Cloning CRISPR gRNA target sequences

gRNA sequences targeting regions of interest should be obtained using an appropriate CRISPR design tool. It is recommended to test multiple gRNA sequences per gene of interest. For cloning, two oligos are designed and synthesized per gRNA. Oligos are then annealed to each other followed by ligation with the desired vector.

Reagent list:

Target sequence oligo design and preparation

The gRNA targeting sequence (without PAM) is synthesized using two complementary oligos with overhangs compatible for cloning.

1. Using the format below, synthesize two oligos for each gRNA design. Oligos can be synthesized at 10nM scale with standard de-salting purification. Resuspend each oligo to a final concentration of 10uM.

   Note: The sequence below includes an underlined G and 19 N's. The design is consistent with a 20 nucleotide target sequence that includes a native G in the first nucleotide. The G is necessary for transcription initiation from a U6 promoter. If target sequences are not available with a native G then insert the select 20 nt design where the 20 N's are shown.

   gRNA oligo 1 - 5' - CACC**G**NNNNNNNNNNNNNNNNNNN
   3' - **C**NNNNNNNNNNNNNNNNNNNCAAA – 5' - gRNA oligo 2

2. Prepare annealing buffer containing the following components:

Component	Final Concentration
Tris (pH 8.0)	10mM
NaCl	50mM

3. Combine the following components in a tube or 96 well PCR plate compatible with a thermal cycler.

Component	Volume (µl)
Annealing Buffer	96
gRNA oligo 1	2
gRNA oligo 2	2

4. Mix oligos and anneal in a thermal cycler using the following program:
   95°C for 5 minutes, ramp down to 25°C at 5°C per minute, hold at 25°C

Vector preparation

1. Digest up to 3 ug plasmid DNA from desired transEDIT vector using BsmBI restriction enzyme (NEB cat#: R0580L). Combine the following components in a tube or 96 well PCR plate compatible with a thermal cycler.

Component	Volume (µl)
pCLIP (plasmid DNA)	--
10X Buffer 3.1	5
BsmBI	1
Water	--
TOTAL	50

2. Incubate in thermal cycler at 55°C for 2 hours.

3. The BsmBI digested plasmid will result in two fragments of 1.9 kB and a larger fragment of ~9-10 kB (depending on transEDIT vector used). Gel purify the larger fragment using an appropriate column purification method.

Ligation of annealed oligos with transEDIT vector

1. Combine the following components for ligation reaction (T4 ligase, NEB cat#: M0202L; T4 PNK, NEB cat# M0201L) in a tube or 96 well PCR plate compatible with a thermal cycler:

Component	Volume (µl)
Digested vector (50ng)	--
Annealed gRNA oligos	2
10X T4 DNA ligase reaction buffer	1
T4 DNA ligase	0.5
T4 PNK	0.5
Water	--
TOTAL	10

2. Incubate in thermal cycler at 16°C for 1 hour.

Transformation and gRNA sequence verification

1. Combine 2 µl from above ligation reaction with 20 µl chemically competent cells (Invitrogen cat #: 10268-019). Follow manufacturer's protocol for transformation with chemically competent cells. After heat shock, keep transformation mixture on ice for at least 2 minutes. Add 50 µl of recovery media followed by incubation at 37°C for 1 hour, shaking at 200 rpm.
2. Plate entire volume from the transformation on LB+ ampicillin agars. Incubate overnight at 30°C.
3. Pick at least 3 colonies per construct for sequence verification.
4. Verify inserted gRNA sequence using the following sequencing primer:
   5' – ACTATCATATGCTTACCGTAAC - 3'