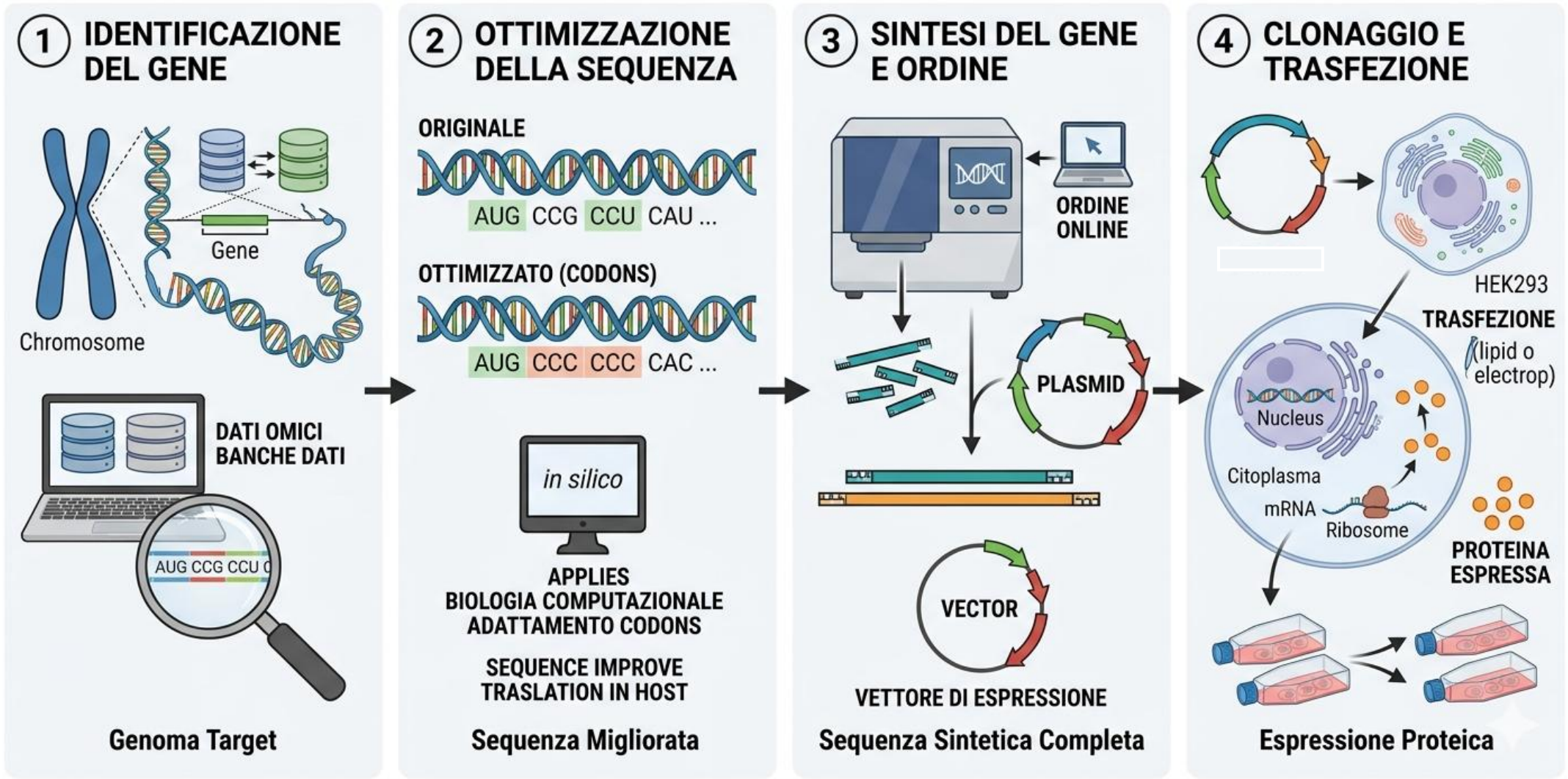
MODERN GENE CLONING FOR EUKARYOTIC EXPRESSION



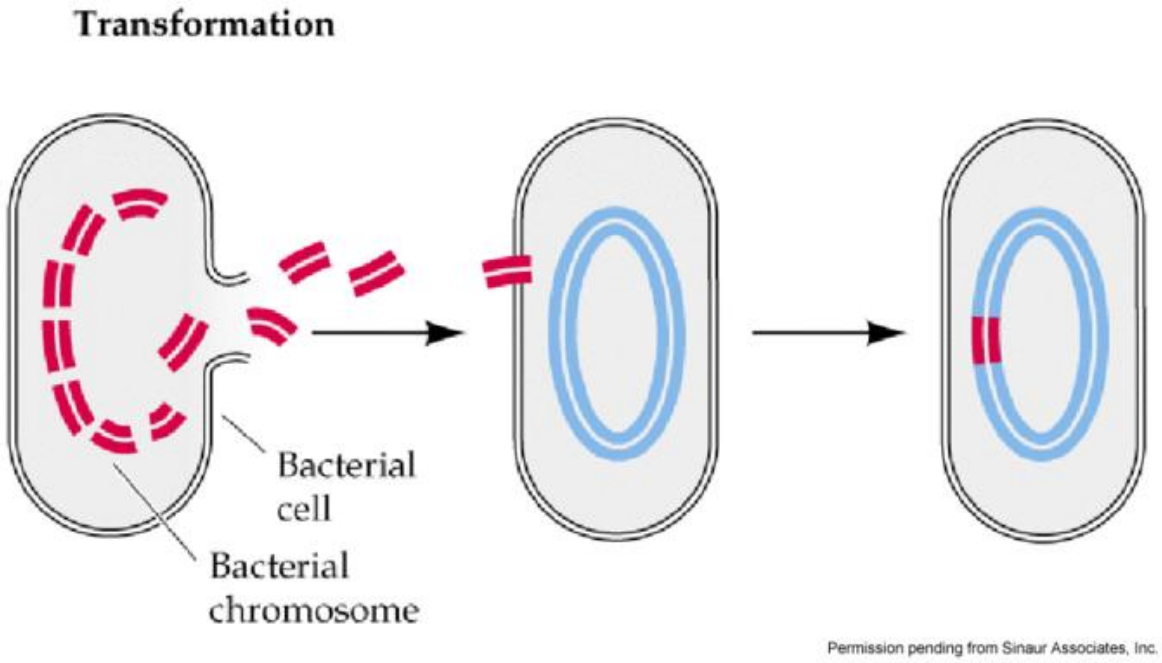
La **trasformazione**
è quel processo di trasferimento nel quale

- **una molecola di DNA**, liberatasi da una cellula batterica "**donatrice**" in seguito **a un processo di estrazione chimica**, oppure liberatasi **spontaneamente** dalla cellula per lisi di questa,

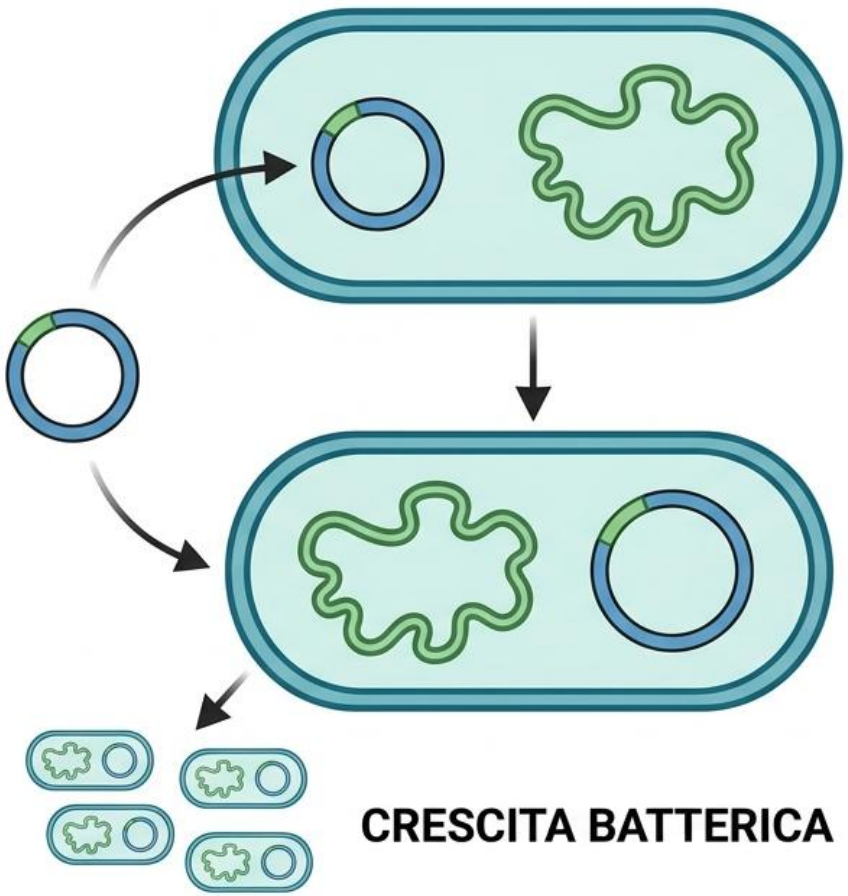
- **penetra in una cellula "recettrice"** e si va a sostituire in corrispondenza della regione cromosomica omologa.

Competenza – capacità cellulare di "catturare" il DNA.

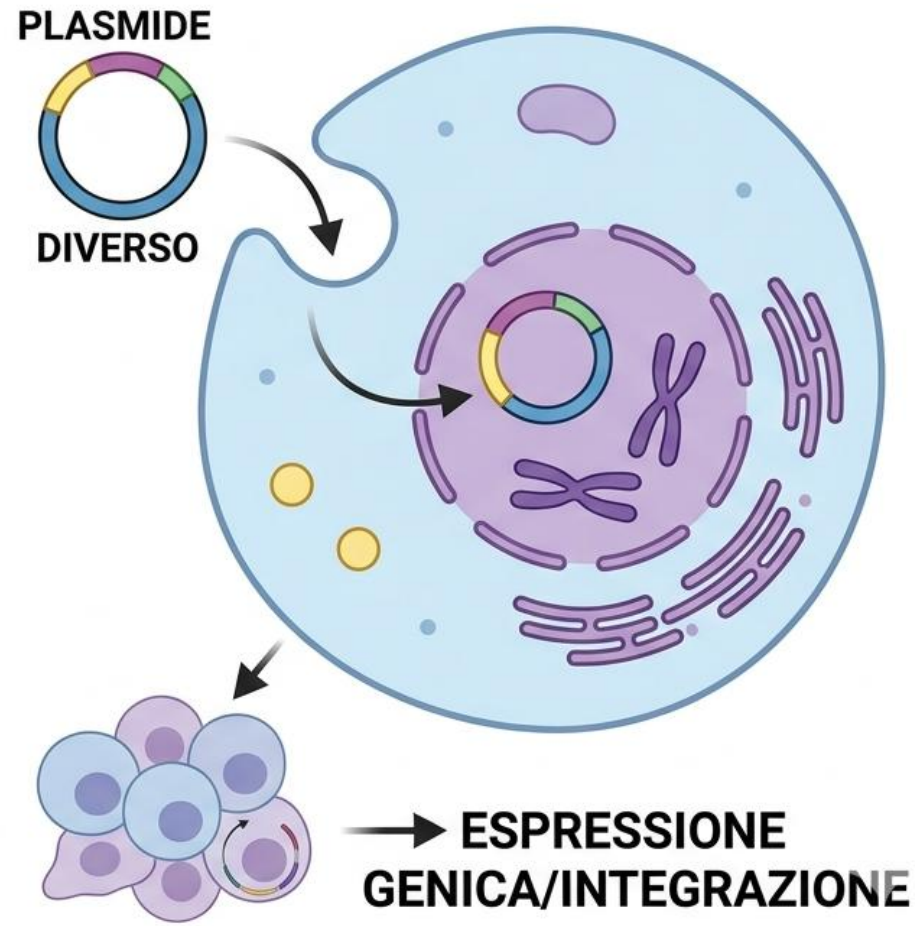
La cellula accettrice, per poter essere **trasformata**, deve trovarsi in una particolare condizione che prende il nome di **competenza**



TRASFORMAZIONE (BATTERI)



TRASFEZIONE (EUCARIOTI)

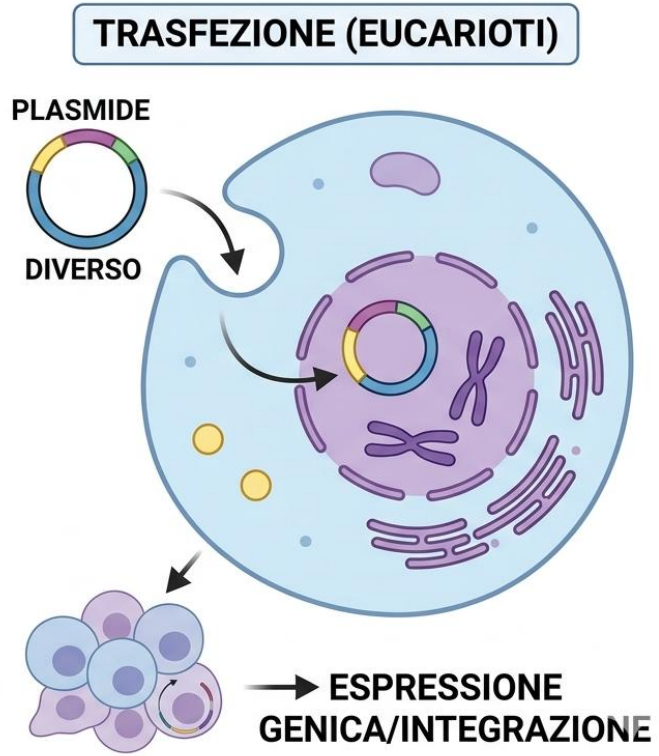


METODI DI TRASFEZIONE

- **PROCESSO:**
  - **Introduzione** del DNA nella cellula
  - Ottenimento dell'**espressione** del gene d'interesse
  - (selezione delle cellule che si sono trasfettate stabilmente)
  - **Caratterizzazione** del gene/ proteina prodotta

- **CARATTERISTICHE:**
  - Elevata efficienza
  - Bassa tossicità
  - Riproducibilità *in vitro* e *in vivo*

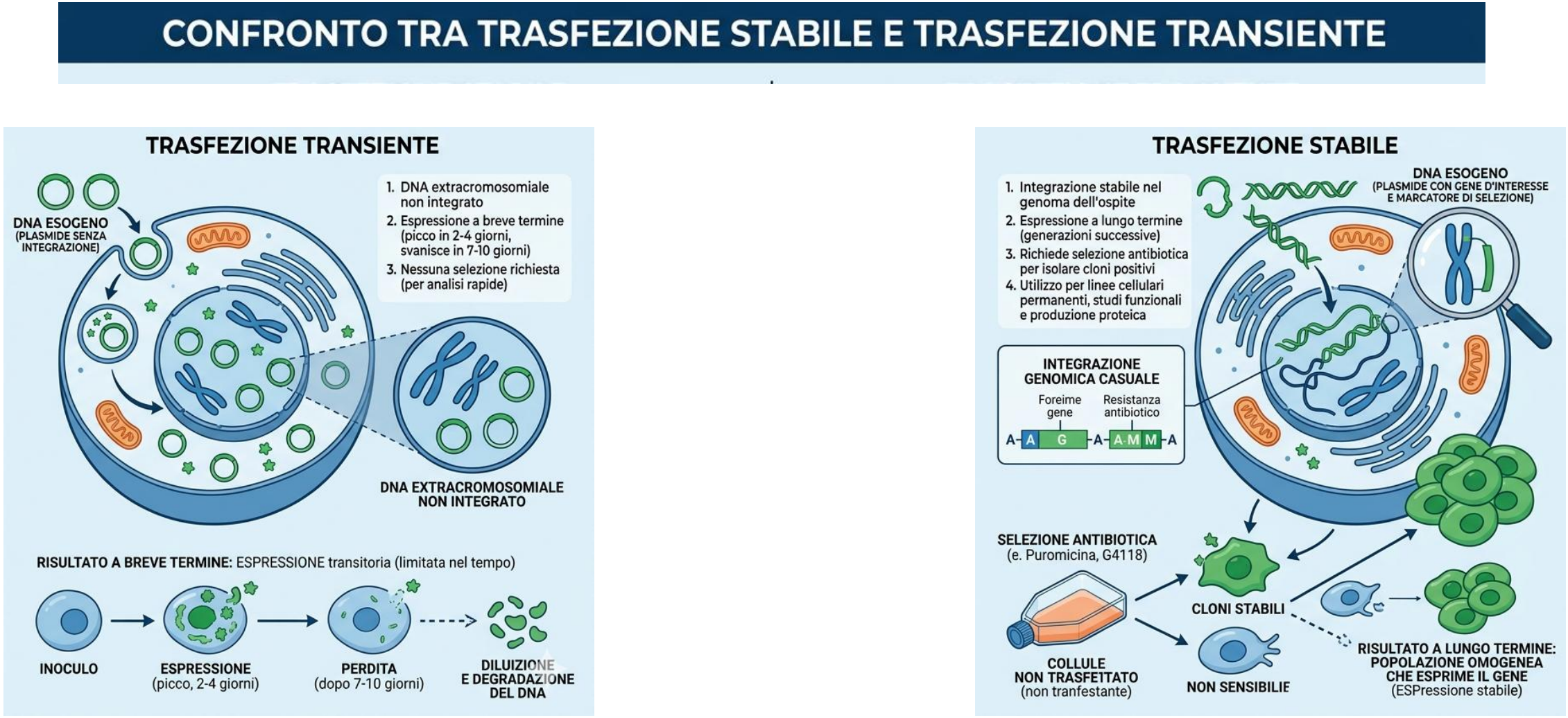
- **PROBLEMATICHE:**
  - Come superare le barriere naturali???
    - **DNA: fortemente POLARE** (carica negativa)
    - **MEMBRANA CELLULARE LIPOFILA**



Il trasferimento genico

- **Tipi cellulari**:
- **Non** tutte le cellule possono essere trasfettate con successo
  - Processo in sé **inefficient**e
  - Necessaria una fonte **abbondante di cellule di partenza** per ottenere un numero utilizzabile di cellule trasfettate

Che tipo di trasfezione?



• TRANSIENTE:

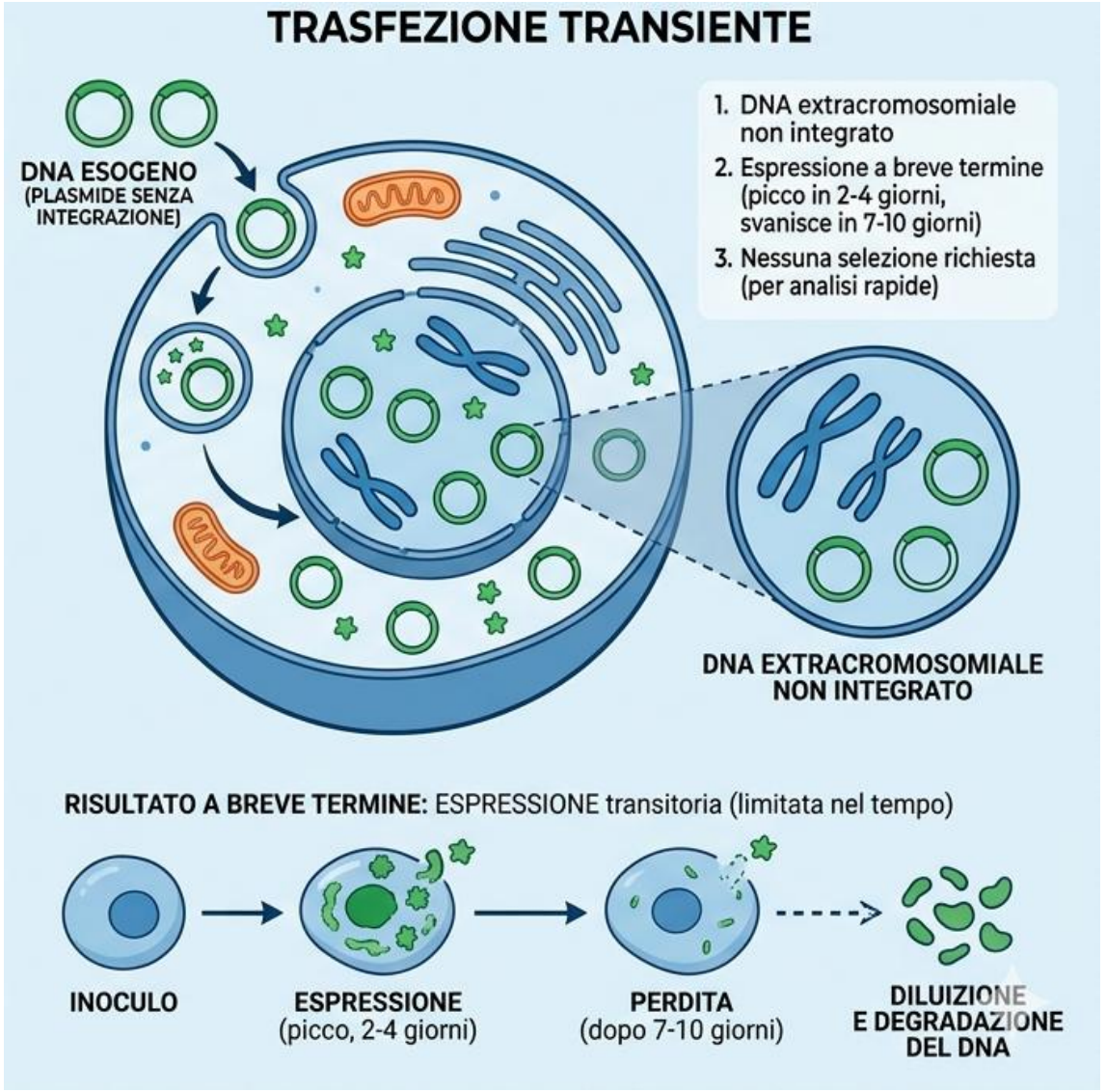
per esperimenti **a breve termine** (es. studio del promotore…)

- le cellule sono normalmente raccolte **48-72 h** dopo la trasfezione

- **over-espressione** genica

- **popolazione cellulare <u>disomogenea</u>**: poche cellule con molto plasmide

- immediata



- Il **DNA trasfettato non si duplica.**

A ogni mitosi raddoppiano le cellule e si dimezza la quantità di DNA trasfettato in rapporto alle cellule.

STABILE:

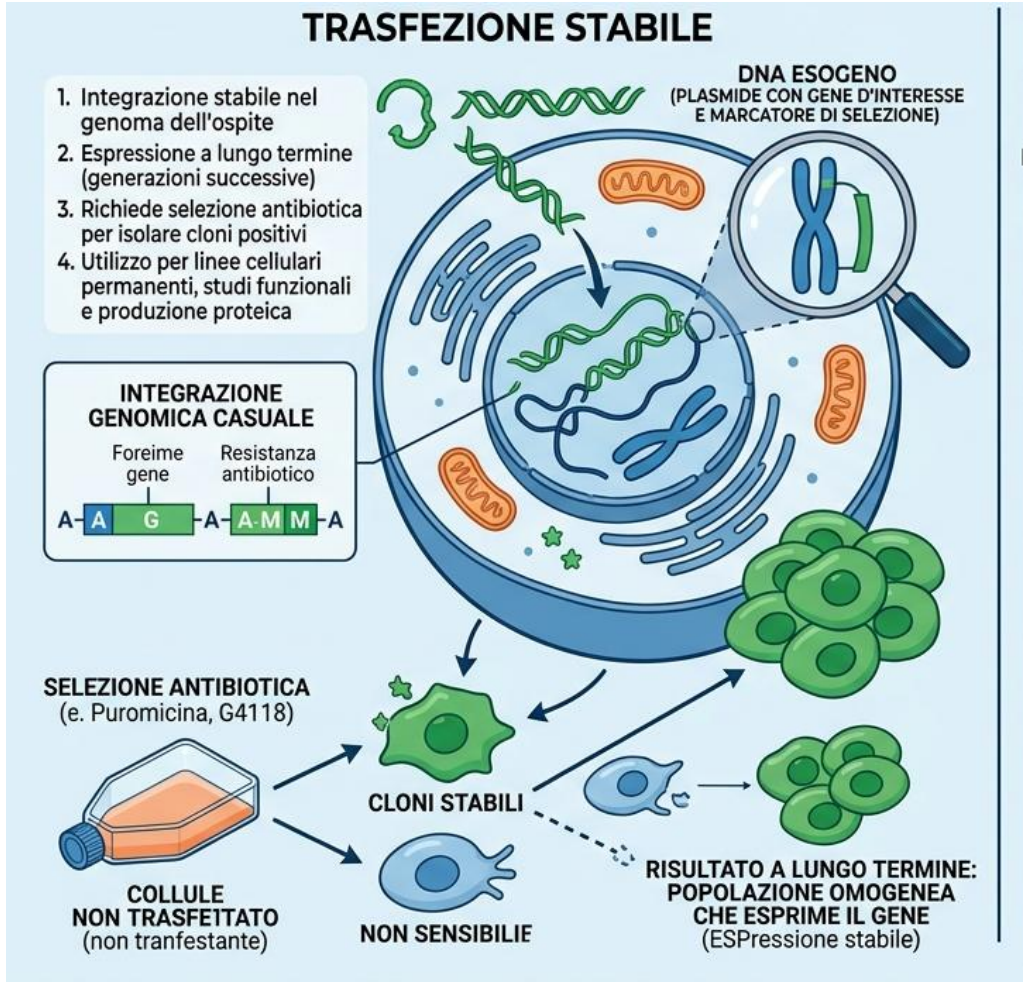
per esperimenti a **lungo termine**

- **il plasmide si integra nel genoma**, processo lungo e laborioso

- **l'integrazione è casuale**

- necessario **marker di selezione** (morfologia, resistenza a sostanze…)

- possibilità di **isolare e propagare singoli cloni** contenenti il DNA trasfettato



popolazione cellulare omogenea: molte cellule con poco plasmide

METODI DI TRASFEZIONE

1) **METODI CHIMICI**

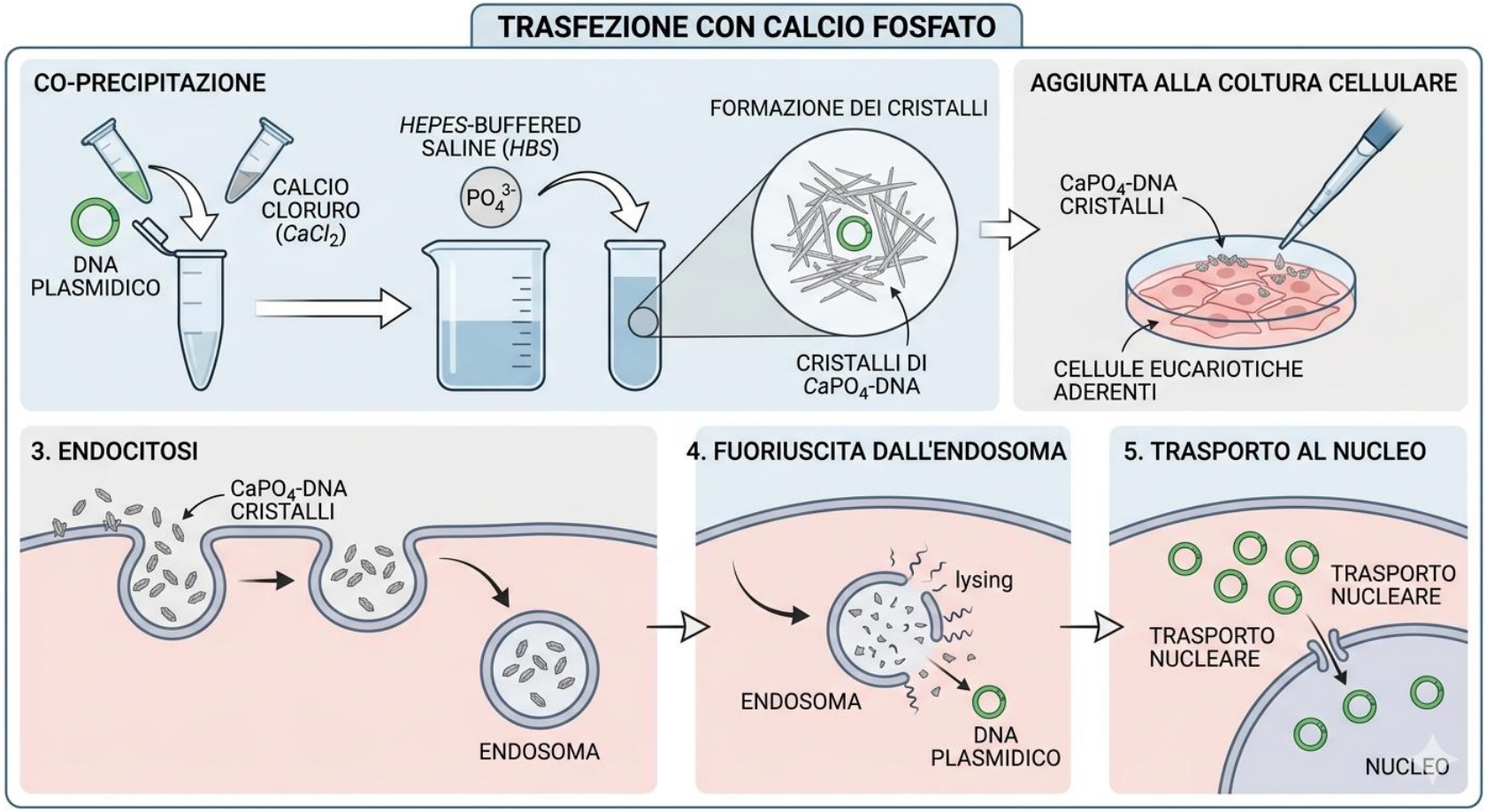
2) **METODI FISICI**

Metodi chimici

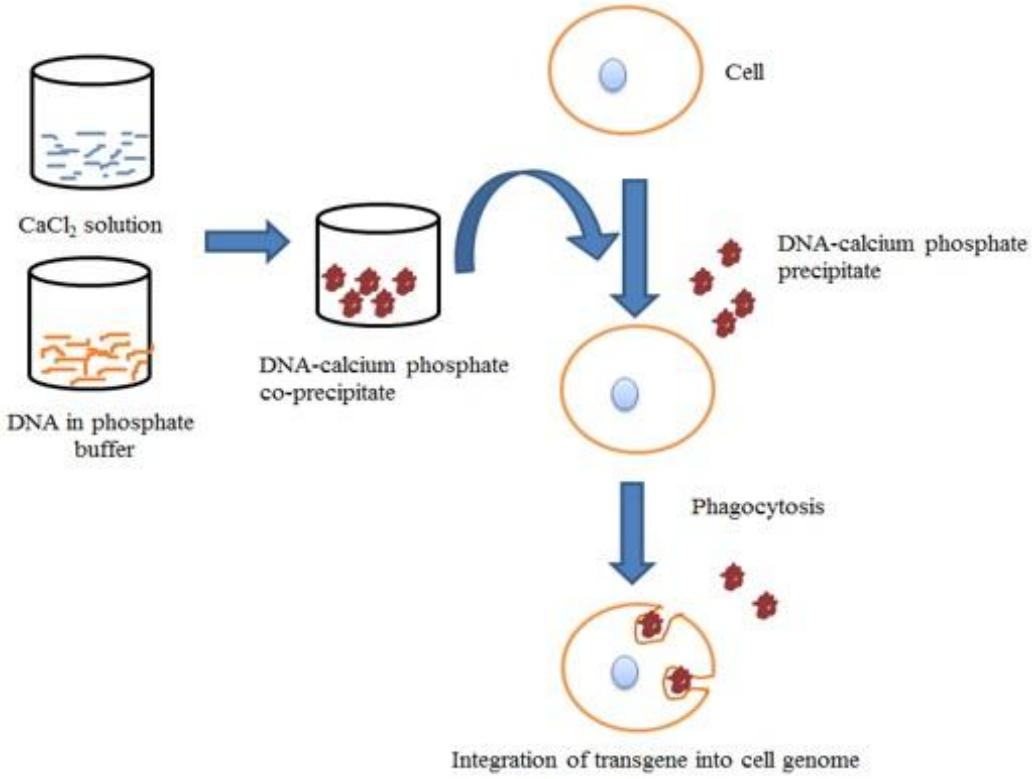
- **CALCIO FOSFATO**

- Il DNA a contatto con una soluzione di **fosfato di calcio** forma dei **precipitati** i quali, con un meccanismo poco noto (probabilmente per endocitosi) entrano nelle cellule

Gli ioni bivalenti possono promuovere l'ingresso di acidi nucleici attraverso le membrane



• CALCIO FOSFATO



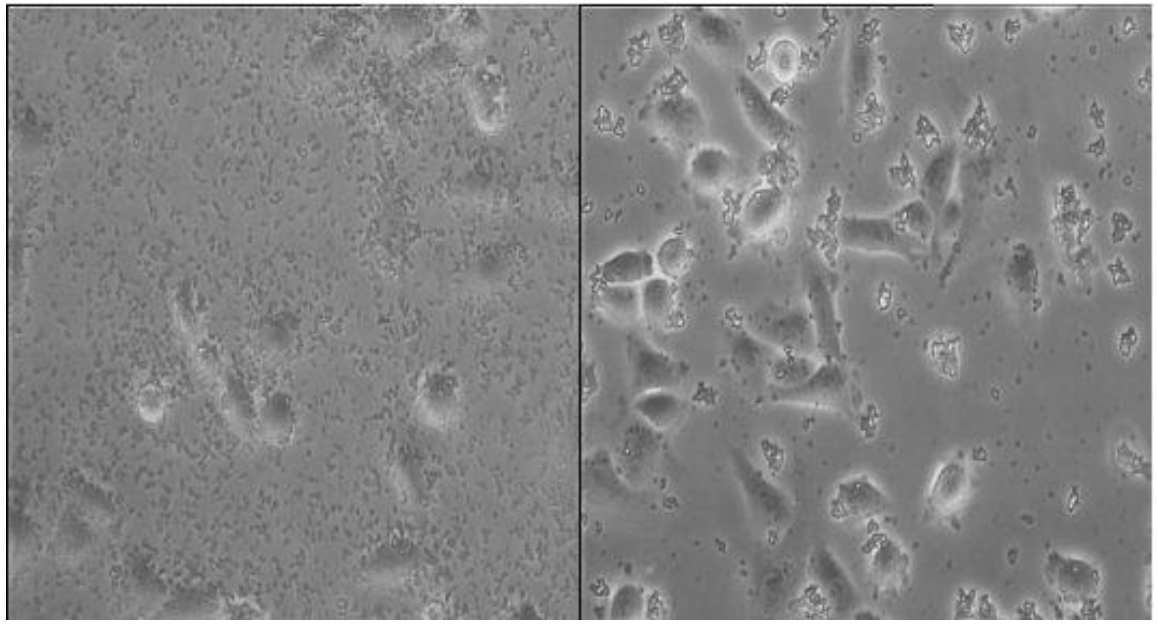


Figure 2. **Small calcium phosphate-DNA precipitates** (left) results in higher reporter gene expression posttransfection than **larger precipitates** (right). Pictures were taken three hours after addition of the transfection complex to the cells.

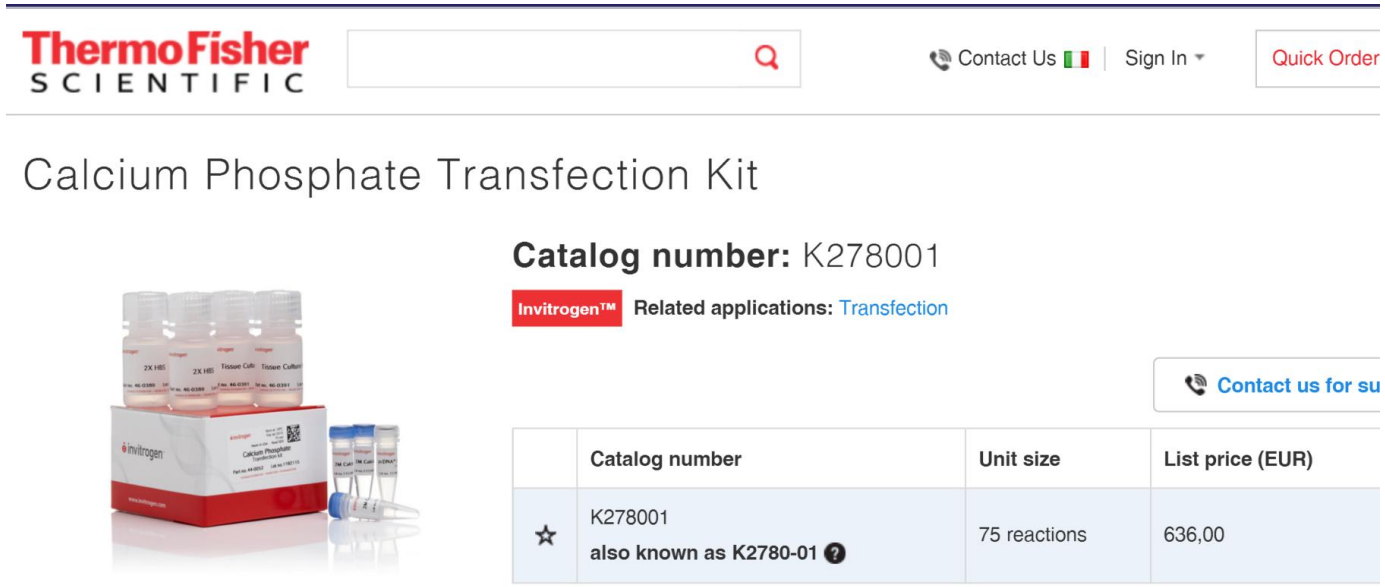
• CALCIO FOSFATO

• *VANTAGGI*

- **Economico**
- Relativamente versatile

• *SVANTAGGI*

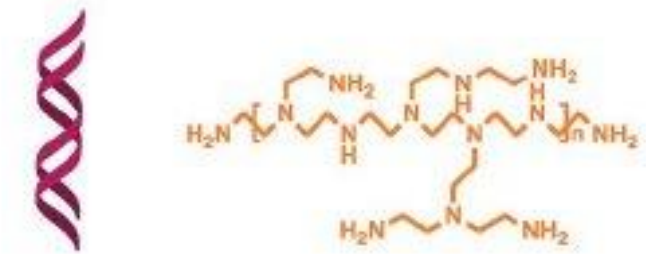
- ✓ Tecnicamente **delicato**
  - forma e dimensioni del precipitato
  - influenzato da variazioni anche minime di pH
- ✓ **Efficienza bassa**
- ✓ Elevata Citotossicità
- ✓ **Non funziona con alcuni tipi cellulari**



Metodi chimici

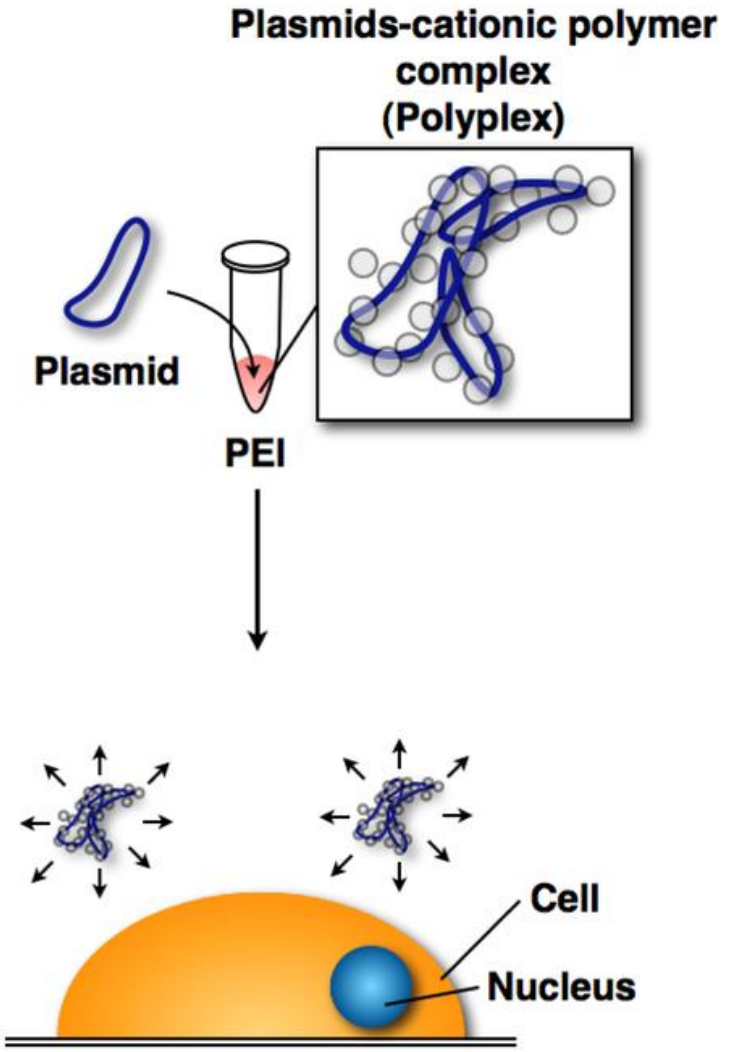
- **polyethylenimine (PEI)**

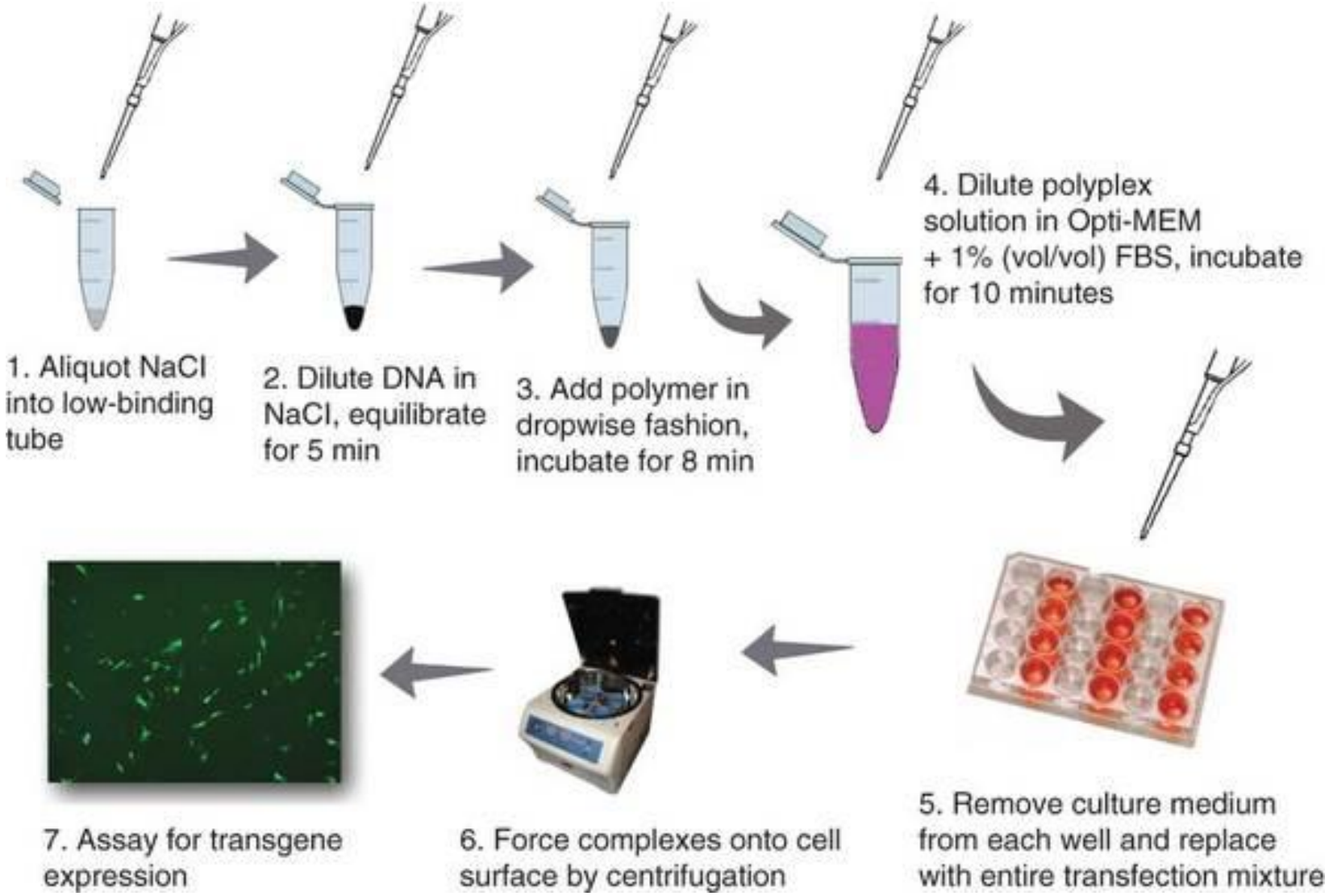
- **PEI** is an organic **polymer** with a **high density of amino groups** that can be protonated.

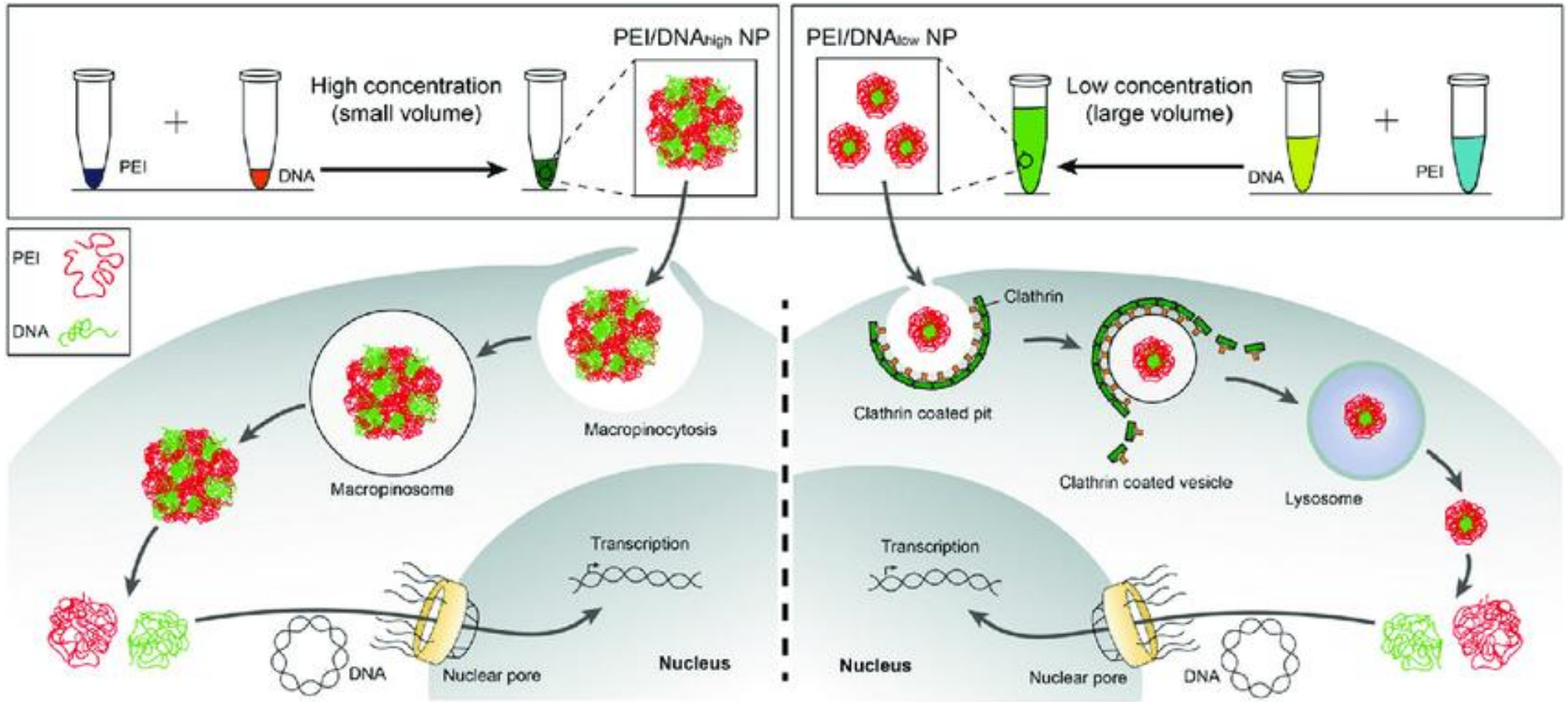


DNA PEI

- At physiological pH, the polycation presents a high affinity for binding DNA and can mediate the transfection of eukaryotic cells

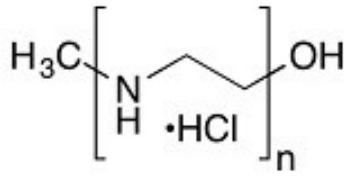



1. Aliquot NaCl into low-binding tube
2. Dilute DNA in NaCl, equilibrate for 5 min
3. Add polymer in dropwise fashion, incubate for 8 min
4. Dilute polyplex solution in Opti-MEM + 1% (vol/vol) FBS, incubate for 10 minutes
5. Remove culture medium from each well and replace with entire transfection mixture
6. Force complexes onto cell surface by centrifugation
7. Assay for transgene expression



small NPs were firmly condensed, whereas the large NPs were bulky and botryoid-shaped. The large NPs entered the tumor cells via the macropinocytosis pathway, and then efficiently dissociated in the cytoplasm and released DNA, thus promoting the intranuclear delivery.

• **PEI:**

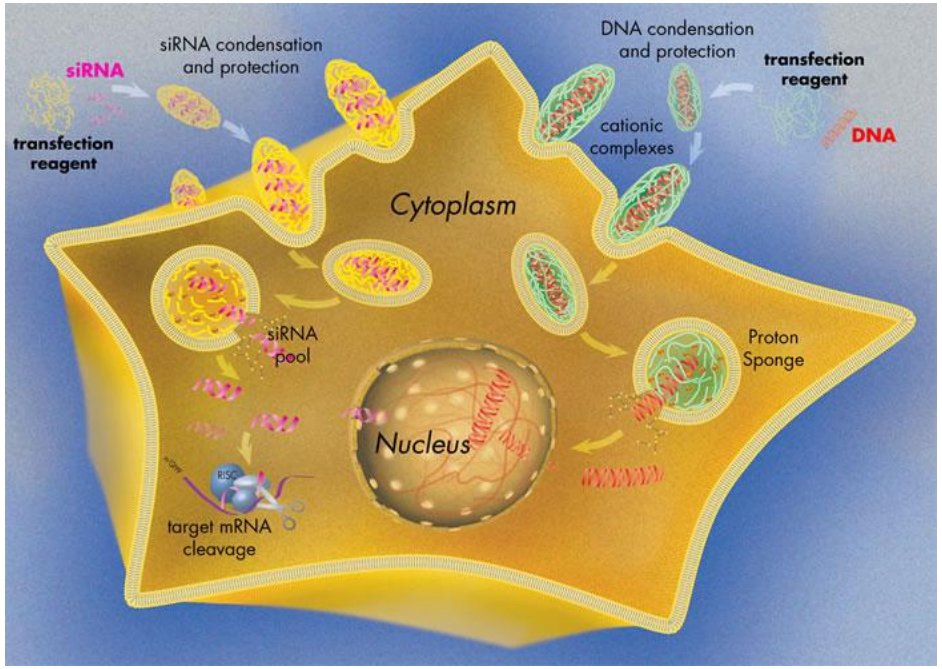


VANTAGGI

- ✓*Molto semplice*
- ✓*Poco costoso*
- ✓*Buona efficienza*

•SVANTAGGI

- ✓ *tarare rapporto PEI/DNA*
- ✓*Condizioni delle cellule*



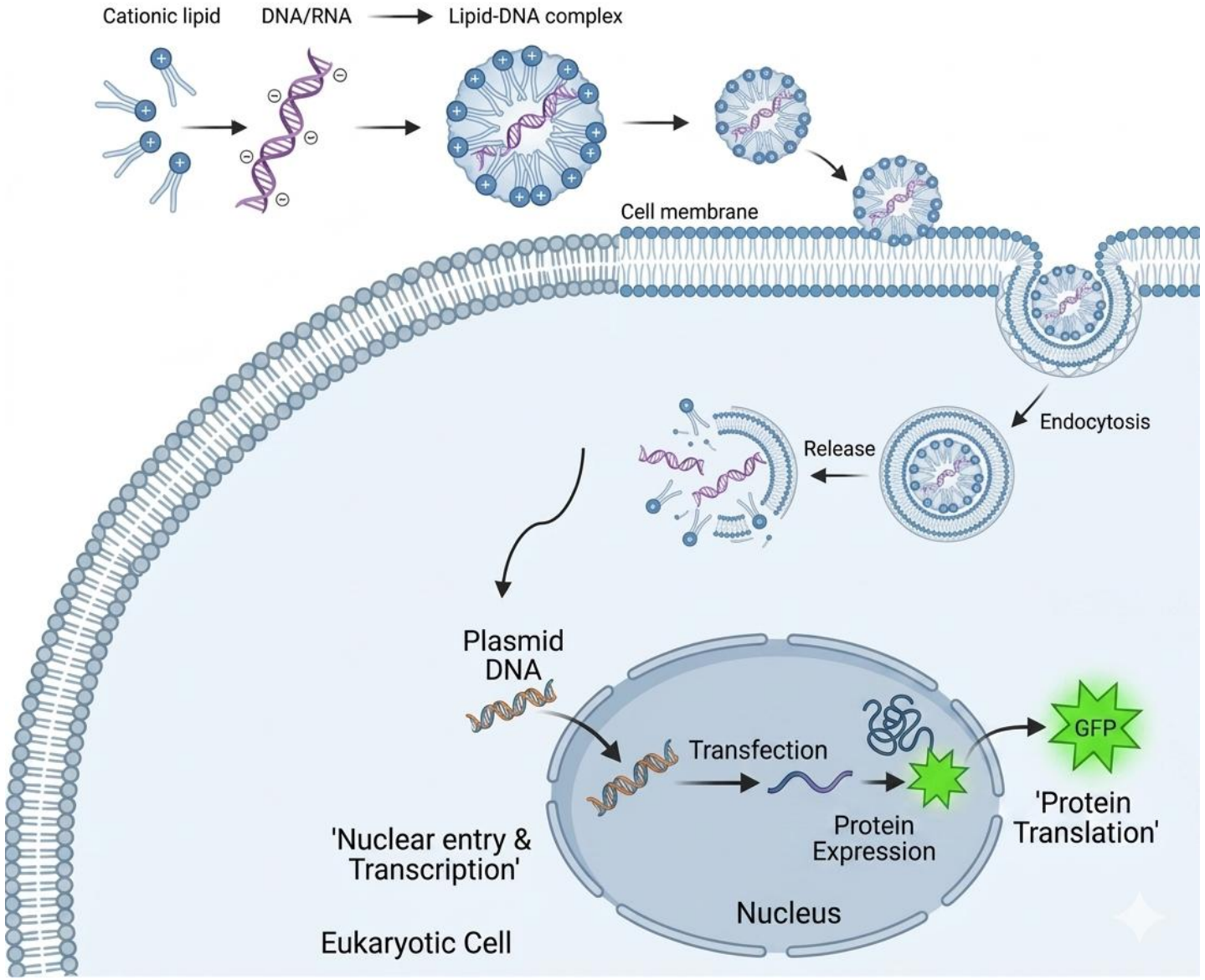
Metodi chimici

- **LIPOSOMI:**

- misto di **lipidi policationici e neutri,** permette la formazione di vescicole liposomiali unilamellari che portano una **carica netta positiva**

- La testa cationica del composto lipidico si associa ai gruppi P negativi dell'acido nucleico

- I complessi lipidi- DNA **si fondono con le membrane cellulari** e rilasciano spontaneamente il loro contenuto nelle cellule




invitrogen
Lipofectamine® Reagent
Qty: 1 mL (2 mg/mL)
1118858
Mar2014
Read SDS
Made in USA
For Research Use Only
5791 Van Allen Way Carlsbad, CA 92008
For Research Use Only. Not intended for any animal or human therapeutic or diagnostic use.
24-hour Medical or Spill Emergency Response (Chemtrec)
North America +1 866 536 0631 / Other +1


invitrogen
L3000-001
Store at: 2°C - 8°C
Lipofectamine®3000
Transfection Kit
Qty: 0.1 mL
0000000
Nov2014
For Research Use Only
5791 Van Allen Way Carlsbad, CA 92008
Read SDS
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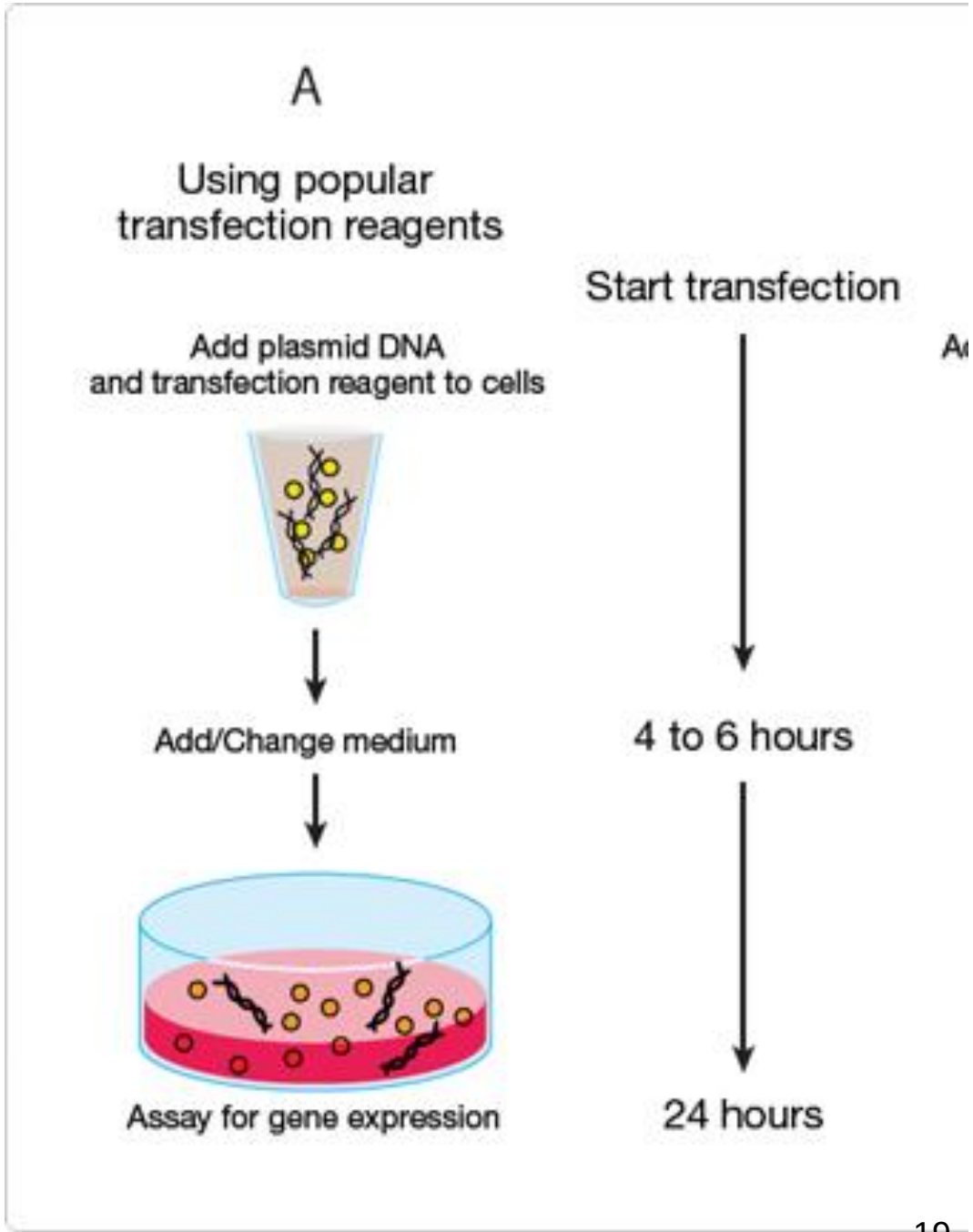
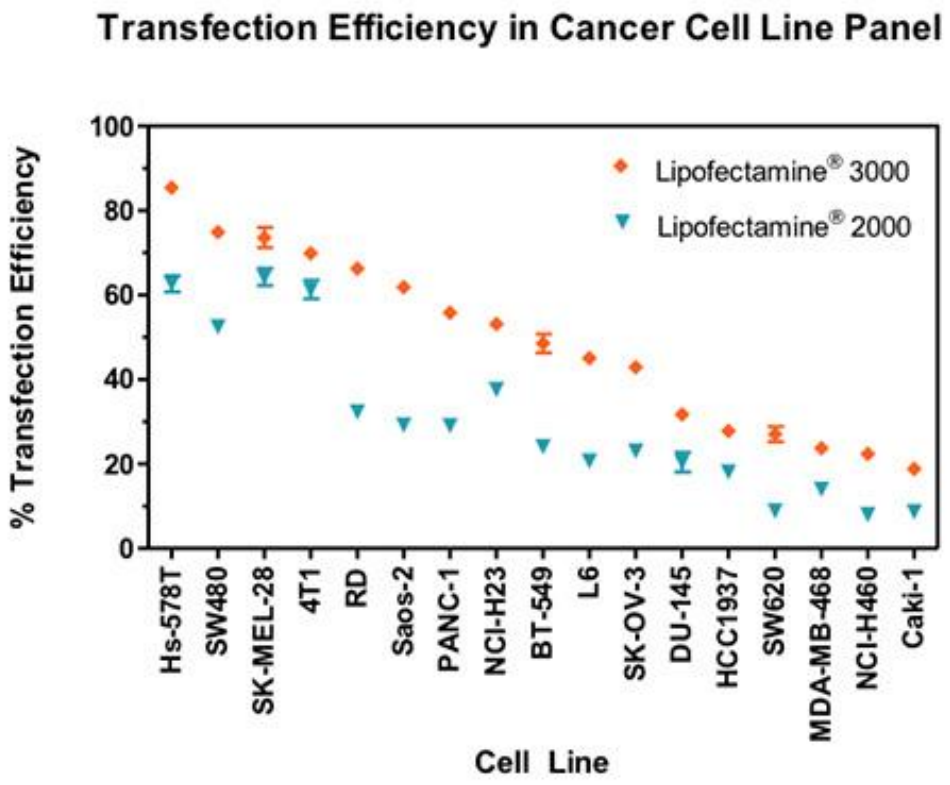
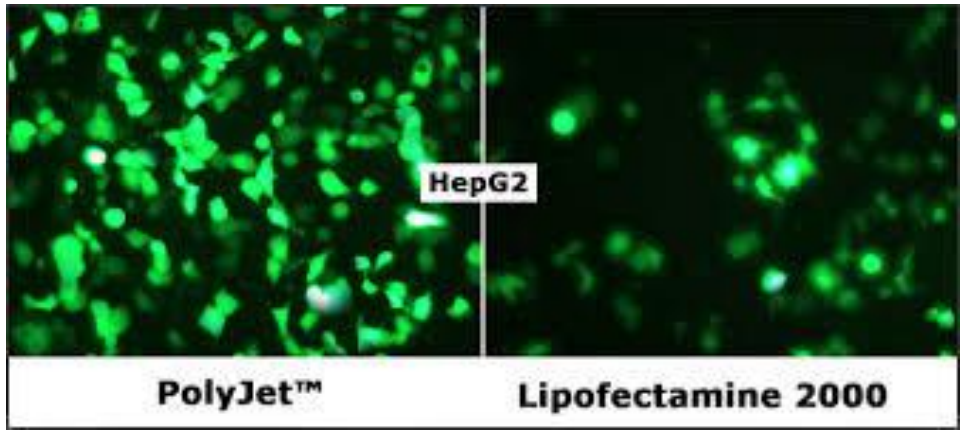

A
Using popular transfection reagents
Add plasmid DNA and transfection reagent to cells
Add/Change medium
Assay for gene expression
Start transfection
4 to 6 hours
24 hours

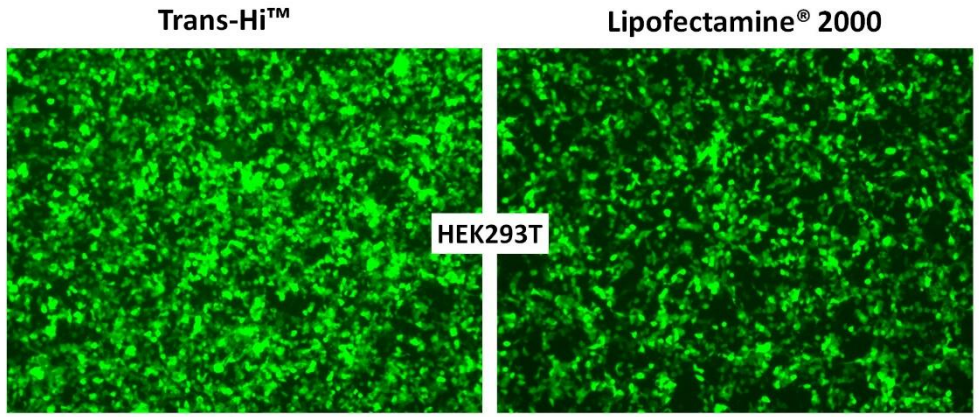
The **advantages** of cationic lipid-mediated transfection are:

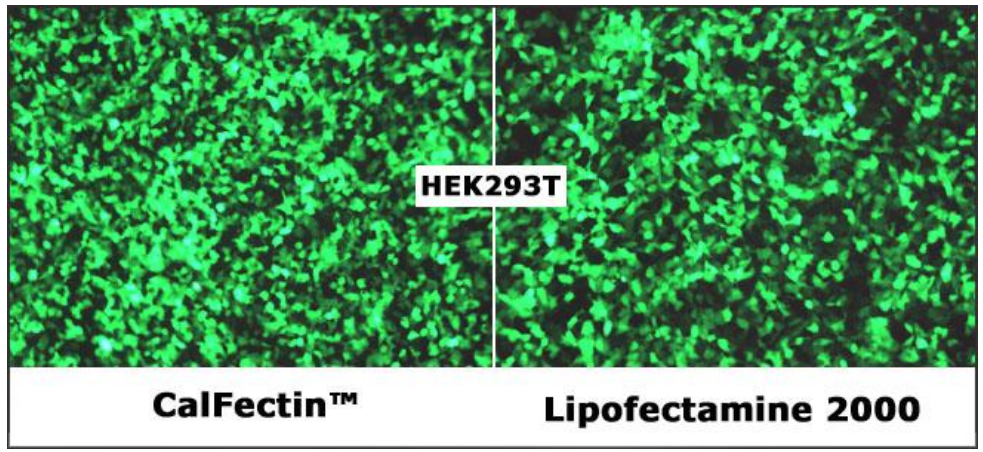
- the ability to transfect **a broad range of cell lines** with high efficiency;
- its applicability to **high-throughput screens;**
- the ability to deliver **DNA of all sizes**, as well as **RNA** and **proteins**.
- In addition, this method can be applied to both **stable and transient**
- expression, and unlike other chemical methods, it can be used for in vivo transfer of DNA and RNA to animals and humans.

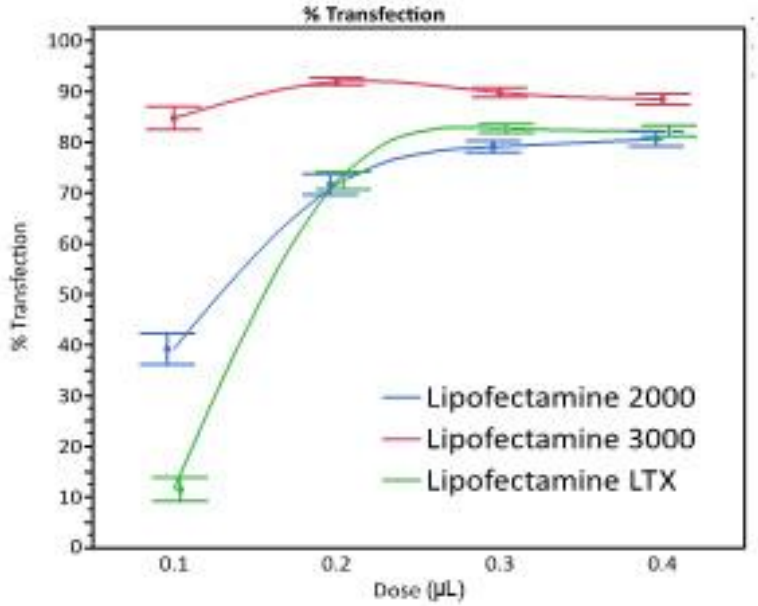
The **main drawback** of cationic lipid-mediated transfection is
the **dependence of transfection efficiency on the cell type and culture conditions**, requiring the optimization of transfection conditions for each cell type and transfection reagent

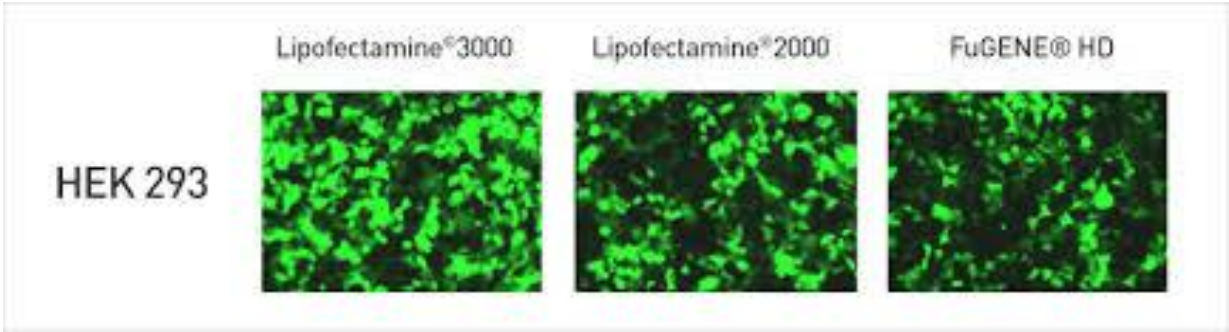



HepG2
PolyJet™
Lipofectamine 2000


Trans-Hi™
Lipofectamine® 2000
HEK293T


HEK293T
CalFectin™
Lipofectamine 2000



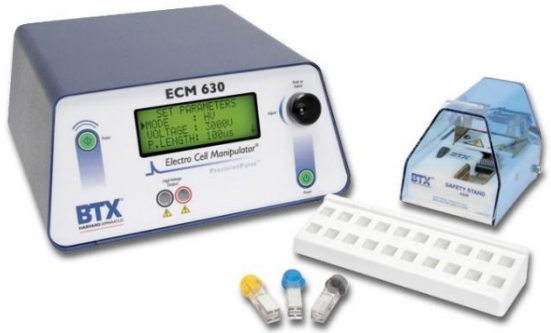


Ordering Information

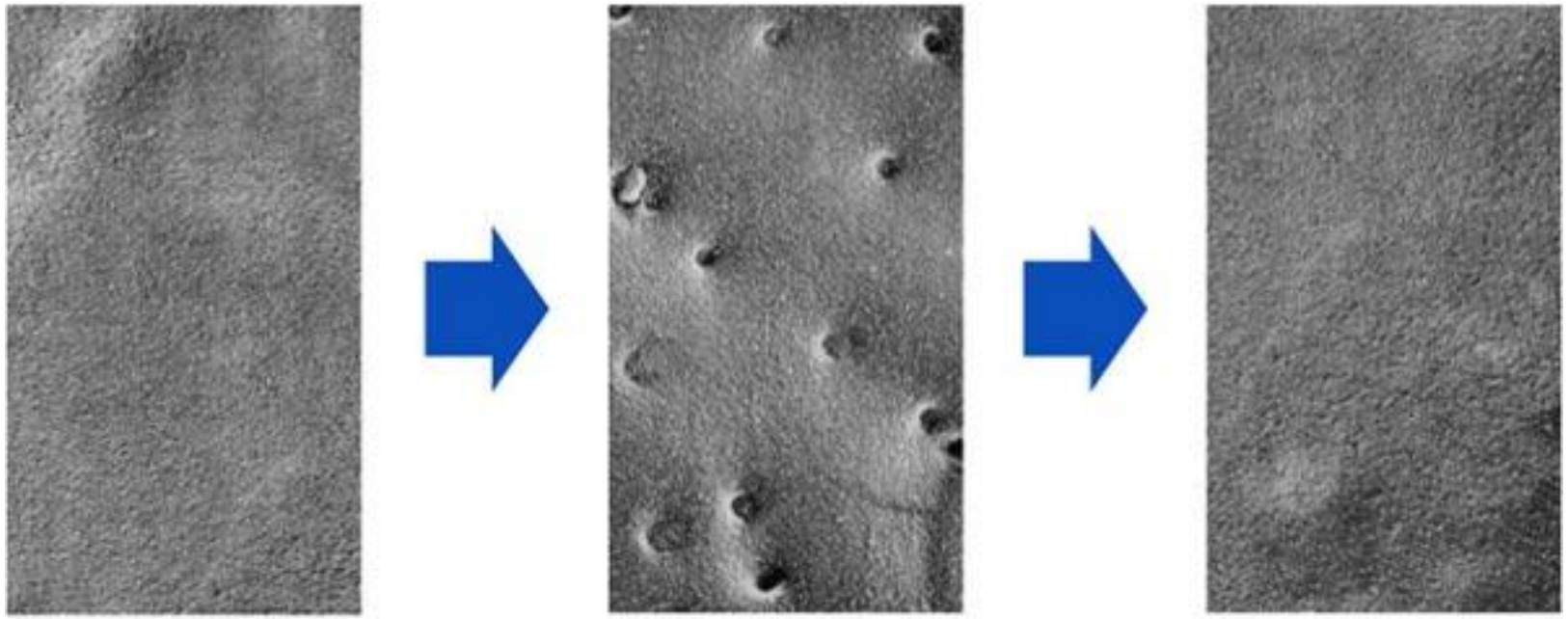
Catalog #	Name	Size	List Price (EUR)
L3000015	Lipofectamine® 3000 Transfection Reagent	1.5 mL	864,00
L3000150	Lipofectamine® 3000 Transfection Reagent	15 mL	7.380,00

Gene Delivery Using Physical Methods

- **ELECTROPORATION:**



- The cells are placed in a solution containing DNA,
- are exposed to a short electrical impulse which transiently produces pores i their membranes



Gene Delivery Using Physical Methods

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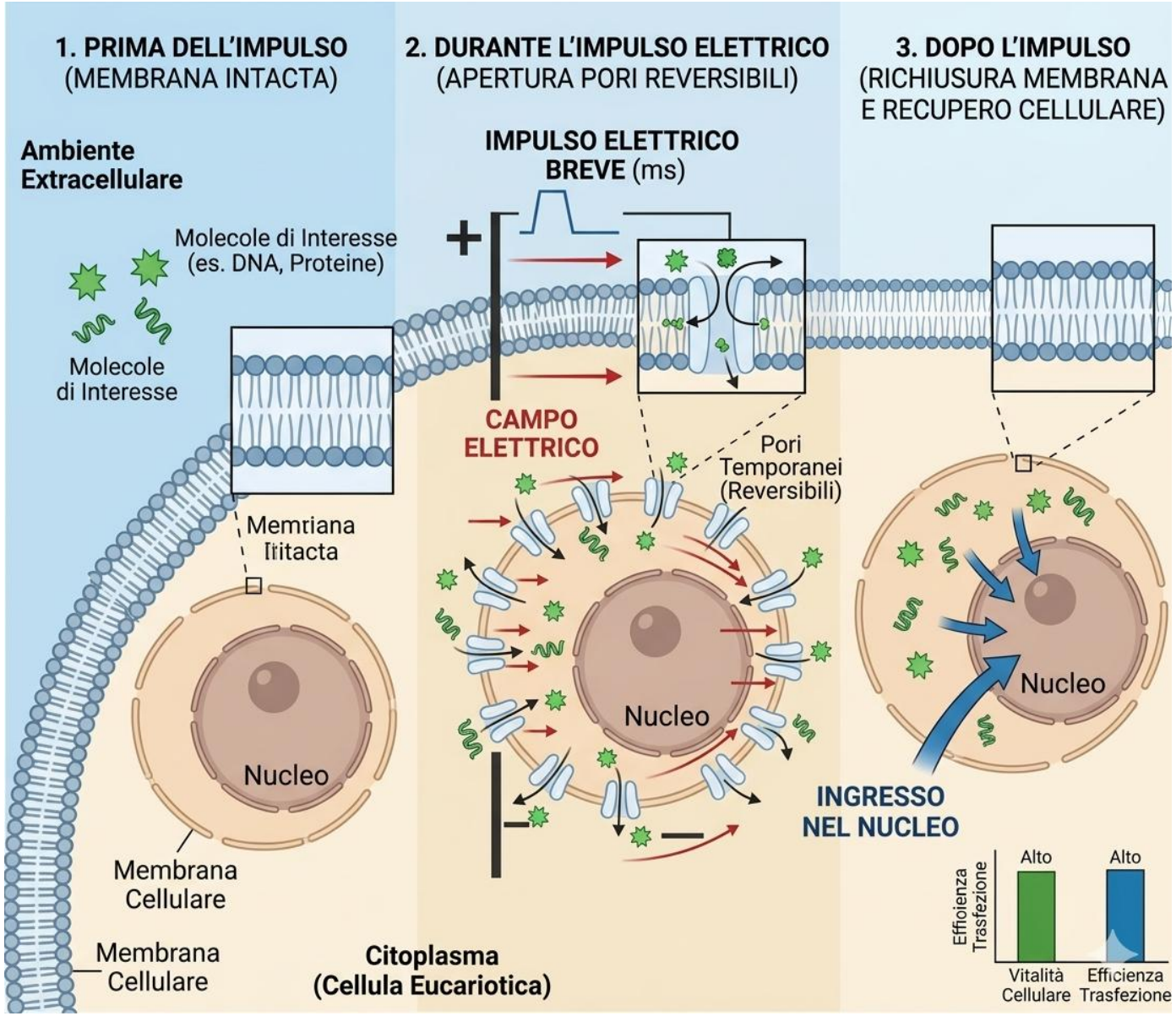
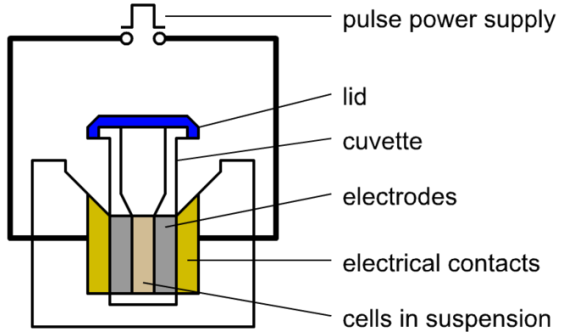
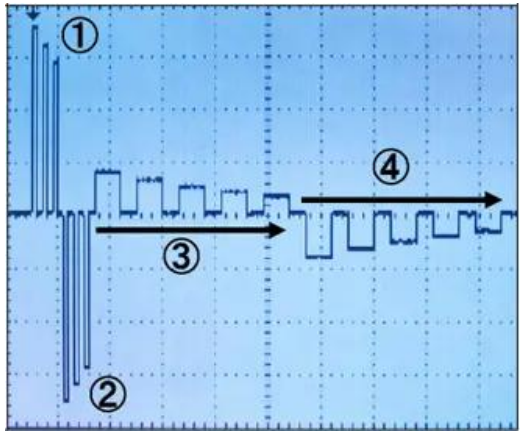


Electroporation is based on a simple process.

- Host cells and selected molecules are **suspended in a conductive solution**, and an electrical circuit is closed around the mixture.

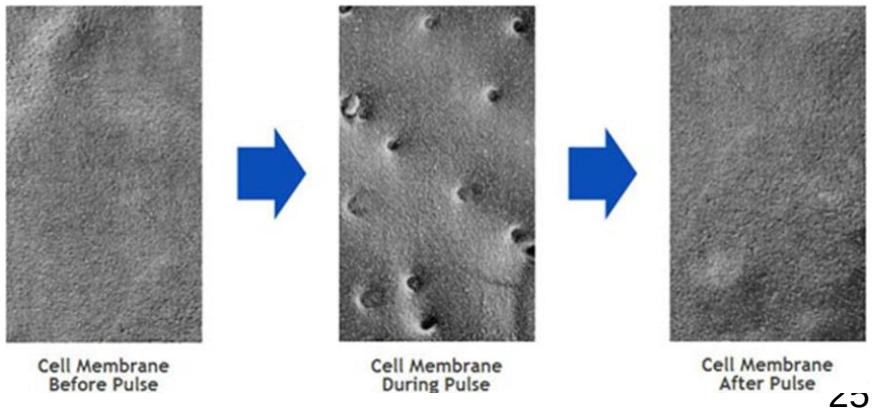


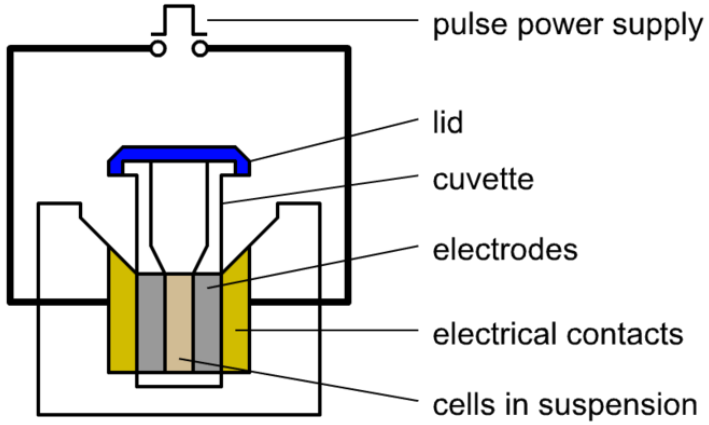
- An electrical pulse at an optimized voltage and only lasting a **few microseconds to a millisecond is discharged through the cell suspension**.

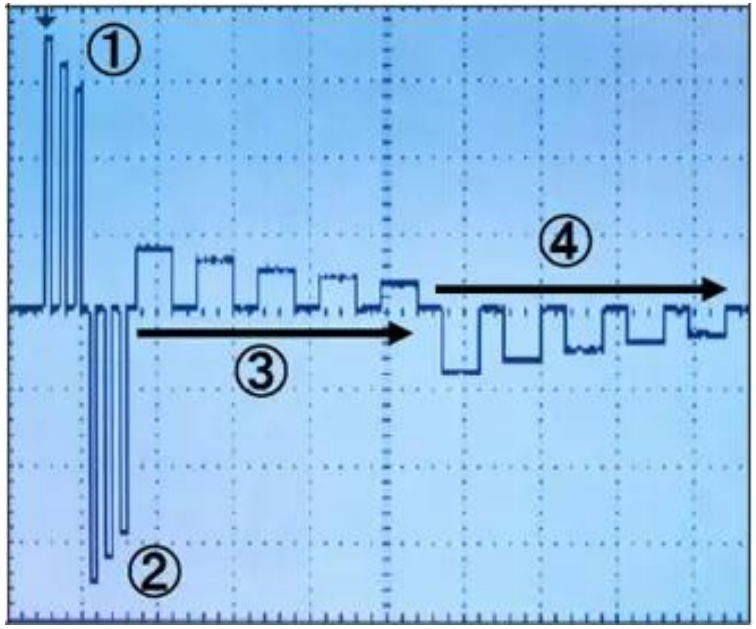


- This disturbs the phospholipid bilayer of the membrane and results in the formation of temporary pores.

- charged molecules like DNA aredriven across the membrane through the pores in a manner similar to electrophoresis







1.Poring Pulse Mode:

1. **Higher voltage, shorter duration**, multiple pulses, voltage decay; This is for forming pores (small holes) in cell membrane with minimum damage.

2.Polarity Exchanged Poring Pulse

1. This can be applied to tissue transfection as well.

3.Transfer Pulse Mode:

1. **Lower voltage, longer duration,** multiple pulses, voltage dcay; This is for delivering the target molecules (DNA, RNA, etc.) into cells with minimum damage.

4.Polarity Exchanged Transfer Pulse

1. This can increase the transfection efficiency

Gene Delivery Using Physical Methods

• ELECTROPORATION:

The **main advantages** of electroporation are:
- its applicability **for transient and stable transfection of all cell types**.

- electroporation is **easy and rapid**,

- it is able **to transfect a large number of cells in a short time** once optimum electroporation conditions are determined.

The **major drawback** of electroporation **is**
- substantial cell death caused by high voltage pulses and only partially successful membrane repair, requiring the use of greater quantities of cells compared to chemical transfection methods.

Gene Delivery Using Physical Methods

• ELETTROPORAZIONE:

VANTAGGI

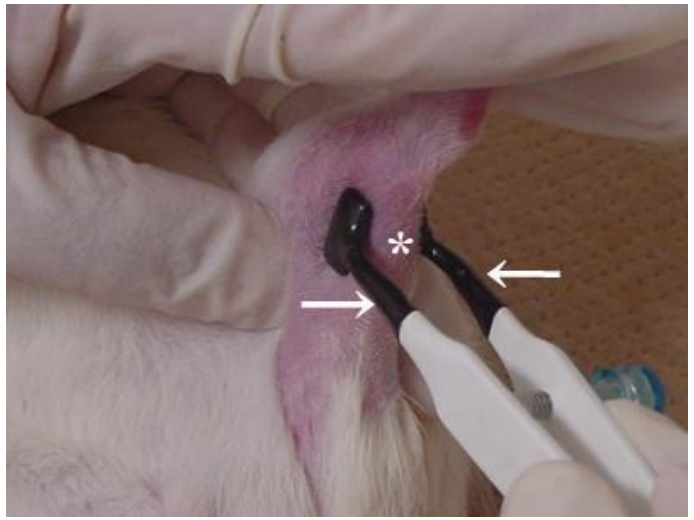
✓ *Può essere utile per quei tipi cellulari in cui non si ottengono risultati con i metodi classici*

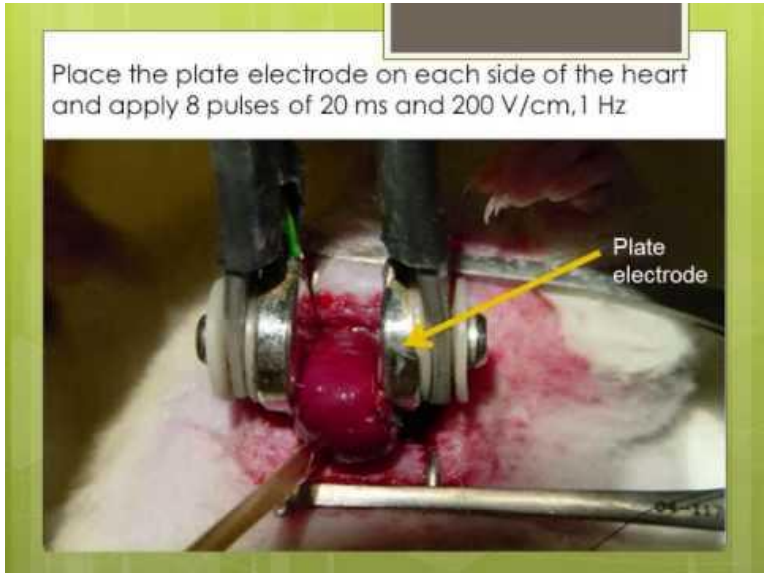
✓ *Il DNA entra direttamente senza passare attraverso il compartimento endosomiale dove può aver luogo la degradazione/**mutazione** dell'acido nucleico*

SVANTAGGI

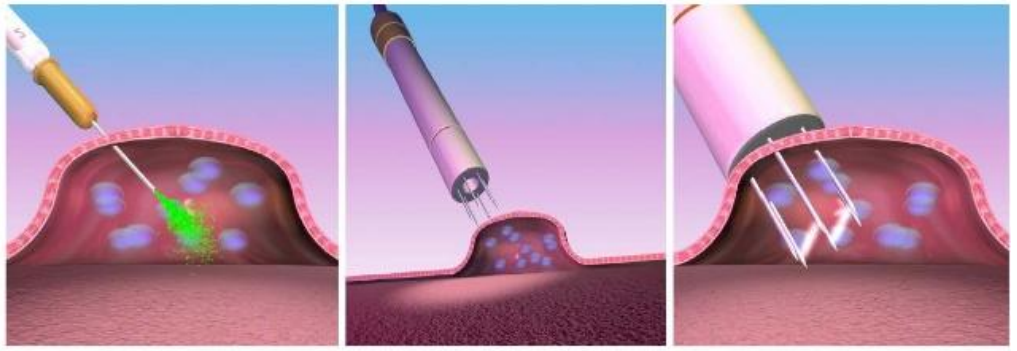
✓ *Necessità di ottimizzare le condizioni del campo elettrico -intensità e durata-*

✓ *Elevato livello di tossicità (50%)*

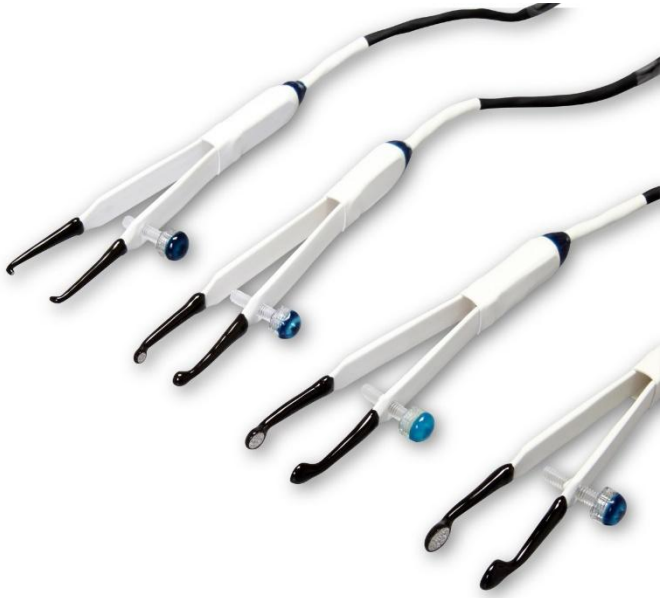


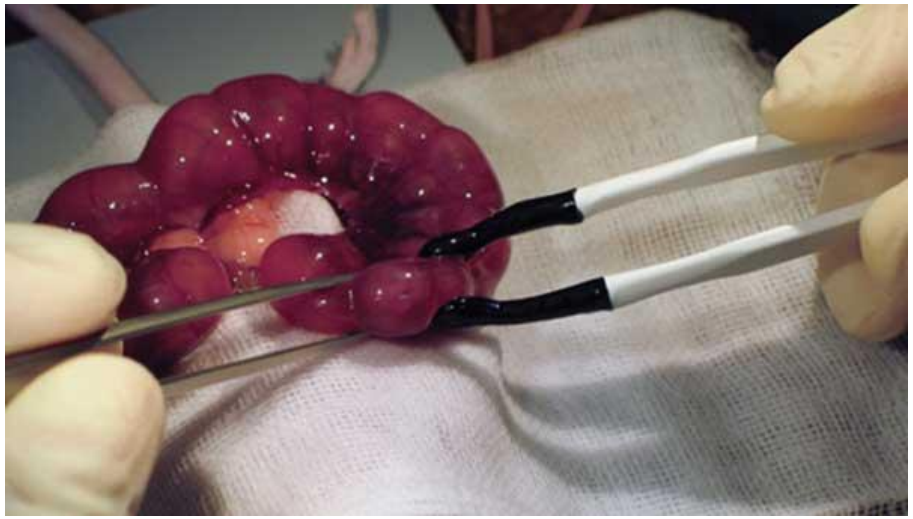


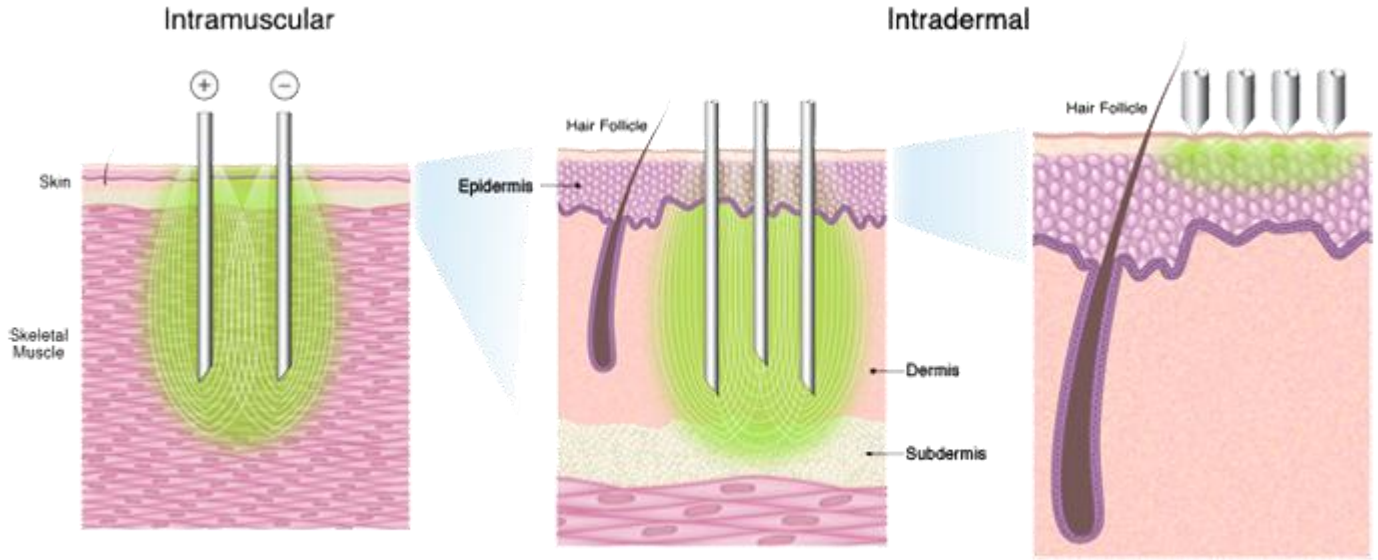
Intratumoral Injection of IL-12 plasmid followed by *in vivo* Electroporation



Injection of plasmid | Electrode Insertion | Electroporation







Gene Delivery Using Physical Methods

- **Microinjection**

Microinjection is a **direct method** to introduce DNA into either **cytoplasm** or **nucleus**.

It is a microsurgical procedure conducted **on a single cell**, using

- a glass needle (i.e., a fine, glass microcapillary pipette)

- a precision positioning device (a micromanipulator) to control the movement of the micropipette

- a microinjector.

Gene Delivery Using Physical Methods

- **MICROINIEZIONE:**

VANTAGGI

✓ *effettuare il trasferimento selettivamente nel citoplasma o nel nucleo*

✓ *aumenta efficienza di espressione*

✓ *modulare facilmente il livello di espressione*

SVANTAGGI

✓ *Tecnicamente impegnativa*

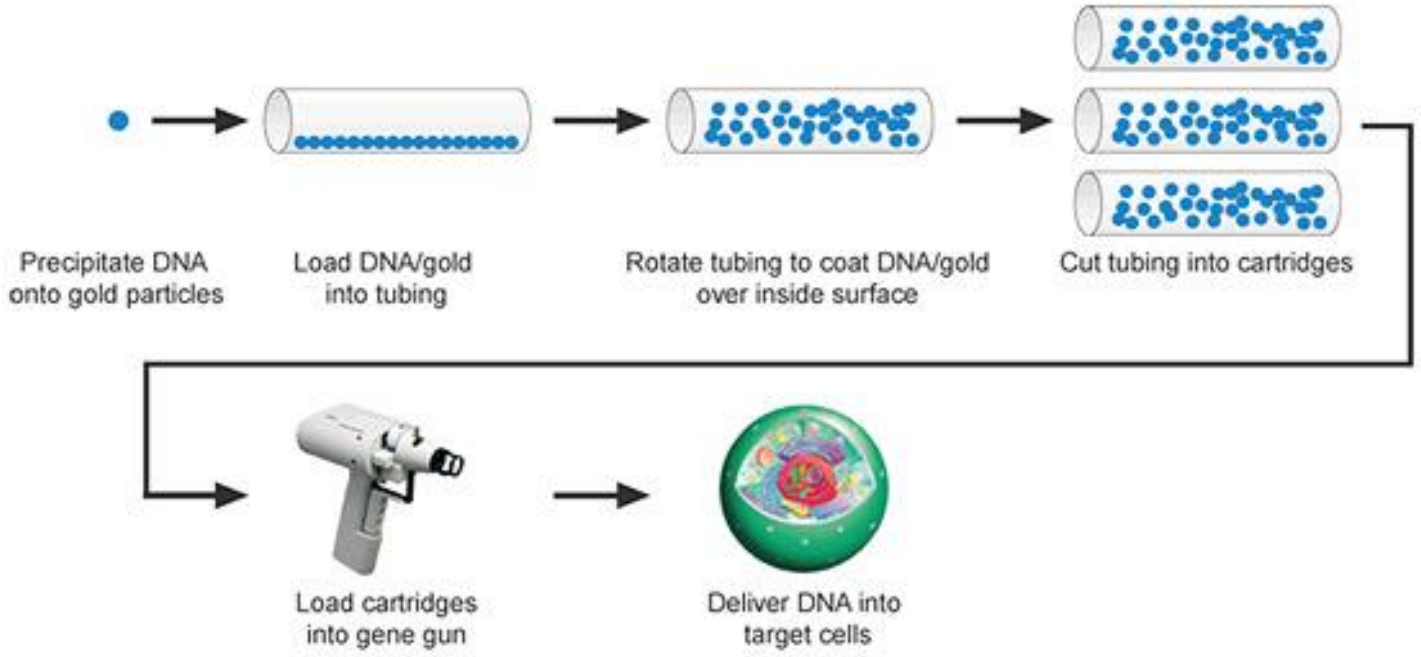
✓ *Poche cellule alla volta*

✓ *COSTOSO*

Gene Delivery Using Physical Methods

• GENE GUN:

- **DNA-coated** gold (or tungsten) microcarriers are prepared
- The particles are distributed on the inner wall of a plastic cartridge
- Use adjustable **helium pressu**re to accelerate gold microbullets and "fire" them directly at the target



Gene gun Helios™ by BioRad is used to transfect cells in cultures and plant leaves

VANTAGGI

✓ *Possibilità di lavorare **in situ***

✓ *Applicabile a quei sistemi cellulari poco responsivi ad altri carrier per il trasferimento genico anche in vivo*

✓ *Possibilità di fare studi di espressione genica in condizioni fisiologiche:*
- *espressione tessuto specifiche*
- *studi sullo sviluppo*

✓ *Possibilità di usare meno DNA e meno cellule di partenza*

SVANTAGGI

✓ *Trattamento invasivo*

✓ *Efficienza fortemente dipendente dal tipo cellulare*

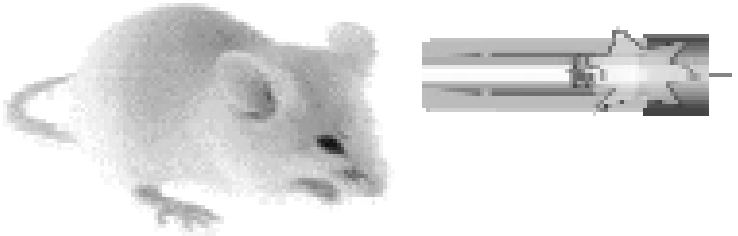
✓ *Molti parametri da controllare*

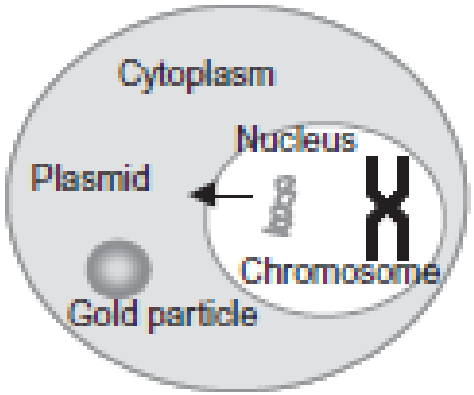
✓ *COSTOSO*

DNA is adsorbed onto gold particles

Particles are placed in a cartridge

The cartridge is coupled to the "gene gun", and the particles are accelerated against the animal's skin





On hitting the cell, the DNA is released from the particle and gets to the nucleus, where it controls the protein synthesis