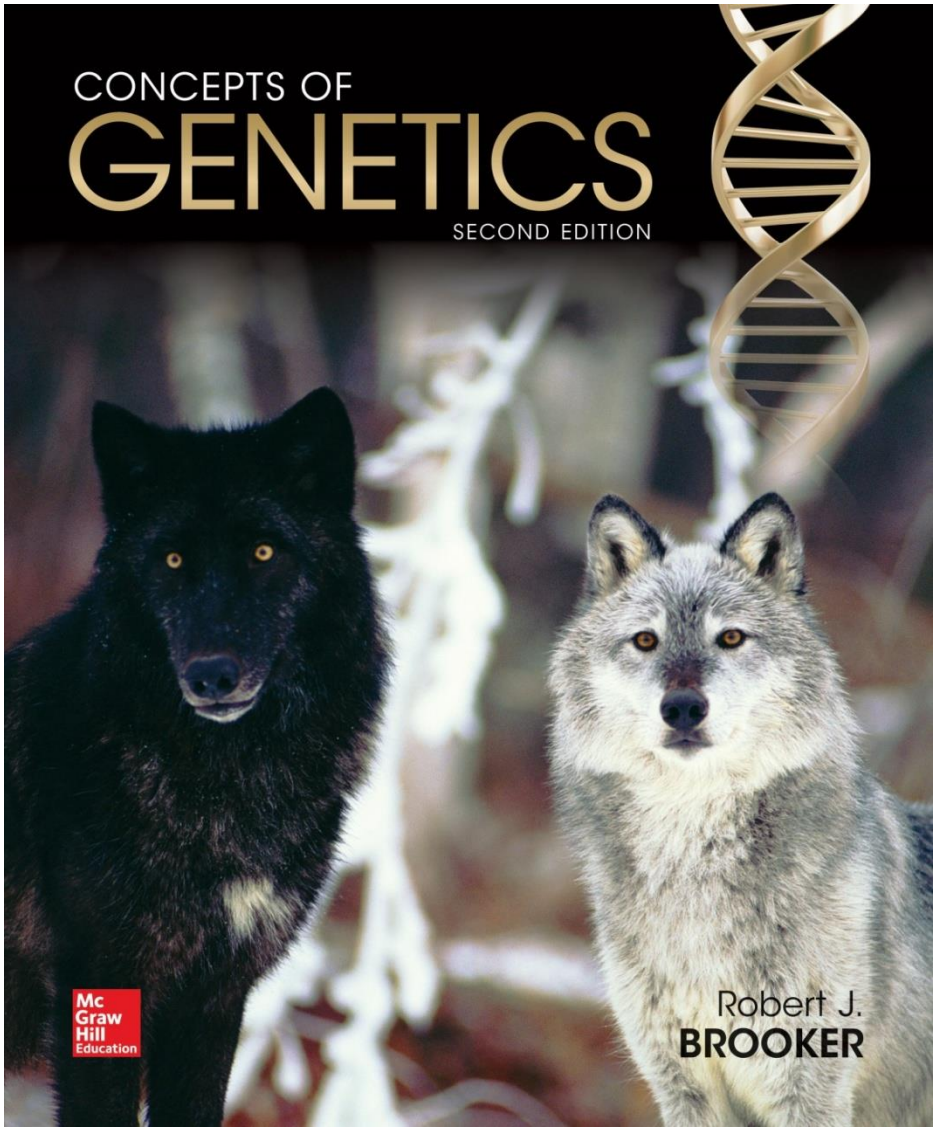
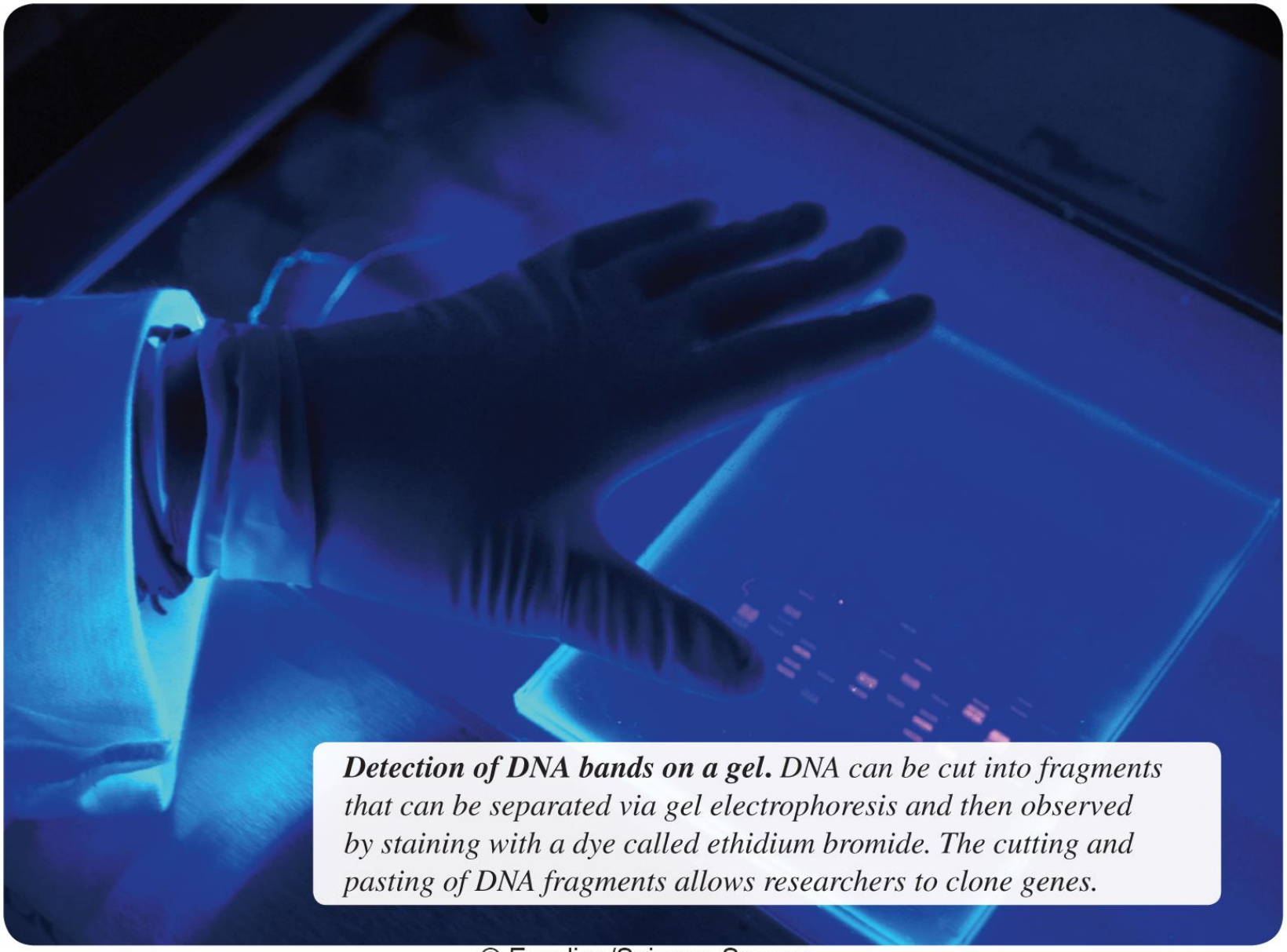


Chapter 20

Lecture Outline



Detection of DNA bands on a gel. *DNA can be cut into fragments that can be separated via gel electrophoresis and then observed by staining with a dye called ethidium bromide. The cutting and pasting of DNA fragments allows researchers to clone genes.*

- **Recombinant DNA technology**
  - Produce new arrangements or combinations of DNA

- In the early 1970s, researchers at Stanford University were able to construct **recombinant DNA molecules**

  - Shortly thereafter, it became possible to introduce such molecules into living cells

  - This achievement ushered in the era of gene cloning

- Recombinant DNA technology can be used for
  - Sequencing the human genome
  - Cloning the open reading frames of genes for study
  - Cloning other fragments of DNA for study (promoters etc.)
  - Expressing proteins in cells to make therapeutic products
    - Human insulin

20.1 Gene Cloning Using Vectors

- The procedure for cloning a gene into a vector
- How cDNA is made
- A genomic library versus a cDNA library

Gene Cloning

- **Gene cloning** refers to isolating, and then making many copies of a particular sequence of DNA
  - Used for whole genes or parts of genes
- A basic method developed in 1970's
  - More refined methods developed since then

- Cloning experiments usually involve the joining or recombining of two kinds of DNA molecules

  - **Chromosomal DNA** – cellular, viral etc.
    - Source of the DNA segment of interest
    - DNA purified using a variety of biochemical techniques from tissues and cells of interest

  - **Vector DNA**
    - DNA that will replicate without integrating into host chromosome – plasmids and viruses
    - Serves as the carrier of the DNA segment to be cloned

- Plasmids and viruses used as **vectors** contain
  - **Origin of replication** that can be used by host cell enzymes
  - Some kind of **selectable marker** to allow for isolation of host cells that carry the vector and force them to maintain the vector– usually antibiotic resistance
  - Site for joining vector DNA to DNA segment to be cloned
  - May have other desirable features such as sequences to allow expression of the cloned DNA

TABLE 20.2

Some Vectors Used in Cloning Experiments

Example	Type	Description
pBluescript	Plasmid	A type of vector like the one shown in Figure 20.2. It is used to clone small segments of DNA and propagate them in *E. coli*.
YEp24	Plasmid	This plasmid is an example of a shuttle vector, which can replicate in two different host species, *E. coli* and *Saccharomyces cerevisiae*. It carries origins of replication for both species.
λgt11	Viral	This vector is derived from the bacteriophage λ, which is described in Chapter 10. λgt11 also contains a promoter from the *lac* operon. When fragments of DNA are cloned next to this promoter, the DNA is expressed in *E. coli*. This is an example of an expression vector. An expression vector is designed to clone the coding sequence of genes so they are transcribed and translated correctly.
SV40	Viral	This virus naturally infects mammalian cells. Genetically altered derivatives of the SV40 viral DNA are used as vectors for the cloning and expression of genes in mammalian cells that are grown in the laboratory.
Baculovirus	Viral	This virus naturally infects insect cells. In a laboratory, insect cells can be grown in liquid media. Unlike many other types of eukaryotic cells, insect cells often express large amounts of proteins that are encoded by cloned genes. When researchers want to make a large amount of a protein, they can clone the gene that encodes the protein into baculovirus and then purify the protein from insect cells.

Table 20.2

Restriction Enzymes

- Insertion of DNA into a vector requires at least two steps
  - Digestion of the vector and chromosomal DNA to create compatible DNA ends
  - Actual joining or **ligation** of the molecules together, uses same ligase used in DNA replication

- The enzymes used to digest DNA are known as **restriction endonucleases** or **restriction enzymes**
  - These bind to specific DNA sequences and then cleave the DNA at two defined locations, one on each strand

- Restriction enzymes bind to specific DNA sequences
  - Many are **palindromic**
    - The sequence is identical when read in the opposite direction in the complementary strand
    - For example, the *Eco*RI recognition sequence is

```
5′ GAATTC 3′
3′ CTTAAG 5′
```

- Phosphodiester backbone of the DNA can be cleaved in two different ways
  - **Staggered** – creates short single-stranded ends or “sticky ends”
    - Ends can only be joined to ends with complementary sequences
  - **Blunt ends** – no single-stranded ends generated, any blunt end can be joined to any other blunt end
- Refer to Figure 20.1

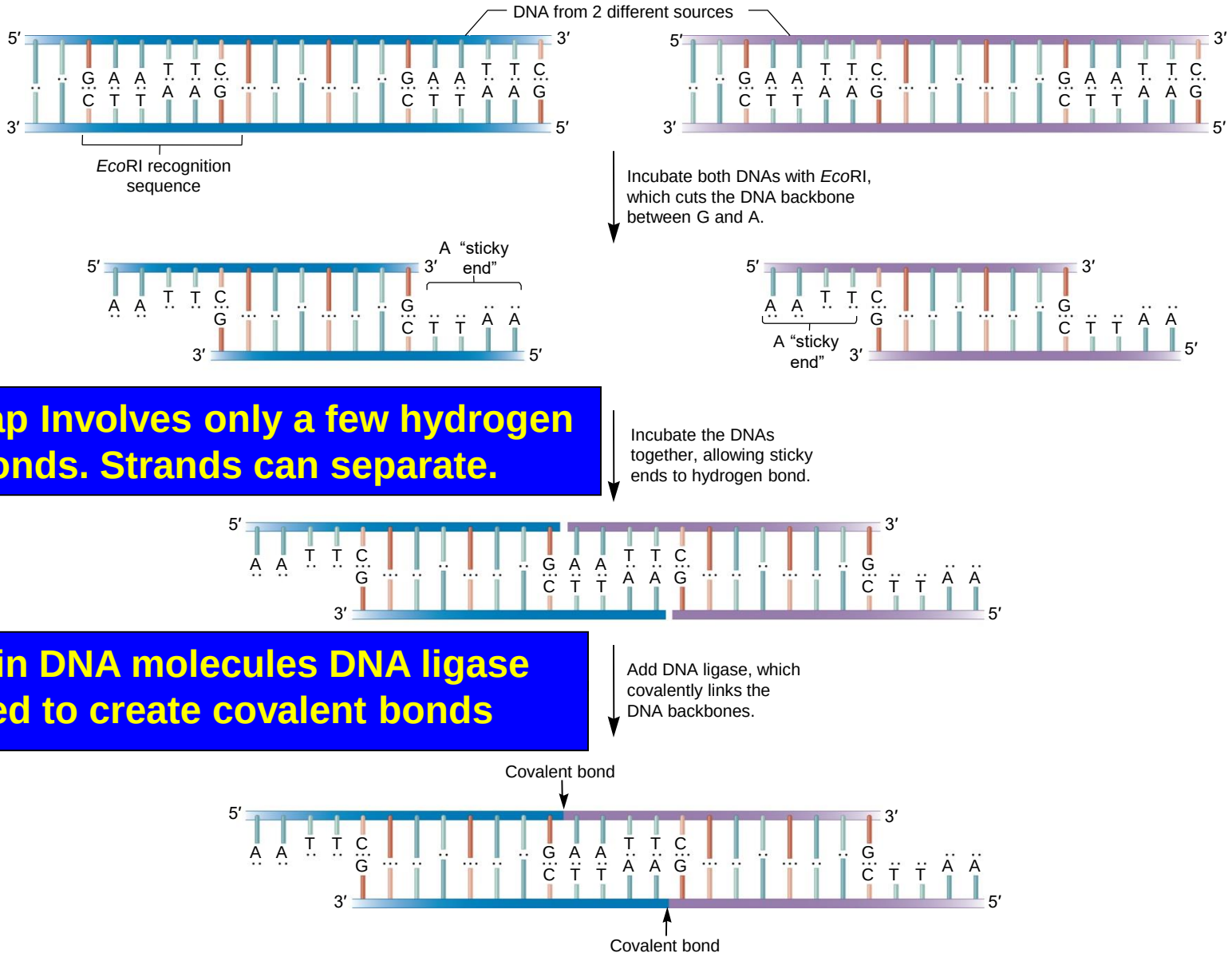


Figure 20.1 A recombinant DNA molecule

- Restriction enzymes are made naturally by many species of bacteria
  - They protect bacterial cells from invasion by foreign DNA, particularly that of bacteriophage
  - Bacteria modify their own DNA so that restriction enzymes that they make aren't able to digest it

- Several hundred different restriction enzymes are available commercially
  - Many different sequences recognized but not all possible combinations
  - Names are derived from genus, species and strain the enzyme is isolated from
    - *Eco*RI
    - *Escherichia coli* strain R
    - The roman numeral "I" indicates in what order the enzymes were discovered – in this case this was the first enzyme discovered from this species and strain

TABLE 20.3

Some Restriction Enzymes Used in Gene Cloning

Restriction Enzyme*	Bacterial Source	Sequence Recognized†
*Bam*HI	*Bacillus amyloliquefaciens* H	5′–GGATCC–3′ 3′–CCTAGG–5′
Sau3A I	*Staphylococcus aureus* 3A	5′–GATC–3′ 3′–CTAG–5′
*Eco*RI	*E. coli* RY13	5′–GAATTC–3′ 3′–CTTAAG–5′
*Nae*I	*Nocardia aerocolonigenes*	5′–GCCGGC–3′ 3′–CGGCCG–5′
*Pst*I	*Providencia stuartii*	5′–CTGCAG–3′ 3′–GACGTC–5′

*Restriction enzymes are named according to the species in which they are found. The first three letters are italicized because they indicate the genus and species names. Because a species may produce more than one restriction enzyme, the enzymes are designated I, II, III, and so on, to indicate the order in which they were discovered in a given species. Some restriction enzymes, like *Eco*RI, produce a sticky end with a 5′ overhang (see Figure 20.1), whereas others, such as *Pst*I, produce a 3′ overhang. However, not all restriction enzymes cut DNA to produce sticky ends. For example, the enzyme *Nae*I cuts DNA to produce blunt ends.

†The arrows show the locations in the upper and lower DNA strands where the restriction enzymes cleave the DNA backbone.

Table 20.3

The Steps of Gene Cloning

- Purify chromosomal DNA
  - Digest with restriction endonuclease

- Purify the plasmid (the vector)
  - Digest with restriction endonuclease that will create ends that are compatible with the digestion products of the chromosomal DNA

- Mix the chromosomal DNA fragments with the digested vector and with DNA ligase to join them together

- Introduce the **recombinant vector** with chromosomal DNA into host bacteria
  - Process called **transformation**
  - Usually *E. coli* that has been chemically treated to allow it to take DNA up from its environment
  - When cells have been treated to allow them to take DNA up from the environment they are called **competent cells**

- Select for cells that
  - Have taken up vector
  - Have a piece of chromosomal DNA in the vector

- Selection of cells that have vector
  - Vectors usually have a gene that confers antibiotic resistance, frequently ampicillin resistance for plasmid vectors (*amp^r)*
  - Cells that do not have vector will die in the presence of the antibiotic
  - Important since transformation very inefficient (<1% of cells will take up DNA)

- Selection of cells with vector that may have chromosomal DNA
  - Several systems
    - *lacZ* - Encodes β-galactosidase
      - Can cleave a substrate to turn colony blue
      - Site for chromosomal DNA ligation in *lacZ* coding region
      - Insertion of chromosomal DNA disrupts *lacZ* expression
        - » Colonies are white
        - » Refer to Figure 20.2

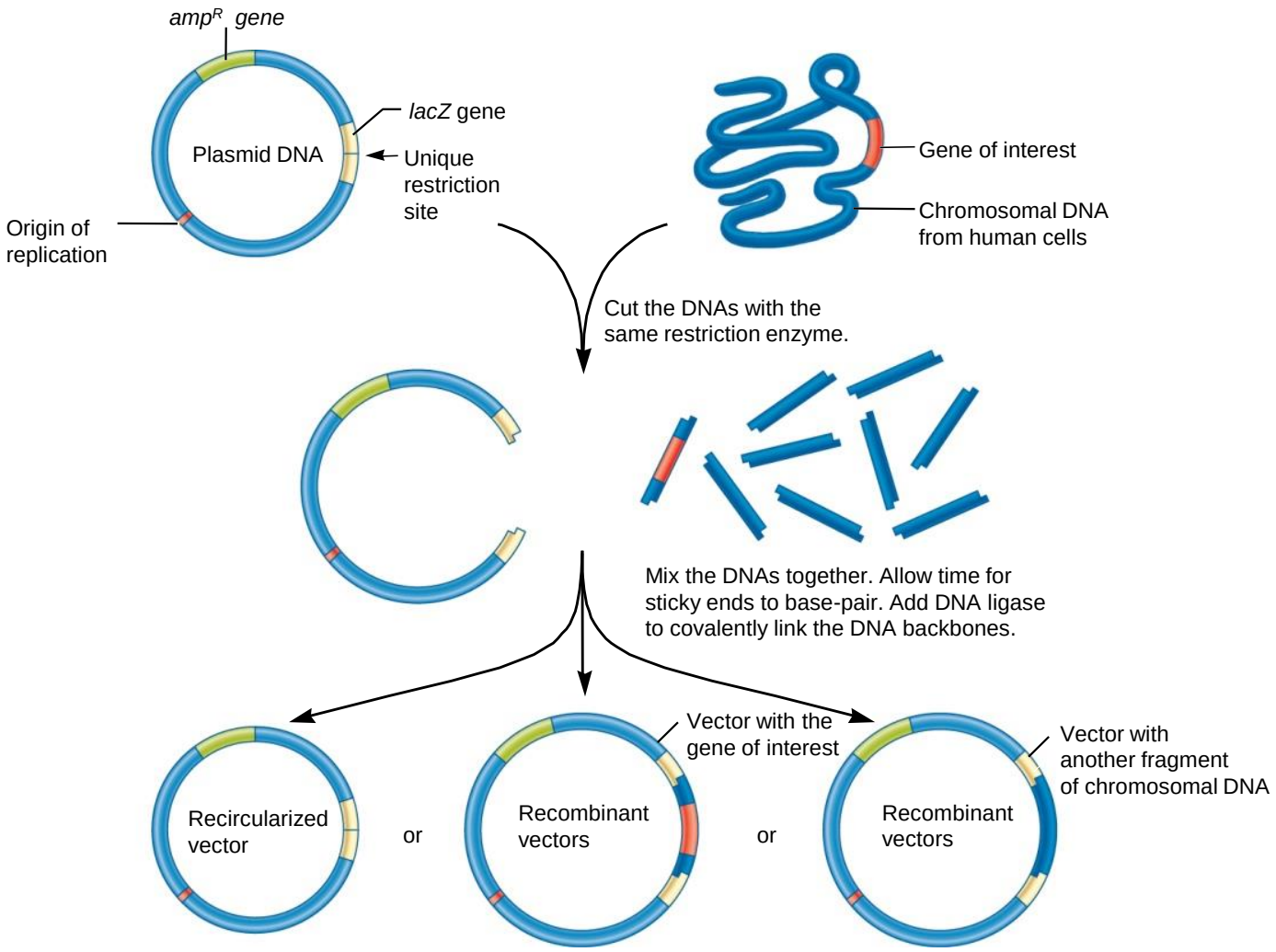


Figure 20.2 (top)

Transformation, when plasmid vectors are used, and transfection, when a viral vector is introduced into a host cell

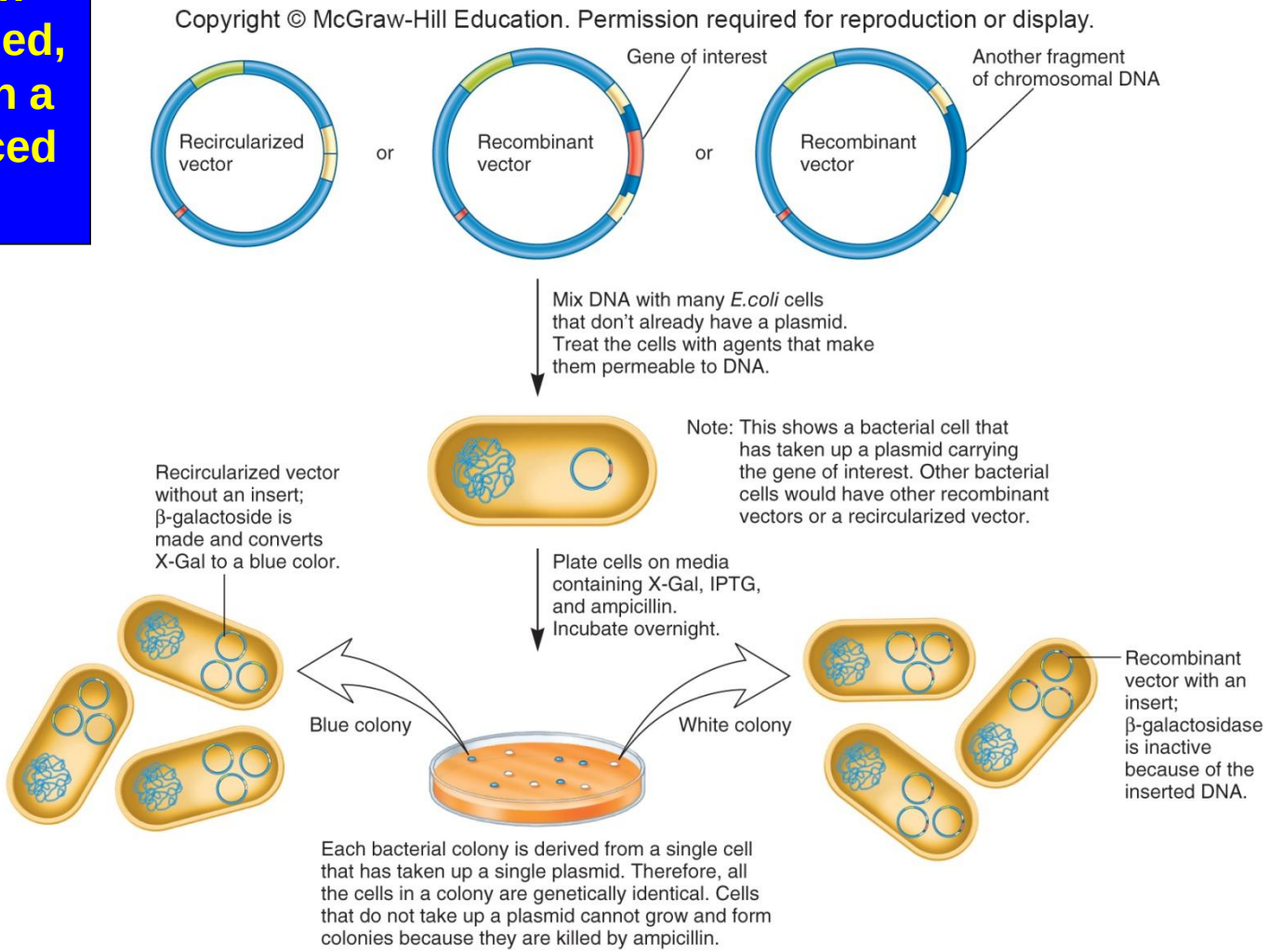


Figure 20.2 (bottom)

- All bacterial colonies growing on the plate had to have picked up the vector and its *amp*^{R} gene
  - This includes vectors that do and do not have chromosomal DNA

- Only those vectors that have an insert will have a disrupted *lacZ* gene

- The growth media contains two relevant compounds:
  - **IPTG** (isopropyl-β-D-thiogalactopyranoside)
    - An allolactose analogue that can induce the *lacZ* gene
  - **X-Gal** (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside)
    - A colorless compound that is cleaved by β-galactosidase into a blue dye
  - The color of bacterial colonies will therefore depend on whether or not the β-galactosidase is functional
    - If it is, the colonies will be **blue** – no insert
    - If not, the colonies will be **white** – insert present

- Gene cloning produces an enormous amount of a single DNA sequence –useful for study and further manipulation

  - A single bacterial cell usually takes up a single copy of the hybrid vector

  - Amplification of the DNA occurs in two ways:
    - Many cells with the construct
      - In a small tube get millions of cells
    - Vector replication
      - Generates many copies per cell

cDNA is Made By Reverse Transcriptase

- It is useful to obtain DNA that is a copy of the mRNA or other RNAs
  - DNA copy of mRNA lacks introns – useful for expression in prokaryotes
  - For studying just those genes that are transcribed

- DNA that is made from RNA is called **complementary DNA** (**cDNA**)
  - It could be single- or double-stranded

- Copying of RNA into DNA is accomplished using **reverse transcriptase**
    - Uses RNA as a template to make a complementary strand of DNA
    - Naturally occurring enzyme in retroviruses to copy their RNA genome to DNA

- Refer to Figure 20.3

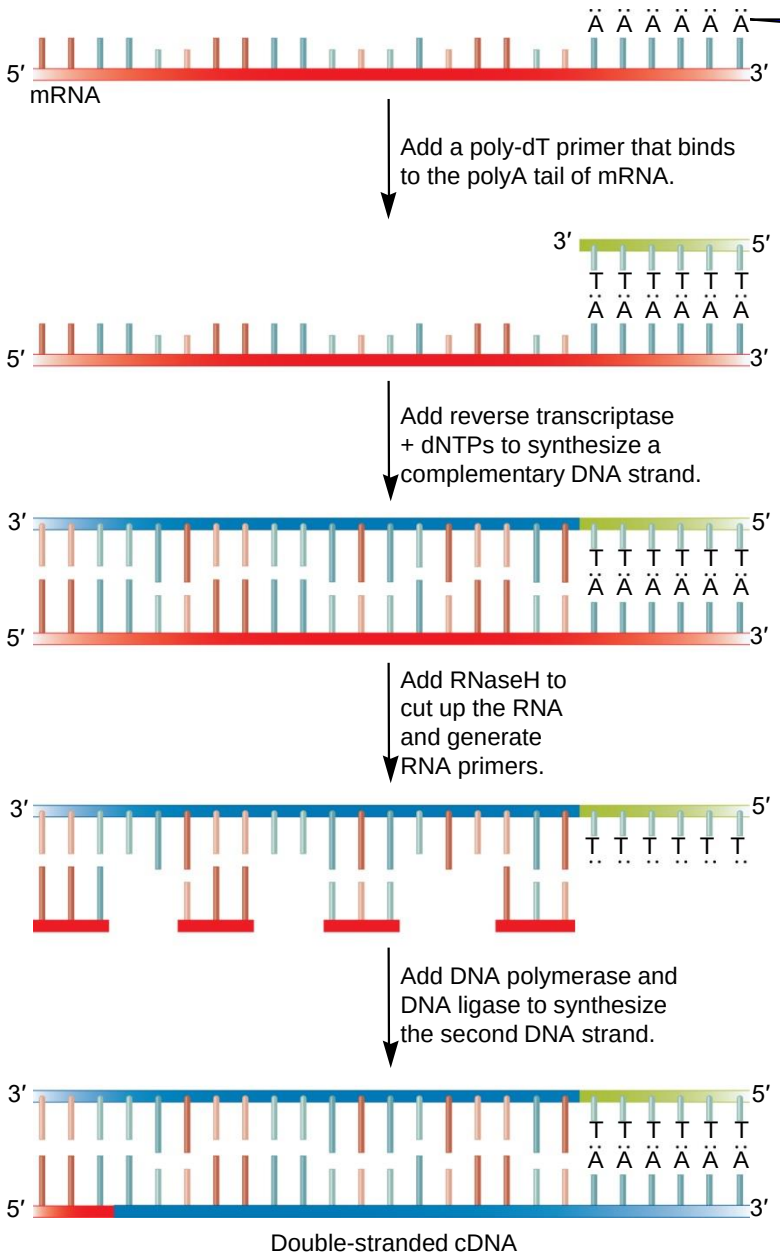
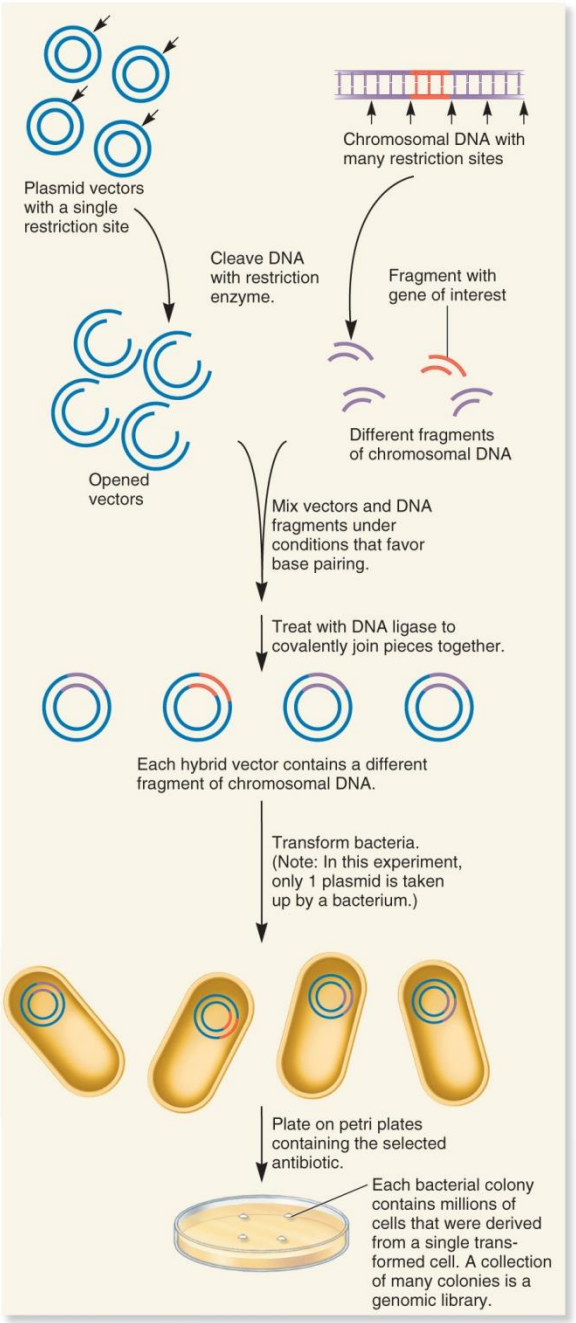


Figure 20.3

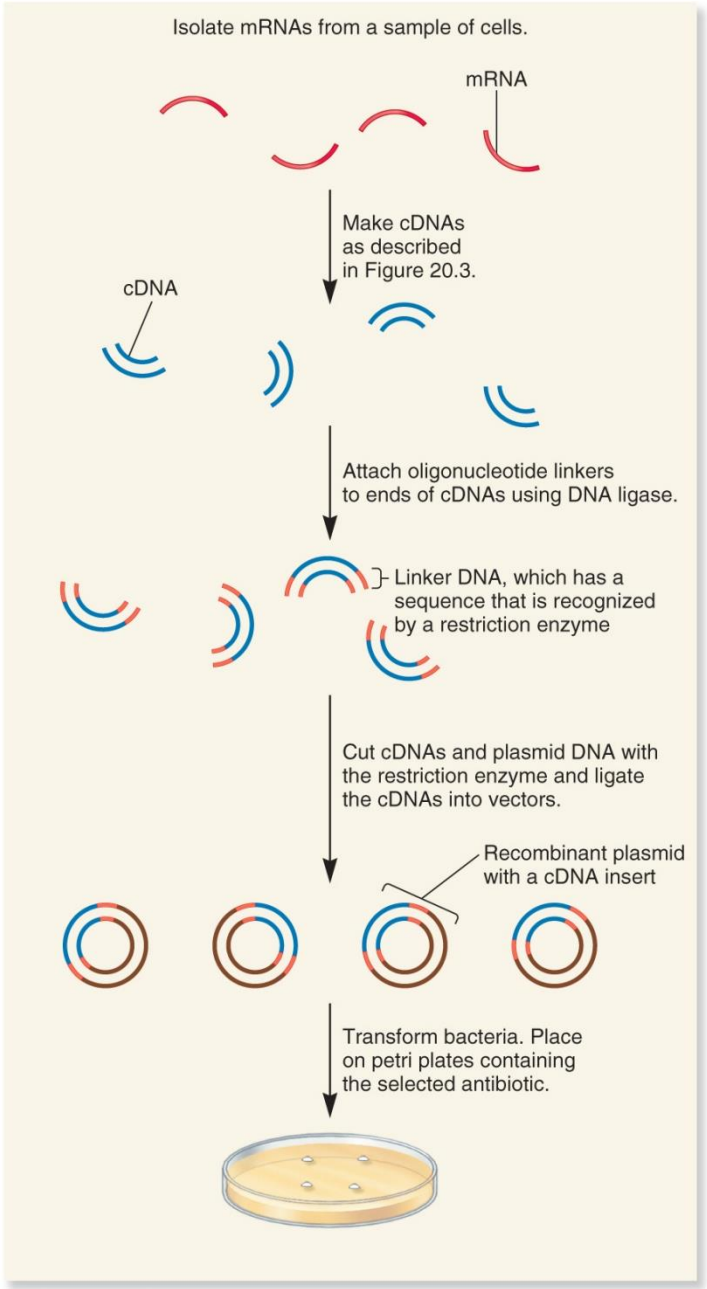
DNA Libraries

- A DNA library is a collection of thousands of different cloned fragments of DNA
  - **Genomic library** composed of chromosomal DNA fragments.
    - Should represent all of the DNA of the cell
  - **cDNA library** composed of cDNA inserts
    - Should represent all of the mRNA in the cell
- Refer to Figure 20.4



(a) Making a genomic library

Figure 20.4a



(b) Making a cDNA library

https://www.jove.com/science-education/5074/molecular-cloning

Figure 20.4b

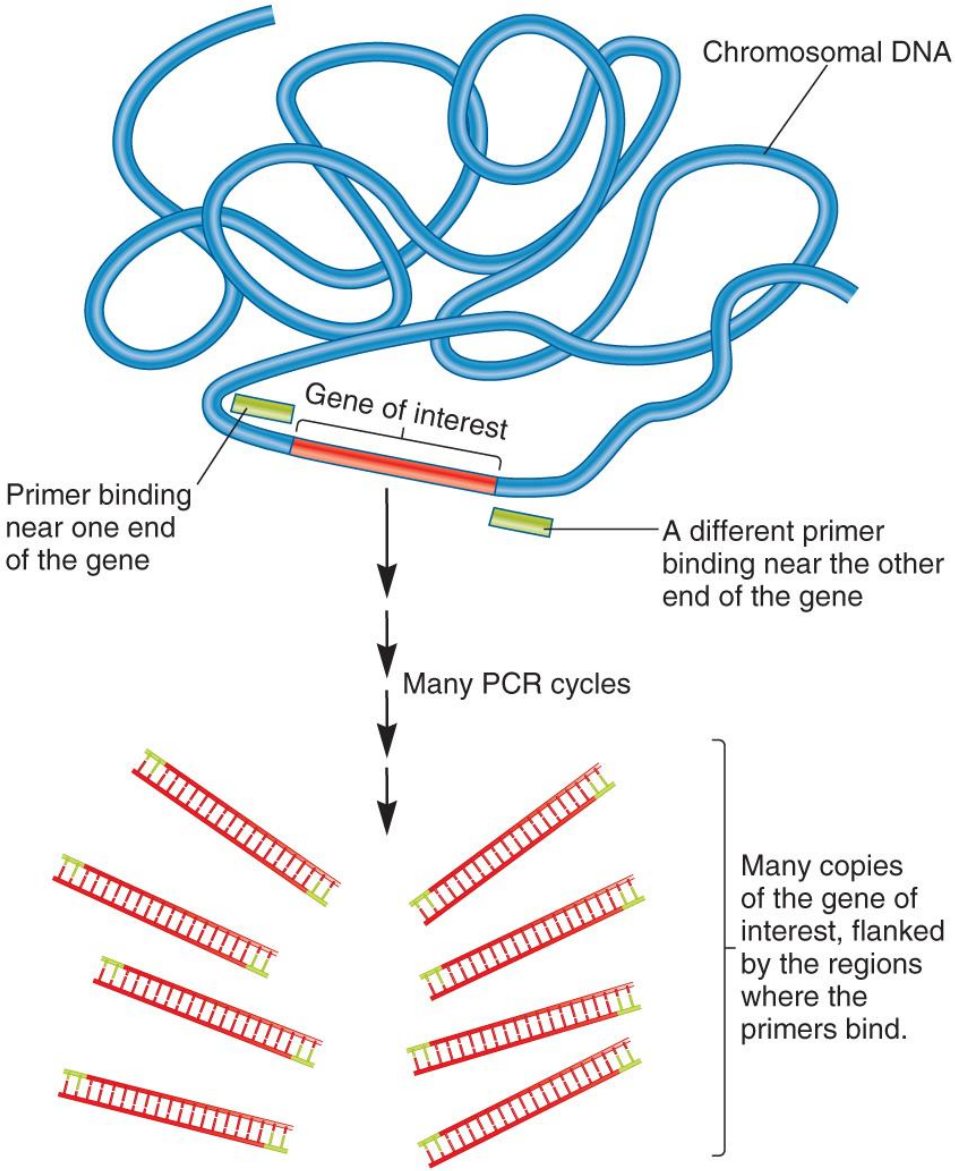
20.2 Polymerase Chain Reaction

- The three steps of a PCR cycle
- How reverse-transcriptase PCR is carried out
- Real-time PCR and why it is used

Polymerase Chain Reaction

- Another way to make many copies of a particular DNA sequence is a technique called **polymerase chain reaction** (**PCR**)
  - It was developed by Kary Mullis in 1985

- Unlike gene cloning, PCR can copy DNA without the aid of vectors and host cells

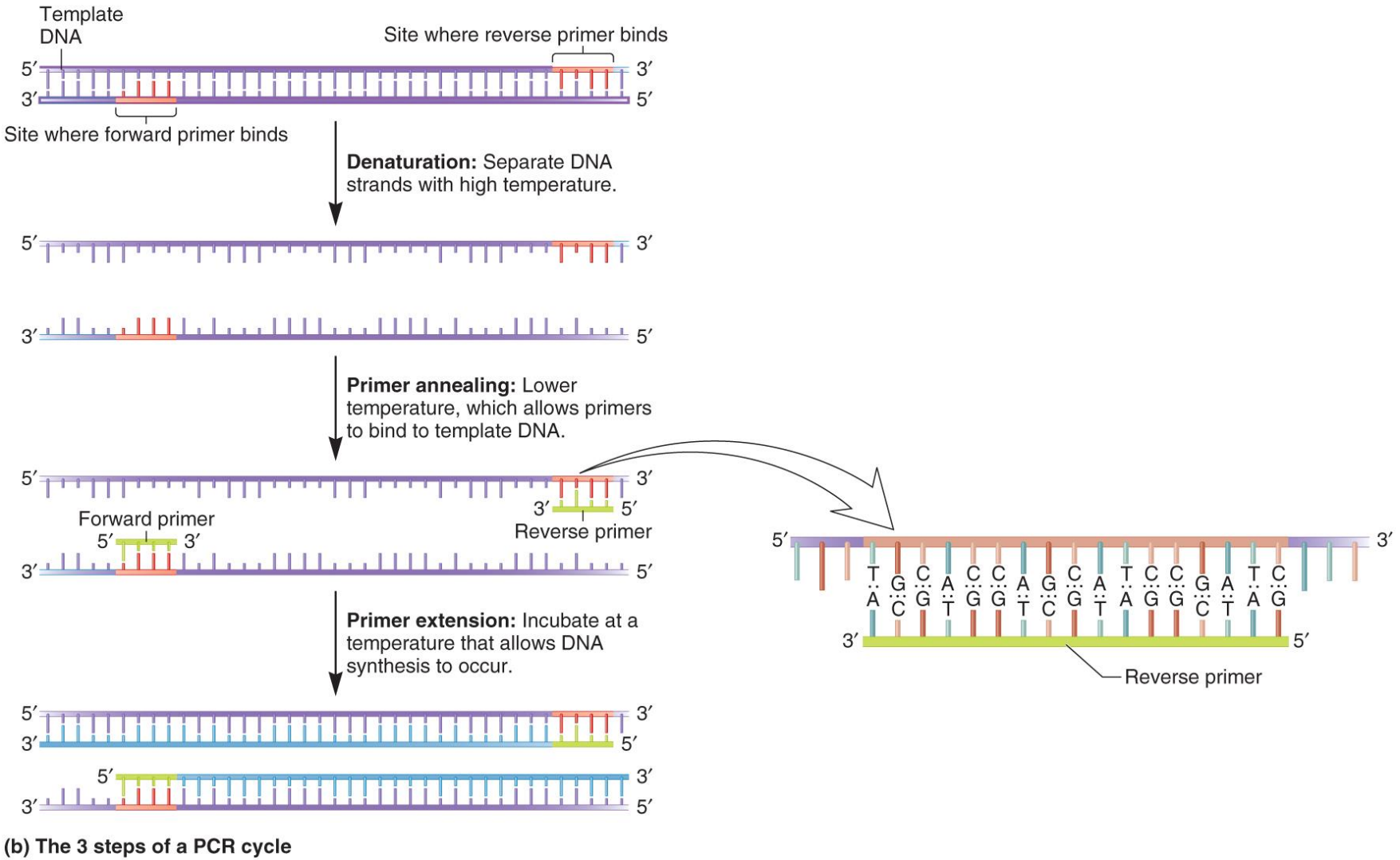


(a) The outcome of a PCR experiment

Figure 20.5a

- The starting material for PCR includes
  - **Template DNA**
    - Contains the region that needs to be amplified
  - **Oligonucleotide primers**
    - Complementary to sequences at the ends of the DNA fragment to be amplified
    - These are synthetic and about 15-20 nucleotides long – need to know sequence of DNA to be bound by primers
  - **Deoxynucleoside triphosphates (dNTPs)**
  - **Thermostable DNA polymerase**
    - Necessary because PCR involves heating steps that inactivate most other DNA polymerases
    - Example: *Taq* polymerase

- Three Steps of PCR
  - **Denaturation** – DNA strands separated
  - **Annealing** – Primers bind to DNA
  - **Synthesis** or **primer extension**
    - Polymerase copies the target sequences
- These take place in a machine called a **thermocycler**
- Refer to Figure 20.5b



(b) The 3 steps of a PCR cycle

Figure 20.5b

- A typical PCR involves 20 to 30 cycles of replication
  - Takes only a few hours to complete

- After 20 cycles, a DNA sample will increase 2^{20}-fold (~ 1 million-fold)

- After 30 cycles, a DNA sample will increase 2^{30}-fold (~ 1 billion-fold)

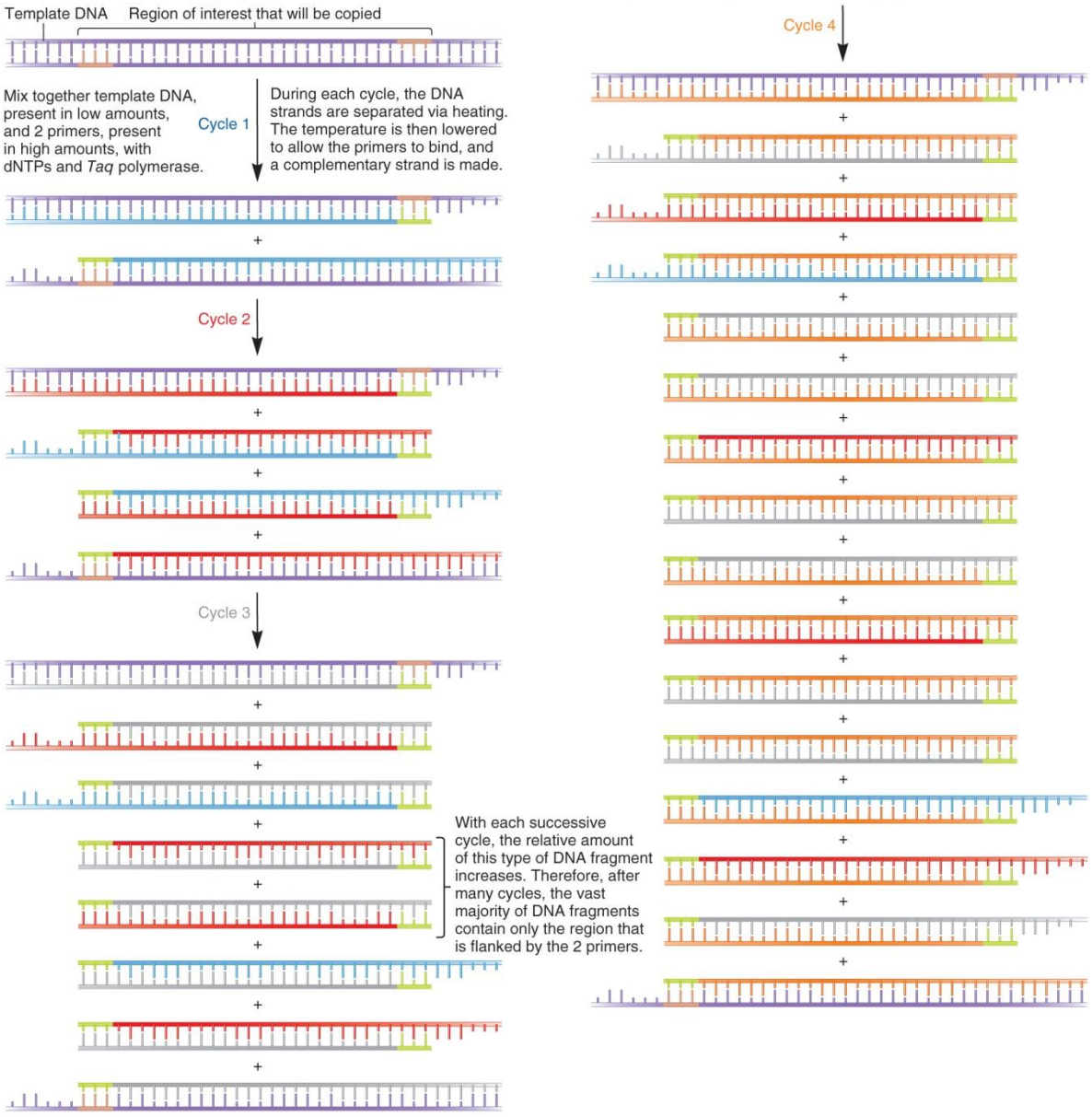


Figure 20.6

Reverse Transcriptase PCR

- PCR can be used to quantify the amount of RNA in living cells
  - The method is called **reverse transcriptase PCR (RT-PCR)**

- RT-PCR is carried out in the following manner
  - RNA is isolated and mixed with deoxyribonucleotides, reverse transcriptase and a primer that anneals near the 3' end of the RNA
  - The single-stranded cDNA produced is used as template DNA in conventional PCR
  - RT-PCR is extraordinarily sensitive
    - Can detect expression of small amounts of RNA in a single cell
    - Refer to Figure 20.7

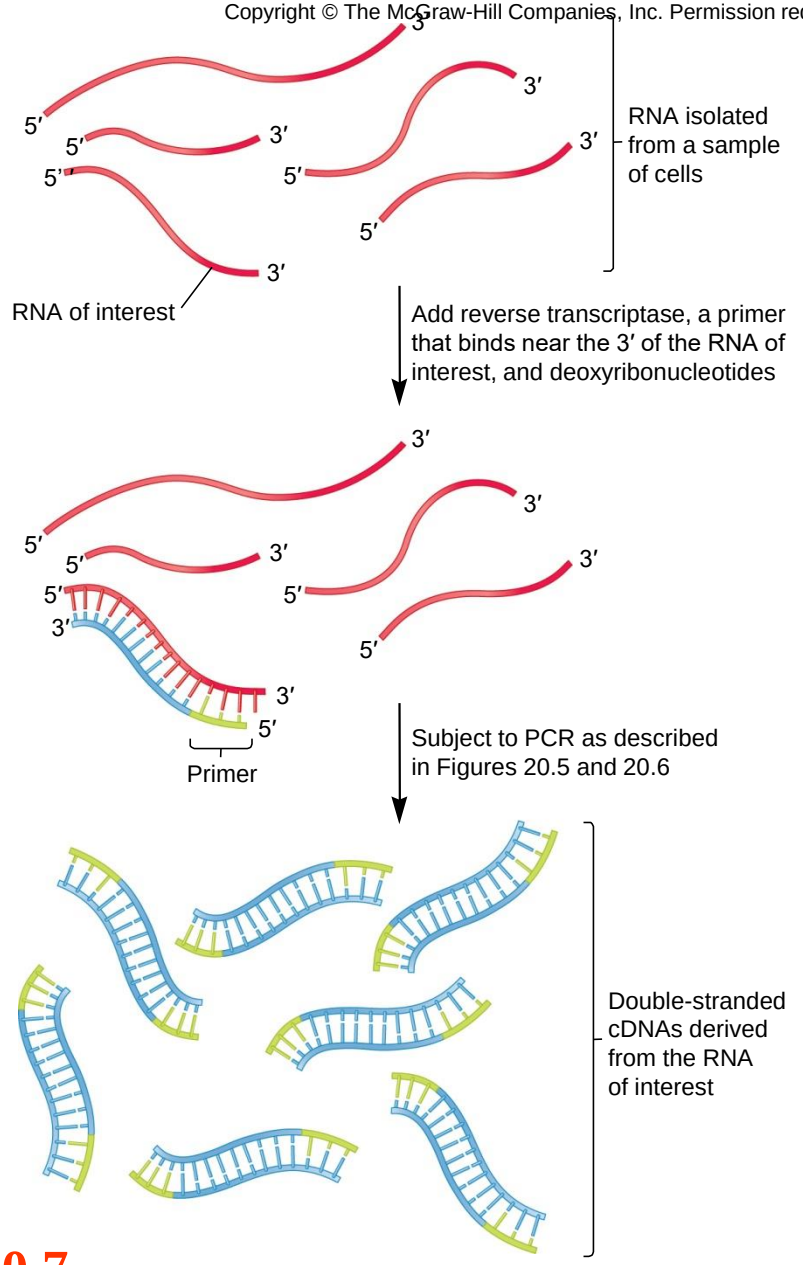


Figure 20.7

https://www.jove.com/science-education/5056/pcr-the-polymerase-chain-reaction

Real-Time PCR

- Real-time PCR
  - Amounts of product are measured in real-time during the PCR process, not just at the end
  - Used for comparing amounts of DNA template
  - Amount of product correlates with amount of target originally in sample
  - Uses a thermocycler that can measure changes in fluorescence from **detector molecules**

- Detector molecules
  - Example: **TaqMan**
    - An oligonucleotide with a reporter at one end, a quencher at the other
    - Designed to be complementary to the PCR product
    - During the PCR extension step, the quencher is cleaved off, increasing the fluorescence

- Refer to Figure 20.8

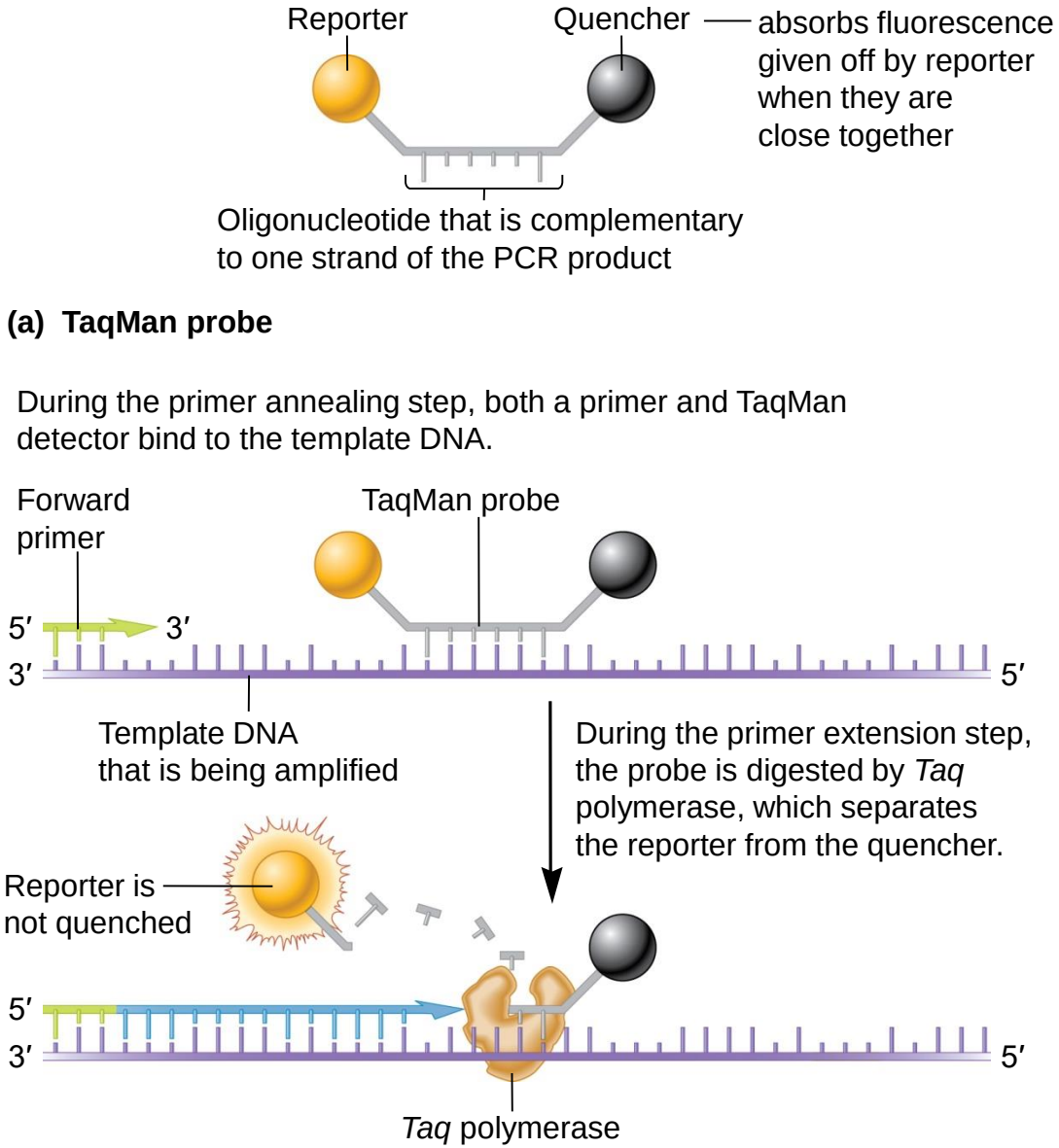


Figure 20.8

- **Cycle threshold method**
  - C_t = cycle threshold
  - Cycle threshold is reached when the accumulation of fluorescence is higher than the background level
    - Product accumulates exponentially
  - Amounts can be compared to a **standard** of known amounts of template
    - Different colored fluorescent molecules distinguish the test product from the standard

- Refer to Figure 20.9

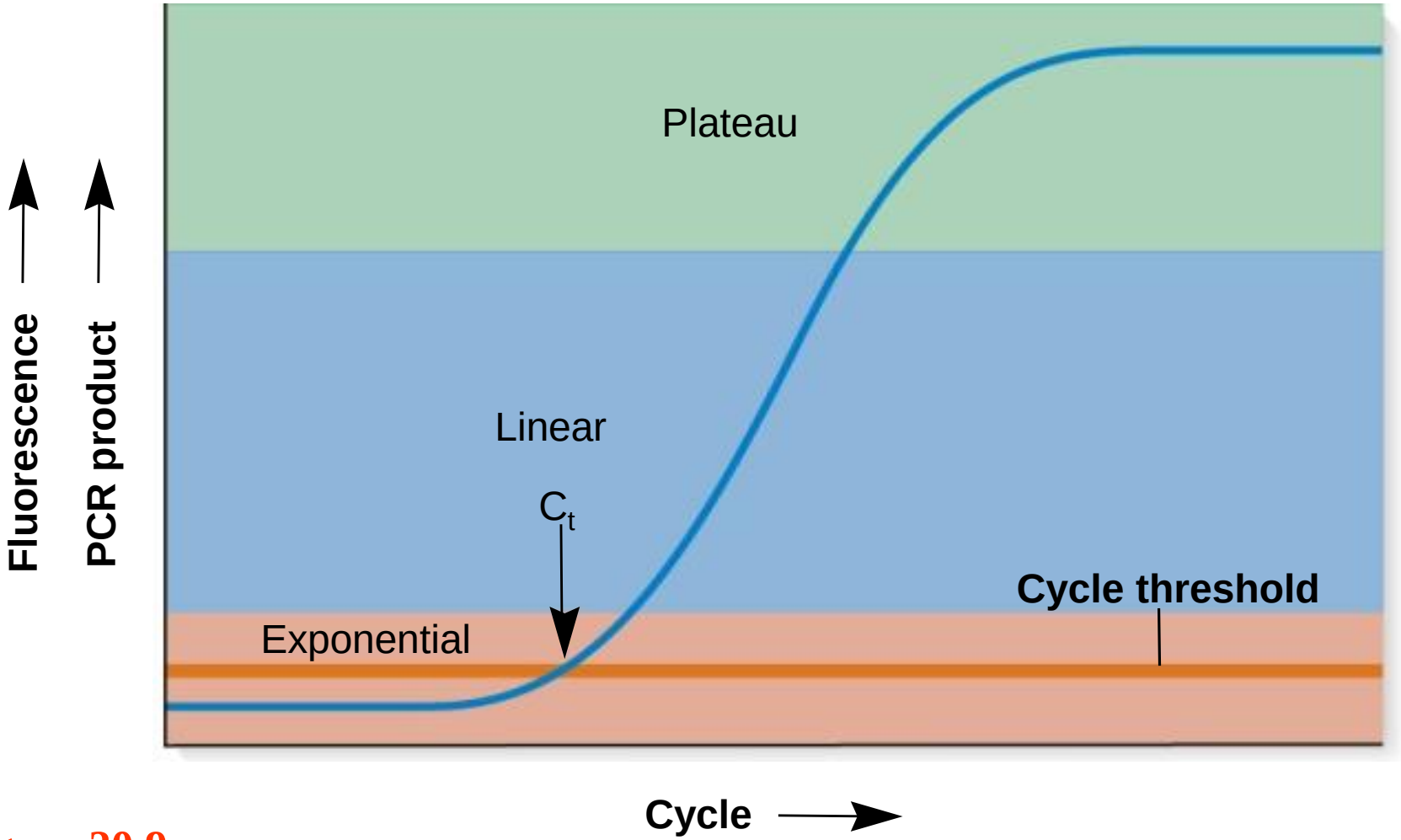


Figure 20.9a

(a) Phases of PCR

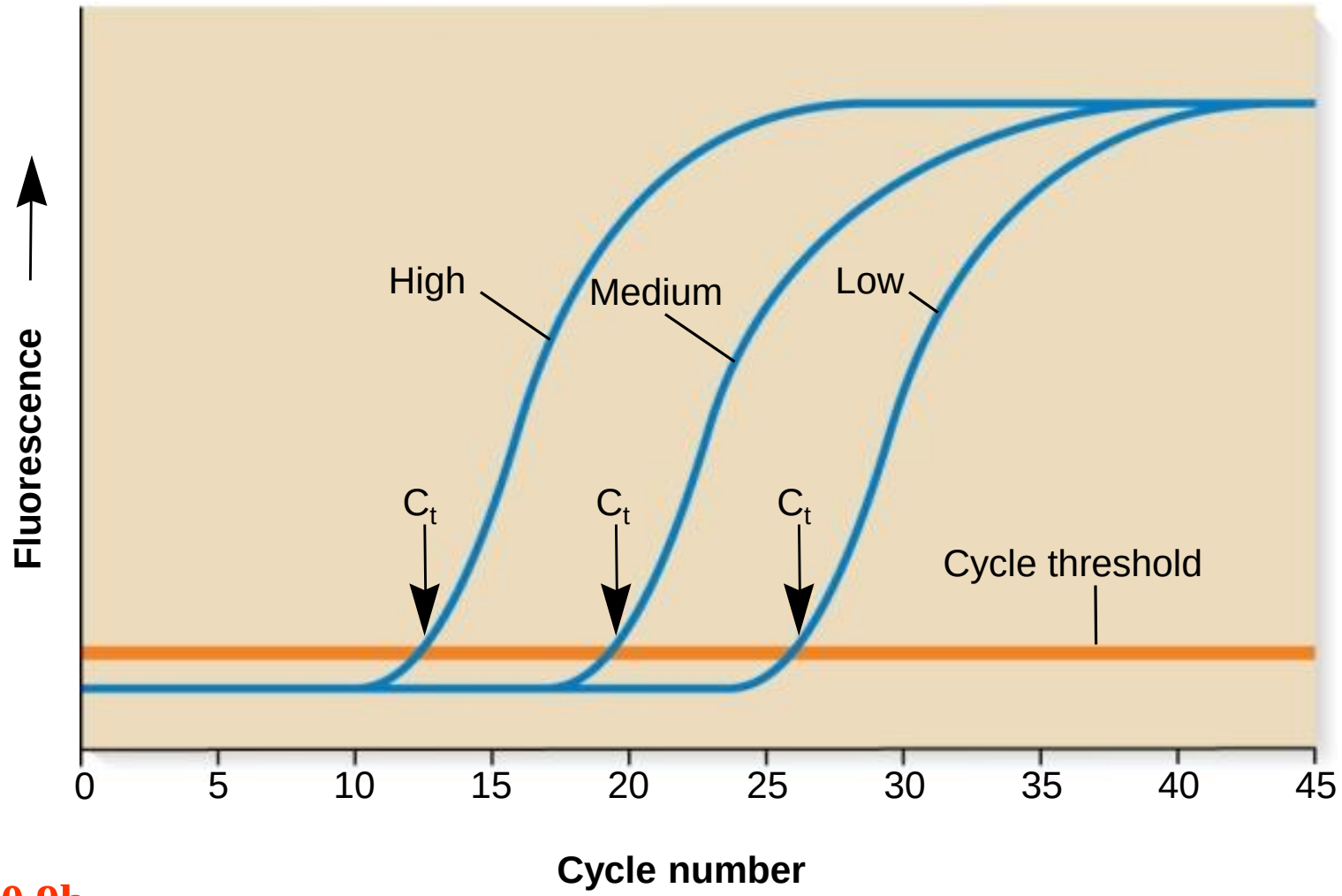


Figure 20.9b

(b) Real-time PCR at high, medium, and low concentrations of the starting template DNA

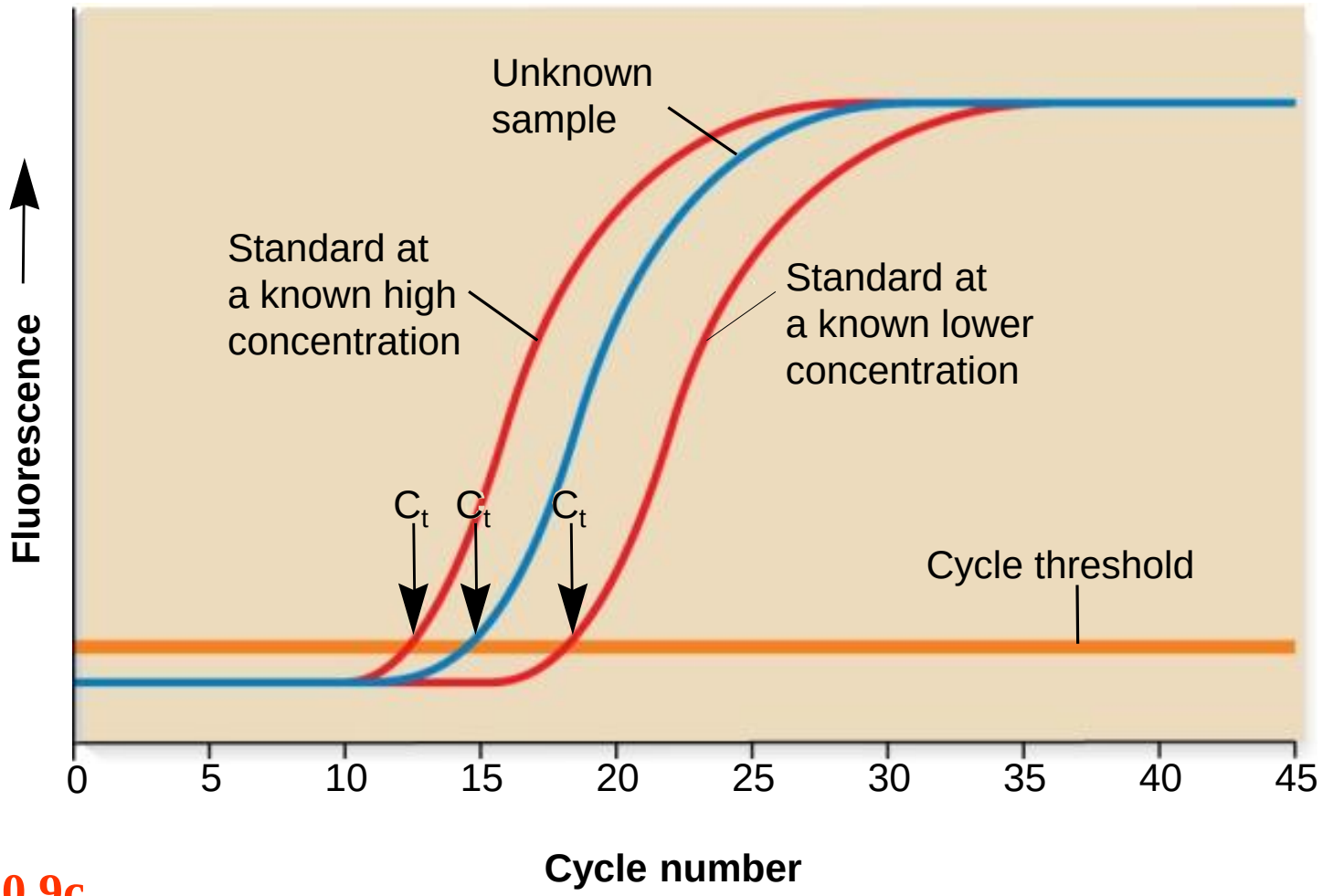


Figure 20.9c

(c) A comparison between an unknown sample and standards of known concentrations

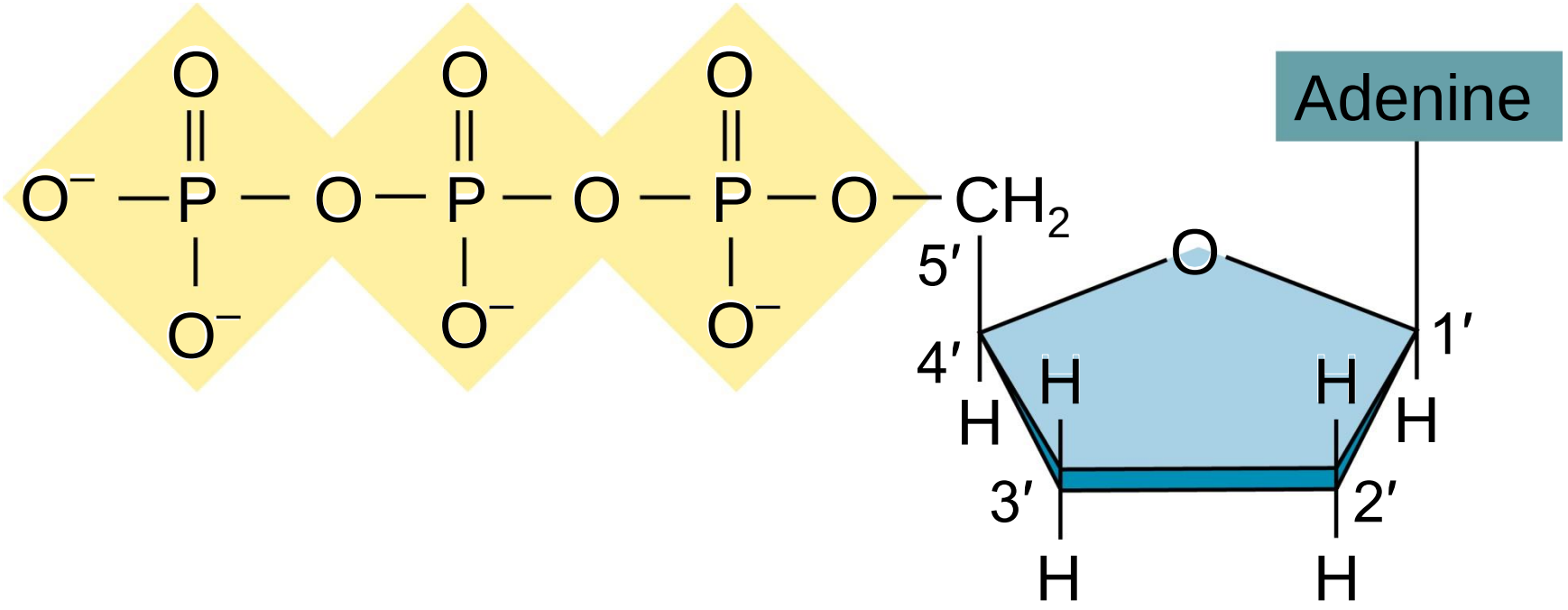
20.3 DNA Sequencing

- The steps in automated DNA sequencing via the dideoxy sequencing method

DNA Sequencing

- During the 1970s two DNA sequencing methods were devised
  - **Base-specific cleavage** - Allan Maxam and Walter Gilbert
  - **Dideoxy sequencing** - Frederick Sanger and colleagues
- The dideoxy method has become the more popular and will therefore be discussed here

- The dideoxy method is based on DNA replication
  - Uses DNA polymerase to copy a strand of DNA
  - However, DNA synthesis is terminated at specific nucleotides by using nucleotide analogs
  - These analogs lack a 3’ hydroxyl group
    - Called **dideoxyribonucleotides (ddNTPs)**
  - Can be added to the growing polynucleotide, but then no new nucleotides can be added – **chain termination**
  - Refer to Figure 20.10



2′, 3′-Dideoxyadenosine triphosphate (ddA)

Figure 20.10

- A mixture of nucleotides, both dideoxy and deoxy, are added to the sequencing reaction along with a primer and DNA polymerase
  - Resulting fragments are separated based on their length

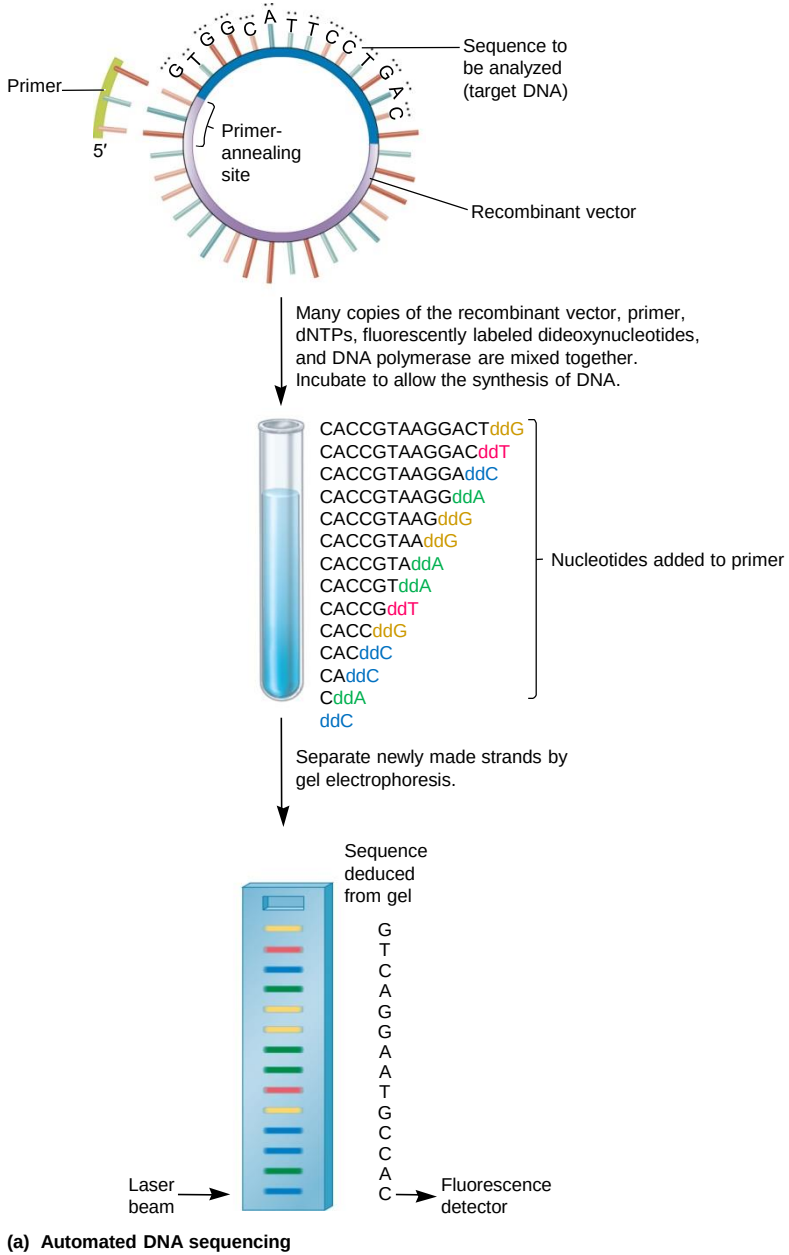
- Originally used radiolabeled ddNTPs
  - Now **automated DNA sequencing** uses four different colors of fluorescent dyes – one for each nucleotide
  - Ex: ddA = green, ddT = red, ddG = yellow, ddC = blue

- Samples are electrophoresed through a polyacrylamide gel in a small capillary tube

- All steps are automated
  - As each band comes off the bottom of the gel, the fluorescent dye is excited by a laser
  - The fluorescence emission is recorded by the fluorescence detector

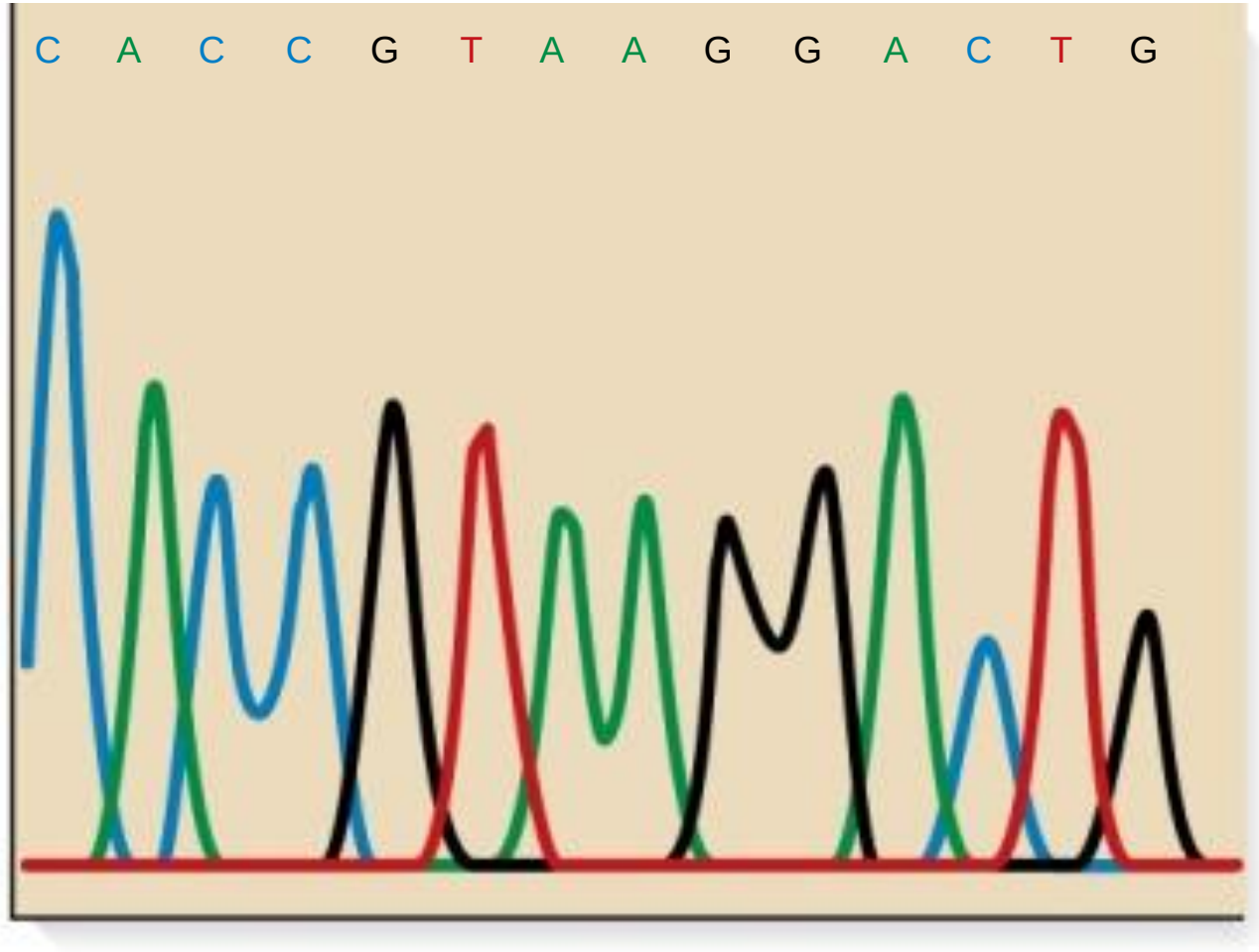
- The sequence of bases is called a **sequencing ladder**

- Refer to Figure 20.11



(a) Automated DNA sequencing

Figure 20.11a



(b) Output from automated sequencing

Figure 20.11b

20.4 Site-Directed Mutagenesis

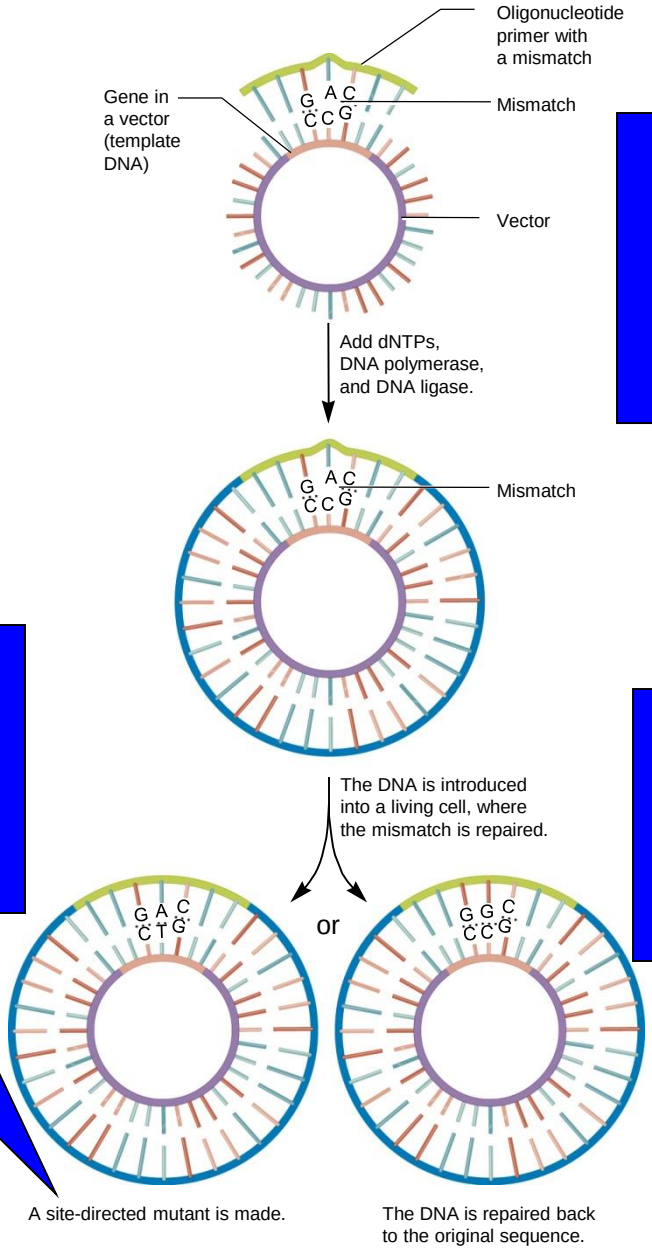
- The method of site-directed mutagenesis
- Reasons why this method is useful

Site-Directed Mutagenesis

- Analysis of mutations can provide important information about normal genetic processes

- Mutations can arise spontaneously, or be induced by mutagens
  - But it may be difficult to isolate a mutant in a specific gene, particularly if the effects are unknown
  - May also want to study the effect of a specific nucleotide or amino acid change

- **Site-directed mutagenesis**
  - Change in designated nucleotide(s)
    - Substitution
    - Insertions
    - Deletions
  - Then test effect of mutation *in vitro* or *in vivo*
    - Expression
    - Function of a protein
    - Phenotype
  - Refer to Figure 20.12



M13 virus which produces a single-stranded DNA as part of its life cycle

Can be identified by DNA sequencing and used for further studies

Depending on which base is replaced, the mutant or original sequence is produced

Figure 20.12

20.5 Blotting Methods to Detect Genes and Gene Products

- The techniques of Southern, Northern, and Western blotting and why they are useful

Southern Blotting

- **Southern blotting** can detect the presence of a particular gene sequence within a mixture of many
  - It was developed by E. M. Southern in 1975

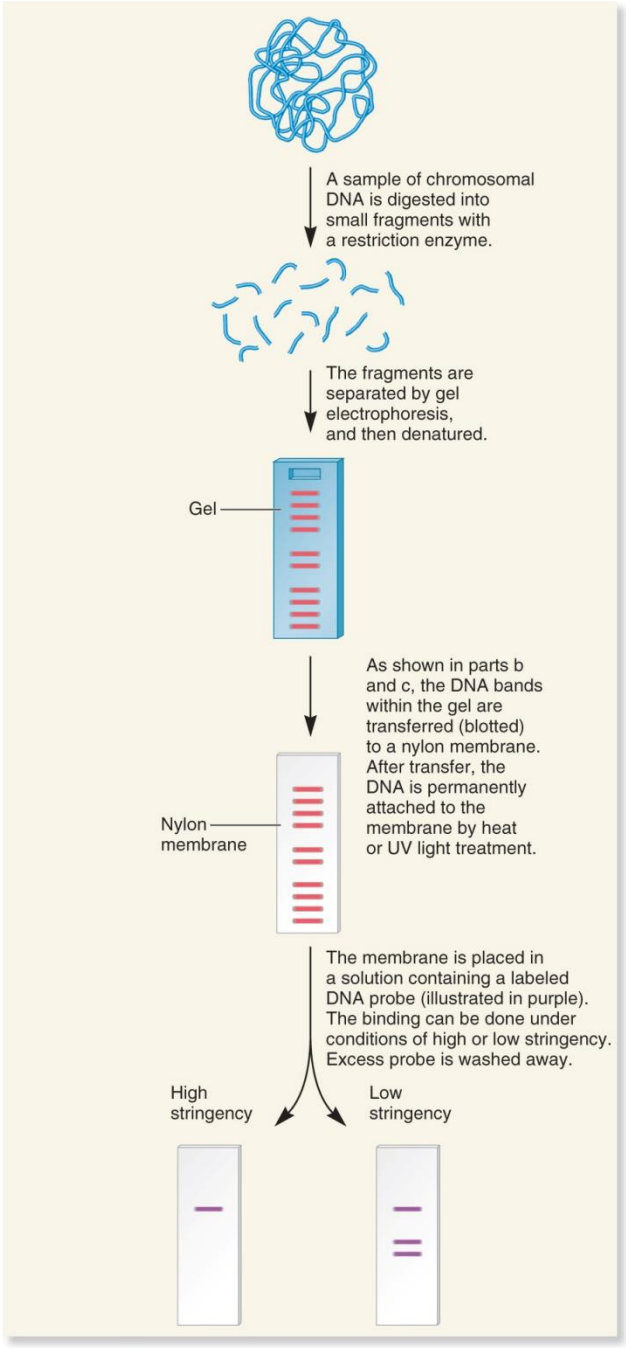
- Southern blotting has several uses
  - It can determine copy number of a gene in a genome
  - It can detect small gene deletions that cannot be detected by light microscopy
  - It can identify gene families
  - It can identify homologous genes among different species

- Uses a piece of nucleic acid as a probe to detect the DNA

- The probe may be
    - A whole gene
    - Part of a gene
    - PCR product
    - Oligonucleotide

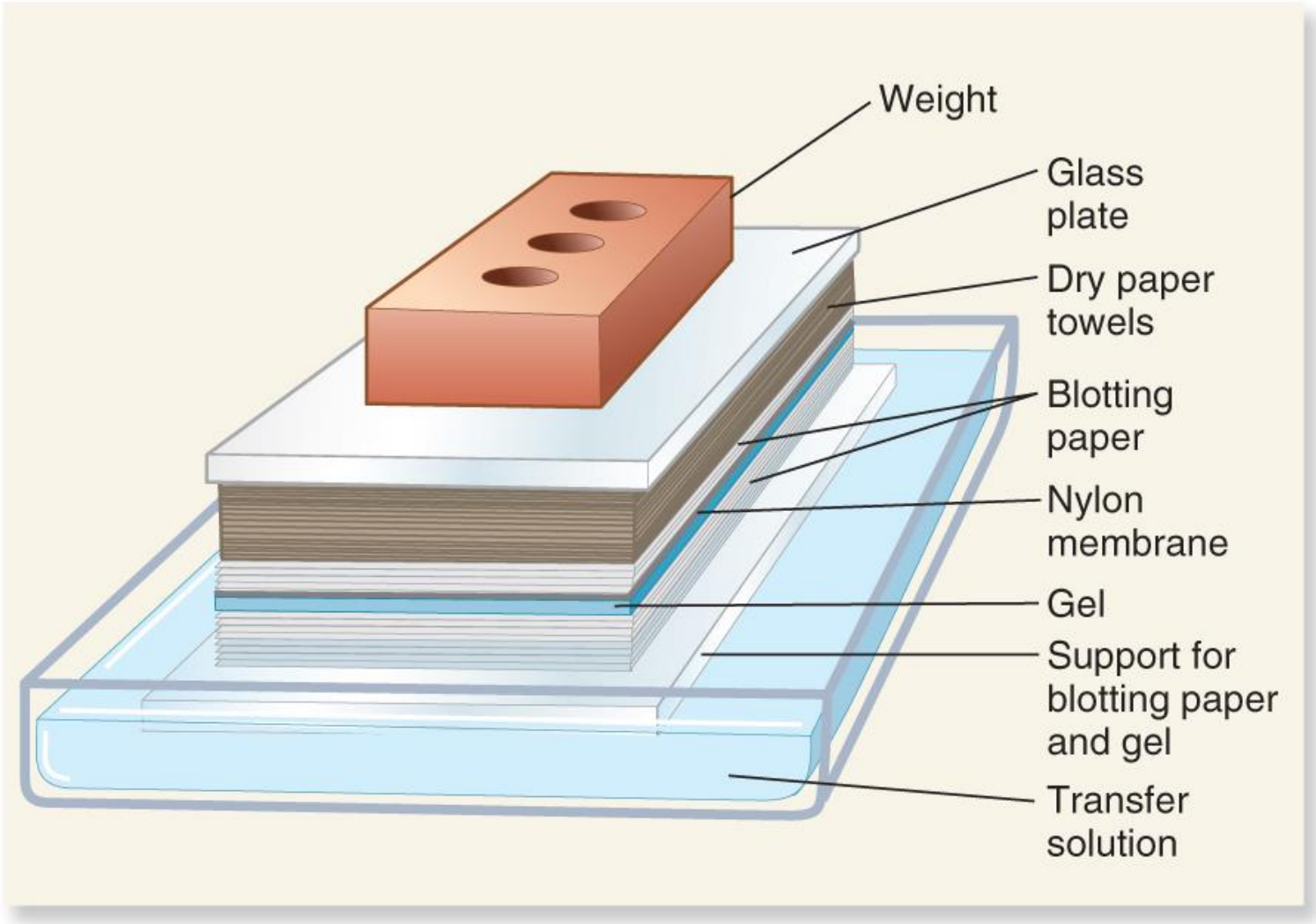
- DNA is labeled (e.g., radiolabeled) and used as a probe

- By varying the annealing conditions, sequences with more or less identity can be detected
    - **High Stringency** – detects nearly identical sequences
    - **Low Stringency** – detects similar sequences
- Refer to Figure 20.13



(a) The steps in Southern blotting

Figure 20.13a



(b) The transfer step (traditional method)

Figure 20.13b

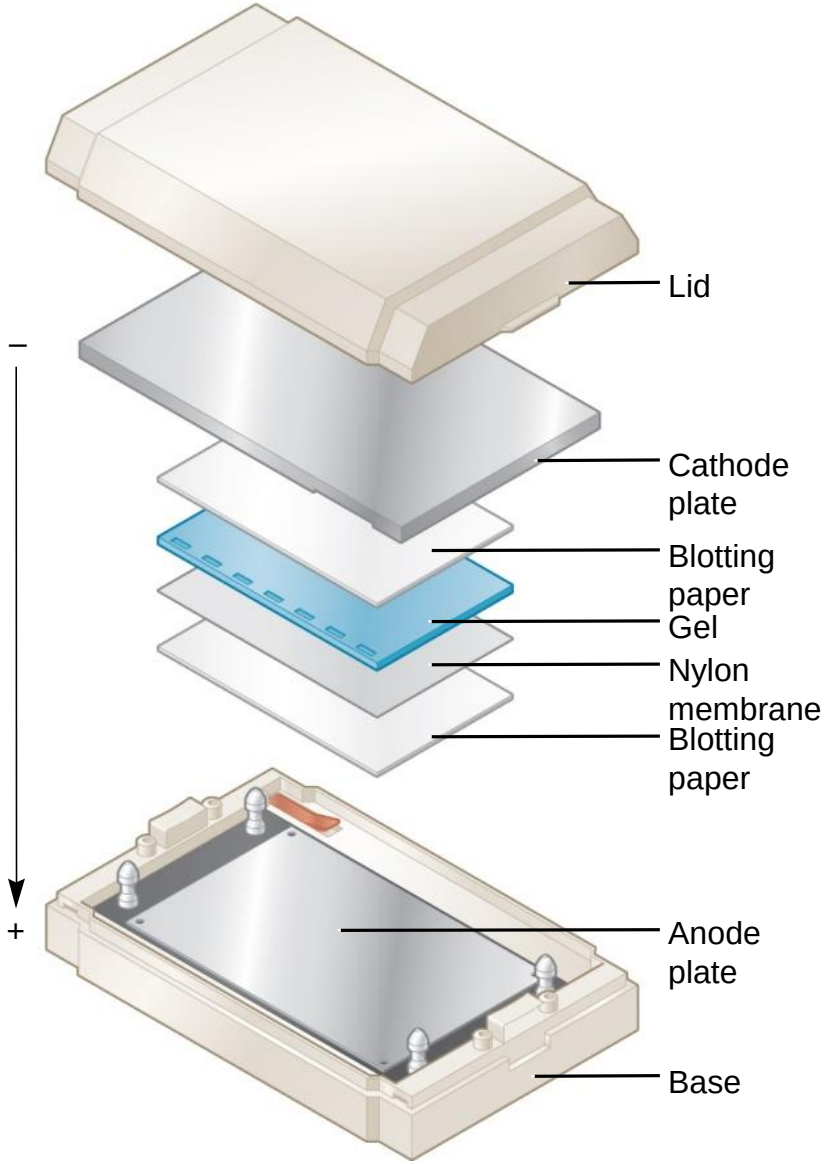


Figure 20.13c

(c) The transfer step via electrophoresis

Northern Blotting

- Northern blotting is used to identify a specific RNA within a mixture of many RNA molecules
  - Similar to Southern blotting
  - RNA is electrophoresed, transferred to membrane, then probed

- Northern blotting has several uses
  - It can determine if a specific gene is transcribed in a particular cell type
    - Nerve vs. muscle cells
  - It can determine if a specific gene is transcribed at a particular stage of development
    - Fetal vs. adult cells
  - It can reveal if a pre-mRNA is alternatively spliced
- Refer to Figure 20.14

Lane 1: Smooth muscle cells

Lane 2: Striated muscle cells

Lane 3: Brain cells

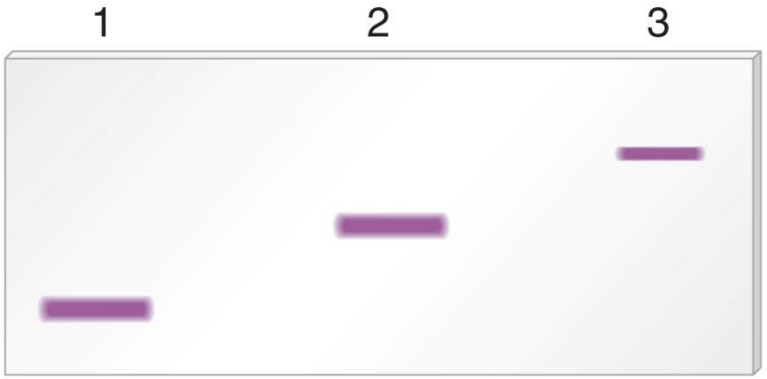


Figure 20.14

- Smooth and striated muscles produce a larger amount of tropomyosin mRNA than do brain cells
  - Tropomyosin plays a role in muscle contraction

- mRNAs have different molecular weights
  - pre-mRNA is alternatively spliced

Western Blotting

- Western blotting is used to identify a specific protein within a mixture of many protein molecules

- Western blotting has several uses
  - It can determine if a specific protein is made in a particular cell type
    - Red blood cells vs. brain cells
  - It can determine if a specific protein is made at a particular stage of development
    - Fetal vs. adult cells

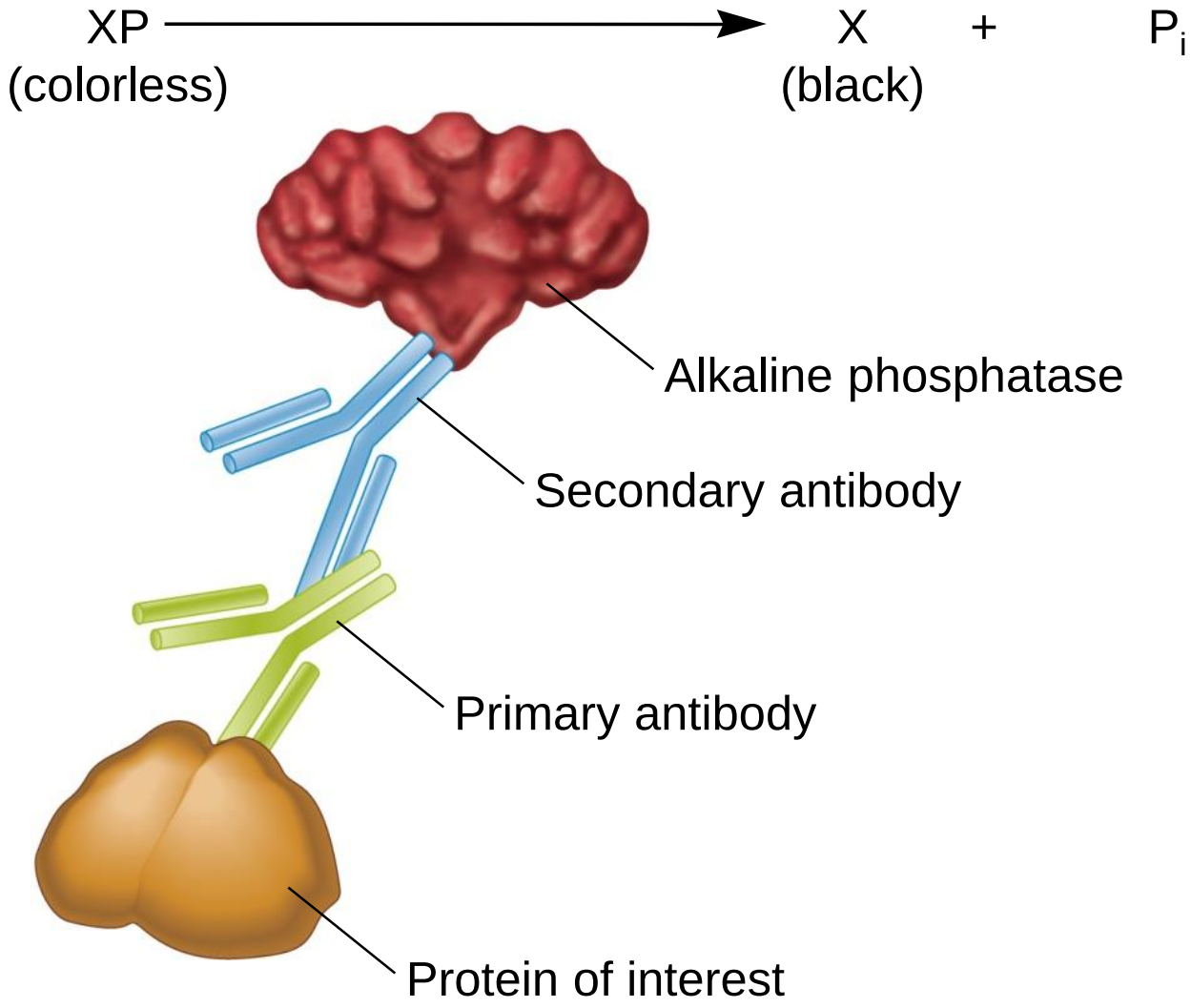
- Western blotting is carried out as follows:
  - Proteins are extracted from the cells and purified
  - Then separated by SDS-PAGE
    - Dissolved in the detergent sodium dodecyl sulfate
      - This denatures proteins and coats them with negative charges
    - The negatively charged proteins are then separated by polyacrylamide gel electrophoresis
  - Transferred to a membrane

- Specific proteins are detected by antibodies
  - **Antibodies** are proteins that are used in the immune system
  - They recognize **epitopes** of an **antigen**
    - An epitope has a three dimensional structure that an antibody recognizes
    - An antigen refers to any molecule that is recognized by an antibody
    - Some examples of antigens: proteins, carbohydrates, nucleic acids, bacteria, viruses, or cells not normally found in the body.

- Filters are placed into a solution containing a primary antibody (recognizes the protein of interest)
  - This antibody usually lacks a label

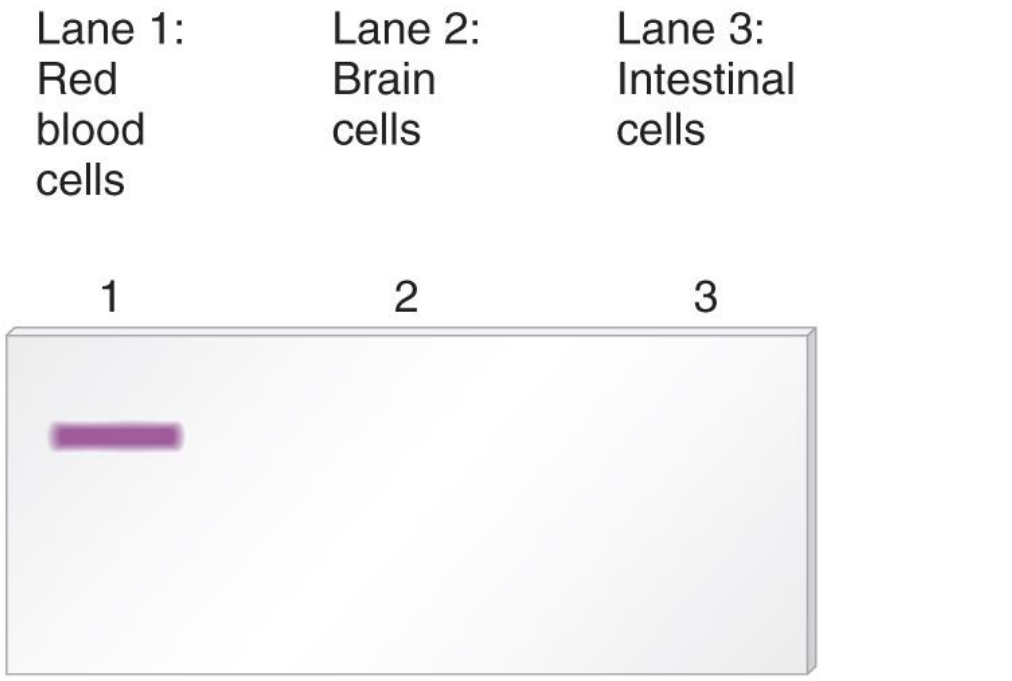
- A second antibody binds to the first antibody
  - Secondary antibody carries an enzyme that creates a visible product

- Refer to Figure 20.15



(a) Interactions between the protein of interest and antibodies

Figure 20.15a



(b) Results from a Western blotting experiment

- This experiment indicates that β-globin is made in red blood cells but not in brain or intestinal cells

Figure 20.15b

20.6 Analyzing DNA- and RNA-Binding Proteins

- How a gel retardation assay is used to determine if a protein binds to DNA or RNA

Detecting the Binding of Proteins to DNA

- Study of DNA-protein interactions useful for understanding
  - Transcription factor activity
  - Histone placement

- **Gel retardation assay**
  - Also called **gel mobility shift assay**

- **Gel retardation assay**
  - Binding of a protein to a fragment of DNA retards (slows) its rate of movement through a gel

- Must be performed under non-denaturing conditions
  - Unfolding of protein would cause it to release DNA
  - Separation of DNA strands would release protein

- Complexes are electrophoresed through a gel
  - Complexes that migrate more slowly indicate that DNA was bound by protein

- Refer to Figure 20.16

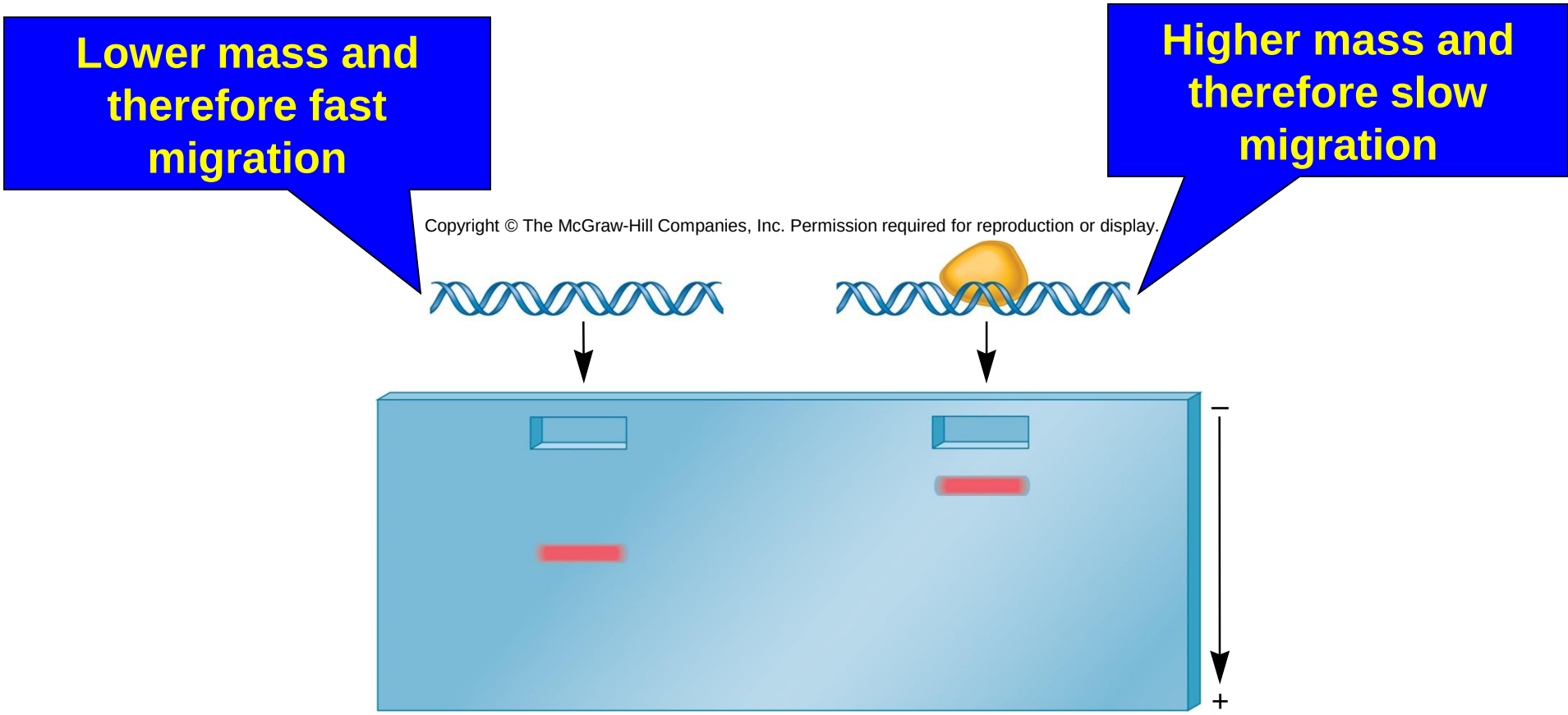


Figure 20.16