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Generation of isogenic iPSC lines through CRISPR/Cas9-Mediated Targeting

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Elena Coccia[1]

[1]Icahn School of Medicine at Mount Sinai

E Elena Coccia
Icahn School of Medicine at Mount Sinai

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We use this protocol and it's working

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Abstract

This protocol describes the generation of genetically modified human pluripotent stem cell (hPSC) lines using CRISPR/Cas9 genome editing. Guide RNAs are cloned into the pX330 Cas9 plasmid and co-delivered with targeting vectors into hPSCs. Following transfection, cells are cultured under supportive conditions to allow recovery and clonal outgrowth. Individual colonies are manually picked, expanded, and screened for successful targeting.

Plasmid preparation

1 Clone CRISPR guide sequences into the pX330 Cas9 plasmid as described in Cong et al., 2013.

Cell preparation

2 Culture hPSCs in Geltrex-coated 6-well plates in Stemflex until they reach 70–80% confluency

Transfection

3 Prepare Lipofectamine Stem transfection mix according to manufacturer's instruction

3.1 Dilute the DNA plasmid mix (1µg per CRISPR plasmid, per well of a 6w plate) in Opti-MEM to a total of 250 µL per well

3.2 Dilute Lipofectamine Stem reagent separately (use a range of 2-3:1 Lipofectamine:DNA) in 250 µL Opti-MEM

4 Combine DNA and Lipofectamine mix dilutions, incubate for 10–15 minutes at room temperature.

5 Add the 500 µL transfection mix dropwise to each well containing 1 mL of Opti-MEM medium supplemented with 10 µM ROCK inhibitor (Y-27632)

6 Gently rock the plate to mix and return it to the incubator

7 After 6 hours, change the medium to fresh StemFlex with ROCK inhibitor

8 After 24 hours, change the medium to fresh StemFlex without ROCK inhibitor

Colony Picking

9 Pre-picking preparation

9.1 Monitor transfected plates daily. If many dead cells are visible, replace the medium with StemFlex + Pen/Strep; otherwise, supplement existing medium with Pen/Strep

9.2 Coat 24-well plates with Geltrex and prepare them with Stemflex supplemented with ROCK inhibitor

9.3 Label PCR strips for genomic DNA extraction

10 In the hood, scrape and aspirate individual colonies using a 200 µL pipette tip

11 Transfer each colony into a non-coated 12-well plate (final volume around 700 µL per well).

12 After picking, pipette each well to break up cell clumps.

13 Transfer ~200 µL to a labeled PCR strip.

13.1 Always transfer to the plate first, then the PCR strip (not sterile).

13.2 Do not overfill PCR strips to allow lids to close properly.

14 Change to fresh StemFlex after 48 hours.

15 Allow colonies to grow to confluency (typically 5–7 days depending on colony size).

gDNA extraction, qPCR and sequencing

16 gDNA extraction

16.1 Prepare lysis mix: Add Proteinase K to Viagen DirectPCR Lysis Reagent (1:40)

16.2 Spin down PCR strips with picked colonies for 2 minutes.

16.3 Aspirate medium and resuspend each pellet in 40–100 µL of lysis mix depending on pellet size.

16.4 Run the following PCR machine program: 55°C for 60 minutes - 85°C for 45 minutes - Hold at 4°C

16.5 Store lysates at 4°C for short term or -20°C for long-term storage.

17 PCR amplification and sequencing

17.1 Design primers to amplify 50–250 bp regions around the target site.

17.2 Run qPCR reaction for amplification

17.3 Analyze PCR products by gel electrophoresis using an agarose concentration suitable for the expected band size.

18 Submit successful reactions for Sanger sequencing using the forward primer.