CRISPR: sgRNA design and synthesis

Helen Willsey Lab
Updated: August 23, 2023 by Jean Dea
Adapted from: NEB Engen sgRNA synthesis protocol

WILLSEY LAB

This protocol was developed with lots of support from the Khokha lab and the NXR. Please cite this, this and this.

Reagents:

- EnGen sgRNA Synthesis Kit (NEB Product # E3322)
- Zymo RNA Clean & Concentrator kit (Genesee Product # 11-352B)
- Cas9-NLS protein (MacroLabs, UC Berkeley)
- Comes at 6.4mg/ml in 20 mM HEPES-KOH pH 7.5, 150mM KCl, 10% glycerol, 1mM DTT in 10µl aliquots at -80oC. To each aliquot, add 10µl pure pure water, then aliquot as 1.05µl single-use tubes (1.05µl = 2.2µg)
- Dextran-Alexa555 (Thermo Fisher Product # D34679)

1. DNA oligo design

5' CTAGCTAATACGACTCACTATAG$(N)_{20}$GTTTTAGAGCTAGAAATAG 3'
T7 promoter, target, crRNA complement

At least one G must be present at the 5' end of the guide (blue)
sgRNA does NOT contain the PAM sequence.

*** go to crisprscan.org, then click genome tracks at the top, then click Xenopus tropicalis. Search your gene. Pick a bright green crispr (bright green = no/few off-targets) that targets an early exon with a high score (>60). Click on it and it will tell you the target sequence and the oligo to order.

2. sgRNA Synthesis (EnGen kit)

☐ At room temperature in the order listed (avoid master mixes):

☐ RNase-free H_2O	2 µl
☐ EnGen 2X sgRNA rxn mix	10 µl
☐ DNA Oligo (**1uM**)	5 µl
☐ DTT (0.1M)	1 µl
☐ Enzyme Mix	2 µl
	20 µl reaction

☐ Incubate at 37°C for at least 4 hours.
☐ Add 30µl RNAse-free H_2O and 2µl DNase I, mix and incubate at 37°C for 15'.

3. RNA purification (Zymo RNA Clean and Concentrator)

☐ Add 2 volumes (100 µl) RNA Binding Buffer to each sample and mix.
☐ Add an equal volume (150 µl) of ethanol (95-100%) and mix.
☐ Transfer to Zymo Column in Collection Tube, spin 30 seconds. Discard flow-through.
☐ Add 400 µl RNA Prep Buffer and centrifuge for 30 seconds. Discard flow-through.
☐ Add 700 µl RNA Wash Buffer and centrifuge for 30 seconds. Discard flow-through.
☐ Add 400 µl RNA Wash Buffer and centrifuge for 2 minutes.
☐ Transfer the column into an RNasefree tube. Add 15 µl RNase-Free H_2O directly to the column matrix and centrifuge for 30 seconds.

☐ Nanodrop, dilute to 830 ng/µl in RNase-free H_2O, run 1 µl on gel, and aliquot in single-use volumes (1.5 µl) at -80°C.

4. Injection

Mix:

☐ Cas9-NLS protein (2.2 µg)	1.05 µl
☐ Dextran-555 (2.5 µg**)	0.5 µl
☐ sgRNA (1.2 µg total; 828ng/ul)	1.45 µl
	3 µl

**adjust KCl of dextran so that final injection mix is ~300 mM salt to prevent needle clogging

We mix 20 µl dextran-555 with 20 µl 3.6 M KCl

(3.6 M KCl is in a 15ml Falcon on Helen's bench)

X. tropicalis: Inject 2nl (8 hashmarks) = 1.5 ng Cas9 + 800 pg sgRNA

X. laevis: Inject 4nl (10 hashmarks) = 3ng Cas9 + 1.6ng sgRNA

Target animal pole, as that is where the nucleus is.

PROTOCOL A (Younger than Stage 44, or if more penetration is needed)
Protocol:

- [ ] 1.
- [ ] 2.
- [ ] 3.

PROTOCOL B (Stage 44+)

Protocol:

1) (but make the blocking step overnight, dilute antibodies in 10% CAS in PBT, and then primary is 2hr RT)

PROTOCOL C (Quick cilia staining)
Protocol: