Evaluation of Transfected HEP-2 Cell Line Using ß-Galactosidase Reporter Assay System

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Abstract:

Liposome-mediated transfection of cancer cells provide a valuable experimental technique to study cellular gene expression and may also be adapted for gene therapy studies. However, the widely recognized advantage of liposome-mediated transfection is high efficiency. Therefore, this study were performed to optimize transfection techniques in human larynx carcinoma cell line Hep-2 using the commercial synthetic lipid TransFast™ Reagent and monitoring the expression efficiency by using the pSV-β-galactosidase Control Vector which encoded β-galactosidase, maximum transfection efficiency were achieved with TransFast™ Reagent used at the Charge ratios of 2:1 and 0.5 µg DNA/ml, this is indicate that TransFast™ Reagent can be used as an efficient transfection agent to deliver foreign DNA into human larynx carcinoma cell line Hep-2 and expression of the transgene efficiently.

Key words: Reporter gene, Hep-2, transfection

Introduction:

The *Escherichia coli lacZ* gene encodes β-galactosidase, a tetrameric enzyme that catalyzes the hydrolysis of b-galactoside sugars such as lactose [1]. The enzymatic activity in cell extracts can be assayed with various specialized substrates that allow quantitation of enzyme activity using a spectrophotometer [2], a fluorometer [3] or a luminometer [4]. A major strength of this reporter gene is the ability to easily assay *in situ* expression with histochemical staining [3]. The enzyme β -galactosidase is widely used as a reporter molecule for both *in vitro* and transgenic applications. [5,6] as well as used with *in vivo* studies that deal with gene transfer to adult mammals especially that used the non-viral vectors [7]. In addition the widely usage of the gene for studying the activity of promoters which is depend on use of cell line for expression [8]. Other studies used this gene to evaluate the transfection methods or reagents in gene transfer [9].

The β -galactosidase reporter gene is frequently used as a control vector for normalizing transfection efficiency when co-transfected with chimeric DNAs linked to other reporter genes [10]. Liposomes are one of a number of chemical reagents used to deliver nucleic acids to eukaryotic cells by a nonviral process referred to as lipofection, other types of chemical compounds used for transfection include calcium phosphate and DEAE dextran[11], cationic liposome designed for transfection, such as the TransFast™ Reagent, are more versatile than many other traditional transfection methods. The advantages include: versatility in the macromolecules that are delivered, in vitro and in vivo applications, ability to more reproducibly transfect cells that are recalcitrant to other methods, and suitability for transient and stable

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transfection [12]. The TransFast™ Transfection Reagent is designed to deliver nucleic acid to many eukaryotic cell lines. To date, we have found that TransFast™ Reagent performs particularly well for DNA delivery to NIH/3T3, CHO, 293, K562, PC12 and Jurkat cell, among other cell types [13, 14, 15, 16, 17 and 18]. The TransFast™ Reagent combines the advantages of cationic liposome-mediated transfection with the features of speed and ease-of-use

Here we use the plasmid vector pSV-ß-galactosidase as an expression vector to evaluate the transfection of Hep-2 cell line using TransFast reagent kit which is a synthetic liposome and monitoring the expression on this cell using β-Galactosidase Enzyme Assay System with Reporter Lysis Buffer kit that depend on spectrophotometric methods and also use in situ histochemical analysis using the substrate X-Gal. this will enable us to find the efficiency of TransFast reagent as a DNA delivery vehicle to this type of cell line by determine the optimum values of the Charge ratios of TransFast™ Reagent:DNA and the DNA concentration, also we inquire the ability of the these cell for expression foreign DNA which is enable us to transfect the cell with other foreign genes that had therapeutic effect on these kind of cancer cell or to produce any other proteins.

Materials and Methods:

The plasmid vector: Promega's pSV-β-galactosidase Control Vector is designed as a positive control vector for monitoring transfection efficiencies of mammalian cells. The SV40 early promoter and enhancer drive transcription of the bacterial *lacZ* gene, which encodes β-galactosidase.

Cell culture: The human larynx carcinoma cell line (Hep-2) was brought faithfully from the Iraqi center of Cancer and medical genetic research. Cell were plated one day before the transfection experiment on RPMI 1640, to get approximately 80% confluent on the day of the transfection according to promega technical bulletin # 260.

Transfection technique: the transfection technique was carried out using TransFast reagent kit (promega), the reagent was warmed to room temperature. 400µl of Nuclease-Free Water were added at room temperature (1mM is the final concentration of the cationic lipid component), vortex the reagent vigorously for 10 seconds to suspend the lipid film. The vial was stored at 15 to 25°C overnight. The optimization for charge ratio and DNA concentration was determined according to promega technical bulletin # 260

For a 24-well plate, the total volume of the medium / DNA and TransFast Reagent should be 200µl per well. The volumes given in Tables 1 and 2 were calculated for 7 wells, indicated amount of medium (serum-free and prewarmed to 37°C) and plasmid DNA were mixed and vortexed then the TransFast Reagent was added and vortex immediately after 15 minutes incubate at room temperature. Growth medium was carefully removed from the wells by aspiration. TransFast Reagent/DNA mixture was added to the plated cells. After 1 hour incubation on 37°C, the medium containing the TransFast Reagent/DNA mixture should be removed, 1ml of complete medium gently overlay the cells, plate were incubated for 24hr.

β –galactosidase activity assay: The standard assay is performed by adding a diluted sample to an equal volume of Assay 2X Buffer that contains the

substrate ONPG (o-nitrophenyl-β- D-galactopyranoside). Samples are incubated for 30 minutes, during which time the β-galactosidase hydrolyzes the colorless substrate to o-nitrophenol, which is yellow. The reaction is terminated by addition of sodium carbonate, and the absorbance is read at 420nm with a spectrophotometer [19].

In situ Staining of Cells for β-Galactosidase Activity: the cells were washed twice with PBS 1X buffer and Fixed by incubating for 15 minutes with 2ml of glutaraldehyde solution, washed three times with PBS 1X buffer to Remove the glutaraldehyde solution, 1ml of X-Gal solution per plate of cells were added . The cells were Incubated at 37°C for 6 hours until the cells are visibly stained, then X-Gal solution was replaced with 1X PBS . Cells now ready to visualize by light microscope, photography in situ-stained cells on the same day of the experiment to obtain a permanent record of the results [20].

Result:

Using the pSV-β-galactosidase vector made possible to express the gene in most of cells because the presence of SV40 early promoter that worked in most animal tissues as a result to taken from SV40 animal virus. Hep-2 cells were plated in 24 well plates, four dilutions were made to find the optimum concentration of the plasmid DNA per well for transfection (0.25µg, 0.5µg, 0.75µg, 1µg) and to optimize the charge ratio of TransFast to DNA two ratios were used as 1:1, 2:1, and with the series of DNA concentration as listed in tables (1). The transfections were performed in the absence of serum for one hour. Six replicate were used for each treatment, control treatment include: treatment without using DNA, treatment without using TransFast and treatment without DNA and TransFast. Negative controls allows a correction for endogenous levels of cellular β-galactosidase or its isozymes and the control for each treatment reduce the statistic error .

Table (1): Optimization Protocol Using a 1:1 and 2:1 Charge Ratio of TransFast™ Reagent to DNA.

Amount of plasmid DNA per well		0.25µg	0.5 µg	0.75 µg	1 µg
Media to final volume		1.4ml	1.4ml	1.4ml	1.4ml
Plasmid DNA		1.8 µg	3.5 µg	5.3 µg	7 µg
TransFast™ Reagent	1:1	5.4µl	10.5 µl	15.9 µl	21 µl
	2:1	10.8µl	21 µl	31.8 µl	42 µl

Detection of the ß-galactosidase enzyme activity was performed by β-Galactosidase Enzyme Assay System with Reporter Lysis Buffer kit (promega) Starting with making standard curve, cell lyses, adding the substrate (o- nitrophenyl- β- D-galactopyranoside (ONPG)) and measuring the O.D. at 420nm, according to instruction in technical bulletin# 97, at first we have to know that: one unit of β-Galactosidase hydrolyzes 1 micromole of o-nitrophenyl -β-D-galactopyranoside (ONPG) to o-nitrophenol and galactose per minute at pH 7.5 and 37°C. Result showed the charge ratio of 2:1 is better than 1:1, which is indicate the quantity of liposome is critical factor not only the quality of the lipid, as well as shown that 0.5µg is the best concentration of the all charge ratios which is give the higher activity of the ß-galactosidase compare with other concentrations, that indicate the use of liposome as a transfection vehicle is not DNA dependent technique as shown in figure (1) it is obviously that: the charge ratio of TransFast reagent to the DNA, and the DNA concentration are most factors that affect the transfection. Form the sheet it was shown the charge ratio 2:1 with DNA concentration 0.5µg is the best treatment.

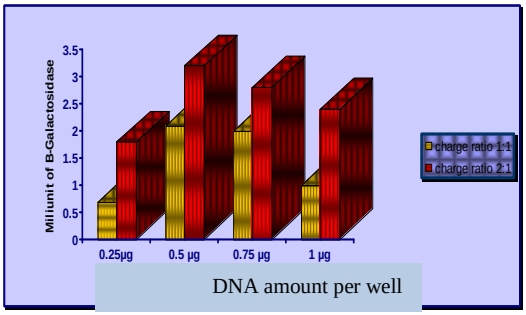

Fig. (1): The β-galactosidase activity measured by miliunit (Y-axis) for four deferent concentration of DNA (0.25µg, 0.5µg, 0.75µg, 1µg) (X-axis) and two deferent charge ratios (blocks in colors), the β-galactosidase activity as a miliunit an indicator for the amount the DNA of the vector that enter the cell and expressed. Form the sheet the charge ratio 2:1 with DNA concentration 0.5µg is the best treatment.

Cells transfected with the pSV-β-Galactosidase control vector and expressing β-galactosidase can be visualized by microscope. The cells appear blue following fixation and incubation with the substrate X-Gal.

Hep-2 cells were plated in 24 well plates and transfected with 0.5µg pSV-β-Galactosidase Control Vector per well. TransFast™ Transfection Reagent was used at a 2:1 TransFast™ Reagent: DNA charge ratio. After fixation by using standard techniques (Promega Technical Bulletin #TB097). The cells expressing b-galactosidase are stained blue. Control treatment without adding DNA and reagent, just to compare the endogenous cellular β -galactosidase activity, clear deference shown between the transfected cell and the control were the endogenous cellular β -galactosidase activity is appear in a few blue spots on cells (which represented the cells that contain the enzyme activity) figure (2: a) in same time a huge blue spots that appear in transfected cells figure (2: b).

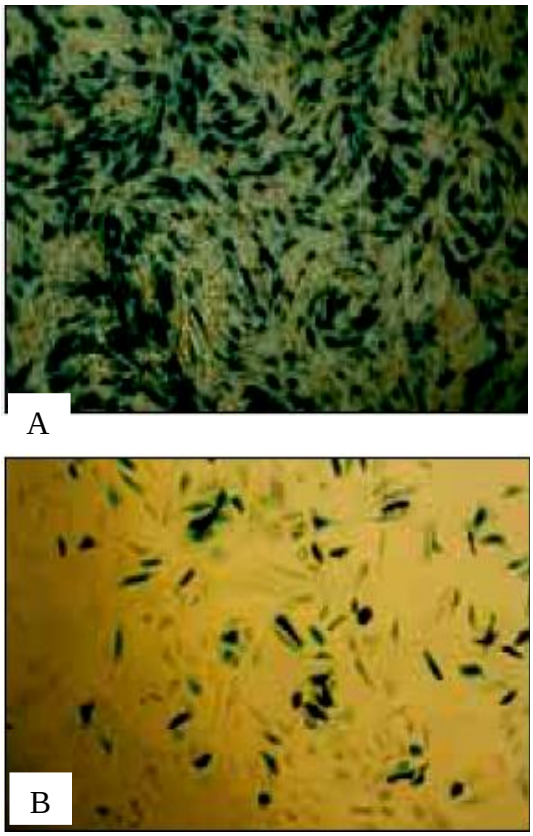

Fig. (2): Histochemical stain of the plated Hep-2 cell after transfected with pSV-β-galactosidase control vector. The picture A represents the optimum treatment (charge ratio of 2:1, DNA Conc. 0.5 µg) which most of cells appear in blue color due to the expression of β-Galactosidase and its activity on X-gal, picture B represents the endogenous cellular b-galactosidase activity as a control to compare with transfected cell, it is clear only a few cells appear in blue color.

Discussion:

With any transfection reagent or method, cell health, degree of confluency, contamination, and DNA quality and quantity are important parameters that can greatly influence transfection efficiency [21]. Plasmid DNA for transfections should be free of protein, RNA and chemical contamination, [22]. The optimal amount of DNA to use in the transfection will vary widely depending upon the type of DNA and

the target cell line [23]. To avoid all above notes, standard vector pSV-β-galactosidase (promega) used directly after diluted to 0.5mg/ml.

It is essential to optimize specific transfection conditions to gain optimal transfection efficiencies, the important parameters to optimize in order to maximize transfection efficiencies are the charge ratio of transfection reagent to DNA and the amount of transfected DNA [24].

The optimal amount of DNA to use in the transfection will vary depending upon the type of DNA circular or linear, the size of DNA and the target cell line used. For adherent cells, it is recommend initially testing 0.25, 0.50, 0.75 and 1µg of DNA in a 24 well plate format at a transfection reagent: DNA ratio of 2:1 and 1:1 for TransFast™ Reagent. Increasing the amount of DNA does not necessarily result in higher transfection efficiencies[25]

Rates of liposome-mediated transfection are dependent on amounts of plasmid DNA and thus on the ratio of DNA/cationic lipids. Our studies revealed that the transfection rate of Hep-2 can be enhanced with an increase in the amount of transfected DNA up to .05 µg DNA/ml. Further increases in the amount of plasmid DNA, and thus alteration of the DNA/liposome ratio, decreased efficiency of transfection. Similar results were obtained in the earlier studies [26]. Therefore, 0.5 µg DNA/ml was the standard amount of plasmid DNA used in the majority of the reported experiments.

Charge ratios of 1:1 to 2:1 TransFast™ Reagent:DNA have worked well with various cultured cells but ratios outside of this range may be optimal for other cell types or applications[27]. It is obvious that our results: charge ratio of 2:1 is better than 1:1, especially the used vector conceder approximately large (6820bp) so the negative charge of the vector need more reagents to neutralized or made the lipoplexe positive charged to complex with the negatively charged cell membrane this is enhance the delivery into the cell.

The type of promoter which regulates the transgene expression can greatly influence transfection efficiency [28]. For example, using a plasmid regulated by cellular promoters especially those regulated with tissue specific expression, show decrease of expression even with good entry of the plasmid to the cell which may give a wrong marker in the evaluation of transfection [29, 30], In contrast, lipofectin-mediated transfection of Hep-2 with a plasmid regulated by a strong viral promoters like CMV, (RSV) promoter, which is able to expression in most animal tissues was approximately high efficient transfection[31,32].

Constructs employed in the present study contained strong promoter (SV40) .the strong promoters use transcription factors which are present in host cells; so they can be constitutively active in transfected cells. These transcription factors remain active at the baseline level even in non-stimulated cells. In addition, one may suggest that cellular stress connected with transfection may further stimulate activation of these transcription factors. It should be noted that the SV40 promoter can provide better transfection rates in Hep-2 compared to the other promoters. This was demonstrated in experiments in which Hep-2 were transfected with plasmids encoding for the same reporter gene but driven either by the SV40 or other promoter [33].

In the present study, transfection conditions were optimized using the pSV-β-galactosidase Control Vector which is designed as a positive control

vector for monitoring transfection efficiencies of mammalian cells. The SV40 early promoter and enhancer drive transcription of the bacterial *lacZ* gene, which encodes β-galactosidase.
B-galactosidase has been recognized to be the reporter gene of choice for transfection studies in cells resistant to uptake of foreign DNA. The transgene is simple to measure even with background levels in animal tissues. Determination of B-galactosidase activity also has the advantage of being several orders of magnitude more sensitive than other common reporter gene assays, such as activities of chloramphenicol acetyltransferase or alkaline phosphatase [34, 35]. However, to determine the efficiency of transfection, the vector encoding for B-galactosidase was used. This experimental approach allowed us to stain and count the transfected cells.

In summary, efficient transfection conditions have been established for a transient transfection of human larynx carcinoma cell line Hep-2. The optimal transfection conditions, resulting in the transfection efficiency, were achieved with cationic liposome TransFast™ Reagent used at the Charge ratios of 2:1 and 0.5 µg DNA/ml, although these transfection conditions were connected with no cytotoxicity, a 48-hour period of maintaining endothelial cells in normal growth medium allowed the cells to recover fully. We conclude that TransFast™ Reagent can be used as an efficient transfection agent to deliver foreign DNA into human larynx carcinoma cell line Hep-2.

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تقويم تحويل خط الخلايا الحنجرة السرطانية (Hep-2)باستعمال نظام تحليل الجين المخبر ß-GALACTOSIDASE

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الخلاصة:

تعتبر الية نقل الجينات الى الخلايا السرطانية باستعمال الجسيمات الدهنية تقنية تجريبية قيمة في دراسة التعبير الجيني والتي يمكن ان توظف في دراسات العلاج الجيني , تمتاز تقنية التحويل باستعمال الجسيمات الدهنية بالكفاءة العالية في النقل ولذلك اجريت هذه الدراسة لايجاد الظروف المثلى لعمليات النقل الجينات الى خط خلايا الحنجرة السرطانية (Hep-2) باستعمال الجسيم الدهني الصناعي التجاري TransFast™ Reagentومراقبة التعبير باستخدام الناقلpSV-β-galactosidase Control Vector والذي يشفر عن انزيم β-galactosidase وتم الحصول على اعلى تحويل عند استخدام TransFast™ Reagen بنسبة تحويل 2:1 وتركيز DNA µg 0.5/ml مما يثبت كفاءة النقل والتعبير في خط خلايا الحنجرة السرطانية (Hep-2) باستعمال الجسيم الدهني TransFast™ Reagen .