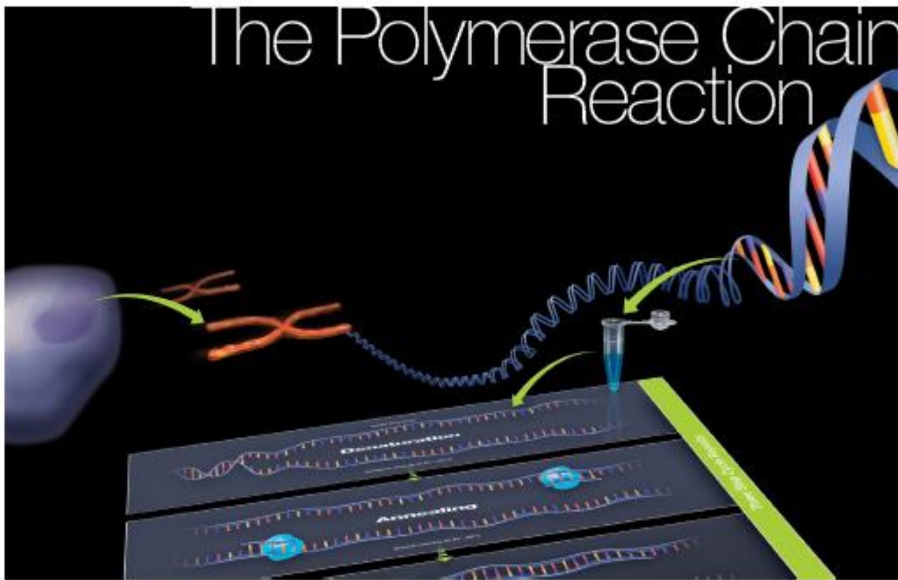


The Polymerase Chain Reaction

Chapter 6: Background

Invention of PCR

- Kary Mullis
  - Mile marker 46.58 in April of 1983
  - Pulled off the road and outlined a way to conduct DNA replication in a tube
  - Worked for Cetus, which was purchased by Chiron and is now part of Novartis

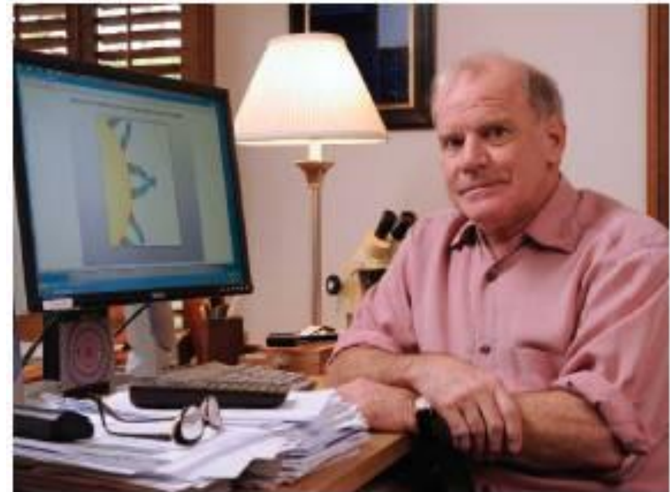
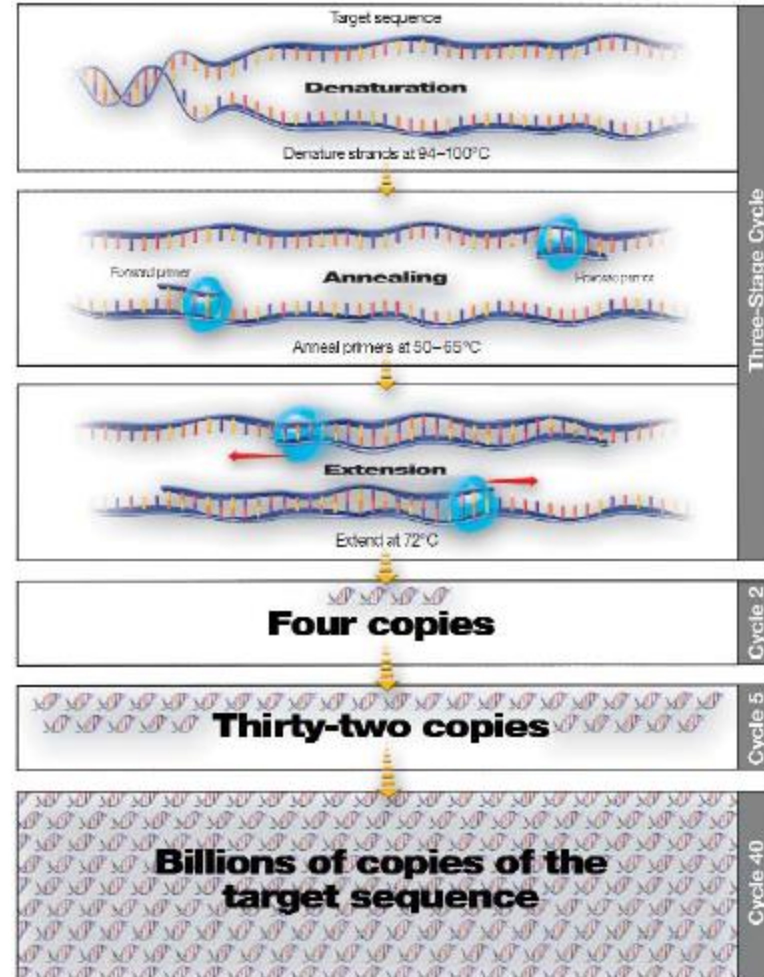


Figure 6.1. **Kary Mullis.** Mullis won the Nobel Prize in 1993 for the development of PCR.

What is PCR?

- Polymerase chain reaction or PCR
  - Simplified version of bacterial DNA replication
  - Copies a specific sequence of DNA
    - Target sequence
    - PCR products
    - Amplicons
  - Target is determined by the primers
    - Short sequences of single-strand DNA
    - To amplify a defined segment, two are required
  - Annealing is when the primers bind to the target sequence
  - The two primers are referred to as forward and reverse primers



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Challenges of DNA Replication in a Tube

- Normal replication uses enzymes that are heat sensitive
- Using heat to denature the double strands of DNA, would damage the enzymes
- Heat-stable DNA polymerases were the key to developing in vitro method of replication
- *Thermus aquaticus* was the bacteria from which the first heat-stable DNA polymerase was isolated and the enzyme was named Taq polymerase

Figure 6.3. **Geyser at Yellowstone National Park.** Taq DNA polymerase was the first thermophilic DNA polymerase used in PCR. This enzyme was isolated from *Thermus aquaticus* bacteria found in hot springs in Yellowstone National Park. Taq is still the most common DNA polymerase used in PCR.

Normal Bacterial Replication

- Steps:
  - Topoisomerase gradually unwinds the DNA helix ahead of the replication fork. Helicase unzips the strands by breaking hydrogen bonds
  - Single-stranded binding proteins prevent DNA from reannealing
  - Primase adds RNA nucleotides in the 5'3' direction
  - DNA polymerase III adds nucleotides to the ends of the RNA primers
  - DNA polymerase I corrects mistakes and removes RNA nucleotides and replaces them with DNA nucleotides
  - Ligase links the leading and lagging strands (Okazaki fragments)

Normal Bacterial Replication

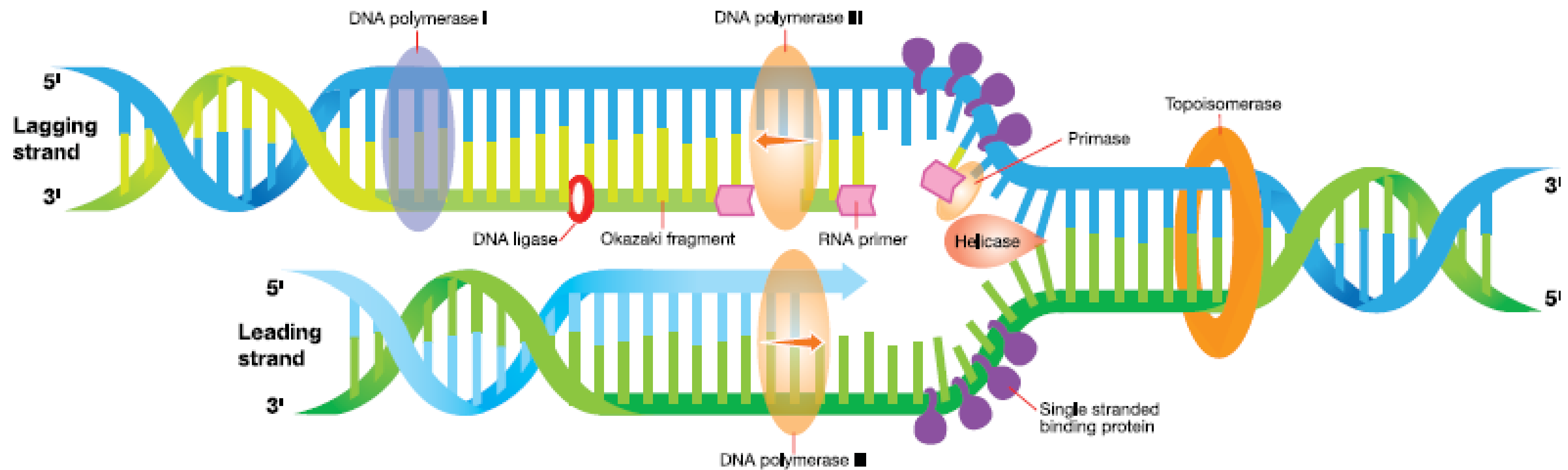

Figure 6.6. **In vivo replication of DNA.** Topoisomerase unwinds DNA and helicase separates the DNA strands that are then kept separate by single-stranded binding proteins. Primase adds RNA primers that are extended by DNA polymerase III on the leading and lagging strands. The lagging strand is made discontinuously and fragments are joined by ligase. DNA polymerase I replaces the RNA primers with DNA.

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Components of a PCR Reaction

- Template DNA
- Nucleotides (dNTPs)
- PCR buffer
- Magnesium chloride ($MgCl_2$)
- Forward and reverse primers
- DNA polymerase

Table 6.2. **Components of a PCR reaction.**

Component	Common Stock Concentration	Final Concentration or Amount per Reaction
DNA template	N/A	Genomic DNA: 50–500 ng, plasmid DNA: 50 pg–50 ng
dNTPs	10 mM	0.2 mM
$MgCl_2$	50 mM	1–6 mM
PCR buffer	10x	1x
Forward primer	100 µM	0.1–1 µM
Reverse primer	100 µM	0.1–1 µM
DNA polymerase	Varies depending on the polymerase and the manufacturer	Usually <1 µl

Thermal Cyclers

- Initially water baths were used, but that was very labor intensive
- Thermal cyclers are electronically controlled heat blocks that can quickly change from one temperature to another
- They are programmable and are in many different configurations depending upon their purpose
- They most commonly have graphical interfaces that make them very easy to program

Types of PCR

- There are many variations of traditional PCR
  - Real-time or quantitative PCR (qPCR)
  - Reverse transcription PCR (RT-PCR)
  - Multiplex PCR
  - Degenerate PCR
  - Nested PCR
  - Random amplification of polymorphic DNA (RAPD)
  - Fast PCR

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Real-Time PCR

- Allows for quantitation of the amount of initial DNA in a sample
- The amount of PCR product is measured at the end of each cycle

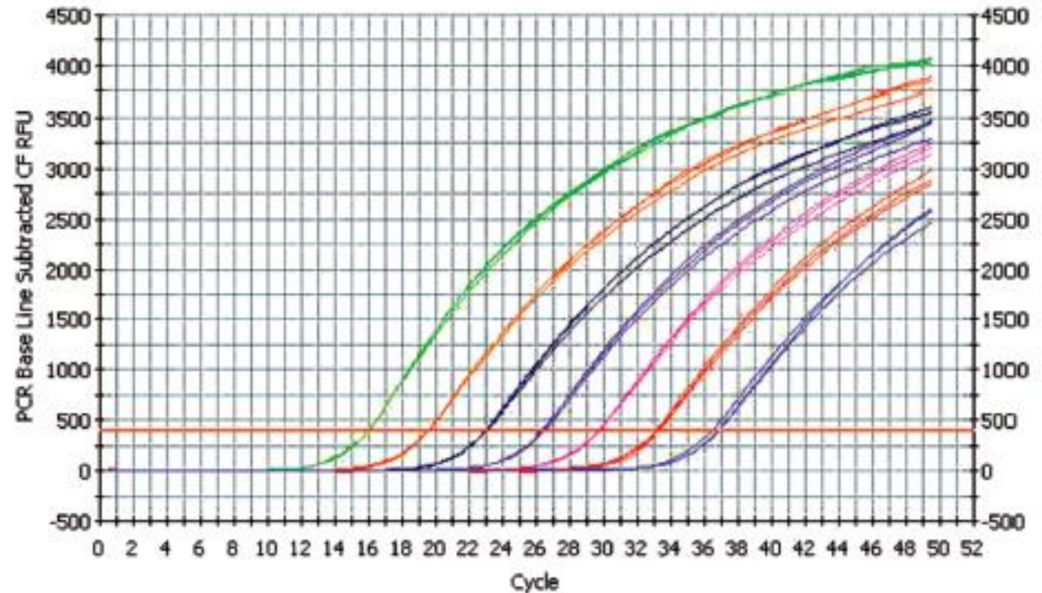

Figure 6.12. **Real-time PCR results.** The fluorescence at each cycle is plotted against the cycle number for each PCR reaction. The cycle at which the fluorescence passes a threshold (called C_q) is determined and used to compare different reactions. In the example, the differently colored lines from left to right represent PCR reactions in triplicate with tenfold reductions in the quantity of starting DNA. The horizontal red line represents the threshold, and the C_q value for each reaction is the cycle where each curve intersects the threshold.

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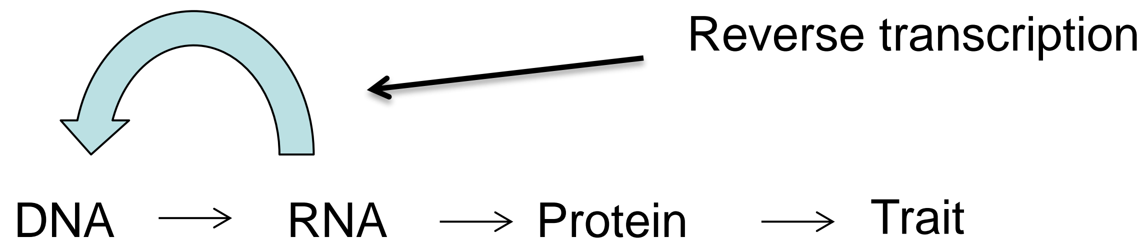
Reverse Transcription PCR

- Uses retroviral enzyme reverse transcriptase to reverse transcribe mRNA into DNA before the PCR begins
- When DNA is made this way it is referred to as cDNA
  - RT-PCR is often used in combination with real-time PCR to measure levels of gene expression



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Other Types of PCR

- Multiplex PCR
  - Multiple sets of primers are used to target and amplify different sequences
  - Often used in GMO detection
- Degenerate PCR
  - Uses sets of primers that have almost the same sequence as each other differing in only 1–3 bases. They are used when working with closely related species and improve the likelihood of amplification

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Other Types of PCR

- Nested PCR
  - Sometimes PCR is not optimal and two rounds are required. The first set of primers might amplify target and non-target DNA. A second set of primers is used that will bind within the target region using the first round as template DNA
- Random Amplification of Polymorphic DNA (RAPD)
  - This is a method of DNA fingerprinting that uses a collection of random primers. These primers are 10 base pairs long (decamers) and will randomly amplify products if they bind close enough on the template DNA. Closely related species will have similar products while others might not

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Other Types of PCR

- Fast PCR
  - Total running time for traditional three-cycle PCR is relatively long (2.5–4 hours)
  - In Fast PCR, the run time can be 0.5–1.5 hours with new advancements
  - This can be achieved by using two steps where the annealing temperature and extension temperature are close to each other
  - This type of reaction must be highly optimized and only works for some reactions

PCR Optimization

- Each reaction is very different and the components and conditions must be adjusted
- Factors that must be taken into account vary with the purpose of the reaction
  - If the goal is to clone a gene, then the PCR product should have no errors so no mutations are introduced
    - Enzymes with proofreading features are used to ensure accuracy

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PCR Optimization

- Conditions and factors to optimize:
  - Template quality
  - Template concentration
  - Primer concentration
  - Primer design
  - Magnesium concentration
  - Annealing temperature
  - Cycling times

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PCR Optimization

- Template quality
  - If DNA is ancient, usually the template is broken into smaller fragments
  - Designing primers to amplify small PCR products should be the goal

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PCR Optimization

- Template concentration
  - Generally there is an optimal concentration of template
  - Too little concentration is often an issue depending upon the length of the target being amplified
  - Too much template can inhibit a reaction
  - Adjusting the concentration to see which concentration yields the best amplification is the goal with template concentration optimization

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PCR Optimization

- Primer concentration
  - The normal range is from 0.1 μM to 1 μM
  - Increasing the concentration can lead to
    - Nonspecific binding
    - Formation of primer dimer
      - Primer dimer is when the primers bind to each other

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PCR Optimization

- Primer design
  - The sequence of the primers is crucial to the success of PCR
    - Primer sequences must be complementary to each end of the target region
    - The melting temperature (T_m) of each primer must be within a few degrees of the other primers
  - The T_m is the temperature at which half of the primers dissociate from the template DNA
  - The annealing temperature is usually 3–5ºC lower than the T_m

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PCR Optimization

- Primer design
  - Primers should be designed without intra- or intercomplementary sequences

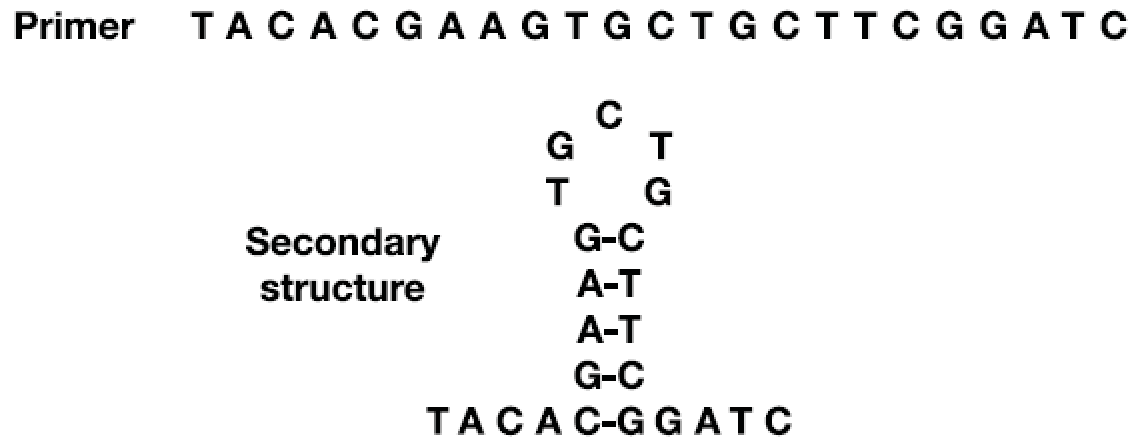

Figure 6.15. **Primer secondary structure.** The primer shown here has intracomplementarity, which results in two complementary sections of the sequence annealing to each other to form a hairpin loop. Primers should be designed without intracomplementary sequences so hairpin loops do not form.

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PCR Optimization

- Magnesium concentration
  - Magnesium is a cofactor for the polymerase enzyme
  - The normal concentration used is between 1–6 mM
  - This should be adjusted to find which concentration yields the best results

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PCR Optimization

- Annealing temperature
  - The temperature that is used for the primers to bind is called the annealing temperature. These are calculated using the formula:

$$T_m = [4°C \times (G+C)] + [2°C \times (A+T)]$$

  - Annealing temperatures are often adjusted up and down a few degrees to optimize them. Many thermal cyclers have a gradient function that will allow multiple annealing temperatures to be tested at one time

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PCR Optimization

- Annealing temperature
  - Many thermal cyclers have a gradient function that will allow multiple annealing temperatures to be tested at one time

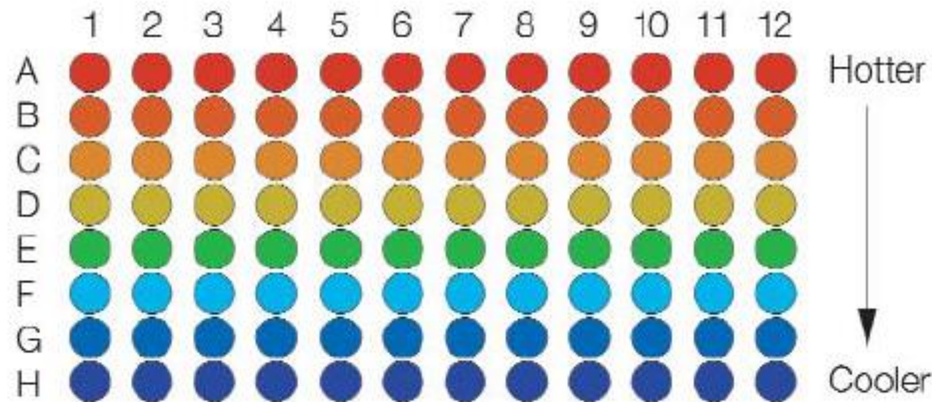

Figure 6.14. **Thermal gradient on a 96-well heat block.** Row A can be set at a higher temperature than row H and the rows in between will form a gradient between the temperatures of A and H, allowing multiple annealing temperatures to be tested with a single PCR run.

PCR Optimization

- Cycling temperatures and times
  - The amount of time that each step is held depends on the length of PCR product and the amount of time it would take to amplify that length. Shorter lengths require shorter times
  - Generally only the annealing temperature is adjusted

Cycle	Step	Temperature	Duration	No. of Repeats of Each Cycle
Initial denaturation	Denature DNA	94°C	5 min	1x
Thermal cycling	Denature	94°C	1 min	40x
	Anneal primers	52°C	1 min	
	Extend	72°C	2 min	
Final extension	Extend	72°C	6 min	1x
Hold	Hold	15°C	∞	1x

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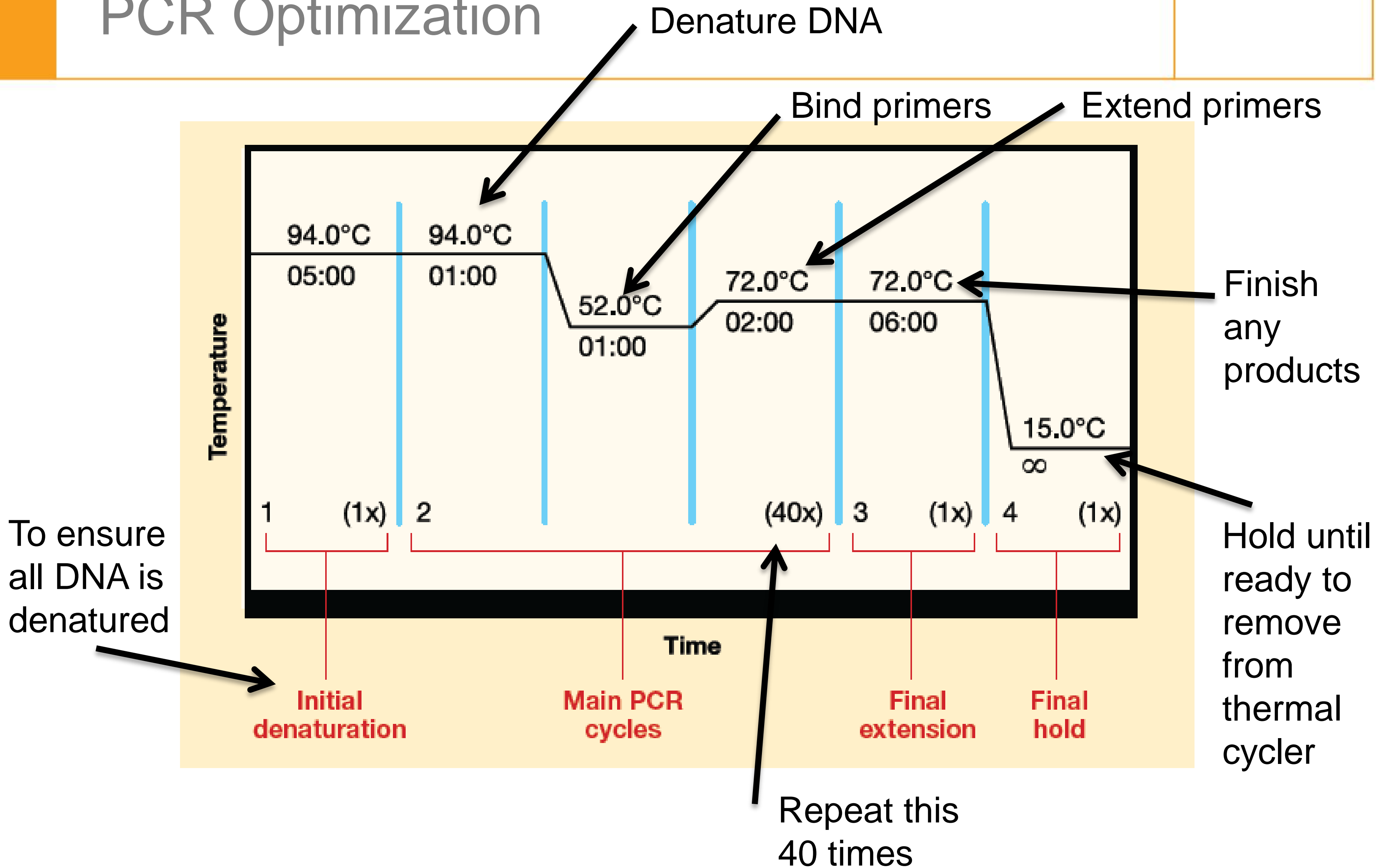
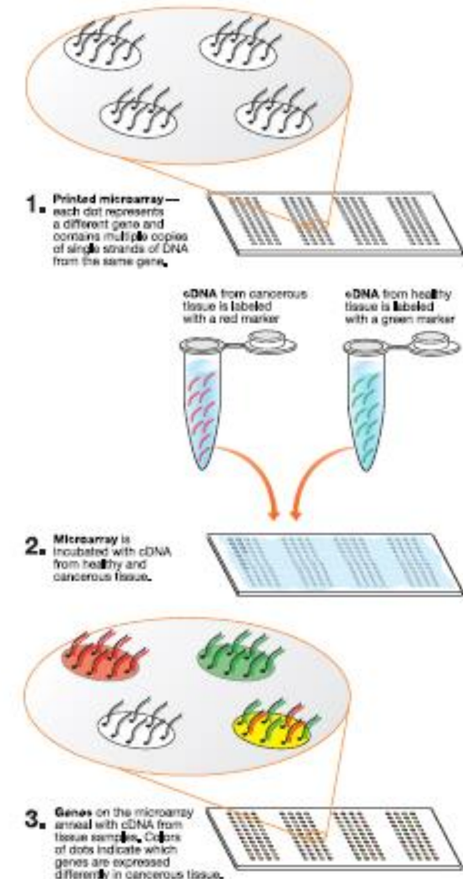

PCR Optimization
Denature DNA
Bind primers
Extend primers
94.0°C
05:00
94.0°C
01:00
52.0°C
01:00
72.0°C
02:00
72.0°C
06:00
15.0°C
∞
Temperature
1 (1x)
2 (40x)
3 (1x)
4 (1x)
Time
Initial denaturation
Main PCR cycles
Final extension
Final hold
Finish any products
Hold until ready to remove from thermal cycler
To ensure all DNA is denatured
Repeat this 40 times

Techniques Based on PCR

- DNA microarrays
  - Small spots of identical pieces of DNA are annealed to a slide and arranged in order to determine which spots had a complementary binding event
  - The spots of DNA fragments can represent variations in gene sequences, mRNA complementary sequences, viral sequences, bacterial sequences, etc.

Microarrays

- PCR is used to amplify the sequence of interest
- They are spotted on a slide
- Sequences from other samples that have been amplified with labeled nucleotides are washed
- The results are detected



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DNA Sequencing

- Modern sequencing techniques use PCR to amplify the sequence to be determined
- A mixture of terminating and non-terminating nucleotides are used in the reaction
- Each terminating nucleotide has a different color dye added to it, a dideoxynucleotide triphosphate (ddNTP). Once the growing chain terminates, the nucleotide will have a unique color associated with it

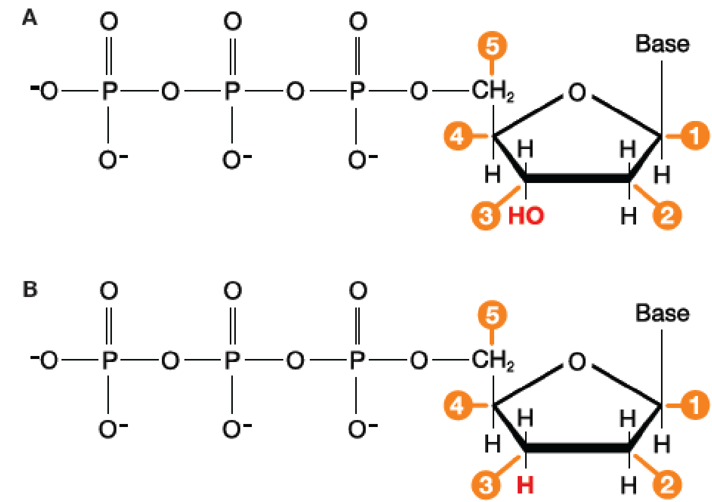

Figure 6.17. **Structure of dNTPS and ddNTPs.** A, dNTPs have a 3'-hydroxyl (3'-OH) group, which is necessary for elongation of DNA. B, ddNTPs do not have a 3'-OH; instead, the 3' position has been modified to have a hydrogen (-H) at that position. When a ddNTP is incorporated into a DNA molecule, the synthesis ends at that nucleotide and the DNA chain is terminated.

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DNA Sequencing

- Since the primer is known, each successive terminated fragment should be one nucleotide longer
- Automated sequencers read the colors as they electrophorese past a detector

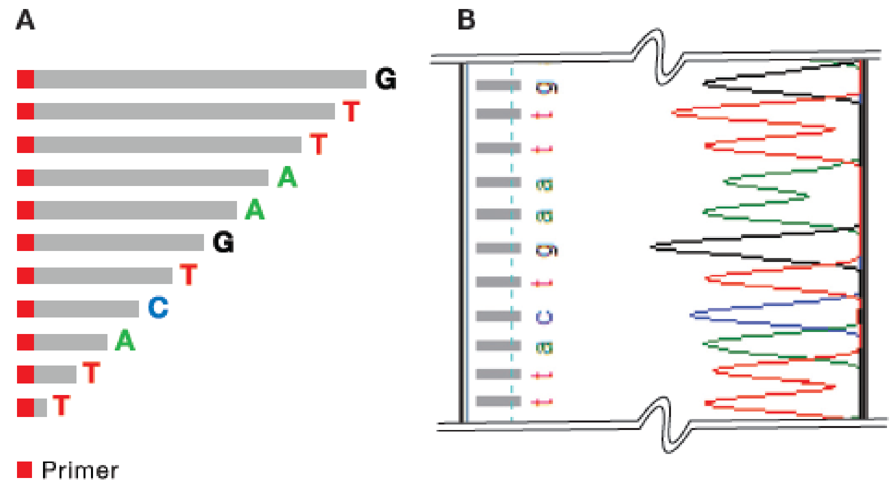

Figure 6.18. **Cycle sequencing.** A, a single primer is extended by copying the template DNA in each round of cycling and may randomly incorporate a fluorescently tagged ddNTP, terminating the strand extension and generating a mixture of fragment sizes; B, when the fragments are electrophoresed, they pass a laser that records the color of the tag on each fragment and presents the results in a chromatogram (also known as an electropherogram). The sequence is determined by reading the terminating nucleotides in order.

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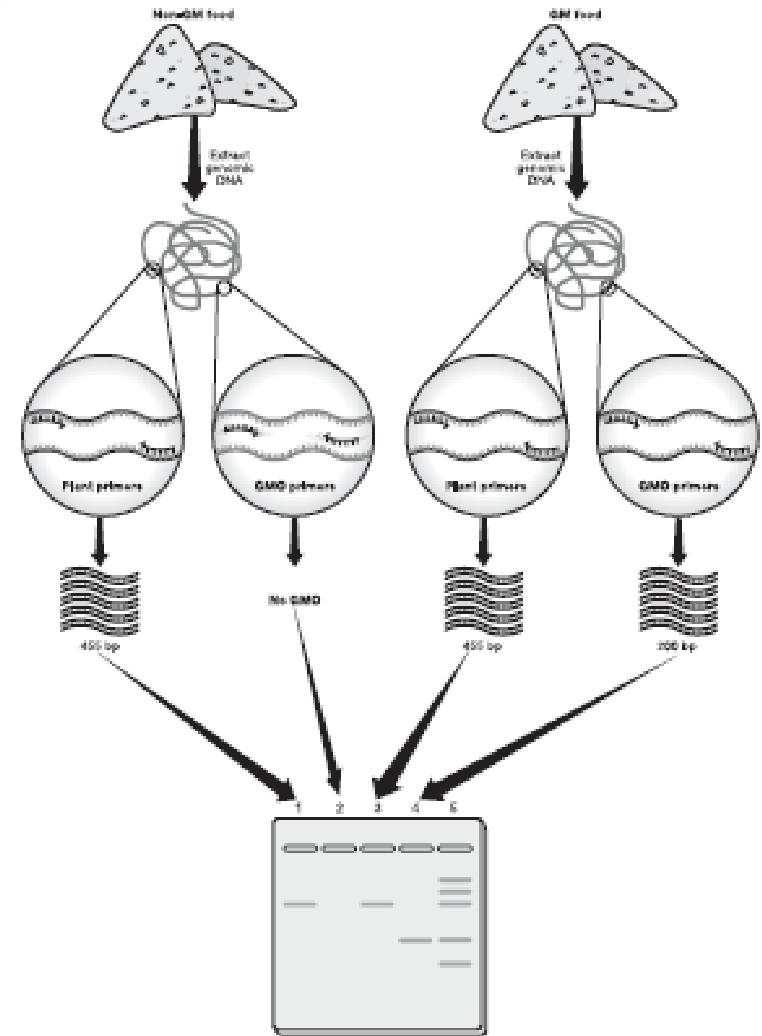
Other Applications of PCR

- PCR in medicine
  - Detection of disease-causing organisms
    - Species-specific primers help isolate genes
  - Amplification of disease-causing organisms for quick diagnosis
    - Real-time PCR confirmed H1N1 outbreak
  - Amplification of genes of interest for cloning
    - Roles of genes in cancer can be explored

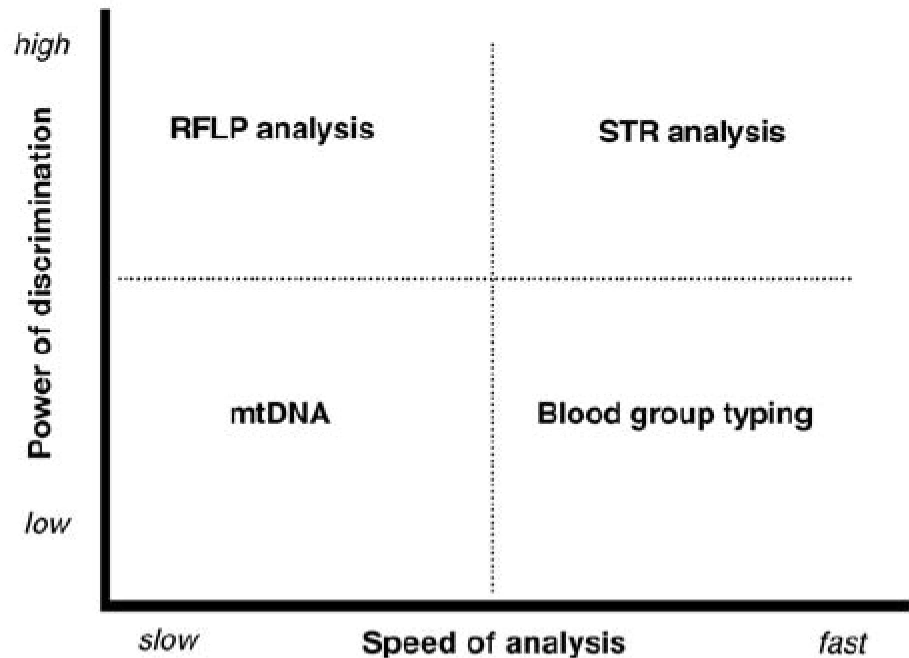
Other Applications of PCR

- PCR in agriculture
  - GMO detection in foods
  - Compliance with reporting requirements
    - Can verify levels of genetic modifications in exports
    - Can identify percentages of genetic modification to meet various countries' reporting requirements



Other Applications of PCR

- PCR in forensics
  - Short tandem repeats (STR) analysis
    - Short tandem repeats are amplified and used as a method of identification
    - They have repetitive regions from 2-6 bp
    - They are inherited from parents
    - They display lots of variety



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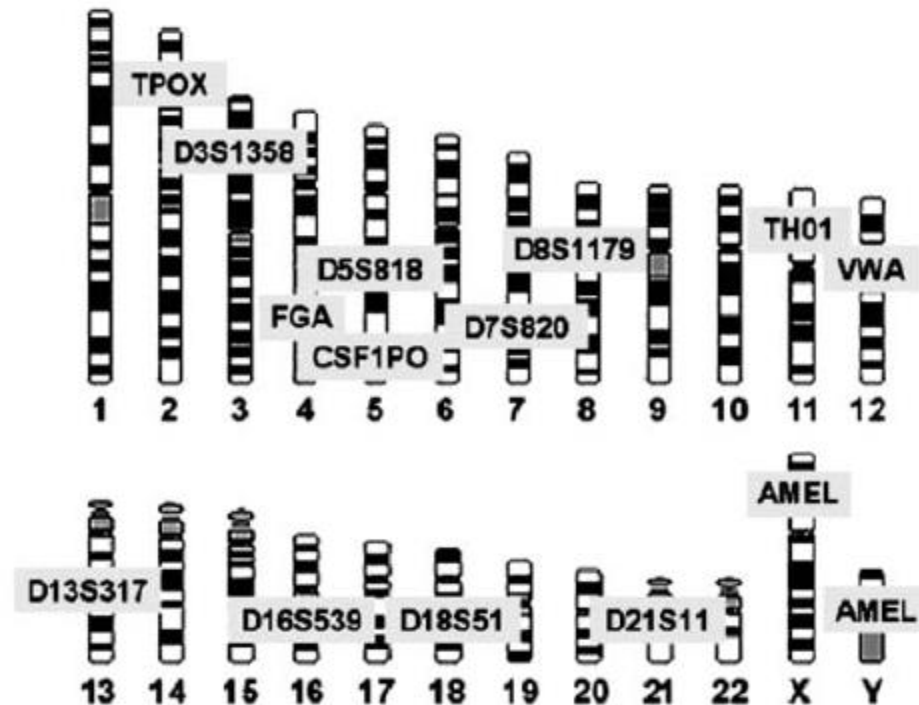
Other Applications of PCR

- PCR in forensics
  - Allele frequencies
    - Vary between different ethic groups
  - Combined DNA Index System (CODIS)
    - Indexes 13 different STR loci
    - Maintains national sample database FBI uses to statistically calculate random match probability



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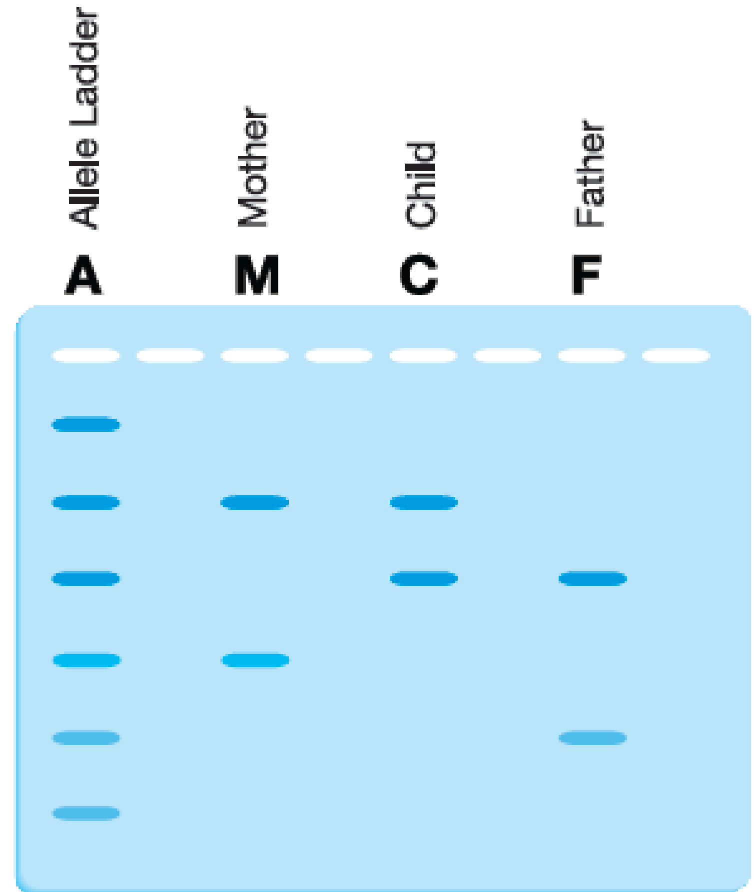
Other Applications of PCR

- PCR in paternity
  - Samples are amplified using STR analysis
    - Children are a combination of parents
    - In example, the child is possible from the mother and father shown
    - Method is often use to exclude paternity



Other Applications of PCR

- PCR in human migration
  - Short interspersed repetitive elements (SINEs) have been inserted randomly into our human genome over millions of years. Alu is one such element
  - PV92 is one specific Alu repetitive element
  - Can be used to trace migration of humans

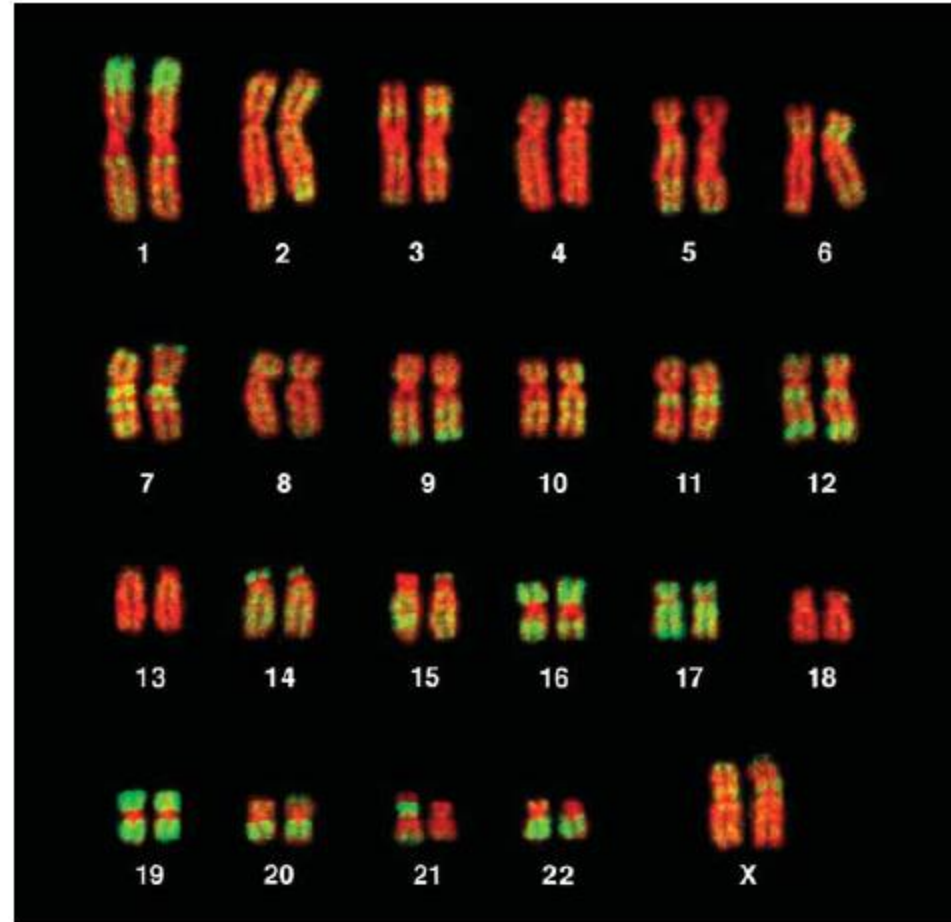


Figure 6.23. **Alu sequences in the human genome.** A karyotype showing human metaphase chromosomes stained with a fluorescent probe to the Alu sequence (green) and counterstained for DNA (red). Photo credit: Bolzer et al. (2005).

Other Applications of PCR

- PCR in wildlife conservation
  - Poaching endangers many animals in the world today
  - STR analysis can determine legality of animal parts to possess or sell

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Chapter 6 Summary