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We use this protocol and it's working

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🌐 Cas9/sgRNA ribonucleoprotein nucleofection using Lonza 4D nucleofector V.7

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MATERIALS

STEP MATERIALS

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

✂ Lonza Nucleofector 4d **Lonza Catalog #AAF-1002X**

BEFORE START INSTRUCTIONS

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

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 containing 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 experience.
 - 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)

- 5

Resuspend RNA oligos (cr and tracr) in IDT duplex buffer to final concentrations of 200 uM.
- 6

Mix the two oligos in equimolar concentrations to a final duplex concentration 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. Allowing 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.

Amata SF Cell Line 4D-Nucleofector Kit S (96 RCT) **Lonza Catalog #V4SC-2096**

- 15 After centrifugation, cell pellets are soft so carefully remove media from cells.
- 16 Resuspend cells in 20 μ L of nucleofector solution (SF cell line solution with added supplement for HEK293T) using a P200.
- 17 Add the entire RNP mix to the 20 μ L resuspension and mix using a P200.
- 18 If using a repair template, add 1 μ L of 100 μ M 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 80 μ L of pre-warmed media to each well. Pipette mixture out with a P200 into your pre-warmed 12-well plate.

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