CRISPR-Cas9 genome editing in human induced pluripotent stem cells

Optimizing results in Essential 8 or KnockOut Serum Replacement media systems

1. Genome editing in human induced pluripotent stem cells (iPSCs) grown in Essential 8 Medium

1.1. Cell culture

Gibco™ Human Episomal iPSC Line (Cat. No. A18945) is cultured in Gibco™ Essential 8™ Medium (Cat. No. A1517001) on tissue culture dishes coated with Gibco™ Geltrex™ LDEV-Free, hESC-Qualified, Reduced Growth Factor Basement Membrane Matrix (Cat. No. A1413302). After thawing, cells are passaged 2–3 times using the recommended protocols. One day prior to transfection, cells are plated as follows:

1. Wash cells grown in a 60 mm plate with 3 mL Gibco™ DPBS (Cat. No. 14190-250).
2. Add 1 mL Gibco™ TrypLE™ Express Enzyme (Cat. No. 12604-013) and swirl to cover the entire surface. Incubate for 2–3 minutes in a 37°C humidified incubator.

   Note: Cells should still attach to plate at this point.
3. Aspirate TrypLE Express Enzyme gently. Add 3 mL Essential 8 Medium and dislodge cells into a single-cell suspension by pipetting up and down a few times.
4. Count cells using the Invitrogen™ Countess™ II Automated Cell Counter (Cat. No. AMQAX1000) or hemocytometer.
5. For transfection with Invitrogen™ Lipofectamine™ CRISPRMAX™ Cas9 Transfection Reagent (Cat. No. CMAX00003, section 1.3), plate 4 x 10^4 cells in a 24-well format in 500 µL of Essential 8 Medium containing 10 µM ROCK inhibitor and Gibco™ RevitaCell™ Supplement (Cat. No. A2644501), then allow cells to recover overnight.
6. For the Invitrogen™ Neon™ Transfection System (Cat. No. MPK5000), refer to sections 1.4 and 1.5.

1.2 Designing and generating gRNA by *in vitro* transcription

Use the Invitrogen™ GeneArt™ CRISPR Search and Design Tool, available at thermofisher.com/crisprdesign, to search our database of >600,000 predesigned gRNA sequences specific to every gene in the human and mouse genomes. GeneArt™ predesigned gRNAs are optimized for gene knockout and typically target the first 3 transcribed exons per gene.

Generate your DNA template containing the T7 promoter and the gRNA sequence using the Invitrogen™ GeneArt™ Precision gRNA Synthesis Kit (Cat. No. A29377).

1.3. Knockout by transfection of GeneArt Platinum Cas9 Nuclease and guide RNA (gRNA)

Transfection of gene-specific gRNA and Invitrogen™ GeneArt™ Platinum™ Cas9 Nuclease (Cas9 protein, Cat. No. B25640) complexes can be used for efficient gene knockout in iPSCs [1,2]. Described below is an optimized transfection protocol utilizing Lipofectamine CRISPRMAX Cas9 Transfection Reagent.

1. On the day of transfection, cells should be ~30% confluent (do not change the cell medium at this point).
2. Prepare Cas9–gRNA ribonucleoprotein (RNP) complex and transfection reagent in separate tubes:

Tube 1:
25 µL Gibco™ Opti-MEM™ I Reduced Serum Medium
1,000 ng GeneArt Platinum Cas9 Nuclease
250 ng gRNA
6 µL Cas9 Plus™ Reagent
→ Mix well → Mix well

Tube 1 mixture will be stable up to 2 hours at room temperature. Always make tube 1 first.

Tube 2:
25 µL Opti-MEM I medium
1.5 µL Lipofectamine CRISPRMAX reagent
→ Mix well, and incubate for 5 min at room temperature

For optimal performance, incubate tube 2 for 5 min (longer incubation will lead to a decrease in transfection efficiency).

3. Add contents of tube 1 to tube 2, briefly vortex, and incubate the resulting complex for 10–15 min at room temperature (the order of addition is important).
4. Add 55 µL complex to one well of a 24-well plate containing cells.
5. Six hours posttransfection, replace the medium with 1 mL of fresh iPSC culture medium without ROCK inhibitor for each well of a 24-well plate and place the cell culture dish back into the incubator.
6. After 48 hours posttransfection, wash the cells once with 500 µL PBS and analyze cleavage efficiency using the Invitrogen™ GeneArt™ Genomic Cleavage Detection Kit (Cat. No. A24372).

Note: We recommend that users optimize transfection conditions for their specific cell type. We optimize our conditions using our in-house HPRT gRNA control, which is available for purchase as custom gRNA (to order, contact us at **GEMServices@thermofisher.com**). Using the protocol described here for Lipofectamine CRISPRMAX reagent, we get >50% cleavage efficiency with the HPRT control in the Gibco Human Episomal iPSC Line.

1.4. Knockout by electroporation of RNP using the Neon Transfection System

1. On the day of electroporation, detach cells using TrypLE Express Enzyme solution and then resuspend cells in growth medium prior to cell counting as described in section 1.1.
2. Prepare electroporation complex by adding 0.5 µL of Cas9 nuclease (3 µg/µL) and 300 ng of gRNA (volume of gRNA should be 0.5 µL or less) to 5 µL of Resuspension Buffer R. Mix gently.
3. Incubate the complex at room temperature for 10 minutes.
4. Meanwhile, transfer 1 x 10^6 cells to a sterile test tube and centrifuge at 300 x *g* for 5 minutes.
5. Aspirate the supernatant and resuspend the pellet in 1 mL of PBS without Ca^{2+} and Mg^{2+}, then centrifuge at 300 x *g* for 5 minutes.
6. Carefully and completely aspirate the PBS, being careful to not disturb the cells.
7. Resuspend the cells in 50 µL Resuspension Buffer R.
8. Transfer 5 µL of resuspended cells to the 6 µL of Cas9–gRNA complexes prepared in steps 2 and 3. Mix gently.
9. Pipette 10 µL of the cell suspension into the Neon™ tip and electroporate with protocol 7 (1,200 V, 30 ms, 1 pulse). Be careful to not introduce bubbles.

Note: We recommend that users optimize electroporation conditions for the Neon Transfection System for their specific cell type. We optimize our conditions using our in-house HPRT gRNA control, which is available for purchase as custom gRNA (to order, contact us at **GEMServices@thermofisher.com**). Using the protocol described here for the Neon Transfection System, we get >80% cleavage efficiency with the HPRT control in the Gibco Human Episomal iPSC Line.

10. Immediately transfer the electroporated cells into a 24-well plate containing 0.5 mL of growth medium containing 10 µM ROCK inhibitor or 1X RevitaCell Supplement. Incubate the cells in a humidified 37°C, 5% CO_2 incubator and change medium the next day. Analyze the cells 48–72 hours after electroporation.
11. Harvest cells and measure cleavage efficiency using the GeneArt Genomic Cleavage Detection Kit, Sanger sequencing, or next-generation sequencing.

1.5. Knockout by electroporation of gRNA and GeneArt CRISPR Nuclease mRNA

The cell harvesting and electroporation method can be followed as described above (sections 1.1 and 1.4, respectively), except for the dosage of RNA and program of the Neon system. When using gRNA and Invitrogen™ GeneArt™ CRISPR Nuclease mRNA (Cat. No. A29378),

200 ng of *in vitro*–transcribed gRNA and 1 µg of GeneArt CRISPR Nuclease mRNA are used per well in a 24-well format. In our hands, we found that out of the 24 preprogrammed optimization conditions of the Neon Transfection System, protocol 17 (850 V, 30 ms, 2 pulses) gave the best cleavage efficiency as detected using the GeneArt Genomic Cleavage Detection Kit.

2. Genome editing in human iPSCs grown in KnockOut Serum Replacement media systems

2.1. Cell culture

Feeder-dependent Gibco Human Episomal iPSC Line (Cat. No. A18945) is cultured on mitotically inactivated MEF feeder cells (EMD Millipore, Cat. No. PMEF-H) in human ESC (hESC) medium containing 20% Gibco™ KnockOut™ Serum Replacement (Cat. No. 10828010), 10 µM MEM Non-Essential Amino Acids Solution (Cat. No. 11140050), 55 µM 2-Mercaptoethanol (Cat. No. 21985023), and 4 ng/mL FGF-Basic (Cat. No. PHG0264) in DMEM/F-12 (Cat. No. 10565018). iPSC cultures are maintained in a 5% CO_2, 37°C humidified incubator with daily media changes and passaged regularly using Gibco™ Collagenase Type IV (Cat. No. 17104019).

2.2. Feeder-free adaptation

Feeder-dependent iPSCs are grown to 80% confluency prior to harvesting with collagenase. Following removal of the cell clusters from the feeder layer, cell clusters are gravity-sedimented to prevent MEF contamination. The cell clusters are then seeded on tissue culture plates coated with Geltrex LDEV-Free, hESC-Qualified, Reduced Growth Factor Basement Membrane Matrix in MEF-conditioned medium supplemented with 4 ng/mL FGF-Basic. MEF-conditioned medium is produced using MEF feeder cells in hESC medium that is harvested on 7 continuous days, sterile-filtered, and frozen until usage. The cultures are allowed to reach 80–90% confluence. The day prior to transfection, cultures should be inspected for signs of differentiation and any contamination from differentiated cells removed via microdissection.

2.3. Harvesting cells for transfection and electroporation

1. One day prior to transfection, rinse cells once with DPBS and then incubate with TrypLE Express Enzyme for about 3 min.
2. Monitor the digestion process periodically under the microscope. When gaps and cracks begin to appear within the large colonies, immediately remove TrypLE Express Enzyme and add 3 mL of growth medium.
3. To avoid cell death, gently dislodge cells into small clumps consisting of approximately triplets of cells. Monitor this step under a microscope. Count an aliquot of cells using the Countess II Automated Cell Counter or hemocytometer.
4. Centrifuge cells at 800 rpm for 3 minutes, then carefully aspirate medium and wash cell pellet with DPBS.
5. Plate 4–5 x 10^4 cells in a 24-well tissue culture plate with medium containing 10 µM ROCK inhibitor and 1X RevitaCell Supplement, then allow cells to recover overnight.
6. Use 1 x 10^5 cells for electroporation with the Neon Transfection System (sections 2.6 and 2.7).

2.4. Knockout by transfection of gRNA and GeneArt CRISPR Nuclease mRNA

1. Prepare dilutions of RNA and transfection reagent in separate tubes:

Tube 1:
25 µL Opti-MEM I medium
500 ng GeneArt CRISPR Nuclease mRNA
150 ng gRNA

Mix well and incubate for 5 min at room temperature.

Tube 2:
25 µL Opti-MEM I medium
1.5 µL Invitrogen™ Lipofectamine™ MessengerMAX™ Transfection Reagent

Mix well and incubate for 5 min at room temperature.

2. Add contents of tube 1 to tube 2, briefly vortex, and incubate the complex for 10–15 min at room temperature (the order of addition is important).
3. Add the entire complex to one well of a 24-well plate containing cells.
4. Six hours posttransfection, replace medium with 1 mL of fresh iPSC culture medium without ROCK inhibitor and return the plate to the incubator.

5. After 48–72 hours posttransfection, wash the cells once with 500 µL PBS and analyze the cleavage efficiency by using the GeneArt Genomic Cleavage Detection Kit.

2.5. Knock-in by transfection of gRNA, GeneArt CRISPR Nuclease mRNA, and single-stranded DNA (ssDNA)

Transfections are performed in a similar fashion as described in section 2.4. For single-nucleotide changes, 10 pmol of 100 bp ssDNA can be added along with gRNA and GeneArt CRISPR Nuclease mRNA (see section 2.4, step 1).

2.6 Knockout by electroporation of RNP using the Neon Transfection System

Electroporation protocol is similar to the procedure described in section 1.4.

2.7. Knock-in by electroporation of RNP and ssDNA using the Neon Transfection System

Electroporation is performed in a similar fashion as described in section 1.4. For gene knock-ins, 10 pmol of oligo (97-base ssDNA) is added along with gRNA and Cas9 protein. The total volume of Cas9 protein, gRNA, and ssDNA should not increase more than 1 µL per electroporation reaction. The best electroporation condition we observed for knock-ins on the Neon Transfection System was optimization protocol 21 (1,300 V, 10 ms, 3 pulses).

References

1. Liang X, Potter J, Kumar S et al. (2015) Rapid and highly efficient mammalian cell engineering via Cas9 protein transfection. *J Biotechnol* 208:44–53.
2. Yu X, Liang X, Xie H et al. (2016) Improved delivery of Cas9 protein/gRNA complexes using Lipofectamine CRISPRMAX. *Biotechnol Lett* 38:919–929.

Recommendations for transfection optimization

- The ratio of GeneArt Platinum Cas9 Nuclease to gRNA (5:1) can be varied to optimize the genome editing efficiency. For example, we found that 1,500 ng protein and 300 ng gRNA works best for electroporation-mediated knockouts in the Gibco Human Episomal iPSC Line.
- To optimize electroporation conditions, follow the 24-well optimization protocol of the Neon Transfection System. Each of the 24 conditions varies in pulse voltage, pulse width, and number of pulses to help you find which conditions work best for your cell line.
- Test different amounts of GeneArt Platinum Cas9 Nuclease and gRNA for various iPSC lines. For the Gibco Human Episomal iPSC Line grown on Geltrex matrix in Essential 8 Medium using 250 ng of HPRT gRNA control and 1,000 ng of Cas9 protein, 30–50% cleavage efficiency was observed.
- Optimize transfection reagent dosage for every new medium or culture condition. Test different dosages of Lipofectamine CRISPRMAX reagent and Cas9 Plus reagent in 24-well plates, such as 1 µL, 1.5 µL, 2 µL, and 3 µL.
- Cell-harvesting reagents influence the transfection and cleavage efficiency. For example, cells collected with TrypLE Express Enzyme showed higher cleavage efficiency compared to cells collected using dispase enzyme.
- Transfection of the Gibco Human Episomal iPSC Line in the presence of ROCK inhibitor showed higher efficiency compared to cells without ROCK inhibitor.
- Dislodging iPSCs into a single-cell suspension is an important factor in achieving higher transfection and cleavage efficiencies.

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