CRISPR-Cas9 Editing in Mammalian Cells

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2020-06-19
The origins of CRISPR-Cas9

https://doudnalab.org/research_areas/crispr-systems/
CRISPR as a genome editing tool

Guide RNA:
Your genome targeted sequence + RNA scaffold for Cas9 (crRNA)

Target sequence:
Complementary to gRNA sequence

Protospacer Adjacent Motif:
-NGG sequences found in genome

Bacterial enzyme from S. pyogenes (or other species)
How CRISPR genome editing works

Non-homologous end joining (NHEJ)

Use HDR for point mutations, adding sequences like affinity tags/RE sites into genome

(HDR happens at a much lower frequency than NHEJ)

NHEJ for knocking out genes

(NHEJ discussed from now on)
• What is the genomic structure of my gene?
• What is the best cell line to use? (human or mouse?)
• What is the best way to deliver my guide RNAs for my cell line?

Best case scenario for CRISPR:
• Genomic region is highly unique with few SNPs
• Transfection method has high efficiency in cell line
  • Cell line has high rates of DNA repair
  • Cell line grows fast in simple media
## Technical considerations: Choosing the best* cell line

<table>
<thead>
<tr>
<th>Well-behaved</th>
<th>More annoying</th>
<th>Hall of Shame</th>
</tr>
</thead>
<tbody>
<tr>
<td>&gt;85% transfection efficiency</td>
<td>OK transfection efficiency</td>
<td>Low transfection efficiency</td>
</tr>
<tr>
<td>Fast growing</td>
<td>Slower/less robust growth</td>
<td>Delicate/slow growing</td>
</tr>
<tr>
<td>Robust &amp; easy to maintain</td>
<td></td>
<td>Low rates of genomic repair</td>
</tr>
</tbody>
</table>

### Well-behaved
- HeLa
- HEK293(T)
- NIH3T3
- C2C12
- RAW264.7
- CHO

### More annoying
- HL-60
- H4
- K562
- SH5Y
- HepG2
- Lymphoblast
- Neuron
- Leukocytes
- Neuron
- Liver

### Hall of Shame
- THP-1
- Jurkat
- Primary cells
- Stem cells
- Monocyte
- T cells
- -
- -

### Other considerations!!
- **Adherent vs. suspension cells?**: either works, suspension is usually easier (esp. for large scale culture)
- **Is there karyotyping data** available for my cell line? (Cell lines can be aneuploid -> more alleles to KO)
- Does your desired cell line express the pathways you need?  
  - *ex.* Some cell lines (e.g. HeLa) don’t have IFN response  
  - *ex.* Some cell lines (e.g. CHO-K1) don’t glycosylate proteins
- If the cell line background you’re using is previously engineered, **does it need to be maintained in Abx?**

*completely unbiased & authoritative rating system from yours truly*
How should I introduce my gRNAs?

<table>
<thead>
<tr>
<th></th>
<th>Plasmid transfection</th>
<th>Ribonuclear proteins</th>
<th>Cas9 mRNA + synthetic gRNA</th>
<th>Lentiviral transduction</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>When to use this</strong></td>
<td>Try this first 😊</td>
<td>- If plasmids don’t work well</td>
<td>For cell lines that are highly resistant to transfection</td>
<td>- For stable expression of Cas9 + gRNA</td>
</tr>
<tr>
<td></td>
<td>Optimized conditions already exist for many cell lines</td>
<td>- Can improve transfection efficiency for tricky cell lines</td>
<td>- Cell lines that are resistant to transfection</td>
<td></td>
</tr>
<tr>
<td><strong>Drawbacks</strong></td>
<td>- Efficiency decreases with plasmid size</td>
<td>- Have to continually make/buy Cas9 protein</td>
<td>- $$$</td>
<td></td>
</tr>
<tr>
<td></td>
<td>- Cas9 lingers in cells -&gt; off targets</td>
<td>- Need to directly synthesize gRNA</td>
<td>- Requires careful RNase-free handling &amp; storage</td>
<td></td>
</tr>
<tr>
<td></td>
<td>- Need to directly synthesize gRNA</td>
<td></td>
<td>- Time consuming</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>- Requires add’l EHS biosafety classification</td>
<td></td>
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<tr>
<td><strong>Reagents</strong></td>
<td>Cationic lipid (e.g. Lipofectamine) or Electroporation system + buffers (e.g. Neon system)</td>
<td>Cationic lipid or Electroporation system</td>
<td>- Synthego (buy gRNAs)</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>- In vitro transcription kits (make gRNAs)</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>- Invitrogen (Cas9 mRNA)</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>- Lipid reagent for delivery</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>HEK293T cells, lentiviral vector</td>
<td></td>
</tr>
</tbody>
</table>

*(Plasmid transfection discussed from now on)*
Generalized CRISPR workflow

**gRNA CLONING**
- Sequence region of interest
- gRNA design
- Cloning

**TRANSFECTION OPTIMIZATION & gRNA VALIDATION**
- Transfect gRNA constructs individually to small batch of cells
- Sort GFP+ by FACS
- Surveyor T7E1 assay

**TRANSFECTION & SORT**
- Transfect 2 gRNA constructs together
- Sort cells into 96-well plates/pool
- Validate gRNA activity in pool with Surveyor assay

**SCREEN & VALIDATE CLONES**
- Propagate clonal cell lines
- PCR of gDNA for KO
- Orthogonal validation of KO

**FUNCTIONAL CHARACTERIZATION**
- METABOLOMICS!! CHEMISTRY!!
gRNA cloning

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### Consider the following for your target:

<table>
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<th>Resource</th>
</tr>
</thead>
<tbody>
<tr>
<td>Is the gene expressed in your cell line of interest?</td>
<td><a href="https://www.proteinatlas.org">Human Protein Atlas</a> (thanks Jaymin!)</td>
</tr>
</tbody>
</table>
| Is this gene alternatively spliced? / Are there multiple mRNA isoforms of this gene? | NCBI Genome Browser for your gene [UCSC Genome Browser](https://genome.ucsc.edu)  
  Avoid using gRNAs that are on exons that get spliced out  
  Avoid putting gRNAs on introns |
| Are there SNPs in your gene of interest?                                | [UCSC Genome Browser](https://genome.ucsc.edu)  
  Avoid using gRNAs that overlap with SNP-heavy regions  
  [SEQUENCE THE AREA OF INTEREST FROM GENOMIC DNA](https://www.ncbi.nlm.nih.gov) |
| Where are the active domains/catalytic residues/binding sites for your protein? | [UniProt](https://www.uniprot.org) |
| Are there pseudogenes/paralogs of your gene in the genome?              | BLAST your gene sequence  
  If present: requires nested PCR protocol for screening  
  Ensure that gRNA target sequence is unique |
| Are there areas of repetitive sequences?                                | Avoid placing gRNAs in repetitive regions |
gRNA Design Parameters

• Select gRNAs in pairs, a minimum of 2 gRNAs per gene of interest
  Space them roughly ~2 kb or less apart
  Design PCR primers >200-300 bp upstream and downstream respectively of gRNA pair

• Place gRNAs on **exons** if possible (earlier exons are best)
  Alternatively: target a specific domain of protein

• Balance between on-target and off-target score
  Highest on-target (Doench) score > 65
  Highest off-target (MIT) score > 65

**IMPORTANT:**
Sequence your genomic region of interest to make sure your gRNA sequence is actually there in the genome!
NOT ALL gRNAs WILL INDUCE CUTTING WITH CAS9!
Designing (& validating) multiple gRNA pairs per gene is optimal to save you time and sadness

https://benchling.com/
Example guide & primer design

- gRNAs
- Whole KO primers
- Surveyor primers

Figure 3: PCR to Identify Clones Containing Deletions

- Wild-type allele
- Deletion allele

PCB primers should flank region to be deleted
Alternative strategy: target catalytic residues

Catalytic triad residues

→ gRNAs

Whole KO primers

Surveyor primers

NATIVE GENOME

Cas9-TREATED GENOME

Catalytic domain
1. Order gRNAs as oligos from Keck & resuspend

2. Phosphorylate oligos

3. Denature & reanneal oligos

4. Golden Gate cloning strategy: BbsI digest and T4 ligation simultaneously

Addgene vector 48138
Modifying gRNA sequences for Keck oligo order*

* instructions apply to gRNAs cloning into pX458 plasmid

1) Copy sequence of gRNA and reverse comp. sequence
2) If seq. does NOT start with G, add one at the 5’ end and add a C at the 3’ end of the reverse comp. seq.
3) Add BbsI site overhangs CACC before your gRNA sequence
   Add AAAC before the reverse complement of that same gRNA sequence

You will need to order:

- Forward sequence of each gRNA modified as above
- Reverse complement of each gRNA
- Set of primers around each gRNA locus only (Surveyor primers)
- Set of primers for detecting the entire knockout (Whole KO primers)
- Sequencing primer for gRNA in plasmid
  (5’-CGTAACTTGAAAGTATTTCGATTTCTTGGC-3’ for U6 promoter)

Example:
Sample gRNA sequence: 5’-ACGCTGAACATGAAGCACCC-3’
Output:
gRNA sequence 5’-CACCACGCTGAACATGAAGCACCC-3’
rev. complement of gRNA 5’-AAACGGGTGCTTCATGTTCAGCGTC-3’
Added G and C + BbsI site compatible overhangs

BLAST YOUR PRIMERS USING NCBI PRIMER BLAST BEFORE ORDERING!
This checks for nonspecific amplification of different areas in the genome
Messy PCRs make gRNA validation difficult
Test transfection & validate gRNAs

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<table>
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<th>Day</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>-2 or -1</td>
<td>Seed cells in 6-well plate Plate 1 well per gRNA construct + 1 well for ‘no DNA’ neg. control</td>
</tr>
<tr>
<td>-1</td>
<td>Change media on cells to culture media <strong>WITHOUT antibiotics</strong> * Abx reduces culture viability with Lipofectamine</td>
</tr>
<tr>
<td>0</td>
<td>Transfect cells with Lipofectamine according to manufacturer’s optimized protocol <strong>Cells should be 80% confluent for highest efficiency</strong></td>
</tr>
<tr>
<td>1</td>
<td>Change media on cells to regular culture media Check GFP+ fluorescence of culture</td>
</tr>
<tr>
<td>2-4</td>
<td>Check GFP+ fluorescence of culture Sort GFP+ cells (helps if transfection efficiency is low)</td>
</tr>
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</table>
Validation of gRNAs: Surveyor Assay

**Purpose:** Test whether your gRNA induces cutting at the desired locus when expressed with Cas9

1. Isolate gDNA from >50k cells
2. PCR
3. Denature
4. Anneal
5. Digest with T7E1

Run gel (ALWAYS RUN UNDIGESTED DNA ALONGSIDE YOUR CUT SAMPLES)

Source: Addgene
Transfect & Sort clones

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Transfection: the real deal

1. Transfect (2) validated guide constructs in same mix

2. ~2-4 days

3. Sort top 5% intensity of GFP+ cells

4. Prepare (4-5) 96-well plates:
   - Many wells never grow!
   - Some wells accidentally get >1 cell (not clonal) -> trash

   Also sort small pool (>100k cells):
   - Extract gDNA -> validate gRNA cutting with Surveyor assay
   - Find out ASAP if transfection was unsuccessful

WAIT TIME: 3 – 8 weeks

- Do that other experiment you put off
- Change culture media 2-3x week
- Cross out wells with >1 cell cluster or contamination as cells grow

Screen clones by PCR for KO

Passage into 24-well plate, then 6-well
Screen & validate clones

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Screening for clones for KO

- **Figure 3: PCR to Identify Clones Containing Deletions**

  - **gRNAs**
  - **Whole KO PCR primers**
  - Extract gDNA
  - PCR

  - **control**
  - **clone1**
  - **clone2**
  - **clone3**
  - **clone4**
  - **clone5**
  - **clone6**
  - **clone7**
  - **clone8**
  - **clone9**
  - **clone10**

  - **Wild-type allele**
  - **Deletion allele**

  - PCR primers should flank region to be deleted

Source: Addgene
Orthogonal validation of your KO clones

- qRT-PCR
- SDS-PAGE:
  - Western blot for your protein
  - Coomassie blue stain for absence of band
- Flow cytometry:
  - Antibody against your protein of interest
- RNA-seq:
  - Check for mutations in off-target locations

(Caveat: Have to ask the grant reviewers very nicely)

After this, it’s finally time for chemistry!
• I only get heterozygous KOs: Can I just transf ect them with the same gRNAs again to get homozygous KOs?
Often the PAM recog. sequence for Cas9 gets mutated during genome editing for much less efficient editing the second time. (Maybe homozygous KOs are lethal?)

• Why can’t I just transf ect 1 gRNA and PCR/sequence the area around the gene?
The sequencing will be messy because there are multiple alleles: i.e. heterozygous KO’s will have multiple overlapping peaks that will be hard to read. This screening protocol is high throughput and robust.

• Will unintentional gene editing by HDR also happen with this protocol? - YES but at a low frequency. NHEJ vs HDR depends on the relative expression of enzymes from either pathway in your cell. HDR will not be useful unless you provide an exogenous DNA template for the cell to use as a reference for repair: thus if HDR happens, the cell will take the remaining WT allele as a template and effectively remove your mutation. This would look like a WT band on your gel during screening. (Transfecting 2 gRNAs also reduces the likelihood that this would be a problem).
• Benchling
  • CRISPR tool overview
  • Primer design in Benchling

• Addgene
  • CRISPR 101
  • Detailed protocol for mammalian cells
  • Validating your KO

• IDT
  • The case for using Cas9 protein over plasmid delivery

• Invitrogen
  • See if your transfection protocol is already optimized with Lipofectamine for your cell line [here](#)
Thanks for your attention!

The CRISPR queen herself (!!!)