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Footprint-Free Genome Editing of iPSC Using Alt-R CRISPR/Cas9

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1 Works for me dx.doi.org/10.17504/protocols.io.bfmmjk46

Neurodegeneration Method Development Community
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Preparing iPSCs for Nucleofection

1 Coat 1 well of a 6-well plate with **1 ml** of Matrigel for **01:00:00** at **37 °C**

2 Add **5 ml** of DMEM/F12 to a 15ml conical tue

3 Thaw vial of cells in **37 °C** water bath for approximately **00:01:00**

4 Wipe off excess water from tube and spray with 70% Ethanol before placing vial into biosafety cabinet

5 Transfer thawed cells into conical tube containing **5 ml** DMEM/F12

If some cells remain in vial use **1 ml** DMEM/F12 to rinse vial clean and transfer into the 15ml conical

6 Swirl to mix cells in the DMEM/F12 and **750 rpm 00:03:00**

Avoid rough mixing or suspension of cells; this helps to keep the cells clustered

7 After centrifugation, aspirate off most supernatant, leaving a small amount in the tube. Do not try to aspirate all the way down to the pellet (may or may not visible)

8 Add **4 µl** of Rock Inhibitor to cell pellet and resuspend in **2 ml** of mTesR1 in order to achieve a

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[M] **10 Micromolar (µM)** solution. Gently swirl to mix

Avoid rough mixing or suspension of cells; this helps to keep the cells clustered

9 Aspirate Matrigel from coated 6-well plate, and pipet cell mixture into well.

10 Incuate overnight at **37 °C**

11 After **24:00:00** change media and allow cells to recover

12 Once cells have reached 80-90% confluency, split culture as single cells into 3 wells of a 6 well tissue culture plate.

12.1 Coat 3 wells of a 6-well plate with **1 ml** of Matrigel for **01:00:00** at **37 °C**

12.2 Aspirate media from well containing cells

12.3 Wash cells with **2 ml** of PBS

12.4 Add **1 ml** of Accutase per well

12.5 Incubate cells and Accutase at **37 °C** for **00:10:00** . Gently tap plate upon removal from incubator to help dislodge cells from Matrigel

12.6 Collect cells in **5 ml** of PBS and transfer to 15ml conical tube

12.7 **750 rpm 00:03:00**

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12.8 Resuspend cell pellet in **6 ml** mTesR1 + **5 Micromolar (µM)** Rock Inhibitor and plate **2 ml** into each freshly coated Matrigel well

13 Perform the single cell passage (Step 12) at least two additional times prior to using cells for Nucleofection

Continuing iPSC Culture

14 Perform a single cell passage approximately **48:00:00** prior to nucleofection

You will need three million cells per nucleofection and one million cells per GFP control. Therefore, three wells of a confluent of a 6-well tissue culture plate are sufficient

15 Coat three wells of a 6-well plate for nucleofection using Matrigel and incubate at **37 °C** for **01:00:00** prior to splitting cells

15.1 Just prior to splitting cells for nucleofection, aspirate Matrigel from coated wells and add **3 ml** of DMEM/F12 + 10% FBS supplemented with **10 Micromolar (µM)** Rock Inhibitor per well

RNP Complex

16 Gather the following reagents for Alt-R Reactions and let them thaw on ice

Reagent	Stability and Storage
Alt-R crRNA	6 months stability at -80°C
Alt-R tracrRNA	6 months stability at -80°C
Alt-R Cas9	6 months stability at -80°C
Electroporation Enhancer (IDT - Catalog # 1075915)	6 months stability at -80°C
sgRNA (if needed)	6 months stability at -80°C

17 Prepare Alt-R Reactions

17.1 Prepare fresh Alt-R gRNA by adding **2.5 µl** of **200 Micromolar (µM)** Alt-R crRNA and **2.5 µl** of **200 Micromolar (µM)** Alt-R tracrRNA in equal volumes. Heat mixture at **95 °C** for **00:05:00** . Let cool to **Room temperature**

The Alt-R gRNA should be prepared fresh before each use

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17.2 Resuspend Alt-R Cas9 and Electroporation Enhancer in PBS to a final concentration of **100 Micromolar (µM)**

17.3 Combine Alt-R gRNA solution and Alt-R Cas9 + Electroporation Enhancer solution together and incubate at **Room temperature** for **00:15:00**

Component	Final Concentration
PBS	-
Alt-R gRNA	120 pmol
Alt-R Cas9	104 pmol
Enhancer	100 µM
Donor ssODN or GFP*	100 µM

*GFP = pMax GFP control in a separate tube

RNP Complex stability limited to **48:00:00** at **4 °C** . Do not freeze

Split Cells for Nucleofection

18 Split cells for nucleofection

18.1 Aspirate media from cells

18.2 Wash each well with **2 ml** of PBS and aspirate

18.3 Add **1 ml** of Accutase per well

18.4 Incubate at **37 °C** for **00:10:00**

18.5 Collect cells in **5 ml** of PBS and transfer to a 15ml conical tube

18.6 **750 rpm 00:03:00**

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Count Cells for Nucleofection

19 Resuspend cell pellet in **1 ml** of PBS in the 15ml conical tube, then dilute cells 1:10 (**10 µl** of cell suspension + **90 µl** of PBS) in a 1.7ml tube

20 Use **10 µl** of diluted cells for cell counts

20.1 Using all four corners of the countess slide, calculate the average number of cells

20.2 Multiply the average by 10,000 (10^4)

20.3 Multiply product from step 20.2 by 3 to get the total number of cells

[Average # of Cells x 10,000 x 3] = Total Number of Cells

20.4 Take total number of cells calculated in step 20.3 and divide by three million

20.5 Take answer from step 20.4 and divide by three to get the volume of cells necessary for nucleofection

Centrifuge Cells for Nucleofection

21 Transfer the desired volume of cells (calculated in step 20.5) to microcentrifuge tube

22 **90 x g 00:05:00**

23 Aspirate PBS from cell pellet

Prepare Lonza Kit Reagents for Nucleofection

24 Make reaction mix from Lonza Kit: P3 Primary Cell 4D (V4XP-3024). Each reaction requires a total of **100 µl** of reaction mix.

If performing more than one reaction, it is best to make a Master Mix

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24.1 Combine **82 µl** of P3 Solution and **18 µl** of Supplement into a 1.7ml microcentrifuge tube

Combining Lonza Kit Reagents and DNA for Nucleofection

25 Combine reaction mix from step 24 (**100 µl**) with previously complexed DNA from step 17

26 Mix reaction mix and DNA with cell pellets (step 23) by pipetting up and down with p200 pipette

Try to pipette as little as possible. Pipette only until mixed

27 Transfer **100 µl** of reaction mix + DNA + cells to a cuvette

Ensure no bubbles are in the transferred mixture of cells in the cuvette. This can interfere with the nucleofection's success

Nucleofection

28 Nucleofect with Lonza Program CA-137 in P3 Solution

29 Let cuvette and cells incubate for **00:10:00** at **Room temperature**

30 Transfer cells/DNA solution to approproate pre-coated well containing **2 ml** of DMEM/F12 + 10% FBS + **10 Micromolar (µM)** Rock Inhibitor

31 Incubate at **37 °C** overnight

Post Nucleofection

32 Continue culturing the iPSC in 1 well of a 6 well plate for 5-7 days post nucleofection, changing mTesR1 daily.

32.1 **24:00:00** post-nucleofection - add mTesR1 with **5 Micromolar (µM)** Rock Inhibitor

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32.2 **48:00:00** post-nucleofection - add mTesR1 with [M] **2.5 Micromolar (µM)** Rock Inhibitor

32.3 **72:00:00** post-nucleofection - add mTesR1 with [M] **1 Micromolar (µM)** Rock Inhibitor

33 Continue culturing cells in mTesR1 until confluent (~5 days)

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