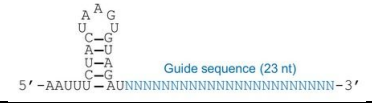
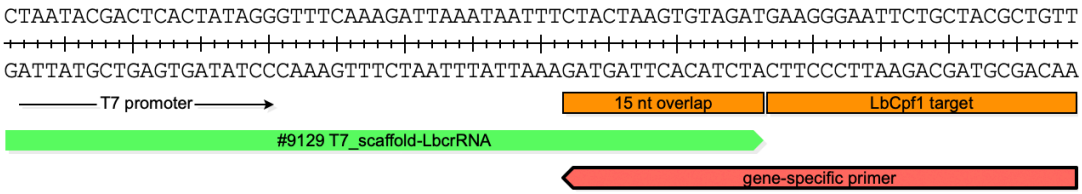
Lawson Lab Masahiro Shin, 10/06/2017

Preparation of crRNA for Cas12a

We use PCR to generate DNA templates, which are then used for *in vitro* synthesis using T7 RNA polymerase. For explanation of "DR-crRNA" and "GC swap", see Liu et al. PMID: 30892626

		tail sequence 5'→3'	5' PAM	Guide length
LbCpf1		GG UAAUUUCUACUAAGUGUAGAU	TTTV (V= G, C or A)	23nt
FnCpf1		GG UAAUUUCUACUGUUGUAGAU	TTN	23nt

Example with LbCas12a and **full-length DR-crRNA** sequence targeting *albino*



Oligos to order:

Top, with T7 promoter and crRNA hairpin:
CTAATACGACTCACTATAGGGTTTCAAAGATTAAATAATTTCTACTAAGTGTAGAT

Bottom, with 15 nucleotide overlap and reverse complement of target site
AACAGCGTAGCAGAATTCCCTTCATCTACACTTAGTAG

Top Oligo for **GC-swap/full-length DR-crRNA**
CTAATACGACTCACTATAGG GTTTCAAAGATTAAATAATT**C**C**C**ACTAAGTGT**G**G**G**T
→ *improved crRNA for LbCpf1, with GC swap*
→ *this is current standard in Lawson Lab*
→ *gene-specific spacer is reverse complement and should include sequence complementary to sequence in green*
→ make sure to incorporate the swap in the bottom oligo!!!

For use with **FnCpf1**
#10000 Fn-crConstant1 (aka CRf13-cr 5p(constant)):
TAATACGACTCACTATAGGGTCTAAGAACTTTAAATAATTCCCACTGTTGTGGGT

Fn-CRf13-cr 3p(target-specific; spacer)
NNNNNNNNNNNNNNNNNNNNNNACCCACAACAGTGGGAATTA

PCR for DNA template

1 µL 200µM #9129 1 µL 200µM your primer 1.2 µL 10mM dNTPs 12 µL 5x Phusion Buffer 44.2 µL H_2O 0.6 µL Phusion 60 µL	1. 98°C 15' 2. 98°C 10' 3. 60°C 15' 4. 72°C 5' 5. 40 cycles (2.3.4.) 6. 72°C 60'

Check 3 µL 3% TBE gel (Agarose 1000, Invitrogen) using small comb.

Precipitate template
57 µL PCR reaction
6 µL 3M NaOAc
150 µL 100%EtOH
1 µL Glycogen (2 mg/mL)

at -80°C for 5min
centrifuge with max speed for 30min at 4°C
aspirate, wash with 70%EtOH, aspirate completely and let air dry
resuspend in 25 µL H_2O
Concentration should be around 400 ng/µL (check on NanoDrop)
Optional: re-check 1µl of crRNA on 3%TBE gel

crRNA synthesis

use 800 ng of DNA template in a half-reaction (10 µL total)
follow instruction from T7 MEGAscript kit (Ambion/Thermofisher) with following modifications:

1. reaction is incubated for at least 5hr
2. perform DNaseTURBO incubation for 40min for complete DNA digestion
3. use recommended phenol/chloroform/isopropanol purification (see Megascript kit protocol)
4. final pellet must be dried completely at RT after isopropanol precipitation
5. make sure pellet is completely suspended in water by pipetting up and down 20 times

add 30 µL H_2O to pellet and check on **fresh** 3% TAE (Agarose 1000, Invitrogen) gel after resuspension; concentration should be range between 1000 and 2000 ng/µL (NanoDrop)

Injection

prepare 5 µL injection solution

1893ng of Dr_crRNA 1 µL LbCas12a* (40µM) 0.5 µL 0.1% Phenol Red X µL H_2O 5 µL injection solution	incubate at room temperature for 20min prior to injection	1nL = 8 fmoles of LbCpf1/DrcrRNA complex (RNP) per embryo

* - purified recombinant protein from **pET-21a-2xNLS-LbCpf1 (Addgene #114366)**; can also use Cas12a from New England Biolabs (M0653), though it is less active than ours.

→ immediately following injection, incubate embryos at 35°C for 4 hours.