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🌐 Cas9/sgRNA ribonucleoprotein nucleofection using Lonza 4D nucleofector with lower amount of RNP (final best version-tested) V.10

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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.

Stock of Cas9 ~ 40uM or higher. gRNA stock ~ 200uM. HDRT ~ 2ug/ul or higher.

Protocol status: Working
We use this protocol and it's working

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Warm up trypsin, media and 1x PBS

1

Prepare Cas9, guide RNA and HDR template

- 2
- Thaw Cas9, sgRNAs and HDR template on ice
 - Add 100pmole of sgRNA to PCR tubes. Usually, stock sgRNA is at 200uM, add 0.5uL.
 - For ssDNA HDRT, add 100pmole of 100uM single-stranded donor DNA (1ul) to different PCR tubes
 - For dsDNA HDRT, 5ug of dsDNA repair template (concentration of 2ug/ul and above ideally so you only use about 2ul)

Prepare cells (part 1)

- 3
- 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 500 x g for 5 mins.
- 4
- Remove media containing trypsin and resuspend cells in an appropriate amount of warm DMEM (usually 7 mL DMEM for an 80-90% confluent 10cm plate).
- 5
- 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 media containing cells in 37C.

Form the crRNA : tracrRNA duplex (if needed)

- 6 Resuspend RNA oligos (cr and tracr) in IDT duplex buffer to final concentrations of 200 uM.
- 7 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.
- 8 Heat at 95C for 5 minutes. Allowing slow cooling to RT by leaving tubes on block before proceeding.

Prepare RNPs mix

- 9 Very slowly, add 50 pmol of Cas9 to PCR tubes previously prepared, containing 100 pmol of gRNA, forming 50pmol of RNP.
-For example, if Cas9 is at 40 uM and gRNA stock at 200 uM, add 1.25 uL of 40 uM Cas9 to 0.5 uL of 200 uM gRNA.
- 10 Incubate at 37C for 10-20 minutes to let RNP complexes form.

Prepare cells (part 2)

- 11 For each nucleofection, pipette media containing 200k cells using a P200 or larger into a 1.5 mL tube.
- 12 Spin 500xg for 6 minutes at RT to pellet cells softly.

- 13 Carefully remove media off of tubes. Add warm 1xPBS to wash cells and spin down again at 500xg for 6 minutes. This step is critical as trypsin and FBS commonly contain RNAses.
- 14 Prepare a 12-well plate containing 1 mL of media per well. Pre-warm at 37C.

Nucleofection

- 15 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. Turn off the instrument and computer then turn them on again in the correct order. Use SF cell line program CM-130 for HEK293T cells.
 - ⊗ Amaxa SF Cell Line 4D-Nucleofector Kit S (96 RCT) **Lonza Catalog #V4SC-2096**
 - ⊗ Lonza Nucleofector 4d **Lonza Catalog #AAF-1002X**
- 16 After 10-20 mins, add RNP complexes to the previously prepared PCR tubes containing HDRTs and allow to incubate together at room temperature for at least 30s, but 10 mins for max efficiency.
- 17 After centrifugation, cell pellets are soft so carefully remove PBS from cells.
- 18 Resuspend cells in 20 uL of nucleofector solution (SF cell line solution with added supplement for HEK293T) using a P200. These cuvette wells can take up to 25-26ul of total reaction mix. Calculate the total amount of cells+nucleofection buffer+cas9+gRNA+HDRT ahead of time. Nucleofection buffer can also be decreased to 18ul if needed.
- 19 Add the entire RNP+HDRT mix to the 20 µL resuspension and mix using a P200.

- 20** Add nucleofection mixes to the multiwell cuvette. Avoid bubbles. Cap.
- 21** Insert cuvette into nucleofector and zap using the configured program.
- 22** Add 80ul warm media into each cuvette well immediately. Allow cells to sit in nucleofection strips for 15 minutes at 37C post-nucleofection. This is supposed to increase viability of cells.
- 23** Pipette mixture out with a P200 into your pre-warmed 12-well plate.
- 24** Allow cells at least 48 hours to settle and recover before downstream analysis.