Morrisey Lab Protocol:
Generating Large (>1kb) Genomic Deletions Using CRISPRs

by Dan Swarr & Dave Frank

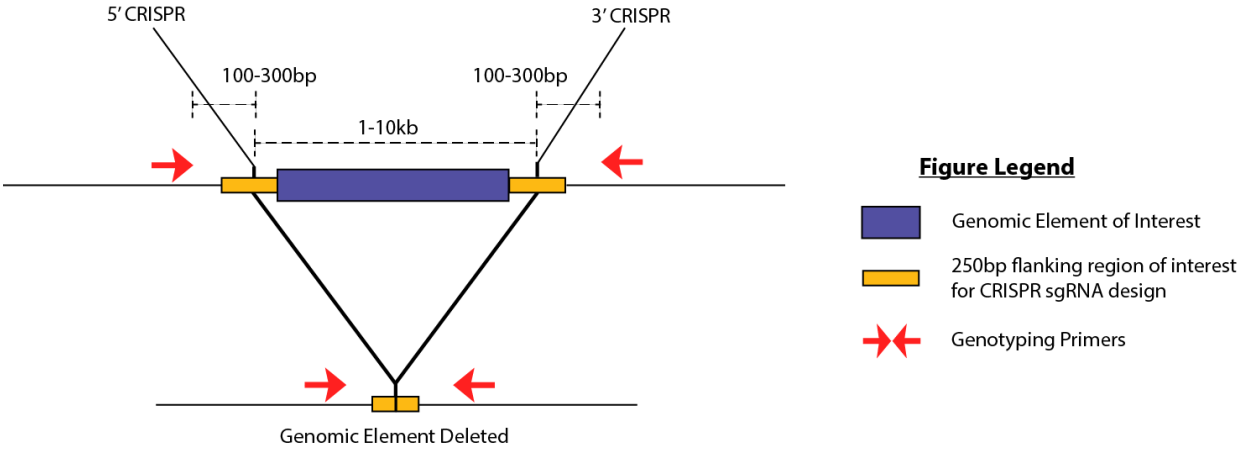
General Comments

- This protocol is intended to use the CRISPR-Cas9 system for generation of large genomic deletions (>1kb), in order to remove or inactivate a genomic element of interest, such as a promoter, enhancer, or portion of a long non-coding RNA (e.g. promoter, TSS, and first exon) by using two guide RNAs to make making two cuts, just upstream and downstream, of the element of interest.
- If you are interested in knocking out a protein-coding gene, you may want to consider using the more standard approach of using a single guide RNA targeted to the first portion of the coding region to introduce a frameshift mutation. As only one guide RNA is required, less cloning and screening is necessary and efficiency may be better. However, this approach of course not suitable for studying the function of non-coding genomic elements.

General Principles

- Generating deletions of several hundred kb has been reported in the literature, although Canver, et al (2014) demonstrated that the efficiency of genome editing is roughly proportional to the size of the deletion being introduced.
- We have had good success with generating deletions of 1-2.5kb, which also facilitates ease of PCR genotyping, but generating larger deletions are certainly possible.
- By cutting 5' to the genomic element of interest with one sgRNA, and 3' to the genomic element of interest with a second sgRNA, the intervening segment of DNA will be removed by non-homologous end-joining (NHEJ) repair in a significant fraction of cells.
- PCR primers flanking this region (see Figure 1) can be used to easily genotype cells using standard PCR.

Figure 1



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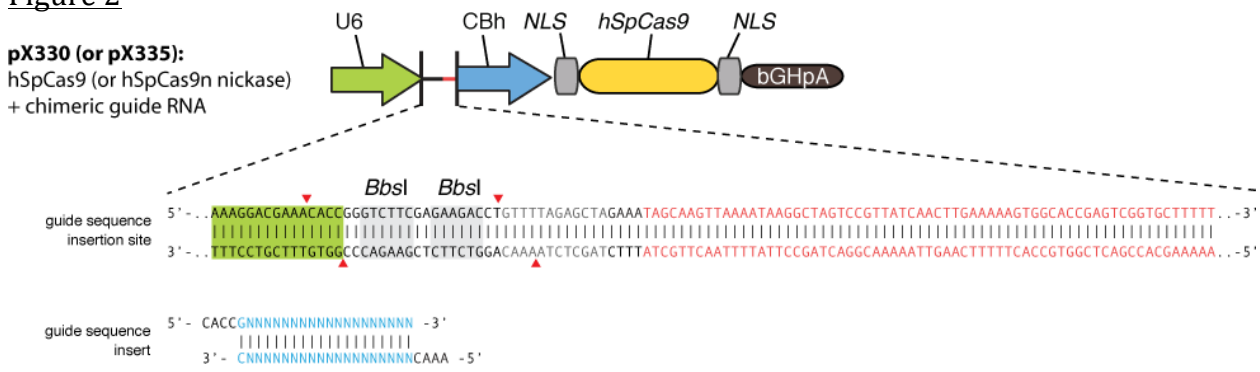
Designing the CRISPR Guide RNAs

1. Choose 250bp of sequence 5' to the region of interest, and 250bp of sequence 3' to the region of interest.
2. Guide RNAs can be designed using the Zheng lab design tool at http://crispr.mit.edu
3. Enter the following info:
    a. Name for sequence
    b. Your e-mail address
    c. Sequence type
    d. Species
4. First paste your 5' sequence into the browser and submit the job.
5. While it is loading, submit a second job with your 3' sequence.
6. Once your job is complete, be sure to save all of the data files, including lists of all potential off-targets for each guide RNA.
7. Choose the top three target sequences on the 5' end, and the top three for the 3' end.

Designing oligos to order (See Figure 2)

1. The MIT CRISPR design tool will input a 21bp target sequence, followed by a 3bp PAM sequence in green letters. **DO NOT** include the PAM sequence when ordering your oligos.
2. For the forward oligo, simply add "CACCG-" to the 5' end of the target sequence.
3. To generate the reverse oligo:
    a. Take the reverse complement of the target sequence
    b. Add "AAAC-" to the 5' end of this sequence, and "-C" to the 3' end.
4. Order these standard oligos for all 6 targets (three pairs for the 5' end, three pairs for the 3' end) from IDT – 25nmol is more than sufficient

Figure 2



Designing Genotyping Primers

- Choose primers 100-300bp upstream and downstream of the 5' and 3' cut sites, respectively.
- This will produce a mutant band in the range of 200-500 bp when the deletion is present.

- For deletions in the 1-5kb range, genotyping should be able to be performed easily with standard PCR conditions. For larger deletions, you may need to consider using TaKaRa LA Taq, or other long-range PCR polymerases, or an alternative genotyping strategy.

Phosphorylating and annealing the oligos

1. Set up the following reaction for each pair of oligos:
   - 2 uL forward oligo (100**µ**M)
   - 2 uL reverse oligo (100**µ**M)
   - 2 uL 10X T4 DNA Ligase Buffer (NEB)
   - 13 uL ddH2O
   - 1 uL T4 PNK (NEB)
   - 20 ul total
2. Anneal in a thermocycler using the following parameters:
   - 37°C 30 min
   - 95°C x 5 min, then ramp down to 25C at 5°C/min
   - 4°C hold

Cut and Dephosphorylate the PX330/PX459 Vector:

1. Digest 1-2ug of PX330 or PX459 with BbsI for 1hr at 37°C.
2. Heat-inactivate the RE at 65C x 15 minutes.
3. Add 1uL of calf intestinal phosphatase (CIP) to the reaction mixture, and incubate for 37C x 1hr.
4. Gel purify digested vector using QIAquick Gel Extraction Kit.

Ligate Oligos into the PX330/PX459 Vector:

1. Set up the following ligation reactions:
   - 150ng of digested/dephosphorylated PX330/PX459
   - 1uL phosphorylated/annealed oligo duplex
   - 4uL 5X T4 DNA ligase buffer (Invitrogen)
   - 1uL T4 DNA Ligase
   - ddH20 to bring reaction volume to 20uL
2. Incubate ligation reaction at 37C x 1hr or 16C overnight

Transformation of Stbl3 Cells

1. Thaw Stbl3 cells on ice
2. Add 2uL of ligation reaction to 20uL of Stbl3 cells
3. Let sit on ice for 30 minutes
4. Heat shock at 42C for 45 s
5. Recover on ice for 2 minutes
6. Add 150uL SOC media
7. Incubate at 37C with shaking for 1 hour
8. Plate entire reaction onto LP/amp plates

Screening Clones

1. After picking colonies and mini-prepping DNA, you can screen for inserts by either

performing double-digest with BbsI & AgeI (single band = oligo successfully annealed into vector; 2 bands indicates vector only without insert) followed by confirmatory sequencing, or proceeding directly to sequencing
2. Sequence positive clones using the human U6 primer: 5' – ACT ATC ATA TGC TTA CCG TAA C – 3'

Screen for Cutting Efficiency

** We recommend screening all 9 combinations of guide RNAs for cutting efficiency in a cell line that is easy to work with and transfect, such as 3T3s (mouse) or 293Ts (human) before proceeding with downstream experiments. Screening can be performed with mini-prepped DNA. **

1. Plate 3T3 or 293T cells into 10 wells of either 6-well or 12-well plates in antibiotic-free media, at a density of 50-70% confluency. This will allow for the 9 combos + GFP only transfection control (we use pMaxGFP+).
2. Transfect each well with 1ug of each 5' and 3' construct (total 2ug DNA) for a 12-well plate, or 2.5ug of each 5' & 3' construct (total 5ug DNA) for a 6 well plate using lipofectamine.
3. For example, the following transfection protocol may be used for 6-well plates:
    a. Combine 2.5ug of each 5' and 3' construct in a single tube, diluted to a final volume of 150ul with OPTI-MEM.
    b. Prepare a Lipofectamine master mix in OPTI-MEM by adding 110uL lipofectamine to 1540uL OPTI-MEM (1X reaction mix: 10uL lipofectamine + 140uL OPTI-MEM). This will provide adequate reagents for all 9 test pairs, plus an additional GFP control. Incubate at room temp x 5 minutes.
    c. Add 150uL of the lipofectamine master mix to each DNA mix (total volume 300uL). Incubate at room temp x 20-30 minutes.
    d. Add all 300uL of lipofectamine:DNA mix dropwise to cells
4. Transfect cells in the late afternoon or evening, and change media the following morning.
5. 24 hours after selection, add puromycin if desired (2ug/mL media works well for 3T3 cells) if you are using the PX459 backbone.
6. Leave cells undisturbed for 36-48 hours.

Genomic DNA Extraction & PCR Genotyping

1. Roughly 3 days after transfection, remove media from cells, place plate on ice and add 300uL of 5-Prime Cell Lysis solution.
2. Scrape cells with cell scraper, pipette cells/cell lysis solution up/down several times with 1000uL pipette, and transfer to clean eppendorf tube.
3. Vortex x 10 seconds to lyse cells. If cell clumps are still visible, incubate at 37C until suspension is homogenous.
4. Add 1.5uL RNaseA to sample, invert several times and then incubate at 37C for 5 minutes.
5. Cool sample to room temperature by placing on ice for 1 minute
6. Add 100uL of 5-Prime Protein Precipitation solution
7. Centrifuge at max rate for 5 minutes

8. Transfer supernatant to clean eppendorf tube, then add 300uL isopropanol to precipitate DNA
9. Invert several times, then centrifuge at max rate x 5 minutes.
10. Wash DNA pellet with 70% ETOH, air dry, and then re-suspend in 20-100uL (goal concentration 200ng/uL).
11. Perform genotype using standard PCR. If your CRISPRs work, you should see a strong mutant band. The relative intensities of the wild-type and mutant band will give you a general sense of how efficient your CRISPR pairs are.
12. Pick your best pair based on their relative efficiency, as well as the best off-target score (from the MIT design tool). Maxi-prep these two plasmids for future experiments.

<u>Generation of Monoclonal Cell Lines using Limiting Dilution*</u>

1. Using your cell line of interest, transfect a single 6-well using the protocol described above (or, transfect an equivalent number of cells using the transfection protocol appropriate to your cell line of interest)
2. Change to fresh media the morning after transfection. If you plan to use puromycin selection, add an appropriate dose 24 hours after transfection.
3. You may proceed to isolation of single clones as early as 60-72 hours after transfection. However, some cell lines may not tolerate the stress of puromycin selection combined with the stress of limiting dilution. Consider changing to fresh media (without puromycin) after 48 hours of puromycin selection, and allowing the cells to recover for 2-3 days before proceeding to limiting dilution.
4. To perform limiting dilution:
    a. Trypsinize the transfected cells, spin down and resuspend in fresh media
    b. Measure cell density with a hemocytometer
    c. Dilute cells to a final concentration of 1 cell per 200uL (5cells/mL)
    d. You will need to do this using a serial dilution approach, transferring at least 1mL media per dilution in order to reduce variability. For example, if you determine your cells are at a concentration of 125,000 cells/mL, dilute them as follows:
        i. Add 1mL cell suspension to 9mL fresh media to produce Dilution 1 at a concentration of 12,500 cells/mL.
        ii. Add 1mL of Dilution 1 to 9mL fresh media to produce Dilution 2, at a concentration of 1,250 cells/mL.
        iii. Add 1mL of Dilution 2 to 9mL fresh media to produce Dilution 3, at a concentration of 125 cells/mL.
        iv. Add 1mL of Dilution 3 to 24mL of fresh media.
    e. Using the final dilution at 5 cells/mL, pipette 200uL into each well of a 96-well plate using a multi-channel pipette. Plate out 1-2 96 well plates.
5. Leave plates undisturbed for at least 3 days. Change to fresh media after 3-5 days. Note that it will be very difficult to see cells/colonies for at least 3-5 days, and it will likely take at least a week before colonies are large enough to be split onto a larger plate.
6. Once colonies are clearly visible (be sure to eliminate wells that have two or more distinct colonies), wash each well with PBS, then trypsinize cells with 50uL trypsin.

7. Add 50uL of cell trypsin suspension to 1mL fresh media in a 24 well plate. Allow cells to grow until they have reached an appropriate density.
8. Trypsinize each well of 24-well plate with 100-200uL trypsin, then split trypsin/cell suspension into two wells of a 12 well plate (per clone) with as much volume as the 12-well will handle (one well will be for genotyping, the 2nd well will be for further expansion of the cell line).
9. One the 12-well cells have reached an appropriate density, extract DNA and perform genotyping PCR.
10. Expand the desired cell lines as appropriate, and proceed to downstream experiments

* Note that not all cell types will tolerate limiting dilution, as it is quite stressful for many cell types to be completely isolated from other cells. You should strongly consider trying this isolation protocol with wild-type cells to assure that they will tolerate this procedure before attempting your CRISPR deletions. Also note that if your deletion significantly affects cell proliferation or viability, you may not be able to successfully isolate cells with the deletion of interest using this procedure.

Generation of KO Mice

1. Purify 10-20ug of each 5' and 3' construct using GeneClean Turbo columns, per the manufacturer's protocol (following the instruction for purification of PCR products)
2. Precipitate DNA with 1/10 volume sodium-acetate and 2-2.5 volumes 100% ethanol.
3. Air-dry DNA pellet, and then resuspend DNA in Injection Buffer (sterile 10mM Tris/0.1 mM EDTA, pH 7.5 prepared with distilled water)
4. Pass the purified DNA through a Millipore purification spin column
5. Again, precipitate the DNA with 1/10 volume sodium-acetate and 2-2.5 volumes 100% ethanol.
6. Air-dry the DNA pellet, and resuspend in DNA Injection Buffer.
7. Submit samples to the Penn Transgenic core to be injected together in a 1:1 ratio (using either a dose of 2.5ng or 5ng per construct, total DNA dose 5ng or 10ng)
8. Genotype F0 generation using genotyping primers as described above

References

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2. Fujii W, et al. Nucleic Acids Res. 2013 Nov 1;41(20):e187.
3. Ran FA, et al. Nat Protoc. 2013 Nov;8(11):2281-308.
4. Canver MC, et al. J Biol Chem. 2014 Jun 6.