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Optimization of transfection conditions for expression of green fluorescent protein in 4T1 mice mammary tumor cells

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Abstract
Various gene expression studies based on transfection of DNA in eukaryotic cells requires suitable transfection method. After transfection procedure selection, there is need to optimize transfection efficiency which varies in each cell type depending on transfection conditions. There are few significant transfection conditions like number of cells seeded 24 hrs before transfection, cell confluence at the time of transfection, optimal transfection reagent/DNA concentration ratio, amount of media added etc., which are required to be optimized individually in each cell type to attain highest transfection efficiency. Transfection with chemical method by using cationic lipids like lipofectamine is commonly used transfection method. Reporter gene like GFP cloned in any vector can be used to optimize transfection for attaining maximum transfection efficiency. In our present study we optimized Lipofectamine 3000®/pVIVO1.GFP plasmid concentration ratio to attain highest transfection efficiency in 4T1 mice mammary tumor cells. Non structural gene of Canine Parvovirus is an oncolytic viral gene reported to be cytotoxic selectively to cancer cells without harming healthy cells. NS1 gene of CPV2 cloned in pVIVO1 vector is already available in our laboratory. To identify different oncolytic mechanisms of CPV2.NS1, there was need to transfect this gene in 4T1 cells and therefore we used GFP as reporter gene cloned in same vector of CPV2.NS1 (pVIVO1.GFP) so as to optimize for Lipofectamine/DNA ratio depicting highest transfection efficiency. The concentration of plasmid DNA (pVIVO1.GFP) was optimized to 3µg in 6 well plate with 6µl of Lipofectamine 3000® by our study. In this concentration of plasmid DNA, the 4T1 cells were showing highest transfection efficiency.

Keywords: Green fluorescent protein, Oncolytic viral gene therapy, 4t1 mice mammary tumor cell line, transfection efficiency, canine parvovirus, non-structural gene1

1. Introduction
Different gene expression studies which are involved in investigations like functional genomics, gene regulation, transcriptomics, pathway analysis and gene therapy requires realible and optimized method of transfection of DNA into different eukaryotic cells. There are various methods of transfection like viral vectored mediated, chemical method, physical method, liposomes mediated etc. Chemical method by using cationic lipids like lipofectamine is most commonly used transfection method in which positively charged cationic lipid forms a complex with negatively charged DNA and complex is fused to negatively charged cell membrane by endocytosis or phagocytosis. Cell confluence and transfection reagent/DNA ratio are important parameters required to be optimized to attain maximum transfection efficiency which extremely vary among different cell type [1]. Either transfection reagent concentration is kept constant with varying DNA concentration or vice versa is used to optimize for transfection reagent/DNA ratio optimization and the one ratio depicting highest transfection efficiency should be selected. A transitory reporter assay system can be used to optimize transfection conditions as it is possible to monitor for transfection efficiency by assessing for the reporter gene product in different situations and cell types [2]. There are various reporter gene which can be used for such purposes like Green fluorescent protein (GFP), luciferase and LacZ gene.
Cancer cases are increasing day by day which makes this disease as foremost cause of death after heart ailments. Breast cancer is frequently reported cancer in women, after skin cancer[3]. The conventional therapies for cancer treatment are all invasive and cause damage to healthy cells along with killing tumor cells. But recently oncolytic viral gene therapy has gained importance to be used as anti cancer therapy.

These oncolytic viral genes on ectopic expression lead to selective killing of neoplastic cells without any toxic effects on normal cells [4]. 4T1 cell line is highly invasive and tumorigenic cell line which when administered in balb/c mice has potential to generate a mammary tumor. This tumor development and metastatic spread induced by 4T1 in mice very closely resemble hBC (human breast cancer) and is considered as a suitable animal model for stage IV human breast cancer[5]. So, this 4T1 cell line can be used to study the effect of oncolytic viral gene. Canine parvovirus non structural gene 1 (CPV2.NS1) is reported to cause cell death by inducing apoptosis in cancer cells through intrinsic pathway [6-8]. To conduct gene expression study by transcriptomics for elucidation of key molecules/pathways involved in oncolytic effects induced by CPV2.NS1 in 4T1 mice mammary tumor cell line, we need to optimize transfection conditions. CPV2.NS1 gene cloned in pVIVO1 vector is already available in our laboratory so in our study we are using GFP as reporter gene cloned in same vector (pVIVO1.GFP) as plasmid DNA and Lipofectamine 3000® as transfection reagent to identify optimal ratio of both which is capable of achieving maximum transfection efficiency.

2. Material and Methods

2.1 4T1 cell culture

This 4T1 cell line was obtained from ATCC (American type culture collection), and was maintained in advanced RPMI-1640 (Roswell park memorial institute) media along with L-glutamine, FBS (Fetal bovine serum) and antibiotic-antimycotic solutions.

2.2 Recombinant DNA plasmid clones

GFP gene cloned in pVIVO1 vector was already available in lab and promoter activity of pVIVO1 vector was confirmed by transfection of this construct in 4T1 mice mammary tumor cell line.

2.3 Transfection of Recombinant plasmid

Promoter activity of pVIVO1 vector was determined in vitro using pVIVO1.GFP as a positive control. Plasmid DNA with an OD260/280 ratio of 1.8-2.0 was used to transfect the cells with Lipofectamine 3000® (Invitrogen, USA). For optimizing ratio of Lipofectamine/DNA depicting maximum transfection efficiency, different concentration of pVIVO1.GFP was used while Lipofectamine 3000® concentration was kept constant in different wells of 6 well plate as depicted in table 1.

Table 1: Concentration was kept constant in different wells of 6 well plate as depicted

Component	6-well	6-well	6-well	6-well	6-well
DNA per well	1.5µg	2µg	2.5µg	3µg	3.5µg
P3000 reagent per well (2µl/µg DNA)	3µl	4µl	5µl	6µl	7µl
Lipofectamine 3000® reagent per well	6µl	6µl	6µl	6µl	6µl

2.4 Protocol for transfection in 4t1 cell line is given below

1. Seed cells 24hrs before undergoing transfection to obtain 70-80% confluence at the time of transfection.
2. Dilute Lipofectamine 3000® in Opti-MEM™ Medium.
3. Prepare mastermix of DNA by diluting DNA in Opti-MEM™ medium, then add P3000 reagent and mix well.
4. Add diluted DNA in each tube of diluted Lipofectamine 3000® reagent and incubate for 15 minutes.
5. Add lipid- DNA complex in each well of 6-well plate with 70-80% confluence.
6. Visualize transfected cells after 24 hours in fluorescence in fluorescent microscope.

3. Results

GFP was used as a reporter gene in our study to confirm promoter activity of pVIVO1 vector and to identify optimal Lipofectamine/DNA ratio to be used in 4T1 cell line for transfection to obtain maximum transfection efficiency. There was seen expression of GFP (green fluorescence) in pVIVO1.GFP transfected cells while no green fluorescence was visualized in empty vector pVIVO1 transfected cells which confirmed the promoter activity of pVIVO1 vector (Fig. 1).

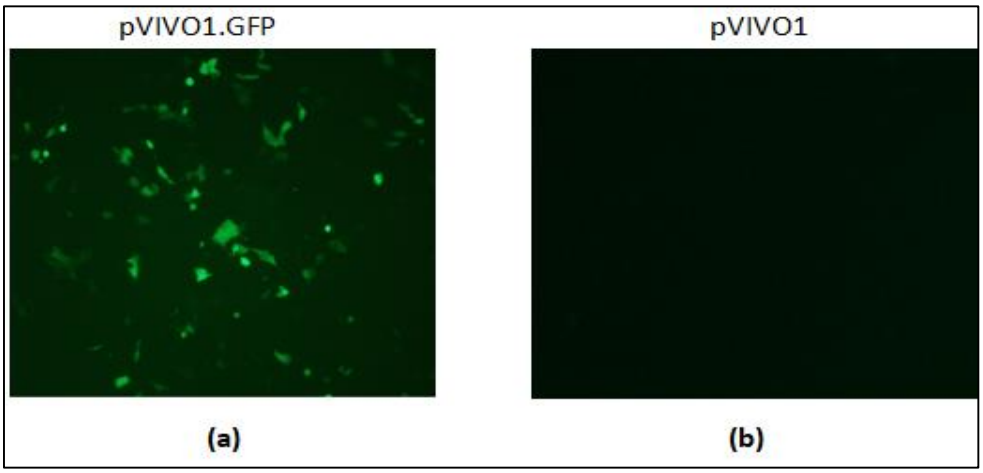

Fig 1: Fluorescent microscopy showing expression of GFP protein cloned into pVIVO1 vector in 4T1 cells

The recombinant plasmid pVIVO1.GFP was isolated using Qiagen Endofree maxi kit. The purity of DNA preparation was assessed by measuring absorbance at 260/280 which was found to be 1.8 demonstrating that the DNA preparation contain pure dsDNA without protein and RNA contamination. To optimize for suitable Lipofectamine 3000®/pVIVO1.GFP plasmid DNA ratio for transfection, different concentration of pVIVO1.GFP was used along with constant amount of Lipofectamine 3000®. The expression of varying pVIVO1.GFP concentration with constant Lipofectamine 3000® is shown in Fig. 2.

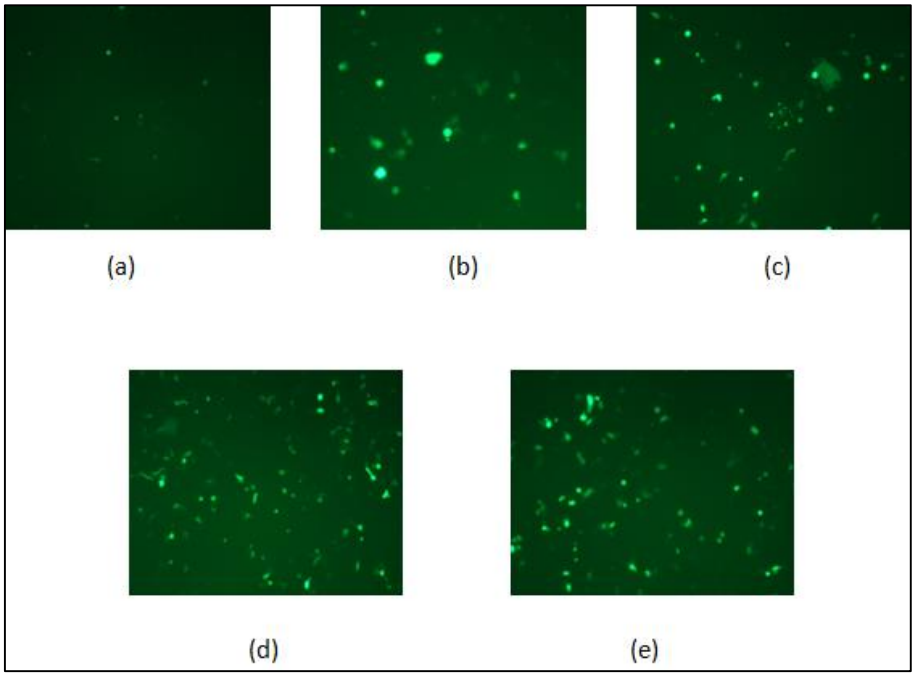

Fig 2: Different concentration of pVIVO1.GFP used for optimization of transfection in 4T1 cell line (a) 1.5µg (b) 2µg (c) 2.5µg (d) 3µg (e) 3.5 µg

The 3µg concentration of pVIVO1.GFP plasmid with 6µl Lipofectamine 3000® showed high amount of bright green fluorescence representing highest transfection efficiency of 40-50% as depicted in fig.2 and this ratio of Lipofectamine/DNA was selected for our further experiments.

4. Discussions

Oncolytic viral gene therapy has recently evolved as promising strategy for cancer treatment with minimal side effects, targeting particularly tumor cells without any damage to healthy cells which mostly occur in other traditional cancer therapeutic measures. Non structural gene 1 of different parvoviruses (PV) like Rat PV (H-1 PV), Human PV (B19v) and Minute virus of mice (MVMv) have been well explored for their oncolytic effects [9]. To identify gene expression changes by transcriptomics in 4T1 mice mammary tumor cells after transfection with CPV2.NS1, the first crucial step is to confirm promoter activity of vector in which NS1 gene is cloned and also need to optimize conditions for transfection in 4T1 cells to identify proper ratio of Lipofectamine/ DNA depicting highest transfection efficiency. Therefore in present study to confirm promoter activity of pVIVO1 vector, we transfected pVIVO1.GFP in 4T1 cells and there was green fluorescence evident in cells in pVIVO1.GFP transfected cells as compared to empty vector pVIVO1 transfected cells when examined under fluorescence microscope after transfection and further 6µl Lipofectamine 3000®/3µg plasmid DNA concentration was identified as optimum ratio depicting highest transfection efficiency of 40-50% in 4T1 cells in our study.

Similarly in other cells like Huh-7 cell line, an immortalized cell line of mammals, is very difficult to transfect and is important for characterization of molecular mechanisms of hepatitis virus. The highest transfection efficiency of 12.2% was observed in Huh-7 cells when 4µl/0.8µg of Lipofectamine 2000®/DNA ratio was optimized after among different ratio of both used for analysis [10]. In *D. melanogaster* Schneider 2 (S2) cells, effect of the lipofectin/DNA ratio on the transfection efficiency was examined by taking seven ratios from 0.5 to 6 of lipofection with constant DNA concentration. It was observed that 5:1 ratio of lipofectin/DNA yielded the maximum GFP concentration in S2 cells which was recognized to be lower than that reported for mammalian CV-1 (10:1) and COS (100:1) cells tested [11]. This all studies supports the notion that transfection efficiency vary in each cell type and with each different transfection reagent/DNA ratio. In summary, apart from optimization of transfection conditions in 4T1 cells, we can utilize this knowledge in our further experiment as if similar ratio of Lipofectamine 3000®/DNA optimized by transfecting pVIVO1.GFP to attain highest transfection efficiency in 4T1 cells, may be used during pVIVO1.NS1 transfection in 4T1 cells then it would denote, more NS1 transfected cells contributing to significant gene expression changes identified by transcriptomics in pVIVO1.NS1 transfected 4T1 cells than control cells (study going on in our laboratory).

5. Conclusions

In conclusion, this study lays subsequent foundation for in vitro transfection based experiments in 4T1 mice mammary tumor cells by providing optimal Lipofectamine 3000®/DNA ratio which can be used to attain highest transfection efficiency.

6. Funding Sources

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7. Conflict of Interest

The authors declare no conflict of interest

8. Acknowledgements

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