Recombinant DNA-based molecular techniques (part I)

DNA cloning

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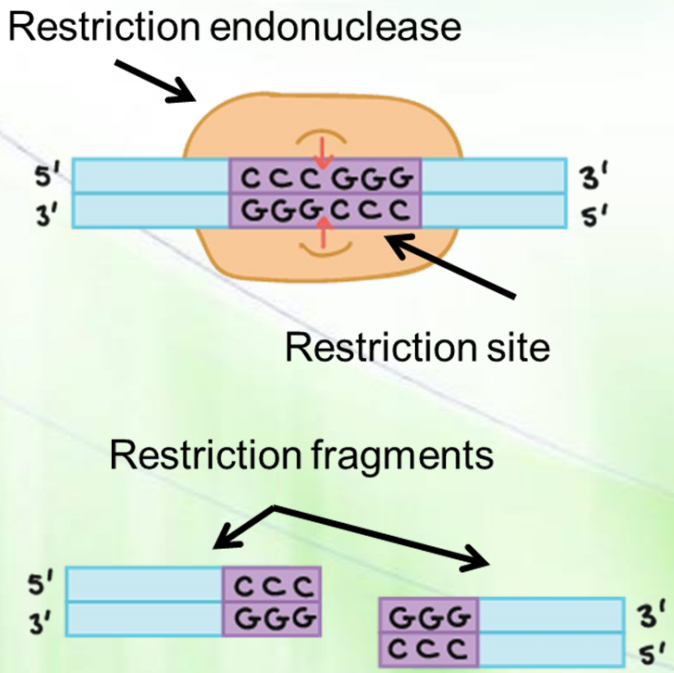
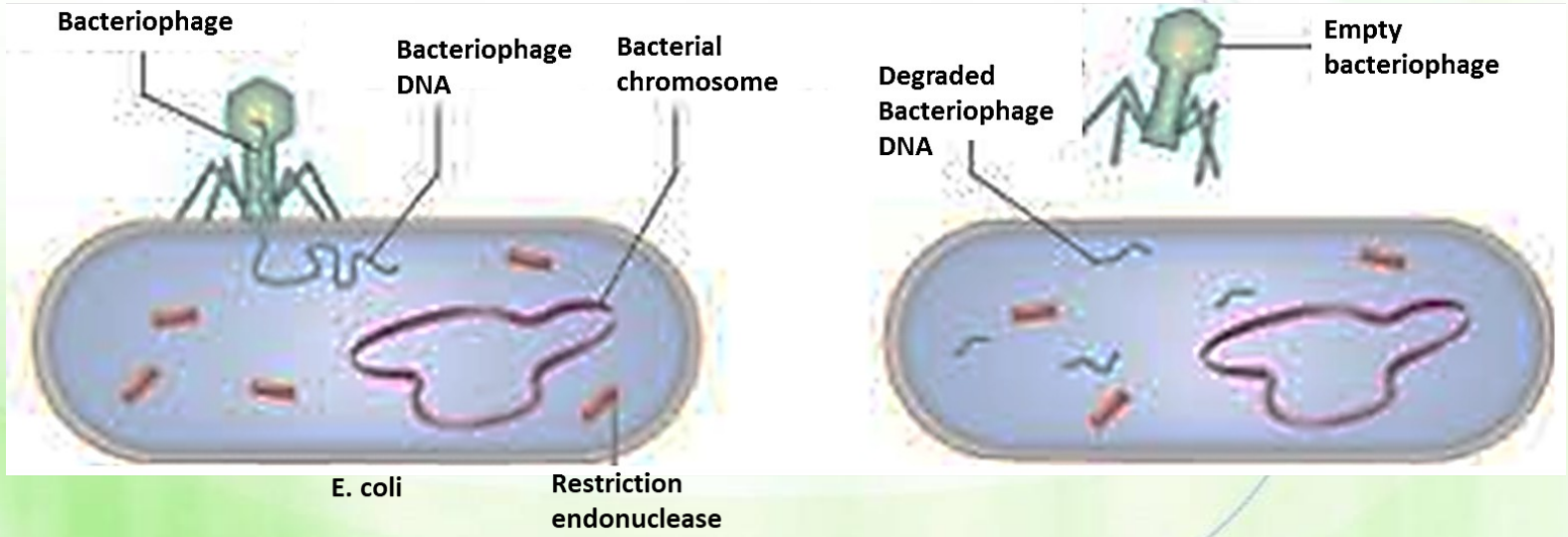
What is DNA cloning?

- DNA cloning is a technique that allows for:
  - amplifying a DNA segment into many, many copies in a biological system.
  - expressing a gene inside a biological system such as bacteria, human cells grown in labs, animals, or even the human body as a whole.
- It usually involves:
  - The formation of a recombinant DNA composed of a vector (a carrier of the gene or the DNA segment of interest; usually a bacterial plasmid) and a gene that encodes a protein or a non-coding RNA using restriction endonucleases.
  - Insertion into the cell(s).

Restriction endonucleases

- Endonucleass are ezymes that degrade DNA within the molecule.
- Restriction endonucleases: Bacterial enzymes that recognize and cut (break) the phosphodiester bond between nucleotides at specific sequences (4- to 8-bp restriction sites) generating restriction fragments.





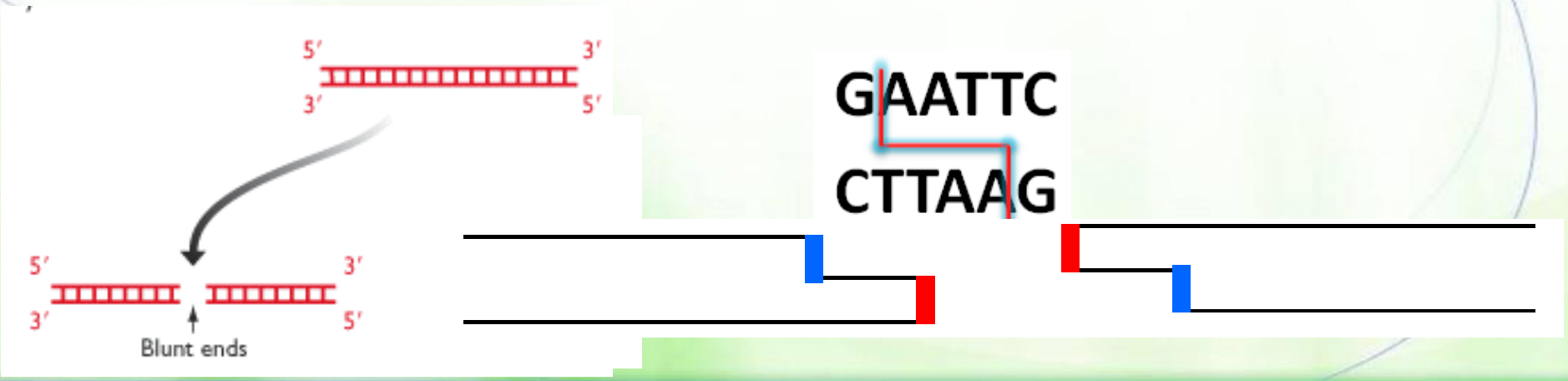
Palindromic sequences

- The sequences recognized by restriction endonucleases—their sites of action—read the same from left to right as they do from right to left (on the complementary strand).

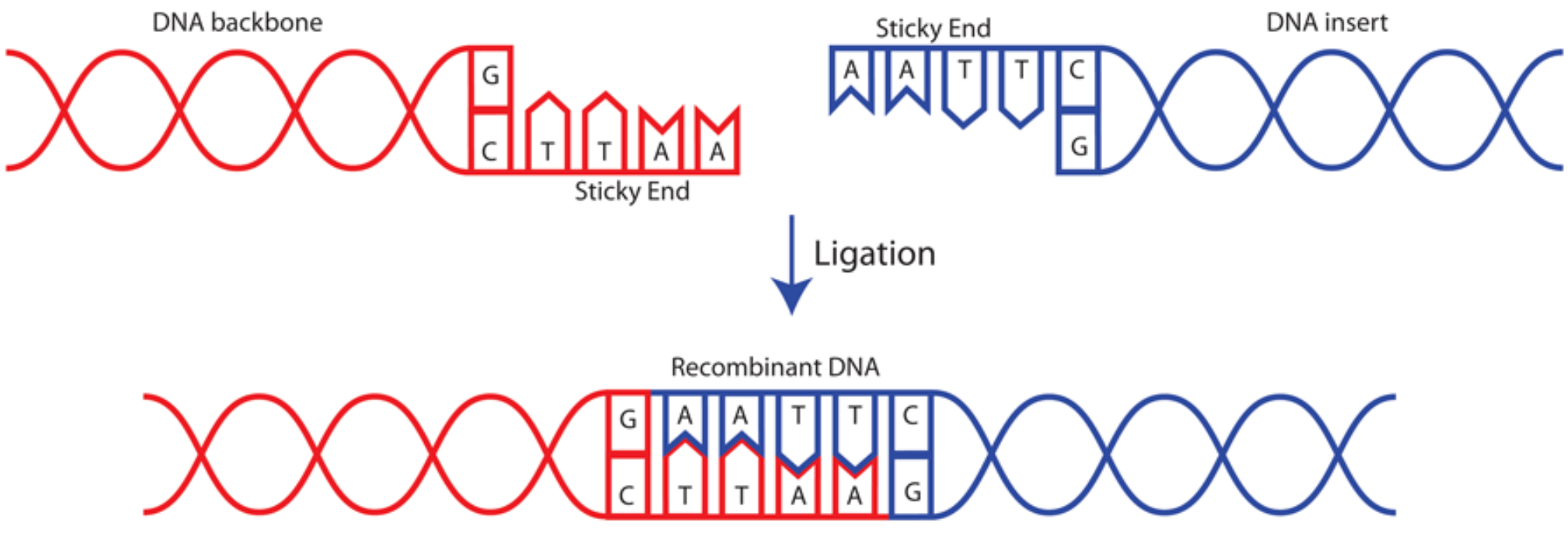
```
EcoRI       5'  GAATTC  3'
             3'  CTTAAG  5'

HindIII     5'  AAGCTT  3'
             3'  TTCGAA  5'

SmaI        5'  CCCGGG  3'
             3'  GGGCCC  5'
```

Types of cuts by restriction endonucleases

- Restriction enzymes cut DNA in two different ways:
  - Blunt: enzymes cut at the same position on both strands giving blunt-ended fragments.
  - Staggered (off-center): enzymes cut the two DNA strands at different positions generating sticky or cohesive ends.
    - The DNA restriction fragments would have short single-stranded overhangs at each end.

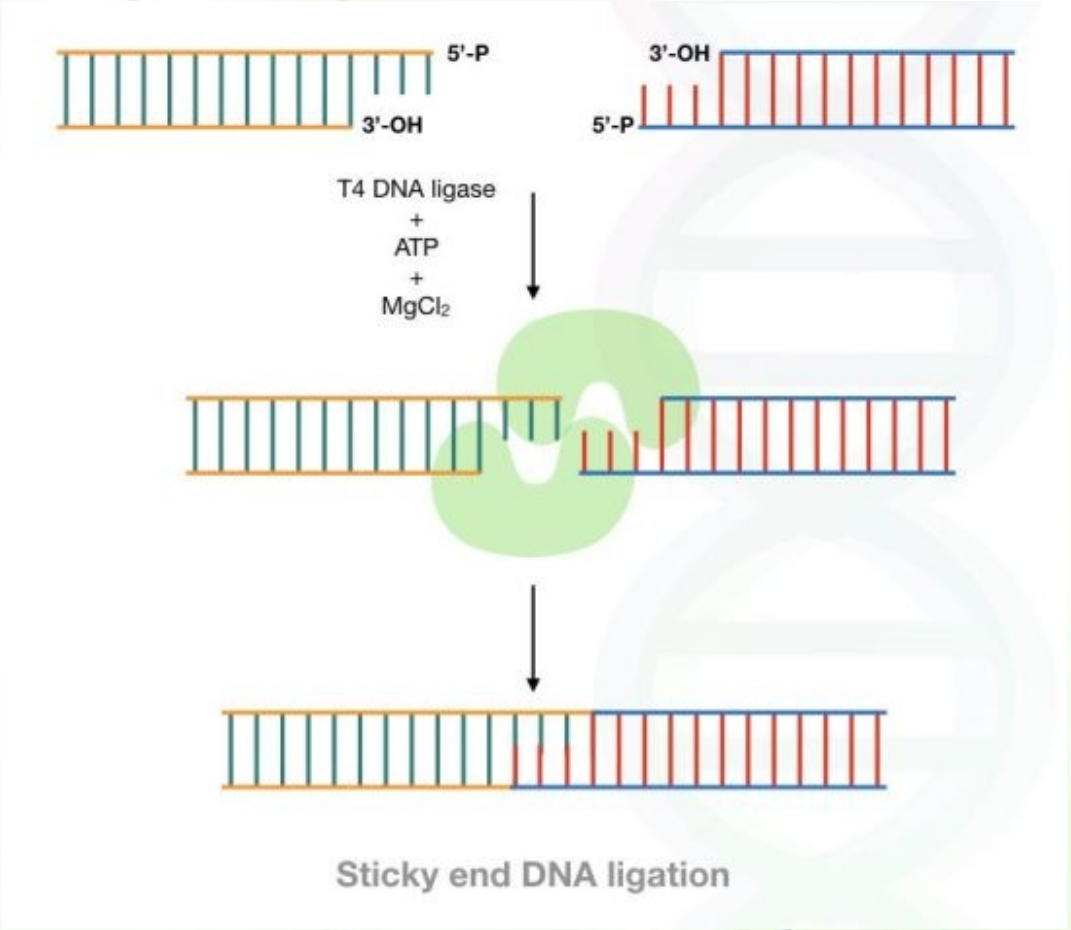


Zoom into the sticky ends



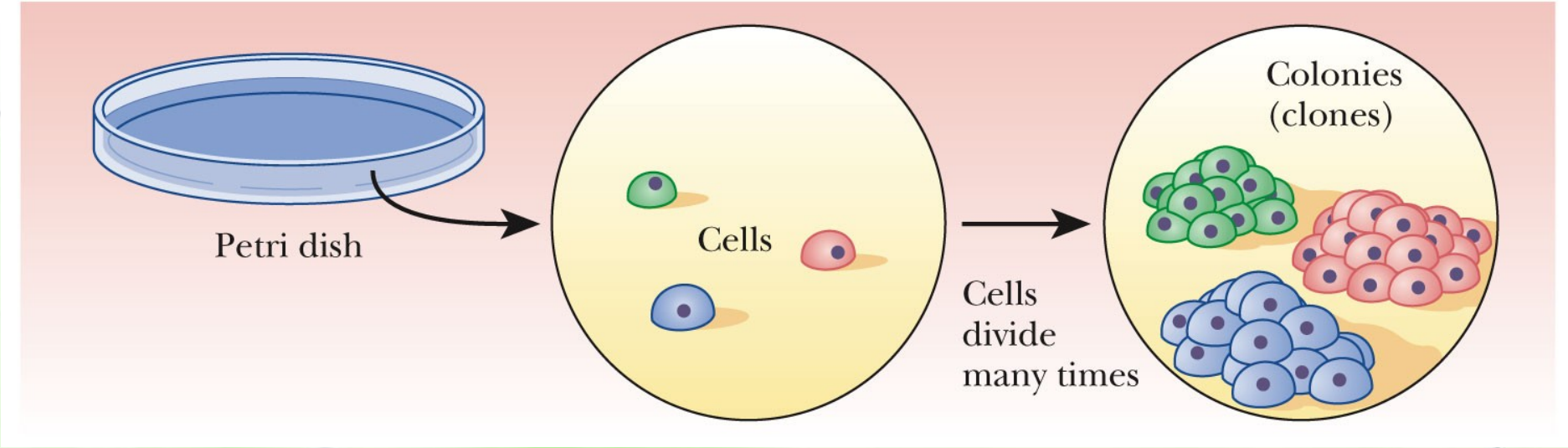
DNA ligase

- It covalently joins DNA ends (example, restriction fragments) by catalyzing the ATP-dependent formation of phosphodiester bonds between the 3’-hydroxyl group of one strand and the 5’-phosphate end of another strand.



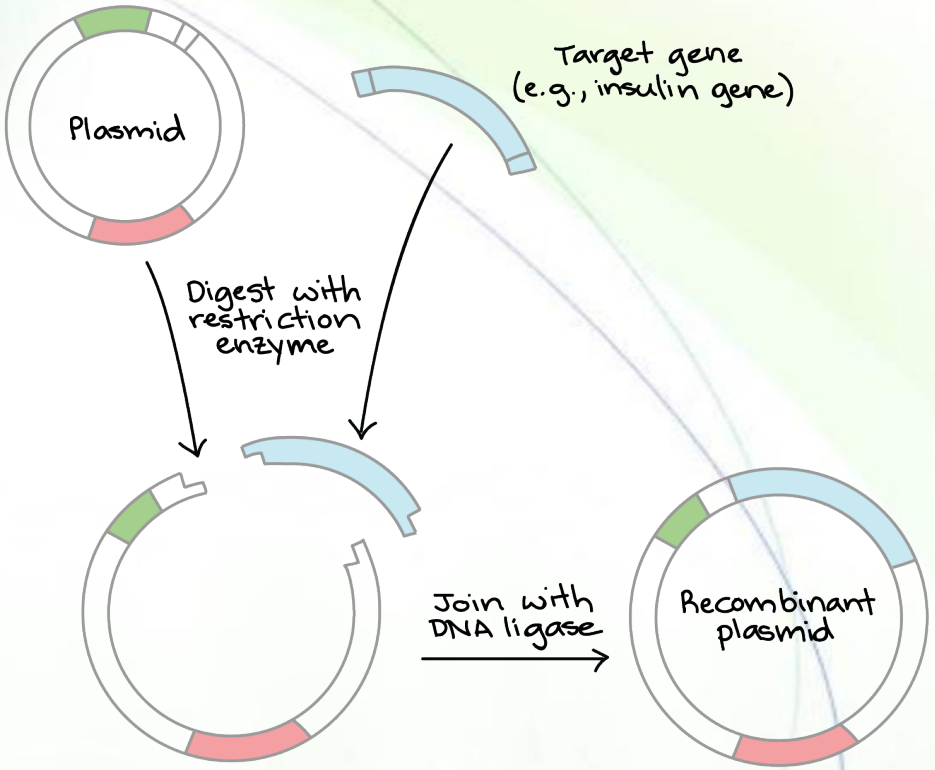
Cloning

- Cloning means that you make several copies of one thing.
- A clone is a genetically identical population, whether of organisms, cells, viruses, or DNA molecules.
- Every member of the population is derived from a single cell, virus, or DNA molecule.



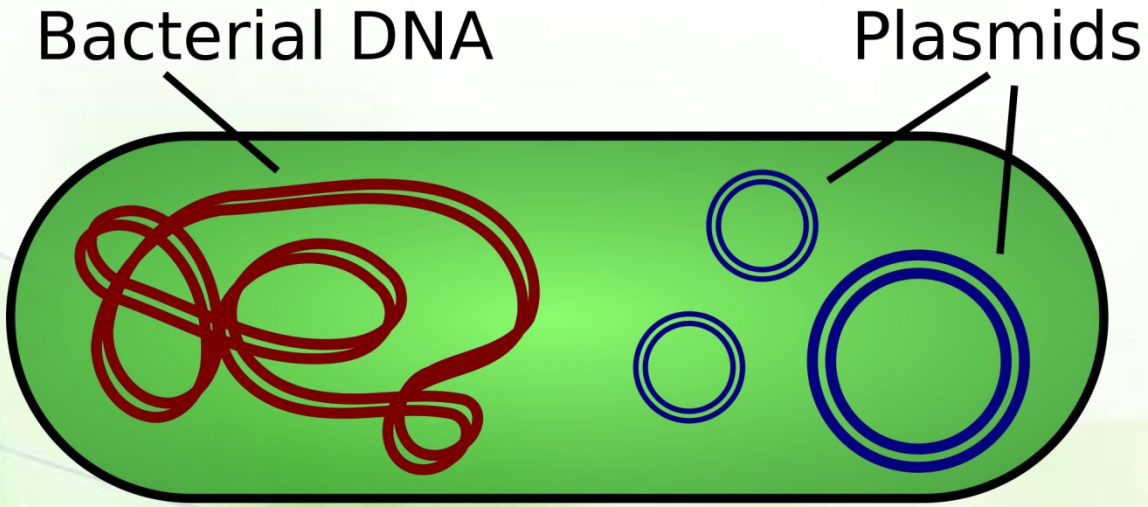
How do we clone a DNA molecule?

- a DNA fragment of interest is inserted into a DNA carrier (called a vector) that can be replicated.
- The resulting DNA molecule is what is known as a recombinant DNA molecule.
- The procedure is known as recombinant DNA technology, which is part of genetic engineering.



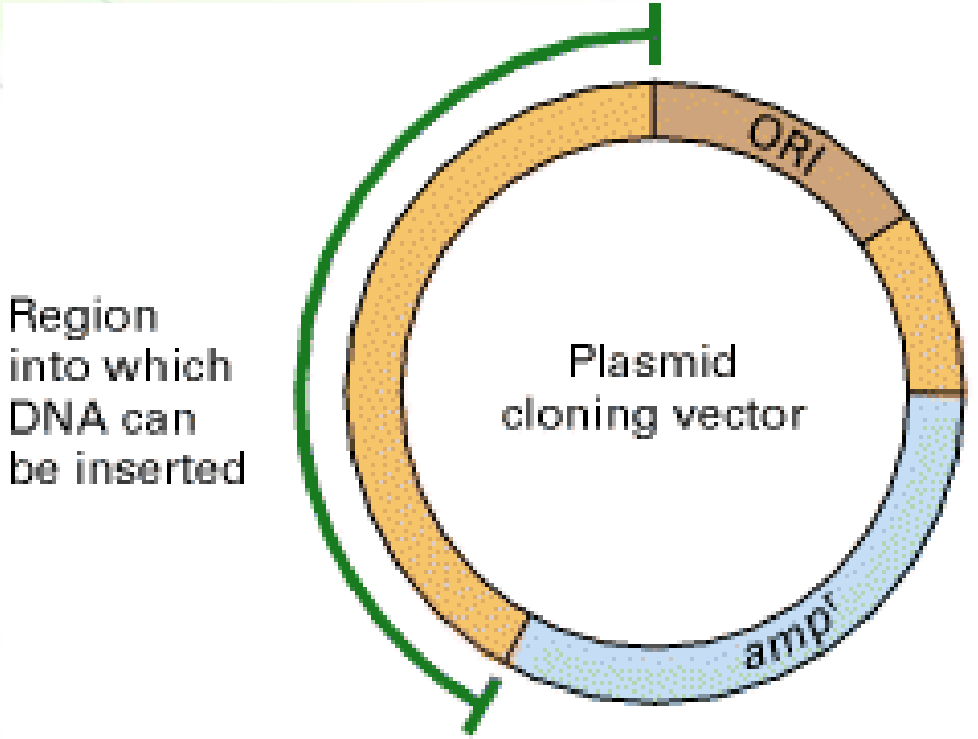
Using plasmids as vectors

- Bacterial plasmids are considered excellent vectors that are used for cloning (cloning vectors) or expression (expression vectors).
- These are natural bacterial circular DNA that is not part of the main circular DNA chromosome of the bacterium.
- A plasmid exists as a closed circle and replicates independently of the main bacterial genome.



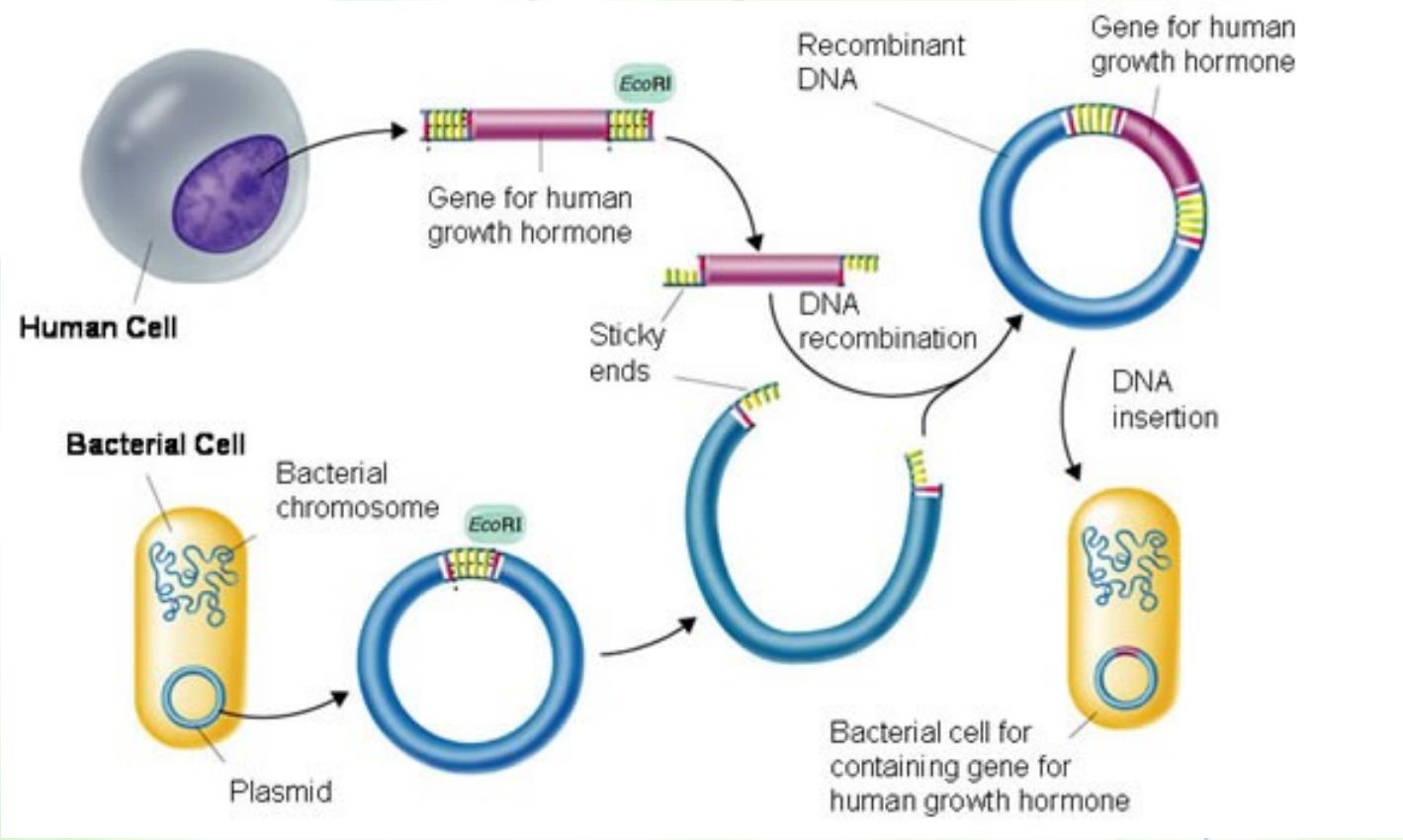
Features of plasmid cloning vectors

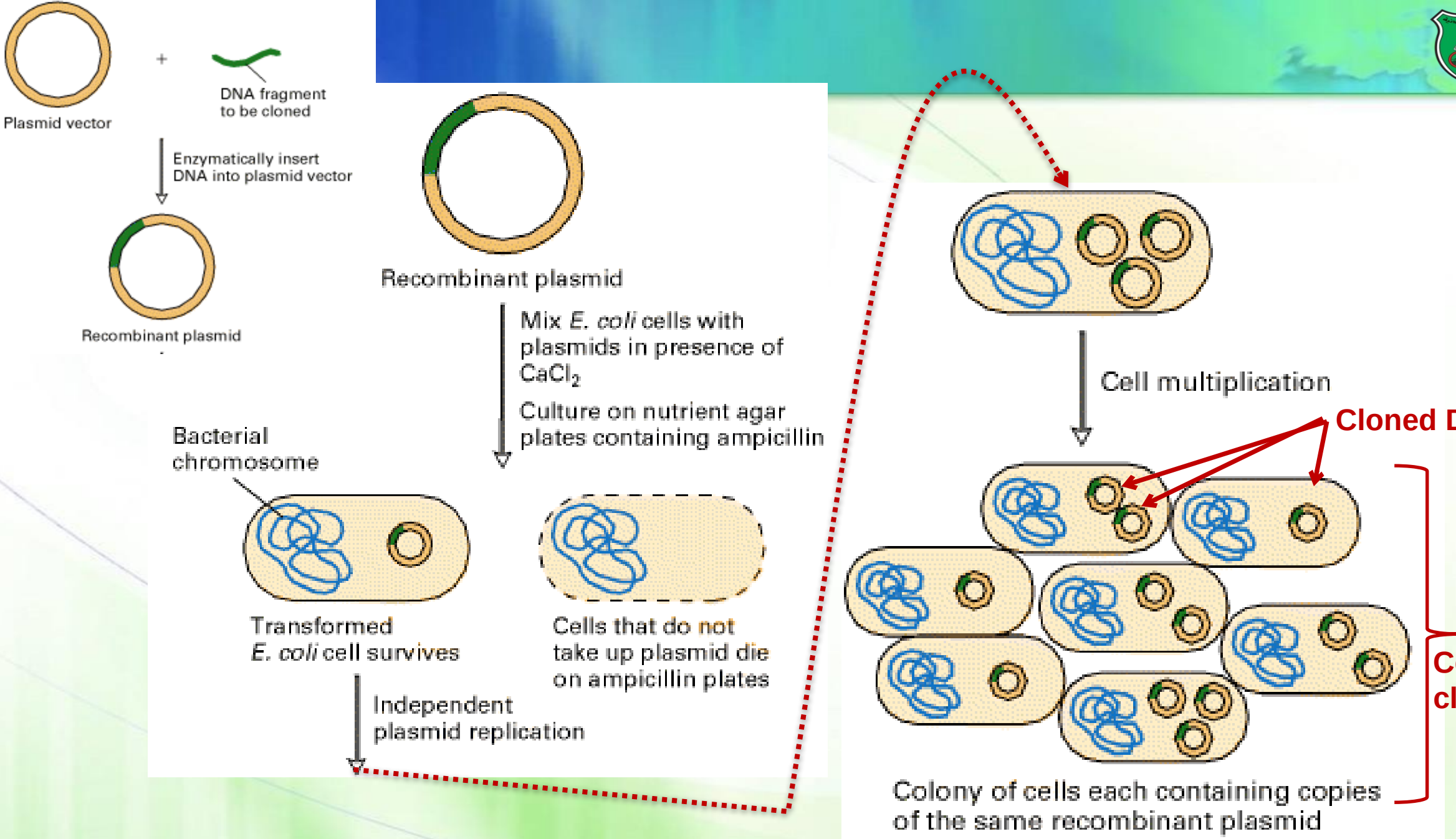
- Plasmid cloning vectors must have the following three components:
  - Their own origin of replication (OriC) that allows them to replicate independently of the bacterial chromosome.
  - Aselectable gene such as an antibiotic resistance gene that allows for selecting for/against the cells that have them.
  - A restriction site that allows for insertion of the DNA segment of interest into the plasmid.



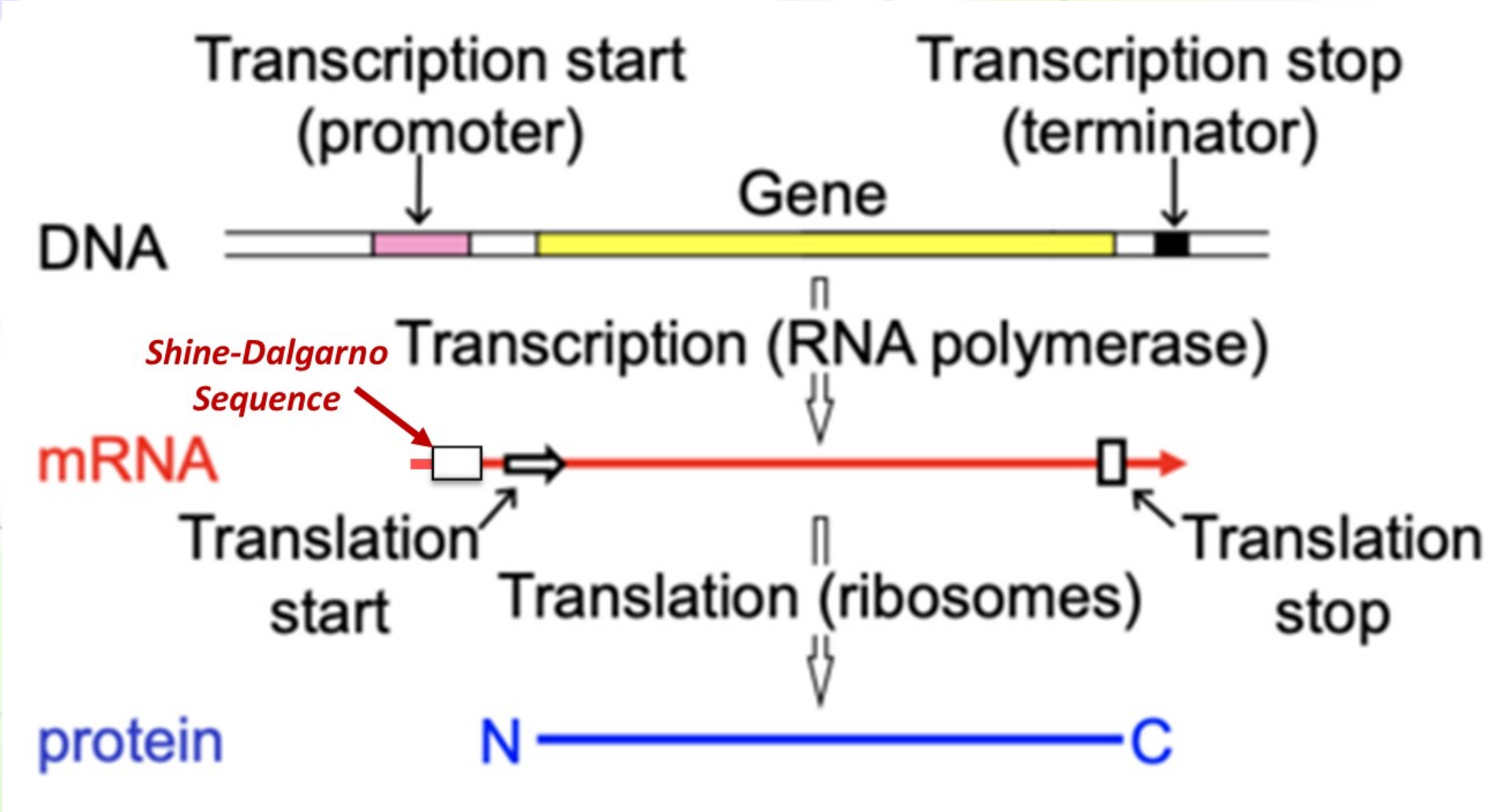
The making of a recombinant DNA

- Both DNA fragments (the DNA to be cloned and a vector) are cut by the same restriction endonuclease that makes DNA fragments with same sticky-ends hybridize (anneal) to each other, when mixed.
- A DNA ligase is added to "close" the plasmid.



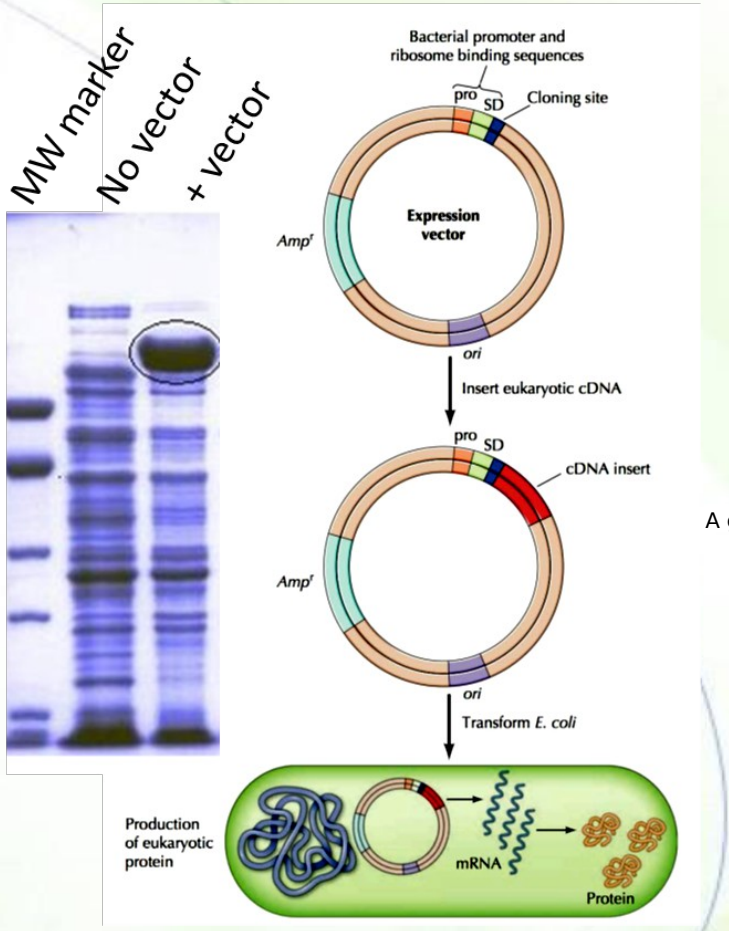

Plasmid vector
DNA fragment to be cloned
Enzymatically insert DNA into plasmid vector
Recombinant plasmid
Recombinant plasmid
Mix E. coli cells with plasmids in presence of $CaCl_2$
Culture on nutrient agar plates containing ampicillin
Bacterial chromosome
Transformed E. coli cell survives
Cells that do not take up plasmid die on ampicillin plates
Independent plasmid replication
Cell multiplication
Cloned DNA
Cell clones
Colony of cells each containing copies of the same recombinant plasmid

Overview of gene expression



Expression vectors

- Expression vectors contain additional sequences:
  - Promoter sequences upstream of gene to be inserted,
  - Ribosomal binding sequences (Shine-Dalgarno [SD] sequences),
  - A transcription termination sequence.
- The protein is expressed and purified.
- Examples: insulin, growth hormone, plasminogen activator, erythropoietin

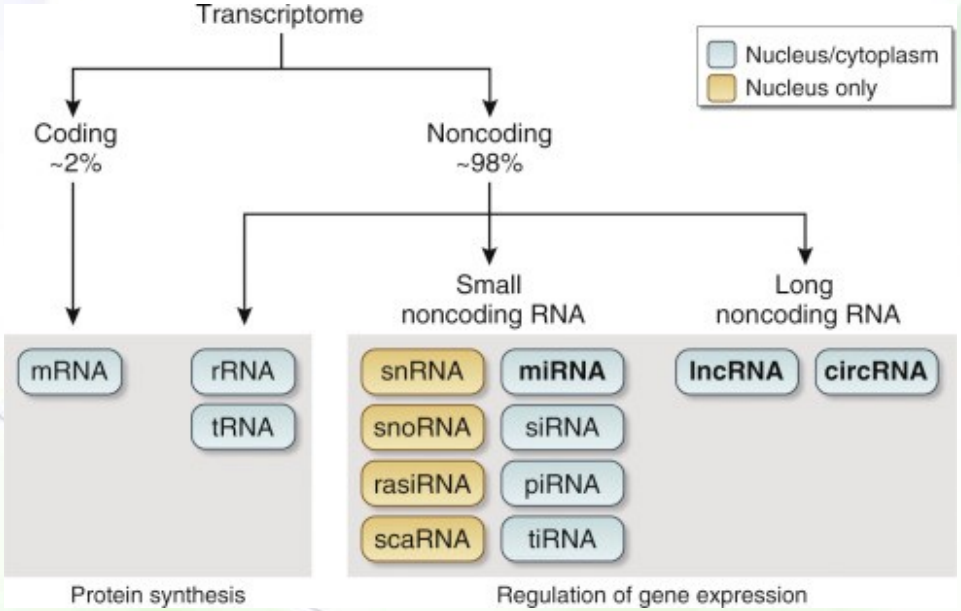


A gene

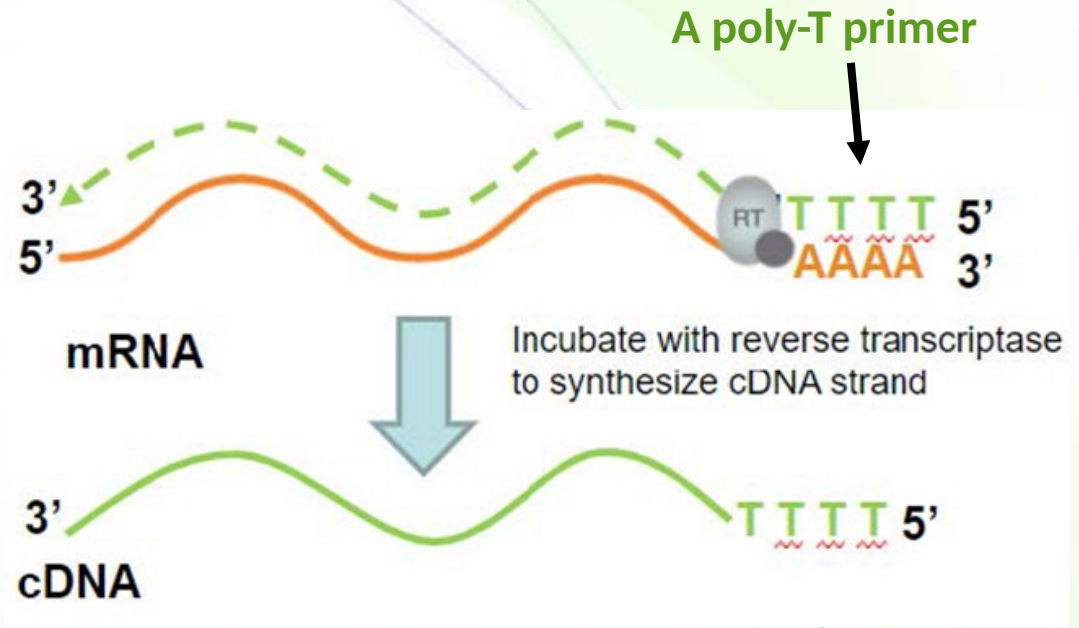
How do we select for human mRNA?

The power of reverse transcriptase (part 1)

The "many types of RNA" challenge



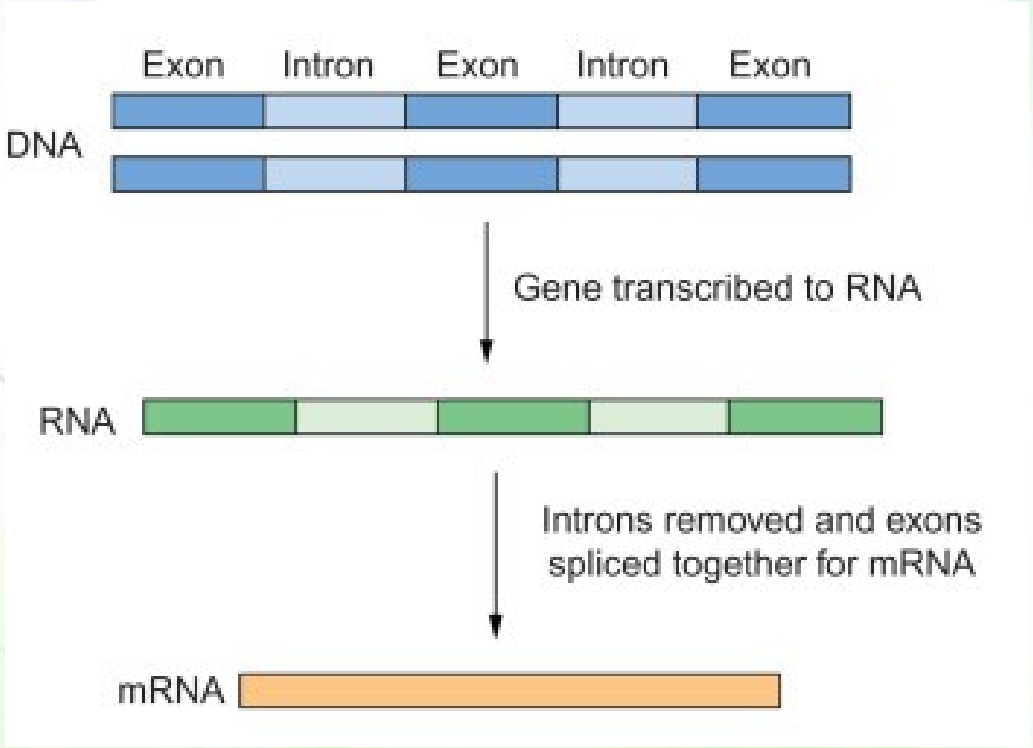
The "poly-T primer" solution



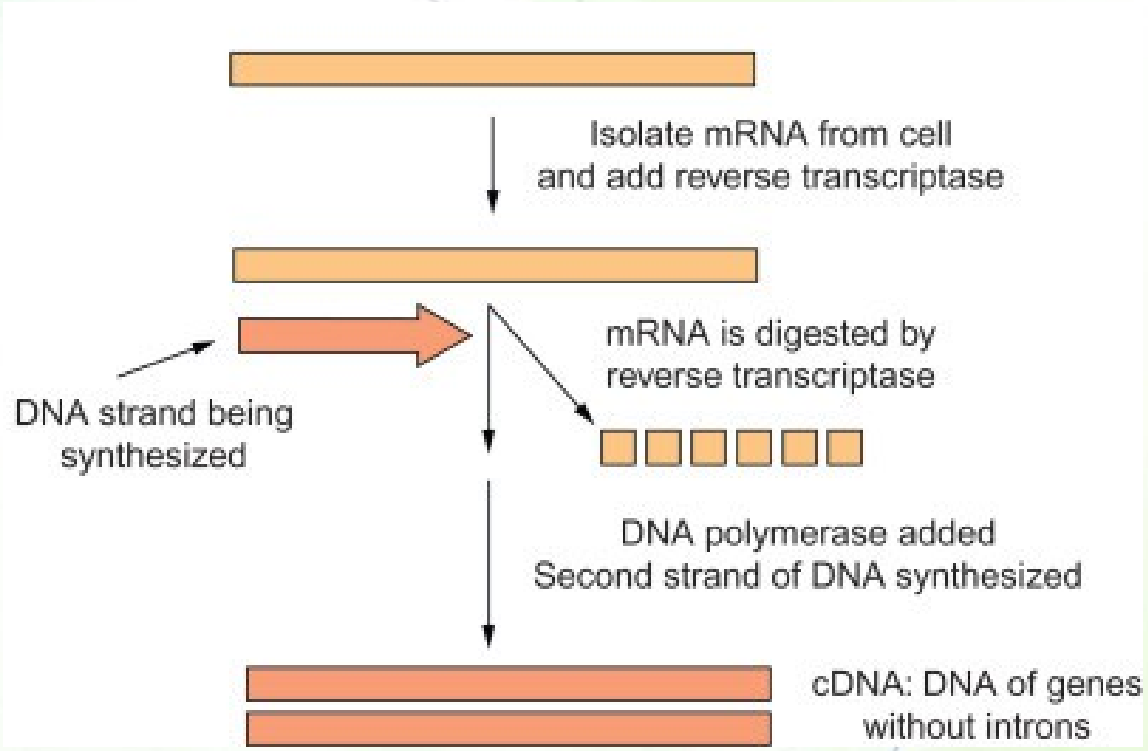
How do we deselect introns?

The power of reverse transcriptase (part 2)

The "intronic" challenge



The "reverse" solution



Challenges of protein expression in bacteria

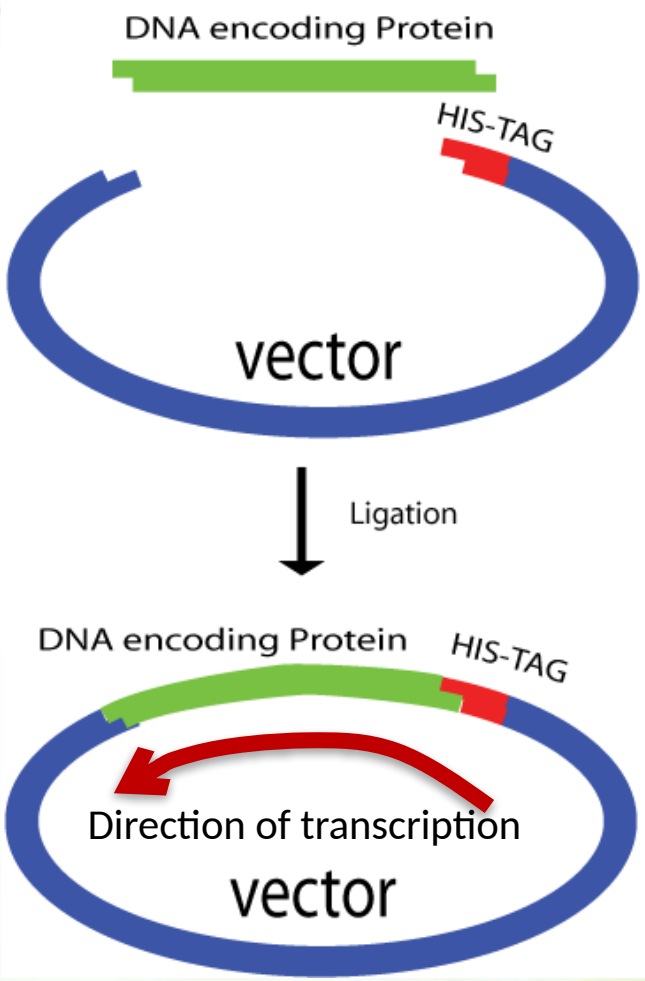
- No internal disulfide bonds
- No post-translational modification (example: glycosylation)
- Protein misfolding
- Protein degradation

- Solution: use a eukaryotic system such as yeast

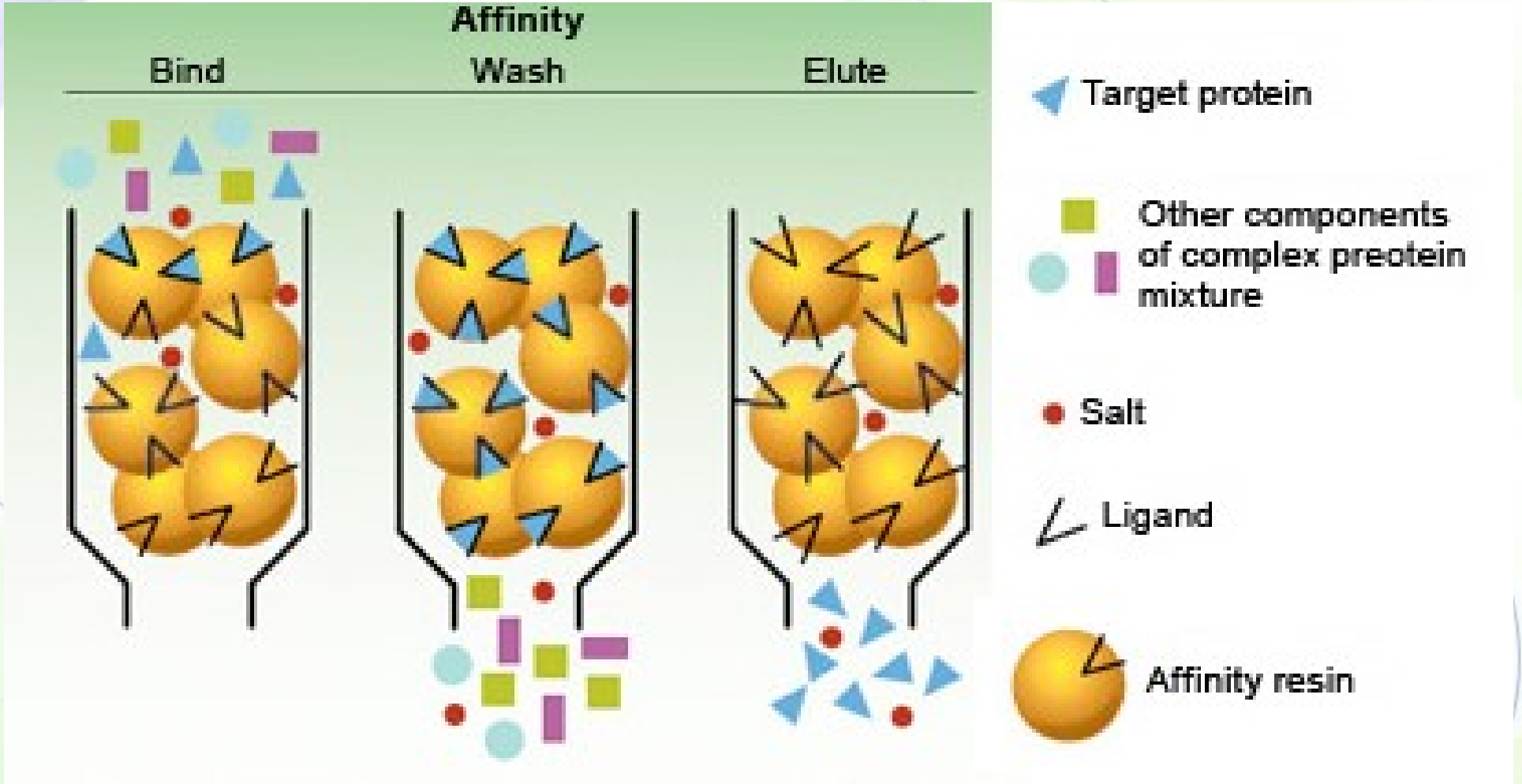
Protein tagging and creation of protein hybrids

Proteins can be "tagged"

- A protein-encoding gene is cloned in a special vector containing a tag gene producing a protein with an extra sequence of amino acids called tags.
- These tags allow easy protein purification and detection.

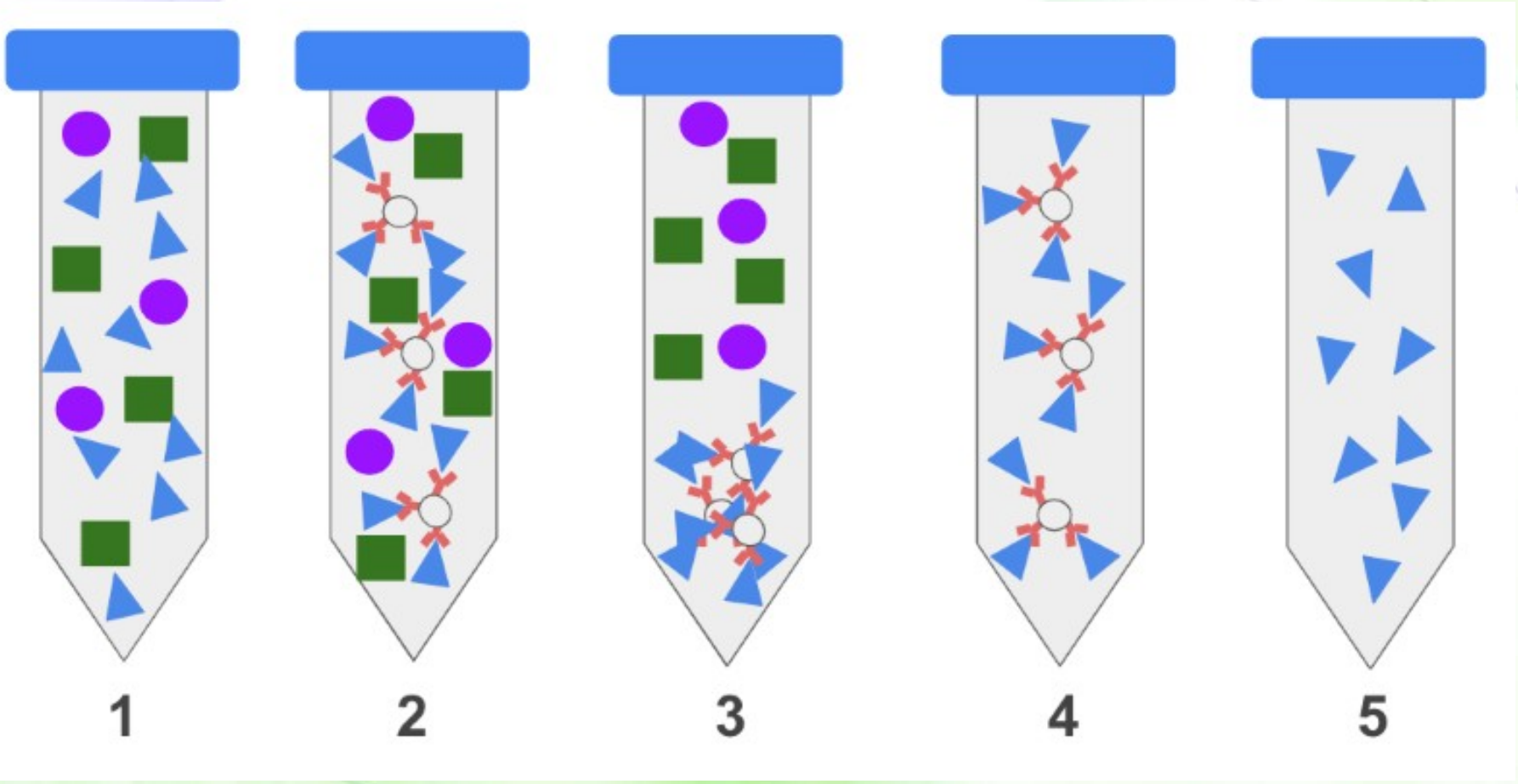


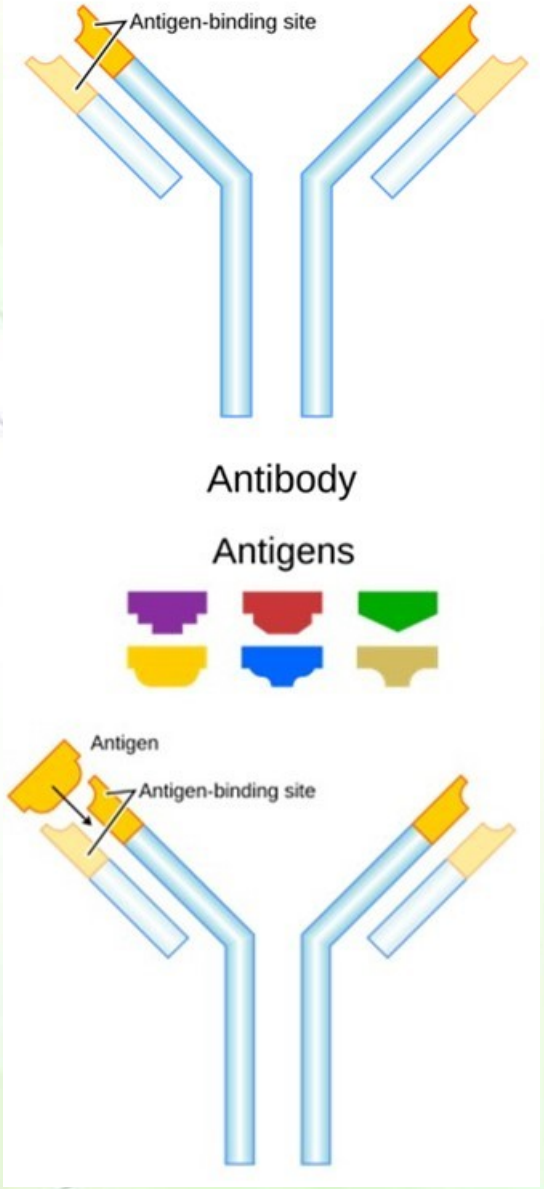
Post-protein tagging…1) Affinity chromatography



https://www.youtube.com/watch?v=8_7cdfNO7OY

Post-protein tagging…2) Immunoprecipitation





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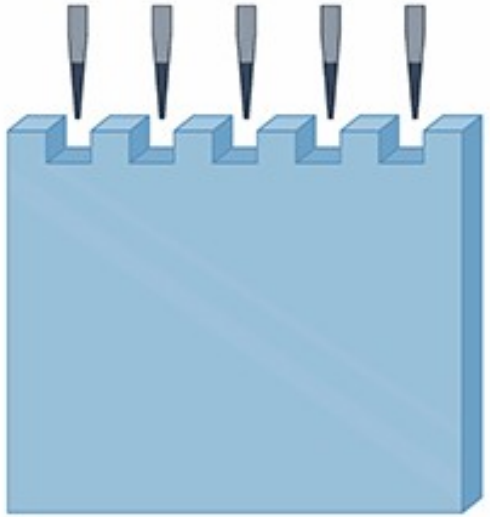
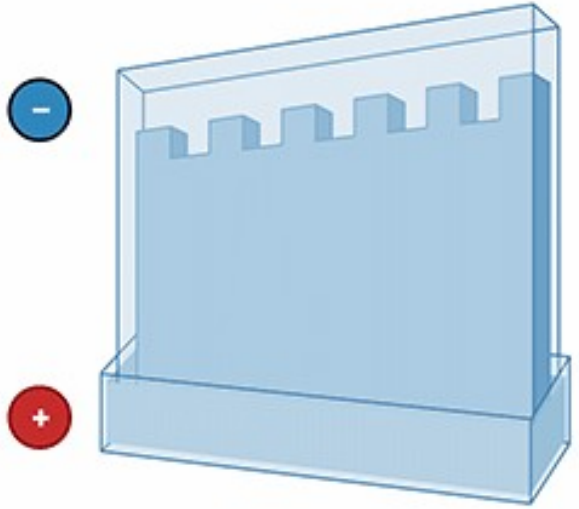
Post-protein tagging…3) Gel electrophoresis (SDS-PAGE)

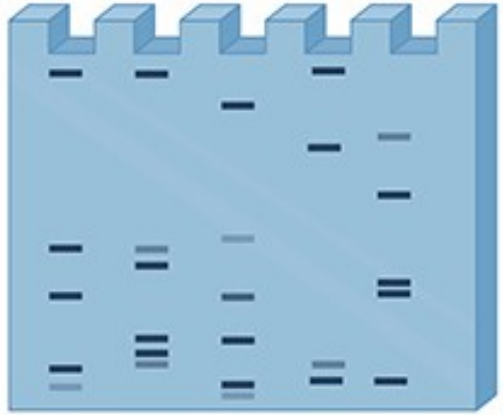
1 **Load protein sample**
into the wells of a gel



2 **Apply current**
to separate proteins
according to size



3 **Analyze gel**
by staining proteins
that look like “bands”



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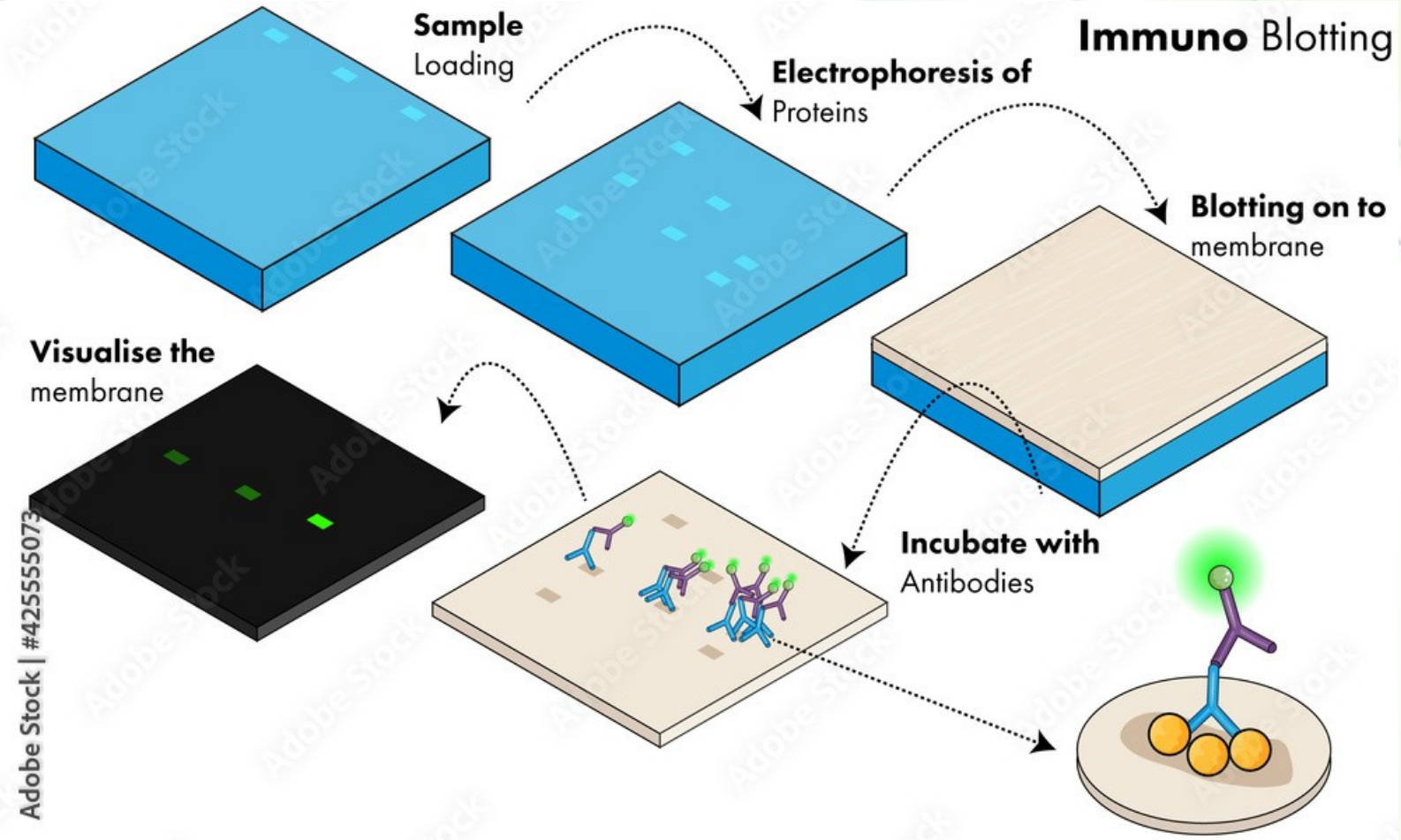
Post-protein tagging…4) Immunoblotting



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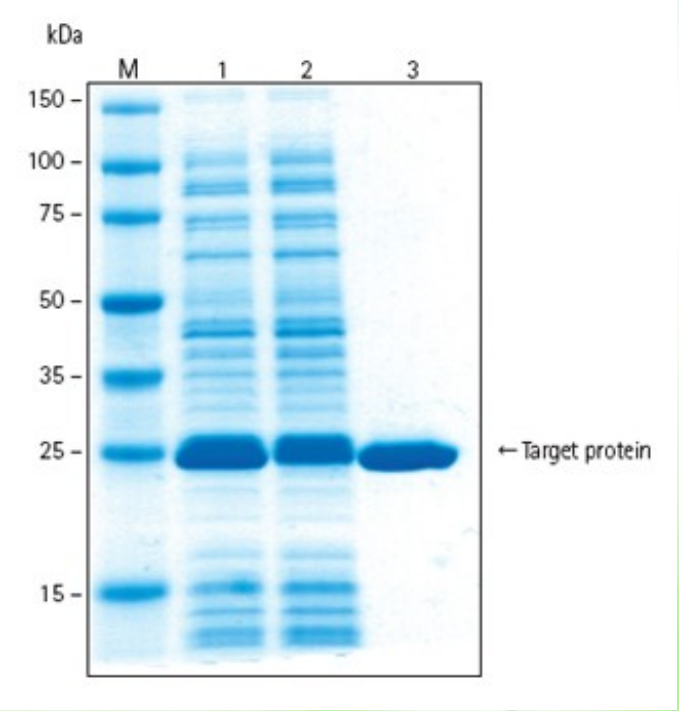
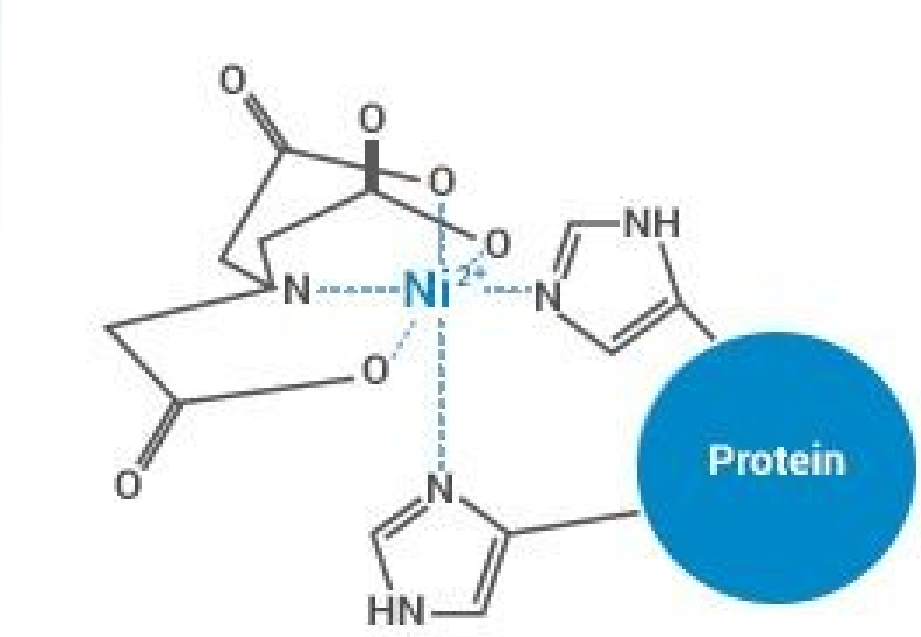
Major protein tags

Name	Amino acids	Detection	Purification
FLAG	DYKDDDDK	antibody	FLAG peptide
Green fluorescent proteins (GFP)	~220 aa protein	antibody or fluorescence	None
Glutathione S transferase (GST)	218 aa protein	antibody	glutathione
HA	YPYDVPDYA	antibody	HA peptide
Poly-His	HHHHHH	antibody	nickel, imidazole
Myc	EQKLISEED	antibody	Myc peptide
V5	GKPIPNPLLGLDST	antibody	V5 peptide

His tag

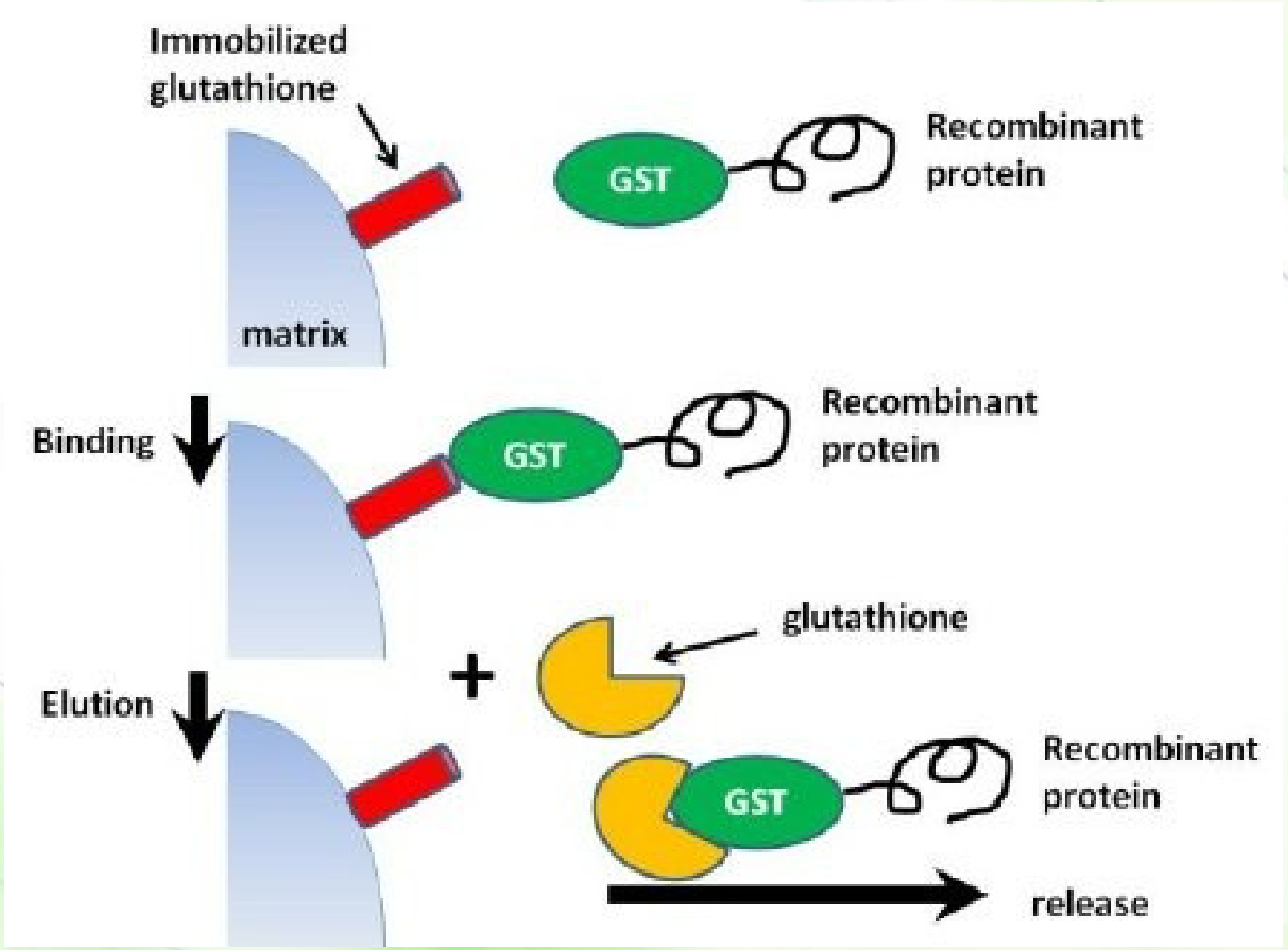
- The addition of six histidines to a protein would allow for purification using breads with bound nickel ions.





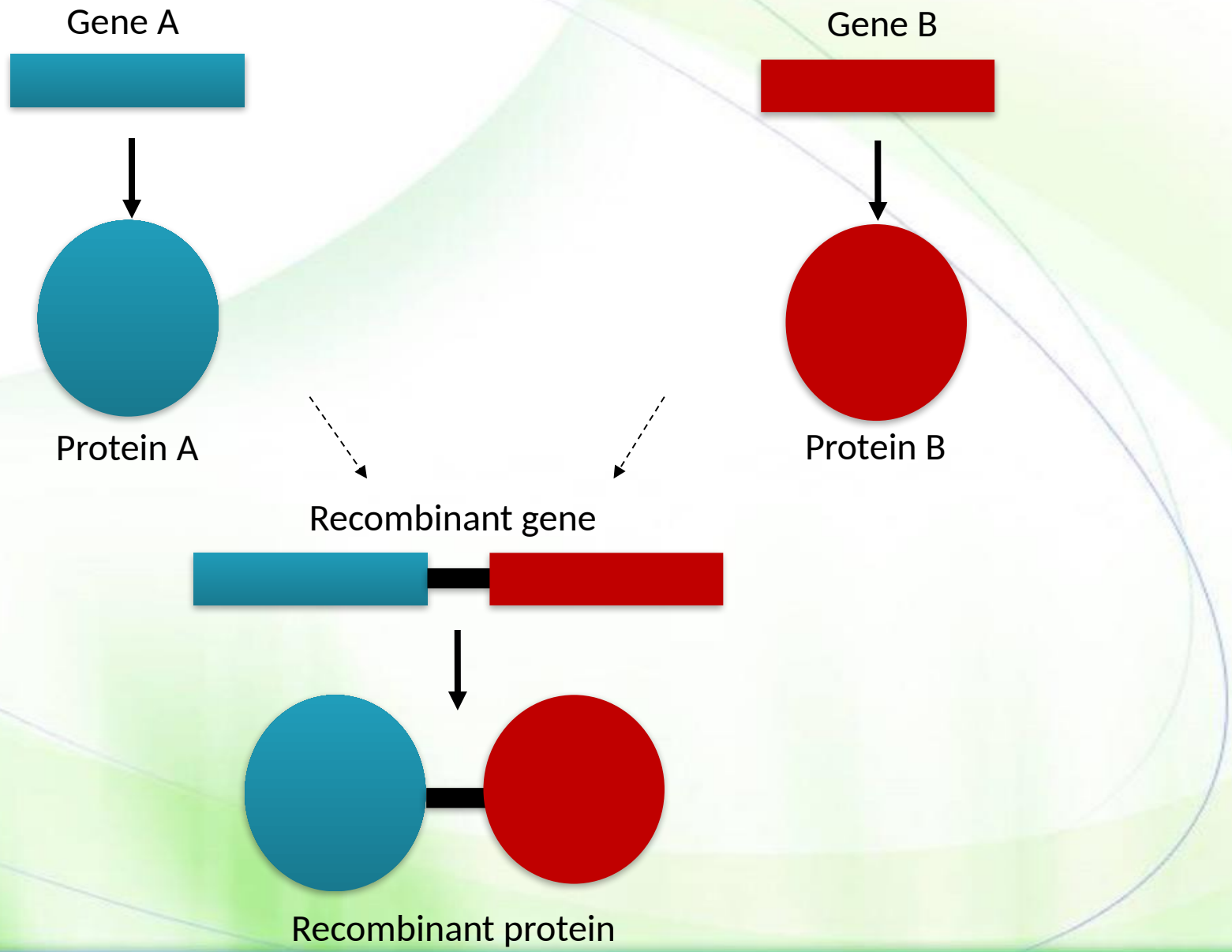
Clone → Express → Purify → Analyze

Purification of GST-tagged proteins

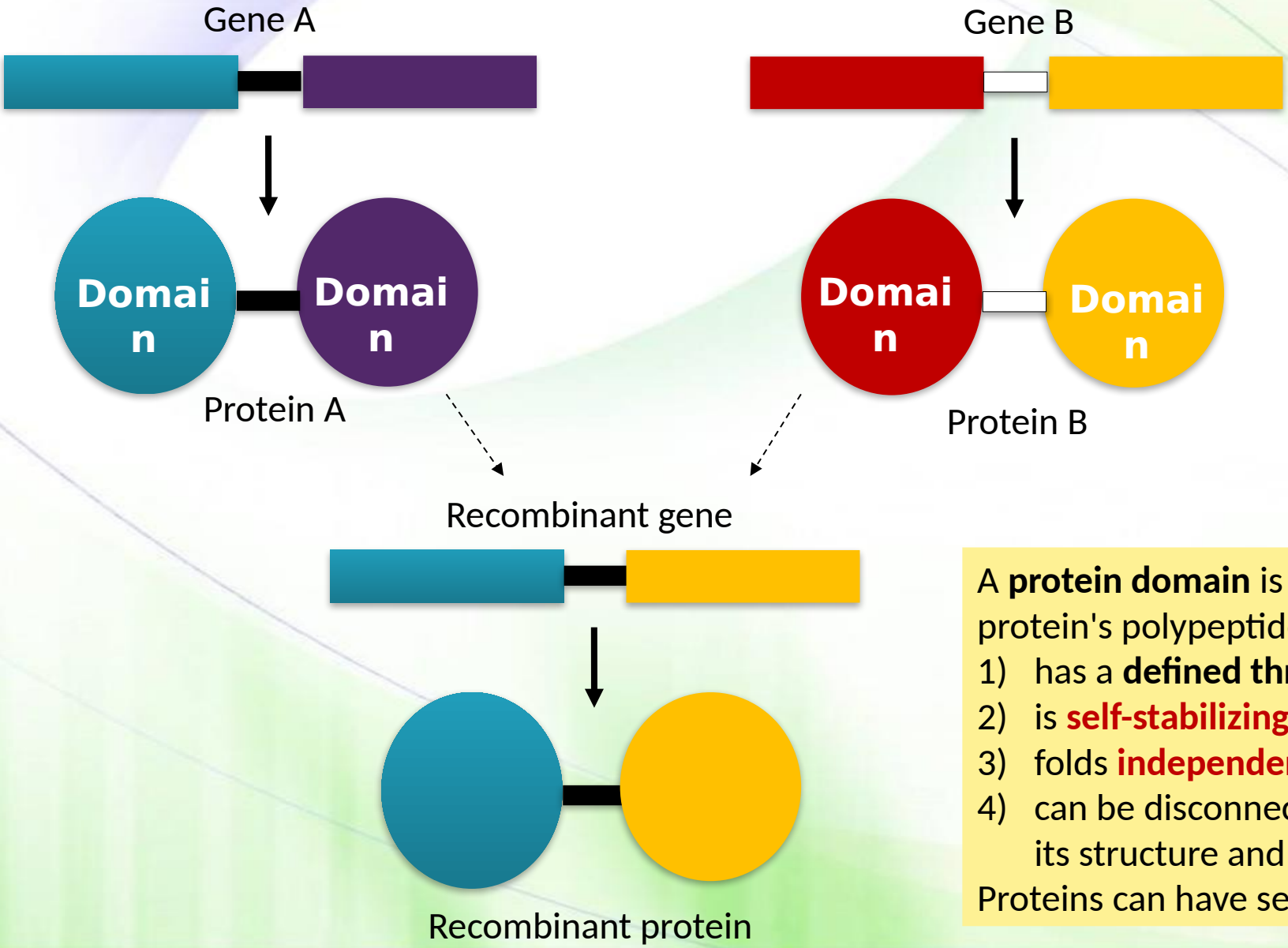


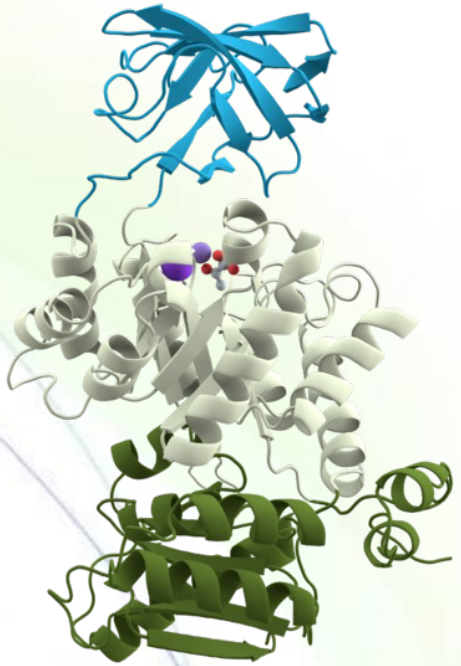
Glutathione S transferase (GST)

Production of a recombinant protein



Production of a recombinant protein…*The power of domains*





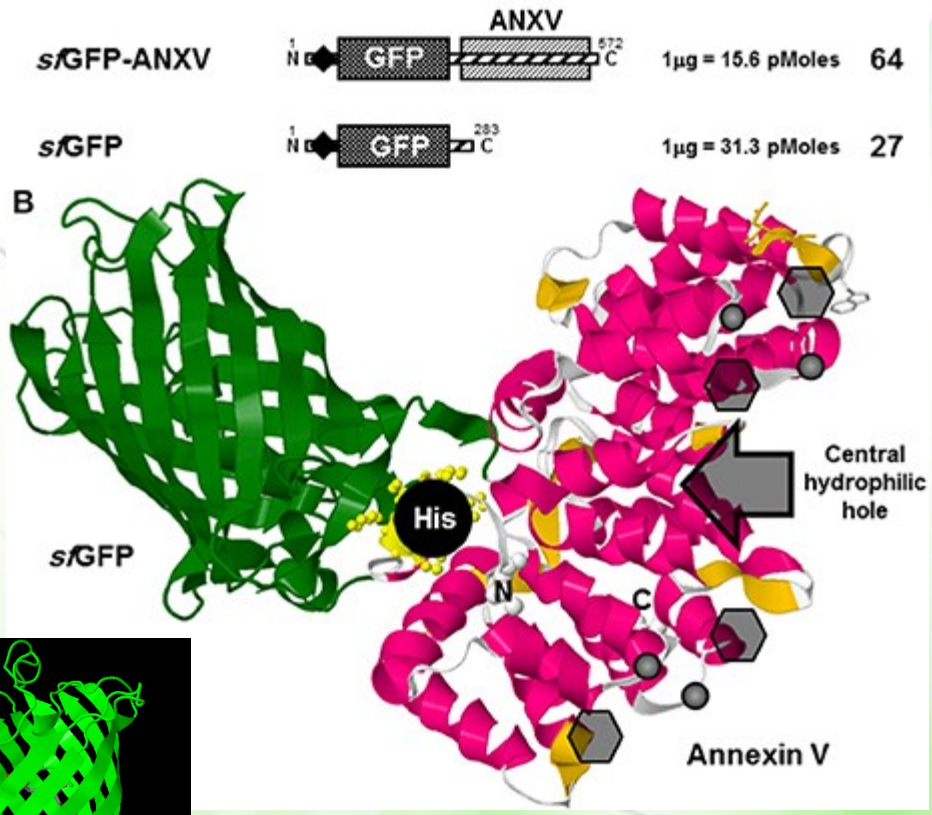
A **protein domain** is a compact region (or part) of the protein's polypeptide chain that:

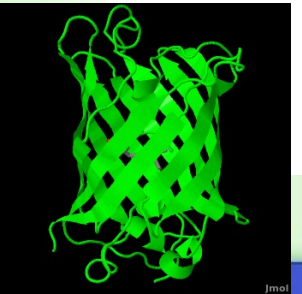
1) has a **defined three-dimensional** structure,
2) is **self-stabilizing,**
3) folds **independently** from the rest.
4) can be disconnected from the protein and, yet, maintains its structure and function.

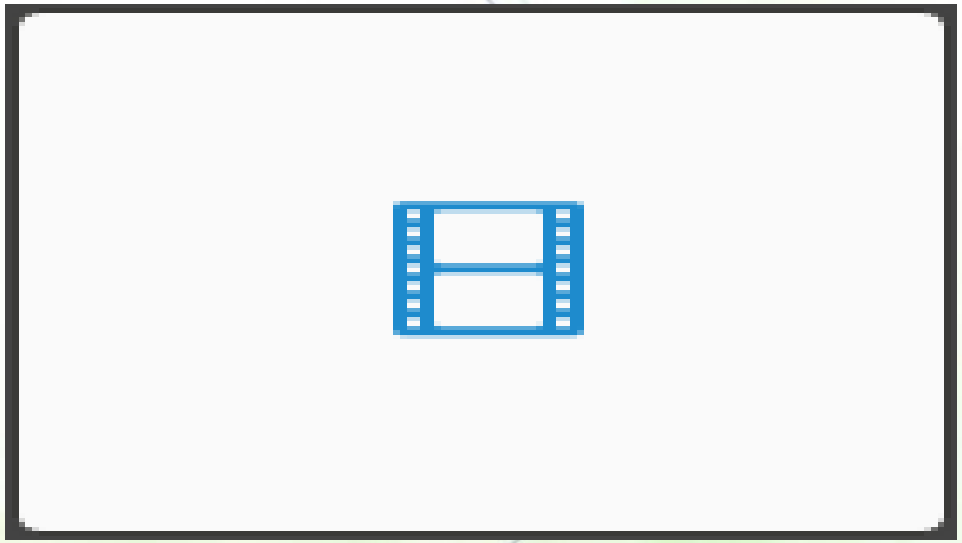
Proteins can have several domains.

GFP-tagged proteins

- Green Fluorescent Protein (GFP) allows for protein detection rather than for purification purposes.







A world of possibilities