Takara Bio USA

Guide-it™ CRISPR/Cas9 Gesicle Production System User Manual

Cat. Nos. 632612, 632613, 632616
(053017)

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Table of Contents

Table of Figures

Table of Tables

I. Introduction

A. Technology Overview

CRISPR/Cas9 gene editing technology has revolutionized the field of genome modification, using two key components that form a complex: Cas9 endonuclease and a single guide RNA (sgRNA) that guides Cas9 to a specific target site in the genomic DNA. Current methods for delivery of the Cas9 and sgRNA components to target cells utilize either transient or stable expression of Cas9 and/or sgRNA via plasmid transfection or lentiviral infection. However, evidence suggests that prolonged expression of these components can lead to undesirable cleavage events at off-target sites (Hsu et al. 2013; Fu et al. 2013). Several groups have achieved direct delivery of the Cas9/sgRNA ribonucleoprotein (RNP) complex, obtaining levels of genome editing that are similar to those of plasmid-based delivery, with the added benefit of fewer off-target effects due to the short lifespan of the Cas9 protein in the cell (Zuris et al. 2014; Lin et al. 2014); however, their methods are not consistent over a broad range of cell types, and can cause cellular toxicity.

To overcome these obstacles, 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 which are able to carry any cargo, such as proteins. 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 1). Once gesicles have been made, they can be harvested, concentrated, and applied to target cells, where the active Cas9/sgRNA complex is transported to the nucleus for efficient gene editing.

The Guide-it CRISPR/Cas9 Gesicle Production System (Cat. No. 632613) is a complete system for producing high yields of gesicles containing target-specific Cas9/sgRNA RNP complexes for use on a wide range of target cells, including cells that are difficult to transfect. 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 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.

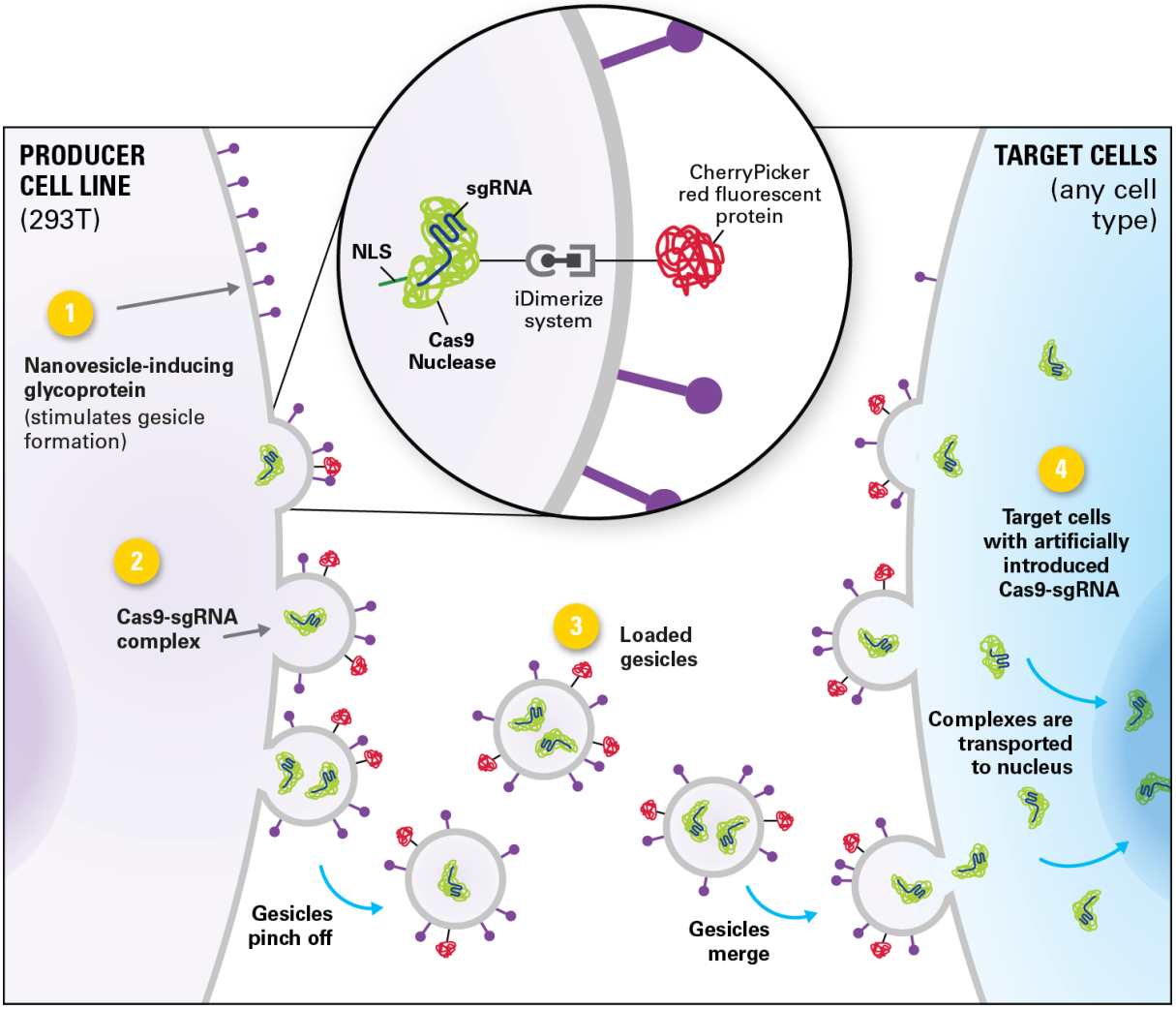
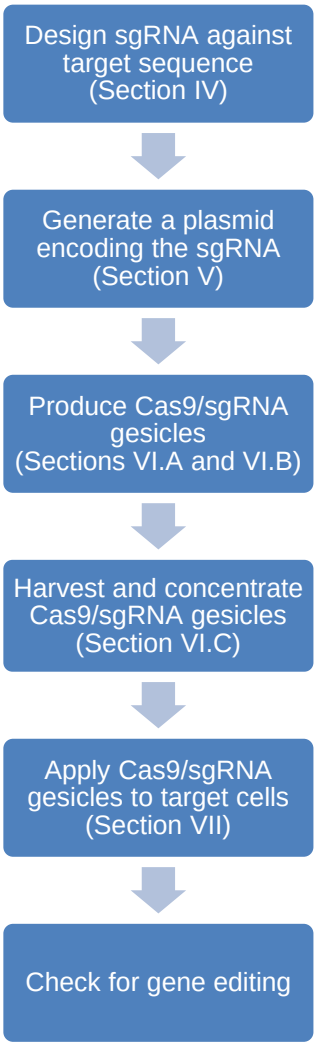


Figure 1. Gesicle Producer 293T cells (left side of image) generate nanovesicles (gesicles) for delivery of Cas9/sgRNA RNP complexes specific to a gene of interest. Once produced, gesicles are harvested from the culture supernatant and concentrated. The gesicles can be applied to target cells immediately (right side of image), or frozen in aliquots for future use.

1. The Gesicle Producer 293T Cell Line (Cat. No. 632617) 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 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 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 and translocated to the nucleus to perform site-specific gene editing in a broad range of cell types.

B. Protocol Overview



sgRNAs can be designed manually, or using online tools. Please visit our website to view a short video about sgRNA design and to review an extensive list of web-based sgRNA design tools.

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.

The cloned plasmid is diluted with dH_2O 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.

48–72 hr later, gesicles containing active Cas9/sgRNA complexes are collected from the medium and concentrated via overnight centrifugation.

Cas9/sgRNA gesicles are applied to the target cells in the presence of protamine sulfate, followed by a 30-min centrifugation step to enhance gesicle-to-cell contact.

Cas9 gene editing activity can be checked using the Guide-it Mutation Detection Kit (Cat. No. 631448).

Figure 2. Workflow of a genome editing experiment using the Guide-it CRISPR/Cas9 Gesicle Production System.

II. List of Components

- Store all components at –20°C.
- Store Guide-it CRISPR/Cas9 Gesicle Packaging Mix 1 and Mix 2 at –20°C in the supplied foil pouch containing the desiccant sachet.
- Make sure to return any unused vials of CRISPR/Cas9 Gesicle Packaging Mix 1 and Mix 2 to the supplied foil pouch containing the desiccant sachet, and store at –20°C.

Guide-it CRISPR/Cas9 Gesicle Production System (Cat. No. 632613)

- **Guide-it CRISPR/Cas9 Gesicle Packaging Set (Cat. No. 632616; 10 rxns)**
  - 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 (500 nM)
  - 200 µl Protamine Sulfate (4 mg/ml)
- **pGuide-it-sgRNA1 Vector System (Cat. No. 632612)**
  - 1 each pGuide-it-sgRNA1 Vector (Linear) (Cat. No. 632614; Not sold separately)
    - 20 µl pGuide-it-sgRNA1 Vector (Linear) (7.5 ng/µl)
  - 1 each Guide-it Ligation Components v2 (Cat. No. 632615; 10 rxns; 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
  - 1 each Stellar™ Competent Cells (Cat. No. 636763; 10 x 100 µl)
    - 10 tubes Stellar Competent Cells (100 µl/tube)
    - 10 tubes SOC Medium (1 ml/tube)
    - 10 µl pUC19 Vector (0.1 ng/µl)

* The DNA Ligation Mighty Mix component is also available separately (100 rxns, Cat. No. 6023).

III. Additional Materials Required

The following materials are required but not supplied:

A. Molecular Biology Supplies

- Target-specific oligos (see Section IV.A.)
- 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 Scientific Nalgene Cat. No. 723-9945 or equivalent)
- Beckman J2-HS Centrifuge with a JS-7.5 swinging bucket rotor (or equivalent)

B. Mammalian Cell Culture Supplies

- 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-Aldrich Cat. No. D5796); 10% Fetal Bovine Serum (FBS); 1% Antibiotic Antimycotic Solution (100x), Stabilized (Sigma-Aldrich Cat. No. A5955)
- Culture medium, supplies, and additives specific for your target cells
- Trypsin/EDTA (e.g., Sigma Cat. No. T4049)
- 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 Cat. No. 354450 or equivalent)
- 24-well collagen I-coated plates (Corning Cat. No. 354408 or equivalent) if the target cell line is adherent
- Guide-it Mutation Detection Kit (Cat. Nos. 631443 & 631448)

C. Plasmid Purification

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

IV. Design sgRNA Against Target Sequence

A. 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 that are 5'of the sequence NGG (also called the **P**roto-spacer **A**djacent **M**otif, or PAM). Given a determined target sequence, sgRNAs can be designed manually or by using an online tool. Please click here to watch a short tutorial on sgRNA design.

1. Designing candidate sgRNAs

- There are several online tools that can be helpful for finding sgRNAs against your target sequence. Please click here to review a list of these tools.
- To design your sgRNAs manually (Figure 3), 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 3), 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.

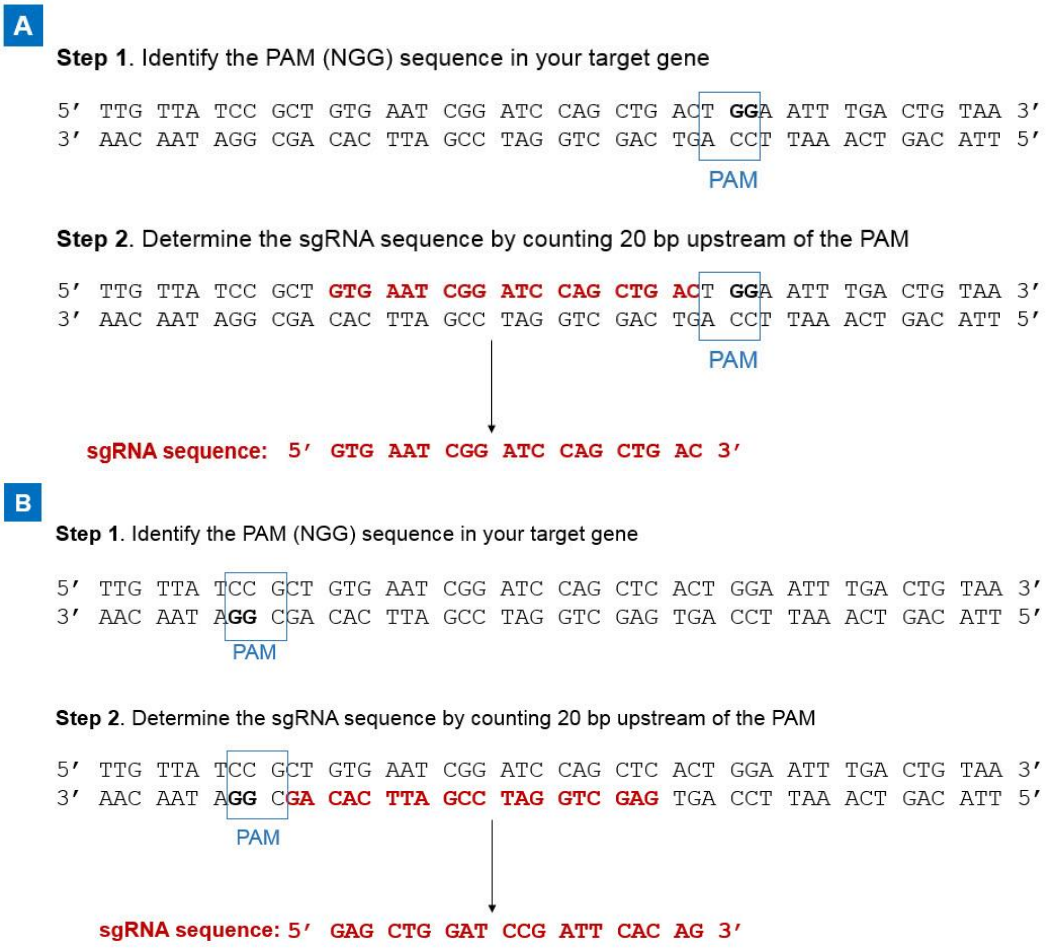


Figure 3. Workflow of sgRNA manual design. The PAM may be located on the sense strand (**Panel A**) or the antisense strand (**Panel B**).

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.

2. 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 in the Guide-it CRISPR/Cas9 Gesicle Production System (Figure 4).**

Optimal sgRNAs	
Target	**sgRNA sequence**
CD81	GCAGCCCTCCACTCCCATGG
CXCR4	GGGCAATGGATTGGTCATCC
EMX1	GAGTCCGAGCAGAAGAAGAA
AcGFP1	GTGAATCGCATCGAGCTGAC
ZsGreen1	GACCATGAAGTACCGCATGG

Figure 4. Examples of optimal sgRNAs for use in the Guide-it CRISPR/Cas9 Gesicle Production System. Each of these example sgRNAs contains a G in position 1 and an A or T in position 17.

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.

3. Minimizing off-target effects

- To minimize off-target cleavage, the entire target sequence including the PAM site should have 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.
- The majority of 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.

B. 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 Step IV.A above, including short 5' overhang sequences complementary to the vector backbone. 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 IV.A, 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 YYY`**C**`-3′`

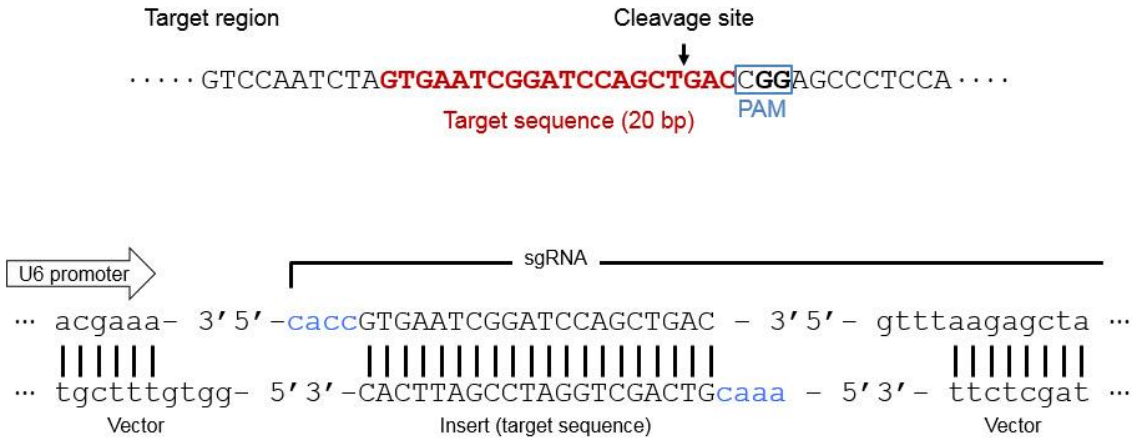


Figure 5. Example of cloning a target sequence using the Guide-it CRISPR/Cas9 Gesicle Production System. This particular example corresponds to the sequence of the Guide-it Control Annealed Oligos v2 included in the kit. The target region (marked in red) is shown in the top half of the figure. Both oligos contain the additional 5' overhang sequences required for cloning into the pGuide-it-sgRNA1 vector, marked in blue (bottom half of figure).

V. Generate 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 of your target oligos (designed according to Section IV 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 6). 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.

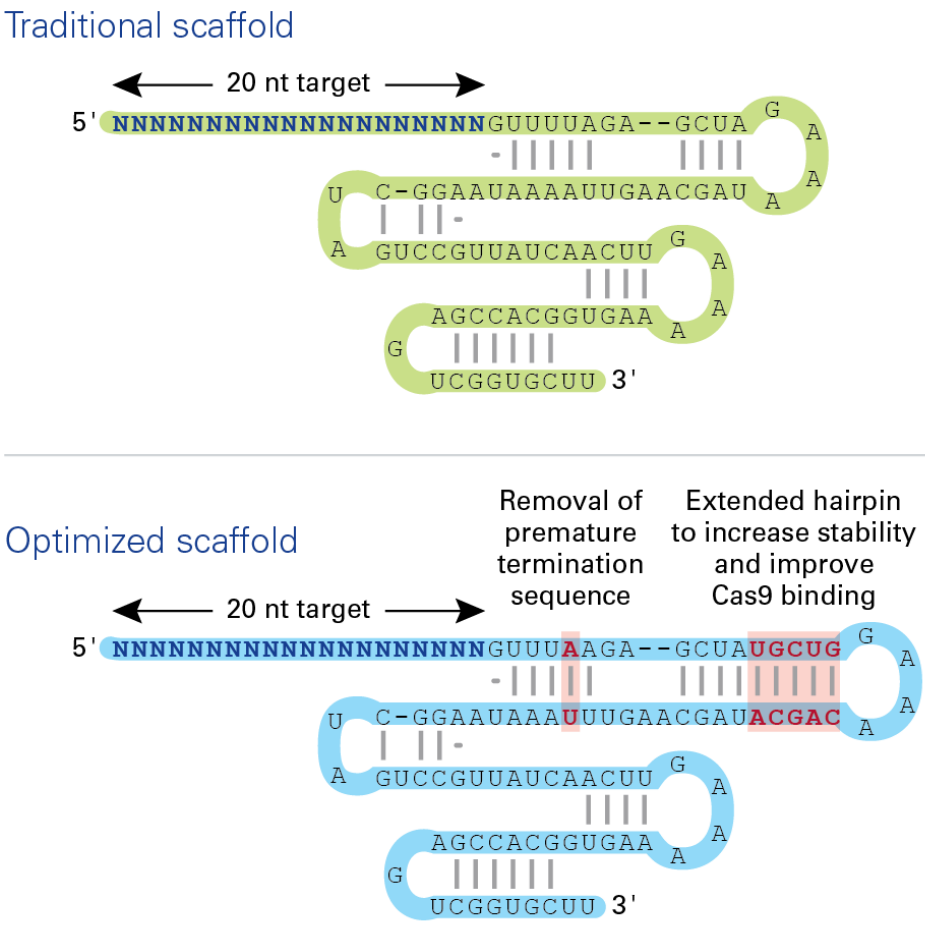


Figure 6. pGuide-it-sgRNA1 includes an improved sgRNA scaffold design for increased editing efficiency. It is *essential* to use this optimized scaffold design for gesicle production.

A. Protocol: Annealing Oligos

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

1 µl	Oligo 1 (100 µM)
1 µl	Oligo 2 (100 µM)
8 µl	Guide-it Oligo Annealing Buffer
10 µl	Total Volume

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

3. Anneal the oligos by using a thermal cycler to denature 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
   15 min slope from 95°C to 25°C
   25°C, hold

4. 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.
5. Store the annealed oligos at –20°C until use.

B. Protocol: Cloning the sgRNA Targeting Sequence Into pGuide-it-sgRNA1

1. 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 V.A, 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

***NOTE:** 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 6).

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

C. Protocol: Isolate and Analyze Plasmids

1. Pick single colonies and inoculate into an appropriate amount of LB medium containing ampicillin (final concentration 100 µg/ml).
2. Incubate with shaking overnight at 37°C.
3. 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).
4. Perform sequencing analysis using the included Guide-it Sequencing Primer 1 and your preferred sequencing protocol.

VI. Produce CRISPR/Cas9 Gesicles

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

A. Protocol Overview

The following steps are required to create target-specific gesicles containing the Cas9/sgRNA complex of interest (Figure 7).

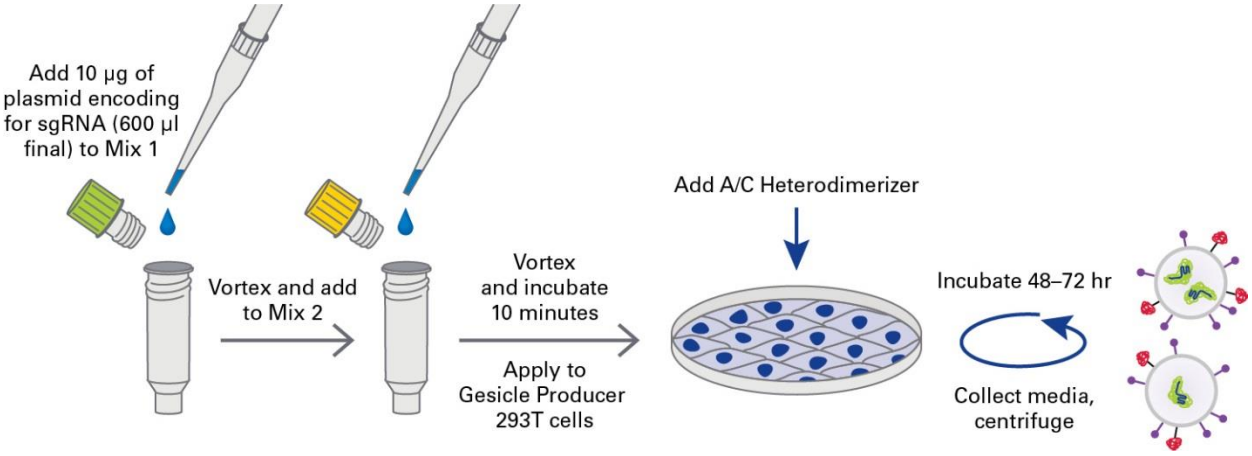


Figure 7. Overview of the CRISPR/Cas9 gesicle production protocol. After cloning the target oligos designed against your gene of interest into the pGuide-it-sgRNA vector, pipette the purified plasmid containing your sgRNA (diluted to 600 µl) into the Guide-it CRISPR/Cas9 Gesicle Packaging Mix 1, vortex, 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 8,000 x g. 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.

B. Protocol: Produce Cas9/sgRNA Gesicles

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

1. 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).
- At the time of transfection, cells should be 40–50% confluent, as shown in Figure 8 and described in Step 2.1 of the protocol below.

NOTE: In order to maximize the level of Cas9/sgRNA gesicle production, it is critical to perform a very good transfection, with high efficiency.

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

1. Approximately 24 hr before transfection, seed 4.5 x 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% CO_2.
2. At the time of transfection, cells should be 40–50% confluent, as shown in Figure 8.

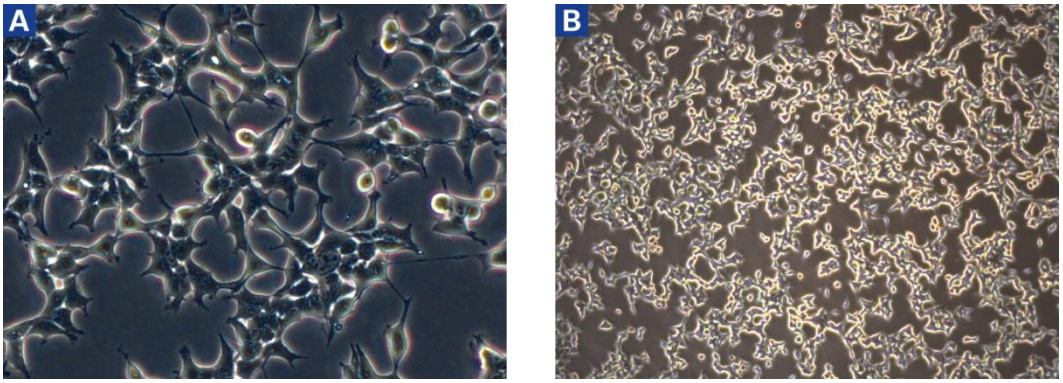


Figure 8. Optimal density of Gesicle Producer 293T cells at the time of transfection. Cells are shown at 20X magnification (**Panel A**) and 5X magnification (**Panel B**).

3. 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.
4. Add 1.25 µl 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.

5. In a sterile microfuge tube, dilute 10 µg of the pGuide-it-sgRNA1 plasmid encoding your sgRNA of choice (from Section V.C, Step 4) with sterile water to a final volume of 600 µl. 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.

6. Add the 600 µl 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.

7. Transfer the 600 µl 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.

8. 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% CO_2.

    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% CO_2 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 9):

    - In successful transfections, the cells will display higher levels of red fluorescence and cellular fusion (Figure 9, 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 9, Panels C–D).

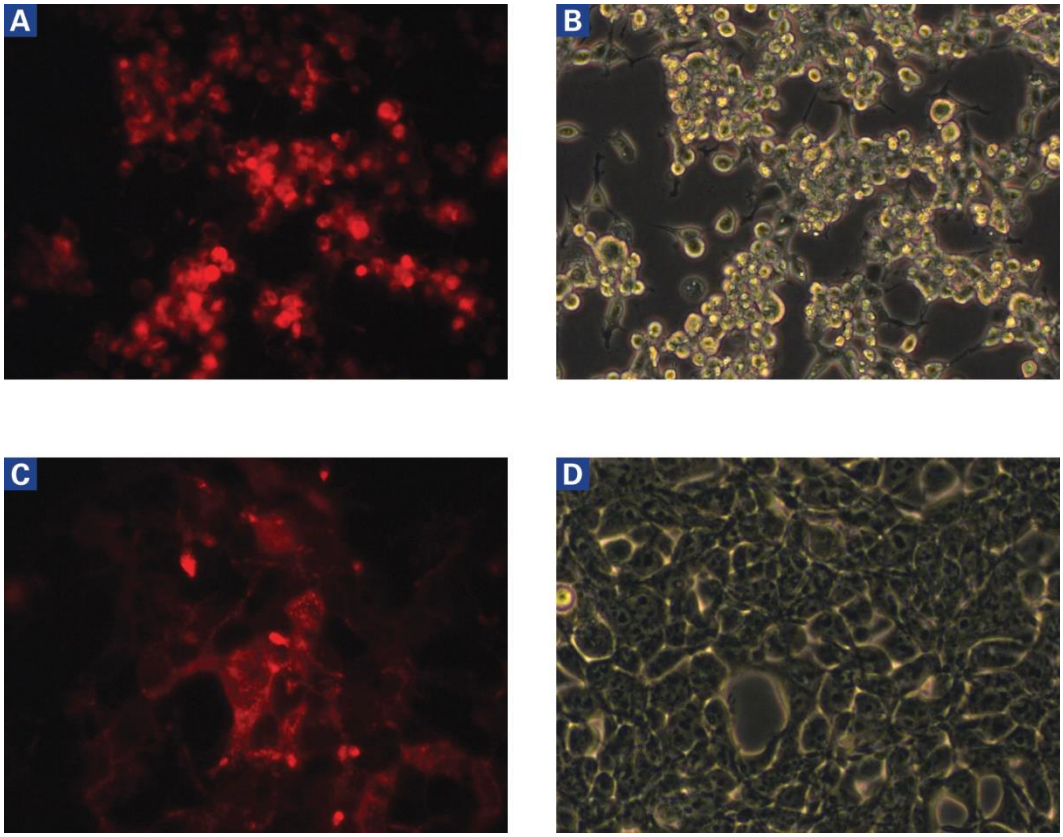


Figure 9. Gesicle Producer 293T cells after transfection. These images (20X magnification) show red fluorescence (left) and phase microscopy (right) results for successfully transfected Gesicle Producer 293T cells (**Panels A and B**) and unsuccessfully transfected Gesicle Producer 293T (**Panels C and D**).

C. Protocol: 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 (500 x g for 10 min) and filter through a 0.45-µm 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 ~8,000 x g (Corning Falcon Cat. No. 352070 or equivalent). Centrifuge the filtered samples at ~8,000 x g 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. Apply Cas9/sgRNA Gesicles to Target Cells

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

A. Protocol: Treating Adherent Target Cells with Cas9/sgRNA Gesicles

Guidelines for Cell Plating

Plate your target cells one day prior to treatment in 24-well collagen I-coated plates (Corning Cat. No. 354408). The cells should be 40–50% confluent on the day of treatment (Figure 10). We advise using 30 µl of gesicles per well in a 24-well plate format. If you want to use 12- or 6-well plates, increase the amount of Cas9/sgRNA gesicles accordingly.

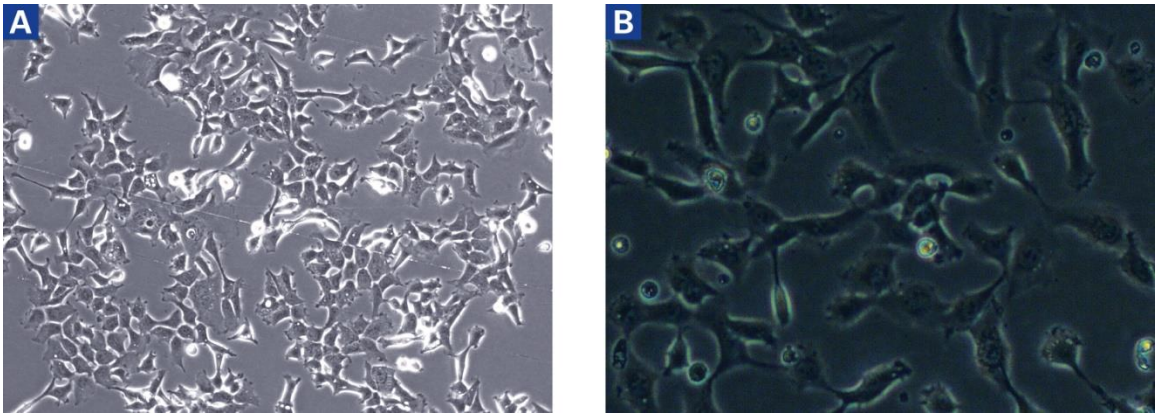


Figure 10. Optimal density of target cells before Cas9/sgRNA gesicle treatment. HEK 293 cells (left; 10X magnification) and HT 1080 cells (right; 40X magnification) plated at 40–50% confluency and suitable for addition of Cas9/sgRNA gesicles.

Application of Cas9/sgRNA Gesicles to Target Cells

Perform the following steps under sterile conditions. The quantities specified here are for a 24-well plate format.

1. Thaw the Cas9/sgRNA gesicles (from Step VI.C.7 above) 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. 400 x g for 15 sec) before adding the gesicles to your cells.

2. Confirm that the cells plated on the previous day are approximately 40–50% confluent.
3. Prepare protamine medium by supplementing the complete medium normally used to culture your target cells with protamine sulfate to a final concentration of 8 µg/ml.
4. Replace the medium in wells that will be treated with gesicles with 500 µl protamine medium.
5. Add 30 µl of the thawed Cas9/sgRNA gesicles from Step 1 to each well containing protamine medium.

6. Centrifuge the 24-well plate at 1,150 x g 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 11, Panel A).
7. After centrifugation, transfer the plate back to a tissue culture incubator and incubate for at least 4 hr at 37°C.
8. After incubating for a minimum of 4 hr, replace the protamine medium with fresh medium that does not contain protamine and continue incubating the cell culture in the tissue culture incubator.
9. After 24 hr, cells can be visualized under a microscope (40X objective) to detect red fluorescence, which indicates gesicle uptake. The fluorescence signal should be detected *inside* the cells, as shown in Figure 11, Panel C.
10. When the cells have reached confluency, they can be expanded.
11. After 72 hr of gesicle treatment, check your cells for gene editing. The cell population can be tested for gene editing using the Guide-it Mutation Detection Kit (Cat. No. 631448).

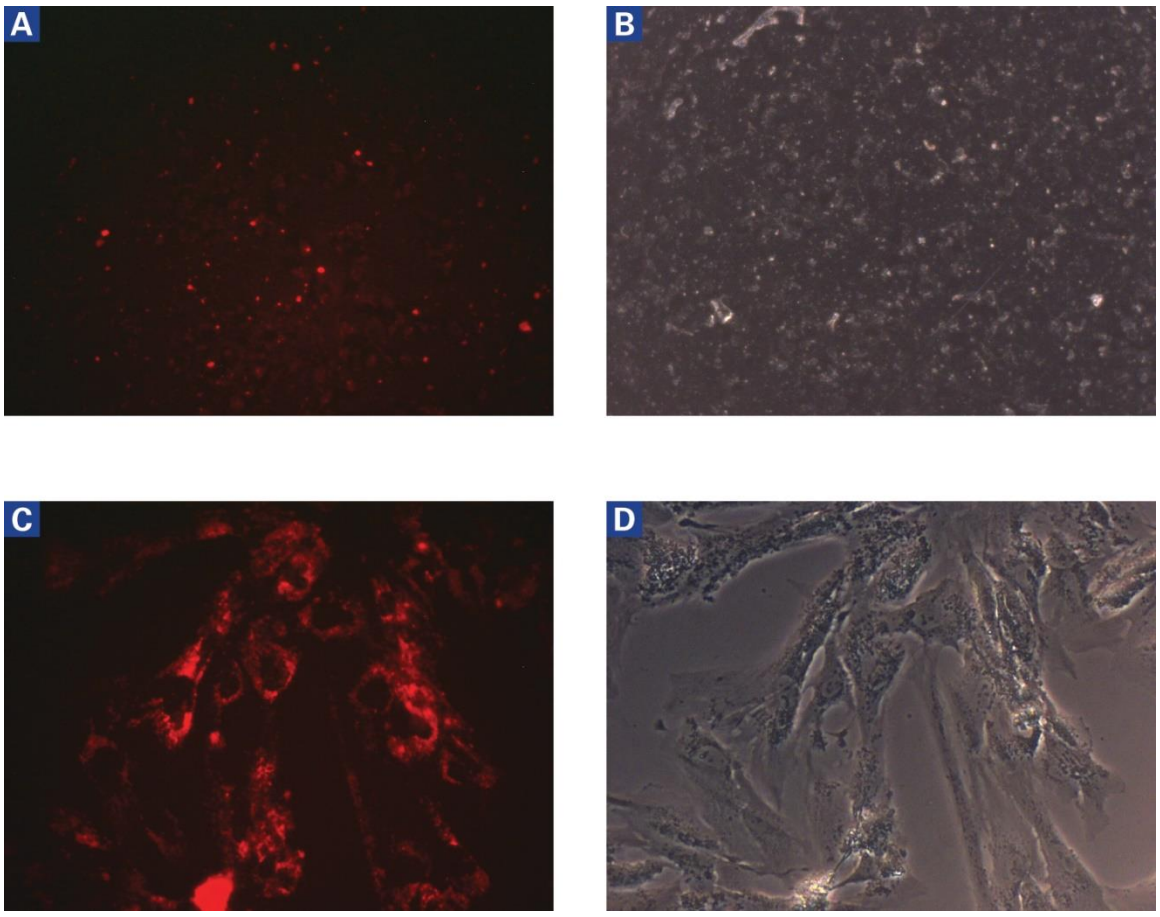


Figure 11. Example of expected results: RPE cells after gesicle treatment. Fluorescence (Panels A and C) and phase (Panels B and D) microscopy images of RPE cells after addition of Cas9/sgRNA gesicles. **Panel A**. Immediately after the centrifugation step, the red signal is spread throughout the well and small aggregates can be detected (5X magnification). **Panel C.** 24 hr later, the fluorescence is localized in the cells (40X magnification).

B. Protocol: Treating Suspension Target Cells with Cas9/sgRNA Gesicles

Guidelines for Cell Plating

Plate your target cells one day prior to treatment. In a 24-well plate format, plate 0.5 ml/well of 1 x 10^5 cells/ml.

Application of Cas9/sgRNA Gesicles to Target Cells

Perform the following steps under sterile conditions. The quantities specified here are for a 24-well plate format.

1. Thaw the Cas9/sgRNA gesicles (from Step VI.C.7 above) 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 gesicle suspension up and down several times and perform a quick spin (e.g., 400 x g for 15 sec) before adding the gesicles to your cells.

2. Add 1.0 µl of protamine sulfate stock to each well containing cells to be treated, for a final concentration of 8 µg/ml protamine sulfate.

   NOTE: Alternatively, the protamine sulfate stock solution can be diluted 1:10 with dH_2O to make a more dilute stock (0.4 mg/ml) protamine solution. 10 µl of this dilute solution can be added to each well to reach a concentration of 8 µg/ml protamine.

3. Add 30 µl of the thawed Cas9/sgRNA gesicles from Step 1 to each well to be treated.
4. Centrifuge the 24-well plate at 1,150 x g for 30 min at room temperature.
5. Incubate the cells for at least 4 hr at 37°C in an appropriate cell culture incubator.
6. After incubation, remove the medium containing protamine and add fresh medium that does not contain protamine. Continue incubating the cell culture at 37°C in an appropriate cell culture incubator.
7. After 24 hr, cells can be visualized under a microscope (40X objective) to detect red fluorescence, which indicates gesicle uptake. The fluorescence signal should be detected *inside* the cells, as shown in Figure 12, Panel A.
8. When the cells have reached confluency, they can be expanded.
9. After 72 hr of gesicle treatment, the cells can be tested for Cas9 activity using the Guide-it Mutation Detection Kit (Cat. No. 631448).

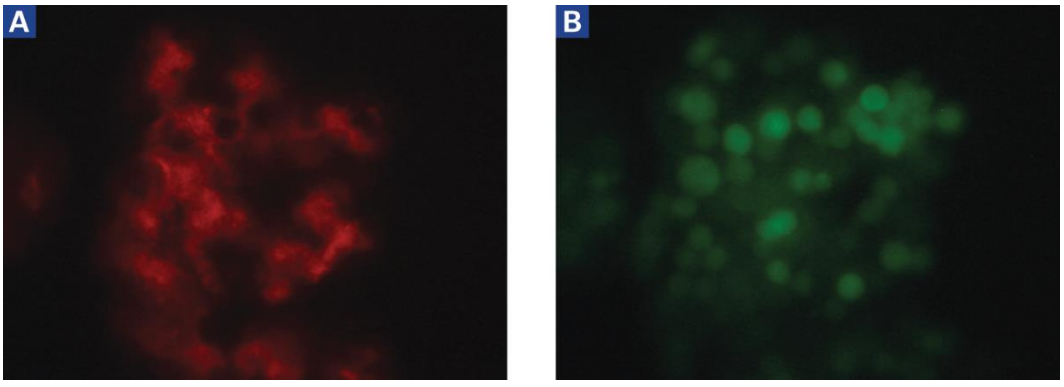


Figure 12. Example of expected results: Jurkat cells 24 hr after treatment with Cas9/sgRNA gesicles. **Panel A.** Red fluorescence is localized inside Jurkat cells (40X magnification) expressing a ZsGreen1 fluorescent protein (green image shown to indicate cell positions; **Panel B**).

VIII. References

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Appendix A. Troubleshooting Guide

Table 1. 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 minutes, then turn off the block and let it cool down to room temperature before removing the oligos • Check for correct sgRNA oligo design (Section IV)
	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.B)
	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.B)
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 V.B and Figure 6) • 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 *in vivo* using HEK 293T cells as the target cells
	Inactive sgRNA	• Use the pGuide-it sgRNA1 vector to clone your sgRNA of choice (see Section V.B and Figure 6) • Confirm the activity of the sgRNA against your target *in vitro* 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 IV, Figure 4) • Confirm the activity of your Cas9/sgRNA gesicles *in vivo* 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.

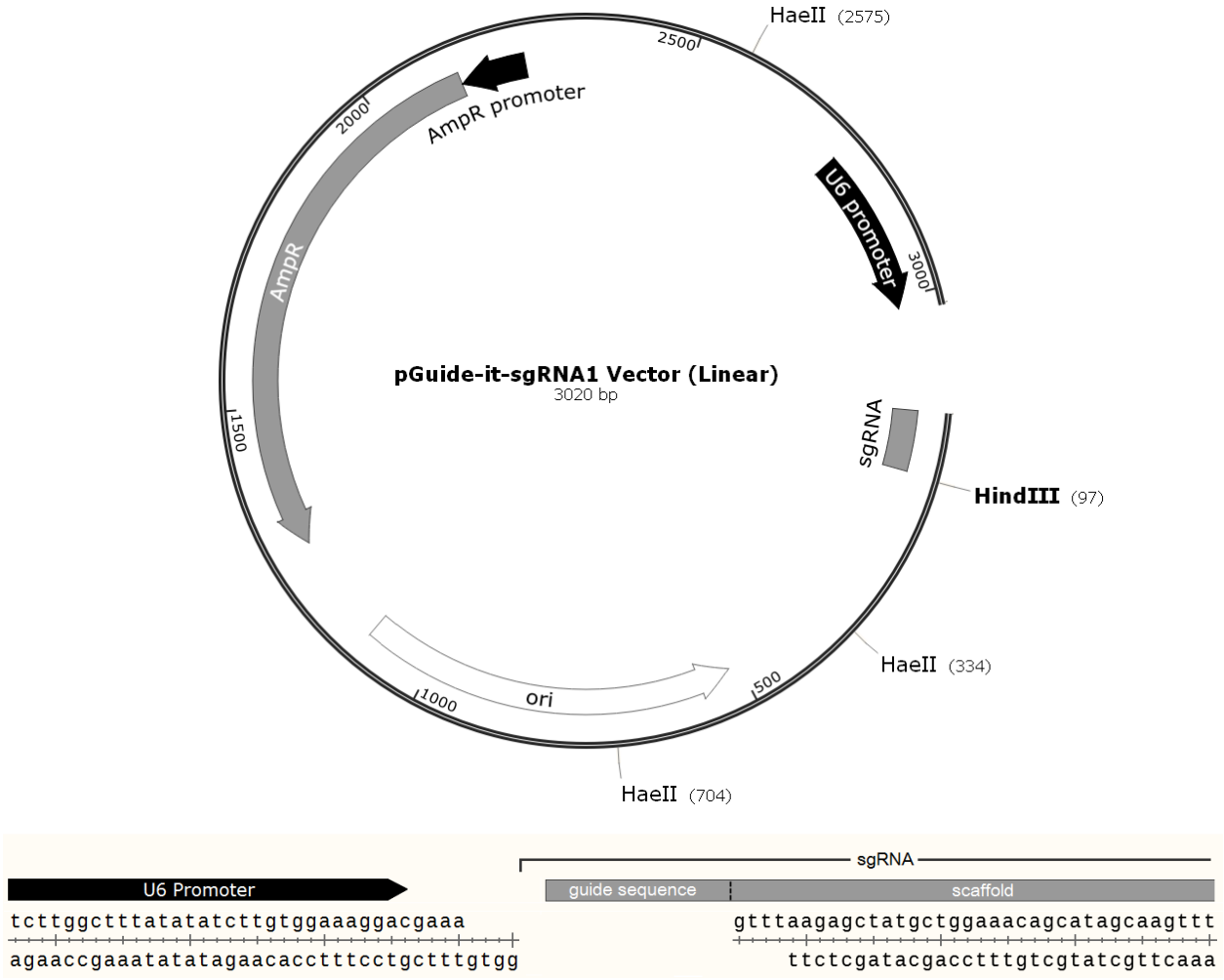

Figure 13. pGuide-it-sgRNA1 Vector (Linear) map and cloning site for user's guide sequence.

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