Microbiology

Chapter 9

Biotechnology and Recombinant DNA

本內容已由授課教師方翠筠修訂

Biotechnology and Recombinant DNA

- **Plasmid vector**: Self-replicating DNA used to carry the desired gene to a new cell
- **Clone**: Population of cells arising from one cell

A Typical Genetic Modification Procedure

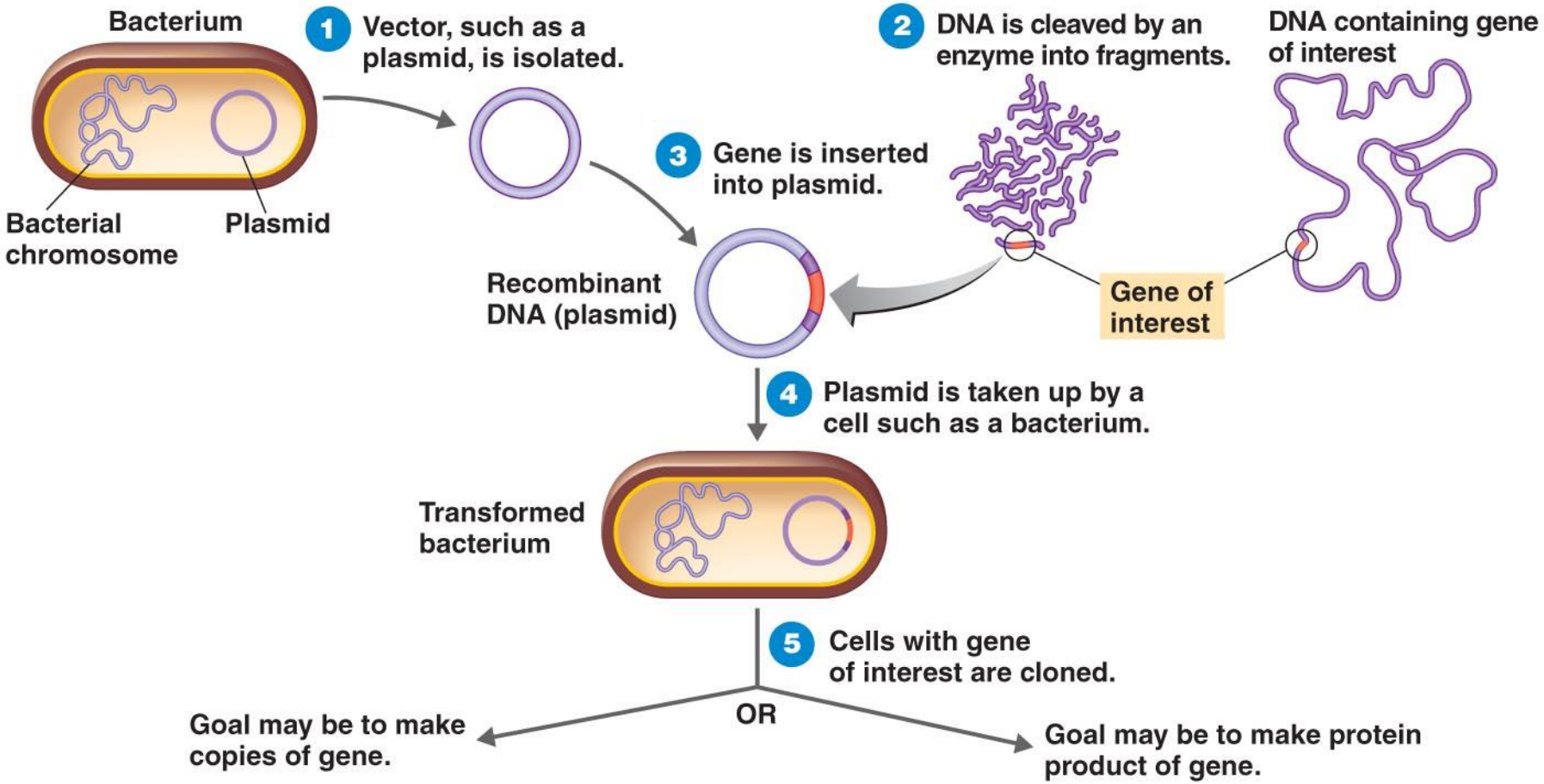


Figure 9.1

A Typical Genetic Modification Procedure

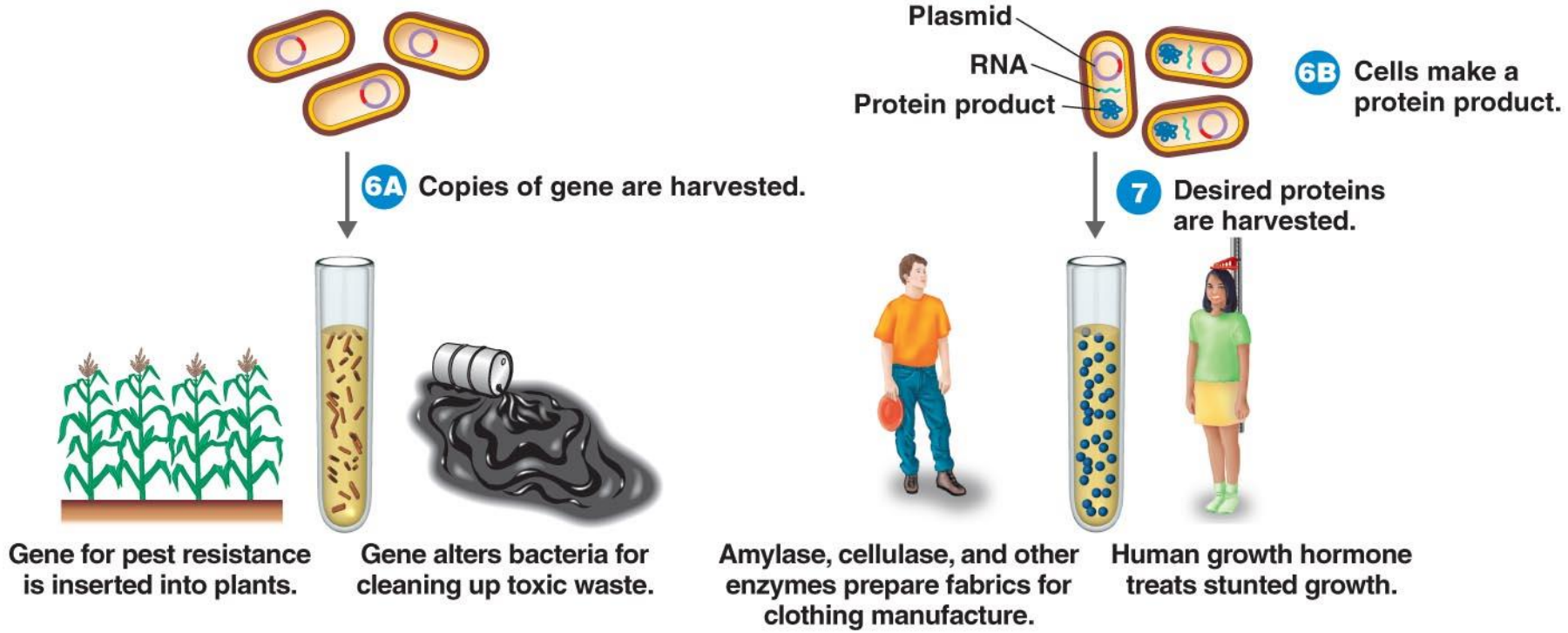


Figure 9.1

An overview of recombinant DNA technologies

- A desired gene is inserted into a DNA vector such as plasmid or viral genome.
- The vector inserts the DNA into a new cell, which is grown to form a clone.
- Large quantities of the gene or the gene product can be harvested from the clone.

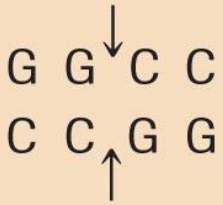
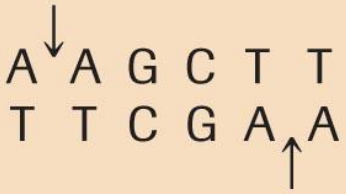
Selection and Mutation

- **Selection**: Culture a naturally occurring microbe that produces desired product
- **Mutation**: Mutagens cause mutations that might result in a microbe with a desirable trait
  - **Site-directed mutagenesis**: Change a specific DNA code to change a protein
  - Select and culture microbe with the desired mutation

Restriction Enzymes

- Recognize and cut specific sequences of DNA
- Destroy bacteriophage DNA (or foreign DNA) in bacterial cells
- The bacteria DNA is protected from digestion because the cell methylates (adds methyl groups to) some of the cytosines in its DNA.
- Restriction enzymes produce DNA fragments with sticky ends or blund ends
- Fragments of DNA produced by the same restriction enzyme usually will join by base pairing. DNA ligase can covalently link the DNA backbones.

Table 9.1 Selected Restriction Enzymes Used in rDNA Technology

Enzyme	Bacterial Source	Recognition Sequence
*Bam*HI	*Bacillus amyloliquefaciens*	
*Eco*RI	*Escherichia coli*	
*Hae*III	*Haemophilus aegyptius*	
*Hin*dIII	*Haemophilus influenzae*	

Restriction Enzyme & Recombinant DNA

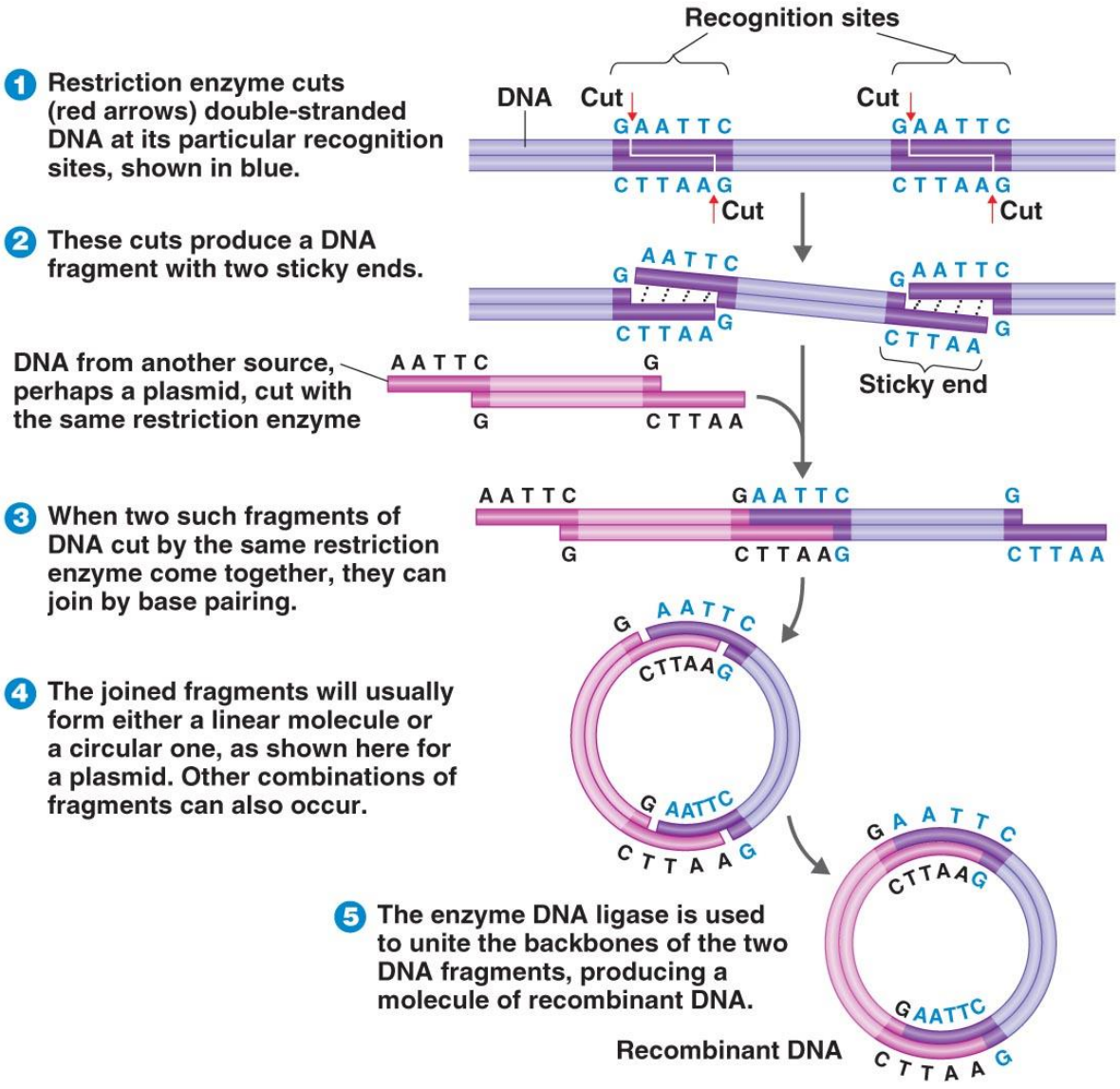


Figure 9.2

Plasmids and viral DNA Vectors

- **Self-replication**
- **Carry desired DNA to cells**
- **Smaller** vector is easily to be manipulated
- When it is necessary to retrieve cells containing the vector, **a marker gene** contained within the vector can often make selection easy.
- A plasmid containing a new gene can be inserted into a cell by transformation.
- A viral vector containing a new gene can be inserted into a cell by transduction.
- Shuttle vectors can exist in several different species

A Plasmid Vector Used for Cloning

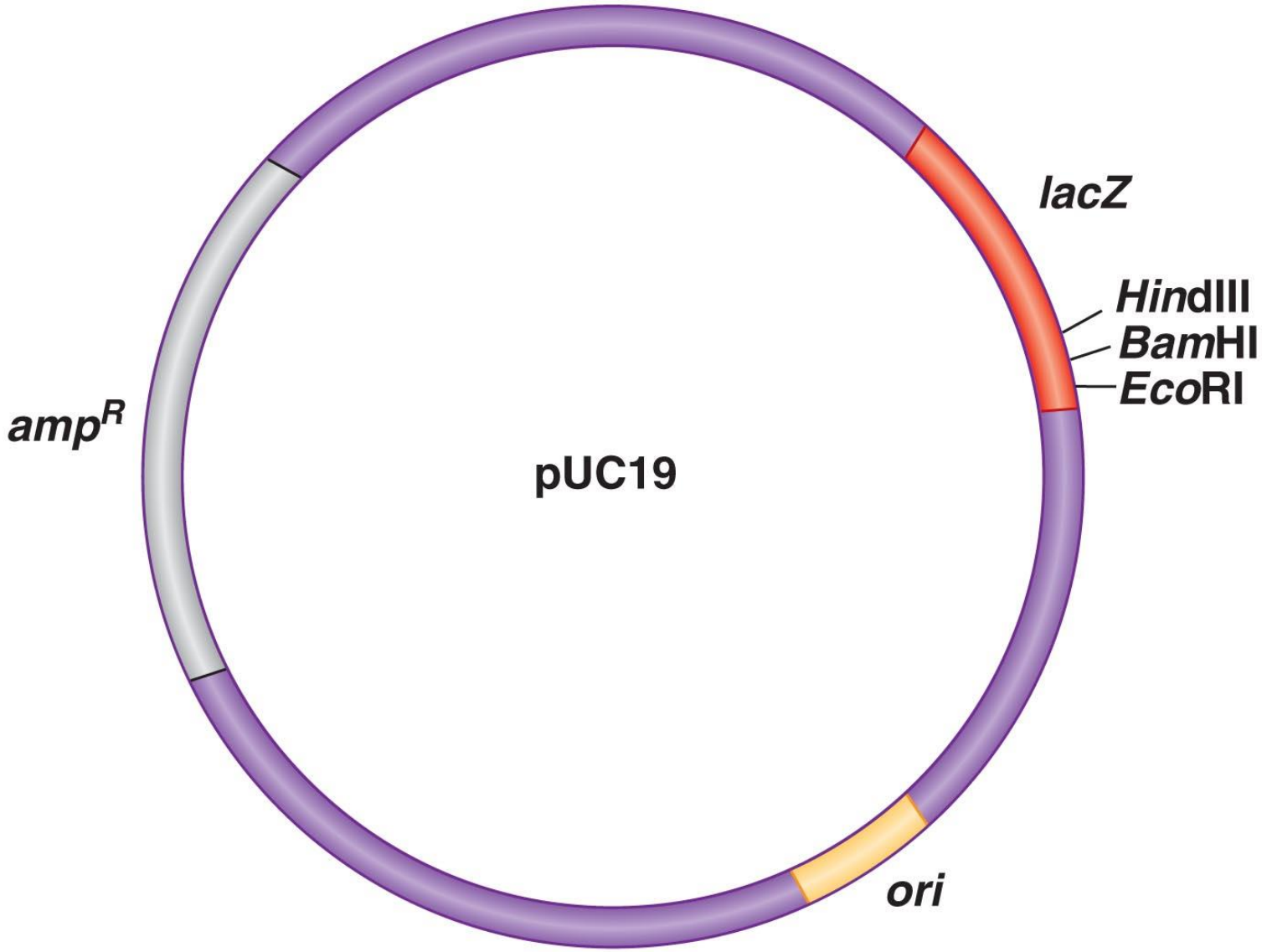


Figure 9.3

Polymerase Chain Reaction (PCR)

- To make multiple copies of a piece of DNA enzymatically➔ increase the amounts of DNA in samples
- Used to
  - Clone DNA for recombination
  - Amplify DNA to detectable levels
  - Sequence DNA
  - Diagnose genetic disease
  - Detect pathogens

PCR

First cycle

1. Incubate target DNA at 94°C for 1 minute to separate the strands.
2. Add primers, nucleotides (deoxynucleotides), and DNA polymerase.

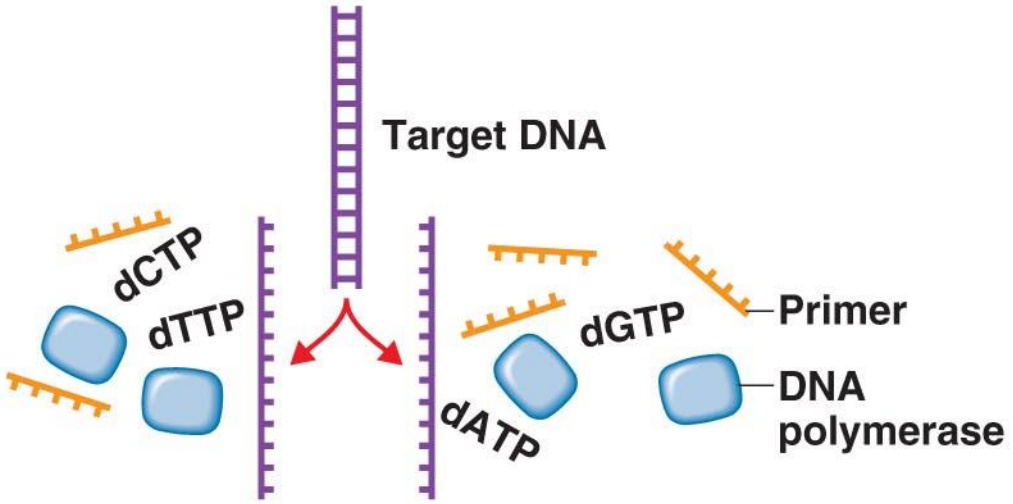


Figure 9.4

PCR

First cycle

3 Primers attach to single-stranded DNA during incubation at 60°C for 1 minute.

4 Incubate at 72°C for 1 minute; during this time, two copies of target DNA are formed.

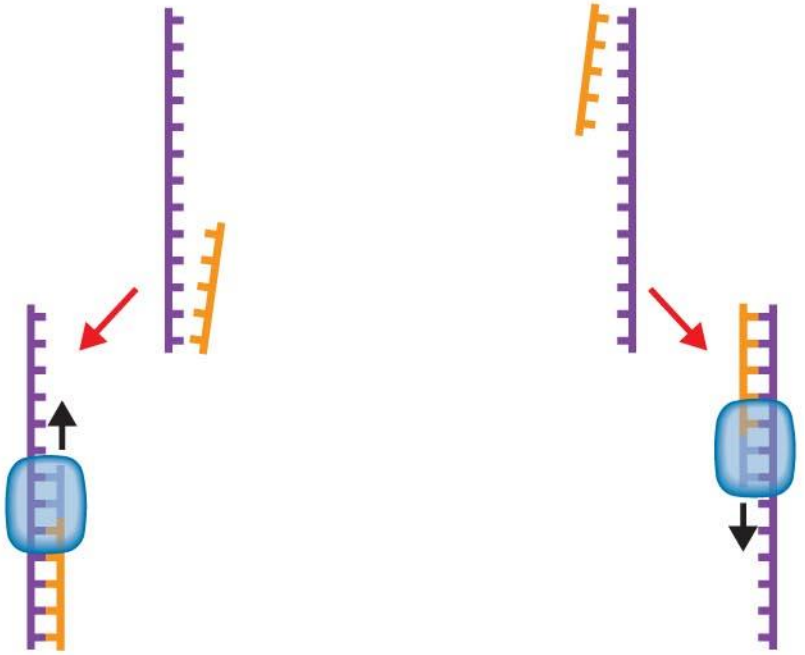
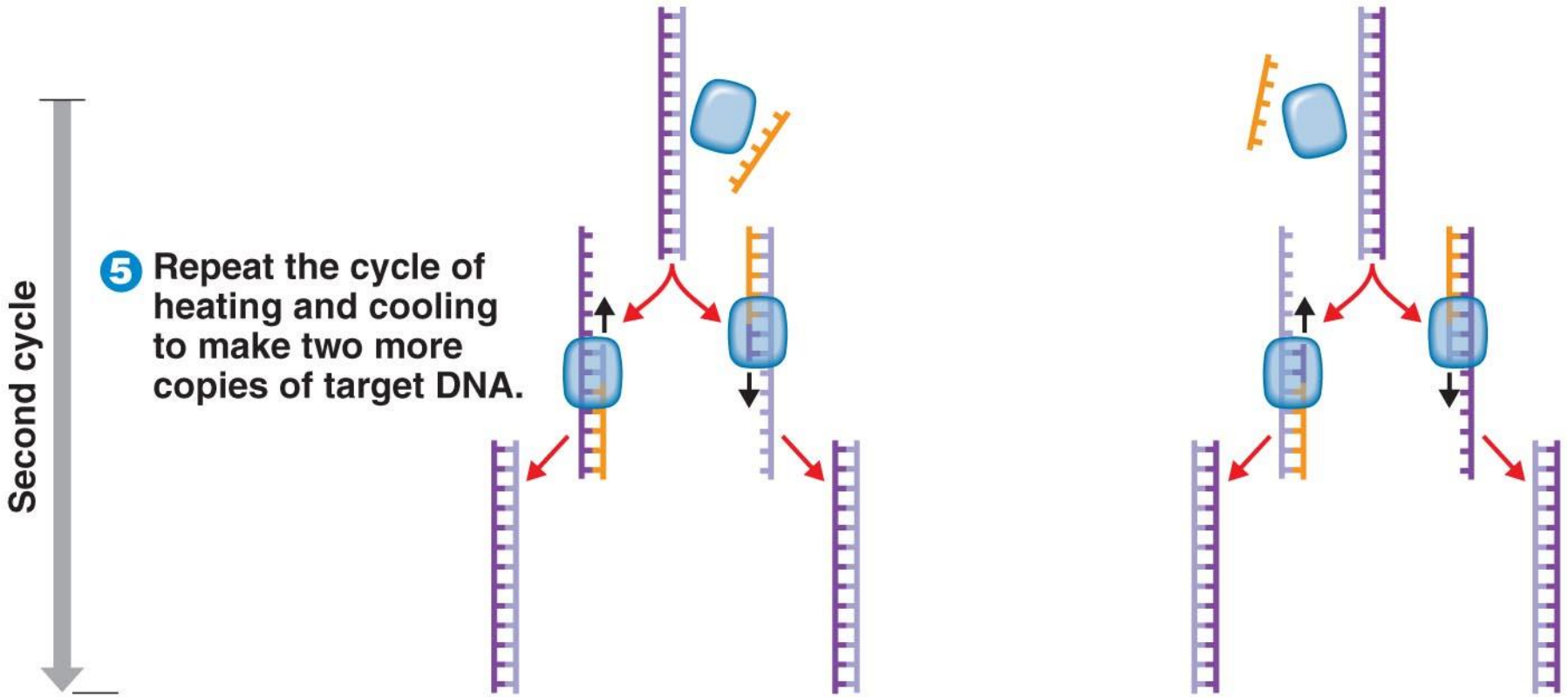


Figure 9.4

PCR



ANIMATION PCR: Process

Figure 9.4

Techniques of Genetic Modification

DNA can be inserted into a cell by:

1. Transformation: bacteria, yeast, mammalian cells

   Chemical treatments are used to make cells that do not naturally transform competent to take up DNA

2. Electroporation: all cells

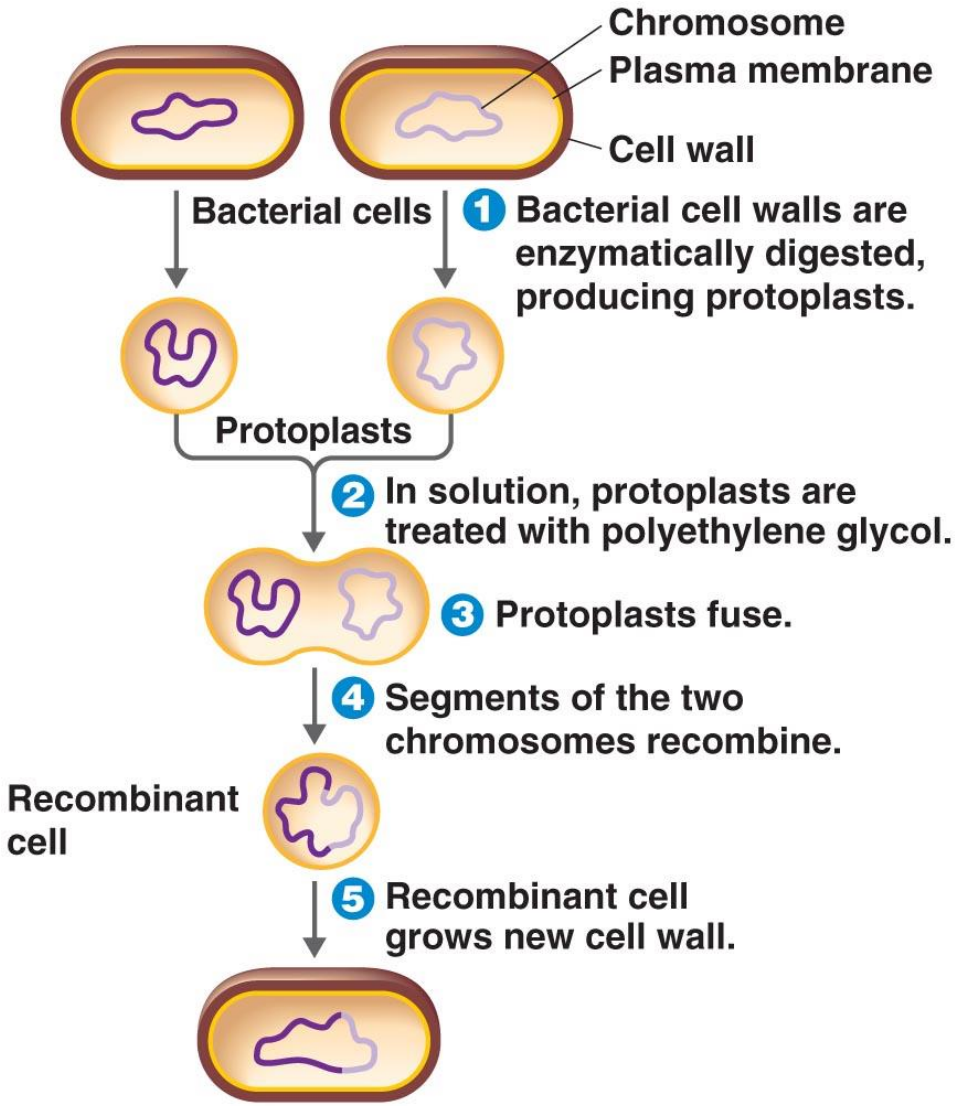
   Pores make in cells by electric current in the process of electroporation can provide entrance for new pieces of DNA.

3. Protoplast fusion (Figure 9.5): plant and algal cells

   Joining of cells whose cell walls have been removed

Figure 9.5b

Process of Protoplast Fusion



(a) Process of protoplast fusion

Figure 9.5a

DNA can be inserted into a cell by:

4. Microinjection

   Foreign DNA can be injected into animal cells by using a fine glass micropipette.

5. Gene gun

   Shooting DNA-coating particles into plant cells.

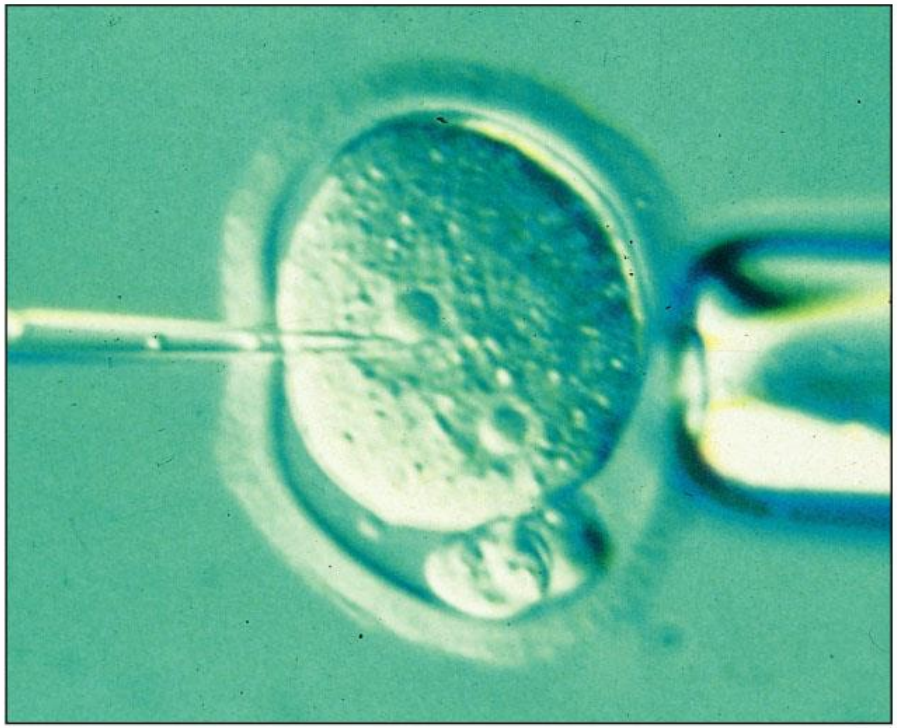


Figure 9.6 & 7

Obtaining DNA

- **Genomic libraries** are made of pieces of an entire genome stored in plasmids or phages

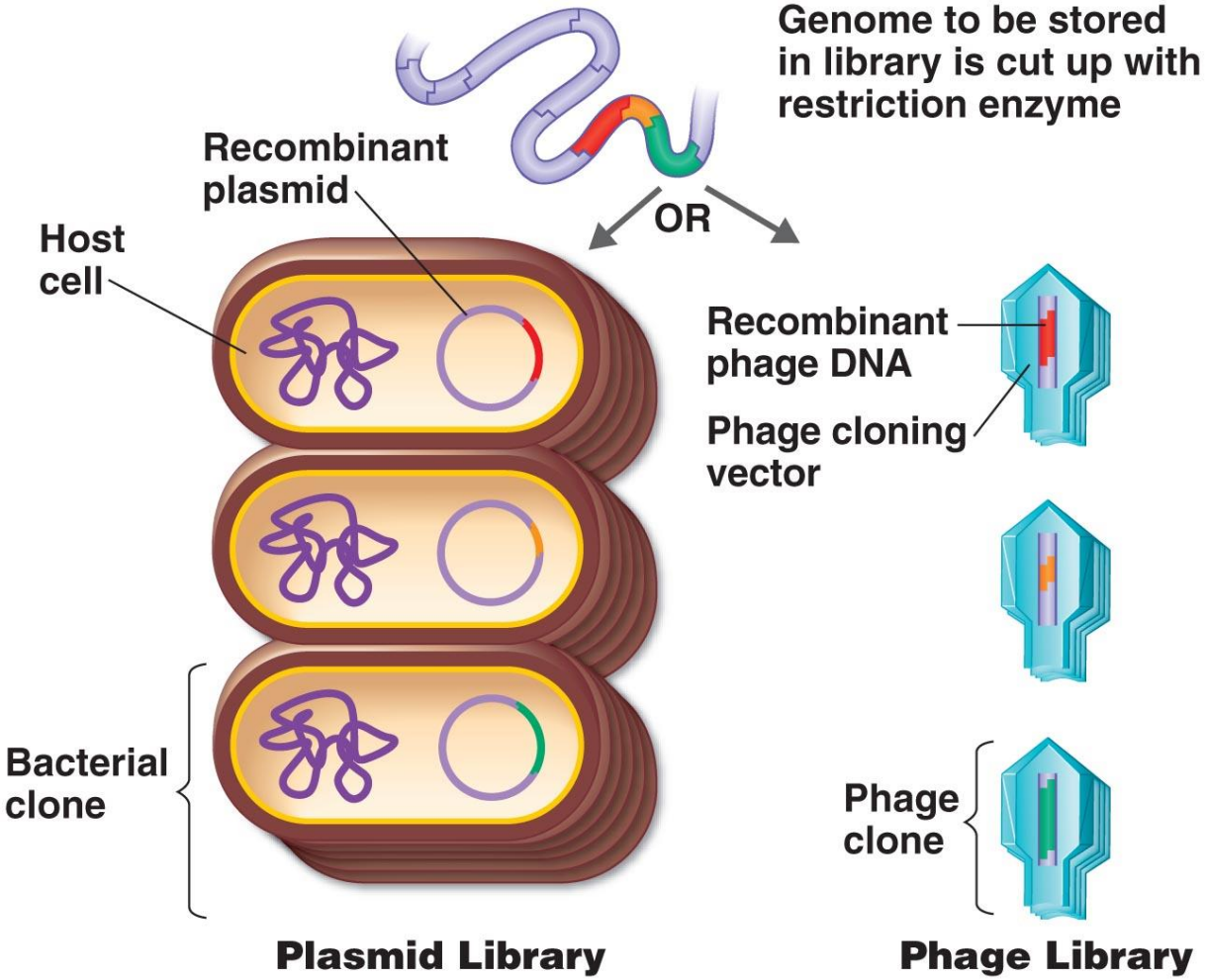


Figure 9.8

Obtaining DNA

- **Complementary DNA (cDNA)** is made from mRNA by reverse transcriptase
  - Genes of eukaryotic cells generally contain both exons, stretches of DNA that code for protein, and introns, intervening stretches of DNA that do not code for protein.
  - The cDNA method is the most common method of obtaining eukaryotic genes.

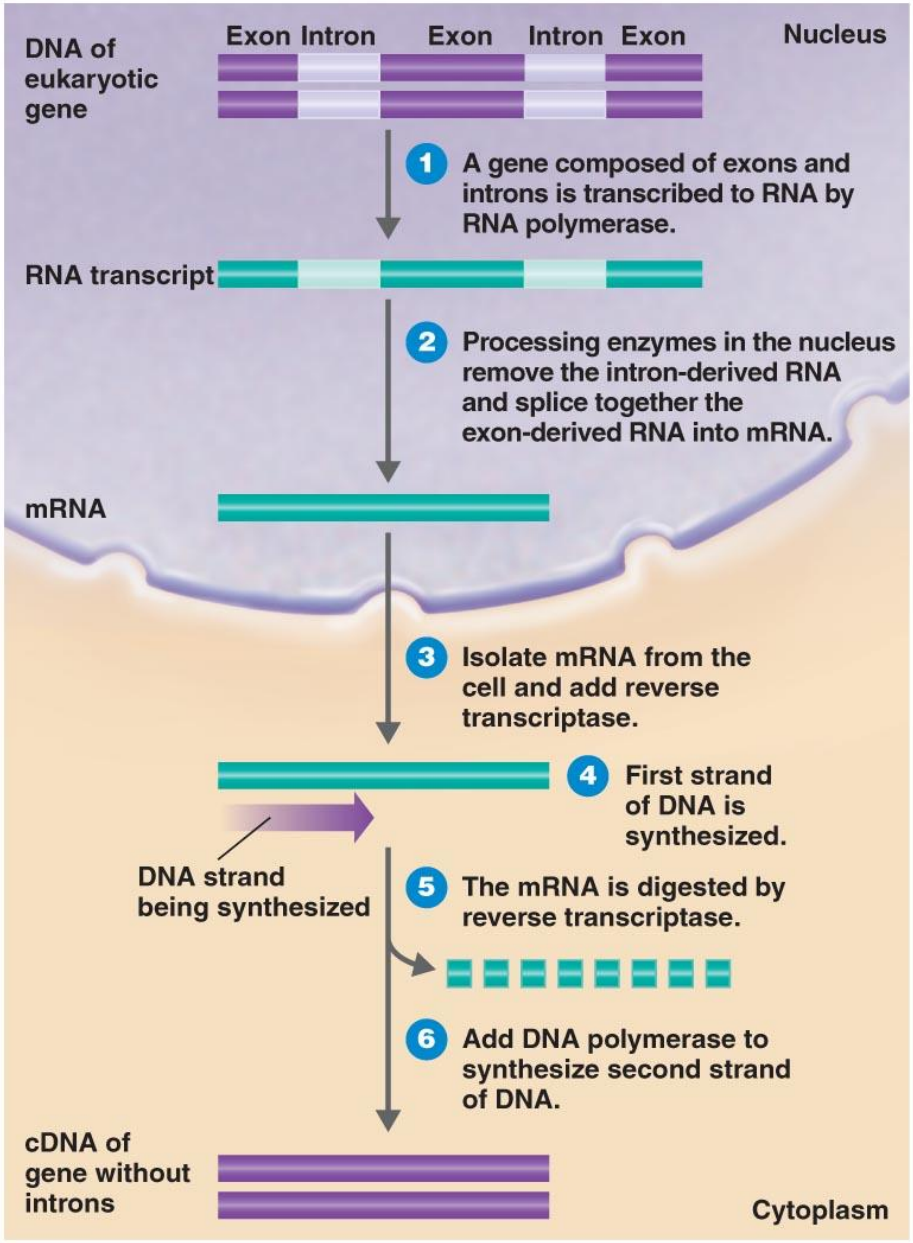


Figure 9.9

Obtaining DNA

- **Synthetic DNA** is made by a DNA synthesis machine
  - A chain of over 120 nucleotides can be synthesized by this method
  - The sequence of the gene must be known before it can be synthesized

Figure 9.10

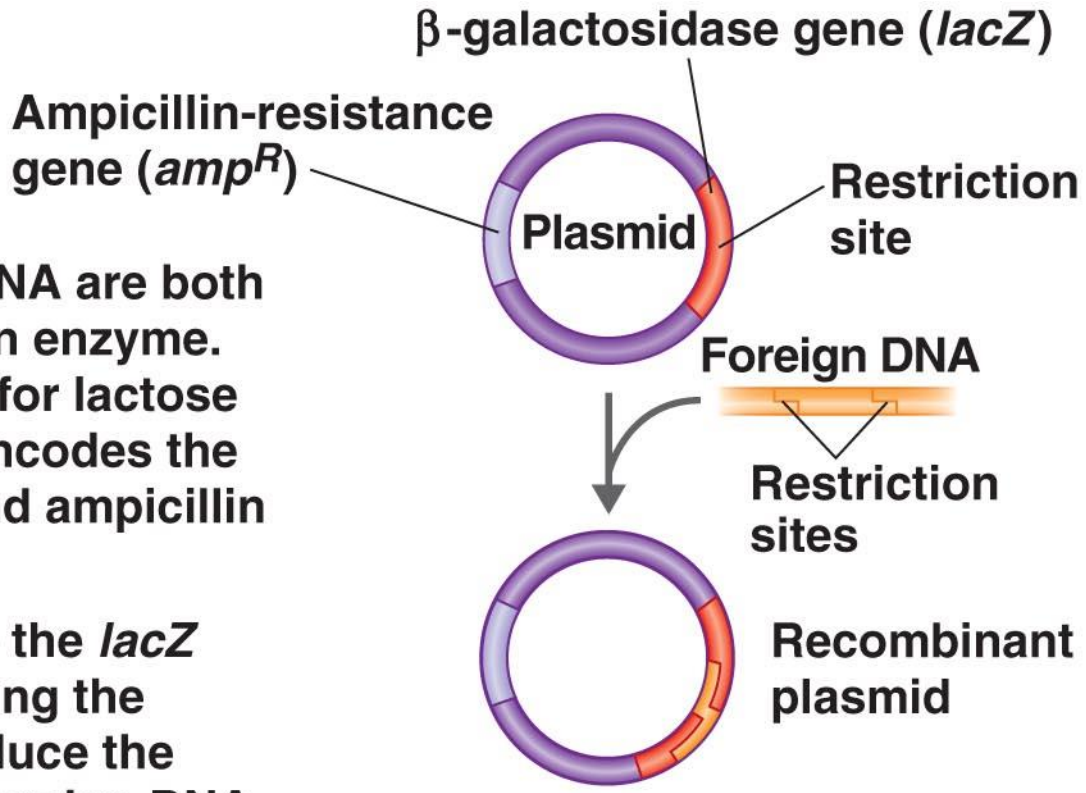
Selecting a Clone

1. Antibiotic-resistance genes
2. Insertion inactivation
   - Blue-white screening (Figure 9.11): the vector contains the genes for amp^R and the α-peptide of β-galactosidase.
     - The desired gene is inserted into the α-peptide-gene site, destroying the gene.
     - Clones containing the recombinant vector will be resistant to ampicillin and unable to hydrolyze X-gal (white colonies).
     - Clones containing the vector without the new gene will be blue. Clones lacking the vector will not grown
3. DNA probes: Colony hybridization (Figure 9.12) is a common method of identifying cells that carry a specific cloned gene.
4. Gene product

Selecting a Clone

Antibiotic-resistance genes

Insertion inactivation



1. Plasmid DNA and foreign DNA are both cut with the same restriction enzyme. The plasmid has the genes for lactose hydrolysis (the *lacZ* gene encodes the enzyme β-galactosidase) and ampicillin resistance.
2. Foreign DNA will insert into the *lacZ* gene. The bacterium receiving the plasmid vector will not produce the enzyme β-galactosidase if foreign DNA has been inserted into the plasmid.

Figure 9.11

Selecting a Clone

3. The recombinant plasmid is introduced into a bacterium, which becomes ampicillin resistant.

4. All treated bacteria are spread on a nutrient agar plate containing ampicillin and a β-galactosidase substrate and incubated. The β-galactosidase substrate is called X-gal.

5. **Only bacteria that picked up the plasmid will grow in the presence of ampicillin. Bacteria that hydrolyze X-gal produce galactose and an indigo compound. The indigo turns the colonies blue. Bacteria that cannot hydrolyze X-gal produce white colonies.**

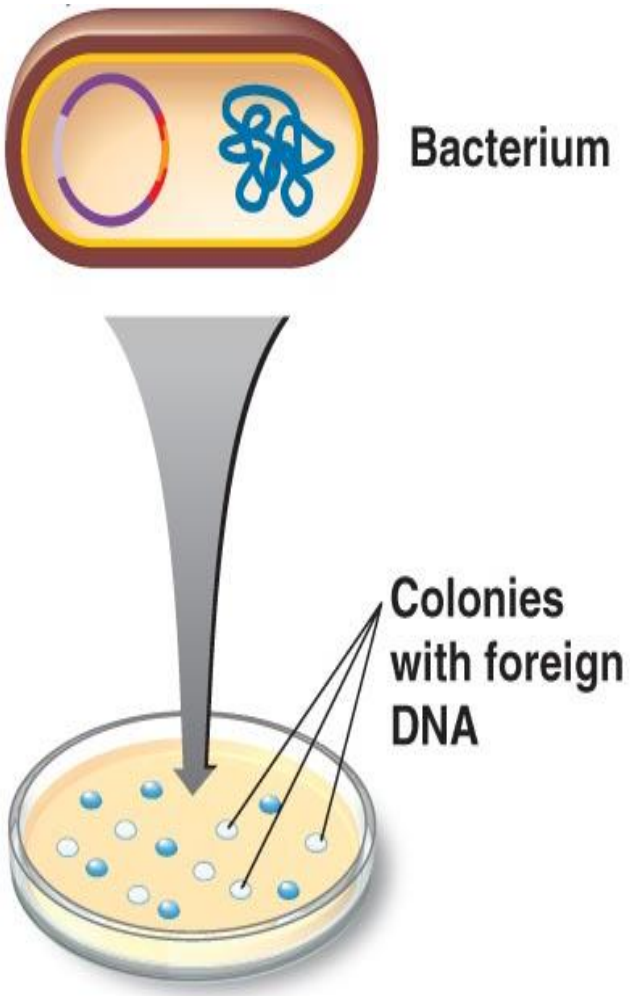


Figure 9.11

Selecting a Clone

Colony hybridization: using a DNA probe

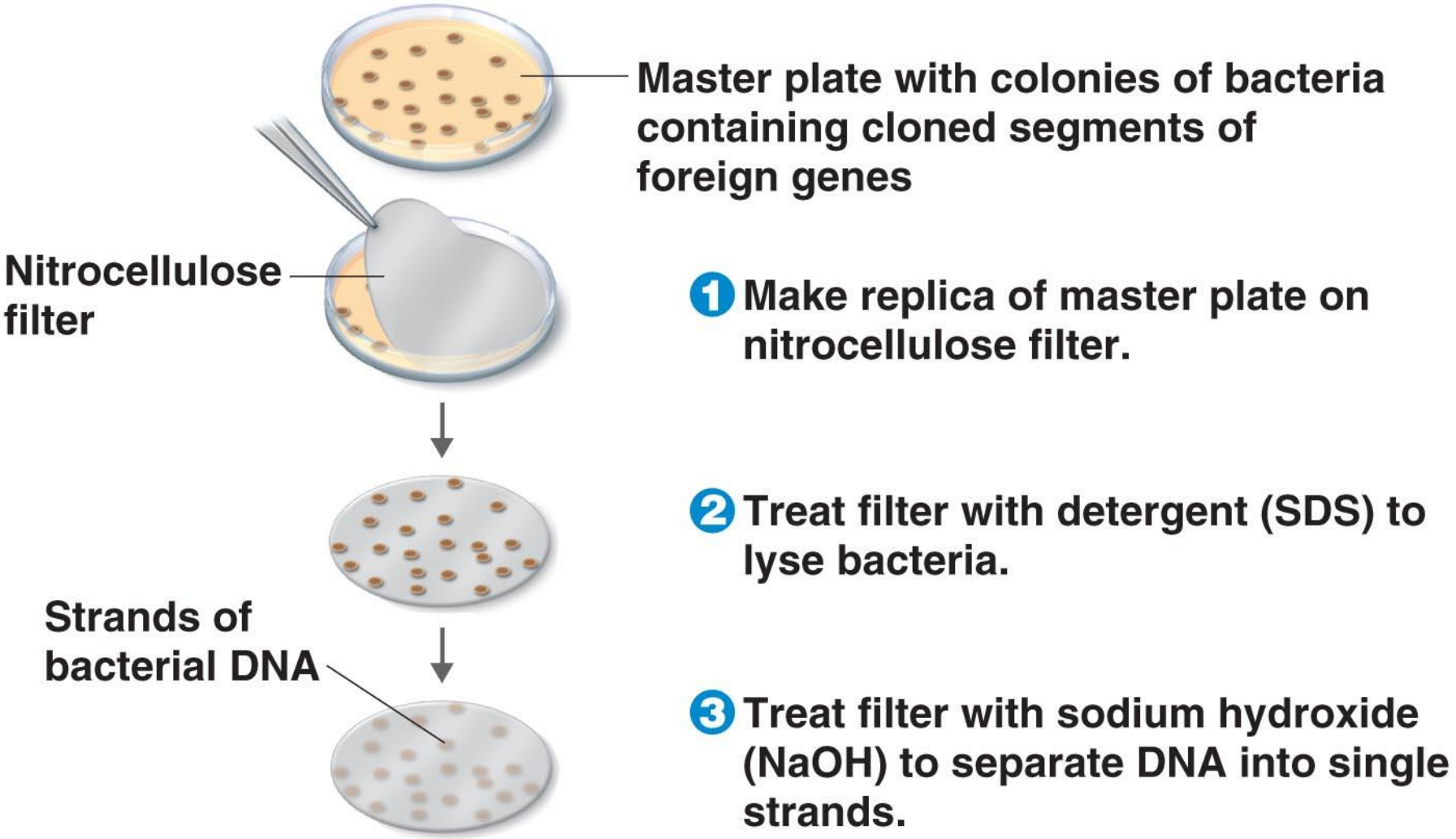
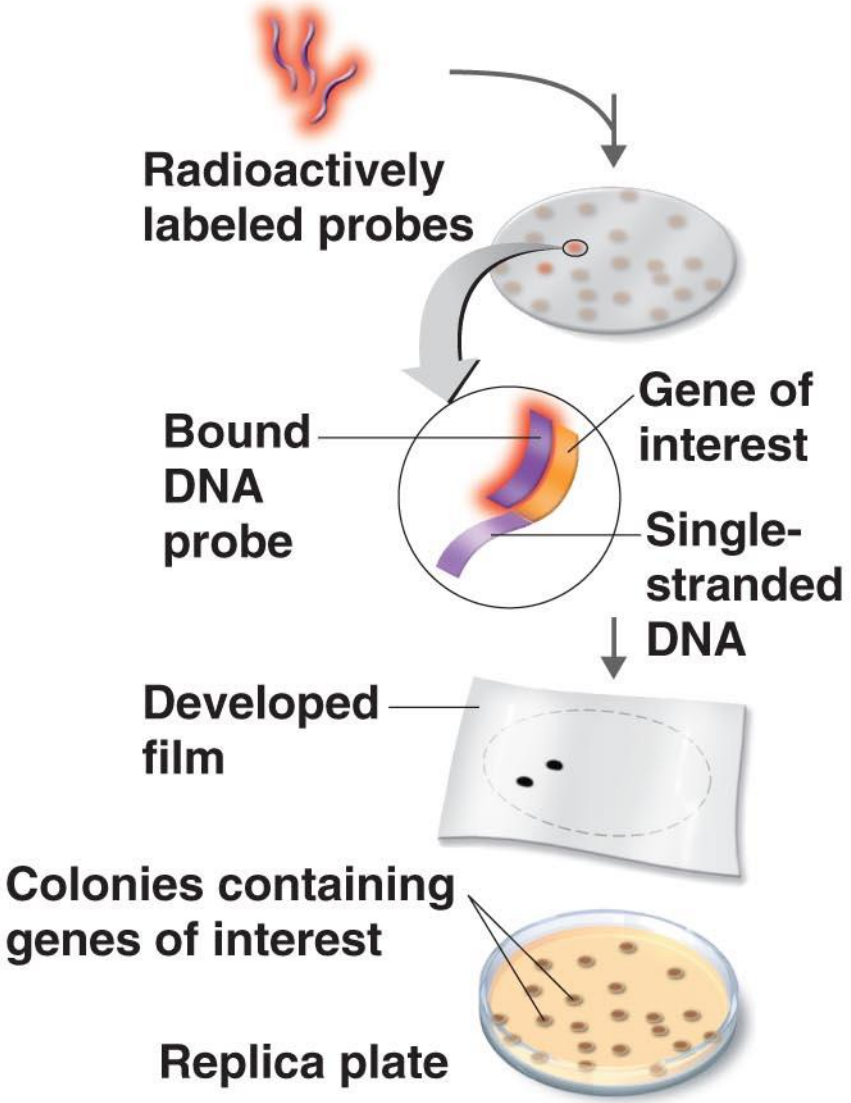


Figure 9.12

Selecting a Clone



4. Add radioactively labeled probes.

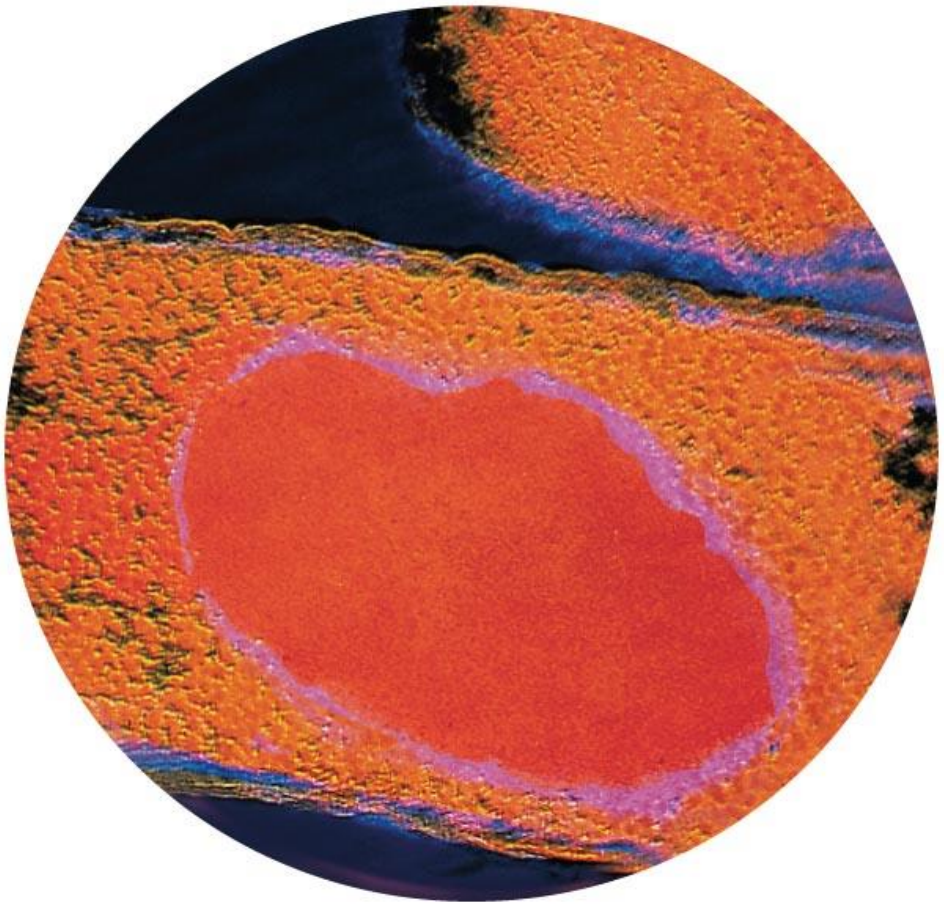
5. Probe will hybridize with desired gene from bacterial cells.

6. Wash filter to remove unbound probe and expose filter to X-ray film.

7. Compare developed film with replica of master plate to identify colonies containing gene of interest.

Figure 9.12

Q&A



- Interferons are species specific, so that interferons to be used in humans must be produced in human cells. Can you think of a way to increase the supply of interferons so that they can be used to treat diseases?

Making a Product

E. coli

Advantage:

- easily grown and its genomics are well known

Disadvantages:

- Need to eliminate endotoxin from products
- Cells must be lysed to get product

Bacillus subtilis

More likely to secrete their products

No endotoxin

Figure 9.13

Making a Product

Saccharomyces cerevisiae

- Used because it is easily grown and its genomics are known
- May express eukaryotic genes easily
- Yeasts are likely to continuously secrete the product

Mammalian cells

- May express eukaryotic genes easily
- Harder to grow
- Best suited to making protein products for medical use

Plant cells and whole plants

- May express eukaryotic genes easily
- Plants easily grown

Applications of rDNA

Produce useful substance more efficiently and less expensively

Obtain information from the the cloned DNA that is useful for either basic research or medical application.

Use cloned genes to alter the characteristics of cells or organisms.

Therapeutic Applications

- **Human enzymes** and other proteins
- **Subunit vaccines:** consisting only a protein portion of a pathogen
  - Made by genetically engineering yeasts
- Nonpathogenic viruses carrying genes for pathogen's antigens as **DNA vaccines**
- **Gene therapy**
  - Replace defective or missing genes
  - Gene silencing by RNA interference (RNAi) : may treat cancer and viral infections
    - Double-stranded RNAs called short (or small) interfering RNAs (siRNA) can bind to the target mRNA causing enzymatic destruction (Fig. 9.14)

Table 9.2 Some Pharmaceutical Products of Genetic Engineering

Product	Comments
Antitrypsin	Assists emphysema patients; produced by genetically modified sheep
Bone Morphogenic Proteins	Induces new bone formation; useful in healing fractures and reconstructive surgery; produced by mammalian cell culture
Cervical Cancer Vaccine	Consists of viral proteins produced by *S. cerevisiae*
Colony-Stimulating Factor (CSF)	Counteracts effects of chemotherapy; improves resistance to infectious disease such as AIDS; treatment of leukemia; produced by *E. coli* and *S. cerevisiae*
Epidermal Growth Factor (EGF)	Heals wounds, burns, ulcers; produced by *E. coli*
Erythropoietin (EPO)	Treatment of anemia; produced by mammalian cell culture
Factor VII	Treatment of hemorrhagic strokes; produced by mammalian cell culture
Factor VIII	Treatment of hemophilia; improves clotting; produced by mammalian cell culture
Interferon	
IFN–α	Therapy for leukemia, melanoma, and hepatitis; produced by *E. coli* and *S. cerevisiae* (yeast)
IFN–β	Treatment for multiple sclerosis; produced by mammalian cell culture
IFN–γ	Treatment of chronic granulomatous disease; produced by *E. coli*
Hepatitis B Vaccine	Produced by *S. cerevisiae* that carries hepatitis-virus gene on a plasmid
Human Growth Hormone (hGH)	Corrects growth deficiencies in children; produced by *E. coli*
Human Insulin	Therapy for diabetes; better tolerated than insulin extracted from animals; produced by *E. coli*

Table 9.2 Some Pharmaceutical Products of Genetic Engineering

Product	Comments
Influenza Vaccine	Trial vaccine made from *E. coli* or *S. cerevisiae* carrying virus genes
Interleukins	Regulate the immune system; possible treatment for cancer; produced by *E. coli*
Monoclonal Antibodies	Possible therapy for cancer and transplant rejection; used in diagnostic tests; produced by mammalian cell culture (from fusion of cancer cell and antibody-producing cell)
Orthoclone OKT3 Muromonab-CD3	Monoclonal antibody used in transplant patients to help suppress the immune system, reducing the chance of tissue rejection; produced by mouse cells
Prourokinase	Anticoagulant; therapy for heart attacks; produced by *E. coli* and yeast
Pulmozyme (rhDNase)	Enzyme used to break down mucous secretions in cystic fibrosis patients; produced by mammalian cell culture
Relaxin	Used to ease childbirth; produced by *E. coli*
Superoxide Dismutase (SOD)	Minimizes damage caused by oxygen free radicals when blood is resupplied to oxygen-deprived tissues; produced by *S. cerevisiae* and *Komagataella pastoris* (yeast)
Taxol	Plant product used for treatment for ovarian cancer; produced in *E. coli*
Tissue Plasminogen Activator (Activase)	Dissolves the fibrin of blood clots; therapy for heart attacks; produced by mammalian cell culture
Tumor Necrosis Factor (TNF)	Causes disintegration of tumor cells; produced by *E. coli*

RNA Interference (RNAi)

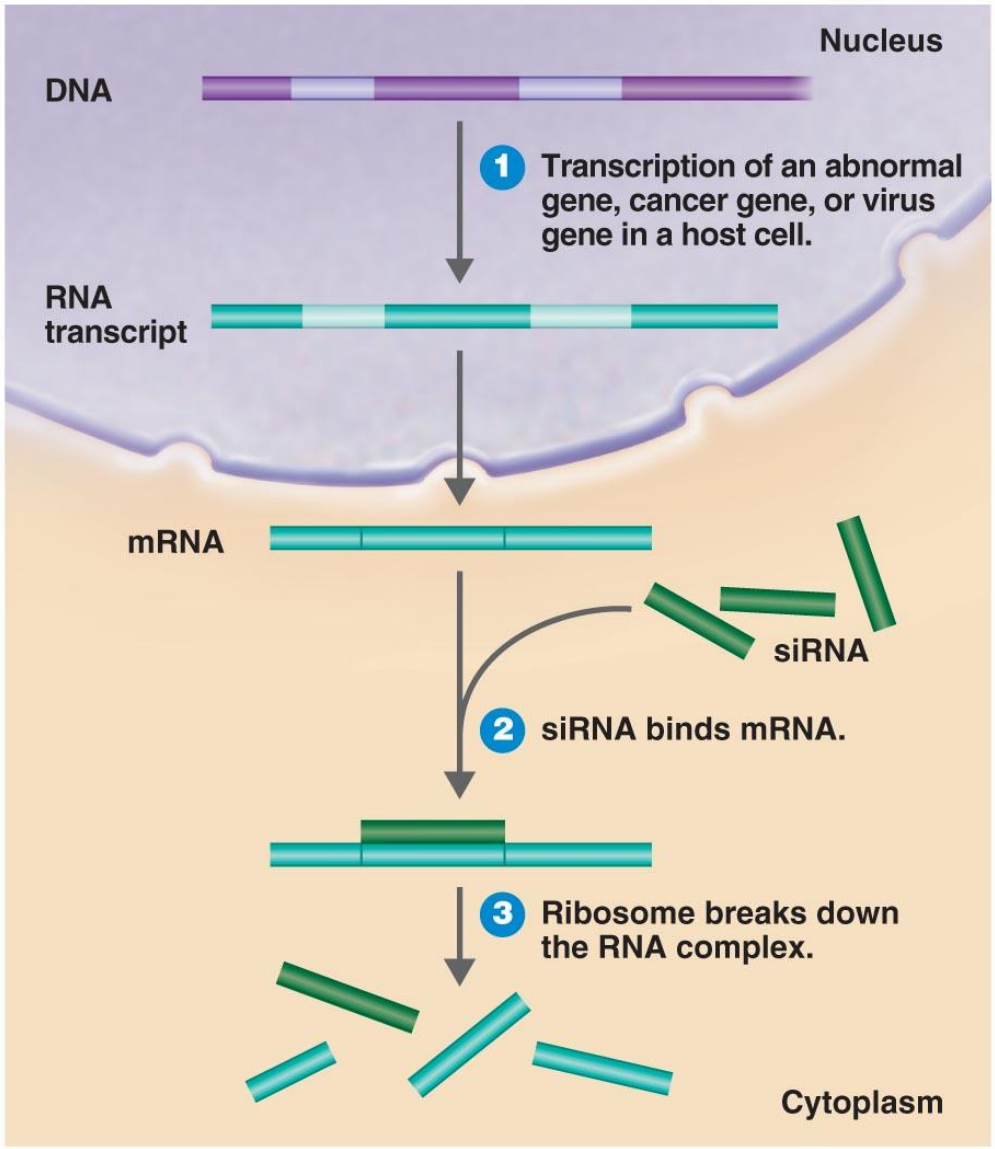


Figure 9.14

The Human Genome Project

- Entire human genome has been sequenced by **random shotgun sequencing (隨機式霰彈槍定序法)** (Fig. 9.15)
  - 20000-25000 genes
- **Human Proteome Project**
  - determine all the proteins expressed in a cell (**proteomics**).
  - may provide diagnostics and treatments of genetic diseases.
  - **Bioinformatics** is the use of computer application to study genetic data
  - **Reverse genetics**: Mutate or block a gene to determine its function

Random Shotgun Sequencing

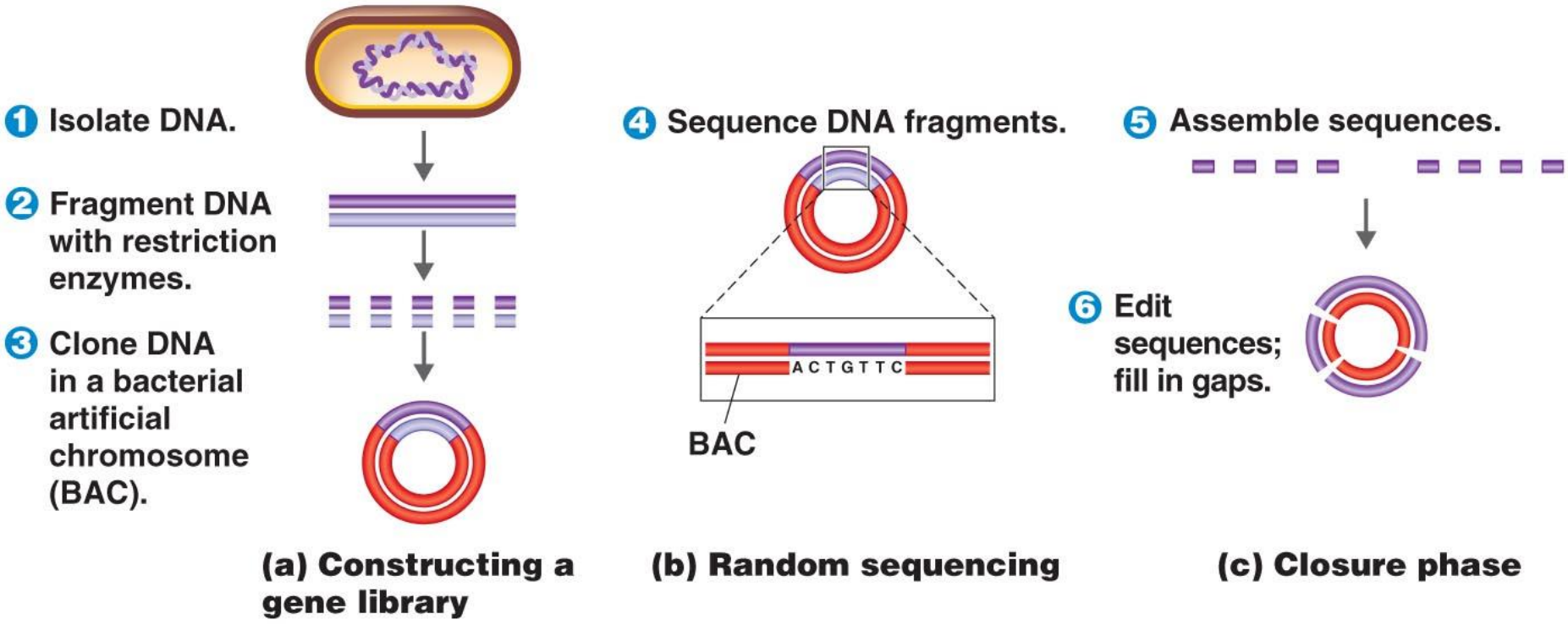


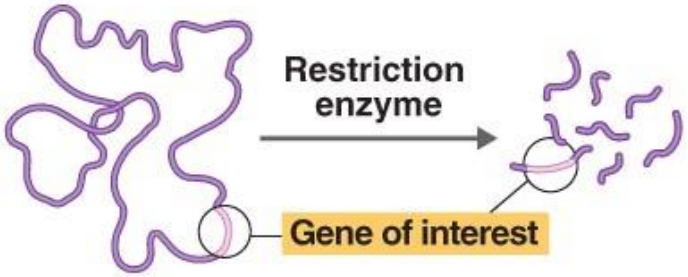
Figure 9.15

Scientific Applications

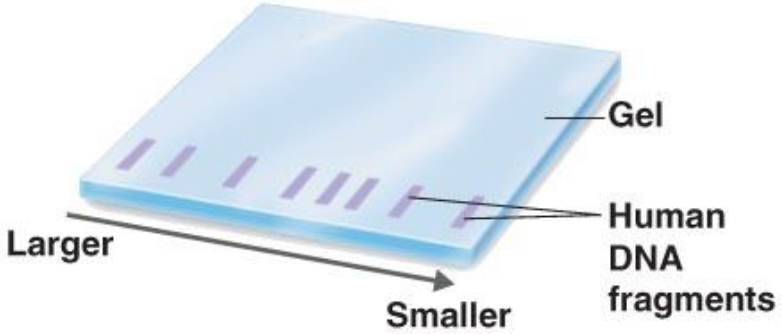
1. Recombinant DNA techniques can be used to **increase understanding of DNA**
   - Sequencing organisms' genomes (random shotgun sequencing, Figure 9.15)
2. **Bioinformatics** is the use of computer application to study genetic data
3. **Proteomic** is the science of studying all of the proteins expressed in a cell.
4. Southern blotting can be used to **locate a specific gene in a cell**.

   Southern blotting is used in DNA fingerprinting to compare DNA recovered from a crime scene with that of a suspect.
5. DNA probes can be used to identify a pathogen quickly in body tissue or food.

Southern Blotting



1 DNA containing the gene of interest is extracted from human cells and cut into fragments by restriction enzymes.

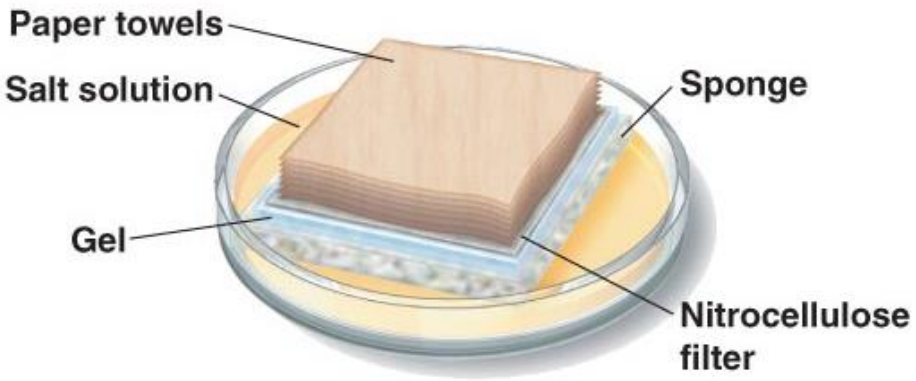


2 The fragments are separated according to size by gel electrophoresis. Each band consists of many copies of a particular DNA fragment. The bands are invisible but can be made visible by staining.

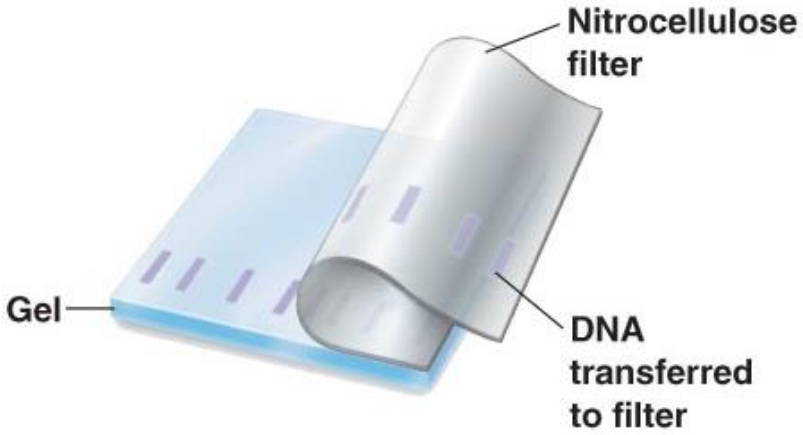
The fragments are called RFLPs (restriction fragments length polymorphisms)

Figure 9.16

Southern Blotting



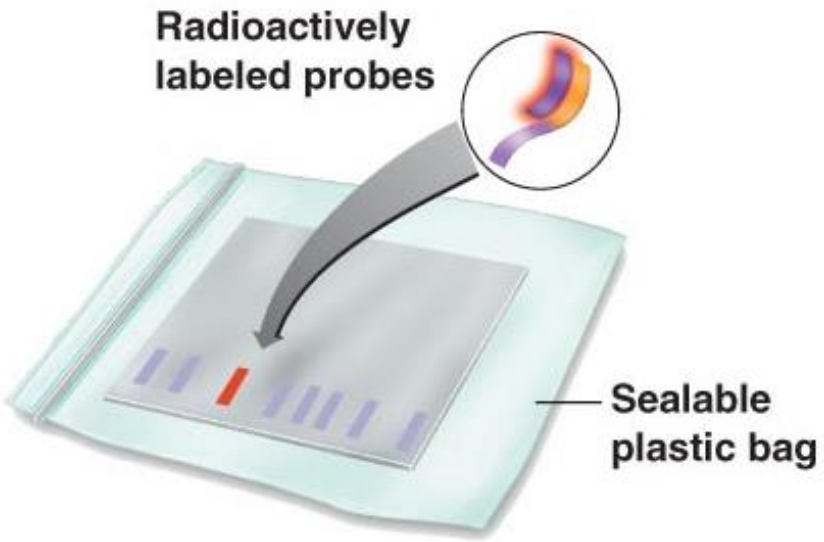
3 The DNA bands are transferred to a nitrocellulose filter by blotting. The solution passes through the gel and filter to the paper towels.



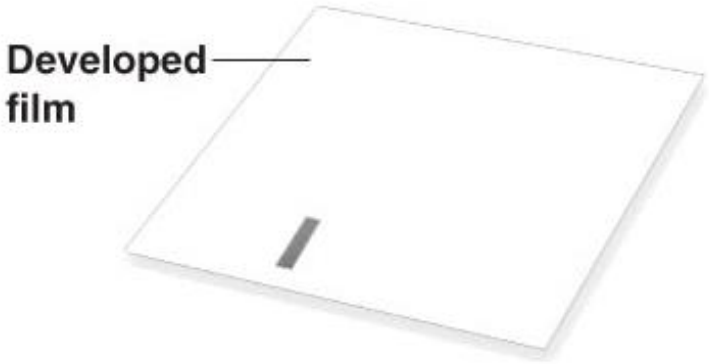
4 This produces a nitrocellulose filter with DNA fragments positioned exactly as on the gel.

Figure 9.16

Southern Blotting



The filter is exposed to a radioactively labeled probe for a specific gene. The probe will base-pair (hybridize) with a short sequence present on the gene.



6 The filter is then exposed to X-ray film. The fragment containing the gene of interest is identified by a band on the developed film.

Figure 9.16

DNA fingerprints for identification

1. DNA fingerprints (using RFLPs for identification) of bacteria that cause foodborne diseases are kept in a database at the Centers for Disease Control and Prevention.
2. Forensic (用於法庭的) microbiology use DNA fingerprinting to identify the source of bacterial or viral pathogens.

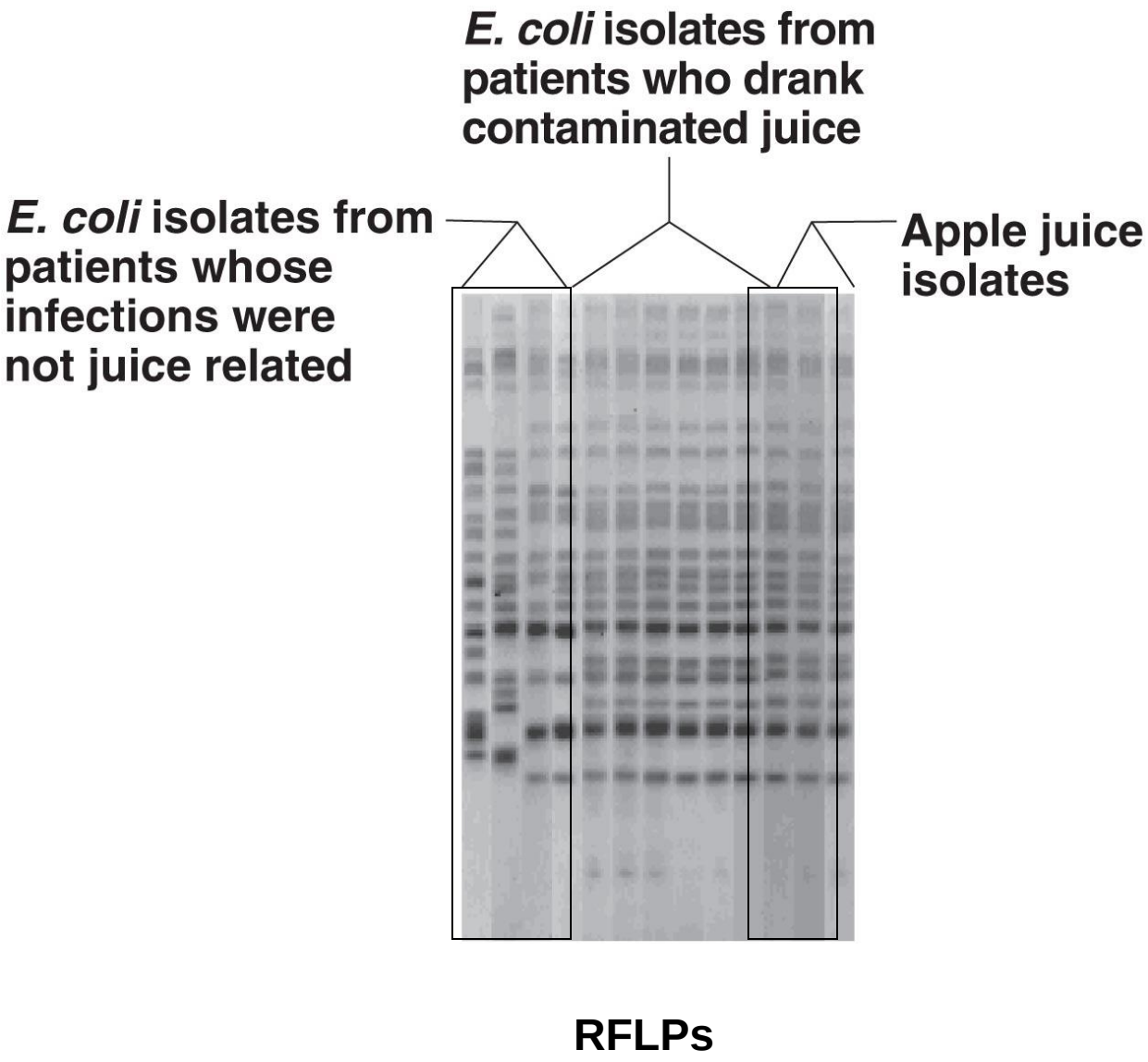


Figure 9.17

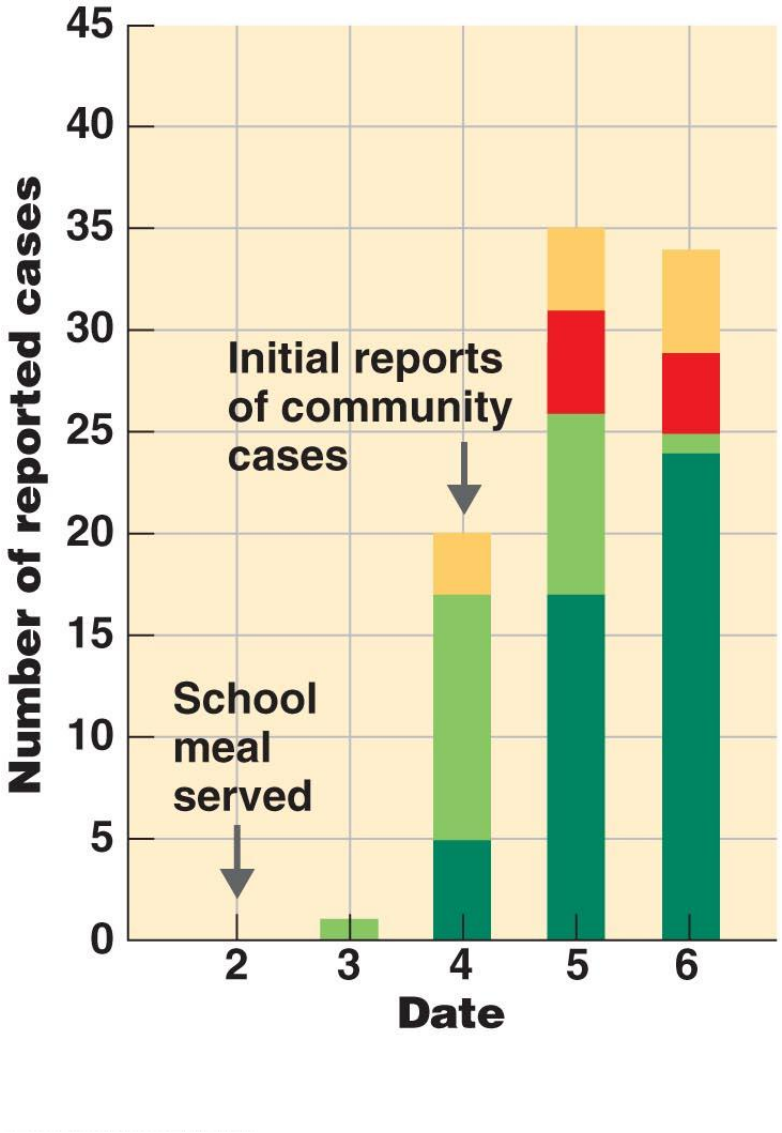
Forensic science

Forensic science is the application of natural sciences to matters of the law. In practice, forensic science draws upon physics, chemistry, biology, and other scientific principles and methods. Forensic science is concerned with the recognition, identification, individualization, and evaluation of physical evidence. Forensic scientists present their findings as expert witnesses in the court of law.

Forensic Microbiology

- PCR can be used to amplify small sample
- Primer for a specific organism will be used to check if that organism is present
- **Real-time PCR**: Newly made DNA tagged with a fluorescent dye; the levels of fluorescence can be measured after every PCR cycle
- **Reverse-transcription PCR (RT-PCR)**: Reverse transcriptase makes DNA from viral RNA or mRNA

Norovirus Outbreak

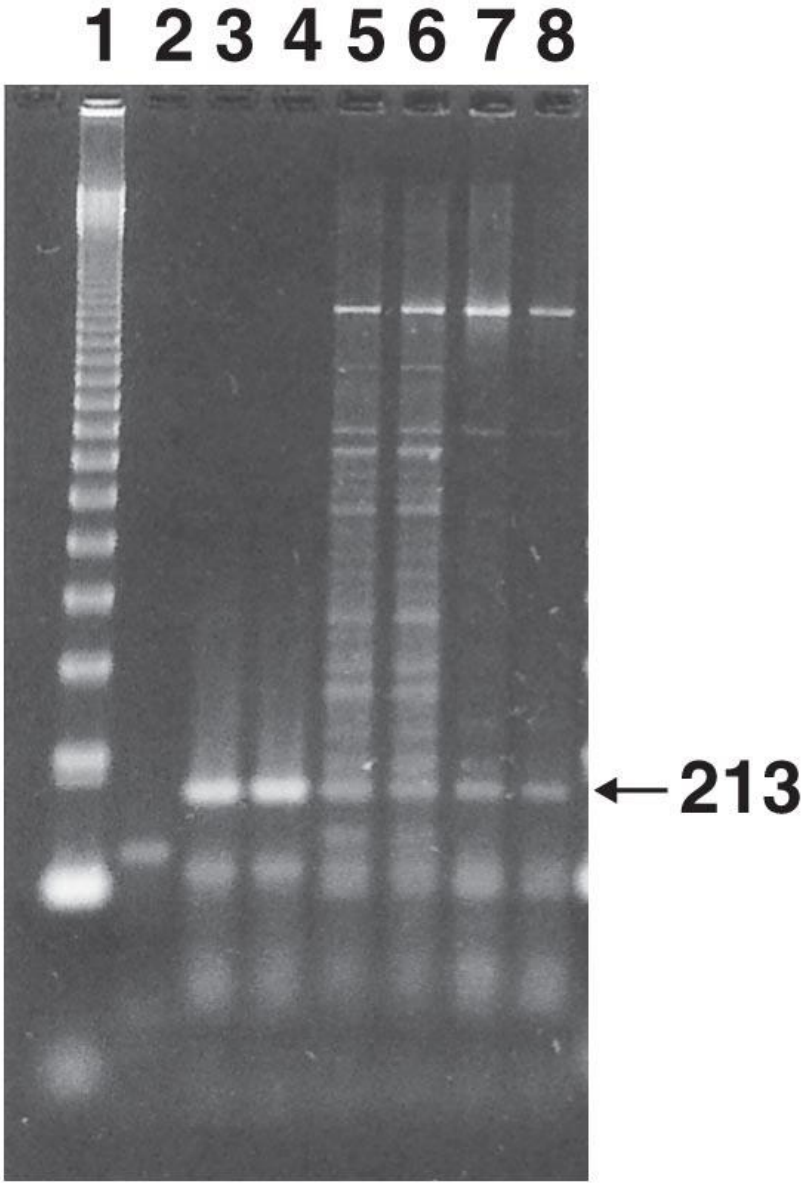


- Are the outbreaks related?
- What is the source?

Clinical Focus, p. 266

Norovirus Outbreak

- RT-PCR with a norovirus primer



Clinical Focus, p. 266

Nanotechnology

- **Nanotechnology**, shortened to "**nanotech**", is the study of the controlling of matter on an atomic and molecular scale. Generally nanotechnology deals with structures of the size 100 nanometers or smaller in at least one dimension, and involves developing materials or devices within that size.
- Bacteria can make molecule-sized particles

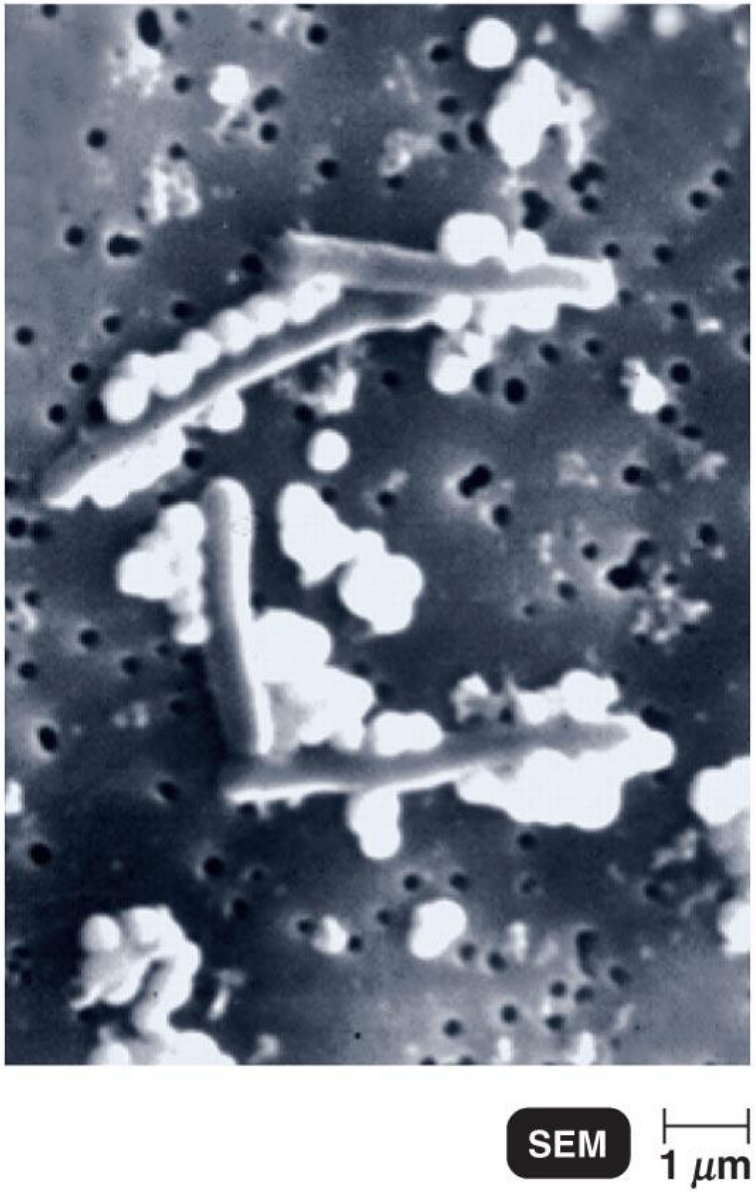


Figure 9.18

Agricultural Applications using *Agrobacterium*

- Bt toxin
- Herbicide resistance
- Suppression of genes
  - **Antisense DNA**
- Nutrition (nitrogen fixation)
- Human proteins

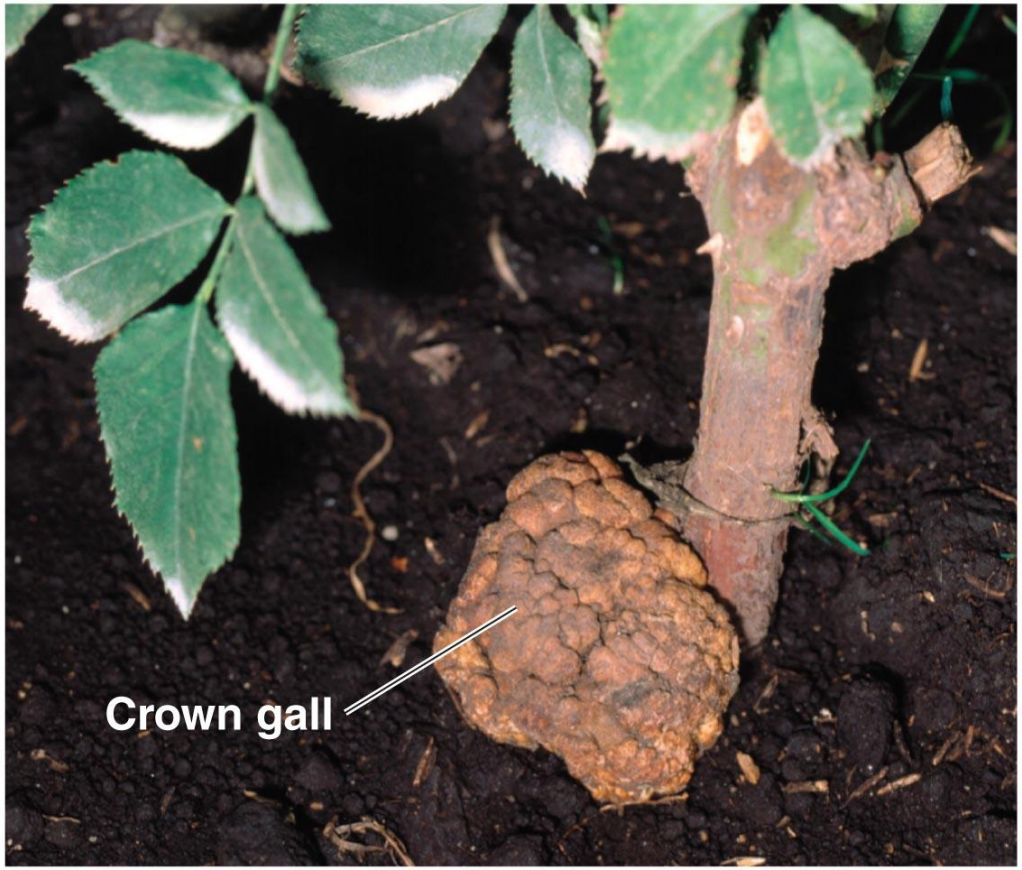
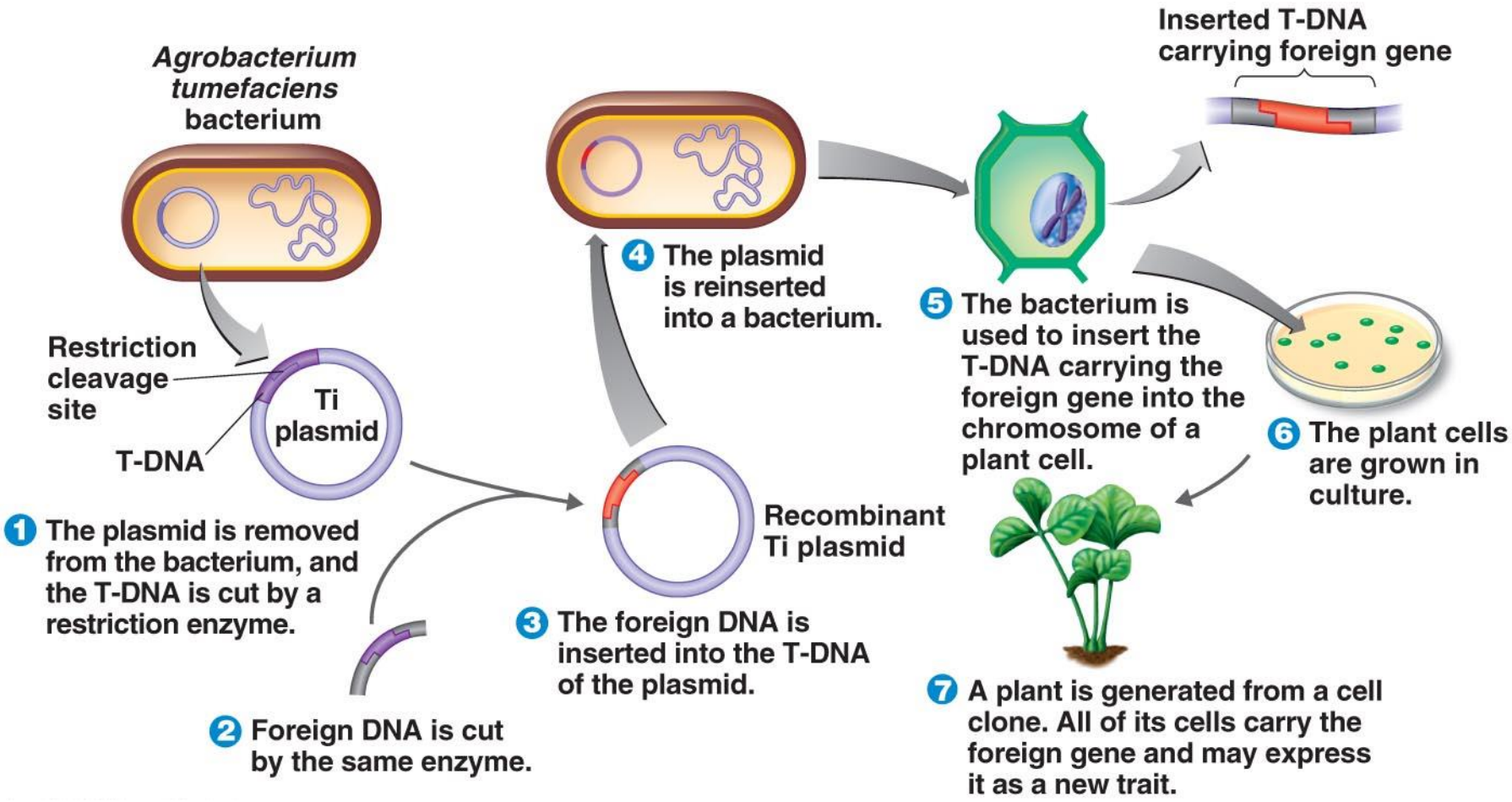


Figure 9.19

Using *Agrobacterium*



Ti stands for tumor-inducing

Figure 9.20

Agricultural Applications

Examples:

1. Plants with resistance to the herbicide glyphosate (Round-Up)
   - Round-Up kills both weeds and crops by inhibiting an enzyme necessary for making aromatic amino acids.
   - Some *Salmonella* bacteria have a mutant enzyme that is resistant to the herbicide.
   - When the gene for this mutant enzyme is introduced to a crop plant, the crop becomes resistant to the herbicide.
2. Plants can produce a *Bacillus thuringeinsis*-derived insecticidal toxin (Bt toxin)
   - Bt toxin is a protein that interferes with the insect digestive tract ➔ when insects eat the engineered crop, they will be killed.

Agricultural Applications

Examples:

3. PG-gene suppression by **antisense DNA technology**
   - MacGregor tomatoes stay firm after harvest because the gene for polygalacturonase (PG), the enzyme that breaks down pectin, is suppressed.
   - First, a length of DNA complementary to the PG mRNA is synthesis.
   - When the antisense DNA is taken up by the cell and binds to the mRNA, the translation is inhibited.
4. *Rhizobium* with enhanced nitrogen fixation ability (N_2 → NH_3)
   - a symbiont on the roots of leguminous plants
   - may be designed to colonize other crop plants.

Agricultural Applications

Examples:

5. *Pseudomonas fluorescens* has been engineered to produce *Bacillus thuringeinsis* toxin against insects.
   - Produce more toxin than *Bacillus thuringeinsis* .
   - Add to plant seeds and then enter the vascular system of the growing plants.
6. Bovine growth hormone (bGH) is being produced by *E. coli*.
   - When bGH is injected into beef cattle, it increases their weight gain; in dairy cows, it also causes a 10% increase in milk production.
   - Will the bGH present in the milk or meat? Harmful to humans?

Table 9.3 Some Agriculturally Important Products of Genetic Engineering

Product	Comments
Agricultural Products	
Bt cotton and Bt corn	Plants have toxin-producing gene from *Bacillus thuringiensis;* toxin kills insects that eat plants
MacGregor tomatoes	Antisense gene blocks pectin degradation, so fruits have longer shelf life
Pseudomonas fluorescens bacterium	Has toxin-producing gene from insect pathogen *B. thuringiensis;* toxin kills root-eating insects that ingest bacteria
Pseudomonas syringae, ice-minus bacterium	Lacks normal protein product that initiates undesirable ice formation on plants
Rhizobium meliloti bacterium	Modified for enhanced nitrogen fixation
Round up (glyphosate)-resistant crops	Plants have bacterial gene; allows use of herbicide on weeds without damaging crops
Animal Husbandry Products	
Bovine growth hormone (bGH)	Improves weight gain and milk production in cattle; produced by *E. coli*
Porcine growth hormone (pGH)	Improves weight gain in swine; produced by *E. coli*
Transgenic animals	Genetic modification of animals to produce medically useful products in their milk
Other Food Production Products	
Cellulase	Enzyme that degrades cellulose to make animal feedstocks; produced by *E. coli*
Rennin	Causes formation of milk curds for dairy products; produced by *Aspergillus niger*

Safety Issues and Ethics of Using rDNA

- Avoid accidental release of genetically engineered MOs.
  - Some microbes used in genetic engineering have been altered so that they cannot survive outside of the laboratory.
- MOs that are intended for use in the environment may be engineered to contain suicide genes so that the organisms do not persist in the environment.
- Genetically modified crops must be safe for consumption and for release in the environment.
- Who will have access to an individual's genetic information?