Lipofection

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I. INTRODUCTION

The introduction of nucleic acids into cells plays an essential role in experimental biology and is becoming an important issue for the development of gene therapy. Gene transfer into tissue culture cells can be accomplished by calcium phosphate precipitation or electroporation, but both these methods can have harmful effects on the cells (Smith *et al.*, 1993). Transfection by use of cationic liposomes (lipofection) is better tolerated by the cells and has the additional advantages of simplicity and high efficiency (see Volume 4, Tilkins *et al.*, "Transfection of Mammalian and Invertebrate Cells Using Cationic Lipids" for additional information). Various types of cationic lipids, including DOTMA, DOTAP, and DDAB, are being used for the preparation of cationic liposomes, often in combination with the neutral lipid dioleyl phosphatidylethanolamine (DOPE). Liposomes for transfection can either be purchased as suspensions ready to use or be prepared from inexpensive constituents. This article describes how to prepare cationic liposomes containing DDAB and DOPE (Rose *et al.*, 1991) and how to use liposomes for the transient and stable transfection of cultured cells.

II. MATERIALS AND INSTRUMENTATION

LipofectAMINE (Cat. No. 18324-012), Lipofectin (Cat. No. 18292-011), LipofectACE (Cat. No. 18301-010), Earle's minimal essential medium (MEM, Cat. No. 041-1090), and trypsin–EDTA solution (Cat. No. 043-5300) are from GIBCO-BRL. DDAB (dimethyldioctadecylammonium bromide, Cat. No. D-2779), DOPE (1,2-di[(*cis*)-9-octadecenoyl]sn-glycero-3-phosphoethanolamine, Cat. No. P-0510), geneticin (Cat. No. G-9516), hygromycin B (Cat. No. H-3274), puromycin (Cat. No. P-8833), and HEPES (Cat. No. H-3375) are from Sigma. DOTAP (*N*-[1-(2,3-dioleyloxy)propyl]-*N,N,N*-trimethylammonium methylsulfate, Cat. No. 1 202 375) is from Boehringer Mannheim.

Glisseal laboratory grease (Cat. No. CH-4528) is from Borer Chemie and is autoclaved in a glass petri dish before use. Cloning cylinders are prepared by cutting the upper 6 mm of yellow pipette tips. They are autoclaved before use. Other equipment includes a 50-ml glass vial, a 50-ml polypropylene tube (Falcon), 60-cm^2 tissue culture flasks (Falcon), 9-cm tissue culture dishes (Falcon), a CO^2 incubator, and a tip sonifier (Branson).

III. PROCEDURES

A. Preparation of Cationic Liposomes

Solutions

1. *DOPE at 10 mg/ml:* Purchased as a ready-made solution in chloroform.
2. *DDAB at 10 mg/ml:* To make 1 ml, dissolve 10 mg DDAB in 1 ml chloroform.
3. *Chloroform.*
4. *Sterile distilled water.*
5. *96% ethanol (v/v).*

Steps

1. Mix 2 ml of the PE solution with 0.8 ml of the DDAB solution in a sterile glass tube. *Warning:* Chloroform vapor is harmful. Use a fume hood.

2. Let the chloroform evaporate at room temperature.

3. Resuspend the lipid in 20 ml of sterile water. Transfer to a 50-ml Falcon tube on ice. Use ethanol to desinfect the tip of a sonicator. Sonify the lipid suspension on ice in pulses of 15 sec, with 45-sec pauses, for 5–30 min, until the suspension appears clear.

4. Aliquot into sterile tubes and store at 4°C.

B. Transient Transfection for Fluorescence Microscopy

Solutions

1. *Transfection medium:* To make 10 ml, mix 9.8 ml MEM with 200 ml 1 *M* HEPES solution under sterile conditions. Make fresh.
2. *Cationic liposomes at 1–2 mg/ml:* LipofectAMINE, Lipofectin, LipofectACE, DOTAP, or the home-made liposomes from Procedure A.
3. *Cell culture medium:* The recommended medium (including serum) for cells that are to be transfected. Store at 4°C.

Steps

1. The cDNA to be expressed should be in a plasmid vector, behind an appropriate promotor working in mammalian cells, e.g., the SV40 or the CMV promotor. Use supercoiled plasmid, prepared via a commercial anion-exchange column, such as Qiagen. Also, miniprep DNA can be used (even when containing RNase), but this will result in some precipitation.

2. Plate cells (ca. 3×10^4/well) on 11-mm glass coverslips in 24-well tissue culture plates. Let the cells incubate overnight at 37°C.

3. Mix ca. 1 μg of the plasmid DNA into 110 μl transfection medium in a polystyrene tube.

4. In a separate tube, mix 5 μg liposomes with 110 μl transfection medium.

5. Mix the diluted DNA with the diluted liposomes. Leave at room temperature for 10 min.

6. Wash the cells once with 1 ml of transfection medium. Then add the DNA/liposome mixture. Incubate at 37°C for 6 hr.

7. Replace the transfection mix with 1 ml of normal, serum-containing medium. Incubate the cells in a CO_2 incubator for 1–2 days.

8. Assay the expression by immunofluorescence microscopy.

C. Preparation of Stable Cell Lines by Lipofection

Solutions

1. *Phosphate-buffered saline (PBS):* To make 4 liters, dissolve 0.632 g $NaH_2PO_4 \cdot H_2O$, 7.93 g $Na_2HPO_4 \cdot 12H_2O$, and 32.4 g NaCl in 4 liters H_2O. Check that the pH is between 7.2 and 7.4. Autoclave and store at 4°C.
2. *Cell culture medium:* Use the complete medium (including serum) recommended for cells that are to be transfected.
3. *Selective medium:* Cell culture medium containing the relevant selective drug(s), such as geneticin, puromycin, or hygromycin B, at an appropriate concentration.
4. *Cationic liposomes at 1–2 mg/ml:* LipofectAMINE, Lipofectin, LipofectACE, DOTAP, or the homemade liposomes from Section A.
5. *Trypsin–EDTA solution at 0.5 g trypsin/liter and 0.2 g EDTA/liter.*

Steps

1. The DNA to be expressed should be cloned behind an appropriate promotor in a plasmid vector. If the plasmid contains a selectable marker gene, conferring resistance to, for instance, geneticin, hygromycin B, or puromycin, then no additional plasmid is needed. If the plasmid vector lacks a selectable marker, then a cotransformation has to be performed. In such cases, use a 10-fold excess of the plasmid vector for the DNA to be expressed, compared to that of a plasmid containing a selectable marker gene.

2. Incubate the cells to be transfected (about 30% confluent) in 3-cm dishes with increasing concentrations of the selective agent(s). Choose the lowest concentration that efficiently kills all cells after 7 days as your working concentration for the selection.

3. Plate approximately $1–2 \times 10^6$ of the cells to be transfected (of low passage number) in a medium-sized (60-cm^2) tissue culture flask. Incubate overnight at 37°C. Optimally, the cells should be about 80% confluent at the time of transfection.

4. Prepare the transfection mix: Mix 50 μg plasmid DNA with 2.5 ml transfection medium in one polystyrene tube and 100 μl cationic liposomes with 2.5 ml transfection medium in another vial. Mix the contents of the two vials and leave at room temperature for 10–20 min.

5. *Optional, for epithelial cells:* Trypsinize the cells with 1 ml trypsin–EDTA until they detach. Add 4 ml of serum-containing medium. Transfer to a 15-ml sterile tube. Centrifuge at 1200 rpm for 5 min in a tissue culture centrifuge.

6. Remove the medium from the cells and add 5 ml of transfection mix. *For epithelial cells:* Resuspend cells carefully using a pipette and transfer to a 60-cm^2 tissue culture flask.

7. Incubate for 6 hr at 37°C.

8. Replace the transfection mix with 10 ml of normal, serum-containing medium. Incubate further at 37°C for about 20 hr.

9. Trypsinize the cells and transfer them into three 9-cm tissue culture dishes at the following dilutions in 10 ml of serum-containing medium containing the appropriate concentration of the selectable agent: (a) 1/100, (b) 1/10, and (c) the rest. Incubate for 10–14 days at 37°C until colonies of about 5 mm in diameter appear. Change medium whenever many dead cells appear.

10. Select a plate with an appropriate (about 10) number of colonies. Wash once with sterile PBS.

11. Dip cloning cylinders into sterile grease, then place them over the colonies. Add 100 μl of trypsin–EDTA into each cylinder. Work quickly, as the cells will dry.

12. When the cells have detached (check by microscopy), use a sterile pipette to carefully transfer the trypsinized cells into 1 ml of selective medium in a 24-well plate.

13. When the cells are confluent, transfer them to 6-well plates. Then expand them

further and assay for protein expression, e.g., by immunofluorescence microscopy or Western blotting.

IV. COMMENTS

The mechanisms involved in liposome-mediated DNA transfer into cells are still obscure. It is believed, however, that the cationic liposomes interact efficiently with negatively charged nucleic acid molecules and that the so-formed complexes bind to the negatively charged cell surface (Smith *et al.*, 1993). It is still not clear if the transfer of nucleic acids into the cytosol and nucleoplasm occurs via direct fusion between the liposome and the plasma membrane or whether endocytosis and subsequent intracellular trafficking are required first. The liposomes in Section IIIA are commercially available under the trade name LipofectACE. Although the described transfection methods are designed for plasmid DNA, they can also be applied for RNA and oligonucleotides. The optimal ratio between liposomes and DNA varies between different batches of liposomes and should ideally be tested in transient transfection experiments. The relative transfection efficiencies obtained with different kinds of cationic liposomes may vary from cell line to cell line.

V. PITFALLS

1. Lipid suspensions that have not been sonicated properly look turbid. Such preparations give poor transfection efficiencies and may be harmful to the cells.

2. Linearized plasmids or plasmids that have been nicked (e.g., by repeated freezing/thawing) result in low transfection efficiencies, particularly in transient transfections. Use supercoiled plasmid DNA for lipofection.

3. Mixing liposomes and DNA before they have been diluted results in precipitation, yielding poor transfection efficiency.

4. Polypropylene tends to adsorb lipid/DNA complexes, and polystyrene tubes should be used whenever an optimal transfection efficiency is required.

5. In some cases, precipitated material can be observed when the transfected cells are viewed in a microscope. Precipitation usually lowers the transfection efficiency and may cause toxic effects to the cells. Precipitates may come from impurities from the DNA preparations, especially when "miniprep" DNA is used. They also occur when an inappropriate ratio between DNA and liposomes is used or when the pH of the transfection medium is too high. Ideally, different amounts of DNA (1–5 μg) and liposomes (2–10 μl) should be tested in combination by transient transfection and immunofluorescence microscopy. The pH of the incubation medium should always be kept below 7.4.

6. Cells growing in colonies or in intact monolayers are inefficiently transfected. Plate the cells as a single-cell suspension, at a maximal density of 80% confluency. For preparing stable lines, we recommend transfecting freshly trypsinized cells (see Section IIIC, step 5).

7. Cells of high passage number or that have been allowed to grow overconfluent are inefficiently transfected.

Acknowledgment

H.S. is a fellow of the Norwegian Cancer Society.

References

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