Constructing Molecular Components for CAR T Cell Engineering

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1. Abstract

Chimeric antigen receptor (CAR) T cells are typically engineered using lentiviral insertion of a CAR construct. To overcome potential oncogenesis resulting from random integration in virally engineered constructs, here, we used Cas9 nucleases along with phagemid-derived circular single-stranded DNA to introduce a red fluorescence marker (mCherry) into the TRAC locus and AAVS1 safe harbor locus as a surrogate for a CAR construct, so that the optimal parameters can be identified for prospective use in engineering CAR T cells.

2. Introduction

2.1. T Cell Therapeutics and CAR T Cells

In the last few years, CAR T cell therapy has shown to be an innovative option for the treatment of various types of cancers. As of 2024, eight clinical trials using CAR T cell technology have been approved by the FDA. These therapeutics have been characterized as “living drugs” due to their ability to use the body’s own re-engineered immune cells, T lymphocytes or ‘T cells’, to recognize and bind to specific tumor-associated antigens. This targeted approach provides a more precise treatment of B cell malignancies such as leukemia and lymphoma and offers new options for patients who do not respond to conventional therapies (Sterner and Sterner, 2021).

T cells are a key part of the body’s natural adaptive immunity. They originate from the bone marrow and migrate to the thymus to reach maturity, and undergo a positive or negative selection process dependent on the functionality of the T cell receptor (TCR). This receptor gives the cell the ability to recognize various protein-based, receptor-bound antigens, which provides a targeted immune response and helps maintain homeostasis. CAR T cells harness this recognition ability through a chimeric antigen receptor (CAR), a transgenic feature added to the cell surface of the T cell. The CAR redirects the function and specificity of the cell and allows it to bind to tumor associated antigens. A CAR consists of two main regions: the endodomain and the ectodomain (as seen in Figure 1). The endodomain contains the intracellular signaling domain. When an antigen-associated protein binds to the antigen recognition region, the endodomain transmits a signal inside the cell (Zhang *et al.*, 2017). The ectodomain is the region outside of the cytoplasm and is exposed to the extracellular space. A key part of the ectodomain is the single-chain of variable fragments (scFv) which allows for antigen recognition outside of the cell (Ahmad *et al.*, 2022).

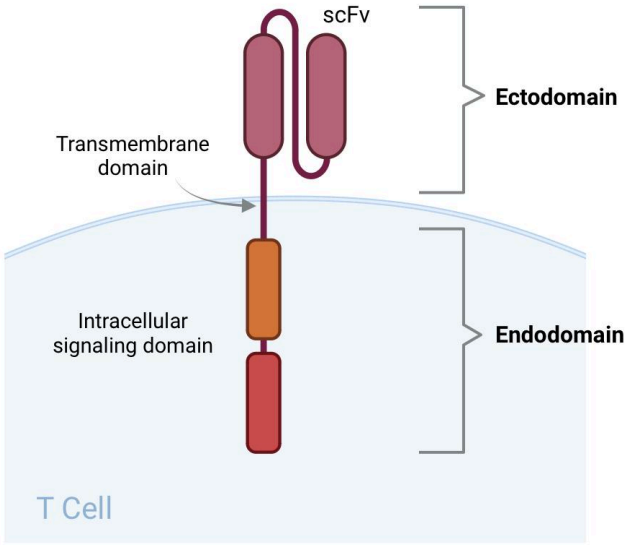


Figure 1. Diagram of a 2nd generation CAR (made with BioRender)

For CAR T cells to be made, T cells need to be removed and isolated from patient blood via leukapheresis. They can then be activated in vitro using CD3 and anti-CD28 antibodies. Following activation, lentiviral or retroviral vectors are used to introduce a CAR construct into the cell. These vectors use viral machinery to attach to the patient cells and introduce RNA, which is then reverse transcribed into DNA so it can be integrated into the cell's genome (Levine *et al.*, 2017). However, the use of viral vectors comes with significant drawbacks including semi-random integration and variable copy number (MacLeod *et al.*, 2017). To combat this, CRISPR/Cas9 and the homology-directed repair pathway can be used to ensure more precise insertion of a CAR construct.

2.2. CRISPR/Cas and Homology-Directed Repair

CRISPR/Cas is a technology that allows for specific, rapid, and versatile gene editing. CRISPR utilizes Cas nucleases, which are enzymes that can bind and create double-strand breaks (DSBs) in DNA. Cas nuclease guidance relies on both CRISPR RNA (crRNA) and trans-activating CRISPR RNA (tracrRNA). The crRNA is complementary to the stored viral spacer from the initial infection in a bacterium, and the tracrRNA functions as a scaffold. In tandem, these two RNAs form a complex known as the guide RNA (gRNA) which is typically 18-20 nucleotides in length, and binds to genomic DNA (Redman *et al*., 2016). The gRNA guides the Cas to the targeted area of the genome, where Cas9 searches for a 3-nucleotide sequence called the protospacer adjacent motif (PAM), found downstream of the target site. Each PAM sequence is specific to the type of Cas protein used and also can vary in nucleotide length, being anywhere from 2 to 5 bases. Once the PAM sequence has been located, Cas will typically begin cutting 3 nucleotides upstream of the PAM sequence. A diagram of these components can be found below in Figure 2. Once the sequence has been completely cut, homology-directed repair (HDR) is initiated to repair the double strand break.

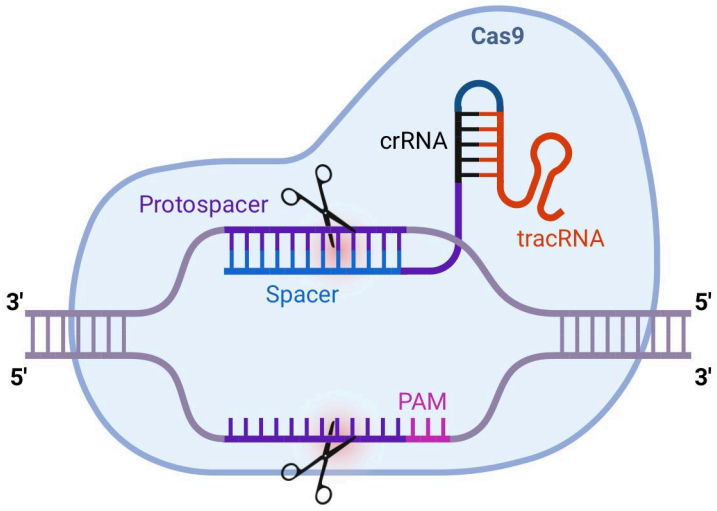


Figure 2. CRISPR/Cas9 mechanism (made with BioRender)

HDR relies on the presence of a homologous donor sequence where the 5’ and 3’ end of the sequence match the broken ends of the genomic DNA, called the homology arms. As long as the donor sequence has the correct homology arms, the sequence in between can be modified to install a desired sequential change into genomic DNA (Chen *et al*., 2024). The presence of a homologous sequence allows for precise genome editing with few errors (Ran, F Ann *et al.*, 2013). The CRISPR/Cas

pathway can introduce DSBs and can then take advantage of HDR through the introduction of a desired cassette with the correct flanking homology arms, allowing for precise insertion of any desired template. HDR has three central steps; the first step is the resection of the 5' end of the break by nucleases to create a ssDNA 3' overhang, which serves as both a substrate for the required proteins for strand invasion as well as a primer for DNA repair synthesis. The ssDNA strand can then displace one strand of the homologous DNA donor and begin to repair based on the other template strand. During this time, the displacement loop is formed, commonly called the D loop. Finally, the recombination intermediates are able to resolve, and the DNA repair process is complete (Devkota, 2018).

2.3. Donor Templates - Plasmid vs Phagemid

Plasmids are extrachromosomal, circular, double-stranded DNA molecules commonly used for genetic cloning due to their versatility. A key feature that allows plasmids to be edited is restriction enzyme sites. Using restriction enzymes, a plasmid can be digested, or separated into fragments, at specific sites and ligated to other DNA fragments with compatible ends to create a recombinant vector. These recombinant vectors allow for the insertion of new genetic material into a cell. However, past research has shown that double-stranded templates can lead to high cellular toxicity and increased risk of off-target integration effects (Iyer *et al*., 2022).

As an alternative, circular single-stranded DNA can be created using phagemids, which offers a safer and less toxic option than double-stranded templates. Phagemids are engineered vectors that contain both plasmid and M13 components, derived from an M13 phage. The M13 phage is a filamentous bacteriophage containing circular single-stranded DNA (cssDNA). Phagemids have the ability to synthesize cssDNA due to their two origins of replication - one of which is derived from M13 and another derived from a plasmid. To synthesize cssDNA, phagemids must be coinfected by helper phages. These helper phage genes allow for the production of cssDNA through coding specific enzymes and coat proteins. They can then be converted into cssDNA molecules which can be delivered into desired cells (Hoy, 2013).

In previous research, the use of single-stranded DNA vectors as opposed to double-stranded has been shown to reduce cytotoxicity and increase the efficiency of targeted insertion into the site of interest. It has also been shown to elicit higher HDR efficiencies relative to equimolar quantities of linear single-stranded DNA (lssDNA), demonstrated by a study showing a two-fold increase in HDR yields using a cssDNA donor compared to a lssDNA donor (Iyer *et al*., 2022).

2.4. Project Objectives

This project aims to create the molecular components necessary for engineering T cells that express mCherry, a red fluorescent protein, as a surrogate for a CAR construct using double-stranded and single-stranded DNA vectors. The successful integration of mCherry could help verify the editing capabilities in various genomic regions with our designed double and single-stranded vectors, which could be applied to later research in CAR T cell engineering. Three mCherry-containing cassettes were designed to target different insertion sites in the T cell genome. Two constructs were created to insert into the T cell receptor α constant (TRAC) locus, and one was created to edit into the AAVS1 genomic safe harbor locus.

Targeted CAR insertion into the TRAC locus has shown to promote uniform expression in peripheral blood T cells, enhance T cell potency, and avert tonic CAR signaling (Eyquem *et al.*, 2017). For our TRAC-specific constructs, the mCherry cassette will be inserted at the 5' end of the first exon to replace the alpha chain of the endogenous T cell receptor, preventing assembly of the native T cell receptor. This knock-out/knock-in approach in the presence of the TRAC promoter allows for stable and robust CAR transgene expression which reflects that of the endogenous TCR (MacLeod *et al.*, 2017).

By adding the appropriate TRAC homology arms from a plasmid backbone (plasmid sources and sequences in Appendix A), homology-directed repair can be achieved to ensure precise insertion into this specific region. This construct will also include a T2A, a polycistronic self-cleaving peptide. The self-cleaving peptide should have little to no effect when

expressing only mCherry, but when a CAR construct is added it would ensure that both regions are in frame and allow both to be expressed simultaneously and driven by a single promoter. The other two features included in this construct are a polyadenylation (or polyA) tail for regulation and stability and a Kozak sequence (added via primers), which is a protein translation initiation site found in eukaryotic mRNA. To evaluate if the addition of a Kozak sequence has a significant impact on mCherry expression, we will create one construct with the Kozak sequence and one without. Based on previous studies, we hypothesize that the addition of a Kozak sequence will promote increased fluorescence of the mCherry protein due to this region's ability to regulate protein translation (Xie *et al.*, 2023). A diagram of the various components of our construct(s) can be found below in Figure 3.

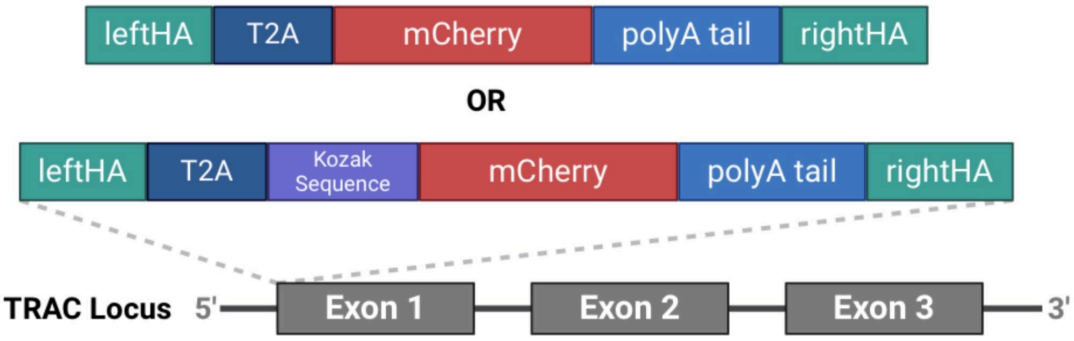


Figure 3. 'Gene insertion' method of editing into the TRAC locus (made with BioRender)

For our 'safe harbor' method, mCherry will be edited into adeno-associated virus site 1 (AAVS1), a well characterized genomic safe harbor (GSH). GSH regions are areas of the genome that can maintain transgene expression without disrupting the function of host cells (Shrestha *et al.*, 2022). The AAVS1 locus is located on chromosome 19 and is a naturally occurring site of integration of adeno-associated virus. (AAV), and is one of the few defined human genomic safe harbor regions. This site in particular has gained popularity in research due to both its targeting and its ability to support transgene expression in multiple cell types. This site has also been shown to have very little genomic interference, safer long-term expression, and more editing flexibility due to its classification as a GSH (Papapetrou & Schamback, 2016). However, there are still concerns regarding editing safety in the AAVS1 locus. Findings show that transgene insertion into the AAVS1 region disrupts the gene phosphatase 1 regulatory subunit 12C (PPP1R12C), as the site for transgene insertion into the AAVS1 region is in the first intron of the PPP1R12C gene (Mizutani *et al.*, 2015). Editing into this locus also cannot support transgene expression in some cell lineages, and transgenes inserted at this site are prone to silencing via DNA methylation (Papapetrou & Schamback, 2016). Inserting the mCherry cassette at this locus could be used to evaluate the safety of AAVS1 transgene integration in T cells for later application in engineering CAR T cells.

This mCherry cassette can be inserted into the AAVS1 region of the T cell genome when guided by the appropriate homology arms corresponding to the target locus. This transgene cassette also requires an exogenous promoter and poly-A tail to allow for complete expression. For this construct, we will be using an exogenous EF-1α promoter. A diagram illustrating all the necessary features of this cassette is included below in Figure 4. Due to the absence of an endogenous promoter in the AAVS1 region, the size of a desired transgene cassette would be larger and potentially more difficult to transfect into cells in comparison to the TRAC construct.

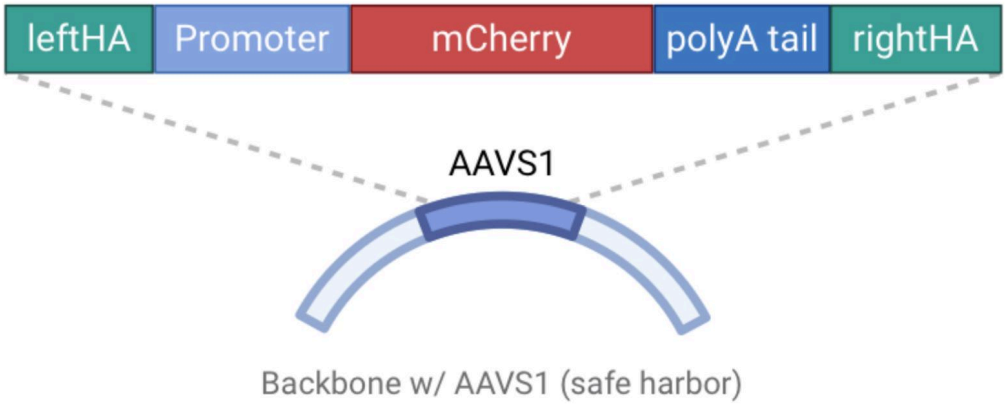


Figure 4. 'Safe harbor' insertion method in the AAVS1 region of a plasmid (made with BioRender)

To assemble these constructs, we used the Gibson assembly method. This cloning strategy uses three enzymatic activities to create an assembled circular DNA product: a 5' exonuclease to generate long overhangs, a polymerase to fill the gaps in the sequence, and a DNA ligase to bind the fragments together (New England Biolabs, Gibson Assembly Protocol). Gibson assembly is a simple and effective procedure that allows for the combining of multiple DNA fragments in a singular reaction, which makes it the ideal method of cloning for this project. Once we created our cloned plasmid constructs, they were used as a template to synthesize our circular single-stranded DNA via phagemid.

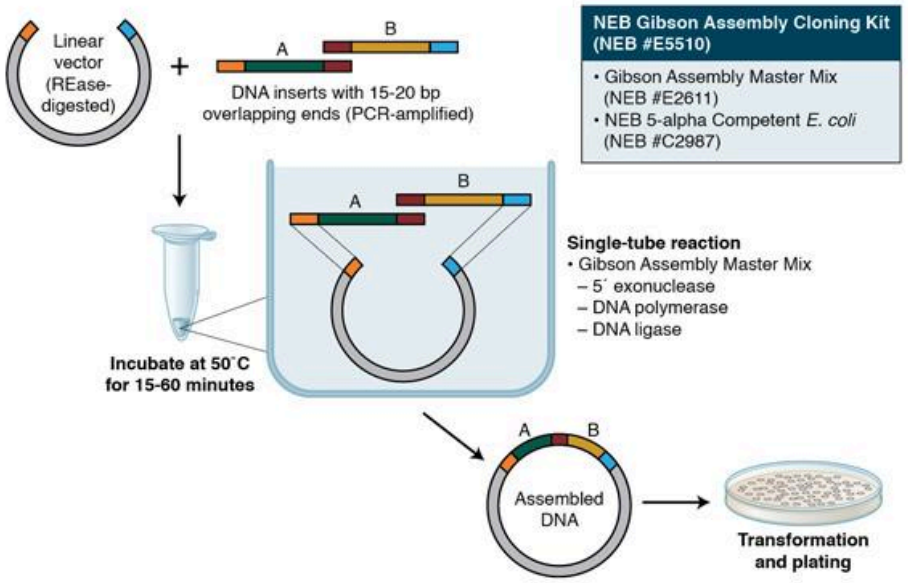


Figure 5. Gibson assembly overview (New England Biolabs)

Along with engineering the three mCherry constructs, we also synthesized the mRNA to code for Cas9 which would allow us to integrate the transgene in T cells using homology-directed repair. This was done via in vitro transcription (IVT). IVT has been used for decades as a way to produce RNA for basic transcriptional mechanism research (Zhang *et al.*, 2023). This

process uses a DNA template, RNA polymerase, and nucleoside triphosphates to allow for the synthesis of template-directed RNA from the DNA template in a safe and versatile way. For this project, we will be using T7 RNA polymerase which recognizes a downstream T7 promoter that catalyzes RNA synthesis.

We predicted that in all constructs, the cssDNA will have higher transfection efficiency than using full plasmid vectors due to the smaller cassette size. Based on past research regarding our insertion regions, we also hypothesize that we may achieve more effective expression in the TRAC locus due to the presence of the endogenous promoter. As mentioned previously, we also theorize that out of the two TRAC constructs, the one containing the Kozak sequence will most likely promote higher fluorescence levels of the edited cells, due to its ability to regulate and promote protein translation (Xie *et al.*, 2023).

3. Methods

3.1. TRAC-mCherry Cloning

Using primers from New England Biolabs (primer sequences shown below in Table 1), an insert and backbone for the +Kozak construct and an insert and backbone for the -Kozak construct were amplified via PCR from the original plasmids available to us in the lab (see Appendix A for primer sources and sequences). This was done according to the NEBNext High Fidelity 2X Master Mix protocol (linked in Appendix B). Briefly, the template DNA sample was combined with its respective forward and reverse primers, NEBNext Master Mix, and nuclease-free water up to 50 µL. DNA was amplified in a thermocycler (Eppendorf Mastercycler Nexus) at times and temperatures recommended in the manufacturer's protocol. Amplified DNA was resolved on a 1% agarose gel to verify length and bands were extracted using a clean scalpel in a blue-light transilluminator. The NEB Monarch DNA Gel Extraction Kits was used to purify the fragments following manufacturer's protocol (linked in Appendix C).

Project	**Purpose**	**Primer #**	**Primer Function**	**Sequence**
'Gene insertion' method into TRAC locus	TRAC mCherry assembly (with Kozak sequence)	1	TRAC + T2A (forward)	gcatggacgagctgtacaagactgtgccttctagttgccagc
		2	TRAC + T2A (reverse)	ggggccggggttctcctc
		3	Kozak + mCherry (forward)	tggaggagaaccccggccccgccaccatggtgagcaagggcgag
		4	Kozak + mCherry (reverse)	cttgtacagctcgtccatgc
	TRAC mCherry assembly (without Kozak sequence)	5	TRAC + T2A (forward)	gctgtacaagactgtgccttctagttgccagc
		6	TRAC + T2A (reverse)	tgctcaccatggggccggggttctcctc
		7	mCherry (forward)	ccccggccccatggtgagcaagggcgag
		8	mCherry (reverse)	aaggcacagtcttgtacagctcgtccatgc
	Colony PCR primers	9	Colony PCR (forward)	tggggcaaagagggaaatga
		10	Colony PCR (reverse)	tcaagtagtcggggatgtcg

Table 1. TRAC-mCherry cloning primers ordered from New England Biolabs (underlined sequence in primer 3 represents Kozak sequence)

The New England Biolabs Gibson assembly protocol (Appendix D) recommends 0.02-0.5 pmols of DNA for a 2-3 fragment assembly, and a 2-3 fold molar excess of each insert. A ratio of 0.15 pmol of backbone and 0.30 pmol of insert was used to ensure that the total molar quantity didn't exceed 0.5 pmol while maintaining a 2:1 insert to backbone molar ratio as recommended by the protocol. The final volume was scaled down x3 to accommodate our sample volume of 20 µL of assembled product, then reactions were halved to 10 µL instead of the recommended 20µL total reaction volume due to material constraints. Full table of calculations shown below (Table 2).

Fragment	**Primer #s**	**Size (bp)**	**Concentration (ng/µL)**	**pmol per µL**	**0.15 pmol backbone**	**0.30 pmol insert**	**Final volumes (scaled down ⅓)**
TRAC backbone 1	1 + 2	4253 bp	27.8 ng/µL	0.010	15.15 µL	N/A	**5.05 µL**
mCherry insert 1	3 + 4	708 bp	26.5 ng/µL	0.034	N/A	5.29 µL	**1.76 µL**
TRAC backbone 2	5 + 6	4253 bp	22.1 ng/µL	0.008	19.23 µL	N/A	**6.41 µL**
mCherry insert 2 (with Kozak sequence)	7 + 8	714 bp	15.9 ng/µL	0.034	N/A	8.89 µL	**2.96 µL**

Table 2. Calculations for TRAC-mCherry Gibson assembly molar ratio (2:1) and reaction volume

The Gibson assembly reaction was set up based on manufacturer protocol. Briefly, the amplified DNA fragments were combined in a PCR tube with the NEB Gibson Assembly Mastermix, then incubated in the thermocycler for 15 minutes at 50°C. The product was then transformed using XL1-blue competent cells.

3.2 AAVS1-mCherry Cloning

Using primers from New England Biolabs (primer sequences in Table 3, shown below), the desired fragments from our original plasmids (see Appendix A) were amplified via PCR. This was done according to the NEBNext High Fidelity 2X Master Mix protocol (linked in Appendix B). Briefly, the template DNA sample was combined with its respective forward and reverse primers, NEBNext Master Mix, and nuclease-free water up to 50 µL. DNA was amplified in a thermocycler (Eppendorf Mastercycler Nexus) at times and temperatures recommended in the manufacturer's protocol. Amplified DNA was resolved on a 1% agarose gel to verify length and bands were extracted using a clean scalpel in a blue-light transilluminator. The NEB Monarch DNA Gel Extraction Kit was used to purify the fragments following manufacturer's protocol (linked in Appendix C).

'Safe harbor' method into AAVS1 locus	AAVS1 Safe Harbor Assembly	1	mCherry + polyA tail - forward	ggtgtcgtgaatggtgagcaagggcgag
		2	mCherry + polyA tail - reverse	caggacagcacgagtgagagacacaaaaaattcc
		3	Left HA + Ef-1α Promoter - forward	cggccgctctagaactagtgcagagcagggccttaggg
		4	Left HA + Ef-1α Promoter - reverse	tgctcaccattcacgacacctgaaatggaag

		5	Right HA - forward	ctctcactcgtgctgtcctgaagtggac
		6	Right HA - reverse	cgaggtcgacggtatcgatagagcacttccttctcggc
	Colony PCR primers	7	Colony PCR - forward	tcactcattaggcaccccag
		8	Colony PCR - reverse	tgctggaccacttgtggatc

Table 3. AAVS1-mCherry cloning primers ordered from New England Biolabs

For a 4-6 fragment assembly, New England Biolabs recommends a 1:1 molar ratio of insert to vector, totaling 0.2 -1 pmols of DNA. The total reaction concentration was 0.2 pmols/uL. The total reaction volume was 20 uL, and the concentration of each fragment was diluted x5, aside from the concentration of the backbone, which was not diluted at all.

Fragment	**Primer #s**	**Size (bp)**	**Concentration (ng/uL)**	**pmol per uL**	**pmol amount**	**Final volumes (uL)**
LHA + Ef-1α	3 + 4	1718	23.4	0.1	0.05	**2.3 uL**
mCherry + polyA tail	1 + 2	850	24.6	0.22	0.05	**1.0 uL**
RHA	5 + 6	502	42.2	0.64	0.05	**0.4 uL**
pBlueScript Backbone	N/A, digested using BamHI and HindIII	2931	79.9	0.041	0.05	**1.1 uL**

Table 4. Calculations for AAVS1-mCherry Gibson assembly molar ratio (1:1:1:1) and reaction volume

The Gibson assembly reaction was set up based on manufacturer protocol (Appendix D). Briefly, the amplified DNA fragments were combined in a PCR tube with the NEB Gibson Assembly Mastermix, then incubated in the thermocycler for 15 minutes at 50°C. The product was then transformed using XL1-blue competent cells.

3.3. Transformation

Plasmids were transformed using E. coli-derived XL1-blue competent cells from Agilent Technologies (amount of cells used was dependent on concentration of plasmid sample). The sample containing competent cells and plasmid was incubated on ice for 30 minutes, then heat shocked in a dry block incubator at 42°C for 45 seconds. Room temperature SOC recovery media was then added to the sample before placing in a shaking incubator at 37°C for 1 hour. During that hour, LB carbenicillin plates were pre-warmed in an incubator. Sample was then plated on the LB carb plates and incubated overnight at 37°C (16-20 hours). Plasmid colonies were picked the following day and added to a tube or flask containing LB broth and carbenicillin (volumes dependent on prep type), samples were left overnight in a shaking incubator at 37°C. The following day, samples were purified using either the QAIprep Spin Miniprep Kit from Qiagen (Appendix E) or Qiagen Plasmid Plus Midiprep Kit (Appendix F).

3.4. Colony PCR

PCR reactions were set up using the APExBIO Taq PCR protocol (linked in Appendix G). Colonies were picked using a pipette tip from the LB carbenicillin plates after an overnight incubation and added directly to the reaction by pipetting up and down. Before colonies were added to the PCR reaction, they were streaked on a new LB carbenicillin plate to be saved for purification after confirming the results of the colony PCR. Purification was performed using the QIAprep Spin Miniprep Kit (Appendix E). Once the results were confirmed through gel electrophoresis, representative samples of each construct were picked from our streaked plates and inoculated in 50 mL of LB broth with 50 µL of carbenicillin. The next day we used a Qiagen Plasmid Plus Midiprep Kit (Appendix F) to purify the plasmids and the DNA amount was quantified using a NanoDrop microvolume spectrophotometer.

3.5. Sequencing

Representative samples of our cloned constructs were sent for whole plasmid sequencing and Sanger sequencing via GeneWiz from Azenta (https://www.genewiz.com) and Plasmidsaurus (https://www.plasmidsaurus.com). Results were analyzed using SnapGene DNA visualization software (https://www.snapgene.com/).

3.6. Single-Stranded DNA Preparation

To generate our single-stranded DNA, we followed the protocol from Iyer, *et al.* (linked in Appendix H). Briefly, our desired plasmids were transformed with XL1-blue competent cells, which were cultured for ~8 hours at 37°C. After culturing, VCSM13 phage was added and incubated at room temperature for about 20 minutes. The cells were then transferred into 2xYT media with ampicillin and cultured for another 1-2 hours. Lastly, kanamycin was added to a final concentration of 75 µg/mL. To prepare our phage pellet, the samples were spun down at 10,000 g for 20 minutes and the supernatant was filtered through a vacuum filter to remove cell debris. Next, DNAse was added and the samples were incubated for 3 hours at 37°C. After the incubation, the phage could be precipitated using PEG-8000 and NaCl and left on ice in a cold room for 1-2 hours. Then the samples were spun down again for 30 minutes at 12,000 g and the supernatant was poured out carefully to retain the pellet. To extract our single-stranded DNA we used a modified Qiagen Plasmid Plus Midiprep Kit (modifications included in Appendix H). DNA amount was quantified using a NanoDrop microvolume spectrophotometer.

3.7. S1 Nuclease

To verify the single-stranded DNA preparation, we used S1 nuclease digestion using the protocol provided by Thermo Fisher Scientific (see Appendix M). DNA was resolved on a 1% agarose gel with ethidium bromide.

3.8. IVT mRNA Synthesis

To isolate Cas9-encoding mRNA, a plasmid template (see Appendix A) with a Cas9-encoding region was linearized using restriction enzyme Esp31 and purified using the NucleoSpin Gel and PCR Cleanup kit from Macherey-Nagel. IVT was accomplished using a modified NEB HiScribe T7 Quick High Yield RNA Synthesis Kit (linked in Appendix I). Full modified reaction protocol with volumes is listed in the appendices (Appendix J). Briefly, a reaction was created with the NEB reagents: NEB HiScribe Buffer, 100 mM ATP/GTP/CTP, pyrophosphatase, RNase inhibitor, and T7 polymerase, along with 100 mM N1-Methylpseudouridine and CleanCap AG from TriLink BioTechnologies. The reaction was placed in a thermocycler (Eppendorf Mastercycler Nexus) to be incubated at 37°C for 15 minutes. To further purify the RNA, we used an RNA cleanup kit from Macherey-Nagel (protocol linked in Appendix K). Product was then analyzed on a denaturing gel.

3.9. Gel Electrophoresis

DNA was resolved on 1% agarose gel with ethidium bromide for imaging. DNA gels were submerged in TAE and run at 120V between 40-50 minutes using a compact power supply from Fisher Scientific. For mRNA analysis, we ran a denaturing gel in the presence of 10x RNA Gel Buffer (MOPS buffer) from Quality Biological Inc. (protocol linked in Appendix L).

4. Results and Discussion

4.1. Generating double-stranded and single-stranded TRAC constructs via plasmid cloning and phagemids

Using plasmids cloned previously in the Keeler lab (sources and sequences of original plasmids in Appendix A), we were able to locate two ideal plasmids to construct our mCherry cassette - a backbone plasmid containing the TRAC homology arms and T2A, and an insert plasmid containing our gene of interest, mCherry. As previously mentioned, fragments were amplified using PCR and their respective primers, then isolated using a gel extraction so that they could be assembled via Gibson assembly. The expected sequence length for the backbone fragment alone was 4253 bp. For the insert without a Kozak sequence, the expected length was 708 bp, and 714 bp for the insert with a Kozak sequence. The gel shown below in Figure 6 was used to confirm that the sizes of our fragment samples matched the expected lengths that were simulated in SnapGene.

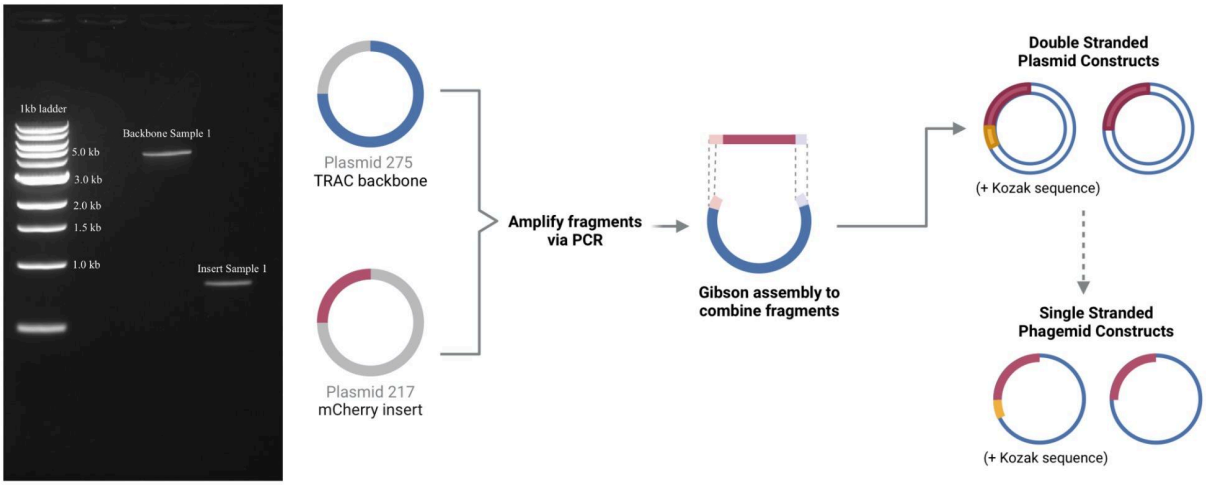
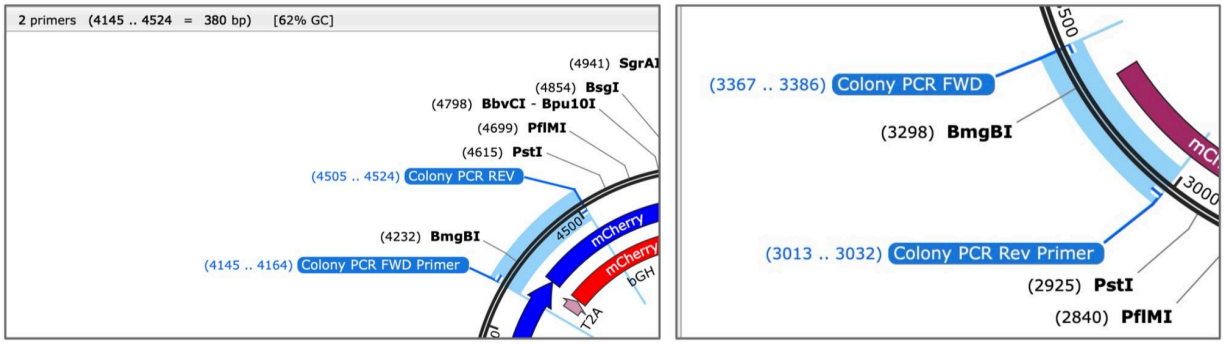


Figure 6. Agarose gel showing backbone and insert fragments (alongside 1kb ladder for comparison) and graphical abstract outlining 'gene insertion' cloning process (made with BioRender)

After the Gibson assembly product had been transformed using competent cells and plated to grow overnight, colony PCR was used to validate the cloned product. Colony PCR is used to quickly screen a plasmid cloning experiment where primers are designed to amplify a region of the cloned plasmid that contains the new fragments. In this case, our forward primer anneals a section of the TRAC backbone, while the reverse primer anneals a section of the mCherry insert (Figure 7).



With Kozak – 380 bp amplicon

Without Kozak – 374 bp amplicon

Figure 7. Colony PCR primer amplicons for TRAC-mCherry cloning (SnapGene)

This primer design allowed us to check for positive clones because if a colony does not possess the expected sequence, the primers would not be able to amplify this genomic region and there would not be a clean band shown in a gel. For this experiment, our expected amplicon sizes were 380 bp with the Kozak sequence and 374 bp without the Kozak sequence. Figure 8 shows a gel where we screened twenty total colonies, ten of our +Kozak construct (samples 6-10 and 16-20) and ten of our -Kozak construct (samples 1-5 and 11-15). All samples showed the expected band size of ~380 bp. Since 6 bp is too small of a difference to detect on a gel, we sent out representative samples (samples 4 and 6) for whole-plasmid sequencing via GeneWiz to confirm the presence of the Kozak sequence.

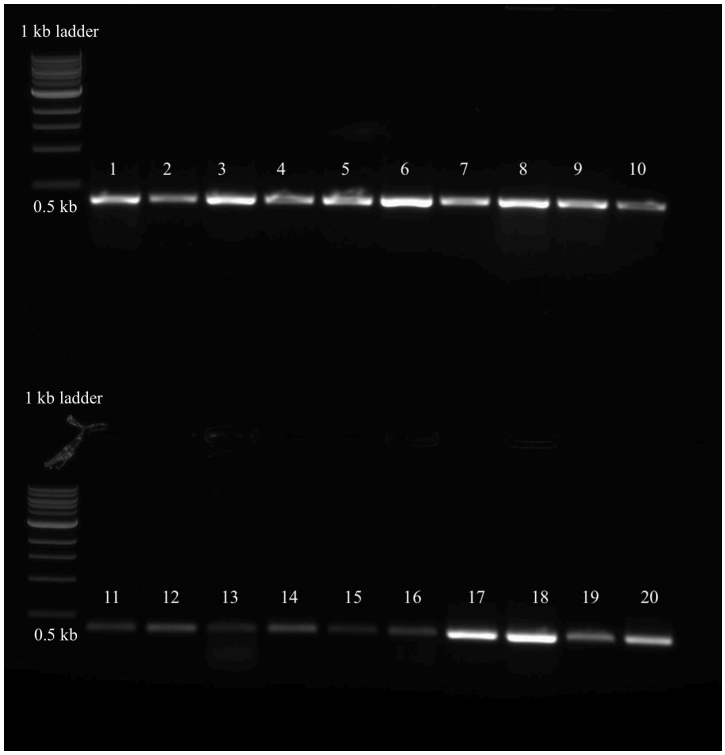


Figure 8. Image of gel after colony PCR showing all positive colonies alongside a 1kb ladder for comparison. Samples 1-5 and 11-15 represent our TRAC-mCherry construct without the Kozak sequence, while 6-10 and 16-20 are our TRAC-mCherry construct with a Kozak sequence. Samples 4 and 6 were sent out for whole-plasmid sequencing through GeneWiz.

After receiving our sequencing results, we aligned the plasmid map to our theoretical plasmid map that we used to design our colony PCR primers in SnapGene. The alignment in Figure 9 verifies that our cloned product had the exact sequence expected from our original simulated plasmid map.

Figure 9. SnapGene alignment of expected cloned sequence and sequencing results from Genewiz of the TRAC-mCherry construct with a Kozak sequence (top) and TRAC-mCherry construct without a Kozak sequence (bottom)

Once we had synthesized the single-stranded versions of these constructs, we set up an experiment using S1 nuclease digestion and analyzed the results via agarose gel to confirm if the synthesis was successful. Figure 10, shown below, outlines the expected outcomes of this experiment, where each column represents a well of the gel. The first two wells contain samples of our undigested dsDNA and ssDNA. It was expected that the undigested ssDNA would be half the size of the undigested dsDNA. The next two wells (3 and 4) contain dsDNA and ssDNA digested with the dual-cutting restriction enzyme Sac1. Because Sac1 is a dual-cutter, the sample containing digested dsDNA would show two distinct bands, whereas the digested ssDNA would only show a band relative to its size since restriction enzymes are unable to cut ssDNA. The last two wells contain dsDNA and ssDNA digested with the S1 nuclease. Due to this nuclease's ability to cleave ssDNA, the well containing ssDNA should not have any bands, as it would've been entirely broken down, and there should be little to no effect on the dsDNA unless the DNA was nicked or supercoiled.

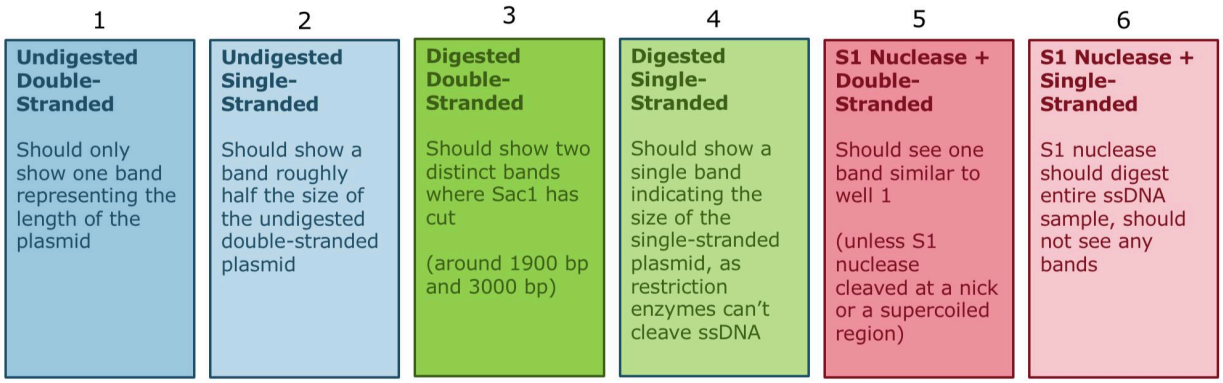


Figure 10. Expected outcomes of S1 nuclease digestion experiment

As seen in the gel below (Figure 11), the expected outcomes of this experiment aligned with what was seen in the gel. It can be assumed that the ssDNA synthesized from our original cloned TRAC-mCherry constructs is pure and does not contain any double-stranded contamination because the S1 nuclease broke down the entire sample and no unexpected bands could be seen in well 6. Two bands can be seen in the well with S1 nuclease and dsDNA (well 5) but this is most likely due to the S1 nuclease cleaving a nick or supercoil in the DNA.

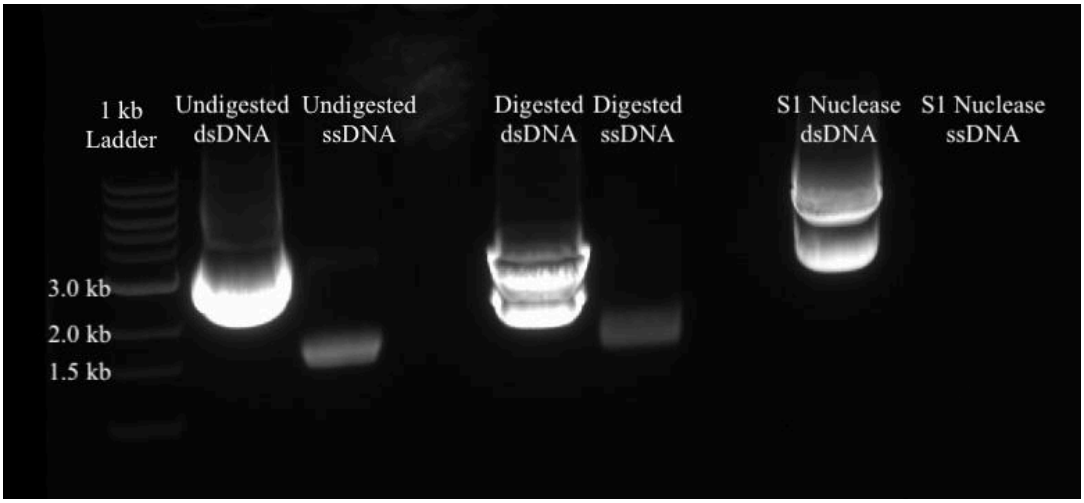


Figure 11. Agarose gel result from S1 nuclease experiment

4.2. Generating double-stranded safe harbor construct via plasmid cloning

To construct an m-Cherry cassette compatible with the AAVS1 safe harbor locus, we identified three plasmids that had been previously cloned by the Keeler lab containing the necessary genetic components (sources and sequences of original plasmids in Appendix A). The backbone plasmid containing the left AAVS1 homology arm and an EF-1α promoter was digested using BamHI and HindIII, and two insert plasmids were identified containing the right homology arm and mCherry gene. Figure 12, seen below, shows an agarose gel confirming the length of the desired fragments to be used in Gibson cloning, as well as the process for cloning the GSH construct.

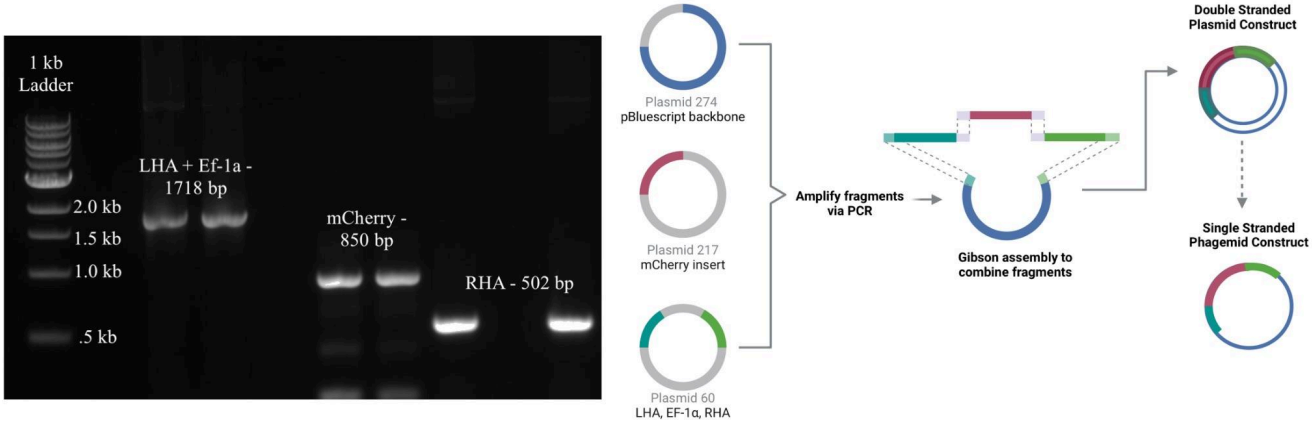


Figure 12. Agarose gel showing backbone and both insert fragments (alongside 1kb ladder for comparison) and graphical abstract outlining 'safe harbor' cloning process (made with BioRender)

To confirm the validity of the cloned product, restriction digestion was performed at sites containing the backbone and the insert using BamHI and HincII. BamHI is a dual cutter and should show two bands in the correct cloned product, while HincII would show one band. The top wells of Figure 13 show the ten colonies that were screened using colony PCR in the top wells of the gel, while the bottom wells show the digested samples of those same colonies. Wells 1 and 11 showed the expected results in both experiments, so the sample was sent out for sequencing using Plasmidsaurus.

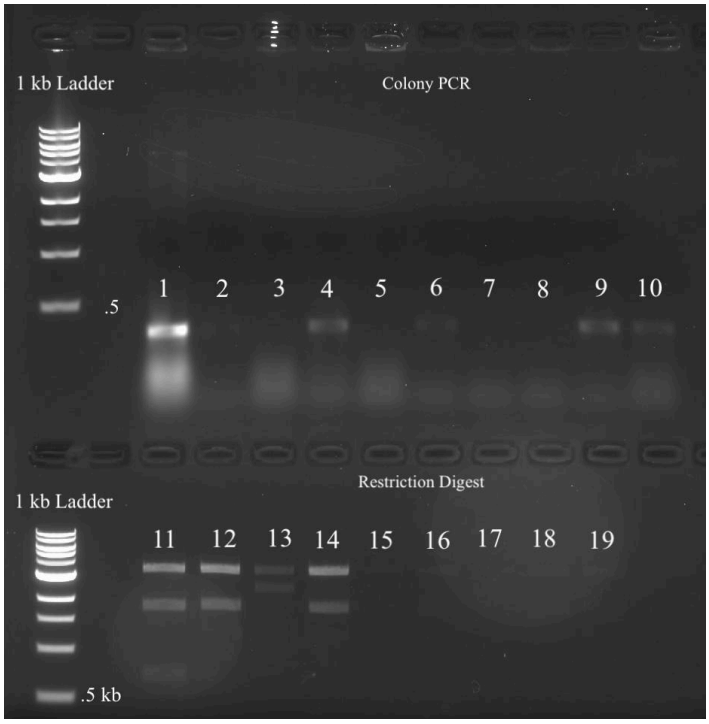


Figure 13. Agarose gel showing the results of both colony PCR and restriction digest on the cloned products from Gibson assembly

After receiving the sequencing results, the sequenced plasmid was aligned with the designed safe harbor insert plasmid to determine sequence similarity and verify the presence of all the necessary components (Figure 14). Two mismatched base pairs were found but there was no effect on the amino acid sequence. Overall, the cloning was considered successful as all necessary components were present.

Figure 14. SnapGene alignment of the expected AAVS1-mCherry sequence with the cloned AAVS1-mCherry sequence, one of the two mismatched base pairs is highlighted

4.4. Synthesis and verification of Cas9-encoding mRNA

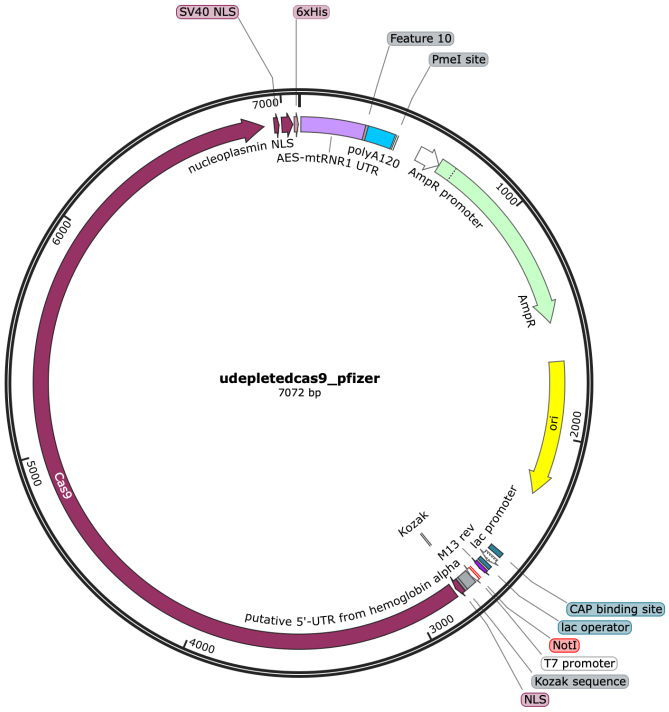


Figure 15. Plasmid map of our original plasmid containing a Cas9-encoding mRNA region

Once we had synthesized the Cas9 mRNA, a denaturing gel was used to verify that the band size matched the size of the original Cas9 encoding region in the plasmid. As seen in Figure 15, the size of this region is ~ 4000 bp. To prepare the denaturing gel, we used the RNA Gel Buffer (MOPS Buffer) from Quality Biological. The result of this gel can be seen below in Figure 16.

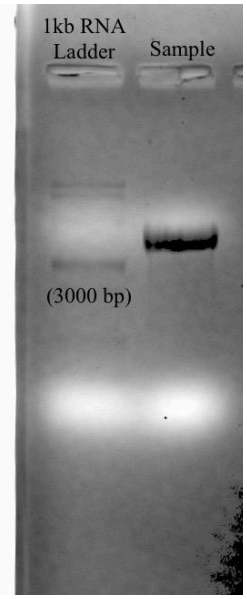


Figure 16. Denaturing RNA gel to confirm size of Cas9 mRNA (alongside 1kb RNA ladder for comparison)

Despite degradation of the RNA ladder, one can conclude that the mRNA was properly synthesized and purified because the band is slightly higher than the 3000 bp band from the RNA ladder, indicating that the size is ~4000 bp.

4.4 Discussion

In summary, we were able to construct and verify the necessary molecular components to transfect T cells with three separate mCherry cassettes as a precursor to the addition of a CAR construct. We successfully cloned two constructs that aim to knock-out the endogenous TCR in the TRAC locus using TRAC homology arms so that expression can be driven by the endogenous TRAC promoter. Of these constructs, one contains a Kozak sequence and one does not. In the next steps of this project, the mCherry fluorescence of both constructs can be compared to evaluate if the addition of a Kozak sequence has a significant impact on protein translation efficiency. We also designed a third construct that aims to edit into the AAVS1 safe harbor region with the appropriate AAVS1 homology arms and an exogenous EF-1α promoter. The sequences for all of these constructs were confirmed through whole plasmid sequencing and alignment with our original predicted sequences.

Along with creating our double-stranded plasmid constructs, we successfully synthesized and verified circular single-stranded DNA templates from both TRAC-mCherry cassettes using phagemids. To confirm that our single-stranded constructs were correctly synthesized, we performed a digestion experiment using S1 nuclease. In this experiment we compared the fragments of our dsDNA and ssDNA constructs in an undigested sample, a dual-cutting restriction enzyme digested sample, and an S1 nuclease digested sample. Due to the S1 nuclease's ability to cleave ssDNA, we expected the S1 nuclease digested ssDNA sample to show no band, which was reflected on our gel.

Lastly, we synthesized the Cas9 mRNA that would allow for homology-directed repair when transfecting T cells. The mRNA-encoding region was isolated from a plasmid and synthesized through in vitro transcription. The size of the mRNA fragment was then confirmed on a denaturing gel.

5. Future Direction

Aside from creating the single-stranded version of the AAVS1 safe harbor construct, our future goals for this project would be setting up electroporation experiments to transfect T cells with the double-stranded and single-stranded constructs using the synthesized Cas9 mRNA. Unfortunately, due to time constraints, we did not make it to electroporation, but the ideal next step would be evaluating the editing efficiency of each construct post-electroporation using FACs, flow cytometry, and genotyping. An ideal cell line for this could be the Jurkat cell line due to its similarity in expression profile to human T cells. If Jurkat cells can be successfully electroporated and transfected, this experiment could then be translated into peripheral blood mononuclear cells (PBMCs) from a human donor. If one or more of these constructs achieve sufficient fluorescence levels in the post-electroporation analysis, the next step would be to utilize these findings to optimize the insertion of a CAR construct.

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Appendices

Appendix A. Plasmid Guide

Plasmid # in lab database	Source	Components used	Sequence
60	Flotte Lab Plasmid	Left AAVS1 HA	cagagcagggccttagggaagcgggaccctgctctgggcggaggaatatgtcccagatagcactggggactctttaaggaaagaaggatg gagaaagagaaagggagtagaggcggccacgacctggtgaacacctaggacgcaccattctcacaaagggagttttccacacggacacc ccctcctcaccacagccctgccaggacggggctggctactggccttatctcacaggtaaaactgacgcacggaggaacaatataaattgggg gactagaaaggtgaagagccaaagttagaactcaggaccaacttattctgattttgtttttccaaactgcttctcctcttgggaagtgtaaggaag ctgcagcaccaggatcagtgaaacgcaccagacagccgcgtcagagcagctcaggttctgggagagggtagcgcagggtggccactgag aaccgggcaggtcacgcatccccccctttccctcccacccccctgccaagctctccctcccaggatcctctctggctt
		EF-1α promoter	cgaagctccggtgcccgtcagtgggcagagcgcacatcgcccacagtccccgagaagttgggggggaggggtcggcaattgaaccggtgc ctagagaagaggtggcgcggggtaaactgggaaagtgatgtcgtgtactggctccgccttttcccgagggtgggggagaaccgtatataagtg cagtagtcgccgtgaacgttcttttttcgcaacgggtttgccgccagaacacaggtaagtgccgtgtgtggttcccgcgggcctggcctctttac gggttatggcccttgcgtgccttgaattacttccacgcccctggctgcagtacgtgattcttgatcccgagcttcgggttggaagtgggtgggag agttcgaggccttgcgcttaaggagccccttcgcctcgtgcttgagttgaggcctggcctgggcgctggggccgccgcgtgcgaatctggtg gcaccttcgcgcctgtctcgctgctttcgataagtctctagccatttaaaatttttgatgacctgctgcgacgcttttttttctggcaagatagtcttgta aatgcgggccaagatctgcacactggtatttcggttttggggccgcgggcggcgacggggcccgtgcgtcccagcgcacatgttcggcga ggcggggcctgcgagcgcggccaccgagaatcggacgggggtagtctcaagctggccggcctgctctggtgcctggcctcgcgccgcc gtgtatcgccccgccctgggcggcaaggctggcccggtcggcaccagttgcgtgagcggaaagatggccgcttcccggccctgctgcagg gagctcaaaatggaggacgcggcgctcgggagagcgggcgggtgagtcacccacacaaaggaaaagggcctttccgtcctcagccgtcg cttcatgtgactccacggagtaccgggcgccgtccaggcacctcgattagttctcgagcttttggagtacgtcgtctttaggttgggggaggg gttttatgcgatggagtttccccacactgagtgggtggagactgaagttaggccagcttggcacttgatgtaattctccttggaatttgccctttttg agtttggatcttggttcattctcaagcctcagacagtggttcaaagtttttttcttccatttcaggtgtcgtga
		Right AAVS1 HA	tgctgtcctgaagtggacatagggcccgggttggaggaagaagactagctgagctctcggacccctggaagatgccatgacaggggct ggaagagctagcacagactagagaggtaaggggggtagggagctgcccaaatgaaaggagtgagaggtgacccgaatccacaggaga acggggtgtccaggcaaagaaagcaagaggatggagaggtggctaaagccagggagacggggtactttggggttgtccagaaaaacggt gatgatgcaggcctacaagaaggggaggcgggacgcaagggagacatccgtcggagaaggccatcctaagaaacgagagatggcaca ggccccagaaggagaaggaaaagggaacccagcgagtgaagacggcatggggttgggtgagggaggagagatgcccggagaggacc cagacacggggaggatccgctcagaggacatcacgtggtgcagcgccgagaaggaagtgctc
217	Given to the Keeler lab from the Wang lab	mCherry	atggtgagcaagggcgaggaggataactccgccatcatcaaggagttcctgcgcttcaaggtgcacatggagggctccgtgaacggccac gagttcgagatcgagggcgagggcgagggccgcccctacgagggcacccagaccgccaagctgaaggtgaccaagggtggccccctg cccttcgcctgggacatcctgtccctcagttcatgtacggctccaaggcctacgtgaagcaccccgccgacatccccgactacttgaagctgt ccttccccgagggcttcaagtgggagcgcgtgatgaacttcgaggacggcggcgtggtgaccgtgacccaggactcctctctgcaggacg gcgagttcatctacaaggtgaagctgcgcggccaccaacttccctccgacggccccgtaatgcagaagaagaccatgggctgggaggcct cctccgagcggatgtaccccgaggacggcgccctgaagggcgagatcaagcagaggctgaagctgaaggacggcggccactacgacgc tgaggtcaagaccacctacaaggccaagaagcccgtgcagctgccccggcgcctacaacgtcaacatcaagttggacatcacctcccacaac gaggactacaccatcgtggaacagtacgaacgcgccgagggccgccactccaccggcggcatggacgagctgtacaag
274	StrateGene	pBluescript backbone SKII+	tatcgataccgtcgacctcgaggggggggcccggtacccaattcgccctatagtgagtcgtattacgcgcgctcactggccgtcgttttacaacg tcgtgactgggaaaaccctggcgttacccaacttaatcgccttgcagcacatccccctttcgccagctggcgtaatagcgaagaggcccgcac cgatcgcccttcccaacagttgcgcagcctgaatggcgaatgggacgcgccctgtagcggcgcattaagcgcggcgggtgtggtggttacg cgcagcgtgaccgctacacttgccagcgccctagcgcccgctcctttcgctttcttcccttcctttctcgccacgttcgccggctttccccgtcaa gctctaaatcgggggctccctttagggttccgatttagtgctttacggcacctcgaccccaaaaaacttgattagggtgatggttcacgtagtgg gccatcgccctgatagacggtttttcgccctttgacgttggagtccacgttctttaatagtggactcttgttccaaactggaacaacactcaaccct atctcggtctattcttttgatttataagggattttgccgatttcggcctattggttaaaaaatgagctgatttaacaaaaatttaacgcgaattttaacaa aatattaacgcttacaatttaggtggcacttttcggggaaatgtgcgcggaacccctatttgtttatttttctaaatacattcaaatatgtatccgctca tgagacaataaccctgataaatgcttcaataatattgaaaaaggaagagtatgagtattcaacatttccgtgtcgcccttattcccttttttgcggca ttttgccttcctgttttttgctcacccagaaacgctggtgaaagtaaaagatgctgaagatcagttgggtgcacgagtgggttacatcgaactggat ctcaacagcggtaagatccttgagagttttcgccccgaagaacgttttccaatgatgagcacttttaaagttctgctatgtggcgcggtattatccc gtattgacgccgggcaagagcaactcggtcgccgcatacactattctcagaatgacttggttgagtactcaccagtcacagaaaagcatcttac ggatggcatgacagtaagagaattatgcagtgctgccataaccatgagtgataacactgcggccaacttacttctgacaacgatcggaggacc gaaggagctaaccgcttttttgcacaacatgggggatcatgtaactcgccttgatcgttgggaaccggagctgaatgaagccataccaaacga cgagcgtgacaccacgatgcctgtagcaatggcaacaacgttgcgcaaactattaactggcgaactacttactctagcttcccggcaacaatta atagactggatggaggcggataaagttgcaggaccacttctgcgctcggcccttccggctggctggtttattgctgataaatctggagccggtg agcgtgggtctcgcggtatcattgcagcactggggccagatggtaagccctcccgtatcgtagttatctacacgacggggagtcaggcaact atggatgaacgaaatagacagatcgctgagataggtgcctcactgattaagcattggtaactgtcagaccaagtttactcatatatactttagatt gatttaaaacttcatttttaatttaaaaggatctaggtgaagatcctttttgataatctcatgaccaaaatcccttaacgtgagttttcgttccactgagc gtcagaccccgtagaaaagatcaaaggatcttcttgagatcctttttttctgcgcgtaatctgctgcttgcaaacaaaaaaaccaccgctaccag cggtggtttgtttgccggatcaagagctaccaactcttttccgaaggtaactggcttcagcagagcgcagataccaaatactgtccttctagtgt agccgtagttaggccaccacttcaagaactctgtagcaccgcctacatacctcgctctgctaatcctgttaccagtggctgctgccagtggcgat

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275	Cloned by Prajakta Ambegaokar and Acadia Kopec in the Keeler Lab, 2023	TRAC backbone	actgtgccttctagttgccagccatctgttgtttgcccctcccccgtgccttccttgaccctggaaggtgccactcccactgtcctttcctaataaaa tgaggaaattgcatcgcattgtctgagtaggtgtcattctattctgggggtgggtggggcaggacagcaagggggaggattgggaagaca atagcaggcatgctggggatgcggtgggctctatgggctagcgcggccgcatatccagaaccctgaccctgccgtgtaccagctgagagac tctaaatccagtgacaagtctgtctgcctattcaccgattttgattctcaaacaaatgtgtcacaaagtaaggattctgatgtgtatatcacagacaa aactgtgctagacatgaggtctatggacttcaagagcaacagtgctgtggcctggagcaacaaatctgactttgcatgtgcaaacgccttcaac aacagcattattccagaagacacctttcttccccagcccaggtaagggcagctttggtgccttcgcaggctgtttccttgcttcaggaatggccag gttctgcccagagctctggtcaatgatgtctaaaactcctctgattggtggtctcggccttatccattgccacccaaaaccctcttttttactaagaaac agtgagccttgttctggcagtccagagaatgacacgggaaaaaagcagatgaagagaaggtggcaggagagggcatcgataccgtcgacc tcgaggggggggcccggtacccaattcgccctatagtgagtcgtattacgcgcgctcactggccgtcgttttacaacgtcgtgactgggaaaac cctggcgttacccaacttaatcgccttgcagcacatccccctttcgccagctggcgtaatagcgaagaggcccgcaccgatcgcccttcccaa cagttgcgcagcctgaatggcgaatgggacgcgccctgtagcggcgcattaagcgcggcgggtgtggtggttacgcgcagcgtgaccgct acacttgccagcgccctagcgcccgctcctttcgctttcttcccttcctttctcgccacgttcgccggctttccccgtcaagctctaaatcgggggg ctcccctttagggttccgatttagtgctttacggcacctcgaccccaaaaaacttgattagggtgatggttcacgtagtgggccatcgccctgatag acggtttttcgccctttgacgttggagtccacgttctttaatagtggactcttgttccaaactggaacaacactcaaccctatctcggtctattcttttg atttataagggattttgccgatttcggcctattggttaaaaaatgagctgatttaacaaaaatttaacgcgaatttaacaaaatattaacgcttacaa tttaggtggcacttttcggggaaatgtgcgcggaacccctatttgtttatttttctaaatacattcaaatatgtatccgctcatgagacaataaccctg ataaatgcttcaataatattgaaaaaggaagagtatgagtattcaacatttccgtgtcgcccttattcccttttttgcggcattttgccttcctgttttg ctcacccagaaacgctggtgaaagtaaaagatgctgaagatcagttgggtgcacgagtgggttacatcgaactggatctcaacagcggtaag atccttgagagttttcgccccgaagaacgttttccaatgatgagcacttttaaagttctgctatgtggcgcggtattatcccgtattgacgccgggc aagagcaactcggtcgccgcatacactattctcagaatgacttggttgagtactcaccagtcacagaaaagcatcttacggatggcatgacagt aagagaattatgcagtgctgccataaccatgagtgataacactgcggccaacttacttctgacaacgatcggaggaccgaaggagctaaccg cttttttgcacaacatggggatcatgtaactcgccttgatcgttgggaaccggagctgaatgaagccataccaaacgacgagcgtgacacca cgatgcctgtagcaatggcaacaacgttgcgcaaactattaactggcgaactacttactctagcttcccggcaacaattaatagactggatgga ggcggataaagttgcaggaccacttctgcgctcggcccttccggctggctggtttattgctgataaatctggagccggtgagcgtgggtctcgc ggtatcattgcagcactggggccagatggtaagccctcccgtatcgtagttatctacacgacggggagtcaggcaactatggatgaacgaaat agacagatcgctgagataggtgcctcactgattaagcattggtaactgtcagaccaagtttactcatatatactttagattgatttaaaacttcatttttt aatttaaaaggatctaggtgaagatccttttgataatctcatgaccaaaatcccttaacgtgagttttcgttccactgagcgtcagaccccgtaga aaagatcaaaggatcttcttgagatcctttttttctgcgcgtaatctgctgcttgcaaacaaaaaaaccaccgctaccagcggtggtttgtttgccg gatcaagagctaccaactctttttccgaaggtaactggcttcagcagagcgcagataccaaatactgtccttctagtgtagccgtagttaggcca ccacttcaagaactctgtagcaccgcctacatacctcgctctgctaatcctgttaccagtggctgctgccagtggcgataagtcgtgtcttaccg ggttggactcaagacgatagttaccggataaggcgcagcggtcgggctgaacgggggttcgtgcacacagcccagcttggagcgaacga cctacaccgaactgagatacctacagcgtgagctatgagaaagcgccacgcttcccgaagggagaaaggcggacaggtatccggtaagcg gcagggtcggaacaggagagcgcacgagggagcttccagggggaaacgcctggtatctttatagtcctgtcgggtttcgccacctctgactt gagcgtcgatttttgtgatgctcgtcaggggggcggagcctatggaaaaacgccagcaacgcggcctttttacggttcctggccttttgctggc ctttgctcacatgttctttcctgcgttatcccctgattctgtggataaccgtattaccgcctttgagtgagctgataccgctcgccgcagccgaacg accgagcgcagcgagtcagtgagcgaggaagcggaagagcgcccaatacgcaaaccgcctctccccgcgcgttggccgattcattaatgc agctggcacgacaggtttcccgactggaaagcgggcagtgagcgcaacgcaattaatgtgagttagctcactcattaggcaccccaggcttt acactttatgcttccggctcgtatgttgtgtggaattgtgagcggataacaatttcacacaggaaacagctatgaccatgattacgccaagcgcg caattaaccctcactaaagggaacaaaagctggagctccaccgcggtggcggccgctctagaactagtggatcctatcaatgagagagcaat ctcctggtaatgtgatagatttcccaacttaatgccaacatacctaaaacctcccattctgctaatgcccagcctaagttggggagaccactccag attccaagatgtacagtttgctttgctgggccttttcccatgcctgcctttactctgccagagttatattgctgggggttttgaagaagatcctattaaa taaaagaataagcagtattattaagtagccctgcatttcaggtttccttgagtggcaggccaggcctggccgtgaacgttcactgaaatcatggc ctcttggccaagattgatagcttgtgcctgtccctgagtcccagtccatcacgagcagctggtttctaagatgctatttcccgtataaagcatgag accgtgacttgccagccccacagagccccgccttgtccatcactggcatctggactccagcctgggttggggcaaagagggaaatgagat catgtcctaaccctgatcctcttgtcccacaggagggcaggggatctctccttacttgtggcgacgtggaggagaaccccggcccc
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(Protocols)

Appendix B: New England Biolabs, PCR Using NEBNext High Fidelity 2X Master Mix
https://www.neb.com/en-us/protocols/2012/08/29/pcr-using-nebnext-high-fidelity-2x-pcr-master-mix-m0541

Appendix C: New England Biolabs, Monarch DNA Gel Extraction Kit Protocol
https://www.neb.com/en-us/protocols/2015/11/23/monarch-dna-gel-extraction-kit-protocol-t1020

Appendix D: New England Biolabs, Gibson Assembly MasterMix - Assembly
https://www.neb.com/en-us/protocols/2012/09/25/gibson-assembly-master-mix-assembly

Appendix E: Qiagen, QIAprep Spin Miniprep Kit
https://www.qiagen.com/us/resources/download.aspx?id=56b0162c-23b0-473c-9229-12e8b5c8d590&lang=en

Appendix F: Qiagen, QIAGEN® Plasmid *Plus* Midi Kit protocol
https://www.qiagen.com/at/resources/download.aspx?id=3da21fc3-a078-4665-aefe-06154db2b6d2&lang=en

Appendix G: APExBIO, Taq PCR Master Mix (protocol)
https://www.apexbt.com/downloader/document/K1034/Protocol.pdf

Appendix H: Iyer, *et al.*, 2022, Preparation of cssDNA (see 'Materials and Methods' section)
https://www.liebertpub.com/doi/10.1089/crispr.2022.0058?url_ver=Z39.88-2003&rfr_id=ori%3Arid%3Acrossref.org&rfr_dat=cr_pub++0pubmed

Appendix I: NEB HiScribe T7 Quick High Yield RNA Synthesis Kit
https://www.neb.com/en-us/products/e2050-hiscribe-t7-quick-high-yield-rna-synthesis-kit

(Continued on next page)

Appendix J: IVT mRNA protocol

IVT mRNA protocol

Template prep: Linearize the plasmid with BsmBI or Esp3I, analyze the product on agarose gel to ensure complete linearization. Purify the reaction using a DNA column, do not gel-purify!

RNase control measures: clean bench and all working surfaces with 10% bleach, 75% EtOH and RNaseZap; change gloves frequently, especially after touching non-decontaminated surfaces; use clean tips/tubes/H2O etc. you'd absolutely trust; preferably, wear a lab coat and a mask.

1. IVT reaction:

10X NEB Hiscribe buffer	1ul
100mM ATP	1ul
100mM GTP	1ul
100mM CTP	1ul
100mM N1-MethylpseudoU	1ul
CleanCap AG (3'OMe)	0.8ul
Pyrophosphatase	0.5ul
RNase Inhibitor	1ul
Linearized DNA template	1000-2000ng
T7 pol mix	2ul
RNase-free water up to	20ul

37C for 2hours

2. Bring up to volume to 50ul by adding 30ul water; add 2ul DNase I, 37C for 15min.

3. Purify the RNA using NEB Monarch columns. You'll expect to get about 100-200ug modified mRNA from the 20ul reaction. Alternatively, you can purify the RNA by LiCl precipitation method (see TriLink protocol).

4. (In some protocols, this step is not included, such as TriLink protocol). Arctic phosphatase (AnP) treatment (adjust the volume according to your elution volume from the previous step):

RNA	~67ul
10X AnP buffer	8ul
AnP	4ul
RNase Inhibitor	1ul
RNase-free water up to	80ul

37C for 30min; Purify the RNA using NEB Monarch columns or LiCl precipitation

5. Analyze the product on 1% formaldehyde/glyoxal agarose gel (see TriLink protocol). If good, the mRNA is ready for use.

Appendix K: Macherey-Nagel, NucleoSpin RNA, Mini kit for RNA purification
https://www.mn-net.com/us/nucleospin-rna-mini-kit-for-rna-purification-740955.50?c=5297

Appendix L: 10X RNA Gel Buffer (10X MOPS Buffer) protocol
https://www.qualitybiological.com/product/rna-gel-buffer-10x-mops-buffer/

Appendix M: S1 Nuclease User Guide
https://assets.thermofisher.com/TFS-Assets%2FLSG%2Fmanuals%2FMAN0013722_S1_Nuclease_UG.pdf