Recombinant DNA technology and DNA cloning

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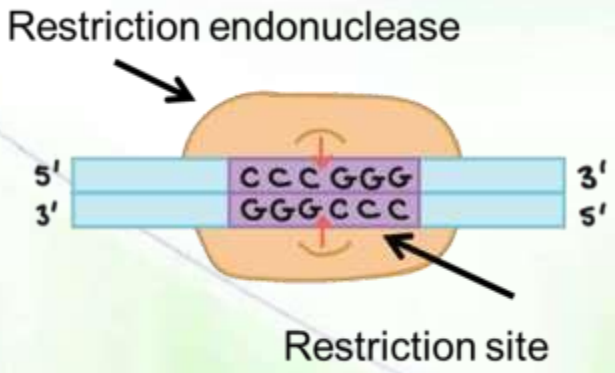
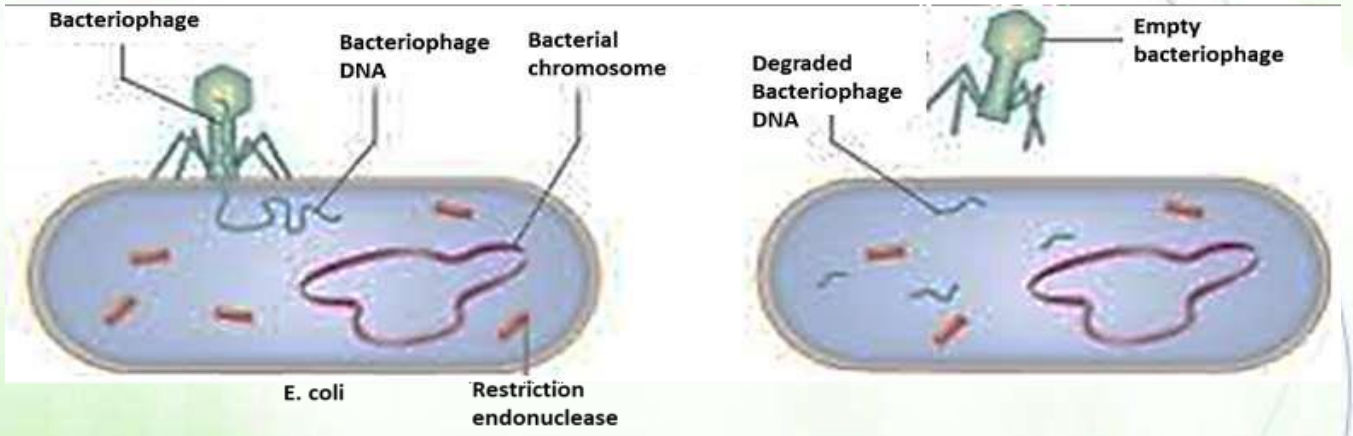
What is DNA cloning?

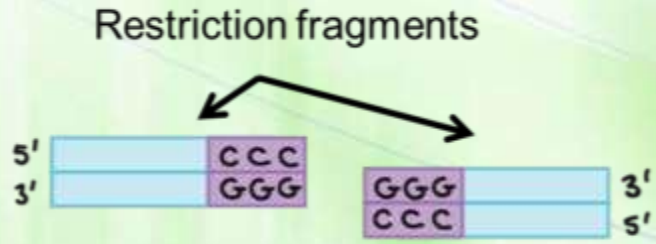
- DNA cloning is a technique that allows for:
  - amplifying a DNA segment into many, many copies in a biological system.
  - expressing a gene inside a biological system such as bacteria, human cells grown in labs, animals, or even the human body as a whole.
- It usually involves:
  - The formation 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).

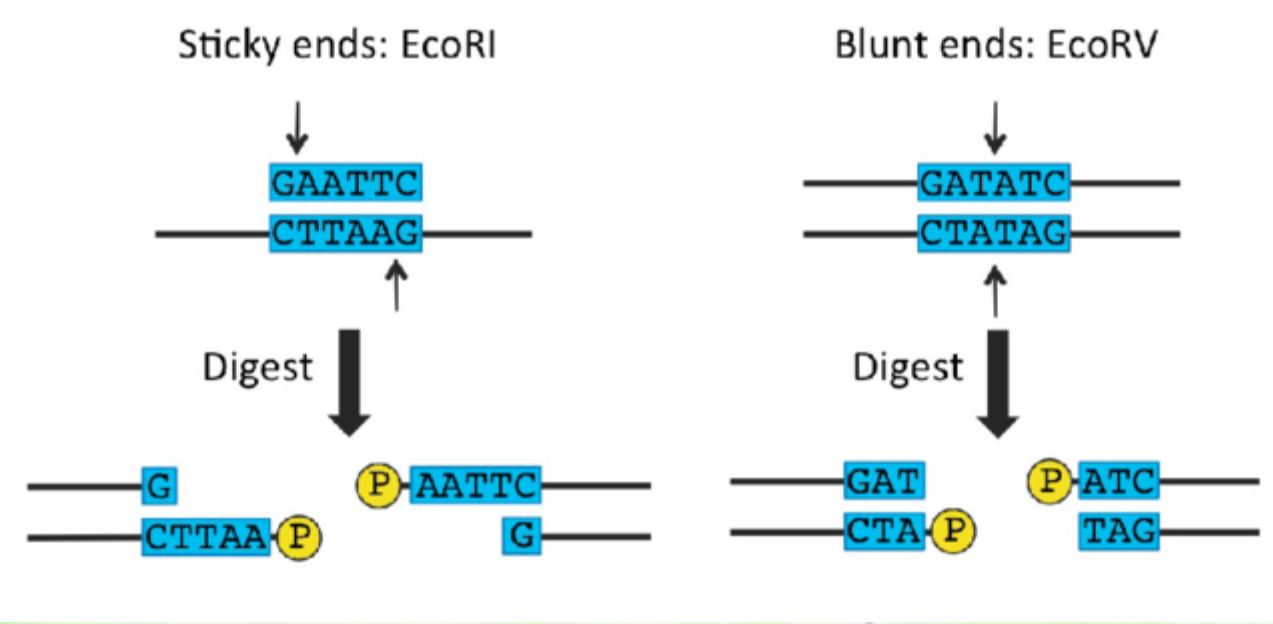
```
EcoRI       5'  GAATTC  3'
             3'  CTTAAG  5'

HindIII     5'  AAGCTT  3'
             3'  TTCGAA  5'

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

Types of cuts by restriction endonucleases

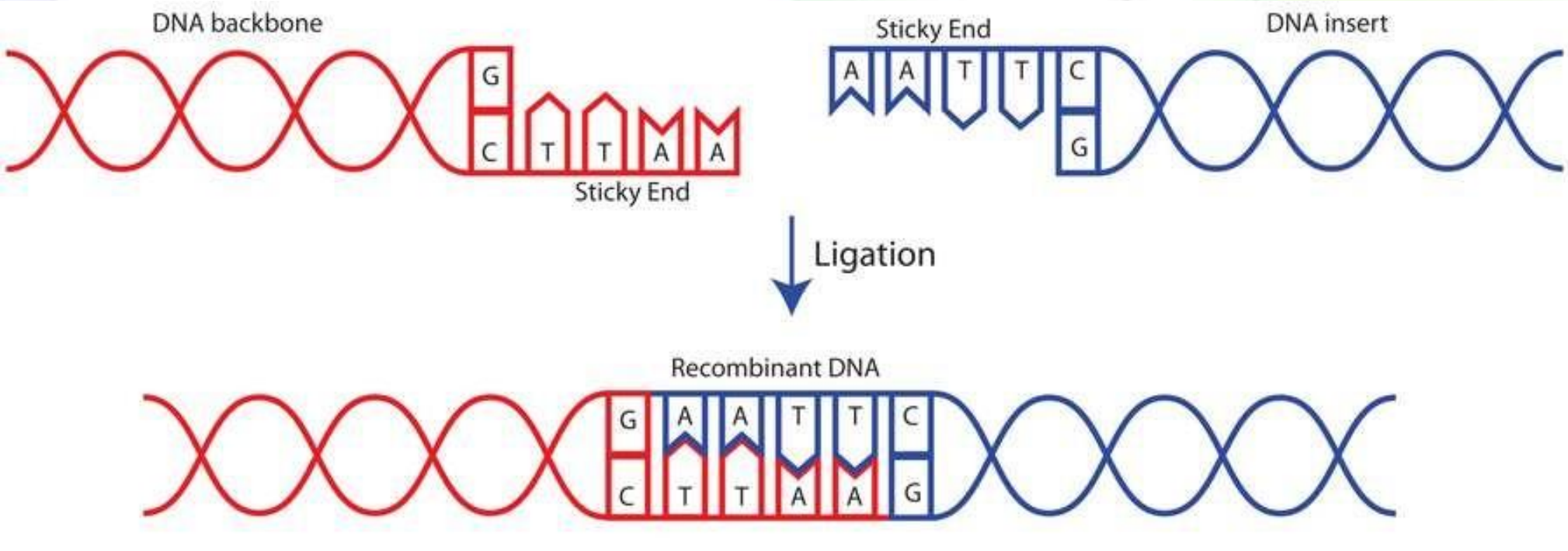
Staggered (off-center): enzymes cut the two DNA strands at different positions generating sticky or cohesive ends.

The DNA restriction fragments would have short single-stranded overhangs at each end.

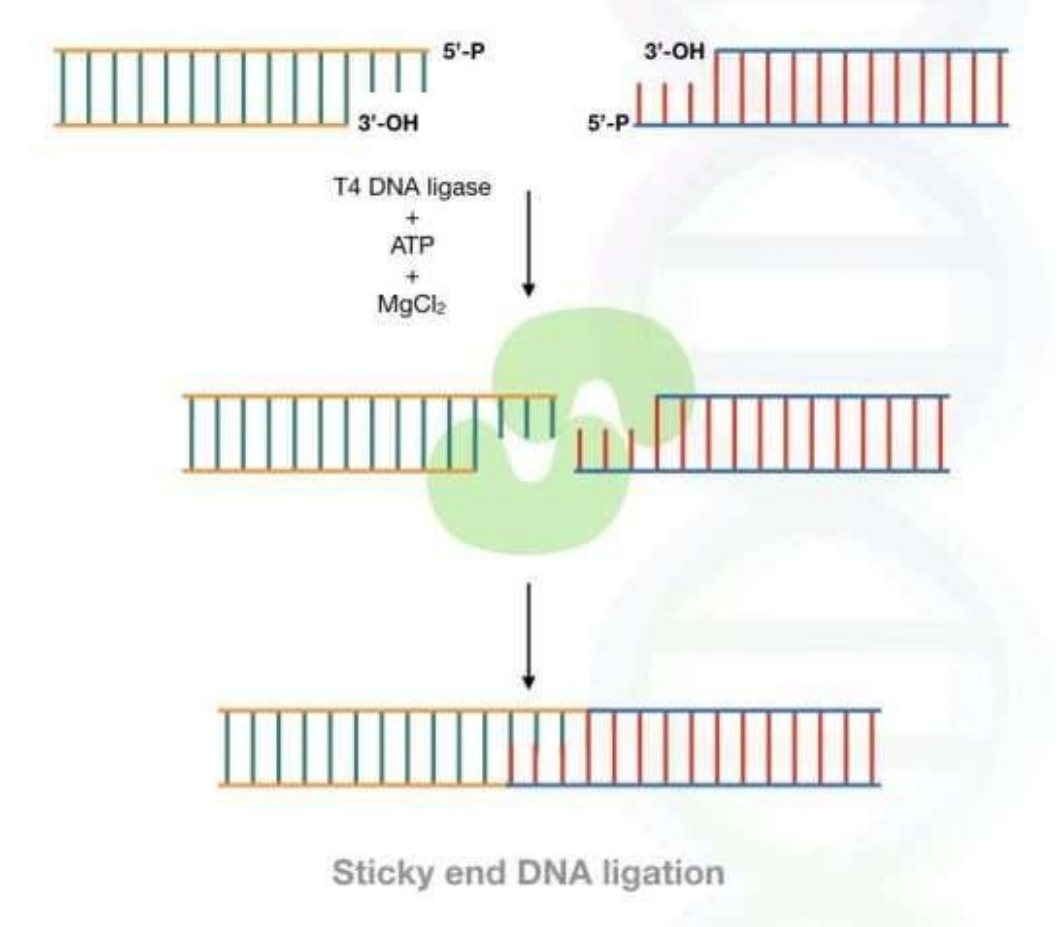
Blunt: enzymes cut at the same position on both strands giving blunt-ended fragments.



Zoom into the sticky ends



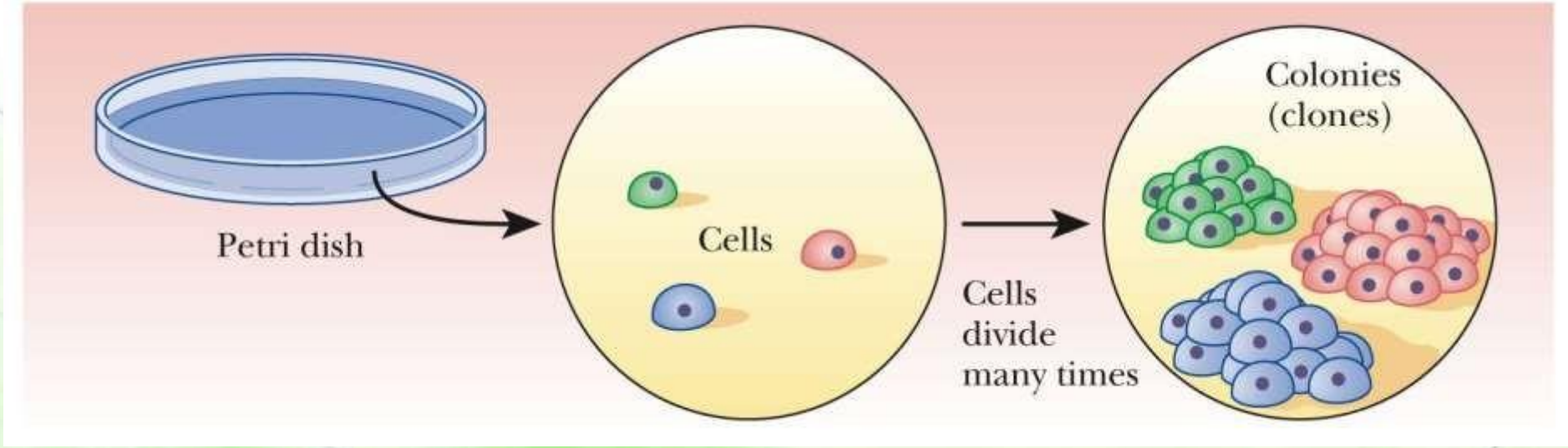
DNA ligase



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.

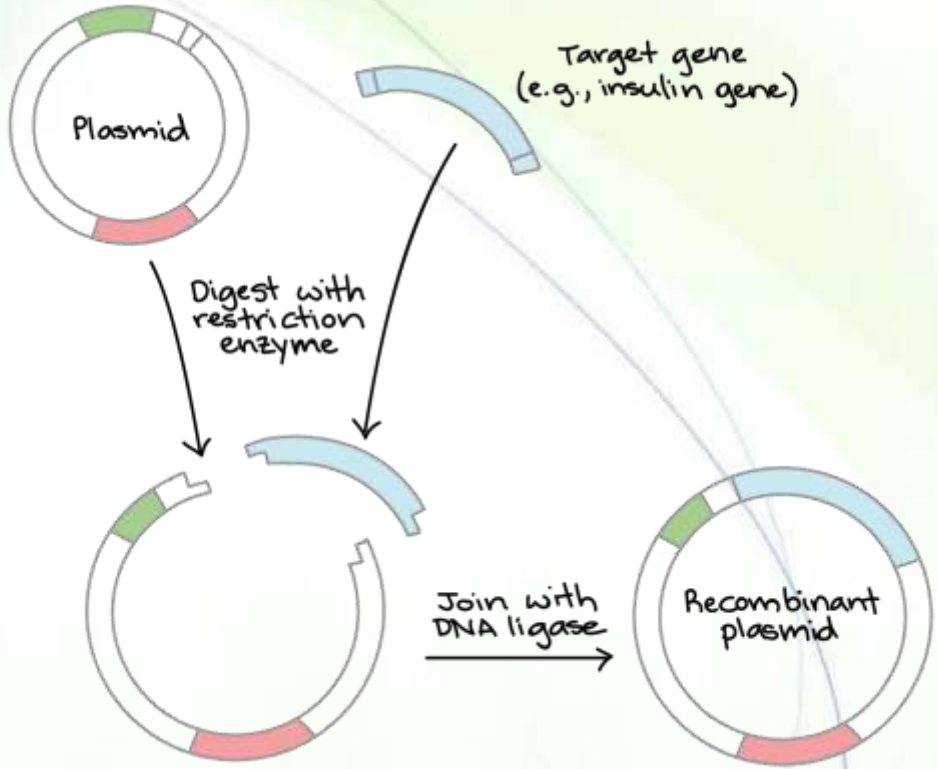
Cloning

- 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.



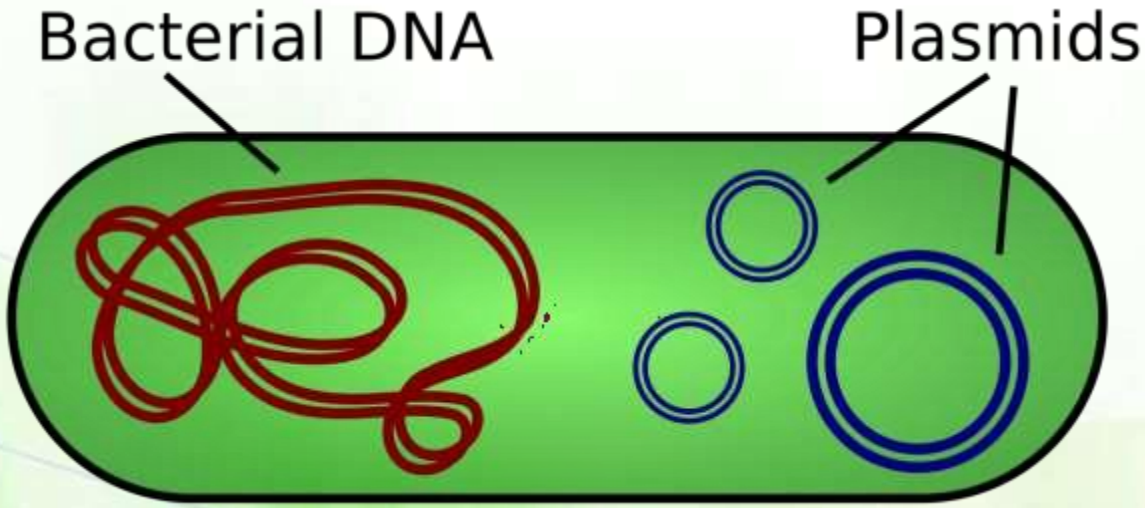
How do we clone a DNA molecule?

- 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.



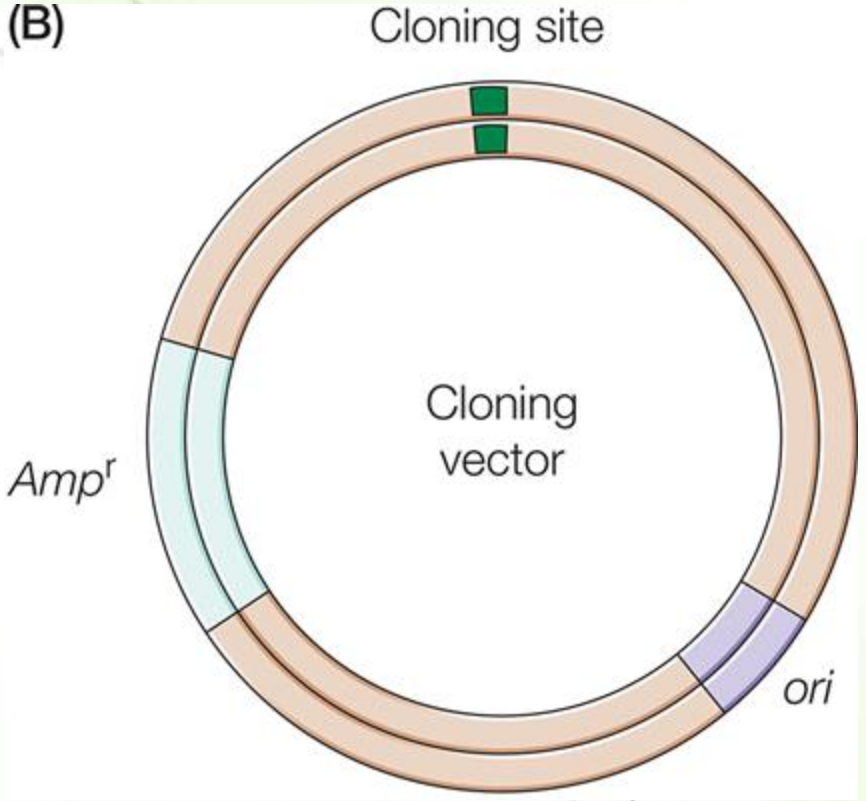
Using plasmids as vectors

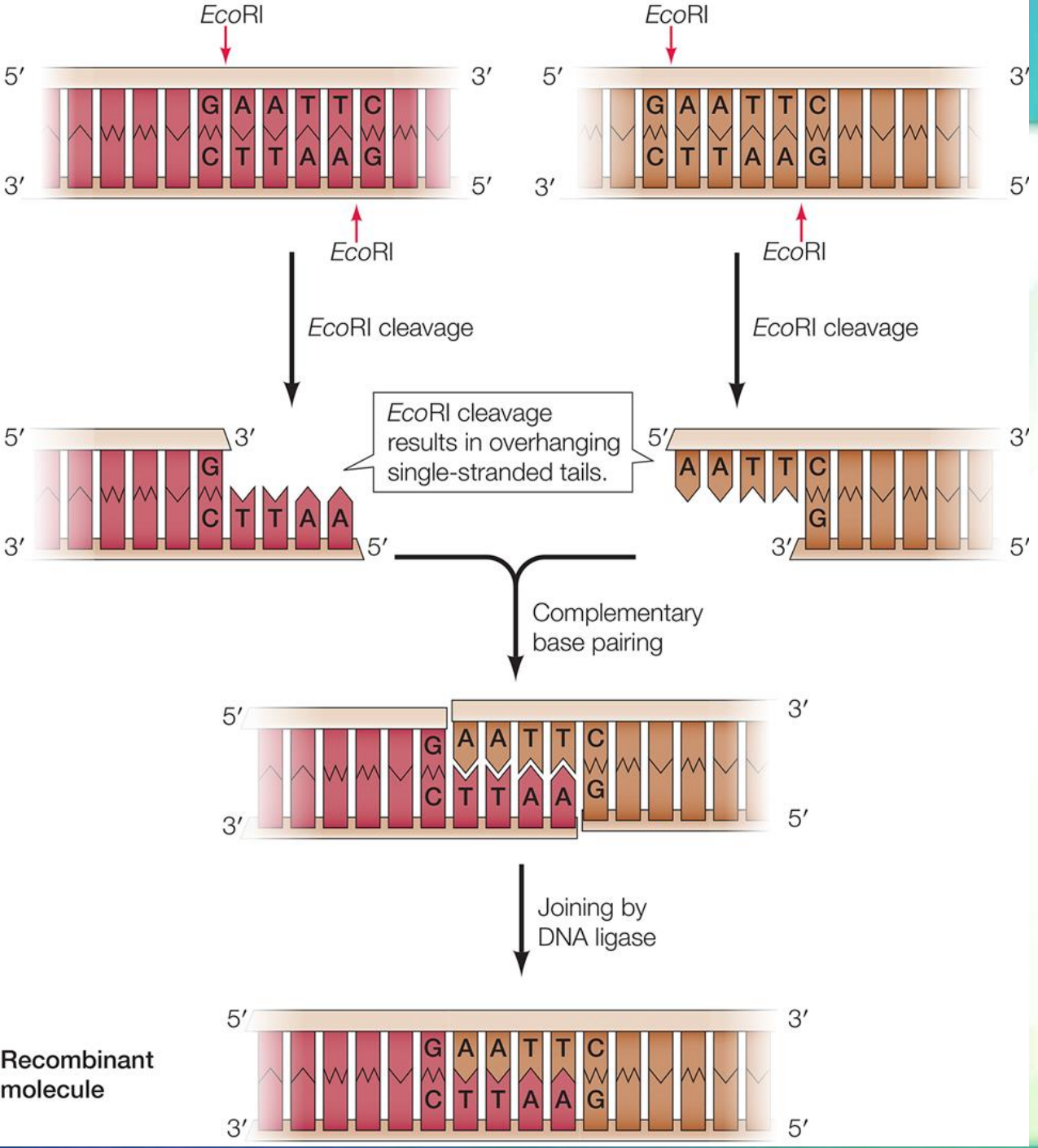
- Bacterial plasmids are considered excellent vectors that are used for cloning (cloning vectors) or expression (expression vectors).
- These are natural bacterial circular DNA that is not part of the main circular DNA chromosome of the bacterium.
- A plasmid exists as a closed circle and replicates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





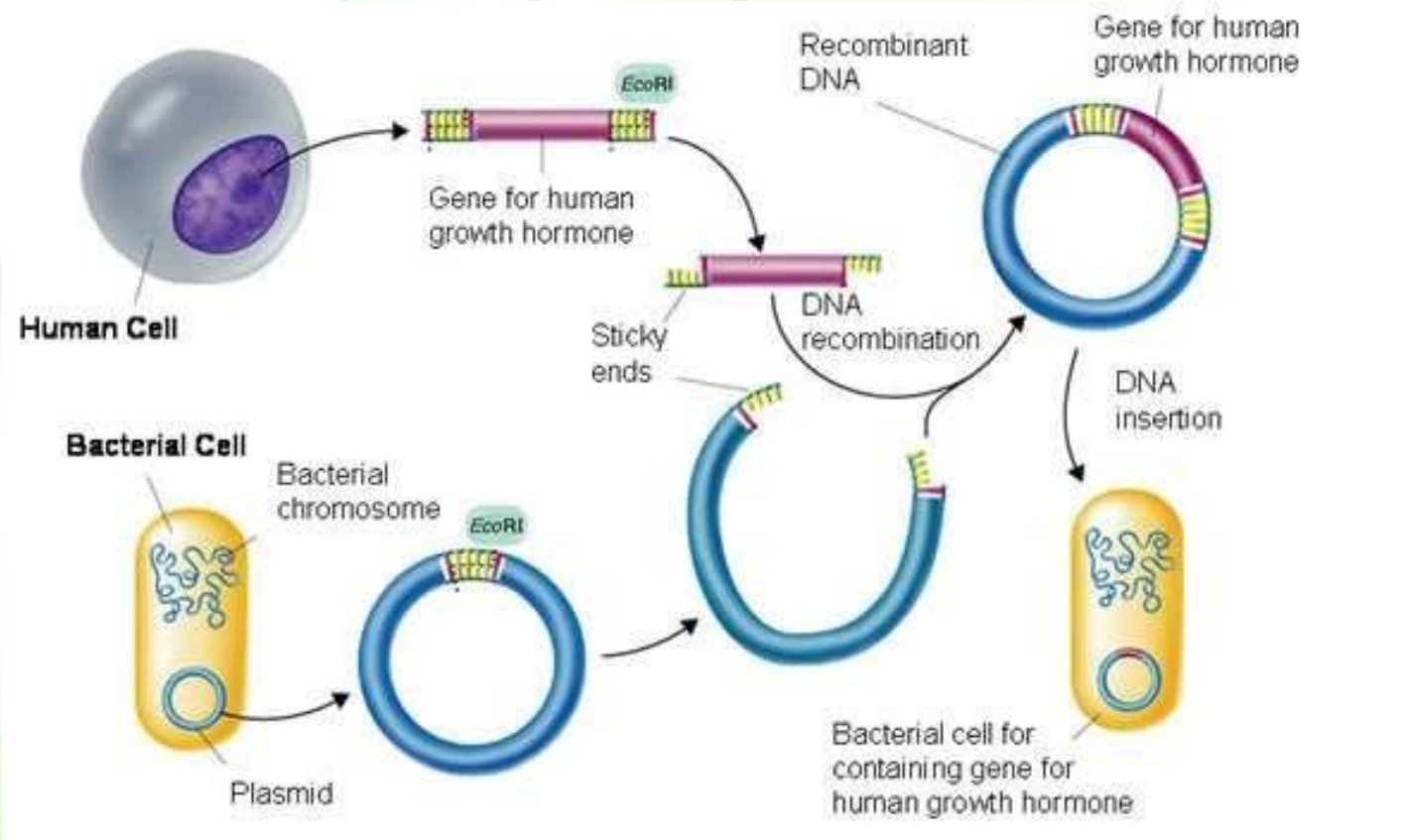
Creation of a recombinant DNA

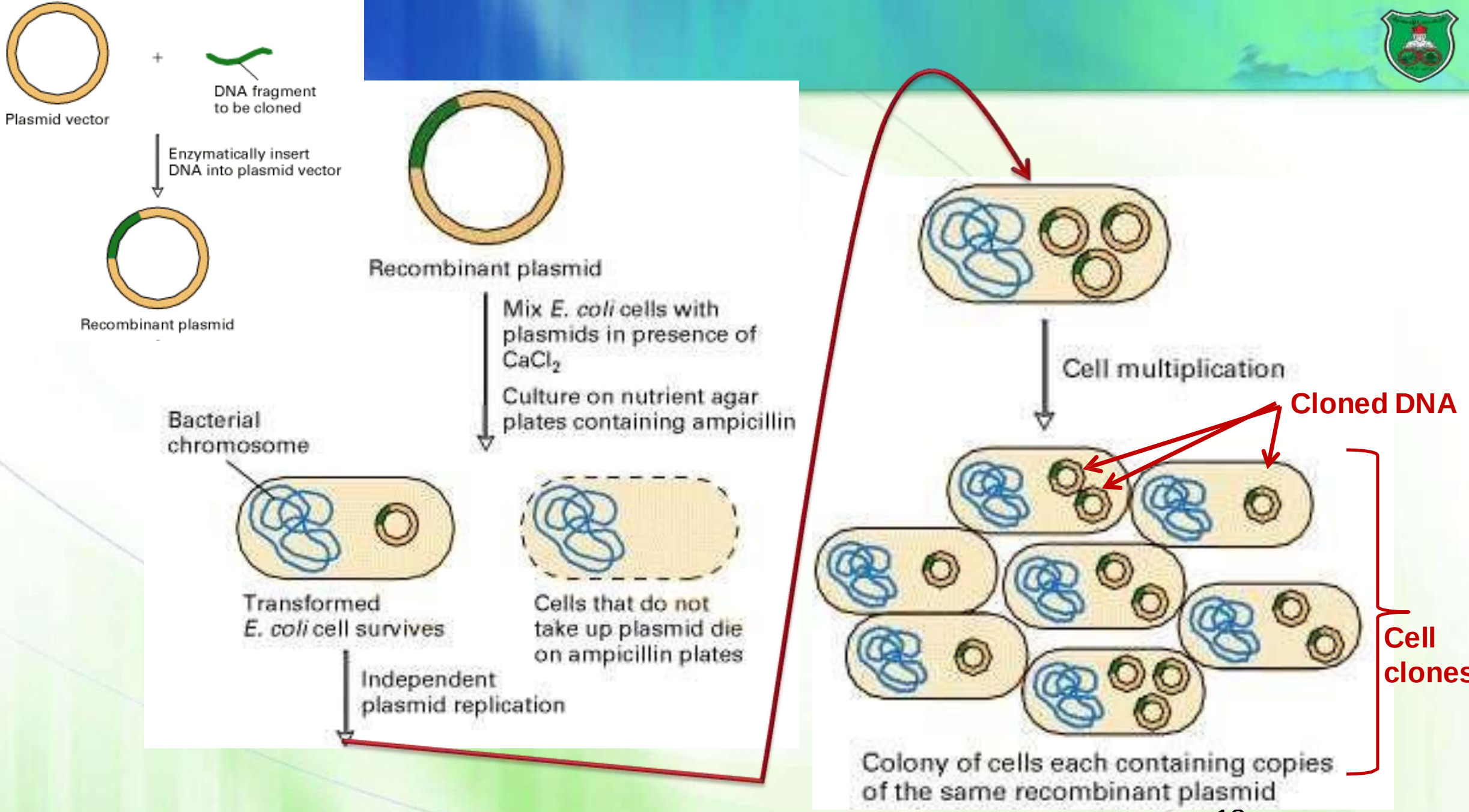
Insert and vector DNAs are digested with a restriction endonuclease (such as EcoRI), which cleaves at staggered sites leaving overhanging single-stranded tails.

Insert and vector DNAs can then associate by complementary base pairing, and covalent joining of the DNA strands by DNA ligase yields a recombinant molecule.

The making of a recombinant DNA

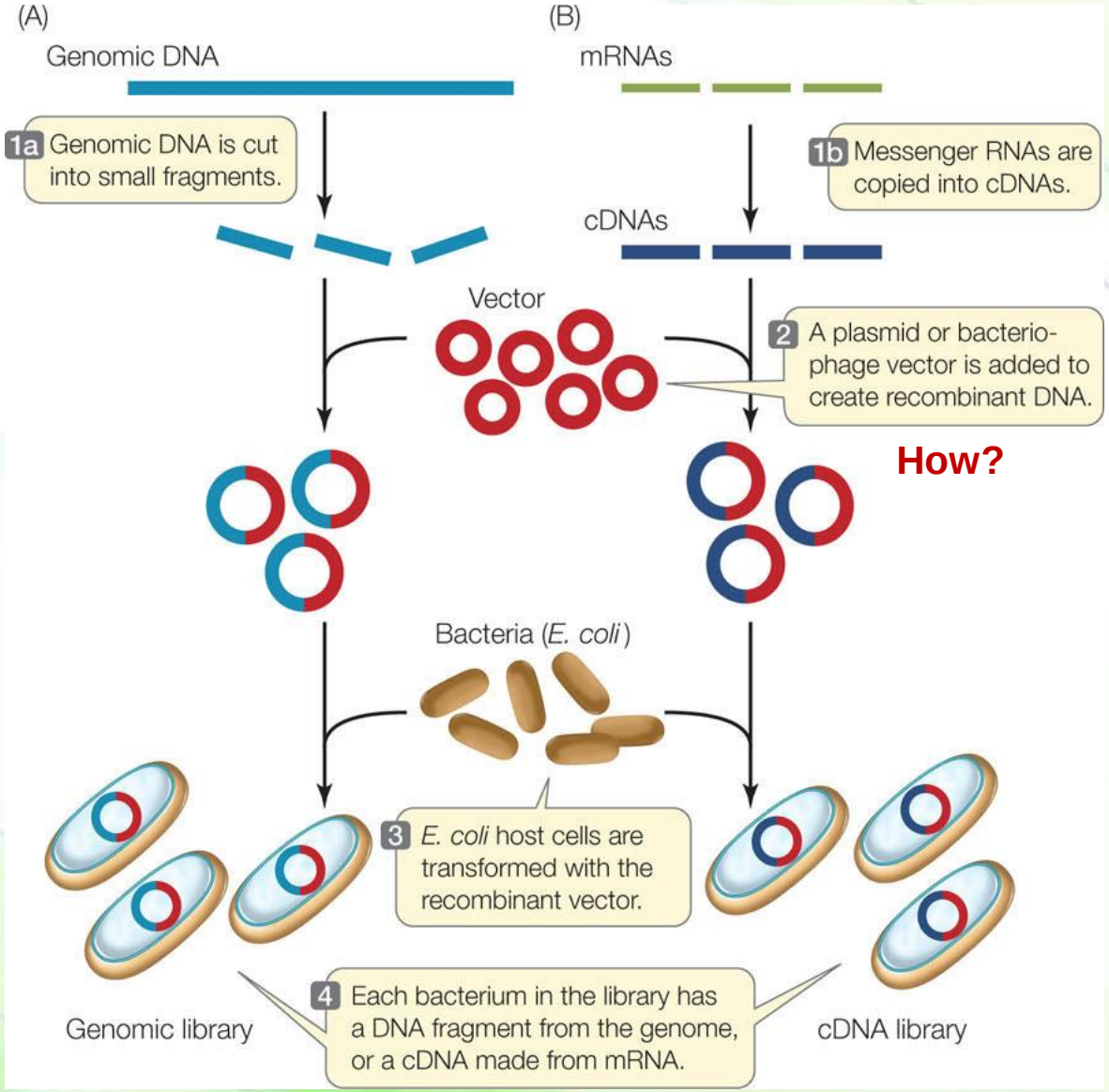
- Both DNA fragments (the DNA to be cloned and a vector) are cut by the same restriction endonuclease that makes DNA fragments with same sticky-ends hybridize (anneal) to each other, when mixed.
- A DNA ligase is added to "close" the plasmid.



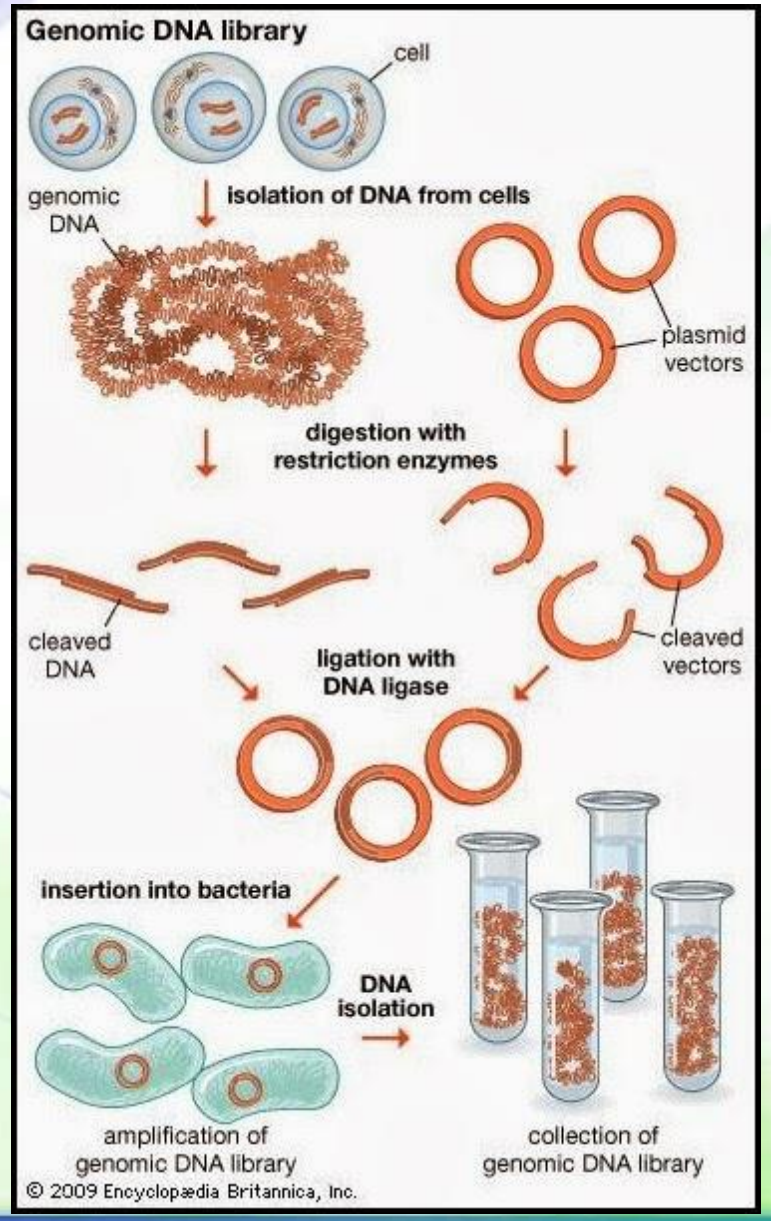

Plasmid vector
+
DNA fragment to be cloned
Enzymatically insert DNA into plasmid vector
Recombinant plasmid
Recombinant plasmid
Mix E. coli cells with plasmids in presence of $CaCl_2$
Culture on nutrient agar plates containing ampicillin
Bacterial chromosome
Transformed E. coli cell survives
Cells that 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

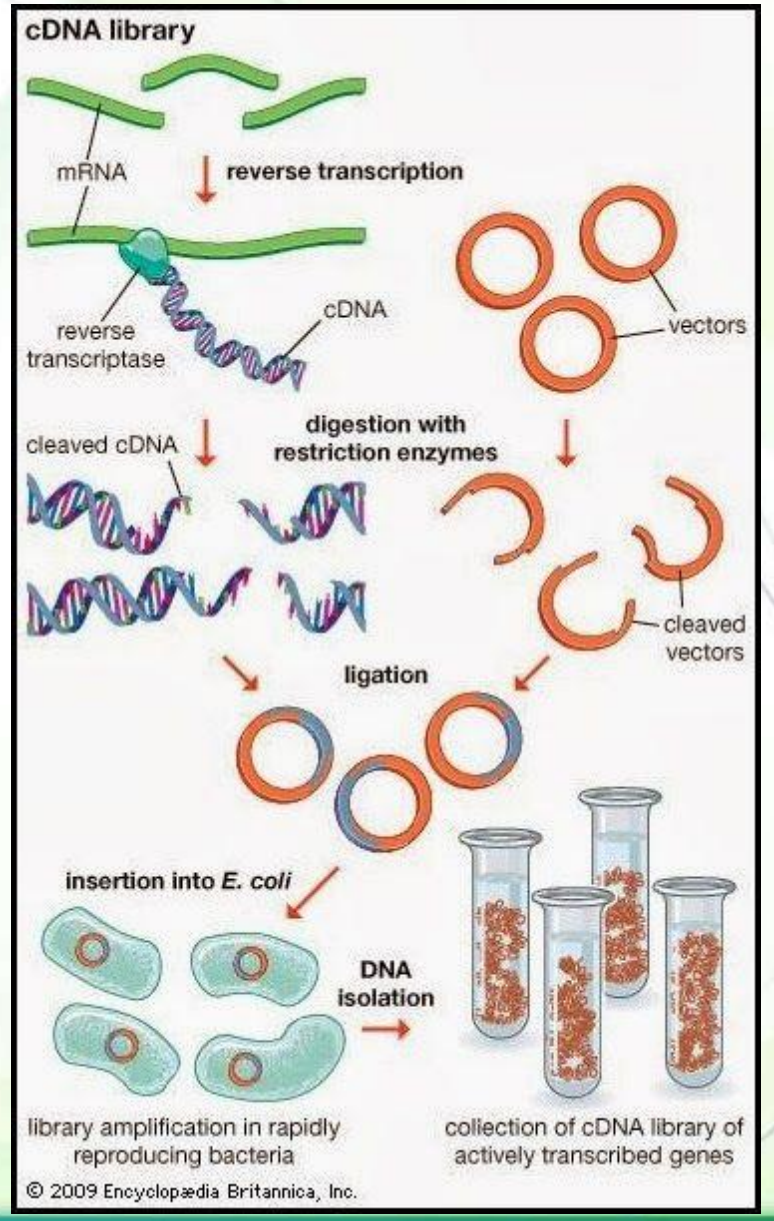
DNA libraries

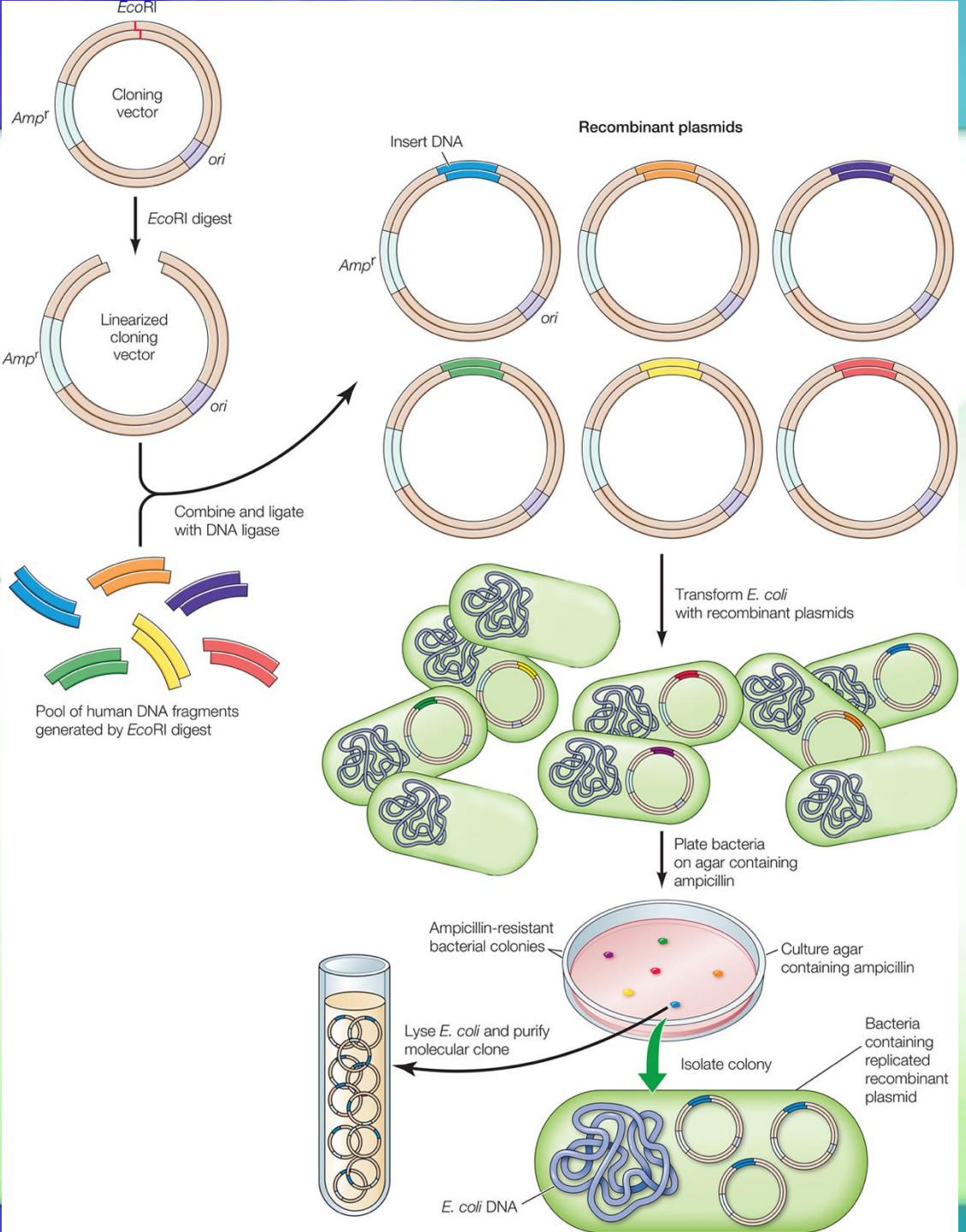
Genomic vs. cDNA libraries



Genomic vs. cDNA libraries



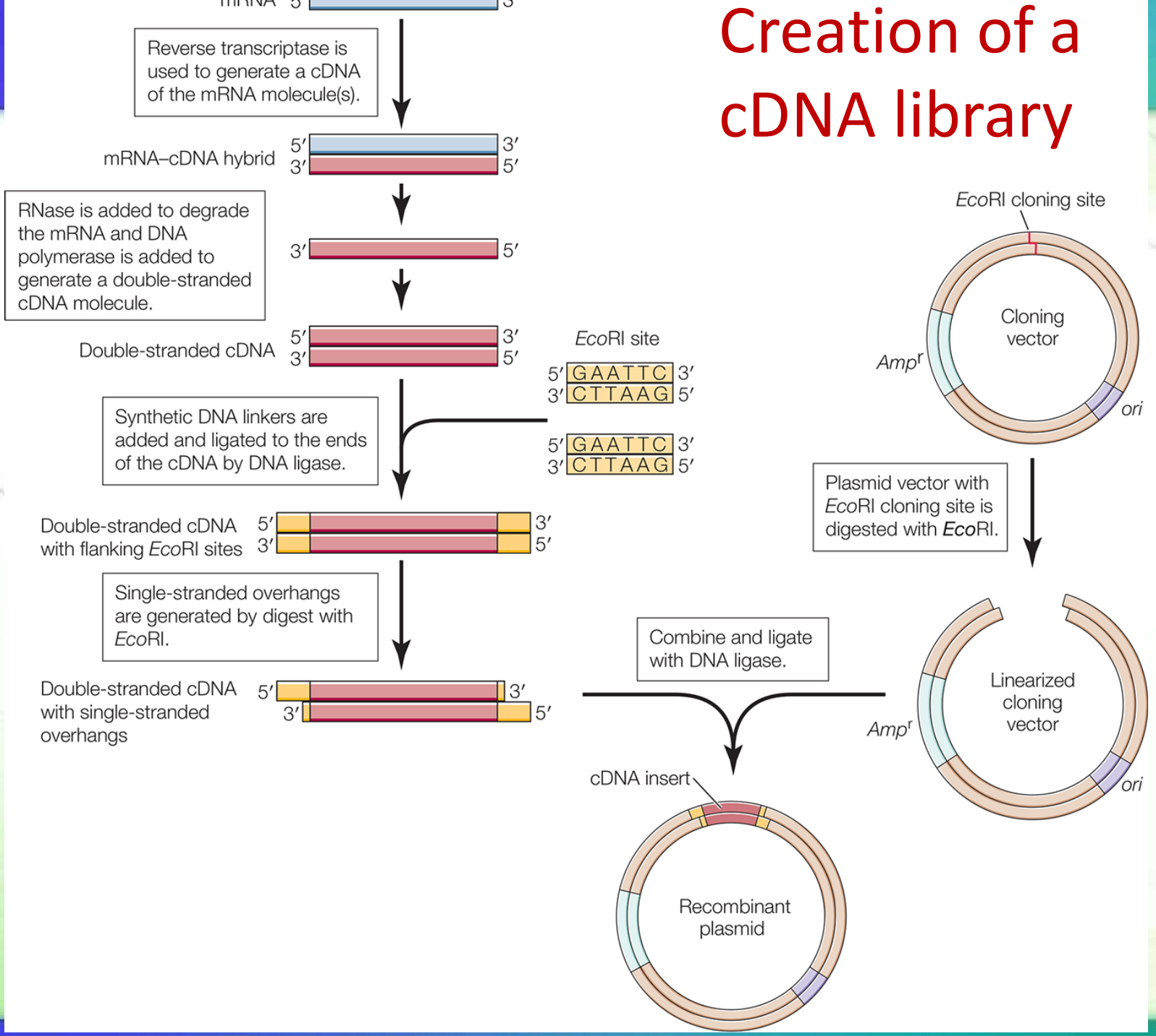




Creation of a genomic library

- A genome is cleaved by the same restriction endonuclease as that used for the vector.
- Each fragment is ligated into a vector.
- Bacterial cells are transformed with the plasmid vector with each cell having one plasmid DNA.
- Each cell can grow into millions of cells and each cell can make multiple copies of every plasmid ending up with billions of copies of plasmid with each plasmid having a specific DNA fragment.

Creation of a cDNA library

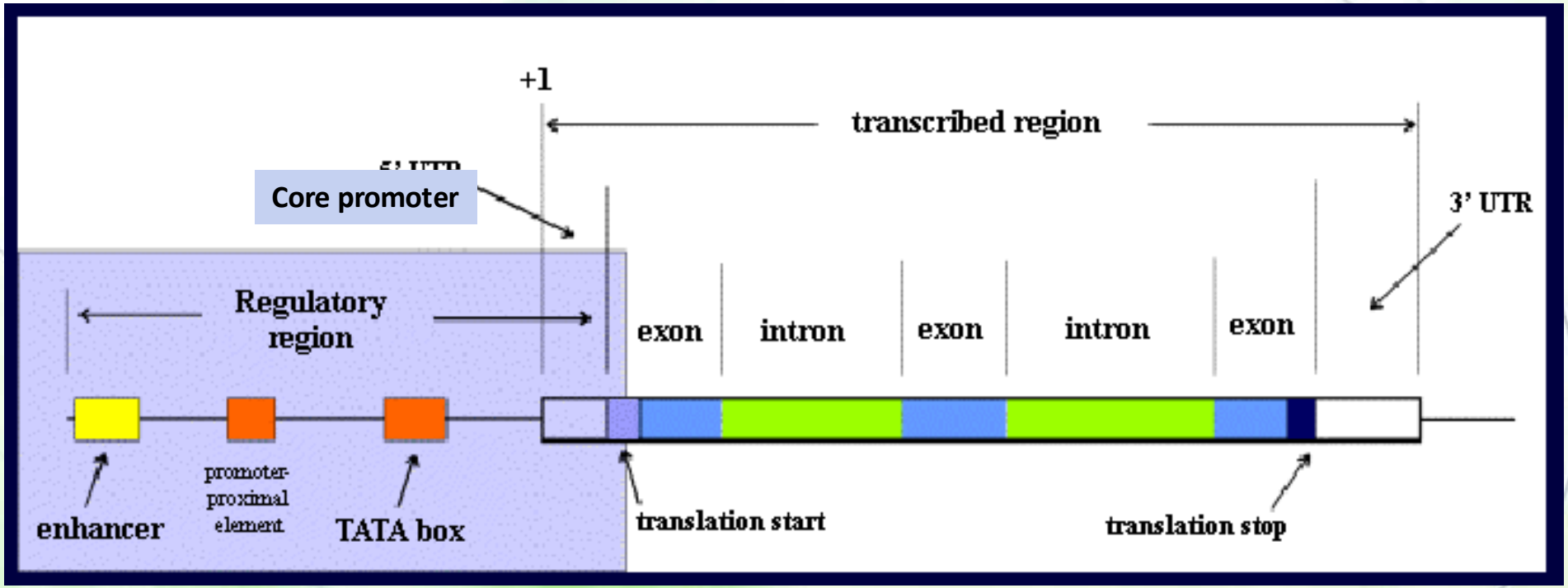


- Messenger RNAs are isolated and reverse transcribed by reverse transcriptase into a cDNA molecule that is replicated by DNA polymerase to form a double-stranded cDNA.
- Synthetic linkers containing a restriction site are ligated to the ends of the cDNAs and then digested with the restriction endonuclease to form overhangs.
- The cDNAs are then cloned into a plasmid.

Analysis of transcriptional regulatory sequences

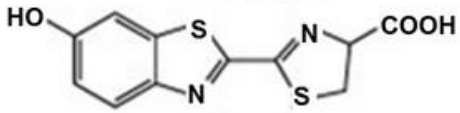
What are transcriptional regulatory sequences?

- Promoter (core promoter): A region of DNA upstream of a gene where relevant proteins (such as RNA polymerase and transcription factors) bind to initiate transcription of that gene.
- Promoter-proximal elements: Any regulatory sequence in eukaryotic DNA that is located close to (within 200 base pairs) a promoter and binds a specific protein thereby modulating transcription of the associated protein-coding gene.
- Enhancers or silencers: Regulatory DNA sequences that, when bound by specific proteins, regulate the transcription of an associated gene. They can be located near, within, after, and/or very far away from the gene, and, if lipped or relocated, are still functional.



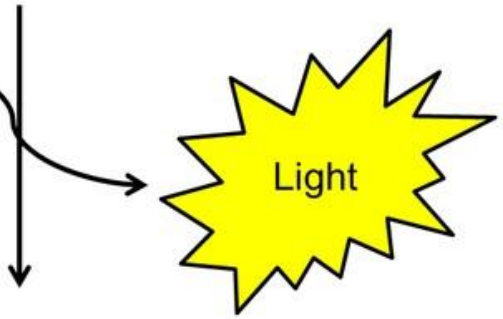
Firefly luciferase

D-Luciferin

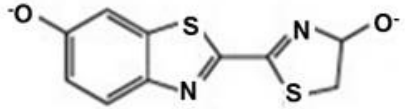


+ ATP + O_2

Firefly Luciferase + Mg^{2+}



Oxyluciferin



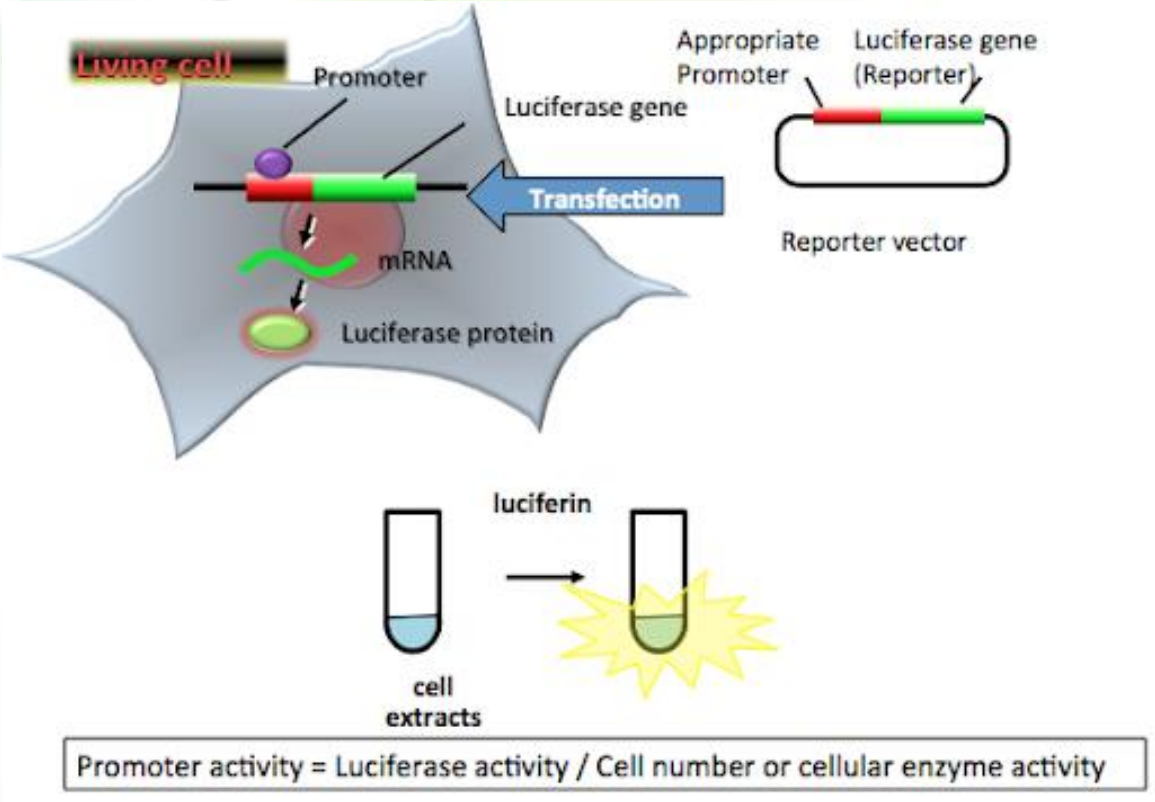
+ PPi + AMP CO_2

Luciferase reporter assay

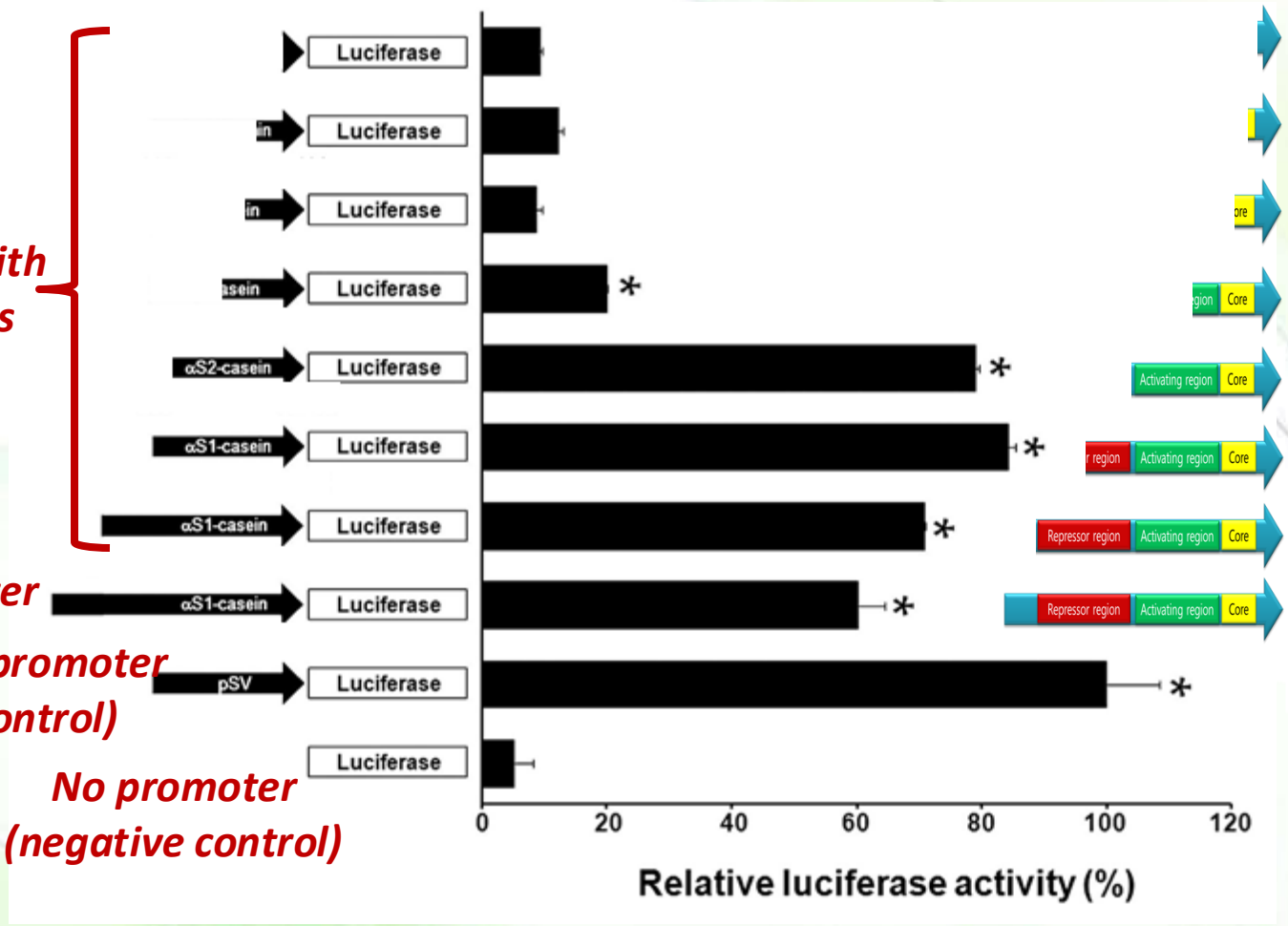
Purpose: study the activity of a gene at certain conditions or identify the function of certain regions of the promoter.

Only the regulatory region (e.g. promoter, PPE, etc.) of the gene is placed upstream of a "reporter gene" such as the luciferase gene in a plasmid.

The plasmid is transfected (inserted) into cells, and the expression level of luciferase (instead of the original gene itself) is measured.



Example


The promoter with deleted regions
The complete promoter
Any good promoter (positive control)
No promoter (negative control)
Luciferase
αS2-casein
αS1-casein
pSV
Activating region
Core
Repressor region
0
20
40
60
80
100
120
Relative luciferase activity (%)


Repressor region
Activating region
Core