

THE OPTIMIZATION OF A COST-EFFECTIVE LENTIVIRAL VECTOR
PRODUCTION PROTOCOL FOR ANIMAL GENE THERAPY TRIALS

by

Brian Hetrick
A Thesis
Submitted to the
Graduate Faculty
of
George Mason University
in Partial Fulfillment of
The Requirements for the Degree
of
Master of Science
Biology

Committee:

_____ Dr. Yuntao Wu, Thesis Director

_____ Dr. Yali Chen, Committee Member

_____ Dr. Kylene Kehn-Hall, Committee
Member

_____ Dr. Iosif Vaisman, Acting Director,
School of Systems Biology

_____ Dr. Donna Fox, Associate Dean,
Office of Student Affairs & Special
Programs, College of Science

_____ Dr. Peggy Agouris, Dean, College of
Science

Date: _____ Summer Semester 2017
George Mason University Fairfax, VA

The Optimization of a Cost-Effective Lentiviral Vector Production Protocol for Animal
Gene Therapy Trials

A Thesis submitted in partial fulfillment of the requirements for the degree of Master of
Science at George Mason University

by

Brian Hetrick
Bachelor of Science
George Mason University 2014

Director: Yuntao Wu, Professor Department of Biology

Summer Semester 2017
George Mason University
Fairfax, VA



This work is licensed under a [creative commons attribution-noncommercial 3.0 unported license](https://creativecommons.org/licenses/by-nc/3.0/).

Table Of Contents

	Page
List of Tables-----	vi
List of Figures-----	viii
List of Abbreviations-----	x
Abstract page-----	xi
Background:	
Vector design strategy page-----	1
Viral Particle Production and Purification page-----	3
Transfection reagents page-----	4
Chapter 1. Initial Optimization and Comparison of Multiple Chemical Transfection methods:	
Introduction-----	8
Materials and Methods	
Experiment 1.1 Plasmid Confirmations-----	10
Experiment 1.2 CaPO ₄ Transfection Reagent Incubation Conditions-----	11
Experiment 1.3 PEI Transfection Reagent Concentrations-----	16
Experiment 1.4 Comparisons of Three Transfection Methods-----	19
Experiment 1.5 Comparisons of Particles From Three Transfection Methods----	26

Results

Experiment 1.1 Plasmid Confirmations-----	27
Experiment 1.2 CaPO ₄ Transfection Reagent Incubation Conditions-----	30
Experiment 1.3 PEI Transfection Reagent Concentrations-----	32
Experiment 1.4 Comparisons of Three Transfection Methods-----	34
Experiment 1.5 Comparisons of Particles From Three Transfection Methods----	35

Chapter 2. PEI Final Optimizations for 10cm dishes

Introduction-----	37
-------------------	----

Materials and Methods

Experiment 2.1 PEI Reagent Incubation Times-----	38
Experiment 2.2 PEI Transfection with Double DNA-----	43

Results

Experiment 2.1 PEI Transfection Reagent Incubation Times-----	48
Experiment 2.2 PEI Transfection with Double DNA-----	50

Chapter 3. Viral Titer Enhancement through Anion Exchange and Size exclusion

Introduction-----	52
-------------------	----

Materials and Methods

Experiment 3.1 Elution of Particles from the Anion Exchange Columns-----	53
Experiment 3.2 Binding Capacity of the Anion Exchange Columns-----	59
Experiment 3.3 Sartorius™ Vivaspin™ 20 Centrifugal Concentrator-----	63

Results

Experiment 3.1 Elution of Particles from the Anion Exchange Columns-----	67
--	----

Experiment 3.2 Binding Capacity of the Anion Exchange Columns-----	68
Experiment 3.3 Sartorius™ Vivaspin™ 20 Centrifugal Concentrator-----	70
Chapter 4. Discussion and The Final Protocol for the Production of Lentiviral	
Particles for Animal Trials	
Discussion-----	72
Final Protocol for the Production of Lentiviral Particles for Animal Trials	
Plasmid Background-----	74
Materials-----	99
Procedure-----	100
Bibliography -----	105
Biography -----	107

List of Tables

Table	Page
Background	
B.1 Transfection Method Cost and DNA Requirements-----	6
Chapter 1	
1.1 Plasmid Digestion Layout-----	11
1.2 CaPO4 Transfection Plasmid DNA Volumes-----	12
1.3 Lipofectamine™ 2000 Transfection Plasmid DNA Volumes-----	14
1.4 PEI Transfection Reagents Concentrations-----	17
1.5 PEI Transfection Plasmid Volume-----	17
1.6 PEI Transfection Tube Layout-----	18
1.7 CaPO4 Transfection Plasmid Volumes-----	20
1.8 Lipofectamine™ 2000 Transfection Plasmid DNA Volumes-----	22
1.9 PEI Transfection Plasmid DNA Volumes-----	24
1.10 Infection of CEMss Cells Tubes Layout-----	26
Chapter 2	
2.1 PEI Transfection Plasmids DNA Volumes-----	39
2.2 PEI Transfection Reagent Medium-----	39
2.3 Infection Well Layout for HEK293T Cells-----	42
2.4 PEI Transfection Plasmids DNA Volumes-----	44

2.5	PEI Transfection Reagent Medium-----	44
2.6	PEI Transfection Tube Layout-----	45
2.7	Infection Well Layout for HEK293T Cells-----	47
Chapter 3		
3.1	PEI Transfection Plasmids DNA Volumes-----	55
3.2	PEI Transfection Reagent Medium-----	55
3.3	Infection Tube Layout for Clone8 Cells-----	58
3.4	PEI Transfection Plasmid Volumes-----	60
3.5	PEI Transfection Reagent Volumes-----	60
3.6	Infection Tube Layout of Clone8 Cells-----	62
3.7	PEI Transfection Plasmid Volumes-----	64
3.8	PEI Transfection Reagent Volumes-----	64
3.9	Infection Tube Layout of Clone8 Cells-----	66
Chapter 4		
4.1	Plasmid Concentrations and Volumes Needed-----	99
4.2	PEI Transfection Plasmid DNA Volumes For Multiple Plates-----	101
4.3	PEI Transfection Reagent Volumes for Multiple Plates-----	102

List of Figures

Figure	Page
Chapter 1	
1. Plasmid Digestions-----	29
2. CaPO ₄ Tranfection Incubation Conditions -----	31
3. PEI Concentrations Transfection-----	33
4. Initially Optimized Transfection Efficiencies-----	35
5. Infection of Particles from Initially Optimized Transfection-----	36
Chapter 2	
6. PEI Transfection Incubation Times -----	49
7. PEI Transfection DNA Concentrations-----	51
Chapter 3	
8. Anion Exchange Column Salt Elution-----	68
9. Anion Exchange Binding Efficiency-----	69
10. Sartorius™ Vivaspin™ 20 Centrifugal Concentrator-----	71
Chapter 4	
11. Plasmid Map of pAD-SIV3+-----	75
12. Plasmid Map of pCGCG-Nef-----	77
13. Plasmid Map of pNL-SIVmac239Env-----	79
14. Plasmid Map of pSIV-TRAF6-RRE-SA-----	80

15. Plasmid Map of pSIV-HSV1tk-RRE-SA-----	82
16. Plasmid Map of pSIV-beta-Gal-GFP-RRE-SA-----	84

List of Abbreviations

CaPO4:	Calcium Phosphate
DMEM:	Dulbecco's Modified Eagle Medium
GFP:	Green Florescent Protein
HEPES:	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)
HIV:	Human Immunodeficiency Virus
HSV1tk:	Herpes Simplex Virus type 1 Thymidine Kinase
NEAA:	Nonessential Amino Acids
PEI:	Polyethylenimine
RPMI:	Roswell Park Memorial Institute medium
RRE:	Rev Response Element
SIV:	Simian Immunodeficiency Virus
VSV:	Vesicular stomatitis Indiana viru

Abstract

THE OPTIMIZATION OF A COST-EFFECTIVE LENTIVIRAL VECTOR PRODUCTION PROTOCOL FOR ANIMAL GENE THERAPY TRIALS

Brian Hetrick, MS

George Mason University, 2017

Dissertation Director: Dr. Yuntao Wu

This thesis describes a cost effective method for the mass production of lentiviral vector particles for the use in animal gene therapy trials. Viral gene therapy is the process by which genes can be introduced, integrated, or up regulated within the genetic messages of viral particles. Due to their relatively small genomes and there affinity for particular cell surface receptors, viral vectors can easily be manipulated to carry genes to a particular cell type. This potentially makes viral vectors a favorable new method to fight diseases. In order to produce replication deficient lentiviral particles safely in a laboratory, the genome, the envelope, and accessory proteins are split into multiple plasmids. Only those cells that have received all the necessary plasmids with all the required genes will form functioning viral particles. Transfections are a tedious and costly procedure that if not standardized will lead to varying results each time virus is produced. To find the most cost effective and

efficient way to produce lentiviral particles, multiple transfections were preformed using three common chemical transfection reagents, Lipofectamine™ 2000, calcium phosphate, and polyethylenimine (PEI). The costs of the procedures and efficiency of the transfections were assed. It was found that the latter of the three methods was the most cost-efficient. In addition to reducing the cost of transfections, anion exchange and size exclusion will be used to amplify the titer and purity of the viral particles produced from these transfections will greatly reduce the need of multiple injections and larger volume of viral particles in animal trials.

Background

Vector design strategy:

Viral vectors are viruses that have been modified to carry a new genome that can cause a cell to express a particular gene of interest. This ability gives them the potential to treat and even cure a wide range of diseases. Viruses are diverse particles that carry specific receptors that allow the virus to enter particular tissue types. For example, Human Immunodeficiency Virus (HIV) carries the receptor for CCR5, a cytokine receptor found on CD4 T cells and macrophages. There are also viruses that infect multiple tissue types like the vesicular stomatitis virus, which enters cells through pH dependent endocytosis (Sun 2005). This ability to target multiple tissues is ideal for developing treatments for disease that only affect certain tissues. To date, most viral gene therapy vectors are based on lentiviruses and adenoviruses. Adenoviruses are non-enveloped double stranded DNA viruses, which can cause mild respiratory infections (Gardner 2015). Lentiviruses are enveloped retroviruses, which carry an RNA genome that gets reverse transcribed into DNA and that DNA is integrated into the host genome. Lentiviruses include viruses like HIV and can infect both dividing and non-dividing cells, making them particularly useful in gene therapy.

In a previous study, a REV dependent viral vector was developed and described by Wu et al in 2007. Rev is an essential protein in the life cycle of HIV. It alters the splicing pattern of the HIV mRNA and allows for the production of late phase proteins. It

does so by binding to the Rev Response Element (RRE) located in the envelope region of the mRNA. The binding of Rev to the RRE modifies the pre-spliced mRNA's confirmation and blocks eukaryotic splicing factors (Wu 2007). As a result of the blocked splicing, the larger un-spliced mRNAs are allowed to be exported from the nucleus and further translated (Wu 2007). The rev dependent system takes advantage of this Rev-splicing pattern and a gene of interest can be placed into the splicing regions. In the case for the animal trial for this project, the genes were for the herpes simplex one-thymidine kinase (TK), human tumor necrosis factor (TRAF6) and a lacZ -TurboGFP. TK is a protein that has been well studied over the past thirty years, which can phosphorylate ganciclovir when it is introduced and causes the cell to go into apoptosis (Tomicic 2002). Phosphorylated Ganciclovir is a competitive inhibitor of dGTP and stops DNA polymerase elongation. When the vector enters a cell that has been infected with HIV, the Rev proteins from the HIV cause the correct splicing of the mRNA region containing the thymidine kinase (Wu 2007). Then, treatment with ganciclovir will kill the cells that have been infected, thus removing viral reservoirs that keep the infection spreading. The second gene, TRAF6 is a tumor necrosis factor receptor associated factor that positively regulates the NF-kappaB pathway, which leads to apoptosis when at high concentrations in the cell (He, 2006). The last vector produced for this study carries a green fluorescent protein (GFP) and LacZ gene, this is used to identify cells that are expressing rev, indicating they are infected with HIV.

Viral Particle Production and Purification:

When producing viral vectors the key is to produce them safely and efficiently. Since viral vectors are modified virus, it is important to make them replication deficient. If they were capable of replicating, then they could spread to non-intended subjects and could mutate into a virulent form. To keep a viral vector from replicating, the viral envelope or capsid proteins are usually removed from the virus genome and put into a separate plasmid. A plasmid is a small circular DNA molecule that is separate from the chromosomal DNA and can be used to transform bacterial cells to confer antibiotic resistant genes. The bacteria cells can be grown under antibiotic selective pressure to grow multiple copies of the plasmid, which can then be extracted and purified. Those plasmids can then be co-transfected into a cell culture, and will express and eukaryotic genes present on the plasmid. The genome of the vector will carry the gene of interest, usually placed in the structural genes of the viral genome, and the remaining viral genes will be knocked out by frame-shift mutation or point mutations. These are then cloned into a plasmid. In order for the virus to be made both plasmids must be present in a cell. These plasmids are introduced into the cell via transfection. Once the transfections are complete, viral particles are released into the supernatant. The supernatant can be spun down to remove cell debris, and virus can be purified and concentrated with an anion exchange column or ultra centrifugation. Ion exchange chromatography works by attracting particles by their opposite charge. Since virus envelopes have a negative charge, they adhere to the column and can be collected using a sodium chloride solution, which is positively charged (Okada 2009). Ultra centrifugation of filtered cell supernatant

can pellet viral particles when spun down at high speeds. The challenge of using ultra centrifugation is other small contaminants can also be spun down with the viral particles, hence for this study only anion exchange columns were used to purify viral particles.

Transfection Reagents:

There are multiple types of transfection into eukaryotic cells. Physical methods include electroporation, which involves running an electric current over the cell culture which forces the DNA into the cells, microinjection, where a small needle injects the cell with the DNA, and laserfection, the use of a laser to create holes in the membrane. Due to their complexity, requiring specific equipment, and extensive cost and maintenance, physical transfection methods are not used in producing viral particles. Chemical transfection methods, the most common being calcium phosphate, LipofectamineTM 2000, and polyethyleneamine(PEI), are a much better choice due to their simplicity and effectiveness on many cell types. Calcium phosphate transfection involves mixing the plasmid DNA with a calcium chloride solution, while aerating a phosphate-containing buffer. The calcium phosphate forms a positively charged complex precipitate around the DNA (Jordan, 2004). The DNA complex sticks to the cell surface and the uptake of DNA happens by endocytosis. The cost of performing a calcium phosphate transfection is fairly low. Calcium chloride cost anywhere between \$50-\$100 and the HEPES buffer with the phosphate will cost around \$60 for a 100 mL bottle (Thermo Fisher). In addition a glycerol shock treatment following the transfection incubation has shown to be beneficial in some cell lines. These will be enough supplies for over several hundred transfections in a 10cm diameter dishes (55cm²). LipofectamineTM 2000 is a commercialized product that uses cationic lipids to form a complex around the DNA. The positive charge of the

particles interacts with the negative charge of the cell. The complex sticks to the surface and then lipid-mediated fusion with the cell surface causes the uptake of the DNA (Felgner 1987). Lipofectamine™ 2000 is a commercial product that is sold by Invitrogen (Dalby 2005). One 1.5mL tube of Lipofectamine™ 2000 cost \$449 and will supply around 25 transfections in 10 cm diameter dishes(Thermo fisher). PEI is a large polymer of repeating amine groups. The nitrogen in the amine groups of the PEI form ionic interactions with the phosphate backbone of nucleic acids forming a positively charged complex which can adhere to the cell membrane and enter through endocytosis (Reed 2006). There are two types of PEI, branched and linear, that can both be used in transfection. The branched is a liquid and the linear is a solid at room temperature. The branched PEI just needs to be mixed in to serum free medium to make workable solutions. The cost of the PEI is less that \$75 for a 100 mL bottle, which is enough to supply thousands of transfections in 10 cm diameter dishes (Sigma). All forms of chemical transfection reagents and DNA itself are cytotoxic in high concentrations. The additional cost to all of three protocols is the amount of purified DNA needed. Calcium phosphate requires around 25-28 ug, Lipofectamine™ 2000 needs about 24 ug and PEI will need 10 ug, the less DNA required the less time and money will be spent to extract and purify plasmid (Reed 2006, Jordan 2004, Dalby 2004). A general plasmid purification kit costs about \$100 and supplies around 50 purifications (Quiagen). By varying the amount of transection reagent, the amount of DNA, and the incubation time will alter the efficiency of the transfection. Previous studies and companies have

developed specific protocols for optimizing the amount of DNA and reagents to add to the cell culture.

Table B.1: Transfection Method Cost and DNA Requirements:

Transfection Type	CaPO4 Transfection	Lipofectamine™ 2000 Transfection	PEI Transfection
DNA	28ug	24ug	10ug
Estimated Cost/ 10cm diameter Dish	\$2.50	\$20.00	\$1.08

Here we compare CaPO₄, Lipofectamine™ 2000 and PEI Transfections efficiencies, and generate one protocol for the production of, animal trial quality, lentiviral particles. For the calcium phosphate transfection the incubation time of the transfection reagents and the addition of glycerol after incubation was evaluated. For PEI transfections, various concentrations of PEI were tested along with the timing of incubation and the amount of DNA added. All the transfections were optimized in HEK293T cells, an embryonic kidney cell line that is used widely for production of viral particles because it can be easily transfected (ATCC). To evaluate the efficiency of the transfection, a pseudotyped lentivirus with the envelope G protein of the vesicular

stomatitis virus (VSV) carrying a GFP genome was produced. The GFP expression was counted by flow cytometry and fluorescent microscopy. Additionally, vHIV-KFS-Mac239Env and vTurboGFP-mac239env particles were produced to test the efficiency of the production and the viability of the particles produced that would closely resemble the particles for the animal trials.

Chapter 1. Initial Optimization and Comparison of Multiple Chemical Transfection methods

Introduction:

To optimize any transfection method for developing lentiviral particles requires a large amount of plasmid DNA. As stated previously, one of the largest costs in transfection procedures is the amount of required plasmid DNA. The following plasmids were used for the optimization of the transfection methods and the amplification of viral titers, pLKO-TurboGFP-Puro, pCMV- Δ R8.2, pNL-4-3-KFS, pNL-deltapsi-SIVmac239env and pHCMVSV-G. The pLKO-TurboGFP-Puro plasmid is a HIV based lentiviral vector genome that carries a TurboGFP protein and a puromycin resistance gene. When transfected or delivered into a cell the cell will produce a GFP signal visible under a fluorescent scope or through a flow cytometer. The pNL-4-3-KFS is a full HIV genome that has been mutated to not express the envelope genes (Iyer, 2009). The helper plasmid, pCMV- Δ R8.2, carries all the HIV-1 genes except for the HIV-1 envelope (Zufferey, 1997). In combination with a genome plasmid and an envelope containing plasmid, the helper will produce all the necessary proteins to produce the viral capsid and package a viral genome into a viable particle. The two envelope plasmids used to optimize and select a transfection method were pNL-deltapsi-SIVmac239env and pHCMVSV-G. The pNL-deltapsi-SIVmac239env was constructed using the pNL-

deltaspi-Env plasmid that carried the HIV-1 envelope gene (Iyer, 2009). The HIV-1 gene was replaced with the SIVmac329 envelope gene from the SIV239 virus. The pHCMVSV-G expresses a vesicular stomatitis virus glycoprotein, which has a very broad host range allowing it to infect a wide range of cell types (Yee 1994).

Once all the plasmids have been purified and passed quality control they can be transfected into mammalian cell cultures. The initial transfection procedures for the CaPO₄ Transfection and the LipofectamineTM 2000 transfections were developed in the Wu lab. The Wu Lab's standard protocol, calls for the use of 28ug of DNA plus 62uL of a 2M calcium chloride solution mixed with HEPES buffer mixed with enough Tris based transfection buffer to equal 500uL per 10cm dish. The DNA, CaCl₂-HEPES, and transfection buffer solution is added drop wise into a well-aerated HEPES solution. This causes the calcium phosphate to form a precipitate around the DNA and will allow for the cell to uptake the plasmids. In a study published by Schenborn et al in 2000 a glycerol shock was suggested to increase the efficiency of CaPO₄ transfections in certain cell types. Changing the conditions of the transfection reagents incubation may greatly impact the transfection efficiency and viral production. When optimizing a protocol the incubation condition and time needs to be resolved.

The LipofectamineTM 2000 protocol follows Thermo Fishers manufacturing advice of using 24ug of plasmid DNA and 60uL of LipofectamineTM 2000 diluted in a total volume of 3mL. Longo et al described a PEI transfection protocol in a paper published in 2013. They indicated that PEI has a working range between 2mg/mL and 0.125mg/mL and uses around 10ug of DNA. The exact concentration of PEI that can form the most effective

DNA-PEI complexes will need to be identified. Comparison of these three transfection methods followed these available generic protocols. The generic protocols have been previously been shown to work and gave a starting point for optimization.

Chapter 1 Materials and Methods:

Experiment 1.1: Plasmid Digestions

Purpose: To confirm plasmid identities for the use in optimization experiments

Materials:

Plasmids for the optimization of Transfections from the Promega PureYield™ Plasmid Midiprep System:

pLKO-TurboGFP-Puro, pCMV-ΔR8.2, pNL-4-3-KFS, pNL-deltapsi-SIVmac239env, pHCMVSV-G.

Restriction Enzymes from New England Biolabs:

EcoRI (R0101S), NdeI, XhoI (R0146S), PstI-HF (R3140S), HindIII-HF (R3104S)
New England Biolab Buffers:

Cutsmart Buffer (B7204S)

Procedure:

Digest the following plasmids with their corresponding enzymes and buffers for 1.5 hours then run on a 1% Agarose gel for 1 hour at 100V

Table 1.1: Plasmid Digestion Layout

Plasmid	Enzyme 1	Enzyme 2	Buffer
pLKO-TurboGFP-Puro	XhoI	PstI-HF	Cutsmart
pCMV-ΔR8.2	NdeI	None	Cutsmart
pNL-4-3-KFS	HindIII-HF	None	Cutsmart
pNL-deltapsi-SIVmac239env	EcoRI	None	Cutsmart
pHCMVSV-G	EcoRI	None	Cutsmart

Experiment 1.2: Calcium Phosphate Transfection Initial Optimization and Lipofectamine™ 2000 Control

Purpose: To test various incubation conditions for CaPO₄ transfections

Materials:

- HEK 293T cell line (2014 ATCC--Liquid Nitrogen Tank2 Rack 3 bottom)
- Hepes Buffer (500mL):

8g NaCl, 0.103g Na₂HPO₄·7H₂O (0.14g if dihydrate; Phosphate must be 1.5mM), 5g HEPES, 0.37g KCl and 1g Dextrose. Bring the pH to 7.0 and bring the

volume to 500mL with distilled water. Re-pH at this point because it can change. Filter and aliquot 525uL into individual 1.7mL Eppendorf tubes. Store at -20°C

- Transfection buffer (50mL):

0.0061g (1mM) Tris, 2.5 uL from a 0.5M stock(0.025mM) EDTA, 50mL

Distilled water, Filter and store at 4°C

- 2M CaCl₂ (50mL)

14.7g CaCl₂-2H₂O and 0.119g HEPES in a final volume of 50mL

distilled water. Filter and store at 4°C

- Micro-centrifuge tubes
- CytoOne 10cm Cell Culture Plates
- 100% Sterile glycerol

Table 1.2: CaPO₄ Transfection Plasmid DNA Volumes:

Plasmids	Amount per well	Volume/well
pLKO-TurboGFP-puro (413ug/mL)	28ug	67.8uL

Procedure:

1. Grow HEK293T cells in DMEM+10% FBS to confluence in T75 flask. One day before infection, dislodge cells from one T75 flask and plate on a 6 well plate (3-5 x 10⁶ cells per plate). Grow at 37°C for 12 to 24 hours to 60-80% confluence.
2. 2-4 hours before transfection remove medium from each flask and replace with 9 mL fresh warm DMEM+10%FBS.
3. 35 minutes before the transfection mix 28ug of plasmid DNA with 62ul of the 2M CaCl₂+HEPES and enough transfection buffer so the total volume for the DNA and CaCl equals 500uL. In a separate tube on a rack put 500uL of HEPES buffer (at 50mM).
4. Bubble the HEPES buffer and at the same time add the CaCl₂+DNA solution dropwise into the HEPES buffer. Incubate at room temperature for 30 minutes.
5. Add 1mL of the DNA-CaPO₄ mixture to the dish dropwise trying to cover the most area (the media in the dish will change color) then rock the plate forwards and backwards gently (not swirling) and incubate at 37°C.
- 6A. 24 hours post transfection carefully remove the transfected medium from the dish and replace the medium with 10 ml of warm 10%FBS·DMEM Plus 100uL of Pen/Strep (antibiotic) medium per well (carefully to the side of the well).
- 6B. 5-8 hours post transfection carefully remove the media from the dish and add 10mL of DMEM serum free with 10% glycerol. Allow the glycerol medium to sit for 2 minutes then replace the medium with 10 ml of warm 10%FBS·DMEM Plus 100uL of Pen/Strep (antibiotic) medium per well (carefully to the side of the well).

6C. 5-8 hours post transfection carefully wash the plate with warm PBS 2-4 times (try to remove all the complexes). Then replace the medium with 10 ml of warm 10%FBS·DMEM Plus 100uL of Pen/Strep (antibiotic) medium per well (carefully to the side of the well).

7. Harvest 10mL supernatant 48 hours post transfection, pellet cells at 2000rpm for 10 minutes and aliquot into 0.5 – 1ml for storage at -80°C.

Lipofectamine™ 2000 Procedure for Control

Materials:

- HEK 293T cell line (2014 ATCC--Liquid Nitrogen Tank2 Rack 3 bottom)
- DMEM+10%FBS+NEAA and DMEM serum free
- Lipofectamine™ 2000 Cat. No. 11668-019 (1.5 mL) ThermoFisher
- CytoOne 10cm Cell Culture Plates
- Micro-centrifuge tubes

Table 1.3: Lipofectamine™ 2000 Transfection Plasmid DNA Volumes:

Plasmids	Amount (24ug) per 10cm Plate	Volume/Plate
pLKO-TurboGFP-puro (413ug/mL)	24ug	58.1uL

Procedure:

1. Grow Hek293 cells in DMEM+10% FBS+NEAA to confluence in T75 flask (3 x 10⁷ cells/per flask). One day before infection, dislodge cells from one T75 flask and plate into 10cm dish (3 ~ 5 x 10⁶ cells per dish).
2. Grow at 37°C for 12 to 24 hours to 80% confluence. Remove medium from each flask and replace with 5ml of warm, serum free medium each dish.
3. In one tube, mix 24ug of plasmid DNA (**58.1uLpKLO-TurboGFP-puro**) with 1.5ml of DMEM serum free medium per dish. In a separate tube mix 60ul of LipofectamineTM 2000 another tube, mix 60ul of Lipofectamine-2000 with 1.5ml DMEM per dish. Mix the DNA and LipofectamineTM 2000 solutions and let them incubate at room temperature for 10mins.
4. Add the DNA mixture to the dish dropwise covering the most area and incubate at 37°C for 5 to 6 hours.
5. Remove the transfection supernatant (carefully with a pipet), add 10ml of warm 10%FBS+NEAA·MDEM Plus 100uL of Pen/Strep (antibiotic) medium per dish (carefully to the side of the dish).
6. 48 hours post transfection harvest 10mL supernatant and pellet cells at 2000 RPM for 10 minutes and aliquot the supernatant in .5mL volumes. Pipet the cells vigorously from the plate and run FACS on the GFP signal.

Experiment 1.3: PEI Transfection Procedure Initial Optimization

Purpose: To find the optimal concentration range of PEI transfection reagent

Materials:

- HEK 293T cell line (2014 ATCC--Liquid Nitrogen Tank2 Rack 3 bottom)
- PEI solutions made from Stock kept at 20mg/mL in 4degree fridge will stay effective for at least a year in DMEM serum free
- DMEM+10%FBS+NEAA and DMEM serum free
- Corning 6 well plate
- Micro-centrifuge tubes
- pKLKO-TurboGFP-puro - from plasmid midi prep

Procedure for making PEI diluted stocks:

1. Make PEI Solutions from Stock (20mg/mL) by first making a 2mg/mL solution by adding 1mL to 9mL of DMEM serum free. Then perform a 1/2 serial dilution from that working solution 5mL into 5mL of serum free medium then repeat

Table 1.4: PEI Transfection Reagents Concentrations:

Tubes	PEI Concentration	Transfer volume in serial dilution
1	2mg/mL	Made from stock 1000uL (in 9mL)
2	1mg/mL	5mL of the 2mg/mL solution into 5mL DMEM serum free
3	0.5mg/mL	5mL of the 1mg/mL solution into 5mL DMEM serum free
4	0.25mg/mL	5mL of the 0.5mg/mL solution into 5mL DMEM serum free
5	0.125mg/mL	5mL of the 0.25mg/mL solution into 5mL DMEM serum free

Table 1.5: PEI Transfection Plasmid Volume:

Plasmids	Amount per well	Volume/well
pLKO-TurboGFP-puro (413ug/mL)	3ug	7.2uL

2. Grow HEK 293T cells in DMEM+10% FBS to confluence in T75 flask (3×10^7 cells/per flask). One day before infection, dislodge cells from one T75 flask and plate into 6 well dish ($3 \sim 5 \times 10^5$ cells per well).
3. Grow at 37°C for 12 to 24 hours to 80% confluence per well. Remove medium from each flask and replace with 1.9ml of warm, serum free medium each well.
4. In separate tubes for each well, mix 3ug (7.2uL) of plasmid DNA with 92.8uL of DMEM serum free medium to equal 100uL per well. In separate tubes mix 18ul of each PEI solution described above with 82uL of serum free medium. Mix the DNA and PEI solutions and let them incubate at room temperature for 15mins.

Table 1.6: PEI Transfection Tube Layout:

Transfection Tubes	Name	DNA Tubes	PEI Tubes
A	2mg/mL	7.2uL of DNA into 92.8uL of DMEM serum free	18ul of tube #1 in 82uL of DMEM serum free
B	1mg/mL	7.2uL of DNA into 92.8uL of DMEM serum free	18ul of tube #2 in 82uL of DMEM serum free
C	0.5mg/mL	7.2uL of DNA into 92.8uL of DMEM serum free	18ul of tube #3 in 82uL of DMEM serum free
D	0.25mg/mL	7.2uL of DNA into 92.8uL of DMEM serum free	18ul of tube #4 in 82uL of DMEM serum free
E	0.125mg/mL	7.2uL of DNA into 92.8uL of DMEM serum free	18ul of tube #5 in 82uL of DMEM serum free

4. Add the DNA mixture to the dish drop wise covering the most area and incubate at 37°C for 24 hours.
5. Remove the transfection supernatant (carefully with a pipet), add 2ml of warm 10%FBS·MDEM Plus 100uL of Pen/Strep (antibiotic) medium per well (carefully to the side of the dish).
6. 48 hours post transfection, vigorously pipette cells from the wells and run FACS to measure GFP signal.

Experiment 1.4: Transfection Procedures After Initial Optimization For Comparison

Purpose: To compare the efficiency of three transfection methods

Calcium Phosphate Procedure after Initial Optimization

Materials:

- HEK 293T cell line (2014 ATCC--Liquid Nitrogen Tank2 Rack 3 bottom)
- Hepes Buffer (500mL):

8g NaCl, 0.103g Na₂HPO₄-7H₂O (0.14g if dihydrate; Phosphate must be 1.5mM), 5g HEPES, 0.37g KCl and 1g Dextrose. Bring the pH to 7.0 and bring the volume to 500mL with distilled water. Re-pH at this point because it can change. Filter and aliquot 525uL into individual 1.7mL Eppendorf tubes. Store at -20°C

- Transfection buffer (50mL):

0.0061g (1mM) Tris, 2.5 uL from a 0.5M stock(0.025mM) EDTA, 50mL

Distilled water , Filter and store at 4°C

- 2M CaCl₂ (50mL)

14.7g CaCl₂-2H₂O and 0.119g HEPES in a final volume of 50mL

distilled water. Filter and store at 4°C

- DMEM+10%FBS+NEAA and DMEM serum free
- Micro-centrifuge tubes
- CytoOne 10cm Cell Culture Plates

Table 1.7: CaPO4 Transfection Plasmid Volumes:

Plasmids	Amount (28ug) per 10cm Plate	Volume/Plate
pCMVdeltaR8.2 (203.9 ug/mL)	10.5ug	51.5
pLKO-TurboGFP-puro (142.5ug/mL)	14ug	98.2
pHCMVSV-G (514.6ug/mL)	3.5ug	6.8

Protocol:

1. Grow Hek293Tcells in DMEM+10% FBS to confluence in T75 flask. One day before infection, dislodge cells from one T75 flask and plate 10cm (3-5 x 10⁶ cells per well).
2. Grow at 37°C for 12 to 24 hours to 60%-80% confluence.
3. Two hours before transfection remove medium from each flask and replace with 9 mL fresh warm DMEM+10%FBS+NEAA.
4. 35 minutes before the transfection mix 28 ug of plasmid DNA (469.5uL) with 62ul of the 2M CaCl₂+HEPES and enough transfection buffer (**968.5mL**) so the total volume for the DNA and CaCl equals 1.5mL. In a separate tube on a rack put 1.5mL of HEPES buffer (at 50mM).
5. Bubble the HEPES buffer and at the same time add the CaCl₂+DNA solution dropwise into the HEPES buffer. Incubate at room temperature for 30 minutes.

6. Add 1mL of the DNA-CaPO₄ mixture to each dish dropwise trying to cover the most area (the media in the dish will change color) then rock the plate forwards and backwards gently (not swirling) and incubate at 37°C:
7. 24 hours post transfection carefully replace the transfection medium with 10mL of warm 10%FBS+NEAA·DMEM Plus 100uL of Pen/Strep.
8. Harvest 10mL supernatant 48 hours post transfection, pellet cells at 2000rpm for 10 minutes. Aliquot into 0.5mL for storage at -80°C. Pipet the cells vigorously from the plate and run FACS on the GFP signal.

Lipofectamine™ 2000 Procedure for Control

Materials:

- HEK 293T cell line (2014 ATCC--Liquid Nitrogen Tank2 Rack 3 bottom)
- DMEM+10%FBS+NEAA and DMEM serum free
- Lipofectamine™ 2000 Cat. No. 11668-019 (1.5 mL) ThermoFisher
- CytoOne 10cm Cell Culture Plates
- Micro-centrifuge tubes

Table 1.8: Lipofectamine™ 2000 Transfection Plasmid DNA Volumes:

Plasmids	Amount (24ug) per 10cm Plate	Volume/Plate
pCMVdeltaR8.2 (203.9 ug/mL)	9ug	44.1uL
pLKO-TurboGFP-puro (142.5ug/mL)	12ug	84.2uL
pHCMVSV-G (514.6ug/mL)	3ug	5.8uL

Procedure:

1. Grow Hek293 cells in DMEM+10% FBS+NEAA to confluence in T75 flask (3 x 10⁷ cells/per flask). One day before infection, dislodge cells from one T75 flask and plate into 10cm dish (3 ~ 5 x 10⁶ cells per dish).
2. Grow at 37°C for 12 to 24 hours to 80% confluence. Remove medium from each flask and replace with 5ml of warm, serum free medium each dish.
3. In one tube, mix 24ug of plasmid DNA (**44.1uL** of pCMVdeltaR8.2, **84.2uL** of pLKO-TurboGFP-puro, and **5.8uL** of pHCMVSV-G) with 1.5ml of DMEM serum free medium per dish. In a separate tube mix 60ul of Lipofectamine™ 2000 another tube, mix 60ul of Lipofectamine™ 2000 with 1.5ml DMEM per dish. Mix the DNA and Lipofectamine™ 2000 solutions and let them incubate at room temperature for 10mins.
4. Add the DNA mixture to the dish dropwise covering the most area and incubate at 37°C for 5 to 6 hours.

5. Remove the transfection supernatant (carefully with a pipet), add 10ml of warm 10%FBS+NEAA+DMEM Plus 100uL of Pen/Strep (antibiotic) medium per dish (carefully to the side of the dish).
6. 48 hours post transfection harvest 10mL supernatant and pellet cells at 2000 RPM for 10 minutes and aliquot the supernatant in .5mL volumes. Pipet the cells vigorously from the plate and run FACS on the GFP signal.

PEI Transfection After Initial Optimization Procedure

Materials:

- HEK 293T cell line (2014 ATCC--Liquid Nitrogen Tank2 Rack 3 bottom)
- PEI solutions made from Stock kept at 20mg/mL in 4degree fridge will stay effective for at least a year in DMEM serum free
- DMEM+10%FBS+NEAA and DMEM serum free
- CytoOne 10cm Cell Culture Plates
- Micro-centrifuge tubes

Table 1.9: PEI Transfection Plasmid DNA Volumes:

Plasmids	Amount (10ug) per 10cm Plate	Volume/Plate	Volume for 3 Plates
pCMVdeltaR8.2 (505.4 ug/mL)	3.75ug	7.4uL	22.2uL
pTurboGFP (543.1ug/mL)	5ug	9.2uL	27.6uL
pHCMVSV-G (712.7ug/mL)	1.25ug	1.8uL	5.4uL
DMEM Serum Free	500uL total /Plate	481.6uL	1.445mL

Protocol:

1. Grow 293 cells in DMEM+10% FBS+NEAA to confluence in T75 flask (3 x 10⁷ cells/per flask). One day before infection, dislodge cells from one T75 flask and plate into 10cm dish (3 ~ 5 x 10⁶ cells per dish).

(With a 100% confluence T75 flask, which can seed five 10cm-dishes.)

2. Grow at 37°C for 12 to 24 hours to 80% confluence. Remove medium from each flask and rinse with serum free medium, then add 9ml of warm, serum free medium each dish.

3a. In one tube, mix 10ug of plasmid DNA (**7.4uL** of pCMVdeltaR8.2, **9.2uL** of pLKO-TurboGFP-puro, and **1.8uL** of pHCMVSV-G) with **0.482ml** of DMEM serum free medium per dish. In a separate tube mix **45ul** of 1mg/mL PEI transfection solution with

0.455mL of serum free medium. Mix the DNA and PEI solutions and let them incubate at room temperature for 15mins.

3b. In one tube, mix 10ug of plasmid DNA (**7.4uL** of pCMVdeltaR8.2, **9.2uL** of pLKO-TurboGFP-puro, and **1.8uL** of pHCMVSV-G) with **0.482ml** of DMEM serum free medium per dish. In a separate tube mix **45ul** of 0.5mg/mL PEI transfection solution with **0.455mL** of serum free medium. Mix the DNA and PEI solutions and let them incubate at room temperature for 15mins.

3c. In one tube, mix 10ug of plasmid DNA (**7.4uL** of pCMVdeltaR8.2, **9.2uL** of pLKO-TurboGFP-puro, and **1.8uL** of pHCMVSV-G) with **0.482ml** of DMEM serum free medium per dish. In a separate tube mix **45ul** of 0.25mg/mL PEI transfection solution with **0.455mL** of serum free medium. Mix the DNA and PEI solutions and let them incubate at room temperature for 15mins.

4. Add the DNA mixtures to the dishes dropwise covering the most area and incubate at 37°C for 24 hours.

5. Remove the transfection supernatant (carefully with a pipet), add 10ml of warm 10%FBS·MDEM Plus 100uL of Pen/Strep (antibiotic) medium per dish (carefully to the side of the dish).

6. 48 hours post transfection harvest 10mL supernatant and pellet cells at 2000 RPM for 10 minutes and aliquot the supernatant in .5mL volumes. Pipet the cells vigorously from the plate and run FACS on the GFP signal.

Experiment 1.5: Infection of CEMss Cells with the Particles Produced from Three Different Transfection Protocols

Purpose: To compare the particles produced from the three different transfection methods

Materials:

- CEMss cells from lab stock in the liquid nitrogen storage
- vVSVG-TurboGFP plasmid in Brian's box at -80°C
- RPMI complete medium (10% FBS, NEAA, gentamycin, and Sodium Pyruvate) stored at 4°C

Table 1.10: Infection of CEMss Cells Tubes Layout:

Infection Tube #	Virus Transfection Number	Volume of particle	Volume of cells
1	Lipo	~200uL	200uL
2	CaPO4 24 hour	~200uL	200uL
3	PEI .5mg/ml	~200uL	200uL
4	PEI 1mg/ml	~200uL	200uL
5	PEI .25mg/ml	~200uL	200uL

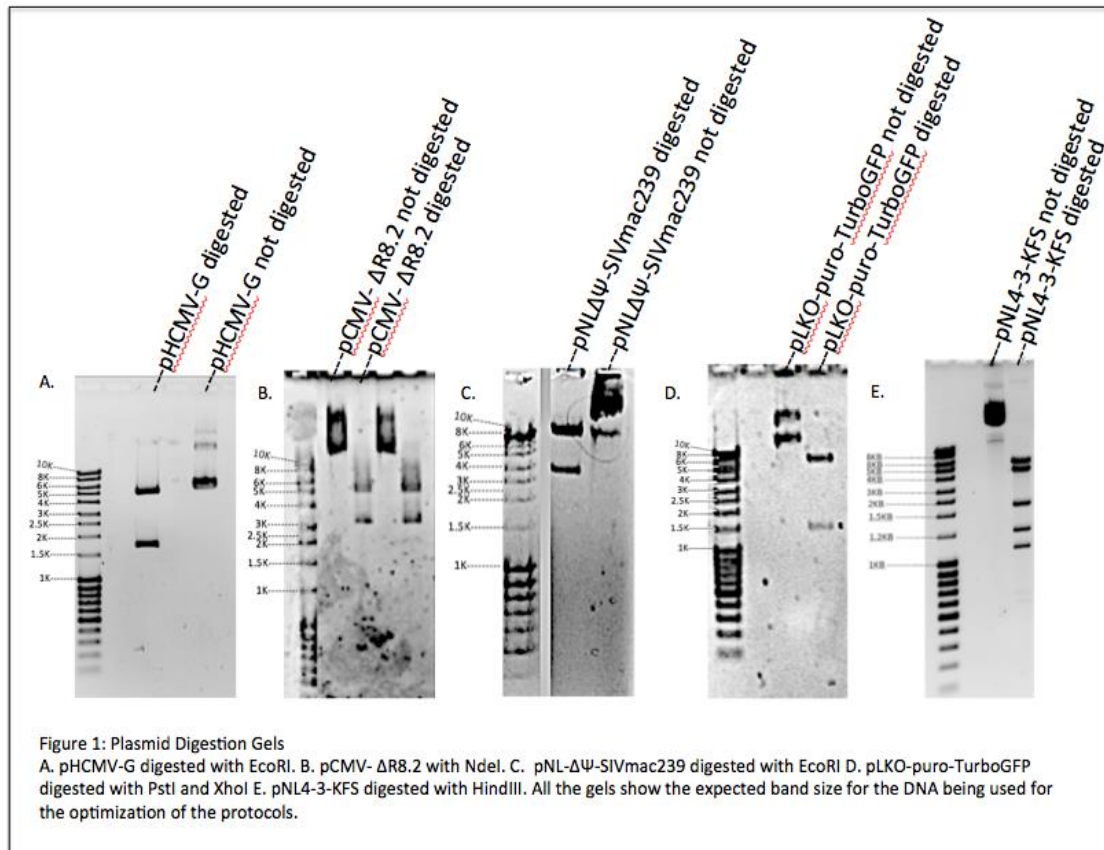
Procedure:

1. Grow CEMss cells in RPMI complete medium until confluent
2. Count CEMss (#/mL)
3. Aliquot of cells (1×10^6) and spin down cells at $350 \times g$ (1,200 RPM) Discard supernatant and re-suspend in 1mL RMPI+10%FBS
4. Transfer 2×10^5 cells in 200 uL into five 5 ml Flacon tubes labeled per chart
5. Infect each 5mL tube with 200uL indicted virus as indicated in the table above.
Tap the tubes vigorously for 30 seconds. Spin the cells for 2 hours at 1200rpm at 37°C then add 2 mL of fresh warm RPMI complete medium
6. Incubate the cells at 37°C overnight
7. Run FACS at 24 hours post infection to look for the GFP signal

Chapter 1 Results:**Experiment 1.1: Plasmid Digestion Results**

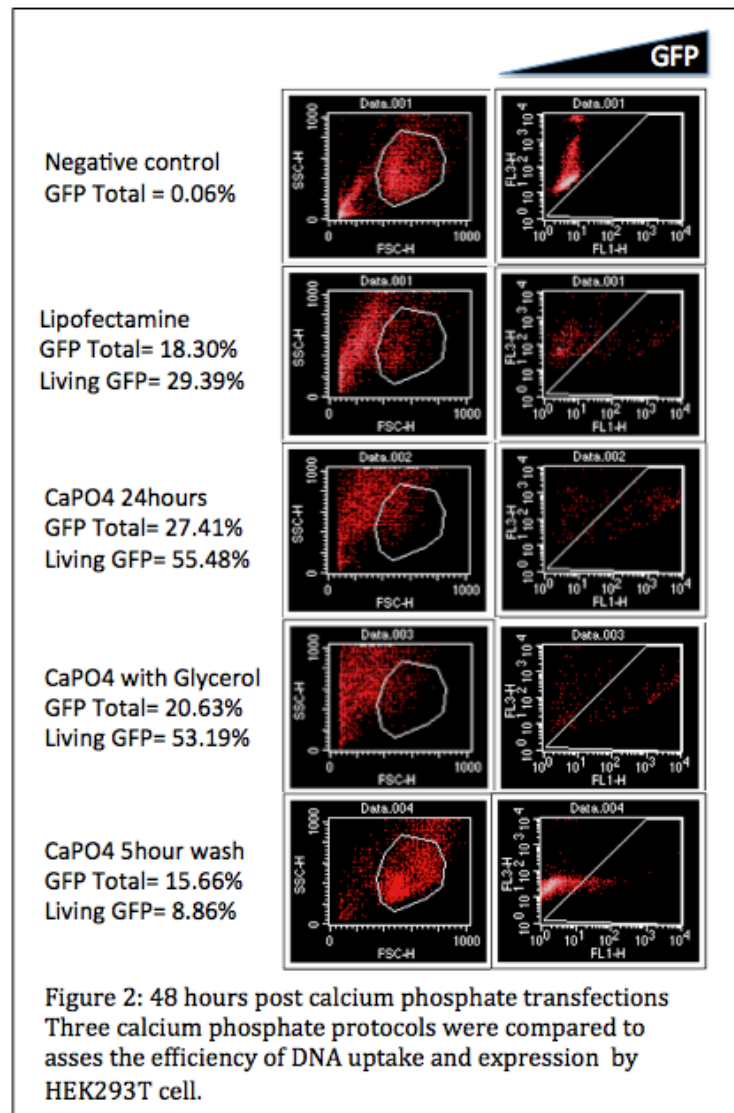
Figure 1 shows the gel electrophoresis plasmid digestions for the plasmids used in this study. For testing transfection methods pLKO-TurboGFP-Puro (8347bp), pCMV- Δ R8.2 (13463bp), pNL-4-3-KFS, pNL-deltapsi-SIVmac239env and pHCMVSV-G were used to make vVSV-G-TurboGFP, vMac239-TruboGFP and vHIV-KFS-mac239Env particles. Plasmids were purified according to the protocol outlined by the Promega PureYield™ Plasmid Midiprep System. Once purified the plasmids were then digested by

restriction enzyme EcoRI for pHCMVSV-G with two bands appearing in the gel electrophoresis around 5000 and 1600 (Figure 1A). Restriction enzymes XhoI and PstI were for pLKO-TurboGFP-puro with two bands appearing in the gel electrophoresis around 7000bp and 1500bp (Figure 1D) and NdeI was used to digest pCMV- Δ R8.2 with two bands around 5000bp and one around 3000bp (Figure 1B). The pNL-deltapsi-SIVmac239env was cut with EcoRI and has two fragments expected at 8KB and 2.8KB (Figure 1C). HindIII was used to cut pNL-4-3-KFS into five bands 5749bp, 4314bp, 2121bp, 1475bp, and 1181bp (Figure 1E). These gels confirm the plasmid identities.



Experiment 1.2: CaPO4 Transfection Incubation Conditions Results

In figure 2 the transfected TurboGFP signal was measured using flow cytometry. The first CaPO4 protocol was a 5-hour incubation with the transfection supernatant, then the cells were washed with warm PBS 2-3 times and the medium was replaced with fresh warm DMEM with 10% FBS. The second protocol required a 5-hour incubation and a glycerol shock treatment prior to changing the medium. The third was a 24-hour incubation with the transfection supernatant before changing the medium. The cells were removed from the plates and analyzed by FACS. The glycerol shock treatment had approximately 20% more total GFP signal versus the 5-hour incubation and 10% more than the 24-hour incubation. However all three had approximately the same GFP signal remaining in the living cells, around 53-54%. Figure 2 examines the three CaPO4 protocols and compares them to the lab standard LipofectamineTM 2000 control. The CaPO4 with glycerol and the 24-hour incubation both had GFP signals higher than the LipofectamineTM 2000 control. The 24-hour incubation had the greatest total GFP around 7% higher GFP than the glycerol treatment and 12% greater than the 5-hour plus wash. The 24-hour incubation was chosen as the standard protocol for the remaining CaPO4 tests due to glycerol having an unknown effect on the quality of viral particles produced. Further testing of the Glycerol procedure should be done.



Experiment 1.3: PEI Transfection Reagent Concentration Results

The GFP signal was measured 48 hours post transfection by FACS shown in figure 3. The 2mg/mL PEI concentration was too high and as a result the cell on the plate died prior to the 24 hour medium exchange. The 2mg/mL data could not be shown since no cells remained for collection after 48 hours. The GFP signal in the 0.5mg/mL and the 0.25mg/mL were within 4% of each other in both the living cells GFP signal and the total GFP signal. The 1mg/mL had lower than half of the total GFP compared to the 0.25mg/mL PEI and had 10% lower living cells with GFP signal than the 0.5mg/mL PEI. The 0.125mg/mL PEI had the lowest total and living cell GFP signal. This experiment was repeated three times. The optimal concentration range of PEI is between 1mg/mL and 0.25mg/mL.

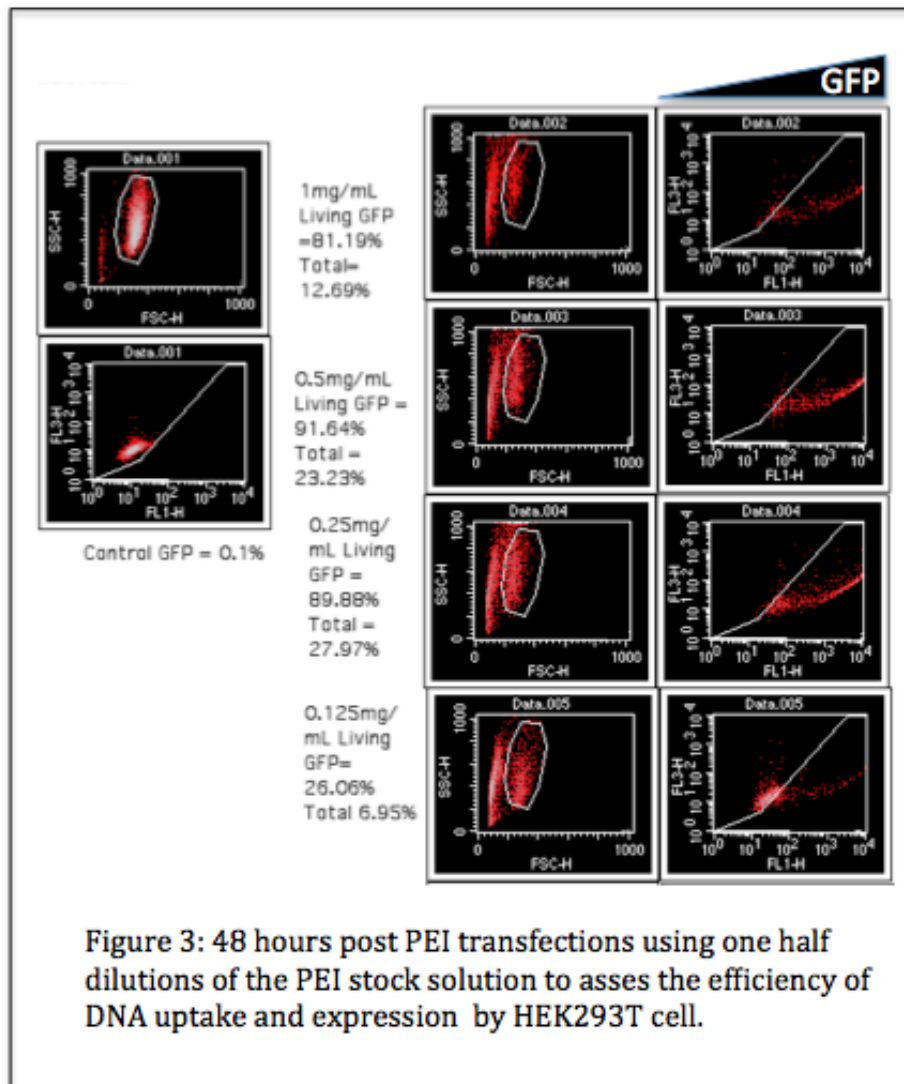
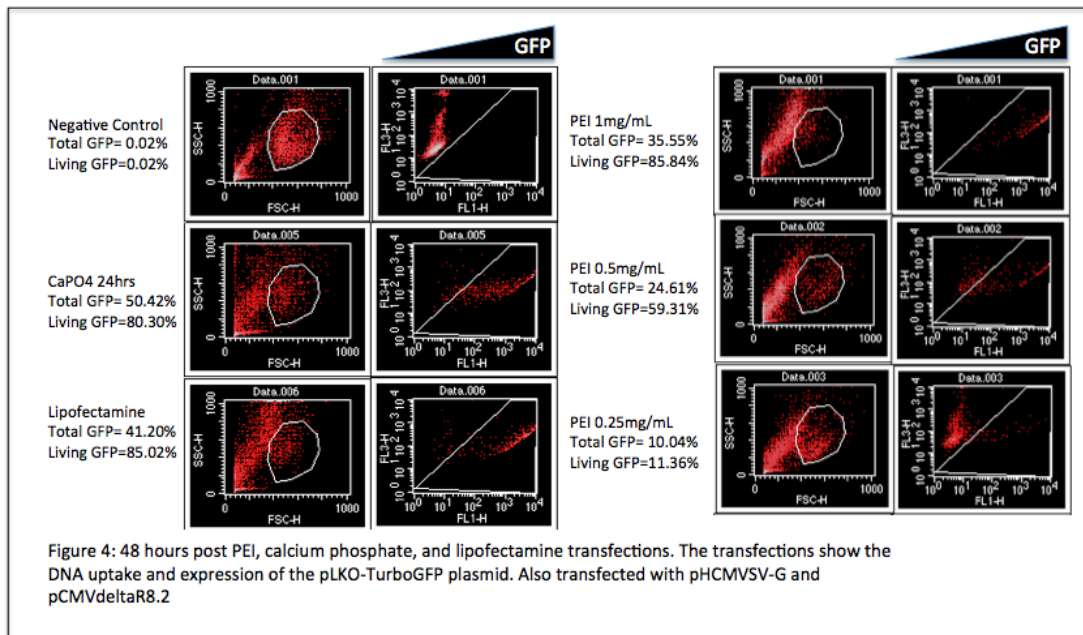


Figure 3: 48 hours post PEI transfections using one half dilutions of the PEI stock solution to asses the efficiency of DNA uptake and expression by HEK293T cell.

Experiment 1.4: Comparison of Three Transfection Efficiencies Results

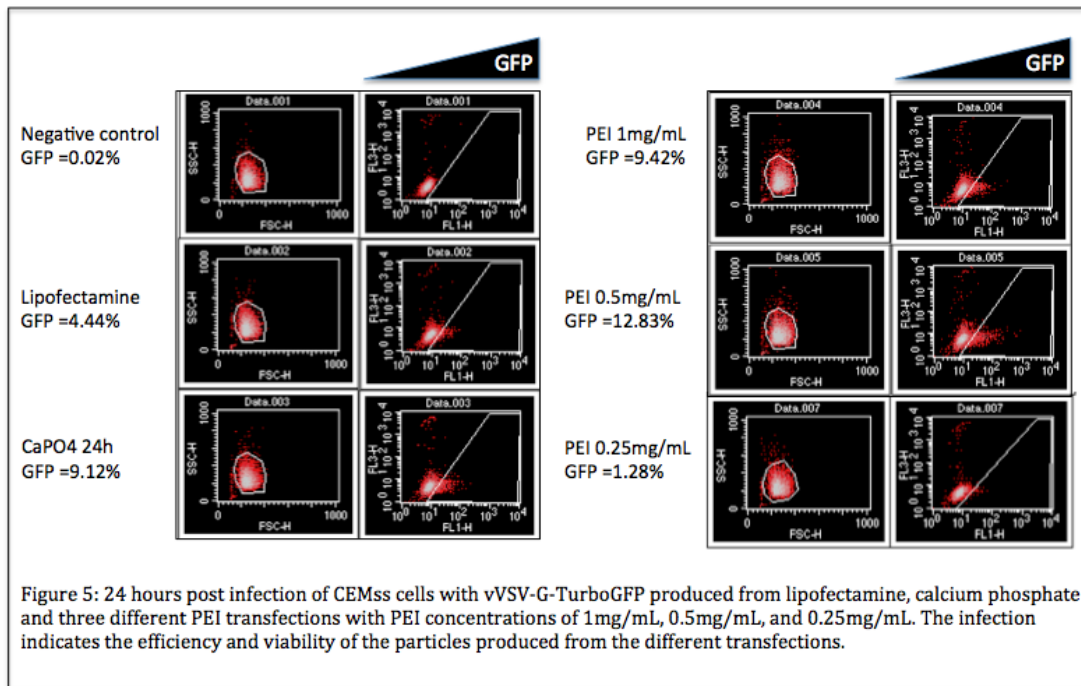
The transfection efficiencies of the calcium phosphate had the highest total GFP signal, around 10-15% higher than the other transfections as shown in figure 4. However the GFP signal was similar, within 5%, among the calcium phosphate, 1mg/mL PEI and Lipofectamine™ 2000 control group. The lowest total GFP signal was observed in the 0.25mg/mL PEI solution about 14% lower than the 0.5mg/mL PEI solution. Similar experiments were conducted comparing the PEI and CaPO₄ experiments and in all attempts both were as good or better than the Lipofectamine™ 2000 control. Although various plate sizes were used so no statistical calculations could be done.



Experiment 1.5: Infection of CEMss cells with Particles Produced From the Three Transfections Results

The infection of the CEMss cells with the vVSV-G-turboGFP particles produced from the previous transfection revealed similar results with the viral titer from the calcium phosphate transfection (9.12% total GFP) and 1mg/mL (9.42% total GFP) and 0.5mg/mL (12.83% total GFP) PEI transfections. All three transfection had higher titer particles than

the Lipofectamine™ 2000 control (4.44% total GFP). PEI at 0.25mg/mL had the lowest yield at 1.28% total presentation. The results of the infection show that the PEI transfections are capable of producing high titer particle with less DNA and cost. In similar experiments both PEI and CaPO4 transfections consistently produced particles at higher titers than the Lipofectamine™ 2000 control.



Chapter 2. PEI Final Optimization for 10cm dishes

Introduction:

Further optimization of the PEI transfection protocol could yield a better production of particle. The previous LipofectamineTM 2000 and CaPO₄ protocols demonstrated that different incubation times could influence particle production. Reed et al describes how the PEI-DNA complexes escape the endosome and enter the cytoplasmic within 3-4 hours post infection. Which means early expression of the plasmids could begin as soon as 6 hours post transfection. Additionally leaving the transfection medium on the cells too long can cause cells to die due to its cytotoxicity (Reed 2006). The transfection medium will need to be changed after the transfection, so optimizing the timing of the medium change can greatly affect the final viral titer. Three time points will be assessed to see if removing the transfection medium earlier greatly reduces or increase viral titers.

One of the reason transfections are cytotoxic is the plasmid DNA themselves in high enough volumes also can cause cell death. However, as previously shown the CaPO₄ and LipofectamineTM 2000 transfections cells are at least capable of handling up to 2.8ug/mL of plasmid DNA. If a cell was to receive a greater copy of plasmids during transfection there might be a greater production of viral particles produced. The plasmid DNA for the PEI transfections will be doubled to assess the cells ability to uptake and

produce the viral particles.

Chapter 2 Materials and Methods:

Experiment 2.1: PEI Transfection Procedure Altering Incubation Times of Transfection Reagent

Purpose: To test at what incubation time is it best to remove the PEI reagent-containing medium from the cells

Materials:

- HEK 293T cell line (2014 ATCC--Liquid Nitrogen Tank2 Rack 3 bottom)
- PEI solutions made from Stock kept at 20mg/mL in 4degree fridge will stay effective for at least a year in DMEM serum free
- CytoOne 10cm Cell Culture Plates
- Corning 6 well plate
- Gibco TrypLE Express
- DMEM+10%FBS+NEAA and DMEM serum free
- Micro-centrifuge tubes

Table 2.1: PEI Transfection Plasmids DNA Volumes:

Plasmids	Amount (ug) per 10cm Plate	V. /Plate (uL)	Volume for 3 plates(uL)
pCMVdeltaR8.2 (428.5ug/mL)	3.375ug	7.8uL	23.4uL
pLKO- TurboGFP- puro(90.8ug/mL)	4.5 ug	49.5uL	148.5uL
pHCMVSV-G (937.1ug/mL)	1.125ug	1.2uL	3.6uL
DMEM Serum FREE Medium	500uL total per plate	441.5uL	1.325mL

Table 2.2: PEI Transfection Reagent Medium:

Transfection Medium	Volume of PEI (0.5mg/mL)	Volume DMEM Serum FREE Medium
1 plate	45uL	455uL
3 plates	135uL	1.365mL

Procedure:

1. Grow Hek293T cells in DMEM+10% FBS to confluence in T75 flask (3×10^7 cells/per flask). One day before transfection, dislodge cells from one T75 flask and plate into 10cm dish ($3 \sim 5 \times 10^6$ cells per dish) (*With a 100% confluence T75 flask, which can seed five 10cm-dishes.*)

2. Grow at 37°C for 12 to 24 hours to 80% confluence. Remove medium from each flask and rinse with serum free medium, then add 9ml of warm, serum free medium each dish.
3. In one tube, mix 10ug/plate of plasmids. **23.4uL** of pCMVdeltaR8.2, **148.5uL** of pLKO-TurboGFP-puro, and **3.6uL** of pHCMVSV-G plasmid DNA with **1.325mL** of DMEM serum free medium. In a separate tube mix **135ul** (45uL/plate) of PEI .5ug/mL with **1.365mL** of DMEM serum free medium. Mix and vortex the DNA and PEI solutions and let them incubate at room temperature for 15mins. (Refer to the table above for volumes of DNA)
4. Label three 10cm plates A B and C. Add the DNA+PEI mixture to each dish dropwise covering the most area rock the plate to ensure the plate is completely covered.
5.
 - a. Incubate plate A. at 37°C for 6 hours. Remove the transfection supernatant (carefully with a pipet), add 10ml of warm 10%FBS·MDEM Plus 100uL of Pen/Strep (antibiotic) medium per dish (carefully to the side of the dish).
 - b. Incubate plate B. at 37°C for 12 hours. Remove the transfection supernatant (carefully with a pipet), add 10ml of warm 10%FBS·MDEM Plus 100uL of Pen/Strep (antibiotic) medium per dish (carefully to the side of the dish).
 - c. Incubate plate C. at 37°C for 24 hours. Remove the transfection supernatant (carefully with a pipet), add 10ml of warm 10%FBS·MDEM Plus 100uL of Pen/Strep (antibiotic) medium per dish (carefully to the side of the dish).

6. Harvest 10mL supernatants on ice 48 hours post transfection. Spin the supernatants down at 1200 rpm for 10 minutes. Collect the cell cleared supernatants in fresh tubes on ice. Filter the supernatant with the .45mm syringe filter. Aliquot out 1mL of each plates harvested supernatant and label each cryotube as VSV-G-TurboGFP A, VSV-G-TurboGFP B, VSV-G-TurboGFP C and store in the VSV-G-turboGFP box at -80 degrees for later use.

Infection of HEK293T cells

Materials:

- HEK 293T cell line (2014 ATCC--Liquid Nitrogen Tank2 Rack 3 bottom)
- vVSVG-turboGFP from previous procedure
- 5mL conical tubes
- 6 well plate
- Cryotubes
- Pippets
- Pen/Step 100X Stock
- DMEM with 10%FBS+NEAA(phenol red free)

Table 2.3: Infection Well Layout for HEK293T Cells:

Well #	Content
1	3X10 ⁵ HEK293T cells + 1.5mL of DMEMw10%FBS + 500uL VSVG TurboGFP A
2	3X10 ⁵ HEK293T cells + 1.5mL of DMEMw10%FBS + 500uL VSVG TurboGFP B
3	3X10 ⁵ HEK293T cells + 1.5mL of DMEMw10%FBS + 500uL VSVG TurboGFP C

Procedure:

1. Grow Hek293T cells in DMEM+10% FBS +NEAA to confluence in T75 flask (3 x 10⁷ cells/per flask). One the day of the infection, dislodge cells from one T75 flask and aliquot 3x10⁵ cells into six wells of a six well plate. Each well should contain 2mL of DMEM+10%FBS+NEAA (spin down the cells to increase their concentration if necessary)
2. 24 hours after plating replace the medium in each well with 1.5mL of DMEM+10% FBS +NEAA+Pen/strep add 500uL of the three harvested VSV-G-TurboGFP viruses to each well indicated in the table above (be sure to cover the most area as possible in each well with the indicated virus.

3. Incubate the plate for 48 hours at 37°C check the GFP signal with FACS by removing the infection supernatant. Add 0.5mL of trypsin to each well and incubate for 3 minutes. Neutralize trypsin with 0.5mL of DMEM+10%FBS and vigorously pipet to remove cells from the well. Transfer 0.5mL of each well to a labeled Flow Cytometer tube and measure the GFP signal with the Flow cytometer.

Experiment 2.2: PEI Transfection Procedure With DNA Totals Doubled and Infection of Hek293T Cells

Purpose: To test if doubling DNA has any effect on the viral titer produced from the transfections

Materials:

- HEK 293T cell line (2014 ATCC--Liquid Nitrogen Tank2 Rack 3 bottom)
- 5mL conical tubes
- 10cm CytoOne dishes
- Cryotubes
- Pippets
- Pen/Step 100X Stock
- DMEM Serum Free and with 10%FBS+NEAA(phenol red free)
- PEI Working stock kept at 0.5mg/mL and 1mg/mL at 4 degrees PEI solutions made from Stock kept at 20mg/mL in 4degree fridge will stay effective for at least a year in DMEM serum free

Table 2.4: PEI Transfection Plasmids DNA Volumes:

Plasmids	Amount (10ug) per 10cm Plate	Amount (20ug) per 10cm plate	Volume for 2 (10ug) plates	Volume for 2 (20ug) plates
pCMVdeltaR8.2 (428.5ug/mL)	3.75ug (16.6uL)	7.5ug (33.2uL)	33.2uL	66.4uL
pLKO- TurboGFP- puro(90.8ug/mL)	5ug (55.1uL)	10ug (110.2uL)	110.2uL	220.4uL
pHCMVSV-G (937.1ug/mL)	1.25ug (1.33uL)	2.5uL (2.66uL)	2.66uL	5.32uL
DMEM Serum FREE Medium	500uL total per plate	500uL total per plate	853.94uL	707.88uL

Table 2.5: PEI Transfection Reagent Medium:

Transfection Medium	Volume PEI	Volume DMEM Serum FREE Medium
2 plates (0.5mg/mL)	90uL	910uL
2 plates (1mg/mL)	90uL	910uL

Procedure:

1. Grow 293 cells in DMEM+10% FBS to confluence in T75 flask (3×10^7 cells/per flask). One day before transfection, dislodge cells from one T75 flask and plate into 10cm dish ($3 \sim 5 \times 10^6$ cells per dish). (*With a 100% confluence T75 flask, which can seed five 10cm-dishes.*)
2. Grow at 37°C for 12 to 24 hours to 80% confluence. Remove medium from each flask and rinse with serum free medium, then add 9ml of warm, serum free medium each dish.
3. In one tube, mix 10ug/plate of plasmids according to the table above.
4. In another tube mix 20ug/plate of plasmids according to the table above.
5. Prepare two PEI solutions according to the transfection reagent table above.
6. Prepare 4 tubes by mixing and vortexing DNA and PEI solutions according to the table below:

Table 2.6: PEI Transfection Tube Layout:

	Tube A	Tube B	Tube C	Tube D
Transfection Reagent	500uL 0.5mg/mL	500uL 0.5mg/mL	500uL 1mg/mL	500uL 1mg/mL
DNA	500uL 10ug of DNA	500uL 20ug of DNA	500uL 10ug of DNA	500uL 20ug of DNA

Let them incubate at room temperature for 15mins. (Refer to the table above for volumes of DNA)

7. Label 4 plates A B C and D. Add the DNA mixture to each dish dropwise covering the most area rock the plate to ensure the plate is completely covered.

8. Incubate the plates at 37°C for 6 hours. Remove the transfection supernatant (carefully with a pipet), add 10ml of warm 10%FBS·MDEM Plus 100uL of Pen/Strep (antibiotic) medium per dish (carefully to the side of the dish)

9. Harvest 10mL supernatant on ice 48 hours post transfection. Spin the supernatants down at 1200 rpm for 10 minutes. Collect the cell cleared supernatants in fresh tubes on ice. Filter the supernatant with the .45mm syringe filter. Aliquot out 1mL of each plates harvested supernatant and label each cryotube as VSV-G-TurboGFP A, VSV-G-TurboGFP B, VSV-G-TurboGFP C and VSV-G-TurboGFP D with the date. Store in the VSV-G-turboGFP box at -80 degrees for later use.

Infection of Hek293T cells

Materials:

- HEK 293T cell line (2014 ATCC--Liquid Nitrogen Tank2 Rack 3 bottom)
- vVSVG-turboGFP from previous procedure
- 5mL conical tubes
- 6 well plate
- Cryotubes
- Pippets

- Pen/Step 100X Stock
- DMEM with 10%FBS+NEAA(phenol red free)

Table 2.7: Infection Well Layout for HEK293T Cells:

Well #	Content
1	3X10 ⁵ HEK293T cells + 1.5mL of DMEMw10%FBS + 500uL VSVG TurboGFP A
2	3X10 ⁵ HEK293T cells + 1.5mL of DMEMw10%FBS + 500uL VSVG TurboGFP B
3	3X10 ⁵ HEK293T cells + 1.5mL of DMEMw10%FBS + 500uL VSVG TurboGFP C
4	3X10 ⁵ HEK293T cells + 1.5mL of DMEMw10%FBS + 500uL VSVG TurboGFP D

Procedure:

1. Grow Hek293T cells in DMEM+10% FBS +NEAA to confluence in T75 flask (3 x 10⁷ cells/per flask). One the day of the infection, dislodge cells from one T75 flask and aliquot 3x10⁵ cells into six wells of a six well plate. Each well should

contain 2mL of DMEM+10%FBS+NEAA (spin down the cells to increase their concentration if necessary)

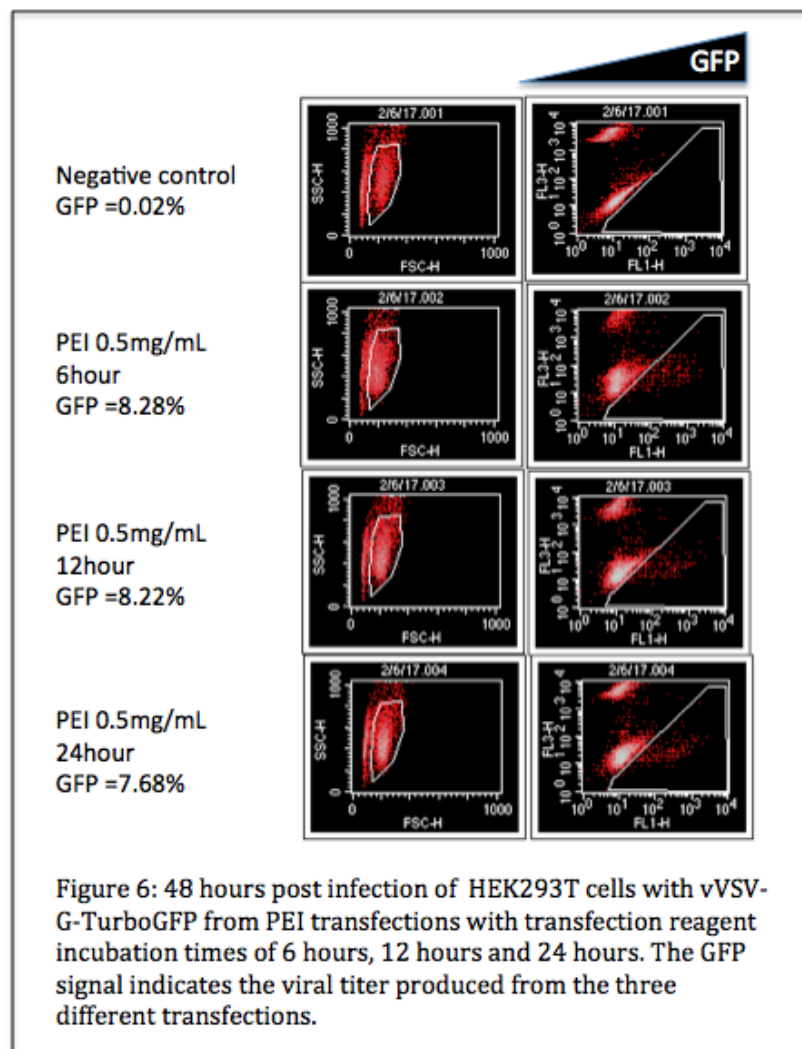
2. 24 hours after plating replace the medium in each well with 1.5mL of DMEM+10% FBS +NEAA+Pen/strep add 500uL of the six harvested VSV-G-TurboGFP viruses to each well indicated in the table above (be sure to cover the most area as possible in each well with the indicated virus.
3. Incubate the plate for 48 hours at 37°C check the GFP signal with FACS by removing the infection supernatant. Add 0.5mL of trypsin to each well and incubate for 3 minutes. Neutralize trypsin with 0.5mL of DMEM+10%FBS and vigorously pipet to remove cells from the well. Transfer 0.5mL of each well to a labeled Flow Cytometer tube and measure the GFP signal with the Flow cytometer.

Chapter 2 Results:

Experiment 2.1: Infection Results of Particles Produced From PEI Transfections with Various Incubation Times

The infection of the HEK293T cells with the vVSV-G-TurboGFP from the PEI transfections with varying incubation times revealed that viral production begins before the 24 hours of incubation. The GFP totals were similar in the 6 hour and 12 hour incubations at 8.28% total GFP and 8.22% total GFP respectively. The 24 hour incubation showed a slightly lower titer of particle at 7.68% total GFP. Other similar test show there is very little difference between 6 and 24 hours. Significance cannot be proven. The results of this infection indicate the best time to change out the transfection

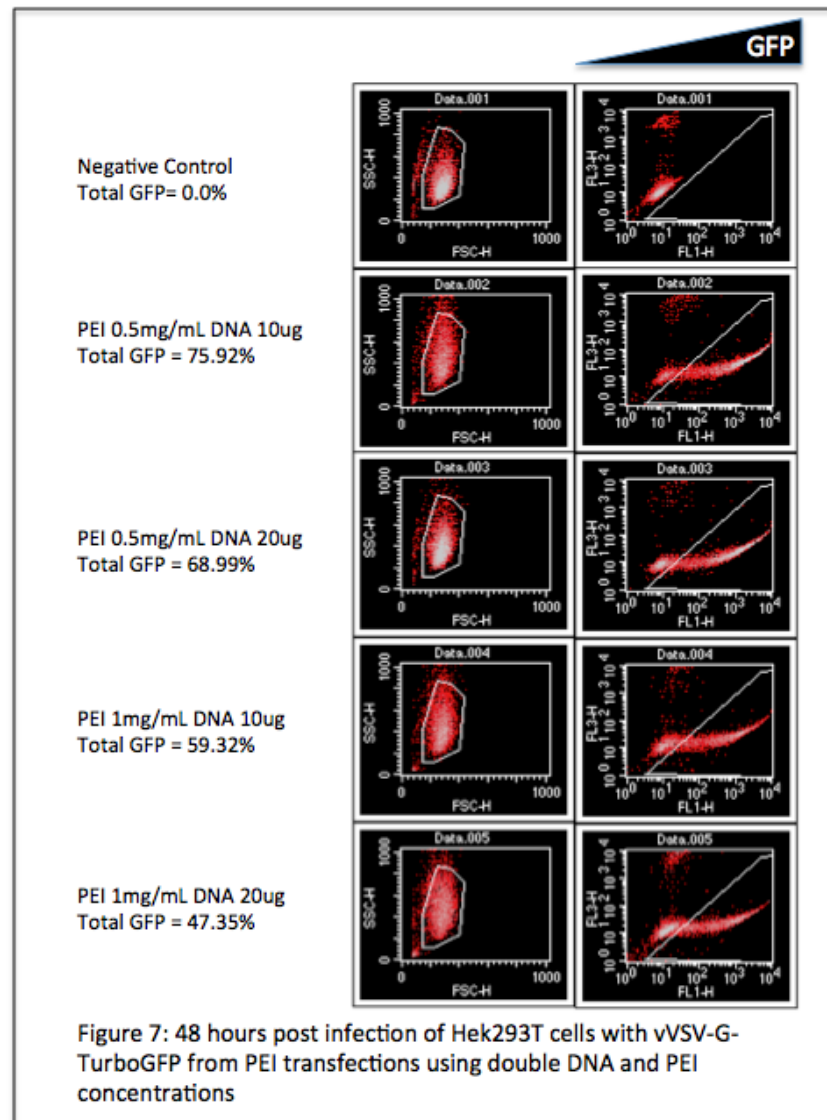
reagent medium from PEI transfections is at 6 hours, this will be sure to prevent any removal of particles produced early after transfection.



Experiment 2.2: Infection Results of Particles Produced From PEI Transfections

Doubling the amount of DNA

The infection of the HEK293T cells indicated that the best combination of PEI and DNA was using the 45ul of 0.5mg/mL PEI and 10ug of plasmid DNA per 10cm dish. This combination had 7% higher than the 20ug of DNA using the same PEI. This indicated that higher levels of DNA may be cytotoxic to the cells and might prevent some viral production. The other combinations of PEI and DNA had smaller titers with the lowest being 45uL of the 1mg/mL PEI solution with 20ug of DNA at 47.35%. To reduce cost the PEI of 0.5mg/mL with 10ug of DNA was chosen. With the best PEI and DNA combination revealed, the titer and purity of the particles being produced needed to be improved for animal trials.



Chapter 3. Viral Titer Enhancement through Anion Exchange and Size exclusion

Introduction:

In order to perform viral vector gene therapy animal trials, high titers of viral particles are needed and at high purity. To enhance viral titer and purity anion exchange column will be tested to demonstrate its ability to concentrate lentiviral particles with a negatively charged SIV envelope. Viral particles that have been purified and concentrated with strong anion exchange columns produce a greater titer of viral particles and can enhance gene transfer (Yamada 2003). The Pierce Strong Anion Ion Exchange Spin Columns work by using a stabilized regenerated cellulose-based membrane with pores around 3,000 nm in size. The viral particles membranes are negatively charged and are capable of binding to the positively charged membranes. To elute particles and proteins from the membrane a salt solution can be added which alters the charge around the particles and they will be released from the membranes. The exact concentration of salt for the dilution will need to be uncovered in order to produce highly purified viral particles. The particles will be vHIV-KFS-SIVmac239ENV, a HIVpseudo-typed virus with the envelope of wild type SIV239 and they will be used to infect the A3R5.7+TurboGFP-RRE (Clone8) cell line created by Todd Hawley in the Wu lab. Clone8 cells are a cell line derived from A3R5.7 cells, a human T-lymphoblastoid cell line that carries the CCR5 receptor, cloned with a REV Response Element (RRE) and a

GFP gene. In the presence of HIV rev the cells will emit a GFP signal. High salt concentrations in solution would be highly cytotoxic to these cells so after the elution of the particles from the columns the viral particles will have to be diluted to PBS levels of .13M before they can be used in culture or injections.

The columns should be capable of binding up to 60-80 mg of protein sample. To identify how much viral particle can bind to anion exchange column viral particles that are able to pass through the column without binding will be collected. The maximum binding capacity should be reached when all the charge of the membrane has been brought to neutral by the negative charge of the particles.

To maximize the injectable particles in animal trials the viral titer will need to be as high as possible. The Sartorius™ Vivaspin™ 20 Centrifugal Concentrator will concentrate any protein or particle that is larger than 100K Daltons up to 100x the original concentration. The viral particles used in these experiments are estimated to be a similar size to HIV, greater than 100,000 Daltons.

Chapter 3 Materials and Methods:

Experiment 3.1: Elution Procedure of SIV Enveloped Particles for the Pierce Strong Anion Exchange Columns

Purpose: To identify the best NaCl solution concentration to elute viral particle from the Pierce Strong Anion Exchange Spin Columns

Materials:

- HEK 293T cell line (2014 ATCC--Liquid Nitrogen Tank2 Rack 3 bottom)
- A3R5.7+Puro-TurboGFP-RRE cell line (Clone8) (Wu Lab--Liquid Nitrogen Tank1 Rack 2) [this is a cell line that turns GFP positive in the presence of the HIV REV protein.]
- PEI solutions made from Stock kept at 20mg/mL in 4 degree fridge will stay effective for at least a year in DMEM serum free
- Pierce Strong Anion Exchange Spin Columns
- CytoOne 10cm Cell Culture Plates
- Gibco TrypLE Express
- DMEM+10%FBS+NEAA and DMEM serum free
- TRIS buffer
- RPMI complete medium (10% FBS, NEAA, gentamycin, Puromycin selection and Sodium Pyruvate) stored at 4°C
- 0.45mm syringe filters
- Micro-centrifuge tubes

Table 3.1: PEI Transfection Plasmids DNA Volumes:

Plasmids	Amount of DNA/plate (ug)	Volume for 4 plates
PNL4-3-KFS (266.5ug/mL)	5ug (18.8uL)	75.2uL
PNL-SIVmac239ENV (348.8ug/mL)	5ug (14.3uL)	57.2uL
DMEM serum free medium	466.9uL	1.868mL

Table 3.2: PEI Transfection Reagent Medium:

Transfection Medium	Volume PEI (0.5mg/mL)	Volume DMEM Serum FREE Medium
4 plates	180uL	1.820mL

Procedure:

1. Grow 293 cells in DMEM+10% FBS to confluence in T75 flask (3×10^7 cells/per flask). One day before transfection, dislodge cells from one T75 flask and plate into 10cm dish ($3 \sim 5 \times 10^6$ cells per dish) (*With a 100% confluence T75 flask, which can seed five 10cm-dishes.*)
2. Grow at 37°C for 12 to 24 hours to 80% confluence. Remove medium from each flask and rinse with serum free medium, then add 9ml of warm, serum free medium each dish.
3. In one tube, mix 10ug/plate of plasmids according to the table above. Mix 75.2uL of pNL-4-3-KFS, 57.2uL of pNL-SIV-mac239ENV and 1.868mL of DMEM serum free medium. In a separate tube mix 180ul (45uL/plate) of PEI .5ug/mL with 1.820 of DMEM serum free medium. Mix and vortex the DNA and PEI solutions and let them incubate at room temperature for 15mins. (Refer to the table above for volumes of DNA)
4. Incubate the plates at 37°C for 6 hours. Remove the transfection supernatant (carefully with a pipet), add 10ml of warm 10%FBS·MDEM Plus 100uL of Pen/Strep (antibiotic) medium per dish (carefully to the side of the dish)

Note: before starting have all tubes on ice and centrifuges at 4 degrees. Equilibrate the anion exchange columns with 20mL of TRIS buffer by centrifugation at 1200rpm (500xg) for 5mins at 4 degrees.

5. Centrifuge the supernatant of the four transfected plates at 1200rpm for 5mins at 4 degrees.
6. Filter the supernatant with the .45mm filter. [Save 2mL for control-aliquot into 5 400uL tubes]. Add the filtered supernatant to 4 anion exchange columns (10mL in each) and centrifuge at 1200rpm (500xg) for 5mins at 4 degrees. Virus will bind to the column (this can be repeated until all the harvested supernates have gone through the column. Discard the flow through.
7. Elute the virus by adding 2mL of .5M, 1M, 1.5M, and 2M of NaCl and centrifuging the columns at 1200rpm (500xg) for 5mins at 4 degrees. [save the eluted solution from each column for infection -500uL each tube]-{can freeze at this point -80}
8. Before the infection, add 100ul of the eluted virus to 400uL of medium repeat for each concentration of the elutants (4 total, 500uL each)
9. Perform an infection on Clone8 cells(at 2×10^5 cells in 1.5mL of Complete RPMI medium) with the pre column solution(500uL) and 4 eluted solutions, diluted previously to 500uL. See table below for infection tube contents. (At this point the highest salt concentration, 2M, will be equivalent to PBS salt concentration, .13M)

Table 3.3: Infection Tube Layout for Clone8 Cells:

Infection Tube #	Viral Elutant	Total volume of diluted particle	Volume of cells
1	None	None	2mL at 2×10^5 cells
2	Pre-Elution	500uL	2mL at 2×10^5 cells
3	0.5M NaCl	500uL	1.5mL at 2×10^5 cells
5	1.0M NaCl	500uL	1.5mL at 2×10^5 cells
6	1.5M NaCl	500uL	1.5mL at 2×10^5 cells
7	2.0M NaCl	500uL	1.5mL at 2×10^5 cells

Mix the contents of each tube by tapping vigorously for 30 seconds.

10. Spin the infection tubes for 2 hours at 1200RPM at 37 degrees.

11. Resuspend the cells in the tubes and incubate at 37 degrees. Run FACS after 48 hours post infection. The higher the infection rate should indicate the higher elution efficiency and if the viral envelope survived the salt concentration and column.

Experiment 3.2: Binding Capacity of SIV Enveloped Particles to the Pierce Strong Anion Exchange Columns

Purpose: To identify the amount of viral containing supernatant each anion exchange column can handle before losing binding capacity

Materials:

- HEK 293T cell line (2014 ATCC--Liquid Nitrogen Tank2 Rack 3 bottom)
- A3R5.7+Puro-TurboGFP-RRE cell line (Clone8) (Wu Lab--Liquid Nitrogen Tank1 Rack 2) [this is a cell line that turns GFP positive in the presence of the HIV REV protein.]
- PEI solutions made from Stock kept at 20mg/mL in 4 degree fridge will stay effective for at least a year in DMEM serum free
- Pierce Strong Anion Exchange Spin Columns
- CytoOne 10cm Cell Culture Plates
- Gibco TrypLE Express
- DMEM+10%FBS+NEAA and DMEM serum free
- TRIS buffer
- RPMI complete selective medium(10% FBS, NEAA, gentamycin, Puromycin and G418 selection and Sodium Pyruvate) stored at 4°C
- 0.45mm syringe filters
- Micro-centrifuge tubes

Table 3.4: PEI Transfection Plasmid Volumes

Plasmids	Amount of DNA/plate (Volume uL)	Volume for 5 plates
pNL4-3-KFS (266.5ug/mL)	5ug (18.8uL)	94uL
pNL-SIVmac239ENV (348.8ug/mL)	5ug (14.3uL)	71.5uL
DMEM serum free medium	466.9uL	2.335mL

Table 3.5: PEI Transfection Reagent Volumes

Transfection Medium	Volume PEI (0.5mg/mL)	Volume DMEM Serum FREE Medium
5 plates	225uL	2.275mL

Procedure:

1. Grow Hek293T cells in DMEM+10% FBS to confluence in T75 flask (3 x 10⁷ cells/per flask). One day before transfection, dislodge cells from one T75 flask and plate into 10cm dish (3 ~ 5 x 10⁶ cells per dish) (*With a 100% confluence T75 flask, which can seed five 10cm-dishes.*)

2. Grow at 37°C for 12 to 24 hours to 80% confluence. Remove medium from each flask and rinse with serum free medium, then add 9ml of warm, serum free medium each dish.
3. In one tube, mix 10ug/plate of plasmids according to the table above. Mix 94uL of pNL-4-3-KFS, 71.5uL of pNL-SIV-mac239ENV and 2.335mL of DMEM serum free medium. In a separate tube mix 225ul (45uL/plate) of PEI .5ug/mL with 2.275mL of DMEM serum free medium. Mix and vortex the DNA and PEI solutions and let them incubate at room temperature for 15mins. (Refer to the table above for volumes of DNA)
4. Incubate the plates at 37°C for 6 hours. Remove the transfection supernatant (carefully with a pipet), add 10ml of warm 10%FBS·MDEM Plus 100uL of Pen/Strep (antibiotic) medium per dish (carefully to the side of the dish)
5. 48 hours post transfection centrifuge the supernatant of the five transfected plates at 1200rpm for 5mins at 4 degrees.

Note: before starting have all tubes on ice and centrifuges at 4 degrees. Equilibrate the anion exchange columns with 20mL of TRIS buffer by centrifugation at 1200rpm (500xg) for 5mins at 4 degrees.

6. Filter the supernatant with the .45mm filter. [Save .5mL for control] into a 50mL tube (save 1mL for testing)
7. Add 10mL of the filtered supernatant to anion exchange column collect the flow through label and store at -80. Repeat, by adding 10mL of filtered supernatant and collecting the flow through. (Spin each cycle at 1200 rpm for 5mins at 4degrees)

8. Once all the flow throughs are collected, elute the virus by adding 2mL of NaCl 1.5M and centrifuging the column at 1200rpm (500xg) for 5mins at 4 degrees. Perform an infection on Clone8 cells(at 2×10^5 cells in 1.5mL of Complete RPMI medium with Puromycin and G418) 5 flow through solutions. Mix the contents of each tube by tapping vigorously for 30 seconds. See table below for infection tube contents.

Table 3.6: Infection Tube Layout of Clone8 Cells:

Infection Tube #	Volume Flow Through	Total volume for infection	Volume of cells (Clone8)
1	None	None	2mL at 2×10^5 cells
2	Flow 1 (10mL)	500uL	1.5mL at 2×10^5 cells
3	Flow 2 (20mL)	500uL	1.5mL at 2×10^5 cells
4	Flow 3 (30mL)	500uL	1.5mL at 2×10^5 cells
5	Flow 4 (40mL)	500uL	1.5mL at 2×10^5 cells
6	Flow 5 (50mL)	500uL	1.5mL at 2×10^5 cells

9. Spin the infection tubes for 2 hours at 1200RPM at 37 degrees.
10. Resuspend the cells in the tubes and incubate at 37 degrees. Run FACS after 48 hours post infection. The higher the GFP signal of the infection rate would indicate a loss of binding efficiency of the column.

Experiment 3.3: Particle Concentration Procedure Using the Sartorius™ Vivaspin™ 20 Centrifugal Concentrator

Purpose: To confirm that the Sartorius™ Vivaspin™ 20 Centrifugal Concentrator can concentrate viral particles without losing infectivity

Materials:

- HEK 293T cell line (2014 ATCC--Liquid Nitrogen Tank2 Rack 3 bottom)
- A3R5.7 cell line (A3R5.7 Parental cellbox-- Liquid Nitrogen Tank 2 rack 1 bottom)
- PEI solutions made from Stock kept at 20mg/mL in 4 degree fridge will stay effective for at least a year in DMEM serum free
- CytoOne 10cm Cell Culture Plates
- Sartorius™ Vivaspin™ 20 Centrifugal Concentrator (Fisher -- VS2042)
- Gibco TrypLE Express
- DMEM+10%FBS+NEAA and DMEM serum free
- RPMI complete medium(10% FBS, NEAA, gentamycin, G418 selection and Sodium Pyruvate) stored at 4°C

- Micro-centrifuge tubes
- Plasmids listed in the table below

Table 3.7: PEI Transfection Plasmid Volumes

Plasmids	Amount of DNA/plate (Volume uL)	Volume for 2 plates(uL)
pCMVdeltaR8.2 (428.5ug/mL)	3.75ug (8.75uL)	17.5uL
pLKO-TurboGFP (90.8ug/mL)	5ug (55.1uL)	110.2uL
PNL-SIVmac239ENV (348.8ug/mL)	1.25ug (3.6uL)	3.6uL
DMEM Serum FREE Medium	500uL total per plate	868.7uL

Table 3.8: PEI Transfection Reagent Volumes

Transfection Medium	Volume of PEI (0.5mg/mL)	Volume DMEM Serum FREE Medium
1 plate	45uL	455uL
2 plates	90uL	910uL

Protocol:

1. Grow Hek 293T cells in DMEM+10% FBS to confluence in T75 flask (3 x 10⁷ cells/per flask). One day before transfection, dislodge cells from one T75 flask and plate into 10cm dish (3 ~ 5 x 10⁶ cells per dish) (*With a 100% confluence T75 flask, which can seed five 10cm-dishes.*)
2. Grow at 37°C for 12 to 24 hours to 80% confluence. Remove medium from each flask and rinse with serum free medium, then add 9ml of warm, serum free medium each dish.
3. In one tube, mix 10ug/plate of plasmids with DMEM serum free medium according to the table above for 2 plates. In a separate tube mix **90ul** (45uL/plate) of PEI .5ug/mL with **910uL** of DMEM serum free medium. Mix and vortex the DNA and PEI solutions and let them incubate at room temperature for 15 mins. (Refer to the table above for volumes of DNA).
4. Add the DNA+PEI mixture to each dish dropwise covering the most area rock the plate to ensure the plate is completely covered.
5. Incubate plate C. at 37°C for 24 hours. Remove the transfection supernatant (carefully with a pipet), add 10ml of warm 10%FBS·MDEM Plus 100uL of Pen/Strep (antibiotic) medium per dish (carefully to the side of the dish).
6. Harvest 20mL supernatant on ice 48 hours post transfection. Spin the supernatants down at 1200 rpm for 10 minutes. Collect the cell cleared

supernatants in fresh tubes on ice. Filter the supernatant with the .45mm syringe filter. Save 1mL aliquots of the filtered particles at –80 degrees

7. Apply 10mL of the supernatant to the Sartorius™ Vivaspin™ 20 Centrifugal Concentrator and spin at 7000rpm for 15 mins at 4 degrees.
8. Collect the concentrate and store at –80 degree.
9. Perform an infection on A3R5.7 cells(at 2×10^5 cells in 1.5mL of Complete RPMI medium with G418 selection) 5 flow through solutions. See table below for infection tube contents.

Table 3.9: Infection Tube Layout of Clone8 Cells:

Infection Tube #	Infection Type	Total volume for infection	Volume of cells (A3R5.7)
1	None	None	2mL at 2×10^5 cells
2	Unconcentrated	500uL	1.5mL at 2×10^5 cells
3	Concentrated	500uL	1.5mL at 2×10^5 cells

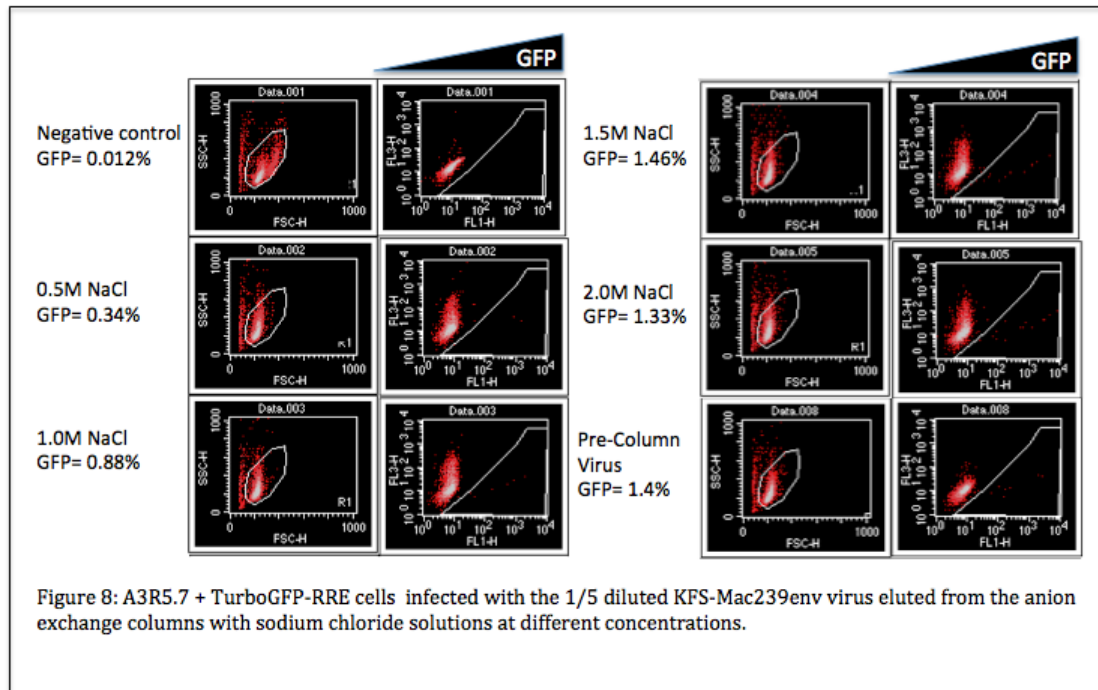
Mix the contents of each tube by tapping vigorously for 30 seconds.

10. Spin the infection tubes for 2 hours at 1200RPM at 37 degrees.
11. Resuspend the cells in the tubes and incubate at 37 degrees. Run FACS after 48 hours post infection. The higher the GFP signal of the infection the larger the titer of concentrated particle.

Chapter 3 Results:

Experiment 3.1: Anion Exchange Elution Buffer Results:

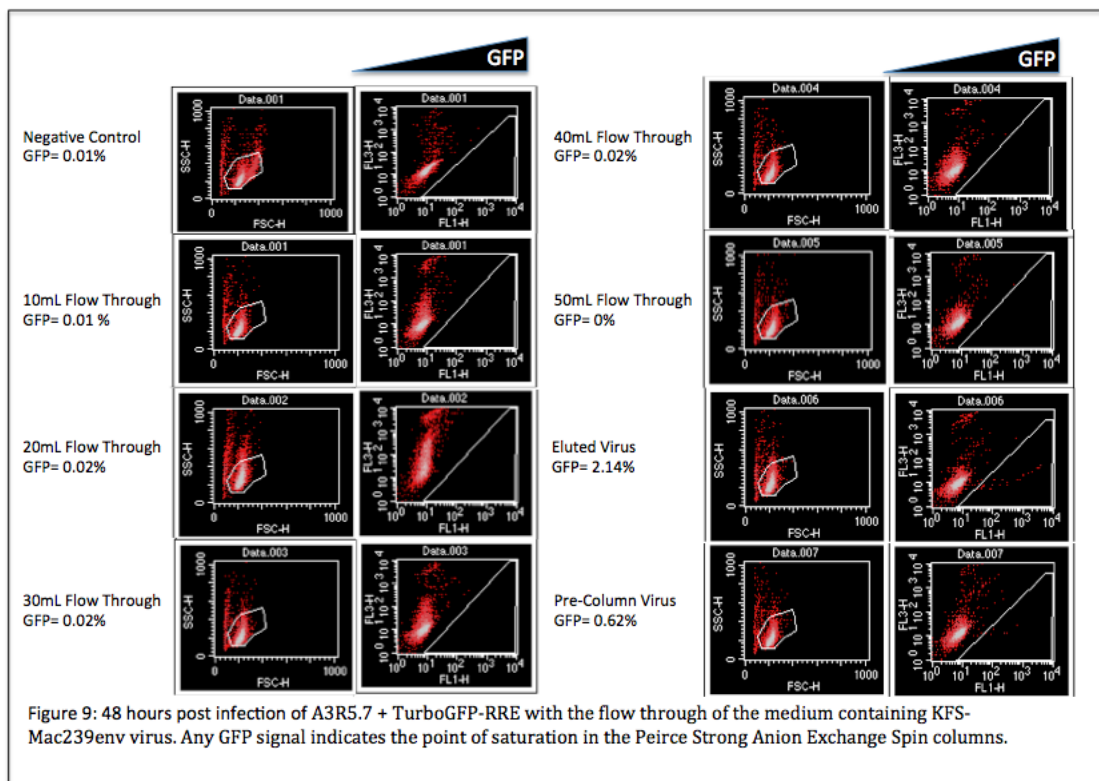
The anion exchange columns allow for the purification and concentration of negatively charged enveloped particles. If the salt elution buffer was successful at harvesting most of the viral particles the infection rate should match the pre-column infection of 1.4% GFP. The infection of the A3R5.7+TurboGFP-RRE cells indicated the highest yield of eluted virus was from the 1.5M sodium chloride solution with an total GFP signal of 1.46% which was 1.6x that concentrated from the 1M and almost 4.3x of the 0.5M eluted particles. The 2M sodium chloride had a slightly lower GFP signal indicating it might be damaging to the particles. Using a sterile 1.5M sodium chloride solution is the most efficient way to elute viral particles from the Pierce Strong Anion Exchange Spin columns. If the eluted virus was left undiluted the high salt concentration would kill the cells during the infection, so all the viruses must go through a dilution step after elution.



Experiment 3.2: Anion Exchange Column Binding Capacity Results:

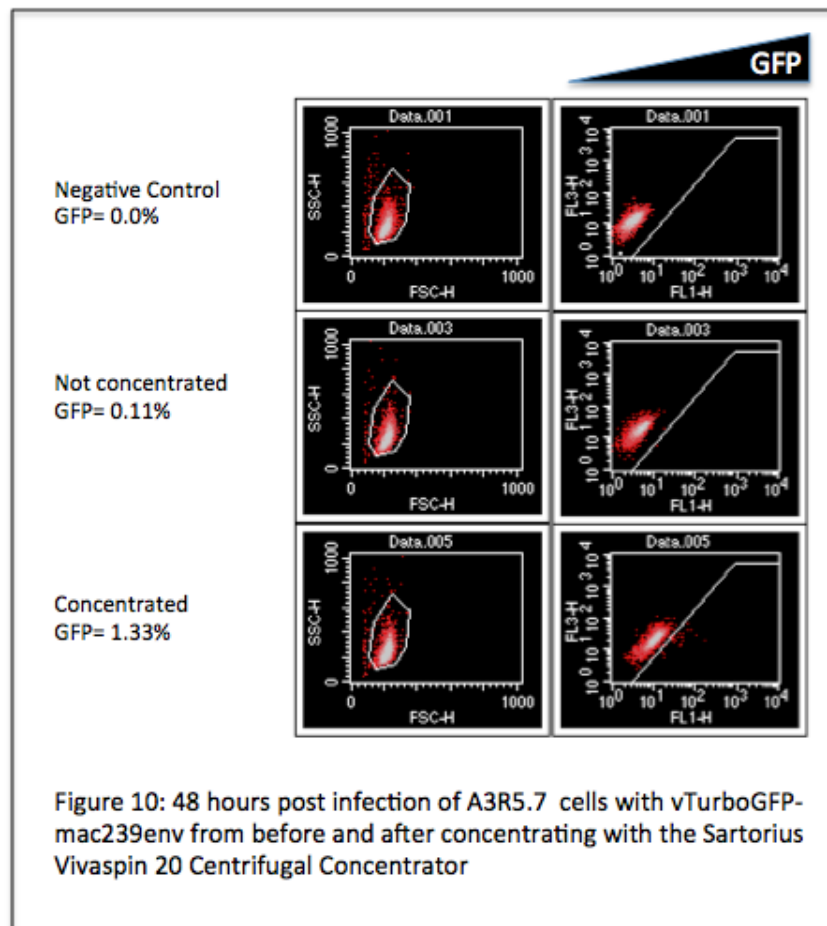
The infection of the Clone8 cells with the flow through of the anion exchange columns showed no infection up to 50 mL of flow through. This indicates that most of the viral particles were able to bind. If they did not there should have been GFP signal in the infection. After the flow through was collected, the particles were eluted and showed a

3.5x infection rate compared to particles harvested directly from the plates. If the columns had a 100% elution rate the final concentration of the particles should have been 5x. These results indicate some viral particles may be lost during the elution process.



Experiment 3.3: Sartorius™ Vivaspin™ 20 Centrifugal Concentrator Results:

The infection of the A3R5.7 cells showed the Sartorius™ Vivaspin™ 20 Centrifugal Concentrator was able to concentrate the vTurbo-GFP-Mac239env particles 12x the original titer. This is highly concentrated and shows that the concentrator does not kill the virus and can deliver much better gene transfer results. In similar experiments the concentrators ranges anywhere from 10x to 30x concentration. The purity of the collected supernatant may influence the column's ability to concentrate. The results show a very low GFP signal compared to previous experiments with vVSV-G-TurboGFP. This is most likely due to the SIVmac349 envelope having a lower affinity for the human CCR5 receptor.



Chapter 4. Discussion and The Final Protocol for the Production of Lentiviral Particles for Animal Trials

Discussion:

Over the past decade many new HIV research has brought around a shift in the way HIV is treatment and prevented which has greatly lowered the incidence of the HIV related deaths. However, millions of people still remain infected and those that are must remain on antiretroviral therapies for their entire life. One of the main issues associated with treating and curing HIV is the virus ability to remain latent in reservoir cells and can become active after stopping anti-retroviral treatment. Targeting the reservoir cells has become a critical fight in the effort to eliminate HIV and AIDS.

In a study conducted by Wu et al in 2007, a rev dependent vector was created that can target cells infected with HIV. The rev protein is a viral protein that binds to the rev response element and alters the splicing of the HIV mRNA. This controls the early and late stage transcription of the viral genome. The three rev dependent vectors carry HSV1-TK, TRAF6 or a b-gal-GFP gene and will be tested at the Tulane National Primate Research center.

The final protocol for the monkey trials will contain six unique plasmid produced by Wu et al in 2007. The characteristics of those plasmids are described below. The final protocol takes advantage of the cost effective PEI transfection protocol, the purification

and concentration ability of the Pierce Strong Anion Exchange Spin columns and the concentrating power of the Sartorius™ Vivaspin™ 20 Centrifugal Concentrator.

Together the protocol will produce cost effective, highly pure and high titer viral particles. For 250 plates of particles will concentrate down to approximately 8mL of medium, approximately 30x concentrated. The approximate cost of the final protocol for 250 plates will be approximately \$2000 before any labor expenses. The most expensive part of the procedure is the anion exchange purification with an approximate cost of \$1600 (ThermoFisher). The total monkey trial will require greater than 1000 10cm plates.

Future work to improve the protocol could also combine some of the multiple helper plasmids into one to help eliminate the need to perform multiple plasmid purifications and help to simplify and bring cost down. A future method to reusing the anion exchange columns or developing a way to remove this step will help to greatly reduce this cost further. This protocol is only optimized for the use in 10cm plates. Larger plates such as the 15cm plates or the even larger Fisher Scientific Cell Factories, which are equivalent to almost 200 10cm plates, may be capable of producing more particles. Additional trials of the PEI, CaPO₄ and Lipofectamine™ 2000 transfection may need to be repeated to confirm, if any, statistically significant differences. However, the CaPO₄ transfections carry a much greater chance of variation due to the protocol requiring the use of far more buffers and salt concentrations, which makes it a riskier transfection that can lead to inconsistencies in viral production. PEI only requires a dissolved buffer of PEI in DMEM

medium. This makes it ideal for keeping conditions consistent from one viral production to the next.

Additionally the low infectivity of the SIVmac239Env particles with the Clone8 cells should be explored further. The human CCR5 receptor on the Clone8 cells may not be fully compatible with the SIVmac239 envelope or there may be an issue with the envelopes ability to infect cells all together. This should be resolved before any large-scale animal trials of the particles are conducted.

The protocol was recently tested and used to produce vSIV-HSV1tk-RRE-SA particles. Those particles were injected into SIV infected Indian rhesus monkeys and are currently being monitored with the hope of controlling the monkeys' viral rebound after antiretroviral therapy is stopped.

Final Protocol for the Production of Lentiviral Particles for Animal Trials

Plasmid Background:

1. Name: pAd-SIV3+ New

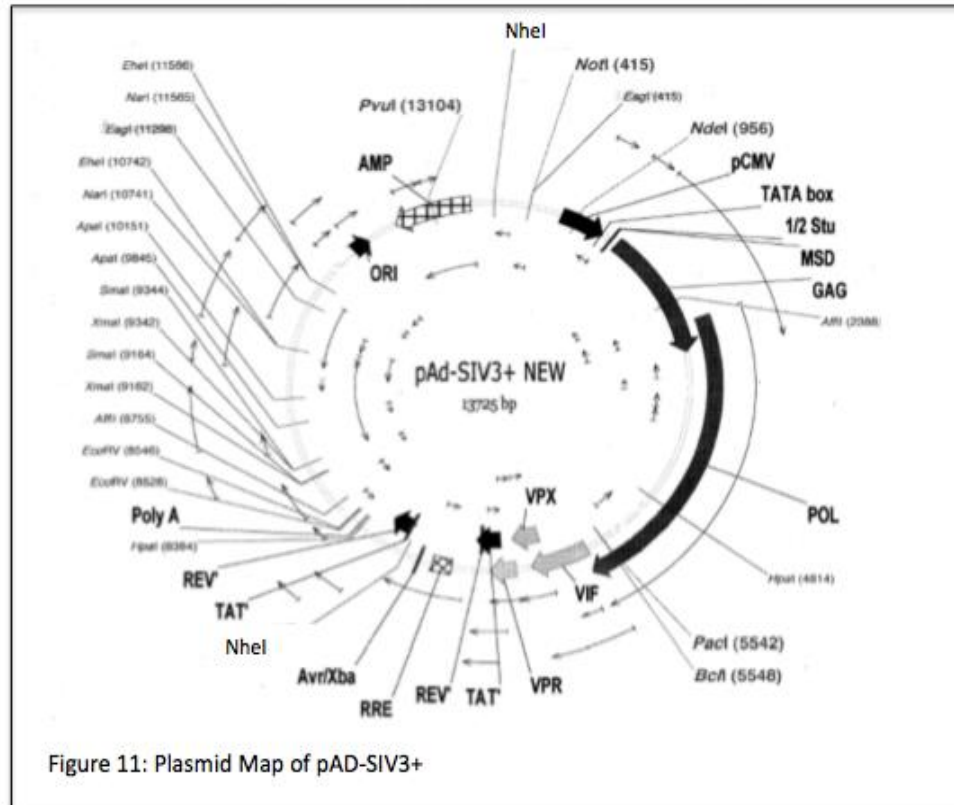
Location of Plasmid: Brian's Plasmid Box 2

Bacteria Stock Location: Brian's Bacteria Box 1

Type of Bacteria: DH5a

Drug Resistance: Amp 1X

Map and Computer File location: No computer file available



Description: Helper Plasmid-carries SIV GAG/POL, Tat and Rev but lacks the packaging signal or the NEF protein.

Origin of Plasmid: This plasmid was given to the Wu lab by Andrea Cara (andrea.cara@iss.it) François-Loïc Cosset (<<mailto:flcosset@ens->

[lyon.fr>flcosset@ens-lyon.fr](mailto:flcosset@ens-lyon.fr)) Didier Negre ([<mailto:Didier.Negre@ens-lyon.fr>Didier.Negre@ens-lyon.fr](mailto:Didier.Negre@ens-lyon.fr))

Additional Notes: Used this plasmid to construct SIV-based particles and test SIV-Rev dependence

2. Name: pCGCG-NEF

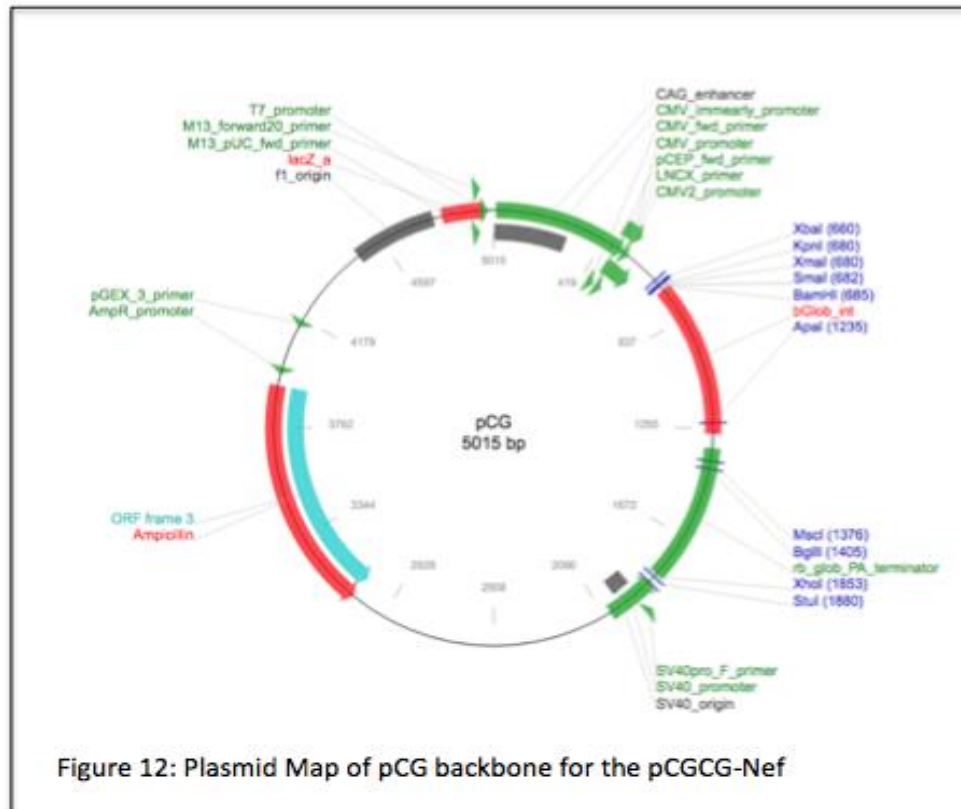
Location of Plasmid: Brian's Plasmid Box 3

Bacteria Stock Location: Brian's Bacteria Box

Type of Bacteria: DH5a

Drug Resistance: Amp 1X

Map and Computer File location: No computer file is available for this plasmid's map. Check back of data sheet for backbone plasmid information.



Description: This is a plasmid that carries the protein nef and is required when making SIV pseudo type viruses in higher titer and higher infectivity

Origin of Plasmid: NEF cloned into Addgene pCG backbone

Additional Notes: Used this plasmid to construct a SIV based particles.

We do not have a plasmid map, however the plasmid backbone is available.

3. Name: pNL-deltapsi- Mac239Env

Location of Plasmid: Brian's Plasmid Box 3

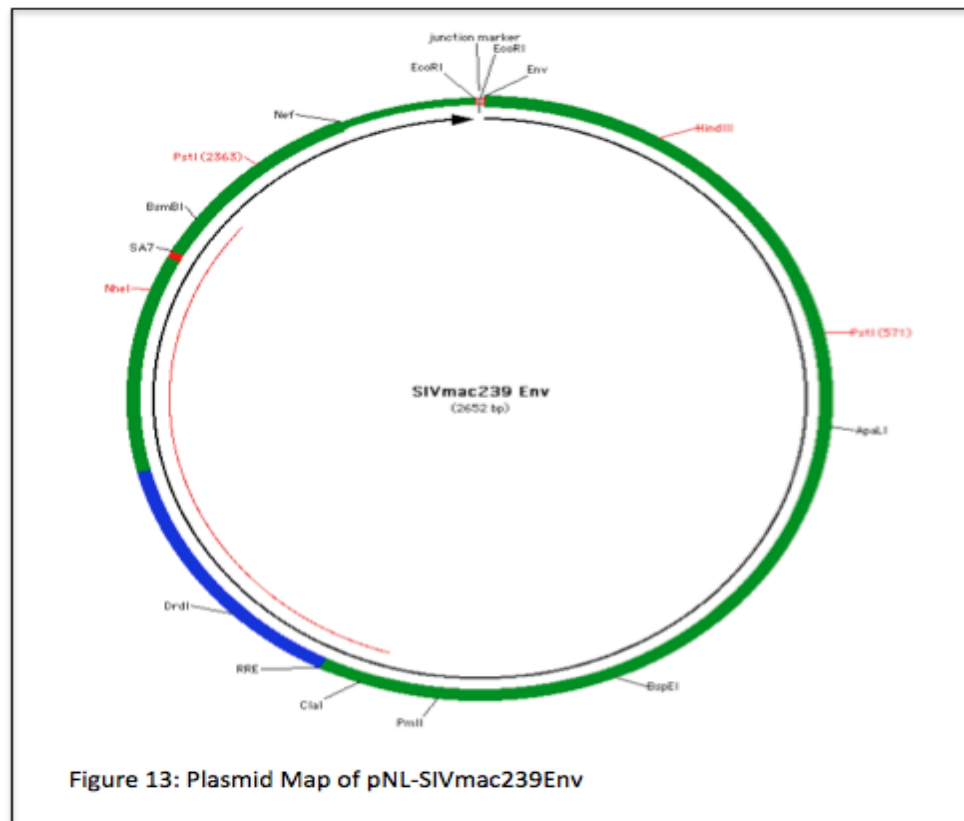
Bacteria Stock Location: Brian's Bacteria Box 3

Type of Bacteria: DH5a

Drug Resistance: Amp 1X

Map and Computer File location:

MiddleLabComputer/Labmember'sFolder/Mark'sFolder



Description: This is a plasmid that carries wild type SIVmac329 envelope gene with a SIV packaging signal. The plasmid will be used to make SIV vector particles for the purpose of targeting SIV infected cells.

4. Name: pSIV-TRAF6-RRE-SA

Location of Plasmid: Brian's Plasmid Box 3

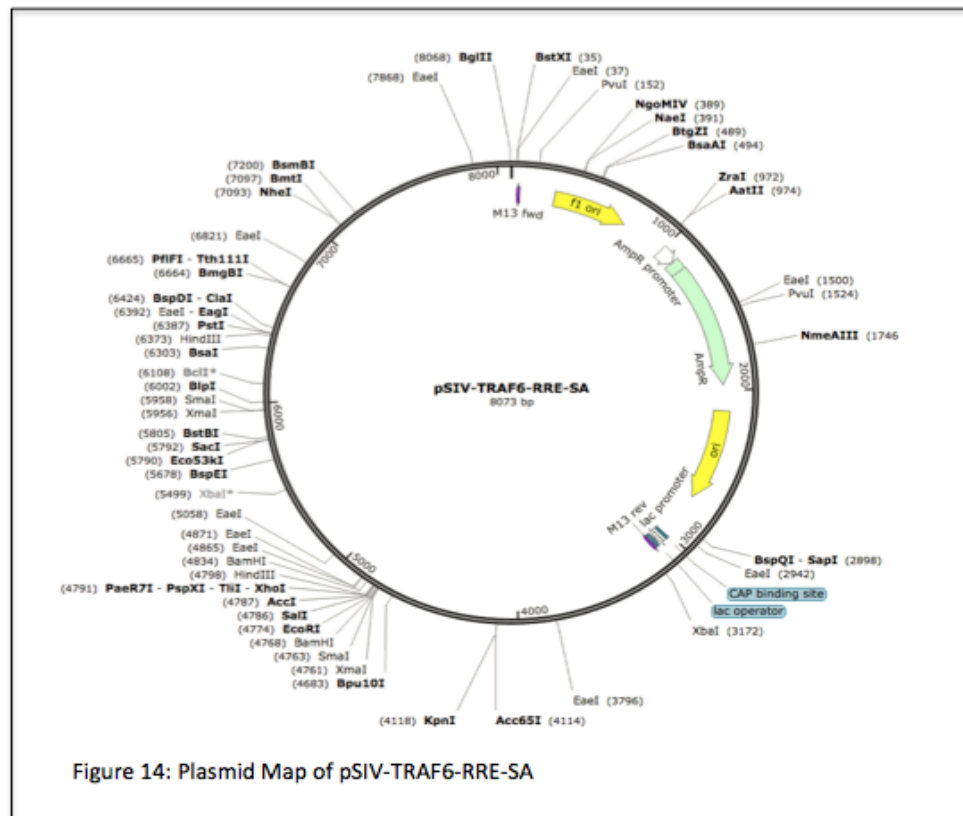
Bacteria Stock Location: Brian's Bacteria Box 1

Type of Bacteria: DH5a

Drug Resistance: Amp 1X

Map and Computer File location:

MiddleLabComputer/Labmember'sFolder/Bac'sFolder



Description: This is a plasmid that carries the tumor necrosis factor attached to a SIV rev response element. Thus, the creation of the tumor necrosis factor protein is SIV dependent. This is one of three genome plasmids being used in the monkey trials at Tulane's Primate Research center.

Origin of Plasmid: Lab Construct: Originally pSIV-RRE-SA and YFP-hTRAF6 (from Dr. Liusheng He)

References: <http://www.ncbi.nlm.nih.gov/pubmed/?term=20410930>

Additional Notes: Used this plasmid to construct a potential particle to attack SIV infected cells.

5. Name: pSIV-HSV-TK-RRE-SA

Location of Plasmid: Brian's Plasmid Box 2

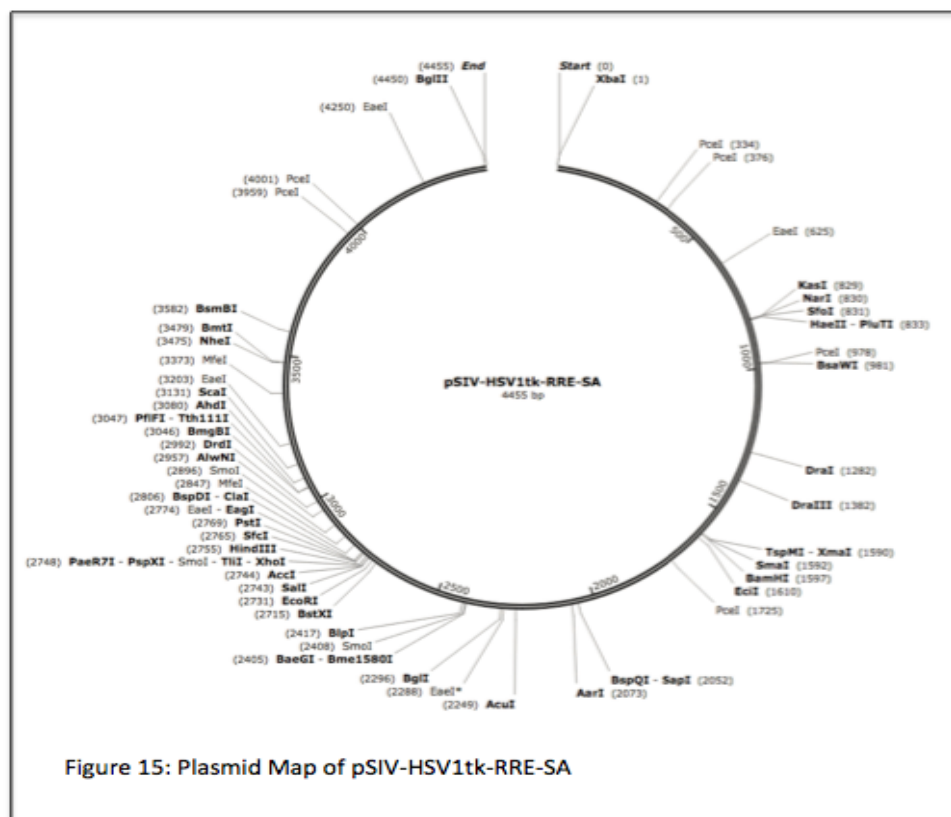
Bacteria Stock Location: Brian's Bacteria Box 1

Type of Bacteria: DH5a

Drug Resistance: Amp 1X

Map and Computer File location:

MiddleLabComputer/Labmember'sFolder/Mark'sFolder



Description: This is a plasmid that carries the herpes simplex I thymidine kinase attached to a SIV rev response element. When an infected cell expresses REV the thymidine kinase will be produced. When ganciclovir is introduced to the cells it will get phosphorylated by the TK and will act nucleoside analog and cause apoptosis. This is one of three genome plasmids being used in the monkey trials at Tulane's Primate Research center.

Origin of Plasmid: Lab Construct: Originally pSIV-RRE-SA and b-gal-IRES –GFP fragment

Additional Notes: Used this plasmid to construct a potential particle to indicate SIV infected cells.

6. Name: pSIV-b-gal-GFP-RRE-SA

Location of Plasmid: Brian's Plasmid Box 3

Bacteria Stock Location: Brian's Bacteria Box 1

Type of Bacteria: DH5a

Drug Resistance: Amp 1X

Map and Computer File location:

MiddleLabComputer/Labmember'sFolder/Mark'sFolder

Sequences of the SIV Vector Genomes:

pSIV-RRE-SA

GATCTTCTAGATGGAAGGGATTTATTACAGTGCAAGAAGACATAGAATCTTA
GACATATACTTAGAAAAGGAAGAAGGCATCATACCAGATTGGCAGGATTACA
CCTCAGGACCAGGAATTAGATACCCAAAGACATTTGGCTGGCTATGGAAATT
AGTCCCTGTAAATGTATCAGATGAGGCACAGGAGGATGAGGAGCATTATTTA
ATGCATCCAGCTCAAACCTCCAGTGGGATGACCCTTGGGGAGAGGTTCTAG
CATGGAAGTTTGATCCAACCTCTGGCCTACACTTATGAGGCATATGTTAGATAC
CCAGAAGAGTTTGGAAGCAAGTCAGGCCTGTCAGAGGAAGAGGTTAGAAGA
AGGCTAACCGCAAGAGGCCTTCTTAACATGGCTGACAAGAAGGAAACTCGCT
GAAACAGCAGGGACTTTCCACAAGGGGATGTTACGGGGAGGTACTGGGGAG
GAGCCGGTCGGGAACGCCCACTTTCTTGATGTATAAATATCACTGCATTTTCGC
TCTGTATTCAGTCGCTCTGCGGAGAGGCTGGCAGATTGAGCCCTGGGAGGTT
CTCTCCAGCACTAGCAGGTAGAGCCTGGGTGTTCCCTGCTAGACTCTCACCAG
CACTTGGCCGGTGCTGGGCAGAGTGACTCCACGCTTGCTTGCTTAAAGCCCTC
TTCAATAAAGCTGCCATTTTAGAAGTAAGCTAGTGTGTGTTCCCATCTCTCCT
AGCCGCCGCCTGGTCAACTCGGTACTCAATAATAAGAAGACCCTGGTCTGTT
AGGACCCTTTCTGCTTTGGGAAACCGAAGCAGGAAAATCCCTAGCAGATTGG
CGCCTGAACAGGGACTTGAAGGAGAGTGAGAGACTCCTGAGTACGGCTGAGT
GAAGGCAGTAAGGGCGGCAGGAACCAACCACGACGGAGTGCTCCTATAAAG

GCGCGGGTCGGTACCAGACGGCGTGAGGAGCGGGAGAGGAAGAGGCCTCCG
GTTGCAGGTAAGTGCAACACAAAAAAGAAATAGCTGTCTTTTATCCAGGAAG
GGGTAATAAGATAGAGTGGGAGATGGGCGTGAGAACTCCGTCTTGTTCAGGG
AAGAAAGCAGATGAATTAGAAAAAATTAGGCTACGACCCAACGGAAAGAAA
AAGTACATGTTGAAGCATGTAGTATGGGCAGCAAATGAATTAGATAGATTG
GATTAGCAGAAAGCCTGTTGGAGAACAAGAAGGATGTCAAAAAATACTTTC
GGTCTTAGCTCCATTAGTGCCAACAGGCTCAGAAAATTTAAAAAGCCTTTATA
ATACTGTCTGCGTCATCTGGTGCATTCACGCAGAAGAGAAAAGTGAAACACAC
TGAGGAAGCAAAACAGATAGTGCAGAGACACCTAGTGTAGAAGCATGCTAT
AACACATGCTATTGTAAAAAGTGTTGCTACCATTGCCAGTTTTGTTTTCTTAA
AAAAGGCTTGGGGATATGTTATGAGCAATCACGAAAGAGAAGAAGAACTCC
GAAAAAGGCTAAGGCTAATACATCTTCTGCATCAAACAAGTAAGTATGGGAT
GTCTTGGGAATCAGCTGCTTATCGCCTAGTAGCACCCGGGCGGATCCGAATTC
GCATGCGTCGACTCGAGGAAGCTTatCTGCAGatTCGGCCGCTGAGTGCAGAG
GTGGCAGAACTGTATCGATTGGAATTGGGAGATTATAAATTAGTAGAGATCA
CTCCAATTGGCTTGGCCCCCACAGATGTGAAGAGGTACCTACTGGTGGCAC
CTCAAGAAATAAAAGAGGGGTCTTTGTGCTAGGGTTCTTGGGTTTTCTCGCAA
CGGCAGGTTCTGCAATGGGCGCGGCGTCGTTGACGCTGACCGCTCAGTCCCG
AACTTTATTGGCTGGGATAGTGCAGCAACAGCAACAGCTGTTGGACGTGGTC
AAGAGACAACAAGAATTGTTGCGACTGACCGTCTGGGGAACAAAGAACCTCC
AGACTAGGGTCACTGCCATCGAGAAGTACTTAAAGGACCAGGCGCAGCTGAA
TGCTTGGGGATGTGCGTTTAGACAAGTCTGCCACCTACTGTACCATGGCCAA

ATGCAAGTCTAACACCAAAGTGGAACAATGAGACTTGGCAAGAGTGGGAGC
GAAAGGTTGACTTCTTGGAAGAAAATATAACAGCCCTCCTAGAGGAGGCACA
AATTCAACAAGAGAAGAACATGTATGAATTACAAAAGTTGAATAGCTGGGAT
GTGTTTGGCAATTGGTTTGACCTTGCTTCTTGGATAAAGTATATACAATATGG
AGTTTATATAGTTGTAGGAGTAATACTGTTAAGAATAGTGATCTATATAGTAC
AAATGCTAGCTAAGTTAAGGCAGGGGTATAGGCCAGTGTTCTCTTCCCCACC
CTCTTATTTCCAGCAGACCCATATCCAACAGGACCCGGCACTGCCAACCAGA
GAAGGCAAAGAAAGAGACGGTGGAGAAGGCGGTGGCAACTAAAAGAAAAG
GGGGGACTGGAAGGGATTTATTACAGTGCAAGAAGACATAGAATCTTAGACA
TATACTTAGAAAAGGAAGAAGGCATCATACCAGATTGGCAGGATTACACCTC
AGGACCAGGAATTAGATACCCAAAGACATTTGGCTGGCTATGGAAATTAGTC
CCTGTAAATGTATCAGATGAGGCACAGGAGGATGAGGAGCATTATTTAATGC
ATCCAGCTCAAACCTCCCAGTGGGATGACCCTTGGGGAGAGGTTCTAGCATG
GAAGTTTGATCCAACCTCTGGCCTACACTTATGAGGCATATGTTAGATACCCAG
AAGAGTTTGGAAAGCAAGTCAGGCCTGTCAGAGGAAGAGGTTAGAAGAAGGC
TAACCGCAAGAGGCCTTCTTAACATGGCTGACAAGAAGGAAACTCGCTGAAA
CAGCAGGGACTTTCCACAAGGGGATGTTACGGGGAGGTACTGGGGAGGAGC
CGGTCGGGAACGCCCACTTTCTTGATGTATAAATATCACTGCATTTGCTCTG
TATTCAGTCGCTCTGCGGAGAGGCTGGCAGATTGAGCCCTGGGAGGTTCTCTC
CAGCACTAGCAGGTAGAGCCTGGGTGTTCCCTGCTAGACTCTCACCAGCACT
TGGCCGGTGCTGGGCAGAGTGACTCCACGCTTGCTTGCTTAAAGCCCTCTTCA
ATAAAGCTGCCATTTTAGAAGTAAGCTAGTGTGTGTTCCCATCTCTCCTAGCC

GCCGCCTGGTCAACTCGGTACTCAATAATAAGAAGACCCTGGTCTGTTAGGA
CCCTTTCTGCTTTGGGAAACCGAAGCAGGAAAATCCCTAGCAAGATC

pSIV-TRAF6-RRE-SA

GAAAGTCCTCTCCACTGACTGTAGCCTCCAATTCCTGGCCGTCGTTTTACAA
CGTCGTGACTGGGAAAACCCTGGCGTTACCCAACCTAATCGCCTTGCAGCAC
ATCCCCCTTTCGCCAGCTGGCGTAATAGCGAAGAGGCCCGCACCGATCGCCC
TTCCCAACAGTTGCGCAGCCTGAATGGCGAATGGCGCCTGATGCGGTATTTTC
TCCTTACGCATCTGTGCGGTATTTACACCGCATACGTCAAAGCAACCATAGT
ACGCGCCCTGTAGCGGCGCATTAAAGCGCGGCGGGTGTGGTGGTTACGCGCAG
CGTGACCGCTACACTTGCCAGCGCCCTAGCGCCCGCTCCTTTCGCTTTCTTCC
CTTCCTTTCTCGCCACGTTTCGCCGGCTTTCCCCGTCAAGCTCTAAATCGGGGG
CTCCCTTTAGGGTTCCGATTTAGTGCTTTACGGCACCTCGACCCCAAAAACT
TGATTTGGGTGATGGTTCACGTAGTGGGCCATCGCCCTGATAGACGGTTTTTC
GCCCTTTGACGTTGGAGTCCACGTTCTTTAATAGTGGACTCTTGTTCCAACT
GGAACAACACTCAACCCTATCTCGGGCTATTCTTTTGATTTATAAGGGATTTT
GCCGATTTTCGGCCTATTGGTTAAAAAATGAGCTGATTTAACAAAAATTTAAC
GCGAATTTTAACAAAATATTAACGTTTACAATTTTATGGTGCCTCTCAGTAC
AATCTGCTCTGATGCCGCATAGTTAAGCCAGCCCCGACACCCGCCAACACCC
GCTGACGCGCCCTGACGGGCTTGTCTGCTCCCGGCATCCGCTTACAGACAAG
CTGTGACCGTCAACGGGAGCTGCATGTGTCAGAGGTTTTACCGTCATCACCG
AAACGCGCGACCCGAAAGGGCCTCGTGATACGCCTATTTTTATAGGTTAATG

TCATGATAATAATGGTTTCTTAGACGTCAGGTGGCACTTTTCGGGGAAATGTG
CGCGGAACCCCTATTTGTTTATTTTTCTAAATACATTCAAATATGTATCCGCTC
ATGAGACAATAACCCTGATAAATGCTTCAATAATATTGAAAAAGGAAGAGTA
TGAGTATTCAACATTTCCGTGTCGCCCTTATTCCCTTTTTTGCGGCATTTTGCC
TTCCTGTTTTTGCTCACCCAGAAACGCTGGTGAAAGTAAAAGATGCTGAAGA
TCAGTTGGGTGCACGAGTGGGTACATCGAACTGGATCTCAACAGCGGTAAG
ATCCTTGAGAGTTTTCGCCCCGAAGAACGTTTTCCAATGATGAGCACTTTTAA
AGTTCTGCTATGTGGCGCGGTATTATCCCGTATTGACGCCGGGCAAGAGCAA
CTCGGTCGCCGCATACACTATTCTCAGAATGACTTGGTTGAGTACTCACCAGT
CACAGAAAAGCATCTTACGGATGGCATGACAGTAAGAGAATTATGCAGTGCT
GCCATAACCATGAGTGATAAACTGCGGCCAACTTACTTCTGACAACGATCG
GAGGACCGAAGGAGCTAACCGCTTTTTTGACAAACATGGGGGATCATGTAAC
TCGCCTTGATCGTTGGGAACCGGAGCTGAATGAAGCCATACCAAACGACGAG
CGTGACACCACGATGCCTGTAGCAATGGCAACAACGTTGCGCAAACCTATTAA
CTGGCGAACTACTTACTCTAGCTTCCCGGCAACAATTAATAGACTGGATGGA
GGCGGATAAAGTTGCAGGACCACTTCTGCGCTCGGCCCTTCCGGCTGGCTGG
TTTATTGCTGATAAATCTGGAGCCGGTGAGCGTGGTTCTCGCGGTATCATTGC
AGCACTGGGGCCAGATGGTAAGCCCTCCCGTATCGTAGTTATCTACACGACG
GGGAGTCAGGCAACTATGGATGAACGAAATAGACAGATCGCTGAGATAGGT
GCCTCACTGATTAAGCATTGGTAACTGTCAGACCAAGTTTACTCATATATACT
TTAGATTGATTTAAAACTTCATTTTTAATTTAAAAGGATCTAGGTGAAGATCC
TTTTTGATAATCTCATGACCAAATCCCTTAACGTGAGTTTTCGTTCCACTGA

GCGTCAGACCCCGTAGAAAAGATCAAAGGATCTTCTTGAGATCCTTTTTTTCT
GCGCGTAATCTGCTGCTTGCAAACAAAAAACCACCGCTACCAGCGGTGGTT
TGTTTGCCGGATCAAGAGCTACCAACTCTTTTTCCGAAGGTAAGTGGCTTCAG
CAGAGCGCAGATACCAAATACTGTTCTTCTAGTGTAGCCGTAGTTAGGCCAC
CACTTCAAGAACTCTGTAGCACCGCCTACATACCTCGCTCTGCTAATCCTGTT
ACCAGTGGCTGCTGCCAGTGGCGATAAGTCGTGTCTTACCGGGTTGGACTCA
AGACGATAGTTACCGGATAAGGCGCAGCGGTCTGGGCTGAACGGGGGGTTCGT
GCACACAGCCCAGCTTGGAGCGAACGACCTACACCGAACTGAGATACCTACA
GCGTGAGCTATGAGAAAGCGCCACGCTTCCCGAAGGGAGAAAGGCGGACAG
GTATCCGGTAAGCGGCAGGGTCGGAACAGGAGAGCGCACGAGGGAGCTTCC
AGGGGGAAACGCCTGGTATCTTTATAGTCCTGTCTGGGTTTCGCCACCTCTGAC
TTGAGCGTCGATTTTTGTGATGCTCGTCAGGGGGGCGGAGCCTATGGAAAAA
CGCCAGCAACGCGGCCTTTTTACGGTTCCTGGCCTTTTGCTGGCCTTTTGCTCA
CATGTTCTTTCCTGCGTTATCCCCTGATTCTGTGGATAACCGTATTACCGCCTT
TGAGTGAGCTGATACCGCTCGCCGCAGCCGAACGACCGAGCGCAGCGAGTCA
GTGAGCGAGGAAGCGGAAGAGCGCCCAATACGCAAACCGCCTCTCCCCGCG
CGTTGGCCGATTCATTAATGCAGCTGGCACGACAGGTTTCCCGACTGGAAAG
CGGGCAGTGAGCGCAACGCAATTAATGTGAGTTAGCTCACTCATTAGGCACC
CCAGGCTTTACACTTTATGCTTCCGGCTCGTATGTTGTGTGGAATTGTGAGCG
GATAACAATTTACACAGGAAACAGCTATGACCATGATTACGCCAAGCTCCT
TCCTCTTCCAGCCCTTCCTCTTTCTCTAGATGGAAGGGATTTATTACAGTGCA
AGAAGACATAGAATCTTAGACATATACTTAGAAAAGGAAGAAGGCATCATA

CCAGATTGGCAGGATTACACCTCAGGACCAGGAATTAGATACCCAAAGACAT
TTGGCTGGCTATGGAAATTAGTCCCTGTAAATGTATCAGATGAGGCACAGGA
GGATGAGGAGCATTATTTAATGCATCCAGCTCAAACCTCCCAGTGGGATGAC
CCTTGGGGAGAGGTTCTAGCATGGAAGTTTGATCCAACCTCTGGCCTACACTTA
TGAGGCATATGTTAGATACCCAGAAGAGTTTGGAAGCAAGTCAGGCCTGTCA
GAGGAAGAGGTTAGAAGAAGGCTAACCGCAAGAGGCCTTCTTAACATGGCT
GACAAGAAGGAAACTCGCTGAAACAGCAGGGACTTTCCACAAGGGGATGTT
ACGGGGAGGTACTGGGGAGGAGCCGGTCGGGAACGCCCACTTTCTTGATGTA
TAAATATCACTGCATTTTCGCTCTGTATTCAGTCGCTCTGCGGAGAGGCTGGCA
GATTGAGCCCTGGGAGGTTCTCTCCAGCACTAGCAGGTAGAGCCTGGGTGTT
CCCTGCTAGACTCTCACCAGCACTTGGCCGGTGCTGGGCAGAGTGACTCCAC
GCTTGCTTGCTTAAAGCCCTCTTCAATAAAGCTGCCATTTTAGAAGTAAGCTA
GTGTGTGTTCCCATCTCTCCTAGCCGCCGCCTGGTCAACTCGGTACTCAATAA
TAAGAAGACCCTGGTCTGTTAGGACCCTTTCTGCTTTGGGAAACCGAAGCAG
GAAAATCCCTAGCAGATTGGCGCCTGAACAGGGACTTGAAGGAGAGTGAGA
GACTCCTGAGTACGGCTGAGTGAAGGCAGTAAGGGCGGCAGGAACCAACCA
CGACGGAGTGCTCCTATAAAGGCGCGGGTCGGTACCAGACGGCGTGAGGAG
CGGGAGAGGAAGAGGCCTCCGGTTGCAGGTAAGTGCAACACAAAAAAGAAA
TAGCTGTCTTTTATCCAGGAAGGGGTAATAAGATAGAGTGGGAGATGGGCGT
GAGAACTCCGTCTTGTCAGGGAAGAAAGCAGATGAATTAGAAAAAATTAG
GCTACGACCCAACGGAAAGAAAAAGTACATGTTGAAGCATGTAGTATGGGC
AGCAAATGAATTAGATAGATTTGGATTAGCAGAAAGCCTGTTGGAGAACAAA

GAAGGATGTCAAAAAATACTTTTCGGTCTTAGCTCCATTAGTGCCAACAGGCT
CAGAAAATTTAAAAAGCCTTTATAATACTGTCTGCGTCATCTGGTGCATTAC
GCAGAAGAGAAAGTGAAACACACTGAGGAAGCAAAACAGATAGTGCAGAGA
CACCTAGTGTAGAAGCATGCTATAACACATGCTATTGTAAAAAGTGTTGCTA
CCATTGCCAGTTTTGTCTTTCTTAAAAAAGGCTTGGGGATATGTTATGAGCAAT
CACGAAAGAGAAGAAGAACTCCGAAAAAGGCTAAGGCTAATACATCTTCTG
CATCAAACAAGTAAGTATGGGATGTCTTGGGAATCAGCTGCTTATCGCCTAG
TAGCACCCGGGCGGATCCGAATTCGCATGCGTCGACTCGAGGAAGCTTATGA
GTCTGCTAAACTGTGAAAACAGCTGTGGATCCAGCCAGTCTGAAAGTGACTG
CTGTGTGGCCATGGCCAGCTCCTGTAGCGCTGTAACAAAAGATGATAGTGTG
GGTGGAACTGCCAGCACGGGGAACCTCTCCAGCTCATTATGGAGGAGATCC
AGGGATATGATGTAGAGTTTGACCCACCCCTGGAAAGCAAGTATGAATGCCC
CATCTGCTTGATGGCATTACGAGAAGCAGTGCAAACGCCATGCGGCCATAGG
TTCTGCAAAGCCTGCATCATAAAATCAATAAGGGATGCAGGTCACAAATGTC
CAGTTGACAATGAAATACTGCTGGAAAATCAACTATTTCCAGACAATTTTGC
AAAACGTGAGATTCTTTCTCTGATGGTGAAATGTCCAAATGAAGGTTGTTTGC
ACAAGATGGAACTGAGACATCTTGAGGATCATCAAGCACATTGTGAGTTTGC
TCTTATGGATTGTCCCCAATGCCAGCGTCCCTTCCAAAAATTCCATATTAATA
TTCACATTCTGAAGGATTGTCCAAGGAGACAGGTTTCTTGTGACAACTGTGCT
GCATCAATGGCATTGTGAAGATAAAGAGATCCATGACCAGAACTGTCCTTTGG
CAAATGTCATCTGTGAATACTGCAATACTATACTCATCAGAGAACAGATGCC
TAATCATTATGATCTAGACTGCCCTACAGCCCCAATTCCATGCACATTCAGTA

CTTTTGGTTGCCATGAAAAGATGCAGAGGAATCACTTGGCACGCCACCTACA
AGAGAACACCCAGTCACACATGAGAATGTTGGCCCAGGCTGTTCATAGTTTG
AGCGTTATACCCGACTCTGGGTATATCTCAGAGGTCCGGAATTTCCAGGAAA
CTATTCACCAGTTAGAGGGTGCCTTGTAAGACAAGACCATCAAATCCGGGA
GCTGACTGCTAAAATGGAACTCAGAGTATGTATGTAAGTGAGCTCAAACGA
ACCATTCTGAACCCCTTGAGGACAAAGTTGCTGAAATCGAAGCACAGCAGTGCA
ATGGAATTTATATTTGGAAGATTGGCAACTTTGGAATGCATTTGAAATGTCAA
GAAGAGGAGAAACCTGTTGTGATTCATAGCCCTGGATTCTACACTGGCAAAC
CCGGGTACAACTGTGCATGCGCTTGACCTTCAGTTACCGACTGCTCAGCGC
TGTGCAAACCTATATATCCCTTTTTGTCCACACAATGCAAGGAGAATATGACAG
CCACCTCCCTTGGCCCTTCCAGGGTACAATACGCCTTACAATTCTTGATCAGT
CTGAAGCACCTGTAAGGCAAAACCACGAAGAGATAATGGATGCCAAACCAG
AGCTGCTTGCTTTCCAGCGACCCACAATCCCACGGAACCCAAAAGGTTTTGG
CTATGTAACCTTTTATGCATCTGGAAGCCCTAAGACAAAGAACTTTCATTAAGG
ATGACACATTATTAGTGCGCTGTGAGGTCTCCACCCGCTTTGACATGGGTAGC
CTTCGGAGGGAGGGTTTTTCAGCCACGAAGTACTGATGCAGGGGTATAGAAGC
TTatCTGCAGatTCGGCCGCTGAGTGCAGAGGTGGCAGAACTGTATCGATTGG
AATTGGGAGATTATAAATTAGTAGAGATCACTCCAATTGGCTTGGCCCCCAC
AGATGTGAAGAGGTACACTACTGGTGGCACCTCAAGAAATAAAAGAGGGGT
CTTTGTGCTAGGGTTCTTGGGTTTTCTCGCAACGGCAGGTTCTGCAATGGGCG
CGGCGTCGTTGACGCTGACCGCTCAGTCCCGAACTTTATTGGCTGGGATAGTG
CAGCAACAGCAACAGCTGTTGGACGTGGTCAAGAGACAACAAGAATTGTTGC

GACTGACCGTCTGGGGAACAAAGAACCTCCAGACTAGGGTCACTGCCATCGA
GAAGTACTTAAAGGACCAGGCGCAGCTGAATGCTTGGGGATGTGCGTTTAGA
CAAGTCTGCCACACTACTGTACCATGGCCAAATGCAAGTCTAACACCAAAGT
GGAACAATGAGACTTGGCAAGAGTGGGAGCGAAAGGTTGACTTCTTGGAAG
AAAATATAACAGCCCTCCTAGAGGAGGCACAAATTCAACAAGAGAAGAACA
TGTATGAATTACAAAAGTTGAATAGCTGGGATGTGTTTGGCAATTGGTTTGAC
CTTGCTTCTTGATAAAGTATATACAATATGGAGTTTATATAGTTGTAGGAGT
AATACTGTTAAGAATAGTGATCTATATAGTACAAATGCTAGCTAAGTTAAGG
CAGGGGTATAGGCCAGTGTTCTCTTCCCCACCCTCTTATTTCCAGCAGACCCA
TATCCAACAGGACCCGGCACTGCCAACCAGAGAAGGCAAAGAAAGAGACGG
TGGAGAAGGCGGTGGCAACTAAAAGAAAAGGGGGGACTGGAAGGGATTTAT
TACAGTGCAAGAAGACATAGAATCTTAGACATATACTTAGAAAAGGAAGAA
GGCATCATACCAGATTGGCAGGATTACACCTCAGGACCAGGAATTAGATACC
CAAAGACATTTGGCTGGCTATGGAAATTAGTCCCTGTAAATGTATCAGATGA
GGCACAGGAGGATGAGGAGCATTATTTAATGCATCCAGCTCAAACCTCCAG
TGGGATGACCCTTGGGGAGAGGTTCTAGCATGGAAGTTTGATCCAACCTCTGG
CCTACACTTATGAGGCATATGTTAGATACCCAGAAGAGTTTGGAAGCAAGTC
AGGCCTGTCAGAGGAAGAGGTTAGAAGAAGGCTAACCGCAAGAGGCCTTCTT
AACATGGCTGACAAGAAGGAAACTCGCTGAAACAGCAGGGACTTTCCACAA
GGGGATGTTACGGGGAGGTACTGGGGAGGAGCCGGTCGGGAACGCCCACTTT
CTTGATGTATAAATATCACTGCATTTTCGCTCTGTATTCAGTCGCTCTGCGGAG
AGGCTGGCAGATTGAGCCCTGGGAGGTTCTCTCCAGCACTAGCAGGTAGAGC

CTGGGTGTTCCCTGCTAGACTCTCACCAGCACTTGGCCGGTGCTGGGCAGAGT
GACTCCACGCTTGCTTGCTTAAAGCCCTCTTCAATAAAGCTGCCATTTTAGAA
GTAAGCTAGTGTGTGTTCCCATCTCTCCTAGCCGCCGCCTGGTCAACTCGGTA
CTCAATAATAAGAAGACCCTGGTCTGTTAGGACCCTTTCTGCTTTGGGAAACC
GAAGCAGGAAAATCCCTAGCAAGATCT

pSIV-HSV1-TK-RRE-SA

TCTAGATGGAAGGGATTTATTACAGTGCAAGAAGACATAGAATCTTAGACAT
ATACTTAGAAAAGGAAGAAGGCATCATACCAGATTGGCAGGATTACACCTCA
GGACCAGGAATTAGATACCCAAAGACATTTGGCTGGCTATGGAAATTAGTCC
CTGTAAATGTATCAGATGAGGCACAGGAGGATGAGGAGCATTATTTAATGCA
TCCAGCTCAAACCTCCCAGTGGGATGACCCTTGGGGAGAGGTTCTAGCATGG
AAGTTTGATCCAACCTCTGGCCTACACTTATGAGGCATATGTTAGATACCCAGA
AGAGTTTGGAAGCAAGTCAGGCCTGTCAGAGGAAGAGGTTAGAAGAAGGCT
AACCGCAAGAGGCCTTCTTAACATGGCTGACAAGAAGGAACTCGCTGAAAC
AGCAGGGACTTTCCACAAGGGGATGTTACGGGGAGGTACTGGGGAGGAGCC
GGTCGGGAACGCCCACTTTCTTGATGTATAAATATCACTGCATTTGCTCTGT
ATTCAGTCGCTCTGCGGAGAGGCTGGCAGATTGAGCCCTGGGAGGTTCTCTC
CAGCACTAGCAGGTAGAGCCTGGGTGTTCCCTGCTAGACTCTCACCAGCACT
TGGCCGGTGCTGGGCAGAGTGACTCCACGCTTGCTTGCTTAAAGCCCTCTTCA
ATAAAGCTGCCATTTTAGAAGTAAGCTAGTGTGTGTTCCCATCTCTCCTAGCC
GCCGCCTGGTCAACTCGGTACTCAATAATAAGAAGACCCTGGTCTGTTAGGA

CCCTTTCTGCTTTGGGAAACCGAAGCAGGAAAATCCCTAGCAGATTGGCGCC
TGAACAGGGACTTGAAGGAGAGTGAGAGACTCCTGAGTACGGCTGAGTGAA
GGCAGTAAGGGCGGCAGGAACCAACCACGACGGAGTGCTCCTATAAAGGCG
CGGGTCGGTACCAGACGGCGTGAGGAGCGGGAGAGGAAGAGGCCTCCGGTT
GCAGGTAAGTGCAACACAAAAAAGAAATAGCTGTCTTTTATCCAGGAAGGGG
TAATAAGATAGAGTGGGAGATGGGCGTGAGAAACTCCGTCTTGTCAGGGAAG
AAAGCAGATGAATTAGAAAAAATTAGGCTACGACCCAACGGAAAGAAAAAG
TACATGTTGAAGCATGTAGTATGGGCAGCAAATGAATTAGATAGATTTGGAT
TAGCAGAAAGCCTGTTGGAGAACAAAGAAGGATGTCAAAAAATACTTTCGGT
CTTAGCTCCATTAGTGCCAACAGGCTCAGAAAATTTAAAAAGCCTTTATAATA
CTGTCTGCGTCATCTGGTGCATTCACGCAGAAGAGAAAGTGAAACACACTGA
GGAAGCAAAACAGATAGTGCAGAGACACCTAGTGTAGAAGCATGCTATAAC
ACATGCTATTGTAAAAAGTGTTGCTACCATTGCCAGTTTTGTTTTCTTAAAAA
AGGCTTGGGGATATGTTATGAGCAATCACGAAAGAGAAGAAGAACTCCGAA
AAAGGCTAAGGCTAATACATCTTCTGCATCAAACAAGTAAGTATGGGATGTC
TTGGGAATCAGCTGCTTATCGCCTAGTAGCACCCGGGCGGATCCATGGCTTCT
TACCCTGGACACCAGCATGCTTCTGCCTTTGACCAGGCTGCCAGATCCAGGG
GCCACTCCAACAGGAGAACTGCCCTAAGACCCAGAAGACAGCAGGAAGCCA
CTGAGGTGAGGCCTGAGCAGAAGATGCCAACCCTGCTGAGGGTGTACATTGA
TGGACCTCATGGCATGGGCAAGACCACCACCTCAACTGCTGGTGGCACTG
GGCTCCAGGGATGACATTGTGTATGTGCCTGAGCCAATGACCTACTGGAGAG
TGCTAGGAGCCTCTGAGACCATTGCCAACATCTACACCACCCAGCACAGGCT

GGACCAGGGAGAAATCTCTGCTGGAGATGCTGCTGTGGTGATGACCTCTGCC
CAGATCACAATGGGAATGCCCTATGCTGTGACTGATGCTGTTCTGGCTCCTCA
CATTGGAGGAGAGGCTGGCTCTTCTCATGCCCCCTCCACCTGCCCTGACCCTGA
TCTTTGACAGACACCCCATTCAGCCCTGCTGTGCTACCCAGCAGCAAGGTA
CCTCATGGGCTCCATGACCCACAGGCTGTGCTGGCTTTTGTGGCCCTGATCC
CTCCAACCCTCCCTGGCACCAACATTGTTCTGGGAGCACTGCCTGAAGACAG
ACACATTGACAGGCTGGCAAAGAGGCAGAGACCTGGAGAGAGACTGGACCT
GGCCATGCTGGCTGCAATCAGAAGGGTGTATGGACTGCTGGCAAACACTGTG
AGATACCTCCAGTGTGGAGGCTCTTGGAGAGAGGACTGGGGACAGCTCTCTG
GAACAGCAGTGCCCCCTCAAGGAGCTGAGCCCCAGTCCAATGCTGGTCCAAG
ACCCACATTGGGGACACCCTGTTACCCCTGTTTCAGAGCCCCCTGAGCTGCTGG
CTCCCAATGGAGACCTGTACAATGTGTTTGCCTGGGCTCTGGATGTTCTAGCC
AAGAGGCTGAGGTCCATGCATGTGTTTCATCCTGGACTATGACCAGTCCCCTGC
TGGATGCAGAGATGCTCTGCTGCAACTAACCTCTGGCATGGTGCAGACCCAT
GTGACCACCCCTGGCAGCATCCCCACCATCTGTGACCTAGCCAGAACCTTTGC
CAGGGAGATGGGAGAGGCCAACGAATTCGCATGCGTCGACTCGAGGAAGCT
TatatCTGCAGatTCGGCCGCTGAGTGCAGAGGTGGCAGAACTGTATCGATTGGA
ATTGGGAGATTATAAATTAGTAGAGATCACTCCAATTGGCTTGGCCCCCACA
GATGTGAAGAGGTACACTACTGGTGGCACCTCAAGAAATAAAAGAGGGGTCT
TTGTGCTAGGGTTCTTGGGTTTTCTCGCAACGGCAGGTTCTGCAATGGGCGCG
GCGTCGTTGACGCTGACCGCTCAGTCCCGAACTTTATTGGCTGGGATAGTGCA
GCAACAGCAACAGCTGTTGGACGTGGTCAAGAGACAACAAGAATTGTTGCGA

CTGACCGTCTGGGGAACAAAGAACCTCCAGACTAGGGTCACTGCCATCGAGA
AGTACTTAAAGGACCAGGCGCAGCTGAATGCTTGGGGATGTGCGTTTAGACA
AGTCTGCCACACTACTGTACCATGGCCAAATGCAAGTCTAACACCAAAGTGG
AACAAATGAGACTTGGCAAGAGTGGGAGCGAAAGGTTGACTTCTTGGAAGAA
AATATAACAGCCCTCCTAGAGGAGGCACAAATTCAACAAGAGAAGAACATG
TATGAATTACAAAAGTTGAATAGCTGGGATGTGTTTGGCAATTGGTTTGACCT
TGCTTCTTGATAAAGTATATACAATATGGAGTTTATATAGTTGTAGGAGTAA
TACTGTTAAGAATAGTGATCTATATAGTACAAATGCTAGCTAAGTTAAGGCA
GGGGTATAGGCCAGTGTTCTCTTCCCCACCCTCTTATTTCCAGCAGACCCATA
TCCAACAGGACCCGGCACTGCCAACCAGAGAAGGCCAAAGAAAGAGACGGTG
GAGAAGGCGGTGGCAACTAAAAGAAAAGGGGGGACTGGAAGGGATTTATTA
CAGTGCAAGAAGACATAGAATCTTAGACATATACTTAGAAAAGGAAGAAGG
CATCATACCAGATTGGCAGGATTACACCTCAGGACCAGGAATTAGATACCCA
AAGACATTTGGCTGGCTATGGAAATTAGTCCCTGTAAATGTATCAGATGAGG
CACAGGAGGATGAGGAGCATTATTTAATGCATCCAGCTCAAACCTCCCAGTG
GGATGACCCTTGGGGAGAGGTTCTAGCATGGAAGTTTGATCCAACCTCTGGCC
TACACTTATGAGGCATATGTTAGATACCCAGAAGAGTTTGGAAGCAAGTCAG
GCCTGTCAGAGGAAGAGGTTAGAAGAAGGCTAACCGCAAGAGGCCTTCTTAA
CATGGCTGACAAGAAGGAAACTCGCTGAAACAGCAGGGACTTTCCACAAGG
GGATGTTACGGGGAGGTACTGGGGAGGAGCCGGTCGGGAACGCCCACTTTCT
TGATGTATAAATATCACTGCATTTGCTCTGTATTCAGTCGCTCTGCGGAGAG
GCTGGCAGATTGAGCCCTGGGAGGTTCTCTCCAGCACTAGCAGGTAGAGCCT

GGGTGTTCCCTGCTAGACTCTCACCAGCACTTGGCCGGTGCTGGGCAGAGTG
 ACTCCACGCTTGCTTGCTTAAAGCCCTCTTCAATAAAGCTGCCATTTTAGAAG
 TAAGCTAGTGTGTGTTCCCATCTCTCCTAGCCGCCGCCTGGTCAACTCGGTAC
 TCAATAATAAGAAGACCCTGGTCTGTTAGGACCCTTTCTGCTTTGGGAAACCG
 AAGCAGGAAAATCCCTAGCAAGATCT

Materials:

Table 4.1: Plasmid Concentrations and Volumes Needed:

	Plasmids	10ug of DNA per plate (volume/plate)	Volume for 50 plates	Volume for 100 plates
1	pSIV-TRAF6- RRE-SA (260.4ug/mL)	10ug (38.5uL)	1925uL	3850uL
2	pSIV3+ (234ug/mL)	5ug (21.4uL)	1070uL	2140uL
3	pCGCG-NEF (983.7ug/mL)	2.5ug (2.5uL)	125uL	250uL
4	pNL-deltapsi- Mac239env (712.5ug/mL)	2.5ug (3.5uL)	175uL	350uL

Volume of DNA = Plate# x Volume per plate

PEI Solution:

To make a stock solution of PEI of 20mg/mL weigh out 0.568mg of PEI and dilute it with 28.4mL of DMEM serum Free. Filter the stock through a .45mm syringe filter and store at 4 degrees. The best working range of PEI concentrations is between 1mg/mL and 0.5mg/mL is an optimal working solution.

Additional Materials

HEK 293T cell line (2014 ATCC--Liquid Nitrogen Tank2 Rack 3 bottom)

10 cm dishes, 5ml polystyrene tubes, 10mL polystyrene tubes and 50mL polystyrene tubes

Tris Buffer (20m, pH 7.2)

Nalgene rapid flow 50mm filter unit (Nalgene cat. 292-4520)

Pen/Strep in -20 by desks

DMEM(+ and - serum) and RPMI with 10%FBS

All plasmids are from the giga kit production in the plasmid box 2 and 3 in the -20 by room 321

Procedure:

1. Grow HEK293T cells to confluence in multiple T75 culture flasks with DMEM+10%FBS. One day before infection, dislodge cells from one T75 flask and plate into 10cm dish (4 x 10⁶ cells per dish). (*With a 100% confluence T75 flask, which can seed five 10cm-dishes.*)

2. Grow at 37°C for 24 hours to 60-80% confluence. Remove medium from each flask and rinse with serum free medium, then add 9ml of warm, serum free medium each dish.
3. In one tube, mix 10ug/plate of the four plasmids into DMEM serum free phenol red free medium according to the chart below (Final volume of DNA+DMEM=500uL per plate).

Table 4.2: PEI Transfection Plasmid DNA Volumes For Multiple Plates:

	Plasmids	20ug of DNA per plate (volume/plate)	Volume for 50 plates	Volume for 100 plates
1	pSIV-TRAF6-RRE-SA (260.4ug/mL)	10ug (38.5uL)	1925uL	3850uL
2	pSIV3+ (234ug/mL)	5ug (21.4uL)	1070uL	2140uL
3	pCGCG-NEF (983.7ug/mL)	2.5ug (2.5uL)	125uL	250uL
4	pNL-deltapsi-Mac239env (712.5ug/mL)	2.5ug (3.5uL)	175uL	350uL
M	DMEM-Serum Free Medium (total volume 500uL/plate)	434.1uL/plate	21.705mL	43.41mL

4. In a separate tube mix 45uL/plate of the PEI (0.5mg/mL) into 455uL/plate of the DMEM serum free phenol red free medium according to the chart below (Final volume of PEI+DMEM =500uL/plate).

Table 4.3: PEI Transfection Reagent Volumes for Multiple Plates:

PEI 0.5mg/mL	45uL/plate	2.250mL/50plates	4.5mL/100plates
DMEM serum free	455uL/plate	22.750mL/50plates	45.5mL/100plates

5. Mix the DNA and PEI solutions in a tube, vortex and let them incubate at room temperature for 15mins.
6. Add 1mL of the DNA+PEI mixture to the dish dropwise covering the most area and incubate at 37°C for overnight to 6 hours. (**Each plate will receive 45uL of PEI and 10ug of DNA per 1mL of DMEM serum free medium**).

7. Remove the transfection supernatant (carefully with a pipet), add 10ml of warm DMEM -**Phenol Red** +10FBS Plus 100uL of Pen/Strep (antibiotic) medium per dish (carefully to the side of the dish).

Note: before harvesting have all tubes on ice and centrifuges **at 4 degrees**. Equilibrate the anion exchange columns (4) with 20mL (80mL needed for 18 plates) of TRIS buffer (20m, pH 7.2) by centrifugation at 1200rpm (500xg) for 5mins at 4 degrees.

8. 48 hours post transfection, harvest supernatant on ice in 50mL tubes. Centrifuge the supernatant at 1200rpm for 5mins at 4 degrees.
9. Filter the supernatant with either the *.45mm syringe filter (<50 dishes)* or the *nalgene rapid flow 50mm filter with vacuum attachment (>50 dishes)* unit into a new flask.
10. Add the filtered supernatant to the equilibrated anion exchange columns 20mL in each and centrifuge at 1200rpm (500xg) for 5mins at 4 degrees. Virus will bind to the column (Columns can each receive an additional 30mL of harvest supernatant before losing binding capacity--Total **50mL** per column) Discard the flow through.
11. Elute the virus by adding 2mL of a 1.5M filtered NaCl solution and centrifuging the column at 1200rpm (500xg) for 5mins at 4 degrees.

12. Filter the eluted virus through a .45mm filter and transfer to the Vivaspın20 columns (be sure to balance two tubes before centrifugation) Centrifuge the Vivaspın20 column at 7500g for 10mins at 4 degrees.
13. Dilute the concentrated virus with filtered DI water) until the NaCl concentration is 0.13M. (**roughly 2mL assuming .2mL remaining in the column**)
14. In the same Vivaspın20 column, spin the diluted virus again. (be sure to balance two tubes before centrifugation) Centrifuge the Vivaspın20 column at 7500g for 10mins at 4 degrees.
15. Store the concentrated virus at -80 degrees until ready for shipment.

Bibliography

- Dalby, Brian, et al. "Advanced transfection with Lipofectamine 2000 reagent: primary neurons, siRNA, and high-throughput applications." *Methods* 33.2 (2004): 95-103.
- Fischer, Ute, et al. "Mechanisms of thymidine kinase/ganciclovir and cytosine deaminase/5-fluorocytosine suicide gene therapy-induced cell death in glioma cells." *Oncogene* 24.7 (2005): 1231-1243.
- Felgner, Philip L., et al. "Lipofection: a highly efficient, lipid-mediated DNA-transfection procedure." *Proceedings of the National Academy of Sciences* 84.21 (1987): 7413-7417.
- Iyer, Subashini R., et al. "Measurement of human immunodeficiency virus type 1 preintegration transcription by using Rev-dependent Rev-CEM cells reveals a sizable transcribing DNA population comparable to that from proviral templates." *Journal of virology* 83.17 (2009): 8662-8673.
- Gardner, Matthew R., et al. "AAV-expressed eCD4-Ig provides durable protection from multiple SHIV challenges." *Nature* (2015).
- He, Liusheng, et al. "TRAF6 regulates cell fate decisions by inducing caspase 8-dependent apoptosis and the activation of NF- κ B." *Journal of biological chemistry* 281.16 (2006): 11235-11249.
- Jordan, Martin, and Florian Wurm. "Transfection of adherent and suspended cells by calcium phosphate." *Methods* 33.2 (2004): 136-143.
- Longo, Patti A., et al. "Transient mammalian cell transfection with polyethylenimine (PEI)." *Methods in enzymology* 529 (2013): 227.
- Okada, Takashi, et al. "Scalable purification of adeno-associated virus serotype 1 (AAV1) and AAV8 vectors, using dual ion-exchange adsorptive membranes." *Human gene therapy* 20.9 (2009): 1013-1021.

- Pyle, Stephen W., et al. "Purification of 120,000 dalton envelope glycoprotein from culture fluids of human immunodeficiency virus (HIV)-infected H9 cells." *AIDS research and human retroviruses* 3.4 (1987): 387-400.
- Reed, Sharon E., et al. "Transfection of mammalian cells using linear polyethylenimine is a simple and effective means of producing recombinant adeno-associated virus vectors." *Journal of virological methods* 138.1 (2006): 85-98.
- Schenborn, Elaine T., and Virginia Goiffon. "Calcium phosphate transfection of mammalian cultured cells." *Transcription factor protocols* (2000): 135-145.
- Sun, Xiangjie, et al. "Role of clathrin-mediated endocytosis during vesicular stomatitis virus entry into host cells." *Virology* 338.1 (2005): 53-60.
- Tomicic, Maja T., Rudolf Thust, and Bernd Kaina. "Ganciclovir-induced apoptosis in HSV-1 thymidine kinase expressing cells: critical role of DNA breaks, Bcl-2 decline and caspase-9 activation." *Oncogene* 21.14 (2002): 2141.
- Wu, Yuntao, Margaret H. Beddall, and Jon W. Marsh. "Rev-dependent lentiviral expression vector." *Retrovirology* 4.1 (2007): 1.
- Yamada, Kaoru, et al. "Lentivirus vector purification using anion exchange HPLC leads to improved gene transfer." *Biotechniques* 34.5 (2003): 1074-1081.
- Yee, Jiing-Kuan, et al. "A general method for the generation of high-titer, pantropic retroviral vectors: highly efficient infection of primary hepatocytes." *Proceedings of the National Academy of Sciences* 91.20 (1994): 9564-9568.
- Zufferey, Romain, et al. "Multiply attenuated lentiviral vector achieves efficient gene delivery in vivo." *Nature biotechnology* 15.9 (1997): 871-875.

Biography

Brian Hetrick grew up in Woodbridge Virginia. He attended George Mason University where he received he Bachelor of Science in Biology in 2014. He went to receive his Master of Science in Biology from George Mason University in 2017. He will be starting his Doctorate in Biology in the fall of 2017.