

Robust genome editing in stem cells

Using GeneArt® CRISPR Nuclease mRNA

Introduction

Genome editing in induced pluripotent stem cells (iPSCs) has been demonstrated to be highly effective for generating disease models for both monogenic and complex genetic disorders. For successful genome editing and downstream application of iPSCs, many factors need to be considered, such as choice of growth media, extracellular matrix, genome editing tools, and nucleic acid (NA) delivery methods (Figure 1). Here we have described feeder-free culture of stem cells and a genome editing protocol that can facilitate efficient disease model generation.

The clustered regularly interspaced short palindromic repeat (CRISPR) system from *Streptococcus pyogenes* has become a powerful technology for genome editing, and it can be used to rapidly generate engineered cell lines and model organisms. It is a simple system comprising a catalytic unit, Cas9, and a short noncoding guide RNA (gRNA) that confers target specificity. It is an

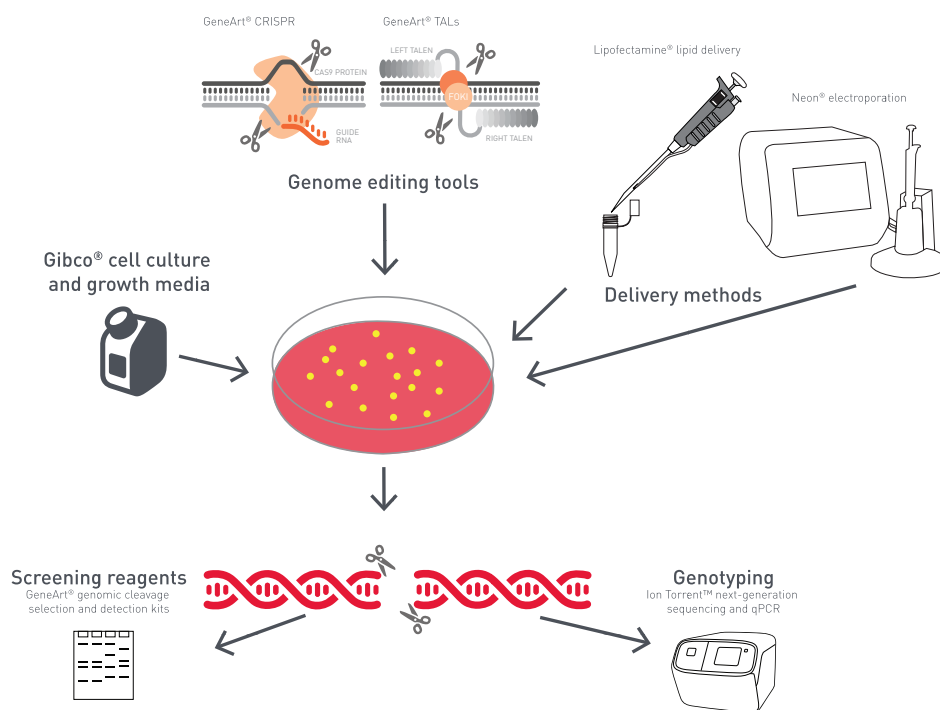


Figure 1. Factors affecting genome editing outcomes in iPSCs.

attractive tool for large-scale genome engineering in a wide variety of hosts. We have developed various CRISPR-Cas9 formats that can be used to edit genomes in a wide variety of cell types, including stem cells. Using these formats we have achieved greater than 50% target-specific DNA cleavage in mouse embryonic stem cells (ESCs) and human iPSCs and ESCs. The methods described here show great potential as highly efficient gene editing tools in stem cells.

Methods

1. *In vitro* transcription (IVT)

The DNA template for an IVT reaction can be ordered either as a synthetic dsDNA fragment containing a T7 promoter sequence (GeneArt® CRISPR T7 Strings™ DNA) or prepared by PCR using a DNA template containing the gRNA coding sequence. Alternatively, ready-to-transfect *in vitro*-transcribed gRNA can be ordered through our custom services team. For details, contact custom.services@lifetech.com. For details on using and ordering synthetic GeneArt® CRISPR T7 Strings™ DNA generated through our GeneArt® gene synthesis service, see the user manuals [3,5]. Other products are listed in the Ordering information at the end of this application note.

dsDNA template containing T7 promoter was amplified from the GeneArt® CRISPR Nuclease Vector with OFP (included in Cat. No. A21174), specific for the human HPRT or mouse *Rosa26* locus, using a target-specific forward primer containing a T7 promoter sequence at the 5' end (5'TAATACGACTCACTATAGG-N19-3', where

N indicates target-specific CRISPR RNA sequence), and a reverse primer specific for the auxiliary tracrRNA (5'AAAAAAGCACCGACTCGG3'). A 50 µL PCR reaction was performed using Phusion™ High-Fidelity DNA Polymerase. The resulting PCR products were purified using the PureLink® PCR Purification Kit. For more information on designing IVT template-specific primers, see the example on page 16 in the GeneArt® CRISPR Nuclease mRNA user guide [3]. IVT was performed as described in the TranscriptAid™ T7 High Yield Transcription Kit. Alternatively, the MEGAshortscript™ T7 Kit can be used for gRNA synthesis. The resulting gRNA was purified using the MEGAclean™ Transcription Clean-Up Kit. gRNA concentration was measured using the Quant-iT™ RNA Assay Kit.

2. Mouse ESCs culture and transfection

2.1 Cell culture

Mouse E14Tg2a.4 ESCs were cultured on mouse inactivated embryonic fibroblasts (MEFs; strain ICR) in the presence of recombinant human leukemia inhibitory factor (LIF) in mouse ESC medium consisting

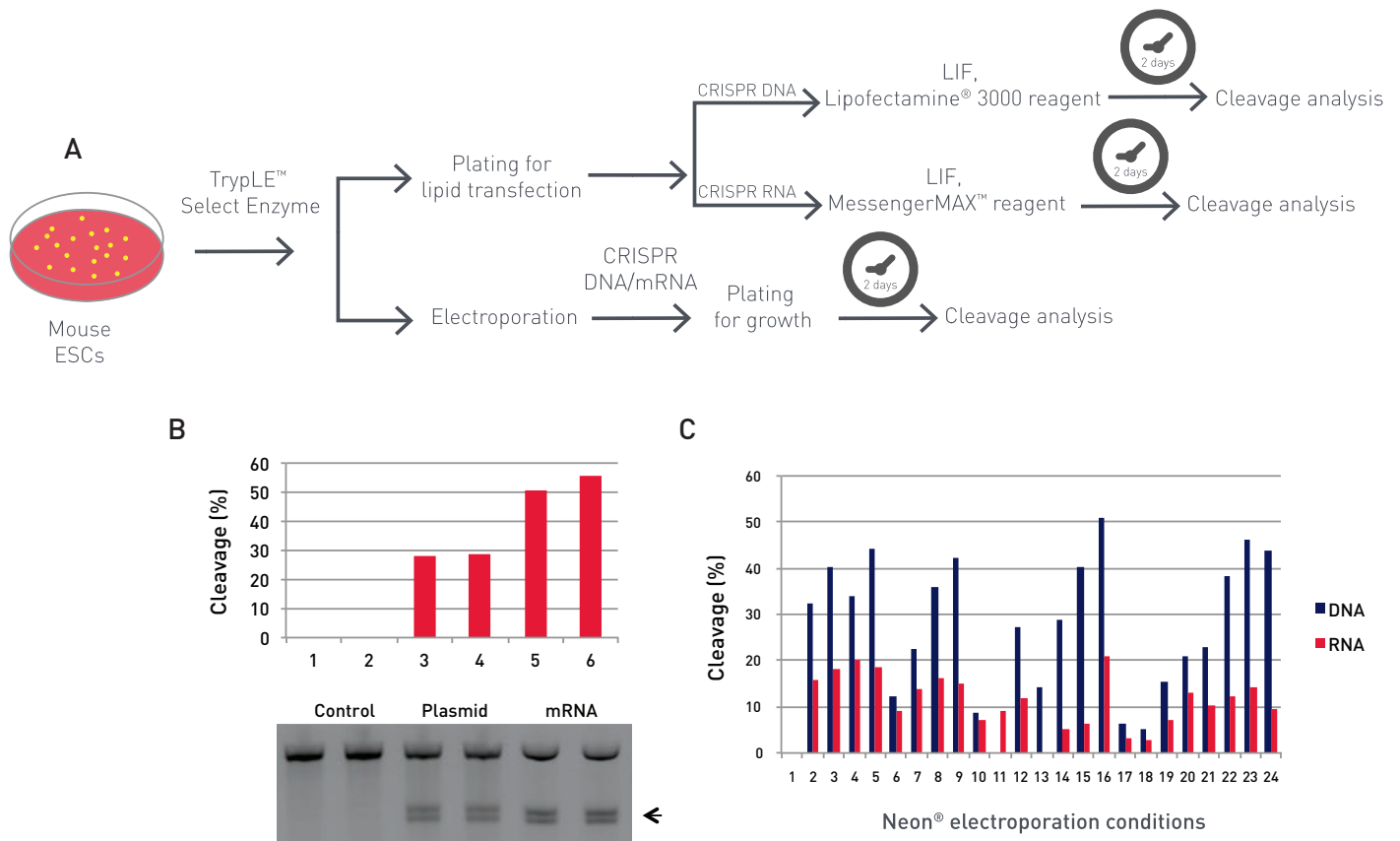


Figure 2. Transfection and electroporation of GeneArt® CRISPR Nuclease Vector with OFP reporter and GeneArt® CRISPR Nuclease mRNA in mouse ESCs. (A) Schematic of mouse ESC transfection using lipid-based transfection and electroporation, and subsequent analysis. **(B)** DNA transfection was performed using Lipofectamine® 3000 and RNA with Lipofectamine® MessengerMAX® reagent, and cells were assayed for genomic cleavage 48 hours after transfection. **(C)** DNA and RNA formats were electroporated using 10 µL tips and 24 Neon® optimization conditions were tested. Highest genomic cleavage was achieved using 1,400 V, 20 ms pulse width, and 2 pulses (Neon® optimization condition No. 16).

of KnockOut™ DMEM, 15% embryonic stem cell-qualified fetal bovine serum, 1X MEM Non-Essential Amino Acids Solution), 1X GlutaMAX™ Supplement, 1X 2-mercaptoethanol, and 10 ng/mL LIF. Before transfection, cells were adapted to feeder-free conditions and maintained on plates coated with attachment factor protein in mouse ESC-conditioned medium. All cultures were maintained in 5% CO₂ at 37°C in a humidified incubator. When setting up the experiments for transfections, 1 x 10⁵ cells were plated per well in a 24-well tissue culture dish coated with attachment factor.

2.2 Transfection

Two options for transfection of mouse ESCs are presented here. Both are shown schematically in Figure 2A. Various factors, including cell seeding density, the health of the cells, media, NA quality, and NA delivery method, affect the transfection efficiency and, in turn, the efficiency of gene editing. Special care should be taken while handling and processing the cells, and the protocols should be strictly followed to maximize efficiency. Refer to our guidelines on DNA or RNA delivery and factors influencing transfection efficiency [1].

Option 1: Lipid-based delivery of CRISPR components

Method when using the DNA format: DNA transfection was performed following the Lipofectamine® 3000 Transfection Reagent user manual. Briefly, 750 ng of GeneArt® CRISPR Nuclease Vector was diluted in 25 µL of Opti-MEM® I Reduced Serum Medium and mixed with 2 µL of P3000 reagent (provided in the Lipofectamine® 3000 Transfection Reagent package). In a separate tube, 3 µL of Lipofectamine® 3000 Transfection Reagent was diluted in 25 µL of Opti-MEM® I Medium. The diluted DNA with P3000 reagent was added to the diluted Lipofectamine® 3000 Transfection Reagent and incubated at room temperature for 5 minutes, and the resulting complex was added to cells in mouse ESC-conditioned medium. Cleavage efficiency of target-specific gRNA was analyzed 48 hours after transfection using the GeneArt® Genomic Cleavage Detection Kit [4].

Method when using the RNA format: For RNA transfection, Lipofectamine® MessengerMAX™ Transfection Reagent was used. Approximately 200 ng of *in vitro*-transcribed gRNA and 1 µg of GeneArt® CRISPR Nuclease mRNA were diluted in 25 µL of Opti-MEM® I Reduced Serum Medium. In a separate tube, 2.5 µL of Lipofectamine® MessengerMAX™ reagent was diluted in 25 µL of Opti-MEM® I Reduced Serum Medium. Diluted RNA and transfection reagent mixes were then combined and incubated for 5 minutes at room temperature, and the resulting 50 µL complex was added to 1 x 10⁵ cells per well in a 24-well plate in complete mouse ESC-conditioned medium. Cleavage efficiency of target-specific gRNA was analyzed 48 hours after transfection using the GeneArt® Genomic Cleavage Detection Kit [4].

Option 2: Delivery of CRISPR components via electroporation into mouse ESCs

Electroporation was performed using the Neon® Transfection System 10 µL Kit with the 10 µL Neon® tips included in the kit. Mouse ESCs were harvested using TrypLE™ Express Enzyme, washed once with PBS, and single-cell suspensions were counted using the Countess® Automated Cell Counter. Then, 1 x 10⁵ cells were resuspended in 10 µL of Neon® R-buffer, and 750 ng of GeneArt® CRISPR Nuclease vector was added to the buffer. The 24 different preset Neon® optimization conditions were used as described in the Neon® user manual [2]. Once the suitable condition has been identified, the user need not use all 24 conditions for subsequent electroporation of the same materials. We found the best efficiency for mouse ESCs using the Neon® optimization condition No. 16 (1,400 V, 20 ms, and 2 pulses). After each electroporation, cells were immediately plated into a single well of a 24-well plate coated with attachment factor protein and containing 500 µL prewarmed mouse ESC-conditioned medium per well. For RNA electroporation, 200 ng of *in vitro*-transcribed gRNA and 1 µg of GeneArt® CRISPR Nuclease mRNA were added to the buffer R-containing cells, and electroporation conditions similar to those described above were followed. In our hands, Neon® optimization condition No. 16 also worked best for the RNA format. Cells were incubated at 37°C for 48 hours, and cleavage efficiency of target-specific gRNA was analyzed 48 hours after transfection using the GeneArt® Genomic Cleavage Detection Kit [4].

3. Human iPSC culture and transfection

3.1. Cell culture

Option 1: Feeder-free adaptation of iPSCs for transfection

Feeder-dependent human episomal iPSC line cells were cultured on MEF feeder cells (strain ICR) in human ESC medium containing 20% KnockOut™ Serum Replacement (Cat. No. 10828-028), 10 μM MEM Non-Essential Amino Acids Solution, 55 μM 2-mercaptoethanol, and 4 ng/mL human fibroblast growth factor basic (FGF-basic) recombinant protein in DMEM/F-12 (Cat. No. 10565-018). All cultures were maintained in 5% CO₂ at 37°C in a humidified incubator. iPSC cultures were maintained with daily medium changes and were passaged regularly using collagenase type IV. For feeder-free adaptation, iPSCs were grown to 80% confluency and harvested using collagenase type IV. Following removal of the cell clusters from the feeder layer, cells were gravity-sedimented to minimize MEF contamination. The cell clusters were then seeded on tissue culture dishes coated with Geltrex® hESC-qualified Reduced Growth Factor Basement Membrane Matrix in MEF-conditioned medium supplemented with 4 ng/mL FGF-basic. MEF-conditioned medium was produced using the abovementioned inactivated feeder cells, supplemented with hESC growth medium, which was harvested for 7 consecutive days, sterile-filtered, and frozen until used. The cultures were allowed to reach 80–90% confluency. On the day of harvest, the cultures were inspected for signs of differentiation, and any contamination of differentiated cells was removed by microdissection. The cultures were washed once with DPBS and then harvested using TrypLE™ Express Enzyme. Single-cell suspensions were counted using the Countess® Automated Cell Counter. For electroporation, the next step was to proceed directly to section 3.2 option 2. For lipid-based transfection, the cells were then seeded onto 24-well tissue culture dishes coated with Geltrex® matrix and using MEF-conditioned medium containing 10 μM ROCK Inhibitor (EMD Millipore, Cat. No. SCM075) and 4 ng/mL FGF-basic. The seeded cells were allowed to recover overnight and then transfected. On the day of transfection, fresh medium without ROCK inhibitor was added prior to proceeding with DNA or RNA delivery using the respective lipid reagent (refer to section 3.2, option 1).

Option 2: Culturing cells in feeder-free conditions in Essential 8® Medium

Gibco® Human Episomal iPSC Line cells were cultured in Essential 8® Medium on tissue culture dishes coated with Geltrex® LDEV-Free, hESC-qualified Reduced Growth Factor Basement Membrane Matrix. After thawing, cells were passaged 2 to 3 times before using them for transfection. Prior to seeding, the cultures were washed once with D-PBS and then harvested using TrypLE™ Express Enzyme by incubating for 2–3 minutes in a 37°C humidified incubator. Leaving the cells in TrypLE™ Express Enzyme for longer than 3 minutes was avoided. After incubation, TrypLE™ Express Enzyme was removed, and cells were washed once with PBS and resuspended in Essential 8® Medium. Single-cell suspensions were counted using the Countess® Automated Cell Counter and electroporated as described in section 3.2, option 2. For lipid-based transfection, a 24-well tissue culture plate coated with Geltrex® matrix was used. Each well was seeded with approximately 1 x 10⁵ cells in 500 μL of Essential 8® Medium containing 10 μM ROCK inhibitor and allowed to recover overnight. On the day of transfection, fresh medium was added before proceeding with DNA or RNA delivery using the respective lipid reagent (refer to section 3.2, option 1).

3.2 Transfection

Two options for transfection are presented here. The lipid-based method is shown schematically in Figure 3A, and the electroporation method in Figure 4A. Various factors, including cell seeding density, the health of the cells, media, NA quality, and NA delivery method, affect the transfection efficiency and, in turn, the efficiency of gene editing. Special care should be taken while handling and processing the cells, and the protocols should be strictly followed to maximize efficiency [1].

Option 1: Lipid-based transfection for iPSCs

Tissue culture dishes for transfection were prepared as described in section 3.1. On the day of transfection, fresh medium without ROCK inhibitor was added to each well prior to proceeding with DNA or RNA formats of CRISPR, using the respective lipid reagent. For the recommended amounts of lipid reagent and DNA or RNA, refer to the instructions and procedure in section 2.2, option 1. At 72 hours after transfection, cells were analyzed for genomic cleavage efficiencies, using the GeneArt® Genomic Cleavage Detection Kit.

Option 2: Electroporation of iPSCs

Electroporation of human iPSCs was performed using a 10 μ L tip of a Neon® Electroporation System (Cat. No. MPK1096). Preparation of single-cell suspensions of human iPSCs was performed as described above in section 3.1. Cells were counted using the Countess® Automated Cell Counter. Electroporation was performed in 24-well plates using 1×10^5 cells per well. When using the DNA format, 750 ng of GeneArt® CRISPR plasmid (OFF reporter) DNA was used. When using the CRISPR RNA format, 200 ng of *in vitro*-transcribed gRNA and 1 μ g of GeneArt® CRISPR Nuclease mRNA was used. Cells were harvested 72 hours after transfection. In our hands, of the 24 different Neon® electroporation conditions, Neon® optimization condition No. 17 (850 V, 30 ms, 2 pulses) gave the best genomic cleavage efficiency.

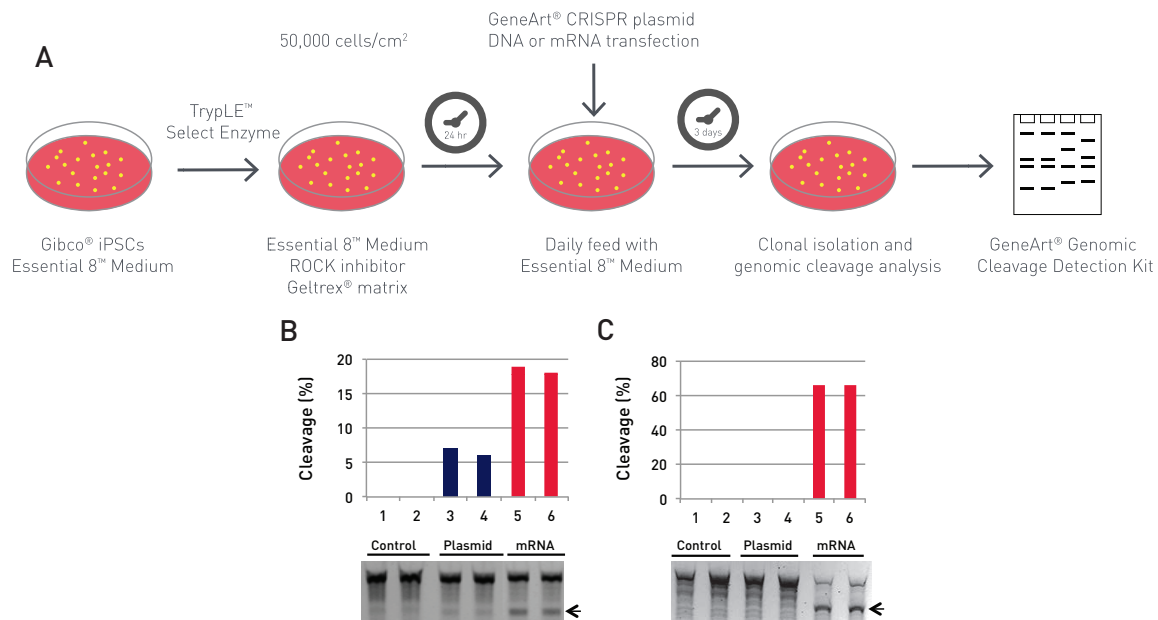


Figure 3. Lipid-mediated transfection and genomic cleavage efficiency of GeneArt® CRISPR Nuclease Vector with OFF Reporter and CRISPR Nuclease mRNA in Gibco® human iPSCs grown in Essential 8® Medium and conditioned medium. (A) Schematic of the lipid-based transfection procedure in a 24-well format. **(B)** Genomic cleavage efficiency for the HPRT locus in Essential 8® Medium. **(C)** Genomic cleavage efficiency for the HPRT locus in Gibco® human iPSCs grown on a feeder layer and, before transfection, transferred to Geltrex® matrix-coated plates in conditioned medium. Plasmid DNA and mRNA were transfected using Lipofectamine® 3000 and Lipofectamine® MessengerMAX™ transfection reagent, respectively. Lanes 1 and 2, control; 3 and 4, GeneArt® all-in-one plasmid vector; 5 and 6, CRISPR Nuclease mRNA. Genomic cleavage efficiency was determined by densitometry of the cleaved band (arrows).

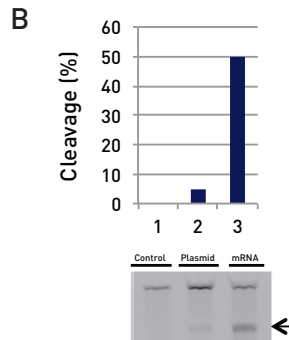
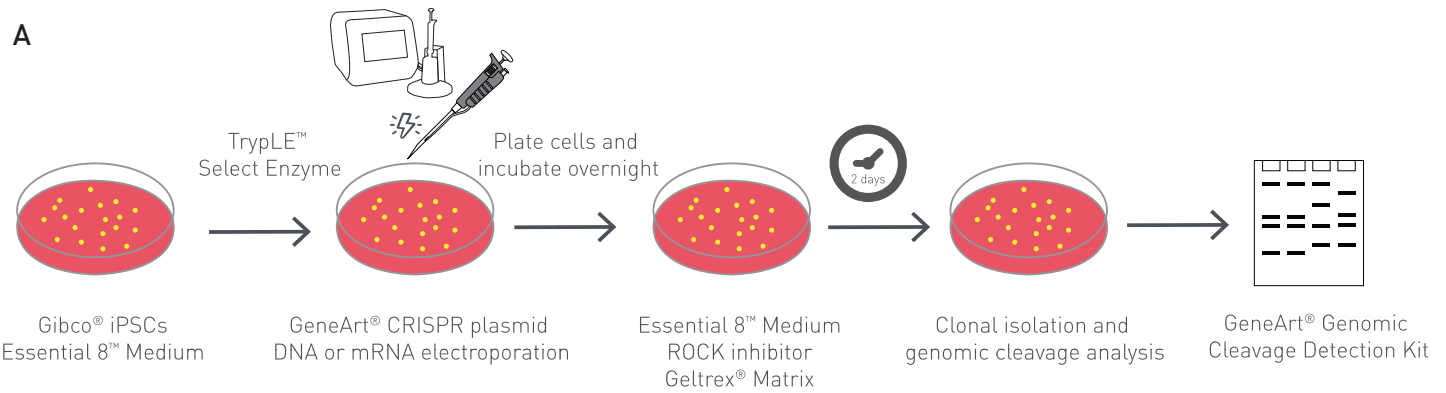


Figure 4. Electroporation of GeneArt® all-in-one plasmid vector and CRISPR Nuclease mRNA in Gibco® human ESCs and iPSCs, and analysis of genomic cleavage efficiency. (A) Schematic of the electroporation procedure. **(B)** Cleavage efficiency of the HPRT locus in human iPSCs grown in Essential 8® Medium. Lane 1, control; 2, GeneArt® all-in-one vector; 3, CRISPR Nuclease mRNA. Genomic cleavage efficiency was determined by densitometry of the cleaved band (arrow).

4. Genomic Cleavage Detection

The GeneArt® Genomic Cleavage Detection Kit provides a simple, reliable, and rapid method for the detection of locus-specific double-strand break formation. After performing the assay, the resulting DNA fragments were analyzed on 2% E-Gel® EX Agarose Gels, and cleavage efficiencies were measured by densitometry analysis [4].

Results

Mouse ESCs

The *ROSA26* locus was targeted using either (1) GeneArt® CRISPR Nuclease OFP Vector specific for the *ROSA26* locus, or (2) *in vitro*-transcribed *ROSA26*-specific CRISPR gRNA and GeneArt® CRISPR Nuclease mRNA. Cell lysates were collected 48 hours posttransfection and analyzed using a genomic cleavage assay. For lipid-mediated delivery, we observed ~30% cleavage efficiency using the DNA format and more than 50% using the RNA format (Figure 2B). We also observed higher cell viability using the RNA format in comparison to the DNA format (data not shown). For electroporation, all of the 24 Neon® optimization conditions were used for the DNA and RNA formats, and we found more than 50% genomic cleavage using the DNA format, but the efficiency of the mRNA format was lower at all the tested electroporation conditions (Figure 2C). Therefore,

we highly recommend using the RNA format with Lipofectamine® MessengerMAX™ reagent for ease of use and to enable high efficiency.

Human iPSCs

Human iPSCs were grown in Essential 8® Medium or in hESC-conditioned medium on Geltrex® matrix-coated plates. Single-cell suspensions of iPSCs were generated by treating cells with TrypLE™ Express Enzyme for 2–3 minutes, as described in the Methods section, and plated on Geltrex® matrix-coated plates for transfection. The next day, DNA (GeneArt® CRISPR Nuclease Vector with OFP) and RNA (*in vitro*-transcribed gRNA and Cas9 mRNA) were transfected using Lipofectamine® 3000 and Lipofectamine® MessengerMAX™ transfection reagent, respectively. Results were analyzed 3 days after transfection. The RNA format yielded between 15% and 20% genomic modification in iPSCs grown in Essential 8® Medium (Figure 3B), and greater than 60% genomic modification in iPSCs grown in conditioned medium (Figure 3C). All-in-one plasmid DNA (the GeneArt® CRISPR Nuclease Vector included with the OFP kit) yielded less than 10% genomic modification in Essential 8® Medium and undetectable genomic cleavage from cells grown in conditioned medium (Figure 3B and C). The CRISPR RNA format delivered through

electroporation resulted in ~50% cleavage efficiency in cells grown in Essential 8[®] Medium and 32% for cells grown in conditioned medium (Figure 4B). Delivery of the GeneArt[®] CRISPR Nuclease Vector with OFP through electroporation resulted in less than 10% efficiency in cells grown in Essential 8[®] Medium and 20% for cells grown in conditioned medium (Figure 4B). The CRISPR RNA format delivered via electroporation resulted in higher cell viability compared to the DNA format (data not shown). We highly recommend using the RNA format with Lipofectamine[®] MessengerMAX[™] Transfection Reagent for ease of use and to enable greater gene editing efficiency.

Conclusion

In this application note, we have described both lipid- and electroporation-based protocols for efficient genomic modifications in mouse ESCs and human iPSCs. ESCs and iPSCs hold great promise in regenerative medicine research and in the study of the mechanics of human diseases. To maximize the potential of these stem cells for research and therapy, efficient and precise genetic engineering techniques are warranted. Using our GeneArt[®] CRISPR-Cas9 system, we can modify mouse and human stem cell genomes with more than 50% efficiency. These methods have the potential to greatly facilitate the generation of genetically modified ESCs for research and therapeutic applications.

Notes: The methods and protocols described here have been specifically optimized for genome editing applications in mouse ESCs or human iPSCs/ESCs using the reagents mentioned here. The electroporation protocols described here have been optimized for 10 µL Neon[®] tips. To use 100 µL Neon[®] tips for electroporation, please follow the Neon[®] Transfection System user manual to optimize the electroporation conditions [2].

References

- Factors influencing transfection efficiency:
<http://www.lifetechnologies.com/us/en/home/references/gibco-cell-culture-basics/transfection-basics/factors-influencing-transfection-efficiency.html>
- Neon[®] Transfection System and optimization protocol:
<http://www.lifetechnologies.com/us/en/home/life-science/cell-analysis/cellular-imaging/imaging-cytometry/tali-image-cytometer/applications-tali-image-cytometer/neon-transfection-tali.html>
- GeneArt[®] CRISPR Nuclease mRNA user guide:
https://tools.lifetechnologies.com/content/sfs/manuals/GeneArt_CRISPR_nuclease_mRNA_man.pdf
- GeneArt[®] Genomic Cleavage Detection Kit:
<http://www.lifetechnologies.com/order/catalog/product/A24372?ICID=search-product>
- Ordering information for GeneArt[®] CRISPR T7 Strings[™] DNA
<https://www.lifetechnologies.com/us/en/home/life-science/genome-editing/geneart-crispr/crispr-nuclease-mrna.html>

Ordering information

Product description	Human iPSCs	Mouse ESCs	Quantity	Cat. No.
Cell culture				
2-Mercaptoethanol	●	●	50 mL	21985-023
Collagenase, Type IV, powder	●		1 g	17104-019
Countess [®] Automated Cell Counter	●	●	1 instrument	C10227
DMEM/F-12, GlutaMAX [™] supplement	●		500 mL	10565-018

Ordering information

Product description	Human iPSCs	Mouse ESCs	Quantity	Cat. No.
Cell culture				
DPBS	●	●	500 mL	14190-144
Essential 8™ Medium	●		500 mL	A1517001
Fetal Bovine Serum	●	●	500 mL	10439-024
Geltrex™ LDEV-Free, hESC-Qualified, Reduced Growth Factor Basement Membrane Matrix	●		5 mL	A1413302
GlutaMAX™ Supplement	●	●	20 x 100 mL	35050-079
Human Fibroblast Growth Factor Basic (FGF-basic) Recombinant Protein	●		50 µg	RFGFB50
Human Episomal iPSC Line	●		1 x 10 ⁶ cells/vial	A18945
KnockOut™ DMEM		●	500 mL	10829-018
MEM Non-Essential Amino Acids Solution	●	●	100 mL	11140-050
Mouse (ICR) Inactivated Embryonic Fibroblasts		●	1 mL	A24903
Opti-MEM® I Reduced Serum Medium	●	●	100 mL	31985062
Recombinant Human Leukemia Inhibitory Factor (LIF)		●	100 µg	PHC9481
TrypLE™ Express Enzyme	●	●	100 mL	12604-013

Product description	Quantity	Cat. No.
Genome editing		
GeneArt® CRISPR Nuclease mRNA	15 µg	A25640
GeneArt® CRISPR Nuclease Vector with OFP Reporter Kit	10 rxns	A21174
GeneArt® CRISPR T7 Strings™ DNA	>200 ng	Order via geneartsupport@lifetech.com
IVT		
MEGAclear™ Transcription Clean-Up Kit	20 preps	AM1908
MEGAshortscript™ T7 Kit	25 rxns	AM1354M
Phusion™ High-Fidelity DNA Polymerase	500 units	F530L
PureLink® PCR Purification Kit	50 preps	K3100-01
Quant-iT™ RNA Assay Kit	1 kit	Q-33140
TranscriptAid T7 High Yield Transcription Kit	50 rxns	K0441
Transfection and delivery		
Lipofectamine® 3000 Transfection Reagent	1.5 mL	L3000015
Lipofectamine® MessengerMAX™ Transfection Reagent NEW	1.5 mL	LMRNA015
Neon™ Electroporation System	1 instrument	MPK5000
Neon® Transfection System 10 µL Kit	96 x 2 rxns	MPK1096
Genome editing screening and detection		
GeneArt® Genomic Cleavage Detection Kit	20 rxns	A24372
GeneArt® Genomic Cleavage Selection Kit NEW	10 rxns	A27663

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