TECHNICAL BULLETIN

GENOME EDITING OF HUMAN PLURIPOTENT STEM CELLS

Using the ArciTect™ CRISPR-Cas9 System

Introduction

The ability of human embryonic stem (ES) and induced pluripotent stem (iPS) cells to self-renew indefinitely and differentiate into all somatic cell types make them an attractive source of human tissue for regenerative medicine. This potential, combined with recent advances in more efficient and accessible genome editing techniques, has opened the door to a wide range of research areas. Disease-causing mutations can now be introduced or corrected in cell lines to create or rescue disease models; work in this area may also pave the way to correcting disease-causing mutations in vivo.

The following protocols provide instructions for the preparation of CRISPR-Cas9 ribonucleoprotein (RNP) complexes, and their transfection into ES or iPS cells via electroporation or chemical transfection. For best results, starting cultures should be of high quality - moderate density, and largely free from differentiated areas. For complete instructions on culturing high-quality ES and iPS cells, including coating plates and use of mTeSR™1 medium (Catalog #85850), refer to the Technical Manual: Maintenance of Human Pluripotent Stem Cells in mTeSR™1 (Document #28315), available at **www.stemcell.com**.

In each case, the materials required are indicated on a per-well basis, for example 1.5 x 10^5 ES or iPS cells per well. These values will need to be scaled up for the actual number of wells in an experiment. It is recommended that each unique crRNA be tested in duplicate wells. Multiple crRNAs are also often tested when a new gene is being targeted, as they will have different efficiencies at the target gene and at off-target sites. Optimization of conditions such as cell density or Cas9:guide RNA ratio may be desired for some difficult-to-transfect cell lines or refractory genomic locations. Multiple cell lines may be desired. Finally, two wells are recommended for positive controls (e.g. ArciTect™ Human HPRT Positive Control Kit, Catalog #76013) and negative (non-transfected) controls. Thus, the total number of wells should be determined before starting, to calculate the total amount of materials required.

This protocol uses the mTeSR™1 supplement CloneR™ (Catalog #05888), which greatly enhances the cloning efficiency and single-cell survival of human pluripotent stem cells (hPSCs). For complete instructions on thawing, preparation, and storage of CloneR™, refer to the Product Information Sheet (Document #DX21725), available at **www.stemcell.com** or contact us to request a copy.

The protocol describes transfection of a single RNP complex into hPSCs, as is typically done in non-homologous end-joining (NHEJ) applications. Application-specific protocol modifications, not detailed here, might include: use of the single-strand endonuclease Cas9-Nickase with two flanking guide RNAs; and/or the addition of single-strand oligodeoxynucleotide (ssODN) targeting vector for homology-directed repair (HDR).

NOTE: Perform all protocols using sterile techniques and a biohazard safety cabinet certified for Level II handling of biological materials.

Preparation of ArciTect™ crRNA and ArciTect™ tracrRNA Stock Solutions

Materials Required

PRODUCT	CATALOG #
ArciTect™ crRNA	76010/76011/76012
ArciTect™ tracrRNA	76016A/76017A/ 76018A
Nuclease-free water	e.g. Sigma W4502
DNase- and RNase-free microcentrifuge tubes	e.g. Axygen® MCT-060-C-S

1. Briefly centrifuge the vials before opening.
2. Add nuclease-free water to give a final concentration of 200 µM, as outlined in Table 1.

Table 1. Resuspension Volumes for 200 µM* ArciTect™ crRNA or ArciTect™ tracrRNA

ArciTect™ crRNA OR ArciTect™ tracrRNA SIZE	VOLUME OF NUCLEASE-FREE WATER (µL)
2 nmol (Catalog #76010)	10
5 nmol (Catalog #76016A)	25
10 nmol (Catalog #76011 or 76017A)	50
20 nmol (Catalog #76012 or 76018A)	100

*200 µM is equal to 200 pmol/µL

3. Mix thoroughly. If not used immediately, aliquot and store at -80°C for up to 6 months. After thawing the aliquots, use immediately. Do not re-freeze.

Transfection of Human ES and iPS Cells Using the TransIT-X2® Dynamic Delivery System

Materials Required

PRODUCT	CATALOG #
ArciTect™ crRNA	76010/76011/76012
ArciTect™ tracrRNA	76016A/76017A/76018A
ArciTect™ Annealing Buffer (5X)	76020
ArciTect™ Cas9 Nuclease OR ArciTect™ Cas9-eGFP Nuclease OR ArciTect™ Cas9 Nickase	76001/76002/76004 OR 76005/76006 OR 76007/76008/76009
Corning® Matrigel® hESC-Qualified Matrix	Corning 354277
mTeSR™1	85850
CloneR™	05888
ACCUTASE™	07920
DMEM/F-12 with 15 mM HEPES	36254
Falcon® 24-Well Flat-Bottom Plate, Tissue Culture-Treated	38021
TransIT-X2® Dynamic Delivery System	Mirus MIR6003
DNase- and RNase-free microcentrifuge tubes	e.g. Axygen® MCT-060-C-S
Falcon® Conical Tubes, 15 mL	38009
Heating block or thermocycler	---

A. Plating Human ES or IPS Cells for Transfection

The following instructions are for harvesting single human ES or iPS cells from a 6-well plate for transfection in 24-well plates. If using other cultureware, adjust volumes accordingly.

1. Coat cultureware with Matrigel® and bring to room temperature (15 - 25°C) for at least 30 minutes prior to use.
2. Warm (15 - 25°C) sufficient volumes of mTeSR™1, CloneR™, DMEM/F-12, and ACCUTASE™.
3. Prepare Single-Cell Plating Medium by adding CloneR™ to mTeSR™1 at a 1 in 10 dilution.

   Example: To prepare 10 mL of Single-Cell Plating Medium, add 1 mL of CloneR™ to 9 mL of mTeSR™1.
4. Aspirate matrix from coated cultureware and add 0.5 mL of Single-Cell Plating Medium to each well to be seeded.
5. Use a microscope to visually identify regions of differentiation (if any) in the wells to be passaged. Mark these using a felt tip or lens marker on the bottom of the plate. Remove regions of differentiation by scraping with a pipette tip or by aspiration.
6. Aspirate the remaining medium from the well and add 1 mL of ACCUTASE™. Incubate the plate at 37°C and 5% CO_2 for approximately 5 minutes (incubation time may vary with different cell lines).
7. Using a micropipettor fitted with a 1000 µL tip, gently wash the cells from the surface of the plate by spraying the solution directly onto the colonies. Pipette the suspension up and down 2 - 3 times to break up small aggregates into single cells.
8. Transfer the cell suspension to a 15 mL conical tube containing at least 5 mL DMEM/F-12 and mix by flicking the tube 2 - 3 times.
9. Centrifuge the suspension at 300 x *g* for 5 minutes. Discard supernatant and resuspend cell pellet in 1 mL of Single-Cell Plating Medium.
10. Count cells using a hemocytometer or automated cell counter.
11. Add 1.25 x 10^5 cells per well to the plate prepared in step 4. Move the plate in several quick, short, back-and-forth, and side-to-side motions to evenly distribute cells.

    NOTE: Optimal cell density may vary with different cell lines and is dependent on when the cells will be harvested (typically 48 - 72 hours after plating).
12. Incubate at 37°C and 5% CO_2 for 24 hours. Refer to Figure 1 for a representative image of cell morphology following 24 hours of incubation.

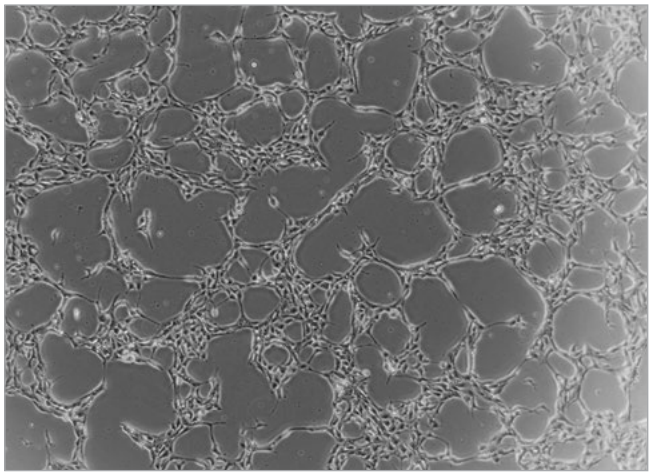

Figure 1. Typical Cell Density of WLS-1C Human iPS Cell Line 24 Hours After Plating 1.25 x 10^5 Single Cells per Well

B. Preparation of ArciTect™ CRISPR-Cas9 RNP Complex for Chemical Transfection

Prepration of RNP and transfection should be performed approximately 24 hours after plating ES or iPS cells.

1. Prepare 5 µM guide RNA by combining crRNA, tracrRNA, and Annealing Buffer in a microcentrifuge tube as indicated in Table 2. The volume below will provide sufficient reagent to transfect 4 wells of a 24-well plate; scale as needed. Mix thoroughly.
2. In a thermocycler or heating block, incubate guide RNA mixture at 95°C for 5 minutes followed by 60°C for 1 minute. Cool to room temperature (15 - 25°C). If not used immediately, store at -80°C for up to 6 months.

Table 2. Preparation of 5 µM Guide RNA

REAGENT	VOLUME (µL) PER 4 TRANSFECTIONS
Nuclease-free water	30
ArciTect™ Annealing Buffer (5X)	8
200 µM ArciTect™ crRNA	1
200 µM ArciTect™ tracrRNA	1
Total	**40**

3. Prepare a 5 µM Cas9 Nuclease solution in a microcentrifuge tube as shown in Table 3. This will provide sufficient reagent to transfect one well; if more transfections are required adjust volumes as needed. Mix thoroughly.

   NOTE: The amount of Cas9 to add will depend on the concentration and molecular weight of the Cas9 purchased, as shown in Table 3.

Table 3. Preparation of 5 µM ArciTect™ Cas9 Nuclease Solution

COMPONENT	STOCK CONCENTRATION	VOLUME PER WELL (µL)			
DMEM/F-12 with 15mM HEPES	n/a	1	4	0.2	3.4
Cas9 Nuclease	1 µg/µL = 6.25 µM	4	-	-	-
Cas9-Nuclease	4 µg/µL = 25 µM	-	1	-	-
Cas9-eGFP	1 µg/µL = 5.26 µM	-	-	4.8	-
Cas9-eGFP	3 µg/µL = 15.8 µM	-	-	-	1.6
Total		**5**	**5**	**5**	**5**

4. To prepare the RNP Complex Mix, combine components in a microcentrifuge tube as indicated in Table 4. Adjust component amounts according to the desired number of transfected wells. Mix thoroughly.

Table 4. Preparation of 5 µM RNP Complex Mix for Chemical Transfection

COMPONENT	VOLUME PER WELL (µL)
DMEM/F-12 with 15 mM HEPES	15.6
5 µM Guide RNA	9.6
5 µM ArciTect™ Cas9 Nuclease	4.8
Total	**30**

NOTE: If two or more guide RNAs are to be used in the experiment e.g. using ArciTect™ Cas9 Nickase, each RNP complex should be prepared separately.

NOTE: May require optimization with different cell lines. A 1:2 (shown) to 1:4 molar ratio of Cas9 to guide RNA is recommended.

5. Incubate the RNP Complex Mix at room temperature (15 - 25°C) for 10 minutes. While RNP complex forms, proceed immediately to section C.

C. Chemical Transfection of Human ES or iPS Cells with RNP Complex Using the TransIT-X2® Dynamic Delivery System

1. To prepare the Transfection Mix, combine components in a microcentrifuge tube as indicated in Table 5. Adjust component amounts according to the desired number of transfected wells. Mix thoroughly by pipetting up and down.

Table 5. Preparation of Transfection Mix

COMPONENT	VOLUME PER WELL (µL)
DMEM/F-12 with 15 mM HEPES	28.8
TransIT®-X2	1.2
Total	**30**

2. Incubate the Transfection Mix at room temperature (15 - 25°C) for 10 minutes.
3. Remove the medium from cells plated onto 24-well plates the previous day, and replace with 450 µL of room temperature (15 - 25°C) mTeSR™1 per well. Place plate in a 37°C and 5% CO_2 incubator.
4. To prepare RNP Transfection Mix, combine components in a microcentrifuge tube in the order listed in Table 6. Volumes shown are for a single well; scale accordingly to accommodate all RNP complexes and replicates.

Table 6. Preparation of RNP Transfection Mix

COMPONENT	VOLUME PER WELL (µL)
Transfection Mix	28
RNP Complex Mix	28
Total	**56**

5. Pipette the RNP Transfection Mix up and down to mix thoroughly.
6. Incubate the RNP Transfection Mix at room temperature (15 - 25°C) for 20 minutes; do not exceed 30 minutes.
7. Add 50 µL of RNP Transfection Mix dropwise per well of the 24-well plate prepared in step 3. Mix by gently moving the plate back and forth 2 - 3 times.
8. Incubate the plate at 37°C and 5% CO_2.
9. Perform a full medium change every 24 hours with 500 µL of room temperature (15 - 25°C) mTeSR™1.
10. If using Cas9-eGFP Nuclease, the transfection efficiency can be assessed 24 hours after transfection by flow cytometry (see Figure 2).

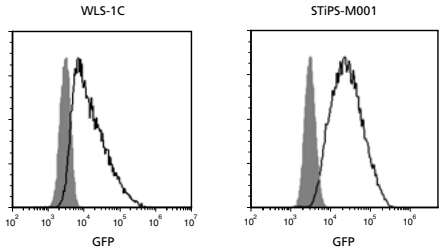

Figure 2. Cas9-eGFP Detection by Flow Cytometry

WLS-1C (left) or STiPS-M001 (right) iPS cells were transfected with RNP complex containing Cas9-eGFP; eGFP was detected by flow cytometry 24 hours after transfection. Filled histogram: Non-transfected control; Solid line histogram: Cas9-eGFP-transfected cells.

11. Culture cells for 48 - 72 hours after transfection to allow genome editing to occur. Assess genome editing efficiency by T7 Endonuclease I Assay (see Figure 3). The cells can then be used to generate clonal ES or iPS cell lines and assess genome editing by T7 Endonuclease I assay and/or DNA sequencing. Refer to the CloneR™ Product Information Sheet (Document #DX21725) for instructions on subcloning ES or iPS cell lines.

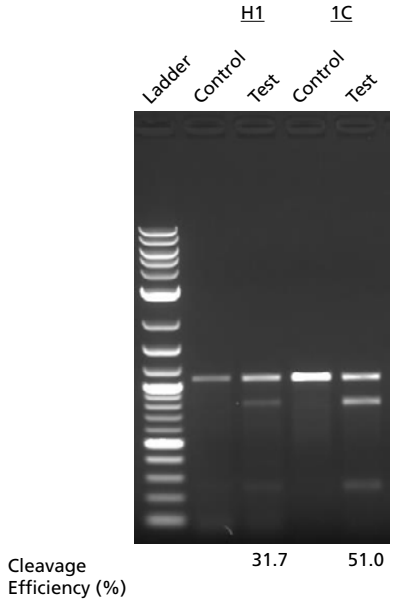

Figure 3. INDEL Detection by T7 Endonuclease I Assay

H1 ES cells or WLS-1C iPS cells were edited using ArciTect™ Human HPRT Positive Control Kit, and INDEL formation was assessed using the T7 Endonuclease I Assay.
Control: Non-transfected cells; Test: HPRT-edited

Transfection of Human ES and iPS Cells Using the Neon® Transfection System

Materials Required

PRODUCT	CATALOG #
ArciTect™ crRNA	76010/76011/76012
ArciTect™ tracrRNA	76016A/76017A/76018A
ArciTect™ Annealing Buffer (5X)	76020
ArciTect™ Cas9 Nuclease OR ArciTect™ Cas9-eGFP Nuclease OR ArciTect™ Cas9 Nickase	76001/76002/76004 OR 76005/76006 OR 76007/76008/76009
Corning® Matrigel® hESC-Qualified Matrix	Corning 354277
mTeSR™1	85850
CloneR™	05888
ACCUTASE™	07920
Falcon® 24-Well Flat-Bottom Plate, Tissue Culture-Treated	38021
Neon® Transfection System 10 µL Kit • Resuspension Buffer R • Electrolytic Buffer E	MPK1025
DNase- and RNase-free microcentrifuge tubes	e.g. Axygen® MCT-060-C-S
Falcon® Conical Tubes, 15 mL	38009
Heating block or thermocycler	---

A. Preparation of Tissue Culture Plates

The following protocol is for electroporation of human ES or iPS cells in a 24-well tissue culture plate. If using other cultureware, adjust volumes accordingly.

1. Coat a 24-well plate with Matrigel® and bring to room temperature (15 - 25°C) for at least 30 minutes prior to use.
2. Warm (15 - 25°C) sufficient volumes of mTeSR™1, CloneR™, and ACCUTASE™.
3. Prepare 5 mL of Single-Cell Plating Medium per transfection by adding CloneR™ to mTeSR™1 at a 1 in 10 dilution.

   Example: To prepare 10 mL of Single-Cell Plating Medium, add 1 mL of CloneR™ to 9 mL of mTeSR™1.
4. Remove Matrigel® from the 24-well plate and replace with 1 mL of Single-Cell Plating Medium per well. Place plate in a 37°C and 5% CO_2 incubator.

B. Preparation of Guide RNA

1. Prepare 60 µM guide RNA by combining crRNA, tracrRNA, and Annealing Buffer in a microcentrifuge tube as indicated in Table 7. This will provide sufficient reagent for approximately 6 transfections in a 24-well plate; for a different number of transfections, adjust volumes accordingly. Mix thoroughly.

Table 7. Preparation of 60 µM Guide RNA

REAGENT	VOLUME (µL) PER 6 TRANSFECTIONS
Nuclease-free water	2
ArciTect™ Annealing Buffer (5X)	2
200 µM ArciTect™ crRNA	3
200 µM ArciTect™ tracrRNA	3
Total	**10**

2. In a thermocycler or heating block, incubate guide RNA mixture at 95°C for 5 minutes followed by 60°C for 1 minute. Cool to room temperature (15 - 25°C). If not used immediately, store at -80°C for up to 6 months.

C. Preparation of a Single-Cell Suspension

1. Use a microscope to visually identify regions of differentiation (if any) in the wells to be passaged. Mark these using a felt tip or lens marker on the bottom of the plate. Remove regions of differentiation by scraping with a pipette tip or by aspiration.
2. Aspirate the remaining medium from the well and add 1 mL of ACCUTASE™ (for 6-well plate). Incubate the plate at 37°C and 5% CO_2 for approximately 5 minutes.
3. Using a micropipettor fitted with a 1000 µL tip, gently wash the cells from the surface of the plate by spraying the solution directly onto the colonies. Pipette the suspension up and down 2 - 3 times to break up small aggregates into single cells.

4. Transfer the cell suspension to a 15 mL conical tube containing at least 2 mL of Single-Cell Plating Medium and mix by flicking the tube 2 - 3 times.
5. Count cells using a hemocytometer or automated cell counter.
6. Add 2 x 10^5 cells to a new 15 mL conical tube for each electroporation condition. Centrifuge at 300 x *g* for 5 minutes.
7. During centrifugation proceed immediately to section D.

D. Preparation of ArciTect™ CRISPR-Cas9 RNP Complex Mix for Electroporation

1. To prepare the RNP Complex Mix, combine components in a microcentrifuge tube as indicated in Table 8. Adjust component volumes according to the desired number of transfections. Mix thoroughly.

Table 8. Preparation of RNP Complex Mix for Electroporation

COMPONENT	VOLUME PER TRANSFECTION (µL)
60 µM guide RNA	1.5
7.2 µg ArciTect™ Cas9 Nuclease (4 µg/µL)*	1.8
Resuspension Buffer R	1.7
Total	**5**

*If using 3 µg/uL ArciTect™ Cas9-eGFP Nuclease add 2.8 µL of Cas9-eGFP and 0.7 µL of Resuspension Buffer R per 5 µL transfection volume. Note that 1 µg/µL Cas9 Nuclease or Nickase cannot be used for 10 µL Neon® electroporation.

NOTE: May require optimization with different cell lines. A 1:2 (shown) to 1:4 molar ratio of Cas9 to guide RNA is recommended.

NOTE: If two or more guide RNAs are to be used in the experiment (e.g. using ArciTect™ Cas9 Nickase), each RNP complex should be prepared separately.

2. Incubate the RNP Complex Mix at room temperature (15 - 25°C) for 10 minutes.

E. Electroporation of Human ES or iPS Cells with RNP Complex

1. Aspirate supernatant from the cell pellet prepared in section C, then flick the tube 2 - 3 times to dislodge and break up the pellet.
2. Resuspend cells in 13 µL of Resuspension Buffer R per electroporation condition and pipette up down to mix.
3. Transfer 10 µL of the cell suspension to a new microcentrifuge tube. Add 5 µL of RNP complex mix (formed in section D) and pipette up and down to mix.

   NOTE: If air bubbles are present in the tip when the cells are electroporated, cell viability and transfection efficiency will be significantly reduced.

4. Using a 10 µL Neon® pipette tip, draw up 10 µL of the mixture, check to see if the capillary is free of bubbles, and place into the electroporation chamber containing 3 mL of Electrolytic Buffer E.
5. Electroporate the mixture using the settings in Table 9.

   NOTE: Refer to the manufacturer's instructions on electroporation. Electroporation conditions may require optimization for different cell lines.

Table 9. Recommended Electroporation Conditions for Human ES or iPS cells Using a Neon® Device

ELECTROPORATION PARAMETER	
Electrical potential	1200 V
Pulse width	30 milliseconds
Number of pulses	1

6. Immediately after electroporation, transfer cells to the pre-warmed plate from section A.
7. Mix by gently rocking the plate back and forth 2 - 3 times.
8. Incubate the plate at 37°C and 5% CO_2.
9. Perform a full medium change every 24 hours with 1 mL of room temperature (15 - 25°C) mTeSR™1.

   Optional: If using Cas9-eGFP nuclease, the transfection efficiency can be assessed 24 hours after transfection by flow cytometry.

10. The cells should be cultured for 48 - 72 hours (or up to 7 days if confluency is low) after transfection for genome editing to occur. The cells can then be harvested to assess genome editing efficiency using a T7 Endonuclease Assay and then used to generate clonal ES or iPS cell lines for downstream applications.

References

1. Gundry MC et al. (2016) Highly efficient genome editing of murine and human hematopoietic progenitor cells by CRISPR/Cas9. Cell Rep 17(5): 1453–61.
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5. Ran FA et al. (2013) Double nicking by RNA-guided CRISPR Cas9 for enhanced genome editing specificity. Cell 154(6): 1380–9.
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