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Improved transfection technique for adherent cells using a commercial lipid reagent

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A variety of techniques have been developed for cell transfection including chemical [e.g., calcium phosphate (1)] and lipid-based methods (2), physical [e.g., electroporation (3)], and viral [e.g., retrovirus (4)] approaches. Nonviral techniques tend to be relatively safe and simple but also tend to be relatively inefficient compared with viral techniques. Lipid-based reagents, including liposomal and nonliposomal lipids, have become increasingly popular for in vitro and in vivo gene transfer (5). Despite the success of lipid-based methods, existing approaches may not be sufficient when an experiment requires transfection of the majority of cells in a population. We have developed an improved method using a commercial nonliposomal lipid reagent (Effectene™; Qiagen, Valencia, CA, USA) to transfect cultured adherent cells that results in improved transfection efficiencies. We transfected C2C12 skeletal myoblasts and NIH-3T3 fibroblasts immediately after trypsinization, while the cells were in suspension; the

standard approach is to transfect adherent cells several hours after they have attached to the culture dish. The transfection efficiency of the new method (70%–80% of cells transfected) may obviate the need for time-consuming stable transfections in many situations.

For the standard transfection procedure, cells were transfected following attachment to plastic culture dishes according to the manufacturer's protocol. C2C12 myoblasts and NIH-3T3 fibroblasts were obtained from American Type Culture Collection (Manassas, VA, USA). Cells were seeded at 2×10^5 cells per well in 6-well plates in 2 mL of growth medium composed of Dulbecco's modified Eagle's medium (DMEM; Sigma, St. Louis, MO, USA) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (GIBCO/Invitrogen, Grand Island, NY, USA). Cells were incubated overnight at 37°C and 5% CO₂. The following morning, lipid-DNA complexes were prepared according to manufacturer's instructions using a 1:8 DNA-to-En-

Figure 1. C2C12 myoblasts transfected with labeled plasmid DNA. C2C12 myoblasts transfected with rhodamine-labeled plasmid DNA showed greater DNA uptake when using the new transfection procedure (A) compared to the standard procedure (B). For both procedures, 2×10^5 cells were transfected with 1 μ g DNA, and cells were viewed using confocal microscopy 24 h after transfection.

hancer ratio and a 1:15 DNA-to-Efectene ratio. DNA was incubated with Enhancer in DNA Condenser buffer for 2 min at room temperature to condense the DNA, and then Effectene reagent was added and the mixture incubated for a further 10 min at room temperature to allow lipid-DNA complex formation. During this incubation period, cell growth medium was replaced with fresh medium. Growth medium was then added to the complexes to a final volume of 600 μ L, and the complexes were added to the cells. Cells were incubated with lipid-DNA complexes

for 24 h at 37°C and 5% CO₂, and then complexes were removed and fresh growth medium added. For experiments testing vector expression in myoblasts and fibroblasts, cells were collected 24 or 48 h posttransfection. For experiments testing expression in myotubes, the medium was switched to differentiation medium (DMEM + 2% horse serum) when cells reached confluency, and cells were allowed to differentiate for 4 days with daily changes in medium before collection.

For the new procedure, cells were transfected immediately after trypsinization and replating. Lipid-DNA complexes were prepared using a 1:8 DNA-to-Enhancer ratio and a 1:15 DNA-to-Efectene ratio, identical to the standard procedure. Myoblasts or fibroblasts grown in 10-cm dishes to 70% confluency in growth medium were trypsinized and then seeded at 2×10^5 cells per well in 6-well plates in 2 mL of growth medium. Immediately after plating cells, while the cells were still in suspension, growth medium was added to the complexes to a final volume of 600 μ L, and the complexes were added to the cells. Cells were incubated with lipid-DNA complexes

while they attached to the culture dish at 37°C and 5% CO₂, and then complexes were removed after 24 h and fresh growth medium was added. Cells were either collected at 24 or 48 h posttransfection or after 4 days of myotube differentiation.

The new transfection procedure resulted in substantially greater uptake of rhodamine-labeled plasmid DNA (Gene Therapy Systems, San Diego, CA, USA) compared with the standard procedure as assessed by confocal microscopy (Figure 1). In these images, nuclei were stained with Hoechst 33258. For both new and standard transfection procedures, the majority of labeled DNA was observed in a punctate vesicle-like pattern, suggesting that DNA may have entered the cell by endocytosis. Previous investigations have supported a primary role for endocytosis in uptake of lipid-complexed DNA (6–8).

When transfecting a cytomegalovirus (CMV)-luciferase vector (Gene Therapy Systems), the new procedure resulted in up to 10-fold greater luciferase activity compared with the standard procedure (Figure 2). The magnitude of the increase in luciferase activity was

Figure 2. C2C12 and NIH-3T3 cells transfected with luciferase expression vectors. C2C12 myoblasts (A) and NIH-3T3 fibroblasts (B) transfected with luciferase expression vectors showed up to 10-fold greater luciferase activity using the new procedure compared with the standard procedure. For both procedures, 2×10^5 cells were transfected with varying amounts of DNA and luciferase activity was measured 48 h after transfection. For assessing expression in C2C12 myotubes (C), 2×10^5 myoblasts were transfected with 1 μ g DNA, grown to confluence, and differentiated for 4 days, and then luciferase activity was measured. Bars represent mean $\pm$ standard errors ($\bar{x} \pm$ SEM; $n = 4$). Results are representative of two independent experiments. Numbers above bars indicate fold increase in transfection efficiency using new procedure compared with standard procedure.

Figure 3. C2C12 and NIH-3T3 cells transfected with green fluorescent protein (GFP) vectors. C2C12 myotubes (A and B) and NIH-3T3 fibroblasts (C and D) transfected with GFP expression vectors showed a greater percentage of cells expressing GFP using the new transfection procedure (A and C) compared with the standard procedure (B and D). For C2C12 myotubes, 2×10^5 cells were transfected with 1 μ g DNA, grown to confluence, and differentiated for 4 days, and then images were recorded. For NIH-3T3 cells, 2×10^5 cells were transfected with 0.5 μ g DNA and images recorded 24 h after transfection.

cell type- and DNA dose-dependent. For C2C12 cells, the largest increase in luciferase activity (approximately 6-fold) was achieved using 1 μ g DNA. For NIH-3T3 cells, the largest increase in luciferase activity (approximately 10-fold) was achieved using 0.5 μ g DNA. When C2C12 myoblasts were transfected with 1 μ g DNA, grown to confluence, and then differentiated for 4 days, the new transfection procedure resulted in an approximately 6-fold increase in luciferase activity compared with the standard procedure. These results indicate that the increased transfection efficiency associated with the new procedure is maintained for at least 6 days after transfection and is maintained throughout differentiation of C2C12 cells.

Using the new transfection procedure to introduce green fluorescent protein (GFP) expression vectors into C2C12 and NIH-3T3 cells resulted in a substantially greater number of cells expressing detectable levels of GFP in C2C12 myotubes and NIH-3T3 fibroblasts (Figure 3). In the figures shown, C2C12 myoblasts were transfected with 1 μ g DNA, grown to confluence, and then differentiated for 4 days, and NIH-3T3 cells were transfected with 0.5 μ g DNA and were approximately 50% confluent when imaged. Qualitative assessments of transfection efficiencies by two independent observers ranged from 10%–20% for the standard procedure and 70%–80% for the new procedure for both NIH-3T3 and C2C12 cells. The transfection efficiencies achieved with the new procedure may be sufficient for many experiments that previously required viral transfection or time-consuming stable transfections.

Our procedure was also effective for introducing an overexpression vector to increase expression of the small heat shock protein HSP25 in C2C12 myotubes and for introducing phosphorothioate antisense oligonucleotide into C2C12 myoblasts to knock down constitutive levels of HSP25. Using the new procedure to introduce 1 μ g of a HSP25 expression vector into C2C12 cells resulted in an approximately 80% increase in HSP25 levels above levels observed following transfection of an empty vector, whereas the standard

procedure resulted in only an approximately 30%–40% increase in HSP25. Using this procedure to introduce 1 μ g of antisense oligonucleotide against HSP25 (TriLink BioTechnologies, San Diego, CA, USA) into C2C12 cells resulted in an approximately 45% decrease in HSP25 levels compared with cells treated with transfection reagent only and cells transfected with a sense oligonucleotide construct, whereas the standard procedure resulted in little to no change in HSP25 levels. These results demonstrate the utility of the new transfection technique for introducing overexpression vectors and antisense oligonucleotides to modulate levels of constitutively expressed cellular proteins.

In summary, transfecting adherent cells immediately after trypsinization and replating improved transfection efficiency in C2C12 muscle cells and NIH-3T3 fibroblasts up to 10-fold. This new transfection procedure was also more effective in modulating levels of a constitutively expressed heat shock protein, HSP25, using overexpression vectors and antisense oligonucleotides. These findings are immediately useful for improving transfection efficiency of adherent cultured cells, and the transfection efficiencies achieved may obviate the need for time-consuming stable transfections in many situations. Further investigation into the mechanisms of improved transfection may reveal strategies to increase transfection efficiency in vivo.

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