Basic Methods in Cellular and Molecular Biology

(DNA)

Northern and Southern Blotting Facilitate Hybridization with Electrophoretically Separated Nucleic Acid Molecule

- In a complex mixture of nucleic acids, DNA probes are often used to detect only those molecules with sequences that are complementary to all or part of the probe.

- Gel electrophoresis can be used to fractionate the many different RNA or DNA molecules in a crude mixture according to their size before the hybridization reaction is performed;

- if the probe binds to molecules of only one or a few sizes, one can be certain that the hybridization was indeed **specific**.

- Moreover, the size information obtained can be invaluable in itself.

An example illustrates this point:
Suppose that one wishes to determine the nature of the defect in a mutant mouse that produces abnormally low amounts of albumin, a protein that liver cells normally secrete into the blood in large amounts.

Northern and Southern Blotting Facilitate Hybridization with Electrophoretically Separated Nucleic Acid Molecule

- First, one **collects** identical samples of liver tissue from mutant and normal mice (the latter serving as controls) and **disrupts** the cells in a strong detergent to inactivate nucleases that might otherwise degrade the nucleic acids.

- Next, one separates the RNA and DNA from all of the other cell components:

-the proteins present are completely denatured and removed by repeated extractions with **phenol**-a potent organic solvent that is partly miscible with water;

-the nucleic acids, which remain in the **aqueous phase**, are then precipitated with alcohol to separate them from the small molecules of the cell.

-Then, one **separates** the DNA from the RNA by their **different solubilities in alcohols**

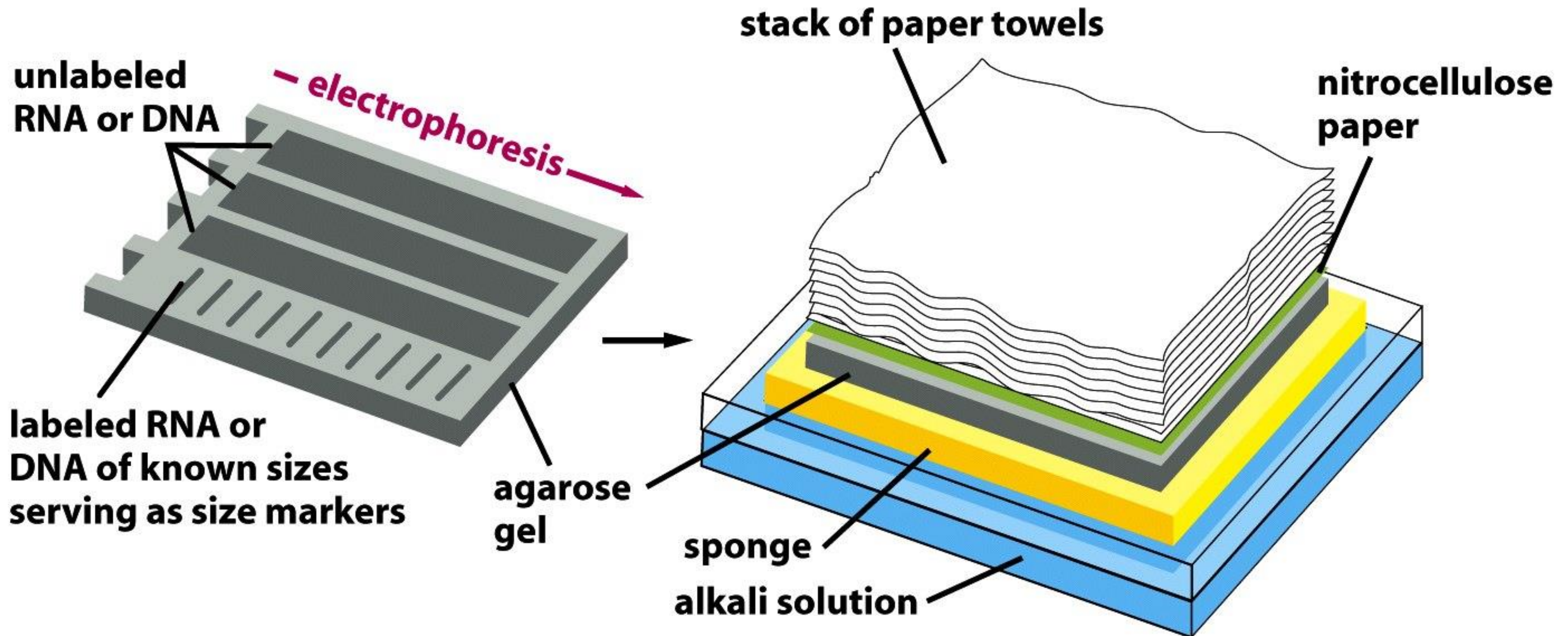
-and **degrades** any contaminating nucleic acid of the unwanted type by treatment with a **highly specific enzyme-either an RNase or a DNase**.

-The mRNAs are typically **separated** from bulk RNA **by retention on a chromatography column that specifically binds the poly-A tails of mRNAs**.

Northern and Southern Blotting Facilitate Hybridization with Electrophoretically Separated Nucleic Acid Molecule

✓ To analyze the albumin-encoding mRNAs, a technique called **Northern blotting** is used.

-First, the intact mRNA molecules **purified** from mutant and control liver cells are fractionated on the basis of their sizes into a series of bands by gel electrophoresis.
-Then, to make the RNA molecules accessible to DNA probes, a replica of the pattern of RNA bands on the gel is made by **transferring** ("blotting") the fractionated RNA molecules onto a sheet of nitrocellulose or nylon paper.



Northern and Southern Blotting Facilitate Hybridization with Electrophoretically Separated Nucleic Acid Molecule

-The paper is then **incubated** in a solution containing a labeled DNA probe, the sequence of which corresponds to part of the template strand that produces albumin mRNA.
-The RNA molecules that **hybridize** to the labeled DNA probe on the paper (because they are complementary to part of the normal albumin gene sequence) are then located by detecting the bound probe by **autoradiography** or **by chemical means**.

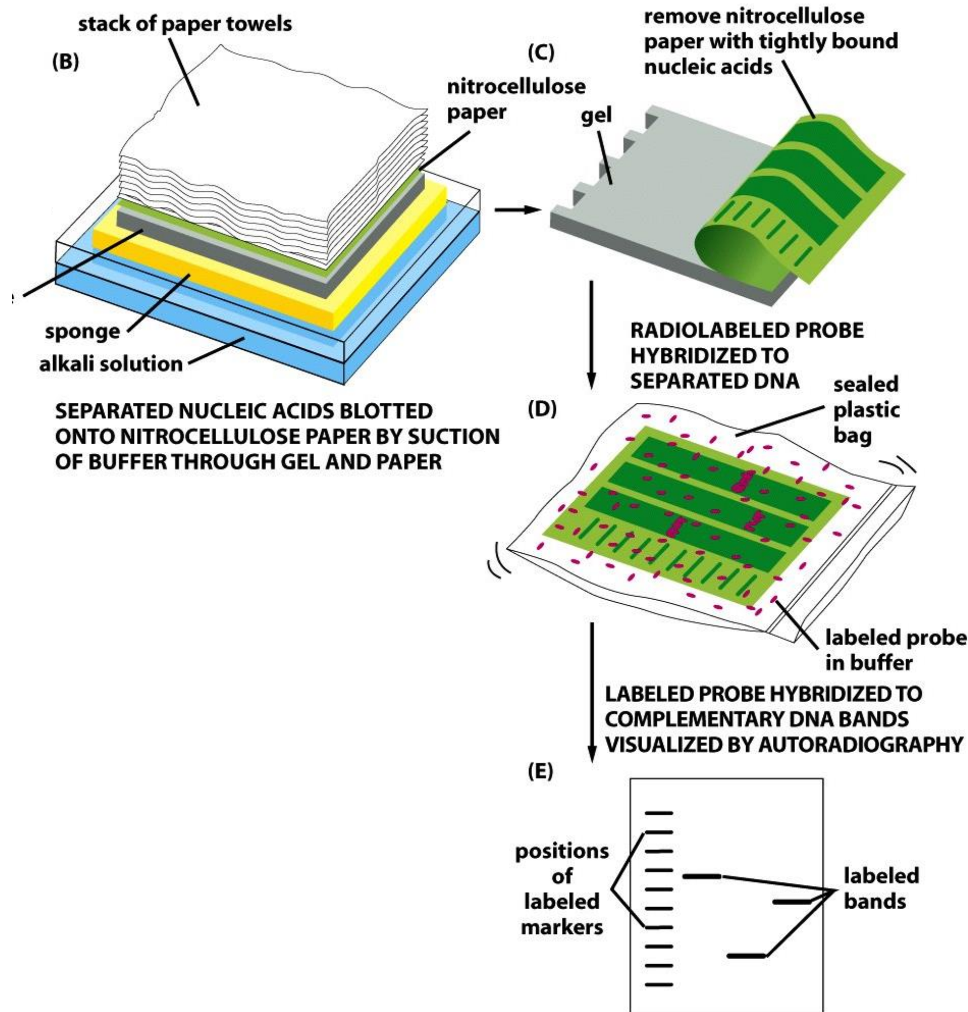
-The sizes of the hybridized RNA molecules can be determined by **reference** to RNA standards of known sizes that are electrophoresed side by side with the experimental sample.

In this way, one might discover:

1-Liver cells from the mutant mice make albumin mRNA in normal amounts and of normal size;

2-you might find that they make it in normal size but in greatly reduced amounts.

3-Another possibility is that the mutant albumin mRNA molecules are abnormally short;
in this case the gel blot could be retested with a series of shorter DNA probes, each corresponding to small portions of the gene, to reveal which part of the normal RNA is missing.



Northern and Southern Blotting Facilitate Hybridization with Electrophoretically Separated Nucleic Acid Molecule

- The original **gel-transfer hybridization** method, called **Southern blotting**, analyzes DNA rather than RNA.

- It was named after its inventor, and the Northern and Western blotting techniques were named with reference to it.

-Here, isolated DNA is first cut into readily separable fragments with restriction nucleases.
-The double-stranded fragments are then separated on the basis of size by gel electrophoresis,
-and those complementary to a DNA probe are identified by blotting and hybridization.

- To characterize the structure of the albumin gene in the mutant mice, an albumin-specific DNA probe would be used to construct a detailed **restriction map** of the genome in the region of the albumin gene (such a map consists of the pattern of DNA fragments produced by various restriction nucleases).

- From this map one could determine if the albumin gene has been rearranged in the defective animals-for example, by the **deletion** or the **insertion** of a short DNA sequence;

- most single-base changes, however, could not be detected in this way.

Genes Can Be Cloned in vitro Using PCR

- **Genomic and cDNA libraries** were once the only route to cloning genes and they are still used for cloning very large genes and for **sequencing whole genomes**.

- However, a powerful and versatile method for amplifying DNA, known as the **polymerase chain reaction (PCR)**, provides a more rapid and straightforward approach to DNA cloning, particularly in organisms whose complete genome sequence is known.

- Invented in the 1980s, PCR revolutionized the way that DNA and RNA are analyzed.

- The technique can amplify any nucleotide sequence selectively and is performed entirely in a test tube.

- Eliminating the need for bacteria makes PCR convenient and rapid—billions of copies of a nucleotide can be generated in a matter of hours.

- Starting with an entire genome, PCR allows DNA from a specified region—selected by the experimenter—to be greatly amplified, effectively "**purifying**" this DNA away from the remainder of the genome, which remains unamplified.

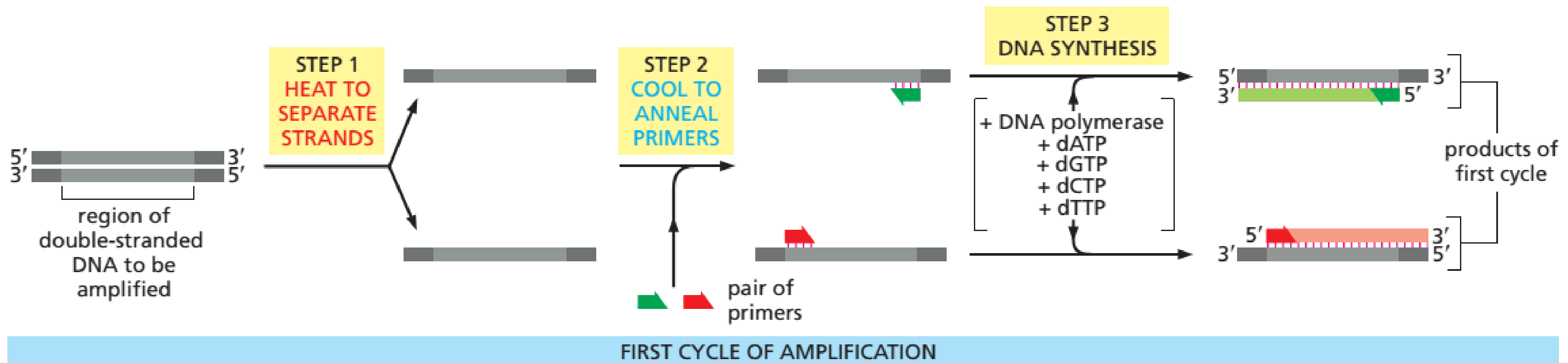
- Because of its power to greatly amplify nucleic acids, PCR is remarkably sensitive: the method can be used to detect the trace amounts of DNA in a drop of blood left at a crime scene or in a few copies of a viral genome in a patient's blood sample.

Genes Can Be Cloned in vitro Using PCR

- The success of PCR depends both on the **selectivity of DNA hybridization** and on the **ability of DNA polymerase to copy a DNA template** faithfully through repeated rounds of replication in vitro.
- **DNA polymerase** adds nucleotides to the 3′ end of a growing strand of DNA.
- To copy DNA, the polymerase requires a **primer**—a short nucleotide sequence that provides a 3′ end from which synthesis can begin.
- For PCR, the primers are designed by the experimenter, synthesized chemically, and, by hybridizing to genomic DNA, "**tell**" the polymerase which part of the genome to copy.
- DNA primers (in essence, the same type of molecules as DNA probes but without a radioactive or fluorescent label) can be designed to **uniquely locate any position on a genome**.
- PCR is an iterative process in which the cycle of amplification is repeated dozens of times.

Genes Can Be Cloned in vitro Using PCR

- At the start of each cycle, the two strands of the double-stranded DNA template are separated and a different primer is **annealed** to each.
- These primers mark the right and left boundaries of the DNA to be amplified.
- DNA polymerase is then allowed to replicate each strand independently.



A pair of primers directs the synthesis of a desired segment of DNA in a test tube. Each cycle of PCR includes three steps:

(1) The double-stranded DNA is **heated** briefly to separate the two strands.

(2) The DNA is exposed to a large excess of a pair of specific primers— designed to bracket the region of DNA to be amplified—and the sample is **cooled** to allow the primers to hybridize to complementary sequences in the two DNA strands.

(3) This mixture is incubated with DNA polymerase and the four deoxyribonucleoside triphosphates so that DNA can be synthesized, starting from the two primers.

To amplify the DNA, the cycle is repeated many times by reheating the sample to separate the newly synthesized DNA strands.

The technique depends on the use of a special DNA polymerase isolated from a **thermophilic bacterium**; this polymerase is stable at much higher temperatures than eukaryotic DNA polymerases, so it is not denatured by the heat treatment shown in step 1. The enzyme therefore does not have to be added again after each cycle.

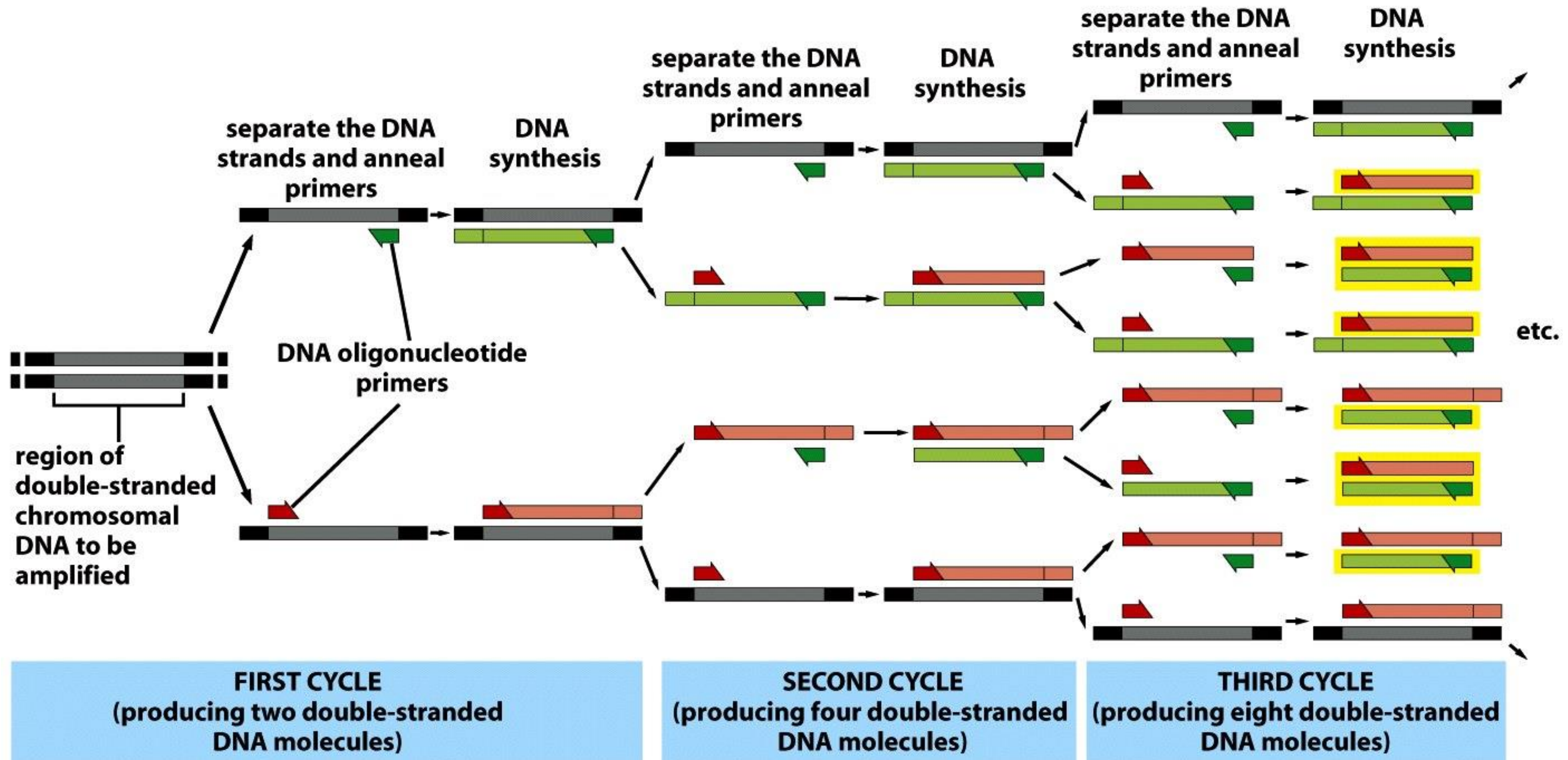
Genes Can Be Cloned in vitro Using PCR

In subsequent cycles, all the newly synthesized DNA molecules produced by the polymerase serve as templates for the next round of replication.

Through this iterative amplification process, many copies of the original sequence can be made—billions after about 20 to 30 cycles.

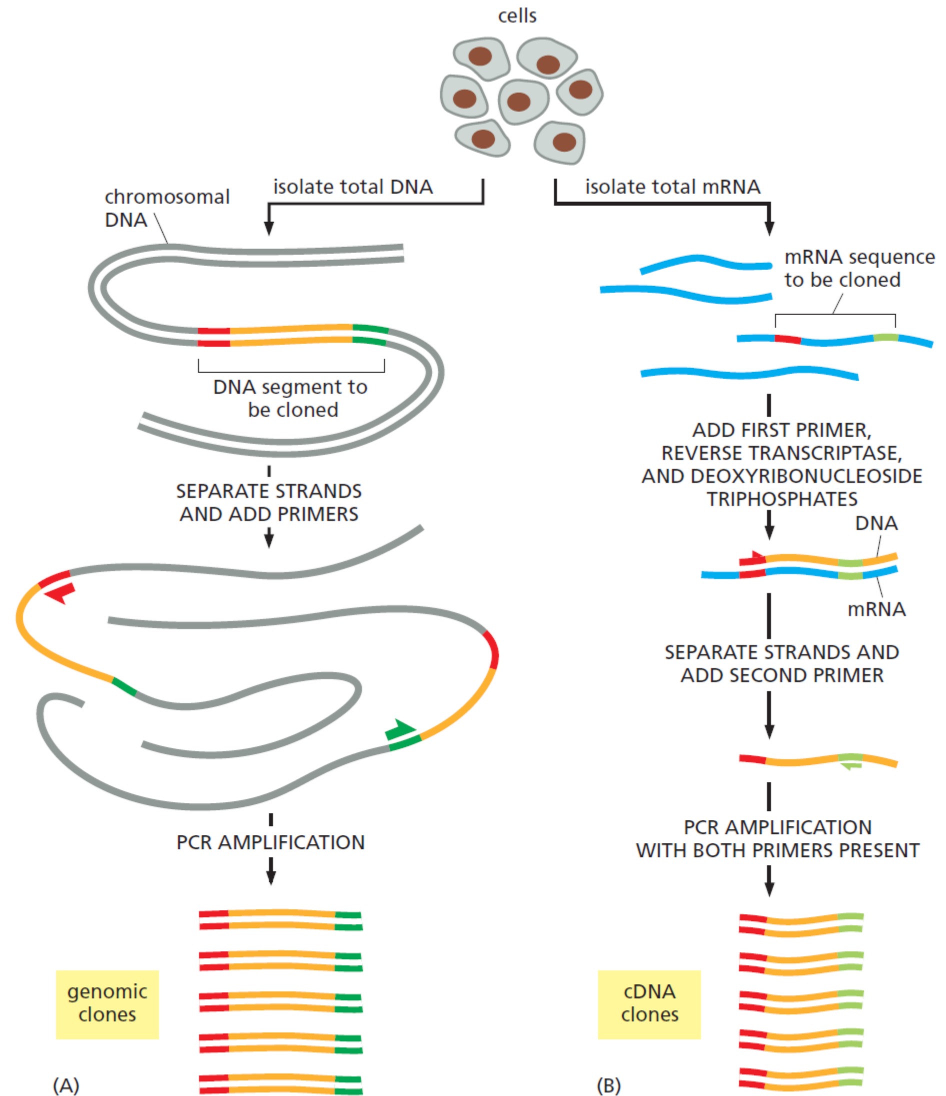
PCR is now the method of choice for cloning relatively short DNA fragments (say, under **10,000** nucleotide pairs).

Each cycle takes only about **five** minutes, and automation of the whole procedure enables cell-free cloning of a DNA fragment in a few hours.



Genes Can Be Cloned in vitro Using PCR

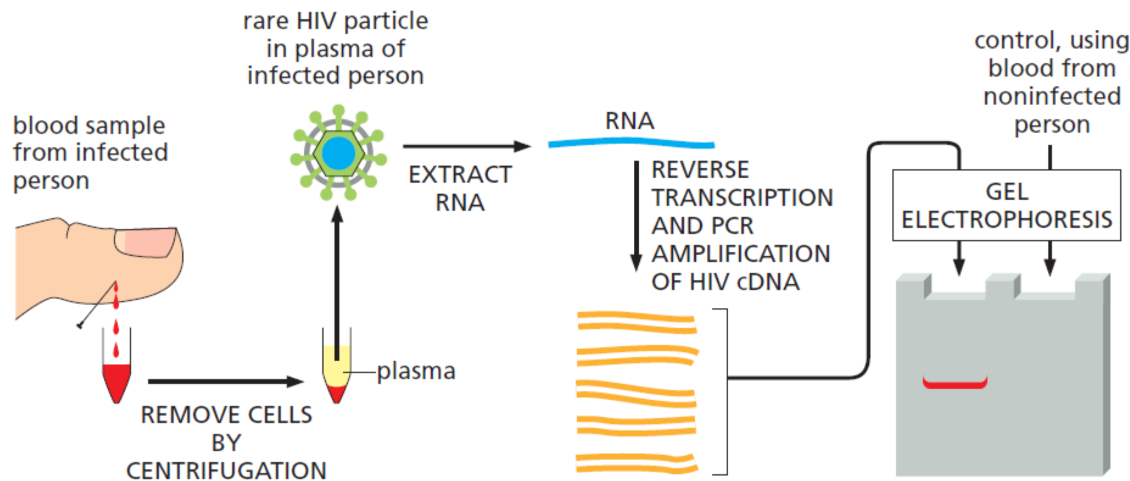
- The original template for PCR can be either DNA or RNA, so this method can be used to obtain either a genomic clone (complete with introns and exons) or a cDNA copy of an mRNA.



(B)To use PCR to obtain a **cDNA clone** of a gene, total mRNA is first purified from cells.
The first primer is added to the population of mRNAs, and reverse transcriptase is used to make a DNA strand complementary to the specific RNA sequence of interest.
The second primer is then added, and the DNA molecule is amplified through many cycles of PCR.

PCR Is Also Used for Diagnostic and Forensic Applications

- The PCR method is extraordinarily sensitive; it can detect **a single DNA molecule** in a sample if at least part of the sequence of that molecule is known.
- Trace amounts of RNA can be analyzed in the same way by first transcribing them into DNA with reverse transcriptase.
- For these reasons, PCR is frequently employed for uses that go beyond simple cloning.
- For example, it can be used to detect invading **pathogens** at very early stages of infection. In this case, short sequences complementary to a segment of the infectious agent's genome are used as primers and following many cycles of amplification, even a few copies of an invading bacterial or viral genome in a patient's sample can be detected.
- For many infections, PCR has replaced the use of antibodies against microbial molecules to detect the presence of the invader.



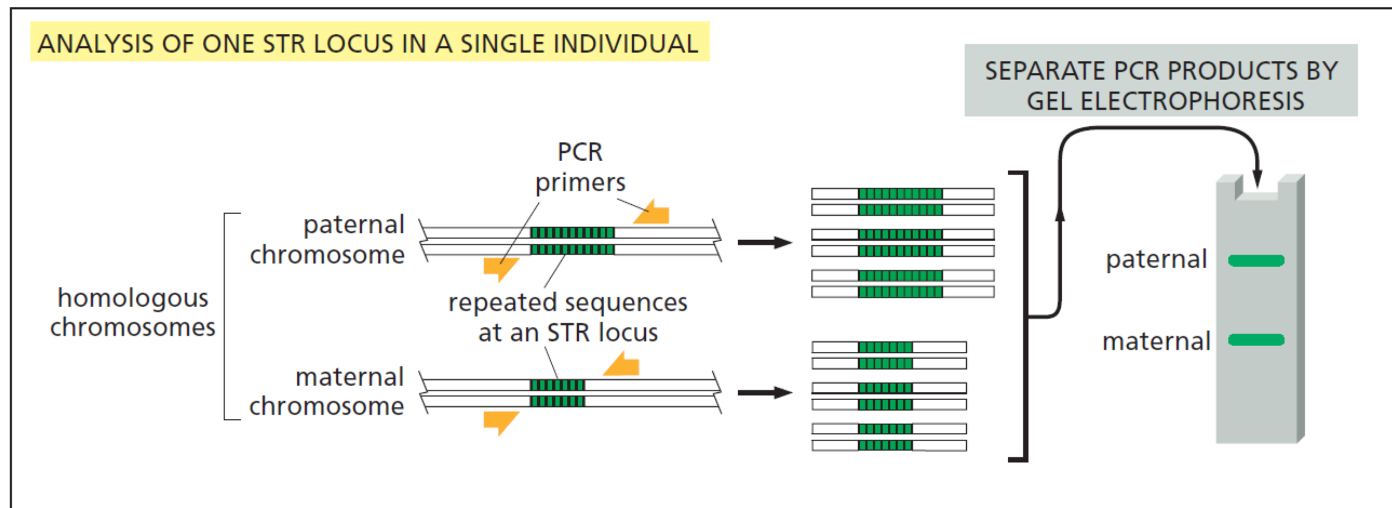
- It is also used to verify the authenticity of a food source—for example, whether a sample of beef actually came from a cow.

PCR Is Also Used for Diagnostic and Forensic Applications

- Finally, PCR is now widely used in forensics. The method's extreme sensitivity allows forensic investigators to isolate DNA from minute traces of human blood or other tissue to obtain a **DNA fingerprint** of the person who left the sample behind.
- With the possible exception of identical twins, the genome of each human differs in DNA sequence from that of every other person on Earth.
- Using primer pairs targeted at genome sequences that are known to be highly variable in the human population, PCR makes it possible to generate a distinctive DNA fingerprint for any individual.

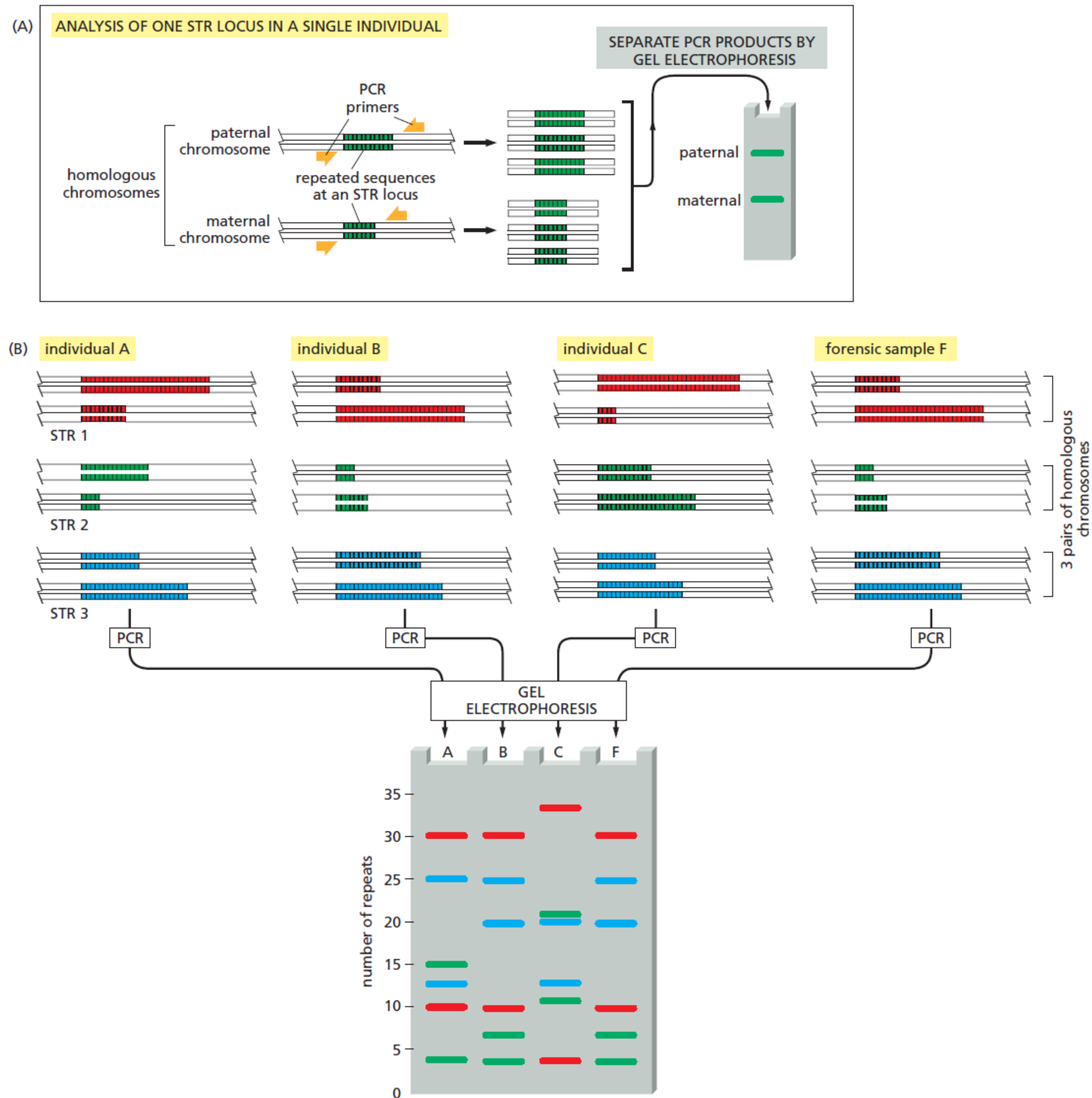
- Such forensic analyses can be used not only to help identify those who have done wrong, but also—equally important—to exonerate those who have been wrongfully accused.

- The DNA sequences analyzed are **short tandem repeats (STRs)** composed of sequences such as CACACA… or GTGTGT… STRs are found in various positions (loci) in the human genome.
- **The number of repeats in each STR locus is highly variable in the population, ranging from 4 to 40 in different individuals**. Because of the variability in these sequences, individuals will usually inherit a different number of repeats at each STR locus from their mother and from their father; two unrelated individuals, therefore, rarely contain the same pair of sequences at a given STR locus.



PCR Is Also Used for Diagnostic and Forensic Applications

- The more loci that are examined, the more confident one can be about the results.
- When examining the variability at **5–10 different STR loci**, the odds that two random individuals would share the same fingerprint by chance are approximately one in 10 billion.
- In the case shown here, individuals A and C can be eliminated from inquiries, while B is a clear suspect.
- A similar approach is used routinely in paternity testing.



Expression of Individual Genes Can Be Measured Using Quantitative RT-PCR

- Quantitative RT-PCR (reverse transcription–polymerase chain reaction) begins with the total population of RNA molecules purified from a tissue or a cell culture.

- It is important that no DNA be present in the preparation; it must be purified away or enzymatically degraded.
- **Two DNA primers** that specifically match the mRNA of interest are added, along with **reverse transcriptase**, **DNA polymerase**, and the **four deoxyribonucleoside triphosphates** needed for DNA synthesis.

- The first round of synthesis is the reverse transcription of the RNA into DNA using one of the primers.
- Next, a series of heating and cooling cycles allows the amplification of that DNA strand by PCR.

- **The quantitative part of this method relies on a direct relationship between the rate at which the PCR product is generated and the original concentration of the mRNA species of interest.**

- By adding chemical dyes to the PCR that fluoresce only when bound to double-stranded DNA, a simple fluorescence measurement can be used to track the progress of the reaction and thereby accurately deduce the **starting concentration of the mRNA that is amplified**.

- Although it seems complicated, this quantitative RT-PCR technique is relatively **fast** and **simple** to perform in the laboratory.

- **It is currently the method of choice for accurately quantifying mRNA levels from any given gene.**

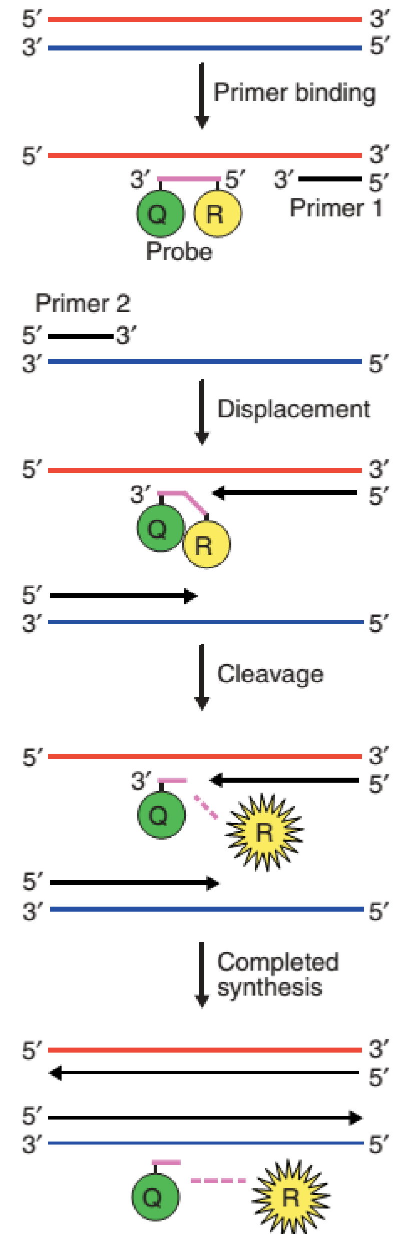
Expression of Individual Genes Can Be Measured Using Quantitative RT-PCR

Quantitative real-time RT–PCR rely upon the detection and quantitation of a fluorescent reporter, whose signal increases in direct proportion to the amount of PCR product in a reaction.

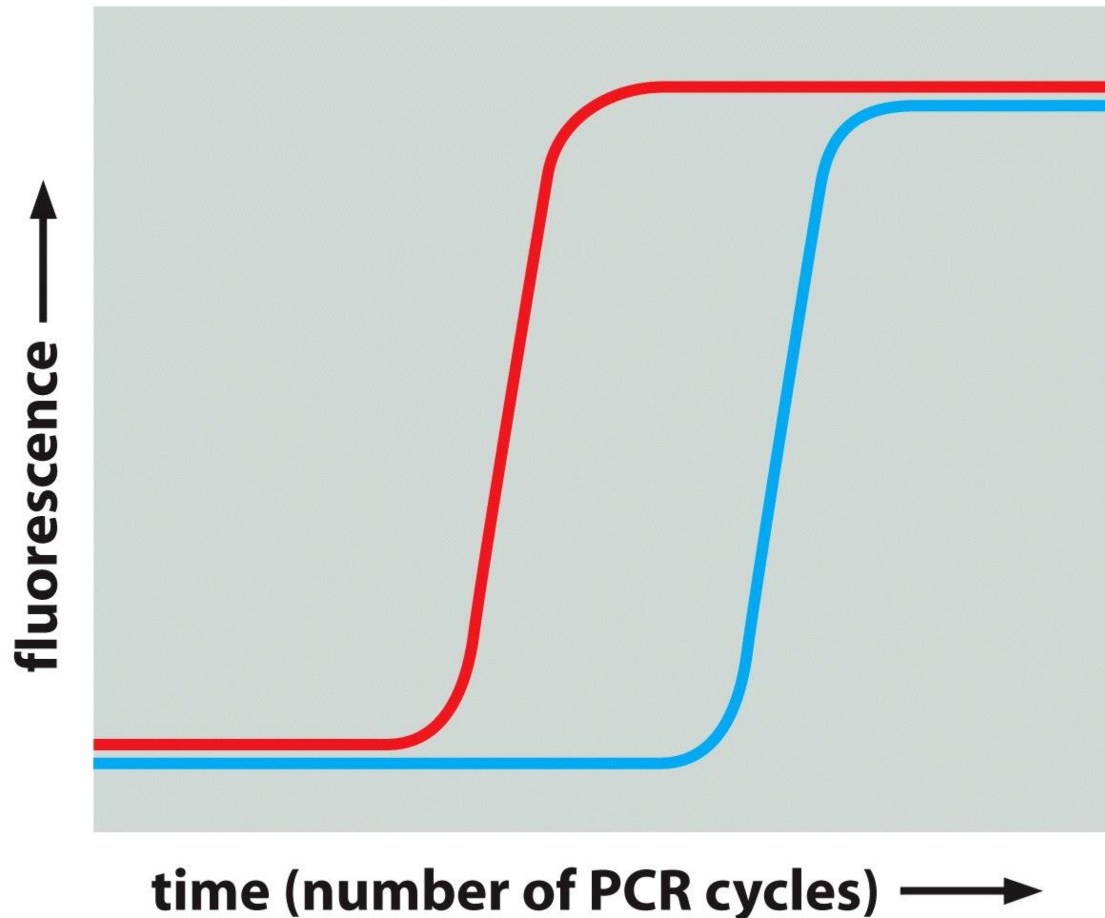
- In the simplest form, the reporter is the double-strand DNA-specific dye SYBR Green.
- SYBR Green binds double-stranded DNA, probably in the minor groove, and, upon excitation, emits light.
- Thus, if the dye is included in a PCR reaction, as a PCR product accumulates the fluorescence increases.
- The **advantages** of SYBR Green are that it is **inexpensive**, **easy** to use, and **sensitive**.
- The **disadvantage** is that SYBR Green will bind to **any double-stranded DNA** in the reaction, including primer dimers and other non-specific reaction products, which can result in an over-estimation of the target concentration.

Expression of Individual Genes Can Be Measured Using Quantitative RT-PCR

- The alternative method for quantifying PCR products is **TaqMan®**, which relies on fluorescence resonance energy transfer (FRET) of hybridization probes for quantitation.
- TaqMan probes are oligonucleotides that contain a fluorescent reporter dye, typically attached to the 5'base, and a quenching dye, typically attached to the 3'base.
- The probe is designed to hybridize to an internal region of a PCR product.
- When irradiated, the excited reporter dye transfers energy to the nearby quenching dye molecule rather than fluorescing, resulting in a **non-fluorescent substrate**.
- During PCR, when the polymerase replicates a template on which a probe is bound, the 5'-3'exonuclease activity of the polymerase cleaves the probe.
- This separates the fluorescent and quenching dyes and FRET no longer occurs.
- Fluorescence increases in each PCR cycle, proportional to the rate of probe cleavage, and is measured in a modified thermocycler.
- Real-time PCR is a powerful quantitative tool, but the cost of reagents and equipment is much higher than that of standard PCR reactions.



Expression of Individual Genes Can Be Measured Using Quantitative RT-PCR



RNA levels can be measured by quantitative RT-PCR.
The fluorescence measured is generated by a dye that fluoresces only when bound to the double-stranded DNA products of the RT-PCR.
The red sample has a higher concentration of the mRNA being measured than does the blue sample, since it requires **fewer PCR cycles to reach the same half-maximal concentration of double-stranded DNA**.
Based on this difference, the relative amounts of the mRNA in the two samples can be precisely determined.