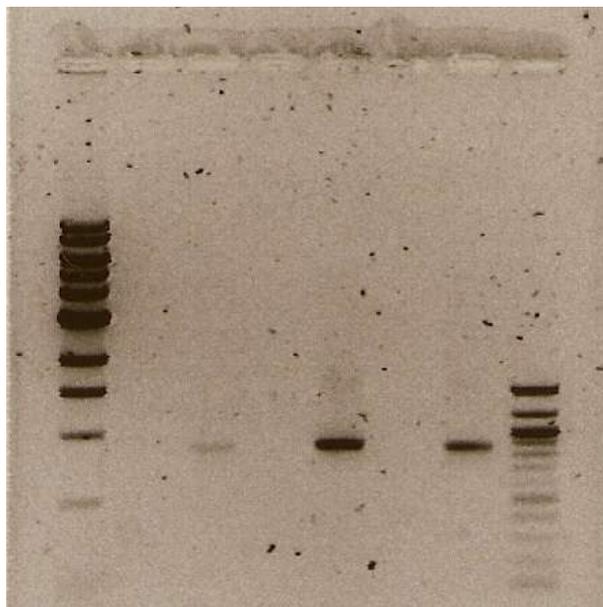


# SPARQ-ed

## *Introduction to Molecular Biology Techniques : The Polymerase Chain Reaction*



SPARQed is a collaboration between The University of Queensland's Diamantina Institute and The Queensland Government's Department of Education and Training. It exists due to the hard work of the SPARQ-ed Regional Reference Group (Regan Neumann, Associate Professor Nigel McMillan, Associate Professor Brian Gabrielli, Dr Peter Darben, Cheryl Capra, Peter Ellerton, Andrew Rhule, Darren Shepherd, Michael Sparks, Patrick Trussler and Jacqui Wilton).

The PCR project is based on the Polo-box Cloning project developed by Associate Professor Brian Gabrielli. The experimental program and all supporting materials were adapted for student use by Dr Peter Darben, under the supervision of Associate Professor Brian Gabrielli and Stephanie Le.

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Risk assessments were developed with the assistance of Paul Kristensen, Maria Somodevilla-Torres and Jane Easson.

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## Introduction

Deoxyribonucleic acid (DNA) is the molecule which carries the genetic instructions for almost every living thing. Its unique chemistry not only allows this information to be copied and passed on to an organism's descendants, it also allows scientists opportunities to investigate and manipulate an organism at a molecular level. As a result, molecular biology techniques are at the forefront of most cutting edge scientific research. In this project you will investigate a number of commonly used molecular biology techniques involving DNA.

### What is Molecular Biology ?

Molecular biology is the study of living things at the level of the molecules which control them and make them up. While traditional biology concentrated on studying whole living organisms and how they interact within populations (a "top down" approach), molecular biology strives to understand living things by examining the components that make them up (a "bottom up" approach). Both approaches to biology are equally valid, although improvements to technology have permitted scientists to concentrate more on the molecules of life in recent years.

Molecular biology is a specialised branch of biochemistry, the study of the chemistry of molecules which are specifically connected to living processes. Of particular importance to molecular biology are the nucleic acids (DNA and RNA) and the proteins which are constructed using the genetic instructions encoded in those molecules. Other biomolecules, such as carbohydrates and lipids may also be studied for the interactions they have with nucleic acids and proteins. Molecular biology is often separated from the field of cell biology, which concentrates on cellular structures (organelles and the like), molecular pathways within cells and cell life cycles.

The molecules which form the basis of life provide scientists with a more predictable and mechanistic tool for scientists to study. Working with whole organisms (or even just whole cells) can be unpredictable, with the outcome of experiments relying on the interaction of thousands of molecular pathways and external factors. Molecular biology provides scientists with a toolkit with which they may "tinker" with the way life works. They may use them to determine the function of single genes or proteins, and find out what would happen if that gene or protein was absent or faulty. Molecular biology is used to examine when and why certain genes are switched "on" or "off". An understanding of each of the factors has granted scientists a deeper understanding of how living things work, and used this knowledge to develop treatments for when living things don't work so well.

## Common Molecular Biology Techniques

The following list covers some of the more commonly used molecular biology techniques – it is by no means exhaustive.

**Electrophoresis** – a process which separates molecules such as DNA or proteins out according to their size, electrophoresis is a mainstay of molecular biology laboratories. While knowing the size of a molecule might not seem like all that much information, it can be used to identify molecules or fragments of molecules and as a check to make sure that we have the correct molecule present.

**Polymerase Chain Reaction (PCR)** – a process used to amplify very small amounts of DNA to amounts which can be used in further experiments. It is used as a basic tool in molecular biology to ensure that we have sufficient DNA to carry out further techniques such as genetic modification, however it has wider practical uses such as in forensics (identification using DNA profiling) and disease diagnosis. PCR can also be used to introduce small point mutations into a gene in a process called site-directed mutagenesis.

**Restriction Digest** – the process of cutting DNA up into smaller fragments using enzymes which only act at a particular genetic sequence.

**Ligation** – the process of joining two pieces of DNA together. Ligation is useful when introducing a new piece of DNA into another genome.

**Blotting** – a technique used to specifically identify biomolecules following electrophoresis. The molecule of interest is indicated using either a labeled probe (a complementary strand of nucleic acid) or a labeled antibody raised against a specific protein.

**Cloning** – the technique of introducing a new gene into a cell or organism. This can be used to see what effect the expression of that gene has on the organism, to turn the organism into a factory which will produce large quantities of the gene or the protein it codes for, or (within the inclusion of a label) to indicate where the products of that gene are expressed in the organism. Insertion of genetic material into a bacterium is called transformation, while insertion into a eukaryotic cell is called transfection. If a virus is used to introduce this material, the process is called transduction.

Each of these techniques is used in conjunction with other techniques to help scientists solve a particular research question. For example, following using **PCR** to create large quantities of a particular gene a scientist may **ligate** a gene for a particular protein into a plasmid vector (a short circular strand of DNA which acts as a carrier), perform a quick **restriction digest** and **electrophoresis** to ensure that the gene has been inserted properly, and then use that plasmid to **transform** a bacterial cell which is used to produce large quantities of the vector. After purification of the vector from the bacteria, it is then used to **transfect** a mammalian cell in culture. The scientist then uses protein **electrophoresis** and **western blotting** to demonstrate the expression of the gene product.

## The Project

Many molecular biology techniques take a significant amount of time to complete. Many of the enzyme-based reactions which underpin these techniques require incubation periods of an hour or more, while cloning and transformation often requires overnight incubation to allow the transformed cells time to recover and multiply. In the limited time we have available, we cannot hope to cover all molecular biology techniques, however you will undertake a mini-project which will expose you to some of the more important DNA-based techniques.

In this project you are tasked with generating multiple copies of a region of a gene using the polymerase chain reaction (PCR). Once the reaction is complete, you will demonstrate the presence of the DNA fragment using agarose gel electrophoresis.

### Getting Started

Before you begin, make sure that you are familiar with the relevant theory behind the techniques we will be performing. This manual contains several appendices which will provide you with this information. Make sure you read this information before proceeding.

#### **Appendix A : DNA**

#### **Appendix B : DNA Replication**

#### **Appendix C : The Polymerase Chain Reaction**

#### **Appendix D : Using a Micropipette**

#### **Appendix E : Glossary of Terms**

Other molecular biology techniques are provided at the SPARQ-ed website at :

<http://www.di.uq.edu.au/sparqedservices#background>

## Theoretical Basis of the Project

### The Polymerase Chain Reaction

Scientists have investigated the nature and role of DNA ever since Watson and Crick reported its structure in 1953. The vital role that this molecule plays in all living organisms means that it can be used in a wide variety of applications, from identification of an individual due to their unique DNA profile, to engineering living things so that they are better suited to survival, or can be used to produce useful products.

Until recently, however, this application of our knowledge of DNA was limited by the amount of DNA we could get our hands on. If we wanted to introduce a gene for a useful protein into another organism, we had to recover that gene initially from large quantities of tissue from the organism where the gene was found. If we wanted to use DNA profiling to identify a suspect in a criminal case, we had to have large quantities of tissue or body fluids from the suspect to be able to demonstrate the profile. This changed with the development of the polymerase chain reaction, or PCR.

In 1983, Kary Mullis reported a technique which utilized DNA's ability to self-replicate in the presence of enzymes called polymerases and the nucleotides which form the building blocks of DNA. Using polymerases from thermophilic ("heat-loving") bacteria which operate at temperatures higher than most organisms can tolerate and custom made primers composed of single strands of DNA complementary to regions around the gene of interest, Mullis' new technique allowed scientists to make billions of copies of a single length of DNA. Now, large quantities of DNA for genetic transformation can be synthesized at a relatively low cost, and enough DNA to produce a profile could be derived from a single hair or drop of blood.

PCR consists of three main stages :

**Denaturation** – heating the DNA to around 95°C to separate the two strands of DNA

**Annealing** – cooling the DNA to 50-60°C to allow the primers to attach, and

**Extension** – heating the DNA to 72°C so that the polymerase enzymes can complete the complementary strands from the nucleotides, starting at the primers

This process is repeated 25-35 times, with the DNA roughly doubling every cycle. When Mullis first developed the procedure he performed each cycle manually using heated waterbaths, however the mechanical nature of the process made it well suited to automation, and most PCR is now performed using PCR cyclers which can be programmed to perform reactions using different combinations of time and temperature. PCR now forms the basis of many molecular biology investigations. For his contribution to science, Kary Mullis was awarded the Nobel prize for chemistry in 1993. A more detailed explanation of PCR can be found at the SPARQ-ed website at <http://www.di.uq.edu.au/spargqpcr>.

In this exercise, you will be amplifying a small region of the gene for a protein called polo-like kinase 1 (PLK1), which is involved in the regulation of the cell cycle and plays an important role in cellular processes which occur during mitosis. The region of the PLK1 gene we will be amplifying codes for a part of the protein called the polobox domain. This domain allows PLK1 to localize to various parts of the cell during the cell cycle. A knowledge of this part of the gene is useful, as disturbances to the normal function and localization of PLK1 is found in some conditions caused by changes to the normal cell cycle, such as cancer.

One way of thinking about what you are doing in this exercise is to imagine the human genome as an enormous library with 23,000 books, each book representing a gene for a particular protein. We have provided you with a copy of the “PLK1 book” (the template DNA, a circular length of DNA called a plasmid which contains the gene for PLK1). Your task will be the equivalent of making billions of copies of the “polobox page” of the PLK1 book. To help you with this, we have also given you “bookmarks” in the form of primers which are upstream (before) and downstream (after) the polobox page and plenty of “copier paper” (nucleotides). You will use the molecular biology equivalent of a photocopier (*Pfu* polymerase and a PCR cycler) to make these copies.

## Agarose Gel Electrophoresis

Most of this procedure involves adding minute quantities (eg. 1 $\mu$ L, the volume enclosed by a cube 1mm on a side) of colourless liquid to minute quantities of other colourless liquids. When the reaction is complete, you are left with a small volume of colourless liquid which looks no different to what you started with. Therefore you will need to use a technique which shows you have made the correct DNA. This is where electrophoresis comes in.

If we wanted to sort sand from gravel from larger rocks, we would use a series of sieves of different sizes. Each sized sieve lets smaller particles pass through but retains the larger fragments. Electrophoresis can be thought of as a sieve for large molecules like DNA or protein.

In agarose gel electrophoresis, a DNA sample is loaded towards one end of a block of a jelly-like substance called agarose. When an electrical current is passed through the gel, the DNA molecules are pushed through the gel away from the negative electrode (DNA has an overall negative charge and like charges repel). Smaller fragments of DNA can move more easily through the gel than larger fragments, so in a given period of time, DNA of different sizes accumulates in regions of the gel. If we include a dye which binds to the DNA, these regions are visible as bands – the further towards the positive electrode a band is located, the smaller the fragments of DNA are found in that band.

To get an idea of the size of a band seen on a gel, we always run a sample consisting of a mixture of DNA fragments of known sizes alongside our test samples. This is called a marker, or a “ladder”, as the multiple bands of DNA seen on the gel resembles the rungs on a ladder. By matching the position of a band in our test sample to those representing DNA of known size in the ladder, we can estimate the size of DNA fragments in our test. The part of the PLK1 gene which codes for the polobox domain is 800 base pairs (bp) long. Therefore, if our PCR has been a success, we should see a band corresponding to our DNA markers which are 800 base pairs long.

More detailed information on electrophoresis can be found at <http://www.di.uq.edu.au/spargDNAelectrophoresis>.

## Experimental Protocol

### How to Use this Manual

Throughout this section you will see a series of icons which represent what you should do at each point. These icons are:



Write down a result or perform a calculation.



Prepare a reaction tube.



Incubate your samples.

When you are asked to deliver a set volume, the text will be given a colour representing the colour of the micropipette used:

- e.g.
- |             |  |
|-------------|--|
| 750 $\mu$ L | Use the blue P1000 micropipette (200-1000 $\mu$ L)       |
| 100 $\mu$ L | Use the strong yellow P200 micropipette (20-200 $\mu$ L) |
| 15 $\mu$ L  | Use the pale yellow P20 micropipette (2-20 $\mu$ L)      |
| 2 $\mu$ L   | Use the orange P2 micropipette (0.1-2 $\mu$ L)           |

## Polymerase Chain Reaction

The first step is a simple one – we need to set up our PCR tubes. Any PCR reaction uses the same basic ingredients :

- Deoxyribonucleoside triphosphates (dNTPs) – adenosine triphosphate, thymidine triphosphate, cytidine triphosphate and guanosine triphosphate – these are the raw materials for making our DNA during PCR.
- A polymerase enzyme to make the DNA (in this case *Pfu* Polymerase)
- Buffer – this is a solution of salts and ions which stabilises the reaction by ensuring that the pH remains constant.
- Primers (forward and reverse).
- Plasmid vector containing the PLK1 gene to act as our template.
- Distilled water to make the volume up to 50µL.

Keep in mind that PCR is a notoriously sensitive procedure – it will amplify **any** DNA it encounters in the reaction mixture, so long as the primers bind. Therefore, it is important to limit contamination in this part of the experiment. We use specially purified water and sterile tips on our micropipettes.

Many of the volumes are quite small and will need the use of the **P2** micropipette. To ensure that the majority of the material is dispensed, add the largest volume (the dH<sub>2</sub>O) first and draw the liquid up and down inside the pipette tip a few times. Make sure you dispose of your tips between samples.

You will be making up two tubes: a *sample* (which has all of the ingredients you need to make your gene fragment) and a *negative control* (which has everything except the DNA vector). The inclusion of the control is a way of testing whether the reaction worked or not.

### What is 10X Buffer ?

Sometimes you will see a solution provided at “10X” concentration. As the name suggests, this means that the solution is ten times more concentrated than it needs to be. Whenever you add one solution to another, each solution is *diluted* by the presence of the other. In order to use a solution appropriately, you will need to add it to the reaction mixture so that it will be properly diluted by the addition of the other reagents. For a 10X solution, this means that the volume of this solution is one tenth of the final volume.

The volumes needed for the PCR reaction are provided below:

Tube Label	dH <sub>2</sub> O	10X Buffer	DNA Template	dNTPs	Forward Primer	Reverse Primer	<i>Pfu</i> II Ultra Polymerase
Sample	35.2 µL	5 µL	4.8 µL	2 µL	1 µL	1 µL	1 µL
Control	40 µL	5 µL	-	2 µL	1 µL	1 µL	1 µL

The thermal cycler in the SPARQed laboratory has been programmed for this experiment and the reaction will run for nearly 2½ hours. For your own information, the details of the program are:

95°C	30 seconds	} 30 Cycles
95°C	30 seconds	
60°C	30 seconds	
72°C	2 minutes	
72°C	10 minutes	
4°C	Until ready	

- Place the tubes containing your test and control solutions into the thermal cycler, close the lid and run the program “pbx001”

## Agarose Gel Electrophoresis

In order to check whether our PCR has been a success, we need to check the size of any fragments of DNA found in our test solution (our control solution, lacking template DNA, should contain no detectable DNA). We do this using agarose gel electrophoresis.

### Preparation of TAE Buffer

Electrophoresis uses an electric field to “push” DNA fragments through the gel. To ensure that this occurs efficiently, all of the DNA must have a negative charge (to ensure that it is pushed away from the negative terminal). This is done using a buffer which keeps the experiment at a pH where all of the DNA is negatively charged.

The buffer most commonly used in DNA analysis is TAE, which stands for Tris – Acetate – EDTA (where EDTA stands for ethylenediaminetetracetic acid). The buffer is usually made up at 50X concentration and then must be diluted when needed (this allows us to make and store large amounts of the buffer without having to remake it as often).

For the whole group, we will only need around 500mL of TAE buffer. You will need to prepare 500mL of 1X TAE buffer from the 50X stock solution provided. Perform the following calculations :

~~✍~~ Total volume = 500mL

~~✍~~ 1/50 of 500mL =  $500 \div 50 =$  \_\_\_\_ mL

∴ volume of 50X stock needed is \_\_\_\_ mL

 Volume dH<sub>2</sub>O needed = Total volume - Volume stock needed

= 500mL – \_\_\_\_ mL

= \_\_\_\_ ml

 Dilute \_\_\_\_ mL stock in \_\_\_\_ mL of dH<sub>2</sub>O



Use the calculations above to prepare 500mL of 1X TAE buffer

## Preparation of Gel

The gel used to studying DNA is made from agarose, a jelly-like substance derived from seaweed. This material is supplied in powder form, and must be dissolved in the TAE buffer. For our experiment, we require a gel containing 0.8% agarose, ie. 0.8g of agarose powder dissolved in 100mL of buffer.



Weigh out 0.8g of agarose powder and suspend in 100mL of TAE buffer in a conical flask. One quantity is sufficient for the entire class

- Microwave the solution on HIGH for 2 minutes (for a small gel). Make sure that the agarose is completely dissolved by swirling the heated mixture roughly every 30 seconds. Allow it to cool for 3 minutes.



**TAKE CARE: Do not put a lid on the flask while microwaving, otherwise the flask may explode.**



**TAKE CARE: The agarose solution is quite hot. Use gloves and be careful not to spill any of the solution.**

- Wipe a plastic gel tray and comb with 70% ethanol and place in the electrophoresis tank so that the rubber tubing forms a seal with the sides of the tank.
- Add 10µL of SYBR-Safe into the melted agarose and swirl to mix. This substance is a dye which binds to the DNA and glows green under ultraviolet light – it allows us to see where the DNA has migrated in the gel.
- Pour the melted agarose into the gel tray. Place the comb into the right position and allow it to set for approximately one hour (this can be done faster by placing the gel tray in the refrigerator).
- Carefully remove the comb from the gel. Rotate the gel tray so that the wells are toward the negative (black) terminals (the top of the tank, assuming that the electrodes are on the right hand side). Cover the gel with 1X TAE running buffer.

## Loading the Gel

The samples must now be loaded into the wells in the gel left by the comb. To make this process easier, we mix the samples with a blue dye and glycerol. The dye migrates before all of the DNA and we can use this to tell when to stop running the gel. The glycerol increases the density of the sample so that it sinks to the bottom of the well on loading. The dye is provided at 6X the required concentration. This means that we have to add it to the sample in a proportion which dilutes it 1 in 6 (ie. five times as much sample as dye). Use the following calculation to find out how much dye is needed to add to a given volume of sample :

- We are only going to use 10µL of our digest product
- if the volume of dye added is “x” :

$$x + \text{Volume of DNA} = 6x$$

 Volume of dye needed to add to 10µL of PCR product = \_\_\_\_\_ µL

 Prepare loading solutions for each of your samples and DNA ladder.

 Load all of the loading solutions into separate wells in the gel (loading the DNA ladder last into a separate well on the left or right hand side of your gel). Use the table below to keep track of where you have loaded each sample:



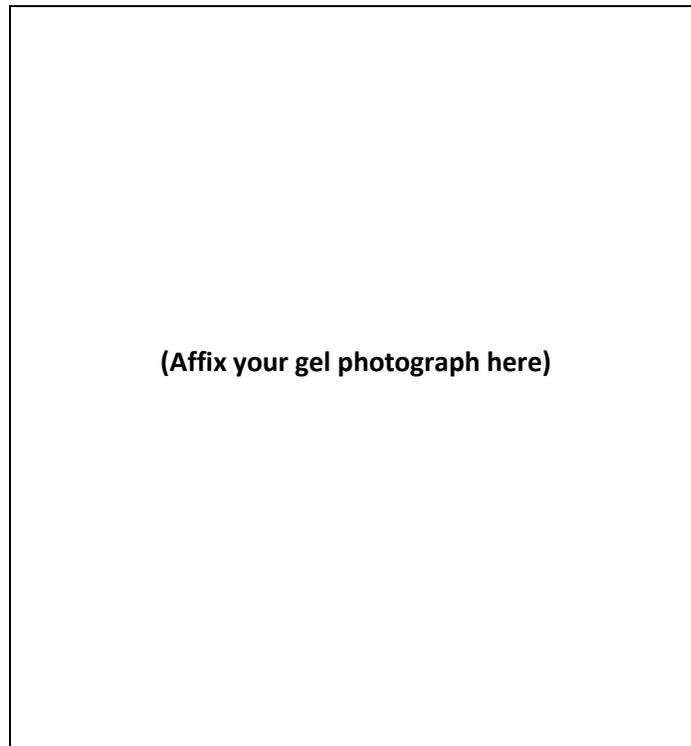
Loading End - Negative (Black) Electrode							
Sample ID #1	Sample ID #2	Sample ID #3	Sample ID #4	Sample ID #5	Sample ID #6	Sample ID #7	Sample ID #8

## Running the Gel

- Run the gel at 80V. There must be small bubbles rising from both ends of the electrophoresis chamber. Check after 5 minutes to make sure the gel is running (i.e. the dye front has moved, is relatively straight and has run the correct direction). Then allow the gel to run for the necessary amount of time (about 1 hour however, check that the dye front has almost run through the gel).

 **TAKE CARE:** While the electrophoresis tanks are well insulated, they still feature high voltages and conductive solutions. Ensure that the power pack is switched off and the leads unplugged before opening the tank.

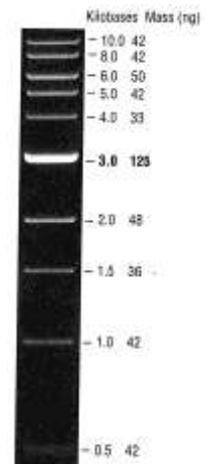
- Switch off the power pack and take the gel to the illuminator. Take a photograph, print off and glue into your workbook in the space below. Annotate the photograph using the ID table you completed above, indicating bands of interest.
- Pour away the buffer from the electrophoresis tank and rinse well with water. Rinse the gel tray and comb as well.



### Interpreting Your Gel

Whenever we run a gel, we should always include a DNA “Ladder” which features fragments of DNA of known size. This ladder serves as a reference point to indicate the size of the DNA fragments in our sample. A map of the ladder we are using in this exercise is provided in Figure 1.

Examine the photograph of your gel and check the sizes of bands. The polobox domain portion of the PLK1 gene is approximately 800 base pairs (or 0.8 kilobases) long. If the PCR has been a success, you should see a single band just below the “1.0kb” marker.



**Figure 1: Map of 1kb DNA Ladder**

## Appendix A : DNA

- Deoxyribonucleic acid (DNA) is a large molecule which stores the genetic information in organisms. It is composed of two strands, arranged in a double helix form. Each strand is composed of a chain of molecules called **nucleotides**, composed of a phosphate group, a five carbon sugar (pentose) called deoxyribose and one of four different nitrogen containing bases.

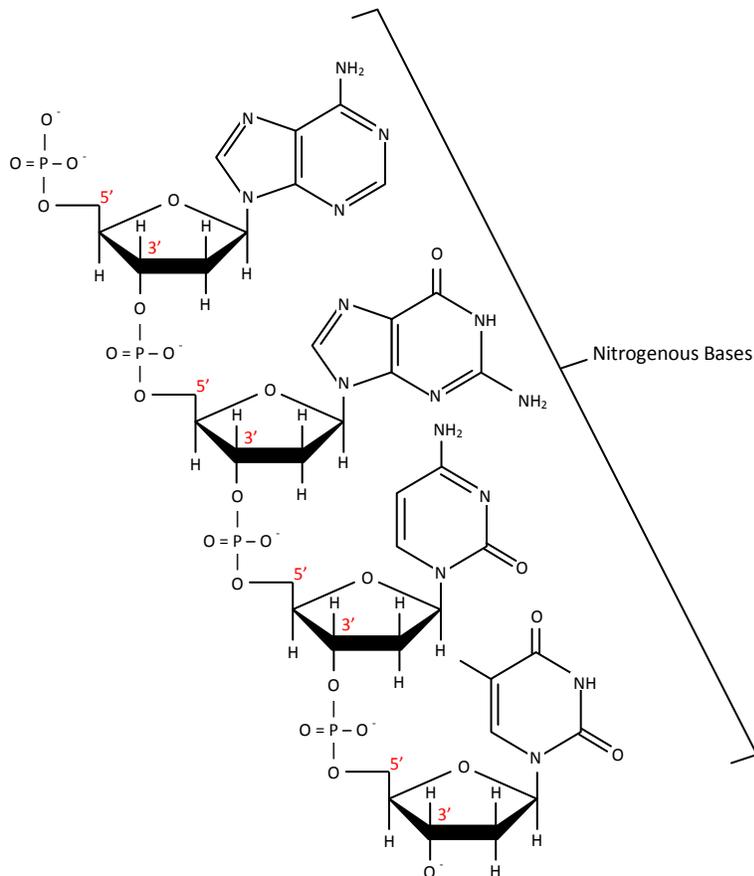
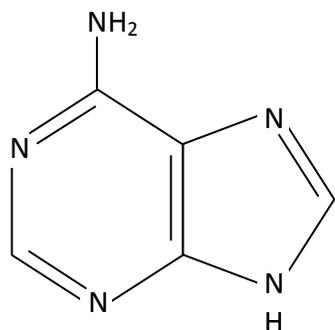


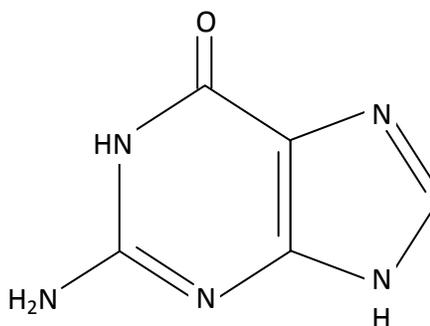
Figure A1 – The Structure of a Single Strand of DNA

- Each nucleotide is connected to the next by way of covalent bonding between the phosphate group of one nucleotide and the third carbon in the deoxyribose ring. This gives the DNA strand a “direction” – from the 5’ (“five prime”) end to the 3’ (“three prime”) end. By convention, a DNA sequence is always read from 5’ → 3’ ends.

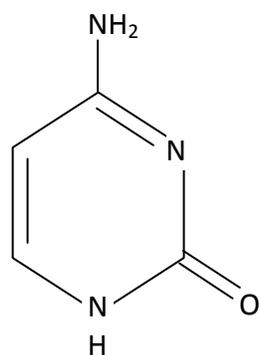
- DNA nucleotides contain one of four different nitrogenous bases:



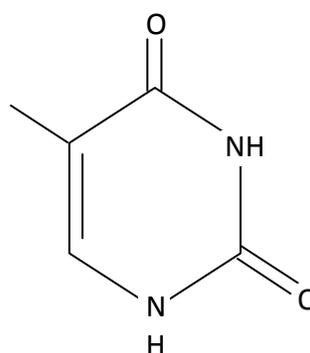
Adenine



Guanine



Cytosine



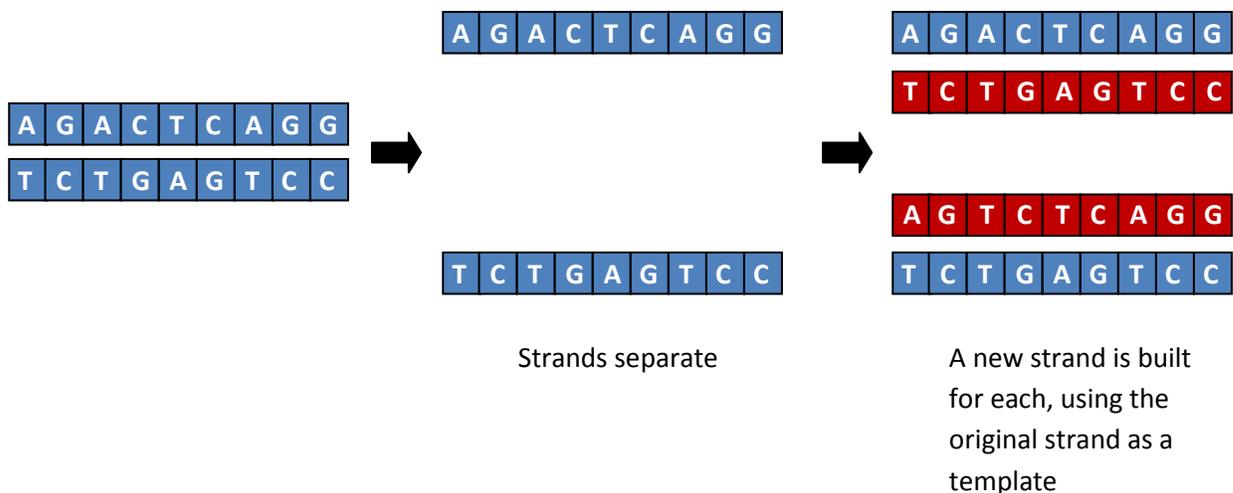
Thymine

Each of these bases jut off the sugar-phosphate “backbone”. If the double helix of the DNA molecule can be thought of as a “twisted ladder”, the sugar-phosphate backbones form the “rails”, while the nitrogenous bases form the “rungs”.

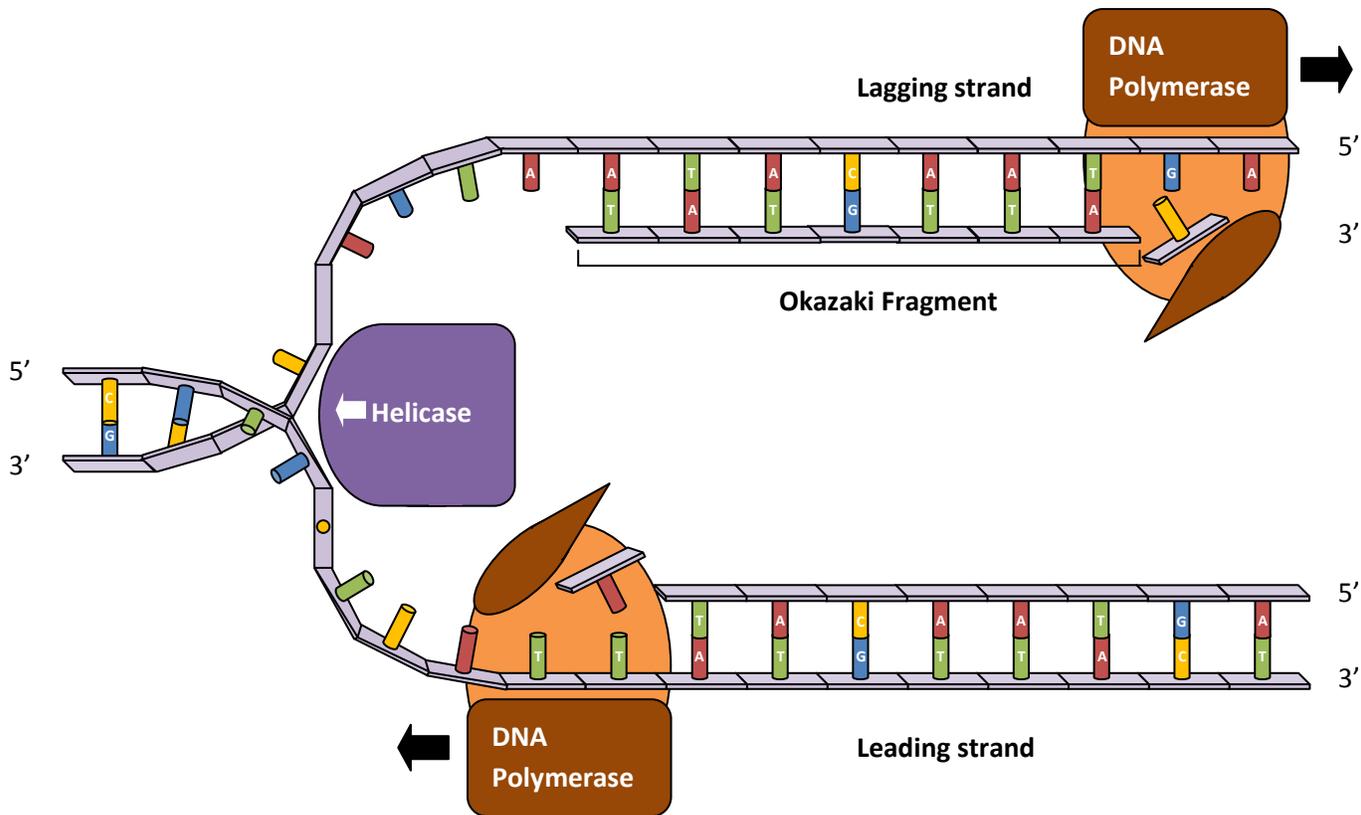
- The two strands of DNA are bound together by hydrogen bonding between the nucleotides. Adenine always binds to thymine and guanine always binds to cytosine. This means that the two strands of DNA are **complementary**. The complementary nature of DNA allows it to be copied and for genetic information to be passed on - each strand can act as a template for the construction of its complementary strand.
- The order of bases along a DNA strand is called the **DNA sequence**. It is the DNA sequence which contains the information needed to create proteins through the processes of transcription and translation.
- Each strand of DNA is **anti-parallel**. This means that each strand runs in a different direction to the other – as one travels down the DNA duplex, one strand runs from 5' → 3', while the other runs 3' → 5'.
- An animation of the structure of DNA can be found at:  
<http://www.johnkyrk.com/DNAanatomy.html>

## Appendix B DNA Replication

- The structure of DNA allows it to carry out two vital functions for the cell :
  - ✦ Encoding the information need to build and regulate the cell, and
  - ✦ Transmission of this information from generation to generation
- In order for the genetic information to be passed on, it must be copied. DNA replication occurs during the S (synthesis) phase of the cell cycle. It only proceeds if the G1 checkpoint is passed, which ensures that the chromosomes have properly segregated during mitosis.
- In simple terms, DNA involves the separation of the two strands of the DNA molecule and the construction of complementary strands for each one, using the A → T, G → C binding rules.



- Because the two new strands of DNA each contain one of the original parental strands, the process of DNA replication is said to be **semi-conservative** (ie. half of the new DNA molecule are strands “saved” from the parental molecule).
- Naturally, the process of replication is a more complicated process than simply matching nucleotide bases. Copying DNA involves the interplay of a series of enzymes and regulatory processes, all kept in check by stringent error checking and repair mechanisms.
- DNA replication begins when the enzyme **helicase** “unwinds” a small portion of the DNA helix, separating the two strands. This point of separation is called the **replication fork**. The two strands are kept separated by **single stranded binding proteins** (SSB) which bind onto each of the strands. A group of enzymes called the **DNA polymerases** are responsible for creating the new DNA strand, however they cannot start the new strand off, only extend the end of a pre-existing strand. Therefore, before the DNA polymerases can start synthesizing the new strand, the enzyme **primase** attaches a short (~60 nucleotides) sequence of RNA called a **primer**. The DNA polymerases then extend this primer, moving along each strand from the 3' end to the 5' end and adding nucleotides to the 3' hydroxyl group of the previous nucleotide base. The order of nucleotides is retained by matching complementary nucleotides on the template strand.



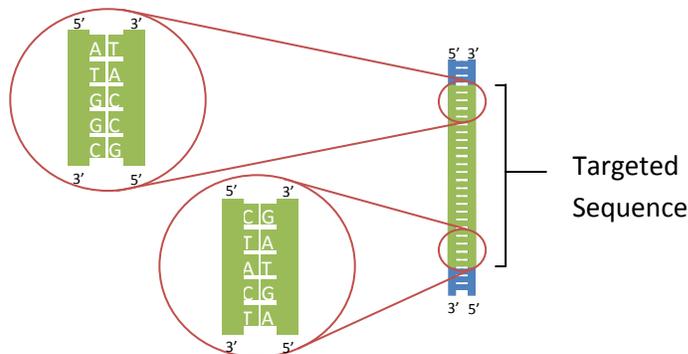
- It's important to realize that the polymerases can only operate in one direction. This works out for one of the DNA strands (the **leading strand**) – the polymerase moves along the strand in the same direction as the replication fork. However the other strand (the **lagging strand**) runs in the opposite direction. As a result the complementary strand to the lagging strand is made in short sections called **Okazaki fragments**. These sections are then later joined together by the enzyme **DNA ligase**.
- Once the complementary strand of DNA has been synthesized, the primers are removed by the enzyme **RNAse H** and the remaining gaps filled with lengths of DNA by DNA polymerase.
- Some excellent animations of DNA replication can be found here :
  - This is a tutorial which takes you through the process step by step.  
[http://www.wiley.com/college/pratt/0471393878/student/animations/dna\\_replication/index.html](http://www.wiley.com/college/pratt/0471393878/student/animations/dna_replication/index.html)
  - This animation is a computer generated movie showing what the process would look like on a molecular level  
<http://www.youtube.com/watch?v=teV62zrm2P0>

## Appendix C THE POLYMERASE CHAIN REACTION (PCR)

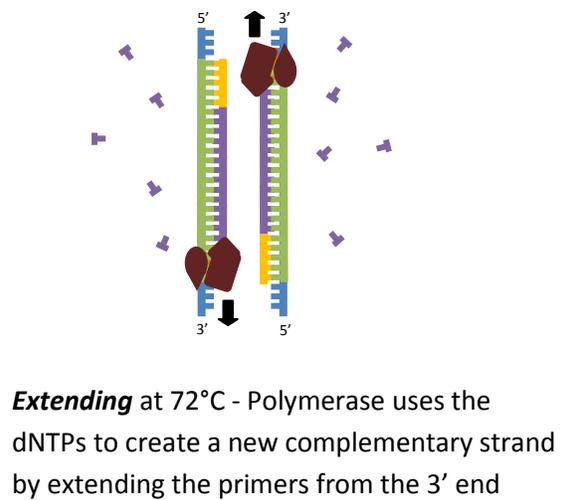
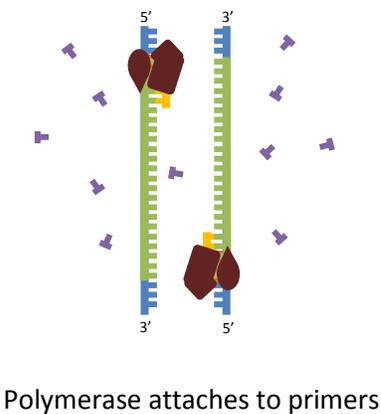
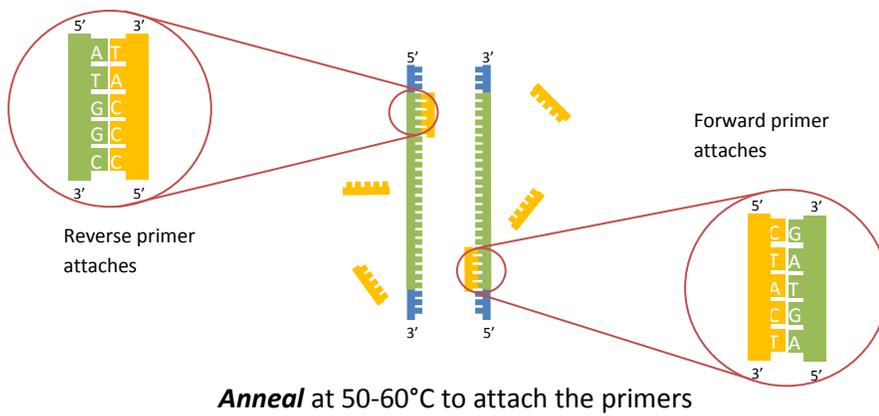
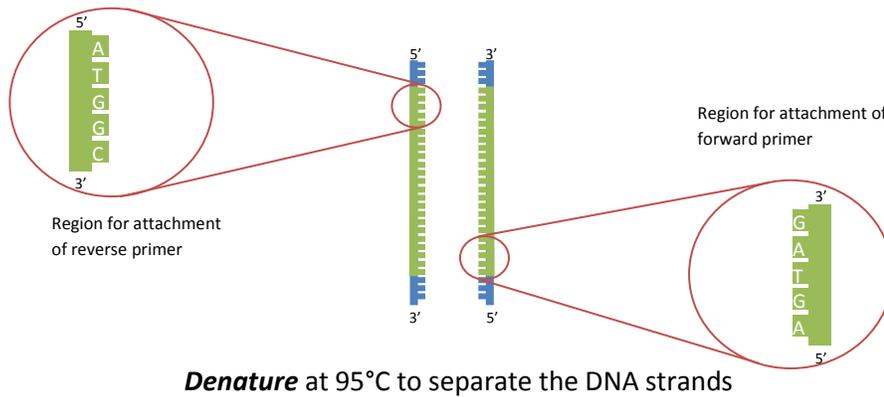
- The polymerase chain reaction is a technique which has revolutionized molecular biology since its development in the early 1980s. It allows researchers to amplify small amounts of DNA to quantities which can be used for analysis. Some of the uses to which PCR has been applied include :
  - ✦ Disease diagnosis, where the causative agent of a disease is identified by its DNA. This is particularly useful when disease agents are difficult to grow in culture or are present in low numbers in a sample
  - ✦ Forensic investigations, where trace amounts of DNA found at crime scenes (eg. in hair, tissue or body fluids) may be amplified up to a level which allows them to be analysed using methods like DNA profiling. PCR may also be used in other areas where the amount of DNA recovered is vanishingly small (eg. archaeology)
  - ✦ Genetic engineering, where genes are introduced into new species – to do this, the genetic material to be introduced must be of a sufficient quantity to ensure efficient transformation of the host cell
- PCR relies on a number of characteristics of the DNA molecule :
  - ✦ The structure of DNA consists of two strands of chains of molecules called **nucleotides**. Each of these nucleotides consists of a phosphate group, a sugar (deoxyribose) and one of four nitrogen containing bases (adenine, thymine, guanine or cytosine). Each strand runs in the opposite direction to the other (ie. they are **anti-parallel**), with only certain bases lying opposite each other (an adenine is always opposite a thymine and a guanine is always opposite a cytosine). This means that the two strands are **complementary** to each other in the order of their bases.
  - ✦ The two strands of DNA are held together by hydrogen bonding between nucleotide base pairs. Hydrogen bonds are much weaker than the covalent bonds which link individual nucleotides within a strand and may be disrupted by heating the DNA. Therefore, we can separate the two strands of DNA without breaking the DNA strands down by heating to around 95°C – this process is called **denaturation**.
  - ✦ **Primers** are short sequences of complementary DNA which bind to certain nucleotide sequences along the DNA strand. They tend to bind onto the single DNA strands at higher temperatures than the entire complementary strand. This means that if the temperature is cooled from 95°C to around 50-60°C, the primers will bind to the single strands before the complementary whole strands do. This process is called **annealing**.
  - ✦ The production of a new complementary strand of DNA using a single strand is performed by a class of enzymes called **polymerases**. These enzymes start off by binding to the primers and then extend the primers by adding new nucleotides to the 3' end, using the single stranded DNA as a template.
  - ✦ Most polymerases function best at the temperatures that their cells operate at (eg. 37°C for human cells). However at this temperature, most of the entire complementary strands will also reattach and interfere with the function of the polymerase (in cells, the DNA strand is kept unwound during replication by enzymes such as helicase). There are some organisms which operate at much higher temperatures – *Thermus aquaticus* is a bacterium which lives in boiling hot

springs. The polymerases which it uses operate best at around 72°C. Therefore these enzymes may be used to ensure that the strands are kept separate during the extension process.

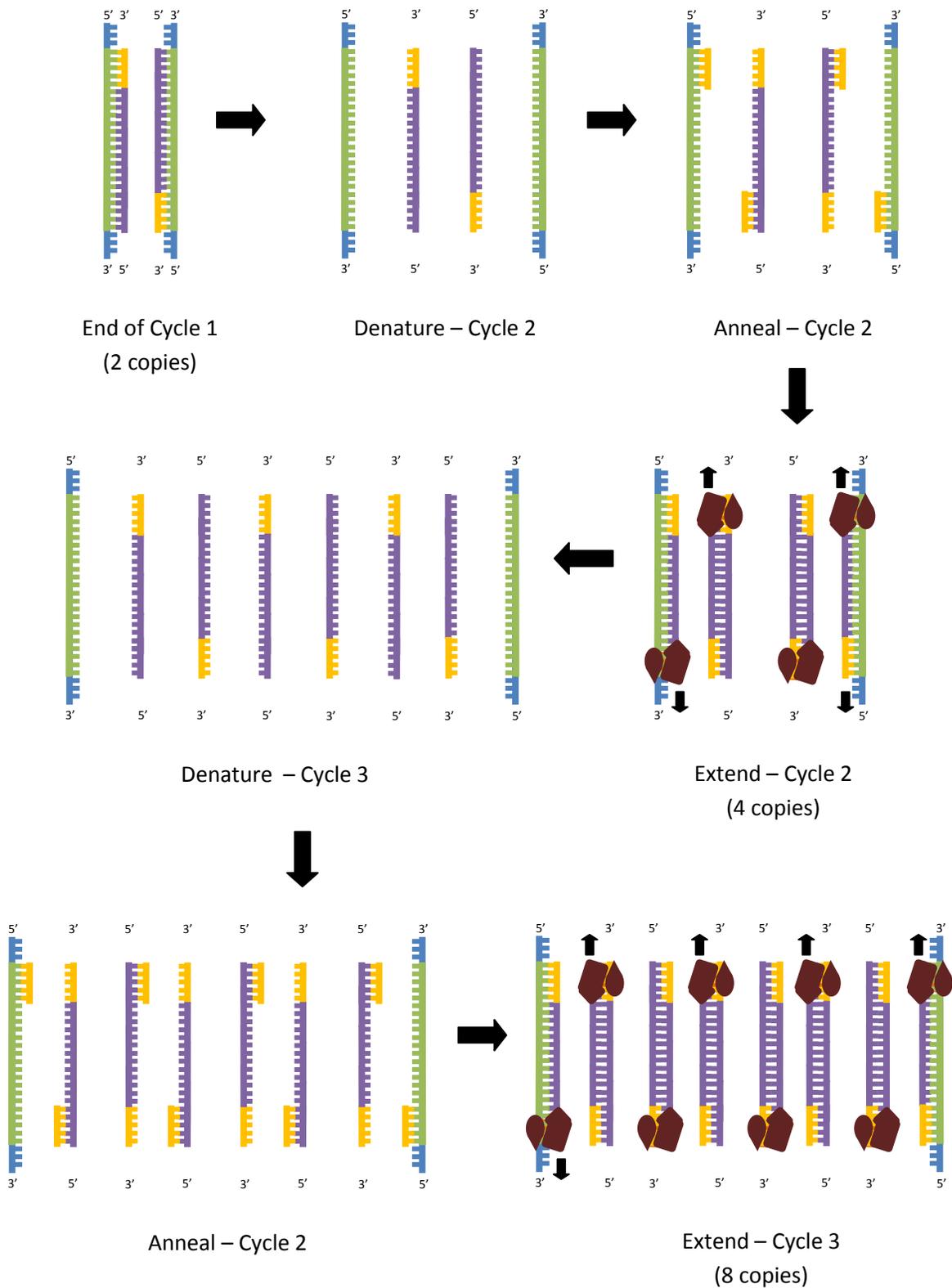
- PCR uses these characteristics to make copies of DNA – basically it is a stripped-down *in vitro* version of the methods that cells themselves use to copy their own DNA.
- A PCR technique needs the following reagents :
  - A DNA sample which acts as the template on which the new DNA will be built
  - 4 deoxyribonucleoside triphosphates (adenosine triphosphate, guanosine triphosphate, thymidine triphosphate and cytidine triphosphate) – these are the “building blocks” from which the new DNA molecules will be made
  - *Taq* Polymerase, or similar polymerase enzyme, which operates best at high temperatures
  - 2 Primers (forward and reverse) to start the process of replication. These primers are designed to be complementary to the nucleotide sequences at the beginning and the end of the section of DNA we want to amplify
  - Buffers and salts to create the correct conditions for the enzyme to function
- A lot of work has to go into designing the primers. Firstly, we need to know the sequence of the section of DNA we are wanting to amplify, particularly the “beginning” (5’) and “ending” (3’) of the sequence. The primers need to be designed so that they are complementary to a unique sequence of nucleotides “upstream” and “downstream of the sequence of interest. They cannot match a sequence within the area of interest (or the PCR will start off too late and miss a portion of the area we want to amplify), and they should also not have complementary regions within themselves (or they will fold over and bind to themselves, forming a “hairpin”. Lastly, the forward and reverse primers should not be complementary, or they will anneal to each other and form a “primer dimer”. We can avoid most of these problems using primers of 15-20 nucleotides in length (note that the examples in the diagrams below use 5 nucleotide primers for simplicity – we would not use these in a real PCR reaction.



- Different protocols need to be developed for each PCR procedure, depending on the primers used, the length of template DNA or the type of polymerase involved. However each protocol has the following basic steps :



- These steps are repeated between 25 and 35 times, with the amount of DNA roughly doubling each time. This might not seem like much, but after 35 cycles, one DNA molecule could theoretically yield in excess of 34,359,738,370 molecules ( $2^{35}$ ).



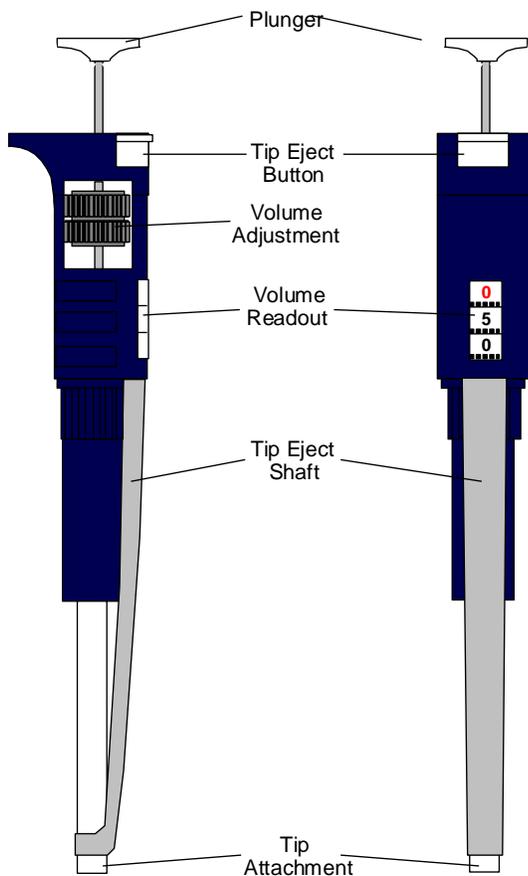
- When PCR was first developed, scientists had to change the temperature manually, swapping the samples between waterbaths kept at just the right temperature. However, now they use PCR cycler machines which heat and cool samples precisely and automatically.
- The best way to learn about how PCR works is to watch it in action. Visit <http://www.dnalc.org/ddnalc/resources/animations.html> and select the “Polymerase Chain Reaction” animation.

You might also want to listen to the “PCR song” at : <http://www.youtube.com/watch?v=x5yPkkCLads>

## Appendix D : Using a Micropipette

When scientists need to accurately and precisely deliver smaller volumes of a liquid, they use a *pipette* – a calibrated glass tube into which the liquid is drawn and then released. Glass and plastic pipettes have been mainstays of chemistry and biology laboratories for decades, and they can be relied upon to dispense volumes down to 0.1mL.

Molecular biologists frequently use much smaller volumes of liquids in their work, even getting down to 0.1 $\mu$ L (that's one ten thousandth of a millilitre, or one ten millionth of a litre!). For such small volumes, they need to use a *micropipette*.



Micropipettes are called a lot of different names, most of which are based on the companies which manufacture. For example, you might hear them called “Gilsons”, as a large number of these devices used in laboratories are made by this company. Regardless of the manufacturer, micropipettes operate on the same principle: a plunger is depressed by the thumb and as it is released, liquid is drawn into a disposable plastic tip. When the plunger is pressed again, the liquid is dispensed.

The tips are an important part of the micropipette and allow the same device to be used for different samples (so long as you change your tip between samples) without washing. They come in a number of different sizes and colours, depending on the micropipette they are used with, and the volume to be dispensed.

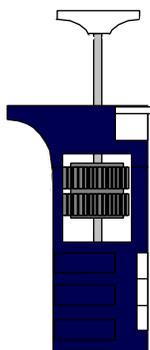
The most commonly used tips are:

- Large Blue – 200-1000 $\mu$ L
- Small Yellow – 2-200 $\mu$ L
- Small White - <2 $\mu$ L

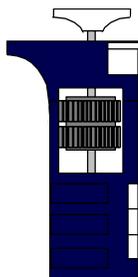
They are loaded into tip boxes which are often sterilised to prevent contamination. For this reason tip boxes should be kept closed if they are not in use. Tips are loaded onto the end of the micropipette by pushing the end of the device into the tip and giving two sharp taps. Once used, tips are ejected into a sharps disposal bin using the tip eject button. Never touch the tip with your fingers, as this poses a contamination risk.

The plunger can rest in any one of three positions:

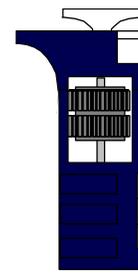
Position 1 is where the pipette is at rest



Position 2 is reached by pushing down on the plunger until resistance is met



Position 3 is reached by pushing down from Position 2



Each of these positions plays an important part in the proper use of the micropipette.

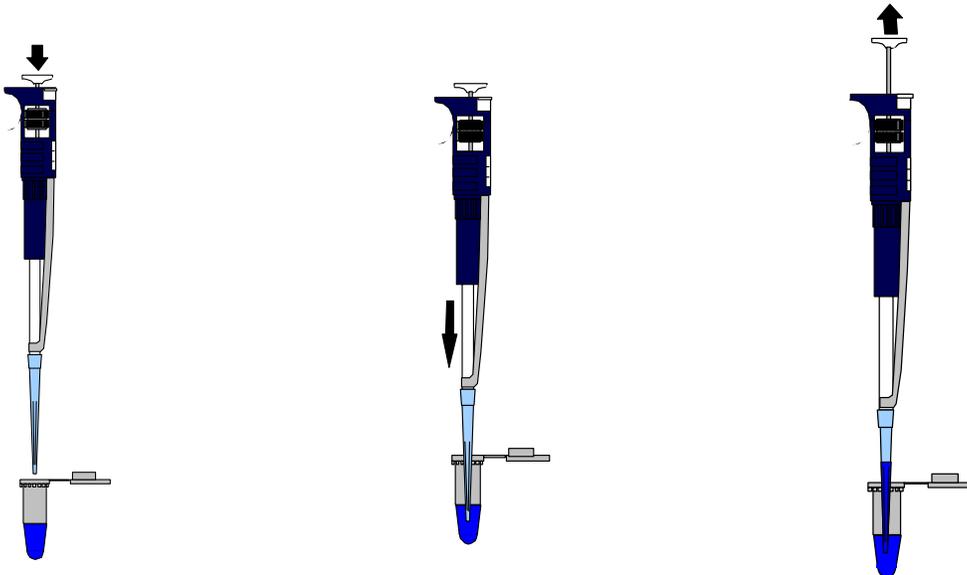
## To Draw Up Liquid:

- Hold the micropipette with the thumb resting on the plunger and the fingers curled around the upper body.

Push down with the thumb until Position 2 is reached.

Keeping the plunger at the second position, place the tip attached to the end of the micropipette beneath the surface of the liquid to be drawn up. Try not to push right to the bottom (especially if you are removing supernatant from a centrifuged pellet), but ensure that the tip is far enough below the surface of the liquid that no air is drawn up.

Steadily release pressure on the plunger and allow it to return to Position 1. Do this carefully, particularly with large volumes, as the liquid may shoot up into the tip and the body of the micropipette. If bubbles appear in the tip, return the liquid to the container by pushing down to Position 3 and start again (you may need to change to a dry tip).



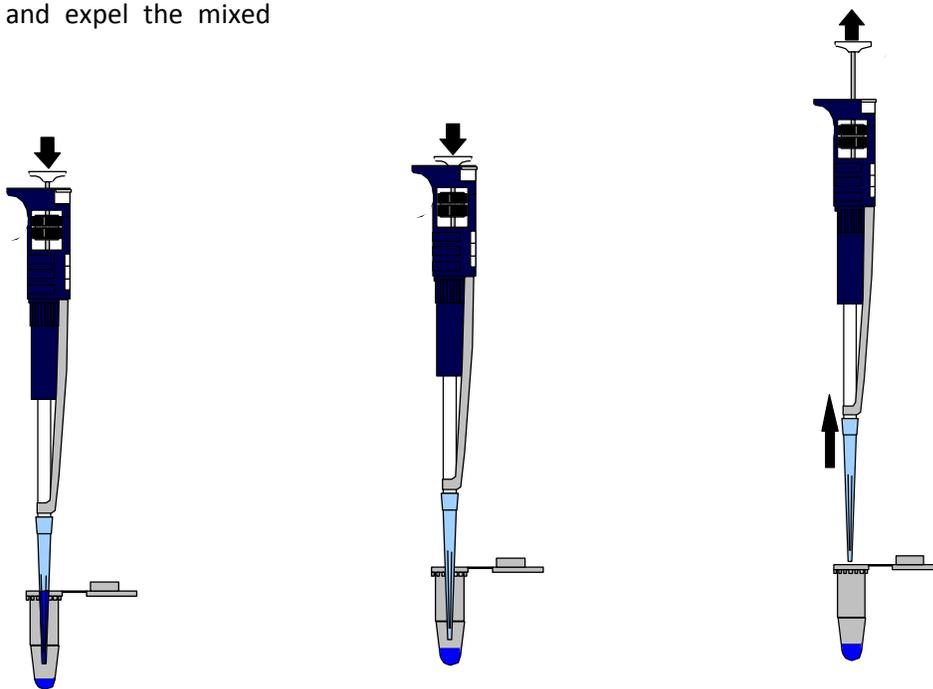
## To Dispense Liquid:

- Hold the micropipette so that the end of the tip containing tip is inside the vessel you want to deliver it to. When delivering smaller volumes into another liquid, you may need to put the end of the tip beneath the surface of the liquid (remember to change the tip afterwards if you do this to save contaminating stock). For smaller volumes you may also need to hold the tip against the side of the container.

Push the plunger down to Position 2. If you wish to mix two liquids together or resuspend a centrifuged pellet, release to Position 1 and push to Position 2 a few times to draw up and expel the mixed liquids

To remove the last drop of liquid from the tip, push down to Position 3. If delivering into a liquid, remove the tip from the liquid before releasing the plunger

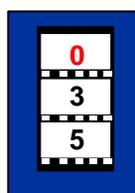
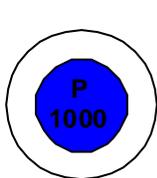
Release the plunger and allow it to return to Position 1



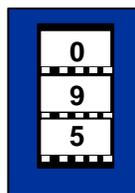
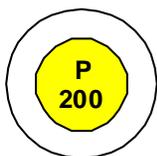
## Changing the Volume:

Some micropipettes deliver fixed volumes, however the majority are adjustable. Each brand uses a slightly different method to do this – Gilsons have an adjustable wheel, others have a locking mechanism and turning the plunger adjusts the volume. All have a readout which tells you how much is being delivered and a range of volumes which can be dispensed. Trying to dispense less than the lower value of the range will result in inaccurate measurements. Trying to dispense over the upper range will completely fill the tip and allow liquid to enter the body of the pipette. Do not overwind the volume adjustment, as this affects the calibration of the micropipette.

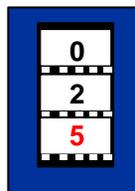
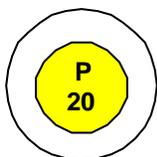
The way to interpret the readout depends on the micropipette used:



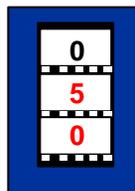
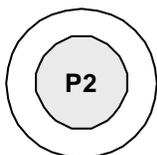
In a 200-1000 $\mu$ L micropipette (e.g. a Gilson P1000) the first red digit is thousands of  $\mu$ L (it should never go past 1), the middle digit is hundreds, while the third is tens. Therefore 1000 $\mu$ L would read as 100, while 350 $\mu$ L would read as 035.



In a 20-200 $\mu$ L micropipette (e.g. a Gilson P200) the first digit is hundreds of  $\mu$ L (it should never go past 2), the second is tens and the third is units. Therefore, 200 $\mu$ L would read as 200, while 95 $\mu$ L would read as 095.



In a 2-20 $\mu$ L micropipette (e.g. a Gilson P20) the first digit is tens of  $\mu$ L (it should never go past 2), the second is units and the third red digit is tenths. Therefore 20 $\mu$ L would read as 200, while 2.5 $\mu$ L would read as 025.



In a 0.2-2 $\mu$ L micropipette (e.g. a Gilson P2) the first digit is units of  $\mu$ L (it should never go past 2), the second red digit is tenths and the third red digit is hundredths. Therefore, 2 $\mu$ L would read as 200, while 0.5 $\mu$ L would read as 050.

## Appendix E : Glossary of Terms

**Agarose** – a substance derived from seaweed which forms a gel when dissolved in water. Agarose gels are used in DNA electrophoresis.

**Band** – a region of a gel containing DNA or protein fragments of a particular size.

**Bases** – the four organic molecules which are found in nucleotides. The bases found in DNA are adenine, thymine, guanine and cytosine. In RNA, thymine is replaced by uracil.

**Biochemistry** – the study of the chemistry of living things.

**Biomolecule** – a complex organic compound which is made as the result of a biological process. Also called **macromolecules**, because most are quite large.

**Blotting** – a technique where bands containing specific proteins are demonstrated using labeled antibodies raised against those proteins.

**Buffer** – a compound which helps to keep the pH of a solution stable and constant.

**Cancer** – a condition characterized by abnormal cell growth and multiplication, as well as migration of affected cells throughout the body.

**Cell** – the basic unit of all living things. Cells are metabolically active membrane bound bodies capable of reproduction.

**Cell Biology** – the study of processes which cells use to survive.

**Cell Cycle** – the progression of stages which a cell passes through in its growth and development. It consists of G1 (Gap 1) phase, where organelles are produced and the cell starts to increase in size, S (Synthesis) phase, where DNA is replicated so that each daughter cell has a complete copy of the genome, G2 (Gap 2) phase, where the cell checks that all is in order for division, and M (Mitosis) phase, where the chromosomes are separated (mitosis) and the cell divides into two daughter cells (cytokinesis). Following M phase, cells return to G1 phase should they need to divide again. Most cells go from G1 phase into G0 phase, where they carry out their normal cellular functions, as most cells do not need to constantly divide. Changes to the cell cycle can lead to a situation where the cells are constantly dividing, a state which may progress to cancer. An understanding of the processes which control the cell cycle can lead to ways to treat cancer, either by stopping the cell cycles of cancerous cells, or preventing cells from turning cancerous in the first place.

**Chromosome** – A length of DNA. Human cells have 46 linear chromosomes, while bacteria have a single circular chromosome.

**Comb** – a device used to create the wells in a gel into which the samples are loaded.

**Dilution** – reducing the concentration of a solution by adding more solvent.

**DNA** – deoxyribonucleic acid – the biomolecule which stores the genetic information in most living things. DNA consists of two strands of deoxynucleotides linked by phosphodiester bonds. The bases in the two nucleotide strands bind in complementary pairs (adenine to thymine, cytosine to guanine) through hydrogen bonds. This gives the molecule the appearance of a twisted ladder, with the sugar-phosphate chains forming the runners and the base pairs forming the rungs. The sugar in the nucleotides which make up DNA is deoxyribose.

**Downstream** – towards the 3' end of a strand of nucleic acid.

**Electrophoresis** – a technique which uses an electric field to separate DNA fragments or proteins by size through a gel.

**Enzyme** – a protein which acts as a biological catalyst – it speeds along reactions which would normally be too slow to be useful.

**Fragment** – a piece of DNA.

**Gel** – a semi-solid material used to separate DNA fragments or proteins by size during the process of electrophoresis.

**Gene** – a small section of DNA which contains the information used to produce a protein, or which controls and regulates the expression of other genes.

**Genome** – the sum total of all of the genes in an organism.

**Incubation** – a waiting period, to allow a reaction time to take place, or organisms time to grow and multiply.

**Kilobase** – a unit representing 1000 bases along a strand of DNA or RNA.

**Ladder** – a collection of bands in a gel produced by including a standard sample of DNA of known sizes. Used to estimate the size of DNA in test samples.

**Micropipette** – a device used to accurately and precisely deliver small quantities (<1mL) of liquid.

**Molecular Biology** – the study of how chemical processes contribute to living systems. Molecular biology concentrates largely on the nature of DNA and proteins.

**Nucleic Acid** – a biomolecule consisting of a chain of nucleotides connected by phosphodiester bonds. DNA and RNA are nucleic acids.

**Nucleoside** – a combination of one of the nitrogenous bases (adenine, guanine, thymine, cytosine or uracil) and a five carbon (pentose) sugar – deoxyribose in DNA or ribose in RNA.

**Nucleotide** – a nucleoside joined to a phosphate (PO<sub>4</sub>) group. Nucleotides make up nucleic acids.

**PCR** – the polymerase chain reaction – a technique which uses the replicative ability of DNA to amplify small amounts of DNA up to quantities suitable for study.

**pH** – the degree of acidity (low pH) or alkalinity (high pH) of a solution.

**Plasmid** – a small, circular “satellite” chromosome found in bacteria and capable of genetic exchange between bacteria.

**PLKI** – pololike kinase I – an enzyme which plays an important regulatory role in the cell cycle.

**Protein** – a biomolecule consisting of polypeptide chains folded up into three dimensional forms. Proteins play many roles in organisms, including being the building blocks of cellular structures, control and regulation of chemical reactions (enzymes), recognition and communication between cells (receptors and hormones) and defense (antibodies).

**Replication** – copying of DNA.

**Stock Solution** – a concentrated solution used to store reagents. Stock solutions are usually made to be a certain number of times more concentrated than the working solutions and so must be diluted by the factor to create the working solution. eg. 50X stock must be diluted 1 in 50 before it can be used.

**TAE** – tris-acetate-EDTA – a buffer used to run DNA gels because it keeps the solution at a pH where all of the DNA is negatively charged.

**Upstream** – towards the 5' end of a strand of nucleic acid.

**Well** – a “hole” cast in a gel using a comb into which the sample is loaded for electrophoresis.

**Working Solution** – the solution which is used in a chemical solution. Working solutions may be made up fresh or diluted from stock solutions. They are normally given the name “1X” to differentiate them from their stock solutions.