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A Generalizable Method To Normalize Luciferase Activity In Transiently Transfected B Cells

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A Generalizable Method to Normalize Luciferase Activity in Transiently Transfected B cells

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Bibliographical Sketch

Carl D. Parson II was born on July 24 1989, in the minute town of Surry County, Virginia to Carl and Lois Parson. He has an eight year younger brother, whom he is devoted to his success. Carl spent the majority of his younger years as a farm hand on his grandfather's (Raymond Parson) farm. There he found his love for livestock and entered his first, and only 4-H Pig Competition where he earned 3rd place for three of a pair and 1st place for best in show. In high school he was a member of Future Business Leaders of America (FBLA), in which he state and national competitions. He graduated with honors and earned a full athletic scholarship to Elizabeth City State University (ECSU), where he received his B.S. in Biology.

While attending ECSU, Carl was a member of the Louis Stokes Alliance for Minority Participation (LSAMP), in which he received recognition for research; "Search for a Diagnostic/Prognostic Biomarker for Brain Cancer Glioblastomamultiforme by 2D-Digems Technique". He also attended the Multicultural Associates Opportunity Program (MAOP) internship where he also received research recognition for research; "HSP90 inhibition by 17-DMAGreduces inflammation in J774 macrophages through suppression of Akt and Nuclear Factor –KB pathways".

In the Spring 2013, Carl enrolled as a Biology Master's candidate at North Carolina Agricultural and Technical State University in Greensboro, NC. While at North Carolina A&T, he was under the direction of Robert Newman and Perpetua Mubweri Muganda. During his time spent in the program, he cultured a number of laboratory skills and techniques. Carl is elated and grateful for the education he received during his tenure at NC A&T.

Acknowledgements

I would like to acknowledge my research advisor, Dr. Robert Newman for his prestige direction, guidance and patience during my tenure at North Carolina Agricultural and Technical State University. He has been an unsung role model and has become a lifelong friend. To Dr. Perpetua Mubweri Muganda for her extensive background knowledge and perception of the study. A special thanks to Jude Akamu, for the experimental construction and also for the constant words of encouragement. Dr. Radiah Minor, for her patience and solid reproach. Lastly, I thank my family for their love and support throughout my educational career.

Table of Contents

List of Figures

List of Tables

Abbreviations and Symbols

β	Greek Letter Beta
µg	Micrograms
µl	Microliter
µm	Micrometer
°C	Degrees Celsius
CO2	Carbon Dioxide
ddH2O	Distilled Deionized Water
g	Gram
kg	Kilogram
mg	Milligram
ml	Milliliter
DEB	Diepoxybutene
GFP	Green fluorescent protein
Fluc	Firefly luciferase
Nluc	Nano-luciferase
Rluc	Renilla luciferase

PEI	Polyethylenimine

Abstract

Cell-based assays are central to life science research. In many cases, these assays require transient, or stable, transfection of an exogenous DNA of the cells under study. Previous studies examining transcriptional regulation mediated by the tumor suppressor, p53, identified several putative p53-regulated promoters involved in diepoxybutene (DEB)-induced cancer progression. However, due to potential differences in transfection efficiencies among different promoter constructs, a direct comparison of these promoters was difficult to assess. Therefore, we sought to develop a generalizable normalization method that would enable direct comparison of promoter activation regardless of transfection efficiency. Specifically, we aimed to use GFP co-transfection as a means of normalizing the signal from transient transfection of a luciferase-based reporter system in TK6 B cells. We focused primarily on cationic lipid-based transfection reagents because 1) they have been widely used to transfect a variety of cell types, 2) they cause less stress on the cells than electroporation, 3) they are safer and faster than viral transduction, and 4) they are more cost effective than specialized transfection methods, such as nucleofection. In this study, we conducted a comparative analysis of the liposome-based transfection reagents, Metafectene Pro, Lipofectamine 2000, Turbofect, and PEI, as well as nucleofection, which is an alternative transfection method based on electroporation. Co-transfection with plasmids encoding luciferase and GFP was achieved using the optimized conditions. These studies demonstrate that GFP co-transfection is an effective means of normalizing for differences in transfection efficiency using luciferase-based reporters.

Chapter 1

Introduction

Cell-based assays, which include assays designed to assess various aspects of cellular physiology such as the cytotoxicity of drugs and environmental factors, the rate of cell proliferation, the induction of apoptosis, and the regulation of cellular signaling pathways, are central to life science research (Alberts 2002). In many cases, cell-based assays require transient or stable transfection of the cells under study. For instance, to measure the regulation of gene expression in different cellular contexts, a variety of transcriptional reporter systems have been developed (Ataei, Torkzadeh-Mahani et al. 2013). Transcriptional reporter systems are composed of a promoter and/or other transcription factor binding sites placed upstream of an easily detectable gene product (i.e. the reporter gene).

While, in theory, the reporter gene can code for any protein whose activity and/or abundance can be measured biochemically, in general, proteins that can be directly detected via fluorescence, such as green fluorescent protein (GFP) family members, or whose enzymatic activity can be easily monitored via colorimetric or luminescence measurements, such as β-galactosidase or luciferase, respectively, are used (Shagoshtasbi, Deng et al. 2015). In these systems, transcriptional activation of the promoter drives the expression of the reporter gene, whose relative levels are measured using one of the aforementioned methods. By placing different promoters upstream of a given reporter gene, the same assay can be used to compare differences in transcriptional activity at various promoters (Reisner, Brauer et al. 2012).

Previous studies examining transcriptional regulation mediated by the tumor suppressor p53 identified several putative p53-regulated promoters involved in diepoxybutene (DEB)-

induced cancer progression (Akamu and Muganda, personal communication). However, due to potential differences in transfection efficiencies among different promoter constructs, a direct comparison of these promoters was difficult to assess. Therefore, we sought to develop a generalizable normalization procedure that would enable direct comparison of promoter activation regardless of transfection efficiency. Specifically, we used GFP co-transfection as a means of normalizing the signal from transient transfection of a luciferase-based reporter system in TK6 B cells. During these studies we focused primarily on cationic lipid-based transfection reagents because 1) they have been widely used to transfect a variety of cell types (Moghimi, Symonds et al. 2005), 2) they cause less stress on the cells than other transfection methods, such as electroporation, 3) they are safer and faster than viral transduction, and 4) they are more cost effective than specialized transfection methods, such as nulceofection (Lonza).

In this study, four commercially available transfection reagents, Lipofectamine 2000 (Life Technologies), Metafectene Pro (Biontex-USA), Turbofect (Life Technologies), and linear polyethylenimine (PEI; Polysciences), were analyzed for their ability to effectively transfect TK6 B cells while maximizing the fluorescence and luminescence signals. We then developed a generalizable normalization protocol that allows comparisons to be made between co-transfected cells regardless of transfection efficiency or luminescence intensity.

Chapter 2

Literature Review

2.1 Luciferase

The emission of light in a biological system stimulated by an enzyme catalyzed biochemical reaction is known as bioluminescence. Due to their low background signal, on the one hand, and their potential for enzymatic amplification of the signal, on the other, bioluminescence approaches have the potential to be more sensitive than similar fluorescent or radionucleotide-based approaches (De, Loening et al. 2007). For most bioluminescent organisms, luminescence is achieved by the oxidation of a luciferin substrate by a family of enzymes known as luciferases. Since luciferases couple each oxidation reaction to the emission of a single photon of light, their activity can be measured effectively by photon counting (Leitão and Esteves da Silva 2010). As a consequence, luciferases have found utility in biotechnology and biomedical research as fusion tags and reporter enzymes.

Most commercially-available luciferases are derived from either luciferases encoded by the firefly, *Photinus pyralis,* or by those encoded by the sea pansy, *Renilla reiniformis*. Nano-luciferase (Nluc) is a newly developed *Renilla* luciferase (Rluc) variant with the brightest bioluminescence reported to date. Nluc exhibits an approximately 150-fold increase in luminescence compared to other firefly (Fluc)- or Rluc-based luciferases that have been similarly configured for glow-type assays. The Nluc enzyme is very stable, retaining its activity following incubation up to 55 °C or in culture medium for >15 h at 37 °C. Importantly, Nluc utilizes the luciferin, coelenterazine, as its substrate. This improves its utility as a bioluminescent tag, since

the conversion of coelenterazine to coelenteramide is achieved in an ATP-independent manner, requiring only molecular oxygen as a co-factor (Kucharikova, Vande Velde et al. 2015).

Due to its stability and robust signal, Nluc has proven to be an effective reporter enzyme in transcriptional reporter systems. These systems may be configured to either achieve maximum sensitivity or, by appending a degradation sequence to reduce intracellular accumulation, to measure dynamic changes in promoter activity (Hall, Unch et al. 2012). Indeed, reporter quantitation can be achieved even at very low expression levels, which allows more reliable coupling with endogenous cellular processes. Like Nluc and other luciferases, green fluorescent protein (GFP) color variants are also effective intracellular reporters. Importantly, GFP fluorescence provides an orthogonal detection method to luminescence, which, as we discuss below, can be used to normalize the luciferase signal in co-transfected cells.

2.2 Green Fluorescent Protein (GFP)

GFP is a well-established tool for non-invasive and real-time studies in living cells. *Aequorea victoria* GFP is the founding member of a large family of autofluorescent proteins isolated from bioluminescent marine organisms, including hydrozoa and reef-building corals of the class Anthozoa (Shimomura 2008). *A. victoria* GFP generates a highly fluorescent *p*-hydroxybenzylidene-5-imidizolinone (*p*-HBI) species from the tripeptide, Ser65-Tyr66-Gly67. It is believed that once the polypeptide chain has folded into a near-native conformation, it undergoes a series of autocatalytic posttranslational modifications to generate the mature fluorophore (Sample, Newman et al. 2009). This process is generally described by a three step mechanism consisting of: (1) internal cyclization of Ser65 and Gly67; (2) dehydration to form an imidizolin-5-one intermediate and (3) dehydrogenation along the Cα-Cβ bond of Tyr66 to conjugate the ring

systems. Dehydrogenation is mediated by molecular oxygen and constitutes the rate-limiting step during fluorophore formation.

The molecular cloning of GFP cDNA and the subsequent expression of GFP as a functional transgene has revolutionized biological research, opening profitable avenues of investigation in cell, developmental and molecular biology (Voss, Larrieu et al. 2013). Over the last twenty years, *A. victoria* GFP has been optimized for efficient use in mammalian cells. GFP optimization, coupled with improvements in fluorescence detection techniques and quantification, has dramatically impacted cell developmental and molecular biology research (Ekberg, Amaya et al. 2011). Indeed, genetically-encoded FP tags have become the basis for a wide range of cell-based applications, including assays designed to assess cytotoxicity, cell proliferation, and the initiation of apoptosis.

2.3 B Lymphocytes

B cells belong to a group of white blood cells known as lymphocytes, making them a vital part of the immune system—specifically the humeral immunity branch of the adaptive immune system (Alberts 2002). The principal functions of B cells are to produce antibodies against foreign antigens and to develop into memory B cells after activation by antigen interaction (Mauri and Bosma 2012).

TK-6 B cells, which were originally isolated from a patient with chronic myelogenous leukemia, are most often used to study mechanisms underlying mutation and to analyze the genotoxicity of diverse xenobiotics in B cells (Schwartz, Jordan et al. 2004). Like most cells, TK-6 B cells undergo apoptosis.

B cells are regarded for their capacity to produce antibody but, recent advances in B cell biology have capitalized on previous findings and demonstrated that B cells also release a broad variety of cytokines (Mauri and Bosma 2012). These cytokines activate specific caspases and downstream pathways. Some of these caspases restrain the excessive inflammatory responses that occur during autoimmune diseases or can be caused by unresolved infections.

2.4 Transfection Methods Comparison and Transfection Reagents

Transfection is the process by which exogenous nucleic acid polymers are introduced into cells. There are four primary methods of transfection: viral, particle-based, non-chemical, and chemical-based. Among chemical-based transfection methods, cationic lipid-mediated transfection remains one of the most widely used in biomedical research. Cationic lipid-based transfection is believed to occur via a three step process involving 1) interaction of the cationic head-groups of the lipids with the negatively-charged backbone of the DNA, 2) endocytosis, and 3) escape from the endosome and subsequent incorporation into the nucleus following M-phase of the cell cycle (Mortimer, Tam et al. 1999).

Meanwhile, electroporation or electropermeabilization, is a technique in which an electrical field is applied to cells in order to increase the permeability of the cell membrane, which in turn, allows genes, drugs, or DNA to be introduced into the cell. Nucleofection is a specialized electroporation-based transfection method that transfers nucleic acids, such as DNA and RNA, directly into the nuclei of cells through the application of a specific voltage program in the presence of specialized buffer systems. Despite superior transfection efficiency and cell viability in many cases, the high cost of nucelofection has discouraged the wide-spread adoption of this new technology (Kang, Ramu et al. 2009).

2.4.1 Cationic liposome

Cationic liposomes are structures that are composed of positively charged lipids and are increasingly being researched for use in gene therapy due to their favorable interactions with negatively charged DNA and cell membranes (Safinya, Ewert et al. 2014). They can be administered efficiently, safely, and repeatedly for direct gene transfer. Liposomes are vesicular structures that can form via the accumulation of lipids interacting with one another in an energetically advantageous manner. Liposomes are generally formed by the self-assembly of dissolved lipid molecules, each of which contains a hydrophilic head group and hydrophobic tails. These lipids take on associations which yield entropically favorable forms of low free energy, in some cases forming bimolecular lipid leaflets. These leaflets are characterized by hydrophobic hydrocarbon tails facing each other and hydrophilic head groups facing outward to associate with aqueous solution (Hattori, Hara et al. 2015). Liposomes can exhibit a range of sizes and morphologies upon the assembly of pure lipids or lipid mixtures suspended in an aqueous medium (Leduc, Wong et al. 2007).

2.4.2 Lipofecatamine 2000

Lipofectamine 2000 is a common transfection reagent used in molecular and cellular biology (Dalby, Cates et al. 2004). It is used to increase the transfection efficiency of RNA or plasmid DNA into in vitro cell cultures (Dalby, Cates et al. 2004). In non-dividing cells, research has shown that Lipofectamine 2000 improves the efficiency of transfection, which suggests that it additionally helps the transfected genetic material penetrate the intact nuclear envelope (Dalby, Cates et al. 2004).

2.4.3 Metafectene Pro

Metafectene Pro, which is purported to be more stable than Lipofectamine 2000, utilizes a specifically designed molecular structure to ensure easy entry of DNA/RNA into cells. This is achieved by condensing DNA/RNA into compact structures (DNA/RNA/lipid-complex) and through endosome buffering. Importantly, metafectene provides persistent expression of the introduced gene. This is beneficial to the production of recombinant proteins and also with the analysis of downstream long-term effects of DNA expression (Iczkowski, Omara-Opyene et al. 2004). Metafectene Pro brings an equilibrium point for transfection efficiency, which makes it the best candidate for transfection of the co-transfection of GFP- and Luciferase-encoding plasmids into B-cells.

2.4.4 Turbofect

Turbofect is a new generation of transfection reagent optimized for nucleic acid delivery into eukaryotic cells (Lee, Kaul et al. 2014). Its base formulation is similar to the cationic lipids described above but it also contains, histone proteins. This lipid/protein blend allows for slower cell membrane penetration that leads to efficient transfection with reduced toxicity (Martin-Montanez 2010). It also has simple application, working well in media containing antibiotic and antimycotic agents. It is ideal for serum containing media; no requirement for media changes.

2.4.5 Polyethylenimine (PEI)

Among cationic polymers, polyethylenimine (PEI) is the most popular synthetic polymer and has a high cationic charge density (Kang, Tachibana et al. 2010). Polyethyleneimines are used in the cell cultures of non-adherent cells to increase attachment, in turn increasing transfection

efficiency. However, it is extremely efficient and also is very cost effective (Moghimi, Symonds et al. 2005).

2.5 Nucleofection

Nucleofection is a transfection method designed to transfer genes into cells quickly and efficiently. The technology is based on electroporation (Muyderman, Yew et al. 2010). The Nucleofector device is essentially a cell electroporator that is programmed with various profiles that are defined electrical parameters for specific cell types. Each cell type is introduced to the DNA (or RNA) in an optimized Nucleofector solution that has been designed for that particular cell type (Kang, Ramu et al. 2009). Since the solutions and the programs are pre-configured by Lonza, there is little optimization by the user. The only variable that exists in the beginning is tittering the amount of nucleic acid that achieves the optimum results, in turn gene expression is achieved in as little as a few hours.

As opposed to most transfection methods, nucleofection introduces the genetic material straight into the nucleus where it is assimilated into the cell independent of cell division. This is advantageous in that it reduces assay time to a couple of hours as opposed to 24 to 48 hours for standard transfection methods using lipid-based reagents. For instance, expression of proteins is achieved in as little as 4 hours using primary T cells (Zhao, Su et al. 2011).

Chapter 3

Methodology

3.1 Transfection Reagents

Lipofectamine 2000 and Opti-MEM serum were purchased from Life Technologies; Metafectene was purchased from Biontex USA; Turbofect was purchased from Thermo Scientific; Polyethylenimine (PEI) was purchased from PolySciences. All cationic lipid transfection reagents were stored at -20 °C, except for Turbofect and Lipofectamine 2000, which were stored at 4 °C. The nucleofection system and buffers are from AMAXA and were stored according to the manufacturer's protocol.

3.2 Cell Culture

TK6 B cells were a gift from Perpetua Mubweri Muganda, Ph.D. The cells were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS; Atlanta Biologicals) at 37 °C in a CO_2 incubator. Passaging was carried out every 36-48 hours. Total cell count was maintained between 0.5-1.1 x 10^6 cells/mL. Cell viability and total count was manually measured using Trypan blue staining and a standard hemocytometer.

3.3 Plasmids and DNA

The pNL1.1 plasmid was obtained from Promega and derivatized by Jude Akamu (Ph.D. candidate in Dr. Muganda's lab) by the insertion of a strong cytomeglia virus (CMV) promoter upstream of the Nluc gene to generate the plasmid, pCMV-LnL1.1. The plasmid encoding GFP-Max was also kindly provided by Perpetua Mubweri Muganda, Ph.D. All plasmids were purified using the DNA Wizard midi-prep kit (Promega), stored at -20 °C and thawed at room temperature, as necessary.

3.4 Transfection using Cationic Lipid-based Transfection Reagents

Approximately 24 hours before transfection, cells were passaged at a density of 0.2 x 10^6 cells/well in RPMI-1640 media supplemented with 10% heat-inactivated FBS into a 6-well tissue culture dish (Greiner) and incubated at 37°C under 5 % CO_2. All cationic lipid-based transfections were done in a similar manner, according to the manufacturers' protocols. Briefly, Transfection Solution A was prepared by diluting 2 µg of plasmid DNA into Opti-MEM serum to a final volume of 100 µL. Likewise, Transfection Solution B was prepared by diluting the appropriate volume of transfection reagent with Opti-MEM serum to a final volume of 100 µL. Transfection Solution A (containing DNA) was then added to Solution B (containing reagent) and mixed gently by manually pipetting a couple of times. The combined A/B transfection mixture was then incubated at room temperature for 15-20 minutes. The DNA to transfection reagent ratio (DNA:Reagent) ranged from 3:1 to 1:3, as indicated. Following incubation, the transfection mixture, containing DNA-lipid complexes, was added drop-wise to the cells. Cells were then incubated at 37 ^oC under CO_2 for 24-48 hours, as indicated. Transfected cells were imaged using a Olympus fluorescence microscope (see Section 3.6) while luminescence and total GFP fluorescence were measured using an Infinite M200 Pro multimode microplate reader (Tecan, USA) using bottom read modes (see Section 3.7).

3.5 Transfection using the Nucleofection System

Nucleofection was done using either an AMAXA 4D or 2D system, according to the manufacturer's protocol. In each case, cells were diluted to a density of 0.7 x 10^6 cells/mL and pre-incubated with 0.4 □g of plasmid DNA dissolved in buffer SE before applying the pulse

program, DS-137. Cells were visualized and assayed 24 and 48 hours after nucleofection, as indicated.

3.6 Fluorescence Microscopy and Calculation of Transfection Efficiency

Twenty-four to forty-eight hours following transfection, fluorescent cells were visualized using an Olympus 1X71 inverted epifluorescence microscope using the GFP filter set (Ex: 469 (35); Em: 525 (39)). In a similar manner, the total number of cells were counted by bright field. The transfection efficiency was then calculated according to the equation:

$$\text{Transection Efficiency} = (\text{fluorescent cells/total cells}) \times 100\%$$

3.7 Total Fluorescence and Luminescence Measurements

3.7.1 Total fluorescence measurements

Before measuring GFP fluorescence, liposome treated cells were pelleted at 500 *x g* for 2 minutes, washed once in Hank's Balanced Salt Solution (HBSS) imaging buffer and pelleted again. The cells were then gently re-suspended in 100 µl of HBSS imaging buffer and transferred to a Bio-One black-walled, flat bottom transparent polystyrol 96-well tissue culture dish (Greiner). Total fluorescence in each well was measured using an Infinite M200 Pro multimode microplate reader. The settings used to measure GFP fluorescence are summarized in Table 1.

Table 1: Fluorescence measurement settings on Infinite M200 PRO

Excitation wavelength	483 nm
Excitation bandwidth	9 nm
Emission wavelength	535 nm
Emission bandwidth	20 nm
Gain	Optimal
Number of flashes	10 flashes
Integration time	20 µs
Lag time	0 µs
Settle time	0 µs

3.7.2 Luciferase Assay

After measuring total fluorescence, total luminescence was measured using the Nano-Glo luciferase assay system (Promega), according to the manufacturer's protocol. Briefly, cells were centrifuged again at 500 x *g* and re-suspended in 100µl of Nano-Glo luciferase buffer containing the coelentrazine substrate. Cells were then incubated for 10 minutes at room temperature followed by luminescence reading on the Infinite M200 Pro system. The settings used to measure luminescence are summarized below:

Table 2: Luminescence measurement settings on Infinite M200 PRO

Attenuation	None
Integration Time	100 ms
Settle Time	0 ms

3.8 Normalization of Luminescence Signal

The luminescence signal was normalized using fluorescence intensity according to the following equation:

$$L_{N,i} = (L_{s,i} - L_{cont})/(F_{s,i}-F_{cont})$$

where $L_{N,i}$ is the normalized luminescene signal, $L_{s,i}$ is the luminescence intensity of sample *i*, L_{cont} is the luminescence intensity of the untransfected control cells, $F_{s,i}$ is the fluorescence intensity of sample *i*, and F_{cont} is the fluorescence intensity corresponding to untransfected controls.

Chapter 4

Results

4.1 Optimization of Transfection and Detection Conditions in B Lymphocytes.

To optimize the detection conditions using the microplate reader format, TK-6 B cells were first transfected with pGFP-Max using a previously developed Metafectene-based transfection protocol (Akamu and Muganda, personal communication). The fluorescence of transfected cells was then measured under different imaging conditions. To this end, transfected cells were first assayed in RPMI-1640, 10% FBS culture medium (containing the pH indicator, phenol red) before being pelleted, re-suspended in an equivalent volume of HBSS imaging buffer, and re-measured (Figure 1). These experiments suggest that the culture media contributes a large amount of background fluorescence compared to the HBSS imaging buffer. Indeed, reading the cells in HBSS imaging buffer causes a substantial reduction in background which, in turn, increases the dynamic range of the assay (Figure 1).).

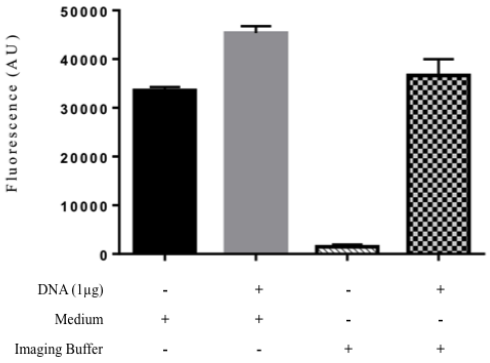


Figure 1. Fluorescence read optimization (RPMI vs Imaging Buffer). B cells were transfected with 2 µg of plasmid DNA encoding GFP-max using Metafectene Pro. The ratio of DNA to transfection reagent was 1:3 (2 µg: 6 µl). Each bar represents the average of at least three independent experiments. Error bars represent standard error.

Next, to determine whether co-transfection with GFP-Max could be used to normalize the luminescence signal produced by NLuc under the control of the strong CMV promoter (pCMV-LnL1.1), pGFP-Max was co-transfected with pCMV-LnL1.1 using a 1:1 DNA:DNA ratio and fluorescence and luminescence intensities were measured 24 and 48 hours after transfection (Figure 2). These experiments showed that, while the luminescence intensity did not exhibit a discernible change between 24 and 48 hours, there was a marked increase in the GFP-Max fluorescence intensity over this same time period. This is likely a function of the maturation time required for the GFP fluorophore. Interestingly, following Metafectene-mediated transfection, the luminescence signal was much lower than anticipated. Therefore, to determine whether this effect was specific to Metafectene or a general property of cationic lipid reagents, we tested a series of cationic lipid-based transfection reagents.

Specifically, we examined Metafectene Pro, Lipofectamine 2000 and linear PEI. In addition, we also assayed Turbofect, which utilizes a related cationic polymer-based formulation supplemented with histone protein. Because B cells are notoriously difficult to transfect, the transfection efficiency of pGFP-Max alone was assessed for each reagent using live cell fluorescence imaging before attempting co-transfection experiments. During these experiments, the total amount of pGFP-Max DNA was varied (while maintaining a 1:1 DNA:reagent ratio) in order to determine the amount of DNA necessary to achieve optimal transfection efficiency without adversely affecting cell viability (Figures 3, 4).

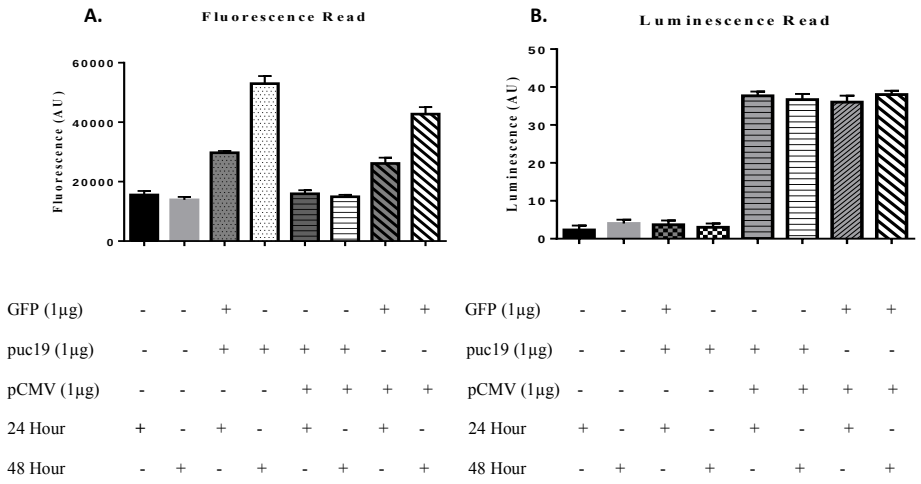


Figure 2. Time trial (24-48hr) Optimization Metafectene Pro. Experimental conditions, including how long after transfection the measurements were taken, are indicated. GFP: pGFP-Max; pCMV: pCMV-LnL1.1; pUC19: empty vector, used to maintain equivalent amounts of DNA across experiments. A) Fluorescence intensity expressed in arbitrary units (AU). B) Luminescence intensity expressed in AU. Error bars represent the standard error.

These experiments suggest that maximal transfection efficiency is achieved between 1 and 2 µg of DNA for all of the reagents tested (Figure 3). Likewise, a marked decrease in viability was observed when > 2 µg of DNA was used (Figure 4). Only PEI, which did not exhibit a substantial decrease in viability until 4 µg of DNA was used, did not appear to follow this trend. Taken together, these results suggest that 1-2 µg of DNA is optimal for the liposome-based transfection reagents tested.

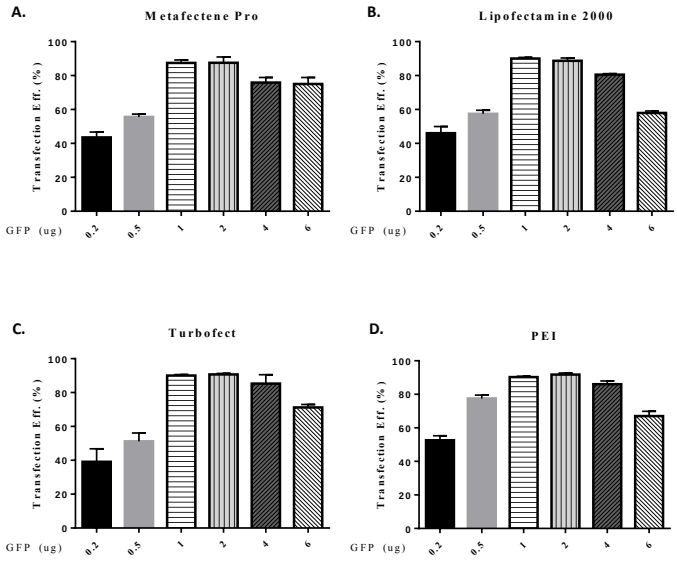


Figure 3. Optimization of Transfection Efficiencies: DNA Titration TK6 B cells were transfected with increasing amounts of DNA and transfection efficiencies were determined. In each experiment, the DNA:reagent ratio was maintained at 1:3. A) Metafectene Pro. B) Lipofectamine 2000. C) Turbofect. D) PEI.

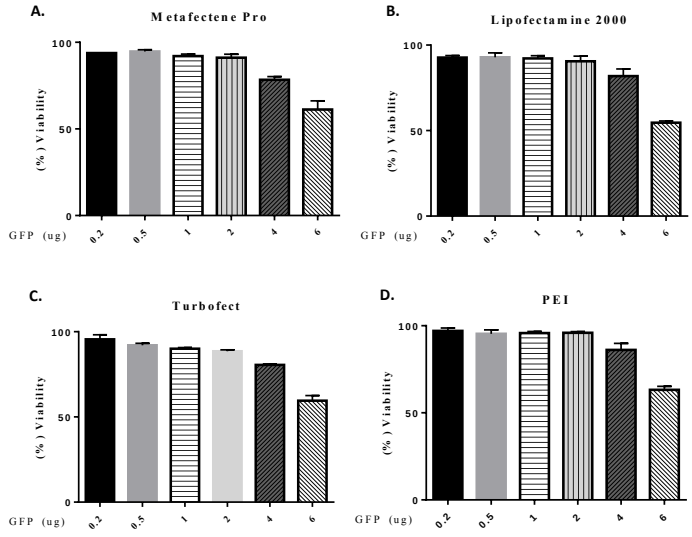


Figure 4. Optimization of Cell Viability: DNA Titration. TK6 B cells were transfected as described in Figure 3. A) Metafectene Pro. B) Lipofectamine 2000. C) Turbofect. D) PEI.

The previous experiments suggested that higher liposome concentrations may adversely affect cell viability—and perhaps cell physiology at even lower concentrations. While the manufacturers suggest using a total DNA:reagent ratio of 1:3 as a starting point, the optimal conditions in our system had yet to be explored. Therefore, we were curious to see if similar results would be obtained at lower DNA:reagent ratios. Each of the four reagents was thus analyzed at various total DNA:reagent ratios, with the total DNA remaining constant (Figure 5). These data indicate that a 1:1 DNA:reagent ratio yields the best results.

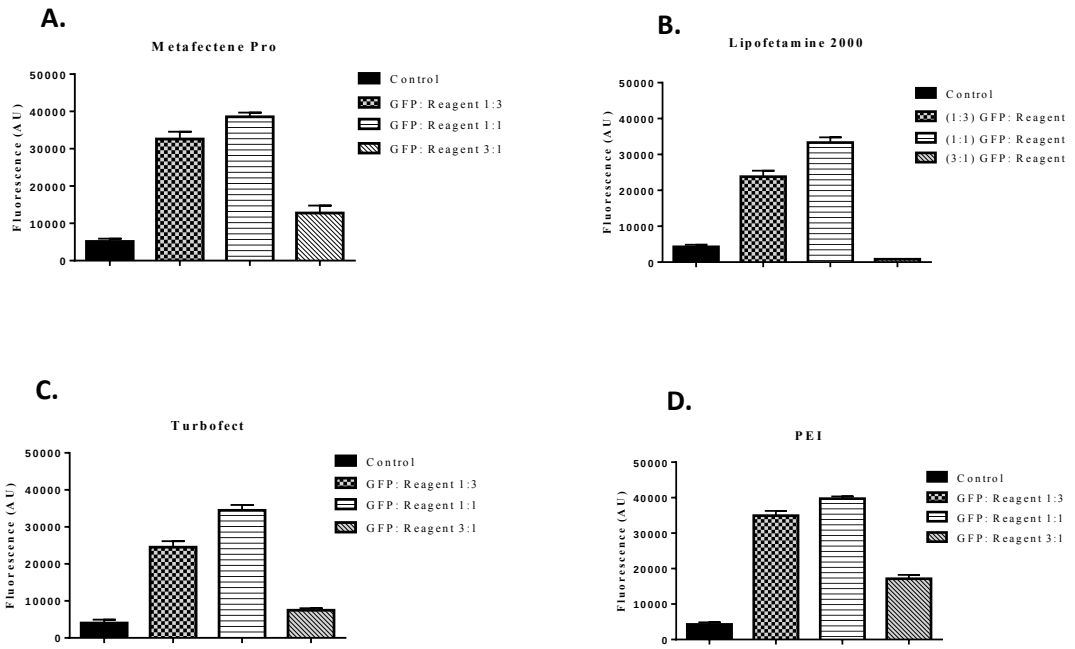


Figure 5. DNA to Reagent Ratio Optimization for Liposome Reagents: Fluorescence Read. 2µg DNA (GFP) with transfection reagent at indicated ratio (m:v); 1:3 (2µg:6µl), 1:1 (2µg:2µl), 3:1 (2µg:0.5µl). A.) Metafectene Pro. B) Lipofectamine 2000. C) Turbofect. D) PEI.

The usage of less reagent is beneficial to the fluorescence. Since the cationic formulation appears to inhibit luciferase activity, it was imperative to see the effects of more luciferase with less transfection reagent. We pursued this by analyzing various DNA:DNA ratios, specifically

weighing more toward luciferase (pCMV-nL1.1). During these experiments, the total DNA:reagent ratio remained constant. As expected, a reduction in pGFP-Max corresponds with a reduction in the fluorescence signal (Figure 6). However, despite an approximately 9-fold increase in pCMV-LnL1.1 DNA, only a marginal increase in luciferase activity was observed (Figure 7). Thus, the optimal DNA:DNA ratio appears to be 1:1. Importantly, during these experiments, Turbofect-mediated transfection resulted in the highest luminescence intensity while PEI exhibited the best fluorescence signal via co-transfection.

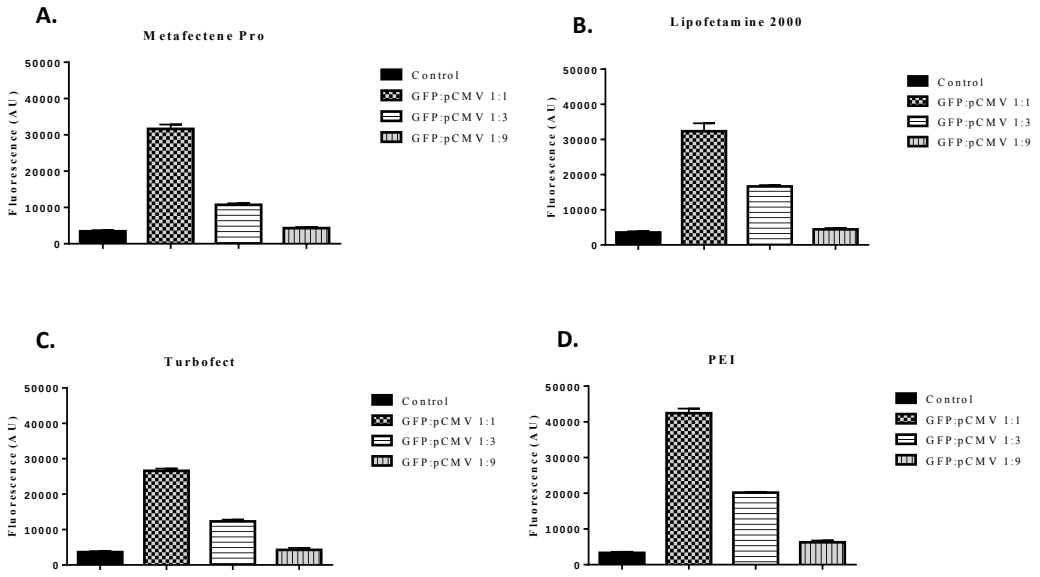


Figure 6. Optimization of DNA to DNA Ratio: Fluorescence Read. Total DNA: reagent ratio remained constant (1:1) (2µg:2µl). DNA:DNA ratio varying at indicated ratio (m:m); 1:1 (1µg:1µg), 1:3 (0.5µg:1.5µg), 1:9 (0.2µg:1.8µg). A) Metafectene Pro. B) Lipofectamine 2000. C) Turbofect. D) PEI.

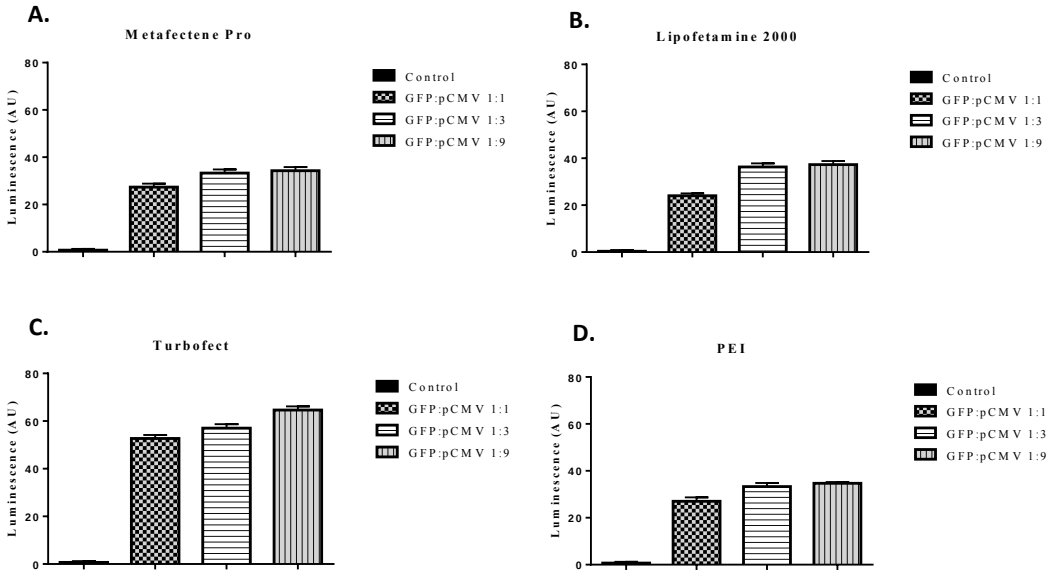


Figure 7. Optimization of DNA to DNA Ratio: Luminescence Read. Total DNA: reagent ratio constant (1:1) (2µg:2µl). DNA:DNA ratio varying at indicated ratio (m:n); 1:1 (1µg:1µg), 1:3 (0.5µg:1.5µg), 1:9 (0.2µg:1.8µg). A) Metafectene Pro. B) Lipofectamine 2000. C) Turbofect. D) PEI.

4.2 Normalization of Luciferase Signal via GFP Co-transfection

Next, with the transfection protocols for TK6 B cells established, the fluorescence and luciferase activity following liposome-mediated transfection could be measured in parallel to normalize the fluorescence/luminescence signal. To this end, B cells were co-transfected with pGFP-Max and pCMV-LnL1.1, using pUC19 as an empty vector so that the total amount of DNA remained constant throughout. These results were consistent with previous co-transfection experiments using Metafectene Pro in TK6 B cells. While Metafectene Pro and Lipofectamine 2000 displayed decent fluorescence intensity, in each case low luciferase activity was observed (Figures 8-9). PEI exhibited the greatest fluorescence signal with a high transfection efficiency. However, just like Metafectene Pro and Lipofectamine 2000, there appeared to be quite a bit of

luciferase interference (Figure 9D). In contrast, though cells transfected with Turbofect showed somewhat less fluorescence activity relative to those transfected with the other liposome-based reagents, they exhibited a consistent luminescence signal that was significantly higher than the other reagents (Figures 10-11). These consolidated results confirmed the hypothesis that cationic lipid-based reagents interferes with luciferase activity, since Metafectene Pro, Lipofectamine 2000, and PEI are of cationic lipid formulation and Turbofect is lipid/histone formulated.

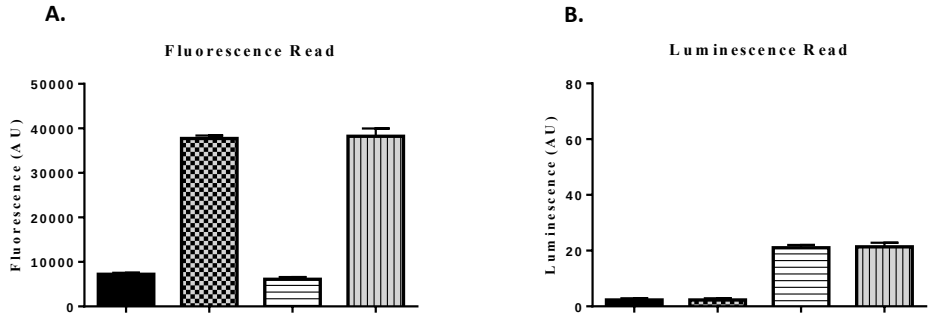
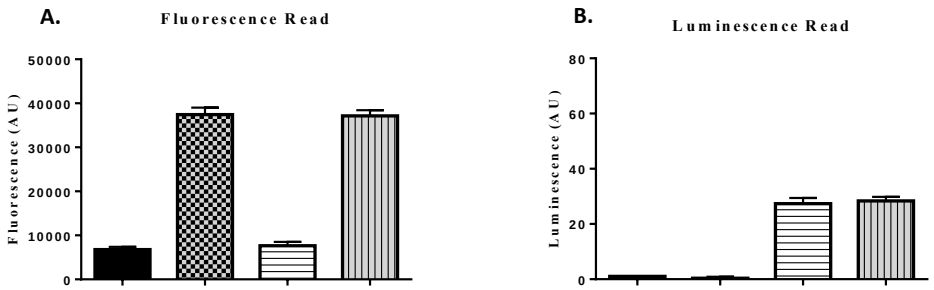


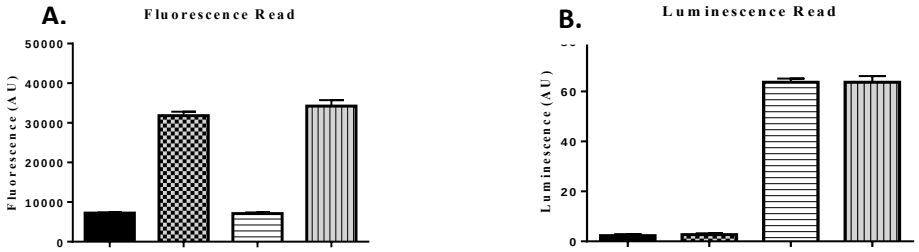
Figure 8. Co-transfection via Metafectene Pro. 48 hour co-transfection GFP/pCMV-LnL1.1. Total DNA: reagent ratio 1:1 (2µg:2µl) with a DNA:DNA ratio 1:1 (1µg:1µg). pUC19 used as empty vector for positive controls. A) Fluorescence read B) Luminescence read



GFP (1µg)	-	+	-	+
pCMV (1µg)	-	-	+	+
puc19 (1µg)	-	+	+	-

GFP (1µg)	-	+	-	+
pCMV (1µg)	-	-	+	+
puc19 (1µg)	-	+	+	-

Figure 9. Co-transfection via Lipofectamine Pro. 48 hour co-transfection GFP/pCMV-LnL1.1. Total DNA:reagent ratio 1:1 (2µg:2µl) with a DNA:DNA ratio 1:1 (1µg:1µg). pUC19 was used as empty vector for positive controls. A) Fluorescence read B) Luminescence read.



GFP (1µg)	-	+	-	+
pCMV (1µg)	-	-	+	+
pUC19 (1µg)	-	+	+	-

GFP (1µg)	-	+	-	+
pCMV (1µg)	-	-	+	+
pUC19 (1µg)	-	+	+	-

Figure 10: Co-transfection via Turbofect. 48 hour co-transfection GFP/pCMV-LnL1.1. Total DNA: reagent ratio 1:1 (2µg:2µl) with a DNA:DNA ratio 1:1 (1µg:1µg). Puc19 used as empty vector for positive controls. A) Fluorescence read B) Luminescence read

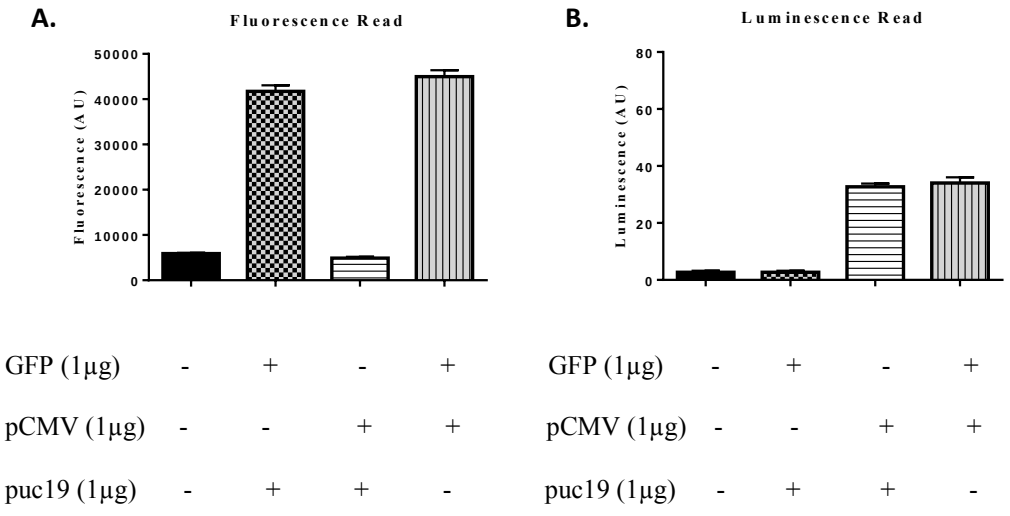


Figure 11: Co-transfection via PEI. 48 hour co-transfection GFP/pCMV-LnL1.1. Total DNA: reagent ratio 1:1 (2µg:2µl) with a DNA:DNA ratio 1:1 (1µg:1µg). Puc19 used as empty vector/filler for positive controls. A) Fluorescence read B) Luminescence read.

To determine whether the liposome-based reagents only inhibit NLuc or whether the inhibition was a general phenomenon among luciferases, pCMV-LnL1.1 (NLuc) was compared to pGL3 encoding FLuc (Figure 12). These results are consistent with previous findings, with Turbofect again showing the least degree of inhibition with both luciferase/luciferin combinations. This was not due to properties intrinsic to the plasmids, since nucleofection resulted in robust luciferase and GFP signals (Figure 13).

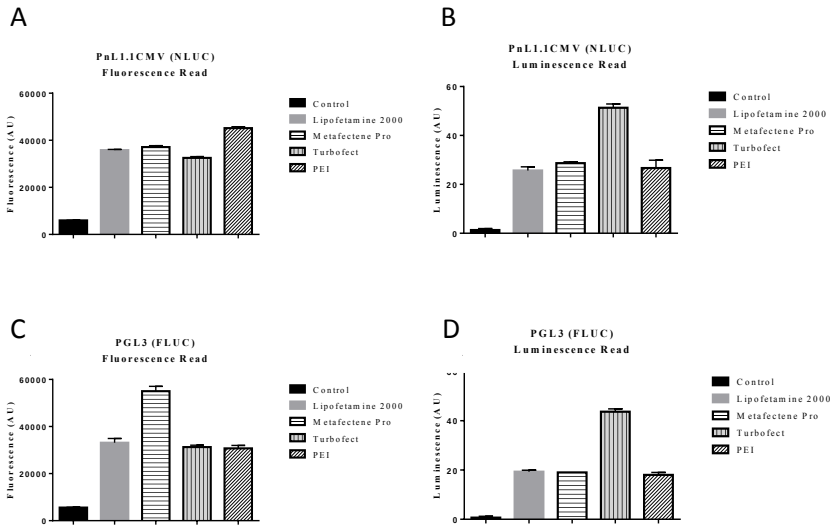


Figure 12: Luciferin Comparison Nano-luciferase vs Firefly Luciferase. 48 hour co-transfection via varying liposome reagents. Total DNA:reagent ratio (1:1) (2µg:2µl) with DNA:DNA (1:1) (1µg:1µg). A) PCMV-LnL1.1 (NLuc) fluorescence read. B) PCMV-LnL1.1(NLuc) luminescence read. C) PGL3 (FLuc) fluorescence read. D) PGL3 (FLuc) luminescence read.

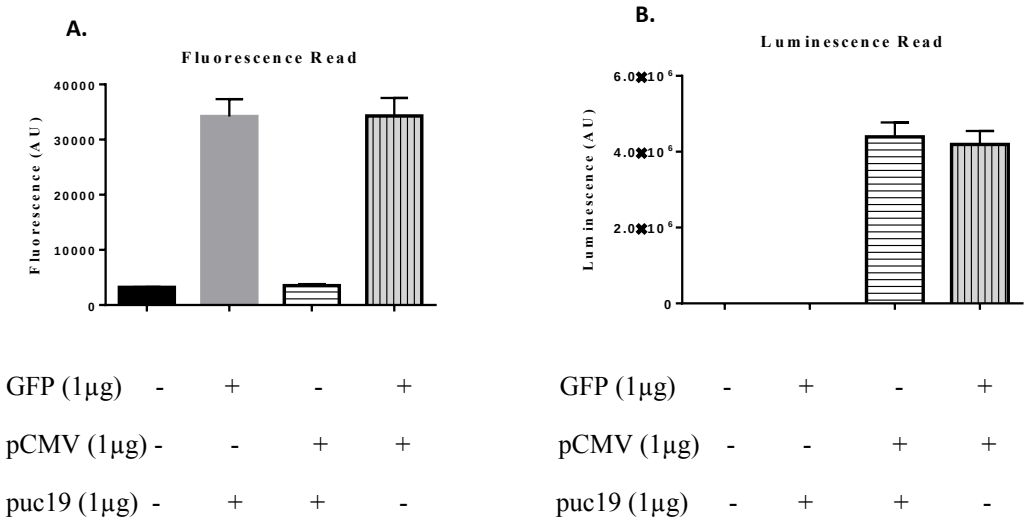


Figure 13: Nucleofection (Lonza) Co-transfection. GFP: pCMV-LnL1.1. pUC19 used as empty vector for positive controls. DNA:DNA 1:1 (0.2µg:0.2µg). (-) Control cells were nucleofected with no DNA. A) Fluorescence read. B) Luminescence read.

Despite the relatively low luminescence signal obtained using liposome-based reagents, the low background luminescence resulted in an approximately 30-fold increase in transfected versus non-transfected cells. We hypothesized that this dynamic range would be sufficient to monitor changes in promoter activity, if differences in transfection efficiency between samples could be eliminated. Therefore, we asked whether GFP normalization would account for differences in transfection efficiency. To this end, transiently transfected TK6 B cells were diluted with untransfected TK6 B cells to simulate differences in transfection efficiency from 100% (undiluted) to 25% (diluted 1:3 with untransfected cells) efficiency. At each dilution, luminescence/fluorescence signal in TK6 B cells was measured and the luminescence signal normalized to the GFP fluorescence. During these studies, we focused on PEI- and Turbofct-mediated transfection. We chose Turbofect because it exhibited the strongest luciferase activity.

Likewise, PEI was chosen because it consistently exhibited robust transfection efficiency with minimal cell death while, at the same time, resulting in strong fluorescence intensity These studies demonstrated that, even if the transfection efficiency varies, the fluorescence/luminescence signal will remain consistent when the corrected luminescence signal is normalized to fluorescence intensity (Figure 14-15). Importantly, normalization also appears to be allow comparisons over a range of luminescence intensities, as evidenced by nucleofection experiments (Figure 16).

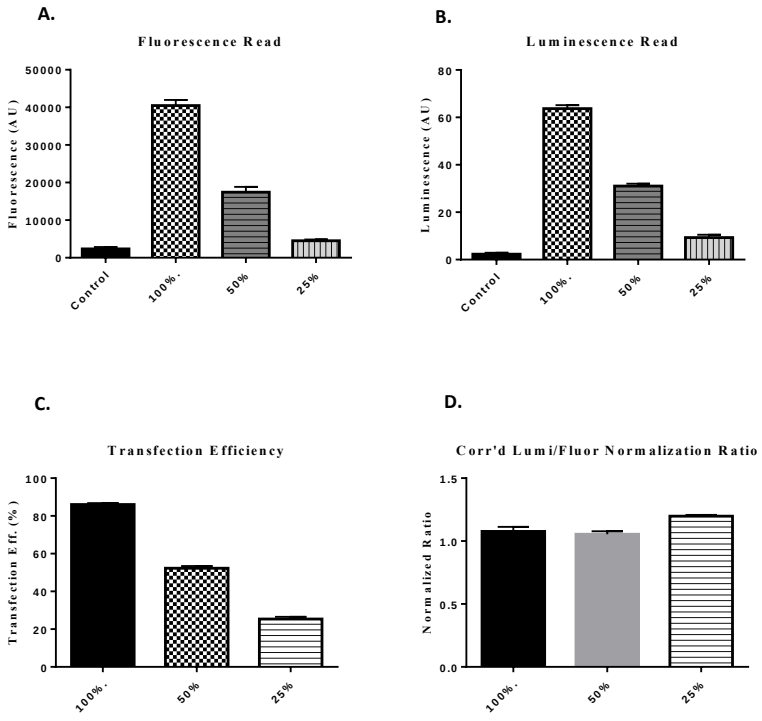


Figure 14: Turbofect Co-transfection Dilution (transfected vs non-transfected cells). GFP: pCMV-LnL1.1. Total DNA: reagent 1:1 (2µg:2µl) with DNA:DNA ratio 1:1 (1µg:1µg). A) Fluorescence read B) Luminescence read C) Transfection efficiency D) Corrected (corr'd) luminescence/fluorescence normalization ratio

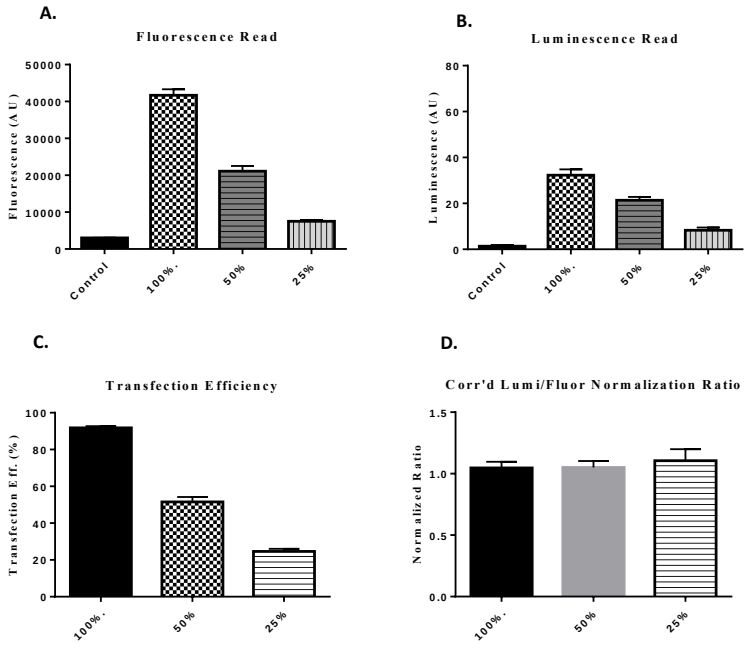


Figure 15: PEI co-transfection dilution ***(transfected vs non-transfected cells)***. GFP: pCMV-LnL1.1. Total DNA: reagent 1:1 (2µg:2µl) with DNA:DNA ratio 1:1 (1µg:1µg). A) Fluorescence read B) Luminescence read C) Transfection efficiency D) Corrected (corr'd) luminescence/fluorescence normalization ratio.

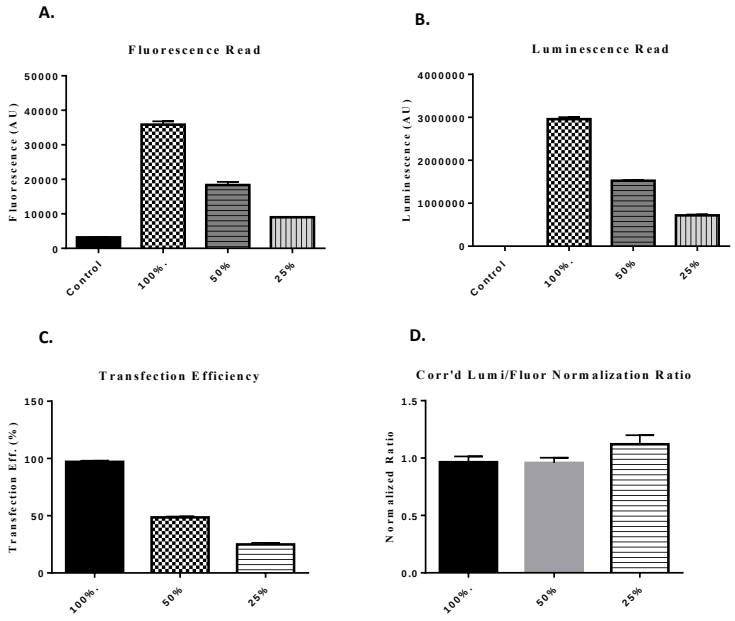


Figure 16: Nucleofection (Lonza) Co-transfection Dilution (transfected vs non-transfected cells). GFP: pCMV-LnL1.1. DNA:DNA ratio 1:1 (0.2µg:0.2µg). A) Fluorescence read B) Luminescence read C) Transfection efficiency D) Corrected (corr'd) luminescence/fluorescence normalization ratio

Chapter 5

Discussion and Future Directions

In this study, we optimized B cell transfection conditions and detection via the liposome-based reagents, Metafectene Pro, Lipofectamine 2000, Turbofect, and PEI. Peak fluorescence intensity was observed 48 hour after transfection while luminescence remained constant between 24 and 48 hours post-transfection. Both the maximum transfection efficiency and cell viability were observed between 1 and 2 µg of DNA. Likewise, a DNA:reagent ratio of 1:1 (2µg:2µl) and a DNA:DNA (pCMV-LnL1.1:GFP) ratio of 1:1 was shown to result in maximal signal using all liposome-based reagents tested..

Interestingly, under our optimized liposome co-transfection conditions, all liposome-based transfection reagents exhibited substantially reduced luminescence signal relative to that observed using nucleofection. Nonetheless, PEI and Turbofect exhibited 20- to 30-fold changes in luminescence intensity relative to untransfected controls and, due to their advantageous attributes, were further pursued. Nucleofection was employed to the cells to compare the commonly used liposome reagents, to a specialized transfection method. In respect to fluorescence activity, the signal remained constant for cells transfected via liposome reagents and nucleofection. On the other hand, nucleofection luciferase activity was outstandingly prominent in comparison to Turbofect and PEI. The transfection efficiency dilution for all (Turbofect; PEI; nucleofection) methods results lead to the overarching goal; the normalization of the luciferase signal no matter the transfection efficiency.

These findings are significant and can be viewed as a two-edged sword. Not only has the transfection of B cells via liposome-based reagents and nucleofection been optimized, but the normalization of the luciferase signal has led to a cost effective means of transfection and detection

in B cells, which are notoriously difficult to transfect. Cost plays a significant role in research, and is often that certain studies are not fully pursued. On a per run basis, nuclofection is very effective but is also ~10x more expensive than Turbofect and ~15x more expensive than PEI (excluding the cost of the nucleofection system, itself). Thus, the development of a robust, generalizable normalization method based on co-transfected GFP enables the use of much less expensive liposome-based reagents.

The co-transfection of GFP with luciferase will help normalize the luciferase signal to investigate p53 promoters stimulated by DEB, the most potent metabolite of 1, 3-butadiene (BD), an environmental chemical found in petrochemical industrial areas. BD is a known mutagen and human carcinogen, and possesses multi-systems organ toxicity (Yadavilli, Martinez-Ceballos et al. 2007). In future studies, the comparison of another promoter with the luciferin, may give insight into whether a change in promoters will increase or decrease luciferase activity via liposome. Furthermore, optimization of the co-transfection protocol may open the door for other types of cell-based assays. For instance, the apoptotic pathway of B cells can be analyzed using fluorescence resonance energy transfer (FRET)- or BRET-based biosensors. DEB-induced cells undergo apoptosis not necrosis. Apoptosis has three phases; initiation, commitment, and execution. The initiation phase was discovered and demonstrates the cytokine c release from the mitochondria, more specifically, the mitochondrial transition pore. This has yet to be seen in TK6 B cells, and the question of do B cells undergo an alternate apoptotic pathway has yet to be answered.

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