Optimization of Transfection of Human Coxsackie and Adenovirus Receptor into Mammalian Cells Mediated by Liposomes-Base Gene Delivery

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Abstract: Cationic liposome-based reagents provide reliable and simple protocol for *in vitro* transfection in a broad range of mammalian cell types. High transfection efficacies and low cytotoxicity of a liposome-based transfection reagent was required to retain adequate viability and high level of transgene of transfected Chinese hamster ovary (CHO) cells for subsequent assay. Three commercial liposome-based transfection reagents were utilized and compared for their aptitude to transfect *in-vitro* CHO cells with reporter gene expressing the red fluorescent (pDsRed-N1) and fusion pDsRed-N1 plasmid containing human cDNA Coxsackie and Adenovirus Receptor (pCAR-DsRed). Xtreme HP Plasmid DNA transfection reagent (Roche) gave the highest transfection efficiencies compared to other tested reagents. The optimal Xtreme HP Plasmid DNA reagent had shown its higher transfection efficiency (39.25±1.00% MFI) and stable increment of DsRed-proportion cells with incubation period (24-72 hours), allowing sufficient yield and shortened the selection stably clone of successful transfected CHO-CAR-DsRed.

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1. Introduction

The biochemical transfection method has been described as a versatile non-viral gene-transfer technique to be utilized for *in vitro* mammalian cells transfection. The cationic liposome-mediated is also known as lipofection technique. Due to its simplicity, suitability, efficiencies, and reproducibility of transfection in a wide range of cell types, this technique has gained wide acceptance in applications for basic research and clinical trials (Maurisse *et al.*, 2010). The ionic interaction between cationic and DNA leads to the formation of lipoplexes that allows the opening of pores or 'holes' in the cell membrane, in which molecules of DNA is transported into the nucleus via endocytic pathway and involving transcribe process (Wagner *et al.*, 2005). Subsequently, the mRNA product is transferred into the cytoplasm and translated into protein.

Even though there are varieties of existing commercial transfection reagents, there is no absolute transfection methods that can be applied to wide types of cells or experiments. Hence, the level of gene expression that can be attained might vary greatly with respect to different methods utilized and titration of DNA concentration (Dahm *et al*., 2008). Each transfer method may have advantages or disadvantages, depending on the type of cells and nucleic acid molecules to be transfected (DNA, RNA, oligonucleotides, proteins), or even the demands of high-throughput or large scale industry applications (Karra and Dahm, 2010). In all scenarios, however, the success of transfection focuses on three factors such as transfection efficiency, low cytotoxicity, and reproducibility (Karra and Dahm, 2010; Butcher *et al.*, 2009). Therefore, to ensure a highly efficient and reliable transfection technology, the reagents that can work optimally under the chosen cell culture condition must first be evaluated. For example; El Said *et al.*, 2013 reported in order to track stem cells in cardiac tissue via the EzWay™ Transfection Reagent, Komabiotech; the optimization transfection was initially carried out to attain the highest transfection ratio prior to testing.

In spite of that, numerous studies utilized fluorescent vectors such as pEmGFP, TagCYP, EGFP-N3, pDsRed-N1, pEYFP-N1 vectors for biological probe in order to monitor the efficiencies of transfected target gene(s); eg. hTERT and SV40LT (Li *et al.*, 2007; Liu *et al.*, 2008), characterization of cells; eg. fibroblast cells (Pei *et al.*, 2014), evaluation of gene alteration on tumorigenic growth properties (Zhai *et al.*, 2013) and gene therapeutic purposes by delivery the target gene recombinant with virus particles, eg. herpes simplex virus type 1 (HSV-1), lentiviral and adenovirus particles (Wang *et al*., 2011; Yan and Shenchao, 2013, Mohamed *et al.*, 2015).

A major determinant for successful Adenovirus type 5 (Ad5) infectivity into target cells depends on the initial interaction between virus and its high affinity receptor, solely Coxsackie and Adenovirus Receptor (CAR) expression and accessibility (Bergelson *et al*., 1997; Carson *et al*., 1997; Tomko *et al.*, 1997). However, the utilization of adenovirus is limited due to lack CAR expression on the cells surface. In order to overcome such limitation, transfection of nucleic acid into host cell via non-viral method was utilized, so that the prime receptor can be taken up by the cell nucleus and expressed (Hassan *et al*., 2009)). To circumvent this problem, Chinese hamster ovary (CHO) cells represent an attractive model for negative Coxsackie and Adenovirus Receptor (CAR) (Bergelson *et al.*, 1997; McDonald *et al.*, 1999). Consequently, the transfection of CAR gene (pCAR) tagged with *Discoscoma* red (DsRed-N1) as a probe into the cultured CHO cells via several liposome-based transfection reagents was performed. Hence, the introduction of CAR gene into the CHO negative CAR as a platform may boost resistant cells to become more sensitive to Ad infection (Giaginis *et al.*, 2008).

The current study investigates the possibility of transfection of CAR gene fused with *Discoscoma* red (DsRed-N1) as a probe into the cultured CHO cell using lipofection method. The analysis of the red fluorescent expression of DsRed protein by inverted fluorescence microscope indicates the successfulness of transfection via lipofection method. This shown that the CAR gene had also been translated into CAR protein as a high affinity receptor for adenovirus attachment.

2. Material and Methods

The cDNA coding for human Coxsackie and Adenovirus Receptor (CAR) were generously given by Prof. Dr. Eric Blair from University of Leeds, United Kingdom and was fused with the pDsRed-Monomer-N1 (Clontech). Transfection reagents such as GeneJuice, Lipofectamine™ 2000, and Xtreme HP Plasmid DNA were obtained from Novagen, Life Technologies, Inc. and Roche, respectively. The Chinese hamster ovary (CHO) cell line was obtained from the American Type Culture Collection (ATCC) (United States). Culture media and supplements were from Life Technologies, Inc. Alamar Blue staining solution was purchased from Biosource (Biosource Euroupe, Nivelle Belgium). Fluorescence images were monitored and captured using inverted fluorescence microscopy and visualized with Image-Pro 6.3 Software (Nikon). The viability of cell was assessed using standard spectrophotometer (Genesys 10S UV-VIS, Thermo Fisher Scientific Inc.). The transfection efficiencies were analyzed via BD FACSVerse flow cytometer and BD FACSuite software (Becton Dickinson, Oxford, UK).

Cell Culture

CHO cells were grown in RPMI 1640, which was supplemented with 1% (v/v) penicillin/streptomycin (10,000 units/mL each), 2% (v/v) L-glutamine (2 mM), and 10% (v/v) fetal bovine serum (FBS) at 37 °C in a humidified atmosphere with 5% CO_2.

Plasmid Preparation and Transfection

Stocks of pDsRed-Monomer-N1 and pCAR fusion with *Discoscoma* red fluorescence (DsRed) transgene (pCAR-DsRed) was isolated respectively using the endotoxin-free Miniprep plasmid kit (Qiagen GmbH, Hilden, Germany). DNA concentration was adjusted to 250 ng/µL in Tris-EDTA and plasmids were stored at -20 °C.

For transfections, CHO cells were seeded in six-well plates at a density of 2 x 10^5 cells/well supplemented with complete growth medium and was allowed to grow for 2 – 4 days until 70 – 80% confluent. Transfection complexes were formed at room temperature in serum-free medium prior to drop-wise addition to CHO cells, followed by 4 to 6 h incubation and replacement with complete medium for 24 h incubated into CO_2 incubator. Antibiotics and antifungal agents were not used during transfection procedures. Three commercially available transfection reagents were tested including GeneJuice (Novagen), Lipofectamine™ 2000 (Invitrogen), and Xtreme HP Plasmid DNA Transfection Reagent (Roche) with a range of diluted DNA (with varying concentration at 0–1000 ng): reagent ratios according to the manufacturers' recommendations. All protocols are per-well of a six-well plate.

Alamar Blue (AB) Assay for Cell Viability

AB was aseptically added directly into the culture media at a final concentration of 10% after transfection incubation and the plate was returned to the incubator. Optical density of the plate was measured at 540 and 630 nm with a standard spectrophotometer at 24 h after adding AB. Because the culture medium was not changed during this

period, the calculated %AB reduction is a cumulative value. As a negative control, AB was added to medium without cells. The %AB reduction of transfected cells via various chemicals were normalized to %AB reduction of negative control. Thus, the percentage viable cells were calculated.

Quantification of DsRed

Transiently transfected CHO cells with pCAR-DsRed were trypsinised at 24-72 h respectively. The cells were harvested, centrifuged, and resuspended in in 100 µL in sheath buffer and incubated on ice for 10 minutes. The samples were kept on ice and analysed using FACScalibre flow cytometer and BD FACSuite software (Becton Dickinson, Oxford, UK). Untransfected CHO cells were used as negative controls. DsRed fluorescence was evaluated at wavelength 505 - 530 nm (PE). The percentage of CHO that expressed DsRed was measured by creating a gated region with 1% of untransfected CHO in the DsRed gate.

Statistical Analysis

Significance of transfection was determined by one-way ANOVA followed by Sidak-Bonferroni and Dunnett test for multiple comparisons or Student's two-tailed t-test as appropriate (* = $p < 0.05$).

3. Results

Our study demonstrated the optimization of several commercial transfection reagents such as GeneJuice (Novagen), Lipofectamine™ 2000 (Invitrogen), and Xtreme HP Plasmid DNA Transfection Reagent (Roche) that was capable of transferring human cDNA gene successfully *in vitro* to CHO cell line. CHO cells were utilized in this study as a model for negative Coxsackie and Adenovirus Receptor (CAR) (Bergelson *et al.*, 1997; McDonald *et al.*, 1999). It has also been reported that the CHO cell line often used in several studies of transfection *in vitro* (Brezinsky *et al.*, 2003; Kim *et al.*, 2004), and even been utilized in a large-scale cultivation of recombinant proteins for medicine purposes (Derouazi *et al.*, 2004). Recent study done by Al-Ashwal and Hamdan (2013) reported on efficiencies transfection of (domain 1 Coxsackie and Adenovirus Receptor fused with EGFP vector (CAR-D1-EGFP) into CHO cells were done with several transfection reagents such as Lipofectamine 2000™, Fugen6, calcium phosphate precipitation and electroporation technique.

Initially, the transfection were optimized by only using pDsRed-N1 expression vector with selected transfection reagents, eg.; GeneJuice (Novagen), Lipofectamine™ 2000 (Invitrogen), and Xtreme HP Plasmid DNA Transfection Reagent (Roche). The titration curve of DNA versus viability was shown in Figure 1a)-c). A dose-dependent increase in the number of cells transfected were observed with the amount of pDsRed/well up to 600 ng (19.77±0.44% MFI; Xtreme HP DNA), 200 ng (11.70±0.10% MFI; GeneJuice), and 1,000 ng (9.53±0.35% MFI; Lipofectamine™ 2000), respectively.

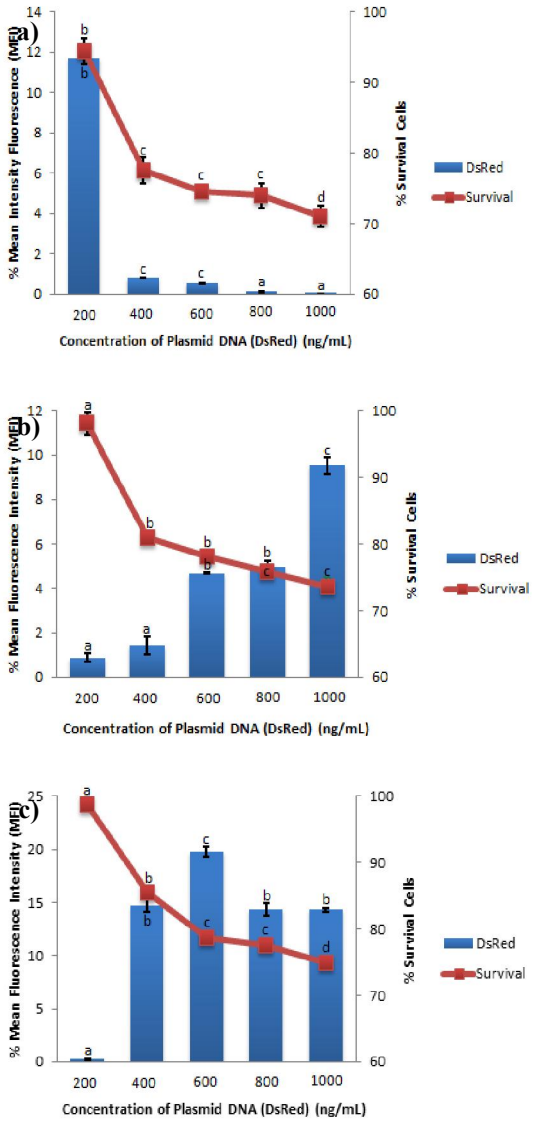

Figure 1. DNA titration and viability cells. Cells were analyzed for 24 h post transfection for DsRed expression and cell viability. The significance was determined by one-way ANOVA followed by Sidak-Bonferroni and Dunnett test for multiple comparisons or Student's two-tailed t-test as appropriate. The level of significance was set at *=$p < 0.05$. Mean Fluorescence Intensity (MFI) was shown pooled from 3 independent experiment ± standard of error mean (SEM). CHO cells were transfected with increasing concentrations of a DsRed expression plasmid using: (a) GeneJuice, (b) Lipofectamine™ 2000, and (c) Xtreme HP DNA transfection reagent. Different letter (a, b, and c) indicates the difference of significant level at $p < 0.05$, whereas the same letter does not show any differences of significant level (*$p < 0.05$).

The optimal condition of transfection reagents: Lipofectamine™ 2000 (1,000 ng), GeneJuice (200 ng), and Xtreme HP (600 ng) were applied on CHO cells by using the pCAR-DsRed accordingly. The protein expressions of CAR-DsRed were observed from 24 h-72 h after post-transfection showed an increment and were evaluated by using flow cytometry (Figure 2) and inverted fluorescence microscopy respectively (Figure 3).

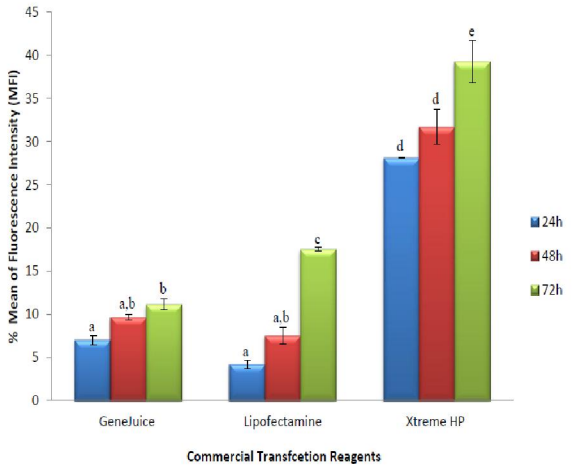

Figure 2. CHO cells were transfected with selected optimum concentrations of a pCAR-DsRed expression plasmid using GeneJuice, Lipofectamine™ 2000, and Xtreme HP transfection reagent respectively. Cells were analyzed 24-72 h post-transfection for CAR-DsRed expression. Mean Fluorescence Intensity (MFI) is shown pooled from 3 independent experiment ± standard of error mean (SEM). Different letter (a, b, c, d, and e) indicates the difference of significant level at $p < 0.05$; whereas the same letter does not shown any differences of significant level ($*p < 0.05$).

4. Discussions

The optimization of pDsRed-N1 vector only was crucial step to perform before preceding the transfection of pCAR-DsRed in order to obtain highest expression of DsRed protein prior the CAR-DsRed protein expression after transfection. The transfection efficacies with various amount of plasmids were deliberately increased until the plateau phase was reached, as shown in Fig. 1a and 1c, whereby the increasing plasmid added did not increase proportionally with DsRed protein expression. It is resulted from insufficient incorporation of increasing amount of nucleic acid into liposome-based compound. The ratio for GeneJuice and Xtreme HP DNA transfection reagent with the amount of plasmid DNA used at 3:1 was performed accordingly to manufacturer's instruction. Thus, to increase the efficiencies of transfection, the ratio of transfection reagent: DNA can be further evaluated. Furthermore, there were intense developments reported in synthesizing liposome - polymer or lipoplexes compound based - to increase the capability of carrying nucleic acid to be delivered to target cells (Chowdhury and Akaike, 2007; Moghaddam *et al.*, 2011). On the contrary, for Lipofectamine™ 2000 transfection, the reagent that did not reach the plateau phase yet was introduced with 1,000 ng pDsRed (Fig. 1b).

The transfection was carried out in serum-free medium due to critical drawbacks which inhibit the transfection efficiencies in the presence of serum (Hunt *et al.*, 2010; Matsui *et al.*, 2006; Zhang *et al.*, 2006). In fact, the presence of anionic proteins in serum subsequently interacts with polycations, resulting in the adsorption of negatively charged proteins on complexes and reduced endocytosis (Sawant *et al.*, 2012). The mock transfection of CHO cells were performed for each transfection reagents and only plasmid vector to assess the presence of autofluorescence of chemical reagents utilized in this study. It is worthy to note that auto-fluorescence in fluorescence and flow cytometry analysis could be resulted from some cationic liposome transfection reagents molecules (Guo *et al.*, 2001). The mock transfection revealed a similar pattern of untransfected CHO cells, in which resulted that those three transfection reagents had no auto-fluorescence detectable.

The three transfection reagents tested mainly formulated from cationic lipids and intensely used in this area (Morrile *et al.*, 2008). The mechanisms proposed transfection via chemical reagents act as a vehicle that package, condense, and deliver the nucleic acids to the nucleus cells, usually engulfed via endocytosis (Wagner *et al.*, 2005). The liposomal-based transfection reagents consist of lipid bilayer that encloses nucleic acid, fused with cells membrane and eventually deliver the nucleic acids to the nucleus' cells. Moreover, the capabilities of these reagents allow the simple, rapid use in high-throughput assays, non-infectious and various types of size nucleic acid molecules that can be transferred to cells (Karra and Dahm, 2010; Ewert *et al.*, 2008). The transfection was performed in semi-confluent CHO cells, whereby the cationic liposome-based transfection reagents reported to be effective in dividing cells due to the capability of uptake nucleic acid as the nuclear membrane which becomes fragmented and condensed during replication (prometaphase) (Kim *et al.*, 2004).

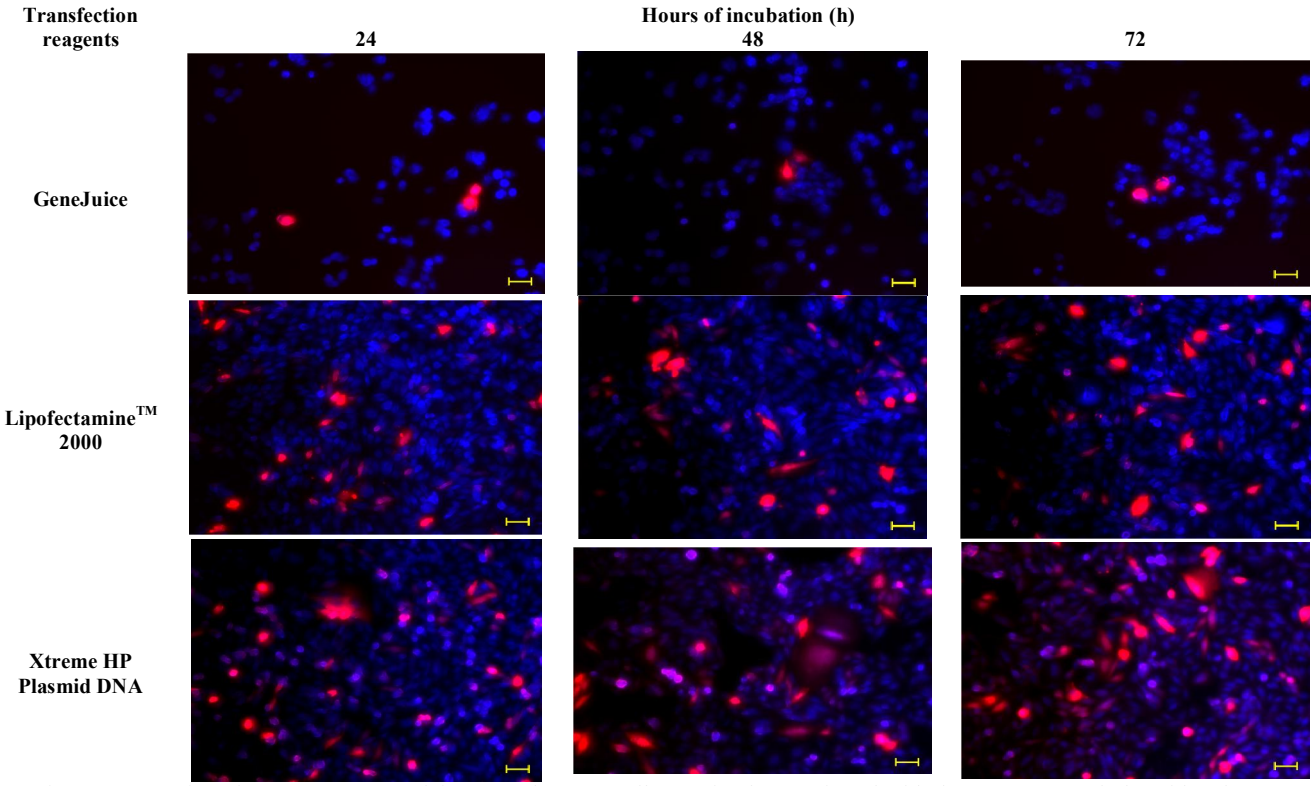

Figure 3. Transfected CHO-CAR-DsRed for 24-72 h. CHO cells transiently transfected with the pCAR-DsRed plasmid and were incubated for 24-72 h. Transfected CHO cells were photographed by inverted fluorescence microscope and visualized via Image-Pro 6.3 software (Nikon) (Magnification 4 x 10X). Merge images of DsRed fluorescence CAR protein (red) with the nucleic stained with Hoechst 33258 (blue). Scale bar (yellow) indicates 100 um in length.

Attaining high efficacy of transfection is associated with toxicity. It has been revealed that evaluating both transfection efficacies and toxicity of transfection reagents towards target cell lines while optimizing the transfection of reagents tested is acquired (Carson *et al.*, 1997; Hunt *et al.*, 2010). Higher tendency for toxicity in increasing ratio of transfection complex: plasmid DNA (200 ng-1,000 ng) might be one of the contributors for toxicity towards cells. Figures 1a-c revealed that toxicity measured via AB assay is equivalent followed by increasing amount of plasmid DNA transfected. Herein, it is reported that the viability is lessen at relatively 25-30% of cytotoxicity for those three transfection reagents.

Transfection of pCAR-DsRed

Previously optimization showed that the Lipofectamine™ 2000 was achieved highest transfected cells compared to GeneJuice and Xtreme HP transfection reagents. Thus, transfection of pCAR-DsRed plasmid was done onto CHO cells by using the optimum concentration plasmid DNA for each transfection reagents such as 200 ng/µL, 800 ng/µL and 1000 ng/µL respectively to GeneJuice, LipofectamineTM 2000 and Xtreme HP. The protein expressions of pCAR-DsRed were observed from 24h-72 h after post-transfection shown an increment and also evaluated by using flow cytometry (Figure 2). Intriguingly those from transfection of pCAR-DsRed plasmids onto CHO cells were resulted transfection using Xtreme HP shows the highest transfection of pCAR-DsRed at 39.25±1.00 MFI compared to LipofectamineTM 2000 (17.1±1.00 MFI) and GeneJuice (11.1±1.00 MFI) after 72 hours incubation.

As described earlier, there were several critical variables that affect the production of proteins. In fact, the occurrence of random integration of plasmids apparently affects partial or complete loss of expression (Schiedner *et al.*, 2008). Even though all the crucial steps have been taken into consideration to overcome such variable, time consuming tests of numerous CHO cell clones over multiple passages cannot be avoided. Moreover it has been reported in studies that DsRed have several critical problems such as a slow and incomplete maturation and also obligate tetramerization (Baird *et al.*, 2000, Campbell *et al.*, 2002) which may affect the observation of transfection efficiency.

The successful transfected CHO cells by pCAR-DsRed were monitored via inverted fluorescence microscopy (Figure 3) for 24 to 72 h. The distribution of DsRed expression on the cell's surface can be

detected clearly. The transfected CHO cells presented by diffusion of CAR-red fluorescence protein from the DsRed were observed in the cytoplasm and nucleus. The Hoechst 33258 staining of the cells' nucleus proved a stable and simple method in observing the viability of adherent cells.

Empirically, for the optimization of transfection reagents to attain optimal expression of transfected genes, critical parameters have been considered to illuminate these dissimilarity of efficiencies such as cell health including cell type, passage history and confluence, purity of vector and size, enhancing promoters, complexion ratio of plasmid DNA to transfection reagent, incubation time, and the presence of serum (Colosimo *et al*., 2000; Hawley-Nelson *et al*., 1993). The most important parameter is healthy cells, which indicate the cells' capability to proliferate promptly after they are plated prior to transfection and the earliest possible passage history (Hawley-Nelson and Shih, 1995). In obtaining the reproducible or consistency of transfection results, the same cell's number seeding should be plated prior for transfection assay.

In conclusion, the three liposome-based commercial transfection reagents investigated in this study revealed that cationic liposomes are capable for practical transfer gene application. The study succeed at 39.25±1.00 MFI of CHO-CAR-DsRed utilizing Xtreme HP Plasmid DNA transfection reagent (Roche) at 600 ng/mL. Furthermore, it was found that different size of plasmid which are pDsRed-N1 (~ 4.7 kb) and human cDNA CAR-DsRed (~ 5.8 kb) transfected at different passages history which resulted in different levels of protein expression regardless similar amount of plasmids utilized. By employing several different parameters, the possibility to achieve successful transfection *in vitro* via cationic liposome transfection reagents are evidently enhanced. Accordingly, the optimization as preliminary stage or platform to evaluate the compatibility of transfection reagents regarding to cell types, size of vector, and passages number of cells is indeed extremely expedient advanced developments of novelty non-viral vehicles for gene transfer techniques.

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