

Introduction

The ProFection® Mammalian Transfection Systems offer the choice of calcium phosphate or DEAE-Dextran mediated transfection procedures. Both of these methods appear to facilitate DNA binding to cell membranes and entry of the DNA into the cell via endocytosis. Calcium phosphate also appears to provide protection against intracellular and serum nucleases (77).

Calcium phosphate transfection may be used for the production of long-term stable transfectants, works well for transient expression of transfected genes and can be used with most adherent cell lines. DEAE-Dextran transfection is also an efficient method for introducing DNA into many cell types, including some cell suspensions. However, its suitability is limited to transient expression studies and it is not recommended for the production of stable transfectants (10). For transient expression studies using a particular cell type, both protocols should be tried in order to determine the most efficient method.

For a list of references using the ProFection® Mammalian Transfection Systems in a variety of cell lines, see Appendix A.

Factors That Affect Efficiency of Gene Transfer

Transfection efficiencies can be increased in many cell types by additional treatments after the primary exposure of the cells to calcium phosphate-DNA or DEAE-Dextran and DNA. The most effective and routinely used agents are glycerol (78,79), dimethyl sulfoxide (DMSO) (79-81), chloroquine (82) and sodium butyrate (83). Since each of these chemicals is toxic to cells, the conditions for transfection of individual cell types must be carefully optimized for reagent concentration and exposure time.

Calcium Phosphate-Mediated Transfection

A precipitate containing calcium phosphate and DNA is formed by slowly mixing a HEPES-buffered phosphate solution with a solution containing calcium chloride and DNA. These DNA precipitates are then taken into eukaryotic cells by an endocytic-type mechanism.

Plating Cells for Transfection

Plate cells the day before the transfection experiment according to the guidelines given in Chapter 3.

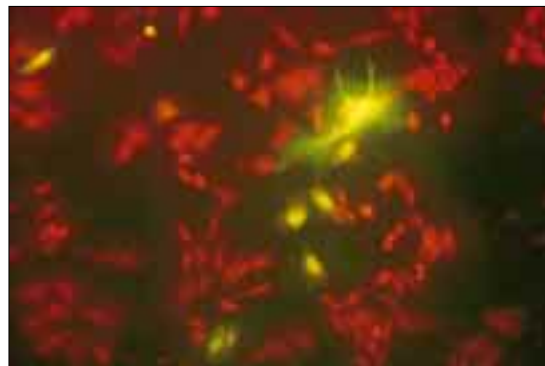


Figure 4.1. CHO cells were transfected with the ProFection® Mammalian Transfection System-Calcium Phosphate and DNA containing the green fluorescent protein reporter gene. Cells were counter-stained with propidium iodide.

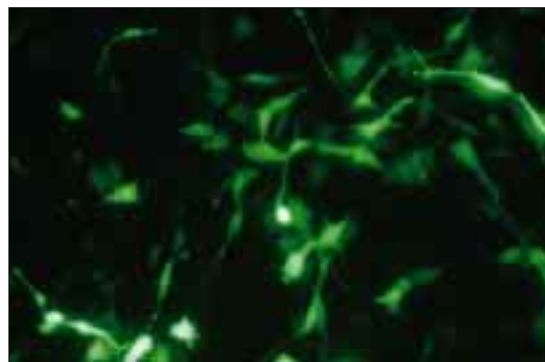


Figure 4.2. NIH/3T3 cells were transfected with the ProFection® Mammalian Transfection System-Calcium Phosphate and DNA containing the green fluorescent protein reporter gene.

Transfection Protocol

The protocol given below is for a 60mm plate. For different size plates scale the volumes and amounts proportionally according to the information given in Chapter 3, Table 3.8.

1. Plate the cells the day before transfection as described in Chapter 3.
2. Three hours prior to transfection, remove the medium from the cells and replace it with fresh growth medium.
3. Thaw all system components and warm them to room temperature. Mix each component thoroughly by swirling the container or vortexing.

4. For each transfection, prepare the DNA and 2X HBS solutions in separate sterile tubes. Add the DNA and water to the first tube, mix well, then add the CaCl₂ and mix again. Add the specified amount of 2X HBS to the second tube.

| | per 60mm dish |
|--------------------------|--------------------------|
| DNA | 6-12µg |
| 2M CaCl ₂ | 37µl |
| sterile, deionized water | to final volume 0.3ml |
| 2X HBS | 0.3ml |

5. Working in a tissue culture hood, gently vortex the tube containing the 2X HBS solution. The speed should be adjusted such that the tube can be vortexed safely with the cap off and can accommodate the addition of the prepared DNA solution. Continue to vortex while slowly adding the prepared DNA solution dropwise to the 2X HBS. (Alternatively, bubble air with a pipet through the 2X HBS while slowly adding the CaCl₂/DNA solution). When the DNA addition is complete, the solution should appear slightly opaque due to the formation of a fine calcium phosphate-DNA coprecipitate. Incubate the solution at room temperature for 30 minutes.
6. Vortex the transfection solution again just prior to adding it to the cells. Add the solution dropwise to the plates. Swirl the plates to distribute the precipitate evenly over the cells. Return the plates to a 37°C CO₂ incubator.
7. When working with sensitive cells, the culture medium should be changed 4-16 hours after transfection. The length of the incubation should be optimized for individual cell lines. Primary cells are particularly sensitive and should not be exposed to calcium phosphate for more than 4 hours.
8. In general cells may be harvested or selective media applied 48-72 hours after transfection.

Tip

Strontium chloride can be used in place of calcium chloride if the cells being transfected are sensitive to the high calcium concentration present in the calcium phosphate/DNA precipitate (84).

Tip

To increase efficiency of transfection of some cell types, glycerol may be added during incubation with the calcium phosphate/DNA precipitate (85).

Glycerol or DMSO Shock**Glycerol Shock**

High transfection efficiencies can be obtained by leaving the DNA/calcium phosphate solution on the cells until the cells are harvested or selective pressure is applied. HeLa cells, for example, respond well to this treatment. However, transfection of some cell lines, such as CHO cells, is enhanced by a glycerol shock step.

The glycerol shock step may be performed 4-16 hours after transfection. In general, if cells can tolerate the calcium phosphate solution, it is best to leave it on for as long as possible and perform the glycerol shock 16 hours after transfection. Cell lines that are more sensitive to the calcium phosphate solution may respond better to a glycerol shock step performed earlier, such as 4 hours after exposure to the DNA. Do not expose the cells to the glycerol solution for more than 2 minutes. The optimum time interval before performing the glycerol shock should be determined empirically for each cell line.

Materials to Be Supplied by the User

(Solution compositions are provided at the end of this chapter.)

- glycerol shock solution (15% glycerol)
 - wash solution: 1X PBS or 1X HBSS
1. Prepare a fresh glycerol shock solution in 1X HBS and warm it to 37°C, along with growth medium and wash solution.
 2. Wash the cells once with 5ml of wash solution per 60mm plate.
 3. Add 2ml of the glycerol shock solution per 60mm plate.
 4. Incubate for up to 2 minutes at room temperature.
 5. Remove the glycerol shock solution and wash the cells twice with 5ml of wash solution per 60mm plate.
 6. Add regular growth medium and return the cells to a 37°C incubator.

DMSO Shock

Certain cell types exhibit enhanced transfection efficiencies after exposure to dimethyl sulfoxide (DMSO). The DMSO step can be added to either the calcium phosphate or DEAE-Dextran transfection protocols. DMSO, like glycerol, is toxic to cells and the concentration and exposure times require careful optimization for each cell type. Most cells should not be exposed to DMSO for more than 2.5 minutes. One representative protocol for a DMSO shock is provided.

Materials to Be Supplied by the User

(Solution compositions are provided at the end of this chapter.)

- DMSO shock solution (10% DMSO)
1. Remove the medium from the cells.
 2. Immediately before use, prepare the DMSO shock solution and warm it to 37°C. Prepare 2ml per 60mm plate.
 3. Add the DMSO shock solution to the cells and incubate for 2.5 minutes. Do not return the plates to an incubator during this time.
 4. Remove the DMSO shock solution, wash the cells twice and add 5ml of regular growth medium per 60mm plate. Return the cells to the 37°C incubator.

DEAE-Dextran-Mediated Transfection

DEAE-Dextran, a polymeric cation, associates tightly with the negatively charged DNA and carries it into the cell. As DEAE-Dextran is toxic to cells, transfection conditions for individual cell lines may require careful optimization of both DEAE-Dextran concentration and exposure times. At higher DEAE-Dextran concentrations, the exposure time to cells can be shortened in order to minimize cell death. This protocol may be inappropriate for certain cell lines for which DEAE-Dextran is highly toxic.

Two different protocols for DEAE-Dextran transfections are given. The standard protocol involves concurrent exposure of cells to DEAE-Dextran and DNA. The second protocol involves pretreatment of the cells with DEAE-Dextran and is a modification of the procedure described by Al-Molish, *et al.* (86). It offers the advantages of limited DEAE-Dextran exposure and longer DNA incubation, allowing maximal DNA uptake. The best protocol for a particular cell line should be determined experimentally. The addition of 80µM chloroquine along with the DNA is an option for both protocols. For some cell lines, chloroquine dramatically increases transfection efficiencies; for others, it has a minimal effect and may be quite cytotoxic. The optimal amount of DNA to use for transfection will vary with the cell line and type of reporter construct being used. Generally, 2-6µg of DNA will be sufficient for a 60mm plate and 4-10µg DNA will be sufficient for a 100mm plate.

Both protocols require a sterile calcium- and magnesium-free salt solution for the wash steps. This wash solution is not provided with the system. 1X PBS or another salt solution such as 1X HBSS works well for this purpose.

Materials to Be Supplied by the User

(Solution Compositions are provided at the end of this chapter.)

- wash solution: 1X PBS or 1X HBSS
- optional: 8mM chloroquine (in sterile water)

Standard DEAE-Dextran Protocol

The protocol given below is for a 60mm plate. For different size plates scale the volumes and amounts proportionally according to the information given in Chapter 3, Table 3.8.

1. Plate cells the day before the transfection experiment according to the guidelines given in Chapter 3.
2. Prepare the wash solution (1X PBS or 1X HBSS) and warm it to 37°C. Ten milliliters of wash solution are required for each 60mm plate. Warm the DEAE-Dextran solution to 37°C.
3. Dilute the 10X PBS stock 10-fold with sterile water. You will need approximately 0.4ml of 1X PBS per 60mm plate. Prepare the transfection solutions as outlined below:

Per 60mm dish: Using a sterile tube, dilute 2-6µg DNA to a final volume of 326µl in 1X PBS. Add 17µl of the 10mg/ml DEAE-Dextran and mix by gently tapping the tube.

4. Remove the medium from the cells. Wash the cells twice with wash solution using 2 x 5ml per 60mm plate.
 5. Add the DNA/DEAE-Dextran mixture and disperse it evenly over the cells. The final concentration of DEAE-Dextran in the salt solution is approximately 0.5mg/ml.
 6. Incubate the plates at 37°C for 30 minutes. Rock the plates occasionally to keep the cells moist.
 7. Gently add 3.5ml of growth medium per 60mm plate. Incubate up to 2.5 hours at 37°C or until cytotoxicity is apparent. Gently change the medium or follow with a DMSO shock.
- Optional:** Add 35µl of 8mM chloroquine per 60mm plate along with the medium during the 2.5 hour incubation step. If chloroquine is added, the culture medium **must be** replaced after 4 hours (or earlier, if signs of cytotoxicity are apparent). The time that the cells are exposed to chloroquine must be empirically determined for each cell line.
8. Harvest the cells 48 hours after transfection.

DEAE-Dextran Pretreatment Protocol

1. Plate the cells the day before the transfection experiment as described in Chapter 3.
2. Prepare the wash solution and warm it to 37°C. Fifteen milliliters of wash solution are required for each 60mm plate.
3. Dilute the 10X PBS stock 10-fold with sterile water. You will need approximately 3ml of 1X PBS per 60mm plate. Prepare the transfection solutions as outlined below:

Dilute the DEAE-Dextran stock solution 1:10 in the 1X PBS solution prepared above. You will need 2ml of diluted DEAE-Dextran per 60mm plate.

Dilute the DNA in 1X PBS to a final volume of 325µl for a 60mm plate.

4. Remove the medium from the cells. Add 5ml of sterile wash solution for 60mm plates. Incubate for 15 minutes at room temperature.
5. Remove the wash solution from the cells. Add 2ml of the diluted DEAE-Dextran solution per 60mm plate. Incubate for 9 minutes at room temperature.
6. Remove the DEAE-Dextran solution. Very gently wash the cells twice with 2 x 5ml of wash solution per 60mm plate. Be careful not to dislodge the cells, which may begin to detach after exposure to DEAE-Dextran.
7. Remove the final wash. Add the diluted DNA and disperse it evenly over the cells. Incubate for 30 minutes in a 37°C CO₂ incubator. Rock the plates occasionally to keep the cells moist.
8. Add 3.5µl of regular growth medium per 60mm plate.

Optional: Add 35ml of 8mM chloroquine per 60mm plate together with the medium. If chloroquine is added, the culture medium **must be** replaced after 4 hours (or earlier, if signs of cytotoxicity are apparent). The time that the cells are exposed to chloroquine must be empirically determined for each cell line.

9. Return the plates to a 37°C CO₂ incubator.
10. Harvest the cells 48 hours after transfection.

Composition of Buffers and Solutions**2X HBS (HEPES-Buffered Saline)**

| | |
|-------|----------------------------------|
| 50mM | HEPES (pH 7.1) |
| 280mM | NaCl |
| 1.5mM | Na ₂ HPO ₄ |

The final pH should be 7.1

1X PBS (Phosphate Buffered Saline)

| | |
|--------|----------------------------------|
| 137mM | NaCl |
| 2.7mM | KCl |
| 4.3mM | Na ₂ HPO ₄ |
| 1.47mM | KH ₂ PO ₄ |

The final pH should be 7.1

1X HBSS (Hanks Balanced Salt Solution)

| | |
|-------|----------------------------------|
| 5mM | KCl |
| 0.3mM | KH ₂ PO ₄ |
| 138mM | NaCl |
| 4mM | NaHCO ₃ |
| 0.3mM | Na ₂ HPO ₄ |
| 5.6mM | D-glucose |

The final pH should be 7.1.

1X Trypsin-EDTA solution

| | |
|-------------|---------|
| 0.05% (w/v) | trypsin |
| 0.53mM | EDTA |

Dissolve these components in a calcium- and magnesium-free salt solution such as 1X PBS or 1X HBSS.

TE buffer

| | |
|------|-------------------|
| 10mM | Tris-HCl (pH 8.0) |
| 1mM | EDTA |

Glycerol shock solution

| | |
|-----|----------|
| 1X | HBS |
| 15% | Glycerol |

DMSO shock solution

| | |
|-----|-----------------------------|
| 1X | PBS |
| 10% | DMSO (tissue culture grade) |

