Polymerase Chain Reaction (PCR)

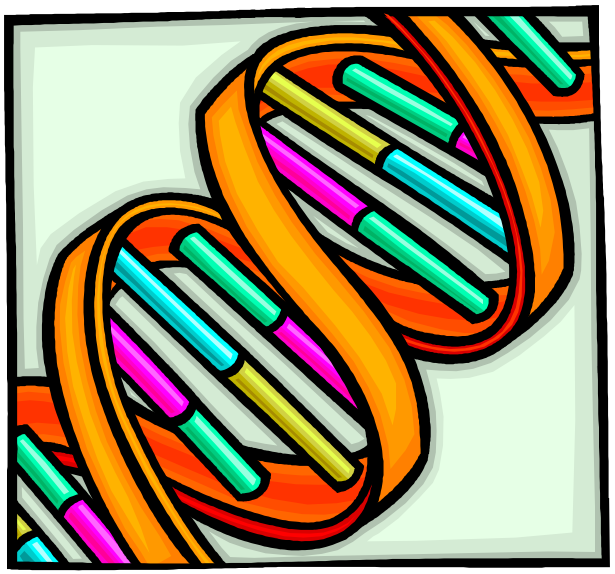
- PCR is a means to amplify a particular piece of DNA
  - *Amplify= making numerous copies of a segment of DNA*
- PCR can make ***billions*** of copies of a target sequence of DNA in a few hours
- PCR was invented in the 1984 as a way to make numerous copies of DNA fragments in the laboratory
- Its applications 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.

DNA Replication in Cells (*in vivo*)

- DNA replication is the copying of DNA
- It typically takes a cell just a few hours to copy all of its DNA
- DNA replication is semi-conservative (i.e. one strand of the DNA is used as the template for the growth of a new DNA strand)
- This process occurs with very few errors (on average there is one error per 1 billion nucleotides copied)
- More than a dozen enzymes and proteins participate in DNA replication



Key enzymes involved in DNA Replication

- DNA Polymerase
- DNA Ligase
- Primase
- Helicase
- Topoisomerase
- Single strand binding protein

DNA Replication enzymes: DNA Polyerase

- catalyzes the elongation of DNA by adding nucleoside triphosphates to the 3’ end of the growing strand
  - A nucleotide triphosphate is a 1 sugar + 1 base + 3 phosphates
  - When a nucleoside triphosphate joins the DNA strand, two phosphates are removed.
- DNA polymerase can *only* add nucleotides to 3’ end of growing strand

Complementary Base-Pairing in DNA

- DNA is a double helix, made up of nucleotides, with a sugar-phosphate backbone on the outside of the helix.
    - *Note: a nucleotide is a sugar + phosphate + nitrogenous base*
- The two strands of DNA are held together by pairs of nitrogenous bases that are attached to each other via hydrogen bonds.
    - The nitrogenous base adenine will only pair with thymine
    - The nitrogenous base guanine will only pair with cytosine
- During replication, once the DNA strands are separated, DNA polymerase uses each strand as a template to synthesize new strands of DNA with the precise, complementary order of nucleotides.

DNA Replication enzymes: DNA Ligase

- The two strands of DNA in a double helix are **antiparallel** (i.e. they are oriented in opposite directions with one strand oriented from 5' to 3' and the other strand oriented from 3' to 5'
    - *5' and 3' refer to the numbers assigned to the carbons in the 5 carbon sugar*
- Given the antiparallel nature of DNA and the fact that DNA ploymerases can only add nucleotides to the 3' end, one strand (referred to as the **leading strand**) of DNA is synthesized continuously and the other strand (referred to as the **lagging strand**) in synthesized in fragments (called **Okazaki fragments**) that are joined together by **DNA ligase.**

DNA Replication enzymes: Primase

- DNA Polymerase *cannot* initiate the synthesis of DNA
    - Remember that DNA polymerase can *only* add nucleotides to 3’ end of an already existing strand of DNA
- **In humans, primase** is the enzyme that can start an RNA chain from scratch and it creates a **primer (a short stretch RNA with an available 3’ end)** that DNA polymerase can add nucleotides to during replication.

    Note that the RNA primer is subsequently replaced with DNA

DNA Replication enzymes:

Helicase, Topoisomerase and Single-strand binding protein

- **Helicase** untwists the two parallel DNA strands
- **Topoisomerase** relieves the stress of this twisting
- **Single-strand binding protein** binds to and stabilizes the unpaired DNA strands

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)

PCR

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

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

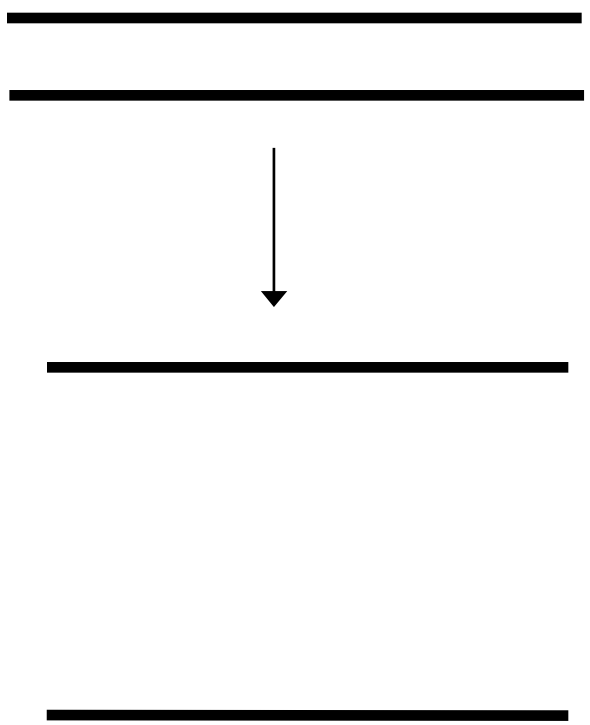
Step 3: DNA polymerase **Extends** the DNA chain

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

Heat-stable DNA Polymerase

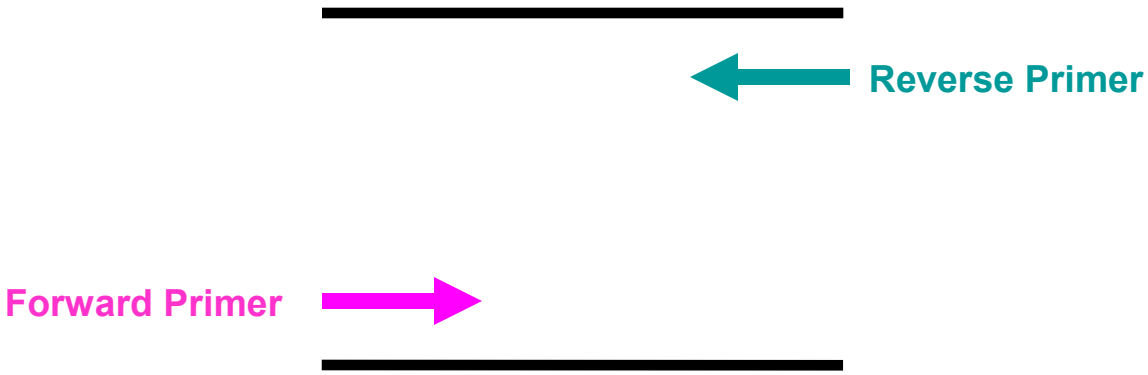
- Given that PCR involves very high temperatures, it is imperative that a heat-stable DNA polymerase be used in the reaction.
    - Most DNA polymerases would denature (and thus not function properly) at the high temperatures of PCR.
- Taq DNA polymerase was purified from the hot springs bacterium *Thermus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

Denaturation of DNA



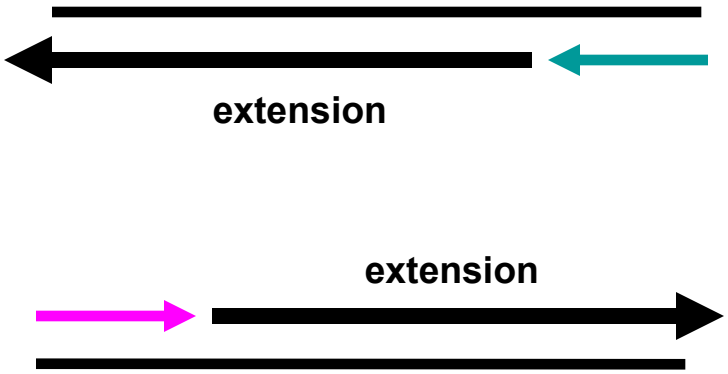
This occurs at 95 ºC mimicking the function of helicase in the cell.

Step 2 Annealing or Primers Binding



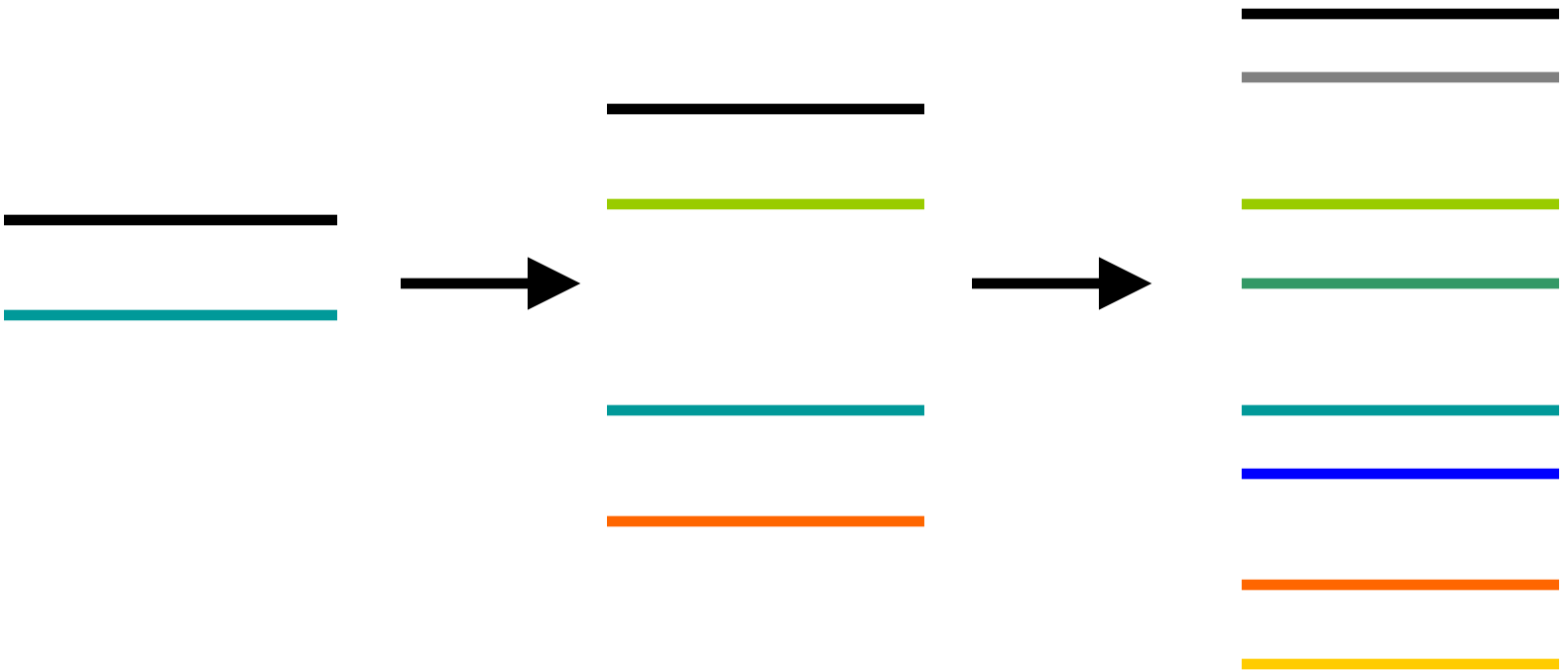
Primers bind to the complimentary sequence on the target DNA. Primers are chosen such that one is complimentary to the one strand at one end of the target sequence and that the *other* is complimentary to the *other* strand at the other end of the target sequence.

Step 3 Extension or Primer Extension



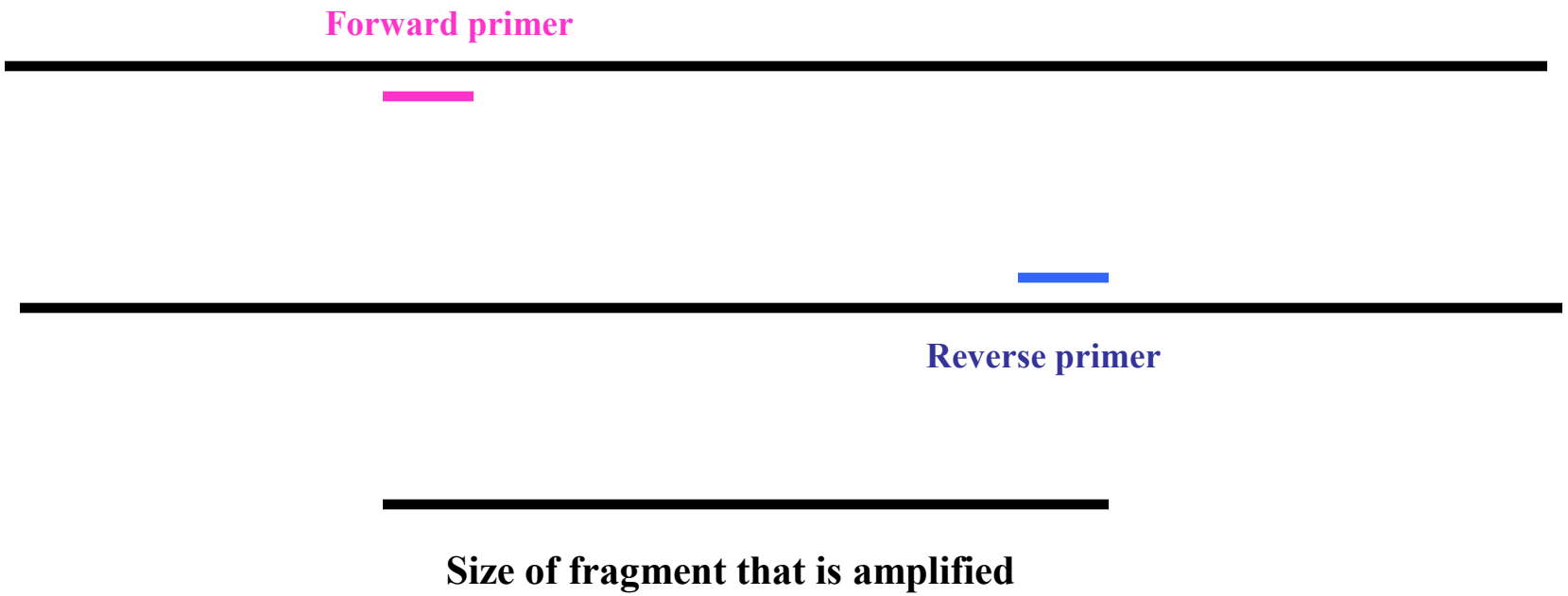
DNA polymerase catalyzes the extension of the strand in the 5-3 direction, starting at the primers, attaching the appropriate nucleotide (A-T, C-G)

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



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.



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) copies of DNA!

PCR has become a very powerful tool in molecular biology

- One can start with a single sperm cell or stand 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.

More about Primers

- 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.

A Review of Probability

A COIN THROW

The probability of a heads (H) or a tails (T) is always 0.5 for every throw. What is the probability of getting this combination of tails in a row?

Event	Probability	
Tails	0.5	= 0.5
T,T	0.5 x 0.5	= 0.25
T,T,T	0.5 x0.5 x 0.5	= 0.125
T,T,T,T,T	$(0.5)^5$	= 0.03125
T,T,T,T,T,T,T,T,T,T,T	$(0.5)^{11}$	= 0.0004883
T,T,T,T,T,T,T,T,T,T,T,T,T,T,T,T	$(0.5)^{16}$	=0.00001526

So it become increasing unlikely that one will get 16 tails in a row (1 chance in 65536 throws). In this same way, as the primer increases in size the chances of a match other than the one intended for is highly unlikely.

Probability in Genetics

- There are 4 bases in the DNA molecule A,C,G,T
- The probability of encountering any of these bases in the code is 0.25 (1/4)
- So let us look at the probability of encountering a particular sequence of bases

Event	Probability	
A	0.25	= 0.25
A,T	0.25 x 0.25	= 0.0625
A,T,A	0.25 x0.25 x 0.25	= 0.015625
A,T,A,G,G	$(0.25)^5$	= 0.0009765
A,T,A,G,G,T,T,T,A,A,C	$(0.25)^{11}$	= 0.000002384
A,T,A,G,G,T,T,T,A,A,C,C,T,G,G,T	$(0.25)^{16}$	=0.0000000002384

So it become increasing unlikely that one will get 16 bases in this particular sequence (1 chance in 4.3 billion). In this same way, one can see that as the primer increases in size, the chances of a match other than the one intended for is highly unlikely.

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 (*CTFR*) gene.
- In non-CF individuals, the *CTFR* 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 *CTFR* gene lead to thick mucous secretions in the lungs and subsequent persistent bacterial infections.
- The presence of *CTFR* 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.

PCR Applications to Forensic Science

- Paternity suits -Argentina's Mothers of the plaza and their search for abducted grandchildren
- Identifying badly decomposed bodies or when only body fragments are found - World trade center, Bosnian , Iraq & Rwandan mass graves

Some cool PCR links

- The Dolan DNA Learning Center:

  Link to Dolan DNA Learning Center's PCR Animation

  This site provides a nice step by step guide to how DNA is copied in PCR reaction.

- DNA Interactive:

  Link to DNAi

  This site is FULL of cool stuff! Two 3-D animation videos relevant to this Powerpoint presentation are:

  – The **DNA Replication** animation (to get to this, click on the above link, then click on "code", then click on "copying the code", then click on "putting it together", and finally click on "replication").

  – The **PCR animation** (to get to this, click on the above link, then click on "manipulation", then click on "techniques", the click on "amplifying" and then finally click on "PCR animation").