
Exp. 2.1 Preparation of Competent Cells and Heat-Shock Transformation

Objective To prepare competent cells of bacteria by chemical treatment and transformation by heat shock.

Introduction

Competent cells are bacterial cells that can accept extra-chromosomal DNA or plasmids (naked DNA) from the environment. The generation of competent cells may occur by two methods: natural competence and artificial competence. Natural competence is the genetic ability of a bacterium to receive environmental DNA under natural or in vitro conditions. Bacteria can also be made competent artificially by chemical treatment and heat shock to make them transiently permeable to DNA. Natural competence dates back to 1928, when Frederick Griffith discovered that prepared heat-killed cells of a pathogenic bacterium could transform the nonpathogenic cells into pathogenic type. Natural competence has been reported in many bacterial strains, i.e. *Bacillus subtilis*, *Streptococcus pneumonia*, *Neisseria gonorrhoeae* and *Haemophilus influenza*. The natural competence phenomenon is highly regulated in bacteria and varies across genera. In some genera, certain portions of the population are competent at a time, and in others, the whole population gains competence at the same time. When the foreign

DNA enters inside the cells, it may be degraded by the cellular nucleases or may recombine with the cellular chromosome. However, natural competence and transformation is efficient for linear molecules such as chromosomal DNA but not for circular plasmid molecules Fig. 2.1.

Artificial competence is not coded by the genes of the bacterial cells. It is a laboratory procedure in which cells are passively made permeable to DNA using unnatural conditions. The procedure of artificial competence is relatively simple and easy and can be used to engineer a bacterium genetically. However, transformation efficiency is very low as only a portion of the cells become competent to successfully take up DNA.

Principle

As DNA is a highly hydrophilic molecule, normally it cannot pass through the cell membrane of bacteria. Hence, in order to make bacteria capable of internalising the genetic material, they must be made competent to take up the DNA. This can be achieved by making small holes in bacterial cells by suspending them in a solution containing a high concentration of calcium. Extra-chromosomal DNA will be forced to enter the cell by incubating the competent cells and the DNA together on ice followed by a brief heat shock that causes the bacteria to take up the DNA (Fig. 2.2). Bacteria no longer become stable when they possess holes on the cell membrane and may

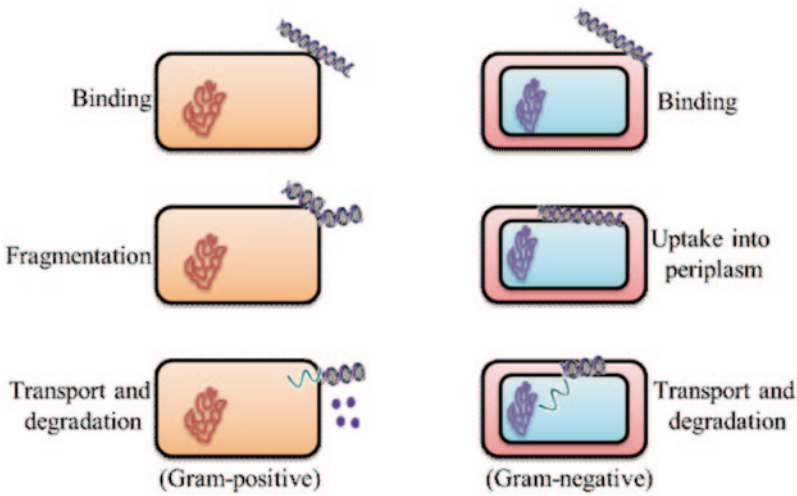


Fig. 2.1 Transformation pathways in Gram-positive and Gram-negative bacteria

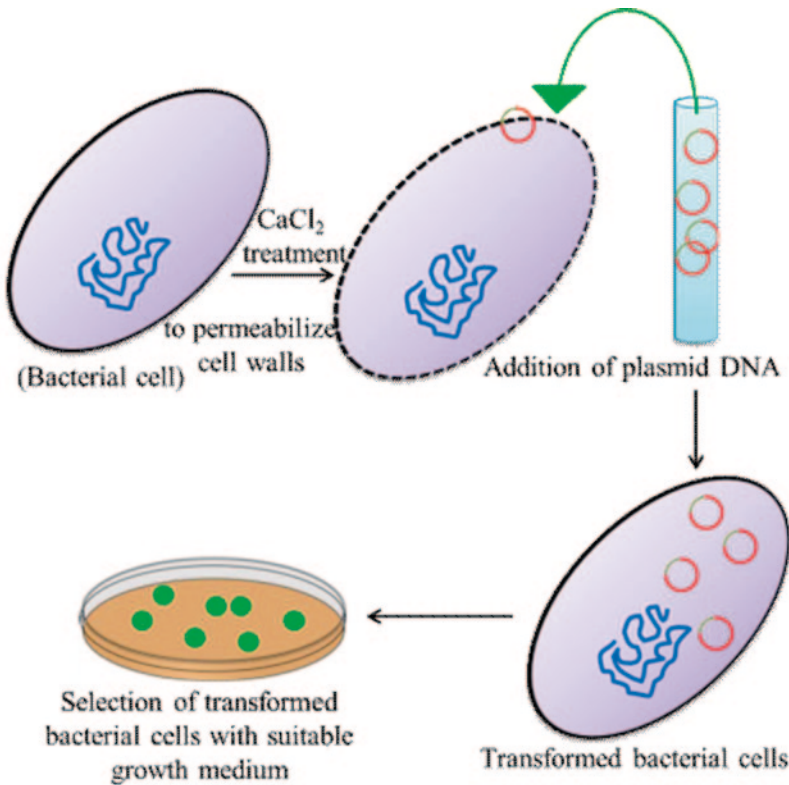


Fig. 2.2 Preparation of competent cells by CaCl_2 treatment and transformation

die easily. Additionally, a poorly performed procedure may lead to not enough competence cells to take up DNA. It has been reported that a naked DNA molecule is bound to the lipopolysaccharide

(LPS) receptor molecules on the competent cell surface. The divalent cations generate coordination complexes with the negatively charged DNA molecules and LPS. DNA, being a larger

molecule, cannot itself cross the cell membrane to enter into the cytosol. The heat shock step strongly depolarizes the cell membrane of CaCl_2 -treated cells. Thus, the decrease in membrane potential lowers the negativity of the cell's inside potential which ultimately allows the movement of negatively charged DNA into the cell's interior. The subsequent cold shock again raises the membrane potential to its original value.

In another method, the transformation storage solution (TSS) buffer method, competence is induced by polyethylene glycol (PEG). This technique is relatively simple and does not require heat shock. Competence of bacterial cells is induced by the addition of low concentrations of divalent cations Mg^{2+} and dimethyl sulfoxide (DMSO). PEG helps in shielding the negative charges on the DNA molecule and host cell membrane; thus, repulsion between them is reduced. The pH of the buffer is maintained at slightly acidic conditions to increase the cell's viability as well as transformation efficiency up to 10^7 – 10^8 .

Reagents Required and Their Role

Luria-Bertani Broth

Luria-Bertani (LB) broth is a rich medium that permits fast growth and good growth yields for many species including *E. coli*. It is the most commonly used medium in microbiology and molecular biology studies for *E. coli* cell cultures. Easy preparation, fast growth of most *E. coli* strains, ready availability and simple compositions contribute to the popularity of LB broth. LB can support *E. coli* growth ($\text{OD}_{600}=2$ – 3) under normal shaking incubation conditions.

Calcium Chloride

Calcium chloride transformation technique is the most efficient technique among the competent cell preparation protocols. It increases the bacterial cell's ability to incorporate plasmid DNA, facilitating genetic transformation. Addition of calcium chloride to the cell suspension

allows the binding of plasmid DNA to LPS. Thus, both the negatively charged DNA backbone and LPS come together and when heat shock is provided, plasmid DNA passes into the bacterial cell. Prepare 2000 ml of 50 mM Calcium chloride stock solution by adding 14.701 g of $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ in 2 l of milli-Q water, autoclave and store at 4°C .

Polyethylene Glycol

PEG is a polyether compound having many functions. In this case, it helps in shielding the negative charges present on the DNA and host cell membrane. This results in lowering of repulsion. In addition, PEG, being a larger molecule, coordinates with the water molecules present in the bacterial suspension. This results in the increased concentration and bioavailability of the plasmid DNA to pass through the membrane of a bacterial competent cell. In other words, a highly effective plasmid concentration results in a more effective DNA transformation into bacteria.

Dimethyl Sulfoxide

DMSO is an organosulfur compound and is a polar aprotic solvent. It can be dissolved both in polar and nonpolar compounds and is miscible with a wide range of organic solvents as well as water. DMSO brings together the reagents that have been added during the reaction. It also acts as a preserving agent as the prepared competent cells need to be stored at -80°C for a longer period without losing their viability.

MgCl_2

MgCl_2 acts in the same way as does CaCl_2 . It induces the ability of the cells to take up DNA by altering the permeability of the membranes. The negatively charged incoming DNA is repelled by the negatively charged macromolecules present on the bacterium's outer surface which is neutralized by the addition of MgCl_2 to neutralize the unfavourable interactions.

Procedure

By CaCl_2 Treatment

1. Inoculate the *E. coli* culture into the LB medium and incubate at 37°C for 24 h with vigorous shaking at 180 rpm.
2. Aliquot 0.5 ml of the grown culture into 50 ml of LB in a 200-ml conical flask. Pre-warm the broth to 37°C.
3. Incubate at 37°C with shaking at 180 rpm.
4. Monitor the growth regularly till the OD_{600} reaches to 0.35–0.4.
5. When suitable growth has been reached, chill the culture on ice.
6. Transfer the culture to an autoclaved centrifuge tube and collect the cell pellets by centrifugation at 6000 rpm for 5 min at 4°C. Discard the supernatant.
7. Resuspend the cell pellets in 20 ml of an ice-cold 50-mM CaCl_2 solution. Incubate the resuspended cells on ice for 20 min.
8. Collect the cell pellets by centrifugation at 6000 rpm for 5 min at 4°C.
9. Resuspend the cells with 2.5 ml of ice-cold 50-mM CaCl_2 . Optionally, if required to store the competent cells for a longer period, resuspend the cells with 2.5 ml ice-cold 50-mM CaCl_2 containing 10% glycerol.
10. Use 100 μl of the prepared competent cells for transformation.
11. Dispense the competent cells into aliquots of 100 μl and store them at -80°C for further use.
4. Split the culture into two 50-ml falcon tubes and incubate on ice for 10 min. All subsequent steps should be carried out at 4°C and the cells should be kept on ice wherever possible.
5. Centrifuge the cells for 10 min at 3000 rpm at 4°C temperature.
6. Discard the supernatant. The cell pellets should be sufficiently solid to pour off the supernatant and pipette out the remaining medium.
7. Resuspend the pellet in chilled TSS buffer. The volume of the TSS to use is 10% of the culture volume that was spun down.
8. Vortex gently to fully resuspend the culture, keep an eye out for the small cell aggregates even after the pellet is completely off the wall.
9. Add 100 μl aliquots to the chilled micro-centrifuge tubes and store at -80°C till further use.

Preparation of Transformation Storage Solution Buffer

To prepare 50 ml of TSS buffer, add 5 g PEG 8000, 1.5 ml 1 M MgCl_2 or 0.30 g $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 2.5 ml DMSO and add LB medium to 50 ml followed by filter sterilization with a 0.22- μm filter.

For Heat-Shock Transformation

By Using Transformation Storage Solution Buffer

1. Grow a 5-ml overnight culture of *E. coli* in LB broth medium. In the morning, dilute the culture into 25–50 ml of fresh LB medium in a 200-ml conical flask to dilute the culture at least by 1/100.
2. Grow the diluted culture to an OD_{600} of 0.2–0.5.
3. Put the micro-centrifuge tubes on ice so that they are cold when cells are aliquoted. If the culture is 'X' ml, 'X' number of tubes will be required. At this point, be sure that the TSS buffer is chilled. It should be stored at 4°C, but if freshly prepared, put it on an ice bath.
4. Split the culture into two 50-ml falcon tubes and incubate on ice for 10 min. All subsequent steps should be carried out at 4°C and the cells should be kept on ice wherever possible.
5. Centrifuge the cells for 10 min at 3000 rpm at 4°C temperature.
6. Discard the supernatant. The cell pellets should be sufficiently solid to pour off the supernatant and pipette out the remaining medium.
7. Resuspend the pellet in chilled TSS buffer. The volume of the TSS to use is 10% of the culture volume that was spun down.
8. Vortex gently to fully resuspend the culture, keep an eye out for the small cell aggregates even after the pellet is completely off the wall.
9. Add 100 μl aliquots to the chilled micro-centrifuge tubes and store at -80°C till further use.
1. Thaw 50–100 μl of competent cells carefully on ice.
2. Add 1 μl of plasmid solution (concentration 1 $\mu\text{g}/\mu\text{l}$) to 100 μl of competent cells.
3. Incubate the cells on ice for 30 min.
4. Quickly transfer the tubes to a water bath previously set at 42°C. Incubate for 1 min, and then quickly transfer to ice.
5. Add 1 ml LB broth medium to the tube.
6. Incubate at 37°C for 30 min to 1 h.
7. Streak out 50–500 μl of culture onto plates containing suitable antibiotic markers.

Observation

Observe the number of colonies grown on the plates after successful transformation. Transformation efficiency (Transformant/ μg of plasmid) may be calculated as the number of colony-forming units (CFU) produced by 1 μg of DNA and is measured by performing a control set of transformation reaction using a known quantity of DNA and then calculating the number of CFU formed per μg of DNA.

3. Keep cells on ice not longer than 3 h; do not use cells again that have been on ice.
4. You may stock-freeze the competent cells in liquid nitrogen. The stock-freezing might keep cells viable for a longer period, but it decreases the transformation efficiency by at least a factor of ten.
5. During preparation of TSS buffer, if non-chemically resistant filters (e.g. cellulose nitrate) are used, add DMSO after sterilization.

$$\text{Transformation efficiency} = \frac{\text{No. of transformants (colonies)} \times \text{Final volume at recovery (ml)}}{\mu\text{g of plasmid DNA} \times \text{Volume plated (ml)}}$$

Troubleshooting

Problems	Possible cause	Possible solutions
Low transformation efficiency with chemically competent cells	Impurities in DNA	Remove phenol, protein, detergents and ethanol by ethanol precipitation
	Excess DNA	Use not more than 1–10 μg of DNA for transformation purpose
	Cells handled improperly	Thaw cells on ice and use immediately; refreezing decreases the efficiency; do not vortex the cells
	Poor cell growth	Incubate cells for a minimum of 90 min during recovery and incubate the transformed colonies for a longer period
	Calculation errors	Ensure to use the correct dilution factor and DNA concentration to calculate efficiency
Few or no colonies	Too little DNA	Maintain the DNA concentration at 1–10 μg
	Wrong antibiotic concentration	Check the optimum antibiotic concentration of the vector
Satellite colonies	Degraded antibiotic	Check expiry date of the antibiotic, avoid repeated freeze-thaw cycle
	Too many colonies on the plate	Plate the transformants from a higher dilution
	Ampicillin use	You may use carbenicillin rather than ampicillin to reduce satellite colonies

Precautions

1. CaCl_2 is a hazardous material for skin, eyes and the respiratory system and may cause burns. Hence, use gloves while using the same.
2. Avoid thawing of cells before use.

FLOW CHART**By CaCl_2 treatment**

Grow the *E. coli* cultures by monitoring the cell growth till OD_{600} reaches to 0.35-0.4.

Chill the culture on ice

Transfer the culture to a centrifuge tube and collect the cell pellet by centrifugation at 6,000 rpm for 5 min at 4°C

Re-suspend the cell pellets in 20 ml of ice cold 50 mM CaCl_2

Use 100 μl of the prepared competent cells for transformation

Dispense the competent cells into aliquots of 100 μl and store them at -80°C till further use

Using TSS buffer

Grow *E. coli* culture to OD_{600} of 0.2 to 0.5

Incubate micro-centrifuge tubes on ice for 30 min to 1 h

Pre-chill the TSS buffer on ice or use the 4°C stored TSS buffer directly

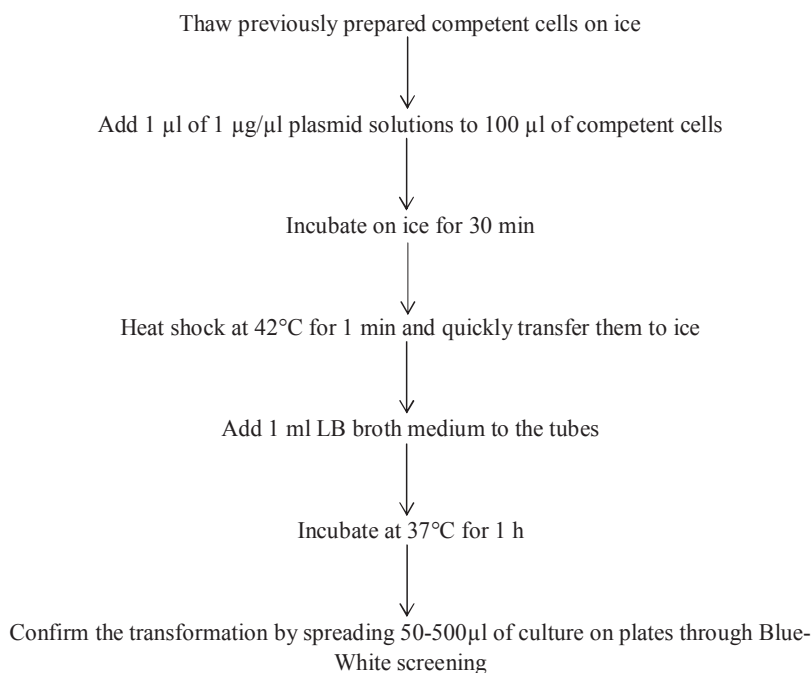
Centrifuge the cells for 10 min at 3,000 rpm and 4°C to collect the cell pellets

Re-suspend the cell pellets in chilled TSS buffer; the volume of TSS buffer should be 10% of the culture volume that have been spun down

Vortex gently to fully re-suspend the culture, make sure for the proper mixing of the small cell aggregates

Add 100 μl aliquots to the chilled micro-centrifuge tubes

Store at -80°C till further use

For transformation

Exp. 2.2 Electroporation

Objective To perform transformation in bacteria by electroporation.

Introduction

Electroporation is the significant increase in electrical conductivity of cell membrane for subsequent increase in its permeability. In most cases, electroporation is used to introduce certain foreign substances into the bacterial cells as a piece of coding DNA and plasmid. An electric pulse of high intensity in kilovolts per centimetre for few microseconds to milliseconds causes a temporary loss of the semipermeable nature of the cell membrane. This phenomenon increases the uptake of drugs, molecular probes and DNA into the cell. Electroporation has many applications such as introduction of plasmids or foreign DNA into living cells for gene transfections, fusion of cells to prepare heterokaryons and hybridomas,

insertion of proteins into cell membranes, improvement of drug delivery, increased effectiveness of chemotherapy of cancerous cells, activation of membrane transporters and enzymes, and alteration of gene expression in living cells.

The electrical device used to porate cells for transformation and gene expression analysis relies upon the discharge of the capacitor by cellular suspension to generate the required electric field. In all the commercially available devices, a capacitor between 2 and 1000 µF is charged to a voltage between 200 and 2000 V and subsequently discharged through the cell suspension by using an electronic or mechanical switch. This ultimately results in a voltage pulse with a rise time of less than 10 µs. Because of their smaller size, bacteria require a much higher electric field to induce poration than mammalian cells or even larger plant cells.

Thus, electroporation is the physical mechanism of allowing cellular introduction of highly charged molecules like DNA through cell membrane. This process is approximately ten times

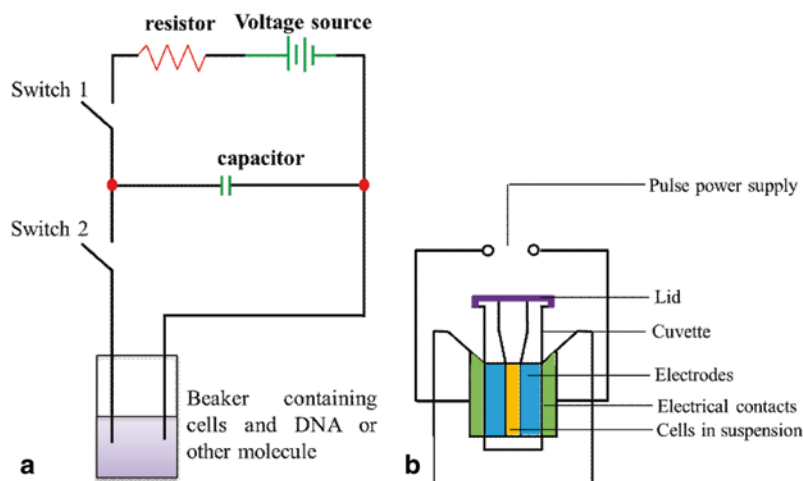


Fig. 2.3 **a** Diagram of the basic circuit setup of an electroporation apparatus, **b** specially designed electroporation cuvette

more effective than that of chemical transformation. During electroporation, the natural barrier function of the membrane is overcome so that ions and water soluble molecules can cross the membrane. Though the microscopic mechanism by which molecular transport occurs is not yet established, significant progress has been made regarding their electrical as well as mechanical behaviour. However, little is known regarding membrane recovery and the ultimate fate of the transformed cells.

Principle

Electroporation capitalizes the weak nature of the cellular membrane and its ability to spontaneously reassemble after disturbance. Ultimately, the quick voltage shock disrupts the membrane temporarily, allowing the polar DNA molecules to pass, and then the membranes reseal quickly to leave the cells intact. During electroporation, the host cell suspension and the DNA molecules to be inserted are kept in the same suspension and the electric field is applied in the electroporation apparatus. Though the electroporation apparatus is commercially produced, the basic process inside this apparatus is quite the same as represented in the diagram in Fig. 2.3.

When the first switch is closed, the capacitor charges up, and a high voltage is stored. When the second switch is closed, the voltage gets discharged through the cell suspension. However, typically 10,000–100,000 V/cm in a pulse lasting for microseconds to milliseconds are necessary for proper electroporation purpose. This, in turn, generates an electric potential across the cell membrane so that the charged molecules such as DNA are driven across the membrane through the pores in a similar fashion to that of electrophoresis. After the charged ions and molecules pass through the pores, the cell membrane discharges, and the pores are closed quickly to reassemble the cell membrane and the intended molecules remain inside the cell for further use (Fig. 2.4).

There are many advantages of using electroporation technique over conventional transformation techniques—technical simplicity, ease of operation, rapidity and reproducibility, greater transformation efficiencies, avoidance of deleterious toxic side effects of chemicals like PEG, no need for pre-incubation of cells and DNA, better control of size and position of the electropores and many others. In order to achieve a good transformation yield, this technique is dependent on several critical factors and parameters that have been categorised into three determinants: (1) cellular factors—growth phase at time of

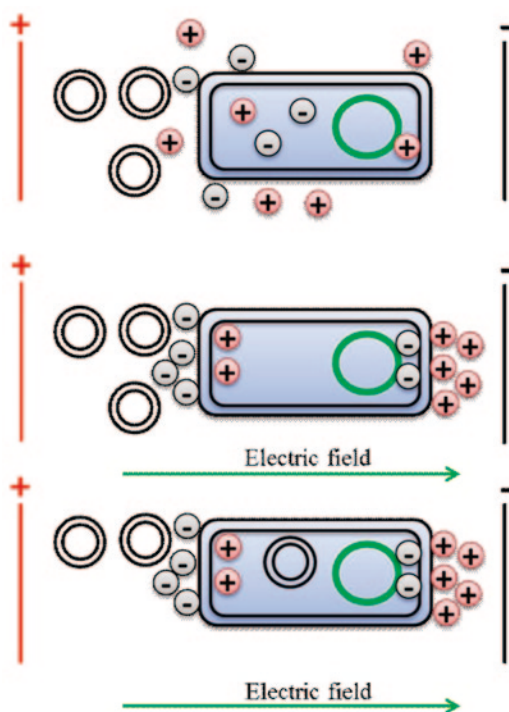


Fig. 2.4 Transfer of foreign genetic material into the cell when electric field is applied, and pathways are formed across the cell membrane allowing DNA to enter the cell

harvesting, cell density, cell diameter, cell wall rigidity and its susceptibility to electroporation; (2) physico-chemical factors—temperature, pH, osmolarity, ionic concentration of electroporation buffer, DNA concentration, etc.; and (3) electrical parameters—optimum field strength, critical voltage, pulse length, number of repetitive pulses, uniform or non-uniform electric fields, etc.

There are many techniques available nowadays for the introduction of foreign DNA into a cell. However, electroporation is the most extensively used technique with huge advantages over other practices such as its versatility, i.e. it is effective with nearly all cell and species types; efficiency, i.e. up to 80% of the foreign DNA can be taken up by the host cell; small scale, i.e. a smaller amount of DNA is required than that of the other techniques; in vivo, i.e. this technique can be performed with intact tissue. Additionally, this method also has certain disadvantages, i.e. cell damage. Similarly, wrong pulse length may

rupture the cell membrane which ultimately fails to close even after discharging the membrane potential. The non-specific transport of materials into the cytoplasm during electroporation may result in ion imbalance and lead to improper cell function and cell death.

Reagents Required and Their Role

Luria-Bertani Broth

Luria-Bertani (LB) broth is a rich medium that permits fast growth and good growth yields for many species including *E. coli*. It is the most commonly used medium in microbiology and molecular biology studies for *E. coli* cell cultures. Easy preparation, fast growth of most *E. coli* strains, ready availability and simple compositions contribute to the popularity of LB broth. LB can support *E. coli* growth ($OD_{600}=2-3$) under normal shaking incubation conditions.

Antibiotics

Prepare antibiotics by dissolving in the appropriate solvents followed by filter sterilization. All the antibiotic solutions can be stored at -20°C till further use. Antibiotics act as the markers for the correct transformation of the plasmid DNA into the bacterial cell. The final concentration of the antibiotics depends on the plasmid and the host. In most of the cases, concentrations of stock solutions for each antibiotic as shown in Table 2.1 are used.

Procedure

Preparation of Electro-competent Cells

1. Streak out *E. coli* cells from the stock culture on fresh LB plates. Incubate at 37°C for 24 h.
2. Use a single colony and inoculate into 10 ml of LB broth. Incubate at 37°C for 24 h with shaking at 250 rpm.
3. Dilute the culture to an OD_{600} of 0.5–1.0.

Table 2.1 List of antibiotics and concentration of final and stock solutions

Antibiotic	Solvent	Stock solution (mg/ml)	Final concentration in medium ($\mu\text{g/ml}$)
Ampicillin	H ₂ O	50	100
Chloramphenicol	C ₂ H ₅ OH	34	34
Gentamycin	H ₂ O	50	50
Kanamycin	H ₂ O	50	50
Rifampicin	CH ₃ OH	50	As required
Streptomycin	H ₂ O	300	10
Tetracycline	C ₂ H ₅ OH	5	300
Timenton	H ₂ O	300	300

- Harvest the cells by centrifuging at 4000 rpm for 15 min at 4°C.
- Remove supernatant and resuspend the cell mass with cold sterile milli-Q.
- Divide the cell suspension to aliquots of 100 μl per tube. Store the cells at -80°C till further use.

Pre-electroporation Procedure

- Gently thaw the cells on ice.
- Add 0.5–2.0 μl of cold plasmid DNA to the cells. Mix well and incubate on ice for 1 min.
- Transfer the cell/DNA mixture into an ice-cold 1-mm electroporation cuvette. Make sure to avoid bubbles or gaps in the cuvette's electrode gap.

Gene Pulser (Bio-Rad, USA) Apparatus Setup

- Turn the gene pulser apparatus on, make sure the display is illuminated and read '0.00'.
- Use the corresponding buttons to set the voltage to 1.8 kV.
- Adjust the capacitor and set the capacitance at 25 μF .
- Adjust the parallel resistor to a resistance of 200 Ω on the gene pulser control panel.

Electroporation

- Wipe the cuvette with tissue paper so that no water bubbles remain on the cuvette.

- Insert the cuvette into the white slide; push the slide into the chamber till the cuvette makes firm contact with the chamber electrodes.
- Charge the capacitor and deliver a pulse, press and hold both the red pulse buttons until a continuous tone sounds. At this point the display should flash 'Chg', indicating that the capacitor is being charged.
- Release the pulse buttons once the display signals the delivery of the pulse.
- Remove the cuvettes from the chamber and add 1 ml of LB broth medium to the cuvette.
- The cells are supposed to be fragile at this stage, transfer the cell culture carefully to a sterile micro-centrifuge tube.
- Turn off the gene pulser apparatus.

Plating of Cells

- Incubate the cells at 37°C on a heat block for 60 min.
- From each transformation, plate 50 μl and 100 μl of cell suspension to the two LB plates with suitable antibiotics.
- Incubate the plates at 37°C for 24 h and observe for the growth of the transformants.

Observation

Transformation efficiency can be calculated after observing the actual number of observed colonies using different amounts of plasmid concentrations. Transformation efficiency is the number of transformants per microgram of supplied plasmid.

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