CRISPR-Cas9 Genome Editing for Research of Human Pluripotent Stem Cells Cultured in StemFlex™ Medium via Electroporation

Pub. No. MAN0016956 **Rev.** A.0

WARNING! Read the Safety Data Sheets (SDSs) and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves. Safety Data Sheets (SDSs) are available from **thermofisher.com/support**.

Introduction

This protocol describes the delivery of Cas9/guide RNA complexes via electroporation to pluripotent stem cells (PSCs) cultured in StemFlex™ Medium, expansion post-editing, and best practices for flow sorting of cultures and subsequent clonal expansion in research applications. For additional information on the StemFlex™ Medium refer to the MAN0016431 for detailed instructions on culturing human PSCs under feeder-free conditions in this culture medium.

Required materials not supplied

Unless otherwise indicated, all materials are available through **thermofisher.com**.

Product	Catalog No.
StemFlex™ Medium Kit	A3349401
Geltrex™ LDEV-Free, hESC-Qualified, Reduced Growth Factor Basement Membrane Matrix[1]	A1413302
Recombinant Human Laminin-521[1]	A29248, A29249
DMEM/F-12, GlutaMAX™ Supplement	10565
TrypLE™ Select Enzyme (1X), no phenol red	12563011
TrypLE™ Express Enzyme (1X), no phenol red	12604013
DPBS, no calcium, no magnesium	14190
DPBS, calcium, magnesium	14040
GeneArt™ Platinum™Cas9 Nuclease (3 µg/ µL)	B25641
GeneArt™ Precision gRNA Synthesis Kit	A29377
Versene Solution	15040
Neon™ Transfection System 10 µL Kit	MPK1025, MPK1096
Qubit™ 3.0 Fluorometer	Q33216
Qubit™ RNA BR Assay Kit	Q10210
(Optional): RevitaCell™ Supplement (100X)	A2644501
(Optional): Human Episomal iPSC Line	A18945
(Optional): TRA-1-60 Alexa Fluor™ 488 Conjugate Kit for Live Cell Imaging	A25618

[1] Use Geltrex™ LDEV-Free, hESC-Qualified, Reduced Growth Factor Basement Membrane Matrix or Recombinant Human Laminin-521

Design and generate gRNA by *in vitro* transcription

1. Use the 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 genome.

   Predesigned GeneArt™ gRNAs are optimized for gene knockout and typically target the first 3 transcribed exons per gene.

2. Generate your DNA template containing the T7 promoter and the guide RNA (gRNA) sequence with the GeneArt™ Precision gRNA Synthesis Kit.

3. Determine gRNA concentration with the Qubit™ 3.0 Fluorometer coupled with the Qubit™ RNA BR Assay Kit.

Prepare CRISPR-Cas9/gRNA Complex

1. Add 0.5 µL of GeneArt™ Platinum™Cas9 Nuclease (3 µg/ µL) and 300 ng of gRNA to 5 µL of Resuspension Buffer R and mix gently.

   Note: The volume of gRNA should be 0.5 µL or less.

2. Determine gRNA concentration using Qubit™ 3.0 Fluorometer.

3. Incubate the complex at room temperature for 10 minutes.

Procedural guidelines

Coat 24-well plates with Geltrex™ LDEV-Free, hESC-Qualified, Reduced Growth Factor Basement Membrane Matrix

1. Dilute Geltrex™ matrix 1:100 in cold DMEM/F-12, GlutaMAX™ Supplement.

2. Add 300 uL per well.

3. Incubate plate(s) at 37°C, 5% CO_2 for >1 hour ahead of seeding of PSCs.

Coat 24-well plates with rhLaminin-521

The optimal working concentration rhLaminin-521 is cell line dependent and ranges from 0.5–2.0 µg/cm^2.

1. To coat plates with 0.5 µg/cm^2, dilute 300 uL of rhLaminin-521 in 12 mL of DPBS, calcium, magnesium, DMEM/F-12, GlutaMAX™ Supplement or StemFlex™ Basal Medium.

2. Add 400 uL of diluted rhLaminin-521 per well.

3. Incubate plates at 37°C, 5% CO_2 ahead of PSC seeding.

Prepare PSCs for electroporation

See "Procedural guidelines" for plate coating information. If using pre-coated plates stored at 2°C to 8°C, pre-warm rhLaminin-521 or Geltrex™ matrix-coated plates to room temperature. Pre-warm StemFlex™ Medium and TrypLE™ Select Enzyme to room temperature.

1. Upon PSCs reaching 40–85% confluency, aspirate spent medium from the culture vessel.

2. Rinse the vessel once with recommended volume of DPBS, no calcium, no magnesium (DPBS –/–). See table for recommended volumes.

Culture vessel (surface area)	6-well (10 cm^2)	12-well (4 cm^2)	24-well (2 cm^2)	35-mm (10 cm^2)	60-mm (20 cm^2)	100-mm (60 cm^2)
DPBS(–/–)	2 mL/well	1 mL/well	0.5 mL/well	2 mL/dish	4 mL/dish	12 mL/dish

3. Aspirate DPBS, no calcium, no magnesium.

4. Add TrypLE™ Select Enzyme to the vessel containing PSCs (see table for recommended volumes), then swirl the vessel to coat the entire well surface.

Culture vessel (surface area)	6-well (10 cm^2)	12-well (4 cm^2)	24-well (2 cm^2)	35-mm (10 cm^2)	60-mm (20 cm^2)	100-mm (60 cm^2)
TrypLE™ Select Enzyme	1 mL/well	0.4 mL/well	0.2 mL/well	1 mL/dish	2 mL/dish	6 mL/dish

5. Incubate the vessel at 37°C, 5% CO_2 for 3–5 minutes.

6. Gently pipette the cells up and down 5–10 times with a 1000 µL pipette to generate a single cell suspension.

7. Transfer the cell suspension to a conical tube containing the recommended neutralization volume of StemFlex™ Medium to dilute the dissociation reagent. See table for recommended volumes.

Culture vessel (surface area)	6-well (10 cm^2)	12-well (4 cm^2)	24-well (2 cm^2)	35-mm (10 cm^2)	60-mm (20 cm^2)	100-mm (60 cm^2)
Neutralization volume, StemFlex™ Medium	3 mL/well	1.2 mL/well	0.6 mL/well	3 mL/dish	6 mL/dish	18 mL/dish

8. Centrifuge the PSCs at 200 × *g* for 4 minutes, then aspirate and discard the supernatant.

9. Flick the tube 3–5 times to loosen the pellet, then resuspend the cells by pipetting them up and down 5–10 times in a resuspension volume of StemFlex™ Medium. See table for recommended volumes.

Culture vessel (surface area)	6-well (10 cm^2)	12-well (4 cm^2)	24-well (2 cm^2)	35-mm (10 cm^2)	60-mm (20 cm^2)	100-mm (60 cm^2)
Resuspension volume, StemFlex™ Medium	2 mL/well	1 mL/well	0.5 mL/well	2 mL/dish	4 mL/dish	12 mL/dish

10. Determine the viable cell density and percent viability using a Countess™ II Automated Cell Counter or similar automated or manual method.

Electroporate CRISPR-Cas9/gRNA Complex via Neon™ Transfection System

1. Transfer 1 million viable cells to a sterile microcentrifuge tube and centrifuge at 200 × *g* for 4 minutes.

2. Carefully and completely aspirate the growth medium.

   Do not disturb the cell pellet.

3. Carefully resuspend the cell pellet in 50 µL of Resuspension Buffer R.

4. Transfer 5 µL of resuspended cells to the 6 µL of CRISPR-Cas9/gRNA complexes prepared in“Prepare CRISPR-Cas9/gRNA Complex“ .

   Mix gently.

5. Pipette 10 µL of the cell suspension into the Neon™ tip and electroporate with protocol 7 (1200 V, 30 ms, 1 pulse) or protocol 14 (1200 V, 20 ms, 2 pulses).

   Be careful to not introduce bubbles.

   We recommend that users optimize electroporation conditions for the Neon™ Transfection System for their specific cell type. HPRT gRNA control, is available for purchase as custom gRNA, for transfection optimization. To order, contact us at **GEMServices@thermofisher.com**.

6. Immediately transfer the electroporated cells into a 24-well plate containing 0.5 mL of StemFlex™ Medium +/- 1X RevitaCell™ Supplement.

7. Move the vessel in several quick side-to-side motions to disperse the cells across the surface of the vessel.

8. Carefully transfer the vessel to a 37°C, 5% CO_2 incubator and incubate the cells overnight.

9. Feed the PSCs the day after electroporation.

10. Analyze the cells 48–72 hours after electroporation.

11. Harvest cells and save a portion for continued propagation and with the other portion measure cleavage efficiency using the GeneArt™ Genome Cleavage Detection Kit.

    With the Neon™ Transfection System we have obtained up to 80% cleavage efficiency with HPRT gRNA control in the Gibco™ Human Episomal iPSC Line expanded on Geltrex™ matrix.

Expand PSCs following genome editing

See "Procedural guidelines" for plate coating information. If using pre-coated plates stored at 2°C to 8°C, pre-warm rhLaminin-521-coated plates to room temperature. Incubate plate(s) at 37°C, 5% CO_2 for >2 hours ahead of seeding of PSCs. Pre-warm StemFlex™ Medium and Versene Solution or 500 µM EDTA solution to room temperature.

1. Aspirate spent medium from the culture vessel.

2. Rinse the vessel once with recommended volume of DPBS, no calcium, no magnesium. See table for recommended volumes.

Culture vessel (surface area)	6-well (10 cm^2)	12-well (4 cm^2)	24-well (2 cm^2)	35-mm (10 cm^2)	60-mm (20 cm^2)	100-mm (60 cm^2)
DPBS (–/–) wash	2 mL/well	1 mL/well	0.5 mL/well	2 mL/dish	4 mL/dish	12 mL/dish

3. Add Versene Solution or 500 µM EDTA to the side of the vessel containing PSCs, see table, then swirl the vessel to coat the entire well surface.

Culture vessel (surface area)	6-well (10 cm^2)	12-well (4 cm^2)	24-well (2 cm^2)	35-mm (10 cm^2)	60-mm (20 cm^2)	100-mm (60 cm^2)
Versene Solution	1 mL/well	0.4 mL/well	0.2 mL/well	1 mL/dish	2 mL/dish	6 mL/dish
500 µM EDTA						

4. Incubate the vessel at room temperature for 5 to 8 minutes or at 37°C for 4 to 5 minutes.

   When the cells start to separate and round up, and the colonies appear to have holes in them when viewed under a microscope, they are ready to be removed from the vessel.

   Note: Do not incubate the cells to the extent that the colonies float off the surface of the culture vessel.

5. Aspirate the Versene Solution or 500 µM EDTA, and add pre-warmed complete StemFlex™ Medium to the vessel. Remove the cells from the well(s) by gently flushing medium over the surface of the well a few times. See table for recommended volumes.

Culture vessel (surface area)	6-well (10 cm^2)	12-well (4 cm^2)	24-well (2 cm^2)	35-mm (10 cm^2)	60-mm (20 cm^2)	100-mm (60 cm^2)
Complete StemFlex™ Medium	2 mL/well	1 mL/well	0.5 mL/well	2 mL/dish	4 mL/dish	12 mL/dish

6. Collect cells in a 15-mL or 50-mL conical tube.

   There may be obvious patches of cells that were not dislodged and left behind. Do not scrape the cells from the dish in an attempt to recover them.

   Note: Depending upon the cell line, work with no more than 1 to 3 wells at a time, and work quickly to remove cells after adding StemFlex™ Medium to the well(s), which quickly neutralizes the initial effect of the Versene Solution or 500 µM EDTA. Some lines re-adhere very rapidly after medium addition, and must be removed 1 well at a time. Others are slower to re-attach, and may be removed 3 wells at a time.

7. Coat culture vessel for 2 hours at 37 °C, 5% CO_2.

8. Aspirate rhLaminin-521 from the culture vessel and discard.

   Do not allow the culture surface to dry out.

9. Immediately add an appropriate volume of pre-warmed complete StemFlex™ Medium to each well of rhLaminin-521-coated plate so that each well contains the recommended volume of complete medium after the cell suspension has been added. See table for recommended volumes.

   Note: The split ratio can vary, though it is generally between 1:6 and 1:18 for established cultures on rhLaminin-521-matrix. Occasionally, cells may recover at a different rate and the split ratio will need to be adjusted.

Prepare PSCs for flow sorting experiments

1. Coat 96-well plates with 0.5–2 µg/cm^2 rhLaminin-521 in 50 µL total volume per well of StemFlex™ Basal Medium.

   Note: Do not use complete StemFlex™ Medium here with the supplement added as the BSA will prevent coating.

2. Incubate plates at 37°C, 5% CO_2 for ≥2 hours.

3. Single cell passage iPSCs as per instructions above in "Prepare PSCs for electroporation".

4. Spin down PSCs and resuspend in DPBS, no calcium, no magnesium and perform flow sorting experiments.

Flow sorting procedural guidelines

- Adaptation onto the rhLaminin-521 for at least 2 passages ahead of fluorescence activated flow sorting is recommended to improve clonal survival following flow sorting.
- Prior to sorting, we recommend staining with Propidium Iodide to facilitate gating of live cells
- We also recommend staining with TRA-1-60 Alexa Fluor™ 488 Conjugate Kit for Live Cell Imaging to ensure sorting of only pluripotent cells.

  Note: This step is not required but useful if working with a iPSC line that tends to show areas of differentiation.
- Sort directly into StemFlex™ Medium.
- Do not aspirate off the rhLaminin-521 matrix coating solution from the well ahead of addition of the cells in StemFlex™ Medium. Rather overlay with 150 uL of 1.33X StemFlex™ Medium (dilute StemFlex™ Supplement 10X to 1.33X with StemFlex™ Basal Medium); e.g., for preparation of 15 mL of 1.33X StemFlex™ Medium add 2 mL of StemFlex™ Supplement 10X to 13 mL of StemFlex™ Basal Medium to give a final concentration of 1X.
- Addition of ROCK inhibitor, RevitaCell™ Supplement to StemFlex™ Medium slightly improves results.
- Do not change the media for the first 3 days, even in the presence of RevitaCell™ Supplement. We find this greatly improves the number of colonies. Thereafter, media changes can be done every 2–3 days for the next two weeks. **Do not** use RevitaCell™ Supplement in these subsequent media changes.
- Evaluate the presence/absence of colonies after 14 days and at this point can be expanded further using routine passage methods. Refer to *StemFlex™ Medium* Kit User Guide (Pub. No. MAN0016431) for further instructions.

Limited product warranty

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