The utilization of CRISPR-Cas9 to establish novel αXβ2 knockout cell lines in mice

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A Senior Honors Thesis Presented to the

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University of Houston

In Partial Fulfillment of the Requirements for

the Degree of Bachelor of Science

By

Daniel Sheng-Zhi Lim

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ABSTRACT

The αXβ2 integrin functions as an important mediator of the complement system, and undergoes inactive to active state by switching its conformation from bent closed to extended open, contingent on binding of different ligands. This extended open conformation has higher affinity for cognate ligands including iC3B, an important molecule of the complement cascade. Thus, the extended open conformation leads to an inflammatory response, and the bent closed conformation suppresses inflammation. Despite the importance of this integrin in mediating the inflammatory response of the human immune system, there have not been studies of the αXβ2 integrin locked in alternate conformational states in a mouse model due to the lack of cell lines. My project has been to create Cas9-plasmid to knock out αX and β2 genes from any mouse cell line. By designing specific oligonucleotides that Cas9 can utilize to make double stranded cuts in the genes for αX and β2, novel cell lines can be created from wild-type DC2.4 and RAW264.7 mouse cells through transfection. These novel cell lines can then be used for future research, such as how the αXβ2 integrin in an alternate state can affect the pathophysiology of diseases such as cancer, inflammatory and autoimmune diseases.

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1 INTRODUCTION

1.1 Function of αXβ2 integrin

The current research focuses on introducing knockout mutations into the genes coding for the αXβ2 integrin, which functions in immune cells as a mediator for the inflammatory response as part of the complement system. This will be done in DC2.4 and RAW264.7 cells, which are immortalized mouse dendritic cells and macrophages, respectively. The αXβ2 integrin functions from αX and β2 subunits which come together to form a functional integrin. αXβ2 functions in innate immunity as a part of the complement receptor, thus dubbed CR4. Innate immunity refers to how the human body responds to foreign matter that is not specific to a particular pathogen, and the αXβ2 integrin is critical in this pathway by binding foreign ic3b-opsonized antigens. The αXβ2 integrin has three distinct major conformations: bent closed, extended closed, and extended open (Fig. 1A-1C). When it is in the extended open state, it has higher affinity for antibodies which bind to it, as they trigger the complement cascade. Thus, the extended open conformation leads to an inflammatory response, and the bent closed conformation suppresses inflammation.

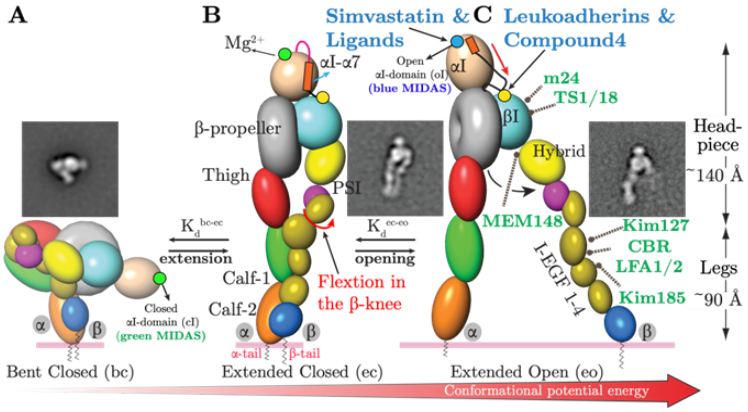


Figure 1. Schematic of integrin dynamism. All states—A) bent/closed (bc), B) extended/closed (ec), and C) extended/open (eo)—in electron microscopy are shown next to their respective artistic rendering.

1.2 Establishment of knockout cell lines

Although the αXβ2 integrin has been studied in the past to determine its function, studies regarding the effects of the αXβ2 integrin in specific conformational state(s) have not been carried out due to the lack of a cell line with knocked out genes coding for the αX and β2 subunits that make up the αXβ2 integrin. The major research field of our laboratory is to investigate the conformational equilibrium of integrins and how each state create a unique functional output. Cell lines with knocked out αXβ2 integrins could allow testing different conformational state αXβ2 integrins and how each state would alter a mouse phenotype. For example, the role of inflammation and its effect on tumorigenesis has been well-studied (1). Thus, the establishment of novel cell lines with the αXβ2 integrin locked into an alternate state could be utilized in future research to investigate this relationship of inflammation and cancer and further help "fine-tune" inflammatory response through integrin signaling pathways. These αXβ2 knockout cell lines in mouse immune cells would be established using CRISPR, a new gene editing technology that can be used to knock out certain portions of genes by introducing mutations.

1.3 Overview of CRISPR

CRISPR is a new technology that can alter DNA sequences and therefore modify gene function. CRISPR evolved from the natural defense mechanisms of bacteria, where the Cas protein associated with CRISPR cuts target strands of DNA. The cutting region is determined by the guide RNA that is complementary to the target sequence. It binds to the PAM, a three nucleotide sequence motif that Cas recognizes and binds to. Cas unwinds the DNA double helix, and if the DNA matches the guide RNA, the DNA and RNA will bind. This triggers Cas's

nuclease activity, which cleaves both DNA strands at a site upstream of PAM. Although cells contain enzymes that repair double-stranded DNA, they are error prone, and will lead to mutations that could inactivate a gene (2). This was utilized in bacteria as defense against foreign DNA such as invading bacteriophages, but because Cas works with any guide RNA that corresponds with an appropriate PAM sequence, CRISPR can also be used to introduce mutations in target genes by creating a specific guide RNA for that target gene to knock out. This has implications involving providing a method for RNA-programmable genome editing. Experiments can then be designed which involve knocking out a specific region of a gene by designing a corresponding guide RNA for Cas to utilize.

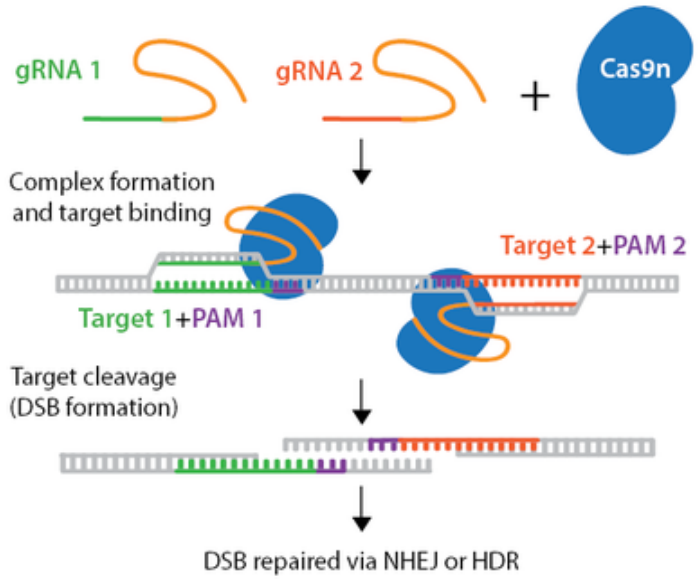
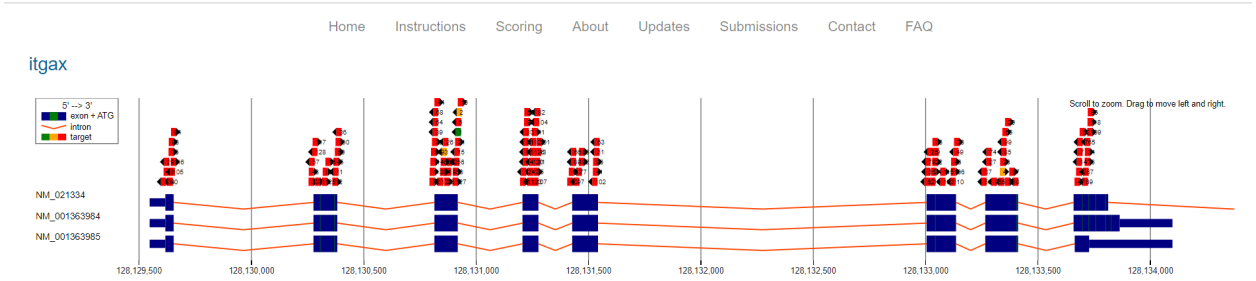


Figure 2. Two guide RNAs allow for two Cas9 enzymes to bind to the target DNA. The Cas9 enzymes cut at the target sequences and result in a double stranded break at each end. Non-homologous end joining (NHEJ) introduces mutations which knock out the section in between the two target sequences. (Figure obtained from Addgene.)

2 METHODS

2.1 CHOPCHOP Web Tool

CHOPCHOP is a web tool that identifies viable CRISPR-Cas9 single guide RNA (sgRNA) targets in a given gene, which was used in designing the oligonucleotides that would be ligated into the Cas9 plasmids (3, 4, 5). The gene names of itgαX and itgβ2 were inputted into the program which then gave viable sgRNAs based on the PAM sequence of NGG. They were ranked based on the least number of possible mismatches, in which the sgRNA binds to transcripts outside of the target gene. Six pairs of upstream and downstream sgRNAs were then chosen from this program, three pairs each for αX and β2. Three pairs were chosen for each subunit to provide redundancy, in case one of them does not successfully knock out the desired gene.



Rank	Target sequence	Genomic location	Strand	GC content (%)	Self-complementarity	MM0	MM1	MM2	MM3	Efficiency
1	GGGCCGTAACTCACCCTGGAGGG	chr7:128130912	-	65	1	0	0	0	0	62.00
2	GGCGGGGCCGTAACTCACCCTGG	chr7:128130916	-	75	1	0	0	0	2	57.44
3	GAGGCCACCTATTTGGTTAGTGG	chr7:128130849	-	50	1	0	0	0	2	39.94
4	GTCAAGCCCTTTAAGTCTGTTGG	chr7:128133333	+	45	1	0	0	0	2	37.47
5	CAGACTTAAAGGGCTTGACGTGG	chr7:128133329	-	50	1	0	0	0	3	68.27
6	GGGGCCGTAACTCACCCTGGAGG	chr7:128130913	-	70	1	0	0	0	3	67.71
7	ATCTTGCCGAGCTCCACTTTGGG	chr7:128133678	-	50	0	0	0	0	3	32.20
8	CATGACATGCTTGATAGCCGAGG	chr7:128133392	-	50	0	0	0	0	4	65.51
9	TGCTACCCGAGCCATCAATCAGG	chr7:128133038	-	55	0	0	0	1	3	51.80
10	CAATCAGGAACACGATGTCTTGG	chr7:128133023	-	45	0	0	0	0	5	52.18
11	CTCACCAGGAACTATCATACTGG	chr7:128130367	-	45	1	0	0	0	5	48.43

Figure 3. Appearance of CHOPCHOP being run on the itgαX gene from *Mus musculus* (house mouse). The possible sgRNAs are ordered based on the number of possible mismatches (MM0, MM1, MM2, MM3) and the PAM sequence of NGG is at the end of the target sequences.

2.2 αXβ2 Integrin Alignment

To ascertain the location at which the Cas9 enzyme will cut when transcribed in the wild-type cells, a pre-existing protein sequence alignment was utilized by translating the chosen target sequences into their respective amino acid sequences. The genes and protein domains contained within the knockout region were also ascertained utilizing this αXβ2 integrin alignment.

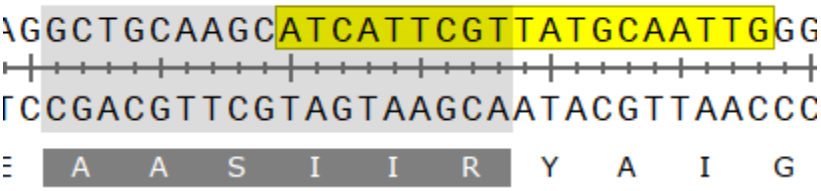


Figure 4. ItgαX oligo 3 as viewed in SnapGene Viewer. The oligonucleotide (sgRNA) is highlighted while the approximate location of double stranded break is in gray.

Figure 5. The AASIIR amino acid sequence is highlighted in blue, as confirmed in the αXβ2 integrin alignment, under αX_Mouse.

2.3 Target Sequence Cloning Protocol

Along with the oligonucleotides chosen from the CHOPCHOP Web Tool, the complementary oligonucleotides were also ordered due to the design necessary, as described by the Zhang Lab Target Sequence Cloning Protocol (4, 5, 6, 7). Thus, each pair of sgRNAs was made up of four oligonucleotides.

5’-CACCGNNNNNNNNNNNNNNNNNN-3’
3’-CNNNNNNNNNNNNNNNNNNCAAA-5’

Figure 6. Generic oligonucleotide design, as described by the Zhang Lab Target Sequence Cloning Protocol. This is the oligo duplex which will be ligated into the Cas9 plasmid.

Table 1. CRISPR-Cas9 sgRNA pair 1 for ItgαX.

Rank	Target sequence	Genomic location	Strand	Cuts at	Upstream or Downstream
1	5'-CACCGGGCCGTAACTCACCCTGGA-3'	chr7:128130912	-	Residue 61 of B-propeller domain (EPI|SLQ)	Up
1c	5'-AAACTCCAGGGTGAGTTACGGCCC-3'	chr7:128130912	+	Residue 61 of B-propeller domain (EPI|SLQ)	Up
3	5'-CACCGATCATTCGTTATGCAATTG-3'	chr7:128133793	+	Residue 262 of a-I domain (AAS|IIR)	Down
3c	5'-AAACCAATTGCATAACGAATGATC-3'	chr7:128133793	-	Residue 262 of a-I domain (AAS|IIR)	Down

Table 2. CRISPR-Cas9 sgRNA pair 2 for ItgαX.

Rank	Target sequence	Genomic location	Strand	Cuts at	Upstream or Downstream
2	5'-CACCGGCGGGGCCGTAACTCACCC-3'	chr7:128130916	-	Residue 62 of B-propeller domain (PIS|LQG)	Up
2c	5'-AAACGGGTGAGTTACGGCCCCGCC-3'	chr7:128130916	+	Residue 62 of B-propeller domain (PIS|LQG)	Up
4	5'-CACCGTTCGTTATGCAATTGGGGT-3'	chr7:128133797	+	Residue 263 of a-I domain (ASI|IRY)	Down
4c	5'-AAACACCCCAATTGCATAACGAAC-3'	chr7:128133797	-	Residue 263 of a-I domain (ASI|IRY)	Down

Table 3. CRISPR-Cas9 sgRNA pair 3 for ItgαX.

Rank	Target sequence	Genomic location	Strand	Cuts at	Upstream or Downstream
5	5’-CACCGAG GCCACCTATT TGGTTAG-3’	chr7:128130849	-	Residue 42 of B-propeller domain (EIK|ATN)	Up
5c	5’-AAACCTAA CCAAATAGGT GGCCTC-3’	chr7:128130849	+	Residue 42 of B-propeller domain (EIK|ATN)	Up
7	5’-CACCGCAT CATTCGTTAT GCAATT-3’	chr7:128133792	+	Residue 262 of a-I domain (AAS|IIR)	Down
7c	5’-AAACAATT GCATAACGA ATGATGC-3’	chr7:128133792	-	Residue 262 of a-I domain (AAS|IIR)	Down

Table 4. CRISPR-Cas9 sgRNA pair 1 for Itgβ2.

Rank	Target sequence	Genomic location	Strand	Cuts at	Upstream or Downstream
4	5’-CACCGGCC CCCGTTGGTC GAACTC-3’	chr10:77546124	-	Residue 65 of hybrid domain (IAN|PEF)	Up
4c	5’-AAACGAG TTCGACCAA CGGGGGCC-3 ’	chr10:77546124	+	Residue 65 of hybrid domain (IAN|PEF)	Up
5	5’-CACCGCGC AATGTCACG AGGCTGC-3’	chr10:77549960	+	Residue 231 of B-1 domain (IGW|RNV)	Down
5c	5’-AAACGCA GCCTCGTGA CATTGCGC-3’	chr10:77549960	-	Residue 231 of B-1 domain (IGW|RNV)	Down

Table 5. CRISPR-Cas9 pair 2 for Itgβ2.

Rank	Target sequence	Genomic location	Strand	Cuts at	Upstream or Downstream
5	5'-CACCGCGC AATGTCACG AGGCTGC-3'	chr10:77549960	+	Residue 231 of B-1 domain (IGW|RNV)	Up
5c	5'-AAACGCA GCCTCGTGA CATTGCGC-3'	chr10:77549960	-	Residue 231 of B-1 domain (IGW|RNV)	Up
3	5'-CACCGGA GTATAGGCAA ATCCCGT-3'	chr10:77557478	+	Residue 370 of hybrid domain (GAS|SIG)	Down
3c	5'-AAACACG GGATTTGCCT ATACTCC-3'	chr10:77557478	-	Residue 370 of hybrid domain (GAS|SIG)	Down

Table 6. CRISPR-Cas9 pair 3 for Itgβ2.

Rank	Target sequence	Genomic location	Strand	Cuts at	Upstream or Downstream
5	5'-CACCGCGC AATGTCACG AGGCTGC-3'	chr10:77549960	+	Residue 231 of B-1 domain (IGW|RNV)	Up
5c	5'-AAACGCA GCCTCGTGA CATTGCGC-3'	chr10:77549960	-	Residue 231 of B-1 domain (IGW|RNV)	Up
1	5'-CACCGGTT GCAAGCCCG GTTACGA-3'	chr10:77560179	+	Residue 541 of EGF-3 domain (GKC|SCK)	Down
1c	5'-AAACTCGT AACCGGGCT TGCAACC-3'	chr10:77560179	-	Residue 541 of EGF-3 domain (GKC|SCK)	Down

Specifically, two plasmids were utilized, one for the upstream genes and one for the downstream genes. These were pSpCas9(BB)-2A-GFP (PX458) and pU6-(BbsI)_CBh-Cas9-T2A-mCherry-H1-(BamHI), respectively (9, 10).

These plasmids were digested with the restriction enzyme BbsI, and then gel purified using a QIAquick Gel Extraction Kit. The chosen oligonucleotides were then phosphorylated and annealed to each other using T4 PNK in T4 ligation buffer, in a thermocycler where the reaction was kept at 37 ºC for 30 minutes, and 95 ºC for 5 minutes which was then ramped down to 25 ºC at 5 ºC per minute. This oligo duplex was then ligated into the BbsI digested plasmid using Quick ligation buffer and quick ligase.

After this, NEB® Stable Competent *E. coli* (C3040I) were used to conduct bacterial transformation, in which 2 uL of the ligation reaction was mixed with 25 uL of *E. coli*, which was then incubated on ice for 30 minutes. The reaction was then heat shocked in a 42 ºC water bath for 30 seconds, after which 200 uL of NEB® 10-beta/Stable outgrowth medium was added to allow the *E. coli* to express the ampicillin resistance gene conferred on both plasmids utilized. 100 uL of the medium was then plated on LB + ampicillin plates and left to grow overnight, after which single colonies were picked for miniprep rapid small-scale plasmid isolation, a protocol developed by Dr. Mehmet Sen's lab.

2.4 Miniprep Rapid Small-scale Plasmid Isolation

In this protocol, the single colonies were grown in 5 mL of LB broth and 5 uL of ampicillin overnight in a shaking incubator, kept at 37 ºC and 220 rpm. The liquid colonies were then spun down in a centrifuge at 4,000 rpm at 4 ºC for 20 minutes. When the bacteria pelleted at the bottom of the tubes, the remaining LB broth was discarded, and the bacterial pellets were resuspended in 300 uL of P1 (+RNAse A) resuspension buffer by gently pipetting up and down. The mixtures were then transferred to new Eppendorf tubes, in which 250 uL of P2 lysis buffer was added. The tubes were then inverted 10 times to ensure proper mixture and were allowed to

rest at room temperature for 1 minute. 300 uL of P3 neutralization buffer was then added to each of the tubes, after which the tubes were inverted 10 times and allowed to rest at room temperature for 1 minute. A white precipitate formed, which is the denatured cellular proteins coming out of solution, allowing the purified plasmid to stay in the clear supernatant. The Eppendorf tubes were centrifuged at 10,000 rpm at 4° C for 10 minutes, in order to pellet the white precipitate.

After this, the supernatant was transferred to a DNA-binding spin column, which was centrifuged at 10,000 rpm at 4 °C for 30 seconds. The liquid in the reservoir was discarded, and 400 uL of PE wash buffer was added at the column to further purify the plasmid DNA. This was then centrifuged at 10,000 rpm at 4 °C for 30 seconds, after which the process of adding 400 uL of PE wash buffer and centrifugation was repeated twice. The column was then transferred to a new Eppendorf tube, after which 50 uL of Milli-Q water was added directly to the membrane. This was allowed to rest for 5 minutes to ensure that the water would collect the bound plasmid DNA, and then the tube was centrifuged at 15,000 rpm at 4 °C for 1 minute to collect the purified plasmid DNA.

2.5 NanoDrop 2000

In order to check the plasmid concentration, a NanoDrop 2000 spectrophotometer was used. The spectrophotometer was blanked using 1 uL of Milli-Q water, and after the pedestal was wiped dry with a Kim wipe, 1 uL of the plasmid DNA sample was pipetted onto the pedestal. The arm of the spectrophotometer was closed onto the sample, after which the NanoDrop 2000 measured the concentration of the plasmid DNA sample in ng/uL utilizing ultraviolet-visible spectrum (UV-Vis) absorbance. Because nucleic acids absorb light with a peak at 260 nm, the

spectrophotometer can ascertain the concentration of the DNA sample from the absorbance. This is done by using the Beer-Lambert law, which states:

$$A = \varepsilon bC$$

Figure 7. Beer-Lambert law, where A = absorbance, ε = wavelength-dependent molar absorptivity coefficient (or extinction coefficient) in $M^{-1}cm^{-1}$, b = path length in cm, and C = concentration in molar (M)

Thus, the spectrophotometer calculates the concentration of DNA in ng/uL using this equation, given that absorbance, molar absorptivity, and path length are known. The 260/280 ratio, which determines the purity of the DNA sample which was prepared from bacteria, can also be calculated by the spectrophotometer. This is because purified proteins absorb light at a peak of 280 nm as opposed to 260 nm, thus presence of unwanted protein will decrease the ratio between the absorbance at 260 nm and 280 nm. Pure nucleic acid is determined to have a 260/280 ratio between 1.8 and 2.

2.6 DNA Extraction from DC2.4 Cells

In preparation for future transfection of the DC2.4 and RAW264.7 cells with the purified CRISPR-Cas9 plasmids ligated with the selected oligo duplexes, an adapted protocol for DNA extraction from mouse tail biopsies was utilized from the Jackson Laboratory. DNA was only extracted from DC2.4 cells due to limitations regarding time and the bacterial contamination of the RAW264.7 cell cultures. When DC2.4 cells reached 90% confluency in the 150 mm cell culture dish, the old media was pipetted out and the cells were washed with 10 mL of PBS buffer. After this, the cells were incubated with 10 mL PBS buffer and 10% trypsin at 37 ^{o}C for 5 minutes. When the cells detached, 5 mL of RPMI-1640 medium was added to deactivate the trypsin. The solution was then centrifuged for 2 minutes at 1.2 rcf to pellet the cells, and the

supernatant was discarded to leave the cell pellet behind. The cells were then resuspended in 2 mL of RPMI-1640, of which 200 uL was pipetted into a fresh 150 mm dish with 15 mL of RPMI-1640 media for continued growth.

The rest of the resuspended cells were centrifuged again for 2 minutes at 1.2 rcf, after which 300 uL of DNA digestion buffer (50 mM Tris-HCl pH 8.0, 100 mM EDTA pH 8.0, 100 mM NaCl, 1% SDS) and 7.5 uL of proteinase K were added to the cell pellet and mixed by pipetting up and down. The mixture was then left in a heat block set to 55^{o} C overnight, to let the cell lysis take place. Chloroform extraction was then utilized to purify the DC2.4 genomic DNA. 300 uL of chloroform was added to the mixture, and inverted ten times to ensure proper mixing. The mixture was then centrifuged at 15,000 rpm for 5 minutes, allowing the upper and lower phases to properly separate.

The upper phase was then pipetted into a new Eppendorf tube, while the lower phase containing the chloroform and any non-DNA contaminants was discarded. 600 uL of 100% ethanol was added to the upper phase and inverted ten times to ensure proper mixing, until the DNA precipitate formed as a white pellet in the tube. After centrifugation at 15,000 rpm for 5 minutes, the supernatant was discarded and 1 mL of 70% ethanol was added, the tube being inverted ten times to wash the DNA. The tube was then centrifuged at 15,000 rpm for 5 minutes, and the supernatant was discarded. The DNA pellet was air dried at room temperature for 30 minutes, before 100 uL of TE buffer was added and incubated in a heat block at 65^{o} C for 15 minutes to resuspend the DNA. The mixture was pipetted up and down gently with a P1000 tip to aid in the resuspension, after which the sample was checked for DNA concentration using the NanoDrop 2000 as outlined previously.

2.7 Primer-BLAST

To pick PCR primers to utilize on the wild-type DC2.4 DNA, Primer-BLAST was used, which is a primer designing tool developed by the National Institutes of Health (11). The range for the forward and reverse primer can be selected, along with the PCR product size. Primer melting temperatures can also be selected for the program to take into account when making viable PCR primers to use. In this case, parameters were selected to amplify a section of DNA which would be knocked out by the Cas9 enzyme. Thus, if the knockout was successful, a smaller DNA band would form when run on an agarose gel via electrophoresis as opposed to the wild type DNA band.

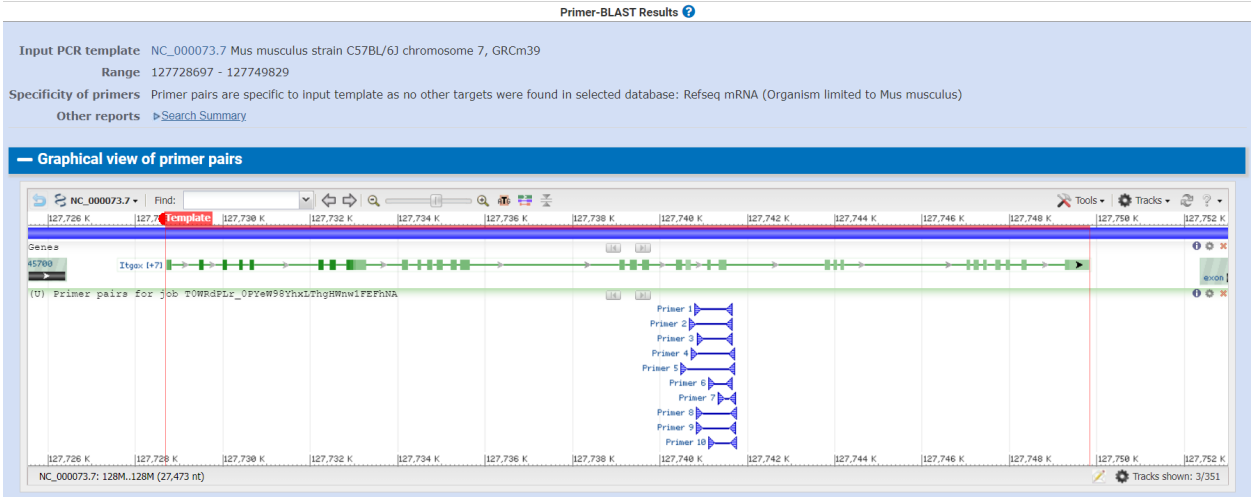
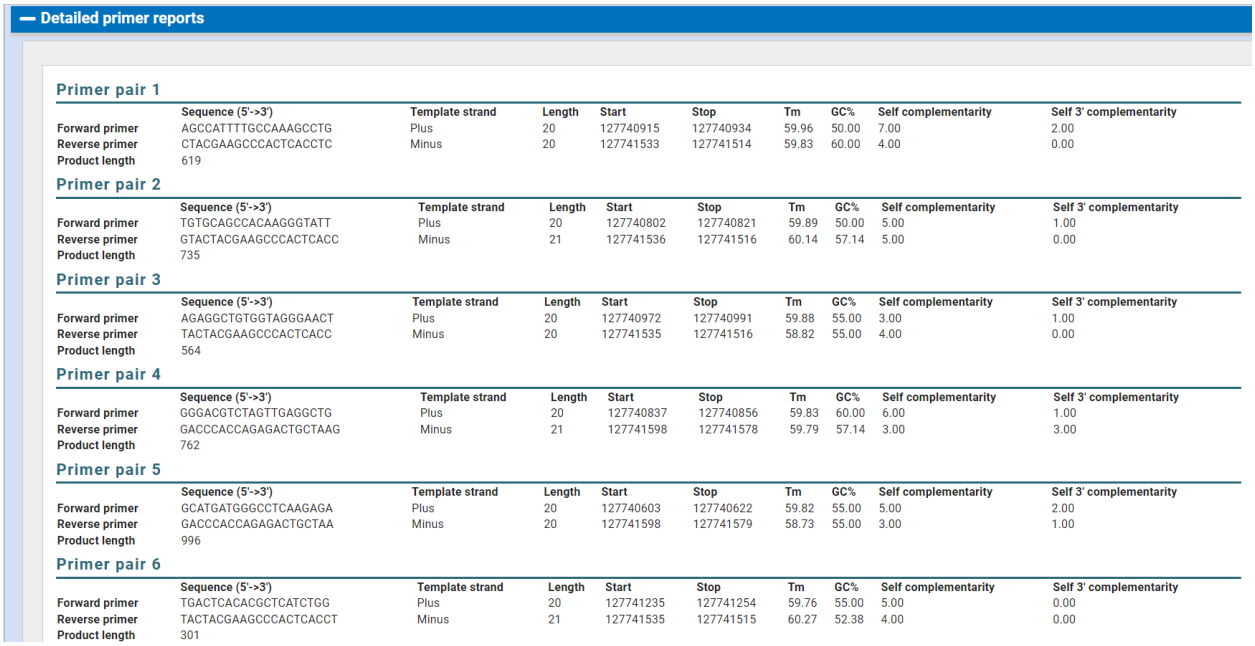


Figure 8. Primer-BLAST running on the gene encoding αX in *Mus musculus*.

Primer-BLAST gives ten primer pairs to choose from, and also lists their important characteristics that should be taken into account when making a decision about which primers to use for PCR. In choosing the PCR primers to use for the αX and β2 genes in DC2.4 cells, this was taken into account, as well as the region which was to be amplified by the primers, to ensure that it fell within the region that was to be knocked out.



— Detailed primer reports

Primer pair 1

	Sequence (5'->3')	Template strand	Length	Start	Stop	Tm	GC%	Self complementarity	Self 3' complementarity
Forward primer	AGCCATTTTGCCAAAGCCTG	Plus	20	127740915	127740934	59.96	50.00	7.00	2.00
Reverse primer	CTACGAAGCCCACTCACCTC	Minus	20	127741533	127741514	59.83	60.00	4.00	0.00
Product length	619								

Primer pair 2

	Sequence (5'->3')	Template strand	Length	Start	Stop	Tm	GC%	Self complementarity	Self 3' complementarity
Forward primer	TGTGCAGCCACAAGGGTATT	Plus	20	127740802	127740821	59.89	50.00	5.00	1.00
Reverse primer	GTACTACGAAGCCCACTCACC	Minus	21	127741536	127741516	60.14	57.14	5.00	0.00
Product length	735								

Primer pair 3

	Sequence (5'->3')	Template strand	Length	Start	Stop	Tm	GC%	Self complementarity	Self 3' complementarity
Forward primer	AGAGGCTGTGGTAGGGAACT	Plus	20	127740972	127740991	59.88	55.00	3.00	1.00
Reverse primer	TACTACGAAGCCCACTCACC	Minus	20	127741535	127741516	58.82	55.00	4.00	0.00
Product length	564								

Primer pair 4

	Sequence (5'->3')	Template strand	Length	Start	Stop	Tm	GC%	Self complementarity	Self 3' complementarity
Forward primer	GGGACGTCTAGTTGAGGCTG	Plus	20	127740837	127740856	59.83	60.00	6.00	1.00
Reverse primer	GACCCACCAGAGACTGCTAAG	Minus	21	127741598	127741578	59.79	57.14	3.00	3.00
Product length	762								

Primer pair 5

	Sequence (5'->3')	Template strand	Length	Start	Stop	Tm	GC%	Self complementarity	Self 3' complementarity
Forward primer	GCATGATGGGCCTCAAGAGA	Plus	20	127740603	127740622	59.82	55.00	5.00	2.00
Reverse primer	GACCCACCAGAGACTGCTAA	Minus	20	127741598	127741579	58.73	55.00	3.00	1.00
Product length	996								

Primer pair 6

	Sequence (5'->3')	Template strand	Length	Start	Stop	Tm	GC%	Self complementarity	Self 3' complementarity
Forward primer	TGACTCACACGCTCATCTGG	Plus	20	127741235	127741254	59.76	55.00	5.00	0.00
Reverse primer	TACTACGAAGCCCACTCACCT	Minus	21	127741535	127741515	60.27	52.38	4.00	0.00
Product length	301								

Figure 9. The description for each primer pair suggested by Primer-BLAST. Important information includes the sequence (5'-3'), template strand, length, start and stop locations in the gene, primer melting temperature (Tm), GC percentage, self complementarity, and self 3' complementarity.

Table 7. Chosen PCR primer pairs for use in amplifying the knockout regions in DC2.4 and RAW264.7 cells. The PCR product spans a wide enough region that all knockout regions created by the three Cas9 plasmid pairs are covered.

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
αX	CTCACGCTTCCTCAGGGTTT	TGCCTAGCTCATGAGGGTCT
β2	CTGTCGGCTCCAGATTGTGT	GTGGTGGACCTCTGACACTG

3 RESULTS

3.1 Plasmid yields

Table 8. CRISPR- Cas9 plasmid concentrations, as determined by the NanoDrop 2000 spectrophotometer.

Plasmid name	Concentration (ng/uL)	260/280 ratio
αX 1+1c	162.0	1.89
αX 3+3c	103.7	1.91
αX 2+2c	303.9	1.86
αX 4+4c	161.5	1.89
αX 5+5c	184.4	1.88
αX 7+7c	104.0	1.82
β2 5+5c	287.9	1.87
β2 3+3c	116.8	1.88
β2 1+1c	224.0	1.88

Table 9. DC2.4 genomic DNA concentration, as determined by the NanoDrop 2000 spectrophotometer.

	Concentration (ng/uL)	260/280 ratio
DC2.4 DNA	3,249.4	2.04

3.2 DNA Sequencing Chromatogram Data

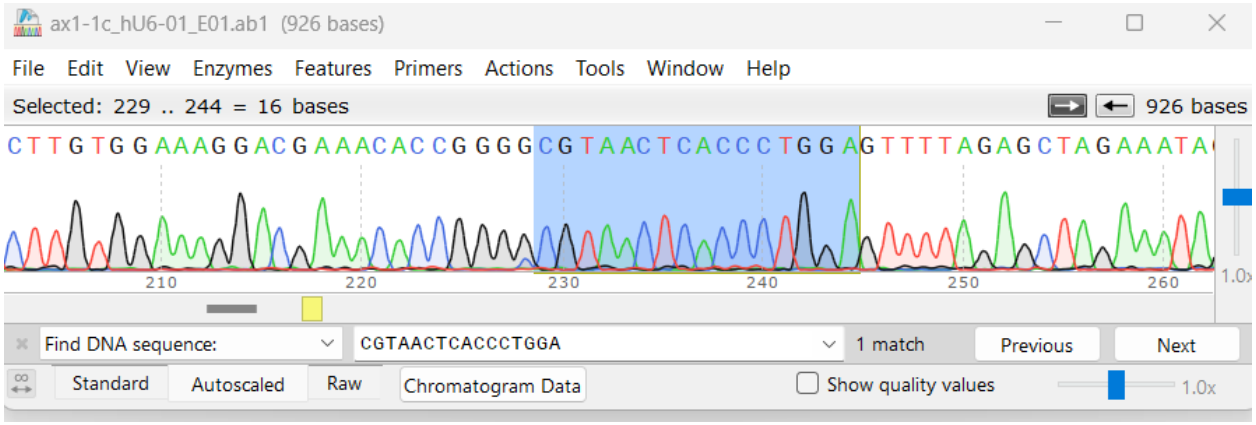


Figure 10. Chromatogram data for αX 1+1c. The identical sequence is highlighted, which confirms the plasmid ligated successfully and the sgRNA is contained in the Cas9 plasmid.

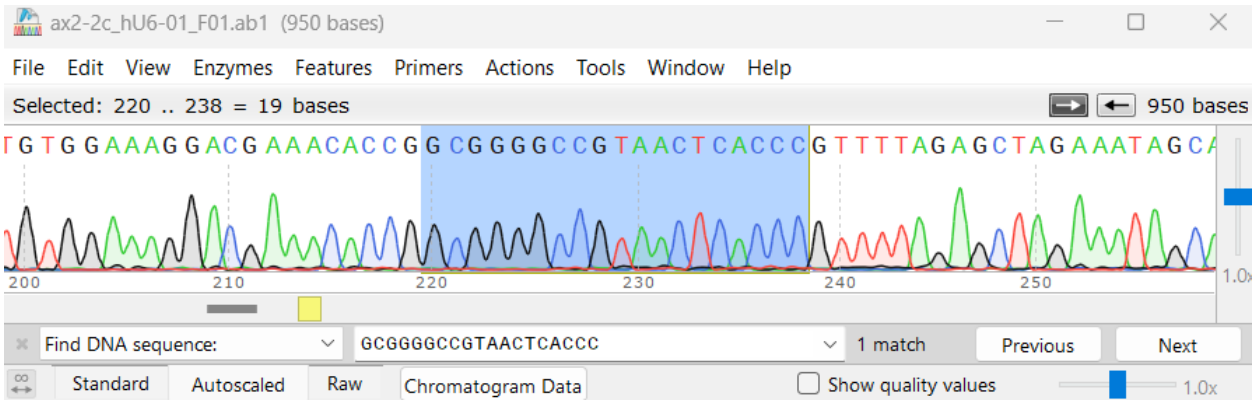


Figure 11. Chromatogram data for αX 2+2c. The identical sequence is highlighted, which confirms the plasmid ligated successfully and the sgRNA is contained in the Cas9 plasmid.

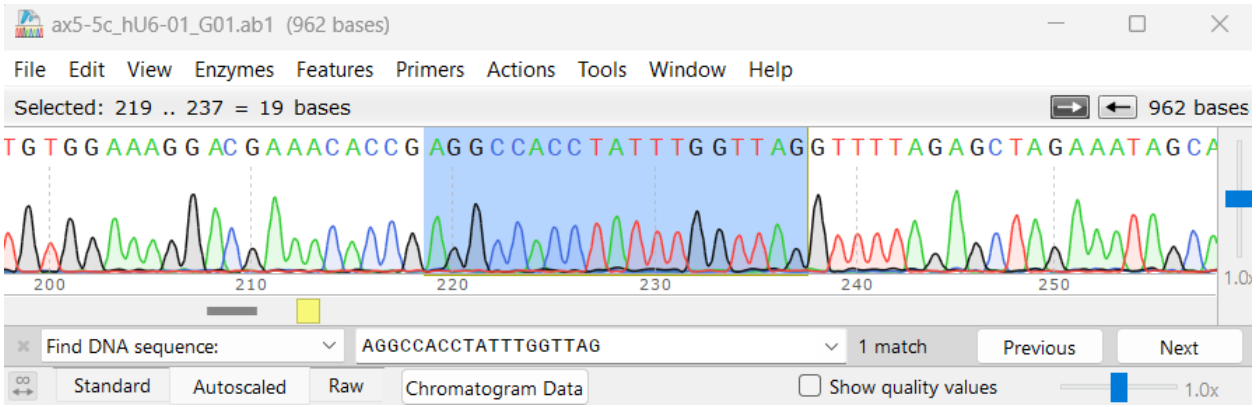


Figure 12. Chromatogram data for αX 5+5c. The identical sequence is highlighted, which confirms the plasmid ligated successfully and the sgRNA is contained in the Cas9 plasmid.

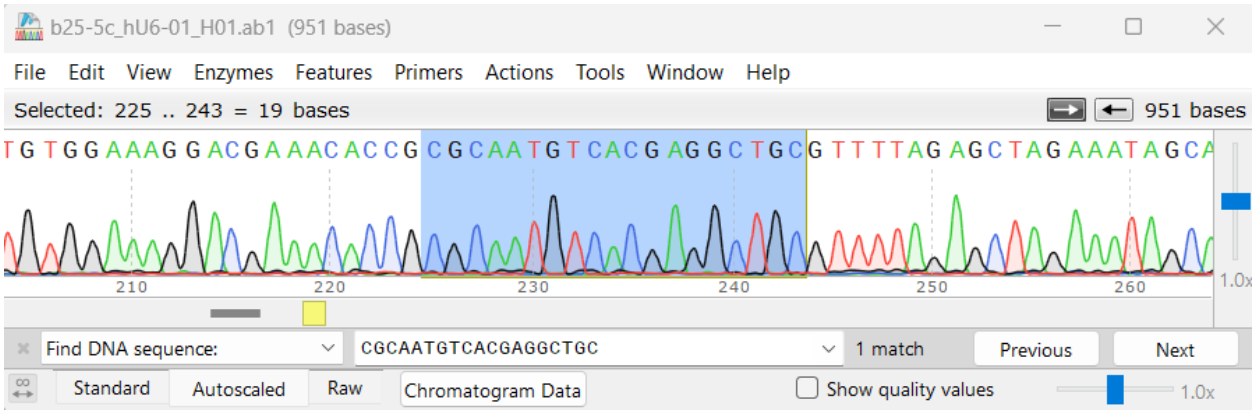


Figure 13. Chromatogram data for β2 5+5c. The identical sequence is highlighted, which confirms the plasmid ligated successfully and the sgRNA is contained in the Cas9 plasmid.

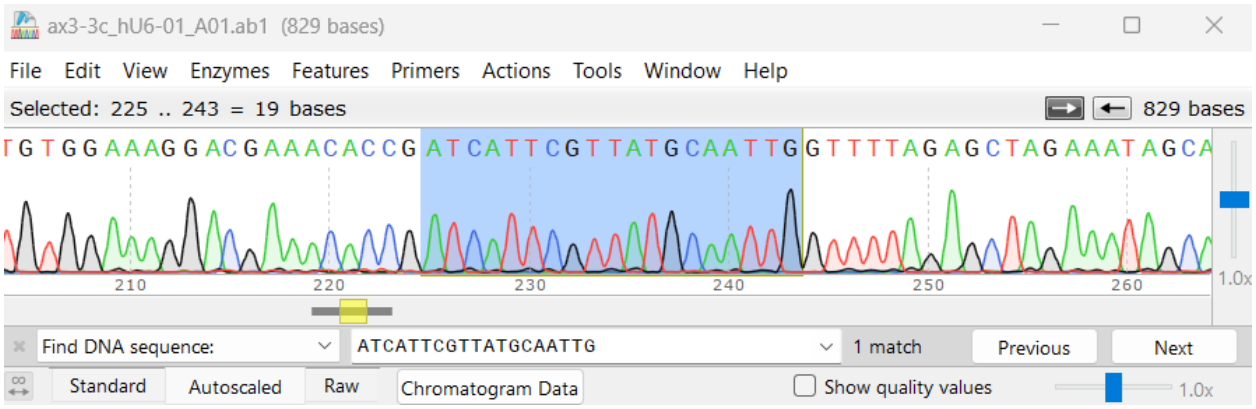


Figure 14. Chromatogram data for αX 3+3c. The identical sequence is highlighted, which confirms the plasmid ligated successfully and the sgRNA is contained in the Cas9 plasmid.

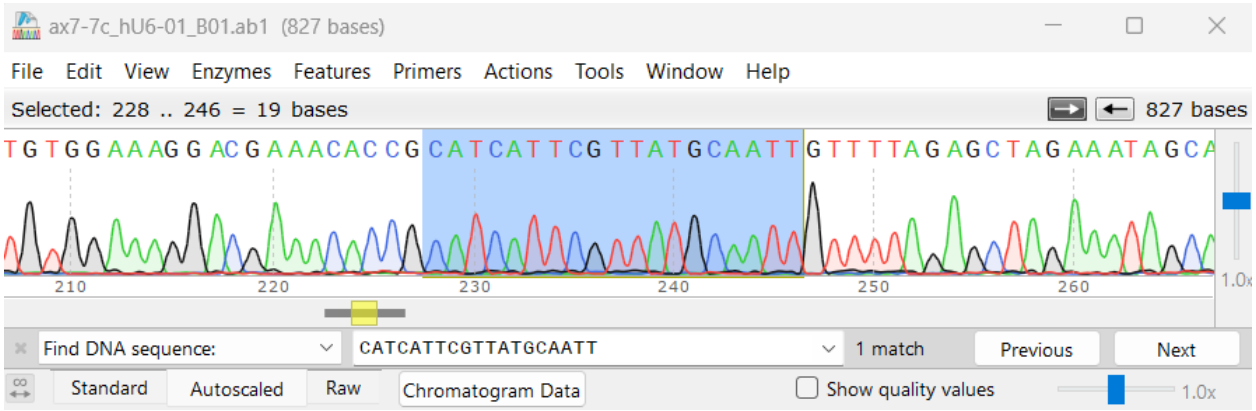


Figure 15. Chromatogram data for αX 7+7c. The identical sequence is highlighted, which confirms the plasmid ligated successfully and the sgRNA is contained in the Cas9 plasmid.

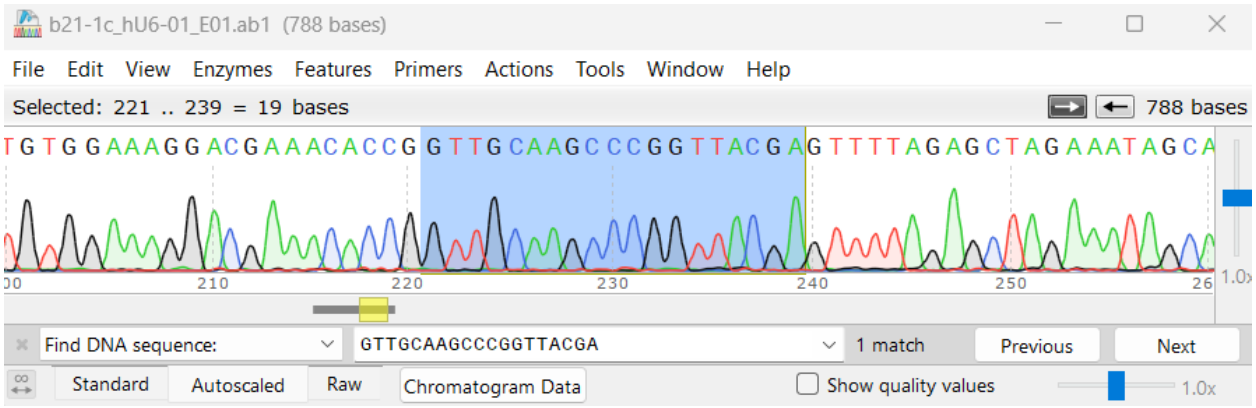


Figure 16. Chromatogram data for β2 1+1c. The identical sequence is highlighted, which confirms the plasmid ligated successfully and the sgRNA is contained in the Cas9 plasmid.

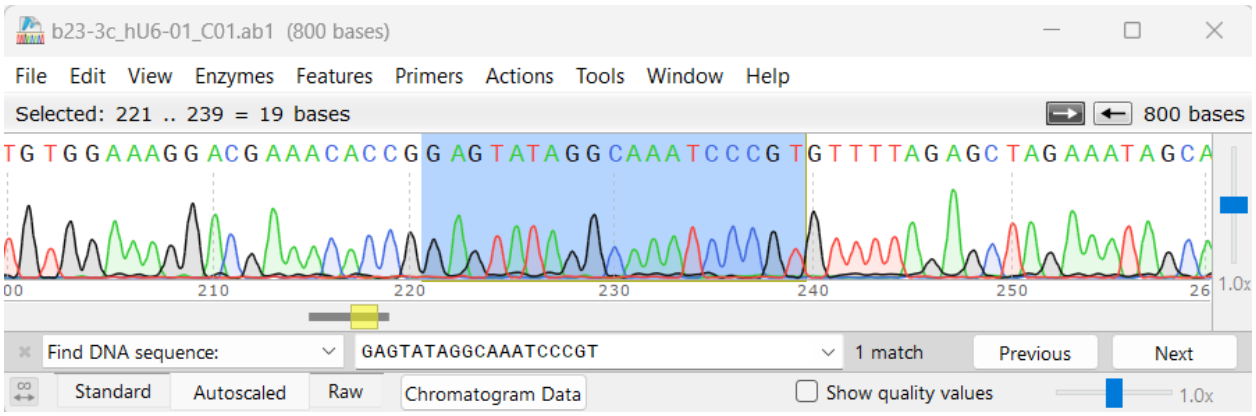


Figure 17. Chromatogram data for β2 3+3c. The identical sequence is highlighted, which confirms the plasmid ligated successfully and the sgRNA is contained in the Cas9 plasmid.

4 DISCUSSION

Overall, there were great plasmid yields for the Cas9 plasmids, and purity was acceptable as determined by the 260/280 ratio being between 1.8 and 2.0. The concentration of genomic DNA purified from DC2.4 cells also appears free of protein contamination, however RNA contamination may exist in the sample due to the 260/280 ratio of 2.04. Considering that the ratio is only slightly above the upper limit of 2.0, this contamination can be assumed to be minor. The contents of these purified plasmids were also confirmed with DNA sequencing, the resulting chromatogram showing a matching sequence to what was ligated into the plasmid. Thus, these purified plasmids are indeed Cas9 plasmids, which can then be transfected into DC2.4 and RAW264.7 cells in the future.

For example, inflammatory immune cells have been implicated as being essential players in cancer-related inflammation in several studies over the last two decades (1). There has been a long standing hypothesis that cancer develops because of unresolved and chronic inflammation, and being able to investigate this further using a mouse model would be beneficial for research on the link between inflammation and the pathophysiology of cancer (12). By knocking out the genes that code for αX and β2, inactivating those subunits would be expected to decrease the inflammatory response because the αXβ2 integrin is critical in the complement system pathway. Without this integrin, it can be assumed that the inflammatory response would decrease. Additionally, we could introduce αXβ2 locked into the alternate states, such as bent state or extended/closed state using well-characterized mutations by our laboratory (13, 14). However, future studies would be necessary to confirm this, utilizing an *in vivo* mouse model for results. Thus, isolating the Cas9 plasmids that allow for the αX and β2 to be knocked out is important.

Potential limitations to this research include that RAW264.7 genomic DNA was not isolated and purified like the DC2.4 genomic DNA, due to time limitations caused by bacterial contamination of the cell cultures. This is hypothesized to be due to the use of 150 mm cell culture plates rather than t75 flasks to culture the cells, as flasks do not become contaminated as easily as plates do. Thus, future research that utilizes the DNA extraction protocol should grow the RAW264.7 cells in flasks as opposed to plates, also ensuring proper cell culturing technique to prevent the incidence of cell culture contamination. Additionally, because transfection of the DC2.4 and RAW264.7 cells has not been successfully done yet, optimization of a protocol to cotransfect the upstream and downstream Cas9 plasmids into cell cultures needs to be done, possibly using polyethylenimine (PEI) or calcium phosphate. This depends on which transfection method is successful.

PCR and Western blotting also need to be utilized to confirm that the transfected cells have knocked out αX or β2 subunits, and single cells need to be picked to ensure that the entire cell culture is made up of αXβ2 knockouts, rather than the wild-type with normal αXβ2 integrins. This is because the wild-type cells may have higher survivability, and if the cell cultures are left to grow, any remaining wild-type cells may dominate the immortalized cell line. Despite these shortcomings, this research has set the foundation for the eventual establishment of novel cell lines that can play a role in future research regarding the relationship between inflammation and cancer, along with any other research projects that may necessitate an inactive and knocked out αXβ2 integrin. Future expansion on this research would be able to utilize the αXβ2 knockout DC2.4 and RAW264.7 cell lines to investigate how they would affect the development of tumors in mice.

5 CONCLUSION

In conclusion, six pairs of ligated Cas9 plasmids with oligo duplexes were purified, with good yield and purity as ascertained by the NanoDrop 2000 spectrophotometer. Additionally, DC2.4 genomic DNA was extracted and purified using a protocol from the Jackson Laboratory, to be used with the designed PCR primers as an indication of the wild-type DNA band when transfected cells are checked for αXβ2 knockout via PCR and agarose gel electrophoresis. The six pairs of ligated Cas9 plasmids, three pairs for αX and three pairs for β2, can be utilized to establish novel αXβ2 knockout cell lines in DC2.4 and RAW264.7 cells, with the aim of utilizing these novel cell lines to investigate the relationship between inflammation and cancer. However, these cell lines can also be used in any other studies that may require nonfunctional αX and β2 subunits of the αXβ2 integrin, which plays a major role in the inflammatory response and therefore is a viable target to use CRISPR-Cas9 to knockout and study its effects. By isolating these plasmids and DC2.4 genomic DNA, this research has set a foundation for future studies that could lead to better understanding of the inflammation-cancer relationship.

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