gRNA synthesis and purification

Material

- Maxiscript T7 transcription kit (Invitrogen AM1312)

I: Synthesis of gRNA through PCR

1. Order 80-mer universal constant oligo (with HPLC purification) for all genes.
   AAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAA
   CTTGCTATTTCTAGCTCTAAAAC

2. Order 60-mer variable oligos for individual genes.
   Example**:** Tyrosinase gene (TYR)
   Target sequence: GGTGTGCACTGATGCTCTGATGG

   Then the variable oligo sequence should be:
   *TAATACGACTCACTATA*GGTGTGCACTGATGCTCTGA*GTTTTAGAGCTAGAAATAGCAAG*

3. Anneal constant and variable oligos via PCR.
   a. PCR reaction composition and program:

1 ul	100 uM constant oligo
1 ul	100 uM variable oligo
1 ul	NEB buffer 2
7 ul	water

95’C	30 sec
72’C	2 min
37’C	2 min
25’C	2 min
12’C	2 min
4’C	forever

b. After reaction, add the following and run reaction for 20 min at 12'C
   0.5 ul dNTPs
   0.25 ul T4 DNA polymerase
   0.2 ul 100x BSA from NEB
   9 ul water

c. Run over PCR cleanup column and elute in 30 ul water
d. Nanodrop: Usually around 200-250 ng/ul

II: *in vitro* transcription

1. RNase-free the area, following 10 ul MAXIscript transcription reaction.
2. Perform everything on the ice, but shift 10x transcription buffer to RT, spermidine can go back into solution (put in 37'C bath for ~5 min, vortexed, and spun down to ensure completely dissolved in solution)
3. (Optional) if plan to pool multiple gRNAs, mix the DNA duplex of each gRNA here, and calculate the final concentration of the mixture.
4. Add the following components in the following order:
   # ul water to make the final volume 10 ul
   # ul DNA duplex, make it 0.5 to 1 ug
   1 ul 10X transcription buffer
   0.5 ul 10 mM ATP
   0.5 ul 10 mM CTP
   0.5 ul 10 mM GTP
   0.5 ul 10 mM UTP
   1 ul Enzyme Mix
5. Mix well, and incubate at 37'C for 1 or 2 hours (1 hour should be enough)
6. Add 0.5 ul TURBO DNase, mix well, and incubate at 37'C for 15 min
7. Transfer solution from PCR tubes to 1.5 ml Eppendorf tubes, for later steps of RNA precipitation and centrifugation
8. Add 15 ul water, 2.5 ul (0.1x volume) ammonium acetate stop solution, and 0.5 ul glycogen blue to the reaction solution.
9. Mix well, then add 90 ul (3x volume) 100% EtOH, and then mix again. Visible precipitation should be observed at this stage
10. Store at -20'C for 4 hours to overnight
11. Centrifuge at high speed at 4'C for 15 min, and then carefully remove supernatant
12. Add 500 ul 70% EtOH for one more wash step. Centrifuge at high speed at 4'C for 5 to 15 min, and then carefully remove supernatant

13. Leave the Eppendorf cap open at room temp for 15 minutes or more, until all remaining EtOH in the tube evaporates.
14. Resuspend in 30 ul water. Be sure all precipitation is dissolved.
15. Nanodrop. Readjust the concentration if needed.
16. Confirm by running on gel
17. Aliquot into PCR strips and freeze at -80’C.

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