

Takara Bio USA, Inc.

Cellartis® iPSC CRISPR/Cas9 Gesicle and Single-Cell Cloning System User Manual

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I. Introduction

The Cellartis iPSC CRISPR/Cas9 Gesicle and Single-Cell Cloning System is a complete system that allows efficient, footprint-free gene editing of human induced pluripotent stem cells (iPSCs) via gesicle delivery of a Cas9/sgRNA complex, followed by successful clonal expansion of single, edited iPSCs. Importantly, this system maintains karyotype and pluripotency during the whole editing 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).

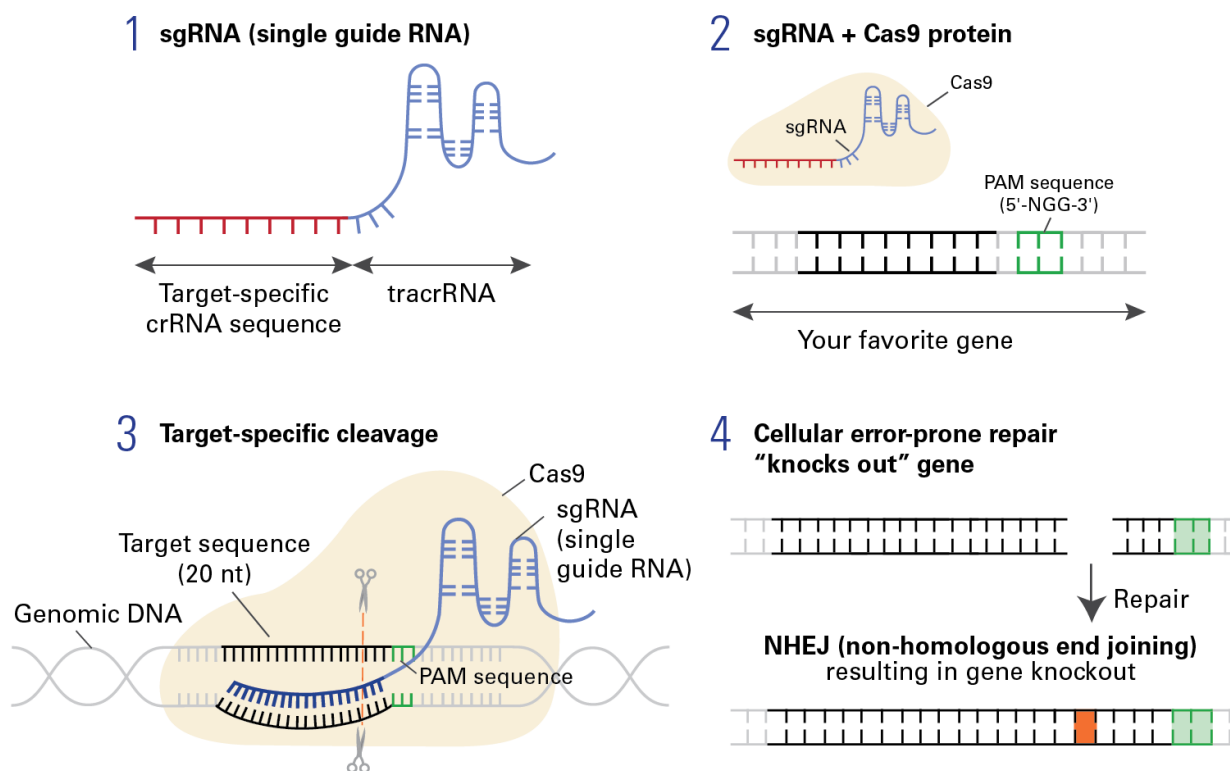


Figure 1. Using Guide-it technology to perform genome editing. The CRISPR/Cas9 system is a simple, RNA-programmable method to mediate gene editing in mammalian cells. The CRISPR/Cas9 system relies on a single guide RNA (sgRNA), which consists of a single RNA strand with a crRNA sequence that binds to a specific DNA target and a tracrRNA sequence that binds to Cas9 protein. When an sgRNA engineered to contain a 20-nucleotide (nt) target sequence of interest binds to a recombinant form of Cas9 protein that has double-stranded DNA endonuclease activity, the resulting complex will produce target-specific double-stranded cleavage. Cellular repair, which is error-prone, will take place at the cleavage site, and may result in a mutation that can knock out a gene.

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 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).

We have developed a method to deliver Cas9/sgRNA RNP complexes using cell-derived nanovesicles called gesicles. Gesicles are vesicles released from the plasma membrane of mammalian producer cells and are capable of carrying any cargo, such as proteins and protein complexes. CRISPR/Cas9 Gesicles are generated by the co-expression of Cas9 protein, a customer-designed sgRNA, and other proteins that stimulate gesicles to be released

from the producer cell membrane (Figure 2). Once gesicles have been made, they can be harvested, concentrated, and applied to target cells, where the active Cas9/sgRNA complex is released and transported to the nucleus for efficient gene editing.

The editing component of the Cellartis iPSC CRISPR/Cas9 Gesicle and Single-Cell Cloning System features a complete system for producing high yields of gesicles containing a Cas9/sgRNA complex targeting a user's gene of interest in human iPS cells. The included Guide-it Gesicle Packaging Mix contains lyophilized Xfect™ Transfection Reagent premixed with an optimized, ready-to-use formulation of plasmids that contain coding sequences for all the elements necessary for Cas9 expression and gesicle production. Gesicle Producer 293T Cells (sold separately; Cat. No. 632617) are transfected with these plasmids plus a target-specific sgRNA plasmid, allowing users to easily generate their own Cas9/sgRNA gesicles for gene editing in target cells.

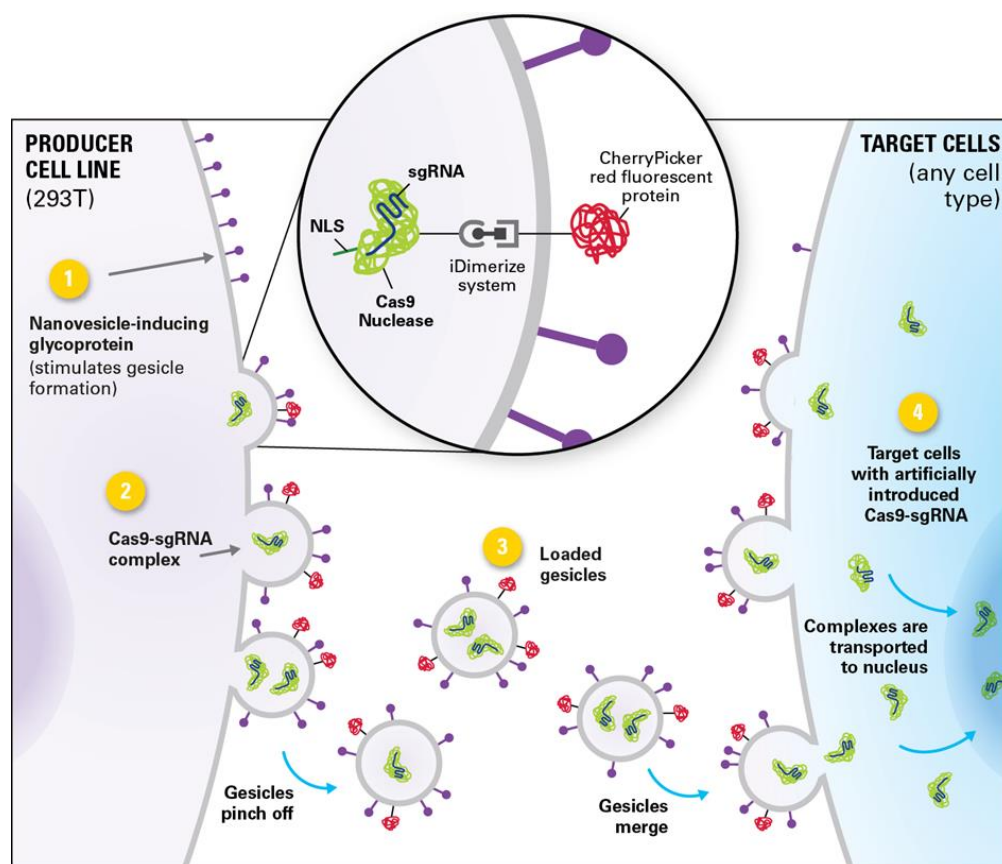


Figure 2. Gesicle Producer 293T cells generate nanovesicles (gesicles) for delivery of Cas9/sgRNA RNP complexes specific to a gene of interest. Once produced (left side of image), gesicles are harvested from the culture supernatant and concentrated. The gesicles can be applied to target cells immediately (right side of image), or frozen in aliquots for future use. Below are detailed descriptions corresponding to the steps in yellow.

1. The Gesicle Producer 293T Cell Line (Cat. No. 632617) (not included) is transfected with a mix of plasmids that induce the formation of gesicles from the surface of transfected cells, together with a target-specific sgRNA expression plasmid (pGuide-it-sgRNA1) and a plasmid encoding for Cas9.
2. Since they are both co-expressed in the packaging cell, the Cas9 protein associates with the sgRNA, forming Cas9/sgRNA complexes. The Cas9 protein is tagged with an inducible dimerization domain (iDimerize™ technology), as is the membrane-bound CherryPicker™ protein. In the presence of a small molecule ligand (the A/C Heterodimerizer), the Cas9/sgRNA complexes are localized with the CherryPicker protein at the plasma membrane and are actively packaged into the forming gesicles.

3. Gesicles loaded with Cas9/sgRNA complexes and labeled with the CherryPicker protein pinch off from the producer cell line, and then they are collected from the supernatant and concentrated via centrifugation to provide a stock of Cas9/sgRNA gesicles.
4. Harvested gesicles can either be frozen in aliquots for later use or applied immediately to the target cells of interest. The gesicles fuse to the target cells, transiently labeling them with the CherryPicker red fluorescent protein. Due to the lack of A/C Heterodimerizer in the culture media of the target cells, the Cas9/sgRNA complex is released from CherryPicker and translocated to the nucleus to perform site-specific gene editing in a broad range of cell types.

Once the Cas9/sgRNA RNP complexes have been delivered by gesicles 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 3). 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 CRISPR/Cas9 Gesicle 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 hiPS clones. The DEF-CS culture system, a monolayer-based culture system, overcomes 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.

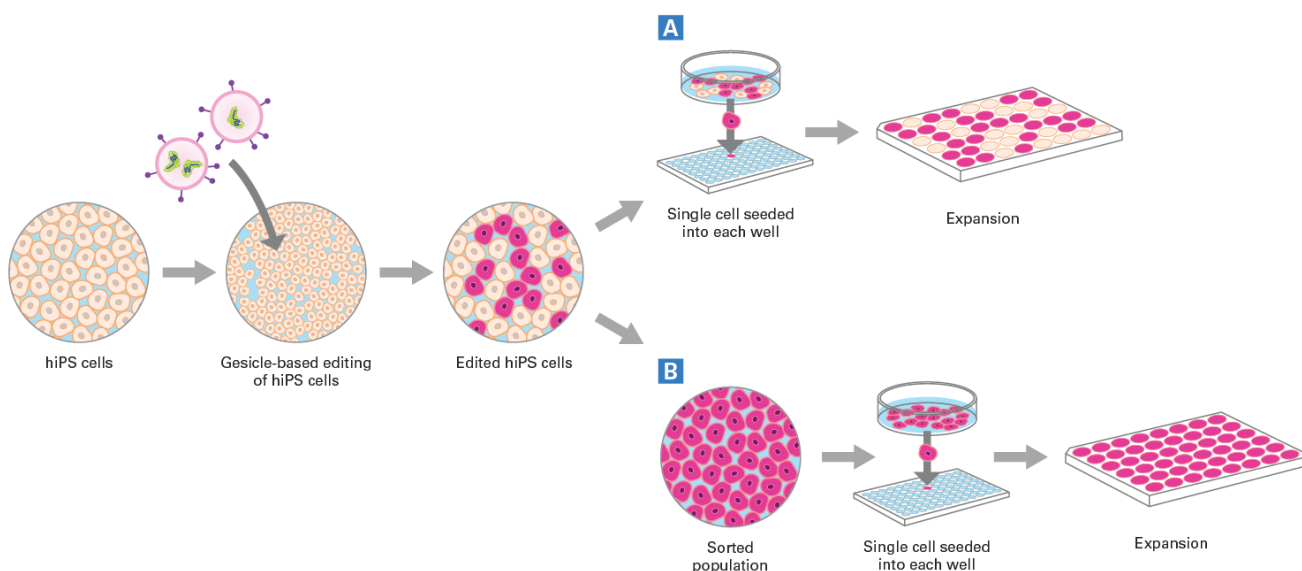


Figure 3. Using DEF-CS technology to generate edited clonal cell lines. Human induced pluripotent stem (hiPS) cells can be cultured, edited, and clonally expanded using the Cellartis iPSC CRISPR/Cas9 Gesicle and Single-Cell Cloning System. Initially, hiPS cells are adapted to the DEF-CS culture system, which maintains cells as a karyotypically stable and pluripotent monolayer. Next, gesicle-based delivery of Cas9 and an sgRNA (together as an RNP) 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 by flow cytometry, then 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 CRISPR/Cas9 Gesicle 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 CRISPR/Cas9 Gesicle and Single-Cell Cloning System (Cat. No. 632642) contains:

- Guide-it™ CRISPR/Cas9 Gesicle Packaging Set (Cat. No. 632616)
 - 10 vials Guide-it CRISPR/Cas9 Gesicle Packaging Mix 1, in a foil pouch (green cap)
 - 10 vials Guide-it CRISPR/Cas9 Gesicle Packaging Mix 2, in a foil pouch (yellow cap)
 - 50 µl A/C Heterodimerizer (0.5 mM)
 - 200 µl Protamine Sulfate (4 mg/ml)
- Guide-it Ligation Components v2 (Cat. No. 632615) (Not sold separately)
 - 50 µl DNA Ligation Mighty Mix*
 - 1.5 ml Guide-it Oligo Annealing Buffer
 - 10 µl Guide-it Control Annealed Oligos v2 (100 fmol/µl)
 - 10 µl Guide-it Sequencing Primer 1 (100 pmol/µl)
 - 1 ml PCR Grade Water

*DNA Ligation Mighty Mix is also available separately (100 rxns, Cat. No. 6023).
- pGuide-it-sgRNA1 Vector (Linear) (Cat. No. 632614) (Not sold separately)
 - 20 µl pGuide-it-sgRNA1 Vector (Linear) (7.5 ng/µl)
- Stellar™ Competent Cells (Cat. No. 636763)
 - 10 tubes Stellar Competent Cells (100 µl/tube)
 - 10 tubes SOC Medium (1 ml/tube)
 - 10 µl pUC19 Vector (0.1 ng/µl)
- 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 CRISPR/Cas9 Gesicle 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:

Cas9/sgRNA Gesicle Production:

- Gesicle Producer 293T Cell Line (Cat. No. 632617)
- Medium for HEK 293 or HEK 293T Cells
Dulbecco's Modified Eagle's Medium (DMEM) with high glucose (4.5 g/L), 4 mM L-glutamine, and sodium bicarbonate (Sigma, Cat. No. D5796 or equivalent)
- 10% Fetal Bovine Serum (FBS) (Takara Bio, Cat. No. 631107 or equivalent)
- 1% Antibiotic Antimycotic Solution (100X), Stabilized (Sigma, Cat. No. A5955 or equivalent)

- Trypsin/EDTA (Sigma, Cat. No. T4049 or equivalent)
- Dulbecco's Phosphate Buffered Saline, Modified, without calcium chloride and magnesium chloride, liquid, sterile-filtered, suitable for cell culture (Sigma, Cat. No. D8537 or equivalent)
- 10-cm tissue culture dishes coated with collagen I or poly-lysine for transfecting Gesicle Producer 293T cells (Corning, 354450 or equivalent)

Single-Cell Cloning:

- 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)

Molecular Biology Supplies

- Target-specific oligos (see Section VI.B)
- TE buffer or molecular biology grade, nuclease-free water
- PCR reaction tubes
- Micropipette tips (with hydrophobic filters)
- 1.5-ml microfuge tubes
- Thermal cycler
- 42°C heat block
- 37°C incubator/shaker
- LB plates containing ampicillin (100 µg/ml)
- Bacteria spreader
- 50 ml conical tubes (Corning Falcon, Cat. No. 352070 or equivalent)
- 20 ml sterile syringes with Luer-Lok tip (BD, Cat. No. 302830 or equivalent)
- 0.45-µm sterile syringe filters (Thermo Fisher Scientific, Cat. No. 723-9945 or equivalent)
- Beckman J2-HS Centrifuge with a JS-7.5 swinging bucket rotor (or equivalent)

B. Recommended

Plasmid Purification Supplies

<u>Cat. No.</u>	<u>Product</u>	<u>Size</u>
740588.50	NucleoSpin Plasmid	50 preps
740410.10	NucleoBond Xtra Midi	10 preps
740414.10	NucleoBond Xtra Maxi	10 preps

Genotype Confirmation Supplies

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

<u>Cat. No.</u>	<u>Product</u>	<u>Size</u>
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 CRISPR/Cas9 Gesicle Packaging Set
 - Store all components at -20°C .
 - Return unused vials of Guide-it CRISPR/Cas9 Gesicle Packaging Mix 1 and Mix 2 to the supplied foil pouches containing the desiccant sachet and store at -20°C .
- Guide-it Ligation Components v2
 - Store at -20°C .
 - Avoid repeated freeze/thaw cycles.
- pGuide-it-sgRNA1 Vector (Linear)
 - Store at -20°C .
 - Spin briefly to recover contents.
 - Avoid repeated freeze/thaw cycles.
- Stellar Competent Cells
 - Store cells at -70°C , and store all other components at -20°C .
- Cellartis iPSC Single-Cell Cloning DEF-CS COAT-1
 - Store at $2-8^{\circ}\text{C}$.
 - Shelf life is specified on the product label.
- Cellartis DEF-CS 500 Basal Medium
 - Store at $2-8^{\circ}\text{C}$.
 - 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 $2-8^{\circ}\text{C}$ 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 CRISPR/Cas9 Gesicle 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 CRISPR/Cas9 Gesicle 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 CRISPR/Cas9 Gesicle and Single-Cell Cloning System provides all materials for generating and isolating edited clonal hiPS cell lines using CRISPR/Cas9 gesicles. The workflow for this system (Figure 4) consists of four main steps:

1. Generation of CRISPR/Cas9 gesicles against your target of interest
2. Application of the CRISPR/Cas9 gesicles to hiPS cells
3. Isolation of gesicle-treated single cells by FACS or limiting dilution
4. Expansion of the clonal cell lines

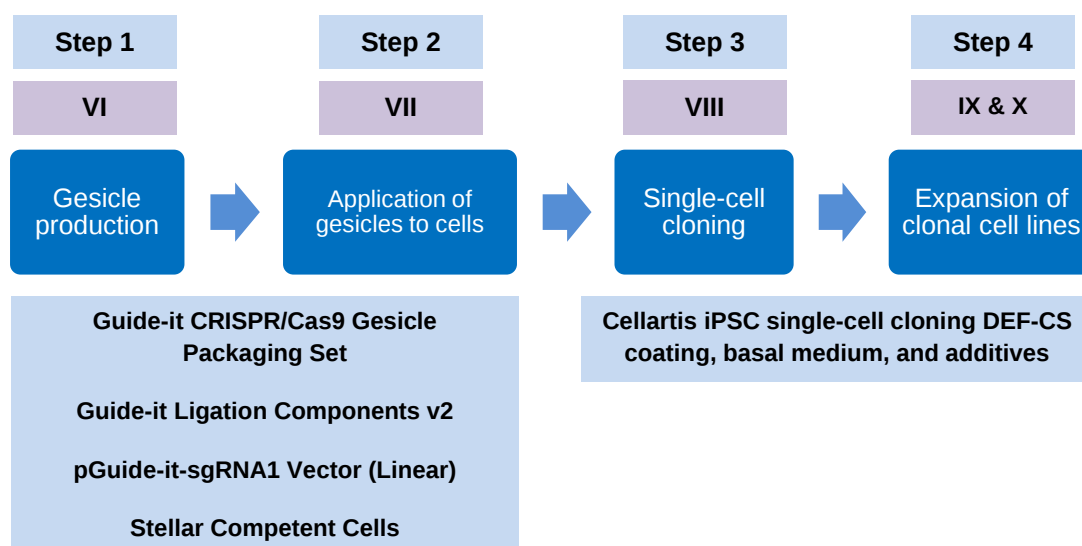


Figure 4. Workflow for gene editing of hiPS cells using components of the Cellartis iPSC CRISPR/Cas9 Gesicle and Single-Cell Cloning System. The purple boxes indicate the sections containing the relevant protocols.

VI. Producing CRISPR/Cas9 Gesicles

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 at non-targeted regions. For many applications, it is advisable to design and test several different sgRNAs against the same genomic target region.

A. Protocol Overview

Figure 4 shows the 4-step workflow for gesicle production:

1. sgRNAs can be designed manually or using online tools. Please visit our website to view a short video about sgRNA design (http://www.takarabio.com/US/Products/Genome_Editing/CRISPR_Cas9/Resources/Designing_sgRNA) and to review an extensive list of web-based sgRNA design tools (http://www.takarabio.com/US/Products/Genome_Editing/CRISPR_Cas9/Resources/Online_tools_for_guide_RNA_design).

2. Oligos corresponding to the target-specific sgRNA designed above are annealed to form a DNA duplex and then cloned into the provided linearized delivery plasmid, pGuide-it-sgRNA1.
3. The cloned plasmid is diluted with dH₂O and added to the gesicle packaging mixes. Following a 10-min incubation, the mix is applied to producer cells in the presence of the A/C Heterodimerizer ligand.
4. 48–72 hr later, gesicles containing active Cas9/sgRNA complexes are collected from the medium and concentrated via overnight centrifugation.

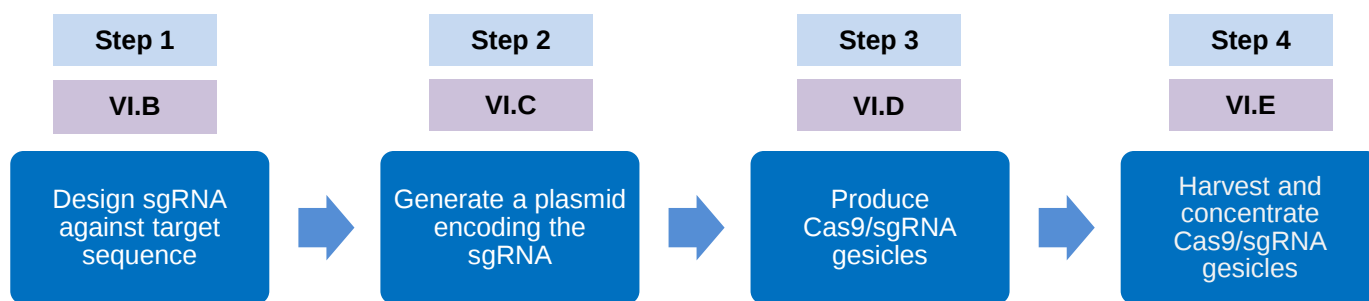


Figure 5. Workflow for CRISPR/Cas9 gesicle production. The purple boxes indicate the sections containing the relevant protocols.

B. Designing sgRNAs Against the Target Sequence

Identifying Candidate sgRNAs Against Your Target Sequence

The first step of a CRISPR/Cas9 experiment is to design candidate sgRNAs that target your gene of interest. Cas9 from *S. pyogenes* cleaves DNA sequences located 5' to the sequence NGG (also called the **Proto-spacer Adjacent Motif**, or PAM). Given a predetermined target sequence, sgRNAs can be designed manually or by using an online tool. Here is a short tutorial on sgRNA design:

<https://www.takarabio.com/learning-centers/gene-function/gene-editing/gene-editing-tools-and-information/how-to-design-sgrna-sequences>

Designing Candidate sgRNAs

- Here is a list of helpful online tools for finding sgRNAs against your target sequence: http://www.takarabio.com/US/Products/Genome_Editing/CRISPR_Cas9/Resources/Online_tools_for_guide_RNA_design
- To design your sgRNAs manually (Figure 6), first identify PAMs in your gene of interest. Then simply count 20 nucleotides upstream of, or 5' to, the PAM. These nucleotides (the target-specific fragment of your sgRNA) will be cloned in front of the sgRNA scaffold to construct the full sgRNA sequence. The PAM itself is not included in your sgRNA. If your PAM is found on the antisense strand (Panel B of Figure 6), be sure to count your 20 nucleotides in a direction that is still 5' to the PAM and remember to read the final sequence in the 5' to 3' direction.

A
Step 1. Identify the PAM (NGG) sequence in your target gene

```

5' TTG TTA TCC GCT GTG AAT CGG ATC CAG CTG ACT GGA ATT TGA CTG TAA 3'
3' AAC AAT AGG CGA CAC TTA GCC TAG GTC GAC TGA CCT TAA ACT GAC ATT 5'

```

PAM

Step 2. Determine the sgRNA sequence by counting 20 bp upstream of the PAM

```

5' TTG TTA TCC GCT GTG AAT CGG ATC CAG CTG ACT GGA ATT TGA CTG TAA 3'
3' AAC AAT AGG CGA CAC TTA GCC TAG GTC GAC TGA CCT TAA ACT GAC ATT 5'

```

PAM

sgRNA sequence: 5' **GTG AAT CGG ATC CAG CTG AC** 3'

B
Step 1. Identify the PAM (NGG) sequence in your target gene

```

5' TTG TTA TCC GCT GTG AAT CGG ATC CAG CTC ACT GGA ATT TGA CTG TAA 3'
3' AAC AAT AGG CGA CAC TTA GCC TAG GTC GAG TGA CCT TAA ACT GAC ATT 5'

```

PAM

Step 2. Determine the sgRNA sequence by counting 20 bp upstream of the PAM

```

5' TTG TTA TCC GCT GTG AAT CGG ATC CAG CTC ACT GGA ATT TGA CTG TAA 3'
3' AAC AAT AGG GAG CAC TTA GCC TAG GTC GAG TGA CCT TAA ACT GAC ATT 5'

```

PAM

sgRNA sequence: 5' **GAG CTG GAT CCG ATT CAC AG** 3'

Figure 6. Workflow of sgRNA manual design. The PAM may be located on the sense strand (**Panel A**) or the antisense strand (**Panel B**).

Whether you use an online tool or design the sequences of potential sgRNAs manually, you will obtain many candidate sgRNAs, because PAM sequences such as NGG occur every 8–12 base pairs in the human genome. In order to narrow the list of potential sgRNAs, you can evaluate the efficiency of each sgRNA and its potential off-target effects.

Testing Candidate sgRNA Efficiency

Effective sgRNAs for gesicle-mediated Cas9/sgRNA editing (i.e., those that result in high levels of gene knockout) contain a G in position 1 and an A/T in position 17 (counting down from the PAM sequence at position 21). **In our experience, only sgRNAs that fulfill both requirements are effective for editing via gesicle-mediated delivery (Figure 7).**

Optimal sgRNAs	
Target	sgRNA sequence
<i>CD81</i>	GCAGCCCTCCACTCCC A TGG
<i>CXCR4</i>	GGGCAATGGATTGGTC A TCC
<i>EMX1</i>	GAGTCCGAGCAGAAGA A GAA
<i>AcGFP1</i>	GTGAATCGCATCGAGC T GAC
<i>ZsGreen1</i>	GACCATGAAGTACCGC A TGG

Figure 7. Examples of optimal sgRNAs targeting common genes. Each of these sequences contains a G in position 1 and an A or T in position 17 (in red).

As an additional check, the Guide-it sgRNA Screening Kit (Cat. No. 632639) enables you to test the efficacy of different sgRNAs *in vitro* prior to using them in studies involving Cas9-mediated gene editing in cells.

Minimizing Off-Target Effects

- To minimize off-target cleavage, the entire target sequence including the PAM site should contain at least three base mismatches with any other, non-targeted genomic sequence (Hsu et al. 2013; Mali et al. 2013); off-target cleavage is especially low if the mismatches are in, or adjacent to, the PAM site.
- Most online tools for sgRNA design also predict the off-target sequences related to each sgRNA. We recommend choosing sgRNAs with minimal predicted off-target effects.

Designing and Ordering Oligos

In order to clone your sgRNA into the provided prelinearized pGuide-it-sgRNA1 Vector, you must order or synthesize a pair of oligos corresponding to the target-specific sequence of the sgRNA of interest, determined in Section VI.B above, including short 5' overhang sequences complementary to the vector backbone (Figure 8). Only the 20-nucleotide sequence corresponding to the target needs to be cloned into the linearized pGuide-it-sgRNA1 vector; the vector already contains the sgRNA scaffold sequence.

1. The sense oligo (Oligo 1) corresponds to the 20-nucleotide sgRNA sequence chosen in Section VI.B, plus the 5' overhang sequence, cacc. Do **not** include the PAM sequence.

Oligo 1: 5' -cacc XXX XXX XXX XXX XXX XXX XX-3'

2. The antisense oligo (Oligo 2) is the complementary sequence of Oligo 1, plus the 5' overhang sequence, aaac.

Oligo 2: 5' -aaac YY YYY YYY YYY YYY YYY YYY-3'

NOTE: If the first base at the 5' end of the sgRNA sequence (Oligo 1) is not a guanine, it is necessary to add an extra guanine at this end, as shown below:

- Oligo 1: 5' -cacc **G**XXX XXX XXX XXX XXX XXX XX-3'
- Oligo 2: 5' -aaac YY YYY YYY YYY YYY YYY **YYC**-3'

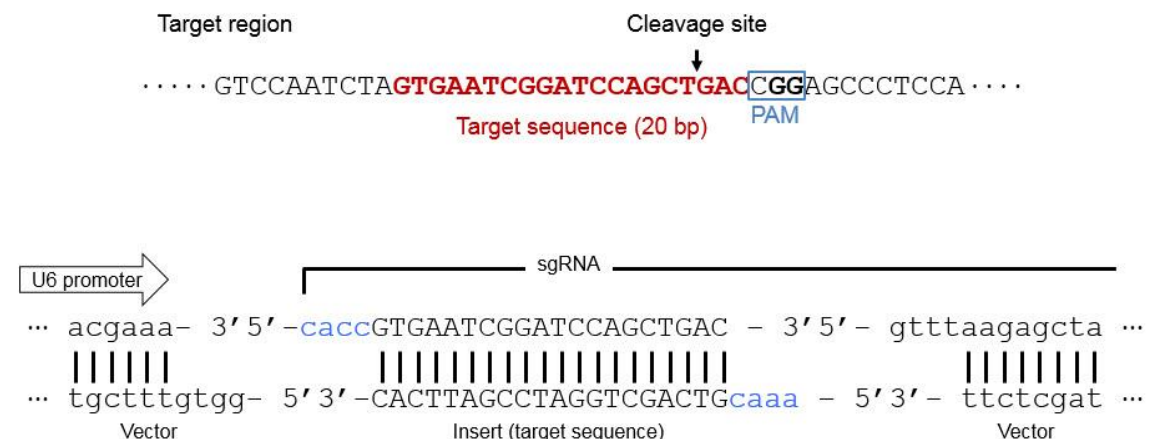


Figure 8. Cloning a target sequence. This example corresponds to the sequence of the Guide-it Control Annealed Oligos v2 included in the kit. The target region (marked in red) is shown in the top half of the figure. Both oligos contain the additional 5' overhang sequences required for cloning into the pGuide-it-sgRNA1 vector, marked in blue (bottom half of figure).

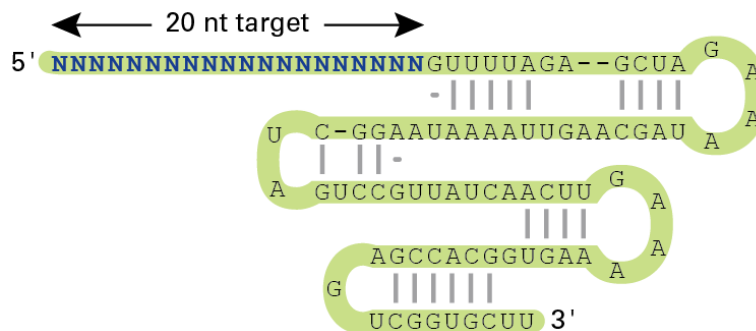
C. Generating a Plasmid Encoding the sgRNA

Please read the protocol completely before starting. Successful results depend on understanding and performing the following steps correctly.

It is *essential* to use the pGuide-it-sgRNA1 Vector for cloning your target oligos (designed according to Section VI.B above), since using other commonly used guide RNA vectors will not result in formation of effective Cas9/sgRNA RNP gesicles. We have modified the sgRNA scaffold in the pGuide-it-sgRNA1

Vector to improve the Cas9/sgRNA interaction, ensuring high editing efficiency (Figure 9). Due to the nature of gesicle production, which requires the Cas9/sgRNA RNP complex to form before it is packaged into the gesicles, it is essential to maximize the efficient binding of the sgRNA to the Cas9 endonuclease by using this modified scaffold.

Traditional scaffold



Optimized scaffold

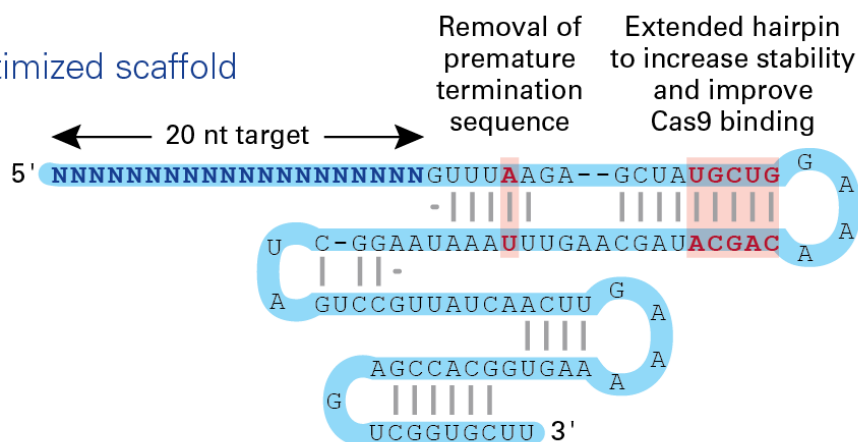


Figure 9. pGuide-it-sgRNA1 includes an improved sgRNA scaffold design for increased editing efficiency. It is *essential* to use this optimized scaffold design for production of functional Cas9/sgRNA gesicles.

1. Annealing Oligos

1. Resuspend each target oligo completely in TE buffer or molecular biology-grade, nuclease-free water such that the concentration is 100 μ M.
2. Mix the oligos in a 200- μ l PCR tube as follows:

1 μ l	Oligo 1 (100 μ M)
1 μ l	Oligo 2 (100 μ M)
8 μ l	Guide-it Oligo Annealing Buffer
10 μ l	Total Volume

NOTE: The concentration of each of the oligos is now 10 μ M.

3. Anneal the oligos by using a thermal cycler to denature them at 95°C, and then reanneal the oligos by slowly reducing the temperature.

Program your thermal cycler with the following cycling conditions:

95°C	2 min
Slope from 95°C to 25°C	15 min
25°C	hold

- Mix 1 µl of the annealed oligo solution with an additional 99 µl of Guide-it Oligo Annealing Buffer to make a 100 nM (fmol/µl) solution.
- Store the annealed oligos at –20°C until use.

2. Cloning the sgRNA Targeting Sequence Into pGuide-it-sgRNA1

- Thaw the necessary reagents at room temperature and set up the reaction as follows:

2 µl	pGuide-it-sgRNA1 Vector (Linear) (7.5 ng/µl)*
3 µl	Target-specific annealed oligos (100 fmol/µl; from Section VI.C.1, Step 5) (For a positive control reaction, use the included Guide-it Control Annealed Oligos v2 (100 fmol/µl))
5 µl	DNA Ligation Mighty Mix
10 µl	Total volume

*Use the pGuide-it-sgRNA1 Vector to clone your target oligos. Other commonly used guide RNA vectors will not result in formation of effective Cas9/sgRNA complexes (see Figure 9).

- Incubate the reaction mix at 16°C for 30 min.
- Meanwhile, thaw one vial of Stellar Competent Cells on ice.
- Add the entire 10-µl ligation mixture to the competent cells and mix gently by tapping.
- Allow the mixture to stand on ice for 30 min.
- Heat shock the cells at 42°C for 45 sec and immediately place on ice. Incubate for 2 min.
- Add 1 ml of SOC medium and incubate at 37°C for 1 hr with vigorous shaking.
- Plate an appropriate amount of the culture on pre-warmed (37°C) LB plates containing ampicillin (final concentration 100 µg/ml).
- Incubate the plates at 37°C overnight.

3. Isolate and Analyze Plasmids

- Pick single colonies and inoculate into an appropriate amount of LB medium containing ampicillin (final concentration 100 µg/ml).
- Incubate with shaking overnight at 37°C.
- Purify plasmid DNA from bacteria. We highly recommend NucleoSpin Plasmid (Cat. No. 740588.50) for rapid, high-yield, and high-purity purification. For transfection-grade plasmid preparation, use NucleoBond Xtra Midi (Cat. No. 740410.10) or NucleoBond Xtra Maxi (Cat. No. 740414.10).
- Perform sequencing analysis using the included Guide-it Sequencing Primer 1 and your preferred sequencing protocol.

D. Produce CRISPR/Cas9 Gesicles

Please read the protocol completely before starting. Successful results depend on understanding and performing the following steps correctly.

Protocol Overview

The following steps are required to create target-specific gesicles containing the Cas9/sgRNA complex of interest (Figure 10).

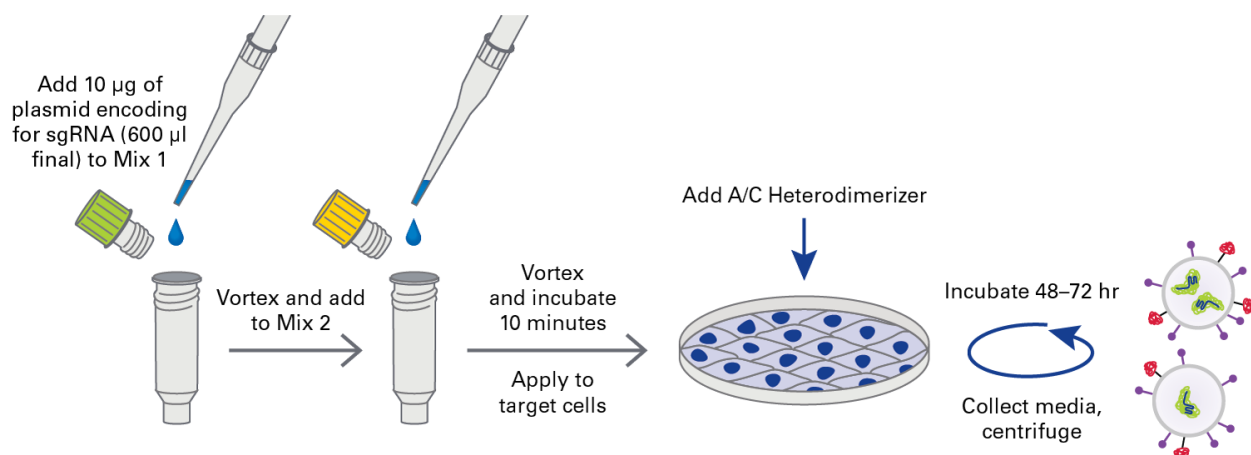


Figure 10. Overview of the CRISPR/Cas9 gesicle production protocol. After cloning the target oligos designed against your gene of interest into the pGuide-it-sgRNA vector, pipette the purified plasmid containing your sgRNA (diluted to 600 µl) into the Guide-it CRISPR/Cas9 Gesicle Packaging Mix 1, vortex, and transfer to Guide-it CRISPR/Cas9 Gesicle Packaging Mix 2. Incubate and apply to Gesicle Producer 293T Cells in a 10-cm dish. Add the A/C Heterodimerizer ligand to the cell media in order to drive active loading of the Cas9/sgRNA complex into the gesicles. 48–72 hr later, centrifuge the cell media overnight at 8,000g. Decant the supernatant and resuspend the pellet (gesicles containing Cas9/sgRNA complexes). The concentrated stock of Cas9/sgRNA Gesicles is now ready to be added to target cells.

Producing Cas9/sgRNA Gesicles

The following steps should be performed in a sterile tissue culture hood. This protocol can be completed in 3–4 days.

General Considerations for Transfection of the Gesicle Producer 293T Cell Line

- We strongly recommend using the Gesicle Producer 293T Cell Line (sold separately; Cat. No. 632617) to produce gesicles containing Cas9/sgRNA complexes with high efficiency.
- Transfections should be performed using 10-cm tissue culture dishes coated with collagen I (see Section III.A).
- At the time of transfection, cells should be 40–50% confluent, as shown in Figure 11 and described in Step 2 of the protocol below.

NOTE: In order to maximize the level of Cas9/sgRNA gesicle production, it is critical to perform a highly efficient transfection.

Producing Gesicles Containing Cas9/sgRNA Complexes Using Gesicle Producer 293T Cells

1. Approximately 24 hr before transfection, seed 4.5×10^6 Gesicle Producer 293T cells into a 10-cm plate coated with collagen I, in 10 ml of growth medium. Make sure to plate the cells evenly, and incubate them overnight at 37°C under 5% CO₂.

- At the time of transfection, cells should be 40–50% confluent, as shown in Figure 11.

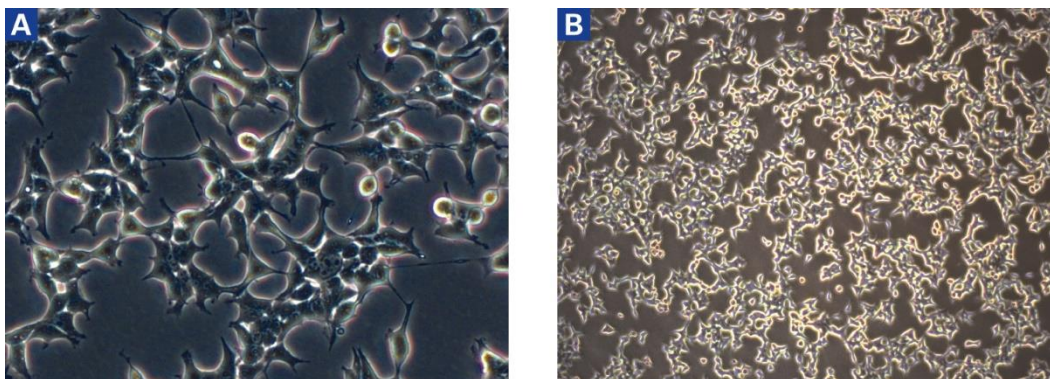


Figure 11. Optimal density of Gesicle Producer 293T cells at the time of transfection. Cells are shown at 20X magnification (**Panel A**) and 5X magnification (**Panel B**).

- Just before transfection, remove 2.5 ml of medium from the 10-cm plate containing the cells and discard it, leaving a total cell culture volume of approximately 7.5 ml.
- Add 1.25 μ l of the A/C Heterodimerizer to the 10-cm plate. Rock the plate gently back and forth to mix.

NOTE: Do not add more than the recommended amount of A/C Heterodimerizer. Its final concentration has been optimized to maximize the number of Cas9/sgRNA complexes packaged inside the gesicles. The number of Cas9/sgRNA complexes will decrease if additional A/C Heterodimerizer ligand is used.

- In a sterile microfuge tube, dilute 10 μ g of the pGuide-it-sgRNA1 plasmid encoding your sgRNA of choice (from Section VI.C.1, Step 5) with sterile water to a final volume of 600 μ l. Mix thoroughly by vortexing.

NOTE: Always dilute your DNA in water prior to adding it to a Guide-it CRISPR/Cas9 Gesicle Packaging Mix. Do not add water and DNA separately; undiluted DNA should **not** be mixed with the Guide-it CRISPR/Cas9 Packaging Mix.

- Add the 600 μ l of diluted pGuide-it-sgRNA1 plasmid to a tube of Guide-it CRISPR/Cas9 Gesicle Packaging Mix 1 (green cap), replace the cap, and vortex well at a high speed for 20 sec. The pellet should dissolve completely.

NOTE: In some cases, some insoluble material may be visible after vortexing. This material does not have a negative effect on transfection efficiency or gesicle production yields.

- Transfer the 600 μ l of Mix 1 to a tube of Guide-it CRISPR/Cas9 Gesicle Packaging Mix 2 (yellow cap), replace the cap, and vortex well at a high speed for 20 sec. The pellet should dissolve completely.

NOTE: In some cases, some insoluble material may be visible after vortexing. This material does not have a negative effect on transfection efficiency or gesicle production yields.

- Incubate the tube for 10 min at room temperature. After the 10-min incubation, centrifuge the tube for 2 sec to collect the complete contents of the tube.

NOTE: Sample tubes can be inserted into 1.5-ml microfuge tubes for centrifugation.

9. Add the entire 600 µl of solution from Mix 2 dropwise to the cell culture (Step 4). Distribute evenly across the culture dish. Rock the plate gently back and forth to mix.

NOTE: It is normal for the medium to change color slightly upon addition of the Guide-it CRISPR/Cas9 Gesicle Packaging Mix solution.

10. Incubate the cells at 37°C under 5% CO₂.

NOTE: A 4-hr incubation with the Guide-it CRISPR/Cas9 Gesicle Packaging Mix is sufficient for optimal transfection. Incubation may be continued overnight for convenience.

11. After incubating the cells (from 4 hr to overnight), add an additional 7.5 ml of fresh complete growth medium containing 1.25 µl of A/C Heterodimerizer to the 10-cm plate (the final cell culture volume will be 15 ml).

NOTE: Do not add more than the recommended amount of A/C Heterodimerizer. Its final concentration has been optimized to maximize the amount of Cas9/sgRNA loaded inside each gesicle. The amount of Cas9/sgRNA in each gesicle will decrease if additional ligand is used.

12. Rock the plate gently back and forth to mix, and incubate at 37°C under 5% CO₂ for an additional 48–72 hr.

13. After 24–36 hr, the cells can be visualized under a microscope to determine if transfection was successful. The CRISPR/Cas9 Guide-it Gesicle Packaging Mix contains a plasmid encoding the CherryPicker red fluorescent protein, and its expression can be used to identify successful transfections (Figure 12):

- In successful transfections, the cells will display higher levels of red fluorescence and cellular fusion (Figure 12, Panels A–B).
- If there is low transfection efficiency, the cells will display only dim fluorescence, with high confluency and no visible cellular fusion (Figure 12, Panels C–D).

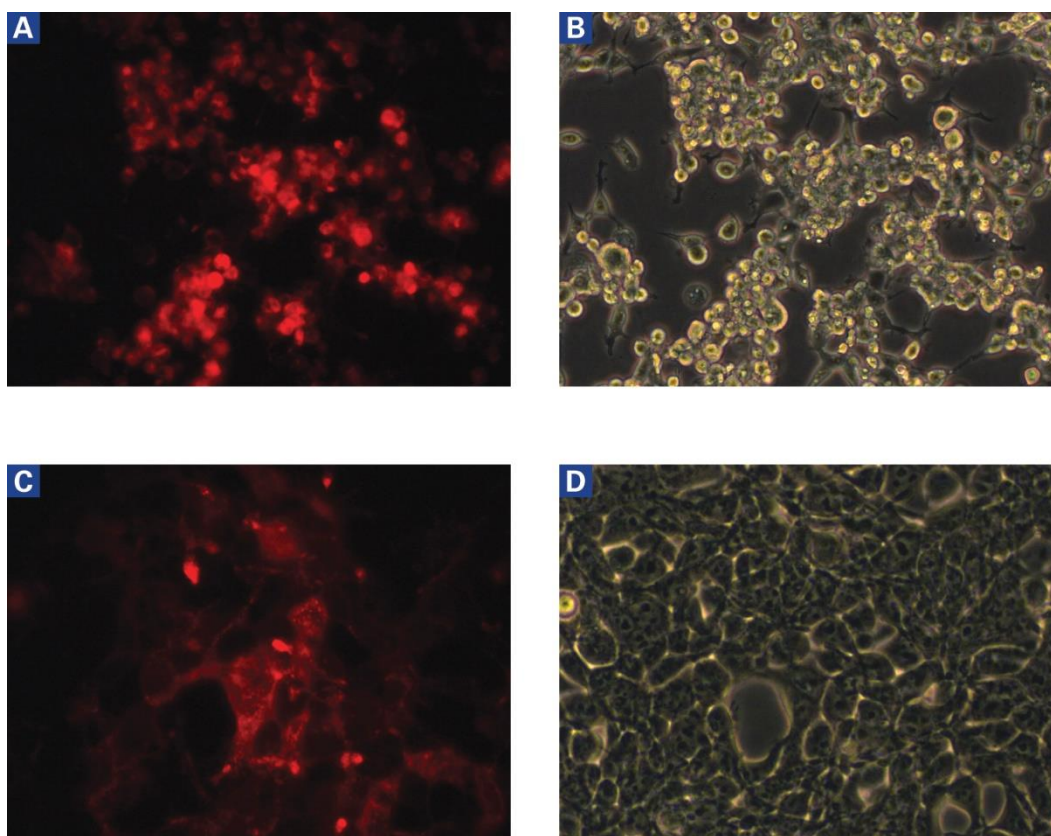


Figure 12. Gesicle Producer 293T cells after transfection. These images (20X magnification) show red fluorescence (left) and phase microscopy (right) results for successfully transfected Gesicle Producer 293T cells (**Panels A and B**) and unsuccessfully transfected Gesicle Producer 293T (**Panels C and D**).

E. Harvest and Concentrate Cas9/sgRNA Gesicles

Collect gesicles from the cell media 48 hr after transfection. If you prefer, you can wait up to 72 hr to collect the gesicles.

1. 48 hr after transfection, centrifuge the cell culture medium briefly (500g for 10 min) and filter through a 0.45- μ m syringe sterile filter made of cellulose acetate or polysulfonate to remove cellular debris.

NOTE: Do not use a nitrocellulose filter.

2. To concentrate gesicles containing the Cas9/sgRNA complexes, transfer the gesicle filtrate to a 50-ml tube that can withstand $\sim 8,000g$ (Corning Falcon Cat. No. 352070 or equivalent). Centrifuge the filtered samples at $\sim 8,000g$ at 4°C for 16 hr to overnight (e.g., 6,500 rpm in a Beckman J2-HS Centrifuge with a JS-7.5 swinging bucket rotor).

IMPORTANT: Do not use a fixed angle rotor. Because gesicles are not visible, it is important to collect them into the smallest possible area at the very bottom of your tube, which requires using a swinging bucket rotor in order to collect of all gesicles in the sample.

3. After centrifugation, gently pour the supernatant (which does NOT contain the gesicles) into another 50-ml tube. Do not aspirate to remove the supernatant, because the gesicle pellet is easily dislodged. Keep the tube tilted while decanting, and use a pipette to remove the medium that remains on the

upper rim of the tube. Do not remove the residual medium (~60 µl) at the bottom of the original tube—it contains the gesicles.

NOTE: The pellet of gesicles is not visible to the naked eye.

4. Add 60 µl of PBS (without calcium or magnesium) to the bottom of the original, centrifuged tube and resuspend the pellet containing the Cas9/sgRNA gesicles by pipetting up and down. Since there is likely to be some residual medium at the bottom of the tube, the total volume of the resuspended pellet will be close to 120 µl.

NOTE: The pellet at the bottom of the tube is usually not visible.

5. Measure the total volume of the suspension and, if necessary, add a 1:1 mixture of 293T cell media:PBS to a final volume of 120 µl. If the volume is higher than 120 µl, no further dilution is necessary.

NOTE: It is important to use a mixture of media and PBS, not PBS alone.

6. Agitate the gesicles on a rocking platform for 2 hr at 4°C.
7. Divide the Cas9/sgRNA gesicle suspension into 30-µl aliquots in separate tubes. Store the Cas9/sgRNA gesicles at –70°C. These gesicles can be stored for more than 1 year.

NOTE: The presence of Cas9 in the gesicles can be verified by Western blot using the Guide-it Cas9 Polyclonal Antibody (Cat. Nos. 632606 & 632607). 2–5 µl of the gesicle suspension is sufficient for Western blot detection using the Guide-it Cas9 Polyclonal Antibody.

VII. Editing hiPS Cells by Gesicle Delivery of Cas9/sgRNA RNPs

IMPORTANT:

- Please read the protocol completely before starting. Successful results depend on understanding and performing all the following steps correctly
- This protocol has been optimized using the Cellartis Human iPSC Cell Line 18 (Cat. No. Y00305)
- The protocol has also been optimized using 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.A).
- 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 CRISPR/Cas9 Gesicle 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

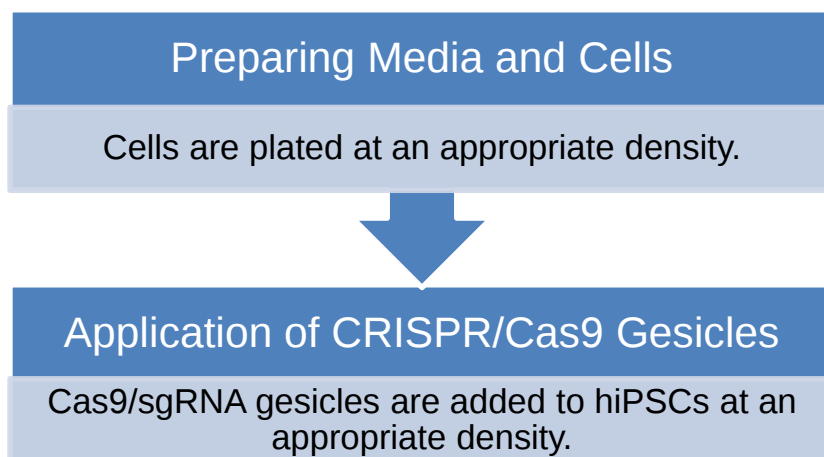


Figure 13. Workflow for gesicle-based delivery of Cas9/sgRNA complexes to hiPSCs.

B. Treating hiPS Cells with Cas9/sgRNA Gesicles

NOTE: If cells have not previously been adapted to growing in DEF-CS, it is strongly recommended to transition cells by passaging five times in DEF-CS prior to performing the editing experiments.

To achieve a correct cell density for the gesicle treatment, cells need to be cultured for 48 hr in a 6-well plate and then transferred to a 48-well plate for gesicle treatment.

Day 1: Plating Cells in the 6-Well Plate for Culture Prior to Gesicle Treatment

Plate hiPS cells in a coated 6-well plate and grow for 48 hr. One 6-well plate will provide enough cells for at least four samples.

Coating the 6-Well Plate

1. Calculate the amount of diluted coating solution required depending on the number of wells to be used (950 µl of diluted DEF-CS COAT-1 solution per well; see Table I).

Table I. Preparation of coating solution for the 6-well plate.

Number of wells (6-well plate)	Volume of diluted coating solution (μl)	Volume of COAT-1 (μl)	Volume of D-PBS +/- (μl)
1	950	47.5	902.5
2	1,900	95	1,805
n	90 x n	(950 x n)/20	(950 x n) – (volume of COAT-1)

2. Dilute the required volume of Cellartis DEF-CS COAT-1 in D-PBS +/- before use (in a 1:20 dilution).
3. Mix the diluted Cellartis DEF-CS COAT-1 solution gently and thoroughly by pipetting up and down.
4. Add the diluted Cellartis DEF-CS COAT-1 solution to a 6-well plate (use 950 μl/well), making sure the entire surface of each well is covered.
5. Place the cell culture plate in an incubator at 37°C ± 1°C, 5% CO₂, and >90% humidity for a minimum of 20 min.
6. Aspirate the Cellartis DEF-CS COAT-1 solution from the cell culture plate just before use.

Preparing DEF-CS Medium for Dissociation and Plating

In this step, you will need medium from the Cellartis DEF-CS 500 Culture System (Cat. No. Y30010) to neutralize the dissociation reagent and passage the cells in the 6-well plate. Prepare the DEF-CS medium using additives from the Cellartis DEF-CS 500 Culture System according to Table II.

Table II. Preparation of medium and dissociation enzyme for culture during gesicle treatment.

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

Cell plating	
Plate type	DEF-CS medium (ml per well)
6 wells	2.5
48 wells	0.3

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. 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.

Plating Cells in the 6-Well Plate

1. Collect the cells from the cell culture flask. First, aspirate the medium from the cell culture flask and wash the cell layer once with D-PBS –/–.
2. Add TrypLE Select to the cell culture flasks (as described in Table II) 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 (as described in Table II) and pipet up and down several times to ensure a single-cell suspension. (The cells will aggregate if left too long in TrypLE Select.)
4. Count cells with the hemocytometer.
5. Plate 4.0×10^4 cells per well in the previously coated 6-well plate.
6. Place the plate in an incubator at $37^\circ\text{C} \pm 1^\circ\text{C}$, 5% CO_2 , and >90% humidity and leave the plate undisturbed for 48 hr.

Day 3: Plating Cells in the 48-Well Plate for Culture During Gesicle Treatment

Plate the cells in the 48-well plate, in which they will be treated with gesicles on Day 4. When the treatment is applied, the cells should be 40–50% confluent and evenly distributed in the well (Figure 14).

Preparing the 48-Well Plate

1. Dilute the required volume of 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 III for guidance).

Table III. Preparation of coating solution for the 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	$90 \times n$	$(90 \times n)/20$	$(90 \times n) - (\text{volume of COAT-1})$

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 $37^\circ\text{C} \pm 1^\circ\text{C}$, 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.

Preparing DEF-CS Medium for Dissociation and Plating

In this step, you will need medium from the Cellartis DEF-CS 500 Culture System (Cat. No. Y30010) to neutralize the dissociation reagent and passage the cells into the 48-well plate. 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. Prepare fresh medium on the day of intended use and warm it to $37^\circ\text{C} \pm 1^\circ\text{C}$ immediately before use. Discard any leftover warmed medium.

Plating Cells

1. Collect the cells from the 6-well plate. First, aspirate the medium from the 6-well plate and wash the cell layer once with D-PBS $-/-$.
2. Add 190 μl of TrypLE Select to each well of the 6-well plate (as described in Table II) 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 plate firmly but gently.
3. Resuspend the cells in DEF-CS medium (as described in Table II) and pipet up and down several times to ensure a single-cell suspension. (The cells will aggregate if left too long in TrypLE Select.)

4. Count cells with a hemocytometer.
5. Plate cells in a 48-well plate at different densities: 3.0×10^4 , 2.5×10^4 , 2.0×10^4 , 1.5×10^4 , and 1.0×10^4 cells per well.
6. Place the plate in an incubator at $37^\circ\text{C} \pm 1^\circ\text{C}$, 5% CO_2 , and >90% humidity, and leave the plate undisturbed overnight.

Days 4 and 5: Applying Cas9/sgRNA Gesicles to hiPS Cells in the 48-Well Plate

Perform the following steps under sterile conditions. The quantities specified here are for a 48-well plate (prepared on Day 3 above). We advise using 30 μl of gesicles per well in a 48-well plate. If you would like to use 12- or 6-well plates, increase the amount of Cas9/sgRNA gesicles accordingly.

Preparing DEF-CS Media for Gesicle Incubation and Recovery

In this step, use components from the Cellartis DEF-CS 500 Culture System (Cat. No. Y30010) in two media preparations:

- Day 4: “DEF-CS gesicle incubation medium,” 300 μl /well, containing DEF-CS GF-1 (dilute 1:333), GF-2 (dilute 1:1,000), and protamine sulfate to a final concentration of 4 $\mu\text{g}/\text{ml}$.
- Day 5: “DEF-CS recovery medium,” 300 μl /well, which contains DEF-CS GF-1 (dilute 1:333) and GF-2 (dilute 1:1,000) without protamine sulfate.

Add supplements and protamine sulfate to Cellartis DEF-CS 500 Basal Medium. Prepare fresh medium on the day of intended use according to the protocol below, and warm it to $37^\circ\text{C} \pm 1^\circ\text{C}$ immediately before use. Discard any leftover warmed medium.

1. Thaw the Cas9/sgRNA gesicles (from Section VI.E, Step 7) on ice for 30 min before adding them to the target cells.

NOTE: Occasionally, small aggregates have been observed in the gesicle suspension. The presence of these aggregates will not affect their function. If you notice such aggregates, pipette the thawed gesicle suspension up and down several times and perform a quick spin (e.g. 400g for 15 sec) before adding the gesicles to your cells.

2. Check the different dilutions plated the day before under a microscope. Confirm that the cells plated on the previous day are approximately 40–50% confluent (Figure 14).

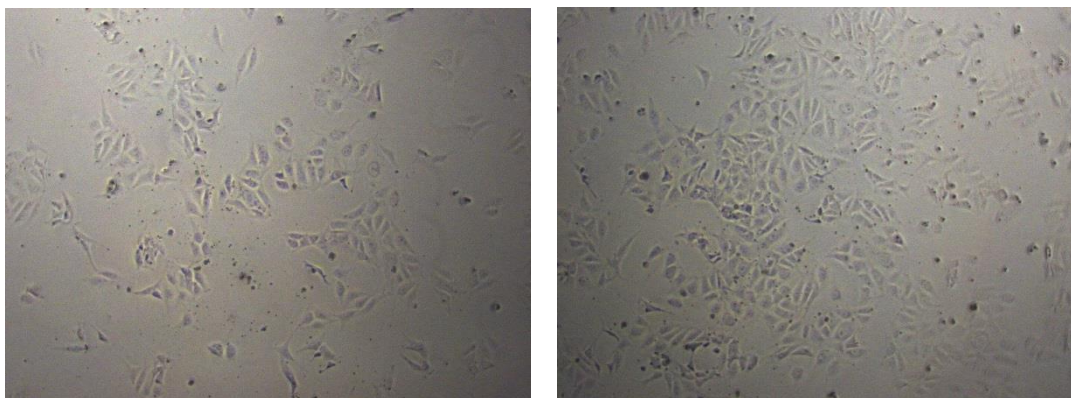


Figure 14. Optimal density of hiPS cells before Cas9/sgRNA gesicle treatment. The left photo shows Cellartis Human iPS Cell Line 18 cells plated at an insufficient density (5X magnification) and the right photo shows the correct density suitable for addition of Cas9/sgRNA gesicles (5X magnification).

3. Prepare DEF-CS gesicle incubation medium and warm to $37^\circ\text{C} \pm 1^\circ\text{C}$ immediately before use.

4. Replace the media in the wells that will be treated with gesicles with 300 µl of DEF-CS gesicle incubation medium.
5. Incubate for 2 hr in an incubator at 37°C ± 1°C, 5% CO₂, and >90% humidity.
6. Add 30 µl of the thawed Cas9/sgRNA gesicles from Step 1 to each well containing DEF-CS gesicle incubation medium.
7. Centrifuge the 48-well plate at 1,150g for 30 min at room temperature. After centrifugation, cells can be visualized under the microscope to detect the red fluorescence signal, which originates from the CherryPicker fluorescent marker on the gesicle surface (Figure 15).
8. After centrifugation, transfer the plate back to a tissue culture incubator and incubate overnight at 37°C.
9. The next day, replace the DEF-CS gesicle incubation medium with fresh, pre-warmed DEF-CS recovery medium and continue incubating the cell culture.
10. When the cells have reached confluency, expand them into a 24-well plate following instructions in the Cellartis DEF-CS 500 Culture System User Manual.

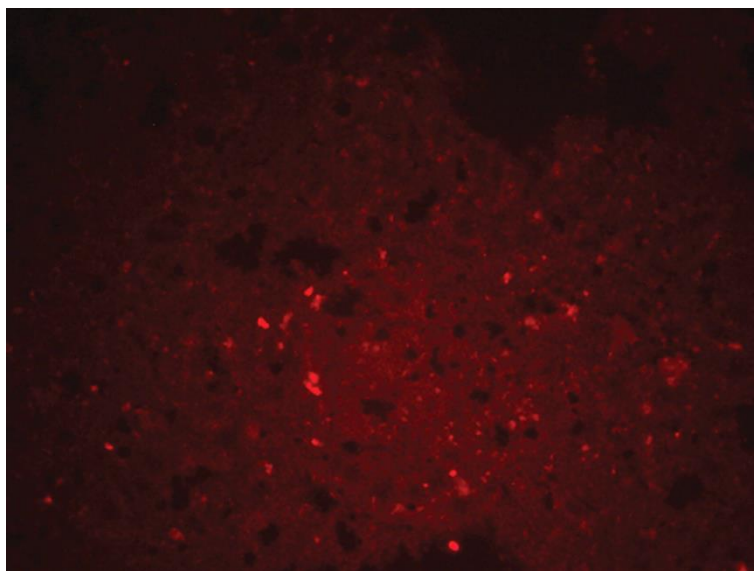


Figure 15. Positive fluorescent signal from gesicle-treated hiPS cells. Fluorescence microscopy image of hiPS cells after addition of Cas9/sgRNA gesicles. Immediately after the centrifugation step, the red signal has spread throughout the well and small, brighter aggregates can be detected (10X magnification).

VIII. Single-Cell Cloning of Gesicle-Treated Cells

A. Protocol Overview

After a recovery period of at least five days, cells can be seeded singly and expanded into 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 CRISPR/Cas9 Gesicle and Single-Cell Cloning System. However, during Protocols VIII–X, use components of the Cellartis iPSC CRISPR/Cas9 Gesicle 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 CRISPR/Cas9 Gesicle 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		100	100
	3		100*	200
	5		150	200
	(6)	GF-1, GF-2, and GF-3	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 $0.8\text{--}1.5 \times 10^5$ cells/cm² (Figure 16).

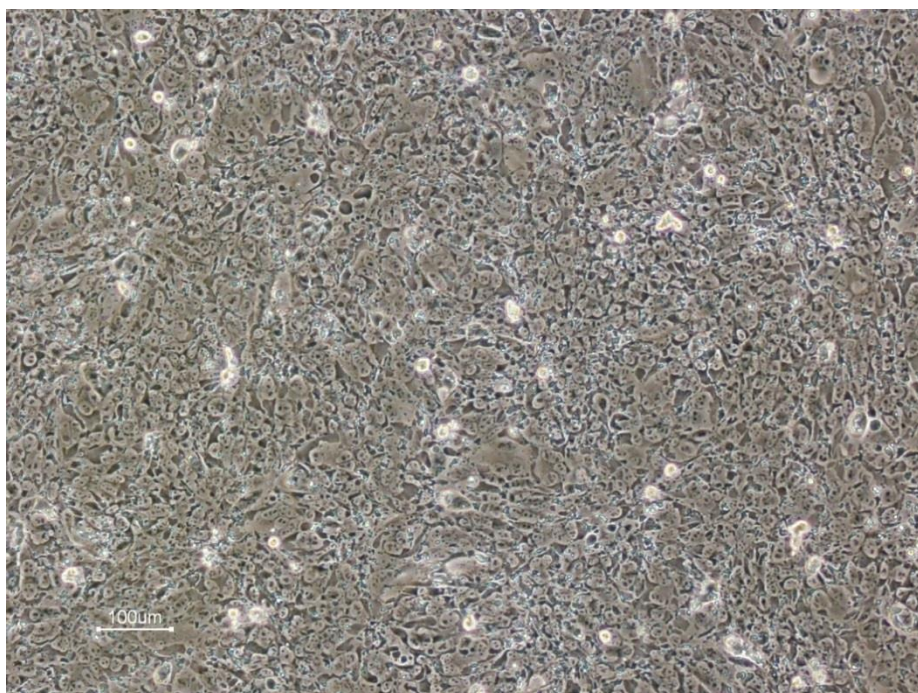


Figure 16. Recommended density of starting culture used for single-cell cloning.

Representative image shows the morphology of cells with a density of 1.5×10^5 cells/cm² at 10X magnification. Scale bar = 100 microns.

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 $37^{\circ}\text{C} \pm 1^{\circ}\text{C}$, 5% CO₂, 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.

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

Single-Cell Seeding**Day 1: Seeding of Single Cells**

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% CO₂, 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% CO₂, and >90% humidity and leave the plate undisturbed for 48 hr. Continue culturing according to Table IV.

Day 3: Adding Media to Wells Containing Single Cells

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 17) that will be passaged for further expansion during scale-up.

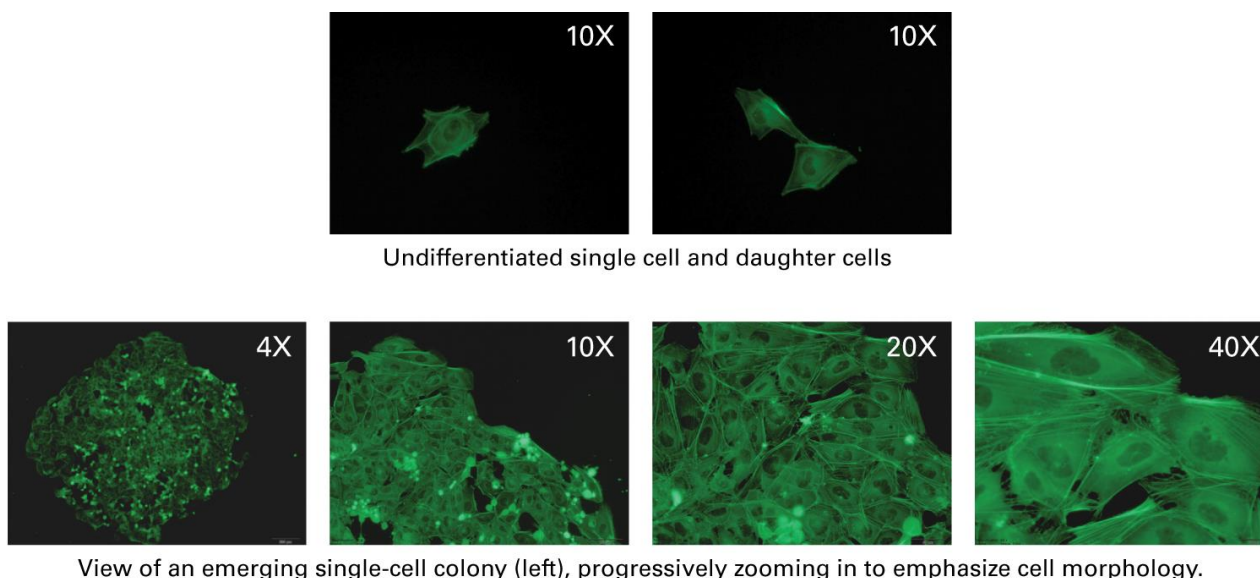


Figure 17. A single cell seeded in one well generates an emerging colony. Representative images of GFP-actin iPS cells taken 24 hr (top) or two weeks (bottom) after single-cell cloning.

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 μ l 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 $37^{\circ}\text{C} \pm 1^{\circ}\text{C}$ immediately before use. Discard any leftover warmed medium.

Changing Media

1. Check cells under the microscope; photo document as necessary.
2. Carefully discard 150 μ l of the medium from each well and add 150 μ l 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 $37^{\circ}\text{C} \pm 1^{\circ}\text{C}$, 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 18).

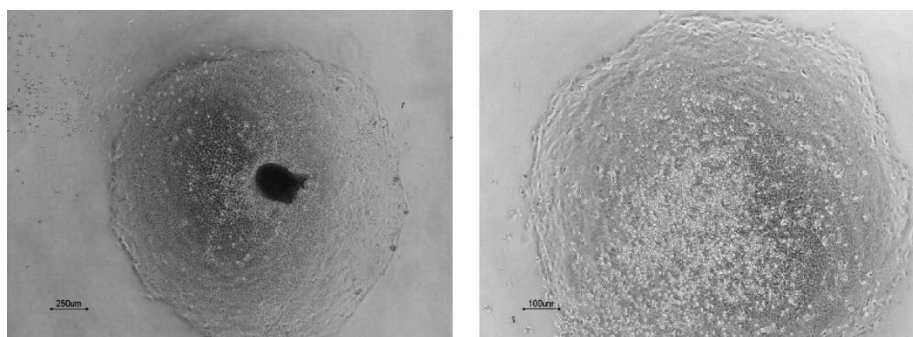


Figure 18. Clonal colonies, ready for transfer to larger wells and scale-up. The cells have the typical undifferentiated stem cell morphology (i.e., high nucleus-to-cytoplasm ratio, defined borders, and prominent nucleoli).

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% CO₂, 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% CO₂, 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% CO₂, 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

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- Mali, P. *et al.* CAS9 transcriptional activators for target specificity screening and paired nickases for cooperative genome engineering. *Nat. Biotechnol.* **31**, 833–838 (2013).
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Appendix A. Troubleshooting Guide

Table VIII. Troubleshooting guide for the Guide-it CRISPR/Cas9 Gesicle Production System.

Problem	Possible Explanation	Solution
Generating a plasmid encoding for sgRNA		
No colonies obtained	Oligos not hybridized	- Check PCR machine - Perform the hybridization step in a heat block at 95°C for 5 min, then turn off the block and let it cool down to room temperature before removing the oligos - Check for correct sgRNA oligo design (Section VI.B)
	Poor transformation efficiency	Use the included Stellar competent cells
Sanger sequencing inconclusive	Poor signal-to-noise ratio	Use alternative primers: M13 forward (GTTGTAAAACGACGGCCAGT) and M13 reverse (TCACACAGGAAACAGCTATGA)
CRISPR/Cas9 gesicle production		
No fluorescence observed in producer cells	Poor transfection efficiency	- Use the Gesicle Producer 293T Cell Line (Cat. No. 632617) - Plate the Gesicle Producer 293T cells at the correct density (Section VI.D, Figure 11)
	Incorrect Guide-it Packaging Mix used	Check that you have used both mixes: one tube containing Packaging Mix 1 (green cap) and one tube containing Packaging Mix 2 (yellow cap)
Dim fluorescence observed in producer cells, or no cellular fusion observed in producer cells	Poor transfection efficiency	- Use the Gesicle Producer 293T Cell Line (Cat. No. 632617) - Plate the Gesicle Producer 293T cells at the correct density (Section VI.D, Figure 11)
Target cell treatment with Cas9/sgRNA gesicles		
No fluorescence detected in the target cells	Microscopy problem	- Use 40X objective or higher - Check microscope fluorescence - Check the excitation/emission of the filter used (it should be 587 nm/610 nm)
	Low gesicle production	- Apply additional gesicles to your target cells (we have not detected problems with cell viability after repeated dosage with gesicles) - Confirm the presence of Cas9 protein in the gesicles via Western blot using the Guide-it Cas9 Polyclonal Antibody (Cat. No. 632607)
	Poor gesicle uptake by target cells	Double-check that protamine was added to the target cells
No gene editing detected in the target cells	Cas9/sgRNA complex is not present in the gesicles	- Use the pGuide-it sgRNA1 vector to clone your sgRNA of choice (see Section VI.C and Figure 9) - Confirm the presence of Cas9 protein in the gesicles via Western blot using the Guide-it Cas9 Polyclonal Antibody (Cat. No. 632607) - Confirm the activity of your Cas9/sgRNA gesicles <i>in vivo</i> using HEK 293T cells as the target cells
	Inactive sgRNA	- Use the pGuide-it sgRNA1 vector to clone your sgRNA of choice (see Section VI.C and Figure 9) - Confirm the activity of the sgRNA against your target <i>in vitro</i> using Guide-it sgRNA Screening Kit (Cat. No. 632639) - Choose an sgRNA with a G in position 1 and an A/T in position 17 (see Section VI.B, Figure 6) - Confirm the activity of your Cas9/sgRNA gesicles <i>in vivo</i> using HEK 293T cells as the target cells
	Poor gesicle uptake by target cells	- Optimize the density of cell plating of the target cells (it may be as low as 30%) - Double-check that protamine was added to the target cells

Appendix B: pGuide-it-sgRNA1 Vector Information

For complete descriptions of the vectors provided with each system, refer to the Certificate of Analysis, which is available at www.takarabio.com.

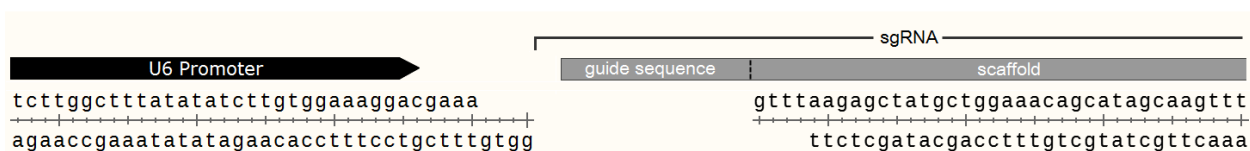
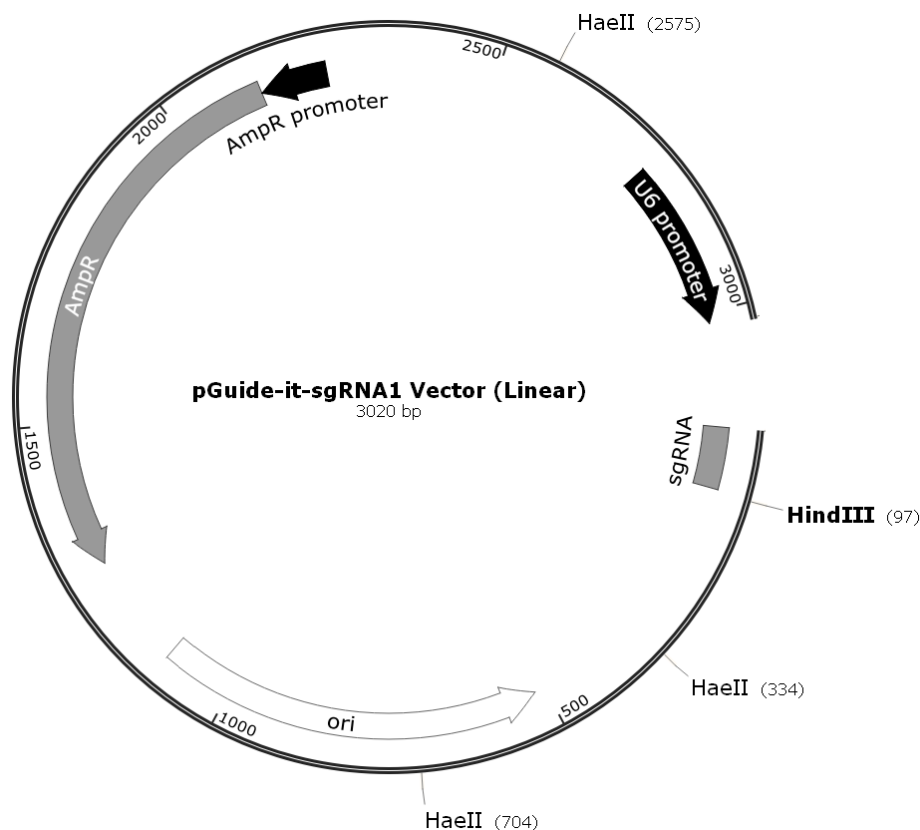


Figure 19. pGuide-it-sgRNA1 Vector (Linear) map and cloning site for user's guide sequence.

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