NUCLEOFECTION OF CAS9/SGRNA RNPS INTO CELLS

ML 10/09/15; based on Lin S. Doudna JA. et al eLife 2014 PMID 25497837

Reagents: **nocodazole**: Sigma #M1404, 200 μg/mL (1000x) stock in DMSO

TCEP-HCl: Sigma #4706, dissolve to 100 mM in {20 mM tris pH 8.0} and adjust pH to 7.5

Cas9 buffer (high salt)

580 mM KCl	for 10 mL:	2.9 mL of 2 M KCl
40 mM Tris pH 7.5		260 μL 1 M Tris pH7.0 + 140 μL 1M Tris pH8.0
20% v/v glycerol		6.7 mL 30 % glycerol
2 mM TCEP-HCl		50 μL of 400 mM stock
2 mM $MgCl_2$		20 μL of 1 M stock

(all RNAse-free, use DEPC H_2O for stocks)

Amaxa 96-wp kit for nucleofection (Lonza #V4SC-2096)

1) Day 0: preparing cells

- Evening before nucleofection: split cells and seed at $0.25x10^6$ cells/mL in media + 200 ng/mL nocodazole

Typically: 12mL 293T @ $0.25x10^6$ cells/mL in 10cm plate; I use same concentration with HeLa or K562 cells and it works well

- Treat with nocodazole for 15-17h prior to nucleofection (shorter might be OK too, >20h seems to hurt viability)

2) Day 1: preparing RNPs

RNP preparation involves:
1) diluting sgRNA into Mg^{2+}-containing Cas9 buffer and heating to "refold" sgRNA
2) adding Cas9 protein to sgRNA to form RNP
3) adding HDR template to mix (if needed) and electroporate into cells

- If needed, dissolve HDR template oligo to 100 μM in DEPC H_2O

Typically add 40 μL to 4 nM Ultramer oligo, heat >5' at 70˚C and pipet ↓↑ to dissolve (sometimes need to heat more, some oligos are hard to get into solution)

- Thaw purified Cas9 on ice (the stock we have is 40 μM), thaw sgRNA (100 μM stock)

Typically we use 1.3x molar excess sgRNA:Cas9. 100 pmol final [RNP] usually gives best results (see Doudna paper), so we would use 130 pmol sgRNA + 100 pmol Cas9. HDR template is added at 100-150 pmol, if needed.

Calculate volumes of components to add:
(Final volume should be 10 μL)

sgRNA	ex: 1.3 μL of 100 μM stock (130 pmol)
Cas9	ex: 2.5 μL of 40 μM stock (100 pmol)
HDR template	ex: 1.5 μL of 100 μM stock (150 pmol)
Cas9 buffer	2 μL
DEPC H2O	to 10 μL, ex. 2.7 μL

- In sterile microcentrifuge tube: mix Cas9 buffer + H_2O + sgRNA, incubate at 70˚C for 5 min to "refold"

NB: heating might not be required here?…

- Slowly add Cas9 protein to diluted sgRNA, mix by pipetting and incubate at 37˚C for 10 min

- If needed: add HDR template to RNP mix and keep at RT until ready for nucleofection

3) Day 1: nucleofection

- Harvest and count nocodazole-treated cells after 17h treatment; will need 0.2 x10^6 cells per sample

NB: for adherent cells: nocodazole-treated cells will float, so trypsin is not necessary. Just shake the culture dish to detach cells and pipet out media to harvest cells. I then pipet ↓↑ the cell suspension a few times to break up clumps before counting. Typically will get ~0.15 x10^6 viable cells per mL from original culture.

- Prepare appropriate amount of Amaxa solution, room temp., in TC hood (need 20µL final per sample)
  For each sample: mix 16.4 µL SF solution + 3.6 µL supplement (make master mix)

NB: Amaxa solution comes in two components: SF solution and supplement; I prepare the required amount of final solution just before nucleofection as the mixed SF solution + supplement has limited shelf life.
I have used successfully the SF cell line kit (recommended for 293T cells) for HeLa and K562 also.

- Wash cells 1x in PBS then resuspend in 1 mL PBS and spin in microcentrifuge tube (5' at 500xg)

- At this point: 1) bring microcentrifuge tubes containing RNPs into TC hood
  2) prepare a 24-wp with 1 mL media/well and pre-warm in 37˚C incubator
  3) make sure Amaxa instruments and computer are turned on and ready to go

NB: Amaxa solution is toxic to cells. The idea here is to have everything ready to minimize the time cells will spend in Amaxa solution. From that point electroporation should be finished within 20 min…

- Resuspend cells in Amaxa solution, then add 20 µL cell resuspension to each 10 µL RNP tube and mix

- Carefully pipet cells/RNP mix into bottom of the nucleofection plate, avoiding bubbles. Tap bottom of plate against hood surface to insure liquid is at bottom.

- Bring to Amaxa, nucleofect cells using CM-130 program

NB: I have used that same program successfully for HeLa and K562 RNP nucleofection…

- Back to TC hood: use 100 µL media from pre-warmed 24-wp plate to resuspend and transfer nucleofected cells to 24-wp

4) Days 2-5: monitor recovery & harvest

Nucleofected cells sometimes look pretty unhappy at first but typically recover after 2-4 days. If splitting is needed: after nucleofection 293T attach only very loosely to the culture dish so be particularly careful not to lose cells while washing. Often I will just pipet cells ↓↑ in 24wp to detach them and split from this.

I usually proceed with gDNA PCR and NHEJ/HDR analysis after 5 days. My feeling is that cutting/repair still goes on 3 or maybe 4 days post-nucleofection, so harvesting cells at day 5 might be a good idea to see maximal effects