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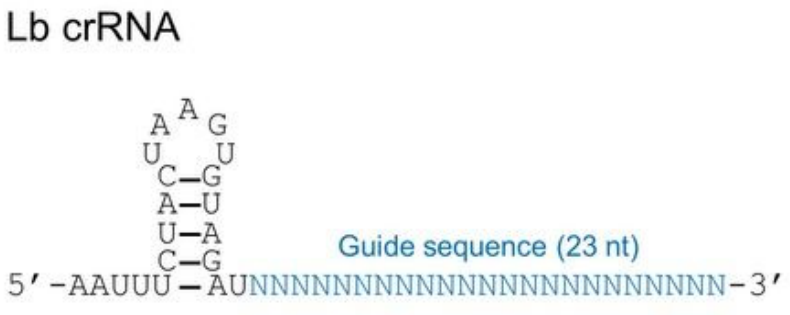
Preparation and injection protocol for crRNA with LbCpf1

	tail sequence 5'=>3'	5' PAM	Guide sequence length
AsCpf1	GG UAAUUUCUACUCUUGUAGAU	TTTN	23nt
LbCpf1	**GG UAAUUUCUACUAAGUGUAGAU**	**TTTV (V= G, C or A)**	**23nt**
FnCpf1	GG UAAUUUCUACUGUUGUAGAU	TTN	23nt

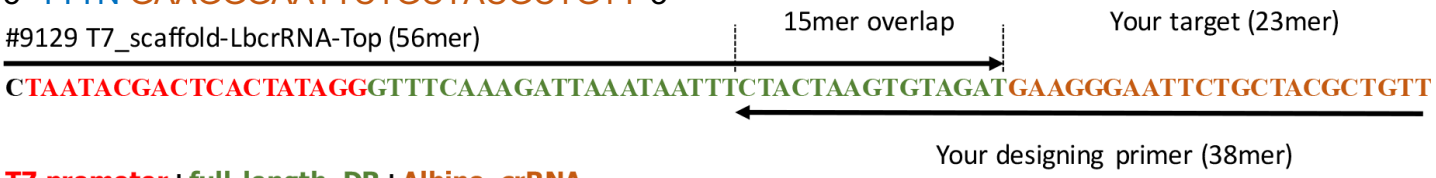
Make DNA template for "dr"-crRNA (see Liu et al., NAR 2020)

- for target design we use CHOPCHOP (http://chopchop.cbu.uib.no)
- we no longer use the "GC swap", as described in Liu et al.
- across multiple targets we have found the extended "dr"-crRNA works best
- example below is for LbCas12a, just swap backbone sequences above as needed if you want to use As or FnCas12a, both of which work in zebrafish.



Example (LbCas12a):

PAM + Target sequence (*albino* locus):
5'-TTTN GAAGGGAATTCTGCTACGCTGTT-3'



T7 promoter+full-length_DR+Albino_crRNA

Top oligo: #9129 T7_scaffold-LbcrRNA-Top (common to all targets):
5'-CTAATACGACTCACTATAGG GTTTCAAAGATTAAATAATTT**CTACTAAGTGTAGAT**-3'
(Primer for making Lb_drcrRNA. Bold letters indicate overlapped to reverse complement primer)

Bottom oligo: *albino*_LbCpf1_drcrRNA Bottom:
5′-AACAGCGTAGCAGAATTCCCTTC **ATCTACACTTAGTAG**-3′

PCR for DNA template

3 µL 200µM top oligo 3 µL 200µM bottom oligo 1.2 µL 10mM dNTPs 12 µL 5x Phusion Buffer 40.2 µL H_2O 0.6 µL Phusion HF (M0530, NEB) 60 µL	Cycling: 1. 98°C 15 sec 2. 98°C 10 sec 3. 60°C 15 sec 4. 72°C 5 sec 5. 40 cycles (2.3.4.) 6. 72°C 60 sec

- run 3 µL on 3% TBE gel (Ultra-pure Agarose 1000, ThermoFisher: 16550100)

Column purify using a Zymogen Gel purification kit (Zymo Research, D4007)
57 µL PCR reaction + 200µL Dissolving buffer, spin at max, 1 minute
Aspirate and add 200µL wash buffer, spin at max 1 minute, repeat 1x
Spin at max to remove residual wash and aspirate.
Elute with 12µL nuclease free dH_2O into RNAse-free tube.
Quantify by NanoDrop (concentration usually around 100~150ng/µL)

crRNA synthesis

use 250~375 ng of DNA template
follow instruction from T7 MEGAscript kit (ThermoFisher, AM1333) with some modifications:

1. Perform half reactions (10 µl)
2. Incubate at 37°C >5hr, overnight is strongly recommended
3. Perform DNaseTURBO (1 µl) incubation for 40 min
4. Clean up with phenol:chloroform and isopropanol precipitation as described in MEGAscript kit
5. final pellet must be dried completely at RT after 80% EtOH wash
6. make sure pellet is completely suspended in water by pipetting up and down 20 times

- resuspend pellet in 20 µL nuclease free dH_2O
- determine concentration by Nanodrop (usually between 1000 and 2000 ng/µL)

Injection

We routinely test activity through injection followed by PCR and enzyme digestion, or TIDE analysis (https://tide.deskgen.com) or ICE analysis (https://ice.synthego.com).

Cas12a MUST be injected as a ribonucleoprotein complex (RNP) to be active in zebrafish.

Prepare ribonucleoprotein complex

1893ng of drcrRNA
1 µL LbCpf1 (diluted to 33µM) (M0653T, NEB, or home-made)
0.5 µL 0.05% Phenol Red
X µL H_2O to 5µL
5 µL injection solution

- incubate at RT for 20min before injection
- 2nL injection gives you 13 fmoles of LbCpf1/crRNA complex (RNP) per embryo
- CRISPR injections are pretty tolerant of variable injection location in the embryo. However, if you are co-injecting with a DNA template for germline transmission we recommend injecting directly into the cell as early as possible.
- **IMPORTANT: Incubate injected embryos at 34ºC for 4hrs immediately following injection, then return to 28°C.**
- **IMPORTANT:** if you are co-injecting Cas12a with a DNA template, make sure to add the DNA template last and do NOT spin after adding the DNA.