

CRISPR transfection protocol for cell line SK-N-SH using Lonza 4D-Nucleofector X kit (V4XC-2024)

ENCODE4 - Version 3

(Replicates split right after transfection, 200 µg/ml G418, 1M cells)

1. Preparation of Nucleofector machine, solutions and cells

1. Power on Lonza Nucleofector machine.
2. Under settings add a customized program for SK-N-SH:
 - a. Pulse codes: DN-100
 - b. Solution: Cell Line SF
 - c. Volume: 100 µl
3. Choose unit "X", 100 µl cuvette, and customized program for SK-N-SH.
4. Fill appropriate number of T-25 flasks with 7 ml of complete MEM medium (without antibiotics). Prepare two flasks per transcription factor, one for each replicate. Put the flasks in the incubator to warm to 37°C while transfecting cells.
5. Prepare 4D-mixed transfection solution by mixing 82 µl of the Nucleofector solution and 18 µl of supplement per transfection.
6. Remove media from the cultured SK-N-SH cells and wash cells once with PBS, incubate cells with TrypLE for 7-10 minutes at 37°C. Add fresh medium to detached cells and resuspend them.
7. Count an aliquot of cells and determine cell density. Transfer 2 million cells into 15 ml tube. Centrifuge the cells at 90 g for 10 minutes at room temperature.
8. Remove supernatant completely.

2. Transfection

1. Resuspend 2 million cell pellet carefully in 200 µl of room temperature 4D-mixed transfection solution. Aliquot into two eppendorf tubes, 1 million cells in 100ul solution in each tube. Considered as two replicates of one transcription factor.
2. Add 1 µg of guide plasmid and 10 µg of donor plasmid to the 100 µl of cell suspension (total volume of plasmids should be less than 10 µl), tap the tube gently to mix.
3. Always include one negative control sample for each batch of transfections. Do everything the same for this samples except not adding plasmids to the cells when doing the transfection.

4. Transfer each replicate of the mixture of cells and plasmids into a 100 μ l Nucleocuvette vessel, and close the lid. Try to avoid bubbles and gently tap the vessel to make sure the sample covers the bottom of the cuvette.
5. Place vessel with closed lid into the retainer of the 4D-Nucleofector X unit. Check for proper orientation of the vessel.
6. Start Nucleofection process by pressing "Start" on the display of the 4D-Nucleofector Core Unit. The turntable will take the vessel away and turn it towards the inside of the machine, you will hear a zapping/buzzing sound, and then it will return the vessel to you.
7. After run completion, carefully remove the vessel from the retainer.
8. Retrieve T-25 flasks from the incubator and set it up in the biosafety cabinet.
9. Pop the vessel top off and transfer 200 μ l of warm medium from one T25 flask to one replicate of 100 μ l cuvette. Mix cells by gently pipetting up and down 2-3 times and transfer 300 μ l into another replicate of 100 μ l cuvette. Now we have 400 μ l cell suspension in one cuvette. Remove 200 μ l of cells from the cuvette and transfer cells into one T-25 flask (replicate 1). Transfer another 200 μ l of cells from the cuvette using the pipettes supplied by Lonza to another T-25 flask (replicate 2). Each flask has 1 million transfected cells and is a independent replicate.
10. Gently shake flasks horizontally to evenly spread the cells.
11. Put the flask at cell culture incubator for 24 hours.

3. Cell upkeep for experiment

1. 24 hours after transfection, aspirate medium in each flask and add 7 ml of fresh complete medium without G418.
2. 48-72 hours after transfection, aspirate medium in each well and add 7ml of fresh complete medium with 200 μ g/ml G418.
3. Continue to change 7 ml medium with 200 μ g/ml G418 for 2-3 times per week. Negative control cells should completely die 10-14 days after transfection.
4. When cells are 50-60% confluent in T-25 flask, trypsinize cells and transfer cells to one T-75 flask, 25ml complete medium with 200 μ g/ml G418 in each flask.
5. When cells are almost confluent in T-25 flask, expand cells to a T-75 flask, 25 ml medium with 200 μ g/ml G418.
6. Continue to passage cells whenever cells are 80% confluent until desired cell numbers are reached for your application such as PCR, IP-Western Blot and cross-linking cells for ChIP-seq.
 - a. Collect 0.5-1 million cells for genomic DNA extraction for PCR validation. Wash cells with PBS once, flash freeze them and store at -80°C .
 - b. Collect 20 million cells for each IP-Western Blot experiment. Wash cells with PBS once, flash freeze them and store at -80°C .
 - c. Cross-linking cells: With 20 million cells suspended in medium, add 37% formaldehyde to a final concentration of 1%, incubate for 10 minutes with rotating. Add 2M Glycine (final concentration is 0.125M) to the cells suspension and incubate for 5 minutes with

rotating. Wash cell pellets once with PBS. Pellets can be snap frozen and stored at -80°C.

NOTE: Before collecting cells for IP-Western Blot and CHIP, SK-N-SH cells might need to be treated with Retinoic Acid (RA). When cells are about 70% confluent, add 20 mM RA (in DMSO) to the culture medium (final concentration 6 μ M). Cover the flasks with aluminum foil. After 48 hours, collect 20 million cells for experiment. Wash cells with PBS once, flash freeze them and store at -80°C. For more details refer to the RA treatment protocol.