CRISPR transfection protocol for cell line MCF-7 using Lonza 4D-Nucleofector X kit (V4XC-1024)

ENCODE4 - Version 2
(Replicates split right after transfection, 600 µg/ml G418)

1. Preparation of Nucleofector machine, solutions and cells

1. Power on Lonza Nucleofector machine.
2. Under settings add a customized program for MCF-7:
    a. Pulse codes: DR-130
    b. Solution: Cell Line SE
    c. Volume: 100 µl
3. Choose unit "X", 100 µl cuvette, and customized program for MCF-7.
4. Prepare 6-well culture plate by filling appropriate number of wells with 1.5 ml of complete MEM medium (without antibiotics). Prepare two wells per transcription factor, one for each replicate. Put the plate 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 MCF-7 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 8 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 8 million cell pellet carefully in 200 µl of room temperature 4D-mixed transfection solution. Split into two 1.5 ml tubes, 100 µl each.
2. Add 1 µg of guide plasmid and 10 µg of donor plasmid to each 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 mixture of cells and plasmids into 100 µl Nucleocuvette vessels, and close the lids. Try to avoid bubbles and gently tap the vessel to make sure the sample covers the bottom of the cuvettes.
5. Place vessels 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 vessels from the retainer.
8. Retrieve the 6-well plate from the incubator and set it up in the biosafety cabinet.
9. Pop one vessel top off and transfer 200 µl of warm medium from one well into the cuvette and mix cells by gently pipetting up and down 2-3 times. Pop the second vessel top off and transfer all 300 µl into the second cuvette which has 100 µl cells. Mix cells by gently pipetting up and down 2-3 times and transfer 200 µl back into the same well of the 6-well plate. Remove the other 200 µl of cells from the cuvette using pipettes supplied by Lonza and transfer cells into a second well. Each well has 4 million transfected cells and is a independent replicate. <u>For the negative control only transfer 25% of the transfected cells from vessel into the well because it has much more alive cells than experimental samples.</u>
10. Gently shake plates horizontally to evenly spread the cells.
11. Put the plate at cell culture incubator for 24 hours.

3. Cell upkeep for experiment

1. 24 hours after transfection, aspirate medium in each well (a lot of dead cells) and add 4 ml of fresh complete medium **<u>without</u>** G418.
2. 72 hours after transfection, replace medium with complete medium with 600 µg/ml G418.
3. Continue to change 4 ml medium with 600 µg/ml G418 for 2-3 times per week. Negative control cells should completely die 10-14 days after transfection.
4. When cells are 30-50% confluent in wells, trypsinize cells and transfer cells to one T-25 flask, 7ml complete medium with 600 µg/ml G418 in each flask.
5. When cells are almost confluent in T-25 flask expand cells to two T-75 flasks, 25 ml medium with 600 µg/ml G418. At this point, we consider each flask as replicate.
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.