Genome editing of pluripotent stem cells cultured in StemFlex Medium via electroporation and lipid-based transfection

Introduction

The availability of technologies for the generation of induced pluripotent stem cells (iPSCs) from somatic cells, such as skin and blood cells, has allowed researchers to generate limitless pools of iPSCs retaining the genetic makeup of the somatic cells from which they were derived. In conjunction with novel tools for gene editing, such as CRISPR-Cas9 systems, iPSCs can be used to generate (1) knockouts to study the impact of genes on cellular processes, (2) knock-ins to assess the impact of reversing point mutations on diseased states, or (3) reporter cell lines. CRISPR-Cas9 systems provide simple and efficient locus-specific editing. Briefly, locus targeting is accomplished by guiding Cas9 nuclease via a variable, locus-specific, 20-base guide RNA (gRNA) sequence to a target site for introduction of a double-strand break (DSB). This break can then be repaired via non-homologous end joining (NHEJ), where small insertions or deletions (indels) are made in the gene of interest, often resulting in a gene knockout, thus impairing its function. DSBs can also be repaired via homology-directed repair (HDR), in which single-nucleotide polymorphisms (SNPs) or larger 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 genome-edited iPSCs cultured under feeder-free conditions in Gibco™ StemFlex™ Medium, since optimal delivery of the CRISPR-Cas9 tools is one of the key factors for efficient genome editing.

Suggested workflow: electroporation of PSCs cultured in StemFlex Medium

Figure 1 highlights the general workflow for electroporation-based delivery of ribonucleoprotein (RNP) complexes containing Cas9 nuclease, gRNA, and possibly a donor for HDR to PSCs cultured in StemFlex Medium. For a detailed protocol, refer to the appendix section on page 9 or **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™ TrueCut™ Cas9 v2 nuclease (Cat. No. A36498) and 300 ng of IVT gRNA or 20 pmol of synthetic TrueCut™ gRNA is delivered to the PSCs via electroporation. If a SNP change is desired, 10 pmol of a 100 bp single-stranded donor (ssDonor) can be included in the electroporation reaction. PSCs are then allowed to recover for 48–72 hours postelectroporation. At this point, some of the PSCs are expanded for use in downstream single-cell plating via fluorescence-activated cell sorting (FACS), while the remaining material is used for detection of editing efficiency using the Invitrogen™ GeneArt™ Genomic Cleavage Detection Kit (Cat. No. A24372) or target-specific next-generation sequencing (NGS). 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 viable cell/well of a 96-well plate. PSC clones are then allowed to recover, sequenced to identify the clones of interest, and subsequently characterized (PluriTest-Compatible PrimeView Assays, Applied Biosystems™ TaqMan® hPSC Scorecard™ Assay, Invitrogen™ Alexa Fluor™ TRA-1-60 live staining kits, or Applied Biosystems™ Karyostat™ assays).

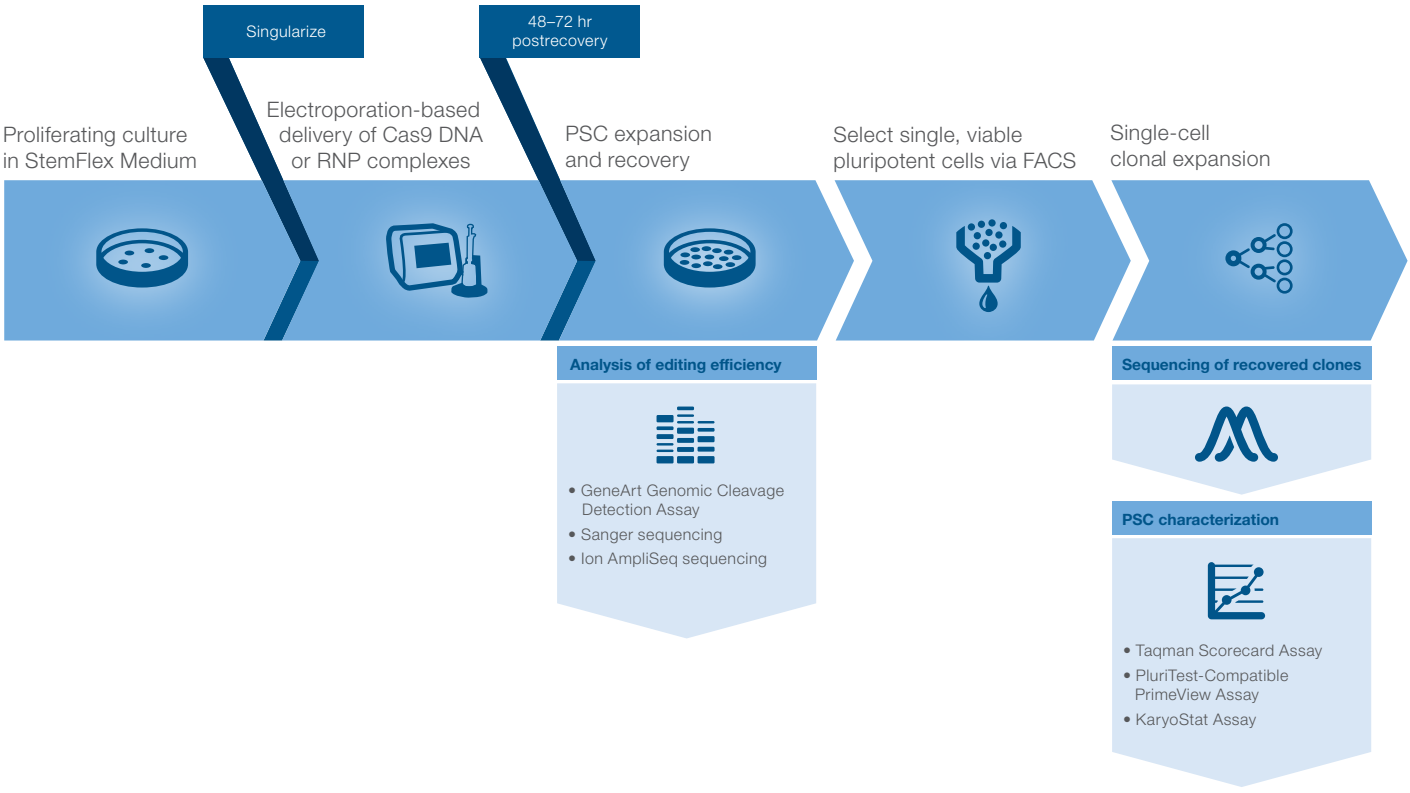


Figure 1. Schematic workflow for CRISPR-Cas9 RNP complex delivery via electroporation with subsequent flow sorting and expansion.

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 Cas9 protein and 300 ng of an *in vitro*–transcribed (IVT) gRNA targeting the *HPRT* gene, cells were added to a well of a Thermo Scientific™ Nunc™ 24-well cell culture–treated 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 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 3A, cleavage efficiency 72 hours postelectroporation was assessed using the GeneArt Genomic Cleavage Detection Kit. These data demonstrate high levels of indel formation following delivery of Cas9–gRNA complexes via the Neon Transfection System.

The following protocols for the Neon Transfection System are recommended for delivery of Cas9 protein–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 human iPSC (hiPSC) line used, the electroporation conditions may need to be further optimized. This can be accomplished by using the 24 preprogrammed optimization parameters provided on the Neon Transfection System (Figure 3B). Refer to the Neon Transfection System User Guide (Pub. No. MAN0001557) for detailed instructions.

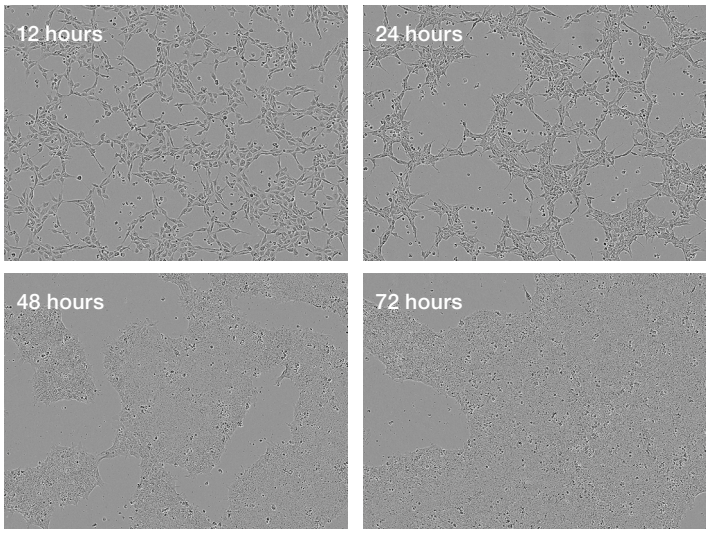


Figure 2. Representative images after electroporation showing robust recovery of iPSCs cultured in StemFlex Medium.

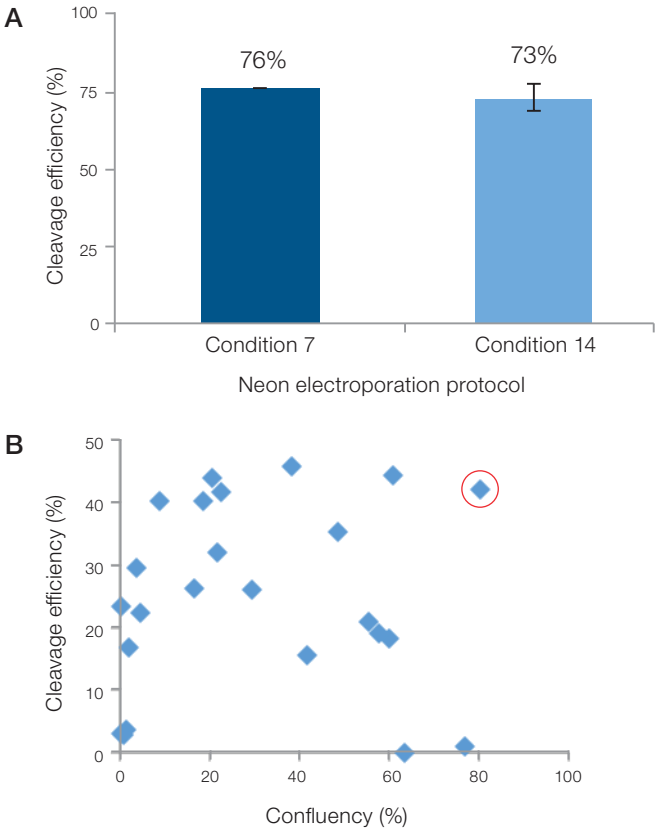


Figure 3. Cleavage efficiency of cultures grown in StemFlex Medium ~72 hours after electroporation with Cas9–gRNA complexes targeting the *HPRT* gene. (A) Electroporation performed using the recommended electroporation conditions demonstrates efficient cleavage. **(B)** Neon system parameters may need to be optimized for efficiency of genome editing and cell survival. An example of editing efficiency and corresponding hiPSCs culture confluency is shown for all 24 optimization conditions on the Neon Transfection System. Confluency is used as a metric of cell survival after electroporation. For a successful experiment, Neon transfection parameters with high editing efficiency and high confluency should be selected (ideal condition circled in red).

While a high percentage of efficient cleavage may be attained, it is incredibly important to ensure that the 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.

In Figure 4, Human Episomal iPSCs that underwent gene editing using Cas9–gRNA complexes targeting the *HPRT* gene, were expanded on Gibco™ rhLaminin-521 or Geltrex matrices. To isolate clonal edited iPSCs following initial expansion, cells were sorted via FACS for live (PI^-) and pluripotent ($TRA\text{-}1\text{-}60^+$) stem cells and seeded as 1 viable cell/well in the presence of 1X Gibco™ RevitaCell™ Supplement (Cat. No. A2644501). Three days postseeding, the medium was exchanged, replacing spent medium with fresh StemFlex Medium without RevitaCell Supplement. Thereafter, medium was exchanged with fresh StemFlex Medium without RevitaCell Supplement every 3 days. Following cell 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 and are pluripotent (Figure 5). 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).

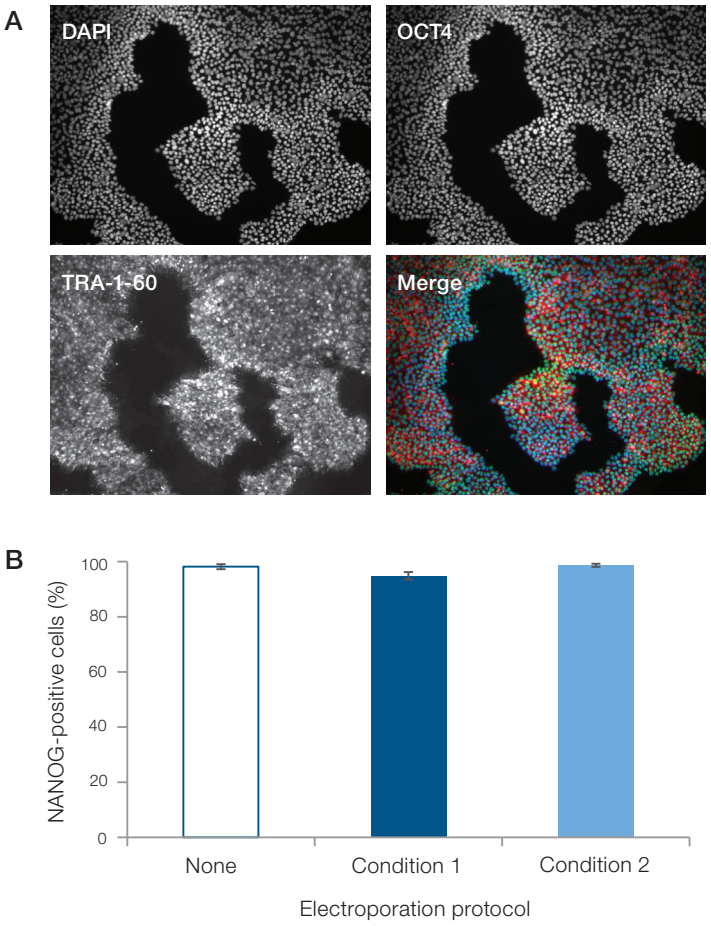


Figure 4. Maintenance of pluripotency of iPSCs cultured in StemFlex Medium after electroporation and recovery. Cultures transfected with Cas9–gRNA complexes targeting the *HPRT* gene were assessed by **(A)** qualitative immunocytochemistry of OCT4 and TRA-1-60 expression and **(B)** quantitative assessment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

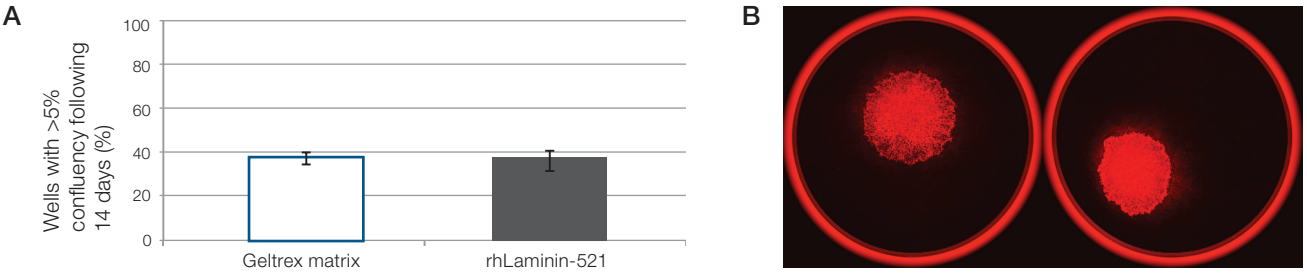


Figure 5. High-efficiency clonal expansion of PSCs in StemFlex Medium following FACS sorting of Cas9-edited cells. (A) PSCs expanded in StemFlex Medium on both Geltrex and rhLaminin-521 matrices demonstrate high clonal expansion. Data are an average of three experiments. **(B)** Clones generated through single-cell cloning via FACS using the culture system described are pluripotent as shown by TRA-1-60 staining (red).

StemFlex Medium is both robust and versatile in its ability to support PSC health during electroporation-based Cas9 delivery for gene editing. As an example, using the Neon system for electroporation and the workflow described, disease-causing mutations were introduced into the genomes of hiPSCs. In this method, the process of HDR was utilized to change single nucleotides that are associated with cardiac and neuronal diseases.

TrueCut Cas9 v2 nuclease along with an IVT gRNA and a 100 bp ssDonor donor carrying the mutation to be introduced were delivered to BS3 and Gibco episomal iPSCs via Neon electroporation. Preset condition 7 on the Neon Transfection System (1,200 V, 30 ms, 1 pulse) was used to electroporate the iPSCs. BS3 and Gibco episomal iPSCs were then expanded in StemFlex Medium.

Briefly, 100,000 cells were diluted into 10 µL of Buffer R with 1.5 µg TrueCut Cas9 Protein v2, 300 ng IVT gRNA, and 10 pmol ssDonor. Cells were electroporated using the Neon Transfection System 10 µL Kit. Following electroporation, cells were plated in StemFlex Medium in the presence of RevitaCell Supplement on rhLaminin-521–coated 24-well plates. Cells were expanded 72 hours postelectroporation in StemFlex Medium using Gibco™ TrypLE™ Express Enzyme on 6-well plates coated with rhLaminin-521. Analysis of genome editing efficiency was then performed via NGS of PCR-amplified genome-edited regions, followed by further clonal expansion. Figure 6 indicates the overall editing efficiency in the pool after delivery of the gene editing tools using the Neon Transfection System.

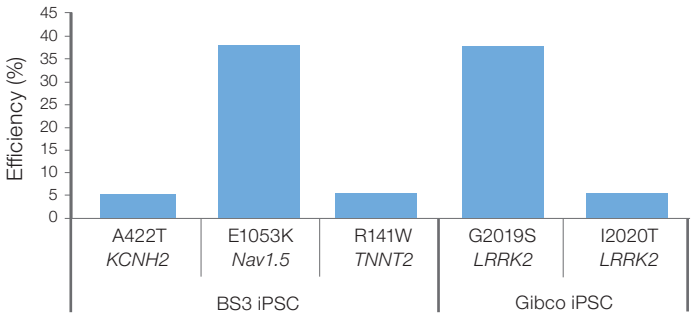


Figure 6. The indicated disease-causing mutations were introduced by delivering the CRISPR-Cas9 tools to iPSCs via the Neon Transfection System as described. The efficiency of SNP introduction at various targets in two hiPSC lines was analyzed using custom Ion Ampliseq™ panel–targeted NGS. Data indicate that editing efficiency is highly target-dependent.

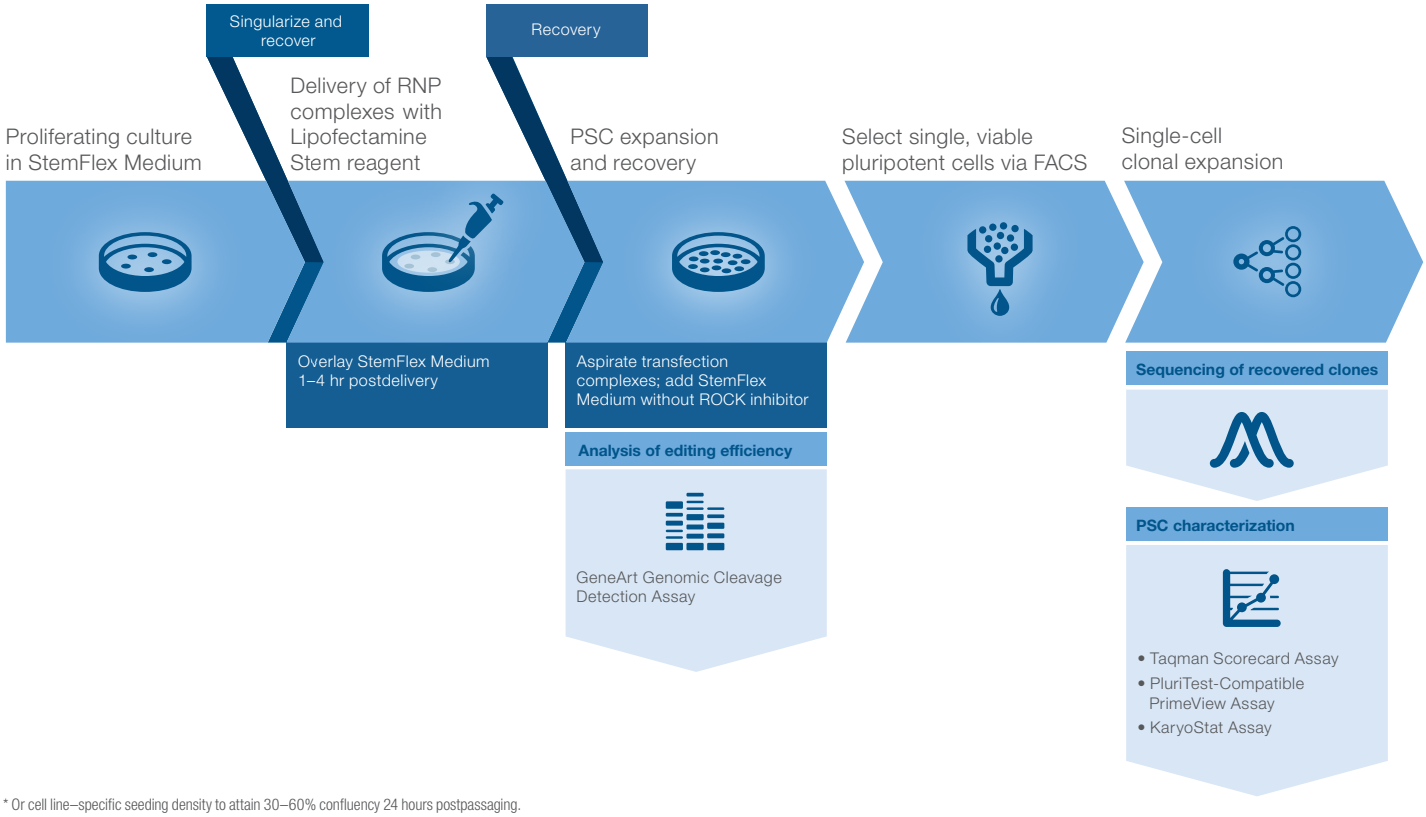
Lipid-based transfection is now also a viable option for some gene editing applications using the Invitrogen™ Lipofectamine™ Stem Transfection Reagent.

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

Figure 7 demonstrates the workflow for an alternative method for gene editing of PSCs through lipid-based delivery of Cas9–gRNA complexes. This method allows for higher-throughput gene editing of PSCs. The Lipofectamine Stem Transfection Reagent (Cat. No. STEM00001) is an efficient lipid-based reagent that provides robust delivery of gene-editing tools such as RNP complexes to PSCs. A detailed protocol can be found in the appendix on page 9 or at **thermofisher.com/lipofectaminestem#protocols** In this application note, we sought to optimize the delivery protocol of Cas9–gRNA complexes to cells maintained in StemFlex Medium.

To transfect PSCs cultured in StemFlex Medium, the medium is first aspirated off of the cells and Gibco™ Opti-MEM™ I Reduced Serum Medium (Cat. No. 31985062) with RevitaCell Supplement is overlaid. The transfection complex containing Cas9 and the gRNA is then delivered to the PSCs with the Lipofectamine Stem reagent, and the cells are incubated for 1–4 hours. Following incubation, transfection complexes are overlaid with StemFlex Medium without ROCK inhibitor or RevitaCell Supplement (Figure 7). The medium is fully exchanged 24 hours posttransfection, and cleavage analysis and downstream expansion for clonal analysis can be initiated 48–72 hours posttransfection.

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



* Or cell line–specific seeding density to attain 30–60% confluency 24 hours postpassaging.

Figure 7. Transfection workflow for delivery of Cas9–gRNA complexes to PSCs cultured in StemFlex Medium using Lipofectamine Stem reagent.

Results: lipid-based transfection

In Figure 8, Human Episomal iPSCs cultured in StemFlex Medium for >15 passages were harvested using TrypLE Select Enzyme and seeded in StemFlex Medium with RevitaCell Supplement. The following day, before Cas9–gRNA complex delivery, the StemFlex Medium was aspirated from the iPSCs and replaced with Opti-MEM I medium with RevitaCell Supplement. Subsequently, the Cas9–gRNA complexes targeting the *HPRT* gene, as well as GFP mRNA 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 StemFlex Medium per well of a 48-well plate, and transfection efficiency was assessed using the IncuCyte ZOOM System—relative %GFP$^+$ confluency = %GFP confluency:%total confluency—24 hours postseeding. When using StemFlex Medium, an increase in transfection efficiency was observed by increasing the time ahead of overlay up to 4 hours (Figure 8).

Assessment of indel formation using the GeneArt Genomic Cleavage Detection Kit indicated high levels of successful cleavage upon Cas9–gRNA complex delivery to Gibco Human Episomal iPSCs. Optimal cleavage was shown when cells were at 30–60% confluency before lipid-based transfection (data not shown). For the experiments shown in Figure 9, cells were seeded at 50,000 or 75,000 viable cells/well of a Nunc 24-well cell culture–treated plate (Cat. No. 142475). 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.

While high transfection efficiency may be observed, it is imperative that the PSCs retain their pluripotency. As shown in Figures 9 and 10, cultures transfected with Lipofectamine Stem reagent demonstrate high cleavage efficiency of up to 42% detected at the *HPRT* locus, while maintaining high expression levels of the intracellular pluripotency marker 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.

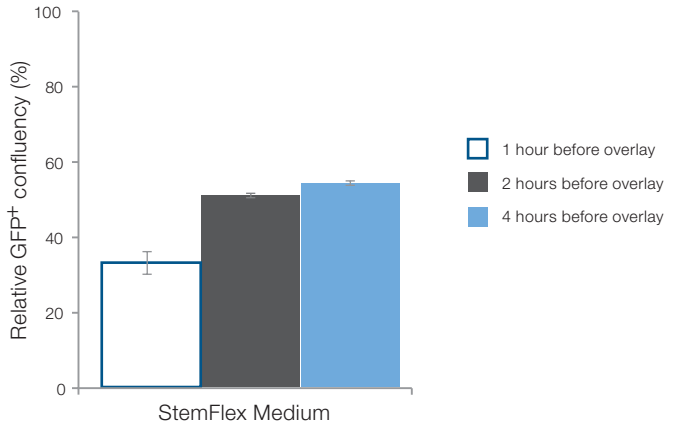


Figure 8. Impact of time ahead of overlay on transfection efficiency as assessed using a GFP mRNA control.

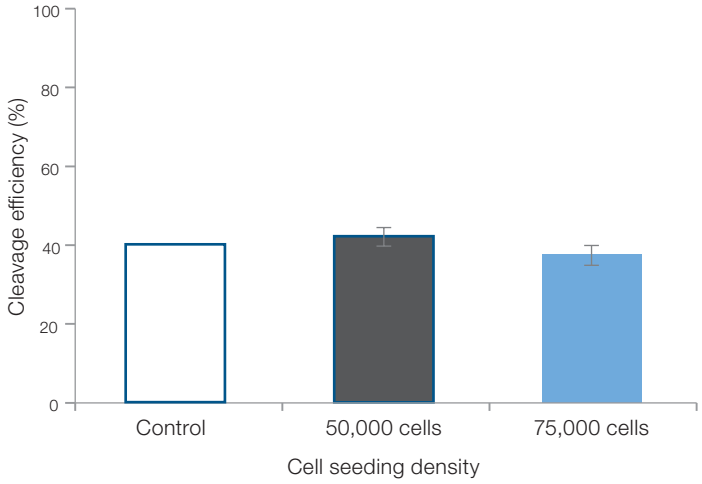


Figure 9. Genomic cleavage efficiency of iPSCs transfected for 4 hours ahead of StemFlex Medium overlay. Average genomic cleavage for 4-hour transfection ahead of StemFlex Medium overlay for seeding densities of 50,000 and 75,000 cells/well. Bars represent mean ±standard deviation of 3 independent transfections.

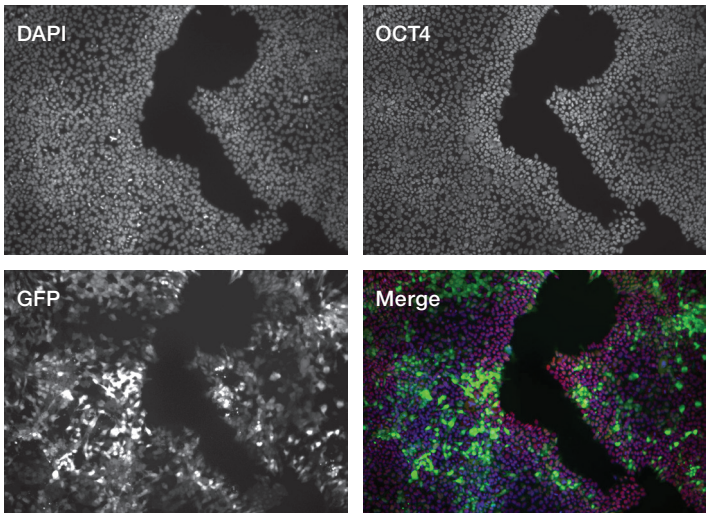


Figure 10. Representative images of transfection efficiency and maintenance of pluripotency.

StemFlex Medium is both robust and versatile in its ability to support PSC health during lipid-based Cas9 delivery for gene editing. Using Lipofectamine Stem reagent delivery and the workflow described, disease-causing mutations were introduced into the genome of hiPSC. In this method, the process of HDR was utilized to change single nucleotides that are associated with cardiac and neuronal diseases.

BS3 iPSC, an internally derived iPSC line generated by reprogramming human dermal fibroblasts using the Invitrogen™ CytoTune™ Reprogramming Kit, and Gibco Episomal iPSCs were expanded in StemFlex Medium. Following dissociation, cells were plated in StemFlex Medium in the presence of RevitaCell Supplement on rhLaminin-521–coated 24-well plates. One day later, Lipofectamine Stem reagent was used to deliver 1.5 µg TrueCut Cas9 Protein v2, 300 ng IVT gRNA, and 10 pmol ssDonor as described above. Three days post-delivery, cells were expanded via passaging using TrypLE Express Enzyme on 6-well plates coated with rhLaminin-521 in StemFlex Medium for genome editing efficiency analysis via NGS of PCR-amplified genome-edited regions, followed by further clonal expansion. Figure 11 indicates the overall editing efficiency in the pool after delivery of the editing tools using the Lipofectamine Stem reagent.

Conclusions

Together, these data demonstrate the utility of two delivery tools that allow reliable genome editing in hiPSCs. The Neon Transfection System for electroporation-based delivery or Lipofectamine Stem reagent for lipid-based delivery allow for efficient cleavage by Cas9 at the guided locus. Gene editing can be obtained without compromising the quality of PSCs when paired with StemFlex Medium.

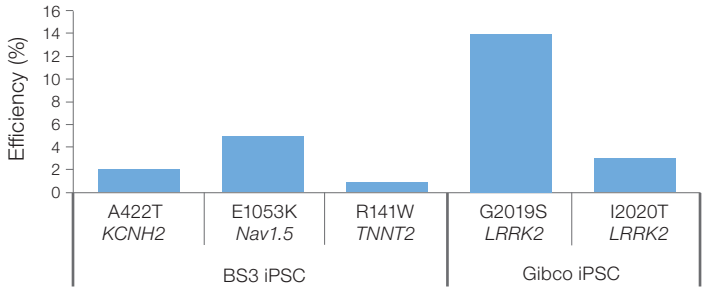


Figure 11. The indicated disease-causing mutations were introduced via CRISPR-Cas9 delivered using Lipofectamine Stem reagent as described. The efficiency of SNP introduction at various targets in two hiPSC lines was analyzed using the custom Ion Ampliseq NGS panel for targeted sequencing. Data indicate that editing efficiency is highly target-dependent.

Genome editing efficiency is highly locus-dependent; loci that are inefficiently targeted are better edited using the Neon Transfection System for Cas9-gRNA complex delivery. Easily targetable loci can be edited using both the Neon and Lipofectamine Stem transfection systems for delivery of Cas9-gRNA complexes (Figure 12). The Lipofectamine Stem reagent provides additional benefits, including use in high-throughput genome editing of hiPSCs, lower cost, and ease of use with a limited amount of equipment.

For a detailed protocol outlining step-by-step instructions for electroporation-based and lipid-based delivery of Cas9–gRNA complexes and guidance for flow sorting of PSCs, please refer to the appendix on page 9.

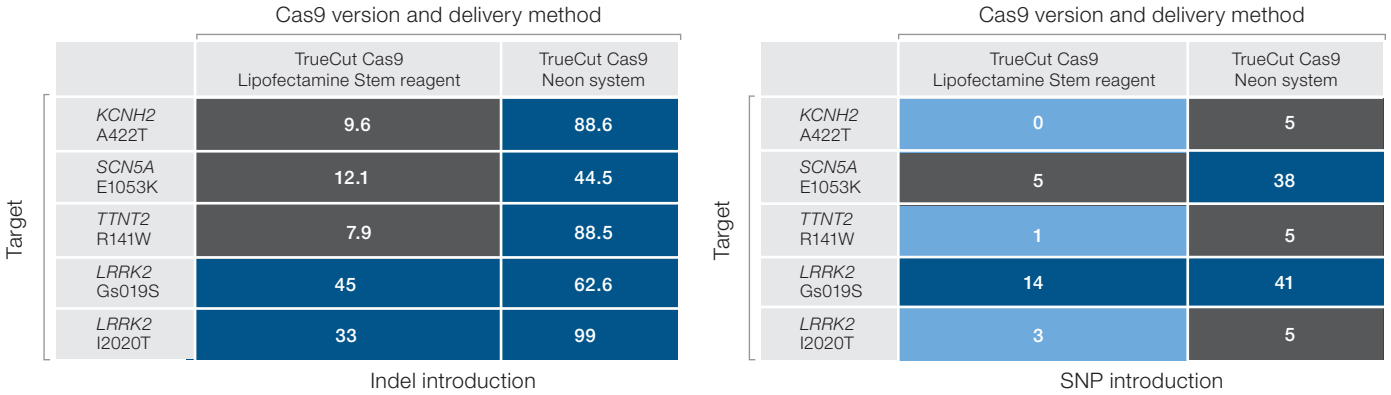


Figure 12. Comparing the use of Neon and Lipofectamine Stem transfection systems in the introduction of indels or SNPs into the genome of hiPSCs via CRISPR-Cas9–mediated genome editing. The numbers indicate genome editing efficiencies (%), and the colors indicate the chance of successful isolation of edited clones (dark blue: high, grey: possible, light blue: impossible).

Appendix

CRISPR-Cas9 genome editing for research of human pluripotent stem cells cultured in StemFlex Medium via electroporation

Introduction

This protocol describes the delivery of Cas9–gRNA complexes via electroporation to PSCs cultured in StemFlex Medium, expansion postediting, and best practices for flow sorting of cultures and subsequent clonal expansion in research applications. Refer to the User Guide (Pub. No. MAN0016431) for detailed instructions on culturing human PSCs under feeder-free conditions in StemFlex Medium.

Product	Cat. No.
StemFlex Medium	A3349401
Geltrex LDEV-Free, hESC-Qualified, Reduced Growth Factor Basement Membrane Matrix	A1413302
Recombinant Human Laminin-521*	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
TrueCut Cas9 Protein v2	A36497
GeneArt Precision gRNA Synthesis Kit	A36498
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

* Use Geltrex LDEV-Free, hESC-Qualified, Reduced Growth Factor Basement Membrane Matrix or Recombinant Human Laminin-521.

Design and generate gRNAs by *in vitro* transcription

1. 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. Predesigned Invitrogen™ 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 gRNA sequence with the Invitrogen™ GeneArt™ Precision gRNA Synthesis Kit.

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

Prepare Cas9–gRNA complex

1. Add 1.5 µL of TrueCut Cas9 Protein v2 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. Determination of 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 Gibco™ DMEM/F-12, GlutaMAX™ Supplement.

2. Add 300 µL per well.

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

Coat 24-well plates with rhLaminin-521

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

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

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

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

Prepare PSCs for electroporation

See "Procedural guidelines" for plate coating information. If using precoated plates stored at 2–8°C, prewarm rhLaminin-521– or Geltrex matrix–coated plates to room temperature. Prewarm 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 the recommended volume of Gibco™ 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 (–/–).
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 with 5% CO_2 for 3–5 minutes.
6. Gently pipette the cells up and down 5–10 times with a 1,000 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 an Invitrogen™ Countess™ II Automated Cell Counter or similar automated or manual method.

Electroporate Cas9–gRNA complexes via the 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 6 µL of Cas9–gRNA complexes prepared in "Prepare Cas9–gRNA complex".
   - **Note:** If using a ssDonor for HDR-mediated editing, add 10 pmol of ssDonor in this step, maintaining the final volume at 11 µL.
   - Mix gently.
5. Pipette 10 µL of the cell suspension into the Neon tip and electroporate with protocol 7 (1,200 V, 30 ms, 1 pulse) or protocol 14 (1,200 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. The *HPRT* gRNA control is available for purchase as a 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 incubator with 5% CO_2 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 the *HPRT* gRNA control in the Gibco Human Episomal iPSC Line expanded on a Geltrex matrix.

Expand PSCs following genome editing

See "Procedural guidelines" for plate-coating information. If using precoated plates stored at 2–8°C, prewarm rhLaminin-521–coated plates to room temperature. Incubate plate(s) at 37°C with 5% CO_2 for >2 hours ahead of PSC seeding. Prewarm StemFlex Medium and Gibco™ 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 (–/–). See table for recommended volumes.

Culture vessel (surface area)	6-well (10 cm²)	12-well (4 cm²)	24-well (2 cm²)	35 mm (10 cm²)	60 mm (20 cm²)	100 mm (60 cm²)
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 cm²)	12-well (4 cm²)	24-well (2 cm²)	35 mm (10 cm²)	60 mm (20 cm²)	100 mm (60 cm²)
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 5–8 minutes or at 37°C for 4–5 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 below for recommended volumes.

Culture vessel (surface area)	6-well (10 cm²)	12-well (4 cm²)	24-well (2 cm²)	35 mm (10 cm²)	60 mm (20 cm²)	100 mm (60 cm²)
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 1–3 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 readhere very rapidly after medium addition and must be removed 1 well at a time. Others are slower to reattach and may be removed 3 wells at a time.

7. Coat culture vessel for 2 hours at 37°C with 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 prewarmed complete StemFlex Medium to each well of an rhLaminin-521–coated plate so that each well contains the recommended volume of complete medium after the cell suspension has been added. See table above for recommended volumes.

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

Transfection of pluripotent stem cells with Lipofectamine Stem Transfection Reagent in StemFlex Medium

Product	Cat. No.
StemFlex Medium	A3349401
Lipofectamine Stem Transfection Reagent	STEM00008
Geltrex LDEV-Free, hESC-Qualified, Reduced Growth Factor Basement Membrane Matrix	A1413302
rhLaminin-521	A29248
Dulbecco's Phosphate Buffered Saline (DPBS) without calcium and magnesium	14190144
Versene Solution	15040066
TrypLE Express Enzyme (1X), no phenol red	12604013
RevitaCell Supplement	A2644501
Opti-MEM I Reduced Serum Medium	31985062

* Use Geltrex LDEV-Free, hESC-Qualified, Reduced Growth Factor Basement Membrane Matrix or Recombinant Human Laminin-521.

Starting with undifferentiated human pluripotent stem cells (PSCs), expanded in a feeder-free culture system such as StemFlex Medium on a Geltrex matrix, or on a defined substrate such as rhLaminin-521, is ideal for efficient transfection.

Passaging

- Maintain PSCs in the format of your choice, such as 6-well plates, 60 cm dishes, or T-75 flasks coated with Geltrex matrix or rhLaminin-521, in StemFlex Medium. Propagating PSCs in 6-well plates and transfecting in 24-well plates are convenient formats used in this protocol.
- Passage PSCs every 3–5 days, before they reach ~85% confluence.
- **Tip:** For routine passaging of PSCs with Versene Solution for expansion, the replating of large clumps of 5–10 cells promotes reattachment and survival in StemFlex Medium without the need to add RevitaCell Supplement. PSCs can be expanded in StemFlex Medium for subsequent transfection on a Geltrex matrix or rhLaminin-521.

Precoating 24-well plates with Geltrex matrix or rhLaminin-521

Coating with Geltrex matrix

1. Prepare a 1:100 dilution of Geltrex matrix in cold DMEM/F-12 Medium with GlutaMAX Supplement.
2. Add 300 µL of diluted Geltrex matrix to each well of a 24-well plate and incubate at 37°C for ≥1 hour, before use.

Coating with rhLaminin-521

1. Prepare a 1:40 dilution of rhLaminin-521 by adding 300 µL of rhLaminin-521 stock solution (0.5 mg/mL) to 12 mL of DPBS, for a final concentration of 2.5 µg/mL.
2. Add 400 µL of diluted rhLaminin-521 to each well of a 24-well plate, and incubate at 37°C for ≥2 hours to coat the wells with 0.5 µg/cm^2 of rhLaminin-521.

- **Important:** The optimal coating concentration of rhLaminin-521 can depend on the PSC line and ranges from 0.5 to 2 µg/cm^2. Increase the concentration if you observe areas of incomplete cellular attachment.
- **Tip:** Plates coated with Geltrex matrix or rhLaminin-521 can be prepared ahead of time and stored for up to 2 weeks at 4°C. Equilibrate at room temperature for 1 hour before plating cells.

Seeding cells for transfection

1. To maximize transfection efficiency, seeding a single-cell suspension of PSCs prepared with TrypLE Select Enzyme is recommended.

- **Important:** As the plating efficiency of PSCs dissociated into single cells is lower than the plating efficiency of clumped cells, we recommend adding RevitaCell Supplement for overnight replating in StemFlex Medium onto a Geltrex matrix or rhLaminin-521 for transfecting the following day.

2. When feeder-free PSC cultures are less than 85% confluent, remove the StemFlex Medium and gently wash the cells twice with 2 mL of DPBS per well in a 6-well plate.
3. Add 1 mL of TrypLE Select Enzyme to each well, swirl to evenly coat the PSCs, and incubate at 37°C for 3–5 minutes.
4. Using a 1 mL pipette, gently triturate the cell suspension 5–10 times to dissociate into single cells.
5. Transfer the cell suspension into a 15 mL conical tube containing 3 mL of StemFlex Medium to inactivate the TrypLE Select Enzyme.
6. Centrifuge the cell suspension at 200 x *g* for 4 minutes.
7. Aspirate the supernatant and resuspend the pellet to a single-cell suspension in 3 mL of StemFlex Medium with RevitaCell Supplement.
8. Perform a total viable cell count with the Countess II Automated Cell Counter or another method.
9. Dilute with additional StemFlex Medium with RevitaCell Supplement to a final concentration of 100,000 cells/mL.
10. Aspirate the Geltrex matrix or rhLaminin-521 from the wells of a precoated 24-well plate.

- **Important:** Proliferating PSC cultures need room to expand during transfection, so plate the recommended starting number of cells (step 11) to achieve 30% confluence on the day of transfection.

11. Add 0.5 mL of the PSC suspension in StemFlex Medium with RevitaCell Supplement to plate 50,000 cells/well in the precoated 24-well plate.
12. Return the plate to the incubator and culture the cells at 37°C with 5% CO_2, overnight.

Changing medium on the day of transfection

Prepare a solution of of Opti-MEM I medium with RevitaCell Supplement. Aspirate the StemFlex Medium and add 0.5 mL of the supplemented Opti-MEM I medium to each well just before transfection.

- **Important:** Transfect in Opti-MEM I Medium with RevitaCell Supplement, not in StemFlex Medium, which can inhibit transfection.

gibco

Ribonucleoprotein (RNP) transfection protocol

RNP complex components:

- TrueCut Cas9 Protein v2
- gRNA (see "Designing and generating gRNA by *in vitro* transcription")

On the day of transfection (1 day after plating PSCs in a single well of a 24-well plate), perform the following steps, which have been optimized for using Lipofectamine Stem reagent in StemFlex Medium (see page 14):

Step	Tube	Complexation component	Amount per well (24-well plate)
1	Tube 1	Opti-MEM I medium	25 µL
		Lipofectamine Stem reagent	2 µL
2	Tube 2	Opti-MEM I medium	25 µL
		Cas9 nuclease	1.5 µg
		gRNA (0.1–0.5 µg/µL)	375 ng
3	Add tube 2 solution to tube 1 and mix well. If using ssDonor for HDR editing, add 10 pmol of ssDonor during this step.		
4	Incubate mixture from step 3 for 10 minutes at room temperature.		
5	Aspirate the StemFlex Medium and add 0.5 mL of Opti-MEM I medium with RevitaCell Supplement per well just before transfection.		
6	Add 50 µL of complex from step 4 to each well; gently swirl plate to ensure even distrbution of the complex across the entire well.		
7	Return culture dish to incubator and culture the cells at 37°C with 5% CO_2 for 4 hours. **Important:** After 4 hours of transfection, add 0.5 mL of StemFlex Medium warmed to room temperature to each well, return plate to incubator, and culture the cells at 37°C with 5% CO_2 overnight.		
8	The following day, aspirate the StemFlex Medium and transfection complexes and add 0.5 mL of fresh StemFlex Medium per well. If PSCs are going to be transfected for 48 hours, passage before they reach 85% confluence.		