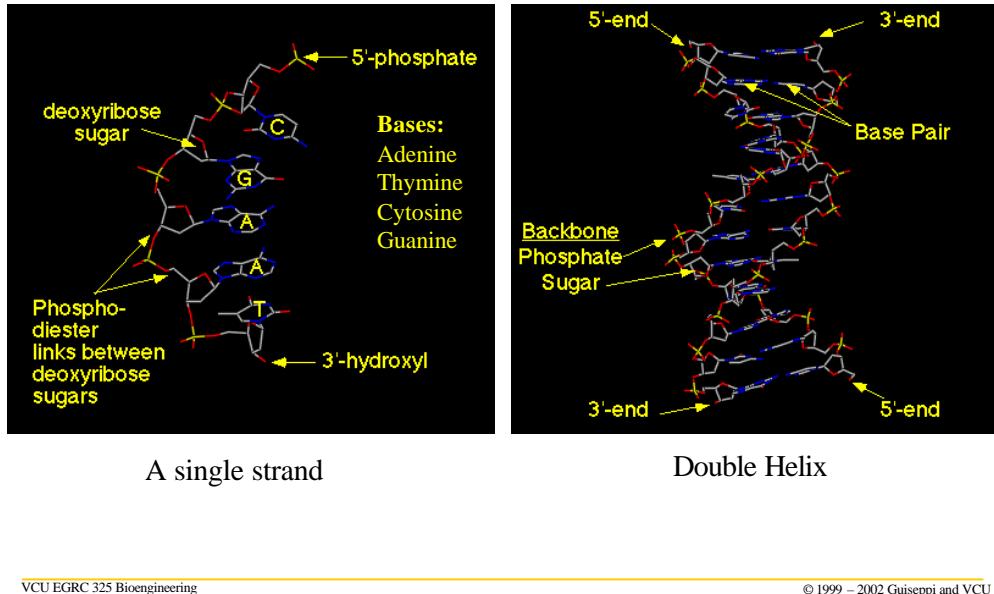
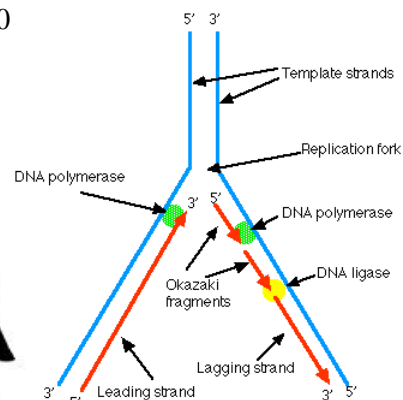
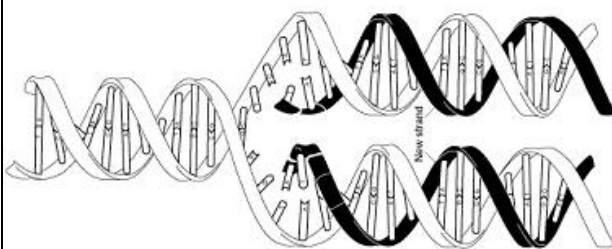


The Molecular Structure of DNA



DNA Replication *in vivo*

- **Replication** begins with **RNA primers** (1 to 60 bases) synthesized by RNA polymerase
- After Primers are manufactured, **DNA Polymerase** replaces RNA polymerase and continues to replicate the template strand



What is Polymerase Chain Reaction?

- ◆ Polymerase Chain Reaction is the amplification of a region of DNA by thermocycling and the use of a special **heat stable** DNA Polymerase (**Taq**) and synthetic primers.
- ◆ Taq was first isolated from the deep-sea hot vent dwelling archaean *Thermus aquaticus*.

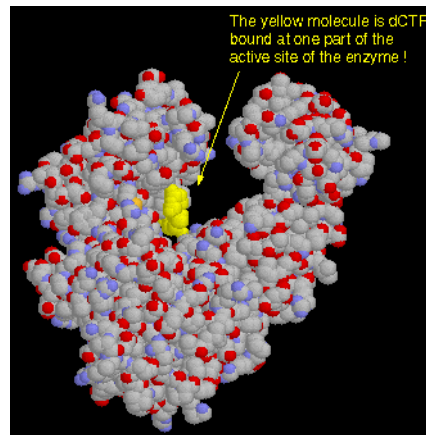
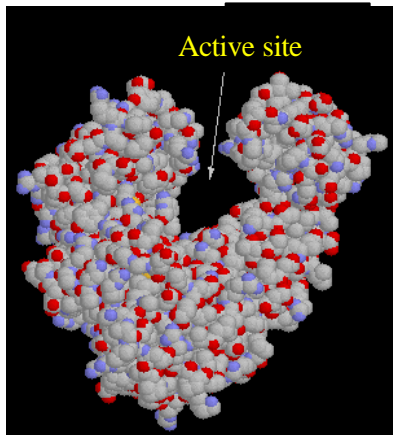
Inventors and Patents

- ◆ Kerri Mullis and colleagues at the Cetus Corp.
- ◆ Khorana and colleagues described the principle about a decade earlier, however, its use at the time was limited until the thermostable Taq polymerase was discovered.
- ◆ U.S. patent #s 4,683,202; 4,683,195; 4,965,188.

Materials and Associated Techniques

- ◆ PCR materials
 - /// Taq polymerase
 - /// DNA template
 - /// Primers
 - /// dNTPs
 - /// Thermocycler
 - /// Mg^{2+} .
- ◆ Associated Techniques
 - /// Gel electrophoresis.
 - /// DNA sequencing.

Taq Polymerase

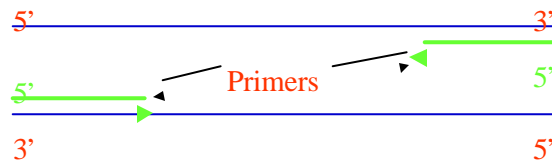


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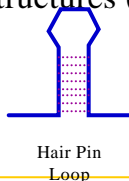
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Primer Design Considerations

- ◆ Taq requires a 3' hydroxyl to begin strand elongation.



- ◆ Taq DNA polymerase “reads” the template in the $3' \Rightarrow 5'$ direction, thus it synthesizes the new strand in the $5' \Rightarrow 3'$ direction.
- ◆ The G/C content of the primers dictates the annealing temp.
- ◆ Potential secondary structures (Hair pins or dimers) must be minimized



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PCR Animation: Step 2 Primer Annealing

Cycle 1

5'-CTAGAATATGAACCTATAGGTACGGTGGCCATTCTATGCTGTGATCCCGTACTACCTACAGAA-3'

3'-GATCTTATACTTTGGATATCCATGCCACCCGGTAGATACAGACTAGGGCCATGATGGATGCTT-5'

PCR Animation: Step 3 Elongation (polymerization)

Cycle 1

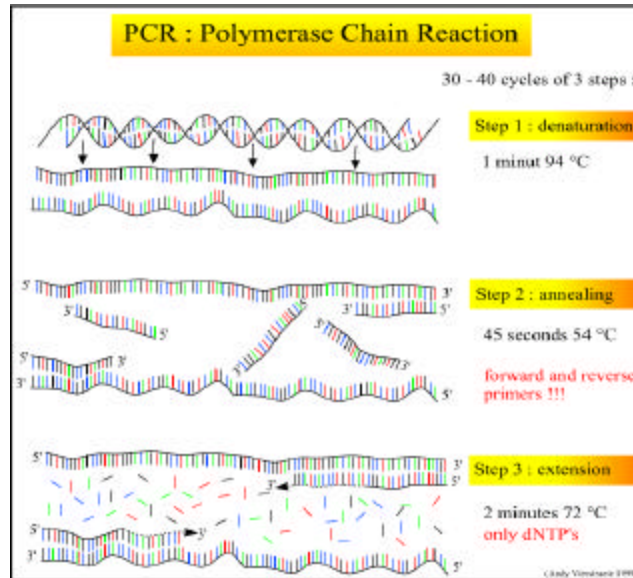
5'-CTAGAATATGAACCTATAGGTACGGTGGCCATTCTATGCTGTGATCCCGTACTACCTACAGAA-3'

|||||
3'-GGGCCATGATGG-5'

5'-ATGAACCTATAG-3'

|||||
3'-GATCTTATACTTTGGATATCCATGCCACCCGGTAGATACAGACTAGGGCCATGATGGATGCTT-5'

Schematic of the PCR reaction

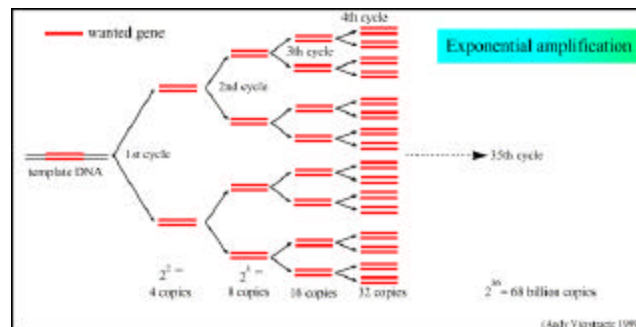


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Amplification

Theoretical yield is 2^n ,
where $n = \#$ of cycles

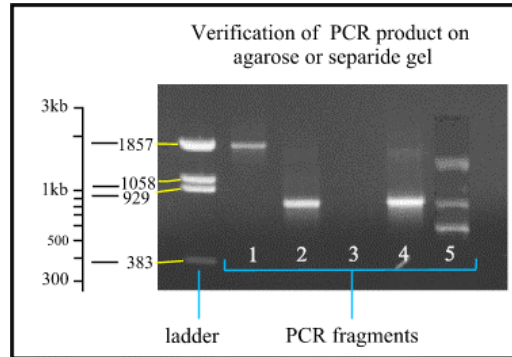
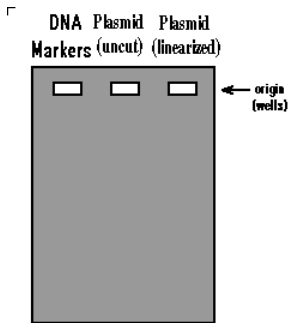


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How do we detect PCR product?

With Gel Electrophoresis!



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Applications of Standard PCR

◆ Amplification with modification. (Gene Cloning)

⚡ Addition of genetic alterations

- Restriction sites
- Single base mutations.

⚡ Addition of fluorescent nucleotides.

◆ Genetic Diagnostics (coupled with sequencing)

⚡ Disease Screening.

⚡ Genetic variation quantification.

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Variations of PCR

◆ Reverse Transcription PCR

/// Amplification of RNA.

/// Microarrays.

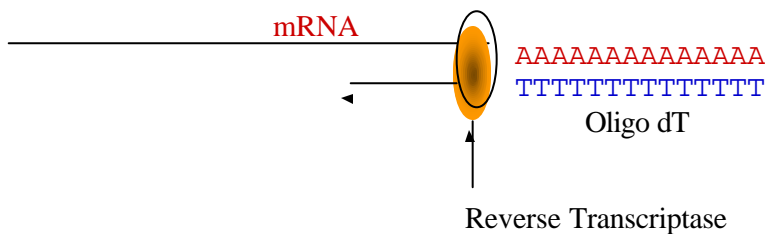
◆ Real Time PCR

/// Dual Labeled Probe.

/// Imaging System.

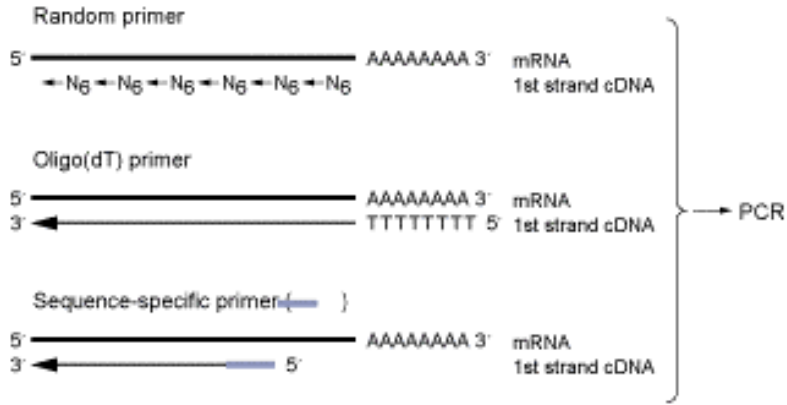
Reverse Transcription

- ◆ This technique takes advantage of the Poly-A-tail of mRNA. Using an Oligo dT primer all cellular mRNA can be amplified. This process is exploited for generating labels for hybridization to DNA microarrays.

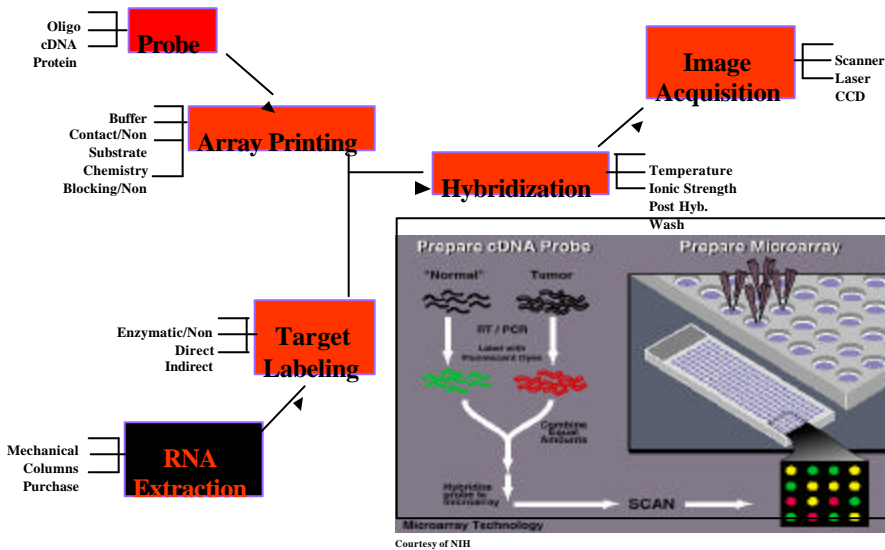


RT PCR Schematic

First Strand Synthesis:



Elements of Experimental Design



Convention: Probe on microarray, target from sample.

After David I. Burke, PE Life Sciences

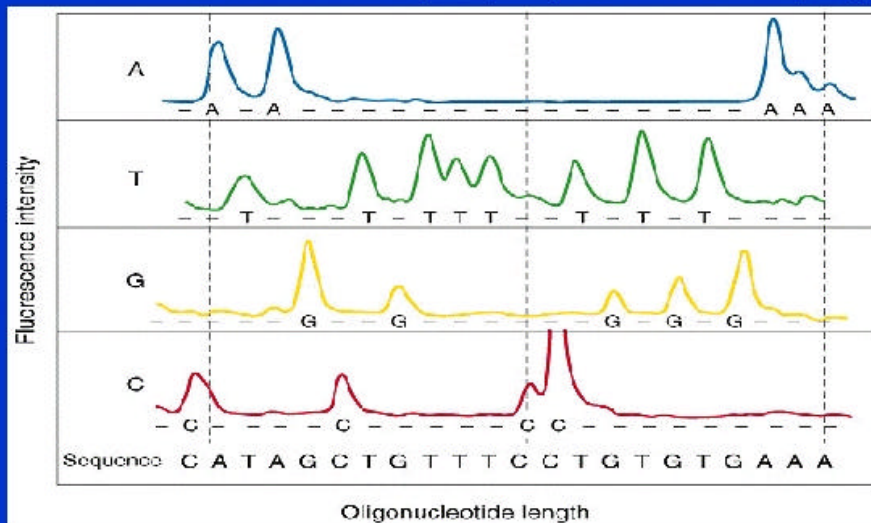
PCR and Gene Sequencing for Diagnostic Purposes

- ◆ You are investigating whether one of your lab rats has a mutation in the p53 gene that may be the cause of cancer.
- ◆ You design primers and do PCR on the p53 allele from a biopsy preparation using fluorescently labeled dNTPs.
- ◆ You extract your fragment from the agarose gel and sequence it.

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Using a Graduate student to scan the gel patterns by hand



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Trouble shooting

◆ Extra Bands.

⚡ Non specific annealing.

- Temperature is not optimized
- Sequence has a high degree of similarity.
- Cycle number is to high.

◆ Low product or no product.

⚡ Primers are not annealing.

⚡ Low Mg

◆ Infidelity in base incorporation

⚡ dNTPs are not in a 1:1:1:1 ratio.

Thermal Cycle Summary

◆ Heat DNA to 95°C to melt double strands

◆ Cool to ~60°C to allow primers to anneal to the template

⚡ At this point primers hybridize to their complementary sequence on DNA. DNA does not re-hybridize, why?

◆ Heat to ~70 °C to allow **Taq** to synthesize a new DNA strand

⚡ This causes extension of the primer sequence to match the DNA sequence or region of interest.

◆ Repeat until desired amplification is obtained.

◆ **The concentration increases 2^n for n cycles.**

Importance of PCR

- ◆ Can amplify specific sequences isolated from a single cell or viral particle.
 - // Search for mutations associated with specific genetically based diseases e.g. **cystic fibrosis (CF)**.
 - // HIV detection before AIDS symptoms appear.
- ◆ Cloning of cDNA based on partial amino acid sequences of proteins.
- ◆ Site directed DNA base changes and mutations.
 - // Investigate how single base changes can affect protein function.
- ◆ Addition of restriction sites into genes.
 - // Applications in molecular cloning, pathway engineering etc.

Principles of PCR Summary

Polymerase Chain Reaction (PCR) is a **synthesis** method used to directly **amplify** specific DNA sequences or regions of interest when, at least, the **ends** of that sequence are **known**.

Process uses:

- ◆ A large excess of two 2 synthetic DNA oligonucleotides (~20-mers), called **primers**. These are complementary to the 3' end of the DNA segment or region of interest.
- ◆ A temperature-resistant polymerase enzyme that is isolated from the bacterium *Thermus aquaticus* (found in hot springs) called *Taq polymerase*
- ◆ A large excess of deoxynucleotides (dNTPs)
- ◆ Temperature cycling enables
 - // **Denaturation** of the template (90 - 95 °C) to allow access for the primers.
 - // **Annealing** (60 - 65 °C) of the synthetic primers to their complementary regions of the template DNA
 - // **Elongation** (68-72 °C) synthesis of the new DNA strand