# CRISPR transfection protocol for iPSC line PGP1 using Lonza 4D-Nucleofector X kit (V4XP-3024)

ENCODE4 - Version 1
(Replicates split right after transfection, 30 µg/ml G418, mTeSR medium)

## 1. Preparation of Nucleofector machine, solutions and cells

1. Change PGP1 cell medium with culture medium with 5uM Y27632 (without antibiotics) 2-3 hours before collecting cells for transfection.
2. Power on Lonza Nucleofector machine.
3. Under settings add a customized program for PGP1:
    a. Pulse codes: CM-150
    b. Solution: Primary Cell P3
    c. Volume: 100 µl
4. Choose unit "X", 100 µl cuvette, and customized program for PGP1.
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 PGP1 cells and wash cells once with PBS, incubate cells with Accutase for 3-5 minutes at 37°C. Add culture medium with 5uM Y27632 (without antibiotics) 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 200 g for 3 minutes at room temperature.
   **NOTE**: You can put 4 million cells in the same tube and resuspend them together with transfection solution before aliquoting them equally out into two different plasmid tubes.
8. Remove supernatant completely.
9. During centrifugation, prepare 6-well **coated** culture plate by filling appropriate number of wells with 2 ml of culture medium (with 5 uM Y27632, **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.

## 2. Transfection

1. Resuspend 2 million cell pellet carefully in 100 µl of room temperature 4D-mixed transfection solution.

2. Add 1 µg of guide plasmid and 10 µg of donor plasmid to each 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 the mixture of cells and plasmids into a 100 µl 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 the 6-well plate from the incubator and set it up in the biosafety cabinet.
9. Pop the vessel top off and transfer 300 µl of warm medium from one well to the cuvette and incubate cuvette 5 mins at RT to recover the 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 1 million transfected cells and is a independent replicate.
   **NOTE**: If the cells become too full in the well before G418 has an effect on the cells, split 100 µl of transfected cells into 4 wells instead of 2 wells. In that case each well has 0.5 million cells and each replicate has two wells.
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 and add 4 ml of fresh complete mTeSR medium **without** G418.
2. 48 hours after transfection, replace medium with complete mTeSR medium with 30 µg/ml G418.
3. Continue to change 4 ml mTeSR medium with 30 µg/ml G418 **every day**. Negative control cells should completely die 5-10 days after transfection.
4. When PGP1 cells under selection have to be passaged, detach cells with EDTA (0.5mM in PBS). Passage cells to 1-2 wells of a 6-well plate or 1 well of 12-well plate depend on the cell colony number.
5. When cells are 70-80% of confluent in the well, expand cells to multiple wells of 6-well plate or a 10 cm dish, 2-4 ml mTeSR medium/well or 10-20 ml mTeSR medium/dish with 30 µg/ml G418. Be sure to change medium **every day**. Remove differentiated cells before changing medium whenever they appear.

6. Continue to passage cells whenever cells are 70-80% 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.