Design and cloning gRNA oligo's into Cas9 vectors from F. Zhang Lab

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Reagents:

Plasmids: pX459, etc.
Guide RNA oligos, designed with Bbs I overhangs, from Feng Zhang http://tools.genome-engineering.org and Shirely Liu's lab (http://crispr.dfci.harvard.edu/SSC/)
Bbs I (NEB R0539S)
Low melt agarose
T4 PNK (NEB M0201S)
High Conc T4 DNA ligase (NEB0202T)
rSAP (NEB M0371S)
T4 DNA Ligase Reaction Buffer (NEB B0202S)
One Shot Stbl3 competent *E.Coli* (Life Tech C7373-03). These are the safe choice, but regular competent bacteria seem to work as well.
Ampicillin, LB, LB-Agar Plates containing 100µg/mL Amp
Sequencing Primer (U6 derived, #1393, GAGGGCCTATTTCCCATGATTCC)

Prior to Starting

1) This protocol is derived from **Ran FA *et al*, Nature Protocols, 2013**. While not identical, it follows it closely. Read that paper for details, as well as check out the genome-engineering.org website. Addgene (addgene.org), where many genomic editing plasmids are deposited, has a number of useful tools, guides, and protocols as well.
2) Design sgRNA oligos (fwd and rev) and order along with U6 fwd primer (standard desalted value oligos at 50N scale of synthesis work fine).
3) Once arrived, dilute oligos to 100 µM in water; store at -20 ^{o}C.

gRNA Design

1) Identify the genomic region you want to target. Get the DNA sequence of the region you wanted targeted. For simply knocking out a gene, I usually identify the second or third coding exon. Targeting the first coding exon can be problematic, since you will make an indel near the initiator ATG- then you can often times get initiation at an internal ATG which will prevent you from making a frame-shift. This can be easily done using the UCSC genome browser (genome.ucsc.edu) if it is a common genome.
2) Use the CRISPR design tool from Shirley Liu's lab. (http://crispr.dfci.harvard.edu/SSC/). It tries to identify gRNAs with the optimal sequence to maximize double stranded breaks (DSBs) while minimizing the off-target effect.
3) Remember to add in the appropriate sequences for cloning into vectors (See Figure 1).
4) Refer to Ran *et al*, *Nature Protocols*, 2013 for details

Screening for editing

1) It is very hard to discuss ALL the possible screening strategies. The T7 assay can be used, which measures non-specific indel formation using a PCT based approach. To design primers for this, I find the genomic region targeted by my gRNA9(s)- typically 50-100bp. I then add on 400-500bp on either side of genomic sequence (from UCSC genome browser) and use the NCBI Primer design tool (www.ncbi.nlm.nih.gov/tools/primer-blast/ ; 60 ^{o}C melting temp, oligo size 18-25 bp) to find the best possible oligo that will contain the indel region AND PCR up a 500-800bp band for agarose gel visualization.
2) Refer to Ran *et al*, *Nature Protocols*, 2013 for details

3) An alternative is to use PAGE electrophoresis on small PCR products (100bp) to detect small changes in size.
4) A final screen is to perform PCR and then sequence the PCR product and simply look for what % have indels. This can be done with TIDE (http://tide.nki.nl), but naturally does not run at the BRI as far as I can tell.

gRNA cloning

•Assumes you are cloning into a px Vector from Feng Zhang or LentiCrispr_v2. Otherwise, will need to check yourself.

Day 1:

1) Digest 3-5µg of the appropriate vector (px459 as an example) in 50µL reactions in 0.2 mL PCR tubes:
   X µL vector
   5 µL 10x NEB Cut Smart Buffer
   2-3 µL 5000U/mL BbsI
   Water to 50 µL

2) 37 ^{o}C for ~3 hours; heat inactivate at 65 ^{o}C for 20 min
3) Dephosphorylate cut vector:

   50 µL BbsI digested vector
   1-2 µL rSAP (any SAP works)
   -incubate in thermocycler at 37 ^{o}C for 30-60 min
   -heat inactivate in cycler at 65 ^{o}C for 5 min if stopping, otherwise load on gel
4) Pour as small and thin a gel as possible for purifying vector (0.7% agarose with TAE)
5) Make sure 6x loading buffer is pH >6.0-8.0; add loading buffer to vector to 10% of volume of sample (ex.: 3 µL buffer to 30 µL sample)
6) Load on gel filling well(s) with as much vector as possible; you should see only one clean band – let it run 1/3 to halfway down the gel. **While gel is running, anneal oligos (step 11).**
7) Carefully, cleanly, and quickly excise band with as little excess agarose as possible in less than 45 seconds.
8) Gel purify the linearized vector using standard IBI kit
9) Quantify gel purified DNA with Qubit Broad Range.
10) Anneal sgRNA oligos:
    a. Mix the following reaction:

sgRNA fwd (100 µM)	1 µL
sgRNA rev (100 µM)	1 µL
10x T4 DNA ligation buffer	1 µL
T4 PNK	1 µL
ddH_20	6 µL
	10 µL

    b. Phosphorylate and anneal oligos in a thermocycler using the following program:

       37 ^{o}C for 30 min
       95 ^{o}C for 5 min
       Ramp down to 25 ^{o}C slowly (0.1 ^{o}C/sec)
       25 ^{o}C for 5 min

c. Dilute phosphorylated and annealed oligos 1:200 (ex.: 1 µL annealed oligos to 199 µL water). Save on ice.

11) Ligate BbsI cut, gel purified, dephosphorylated vector and phosphorylated, annealed oligos using Quick T4 DNA ligase; also, do a vector only (NO oligo) ligation control to ensure the vector was adequately digested and dephosphorylated:
    25-50 ng of BbsI cut, gel purified, dephosphorylated vector
    2 µL of annealed Oligo's
    1 µL of 10x DNA ligase Buffer
    1 µL of High Conc T4 DNA Ligase
    Water to 10 µL
    16 ^{o}C overnight
12) Transform into Stbl3 bugs as that protocol indicates. Briefly:
    a. Add 3 µL of each ligation (step 12) to a tube of bacteria. Mix gently; DO NOT pipet up and down.
    b. Incubate on ice for 30 min
    c. Heat shock at 42 ^{o}C for 45 sec
    d. Incubate on ice for 2 mins
    e. Add 250 µL of prewarmed SOC media
    f. Shake at 37 ^{o}C for 1 hr while shaking at 225 rpm. Prewarm the plates at 37 ^{o}C.
    g. Plate on LB/100µg/mL Amp plates at 200 µL and 50 µL; plate vector only control at 200 µL and pUC19 transformation control(if done) per protocol
    h. Grow overnight at 37 ^{o}C.
    i. COMMENT: We have used non Stbl3's with good results- but I would be careful to check and make sure your plasmid did not go through some type of recombination.

Day 2:

13) Compare # of colonies between the vector control(no insert) and ligation plates. Should see a LOT more colonies on the vector+insert plate than on vector alone.
14) Pick a reasonable # of colonies per construct. I like to pick so the final number is a multiple of 6 (6, 12, 18, 24, etc.); makes it easier to process.
15) Grow colonies in LB/100µg/mL Amp
16) Shake overnight 37 ^{o}C.

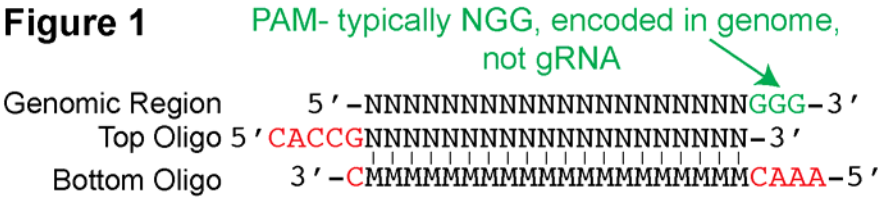
Day 3:

17) Miniprep each culture
18) Screen by PCR using U6 fwd + sgRNA rev primers; you should see a strong 273bp band for a positive
19) Sequence clones using U6 fwd primer
20) COMMENT: Because the cloning rate is so ridiculously high, I don't check for an insert. I miniprep and send for sequencing, 1-2 minipreps per insert.

Day 4:

21) Maxiprep positives and screen with PCR as above.

Figure 1



N and M are complementary bases.N's are the "output" of CRISPR Design Tool

Top Oligo 5′-CACCGNNNNNNNNNNNNNNNNNNN-3′

Bottom Oligo 5′-AAACMMMMMMMMMMMMMMMMMMMC-3′