July 8, 2020

dbGuide – Protocol reference for designed oligos

Upon clicking the "Design" button, a text file will be downloaded onto your computer with the following format:

```
# oligos for ligation
EGFR-Guide-1-F  CACC G TCTTAATTCCTTGATAGCGA
EGFR-Guide-1-R  AAAC TCGCTATCAAGGAATTAAGA C
EGFR-Guide-2-F  CACC G GAGGATGTTCAATAACTGTG
EGFR-Guide-2-R  AAAC CACAGTTATTGAACATCCTC C


# oligos for in vitro transcription
T7_Rev_Long
AAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAAC
T7_Fwd_Amp      GGATCCTAATACGACTCACTATAG
T7_Rev_Amp      AAAAAAGCACCGACTCGG
EGFR-Guide-1-IVT-F      GGATCCTAATACGACTCACTATAG TCTTAATTCCTTGATAGCGA GTTTTAGAGCTAGAA
EGFR-Guide-2-IVT-F      GGATCCTAATACGACTCACTATAG GAGGATGTTCAATAACTGTG GTTTTAGAGCTAGAA


# target site information
EGFR-Guide-1    TCTTAATTCCTTGATAGCGA    hg38:chr7:55174759-55174781
EGFR-Guide-2    GAGGATGTTCAATAACTGTG    hg38:chr7:55142353-55142375
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For each guide selected, the oligonucleotide sequences are listed for either using the guides in a plasmid format or using them in a way to generate a PCR template for in vitro transcription (IVT). The final listing below gives the guide RNA spacer sequences along with the genomic location of the target site.

Cloning of guide RNAs into plasmids

Table 1. List of some compatible sgRNA backbones to clone for SpCas9

Addgene#	*Description*	*Cas9*	*Selection marker*	*Enzyme*
52962	Lenti-CRISPR-V2	Yes	Puromycin	BsmBI
98291	Lenti-CRISPR-V2-Hygromycin	Yes	Hygromycin	BsmBI
98292	Lenti-CRISPR-V2-Neomycin	Yes	Neomycin	BsmBI
98293	Lenti-CRISPR-V2-Blasticidin	Yes	Blasticidin	BsmBI
82416	Lenti-CRISPR-V2-2A-GFP	Yes	GFP	BsmBI
99154	Lenti-CRISPR-V2-2A-mCherry	Yes	mCherry	BsmBI
48138	pX458 - Cas9-2A-GFP	Yes	GFP	BbsI
52963	Lenti-Guide-Puro	No	Puromycin	BsmBI
64046	pSB700 - Lenti-Guide-mCerulean	No	mCerulean	BsmBI

Table 2. List of necessary reagents

Reagent	*Vendor*	*Catalog #*	*Alternatives*
T4 Polynucleotide kinase (PNK)	NEB	M0201S	
Quick/rapid T4 ligase	NEB	M2200S	Enzymatics (
BsmBI-v2	NEB	R0739S	Thermo Fisher
BbsI-HF*	NEB	R3539S	Thermo Fisher
Gel extraction kit	Zymo	D4007	Qiagen (
NEB Stbl	NEB	C3040H	Invitrogen (

*for pX458

Step 1: Choose backbone plasmid and digest/gel extract

Table 1 lists some backbones present in the Addgene collection that are compatible with the oligos designed.

Step 2: Digestion and gel extraction of plasmid backbone

Digest plasmid with appropriate enzyme listed next to the backbone plasmid. Digest for 1-2hrs and run on a 1% agarose gel and cut out of gel. A recommended amount of plasmid to digest is 1 to 2 ug. Table 3 lists a digestion reaction with BsmBI.

Table 3. Plasmid digestion

Component	*Volume*
1 – 2 ug of plasmid	x
Buffer 3.1	5 uL
BsmBI-v2 enzyme	1 uL
Water	44 - x
Total	50 uL

55C for 1-2hrs

Step 3: Annealing and phosphorylation of oligos

For each guide RNA to clone, anneal and phosphorylate the “F” and “R” oligos for subsequent ligation. First, re-suspend oligos in TE buffer to a final concentration of 100 uM. Use the following reaction mixture:

Table 4. Annealing and phosphorylation of oligos

Component	*Volume*
100 uM F oligo	1 uL
100 uM R oligo	1 uL
T4 PNK	0.5 uL
10X T4 PNK buffer	1 uL
Water	6.5 uL

Protocol: 37C for 30mins; 95C for 5mins; Ramp down 0.1C/s to 25C

Step 4: Ligation of annealed product into digested backbone

First, dilute the annealed oligo by 1:150 with water or TE buffer. Ligate annealed product into digested backbone using the following reaction:

Table 5. Quick/rapid T4 ligation reaction

Component	*Volume*
10X buffer	1 uL
Quick T4 ligase	0.5 uL
Annealed oligo (1:150)	1 uL
Digested backbone (25-50ng)	x uL
Water	7.5-x uL
Total	10 uL

Incubate at room temperature for 5mins

Step 5: Transformation of competent cells

Take 1 uL of ligation reaction and transform NEB Stable/Invitrogen Stbl3 cells and follow manufacturer's transformation protocol.

It is also recommended to do a transformation of the digested backbone (with no insert) to validate digest was complete and no colonies are seen the next day. It is also recommended to plate different dilutions of your recovered cells as you may see different transformation efficiencies with each batch of digested plasmid.

In vitro transcription of guide RNAs for Cas9 RNP production

Protocols for the production of *in vitro* transcribed guide RNAs by run-off transcription can be found here:

https://www.protocols.io/view/In-vitro-transcription-of-guide-RNAs-dwr7d5

https://www.protocols.io/view/in-vitro-transcription-of-guide-rnas-and-5-triphos-bfxfjpjn