Lecture 3

DNA recombinant techniques (part 1):

- Restriction enzymes
- Cloning vectors
- Restriction mapping
- BACs, YACs
- gDNA/cDNA libraries
- DNA/RNA hybridization

Required reading for lectures 3-5: Chapters 7 and 8

Isolating genes for study aka gene cloning, involves a number of different enzymes

TABLE 7-1

Some Enzymes Used in Recombinant DNA Technology

Enzyme	*Function*
Type II restriction endonuclease	Cleaves DNA at specific base sequences
DNA ligase	Joins two DNA molecules or fragments
DNA polymerase I (*E. coli*)	Fills single-stand gaps in duplex DNA by stepwise addition of nucleotides to 3′ ends
Reverse transcriptase	Makes a DNA copy of an RNA molecule
Polynucleotide kinase	Adds a phosphate to the 5′-OH end of a polynucleotide, to label it or permit ligation
Terminal transferase	Adds homopolymer tails to the 3′-OH ends of a linear duplex
Exonuclease III	Removes nucleotide residues from the 3′ ends of a DNA strand
Bacteriophage λ exonuclease	Removes nucleotides from the 5′ ends of a duplex to expose single-stranded 3′ ends
Alkaline phosphatase	Removes terminal phosphates from the 5′ end, the 3′ end, or both

Table 7-1
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Genes are cloned by insertion into cloning vectors

DNA cloning involves:

- separating a specific gene or DNA segment from a larger chromosome
- Attaching it to a small molecule of carrier DNA
- Introducing this modified DNA into a host cell
- Produce more copies of DNA
- Selective amplification of gene of interest

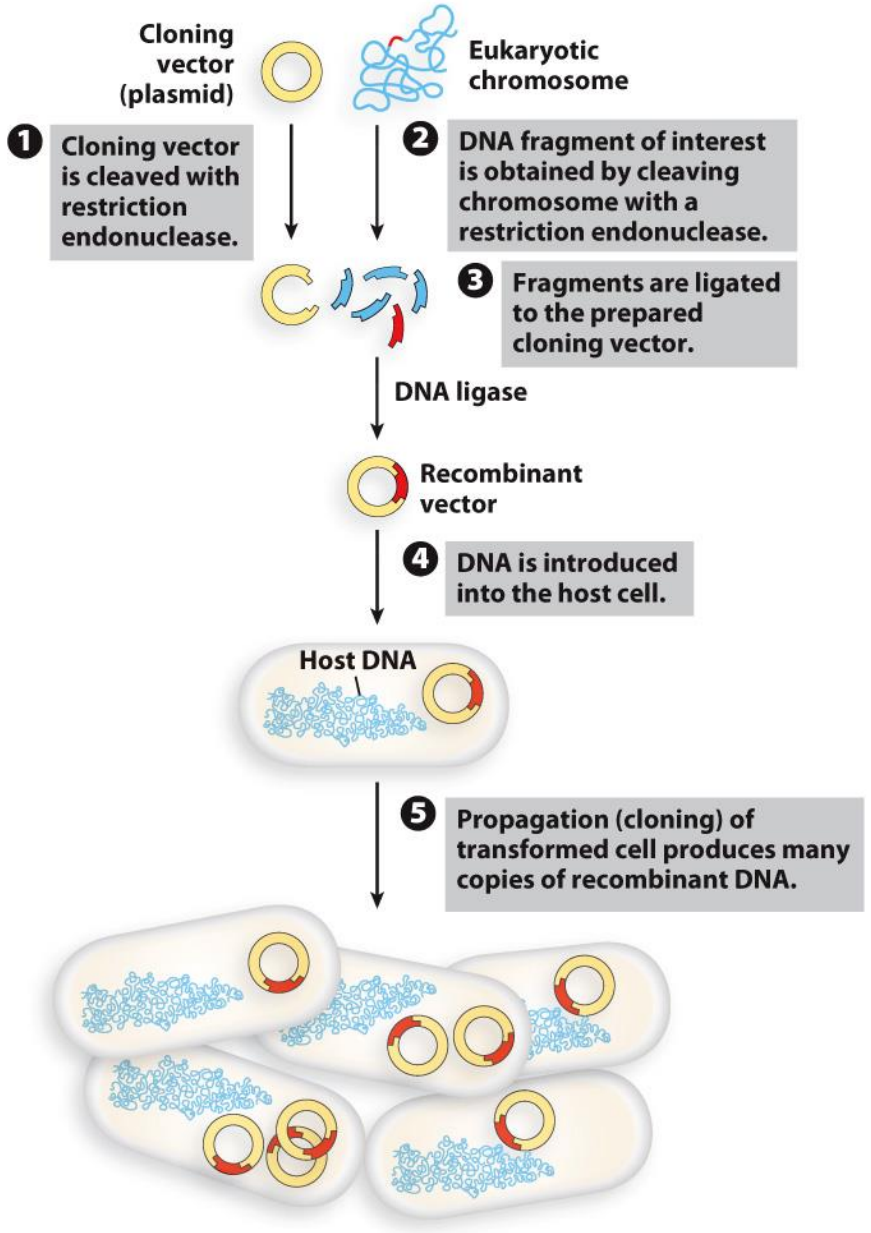

Figure 7-1
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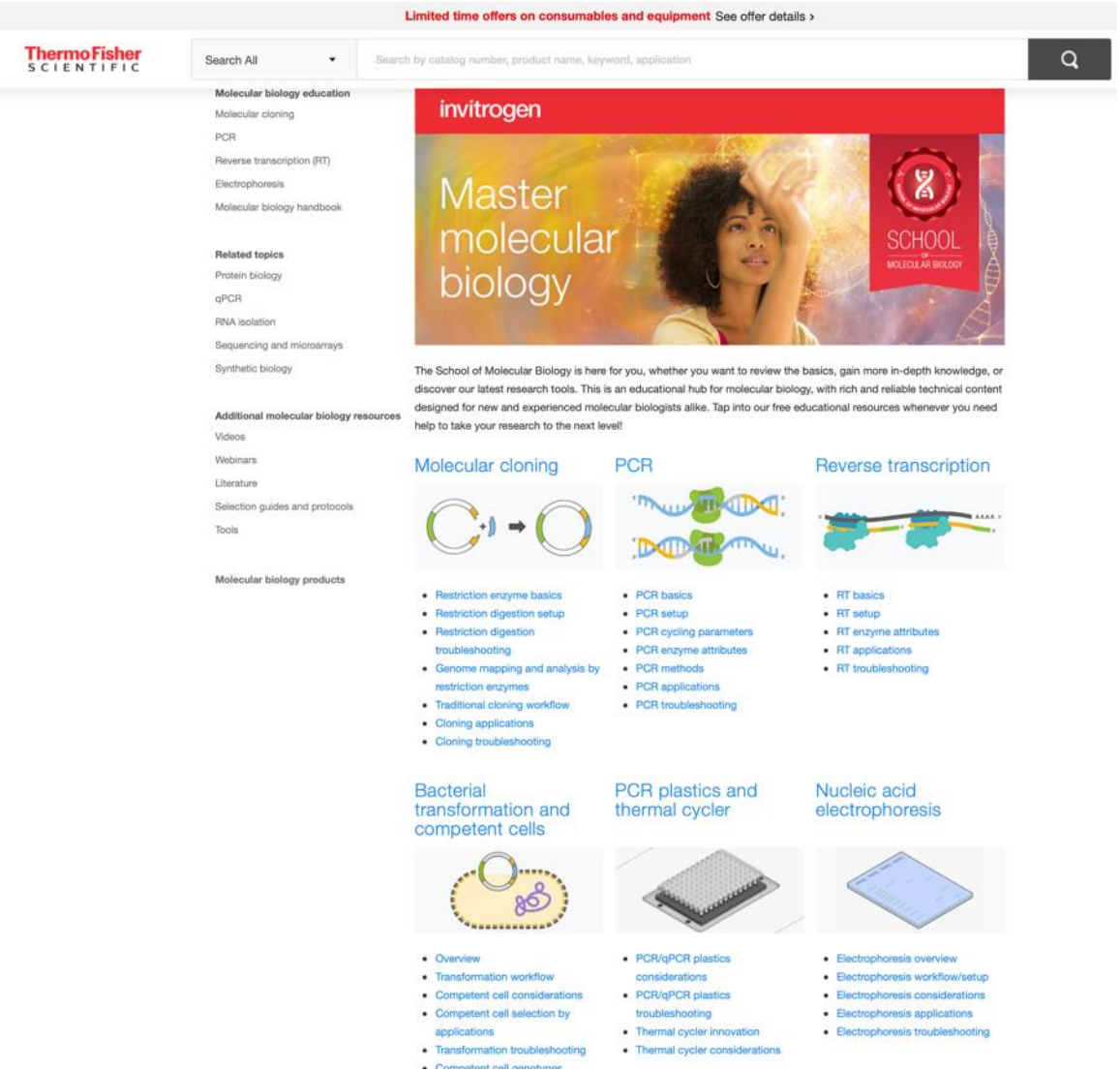
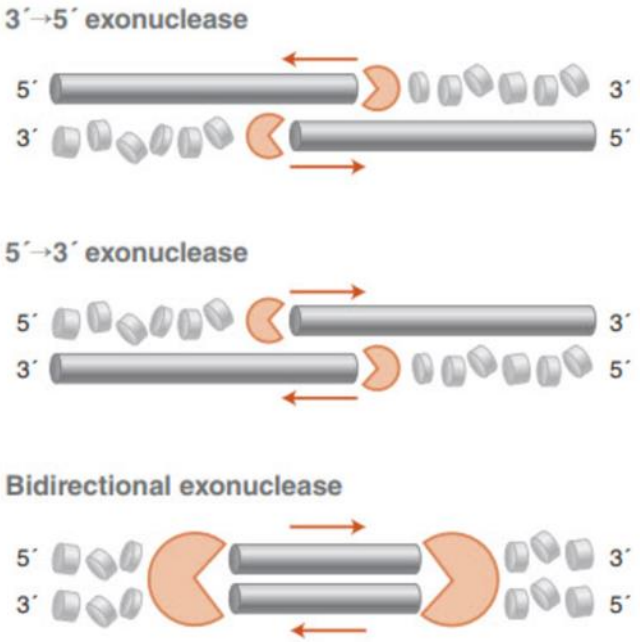
There are many online resources and tools made available by the companies that manufacture and sell products/enzymes/reagents used in MB



examples:
thermofisher.com
https://international.neb.com



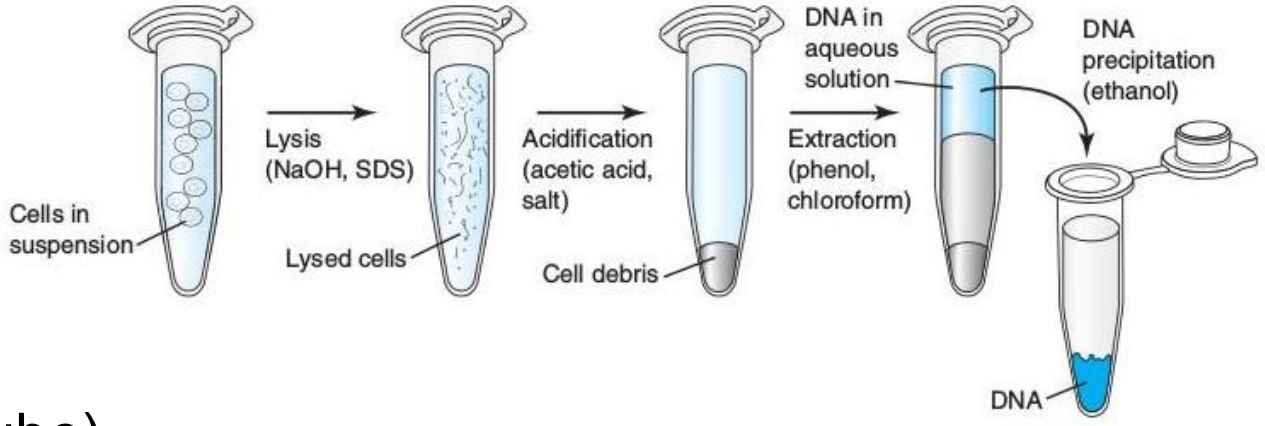
DNA ISOLATION

All DNA molecules (regardless of what RNA/proteins they encode) have the same chemical nature (A, T, C, G, phosphate backbone, two strands in double helix)

So, to purify any DNA from cells:

1. Lyse cells (use detergent, soap)

2. Remove debris (larger organelles, unbroken cells, membranes) after centrifugation, or by simply let heavy particles settled on the bottom of the tube)



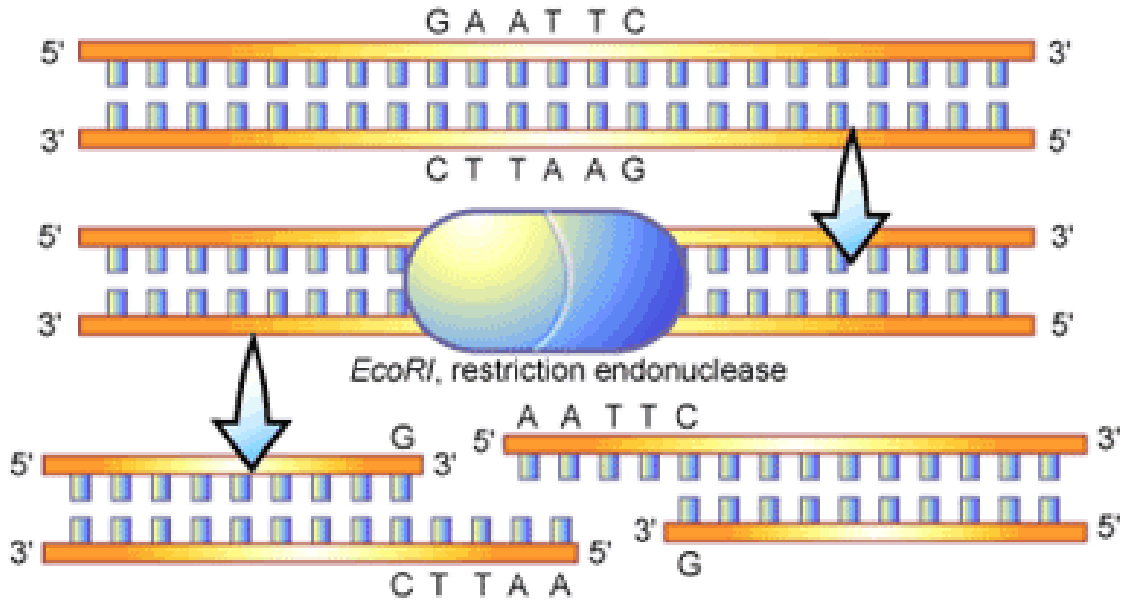
3. Separate DNA from protein (e.g. phenol extraction & CsCl gradient centrifugation)

4. Concentrate by ethanol precipitation (ice cold 95%) and centrifugation

RECOMBINANT DNA METHODS

Restriction Endonucleases

- If you wanted to study a gene of interest, you would need to perform some type of gene cloning
- This involves isolating your gene of interested, cutting it and joining it with some other DNA such as a vector
- Restriction endonucleases allow us to cut DNA at sequence specific locations



Why are they called <u>ENDO</u>nucleases?

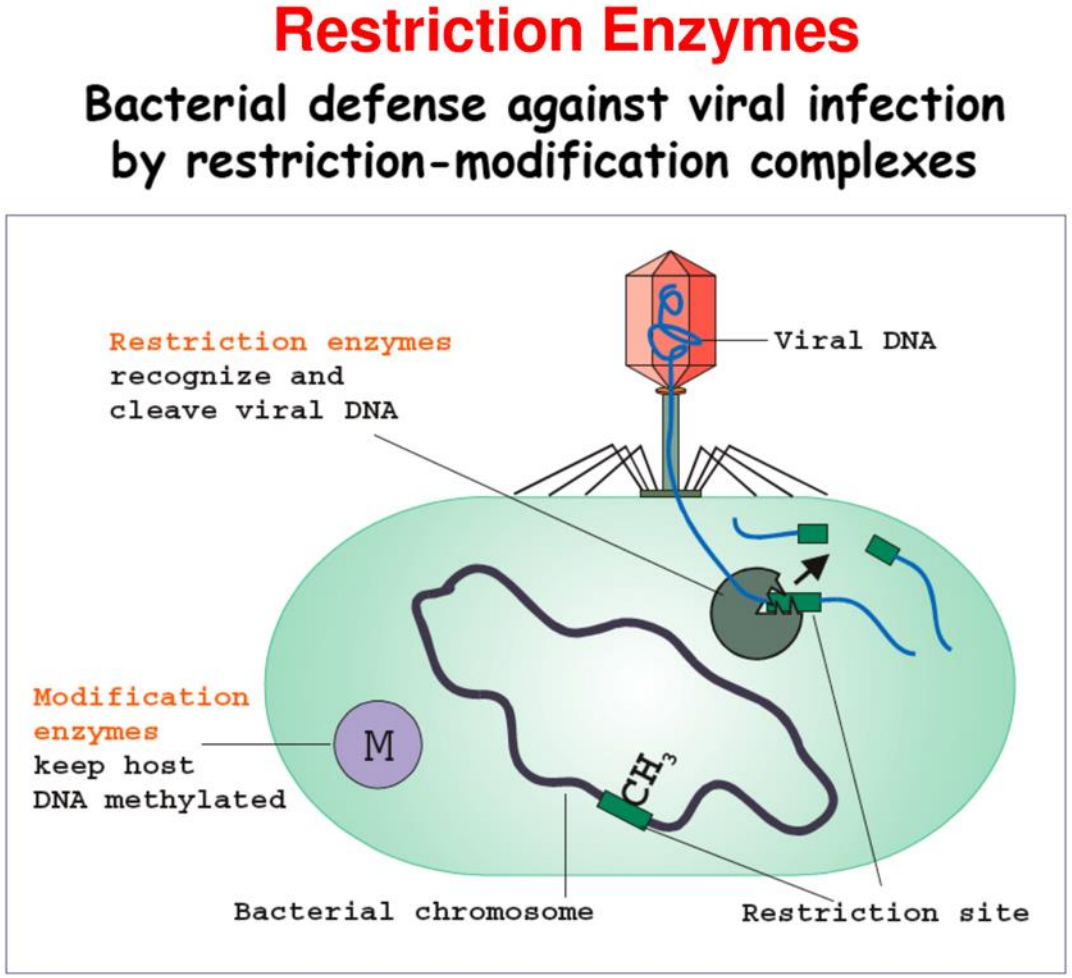
RESTRICTION ENDONUCLEASES (REs)

First isolated in 1962 by Steward Linn and Werner Arber. Their work was based on observations of bacteriophage infection cycles where it was noted that **some *E. coli* strains were not susceptible to phage infection**.

The bacteriophages had a restricted host range and could only replicate in certain strains of bacteria.

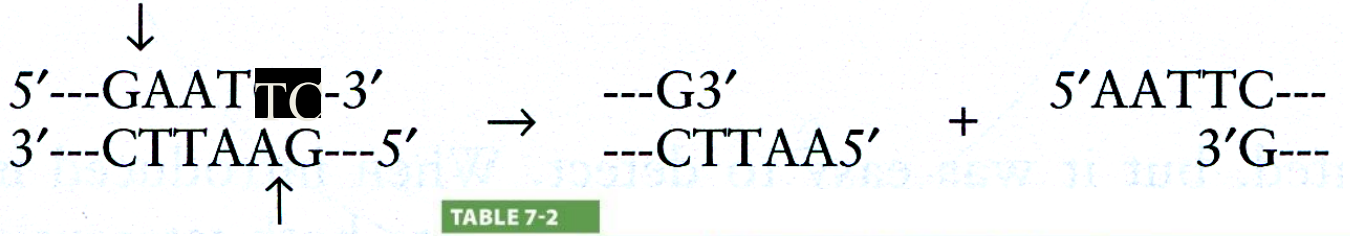
Using extracts from *E. coli*, Arber's research group showed that a bacterial (i.e. *E. coli*) **enzyme cut the phage DNA** into fragments, thus disrupting viral replication.

Named because they prevent invasion by foreign DNA by cutting phage DNA upon infection



Watch: https://www.youtube.com/watch?v=aA5fyWJh5S0 and https://www.youtube.com/watch?v=rhd_fBPyzSM

RESTRICTION ENDONUCLEASES (REs)



REs cut DNA like scissors cut paper = generate DNA fragments

- *E. coli* type II endonucleases cleave DNA at specific sites in bacteria
- Type II restriction enzymes cut specific 4 or 6 bp sequences - usually palindromes (*e.g.* GAATTC)
- Type I and Type III endonuclease also exist and are used but they cut at sites a certain distance away from the recognition sequence.

TABLE 7-2

Recognition Sequences for Some Type II Restriction Endonucleases

BamHI	5′-GGATCC-3′ CCTAGG	HindIII	5′-AAGCTT-3′ TTCGAA
ClaI	5′-ATCGAT-3′ TAGCTA	NotI	5′-GCGGCCGC-3′ CGCCGGCG
EcoRI	5′-GAATTC-3′ CTTAAG	PstI	5′-CTGCAG-3′ GACGTC
EcoRV	5′-GATATC-3′ CTATAG	PvuII	5′-CAGCTG-3′ GTCGAC
HaeIII	5′-GGCC-3′ CCGG	Tth111I	5′-GACNNNGTC-3′ CTGNNNCAG

Note: **Arrows denote phosphodiester bonds cleaved by each restriction endonuclease. Asterisks mark bases that are methylated by the corresponding methyltransferase (where known). N denotes any base. Each enzyme name consists of a three-letter abbreviation of the bacterial species from which it is derived, sometimes followed by a strain designation and a Roman numeral to distinguish restriction endonucleases isolated from the same bacterial species or strain. Thus, BamHI is the first (I) restriction endonuclease characterized from *Bacillus amyloliquefaciens*, strain H.**

Table 7-2
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RESTRICTION ENZYMES TERMINOLOGY

- ***How are they named?*** The first 3 letters in an enzyme's name are italicized and indicate species of bacterium from which this enzyme has been isolated; other letters denote strain of this bacterium

- Roman numerals indicated enzyme no. (usually, order of isolation)

"Sticky end" generators

***Eco* RI** (from *Eschericia coli* strain R, first enzyme isolated, so I)

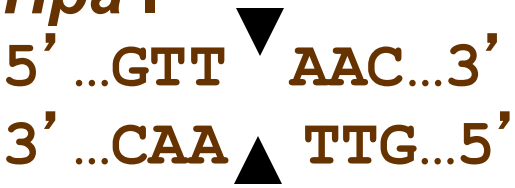
leaves 5' overhangs

***Kpn* I**

leaves 3' overhangs

"Blunt-end" generators

***Hpa* I**



leaves no overhang

At home: PRACTICE QUESTION

Pick two enzymes to cut the following DNA sequence:

5’ AGCGTGCGATTGAATTCAAGCTTCCCGGG 3’

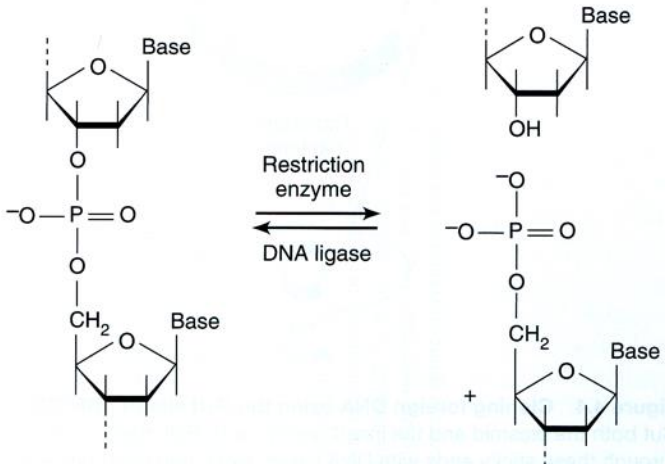
Note that by convention a sequence of DNA is given as ss (its complement should be obvious).

Restriction enzymes work ONLY with dsDNA, so their recognition sequences are also written (by convention) as single stranded sequence and always in the 5’ to 3’ direction.

Enzyme	Recognition Sequence
*Alu*I	A G ↓ C T
*Bam*HI	G ↓ G A T C C
*Bgl*II	A ↓ G A T C T
*Cla*I	A T ↓ C G A T
*Eco*RI	G ↓ A A T T C
*Hae*III	G G ↓ C C
*Hin*dII	G T Py ↓ Pu A C
*Hin*dIII	A ↓ A G C T T
*Hpa*II	C ↓ C G G
*Kpn*I	G G T A C ↓ C
*Mbo*I	↓ G A T C
*Pst*I	C T G C A ↓ G
*Pvu*I	C G A T ↓ C G
*Sal*I	G ↓ T C G A C
*Sma*I	C C C ↓ G G G
*Xma*I	C ↓ C C G G G
*Not*I	G C ↓ G G C C G C

What do we do with DNA that has been cut with restriction enzymes?

Sticky and blunt ends generated by restriction enzymes can be ligated by DNA ligase (requires ATP)



***Eco* RI**

5’ …G AATTC…3’
3’ …CTTAA G…5’

Ligase

Specific ligation

***Kpn* I**

5’ …GGTAC C…3’
3’ …C CATGG…5’

Ligase

Specific ligation

***Hpa* I**

5’ …GTT AAC…3’
3’ …CAA TTG…5’

Ligase

Non-specific ligation

What pieces of DNA are ligating together?

Generally speaking, a gene of interest is ligated to a plasmid/vector cut with the same restriction enzymes

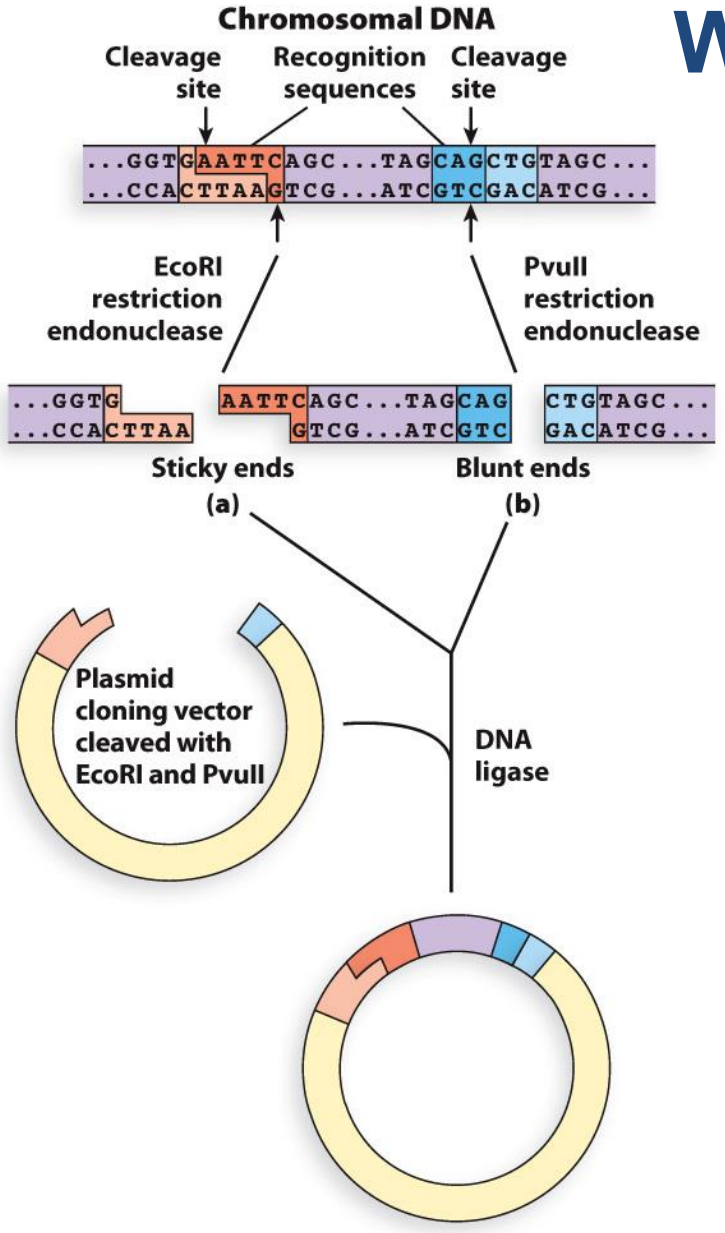


Figure 7-2
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Cloning vectors contain a region known as a **polylinker** or **multiple cloning site (MCS)** that contains recognition sequences for several restriction endonucleases.

These are often (almost always) included into plasmids to aid in cloning.

Provide the site in the vector where you will be inserting your DNA of interest.

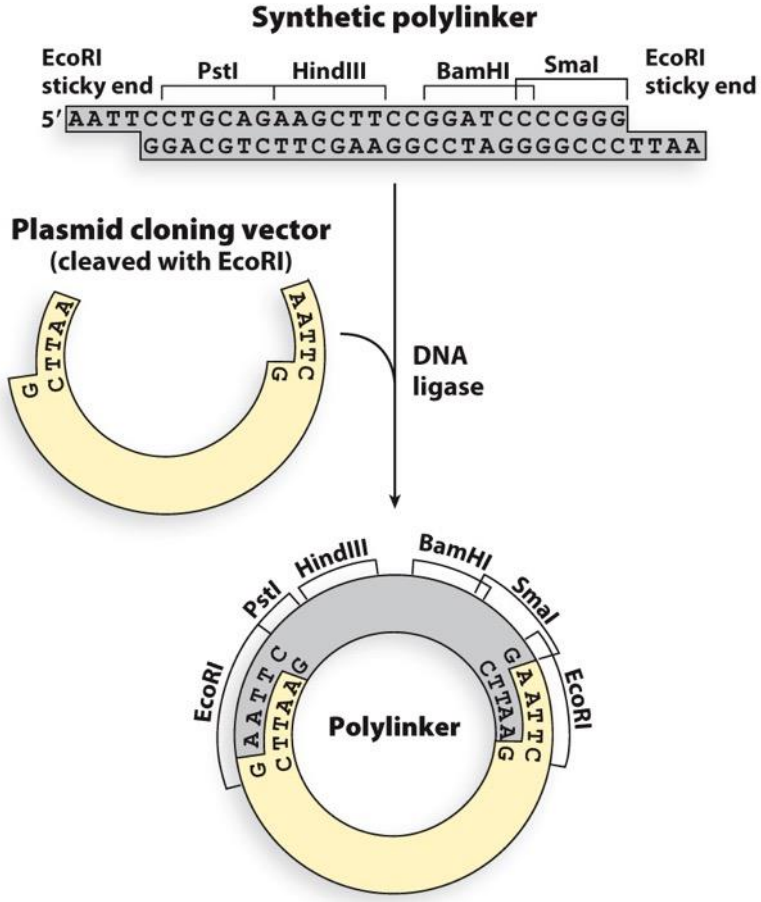

Figure 7-3
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Plasmids

- A plasmid is a circular piece of dsDNA that replicates separately from the host chromosome
- Plasmids are naturally occurring but the ones used for gene cloning are engineered
- Plasmids have features that enable them to grow and survive in a bacterial or eukaryotic host
- This allows them to be extremely useful to researchers

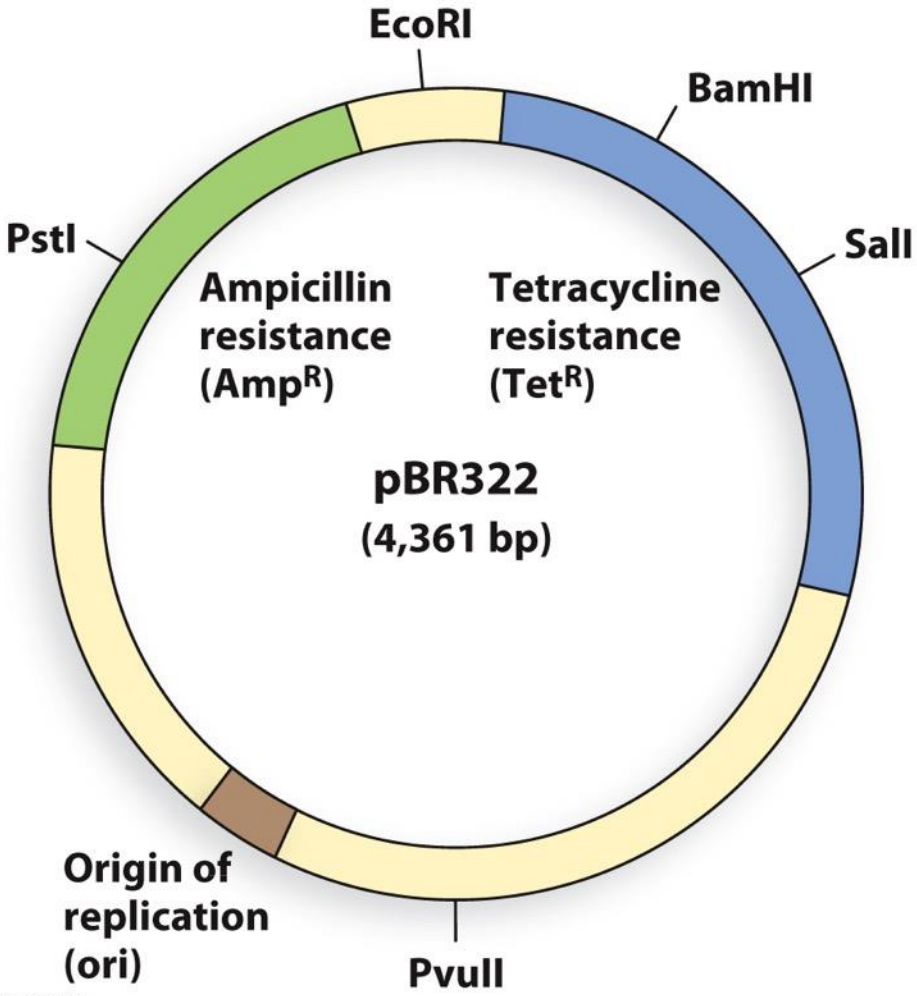

Figure 7-4
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Plasmids

Typical features of plasmids:

- Replication origin
- Plasmid selection markers
- Recognition sequences that are targets for restriction endonucleases
- Small in size

The basepairs within the plasmid are numbered and this is use to help determine position

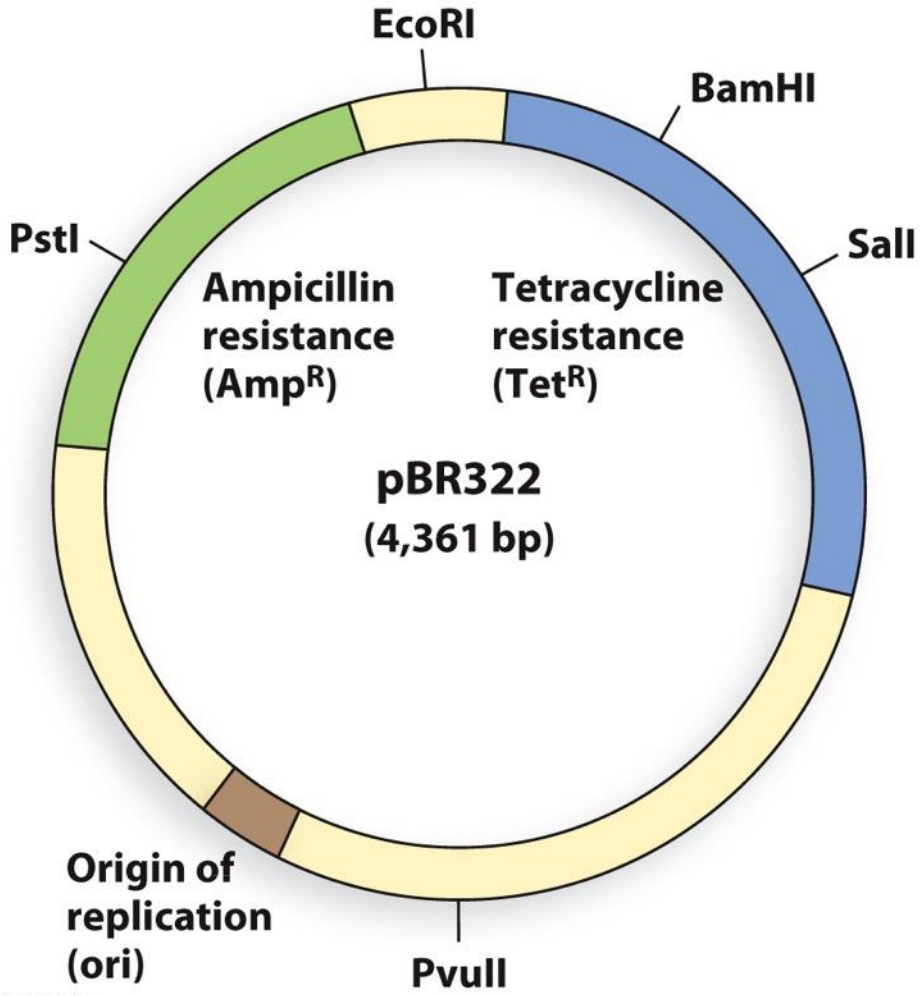

Figure 7-4
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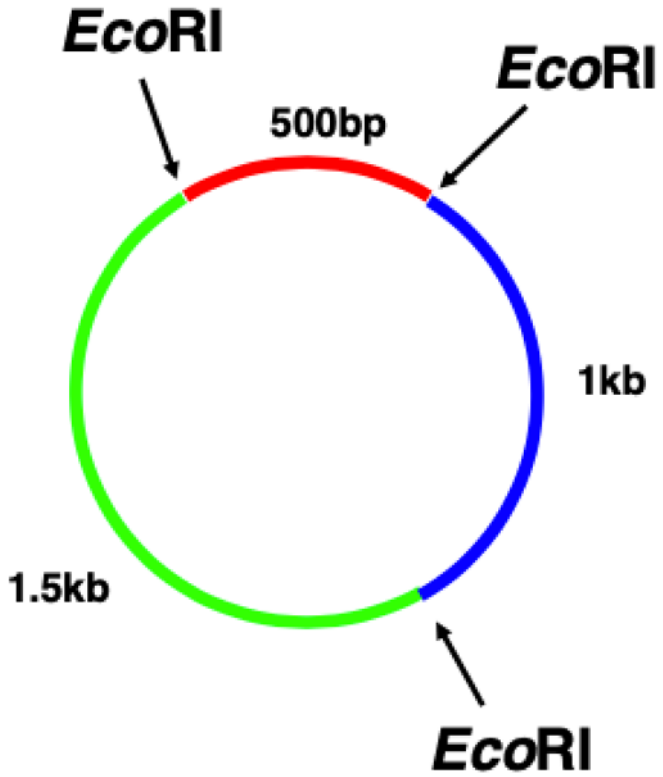
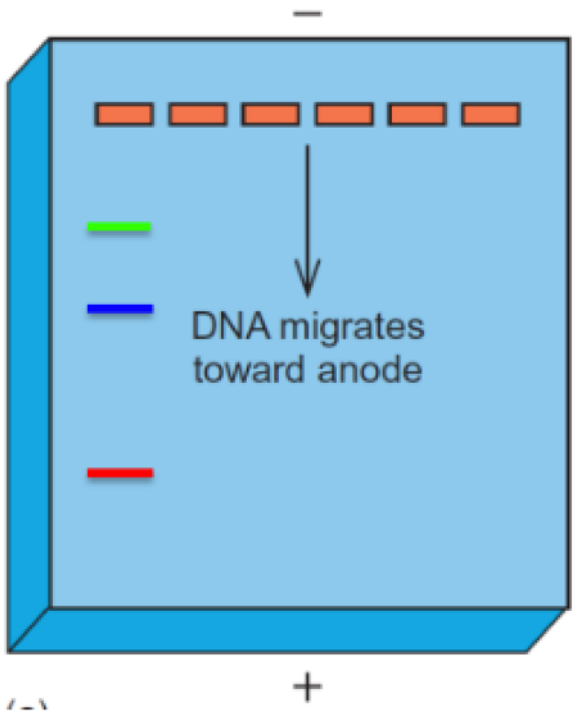
Insertional inactivation of antibiotic resistant genes is possible

Gel electrophoresis is used to solve cloning problems

Recall that DNA is negatively charged because of the phosphates and so migrates toward the anode in an agarose gel



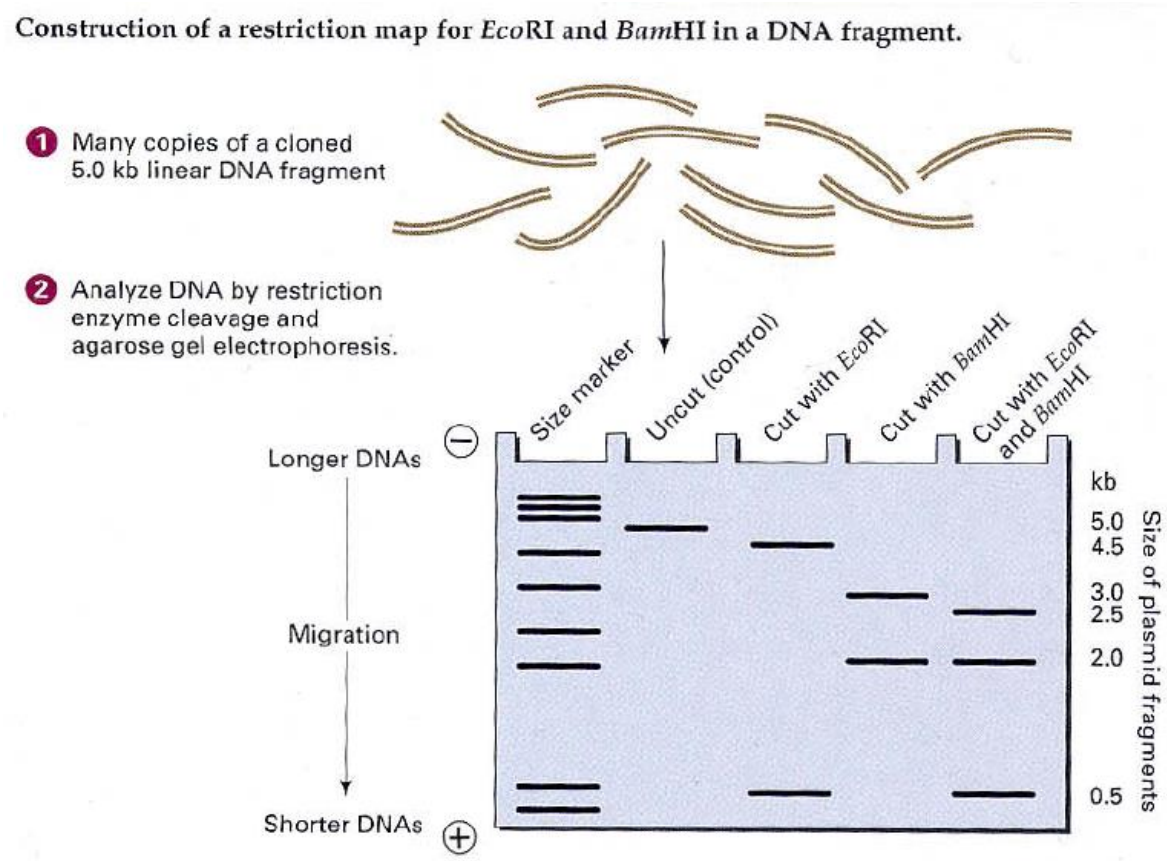


RESTRICTION MAPPING

- In the early days of molecular biology, for un-sequenced DNA, restriction maps generated a useful, **physical representation of linear or circular DNA fragments and pointed the location of various genes** (hence the term “mapping”)

- General procedure: choose restriction enzymes and complete single digests of your DNA with each enzyme as well as all possible combination of digests

- Run all digests on the agarose gel and compare with size standards



Russell, Fundamentals of Genetics, 2nd edition

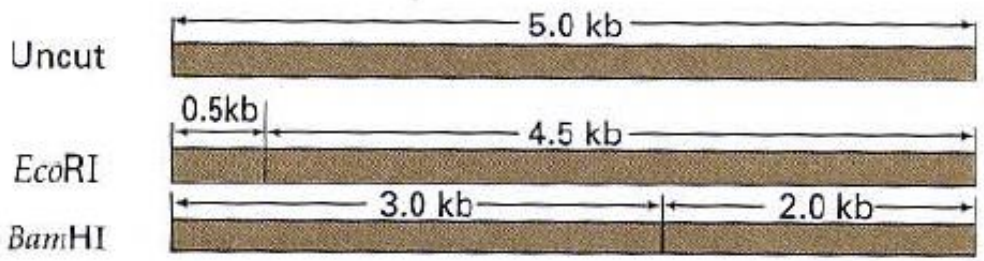
RESTRICTION MAPPING

3 Calculate fragment size from a calibration curve of marker fragment size vs. distance.

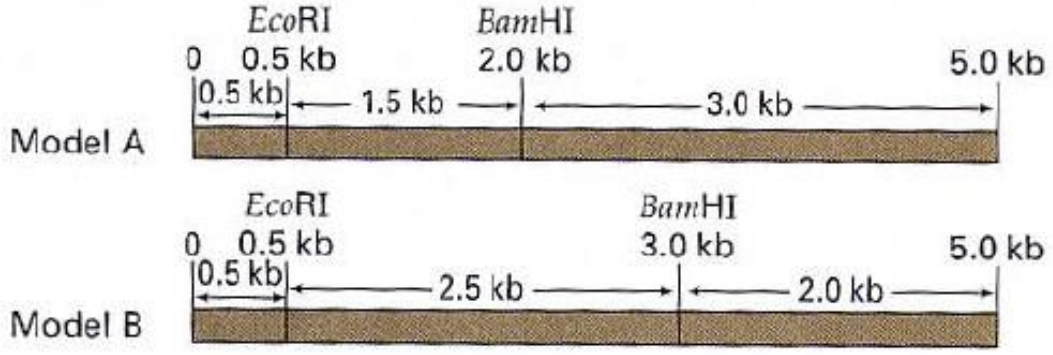
Results:

Uncut	*Eco*RI	*Bam*HI	*Eco*RI + *Bam*HI
5.0 kb	4.5 kb 0.5 kb	3.0 kb 2.0 kb	2.5 kb 2.0 kb 0.5 kb

4 Interpretation



5 Construct models



Predicted *Eco*RI and *Bam*HI fragments

3.0, 1.5, and 0.5 kb

2.5, 2.0, and 0.5 kb

6 Conclusion

*Eco*RI and *Bam*HI data indicate model B is correct.

Russell, Fundamentals of Genetics, 2nd edition

Practice problem

A 5000 bp circular DNA was treated with *Hind*III and *Eco*RI restriction enzymes. *Eco*RI cuts at position 100. *Hind*III cuts at positions 600 and 2500. Which of the following represents the fragments that would be observed if the restricted DNA were analyzed using agarose gel electrophoresis?

A. 100, 600, 2500
B. 400, 2500, 1100
C. 400, 1900, 2600
D. 500, 1900, 2600
E. 500, 1100, 600

DNA CLONING and VECTORS – *USING THE "TRANSFORMING PRINCIPLE"*

Transformation: *competent* cells take-up DNA – two methods used in the lab:

1. Chemical (*e.g.* $CaCl_2$) or 2. electroporation (voltage)

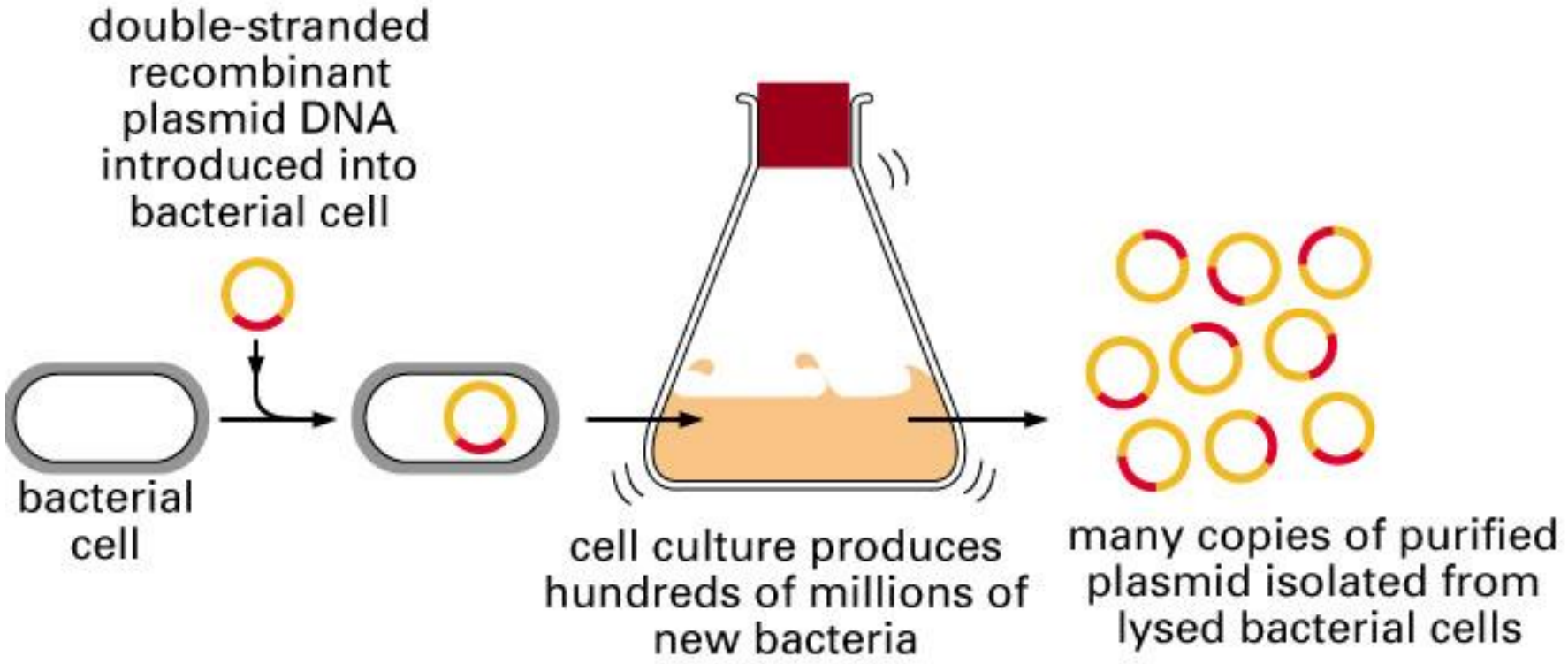


Figure 8–31. Molecular Biology of the Cell, 4th Edition.

Competence is the ability of a cell to take up extracellular ("naked") DNA from its environment

Natural (ability under natural conditions) and induced or artificial competence which arises when cells in laboratory cultures are treated to make them transiently permeable to DNA. Natural competence was discovered by Griffith (Avery & collegues showed that DNA was responsible for "transforming" cells).

Artificial competence* involves making cells passively permeable to DNA by exposing them to conditions that do not normally occur in nature.(i.e. **cells are "forced" to take up DNA**). Typically occurs in one of two ways: 1) Cold cation treatment, followed by a heat shock, which in short, allows the negatively charged plasmid DNA to enter the cells. 2) Electroporation. Cells are briefly shocked with an electric field, which is thought to create holes in the cell membrane through which the plasmid DNA may enter. After the electric shock the holes are rapidly closed by the cell's membrane-repair mechanisms.

details here of how artificial competence is achieved are not important to commit to memory. You will revisit this in MBB 308.

Antibiotic selection for successful transformations

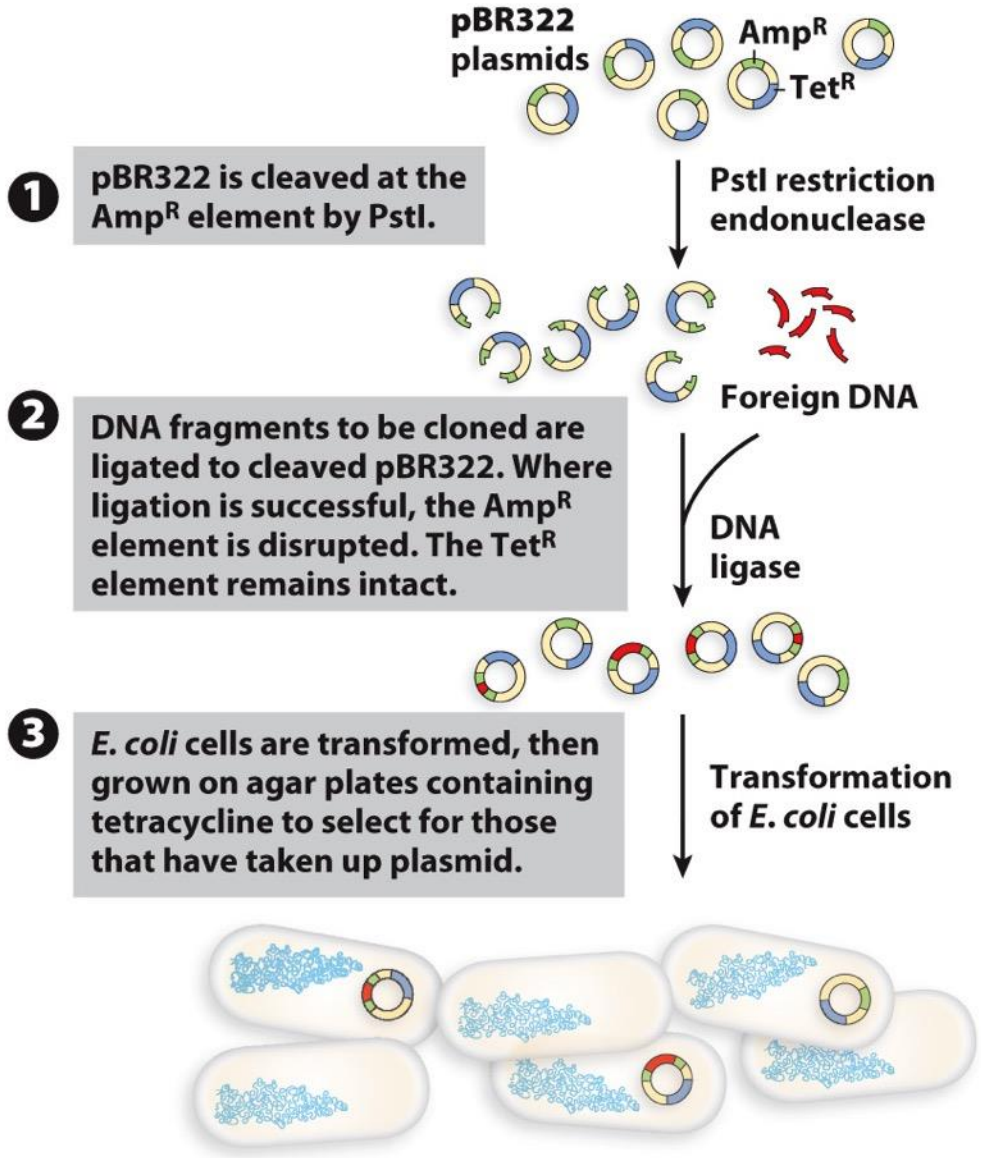
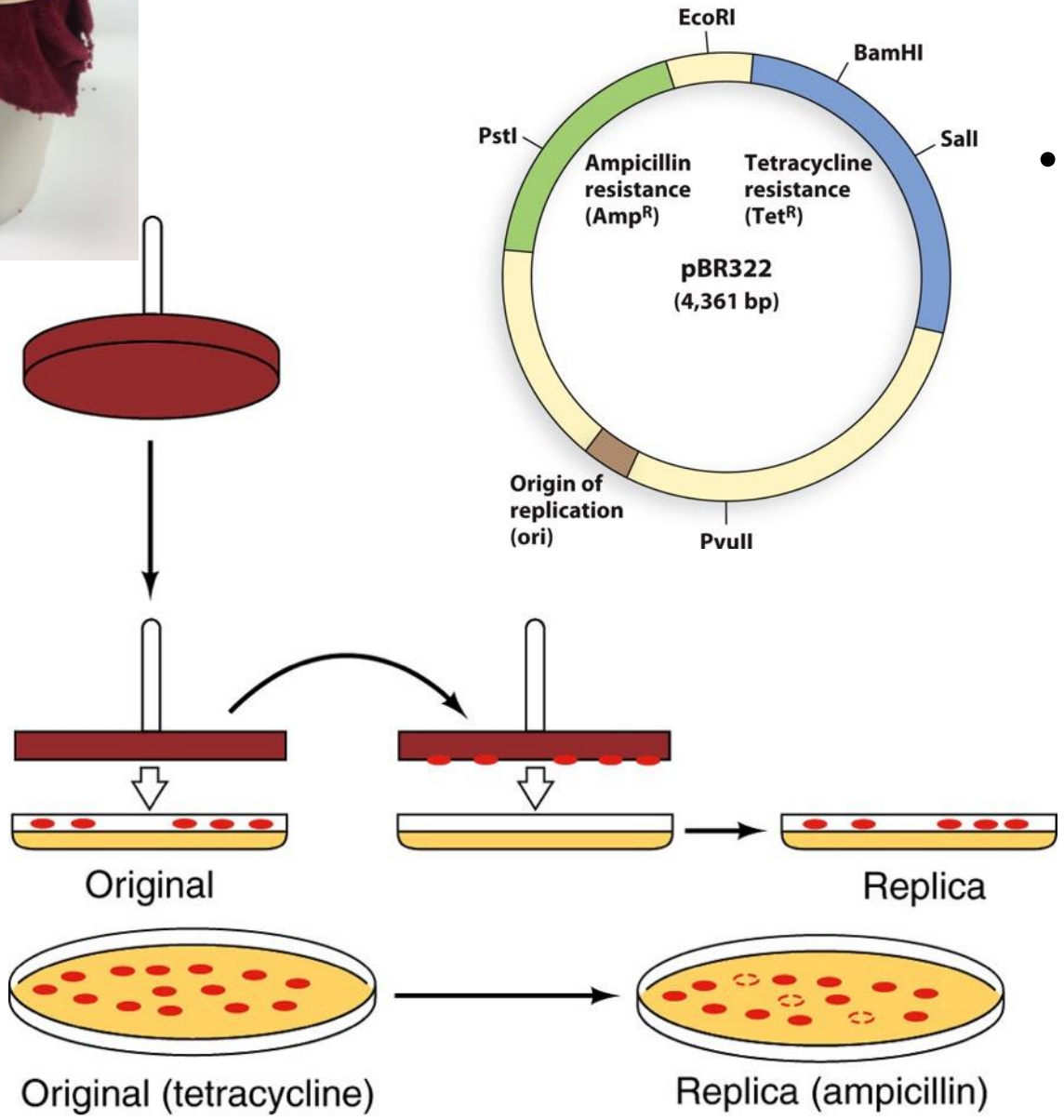


Figure 7-5 part 1
Molecular Biology: Principles and Practice, Second Edition

- Remember that a key requirement for successful cloning is having and utilizing a **selectable marker**
- Insertion into an antibiotic resistance gene, knocks out its function
- You can therefore set up a series of steps to screen for cells that contain your gene of interest.

SCREENING BY REPLICA PLATING



- Touching velvet to surface of original plate, cells from each colony transferred to new plate (*e.g.* one containing a different antibiotic)

EXAMPLE – transformants with DNA inserted into amp^R (β-lactamase) gene on plasmid (also carrying tet^R) do not grow when replica printed on amp plate

Antibiotic selection for successful transformations

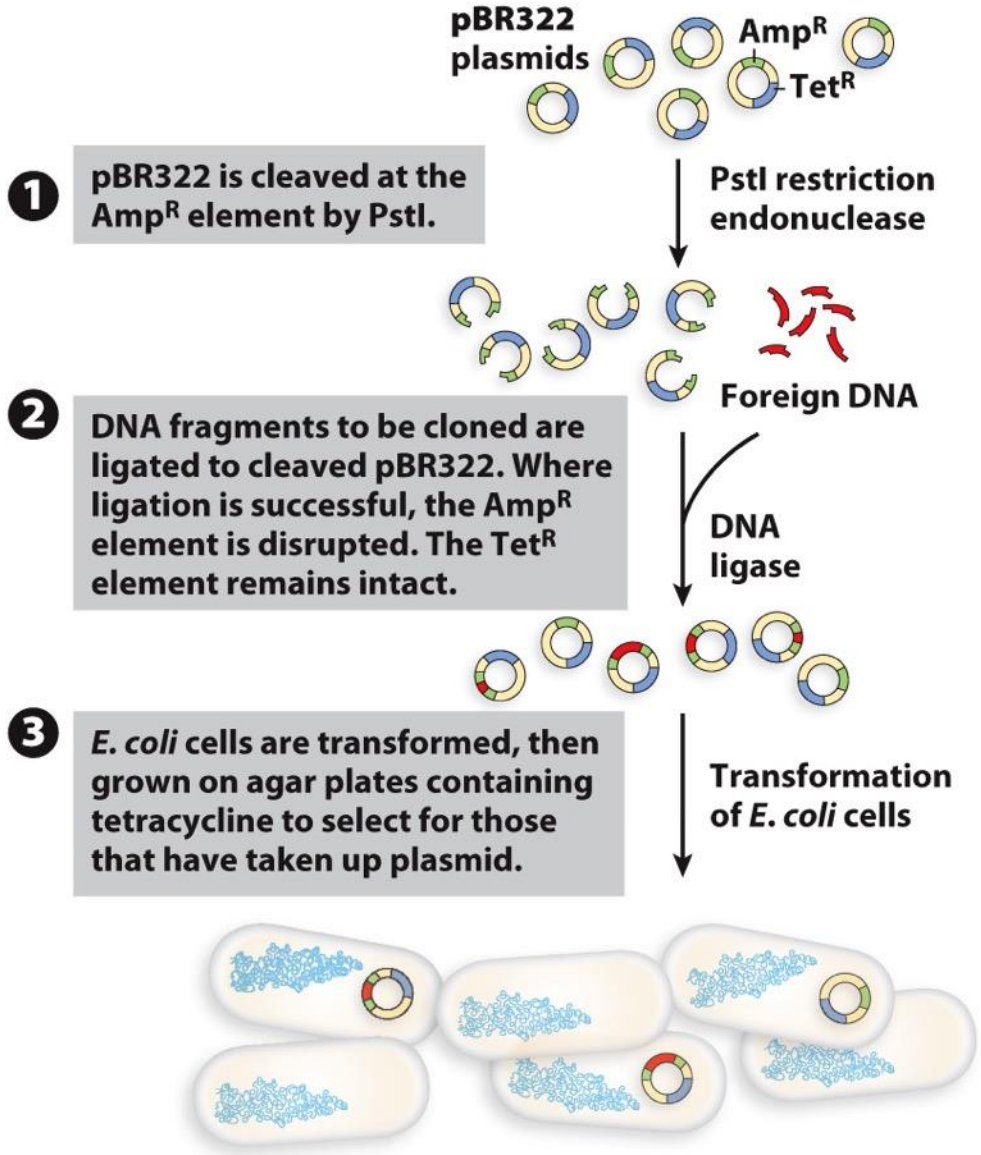


Figure 7-5 part 1
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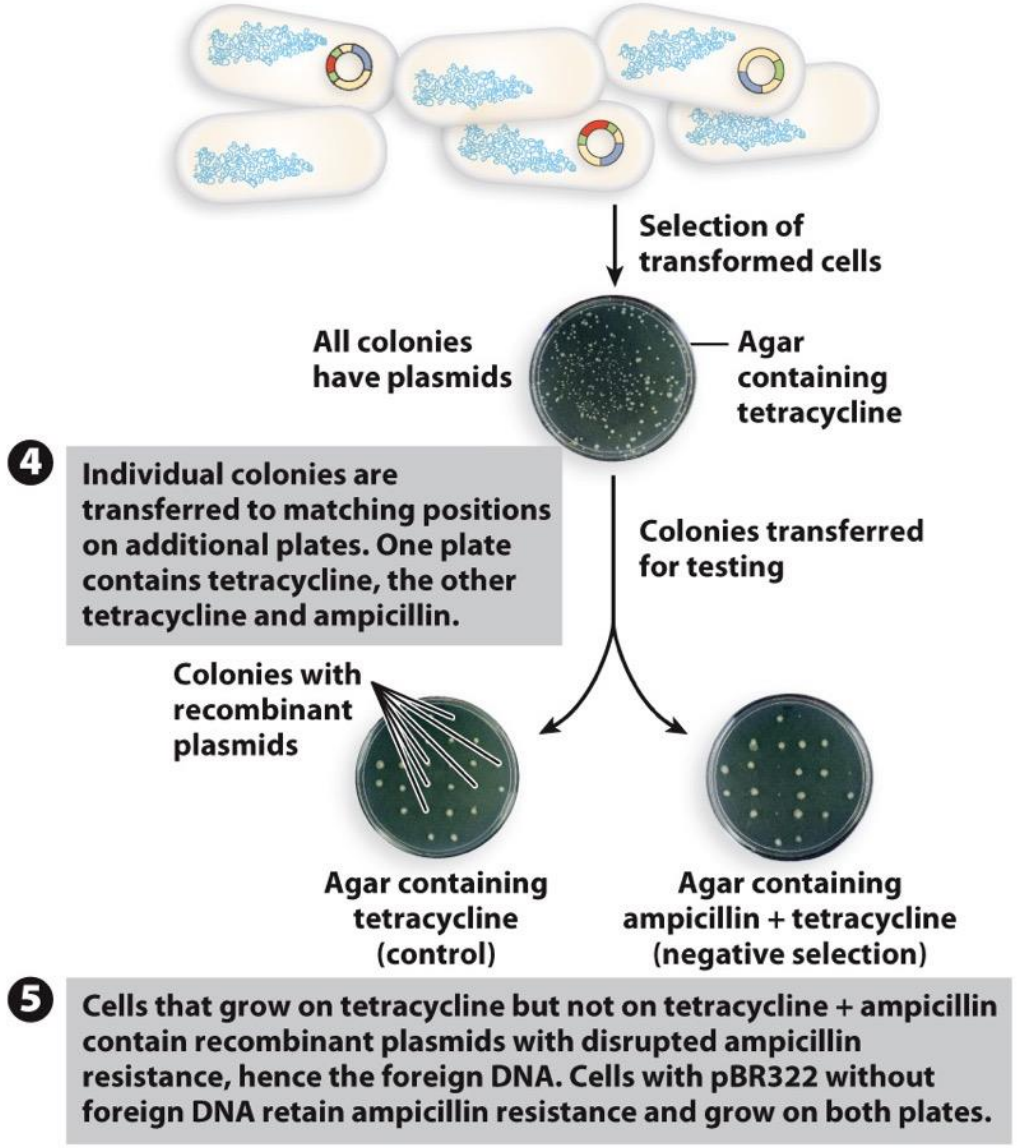

Figure 7-5 part 2
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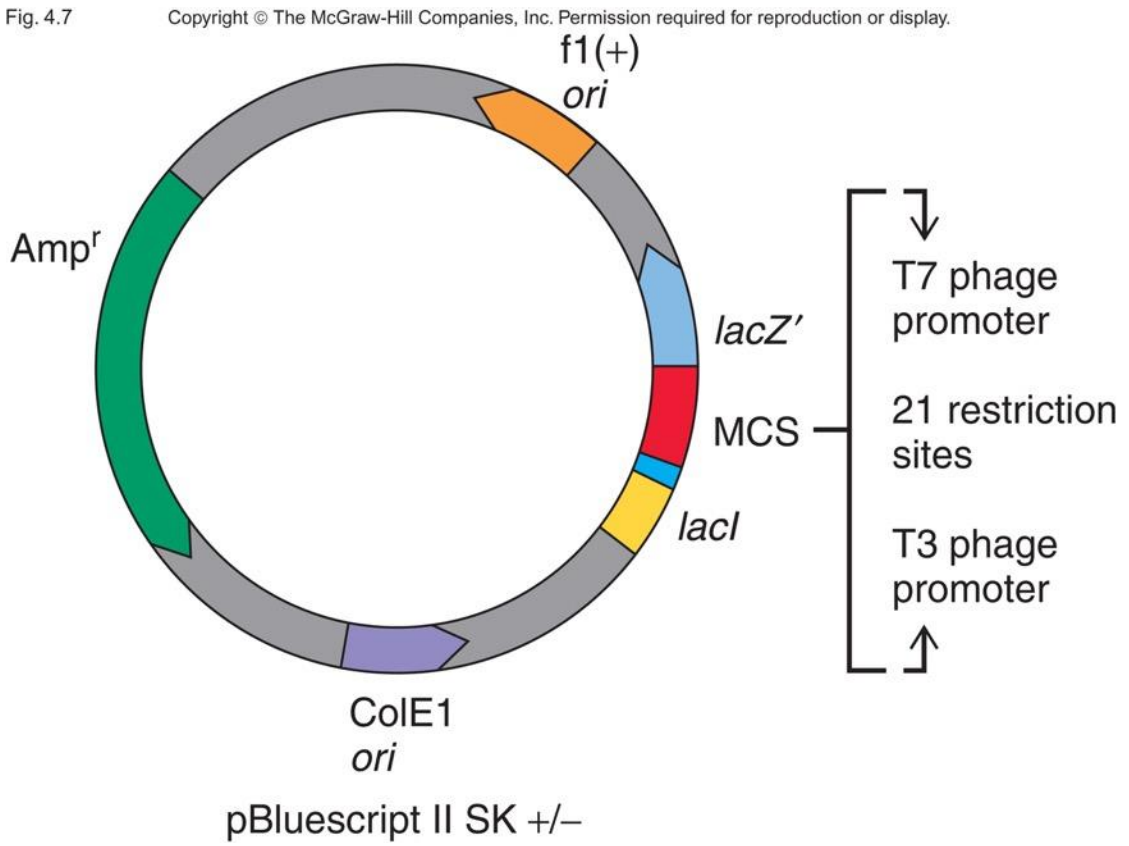
"MODERN" PLASMID VECTORS

USEFUL FEATURES OF CLONING VECTORS:

- **Origin of replication (*ori*)**
- **Antibiotic resistence gene**
- **Promoter sites**
- **Multiple cloning sites (MCS) -** many different restrict sites within small region of *E. coli* lacZ gene (pBluescript)
- **lacZ' gene**



"MODERN" PLASMID VECTORS

USEFUL FEATURES OF CLONING VECTORS:

- **Multiple cloning sites (MCS) -** many different restrict sites within small region of *E. coli* lacZ gene (pBluescript)
- **lacZ' gene** (also known as a screenable marker)

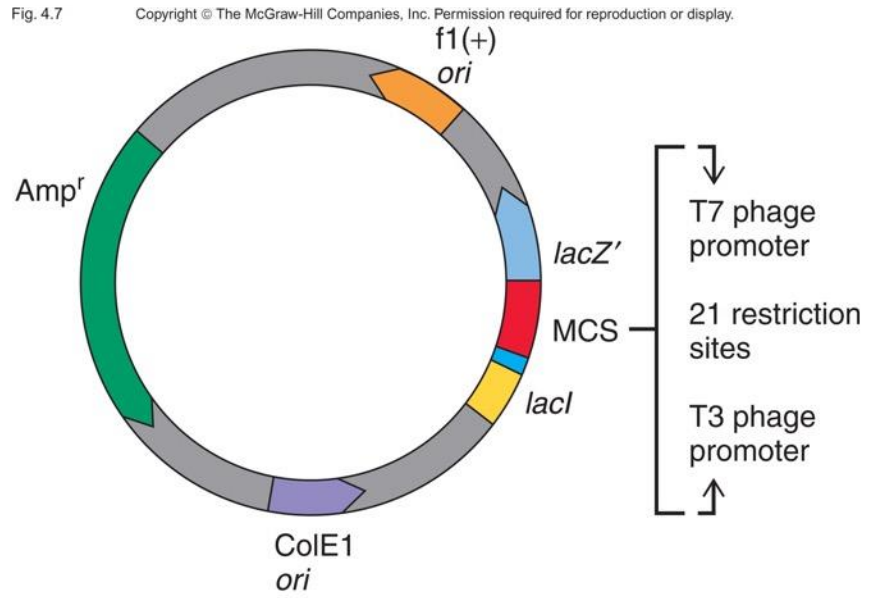
lacZ' encodes β-galactosidase

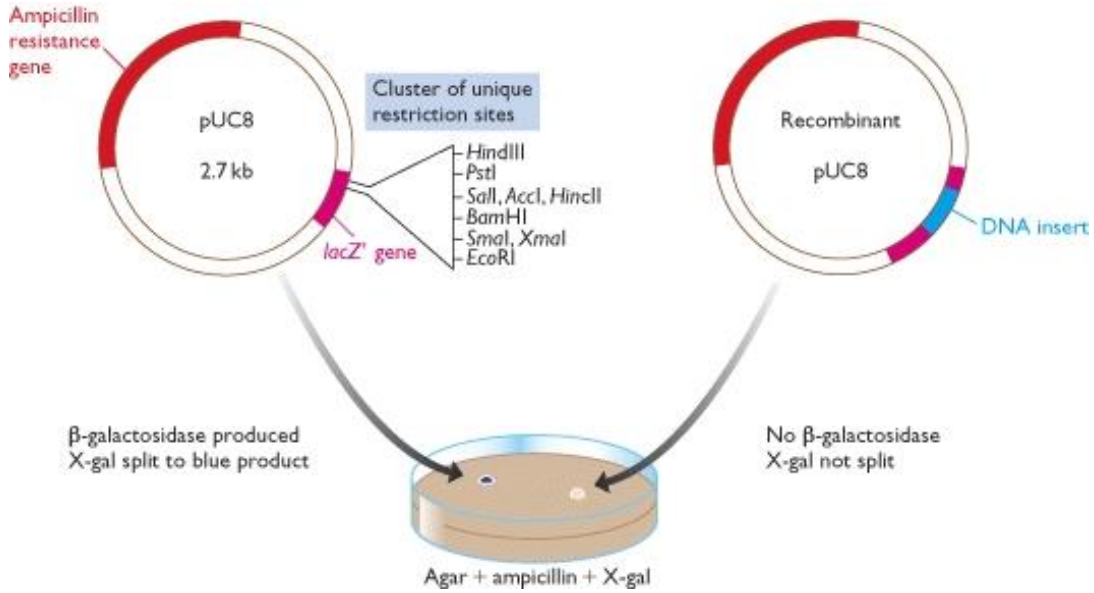
+ X-gal ⟶ BLUE

cloning DNA into MCS - lacZ DISRUPTED

+ X-gal ⟶ WHITE

This is known as **insertional inactivation**





Bacterial Artificial Chromosomes (BACs)

Size matters!

Can handle 150 – 350 kbp of DNA

Used in the Human Genome Project

- stable *ori*
- ***par* genes**
- **selectable marker**
- **screenable markers**

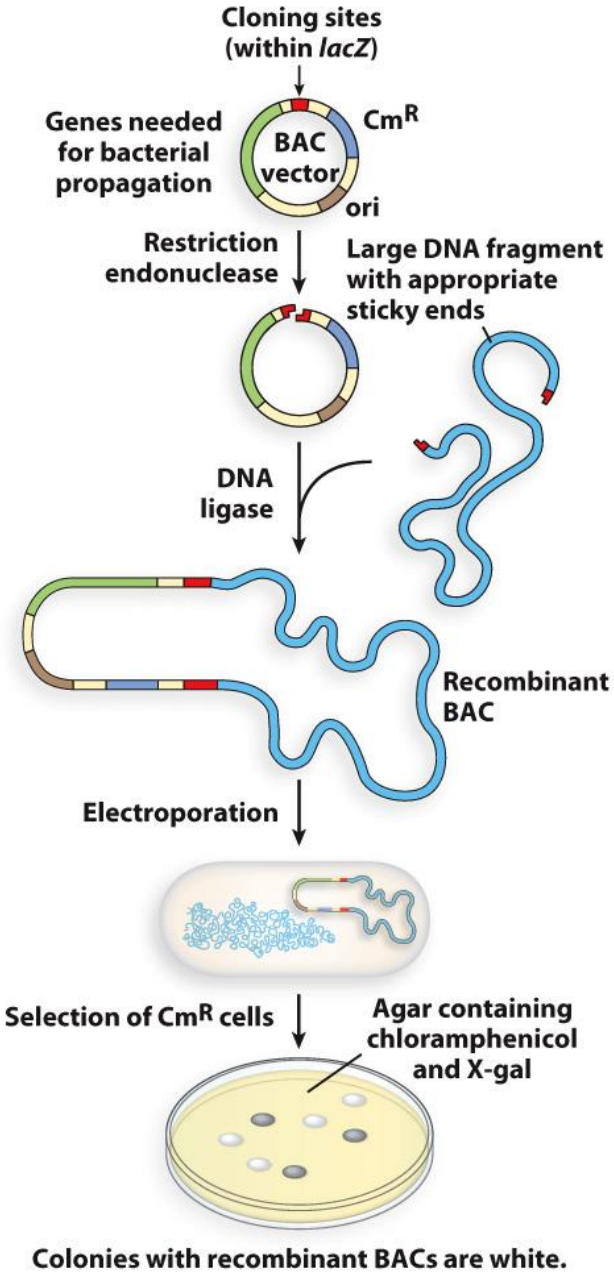


Figure 7-6

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Yeast Artificial Chromosomes (YACs)

Size matters!

Can handle 100 – 1000 kbp!!!

Shuttle vector (plasmids that can be propagated in cells of two or more species)

ori

TEL

CEN

Vector arms with selectable markers **X** and **Y**

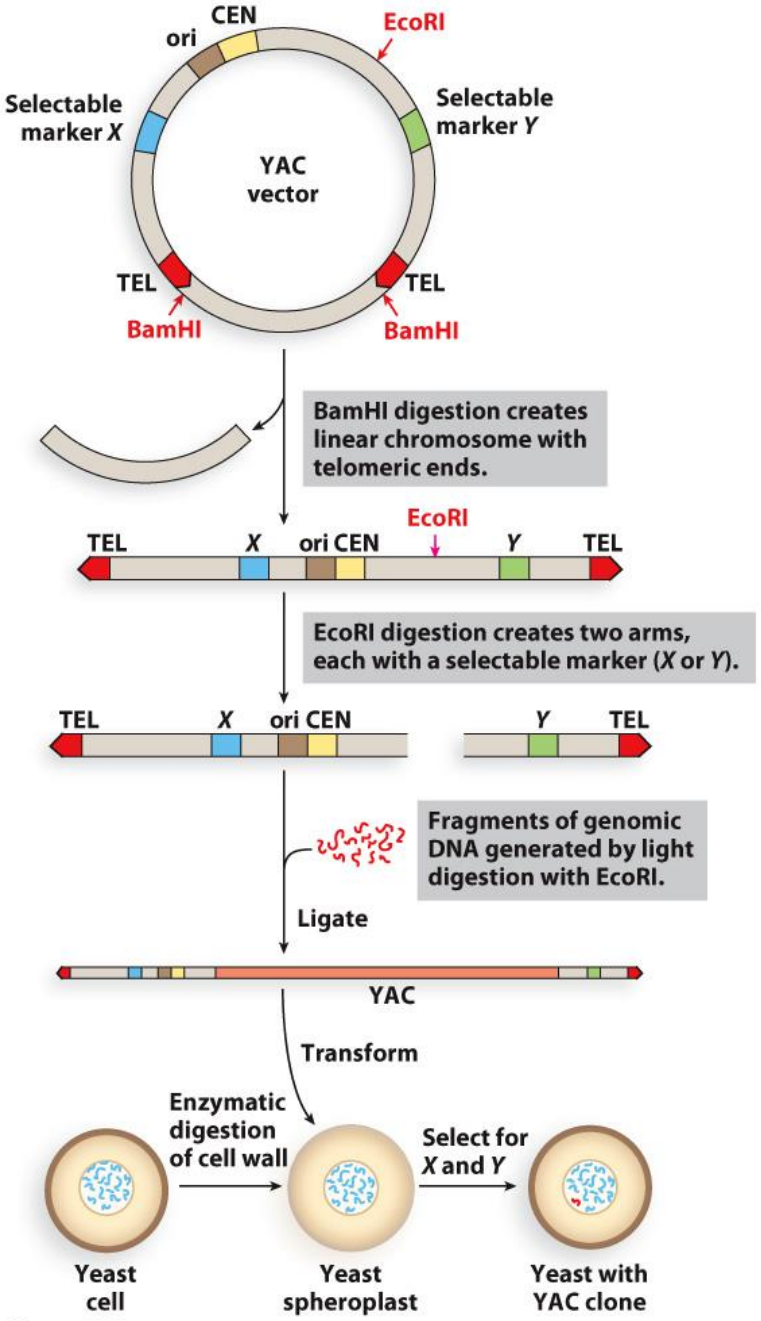

Figure 7-7

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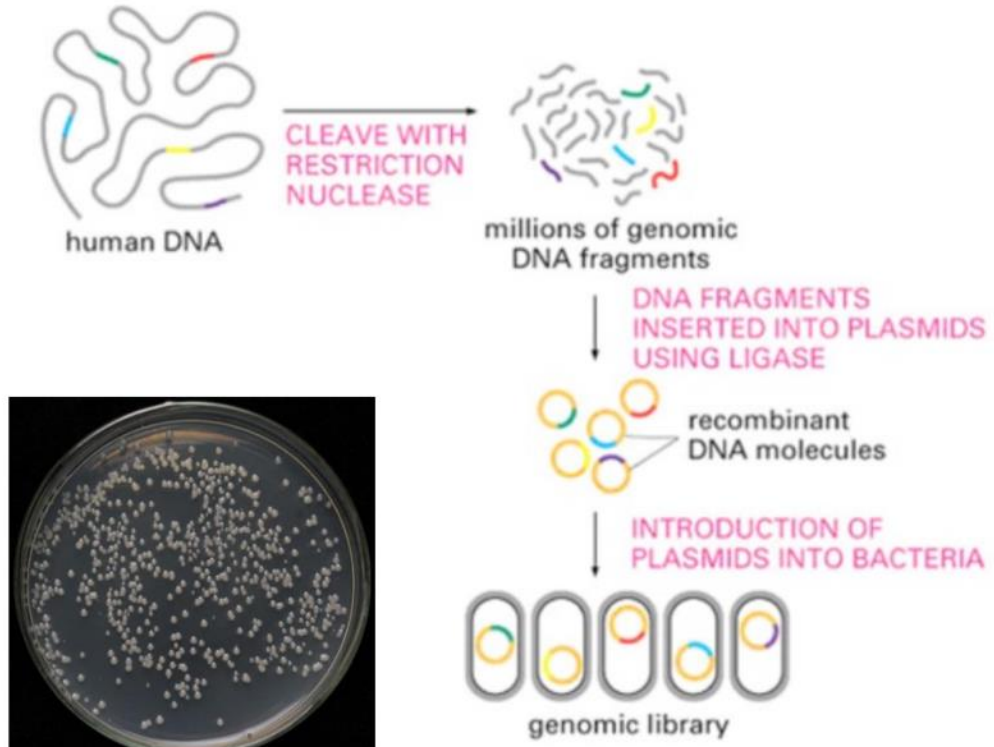
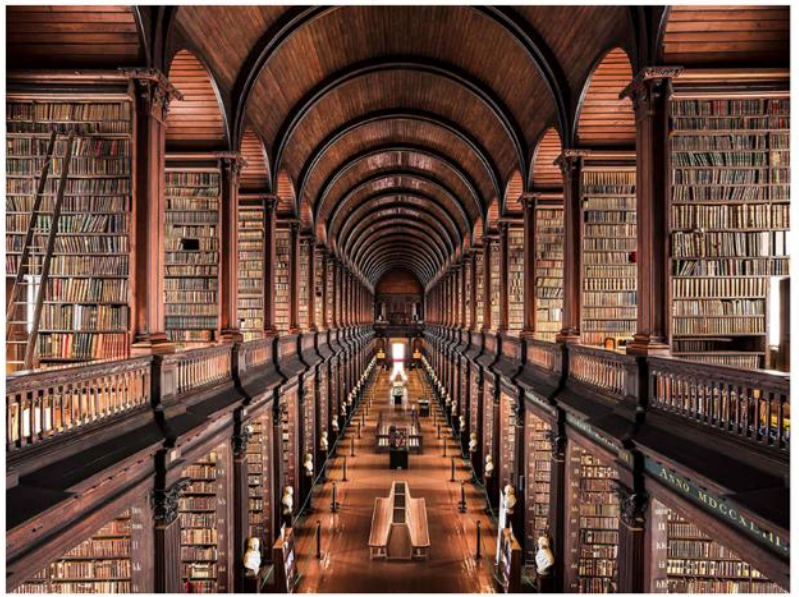
Genomic Library

- Plasmid vectors can handle only small DNA fragments (<100 kbp), so BACs and YACs have advantages of size and have been therefore used in early (historically) genome sequencing projects, like *E. coli* and the HGP

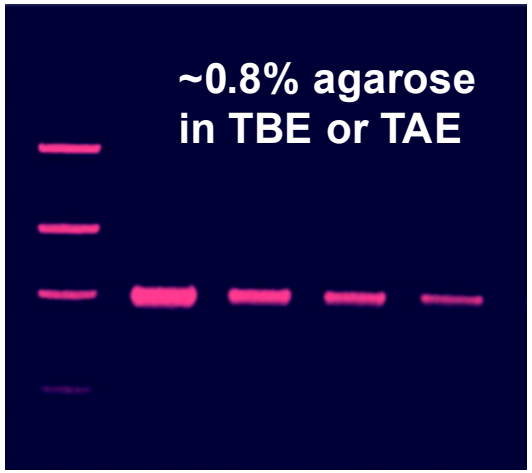
- **A genomic library**:
  A set of clones containing DNA fragments derived directly from the genome of a single organism
  - Genomes are cleaved into thousands of fragments and ALL of them are cloned by insertion into a cloning vector
  - Building libraries like this is traditional and preludes large, more current, sequencing projects



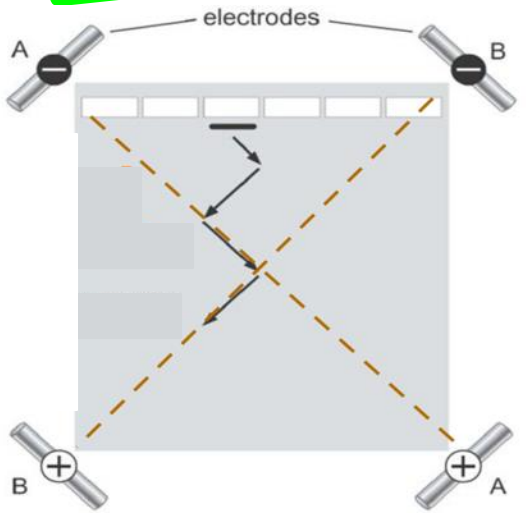


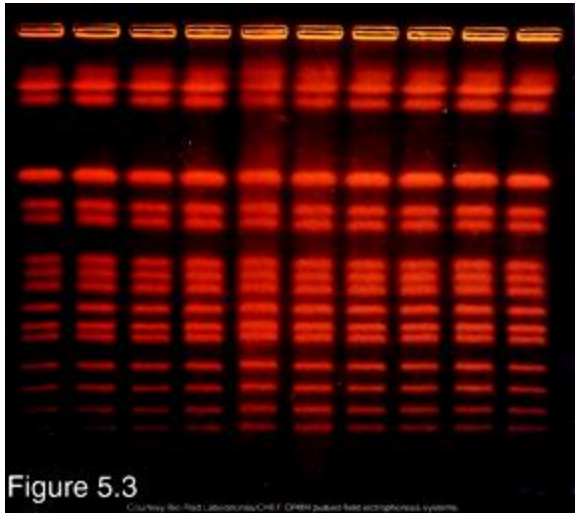
Standard vs. PULSE-FIELD GEL ELECTROPHORESIS

Relatively small DNA fragments resolved (<20kb) by **standard agarose gel electrophoresis**



Large DNAs (>2Mb) separated by **PULSE-FIELD agarose gel electrophoresis**

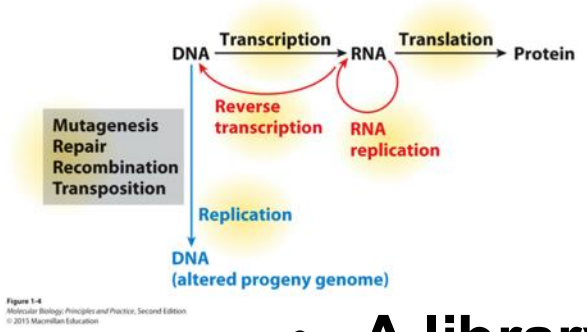




Identical samples of yeast chromosomes were electrophoresed in 10 parallel lanes and stained with EtBr.

The bands represent chromosomes having sizes ranging from 0.2 Mb (at the bottom) to 2.2 Mb (at top).

Original gel is about 13 cm wide by 12.5 cm long.



cDNA Library

- **A library that includes only those sequences of DNA that are *expressed/transcribed* into RNA**
- Your starting material is therefore _____ not gDNA in creating a genomic library

- **mRNA** "reverse transcribed" into DNA

- **REQUIRES:**
  – poly(A)tailedmRNA
  – oligo(dT)primer(deoxythimine)
  – reversetranscriptase

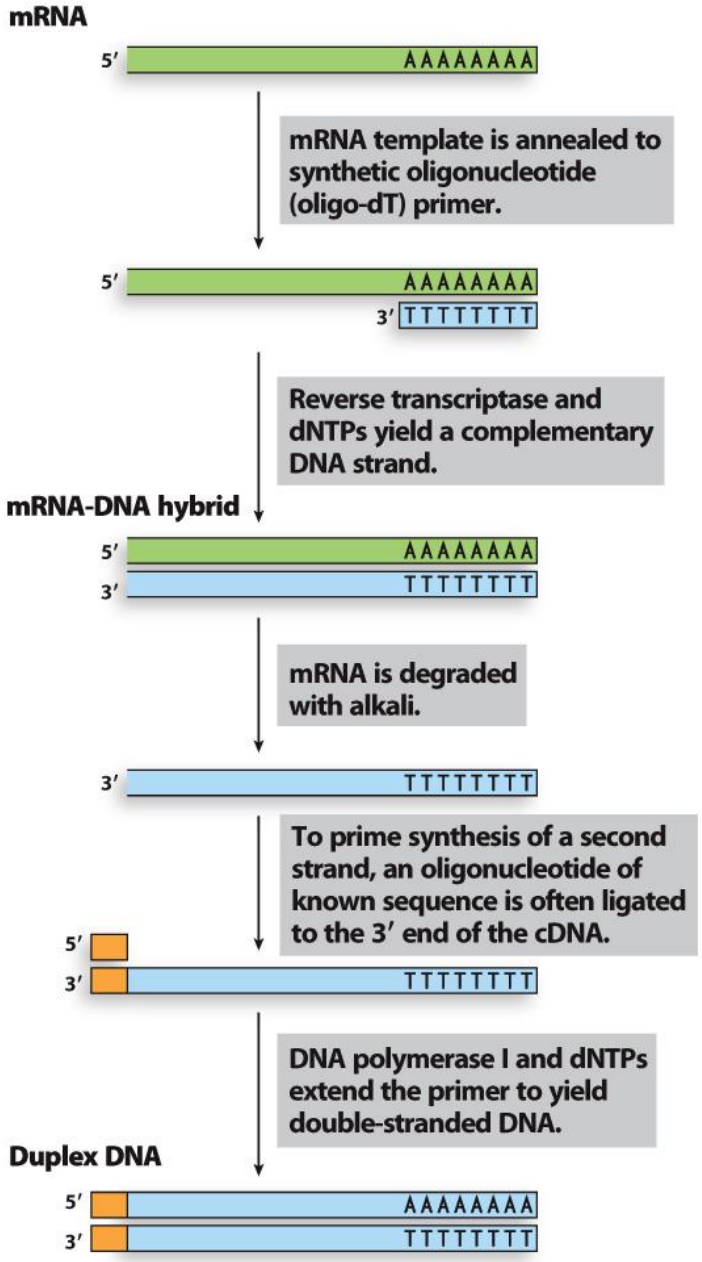


Figure 7-8
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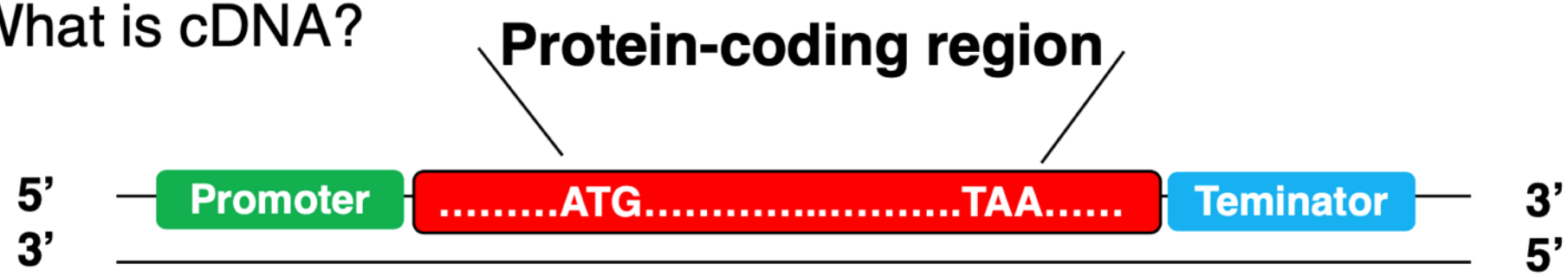
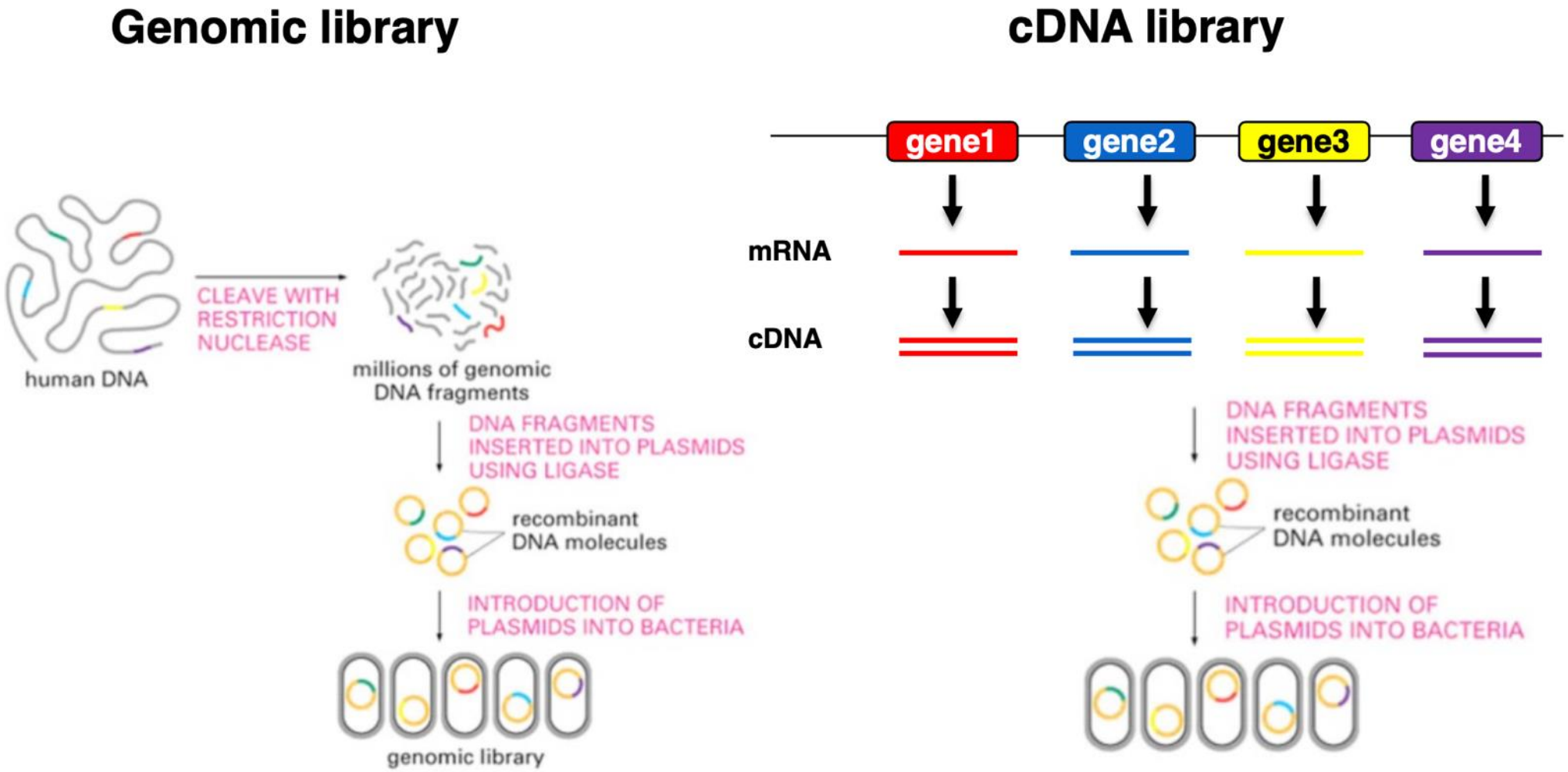
cDNA Library

- What is cDNA?



mRNA	5’	……AUG……UAA……	3’
cDNA (first strand)	5’	……AUG……UAA……	3’
	3’	……TAC……ATT……	5’
cDNA (double-strand)	5’	……ATG……TAA……	3’
	3’	……TAC……ATT……	5’

Difference between genomic and cDNA library



Practice Question

In constructing a cDNA library, which of the following would you use to generate rare restriction sites on the cDNA strands? Explain your answer.

A. restriction enzymes
B. DNA methylases
C. *Bam*HI restriction followed by ligation
D. ligate cDNA to specific linkers
E. digestion of RNA-DNA ends

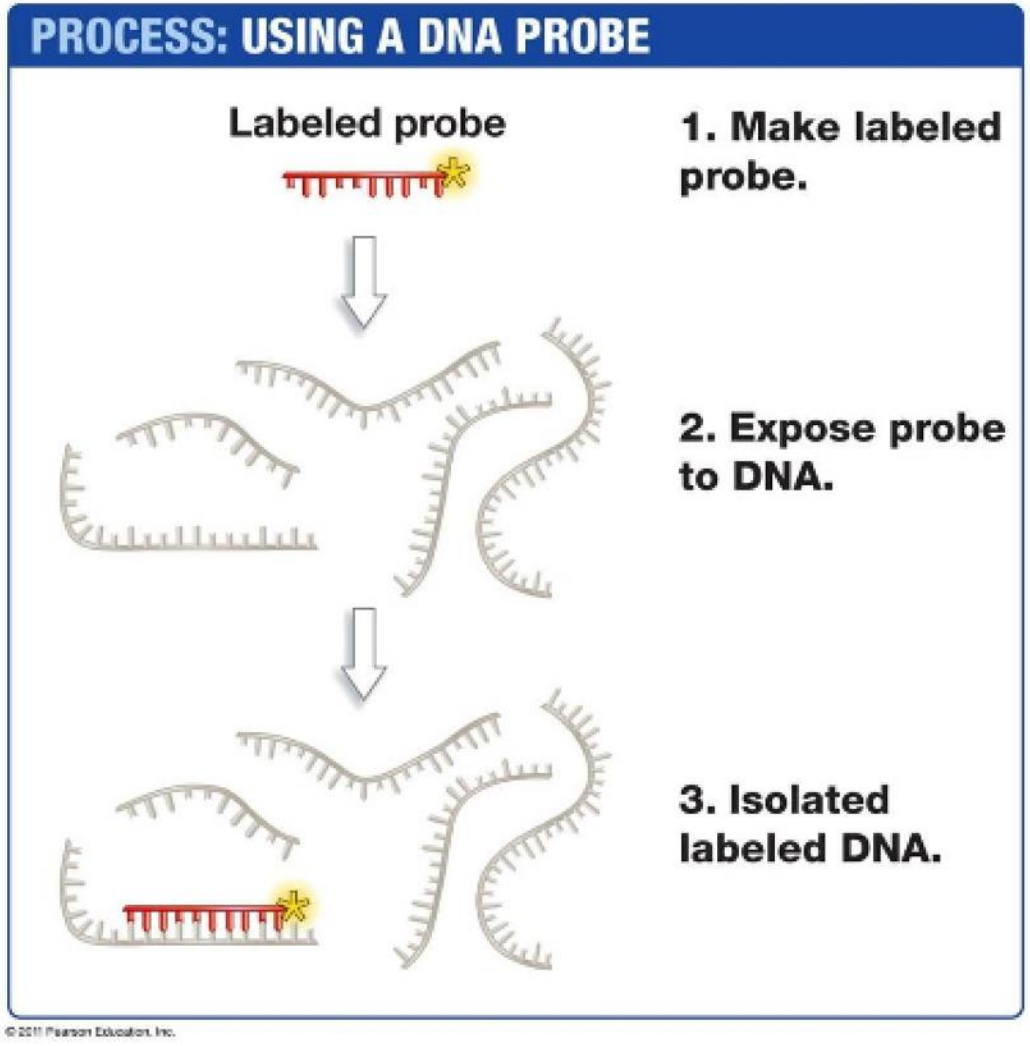
Given all the techniques we discussed today, how would you screen for the presence of a specific sequence (i.e., gene of interest)?

HYBRIDIZATION using DNA/RNA probes

Hybridization/annealing: one strand of nucleic acid forms a double helix with another strand of complementary sequence

- Plaque hybridization: DNA-DNA
- Southern Blots: DNA-DNA
- Northern Blots: RNA-RNA or RNA-DNA/RNA

- Use RADIOACTIVELY LABELED PROBES to DETECT SPECIFIC DNA FRAGMENTS
- Probes must be **SINGLE-STRANDED DNA PROBE** & **COMPLEMENTARY** TO TARGET ssDNA to anneal

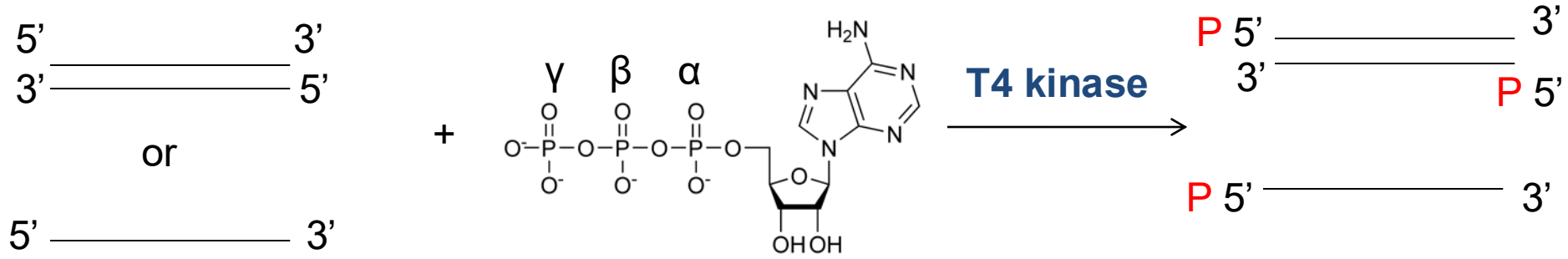


How to make a DNA/RNA radioactive probe?

Which enzyme do we use?

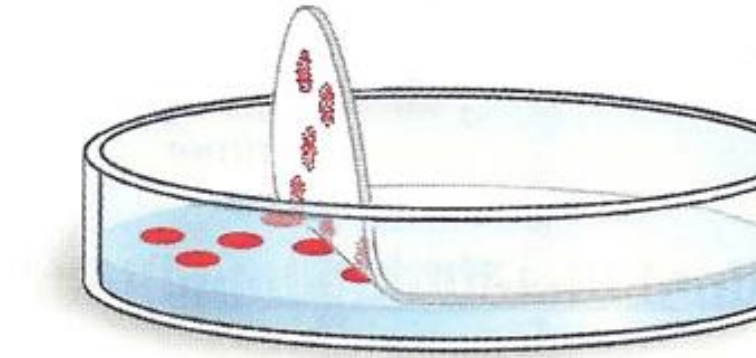
Use **T4 polynucleotide kinase**: it catalyzes the transfer of a phosphate group from the γ position of ATP to the 5' hydroxyl terminus of double stranded and single stranded DNA or RNA. This enzyme will also remove 3' phosphoryl groups.

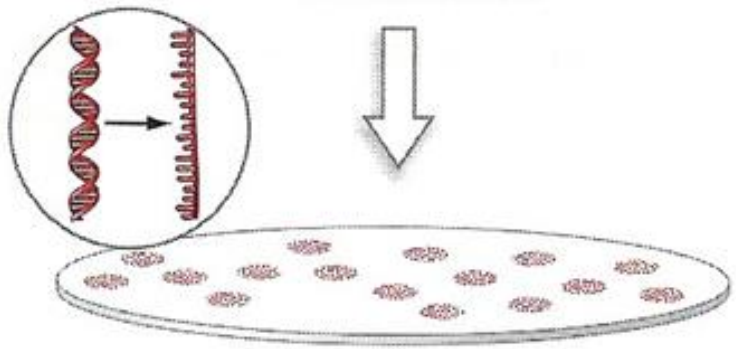
Note that oligonucleotides that are obtained from automated synthesizers lack a 5' phosphate group, and thus cannot be ligated to other polynucleotides. T4 polynucleotide kinase can be used to phosphorylate the 5' end of such polynucleotides.



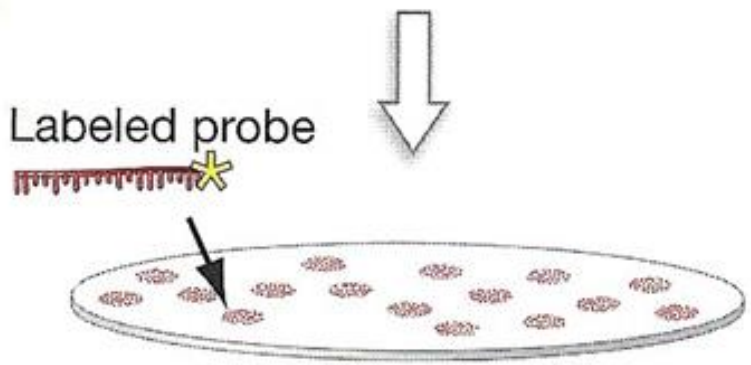
P32-labeled- ATP

Hybridize probe to filters

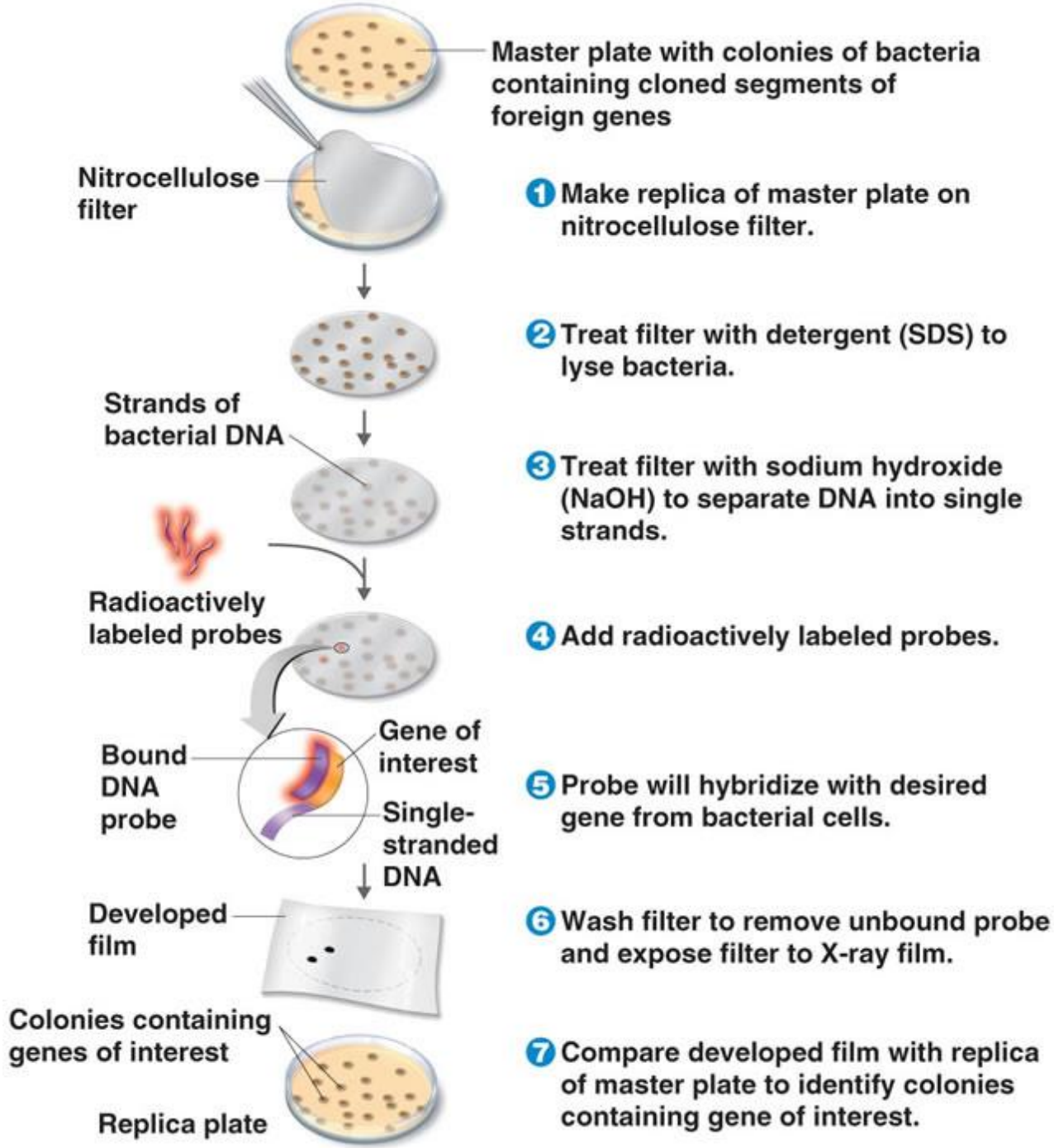




Lyse bacteria,
denature DNA



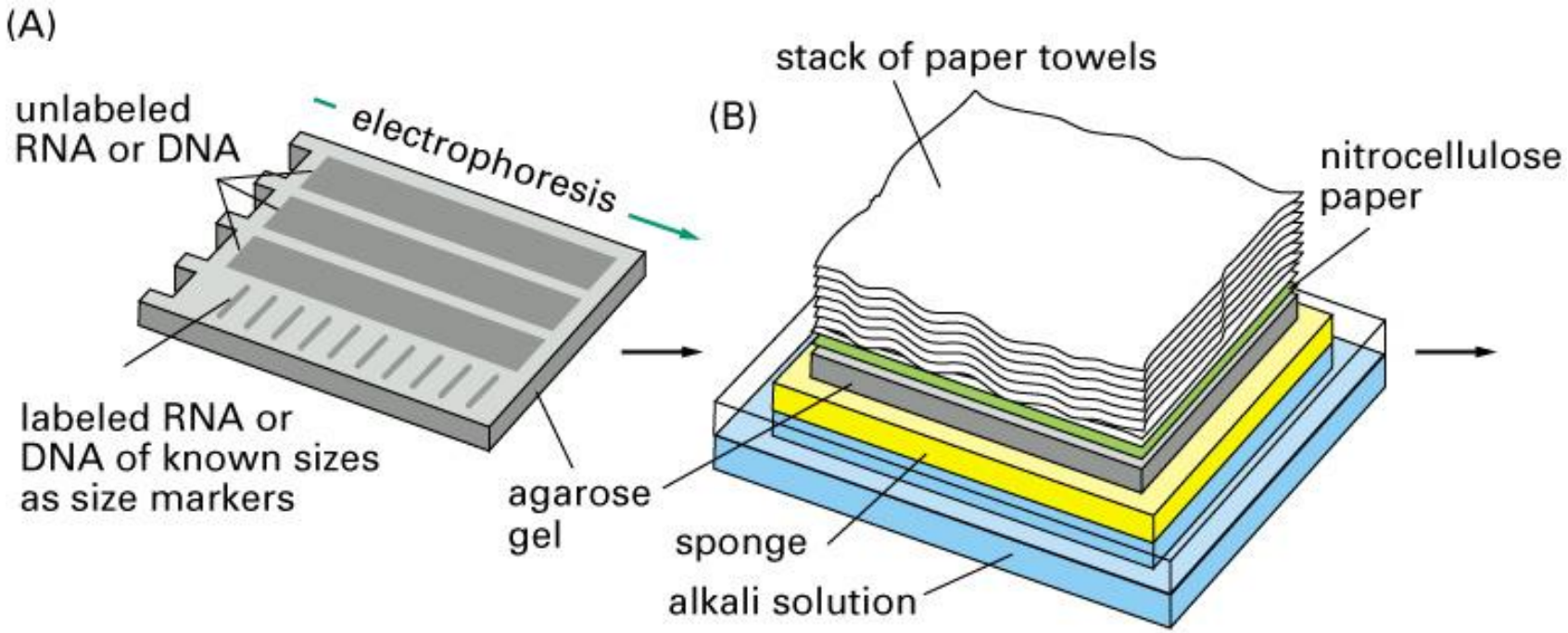
Hybridize labeled
probe to filters



DNA BLOTS SOUTHERN TRANSFER

(Ed Southern):

A. DNA Electrophoresis
B. Transfer out of gel to nitrocellulose paper (blot)



The gel is submerged in denaturation buffer, which separates the dsDNA into ssDNA. Then, the gel is placed into the neutralization buffer, which lowers the pH of the gel and the DNA within it so that the DNA can bind to the membrane.

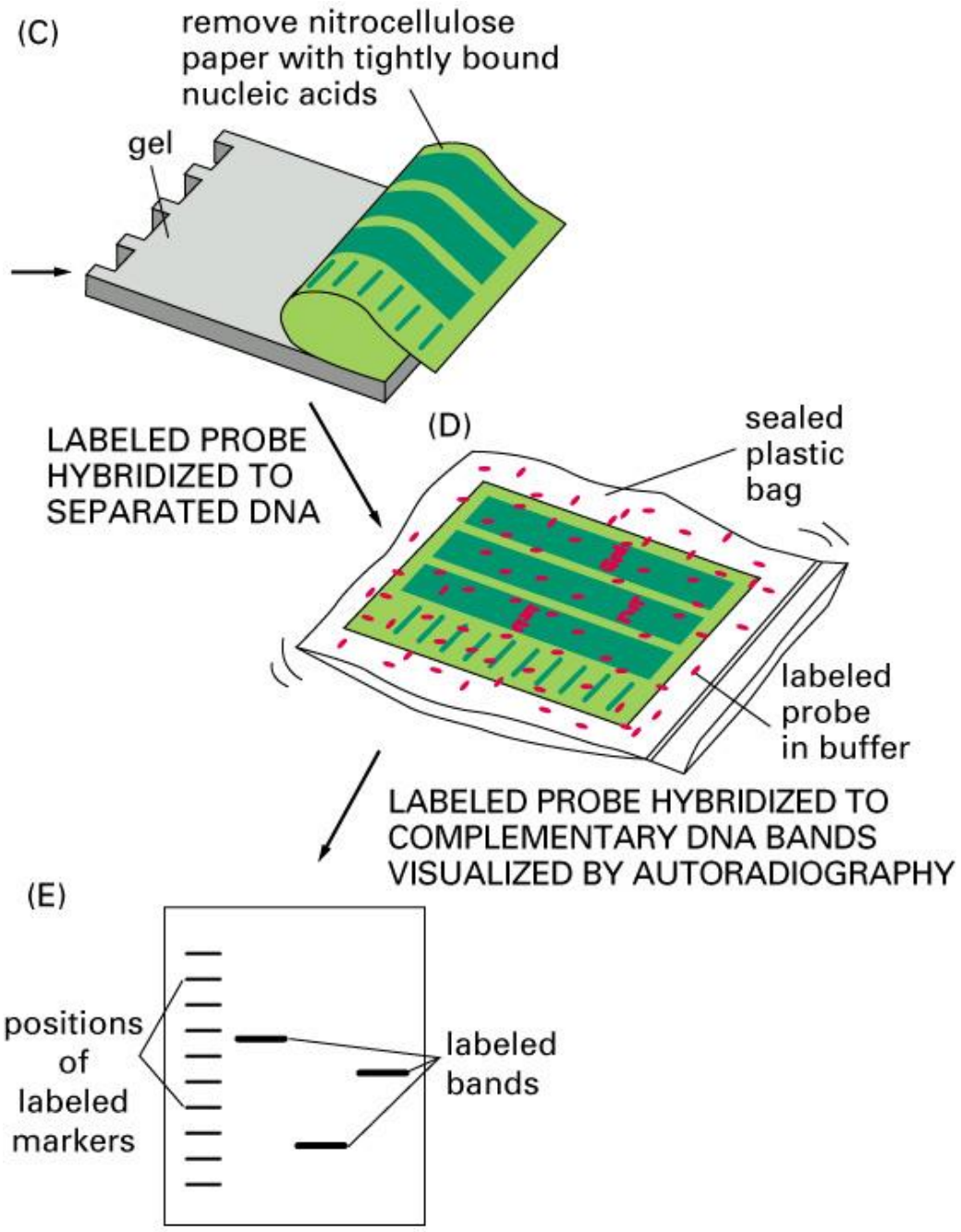
The DNA is transferred out of the gel by capillary action. Salt buffer moves through the gel toward the membrane, carrying ssDNA to the membrane where binding takes place.

HYBRIDIZATION SOUTHERN TRANSFER:

C. Probes anneal with DNA on blot

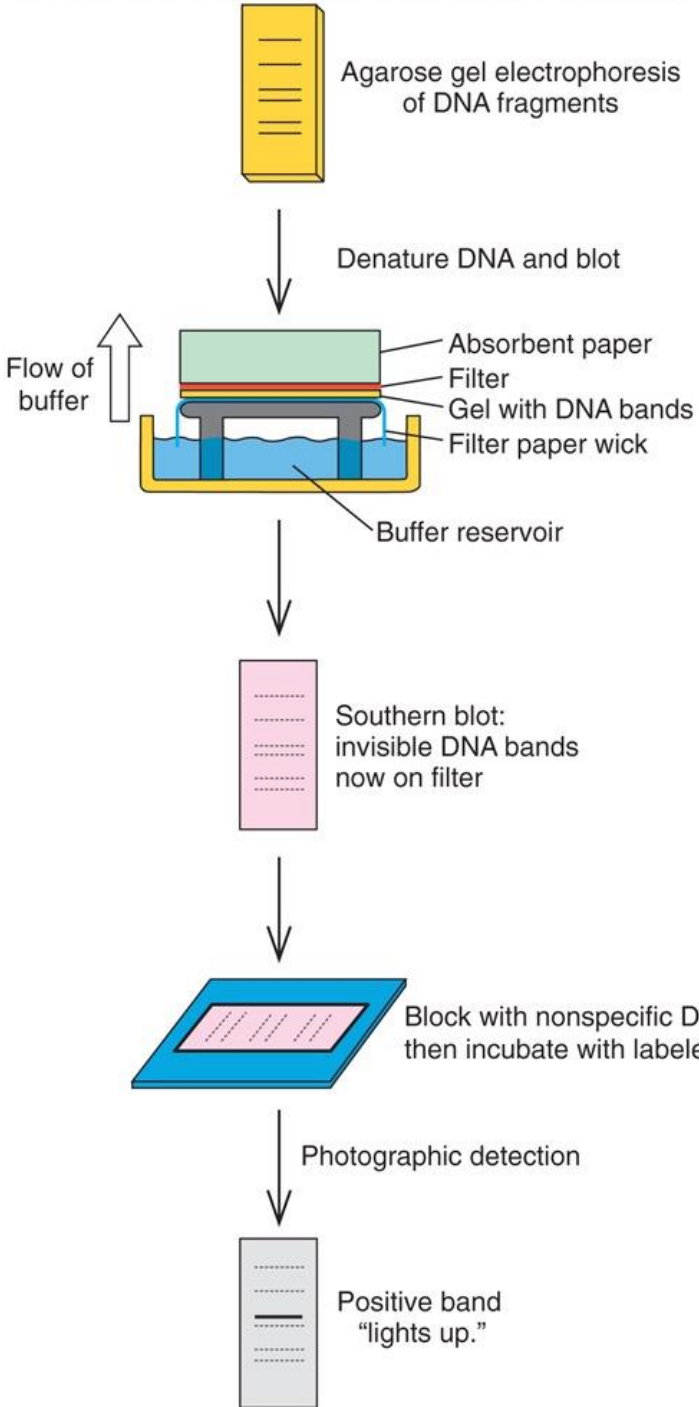
D. Place blot on autoradiography film to visualize radioactive probe bound to DNA

When the transfer is complete, the DNA is irreversibly bound to the membrane by UV cross-linking of DNA to the membrane.



Hybridization stringency: stringency/strength of base-pairing matches between probe and target sequence may be controlled by… ?

If you use [high salt] and low temp in your hybridization experiment, what kind of sequences will anneal?

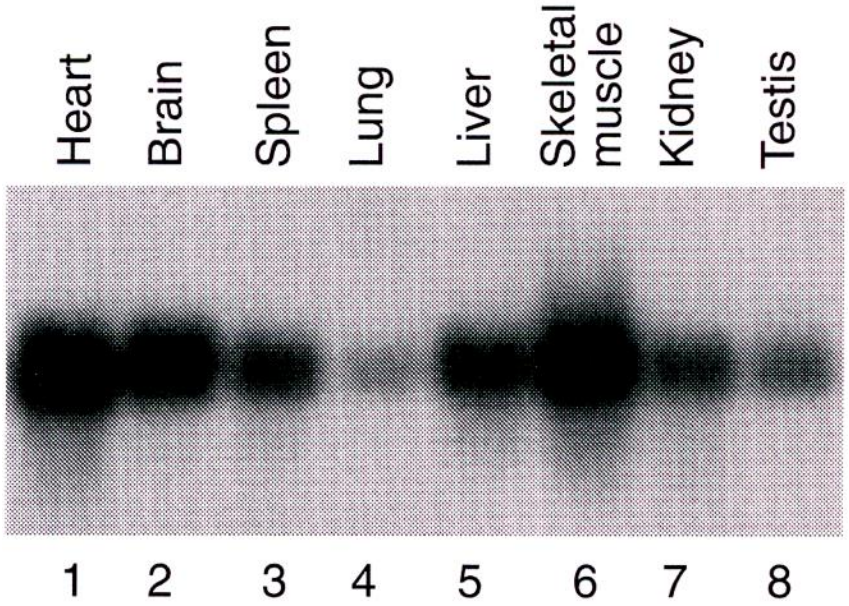


To increase probe-DNA specificity

RNA BLOTS and HYBRIDIZATION (aka NORTHERN Blot/Transfer)

RNA transcripts anneal with radiolabeled DNA probe (^{32}P)

This technique measures mRNA (gene) expression.



labeled probe: rat glyceraldehyde-3-phosphate dehydrogenase (G3PDH) gene.

The bands represent the G3PDH mRNA, and their intensities are indicative of the amounts G3PDH mRNA in each tissue.

A Northern blot is similar to a Southern blot, but it contains electrophoretically **separated RNAs instead of DNAs**. The RNAs on the blot can be detected by hybridizing them to a labeled probe. **The intensities of the bands reveal the relative amounts of specific RNA in each.**

Nowadays, investigators can use non-radioactive probes

DNA FINGERPRINTING originally used specific DNA probes on Southern blots

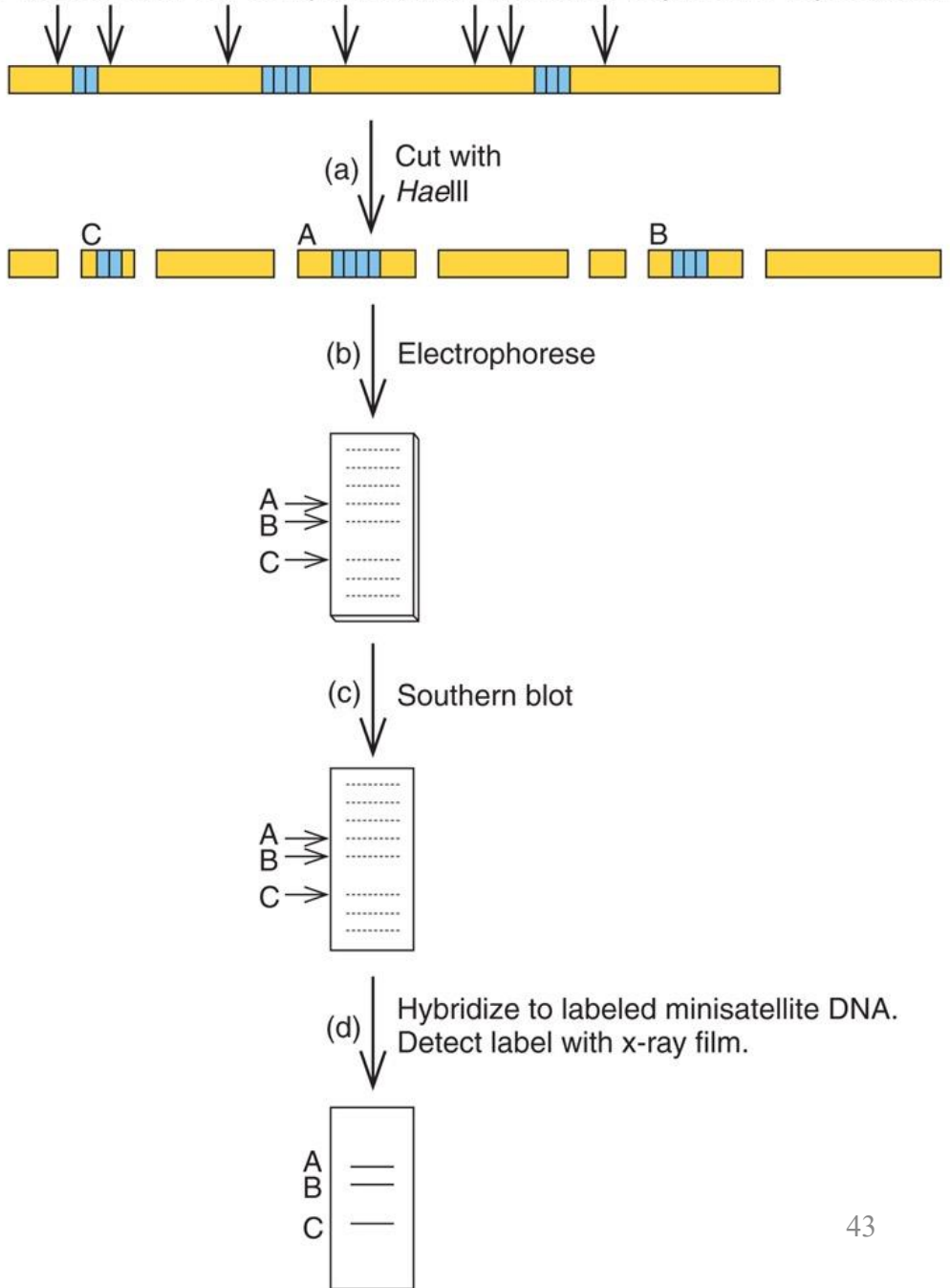
A DNA fingerprint is really just a Southern blot. Investigators first cut the DNA under study with a restriction enzyme (*Hae*III).

Works because of the presence of minisatellites present in the human genome

*Hae*III will cut on either side of the minisatellite regions, but not inside.

Probe designed against minisatellite sequence

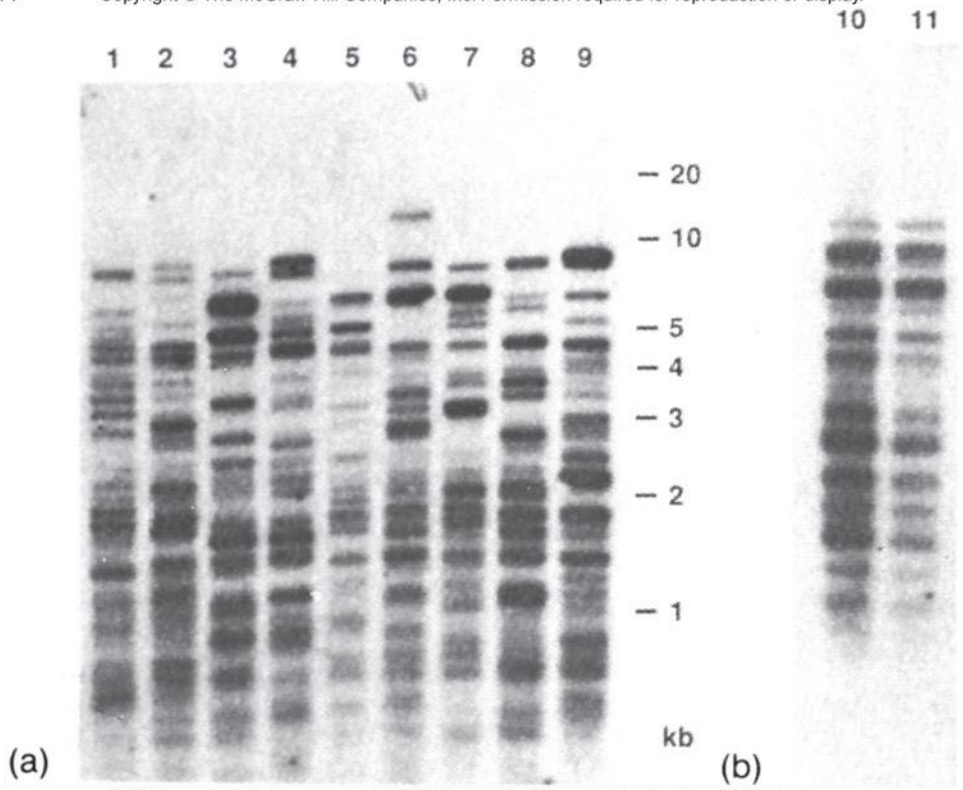
In this example, the DNA has three repeated regions, containing four, three, and two repeats. Thus, three different-size fragments bearing these repeated regions will be produced.



Animal genomes will contain many more than three fragments that contain minisatellite sequence that will react with the minisatellite probe.

This Figure shows an example of the DNA fingerprints of unrelated people (lanes 1-9).
The patterns are extremely complex for two individuals to have identical pattern, unless ???

Fig. 5.14



© G. Vassart et al., A sequence in M13 phage detects hypervariable minisatellites in human and animal DNA. Science 235 (6 Feb 1987) p. 683, f. 1. © AAAS.

DNA fingerprints are very complex. They contain dozens of bands, some of which smear together, which can make them hard to interpret, so…