Molecular Biotechnology

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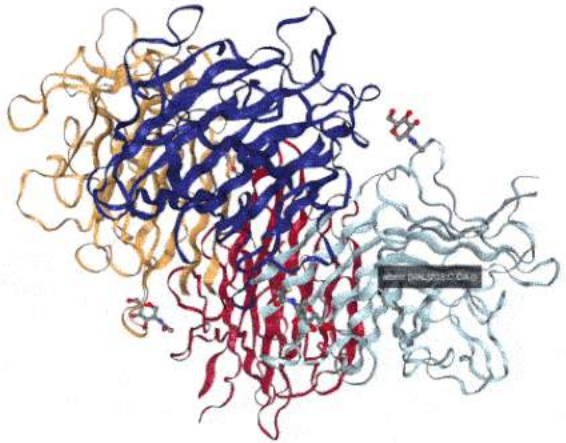
Department Of Biology And Biochemistry, BZU



Biotechnology
BIOC438

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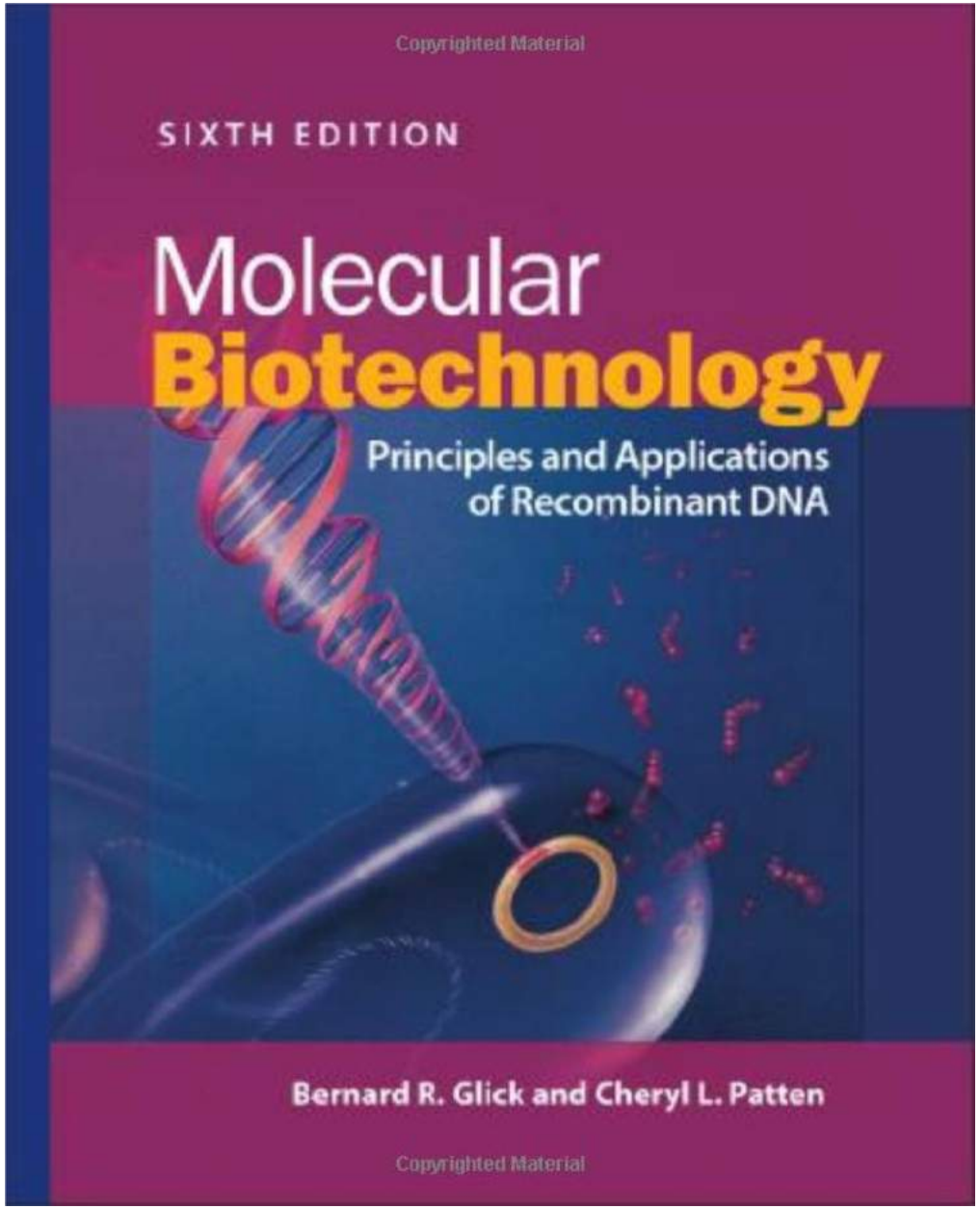
Time: Sat, Wed 10:00 am – 11:15 pm
Pre-requisite: BIOL333



Biotechnology
BIOC438

What is Science ?

- **Science** is a systematic endeavor that builds and organizes knowledge in the form of testable explanations and predictions about the universe.

- Science is **the pursuit and application of knowledge and understanding of the natural and social world following a systematic methodology based on evidence**.

What is Knowledge?

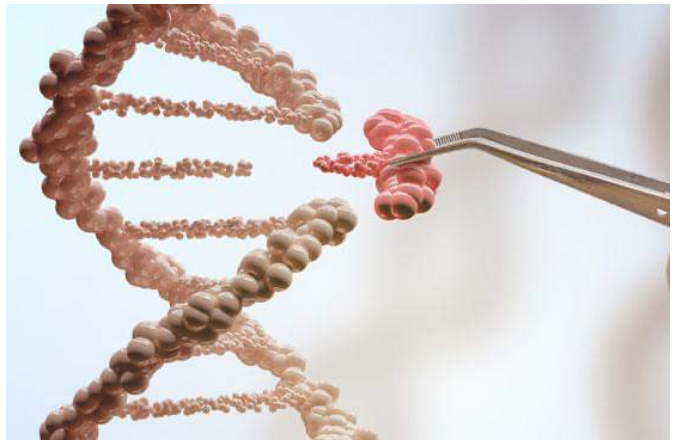
- **Awareness, understanding, or information that has been obtained by experience or study, and that is either in a person's mind or possessed by people generally**

What is Technology?

- The application of scientific knowledge for practical purposes, especially in industry.

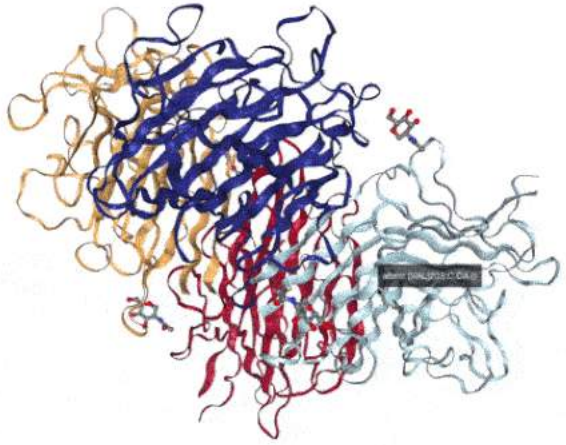
What is Biotechnology ?

Biotechnology is **technology that utilizes biological systems, living organisms or parts of this to develop or create different products**. Brewing and baking bread are examples of processes that fall within the concept of biotechnology (use of yeast (= living organism) to produce the desired product).



Molecular Biotechnology

Chapter 2, DNA, RNA, and Protein Synthesis



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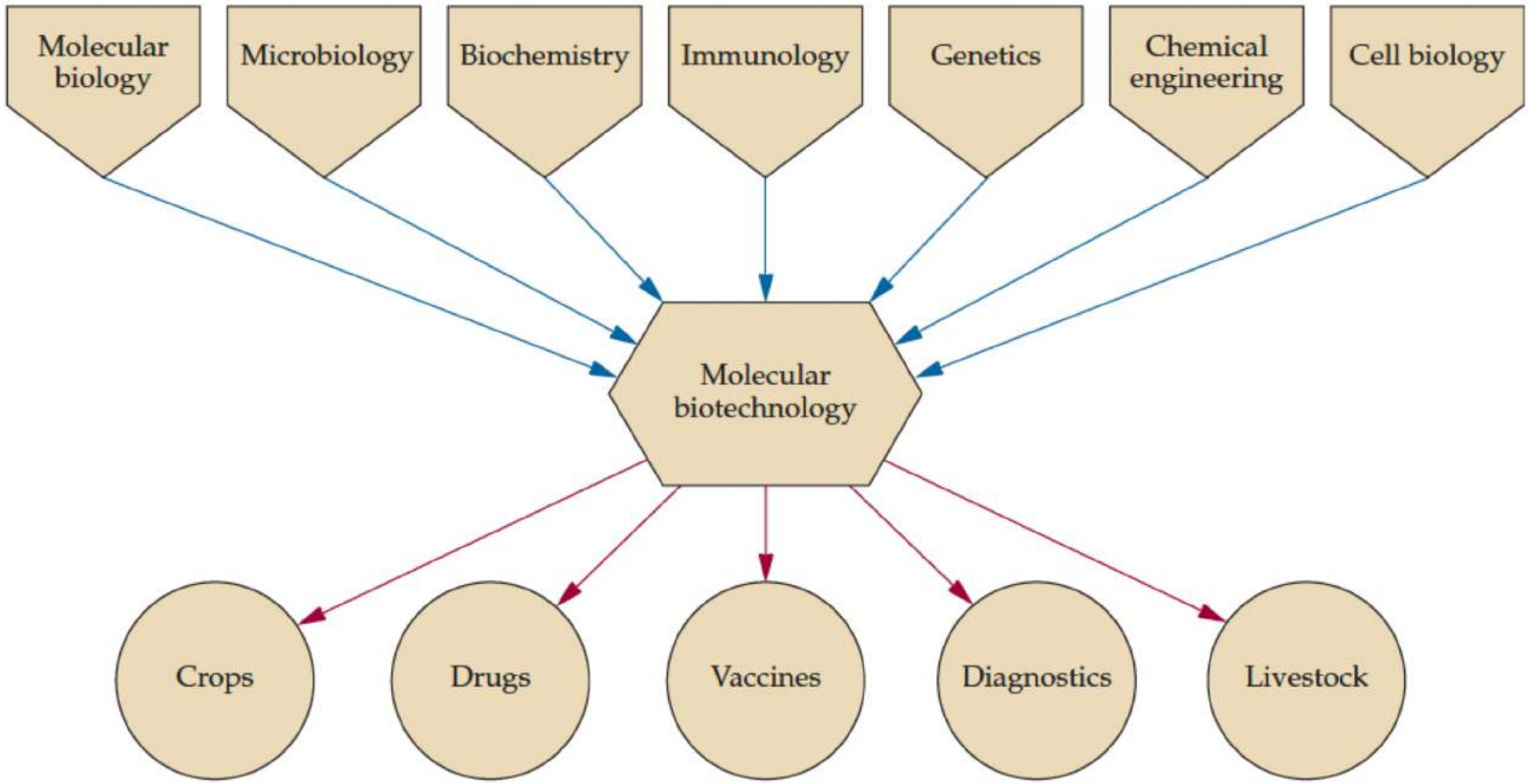
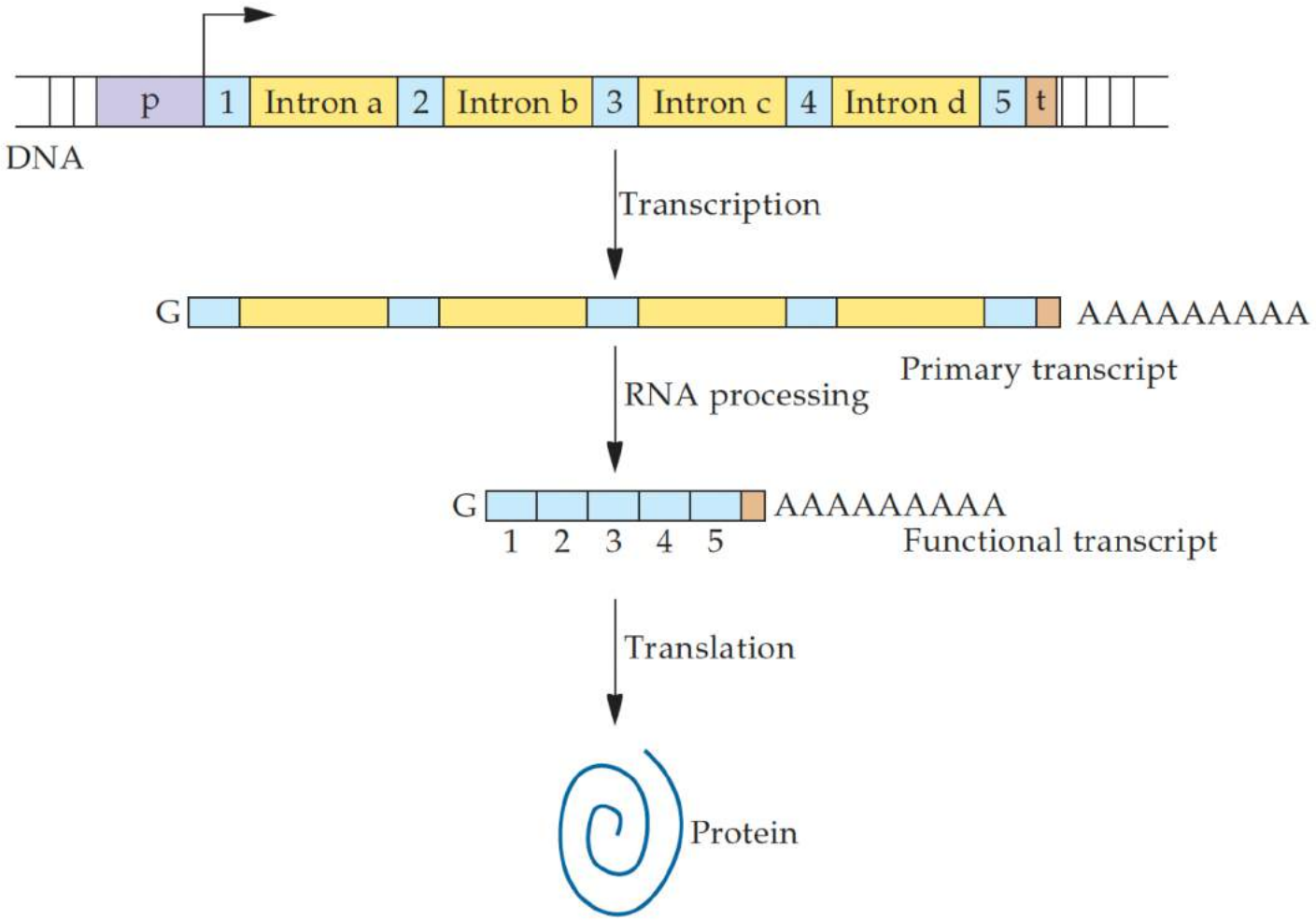
Many Scientific disciplines contribute to molecular biotechnology



Schematic representaion of aa Eukaryotic structural gene



Eukaryotic gene structure

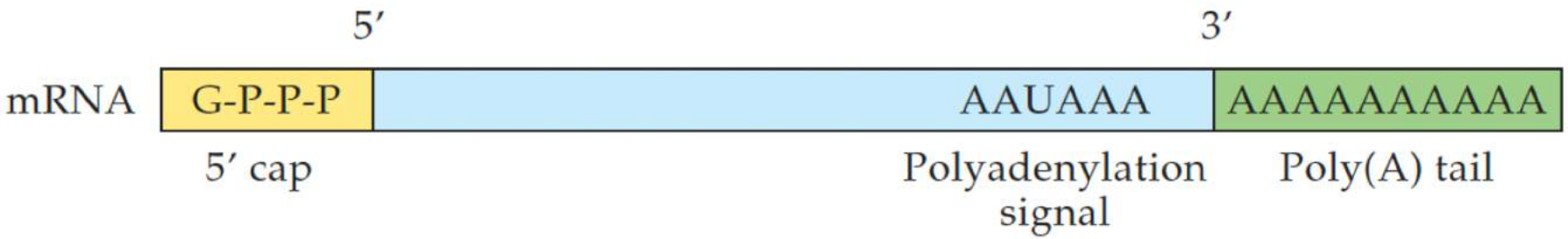
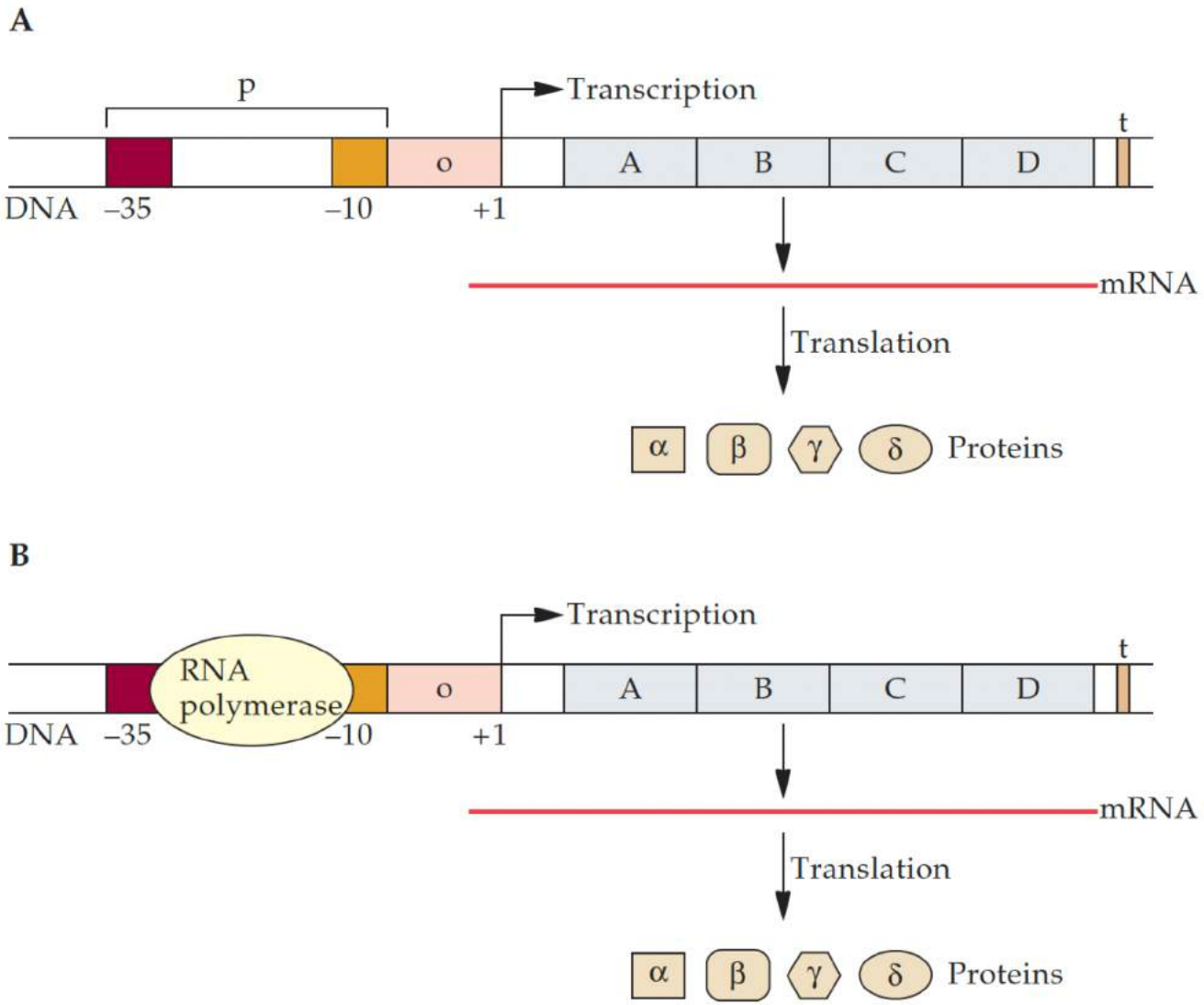
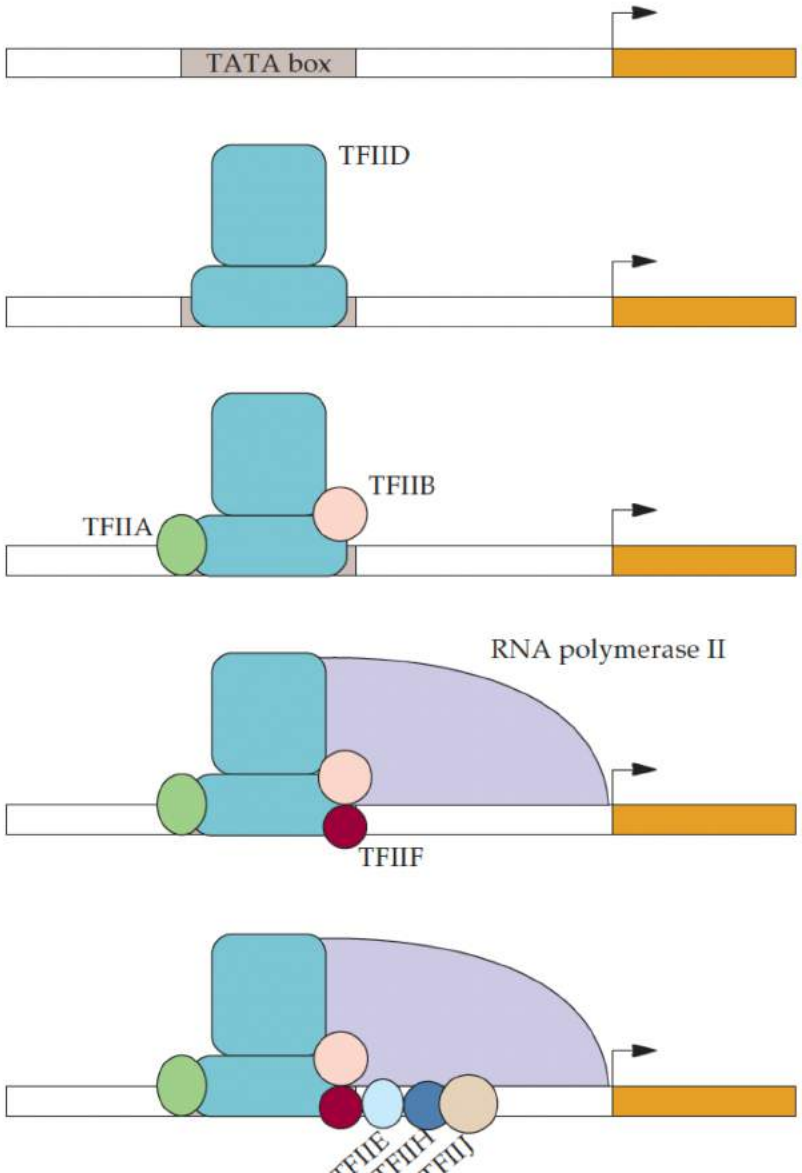


FIGURE 2.18 Modification of the ends of a primary RNA transcript in the nucleus. A modified guanine nucleotide cap is added to the 5′ end of the transcript, and a polyadenylation signal in the RNA sequence specifies the addition of a polymer of 50 to 250 adenine (A) nucleotides to the 3′ end to form a poly(A) tail. The modified ends aid in the transport of the mature mRNA from the nucleus and in the binding of ribosomes to the mRNA and increase the stability of the mRNA.

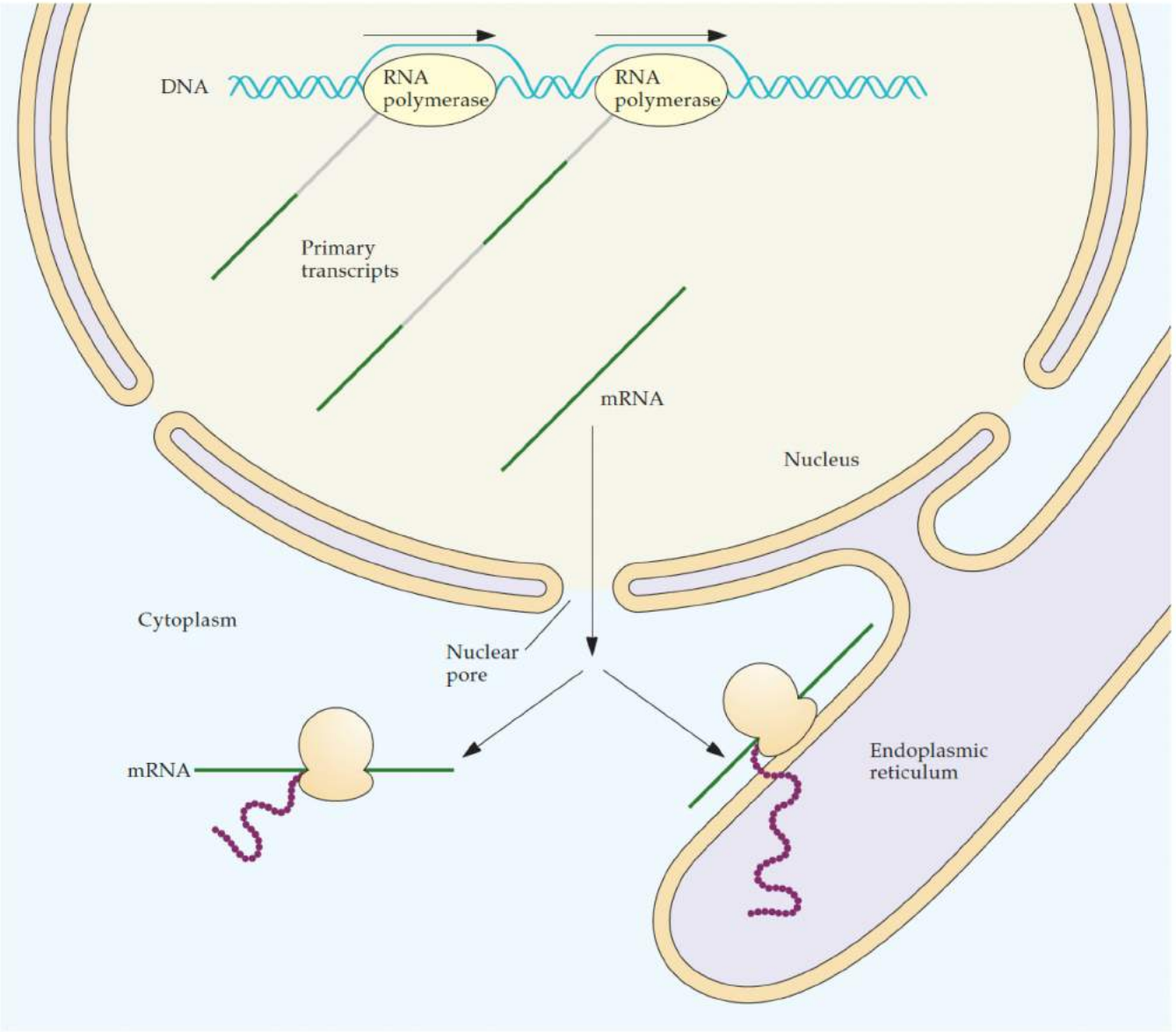
Prokaryotic gene structure



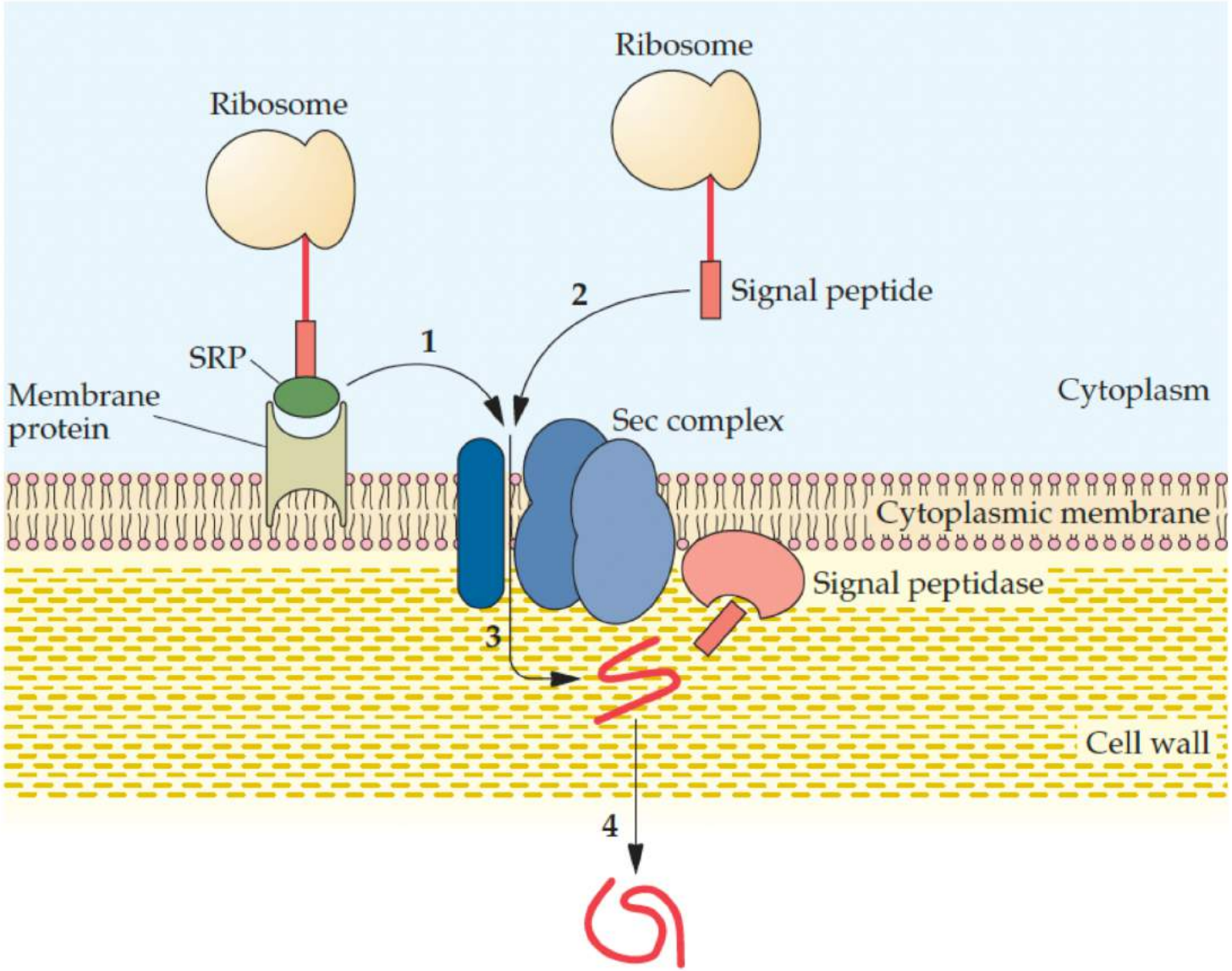
Formation of an RNA polymerase II transcription initiation complex



Transcription and translation in eukaryoutes



Secretion in gram positive bacteria



Secretion in gram negative bacteria 1

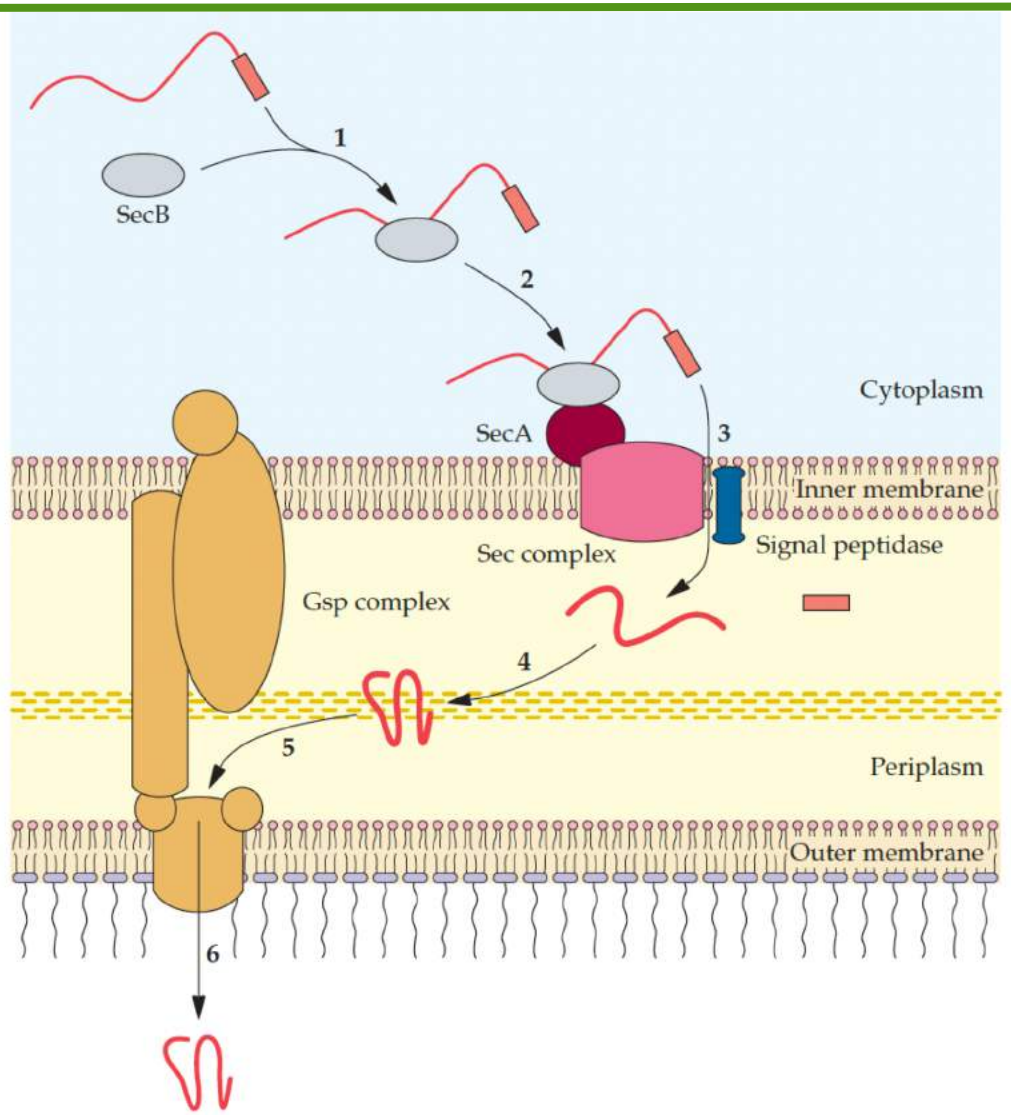


FIGURE 2.30 Schematic representation of a type II secretion pathway in gram-negative bacteria. The SecB protein binds to a secretory protein in the cytoplasm (1), SecB attaches to the SecA protein that is part of the Sec complex of the inner mem-

Secretion in gram negative bacteria 1

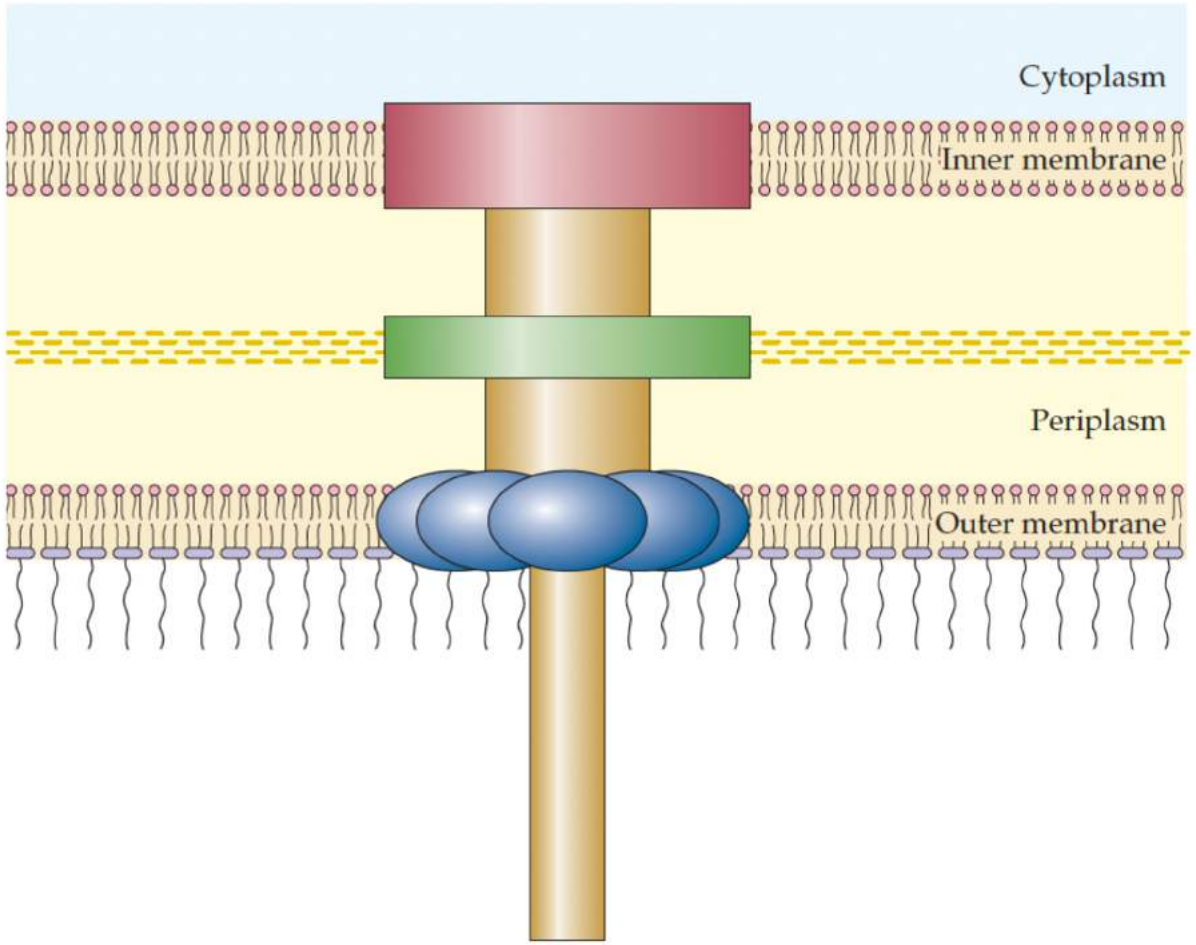


FIGURE 2.31 The type III secretion system is made up of about 20 different proteins that form a continuous channel through the inner and outer membranes of gram-negative bacteria. The type III secretion system is used by bacterial pathogens to secrete toxins and other proteins into plant and animal host cells. A hollow needle-like protein structure extends from the bacterial surface into the host cell.

Secretion pathway in eukaryoutes

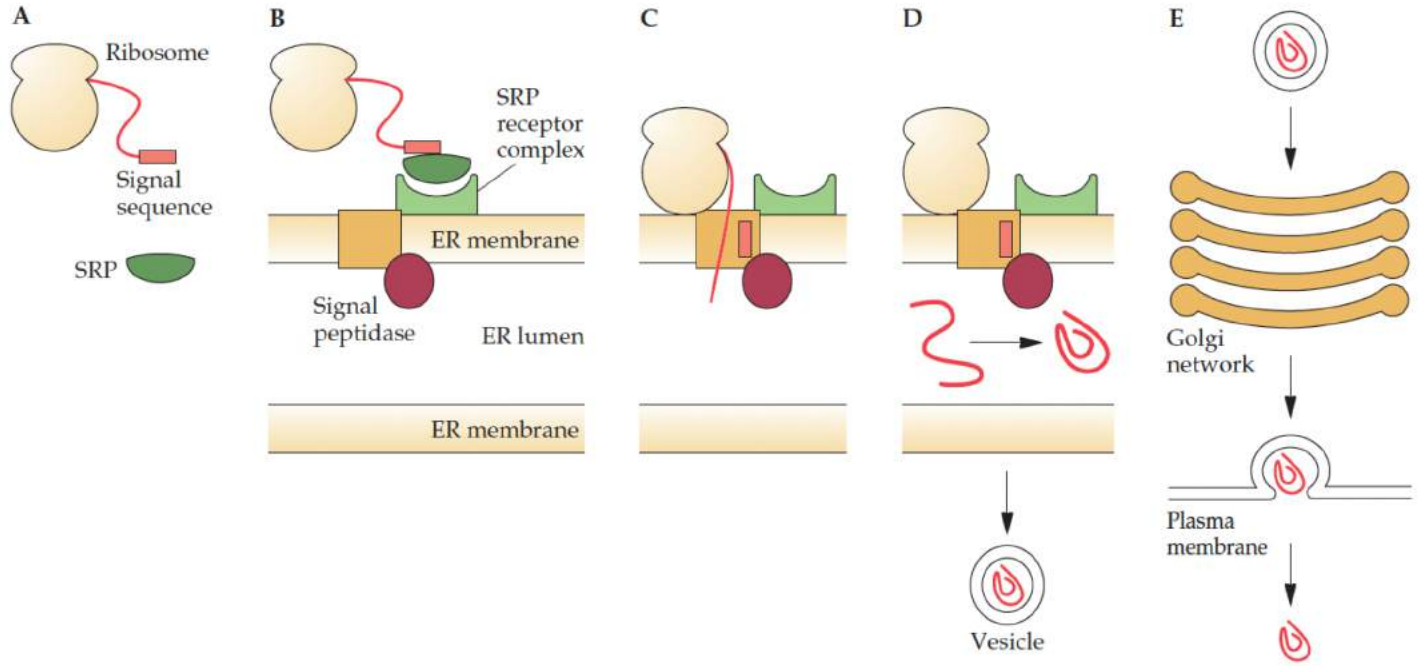


FIGURE 2.32 Schematic representation of the secretion pathway in eukaryotes. **(A)** A signal recognition particle (SRP) binds to the signal sequence of a secretory protein. **(B)** The SRP attaches to an SRP receptor on the endoplasmic reticulum (ER) membrane. **(C)** The secretory protein is translocated into the lumen of the ER, and a signal peptidase removes the signal sequence. **(D)** The secretory protein is folded, partially modified, and packaged in a transport vesicle intended for the Golgi network. **(E)** The ER-released vesicle carrying the secretory protein enters the Golgi network at the *cis* face and passes through the Golgi stack, where it is further modified; after it is sorted, a plasma membrane-specific vesicle is formed at the *trans* face of the Golgi network. The secretory transport vesicle fuses with the plasma membrane and releases the secretory protein to the extracellular environment.

Molecular Biotechnology

Chapter 3, Recombinant DNA Technology

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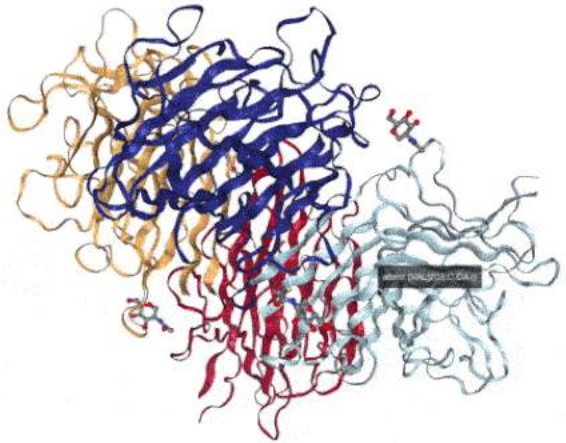
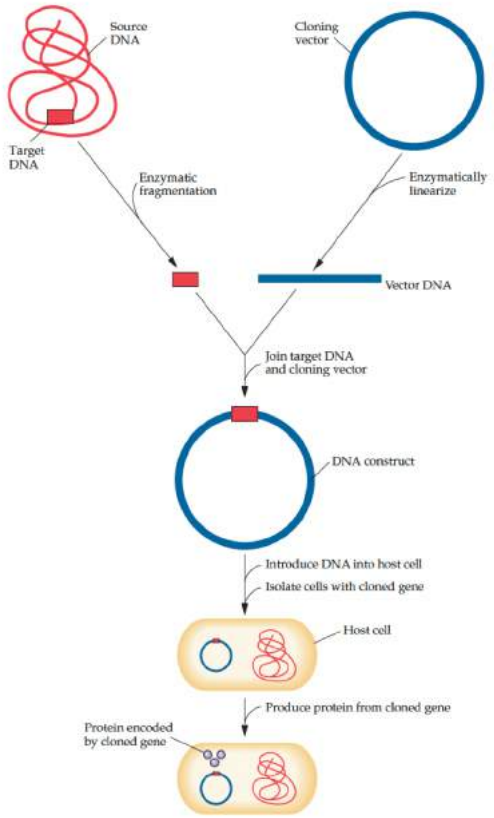


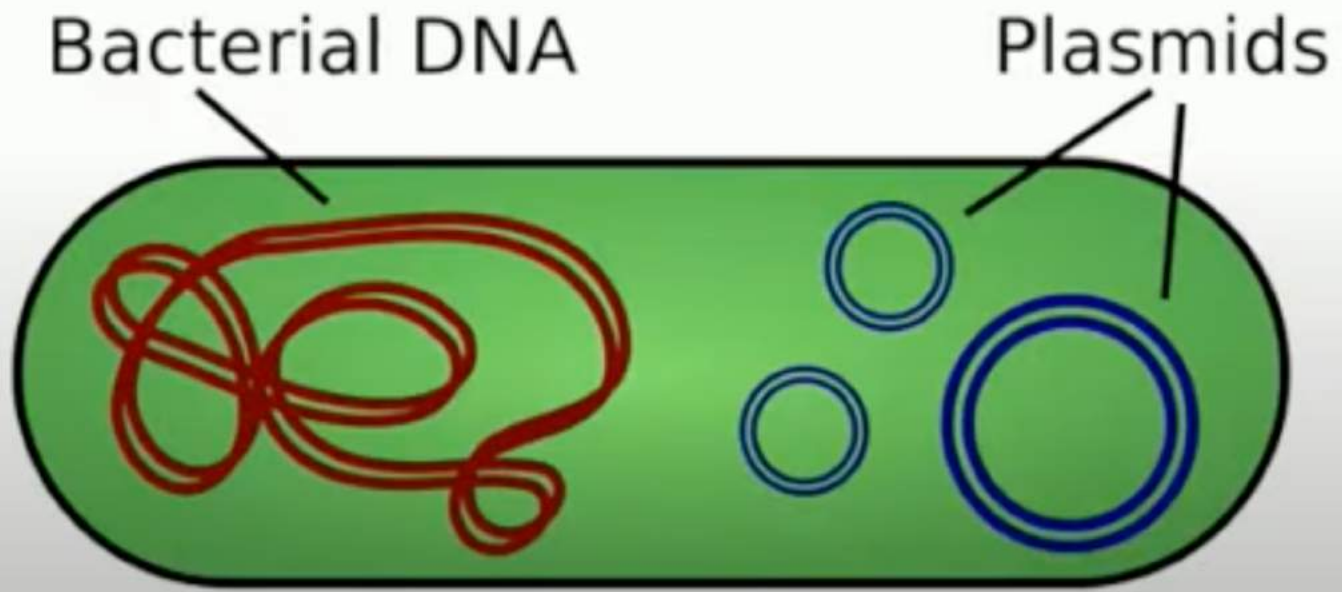
Recombinant DNA technology

Recombinant DNA technology, which is also called gene cloning or molecular cloning, is a general term that encompasses a number ofexperimental protocols leading to the transfer of genetic information (DNA) from one organism to another.



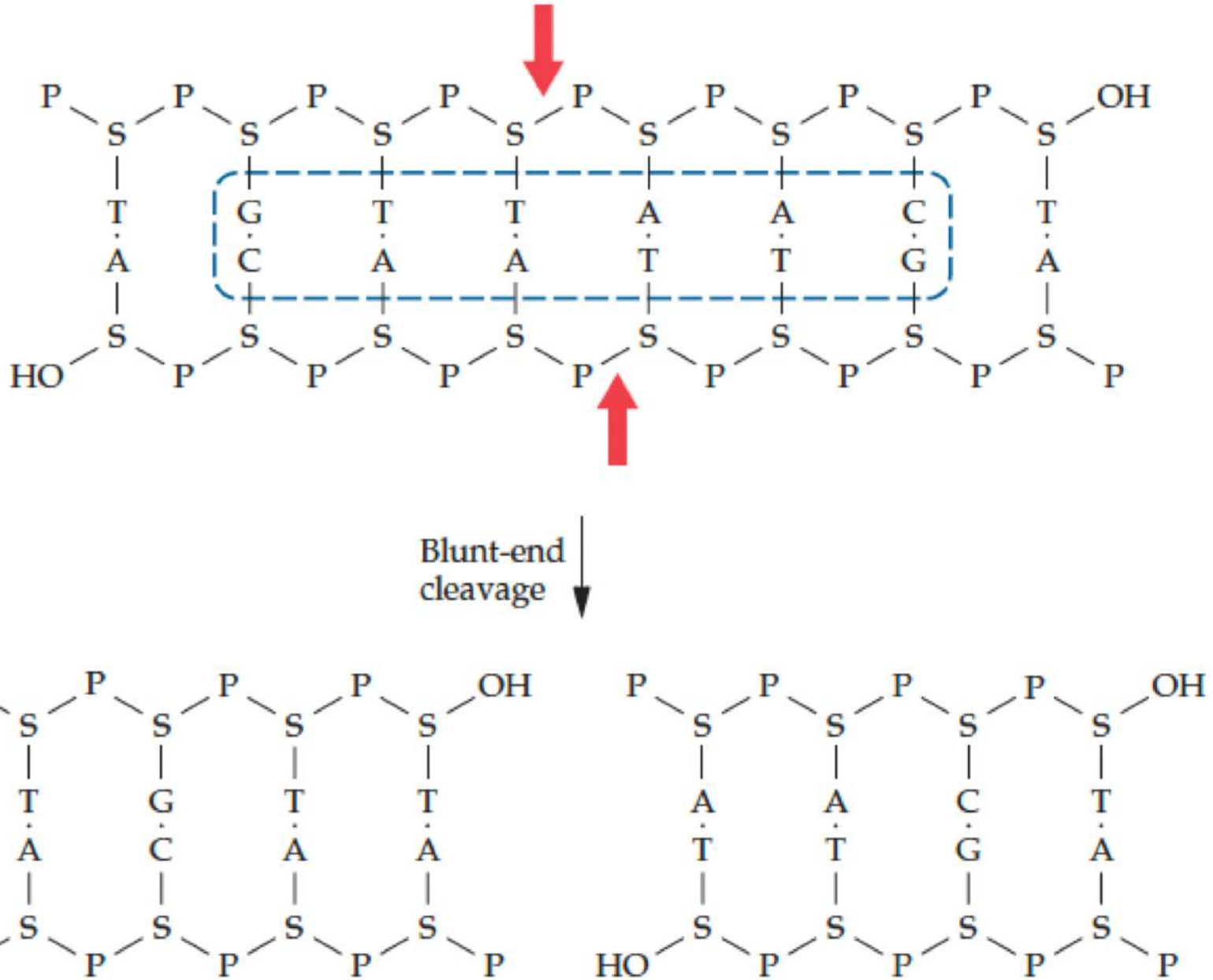
Some vocabulary

- **Plasmids-** A circular self-replicating form of DNA found in bacteria.



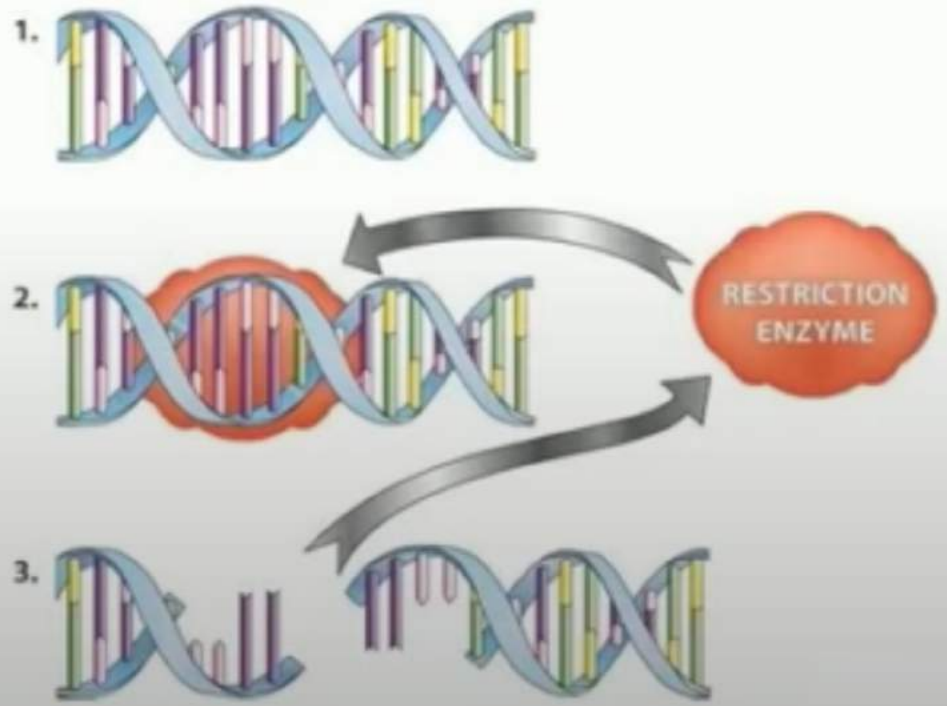
Recombinant DNA technology

Scientists have taken advantage of plasmids to use them as tools to clone, transfer, and manipulate genes. **Plasmids that are used experimentally for these purposes are called vectors**. Researchers can insert DNA fragments or genes into a plasmid vector, creating a so-called recombinant plasmid.

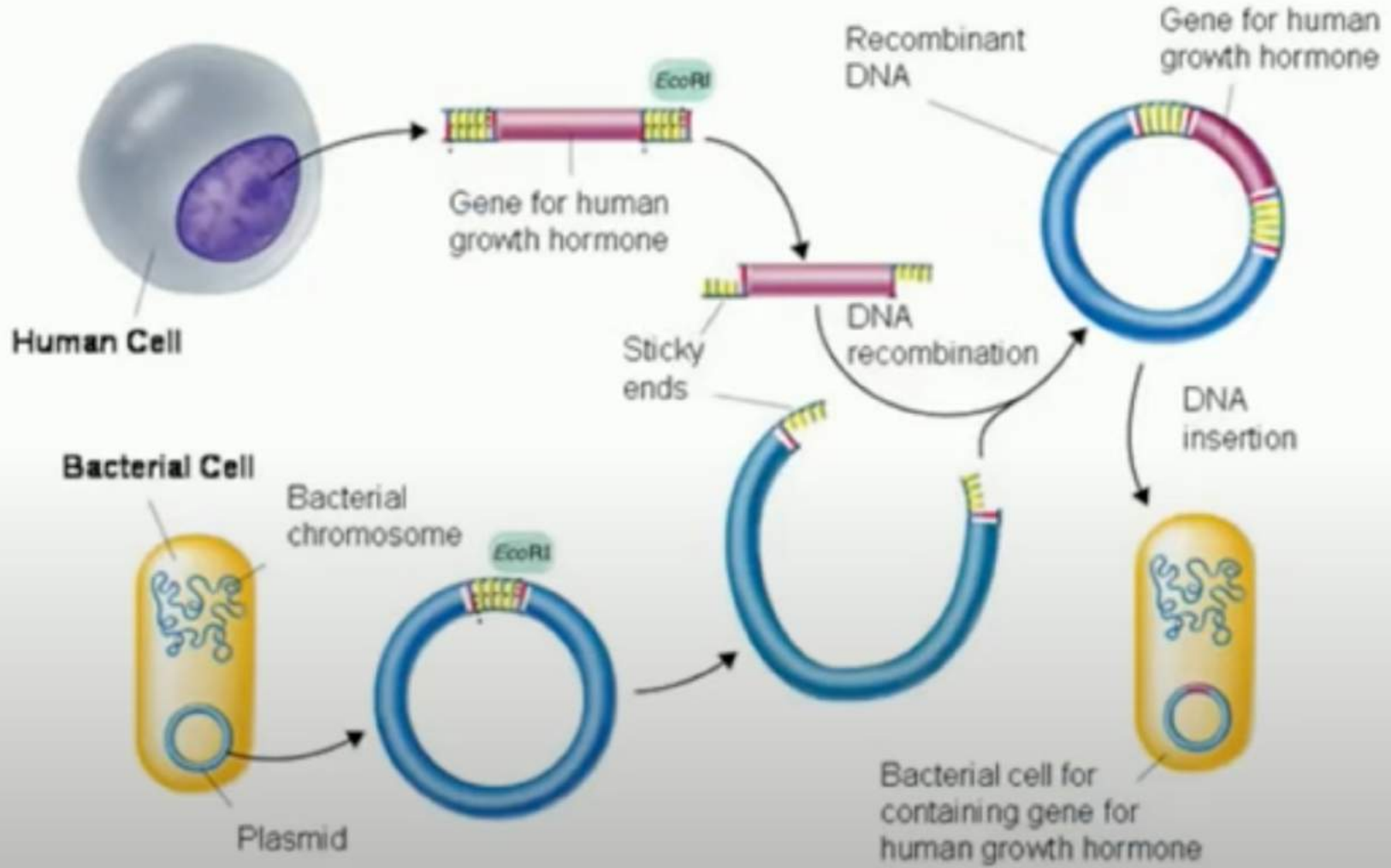


Some vocabulary

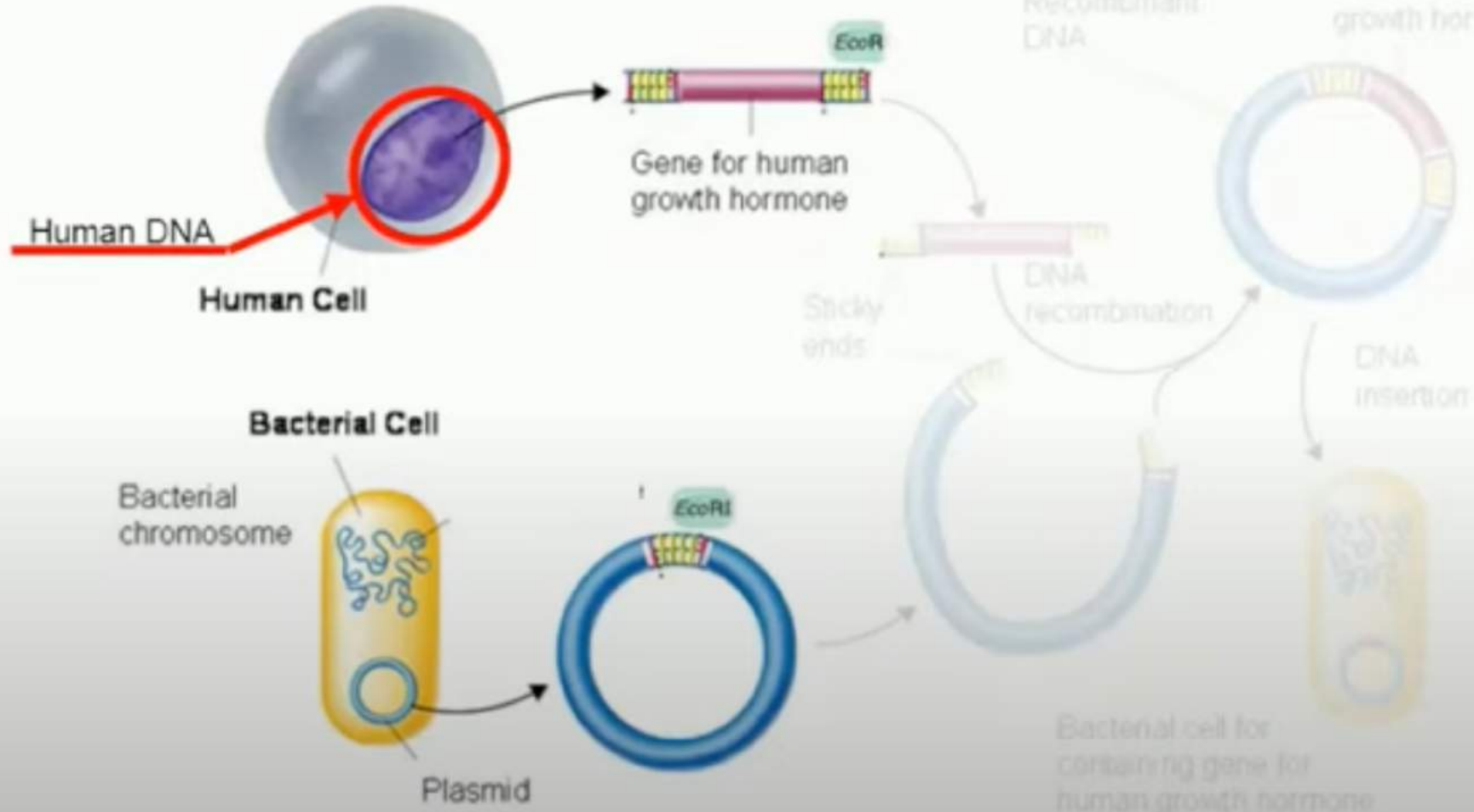
- **Enzymes**- Used as a catalyst to cut a specific DNA sequence. This process is often called **digestion.**
  - **Think of them as scissors to cut a specific DNA sequence.**



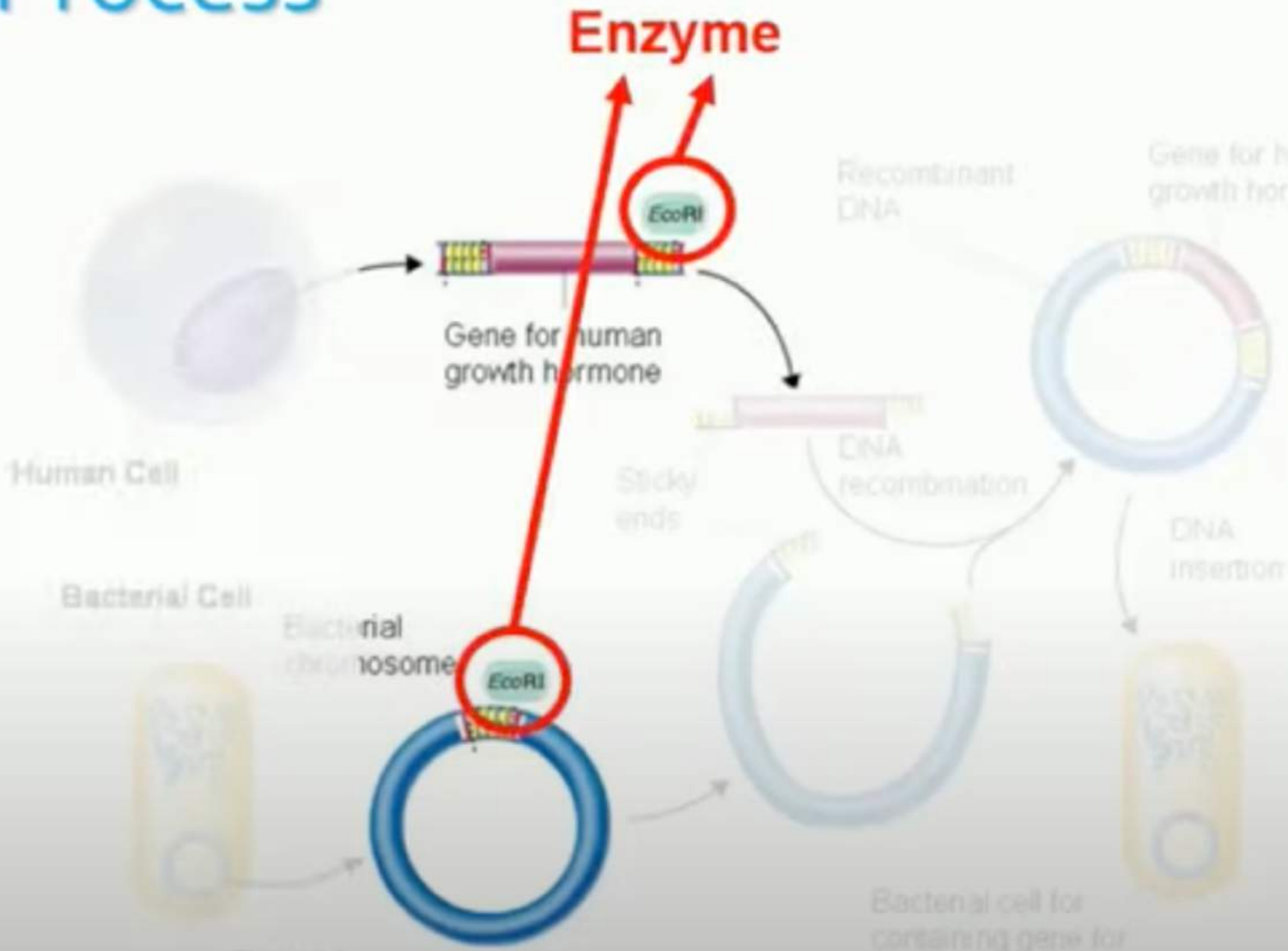
The Process



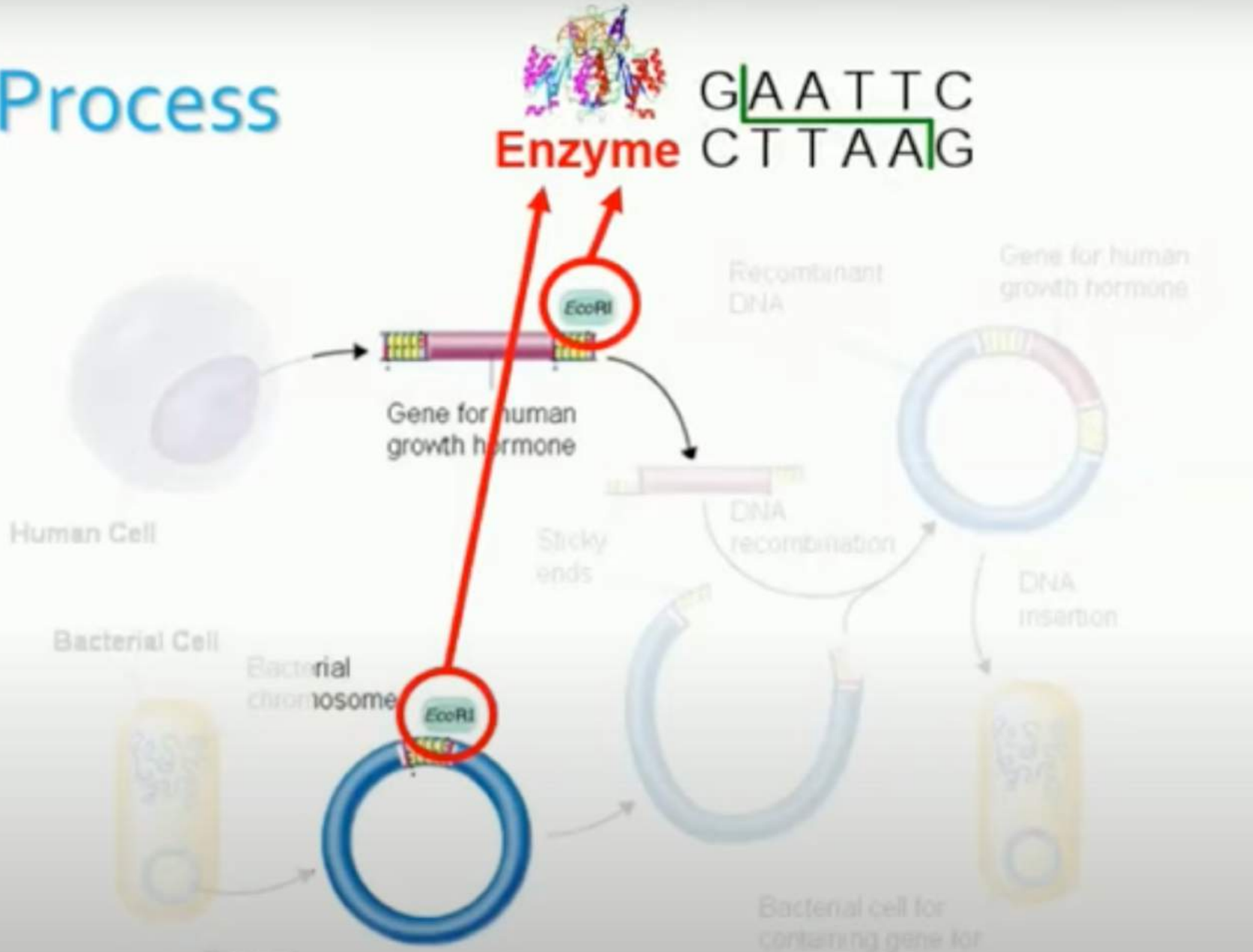
The Process



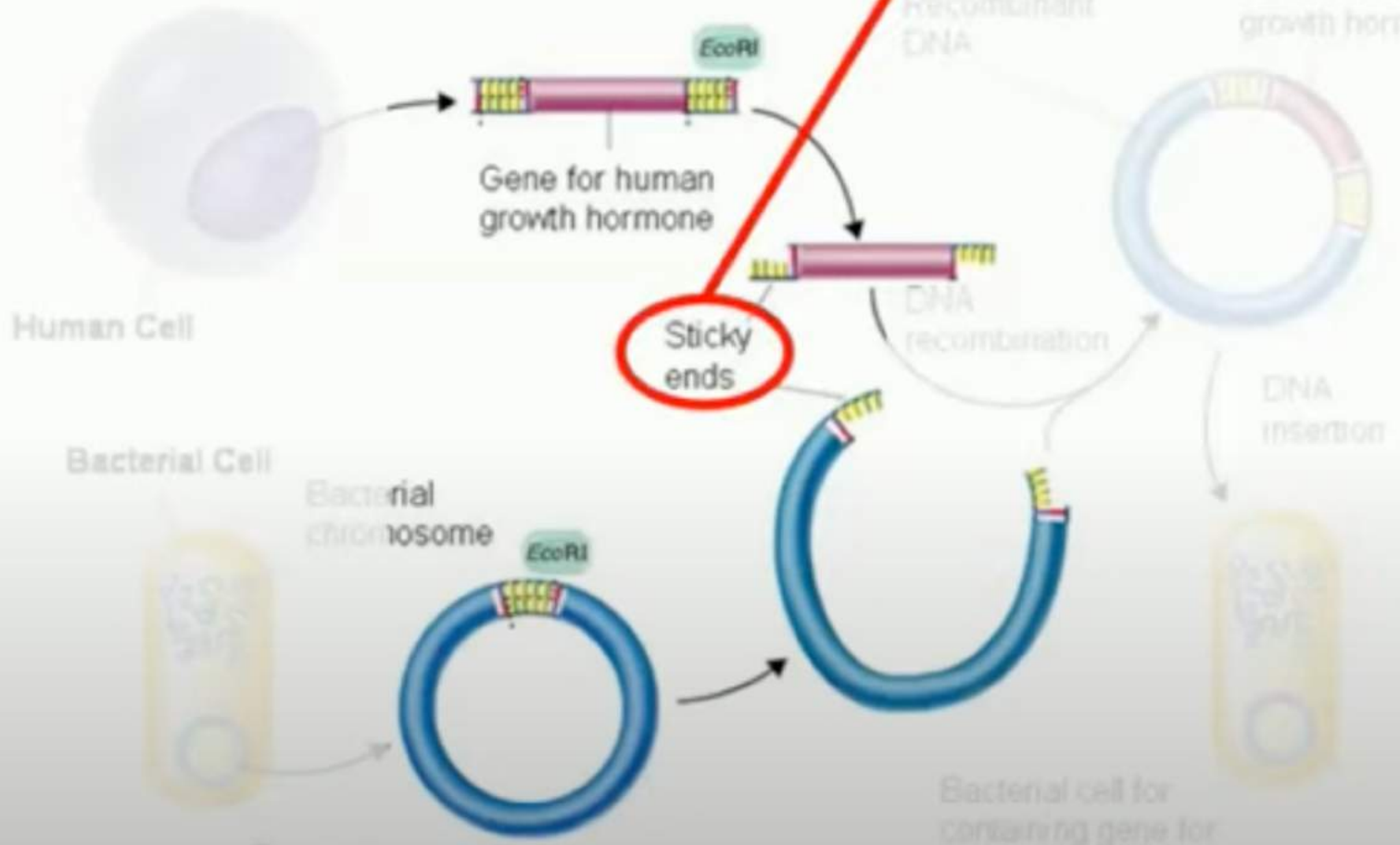
The Process



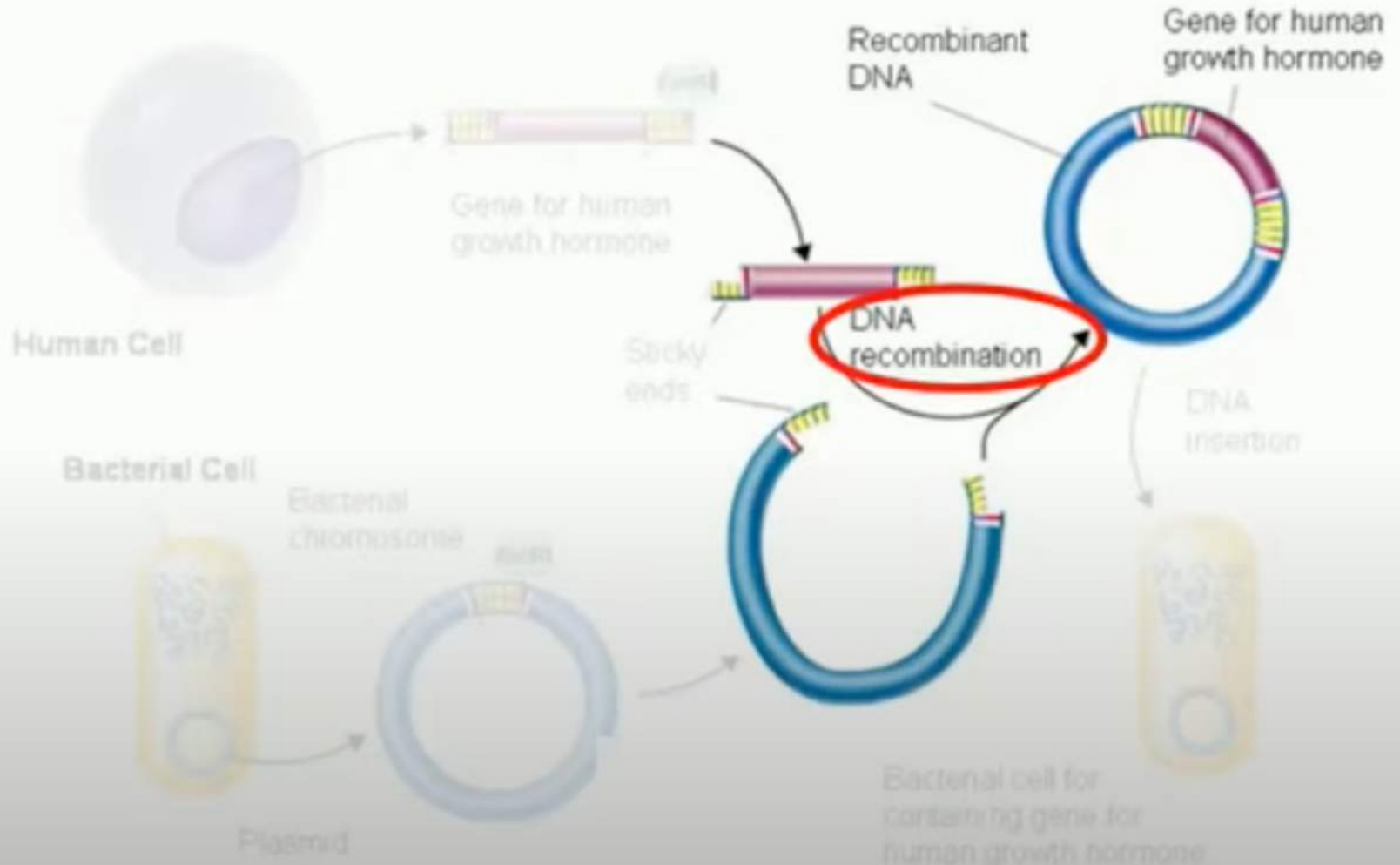
The Process



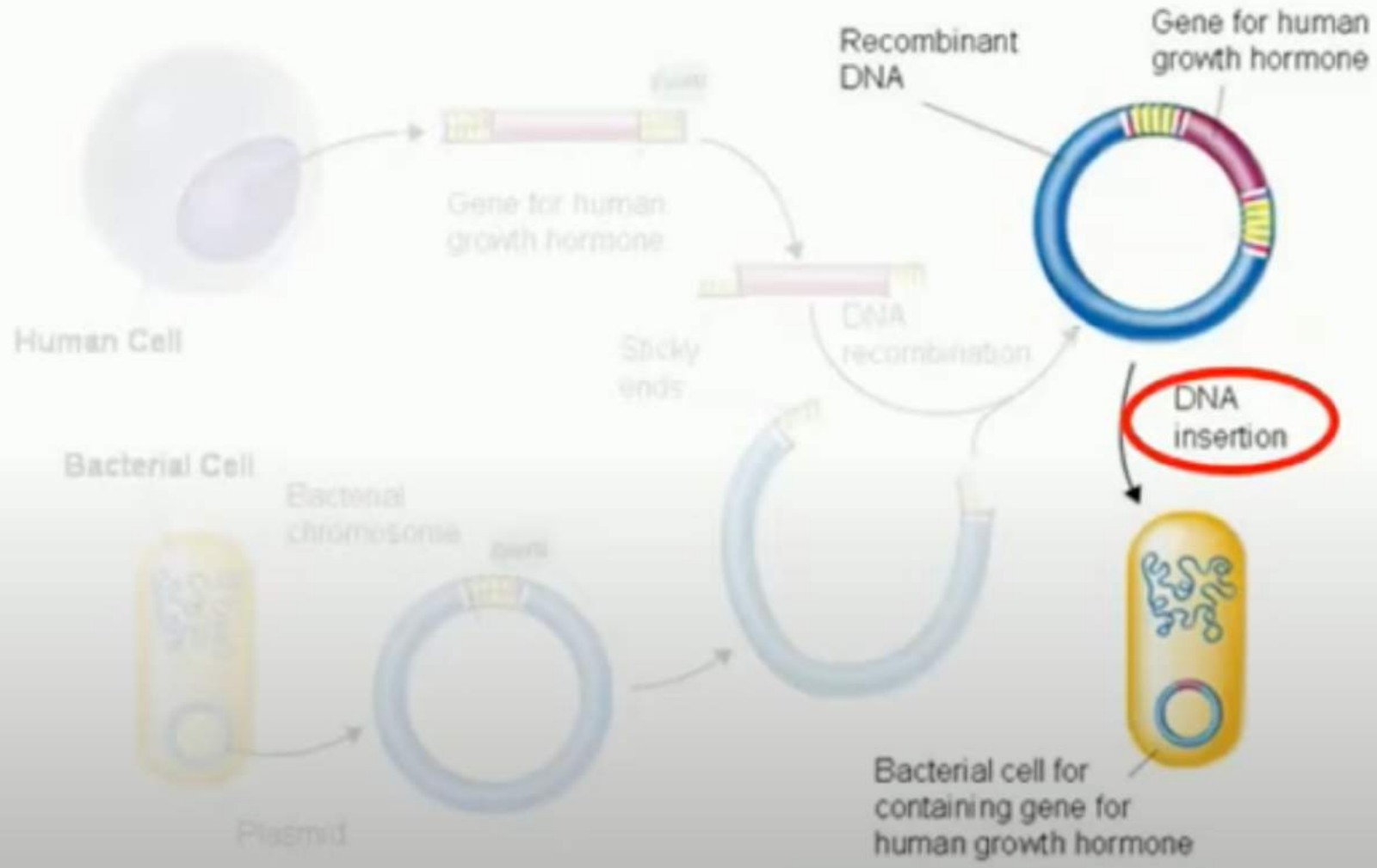
The Process



The Process



The Process



History of insulin:
A story of recombinant DNA

- Animal source insulin was the only type available until 1977 when…
- The first genetically-engineered insulin was produced in a laboratory using E. coli by Dr. Riggs, Dr. Itakura and Dr. Boyer.
- The vast majority of insulin used today is produced by using recombinant DNA

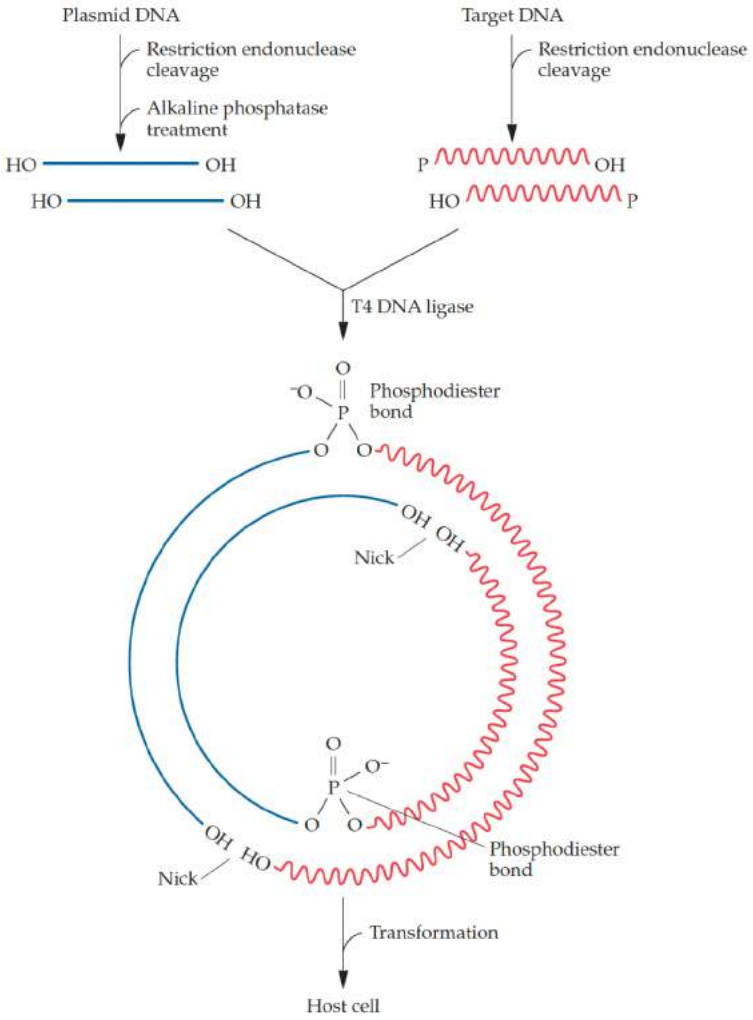
Insulin from pigs

One of the many synthetic insulins on the market

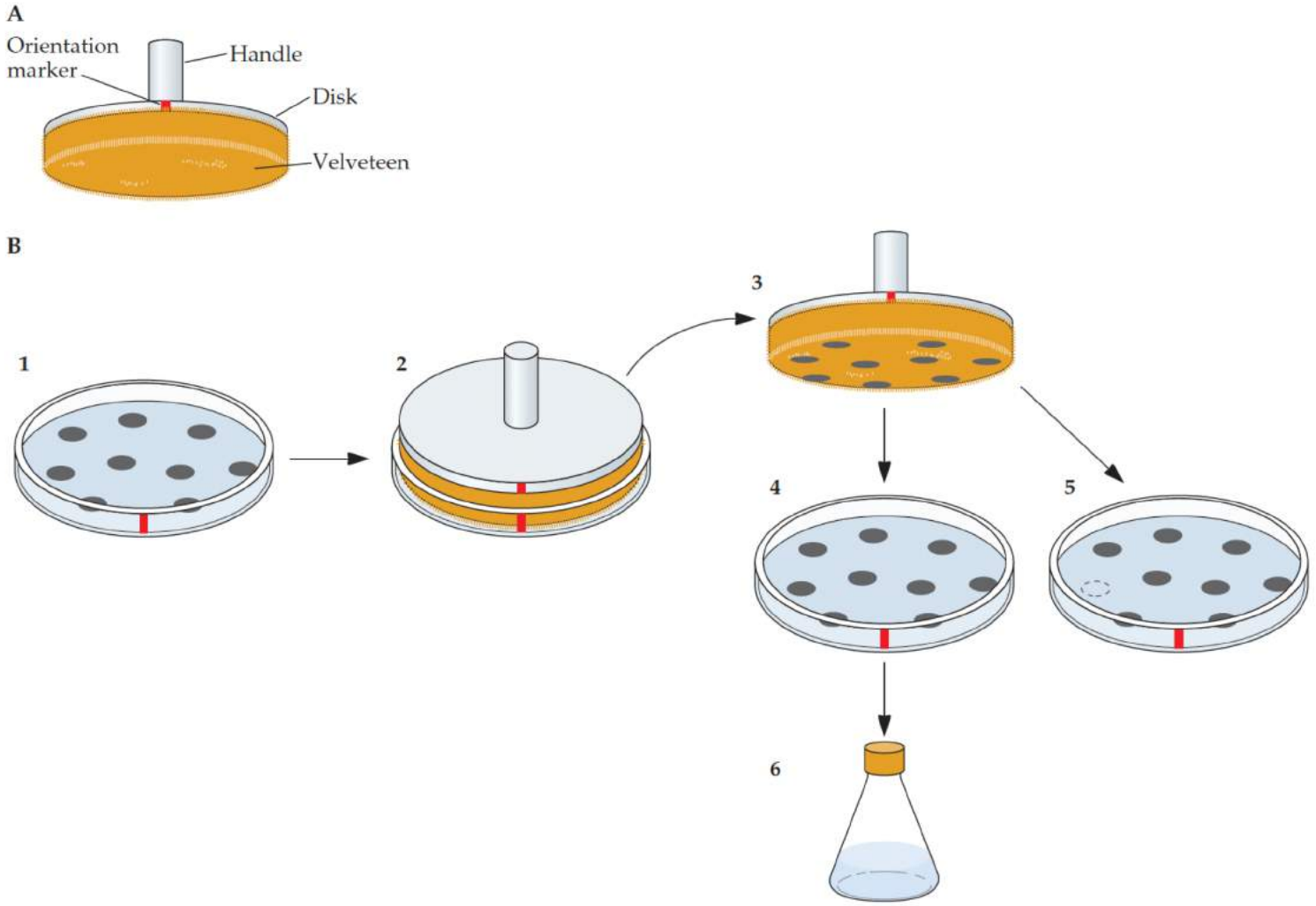
TABLE 3.1 Recognition sequences of some restriction endonucleases

Enzyme	Recognition site	Type of cut end
EcoRI	G↓A–A–T–T–C C–T–T–A–A↑G	5′ phosphate extension
BamHI	G↓G–A–T–C–C C–C–T–A–G↑G	5′ phosphate extension
PstI	C–T–G–C–A↓G G↑A–C–G–T–C	3′ hydroxyl extension
Sau3AI	↓G–A–T–C C–T–A–G↑	5′ phosphate extension
PvuII	C–A–G↓C–T–G G–T–C↑G–A–C	Blunt end
HpaI	G–T–T↓A–A–C C–A–A↑T–T–G	Blunt end
HaeIII	G–G↓C–C C–C↑G–G	Blunt end
NotI	G↓C–G–G–C–C–G–C C–G–C–C–G–G–C↑G	5′ phosphate extension

Restriction Endonucleases

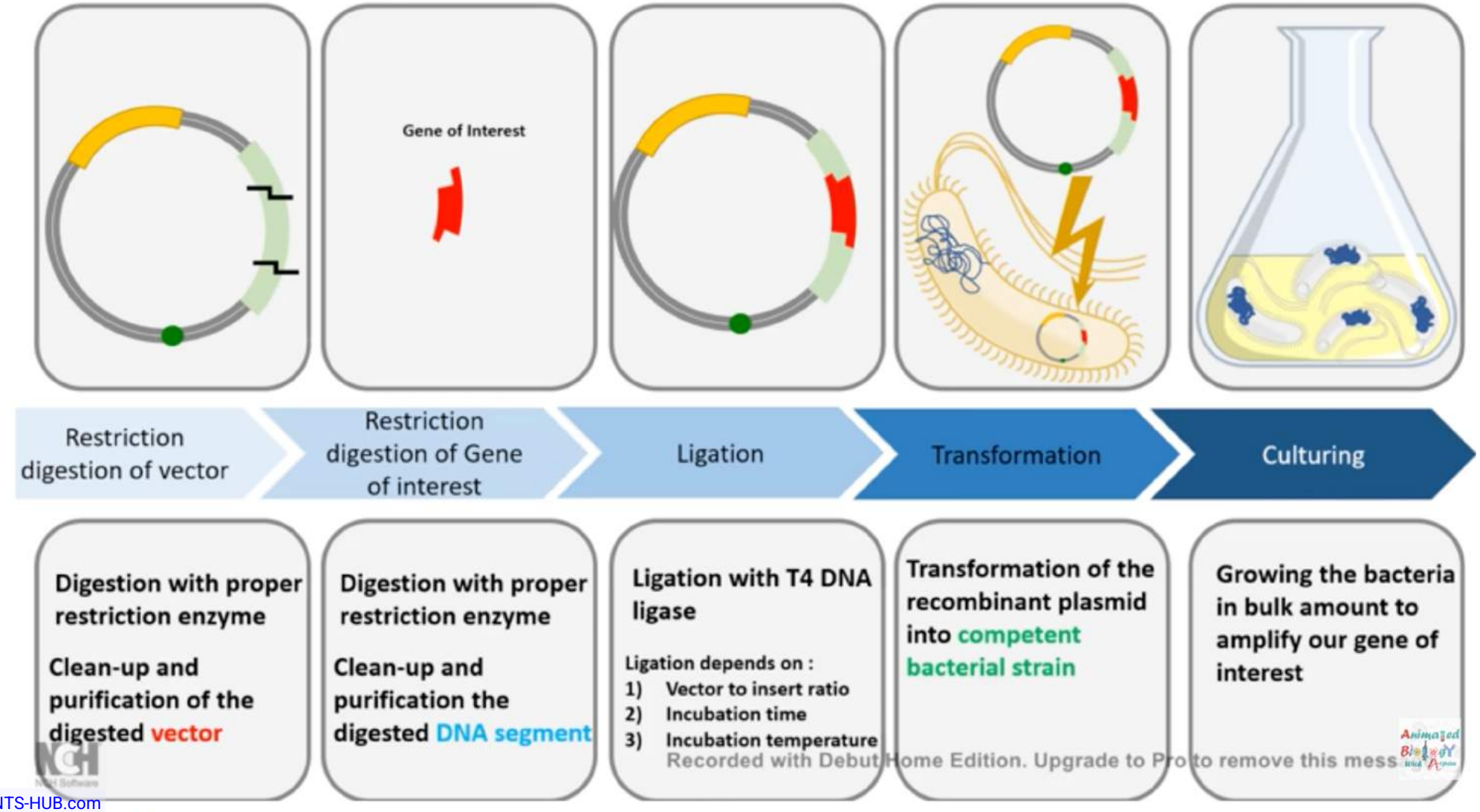


Screening bacterial colonies for mutant strains by replica plating

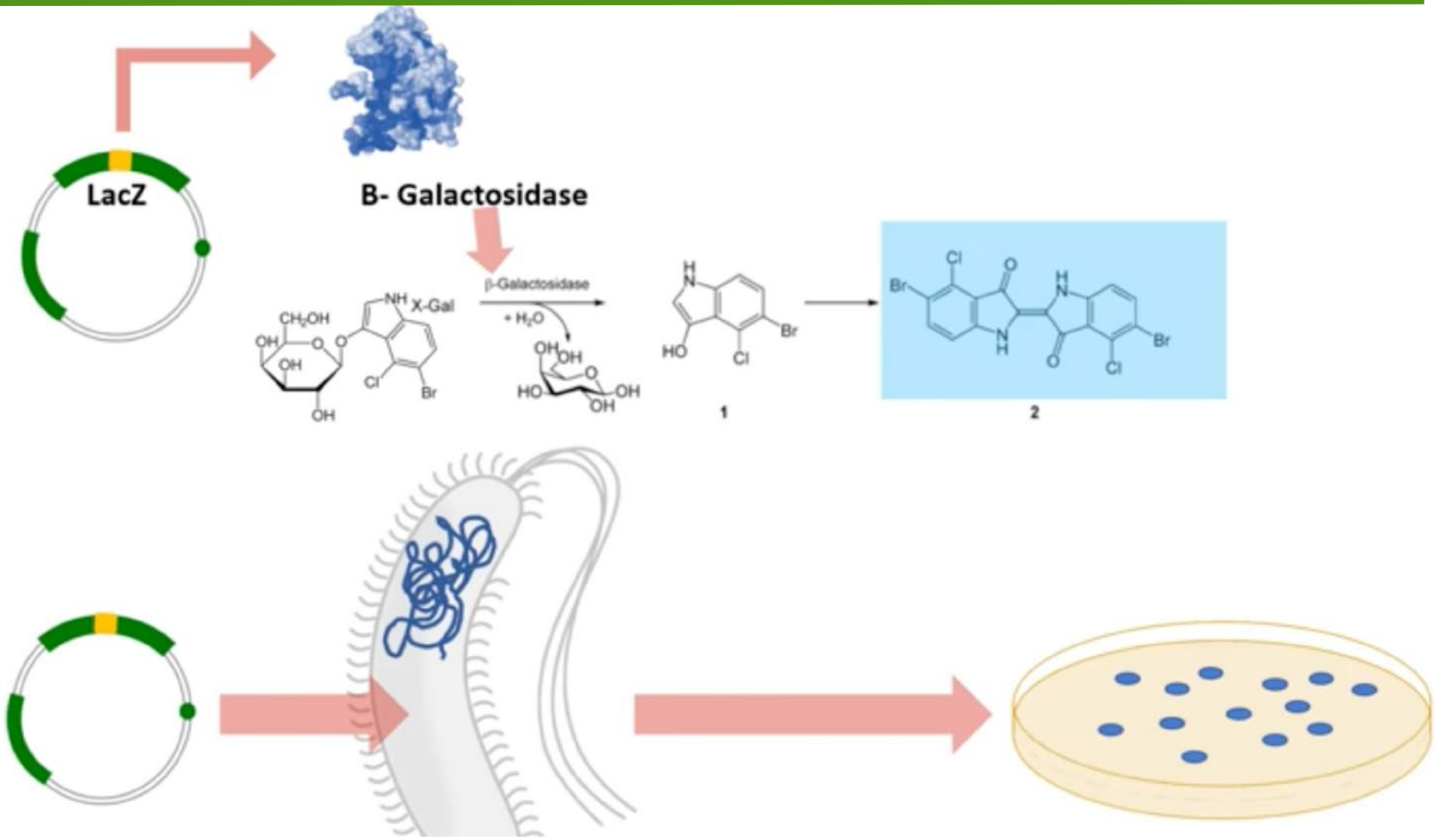


- used to identify colonies that require a nutritional supplement for growth, i.e., auxotrophic
- mutants. The missing colony (dashed circle) on the minimal medium (5)

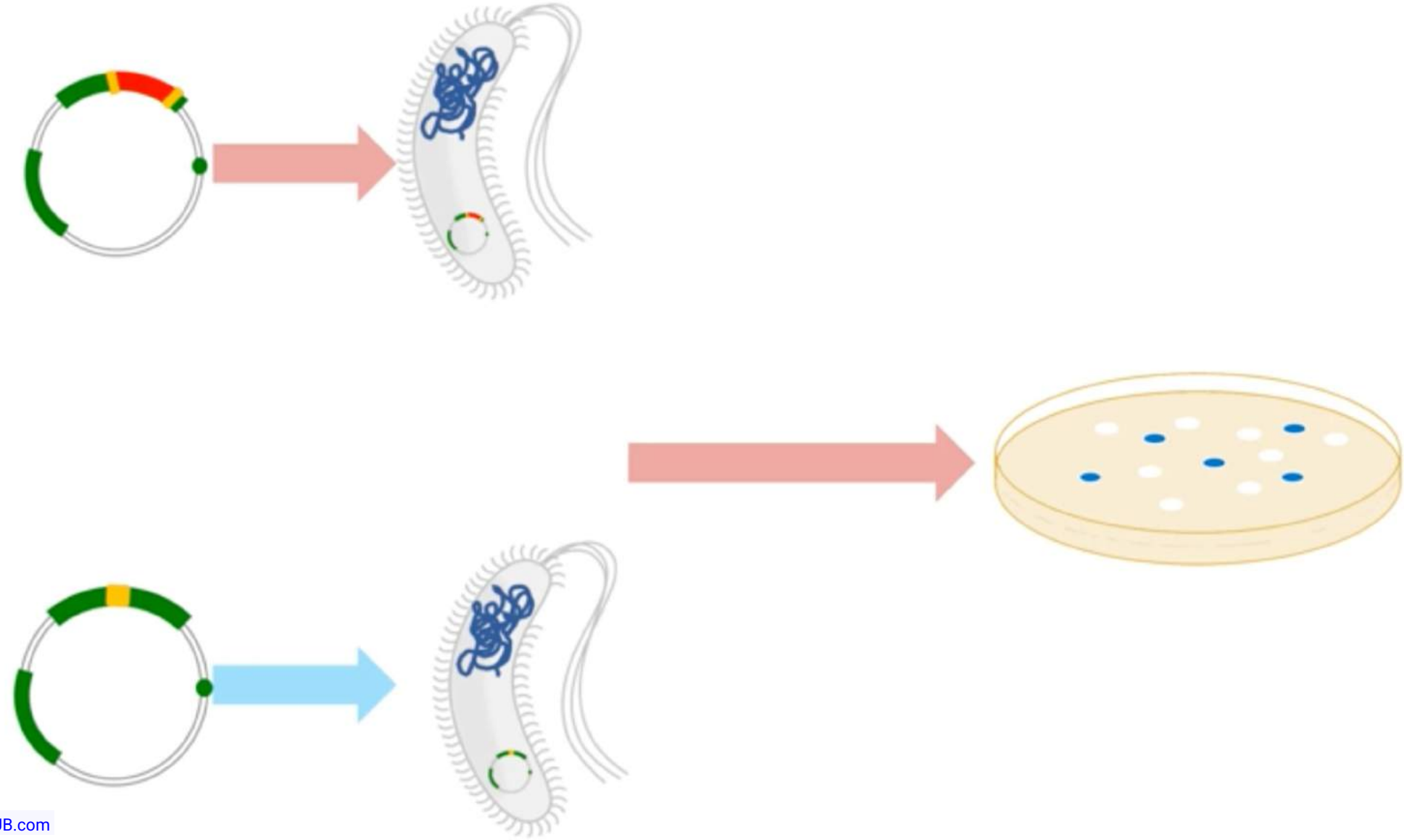
Blue white screening



Blue white screening

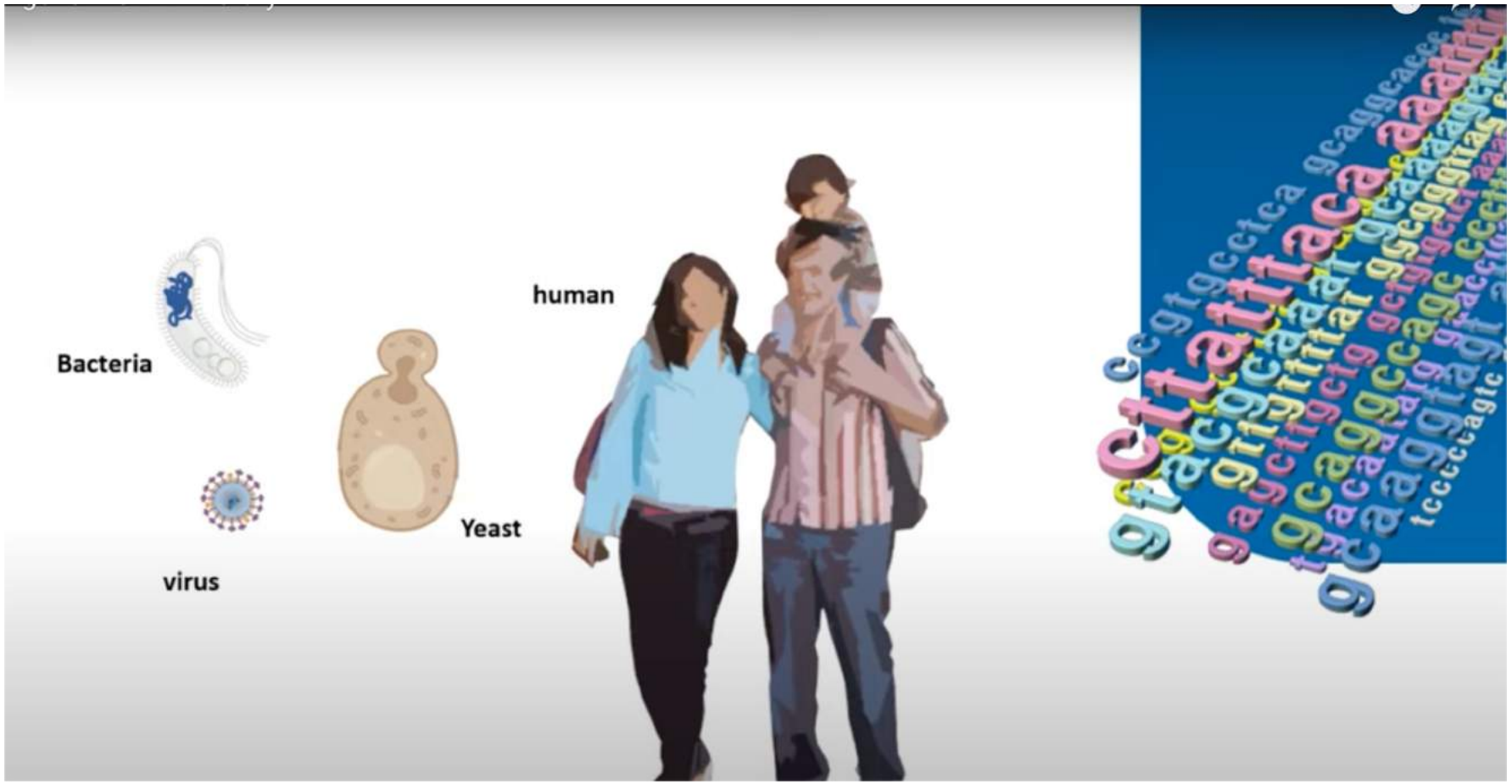


Blue white screening



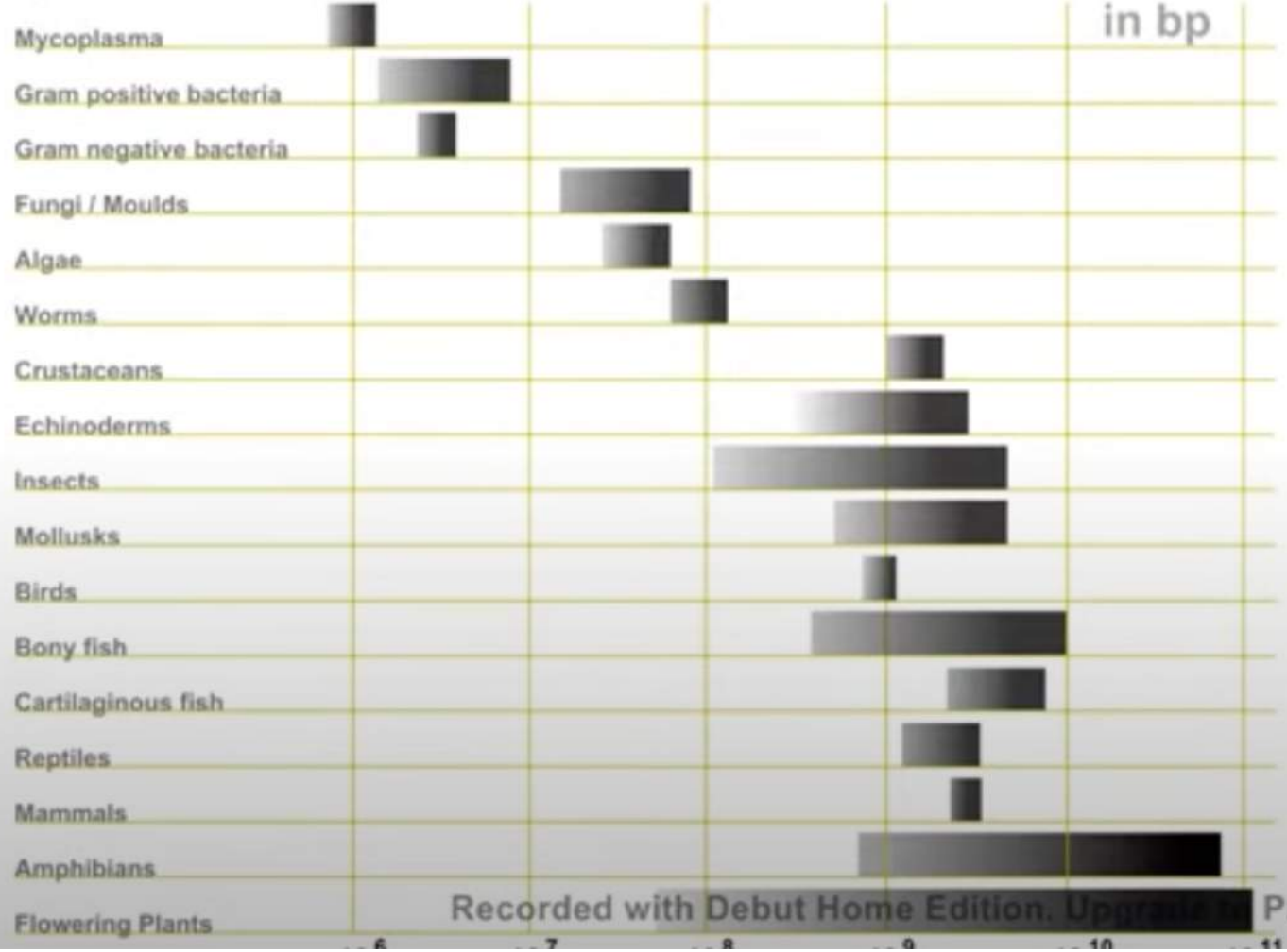
Creating and Screening a Library

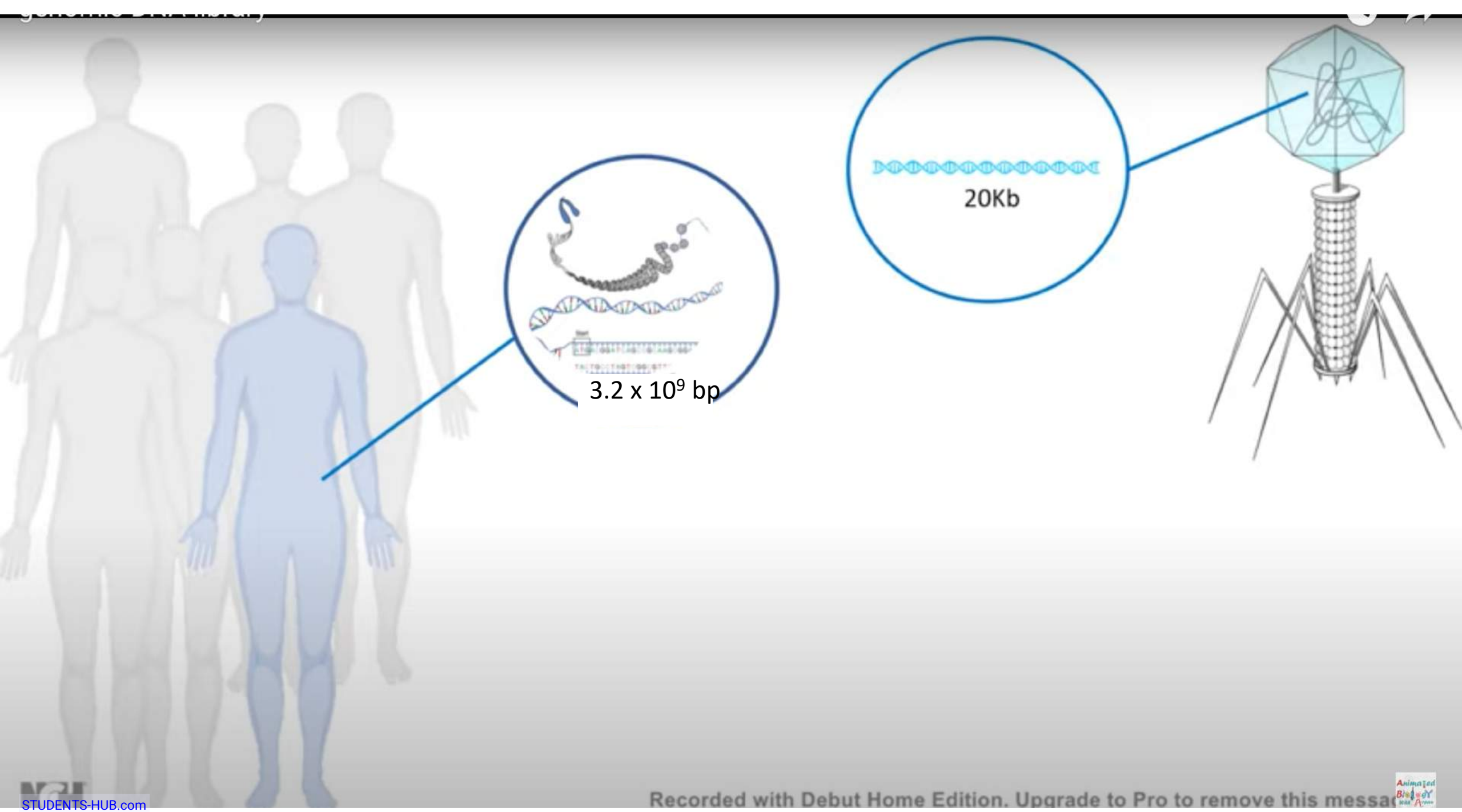
Making a Genomic Library

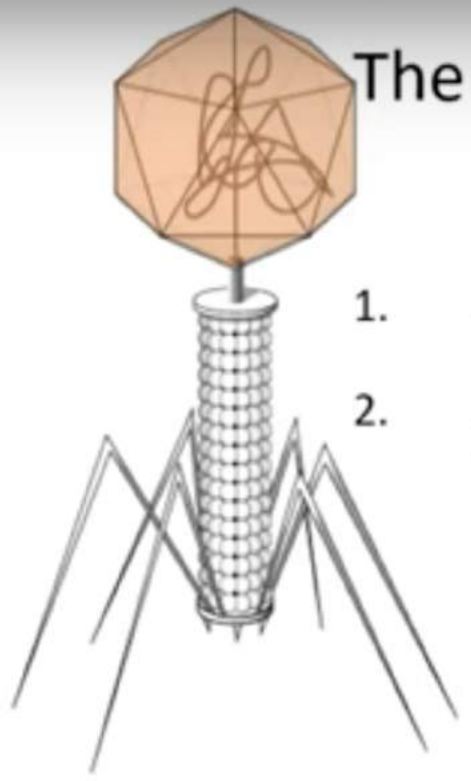


3 Big problems:

How to clone such big DNA regions?
Where to clone?
How to screen desired sequence from a collection of sequence?

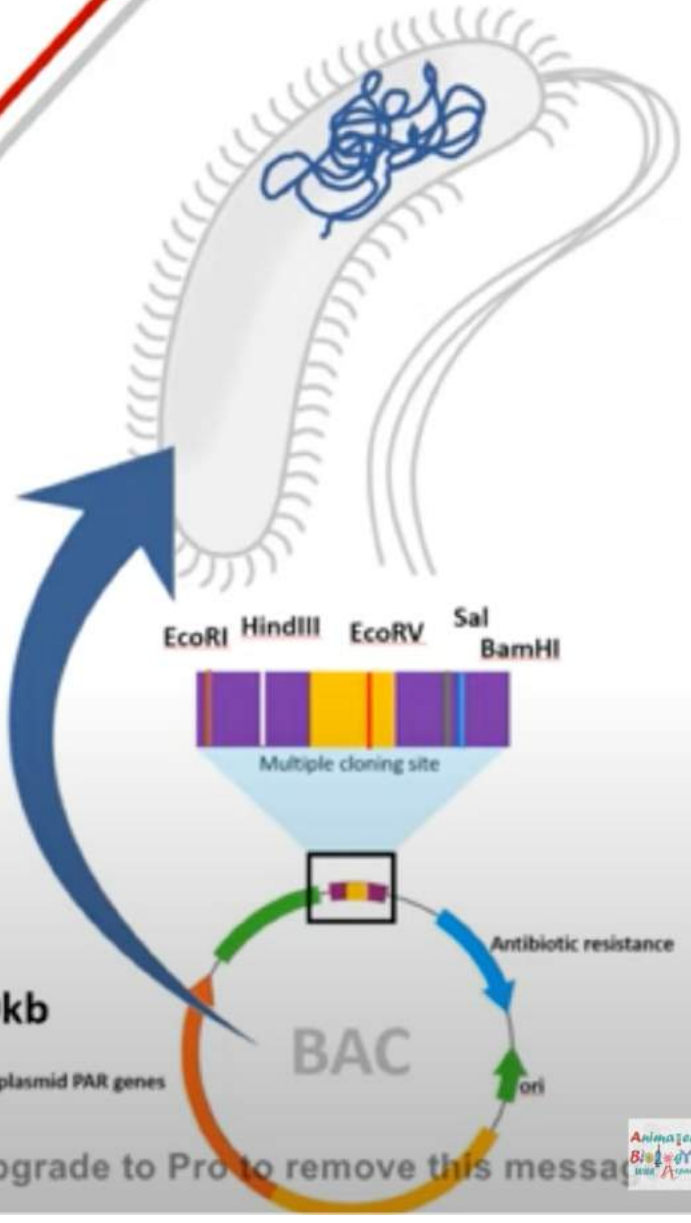






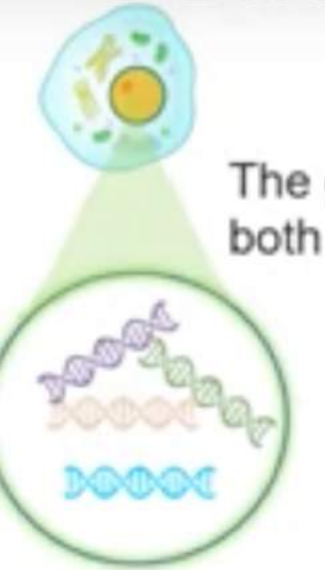
The genomic library is generated by using λ-phage for the following reasons.

1. A large number of λ phage can be screened simultaneously (5 x 10E4 phage plagues).
2. λ phage as a higher transformation efficiency about 1000 times higher compared to a plasmid.



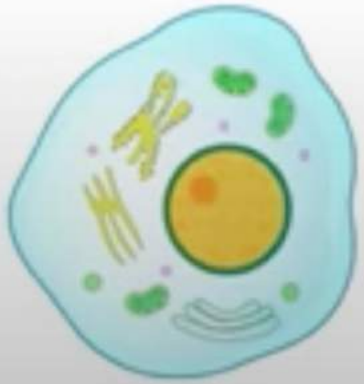
Carrying capacity= 100kb to 300kb

Genomic DNA library

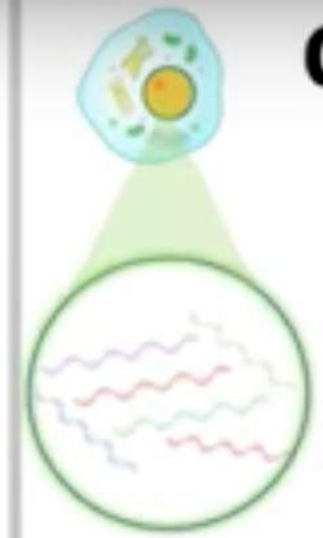


The **genomic DNA** contain the sequences for both intron and exon.

Genomic DNA library would contain representation for all the genes

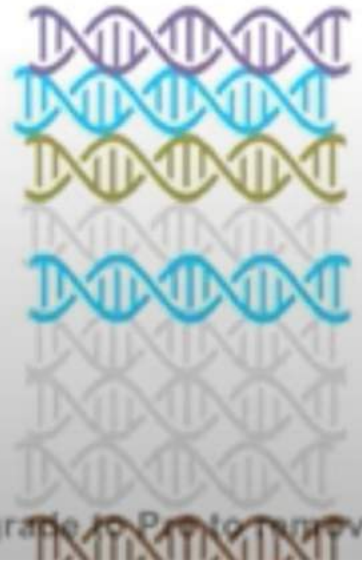


cDNA library



The **cDNA** clone will only contain the sequences found in the mRNA, not the entire gene while the **genomic** clone could have the sequences of the entire gene

cDNA library would contain representation for all the genes that are expressed in that cell in a given point of time



Molecular Biotechnology

Chapter 3, Recombinant DNA Technology

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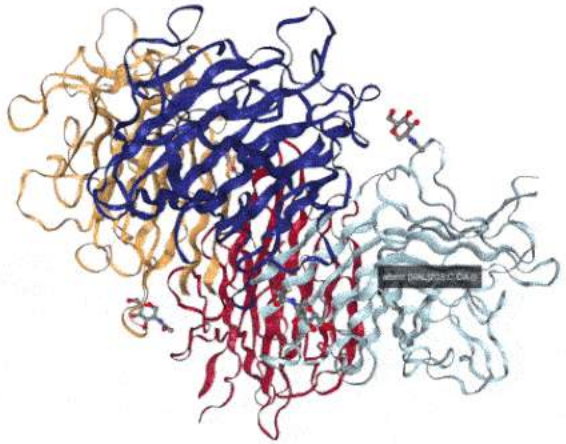
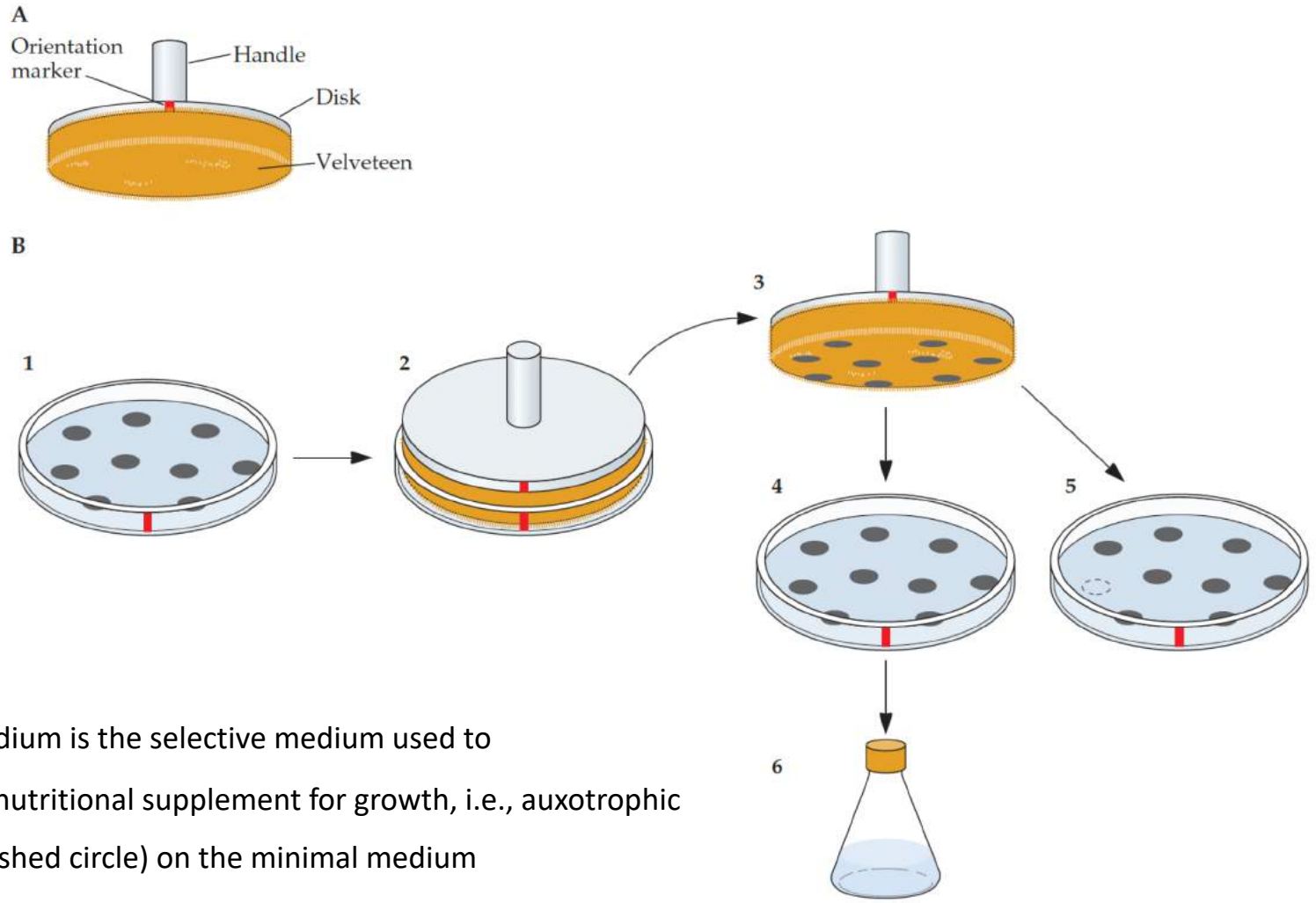


Screening bacterial colonies for mutant strains by replica plating



- In this example, minimal medium is the selective medium used to identify colonies that require a nutritional supplement for growth, i.e., auxotrophic mutants. The missing colony (dashed circle) on the minimal medium (5) denotes an auxotrophic mutation.

Effect of increasing the time of restriction endonuclease digestion

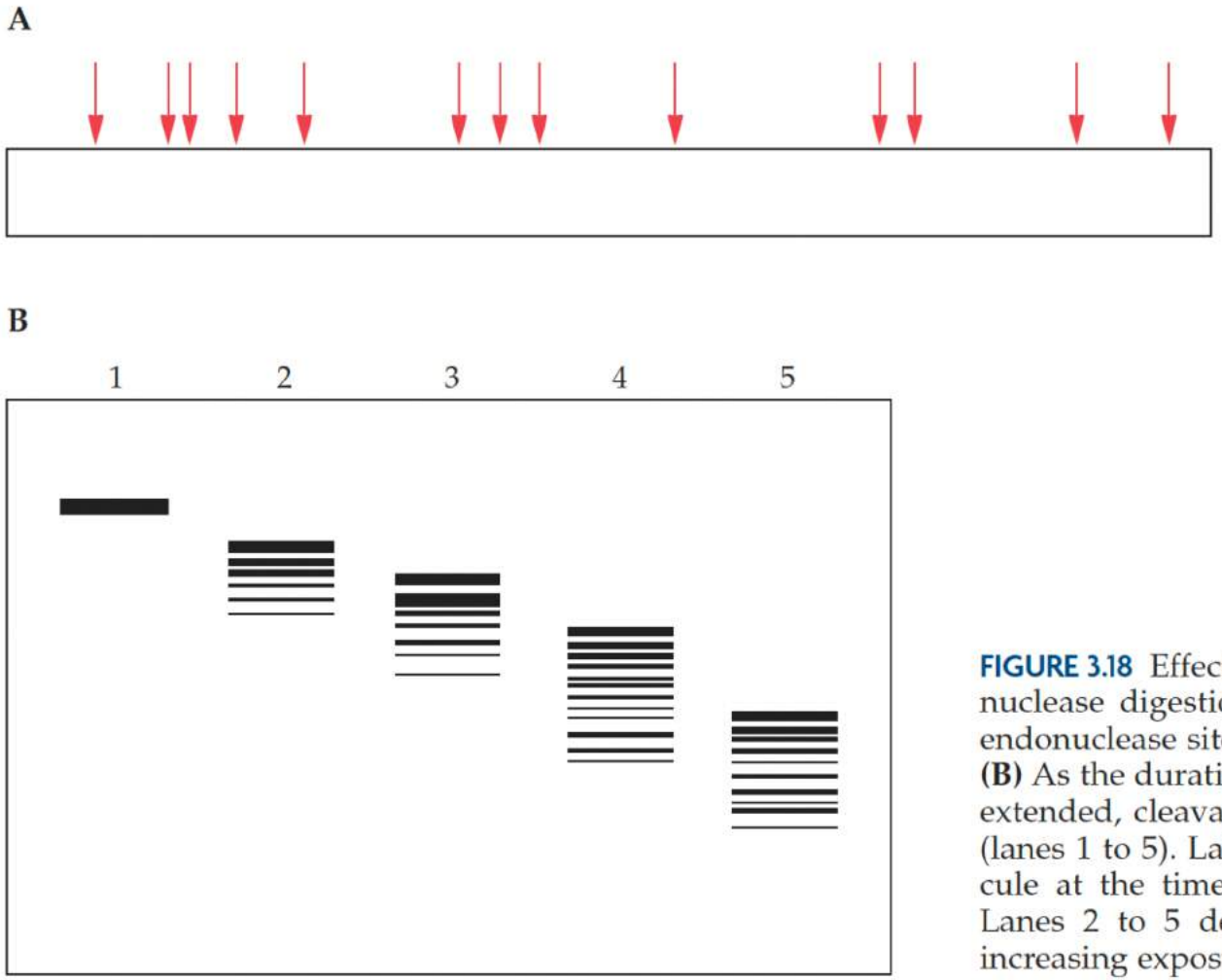


FIGURE 3.18 Effect of increasing the tim
nuclease digestion of a DNA sample
endonuclease sites (arrows) of a DNA
(B) As the duration of restriction endo
extended, cleavage occurs at an incre
(lanes 1 to 5). Lane 1 represents the si
cule at the time of addition of resti
Lanes 2 to 5 depict the extents of
increasing exposures to restriction enc

Vector backbone exchange

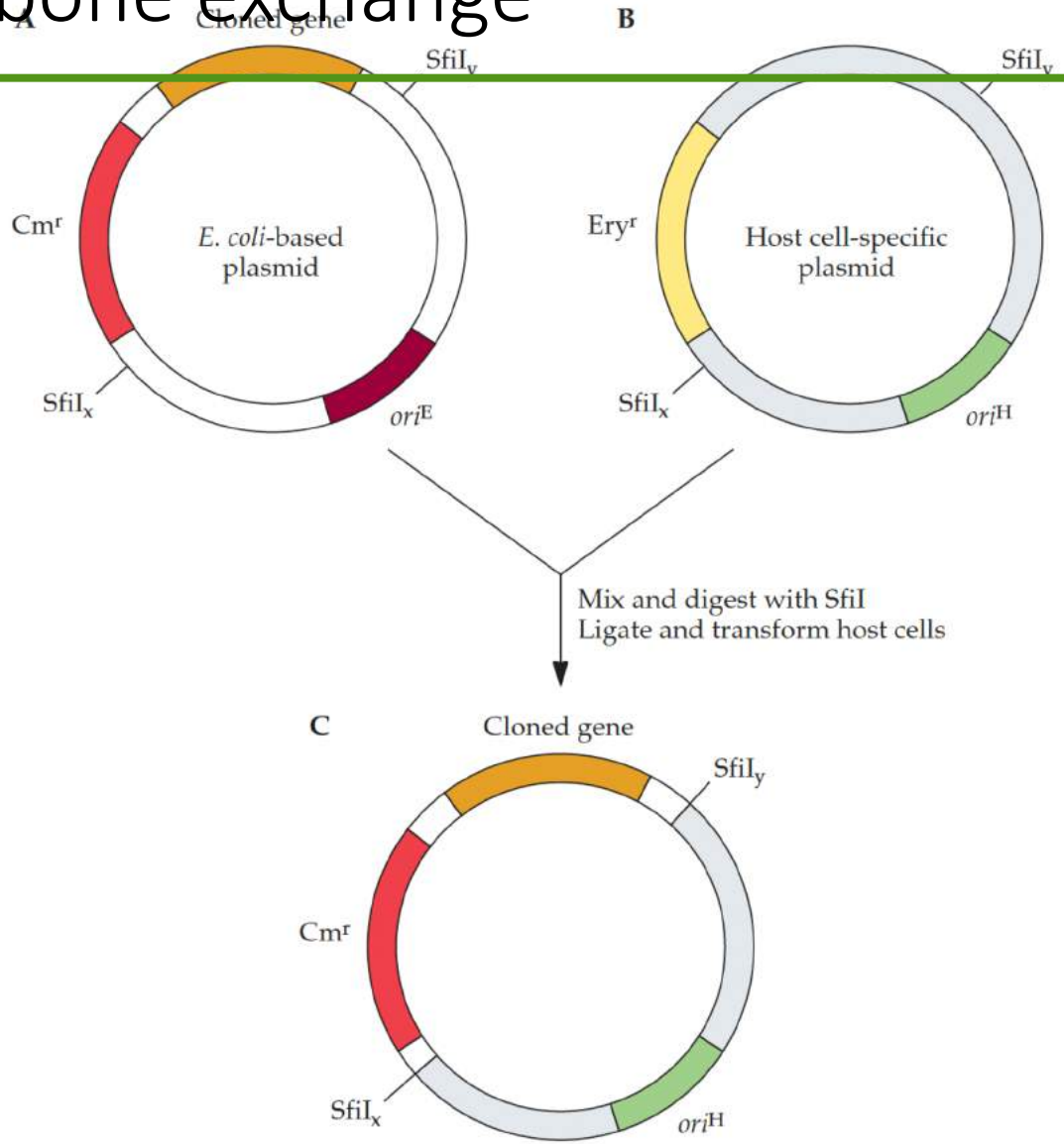


FIGURE 3.16 Vector backbone exchange. Shown are an *E. coli*-based plasmid with a cloned DNA sequence, a chloramphenicol resistance gene (Cm^r), and an *E. coli*-spe-

Screening a library with a labeled DNA probe (colony hybridization)

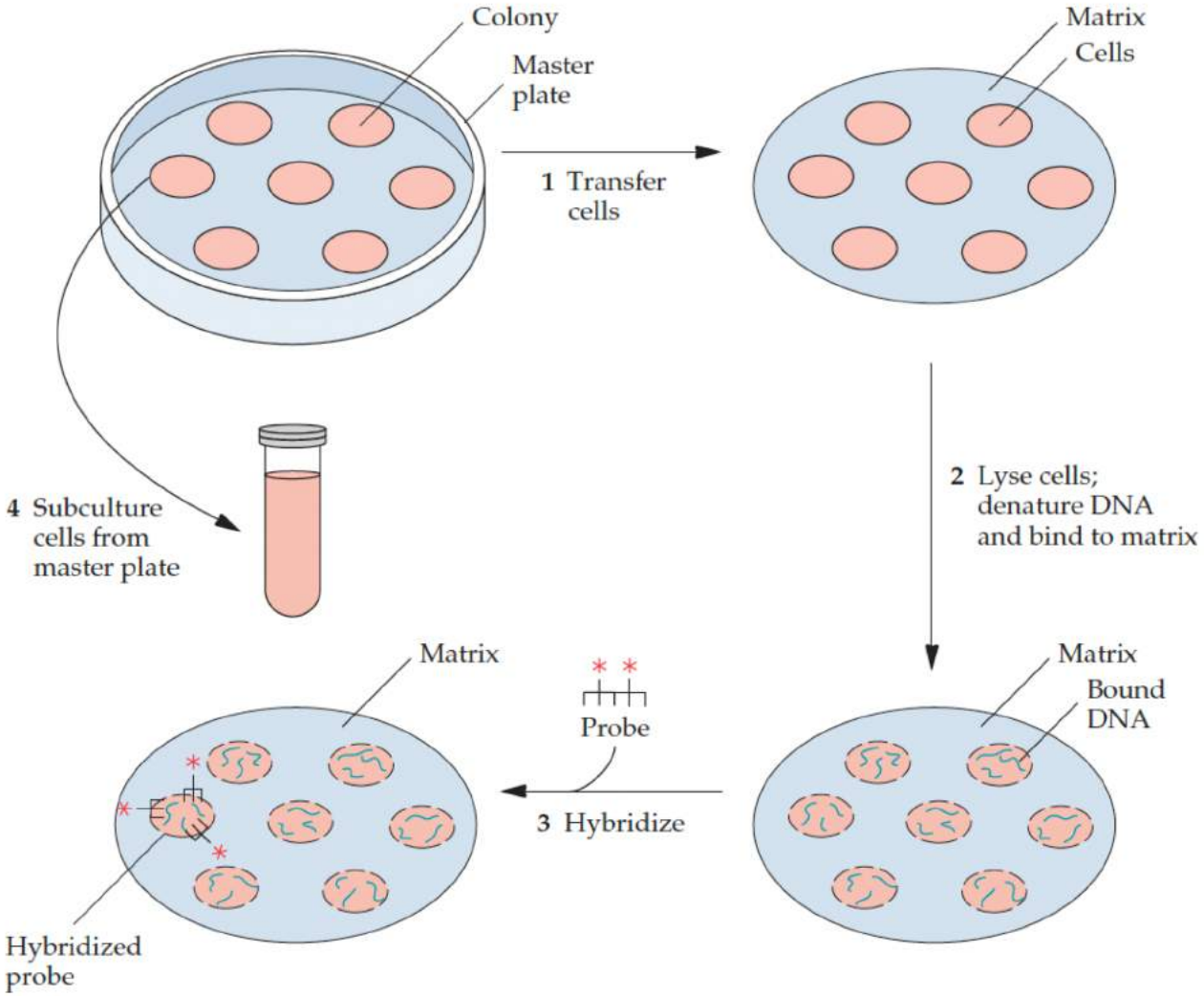
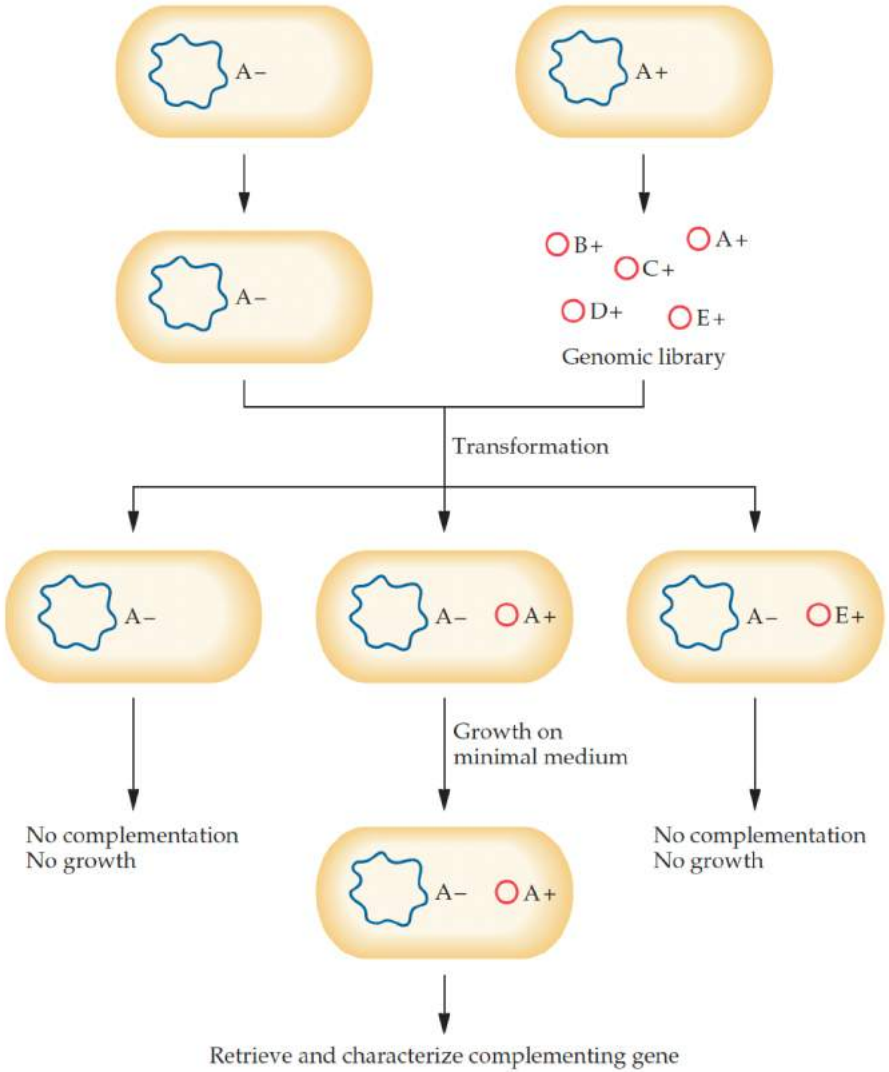


FIGURE 3.22 Screening a library with a labeled DNA probe (colony hybridization). Cells from the transformation reaction are plated onto solid agar medium under conditions that permit transformed, but not nontransformed, cells to grow. (1)

Gene cloning by functional complementation.



A cosr

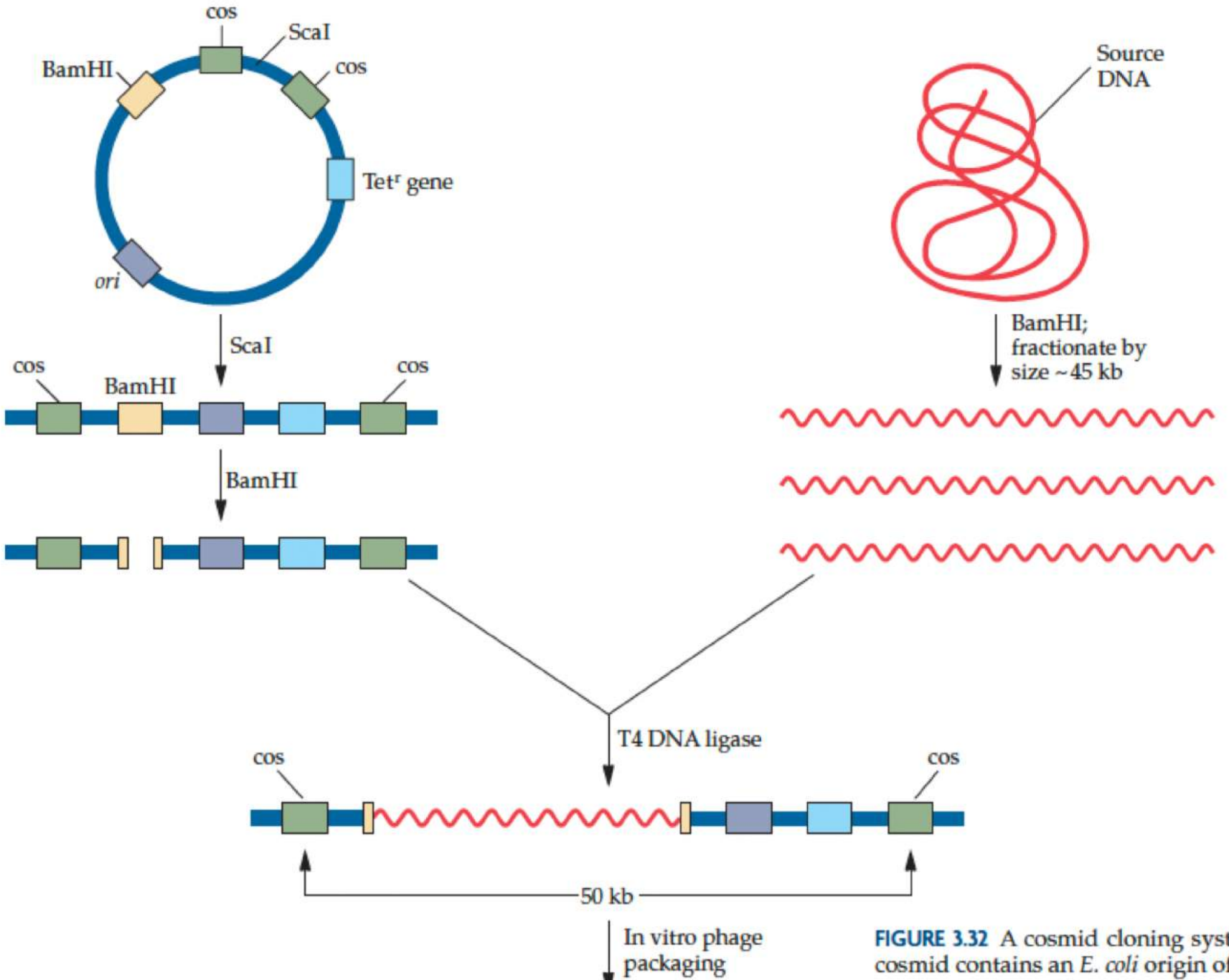
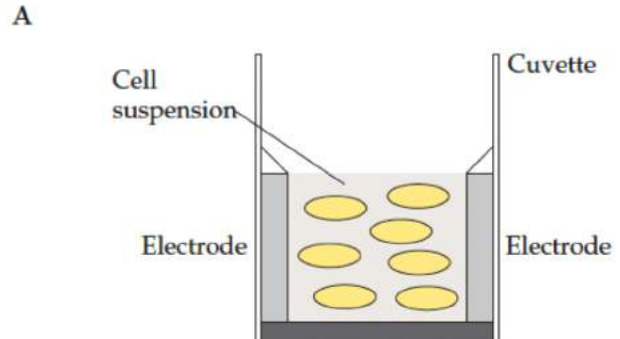


FIGURE 3.32 A cosmid cloning system. The cosmid contains an *E. coli* origin of replica-

Genetic Transformation of Prokaryotes

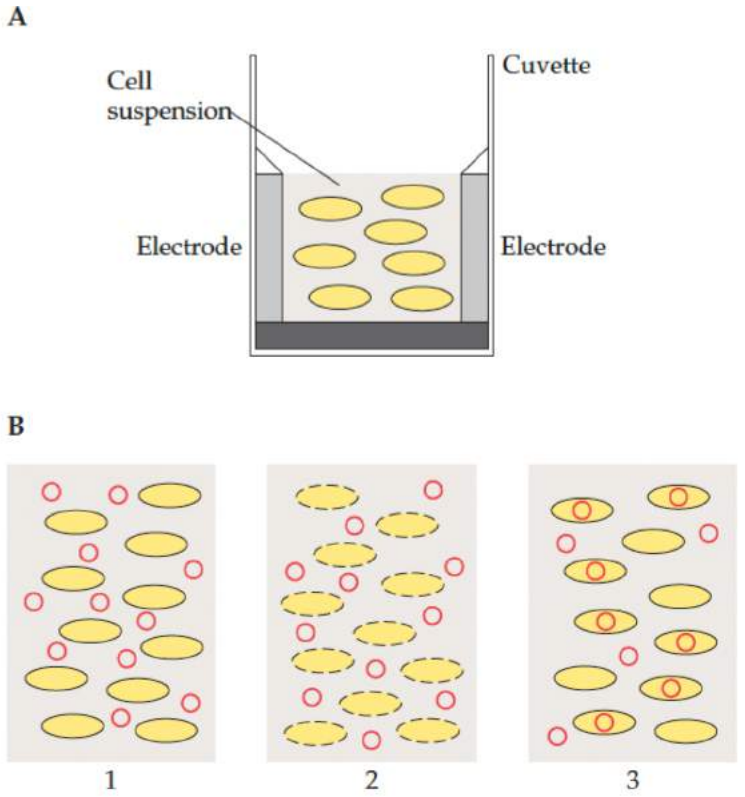
- Heat Shock
  - by treating mid-log-phase cells with ice-cold calcium chloride (CaCl2) and then exposing them for 2 minutes to a high temperature (42ÅãC). This treatment creates transient openings in the cell wall that enable DNA molecules to enter the cytoplasm.

  - transformation frequency of about 1 transformed cell per 1,000 cells (i.e., 10−3). The transformation efficiency is approximately 107 to 108 transformed colonies per microgram of intact plasmidDNA.



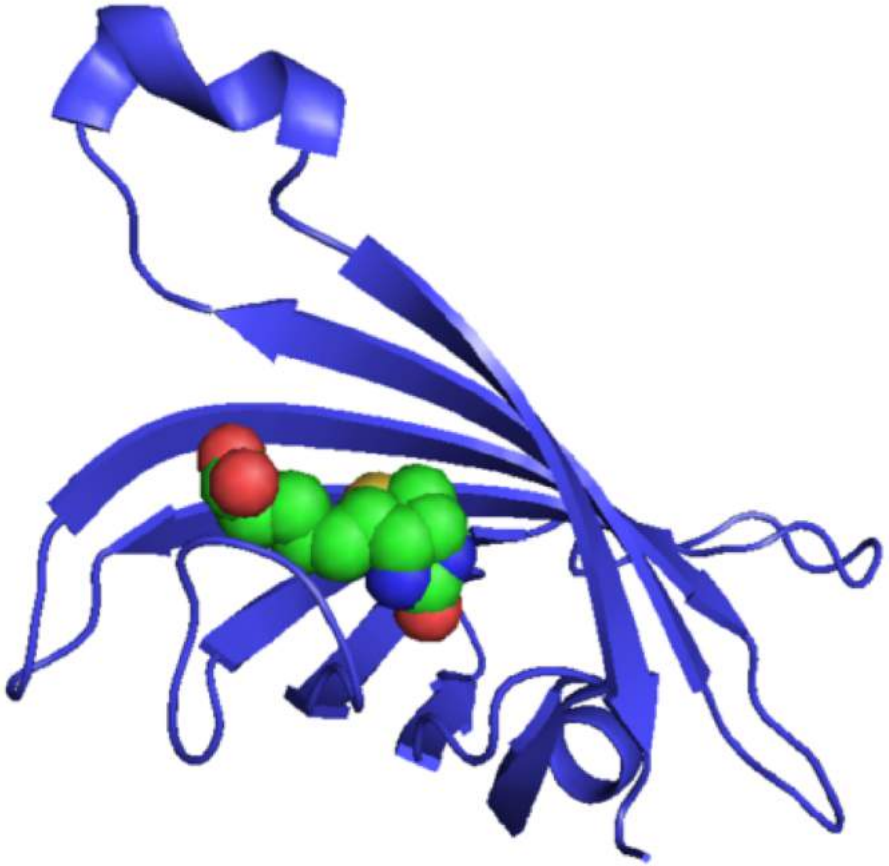
Electroporation

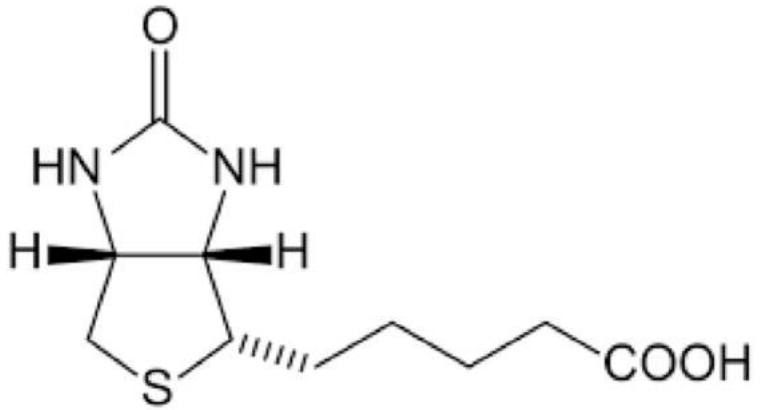
- By subjecting bacteria to a highvoltage
- For E. coli, the cells (~50 microliters) and DNA a single pulse of 25 microfarads, 2.5 kilovolts, and 200 ohms is administered for about 4.6 milliseconds.
- Transformation efficiencies of 109 transformants per microgram of DNA for small plasmids (~3 kb) and 106 for large plasmids (~136 kb).
- Similar conditions are used to introduce BAC vector DNA
- Plasmids containing inserts that are longer than 100 kb. Very little is known about the mechanism



- **Streptavidin** /ˌstrɛpˈtævɪdɪn/ is a 66.0 (tetramer) kDa protein purified from the bacterium *Streptomyces avidinii*. Streptavidin homo-tetramers have an extraordinarily high affinity for biotin (also known as vitamin B7 or vitamin H). With a dissociation constant (K_d) on the order of $\approx 10^{-14}$ mol/L,[1] the binding of biotin to streptavidin is one of the strongest non-covalent interactions known in nature. Streptavidin is used extensively in molecular biology and bionanotechnology due to the streptavidin-biotin complex's resistance to organic solvents, denaturants (e.g. guanidinium chloride), detergents (e.g. SDS, Triton X-100), proteolytic enzymes, and extremes of temperature and pH.

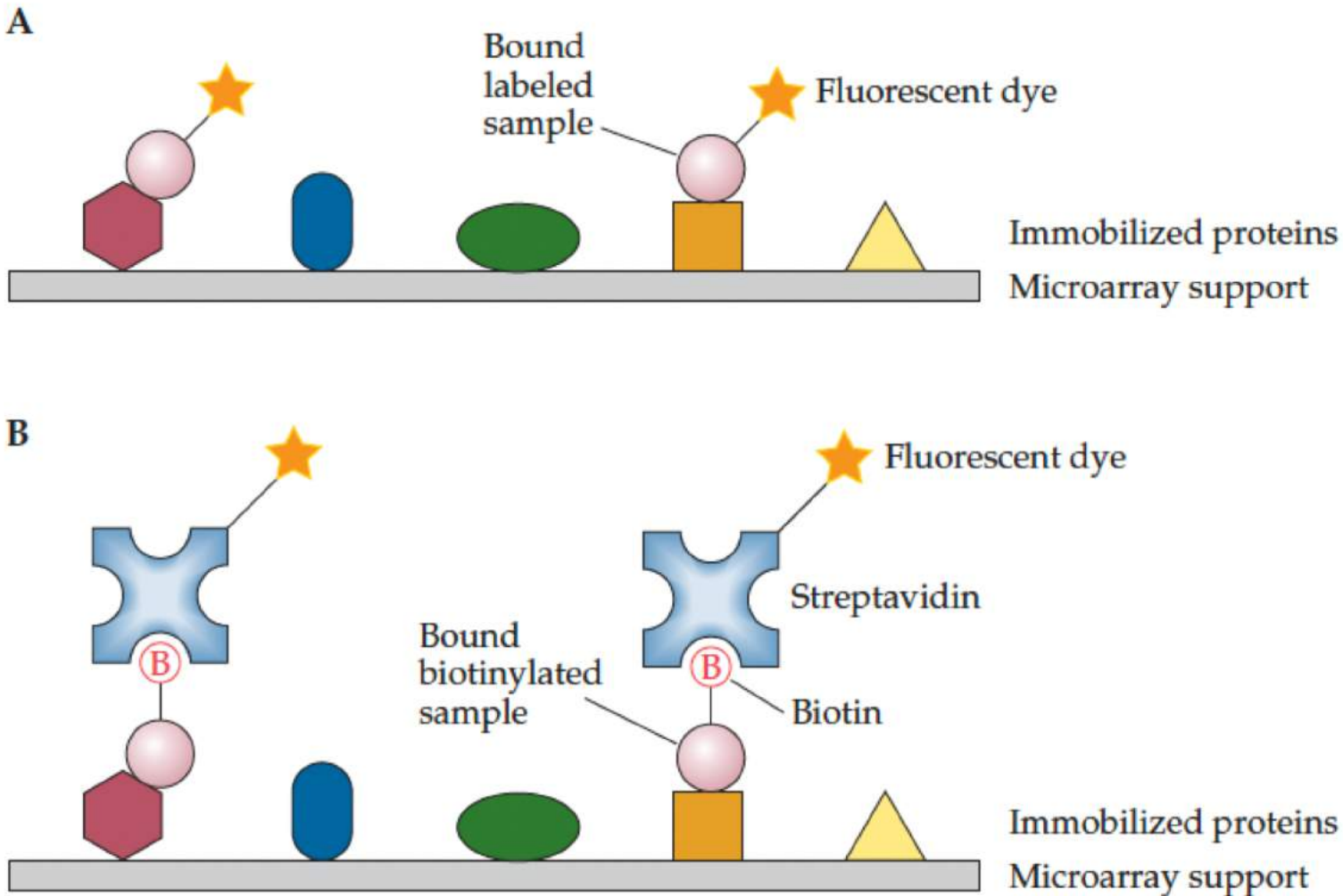
Streptavidin Bitoin





- Broadly speaking, there are three types of protein microarrays: analytical (capture), reverse phase, and functional.

FIGURE 5.15 Protein microarray detection methods. **(A)** Direct labeling. The sample molecules are labeled with a detector reagent, e.g., fluorescent dye. **(B)** Sandwich style assay. The sample molecules are biotinylated, and after the initial incubation, a streptavidin–fluorescent-dye conjugate that binds to biotin to facilitate the detection of sample molecules is applied.



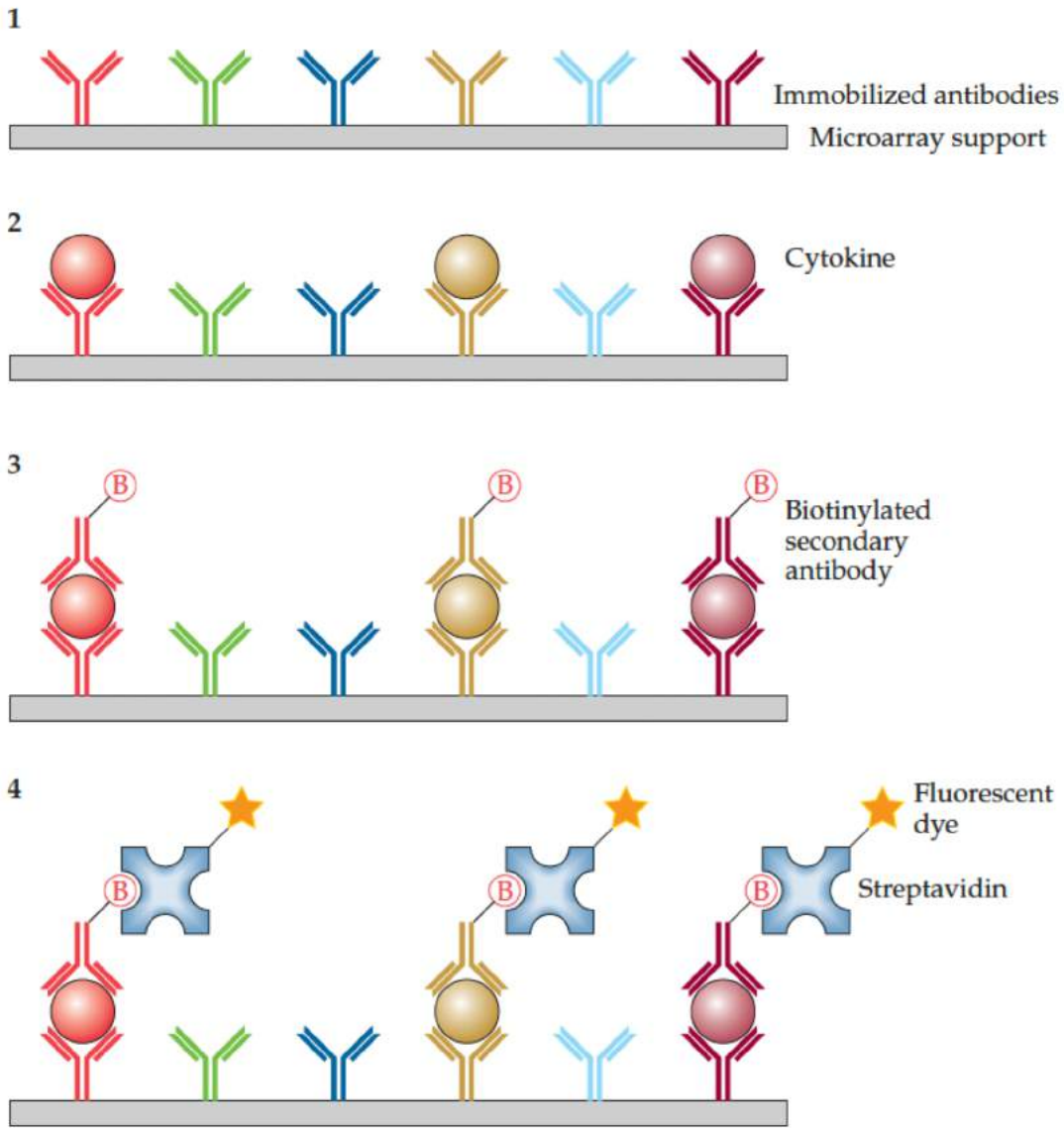


FIGURE 5.16 Detection of cytokines with a cytokine antibody microarray. A cytokine antibody microarray **(1)** is incubated with a sample, and cytokines (solid circles) bind to specific antibodies **(2)**. Free biotinylated cytokine antibodies are added and bind to the corresponding captured cytokine **(3)**. For visualization, a streptavidin–fluorescent-dye conjugate attaches to the biotin of the secondary antibody **(4)**.

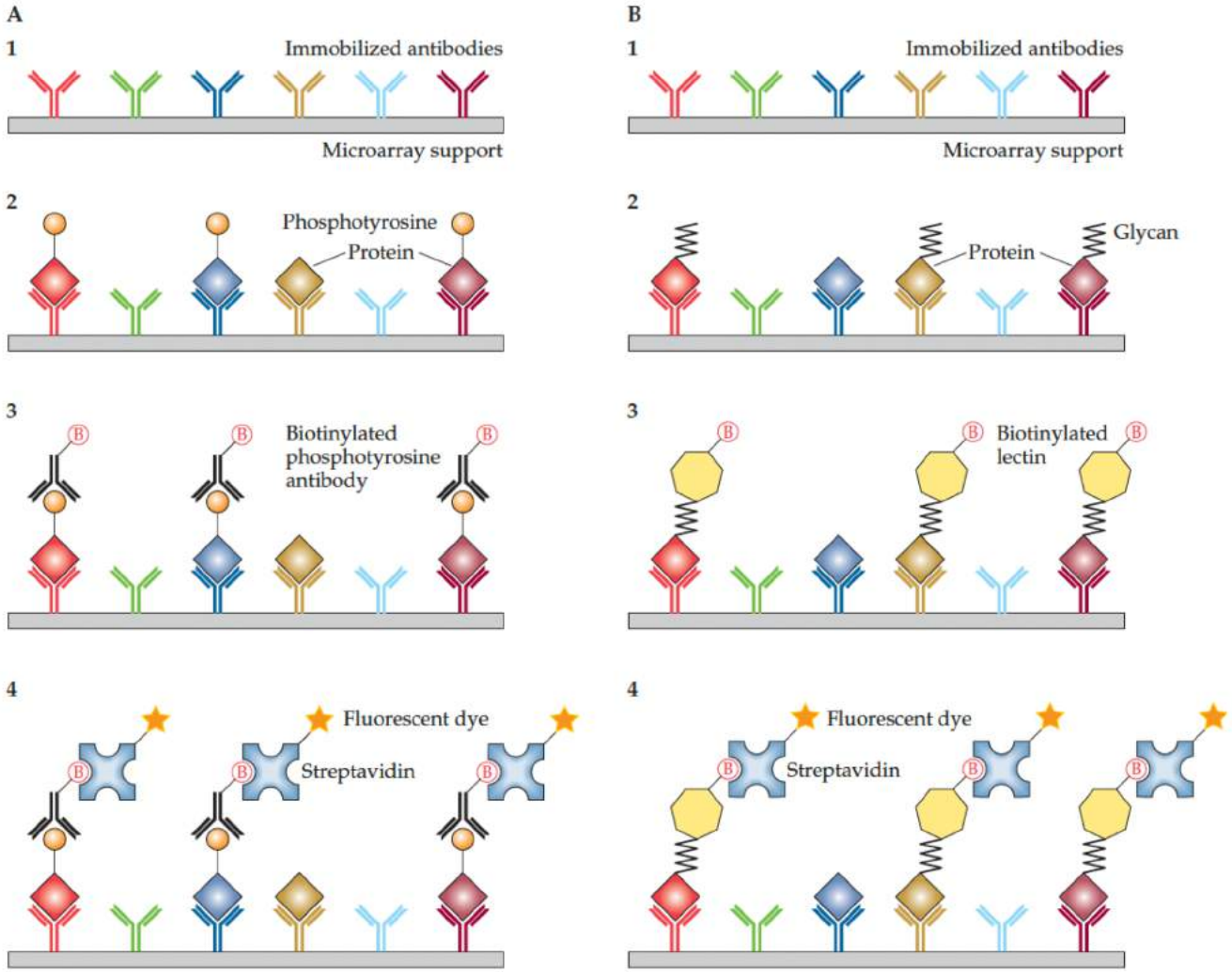


FIGURE 5.17 Detection of post-translational modifications with antibody microarrays. **(A)** Detection of tyrosine phosphorylation. An antibody microarray (1) is incubated with a protein sample (2). Biotinylated phosphotyrosine antibody is added (3), and for visualization, a streptavidin–fluorescent-dye conjugate attaches to the biotin of the phosphotyrosine antibody (4). **(B)** Detection of glycan groups. An antibody microarray (1) is incubated with a protein sample (2). A biotinylated molecule (e.g., lectin) that binds to a specific glycan is added (3), and for visualization, a streptavidin–fluorescent-dye conjugate attaches to the biotin of the lectin (4).

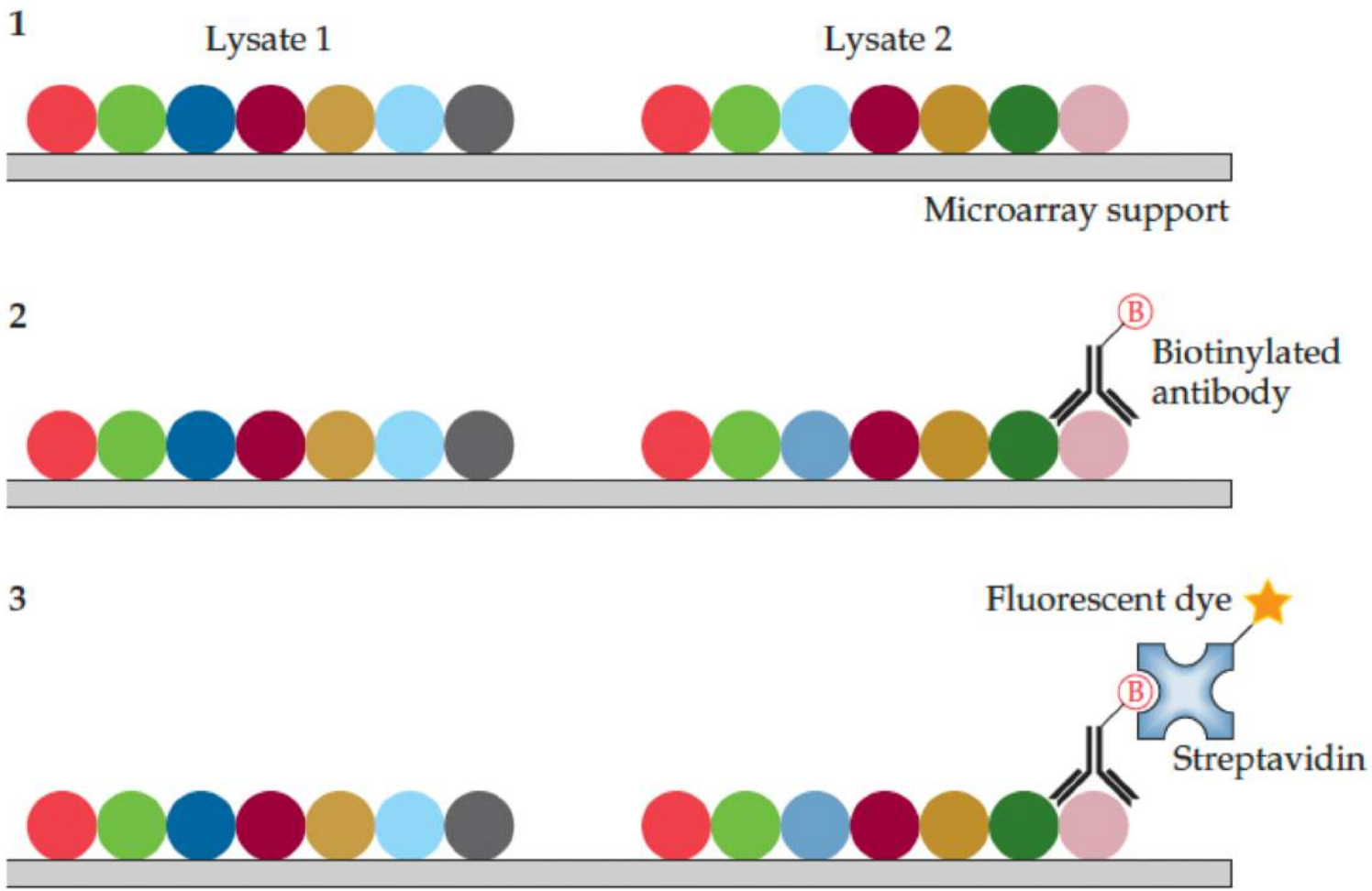


FIGURE 5.18 Reverse-phase microarray format. Multiprotein samples, e.g., cell lysates, are spotted on a solid support (1) and incubated with a known biotinylated antibody (2). A streptavidin–fluorescent-dye conjugate is used to identify samples with bound antibody (3).

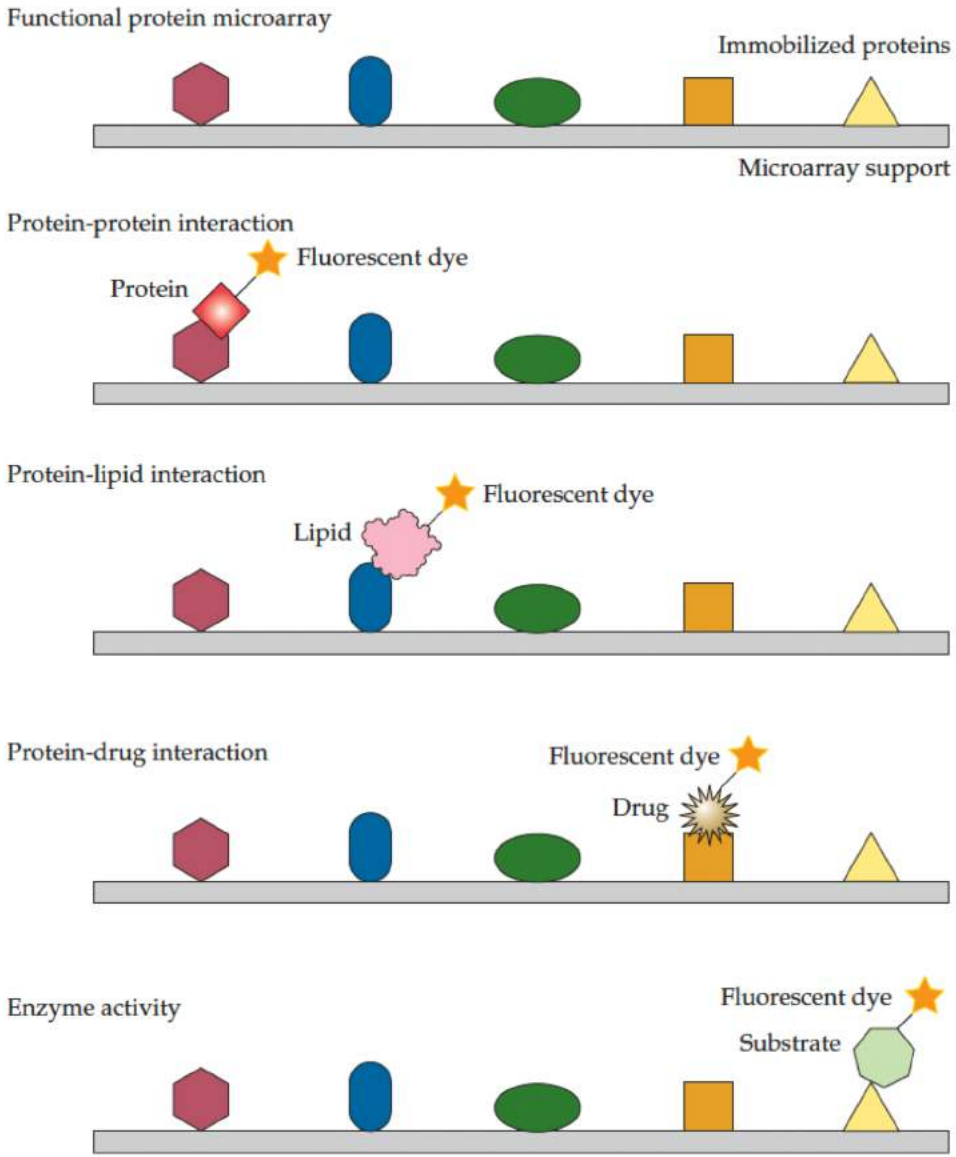


FIGURE 5.19 Functional protein microarray platform. The individual proteins of a functional protein microarray can be examined for interactions with proteins, lipids, and drugs, among other compounds, and tested for substrate binding and enzyme activities. Here, for convenience, direct labeling of the input sample is depicted as the detection method. However, there are a variety of visualization protocols for different types of samples.

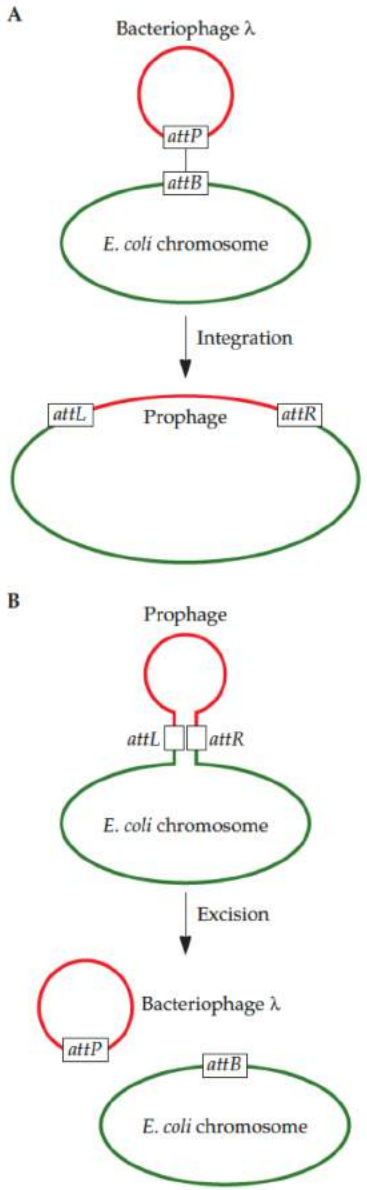
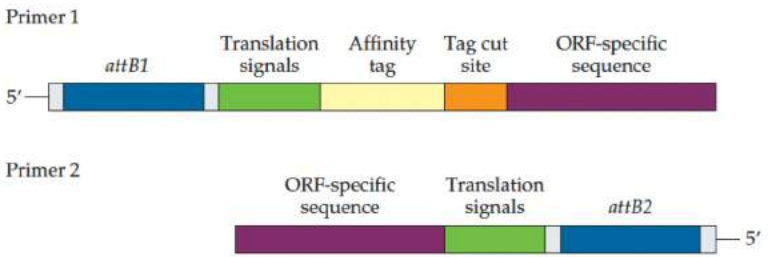


FIGURE 5.20 Integration **(A)** and excision **(B)** of bacteriophage λ into and from the *E. coli* genome via recombination between attachment (*att*) sites in the bacterial and bacteriophage DNAs.

sequences are mixed with a vector (donor vector) that has *attP1* and *attP2* sites flanking a negative selection gene *(ccdB)* (Fig. 5.22A). When present and expressed, the product of the *ccdB* gene interferes with DNA replication and is toxic to bacterial cells. Integration host factor and integrase are added to the mixture of DNA molecules to catalyze in vitro recombination between the *attB1* and *attP1* sites and between the *attB2* and *attP2* sites. As a consequence of the two recombination events, the *ccdB* gene sequence between the *attP1* and *attP2* sites on the donor vector is replaced by the ORF. The recombination events create new attachment sites flanking the ORF sequence (designated *attL1* and *attL2*), and the plasmid with the *attL1*-ORF-*attL2* sequence is referred to as an entry clone. The mixture of original and recombinant DNA molecules is transformed into *E. coli*, and cells that are transformed with donor vectors that have not undergone recombination retain the *ccdB* gene and therefore do not survive. Host cells carrying the entry clone are positively selected by the presence of a selectable marker. This procedure is repeated to clone each of the ORFs in the proteome.

The next step to obtain functional proteins is the expression of each cloned ORF. For expression, the ORF is transferred from the entry vector to a destination vector that carries a promoter and other expression signals. An entry clone is mixed with a destination vector that has *attR1* and *attR2* sites flanking a *ccdB* gene (Fig. 5.22B). In the presence of integration host factor, integrase, and bacteriophage λ excisionase, the *attL1* and *attL2* sites on the entry clone recombine with the *attR1* and *attR2* sites, respectively, on the destination vector. This results in the replacement of the *ccdB* toxin gene on the destination vector with the ORF from the entry clone, and the resultant plasmid is designated an expression clone. The reaction mixture is transformed into *E. coli*, and a selectable marker is used to isolate transformed cells that carry an expression clone. Cells that carry an intact destination vector or the exchanged entry plasmid (known as a by-product plasmid) will not survive because they carry the *ccdB* gene. Destination vectors are available for maintenance and expression of the ORF in various host cells, such as *E. coli* and yeast, insect, and mammalian cells. For construction of a microarray, each protein encoded by an ORF is isolated by affinity purification using the affinity tag that was encoded on the initial

FIGURE 5.21 Primer pair used to amplify ORFs for recombinational cloning to generate an ORFeome.



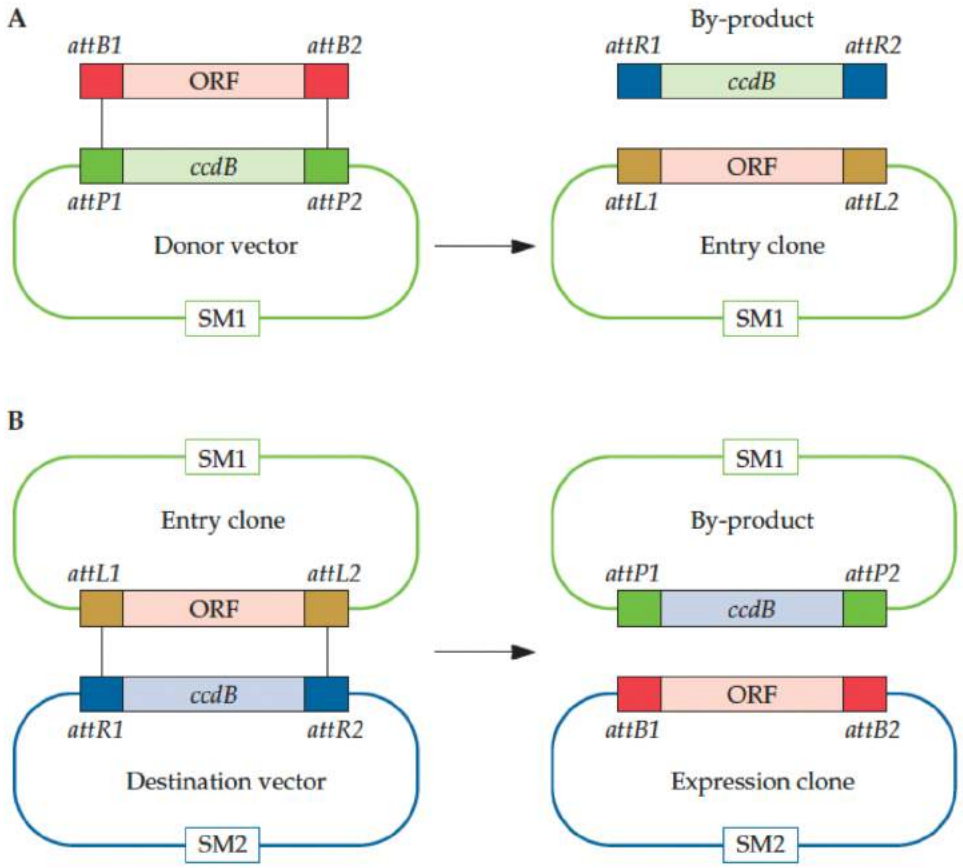
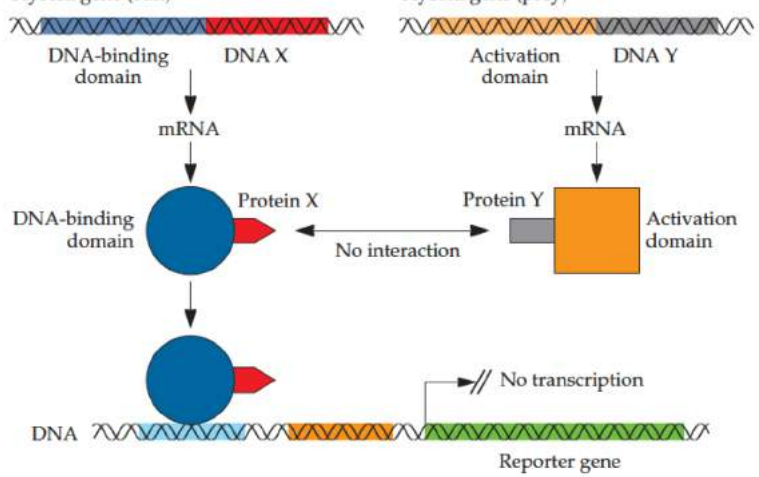


FIGURE 5.22 Recombinational cloning. **(A)** Recombination (thin vertical lines) between a PCR-amplified ORF with attachment sites *(attB1* and *attB2)* and a donor vector with *attP1* and *attP2* sites on either side of the *ccdB* gene results in an entry clone in which the ORF is flanked by *attL1* and *attL2* sites. The selectable marker (SM1) selects transformed cells with an entry clone. The protein encoded by *ccdB* is toxic to transformed cells with nonrecombined donor vector molecules. The origin of replication of the donor vector is not shown. **(B)** Recombination (thin vertical lines) between the entry clone with *attL1* and *attL2* sites and a destination vector with *attR1* and *attR2* sites results in an expression clone with *attB1* and *attB2* sites flanking the ORF. The selectable marker (SM2) selects transformed cells with an expression clone. The second plasmid, designated a by-product, has the *ccdB* gene flanked by *attP1* and *attP2* sites. Cells with an intact destination vector that did not undergo recombination or that retain the by-product plasmid are killed by the CcdB protein. Transformed cells with an entry clone, which lacks the SM2 selectable marker, are selected against. The origins of replication and the sequences for expression of the ORF are not shown.

both constructs (hybrid genes) are introduced into a cell. After translation, the DNA-binding domain–protein X fusion protein binds to the regulatory sequence of a reporter gene. However, protein Y (prey) does not interact with protein X (bait), and the reporter gene is not transcribed because the activation domain does not, on its own, associate with RNA polymerase. (C) The coding sequence for the activation domain is fused to the DNA for protein Z (DNA Z) and transformed into a cell containing the DNA-binding domain–DNA X fusion construct. The proteins encoded by the cDNAs of the hybrid genes interact, and the activation domain is properly oriented to initiate transcription of the reporter gene, demonstrating a specific protein–protein interaction.



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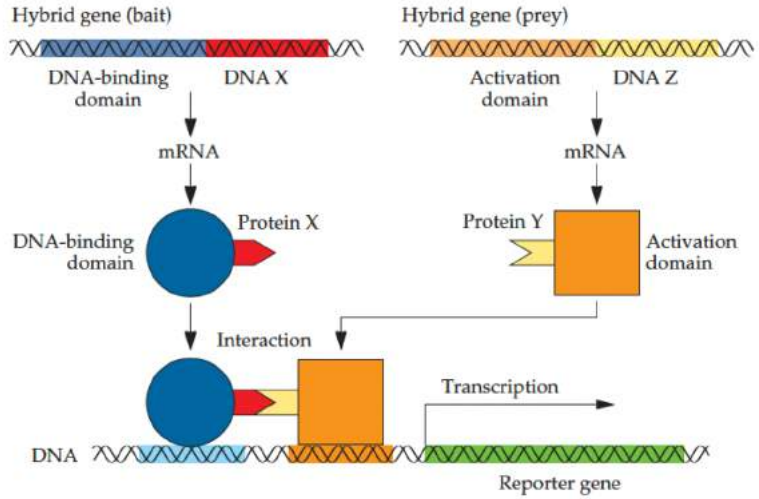

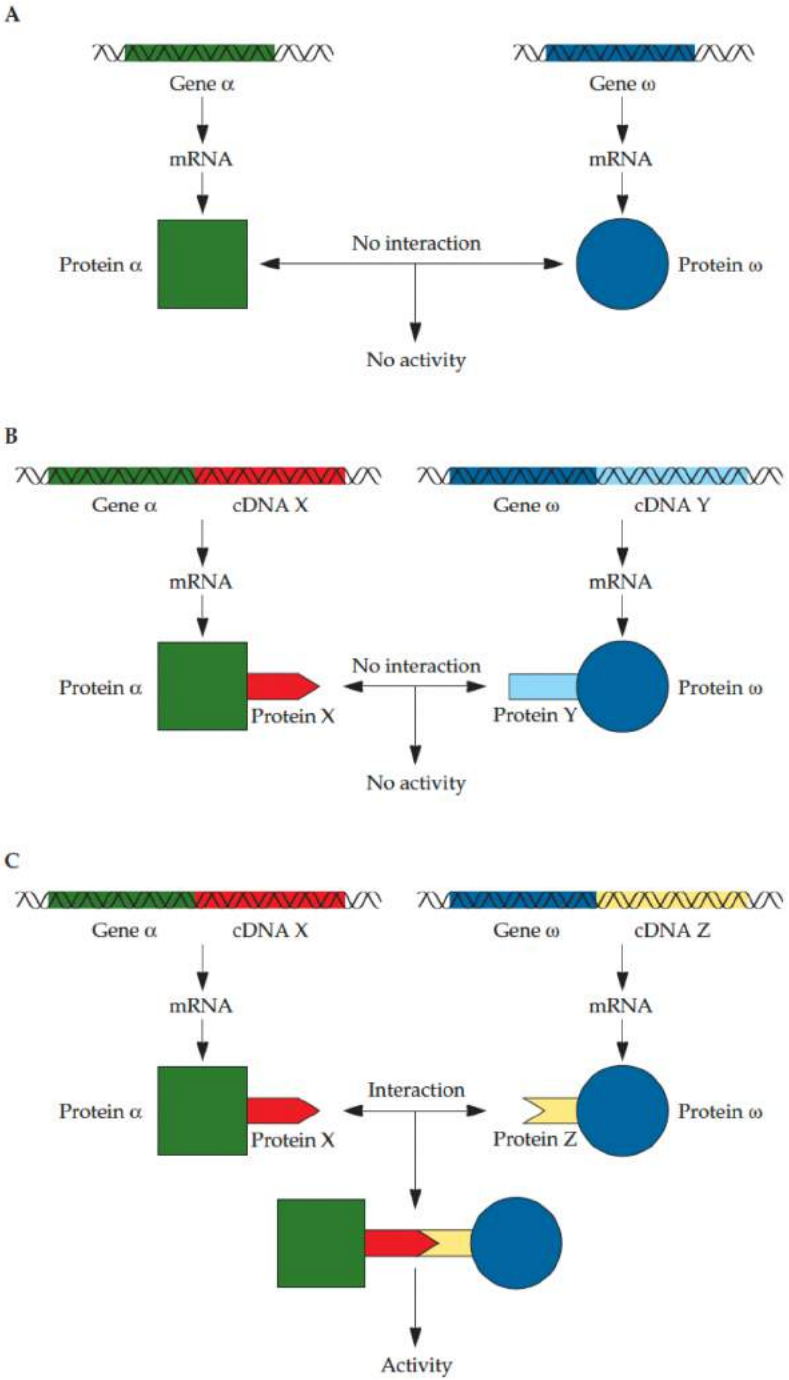

FIGURE 5.24 Complementation assay for detecting pairwise protein interactions in mammalian cells. **(A)** Proteins α and ω must combine to produce an active enzyme but are not able to interact spontaneously due to mutations. **(B)** DNA fusion constructs of the gene encoding protein α (gene α) with a cDNA (cDNA X) and the gene encoding protein ω (gene ω) with a cDNA (cDNA Y) are introduced into a cell. Since proteins X (bait) and Y (prey) do not interact, proteins α and ω do not associate, and the activity specified by the α:ω combination is not observed. **(C)** DNA fusion constructs of the gene encoding protein α (gene α) with a cDNA (cDNA X) and the gene encoding protein ω (gene ω) with a cDNA (cDNA Z) are introduced into a cell. Proteins X and Z interact, bringing together proteins α and ω, and the activity specified by the α:ω combination is observed.

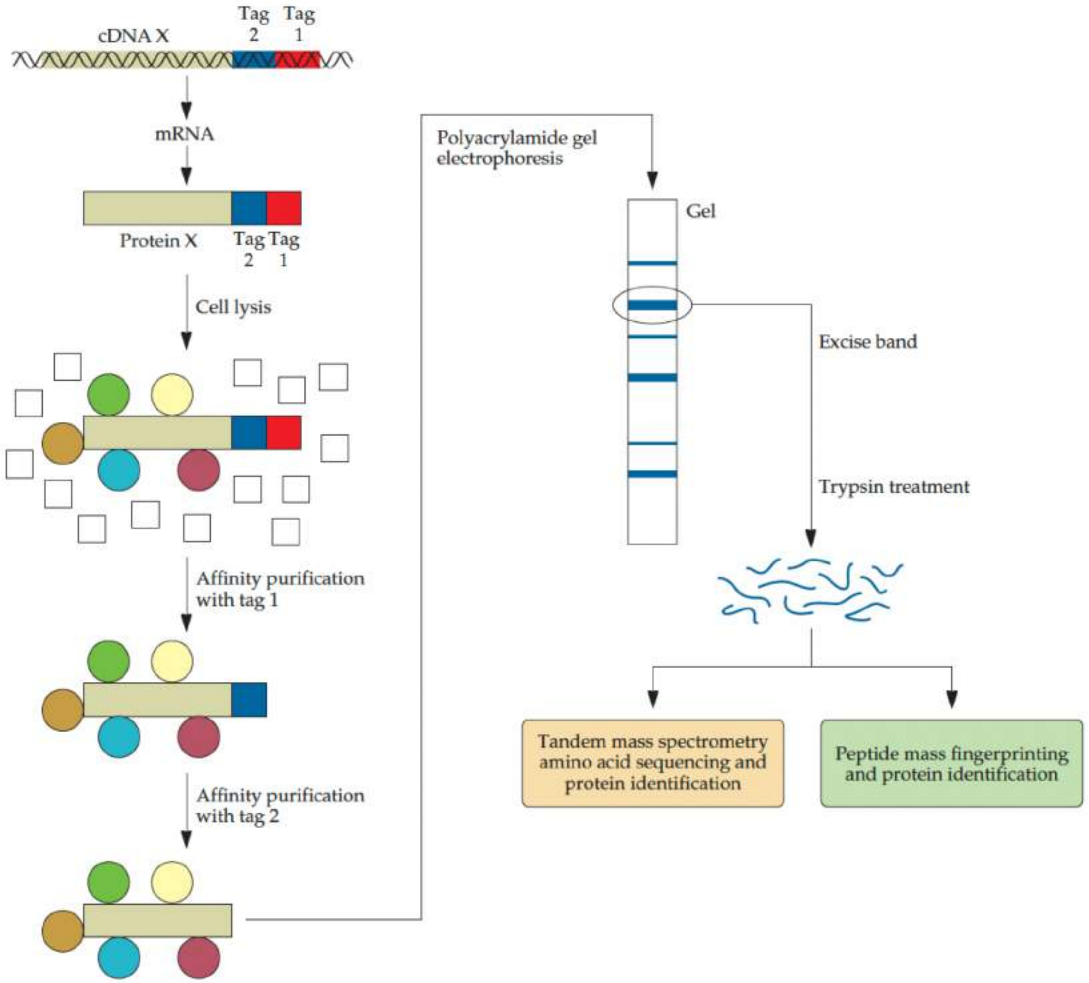

FIGURE 5.27 TAP tag procedure for detecting protein–protein interactions. Two DNA sequences (tag 1 and tag 2), each encoding a short amino acid sequence with high affinity for a specific molecule, are cloned together and fused in frame to the 3′ end of the coding region of a cDNA (cDNA X). The tagged cDNA construct is introduced into a host cell, where it is transcribed and the mRNA is translated. Other cellular proteins bind to the protein encoded by cDNA X (protein X). The cluster consisting of protein X and its associated proteins is separated from cell components (squares) by the binding of tag 1 to its affinity partner, which is usually fixed to a column that retains the cluster while allowing all noninteracting proteins to flow through. The cluster is eluted from the affinity partner, typically by cleaving off tag 1, and a second purification step is carried out with tag 2 and its affinity partner. The proteins of the cluster are separated by one-dimensional PAGE. Single bands are excised from the gel and treated with trypsin. The protein represented by the tryptic peptides is identified by either peptide mass fingerprinting or searching a protein database with peptide amino acid sequences obtained with ESI–MS-MS.