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Endogenous N-terminal 3X FLAG Tagging of SLC45A1 in iPSCs (WTC11-Ngn2 Line)

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We use this protocol and it's working

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Abstract

This protocol describes the steps for endogenously tagging the N-terminal of the SLC45A1 gene in WTC11-Ngn2 iPSCs with a 3X FLAG tag. It utilizes a Cas9-sgRNA ribonucleoprotein (RNP) complex and a single-stranded donor oligonucleotide (ssODN) template for homology-directed repair (HDR). The procedure can be adapted for generating point mutations or inserting sequences up to 130 bp. In this case, a 66 bp 3X FLAG tag is inserted after the start codon.

Materials

- **iPSC Line:** WTC11-Ngn2 iPSCs
- **Reagents:**
- Thiazovivin (ROCK1 inhibitor), 10 µM
- ReLeSR™ for cell dissociation
- Cas9 enzyme (IDT Alt-R™ S.p. Cas9 Nuclease V3), 10 µg/µL
- sgRNA (Synthego - 2'-O-methyl modified), 3.2 µg/µL
- ssODN donor template (IDT Alt-R HDR donor oligonucleotide), 6 µg/µL
- P3 Primary Cell Nucleofector™ Solution (Lonza, V4XP-3032)
- 16-well Nucleocuvette™ Strips
- 4D-Nucleofector™ system (Lonza)
- AZD7648, 1 mM stock (final concentration 1 µM)
- Growth medium for iPSCs
- PBS, sterile
- 24-well culture plates

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Designing Donor Template (ssODN) Sequence

2 5'- agcagcccagcggggacagggatgcctgccgtctccacccacagggacgcccaccagccctccccacg ATGGACTACAAAGACCATGACGGTGATTATAAAGATCATGACATCGATTACAAGGATGACGAT GACAAG ATCCCCGCAGCCAGCAGCACCCCGCCGGGAGATGCCCTCTTCCCCAGCGTGGCCCCACA GGAC -3'

- **Left homology arm**: 71 bp
- **3X FLAG tag**: GACTACAAAGACCATGACGGTGATTATAAAGATCATGACATCGATTACAAGGATGACGAT GACAAG (66 bp)
- **Right homology arm**: 63 bp
- **Total:** 200 bp

- **Guide sequence**: CTGGCTGCGGGGATCATcg

2.1 **Key Considerations:**

- Homology arms should be at least 35 bp long.
- Ensure the guide RNA contains at least 2 mismatches within the first 7 bp following the PAM sequence in the template (leveraging genetic code redundancy) to prevent Cas9 from re-cleaving the template after HDR.

Pre-treatment of iPSCs (Day -1)

3 **Objective:** Enhance iPSC viability during nucleofection.

3.1 Plate iPSCs in regular growth medium at approximately 50% confluence.

3.2 Add **10 µM Thiazovivin** to the medium.

3.3 Incubate cells overnight at 37 °C with 5% CO_2.

Preparation of Cas9-sgRNA RNP Complex (Day 0) 15m

4 **Objective:** Form the Cas9-sgRNA ribonucleoprotein (RNP) complex for delivery into iPSCs.

4.1 Mix **6 µg Cas9 enzyme** with **3.2 µg sgRNA** in a PCR tube.

4.2 Incubate at 37 °C for 00:15:00 to form the RNP complex. 15m

4.3 Keep the RNP complex on ice until nucleofection.

Dissociation of iPSCs (Day 0) 8m

5 **Objective:** Prepare a single-cell suspension of iPSCs for nucleofection.

5.1 Aspirate the culture medium from iPSCs and add **ReLeSR™** (Stemcell Technologies), incubate for 00:03:00 to 00:05:00 37 °C 8m

5.2 Gently tap the plate to detach cells and resuspend cells in growth medium (StemFlex,Stemcell Technologies).

5.3 Count cells to ensure **0.3 × 10^6 viable cells** per nucleofection reaction.

5.4 Centrifuge at 1000 rpm , 00:03:00 . Wash the cell pellet with sterile PBS and centrifuge again 1000 rpm, 00:03:00 6m

5.5 Resuspend the cell pellet with **20 µL P3 Nucleofector™ Solution** per nucleofection.

Nucleofection Setup (Day 0)

6 **Objective:** Introduce Cas9 RNP and ssODN template into iPSCs via nucleofection.

6.1 Add **6 µg ssODN** to the **20 µL** cell suspension (from **step 5.5**).

6.2 Gently transfer the cell suspension and ssODN (**step 6.1**) to RNP complex (from **step 4.3**).

6.3 Transfer the mixture to a well of the **16-well Nucleocuvette™ Strip**.

6.4 Insert the strip into the **4D-Nucleofector™ system**.

6.5 Run the program **CA137** for iPSC nucleofection.

6.6 Immediately add **80 µL pre-warmed growth medium** with **10 µM Thiazovivin** to each nucleofected well.

Post-Nucleofection Culture (Day 0) 1d

7 **Objective:** Ensure survival and recovery of nucleofected iPSCs.

7.1 Plate nucleofected cells in a **pre-coated 24-well plate** (already coated with rhLaminin-521 or Matrigel) with pre-warmed growth medium.

7.2 Add **1 µM AZD7648** and **10 µM Thiazovivin** to the medium.

Treatment with AZD7648, a DNA-PK inhibitor, aims to enhance HDR efficiency for improved integration.

7.3 Incubate cells at 37 °C, 5% CO_2 for 24:00:00 1d

Post-Transfection Treatment (Day 1) 3d

8 **Objective:** Facilitate cell recovery and discontinue AZD7648 treatment

8.1 After 24 hours, replace the culture medium with fresh iPSC growth medium.

8.2 Remove **AZD7648**, but continue treatment with **10 µM Thiazovivin**.

8.3 Culture cells for an additional 72:00:00 to allow for recovery and expansion. 3d

Validation of 3X FLAG Tag Integration

9 **Objective:** Confirm successful integration of the 3X FLAG tag using PCR and Sanger sequencing.

9.1 Design PCR primers flanking the 3X FLAG insertion site, approximately 250–300 bp upstream and downstream of the insertion. Perform PCR for both edited and wildtype cells

9.2 Run the PCR products on a gel to confirm successful amplification. If the correct band is observed, send the PCR product for Sanger sequencing.

9.3 Analyze the sequencing data to confirm the presence of the 66 bp 3X FLAG sequence at the correct site, compared to wildtype cells, using SnapGene or similar tools

9.4 Optional: If integration is suboptimal, perform single-cell selection to isolate clones with the correct integration.