Can CRISPR/Cas9 off-target genomic editing be avoided? Ways to improve target specificity.

Maxine Chen, PhD

GenScript
Make Research Easy

1. What is CRISPR?
2. How do we use CRISPR?
3. Avoiding off-target effects
4. Case study
5. GenCRISPR™ service

About GenScript

Gene Services

Peptide Services

Protein Services

Antibody Services

Discovery Biology Services

Catalog Products

Discovery Biology Services

Antibody Engineering

- Single domain antibody generation
- Antibody sequencing
- Affinity maturation and humanization

In-vitro Pharmacology

- CellPower™ custom stable cell line for assays
- Cell-based assays
- Ion channel and GPCR assays

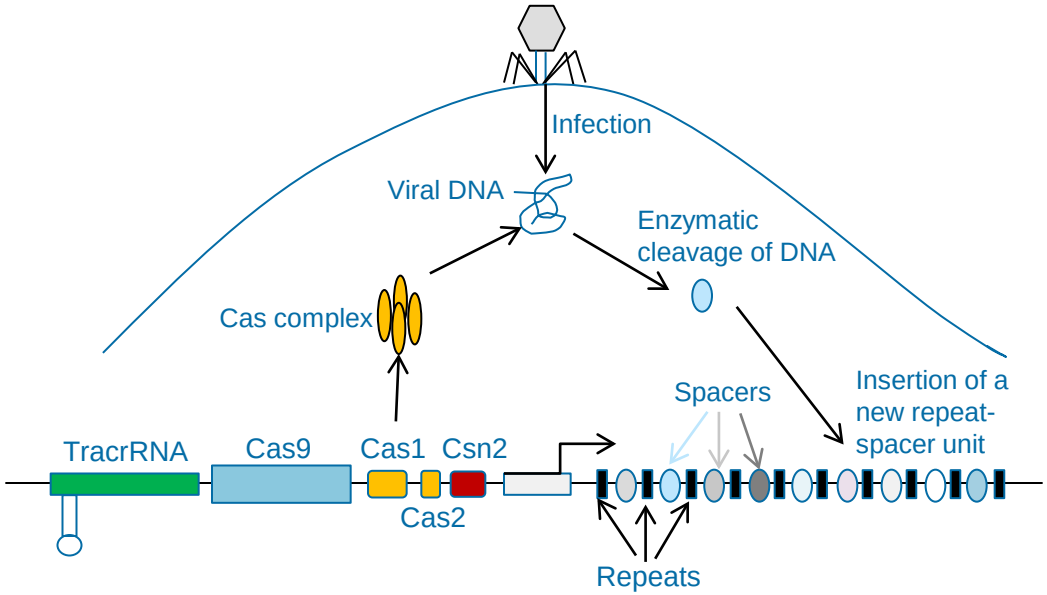
In-vivo Pharmacology

- Tumor models including SC xenograft, orthotopic and syngeneic
- Bioluminescence imaging of tumors
- Fibrosis models

What is CRISPR?

CRISPR – **C**lustered **r**egularly **i**nterspaced **s**hort **p**alindromic **r**epeats

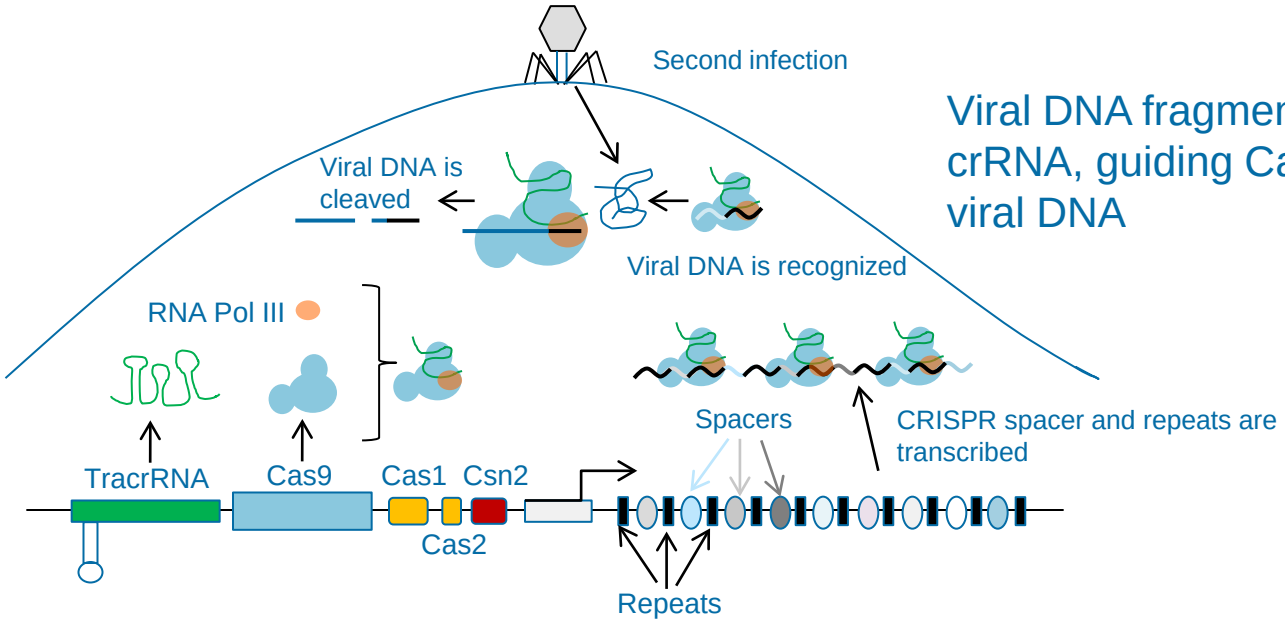
Cas9 – **C**RISPR **a**ssociated **s**ystem. RNA-guided dsDNA-binding protein that has nuclease activity



Fragments of viral DNA are stored in CRISPR locus

Adapted from: Mali P. et al. **Cas9 as a versatile tool for engineering biology**. *Nat. Methods* (2013), 10(10):957-963

What is CRISPR?



Viral DNA fragment now part of the crRNA, guiding Cas9 to the target viral DNA

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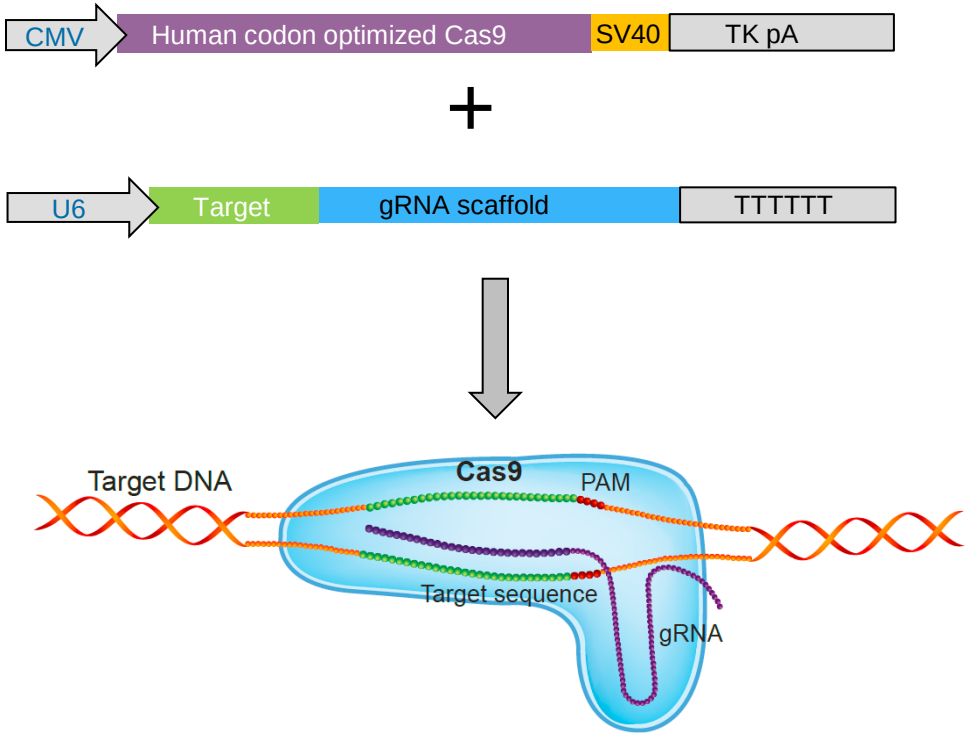
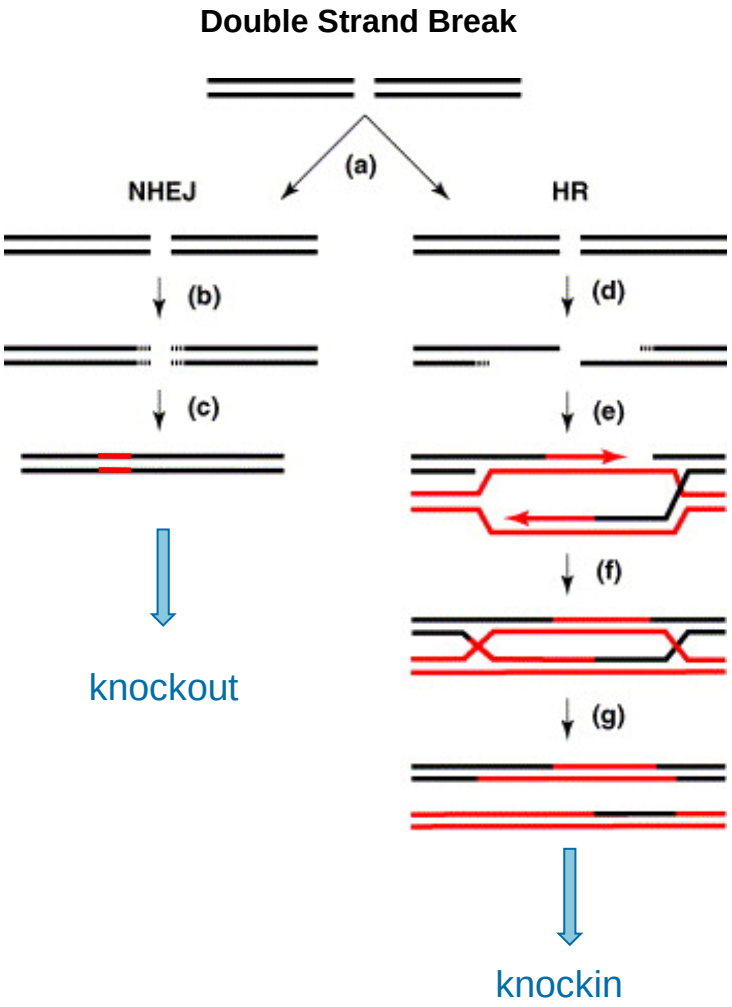
How is CRISPR Used in Mammalian Cells?

- Cas9: nuclease activity
- gRNA: targeting sequence



Adapted from:Mali P. et al. **RNA-Guided Human Genome Engineering via Cas9**. *Science* (2013), 339(823); DOI: 10.1126/science.1232033

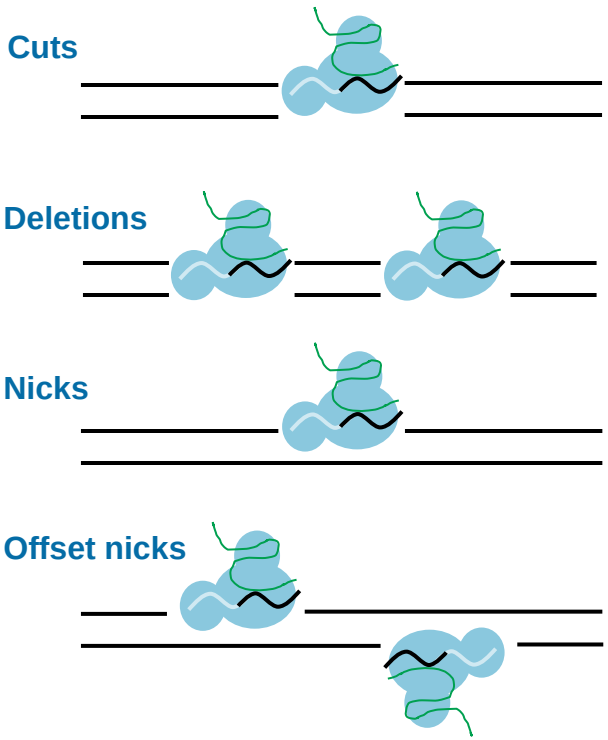
How is CRISPR Used in Mammalian Cells?



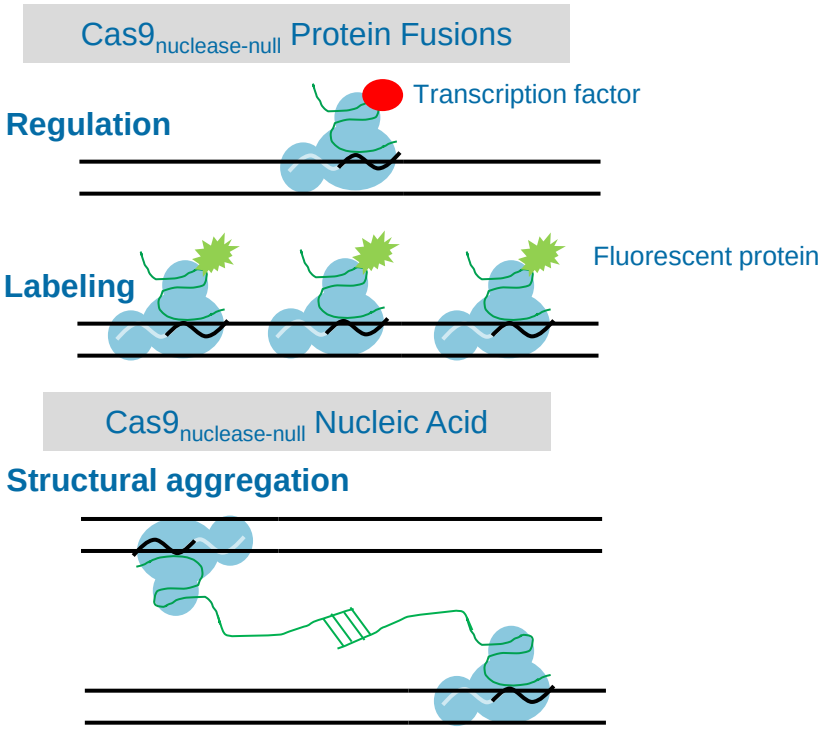
- Non homologous end joining (NHEJ) can generate a gene knockout
- Homologous recombination (HR) can generate a knock-in

Potential applications for CRISPR-Cas9

Genome editing

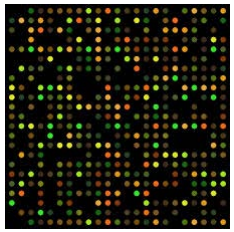


Genome regulation, reorganization and visualization



Adapted from: Mali P. et al. **Cas9 as a versatile tool for engineering biology**. *Nat. Methods* (2013), 10(10):957-963

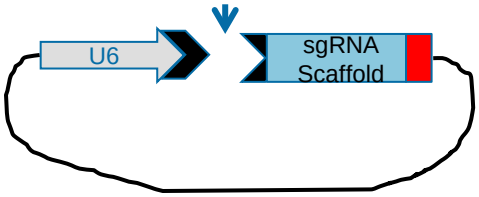
Multiplex Biological Screens



DNA arrays

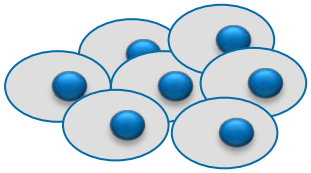
Harvest DNA, amplify and insert

Oligonucleotide spacer library

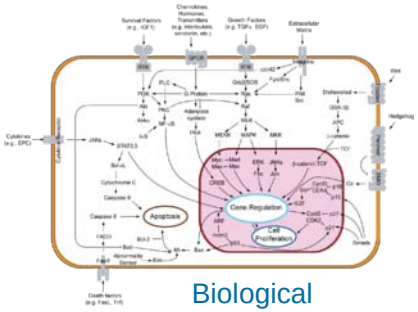


Deliver sgRNA libraries via viruses or nanoparticles

Cas9 cell line



Functional screens



Biological Pathways

- Generate libraries with hundreds of single gene knockouts
- Screen for functional alterations in pathways of interest

Mali P. et al. **Cas9 as a versatile tool for engineering biology**. *Nat. Methods* (2013), 10(10):957-963

Limitations with CRISPR-Cas9

- Since Cas9 induces double stranded breaks, any off target nuclease activity can cause mutations in those genes, leading to possible oncogenesis

- CRISPR/Cas9 can tolerate 1-3 mismatches in their target, which can lead to off target nuclease activity

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Enhancing Specificity By Modifying sgRNA Length

- Extension of guide sequence from 20-30 bp
  - Did not work because cells processed guide sequence back down to 20 bp

Ran AF. et al. **Double nicking by RNA-guided CRISPR Cas9 for enhanced genome editing specificity**. *Cell* (2013). 154:1380-1389

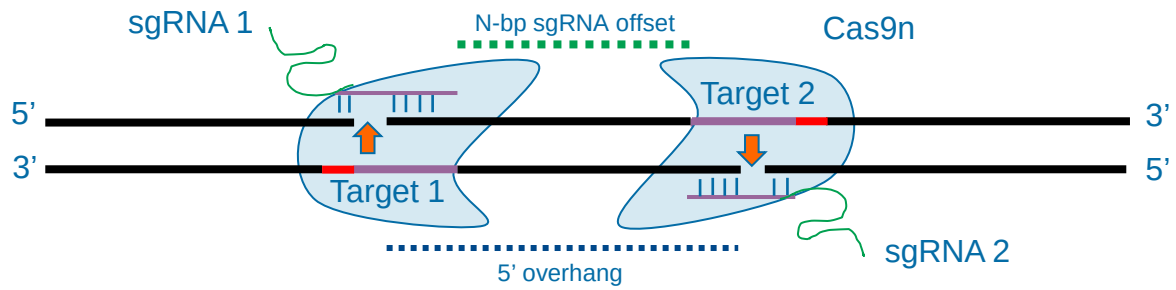
- sgRNA sequences can be 17-20 nt in length to achieve similar levels of on-target gene editing
- Up to 10,000 fold improvement in target specificity when truncated (17 or 18 base pair) sgRNA is used

Fu Y. et al. **Improving CRISPR-Cas nuclease specificity using truncated guide RNAs**. *Nat. Biotech*. (2014). 32:279-284

- Using a shorter sgRNA (17 or 18 nt) can greatly improve off-target specificity

Double Nicking Cas9n Complex

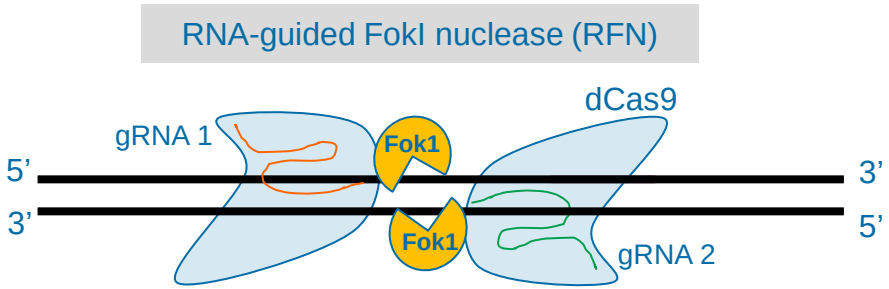
- D10A mutation on Cas9 allows for single strand nicking
- One sgRNA on each strand Cas9n would cause a single stranded break.
- Requires an sgRNA offset which generates a 5’ overhang

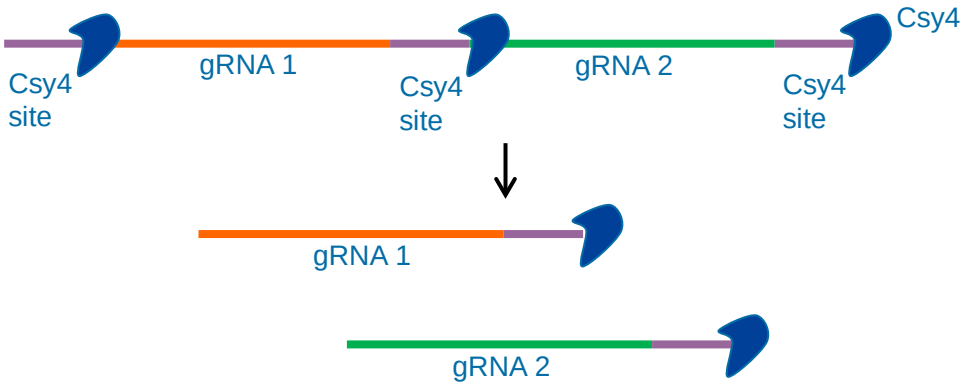


- Up to 1500-fold increase in specificity compared with wildtype Cas9 and single sgRNA

Adapted from Ran AF. et al. **Double nicking by RNA-guided CRISPR Cas9 for enhanced genome editing specificity**. *Cell* (2013). 154:1380-1389

RNA-guided Fok1 Nuclease





- FokI was fused to a catalytically inactive Cas9 (dCas9) mutant
- Addition of Csy4 site on gRNA sequence allows for two gRNAs to be transcribed and processed from a single expression cassette

Adapted from: Tsai SQ et al**. Dimeric CRISPR RNA-guided FokI nucleases for highly specific genome editing**. *Nat. Biotech.* (2014). 32:569-575

Single gRNA Fok1-dCas9 Has Less Mutagenic Activity

- Up to 10,000 fold less mutagenic activity of Fok1-dCas9 with single sgRNA
- Single Cas9 nickase can introduce point mutations at high efficiencies into their target sites

Tsai SQ et al**. Dimeric CRISPR RNA-guided FokI nucleases for highly specific genome editing**. *Nat. Biotech.* (2014). 32:569-575

Genome Wide Analysis of Off Target Sites

Methodology

- Cells transfected with HA-tagged dCas9 and 12 different sgRNA targeted to different chromatin states
- ChIP of HA-tagged dCas9 reveals different binding sites

PAM and Proximal Region

- Sequences proximal to PAM are preserved in off target sequences, indicating that these are important in dCas9 binding specificity
- Third position in the PAM site is most important, followed by the second and first

Chromatin region

- More than 30% of Cas9 off target sites are in open chromatin regions
- Off-target sites are concentrated in the promoter, 5'UTR and exon regions

Kuscu C et al. **Genome-wide analysis reveals characteristics of off-target sites bound by the Cas9 endonuclease**. *Nat Biotech.* (2014). doi: 10.1038/nbt.2916

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GenCRISPR™ Workflow

gRNA design (1–2 days)
Construct and Plasmid prep (2 weeks)

↓

Transfection with gRNA and Cas9 (3 days)

↓

Cell pool sequencing (2–3 days)

↓

Single clone generation (10x 96 well plates)
(4 weeks)

↓

Single cell clone sequencing (3 days) and
cell banking (20 days)

Total turnaround time: 13 weeks

Cost Analysis: DIY vs GenScript

3 months of postdoc salary: ~$13,360

CRISPR kit, plasmid prep and transfection materials: ~$1350

Cell culture reagents, including cell line: ~$1390

FACS sorting and sequencing:~$1050

Total cost: ~$17,150

GenCRISPR™ cell line service costs roughly half!!

Case Study: Development of a Glutamine Synthetase Knockout Cell Line

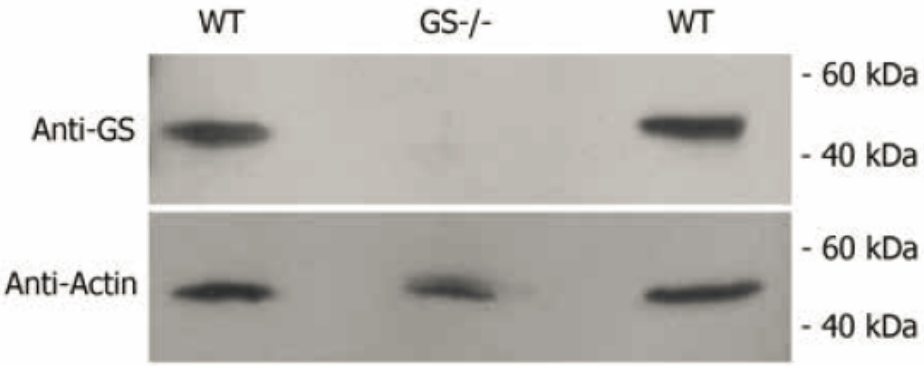
Figure 1. Deletion on GS allele causes frame shift mutation

```
Wild-type CAAATAGGACCCTGTGAAGGAATCCGCATGGGAGATCATCTCTGGGTGGCC
GS-/-     CAAATAGGACCCTGTGAAGGAATCCGCATGGGAGATCATCT--GGGTGGCC
```

- A sequence optimized gRNA was designed and synthesized to target a specific region on the GS allele. DG44 cells were transfected with the construct and the cell pool was analyzed by Sanger sequencing.
- Several hundred clones were derived from the cell pool and Sanger sequence analyzed. A single clone containing a frame shift mutation was carried forward.

Case Study: Development of a Glutamine Synthetase Knockout Cell Line

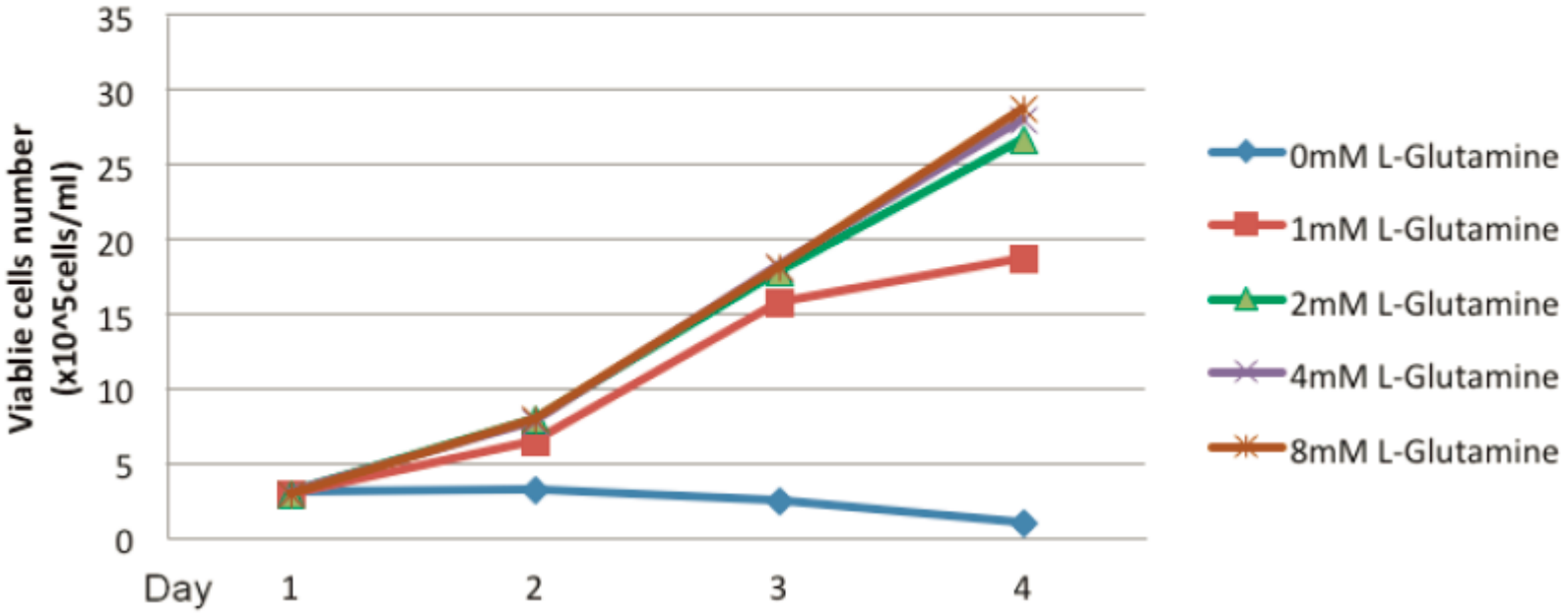
Figure 2. Glutamine synthetase is not detected by an anti-GS antibody in GS knockout cell lysate



- Western blot analysis shows that GS protein is not detectable in GS knockout cells

Case Study: Development of a Glutamine Synthetase Knockout Cell Line

Figure 3. L-Glutamine Dependence of DG44 (GS-/-)



- Functional analysis of GS knockouts show that the cells were unable to grow in the absence of glutamine

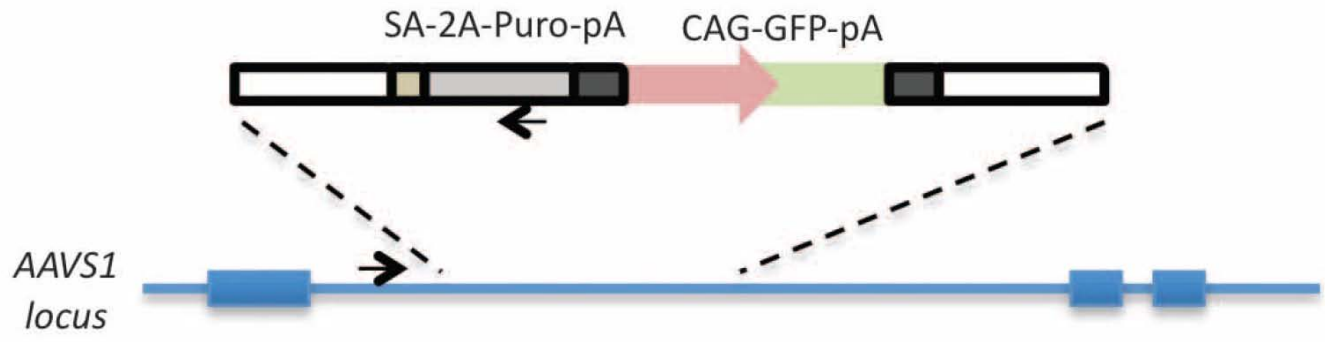
Case Study: Off-Target Validation of a GS Knockout Cell Line

Potential off-target site and alignment to gRNA-targeting site		Identity(%) in GS-KO clone
GS-T1	GTGTAAACGGATAATGGACATGG	100%
Cpne2	acccacaatgataatggacatgg	
GS-T1	GTGTAAACGGATAATGGACATGG	100%
LOC100768348	gtcaaacggataatggacatgg	
GS-T1	GTGTAAACGGATAATGGACATGG	100%
Klhl8	cggaatagacctaatggacatgg	
GS-T1	GTGTAAACGGATAATGGACATGG	100%
Ubap2	ggctctgttggtaatggacatgg	
Gene(intron)	tgttctttgtagaatggacatgg	
GS-T1	GTGTAAACGGATAATGGACATGG	100%
LOC100752546	taggacctagttaatggacatgg	
GS-T1	GTGTAAACGGATAATGGACATGG	100%
Entpd7	gacggtgggataatggacatgg	
GS-T1	GTGTAAACGGATAATGGACATGG	100%
LOC100754264	gtcctttggataatggacatgg	
GS-T1	GTGTAAACGGATAATGGACATGG	100%
Adamts1	tcattcctggataatggccatgg	
GS-T1	GTGTAAACGGATAATGGACATGG	100%
Lmbr1l	gtgtaaacggatttggggacaagg	
GS-T1	GTGTAAACGGATAATGGACATGG	100%
LOC100761973	accatggtggataatggacaggt	
GS-T1	GTGTAAACGGATAATGGACATGG	100%
LOC100750752	gtgattagtcaccaatggacatgg	

- gRNA targeting region sequence is blasted in NCBI, and top 11 off-target hits were identified
- Off target sites were Sanger sequenced in GS-KO clones: None of the top 11 off-target sites had mutagenesis

Case Study: Development of a Knock-in Cell Line

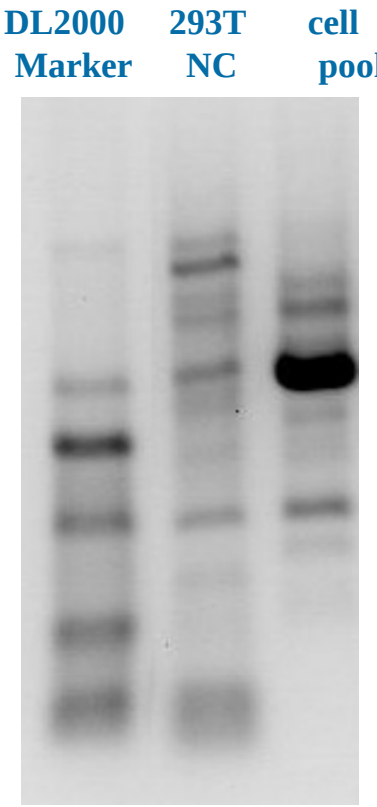
- **Homologous directed integration of Puro-GFP gene into native AAVS1 locus in HEK293 cells**

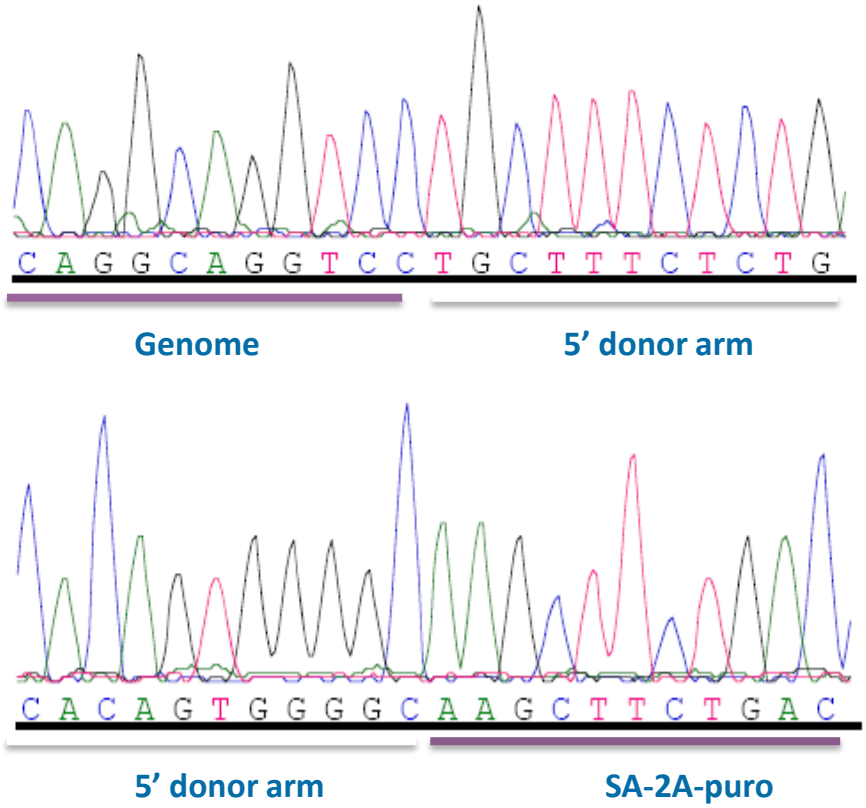


Mali P. et al. **RNA-Guided Human Genome Engineering via Cas9**. *Science* (2013), 339(823); DOI: 10.1126/science.1232033

- **gRNA was selected to target a specific region on the AAVS1 locus.**
- **Homologous repair template was designed to insert Puro-GFP into AAVS1 locus.**

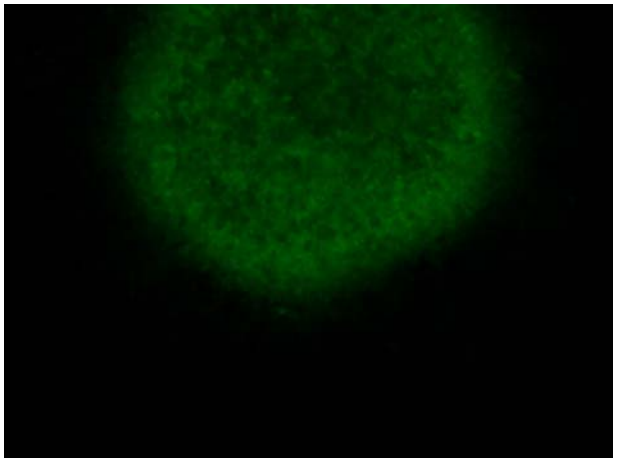
Case Study: Development of a Knock-in Cell Line

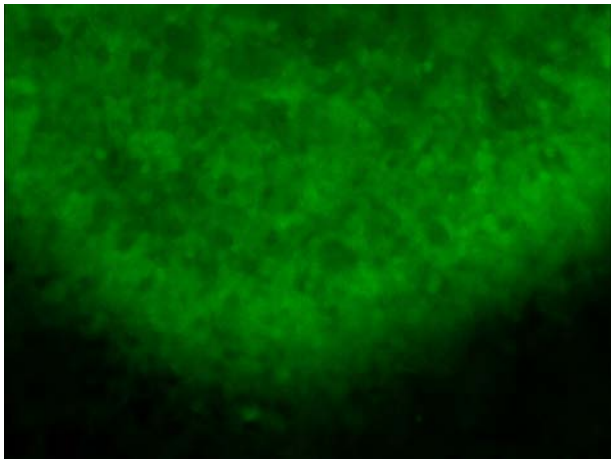




- HEK293 cells were transfected with the constructs and analyzed by PCR.
- A single clone containing Puro-GFP at AAVS1 loci was confirmed by sequencing of the PCR amplicon

Case Study: Development of a Knock-in Cell Line





- Cells were selected with puromycin for 2 weeks.
- Above is a representative GFP positive clone

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GenCRISPR™ Gene Editing Services

Custom Cell Line Service

Service Steps	Service Features	Optional Services
gRNA design for a single target gene and plasmid prep	**Turnaround time**: ~13 weeks **Deliverables**: Single clone, target sequence validated and detailed report	Additional target sequenced clones
Transfection and cell pool evaluation		Additional targeted genes Functional validation of a single clone
Single cell clone generation and validation		Off-target analysis using Sanger or next generation sequencing

What Sets GenCRISPR™ Apart?

Full service includes everything, from target gRNA design to single clone isolation and characterization (sequence validation) and wide variety of functional assays as well

Technology is licensed from a prominent institution

- Vectors used are the original licensed

We do not use additional reporter genes (ie. CD4, or eGFP), thereby maintaining integrity of pathways to be studied

Gene synthesis and cloning optimization completed in house, using industry-leading technology to ensure success

Clients have access to over 250 human tumor cell lines, and common cell lines (additional fees may apply)

In house expertise on a wide range of functional assays to analyze single clones (additional fees apply)

GenCRISPR™ Gene Editing Services

gRNA Construct Service

Service Steps	Service Features
Customer provides gRNA target sequence, or GenScript can design gRNA for a single target gene Synthesis and cloning into vector	**Turnaround time**: 10 days **Deliverables**: 4 µg of plasmid DNA for each gRNA construct. Final report with QC data.
Validation by PCR, enzyme digest and sequencing	

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Service Steps	Service Features
Customer provides gRNA target sequence, or GenScript can design gRNA for a single target gene Synthesis and clonin	**D**: 10 days **ables**: 4 µg of plasmid DNA for each gRNA construct. Final report with QC data.
Validation by R yme digest and sequencing	

Coming soon! In 1-2 weeks

Pricing starts at $159!!

Summary

- CRISPR Cas9 is an efficient and easy to implement form of genome editing
- CRISPR Cas9 can tolerate mismatches and generate off-target mutations
- Careful gRNA design, by truncating sequence to 17 or 18nt and picking sequences with fewer off-target mismatches
- Using Cas9n-Fok1 system can increase specificity
- No off-target mutations observed in GenCRISPR™ developed knockout cell line
- GenScript offers GenCRISPR™: a complete gene editing solution including custom cell line development and gRNA construct service

Thank you for your participation

We wish you all success in your research

Email me: Maxine.Chen@GenScript.com

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Optimizing conditions for recombinant soluble protein production in *E. coli* - *Keshav Vasanthavada*

June 26, 2014/ 8:00 am EST (2:00 pm CET)

Protein or peptide antigen: choosing the optimal immunogen for antibody production - *Liyan Pang, Ph.D.*