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

Pub. No. MAN0016956 **Rev.** B.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. See *StemFlex™ Medium Kit User Guide* (Pub. No. 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	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 (DPBS –/–)	14190
DPBS, calcium, magnesium (DPBS +/+)	14040
TrueCut™ Cas9 Protein v2	A36498
GeneArt™ Precision gRNA Synthesis Kit	A29377
Versene Solution	15040
Neon™ Transfection System 10 µL Kit	MPK1025, MPK1096
Neon™ Transfection System	MPK5000
Qubit™ 3.0 Fluorometer	Q33216
Qubit™ RNA BR Assay Kit	Q10210
RevitaCell™ Supplement (100X)	A2644501
TRA-1-60 Alexa Fluor™ 488 Conjugate Kit for Live Cell Imaging	A25618
Gentamicin	15750078

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

Product	Catalog No.
Propidium Iodide	P3566
PSC Cryopreservation Kit	A2644601
(Optional): Human Episomal iPSC Line	A18945

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 0.3 µL of TrueCut™ Cas9 Protein v2 (5 µg/ µL) and 300 ng of gRNA to 5 µL of Resuspension Buffer R and mix gently.

   Note: It is recommended to dilute the Cas9 nuclease in Resuspension Buffer R to a concentration of 0.5 µg/ µL, and then adding 3 µL, still maintaining a final volume of 5 µL.

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

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

   Note: While complex formation is occurring, the cells can be prepared for electroporation as outlined below.

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 2 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.

   Note: If a SNP change is being made, 10 pmol of a 100 bp single stranded oligo donor carrying the desired SNP change can be added in this step by adding 1 µL of oligo donor diluted in Resuspension Buffer R.
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 with or without 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 with StemFlex™ Medium 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™ Genomic 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.
12. Alternatively, when making SNP changes, use a target specific NGS analysis to estimate genome editing efficiency.

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.

   Note: TrypLE™ Select Enzyme can be used as described in "Prepare PSCs for electroporation" on page 3.

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.

10. If desired, the edited pools can be cryopreserved at this stage using the PSC Cryopreservation Kit.

Procedural guidelines for pool recovery, clonal isolation and screening

If the desired edit was detected in the cell pool, clonal isolation is needed to obtain a homogenous cell line. A stringent sorting protocol should be used to ensure seeding of single, viable and pluripotent iPSC. Before you begin preparing your samples, ensure the sorter is available since prepared cells should be sorted as soon as possible.

The number of 96-well plates needed to generate single cell clones depends on the edit incidence and the need for heterozygous or homozygous clones. For example, if you have a 5% editing efficiency and you need a homozygous cell line, you have a 1/40 chance to find a homozygous clone, which means you would need to screen at least 40 colonies to find it. When estimating the number of 96-well plates needed, expect about 15–50 colonies (PSC line dependent), but do generate a couple extra plates to ensure you obtain the appropriate number of clones.

Recover the cell pool (if the electroporated pool was frozen

Thaw the preferred pool on rhLaminin-521 in StemFlex™ Medium Kit and perform one passage before cell sorting.

Prepare plates for sorting

1. Prepare coated 96-well plates by adding 50 µL rhLaminin-521 solution per well (1:40 dilution in DPBS +/+).
2. Incubate rhLaminin-521-coated plates at 37°C, 5% CO_2 for 2 hours.
3. Add 150 µL of iPSC medium containing 1X RevitaCell™ Supplement and Gentamicin (100 µg/mL final concentration).

   Note: Gentamicin is used to prevent any contamination which may be introduced during cell sorting.

Prepare cell samples for clonal sorting

1. For single cell sorting, aspirate the medium from two 2 wells of a 6-well plate of a ~70% confluent culture and wash with DPBS –/–.
2. Add 1 mL of TrypLE™ Express Enzyme and place in the 37°C, 5% CO_2 incubator for 3 minutes.
3. Aspirate the TrypLE™ Express Enzyme and resuspend the cells in iPSC medium plus RevitaCell™ Supplement, use 2 mL well.
4. Distribute the 4 mL into four 15 mL sterile tubes: pipet 0.5 mL dissociated cells into three tubes (for unstained, TRA-1-60 only, and Propidium Iodide only sort controls) and 2.5 mL into one tube (for sorting single cells).
5. Add 2.5 µL Propidium Iodide to the Propidium Iodide control tube, add 5 µL Alexa Fluor™ 488 Mouse Anti-Human TRA-1-60 to the TRA-1-60 control tube, and add 12.5 µL Propidium Iodide and 25 µL Alexa Fluor™ 488 Mouse Anti-Human TRA-1-60 to the 2.5 mL tube.
6. Stain on ice for 30 minutes.
7. Wash 3 times with DPBS –/–.
8. Resuspend each sample in 2 mL of DPBS –/– plus RevitaCell™ Supplement and transfer to a round bottom tube with a cell strainer cap for sorting.
9. Incubate the cells on ice while setting up the controls on the sorter.
10. Sort single cells into the 96-well plates prepared above, through gating out doublets (forward and sideward scatter based), dead cells (Propidium Iodide positive cells), and differentiated cells (TRA-1-60 negative cells).

Expansion of single cells post sorting

1. Right after sorting, incubate plates at 37°C, 5% CO_2 for 72 hours.
2. Perform a medium change with 175 µL medium plus Gentamicin.
3. Perform media changes every three days until colonies are observed, maintaining Gentamicin addition for the first week of culture after sorting.

   After one week, StemFlex™ Medium alone should be used.
4. Check on the colonies, which should begin emerging on day 8 and reach a good size by day 14.
5. On Day 14, scan plates using whole-well imaging and confluency analysis to identify clones.

   Wells with confluency greater than 5% will typically contain a colony.

Consolidate single cell clones post sorting

After sorting single cells into 96-well plates, clones will emerge in random wells across the plates. To facilitate downstream processing, consolidate identified clones into a new rhLaminin-521-coated 96-well plate.

1. Aspirate the media from the 96-well plates, wash with DPBS –/–, add 50 µL TrypLE™ Express Enzyme and incubate at 37°C, 5% CO_2 for 3 minutes.
2. Aspirate the TrypLE™ Express Enzyme, resuspend the wells containing clones in 150 µL medium plus RevitaCell™ Supplement, and add to a 96-well plate coated with rhLaminin-521.
3. Mix the cells well and transfer half to a PCR plate.
4. Retain the other half in the rhLaminin-521-coated plate and move the rhLaminin-521-coated plate into a humidified 37°C, 5% CO_2 incubator overnight.
5. Spin down the cells in the PCR plate and aspirate the supernatant, leaving behind about 20 µL per well to avoid aspirating the cells.

   The PCR plate can then be frozen until ready for PCR processing and Sequencing.

6. The next day, change the medium in the rhLaminin-521-coated plate with 175 µL medium and allow the cells grow until ~80% confluence.

7. When confluent, cryopreserve the clones as follows: aspirate the media, wash with DPBS −/−, add 50 µL TrypLE™ Express Enzyme, and incubate at 37°C, 5% CO_2 for 3 minutes.

8. Aspirate the TrypLE™ Express Enzyme and resuspend in 200 µL PSC Cryopreservation Medium.

9. Wrap the plate(s) with Parafilm™ wrapper and store in a zippered plastic bag in −80°C.

Clone recovery and expansion

The clones will be frozen while screening is undergoing by Sanger Sequencing (or NGS). As positive clones have been identified, follow this protocol to thaw and expand the desired clones.

1. Prepare rhLaminin-521-coated 12-well plates, count one well per clone to be recovered.

2. Thaw the frozen 96-well plate inside an incubator.

   This typically takes about 20 minutes.

3. Identify the clones to be recovered by circling the positive wells on the lid.

4. Resuspend each of thawed wells and add into a 15-mL conical containing 2 mL medium plus RevitaCell™ Supplement.

5. Spin down cells and resuspend in 1 mL medium plus RevitaCell™ Supplement and plate in one well of a 12-well plate.

   Repeat for as many clones as needed.

6. Expand cells until at least 15×10^6 cells have been obtained for characterization (Sequence confirmation by Sanger and/or NGS, TaqMan® PSC Scorecard, ICC TRA1-60/SOX2, karyotype and directed differentiation) and banking.

Limited product warranty

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