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CRISPR/Cas9 gene editing in hiPSC

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We use this protocol and it's working

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Abstract

This protocol is adapted from Skarnes et al., 2019 and describes the steps to follow for knocking-in point mutation via CRISP/Cas9 in humain inducible pluripotent cells (hiPSC)

A. Pre-nucleofection

1 Resuspend the sgRNA overnight at 4C in TE buffer at a concentration of [M] 4 µg/µL

2 Resuspend the Alt-R HDR modified ssODN overnight in D-PBS at a concentration of [M] 200 pmol/ul

3 For each nucleofection, prepare 6ml StemFlex + Revitacell or Rock inhibitor and coat 1× 6-well plate with geltrex/synthemax/matrigel for 01:00:00 at 37 °C

4 For each nucleofection, prepare complete P3 solution by adding 20 µL of Supplement to 90 µL of P3 solition in a 1.5ml Eppendorf

B. Pre-assembly of Cas9 RNP

5 For each nucleofection, mix 4 µL of sgRNA (16 µg total) and 2 µL of recombinant Cas9 protein (20 µg total), in 1.5ml Eppendorf tube

6 Incubate at RT for 00:30:00 to 00:45:00 . *Note: begin harvesting iPSC after 15mins*

7 Immediately prior to nucleofection, add 1 µL (200 pmol) of Alt-R HDR modified ssODN to the pre-assembled Cas9 RNP.

C. Delivery of Cas9 RNP and ssODN using Nucleofector

8 Switch on the nucleofector unit and select “X-unit” and single cuvette. Use the “Primary Cell P3” program and set the pulse code to “**CA-137**”

9 Collect the iPSC from an 80% confluent well with pre-warmed accutase. Count cells and pellet 8 × 10^5 cells by centrifuging at 300 x g, 20°C, 00:05:00 and aspirate supernatant.

10 While the cells are spinning, add the 1 µL of ssODN to the pre-assembled Cas9 RNP Eppendorf.

11 After pelleting cells and supernatant removed, gently resuspend the cell pellet in 100 µL complete P3 Solution and transfer the cells to the Eppendorf containing Cas9 RNP+ssODN. Transfer the cell suspension to the nucleocuvette. Tap the cuvette on a flat surface to eliminate bubbles and nucleofect immediately using the “Primary Cell P3” program and “CA-137” pulse code.

12 Immediately after the nuclefection, transfer the cells into one well of pre-coated, warm 6-well plate containing 2-3 ml of Stemflex + revitacell/Rock. Inh.

13 Add HDR enhancer to a final concentration of [M] 30 micromolar (µM) (1:100 from the 3mM stock
solution). Culture the cells in a 32 °C incubator (cold shock for 2 days: important for homozygous gene editing).

14 Change media the next day to StemFlex without revitacell/rock inh and HDR enhancer

15 Return cells to 37 °C incubator on day 3

D. Plating single cells to isolated clones

16 Once the cells in the 6-well plate are 80% confluent, dissociate them with accutase into a single cell suspension, centrifuge at 210 x g, 20°C, 00:05:00. Remove supernatant and resuspend cell pellet in StemFlex + revitacel/rock.inh

17 Count the cells and plate between 800 – 1000 single cells in geltrex/synthemax/matrigel-coated 10cm
petri dishes containing 10ml Stemflex + revitacel/rock inh.

18 Transfer cells to 37 °C incubator.

19 Change the media the next day and daily with Stemflex to remove revitacell/rock inhibitor

E. Picking single cell-derived clones

20 With the aid of a dissecting microscope and a p200, individual colonies are picked and transferred into 2× 96-well plates, one for colonies expansion, the other for DNA lysis and genotyping.

21 For each 96 colonies to be picked, prepare 1x geltrex coated 96-well and 15ml of Stemflex+Rock inh/revitacell and warm to RT in the hood.

22 Use the p200 tip to break apart the colony from the plate and collect the cell fragments in a volume of 50 µL of media. Transfer each colony to a well of a U-bottom 96-well plate.

23 Once 96 colonies are picked, add 150 µL of Stemflex+rock inh/revitacell to each well of the U-bottom plate with a multichannel.

24 With the use of the multichannel set to 100ul, pipette cells up and down 10 times in the U-bottom plate. Transfer 100 µL of the cell suspension to geltrex coated 96-well plate (for colony expansion) and 100 µL to 96-well plate for lysis and DNA extraction.

25 The following day, replace the media in the expansion 96-well plate with fresh Stemflex without revitacell/rock inh.

F. Genomic DNA extraction from 96-well plates

26 For each 96-well plate to be lysed, add 10 mg of proteinase K powder (Sigma-Aldrich, P6556) to 10 mL of lysis buffer ([M] 50 millimolar (mM) KCl, [M] 10 millimolar (mM) Tris-HCl pH 8, [M] 2 millimolar (mM) MgCl2, [M] 0.45 % volume (v/v) NP40, [M] 0.45 % volume (v/v) Tween20).

27 With a multichannel pipettor, remove media from confluent wells of the Synthemax-coated 96-well plate and wash each well once with 150 µL room temperature D-PBS.

28 Add 100 µL of lysis buffer containing 1 mg/ml proteinase K, seal the 96-well plate andcincubate at 60 °C for at least 04:00:00 .

29 While the plate is still warm, transfer lysates to a 96-well PCR plate, seal the plate and heat inactivate the proteinase K by incubating the plate at 95 °C for 00:10:00 in a thermocycler.

30 While the samples are warm, prepare a 1:20 dilution of the lysates in water for setting up PCR reactions. Store the undiluted lysates at -80 °C . The diluted samples can be kept at 4 °C for a few days.