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Original Article

Evaluation and Optimization of Lipofectamine 3000 Reagents for Transient Gene Expression in KYSE-30 Esophagus Cancer Cell Line

Reihaneh Alsadat Mahmoudian[1], Moein Farshchian[2], Mohammad Reza Abbaszadegan[3]*

1- Immunology Research Center, Mashhad University of Medical Sciences, Mashhad, Iran.
2- Stem Cell and Regenerative Medicine Research Group, Academic Center for Education, Culture, and Research-Khorasan Razavi, Mashhad, Iran.
3- Medical Genetics Research Center, School of Medical Sciences, Mashhad University of Medical Sciences, Mashhad, Iran.

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Abstract

Background and Aim: Transfection of DNA/RNA sequence into eukaryotic cells has a major effect on scientific studies. Various methods are used to transfer the DNA/RNA sequence into cells, such as lipid-based carriers as the available and easy procedure. Transfection with cationic lipid liposome is introduced as a simple and efficient procedure for monitoring the DNA/RNA sequence through gene function analysis, including fluorescence imaging RNA and protein expression. This study aimed to investigate the transfection efficiency and cell death through GFP expression in human esophageal squamous cell carcinoma (ESCC) cell line KYSE-30 using Lipofectamine 3000 reagent.

Methods: The pCDH-513b plasmid DNA was transfected into KYSE-30 cells using Lipofectamine 3000 in different concentrations of the plasmid DNA and reagent. The transfection efficiency was evaluated by fluorescence microscope and flow cytometry analysis to determine the percentage of GFP-expressing cells. Moreover, the viability and death of transfected KYSE-30 cells were evaluated using a trypan blue exclusion assay.

Results: The transfection efficiency of KYSE-30 with Lipofectamine 3000 was increased with higher plasmid DNA concentration and a lower amount of Lipofectamine 3000 reagent. The Optimized concentration of 1.5 µg plasmid DNA and volume of one µl of lipofectamine 3000 reagents were identified for 95% transfection efficiency in the KYSE-30 cell line. The viability and death of transfected cells were 43% and 58% after transfection, respectively.

Conclusion: The results indicated that Lipofectamine 3000 might not be suitable for transfection in KYSE-30 cells due to increased cell death.

Keywords: Non-Viral Vector, Transfection Efficiency, Cationic Lipid, Lipofectamine 3000.

***Corresponding Author:** Mohammad Reza Abbaszadegan; **Email:** abbaszadeganmr@mums.ac.ir

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Introduction

Gene delivery is introduced as a key and valuable procedure for transfer a sufficient amount of genetic materials like nucleic acid fragments (DNAs or RNAs) into the target somatic or germ cells. Gene the transfer can help to express or suppress the gene of interest (GOI) in cells for the desired time to investigate the cellular and molecular mechanisms underlying gene transfer (1-3). Delivery of foreign DNA or RNA into the mammalian cells for genetic modification can have transient or stable integration into the host genome, depending on the type of GOI and how it is transmitted (4). The transient versus stable gene expression is defined as a quick and affordable process in producing recombinant proteins (rProteins) for a limited period (5). There are several beneficial factors to determine the high

efficiency of GOI expression in a specific tissue or cell and low level of death cells. Among these factors can mention to the expression level of GOI, common biosafety, desired delivery efficiency, cellular context, cell division, and cell viability (6). There are two approaches for the delivery of foreign DNA or RNA into the target cells, including natural and artificial-mediated techniques (7).

Non-viral gene transfer methods have led to transient gene expression with remarkably less efficiency than viral techniques. Nevertheless, new non-viral techniques overcome these limitations and improve the rProtein production efficiency (6, 8, 9). The non-viral methods are divided into physical and chemical approaches, which are generally dependent on the synthetic cellular barriers, like liposomes or nanoparticles for transferring the genetic material (2). The key factors in the effective non-viral gene transfer can refer to the type of target cell, the optimal cell confluency, the number of cell passages, existence or absence of serum in culture media, the optimal amount of foreign genetic material, the appropriate time for transfection complex formation, and expression of GOI in various cells (10). Among non-viral gene transfer vehicles are cationic lipids such as a liposome. Generally in this delivery system, positively charged liposome plays an important role, in contrast, the negative charge of both nucleic acid and cell membrane to overcome the electrostatic force of the cell membrane (10, 11). The formation of cationic liposome-nucleic acid complex leads to cellular uptake of GOI through the ionic interaction of the complex with the plasma membrane of the host cell (10, 12). The advantages of liposome-mediated gene transfer are the high efficiency of gene delivery into cells, fast and convenient in the procedure (9). Among liposome-based transfection reagents can use the Lipofectamine 3000 reagent (13). Lipofectamine 3000 is currently considered in different types of cells to obtain the appropriate transfection efficiency; however, it demonstrates toxicity and apoptotic effects for some eukaryotic cell lines. Consequently, type and number of target cells, size of plasmid, and transfection reagents are key factors for the best yield of transfection (14).

Hence, this study aimed to optimize the transfection efficiency and cell death of the chemical-based transfection method for human esophageal squamous cell carcinoma (ESCC) cell line KYSE-30 to find the best transfection conditions. Therefore, Lipofectamine 3000 reagents as liposome-based transfection reagents were used to transfect KYSE-30 cells.

Methods

Plasmid preparation

The pCDH-513b (System Bioscience, Palo Alto, CA) plasmid DNA as a green fluorescent protein (GFP) expression vector was applied to compare the transfection efficiency. The plasmid DNA firstly amplified in competent *E. coli* Stbl cells, then isolated and purified using the Qiagen Plasmid Maxiprep Kit (Qiagen, UK) according to the manufacturer's instructions. The integrity of plasmid DNA was confirmed by electrophoresis on 0.8% agarose gel, and the plasmid DNA was diluted to a final concentration of 1.5 µg/µL DNA in sterile water.

Cell culture

Human ESCC (KYSE-30) cell line was obtained from the Pasteur Institute Cell Bank of Iran (Tehran, Iran) (http://en.pasteur.ac.ir/) and cultured under the standard condition as follows in RPMI 1640 medium (Biosera) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco, USA) with penicillin /streptomycin (100 U/mL) (Gibco, USA) at a humidified 37 °C incubator with 5% CO2.

Liposome-mediated transfection using Lipofectamine 3000

All transfection procedures were performed in 24-well plates using Lipofectamine 3000 (Invitrogen, USA, L3000015). There are two components in Lipofectamine 3000 kit, including lipofectamine 3000 (LFN3000) and P3000 reagents. KYSE-30 cells were seeded at a density of 0.25×10^6 cells per well, 20-24 hours prior to transfection in 0.5 mL of culture medium with 10% FBS to obtain 70-80% confluency at the time of transfection.

Firstly, to determine the efficient amount of the LFN3000 reagent, a mixture reaction was prepared, containing 1 µg plasmid DNA, 1 µL P3000 reagent, 50 µL Opti-MEM serum-free medium (Gibco, USA), along with the different amount of LFN3000 (including 0.5, 1, 1.5, and 2 µL) according to the manufacturer's instructions. Afterward, this cocktail was incubated at room temperature for 20 minutes to form the DNA-LFN3000 complex. Finally, the cell supernatant was replaced with serum and antibiotic-free media, and the prepared DNA-LFN3000 complex was diluted to each well and the mixture was incubated at 37 °C for 36 hours. After determining the efficient amount of LFN3000 reagent, the amount of 0.5, 1, and 1.5 µg plasmid DNA were evaluated according to the procedure described above to obtain optimal plasmid DNA concentration.

Green fluorescence protein (GFP) assay

The transfection efficiency was assessed through the expression of the integrated GFP as a reporter gene using wavelengths 450-490 nm ultraviolet light 36 hours after transfection. The GFP expression was visualized by an inverted fluorescence microscope with different magnifications. The transfection efficiency was delineated according to the percentage of GFP-positive cells. Image analysis of GFP-positive cells was performed with the ImageJ software package (15) . Briefly, we have selected the area integrated intensity of image using ImageJ, then the GFP-positive cell counting from fluorescence images in a final magnification of 10X microscope was performed randomly with four repeats using the ImageJ cell counter plugin. Afterward, we calculated the number of cells in the field of view (FOV) of 10X magnifying Optika microscope for a 24-well plate as follow:

Number of cells = [0.025 (FOV of 10X magnifying microscope) × (0.25×10^6) cells]/20 mm surface area for 24-well plate

Finally, the number of GFP-positive cells versus the total cell quantity was estimated in a different amount of the plasmid DNA and LFN3000 as follow:

% Transfection efficiency = (number of GFP-positive cells by ImageJ/total number of cells in FOV of 10X magnifying microscope) × 100

Flow cytometry analysis

The transfection efficiency was examined by flow cytometry analysis 36 hours post-transfection to determine the percentage of GFP-expressing cells. Briefly, cells were washed twice with phosphate-buffered saline (PBS; pH = 7.4) and then trypsinized by 0.25 % trypsin-EDTA for 5 min at 37 °C. The cell suspensions were centrifuged at 2000 rpm for 6 min and the samples were detected by a FACSCalibur flow cytometer (BD Bioscience, San Jose, CA, USA) for FL1 (GFP) fluorescence. Then data analysis was performed using FlowJo 7.6 software (Tree Star, Ashland, OR) to determine the percentage of GFP-expressing cells.

Trypan blue exclusion test of cell viability

The cell viability of transfected KYSE-30 cells by Lipofectamine 3000 reagents was evaluated using a trypan blue exclusion assay (16). The transfected cells were first trypsinized and mixed with trypan blue dye, then visually examined the uptake (stained or nonviable) and exclude (unstained or viable) of dye into cells (17). Although Lipofectamine 3000 indicated high transfection efficiency, perturbation due to dead cells may interfere with future functional studies (18). The percentage of nonviable and viable cells was calculated as follows:

% Viable cells = (number of viable cells/ total number of cells) × 100

% Dead cells = 100 - % viable cells

Statistical analysis

All statistical analyses were carried out using GraphPad Prism 5 (GraphPad Software, La Jolla, CA, USA). The Student T-test was applied for the transfection efficacy of the serially diluted LFN3000 and the plasmid DNA. All data was obtained from four biological experiments and values were reported as mean. The value of statistical significance was indicated as a *P* value less than 0.05.

Results

Assessment of transfection efficiency

Results of GFP assay after 36 hours

The ESCC cell line KYSE-30 was transfected with pCDH-513b vector by different volumes of the LFN3000 and plasmid DNA concentrations. The results indicated that the most efficient GFP-expressing cells were achieved with a volume of one µl among the four different LFN3000 volumes that select for later experiments (data not showed). These results suggested that the transfection efficiency improved in KYSE-30 cells with a smaller amount of LFN3000. As indicated in Figure 1 (A-H), green fluorescence expression observed in different plasmid DNA concentrations. The expression fluorescence intensity increased with the increase of plasmid DNA quantity, and the green fluorescence quantity of 1.5 µg of DNA was maximum. The number of transfected cells and the percentage of GFP-positive cells are demonstrated in Figure 2.

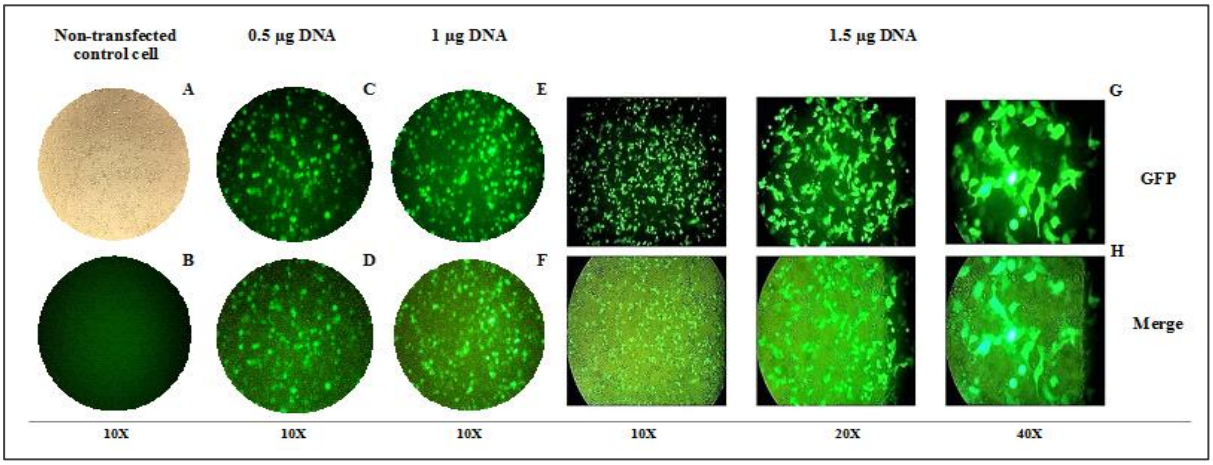
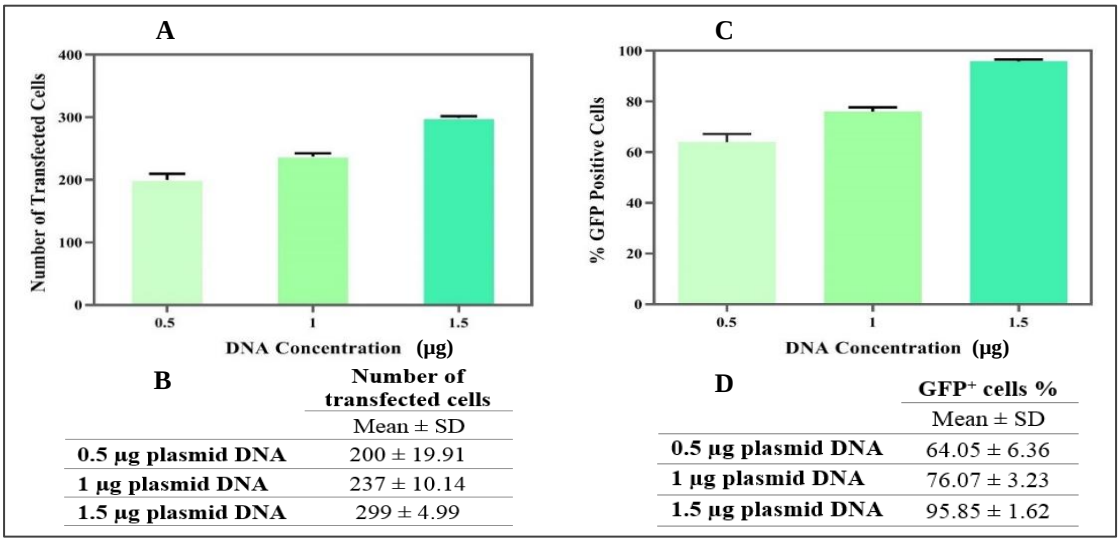


Figure 1. The fluorescence microscopy images of the GFP expression profile in KYSE-30 cell transfected by Lipofectamine 3000. (A & B) non-transfected cells, (C & D) 0.5 µg of transfected GFP plasmid, (E & F) 1 µg of transfected GFP plasmid, (G & H) 1.5 µg of transfected GFP plasmid with different magnification. The down panels demonstrated the corresponding merge images of the fluorescence images. The scale bar is 200 µm. The total of images was obtained using an inverted fluorescence microscope.



B

	Number of transfected cells
	Mean ± SD
0.5 µg plasmid DNA	200 ± 19.91
1 µg plasmid DNA	237 ± 10.14
1.5 µg plasmid DNA	299 ± 4.99

D

	GFP⁺ cells %
	Mean ± SD
0.5 µg plasmid DNA	64.05 ± 6.36
1 µg plasmid DNA	76.07 ± 3.23
1.5 µg plasmid DNA	95.85 ± 1.62

Figure 2. The transfection efficiency was measured in KYSE-30 cells after the transfection of the pCDH-513b vector using Lipofectamine 3000. (A & B) the number and mean of transfected cells in different plasmid DNA concentrations, (C & D) percentage and mean of GFP positive cells in different plasmid DNA concentrations. SD: standard deviation.

The mean of GFP-positive cells was 64.05%, 76.07%, and 95.85% for 0.5 µg, 1 µg, and 1.5 µg of DNA, respectively, as depicted in Figure 2C and D. The highest level of GFP expression (98.3%) was detected with 1.5 µg plasmid DNA. Moreover, the average ± standard deviation (SD) of the number of transfected cells were 200 ± 19.91, 237 ± 10.14, and 200 ± 4.99 cells for 0.5 µg, 1 µg, and 1.5 µg of DNA, respectively (Figure 2A and B). These results proposed that the transfection efficiency significantly increased in KYSE-30 cells with increasing higher plasmid DNA concentration (*P*-value < 0.0001).

Results of flow cytometry

Flow cytometric analysis was used to directly quantify the transfection efficiency in KYSE-30 cells (Figure 3). KYSE-30 cells without GFP plasmid was used as a control or unstained cells (Figure 3A and B).

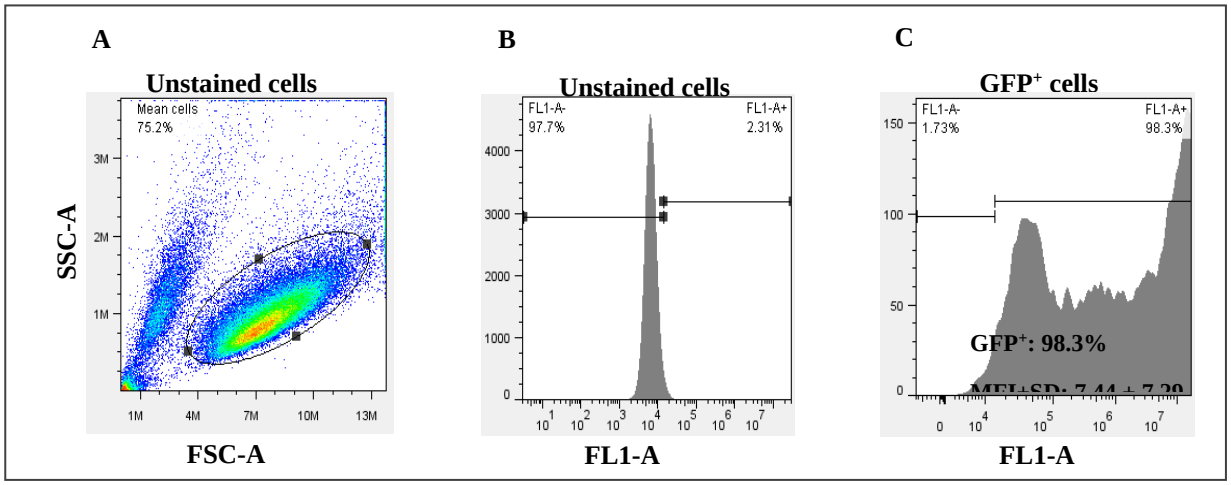
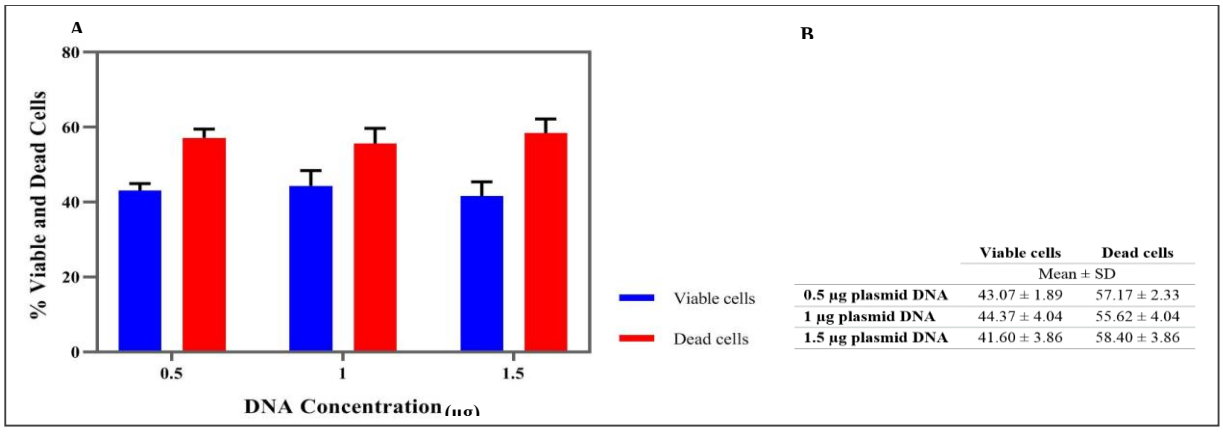


Figure 3. The transfection efficiency analysis of pCDH-513b-transfected KYSE-30 cells using 1.5 µg of plasmid DNA. The Flow cytometry was used to analyze the GFP expression, (A) gating for living cells in untransfected cells (B) untransfected cells for GFP expressing cells (C) percentage and MFI of GFP expressing cells. FSC: forward scatter; SSC: side scatter; FL: fluorescence scatter; MFI: mean fluorescence intensity.

This study only analyzed the cells in a concentration of 1.5 µg plasmid DNA and one µl LFN3000 volume. The percentage of GFP-positive cells and mean fluorescence intensity (MFI) was estimated at approximately 98.3% and 7.44±7.29, respectively, as indicated in Figure 3C (the results of flow cytometry analysis are shown only for the concentration of 1.5 µg plasmid DNA). The flow cytometry data was indicated that the highest yield of transfection efficiency achieves with a concentration of 1.5 µg plasmid DNA and one µl LFN3000.

Assessment of cell viability and cell death

The viability and general health of cells were maintained high at the time of transfection. Trypan blue assay indicated that the mean ± SD of viable cells 36 hours after transfection were decreased from 43.07±1.89 for 0.5 µg to 41.60±3.86 for 1.5 µg of the plasmid DNA (Figure 4). Moreover, the mean ± SD of dead cells were increased from 57.17±2.33 for 0.5 µg to 58.40 ± 3.86 for 1.5 µg of the plasmid DNA. The data was exhibited that LFN3000 reagent induced a high relative level of cell death in KYSE-30 cells. As shown in Figure 4, the difference in cell viability and cell death in different concentrations of plasmid DNA is very low.



	Viable cells	Dead cells
	Mean ± SD	
0.5 µg plasmid DNA	43.07 ± 1.89	57.17 ± 2.33
1 µg plasmid DNA	44.37 ± 4.04	55.62 ± 4.04
1.5 µg plasmid DNA	41.60 ± 3.86	58.40 ± 3.86

Figure 4. Cell viability and cell death were measured by the trypan blue assay in KYSE-30 cells after the transfection of the pCDH-513b vector using Lipofectamine 3000. (A) comparison of viable and dead cells in different DNA concentrations, (B) table shows the mean of viable and dead cells in different DNA concentrations. SD: standard deviation.

Discussion

Development of gene delivery systems is required not only in cell biology researches and clinical gene therapy but also in the evaluation of genes function analysis (19). The outcomes of DNA/RNA cargo delivery depend on the delivery systems for controlling the gene expression within any type of cell and function of the target genes of cargo. Among other factors that affect the transfer efficiency, including amount and type of cargo, timely escape of the biomolecule from endocytosis machinery, cell viability after transfection of cargo/reagent into cells, and technical aspects of gene delivery (1, 20, 21). The efficient amount of the transfection reagent and DNA/RNA cargo are vital matters in the transfection efficiency, as this study indicated that the different amounts of LFN3000 reagent and plasmid DNA had a considerable role in the high-level expression of the transgene and cell viability *in vitro* (1).

In the present study, we performed a comparative analysis of the transfection efficiency of GFP plasmid DNA containing a CMV-promoter by Lipofectamine 3000 in adherent KYSE-30 cells for determination of the optimal DNA/LFN3000 ratio and cell viability. We reported the efficient transfer of DNA into KYSE-30 cancer cells by LFN3000 reagents. The transfection improved with higher plasmid DNA concentration; although cell death was relatively high through LFN3000 in KYSE-30 cells. Consequently, the high amount of plasmid DNA and low volume of transfection reagents could be an important factor for optimal transfection efficiency.

Generally, lipofectamine reagents as lipid-based components are gold-standard systems for transferring DNA and siRNA/miRNA into the widest spectrum of cell types (22). The advantages of an effective transfection reagent are transgene delivery to more validated cell types, low toxicity, non-oncogenic, non-immunogenic, simplicity, protection against transgene degradation, high transfection efficiency, and no negative effects on downstream experiments (4, 6, 7). Lipofectamine 3000 is a novel cationic liposome mediated-transfection reagent with two components, including LFN3000 and P3000, leading to an efficient gene entry into the cells. According to numerous studies, Lipofectamine 3000 indicated successful nucleic acid transfer with a population of viable cells in various eukaryotic cell types (13, 18, 23, 24). It has been reported that various cells can transfect with high efficiency using Lipofectamine 3000, including easy and recalcitrant cells in transfection, such as embryonic stem cells (ESCs), induced pluripotent stem cells (iPSCs), primary cell cultures, and fibroblasts (25, 26). Lipofectamine 3000 can apply for the transfection of cargo types, including plasmid DNA, shRNA, miRNA, siRNA, CRISPR/Cas9, and protein (24). Lipofectamine 3000 reagents can deliver exogenous proteins like translation-inhibiting enzymes into the cytosol of various cancer cell lines for evaluating their

intracellular functions (27). Besides, the concomitant transfection of several plant cryptochrome linear DNAs were accomplished with stable transfection efficacy in HEK293T cells using Lipofectamine 3000 (28). Both plasmid DNA and siRNA were transfected to mouse embryonic stem cells (mESCs) by Lipofectamine 3000, resulting in a good transfection efficiency with siRNA-mediated 70% downregulation (24). Furthermore, Cas9-mediated gene silencing in mESCs was performed by some transfection methods, suggesting good transfection efficiency by Lipofectamine 3000 in CRISPR-Cas9 genome editing (29). It has been shown that miRNA-145 was successfully silenced using Lipofectamine 3000 in numerous human colorectal cancer cell lines (HCT-8, HCT-116, SW620, SW48, LoVo, and HT-29) for evaluating cell invasion, proliferation, and migration (30). Besides, knock-out/knock-down experiments of various types of coding and non-coding RNAs (PLCE1, miR503, miR543, miR107, PIM1, GAS5, WASH, and SIX1) were performed using Lipofectamine 3000 in various esophageal cancer cell lines (EC9706, Eca109, TE1/3/12, KYSE510/140/150/180/410/450, YES-2, and Het-1A), indicating a successful transfection for downstream analysis (31-35). In line with the previous study, our results indicated good transfection efficiency in KYSE-30 cells with pCDH-513b plasmid. This data confirmed the role of transfection reagent and DNA cargo in the final efficiency of transfection. The higher and lower amounts of DNA and LFN3000 reagent, respectively, can lead to high transfection efficiency. The probability of success of DNA-LFN3000 complexes delivery into cells by endocytosis depends on the amount of DNA delivered into cells. There is a direct relation between transfection efficacy and toxicity of transfection reagents based on the type of cell. The design of the non-viral delivery agents is based on increase transfection efficiency and reduces toxicity effects on cell viability after cargo delivery (36). The transfection process may lead to decreased living cells, increased cell death, cytotoxicity, and the morphological abnormalities of cells (37). These adverse effects can nonspecifically affect the expression of certain genes (38). Consequently, the optimization of the DNA/transfection reagents ratio has a critical role in the balance between the transfection efficiency and side effects (39). Transfection of single-strand oligonucleotide molecules like antisense oligonucleotide in different types of cell lines was demonstrated good transfection efficiency, high cytotoxicity, and decrees cell viability, resulting in that additional amounts of LFN3000 reagent may have led to side effects (18). Our data demonstrated that the use of smaller quantities of LFN3000 led to an increase in the percentage of GFP-expressing cells. However, it has been demonstrated that the transfection of miRNA and plasmid DNA into HEK293 cells only by LFN3000 reagent (without P3000 reagent) can reduce toxicity. Nevertheless, the usage of LFN3000 without P3000 reagent is usually not suitable for transfection efficiency (40). There are no studies that have evaluated the function and role of P3000 reagent in the transfection processes. The majority of previous studies have shown low toxicity of Lipofectamine 3000 reagents in different cell types. It is noteworthy that cytotoxicity and transfection efficiency are extremely dependent on cell type (13, 18, 24). Differences in cargo type and formulation of transfection reagents can lead to different levels of cytotoxicity in cells (41). Our results indicated that increasing the plasmid DNA concentration not only increased the number of transfected cells and the percentage of GFP-positive cells but also led to 59% cell death in KYSE-30. It has been indicated that variation at different concentrations of the plasmid DNA generally did not show a significant difference in cell viability and cell death, but can improve the transfection efficiency between 20-30% (18, 42). Consistent with previous studies, our results exhibited the 20-30% increase in GFP-positive cells, very slight differences in average of cell death and viability at different DNA/LFN3000 concentrations in KYSE-30 cells. Moreover, the cell seeding density is a key factor in the cargo expression and yield of transfection, accordingly in our study, cells were seeded 24 hours before transfection to reach the

suitable cell density (70-80% confluency) at the time of transfection (10). We also applied pCDH-513b plasmid DNA under the control of the cytomegalovirus (CMV) promoter for DNA transfection. This promoter can active in the undifferentiated embryonic stem cells and various differentiated cell types (43). CMV promoter leads to high levels of stable and transient expression of cargo in different cells (44). Taken together, our findings consider that these reagents may not offer a feasible approach for gene delivery into KYSE-30 cells and require further functional gene analysis.

Conclusion

In conclusion, the transfection efficiency of KYSE-30 cells with Lipofectamine 3000 was improved with higher DNA concentration and a lower amount of LFN3000 reagent. Taken together, our findings revealed high transfection efficiency along with cell death of approximately 50% at different DNA concentrations. The cell type, amount of DNA, and LFN3000 reagent were recognized as key factors for prosperous transfection into KYSE-30. Therefore, transfection KYSE-30 cells using Lipofectamine 3000 need more attention for later analysis of genes function.

Conflict of Interest

The authors declared no conflict of interest.

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Not declared.

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