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!!! RNase-free working environment is required for the whole procedure!!!

sgRNA preparation

. Amplify the template for *in vitro* sgRNA transcription

1) Oligos need to be ordered:

Template: TAATACGACTCACTATAGGGGCCACTAGGGACAGGATGTTTT AGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAA AGTGGCACCGAGTCGGTGCTTTTTT

Fw: ggcactcTAATACGACTCACTATAGG

Rv: cggagcgAAAAAAGCACCGACTC

2) PCR reaction mix:

!! 8-16 reactions will be pooled together.

Template (10ng/μL)	1μL
Fw (10μM)	1μL
Rv (10μM)	1μL
5x HF buffer	4μL
dNTP (10mM)	0.4μL
DMSO	0.6μL
Phusion	0.2μL
H_2O	11.8μL
Total reaction volume	20μL

3) PCR cycles:

98°C	20s	
98°C	10s	|
60°C	25s	|x34
72°C	5s	|
72°C	3m	
4°C	∞	

4) PCR purification

! Use one column to bind DNA.

! Elute with 20μL Nuclease-free water.

!! The expected concentration should be more than 300ng/μL.

! Adjust the concentration to 250ng/μL as the template of the sgRNA synthesis.

. *In vitro* sgRNA synthesis using MEGAshortscript™ T7 Kit

Thaw the T7 10X Reaction Buffer at room temperature by vortexing.

CRITICAL!!! Make sure there is NO DTT crystal remaining in the T7 10X Reaction Buffer.

When a new kit is opened, thaw the T7 nTP Solutions, transfer 50μL of each T7 nTP Solution into a new tube, well-mixed and labeled as 'T7 nTP Mix'

!! Assemble the reaction mix (in PCR tube) at room temperature, in the following order:

!! 3 reactions will be pooled together.

Nuclease-free water	7μL
T7 10X Reaction Buffer	2μL
T7 nTP Mix	8μL

Template DNA (250ng/μL)	1μL
T7 Enzyme Mix	2μL
Total reaction volume	20μL

Mix thoroughly by pipetting or flicking the tube, then centrifuge the tube briefly.
Incubate in a thermocycler at 37°C for 4 hours.

. DNase treatment

sgRNA synthesis reaction	20μL
TURBO DNase	1μL

Mix well by pipetting or flicking. Incubate at 37°C for 15min
! After DNase treatment, proceed immediately to the purification procedure. No DNase inactivation needed!

. sgRNA purification using MEGAclear™ Kit
Set the heating block to 95°C, pre-heat elution buffer in a 1.5mL eppendorf.

1. Pool 3 DNase-treated samples together (in total 60μL), add 40μL of Elution Solution (4°C, NOT the pre-heated!!!). Vortex briefly to mix thoroughly. Briefly spin the tube.
2. Add 350μL of Binding Solution Concentration (4°C) to the sample. Mix gently by pipetting up and down immediately after adding.
3. Take 100% Ethanol from -20°C. Add 250μL of cold 100% ethanol to the sample. Invert the tube few times to mix thoroughly. Stand on ice for 1min, and invert again for few times.
   NOTE: This is the critical step, directly affecting the final yield!!!
   1). The cold ethanol is very important for this step, to precipitate the RNA!!! So, take your 100% ethanol from -20°C just before you start on this step
   2)Because ethanol precipitates the RNA, after thoroughly mixing, the solution can become opaque suspension. DO NOT SPIN THE SAMPLES AFTER MIX!!!
4. Load the sample to a filter with a collection tube.
5. Centrifuge for 30s at 12000xg at r/t. Remove the flow-through.
6. Wash the filter 2 times with 500μL of Washing Solution (4°C). Centrifuge for 30s at 12000xg at r/t. Discard the flow-through.
7. Centrifuge again for 1min at 12000xg to completely remove the Washing Solution.
8. Remove the filter to a new collection tube.
9. Apply 50μL of pre-heated Elution Solution to the center of the filter. Centrifuge for 1min at 12000xg at r/t.
   NOTE: pipetting the pre-heated elution buffer at the heating block, in stead of taking the pre-heated elution solution to the RNA hood.
10. Apply another 50μL of pre-heated Elution Solution to the same filter. Centrifuge for 1min at 12000xg at r/t.
11. Add 10μL of 5M NH_4Ac to 100μL of the eluted sample. Vortex to mix well and spin down briefly.
12. Add 275μL of cold 100% ethanol (from -20°C). Mix well by inverting the tube for few times.
13. Stand the tube at -20°C for at least 30min. (Normally I do it for 45 min)
14. Centrifuge at maximum speed for 15min at 4°C.
15. Carefully remove the supernatant by pipetting, without disturbing the pellet.
16. Add 500μL of cold 70% ethanol (from -20°C). Centrifuge at maximum speed for 5min at 4°C.

17. Remove most of the supernatant by pipetting. Centrifuge again, at maximum speed for 1min at 4°C. Carefully remove the rest of supernatant by pipetting.
18. Air-dry the pellet, 5-8 mins.
    !!! DO NOT over-dry the pellet!!! When the pellet is dried, the body of the pellet should remain white, whereas the edge of the pellet turns transparent.
19. Dissolve the pellet in 40µL (varies depending on the size of your pellet) Nuclease free water.
20. Store sgRNA samples at -80°C, after checking the concentration*.

*For checking the concentration with Nanodrop:

sgRNA	2µL
H_2O	38µL
In total	40µL

Mix thoroughly, and spin down briefly.
Check the concentration with Nanodrop (RNA-40).

!!! From 3 reactions, the expected yield should be more than 150µg, or with the minimum concentration above 3.5µg/µL.

. Prepare sgRNA for the assay

Dilute the sgRNA to 1µM (1pmol/µL, 31ng/µL).
To cut 100fmol substrate with 1pmole CAS9, 3-4pmol of sgRNA is recommended.
NOTE: repeatedly freeze and thaw the sgRNA samples can sharply decrease quality. We suggest to aliquot you sgRNA samples in working concentration and store at -80°C.