Farnham Lab Protocol for CRISPR/Cas 9-mediated enhancer deletion using a puromycin selection vector

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Part A. Design gRNA oligos

1. Design gRNAs
   Go to Optimized CRISPR design website: http://crispr.mit.edu/. Input 23-500nt genomic sequence, choose the correct target genome and submit the job. Choose gRNA that has a score greater then 85.
2. gRNA specificity check
   a) Go to CRISPR RGEN tool Cas-OFFinder website: http://www.rgenome.net/cas-offinder/. Select PAM type (5'-NGG-3') and target genome. Input the 20nt gRNA w/o PAM as query sequence. Allow mismatch number equal or less than 2. Submit the job and check result. Avoid using a gRNA that has 1 or 2 mismatch sequences.
   b) Back to Cas-OFFinder again, input the 15nt gRNA that close to PAM (w/o PAM) as query sequence. Allow mismatch number equal or less than 2. Submit the job and check result. Avoid using gRNA has other 0 mismatch sequences since the sequence near PAM is critical for CRIPSR specificity.
3. Order oligos
   For pSpCas9(BB)-2A-Puro (PX459) V2.0 *(Addgene plasmid #62988)* backbone vector, order reverse complement oligos that contains 5' and 3' overhangs as shown below:
   e.g.
   20nt gRNA sequence: TGTTCTTCTTATCGTGCCAT
   Forward oligo: CACCGTGTTCTTCTTATCGTGCCAT
   Reverse oligo: AAACATGGCACGATAAGAAGAACAC

Part B. Plasmid cloning

1. Annealing of oligo
   Option A
   a) In a PCR tube, mix:

100µM Forward oligo	15 µl
100µM Reverse oligo	15 µl
Annealing buffer	45 µl
Total Reaction Volume	75 µl

   **Annealing buffer (10mM Tris-HCl pH8.0, 1mM EDTA, 100Mm NaCl).
   b) Incubate in thermal cycler 92°C 2min, 72°C 2min, 55°C 2min, 37°C 2min, hold at 10°C.

   OR

Option B

a) In a PCR tube, mix:

100µM Forward oligo	1 µl
100µM Reverse oligo	1 µl
10X T4 Ligation Buffer (NEB)	1 µl
ddH20	6.5 µl
T4 PNK	0.5 µl
Total Reaction Volume	10 µl

b) Incubate in thermal cycler 37°C 30min, 95°C 5min, ramp down to 25°C at 5°C/min, hold at 10°C.

2. Digest pSpCas9(BB)-2A-Puro (PX459) V2.0 *(Addgene plasmid #62988)* with BbsI endonuclease *(NEB Cat# R0539S/*R0539L) or FastDigest BbsI *(Thermo Cat.#FD1014)*, reaction system:

	NEB BbsI	FastDigest BbsI
Restriction Enzyme	1ul (or 10 units)	1ul
PX459 Vector	1 µg	2ul (up to 1 µg)
10X Buffer	5 µl	2 µl
ddH_20	x µl	x µl
Total Reaction Volume	50 µl	20 µl

Incubate at 37°C for 1-2 hrs for NEB enzyme, 5-30 mins for FastDigest enzyme.

3. Gel purification of BbsI-digested PX459 vector using QIAquick Gel extraction kit *(QIAGEN, Cat# 28704/28706)*.

4. Ligate annealed oligo duplex with digested PX459 vector
   a. If using **Option A,** use T4 ligase *(NEB #M0202)* in this step.

Diluted oligo duplex from step 1 (1:20)	1 µl
Digested PX459 vector	50 ng
10X T4 ligase buffer (NEB)	2 µl
T4 ligase	1 µl
ddH_20	x µl
Total Reaction Volume	20 µl

Incubate at 16°C overnight

If using **Option B**, use Quick Ligase *(NEB #M2200)* in this step:

Diluted oligo duplex from step 1 (1:250)	1 µl
Digested PX459 vector	50 ng
2X Quick Ligation buffer (NEB)	5 µl
Quick ligase	1 µl
ddH_20	x µl
Total Reaction Volume	11 µl

Incubate at 37°C for 10 min

5. OPTIONAL: Treat ligation reaction with PlasmidSafe exonuclease to digest any residual linearize DNA:

Ligation product from previous step	11 µl
10X PlasmidSafe Buffer	1.5 µl
10Mm ATP	1.5 µl
PlasmidSafe exonuclease	1 µl
Total Reaction Volume	15 µl

Incubate at 37 °C for 30 min, followed by 70 °C for 30 min. After PlasmidSafe treatment, the reaction can be stored at −20 °C for at least 1 week.

6. Transformation of PX459 ligation product by using XL-10 gold Ultracompetent cells *(Agilent Technologies, Cat# 200315)*
    a) Pre-chill several 14ml BD Falcon polypropylene round-bottom tubes on ice. (Remember to include one tube for negative control and one tube for the pUC18 positive control.) Preheat SOC medium to 42°C.
    b) Thaw the competent cells on ice. When thawed, gently mix and aliquot 100 µl of cells into each of the pre-chilled tubes.
    c) Add 4 µl of the β-ME mix provided with this kit to each aliquot of cells.
    d) Swirl the tubes gently. Incubate the cells on ice for 10 minutes, swirling gently every 2 minutes.
    e) Add 0.1-50 ng of the experimental DNA (or 2 µl of a ligation mixture) to one aliquot of cells. Dilute the pUC18 control DNA (provided in the kit) 1:10 with sterile dH2O, then add 1 µl of the diluted pUC18 DNA to the other aliquot of cells as positive control. Add 2ul of sterile dH2O to negative control
    f) Swirl the tubes gently, then incubate the tubes on ice for 30 minutes.
    g) Heat-pulse the tubes in a 42°C water bath for 30 seconds. The duration of the heat pulse is critical.
    h) Incubate the tubes on ice for 2 minutes.
    i) Add 0.9 ml of preheated (42°C) SOC medium and incubate the tubes at 37°C for 1 hour with shaking at 225-250 rpm.
    j) Plate ≤200 µl of the transformation mixture on LB agar plates containing the appropriate antibiotic (in this case, ampicillin). For the pUC18 control transformation, plate 5 µl of the transformation on LB-ampicillin agar plates.
    k) Incubate the plates at 37°C overnight.
    l) For the pUC18 control, expect 250 colonies (≥5 × 109 cfu/ µg pUC18 DNA). For the experimental DNA, the number of colonies will vary according to the size and form of the transforming DNA, with larger and non-supercoiled DNA producing fewer colonies.
7. Isolation of recombinant plasmid by using QIAprep Spin Miniprep Kit(250) *(Qiagen, Cat# 27106)*.
8. Send plasmid for Sanger sequencing to confirm successful cloning.

Part C. Isolation of CRISPR Knock out clonal cell line

1. Transfection of recombinant plasmid to cells using Lipofectamine LTX reagent *(Invitrogene, Cat# 15388-100)* or other suitable transfection reagents.
    a) Plate cells($3.0X10^5$/well) in 6well plate 1-2 days to reach 60-70% confluence.

**Make sure you have one well for no gRNA negative control PX459 plasmid.
**GFP plasmid can be used to test transfection efficiency.

b) One the day of transfection, in a tube dilute Lipofectamine LTX reagent 5-12.5ul/well in Opti-MEM reduce serum medium *(ThermoFisher, Cat.# 31985062)* 125ul/well.

**5ul/well works for C4-2B and LNCaP cells

c) In a tube, dilute recombinant plasmid(s) or negative control PX459 plasmid 2500ng/well in Opti-MEM medium 125 ul/well, add Plus Reagent 2.5 ul/well.

**If using multiple gRNAs, add plasmids at a 1:1 ratio to a total DNA amount of 2500ng/well

d) Add diluted DNA to diluted Lipofectamine LTX reagent at 1:1 ratio (125ul+125ul/well).
e) Incubate 5min at RT to make DNA-lipid complex.
f) Take out old medium from the wells
g) Add DNA-lipid complex 250ul/well to cells with 2ml/well fresh complete growth medium
h) Incubate at 37°C overnight

2. Gently replace medium with 1ml of fresh complete growth medium + puromycin
   **For C4-2B cells, use 400-900ng/ml of puromycin
3. Incubate at 37°C for 48-72hrs, until most of cells in un-transfected well are dead
4. Disassociate the transfected cells to single cell suspension
5. Count cells and serially dilute the cells to 0.3-0.5 cell/ 100ul (that's 3-5 cells/ml), plate the single cell suspension into several 96well plates (I usually do 2-3 plates) 100ul/well, return to incubator.
   **Conditional medium (40% old growth medium + 60% complete medium) might be needed for some cell lines at the last dilution.
   e.g. RWPE-1, RWPE-2

   OR

   Sort live cells into 96 well plates contain 100ul/well growth medium at 1cell/well, return to incubator.
6. Freeze down the leftover transfected cell population for future potential use.
7. OPTIONAL: Test deletion efficiency on the population using leftover cells.
   a) Dissociate transfected cell population and spin them down at 200g for 5 min at room temperature.
   b) Aspirate the medium completely
   c) Extract DNA by adding QuickExtract DNA extraction solution *(Epicentre, cat.#QE09050)*, 50 µl or 10 µl of the solution for each well of a 24-well or 96-well plate, respectively, is typically used.
   d) Vortex mix for 15 seconds.
   e) Transfer the tube to 65°C and incubate for 6 minutes.
   f) Vortex mix for 15 seconds.
   g) Transfer the tube to 98°C and incubate for 2 minutes.
   h) Store the DNA at –20°C, or at –70°C for long term storage.
   i) PCR using primers flank the deletion region, use 5 µl or less of the extracted DNA for each PCR amplification (1 µl is usually used).

8. Allow several weeks (2-3 weeks usually) for each single cell to grow into a visible colony, inspect cells every week and change medium when necessary.
9. Disassociate the colonies and re-plate half of them into 96well plates to allow cells to spread out and avoid clumping.
10. Harvest other half of cells to test for deletion
    **Make sure to label the tubes correspondingly to the wells
    a) Spin them down at 200g for 5 min at room temperature.
    b) Aspirate the medium completely.
    c) Extract DNA by adding 10 µl QuickExtract DNA extraction.
    d) Vortex mix for 15 seconds.
    e) Transfer the tube to 65°C and incubate for 6 minutes.
    f) Vortex mix for 15 seconds.
    g) Transfer the tube to 98°C and incubate for 2 minutes.
    h) Store the DNA at –20°C, or at –70°C for long term storage.
    i) PCR using primers flank the deletion region as well as primers inside of deletion region, use 5 µl or less of the extracted DNA for each PCR amplification (1 µl is usually used).
    j) For colonies that show no inside band and a clean deletion band, congratulations, you just got yourself a complete deletion clone!
11. Keep growing and expanding the deletion clone for storage and future functional assays.

REFERENCE

Ran, F. Ann, et al. "Genome engineering using the CRISPR-Cas9 system."Nature protocols 8.11 (2013): 2281-2308.