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Electroporation Technique of DNA Transfection

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INTRODUCTION

Electroporation is a simple and rapid procedure by which DNA may be transferred into cells. Essentially, a high voltage pulse is applied to a suspension of cells and DNA placed between electrodes in a suitable cuvet. It is thought that this pulse induces local areas of cell-membrane breakdown, or pores, through which the DNA then enters the cell. Once these pores have resealed, normal cell functions can continue. Since this transfection method involves a physical effect of the delivered pulse on the cell membrane, it can potentially be used for most cells, independent of their phagocytic capacity as required for transfection by the calcium phosphate coprecipitation technique.

The following range of cells have all been successfully electroporated: Mouse fibroblasts (L, NIH 3T3), pre-B cells (Abelson virus transformed), thymomas (RIA), T-cell clones (D10.G4.1); human lymphoblastoid cells (Epstein-Barr virus transformed), hematopoietic stem cells, peripheral blood T-cells, T-cell lines (HSB-2, TALL-1), a monocytic cell line (U937), an erythroleukemia line (K562), and Chinese Hamster Ovary cells (CHO) (*1–6*).

Electroporation can be used to obtain both short-term (transient) or long-term (stable) transfectants. For some cells, the efficiency of transfection is at least as good as that obtained by calcium phosphate (*2*); in other cells, efficiencies may be greater or less. Transfection by electroporation offers certain advantages over other methods. Insertion by electroporation

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can result in gene copy numbers ranging from one to 20, differing from the large tandem arrays frequently seen following calcium phosphate (*7*). Achieving such low gene copy numbers is useful when the transfected cells are to be used for gene expression studies. In addition, following electroporation, nuclear uptake and incorporation of DNA takes place without rearrangement or extensive modification of the termini (*7*). Although plasmid DNA is generally transfected, genomic DNA (>65 kb) can also be used (*8*).

Although many parameters should be considered when attempting to electroporate a cell or cell line for the first time, published conditions that work for a similar cell type are a useful starting point. Two examples (a fibroblast line and a thymoma line) will be described in the Methods Section below. To achieve optimal transfection efficiencies, these variables would need to be finely adjusted for each cell line. These variable aspects will be briefly described here.

Several electroporation machines that are safe and easy to use are currently available, offering different types of pulses. Firstly, capacitor discharge devices (e.g., Bio-Rad Gene Pulser) can be used to deliver pulses that then decay exponentially. A range of capacitance settings can be selected, as can the applied voltage; the machine will then indicate the actual time of the discharge. Both high-voltage low-capacitance (hence short-time) and low-voltage high-capacitance (hence long-time) conditions may be selected. Special disposable cuvets (with covers to maintain sterility) are needed, although the volume of cell suspension to be pulsed can be varied. Other electroporation devices can deliver square wave pulses (e.g., Hoefer Pro-Genetor pulse controller). In this particular device, the pulse voltage and duration can be selected. Cuvets are not needed, since the electrode fits directly into the wells of a tissue-culture plate. Other square-wave-type machines offer the facility for accurately delivering more than one pulse, possibly multiple pulses separated by a predetermined time interval (e.g., Baekon 2000). It is not yet clear whether one of these machine types is best for electroporation of a particular type of cell. Whichever machine is available or selected, successful transfection may be possible only within a narrow voltage range.

The choice of electroporation buffer is critical for this technique, since the composition and electrical resistance of the electroporation medium will greatly affect the time of pulse that can be delivered. Cells in high-ionic-strength media (e.g., phosphate-buffered saline) manifest shorter time constants than those in HEPES-buffered isotonic sucrose media, if all other parameters are fixed. In addition, the salt conditions around the cells influence cell survival. An ideal medium would lead to minimal detrimental effects during the critical period when the pores are induced. Such a medium would reassemble the cytosol and would also facilitate rapid resealing to restore cell function. (For a detailed assessment, *see* ref. *9*.).

Electroporation may be carried out either at room temperature or, more usually, with incubations on ice. Prechilling avoids cell damage through local heating during pulsing, whereas resting on ice after pulsing prolongs the period when the pores are open so that DNA may enter over an extended time span. However, raising the temperature after pulse delivery reseals the pores and enables the cells to survive unfavorable media. The latter can also be achieved by addition of a large volume of culture medium.

The form of DNA used for transfection is important. Unlike the use of circular plasmid DNA for the calcium phosphate technique, in order to obtain stable transfectants by electroporation, it is usually necessary to linearize plasmids at a side outside the transcriptional unit by digestion with a suitable restriction enzyme. For transient expression this is not desirable, since it results in decreased transient efficiency (*1*). In either case, DNA concentrations in the range 1–80 μg/mL are effective. For at least some cells it is possible to cotransfect two linearized plasmids, provided a suitable DNA ratio is used to achieve stable selection of both genes. Thus, it is not essential to link genes covalently to selectable markers, although for cells with low transfection efficiencies, this step would be advisable. For some cells, the use of carrier DNA can increase transfection efficiency. Again, the amount used should be titrated; sonicated salmonsperm DNA can be effective at 300 μg/mL, but is toxic at high concentrations (*2*).

The best results are obtained with electroporation if the cells to be transfected are actively growing and dividing, rather than from confluent cultures. In the case of resting cells that do not normally divide, using a stimulus to induce proliferation (e.g., phytohemagglutinin for human T-cells) could be one way to make them transfectable (*4*). The density of cells in the cuvet is also important and should be in the range 10^6–10^7/mL, since at lower concentrations, poor cell recovery occurs, and at higher concentrations, undesirable cell fusion starts to take place.

A method will be described for electroporation leading to stable transfection of two genes (a murine Major Histocompatibility Complex class I gene and neomycin selectable gene) in mouse L-cells (fibroblast) and mouse R1A cells (thymoma). For the L-cells, a low capacitance condition is needed, but a harsher, higher capacitance is applied for the smaller R1A cells (the latter cannot be transfected by calcium phosphate).

MATERIALS

1. Plasmids containing the bacterial neomycin phosphotransferase gene (pSV2*neo*) and the murine MHC class I D^d gene purified by cesium chloride gradient ultracentrifugation.
2. Restriction enzymes and appropriate digestion buffers as recommended by the manufacturer.

3. Phenol: Equilibrated with 0.1*M* Tris, pH 8.0, and 0.2% 2-mercaptoethanol; containing 8-hydroxyquinoline (Sigma).
4. 3*M* sodium acetate, pH 5.2, and 100% ethanol for DNA precipitations.
5. TE buffer: 10 m*M* Tris-HCl, 1 m*M* EDTA, pH 7.4; sterilized by passage through a 0.22 μm filter.
6. Carrier DNA: Salmon-sperm DNA (Sigma D-1626) at 10 mg/mL in TE buffer (dissolved overnight at 37°C) sonicated to less than 2 kb.
7. Phosphate-buffered saline: Dulbecco's "A", containing 8 g NaCl, 0.2 g KCl, 0.2 g KH_2PO_4, 1.15 g Na_2HPO_4/L, sterilized by autoclaving.
8. Complete culture medium: Dulbecco's Modified Eagle's Medium supplemented with glutamine, penicillin, streptomycin, bicarbonate, HEPES, 2-mercaptoethanol, and 10% heat-inactivated fetal calf serum.
9. Tryspin/EDTA for harvesting adherent cells.
10. Geneticin sulfate (G418) stock solution, 2 mg/mL in complete medium (sterilized by passage through a 0.22-μm filter). The minimum concentration found to kill untransfected L-cells is 0.8 mg/mL (for R1A cells, 1.8 mg/mL), although the concentration required must be determined for each batch of G418.
11. Actively dividing cell cultures: L-cells should be approaching confluence (1–1.5 × 10^7 from a 75 cm^2 culture flask), R1A cells at 1–2 × 10^7/mL culture. For each transfection, 2 × 10^6 cells are needed.
12. Bio-Rad Gene Pulser (with Capacitance Extender fitted) and cuvets (these can be reused up to five times if thoroughly washed with dH_2O, sterilized with 70% alcohol, and rinsed five times with PBS before use).

METHODS

Electroporation of L-Cells

1. In separate Eppendorf tubes, linearize 5 μg pSV2*neo* and 20 μg D^d in the appropriate digestion buffer in a vol of 100 μL with appropriate restriction enzymes. (It is a good idea to check that linearization is completed by analysis on a mini-gel; *see* Note 1).
2. To remove the restriction enzymes (and hence avoid further DNA digestion when both DNAs are later mixed), add 100 μL of phenol to each digest. Mix until an emulsion forms and then centrifuge each for 10 min in an Eppendorf centrifuge at 4°C.

3. Pipet the upper (aqueous) layers to fresh tubes with caps. To concentrate and sterilize each, add one-tenth of the 3*M* sodium acetate, mix, and then add 2 vol ice-cold ethanol. Store for a minimum of 1 h at −70°C to allow the DNAs to precipitate. Centrifuge as before.
4. In a laminar flow hood, carefully remove the supernatants with a needle and syringe and discard. Allow the pellets to air-dry for 30 min, then thoroughly dissolve them in sterile TE buffer to 2 μg/μL (*see* Note 2).
5. Similarly sterilize 250 μg (25 μL of 10 mg/mL) sonicated salmon-sperm DNA by addition of 75 μL distilled H_2O, 10 μL 3*M* sodium acetate, and 200 μL ethanol. Precipitate and resuspend in 25 μL sterile TE buffer.
6. Under sterile conditions, harvest the L-cells with trypsin/EDTA, add complete medium, and spin down. Wash the cell pellet thoroughly with PBS (4 washes, 5 mL each) and resuspend the pellet to 2.5×10^6 viable cells/mL in PBS. Cell viability should be 90–95%.
7. Transfer 0.8 mL of cell suspension to a cuvet. Add the sonicated salmon-sperm DNA (250 μg in 25 μL), pSV2*neo* (5 μg in 2.5 μL) and D^d (20 μg in 10 μL), mixing thoroughly after each addition. Cap the cuvet and place on ice for 10 min.
8. Meanwhile, place 15 mL of complete medium at room temperature into a 75-cm^2 flask.
9. Mix the cuvet contents briefly with a graduated 1-mL plastic pipet to ensure that the cells have not settled out. Place in the cuvet holder and deliver a pulse of 1000 V at 25 μF. A release of gas may be seen on the electrodes (*see* Note 3).
10. One minute after pulsing, gently tranfer the cuvet contents to the medium in the flask, and then incubate for 48 h (37°C, 5% CO_2).
11. Discard the medium and give fresh medium containing 0.8 mg/mL G418.
12. After 10–14 d, G418-resistant colonies should be visible by eye (20–200/flask). These can be harvested separately as clones or collectively (bulk) and analyzed with the fluorescence-activated cell sorter after staining with a suitable monoclonal antibody reactive with surface D^d antigen. In one experiment, all G418-resistant cells were also expressing the D^d antigen (*see* Notes 4–6).

Electroporation of R1A Cells

1. The plasmid DNAs are linearized and sterilized as described above. Optimal transfection requires 12 μg pSV2*neo* and 50 μg D^d, and these should be redissolved in TE buffer to 5 μg/μL.

2. The required number of cells is washed free of FCS and resuspended to 2.5×10^6/mL in PBS. Following DNA addition and prechilling on ice, the electroporation machine should be set at 350 V, 960 μF for pulsing. This will give considerable frothing!
3. After 1 min, gently tranfer the cell suspension (but not the froth) to 4 mL of complete medium in one well of a 12-well culture plate and incubate for 48 h at 37°C, 5% CO_2.
4. Harvest the cells, pellet them, and resuspend in 24 mL complete medium containing 1.8 mg/mL G418. Plate six wells (4 mL each).
5. By day 10, colonies of G418-resistant cells are visible by eye. These cells divide more frequently than many other lines and can soon be analyzed for surface D^d antigen expression, as indicated above. In one experiment, 50% G418-resistant R1A cells were expressing the D^d antigen. In a separate transfection of a K^dK^k hybrid gene, 100% G418-resistant cells were also expressing surface K^d antigen.

NOTES

1. It is usually more convenient to linearize and sterile a larger amount of pSV2*neo* (for example, 100 μL at 2 μg/mL), and sonicated salmon-sperm DNA (for instance, 200 μL at 10 mg/mL). These can be stored at −20°C and thawed as required, refreezing after use.
2. It is most convenient and easiest to resuspend the pelleted DNA in TE buffer as described, but it should be remembered that this is not isotonic. The above volumes recommended for resuspending the DNAs are tolerated by these cells, but should not be exceeded, especially for osmotically fragile cells. It is possible that the slightly hyposmotic conditions that result contribute to successful electroporation (2).
3. The conditions described above, with the pulse delivered from a capacitor discharge device, result in only 40–80% cell recovery when assessed at 24 h by dye exclusion (*see* Note 6). Conditions causing some cell death are generally needed for successful transfection of the remaining cells with this type of device.
4. When attempting electroporation for the first time, confirm these conditions for transfection of L-cells with linearized selector plasmid alone. When other cells are considered, the initial conditions to be tested (e.g., capacitance setting) could be based on those published for a similar cell type, provided that the type of electroporation machine to be used is similar.

Unfortunately, the effective voltage intensity may be so cell-dependent that transfection occurs only if the exact voltage from a narrow range is selected. Suitably small voltage increments would be needed to locate this. Cell death being observed only after a certain voltage is exceeded would help to narrow down the possibilities.

5. As mentioned in the Introduction Section, several other parameters can also be altered, including the electroporation buffer, temperature and timing, and cell and DNA concentration.
6. Once colonies of stable transfectants can be obtained, it is comparatively straightforward to determine whether the transfection efficiency can be increased. If no transfection at all is occurring, it might be useful to monitor the induction of a state of permeability to dyes in the electroporated cells. For example, permeabilized cells take up the fluorescent dye Lucifer yellow (M_r 457 dalton) (*10*). Since not all permeabilized cells fully recover, it is important to also record cell survival after resealing or at 24 h. Both aims may be achieved in one approach if access to a fluorescence-activated cell sorter is possible (*11*). Propidium iodide (PI) is added to the cell suspension immediately after pulsing and the cells held on ice for 10 min. After a period of resealing in complete medium at 37°C, fluorescein diacetate (FDA) is added. Permeabilized viable cells fluoresce both red (following uptake of PI) and green (since live cells hydrolyze the FDA to fluorescein). Permeabilized cells that have failed to reseal are red only. Achieving doubly stained cells is a fast way to monitor early electroporation attempts with a new cell line.

   If these dyes affect the ionic strength of the buffer, they are best added during a postpulsing incubation on ice. It should be remembered that dye uptake will differ from DNA uptake —it has been suggested that DNA enters the permeabilized cells electrophoretically during the pulse itself, rather than by simple diffusion (*12*).

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