

Transfection of Mammalian cell with plasmid DNAs by Calcium phosphate precipitation method

Dr Eftekhar (PhD)

Associate professor of Clinical Biochemistry

Transfection vs Transformation

- Introduction of foreign DNA into eukaryotic cell is often called **transfection**
- Introduction of DNA into bacterial cells for genetic manipulation is called **transformation.**

The transfer of a gene from one bacterium to another by a bacteriophage is called **Transduction.**

Transfection Methods

- **Biochemical methods:** DEAE-dextran, calcium phosphate, and liposome-mediated transfection methods.
- **Physical methods:** direct micro-injection of materials and electroporation.

Stable Vs Transient Transfection

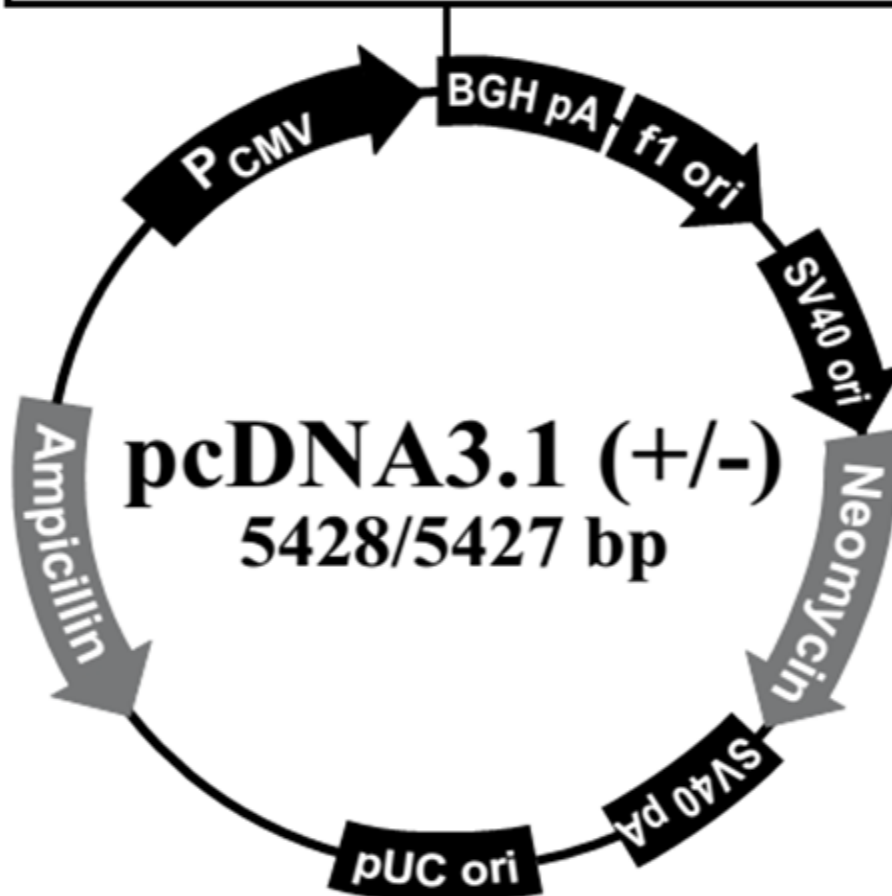
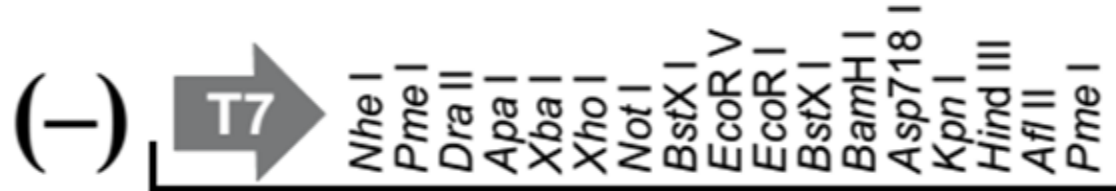
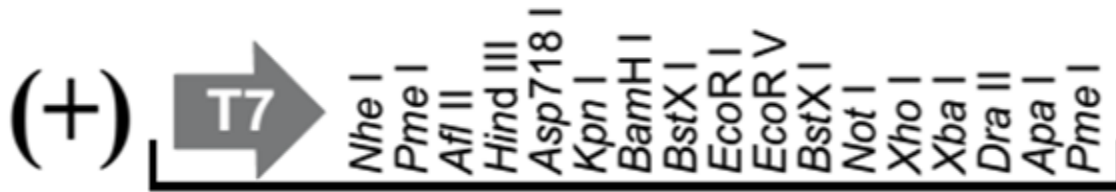
- **Transient Trans.:** DNA introduced into the cell is not integrated into the nuclear genome, the foreign DNA will be diluted through mitosis or degraded.
- **Stable Trans.:** transfected gene actually integrate in the genome of the cell and its daughter cells.

Transfection Applications??

Requierments for successful Transfection

1- High quality DNA

- Ratio $260/280 = 1.8$
- Purify with invitrogene kit (miniprep)
- Should be concentrated



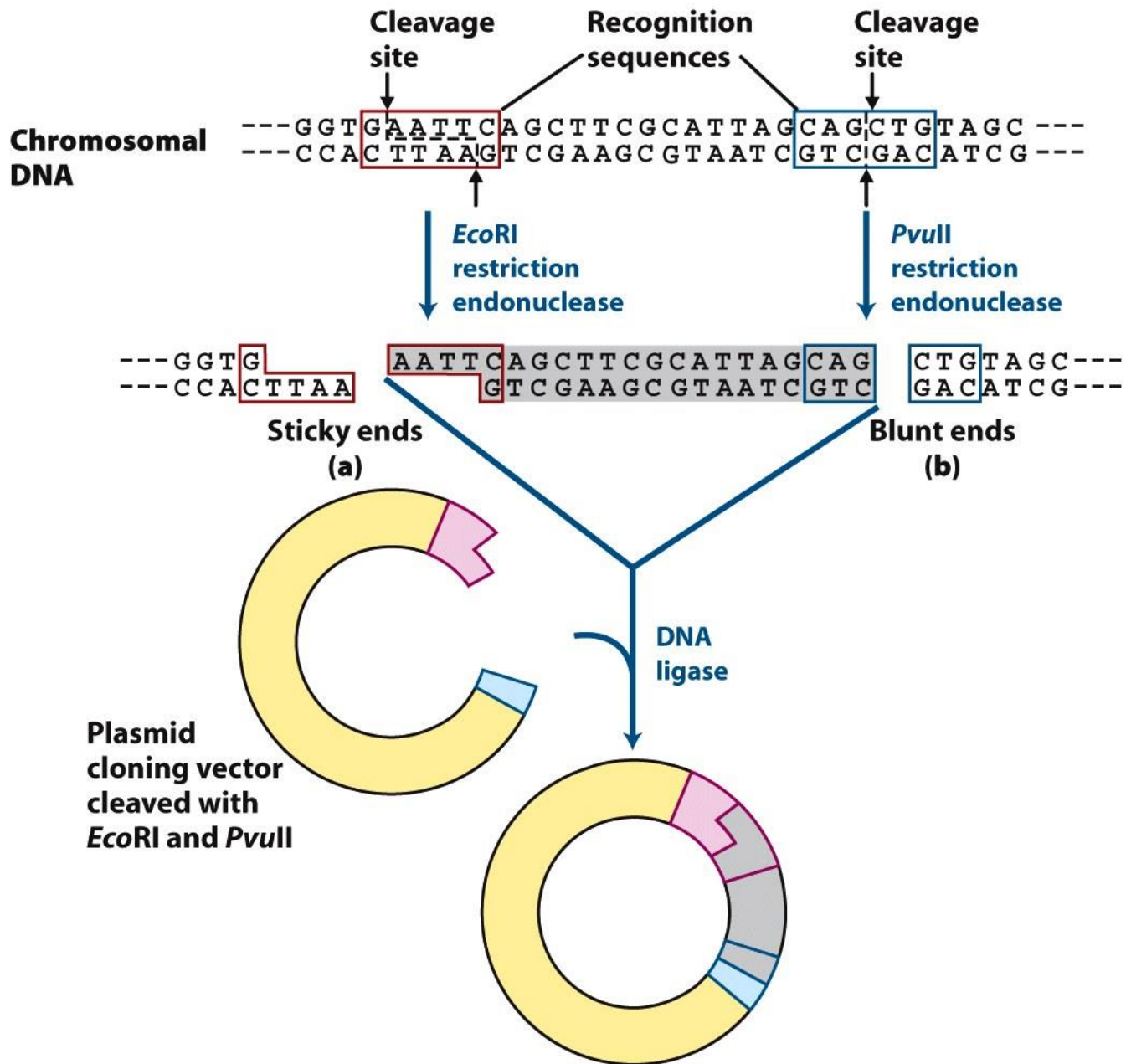
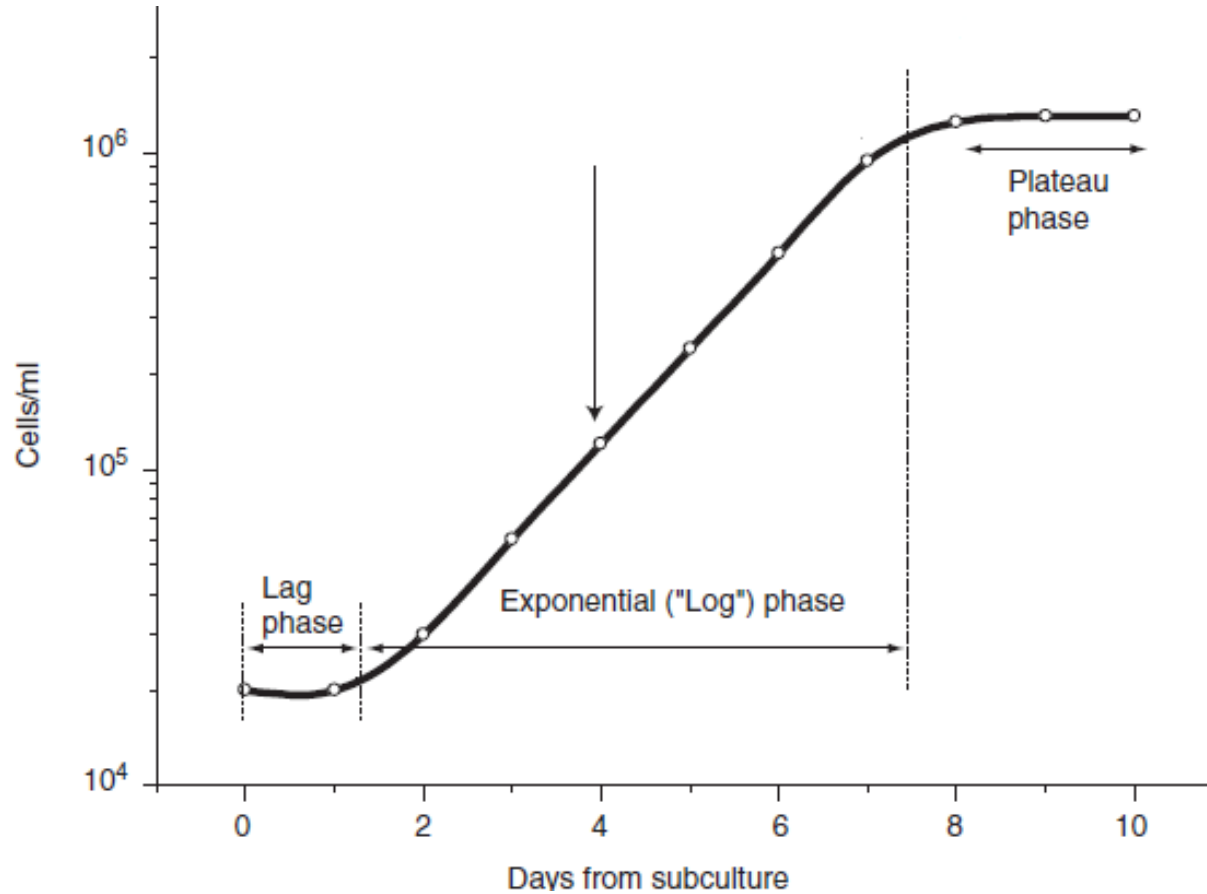


Figure 9-2ab
Lehninger Principles of Biochemistry, Fifth Edition
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2- Growth cell condition



Cell growth curve

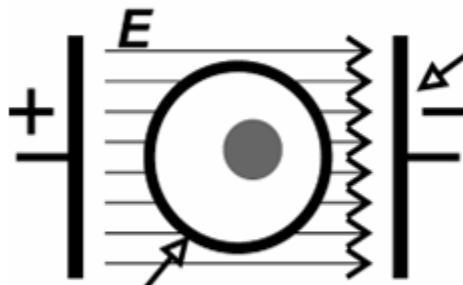
3-Determination of antibiotic sensitivity for stable transfection??

Electroporation

- Electroporation is a method which utilizes an electric field to introduce DNA or other macromolecules into cells .
- When a cell is exposed to a pulse of high electric field, its cell membrane quickly becomes permeabilized.
- During this permeabilized state, macromolecules from external medium can enter the cell.

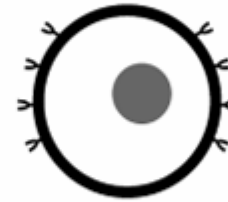
exposure of cell to electric field

electrode



cell

permeabilized cell membrane



insertion of proteins into cell membrane



introduction of small molecules



introduction of large molecules

destruction of cell membrane



Transfection by Electroporation

Advantages of electroporation

- 1-applicable to perhaps **all cell** types (animal, plant, yeast, bacteria).
- 2-yields a high frequency of stable transformants and has a high efficiency of transient gene expression
- 3-is simple. takes only minutes
- 4-No special treatment of DNA is required

Equipment



Materials and Equipment

1- Pulse Generator

2- Sample Chamber

3- solutions

4-DNA

5-Cell

1-Pulse Generator (PG)



- The basic equipment required for ele. is a high-power PG for applying the electric field to the target cells.
- The major function of the PG is to generate a high voltage pulse.
- When the cells are placed between a pair of electrodes with a gap distance d , the electric field is equal to:

- $E=V/d$

V= voltage of applied pulse

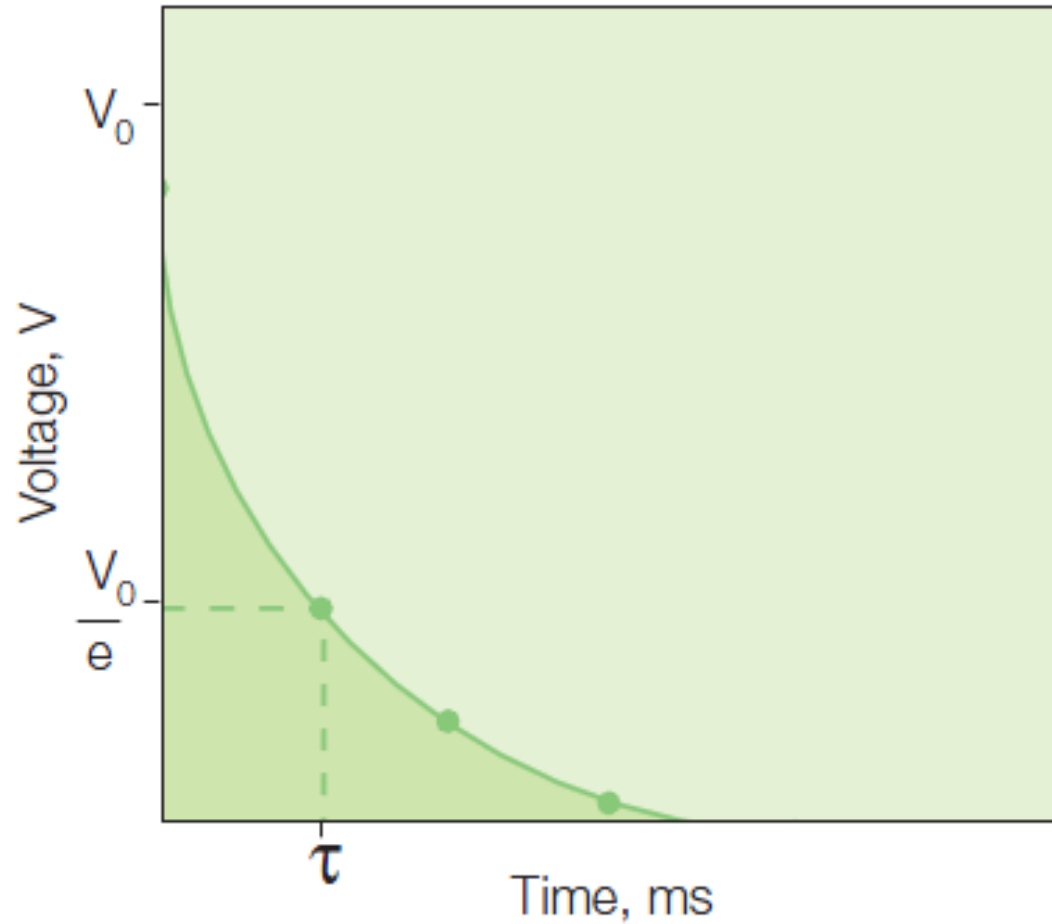
d=electrode gap distances (0.1-0.4 cm)

- Since the field strength required to electroporate animal cells is on the order of 1-2 kV/cm, the PG must be capable of supplying adequate voltage

Two different types of waveforms have been used to electroporate cells:

A: Exponential decay pulse

- Generated by discharging a capacitor which is precharged at a high voltage; thus, it is also called the “capacitor-discharge” (CD) pulse.
- When a capacitor is discharged into sample, the V across the electrodes raised rapidly to the peak voltage then decline over time, t , with an exponentially decay wave form.



Exponential decay pulse

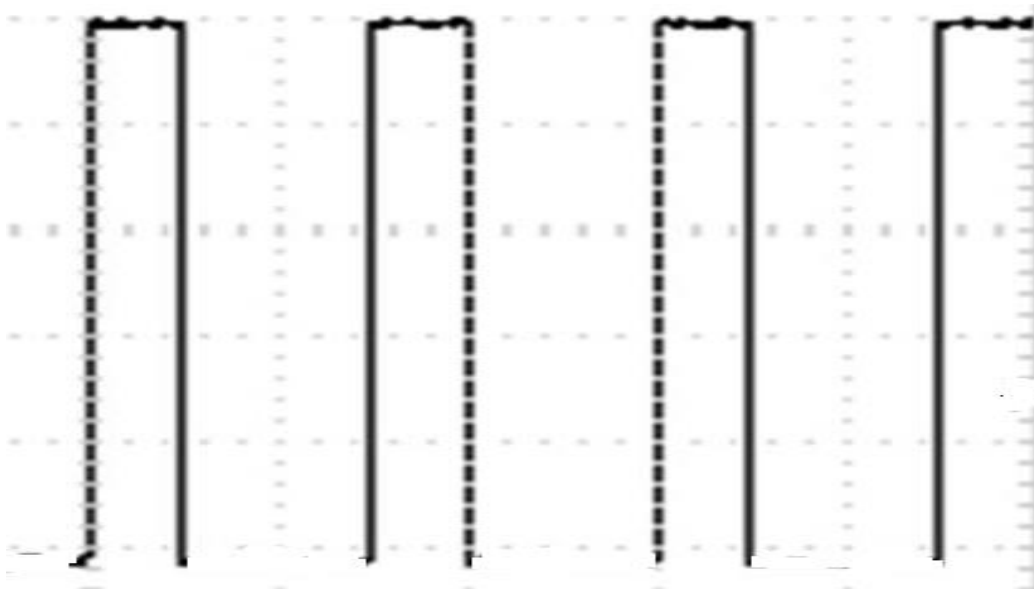
- The ele. is usually accomplished with only one pulse.
- The following combination of parameters may be entered:
 - 1- Capacitance + Voltage
 - 2- Time constant + Voltage

Time constant (τ) (msec)=(Capacitance x Resistance)

At present, most of the commercially available Ele. devices are generators of CD pulses.

B: Rectangular pulse or square wave pulse (SW)

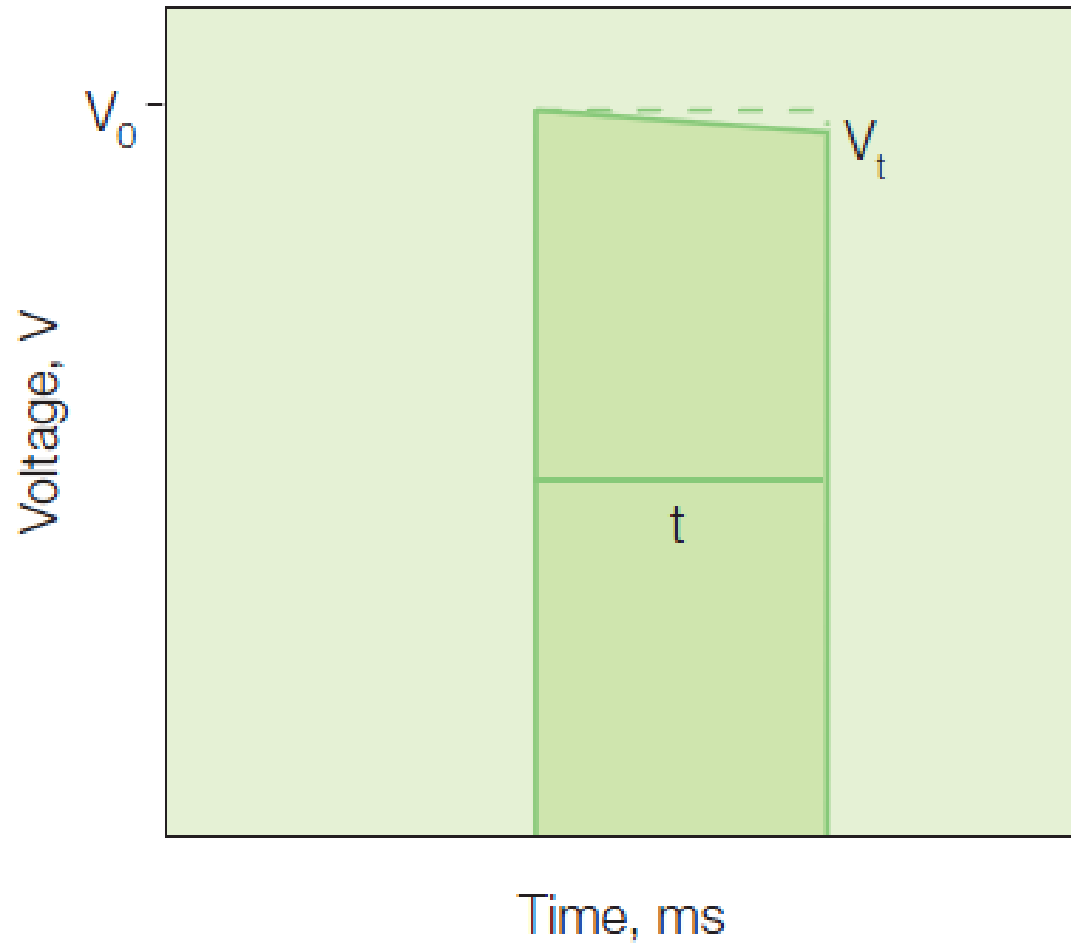
Truncating the pulse from a capacitor after discharging it into the sample generates SW.



Electroporation using SW maybe used to deliver a series of multiple pulses to the cells.

For sensitive and hard to transfect cell line increase efficiency and viability.

SW has the same V at the end as at the beginning of the pulse.



Square wave pulses

Square wave pulses are characterized by:

1- Voltage

2- Length of each pulse (msec)

3- The number of pulses

4- The length of the interval between pulses (Sec)

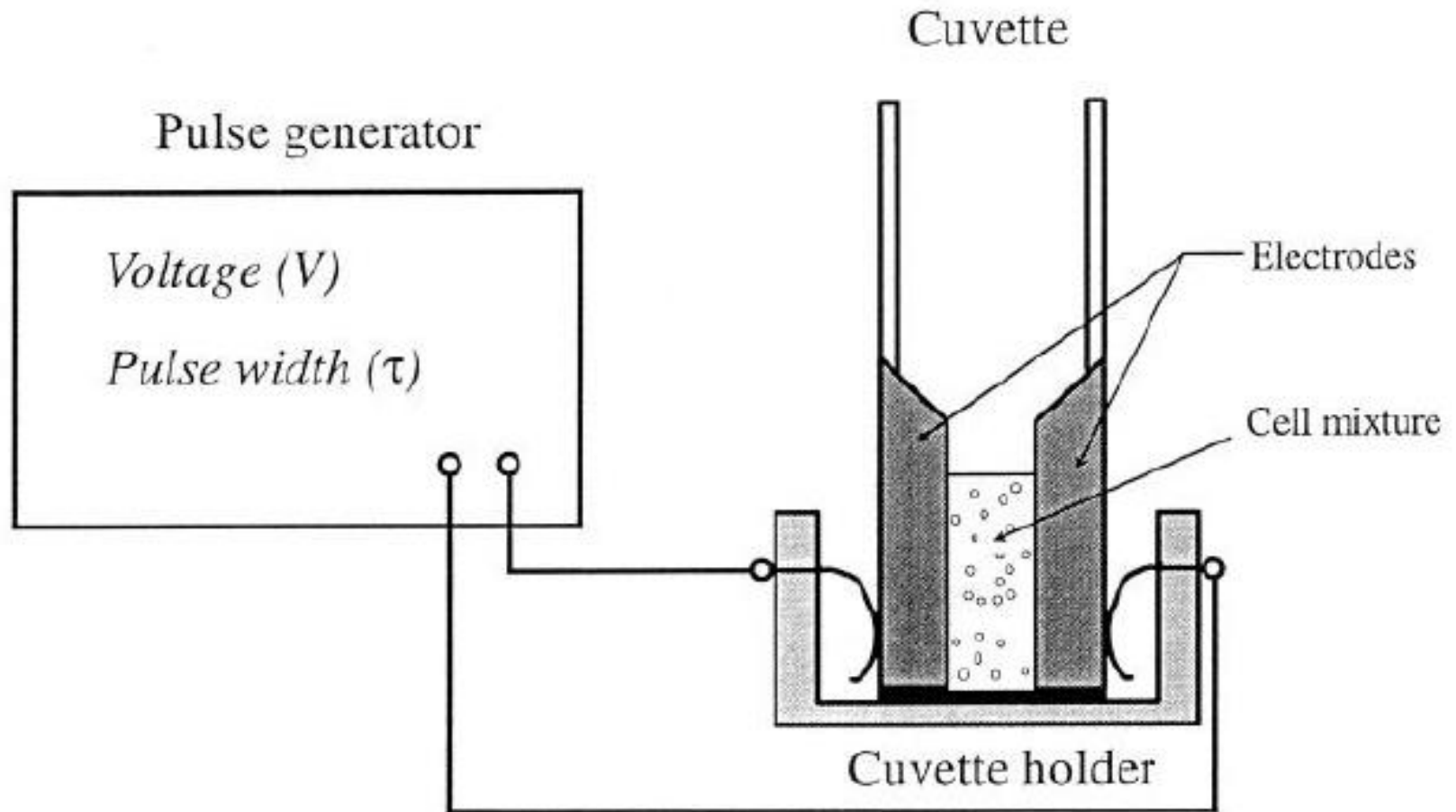
2-Sample Chamber



- has two functions: to contain the cell mixture, and to provide a pair of electrodes for applying the external electric field
- The main body of the chamber is made of transparent nonconducting material, such as polystyrene.
- The electrodes are made of metal.



Three different Cuvettes for electroporation



simplified diagram showing the basic components of the electroporation device and their arrangement.

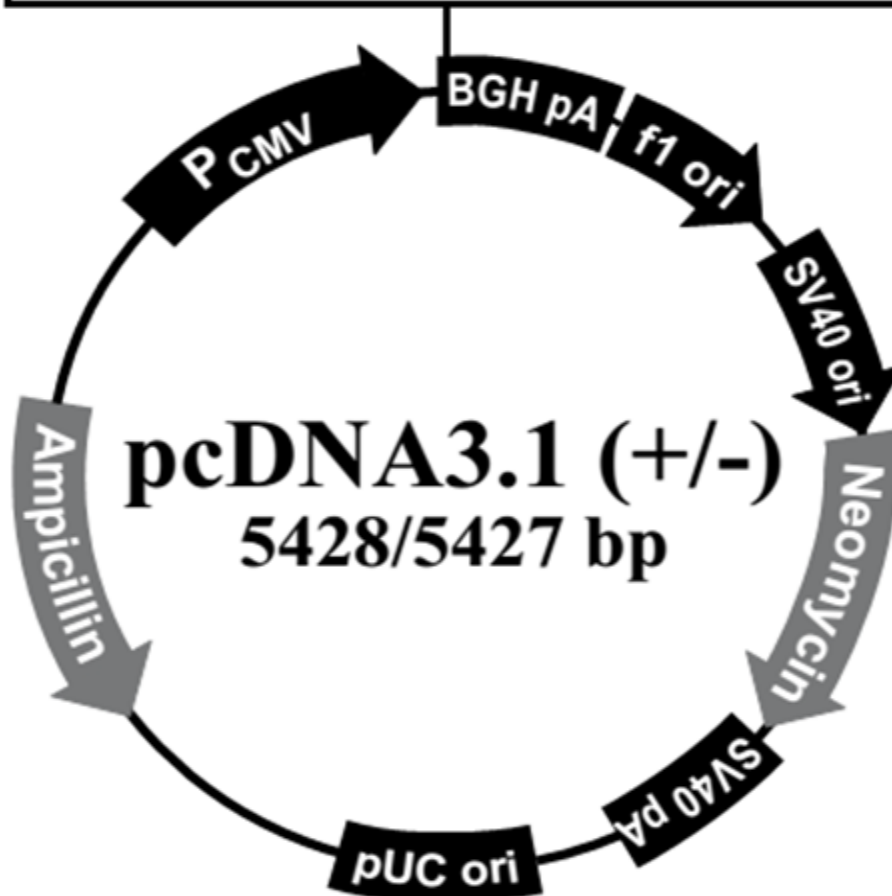
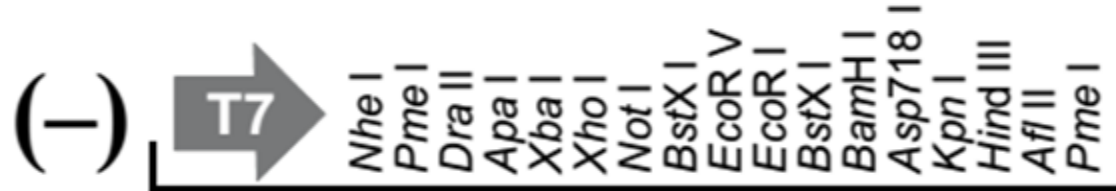
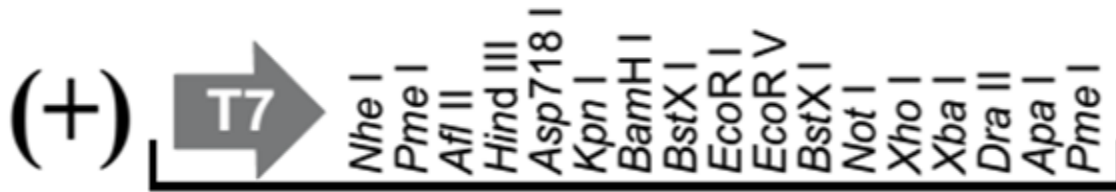
Electroporation Cuvette Selection Guide

0.4 cm gap cuvettes	Wider gap for low field strength, used for mammalian and other eukaryotic cells
0.2 cm gap cuvettes	Narrow gap for high field strength, used for yeast, bacterial, and eukaryotic cells
0.1 cm gap cuvettes	Narrowest gap and shallow bottom for small sample volumes (40–80 μ l) and very high field strength, used for yeast and bacterial transformation

- The solution should be made with triple distilled water and filtered in 0.2 um filter for sterilization.
- The poration medium can be stored at 4°C and used over a period of several months.

4-DNA

- The DNA can be used directly in its normal buffer, such as Tris buffer or distilled water
- *For stable trans., DNA should be linearized by cleavage with a restriction enzyme that cuts in a nonessential region and purified by phenol extraction and ethanol precipitation .*
- *For transient expression, the DNA may be left supercoiled.*



- 4-20 μ g DNA per transfection

- *Cotransfection can be done with 1 μ g of a selectable marker containing DNA and 10 μ g of the DNA containing the gene of interest.*

Selecting the Proper Electrical Parameters

- Both SW and Exponential decay pulses are used for ele. of mammalian cells.
- Two key electroporation parameters are:

1-The maximum voltage of the shock

2-The duration of the current pulse

- For sensitive cell line try a capacitance between 950 and 1050 μF and test a range of voltages from 200 to 350 V in HeBS buffer.
- some cells (especially primary cells) are very easily killed and thus electroporate poorly at the high voltages needed for PBS or HeBS electroporation buffers. (Use tissue culture medium especially RPMI 1640).

Procedures for Gene Transfer by Electroporation

- 1- harvest cells in midlog growing phase, using a trypsin treatment and centrifuge it.
- 2- resuspend cell in poration medium and add plasmid DNA
- 3- transfer to cuvette and mix
- 4-Set elec. Parameters and pulse it.

5-transfer to plate using 0.5 ml media and incubate in 37°c.

6- assay transient gene expression 24-48h

7- For stable transfection, allow the cells to double twice (48 -55 hours) before plating in selective medium.

Important notes

- 1-To achieve high efficiency, cells must be harvested during the midlog phase (replate the cultured cells within 2 d before ele.)
- 2- concentration of DNA ($1\mu\text{g}/\mu\text{l}$)
- 3- carrier DNA, is added to the cell mixture during ele.
- 4- temperature
- 5- transfection efficiency can be enhanced by using a recovery treatment

6- cuvette

7-like other molecular biology procedures, protocols for ele. can vary significantly among different laboratories.

- If the result of ele. is less than satisfactory in spite of the efforts to optimize the voltage and pulse width, one may try to change the other experimental parameters, such as the poration medium, temperature, cell treatment, addition of carrier DNA, etc.

- **Lipofection** (or liposome transfection) is a technique used to inject genetic material into a cell by means of liposomes, which are vesicles that can easily merge with the cell membrane since they are both made of a phospholipid bilayer.
- Lipofection generally uses a positively charged (cationic) lipid to form an aggregate with the negatively charged (anionic) genetic material.
- A net positive charge on this aggregate has been assumed to increase the effectiveness of transfection through the negatively charged phospholipid bilayer

- **Lipofectamine** or **Lipofectamine 2000** is a common transfection reagent, produced and sold by invitrogen.
- It is used to increase the transfection efficiency of RNA (including mRNA and siRNA) or plasmid DNA into in vitro cell cultures by lipofection.
- Lipofectamine reagent contains lipid subunits that can form liposome in an aqueous environment, which entrap the transfection materials, i.e. DNA plasmids.

- Calcium phosphate–based eukaryotic cell transfection can be used for both transient and stable transfections.
- A precipitate containing calcium phosphate and DNA is formed by slowly mixing a HEPES ((N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic)-buffered saline solution with a solution containing calcium chloride and DNA.

- This precipitate adheres to the surface of cells and should be visible in the phase contrast microscope the day after transfection.
- Depending on the cell type, up to 10% of the cells on a dish will take up the DNA precipitate through an as yet undetermined mechanism.

Transfection using CP-DNA precipitate formed in HEPES

Solution A
Plasmid DNA
calcium solution

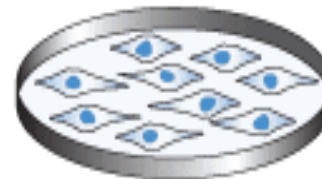


Add
Solution A
to Solution B
while
vortexing



Incubate 20 min

Apply
transfection
solution to
subconfluent
cell culture



Incubate 2–12 hr

Replace
transfection
solution with
complete
growth medium

Assay for transient
gene expression
or
Begin selection for
stable transformants
(24–72 hr
post transfection)

Solution B
2X HBS

Glycerol and DMSO shock of mammalian cell

- Transfection efficiency in some cell lines is dramatically increased by “shocking” the cells with either 10% glycerol or DMSO.
- Precipitates are left on the cell for only 4 to 6 hr, and the cells are shocked immediately after removal of the precipitate.

2- BES- buffer method

- A solution of CaCl_2 , plasmid DNA, and N,N-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid (BES) buffer, pH 6.95, is added to a plate of cells containing culture medium.
- The plates are incubated overnight while a calcium phosphate–DNA complex forms gradually in the medium under an atmosphere of 3% CO_2 .
- With this method, 10% to 50% of the cells on a plate stably integrate and express the transfected DNA.
- Transient expression under these conditions is comparable to that obtained with the Basic Protocol.
- Glycerol or DMSO shock does not increase the number of cells transformed.

Critical Parameters and Troubleshooting

- CP transfections do not always work. the most common reason for failure is a 2×HEPES solution that is no longer at the appropriate pH.
- Efficiency test should be done. How?
- The pH of the solution can change during storage and an old 2× HEPES solution may not work well.

3-Replenish the selective media every 3-4 days, and observe the percentage of surviving cells.

4- the appropriate level of G418 for selection is the lowest con. that kills the cell within 10-14 days.