Annealed Oligo Cloning of gRNAs – Donaldson Lab 08/01/2023

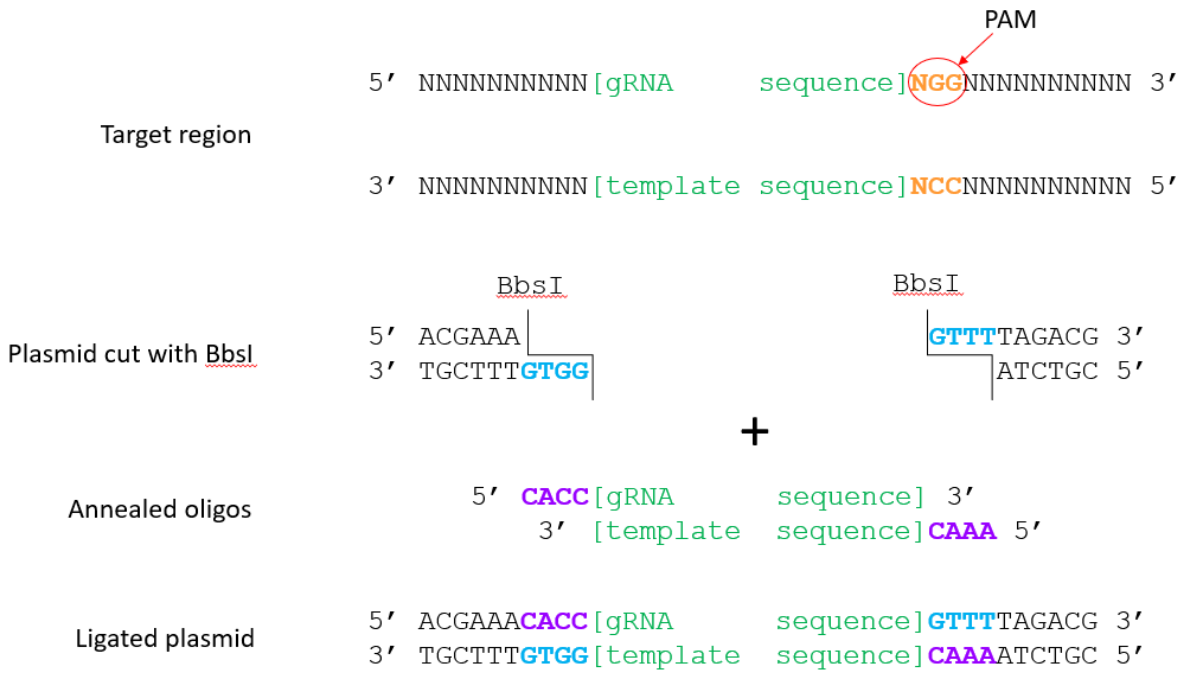
You can find the full protocol at http://www.zdonaldsonlab.com/protocols

Design oligos for lenti U6-sgRNA-EF1a-mCherry plasmid (Addgene #114199) or AAV-U6-gRNA-EmGFP (Addgene #89060)

Benchling Project folder can be accessed via: https://benchling.com/drzoephd/f_/0gpl9ssR-grna-design-and-cloning-workshop/ (Project is set to public but you may need a benchling account to access it).

Guide RNA (gRNA) should be designed as follows:

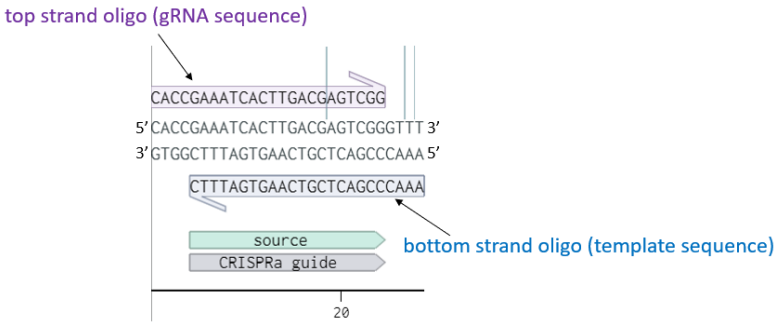
- PAM sequence (NGG) must be at the 3' end of your desired gRNA sequence



1. Determine ideal gRNA using algorithm like on Benchling
    - Go to NCBI gene: https://www.ncbi.nlm.nih.gov/gene
    - Type in "[your gene] microtus ochrogaster" in the search bar
    - Click relevant link that comes up
    - Scroll down to the heading that says "Genomic regions, transcripts, and products." This gives you a visual representation of where the gene/transcript are in the genome
    - Tracks with numbers that start "NM" or "XM" are mRNA transcripts
    - Zoom in using the (+) magnifying glass to the region where the mRNA transcript starts

- Highlight the region ~1 kb upstream to ~1 kb downstream of the beginning of the mRNA transcript (transcription start site)
- Scroll over highlighted part and download the sequence as a GenBank Flat File
- Open Benchling
- Click the + sign and click "Import DNA sequences." Import the GenBank Flat file you just downloaded
- Transcription start site (TSS) is where the mRNA track begins
- For CRISPRa, design guide RNA (gRNA) from -50 to -400 bp upstream of the TSS.
  For CRISPRi, design gRNA from +50 to +100 downstream of TSS
  For CRISPR + Cas9 nuclease activity, design gRNA in first exon within 1st 100 amino acids. Alternately, targeting an exon that is essential for function can be a good alternative.
  For transgene insertion, it will depend where you are trying to insert the new gene.
- Highlight desired region for either CRISPRa or CRISPRi
- Click target sign on right side of the screen
- Click "Design and analyze guides;" Design type: Single guide; Guide length: 20; Genome: microtus ochrogaster; PAM: NGG; Finish
- Click (+) sign next to target region
- Scroll down to see different gRNAs. You want a gRNA with an on-target score >60 and an off-target score >50. If there are none that meet these criteria, see if any are close. If none are close, increase range selection to -500 bp upstream for CRISPRa or +150 downstream for CRISPRi
- Click checkbox next to desired guides. This will show where they are in sequence
- Create annotation to label them (make sure the orientation of the annotation is correct: To do so, NGG should come at the 3' end of your gRNA in the genomic sequence – see Target region diagram.)
- Copy the guide into a new DNA file with orientation so that guide is in the forward direction. As soon as you select copy, a box will come up showing the original and the reverse complement.

2. Add 5'CACC3' at 5' end of top (gRNA) strand
3. Add 5'GTTT3' at 3' end of top (gRNA) strand (you can only type letters into the top strand)
4. Top oligo is gRNA sequence 5'CACC[gRNA]3' (I call this the "forward" oligo or F)
5. Bottom oligo is template sequence 5'AAAC[template]3' (make sure your template sequence comes from the bottom strand and is in the correct 5'→3' direction!) (I call this the "reverse" oligo, or R)
6. A visual for what it should look like in Benchling (save this short guide fragment sequence as its own DNA sequence in Benchling):



7. Order oligos from IDT

In silico cloning

1. Go to backbone plasmid file (pAAV-U6-gRNA-EmGFP)
2. Click "Assembly Wizard" in bottom right corner > "Create New Assembly" > "Gibson Assembly" (we are not really doing Gibson Assembly, but it's the easiest way to design the plasmid in silico)
3. To highlight the part you want to use as the backbone plasmid: click one BbsI site, and shift+click the other. Right click and "invert selection." Click "Backbone" at bottom left of assembly wizard panel. Click "Set Fragment"
4. Go to your short guide fragment sequence. Highlight the whole sequence and click "Insert" at bottom of assembly wizard panel. Click "Set Fragment."
5. Name your assembly and click "Assemble"
6. Note: this will create your assembled plasmid and also two primers called [your guide] FWD and REV. Ignore these – they're just for Gibson Assembly so you don't need them

Digest plasmid

1. Set up reaction as follows:

Component	**50 µL reaction**
Plasmid DNA (pAAV-U6-gRNA-EmGFP or pLV-U6-gRNA-EF1a-mCherry)	1 µg
10X CutSmart Buffer	5 µL (1X)
BbsI-HF	1.0 µL (or 10 units)
Nuclease-free water	To 50 µl

2. Incubate at 37°C for 2 hours, as BbsI-HF is Time-Saver qualified.
3. Inactivate BbsI-HF by heating at 65°C for 20 mins

Check digest of plasmid in gel

1. Pour 0.7% agarose gel (in TAE) – 200 mL gel, 1.4 g agarose, 1 µL EtBr
    a. Weigh out 1.4 g agarose using the scale
    b. Add 200 mL TAE buffer
    c. Microwave for ~2 mins (watch it to make sure it doesn't boil!)
    d. Add 1 µL EtBr
    e. Check gel box with water to ensure there are no leaks.
    f. Place comb at end of gel box
    g. Pour gel in hood if still hot (fumes are toxic). Ensure no bubbles.
    h. Wait 30 mins for gel to set
2. Add dye to digested DNA (6X dye: 8.5 µL dye in 50 µL reaction)
3. Load gel with 1 kb ladder and sample(s) (10 µL should be sufficient)
4. Run gel at 110V for 1 hour
5. Check bands to see if right size
6. If only one band of right size, proceed to column purification.

Column purify digested plasmid DNA using Zymo DNA Clean & Concentrator

Before starting: add 24 mL ethanol to 6 mL wash buffer

All centrifugation steps should be performed at 10,000 x g.

1. In a 1.5 mL microcentrifuge tube, add 2-7 volumes of DNA Binding Buffer to each volume of DNA sample (see table below). Mix briefly by vortexing.

Application	DNA binding buffer:sample	Example
Plasmid, genomic DNA (>2 kb)	**2:1**	**200 µL : 100 ul**
PCR product, DNA fragment	5:1	500 µL : 100 ul
ssDNA (eg. cDNA, M13 phage)	7:1	700 µL : 100 ul

2. Transfer mixture to a provided Zymo-Spin™ Column in a Collection Tube.
3. Centrifuge for 30 seconds. Discard the flow-through.
4. Add 200 µl DNA Wash Buffer to the column. Centrifuge for 30 seconds. Repeat the wash step.
5. Add ≥ 6 µl DNA Elution Buffer or water directly to the column matrix and incubate at room temperature for one minute. For DNA >10 kb, total yield may be improved by eluting the DNA with 60-70°C DNA Elution Buffer. Transfer the column to a 1.5 mL microcentrifuge tube and centrifuge for 30 seconds to elute the DNA. Ultra-pure DNA is now ready for use.

Anneal oligos

1. Resuspend oligos in Duplex Buffer (BioWorld) to 100 μM (see oligo data sheet for volume). Pipette up and down and vortex to mix.
2. Mix oligos in 1:1 molar ratio (25 μL top strand + 25 μL bottom strand) in an 8-tube strip
3. Place tubes in thermocycler at 95°C for 5 mins.
4. Turn off the thermocycler and allow annealed oligos to cool slowly to room temp (~1 hour).
5. Can store at -20°C

FOR CONTROL: blunt (some) vector (*optional)

This is to make a version of the plasmid that has NO gRNA in it. It should be a positive control for the digest and a negative control for the gRNA insertion. You should have colonies but when you sequence them, there should be no DNA in the gRNA site.

Quick Blunting kit NEB **makes 5' phosphorylated ends (has both 3' → 5' exonuclease activity and 5' → 3' polymerase activity)

1. Mix the following components in a sterile microfuge tube:

Component	Amount
Purified DNA (up to 5 ug)	1-19 μL
10X Blunting Buffer	2.5 μL
1 mM dNTP Mix	2.5 μL
Blunt Enzyme Mix	1.0 μL
Sterile water	variable
Total	25 μL

2. Reactions containing restriction enzyme digested DNA are incubated at room temperature for 15 minutes.
3. Immediately inactivate enzyme in the blunting reaction by heating at 70°C for 10 minutes.
4. Proceed directly to the ligation step using T4 DNA Ligase. Blunt ligation reactions using standard T4 DNA Ligase should be incubated overnight at room temperature.

Ligate oligos with plasmid (or ligate empty blunted vector if including)

1. Set up the following reaction in a microcentrifuge tube on ice.
   (The T4 DNA Ligase Buffer should be thawed and resuspended at room temperature. T4 DNA Ligase should be added last.)

Component	20 μL Reaction
T4 DNA Ligase Buffer (10X)	2 μL
Vector DNA (9 kb) (for 10:1 molar ratio of insert:vector)	0.02 pmol
Insert DNA (30 bp)	0.20 pmol

Nuclease-free water	To 20 µL
T4 DNA Ligase	1 µL

2. Gently mix the reaction by pipetting up and down and microfuge briefly.
3. For cohesive (sticky) ends, incubate at 16°C overnight. For blunt ends from Quick Blunting Kit, incubate at room temp overnight.
4. Heat inactivate at 65°C for 10 minutes.
5. Chill on ice and transform 1-5 µL of the reaction into 25 µL competent cells.

Transformation

1. Take out NEB Stable cells to thaw (thaw in ice!)
2. Take out NEB Stable outgrowth medium from 4°C and place in bacterial incubator to warm
3. Take out appropriate number of LB/agar plates (1 per reaction) and place in bacterial incubator to warm. Make sure they are the correct antibiotic
4. Add 1 µL ligation reaction to 10 µL competent cells. Tap tube to mix
5. Incubate cells/DNA on ice for 30 mins
6. Heat shock at 42°C for 1:15 (1 min 15 seconds)
7. Immediately place back on ice for 5 mins
8. Add 190 µL pre-warmed NEB Stable/10-beta outgrowth medium to each reaction
9. Shake cells horizontally in the shaker incubator at 30°C, 120 rpm, for 1 hr
10. Plate 20-200 µL of each reaction on a pre-warmed LB/agar + carbenicillin plate
11. Incubate overnight at 30°C

Picking and culturing colonies

1. Pick single colony and culture in 2-5 mL of LB + carbenicillin (in 15 mL snap cap falcon tube) overnight in 30°C shaker incubator
2. For glycerol stock: 500 µL culture + 500 µL 50% glycerol. Store at -80°C
3. Spin down remaining culture 10 mins at 4000 g

Mini prep (Omega Biotek Kit) from ML

EZNA Plasmid DNA Mini Kit I (D6942-02)

1) To pellet, add 250 µL of Solution I (with RNase A, stored at 4°C). Vortex
2) Add 250 µL of Solution II (room temp) invert and rotate until clear, about 2-3 minutes. Do not exceed 5 minutes. Do not vigorously mix
3) Add 350 µL of Solution III. Invert until white precipitate forms.
4) Centrifuge at 14,000 g for 10 minutes
5) Set up 2 mL tubes with HiBind DNA Mini Column

6) Transfer supernatant into HiBind Columns
7) Centrifuge at maximum speed for 1 minute. Discard filtrate (use pig if many samples)
8) Add 500 µL HBC buffer (room temp. Should have isopropanol already added to it)
9) Centrifuge at maximum speed for 1 minute. Discard filtrate
10) Add 700 µL DNA Wash Buffer (should have ethanol already added to buffer). Centrifuge at maximum speed for 30 seconds. Discard filtrate and reuse collection tube
11) Perform second DNA Wash buffer step: add 700 µL DNA wash buffer. Centrifuge at maximum speed for 30 seconds. Discard filtrate
12) Centrifuge empty HiBind DNA Mini Column at maximum speed for 2 minutes to dry the column.
13) Put HiBind column into nuclease-free 1.5 mL microcentrifuge tube.
14) Add 50 µL of Elution Buffer or sterile deionized water. Ensure covers filter. Let sit at RT for 1 minute. Centrifuge at maximum speed for 1 minute.
15) Perform second elution step: add 50 µL of EB or water. Let sit at RT for 1 minute. Centrifuge at max speed for 1 minute.
16) Store eluted DNA at -20 C. (If measuring DNA concentration next morning, store at 4°C).

Sequencing – You can use your favorite facility but we recommend full plasmid sequencing, such as plasmidsaurus

Reagent Preparation (ML):

1) Terrific broth:
    a. 23.8 g in 500 mL with 2 mL glycerol (as per package instructions)
    b. Heat to 37 C to dissolve.
    c. Autoclave on liquid setting. N.B. keep caps loose

2) Agar plates:
    a. 1 L H2O with 40 g of agar
    b. Heat so goes into solution or autoclave immediately
    c. Label plates with date and antibiotic.
    d. Remove from autoclave and while on stir plate cool to around 55 C (when it doesn't hurt hands to touch. Can use tub of water to cool faster: first tub 2 minutes, switch water to cool water again and cool for 1 minute)
    e. Add antibiotic (carbenicillin) (1 mL of 1000X stock for 1X final concentration).
    f. Pour plates (about half full). Do with Bunsen flame
    g. Stack plates to minimize condensation on plates)
    h. Let cool. Should solidify in about an hour.
    i. Store in cold room in sleeve or with parafilm.

    Plates should be under 3 weeks old, ideally less than 2 weeks old

3) Carbenicillin stock (1000X solution = 100 mg/mL)
    a. 1000 mg in 10 mL of H2O
    b. ZRD: no need to filter sterilize

4) 1% agarose gel
    a. 200 mL 1X TAE buffer. [Replace TAE in electrophoresis about every week]
    b. 2 g of agarose (use agarose spatula). Agarose is toxic!!!
    c. Heat in microwave by electrophoresis machine/engineering area. Shake periodically until solution is clear without any flecks.
    d. Add 1 µL of ethidium bromide. EtBr is also toxic!!! Swirl to mix
    e. Check with water to ensure there are no leaks in apparatus you will eventually pour the gel in. Easier than cleaning up the gel with EtBr if there is a leak ☺
    f. Pour gel in hood if still hot (fumes are toxic). Ensure no bubbles.

Mira prep (Omega Biotek Kit):

1) Set up a 5 mL bacterial culture with 1 colony and incubate at 37 C on shaker at 250 rpm for about 8 hrs

2) Pour 5 mL of bacterial culture into 45 mL of broth and incubate at 37 C on shaker at 250 rpm overnight
3) Transfer bacteria to 50 mL tube and spin at 4K g for 10 minutes
4) Discard supernatant and resuspend pellet in 2 mL resuspension buffer (Solution I) with 50 ug/mL RNase freshly added
5) Add 2 mL of lysis buffer (Solution II), invert 3-4 times and incubate for 3 minutes at RT
6) Add 2 mL of neutralization buffer (Solution III) and invert 3-4 times
7) Distribute lysate into 1.5 mL Eppendorf tubes by pouring, not by pipetting
8) Spin at 13,200 g at RT for 10 min
9) Collect supernatants in a 15 mL tube and discard pellets
10) Add 1X volume of 96% ethanol (~5 mL)
11) Mix thoroughly for 5 seconds
12) Loads the sample/ethanol mix onto 5 spin columns in 3 sequential ~700 µL aliquots. After the addition of each aliquot, spin the column 30 sec at 13,200 g
13) Discard the flow-through
14) Repeat until entire sample is run through spin columns
15) Wash with 500 µL HBC washing buffer and spin at 13,200 g at RT for 30 seconds.
16) Discard flow through
17) Repeat DNA washing step
18) Do a final spin at 13,200 g at RT for 1.5 minutes
19) Discard old tube and put column in new tube
20) Add 30-35 µL of ddH2O or elution buffer and incubate for 2 minutes at RT
21) Spin at 13,200 g for 2 minutes to elute DNA from colum.
22) Perform elution step a second time (with less volume if desired)
23) Combine eluted DNA from all 5 columns in 1 tube (~175 uL-300uL)
24) Measure DNA concentration
25) Store samples at -20 C.