A simple, rapid method for evaluation of transfection efficiency based on fluorescent dye

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ABSTRACT

Enhanced transfection efficiency of transient gene expression (TGE) and electroporation is a useful approach for improvement of recombinant therapeutic proteins in mammalian cells. A novel method is described here in which CHO cells expressing recombinant FVII (rFVII) were labeled with fluorescent dye and analyzed by confocal microscopy. Cells with or without rFVII encoding gene were detectable by flow cytometry. Thus, we were able to distinguish positive cells (with rFVII encoding gene) and quantify their percentages. We evaluated the effects of varying electroporation conditions (voltage, number of repetitions, plasmid amount, carrier DNA) in order to optimize transfection efficiency. The highest transfection efficiency achieved was ~86%. The method described here allows rapid evaluation of transfection efficiency without co-expression of reporter genes. In combination with appropriate antibodies, the method can be extended to evaluation of transfection efficiency in cells expressing other recombinant proteins.

KEYWORDS

CHO cells; electroporation; fluorescent dye; transfection efficiency

Introduction

Since the FDA approval of recombinant tissue plasminogen activator (rt-PA) ("Activase"; Genentech, Inc.) in 1987,¹ researchers have expressed a steadily increasing number of recombinant therapeutic proteins in mammalian cells, which display post-translational modifications (PTMs) and glycosylation similar to those of human cells.²,³ Transient gene expression (TGE) and electroporation are the primary methods used for expression of recombinant proteins in mammalian cells following delivery of exogenous encoding genes into the cells. TGE is commonly used to produce small amounts of proteins for pre-clinical and clinical evaluation of potential therapeutic drugs. Transfection efficiency affects the percentage of positive cells and is thus related to final production of recombinant proteins in subsequent culture.⁴,⁶ In contrast to TGE, exogenous genes delivered to cells are randomly integrated into host cell chromosomes following electroporation. The variety of genomic integration sites on DNA results in varied expression of target proteins. High transfection efficiency is therefore desirable for isolation of high expression clones from cells with different genomic integration sites.

Reporter genes having measurable and distinctive characteristics are routinely used by researchers. In studies with these genes, transfection processes and conditions are often analyzed using fluorescent proteins with different colors. Green fluorescent protein (GFP) can be co-expressed with a target protein through either integration with vector or driving by internal ribosome entry sequence (IRES).⁴,⁵,⁷ Light and heavy chain antibody expression can be detected simultaneously by green and yellow fluorescent reporter proteins driven by IRES.⁷ After transfection, positive cells expressing the target protein can be easily distinguished by the presence of intracellular fluorescent protein, and transfection efficiency can be evaluated by flow cytometry. Other techniques can be
combined with use of intracellular fluorescent reporters to evaluate transfection efficiency or for other purposes, e.g., cellular imaging of DNA delivery by high-content screening (HCS), analysis of biochemical composition and metabolism, and fluorescence-activated cell sorting.

We describe here a new method for analysis of transfection efficiency that does not involve co-expression of reporter genes. CHO cells with or without rFVII encoding gene were examined by confocal microscopy and flow cytometry to assess fluorescent dye levels, and various electroporation conditions were optimized to enhance transfection efficiency. Our novel method allowed rapid and accurate evaluation of transfection efficiency.

Results

Detection of rFVII-expressing cells by confocal microscopy

For detection of rFVII-expressing cells, CHO cells with vs. without rFVII encoding gene were examined by confocal microscopy (Fig. 1). rFVII-expressing CHO cells were constructed and selected by dot blot and western blot as described in our previous study. Adherent cells were treated with Triton X-100 to increase membrane permeability, incubated with FVII antibody, conjugated with secondary antibody with green fluorescent dye, and examined by confocal microscopy. Typical images are shown in Fig. 1. Nuclei were stained with DAPI (blue color), and rFVII in cells was stained green. We were able to easily distinguish cells with vs. without rFVII encoding gene using the green fluorescent dye. Only small amounts rFVII were remaining in ER and hardly detected. Therefore, the detected rFVII in Fig. 1 may be the transporting rFVII.

Method for analysis of transfection efficiency based on flow cytometry

Because CHO cells with vs. without rFVII encoding gene could be distinguished by confocal microscopy, we were able to quantify transfection efficiency by flow cytometry using green fluorescent dye. To rule out possible interference effect, we incubated rFVII-expressing cells without FVII antibody (NC-1), without secondary antibody (NC-2), or with both antibodies (PC). Flow cytometry revealed no green fluorescence for either NC-1 or NC-2 (Fig. 2a, 2b). This method allowed us to easily distinguish positive cells (labeled with green fluorescent dye) (Fig. 2c). We therefore used this method to evaluate transfection efficiency under various conditions in subsequent experiments.

Effects on transfection efficiency of electroporation voltage, number of repetitions, and plasmid amount

Transfection efficiency (positive cell percentage) under various conditions was analyzed by flow cytometry as described above. Among the 5 Vages tested, transfection efficiency was highest (86.5%) at
400 V (Fig. 3a). Positive cell percentage increased as number of repetitions increased from 1 to 3 (Fig. 3b), but dropped with 4 repetitions. With 4 repetitions, density of viable cells in the electroporation mixture was also reduced (data not shown). Among the 4

![Figure 2](image1.png)

**Figure 2.** Analysis of CHO cells with or without rFVII encoding gene by flow cytometry. (A) NC-1: without FVII antibody. (B) NC-2: without secondary antibody. (C) PC: with both antibodies.

![Figure 3](image2.png)

**Figure 3.** Effects of variation in 3 electroporation conditions on transfection efficiency of CHO cells. (A) voltage. (B) number of repetitions. (C) plasmid amount.
plasmid amounts tested, transfection efficiency was highest (85.7%) for 20 µg plasmid (Fig. 3c).

**Effects on transfection efficiency of carrier DNA addition and G418 selection**

Salmon sperm DNA (used as carrier DNA) was added to the electroporation mixture. Transfection efficiency was significantly higher with carrier DNA (85%) than without carrier DNA (48.2%) (Fig. 4), demonstrating the important role of carrier DNA in electroporation of mammalian cells. Transfected cells were further purified by G418 selection to eliminate non-transfected cells. After 2 d of culture with G418, all cells were rFVII-expressing (Fig. 5).

**Discussion**

Increasing transfection efficiency is a key strategy for construction of recombinant protein-expressing cells. In order to analyze transfection efficiency, certain reporter genes have been co-expressed with target proteins, and many studies have used green fluorescent protein (GFP) to mark positive cells after transfection. Transfection efficiency has been enhanced by various strategies based on reporter genes, or by optimization or addition of conditions or factors such as transfection reagents, DNA topology of plasmid, cell cycle distribution of host cells, and non-encoding carrier DNA. The Epstein-Barr virus nuclear antigen-1 (EBNA-1) gene was shown to accelerate gene delivery and promote transcription. Transfection efficiency and protein expression in electroporation were enhanced when this gene was inserted in the expression plasmid. The above efficiency-enhancing strategies involved intracellular co-expressed fluorescent reporters. Fluorescent protein-encoding genes can be integrated with plasmid and thus co-expressed with recombinant protein. Expression of fluorescent proteins is also driven by internal ribosome entry sequence (IRES), which is responsible for cap-independent translation of fluorescent proteins following antibody expression. Sleiman et al. used this method to isolate high recombinant antibody-expressing cells based on light and heavy chain-specific fluorescent reporters.

Despite its wide application, co-expression of reporter genes has several disadvantages, notably the silencer effect of CAT, spontaneous mutations caused by luciferase, and occupation of limited space in plasmid by fluorescent protein. Fluorescent dyes are commonly used in confocal microscopy for localization of labeled molecules or organelles in cells. We established an effective method based on fluorescent dye for evaluating transfection efficiency of electroporation. This method allowed us to distinguish positive cells (expressing FVII) from negative cells (not transfected with plasmid). We tested various transfection conditions, and achieved a maximal transfection efficiency of ~86%. The method also allowed rapid analysis of the purity of recovered cells. In the present study, we evaluated transfection efficiency of electroporation and the effects of various transfection...
conditions. The method is applicable for optimization of transient transfection without construction of a vector containing the fluorescent protein-encoding gene. The method requires a short total duration (~3–5 h) and simple procedure (incubation with antibody).

Certain current techniques applied in combination with fluorescent labeling have other purposes. After transfection, vector containing the encoding gene may enter the cell and then integrate with the host cell chromosome. The delivery process and cytotoxicity of vector in such cases are difficult to measure and visualize. In a study by Wongrakpanich’s group, PEI and vector were both labeled with fluorescent marker, and the relationship between transfection efficiency and cellular polyplex was evaluated by high-content screening (HCS). Rosa et al. used Fourier transform mid-infrared (FT-MIR) spectroscopy with GFP to assess the effect of the transfection process on biochemical and metabolic responses of host cells. Confocal laser scanning microscopy is commonly used in several applications, including monitoring of drug uptake and delivery, measurement of physiological parameters, and detection of cell functions that involve subcellular compartments and ligands. Biological specimens (cells, tissues, or organisms) can be easily visualized when combined with or linked to fluorescent markers. A variety of fluorescent indicators (dyes, luciferases, enzymes) can be detected among intracellular mixtures of molecules, thus allowing localization of target molecules in natural environments. The method for analysis of transfection efficiency described here will also be useful for investigation of intracellular synthetic processes.

Methods and materials

Cell culture and plasmid

Chinese hamster ovary (CHO) cells and plasmid pMH3 were from AmProtein Co. (Hangzhou, China). Transfected plasmid with human FVII encoding gene was constructed as described previously. CHO cells were cultured in DMEM/F-12 (Invitrogen; Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Invitrogen) at 37°C in 5% CO₂ atmosphere.

Confocal microscopy

rFVII-expressing CHO cells, produced in our previous study, were used as positive cells for confocal microscopy. CHO cells with and without rFVII encoding gene were cultured in separate dishes. Cells were treated with 4% (v/v) paraformaldehyde for 30 min, washed with PBS, treated with 0.2% (v/v) Triton X-100 for 10 min, washed with PBS, blocked with 5% (w/v) bovine serum albumin for 1 h, washed with PBS, incubated with sheep anti-human factor VII antibody (Cedarlane Labs; Burlington, Canada) at 4°C for 1 h, labeled with Alexa Fluor 488 donkey anti-goat antibody (1:200) as secondary antibody for 30 min, and examined by confocal laser scanning microscopy (model LSM 510, Zeiss).

Transfection and electroporation of cells

Plasmid was transfected into CHO cells by electroporation using 400V, 40 μsec, 3 times (control). The electroporation mixture contained 3 × 10⁶ cells, 20 μg plasmid, and 5 μg salmon sperm DNA. Various electroporation parameters (voltage 100, 200, 300, 400, 500 V; plasmid amount 5, 10, 15, 20 μg; number of repetitions 1, 2, 3, 4) were tested in order to achieve optimal transfection efficiency. In addition, electroporation mixtures with vs. without salmon sperm DNA were analyzed. During analysis of a given parameter, other parameters were maintained at control value. Following electroporation, cells were collected and cultured for 4 d, and transfection efficiency was analyzed.

Analysis of transfection efficiency by flow cytometry

Collected CHO cells as above were detached by 2 mM EDTA and suspended in PBS solution to obtain a single cell suspension. Processing steps for flow cytometry were similar to those described above for confocal microscopy. Treated cells were analyzed with a FACS-Calibur flow cytometer (BD Biosciences) and positive cell percentage was calculated using the FlowJo 7.6 software program. The negative controls were cells incubated without FVII antibody (NC-1) or without secondary antibody (NC-2). The positive control (PC) was the rFVII-expressing cell line described in our previous study.

Selection of transfected CHO cells using G418

Transfected cells were cultured with DMEM/F-12 supplemented with 10% FBS at 37°C, 5% CO₂ atmosphere, for 2 d. Two mg/mL of the aminoglycoside antibiotic G418 (a selection agent) was added to
culture medium for 2 d, and cells were washed with PBS solution 3 times. Remaining adherent cells were cultured with DMEM/F-12 with 10% FBS for 1 d, collected, and positive cell percentage was measured by flow cytometry.

**Disclosure of potential conflicts of interest**
No potential conflicts of interest were disclosed.

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