

Optimization for Expression of Coxacki Adenovirus Receptor on Chinese Hamster Ovary Cell line Using Different Transfection Methods

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Abstract: Adenovirus cells with little or no expression of Coxacki Adenovirus Receptor have been shown to be inefficient for gene transfer protocols. This paper presents the results of four transfection methods for D1 domain of CAR into the CAR- negative Chinese Hamster Ovary cells. The investigated methods were, Lipofectamine2000 (Invitrogen), Fugen 6(Roche), calcium phosphate precipitation technique and electroporation using 1ul: 2ul of DNA: reagents and 1ul: 2 pulses for electroporation. A reporter gene of enhanced green fluorescent protein and red fluorescent proteins were used to determine the percentage of transfected cells under inverted fluorescent microscope. Lipofectamine 2000 conferred the strongest expression of the D1 domain of CAR gene and transfection efficiency where the highest number of healthy and good shape of transfected cells was counted. The optimum transfection efficiency was achieved when DNA: Lipofectamine 2000 reagent ratio was 6 ug: 6uL (1:1 ug /ul), healthy proliferating cells plated at range of 5 x10⁵ 1x10⁶ cells density, 50-70% confluent on the day of transfection and supplemented with serum for 48hours of incubation. An optimum ratio could be used to prepare stable engineered CHO cells, which express D1 CAR receptor and could enhance the infectability of adenovirus into engineered CHO cells.

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1. Introduction

Gene therapy could be a powerful treatment for both congenital and acquired diseases. The success of gene therapy is largely dependent on the development of the gene delivery vector [1]. Adenovirus had been studied as a potential gene therapy vector for lot of diseases [2, 3]. Adenoviruses have certain features, which make them attractive vectors for gene transfer to target cells. Some of these properties include their ability to infect a wide range of cell types, like dividing and non-dividing cells, the ease with which adenovirus genome can be manipulated, and the ability to obtain high titers [4]. Human adenoviruses (Ads) especially serotypes 2 and 5 (Ad2/5), have been widely used as gene transfer vectors for acquired and genetic diseases, including cystic fibrosis CAR expression, which is often low on primary human tumors, is an important determining factor for adenoviral gene transfer efficiency. Expression of receptors may determine whether tissues are susceptible to transduction by adenovirus-mediated gene delivery (Bergeson; 2005). CAR extracellular domain is sufficient to permit virus attachment and entry and the amino-terminal immunoglobulin domain (D1) of CAR is necessary and sufficient for adenovirus binding [7]. Transfection, which is the introduction of foreign DNA into cell, is an important tool for study the

regulation of gene expression as well as protein expression and function. There are two types of transfection stable and transient, in stable transfection, the foreign DNA becomes integrated into the genomic DNA of the cell so that it is passed in the cell lineage and continues to express the encoded gene of interest. Stable transfection of mammalian cells is a widely used technique for the study of gene expression and protein purification. Transient transfection is more commonly used in which higher copy numbers of the foreign DNA and hence higher levels of gene expression are present in the cell for a brief period of time [8].

Transfection efficiency, defined as the percentage of cells that take up exogenous DNA, the transfection process depends on cell line, plasmid and its preparation as well as type of transfection reagent and its concentration [9]. The low transfection efficiencies and cytotoxicity effects in different protocols were a common problem. With presence of many different methods of gene transfer, the best transfection reagent or technique to give the highest transfection efficiency and the optimal conditions for transfection should be determined as a model for cells that lacks of CAR receptor expression. In this study four different methods were used to transfect D1 domain of CAR receptors into CHO cell lines and EGFP and ds RED were used as reporter genes to

investigate their expression on CHO cells using the inverted fluorescence microscope then the best transfection method was optimized.

2. Material and Methods

2.1 Chemicals and Reagents

All chemicals and reagents that have been used for this project were provided by the Biology Department, Faculty of Bioscience and Bioengineering, Universities Technology Malaysia and were supplied by various companies. The plasmid DNA preparation was used QIA prep spin miniprep kit from Qiagen, Hyper Ladder I which included 5X loading buffer and DNA marker ladder were purchased from Bionline Company, 1kb plus DNA marker and loading buffer and Fetal bovine serum provided by Invitrogen. The cultural components such as Trypsin, Penicillin and Streptomycin were supplied by GIBCO.

2.2 Bacterial Strain and Plasmids

Transformed *E.coli* DH5 α cells, DNA plasmid constructs were used for transfection. Plasmid pIRES-D1CAR-EGFP and Plasmid p ds RED D1CAR- were prepared. The cloned plasmid was purified from the strain to be transfected into the CHO cells.

2.3 Cell Culture

Chinese Hamster Ovary (CHO) cells, which supplied by University of Leeds, United Kingdom (American Type Culture Collection) were used to be transfected with pD1CAR EGFP, pD1CAR ds RED genes.

2.4 Primers for PCR

All the primers used for PCR amplification were synthesized by 1st Base laboratory SDN BHD Ltd, Master Mix (M712, Gotaq Green), master mix that used was supplied by Promega; (BIO-RAD Thermocycler) was used for PCR amplification.

2.5 Culturing Transformed Cells

Plating the transformed *E. coli* DH5 α was done on LB agar with 30 μ g/mL Kanamycin; streaking plate with the bacteria was done. The inoculate were cross-streak with 60° from the first streak then the cross-streak was repeated. The plate was labeled and incubated overnight at 37°C incubator. A single colony of *E. coli* strains DH5 was used to inoculate 10mL of LB medium and incubated overnight at 37°C in an orbital shaker to an optical density (OD) of 0.8 using spectrophotometer at 680 nm.

2.5.1 Small Scale Plasmid Isolation (Mini-prep)

The isolation of plasmid was done using QIA prep spin Miniprep kit from Qiagen manufacturer protocol. Isolated DNA was free of RNA, proteins and salt and is ready to use for further analysis. Finally, the column was centrifugation at 13 000 rpm for 2 minutes to obtain purified plasmid.

2.5.2 Gel Electrophoresis

Gel electrophoresis was performed in this project for analytical purpose to separate DNA molecules according to molecular size and the concentrations of the agars in the gel may vary according to purpose and DNA molecular sizes (E.M. Southern 1975), 1.2% agarose (w/v) with Ethidium bromide at a final concentration of 0.5 μ g/ml was used for analytical purpose (0.4 to 6 KB). Samples containing DNA pre-mixed with 7 μ L sample was pre-mix with 1 μ L loading buffer loading buffer then pipetted into the sample wells. An appropriate marker ladder was also loaded in order to calibrate DNA samples. Gel electrophoresis was performed at 85V, 80mA for 45 minutes. The loading buffer used to increase the density of the sample to insure that the DNA sinks evenly into the plates well where it adds color to the sample, thereby simplifying the loading process. The gel was visualized under UV Transilluminator (SYN GIENE FLASH) and the photos were taken.

2.5.3 PCR amplification

PCR amplification was carried out using *GoTaq® Green Master Mix* from 1st BASE in DNA Thermocycler (BIO-RAD Thermocycler). The PCR Master Mix contains bacterially derived Taq DNA polymerase, dNTPs, MgCl₂ and reaction buffers at optimal concentration for efficient amplification of DNA templates using PCR. *Gotaq® Green Master Mix* contains two dyes (blue and yellow) that allow monitoring of progress during electrophoresis.

PCR amplification was carried out in a total volume of 50 μ L for 35 cycles, in a PCR tube; the following were added forward primer 1 μ L, reverse primer 1 μ L, plasmid DNA 1 μ L master Mix 25 μ L Nucleus free water to 50 μ L total volume. The optimal conditions for PCR reaction are summarized in Table 2.1 The isolated DNA was separated on a 1% agarose gel for 1 hour at 80 V once the electrophoresis was completed the picture was taken using digital camera.

Table 2.1: The Optimal Conditions for PCR Reactions

PCR Product	CAR extra-cellular domain (CAR D1)	Full length CAR
Denaturing	94 °F, 30 sec	94 °F, 30 sec
Annealing	59 °F, 30 sec	60 °F, 1 min
Extending	72 °F, 1 min	72 °F, 1 min
Final Extension	72 °F, 10 min	72 °F, 10 min
Cycles	35	35

DNA	pD1CAR (EGFP)		pD1CAR (DSRED)	
	CONCENTRATION (ng/ul)	PURITY (260/280)	CONCENTRATION(ng/ul)	PURITY(260/280)
1	40.0	1.8	40.2	1.89
2	27.9	1.91	30.0	1.75
3	30.3	1.81	15.1	1.63
4	50.7	1.92	50.6	1.88

2.5.4 Determination of Plasmid DNA Concentration

Nucleic acid samples were checked for concentration and quality using the NanoDrop® ND-1000 Spectrophotometer. The plasmid DNA concentration was determined; A 1 ul sample pipette onto the end of a fiber and the measurements for samples varying by more than 1000 fold in concentration. The instrument controlled by special software run from a PC, and the data were logged in an archive file on the PC. The ratio of absorbance at 260 and 280 nm was used to assess the purity of DNA and RNA. A ratio of ~1.8 was accepted as "pure" for DNA; a ratio of ~2.0 was accepted as "pure" for RNA. If the ratio is appreciably lower in either case, it may indicate the presence of protein, phenol or other contaminants that absorb strongly at or near 280 nm, 260/230: ratio of sample absorbance at 260 and 230 nm, was a secondary measure of nucleic acid purity. The 260/230 values for "pure" nucleic acid are often higher than the respective 260/280 values. They are commonly in the range of 1.8-2.2. If the ratio is appreciably lower, this may indicate the presence of copurified contaminants, sample concentration in ng/ul based on absorbance at 260 nm and the selected analysis constant (11, 12).

2.5.5 Subculture of Chinese Hamster Ovary Cells

CHO cells culture was done using RPMI1640 media. The media were supplemented with 10% of fetal bovine serum, 2%L- Glutamine and 1% antibiotics. Keeping cells viable was done by sub-culturing of the cells every 3 days, media was discarded and been washed with 10ml PBS solution then Trypsin was added to flask, kept for 5minutes in 5% CO₂ incubator, followed by removal of the trypsenized cells, the 1ml left in the flask plus 15ml of medium were added and finally kept at 37oC atmosphere 5% of CO₂ incubator. Plastic vial and six-well plate were used as a culture surface to subculture the cancer cells, which is always, have been handled in sterile working laminar flow.

2.5.5.1 Counting Cells Using Hemocytometer

CHO cells were plated in 6-well dishes one to two days before transfection using different cell numbers. A clean hemocytometer with cover slip and other materials that required for the operation were prepared. After the Trypsin neutralization for the CHO cells, the cell suspension placed in a suitably

sized conical centrifuge tube. To obtain an accurate cell count, a uniform suspension containing single cells was prepared.

The cell suspension was pipeted up and down in the tube 5-7 times using a pipette with a small-bore 20 microliters of cell suspension that was charged into the chambers of the hemocytometer. Therefore, 30-50 microliters of (1:2) of dilution of the cell suspension in trypan blue was prepared. Combined the 15 microliters of cell suspension with the 15 microliters of trepan blue in the microfuge tube, then coverslips Placed over the counting chambers, the entire volume of the chamber was filled. The cells viewed under a microscope at 100x magnification. The cells should be visible above the grid of the counting chamber for an accurate determination, the total number of cells overlying one 1 mm² should be between 15 and 50. If the number of cells per 1 mm² exceeds 50, the sample diluted and counted again. If the number of

Cells per 1 mm² are less than 15, a less diluted sample used. If less dilutes samples were not available, cells counted on both sides of the homocytometer. The volume of fluid over one of the 1 mm² areas of the grid is 0.1 cubic mm or 0.0001 ml Count all of the cells contained within the four 1 mm² areas, e.g. $100/0.5=50*1000=50000$ The total (or viable) cell number was calculated as follows: the total number of cells Counted in the four large corner squares plus the middle combined. Each square has surface area of 1 mm squared and a depth of 0.1 mm, giving it a volume of 0.1 mm cubed when we counted 125 cells (total) in the five squares, means that we have 125 cells per 0.5 mm cubed, which are 250 cells/mm-cubed. Again, multiply by 1000 to determine cell count per ml (250,000). Diluted a cell suspension to get the cell density low was enough for counting.

2.6 Transfection

2.6.1 Lipofectamine

DNA Transfection using Lipofectamine TM 2000 Reagent and Chinese Hamster Ovary cells were plated in 6-well tissue culture dish one day before the transfection and incubated until the confluency of the cells reached up to 70% -80%. Solution A was prepared (X µg of plasmid DNA diluted in 50µL Serum Free Medium) then after 5 minutes was mixed with solution B (X µL of Lipofectamine2000reagent

diluted in 50µL Serum Free Medium) and incubated for 30 minutes at room temperature, then added to the cells that previously washed with PBS. The mixture was incubated for another 5 hours, with serum free medium the cells was washed and cultured for 42 to 72 hours in FCM.

2.6.2 FugenR6 Transfection

DNA Transfection using Fugen R6 Reagent and Chinese Hamster Ovary cells were plated in 6-well tissue culture dish one day before the transfection and incubated until the confluency of the cells reached up to 70% -80%. Solution A was prepared (plasmid DNA diluted in Serum Free Medium) then after 5 minutes was mixed with solution B (fugen^R6 reagent diluted in Serum Free Medium) and incubated for 15-30 minutes at room temperature, then added to the cells that previously washed with PBS. The mixture was incubated for another 5 hours, with serum free medium the cells was washed and cultured for 24 to 72 hours in Fresh Complete Medium. After 24-48 hours cells were analyzed by and immune-fluorescence microscope.

2.6.3 Calcium Phosphate Co-Precipitation

DNA Transfection using Calcium Phosphate Co-Precipitation- One day before the experiment CHO cells seeded at a 2 ml medium for each well of 6 wells plate. Plasmid DNA is mixed in a solution of calcium chloride, and then is added to a phosphate-buffered solution. Over a period of 20 minutes a fine precipitate forms in the solution, and this solution is then added directly to the cells in culture. Two hours before the transfection, the medium aspirated and added 2 ml of fresh DMEM supplemented with 10% fetal bovine serum, 2% L-glutamine, 1% pen/strep. In a sterile 1.5 ml microcentrifuge tube, the following was added in the order: 5 ml DNA (1mg/ml in sterile molecular biology water or TE), 49 ml molecular biology water, 6 ml 2.5 M CaCl₂ (Note: DNA that has been ethanol precipitated is considered sterile.) and Mixed gently by pipetting. In a second sterile 1.5 ml microcentrifuge tube, 60 ml of 2 X HEPES-Buffered Saline (HBS) was added, pH 7.05, Bubble the 2 X HBS Buffered. Saline using a pipette, while bubbling the HBS, CaCl₂/DNA solution from previous steps added drop wise with a sterile pipette tip, Vortex for 2-4 seconds, finally Allow the precipitate to sit undisturbed for 20 minutes at room temperature before Distribute the precipitate over the cells in the culture dish and gently agitated to mix the precipitate and medium, Incubated the cells for 5 hours in a cell culture incubator (37 °C).

2.6.4 Electroporation

DNA Transfection using electroporation - Adherently growing cells with identical passage numbers were harvested in the exponential growth phase, Washed with 10ml PBS after trypsinization

with 2.5 ml trypsin, counted then resuspended in the growth medium (to inactivate Trypsin) and centrifuged (at room temperature and 1500 rpm for 5 min at 4°C) and resuspended in with PBS or media till 5×10^5 – 1.5×10^6 cells density. The electroporation was performed in the plastic cuvettes with the volume of 400 µl using Eppendorf Multiporator transfection Protocols for eukaryotic Cells i.e., at 100 V, 70µS m pulse. The distance between the electrodes in the cuvettes was 2 mm. In one electroporation cuvettes 400 µl of cell suspension of 5×10^5 – 1.5×10^6 cells/ml of cell density was poured.

After electroporation the cells were transferred, growth medium, the cells and the viability was determined by visual inspection 24-72 hours after transfection. The successful of transfection was determined by using inverted fluorescence microscope. The transfected cells were grown on sterile cover slips on 6 wells plates. This microscope uses a much higher intensity light to illuminate the sample. This light excites fluorescence species on sample, which then emit light of a longer wavelength. The expression of EGFP and ds RED protein in the cells was observed as green, red fluorescence respectively. This showed that the gene of interest, CAR D1 had been successfully transfected into the CHO cells along with the EGFP and ds RED. The cells viability was determined by visual inspection 72 hours after transfection.

2.7 Staining of the Cells

CHO cells grown on 6 well plates over cover slips were washed once with PBS, fixed with 100µl Para formaldehyde for each well 15 minutes over ice, washed 3X with PBS the cover slip put inverted over one drop of Vectashield^R mounting medium staining with (DAPI) for EGFP, iodide for ds RED Nuclei were stained blue with Vectashield mounting media (Vector Laboratories, Inc., Burlingame, CA). Finally the edges were sealed with clear nail varnish and stored at 4°C. Then the cells can view after that and The picture can be taken for the living transfected cells.

3. Results

3.1 Verification of Plasmid DNA

D1 CAR EGFP and D1 CAR ds RED plasmid constructs were prepared in-house. The presence of the gene of interest was verified to ensure the validity of the clone during storage.

3.2 Culture of Transformed E. coli DH5α Cells

A stored stock of bacteria has been streaked onto selective plates with Kanamycin, its give rise to healthy colonies carrying the appropriate antibiotic resistance (figure 1). *E. coli* dh5α that carry the plasmid DNA have the ability to metabolize Kanamycin, so the growing colonies on the agar plate means that bacterial cells containing desired plasmid

DNA have been selected. A liquid culture was prepared for plasmid isolation.



Figure 1 – Culture of Transformed *E. coli* DH5 α cells

By transferring one colony of plated bacteria's that contains (plasmid D1 CAR EGFP and D1 CAR ds RED) to 10 mL LB broth, grown at 37 °C for ~24 h with vigorous shaking overnight culture. Cloudy culture in the solution indicates the growths of *E. coli* DH5 α . In order to determine the optimal harvesting time for the bacterial cells the growth curve of a bacterial culture was monitored photometrically by measuring the optical density at 680 nm.

It was found that the growth of bacteria containing ds RED was obviously slower than that cloned with EGFP. A lag phase occurred after dilution of the starter culture into fresh medium, cell division was slow (0 -6 hours) for pD1CAR EGFP and pD1CAR ds RED, that might be occurred due to the time, where the bacteria needed time to be adopted to the fresh medium. Growth of cultures was affected by host strain, plasmid insert or culture medium. Within (6-28 hours) the pEGFP culture enters logarithmic (log) phase, p D1CARdsRED (6-36 hours) where bacteria grew exponentially. After that cells changed from slow growth phase to stationary phase (~30, 36hours) respectively for pD1CAREGFP and pD1CARdsRED when the available nutrients were used up. The OD reading remained nearly constant in this phase, the culture entered the phase of decline, once cells started to lyse the number of viable bacteria falls and DNA becomes partly degraded. Growth for 18-28hr was corresponds to the transition from logarithmic into stationary growth phase when OD reading was high (0.5-0.9) in pEGFP, (18-36) hrs in pdsRED.

3.3 Isolation of Plasmid DNA

Miniprep of pD1CAR EGFP and pD1 CAR dsRED were performed and run on gelelectrophoresis. It was found that the size pD1CAR EGFP and pD1CAR dsRED were approximately 5.2 Kb and 4.7 Kb respectively which were approximately

corresponding to the plasmid D1CAR EGFP, pD1CAR dsRED.

3.4 PCR Analysis of the Prepared DNA

To confirm the insert size of DNA plasmid D1CAR, polymerase chain reaction was performed using forward and reverse primers that specifically targets D1 region. Electrophoresis analysis was performed; a clear band at 500bp was detected that gave the molecular weight of DNA fragment encoding the D1 domain of CAR (figure 2). Thus, verified the presence of D1 CAR through gel electrophoresis analysis of PCR amplification product which was ~500bp.

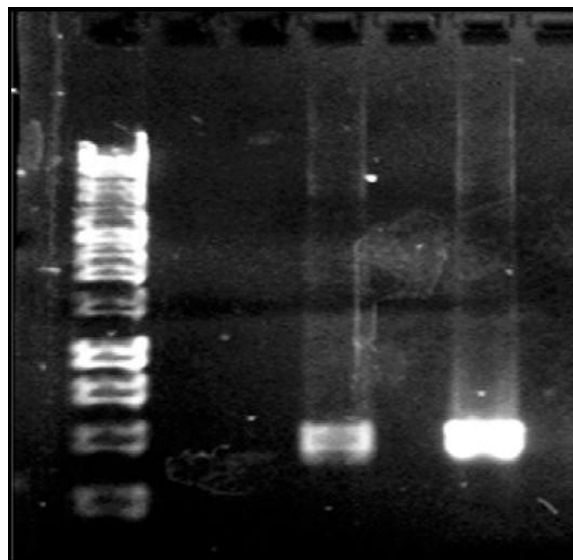


Figure 2- PCR Analysis of the Prepared DNA

3.5 Determination of Plasmid DNA Concentration and Purity

DNA concentration was measured by Nanodrop® ND-1000 Spectrophotometer to determine the purity of plasmid. Purity of plasmid DNA was calculated on the ratio of absorbance between OD260 and OD280. All the plasmids isolated were measured for its purity and concentration as shown in table 1; a ratio of ~1.8 was accepted to be transfected into CHO cells. A ratio of ~1.8 is generally accepted as "pure" for DNA and a ratio of ~2.0 is generally accepted as "pure" for RNA. If the ratio is appreciably lower in either case, it may indicate the presence of protein, phenol or other contaminants that absorb strongly at or near to 280nm ng/ul: sample concentration in ng/ul based on absorbance at 260 nm and the selected analysis constant.

Table 2: OD reading of Miniprep plasmids using Nanodrop ® ND-1000 Spectrophotometer DNA

D N A	P D1CAR (EGFP)		pD1CAR (DSRED)	
	CONCENTRTION (ng/ul)	PURITY (260/280)	CONCENTRATION(ng/ul)	PURITY(260/280)
1	40.0	1.8	40.2	1.89
2	27.9	1.91	30.0	1.75
3	30.3	1.81	15.1	1.63

3.6 Culturing of Chinese Hamster Ovary Cells

A subculture of CHO cells was done every three days to maintain the viability of the Cells. Trypsinization step was an important step where the best technique of trypsinization and detached the clumped cells made a difference until a good shape of cells and less dead cells were observed. After counting the cells using the homocytometer using different dilutions the best confluence were observed by 1mL trypsin diluted with 10ml of the medium. CHO cells were plated in six-well plates at a density of 4×10^5 - 1×10^6 cells per well. By the next day 70% - 80% Confluency was observed and ready.

3.7 Transfection

To accomplish expression of the transgene, DNA must reach the nucleus of the cell and become accessible to the transcriptional machinery. The cells that used were healthy, proliferating well and plated at a consistent density to minimize both intra and inter-experimental variance in transfection efficiency. At the beginning of the trial, the preliminary transfection study was carried out with 1 μ L:2 μ L of DNA: (lipofectamine2000, fugen6, calcium phosphate and electroporation) from the 30 μ g/ μ L DNA concentration on 80% confluent CHO cells.

The number of fluorescent cells was counted after 24 hours of Some of DNA sample concentrations gave lower transfection efficiencies than others, making it difficult to draw a conclusion. Low cell viability or slow growth rates with pD1CAR ds RED might be the reason of the high levels of protein expression, as the cells' metabolic resources were directed toward production of the heterogeneous protein. The expressed protein may also be toxic to the cell.

The highest viability number of transfected cells was counted with Lipofectamine 2000 reagent (Figure 4). However, deaths of the cells were very obvious with calcium phosphate co-precipitation methods where flower clumping like of cells can be seen. With fugen 6 there were so many dead cells with bad conditions of shape of cells and huge clumps of cells was noticed.

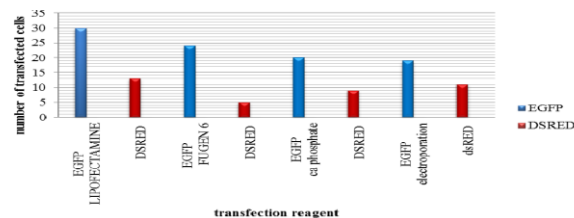


Figure 3-preliminary transfection experiment

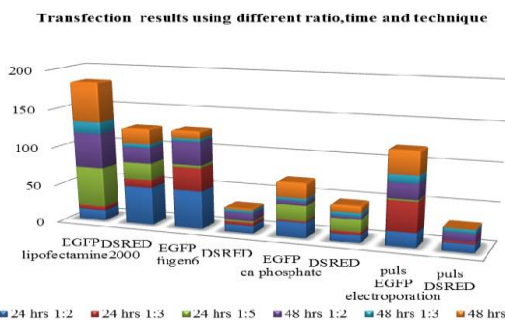


Figure 4- Four method of transfection after 24 and 48 hrs.

The second trial was performed using 40 μ g/ μ L DNA, on 70% confluent CHO cells and the number of transfected cells obtained with different methods of transfection after 24 hours and 48 hours of incubation (figure 5).

For the electroporation the main variable to change was the number of pulses where using 1, 2, 3 μ L DNA: 2, 3, 5 pulses at 100 V, 70 μ s m. As shown in figure 4 the number of transfected cells were the best with Lipofectamine 2000 for either pD1CAR EGFP or pD1CARdsRED, the fugen 6 presented the second better results, the electroporation was the third and the last method was the calcium phosphate.

It may be due to less toxicity of Lipofectamine 2000 reagent on cells; compared to others were a lot of dead cells observed. The number of transfected cells seemed affected by the incubation time hence number of cells decreased after 48 hours of incubation that may be happened due to the over stress on cells because of over expression of transfected genes (pEGFP or pdsRED), toxicity of the reagents or the confluency of cells reached to

100% . Another investigation was done to overcome the expected factors of weak transfection; utilizing the same concentrations of DNA (4 μ L DNA: 10, 12, 15 μ L of Lipofectamine 2000 and fugen6 reagents) and Calcium phosphate and the electroporation were excluded because they gave low transfection on the trials previously. The comparison restricted between the Lipofectamine2000 and fugen 6 reagents and has been done under the same conditions, Confluency of CHO cells and the DNA concentration was 40 μ g/ μ l then the results were observed after 24, 48 and 72 hrs as revealed in figure 5.

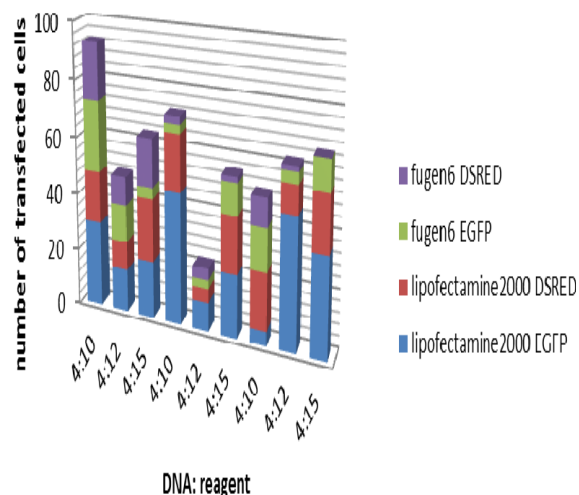


Figure 5: Transfection, number of transfected cells obtained using Fugen 6 and Lipofectamine2000 reagents.

Lipofectamine presented the best result, where a good condition, high number of transfected cells were achieved while in fugen 6 the problem probably was the high toxicity of fugen 6 reagents that led to modulation of the shape of cells and death. The number of transfected cells decreased with time that could be due to the depletion of nutrition, over expression of reporter genes and the confluency of cells. As a result, Lipofectamine 2000 (Invitrogen) was the best transfection method for delivering D1 CAR into CHO cells.

3.8 Optimization of Lipofectamine 2000 transfection conditions

Lipofectamine 2000 is a cationic liposome formulation that functions by complexing with nucleic acid molecules, allowing them to overcome the electrostatic repulsion of the cell membrane and to be taken up by the cell. Lipofectamine is like other reagent needs the optimization of the parameters that control the experiment to maximize transfection efficiencies of D1CAR gene to CHO cells. The parameters that were optimized were the DNA (μ g):

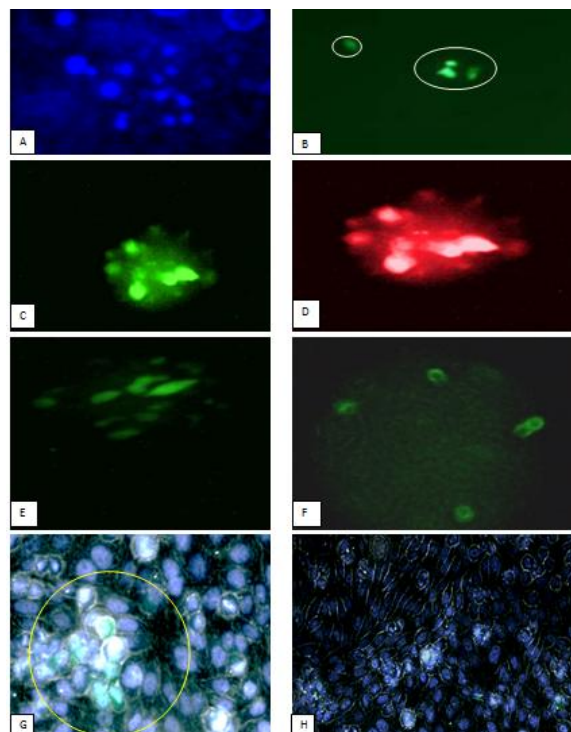


Figure 6- Transfection result under microscope Photograph of the EGFP-transfected cell obtained with a fluorescence microscope for transfected CHO cells, A: stained cells with DAPI blue fluorescent nucleus indicate the living cells, B, E and F: D1CAR EGFP transfected cells, C and D: dsRED transfected CHO cells. G and H photos composed 3 layer of phase contrast.

Lipofectamine™ 2000 (μ l) ratios, the time of the cells were incubated after transfection and the presence or absence of serum in the culture medium. In the optimization experiments the pEGFP were used as a reporter gene because the p dsRED was not accurate in this study, since it fluorescent green and red on the same time may be due to artifact on the appearance of fluorescent cells that was either due to the instability of the new reporter gene (dsRED) or the toxic effect of dsRED gene on cells (Figure 6). DAPI stained cells and green fluorescent cell's nucleus indicate the living cells, B, C, E and F: transfected cells with D1CAR EGFP, D: dsRED transfected CHO cells.

3.9 Transfected Ratio of Nucleic Acid to the Lipofectamine 2000 (Invitrogen)

The first parameter to be optimized was the ratio of transfected nucleic acid to the Lipofectamine 2000 reagent. First the ratio depended on the volume, the amount of DNA was used on μ l, the DNA concentration was 30 μ g/ μ L and Lipofectamine 2000 reagent concentration was 1 μ g/ μ L,. The best result was obtained when the ratio was 2 μ l:9 μ l (5:1 μ l/ μ l)

DNA: Lipofectamine 2000 reagent as presented in the Figure 7.

The vast amount of DNA was 6 µg after 48hrs of transfection as shown in Figure 8 the best ratio was 6µg:10 µl (3:5) of DNA concentration: Lipofectamine 2000 reagent, but still not optimized.

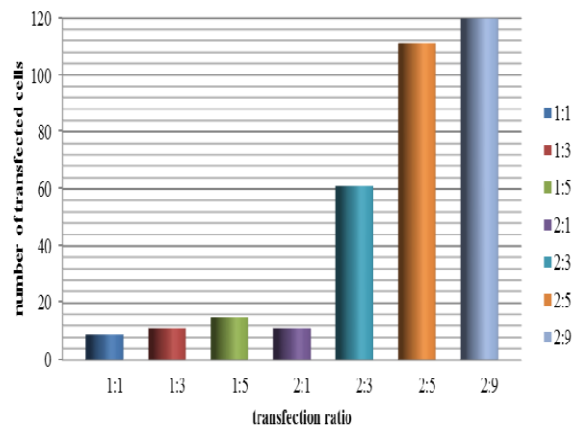


Figure 7-Optemisation of reagent concentration: Number of transfected cells using 1, 2 µL of DNA: Lipofectamine 2000 transfection, DNA concentration (30 µg /µL) 24hrs.

Another investigation was done with fixing the DNA concentration on 6 µg and varying the Lipofectamine2000 reagent volume, where 6 µg:6 µl (1:1) ratio of DNA: Lipofectamine2000 reagents gave the optimum ratio after 48hours of incubation.

When the DNA: lipofectamine2000 concentration was below or above this value the transfection efficiencies decreased may be due to too little DNA so few numbers of transfected cells presented and with the too much DNA few cells transfected and weak expression was presented may be due to toxicity of excess DNA or toxicity of Lipofectamine 2000 reagent on the cells . The ratio of (1:1) of DNA (µg): Lipofectamine2000 (µl) of giving the optimal ratio for transfection of D1CARA to CHO cells on 6-well culture plates (Figure 9).

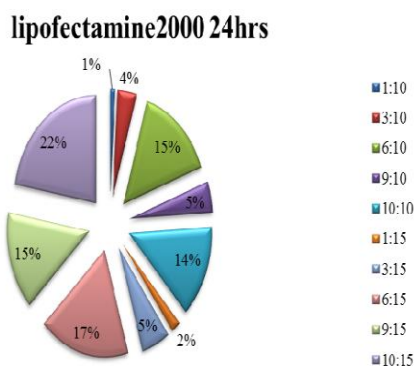


Figure 8- Optimization of DNA concentration: Number of transfected cells using 1,3, 6, 9 and 10 µg of DNA: 10 and 15 µl Lipofectamine 2000 transfection reagents.

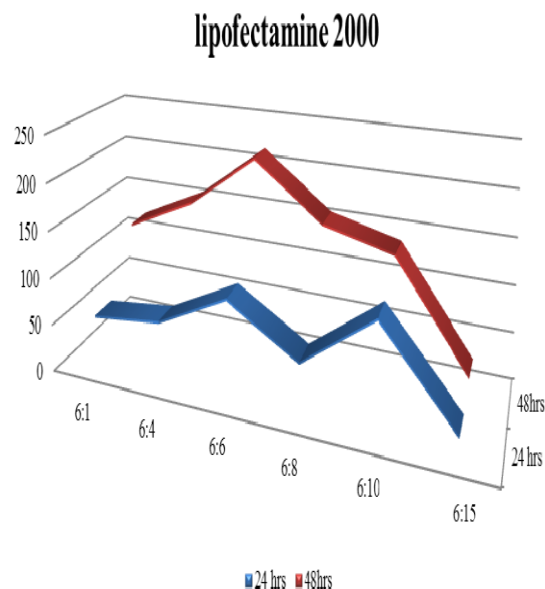


Figure 9: Optimization Number of transfected cells using 6 µg of DNA with 1, 4, 6,8and 15 µl of Lipofectamine 2000 reagent after 24 and 48 hours of incubation, 6 µg:6 µl ratio was the optimized ratio.

3.10 Effect of Serum on Transfection Efficiency:

To obtain optimal results different concentrations of DNA have been used with different concentrations of reagents, the number of transfected cells was counted after 24, 48 of incubation. Serum-free medium used during the transfection procedure, replaced with serum-containing medium after five hours of incubation, the same concentrations of DNA and Lipofectamine2000 reagent used with serum containing medium without removal. Interpretation of the results was done after 24, 48 and 72, under inverted fluorescent microscope. The data as explained in Figure 10 showed that the number of transfected cells was higher in the of presence serum for most of the ratios.

Effect of serum on transfection efficiency

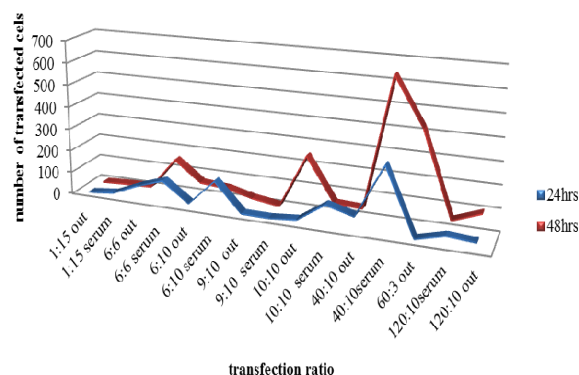


Figure 10: Optimization of medium: Number of transfected cells in the presence and absence of serum in the culture medium.

3.11 Effect of Incubation Time on Transfection Efficiency:

As noticed from most of the previous analysis that the best transfection efficiency was accomplished after 48hrs of incubation during transfection.

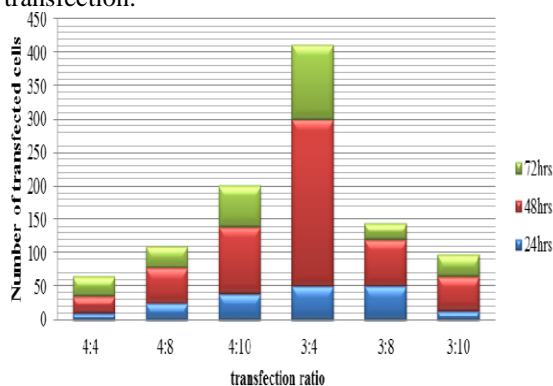


Figure 11-Optemisation of reporter gene: Number of Transfected cells (D1CAR) using Lipofectamine 2000 Reagent and EGFP reporter gene after 24 hrs, 48 hrs and 48 hours of incubation.

A Further study was carried out to investigate the incubation time effect on the transfection efficiency. Yet it was found that the 48 hours of incubation time prevailed the highest number of transfected cells. However, expression of cells debilitated after 72 hours of incubation and it may be occurring due to the unhealthy condition of the too confluent cells. Confluent cells may lead to apoptosis process or loss of the expression upon them because of the cells metabolism that followed the

transfection (Figure 11).

4.4 Discussions

In this research the plasmid that contained D1CAR insert was transfected into CHO cells using four different methods of transfection, green and red fluorescence that expressed on the surface of cells which gave an indirect indication for the successful transfer of the D1CAR DNA and expression of D1CAR on the surface of CHO cells. The expression of pdsRED in CHO cells was unstable while pEGFP gave a good and stable expression as a consequence pEGFP was chosen as a favorite for further analysis and optimization. Moreover Lipofectamine2000 was chosen as the best transfection reagents out of four different transfection methods used, i.e. Lipofectamine 2000 (Invitrogen), fugenR6 (Roche), calcium phosphate Co precipitation, Electroporation (Eppendorf multiporator) because it gave the highest number of transfected cells as well as healthy and good shape of transfected cells (Figure 6,7). Furthermore, to maximize transfection efficiencies, the amount of nucleic acid (DNA): Lipofectimine reagent, the length of incubation time during transfection and the presence or absence of serum were manipulated. The optimum transfection efficiency was achieved when DNA: Lipofectamin 2000 reagent ratio was 6 µg :6µL (1:1 µg /µl), healthy proliferating cells plated at a range of 5 x10⁵ 1x10⁶ cell density, 50-70% confluent on the day of transfection and supplemented with serum for 48hours of incubation as explained at figure 8,9,10 and 11.

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