Recombinant DNA Technology

Lectures by Lara Dowland
Chapter Contents

• 3.1 Introduction to Recombinant DNA Technology and DNA Cloning
• 3.2 What Makes a Good Vector?
• 3.3 How Do You Identify and Clone a Gene of Interest?
• 3.4 What Can You Do with a Cloned Gene? Applications of Recombinant DNA Technology
3.1 Introduction to Recombinant DNA Technology and DNA Cloning

• Recombinant DNA tech is used to clone
  – Genetic engineering relies on recombinant DNA tech and cloning.

• 1970s gene cloning became a reality
  – **Clone** – a molecule, cell, or organism that was produced from another single entity.
    • Word derived from Greek (cutting of a twig)

• Made possible by the discovery of
  – **Restriction Enzymes** – DNA cutting enzymes
  – **Plasmid DNA Vectors** – circular form of self-replicating DNA
    • Can be manipulated to carry and clone other pieces of DNA
3.1 Introduction to Recombinant DNA Technology and DNA Cloning

• Restriction Enzymes
  – Primarily found in bacteria in 1960s (Werner Arber).
  – Cut DNA phages and prevent their replication.
  – Cut DNA by cleaving the phosphodiester bond that joins adjacent nucleotides in a DNA strand
  – Bind to, recognize, and cut DNA within specific sequences of bases called a recognition sequence or restriction site.
  – 1970, *Hind III* isolated; first restriction enzyme to be used for DNA cloning.
    • Endonuclease: Cuts within DNA sequences.
      – Exonucleases cut from the ends of DNA sequences
3.1 Introduction to Recombinant DNA Technology and DNA Cloning

• Restriction Enzymes
  – Named based on the bacteria where they were isolated.
    • E.g: EcoRI discovered in *E. coli* strain RY13.
  – Recognition sequences are *palindromes (read the same front and back)*
    • *Recognition sites or restriction sites (substrate specific).*
      – 4 to 8 base pair cutters.
  – Cohesive (sticky) ends – overhanging single-stranded ends (e.g. EcoRI)
  – Blunt ends – double-stranded, non-overhanging ends
3.1 Introduction to Recombinant DNA Technology and DNA Cloning

- 1970s Paul Berg isolated Chromosomal DNA from *E. coli* and DNA from simian virus 40 (SV40), cut both DNA samples with *EcoRI* and successful ligate them together.
- Plasmid DNA – small circular pieces of DNA found primarily in bacteria (extrachromosomal DNA)
- Can be used as vectors *(Stanley Cohen)* – pieces of DNA that can accept, carry, and replicate other pieces of DNA
- 1975- NIH formed Recombinant DNA Advisory Committee (RAC) to set guidelines for working with recombinant organisms.
1) Restriction enzyme cuts double-stranded DNA at its particular recognition sequence.

2) These cuts produce DNA fragments with cohesive ends.

DNA from another source, perhaps a bacterial plasmid

3) When two such fragments of DNA cut by the same restriction enzyme come together, they can join by base pairing.

4) The joined fragments will usually form either a linear molecule or a circular one, as shown here for a plasmid. Other combinations of fragments can also occur, however.

5) The enzyme DNA ligase is used to unite the backbones of the two DNA fragments, producing a molecule of recombinant DNA containing human and plasmid DNA.
Restriction enzyme recognition sequence

DNA 5' GAATTC 3'
3' CTTAAG 5'

Restriction enzyme cuts the DNA

Addition of a DNA fragment from another source; fragments stick together by base pairing

DNA fragment produced by the same restriction enzyme

One possible combination

DNA ligase seals the strands

Recombinant DNA molecule
Sticky ends: EcoRI
5’-GAATTC-3’ → G + AATTC
3’-CTTAAG-5’ → CTTAA-5’

PstI
5’-CTGCAG-3’ → CTGCA-3’ + G
3’-GACGTC-5’ → G 3’-ACGTC

Blunt ends: SmaI
5’-CCCGGG-3’ → CCC + GGG
3’-GGGGCC-5’ → GGG CCC
Sticky ends: SpeI

5’-ACTAGT-3’ → A + CTAGT
3’-TGATCA-5’ → TGATC-5’ A

HindIII

5’-AAGCTT-3’ → A + AGCTT
3’-TTGCAG-5’ → TTCGA-5’ A

Partial Filling:

SpeI

A
TGATC-5’ + dC,dT

ACT
TGATC-5’

HindIII

5’-AGCTT A + dA,dG

5’-AGCTT GAA
3.1 Introduction to Recombinant DNA Technology and DNA Cloning

- Transformation of Bacterial Cells (Stanley Cohen)
  - A process for inserting foreign DNA into bacteria
    - Treat bacterial cells with calcium chloride
    - Add plasmid DNA to cells chilled on ice
    - Heat the cell and DNA mixture
    - Plasmid DNA enters bacterial cells and is replicated and expressed
  - More modern method of transformation is electroporation
Cloning a human gene in a bacterial plasmid: a closer look (Layer 1)

1. Isolate plasmid (vector) DNA and human DNA.

2. Insert human DNA into plasmids:
   - Cut both DNAs with same restriction enzyme.
   - Mix the DNAs; they join by base pairing. (Some plasmids, like this one, join with the gene of interest.)
   - Add DNA ligase to bond covalently.

Recombinant plasmid

Human DNA fragment containing gene of interest

Nonfunctional lacZ gene
Cloning a human gene in a bacterial plasmid: a closer look (Layer 2)

1. Isolate plasmid (vector) DNA and human DNA.
2. Insert human DNA into plasmids.
3. Mix the DNAs; they join by base pairing. (Some plasmids, like this one, join with the gene of interest.)
4. Add DNA ligase to bond covalently.
5. Put plasmids into E. coli cells by transformation.

Cells are spun down and broth is discarded.

Proteins are purified from cells.

bacteria cells lacking the characteristic of interest

Cold calcium chloride is added to cells to make their membranes porous.

Transformed cells are grown on agar that allows expression of new genes and protein production and then in large volumes of broth

Recombinant plasmid is added and some plasmids enter cells.

Plasmid genes are expressed. New proteins are made.

A heat shock enlarges pores and “sucks” more plasmids into cells.
Cloning a human gene in a bacterial plasmid: a closer look (Layer 3)

1. Isolate plasmid (vector) DNA and human DNA.
   - Human DNA containing gene of interest

2. Insert human DNA into plasmids:
   - Cut both DNAs with same restriction enzyme.
   - Mix the DNAs; they join by base pairing. (Some plasmids, like this one, join with the gene of interest.)
   - Add DNA ligase to bond covalently.

3. Put plasmids into \( \text{lacZ}^- \) bacteria by transformation.

4. Clone cells:
   - Plate cells onto medium with ampicillin and X-gal.

5. Identify clones of cells containing recombinant plasmids by their ability to grow in presence of ampicillin and their white color.

6. Identify clone carrying gene of interest.

Copyright © Pearson Education, Inc., publishing as Benjamin Cummings.
3.1 Introduction to Recombinant DNA Technology and DNA Cloning

- Selection of Recombinant Bacteria
  - **Selection** is a process designed to facilitate the identification of recombinant bacteria while preventing the growth of non-transformed bacteria
    - Antibiotic selection- Ampicillin on agar plate
    - Blue-white selection- DNA is cloned into a restriction site in the lacZ gene. It encodes β-gal enzyme that breaks down lactose. When interrupted
The molecular mechanism for blue white screening is based on the Lac operon. The vector (e.g. pUC19) contains the Lac Z gene with an internal Multiple cloning site (MCS). The MCS can be cleaved by different restriction enzymes so that the foreign DNA can be inserted within Lac Z gene, thus disrupting the activity of the β-galactosidase when the protein is expressed. The chemical required for this screen is X-gal, which functions as indicator, and Isopropyl β-D-1-thiogalactopyranoside (IPTG), which functions as the inducer of the Lac operon. The hydrolysis of colourless X-gal by the β-galactosidase causes the characteristic blue color in the colonies; it shows that the colonies contain unligated vector. White colonies indicate insertion of foreign DNA and loss of the cells' ability to hydrolyse the marker.
Electroporation is done with electroporators, appliances which create the electric current and send it through the cell solution (typically bacteria, but other cell types are sometimes used). The solution is pipetted into a glass or plastic cuvette which has two aluminum electrodes on its sides.
For bacterial electroporation, a suspension of around 50 microliters is usually used. Prior to electroporation it is mixed with the plasmid to be transformed. The mixture is pipetted into the cuvette, the voltage is set on the electroporator (240 volts is often used) and the cuvette is inserted into the electroporator. Immediately after electroporation 1 milliliter of liquid medium is added to the bacteria (in the cuvette or in an eppendorf tube), and the tube is incubated at the bacteria's optimal temperature for an hour or more and then it is spread on an agar plate.

The success of the electroporation depends greatly on the purity of the plasmid solution, especially on its salt content. Impure solutions might cause a small explosion (known as arcing), in which case the bacteria are dead and the process needs to be redone. If this happens often, an additional precipitation may be performed prior to electroporation.
3.2 What Makes a Good Vector?

- Practical Features of DNA Cloning Vectors
  - Size
  - Origin of replication (ori)
  - Multiple cloning site (MCS)
  - Selectable marker genes
  - RNA polymerase promoter sequences
  - DNA sequencing primers
3.2 What Makes a Good Vector?

• Types of Vectors
  – Bacterial plasmid vectors
  – Bacteriophage vectors
  – Cosmid vectors
  – Expression vectors
  – Bacterial Artificial Chromosomes (BAC)
  – Yeast Artificial Chromosomes (YAC)
  – Ti vectors
Plasmids

• Conjugative plasmid: Carries genes for sex pili and transfer of the plasmid
• Dissimilation plasmids: Encode enzymes for catabolism of unusual compounds
• R factors: Encode antibiotic resistance
Plasmid Vector
DsRed Antibodies:
Living Colors® DsRed Antibodies recognize wild-type *Discosoma* sp. red fluorescent protein (DsRed) and most variants, including DsRed1, DsRed2, DsRed1-E5 (Fluorescent Timer), and DsRed-Express. The DsRed Polyclonal antibody is used for detection of the DsRed-Monomer. Both C-terminal and N-terminal fusions to the red fluorescent protein can be detected. On Western blots, these antibodies exhibit no cross-reactivity with A.v. GFP or its variants or with HcRed, the far-red fluorescent protein from *Heteractis crispa*. The DsRed Polyclonal Antibody can be used at high dilutions (1:1,000) in Western blots.
Plasmid Vector

Restriction Map and Multiple Cloning Site (MCS) of pDsRed2. Unique restriction sites are in bold.
3.3 How Do You Identify and Clone a Gene of Interest?

- Creating DNA Libraries
  - Collections of cloned DNA fragments from a particular organism contained within bacteria or viruses as the host
  - Screened to pick out different genes of interest

- Two Types of Libraries
  - Genomic DNA libraries
  - Complementary DNA libraries (cDNA libraries)
3.3 How Do You Identify and Clone a Gene of Interest?

- Genomic Libraries
  - Chromosomal DNA from the tissue of interest is isolated and digested with restriction enzyme
  - Vector is digested with same enzyme and DNA ligase is used to ligate genomic DNA fragments and vector DNA
  - Recombinant vectors are used to transform bacteria
  - Disadvantages
    - Non-protein coding pieces of DNA (introns) are cloned in addition to exons; majority of genomic DNA is introns in eukaryotes so majority of the library will contain non-coding pieces of DNA
    - Many organisms have very large genome, so searching for gene of interest is difficult at best
3.3 How Do You Identify and Clone a Gene of Interest?

- **cDNA Libraries**
  - mRNA from tissue of interest is isolated
  - Converted to a double-stranded DNA by using the enzyme **reverse transcriptase**
    - Called complementary DNA (cDNA) because it is an exact copy of the mRNA
  - mRNA is degraded
  - DNA polymerase used to create the second strand of DNA
  - Short linker sequences are added to the end of the cDNA
    - Contain restriction enzyme recognition sites
  - Cut with restriction enzyme, cut vector with same enzyme, ligate fragments to create recombinant vectors
  - Vectors used to transform bacteria
3.3 How Do You Identify and Clone a Gene of Interest?

• cDNA Libraries
  – Advantage
    • Collection of actively expressed genes in the cells or tissues from which the mRNA was isolated
    • Introns are NOT cloned
    • Can be created and screened to isolate genes that are primarily expressed only under certain conditions in a tissue
  – Disadvantage
    • Can be difficult to make the cDNA library if a source tissue with an abundant amount of mRNA for the gene is not available
(a) Human DNA

Millions of genomic DNA fragments

Recombinant DNA molecules

DNA fragments inserted into plasmids by DNA ligase

Genomic library containing all restriction fragments of human DNA

(b) mRNA

A primer is annealed to the poly (A) tail, which provides a free 3' end that can be used for extension by reverse transcriptase.

Reverse transcriptase

The mRNA is digested away with NaOH or RNase, an enzyme that degrades RNA.

A second strand is synthesized by DNA polymerase using random short sequences as primers.

Linker

Double-stranded DNA

DNA polymerase

Linkers containing a restriction site are added. cDNA and plasmid vectors with EcoRI are digested and then ligated with DNA ligase.

Recombinant DNA molecules

Plasmids are introduced into bacteria
Making complementary DNA (cDNA) for a eukaryotic gene

1. Transcription
2. RNA splicing (removes introns)
3. Isolation of mRNA from cell and addition of reverse transcriptase; synthesis of DNA strand
4. Degradation of RNA
5. Synthesis of second DNA strand

Cell Nucleus:
- DNA of eukaryotic gene:
  - Exon
  - Intron
  - Exon
  - Intron
  - Exon

RNA primary transcript:
- Exon
- Intron
- Exon

mRNA:
- Exon
- Intron
- Exon

Test Tube:
- Reverse transcriptase
- cDNA strand:
  - cDNA of gene (no introns)

CDNA libraries
Genomic libraries

(c) YAC library

Cloning into a yeast Artificial Chromosome (YAC) – (75-260 kb DNA inserts).

Why is it so important to be able to clone such large sequences? ... In principle, the human genome could be represented in about 10,000 YAC clones.
3.3 How Do You Identify and Clone a Gene of Interest?

- Libraries are “screened” to identify the gene of interest
- Colony hybridization
  - Bacterial colonies containing recombinant DNA are grown on an agar plate
  - Nylon or nitrocellulose filter is placed over the plate and some of the bacterial colonies stick to the filter at the exact location they were on the plate
  - Treat filter with alkaline solution to lyse the cells and denature the DNA
  - Denatured DNA binds to filter as single-stranded DNA
  - Filter is incubated with a probe
    - DNA fragment that is complementary to the gene of interest
  - Probe binds by hydrogen bonding to complementary sequences on the filter
3.3 How Do You Identify and Clone a Gene of Interest?

• Colony Hybridization
  – Filter is washed to remove excess unbound probe
  – Filter is exposed to film – autoradiography
    • Anywhere probe has bound, light will be emitted and expose silver grains in the film
    • Film is developed to create a permanent record of the colony hybridization
  – Film is then compared to the original agar plate to identify which colonies contained recombinant plasmid with the gene of interest
1) Transfer cells to filter.

2) Treat filter with detergent and NaOH to lyse bacteria and denature DNA.

3) Fix DNA to filter by baking or exposing to UV light.

4) Add radioactivity labeled probe to filter.

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

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

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

8) Cells containing gene of interest can be grown in liquid culture and processed to isolate recombinant plasmid DNA.
3.3 How Do You Identify and Clone a Gene of Interest?

- Colony Hybridization
  - Type of probe used depends on what is already known about the gene of interest

- Rarely results in the cloning of the full-length gene
  - Usually get small pieces of the gene; the pieces are sequenced and scientists look for overlapping sequences
  - Look for start and stop codons to know when the full length of the gene is obtained
Using a nucleic acid probe to identify a cloned gene

1. Transfer cells to filter
2. Treat cells on filter to denature DNA
3. Add probe to filter
4. Autoradiography
5. Compare autoradiograph with master plate

Bacterial colonies containing cloned segments of foreign DNA

Radioactive DNA

Solution containing probe

Probe DNA

Gene of interest

Single-stranded DNA from cell

Hybridization on filter

Master plate

Colonies containing gene of interest

Developed film
3.3 How Do You Identify and Clone a Gene of Interest?

- **Polymerase Chain Reaction**
  - Developed in the 1980s by Kary Mullis
  - Technique for making copies, or amplifying, a specific sequence of DNA in a short period of time
  - Process
    - Target DNA to be amplified is added to a tube, mixed with nucleotides (dATP, dCTP, dGTP, dTTP), buffer, and DNA polymerase.
    - Primers are added – short single-stranded DNA oligonucleotides (20–30bp long)
    - Reaction tube is placed in an instrument called a thermocycler
3.3 How Do You Identify and Clone a Gene of Interest?

• Process
  – Thermocycler will take DNA through a series of reactions called a **PCR cycle**
  – Each cycle consists of three stages
    • Denaturation
    • Annealing (hybridization)
    • Extension (elongation)
  – At the end of one cycle, the amount of DNA has doubled
  – Cycles are repeated 20–30 times
3.3 How Do You Identify and Clone a Gene of Interest?

**Starting Materials**
- DNA polymerase
- Primers:

**Denaturation Stage**
1) Heat to denature DNA

**Hybridization/Annealing Stage**
2) Cool to allow primers to bind (hybridize)

**Extension Stage**
3) DNA polymerase extends the 3’ end of each primer

**Target DNA**
5’ 3’

**Nucleotides:**
- dATP
- dCTP
- dGTP
- dTTP

**Cycle 1**
- Yields 2 molecules

**Cycle 2**
- Yields 4 molecules

**Cycle 3**
- Yields 8 molecules

Copyright © 2009 Pearson Education, Inc.
3.3 How Do You Identify and Clone a Gene of Interest?

- The type of DNA polymerase used is very important
  - *Taq* DNA polymerase – isolated from a species known as *Thermus aquaticus* that thrives in hot springs
- Advantage of PCR
  - Ability to amplify millions of copies of target DNA from a very small amount of starting material in a short period of time
- Applications
  - Making DNA probes
  - Studying gene expression
  - Detection of viral and bacterial infections
  - Diagnosis of genetic conditions
  - Detection of trace amounts of DNA from tissue found at crime scene
3.3 How Do You Identify and Clone a Gene of Interest?

- Cloning PCR Products
  - Is rapid and effective
  - Disadvantage
    - Need to know something about the DNA sequence that flanks the gene of interest to design primers
    - Includes restriction enzyme recognition sequences in the primers
  - Uses T vector
    - *Taq* polymerase puts a single adenine nucleotide on the 3’ end of all PCR products
The polymerase chain reaction (PCR)

1983
Kary Mullis, a biochemist invented the polymerase in reaction or polymerase chain reaction which is a technique enabling scientists to reproduce bits of DNA faster than ever before. (Mullis was awarded the Nobel Prize for this in 1993)
The polymerase chain reaction (PCR)

Step 1: 30 sec  95°C
Step 2: 30 sec  45-55°C
Step 3: 1-2 min 72°C
The polymerase chain reaction (PCR)

Primers

Direct primer for *red fluorescent* protein gene:
5’- CTC TAG AGG ATC CCC GGG TAC – 3’

Reverse primer for *red fluorescent* protein gene:
5’ – CGG CGC TCG AGT TGG AAT TCT AGA GTC GCG – 3’
Plasmid Vector

Restriction Map and Multiple Cloning Site (MCS) of pDsRed2. Unique restriction sites are in bold.
Cloning a GFP gene in a bacterial plasmid

1. Isolate plasmid (vector) DNA and human DNA.
2. Cut both DNAs with the same restriction enzyme.
3. Mix the DNAs; they join by base pairing. (Some plasmids, like this one, join with the gene of interest.)
4. Add DNA ligase to bond covalently.
5. Insert human DNA into plasmids.
6. Mix plasmids into E. coli bacteria by transformation.
7. Clone cells:
8. Clone cells carrying many copies of human gene of interest in the bacterial clone.
9. Plate cells onto medium with ampicillin and X-gal.
10. Identify clones of cells containing recombinant plasmids by their ability to grow in presence of ampicillin and their white color.
11. Identify clone carrying gene of interest.

Restriction enzymes used:
- SpeI-Partial Filling-NcoI
- HindIII-Partial Filling-NcoI
Plasmid Vector

HindIII-Partial Filling-NcoI

CHECK: XhoI+PstI

SpeI-Partial Filling-NcoI

2 NcoI sites
2 PstI sites
No XhoI site

1 NcoI site
1 PstI site
1 XhoI site
Express protein and study protein structure and function in vivo; isolate and purify protein to study protein structure and function in vitro.

Use purified protein to make antibodies for medical purpose and/or make vaccines for the treatment of disease.

Find chromosomal location of cloned gene, determine gene copy number, and study gene structure.

Mutate gene and study function of altered protein produced.

Create transgenic animals and gene knock-out animals to study gene function.

Scale-up production, isolation, and purification of therapeutic proteins (i.e., insulin, human growth hormone, and clot-dissolving proteins used to treat heart attacks) for use in humans as recombinant DNA products.

Recombinant plasmids with cloned gene of interest.

Create new, genetically engineered microorganisms, animals, and plants with a range of applications from waste-degrading microorganisms to disease-resistant plants and animals.

Gene used to alter bacteria for cleaning up toxic waste.

Use in human gene therapy.

Use in forensic applications such as DNA fingerprinting.

Diagnose human genetic disorders and infectious disease conditions.
3.4 What Can You Do with a Cloned Gene?
Applications of Recombinant DNA Technology

- Gel Electrophoresis and Gene Mapping
  - Map of the gene
  - Determine which restriction enzymes cut the gene and pinpoint the exact location of the sites
    - Restriction map
3.4 What Can You Do with a Cloned Gene?  
Applications of Recombinant DNA Technology

• Agarose Gel Electrophoresis
  – Agarose melted in a buffer and poured into a horizontal tray
  – When solidified, the gel contains small holes or pores through which the DNA fragments will travel
  – The percentage of agarose used to make the gel determines the ability of the gel to separate DNA fragments of different sizes
    • Most common is 0.5–2% agarose
    • Higher percentage will separate small DNA fragments better
    • Lower percentage is better for separating large fragments
3.4 What Can You Do with a Cloned Gene?
Applications of Recombinant DNA Technology

- Agarose Gel Electrophoresis
  - To run a gel, it is submerged in a buffer solution that conducts electricity
  - DNA is loaded into wells at the top of the gel
  - Electric current is applied to the ends of the gel
    - DNA migrates according to its charge and size
    - Rate of migration through the gel depends on the size of the DNA
      - Large fragments migrate slowly; smaller fragments migrate faster
  - Tracking dye is added to the samples to monitor DNA migration during electrophoresis
  - DNA can be visualized after electrophoresis by the addition of DNA staining dyes
3.4 What Can You Do with a Cloned Gene?  
Applications of Recombinant DNA Technology

(a) Mixture of DNA fragments of different sizes

Cathode → Wells

Power source

Anode

Mixture of DNA fragments

Gel

Stain gel with DNA-binding dye (ethidium bromide)

Visualize bands by fluorescence under UV light

(b) Longer fragments

Shorter fragments

Completed gel

Marker DNA

λ DNA Uncut

λ DNA + Hind III

E. coli DNA Uncut

E. coli DNA + Hind III

Marker DNA

8000 bp

4000 bp

2000 bp

1000 bp

500 bp
Gel electrophoresis of macromolecules

Agarose Gel Tray. Gel trays differ depending on the manufacturer. Each has some method of sealing the ends so that liquid agarose can mold into a gel. Some gel trays, such as those made by Owl Separation Systems, make a seal with the box, so casting a gel is simple. Other trays require masking tape on the ends to make a mold. Still others, like the one shown here, have gates that screw into position: up for pouring the gel and down for running the gel.

Molecules in a Gel Box. If negatively charged molecules are loaded into the wells and run on the gel, the smaller ones run faster and farther than the larger ones toward the positive electrode. This is because smaller molecules pass more easily through the tiny spaces of the gel network.
Gel electrophoresis of macromolecules
Plasmid DNA Isolation

**Preparation** – the process of extracting plasmid DNA from cells

**Miniprep** – a small DNA preparation yielding approximately 40 µg/mL of plasmid DNA

**Midiprep** – a DNA preparation yielding approximately 800 µg/mL of plasmid DNA

**Maxiprep** – a DNA preparation yielding approximately 1 mg/mL or more of plasmid DNA
**Restriction Map** - Physical map of the gene is created to determine which restriction enzymes cut the cloned gene.

**Procedure:** Cut with different restriction enzymes and characterize with gel electrophoresis.
Uncut DNA: Lane 1: 7 kb
BamHI, Lane 2: 4.5 kb, 2.5 kb
PstI, Lane 3: 5.5 kb, 1.5 kb
Bam HI + PstI, Lane 4: 3.0 kb, 2.5 kb, 1.5 kb

DNA pieces are negatively charged and separate by size with the smallest pieces running the fastest.
Using restriction fragment patterns to distinguish DNA from different alleles

(a) DNA from two alleles

(b) Electrophoresis of restriction fragments

(c) Completed gel

Copyright © Pearson Education, Inc., publishing as Benjamin Cummings.
Biotechnology

RFLP markers close to a gene

DNA

RFLP marker

Restriction sites

Disease-causing allele

Normal allele
Biotechnology

DNA fingerprints from a murder case

Defendant’s blood

Blood from defendant’s clothes

Victim’s blood
3.4 What Can You Do with a Cloned Gene? Applications of Recombinant DNA Technology

- DNA Sequencing
  - Important to determine the sequence of nucleotides of the cloned gene
  - Chain termination sequencing (Sanger method)
  - Computer automated sequencing
    - ddNTP’s are each labeled with a different fluorescent dye
    - Samples are separated on a single-lane capillary gel that is scanned with a laser beam
    - Creates different color patterns for each nucleotide
    - Converted by computer to the sequence
1977
Walter Gilbert and Frederick Sanger worked out methods to determine the sequence of bases in DNA. The same year Frederick Sanger determined the base sequence of an entire viral genome (f X174).

1. **Maxam-Gilbert** sequencing (chemical cleavage method using double-stranded (ds) DNA).
2. **Sanger-Coulson** sequencing (chain termination method using single-stranded (ss) DNA).

Autoradiogram of part of a sequencing electrophoresis gel showing a lane for each of the four DNA bases A, C, G & T.
Sequencing of DNA by the Sanger method

1. Single-stranded DNA with unknown sequence (blue) serves as a template
2. DNA polymerase
3. dATP, dCTP, dTTP, and dGTP
4. Radioactively labeled primer
Biotechnology

Sequencing of DNA by the Sanger method (Layer 2)

1. Single-stranded DNA with unknown sequence (blue) serves as a template
   + DNA polymerase
   + dATP, dCTP, dTTP, and dGTP
   + Radioactively labeled primer

2. Prepare four reaction mixtures
   + dATP
   + ddCTP
   + dTTP
   + ddGTP

Copyright © 2009 Pearson Education, Inc., publishing as Benjamin Cummings.
Sequencing of DNA by the Sanger method (Layer 3)

1. Single-stranded DNA with unknown sequence (blue) serves as a template.
   - DNA polymerase
   - dATP, dCTP, dTTP, and dGTP
   - Radioactively labeled primer

2. Prepare four reaction mixtures:
   - ddATP
   - ddCTP
   - ddTTP
   - ddGTP

3. DNA synthesis and Gel electrophoresis followed by autoradiography:
   - Longer fragments
   - Reaction products
   - Shorter fragments

Copyright © 2009 Pearson Education, Inc.
Biotechnology

Sequencing of DNA by the Sanger method (Layer 4)

1. Single-stranded DNA with unknown sequence (blue) serves as a template
   + DNA polymerase
   + dATP, dCTP, dTTP, and dGTP
   + Radioactively labeled primer

2. Prepare four reaction mixtures
   + ddATP
   + ddCTP
   + ddTTP
   + ddGTP

3. DNA synthesis followed by gel electrophoresis

4. Longer fragments
   ddATP
   ddCTP
   ddTTP
   ddGTP

   Read sequence of new strand

   and deduce sequence of template

   Reaction products
   ddG
   ddA
   ddT
   ddC

   Shorter fragments
Biotechnology

Sequencing of DNA by the Sanger method (Layer 4)
Biotechnology

Sequencing of DNA by the Sanger method (Layer 4)
Sequencing of DNA by the Sanger method (Layer 4)
The Human Genome Project

Original idea was:

- Determining the human DNA sequence
- Understanding the function of the human genetic code
- Identifying all of the genes
- Determining their functions
- Understanding how and when genes are turned on and off throughout the lifetime of an individual
# The Human Genome Project

## Genome Sizes and Numbers of Genes

<table>
<thead>
<tr>
<th>Organism</th>
<th>Genome Size</th>
<th>Estimated Number of Genes</th>
<th>Genes per Mb*</th>
</tr>
</thead>
<tbody>
<tr>
<td>H. influenzae (bacterium)</td>
<td>1.8 Mb*</td>
<td>1,700</td>
<td>950</td>
</tr>
<tr>
<td>S. cerevisiae (yeast)</td>
<td>12 Mb</td>
<td>6,000</td>
<td>500</td>
</tr>
<tr>
<td>C. elegans (nematode)</td>
<td>97 Mb</td>
<td>19,000</td>
<td>200</td>
</tr>
<tr>
<td>A. thaliana (plant)</td>
<td>100 Mb</td>
<td>25,000</td>
<td>200</td>
</tr>
<tr>
<td>D. melanogaster (fruit fly)</td>
<td>180 Mb</td>
<td>13,000</td>
<td>100</td>
</tr>
<tr>
<td>H. sapiens (human)</td>
<td>3,200 Mb</td>
<td>30,000–40,000</td>
<td>10</td>
</tr>
</tbody>
</table>

*Mb = million base pairs

Productivity of new generation of sequencing machines - 20Mb per 4 hour run
Completed in 2003, the Human Genome Project (HGP) was a 13-year project coordinated by the U.S. Department of Energy and the National Institutes of Health. During the early years of the HGP, the Wellcome Trust (U.K.) became a major partner; additional contributions came from Japan, France, Germany, China, and others.
Project goals were to:

*Identify* all the approximately 20,000-25,000 genes in human DNA,

*Determine* the sequences of the 3 billion chemical base pairs that make up human DNA,

*Improve* tools for data analysis,

*Transfer* related technologies to the private sector, and

*Address* the ethical, legal, and social issues (ELSI) that may arise from the project.
The Human Genome Project

Though the HGP is finished, analyses of the data will continue for many years.

An important feature of the HGP project was the federal government's long-standing dedication to the transfer of technology to the private sector.

By licensing technologies to private companies and awarding grants for innovative research, the project catalyzed the multibillion-dollar U.S. biotechnology industry and fostered the development of new medical applications.
3.4 What Can You Do with a Cloned Gene? Applications of Recombinant DNA Technology

• Chromosome Location and Copy Number
  – Identify the chromosome location of the cloned gene
  – Determine if the gene is present as a single copy in the genome
  – Fluorescence *in situ* hybridization (FISH)
    • Chromosomes are isolated from cells and spread out on glass slide
    • cDNA probe for gene of interest is labeled with fluorescent nucleotides and incubated with slides
    • Probe will hybridize with complementary sequences on slide
    • Slide is washed and exposed to fluorescent light
    • Wherever probe bound, it is illuminated to indicate the presence of that gene
3.4 What Can You Do with a Cloned Gene?
Applications of Recombinant DNA Technology

• Chromosome Location and Copy Number
  – Southern blotting (Dr. Ed Southern)
    • Digest chromosomal DNA into small fragments with restriction enzymes
    • Fragments are separated by agarose gel electrophoresis
    • Gel is treated with alkaline solution to denature the DNA
    • Fragments are transferred onto a nylon or nitrocellulose filter (called blotting)
    • Filter (blot) is incubated with a probe and exposed to film by autoradiography
    • Number of bands on film represents gene copy number
Restriction fragment analysis
by Southern blotting

1. Restriction fragment preparation
2. Electrophoresis
3. Blotting
4. Hybridization with radioactive probe
5. Autoradiography
3.4 What Can You Do with a Cloned Gene?  
Applications of Recombinant DNA Technology

• Studying Gene Expression
  – Techniques involve analyzing mRNA produced by a tissue
  – **Northern blot analysis**
    • Basic method is similar to Southern blotting
    • RNA is isolated from a tissue of interest, separated by gel electrophoresis, blotted onto a membrane, and hybridized to a probe
  – **Reverse transcription PCR**
    • Reverse transcription of mRNA is performed – converted into double-stranded cDNA
    • cDNA is then amplified with a set of primers specific for the gene of interest
    • Products electrophoresed on agarose gel
3.4 What Can You Do with a Cloned Gene?  
Applications of Recombinant DNA Technology

- Studying Gene Expression
  - *In situ* hybridization
    - Used to determine the cell type that is expressing the mRNA
    - Tissue of interest is preserved in a fixative solution and embedded in a wax-like substance
    - Tissue can be sliced into very thin sections attached to microscope slides
    - Slides are incubated with a probe to the gene of interest
    - Probe hybridizes with mRNA in cells
    - Probe is detected
3.4 What Can You Do with a Cloned Gene?
Applications of Recombinant DNA Technology

- Studying Gene Expression
  - Gene microarrays
    - DNA microarray analysis
    - Single-stranded DNA molecules are attached onto a slide using a robotic arrayer fitted with tiny pins
    - Can have over 10,000 spots of DNA
    - Extract mRNA from tissue of interest, tag it with fluorescent dye, and incubate overnight with the slide
    - mRNA will hybridize to spots on the microarray that have complimentary DNA sequences
    - Slide is scanned with a laser that causes the spots to fluoresce
1) Isolate mRNA.

2) Make cDNA by reverse transcription, using fluorescently labeled nucleotides, red in this example.

3) Hybridization: Apply the cDNA mixture to a DNA microarray, cDNA hybridizes to DNA on microarray.

4) Rinse off excess cDNA, put the microarray in a scanner to measure fluorescence of each spot. Fluorescence intensity indicates the amount of gene expressed in the tissue sample.
DNA microarray assay for gene expression

1. Isolate mRNA.

2. Make cDNA by reverse transcription, using fluorescently labeled nucleotides.

3. Hybridization: Apply the cDNA mixture to a DNA microarray.

4. Rinse off excess cDNA; scan microarray for fluorescence.

Each fluorescent spot represents a gene expressed in the tissue sample.

Size of an actual DNA microarray with all the genes of yeast (6,400 spots)

(a) Procedure using labeled cDNA prepared from a tissue sample

Copyright © Pearson Education, Inc., publishing as Benjamin Cummings.
DNA microarray assay for gene expression
Biotechnology

Alternative strategies for sequencing an entire genome

1. Genetic (linkage) mapping (distances in centimorgans)

2. Physical mapping (distances in nucleotide bases)

3. DNA sequencing

(a) Hierarchical, three-stage approach used by the public consortium

(b) Whole-genome shotgun approach used by Celera Genomics
Chromosome walking

1. Starting DNA: Known gene (3' end of gene) and DNA to be mapped.
2. Library I: DNA of cloned fragments.
3. Library II: Probes 1, 2, and 3.
Biotechnology

An overview of how bacterial plasmids are used to clone genes

1. Isolation of plasmid DNA and DNA containing gene of interest
2. Gene inserted into plasmid
3. Plasmid put into bacterial cell
4. Cells cloned with gene of interest
5. Identification of desired clone
6. Various applications

- Gene for pest resistance inserted into plants
- Gene used to alter bacteria for cleaning up toxic waste
- Protein dissolves blood clots in heart attack therapy
- Human growth hormone treats stunted growth

Basic research on gene
Basic research on protein
Biotechnology

One type of gene therapy procedure

1. Insert RNA version of normal allele into retrovirus.
2. Let retrovirus infect bone marrow cells that have been removed from the patient and cultured.
3. Viral DNA carrying the normal allele inserts into chromosome.
4. Inject engineered cells into patient.
Biotechnology

Expression and Transformation Systems

- **Transient System (Research Scale)**
  
  Genes in vector $\rightarrow$ *Agrobacterium* $\rightarrow$ Plant (Lettuce) $\rightarrow$ 3 day $\rightarrow$ Extract and purify antibody for characterization (mg level)

- **Stable Transgenic Plants (Commercial Production)**
  
  Genes in vector $\rightarrow$ *Transform Plant* $\rightarrow$ Seeds $\rightarrow$ Harvest Seed $\rightarrow$ Large scale Planting and Biomass harvest $\rightarrow$ Purify (g-kg level)

  Plant / Screen
**Biotechnology**

**Transient Expression System**

1. Whole plant
2. Dip & vacuum infiltrate with Agrobacterium
3. Extract & purify
4. 3 days incubation
Biotechnology

Using the Ti plasmid as a vector for genetic engineering in plants

1. Ti plasmid
   - T DNA
   - Site where restriction enzyme cuts

2. Recombinant Ti plasmid
   - Incubation with restriction enzyme and DNA containing the gene of interest

3. Introduction into plant cells in culture
   - Regeneration of plant
   - Inserted T DNA carrying new gene

Plant with new trait
Biotechnology

Plant Binary Vector
Biotechnology

A DNA gun

(a) 
(b) 

Copyright © Pearson Education, Inc., publishing as Benjamin Cummings.
Biotechnology
Protoplasts
Biotechnology
Injecting DNA
Biotechnology

Test-tube cloning of carrots
Stable Transgenic Lettuce

Transgenic lettuce ready for transfer in soil
Biotechnology

“Golden” rice contrasted with ordinary rice

“Golden Rice” → High Content of Vitamin A
Biotechnology

“Pharm” animals
The Food and Drug Administration Is Now Drafting Rules For Letting Genetically Altered Animals Into The Food Supply.

Bioengineers In Canada And The United States Have Created And Tested Transgenic Creatures, Including These:
Genetically modified (GM) fishes are Atlantic salmon that have been given a growth-hormone gene from the Chinook salmon. They have also been equipped with a genetic on-switch from a fish called the ocean pout, a distant cousin of the salmon. Normally, salmon produce growth hormone only in warmer months, but the pout gene’s on-switch keeps the hormone flowing year round. That enables the (GM) fish to grow faster, reaching their market weight in about 18 months instead of 30.
Biotechnology

Farming With Borrowed Genes

**Cleaner-manure pigs**
Manure from these pigs has less phosphorus, a pollutant that runs off into lakes and rivers, where it can cause algal blooms and kill fish.

**Implanted gene:** A bacterial gene for the enzyme phytase helps the pig digest more phosphorus, leaving less in the manure.

**Status:** Looking for commercial partner

**Developer:** University of Guelph, Ontario

**Omega-3 enriched pigs**
Meat from these pigs contains omega-3 fatty acids, which are normally obtained from eating fish and known to be good for the heart.

**Implanted gene:** A roundworm gene for omega-3 acids production will make the fatty acids available in the animal’s meat.

**Status:** Looking for commercial partner

**Developer:** Harvard University, University of Missouri, University of Pittsburgh

**Extra milk-producing pigs**
These pigs have increased milk production, allowing for larger litters and faster-growing piglets.

**Implanted gene:** A cow gene that increases the production of lactose, a milk component, will do the same for pigs.

**Status:** Research

**Developer:** University of Illinois, Urbana-Champaign
Biotechnology

Farming With Borrowed Genes

**Infection-resistant cows**

These cows produce a protein that can prevent hard-to-treat udder infections that are costly to the dairy industry.

**Implanted gene:**
A bacterial gene for the protein lysostaphin kills Staphylococcus aureus, a bacterium that causes udder infections.

**Status:** Research

**Developer:** U.S. Department of Agriculture, Beltsville, Md.

**Antibacterial goat milk**

Milk from these goats may help prevent infections in young children who drink it, particularly in developing countries.

**Implanted gene:**
A human gene for lysozyme allows for production of this antimicrobial protein, found in human tears and breast milk.

**Status:** Research

**Developer:** University of California, Davis
Express protein and study protein structure and function \textit{in vivo}; isolate and purify protein to study protein structure and function \textit{in vitro}.

Use purified protein to make antibodies for medical purpose and/or make vaccines for the treatment of disease.

Find chromosomal location of cloned gene, determine gene copy number, and study gene structure.

Study gene structure, gene sequence, and gene expression in organs, tissues, and individual cells.

Mutate gene and study function of altered protein produced.

Create transgenic animals and gene knock-out animals to study gene function.

Create new, genetically engineered microorganisms, animals, and plants with a range of applications from waste-degrading microorganisms to disease-resistant plants and animals.

Scale-up production, isolation, and purification of therapeutic proteins (i.e., insulin, human growth hormone, and clot-dissolving proteins used to treat heart attacks) for use in humans as recombinant DNA products.

Gene used to alter bacteria for cleaning up toxic waste.

Use in forensic applications such as DNA fingerprinting.

Gene for pest resistance inserted into plants.

Use in human gene therapy.

Diagnose human genetic disorders and infectious disease conditions.

Copies of protein product isolated.
<table>
<thead>
<tr>
<th>Organism (scientific name)</th>
<th>Approximate Size of Genome (date completed)</th>
<th>Number of Genes</th>
<th>Approximate Percentage of Genes Shared with Humans</th>
<th>Web Access to Genome Databases</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacterium (Escherichia coli)</td>
<td>4.1 million bp (1997)</td>
<td>4,403</td>
<td>Not determined</td>
<td><a href="http://www.genome.wisc.edu/">www.genome.wisc.edu/</a></td>
</tr>
<tr>
<td>Chicken (Gallus gallus)</td>
<td>1 billion bp (2004)</td>
<td>~20,000–23,000</td>
<td>60%</td>
<td><a href="http://genomeold.wustl.edu/projects/chicken">http://genomeold.wustl.edu/projects/chicken</a></td>
</tr>
<tr>
<td>Dog (Canis familiaris)</td>
<td>6.2 million bp (2003)</td>
<td>~18,400</td>
<td>75%</td>
<td><a href="http://www.ncbi.nlm.gov/genome/guide/dog">http://www.ncbi.nlm.gov/genome/guide/dog</a></td>
</tr>
<tr>
<td>Chimpanzee (Pan troglodytes)</td>
<td>~3 billion bp (initial draft, 2005)</td>
<td>~20,000–24,000</td>
<td>96%</td>
<td><a href="http://www.nature.com/nature/focus/chimpgenome/index.html">http://www.nature.com/nature/focus/chimpgenome/index.html</a></td>
</tr>
<tr>
<td>Fruit fly (Drosophila melanogaster)</td>
<td>165 billion bp (2000)</td>
<td>~13,600</td>
<td>50%</td>
<td><a href="http://www.fruitfly.org">www.fruitfly.org</a></td>
</tr>
<tr>
<td>Humans (Homo sapiens)</td>
<td>~2.9 billion bp (2004)</td>
<td>~20,000–25,000</td>
<td>100%</td>
<td><a href="http://www.doegenomes.org">www.doegenomes.org</a></td>
</tr>
<tr>
<td>Mouse (Mus musculus)</td>
<td>~2.5 billion bp (2002)</td>
<td>~30,000</td>
<td>~80%</td>
<td><a href="http://www.informatics.jax.org">www.informatics.jax.org</a></td>
</tr>
<tr>
<td>Plant (Arabidopsis thaliana)</td>
<td>119 million bp (2000)</td>
<td>~26,000</td>
<td>Not determined</td>
<td><a href="http://www.arabidopsis.org">www.arabidopsis.org</a></td>
</tr>
<tr>
<td>Rat (Rattus norvegicus)</td>
<td>~2.75 billion bp (2004)</td>
<td>~22,000</td>
<td>80%</td>
<td><a href="http://www.hgsc.bcm.tmc.edu/projects/rat">www.hgsc.bcm.tmc.edu/projects/rat</a></td>
</tr>
<tr>
<td>Roundworm (Caenorhabditis elegans)</td>
<td>97 million bp (1998)</td>
<td>19,099</td>
<td>40%</td>
<td>genomeold.wustl.edu/projects/celegans</td>
</tr>
<tr>
<td>Yeast (Saccharomyces cerevisiae)</td>
<td>12 million bp (1996)</td>
<td>~5,700</td>
<td>30%</td>
<td>genomeold.wustl.edu/projects/yeast.index.php</td>
</tr>
</tbody>
</table>