Generation of gRNAs for multiplexed targeting

This protocol is for synthesizing multiple guide RNAs for G0 phenotypic screening. It is adapted from PMID: 27809318 and uses Phusion polymerase, the Zymo DNA Clean and Concentrator-5 kit, and the Zymo RNA Clean and Concentrator-5 kit.

Note: Please ensure your top strand oligos are compatible with the bottom strand ultramer. The bottom strand ultramer sequence is:
`AAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAAC`

Assemble oligos

1. Prepare working stocks of top oligos with water to a concentration of 10 μM.
2. Mix equimolar concentration of top oligos (10 μM each). Up to 12 can be used in one reaction. E.g. mix 10 μl of each oligo together. Vortex and brief centrifuge.
3. Set up the following oligo assembly reaction in a PCR strip:

2 x Phusion buffer +dNTPs	25 μl	1x
Top strand oligo mix (10 μM)	1 μl	0.2 μM
Bottom strand ultramer (10 μM)	1 μl	0.2 μM
Phusion DNA polymerase	0.5 μl	
Nuclease-free water	22.5 μl	
Final	**50 μl**	

Note: the Bottom strand ultramer is found in the communal gRNA reagents box.
If synthesizing several, first make up a mastermix containing everything except the top strand oligo mix.

4. Anneal and extend using the thermocycler:

98°C	2 min
50°C	10 min
72°C	10 min
4°C	hold

Note: this program is in the **General** folder, named **GRNA_ASS**

Purify assembled oligos (DNA Clean & Concentrator-5 kit, Zymo Research)

5. Transfer assembled oligo from PCR strip to 1.5 ml tube and add 250 μl of DNA binding buffer.
6. Mix briefly by vortexing then spin down briefly to collect contents in bottom of tube.
7. Transfer mixture to a Zymo-Spin Column in a collection tube.
8. Centrifuge in desktop centrifuge at max speed for 30 seconds. Discard flow through.

9. Add 200 µl of DNA Wash Buffer to the column (check ethanol has been added to the wash buffer). Centrifuge at max speed for 30 seconds and discard flow through.
10. Add another 200 µl of DNA Wash Buffer, centrifuge for 30 seconds and discard flow through.
11. Transfer column to a fresh 1.5 ml tube (RNase-free); check there is no residual ethanol by dabbing the bottom of the column on your glove. If there is, spin again.
12. Add 6 µl of RNase-free water directly to the column matrix. Incubate at room temperature for one minute.
13. Centrifuge for 30 seconds to elute the DNA.

Quality Control: agarose gel

14. Run 1 µl of purified assembled oligos on a 2% (wt/vol) agarose gel alongside 100 bp or 1 kb plus ladder. Correctly assembled oligos appear as 117-120 bp bands. This is an optional step and not necessary for experienced users.

In vitro RNA synthesis

From this point forward, use RNase-free solutions, tips, and technique. Clean work space with RNaseZAP, use fresh gloves etc.

15. Using NEB T7 High Yield kit set up an NTP Buffer Mastermix containing buffer and each of the NTPs:

10 x buffer	1 µl	2x
NTPs (100 mM)	1 µl	20 mM each
Final	**5 µl**	

16. Set up T7 reaction mix in a PCR strip:

2 x NTP buffer mix	5 µl	1x
Assembled oligo	4 µl	
T7 RNA polymerase	1 µl	
Final	**10 µl**	

17. Incubate for 4 hours at 37°C.

17. Add 0.5-1 µL DNase

18. Incubate at 37°C for 15 mins, then bring volume to 50 µL with nuclease-free water

Purify sgRNA (RNA Clean & Concentrator-5 Kit, Zymo Research)

18. Transfer the contents of the in vitro RNA synthesis to a 1.5 ml RNase-free microfuge tube.
19. Add 2 volumes of RNA binding buffer (e.g., 100 µL into a 50 µL rxn)

20. Add an equal volume of 200 proof EtOH (e.g., 150 µL). Mix by agitating the solution. Briefly spin to collect contents.
21. Transfer to a Zymo-spin column and collection tube, centrifuge for 30 s, discard flow-through
22. Add 400 µL of RNA prep buffer, spin 30 s, discard flow-through
23. Add 700 µL RNA wash buffer (check ethanol has been added), spin 30 s, discard flow-through
24. Add 400 µL RNA wash buffer, spin 30 s, discard flow through
25. Spin dry for 2 min
26. Transfer column to a fresh RNase-free microfuge tube (check there is no residual ethanol), and elute in 15 µL DNase/RNase free H_2O by 30 s centrifugation.

27. Separate into aliquots (e.g., 5 aliquots 3 µL each), flash freeze all but 1 or 2 in liquid N_2 or dry ice and store at -80ºC.

Quality Control: agarose gel and nanodrop

28. Run 1 µL (or 1 aliquot) of purified RNA on a 2% (wt/vol) agarose gel alongside 100 bp or 1 kb plus ladder. Correctly assembled RNA appears as two 117-120 bp bands.

29. Nanodrop 1 µL of purified RNA to obtain concentration. 260/280 should be ≥2.