HEK293T Transfection Protocol

1. Make HEK293T growth media:

Reagent	Manufacturer	Catalog No.	Volume
GlutaMAX	Gibco	35050	5 mL
Penicillin-Streptomycin	Gibco	15140-122	5 mL
FBS	Gibco	26140-079	50 mL (10% final)
DMEM	Gibco	11965-084	Fill to 500 mL

   Use a 0.2 µm filter to sterilze media. Store at 4 °C protected from light.

2. Day -1: Plate 350,000 HEK293T cells/plate in 60 mm tissue culture dishes. Incubate at 37 °C, 5% CO_2.
   a. To prevent cells from concentrating in the center of the dish, we allow the cells to settle for up to one hour before placing them in the incubator.
   b. We found that harvesting DNA/RNA/Protein from an individual 60 mm dish provided sufficient sample to analyze.
3. Day 0: Transfect the cells.
   a. Check the cells. The cells should be about 30% confluent (or slightly higher) and have a normal morphology. This is lower than most transfection protocols call for; however, 293T cells grow very quickly and this low confluence is necessary to reach Day 4 time points without passaging the cells.
   b. Dilute the plasmid DNA in OptiMEM (Gibco 31985-070). For each 60 mm dish use a total of 5.5 ug of DNA. Reserve 0.5 or 0.7 ug of plasmid mass for the pMaxGFP plasmid; the lower mass is used when transfecting many sgRNA so more sgRNA plasmid copies are available for transfection. Divide the remaining mass equally between the dCas9 fusion plasmid and the sgRNA. When transfecting more than sgRNA, divide the mass allocated for sgRNA equally amongst each sgRNA plasmid will use. This plasmid master mix will be used to transfect all plates for this particular experimental condition.

   An example master mix for one 60 mm dish with three sgRNA (g1A, g33A, and g7A) appears below.

sgPool + Cas9-DNMT3A		ug for	uL for
Plasmid	ug/uL	each dish	each dish
Cas9-DNMT3A WT	0.455	2.400	5.27
g1A	0.22	0.800	3.64
g33A	0.13	0.800	6.15
g7A	0.153	0.800	5.23
pMax-GFP	1	0.700	0.70
OptiMEM			1059.76
Lipofectamine LTX			19.25
Total		5.500	1100.00
ug/mL	0.005		
Lipo LTX:DNA	3.5	(uL/ug)	

   c. While diluting the DNA, warm Lipofectamine LTX to room temperature. Then add an appropriate volume of Lipofectamine to each plasmid master mix. Do not add Plus reagent. Final Lipofectamine:DNA (ul:ug) ratio is 3.5. Total master mix volume should have 1100 uL/plate with a little extra to prevent pipetting error on the final pipetting step.
   d. Mix the master mixes well by inversion. Allow them to sit for 30 min.
   e. Add the Lipofectamine/DNA complexes to the cells dropwise. Then rock the plates gently by hand to distribute evenly.
   f. Place the cells back in the incubator. You do not need to change the media.

4. Day 1: Check on the cells. Take pictures as needed. This is a good time to gauge transfection efficiency via estimating the amount of GFP positive cells.
5. Day 2: Feed the cells by changing their media.
6. Day 4: Harvest samples. Cut the cells if continuing with the time course.
    a. Harvest DNA using 1 mL of gDNA lysis buffer from Zymo Research Quick gDNA Miniprep kit. Scrape lysate down with pipette tip. Transfer to a microcentrifuge tube and store at -20 °C until extraction.
    b. Harvest RNA using 600 uL of RNA lysis buffer from Zymo Research Quick RNA minprep kit. Scrape lysate down with pipette tip. Transfer to a microcentrifuge tube and store at -20 °C until extraction.
    c. Harvest Protein using Santa Cruz RIPA buffer (sc-24948). Trypsinize cells. Wash with 1 mL ice cold DPBS. Lyse with 600 uL or 1 mL of complete RIPA buffer (all additives diluted 1:100). Sonicate 5 times with 30 sec ice incubations between round of sonication. Pellet cell debris at 20,000 xg for 20 min. Remove lysate to a microcentrifuge tube and store at -80 °C.