CRISPR-Cas9 editing of pluripotent stem cells cultured in StemFlex Medium

Introduction

The emergence of technology for development of induced pluripotent stem cells (iPSCs) from somatic cells, such as skin and blood cells, has resulted in the ability of researchers to have limitless pools of iPSCs retaining the genetic makeup of the somatic cells from which they were derived. In conjunction with tools for downstream gene editing, such as CRISPR-Cas9 systems, iPSCs can be used to generate (1) knockouts to study the impact of genes on cellular processes, or (2) knock-ins to assess the impact of reversal of point mutations on diseased states, or furthermore, for the generation of reporter cell lines. Briefly, CRISPR-Cas9 systems provide simple and efficient site-specific targeting that is accomplished by guiding Cas9 nuclease via a variable 20-base guide RNA (gRNA) sequence to a target site for formation of a double-strand break. This break can then be repaired via nonhomologous end joining (NHEJ), where small insertions or deletions are made in the gene of interest to knock out its function, or via homology-directed repair (HDR) in which single-nucleotide changes or knock-ins can be accomplished using a donor DNA template for repair.

Together, iPSCs and CRISPR-Cas9 systems provide researchers with effective *in vitro* tools for assessing gene function, disease modeling, and regenerative therapy. In this application note, we discuss the electroporation and lipid-based delivery workflows available for generating double-strand breaks for repair in iPSCs cultured under feeder-free conditions in Gibco™ StemFlex™ Medium. Nuances to the proposed protocol are also provided to demonstrate the versatility of StemFlex Medium in supporting cell health during workflows that are stressful to cells.

Suggested workflow: electroporation of PSCs cultured in StemFlex Medium

Figure 1 highlights the general workflow for electroporation-based delivery of ribonucleoprotein (RNP) complex containing Cas9 nuclease and gRNA to PSCs cultured in StemFlex Medium. For a detailed protocol, refer to **thermofisher.com/stemflexedit**. Briefly, proliferating cultures in StemFlex Medium are passaged using Gibco™ TrypLE™ Select Enzyme to singularize the cells. Following singularization, neutralization, and resuspension of cells in Buffer R, a complex of 1.5 µg of Invitrogen™ GeneArt™ Platinum™ Cas9 Nuclease (Cat. No. B25641) and 300 ng of gRNA is delivered to the PSCs via electroporation. Electroporated PSCs are then recovered for 48–72 hours post-electroporation.

At this point, some of the PSCs are used for continued expansion for downstream fluorescence-activated cell sorting (FACS), while the remaining material is used for detection of cleavage efficiency using the Invitrogen™ GeneArt™ Genomic Cleavage Detection Kit (Cat. No. A24372). Once PSCs are sufficiently expanded, viable (propidium iodide (PI) negative) and pluripotent (TRA-1-60 positive) stem cells are sorted via FACS analysis and seeded at 1–5 viable cells/well of a 96-well plate. PSC clones are then allowed to recover and subsequently sent for downstream sequencing analysis.

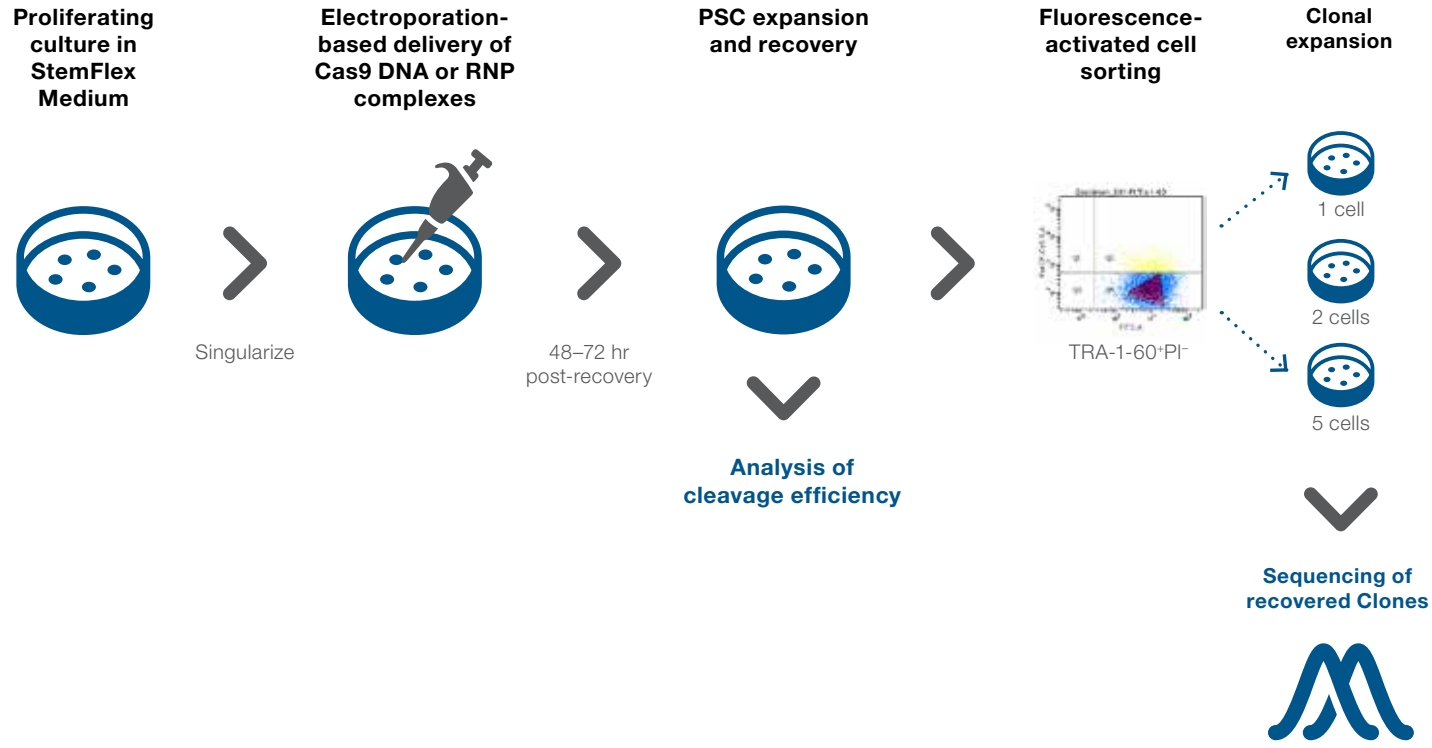


Figure 1. Schematic workflow for CRISPR-Cas9 RNP complex delivery via electroporation with subsequent flow sorting and expansion.

Results: electroporation

Gibco™ Human Episomal iPSCs (Cat. No. A18945) were adapted into StemFlex Medium for >3 passages. Electroporation was subsequently conducted using the Invitrogen™ Neon™ Transfection System (Cat. No. MPK5000) with the Neon™ Transfection System 10 µL Kit (Cat. No. MPK1096). Following electroporation of 100,000 viable cells resuspended in Buffer R with a complex prepared using 1.5 µg of GeneArt Platinum Cas9 Nuclease (Cat. No. B25641) and 300 ng of *in vitro*–transcribed (IVT) gRNA targeting the *HPRT* gene, cells were added to a well of a 24-well plate coated with Gibco™ Geltrex™ matrix in StemFlex Medium. Figure 2 shows representative images of cultures monitored with the IncuCyte™ ZOOM System after electroporation (1,200 V, 30 ms, 1 pulse). The images demonstrate robust recovery of iPSCs cultured in StemFlex Medium on Thermo Scientific™ Nunc™ 24-well cell culture–treated plates (Cat. No. 142475) coated with Geltrex matrix. Furthermore, these images indicate the expected morphology changes over time, resolving in normal PSC morphology. In Figure 3, cleavage efficiency 72 hours post-electroporation was assessed using the GeneArt Genomic Cleavage Detection Kit (Cat. No. A24372). These data demonstrate high levels of insertion or deletion (indel) formation following delivery of Cas9–gRNA complexes.

The following protocols for the Neon Transfection System are recommended for delivery of Cas9–gRNA complexes to PSCs cultured in StemFlex Medium: electroporation condition 7 (1,200 V, 30 ms, 1 pulse) or electroporation condition 14 (1,200 V, 20 ms, 2 pulses). Depending upon the content being delivered (RNP complex plus donor DNA, plasmid DNA, or mRNA plus gRNA), the electroporation conditions may need to be optimized. This can be accomplished by using the 24 preprogrammed optimization parameters provided on the Neon Transfection System. Refer to the Neon Transfection System User Guide (Pub. No. MAN0001557) for detailed instructions.

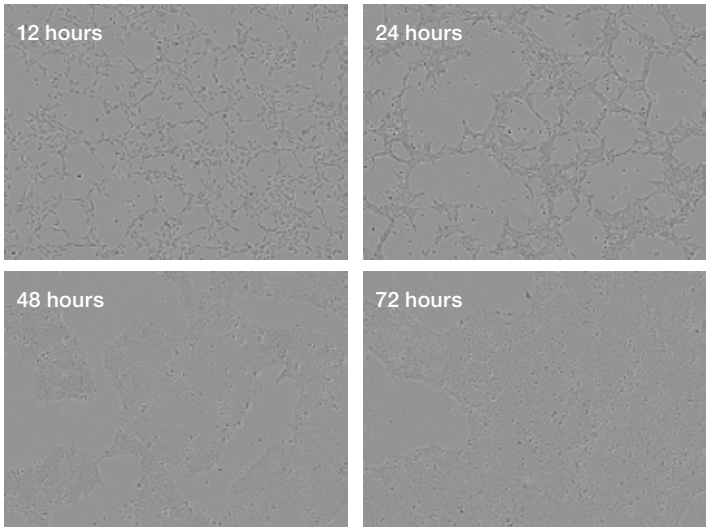


Figure 2. Representative images after electroporation showing robust recovery of iPSCs cultured in StemFlex Medium.

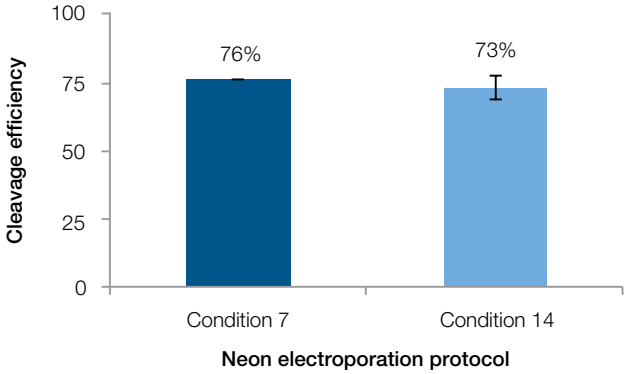


Figure 3. Cleavage efficiency of cultures grown in StemFlex Medium ~72 hours after electroporation with Cas9–gRNA complexes targeting the *HPRT* gene.

While a high percentage of cleavage efficiency may be attained, it is incredibly important to ensure that PSCs maintain pluripotency after editing, as harsh manipulation of cells can result in aberrant differentiation. Figure 4 indicates that PSCs recovered in StemFlex Medium following electroporation of Cas9–gRNA complexes maintain high levels of pluripotency as assessed by immunocytochemistry and flow cytometry.

In Figure 5, Human Episomal iPSCs, which underwent gene editing using Cas9–gRNA complexes targeting the *HPRT* gene, were expanded on Gibco™ rhLaminin-521 or Geltrex matrices. Following expansion, cells were sorted via FACS for live (PI negative) and pluripotent (TRA-1-60 positive) stem cells and seeded as 1 viable cell/well in the presence of 1X Gibco™ RevitaCell™ Supplement (Cat. No. A2644501). Three days post-seeding, the medium was exchanged, replacing spent medium with StemFlex Medium in the absence of RevitaCell Supplement. Medium was then exchanged with StemFlex Medium in the absence of RevitaCell Supplement every 3 days thereafter. Following recovery for 2 weeks, whole-well imaging using the IncuCyte ZOOM System was performed and the percentage of wells with >5% confluency, indicative of successful clonal expansion, was documented. As shown in Figure 5, PSCs expanded in StemFlex Medium on both Geltrex and rhLaminin-521 matrices demonstrate high clonal expansion. For leaner media systems, such as xeno-free Gibco™ Essential 8™ Medium, a benefit of using rhLaminin-521 over Geltrex matrix has been observed (data not shown).

To demonstrate the robustness and versatility of StemFlex Medium in supporting PSC health during electroporation-based Cas9 gene editing, a case example is provided. Using an alternative electroporation-based delivery protocol, a QuasAR2 reporter PSC line was developed. In this method, the process of homology-directed repair was utilized to insert a cassette at the *AAVS1* safe harbor locus. Invitrogen™ GeneArt™ CRISPR Nuclease Vector with OFP Reporter (Cat. No. A21174) along with donor plasmid carrying the QuasAR2 expression cassette and a puromycin resistance gene were delivered to the ESI-017 human embryonic stem cell line (ESI BIO) via electroporation. The electroporation condition that was used for this study was preset condition 2 on the Neon Transfection System (1,400 V, 20 ms, 1 pulse). ESI-017 cells were expanded in mTeSR™1 Medium or StemFlex Medium.

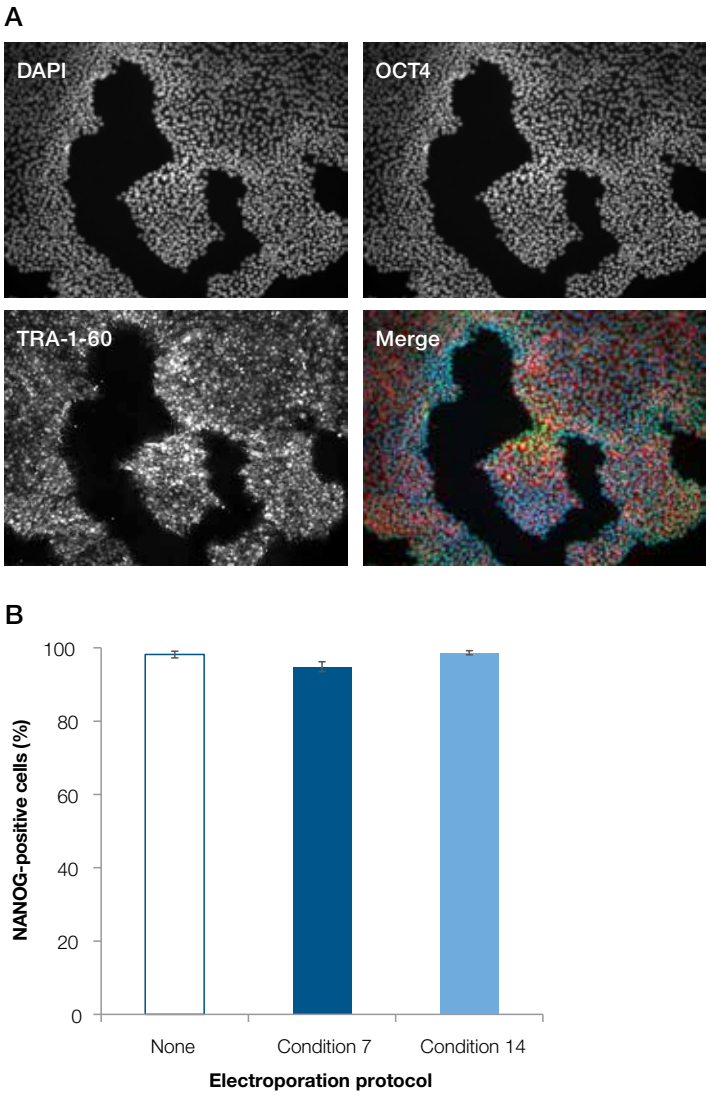


Figure 4. Maintenance of pluripotency of iPSCs cultured in StemFlex Medium after electroporation and recovery. Cultures transfected with Cas9–gRNA complexes targeting the *HPRT* gene were assessed by **(A)** qualitative immunocytochemistry of OCT4 and TRA-1-60 expression and **(B)** quantitative assessment of NANOG expression via flow cytometric analysis.

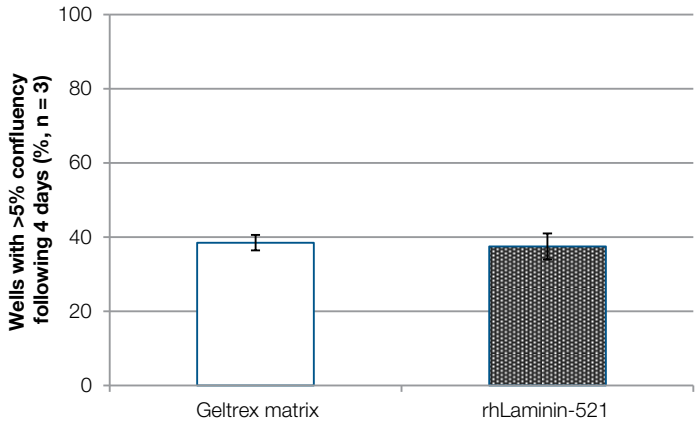


Figure 5. High-efficiency clonal expansion of PSCs in StemFlex Medium following FACS sorting of Cas9-edited cells.

For electroporations, 1 million cells were diluted into 100 µL of Buffer R with 5 µg total DNA (2.5 µg Cas9 plasmid, 2.5 µg donor vector), and cells were electroporated using the Invitrogen™ Neon™ Transfection System 100 µL Kit (Cat. No. MPK10096). Following electroporation, cells were plated at 250,000 viable cells in the respective medium (mTeSR1 or StemFlex Medium) in the presence of RevitaCell Supplement on rhLaminin-521–coated 24-well plates. Figure 6 indicates the relative cell survival in each system using the average of 3 independent experiments.

72 hours post-electroporation, cells were passaged using Gentle Cell Dissociation Reagent (STEMCELL Technologies) at 14,000 viable cells/cm^2 onto a 10 cm dish coated with rhLaminin-521 in the respective medium (mTeSR1 or StemFlex Medium) in the presence of 0.2 µg/mL puromycin. Culture medium was then exchanged daily for up to 10 days after seeding. Cell imaging of colonies in Figure 7 indicates robust recovery of PSCs in StemFlex Medium, with more abundant and larger-size colonies for further expansion.

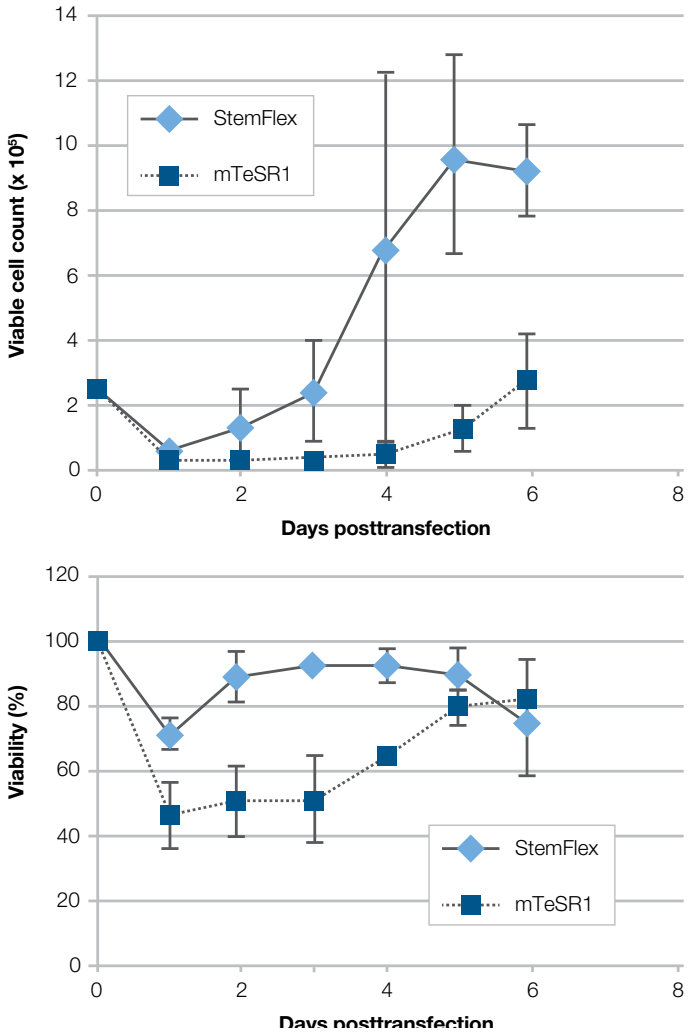


Figure 6. Cell growth and viability following electroporation of Cas9 plasmid and donor plasmid into ESI-017 cells.

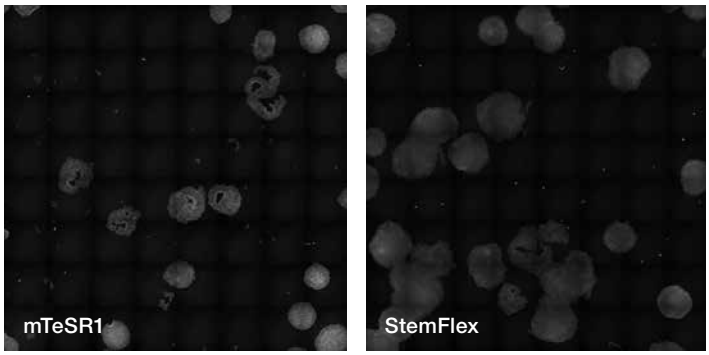
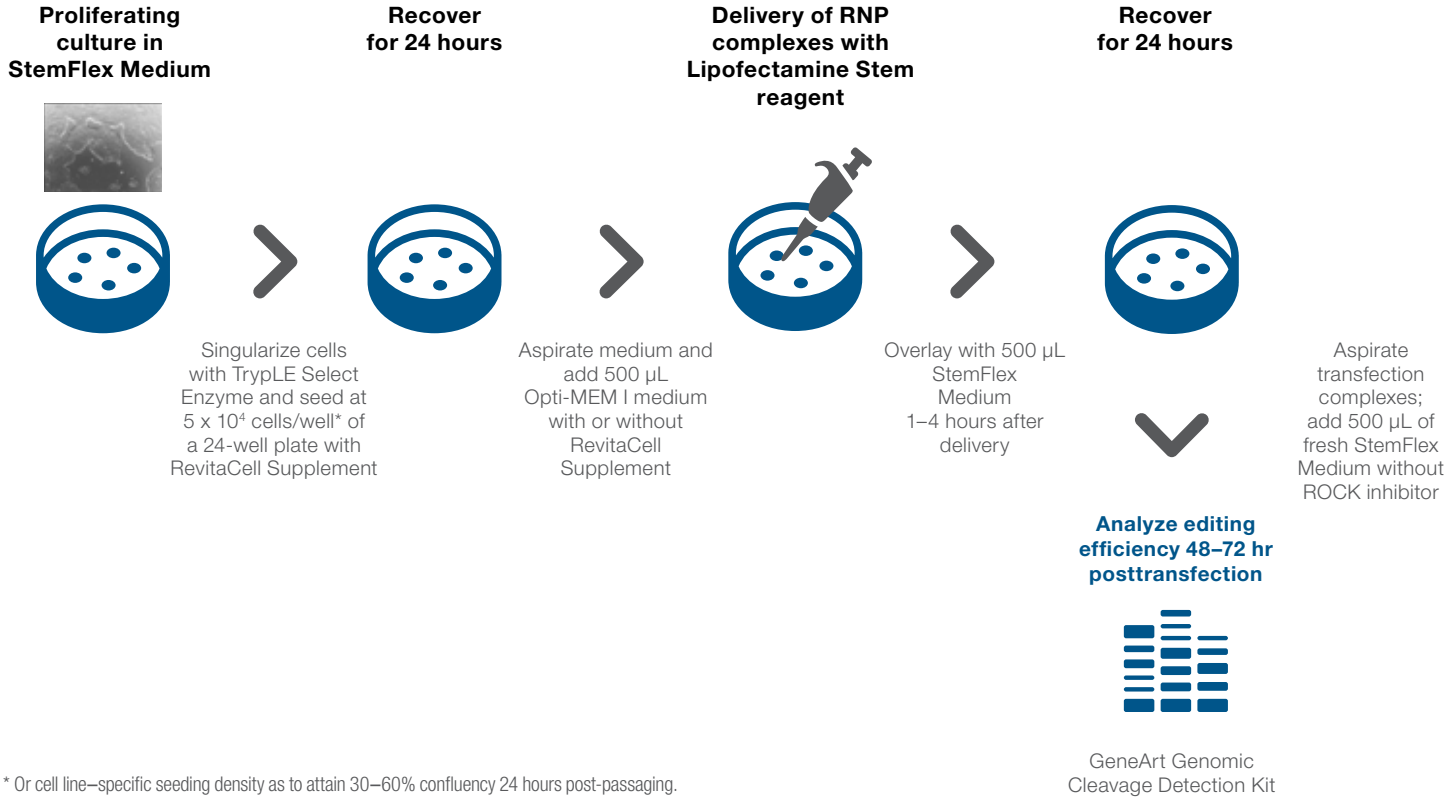


Figure 7. Clonal colony growth following electroporation-based editing of ESI-017 human embryonic stem cells.

Suggested workflow: lipid-based transfection of PSCs cultured in StemFlex Medium

An alternative method for gene editing of PSCs is lipid-based delivery of Cas9–gRNA complexes or plasmid DNA. This method allows for higher-throughput gene editing of PSCs. Invitrogen™ Lipofectamine™ Stem Transfection Reagent (Cat. No. STEM00001) is an efficient lipid-based reagent that provides robust delivery of gene editing content to PSCs, including RNP complexes, plasmid DNA, or Cas9 mRNA with gRNA. In this application note, we sought to optimize the delivery protocol of Cas9–gRNA complexes to cells maintained in StemFlex Medium (refer to Figure 8 for workflow and protocol at the end of this document). For PSCs cultured in StemFlex Medium, the medium is aspirated off of the cells, and cells are overlaid with Gibco™ Opti-MEM™ I Reduced Serum Medium (Cat. No. 31985062) with RevitaCell Supplement (Cat. No. A2644501). The transfection complex containing Cas9 and gRNA is then delivered to the PSCs with Lipofectamine Stem reagent and incubated for 1–4 hours. Following incubation, transfection complexes are overlaid with StemFlex Medium without ROCK inhibitor or RevitaCell Supplement (Figure 8). The medium is fully exchanged 24 hours posttransfection, and cleavage analysis and downstream expansion for clonal analysis can be initiated 48–72 hours posttransfection.



* Or cell line–specific seeding density as to attain 30–60% confluency 24 hours post-passaging.

Figure 8. Transfection workflow for delivery of Cas9–gRNA complexes to PSCs cultured in StemFlex Medium using Lipofectamine Stem reagent.

Results: lipid-based transfection

In Figure 9, Human Episomal iPSCs cultured in StemFlex, Essential 8, or Essential 8 Flex Medium for >15 passages were harvested using TrypLE Select Enzyme and seeded in StemFlex Medium with RevitaCell Supplement. The day following seeding of PSCs and immediately ahead of Cas9–gRNA complex delivery, PSC medium was aspirated from the PSCs and replaced with Opti-MEM I medium with RevitaCell Supplement. Subsequently, Cas9–gRNA complexes targeting the *HPRT* gene, as well as GFP mRNA, used as a transfection control, were delivered using 1 µL of Lipofectamine Stem reagent per reaction in a Thermo Scientific™ Nunc™ 48-well tissue culture–treated plate (Cat. No. 150687). Following incubation for 1–4 hours, transfection complexes were overlaid with 250 µL of respective feeder-free growth medium (StemFlex Medium, Essential 8 Medium, or Essential 8 Flex Medium) per well of 48-well plate and transfection efficiency was assessed using the IncuCyte ZOOM System (relative % GFP-positive confluency = % GFP confluency/% phase-contrast monitored confluency) 24 hours post-seeding. For 1-hour delivery ahead of overlay, all media demonstrated comparable transfection efficiency (Figure 9). For StemFlex Medium, an increase in transfection efficiency was observed by increasing the time ahead of overlay up to 4 hours.

Assessment of indel formation using the GeneArt Genomic Cleavage Detection Kit indicated high levels of successful cleavage upon Cas9–gRNA complex delivery to Human Episomal iPSCs. Optimal cleavage was shown for cells attaining confluencies of 30–60% ahead of lipid-based transfection (data not shown). For the experiments shown in Figure 10, cells were seeded at 50,000 or 75,000 viable cells/well of a Nunc 24-well cell culture–treated plate (Cat. No. 142475). Ahead of transfection, wells seeded at 50,000 viable cells/well were at 50.18 ±1.34% confluency (within the recommended range of starting confluency), whereas wells seeded at 75,000 viable cells/well were at 75.90 ±1.30% confluency (outside the recommended range of starting confluency). These data indicate that there is a small decline in performance outside of the recommended range of confluency.

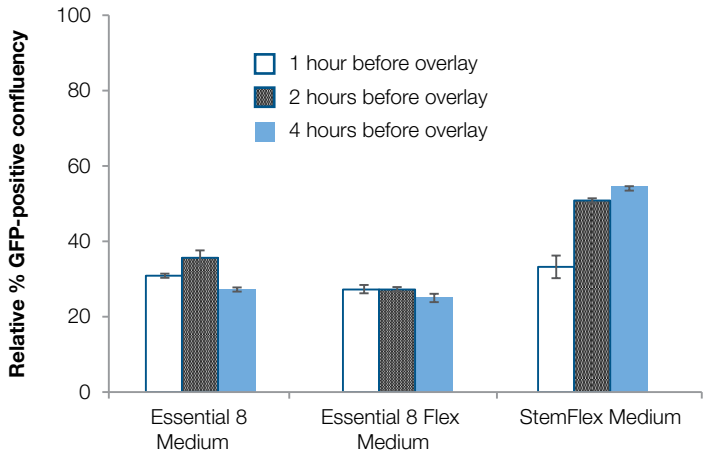


Figure 9. Impact of time ahead of overlay on transfection efficiency as assessed using a GFP mRNA control.

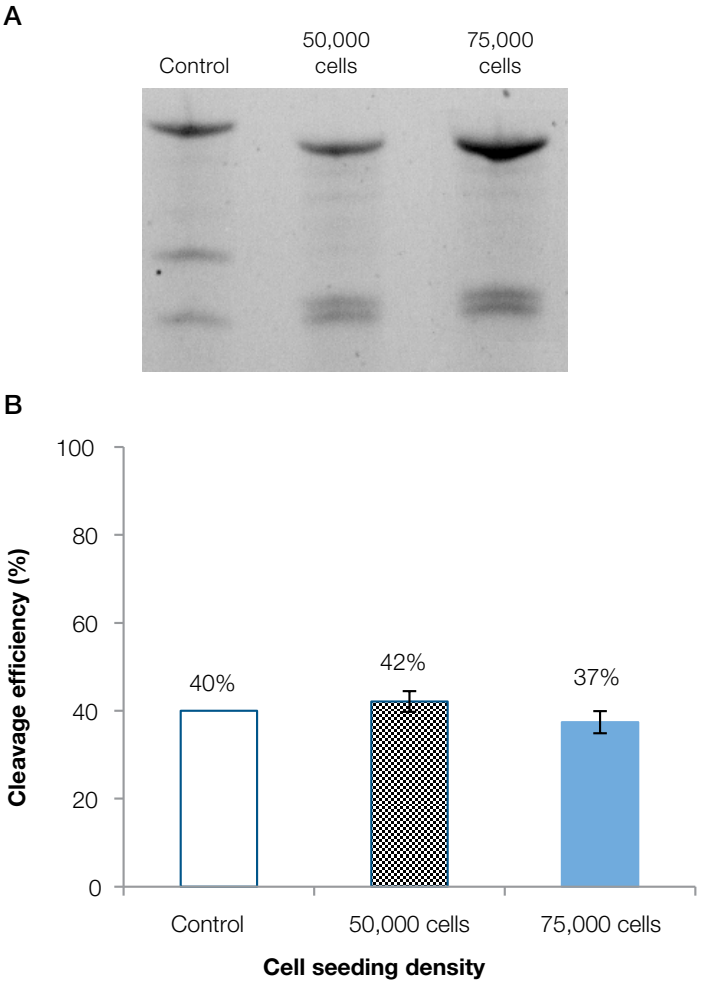


Figure 10. Genomic cleavage efficiency of PSCs transfected for 4 hours ahead of StemFlex Medium overlay. (A) Representative genomic cleavage results from cultures seeded at 50,000 and 75,000 viable cells/well and subsequently transfected with Cas9–gRNA complexes using 2 µL of Lipofectamine Stem reagent per the workflow in Figure 8. The GeneArt Genomic Cleavage Detection Kit control is also shown. **(B)** Average genomic cleavage for 4-hour transfection ahead of StemFlex Medium overlay for seeding densities of 50,000 and 75,000 cells/well. Bars represent mean ±standard deviation of 3 independent transfections.

While high transfection efficiency may be observed, it is imperative that the PSCs retain their pluripotency. As shown in Figures 10 and 11, cultures transfected with Lipofectamine Stem reagent demonstrate high cleavage efficiency of up to 42% detected at the *HPRT* locus, while maintaining high levels of expression of the intracellular marker of pluripotency OCT4. Qualitative assessment of OCT4 staining indicates 98.95 ±0.05% staining for cells seeded at 50,000 viable cells/well and 96.59 ±0.48% staining for cells seeded at 75,000 viable cells/well of a 24-well plate.

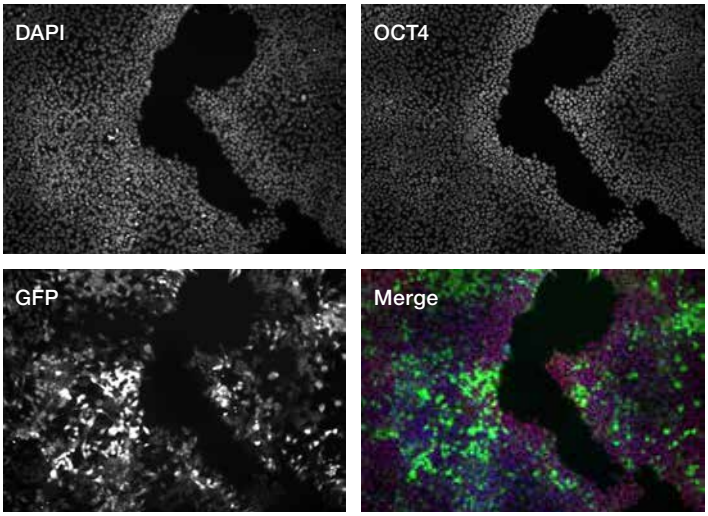


Figure 11. Representative images of transfection efficiency and maintenance of pluripotency.

Conclusions

Together, these data demonstrate the utility and versatility of StemFlex Medium in supporting PSC health during the CRISPR-Cas9 gene editing workflow. When paired with the Neon Transfection System for electroporation-based delivery or Lipofectamine Stem reagent for lipid-based delivery, efficient cleavage by Cas9 at the guided locus can be obtained without compromising the quality of PSCs.

For a detailed protocol outlining step-by-step instructions for electroporation-based delivery of Cas9–gRNA complexes and guidance for flow sorting of PSCs, please refer to the following user bulletin: CRISPR-Cas9 Genome Editing for Research of Human Pluripotent Stem Cells Cultured in StemFlex Medium via Electroporation (Pub. No. MAN0016956, available at **thermofisher.com**). For a detailed protocol for lipid-based transfection, please refer to the following section.

For information on lipid based delivery of alternative formats (e.g., protein, DNA, and/or mRNA) refer to **thermofisher.com/stemflexlipofectaminestem**

CRISPR-Cas9 genome editing of human PSCs cultured in StemFlex Medium via lipid-based transfection

This protocol describes delivery of Cas9–gRNA complexes using Lipofectamine Stem reagent to PSCs cultured in StemFlex Medium. Refer to the StemFlex Medium Kit User Guide (Pub. No. MAN0016431) for detailed instructions on culturing human PSCs under feeder-free conditions in this culture medium.

Reagents

- StemFlex Medium (Cat. No. A3349401)
- Geltrex LDEV-Free, hESC-Qualified, Reduced Growth Factor Basement Membrane Matrix (Cat. No. A1413302) or rhLaminin-521 (Cat. No. A29248)
- Gibco™ DMEM/F-12, GlutaMAX Supplement (Cat. No. 10565)
- TrypLE Select Enzyme (1X), no phenol red (Cat. No. 12563011) or TrypLE Express Enzyme (1X), no phenol red (Cat. No. 12604013)
- Gibco™ DPBS, no calcium, no magnesium (Cat. No. 14190144)
- GeneArt Platinum Cas9 Nuclease (Cat. No. B25641)
- gRNA (refer to "Designing and generating gRNA by *in vitro* transcription" section)
- Opti-MEM I Reduced Serum Medium (Cat. No. 31985062)
- Lipofectamine Stem Transfection Reagent (Cat. No. STEM0001)
- RevitaCell Supplement (Cat. No. A2644501)
- Optional: Human Episomal iPSC Line (Cat. No. A18945)

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 genome. These 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). gRNA concentration should be determined using the Invitrogen™ Qubit™ 3 Fluorometer (Cat. No. Q33216) coupled with the Invitrogen™ Qubit™ RNA BR Assay Kit (Cat. No. Q10210).

Transfection of adherent cells with Cas9–gRNA complexes via Lipofectamine Stem reagent

Approximately 24 hours before delivery of transfection complexes, detach cells from the culture vessel using TrypLE Select Enzyme and resuspend cells in growth medium with or without RevitaCell Supplement prior to cell counting:

1. If using precoated plates stored at 2–8°C, prewarm plates coated with rhLaminin-521 or Geltrex matrix to room temperature. Prewarm StemFlex Medium and TrypLE Select Enzyme to room temperature.

2. Precoat 24-well plates with Geltrex matrix or 0.5–2 µg/cm^2 rhLaminin-521:

   – **Instructions for coating with Geltrex matrix:** Prepare a 1:100 dilution of Geltrex matrix in cold DMEM/F-12 with GlutaMAX Supplement and add 300 µL/well of a 24-well plate. Incubate plates at 37°C in 5% CO_2 for >1 hour ahead of seeding of PSCs.

   – **Instructions for coating with rhLaminin-521:** The optimal working concentration for rhLaminin-521 is cell line–dependent and ranges from 0.5 to 2.0 µg/cm^2. Briefly, for coating with 0.5 µg/cm^2 rhLaminin-521, dilute 300 µL of rhLaminin-521 in 12 mL of Gibco™ DPBS with calcium and magnesium (Cat. No. 14040), DMEM/F-12 with GlutaMAX Supplement (Cat. No. 10565), or StemFlex Basal Medium (part of Cat. No. A3349301). Add 400 µL of the diluted rhLaminin-521 per well of a 24-well plate. Incubate plates at 37°C in 5% CO_2 for >2 hours ahead of seeding of PSCs.

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

4. Rinse the vessel once with 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, no calcium, no magnesium	2 mL/well	1 mL/well	0.5 mL/well	2 mL/dish	4 mL/dish	12 mL/dish

5. Aspirate DPBS, no calcium, no magnesium and add TrypLE Select Enzyme to the vessel containing PSCs, then swirl the vessel to coat the entire well surface. 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)
TrypLE Select Enzyme	1 mL/well	0.4 mL/well	0.2 mL/well	1 mL/dish	2 mL/dish	6 mL/dish

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

7. Gently pipet the cells up and down 5–10 times with a 1,000 µL pipette to generate a single-cell suspension.

8. 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	1.2 mL	0.6 mL	3 mL	6 mL	18 mL

9. Centrifuge the PSCs at 200 x *g* for 4 minutes.

10. Aspirate and discard the supernatant, flick the tube 3–5 times to loosen the pellet, and resuspend the cells by pipetting them up and down 5–10 times in the recommended resuspension volume of StemFlex Medium with or without RevitaCell Supplement. 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	1 mL	0.5 mL	2 mL	4 mL	12 mL

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

12. Seed PSCs at 50,000 viable cells/well of a 24-well plate in the presence of RevitaCell Supplement ~24 hours ahead of transfection complex delivery.

Prepare RNP editing complexes and perform transfection (the corresponding instructions are for a 24-well plate; reagent volumes may be scaled to accommodate alternative plate formats):

1. Cell confluency on the day of complex delivery should be ~30–60%.
   Note: This percentage confluency should be obtained within 24 hours post-seeding. For some lines, the recommended seeding density may need to be adjusted to achieve 30–60% confluency within the desired time frame.

2. Prepare an editing complexes in Opti-MEM I medium:
   Note: A 1:1 molar ratio of Cas9 nuclease to gRNA is recommended, so gRNA concentration may need to be adjusted. This example is shown for HPRT gRNA.

   – 50 µL of Opti-MEM I medium

   – 1.5 µg of GeneArt Platinum Cas9 Nuclease

   – 375 ng of gRNA (volume of gRNA should be 0.5 µL or less; determine gRNA concentration using the Qubit 3 Fluorometer)

3. Mix the Cas9–gRNA complex well and add 2 µL of Lipofectamine Stem reagent.

4. Vortex to mix well and incubate at room temperature for 10–15 minutes.
5. Immediately ahead of complex delivery, aspirate the StemFlex Medium with RevitaCell Supplement from the well and add 500 µL of Opti-MEM I medium with or without RevitaCell Supplement (Note: We have observed improved transfection efficiency via inclusion of RevitaCell Supplement at this step.)
6. Following the 10–15 min incubation period of the complex with Lipofectamine Stem reagent, add the transfection complex to the cells.
7. Move in north-south and east-west motions to distribute the transfection complex over the surface of the cells and transfer the plate to a 37°C, 5% CO_2 incubator.
8. Following 1–4 hours incubation, overlay the transfection complex with 500 µL of StemFlex Medium per well.
9. Gently place the vessel into a 37°C, 5% CO_2 incubator and incubate the cells overnight.
10. Feed the PSCs the day after transfection. Analyze the cells 48–72 hours after electroporation.

Harvest cells and save a portion for continued propagation. Use the other portion to measure cleavage efficiency with the GeneArt Genomic Cleavage Detection Kit. Using the protocol described herein for Lipofectamine Stem reagent, we have obtained up to 48% cleavage efficiency with control gRNA targeting the *HPRT* gene in Human Episomal iPSCs that were expanded on Geltrex matrix in the presence of RevitaCell Supplement.

For additional protocols for delivery of DNA and mRNA to PSCs cultured in StemFlex Medium, refer to **thermofisher.com/stemflexlipofectaminestem**

gibco

Find out more at **thermofisher.com/stemflex**

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