University Children's Hospital Zurich 2016
Leukemia Lab, Bornhauser Cell Death Group

Protocol for Cloning of single guide RNAs for Cas9 genome editing using LentiCRISPR

Materials

- Tango Buffer
- Oligos (standard synthesis, purification)
- Screw cap tubes or Eppendorf tubes (e.g. 1.5 ml)
- CRISPR target plasmid with BsmBI insert site (eg LentiCRISPR-EGFP, mCherry etc…)
- T4 DNA ligase (NEB, M0202M or M202L**, make aliquots of T4 DNA ligase buffer and use only freshly thawed aliquots for reaction**)
- Esp3I restriction enzyme (Thermo Scientific, ER0451; **NOT NEB BsmBI**, BsmBI requires 55°C for efficient cleavage – not compatible with the reaction here)
- Forward test primer for colony PCR: 5'-GACTATCATATGCTTACCGT-3' (specific for U6 promoter in LentiCRISPR)
- Mini- and/or Midi-Prep kits (e.g. Qiagen)

Preparation

- screen your target genomic locus for CRISPR guide RNA binding sites (e.g. http://crispr.mit.edu)
- use the target designer excel sheet to get the forward and reverse primers for your sgRNA
- paste the 20 nucleotide sequence of your favourite guide RNA(s) (**with PAM** into the target designer generator
- order the generated forward and reverse oligos (lyophilized or at a concentration of 100 µM, desalted is fine)

Day 1 – Annealing of single-stranded oligos and LIgating of double-stranded oligos into LentiCRISPR

- for lyophilized oligos elute in an appropriate volume of H2O to obtain an oligo concentration of 100 µM
- pipette the following reaction for each pair of forward and reverse oligos into a screw cap tube and close tightly:

  10 µl oligo forward
  10 µl oligo reverse
  10 µl Tango Buffer (or similar)
  70 µl H2O

- bring about up to 95°C for 5 mins
- Let oligos return to room temperature over 1-2h

Inserting annealed oligos into LentiCRISPR

- set up following reaction for ligation of annealed double-stranded oligos into LentiCRISPR:

  150 ng of LentiCRISPR plasmid
  1 µl of double-stranded oligos
  2µl NEB T4 ligase buffer
  to 18 µl with H2O
  0.5 µl Esp3I
  0.5 µl T4 ligase

- run the following reaction in a thermocycler

  37°C 5 min
  16°C 10 min 10 cycles (can be extended to 50 cycles over night)
  37°C 15 min
  80°C 5 min

- Note: There is an additional BsmBI(Esp3I) site in the tagBFP sequence in the LC-BFP vector. For this vector the final 37°C step should be removed from the cycle protocol to improve yield

- transform ligation mix into competent bacteria (electroporation or heat shock)
- plate transformed bacteria on LB-Agar + Ampicillin

Day 2 – Identify bacterial clones with LentiCRISPR + double-stranded oligo insert

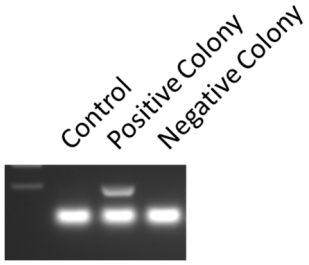
- run a colony PCR for 3-5 colonies per transformed LentiCRISPR (3 colonies is almost always enough as this procedure should have 80-100% efficiency)
- Colony PCR program:

95°C	3 min	
95°C	30 sec	
55°C	30 sec	
72°C	10 sec	25 cycles
72°C	10 min	

- prepare separate LB-agar Amp plate with a number for each clone that will be picked
- prepare PCR reaction mix (20 µl) with the primers

  Forward: Forward Test Primer (GACTATCATATGCTTACCGT)
  Reverse: reverse single-stranded guideRNA oligo that was used for annealing of double-stranded oligos (day 1)

- pick single colonies with toothpick or pipette tip and streak on separate numbered plate and subsequently submerge toothpick/pipette tip in one well of PCR reaction buffer
- after picking all colonies, remove toothpicks/pipette tips, run PCR and put numbered plate with streaks at 37°C for 2-5h
- load PCR reactions on 2% agarose gel
  - clones with LentiCRISPR + double-stranded oligo insert should give a band around 100 bp
  - clones with empty LentiCRISPR should give no band



- pick positive clone(s) from numbered plates to start overnight cultures for mini or midi preps
- You can also sequence plasmid using the forward vector if you wish to confirm insert
- Proceed to Lentiviral production using ps.Pax2 and p.VSV.G in 293T Cells
- Some hints for Lentiviral work:
  - LentiCRISPR should only be handled by those with working knowledge and safety training in the production and use of Lentivirus.
  - LentiCRISPR is known to have very low viral yields due to the size of the plasmid.
  - We have improved our viral production by increasing the amount of viral plasmid transfected into 293T cells
  - We use 4ug LentiCRISPR + 1.5 ug ps.Pax2 + 1.5ug p.VSV.G in 3mL plated in a 6 well dish
  - We have had success using both PEI and CaCl2/HEBS transfections
  - We change the media at 6 hours after transduction, then collect viral supernatant at 24 and 48 hours.
  - Don't expect to get 50-100% transduced cells using raw supernatant. If you wish to get high transduction you will need to concentrate.
  - We generally aim for 5% transduction of cells and then sort from there.