

# **Gene Delivery to Mammalian Cells**

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METHODS IN MOLECULAR BIOLOGY™

# Gene Delivery to Mammalian Cells

*Volume 1: Nonviral Gene Transfer Techniques*

Edited by

**William C. Heiser**

*Bio-Rad Laboratories  
Hercules, CA*


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# Preface

The efficiency of delivering DNA into mammalian cells has increased tremendously since DEAE dextran was first shown to be capable of enhancing transfer of RNA into mammalian cells in culture. Not only have other chemical methods been developed and refined, but also very efficient physical and viral delivery methods have been established. The technique of introducing DNA into cells has developed from transfecting tissue culture cells to delivering DNA to specific cell types and organs *in vivo*. Moreover, two important areas of biology—assessment of gene function and gene therapy—require successful DNA delivery to cells, driving the practical need to increase the efficiency and efficacy of gene transfer both *in vitro* and *in vivo*.

These two volumes of the *Methods in Molecular Biology*<sup>TM</sup> series, *Gene Delivery to Mammalian Cells*, are designed as a compendium of those techniques that have proven most useful in the expanding field of gene transfer in mammalian cells. It is intended that these volumes will provide a thorough background on chemical, physical, and viral methods of gene delivery, a synopsis of the myriad techniques currently available to introduce genes into mammalian cells, as well as a practical guide on how to accomplish this. It is my expectation that it will be useful to the novice in the field as well as to the scientist with expertise in gene delivery.

*Volume 1: Nonviral Gene Transfer Techniques* discusses delivery of DNA into cells by nonviral means, specifically chemical and physical methods. *Volume 2: Viral Gene Transfer Techniques* details procedures for delivering genes into cells using viral vectors. Each volume is divided into sections; each section begins with a chapter that provides an overview of the basis behind the delivery system(s) described in that section. The succeeding chapters provide detailed protocols for using these techniques to deliver genes to cells *in vitro* and *in vivo*. Many of these techniques have only been in practice for a few years and are still being refined and updated. Some are being used not only in basic science, but also in gene therapy applications.

I wish to express my thanks to all of the authors who made *Gene Delivery to Mammalian Cells: Volume 1: Nonviral Gene Transfer Techniques* and *Volume 2: Viral Gene Transfer Techniques* possible. I would especially like to thank those who contributed the overview chapter to each section. They provided invaluable discussions, suggestions, and assistance on organizing those sec-

tions. I would particularly like to mention Joanne Douglas, Tom Daly, and Bill Goins for their suggestions on topics and authors, Dexi Liu and Shan Lu for their helpful discussions, and Mark Jaroszeski for his suggestions on organizing the entire editing process.

***William C. Heiser***

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## Gene Transfer into Mammalian Cells Using Calcium Phosphate and DEAE-Dextran

Gregory S. Pari and Yiyang Xu

### 1. Introduction

Simplicity and cost are just two of the factors that have sustained the popularity of calcium phosphate and, to a lesser extent, DEAE-dextran transfection methods. However, notwithstanding these factors, the calcium phosphate method, especially use of *N,N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid (the BES variation), has proven to be the only method sufficient for the co-transfection of multiple plasmids into a wide variety of cell types. Although not as widely used today, DEAE-dextran-mediated transfection is a highly reproducible method for transient expression of a foreign gene.

The DEAE-dextran-mediated transfection method was widely used in the early to mid-1980s because of the simplicity, efficiency, and reproducibility of the procedure (1–5). One major drawback of this method is the poor efficiency in forming stable cell lines. In addition, cellular toxicity is high because it is necessary to expose the cells to dimethyl sulfoxide (DMSO). Consequently, DEAE-dextran-mediated transfection has fallen out of favor with many investigators, giving way mostly to lipid-mediated transfection. However, because lipid-mediated transfection can be costly and inefficient in some cell types, many laboratories may want to consider the DEAE-dextran method. Some investigators have found DEAE-dextran-mediated transfection to be highly effective in certain cell lines. Several reports demonstrated that this is the method of choice for delivering DNA to primary cultured human macrophages (6,7). In addition, it appears that DEAE-dextran enhances the transfection efficiency of mammalian cells when using electroporation (8).



In contrast to DEAE-dextran, the calcium phosphate co-precipitation procedure has remained a popular method to efficiently deliver DNA to a wide variety of cell types. The main advantage of calcium phosphate DNA transfection is the high efficiency for the generation of constitutively expressing cell lines. Calcium phosphate is the method of choice for the simultaneous transfection of multiple plasmids. In our laboratory, we routinely co-transfect as many as 12 different plasmid constructs at the same time into mammalian cells. Plasmid DNA to be transfected must be of the highest purity, usually only double-banded CsCl DNA is used for transfection.

The original calcium phosphate method used a HEPES-based buffer system (9). This method is simple to use, but is limited in the range of cell lines that can be efficiently transfected. Many variations of the HEPES-based system exist, and some have optimized this method for a particular cell type (10). A variation of the original calcium phosphate transfection method, one that uses BES buffer, has emerged as a very versatile and highly efficient transfection method (11). The BES method uses a different buffer system in which the pH is lower than the HEPES-based procedure. A lower pH, coupled with incubation in a reduced CO<sub>2</sub> atmosphere for 15 h, allows the DNA-calcium phosphate precipitate to form slowly on the cells. This results in a significant increase in the efficiency of transfection and a higher percentage of stably expressing cell lines than the HEPES-based procedure. Co-transfection efficiencies are also much higher using the BES method versus the original HEPES-based buffer transfection method. This feature is of particular importance to establish a co-transfection replication assay (12,13). Because of these advantages, we present only the BES method in this chapter.

The following are representative protocols for DEAE-dextran transfection of adherent and suspension cells, as well as a protocol for the BES method for calcium phosphate-mediated transfection.

## 2. Materials

### 2.1. DEAE-Dextran Transfection

1. Tris-buffered saline (TBS): Prepare the following sterile solutions: Solution A: 80 g/L NaCl, 3.8 g/L KCl, 2 g/L Na<sub>2</sub>HPO<sub>4</sub>, 30 g/L Tris base. Adjust pH to 7.5. Solution B: 15 g/L CaCl<sub>2</sub>, 10 g/L MgCl<sub>2</sub>. Filter-sterilize both solutions and store at -20°C. To make 100 mL of working solution, add 10 mL of Solution A to 89 mL of water and then add 1 mL of Solution B, filter-sterilize and store at 4°C.
2. Suspension Tris-buffered saline (STBS): 25 mM Tris-HCl, pH 7.4, 137 mM NaCl, 5 mM KCl, 0.6 mM Na<sub>2</sub>HPO<sub>4</sub>, 0.7 mM CaCl<sub>2</sub>, 0.5 mM MgCl<sub>2</sub>. Dissolve in distilled H<sub>2</sub>O and filter-sterilize.

3. Phosphate-buffered saline (PBS): 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.4 mM KH<sub>2</sub>PO<sub>4</sub>, pH 7.3.
4. Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS).
5. DEAE-dextran: 10 mg/mL in TBS.
6. 10% DMSO.
7. For suspension cultures, cells are grown in RPMI 1640 medium supplemented with 10–20% FBS.

## 2.2. Calcium Phosphate Co-Precipitation

1. DMEM supplemented with 10% FBS.
2. CsCl-purified double-banded DNA.
3. 2.5 M CaCl<sub>2</sub> filter sterilized through a 0.45- $\mu$ m filter.
4. 2X BES-buffered saline (BBS): 50 mM N,N-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid, 1.5 mM Na<sub>2</sub>HPO<sub>4</sub>, 280 mM NaCl. Adjust pH to 6.95 with 1 N NaOH (*see Note 1*).
5. 35°C 3% CO<sub>2</sub> humidified incubator.

## 3. Methods

### 3.1. DEAE-Dextran Methods

Two DEAE-dextran methods are commonly used. The first is the basic protocol, which can be used on all anchorage-dependent cells. The second can be used on cells that normally grow in suspension or with anchorage-dependent cells that have been trypsinized and are in suspension. This procedure may increase transfection efficiency in some cells. For adherent cells, it is advisable to try the basic protocol first, then if transfection efficiency is low, try the suspension procedure.

#### 3.1.1. Anchorage-Dependent Cells

1. Plate  $5 \times 10^5$  cells in a 10 cm tissue culture dish (*see Note 2*). Cells should be plated at least 24 h before transfection and should be no more than 40–60% confluent.
2. For each plate of cells to be transfected, ethanol precipitate 5  $\mu$ g of DNA in a 1.5-mL microcentrifuge tube and resuspend the pellet in 40  $\mu$ L of TBS. If the same DNA is used for multiple plates, precipitate all the DNA in one tube. Ethanol precipitation sterilizes DNA.
3. Remove media from the cells and wash cells with 10 mL of PBS. After washing, add 4 mL (for a 10-cm dish) of DMEM supplemented with 10% FBS.
4. Aliquot 80  $\mu$ L of DEAE-dextran into 1.5-mL microfuge tubes and warm to 37°C in a water bath. Add the resuspended DNA (5  $\mu$ g of DNA per 80  $\mu$ L of DEAE-dextran) slowly to the tube while vortexing gently.

5. Add 120  $\mu\text{L}$  of the DNA/DEAE-dextran mixture to the plate in a dropwise fashion using a 200  $\mu\text{L}$  pipet tip. Swirl the plate after each drop is applied to ensure that the mixture is distributed evenly (*see Note 3*).
6. Incubate the plates for 4 h in a 37°C incubator with a 5%  $\text{CO}_2$  atmosphere. This incubation time can be shortened for some cell types.
7. Remove the medium. At this point, the cells may look a little sick but this is normal.
8. Add 5 mL of 10% DMSO in PBS. Incubate for 1 min at room temperature. Remove the DMSO and wash the cells with 5 mL of PBS. Replace the PBS with 10 mL of DMEM supplemented with 10% FBS.

### 3.1.2. Cells in Suspension

1. Ethanol precipitate DNA and resuspend the pellet in 500  $\mu\text{L}$  of STBS (*see Subheading 2*). Use 10  $\mu\text{g}$  of DNA per  $2 \times 10^7$  cells. Cells can be either normally growing suspension cells, for example B-cells, or trypsinized anchorage-dependent cells.
2. Pellet cells in a 50-mL conical centrifuge tube.
3. Resuspend cells in 5 mL of STBS and re-pellet as in **step 2**.
4. Prepare a 2X solution of DEAE-dextran (200–1000  $\mu\text{g}/\text{mL}$ ) in STBS and add 500  $\mu\text{L}$  of this solution to 500  $\mu\text{L}$  of the DNA resuspended in 500  $\mu\text{L}$  of STBS from **step 1**. Mix well. Resuspend pelleted cells with this DEAE-dextran/DNA solution. Use a final concentration of 100–500  $\mu\text{g}/\text{mL}$  of DEAE-dextran.
5. Incubate cells in a  $\text{CO}_2$  incubator for 30–90 min. Tap cells occasionally to keep them from clumping. Incubation times vary and should be determined experimentally.
6. Add DMSO to cells dropwise to a final concentration of 10%, mix well while adding.
7. Incubate cell with DMSO for 2–3 min. Add 15 mL of STBS to cell.
8. Pellet cells, wash with 10 mL of STBS and pellet again. Wash cells in medium with serum and pellet. After this centrifugation, resuspend cells in complete medium (RPMI plus 10–20% FBS). If cells are normally anchorage-dependent, re-plate on a 10 cm dish or in a 75-cm<sup>2</sup> flask. If cells are normally grown in suspension, incubate cells in normal growth media in 25-cm<sup>2</sup> flasks. The onset of expression from transfected plasmids varies depending on cell type. Usually expression begins between 24–48 h post-transfection.

### 3.2. Calcium Phosphate Co-Precipitation Method

Like DEAE-dextran transfection, two calcium phosphate transfection methods are routinely used: a HEPES-based method and a BES buffer method. Both are good for transient expression, but the BES-buffer procedure is much more efficient for making established constitutively expressing cell lines, in some

cells 50% efficiency can be achieved. In addition, this procedure works better on a wider variety of cell types, is excellent for co-transfection, and is easier to perform than the traditional HEPES-based method. Because the BES method is so versatile and offers these advantages, this method is presented here.

1. Plate approx  $5 \times 10^5$  cells on a 10-cm tissue culture dish 24 h before transfection. The cells should be no more than 50% confluent for making established cell lines and about 70% confluent for transient expression. Smaller plates (e.g., 6 cm) may be used and in some cases this is actually sufficient and easier.
2. Dilute the 2.5 M  $\text{CaCl}_2$  solution to 0.25 M with sterile water.
3. Precipitate plasmid DNA in  $17 \times 100$  mm polypropylene tubes as follows: Add 20–30  $\mu\text{g}$  of plasmid DNA per tube (Falcon # 2058) or 10–20  $\mu\text{g}$  for a 6-cm plate (*see Note 4*). For transfecting cells in a 100-mm dish or in two 60-mm dishes, add 20–30  $\mu\text{g}$  of DNA to the tube followed, in order, by 500  $\mu\text{L}$  of 0.25 M  $\text{CaCl}_2$ , then 500  $\mu\text{L}$  of 2X BBS. Use one-half of this mixture on each plate when using 60-mm dishes. For transfecting cells in one 60-mm dish, add 10–20  $\mu\text{g}$  of DNA followed, in order, by 250  $\mu\text{L}$  of 0.25 M  $\text{CaCl}_2$ , then 250  $\mu\text{L}$  of 2X BBS. In both cases, mix well and incubate at room temperature for 10–20 min (*see Note 5*).
4. Add the calcium phosphate/DNA mixture to cells in a dropwise fashion, swirling the plate after each drop. Incubate the cells overnight in a 35°C 3%  $\text{CO}_2$  incubator (*see Note 6*).
5. Wash cells twice with 5 mL of PBS, then add 10 mL of DMEM with 10% FBS. Incubation of cells from this point on is done in a 5%  $\text{CO}_2$  37°C incubator.
6. For transient expression, harvest cells 48 h post-transfection. For selection of stably integrated expression clones, split cells (1:10) 48 h post-transfection into selection medium. For co-transfection *see Note 7*. Many investigators have used the BES method to elucidate genes required for viral DNA replication. The BES method is well-suited for this purpose because of the high co-transfection efficiency (*see Note 8*).

#### 4. Notes

1. BBS pH is critical. Make three solutions ranging in pH from 6.93–6.98. Usually a visual inspection of cells after an overnight transfection will indicate which BBS mixture and DNA concentration works best. A coarse and clumpy precipitate will form when DNA concentrations are too low, a fine, almost invisible precipitate will form when DNA concentration are too high. An even granular precipitate forms when the concentration is just right. This usually correlates to the highest level of gene expression or formation of stably integrated cell lines.

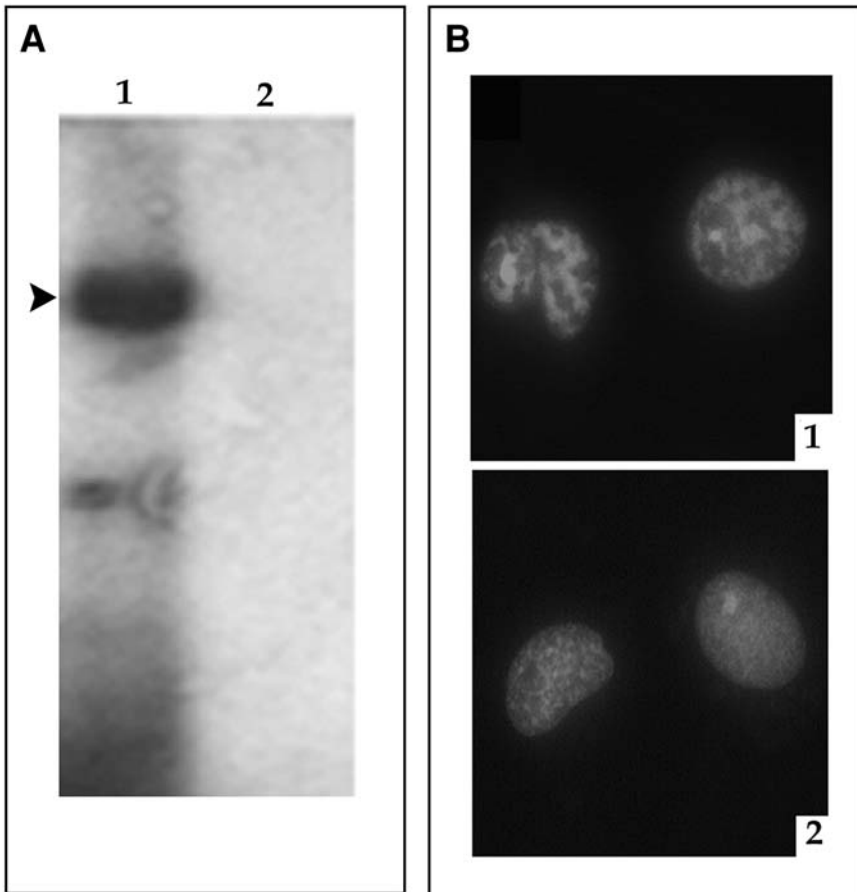


Fig. 1. Cotransfection of 11 plasmids using BES calcium phosphate coprecipitation. **(A)** Replication assay. Human foreskin fibroblasts (HFF) were transfected with 10 plasmids encoding human cytomegalovirus (HCMV) replication genes along with a plasmid encoding the cloned origin of lytic replication (oriLyt). Total cellular DNA was harvested 5 d post-transfection and cleaved with *EcoRI* and *DpnI*. *DpnI*, a four base-pair recognition enzyme, will cleave input DNA (unreplicated DNA) multiple times and *EcoRI* will linearize the HCMV oriLyt. Replicated plasmid is *DpnI*-resistant and is indicated by the arrow. DNA is separated on an agarose gel and hybridized with the parent plasmid vector. Lanes: 1, All required plasmids plus HCMV oriLyt; 2, omission of one plasmid required for oriLyt replication. **(B)** HCMV replication compartment formation requires cotransfection of essential replication proteins. Cotransfections included a replication protein fused in frame with EGFP. HFF cells were transfected with 10 plasmids encoding HCMV replication proteins along with a plasmid encoding a replication protein fused in-frame with EGFP. Transfected cells were fixed and visualized using a confocal microscope. Panel 1: cotransfections were performed the same as in A sample number 1, cells were fixed and visualized using a confocal microscope.

2. Smaller dishes may be used; adjust the number of cells and DNA used proportionally. Higher densities of some cell types may be necessary to achieve good transfection efficiencies. If cell death is too high owing to the toxicity of DEAE, then try plating cells at a higher density.
3. It may be necessary to determine the optimal concentration of DEAE-dextran needed for good transfection efficiencies. Vary the volume of TBS used to resuspend DNA and the amount of DEAE-dextran. For example:

DNA in TBS, $\mu\text{L}$	DEAE-dextran, mg/mL
80	160
40	80
20	40

4. Only high-quality plasmid DNA will work. Use only double-banded CsCl purified DNA. Carrier DNA is not necessary and actually will decrease efficiency. Also, linear DNA does not transfect well.
5. At this point no precipitate should be visible. Use three different concentrations of DNA to help identify the DNA concentration necessary for optimal transfection.
6.  $\text{CO}_2$  level is critical. Measure the level with a Fyrite gas analyzer. Temperature is somewhat less critical. A  $37^\circ\text{C}$  incubator can be used.
7. When performing cotransfections, vary the amount of effector plasmid in relation to the other plasmids in the mix keeping the total amount of DNA the same. The ratio of plasmids used in the mixture can be the difference between success and failure. We routinely find that a higher concentration of effector plasmid in the mix yields better results. We commonly use this cotransfection method to assay the level of promoter activity effected by certain vital transactivators.
8. The co-transfection-replication assay involves the co-transfection of several different plasmids (in the case of HCMV 11 different plasmids) each encoding a gene required for DNA replication. Depending on the number of plasmids in the transfection mixture, vary the amount of transactivators and effector plasmid. As many as 11 plasmids can be transfected at one time. Each plasmid can contain one or many genes required for replication of a cloned origin of DNA replication (**Fig. 1**).

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Panel 2: cotransfections were the same as in (A), sample number 2, in which one plasmid encoding an essential protein was omitted from the transfection mixture. Inclusion of all of the essential proteins results in a more organized pattern of fluorescence typical of DNA replication compartments.

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