Cas9/sgRNA ribonucleoprotein nucleofection using Lonza 4D nucleofector

Version 7

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PROTOCOL STATUS

Working

We use this protocol in our group and it is working

STEPS MATERIALS

NAME	CATALOG #	VENDOR
Amaxa SF Cell Line 4D-Nucleofector Kit S (96 RCT)	V4SC-2096	Lonza
Lonza Nucleofector 4d	AAF-1002X	Lonza

BEFORE STARTING

Grow cells to 80-90% confluency. Maintain cells very healthy before transfection by changing media frequently. Plate enough cells for 200K cells per nucleofection reaction.

Mg2+ is required for cleavage of DNA by Cas9. Avoid buffer containing high concentration of EDTA as it can chelate Mg2+.

Use SF cell line solution with added supplements as nucleofection solution for HEK293T cells. Don't leave cells in nucleofection solution for a long time as it might be toxic to the cells.

Warm up trypsin, media and 1x PBS

1

Prepare cells (part 1)

2 -Trypsinize cells: Leave cells in trypsin (2 mL for a 10cm plate) at 37C for 3-5 minutes.
Note: don't leave cells in trypsin for a long period of time.
-Add in warm media to neutralize trypsin (8 mL for a 10cm plate).
-Pellet cells at 200 x g for 10 mins.

3 Remove media containging trypsin and resuspend cells in an appropriate amount of warm 1x PBS (usually 7 mL of 1x PBS for an 80-90% confluent 10cm plate). This step is critical as trypsin and FBS commonly contain RNAse activity.

4 -Count cells: Use the hemocytometer to count as it is more accurate and consistent in our experienece.
-Add 20ul of trypan blue with 20ul of media containing cells. Mix well.
NOTE: do not leave cells in trypan blue for more than 5 minutes as it is very toxic to the cells.
-Add about 15uL of the cell:trypan blue mixture to the hemocytometer. Count 5 squares and average them out.
-Record the cell concentration (cells/uL). In the meantime, put solution containing cells in 37C.

Form the crRNA : tracrRNA duplex (if needed)

Resuspend RNA oligos (cr and tracr) in IDT duplex buffer to final concentrations of 200 uM.

5

6 Mix the two oligos in equimolar concentrations to a final duplex concentraition of 100 uM. For example, mixing 1.25 uL of 200 uM crRNA and 1.25 uL of 200 uM tracrRNA yields 2.5 uL of 100 uM guide duplex.

7 Heat at 95C for 5 minutes. Alowing slow cooling to RT by leaving tubes on block before proceeding.

Prepare ribonucleoproteins RNPs mix

8 Add 100 pmol of Cas9 to 200 pmol of gRNA very very slowly:
-For example, if Cas9 is at 40 uM and gRNA stock at 200 uM, add 2.5 uL of 40 uM Cas9 to 0.6 uL of 200 uM gRNA.

9 Incubate at RT for 10-20 minutes to let RNP form.

Prepare cells (part 2)

10 For each nucleofection, pipette 200k cells using a P200 or larger into a 1.5 mL tube.

11 Spin 200 x g for 10 minutes at RT to pellet cells softly.

12 Carefully remove media off of tubes.

13 Prepare a 12-well plate containing 1 mL of media per well. Pre-warm at 37C.

Nucleofection

14 Prepare and label wells on nucleofection cuvettes. To avoid cells staying in nucleofection solution for a long period of time in the subsequent steps, configure Lonza 4D ahead of time using the recommended cell-type program. Use SF cell line program CM-130 for HEK293T cells.

Amaxa SF Cell Line 4D-Nucleofector Kit S (96 RCT)
by Lonza
Catalog #: V4SC-2096

Lonza Nucleofector 4d
by Lonza
Catalog #: AAF-1002X

15 After centrifugation, cell pellets are soft so carefully remove media from cells.

16 Resuspend cells in 20 uL of nucleofector solution (SF cell line solution with added supplement for HEK293T) using a P200.

17 Add the entire RNP mix to the 20 µL resuspension and mix using a P200.

18 If using a repair template, add 1uL of 100uM single-stranded donor DNA (100 pmoles) and mix well.

19 Add nucleofection mixes to the multiwell cuvette, and cap.

20 Insert cuvette into nucleofector and zap using the configured program.

21 Allow cells to sit in nucleofection strips for 10 minutes post-nucleofection. This is supposed to increase efficiency.

22 Add 80uL of pre-warmed media to each well. Pipette mixture out with a P200 into your pre-warmed 12-well plate.

23 Allow cells 24 hours - 48 hours to settle and recover before attempted downstream analysis. Consider including un-zapped controls to test viability.