Takara Bio USA, Inc.

Lenti-X™ Tet-On® 3G CRISPR/Cas9 System User Manual

Cat. No. 632633
(013023)

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I. Introduction

A. Summary

CRISPR/Cas9 gene editing technology has revolutionized the field of genome editing, using two key components that form a complex: Cas9 endonuclease and a single guide RNA (sgRNA) that directs Cas9 to a specific target site in genomic DNA (Jinek et al. 2012). The **Lenti-X Tet-On 3G CRISPR/Cas9 System** (Cat. No. 632633) is a complete system for producing high yields of lentiviruses encoding the components necessary for doxycycline-inducible CRISPR/Cas9-mediated genome editing in mammalian cells that are difficult to transfect, including dividing and non-dividing cell types. By enabling tight control of Cas9 expression in target cells, this kit enables users to minimize the likelihood of phenomena associated with constitutive Cas9 expression, including off-target effects and cellular toxicity (Dow et al. 2015; Lin et al. 2014; Gonzalez et al. 2014). This system also contains necessary controls and sufficient reagents for construction of 10 different sgRNA expression plasmids, including pre-linearized pLVX-hyg-sgRNA1 vector, ligation components, and Stellar™ Competent Cells.

sgRNA construction
The kit workflow (Figure 1, next page) begins with insertion of a user-designed sgRNA-targeting sequence into the pre-linearized pLVX-hyg-sgRNA1 plasmid, which includes the human U6 promoter for constitutive sgRNA expression in target cells. In this step, a pair of user-provided oligos corresponding to the target genomic sequence of interest are annealed to form a duplex, which is then cloned into the pre-linearized vector using the included high-efficiency ligation mix.

Lentiviral production
Lentiviruses encoding the user-designed sgRNA, Tet-On 3G transactivator protein, and Cas9 endonuclease, respectively, are then generated using Lenti-X Packaging Single Shots (VSV-G). Lenti-X Packaging Single Shots (VSV-G)—pre-aliquoted, lyophilized, single tubes of Xfect™ Transfection Reagent premixed with an optimized formulation of Lenti-X lentiviral packaging plasmids—provide the highest titers and widest tropism for viral transduction.

Transduction of system components and screening of clones
Target cells are first transduced with the Tet-On 3G-encoding lentivirus, and G418 is used to select for positive clones. Following antibiotic selection, induction of the constitutively expressed Tet-On 3G transactivator protein is assayed in independent clones via introduction of a plasmid encoding a luciferase reporter under the P_{TRE3GV} promoter, and cells demonstrating the highest-fold induction of reporter activity (i.e., the highest ratio of induced luciferase expression to basal luciferase expression) are chosen.

Selected Tet-On 3G-positive cells are then transduced with the Cas9-encoding lentivirus, and puromycin is used to select for Cas9-positive clones. Inducibility of Cas9 expression (under the P_{TRE3GV} promoter) in selected clones can be pre-screened by Western blot (optional) and assayed by qPCR using the provided primers (recommended). As with the luciferase assay, clones demonstrating the highest-fold induction potential are selected for further use.

Selected Tet-On 3G-Cas9-positive cells are then transduced with the sgRNA-encoding lentivirus, and selection of sgRNA-positive clones can be performed using hygromycin (optional). Following induction of Cas9 expression with doxycycline, editing of genomic targets can be assayed using a variety of approaches (see Appendix A for examples of data generated from cells that were edited using this system).

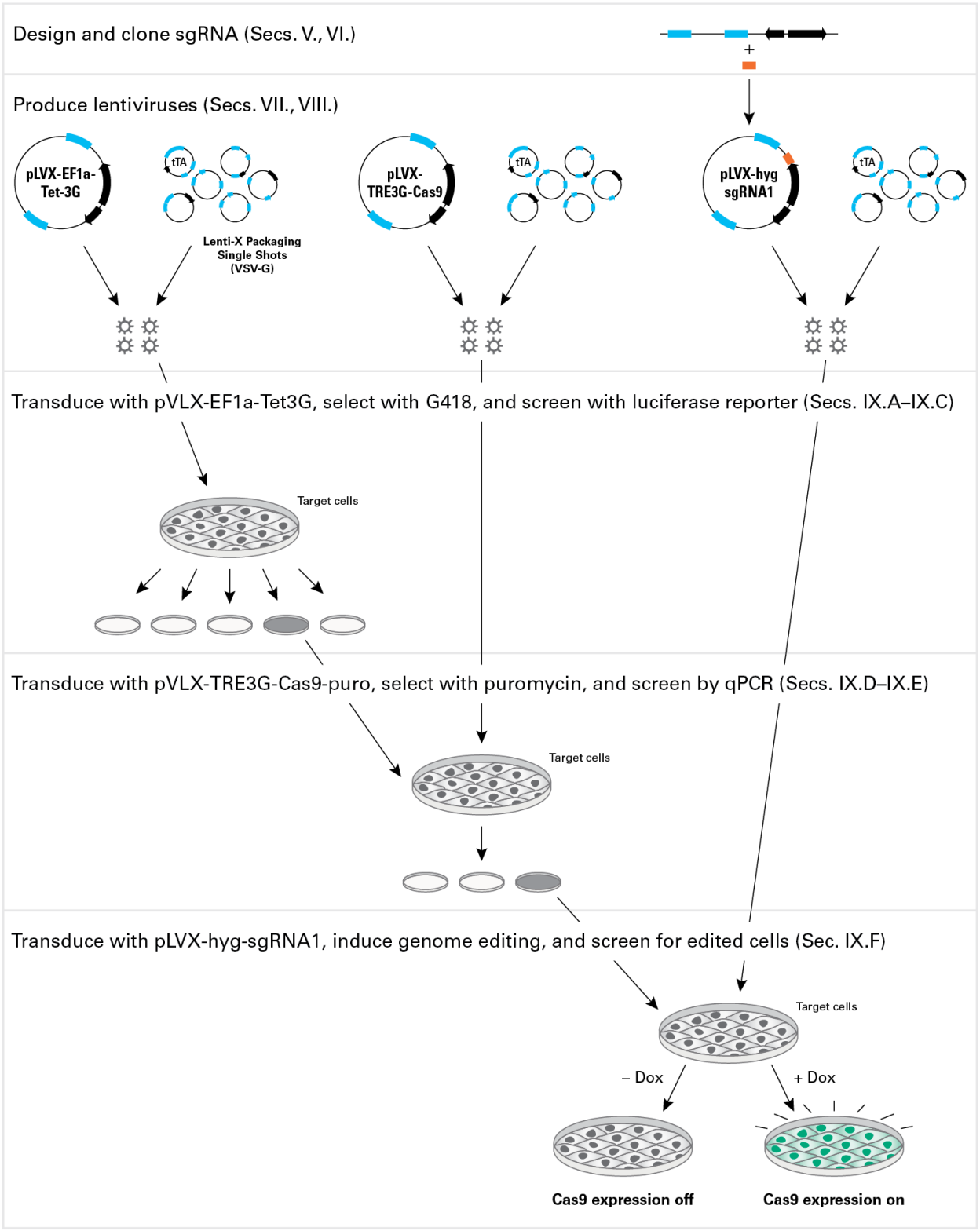


Figure 1. Protocol overview for the Lenti-X Tet-On 3G CRISPR/Cas9 System.

B. Elements of Lenti-X Tet-On 3G CRISPR/Cas9 System

pLVX-hyg-sgRNA1 Vector (Linear): cloning and constitutive expression of user-designed sgRNA
pLVX-hyg-sgRNA1 Vector (Linear) is a prelinearized vector ready for insertion of a user-designed sgRNA sequence for expression under the constitutive human U6 promoter. Design of the sgRNA sequence is discussed in detail in Section V. This vector also includes all of the elements necessary for lentiviral production and antibiotic selection of transduced cells with hygromycin.

pLVX-EF1a-Tet3G Vector: constitutive expression of Tet-On 3G transactivator protein
pLVX-EF1a-Tet3G encodes the Tet-On 3G transactivator protein under the control of the constitutive EF-1 alpha promoter and includes all of the elements necessary for lentiviral production and antibiotic selection of transduced cells with G418. Originating from a series of inducible transcription factors developed previously (Gossen and Bujard 1992; Gossen et al. 1995; Urlinger et al. 2000), Tet-On 3G is a modified form of the Tet-On Advanced transactivator protein, which was evolved to have a far higher sensitivity to doxycycline (Zhou et al. 2006). Constitutive expression of Tet-On 3G is driven by the EF-1 alpha promoter, which was derived from *EEF1A1*, the human gene encoding the alpha subunit of the eukaryotic elongation factor-1 complex. In contrast with the commonly used CMV promoter, EF-1 alpha is less susceptible to silencing by certain cell types (including various types of stem cells), enabling the establishment of inducible cell lines that are suitable for long-term use.

pLVX-TRE3G-Cas9-puro Vector: inducible expression of Cas9 endonuclease
pLVX-TRE3G-Cas9-puro encodes a modified version of the Cas9 endonuclease under the control of the inducible P_{TRE3GV} promoter and includes all of the elements necessary for lentiviral production and antibiotic selection of transduced cells with puromycin. The Cas9 sequence encoded in pLVX-TRE3G-Cas9-puro was originally derived from the bacterium *S. pyogenes*, and has been codon optimized for expression in mammalian cells. Additional Cas9 modifications include the incorporation of a nuclear localization signal (NLS) and a tag comprising the amino acid sequence DYKDDDDK. When expressed in target cells, Cas9 is directed to cleave DNA at specific locations in the genome via physical association with corresponding sgRNA molecules (encoded by pLVX-hyg-sgRNA1 in this system).

The inducible promoter P_{TRE3G} provides for very low basal expression and high maximal expression after induction (Loew et al. 2010). It consists of seven repeats of a 19-bp tet operator sequence located upstream of a minimal CMV promoter. P_{TRE3GV} is a version of P_{TRE3G} that was modified for higher performance in lentiviruses and retroviruses. In the presence of doxycycline, Tet-On 3G binds specifically to P_{TRE3GV} and activates transcription of the downstream Cas9 enzyme. P_{TRE3GV} lacks binding sites for endogenous mammalian transcription factors, so it is virtually silent in the absence of induction.

By allowing for tight control of Cas9 expression via the inducible P_{TRE3GV} promoter, the Lenti-X Tet-On 3G CRISPR/Cas9 system enables users to minimize the occurrence of undesired genomic cleavage events (off-target effects) associated with constitutive Cas9 endonuclease activity (Hsu et al. 2013; Fu et al. 2013; Gonzalez et al. 2014; Dow et al. 2015). While several groups have overcome the challenge of off-target effects via direct delivery of Cas9-sgRNA ribonucleoprotein (RNP) complexes to target cells (Zuris et al. 2014; Lin et al. 2014), this method does not provide consistent performance for a broad range of cell types, and can cause cellular toxicity.

4th Generation Lentiviral Packaging System
Lenti-X Packaging Single Shots (VSV-G), provided with the **Lenti-X Tet-On 3G CRISPR/Cas9 System** (Cat. No. 632633), can generate lentiviral titers that are superior to most other commercially available lentiviral packaging systems. The concerted effects of multiple components in an optimized

five-vector plasmid mix, pre-aliquoted and lyophilized with Xfect Transfection Reagent, allow **Lenti-X 293T Cells** (sold separately; Cat. No. 632180) to produce the highest amounts of safe, replication-incompetent lentivirus (see takarabio.com).

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 Tet-On 3G CRISPR/Cas9 System (Cat. No. 632633)

- 20 µl pLVX-hyg-sgRNA1 Vector (Linear) (Cat. No. 632632; 7.5 ng/µl; not sold separately)
- 20 µl pLVX-EF1a-Tet3G Vector (Cat. No. 631359; 500 ng/µl; not sold separately)
- 1 each pLVX-TRE3G-Cas9-puro Vector Set (Cat. No. 632634; not sold separately)
    - 20 µl pLVX-TRE3G-Luc Control Vector (500 ng/µl)
    - 20 µl pLVX-TRE3G-Cas9-puro Vector (500 ng/µl)
    - 50 µl Cas9 qPCR Primer Mix (10 µM)
- 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)
- 1 each Lenti-X Packaging Single Shots (VSV-G) (Cat. No. 631275; 16 rxns)

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)
631367	Tet System Approved FBS (3 x 500 ml)
631101	Tet System Approved FBS, US-Sourced (500 ml)
631105	Tet System Approved FBS, US-Sourced (50 ml)
631368	Tet System Approved FBS, US-Sourced (3 x 500 ml)

B. Antibiotics for Selecting Stable Cell Lines

pLVX-hyg-sgRNA1, pLVX-EF1a-Tet3G, and pLVX-TRE3G-Cas9-puro contain hygromycin, G418, and puromycin resistance markers, respectively, for selection of stable clones or populations. Use the following recommended antibiotic concentrations:

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

		Recommended Concentration (µg/ml)	
Cat. No.	**Antibiotic**	**Selecting Colonies[1]**	**Maintenance**
631309	Hygromycin B (1 g)	50–400	100
631307	G418 (1 g)	100–800	200
631308	G418 (5 g)		
631305	Puromycin (25 mg)	0.25–10	0.25
631306	Puromycin (100 mg)		

[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 (G418 & hygro). If all of 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, Cat. No. D5796); 10% Fetal Bovine Serum (FBS).
- Culture medium, supplies, and additives specific to your target cells
- Trypsin/EDTA (Sigma-Aldrich, Cat. No. T4049)
- Cloning cylinders or discs for isolating colonies of adherent cell lines (Sigma-Aldrich, Cat. No. C1059*)*
- Cell freezing medium, with or without DMSO (Sigma-Aldrich, Cat. Nos. C6164 or C6039), for freezing stable clones or 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 meet 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. 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)

F. 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)

G. Transduction Enhancers

Use polybrene (hexadimethrine bromide; Sigma-Aldrich, Cat. No. H9268), Lenti-X Accelerator (see below), or RetroNectin® reagent (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 reagent is a multivalent molecule that simultaneously binds virus particles and cell surface proteins, maximizing cell-virus contact. RetroNectin reagent, in particular, is recommended for increasing the transduction efficiency of suspension cells and 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

H. 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

I. Doxycycline

- 5 g Doxycycline (Cat. No. 631311)
  Dilute to 1 mg/ml in double distilled H_2O. Filter sterilize, aliquot, and store at –20°C in the dark. Use within one year.

J. Luciferase Assay and Luminometer

These items are required when using pLVX-TRE3G-Luc to test for induction (Section IX.C). Any standard firefly luciferase assay system and luminometer can be used, but we recommend the Luciferase Assay System (Promega, Cat. No. E1500).

K. Cas9 Antibody

Guide-it Cas9 antibodies are raised against recombinant Cas9 protein from *Streptococcus pyogenes*. These antibodies recognize the modified version of Cas9 included with the Lenti-X Tet-On 3G CRISPR/Cas9 System, and are suitable for Cas9 detection by Western blot.

Cat. No.	Product	Size
632628	Guide-it Cas9 Monoclonal Antibody	100 µl
632627	Guide-it Cas9 Monoclonal Antibody	3 x 100 µl
632607	Guide-it Cas9 Polyclonal Antibody	100 µl
632606	Guide-it Cas9 Polyclonal Antibody	3 x 100 µl

L. qRT-PCR

To quantify Cas9 expression in the presence and absence of doxycycline using the included primer set, we recommend the following qRT-PCR reagents:

Cat. No.	Product	Size
RR82LR	TB Green® *Premix Ex Taq*™ II (Tli RNase H Plus), ROX Plus	200 rxns
2680A	PrimeScript™ Reverse Transcriptase	10,000 U
2313A	Recombinant RNase Inhibitor	5,000 U

M. Assays for Determining Editing Efficiency

These items are required for determining the efficiency of gene editing and the nature of the edits:

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

N. TetR Monoclonal Antibody

If you wish to confirm that Tet-On 3G is expressed in your cells, we recommend that you use the following antibody and detect the protein via Western blot:

Cat. No.	Product	Size
631131	TetR Monoclonal Antibody (Clone 9G9)	40 µg
632606	TetR Monoclonal Antibody (Clone 9G9)	200 µg

O. Molecular Biology Supplies

- Target-specific oligos (see Section V.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, 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 Fisher Scientific, 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-Blackwell, 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 Institutes of Health and the Centers for Disease Control and Prevention have designated recombinant lentiviruses as Level 2 organisms. This requires the maintenance of a Biosafety Level 2 (BSL-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 (BMBL), Fifth Edition (December 2009) HHS Pub. No. (CDC) 93-8395. U.S. Department of Health and Human Services Centers for Disease Control and Prevention and NIH.

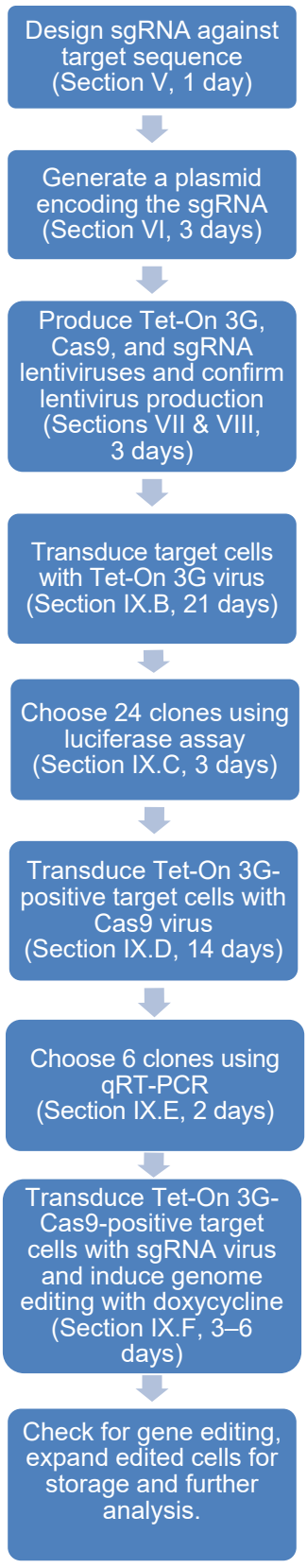
Available on the web at http://www.cdc.gov/biosafety/publications/bmbl5/

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 unrecirculated
  - PPE: protective laboratory coats, gloves, face protection as needed
- **Facilities:**
  - Autoclave available for waste decontamination
  - Chemical disinfectants available for spills

C. Workflow 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, pLVX-hyg-sgRNA1 Vector (Linear).

pLVX-EF1a-Tet3G, pLVX-TRE3G-Cas9-puro, and pLVX-hyg-sgRNA1 are added individually to Lenti-X Packaging Single Shots. Following a 10-min incubation, each mix is applied to separate plates of 293T producer cells and 48–72 hr later, lentivirus particles are collected from the media and virus titers are measured.

pLVX-EF1a-Tet3G virus is applied to target cells in the presence of polybrene, followed by a 30-min centrifugation step to enhance virus-to-cell contact. Cells are selected in G418 until a stable population or clones are obtained

Introduction of a luciferase reporter (pLVX-TRE3G-Luc) allows for identification of clones with the lowest residual activity and highest-fold induction of the P_{TRE3GV} promoter. Optimal clones are expanded into stable cell lines for storage and subsequent use.

pLVX-TRE3G-Cas9-puro virus is applied to chosen Tet-On 3G-positive target cells in the presence of polybrene, followed by a 30-min centrifugation step to enhance virus-to-cell contact. Cells are selected in puromycin until a stable population or clones are obtained.

Analysis of Cas9 induction by qRT-PCR allows for identification of optimal clones, which are expanded into stable cell lines for storage and subsequent use. Western blot can be used to pre-screen for Cas9 inducibility.

pLVX-hyg-sgRNA1 virus is applied to chosen Tet-On 3G-Cas9-positive target cells in the presence of polybrene, followed by a 30-min centrifugation step to enhance virus-to-cell contact. Beginning on the following day, Cas9 expression is induced by culturing cells in doxycycline (500 ng/ml).

Cas9 gene editing activity can be checked using the Guide-it Mutation Detection Kit (Cat. No. 631448). Cells that have been modified successfully are expanded for storage and further analysis.

Figure 2. Summary of a genome editing experiment using the Lenti-X Tet-On 3G CRISPR/Cas9 System.

V. Design sgRNA Against Target Sequence

A. Protocol: 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 for manual sgRNA 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 CRISPR/Cas9 gene 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 Tet-On 3G 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 Tet-On 3G 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 Complete sgRNA Screening System (Cat. No. 632636) 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. Protocol: 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 Section 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 V.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 (G), it is necessary to add an extra G 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′`

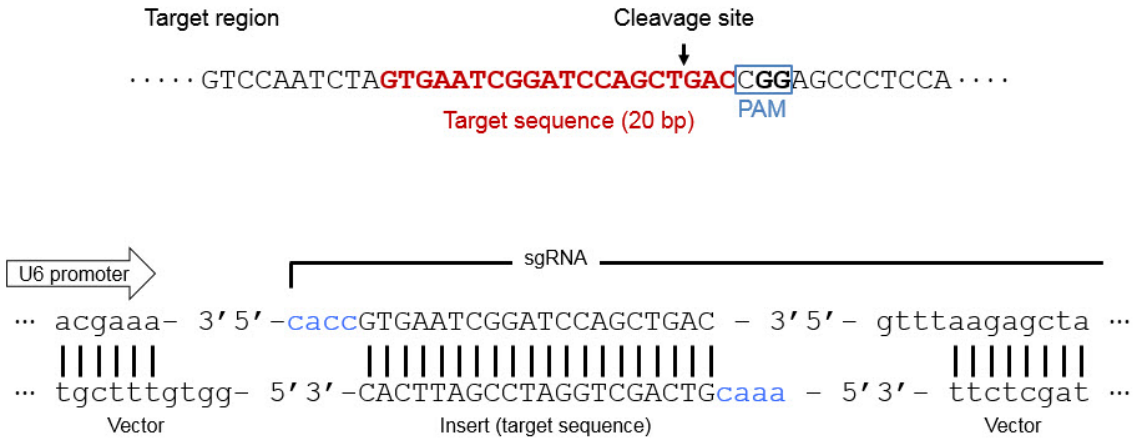


Figure 5. Example of cloning a target sequence for use in the Lenti-X Tet-On 3G CRISPR/Cas9 System. This particular example corresponds to the sequence of the Guide-it Control Annealed Oligos v2 included in the kit. The target region (marked in red) is shown in the top half of the figure. Both oligos 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.

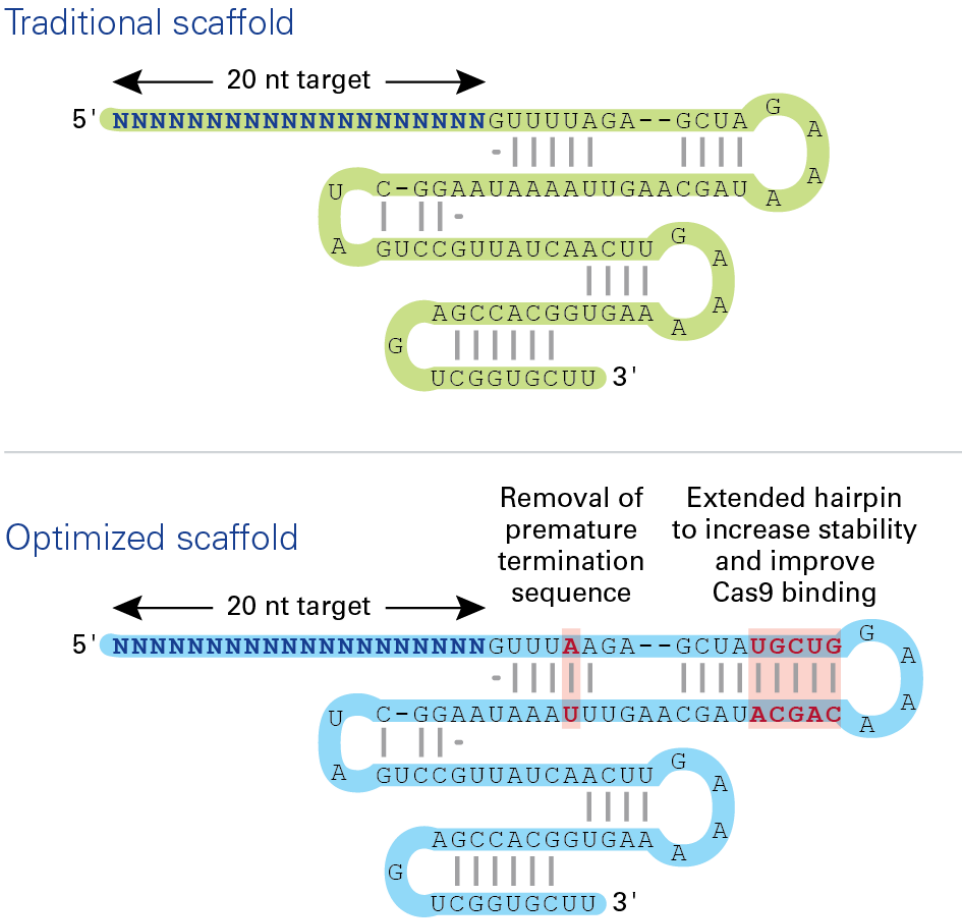


Figure 6. pLVX-hyg-sgRNA1 includes an improved sgRNA scaffold design for increased editing efficiency.

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: Isolating and Analyzing 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 Tet-On 3G, Cas9, and sgRNA Lentiviruses

For production of lentiviruses encoding Tet-On 3G, Cas9, and user-designed sgRNAs, follow the **Lenti-X Packaging Single Shots (VSV-G) Protocol-At-A-Glance**. (Locate this protocol by searching at takarabio.com/manuals).

NOTE: If you plan to assay induction of Tet-On 3G-positive clones via transduction of the luciferase reporter pLVX-TRE3G-Luc (Section IX.C), you should also produce lentivirus with this vector.

VIII. Lentivirus Titration

A. Summary

Determining the viral titer is necessary for obtaining 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 = number of infectious virus particles per target cell
- The maximum number of target cells that can be transduced by a given virus volume.

1. Instant Qualitative Titer Test

You can assess the quality of your lentivirus stock in ten minutes with **Lenti-X GoStix** (Cat. Nos. 631243 & 631244). 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 three-prep sample is included with many of the Lenti-X systems.

2. Quantitative Titer Tests

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 (or other) cells 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 tube 1. Mix.
   c. Transfer 150 µl of the dilution from 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*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 four colonies in the 10^6 dilution would represent a viral titer of 4 x 10^6 colony forming units (cfu).

IX. Transducing Target Cells

A. Summary

Creating a population of Tet-On 3G-Cas9-sgRNA-expressing cells

Genome editing can be performed successfully with few off-target effects when Cas9 expression is tightly controlled. Development of a stable Tet-On 3G-Cas9 cell line with optimal induction characteristics (i.e., lowest residual expression of Cas9 in the absence of doxycycline and highest-fold induction of Cas9 in the presence of doxycycline) is imperative to achieving this objective.

Following transduction of pLVX-EF1a-Tet3G and selection of positive clones with G418, the included pLVX-TRE3G-Luc Control Vector can be introduced into candidate cells via transfection or transduction, allowing for identification of optimal clones with a simple luciferase assay. Chosen target cells are then transduced with pLVX-TRE3G-Cas9-puro, and positive clones are selected with puromycin.

Following selection of Cas9-positive clones, induction of Cas9 expression with doxycycline can be pre-screened by Western blot (optional) and assayed via qRT-PCR with provided primers (recommended). Chosen Tet-On 3G-Cas9-positive target cells are then transduced with pLVX-hyg-sgRNA1, followed by selection of positive clones with hygromycin, and induction of genome editing with doxycycline.

NOTES:

- We do not recommend co-transduction with all three viruses as this results in little controlled editing.
- In addition to qRT-PCR and Western blot, optimal Tet-On 3G-Cas9-positive clones can be identified by transducing candidate cells with pLVX-hyg-sgRNA1 and assaying genome editing. We recommend the Guide-it Mutation Detection Kit (Cat. Nos. 631443 & 631448) for determining the efficiency of genome editing.

B. Protocol: Transducing Target Cells to Create Stable Tet-On 3G Clones

NOTE: This protocol can be completed in ≤3 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-EF1a-Tet3G 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) or **RetroNectin** (Cat. Nos. T110A, T100B & T100A) may be used as transduction enhancers instead of polybrene (see Section III.G).
4. Transduce 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*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 48 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 with fresh growth medium. Do not add G418 yet.
8. At 48 hr post-splitting, remove medium and replace with fresh growth medium supplemented with G418 at the appropriate concentration (this is usually 400–500 µg/ml of G418, please refer to Section III.B**).**
9. Remove medium and replace with fresh growth medium containing G418 every 4 days, or more often if necessary.
10. After selecting with G418 for 2–3 weeks, 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. Isolate as many clones as feasible, so that at least 24 clones are available for testing. Suspension cultures must be cloned using a limiting dilution technique.
11. When cells reach >90% confluency, subculture the cells in a 12-well plate using fresh growth medium supplemented with 100–200 µg/ml of G418.
12. When cells reach >90% confluency, subculture the cells in a 6-well plate using fresh growth medium supplemented with 100–200 µg/ml of G418.

C. Protocol: Selecting 24 Independent Clones and Testing Induction

NOTE: This protocol can be completed in 3 days.

IMPORTANT: Selection of Tet-On 3G-positive clones with optimal induction characteristics is critical for success with this system. Tight control of Cas9 expression is necessary to minimize the occurrence of off-target effects and cellular toxicity.

1. For each clone to be tested, from a 6-well plate (Section IX.B, Step 12) seed 1/3 of the total amount of cells into a single well of a 6-well plate containing fresh growth medium without antibiotics. The cells in this "stock plate" may be propagated, depending upon the results of the screening assay.
2. Divide the remaining 2/3 of the cells between duplicate wells of a second 6-well plate containing fresh growth medium without antibiotics. Allow the cells to adhere overnight, and then either transduce the cells with pLVX-TRE3G-Luc lentivirus at an MOI of 5–10 (using the protocol in Section IX.B, Steps 1–5), or transiently transfect the cells in each well with 3 µg of pLVX-TRE3G-Luc plasmid using Xfect transfection reagent.
3. After 6–8 hr, replace the culture medium with fresh medium and add doxycycline (100–1,000 ng/ml) to one of the duplicate wells, while leaving the second well doxycycline-free. At 24 hr post-induction, assay for luciferase activity and calculate fold induction (e.g., +Dox RLU/–Dox RLU).
4. Select clones with the highest fold induction (ratio of maximal to basal luciferase activity) for propagation and further testing.

   NOTE: When testing clones via transient transfection of pLVX-TRE3G-Luc, you can expect lower-fold induction levels than in double-stable clones. This is because transiently transfected cells contain more copies of the pLVX-TRE3G-Luc plasmid than do stable cell lines.
5. Expand promising Tet-On 3G-positive clones for the next round of transduction, and prepare frozen stocks for each as soon as possible.

   NOTE: Based on the results of luciferase assay, choose the three best clones and prepare stocks for each. Continue culturing cells from one of the chosen clones for the next step.

D. Protocol: Generating Stable Tet-On 3G-Cas9 Clones

NOTE: This protocol can be completed in 2 weeks.

1. Plate chosen target cells in complete growth medium (without G418) 12–18 hr before transduction. The cells should be 70–80% confluent at the time of transduction.
2. Thaw aliquots of your pLVX-TRE3G-Cas9 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) or **RetroNectin** reagent (Cat. Nos. T110A, T100B & T100A) may be used as transduction enhancers instead of polybrene (see Section III.G).
4. Transduce 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*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 48 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 with fresh growth medium, do not add puromycin yet.
8. At 48 hr post-splitting, remove medium and replace with fresh growth medium supplemented with puromycin at the appropriate concentration (this is usually 0.5–2 µg/ml of puromycin, please refer to Section III.B).
9. Remove medium and replace with fresh growth medium containing puromycin every 4 days, or more often if necessary.
10. After selecting with puromycin for ~2 weeks, 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. Isolate as many clones as feasible, so that at least 6 clones are available for testing. Suspension cultures must be cloned using a limiting dilution technique.
11. When cells reach >90% confluency, subculture the cells in a 12-well plate using fresh growth medium supplemented with 0.25 µg/ml of puromycin.
12. When cells reach >90% confluency, subculture the cells in a 6-well plate using fresh growth medium supplemented with 0.25 µg/ml of puromycin.

E. Protocol: Selecting 6 Independent Clones and Testing Cas9 Induction

NOTE: This protocol can be completed in 4 days. Alternatively, users who wish to assay Cas9 induction by performing genome editing may skip this protocol and proceed to Section IX.F.

IMPORTANT: Stable Tet-On 3G cell lines that performed well in the luciferase assay should be suitable for induction of Cas9 as well. Therefore, screening 6 clones at this stage should be sufficient for obtaining a promising Tet-On 3G-Cas9 cell line. Alternatively, users who wish to assay Cas9 induction by performing genome editing may skip this protocol and proceed to Section IX.F.

1. For each clone to be tested, from a 6-well plate (Section IX.D, Step 12) seed 1/3 of the total amount of cells into a single well of a 6-well plate containing fresh growth medium without antibiotics. The cells in this "stock plate" may be propagated, depending upon the results of the screening assay.
2. Divide the remaining 2/3 of the cells between duplicate wells of a second 6-well plate and add doxycycline (100–1,000 ng/ml) to one of the duplicate wells, while leaving the second well doxycycline-free.
3. At 48 hr post-induction, assay Cas9 induction by qRT-PCR using the provided primers and parameters indicated below. Select clones with the highest-fold induction (ratio of maximal to basal Cas9 expression) for propagation and further use. Inducibility of Cas9 expression can also be pre-screened by Western blot using Guide-it Cas9 Antibody (Cat. Nos. 632628, 632627, 632607, and 632606).

   NOTE: qRT-PCR is recommended over Western blotting for choosing the best clones. While Western blotting can be used for pre-selecting clones, CRISPR/Cas9-mediated editing is so efficient that it can occur even when Cas9 protein is undetectable by Western blot. qRT-PCR is sensitive enough to determine which clones express Cas9 at levels low enough to avoid detectable genome editing in the absence of doxycycline (see Figure 7, next page).

qRT-PCR master mix (per reaction):

12.5 µl	2X TB Green *Premix Ex Taq* II (Tli RNase Plus), ROX Plus
2.5 µl	Cas9 qPCR Primer Mix (diluted to 4uM in water prior to use)
0.125 µl	PrimeScript RT (Cat. No. 2680A; diluted to 20 U/µl in 2X TB Green *Premix Ex Taq* II (Tli RNase Plus), ROX Plus)
0.625 µl	Recombinant RNase Inhibitor (Cat. No. 2313A; 40 U/µl)
2 µl	RNA sample (0.01 ng/µl)
7.25 µl	Nuclease-Free Water
25.0 µl	**Total volume added per reaction**

qRT-PCR cycling parameters:

50°C	5 min
95°C	1 min
40 cycles:	
95°C	5 sec
60°C	1 min
4°C	forever

Cas9 qPCR Primer Mix primer sequences:

Forward: 5′-ACTACAAGGTGCCGAGCAAAA-3′
Reverse: 5′-CGCCAATGAGGTTCTTCTTTATGCT-3′

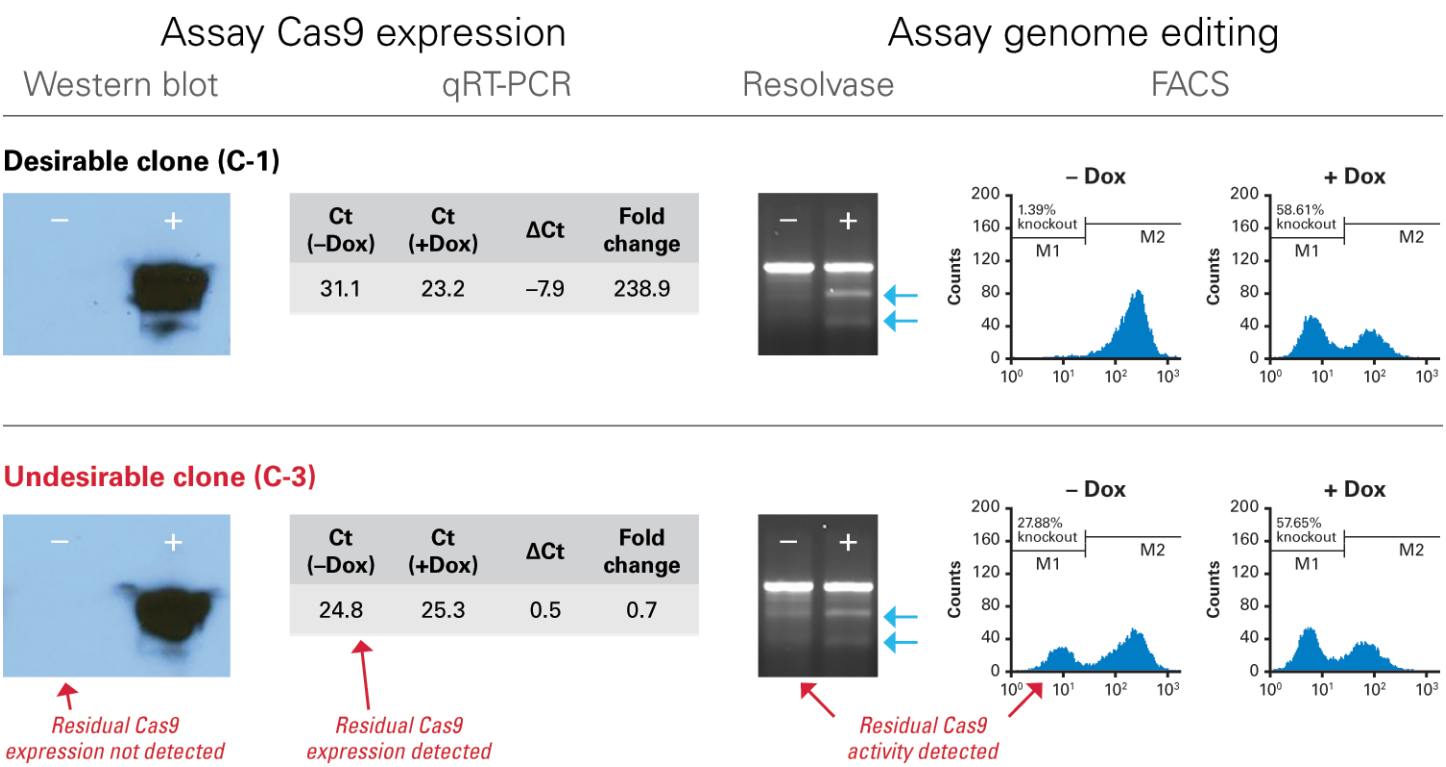


Figure 7. Choosing an optimal clone for inducible genome editing. Clones may be selected by assaying Cas9 expression or genome editing in both uninduced and induced cell populations. However, while Western blot detection of Cas9 is helpful for pre-screening clones, CRISPR/Cas9-mediated editing is so efficient that even when Cas9 protein is undetectable by Western blot, genome editing can still occur in corresponding clones. We recommend qRT-PCR for identifying clones in which the residual expression of Cas9 is low enough for editing to be avoided in the absence of doxycycline. Data in the upper row is from a desirable clone (C-1), which exhibits robust expression of Cas9 protein in induced cells (Western blot, +), and the lowest residual levels of Cas9 mRNA (qRT-PCR). Genome editing occurs at much higher frequencies upon induction of Cas9 expression in cells derived from this clone (+ or +Dox) relative to uninduced cells (– or –Dox), as evidenced by the increase in smaller-sized bands in the Resolvase assay (blue arrows) and the greater proportion of cells in the "knockout" category as determined by FACS, with the lowest residual editing in uninduced cells. In contrast, data in the lower row is from an undesirable clone (C-3), which exhibits residual transcription of Cas9 (qRT-PCR) and detectable frequencies of genome editing (Resolvase, FACS) in the absence of doxycycline, even though Cas9 protein expression in uninduced cells (-) is undetectable by Western blot.

F. Protocol: Inducible Genome Editing

NOTE: This protocol can be completed in 3 days.

1. Distribute chosen target cells between duplicate wells 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-hyg-sgRNA1 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/or **RetroNectin** reagent (Cat. Nos. T110A, T100B & T100A) may be used as transduction enhancers instead of polybrene (see Section III.G).
4. Transduce 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, replace the culture medium with fresh medium and add doxycycline (100–1,000 ng/ml) to one of the duplicate wells, while leaving the second well doxycycline-free.

   CAUTION: Discarded medium contains infectious lentivirus.

   OPTIONAL: Successful genome editing with this system typically does not require selection of sgRNA-positive clones prior to induction of Cas9 expression. However, if isolation of Tet-On 3G-Cas9-sgRNA clones is desired, proceed as follows: At 48 hr post-transduction, split the cells. At 48 hr post-splitting, replace medium with medium containing hygromycin at an appropriate concentration (this is usually 50–400 µg/ml, please refer to Section III.B). Select with hygromycin for 2 weeks, replacing the growth medium with fresh medium containing hygromycin every 4 days, or more often if necessary. After 2 weeks, isolate large, healthy colonies for expansion, storage, and induction of genome editing.

7. After more than 2 days, use your preferred method to determine whether genome editing has occurred (see Appendix A for sample data from cells that were edited using this system).

   NOTE: The time necessary for genome editing to occur varies for different loci.

X. References

Takara Bio's Tet systems were developed in cooperation with Dr. Bujard and his colleagues at the Center for Molecular Biology in Heidelberg (ZMBH) and in Dr. Wolfgang Hillen's laboratory at the University of Erlangen, Germany. Additional background information on Tet-regulated gene expression systems and an extensive bibliography are available at the website maintained by TET Systems: http://www.tetsystems.com (please note that Takara Bio is not responsible for the information contained on this website).

Dow, L. E. *et al.* Inducible in vivo genome editing with CRISPR-Cas9. *Nat. Biotechnol.* **33,** 390–4 (2015).

Fu, Y. *et al.* High-frequency off-target mutagenesis induced by CRISPR-Cas nucleases in human cells. *Nat. Biotechnol.* **31,** 822–6 (2013).

Gonzalez, F. *et al.* An iCRISPR platform for rapid, multiplexable, and inducible genome editing in human pluripotent stem cells. *Cell Stem Cell* **15,** 215–226 (2014).

Gossen, M. *et al.* Transcriptional activation by tetracyclines in mammalian cells. *Science* **268,** 1766–1769 (1995).

Gossen, M. & Bujard, H. Tight control of gene expression in mammalian cells by tetracycline-responsive promoters. *Proc. Natl. Acad. Sci. U. S. A.* **89,** 5547–51 (1992).

Hsu, P. D. *et al.* DNA targeting specificity of RNA-guided Cas9 nucleases. *Nat. Biotechnol.* **31,** 827–32 (2013).

Jinek, M. *et al.* A Programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science* **337,** 816–822 (2012).

Lin, S., Staahl, B., Alla, R. K. & Doudna, J. A. Enhanced homology-directed human genome engineering by controlled timing of CRISPR/Cas9 delivery. *Elife* **3,** 1–13 (2014).

Loew, R., Heinz, N., Hampf, M., Bujard, H. & Gossen, M. Improved Tet-responsive promoters with minimized background expression. *BMC Biotechnol.* **10,** 81 (2010).

Mali, P. *et al.* CAS9 transcriptional activators for target specificity screening and paired nickases for cooperative genome engineering. *Nat. Biotechnol.* **31,** 833–8 (2013).

Urlinger, S. *et al.* Exploring the sequence space for tetracycline-dependent transcriptional activators: novel mutations yield expanded range and sensitivity. *Proc. Natl. Acad. Sci. U. S. A.* **97,** 7963–8 (2000).

Zhou, X., Vink, M., Klaver, B., Berkhout, B. & Das, A. T. Optimization of the Tet-On system for regulated gene expression through viral evolution. *Gene Ther.* **13,** 1382–1390 (2006).

Zuris, J. A. *et al.* Cationic lipid-mediated delivery of proteins enables efficient protein-based genome editing in vitro and in vivo. *Nat. Biotechnol.* **33,** 73–80 (2014).

Appendix A. Sample Data

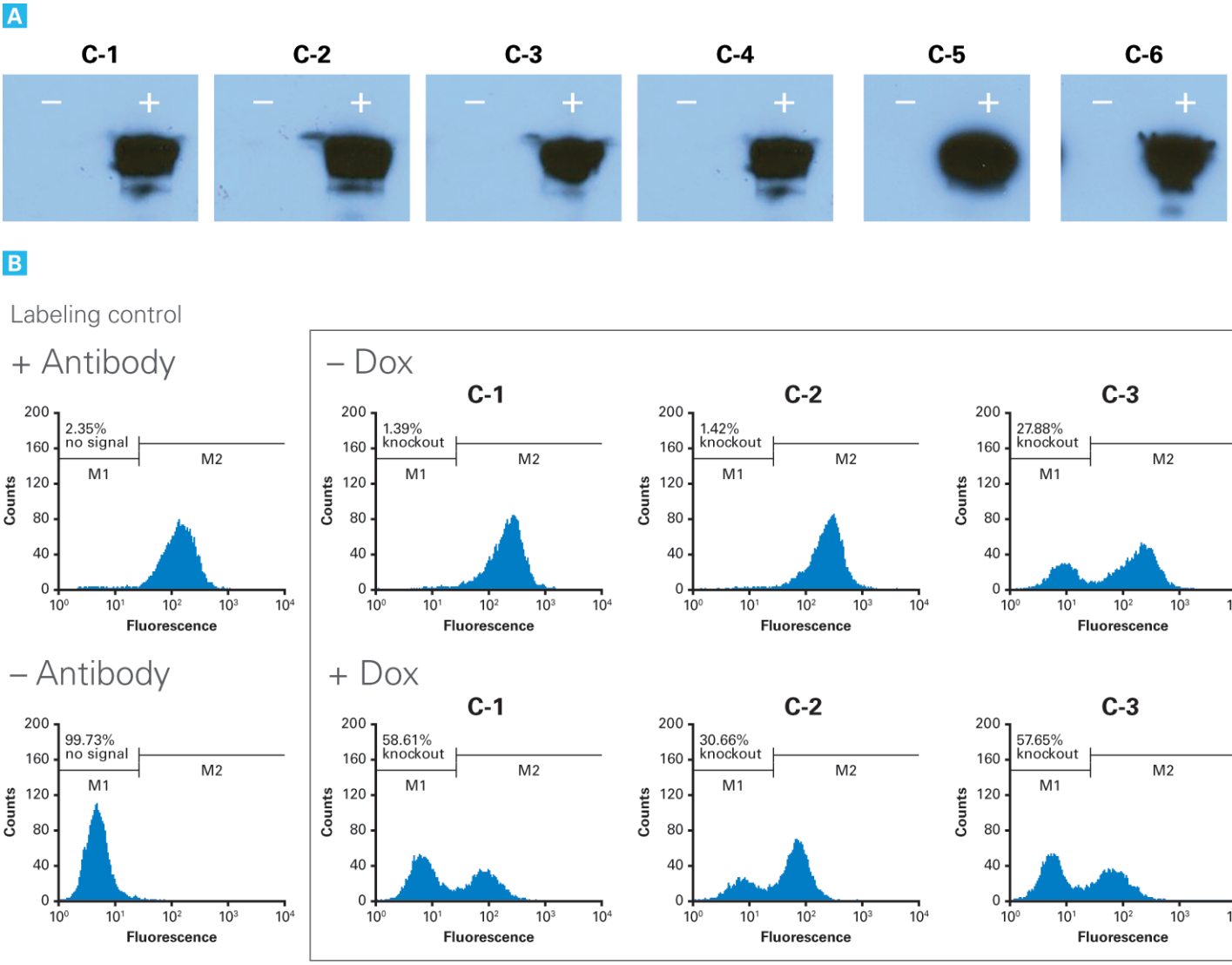


Figure 8. Inducible knockout of *CD81* in HEK293 cells. Target cells were first transduced with Tet-On 3G lentivirus and selected with G418. 24 independent Tet-On 3G-positive clones were assayed for induction of the luciferase reporter, and one clone exhibiting the lowest residual expression was selected and transduced with Cas9 lentivirus at an MOI of 5. Target cells were selected with 1 µg/ml puromycin for two weeks, and six stable clones were randomly selected for analysis of Cas9 induction. Cells from each clone were divided between two wells of a 12-well plate, and incubated either in the presence or absence of doxycycline (0.5 µg/ml) for two days. Cas9 induction was then assayed by Western blot using polyclonal anti-Cas9 antibody (Cat. No. 632607), diluted 1:1,000, and ECL reagent. Cells derived from three of the Tet-On 3G-Cas9-positive clones (C-1, C-2, and C-3) were then divided between two wells of a 12-well plate and transduced with CD81-sgRNA lentivirus at an MOI of 5. Eight hours after transduction, doxycycline was added to one well for each clone at a concentration of 0.5 µg/ml, and cells were cultured for six days. Cells were then harvested and stained with a FITC-tagged anti-human CD81 antibody, and scanned for CD81 expression by FACS. **Panel A.** Western blot results for six independent Tet-On 3G-Cas9-positive cell populations cultured in the absence (-) or presence (+) of 0.5 µg/ml doxycycline, assayed with anti-Cas9 antibody. Cas9 expression is either undetectable or highly elevated in the absence or presence of doxycycline, respectively. **Panel B.** FACS results for three independent Tet-On 3G-Cas9-positive 293T cell populations transduced with CD81-sgRNA lentivirus and cultured in the absence (-Dox) or presence (+Dox) of 0.5 µg/ml doxycycline. Two of the populations (C-1 and C-2) exhibited no residual editing in the absence of doxycycline, and knockout of *CD81* in either 58.6% or 30.7% of cells, respectively, while the third population (C-3) exhibited residual editing (27.9% of cells) in the absence of doxycycline.

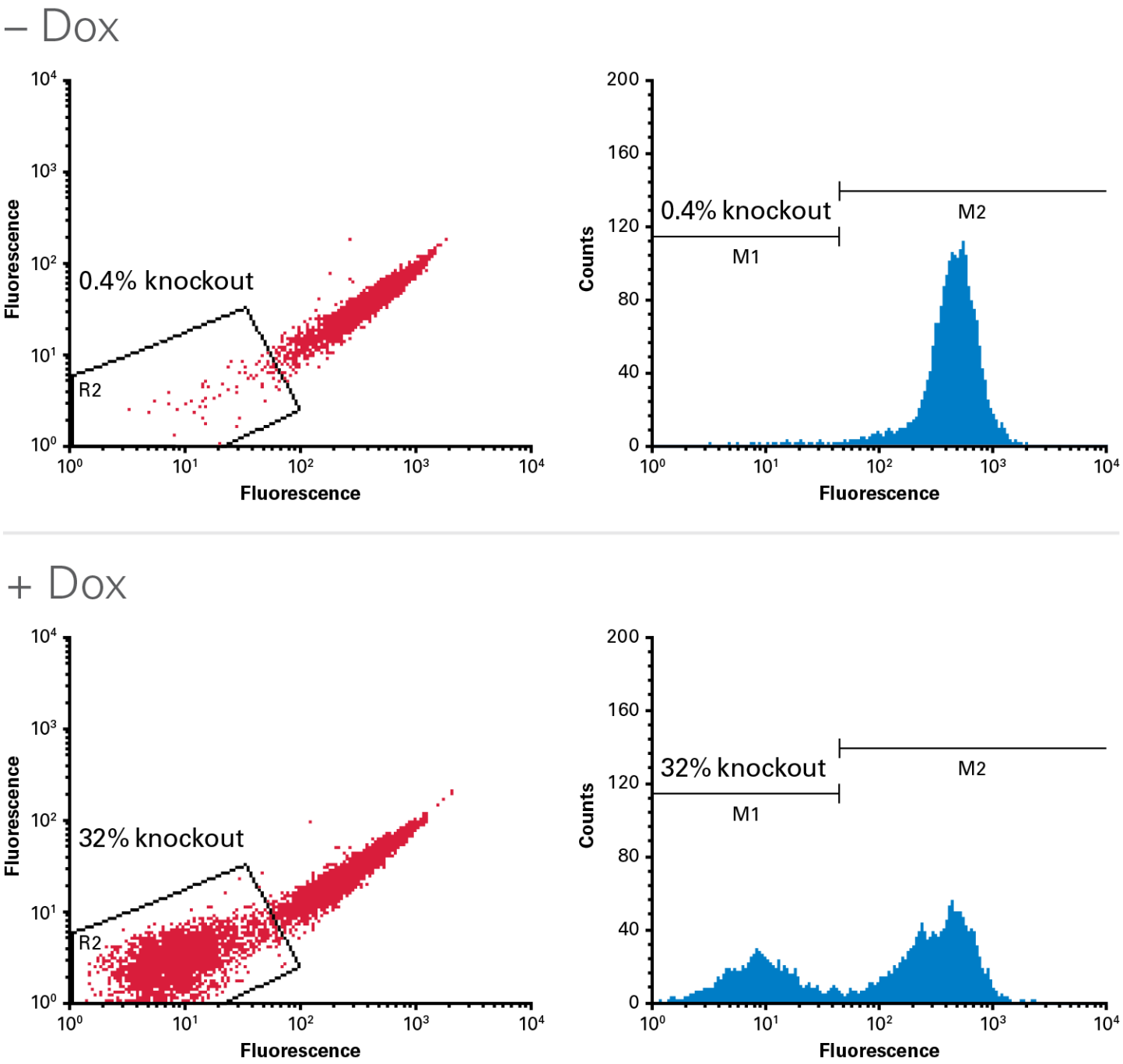


Figure 9. Inducible knockout of *CD81* in Jurkat cells. Tet-On 3G-Cas9-positive Jurkat cells with low residual Cas9 expression were generated using the established protocol and transduced twice with CD81-sgRNA lentivirus at an MOI of 5 on successive days. Cells were then divided between two wells of a 12-well plate and cultured in the absence (-Dox) or presence (+Dox) of doxycycline at a concentration of 0.5 µg/ml for seven days. Cells were then treated with FITC anti-human CD81 antibody and analyzed by FACS. Only a small proportion of cells (0.4%) cultured in the absence of doxycycline (-Dox; top) exhibit residual editing. A significant proportion of cells (32.0%) cultured in the presence of doxycycline (+Dox; bottom) exhibit knockout of *CD81*.

A

	Cas9 expression			
Clone	Ct (–Dox)	Ct (+Dox)	ΔCt	Fold change
1	31.1	23.2	–7.9	238.9
2	32.6	26.6	–6.0	64.0
3	24.8	25.3	0.5	0.7
4	30.7	25.4	–5.3	39.4
5	29.4	24.2	–5.2	36.8
6	31.2	24.3	–6.9	119.4

B

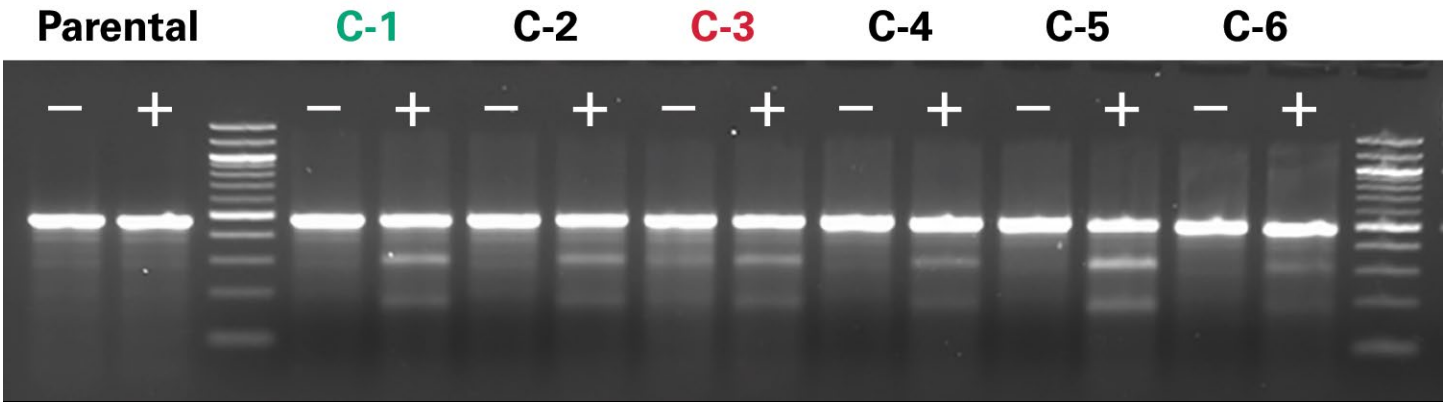


Figure 10. Inducible editing of *AAVS1* in HEK293 cells. Induction of Cas9 expression in six stable Tet-On 3G-Cas9-positive HEK293 cell lines was assayed by qRT-PCR. Cells from each line were then divided between two wells of a 12-well plate and transduced with AAVS1-sgRNA lentivirus at an MOI of 5. Eight hours after infection, doxycycline was added to one well for each cell line at a concentration of 0.5 µg/ml, and the cells were cultured for six days. The Guide-it Mutation Detection Kit (Cat. No. 631443) was then used to screen for editing of *AAVS1*. **Panel A.** qRT-PCR results for Cas9 expression in uninduced (-Dox) and induced (+Dox) cells for each clone. Ct and ΔCt values for uninduced and induced clones are indicated in the corresponding columns and the calculated fold-difference in expression for each clone is indicated in the column on the right. Of the various clones tested, Clone 1 (numbers in green) exhibited the highest-fold inducibility, while only Clone 3 (numbers in red) exhibited residual expression of Cas9 in the absence of doxycycline and low inducibility. **Panel B.** Results of Guide-it resolvase assay for detection of *AAVS1* gene editing in uninduced (-) and induced (+) clones. In contrast with the assay of the parental line, which yielded bands of equivalent size for uninduced and induced cells, assays of the induced clones yielded additional bands of smaller sizes, indicating the occurrence of gene editing at *AAVS1* for all clones tested. Cleavage products are also visible for the uninduced cells from Clone 3, consistent with the residual expression of Cas9 observed in the qRT-PCR assay.

Appendix B. Troubleshooting Guide

Table 2. Troubleshooting Guide for the Lenti-X Tet-On 3G 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 V).
	Poor transformation efficiency	Use the included Stellar Competent Cells.
Sanger sequencing inconclusive	Poor signal-to-noise ratio	Use the following sequencing primer (binds in U6 promoter): Guide-it Sequencing Primer 1, `5′-AATGGACTATCATATGCTTACCGT-3′`
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 III.C. 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 10^6 cells/100 mm plate, or fewer if the cells divide rapidly. Use at 50–80% confluency.
	Transfection is toxic to cells.	Use the optimized conditions specified in the Lenti-X Packaging Single Shots Protocol-at-a-Glance.
	Cells analyzed too soon after transfection.	Wait 48 hr after transfection for maximal expression of GOI or marker to determine efficiency.
Low titers (<10^5 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 VIII or use the Lenti-X Concentrator (Section III.F) to increase your available titer up to 100-fold without ultracentrifugation.
	Transduction protocol not optimized	See the Lenti-X Lentiviral Expression Systems User Manual for references to help with optimizing transduction protocols.
	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 (Cat. No. T100B) and the RetroNectin-Bound Virus transduction protocol or purify your virus prior to transduction using the **Lenti-X Maxi Purification Kit** (Cat. Nos. 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 reagent-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. Nos. 631233 & 631234).
Infection is toxic to target cells.	MOI too high (i.e. too much virus used)	Titrate the virus; dilute virus stock.
	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. Nos. 631233 & 631234).
E. Target cell treatment with Cas9/sgRNA Lentivirus		
No gene editing detected in the target cells.	Inactive sgRNA	• Confirm the activity of the sgRNA against your target *in vitro* using the **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 V, Figure 4).
F. Inducing Expression		
Low fold induction (ratio of maximal to basal expression of Cas9)	Cells were harvested and analyzed too soon or too late.	Harvest and analyze cells between 18–48 hr after addition of doxycycline
	Poor infection efficiency	• Confirm virus titers using a titration kit (Section VIII.A). • Increase amount of virus applied to target cells. • Optimize density of cells when transducing.
	Poor target cell viability	• Optimize passage number of target cells. • Optimize culture conditions of target cells. • Optimize tissue culture plasticware.
	The FBS used in the cell culture medium contains tetracycline derivatives.	Use our Tet System Approved FBS (Section III.A), which was functionally tested with our double-stable CHO-AA8-Luc Tet-Off® Control Cell Line.
Decrease in fold induction after several passages **or** Loss of inducibility after passaging of a (previously frozen) double-stable cell line	The appropriate antibiotics are missing from the cell culture medium.	Maintain optimal antibiotic concentrations (Section III.B).
	Mixed cell population	Reselect the current cell line through single colony selection using selective concentrations of puromycin and G418, (Section III.B).

G. Establishment of Stable Cell Lines		
Untransduced cells do not die at the high antibiotic concentration established via titration in Section III.B.	• The cells have not been recently passaged, so they remain well-attached to the plate surface even when they are dead. • You have achieved 100% transduction efficiency.	To determine the appropriate antibiotic concentration, use cells that have been split within the last 2–3 days.
There are no surviving cells after transduction followed by selection.	The antibiotic concentration which caused massive cell death when determining the appropriate dose via titration could be too high.	Use a lower antibiotic concentration for selection of stably transfected cell clones.
H. Detection of Expression		
No detectable Cas9 expression by Western Blot.	Low sensitivity of detection method.	Check sensitivity of primary and secondary antibodies. Analyze Cas9 expression by qRT-PCR, using the provided primers.
I. Detection of Genome Editing		
No detectable genome editing	Unsuccessful sgRNA design	Try alternative sgRNA guide sequence.
	Genomic target sequence contains SNPs	Sequence genomic target region and align with chosen sgRNA to confirm specificity.
Editing occurs without doxycycline.	Leaky Cas9 expression	Perform qRT-PCR with provided primers to identify Tet-On 3G-Cas9-positive clones with minimal residual expression of Cas9.

Appendix C. pLVX-hyg-sgRNA1 Vector Information

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

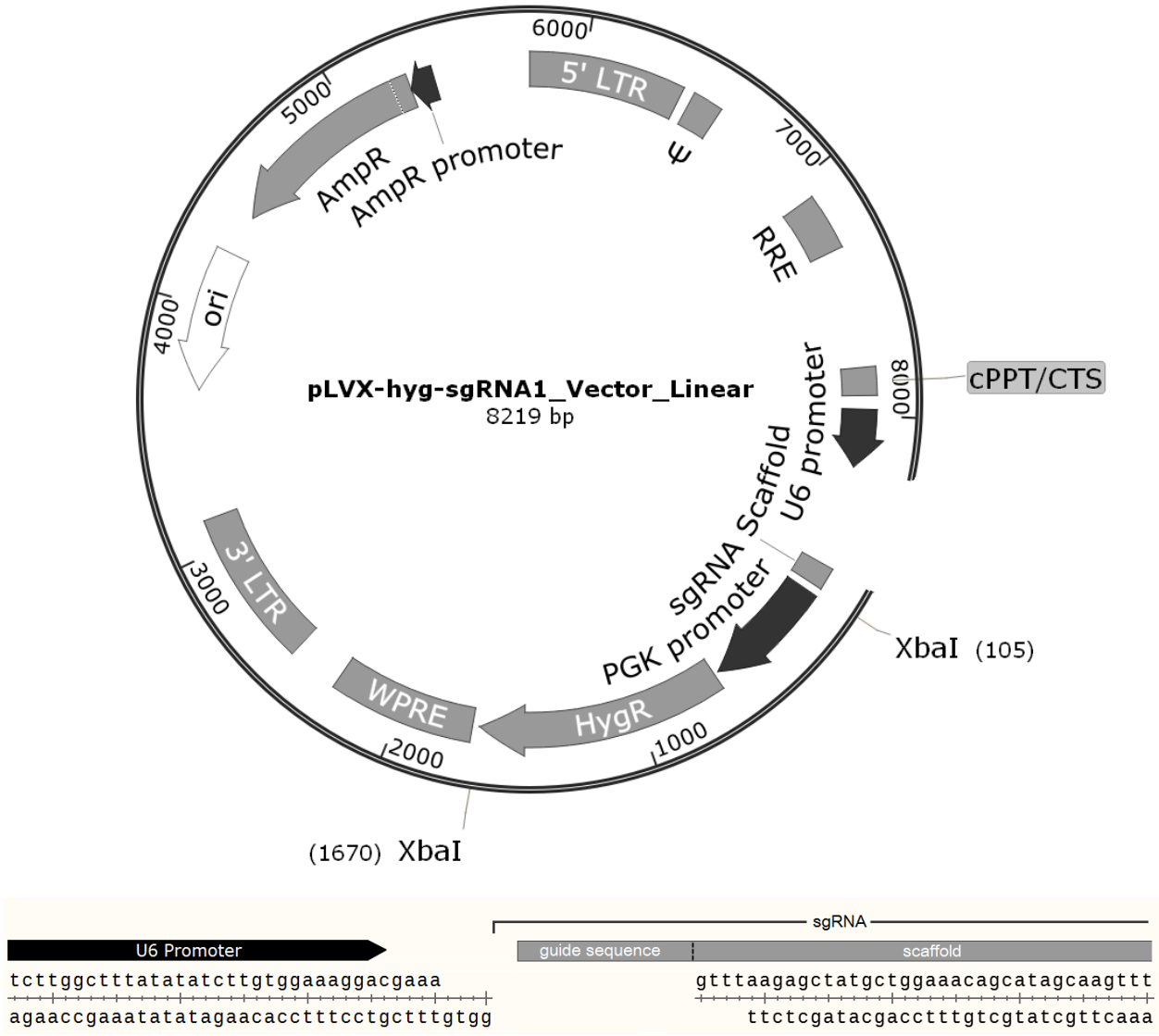


Figure 11. pLVX-hyg-sgRNA1 Vector (Linear) map and cloning site for user's guide sequence.

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