Optimizing Cre Recombination to Develop *in vitro* Models for ABCA3 Deficiency

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Introduction

Advances in genetic engineering techniques could be the key to treating and immunizing people for the vast spectrum of DNA mutation-driven diseases. Genetic mutations can cause or significantly increase patients' chances of developing many of the world's leading causes of death, including Heart Disease, Cancer, certain Pulmonary Diseases, and Neurodegenerative Disorders.[1,2] With many recent breakthroughs in gene editing technology such as CRISPR-Cas9 techniques, and later base editing and prime editing, we have access to a plethora of new methods of modifying deadly disease-causing DNA patterns.[3,4]

With the lungs being a vital organ, DNA abnormalities in genes that encode for essential lung structure and function can have far-reaching effects on one's health. A vital gene for the lungs' ability to produce and transport surfactant is the ATP-binding cassette subfamily A member 3 (ABCA3) gene. Pulmonary surfactant, a substance made up of lipids and proteins, "lowers surface tension at the air-liquid interface" of the alveoli, protecting against atelectasis.[5] ABCA3 is located in the membrane of intracellular vesicles and lamellar bodies in Alveolar Type II (AT2) cells. The ABCA3 glycoprotein plays an integral part in surfactant production as a lipid pump. Specifically, ABCA3 is necessary for packaging and secreting surfactant lipids. Thus, when infants are born with a mutation to their ABCA3 gene, the condition is typically fatal.[6] Previous literature cites that symptoms before mortality due to knockout of the ABCA3 gene in a murine model include pulmonary inflammation, hyperemia, and alveolar wall-thickening. Currently, the only treatment option for such a disease is lung transplantation, which is not easily accessible to most patients, especially infants.[7] Thus, developing gene engineering techniques that can be used to treat infants who have this fatal condition can significantly reduce the high mortality rate of a very lethal disease.

All of these factors make it imperative to have access to a model that offers thorough assessment of genetic-engineering technology for ABCA3 mutation correction. Possible methods of editing, excising, and swapping gene sequences are rapidly developing with the recent surge in attention towards gene editing techniques. This study focuses on the use of Cre Recombinase, an enzyme that facilitates excision or swapping of genetic sequences flanked by "loxp" sites or mutant versions of loxp sites, which are specific DNA patterns that are recognized by the Cre Recombinase enzyme.[8] We use Cre recombination methods to test developed cell lines as models for assessing performance of gene editing technology on lung AT2 cells. One of the key innovations introduced in this project is the integration of a certain "landing pad" DNA sequence in the cell genome, similar to research done by Cooney *et al.*[9] Our landing pad sequence is flanked by either two loxp sites or one loxFAS site and one loxp site. Work by Lanza *et a*l. has shown that mutating lox-site sequence can alter the ratio of swapping to excision events. Specifically, they found a mutated lox site sequence known as loxFAS that increases the ratio of swapping to excision.[10] This is a finding we implement into our experimental strategy to work towards the goal of finding an optimized method of swapping the ABCA3 gene or its mutants into a chosen cell line. The application of this is to create an *in vitro* model cell line that can be modified to contain ABCA3 or its mutant versions to test effects of gene therapy techniques for ABCA3 deficiency. Our study uses a system of donor and integrant plasmids. After an integrant sequence containing lox sites has been transfected or transduced into a cell, the presence of Cre enzyme has the potential to swap that genetic sequence with another sequence provided by donor DNA. We seek to show how Cre Recombinase enzyme can be used to swap ABCA3 or a mutant version of it into lung AT2 cells by using a RFP and EGFP based negative reporter system.

Methodology

Plasmid Creation

To conduct this study, a number of plasmids were prepared to test Cre Recombination events. Plasmids created include integrants containing landing pads, donors to deliver certain genetic sequences, and plasmids that express a known element (commonly used as a positive control condition). All plasmids used were created in house except for pGM1247, which was obtained from Addgene.

Plasmid Identities

Plasmid Name	Type	Expresses or Includes	Configuration	Purpose/Use	Created in Lab
pGM131	Intermediate Donor	Wild Type ABCA3	LoxFAS/loxp	Delivering Wild Type ABCA3 gene to loxFAS/loxp sites	Yes
pGM1382	Intermediate Donor	Wild Type ABCA3	Loxp/loxp	Delivering Wile Type ABCA3 gene to loxp/loxp sites	Yes
pGM1383	Intermediate Donor	ABCA3 with E292V mutation	LoxFAS/loxp	Delivering ABCA3 with E292V mutation gene to loxFAS/loxp sites	Yes
pGM1384	Intermediate Donor	ABCA3 with L101P mutation	LoxFAS/loxp	Delivering ABCA3 with L101P mutation gene to loxFAS/loxp sites	Yes
pGM1385	Enzyme	Cre	N/A	Used to facilitate Cre Recombination	Yes
pGM1414	Final Donor (no CMV promoter)	Enhanced Green Fluorescent Protein (EGFP)	Loxp/loxp	Used to test Cre Recombination efficiency via Luciferase expression	Yes

pGM1415	Intermediate Donor	Firefly Luciferase	Loxp/loxp	Used to test Cre Recombination efficiency via Luciferase expression	Yes
pGM1417	Intermediate Donor	ABCA3 with L101P mutation	Loxp/loxp	Delivering ABCA3 with L101P mutation gene to loxp/loxp sites	Yes
pGM1419	Intermediate Donor	Enhanced Green Fluorescent Protein (EGFP)	N/A	Used to test Cre recombination swapping event frequency	Yes
pGM1380	Integrant (Lentiviral Vector Genomes)	dsRed flanked by loxFAS/loxp sites followed by EGFP and puromycin resistance	loxFAS/loxp	Used to assess frequency of swapping versus excision events with a loxFAS/loxp configuration	Yes
pGM1247	Integrant (Lentiviral Vector Genomes)	dsRed flanked by loxp sites followed by EGFP and puromycin resistance	loxp/loxp	Used to assess frequency of swapping versus excision events with a loxp/loxp configuration	No
pGM963	Stuffer	Empty plasmid	N/A	Used as a control in transfection experiments	Yes
pGM356	Lentiviral Vector Genomes)	Firefly Luciferase	N/A	Used to create pGM1415	Yes
pGM357	Lentiviral Vector Genomes	EGFP	N/A	Used to create pGM1414	Yes
pGM967	Expressing plasmid	EGFP	N/A	Used as a control for EGFP	Yes

				expression	

Note: The above figure identifies all plasmids used in this project, what they encode, their loxp/loxp vs loxFAS/loxp configuration (if applicable), purpose, and whether or not they were created in house.

To prepare the above plasmids, the CMV LTR Fusion promoter needed to be removed from pGM1381, pGM1382, pGM1383, pGM1384 plasmids. The promoter was located upstream of the region of Cre Recombination, meaning that proteins encoded by the donor plasmids or proteins encoded by genes swapped into donor plasmids during recombination would be expressed, unless the promoter was removed. Restriction enzyme cloning techniques were used with SalIHF and PSPX enzymes to excise the promoter. 5 ug of each plasmid was mixed with 2 uL of each enzyme, 2 uL CutSmart Buffer from New England Biolabs, and then solution was brought up to 20 uL final volume using nuclease free water. Samples were kept in a thermocycler for 1 hour and 20 minutes in 37°C and then stored at 4°C. 1% agarose gel was prepared including 1x Cybersafe from invitrogen. This same process was used for all restriction enzyme digests for gel extraction in this study. Loading dye was added for a 1x concentration for all samples, and the samples were loaded onto the gel and run at 100 V for 120 minutes. The bands produced were then excised and purified using Qiagen gel Purification Kit following the standard protocol. Then the plasmids were ligated via standard protocol with New England Biolabs Quick Ligation kit, using 50 ng of backbone DNA and 3x that molecular weight worth of insert DNA. The resulting plasmids were transformed into bacterial cells via standard protocol using One Shot Stbl3 cells. After leaving the colonies to incubate overnight, five colonies were chosen from each sample (larger colonies were favored in choice) to inoculate. The selected colonies were kept in 5 mL LB broth in a shaking incubator at 225 RPM overnight.

Consequently, 1.5 mL of each sample was used to generate stock solutions of each plasmid via the Wizard Plus SV Miniprep DNA Purification protocol. Results of all samples were assessed via restriction enzyme diagnostic digestion using Nhe1 and ClaI, to confirm that the produced plasmids reflect the correct band sizes. All diagnostic digests in this study use samples of 0.5 uL of each enzyme, 200 ng of each plasmid, 2 uL cutsmart buffer, and then enough nuclease free water to bring the solution to 20 uL. To move forward with constructing more plasmids, pGM1382 intermediate 4, pGM1384 intermediate 4, and pGM1384 clone 5 were cut with NheI and HindIII to isolate inserts and backbones of the plasmids. Next, pGM356 and pGM357 were cut with just NheI to differentiate between the linear fragment and uncut band. Then they were afterwards cut with HindIII to isolate the firefly luciferase and EGFP inserts. Next, T4 ligation reactions were done to construct pGM1414, pGM1415, pGM1417, and pGM1419. The ligation reaction combinations were as follows: pGM1382 colony 5 + pGM357 insert = pGM1414, pGM1382 colony 5 + pGM356 insert = pGM1415, pGM1382 backbone + pGM1384 insert = pGM1417,and pGM1382 intermediate 4 + pGM357 insert = pGM1419. All samples were then again cut with NheI and HindIII restriction enzymes for a diagnostic digest. Then the samples were transformed via the aforementioned Stbl3 cell One Shot protocol. A diagnostic digest was done cutting the samples with PspOMI to ensure that plasmids were created correctly. Midi prep techniques were used to produce stock solutions of pGM1414, pGM1415, pGM1417, and pGM1419. All midi preps were done as follows, using reagents from Qiagen Plasmid Midi kit. After bacterial cell cultures were incubated in 1.5 mL broth, 400 uL was transferred into a 2 L Erlenmeyer flask containing LB Broth and left to grow overnight. Then bacterial cultures were centrifuged at 6000 x g for 15 minutes at 4ºC. All supernatant was aspirated and the bacterial cell pellet was resuspended in 4 mL of Buffer P1. Then, 4 mL of Buffer P2 was added followed by

vigorous inverting of the mixture 4-6 and a 5 minute incubation at room temperature. Next, 4 mL of prechilled Buffer P3 was added to the mixture and again followed by vigorous inversion 4-6 times. The resulting mixture was then centrifuged at 20,000 x g for 30 minutes at 4ºC. Then equilibrate a Qiagen-tip by allowing 4 mL of buffer QBT to pass through via gravity flow. Apply the supernatant from the previous centrifuge Qiagen-tip and allow it to pass via gravity flow. Then, was the Qiagen tip with 10 mL of Buffer QC twice. Then, elute DNA by passing 5mL of Buffer QF through the Qiagen-tip into a clean vessel. Precipitate the DNA by adding 3.6 mL of 70% isopropanol to the eluted DNA and mix. Then, centrifuge the sample at 15,000 x g for 30 minutes at 4ºC. Afterwards, carefully decant the supernatant. Wash the DNA pellet with 2 mL of room temperature 70% ethanol and centrifuge at 15,000 x g for 10 minutes and again carefully decant the supernatant. Finally, air-dry the pellet for 5-10 minutes and redissolve DNA.

Cell Culture

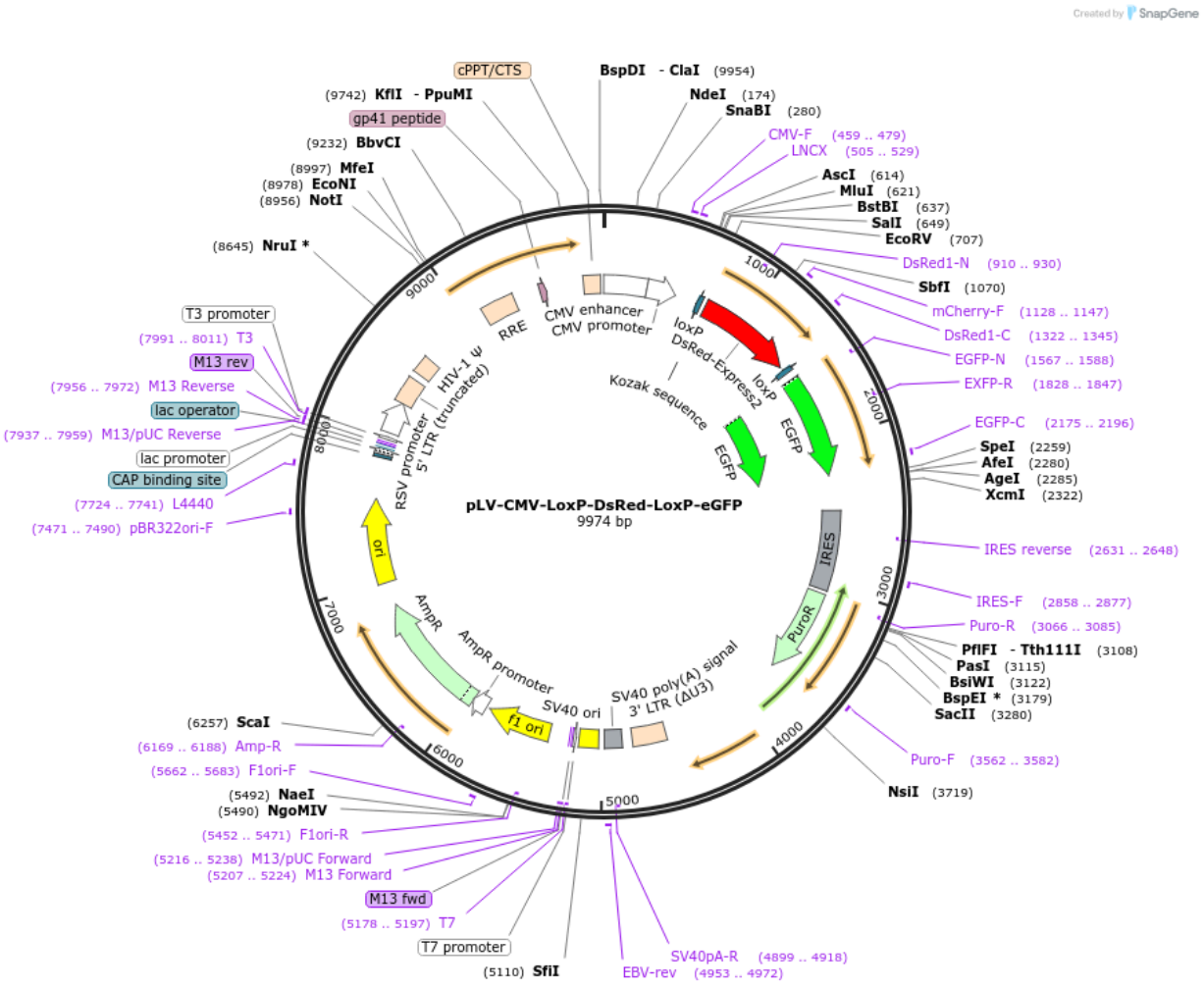
A549 human alveolar basal epithelial cells and HEK293T cells were used to generate genetically modified cell lines to assess landing pad technology. A549 cells were chosen as a model AT2 cell line as they are used as a gold standard for this purpose in previous literature. Meanwhile, HEK293T cells were frequently used to optimize recombination techniques due to their previously established high capacity for transfectability. Experimental methods were often first tested on HEK293T cells as a proof of concept for the technique and then redone on A549 cells with an integrated landing pad to test the landing pad functionality.

Lentiviral transduction was done on all cells (except A549 positive control landing pad cells) using a HIV VSVG pseudotype vector with pGM1247, which encodes for dsRed, a stop codon, EGFP, and puromycin resistance. A549 clone 2f3 original landing pad cells were transduced with pGM1380 (the loxFAS:loxp version of pGM1247), so everything remained

constant in the integrant plasmid except for switching the first loxp site into a loxFAS site. To select for cells that were sufficiently transduced, puromycin was added to the media of all A549 and HEK293T cell cultures at the concentration of 1.5 ug/mL for three days. Afterwards, single cell cloning via lenti dilution was done to isolate separate cell line samples.

Flow Cytometry was used to test if all cell lines derived from clones had the intended gene expression after the lentiviral transduction. The Flow Cytometry data revealed that some cell lines were not 100% dsRed expressing, so to re-select for cells that were sufficiently transduced, another 3 day puromycin selection was done using 1.5 ug/mL concentration.

pGM1247 Map from Addgene



Note: The above figure is a plasmid map of pGM1247: the integrant plasmid for loxp/loxp configuration.

The above plasmid was obtained from AddGene (catalogue code #65726). All integrants used include a genetic pattern that encodes for dsRed expression followed by a stop codon, EGFP expression, and puromycin resistance. The loxp/loxp sites flanking the dsRed gene and stop codon allows us to fluorescently mark the different types of Cre recombination. When the dsRed and stop codon sequence is excised via Cre recombination, EGFP will fluoresce instead of dsRed. However, when the Cre Recombinase enzyme swaps the dsRed and stop codon sequence with a donor plasmid that contains a stop codon as well, there should be no fluorescence of RFP or GFP. To test this in loxp/loxp and loxFAS/loxp conditions, the original plasmid was edited with double stranded primers to produce pGM1380, a plasmid identical to pGM1247 but with loxFAS/loxp configuration instead of loxp/loxp.

Transfection studies were done to use Cre Recombination as a tool to assess the cell lines' potential for use as an *in vitro* model for gene therapy on AT2 cells with ABCA3 deficiency. The first transfection was to assess the performance of Cre Recombination by isolating the variables of loxp/loxp sites versus loxFAS/loxp sites and the addition or absence of Cre enzyme. These were done on HEK293T negative control cells to first test validity of the method.

	Condition 1	Condition 2	Condition 3	Condition 4	Condition 5	Condition 6
pGM963 (stuffer)	no	yes	yes	no	yes	no
pGM967 (EGFP expressing)	no	yes	no	no	no	no

pGM1247 (loxp/loxp dsRed gene)	no	no	yes	yes	no	no
pGM1380 (loxFAS/loxp dsRed gene)	no	no	no	no	yes	yes
pGM1385 (Cre enzyme)	no	no	no	yes	no	yes

Note: "Transfection 1:" The above figure maps the DNA transfected into each condition.

1.5 mL centrifuge tubes were assigned to each of the 6 conditions, and 500 ng of each plasmid was dissolved in Opti-Mem from Thermofisher Scientific. Then, lipofectamine reagent was diluted in six times the volume of Opti-Mem that the DNA was, to create a stock solution that can be added to all conditions. The ratio of 1 ug total DNA per well: 3 uL lipofectamine was used. All mixtures were left to incubate at room temperature for five minutes. Then an equal volume of the lipofectamine/Opti-Mem mix was added into each of the six tubes containing DNA. These new mixtures were left at room temperature to incubate for another 15 minutes. Finally, the DNA, lipofectamine, and OptiMem mixtures were added to corresponding wells with HEK293T negative control cells. Results were read 48 hours after transfection. This same framework was used for all HEK293T cell transfections in this project.

	Cond. 1	Cond. 2	Cond. 3	Cond. 4	Cond. 5	Cond. 6	Cond. 7	Cond. 8
pGM963	no	no	no	no	no	no	no	no
pGM967	no	no	no	no	no	yes	no	no
pGM1247	no	yes	no	yes	no	no	no	no
pGM1380	no	no	yes	no	yes	no	no	no
pGM1385	no	no	no	yes	yes	no	no	no

pGM1381 (loxFAS/ loxp donor)	no	no	yes	no	yes	no	no	no
pGM1382 (loxp/loxp donor)	no	yes	no	yes	no	no	no	no
pGM1414 (EGFP donor with no promoter)	no	no	no	no	no	no	yes	no
pGM1419 (EGFP donor)	no	no	no	no	no	no	no	yes

Note: "Transfection 2:" The above figure maps the DNA transfected into each condition

The same transfection techniques as Transfection 1 were used, except conditions with just one plasmid (conditions 6,7, and 8) used 500 ng of total DNA, which the lipofectamine concentration was adjusted to according to the same 1 ug DNA: 3 uL lipofectamine ratio.

Transfection 3

All conditions with two plasmids were transfected with 350 ng of each plasmid. Conditions with three plasmids were transfected with 333.33 ng of each plasmid. A ratio of 1 ug DNA: 3 uL lipofectamine was used following the same transfection protocol as previously used. All conditions were repeated in triplicates. The lipofectamine/OptiMem mix used for the two plasmid condition was added to the Sham well.

Condition	**1**	**2**	**3**	**4**	**5**	**6**	**7**
	No plasmid	pGM1247 pGM1382	pGM1247 pGm1385	pGM1247 pGM1382 pGM1385	pGM1380 pGM1381	pGM1380 pGM1385	pGM1380 pGM1381 pGM1385

Note: "Transfection 3:" The above figure lists the plasmids that were added into each of the wells based on what condition they were assigned.

Transfection 4, Luciferase Assay, and Protein Assay

All transfections were single transfections with 500 ng of each plasmid added.

Condition	**1**	**2**	**3**	**4**	**5**	**6**
Time = 0 h	No plasmid	pGM1415	pGM1385	pGM1415	pGM1415	pGM356
Time = 24 h	No plasmid	pGM963	No plasmid	pGM1385	No plasmid	No plasmid

Note: "Transfection 4:" The above table shows the plasmids that were transfected at each time point for cells used for the Luciferase Assay.

Each condition for this experiment was done in triplicates that were kept separate throughout the transfection, Luciferase Assay, and Protein Assay. For the Luciferase Assay, cells were harvested after washing with PBS and dissociation using TrypLE. Cells were then centrifuged at 600 x g for 5 minutes and resuspended in PBS thrice. After the third centrifuge, cells were resuspended in Reporter Lysis Buffer and frozen in -80 degrees Celsius. The next day, cells were left to thaw on ice for a few hours before they were centrifuged again at 600 x g for five minutes and the supernatant from each tube was moved to a new clean tube. Then 20 uL of each sample was added to a microplate and 100 uL of Luciferase Assay Reagent was added to each well as the plate was being read for luminescence. The Protein Assay was done using the standard Microplate Assay protocol (with addition of Reagent S to Reagent A) from DC Protein Assay Instruction Manual. Luminescence values for each condition were divided by the protein quantification value collected for that respective condition to normalize luminescence values to protein content.

Condition	1	2	3	4	5	6	7	8
Time = 0 hr	No plasmid	pGM967	ABCA3 expressing plasmid	pGM1380 pGM1381	pGM1380 pGM1381 pGM1385	pGM1380 pGM1381	pGM1380 pGM1381 pGM963	pGM1380 pGM1385
Time = 24 hr	No plasmid	No plasmid	No plasmid	pGM1385	pGM963	pGM963	pGM963	No plasmid

Note: "Transfection 5:" The above table shows which plasmids were transfected into A549 original clone 2f3 landing pad cells during each time point

When transfecting A549 cells, a different protocol was used since A549 cells do not have the high transfectability that 293T cells have. A549 cells were seeded at a density of 80,000 cells per well in a standard 24 well plate. After 24 hours, all DNA was mixed in 27.5 uL of OptiMem per condition and left to incubate at room temperature for 5 minutes. Then, a ratio 3 uL Fugene HD Transfection Reagent per 1 ug of DNA was added to each of the tubes. Then, these mixtures were left to incubate at room temperature for another 15 minutes. Lastly, 25 uL of each mixture of DNA and Fugene reagent were added to their corresponding cells. Transfections were read 48 hours after the first transfection.

Quantitative Polymerase Chain Reaction

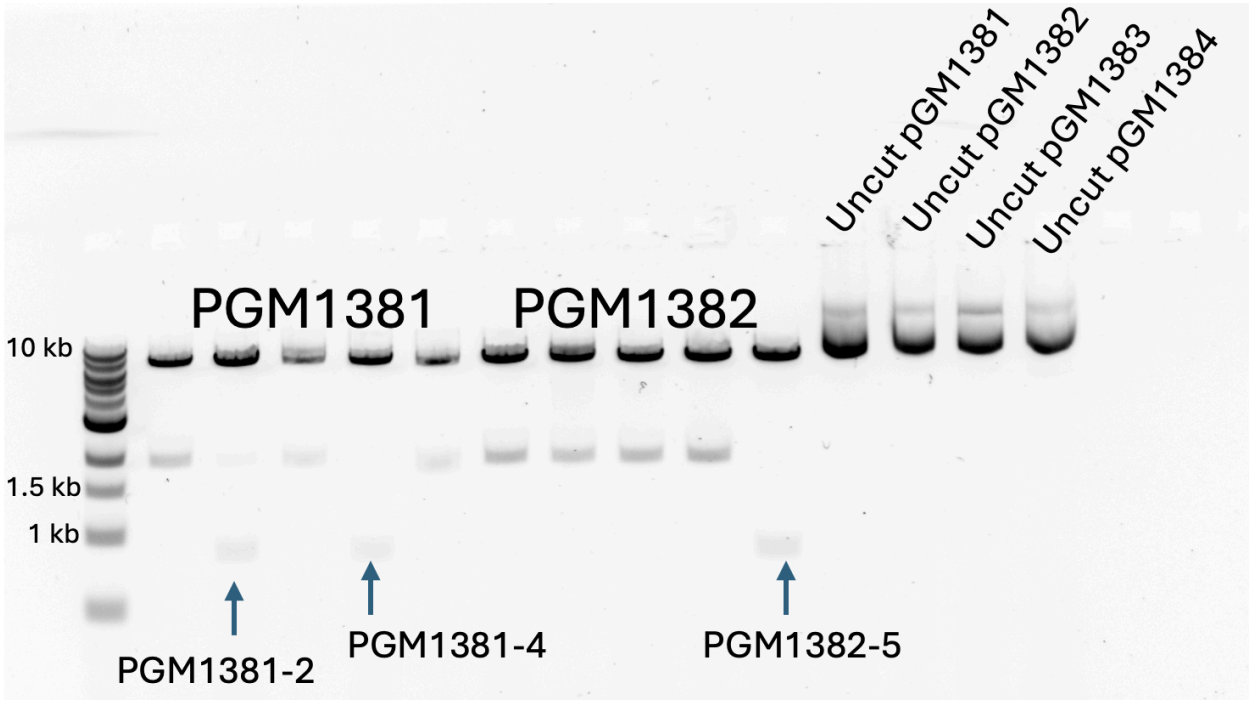
Genomic DNA was isolated from each clonal cell line using standard protocol with Qiagen Genomic DNA Isolation kit. Then, DNA samples were diluted to 20 ng per microliter. PCR samples were loaded in a 384 well plate mixing 4 uL of sample with 6 uL of qPCR mastermix for EGFP expression. The mastermix ratio was 5 uL of 2x Fast Advanced mastermix, 0.03 uL DNAVEC forward 100 uM primer, DNAVEC reverse 100 uM primer, 0.2 uL DNAVEC WPRE probe 5 uM, and 0.74 uL Nuclease Free Water. Standards were produced by serial diluting 327680 copies of pGM1247 by a fourth 8 times.

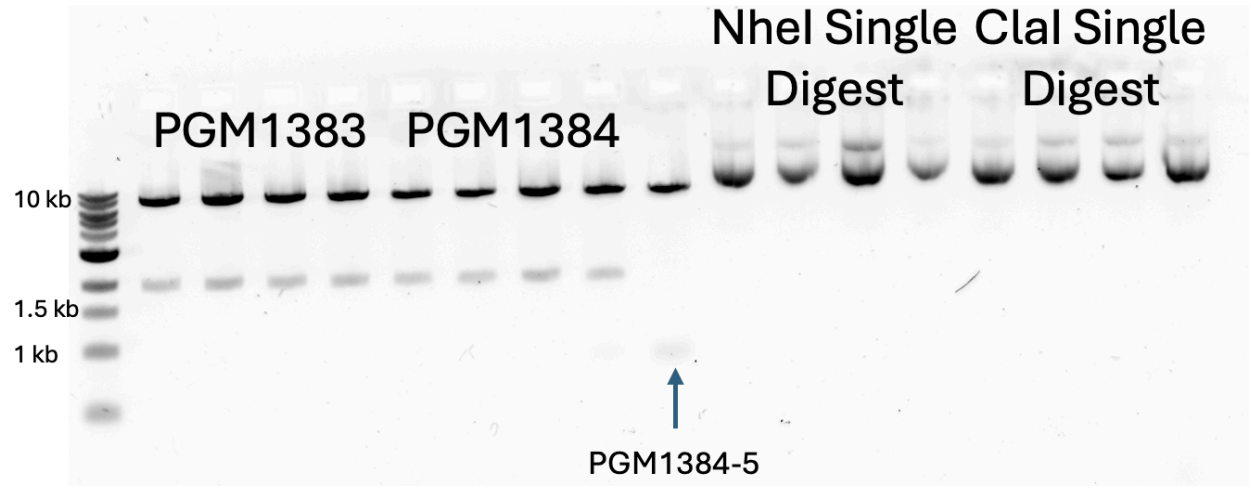
Cell Line Selection

Based on data between qPCR results and fluorescence microscopy, cell lines showing the best transduction efficiency were chosen to be preserved for future experimentation.

Results

Diagnostic Digest: Nhe1 and Clal – Isolating Backbones and inserts

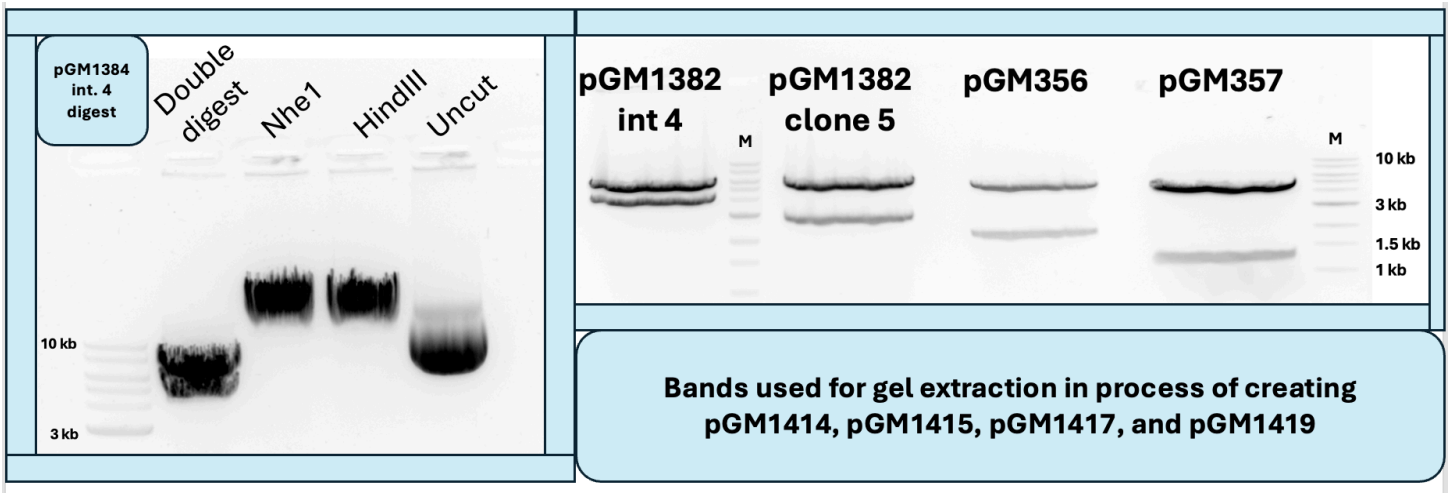




Note: The above figure shows chosen clones of each donor plasmid for pGM1381, pGM1382, and pGM1384. Single digests are shown to ensure that each enzyme was cutting appropriately.

The plasmids were expected to be cut into 7,807 base pairs (BP) and 902 BP fragments. Based on this diagnostic digest, we isolated pGM1381 clone 2, pGM1382 clone 4, and pGM1384 clone 5 as they showed bands at the expected positions of the gel. pGM1383 was not used for the experiments discussed in this report.

Gel Extraction and Diagnostic Digests: NheI and HindIII

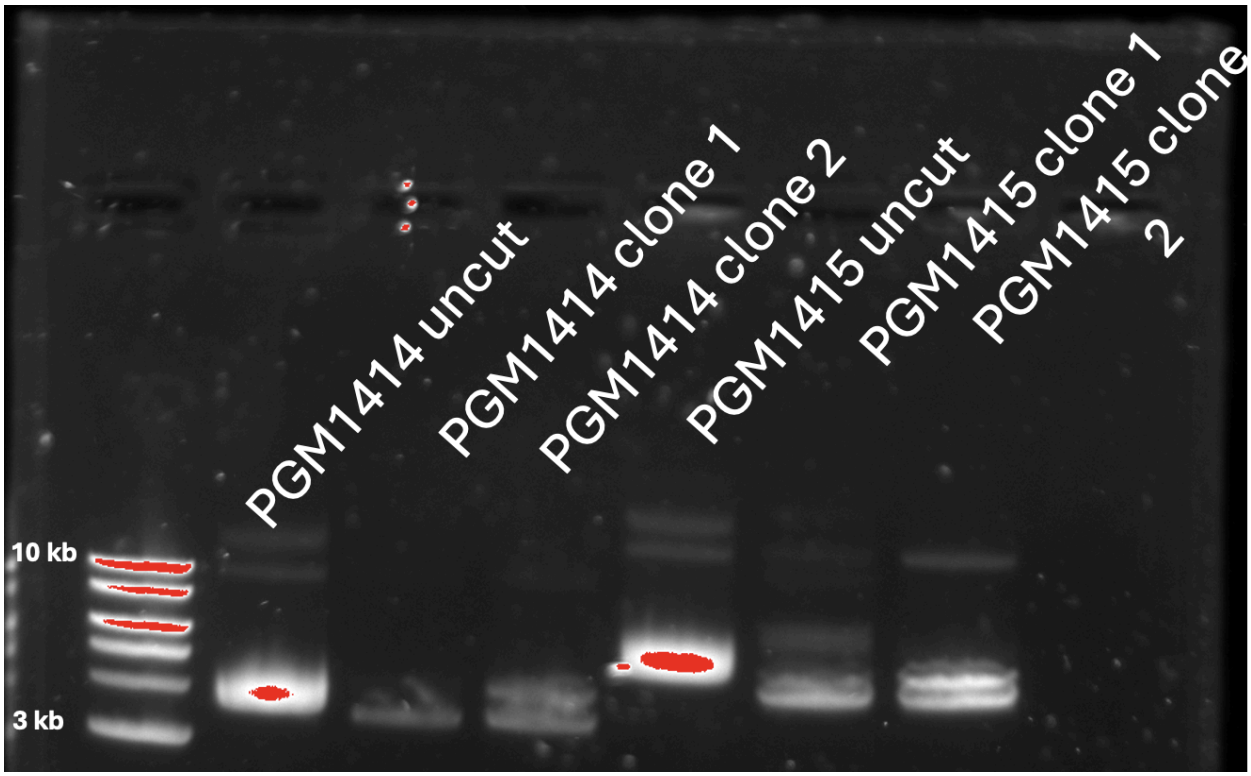


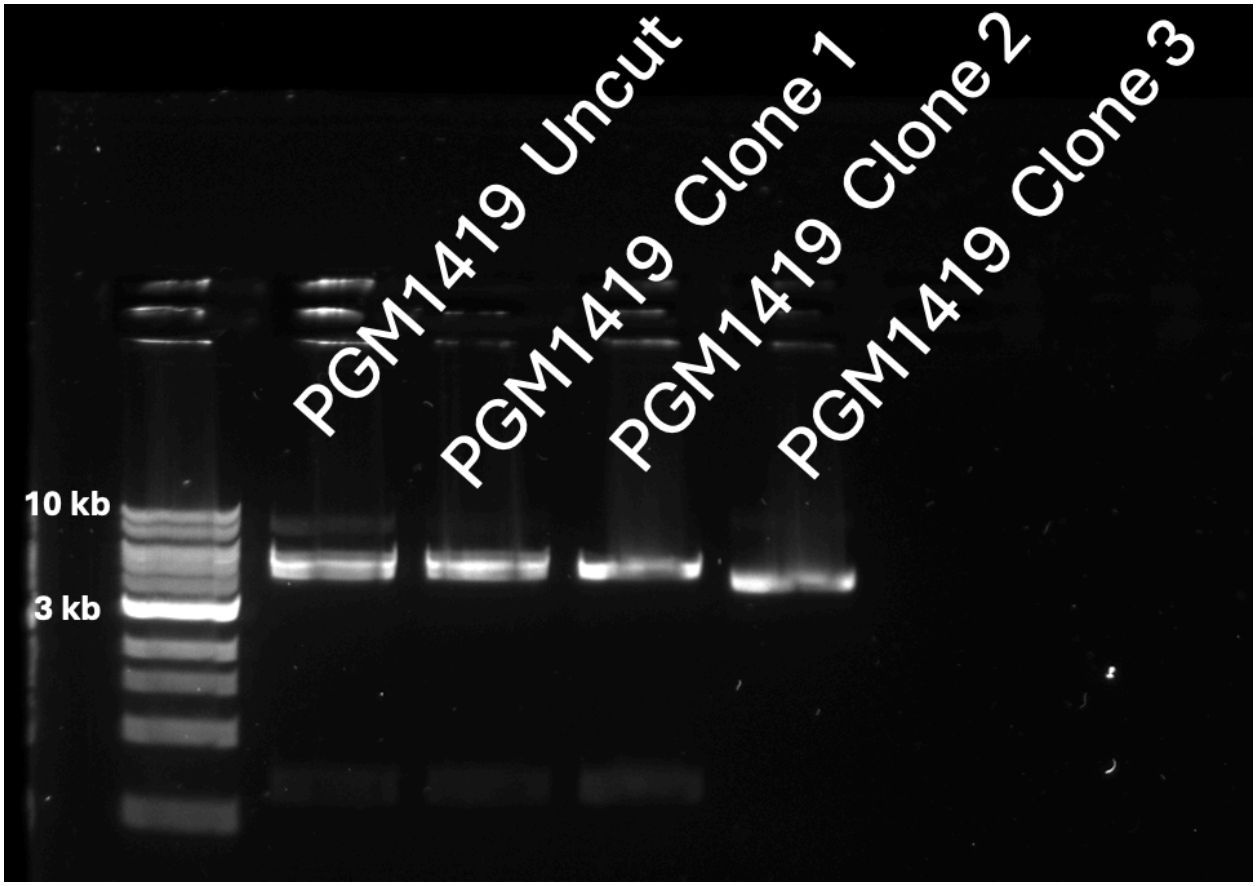
Note: The above figure shows the final bands of pGM1382 intermediate 4, pGM1384 intermediate 4, pGM1382 clone 5, pGM356, and pGM357 following enzyme digests that separated their DNA backbones and inserts.

NheI and HindIII cuts were expected to cut pGM1382 and 1384 intermediates into fragments of 5270 bp and 2989 bp. For pGM1382 clone 5, a clone with a region of the original plasmid excised to remove the CMV promoter, the fragments were expected to be 5270 bp and 1989 bp. For pGM356, the expected fragment sizes were 2272 bp for the insert and 5450 bp for

the backbone. Lastly, pGM357 was expected to yield fragments of sizes 1333 bp for the insert and 5457 bp for the backbone. All bands were located at the expected regions before they were purified and used for further cloning.

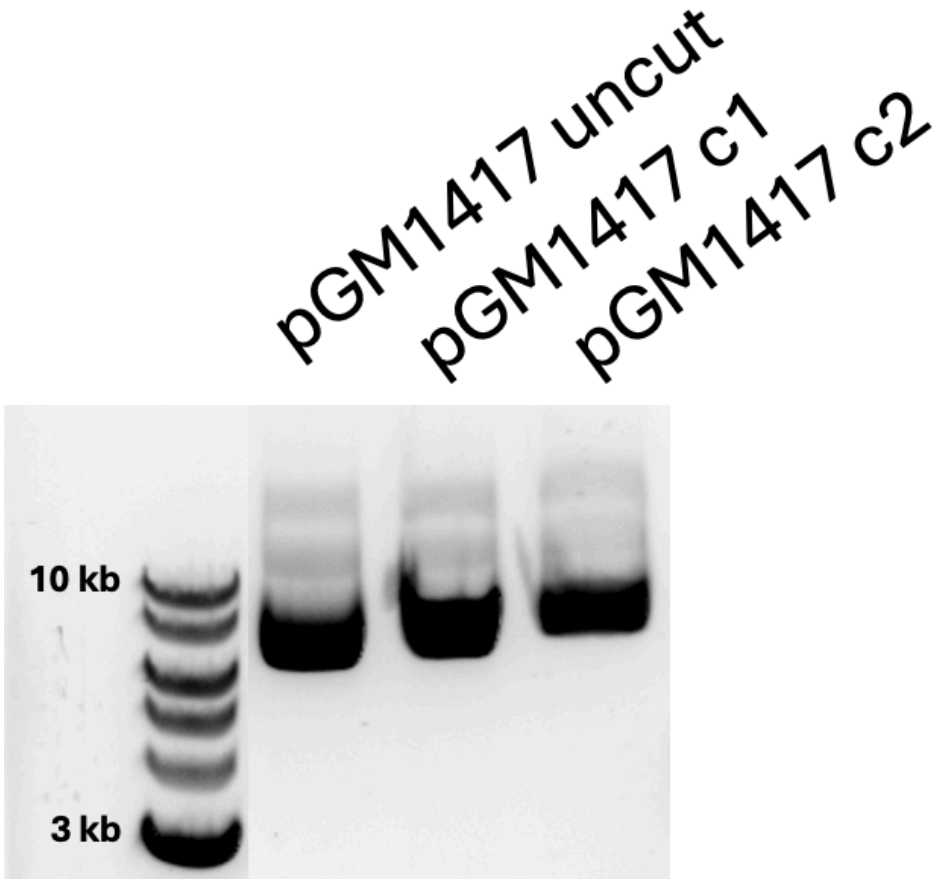
Diagnostic Digest: PspOMI





Note: The above figure shows plasmids cut with PspOMI to ensure that the new plasmids have intended characteristics.

Plasmids were expected to produce the following fragment sizes following PspOMI restriction enzyme digest: pGM1414– 660 bp and 3662 bp, pGM1415– 660 bp and 4601 bp, and pGM1419– 660 bp and 4778 bp. Based on the small size of the 660 bp fragments, they were run off the gel within the electrophoresis time. Based on these parameters, pGM1414 clone 2, pGM1415 clone 1, and pGM1419 clone 2 were chosen to prepare the plasmids.

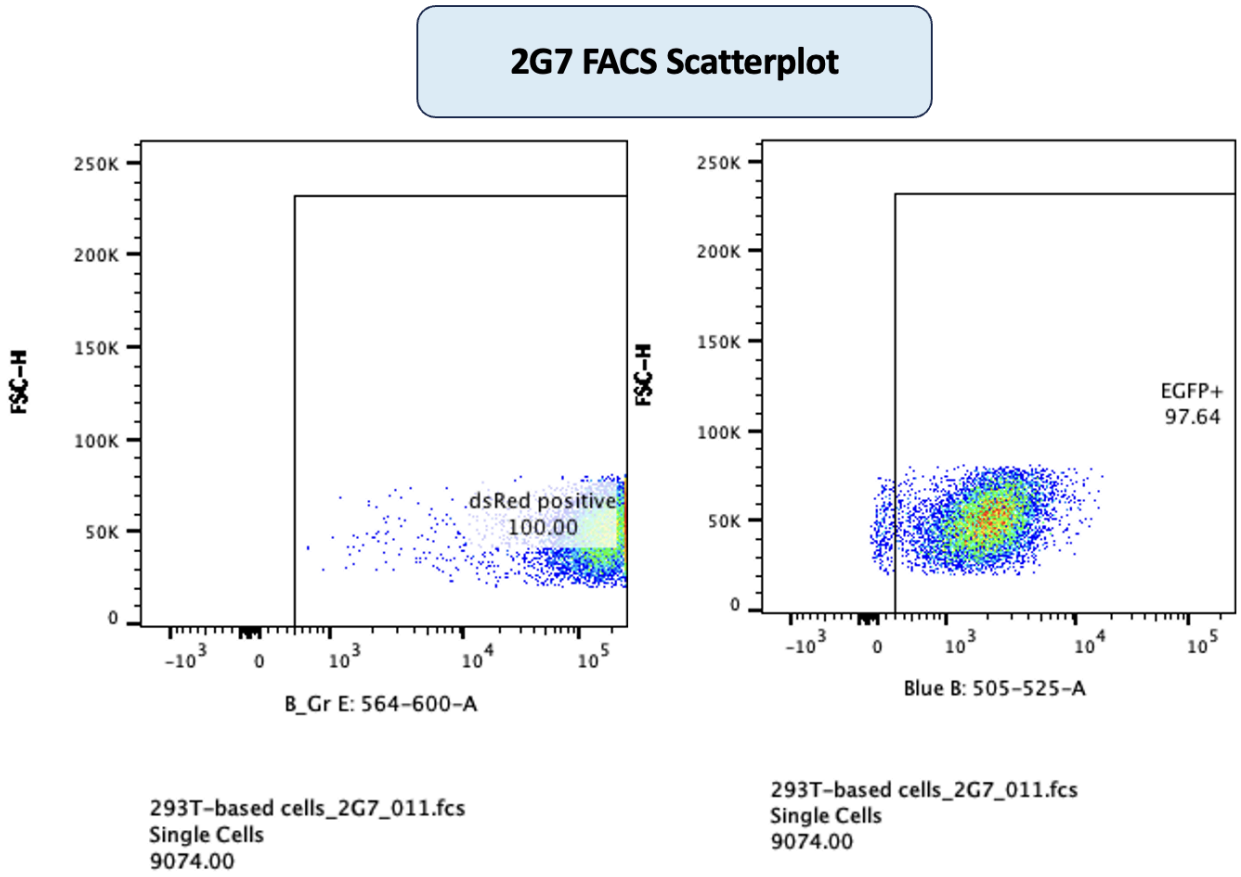


Note: The above figure shows pGM1417 colonies ("c1" and "c2") cut with PspOMI to ensure that the new plasmids have intended characteristics.

The expected fragment sizes from pGM1417 after PspOMI restriction enzyme digest are 660 bp and 8049 bp. Based on the electrophoresis results, pGM1417 clone 1 was used to move forward with the plasmid preparation project.

Assessing Transduction Efficiency

Fluorescence Activated Cell Sorting: Scatterplot Gating



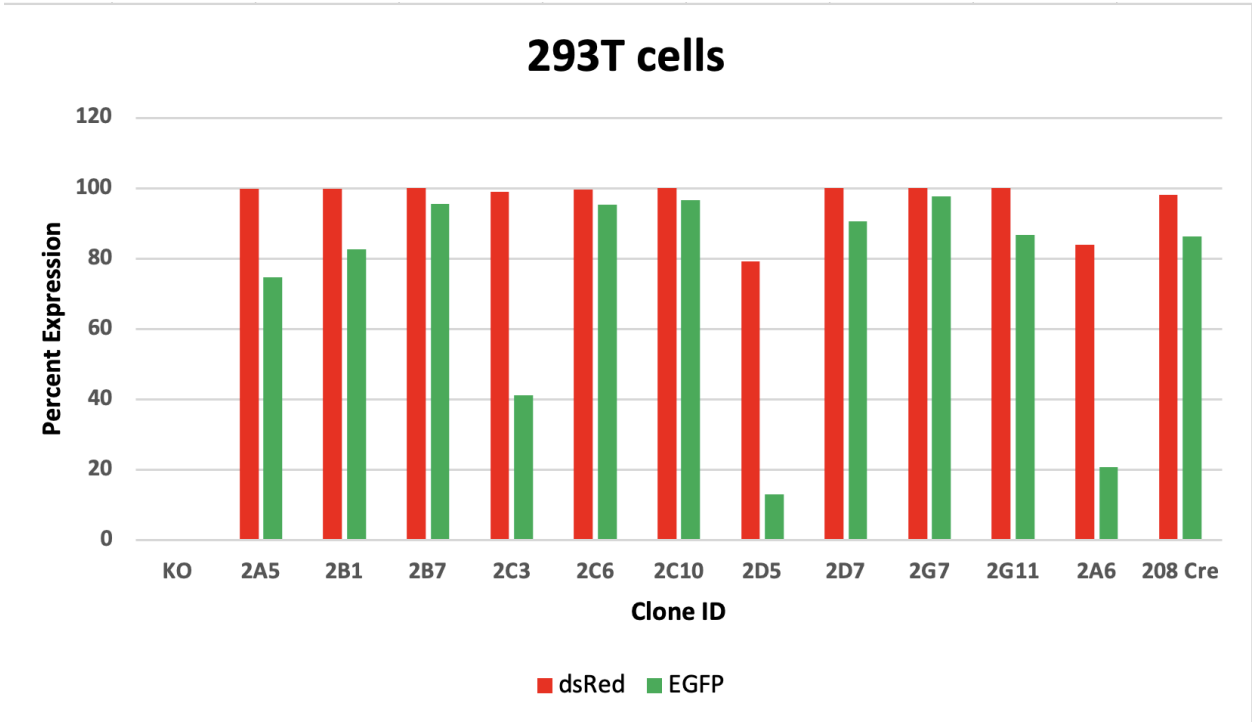
Note: The above figure displays an example of a 293T cloned cell line, 2G7, and how it was gated for dsRed and EGFP expression.

2G7 was identified as a cell line with 100% dsRed expression and high EGFP expression.

Flow Cytometry

To assess the quality of lentiviral transduction of pGM1247 and pGM1380, FACS was used to assess dsRed and EGFP expression.

Flow Cytometry: 293T cell Transduction Efficiency



Note: The above figure shows %RFP expressing and %GFP expressing cells in each generated cell line of 293T cells

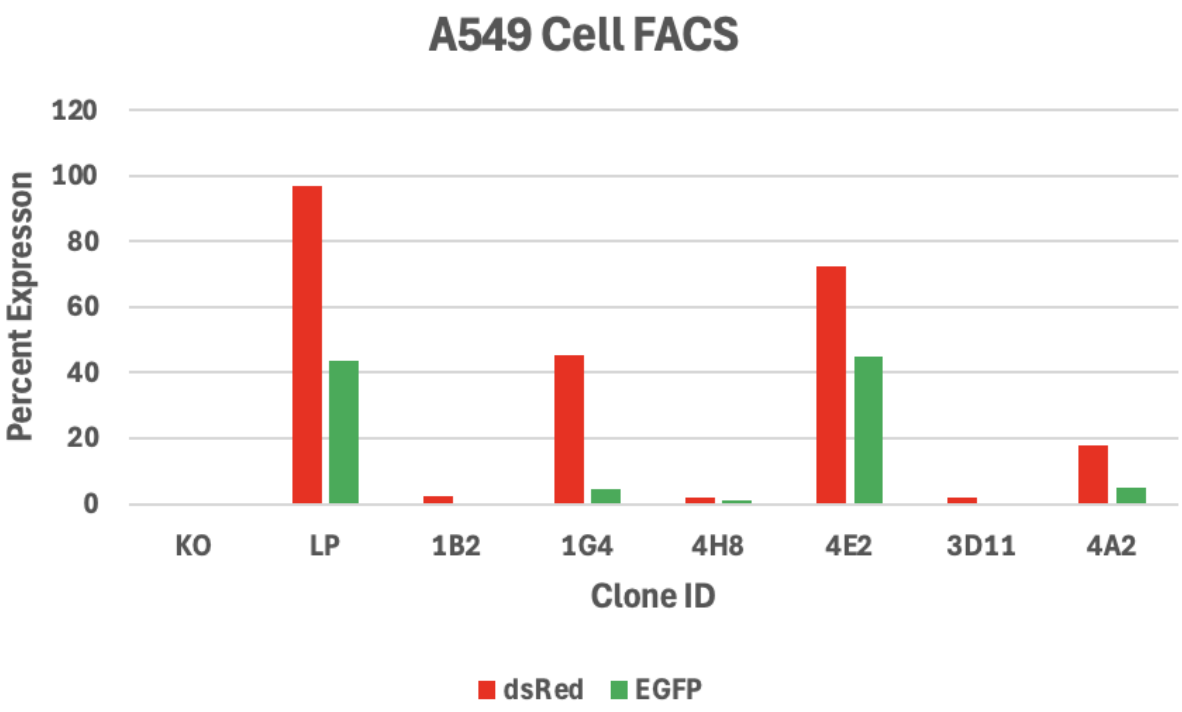
All 293T cells showed near or at 100% dsRed expression except for clone line 2D5, which was no longer used for future experiments based on this data

*Flow Cytometry: 293T cells Transduction Efficiency*Median Fluorescence Intensity*

Note: The above figure is a log transformed graph of transduction efficiency multiplied by median fluorescence intensity for dsRed and EGFP expression in each clonal cell line for 293T cells

To visualize the difference in dsRed and EGFP expression levels, transduction efficiency values were multiplied by median fluorescence intensity for each cell line. The data shows that for all cell lines, EGFP fluorescence intensity is markedly lower than dsRed expression.

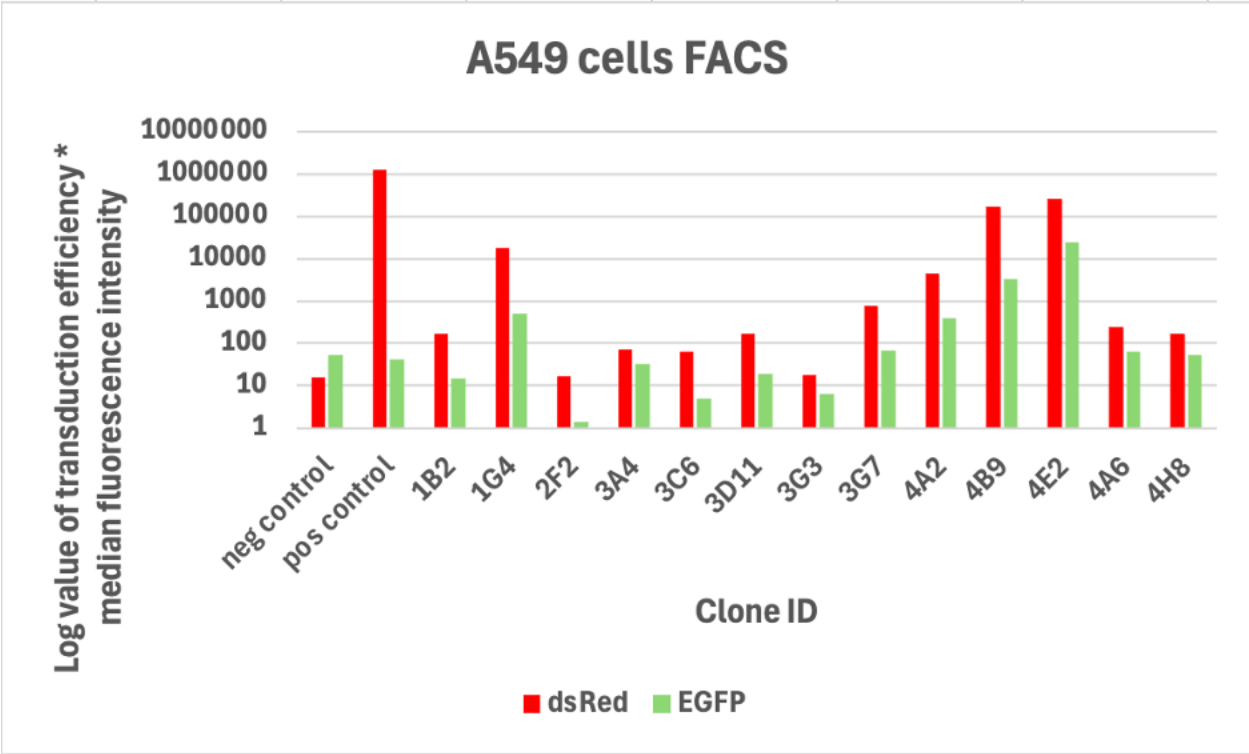
Flow Cytometry: A549 Cell Transduction Efficiency



Note: The above figure shows %RFP expressing and %GFP expressing cells in each generated cell line of A549 cells

A549 cells overall did not show as high transduction efficiency as 293T cells. Due to this, selection was done for transduced cells again with another round of puromycin treatment for all A549 and HEK293T cells.

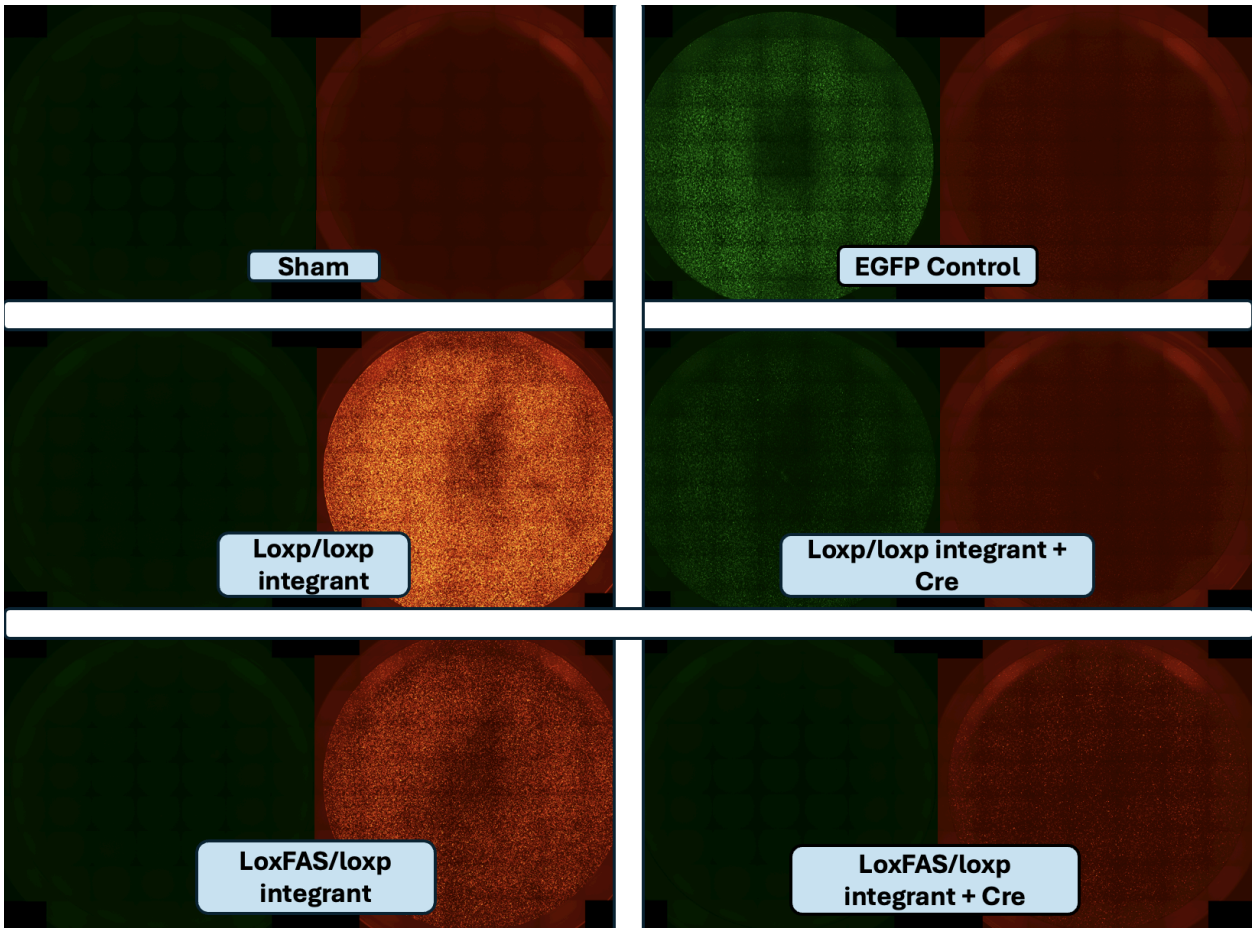
*Flow Cytometry: 293T cells Transduction Efficiency*Median Fluorescence Intensity*



Note: The above figure is a log transformed graph of transduction efficiency multiplied by median fluorescence intensity for dsRed and EGFP expression in each clonal cell line

As was done for the HEK293T cells, transduction efficiency for each A549 cell line was multiplied by median fluorescence intensity to demonstrate that overall EGFP expression intensity is substantially lower than RFP expression intensity.

First Transfection: Cre Recombination on 293T Negative Control Cells

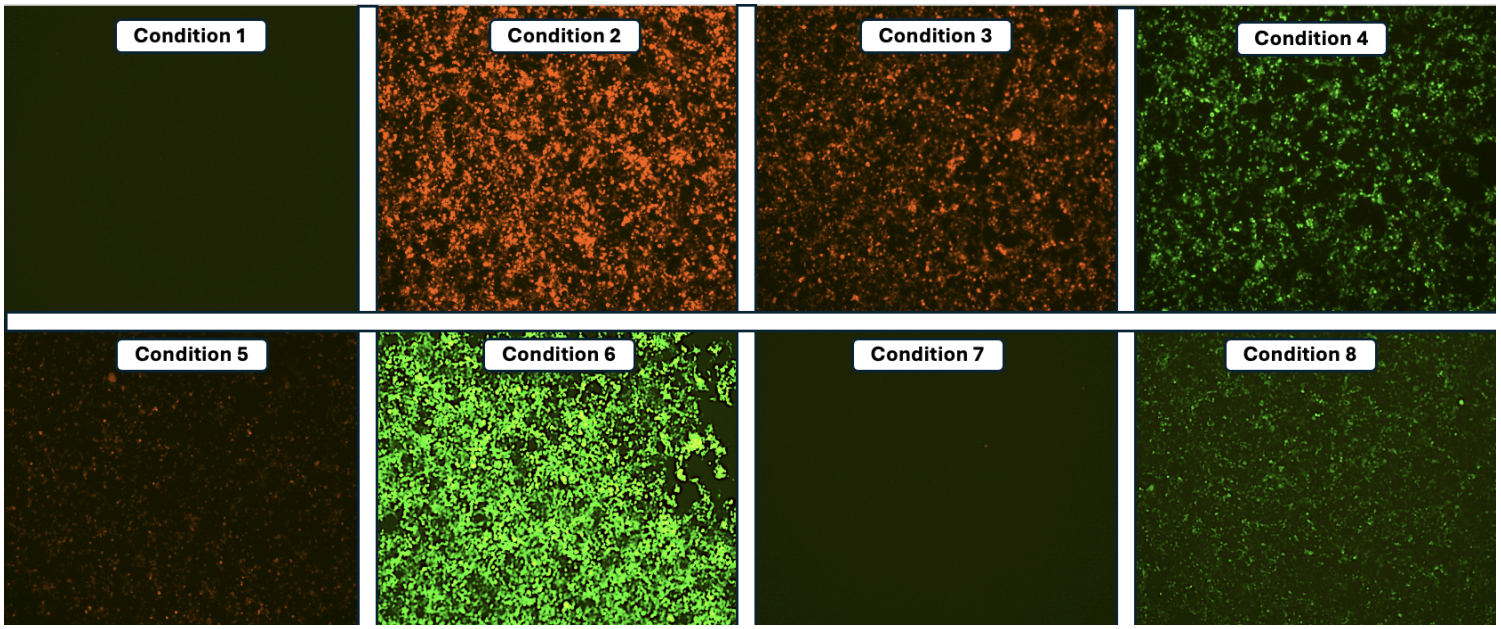


Note: The above figure shows images obtained through fluorescence microscopy on the HEK293T negative control cells used in Transfection 1. Each condition shows the EGFP channel image on the left and dsRed channel image on the right.

As expected the control cells in condition 1 show no fluorescence as they were not transfected with any plasmids. Accordingly, cells transfected with an EGFP expressing plasmid in condition 2 show full EGFP expression and no dsRed expression. Condition 3 shows only dsRed fluorescence, as expected, due to integration of the pGM1247 insert plasmid into the cell genome. Meanwhile, condition 4 shows EGFP expression only, due to excision of the dsRed from the pGM1247 integrant after Cre enzyme was added. Condition five showed some dsRed

expression after transfection with the loxFas/loxp configuration dsRed plasmid, but not as much as the condition that was transfected with the loxp/loxp dsRed plasmid. However, the image still suggests that a significant amount of the cells were transfected. Condition 6 shows no EGFP expression and traces of dsRed expression. Tracing steps back to the original plasmid design revealed that the pGM1380 loxFAS and loxp sites are facing opposite directions, meaning that a third Cre Recombination event known as inversion is likely occurring. Inversion via Cre Recombination occurs when the sequence of a lox site is flipped, leading to the Cre enzyme facilitating flipping of the DNA sequence that is flanked by lox sites. This is the most plausible cause for the loss of dsRed and no gain of EGFP expression. This transfection can be redone after pGM1380 is re-engineered with an adjusted DNA sequence for its loxFAS site.

Second Transfection: Cre Recombination on 293T Negative Control Cells

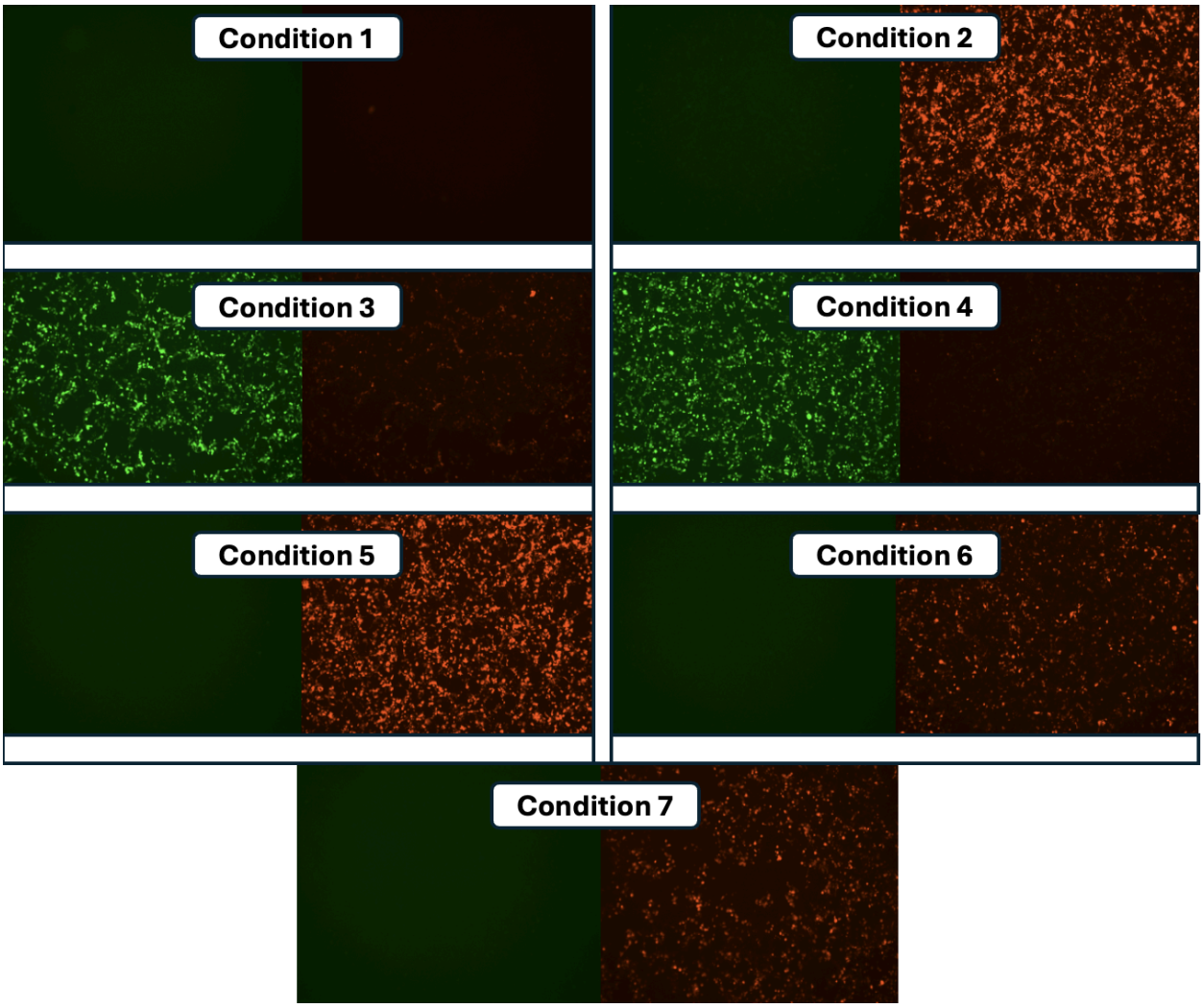


Note: The above figure shows images obtained through fluorescence microscopy on the HEK293T negative control cells used in the Transfection 2. Each condition shows a view of the according well with merged EGFP and dsRed channel images.

As expected, Condition 1, the control condition, is showing no fluorescence. Condition 2 shows dsRed expression, explained by the presence of the dsRed integrant and absence of Cre to facilitate excision or swapping. Condition 3 shows the same results as Condition 2 but with the loxFAS/loxp configuration instead of loxp/loxp. Condition 4 shows faint traces of EGFP expression, showing that with integrant, donor, and Cre in a loxp/loxp configuration Cre recombination does happen mostly through swapping events but with a small amount of excision events, marked by the EGFP. Meanwhile, Condition 5 shows that with the presence of integrant, donor, and Cre in the loxFAS/loxp configuration, there is a significant decrease in dsRed expression with no EGFP expression. While this does mean frequency of Cre excision is reduced by the losFAS:loxp configuration, it is not possible to tell if Cre Recombination events are in the form of swapping or inversion. However, the slight traces of dsRed expression show that the swapping is just below 100% efficient.

Conditions 6, 7, and 8 were done to assess if the excision of the CMV promoter was necessary. To do this we compare the conditions of transfection with an EGFP expression plasmid, an EGFP donor without the CMV promoter, and an EGFP donor with the promoter. The strong EGFP expression in Condition 6 reflects the intended effect of the EGFP expressing plasmid that the cells were transfected with. Condition 7 shows no fluorescence, which is expected due to pGM1414 lacking the CMV promoter before the EGFP encoding sequence. Finally, condition 8 shows some EGFP expression after being transfected with pGM1419, the EGFP donor plasmid. This shows that excising the CMV promoter was a necessary step to take for this study.

Third Transfection: Cre Recombination on 293T Negative Control Cells (adding integrant + cre condition)

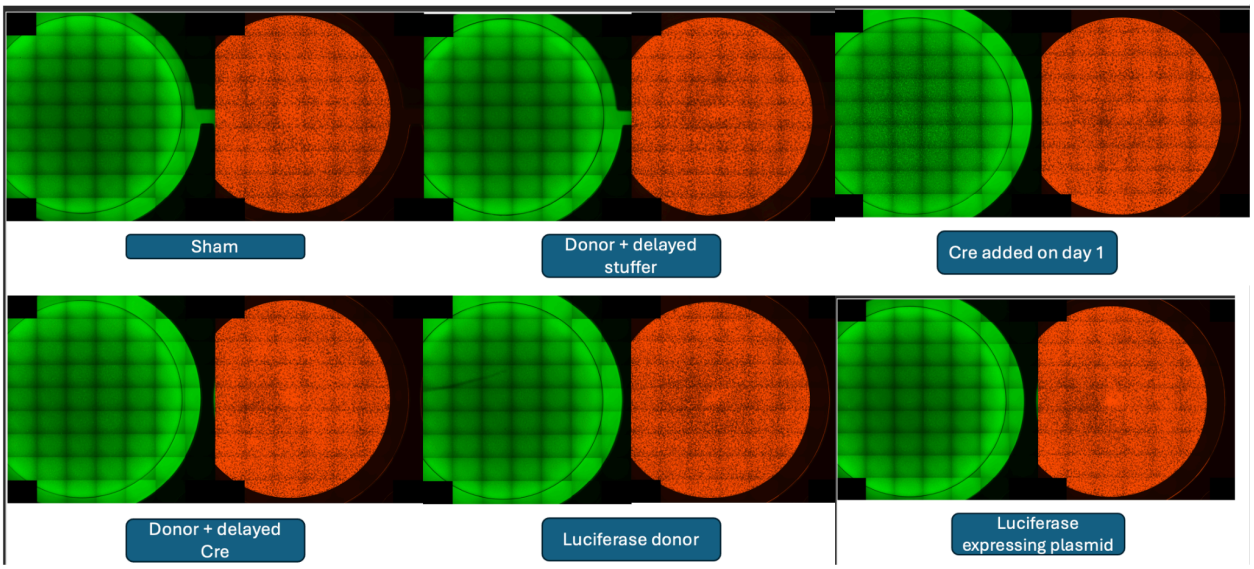


Note: The above figure shows GFP and RFP channels for each condition of Transfection 3.

The third transfection was done to introduce the new control condition of adding Cre enzyme and integrant with no donor, to test for the frequency of excision events rather than swapping events when the Cre enzyme is present. This experiment was designed before we realized the flipped orientation of loxFAS in pGM1380, which created potential for inversion. Condition 1 expectedly shows no fluorescence as the untransfected control. Condition 2 involved

transfection with donor and integrant for loxp/loxp configuration and Condition 5 shows the same for loxFAS/loxp configuration. Both show integration of the respective insert plasmids with no signs of Cre Recombination. Conditions 3 and 6 represent presence of integrant and Cre for loxp/loxp and loxFAS/loxp, respectively. Condition 3 shows clear EGFP expression with some traces of dsRed, showing that Cre excision works with a high efficiency with a loxp/loxp configuration. Condition 6 shows a reduction in dsRed expression and no EGFP expression, suggesting the occurrence of inversion. Conditions 4 and 7 were transfected with Cre enzyme, integrant, and donor, for loxp/loxp and loxFAS/loxp respectively. The loxp/loxp condition, similar to the previous transfection, shows clear EGFP expression, suggesting that there are significant levels of excision events instead of swapping events, even with the donor and Cre enzyme present. Meanwhile, the loxFAS/loxp configuration shows some dsRed expression and no EGFP expression suggesting that there is no significant number of excision events while there is Cre recombination in the form of swapping or inversion occurring.

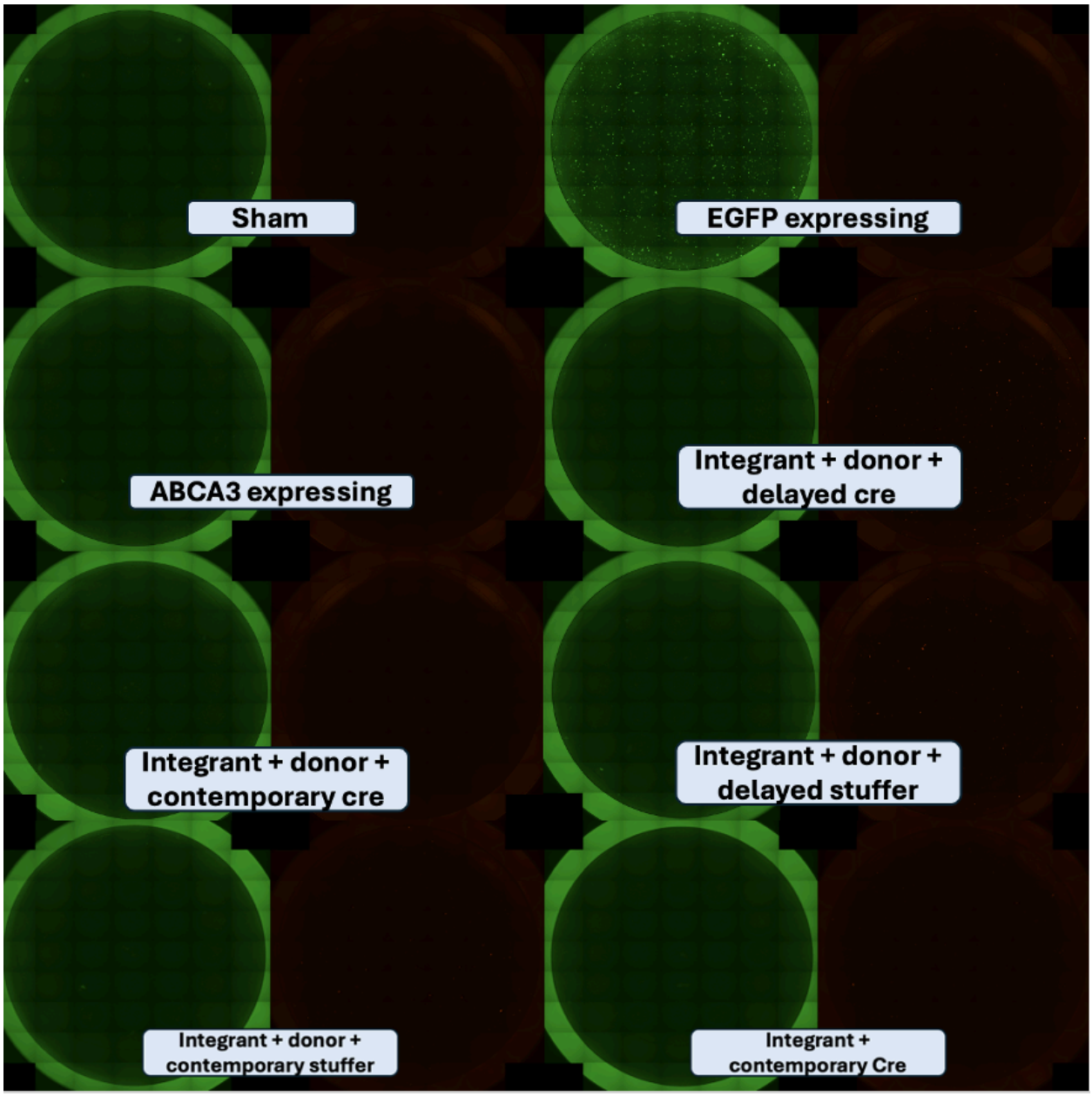
Fourth Transfection and Luciferase Assay with loxp:loxp sites



Note: The above figure displays RFP and GFP channel fluorescent images for each transfection condition.

Previous research suggests that delaying addition of Cre by 24 hours can increase ratio of swapping to excision Cre Recombination events.[10] Thus, when Cre was added for this experiment, it was added after a 24 hour gap after initial transfection. All transfected cells showed expected imaging results, except it is not clear whether or not there was a loss of dsRed or increase of EGFP between the donor + delayed stuffer group and the donor + delayed Cre group. These landing pad cells were previously transduced with pGM1247, so their dsRed expression level is significantly high. To test if there was an increase in Cre Recombination between the aforementioned groups, a t test was done on luminescence values obtained from a luciferase assay. The t test yielded a p-value of 0.06 for a difference in means in luciferase luminescence between the group that received donor + delayed stuffer vs the group that received donor + delayed Cre, suggesting that there was not a statistically significant increase in Cre Recombination.

Fifth Transfection: Assessing Delay of Cre Addition on A549 Cells

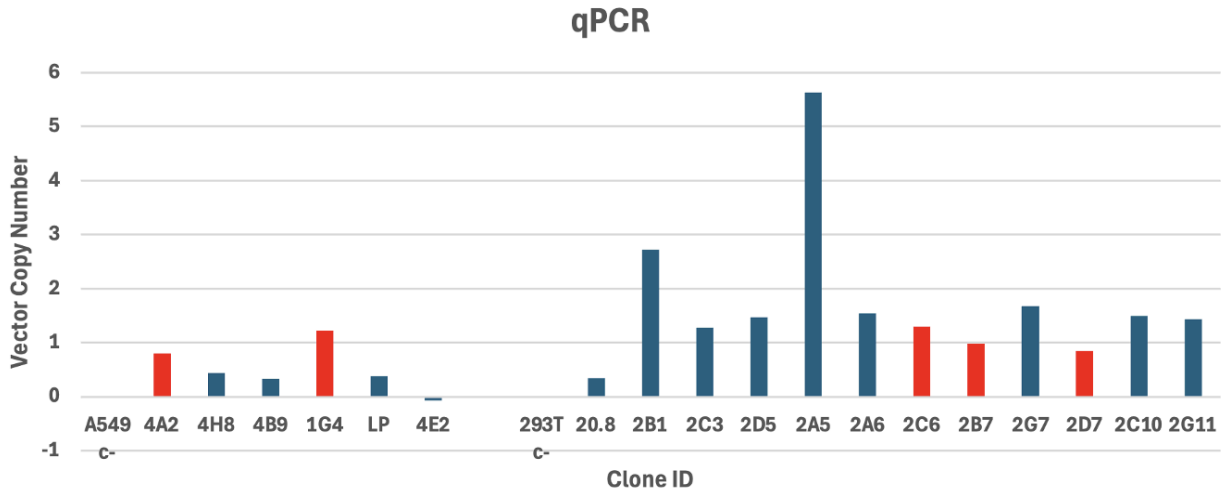


Note: The above figure displays GFP and RFP channels for each transfection condition using A549 landing pad cells.

Generally, as anticipated, transfection efficiency was not as high for A549 cells compared to HEK293T cells. This is most clearly exemplified in the EGFP Expressing Plasmid condition in which eGFP fluorescing cells are visible but sparse. Changes between cells that were

transfected at time point 0 versus time point 24 hours are not intelligible. Before testing this hypothesis, it is necessary to optimize transfection efficiency for A549 cells.

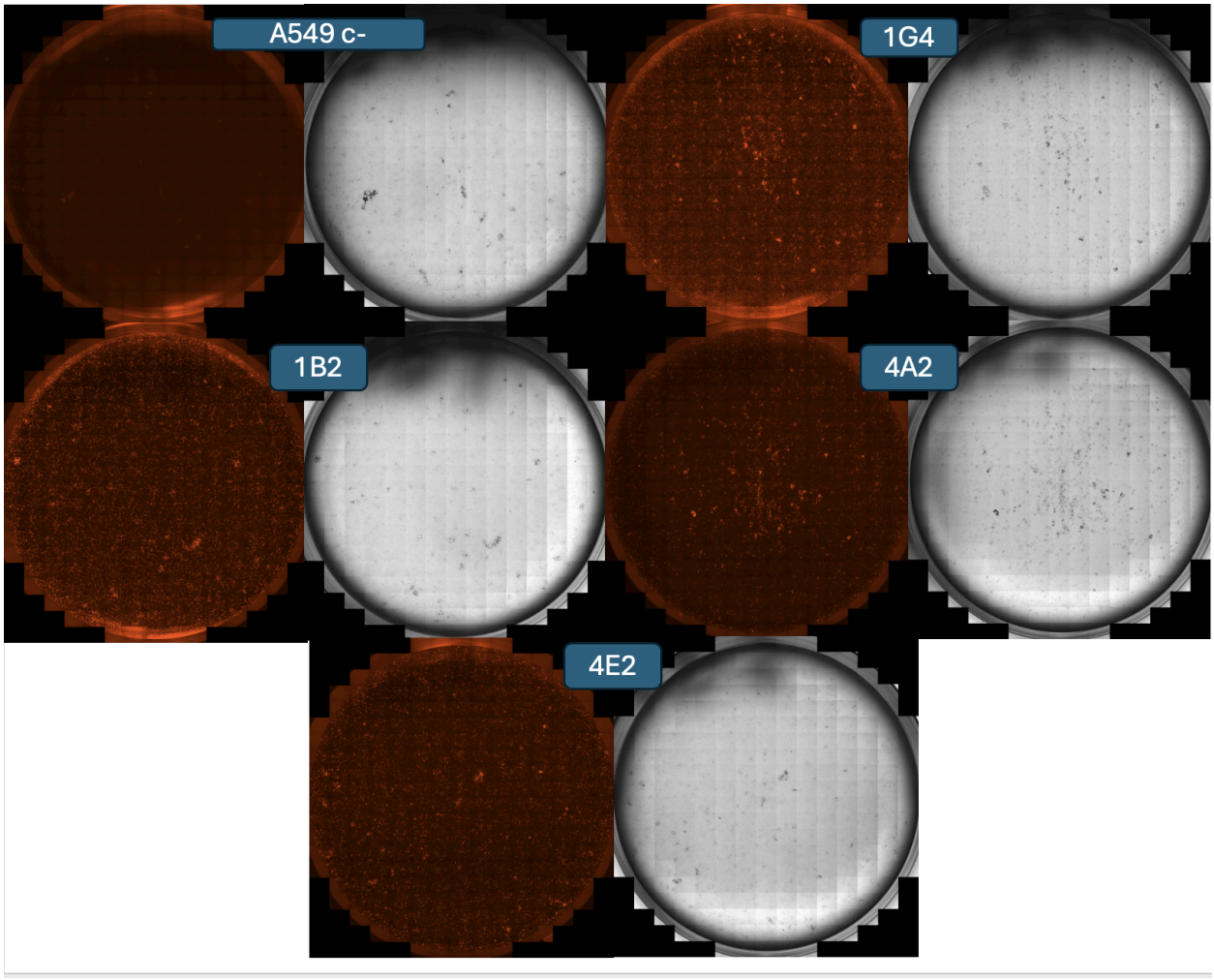
Quantitative PCR



Note: To assess vector copy number of EGFP in transduced cells, quantitative polymerase chain reaction analysis was done with the results shown in the figure above. Cell lines within +/- 0.3 of one vector copy number are highlighted.

Based on results from qPCR, cloned cell lines 4A2, 1G4, 2C6, 2B7, and 2D7 were chosen to save for future experiments. These choices were corroborated with fluorescence microscopy images that showed these cell lines have high dsRed expression levels. The results shown for the A549 landing pad clonal cell line and 20.8 Cre 293T cell line need to be repeated in the future, seeing as flow cytometry data showed 100% dsRed expression, which does not align with the <1 vector copy numbers of those cell lines suggested by this specific qPCR dataset.

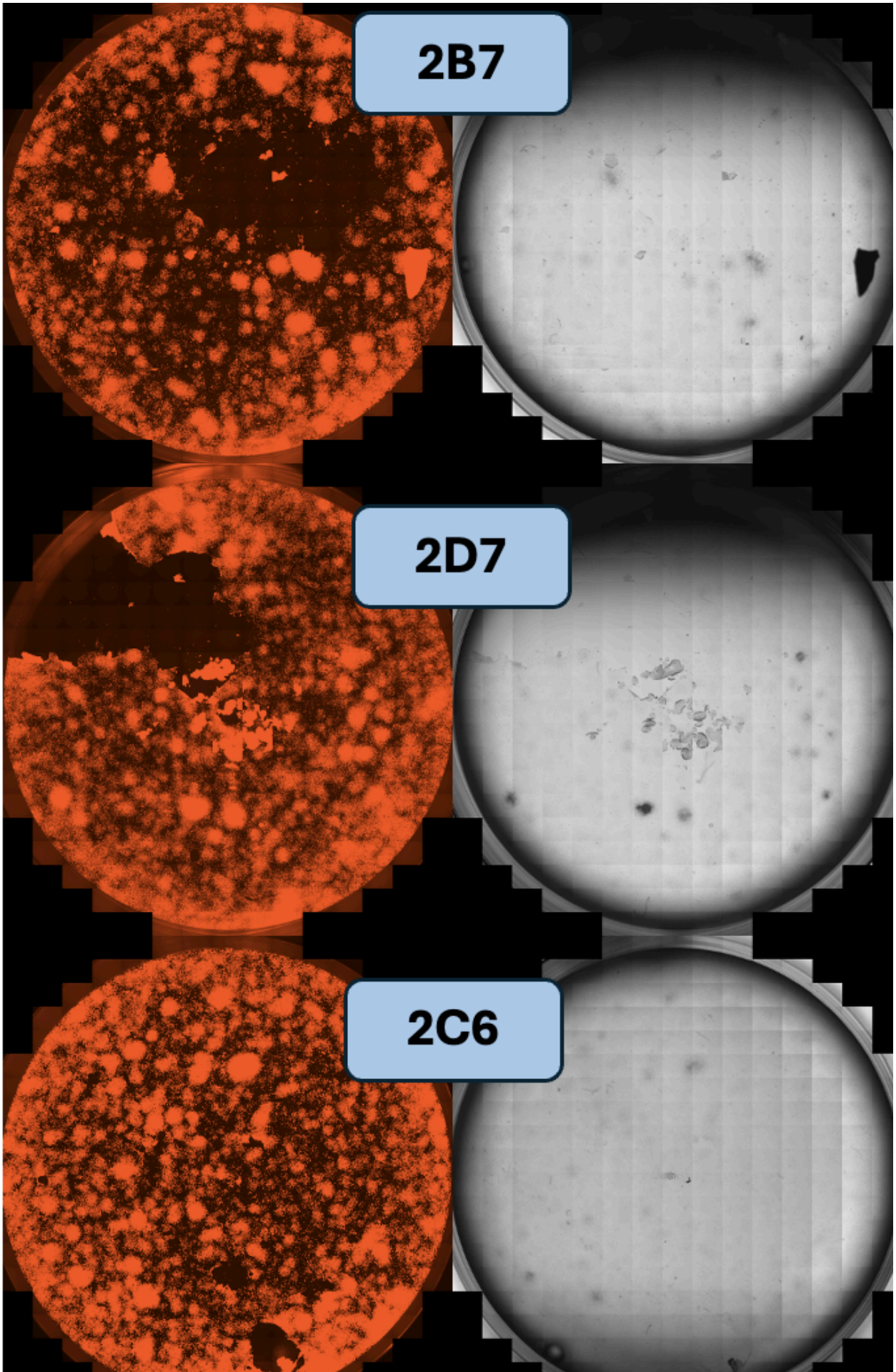
Fluorescence Microscopy of A549 Candidate Cell Lines



Note: The above image shows first an RFP channel image of each candidate A549 clonal cell line followed by a transilluminator image

Cell lines 1G4 and 4E2 show significant dsRed expression when comparing the RFP and transilluminator channels, suggesting that they are strong candidate model cell lines with high transduction efficiency. These cell lines were banked for future experimentation.

Fluorescence Microscopy of 293T Selected Cell Lines


2B7
2D7
2C6

Note: The above image shows the three cell lines with vector copies within +/-0.3 of 1 based on qPCR analysis.

The above HEK293T cell lines showed promising data for high transduction efficiency and vector copy number close to 1, so they were banked for future experiments.

Conclusions

Removal of the CMV promoter from donor plasmids was a necessary step to test Cre Recombination in cells using this reporter system. 2 A549 cell lines and 3 HEK293T cell lines were banked for future experimentation after a candidate selection process involving fluorescence microscopy, flow cytometry, and qPCR. Due to the inverted loxFAS sequence on pGM1380, this data cannot be used to conclude whether or not the loxFAS/loxp configuration increases the ratio of swapping to excision compared to the loxp/loxp configuration. Due to low transfection efficiency, we are unable to decipher if delay of Cre addition to A549 cells increases the ratio of swapping to excision. Near 100% transfection efficiency is necessary to test this so cells being transfected with donor at time point 0 hours are also the same cells being transfected with Cre at time point 24 hours. Data from the luciferase assay suggests that there was not a statistically significant increase in Cre swapping between the cells that were transfected with donor plasmid and Cre compared to the cells that were transfected with just donor plasmid.

Future Directions

Repeating transfections 1, 2, and 3 with pGM1380 after the loxFAS site is oriented in the correct direction will give insight on how the loxFAS/loxp configuration compares to loxp/loxp in terms of ratio of swapping to excision. Exploring methods of optimizing transfection efficiency of A549 cells can allow us to revisit the hypothesis that delaying Cre addition by 24 hours increases ratio of swapping to excision. Alternative methods of delivering Cre, besides

transfection, can be used, such as viral vector delivery. Once ABCA3 in a wild type or mutated form is successfully swapped into the cell lines, functional assays can be done to test ABCA3-based activity. This includes assays for ATPase activity, staining for lamellar bodies using Nile Red. Tests can also be done correcting mutated ABCA3 with gene therapy approaches such as CRISPR.

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