

Polymerase Chain Reaction (PCR)

- PCR is a means to amplify a particular piece of DNA
 - *Amplify= making numerous copies of a segment of DNA*
- PCR can make **billions** of copies of a target sequence of DNA in a few hours
- PCR was invented in the 1984 as a way to make numerous copies of DNA fragments in the laboratory
- Its applications are vast and PCR is now an integral part of Molecular Biology

DNA Replication vs. PCR

- PCR is a laboratory version of DNA Replication in cells
 - The laboratory version is commonly called “*in vitro*” since it occurs in a test tube while “*in vivo*” signifies occurring in a living cell.

PCR: the *in vitro* version of DNA Replication

The following components are needed to perform PCR in the laboratory:

- 1) DNA (your DNA of interest that contains the target sequence you wish to copy)
- 2) A heat-stable DNA Polymerase (like Taq Polymerase)
- 3) All four nucleotide triphosphates
- 4) Buffers
- 5) Two short, single-stranded DNA molecules that serve as primers
- 6) Thin walled tubes
- 7) Thermal cycler (a device that can change temperatures dramatically in a very short period of time)

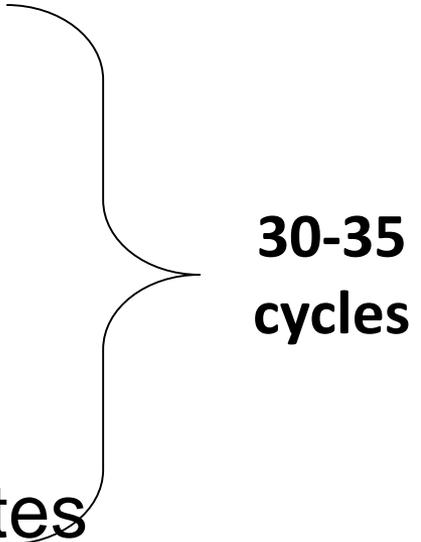
How PCR Works

- Reagents Needed
 - DNA sample which you want to amplify
 - DNA polymerase
 - Taq DNA polymerase – Works at high temps (explained in a minute)
 - Nucleotides
 - Called dNTPs
 - Pair of primers
 - One primer binds to the 5' end of one of the DNA strands
 - The other primer binds to the 3' end of the anti-parallel DNA strand
 - Delineate the region of DNA you want amplified
 - Water
 - Buffer

How PCR Works

- Protocol
 - Put all reagents into a PCR tube
 - Break the DNA ladder down the middle to create two strands, a 5' to 3' strand and a 3' to 5' strand
 - Melting or heat denaturation
 - Bind each primer to its appropriate strand
 - 5' primer to the 5' to 3' strand
 - 3' primer to the 3' to 5' strand
 - Annealing
 - Copy each strand
 - DNA polymerase
 - Extending

How PCR Works

- Temperature Protocol
 - Initial Melt: 94°C for 2 minutes
 - Melt: 94°C for 30 seconds
 - Anneal: 55°C for 30 seconds
 - Extend: 72°C for 1 minute
 - Final Extension: 72°C for 6 minutes
 - Hold: 4°C
- 
- 30-35
cycles**

PCR THERMOCYCLER

The DNA, DNA polymerase, buffer, nucleoside triphosphates, and primers are placed in a thin-walled tube and then these tubes are placed in the PCR thermal cycler.

The thermal cycler heats and cools the reaction tubes to achieve the temperatures required at each step of the reaction .

Many modern thermal cyclers make use of the Peltier effect, which permits both heating and cooling of the block holding the PCR tubes simply by reversing the electric current.

Thin-walled reaction tubes permit favorable thermal conductivity to allow for rapid thermal equilibration.



PCR Thermocycler

The three main steps of PCR

- The basis of PCR is **temperature changes** and the effect that these temperature changes have on the DNA.
- In a PCR reaction, the following series of steps is repeated **20-40 times**
(note: 25 cycles usually takes about 2 hours and amplifies the DNA fragment of interest 100,000 fold)

Step 1: Denature DNA

At **95°C**, the DNA is denatured (i.e. the two strands are separated)

Step 2: Primers Anneal

At **40°C- 65°C**, the primers anneal (or bind to) their complementary sequences on the single strands of DNA

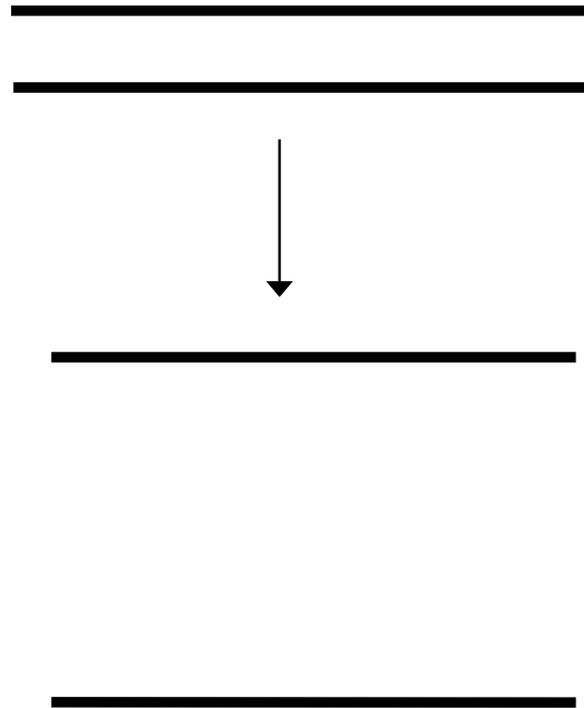
Step 3: DNA polymerase Extends the DNA chain

At **72°C**, DNA Polymerase extends the DNA chain by adding nucleotides to the 3' ends of the primers.

Heat-stable DNA Polymerase

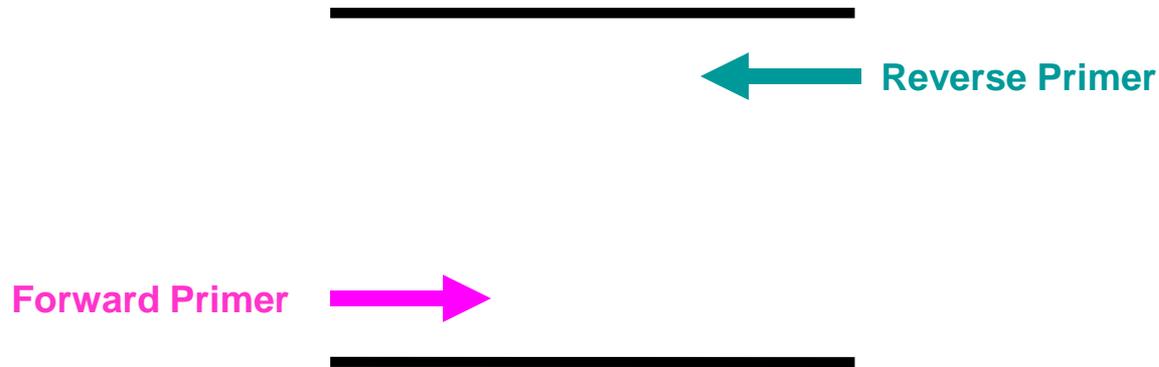
- Given that PCR involves very high temperatures, it is imperative that a **heat-stable DNA polymerase be used in the reaction.**
 - Most DNA polymerases would denature (and thus not function properly) at the high temperatures of PCR.
- Taq DNA polymerase was purified from the hot springs bacterium *Thermus aquaticus* in 1976
- Taq has maximal enzymatic activity at 75 °C to 80 °C, and substantially reduced activities at lower temperatures.

Denaturation of DNA



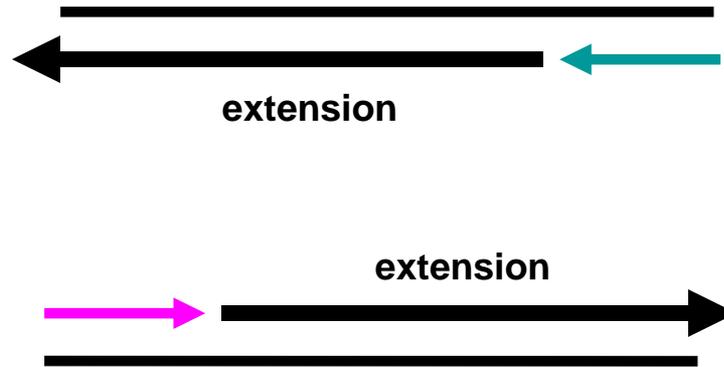
This occurs at 95 °C mimicking the function of helicase in the cell.

Step 2 Annealing or Primers Binding



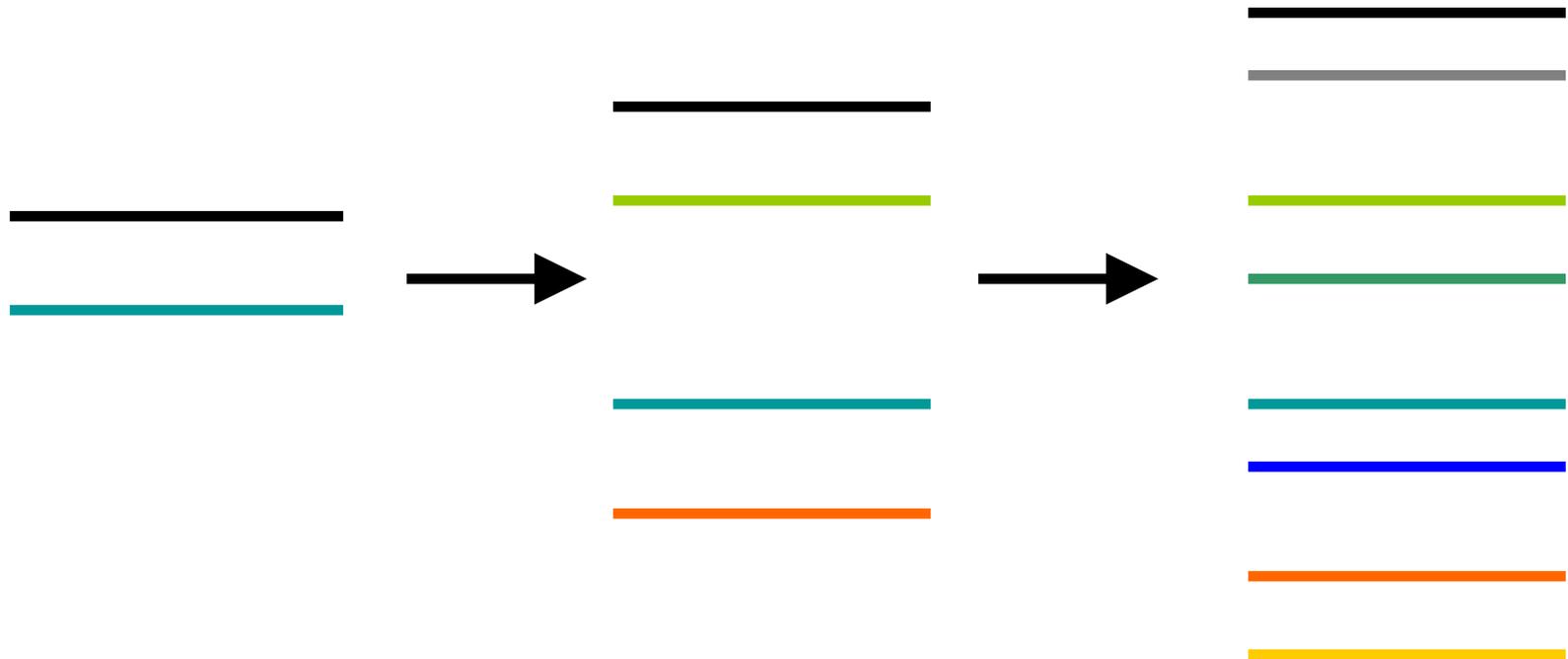
Primers bind to the complementary sequence on the target DNA. **Primers** are chosen such that one is complementary to the one strand at one end of the target sequence and that the *other* is complementary to the *other* strand at the other end of the target sequence.

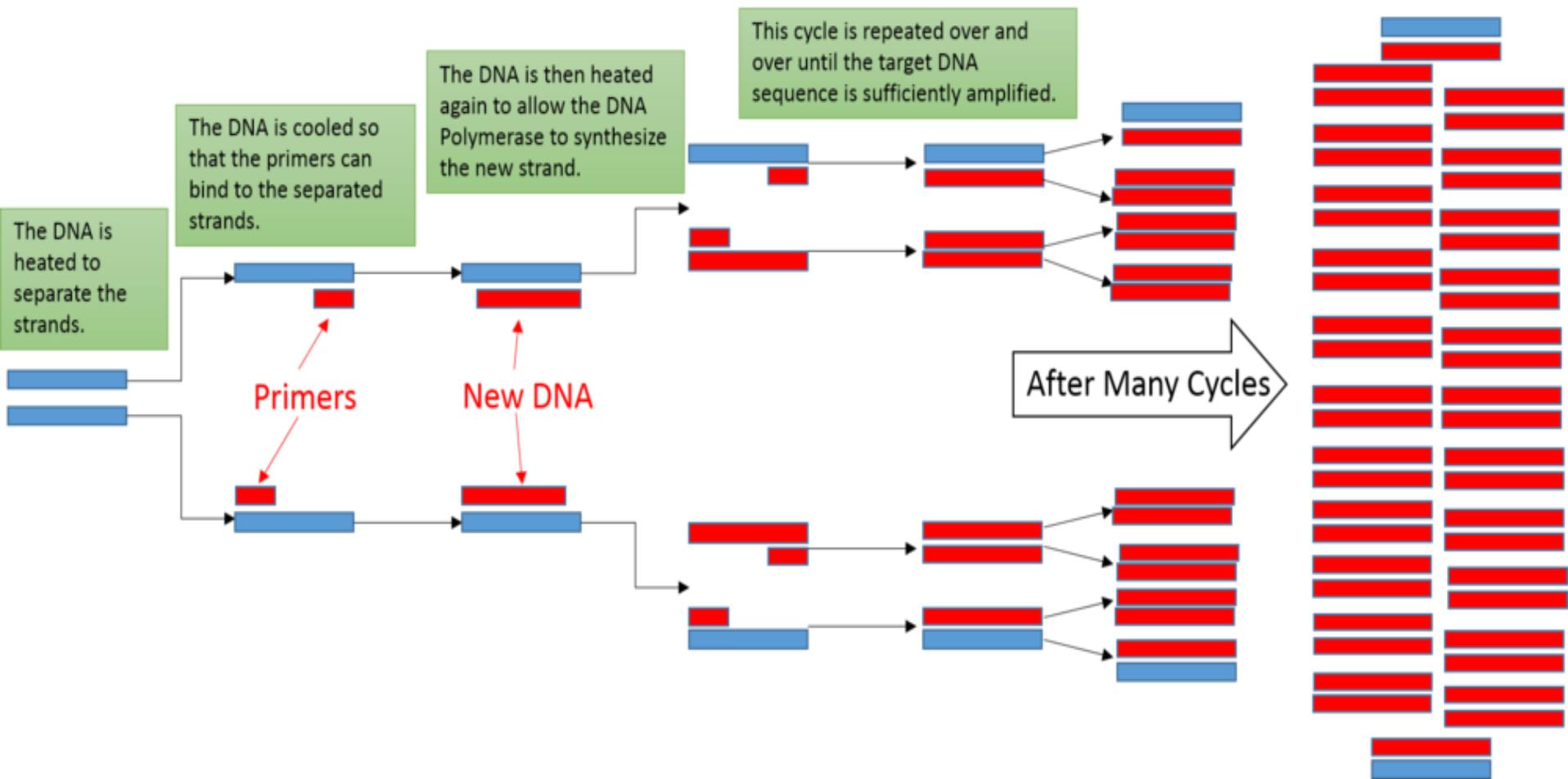
Step 3 Extension or Primer Extension



DNA polymerase catalyzes the extension of the strand in the 5-3 direction, starting at the primers, attaching the appropriate nucleotide (A-T, C-G)

- The next cycle will begin by denaturing the new DNA strands formed in the previous cycle





Theoretical amplification rate: 2^n

The Size of the DNA Fragment Produced in PCR is Dependent on the Primers

- The PCR reaction will amplify the DNA section between the two primers.
- If the DNA sequence is known, primers can be developed to amplify any piece of an organism's DNA.

Forward primer



Reverse primer



Size of fragment that is amplified

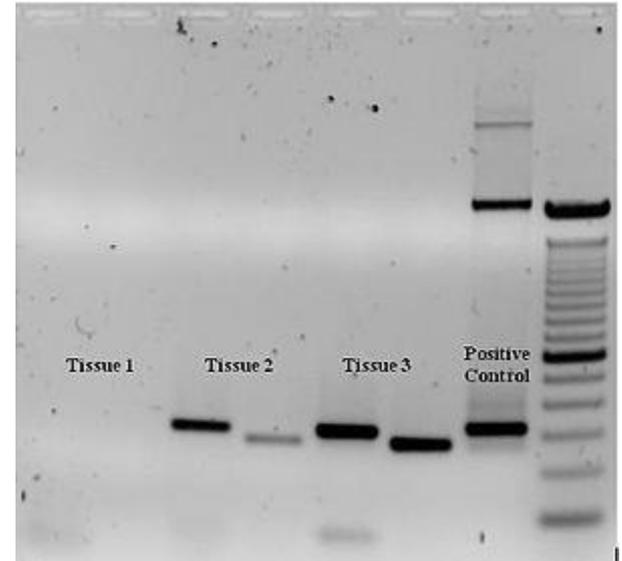
The DNA of interest is amplified by a power of 2 for each PCR cycle

For example, if you subject your DNA of interest to 5 cycles of PCR, you will end up with 2^5 (or 64) copies of DNA.

Similarly, if you subject your DNA of interest to 40 cycles of PCR, you will end up with 2^{40} (or 1,0995116¹²) copies of DNA!

Gel Electrophoresis of DNA

- To check whether the PCR generated the anticipated DNA fragment (also sometimes referred to as the amplicon or amplicon), agarose **gel electrophoresis** is employed for size separation of the PCR products.
- The size(s) of PCR products is determined by comparison with a DNA ladder (a molecular weight marker), which contains DNA fragments of known size, run on the gel alongside the PCR products



Gel Electrophoresis of DNA

- Gel electrophoresis detects the presence of DNA in a sample
- Gel electrophoresis detects the number of nucleotides in a fragment of DNA
 - e.g., the number of nucleotides in a DNA region which was amplified by PCR
 - Is a rough estimate, is not exact, need more sophisticated sequencing techniques to get an exact number of nucleotides
 - Can be used to tentatively identify a gene because we know the number of nucleotides in many genes

How Gel Electrophoresis of DNA Works

- A sample which contains fragments of DNA is forced by an electrical current through a firm gel which is really a sieve with small holes of a fixed size
 - Phosphate group in DNA is negatively charged so it is moved towards a positive electrode by the current
 - Longer fragments have more nucleotides
 - So have a larger molecular weight
 - So are bigger in size
 - So aren't able to pass through the small holes in the gel and get hung up at the beginning of the gel
 - Shorter fragments are able to pass through and move farther along the gel
 - Fragments of intermediate length travel to about the middle of the gel
- DNA fragments are then visualized in the gel with a special dye
- The number of nucleotides are then estimated by comparing it to a known sample of DNA fragments which is run through the gel at the same time

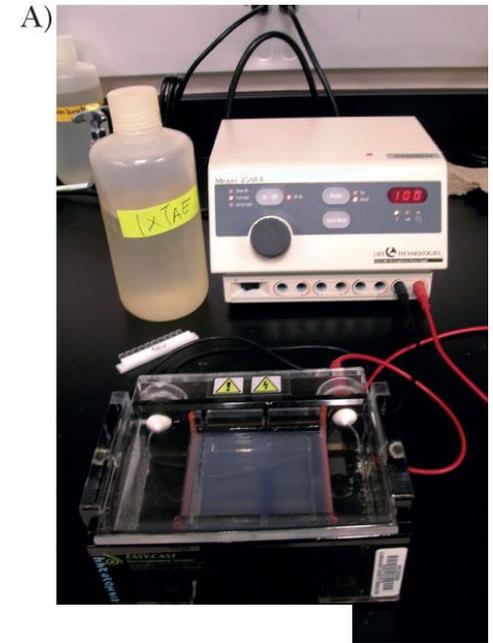
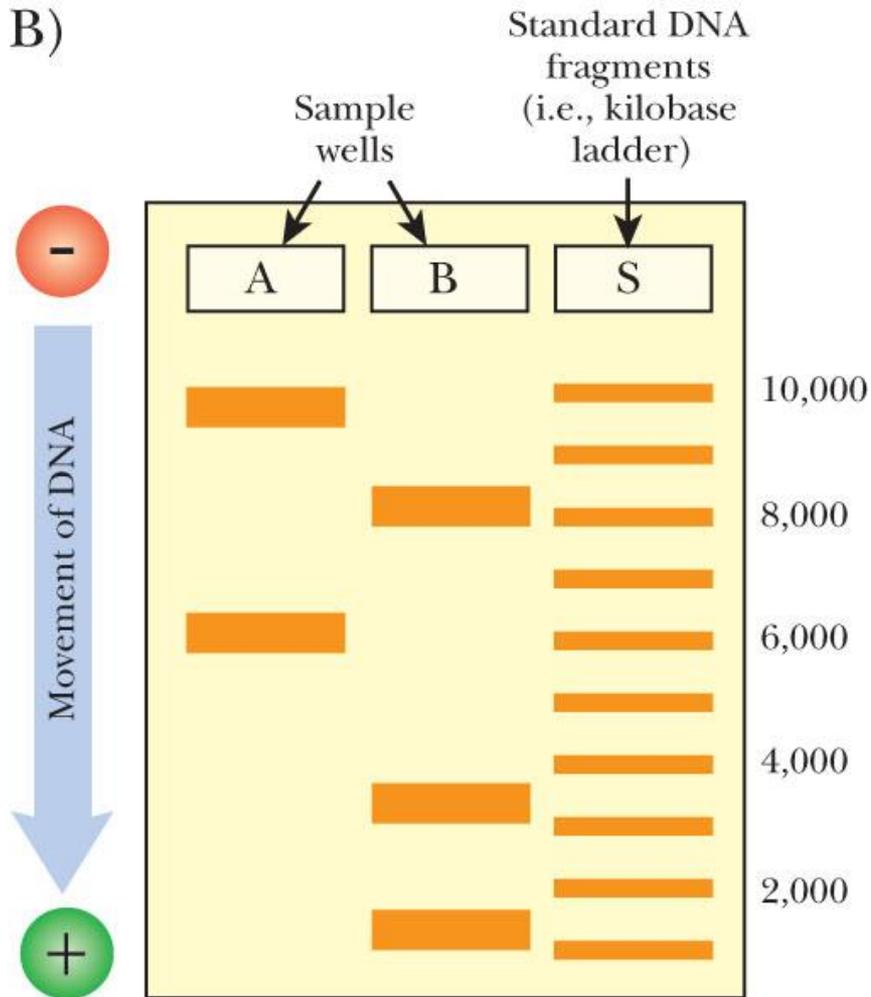
How Gel Electrophoresis of DNA Works

- Reagents Needed
 - Sample of DNA fragments
 - Known sample of DNA fragments
 - DNA ladder
 - Gel
 - Agarose
 - Dye to visualize the movement of the sample as it is traveling through the gel
 - Loading dye – Blue juice
 - So know when to stop so sample doesn't just run out of the gel
 - Dye to visualize DNA after it has traveled to its final spot in the gel
 - Syber® Safe
 - Buffer

How Gel Electrophoresis of DNA Works

- Equipment Needed
 - Box to hold the gel
 - Comb to create small wells in the agarose gel to put the DNA sample into at the beginning of the gel
 - Positive and negative electrodes to create the electrical current
 - Power supply
 - Gel photo imaging system

How Gel Electrophoresis of DNA Works

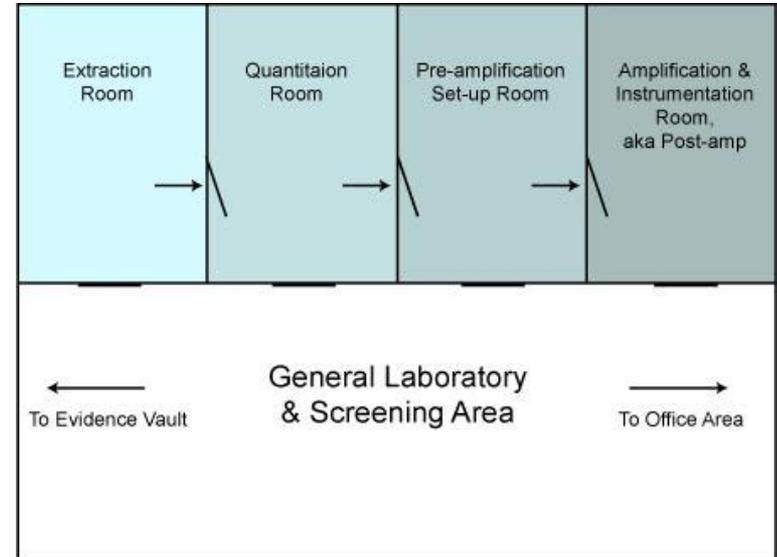


PCR has become a very powerful tool in molecular biology

- One can start with a single sperm cell or strand of hair and amplify the DNA sufficiently to allow for DNA analysis and a distinctive band on an agarose gel.
- One can amplify fragments of interest in an organism's DNA by choosing the right primers.
- One can use the selectivity of the primers to identify the likelihood of an individual carrying a particular allele of a gene.

PCR optimization : lab organization and cleaning

- PCR can fail for various reasons: due to its sensitivity to contamination causing amplification of spurious DNA products.
- Contamination with extraneous DNA is addressed with lab protocols and procedures that separate pre-PCR mixtures from potential DNA contaminants.
- This usually involves spatial separation of PCR-setup areas from areas for analysis or purification of PCR products, use of disposable plasticware, and thoroughly cleaning the work surface between reaction setups.



PCR optimization : Primers

- Primer-design techniques are important in improving PCR product yield and in avoiding the formation of spurious products, and the usage of alternate buffer components or polymerase enzymes can help with amplification of long or otherwise problematic regions of DNA
- PCR primers are short, single stranded DNA molecules (15-40 bp)
- They are manufactured commercially and can be ordered to match any DNA sequence
- Primers are sequence specific, they will bind to a particular sequence in a genome
- As you design primers with a longer length (15 → 40 bp), the primers become more selective.
- DNA polymerase requires primers to initiate replication

Selectivity of Primers

- Primers bind to their complementary sequence on the target DNA
 - A primer composed of only 3 letter, ACC, for example, would be very likely to encounter its complement in a genome.
 - As the size of the primer is increased, the likelihood of, for example, a primer sequence of 35 base letters repeatedly encountering a perfect complementary section on the target DNA become remote.

PCR and Disease

- Primers can be created that will only bind and amplify certain alleles of genes or mutations of genes
 - This is the basis of genetic counseling and PCR is used as part of the diagnostic tests for genetic diseases.
- Some diseases that can be diagnosed with the help of PCR:
 - Huntington's disease
 - cystic fibrosis
 - Human immunodeficiency virus

Huntington's Disease (HD)

- HD is a genetic disorder characterized by abnormal body movements and reduced mental abilities
- HD is caused by a mutation in the Huntingtin (*HD*) gene
- In individuals with HD, the *HD* gene is “expanded”
 - In non-HD individuals, the *HD* gene has a pattern called **trinucleotide repeats** with “CAG” occurring in repetition *less than* 30 times.
 - IN HD individuals, the “CAG” trinucleotide repeat occurs more that 36 times in the *HD* gene
- PCR can be performed on an individual's DNA to determine whether the individual has HD.
 - The DNA is amplified via PCR and **sequenced** (a technique by which the exact nucleotide sequence is determined) and the number of trinucleotide repeats is then counted.

Cystic Fibrosis (CF)

- CF is a genetic disease characterized by severe breathing difficulties and a predisposition to infections.
- CF is caused by mutations in the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene.
- In non-CF individuals, the *CFTR* gene codes for a protein that is a chloride ion channel and is involved in the production of sweat, digestive juices and mucus.
- In CF individuals, mutations in the *CFTR* gene lead to thick mucous secretions in the lungs and subsequent persistent bacterial infections.
- The presence of *CFTR* mutations in a individual can be detected by performing PCR and sequencing on that individual's DNA.

Human Immunodeficiency Virus (HIV)

- HIV is a retrovirus that attacks the immune system.
- HIV tests rely on PCR with primers that will only amplify a section of the viral DNA found in an infected individual's bodily fluids.

Therefore if there is a PCR product, the person is likely to be HIV positive. If there is no PCR product the person is likely to be HIV negative.

- Protein detection based tests are available as well but all US blood is tested by PCR.

PCR and Forensic Science

- Forensic science is the application of a broad spectrum of sciences to answer questions of interest to the legal system. This may be in relation to a crime or to a civil action.
- It is often of interest in forensic science to identify individuals genetically. In these cases, one is interested in looking at variable regions of the genome as opposed to highly-conserved genes.
- PCR can be used to amplify highly variable regions of the human genome. These regions contain runs of short, repeated sequences (known as variable number of tandem repeat (VNTR) sequences). The number of repeats can vary from 4-40 in different individuals.
- Primers are chosen that will amplify these repeated areas and the genomic fragments generated give us a unique “genetic fingerprint” that can be used to identify an individual.