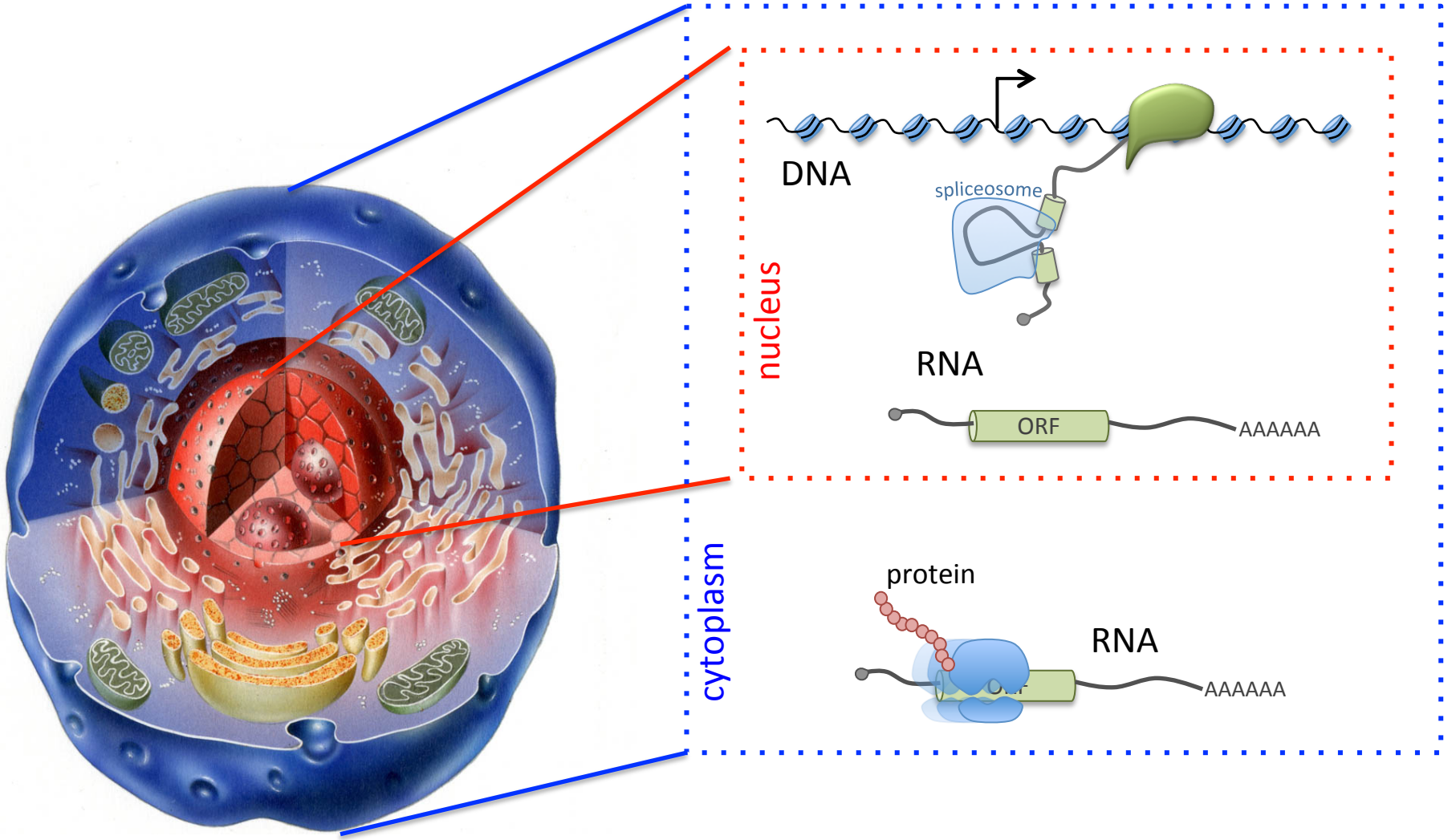
Nucleic acid subcellular localization



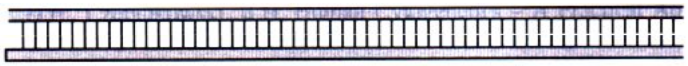
Eukaryotic cell

1. DNA elettrophoresis for separating DNA fragments based on lenght (PCR products….)

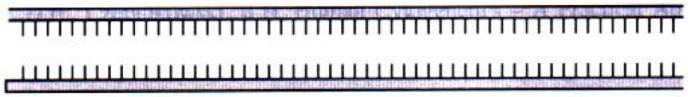
2. DNA elettrophoresis and blot for analysing the presence and the structure of a specific gene (Southern blot)

3. RNA elettrophoresis and blot for analysing gene expression or to check the presence of a specific RNA molecules in a cell type/ tissue (Northern blot

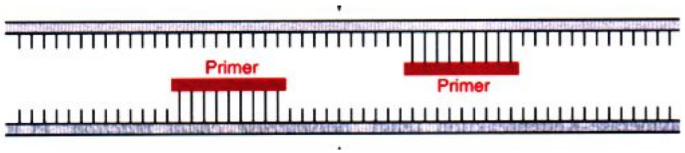
I cycle…



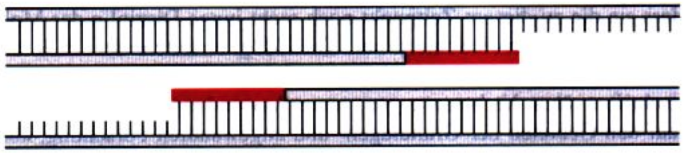
dsDNA



DENATURATION

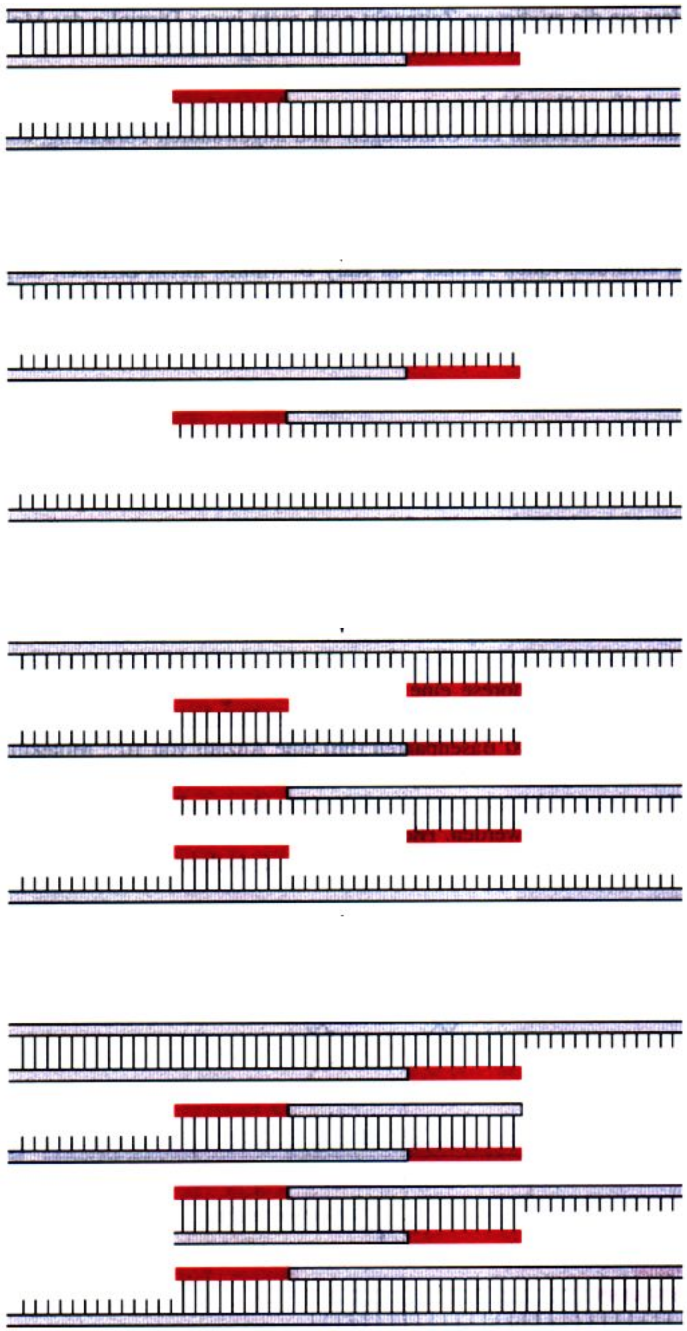


ANNEALING



SYNTHESIS

..II cycle



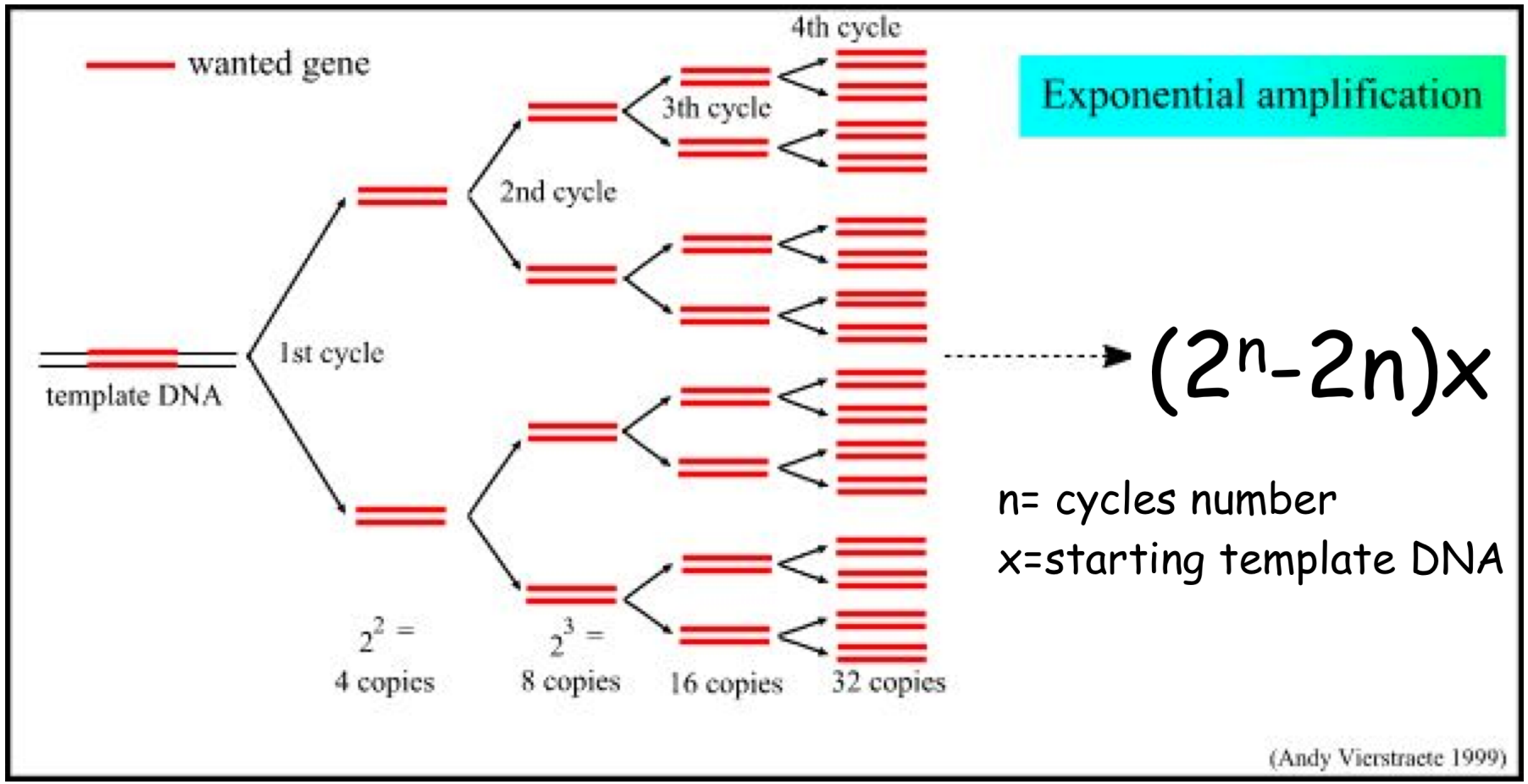
dsDNA

DENATURATION

ANNEALING

SYNTHESIS

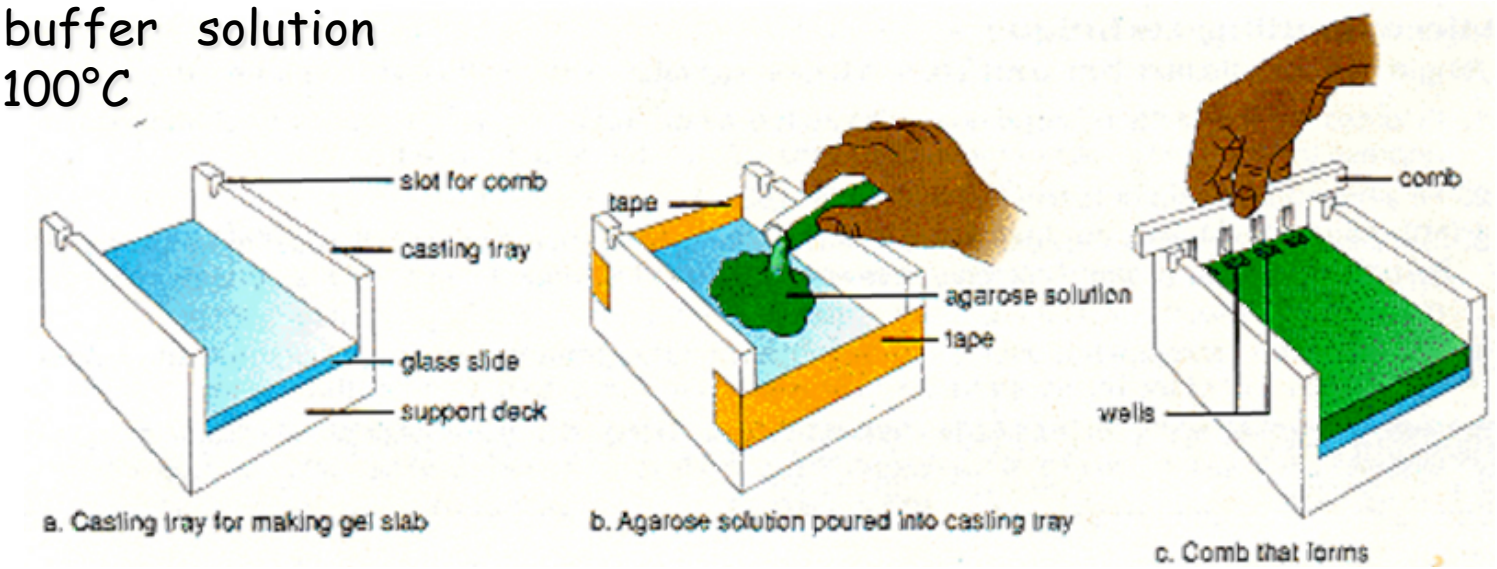
...additional cycles

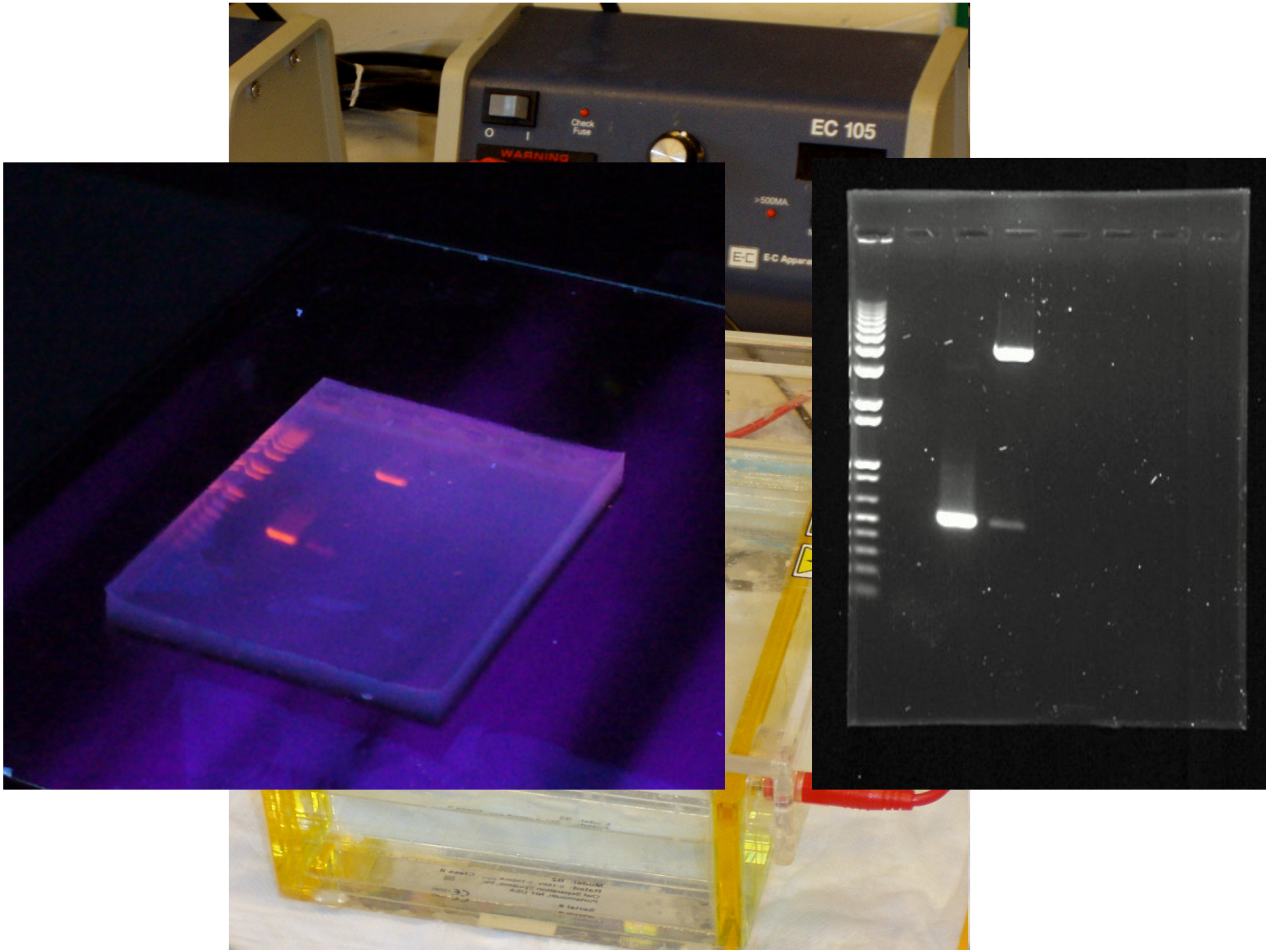


How to prepare agarose gel:

dissolve the agarose powder in a buffer solution at 100°C

add ethidium bromide




EC 105
Check Fuse
WARNING
>500MA

1. DNA elettrophoresis for separating DNA fragments based on lenght (PCR products….)

2. DNA elettrophoresis and blot for analysing the presence and the structure of a specific gene in one genome (Southern blot)

3. RNA elettrophoresis and blot for analysing gene expression or to check the presence of a specific RNA molecules in a cell type/ tissue (Northern blot)

Southern blot

- **Procedure**

- Genomic DNA isolation

- Digestion with restriction enzymes for generating short fragments (from hundreds to some Kilo bases)

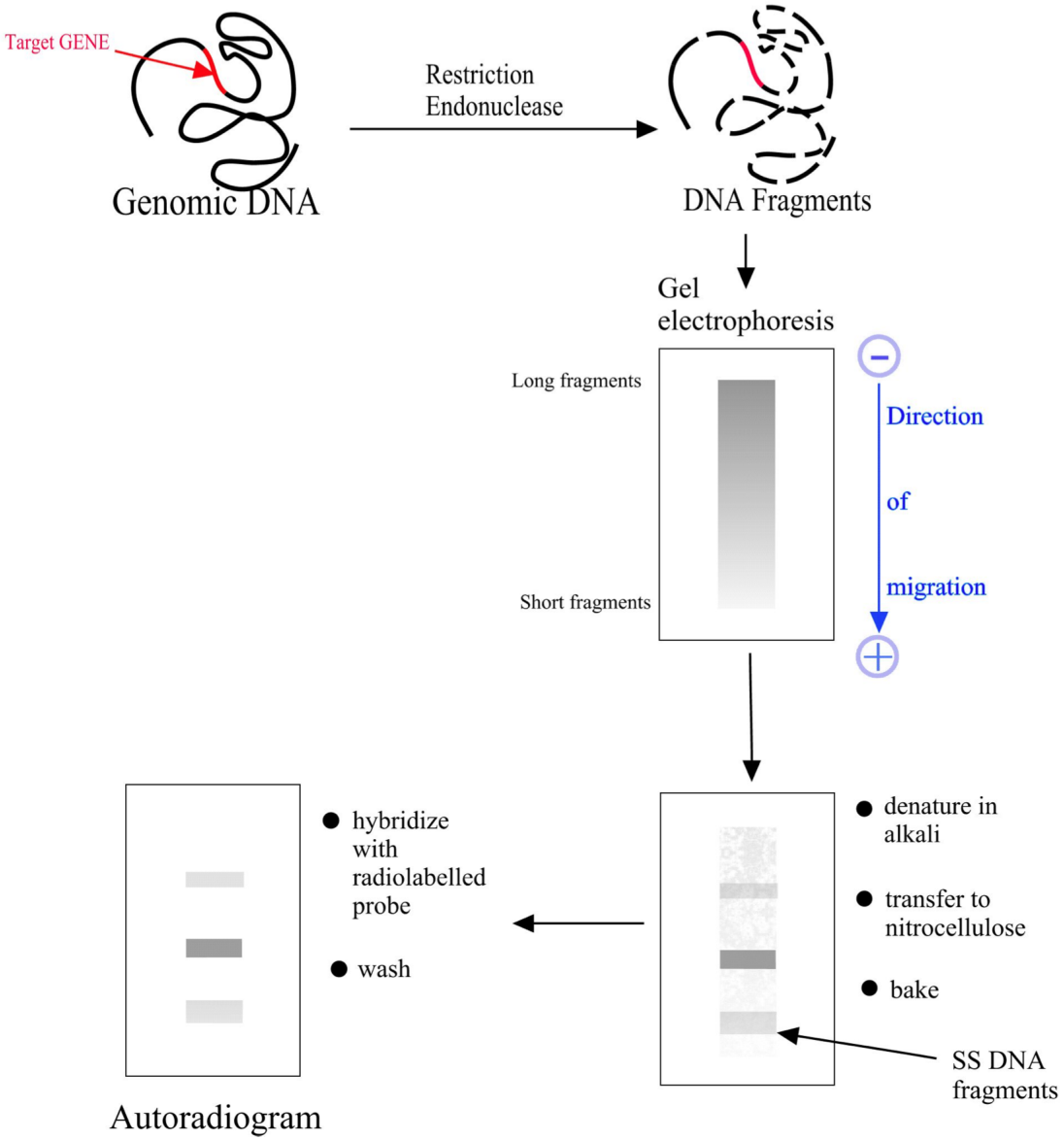
- Electrophoresis of DNA fragments on agarose gel

- Denaturation of complementary DNA filaments

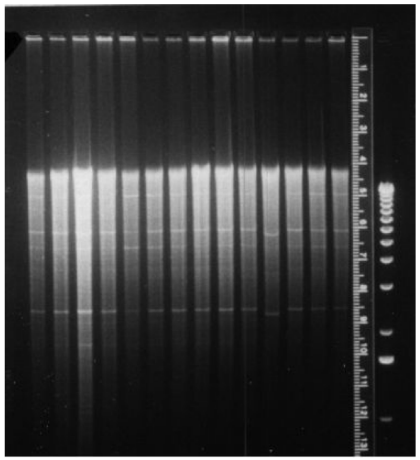
- Transfer the DNA from the gel to the membrane

- Hybridation with specific labelled probe

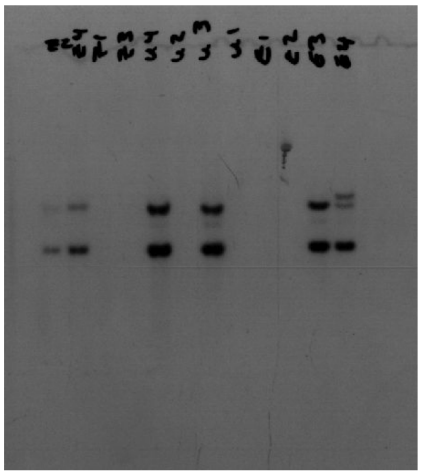
Southern blot



Gel



X-ray film



1. DNA elettrophoresis for separating DNA fragments based on lenght (PCR product….)

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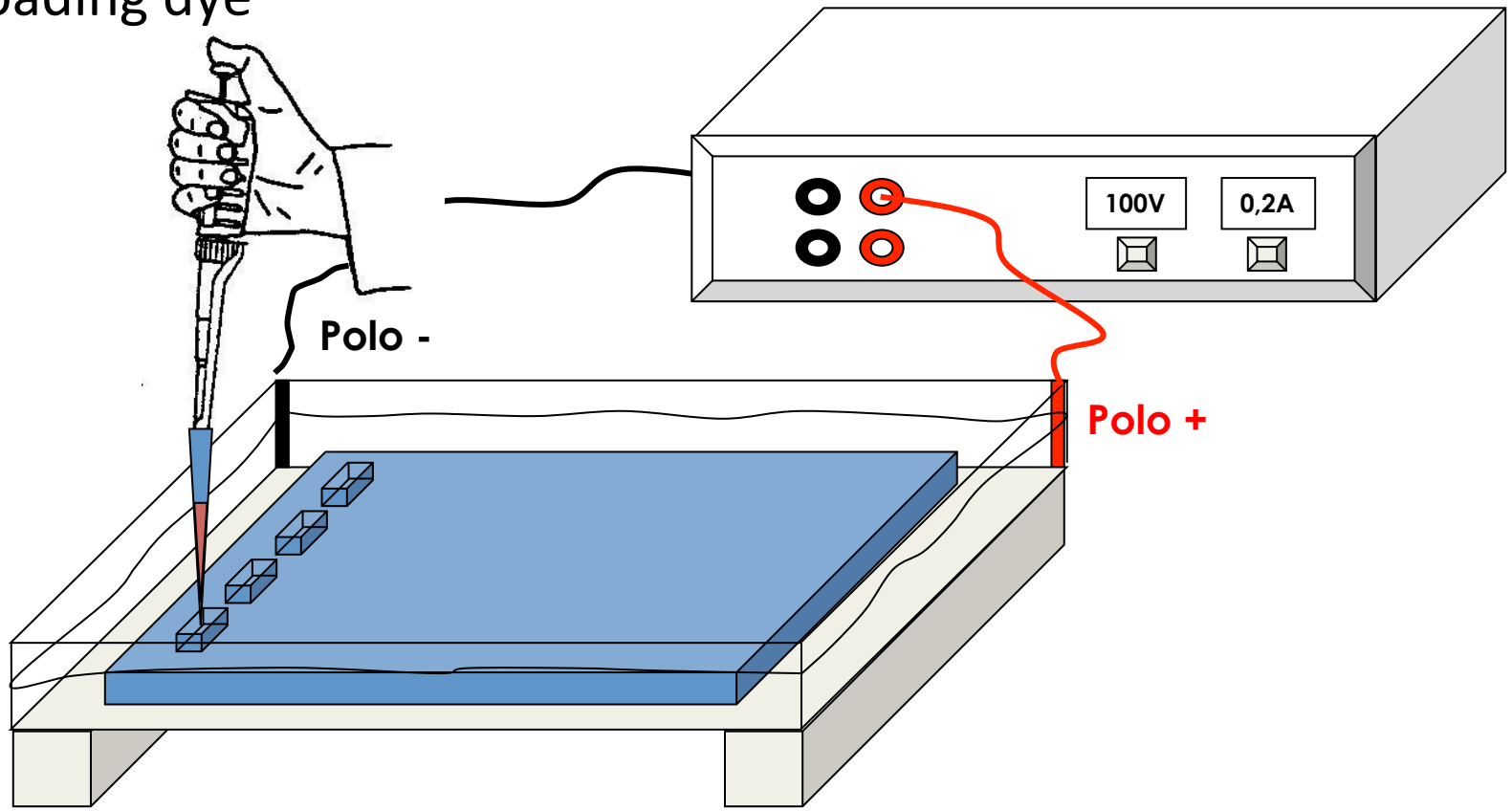
Northern blot

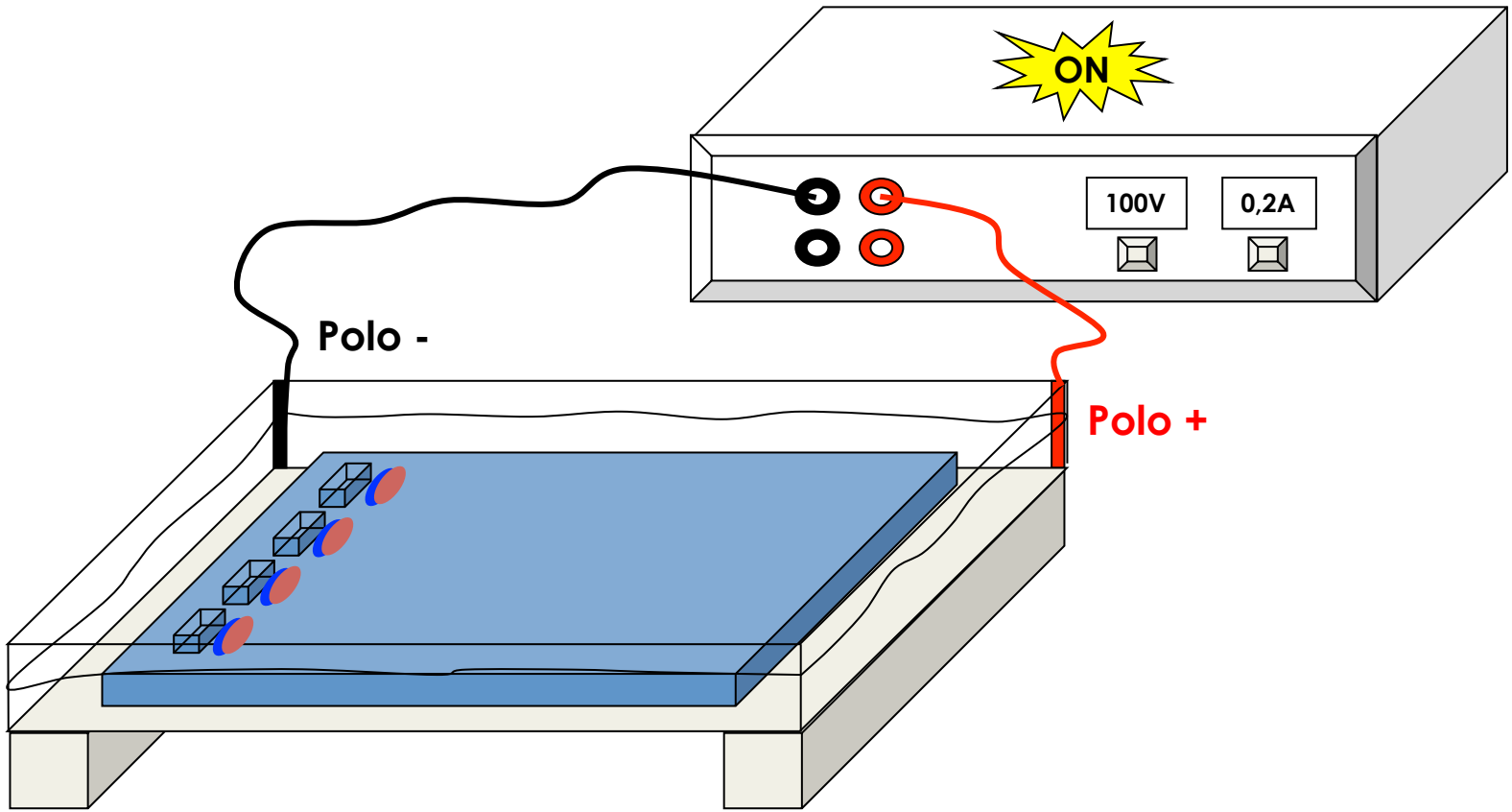
- **Procedure**
  - RNA extraction
  - Remove secondary structures 
  - Electrophoresis (agarose or acrylamide)
  - Transfer the RNA on membrane (nylon)
  - hybridization with labelled complementary probe

+ Loading dye

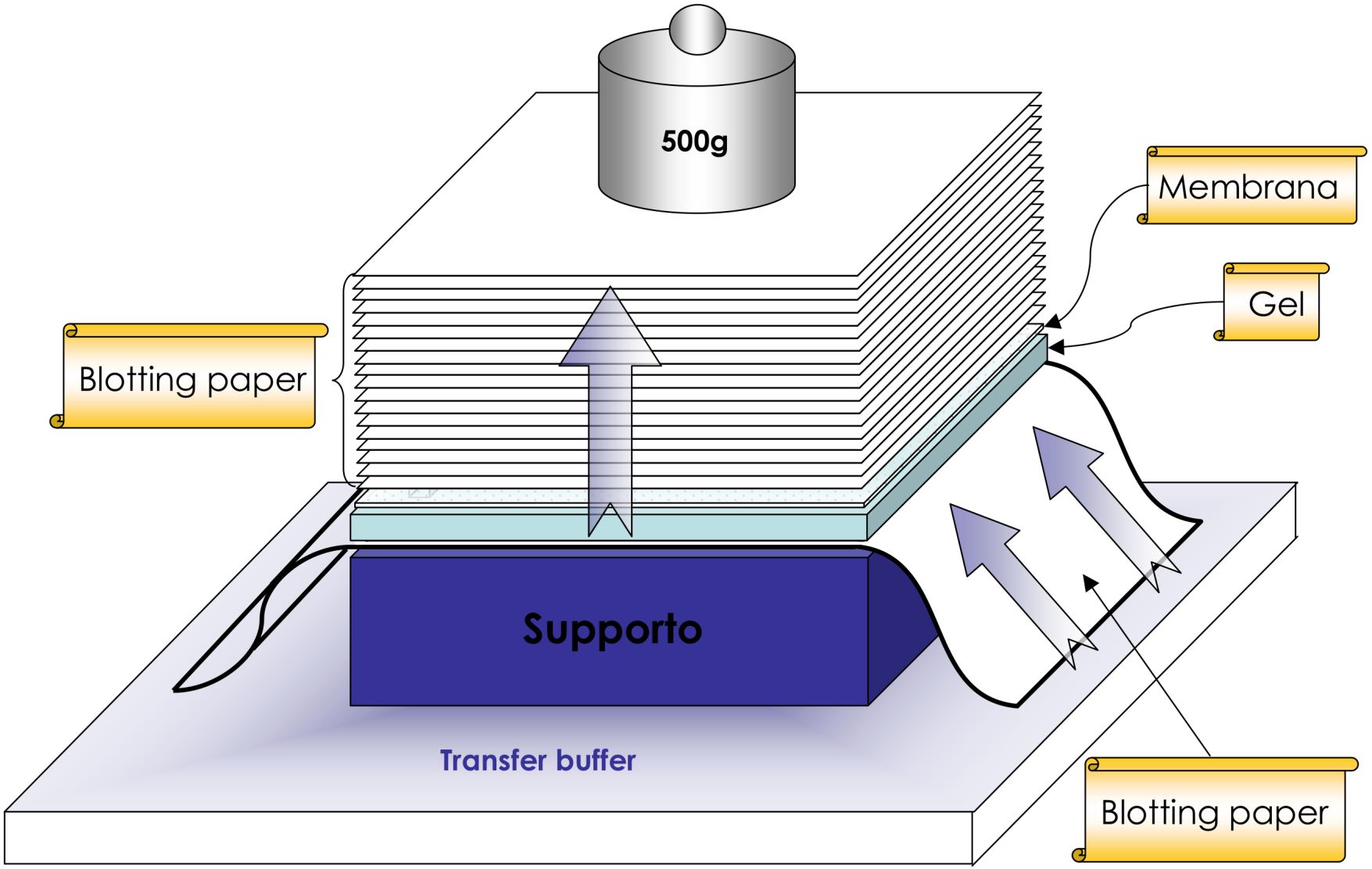


Loading dye helps the sample loading in the well

It contains glycerol, bromophenol Blu and xilencianol Blu that migrate in the gel at different speeds.

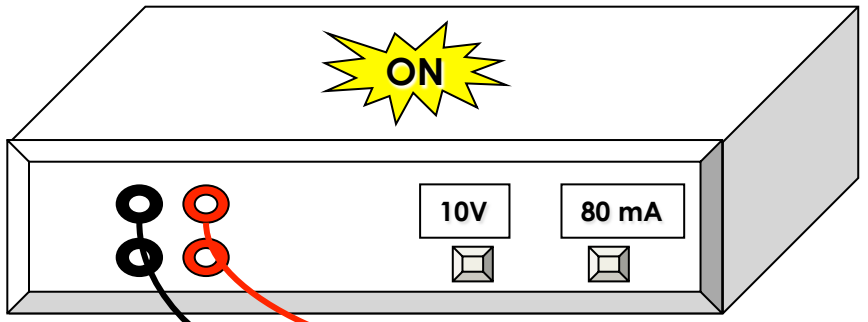

ON
100V
0,2A
Polo -
Polo +

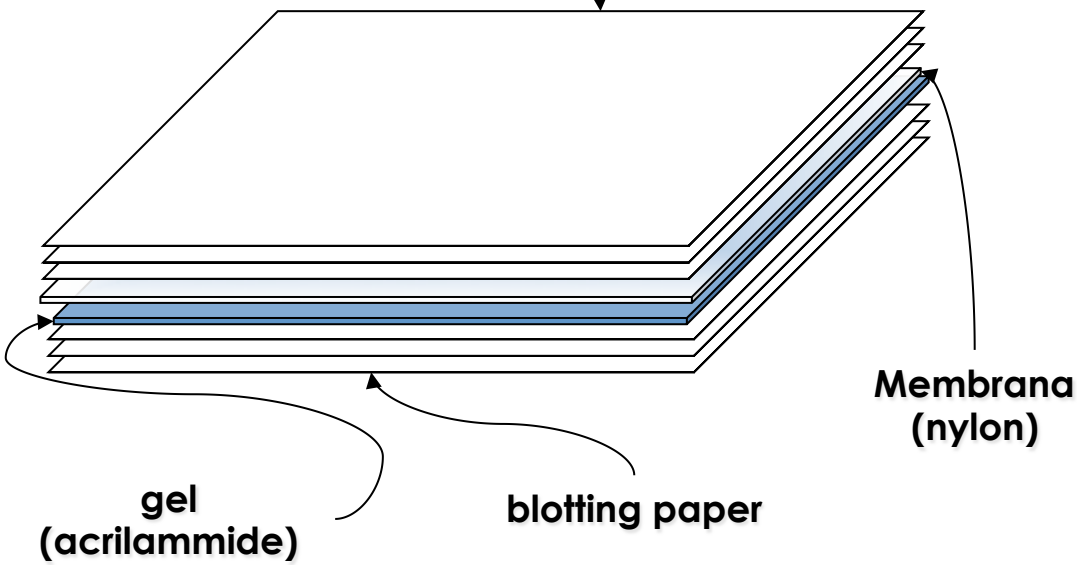
BLOT: transfer the nuclei acids from the gel to the membrane by diffusion (for agarose gel)

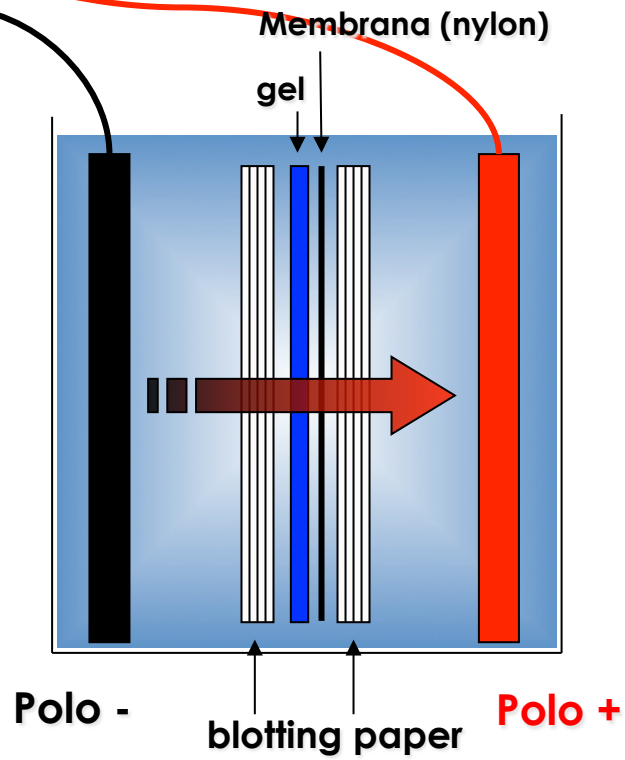


Elettroblot

(for acrylamide gel)

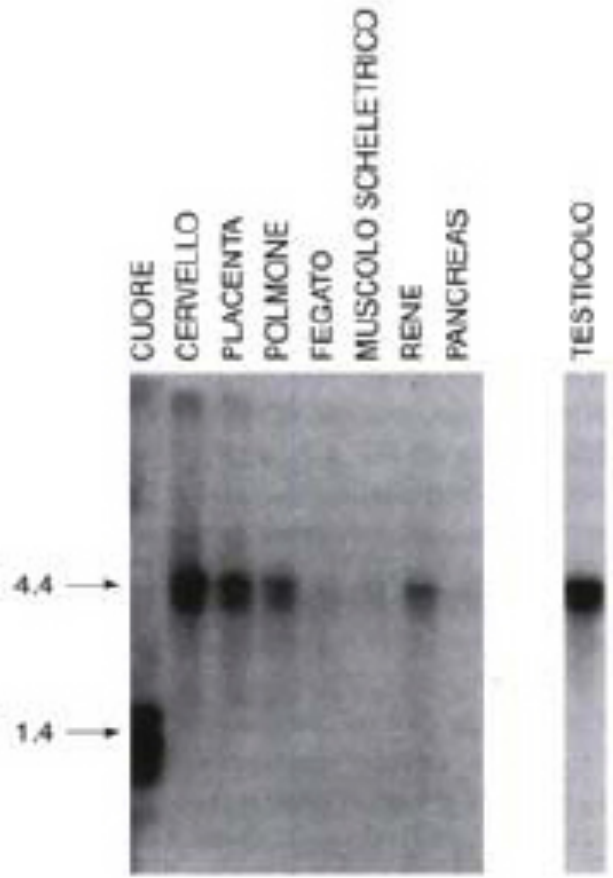






Applications

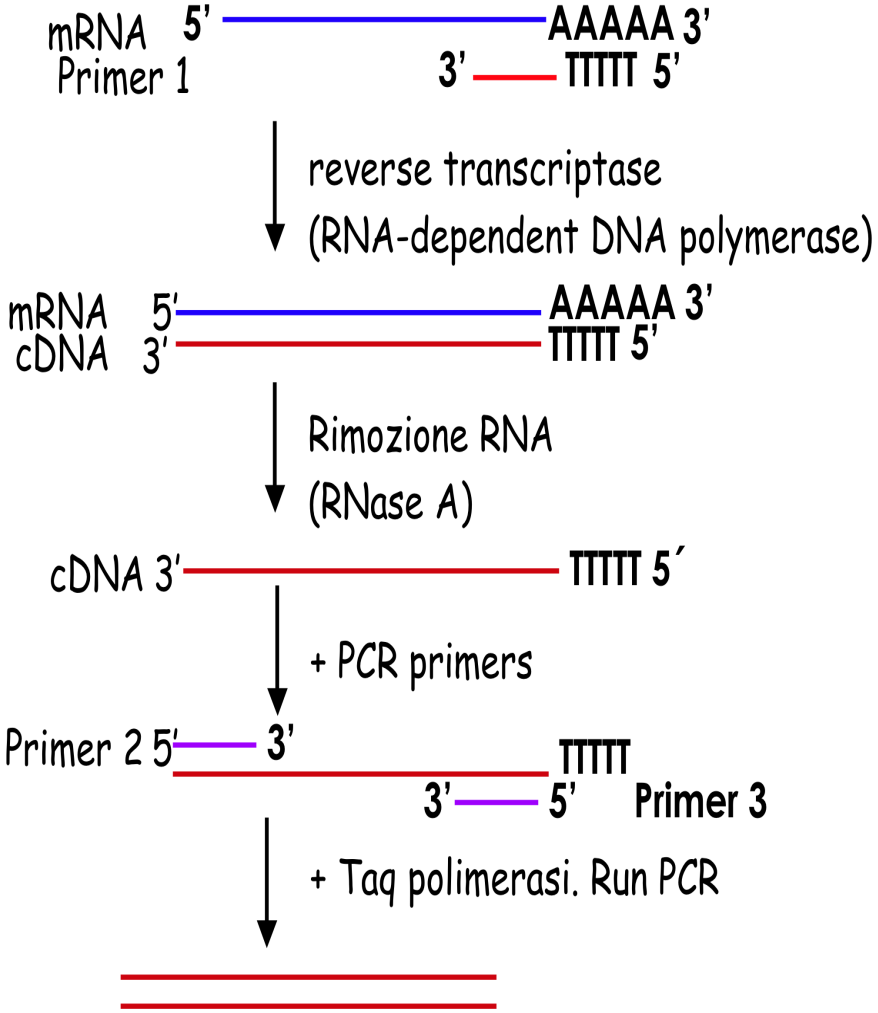
1. In which tissue is the RNA expressed?

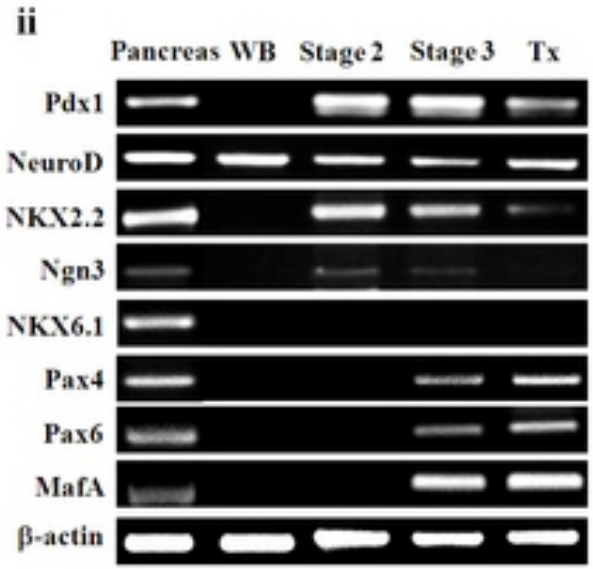


Many information will be obtained:

1- expression levels in many tissues
2- lenght of produced RNA

gene expression can be studied by rt-PCR





Molecular cloning

Milestones for genetic ingeneering

1967:
Discovery of
DNA ligase

1968:
Discovery of
restriction enzymes

1972:
Advancement of DNA
transfer and use of the
plasmid

Molecular Cloning

The usual procedure in a gene cloning experiment is to place a foreign gene into bacterial cells, separate individual cells, and grow colonies from each of them. All the cells in each colony are identical and will contain the foreign gene.

Thus, as long as we ensure that the foreign gene can replicate, we can clone the gene by cloning its bacterial host. Stanley Cohen, Herbert Boyer, and their colleagues performed the first cloning experiment in 1973.

Even vertebrates can be cloned.
A sheep named Dolly was cloned in Scotland in 1997 using an enucleate egg and a nucleus from an adult sheep mammary gland. Identical twins constitute a natural clone.

Molecular cloning

The **molecular cloning**, that is the isolation and amplification of a specific DNA sequence, for instance the sequence corresponding to a gene, is the base of the genetic ingeneering..

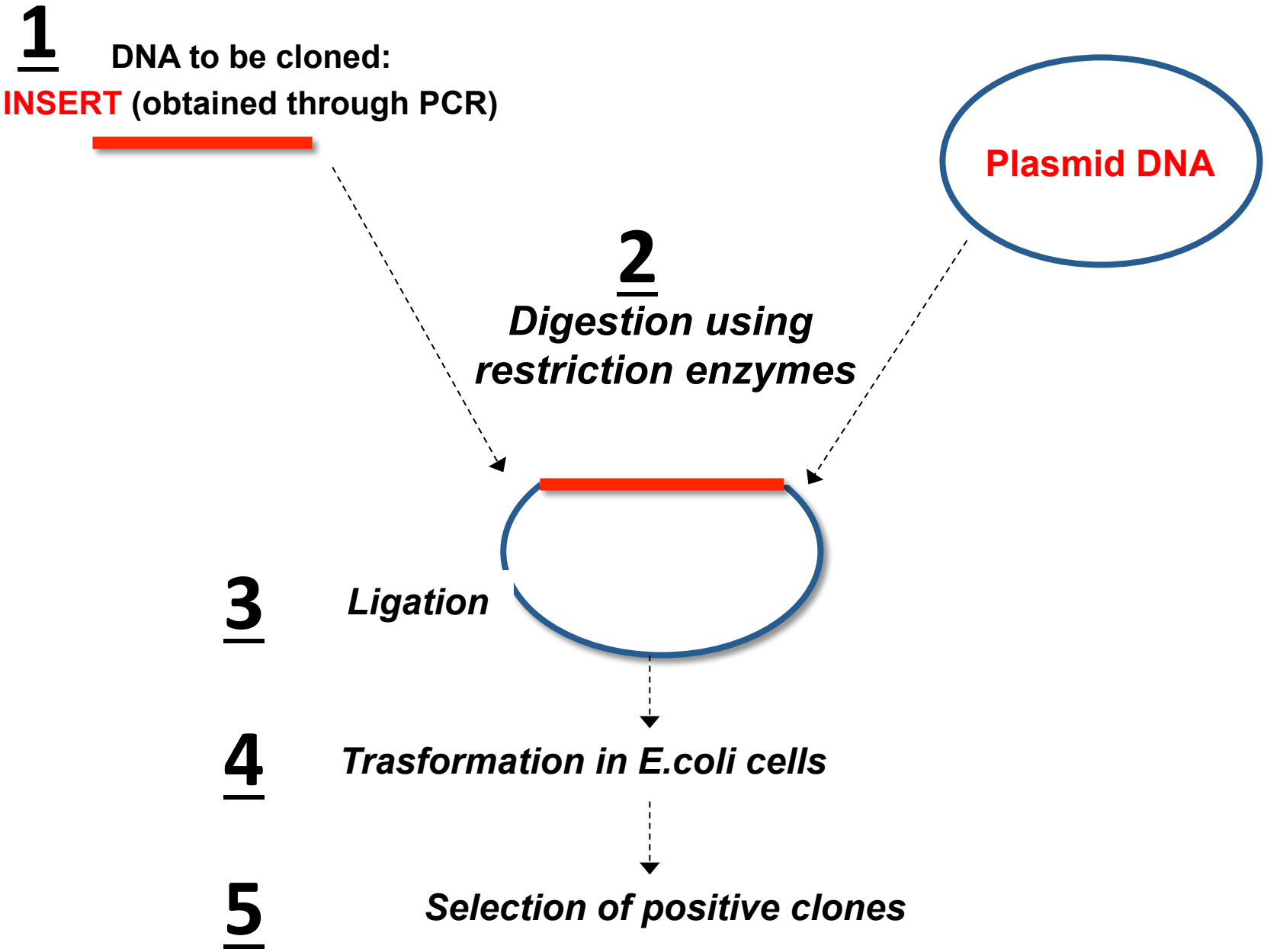
The **molecular cloning** consists of 4 phases:

1) *Gene Isolation*

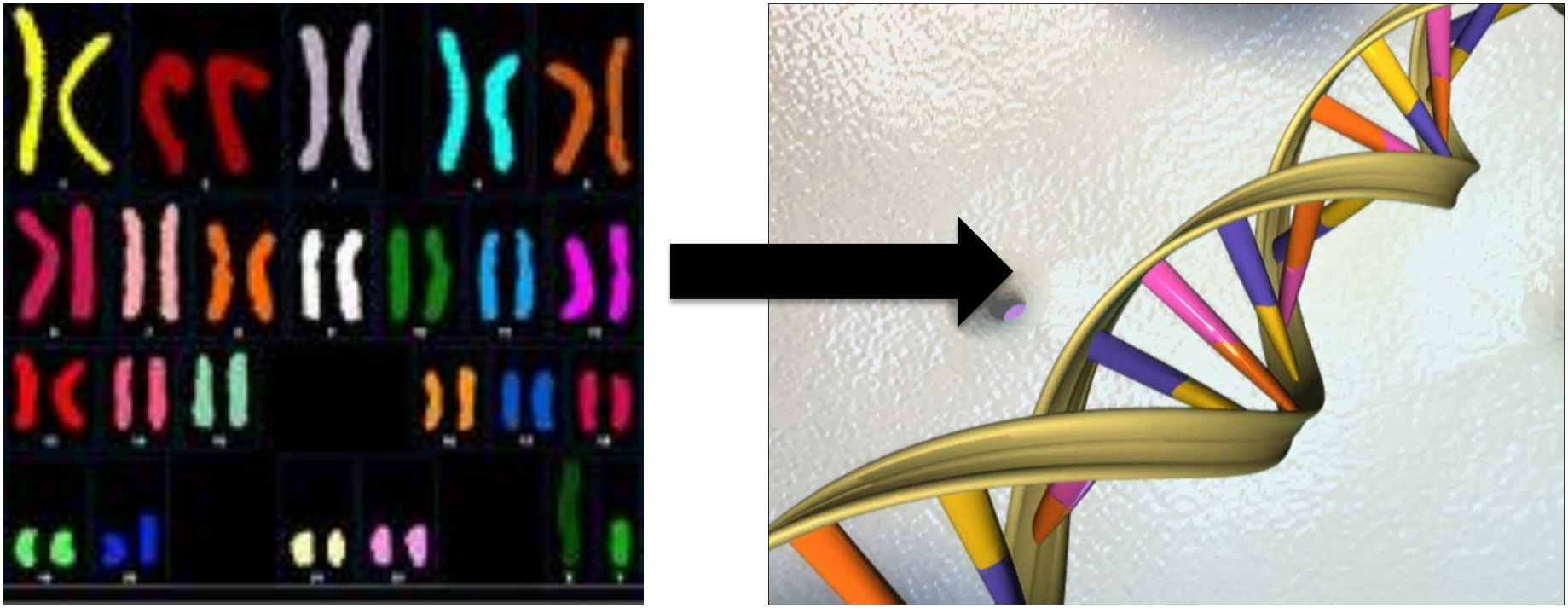
2) *Insertion in a vector*

3) *Insertion in a host organism*

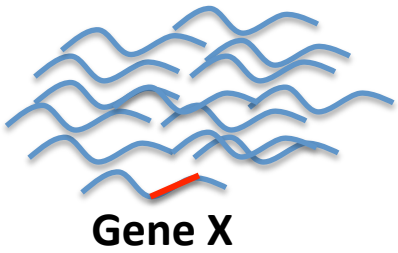
4) *Selection of the positive clones*


1
DNA to be cloned:
INSERT (obtained through PCR)
Plasmid DNA
2
Digestion using
restriction enzymes
3
Ligation
4
Trasformation in E.coli cells
5
Selection of positive clones

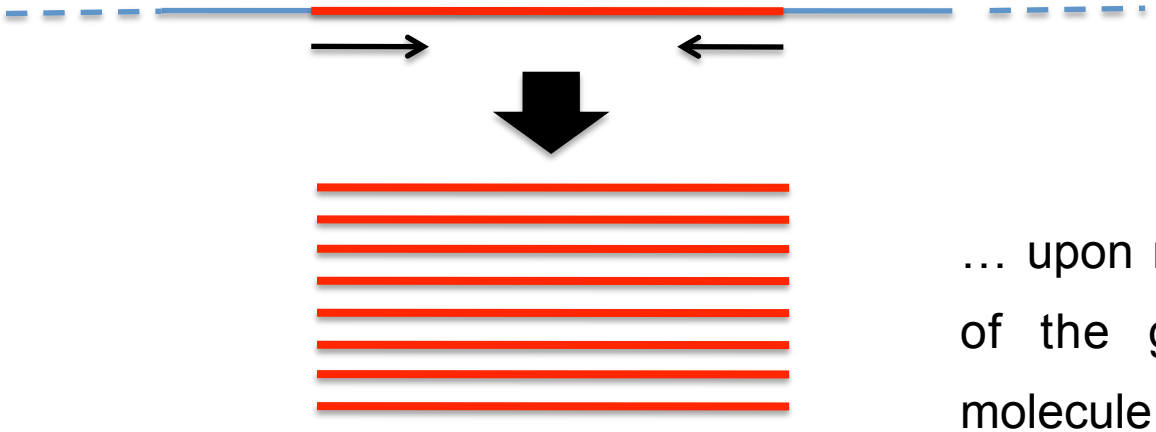
Step 1: How to select a specific DNA seuquece from bilion of different sequences?



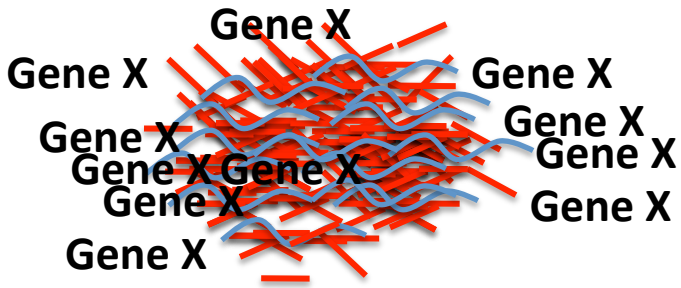
PCR for selecting specific sequences



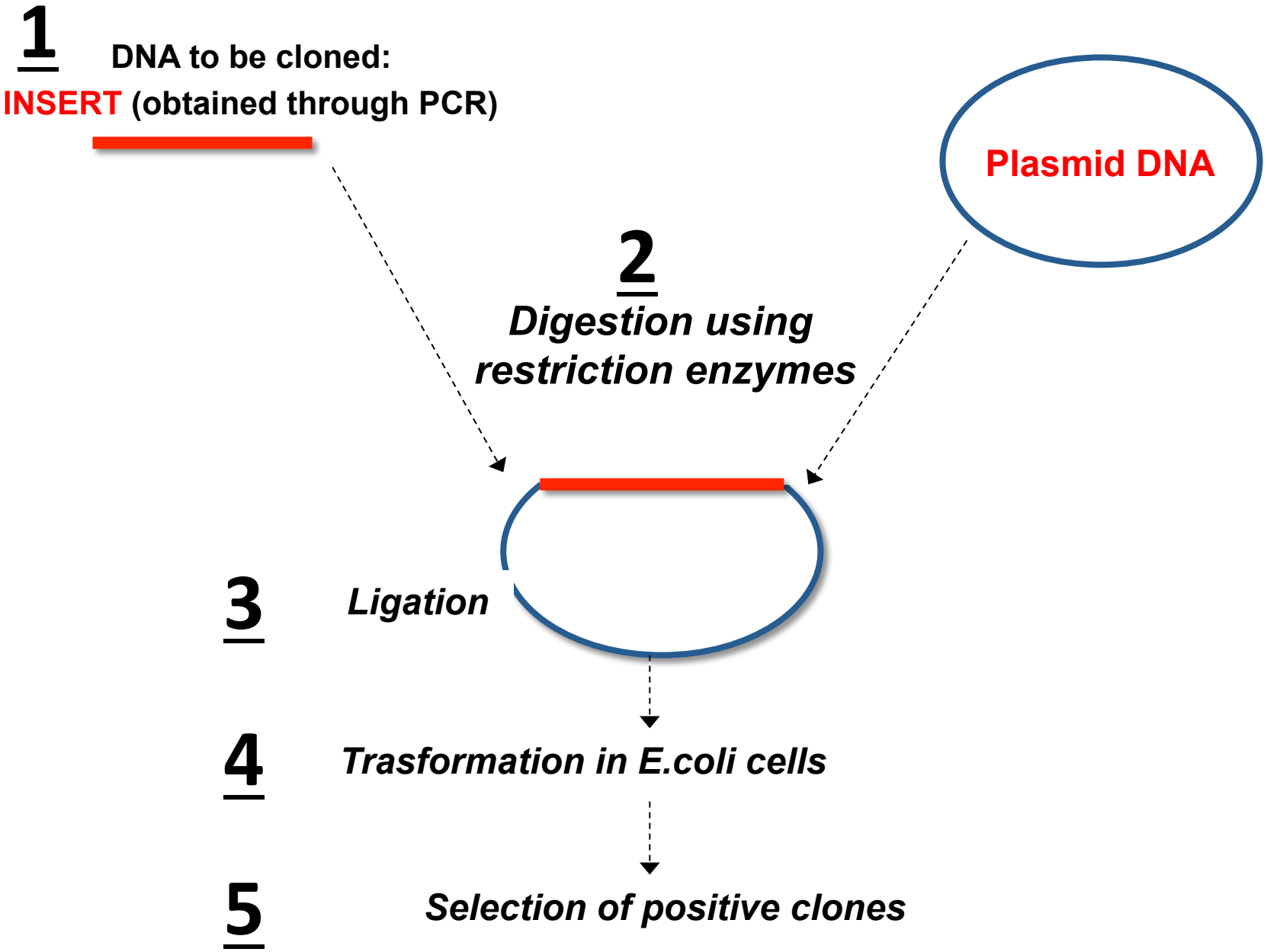
The gene X is only one of many equences that form the genome



… upon n cycles of PCR 2^n copies of the gene X for each initial molecule will be obtained….



…a huge enrichment of the selected sequence will be obtained.!


1
DNA to be cloned:
INSERT (obtained through PCR)
Plasmid DNA
2
Digestion using
restriction enzymes
3
Ligation
4
Trasformation in E.coli cells
5
Selection of positive clones

Step 2: Restriction enzyme activity

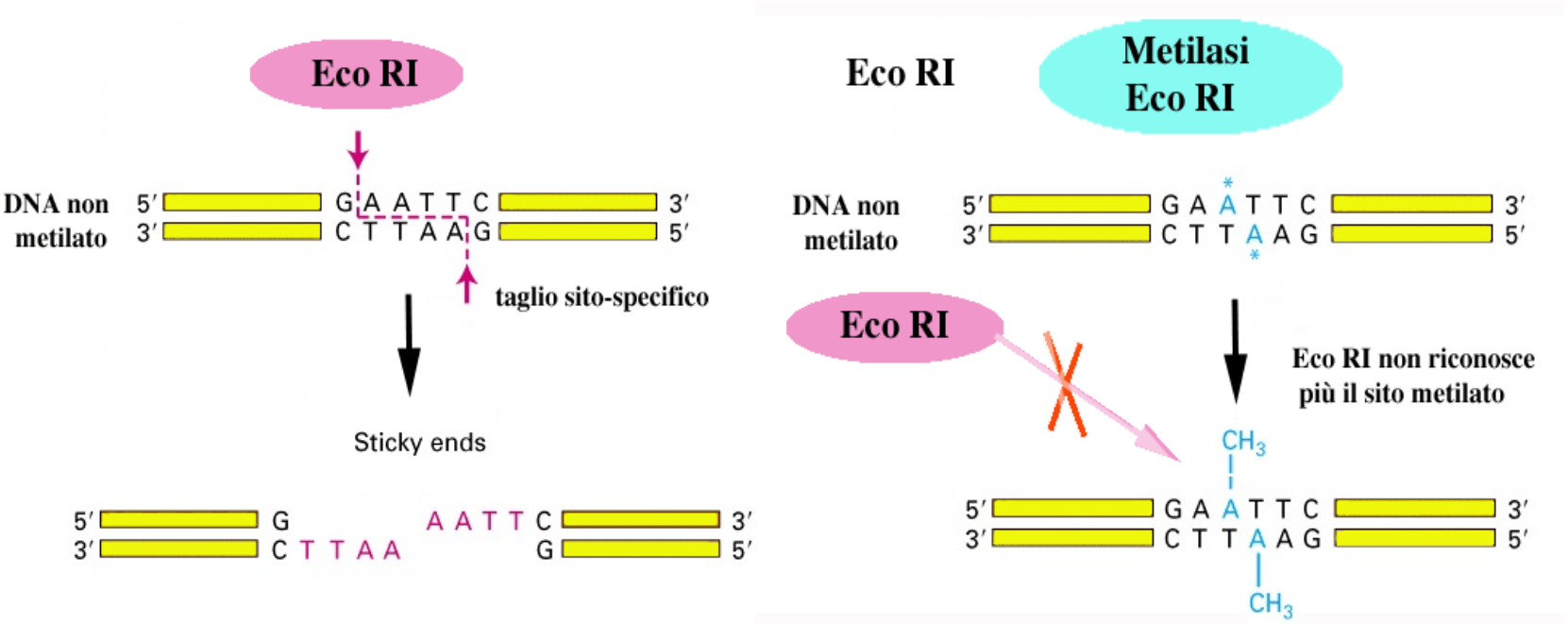
Stewart Linn and Werner Arber discovered restriction endonucleases in E. coli in the late 1960s. These enzymes get their name from the fact that they prevent invasion by foreign DNA, such as viral DNA, by cutting it up. Thus, they "restrict" the host range of the virus. Furthermore, they cut at sites within the foreign DNA, rather than chewing it away at the ends, so we call them endonucleases

Arber, Nathans e Smith, get the Nobel prize for Fisiology and Medicine

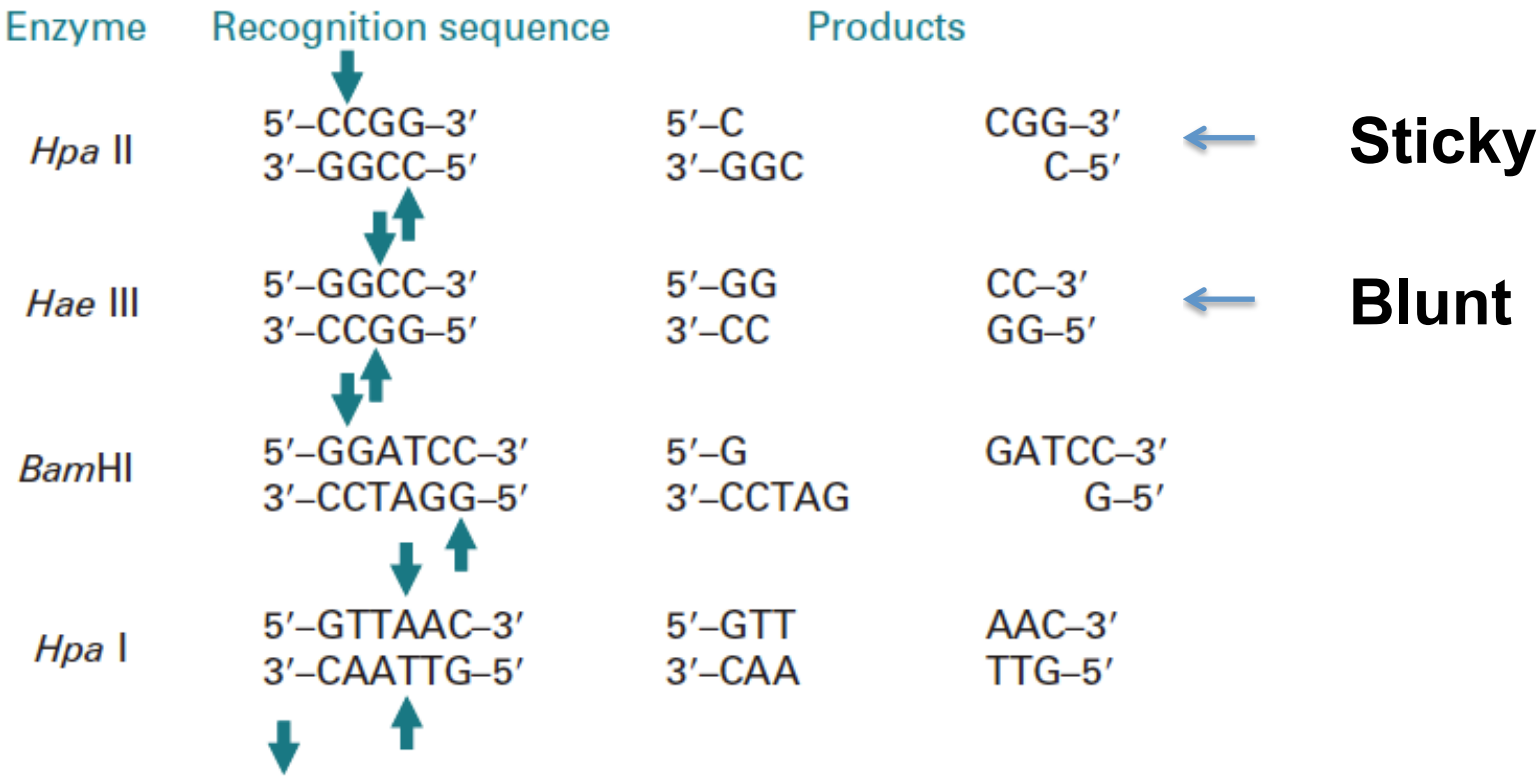
Restriction enzymes are site specific endonucleases able to recognise short target sequences usually palindromic that is read the same in both directions on each strand.

For instance:

Bacterial protection is afforded by the use of a restriction modification system based on differential methylation of host DNA, so as to distinguish it from foreign DNA such as viruses. Therefore only the exogenous DNA will be recognised and cut by restriction enzymes because not methylated.

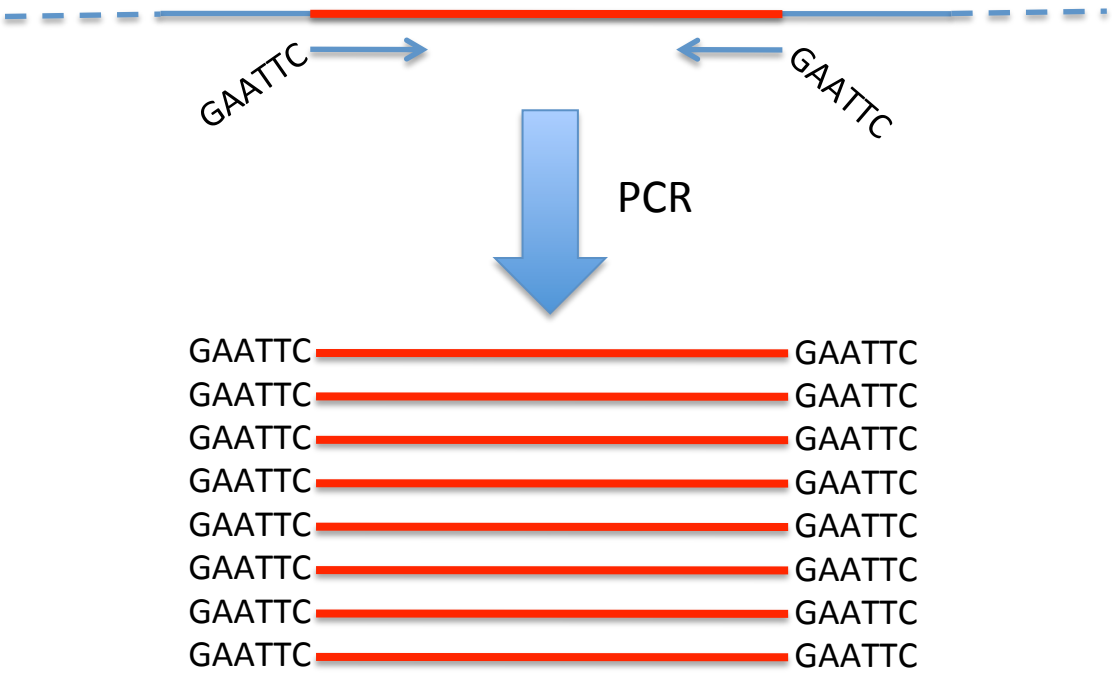


When cleave they can leave a blunt-ended or sticky-ended fragment depending on the particular enzyme used.



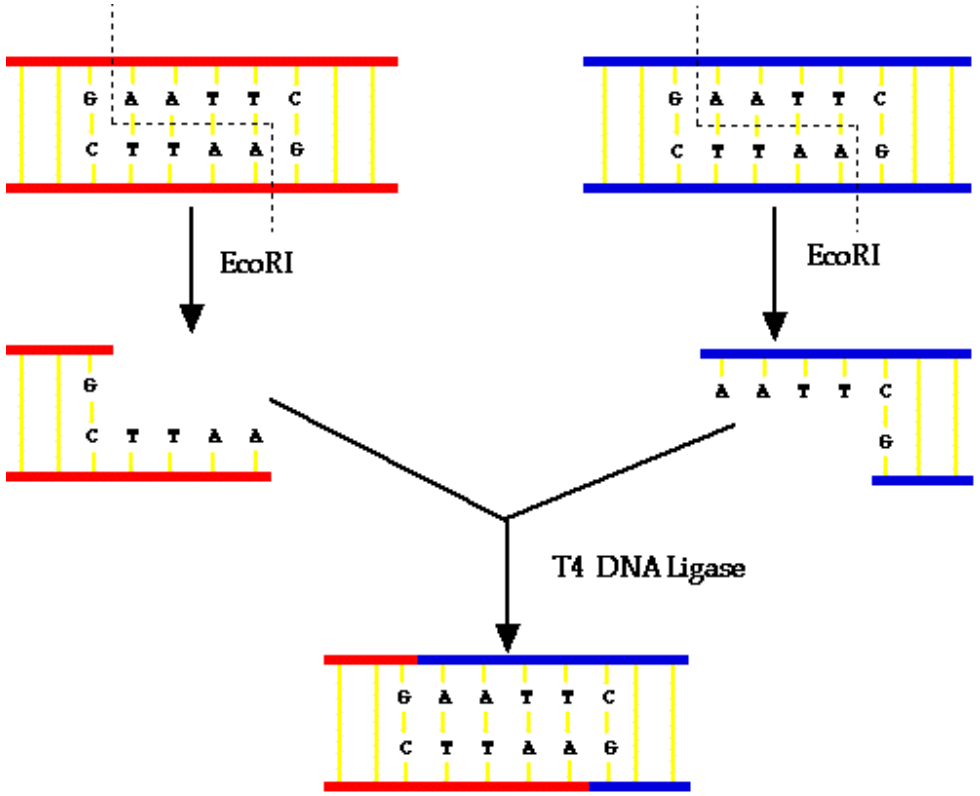
Note: after cleavage the two ends (from different molecules cleaved with the same enzyme or from the same molecule) can be joined again and the restriction site is re-formed.

DNA fragments obtained by PCR can be cleaved by restriction enzymes if PCR primers contain an additional sequence at the 5'end corresponding to the sequence of a specific restriction enzyme.



An important property of sticky ends is that those produced from different molecules by the same enzyme are complementary and so will anneal to each other easier than blunt ends.

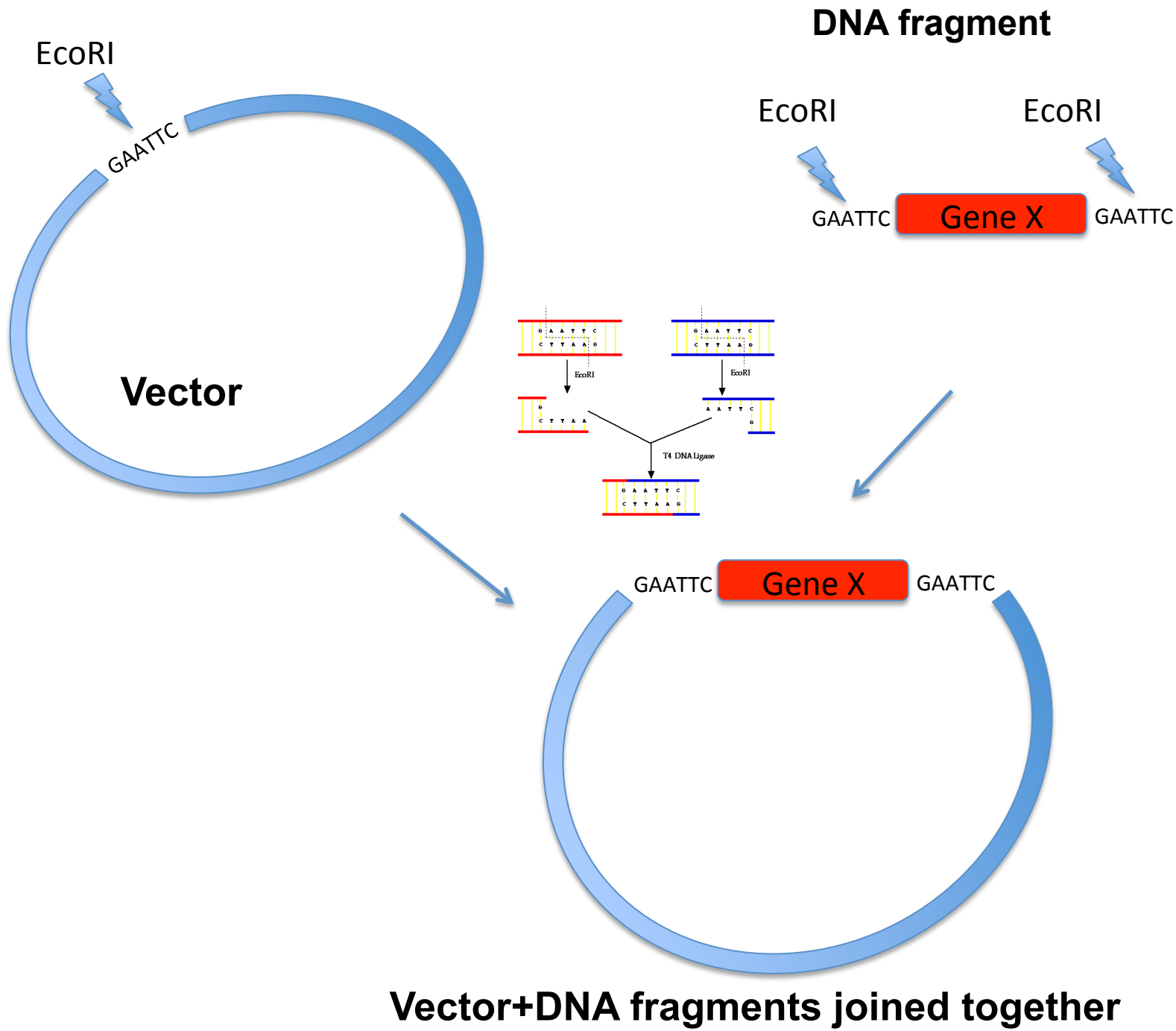
The annealed strands are held together only by hydrogen bonding between complementary bases on opposite strands. Covalent joining of ends on each of the two strands may be brought about by the enzyme DNA ligase



This is widely exploited in molecular biology to enable the construction of

recombinant DNA

i.e. the joining of DNA fragments from different sources.


DNA fragment
EcoRI
EcoRI
EcoRI
GAATTC
Gene X
GAATTC
GAATTC
Vector
EcoRI
EcoRI
T4 DNA Ligase
GAATTC
Gene X
GAATTC
Vector+DNA fragments joined together

Plasmids

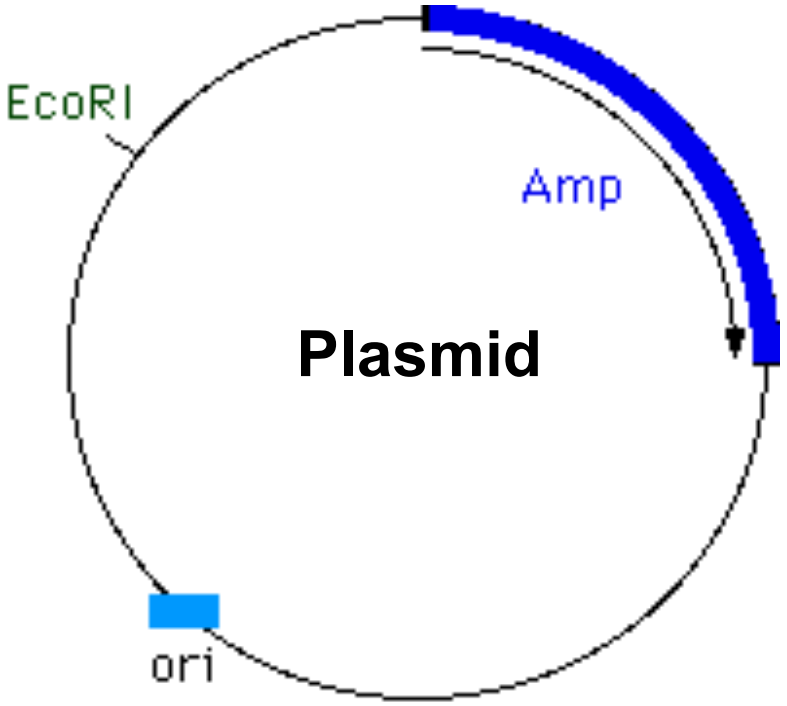
Cloning a gene means isolating it from the whole genome and inserting it into a specialised DNA carrier molecule termed a vector which allows the recombined DNA to then be replicated indefinitely within microbial cells

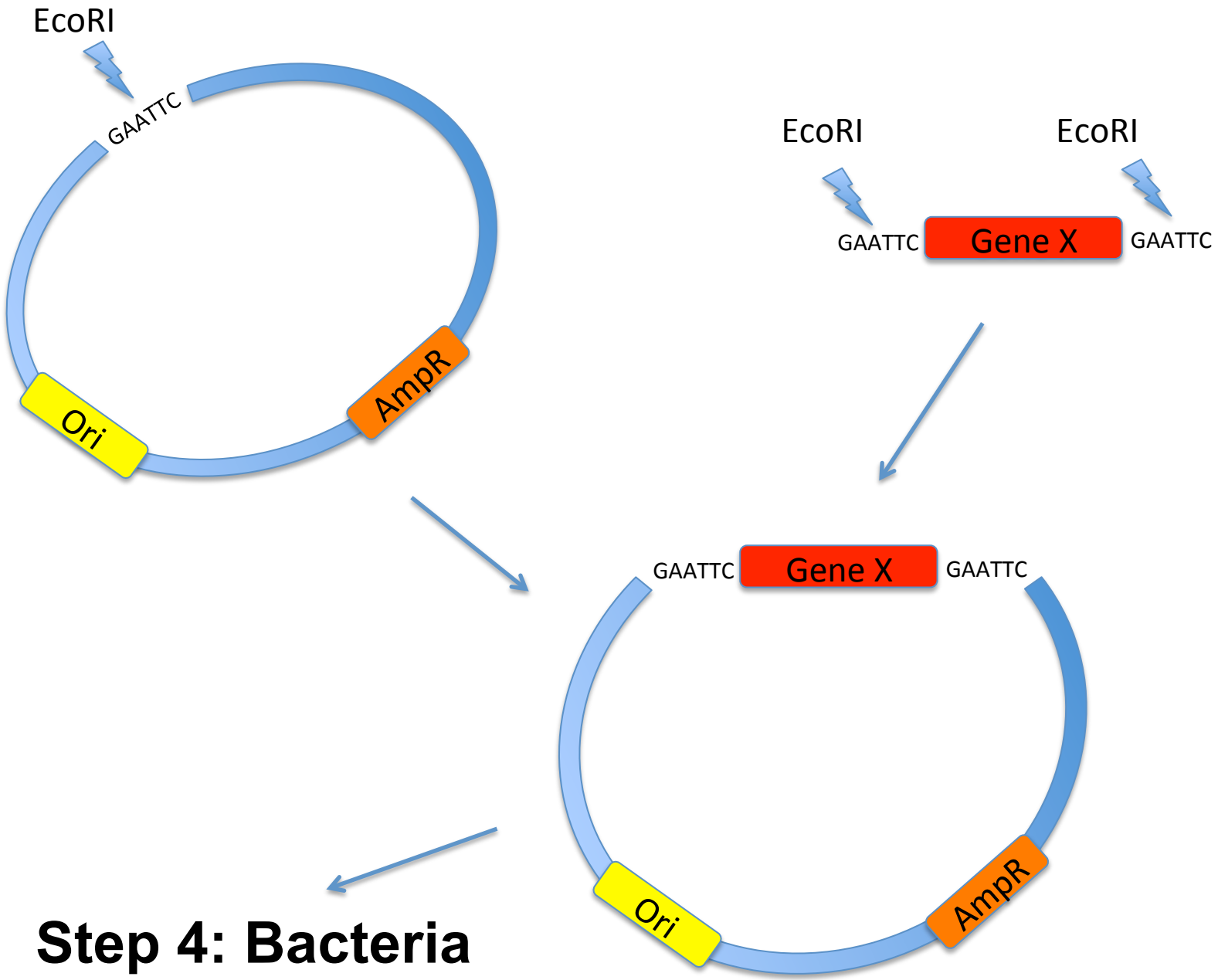
The vectors used for cloning vary in their complexity, their ease of manipulation, their selection and the amount of DNA sequence they can accommodate (the insert capacity). Vectors have in general been developed from naturally occurring molecules such as bacterial **plasmids**

Plasmid is an extrachromosomal element of DNA, which is a relatively small, covalently closed circular molecule.

A typical Plasmid vector is caracterised by:

- origin of DNA replication
- genes encoding for resistance to antibiotics
- single recognition sites for a number of restriction enzymes



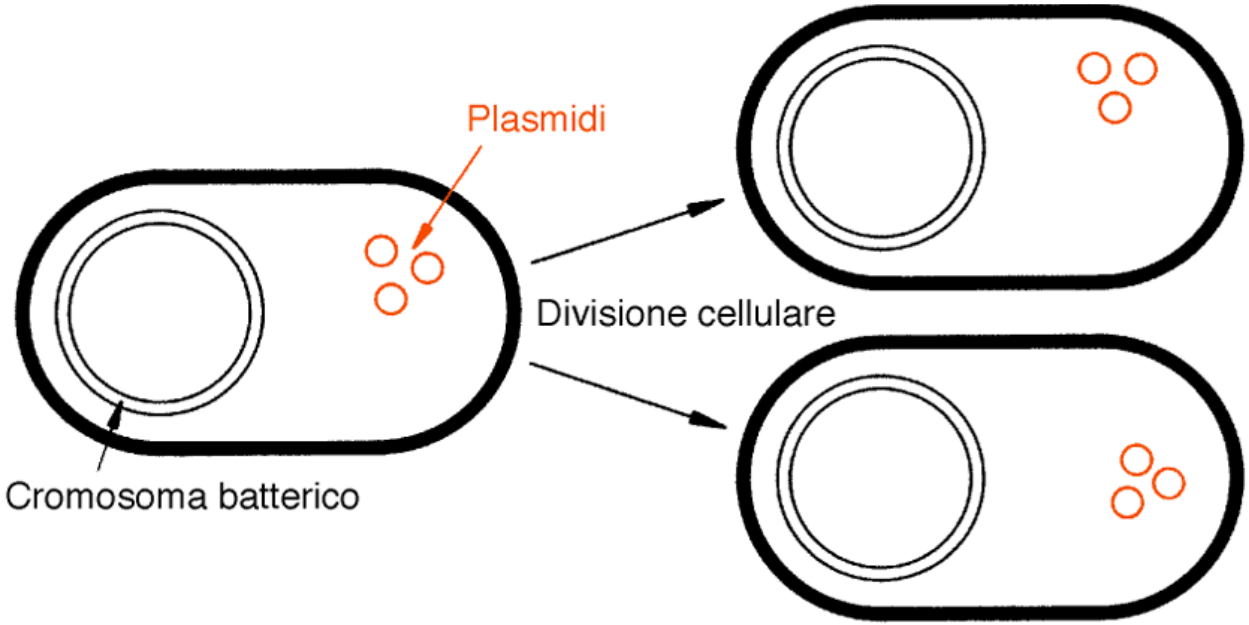


Step 4: Bacteria transformation

The bacterial **transformation** was developed to facilitate the introduction of the plasmids in bacteria, a process that occurs in nature to a minimal extent.

This is achieved by modifying certain physico-chemical properties of the walls and cell membranes with the use of **chemicals** (CaCl2), associated with **rapid changes in temperature** or **electrical discharges** high voltage (electroporation); resulting in a temporary permeabilization of the cells to the exogenous DNA.

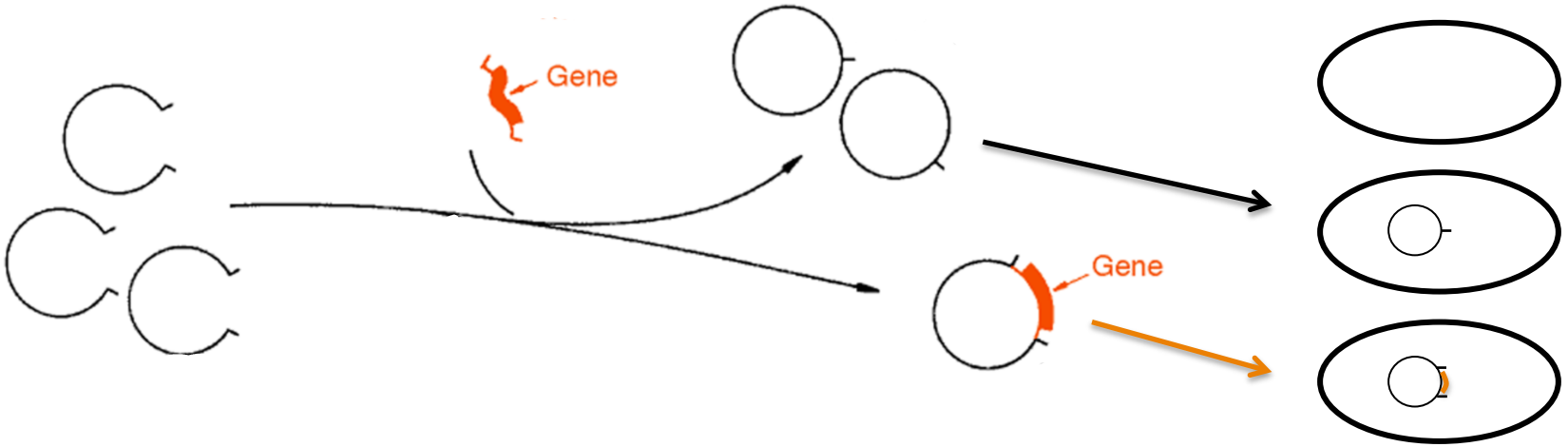
The bacterium is a sort of **"biomolecular factory "**, each time it divides itreplicates also the plasmid with our gene of interest



Step 5: Selection of recombinant clones

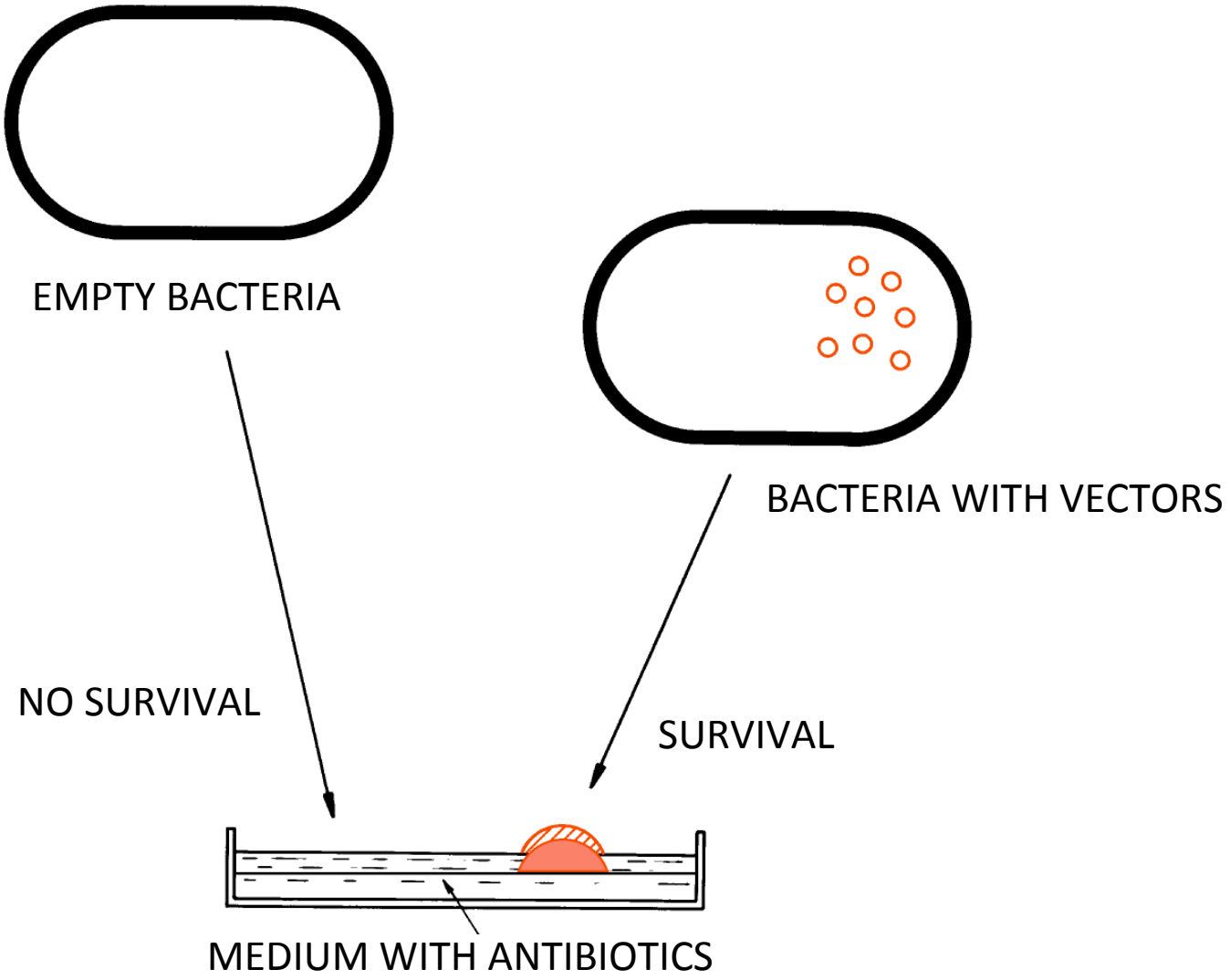
Molecular cloning consists in the ligation of an insert with a plasmid vector and the introduction of this recombinant DNA in a bacterial host.
This transformation produces three types of bacteria:

- *empty* bacteria
- bacteria containing *empty plasmid vector* (plasmid without insert)
- bacteria containing the *recombinant DNA* (plasmid plus insert)



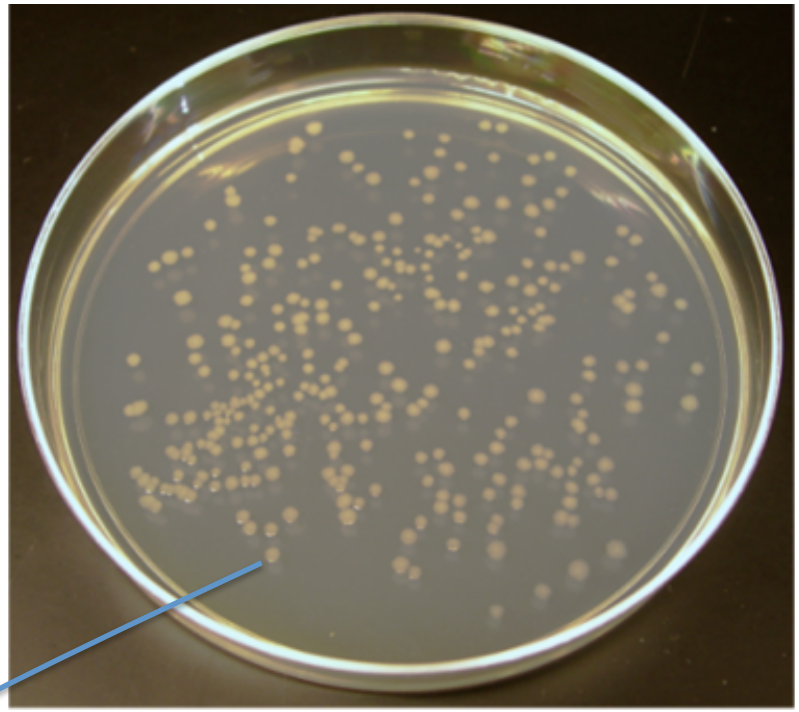
How we discriminate between these three options????

The use of selectable markers, for example, resistances to antibiotics, easily allows to distinguish empty bacteria, sensitive to the antibiotic, from bacteria containing the vector, with or without the insert, resistant to the antibiotic.



Each single colony is a CLONE, namely a set of bacteria "twins" coming from a single bacterial cell transformed.

Duplication time in E. coli in around 20-25 minutes

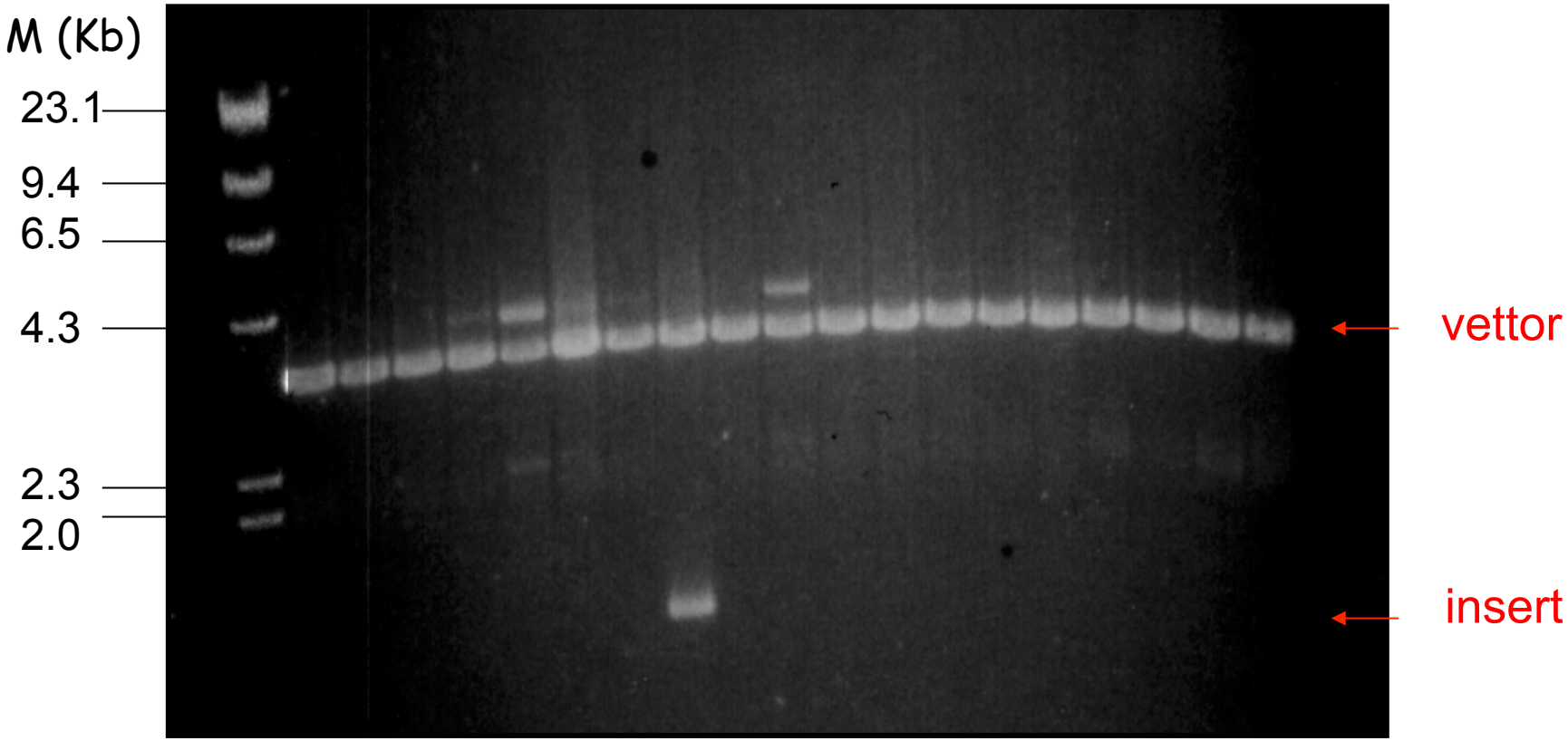


These colony will appear after 16hrs on incubation at 37°C

A single clone can be therefore expanded in a liquid culture

Restriction analysis

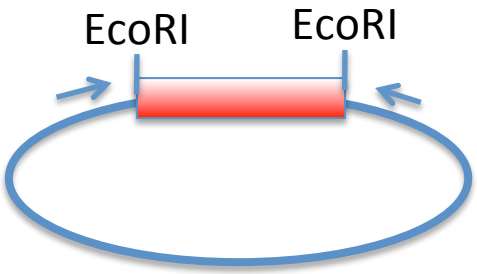
The easiest way to distinguish bacteria with empty vector from those with recombinant DNA is to extract the plasmid DNA from the transformants, and digesting it with appropriate restriction enzymes



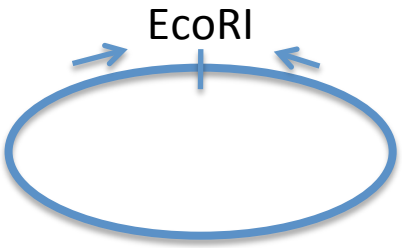
In this example the insert was 1500bp long, it has been digested by *Hind*III/*Eco*RI and inserted in a vector. After ligation and transformation the transformants were analysed by *Hind*III/*Eco*RI digestion. Among 19 clones, 18 corresponded to empty vectors while only one contained the insert.

Analysis by PCR

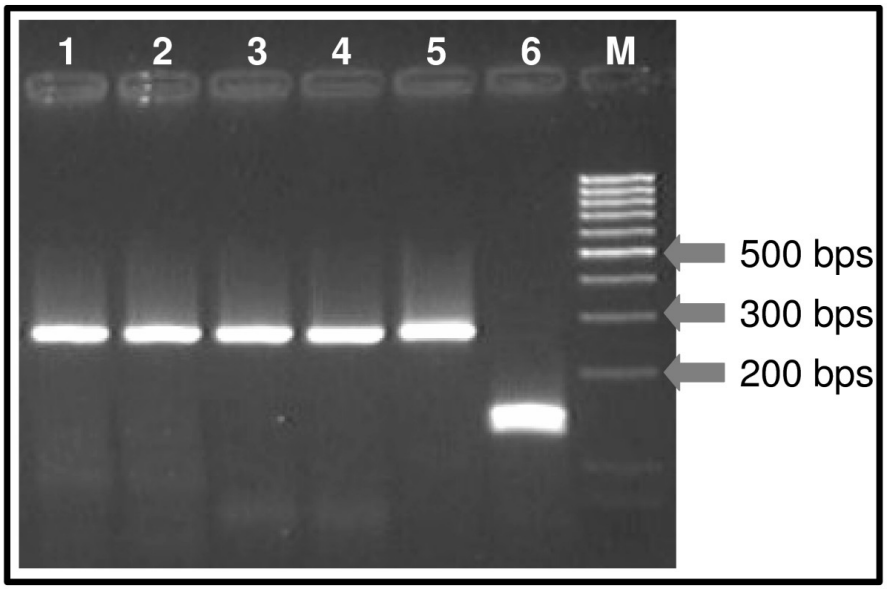
Amplification of DNA plasmids in bacteria using specific primers



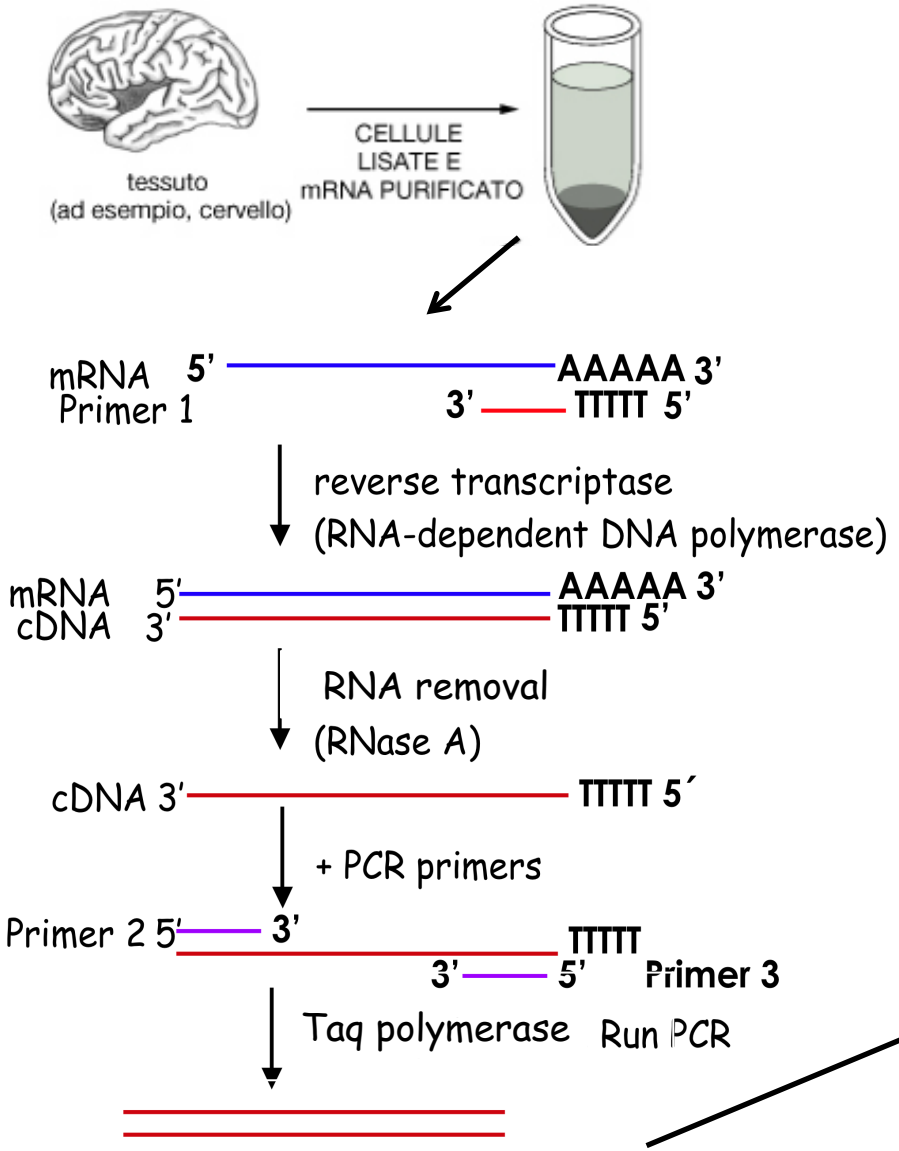
Plasmid+insert



Empty plasmid

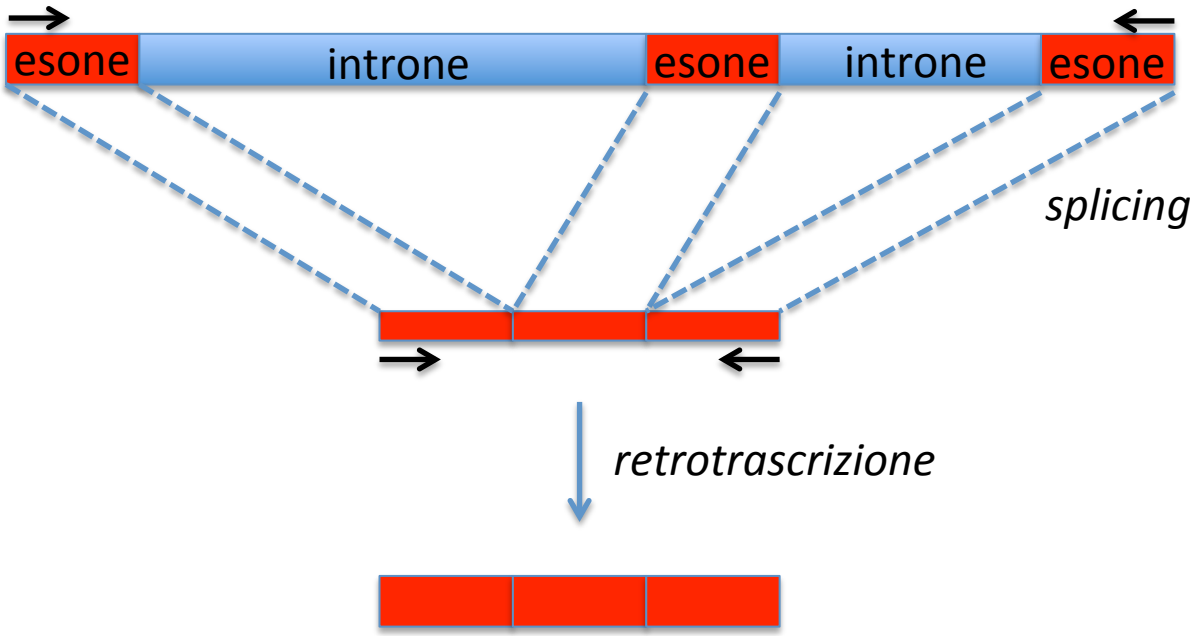


cDNA clonig



cDNA can be used as an insert and inserted into a vector

cDNA does not have introns



Genomic DNA

mRNA

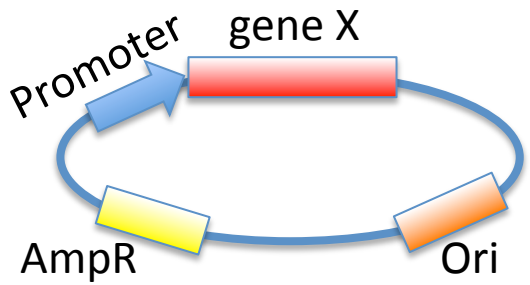
cDNA

Different types of plasmid vectors

Plasmid vectors can contain additional elements:

- A *promoter* to allow the expression of the cloned gene (expression vectors)
- A *reporter* gene that gives rise a product easly to be detected
- genes encoding for resistance to antibiotics working in eucaryotes for selecting this type of cells.

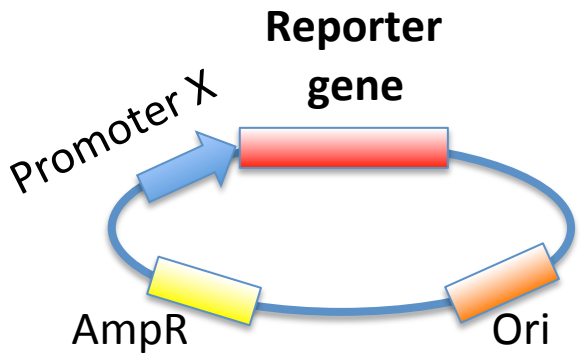
Expression vectors



different promoters for different applications:

- *Constitutive promoter*: allow the ubiquitous expression of the gene
- *tissue-specific promoter*: only active in specific tissue/type of cells or at specific time points during development
- *inducibile/repressive promoters*: their activity can be modulated in time

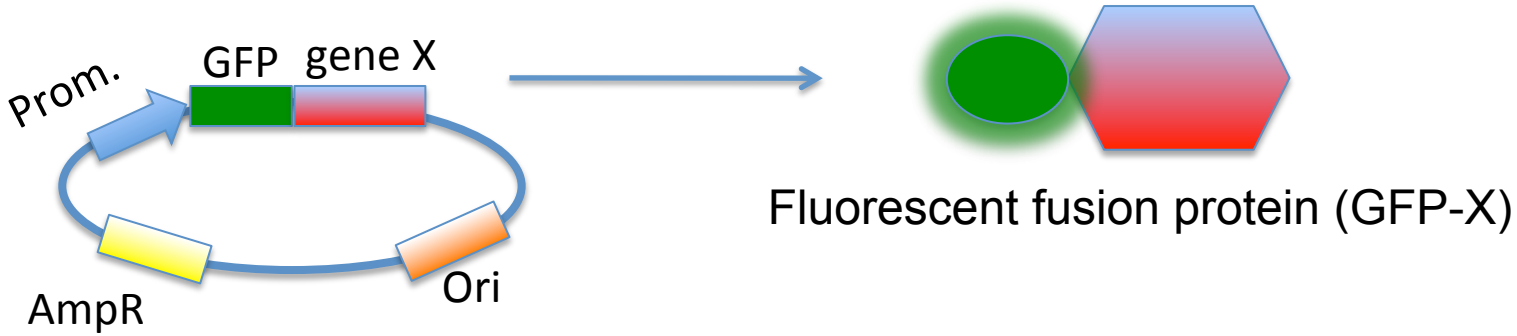
reporter genes

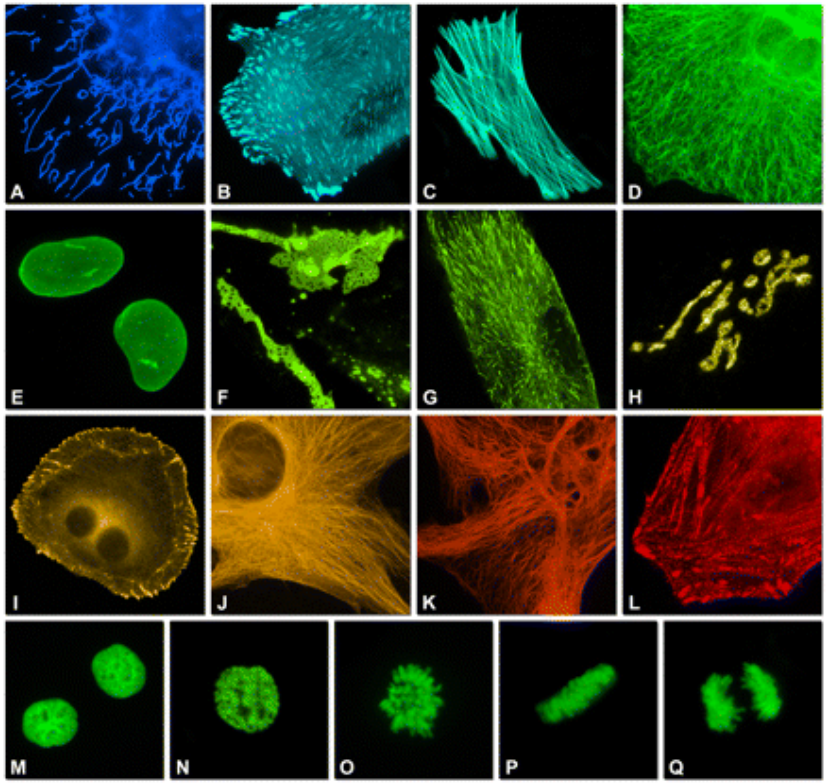


Reporter genes encode for a product easly to be detected

- *Fluorescent protein* : GFP (505 nm), CFP, YFP, BFP; Cherry, Venus, DsRed, etc.
- *Luciferase*: Firefly , Renilla. Enzymes that convert a specific substrate in a luminescence product
- *β-Galattosidase*: converts X-gal substrate in a blue colored product.

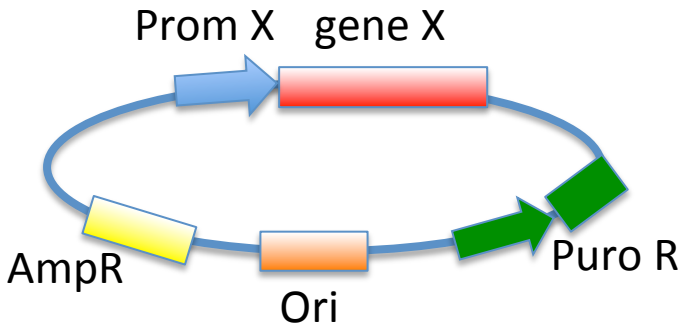
Generation of fusion proteins





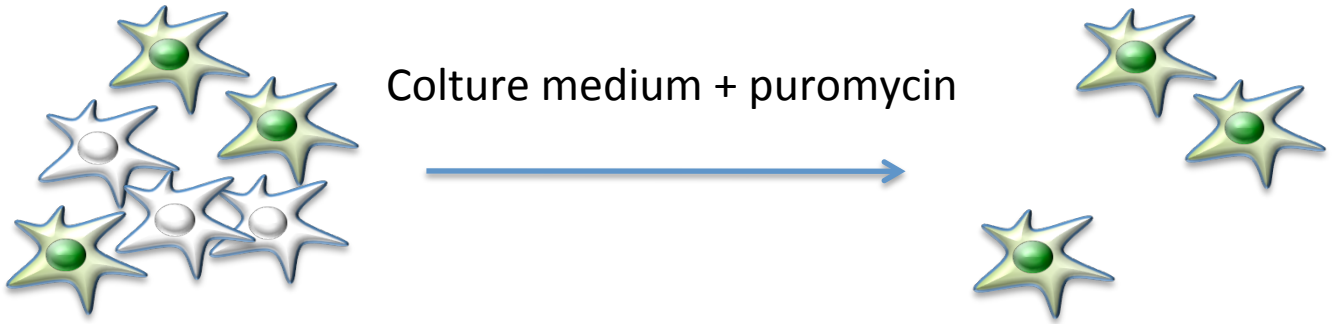
Subcellular structures visualised in vivo by using florescent fusion proteins.

genes coding for resistance to antibiotics working in eucaryotes



Tese genes allow the selection of eucoaryotic cells **TRANSFECTED** with the vectors.

Example: transfection with a vector for the expression of GFP and containing the gene for puromycine resistance.



Mixed population (transfection efficiency:50%)

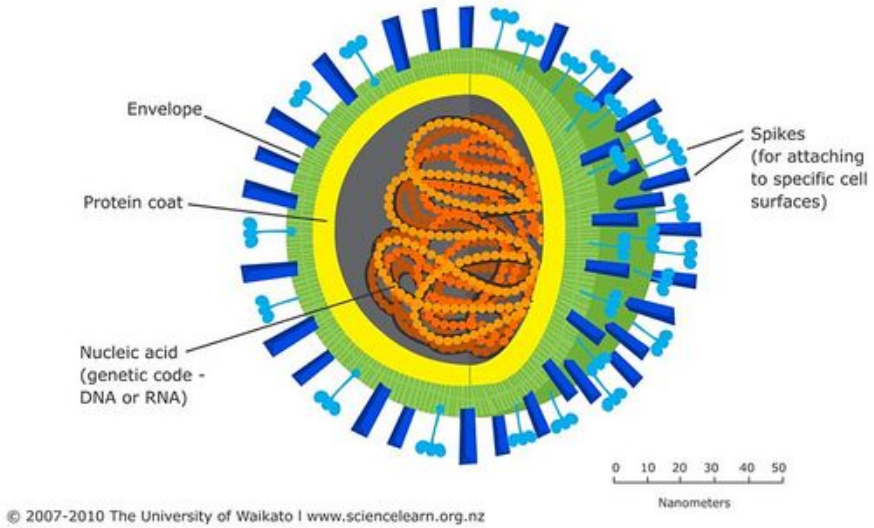
Pure population (GFP+/PuroR)

Theses antibiotics interfere with essential functions in eucaryotes such as protein synthesis.

Antibiotico	Attivita’
Puromycin	Induces premature translation termination
Neomycin (G418)	Inhibits the elongation phase during translation
Igromycin	Interferes with tRNA loading and ribosome traslocation
Blasticidin	Inhibits the formation of peptidic bond
Zeocin	Interposes into DNA and allow the nick

Viral vectors

Sometimes the simple transfection is not sufficient to transfer genetic material into cells. Some cell types are particularly refractory, making the transfection efficiency extremely low. To overcome this problem it is possible to use the property of virus to transduce their own genetic material into the cell (infection)



What is a virus? It is a parasite that requires a host cell in order to replicate. Viruses consist of a genome (double or single stranded DNA or RNA) and a capsid, an envelop that contains the genome and allows the infection of the host cell.

Viruses have specialized during evolution by developing specific mechanisms to efficiently transfer its genome into the host cell.

These mechanisms are:

- *interaction* with cells by binding membrane receptors
- *internalization* through endocytosis mediated by receptor
- integration into the host genome (in a sequence specific manner for some viruses)

Types of viral vectors

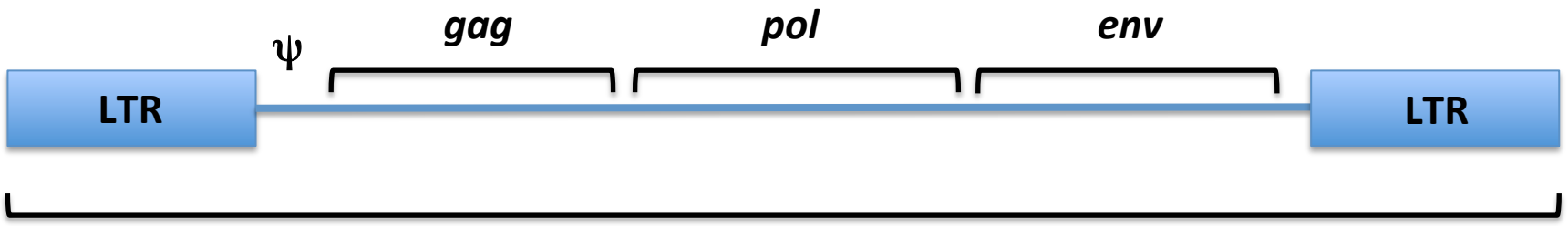
Nome	Genoma	Integrazione	Cargo
Retrovirus e lentivirus	ssRNA	YES	8-10 Kb
Adenovirus	dsDNA	No	35 Kb
Adeno-Associated Virus (AAV)	ssDNA	YES/NO	4 Kb

Different viruses for different applications

Parameters to be considered:

- **Integration capacity**
- **Cargo capacity:** maximum length of the insert

Retroviral and Lentiviral vectors



around 10 kb

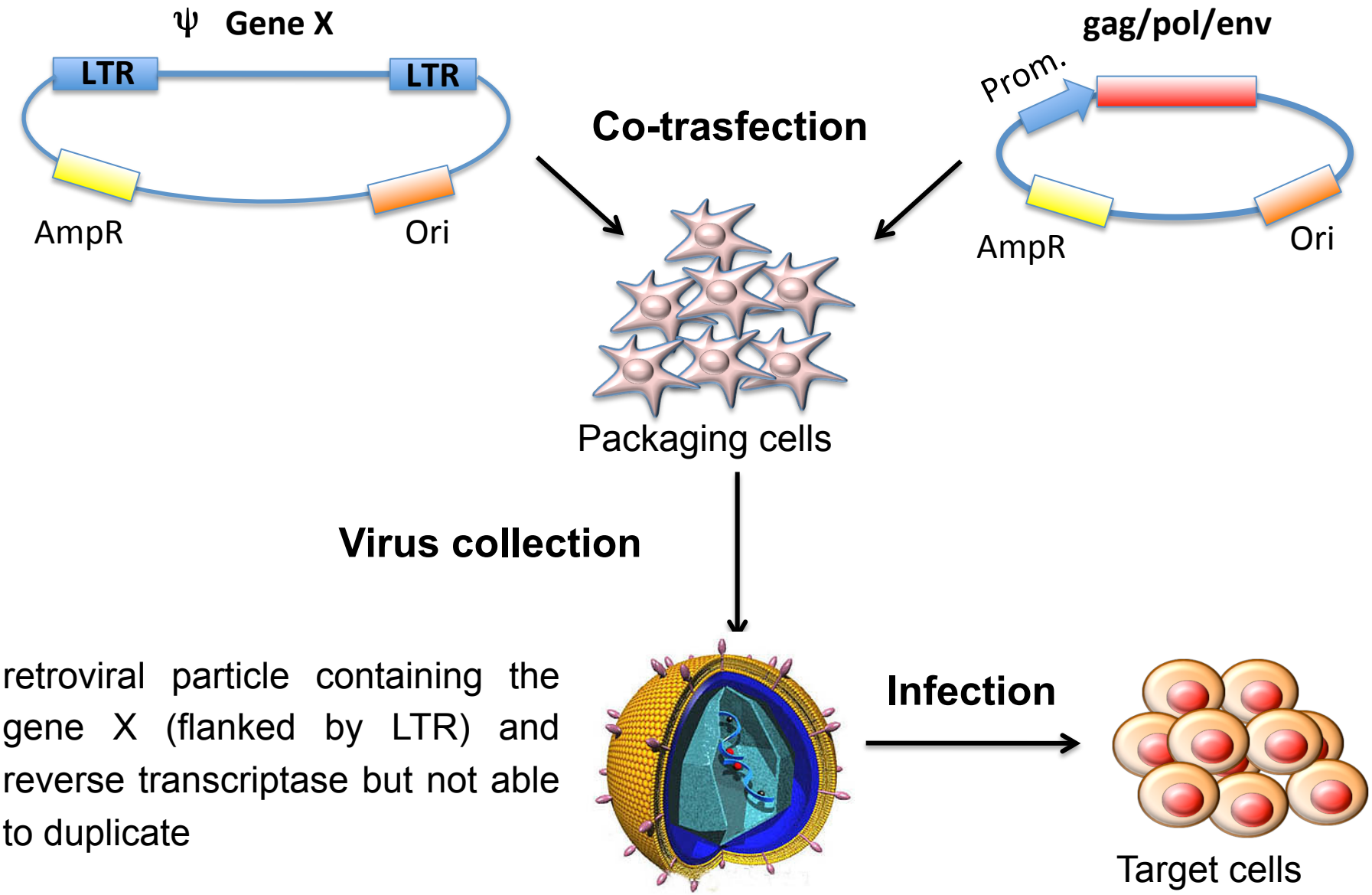
LTR: long terminal repeats, acting as *promoters*

ψ: sequence for encapsidation

gag, **pol**, **env**: structural genes that encode factors necessary for the retrotranscription of the viral RNA genome and production of the proteins of the envelop

The sequence for structural genes (8 Kb) are replaced by the insert in the viral vectors

Viral vector production



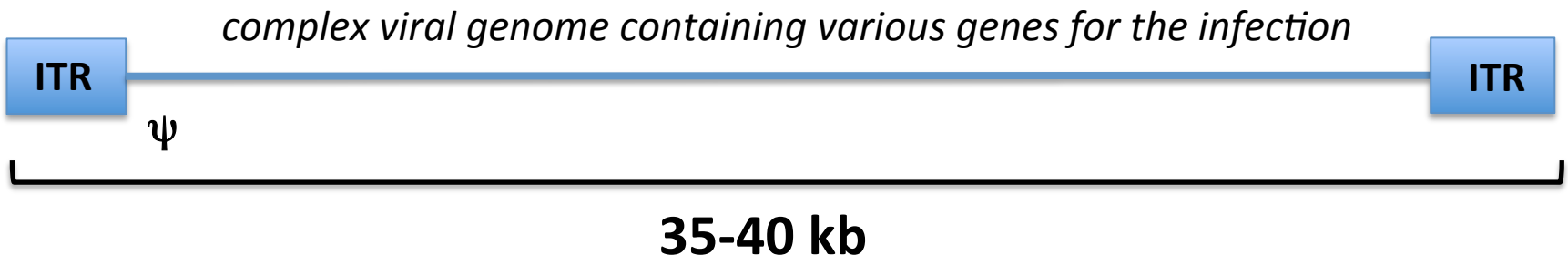
retroviral particle containing the gene X (flanked by LTR) and reverse transcriptase but not able to duplicate

Retroviruses have a high transduction efficiency, which can reach 100%, but only if the target cells are actively proliferating. This limits the application in cells that do not enter into mitosis, such as neurons.

Lentiviruses

Lentiviruses constitute a subgroup of retroviruses characterized by the ability of infecting non-dividing cells (quiescent). They contain some accessory genes. The human immunodeficiency virus (HIV) is a lentivirus.

Adenoviral Vectors (Adenoviruses)

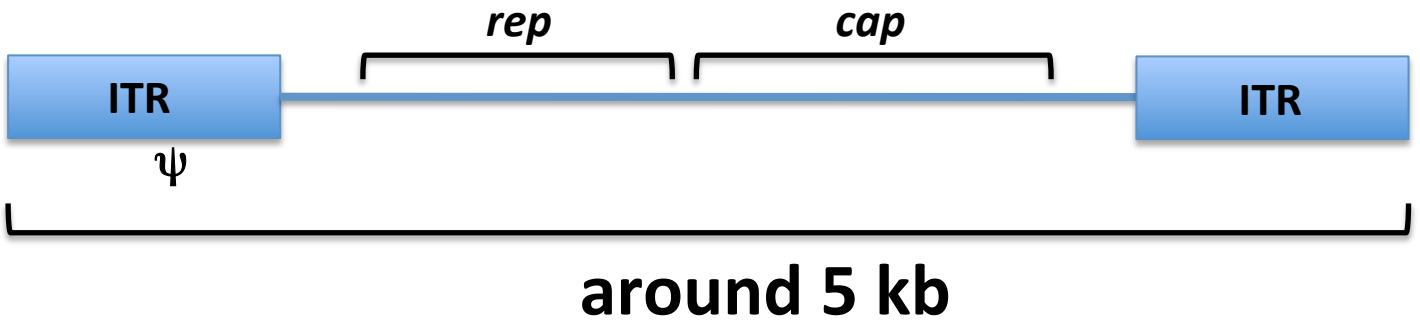


ITR: Inverted terminal repeats.

ψ: sequence for encapsidation

Benefit: large capacity of cargo (up to 35 kb in the vectors of the latest generation, in which all the genomic sequences, except ITR were eliminated). These vectors infect with high efficiency also non-dividing cells and do not integrate into the genome, remaining episomal.

Adeno-Associated viral vectors (AAV)



ITR: Inverted terminal repeats.

ψ: sequence for encapsidation

rep: gene encodes proteins for viral replication

cap: gene encodes for envelop proteins

The AAV viruses are unable to replicate autonomously, but require a helper virus, usually an adenovirus.

Adeno-Associated viral vectors (AAV)

- The virus AAV wild-type is able to integrate in a specific site in the host genome, in a region of chromosome 19. This integration does not seem to have consequences in the target cell (for example, interference with host genes or activation of oncogenes).

- One disadvantage in the use of AAV vectors is the low capacity of cargo, about 4 kb, which limits their use to small genes

- However, the **low immunogenicity** and the **persistence** for years into the host cells without the integration, make AAV a very promising tools in gene therapy.