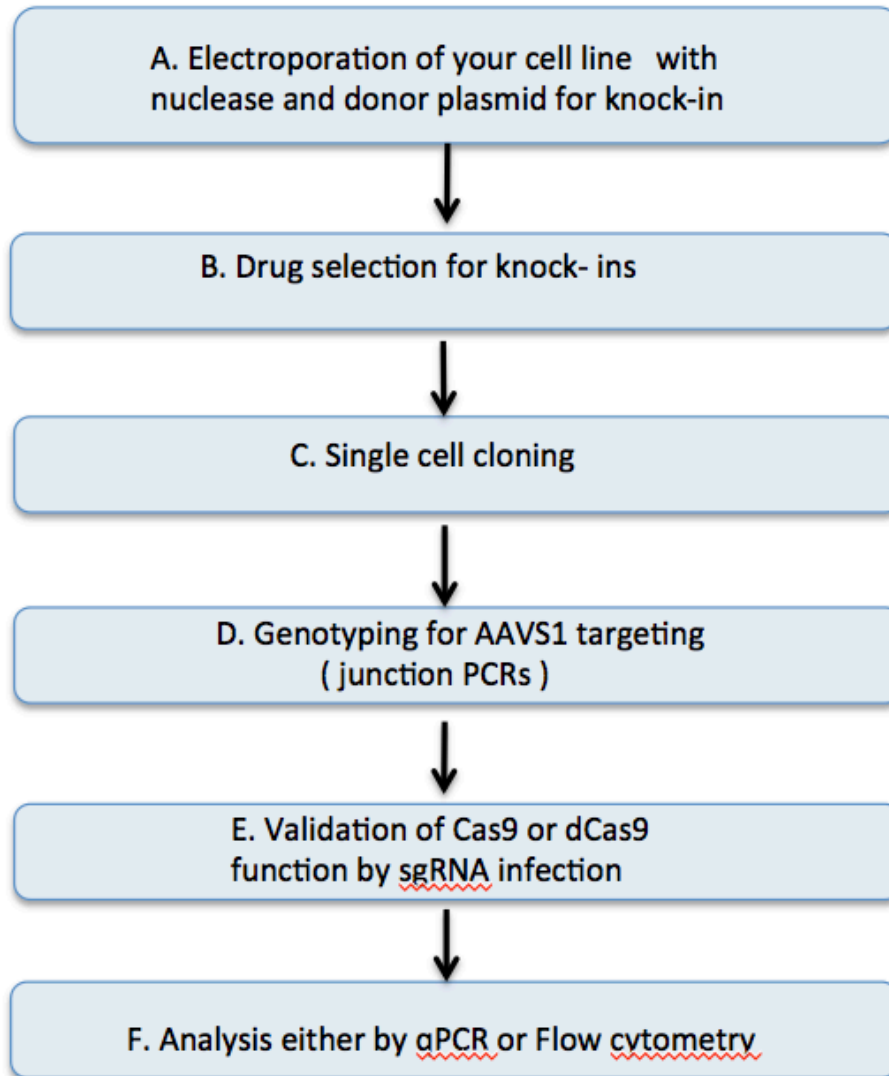


Generating stable cell lines expressing various Cas9 proteins from AAVS1 locus

- This is modified from original protocol from the Conklin laboratory (Mandegar et al. submitted)
- Outline of experiment is shown below



A. Electroporation of your cell line with nuclease and donor plasmid

Mandegar et al created donor plasmids to used for AAVS1 targeting. All donors have same 5' and 3' homology arms (~800bp). Inserts are shown below.

pAAVS1-NC-CRISPRi: constitutively active dCas9-Krab version

pAAVS1-NDi-CRISPRi-: inducible dCas9-Krab version without mcherry fusion

pAAVS1-NDi-CRISPRi+: inducible dCas9-Krab version with mcherry fusion

pAAVS1-PTi-CRISPRn: inducible wt Cas9 nuclease

Mandegar et al used TALEN nuclease to create double-strand breaks. Plasmids are called.

pAAVS1-TALEN F

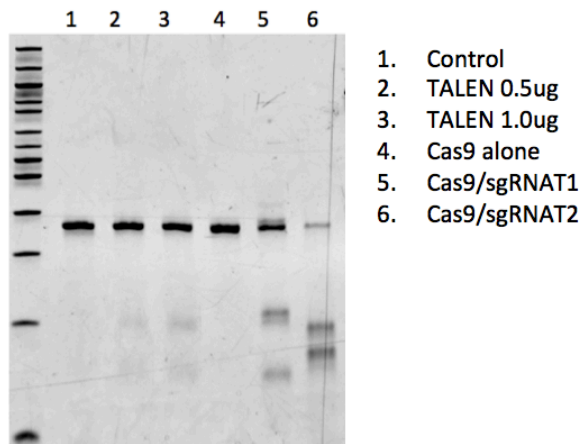
pAAVS1-TALEN R

If you wish, you can use Cas9 protein instead of TALENs. sgRNA sequences are shown below (from Church lab 2013 paper). Cutting sites of TALENs and sgRNAs are very close and all work with the same donor plasmids.

sgRNA_T1: gtcccctccaccccacagtg

sgRNA_T2: ggggccactagggacaggat

T7E1 assay was done in 293T cells to compare their cutting efficiencies. For Cas9, we used cas9 protein and sgRNAs for electroporation (based on Doudna's eLife paper). As shown below, Cas9 RNP works more efficiently in cutting compared to TALEN plasmid electroporation at AAVS1 site. The RNPelectroporation Protocol is available from IGI along with in vitro sgRNA synthesis protocol (request to Chong).



T7E1 assay in 5% TBE gel

B & C. Drug selection for knock-ins and single cell cloning

Choose delivery method based on your cell line and follow the manufacturer's protocol.

We use Neomycin and Puromycin for selection. Neomycin- and puromycin-resistant gene products are expressed under the endogenous AAVS1 promoter. Optimize your drug dose ahead of time. Test multiple doses on multiple cell density to get an optimal dose. For Jurkat and HeLa cell lines, we used 1mg/ml and 0.75ug/ml of Geneticin and Puromycin respectively.

For suspension cell lines, we select cells as pool and then did dilution cloning in 96 well plates. For adherent cell lines, we plate cells sparsely and pick single colonies under microscope.

For HeLa cells, more than 90 out of 96 colonies were positive for junction PCRs. 1~2 out of 12 clones have Knockins on both alleles. You may be able to get a couple of lines by picking 24 clones in our experience.

D. Genotyping for AAVS1 targeted clones**a. Junction PCR primer information**

Wt AAVS1 locus (336bp)

AAVS1 panF1: ctatgtccacttcaggacagca

Wt AAVS1 R: AGGATCCTCTCTGGCTCCAT

AAVS1 5' junction PCR for inducible dcas9-krab/cas9 nuclease

AAVS1 5' JF: CTGCCGTCTCTCCTGAGT

Neo JR: CTCGTCCTGCAGTTCATTCA (1258bp)(both NDi-CRISPRi &NCi-CRISPRi)

Puro JR:GTGGGCTTGTA CTGGTCAT (1068bp) (PTi-CRISPRn)

AAVS1 3' junction PCR for inducible dcas9-krab (1382bp)

TRE3G 1F: GTGTTGTGGAATTGCTCCAG

3JR2: aagagtgagtttgccaagcagt

AAVS1 3' junction PCR for constitutively active dcas9-krab (1135bp) (NC-CRISPRi)

3JF3: tctcttatggagatccctcgac

3JR2: aagagtgagtttgccaagcagt

b. Genomic PCR condition

Lyse cells in QuickExtract DNA extract solution from epicenter (<http://www.epibio.com/applications/nucleic-acid-purification-extraction-kits/buccal-swabs-and-dna-extraction/quickextract-dna-extraction-solution?details>). I modified a bit from its protocol to save reagents.

- Collect about 10,000~100,000 cells and spin down to make pellet Then take media off.
- Add 50ul of QE solution to cell pellet and vortex well to mix.
- Incubate the plates/tubes at 65 degree, 10min and then 98 degree, 5min. Then store on ice until use.

I used 2 ul of samples for 20ul PCR reaction (no more than 1/10th). From 10000 and 100000 cells, I got ~20ng/ul and ~100ng/ul readings from Nanodrop. Both concentration gave me a clean band. It seems to work okay in this range.

I use Phusion high-fidelity Taq polymerase with DMSO. Other enzymes will work too. If you like to use other Taq polymerase, you need to test temperature gradient for optimal annealing temperature. My conditions are below. I used the same forward primer to sequence the PCR product initially.

component	in 20ul	final
genomic DNA (~50ng/ul)	2	~100ng
5XGC buffer	4	1X
DMSO	0.6	3%
F-primer (10uM)	1	0.5uM
R-primer (10uM)	1	0.5uM
dNTPs (10mM each)	0.4	200uM
Phusion polymerase	0.2	
water	X	
	total 20ul	

STEP	TEMP	TIME
initial denaturation	98	1 min
35 cycles	98	10 sec
	63/65*	20 sec
	72	30 sec
final extention	72	5 min
hold	12	

* 63 degree for 5' junction PCR and 65 degree for 3' junction PCR

E & F. Validation of cell lines

This can be done by checking knockdowns of target gene expression via qRT-PCR or flow cytometry after a sgRNA delivery by lentiviral transduction.

a. qRT-PCR method to look at knock

Shown below are sgRNAs from Gilbert et al, 2014 Paper, which work well in K562 cell line. Plasmids/viruses are available from IGI. These are in pSLQ1371-BFP version.

TSS-I #	target	sgRNA protospacer sequence	CRISPRi qPCR	qPCR primer for	qPCR primer rev
1	SEC23B-1	GGTGCGGGACGACATCCGCA	0.048526549	CGATGCCACGTTACATCAAC	CTGTCCCAAGCATAACAGGT
17	ST3GAL4-4	GCCGCCGGTCCGGTGCGCCTAG	0.022929431	GATCACGCTCAAGTCCATGG	CTTGCCCAGGTCAGAAGGA
27	RAB1A-7	GTGTTCTAGGGACGGAGTA	0.081252632	CATTTGTGTGCACAATGC	ACAGCACAGAAGCCCCTCTA
32	SEL1L-1	GCAGGAAGAGCAGCGGCGAGG	0.007948288	GAGGGGAAAGTGCACAGA	GGTCAAAGCTGGTTCCGTA
37	SYVN1-3	GCACCGCGTCTGAGGTCTC	0.019770804	ACCAGATCCCTAGCTCAGA	TCCTCAGGCATCTCCTCTGT
38	CANX-10	GCGCCGAGTAAGAGAGAGG	0.00358756	GATCCAGACGAGAGAAACC	CATCCAGGAGCTGACTCACA
negative controls	Gal4-4	GAACGACTAGTTAGCGCTGTA			

b. Flow cytometry method to look at knockdown of cell surface marker expression

The protocol was established by the Martin Kampmann Lab. You need to identify a surface marker that is highly expressed in your cell line. We have been successfully used CD38 for Jurkat and E-cadherin for some adherent cells. The sgRNAs can be cloned into pSLQ1371-GFP version.

sgRNA-CD38-3: GGGCTGGGCGAAGATGAGGC

sgRNA-Ecadherin-2: GCTGGCCGGGACGCCGAGCG