CRISPR knock-in strategy:

**Rules for sgRNA design:**

1. Use 20-mer sgRNA
2. PAM sequences
	1. *S. pyogenes* Cas9 PAMs: NGG
	2. xCas9 PAMs: NGG, NGA, NGC, NGT, GAA, GAT
3. Chose guide sequences with
	1. least number of off-targets
	2. GC content of sgRNA of 40-60%
	3. corresponding PAM motif
	4. high Cas9 loading score if possible

**CRISPR sgRNA design:**

1. Obtain annotated gene:
	1. <https://www.ncbi.nlm.nih.gov/gene>
	2. Click on primary assembly, Genbank (should open new page)
	3. Copy all information, paste in text edit file
	4. Save as plain text
	5. Save as “.gbk” file type
	6. Open with SnapGene program
2. Finding guide RNA
	1. <https://crispr.ybzhao.com>
	2. Use parameter hg19 for human cells to get proper off target locations
	3. Use guide RNA that cuts close to mutation site



* 1. Synthesize sgRNA primers for easy cloning
		1. 5’ – CACCGNNNNNNNNNNNNNNNNNNN – 3’
		2. 3’ – CNNNNNNNNNNNNNNNNNNNCAAA – 5’
	2. Guide RNA will have a G at beginning of sequence (added by CRISPR tool)
1. Verify that guides have unique genomic binding site (Blast)

**Design construct**

1. For mutagenesis
	1. Use common codon usage
		1. <https://www.kazusa.or.jp/codon/cgi-bin/showcodon.cgi?species=9606&aa=1&style=N>
	2. Codon optimization tool
		1. <http://www.idtdna.com/codonopt>
	3. If possible, add restriction site to make confirmation easier
		1. <http://watcut.uwaterloo.ca>
2. Mutate site corresponding to gRNA and/or PAM in knock-in construct so Cas9 won’t cut (codon optimized)
3. Check mouse models
	1. <http://omim.org>
4. To knock in, create homology arm using genomic DNA sequences
	1. Use a two-primer set approach, one set for left arm and one for right
	2. The more internal primer should carry the mutation/tag/etc.

**Designing homology arms:**

Peng et al. Potential pitfalls of CRISPR/Cas9‐mediated genome editing

<https://febs.onlinelibrary.wiley.com/doi/full/10.1111/febs.13586>

Richardson et al. Enhancing homology-directed genome editing by catalytically active and inactive CRISPR-Cas9 using asymmetric donor DNA

* Overlap the Cas9 cut site with 36 bp on the PAM-distal side, and with a 91-bp extension on the PAM-proximal side of the break
* Protect DNA ends with two phosphorothioate modifications on each end N\*N\*[N]n\*N\*N
* Use donor DNA from non-target strand (not-PAM strand)



Liang et al. Enhanced CRISPR/Cas9-mediated precise genome editing by improved design and delivery of gRNA, Cas9 nuclease, and donor DNA

* Good comprehensive paper with optimizations
* For small changes such as SNPs or short insertions, asymmetric single stranded donor molecules with 30 base homology arms 3′ to the insertion/repair cassette and greater than 40 bases of homology on the 5′ end seems to be favored

Renaud et al., *Cell Reports* 2016, Improved Genome Editing Efficiency and Flexibility Using Modified Oligonucleotides with TALEN and CRISPR-Cas9 Nucleases

* Phosphorothioate modified ssODNs have improved efficiency and flexibility
	+ http://blog.biosearchtech.com/know-your-oligo-mod-phosphorothioate-bonds
* ssODN mediated genome editing likely not by classical HR, but rather two steps of single-strand annealing
* Cas9 cuts 3-4bp upstream of PAM sequence
* For small changes (<50bp) use single stranded DNA (ssDNA, ssODN)

**Cloning sgRNA into vector:**

1. Digest 5ug of plasmid with BbsI for 3h-ON at 37°C:

5 ug Plasmid

2.5 ul BbsI-HF (add 50 U, enzyme is at 20 U/ul)

5 ul 10X Cutsmart Buffer

X ul ddH2O .

50 ul total

2. Phosphorylate digested DNA for 90m at 37°C with alkaline phosphatase

50 ul digest

1-2.5 ul CIP

3. Gel purify digested plasmid using QIAquick Gel Extraction Kit and elute in EB.

4. Phosphorylate and anneal each pair of oligos:

0.75ul oligo 1 (100uM)

0.75ul oligo 2 (100uM)

1ul 10X T4 Ligation Buffer (NEB) \*with ATP added (if buffer is old, can add fresh ATP)

6.5 ul ddH2O

1 ul T4 PNK (NEB) .

10 ul total

Phosphorylate and anneal in a thermocycler using the following parameters:

37oC 30 min
95oC 5 min and then ramp down to 25oC at 5oC/min

\*T4 PNK heat inactivates at 65oC 20 min, if annealing is done followed by incubation with PNK, add in a heat activation step

5. Dilute annealed oligos from **Step 4** by adding 90 ul ddH2O

6. Set up ligation reaction and incubate ON 16oC or RT 10 min followed by heat inactivation at 65oC 10 min: (Alternative: use QuickLigase 5 min RT)

X ul BbsI digested plasmid from step 2 (50ng)

1 ul phosphorylated and annealed oligo duplex from step 4 (dilution)

1 ul T4 DNA Ligase buffer

1 ul T4 DNA Ligase (NEB)

X ul ddH2O .

10 ul total

7. (optional) Treat ligation reaction with PlasmidSafe exonuclease to prevent unwanted recombination products:

11 ul ligation reaction from step 5

1.5 ul 10X PlasmidSafe Buffer

1.5 ul 10mM ATP

1 ul ddH2O

15 ul total
Incubate reaction at 37C for 30 min.

8. Transformation STBL3

Mix 1-2.5 ul DNA with 20 – 50ul competent cells on ice, incubate 20 – 30 min

Heat shock 42C 1 min

Place on ice 2 min

Add SOM (media)

Incubate 37C for 40 – 60 min depending on resistance

9. Miniprep two colonies per sgRNA

10. Confirm successful cloning via sequencing

\*make sure cell line to be used is able to grow from single cell clones

**Nucleofection of cells with Lonza Nucleofection SF:**

1. The day before: split and amplify cells, 2-5 x 105 cells/ml (for suspension cells)
2. The next day: prepare 12 well plates with 2.5 ml/well of media, place in incubator to prewarm
3. Count, spin down at 90g for 10 minutes, and wash cells with PBS
4. Use 1 million cells per condition resuspended in 20 ul SF nucleofection reagent (don’t do too many at a time)
	1. Add 1ug sgRNA plasmid DNA
	2. Add 1ug ssODN donor (test different concentrations)
	3. Do 3 wells when generating a knock-in cell line
5. Pipette cells into 16-well Nucleocuvette Strip
	1. Avoid bubbles
6. For DG75 cells, nucleofect with Custom program designed for CH12 cells
7. Add 100 ul of media to each well and allow recovery for 1 minute
8. Pipette each well into a well of 12-well plate with specialized plastic disposable pipettes, washing the nucleofection well with media to get maximum recovery
	1. Include one well of non-transfected cells
9. Incubate for 24-48 hours before recovering cells

**Test sgRNA efficiency:**

Perform either T7E1 ampligase assay or double cutting efficiency

1. Nucleofect cells as described
2. Pick up cells
	1. Cells will be at bottom of well, can remove media on top then pick up cells in 1.5 ml tube
	2. Don’t need to count nor PBS wash cells
	3. 2000 rpm 5 min to pellet cells
3. Extract genomic DNA
	1. DNeasy Quiagen, elute in 50 ul EB or water
4. PCR with primers that only amplify double cutter
	1. 50 ng of DNA in 20 ul reaction, 35 cycles (if 1 clone is used, use 10 ng DNA)
	2. also include gDNA without sgRNA to make sure no amplification
	3. can also include WT primers

**Selecting clones:**

1. Sort for GFP positive clones
	1. Prepare flat bottomed 96 well plates with 200 ul warmed media, use multichannel pipette
	2. Spin down and concentrate cells, resuspend in media
	3. Filter cells into FACs tube
	4. Include control of non-transfected cells
2. After a few days, check and make sure colonies in each well are growing from one clone only
3. Grow sorted clones for 1-2 weeks before picking
4. Pick clones and test
	1. Take majority of well for gDNA extraction, plate one drop in fresh 96-well plate
	2. Perform PCR
	3. Sequence positive clones
5. Expand and freeze clones
	1. Either plate format (with parafilm) or in tubes

***ssDNA-specific nuclease treatment to eliminate residual ssODN donors and avoid PCR artifacts.***Nuclease treatment was performed when necessary before the PCR reaction to eliminate residual ssODN and potential PCR artifacts. Three ssDNA-specific nucleases, Mung Bean, S1 and Bal31 nucleases, were used at 0.5 or 5U, 10 minutes at 25°C in 10μL containing 5μL genomic DNA at 150 ng/μL. The digestion was then purified using EZNA cycle pure kit (Omega Biotek) and 50 ng ssDNA nuclease-treated genomic DNA used in a 25 μL PCR reaction.

S1 nuclease: Inactivated by heating at 70 °C for 10 min in the presence of EDTA.

Exo1:

Mung bean nuclease: can’t heat inactivate

Enzymatic PCR Cleanup Protocol

 Add 0.5 μl of Exo I and 1 μl of rSAP to 5 μl of PCR product.

(Note that the Quick Dephosphorylation Kit (NEB #M0508), which contains Quick CIP, can be used in the same volume.)

Incubate the mix at the 37°C for 15 minutes.

Inactivate both enzymes at 80°C for 15 minutes.

PCR products are ready for downstream application.

Amount of ssDNA to use:

12 ug

Dilute ssODN to 3 uM in water, want final [] of 18nM

-some ppl use 1 ul of that for transfection

-one person uses SF kit like us, for 200,000 cells adds 1 ul of 10 uM ssDNA (this is 0.429 ug)

For gene editing using ssODNs, 1 × 107 cells were electroporated with 7.5 μg of each ZFN encoding plasmid and 15 μg of pEGFP-N1 (Clontech) together with 30 μg of ssODNs (5′-GACTTATGTCTTGAATTTGTTTTTGTAGGCTCCAAAACCAAGA AGGGAGTGGTGCATGGTGTGGCAACAGGTAAGCTCCATTGTGCTTATATCCA AAGATGATATTTAAAGTAT-3′; Integrated DNA Technologies, Iowa).

 =>1 × 106 cells, 3ug

10 pmol

2.7um

\*try same amount of ug of ssODN as plasmid DNA (1 ug)