Molecular Genetics Techniques

BIT 220

Chapter 20

What is Cloning?
Recombinant DNA technologies

1. Producing Recombinant DNA molecule

Incorporate gene of interest into plasmid (cloning vector)

2. Recombinant molecule is cloned (replicated)

Amplification of plasmid by replication in host cell

Restriction Endonucleases

TABLE 20.1

•enzymes that cuts double-stranded DNA at RECOGNITION SEQUENCE

4 base cutters

6 base cutters

•Naming Protocol – after bacteria

•Staggered Cleavage vs. Blunt-end Cleavage

•Palindromes

•NOT species specific

•Need Ligase (T4 bacteriophage)

•forms phosphodiester linkage

FIGURE 20.1

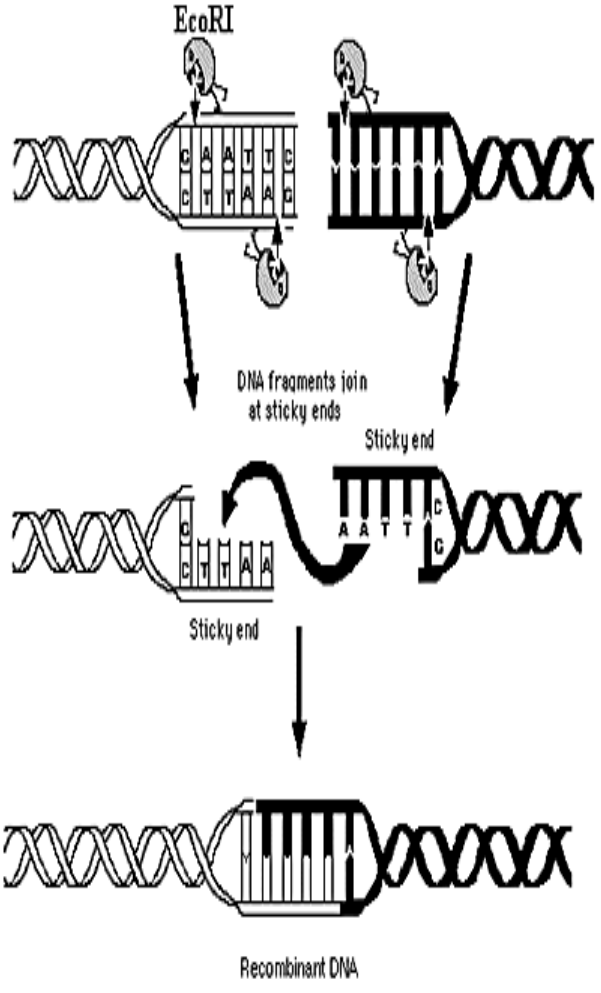
Why use REs?

1)Cloning a gene into a plasmid

2) Restriction Maps

Gene of interest can not have restriction site within its sequence

FIGURE 20.9



Restriction Enzyme
Action of EcoRI

Plasmids

- Naturally found in bacteria
- extrachromosomal
- small circular DNA
- self-replicating each time bacteria divides
- double stranded
- can hold extra genes – YOUR gene of interest

Cloning Vectors

FIGURE 20.2

Derived from naturally occurring plasmids or viruses

Scientists have engineered plasmids to carry these characteristics

FEATURES

A. Selectable marker

B. Unique restriction site

Multiple cloning site (polylinker)

FIGURE 20.3

C. Origin of replication

Plasmid Vector

1) small size (<10 kb for plasmid, also for size of insert in can hold)

2) pBR322 **FIGURE 20.4**

Ampicillin resistance gene
Tetracycline resistance gene
4361 bp
origin of replication -
(these are specific to species)
high copy number

High-Copy Number Plasmids
10-100 copies per host cell
growth vectors

Low-Copy Number
1-4 copies per cell
expression vectors

Other vectors

1. Bacteriophage Vectors

FIGURE 20.5

Vector is 45 kb
Accommodates inserts 10-15 kb

2. Cosmids

combination of lambda phage and plasmid

hold inserts 35-45 kb

FIGURE 20.6

3. Artificial Chromosomes YAC (yeast artificial Chromosomes - 500 kb inserts; BAC's also

Shuttle vectors

Species use different regulatory sequences
transcription and translational
ori – can vary
promoters also different

Shuttle Vectors often have regulatory elements for both prokaryotic and eukaryotic use

- created and amplified in E. coli
- expressed in mammalian cells

- **Figure 20.7- example of use**

Selection for *E coli* which contain plasmid

Insert at BamH1 site
disrupt amp^R gene

Transform into *E coli*

Grow on agar that contains amp.
If colonies grow cells contain plasmid

Types of Genes

A. Structural Genes transcribed and translated to make enzymatic protein

B. Operator Genes control structural genes

C. Regulator Genes indirectly control operator genes

OPERON

No Lactose Present

Lac Repressor protein

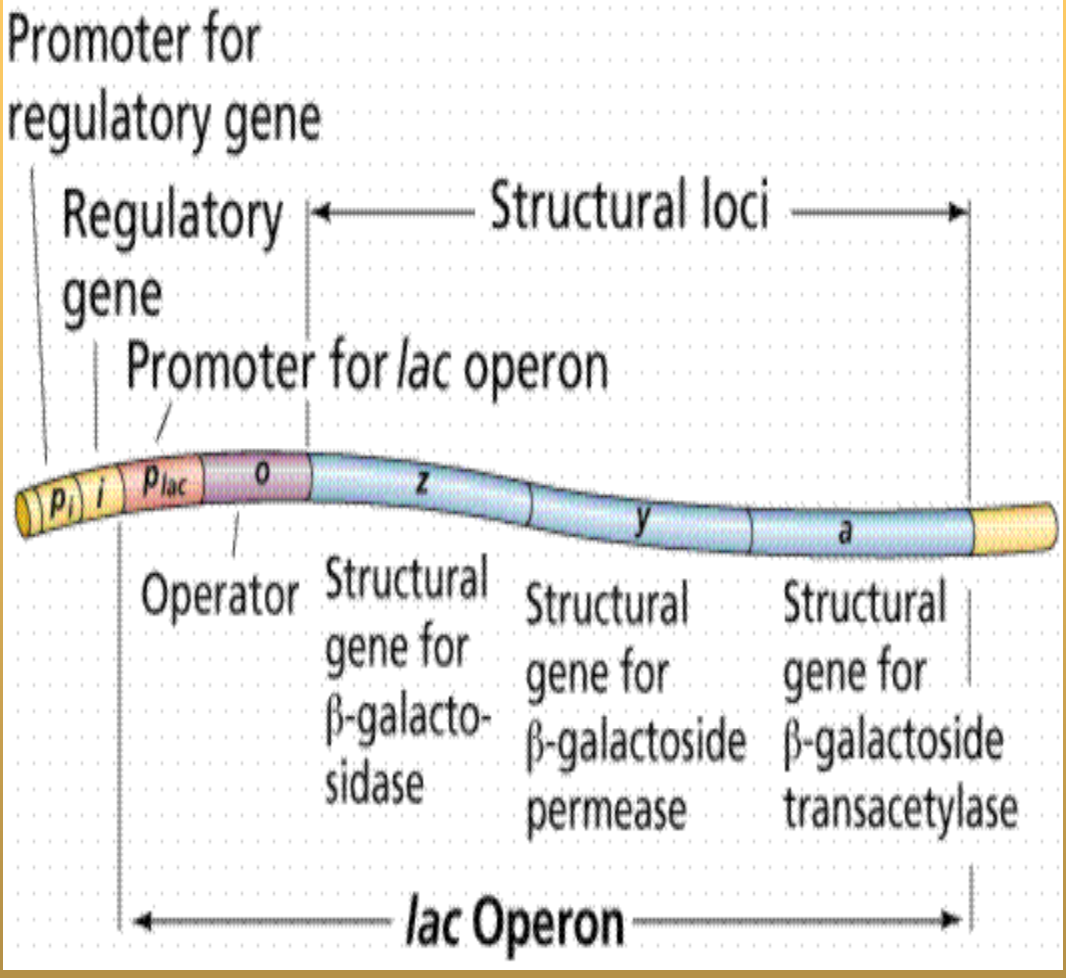
- made from regulatory gene (I)
- binds to operator
- RNA polymerase can NOT bind
- inhibits B-galactosidase transcription

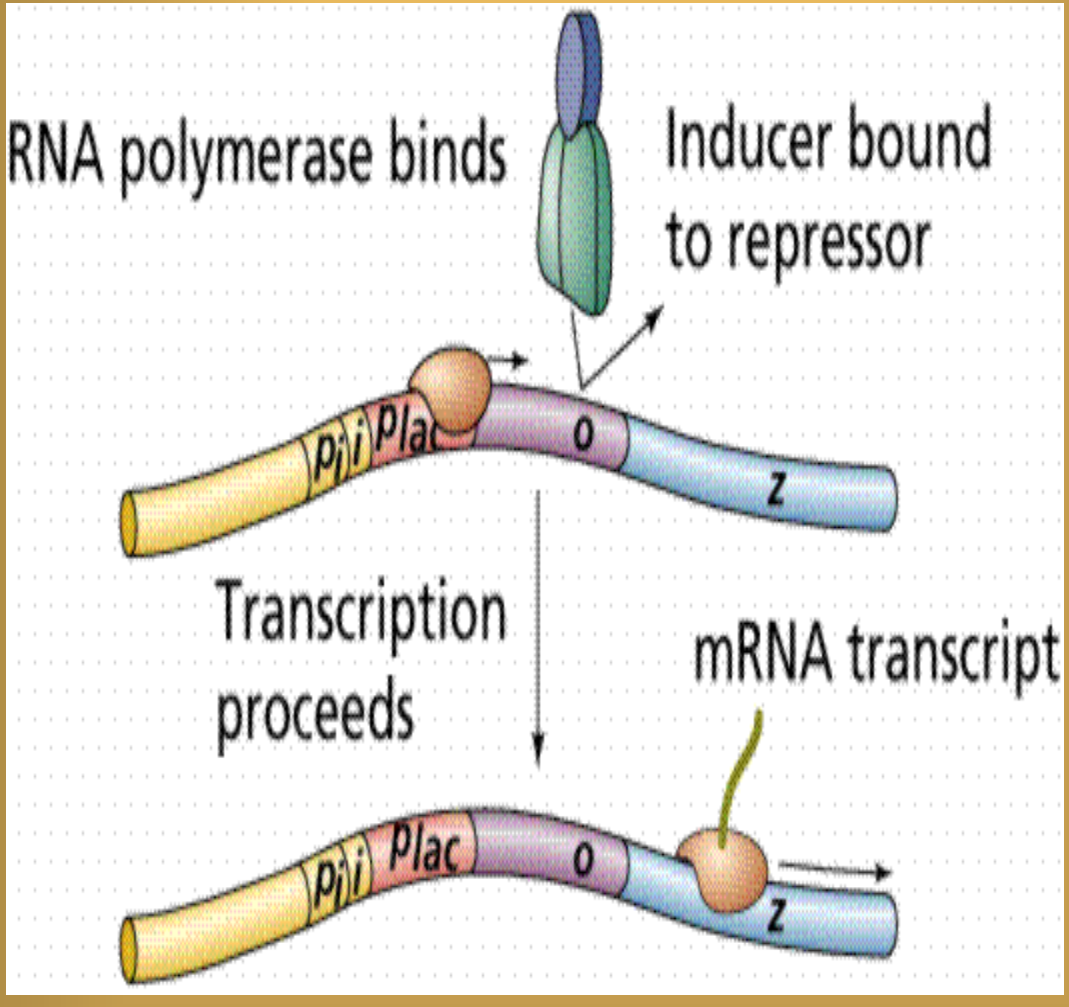
Lactose Present

Lactose - Inducer molecule

- Lactose (IPTG) binds to lac repressor protein
- lac repressor protein can not bind to operator
- RNA polymerase binds to promoter
- B galactosidase is transcribed/translated
- X-gal is cleaved
- Cells turn BLUE

Transcriptional Control




RNA polymerase binds
Inducer bound to repressor
Transcription proceeds
mRNA transcript
Plac
O
Z
Plac
O
Z

pUC19

Amp R

lacI gene: Repressor product

lac Z gene: B-galactosidase

IPTG: inducer of lac operon

A. Grow on
- Ampicillin those with plasmid (transformed cells) grow
- IPTG
- X gal

B. Unmodified plasmid - blue colonies
- With insert - white colonies
- insert disrupts lacZ gene

No Insert

IPTG induces the lac operon

Lac Z gene produces part of β gal

β -gal cleaves X gal

Colonies Turn Blue

WITH INSERT

Gene of interest inserted at MCS

Interrupts LacZ gene

B gal can NOT be made

X gal can NOT be cleaved

White Colonies

Genomic Library

Definition:

DNA clones which collectively contain all of the genomic DNA of the source organism

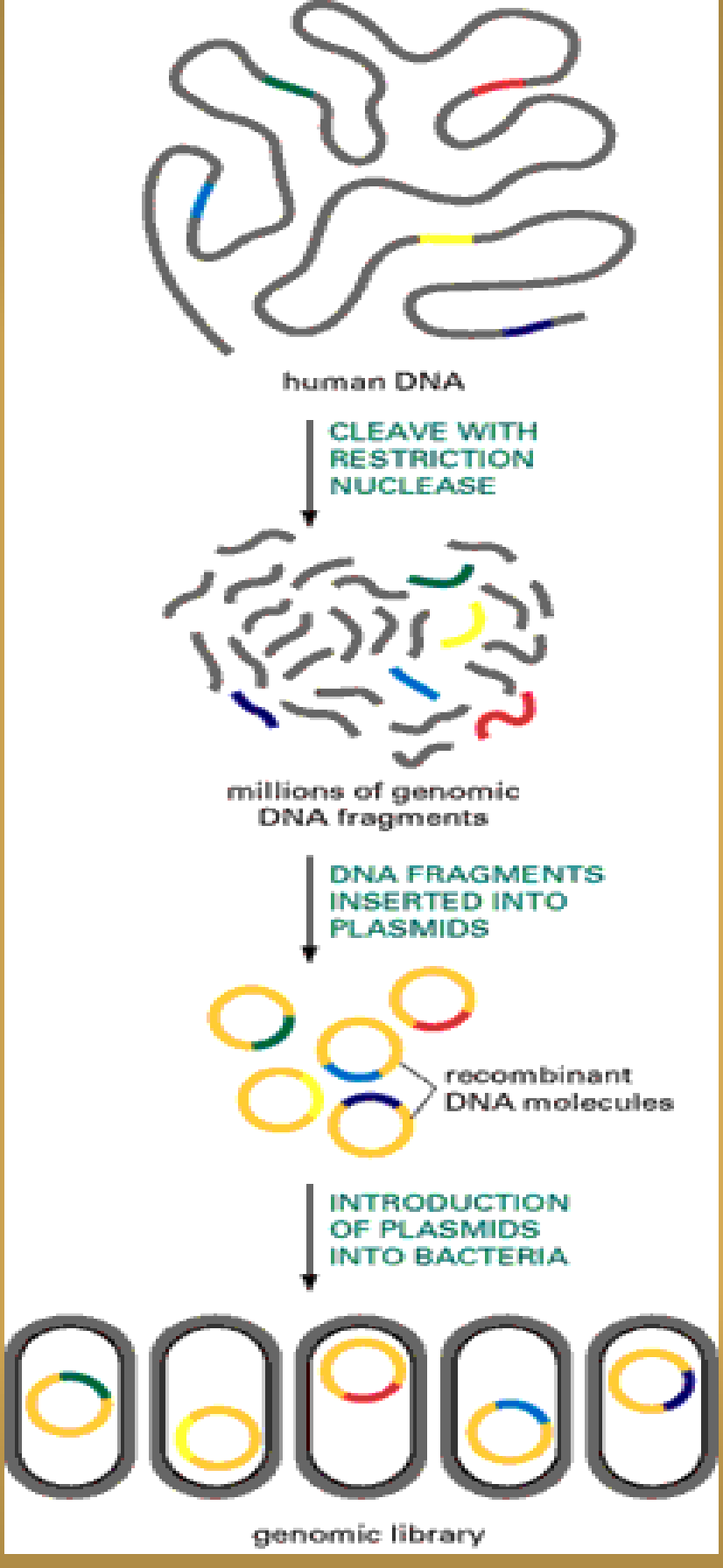
A. Genomic DNA library
B. cDNA library **FIGURE 20.11**

Procedure:

A. Cut entire genome with RE
B. Clone all fragments into vectors
C. Transform cells

Identification of Genes???

A. DNA hybridization
B. Immunological screening
antibody against protein
C. Gene Selection
Complementation Screening


human DNA
CLEAVE WITH RESTRICTION NUCLEASE
millions of genomic DNA fragments
DNA FRAGMENTS INSERTED INTO PLASMIDS
recombinant DNA molecules
INTRODUCTION OF PLASMIDS INTO BACTERIA
genomic library

Hybridization-Screening Libraries **FIGURE 20-12**

1. Plate bacteria on agar

2. Replica plate

3. Lyse cells

4. Denature double-stranded DNA

5. Transfer to filter (Nitrocellulose)

6. Incubate with a labeled probe
   - 100-1000 bp
   - 80% match over 50 base pairs

Where do we get probe?

DNA from related organism

Chemically Synthesize it from AA sequence

Chemical Synthesis of DNA

Gene Machines OR DNA synthesizers

-automated chemical reactions which synthesize single-stranded oligonucleotides (50)

USES:

1) hybridization probes

2) primers for PCR

3) linkers for cloning

4) alter sequences of clones genes
   mutagenesis
   codon optimization

Sequencing of Nucleic Acids

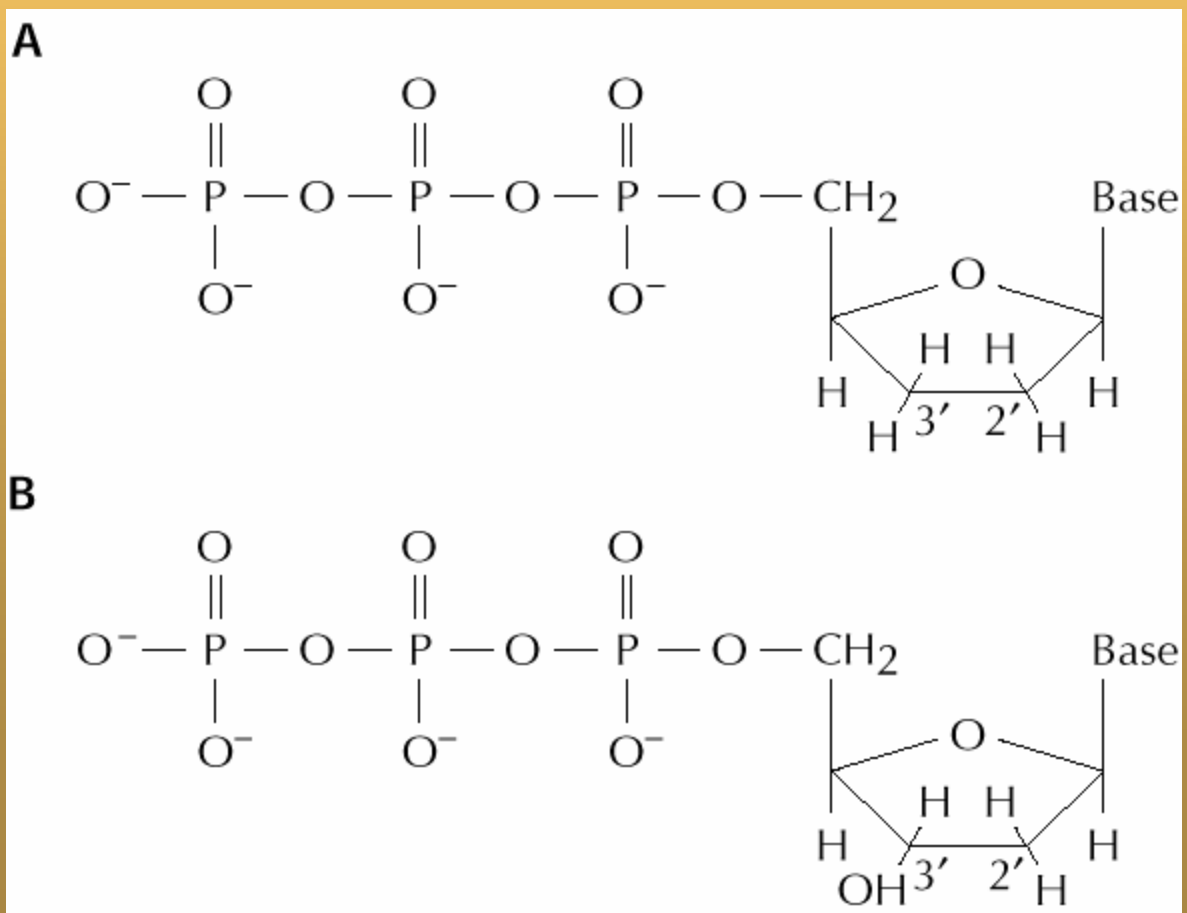
Sanger Method

enzymatic

dideoxynucleotide method

Maxam and Gilbert

chemical procedure



Sequencing protocol

DNA template to be sequenced

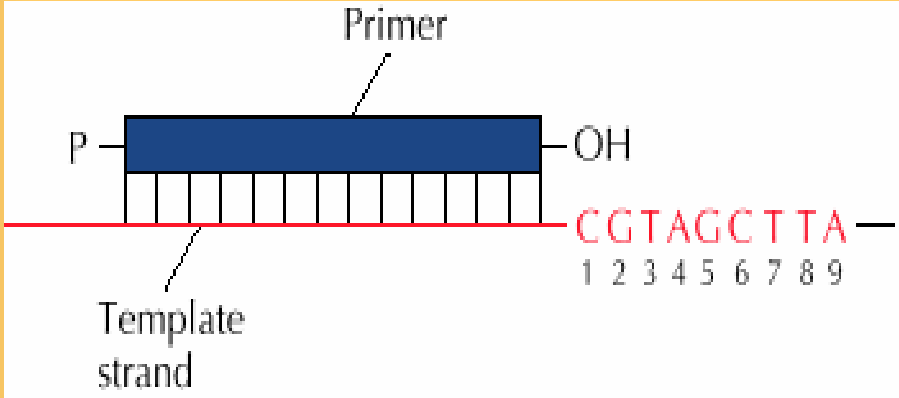
Primer - complementary sequence (17-24-mer)
to beginning of template

DNA polymerase

4 dNTPs

One radioactive dNTP

All tubes have all previously mentioned reactants
Did- dideoxynucleotide – missing other oxygen
In Tube 1: didATP
Tube 2 : didTTP
Tube 3 : didGTP
Tube 4 : didCTP

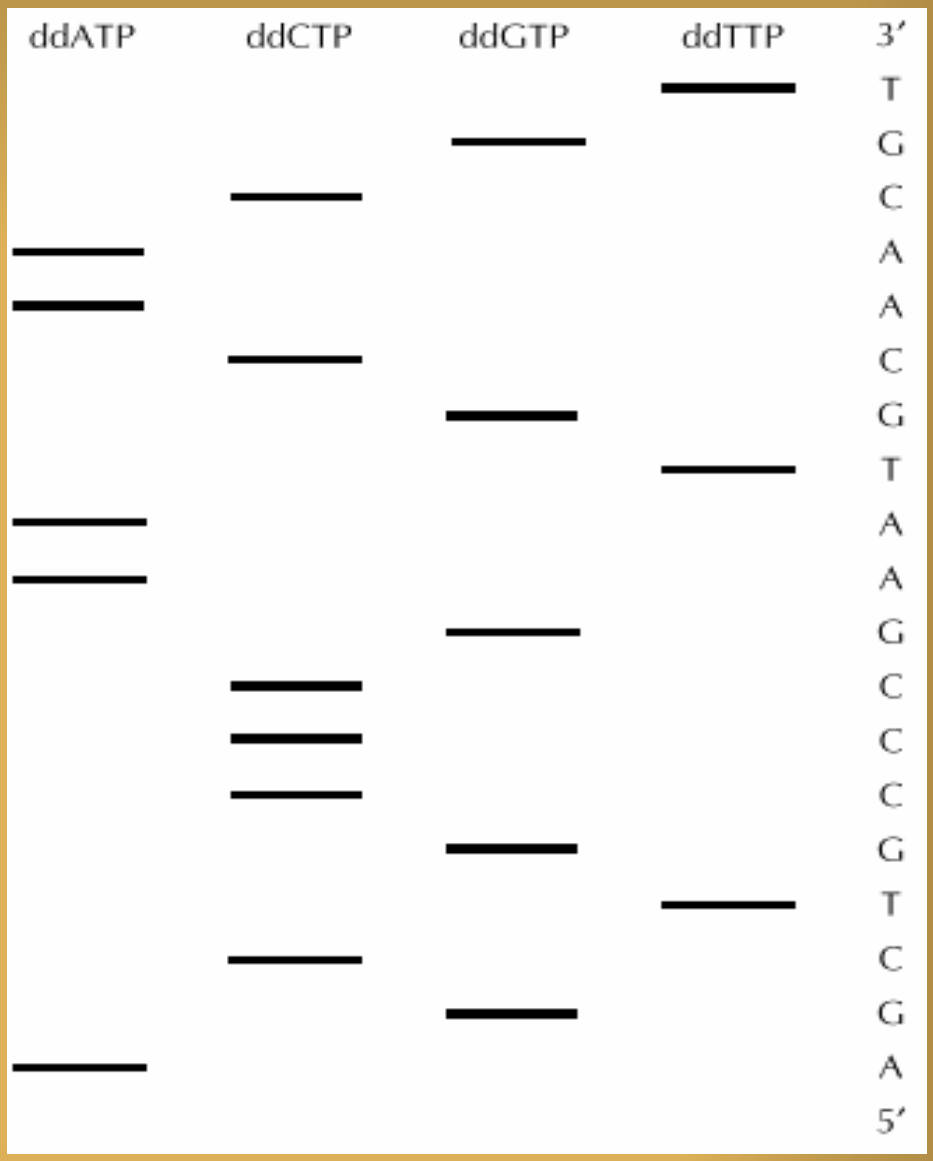


Contents of reaction tube	Size of primer and extension	Primer and sequence of extension
ddATP + four dNTPs	Primer + 3	Primer – dGdCddA
	Primer + 7	Primer – dGdCdAdTdCdGddA
	Primer + 8	Primer – dGdCdAdTdCdGdAddA
ddCTP + four dNTPs	Primer + 2	Primer – dGddC
	Primer + 5	Primer – dGdCdAdTddC
ddGTP + four dNTPs	Primer + 1	Primer – ddG
	Primer + 6	Primer – dGdCdAdTdCddG
ddTTP + four dNTPs	Primer + 4	Primer – dGdCdAddT
	Primer + 9	Primer – dGdCdAdTdCdGdAdAddT

Autoradiograph

Read bottom to top

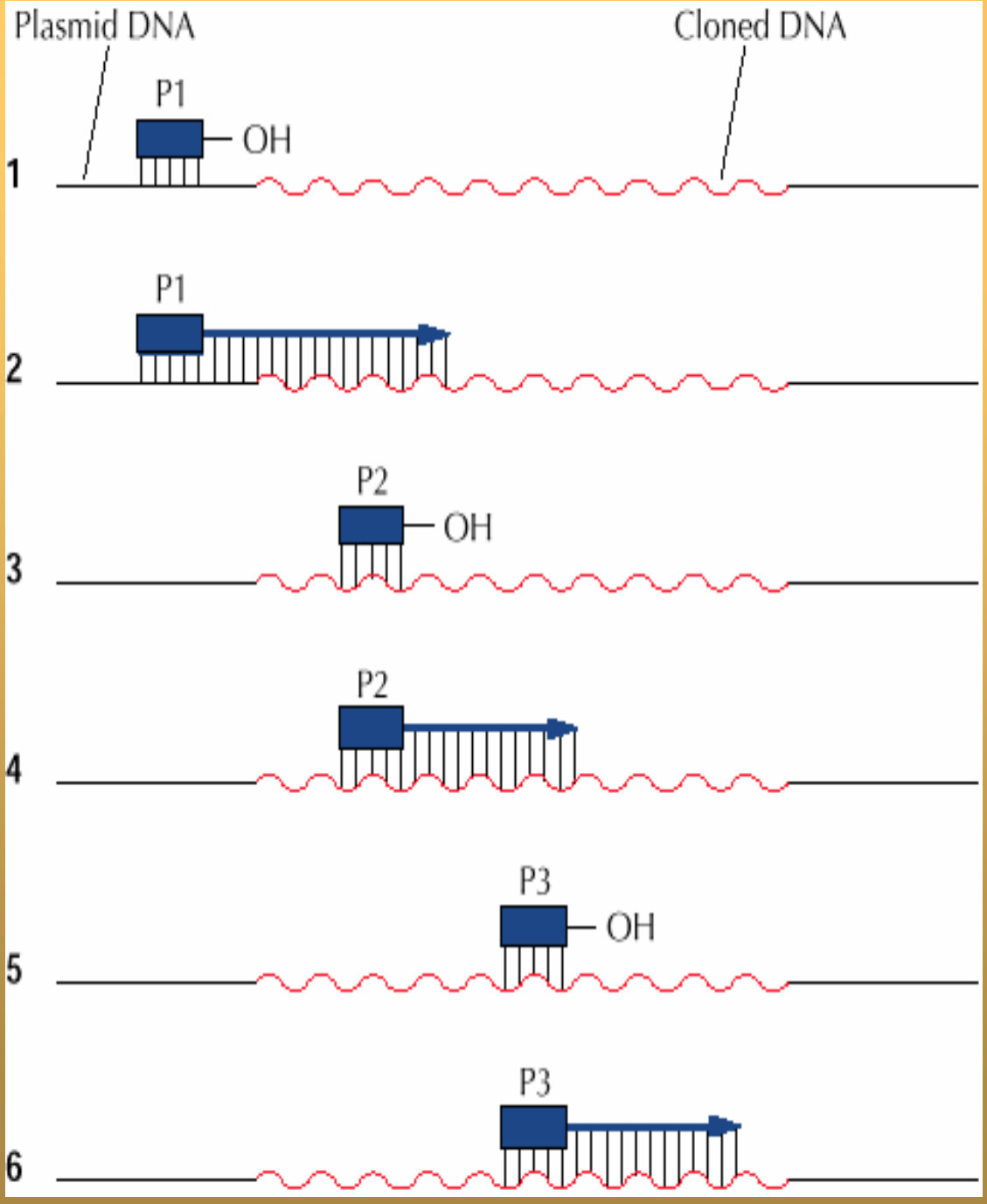
Deduce complement strand



250-350 nt can be sequenced per autoradiograph

For very large pieces of DNA (5000 bp) -
use PRIMER WALKING

Primer Walking



Polymerase Chain Reaction

Amplify a single piece of DNA to make rare sequences abundant

Reactants

- original piece of DNA (double stranded)
- primer (second strand)
- nucleotides
- DNA polymerase (Taq)
  - isolated from bacterium
  - thermostable

PCR
Figure 20.24

Procedure:

1. Denature double stranded DNA with high temp
   95^{o}C for 1 minute

2. Renature (Anneal)
   Cool reaction 55^{o}C
   primers attach

3. Synthesis:
   Raise temp to 75^{o}C
   complementary strands are synthesized

4. Repeat Heat /Cooling Cycle
   (each cycle 3-5 minutes)

Uses of PCR

1. Generate cDNA from mRNA
2. Detect mutations
3. To produce mutations
4. For DNA sequencing
5. Assemble whole genes from synthetic oligo

Blots

1. Electrophoreses
   agarose, acrylamide (smaller)

2. Transfer **FIGURE 20.19**
   nitrocellulose
   nylon

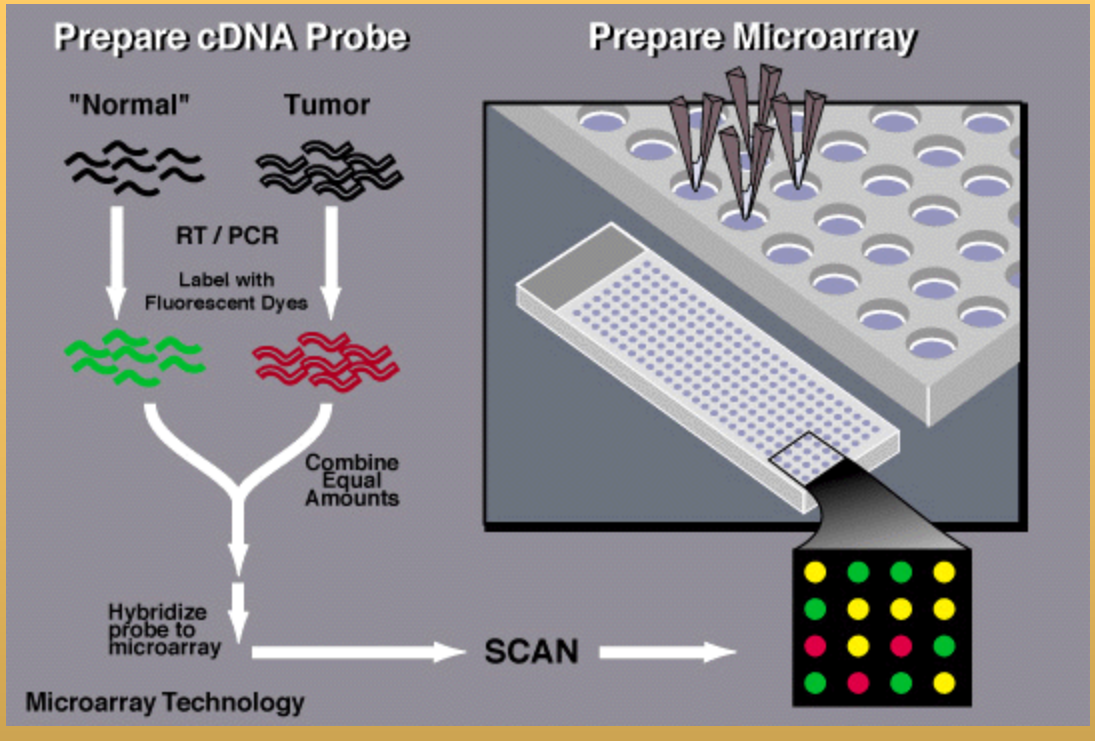
3. Probe

A. Southern-DNA

B. Northern RNA
   which genes are being expressed

C. Western-protein
   FIGURE 20.22

Microarray Technology



RFLPs

- Restriction fragment length polymorphisms
- Help find a change in the sequence by adding/eliminating a restriction site
- E.g., GAATTC –Glu/Phe also site for Eco RI
- GAATAC – eliminates EcoR1 site and also now amino acids are Glu/Tyr
- Can predict changes in sizes expected when probed
- Do example on board

Double Digests of DNA

- Go over Figure 20.25 and Problem 20.25, page 512.

- How to determine order of restrictions sequences when DNA is digested with one or more restriction enzymes.