Takara Bio USA, Inc.

Cellartis® iPSC rCas9 Electroporation and Single-Cell Cloning System User Manual

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I. Introduction

The Cellartis iPSC rCas9 Electroporation and Single-Cell Cloning System allows efficient, footprint-free gene editing of human induced pluripotent stem cells (hiPSCs) using the CRISPR/Cas9 system, followed by successful clonal expansion of single, edited human iPSCs. Importantly, this system maintains karyotype and pluripotency during the whole editing and culturing process.

The CRISPR/Cas9 system has emerged as a powerful tool for gene editing because of its high targeting specificity, editing efficiency, and ease of use. The power of this technology derives from its simplicity, since all it requires is a Cas9 nuclease enzyme combined with a single guide RNA (sgRNA) that determines its target specificity (Jinek et al. 2012). This RNA-programmable method exploits the error-prone nature of the non-homologous end joining DNA repair pathway (NHEJ) to generate gene knockouts (via insertion/deletion). The method can also be used to generate knockins via the homology-directed repair (HDR) pathway (Figure 1).

CRISPR/Cas9 system components have been delivered successfully into target cells through a variety of approaches, including vector-based expression systems, transfection of RNA, and introduction of Cas9-sgRNA ribonucleoprotein (RNP) complexes (Sander and Joung 2014). Delivery of Cas9-sgRNA RNPs via electroporation, the method described in this manual, provides a fast turnaround for gene-editing experiments while minimizing the likelihood of off-target effects compared to vector-based approaches (Kim et al. 2014).

The editing component of the Cellartis iPSC rCas9 Electroporation and Single-Cell Cloning System contains:

- Recombinant wild-type Cas9 nuclease [Guide-it™ Recombinant Cas9 (Electroporation-Ready)]
    - This recombinant wild-type *Streptococcus pyogenes* Cas9 nuclease was expressed with a C-terminal nuclear-localization signal (NLS) and purified from *E. coli* for use in CRISPR/Cas9-mediated gene editing experiments.
    - The rCas9 protein solution has been verified to be sterile and well-tolerated by mammalian cells when electroporated as an RNP with a sgRNA for knockout experiments, or as an RNP with a donor repair template for knockin experiments.
- The Guide-it sgRNA In Vitro Transcription Components v2 is used to produce high yields of sgRNAs from *in vitro* transcription (IVT) reactions followed by the Guide-it IVT RNA Clean-Up Kit, used to quickly purify sgRNA in a phenol-free manner.

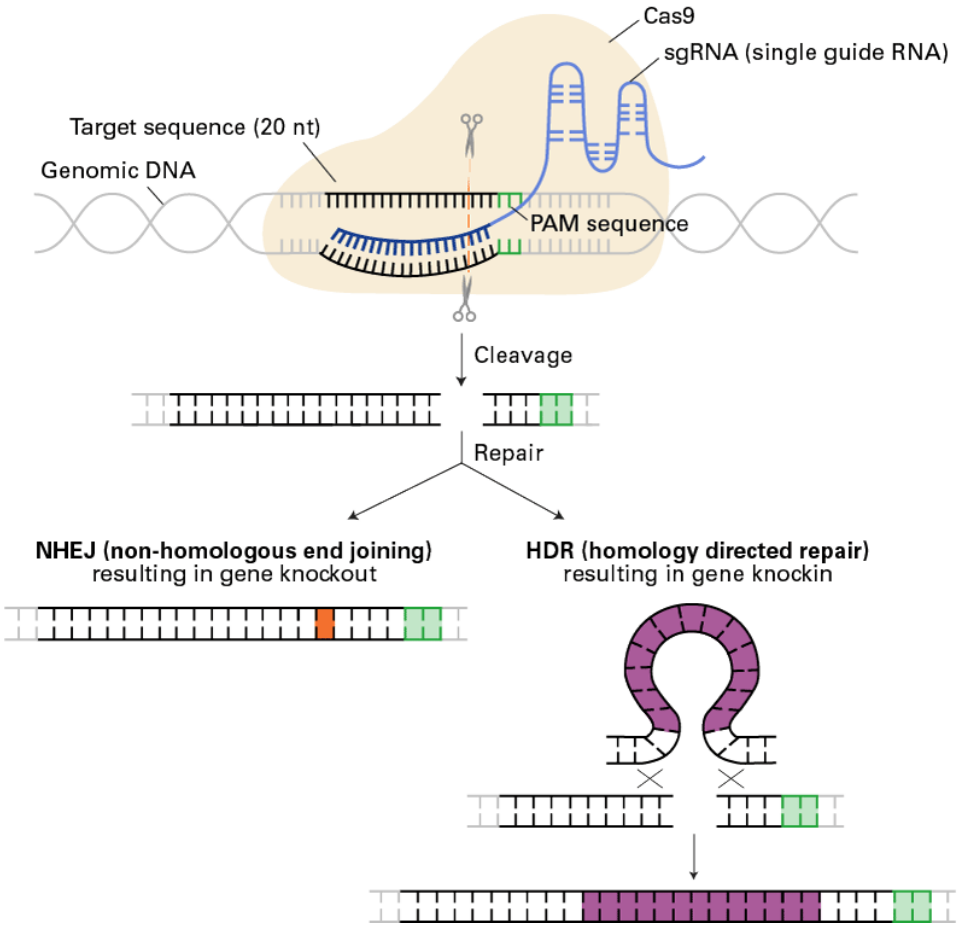

Figure 1. Schematic of CRISPR/Cas9-based editing to create gene knockouts and knockins. The CRISPR/Cas9 system is a simple, RNA-programmable method to mediate gene editing in mammalian cells. The CRISPR/Cas9 system relies on a single guide RNA (sgRNA) directing the rCas9 endonuclease to induce a double-strand break at a specific target sequence three base-pairs upstream of a PAM sequence in genomic DNA. This DNA cleavage can be repaired in one of two ways: 1) non-homologous end joining (NHEJ), which can result in gene knockout due to error prone repair (orange), or 2) homology directed repair (HDR), which can result in gene knockin if a homologous repair template (purple) is co-delivered.

Once the rCas9/sgRNA RNP complexes have been delivered by electroporation and cells have recovered, single cells must be isolated and expanded into clonal cell lines in order to isolate and screen for the genotype of interest (Figure 2). Traditionally, the establishment of a clonal population from hiPS cells grown and passaged as colonies is inefficient, challenging, and time-consuming; often, it results in cell death or premature differentiation. However, the cell culture component of the Cellartis iPSC rCas9 Electroporation and Single-Cell Cloning System contains a defined culture system (the Cellartis DEF-CS™ culture system, composed of basal medium, coating, and additives) for efficient single-cell cloning and expansion of edited hiPSC clones. The DEF-CS culture system, a monolayer-based culture system, bypasses the challenges of colony-based culture by allowing single-cell passaging, promoting survival and further expansion of plated single cells, and preserving the pluripotency of these cells.

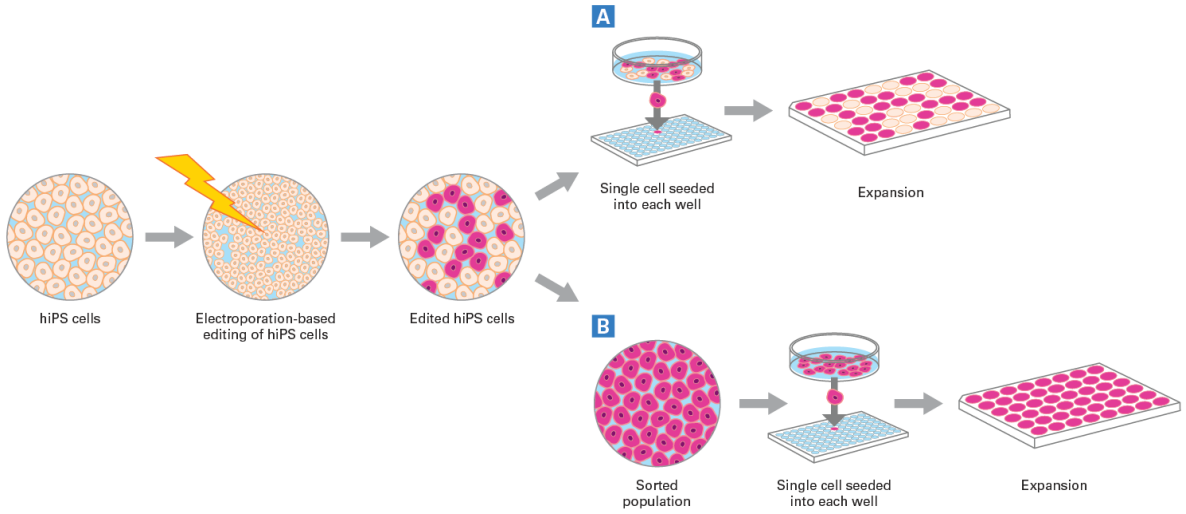

Figure 2. Using DEF-CS technology to generate edited clonal cell lines. Human induced pluripotent stem (hiPS) cells can be cultured, edited, and clonally expanded using the Cellartis iPSC rCas9 Electroporation and Single-Cell Cloning System. Initially, hiPS cells are adapted to the DEF-CS culture system, which maintains cells as a karyotypically stable and pluripotent monolayer. Next, electroporation-based delivery of rCas9 and an sgRNA (together as a ribonucleoprotein complex) are used to edit the cells. Using FACS or limiting dilution, edited cells can be individually seeded into wells of a 96-well plate (**Panel A**) and expanded into clonal lines. If desired, edited cells can be first sorted as a population by flow cytometry, prior to being seeded into wells of a 96-well plate, and finally expanded into clonal lines (**Panel B**).

All procedures described in this manual are optimized for Cellartis human iPS cell lines. If you wish to use the Cellartis iPSC rCas9 Electroporation and Single-Cell Cloning System for other human iPS cell lines or for Cellartis iPS cells grown in another system, please be aware that these cell lines will need to be adapted to the DEF-CS culture system before editing (see Section IV.B).

II. List of Components

The Cellartis iPSC rCas9 Electroporation and Single-Cell Cloning System (Cat. No. 632643) contains:

- 100 µg Guide-it Recombinant Cas9 (Electroporation-Ready) (Cat. No. 632641) (3 µg/µl)
- Guide-it sgRNA In Vitro Transcription Components v2 (Cat. No. 632637) (Not sold separately)
    - 50 µl Guide-it Scaffold Template (1 ng/µl)
    - 350 µl Guide-it In Vitro Transcription Buffer
    - 1 ml RNase Free Water
    - 150 µl Guide-it T7 Polymerase Mix (33 U/µl)
    - 625 µl PrimeSTAR® Max Premix (2X)
    - 100 µl Recombinant DNase I (RNase-free) (5 U/µl)
- Guide-it IVT RNA Clean-Up Kit (Cat. No. 632638)
    - 13 ml RNase Free Water
    - 3 ml IVT Binding Buffer
    - 50 IVT RNA Clean-Up Spin Columns
    - 2 x 6 ml IVT Wash Buffer
    - 50 Collection Tubes (2 ml)
- 2 x 800 µl Cellartis iPSC Single-Cell Cloning DEF-CS COAT-1 (Cat. No. Y30018) (Not sold separately)
- 500 ml Cellartis DEF-CS 500 Basal Medium (Cat. No. Y30011) (Not sold separately)
- Cellartis iPSC Single-Cell Cloning DEF-CS Additives (Cat. No. Y30019) (Not sold separately)
    - 2 x 750 µl DEF-CS GF-1
    - 500 µl DEF-CS GF-2
    - 500 µl DEF-CS GF-3

III. Additional Materials Not Supplied

A. Required

Mammalian Cell Culture Supplies

Use the Cellartis DEF-CS 500 Culture System (Takara Bio, Cat. No. Y30010) for maintaining hiPS cell lines 1) prior to using the Cellartis iPSC rCas9 Electroporation and Single-Cell Cloning System and 2) for scaling up clonal lines created using this system.

The following tissue culture materials are required but not supplied:

- 96-well plates, flat bottom, cell-culture treated (Corning, Cat. No. 3595 or equivalent)
- 48-well plates, flat bottom, cell-culture treated (Corning, Cat. No. 3548 or equivalent)
- TrypLE Select Enzyme (1X), no phenol red (Thermo Fisher Scientific, Cat. No. 12563011)
- PBS Dulbecco's with Ca^{2+} & Mg^{2+} (D-PBS +/+) (Sigma, Cat. No. D8662 or equivalent)
- PBS Dulbecco's w/o Ca^{2+} & Mg^{2+} (D-PBS –/–) (Sigma, Cat. No. D8537 or equivalent)

General Supplies

- 96–100% ethanol (Sigma, Cat. No. E7023 or equivalent)
- Isopropanol (Sigma, Cat. No. I9516 or equivalent)
- NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Cat. No. ND-2000 or equivalent)

Electroporation Supplies

Use of this product requires an electroporator, electroporation chamber (typically cuvettes or tips), and an electroporation buffer that is suitable for hiPSCs. In this manual, we provide a protocol for electroporation using the Neon Transfection System (Thermo Fisher Scientific, Cat. No. MPK5000). If using the 4D-Nucleofector System (Lonza, Cat. No. AAF-1002B), please refer to the manufacturer's website for operating instructions.

B. Recommended

sgRNA Efficacy Testing Supplies

If you to wish to test the efficacy of different sgRNAs *in vitro* prior to using them in editing experiments, try the Guide-it sgRNA Screening Kit (Cat. No. 632639).

Genotype Confirmation Supplies

These items are recommended for determining the efficiency of gene editing after electroporation (Cat. Nos. 631443 & 631448), the genotype (Cat. No. 632611), and the sequence of the edits (Cat. No. 631444) in the clonal cell lines:

Cat. No.	Product	Size
631443	Guide-it Mutation Detection Kit	100 rxns
631448	Guide-it Mutation Detection Kit	25 rxns
632611	Guide-it Genotype Confirmation Kit	100 rxns
631444	Guide-it Indel Identification Kit	10 rxns

IV. General Considerations

A. Storage and Handling

- Guide-it sgRNA In Vitro Transcription Components v2
  - Store at –20°C.
  - Avoid repeated freeze/thaw cycles.
- Guide-it IVT RNA Clean-Up Kit
  - Store at room temperature.
- Guide-it Recombinant Cas9 (Electroporation-Ready)
  - Store at –70°C.
  - At first use, thaw and aliquot.
  - Avoid repeated freeze/thaw cycles.
- Cellartis iPSC Single-Cell Cloning DEF-CS COAT-1
  - Store at 2–8°C.
  - Shelf life is specified on the product label.
- Cellartis DEF-CS 500 Basal Medium
  - Store at 2–8°C.
  - Contains penicillin and streptomycin.
- Cellartis iPSC Single-Cell Cloning DEF-CS Additives
  - Store at –20°C.
  - Shelf life is specified on the product label.
  - At first use, thaw provided vials, mix each vial gently, and aliquot each component separately into appropriate volumes. Store aliquots at –20°C until the expiration date on the original vial. Thawed vials may be stored at 2–8°C for up to one week. Do not re-freeze aliquots after thawing.

B. Transferring Human iPS Cells to the DEF-CS Culture System

It is strongly recommended to transfer cells from other culturing systems to the Cellartis DEF-CS 500 Culture System (Cat. No. Y30010) before editing and single-cell cloning with the Cellartis iPSC rCas9 Electroporation and Single-Cell Cloning System. Human iPS cells maintained in other culture systems can be readily transferred: fresh cultures can be transferred at passage and cryopreserved cultures can be thawed directly into the Cellartis DEF-CS 500 Culture System. Cells should be passaged at least five times in the DEF-CS system prior to using the Cellartis iPSC rCas9 Electroporation and Single-Cell Cloning System.

Expected Morphology of Human iPS Cells in the DEF-CS System

DEF-CS technology uses enzyme-based passaging in conjunction with a coating to promote single-cell survival, rapid expansion, and easier passaging. When transferring iPS cells to this system, you will notice that some cell characteristics differ from those of iPS cells cultured in your previous system. In contrast to commonly used colony-based culture systems, the DEF-CS culture system yields a monolayer of evenly spaced cells. Newly passaged cells grown in the DEF-CS culture system tend to spread out; however, as cells proliferate, the culture gets more confluent, and cells display typical undifferentiated stem cell morphology (i.e., high nucleus to cytoplasm ratio, defined borders, and prominent nucleoli).

V. Complete Experimental Workflow

The Cellartis iPSC rCas9 Electroporation and Single-Cell Cloning System provides all materials for generating and isolating edited clonal hiPS cell lines using CRISPR/Cas9. The workflow for this system (Figure 3) consists of four main steps:

1. Generation and purification of the sgRNA via *in vitro* transcription
2. Formation of the rCas9/sgRNA RNP followed by its electroporation into hiPS cells
3. Isolation of edited single cells by FACS or limiting dilution
4. Expansion of the clonal cell lines

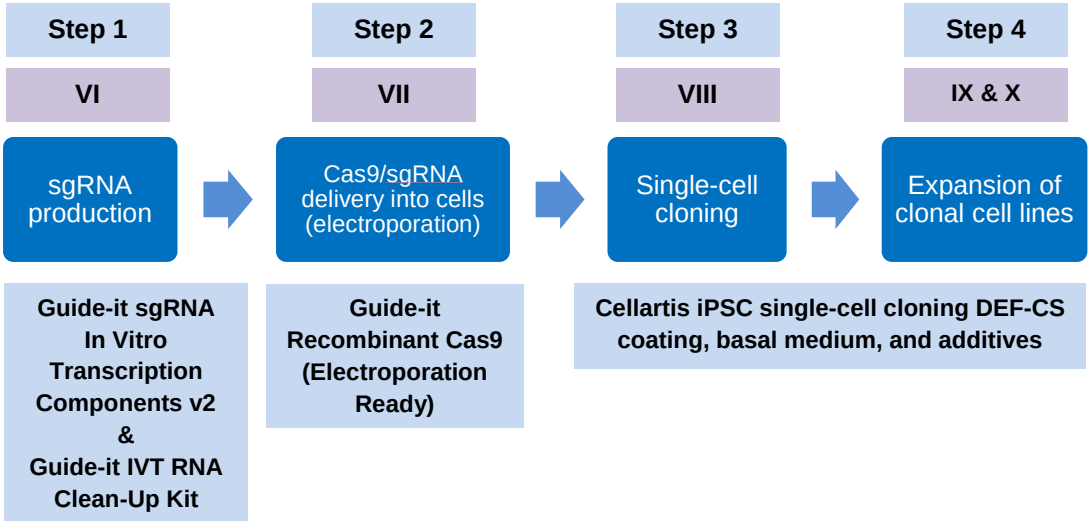

Figure 3. Workflow for gene editing of hiPS cells using components of the Cellartis iPSC rCas9 Electroporation and Single-Cell Cloning System. The purple boxes indicate the sections containing the relevant protocols.

VI. *In Vitro* Transcription of an sgRNA Containing the Desired Target Sequence

CRISPR/Cas9 gene editing requires a custom sgRNA with a user-designed targeting sequence that is homologous to the target gene or genomic region of interest. Selecting an appropriate DNA sequence at the target region is critical for maximizing the potential for efficient cleavage at the target site and for minimizing the likelihood of non-specific cleavage events. For many applications, it is advisable to design and test several different sgRNAs against the same genomic target region. Candidate sgRNAs must be produced in sufficient quantity for the generation of functional rCas9/sgRNA ribonucleoproteins.

A. Protocol Overview

The Guide-it sgRNA In Vitro Transcription Components v2 and Guide-it IVT RNA Clean-Up Kit can be used to synthesize sgRNAs as follows (Figure 4):

1. Generate a DNA template that contains your sgRNA-encoding sequence under the control of a T7 promoter by performing a PCR reaction with the included Guide-it Scaffold Template and a primer you design.
2. *In vitro* transcribe this template with the included Guide-it T7 Polymerase Mix to create an sgRNA containing your target sequence.

3. Purify your sgRNA after digestion with Recombinant DNase I (RNase-Free) using the Guide-it IVT RNA Clean-Up Kit; measure its concentration using a NanoDrop 2000 spectrophotometer or equivalent.

4. If you wish to determine cleavage efficiency before proceeding with editing (Figure 4, gray boxes), we recommend using the Guide-it sgRNA Screening Kit (sold separately).

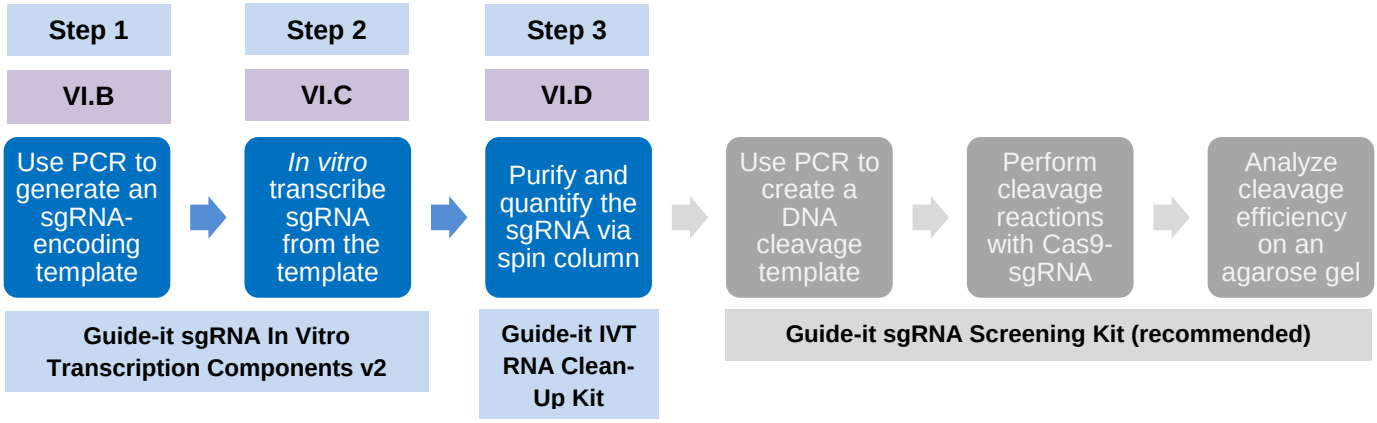

Figure 4. Workflow for generating sgRNA using components of the Cellartis iPSC rCas9 Electroporation and Single-Cell Cloning System. Steps 1–3 describe the workflow for using Guide-it sgRNA In Vitro Transcription Components v2 and the Guide-it IVT RNA Clean-Up Kit to synthesize and purify sgRNAs. The purple boxes indicate the sections containing the relevant protocols. The gray boxes indicate optional steps of *in vitro* sgRNA screening.

B. Generating the DNA Template

Guidelines for Designing PCR Primers

Use the following guidelines to design a forward primer to be used in a PCR reaction with the included Guide-it Scaffold Template to create a DNA template for *in vitro* transcription of your sgRNA. This primer should contain the T7 promoter sequence, followed by your sgRNA target sequence, and the Guide-it Scaffold Template-specific sequence (Figure 5).

Choosing the Correct DNA Target Sequence

Choose the DNA target sequence that will correspond to your actual sgRNA target sequence as shown in Figure 5, Panel A, according to the following guidelines:

a. The DNA target sequence you choose must end with the proto-spacer adjacent motif (PAM) sequence, NGG, on its 3' end. Only DNA sequences that are 20 nucleotides upstream of a PAM sequence can be used for CRISPR/Cas9.

b. Any target sequence can be used as long as the sequence is followed by the PAM sequence, NGG. However, to minimize off-target cleavage events, the entire target sequence (including the PAM) should have at least three base mismatches with any other non-targeted genomic sequence. Off-target events should be especially low if the mismatches are in, or adjacent to, the PAM. Most online tools for sgRNA design will predict off-target sequences for a given sgRNA target sequence. To learn more, visit http://www.takarabio.com/sgRNA-design-tools.

Designing a 56- to 58-nt Forward PCR Primer

The forward (sense) primer must contain the following four sequence elements, in the order shown in Figure 5, Panel B.

a. A T7 promoter sequence plus four extra bases (21 total nt) at the 5' end of the primer.
b. A transcription initiation site (0–2 guanine (G) residues): The number of Gs added is dependent on the 5' end of the target sequence. The T7 promoter requires at least two Gs for efficient transcription.

NOTE: If your specific target sequence (see item c, below) already contains two Gs, there is no need to add extra Gs for transcription initiation. Extra Gs could reduce cleavage efficiency.

c. Your specific sgRNA target sequence (20 nt).
d. The Guide-it Scaffold Template-annealing sequence (15 nt at the 3'end of the primer)

NOTES:

- A reverse (antisense) primer comes premixed with the Guide-it Scaffold Template.
- The forward primer should be subjected to salt-free purification following synthesis and diluted to a concentration of 10 µM in PCR-grade water.

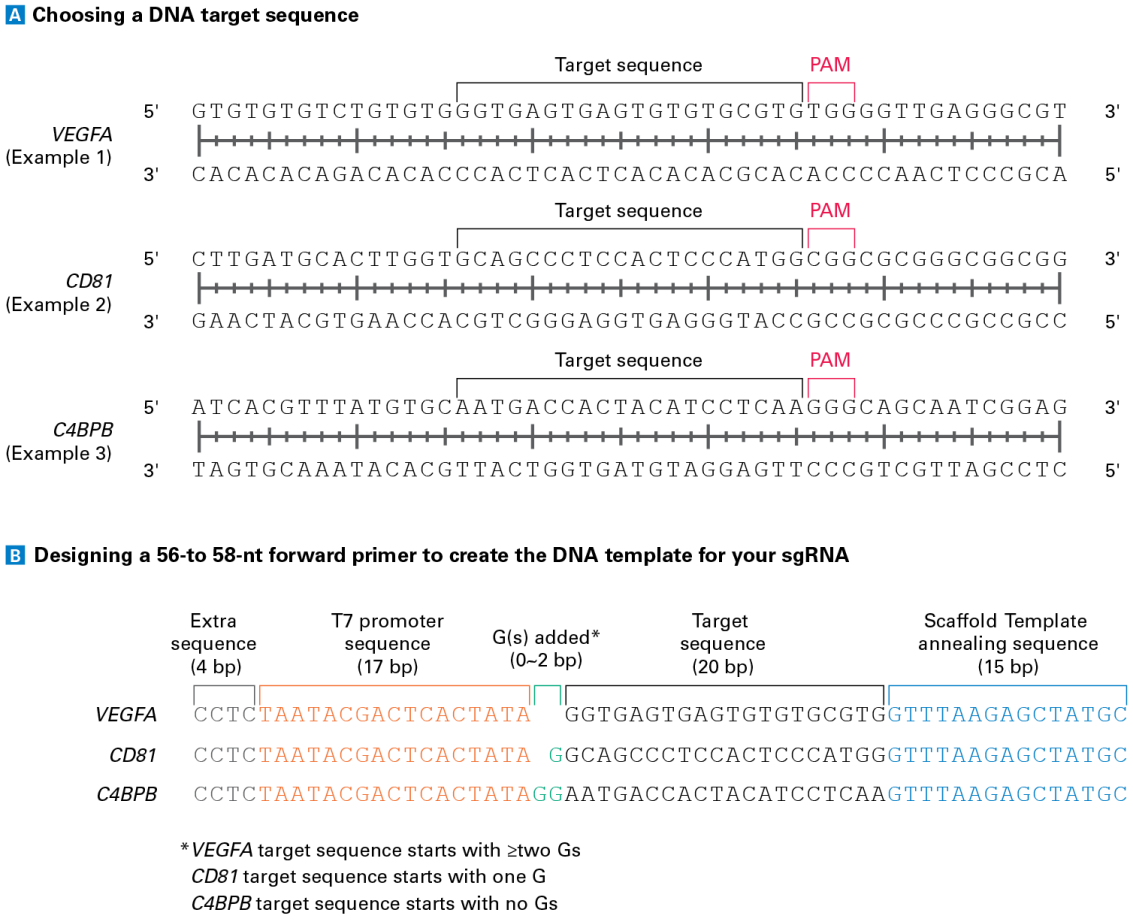

Figure 5. Designing a forward PCR primer to generate a DNA template for an sgRNA containing your target sequence. Panel A. Choose the DNA target sequence that will correspond to your actual sgRNA target sequence. **Panel B.** Design the forward primer to create the *in vitro* transcription template you will use to generate your sgRNA. [**NOTE:** The T7 promoter sequence (with four extra bases) and the Scaffold Template-specific sequence do **not** change.]

PCR-Amplifying the sgRNA Template

For use with the Guide-it sgRNA In Vitro Transcription Components v2.

1. Combine the following components in a 200-µl PCR tube. Briefly vortex and spin down to collect the reagents at the bottom of the tube.

Reagent	Amount (µl)
PrimeSTAR Max Premix (2X)	12.5
Guide-it Scaffold Template	1
Your forward primer (10 µM)	0.5
RNase Free Water	11
Total	25

2. Place reactions in a preheated thermal cycler with a heated lid and run the following program:

33 cycles:
98°C 10 sec
68°C 10 sec
4°C forever

3. Run and analyze 5 µl of the PCR product on a 2% agarose gel with a 100-bp DNA ladder. You should see a single band at ~130 bp (see Figure 6).

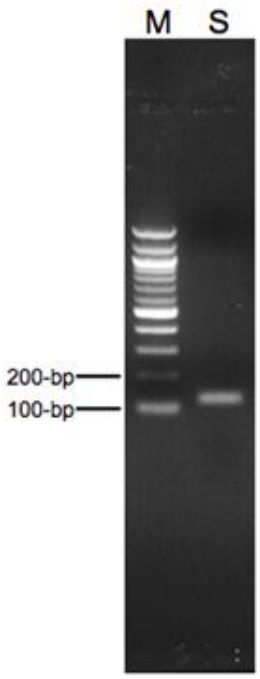

Figure 6. Gel electrophoresis of the PCR product. M = 100-bp DNA ladder. S = 5 µl of sample.

C. Performing the *In Vitro* Transcription (IVT) Reaction

The PCR product amplified in the previous section is directly used as template for the IVT reaction without purification.

1. Combine the following components in a 200-µl PCR tube. Briefly vortex and spin down to collect the reagents at the bottom of the tube.

Reagent	Amount (µl)
sgRNA PCR template (from Step B)	5
Guide-it In Vitro Transcription Buffer	7
Guide-it T7 Polymerase Mix	3
RNase Free Water	5
Total	20

NOTE: If you require a higher amount of sgRNA, you can scale up the total reaction size (e.g., to 50 µl) without affecting the quality of the sgRNA.

2. Place reactions in a preheated thermal cycler with a heated lid and run the following program:

37°C	4 hr
4°C	forever

NOTE: We recommend a 4-hr incubation, but a shorter incubation time is acceptable if you do not need to maximize your sgRNA yield (see Figure 7). We have observed a clear, sharp band via Agilent Bioanalyzer following an 8-hr incubation, indicating no drop in sgRNA quality.

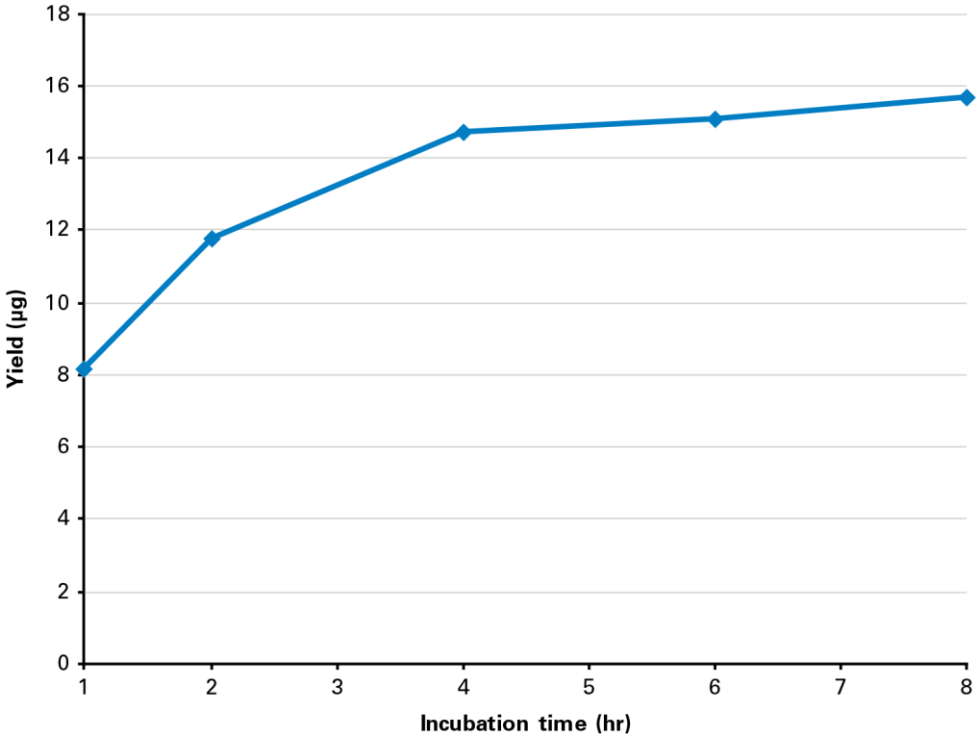

Figure 7. sgRNA yield over time. 20 µl of sgRNA IVT reactions were incubated for different times at 37°C. The reactions were purified using the Guide-it IVT RNA Clean-Up Kit and quantified using a NanoDrop spectrophotometer.

3. Following incubation, add 2 µl of Recombinant DNase I (RNase-Free) to the 20-µl IVT reaction. Briefly vortex and spin down to collect the reagents at the bottom of the tube.

4. Place reactions in a preheated thermal cycler with a heated lid and run the following program:

37°C	15 min
4°C	forever

D. Purifying the Transcribed sgRNA

For use with the Guide-it IVT RNA Clean-Up Kit.

NOTE: Before purifying your sgRNA, prepare the IVT Wash Buffer by adding 24 ml of 96–100% ethanol.

1. Add 78 µl of RNase Free Water to the reaction mixture (from Step C) for a total volume of 100 µl. Transfer all 100 µl to a 1.5-ml microcentrifuge tube.
2. Add 30 µl of IVT Binding Buffer and vortex for 5 seconds.
3. Add 130 µl of isopropanol and vortex for 5 seconds.
4. Place an IVT RNA Clean-Up Spin Column in a Collection Tube and load the sample from Step 3 onto the column. Centrifuge at 11,000*g* for 30 seconds at room temperature.
5. Discard the flowthrough from the Collection Tube and place the column back in the same Collection Tube.
6. Add 600 µl of IVT Wash Buffer and centrifuge at 11,000*g* for 30 seconds at room temperature.
7. Discard the flowthrough from the Collection Tube and place the column back in the same Collection Tube.
8. Add 250 µl of IVT Wash Buffer and centrifuge at 11,000*g* for 2 min at room temperature.
9. Place the IVT RNA Clean-Up Spin Column in a new 1.5-ml microcentrifuge tube.
10. Add 20 µl of RNase Free Water directly onto the silica membrane of the Spin Column and incubate for 1 min at room temperature.

    NOTE: With an elution volume of 20 µl, a concentration of >0.5 µg/µl is expected. This is appropriate for applications such as transfection, electroporation, and *in vitro* cleavage assays. If you need a higher concentration of sgRNA, the elution volume can be reduced to 5 µl (see Table I).

    Table I. Examples of sgRNA concentration and yield using different elution volumes.

Elution volume*	5 µl	10 µl	20 µl
Concentration	2.2 µg/µl	1.3 µg/µl	0.7 µg/µl
Yield	11.2 µg	12.8 µg	13.2 µg

    *Different elution volumes were used for purification of sgRNA using the Guide-it IVT RNA Clean-Up Kit. A smaller elution volume can be used without a significant loss of total yield of sgRNA if the higher sgRNA concentration is required for your experiment.

11. Centrifuge at 11,000*g* for 1 min at room temperature.
12. Use 1 µl to measure the OD using a NanoDrop Spectrophotometer (or equivalent). A yield of 10–20 µg of sgRNA is expected.
13. Store sgRNA at –80°C until it is used to form the rCas9/sgRNA RNP complex.

VII. Editing hiPS Cells by Electroporation

Once your sgRNA has been prepared, the electroporation protocol can begin. We recommend using the Neon Transfection System or the Amaxa 4D-Nucleofector System to electroporate cells. Our electroporation protocol has been optimized using the Neon Transfection System to electroporate cells from Cellartis Human iPS Cell Line 18 (Cat. No. Y00305) and Cellartis Human iPS Cell Line 22 (Cat. No. Y00325) cultured in the DEF-CS culture system (Cat. No. Y30010). Please refer to the Neon Transfection System User Manual and manufacturer's website for detailed operating instructions for the Neon Transfection System.

Likewise, please refer to the Amaxa 4D-Nucleofector System manufacturer's website for detailed operating instructions: http://www.lonza.com/products-services/bio-research/transfection/genome-editing.aspx

IMPORTANT:

- Use the Cellartis DEF-CS 500 Culture System (Cat. No. Y30010) during the gene editing process as well as the cell recovery step (prior to single-cell cloning, Section VIII).
- Please note that when the following protocol specifies use of "COAT-1," it is referring to the DEF-CS COAT-1 from the Cellartis DEF-CS 500 Culture System. Do not use the coating contained in the Cellartis iPSC rCas9 Electroporation and Single-Cell Cloning System, which is abbreviated as "SCC-COAT-1" due to its sole use for the single-cell cloning and expansion protocols.
- If cells have not been previously adapted to growing in the DEF-CS system, we strongly recommend transitioning cells by passaging five times in the DEF-CS system prior to performing the editing experiments.

A. Protocol Overview

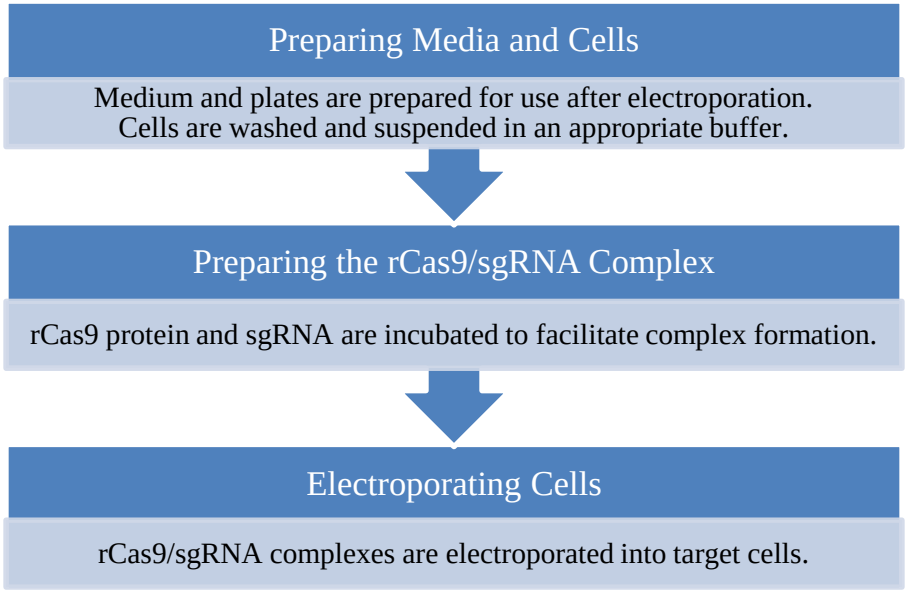

Figure 8. Workflow for electroporation-based delivery of rCas9/sgRNA complexes.

B. Preparing Media and Cells

Prior to beginning the electroporation protocol, prepare a starting culture of hiPSCs. You will need 1.5 x 10^5 cells per sample to be electroporated. (Please see the NOTE under Section VII.B.3 for details.)

1. Preparing 48-Well Plates

These plates will be used for seeding the electroporated cells. One well of a 48-well plate is needed for each electroporated sample.

1. Dilute the required volume of COAT-1 (not SCC-COAT-1) in D-PBS +/+ prior to use. Make a 1:20 dilution. Calculate the amount of diluted coating solution required depending on the number of wells to be used (90 µl of diluted coating solution per well; see Table II for guidance).

Table II. Preparation of coating solution for a 48-well plate.

Number of wells (48-well plate)	Volume of diluted coating solution (µl)	Volume of COAT-1 (µl)	Volume of D-PBS +/+ (µl)
1	90	4.5	85.5
2	180	9	171
n	90 x n	(90 x n)/20	(90 x n) – (volume of COAT-1)

2. Mix the diluted COAT-1 solution gently and thoroughly by pipetting up and down.
3. Add the diluted COAT-1 solution to a 48-well plate (using 90 µl/well), making sure the entire surface of each well is covered.
4. Place the plate in the incubator at 37°C ± 1°C, 5% CO_2, and >90% humidity for a minimum of 3 hr.
5. Aspirate the diluted COAT-1 solution from the 48-well plate immediately before use.

2. Preparing DEF-CS Medium for Dissociation and Recovery After Electroporation

For harvesting (before electroporation) and plating (recovery after electroporation), use components of the Cellartis DEF-CS 500 Culture System to prepare enough DEF-CS medium to 1) neutralize the TrypLE Select Enzyme (1X) used to dissociate cells from the initial culture vessel (a 1:10 dilution) and 2) seed the electroporated cells into the coated 48-well plate using 300 µl/well of medium.

NOTE: Prepare fresh medium on the day of intended use and warm it to 37°C ± 1°C immediately before use. Discard any leftover warmed medium.

- Prepare the appropriate volume of DEF-CS medium by adding DEF-CS GF-1 (dilute 1:333), GF-2 (dilute 1:1,000), and GF-3 (dilute 1:1,000) to Cellartis DEF-CS 500 Basal Medium.
- Use Table III as a guide to ensure there is sufficient medium for dissociation and seeding.

Table III. Preparation of DEF-CS medium for dissociation and recovery.

Cell dissociation

Plate type	TrypLE Select Enzyme (1X) (µl per well)	DEF-CS medium (µl per well)
48 wells	18	200
24 wells	38	400
12 wells	76	800
6 wells	190	2,000
10 cm	1,100	12,000

Recovery

Plate type	DEF-CS medium (µl per well)
48 wells	300

3. Preparing Cells for Electroporation

To prepare cells for electroporation, harvest enough fresh cells for your experiment, wash them, and resuspend them in the appropriate electroporation buffer.

NOTE: For electroporation, each sample requires 1 x 10^5 cells. However, due to the potential variation of pipette and tip volumes, we recommend preparing 1.5X the necessary volume of cell suspension (i.e., 1.5 x 10^5 cells) for electroporation with a 10-µl Neon Tip to ensure that there is sufficient volume.

1. Aspirate the media from the starting culture vessel and wash the cell layer once with D-PBS –/–.
2. Add TrypLE Select Enzyme (1X) to the initial culture vessel (see Table III) and incubate for 5 min at 37°C, or until the cell layer has detached. Detachment can be aided by swirling the cell culture flask or by tapping the side of the cell culture flask firmly but gently.
3. Resuspend the cells in DEF-CS medium (see Table III) and pipet up and down several times to ensure a single-cell suspension. (The cells will aggregate if left too long in TrypLE enzyme.)
4. Take an aliquot of the cell suspension and measure the cell density using your preferred method.
5. Centrifuge the cells at 400*g* for 5 min in a 15-ml conical tube.
6. Wash the cells once with D-PBS –/–, then resuspend cells in Buffer R (included with Neon kits) at a concentration of 2 x 10^7 cells/ml (i.e., 1.5 x 10^5 cells in 7.5 µl).
7. Keep the cell suspension on ice until use.

C. Preparing the rCas9/sgRNA RNP complex

Next, rCas9 and sgRNA components are combined to form RNP complexes for electroporation.

1. Thaw Guide-it Recombinant Cas9 (Electroporation-Ready) and sgRNA solutions at room temperature.

   NOTE: We recommend preparing aliquots upon initial thawing of Guide-it Recombinant Cas9 (Electroporation-Ready) to avoid repeated freeze/thaw cycles.

2. Combine the following components in a 200-µl PCR tube to mix the rCas9 protein and sgRNA at a 5:1 mass ratio. The molar ratio of rCas9 protein to sgRNA will be approximately 1:1 in this mixture, and the total volume will be 7.5 µl. Be sure to use the same buffer that was used to resuspend the cells.

   NOTE: The reaction volume indicated below is 1.5X the required volume.

   Per reaction:

0.45 µl*	sgRNA (e.g., 1 µg/µl)
0.75 µl	Guide-it Recombinant Cas9 (Electroporation Ready) (3 µg/µl)
6.3 µl*	Resuspension Buffer R or T
7.5 µl	Total volume

   *The added volume of sgRNA will vary depending on sgRNA concentration, and the added volume of Resuspension Buffer should be adjusted such that the total reaction volume is 7.5 µl. The volumes indicated above are based on a sgRNA concentration of 1 µg/µl.

   NOTES:

   - Make a master mix if you are performing multiple electroporations.
   - To maximize electroporation efficiency, the combined volume of the rCas9 and sgRNA solutions should be ≤20% of the total volume of the rCas9/sgRNA RNP complex reaction (e.g., for the 7.5-µl reaction specified above, the combined volume of the sgRNA and rCas9 solutions should be ≤1.5 µl).
   - If you plan to use donor DNA to induce HDR-mediated knockin, add the DNA **after** the subsequent incubation step (Step 3). We recommend using ≤1 µg of DNA for knockin experiments. Adjust the volume of Resuspension Buffer R or T included in the reaction such that the final volume upon addition of donor DNA is 7.5 µl.

3. Mix the reaction well by gently pipetting up and down. Incubate using a thermal cycler preheated to 37°C with the following program:

37°C	5 min
4°C	hold

4. **OPTIONAL:** Add donor DNA and keep on ice until use.

D. Electroporating Cells Using the Neon Transfection System

1. Aspirate the COAT-1 from each well of the 48-well plate (prepared in Section VII.B.1) and add 300 µl of pre-warmed DEF-CS medium (prepared in Section VII.B.2) to each well.
2. Fill the Neon Tube with 3 ml of Buffer E (included with Neon kits) and insert the Neon Tube into the Neon Pipette Station.
3. Using the touchscreen on the Neon system, set up the electroporation parameters as follows: Pulse voltage / Pulse width / Pulse number = 1,100 v / 20 ms / 2 pulses
4. Gently resuspend the cells by tapping, and then transfer 7.5 µl of the cell suspension into the tube containing the 7.5 µl of rCas9/sgRNA solution.
5. Mix well by gently pipetting up and down.
6. Insert the Neon Pipette into the Neon Tip and confirm that the pipette and tip are tightly connected.
7. Using the Neon Pipette, pipet the mixture slowly into the Neon Tip.

   NOTE: Avoid any air bubbles in the tip. If you notice air bubbles, place the sample back into the tube and aspirate again into the tip without any air bubbles.
8. Insert the Neon Pipette into the Neon Tube placed in the Neon Pipette Station and run the program.
9. Remove the pipette very carefully and transfer the cells into the 48-well plate containing pre-warmed medium. Repeat Steps 4–9 until all the samples have been electroporated.
10. Shake the plate appropriately to disperse the cells and incubate at 37°C ± 1°C, 5% CO_2, and >90% humidity.

    NOTE: Allow cells to recover for at least five days prior to conducting single-cell cloning (Section VIII).

VIII. Single-Cell Cloning of Edited Cells

A. Protocol Overview

After a recovery period of at least five days, cells can be seeded singly and expanded into edited clonal cell lines. As Cellartis iPSC culture systems are designed for a seamless transition between the various stages of an editing experiment, there is no special transition needed from the Cellartis DEF-CS 500 Culture System to the Cellartis iPSC rCas9 Electroporation and Single-Cell Cloning System. However, during Protocols VIII–X, use components of the Cellartis iPSC rCas9 Electroporation and Single-Cell Cloning System, and then transition back to the Cellartis DEF-CS 500 Culture System for further upscaling.

Table IV describes a schedule of all media changes (volume and composition) necessary to create clonal lines in 24-well plates that are ready for culture with the Cellartis DEF-CS 500 Culture System.

Table IV. Workflow for single-cell cloning and expansion using the Cellartis iPSC rCas9 Electroporation and Single-Cell Cloning System.

Plate type	Day	Additives used	Volume of medium (µl per well)	Total volume (µl per well)
96-well plate	1	GF-1, GF-2, and GF-3	100	100
	3		100*	200
	5		150	200
	(6)		150	200
	7		150	200
	(8)		150	200
	9**		150	200
48-well plate	Passaging	GF-1, GF-2, and GF-3	500	500
	Medium Change	GF-1 and GF-2	500	500
24-well plate	Transition to Cellartis DEF-CS 500 Culture System			

*Add medium; do not replace.
**Use the same volumes for subsequent days until the cells are ready for passaging.
( ) Media change is only necessary if media is yellow due to high metabolic activity.

Once stable clonal lines have been developed, proceed to verification of your desired edits.

B. Single-Cell Seeding into a 96-Well Plate

To optimize the survival rate and expansion potential during single-cell seeding, use cells that are in an early proliferative state. We recommend starting with a *confluent but not dense* culture, corresponding to a density of 0.8–1.5 x 10^5 cells/cm^2 (Figure 9).

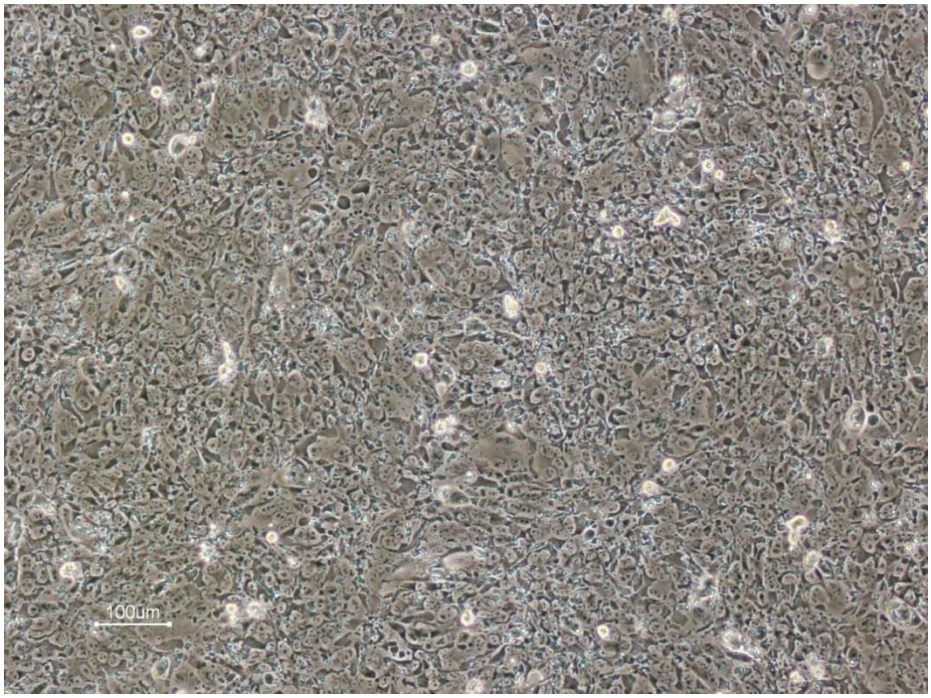

Figure 9. Recommended density of starting culture used for single-cell cloning.
Representative image shows the morphology of cells with a density of 1.5 x 10^5 cells/cm^2 at 10X magnification. Scale bar = 100 microns.

1. Coating a 96-Well Plate

1. Dilute the required volume of Cellartis iPSC Single-Cell Cloning DEF-CS COAT-1 (SCC-COAT-1) in D-PBS +/+ prior to use. Make a 1:10 dilution. Calculate the amount of diluted coating solution required depending on the number of wells to be used (50 µl of diluted coating solution per well: see Table V for guidance).

Table V. Preparation of coating solution for a 96-well plate.

Number of wells (96-well plate)	Volume of diluted coating solution (µl)	Volume of SCC-COAT-1 (µl)	Volume of D-PBS +/+ (µl)
1	50	5	45
2	100	10	90
96	4,800	480	4,320
n	50 x n	(50 x n)/10	(50 x n) – (volume of SCC-COAT-1)

2. Mix the diluted SCC-COAT-1 solution gently and thoroughly by pipetting up and down.
3. Add the diluted SCC-COAT-1 solution to a 96-well plate for single-cell seeding, making sure the entire surface of each well is covered.
4. Place the cell culture plate in an incubator at 37°C ± 1°C, 5% CO_2, and >90% humidity for a minimum of 3 hr.
5. Aspirate the diluted SCC-COAT-1 solution from the cell culture plate just before use.

2. Preparing DEF-CS SCC Medium for Single-Cell Seeding

Prepare the appropriate volume of DEF-CS SCC medium by adding DEF-CS GF-1 (dilute 1:333), GF-2 (dilute 1:1,000), and GF-3 (dilute 1:1,000) to Cellartis DEF-CS 500 Basal Medium according to Table VI. Prepare fresh medium on the day of intended use and warm it to 37°C ± 1°C immediately before use. Discard any leftover warmed medium.

It is important to prepare enough DEF-CS SCC medium to 1) neutralize the TrypLE Select Enzyme (1X) used to dissociate cells from the initial culture vessel (a 1:10 dilution) and 2) seed the detached cells using 100 µl/well into a 96-well plate. Use Table VI as a guide to ensure there is sufficient medium for dissociation and seeding.

Table VI. Preparation of medium for dissociation and single-cell seeding.

Cell Dissociation

Plate type	TrypLE Select Enzyme (1X) (µl per well)	DEF-CS SCC medium (µl per well)
48 wells	18	200
24 wells	38	400
12 wells	76	800
6 wells	190	2,000
10 cm	1,100	12,000

Single-Cell Seeding

Plate type	DEF-CS SCC medium (µl per well)
96 wells	100

3. Single-Cell Seeding

Seeding of Single Cells (Day 1)

1. Check cells under a phase contrast microscope; photo document as necessary.
2. Aspirate the medium from the culture vessel and wash the cell layer once with D-PBS –/–.
3. Add TrypLE Select Enzyme (1X) to the culture vessel, using the amount indicated in Table VI. Place the vessel in an incubator at 37°C ± 1°C, 5% CO_2, and >90% humidity for 5 min, or until the cell layer has detached. Detachment can be aided by tapping the side of the vessel firmly but gently. It is not recommended to tilt or swirl the cell culture vessel.

   NOTE: If starting from multiple samples in the same plate, please make sure to replace the culture vessel lid after removing a sample from a well, then gently tap the side of the vessel. This redistributes the dissociation enzyme and minimizes the risk of the other samples drying out.
4. Resuspend the cells in DEF-CS SCC medium (using the volume indicated in Table VI) and pipet up and down several times to ensure a single-cell suspension. (The cells will aggregate if left too long in TrypLE enzyme.)
5. Use your preferred method to isolate single cells: FACS or limiting dilution. For limiting dilution, we recommend using a final dilution of 0.5 cells per well of a 96-well plate.
6. Place the plate in an incubator at 37°C ± 1°C, 5% CO_2, and >90% humidity and leave the plate undisturbed for 48 hr. Continue culturing according to Table IV.

Adding Media to Wells Containing Single Cells (Day 3)

Without discarding any medium, carefully add 100 µl of fresh DEF-CS SCC medium per well. (See Table IV for guidelines.) There should now be a total of 200 µl per well.

C. Culturing Single-Cell Colonies

After single-cell cloning, cells will proliferate into emerging colonies (Figure 10) that will be passaged for further expansion during scale-up.

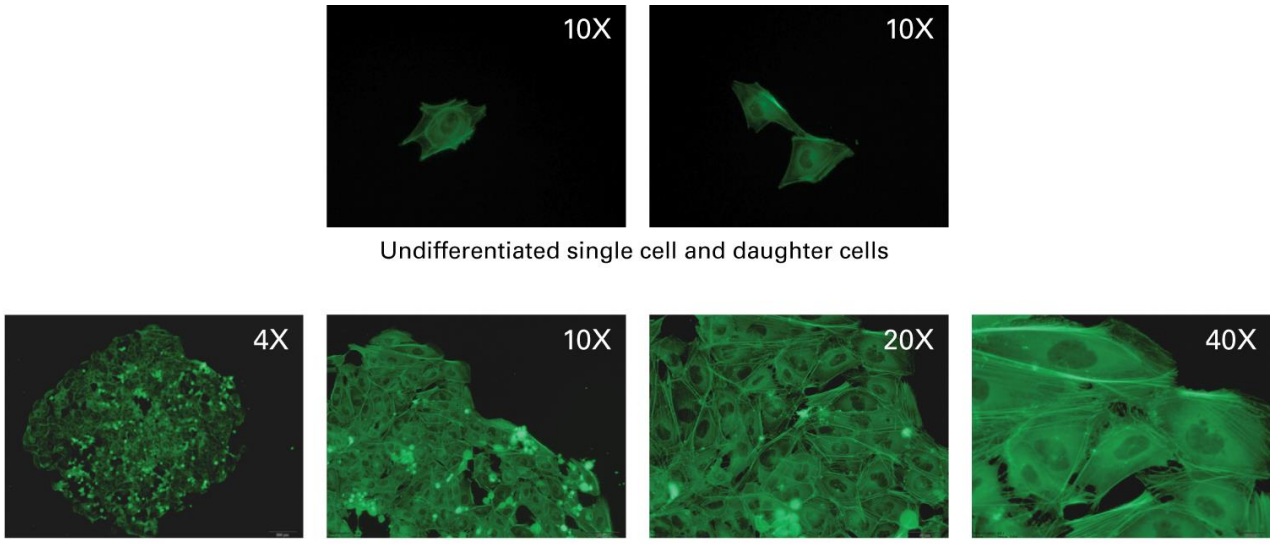

Figure 10. A single cell seeded in one well generates an emerging colony. Representative images of GFP-actin iPS cells taken 24 hr (top) or two weeks (bottom) after single-cell cloning.

Changing Media on Single-Cell Colonies in the 96-Well Plate

NOTE: A media change in the 96-well plate is recommended on Day 5 post-seeding and then every other day (see Table IV for guidance). If the medium turns yellow due to high metabolic activity, change the media every day.

Preparing DEF-CS SCC Medium for Establishment of Single-Cell Colonies

1. Prepare 150 µl per well of DEF-CS SCC medium by adding DEF-CS GF-1 (dilute 1:333), GF-2 (dilute 1:1,000), and GF-3 (dilute 1:1,000) to Cellartis DEF-CS 500 Basal Medium according to Table IV.
2. Prepare fresh medium on the day of intended use and warm it to 37°C ± 1°C immediately before use. Discard any leftover warmed medium.

Changing Media

1. Check cells under the microscope; photo document as necessary.
2. Carefully discard 150 µl of the medium from each well and add 150 µl of newly warmed medium into each well of the plate. It is recommended to always use manual pipetting (not a vacuum pump). **Avoid flushing medium directly onto the cell layer.**
3. Place the cell culture plate in an incubator at 37°C ± 1°C, 5% CO_2, and >90% humidity.

NOTE: The colonies will be ready to passage from the 96-well plate to a 48-well plate after 8–14 days, depending on the generation time of the specific iPS cell line (Figure 11).

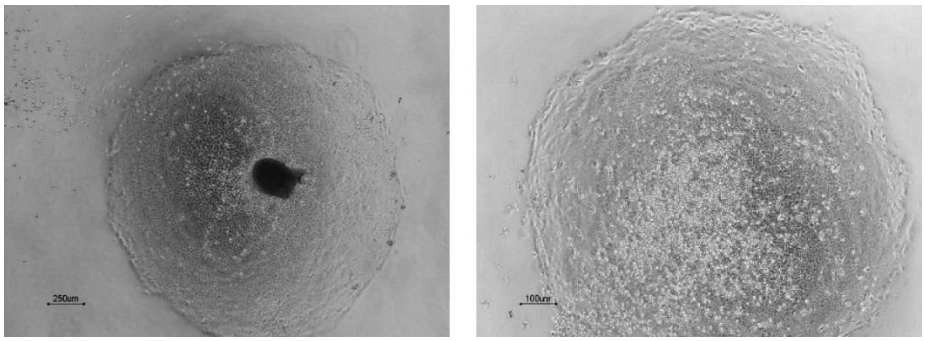

Figure 11. Clonal colonies, ready for transfer to larger wells and scale-up. The cells have the typical undifferentiated stem cell morphology (i.e., high nucleus-to-cytoplasm ratio, defined borders, and prominent nucleoli).

IX. Passaging Cells from the 96-Well Plate to a 48-Well Plate

A. Coating a 48-Well Plate

1. Dilute the required volume of SCC-COAT-1 in D-PBS +/+ prior to use. Make a 1:10 dilution. Calculate the amount of diluted coating solution required depending on the number of wells to be used (200 µl of diluted coating solution per well of a 48-well plate; see Table VII for guidance).

Table VII. Preparation of coating solution for a 48-well plate.

Number of wells (48-well plate)	Volume of diluted coating solution (µl)	Volume of SCC-COAT-1 (µl)	Volume of D-PBS +/+ (µl)
1	200	20	180
2	400	40	360
48	9,600	960	8,640
n	200 x n	(200 x n)/10	(200 x n) – (volume of SCC-COAT-1)

2. Mix the diluted SCC-COAT-1 solution gently and thoroughly by pipetting up and down.
3. Add the diluted SCC-COAT-1 solution to a 48-well plate (using 200 µl/well), making sure the entire surface of each well is covered.
4. Place the plate in the incubator at 37°C ± 1°C, 5% CO_2, and >90% humidity for a minimum of 3 hr.
5. Aspirate the diluted SCC-COAT-1 solution from the 48-well plate immediately before use.

B. Preparing DEF-CS SCC Medium for Passaging

1. Prepare the appropriate volume of DEF-CS SCC medium by adding DEF-CS GF-1 (dilute 1:333), GF-2 (dilute 1:1,000), and GF-3 (dilute 1:1,000) to Cellartis DEF-CS 500 Basal Medium according to Table IV. The volume of medium needed for each well of the 48-well plate is 500 µl. Calculate the amount of medium needed depending on the number of clonal lines to be expanded.
2. Prepare fresh medium on the day of intended use and warm it to 37°C ± 1°C immediately before use. Discard any leftover warmed medium.

C. Passaging

1. Check the cells under the microscope; photo document as necessary.
2. Aspirate the media from the wells and wash the cell layer with D-PBS –/–.
3. Add 50 µl per well of room-temperature TrypLE Select Enzyme (1X) to the cells. Make sure the whole colony in the well is covered. Place the plate in an incubator at 37°C ± 1°C, 5% CO_2, and >90% humidity, and incubate for 5 min or until all cells have detached.

4. Resuspend the cells in 500 µl per well of pre-warmed DEF-CS SCC medium. Transfer all of the cell suspension to a newly coated well in a 48-well plate.

   NOTE: To prevent cell loss, counting the cells at this stage is not recommended.

5. Tilt the dish backwards and forwards gently to ensure that the cell suspension is dispersed evenly over the surface, then place it in an incubator at 37°C ± 1°C, 5% CO_2, and >90% humidity.

X. Scaling up from the 48-Well Plate

Once the cells have been passaged into a 48-well plate, **DEF-CS GF-3 is no longer needed in the growth medium** used when changing the media. Prepare "DEF-CS medium for maintenance" by adding DEF-CS GF-1 (dilute 1:333) and GF-2 (dilute 1:1,000) to Cellartis DEF-CS 500 Basal Medium. When the cells are ready to be scaled up to a 24-well plate, they can be cultured with the Cellartis DEF-CS 500 Culture System (Cat. No. Y30010).

XI. References

Jinek, M. *et al.* A Programmable Dual-RNA–Guided DNA Endonuclease in Adaptive Bacterial Immunity. *Science.* **337,** (2012).

Kim, S., Kim, D., Cho, S. W., Kim, J. & Kim, J.-S. Highly efficient RNA-guided genome editing in human cells via delivery of purified Cas9 ribonucleoproteins. *Genome Res.* **24,** 1012–9 (2014).

Sander, J.-D. & Joung, J.-K. CRISPR-Cas9 systems for genomic editing, regulation and targeting. *Nat. Biotechnol.* **32,** 347–55 (2014).

For information on using the 4D Nucleofector System to electroporate cells, please visit:
http://www.lonza.com/products-services/bio-research/transfection/genome-editing.aspx

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