Biochem 717
Misc.

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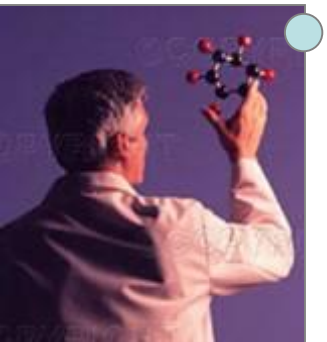
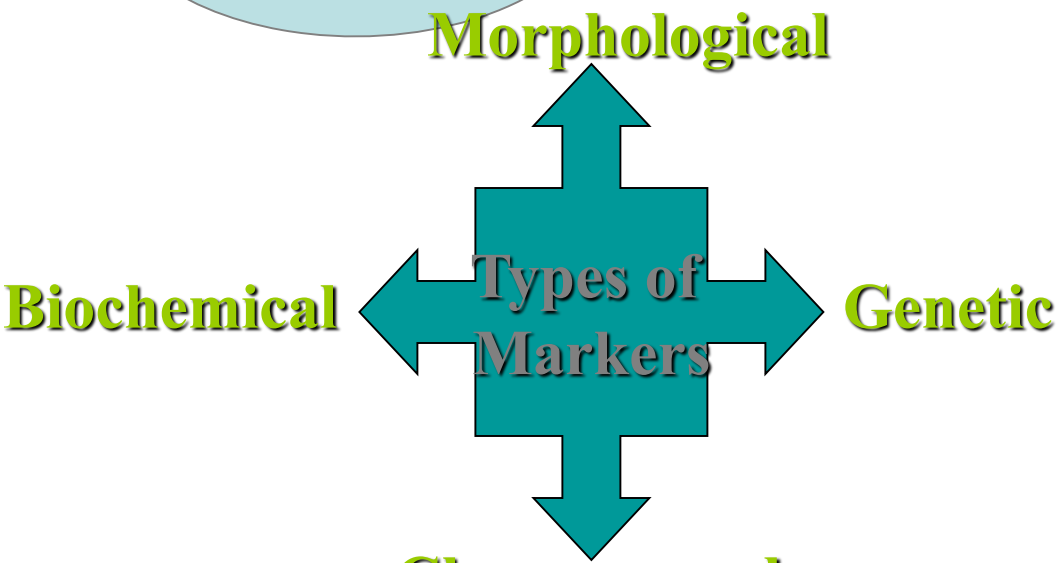
What is Marker?

Marker is a piece of DNA molecule that is associated with a certain trait of a organism





Morphological Markers

Animals are selected based on appearance

Eg. PIGMENTATION

Disadvantage: lack of polymorphism

Biochemical Markers

Animals are selected based on biochemical properties

Eg. Hb, AMYLASE, BLOOD GROUPS ETC.

Disadvantage:

Sex limited

Age dependent

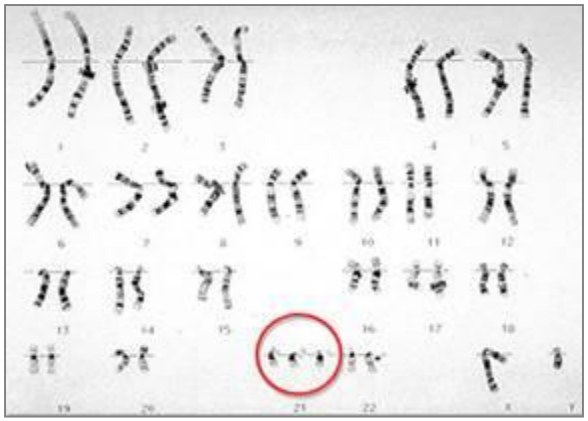
Influenced by environment

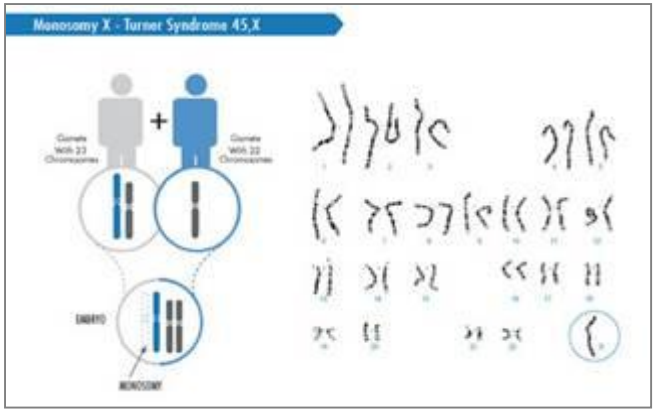
It covers less than 10% of genome

Chromosomal Markers

Animals are selected based on structural & numerical variations

Eg. Structural and Numerical Variations
Structural- Deletions, Insertions etc.
Numerical- Trisomy, Monosomy, Nullysomy

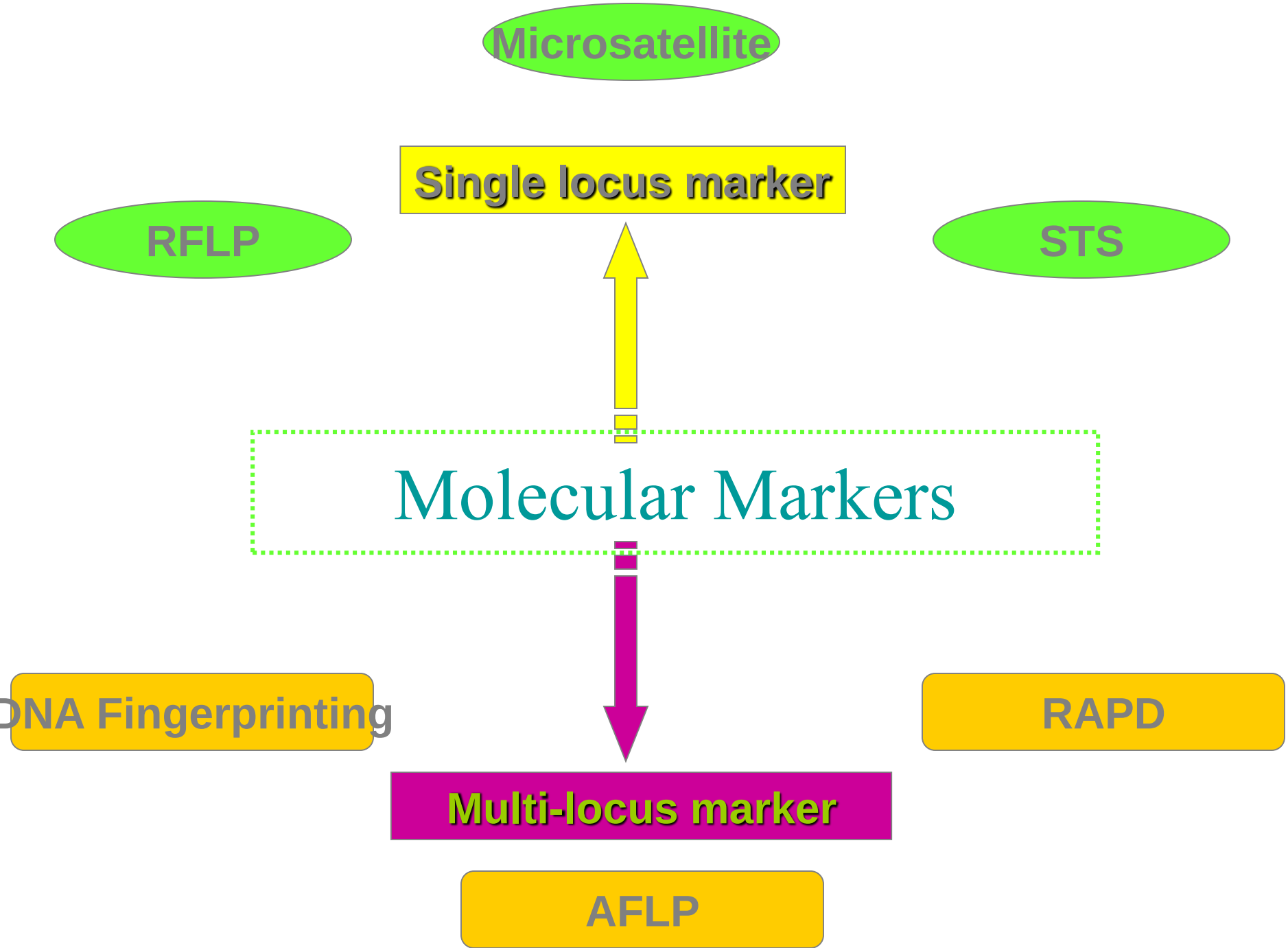




Disadvantage: low polymorphism

Molecular Marker

- Revealing variation at a DNA level
- Characteristics:
  - Co-dominant expression
  - Nondestructive assay
  - Complete penetrance
  - Early onset of phenotypic expression
  - High polymorphism
  - Random distribution throughout the genome
  - Assay can be automated


Microsatellite
Single locus marker
RFLP
STS
Molecular Markers
DNA Fingerprinting
RAPD
Multi-locus marker
AFLP

Randomly Amplified Polymorphic DNA (RAPD)

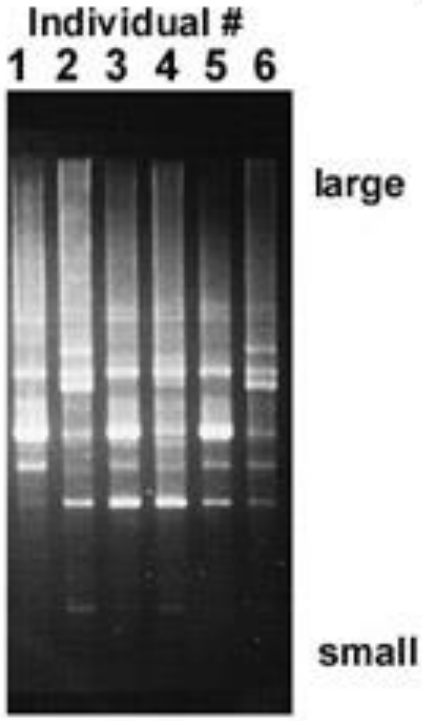
- PCR based marker with 10-12 base pairs
- Random amplification of several fragments
- Amplified fragments run in agarose gel detected by EtBr
- Unstable amplification leads to poor repeatability

RAPD (Randomly Amplified Polymorphic DNA) marker

Basic technique

Half arrows: 10-nucleotide primer that will find an identical matching site at many different locations in the whole genome (black blob). Only primers that point towards eachother AND are in close enough proximity will result in a product during PCR-amplification reactions.

PCR amplified fragment (RAPD marker linked to YFG, Your Favorite Gene)



Example of a RAPD agarose gel. A mixture of many different PCR-amplified fragments has been separated in size by electrophoresis.

DNA Fingerprinting and DNA Typing

- Southern blots are used in forensic labs to identify individuals from DNA-containing materials
- Minisatellite DNA is a sequence of bases repeated several times, also called a DNA fingerprint
  - Individuals differ in the pattern of repeats of the basic sequence
  - The difference is large enough that 2 people have only a remote chance of having exactly the same pattern of repeats

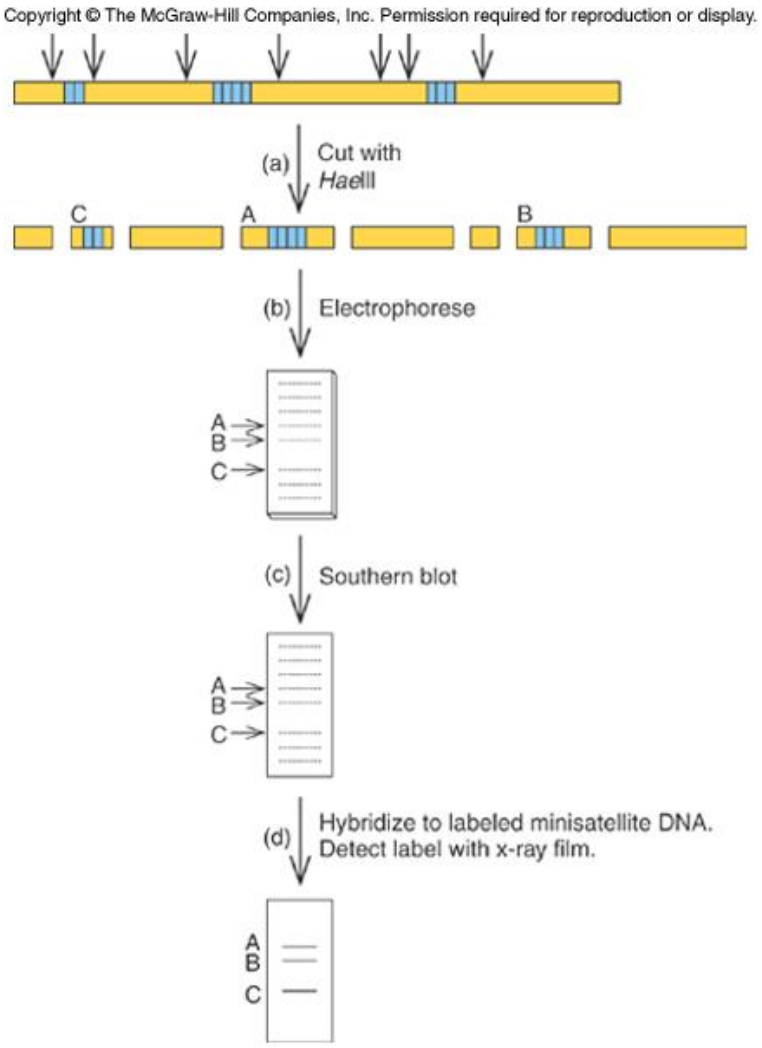
Restriction Fragment Length Polymorphism (RFLP)

- Genomic DNA digested with Restriction Enzymes
- DNA fragments separated via electrophoresis and transfer to nylon membrane
- Membranes exposed to probes labelled with P^{32} via southern hybridization
- Film exposed to X-Ray

DNA Fingerprinting

Process is a Southern blot

- Cut the DNA under study with restriction enzyme
  - Ideally cut on either side of minisatellite but not inside
- Run the digested DNA on a gel and blot
- Probe with labeled minisatellite DNA and image
  - Note that real samples result in very complex patterns

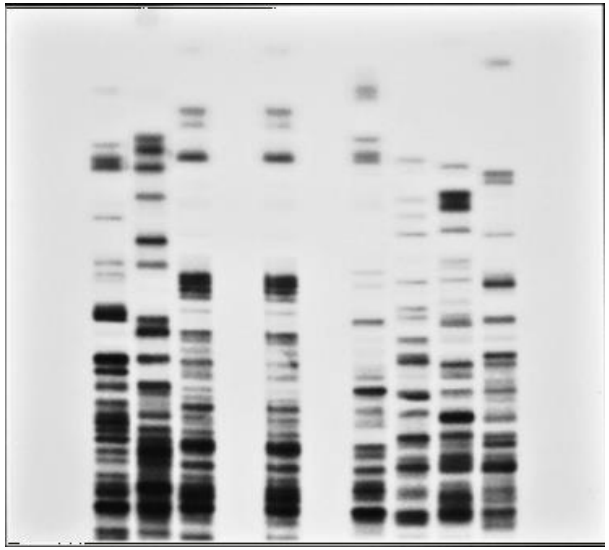


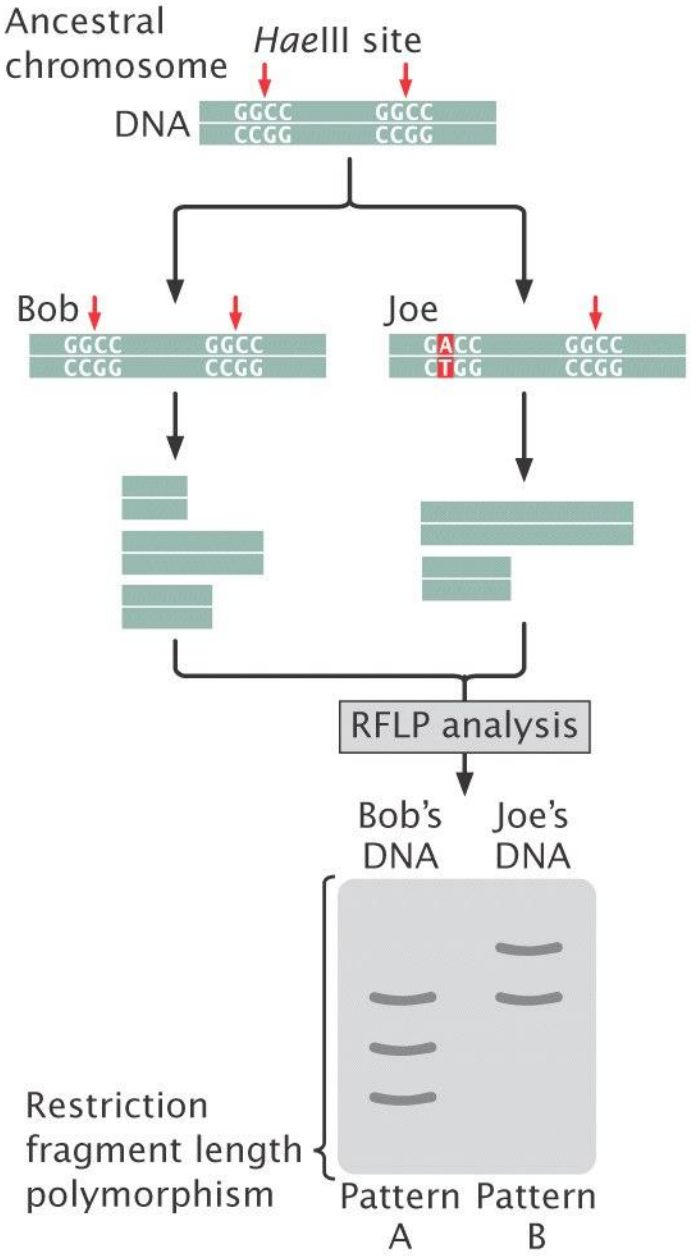
Forensic Uses of DNA Fingerprinting and DNA Typing

- While people have different DNA fingerprints, parts of the pattern are inherited in a Mendelian fashion
  - Can be used to establish parentage
  - Potential to identify criminals
  - Remove innocent people from suspicion
- Actual pattern has so many bands they can smear together indistinguishably
  - Forensics uses probes for just a single locus
  - Set of probes gives a set of simple patterns

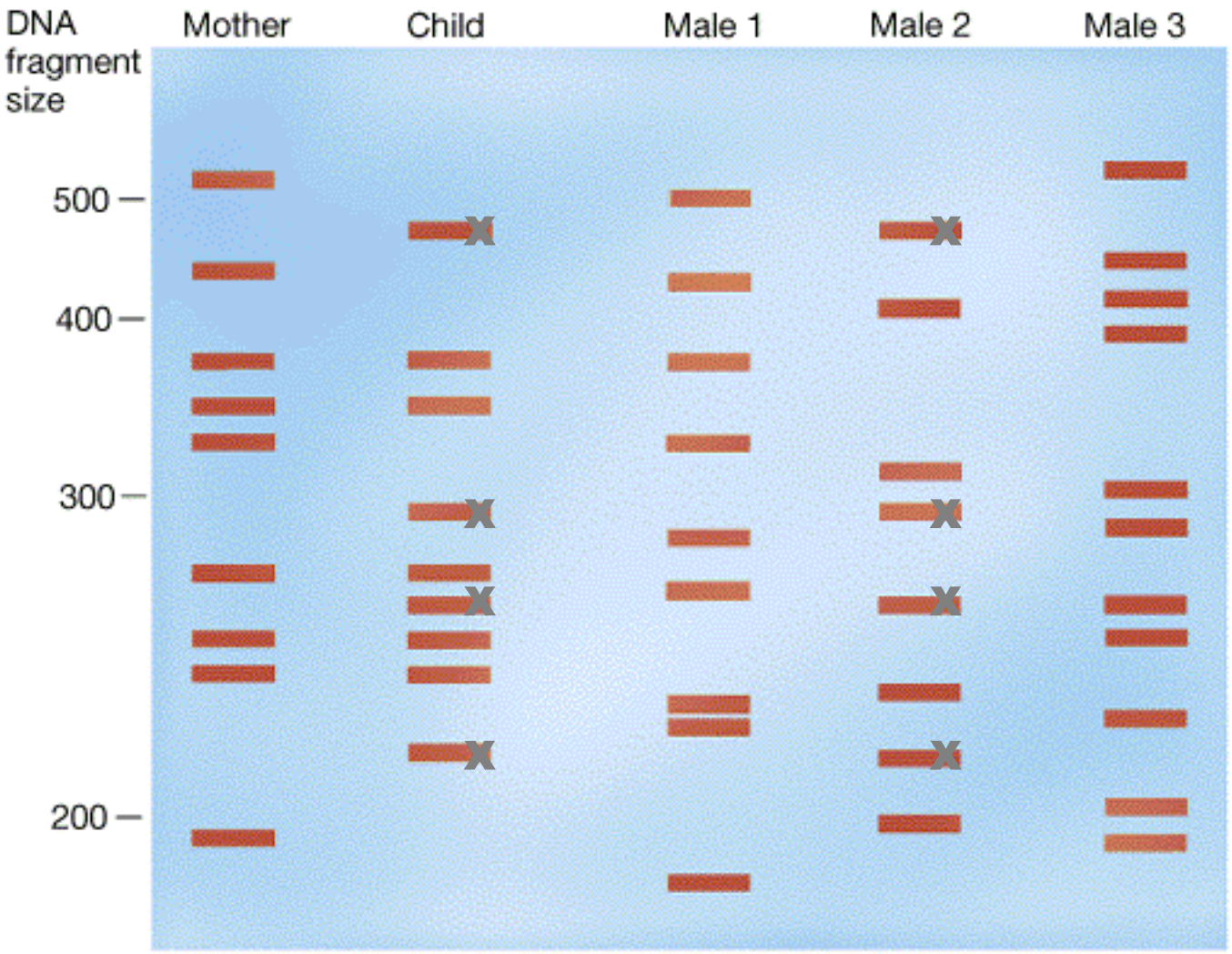
RFLP An

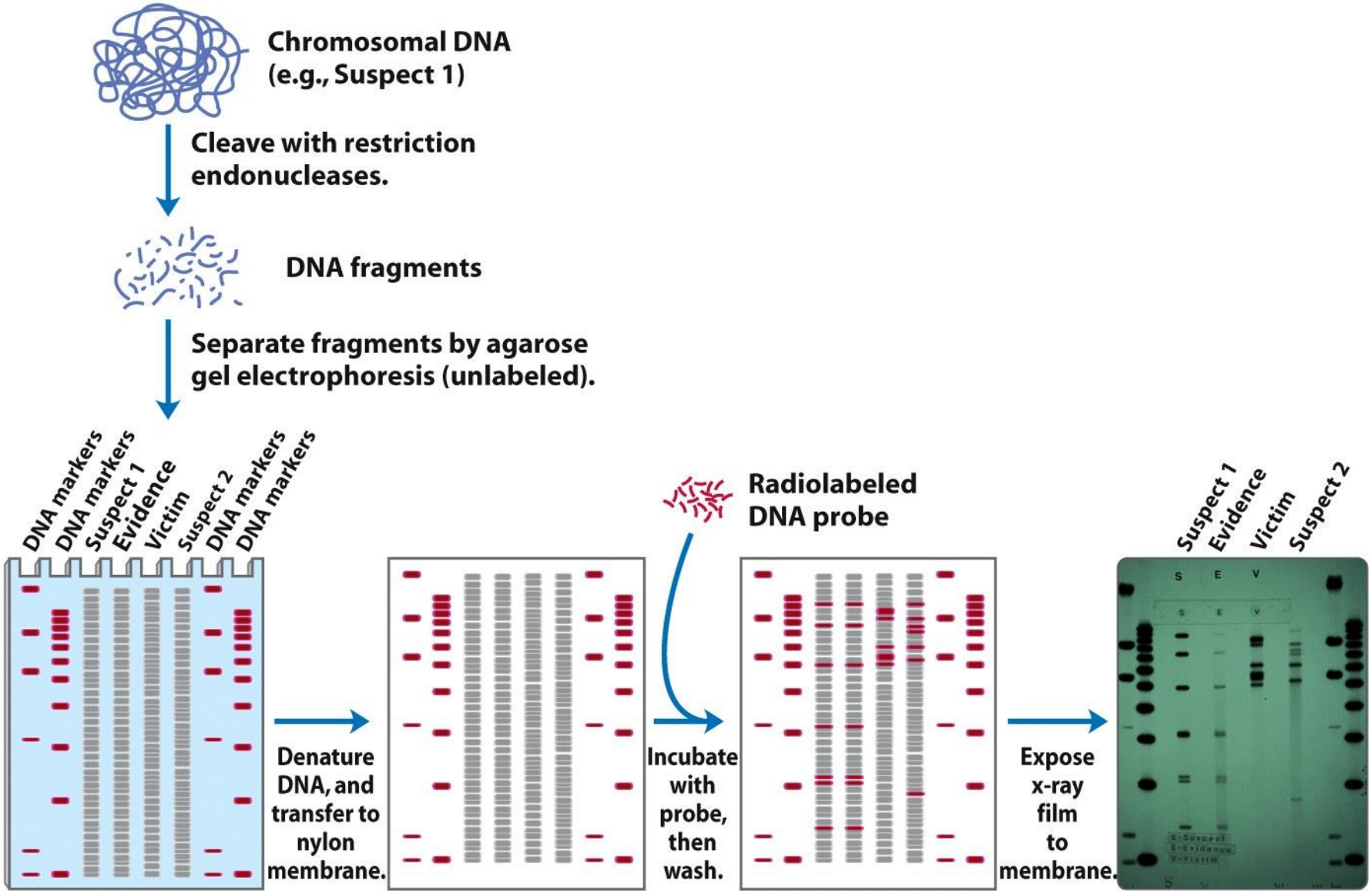
- RFLP = Restriction Fragment Length Polymorphism
- Identifies differences in the length of restriction fragments derived from similar DNA sequences
- Analyzed by Southern Blotting
- Used in gene mapping





RFLP Analysis in Paternity Testing





Box 9-1 figure 1

Lehninger Principles of Biochemistry, Fifth Edition

TABLE 1 Properties of the Loci Used for the CODIS Database

Locus	Chromosome	Repeat motif	Repeat length (range)*	Number of alleles seen†
CSF1PO	5	TAGA	5–16	20
FGA	4	CTTT	12.2–51.2	80
TH01	11	TCAT	3–14	20
TPOX	2	GAAT	4–16	15
VWA	12	[TCTG][TCTA]	10–25	28
D3S1358	3	[TCTG][TCTA]	8–21	24
D5S818	5	AGAT	7–18	15
D7S820	7	GATA	5–16	30
D8S1179	8	[TCTA][TCTG]	7–20	17
D13S317	13	TATC	5–16	17
D16S539	16	GATA	5–16	19
D18S51	18	AGAA	7–39.2	51
D21S11	21	[TCTA][TCTG]	12–41.2	82
Amelogenin	X,Y	Not applicable		

Source: Adapted from Butler, J.M. (2005) *Forensic DNA Typing,* 2nd edn, Academic Press, San Diego, p. 96.

*Repeat lengths observed in the human population. Partial or imperfect repeats can be included in some alleles.

†Number of different alleles observed to date in the human population. Careful analysis of a locus in many individuals is a prerequisite to its use in forensic DNA typing.

Box 9-1 table 1

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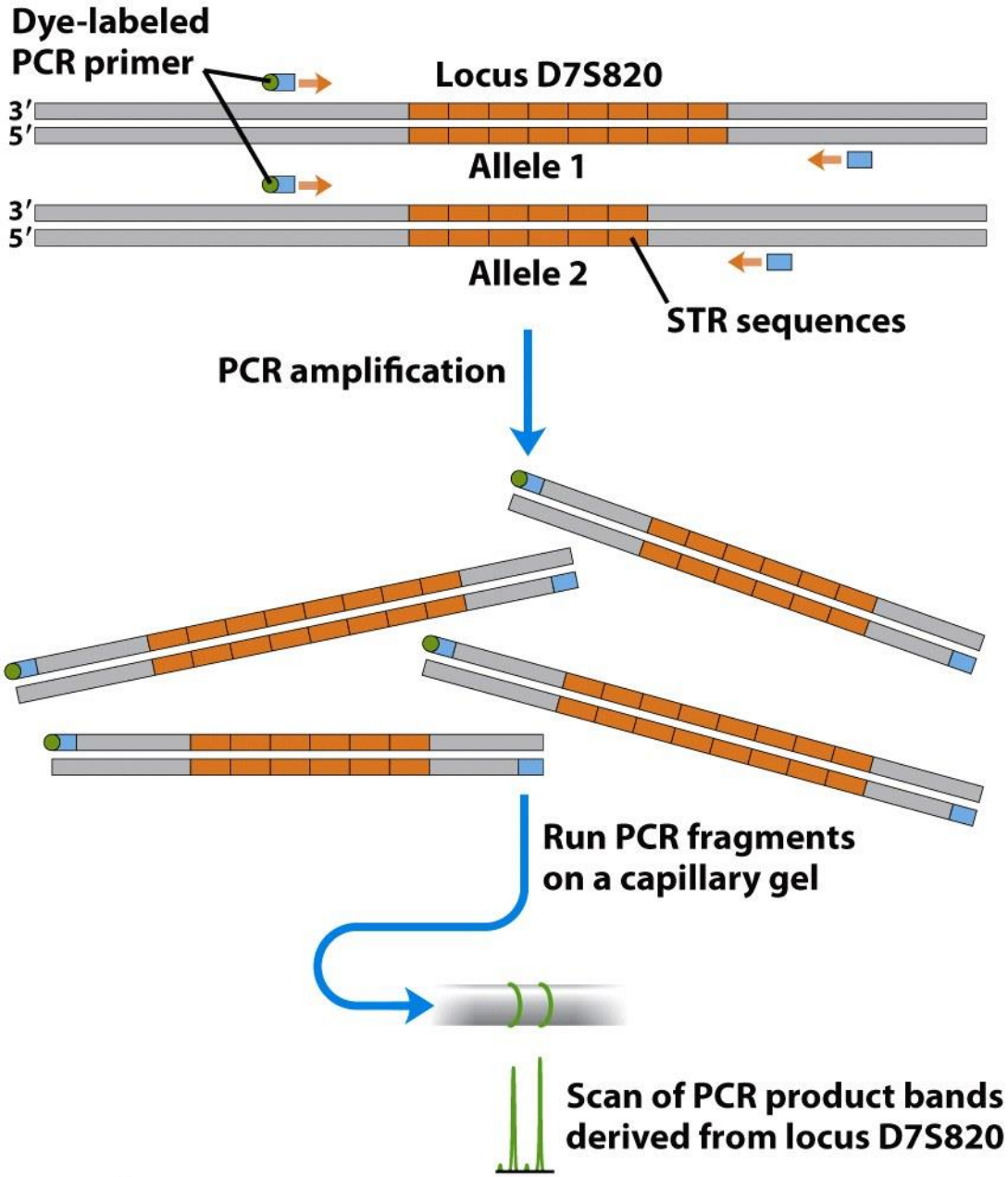

Box 9-1 figure 2a

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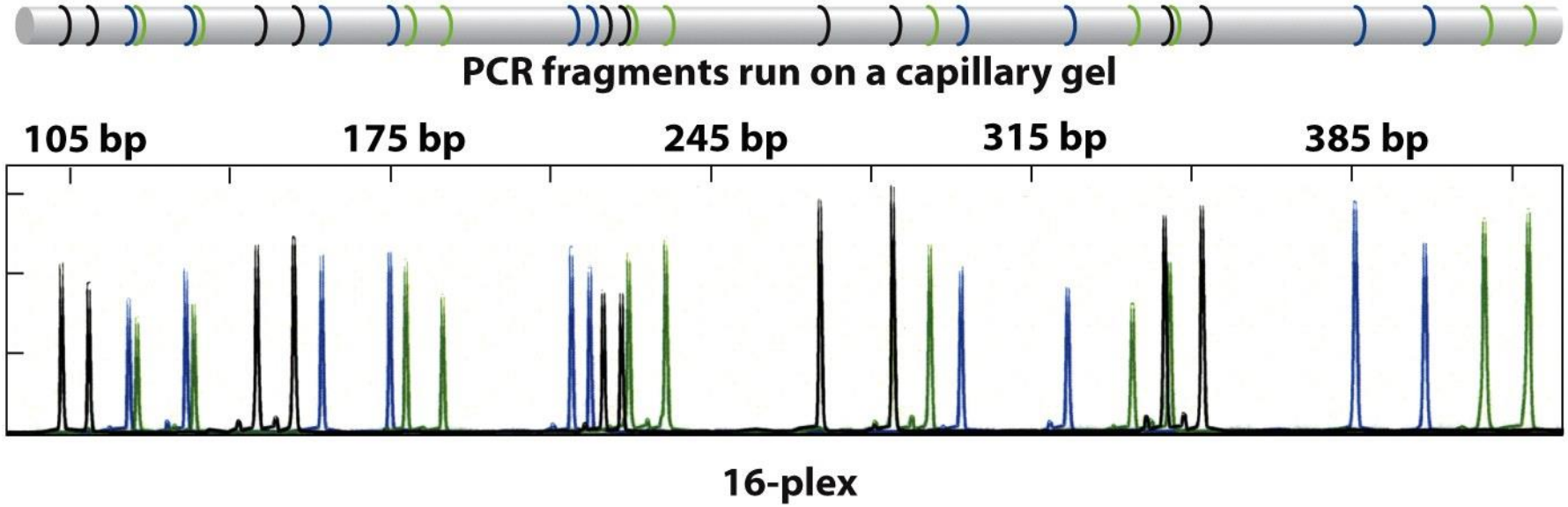

Box 9-1 figure 2b
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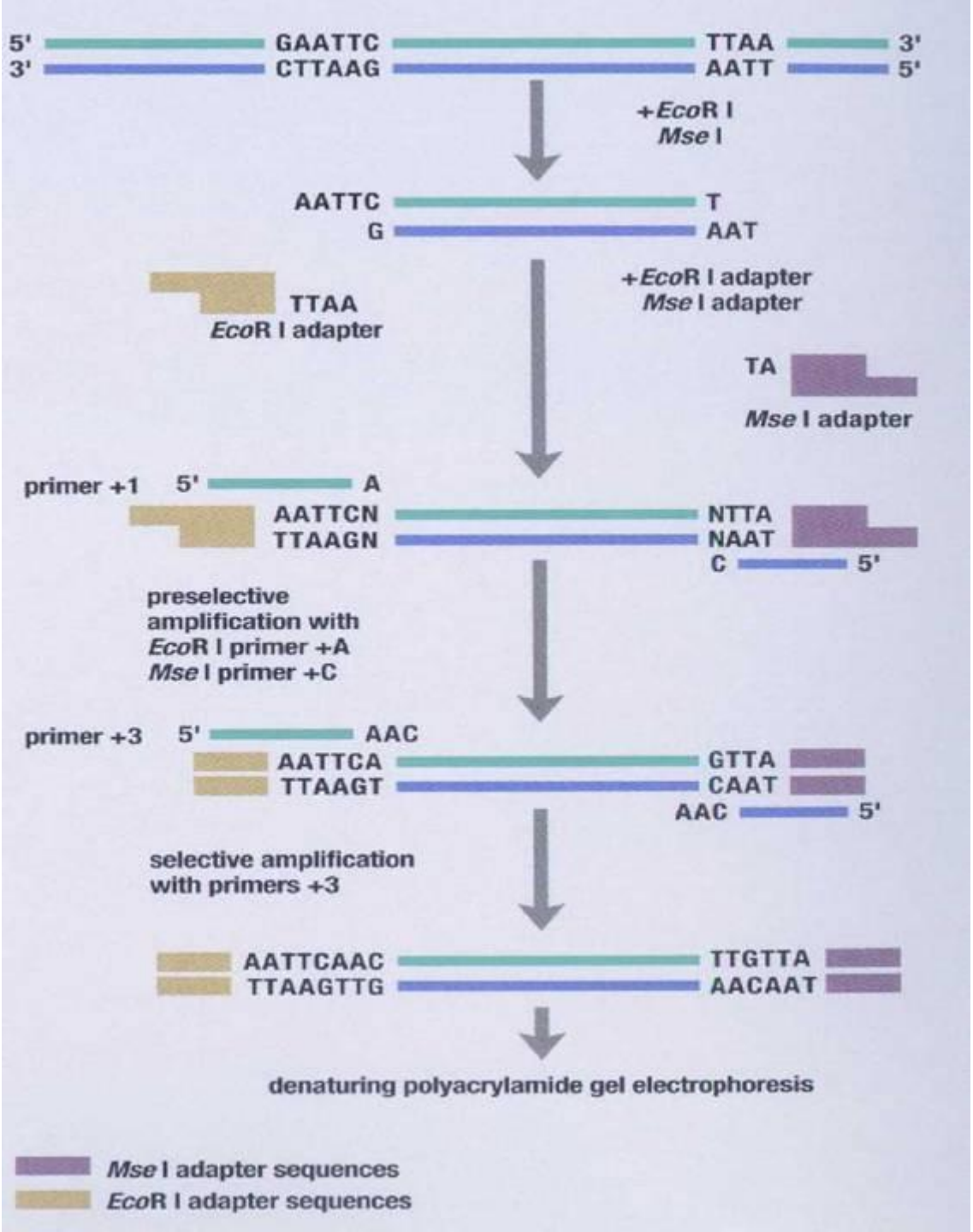
Amplified Fragment Length Polymorphism (AFLP)

- Restriction endonuclease digestion of DNA
- Ligation of adaptors
- Amplification of ligated fragments
- Separation of the amplified fragments via electrophoresis and visualization
- AFLPs have stable amplification and good repeatability


5' GAATTC TTAA 3'
3' CTTAAG AATT 5'
+EcoR I
Mse I
AATTC T
G AAT
TTAA
EcoR I adapter
+EcoR I adapter
Mse I adapter
TA
Mse I adapter
primer +1 5' A
AATTCN NTTA
TTAAGN NAAT
C 5'
preselective
amplification with
EcoR I primer +A
Mse I primer +C
primer +3 5' AAC
AATTCA GTTA
TTAAGT CAAT
AAC 5'
selective amplification
with primers +3
AATTCAAC TTGTTA
TTAAGTTG AACAAT
denaturing polyacrylamide gel electrophoresis
Mse I adapter sequences
EcoR I adapter sequences

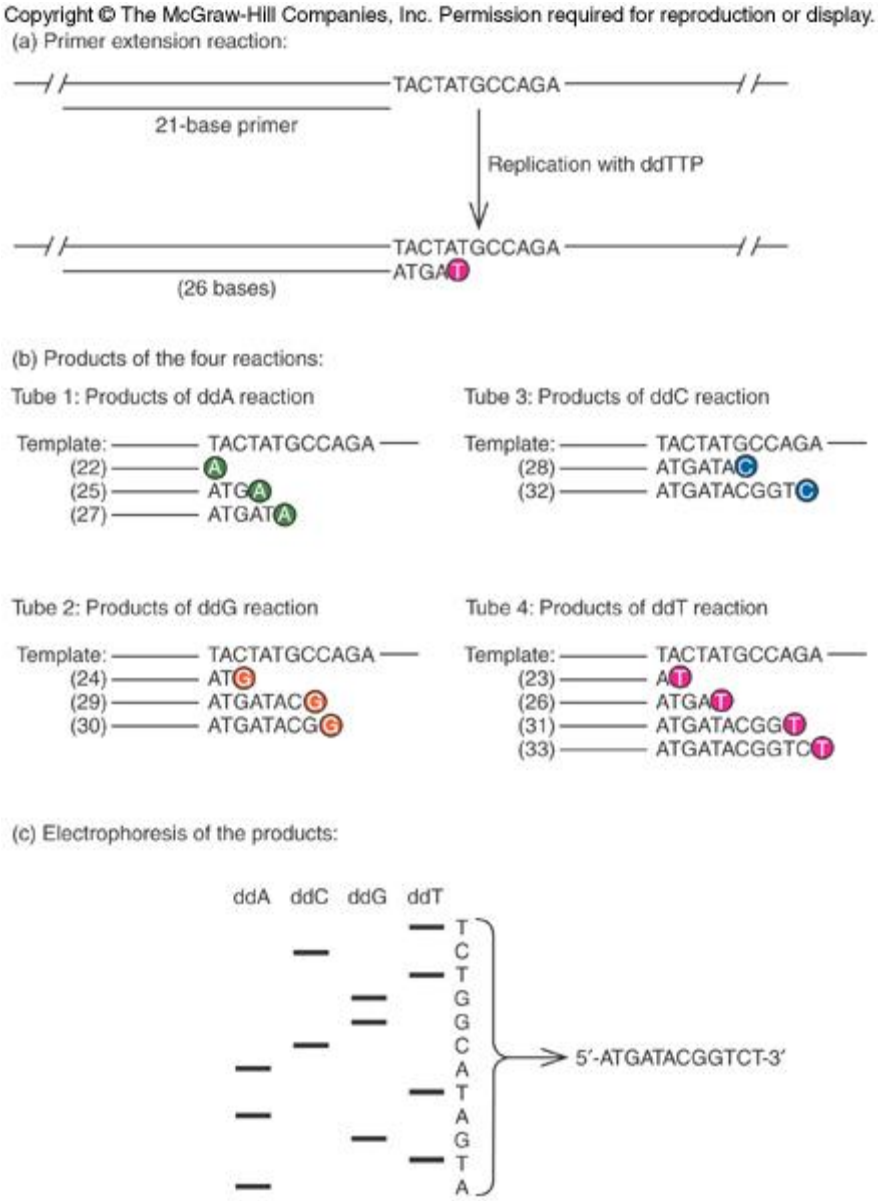
Properties of Different MM

Features	RFLP	PCR-RFLP	DFP	RAPD	Microsatellite	SNP
Detection method	Hybridization	PCR	Hybridization	PCR	PCR	PCR
Type of probe/primer used	g DNA/ cDNA sequence of structural genes	Sequence specific primers	Mini satellite synthetic oligos	Arbitrarily design primer	Sequence specific primers	Sequence specific primers
Requirement of radioactivity	Yes	No/Yes	Yes	No/Yes	No/Yes	No/Yes
Extant of genomic coverage	Limited	Limited	Extensive	Extensive	Extensive	Extensive
Degree of polymorphisms	Low	Low	High	Medium to High	High	High
Phenotype expression	Co dominant	Co dominant	Co dominant	Co dominant/Dominant	Dominant	Co dominant
Possibility of automation	No	Yes	No	Yes	Yes	Yes

5.4 DNA Sequencing

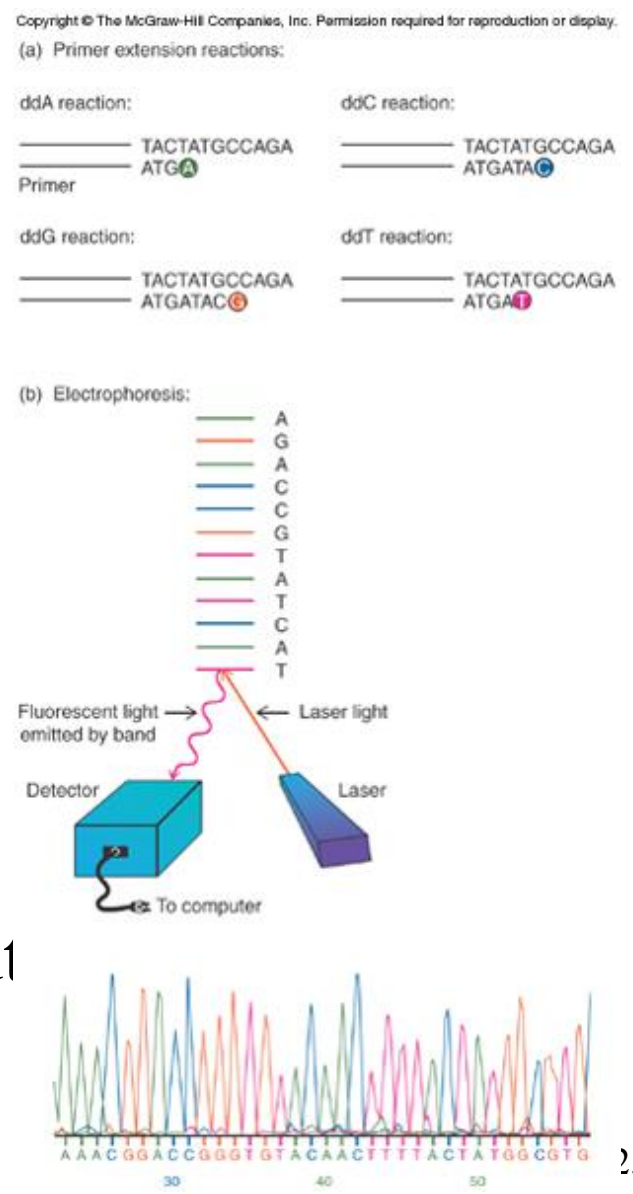
- Sanger, Maxam, and Gilbert developed 2 methods for determining the exact base sequence of a cloned piece of DNA
- Modern DNA sequencing is based on the Sanger method and uses dideoxy nucleotides to terminate DNA synthesis
  - The process yields a series of DNA fragments whose size is measured by electrophoresis
  - The last base in each fragment is known as that dideoxy nucleotide was used to terminate the reaction
  - Ordering the fragments by size tells the base sequence of the DNA

Sanger Method of DNA Sequencing

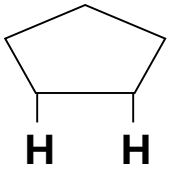


Automated DNA Sequencing

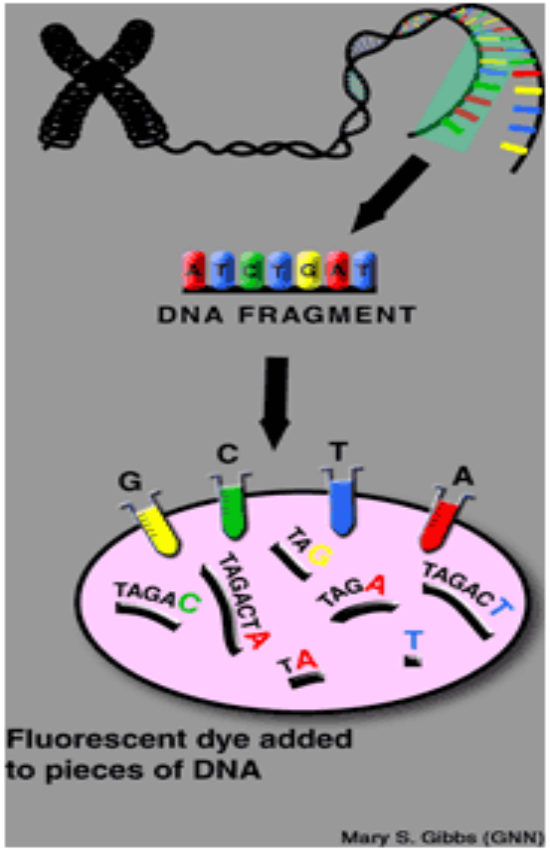
- Manual sequencing is powerful but slow
- Automated sequencing uses dideoxynucleotides tagged with different fluorescent molecules
  - Products of each dideoxynucleotide will fluoresce a different color
  - Four reactions are completed, then mixed together and run out on one lane of a gel

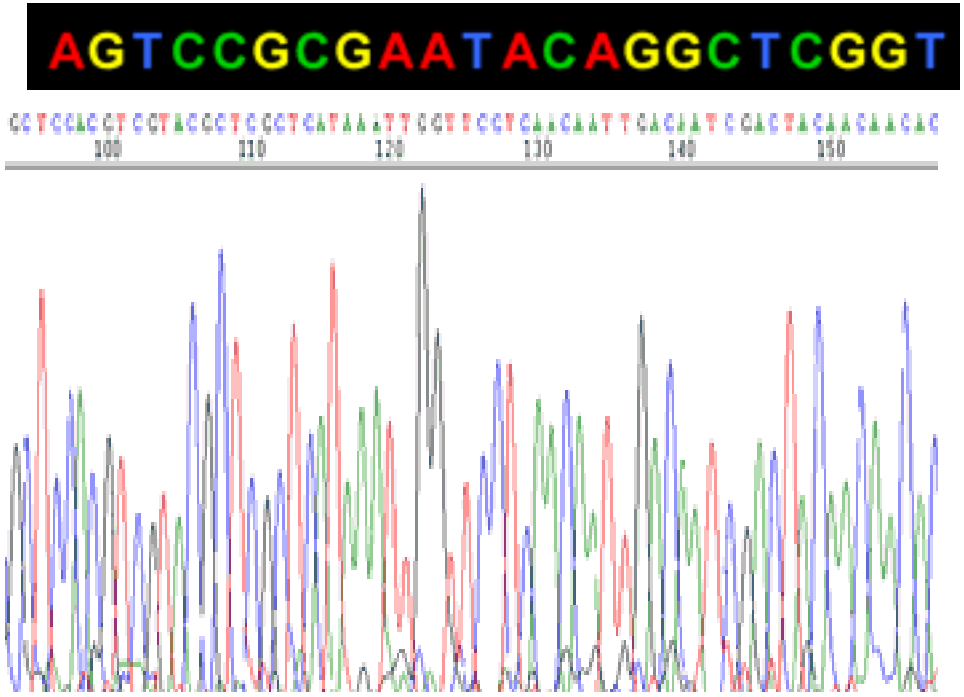


DNA Sequencing by the Sanger Method



dideoxyribose





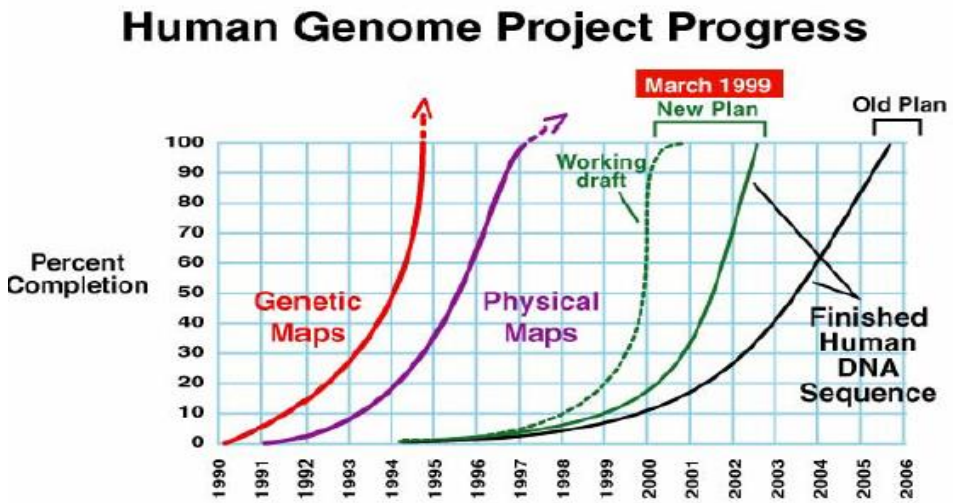
Human genome project

The most challenging quest ever undertaken by science

- **Initiated in 1990**
- **Completed in 2003**
- **Estimated cost: US$ 3 billion.**
- **plans were ;**
  Mapping and sequencing of human genome and model organisms, Data collection and distribution, Technology development and transfer,
- **Functional genomics**

Human genome project

- Sequencing procedure
  - DNA Isolation
  - Break the large pieces of DNA into smaller pieces
  - Make a library
  - Sequencing
  - Analyze the data
  - Fixing the gaps



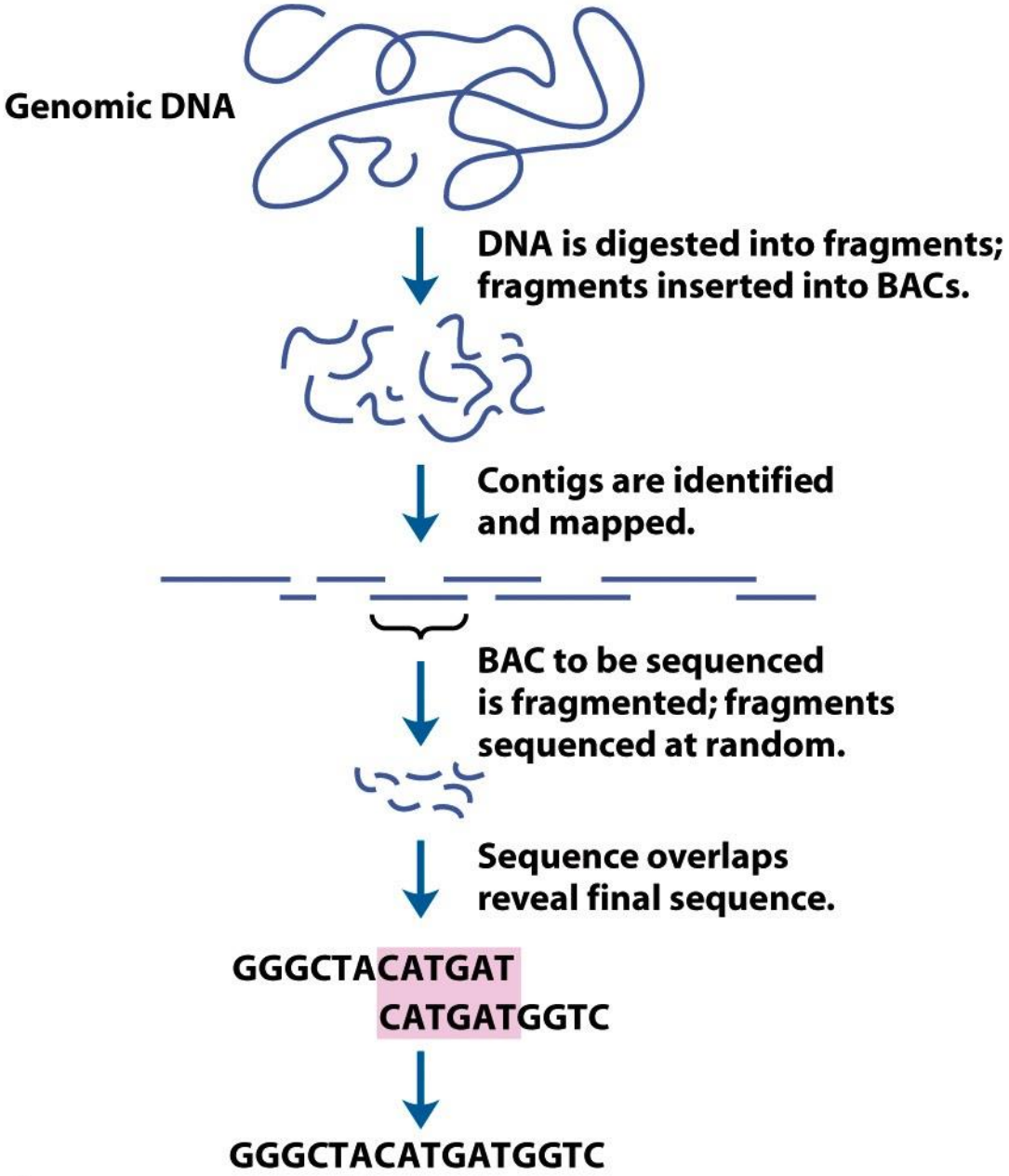


Figure 9-17
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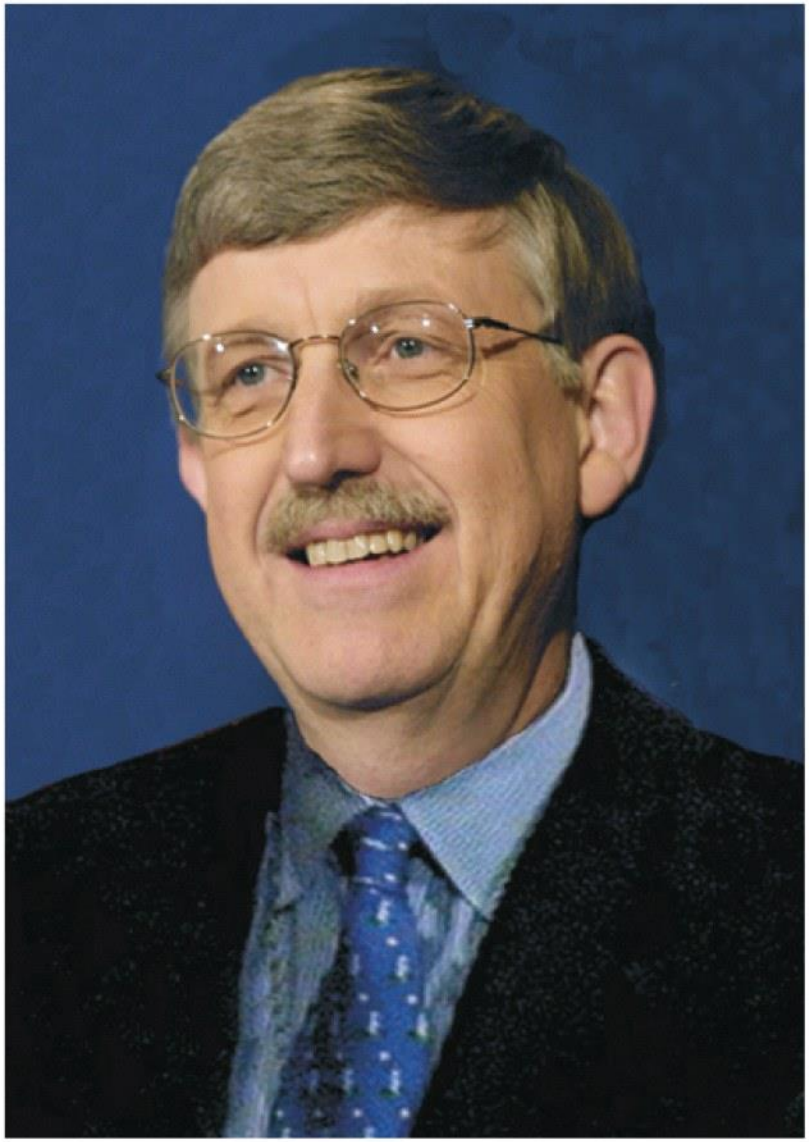
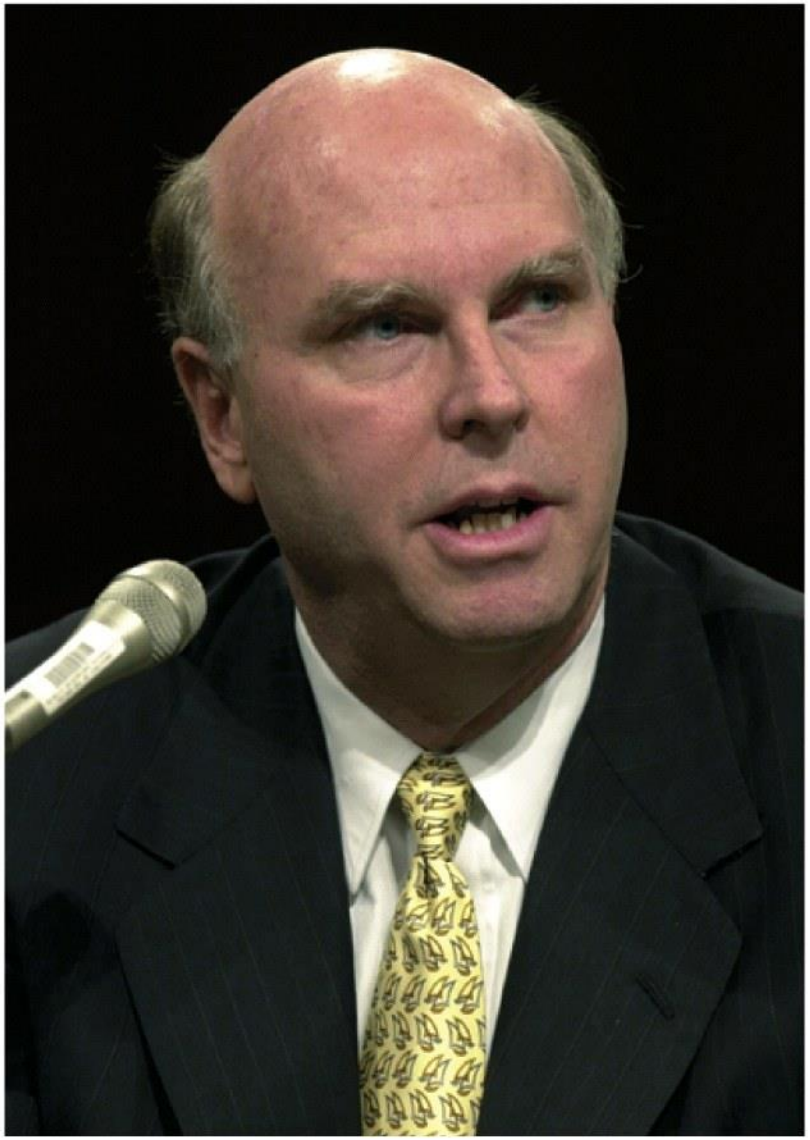


Francis S. Collins



J. Craig Venter

Unnumbered 9 p322

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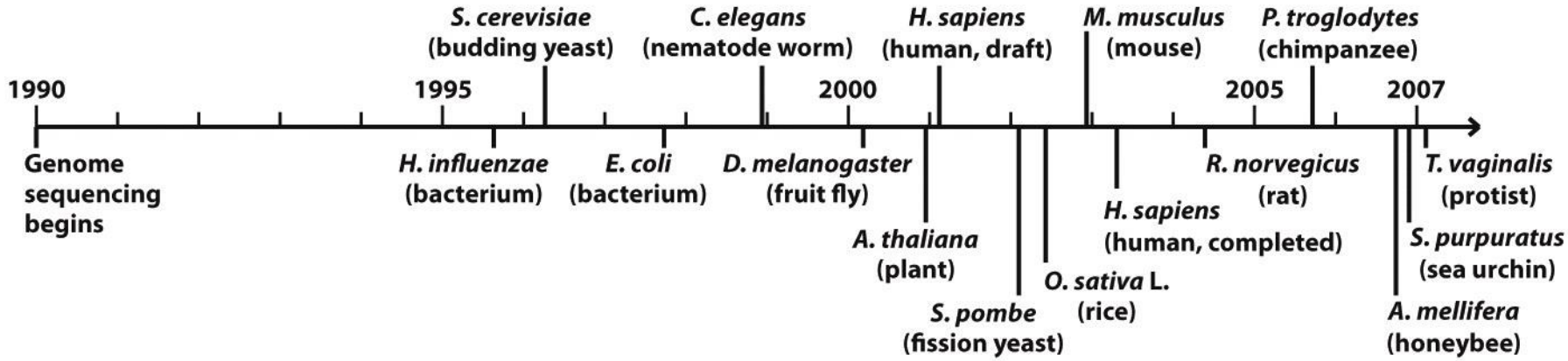

Figure 9-18
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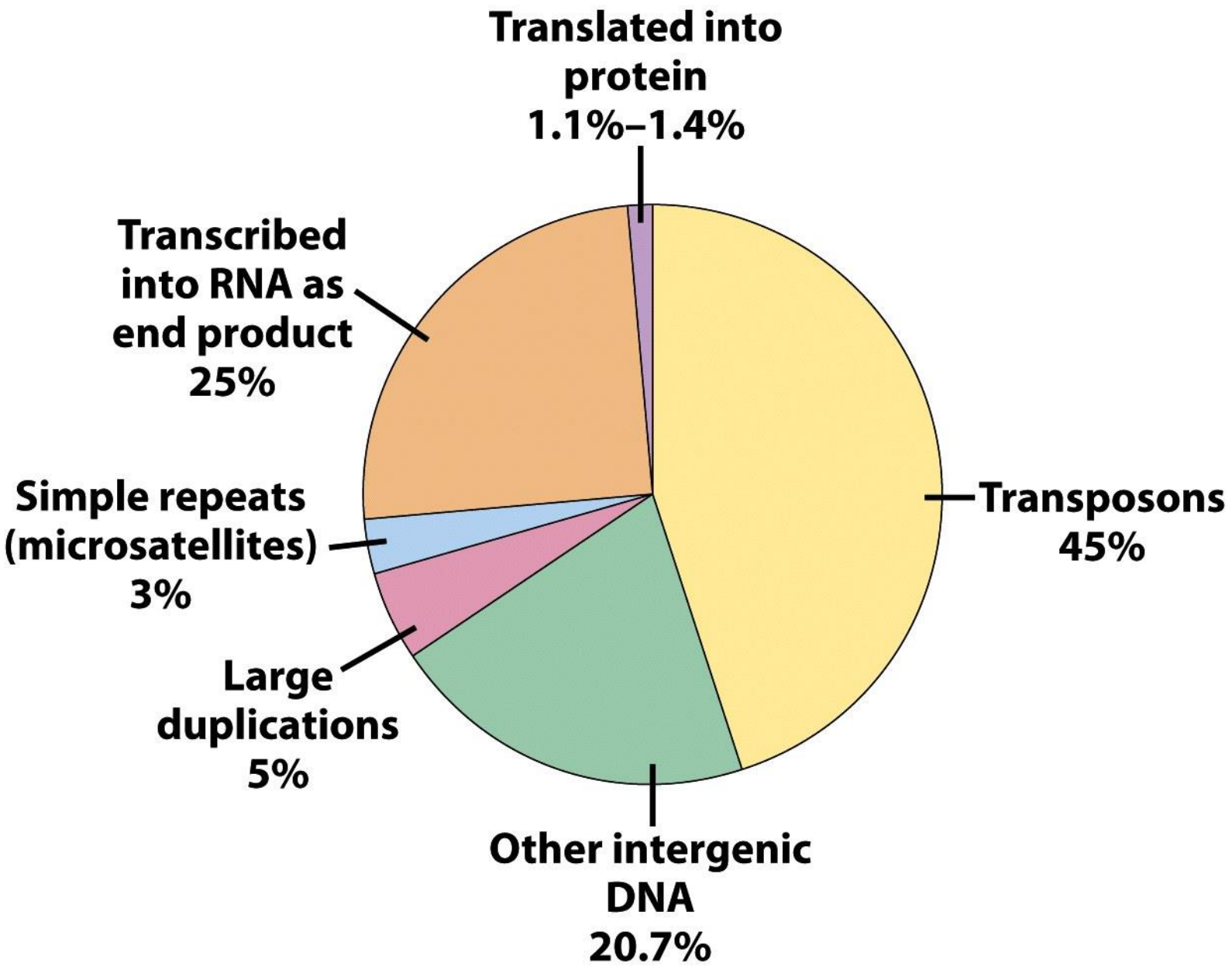

Figure 9-19
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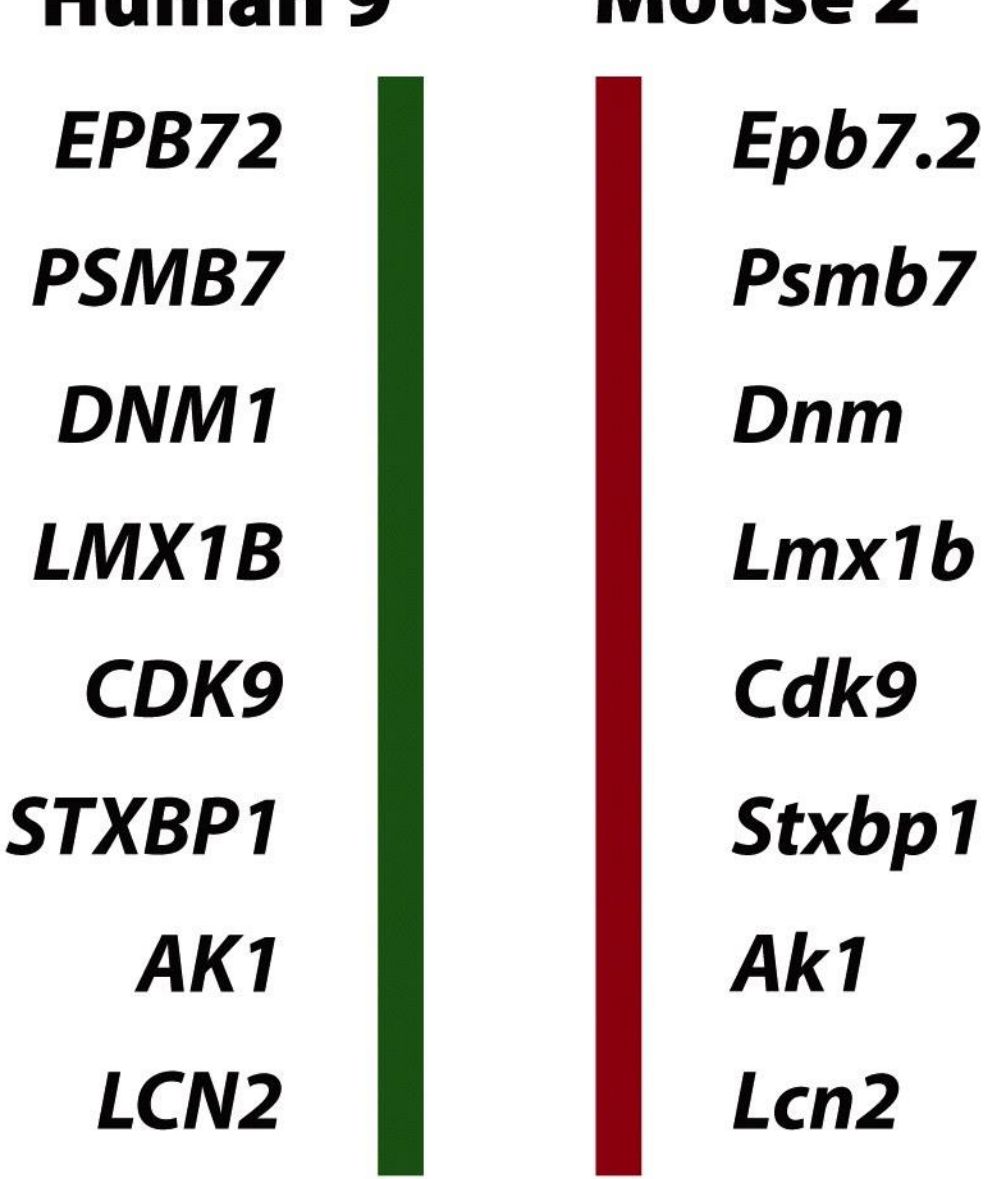

Figure 9-20
Lehninger Principles of Biochemistry, Fifth Edition

High Throughput Sequencing

- Once an organism's genome sequence is known, very rapid sequencing techniques can be applied to sequence the genome of another member of the same species
- Produces relatively short reads or contiguous sequences (25-35bp or 200-300bp, depending on the method) that can easily be pieced together if a reference sequence is available

High Throughput Sequencing

- **Pyrosequencing** is one example that is an automated system with the advantages of speed and accuracy

  - nucleotides are added one by one and the incorporation of a nucleotide is detected by a release of pyrophosphate, which leads to a flash of light

- **Another method** (Illumina company) starts by attaching short pieces of DNA to a solid surface, amplifying each DNA in a tiny patch on the surface, then sequencing the patches together by extending them one nucleotide at a time using fluorescent chain-terminating nucleotides, whose fluoresce reveals their identity

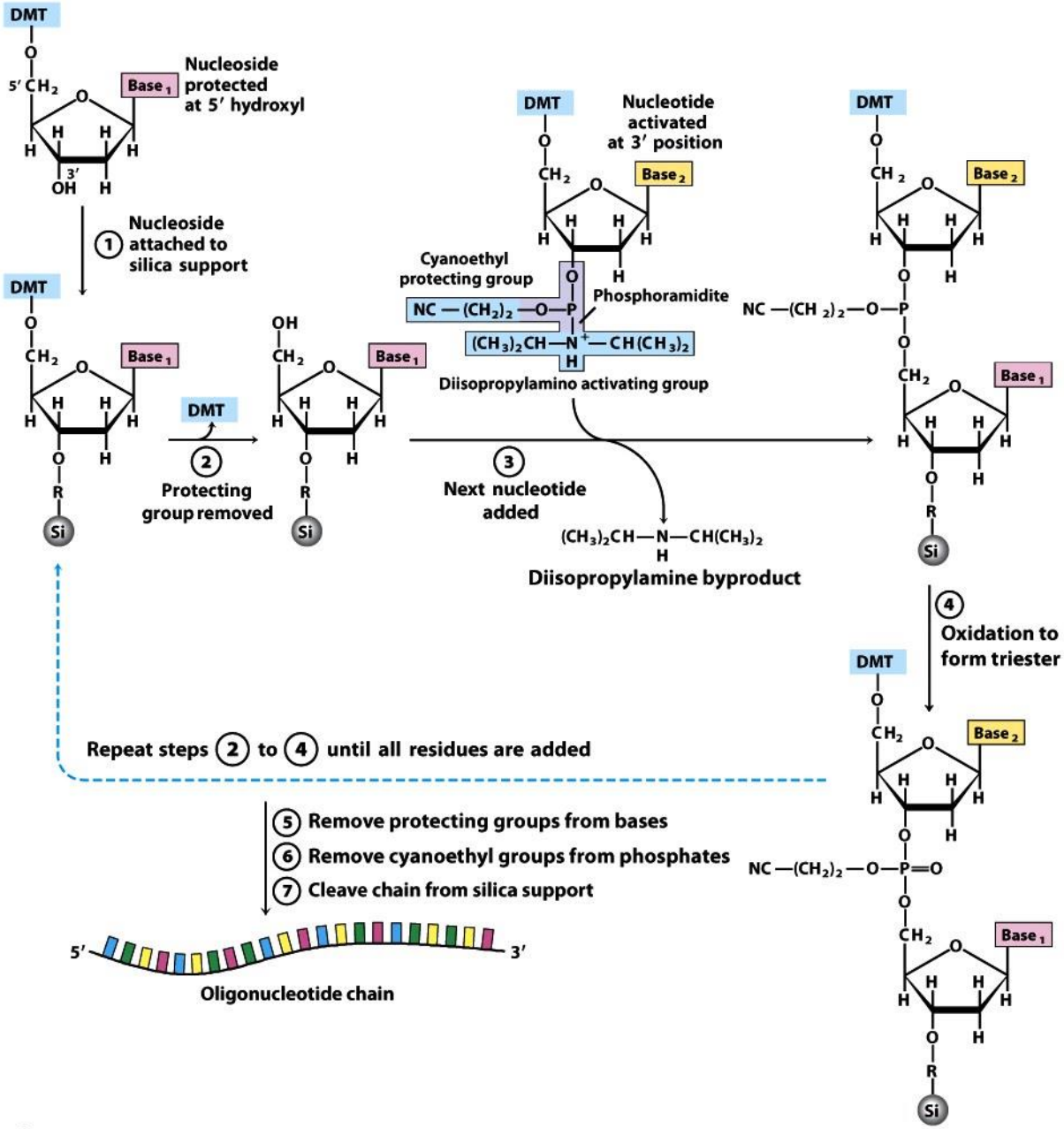

Figure 8-35

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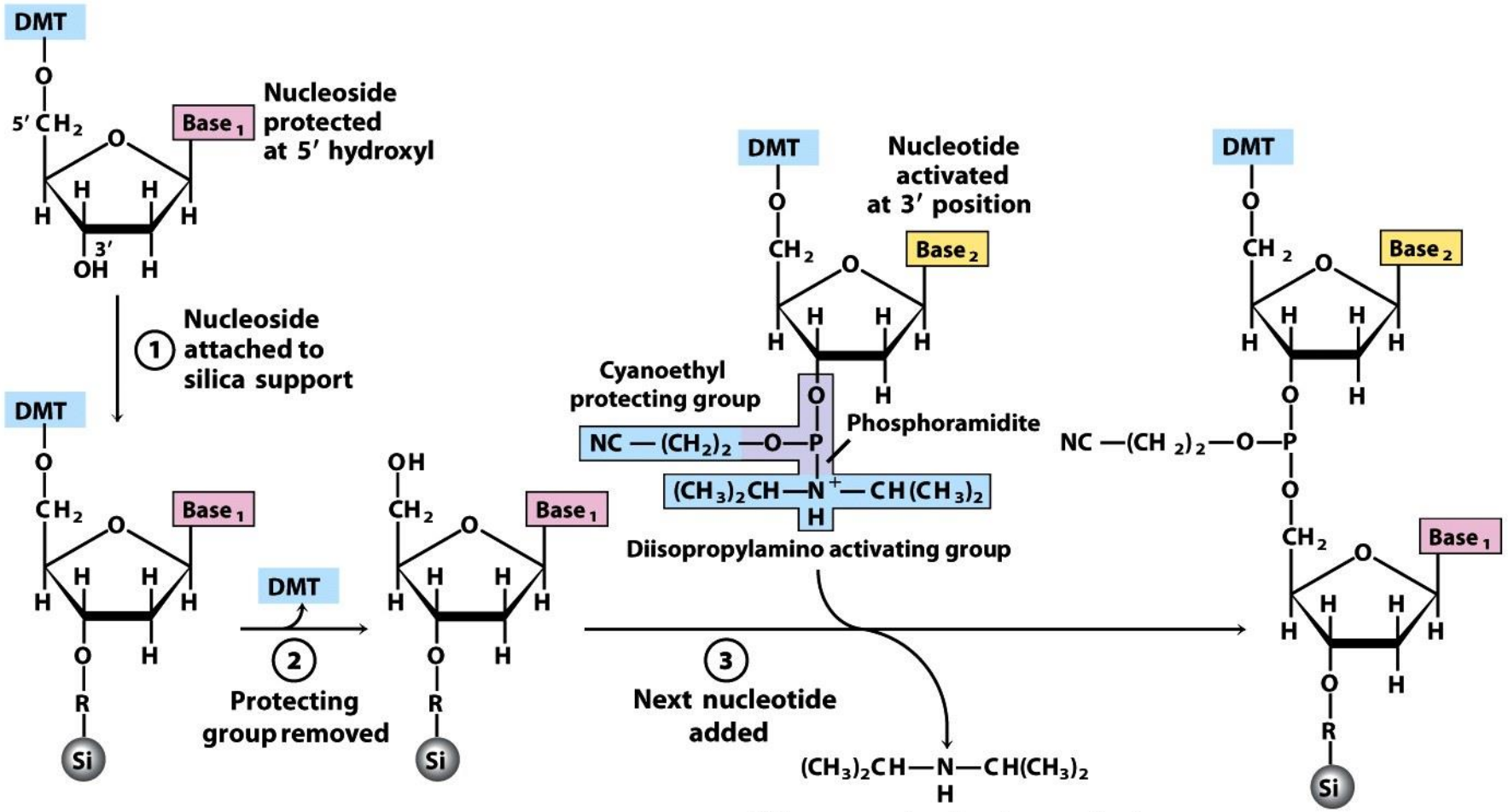

Figure 8-35 part 1

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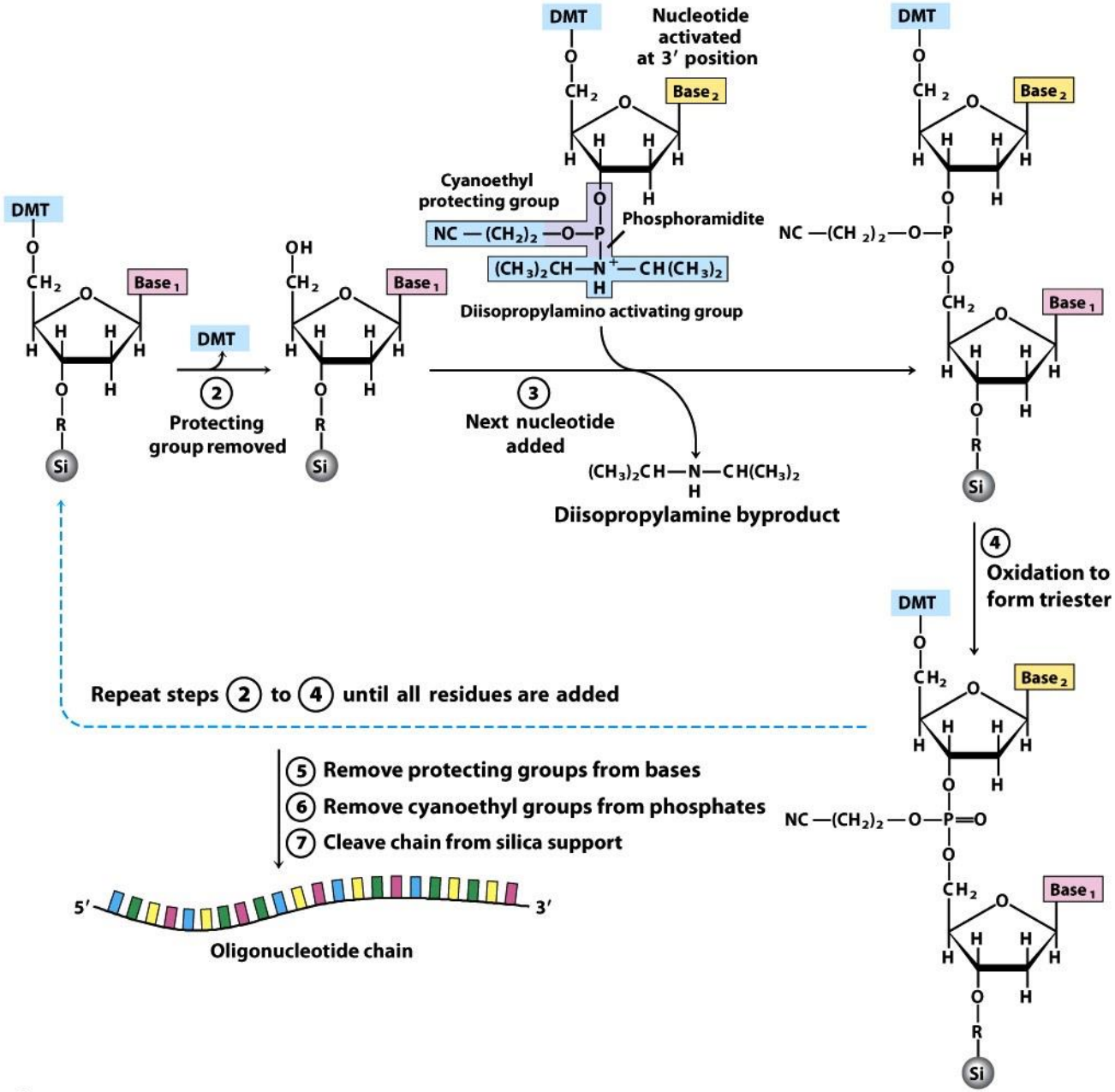

Figure 8-35 part 2

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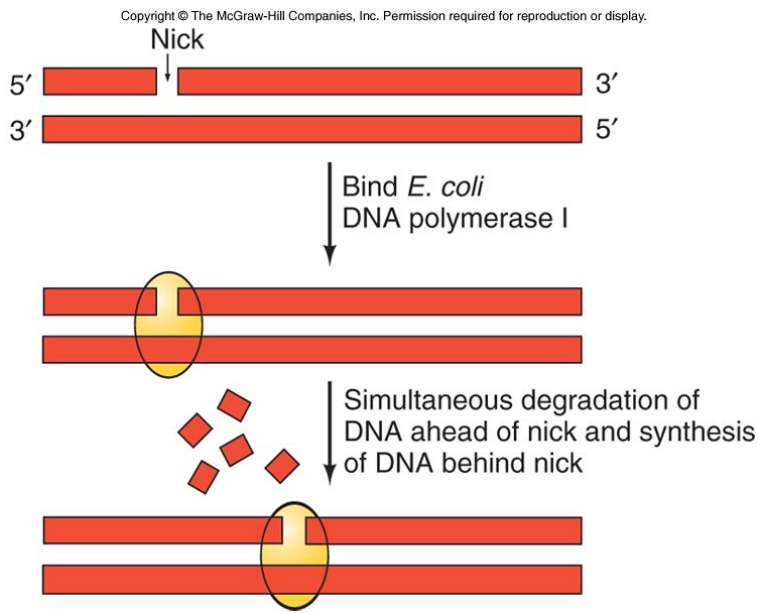
Nick Translation

- The nick translation process simultaneously:
  - Removes DNA ahead of a nick
  - Synthesizes DNA behind nick
  - Net result moves the nick in the 5’ to 3’ direction
- Enzyme often used is *E. coli* DNA polymerase I
  - Has 5’ to 3’ exonuclease activity
  - Allows enzyme to degrade DNA ahead of the nick



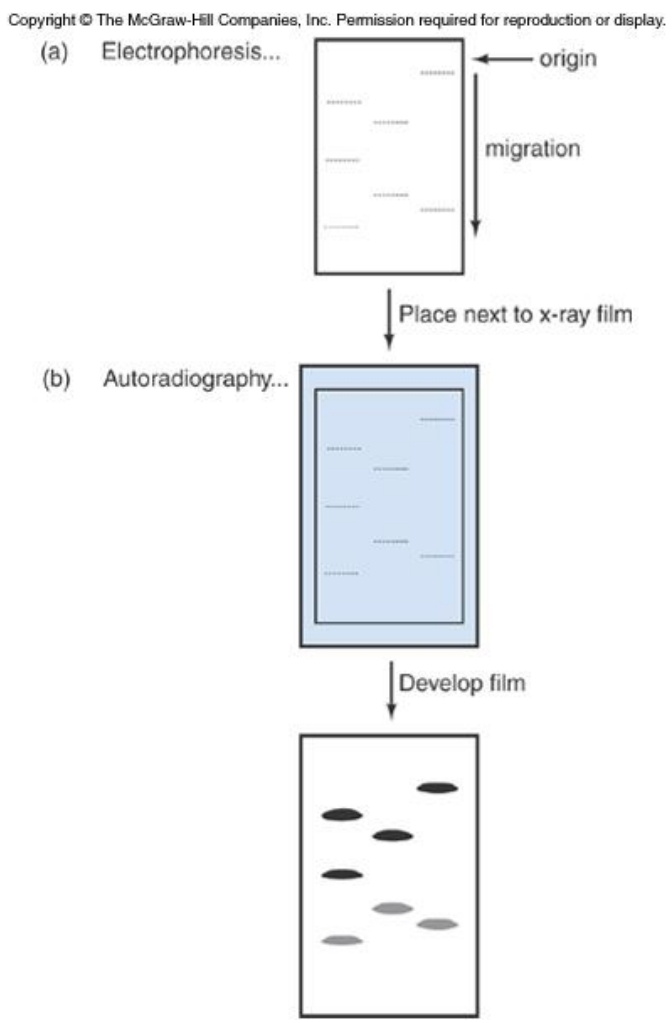
5.2 Labeled Tracers

- For many years “labeled” has been synonymous with “radioactive”
- Radioactive tracers allow vanishingly small quantities of substances to be detected
- Molecular biology experiments typically require detection of extremely small amounts of a particular substance

Autoradiography

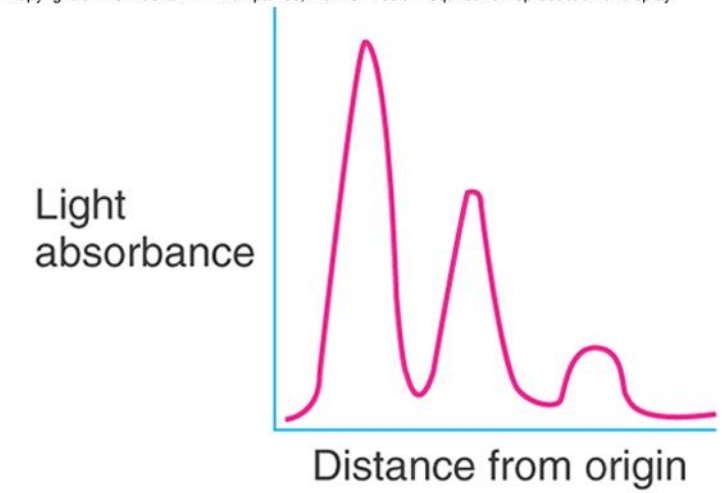
Autoradiography is a means of detecting radioactive compounds with a photographic emulsion

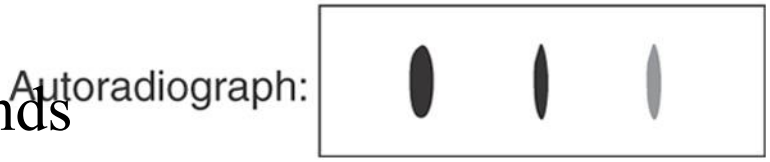
- Preferred emulsion is x-ray film
- DNA is separated on a gel and radiolabeled
- Gel is placed in contact with x-ray film for hours or days
- Radioactive emissions from the labeled DNA expose the film
- Developed film shows dark bands



Autoradiography Analysis

- Relative quantity of radioactivity can be assessed looking at the developed film
- More precise measurements are made using a densitometer
  - Area under peaks on a tracing by a scanner
  - Proportional to darkness of the bands on autoradiogram





Phosphorimaging

This technique is more accurate in quantifying amount of radioactivity in a substance

- Response to radioactivity is much more linear
- Place gel with radioactive bands in contact with a phosphorimager plate
- Plate absorbs β electrons that excite molecules on the plate which remain excited until plate is scanned
- Molecular excitation is monitored by a detector

Liquid Scintillation Counting

Radioactive emissions from a sample create photons of visible light are detected by a photomultiplier tube in the process of liquid scintillation counting

- Remove the radioactive material (band from gel) to a vial containing scintillation fluid
- Fluid contains a fluor that fluoresces when hit with radioactive emissions
- Acts to convert invisible radioactivity into visible light

Nonradioactive Tracers

- Newer nonradioactive tracers now rival older radioactive tracers in sensitivity
- These tracers do not have hazards:
  - Health exposure
  - Handling
  - Disposal
- Increased sensitivity is from use of a multiplier effect of an enzyme that is coupled to probe for molecule of interest

Detecting Nucleic Acids With a Nonradioactive Probe

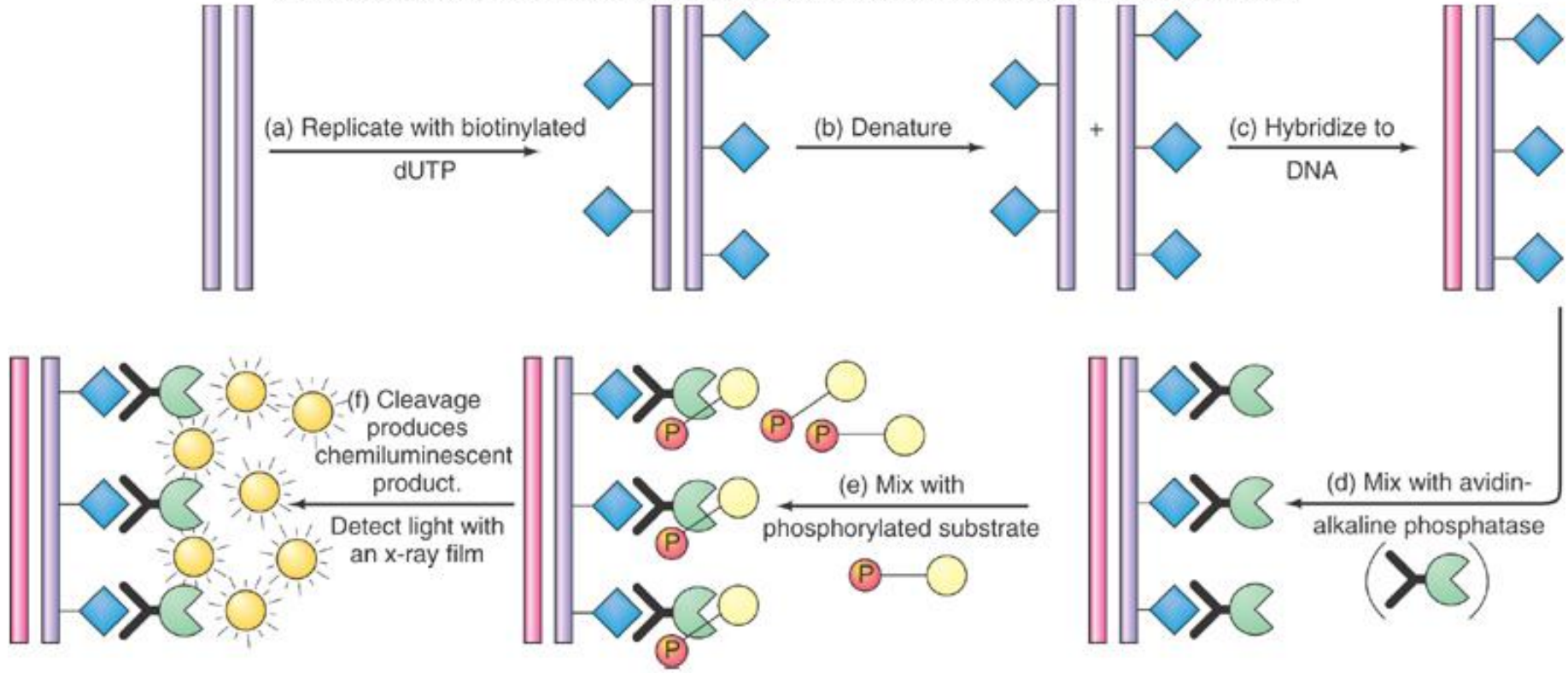
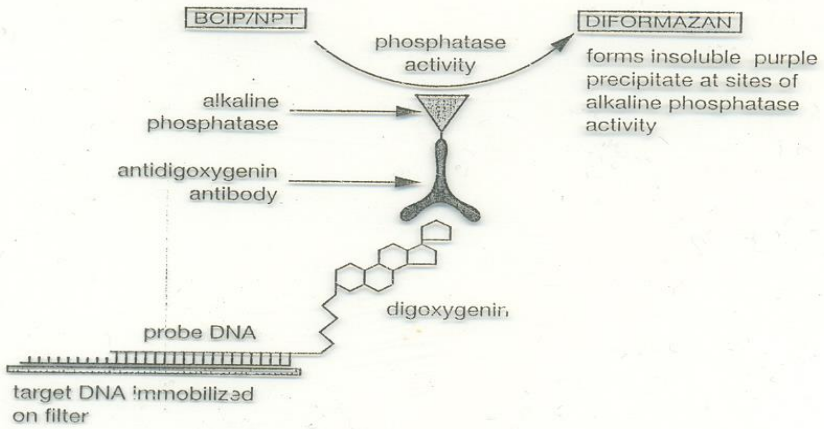


TABLE A9-6 Methods of Labeling Nucleic Acids with Digoxygenin

Method of Labeling	Enzyme	Number of Digoxygenin Molecules Incorporated	Reference
Random priming	Klenow fragment	1 per 25–36 nucleotides	Kessler et al. (1990)
Nick translation	*E. coli* DNA polymerase I	1 per 25–36 nucleotides	Höltke et al. (1990)
Tailing	Terminal transferase	1 per 12 nucleotides	Schmitz et al. (1991)
Amplification by PCR	*Taq* and other thermostable polymerases	1 per 25 nucleotides	Seibl et al. (1990)
Transcription	T3, T7, and SP6 RNA polymerases	1 per 25–36 nucleotides	Höltke and Kessler (1990)
cDNA synthesis	Reverse transcriptase	1 per 25–36 nucleotides	McCracken (1989)

For review, please see Kessler (1991).



[2'-spiroadamantane]-4-methoxy-3-[3''-(phosphoryl)phenyl]
1,2-dioxetane

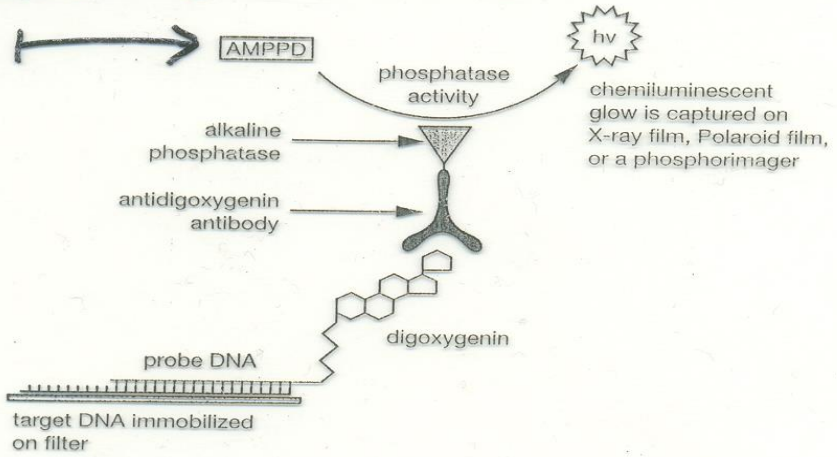


FIGURE A9-6 Detection of Digoxygenin-labeled Nucleic Acid Probes with BCIP/NBT or AMPPD

TABLE A9-5 Excitation and Emission Wavelengths of Fluorochromes

FLUOROCHROME	EXCITATION WAVELENGTH (nm)	EMISSION WAVELENGTH (nm)
Fluorescein	495 blue	524 greenish-yellow
Rhodamine	540 green, visible	575 orange-red
Texas Red	595 orange-red, visible	620 red
Oregon Green	496 blue	524 greenish-yellow
R-phycoerythrin	480, 545, 565 green, visible	574 orange-red

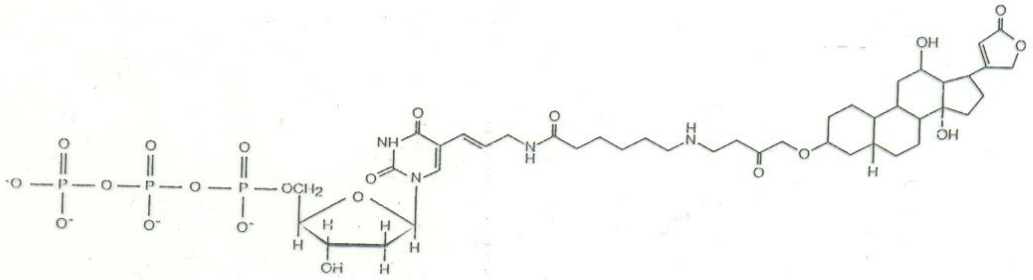
TABLE A9-3 Chemiluminescent Assays for Immunoassay and Nucleic Acid Hybridization Labels

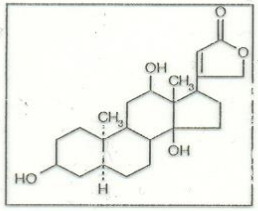
ENZYME	SUBSTRATE	DETECTION LIMIT (ZEPTOMOLES)
Acridinium ester	NaOH + peroxide	500
Alkaline phosphatase	AMPPD	1
β-D-galactosidase	AMPGD	30
Horseradish peroxidase	luminol + perborate + 4-iodophenol	5,000
Isoluminol	microperoxidase + peroxide	50,000
Xanthine oxidase	luminol + Fe EDTA	3,000

Bronstein and Kricka (1989); Kricka (1991).

Labeling Nucleic Acids with Digoxygenin

For labeling of nucleic acids, digoxygenin is supplied by the manufacturer (Boehringer Mannheim) in two forms: digoxygenin-11-dUTP (DIG-11-dUTP) and digoxygenin-11-UTP (DIG-11-UTP). In each form, digoxygenin is coupled by an alkali-stable linkage and a spacer arm to deoxyuridine triphosphate and uridine triphosphate, respectively.





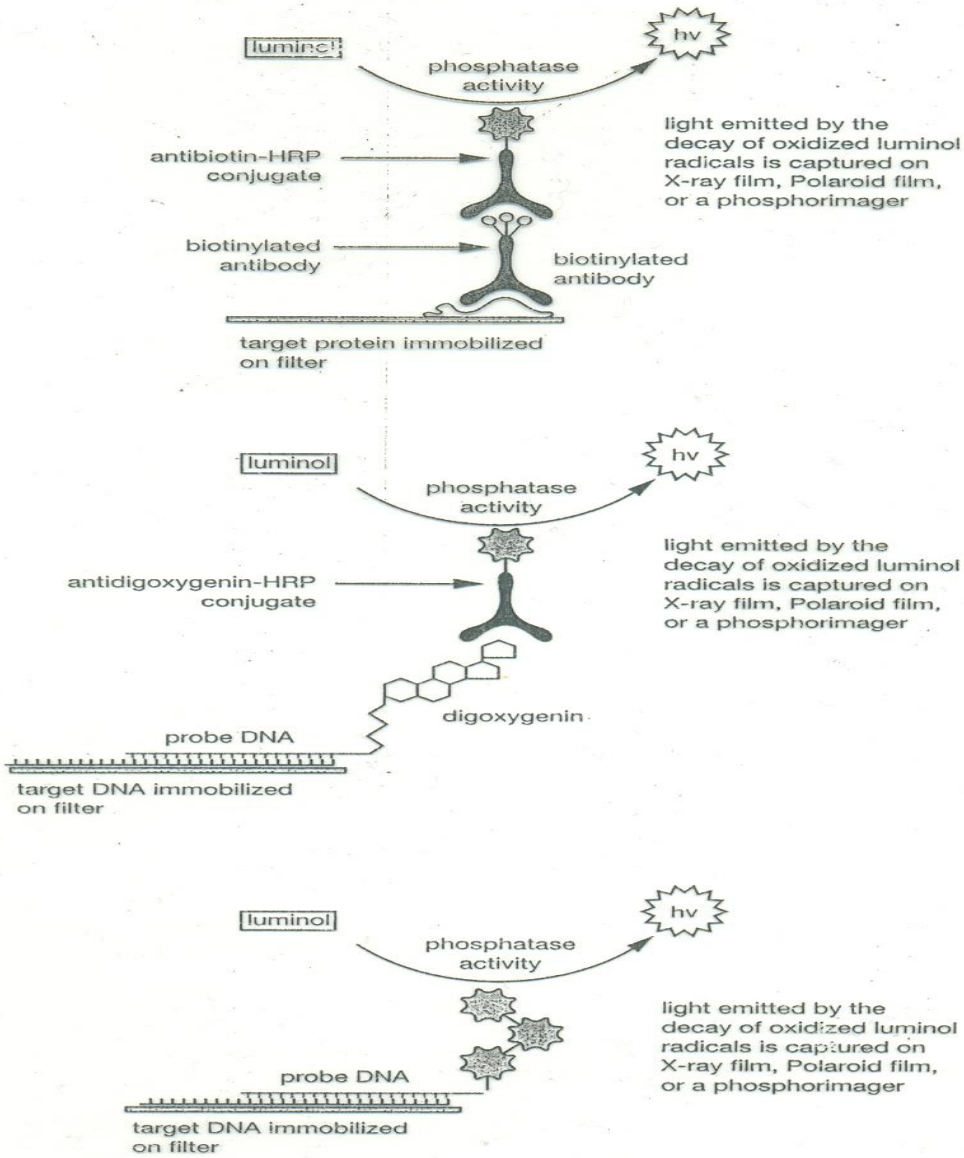


FIGURE A9-4 Experimental Formats for Detection of Immobilized Nucleic Acids and Proteins with Horseradish Peroxidase (HRP)

Light emitted by the decay of oxidized luminol radicals is captured on X-ray film or by a CCD camera.

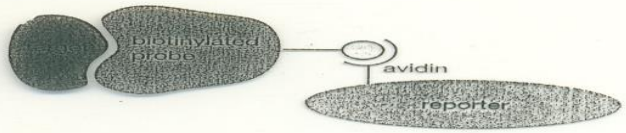
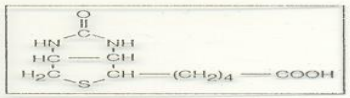


FIGURE A9-12 The Avidin-Biotin Complex



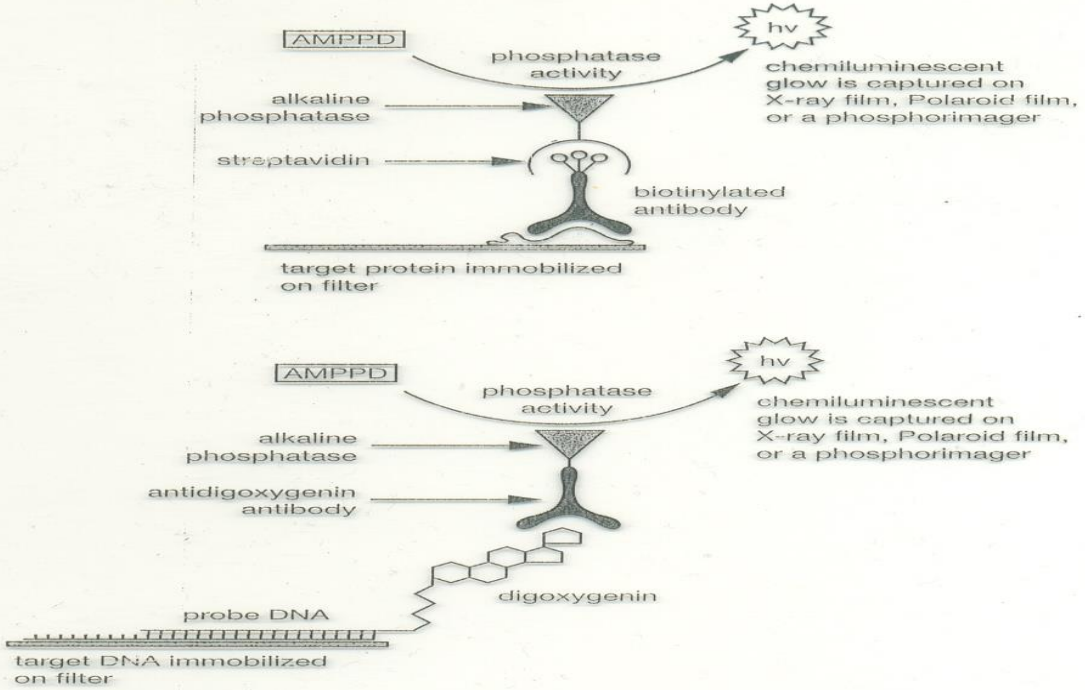
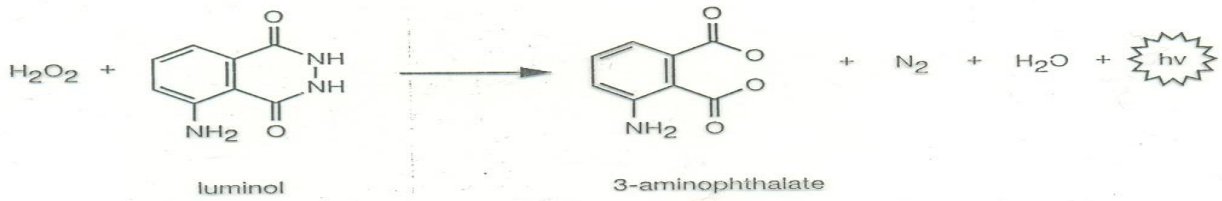


FIGURE A9-9 Detection of Immobilized Nucleic Acids and Proteins with AMPPD

(*Top*) Detection of target protein by western blotting; (*bottom*) detection of nucleic acid sequence in Southern or northern blotting.



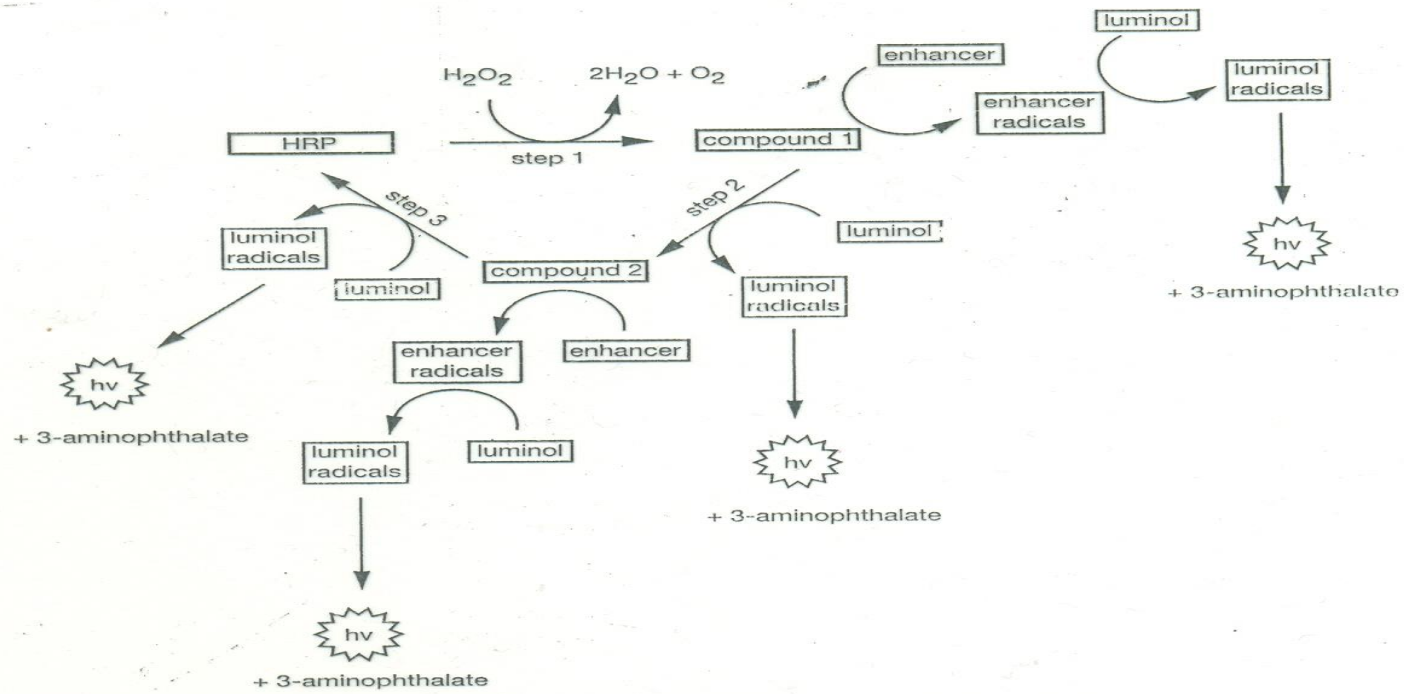


FIGURE A9-5 Proposed Cyclic Reaction for the Generation of Light by Oxidation of Luminol by HRP

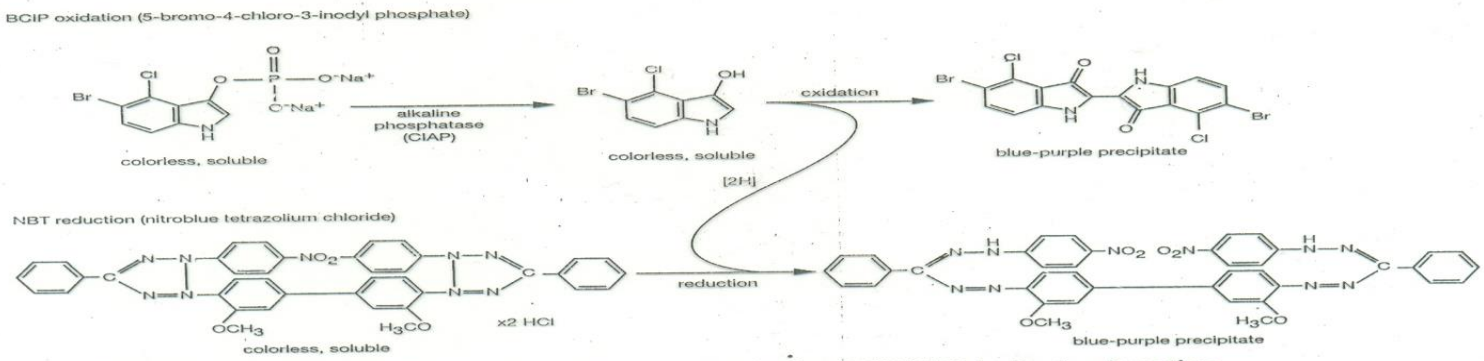


FIGURE A9-7 Oxidation of BCIP and Reduction of NBT in the BCIP/NPT Indicator Reaction

(Reprinted, with permission, from Kessler 1991 [Copyright 1991 ©Academic Press London].)

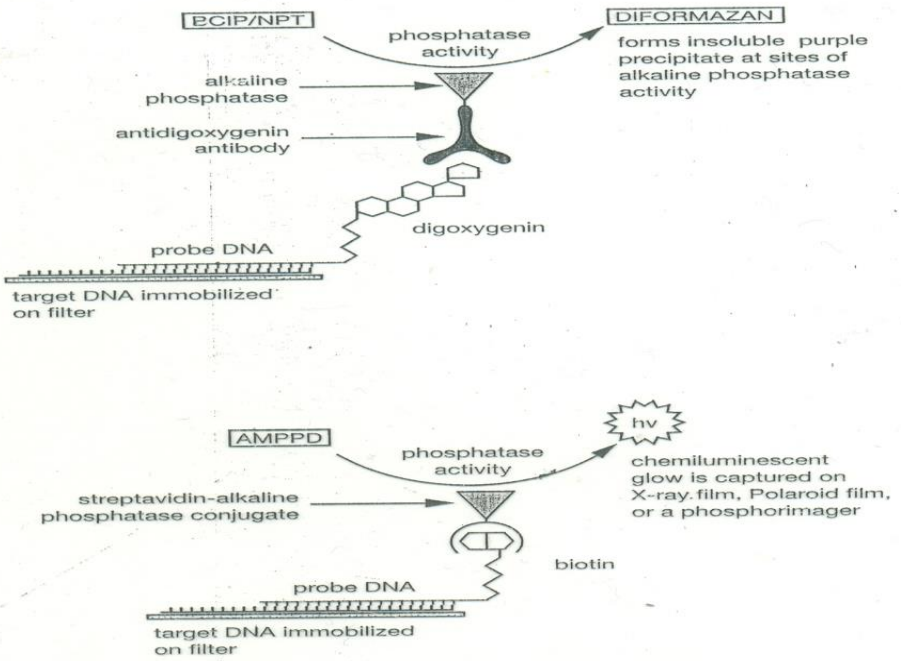
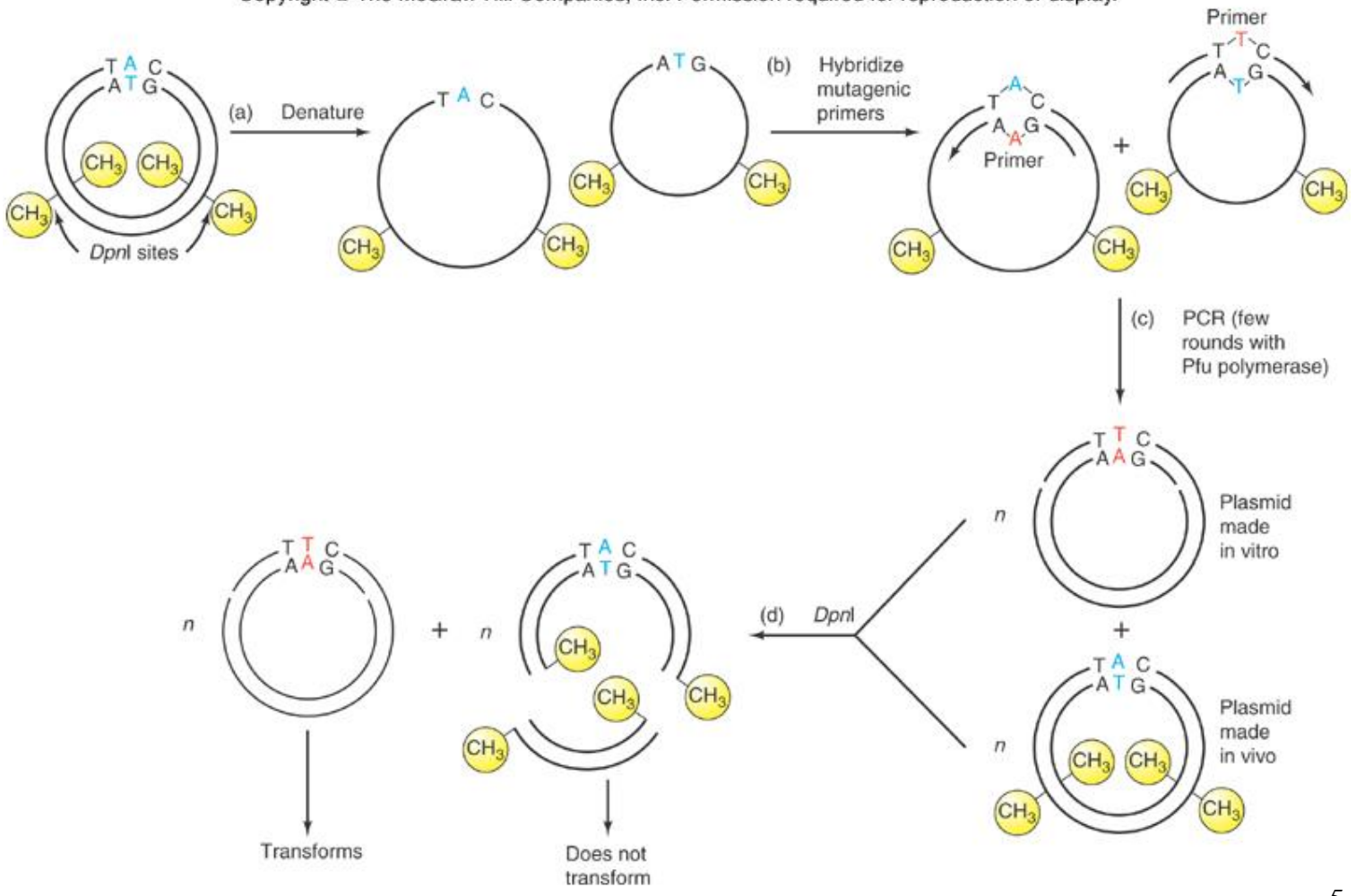


FIGURE A9-8 Detection of Digoxygenin- and Biotin-labeled Nucleic Acid Probes with BCIP/NPT

5.5 Protein Engineering With Cloned Genes: Site-Directed Mutagenesis

- Cloned genes permit biochemical microsurgery on proteins
  - Specific bases in a gene may be changed
  - Amino acids at specific sites in the protein product may be altered as a result
  - Effects of those changes on protein function can be observed

PCR-based Site-Directed Mutagenesis



Transgenic Plants

Round Up Ready Soybeans are resistant to herbicide

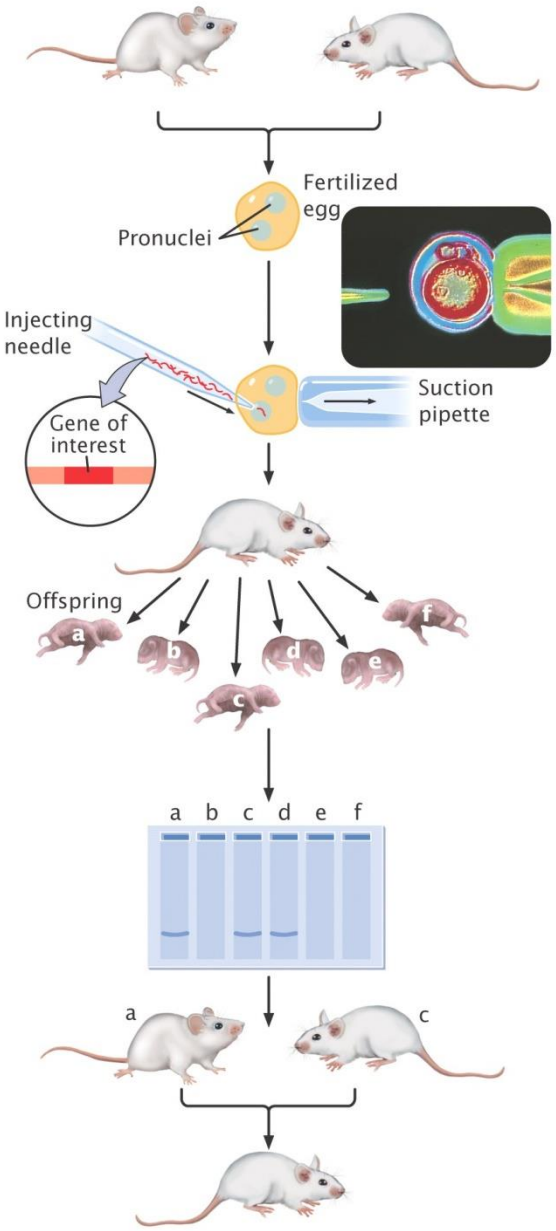
Herbicide Tolerance, Insect Resistance, quality traits
Soybean, Corn, Cotton, Canola Tomato

“Golden” rice with beta-carotene and extra iron

Bt Corn produces its own pesticide

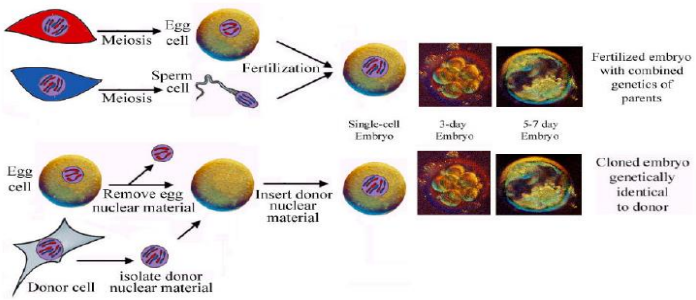
Genetically Modified Organisms (Transgenic)

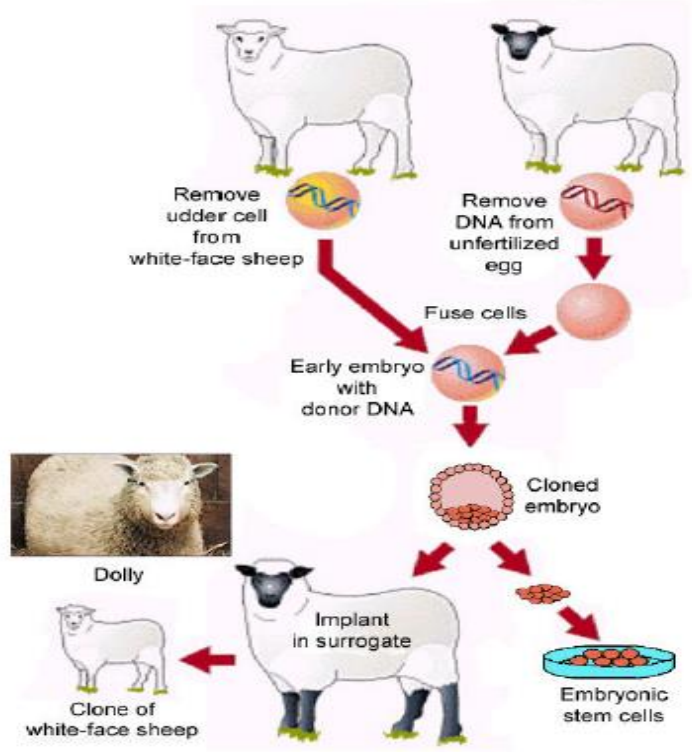
- Introducing or modifying specific genes to alter the phenotype of an organism
- Transgenic: organism that contains a gene from another species in all of its cells
- Transgenic Animals
  - Models of Human Disease
  - Produce Pharmaceuticals



Dolly - 1996

Fertilization *vs*. Cloning **(somatic cell nuclear transfer)**





Cloning since Dolly

Cloning of this sort has now been done on cattle, pigs and mice also.
The success rate has improved considerably.

Guar: First cloned endangered species dies 2 days after birth

Snuppy

Figure 9-33
Lehninger Principles of Biochemistry, Fifth Edition

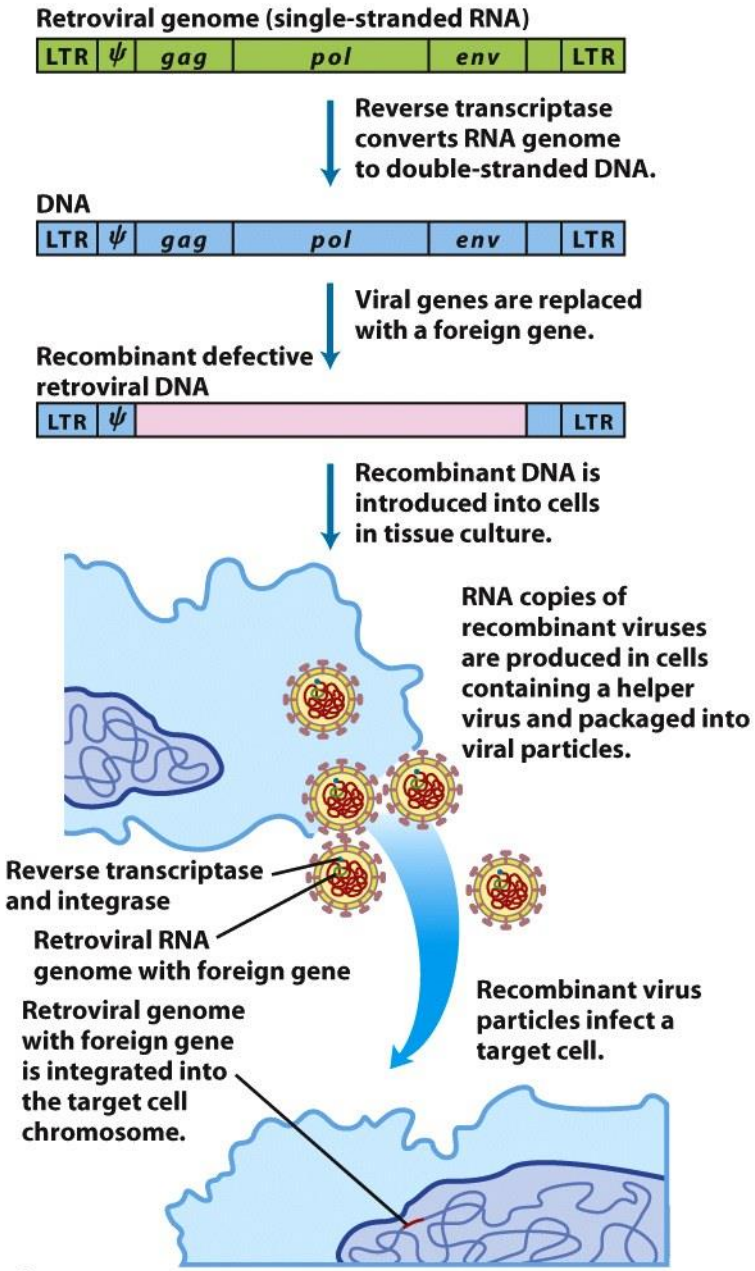


Figure 9-32

Lehninger Principles of Biochemistry, Fifth Edition

Some recombinant DNA products

Product category	*Examples/uses*
Anticoagulants	Tissue plasminogen activator (TPA); activates plasmin, an enzyme involved in dissolving clots; effective in treating heart attack patients.
Blood factors	Factor VIII; promotes clotting; it is deficient in hemophiliacs; treatment with factor VIII produced by recombinant DNA technology eliminates infection risks associated with blood transfusions.
Colony-stimulating factors	Immune system growth factors that stimulate leukocyte production; treatment of immune deficiencies and infections.
Erythropoietin	Stimulates erythrocyte production; treatment of anemia in patients with kidney disease.
Growth factors	Stimulate differentiation and growth of various cell types; promote wound healing.
Human growth hormone	Treatment of dwarfism.
Human insulin	Treatment of diabetes.
Interferons	Interfere with viral reproduction; used to treat some cancers.
Interleukins	Activate and stimulate different classes of leukocytes; possible uses in treatment of wounds, HIV infection, cancer, and immune deficiencies.
Monoclonal antibodies	Extraordinary binding specificity is used in: diagnostic tests; targeted transport of drugs, toxins, or radioactive compounds to tumors as a cancer therapy; many other applications.
Superoxide dismutase	Prevents tissue damage from reactive oxygen species when tissues briefly deprived of O_2 during surgery suddenly have blood flow restored.
Vaccines	Proteins derived from viral coats are as effective in "priming" an immune system as is the killed virus more traditionally used for vaccines, and are safer; first developed was the vaccine for hepatitis B.