Takara Bio USA

Lenti-X™ CRISPR/Cas9 System User Manual

Cat. No(s). 632629, 632630

(090622)

Takara Bio USA, Inc.
2560 Orchard Parkway, San Jose, 95131, USA
U.S. Technical Support: techUS@takarabio.com

Table of Contents

I. Introduction 4
A. Summary 4
B. Elements of Lenti-X CRISPR/Cas9 System 5
II. List of Components 6
III. Additional Materials Required 7
A. Tetracycline-Free Fetal Bovine Serum 7
B. Antibiotic for Selecting Stable Cell Lines 7
C. Mammalian Cell Culture Supplies 7
D. Lenti-X 293T Cells 7
E. High-Titer Packaging System 8
F. Lentiviral Titer Determination 8
G. Lentivirus Concentration 8
H. Transduction Enhancers 8
I. Plasmid Purification (Transfection-Grade) 9
J. Assays for Determining Editing Efficiency 9
K. Molecular Biology Supplies 9
IV. Protocol Overview 9
A. General Cell Culture 9
B. Safety Guidelines for Working with Lentiviruses 9
C. Protocol Overview 11
V. Design sgRNA Against Target Sequence 11
A. Identifying Candidate sgRNAs Against Your Target Sequence 11
B. Designing and Ordering Oligos 13
VI. Generate a Plasmid Encoding the sgRNA 14
A. Protocol: Annealing Oligos 15
B. Protocol: Cloning the sgRNA Targeting Sequence Into pLVX-hyg-sgRNA1 16
C. Protocol: Isolate and Analyze Plasmids 16
VII. Produce Cas9 and sgRNA Lentiviruses 16
VIII. Lentivirus Titration 17
A. Summary 17
B. Protocol: Determining Viral Titer Using Antibiotic Selection 17
IX. Transducing Target Cells 18
A. Summary 18

B. Protocol: Transducing Target Cells for sgRNA Expression 18
C. Protocol: Transducing Target Cells for Cas9 Editing 19
X. References 20
Appendix A. Troubleshooting Guide 20

Table of Figures

Figure 1. An overview of the Lenti-X CRISPR/Cas9 System. 5
Figure 2. Workflow of a genome editing experiment using the Lenti-X CRISPR/Cas9 System. 11
Figure 3. Workflow of sgRNA manual design. 12
Figure 4. Examples of optimal sgRNAs for use in the Lenti-X CRISPR/Cas9 System. 13
Figure 5. Example of cloning a target sequence using the Lenti-X CRISPR/Cas9 System. 14
Figure 6. pLVX-hyg-sgRNA1 includes an improved sgRNA scaffold design for increased editing efficiency. 15

Table of Tables

Table 1. Recommended Antibiotic Concentrations for Selecting & Maintaining Stable Cell Lines 7
Table 2. Troubleshooting Guide for the Lenti-X CRISPR/Cas9 System. 20

I. Introduction

A. Summary

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. The Lenti-X CRISPR/Cas9 System (Cat. No. 632629) is a complete system for producing high yields of lentiviruses encoding the components necessary for CRISPR/Cas9-mediated genome editing [i.e., single guide RNA (sgRNA) and Cas9 nuclease] for delivery to mammalian cells that are difficult to transfect. This system also contains necessary controls and sufficient reagents for construction of 10 different target (sgRNA) expression plasmids. The pLVX-hyg-sgRNA1 Vector System provides additional linearized plasmids, ligation components, and Stellar™ Competent Cells.

This protocol is provided for transfection and lentivirus production with Lenti-X Packaging Single Shots, pre-aliquoted, lyophilized, single tubes of Xfect™ Transfection Reagent premixed with an optimized formulation of Lenti-X lentiviral packaging plasmids. Lenti-X Packaging Single Shots (VSV-G) provide the highest titers and widest tropism.

A target sgRNA expressed from the human U6 promoter is introduced into the pLVX-hyg-sgRNA1 plasmid. To construct the vector, a pair of user-provided oligos corresponding to the target genomic sequence of interest are annealed to form a duplex. Then, the duplexed DNA is cloned into the pre-linearized vector using the included high-efficiency ligation mix.

As seen in Figure 1, target cells are first transduced with the sgRNA-expressing lentivirus with the U6 promoter and integration is selected for using hygromycin. Then the second lentivirus with CMV promoter driving Cas9 expression is added to cells expressing Cas9. Integration of the Cas9-encoding DNA is selected for using puromycin and stable clones are screened for genome editing.

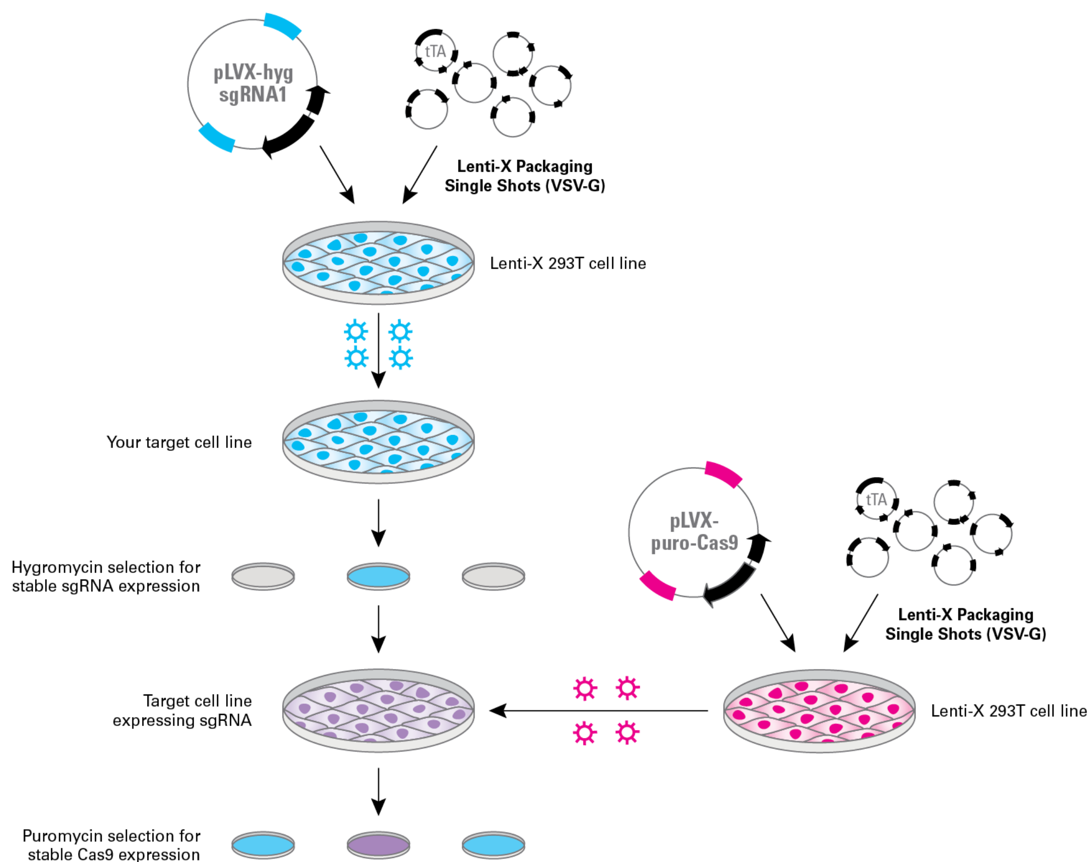


Figure 1. An overview of the Lenti-X CRISPR/Cas9 System. In this system, two types of lentivirus are generated: a virus encoding for Cas9 expression under the CMV promoter and a second virus encoding for expression of your custom sgRNA under the U6 promoter. First, your target cells are transduced with the sgRNA-expressing lentivirus and selected for integration using hygromicin. Then, the Cas9 lentivirus is transduced into this cell line and selected for using puromycin. The final result is a cell line stably expressing both Cas9 and your custom sgRNA.

B. Elements of Lenti-X CRISPR/Cas9 System

pLVX-puro-Cas9 Vector

The pLVX-puro-Cas9 Vector contains all of the elements necessary for lentiviral production. The vector also contains puromycin resistance and Cas9 driven under the CMV promoter.

pLVX-hyg-sgRNA1 Vector (Linear)

The pLVX-hyg-sgRNA1 Vector (Linear) is a prelinearized vector ready for insertion of your custom sgRNA sequence expressed under the U6 promoter. Design of the sgRNA sequence is discussed in section V. This vector also contains all of the elements for lentiviral generation and hygromycin resistance.

NOTE: We do not recommend co-transduction with both viruses at the same time as this results in little to no editing.

II. List of Components

- Store Stellar Competent Cells (Cat. No. 636763) at –70°C .
- Store all other components at –20°C.
- Store Lenti-X Packaging Single Shots at –20°C in the supplied foil pouch containing the desiccant sachet.
- Make sure to return any unused Lenti-X Packaging Single Shots to the supplied foil pouch containing the desiccant sachet, and store at –20°C.

Lenti-X CRISPR/Cas9 System (Cat. No. 632629)

- 20 µl pLVX-hyg-sgRNA1 Vector (Linear) (Cat. No. 632632; not sold separately)
- 20 µl pLVX-puro-Cas9 Vector (Cat. No. 632631; 500ng/ul)
- 10 rxns 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)
- 1 each Lenti-X Packaging Single Shots (Cat. No. 631275; 16 rxns)

pLVX-hyg-sgRNA1 Vector System (Cat. No. 632630)

- 20 µl pLVX-hyg-sgRNA1 Vector (Linear) (Cat. No. 632632; not sold separately)
- 10 rxns 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)

III. Additional Materials Required

The following reagents are required but not supplied.

A. Tetracycline-Free Fetal Bovine Serum

Tetracycline-free FBS should be used in the transfection medium and in the medium used to collect the virus. The following functionally tested tetracycline-free sera are available from Takara Bio:

Cat. No.	Serum Name
631106	Tet System Approved FBS (500 ml)
631107	Tet System Approved FBS (50 ml)
631101	Tet System Approved FBS, US-Sourced (500 ml)
631105	Tet System Approved FBS, US-Sourced (50 ml)

B. Antibiotic for Selecting Stable Cell Lines

The pLVX-puro-Cas9 Vector contains a puromycin resistance marker for selection of stable clones or populations (Section IX.C). The pLVX-hyg-sgRNA1 Vector contains a hygromycin resistance marker (Section IX.B). Use the following recommended puromycin and hygromycin concentrations:

Table 1. Recommended Antibiotic Concentrations for Selecting & Maintaining Stable Cell Lines

		Recommended Concentration (µg/ml)	
Cat. No.	**Antibiotic**	**Selecting Colonies[1]**	**Maintenance**
631306	Puromycin (100 mg)	0.25–10	0.25
631305	Puromycin (25 mg)		
631309	Hygromycin B (1 g)	50–400	100

[1] When selecting for single colonies, the appropriate dose must be determined empirically for your specific cell line. Test a dosage range using dishes of untransfected cells and choose the dose that kills all of the cells in 3–5 days (puro) or 7-10 days (hygro). If all the cells die in less than 24 hr, you should use a lower dose.

C. Mammalian Cell Culture Supplies

- **Medium for Lenti-X 293T Cells:**
  90% Dulbecco's Modified Eagle's Medium (DMEM) with high glucose (4.5 g/L), 4 mM L-glutamine, and sodium bicarbonate (Sigma-Aldrich, D5796); 10% Fetal Bovine Serum (FBS); 100 units/ml penicillin G sodium & 100 µg/ml streptomycin sulfate.
- Culture medium, supplies, and additives specific for your target cells
- Trypsin/EDTA (e.g., Sigma, Cat. No. T4049)
- Cloning cylinders or discs for isolating colonies of adherent cell lines (Sigma, Cat. No. C1059*)*
- Cell Freezing Medium, with or without DMSO (Sigma, Cat. Nos. C6164 or C6039), for freezing stable clones and Lenti-X 293T cell lines.
- 6-well, 12-well, 24-well and 96-well cell culture plates; 10 cm cell culture dishes

D. Lenti-X 293T Cells

- Lenti-X 293T Cell Line (Cat. No. 632180)

Getting the most from any lentiviral packaging system requires a host 293T cell line that transfects easily and supports high-level expression of viral proteins. Our Lenti-X 293T Cell Line was clonally selected to meets these requirements, allowing you to produce the highest possible lentiviral titers when combined with Lenti-X Packaging Single Shots (VSV-G), an optimized fourth-generation packaging system, pre-mixed and lyophilized with Xfect Transfection Reagent.

E. High-Titer Packaging System

Cat. No.	Lentiviral Packaging System
631275	Lenti-X Packaging Single Shots (VSV-G) (16 rxns)
631276	Lenti-X Packaging Single Shots (VSV-G) (96 rxns)

F. Lentiviral Titer Determination

For accurate and consistent transductions, we highly recommend titrating your lentiviral stocks. Various technologies are available from Takara Bio; visit **takarabio.com** for details.

Cat. No.	Lentiviral Titration Technology
632200	Lenti-X p24 Rapid Titer Kit (96 rxns)
631235	Lenti-X qRT-PCR Titration Kit (200 rxns)
631243	Lenti-X GoStix (20 tests)

G. Lentivirus Concentration

Use Lenti-X Concentrator to simply increase your available titer up to 100-fold or reduce sample volume, without ultracentrifugation—visit **takarabio.com** for details.

Cat. No.	Concentrator
631231	Lenti-X Concentrator (100 ml)
631232	Lenti-X Concentrator (500 ml)

H. Transduction Enhancers

Use Polybrene (hexadimethrine bromide; Sigma-Aldrich, No. H9268), Lenti-X Accelerator (see below), or RetroNectin® (see below).

- Lenti-X Accelerator is a magnetic bead-based technology designed to accelerate lentiviral and retroviral transduction experiments; visit **takarabio.com** for details.
- RetroNectin is a multivalent molecule that simultaneously binds virus particles and cell surface proteins, maximizing cell-virus contact. RetroNectin, in particular, is recommended for increasing the transduction efficiency of suspension cells and hematopoietic stem cells; visit **takarabio.com** for details.

Cat. No.	Transduction Enhancer	Size
631256	Lenti-X Accelerator	400 µl
631257	Lenti-X Accelerator	1,000 µl
631254	Lenti-X Accelerator Starter Kit	each
T110A	RetroNectin Precoated Dish	10 dishes
T100B	RetroNectin Recombinant Human Fibronectin Fragment	2.5 mg
T100A	RetroNectin Recombinant Human Fibronectin Fragment	0.5 mg

I. Plasmid Purification (Transfection-Grade)

Cat. No.	Product	Size
740412.10	NucleoBond Xtra Midi Plus	10 preps
740416.10	NucleoBond Xtra Maxi Plus	10 preps
740422.10	NucleoBond Xtra Midi EF Plus	10 preps
740426.10	NucleoBond Xtra Maxi EF Plus	10 preps

J. Assays for Determining Editing Efficiency

These items are required when determining the efficiency of gene editing and the nature of those edits

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

K. Molecular Biology Supplies

- Target-specific oligos (see Section V.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. 352017 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)

IV. Protocol Overview

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

A. General Cell Culture

This user manual provides only general guidelines for mammalian cell culture techniques. For users requiring more information on mammalian cell culture, transfection, and creating stable cell lines, we recommend the following general reference:

Freshney, R.I. (2010). *Culture of Animal Cells: A Manual of Basic Technique, 6th Edition* (Wiley-Liss, Hoboken, NJ).

B. Safety Guidelines for Working with Lentiviruses

The protocols in this User Manual require the production, handling, and storage of infectious lentivirus. It is imperative to fully understand the potential hazards of, and necessary precautions for, the laboratory use of lentiviruses.

The National Institute of Health and Center for Disease Control have designated recombinant lentiviruses as Level 2 organisms. This requires the maintenance of a Biosafety Level 2 facility for work involving this virus and others like it. The VSV-G pseudotyped lentiviruses packaged from the HIV-1-based vectors described here are capable of infecting human cells. The viral supernatants produced by these lentiviral systems could, depending on your insert, contain potentially hazardous recombinant virus. Similar vectors have been approved for human gene therapy trials, attesting to their potential ability to express genes *in vivo*.

IMPORTANT: For these reasons, due caution must be exercised in the production and handling of any recombinant lentivirus. **The user is strongly advised not to create VSV-G pseudotyped lentiviruses capable of expressing known oncogenes.**

For more information on Biosafety Level 2 agents and practices, download the following reference:

> Biosafety in Microbiological and Biomedical Laboratories. *Public Heal. Serv. Centers Dis. Control Prev. Natl. Institutes Heal. HHS Publ. No.* 21–1112.

Available on the web at **https://www.cdc.gov/labs/BMBL.html**

Biosafety Level 2: The following information is a brief description of Biosafety Level 2. *It is neither detailed nor complete.* Details of the practices, safety equipment, and facilities that combine to produce a Biosafety Level 2 are available in the above publication. If possible, observe and learn the practices described below from someone who has experience working with lentiviruses.

Summary of Biosafety Level 2:

- **Practices:**
  - Standard microbiological practices
  - Limited access to work area
  - Biohazard warning signs posted
  - Minimize production of aerosols
  - Decontaminate potentially infectious wastes before disposal
  - Use precautions with sharps (e.g., syringes, blades)
  - Biosafety manual defining any needed waste decontamination or medical surveillance policies
- **Safety equipment:**
  - Biological Safety Cabinet, preferably a Class II BSC/laminar flow hood (with a HEPA microfilter) used for all manipulations of agents that cause splashes or aerosols of infectious materials; exhaust air is not recirculated
  - PPE: protective laboratory coats, gloves, face protection as needed
- **Facilities:**
  - Autoclave available for waste decontamination
  - Chemical disinfectants available for spills

C. Protocol Overview

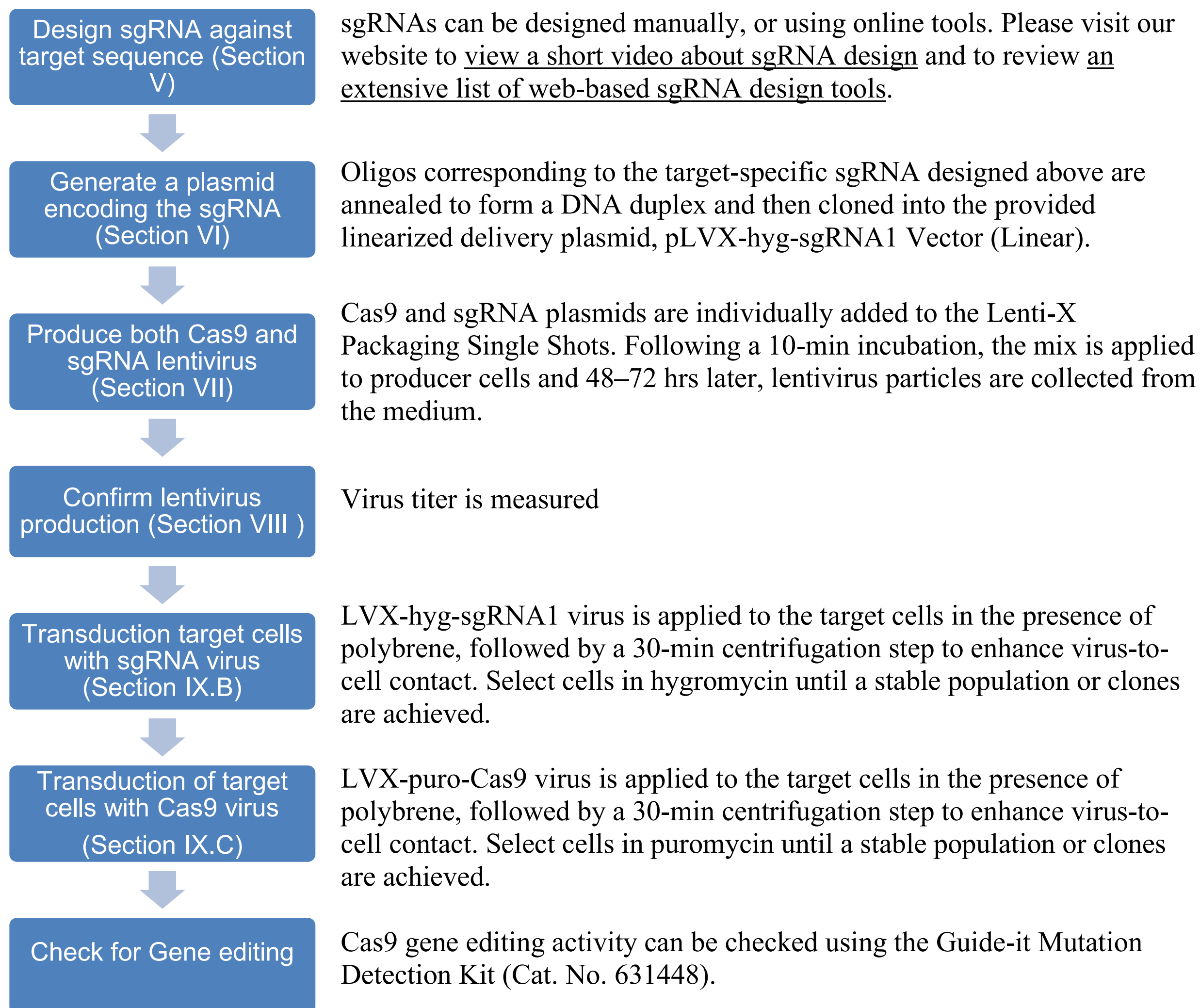


Figure 2. Workflow of a genome editing experiment using the Lenti-X CRISPR/Cas9 System.

V. 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.

A

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

5′ TTG TTA TCC GCT GTG AAT CGG ATC CAG CTG ACT **GG**A 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 AC**T **GG**A 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 A**GG** 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 A**GG** C**GA 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 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, sgRNAs that fulfill both requirements are most effective in the Lenti-X CRISPR/Cas9 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 Lenti-X CRISPR/Cas9 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 pLVX-hyg-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 V.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 pLVX-hyg-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 GXXX XXX XXX XXX XXX XXX XX-3'`
- Oligo 2: `5'-aaac YY YYY YYY YYY YYY YYY YYYC-3'`

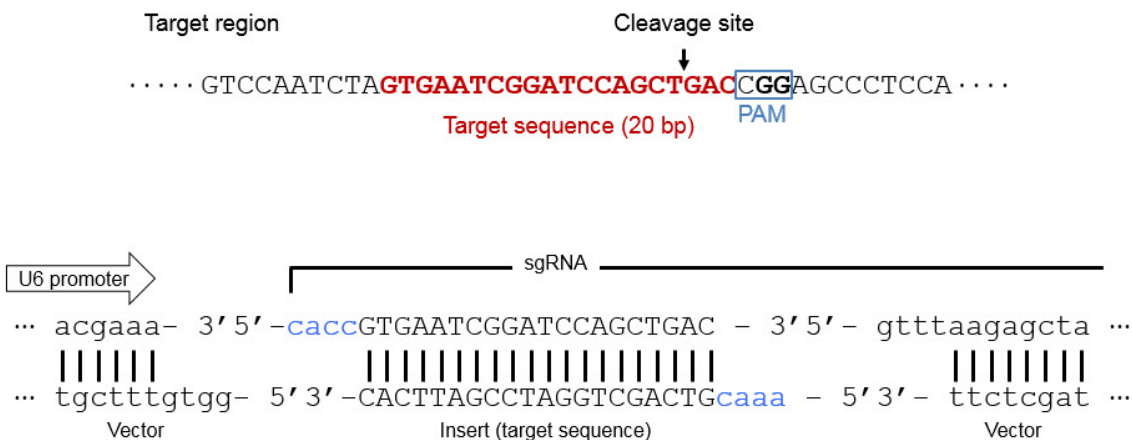


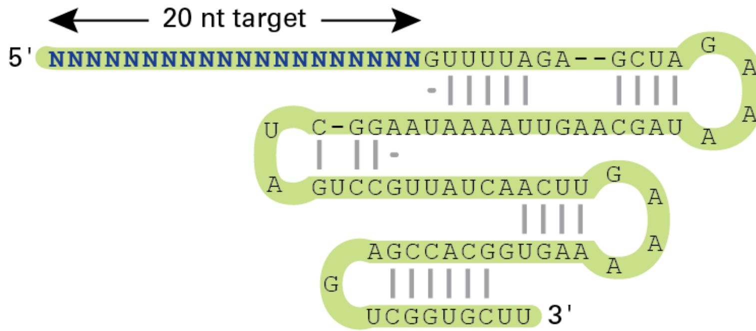
Figure 5. Example of cloning a target sequence using the Lenti-X CRISPR/Cas9 System. This particular example corresponds to the sequence of the Guide-it Control Annealed Oligos v2 included in the kit. The target region (marked in red) is shown in the top half of the figure. Both oligos contain the additional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

VI. Generate a Plasmid Encoding the sgRNA

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

We have modified the sgRNA scaffold in the pLVX-hyg-sgRNA1 Vector to improve the Cas9/sgRNA interaction, ensuring high editing efficiency (Figure 6). Due to the nature of the Cas9/sgRNA complex, it is important to maximize the efficient binding of the sgRNA to the Cas9 endonuclease by using this modified scaffold.

Traditional scaffold



Optimized scaffold

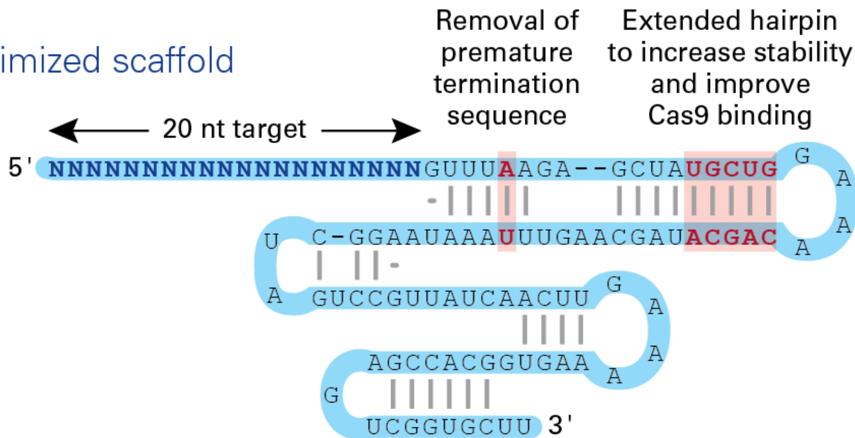


Figure 6. pLVX-hyg-sgRNA1 Vector (Linear) includes an improved sgRNA scaffold design for increased editing efficiency.

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 pLVX-hyg-sgRNA1

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

2 µl	pLVX-hyg-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

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.

VII. Produce Cas9 and sgRNA Lentiviruses

For production of lentivirus expressing your guide RNA and Cas9, follow the **Lenti-X Packaging Single Shots (VSV-G) Protocol-At-A-Glance**. (Locate this protocol by searching at **takarabio.com/manuals**).

VIII. Lentivirus Titration

A. Summary

1. Instant Qualitative Titer Test

You can assess the quality of your lentivirus stock in ten minutes with Takara Bio **Lenti-X GoStix** (Cat. Nos. 631241, 631243 & 631244). The GoStix detect lentiviral p24 in only 20 µl, and can be used to determine whether virus production is within a usable range or for selecting the best time to harvest your virus. A 3 prep sample is included with many of Takara Bio's Lenti-X systems.

2. Quantitative Titer Test

Determining the viral titer is necessary to obtain the following information:

- Confirmation that viral stocks are viable
- The proper transduction conditions for your particular cell type by adjusting the MOI for the desired transduction efficiency. MOI = No. of infectious virus particles per target cell
- The maximum number of target cells that can be transduced by a given virus volume.

To transduce using a known multiplicity of infection (MOI), it is necessary to titrate your lentiviral stocks. We recommend the **Lenti-X qRT-PCR Titration Kit** (Cat. No. 631235) or **Lenti-X p24 Rapid Titer Kit** (Cat. No. 632200) for very rapid quantitative titrations of virus stocks (~4 hr), or a standard method that relies on infection.

The **standard viral titration protocol** consists of infecting cells with serial dilutions of the stock, selecting for stable transductants with antibiotic and counting the resulting cell colonies (Section VIII.B).

- Freshly harvested virus can be titrated immediately, concentrated, or frozen in aliquots at –80°C and then titrated. Note that each freeze-thaw cycle can reduce the functional titers of infectious virus by up to 2–4 fold.
- Absolute titers will depend heavily on the cell type used for titration, and there may be significant differences between the titer values determined in cells typically used for lentiviral titration (i.e. HT-1080) and the number of target cells transduced by the titrated virus. However, titrations serve to determine the relative virus content of different viral stocks prepared from different vectors.

B. Protocol: Determining Viral Titer Using Antibiotic Selection

NOTE: This protocol can be completed in 7–14 days.

1. Plate HT-1080 cells (or other) in 6-well plates the day before performing the titration infections. Plate 2 x 10^5 cells/well, in 2 ml of medium. Allow at least one well to be used as a "no infection" control.
2. Prepare 20 ml of complete medium and add 60 µl of 4 mg/ml Polybrene. This will be diluted 3-fold for a final Polybrene concentration of 4 µg/ml.

NOTE: Polybrene is a polycation that reduces charge repulsion between the virus and the cellular membrane. The optimum final concentration of Polybrene may be determined empirically but

generally falls within a range of 2–12 µg/ml. Excessive exposure to Polybrene (>24 hr) can be toxic to cells.

3. Prepare filtered viral supernatant from packaging cells (Section VII). This is the virus stock.
4. Prepare six 10-fold serial dilutions of the virus stock as follows:
   a. Add 1.35 ml of medium containing Polybrene (Step 2) to each of six sterile and numbered 1.5 ml microfuge tubes.
   b. Add 150 µl of the virus stock (Step 3) to the tube 1. Mix.
   c. Transfer 150 µl tube 1 to tube 2 and mix. Continue making serial dilutions by transferring 150 µl from each successive dilution into the next prepared tube.
5. Infect the HT-1080 cells by adding 1 ml of each viral dilution (Step 4) to each appropriate well. The final Polybrene concentration will be 4 µg/ml in ~3 ml. Centrifuge the cultures to improve infection efficiency*.

   ***NOTE:** CULTURE CENTRIFUGATION INCREASES INFECTION EFFICIENCY. Centrifuging the plate at 1,200 x g for 60–90 min at 32°C can significantly increase infection efficiency. A room temperature centrifuge is acceptable if a 32°C unit is not available.

6. After infecting for 8–24 hours, remove supernatants and subject the cells to antibiotic selection using the selection concentrations that are optimal for your cell line (Section III.B).
7. Allow colonies to form for 7–14 days. Stain the colonies with 1% crystal violet solution (in 10% ethanol) and count.
8. The titer of virus corresponds to the number of colonies generated by the highest dilution, multiplied by the dilution factor. For example, the presence of 4 colonies in the 10^6 dilution would represent a viral titer of 4 x 10^6 colony forming units.

IX. Transducing Target Cells

A. Summary

- **Creating a population of sgRNA expressing cells:** It is our experience that the most efficient editing occurs when the sgRNA and Cas9 are introduced sequentially. Once the sgRNA is introduced and a population or clones are selected, these can then be used for transduction with the Cas9 lentivirus.

NOTE: We do not recommend co-transduction with both viruses as this results in little to no editing.

B. Protocol: Transducing Target Cells for sgRNA Expression

NOTE: This protocol can be completed in ~2 weeks.

1. Plate target cells in complete growth medium 12–18 hr before transduction. The cells should be 70–80% confluent at the time of transduction.
2. Thaw aliquots of your pLVX-hygro-sgRNA1 Vector (Linear) lentiviral stock, or use filtered virus stocks freshly prepared from packaging cells (Section VII).
3. Add polybrene to the cell cultures to obtain the desired final concentration during the transduction step (e.g., 4 µg/ml).

NOTE: Lenti-X Accelerator (Cat. Nos. 631256, 631257 & 631254) and **RetroNectin** (Cat. Nos. T110A, T100B & T100A) may be used as transduction enhancers instead of Polybrene (see Section III.G).

4. Transduce your target cells at an MOI of 1–10 so that every cell is transduced at least once. Make sure that the total volume of viral supernatant used makes up no more than 1/3 the final volume of culture medium used in the transduction. Centrifuge the cultures to improve transduction efficiency.

   ***NOTE:** CULTURE CENTRIFUGATION INCREASES INFECTION EFFICIENCY. Centrifuging the plate at 1,200 x g for 60–90 min at 32°C can significantly increase infection efficiency. A room temperature centrifuge is acceptable if a 32°C unit is not available.

5. Transduce the cells for 8–24 hr. If you are concerned that exposure to either the Polybrene or to the viral supernatant (which contains medium conditioned by the packaging cells) may adversely affect your target cells, limit the transduction to 6–8 hr.
6. Remove and discard the virus-containing medium and replace it with fresh growth medium, as appropriate.

   CAUTION: Discarded medium contains infectious lentivirus.

7. At 24 hr post-transduction, remove medium, trypsinize and split the cells. Cells from a single well of a 6-well plate should be split into 10 cm dishes containing complete growth medium supplemented with hygromycin at the appropriate concentration (Section III.B)
8. After ~2 weeks, hygromycin-resistant colonies should begin to appear.
9. When the colonies are large enough to transfer, use cloning cylinders or disks to harvest (i.e., “pick”) large, healthy colonies, and transfer each into a separate well of a 24-well plate.
10. Isolate 10–20 colonies and culture in a maintenance concentration of hygromycin (Section III.B).
11. Alternately, clones may be trypsinized and pooled to create a transduced population of sgRNA-expressing cells.

C. Protocol: Transducing Target Cells for Cas9 Editing

NOTE: This protocol can be completed in ~2 weeks.

1. Plate target cells selected for sgRNA expression in a well of a 6-well plate in complete growth medium 12–18 hr before transduction. The cells should be 70–80% confluent at the time of transduction.
2. Thaw aliquots of your pLVX-puro-Cas9 Vector lentiviral stock, or use filtered virus stocks freshly prepared from packaging cells (Section VII).
3. Add Polybrene to the cell cultures to obtain the desired final concentration during the transduction step (e.g., 4 µg/ml).

   NOTE: Lenti-X Accelerator (Cat. Nos. 631256, 631257 & 631254) and **RetroNectin** (Cat. Nos. T110A, T100B & T100A) may be used as transduction enhancers instead of Polybrene (see Section III.G).

4. Transduce your target cells at an MOI of 1–10. Make sure that the total volume of viral supernatant used makes up no more than 1/3 the final volume of culture medium used in the transduction.

5. Transduce the cells for 24 hr. If you are concerned that exposure to either the Polybrene or to the viral supernatant (which contains medium conditioned by the packaging cells) may adversely affect your target cells, change the medium at 6–8 hr.
6. At 24 hr post-transduction, remove medium, trypsinize and split the cells. Cells from a single well of a 6-well plate should be split into 4 x 10 cm dishes containing complete growth medium supplemented with 0.1–1 μg/ml puromycin (Section III.B).

   CAUTION: Discarded medium contains infectious lentivirus.
7. After 1–2 weeks, puromycin-resistant colonies should begin to appear.
8. When the colonies are large enough to transfer, use cloning cylinders or disks to harvest (i.e., "pick") large, healthy colonies, and transfer each into a separate well of a 24-well plate.
9. Isolate 10–20 colonies and culture in a maintenance concentration of puromycin (Section III.B).
10. When confluent, split the cells from each well into three wells of a 6-well plate for testing and maintenance (Section IX.E).
11. Alternately, clones may be trypsinized and pooled to create a transduced population of both sgRNA and Cas9-expressing cells.
12. Genome editing can be tested for using the Guide-it Mutation Detection Kit (Cat. No. 631448)

    NOTE: You may wish to use Cas9 monoclonal antibody (Section III.L) to determine, via Western blot, which clones express the Cas9 protein. However, Western analysis should not be used to substitute for a functional test for genome editing (Section IX.E), since the highest expressing Tet-On 3G clones often do not provide the highest fold inducibility.

X. References

Hsu, P. D. *et al.* DNA targeting specificity of RNA-guided Cas9 nucleases. *Nat. Biotechnol.* **31,** 827–832 (2013).

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Appendix A. Troubleshooting Guide

Table 2. Troubleshooting Guide for the Lenti-X CRISPR/Cas9 System.

Problem	Possible Explanation	Solution
A. 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
B. Lenti-X 293T Packaging Cells		
Poor viability upon thawing	Improper thawing techniques	Use thawing procedure in Section V.B, and/or consult the Lenti-X 293T Cell Line Protocol-at-a-Glance

	Incorrect culture medium	Use DMEM with additives listed in Section II.B. Use 10% Tet System Approved FBS (Tc-free).
	Improper tissue culture plasticware	Use collagen I-coated plates to aid cell adherence during initial seeding.
Slow growth	Incorrect culture medium	Use DMEM with additives listed in Section II.B. Use 10% Tet System Approved FBS (Tc-free).
Cells do not attach to plate	Improper tissue culture plasticware	Use collagen I-coated plates to aid cell adherence during initial seeding.
Cells appear morphologically different	Passage of cell culture is too high (old cells)	Thaw/purchase new aliquot of Lenti-X 293T cells.
C. Virus Production		
Poor transfection efficiency (as determined by GOI or marker expression in the Lenti-X 293T cell line)	Cells plated too densely	Plate 4–5 x 106 cells/100 mm plate, or fewer if the cells divide rapidly. Use at 50–80% confluency. See Section VI.
	Transfection is toxic to cells	Use the optimized conditions provided in Section VI.
	Cells harvested or analyzed too soon after transfection	Wait 48 hr after transfection for maximal expression of GOI or marker to determine efficiency.
Low titers (<105 cfu/ml)	Serum in medium contains tetracycline contaminants	Use **Tet System Approved FBS** (Cat. Nos. 631101, 631105, 631106 & 631107) in the 293T culture medium.
	Poor transfection efficiency	See above section. Concentrate the virus using centrifugation (see Appendix A) or use the **Lenti-X Concentrator** (Cat. Nos. 631231 & 631232) to increase your available titer up to 100-fold without ultracentrifugation.
	Virus was harvested too early	Harvest virus 48–72 hr after the start of transfection.
	Polybrene is missing or at suboptimal concentration	Add Polybrene (4 µg/ml) during transduction or optimize the concentration (2–12 µg/ml)
	Virus was exposed to multiple freeze-thaw cycles	Each cycle reduces titer by approximately 2–4 fold. Limit the number of freeze-thaws.
	Suboptimal selection procedure during titration	Perform an antibiotic kill curve on the cell line prior to using it for titration.

D. Transduction of Target Cells		
Poor transduction efficiency	Low titer	See Section C or use the Lenti-X Concentrator (Section II.E) to increase your available titer up to 100-fold without ultracentrifugation.
	Low viability of target cells during transduction	Optimize culture conditions for target cells prior to infection
		Packaging cell line-conditioned media may affect cell growth; dilute viral supernatant or shorten exposure time to viral supernatant. Consider using RetroNectin Reagent and the RetroNectin-Bound Virus transduction protocol or purify your virus prior to transduction using the **Lenti-X Maxi Purification Kit** (Cat. #s 631233 & 631234).
		Excessive exposure to Polybrene: optimize amount (titrate) or shorten exposure time to viral supernatant
	Viral supernatant contains transduction inhibitors	Use RetroNectin Reagent or RetroNectin-coated plates in the RetroNectin-Bound Virus transduction protocol, which allows virions to bind the RetroNectin substratum and be washed free of inhibitors prior to target cell infection; or, purify your virus prior to transduction using the Lenti-X Maxi Purification Kit (Cat. #s 631233 & 631234).
Infection is toxic to target cells	MOI too high (i.e. too much virus used)	Dilute virus stock; titrate the virus.
	Polybrene toxicity	Reduce or optimize Polybrene concentration; reduce infection time.
	Packaging cell supernatant or medium is toxic to cells	Dilute virus stock using target cell culture medium; harvest virus from packaging cells using target cell medium. Consider purifying your virus prior to transduction using the **Lenti-X Maxi Purification Kit** (Cat. #s 631233 & 631234).
Target cell treatment with Cas9/sgRNA Lentivirus		
No gene editing detected in 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

	Cas9 may be toxic to target cells	• Use a lower MOI for transducing the target cells with pLVX-puro-Cas9 and select for clones that survive

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