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Melissa Querrey March 30, 2016

Optimizing the Testing of VLR-CAR in Lymphoid Cells

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Abstract

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By Melissa Querrey

Gene therapy has not only become an increasingly popular method for treating and curing genetic disorders but also in engineering cells for cancer treatment. Through utilization of chimeric antigen receptors (CAR), which include variable lymphocyte receptors (VLR), immunocompetent cells can be directed to target specific antigens common to malignant tumors. To aid in the advancement of this technology, we sought to optimize the method used to efficiently test various VLR containing CAR constructs by looking at gene transfer methods involving transient transfection methods and viral transduction using both AAV and lentivirus-based vectors into lymphoid cells. We have determined an efficient method to rapidly test expression and function of several VLR structures through cells via lentiviral transduction. The ability to use a high-throughput screening method to test for functional VLR-CAR constructs will enhance the process for VLR-CAR characterization and possible selection for clinical translation. However, whether this method is a high-throughput system is yet to be determined. Due to low transduction efficiency of VLR-CAR constructs into more clinically relevant immunocompetent cells with lentivirus, current efforts are focused on an *in vivo* bone marrow transplant model to proceed testing of the functionality of the VLR-CAR constructs. This study is a gateway to the development of alternative cancer immunotherapies with immunocompetent cells.

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Tables, Graphs, and Figures

Introduction

The capability to introduce DNA into human cells is the foundation for the field of gene therapy.[1] Gene therapy introduces genetic information into the cells of patients, which can encode for a variety of constructs, such as engineered gene constructs or genes endogenous to the body that are lacking because of a certain disorder. The expression of these gene products can subsequently cure or effectively treat disorders.[2] Globally, gene therapy has gained increasing support and funding, making it a front-runner for the development of new therapies in a widening range of diseases it can treat.

One of the most recent applications for gene therapy is cancer treatment. Gene therapy for cancer treatment has become a priority because of its potential to combat the negative side effects associated with current therapeutic interventions, including chemotherapy. Many problems with chemotherapy arise from toxic effects on rapidly dividing cells, which not only affect tumor cells but also affect other cell types that are important to normal body function, such as epithelial cells and white blood cells. With chemotherapy, the decrease in rapidly dividing cells involved in normal body function can cause several acute side effects, such as fatigue or nausea, or severe toxicities, such as peripheral neuropathy and leukopenia, the reduction of white blood cells.[3]

The toxicities caused by chemotherapy on the body raises questions about the consequences it may have on patient quality of life and the ethical implications of administering chemotherapy as the only treatment option for cancer patients. Several studies have shown that in addition to acute and severe side effects caused by treating cancer with chemotherapy, there is also a radical change in the psychology of patients, such

as an increased risk of depression.[4,5,6] In an effort to address these concerns, alternative treatment methods have become increasingly popular in the past decade, one being gene therapy by way of immunotherapy for cancer.

Immunotherapy for cancer utilizes gene therapy methods to make the cells of the immune system re-recognize cancer cells or enhance the immune cells with engineered constructs.[7] Several immunotherapy strategies have been established, such as vaccines and checkpoint inhibitors, to produce anti-tumor immunity in the body.[8] Another cancer immunotherapy method that has become more advanced in the past decade is the introduction of chimeric antigen receptors (CAR) into T cells.[9]

The CAR frenzy started with its creation in Israel by Dr. Zelig Eshar, who later teamed up with Dr. Steven Rosenberg at the University of Pennsylvania to develop the first CAR treatments for melanoma.[10] Since then, the spotlight has been on the rapidly developing market for CAR therapies, with companies such as Juno, that target CD19 for Acute Lymphocytic Leukemia (ALL), Non-Hodgkins Lymphoma, and Chronic Lymphocytic Leukemia (CLL). In addition, MD Anderson is using CAR therapies to target PD-1, a death receptor found on tumor cells.[11,12] Chimeric antigen receptors (CAR) are engineered proteins that combine an antigen-binding domain, which target a tumor-specific antigen, with transmembrane and cytoplasmic domains of the T cell receptors.[13] The chimeric antigen structure used in many labs, including ours, consists of a single-chain fragment variable (scFv), a heavy and light chain linked protein from immunoglobulin as the antigen binding domain, a CD28 transmembrane region, which assists in T cell activation, and a CD3ζ cytoplasmic region to couple antigen recognition to signaling pathways in T cells

(figure 1).[14,15] In essence, a CAR combines the effective antigen binding and specificity found in humoral immunity with the anti-tumor capabilities of a cytotoxic T cell.[16]

Introducing a CAR construct into T cells has several advantages, including increased antigen specificity and propagation of T cells to the tumor site.[17] One of the amazing and yet most horrible aspects of cancer is not only its ability to immortalize and continuously divide but also its ability to evade the immune system by down-regulation of target molecules or decreasing expression of target proteins. CAR mediated T cells utilize antibody-like binding, which can avoid tumor evasion mechanisms and target the tumor specific antigens found on the cell surface.[13]

Even with the added specificity that CARs have, there are still issues with off-site targeting and activation of T cells. A novel idea to increase antigen specificity and recognition is to utilize the functional component in immune system of jawless vertebrates, variable lymphocyte receptors (VLR). Discovered by Dr. Max Cooper at Emory University, VLRs are derived from the adaptive immune system of jawless vertebrates, like lamprey and hagfish.[18] Jawless vertebrate immune systems have cells very similar to human based immune cells; however, instead of being immunoglobulin based like jawed-vertebrates the VLR is the functional unit. Each VLR have variable numbers of leucine rich repeats (LRRs), which upon maturation, somatically rearrange to form complex and unique sequences.[19] The number of LRRs contribute to the concavity of the receptor and the variability of the antigen binding region of the VLR (figure 2).[20,21,22]

VLRs have been found to have high specificity towards particular antigens and bind with high avidity, the interaction strength between the antigen and the VLR, even more so than T cell receptors (TCR), because VLRs bind to antigen via β-pleats, whereas

immunoglobulin based receptors bind to antigen with an extended loop structure.[23] In our CAR construct, we use the VLR as the antigen-binding domain, instead of the scFv. By using the VLR, we can target a larger repertoire of tumor specific antigens than with an scFv binding-domain. The addition of the VLR will hopefully facilitate less off-site targeting in addition to effectively activating T cells through the CAR construct (figure 3)[20]. However, we need an effective way to analyze new VLRs.

By working with Dr. Max Cooper, we have developed several VLRs to target specific tumor antigens, ranging from Neuroblastoma, T cell leukemia, and murine B cell lymphoma (BCL). To develop the VLRs for an antigen, we first immunize lampreys with a specific antigen, which in our case, are tumor antigens. We then isolate, amplify, and create a VLR yeast library. Lastly we clone the yeast, and eventually screen and sequence all VLRs that actively bind to the antigen. For each particular antigen there can be a large quantity of VLRs that bind. However, we need to determine the VLR with the best expression and function. In order to do this, we need a high throughput screening method to test the expression and functionality of all the VLR-CAR cassettes produced by a rapid and efficient method. The VLR sequence that we will be focusing on is the BCL VLR-CAR cassette.[24]

Screening Methods Background

In order to determine which VLR-CAR cassettes to further develop, we need a rapid high throughput system to test the expression and functionality of the cassettes in cells. The most common system used in gene therapy to introduce a gene construct into cell lines is a viral vector delivery system. Viral gene transfers, specific to lentivirus and γ-retrovirus, utilize a self-inactivating viral vector with a plasmid containing the gene of

interest. The engineered virions then infect the cells that are to be transduced with the virus.[25] The type of viral system used for gene transfer is usually dependent on the specific tropism, or what specific cell receptor the virus targets, whether the respective receptor is on the cells of interest, and how that particular virus introduces the DNA or provirus containing the transgene into the cell. This is usually done by either integration or leaving the proviral sequence, DNA, or RNA, episomal.

A common viral vector used is the lentivirus system. Dependent on what the lentivirus is pseudotyped with, the virus will recognize different receptors. Our lentivirus is pseudotyped with Vesicular Stomatitis Virus glycoprotein, or VSV-G, which targets the low-density lipoprotein receptor (LDLR) on cells and subsequently integrates the proviral sequence into the genome upstream of transcriptional start sites.[26, 27] In addition to the lentiviral method of gene transfer, there are other viral vectors that can be used, such as recombinant γ-retrovirus, and adeno-associated virus (AAV). There have been several gene therapy studies utilizing γ-retrovirus as the gene transfer vector.[28-30] Similarly to lentiviral method of gene transfer, γ-retrovirus integrates the DNA into the genome; however, the DNA is integrated into actively transcribing regions. In addition, since our γ-retrovirus is pseudotyped with an amphophile envelope, the vector targets Pit-2 receptors, a phosphate transporter.[31] Unlike both the γ-retrovirus and lentivirus delivery systems, recombinant AAV does not integrate the DNA into the genome because the proteins necessary for effective integration are absent. The DNA is subsequently left episomally in the cell, which will gradually be lost if the cell rapidly divides. The tropism of AAV is dependent on the serotype and is not well characterized.[32, 33]

Even though viral delivery systems for gene transfer are common, the process itself is rather slow, with the rate-determining factor being the viral construct production and subsequent titering of the virus; a process which could take a couple of weeks. In order to test our VLR-CAR constructs, we studied other methods that could potentially have a more rapid and efficient gene transfer system.

Transient methods of transfection are notably quicker because instead of a viral intermediate, these methods utilize only plasmid. In addition, when compared to viral gene transfer, transient methods do not rely on specific cell receptors, do not have Brownian motion as a factor, nor viral decay. Even though the method of gene transfer into cells is much less time consuming than viral gene transfer, transient transfection leaves DNA in the cell episomally. If the cell targeted for gene transfer divides, the episomal expression of the transgene will gradually decrease. A common method of transient transfection is electroporation. Electroporation creates momentary pores in the plasma membrane by disrupting the lipid bilayer with a high voltage electric shock. DNA plasmids can then enter the cell because of a rise in electrical potential of the cell.[34, 35] An advantage of electroporation is being able to rapidly and stably transfect a large quantity of cells. However, electroporation tends to be toxic to the cells resulting in lower cell viability, even after transfer to an appropriate growth medium.[36]

Another method related to electroporation is nucleofection. Nucleofection is a form of electroporation in that it is a plasmid plus cell technology that creates momentary pores; however, it is optimized from standard electroporation methods. Nucleofection also creates momentary pores in the plasma membrane, but nucleofection technology utilizes an optimized electrical charge per cell type with a cell-specific solution that directs DNA

plasmid not only into the cell itself, but also directly into the nucleus.[37, 38] This is a more efficient method to introduce plasmid DNA into cells for a rapid, large quantity method of gene transfer. In addition, due to the optimized electrical pulses and specific solutions, the cells experience substantially less toxicity and physiological changes after the shock than electroporation.[36]

Potential Cell Targets

One of the primary aims of this study was to determine and optimize the most efficient gene transfer method for lymphoid cells in order to test the expression and function of several VLRs in a library. For laboratory testing purposes, the cell line used to optimize the methods was the Jurkat cell line, an immortalized T cell line. However, as the project progressed, a gene transfer method needed to be optimized for more clinically relevant cell lines, such as the various lineages of primary innate and αβ T cells.

One of the most well studied cell type that has been show to target tumor with CAR constructs is the T cell.[9, 12, 39, 40] Most CAR studies with T cells use a particular type of T cell, the αβ T cell, where α and β are the type of T cell receptor expressed on the surface of the cell. Another type of T cell used in gene therapy is the γδ T cell. The main difference between the two receptor types is that αβ T cells function mostly as cytotoxic or helper cells that classically require antigen display from an MHC molecule, whereas γδ T cells do not require antigen presentation by an MHC molecule.[41] In addition, γδ T cells recognize several different molecules when compared to αβ T cells, which are mainly classes of stress related proteins. γδ T cells have been shown to have a variety of immunotherapy

applications, including antitumor activity *ex vivo* and *in vitro*.[42-44] A drawback to the γδ T cells for laboratory testing is difficulty in expanding cell populations.

A cell type that does expand well, but less studied in gene therapy, are natural killer (NK) cells. NK cells also do not rely on MHC recognition but can be activated by complexes through CD3ζ, which CARs contain.[45] CAR modified NK cells can target antigens associated with several malignancies by expressing VLR-CAR, which can also activate the cytotoxic capabilities of the NK cell at the same time. There have been several studies looking at NK cells for their potential role in cancer immunotherapies and the possibility of acquiring more efficient anti-tumor properties through the expression of CAR constructs.[46-48]

Aims of This Study

In the current study, we focus on three main aims: i) to determine an effective method of gene transfer of VLR-CAR into immunocompetent cells by investigating transient and viral transfection methods, ii) to employ the optimized method to test the expression and function of the VLR-CAR, and iii) initiate murine bone marrow transplant protocols to test VLR-CAR expression in primary murine lymphocytes. Overall, the main goal is to determine and optimize a high throughput screening method for laboratory testing of VLR-CAR constructs, which could rapidly accelerate our efforts with VLR-CAR as a potential cancer therapy. To accomplish this, we first investigated gene transfer efficiency into the Jurkat cell line by transient transfection and viral transduction methods. We then tested the expression and function of VLR-CAR constructs by two optimized methods, nucleofection and viral transduction into Jurkat cells as well as primary lymphocytes. Lastly, we initiated the process of an *in vivo* murine bone marrow transplant model by

transducing bone marrow cells with recombinant lentiviral vector and then transplanting the genetically engineered cells into mice.

Methods

Electroporation

To determine the optimal gene transfer system, we first looked at a transient transfection via electroporation. We used a BTX electroporator and BTX protocol number 542. For the experimental group, the electroporator parameters were low voltage (LV), voltage of 320V, resistance of 250μF, and no resistance. Per each sample, we harvested 10^7 Jurkat cells, washed twice with ice-cold PBS, and then transferred the Jurkat cells to 500μL of chilled PBS with various concentrations (between 5-40μg) of green fluorescent protein (GFP) plasmid in a 4mm gap BTX disposable cuvette. The cuvette was then electroporator with the protocol parameters. Cells incubated in the cuvette for 10 minutes at room temperature and then carefully pipetted into a 6-well plate with up to 2mL of complete DMEM medium containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (P/S).

Several other parameters were tested with the electroporator to test for the transfer of GFP plasmid into Jurkat cells. Results found in table 1.

Expression of the plasmid was quantified via flow cytometry 48 hours post transfection.

Nucleofection

Another method of transient transfection that was tested was Lonza Amaxa Nucleofector. We used a protocol optimized specifically for Jurkat cells, Amaxa Cell Line Nucelofector Kit V. Per sample, 10^6 Jurkat cells were harvested, spun, and aspirated. The next steps were

done in less than 10 minutes because the amount of time the cells are in the nucleofector solution was time sensitive (as written in protocol V). The cells were resuspended in 100µL of room temperature Lonza Nucleofector Solution. The solution was combined with 2µg of plasmid DNA, either pmaxGFP (a GFP plasmid provided by Lonza) or a VLR-CAR construct. The solution was transferred to a supplied cuvette and shocked in the nucleofector with program X-001, parameters specific to Jurkat cell lines. Complete DMEM at a volume of 500µl was added to the cuvette and the sample was transferred to 12-well plate with a volume of 1.5ml. Flow cytometry was used to quantify the expression of the plasmid 48 hours post transfection.

Lentiviral Vector Transduction

In order to determine the efficiency of viral gene transfer, we used lentiviral constructs to transduce cell lines with lenti-GFP and lenti-VLR-CAR constructs. The virus was produced at Expression Therapeutics (ET). In a 6-well plate, K562 cells were plated at 10^6 in 1mL in complete DMEM per well. The cells were then statically transduced with the virus at an MOI of 5. Media was changed 24 hours post transduction. Flow cytometry and fluorescent microscopy was used to quantify GFP expression.

Jurkats cells were plated in a 6-well plate at 10^6 cells in 1mL in complete DMEM per well. The cells were then statically transduced with lentiGFP virus at an MOI of 5. Media was changed 24 hours post transduction. Flow cytometry was used to quantify GFP expression.

Again, Jurkats cells were plated in a 6-well plate at 10^6 cells in 1mL in complete DMEM per well. The cells were then statically transduced with the BCL-CAR virus at MOIs of 2 and 10. Media was changed 24 hours post transduction. Flow cytometry was used to quantitate viral transgene expression at 48 hours post transduction.

BCL Assay

After lentiviral transduction of the BCL-CAR construct, 300k transduced cells were cultured with $3x10^5$ BCL cells for 4 hours. After incubation, cells were stained with anti-CD45, anti-myc, and anti-CD69 antibodies. Flow cytometry was used to determine whether or not the presence of BCL cells activated the BCL-CAR construct in Jurkat cell line.

Plasmid Production for Gamma-Retrovirus

MSCV-CAR

A) MSCV-neo plasmid and BCL-CAR plasmid were digested using enzymes AgeI/SalI and AgeI/XhoI respectively to isolate the MSCV promoter and BCL-CAR sequences (figure 4). The plasmid DNA at 1µg was digested with the 10 unites of each enzyme, 5µl of buffer, and water up to 20µl. The sample was incubated for 45 minutes at 37°C.

B) The digested plasmid was run on a 1% agarose gel at 115V to check for correct digest fragments.

C) The correct plasmid size band corresponding to the MSCV promoter and the BCL-CAR solely, was cut out of the gel and DNA fragments were collected via Qiagen Gel Extraction protocol.

D) The plasmid fragments were ligated using NEB ligation protocol for both a 3:1 ligation and 7:1 ligation.

E) The ligated plasmid was amplified via cloning by transformation into competent Stbl3 cells. The media was spread onto LB-Ampicillin plates to select for correct clones. The plates were incubated overnight at 37°C.

F) Bacterial colonies were selected from the LB plates and incubated in LB-ampicillin broth overnight at 37°C.

G) DNA was extracted from the bacterial stock grown using Qiagen Mini Prep Spin Kit and eluted in water.

H) DNA extracted from all samples was screened with NheI to check for ligation success following the restriction digest protocol from step A and subsequently run on a gel for quantification.

I) One colony was picked to form larger bacterial stock based off of step H digest. The stock was grown overnight at 37°C. The DNA was extracted from the stock by Qiagen Midi prep kit.

J) The MSCV-CAR plasmid was screened using a restriction digest protocol from step A using plasmids AscI, SgrAI, BslEII, and SphI. The digested plasmid was then run on a gel at 115V. The plasmid was rescreened using the enzymes DrdI, EcoRV, NaeI, and XmaI and then run on a 1% agarose gel after digest.

MSCV-GFP plasmid

A) MSCV-neo plasmid and SIV-GFP plasmid were digested using enzymes AccI/AgeI and ClaI/AgeI respectively to isolate the MSCV promoter and GFP sequences (figure 5). The

plasmid DNA at 1µg was digested with the 10 unites of each enzyme, 5µl of buffer, and water up to 20µl. The sample was incubated for 45 minutes at 37°C

B) Digested plasmid was run on a 1% agarose gel at 115V to check for correct digest fragments.

C) The correct plasmid size band corresponding to the MSCV promoter and the BCL-CAR solely, was cut out of the gel and the DNA fragments were collected via Qiagen Gel Extraction protocol.

D) The plasmid fragments were ligated using NEB ligation protocol for both a 3:1 ligation and 7:1 ligation.

E) The ligated plasmid was amplified via cloning by transformation into competent cells using Stbl3 cells. The media was spread onto LB-Ampicillin plates to select for the correct clones. The plates were incubated overnight at 37°C.

F) Bacterial colonies were selected from the LB plates and incubated in sterile LB broth overnight at 37°C.

G) DNA was extracted from the bacterial stock grown using Qiagen Mini Prep Spin Kit and eluted in water.

H) DNA extracted from all samples was digested with SphI to check for ligation success following the restriction digest protocol from step A and subsequently run on a gel for quantification.

I) One colony was picked to form larger bacterial stock based off of step H digest. The stock was grown overnight at 37°C. The DNA was extracted from the stock by Qiagen Midi prep kit.

J) The MSCV-CAR plasmid was screened using a restriction digest protocol from step A using plasmids DrdI, BsrGI, and EagI. The digested plasmid was then run on a 1% agarose gel to check for correct plasmid sequence.

γ-Retrovirus Production

To further optimize the viral transduction method, we looked into a different viral type, γ-retrovirus. We produced the virus in-house. Phoenix Ampho cells were cultured and then split into ten, 10cm plates with complete DMEM. This cells were allowed to reach ~70% confluence. In order to make the virus, we used a calcium phosphate transfection using the in-house made MSCV-GFP plasmid. The supernatant on the plates were collected 48 hours, 72 hours, and 96 hours post transfection. The supernatants were then filtered with a 0.22μm filter and then concentrated by spinning overnight at 10,000g. The pellet was then resuspended into 1mL of complete DMEM and stored at -80°C. This production was repeated again using Addgene plasmid MSCV-IRES-eGFP.

Rough test of γ-Retrovirus MSCV-GFP

To test if the in-house γ-retrovirus with MSCV-GFP was functional virus, we transduced Jurkat cell lines. We plated 300,000 Jurkat cells in a 24-well plate and then statically transduced the Jurkat cells with 300μL of unconcentrated γ-retro-MSCV-GFP virus with polybrene. The cells incubated overnight at 37°C. At 24 hours post transduction, media was changed to complete DMEM. Quantification of viral transduction success was measured by fluorescent microscopy of the GFP 48 hours post transduction.

Lipofectamine with MSCV-GFP

To test whether the viral production or the MSCV-GFP plasmid was correct, Jurkat cells were transfected with MSCV-GFP by Invitrogen's Lipofectamine 2000 protocol. Jurkats cells were plated at 10^6 in a 6 well plate and then transfected with respective lipofectamine solution volume with the recommended 4μg of MSCV-GFP. Another sample was transfected with two times the amount of DNA at 8μg.

Fluorescent microscopy was used 48 hours post transfection to check for successful gene transfer.

Sequencing of MSCV-GFP Plasmid

In order to compare the in-house made MSCV-GFP plasmid with the gene map of the plasmid, we sent the plasmid to Beckman-Coulter for sequencing.

γ-Retrovirus MSCV-IRES-eGFP Titering

The in-house made γ-retrovirus with MSCV-IRES-GFP was titered by transducing HEK-293T cell line and 3T3 cell line for a control. In a 6-well plate, 150,000 cells per sample were cultured in complete DMEM and complete RPMI respectively. Cells incubated and expanded overnight to ~200,000 cells per well. For each cell type, 0μl, 10μl and 100μl volumes of γ-retrovirus MSCV-IRES-eGFP was added to different wells to statically transduce. Cells were incubated with the virus overnight. At 24 hours post transduction, media was changed on the cells.

48 hours post-transduction, flow cytometry was used to quantify the expression of the GFP plasmid in cells. Viability counts of each sample were also recorded. This procedure was repeated twice.

AAV-GFP Transduction of PBMCs

In order to further optimize viral transduction method, Adeno-Associate Virus was also used to transduce pan T cells isolated, which was done by others in the lab, from peripheral blood mononucleocytes (PBMC). Three AAV strains were used to transduce PBMCs: 6wt, 6.663, and 6m3. In a 6-well plate, 200,000 PBMCs were plated in each sample. The PBMCs were statically transduced with each type of AAV with both volumes of 5µl and 50µl MOI 5000 and 50,000 respectively. As comparison, 200,000 PBMCs in a 6-well plate and transduced with 15µl of lentiGFP, MOI 15. After 24-hour post transduction, the media was changed to fresh media.

Flow cytometry was used both 6 days and 8 days post transduction to quantify GFP expression in the PBMCs. Data from day 8 is shown.

AAV-GFP Transduction of NK92 Cells

300k NK-92 cells were plated in a volume of 300µl of RPMI with 20% FBS in a 48 well plate. NK-92 cells were transduced with volume of 5µl or MOI 50,000, by different AAV viruses, 6wt, 6.663, 6m3, to a well respectively. Media was changed 24 hours post

transduction. Flow cytometry was done day 3 post-transduction and day 7 post-transduction. Fluorescent microscopy was done from day 3 to day 6 post-transduction. Doubling time was calculated by (Cells count at time point)=(Initial Cell Count)*2^(time*frequency in time unit).

Sample Size Determination

In order to determine the amount of Balb/c mice needed for the experiment, a power analysis was performed via an online calculator, http://powerandsamplesize.com. The true mean, null hypothesis mean, and standard deviation were determined by IACUC standards for tumor growth in mice. Null hypothesis mean was equivalent to the maximum size a tumor can be in a mouse, $2cm^2$, standard deviation was determined by a 70% decrease in tumor size, $1.4cm^2$, and true mean was determined by subtracting the standard deviation from null hypothesis mean, $0.6cm^2$.

Bone Marrow Extraction

In order to extract bone marrow cells (BMC), Balb/c mice were euthanized according to IACUC standards. Bone marrow was isolated from the tibia and femur by flushing with PBS. Cells were mixed well and then placed through a 40µm nylon mesh to remove cell clumps. The BMCs collected were spun at 300g for 10 minutes at 4°C, supernatant was aspirated, and the cells resuspended in 1mL of buffer per 10^8 cells. To the cells, 5µg Biotin anti-Sca antibody (0.5µg/µl) was added per 10^8 cells, incubated at 4°C for 20 minutes, vortexing every 5 minutes. Cells were washed and resuspended in buffer. To the cells, 20µl of anti-biotin microbeads were added and incubated for 15 minutes at 4°C. Cells were

washed again in buffer, spun at 300g for 10 minutes, and the supernatant was completely removed. The pellet was resuspended in 3mL of PBS with 1% BSA and applied to a separation column. Column was washed three times with buffer and the effluent was collected as the negative fragment. Column had buffer applied again twice and cells were firmly flushed out as the Sca+ fraction. Cells were spun and brought up in StemPro supplemented with cytokines and then cultured at 37°C in 10cm dish overnight.

Transduction of Bone Marrow Cells with Lenti-BCL-CAR

After 3 days post Sca+ isolation, cells were collected from the plate, spun down, and then resuspended in: mSCF, mIL-3, hIL-1l, hFlt-3, L-glutamine, P/S, polybrene, and 300µl of lentiBCL-CAR. Cells were incubated overnight at 37°C. Another 300µl of virus was added to cells. Cells incubated for 12 hours, spun down, and resuspended in 10uL of sterile PBS per 10^6 cells.

Balb/c Mouse Bone Marrow Transplant

On the fourth day post Sca-cell selection, the Balb/c mice were irradiated with a lethal dose of radiation. Radiation was split into two doses at 550 rad each dose, one in the morning and the other in the afternoon. Three mice were then subjected to a retro-orbital injection of $1x10^6$ transduced bone marrow cells with the lenti-BCL-CAR. Three mice were also injected with $1x10^6$ naïve Sca+ cells. Mice were then placed in sterile facility.

Results

Electroporation Studies

In this study, the electroporation protocol was established using Jurkat cells with a GFP expression plasmid. Several parameters were tested in an effort to optimize the electrical pulse delivered to the cells (table 1). Cell morphology was visibly altered after many of the pulses were applied and the viability of the cells was drastically reduced (table 2). In addition, when each sample was quantified via flow cytometry, all samples had a similar result of <3% gene expression of the GFP plasmid. The first parameter results as assessed by flow cytometry had the best viability count and gene expression out of all the trials, with viabilities close to 80% and gene expression around 5% in a DNA concentration dependent gradient. GFP expression was 0.5%, 1.0%, 2.1%, and 6.7% for naïve Jurkat cells and Jurkat cells electroporated at 5μg, 20μg, and 40μg DNA respectively (figure 6 and 7). Not all flow cytometry data shown.

Nucleofection Studies

Jurkat Cells with GFP plasmid

Jurkat cells were nucleofected with 5μg of pmaxGFP plasmid with the X-001 program from Lonza. GFP expression was quantified via flow cytometry and yielding 22.9% GFP expression (figure 8).

Jurkat cells with VLR-CAR Plasmids

Jurkat cells were nucleofected using the X-001 program with 5μg of pmaxGFP, Neuroblastoma VLR-CAR (Nbl-CAR), and CD5-CAR. Flow cytometry results showed that

the pmaxGFP was expressed in 85.9% of the cell population, a substantial improvement compared to the previous nucleofection with pmaxGFP. The VLR-CAR plasmid expression levels were measured using flow cytometry against the myc-tag in the CAR construct. Both the Nbl-CAR and CD5-CAR quantified expression around 0%. Surprisingly, there was still a moderate yield of activation from the CD5-CAR, which because Jurkat cells express CD5 on their surface, can be activated when expressed in Jurkat cells VLR-CAR construct. However, the Nbl-CAR also had a slight amount of activation, which is not a self-activating VLR-CAR because Jurkat cells do not express Neuroblastoma antigen (figure 9).

Using the same plasmid system, pmaxGFP, Nbl-CAR, and CD5-CAR, the concentration of DNA used was varied. For Nbl-CAR and CD5-CAR, the amounts were doubled, so the Jurkat cells were nucleofected with program X-001 with 10μg of plasmid DNA. However, since the pmaxGFP yielded already around 90% expression with only 5μg of plasmid DNA, the concentration was halved to 2.5μg of plasmid DNA. Both of the VLR-CAR constructs still yielded 0% expression. CD5-CAR activation also did not change regardless of the doubled plasmid DNA concentration. However, the Nbl-CAR activation increased 2-fold. (figure 10).

Lentiviral Transduction Studies

Lentivirus-GFP with K562 cells and Jurkat cells

K562 cells were statically transduced with Lenti-GFP virus at an MOI of 5. Flow cytometry showed GFP expression at 80.2% with visibly bright GFP expression in the cells as seen by fluorescent microscopy (figure 11).

We also transduced Jurkat cells with LentiGFP at MOI 5. Flow cytometry quantified expression of GFP in 99.5% of Jurkat cells (figure 12). Regardless of the inadequate shifting of the GFP fluorescent channel on the flow cytometer, this should not affect the GFP expression quantification in the table.

Lenti-BCL-CAR with Jurkat Cells Expression and Activation via Co-Culture Assay

Jurkat cells were transduced with lenti-BCL-CAR at MOI 2 and 10. Flow cytometry quantified the expression of the CAR on Jurkat cells by measuring Myc tag expression on the Jurkat cells. Jurkat cells transduced at an MOI 2 had 65.3% CAR expression while MOI 10 transduced cells had 89.3% CAR expression (figure 13).

Again, Jurkat cells were transduced with Lenti-BCL-CAR at MOI 2 and 10. The transduced cells were then cultured with BCL cells to show cell activation when subjected to the BCL antigen. Via flow cytometry, Jurkat cells were selected from the cell population by human-CD45 expression. Jurkat cells expressing the CAR construct were quantified by Myc-tag expression on the cells: naïve Jurkat cells had 0.2% CAR expression, Jurkat cells transduced at MOI 2 had 40.0% CAR expression, and MOI 10 transduced Jurkat cells had 90% CAR expression. Finally, Jurkat cells with the CAR were measured for activation by CD69 expression: naïve Jurkat cells were activated by CAR 0.2% of cells, MOI 2 transduced Jurkat cells were activated by CAR 20.7% of cells, and MOI 10 transduced Jurkat cells were activated by CAR in 42.3% of cells (figure 14). This shows a dose dependent expression and activation in Jurkat cells when transduced with increasing amounts of lenti-BCL-CAR.

This was repeated several times. A summary of the results is reported (table 3, graph 1).

γ-Retrovirus Studies

Production of MSCV-BCL-CAR Plasmid

MSCV-neo and BCL-CAR plasmid were digested according to protocol and screened (figure 15). According to the screening map and confirmed by the gel from the digest, with DrdI there are 2 DNA fragments at 1869 and 4712 base pairs, with EcoRV there are 3 DNA fragments at 3385, 253, and 2943 base pairs, with NaeI there are 2 fragments at 799 and 5782 base pairs, and with XmaI there are 3 fragments at 1586, 3638, and 1357 base pairs (figure 16). The final screening gel is shown to confirm the accuracy of the plasmid.

Production of MSCV-GFP Plasmid

MSCV-neo and GFP plasmid were digested according to protocol and screened (figure 17). According to the screening map and confirmed by the gel from the digest: BsrGI yields 1 fragment at 6467 base pairs, DrdI yields 3 fragments, and EagI yields 2 fragments at 1269 and 5198 base pairs (figure 18). The final screening gel is shown to confirm the accuracy of the plasmid.

Unconcentrated γ-retrovirus -MSCV-GFP transduction on Jurkat cells

The γ-retrovirus -MSCV-GFP virus transduced on Jurkat cells showed no gene expression or GFP illumination by fluorescent microscopy (figure 19).

This process was repeated again and yielded the same results. Data not shown.

Lipofectamine with MSCV-GFP

Jurkat cells transfected with Lipofectamine 2000 with the MSCV-GFP plasmid also showed no GFP expression via fluorescent microscopy, even when transfected at 2-fold increase in plasmid concentration (figure 20).

Sequencing Results of MSCV-GFP Plasmid

The in-house MSCV-GFP plasmid was screened to confirm sequence accuracy. There were large regions of the sequence omitted from the in-house plasmid. Data not shown. This confirmed that the plasmid made was inaccurate and not able to sufficiently express GFP when transfected into γ-retrovirus vectors. Looking at the plasmid map (figure 5), there was an unlabeled Pkg promoter on the MSCV-neo plasmid and the digest protocol cuts in the sequence by AccI enzyme. Due to the cut in the Pkg promoter sequence, there is subsequently less transcription of the GFP sequence, yielding insufficient expression of the protein.

Screening of Purchased Addgene MSCV-IRES-GFP

After purchasing the MSCV-IRES-GFP plasmid from Addgene, we screened the plasmid for accuracy. According to the DrdI digest, there are 3 bands expected and confirmed at 1869, 1247, and 3372 base pairs (figure 21).

Production of Gamma-Retro-MSCV-IRES-GFP and Titer

After producing the gamma-retro-MSCV-IRES-GFP with calcium phosphate transfection, the virus was concentrated and then titered by transducing HEK-293T cells and 3T3 cells

as a control. The transduction of the HEK-293T cells at 10µl of virus and 50µl of virus was 0.1%, 0.2%, and 0.4% respectively. 293T cells are easily transducible and were expected to be transduced by the γ-retrovirus. The 3T3 cells were transduced as well with the same volumes of virus, yielding 0.2%, 1.0%, and 1.4% expression of GFP, although, low-level transduction was anticipated (figure 22). Titer of the virus could not be calculated because the γ-retrovirus was unable to infect and subsequently express GFP in 293T cells.

AAV Studies

Transduction of PBMCs with different AAV-GFP vectors Compared to Lenti-GFP

After receiving 3 different AAV-GFP constructs from collaborators at the University of Florida, we statically transduced PBMCs cells with each at 5µl and 50µl, or MOI 5,000 and 50,000, of virus. Day 8 post transduction at 5µl volume yielded 5.4%, 4.2%, and 11.0% GFP expression for 6m3, 6wt, and 6.663 AAV vectors respectively. At a volume of 50µl, the GFP expression was 38.6%, 34.0%, and 55.1% for 6m3, 6wt, and 6.663 AAV vectors respectively. Compared to lenti-GFP transduction at an MOI 15 into PBMCs 8 days post-transduction, lenti-GFP yielded only 11.6% GFP expression (figure 23 and 24).

Transduction of NK92 with different AAV-GFP Vectors

We used the same AAV viruses, 6wt, 6.663, and 6m3 to transduce NK92 cells at MOI 50,000. Flow cytometry and fluorescent microscopy was used to quantify the GFP expression. Day 3 post transduction, 6m3 yielded 97.9% GFP expressing cells, 6wt expressing 96.0% GFP expressing cells and 6.663 yielded 99.6% GFP expressing cells (figures 25 and 26).

AAV exists episomally in cells. In order to determine the loss of GFP expression by cell divisions, we again used flow cytometry to quantify GFP expression in the NK-92 cells day 7 post-transduction. Indeed, there was a loss in GFP expression: 6m3 yielded 45.7% expression, 6wt yielded 64.7%, and 6.663 yielded 94.5% expression. AAV vector 6.663 still yielding the most GFP expression and least amount of decrease (figure 26). In addition, fluorescent microscopy was used again to qualitatively show the loss in GFP daily from day 4 post-transduction to day 6 post-transduction (figures 27-30). To correlate cell division with GFP expression loss, cell counts of each NK-92 sample were recorded from day 3 to day 6 and doubling times were calculated. Naïve NK-92 cells doubled in 18.06 hours, 6wt transduced cells doubled at a rate of 30.5 hours, 6m3 transduced cells doubled at a rate of 41.8 hours, and 6.663 transduced cells doubled at a rate of 29.07 hours (graph 2). It is interesting to note that NK-92 cells with a longer and higher duration of GFP expression had a faster doubling time. This also correlated cell division with loss of episomal DNA with AAV vectors in NK-92 cells.

Discussion

Our first aim was to determine and optimize a high throughput method to rapidly test several VLR-CAR constructs in lymphoid cells. To test this, we experimented with several transient transfection methods, nucleofection, electroporation, and viral transduction methods through lentiviral and AAV vectors to see the efficiency of gene transfer in Jurkat cells.

We first tested the transient gene transfer of a GFP plasmid into Jurkat cells. We experimented with the electroporation protocol from BTX and several parameters in an effort to optimize the machine for Jurkat cells. Every sample that was subjected to the electric pulse in the electroporator had little to no gene transfer. In addition, the cells electroporated had significantly lower cell viability both qualitatively and quantitatively. The toxicity of the electric pulse was expected because cellular damage is one of the disadvantages of electroporation. However, there are several studies suggesting that electroporation is a sufficient method for efficient gene transfer. It is likely that the electrical pulse caused changes in the cell morphology and that gene transfer itself via electroporation killed the cells. This was confirmed by fluorescent microscopy, showing a large quantity of fluorescing cell debris from GFP transfer whereas the living cells were not fluorescing.

We then tested another transient gene transfer method by nucleofection of GFP plasmid into Jurkat cells. Following the Lonza protocol and program for nucleofecting Jurkat cells, gene transfer of the GFP plasmid ranged from approximately 20% to 90%. Regardless of the variability, the gene transfer efficiency was greater than electroporation and the toxicity of the shock to the cells was less than electroporation, as seen by the

increased viability in the cell samples. Following the transient transfection experiments with GFP plasmid and Jurkat cells, we nucleofected Jurkat cells with two VLR-CAR constructs, Nbl-CAR and CD5-CAR. Since we knew that gene transfer into Jurkat cells was possible, the results from the VLR-CAR nucleofections were not expected. For both constructs, using the Myc tag to quantify the gene transfer through flow cytometry, we quantified little to no gene transfer of the VLR-CAR construct. However, using CD69 as a marker for activation of the construct, there was dose-dependent activation for CD5-CAR, which was expected. Jurkat cells are CD5+, so Jurkat cells expressing a CD5-CAR will "self-activate." In addition, there was also activation of the Nbl-CAR, which was not expected because the Nbl-CAR is not self-activating nor was Neuroblastoma antigen present in the sample. Due to the insignificance between Nbl-CAR and CD5-CAR activation and the lack of Myc expression in both VLR-CAR constructs, we could not conclude that nucleofection successfully transferred the VLR-CAR constructs into Jurkats cells nor caused the activation quantified.

After exhaustion of transient transfection methods, we next tested lentiviral vector mediated gene transfer into Jurkat cells with lenti-GFP and lenti-BCL-CAR. Both the GFP and the BCL-CAR construct showed a considerable increase in gene transfer in comparison to transient transfection methods. These studies successfully concluded that the most efficient gene transfer method for VLR-CAR constructs into lymphoid cells, like Jurkat cells, is via viral transduction.

However, when others in the lab tested lentiviral gene transfer of NK92 cells, there was little to no gene transfer of GFP or the VLR-CAR. In order to overcome this obstacle, we experimented with other viral systems such as γ-retrovirus, which literature suggests

should have efficient gene transfer into lymphoid and natural killer cells. However, there were several issues with the production of the γ-retrovirus. When designing the expression plasmids, MSCV-GFP and MSCV-BCL-CAR, there was an unlabeled PKG promoter within the plasmid map that was cut during the restriction digest. This was confirmed with sequencing results, which showed a large portion of the expected sequence missing. Cutting through this promoter affected the transcription of the gene of interest, in our case, GFP, causing lower expression. Even after we purchased the MSCV-IRES-GFP plasmid and successfully produced γ-retrovirus, the titer of the virus was too low to quantify and use.

It has been suggested by others that AAV is a potential gene transfer system that could efficiently transduce immunocompetent cells.[49-51] More recently, we experimented with different AAV-GFP vectors for gene transfer into Jurkat cells and NK92 cells. For both cell types, there was a drastic increase in gene transfer when compared to lentiviral constructs. The most surprising was the gene transfer efficiency into NK92 cells, which reached, at maximum, 97.9% expression of GFP for the 6.663 AAV-GFP construct.[52] One shortcoming of AAV vectors instead of other viral vectors like γ-retrovirus and lentivirus is that AAV does not integrate the gene of interest into the genome. Our data supports that if AAV were to be used in rapidly dividing cells, gene expression would decrease after several divisions. These preliminary studies involving AAV-GFP suggest that this may be a better viral gene transfer system than lentivirus for VLR-CAR constructs. However, AAV could be a more permanent gene transfer for slower dividing or non-dividing cell types.

This study concluded that the most efficient method of gene transfer into lymphoid cells for VLR-CARs is via viral transduction, however, we can not conclude that we have

determined and optimized a *rapid high throughput screening system*. One of the shortcomings of a viral transduction system to test VLR-CARs is the length of time needed to produce and experiment with the virus. At this point, it is undetermined whether or not viral transductions can be optimized and used as a rapid screening system. The transduction of cells by virus is not the rate-limiting step, however the process to produce the viral constructs is, which could take around a couple weeks to produce the amount of virus needed for experimentation. Given that the VLR-CAR library that we can produce for a particular antigen can be upwards to several hundred VLR-CARs in one library, making several batches of viral construct may not be the most optimal system to rapidly test VLR-CARs. Transient transfections, in particular nucleofection, were a promising to rapidly screen VLR-CARs, but because of difficulty quantifying Myc tag in the CAR construct with flow cytometry, we did not proceed with these methods. Others in the lab have confirmed similar issues with the Myc tag, concluding the Myc is not an ideal quantification tag. In the future, nucleofection should be re-tested and optimized as a high throughput method if VLR-CAR constructs use other methods for quantification, such as a GFP reporter sequence instead of a Myc tag.

Although determining and optimizing a high throughput screening method would be relevant for rapidly testing VLR-CARs in the laboratory, a rapid method would be less relevant in the clinic because the more important issue for a clinic setting is which method allows for the best expression and function of VLR-CARs in human patients. At that point, the correct VLR-CAR would already be determined. In order to investigate the expression and function capabilities of a VLR-CAR when transferred by a viral transduction, we are in the process of a bone marrow transplant of transduced bone marrow cells with the BCL-

CAR construct. Thus far we have virally transduced bone marrow cells of Balb/c mice with lenti-BCL-CAR, lethally irradiated Balb/c experimental mice and then introduced the modified bone marrow cells back into the mice. The long-term goal of this project is to have successful engraftment of the modified chimera and efficient prevention of tumor growth after injection of BCL cells. These results will confirm the expression and function of the BCL-CAR gene transfer with lentiviral transduction in an *in-vivo* model. The results for this study will take some time to produce. The short-term goal of this project will be to determine the expression of the CAR in murine lymphoid cells, which will take less time to produce results. A successful bone marrow transplant takes six to eight weeks to engraft and then a few more weeks after the BCL cell injection to see if there was tumor growth or not. These studies will provide a conclusion attesting to the expression and function of a VLR-CAR when introduced virally to clinically relevant cells.

Overall, these studies confirmed that the best method to test the expression and function of VLR-CARs is with viral transduction. We have shown that transient transfection methods are not successful for measuring the expression and function of VLR-CARs in Jurkat cells. In addition, we have shown that the BCL-CAR construct can be expressed in both lymphoid and clinically relevant cells, while also activated in lymphoid cells. Pending the results of the *in vivo* mouse model, the function of the BCL-CAR in a living organism is still to be determined. Unfortunately, we cannot confirm whether or not viral transduction is a high throughput screening system for VLR-CARs. Due to the length of time it takes to make viral constructs with VLR-CAR cassettes, viral transduction may not be the most rapid method of experimentation, however it is the best to determine expression and function of VLR-CAR constructs in lymphoid and other immunocompetent cells.

For long term and clinical settings, viral transductions are still the method of choice to transduce cells for patient transfer. The importance of this study was to find a more rapid method to test several VLR-CARs in a large database in order to determine the best VLR-CAR for a specific antigen. In the clinical setting, the rapid testing of several VLR-CARs is not the primary goal, but rather the expression and function of the selected VLR-CAR. This study points towards viral transduction being both the best method to test several VLR-CARs in the VLR library as well as the most efficient gene transfer system for the expression and function of VLR-CARs in some clinically relevant cells. However, whether this is or isn't a *rapid* high throughput screening method to test VLRs in a library was not determined. Even as the best method for VLR-CAR screening, viral transduction, in relation to other methods, is not a rapid method for screening. This study can be used as the basis to continue using viral transfer to develop a method to test a complex VLR library.

This study presented is a small step in the development of the field of immunotherapy for cancer using gene therapy with VLR-CARs. Even though this study is a small step within the field of gene therapy, this study still has several ethical issues involved that need to be addressed. How far *is* too far when it comes to gene modification? There is a broad societal notion of "normalcy" and even though the term may be arbitrary, it is still one of the primary reasons we consider the ethical implications of sciences, such as gene therapy, may have.[53] For example, let's say that VLR-CARs become a standard treatment for several cancer malignancies. Are the individuals with modified "super" immune cells that can re-recognize, target, and effectively destroy cancer cells still normal when other individuals that cannot are the standard for normalcy? Perhaps these people

with an improved immune system will be viewed slightly outside of what is considered a "normal" human; thus losing humanness.

Losing what we currently define as humanness is neither good nor bad by fact, but societal groups will have their opinions. However, it is most likely that, regardless of opinion, science, and gene therapy for that matter will continue to discover new ways to improve what characteristics we, as humans, currently have. "We, as a society, need to brace ourselves for a radical shift in the evolution of our biology and of our Self, whether that is characterized by humanness or not.[53]"

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Tables and Figures

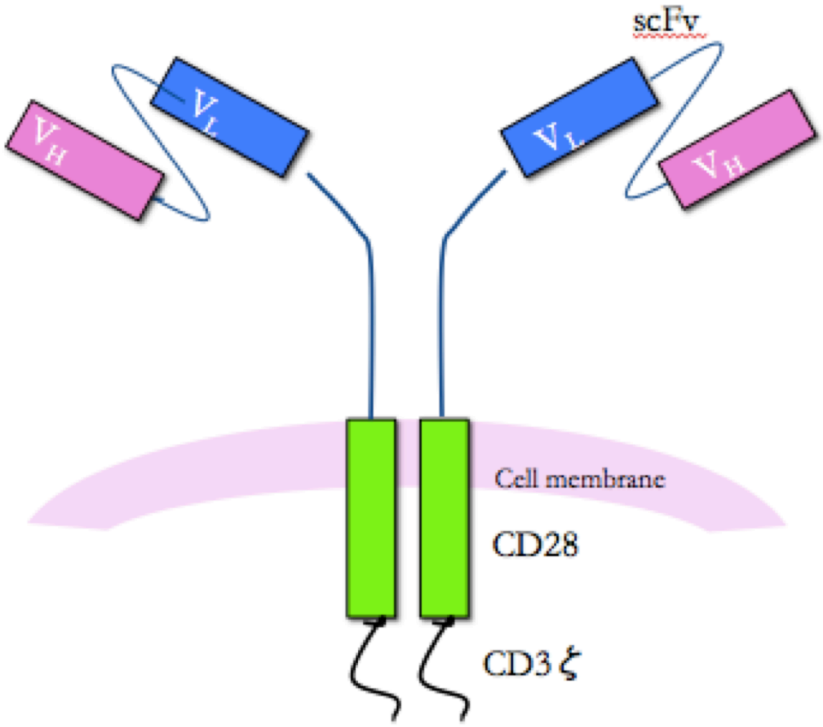


Figure 1 A protein diagram of a chimeric antigen receptor (CAR) containing antigen-binding domain, single chain variable fragment (scFv) from heavy and light chains, a transmembrane CD28 region, and an intracellular CD3ζ.

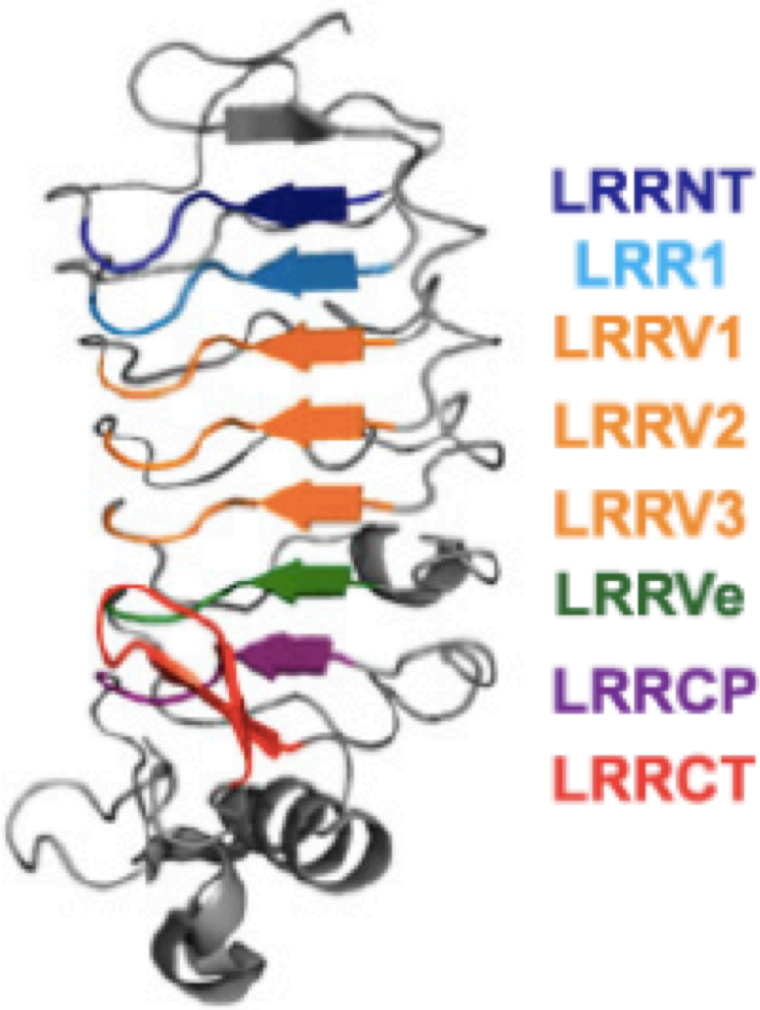
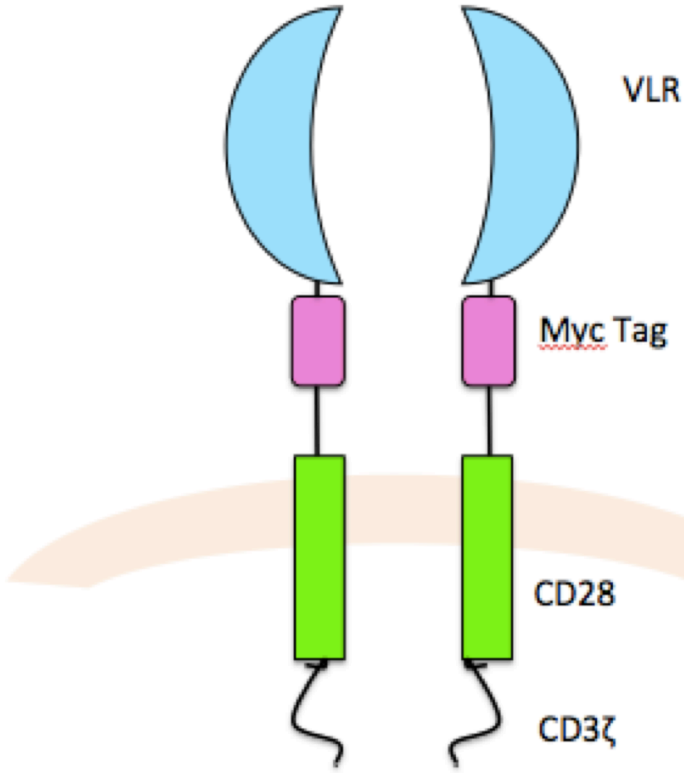


Figure 2. A protein crystallography depiction of a variable lymphocyte receptor.

Reprinted from Herrin BR. And Cooper MD., Alternative adaptive immunity in jawless vertebrates. J Immunol. 2010 Aug 1;185(3):1367-74. Copyright 2010 by The American Association of Immunologists, Inc. http://www.jimmunol.org/content/185/3/1367.long

A



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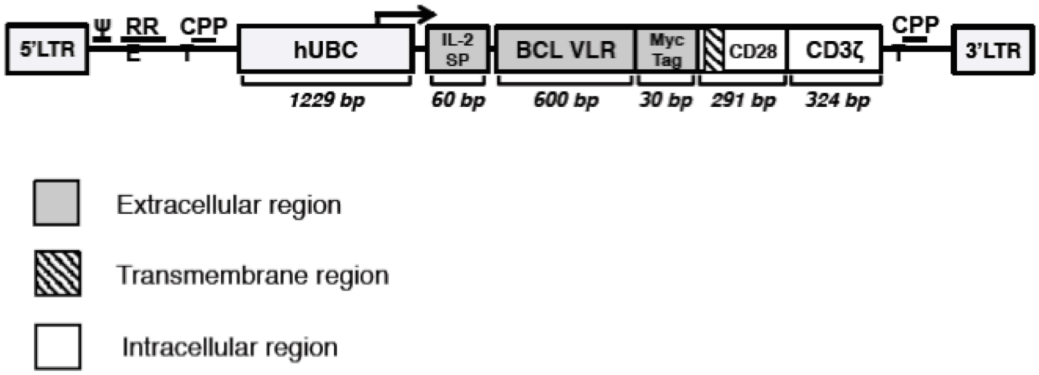


Figure 3a A protein diagram of our VLR-CAR construct containing VLR structure, a myc tag region, transmembrane CD28 protein, and intracellular CD3**ζ.**
Figure 3b Our BCL-CAR construct sequence driven by a human ubiquitin promoter and contains the VLR-CAR, BCL-CAR that targets B cell lymphoma antigen, Myc tag for quantification, and CD28 and CD3ζ that assist in T cell proliferation, differentiation, and signaling.

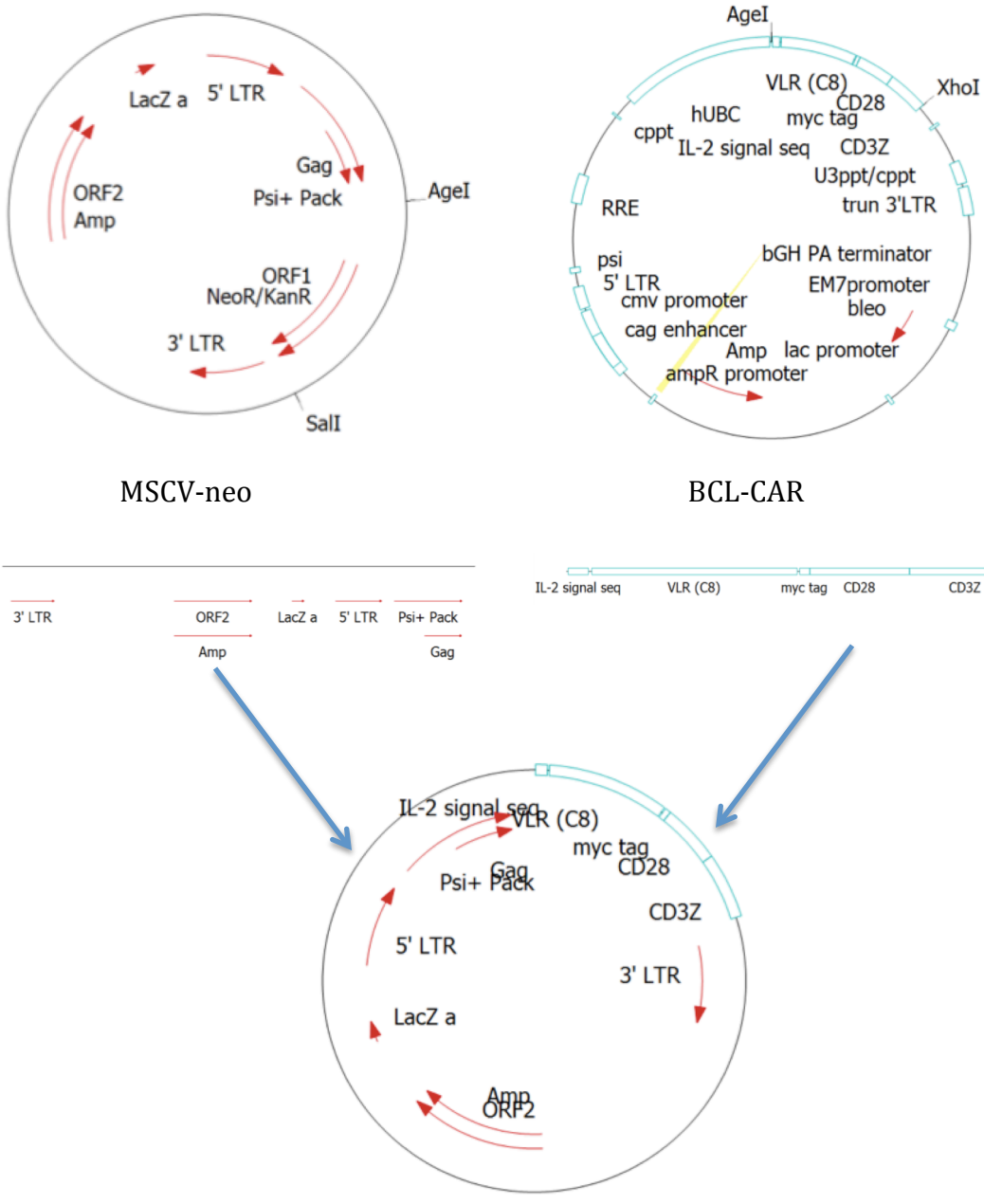


Figure 4. Cloning strategy of the MSCV-BCL-CAR plasmid. MSCV-neo was digested with AgeI and SalI while BCL-CAR was digested with AgeI and XhoI enzymes. The two plasmids were ligated to form MSCV-BCL-CAR.

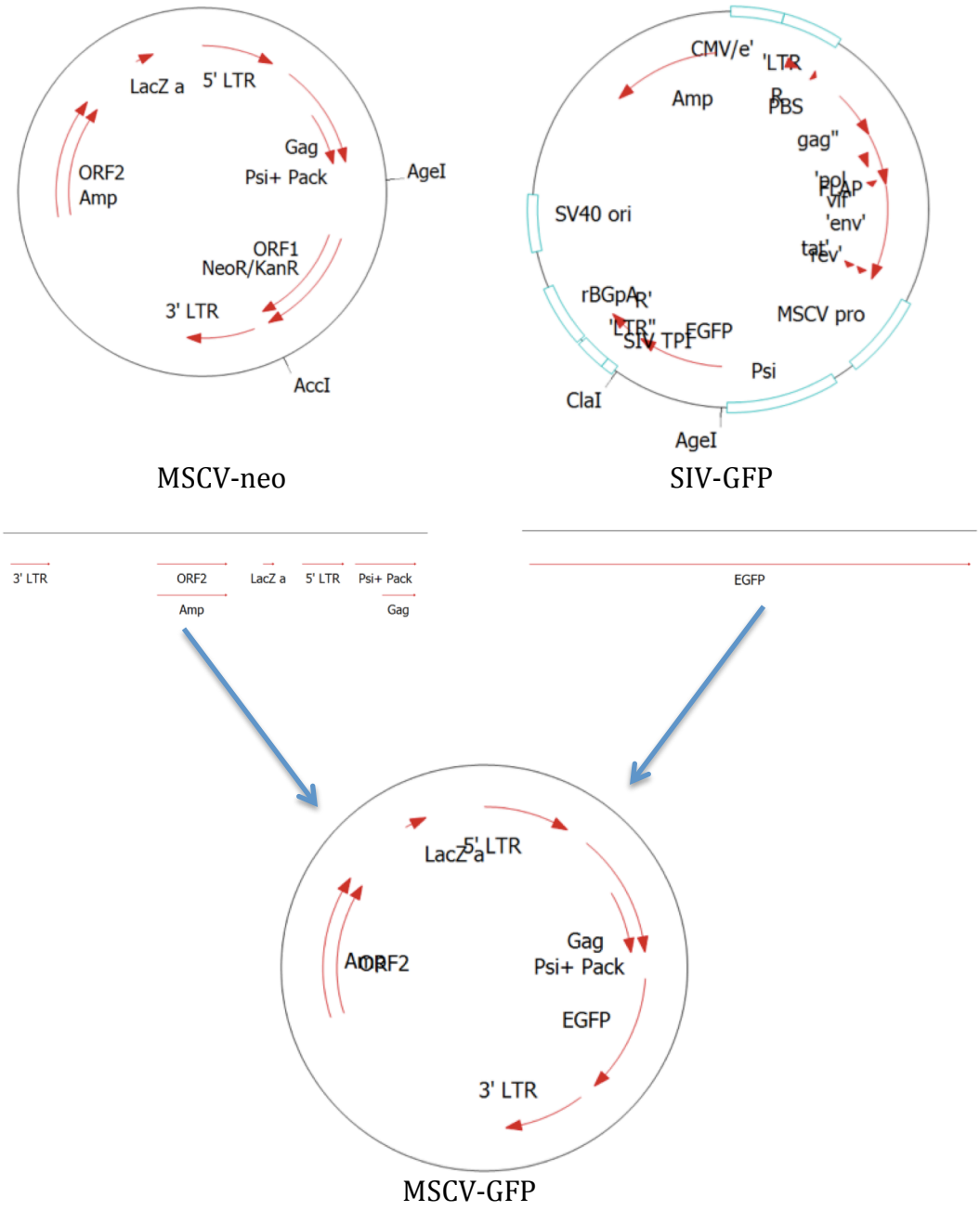


Figure 5. Cloning strategy of the MSCV-GFP plasmid. MSCV-neo was digested with AgeI and ClaI while SIV-GFP plasmid was digested with AgeI and AccI enzymes. The two plasmids were ligated to form MSCV-GFP.

Trial	Total Volume (μL)	Cell Density (μg)	Cuvette Width (mm)	Media	DNA (μg)	Voltage (V)	Capacitance (μF)	Resistance (Ω)	Pulse
I BTX 542	250	10^7	2	PBS	0, 5, 20, 40	160	175	0	1
II	250	10^6	2	PBS	20	100, 130	175	0	1
III	250	10^7	2	PBS	20	160	175	0	2
IV	250	10^7	2	PBS	20	150	950	0	1
V	150	10^6	2	PBS	20	250, 200, 250	300, 500, 350	1000	1
VI	250	10^7	2	PBS DMEM RPMI	20	160	175	0	1
VII	250	10^7	2	PBS	0	160	175	0	1

Table 1. Parameters of the electroporation trials. Experiment I tested different DNA concentrations at 0μg, 5μg, 20μg, and 40μg. Experiment II tested different voltage settings at 100V and 130V. Experiment III tested the standard setting for experiment I again at 20μg of DNA, a standard amount of DNA for electroporation. Experiment IV tested a setting suggested by a lab member who has done electroporation before. Experiment V examined the ratio of voltage to capacitance by testing the following pulses at 1000Ω, 250V with 300μF, 200V with 500μF, and 250V with 350μF. Experiment VI tested the efficiency of electroporation with the media the pulse was performed in, PBS, complete DMEM, and complete RPMI. The last experiment tested the standard setting for protocol I, BTX 542, pulse settings without DNA, to see if the DNA transfer into the cell was causing toxicity instead of the pulse itself.

Experiment	Sample	Percent GFP expression	Percent Viability
IA	0μg	0.5	94.8
	5μg	1.3	88.7
	20μg	2.1	82.0
	40μg	3.5	94.9
IB	0μg	0.5	82.9
	5μg	1.0	89.2
	20μg	2.1	85.9
	40μg	6.7	94.5
II	100V	0.0	62.4
	130V	0.0	59.8
III	1 parameter	0.2	85.6
IV	1 parameter	0.0	55.0
V	250V/300μF	0.0	45.6
	200V/500μF	0.0	34.3
	250V/350μF	0.0	44.5
VI	PBS	1.2	89.5
	DMEM	0.0	85.4
	RPMI	0.0	86.0
VII	0μg DNA	0.0	92.3

Table 2. A correlated table of results measuring GFP expression from the electroporation experiments found in Table 1 showing the percentage of GFP expression and the percentage of cell viability post electroporation. All experiments had GFP expression lower than 5%, however, some parameters had better cell toxicities than others.

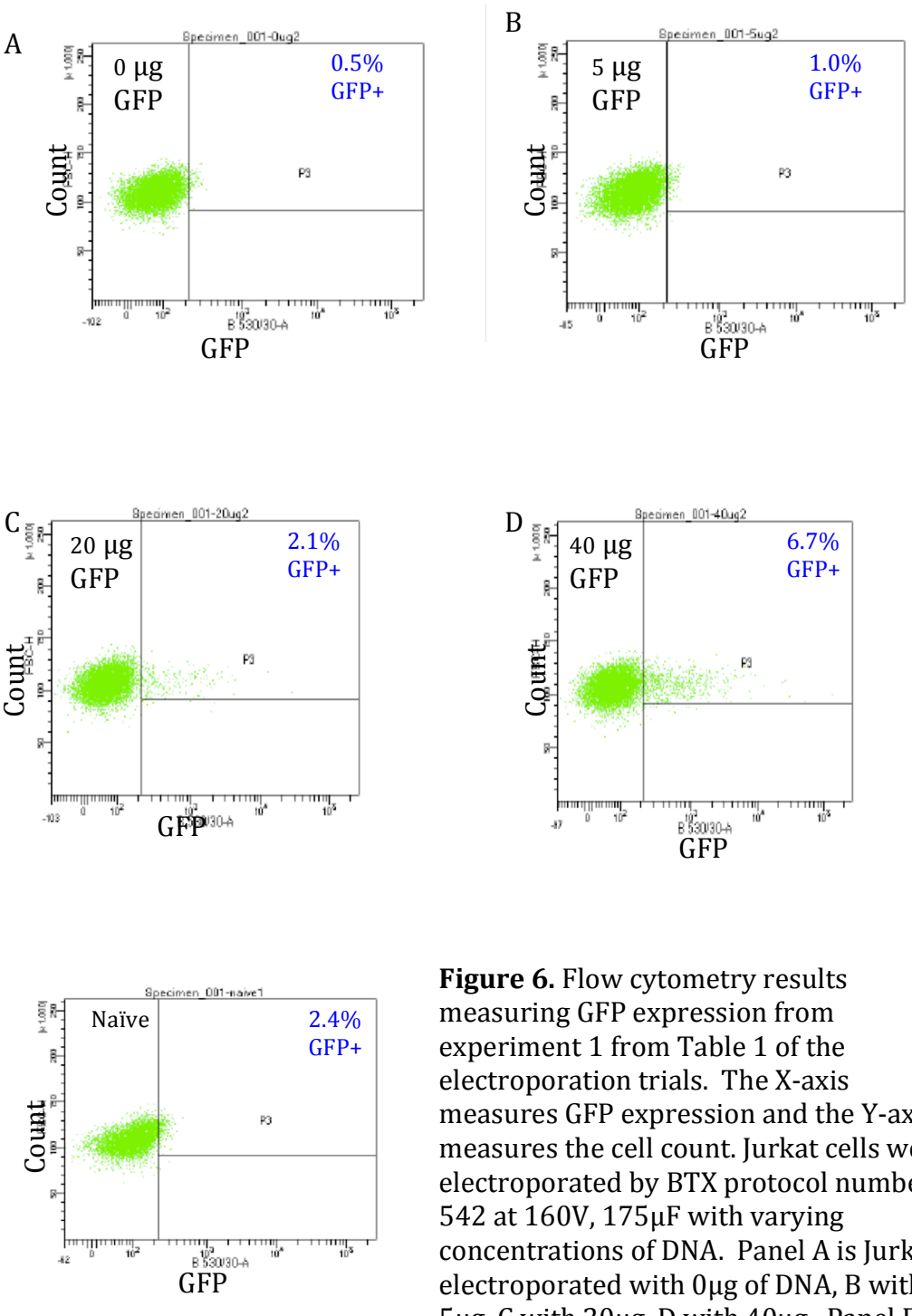


Figure 6. Flow cytometry results measuring GFP expression from experiment 1 from Table 1 of the electroporation trials. The X-axis measures GFP expression and the Y-axis measures the cell count. Jurkat cells were electroporated by BTX protocol number 542 at 160V, 175µF with varying concentrations of DNA. Panel A is Jurkats electroporated with 0µg of DNA, B with 5µg, C with 20µg, D with 40µg. Panel E is non-pulse naïve Jurkat cells. The amount of GFP expressed is 0.5%, 1.0%, 2.1%, 3.2%, and 2.4% respectively.

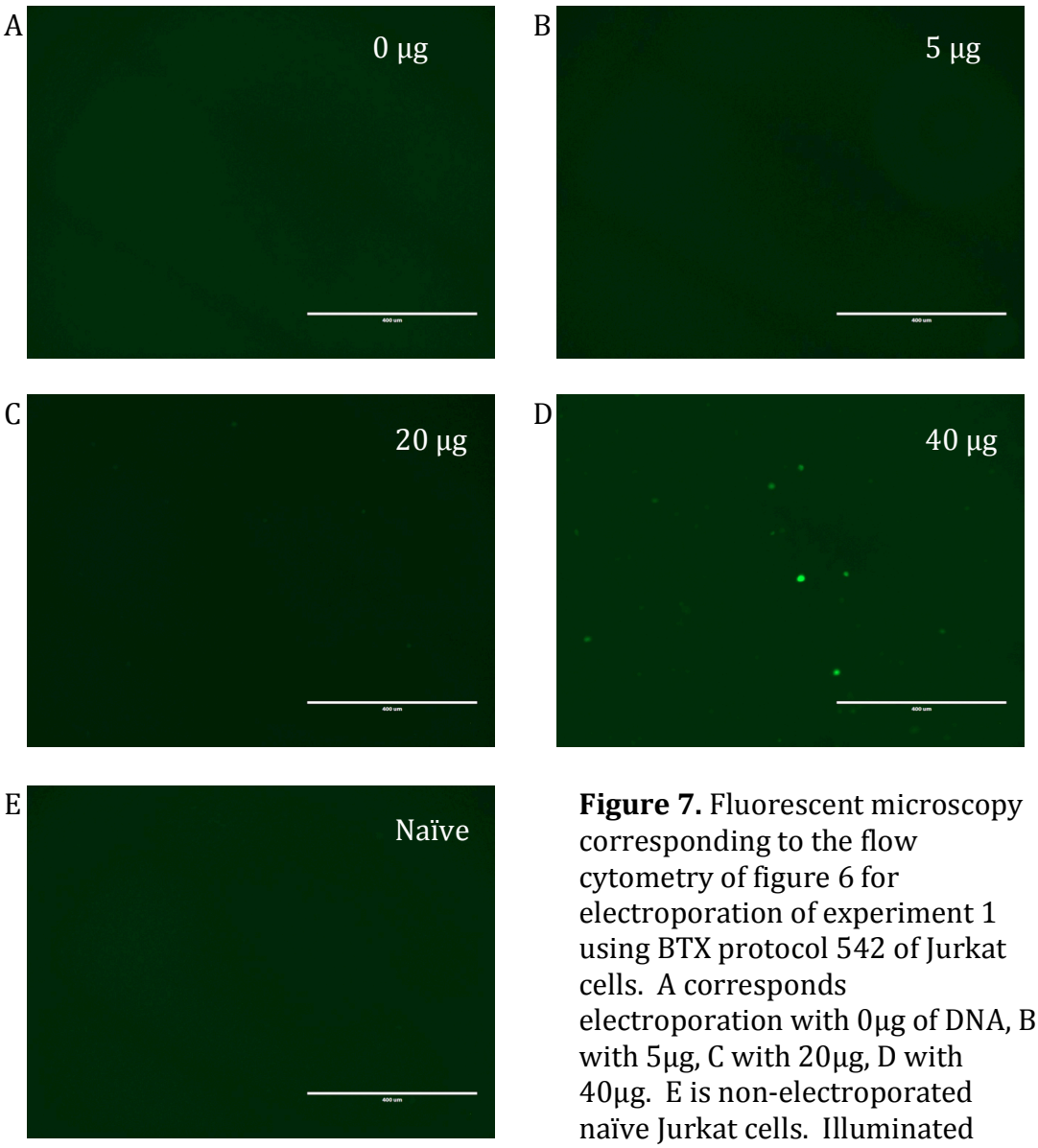
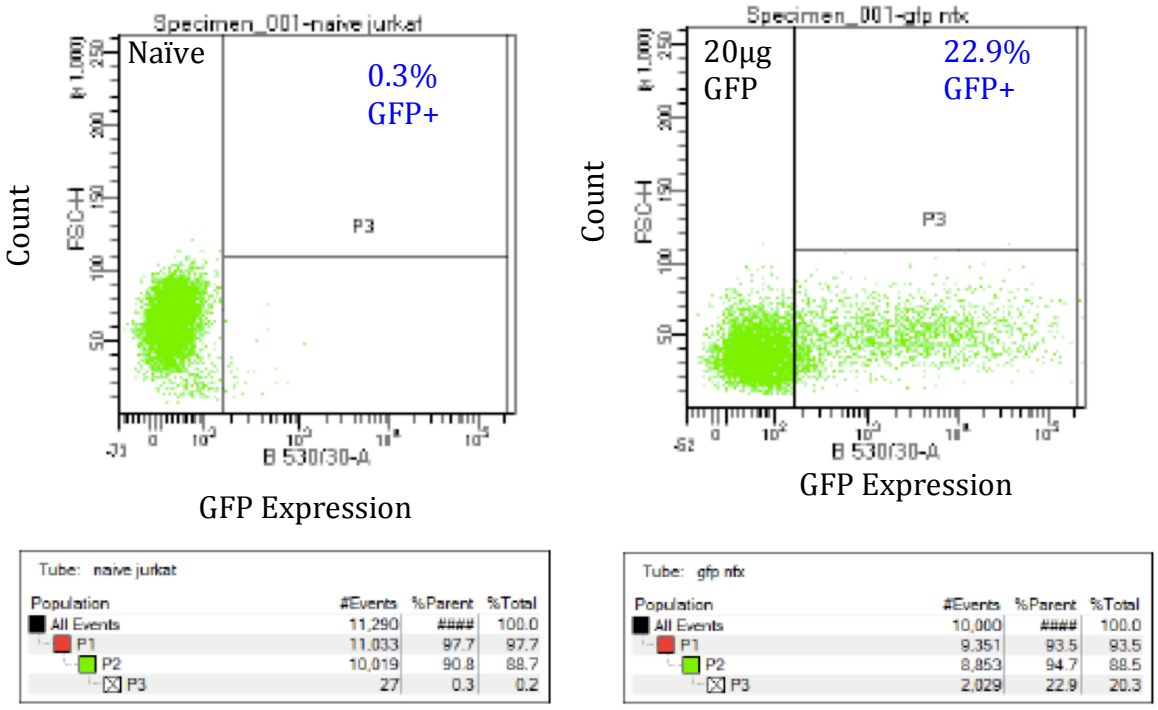


Figure 7. Fluorescent microscopy corresponding to the flow cytometry of figure 6 for electroporation of experiment 1 using BTX protocol 542 of Jurkat cells. A corresponds electroporation with 0µg of DNA, B with 5µg, C with 20µg, D with 40µg. E is non-electroporated naïve Jurkat cells. Illuminated green cells are those that are expressing GFP.



Tube: naive jurkat

Population	#Events	%Parent	%Total
All Events	11,290	####	100.0
P1	11,033	97.7	97.7
P2	10,019	90.8	88.7
P3	27	0.3	0.2

Tube: gfp ntx

Population	#Events	%Parent	%Total
All Events	10,000	####	100.0
P1	9,351	93.5	93.5
P2	8,853	94.7	88.5
P3	2,029	22.9	20.3

Figure 8. Flow cytometry results measuring GFP expression (X-axis) of Jurkat cells were nucleofected according to Lonza protocol under program X-001 with 20µg of DNA. The X-axis corresponds to GFP expression and the Y-axis corresponds to cell count. There is 22.9% GFP expression in the experimental group that was electroporated.

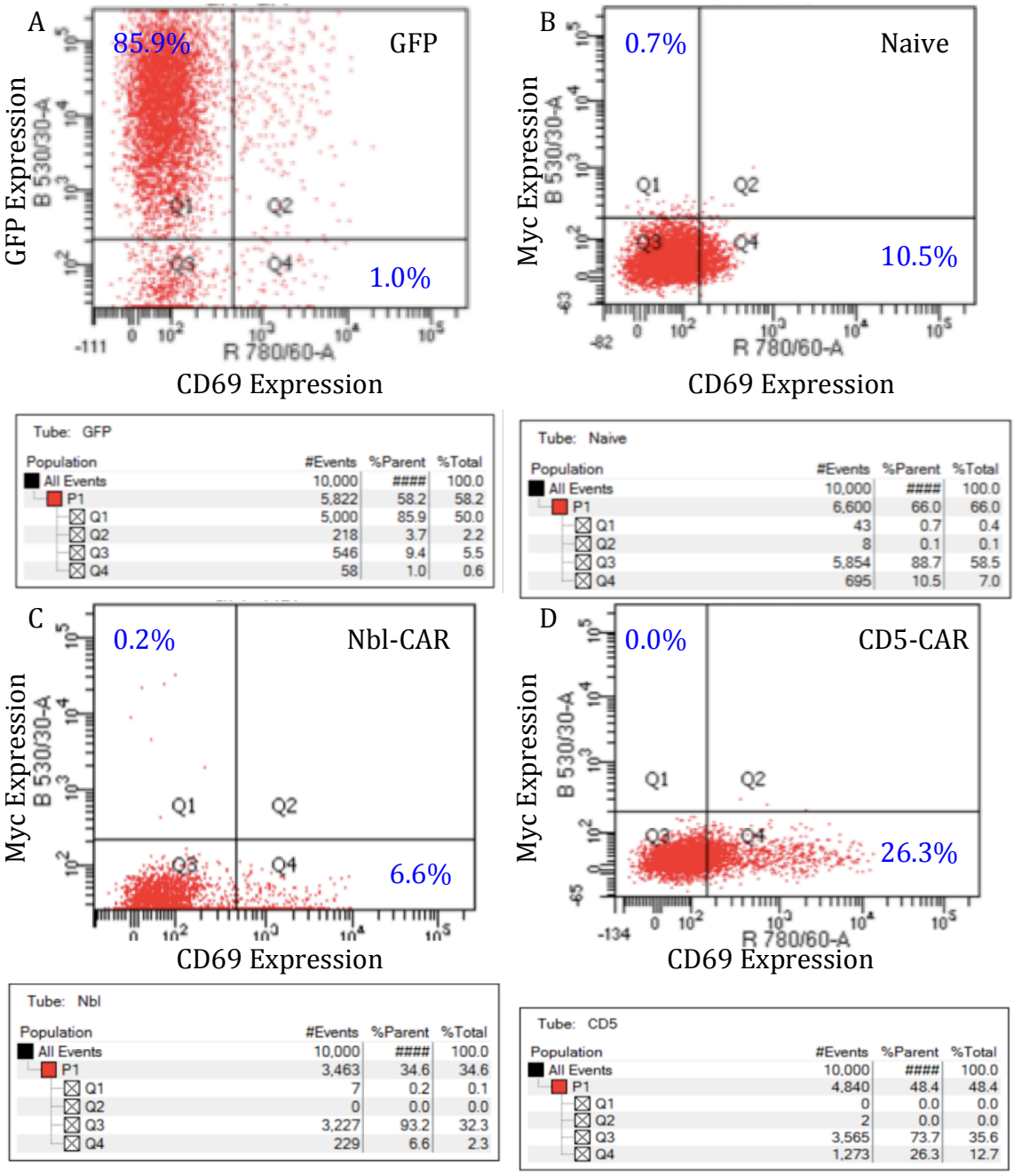
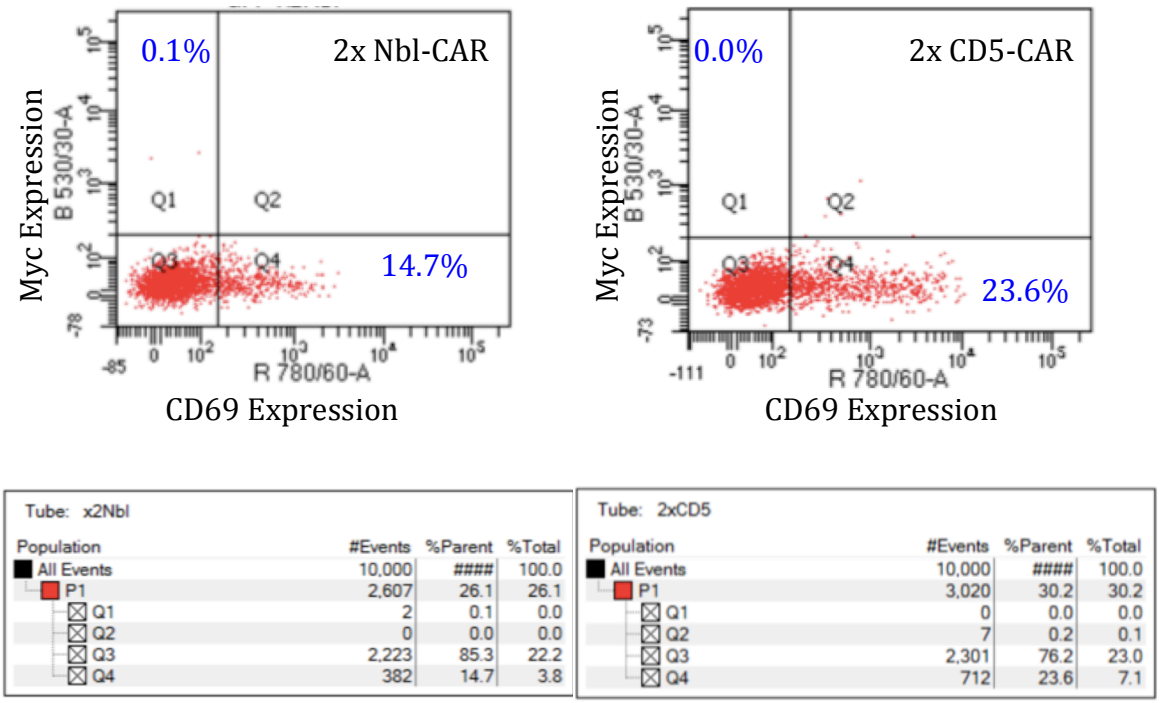


Figure 9. Flow cytometry results measuring CAR expression and activation of Jurkat cells nucleofected with GFP as control, Neuroblastoma CAR and CD5 CAR. The X-axis measures CAR activation by CD69 expression and the Y-axis measures CAR expression by Myc expression. Both NBL-CAR and CD5-CAR show activation at 6.6% and 26.3% respectively, however there is no Myc tag expression for either. Quandrant 1 in the GFP control is showing expression of GFP, not Myc. The Myc antibody is attached to FITC, which illuminates the same channel as GFP, B530/30-A. Therefore, even though there is expression in quadrant 1 of the GFP control, there is not Myc tag nor should there by activation seen in quadrant 3 by CD69 up-regulation.



Tube: x2Nbl

Population	#Events	%Parent	%Total
All Events	10,000	####	100.0
P1	2,607	26.1	26.1
Q1	2	0.1	0.0
Q2	0	0.0	0.0
Q3	2,223	85.3	22.2
Q4	382	14.7	3.8

Tube: 2xCD5

Population	#Events	%Parent	%Total
All Events	10,000	####	100.0
P1	3,020	30.2	30.2
Q1	0	0.0	0.0
Q2	7	0.2	0.1
Q3	2,301	76.2	23.0
Q4	712	23.6	7.1

Figure 10. Flow cytometry results measuring expression of the CAR through the Myc tag (Y-axis) and subsequent activation of the CAR and Jurkat cell (X-axis) of Jurkats nucleofected again with NBL-CAR and CD5-CAR at two times the amount of DNA, 40µg. In comparison to Figure 9, there is no change in the 0% expression of the Myc tag and insignificant change in CD-69 up-regulation at 14.7% and 23.6% respectively.

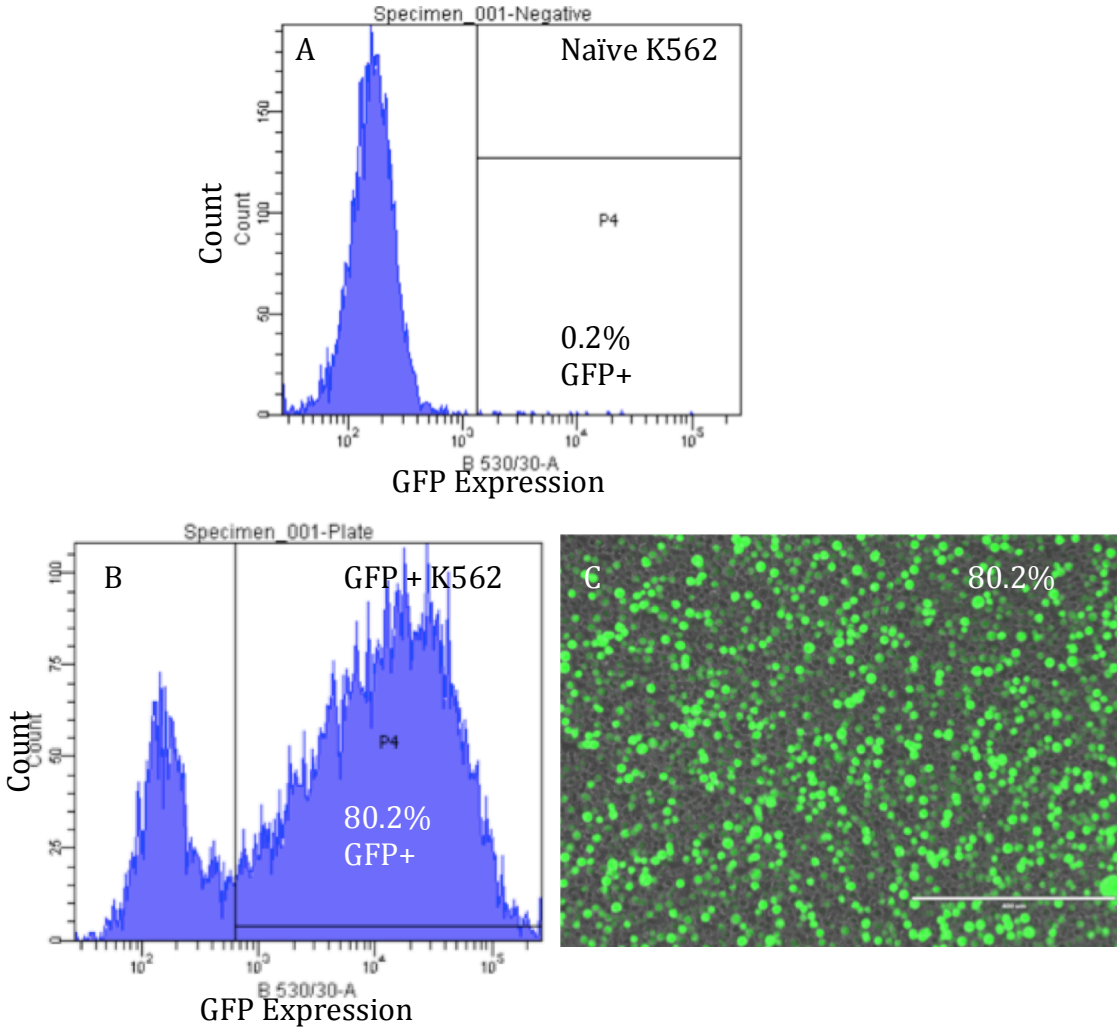
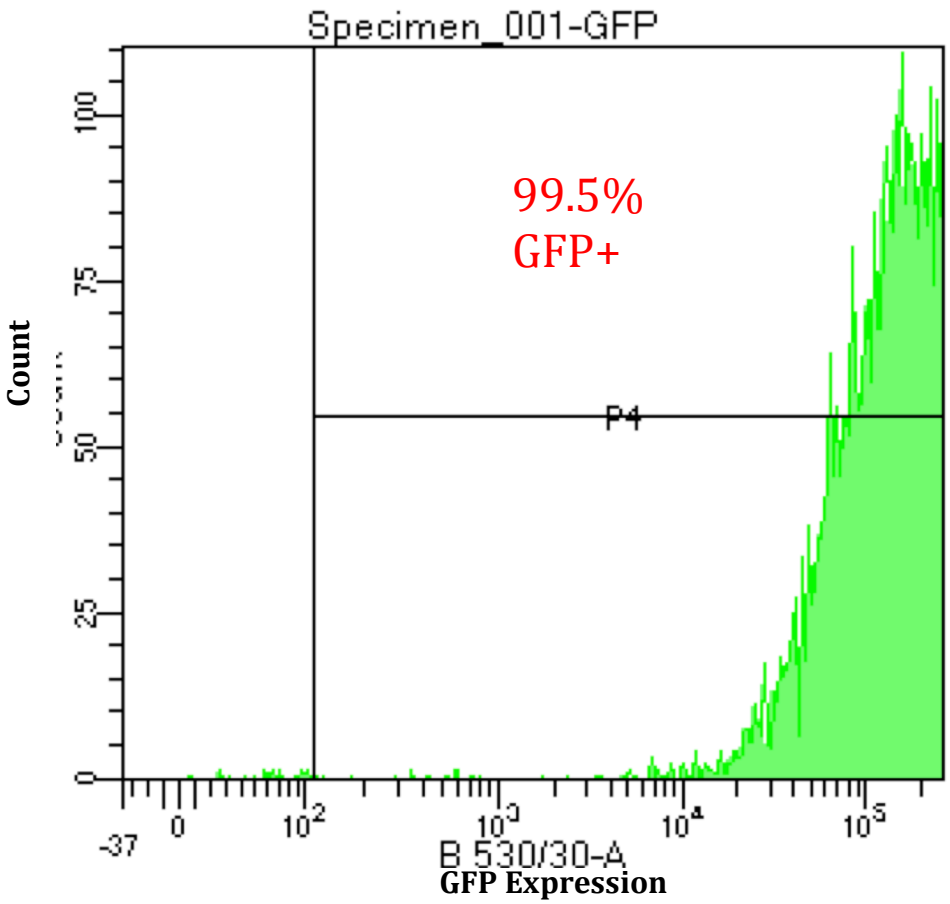


Figure 11. K562 cells were viral transduced at an MOI of 5 using Lenti-GFP. Panel A and B are flow cytometry results measuring GFP positive K562 cells (X-axis). The Y-axis is measuring the cell count. There was 80.3% GFP expression shown in panel B. Panel C is the fluorescent microscopy of LentiGFP transduced K562 cells with the green illumination being cells expressing GFP. Panel A is a naïve control.



Tube: GFP

Population	#Events	%Parent	%Total
All Events	8,977	####	100.0
P1	5,064	56.4	56.4
P2	4,865	96.1	54.2
P3	4,847	99.6	54.0
P4	4,843	99.5	53.9

Figure 12. Jurkats cells were transduced with LentiGFP at an MOI of 5 and analyzed via flow cytometry for GFP expression (X-axis). The count of events, cells expressing or not expressing the CAR is measured on the Y-axis. Gene transfer of GFP into Jurkats by LentiGFP at 99.5%.

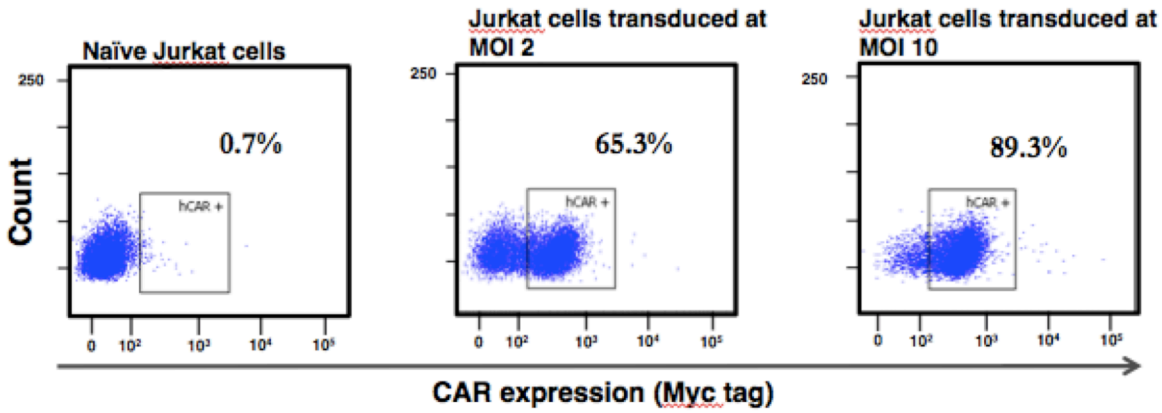


Figure 13. Jurkats cells were transduced with Lenti-BCL-CAR at an MOI of 2 (central panel) and 10 (right panel) and analyzed via flow cytometry with an anti-myc tag antibody for CAR expression (X-axis). Gene expression of the CAR is 65.3% and 89.3% at an MOI of 2 and 10 respectively.

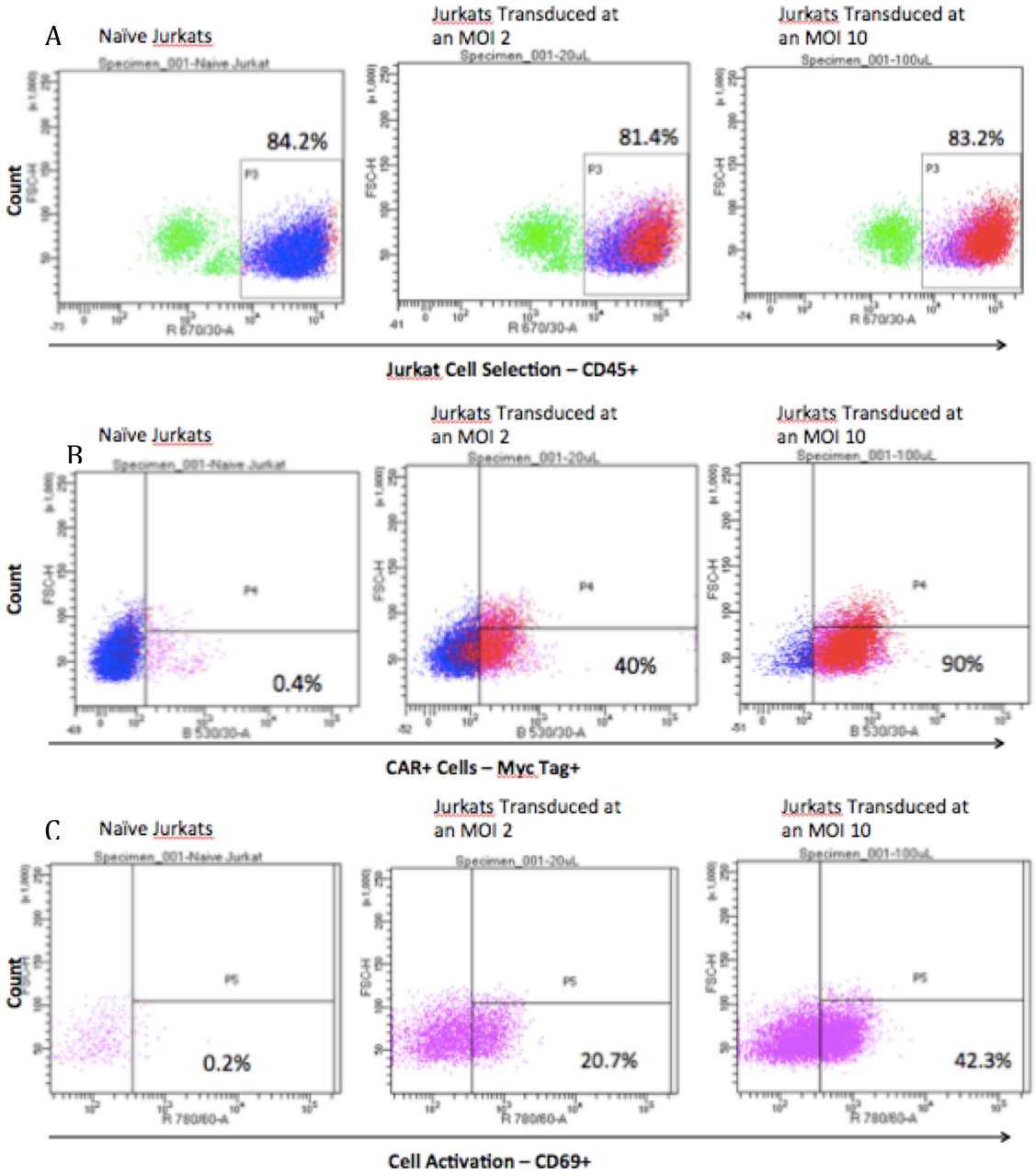


Figure 14. Flow cytometry result of BCL assay of naïve and transduced Jurkat cells with Lenti-BCL-CAR and then assayed with 300,000 BCL cells at 37C for 4 hours. Row A measures CD45+ cells (X-axis), for Jurkat cell selection. Row B measures Myc tag positive cells in Jurkat cells (X-axis) for CAR expression in naïve and Jurkats transduced at MOI of 2 and 10 of Lenti-BCL-CAR yielding 0.4%, 40%, and 90% respectively. Row C measures cell activation by CD69 expression (X-axis) of Jurkats that were CAR positive from naïve and Jurkats transduced at MOI 2 and 10 yielding 0.2%, 20.7%, and 42.3% respectively.

	Sample	% of Jurkats	% BCL-CAR+ Jurkats	% Activated BCL-CAR Jurkats
1	Naïve	84.2	3.2	2.1
	MOI 2	81.4	40.0	20.7
	MOI 10	83.2	90.0	42.3
2	Naïve	84.0	2.7	0.6
	MOI 2	81.3	30.9	20.7
	MOI 10	83.0	73.4	55.4
3	Naïve	-	-	1.2
	MOI2	-	-	35.9
	MOI 10	-	-	96.3

Average BCL Assay Results

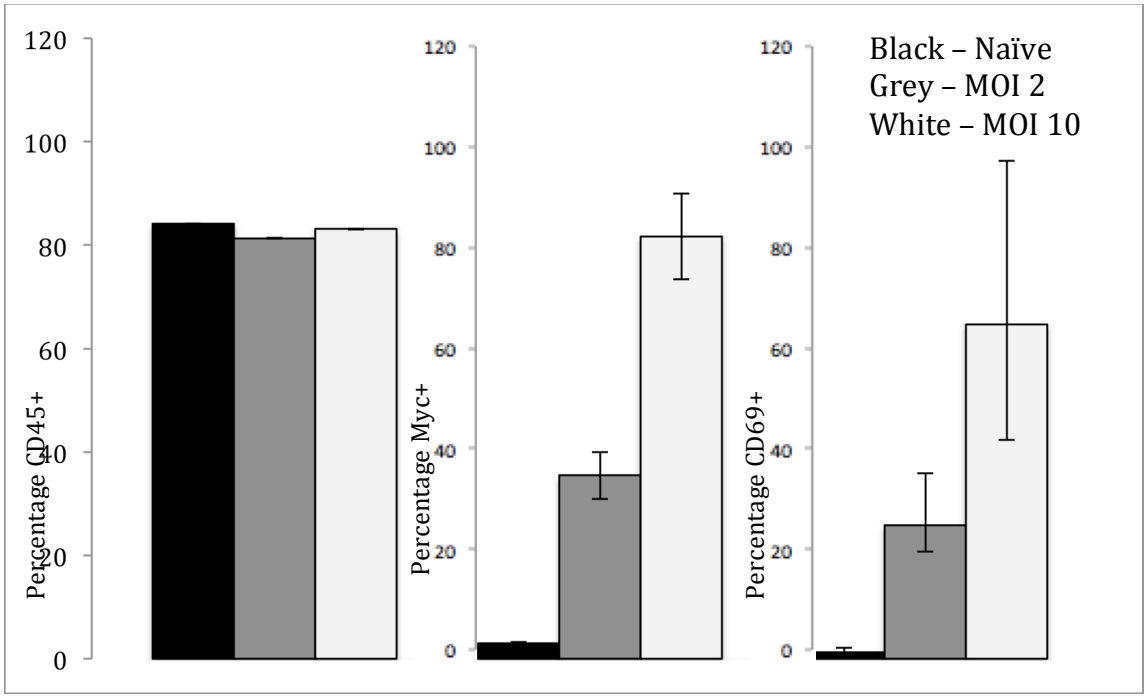


Table 3. Collated BCL assay results from three trials showing % of cells that are Jurkats, BCL-CAR expressing Jurkats, and Activated BCL-CAR Jurkats. Trial three only measured the activation percentage of the BCL-CAR Jurkats.

Graph 1. Averages of the BCL assay results from Table 3.

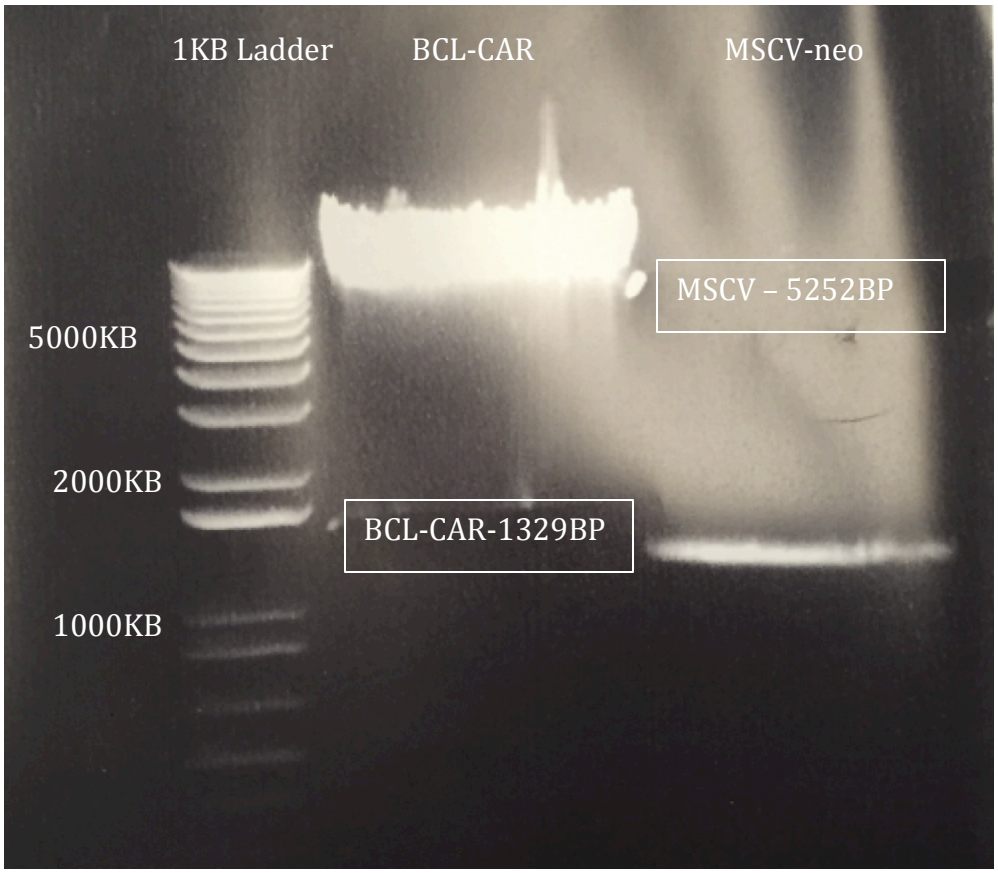
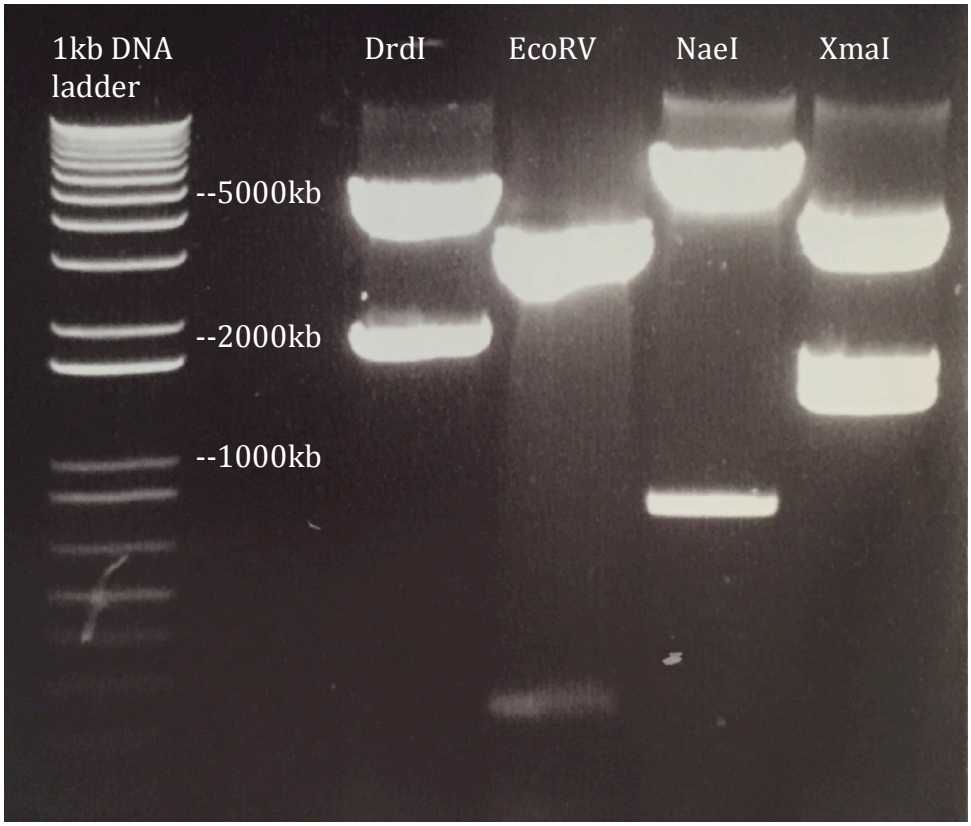


Figure 15. Restriction digest of MSCV-neo and BCL-CAR gel after the MSCV-neo was cut with AgeI and SalI and BCL-CAR was cut with AgeI and XhoI. The fragments of interest (MSCV – 5252 BP and BCL-CAR – 1329 BP) were cut out for a gel extraction.



Enzyme	Fragments BP
DrdI	1869, 4712
EcoRV	3385, 253, 2943
NaeI	799, 5782
XmaI	1586, 3638, 1357

Figure 16. The final restriction enzyme screen of MSCV-BCL-CAR. The table below shows the fragments expected for an accurate plasmid construct corresponding with the gel above.

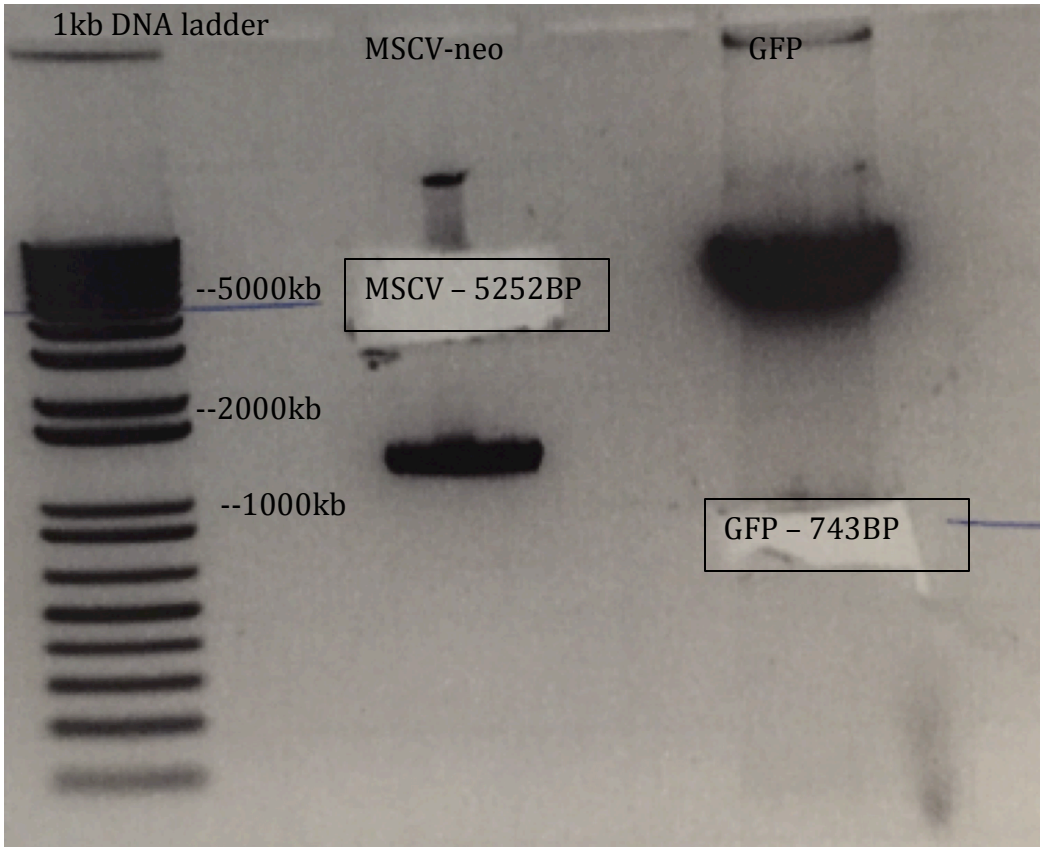
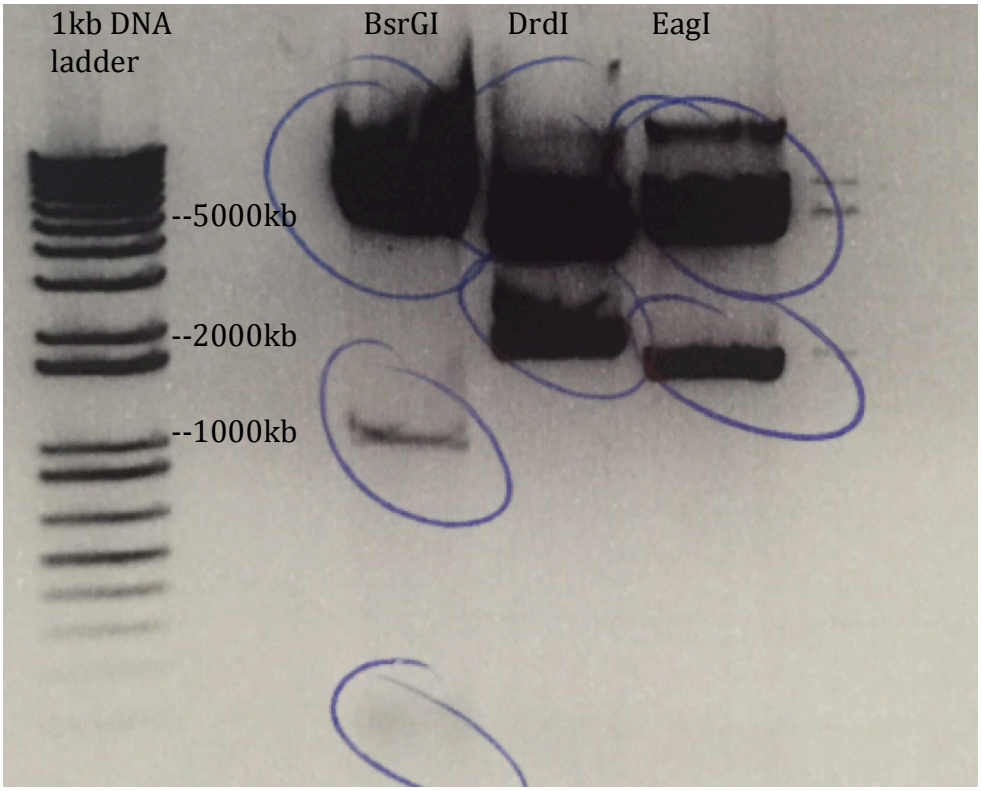


Figure 17. Restriction digest of MSCV-neo and GFP after they were digested with AccI/AgeI and ClaI/AgeI respectively. The fragments of interest (MSCV-5252BP and GFP – 743BP)were cut out for a gel extraction.



Enzyme	Fragments BP MSCV	Fragments BP GFP	Fragments BP MSCV GFP
BsrGI	1 frag.	881, 7321	987, 5007
DrdI	3 frags	4469,3733	1869, 4125
EagI	1269, 5198	1452, 6750	1558, 4436

Figure 18. The final restriction enzyme screen of MSCV-GFP. The table below shows the fragments expected for an accurate plasmid construct corresponding with the gel above versus fragments of unligated plasmids.

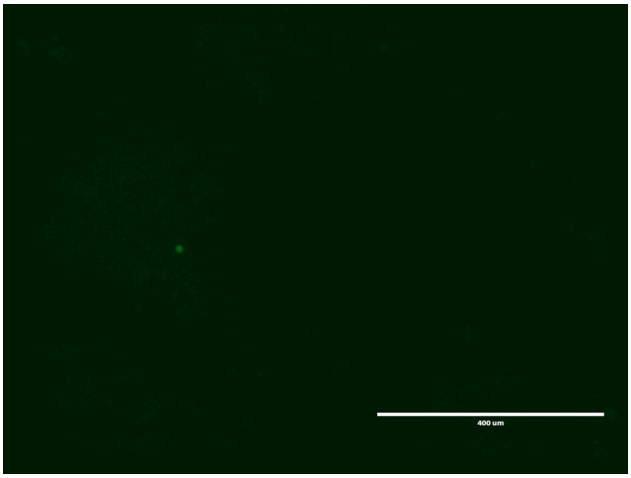


Figure 19. Fluorescent microscopy of Jurkats transduced with 100% unconcentrated in-house gamma-retro MSCV-GFP plasmid. There is one cell with green illumination, meaning GFP gene expression.

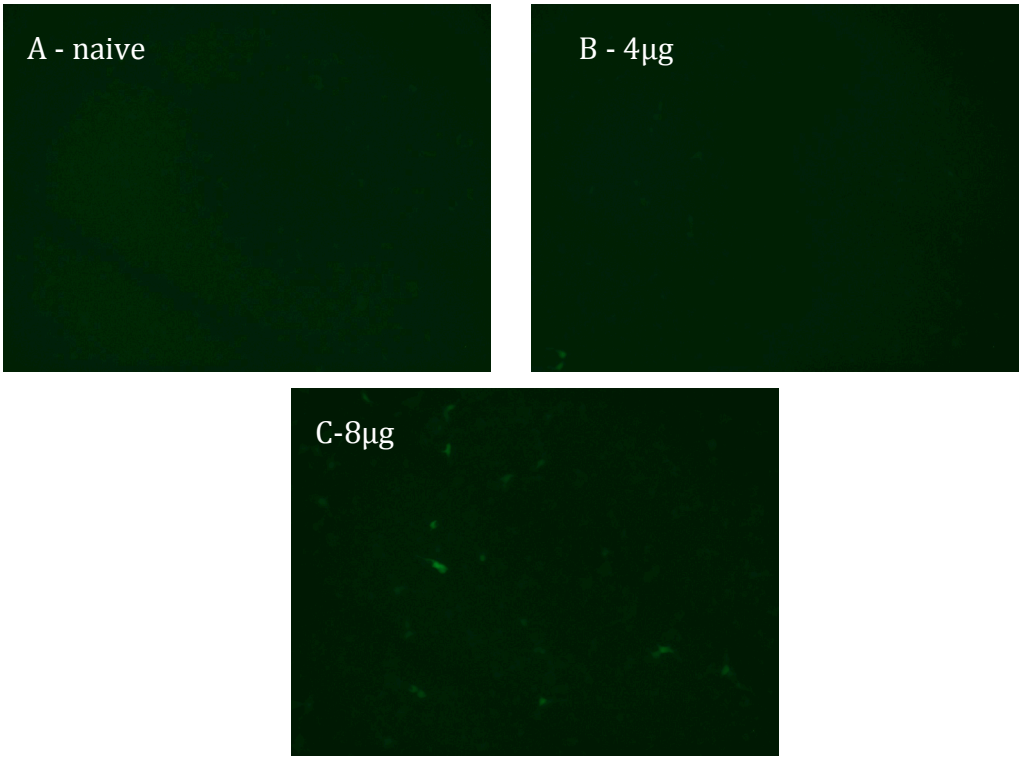
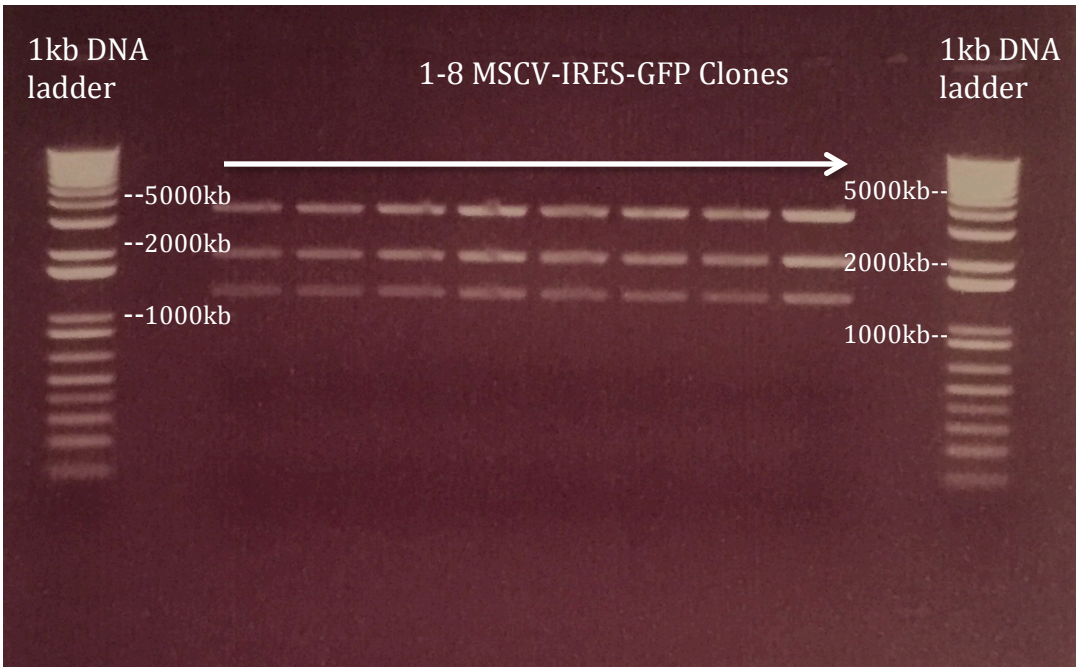
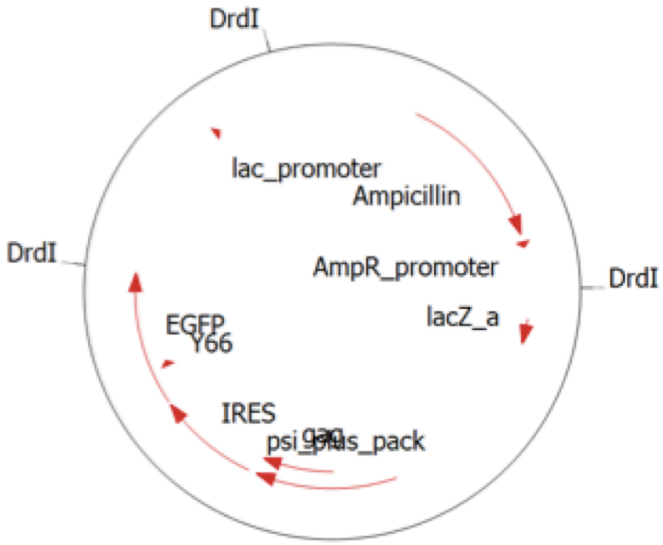


Figure 20. Fluorescent microscopy of Jurkat cells that were transfected via lipofectamine with the in-house MSCV-GFP plasmid. Panel A is naïve, Panel B shows the standard amount of DNA for lipofectamine at 4µg DNA, and panel C is two times the amount of DNA at 8µg. Gene expression of the GFP plasmid is shown as illuminated green.





MSCV-IRES-GFP

Figure 21. Restriction enzyme screen of Addgene construct MSCV-IRES-GFP with DrdI. The expected bands were 1869, 1247, and 3372 BP. Below is the plasmid map for the MSCV-IRES-GFP plasmid.

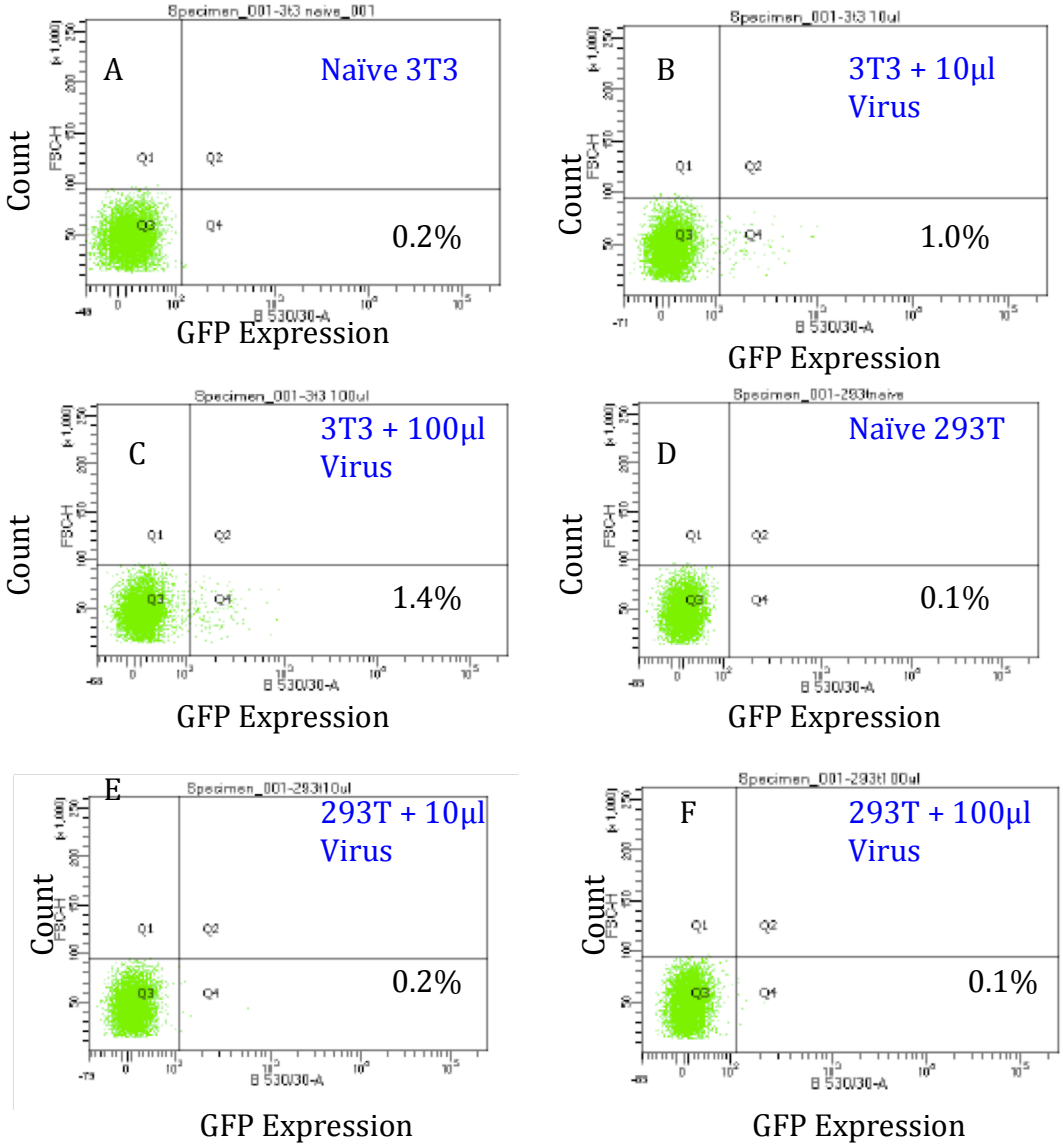


Figure 22. Titering of gamma-retro-MSCV-IRES-GFP virus. Panel A-C are 3T3 cells transduced with 0µl, 10µl, and 100µl and panels D-F are 293T cells transduced with 0µl, 10µl, and 100µl. All gene expression of GFP was under 2%. Titer could not be calculated.

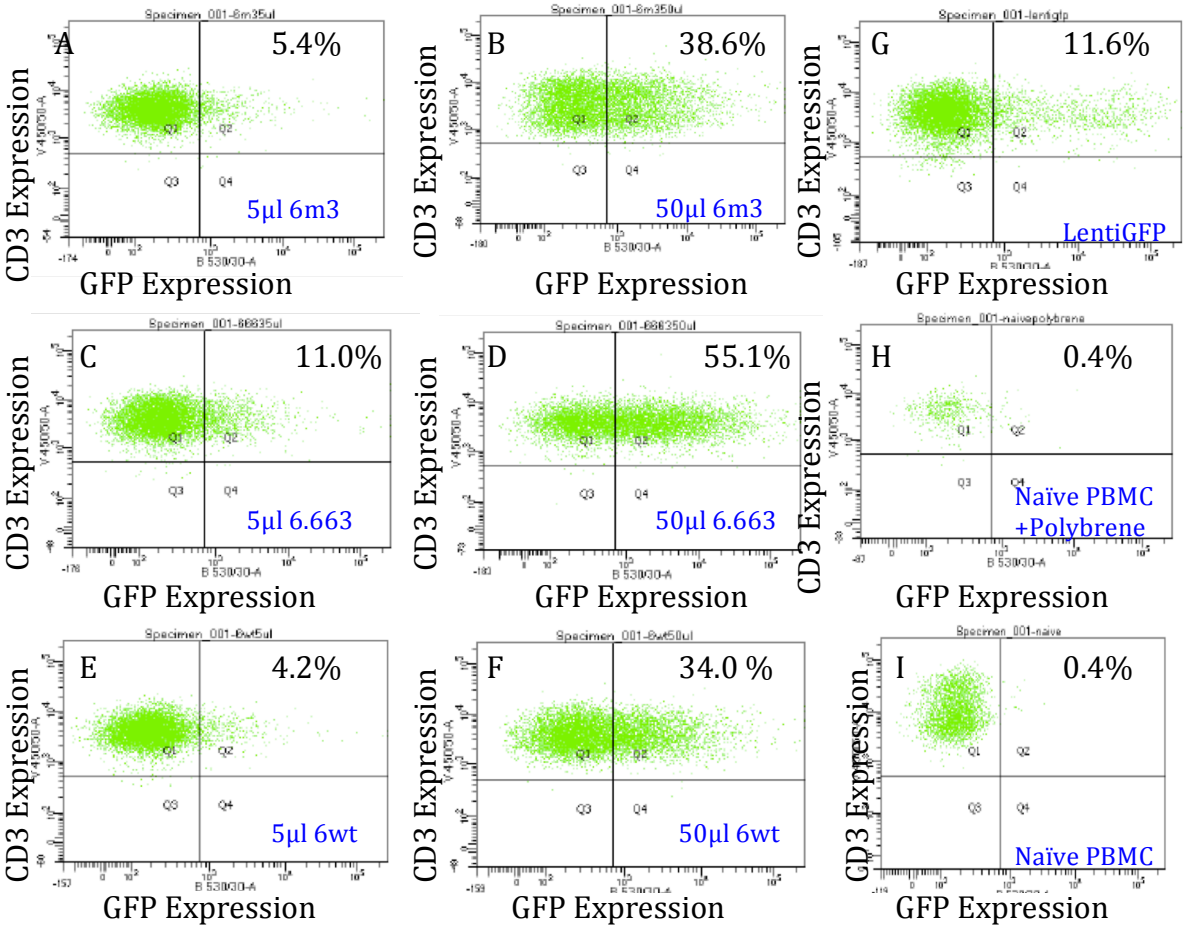


Figure 23. Flow cytometry results 8 days post transduction of PBMCs with different AAV-GFP vectors and concentrations measuring GFP expression. The X-axis measures GFP expression and the Y-axis measures CD3 expression, signifying which cells are T cells. A-B are PBMCs with 6m3 AAV-GFP transduced PBMCs with 5µl yielding 5.4% GFP expression and 50µl yielding 38.6% GFP expression. C-D are 6.663 AAV-GFP transduced PBMCs with 5µl yielding 11% GFP expression and 50µl yielding 55.1% GFP expression. E-F are 6wt AAV GFP transduced PBMCs with 5µl yielding 4.2% GFP expression and 50µl yielding 34% GFP Expression. Panel I is lenti-GFP transducing PBMCs yielding 11.6% GFP expression. Panels G-H are controls

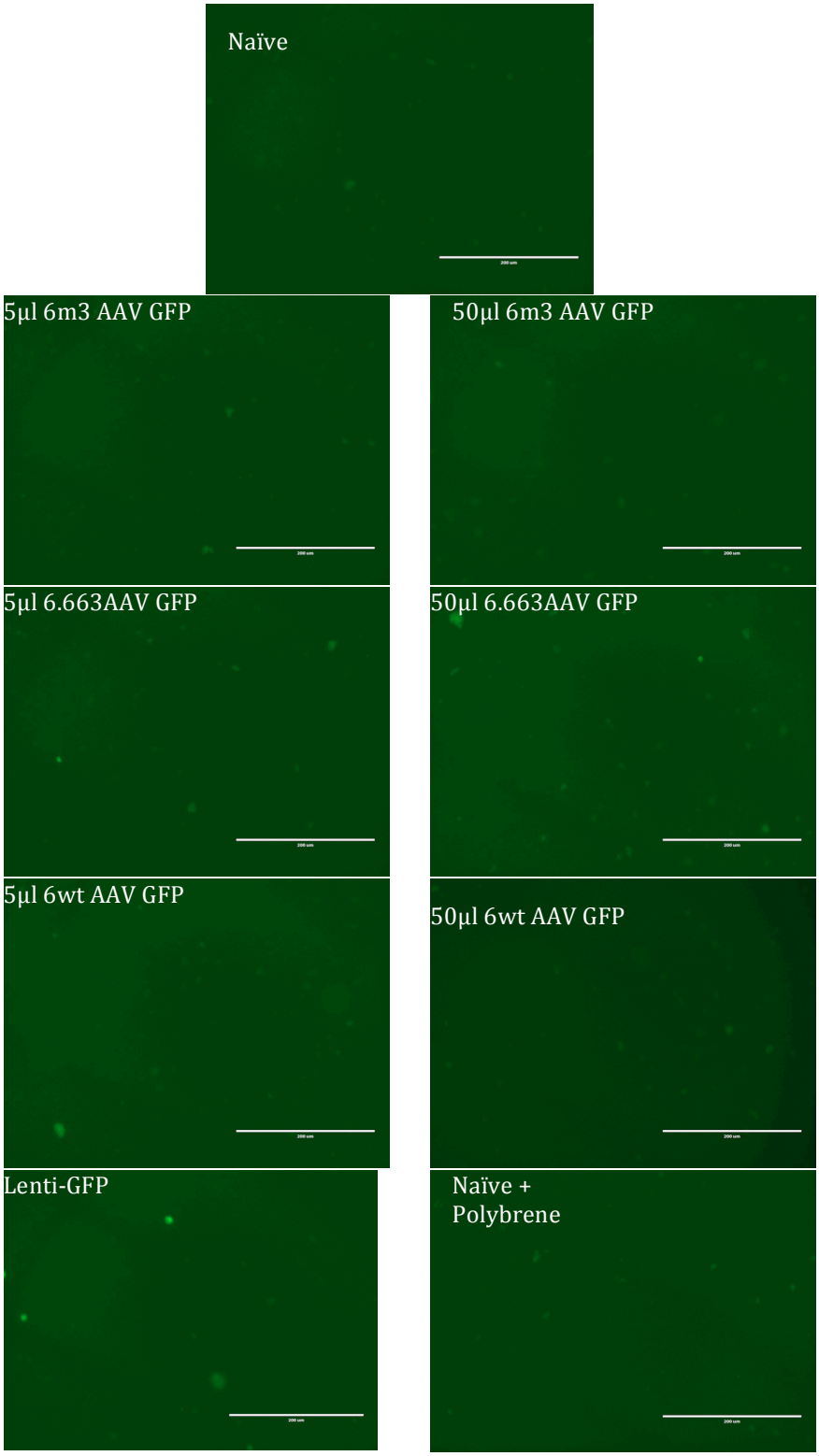


Figure 24. Fluorescent microscopy of figure 25, day 8 PBMCs transduced with varied amounts and AAV-GFP constructs compared to Lenti-GFP. Illuminated green cells are expressing GFP.

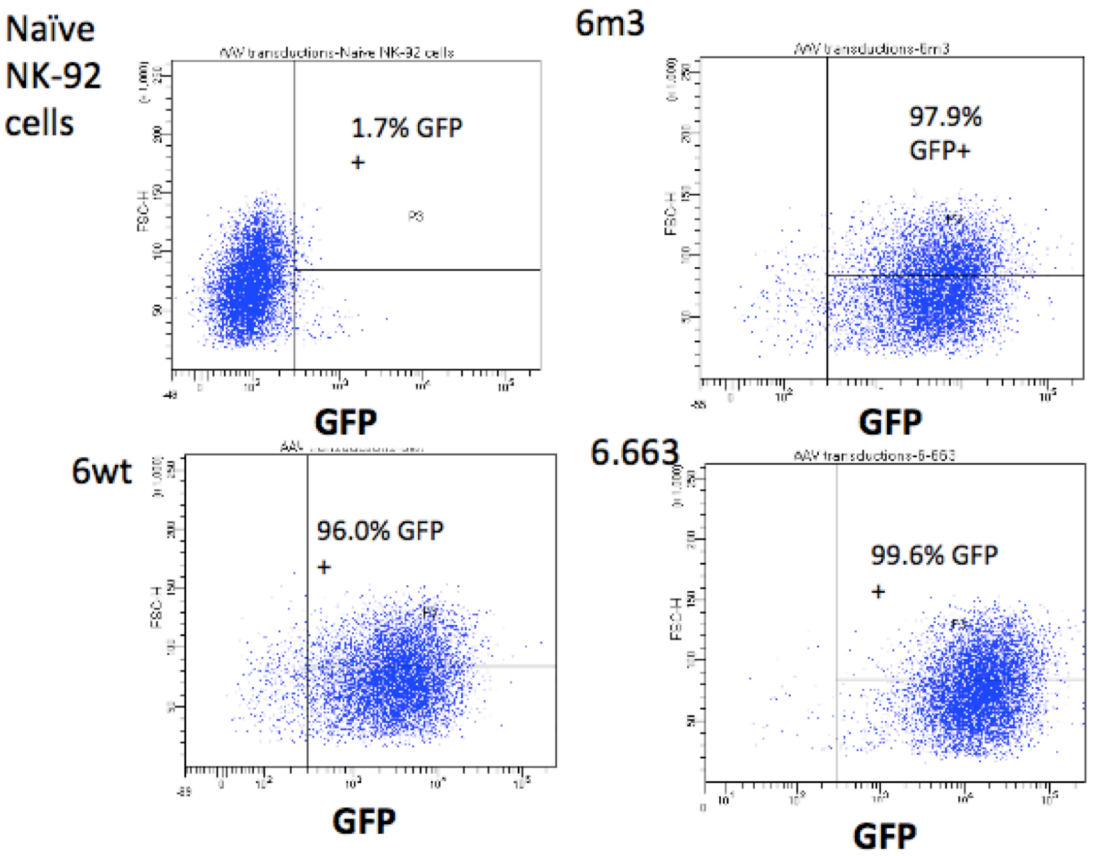


Figure 25. Flow cytometry measurement of GFP expression. NK-92 cells transduced with 5µl (MOI 50,000) of the AAV-GFP constructs 6m3, 6wt, and 6.663 day 3 post-transduction. The X-axis measures GFP expression and Y-axis measures the cell number. Gene expression in NK-92 cells is 97.9%, 96.0%, and 99.6% respectively. The top left panel is a naïve control.

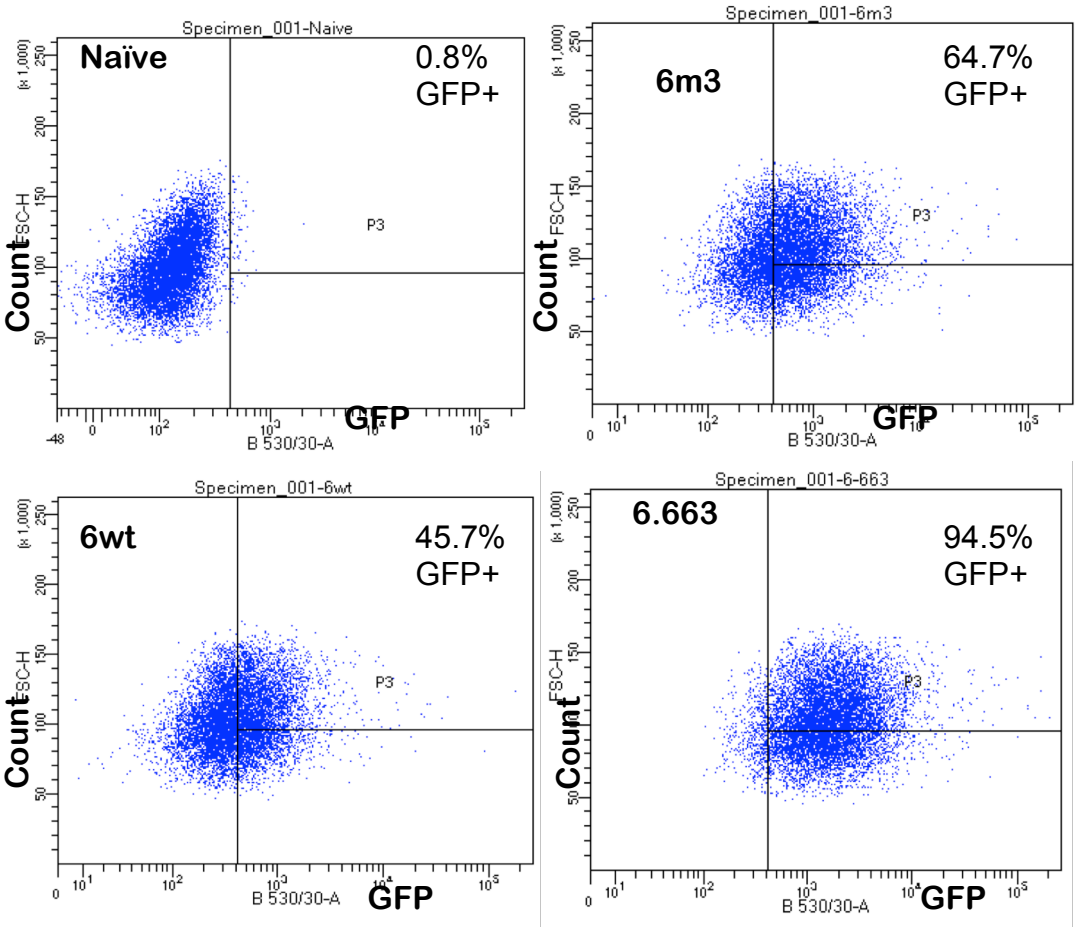


Figure 26. Flow cytometry measurement of GFP expression. NK-92 cells transduced with 5µl (titer of $1x10^{12}$) of the AAV-GFP constructs 6m3, 6wt, and 6.663 day 7 post-transduction. The X-axis measures GFP expression and Y-axis measures the cell number. Gene expression in NK-92 cells is 64.7%, 45.7%, and 94.5% respectively. The top left panel is a naïve control.

Day 3

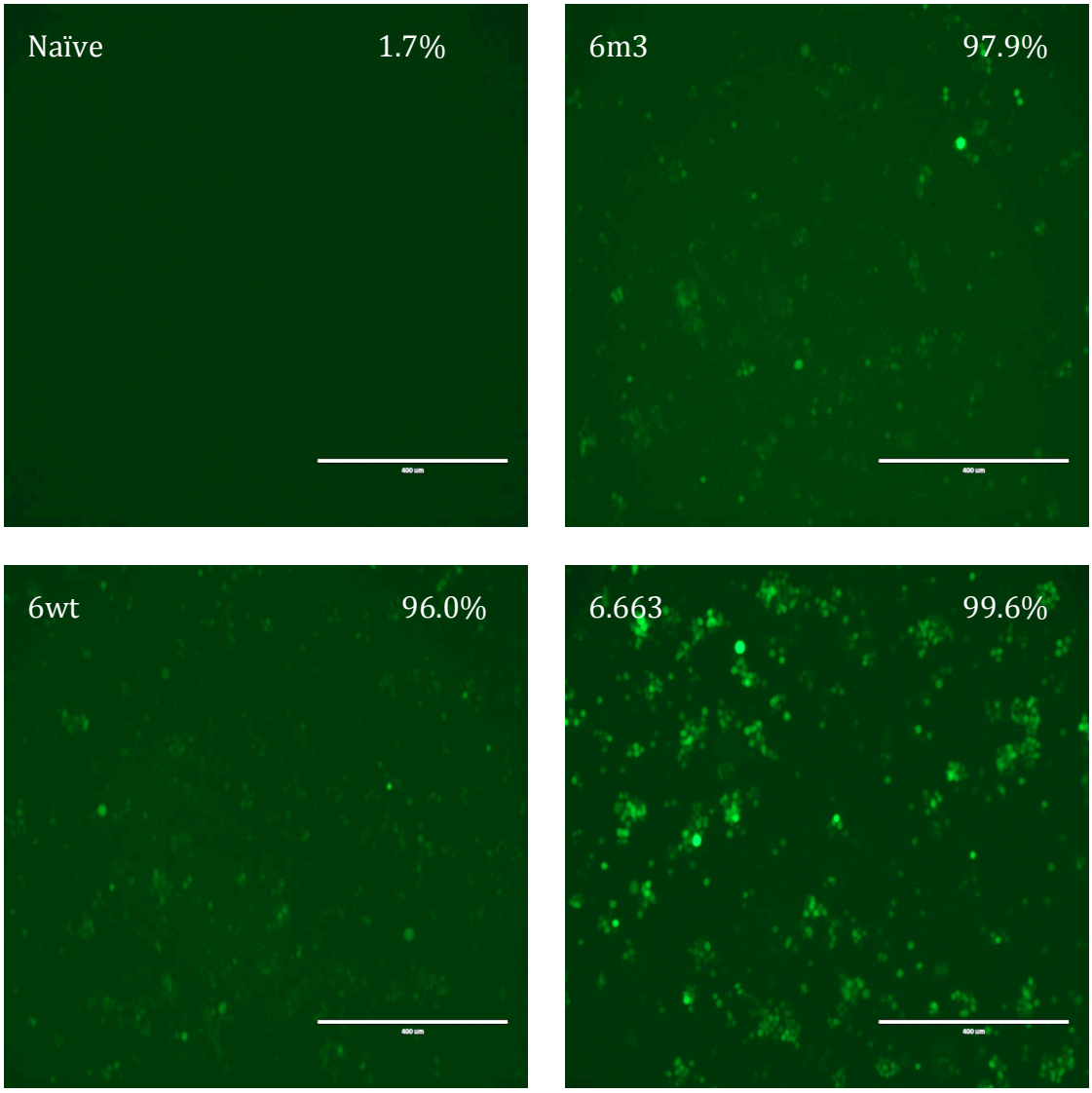


Figure 27. Fluorescent microscopy day 3 post- transduction of NK-92 with AAV-GFP vectors 6m3, 6wt, and 6.663. These images correspond with the flow cytometry results from figure 25. Illuminated green cells are expressing GFP.

Day 4

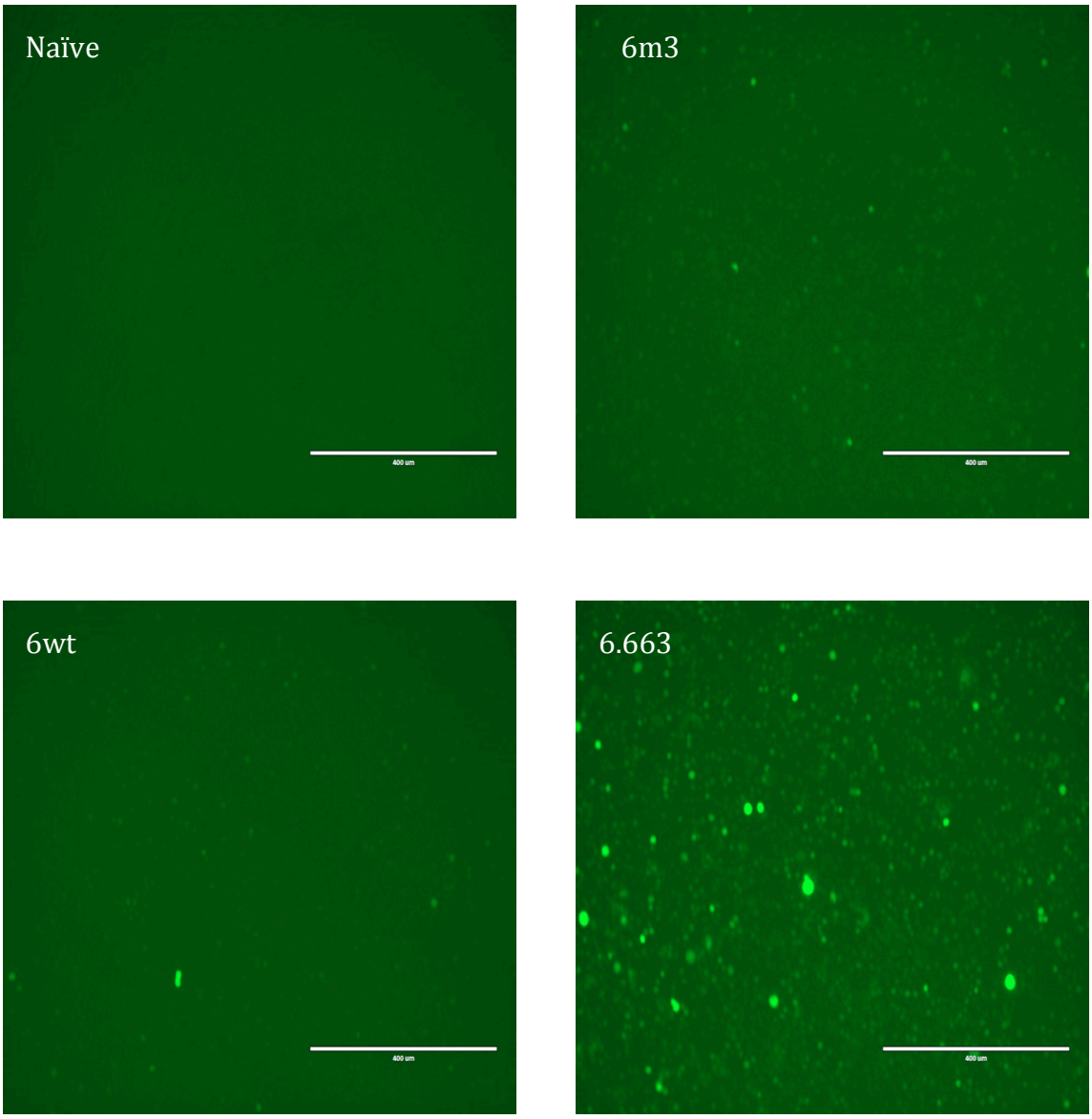


Figure 28. Fluorescent microscopy day 4 post- transduction of NK-92 with AAV-GFP vectors 6m3, 6wt, and 6.663 Illuminated green cells are expressing GFP.

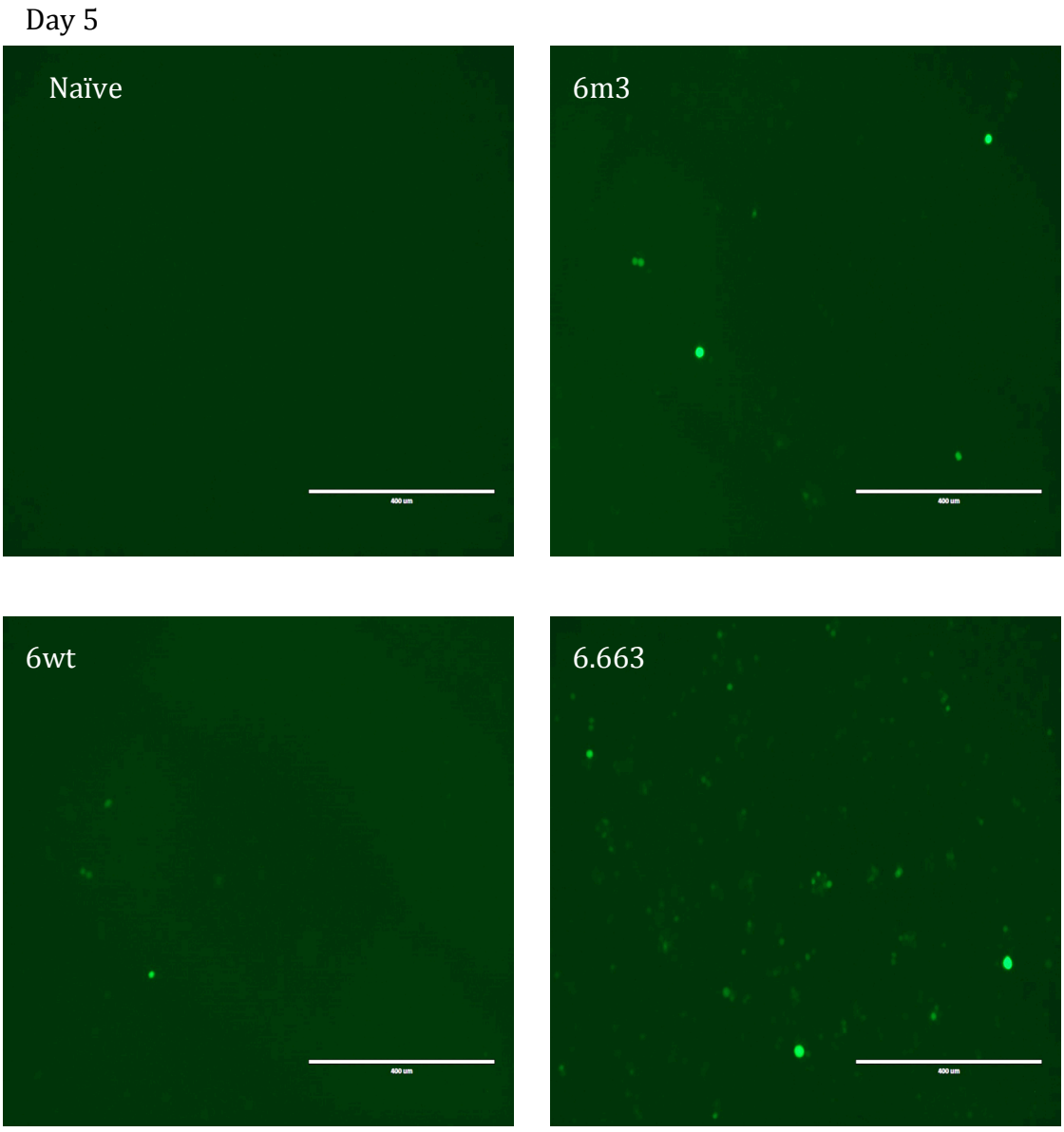


Figure 29. Fluorescent microscopy day 5 post- transduction of NK-92 with AAV-GFP vectors 6m3, 6wt, and 6.663. Illuminated green cells are expressing GFP.

Day 6

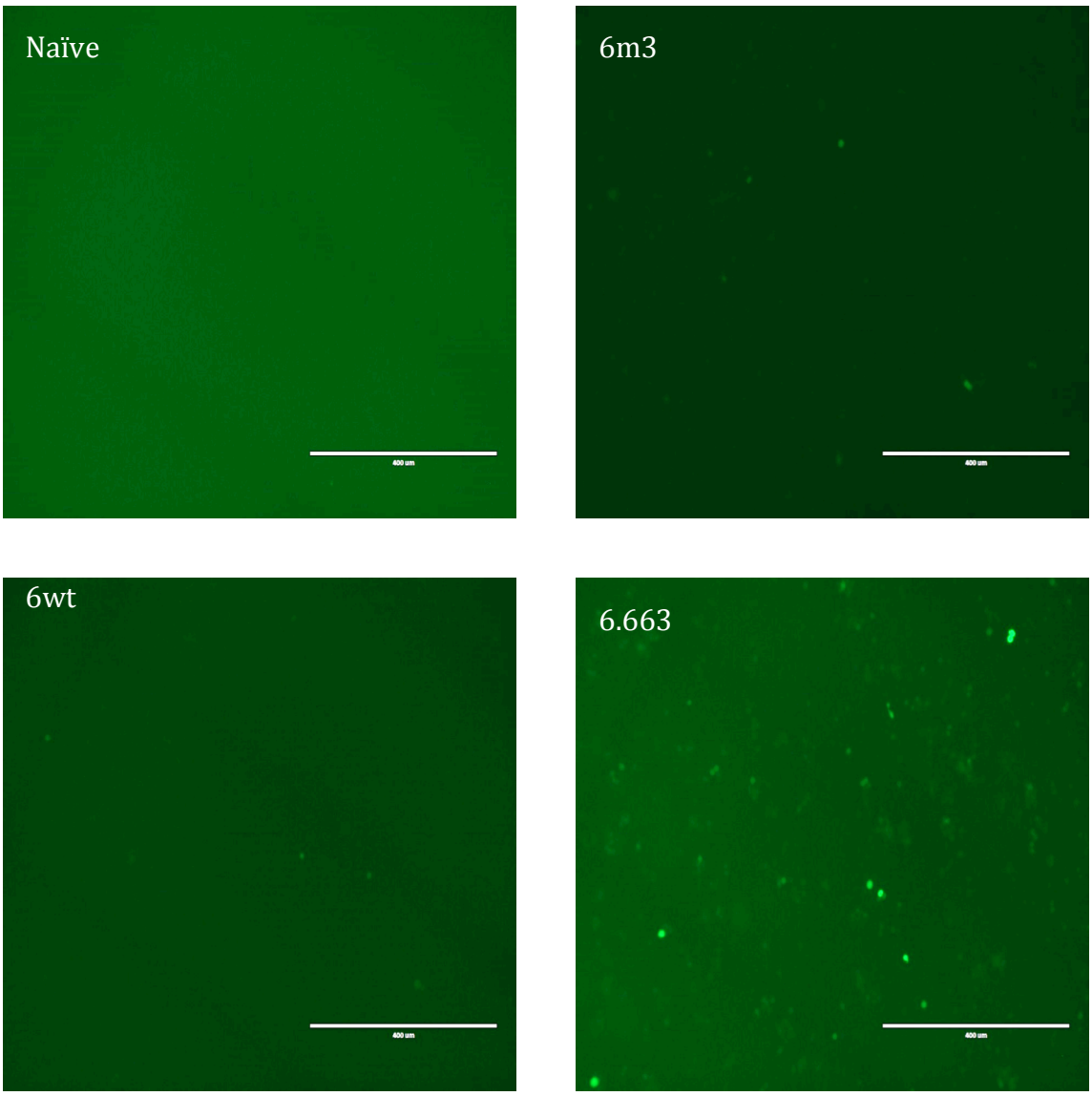
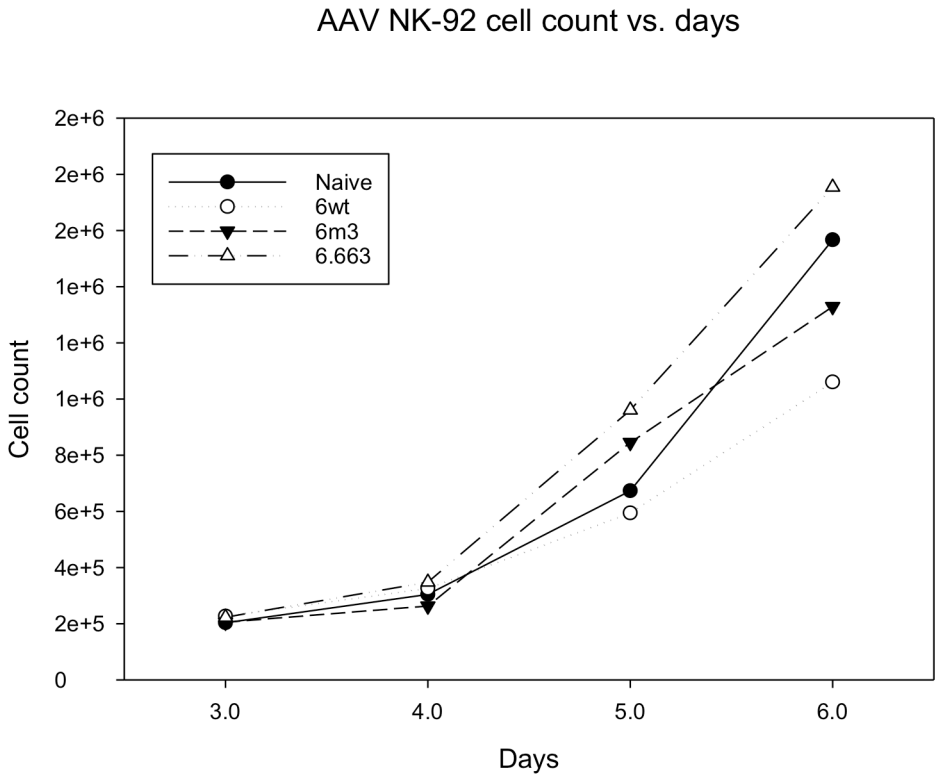


Figure 30. Fluorescent microscopy day 6 post- transduction of NK-92 with AAV-GFP vectors 6m3, 6wt, and 6.663. Illuminated green cells are expressing GFP.



Doubling time:
Naïve: 18.06h
6wt: 30.5h
6m3: 41.8h
6.663: 29.07h

Graph 2. Graph of cell counts of AAV transduced NK-92 cells over the course of 4 days. The doubling times for each group of transduced cells, 6m3, 6wt, and 6.663 at 41,8 hours, 30.5 hours, and 29.07 hours respectively, were calculated alongside naïve NK-92 cell doubling time of 18.06 hours.