PrecisionX™ LentiCas9 SmartNuclease System

Catalog#s CASLVxxx series

User Manual

Store at -20°C or -80°C

Please check storage conditions for each product

A limited-use label license covers this product. By use of this product, you accept the terms and conditions outlined in the Licensing and Warranty Statement contained in this user manual.

(ver. 1-09242014)

Contents

I. Introduction

A. Overview of CRISPR/Cas9 system

In the past decade, a great deal of progress has been made in the field of targeted genome engineering. Technologies such as designer zinc finger nucleases (ZFNs), transcriptional activator-like effector nucleases (TALENs), and homing meganucleases have made site-specific genome modifications a reality in many different model organisms ranging from zebrafish to mammalian cells. Based on the results to date, however, genome editing tools that are efficient, flexible, and cost-effective have remained elusive to the general research community. The recent discovery of the type II prokaryotic CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats)

system, originally discovered in the bacterium *Streptococcus pyogenes* as a mechanism to defend against viruses and foreign DNA, has provided yet another tool for targeted genome engineering, this time taking advantage of a system that uses small RNAs as guides to cleave DNA in a sequence-specific manner. With its ease in designing guide sequences to target specific sequences (unlike ZFNs and TALENs where construct assembly can be laborious and time-consuming), as well as its targeting efficiency, this system has the potential to be a disruptive technology in the field of genome-engineering.

The CRISPR/CRISPR-associated (Cas) system involves 1) retention of foreign genetic material, called "spacers", in clustered arrays in the host genome, 2) expression of short guiding RNAs (crRNAs) from the spacers, 3) binding of the crRNAs to specific portions of the foreign DNA called protospacers and 4) degradation of protospacers by CRISPR-associated nucleases (Cas). A well-characterized Type II CRISPR system has been previously described in the bacterium *Streptococcus pyogenes*, where four genes (*Cas9, Cas1, Cas2, Csn1*) and two non-coding small RNAs (pre-crRNA and tracrRNA) act in concert to target and degrade foreign DNA in a sequence-specific manner [Jinek *et. al.* 2012]. The specificity of binding to the foreign DNA is controlled by the non-repetitive spacer elements in the pre-crRNA, which upon transcription along with the tracrRNA, directs the Cas9 nuclease to the protospacer:crRNA heteroduplex and induces double-strand breakage (DSB) formation. Additionally, the Cas9 nuclease cuts the DNA only if a specific sequence known as protospacer adjacent motif (PAM) is present immediately downstream of the protospacer sequence, whose canonical sequence *in S. pyogenes* is 5' - NGG -3', where N refers to any nucleotide.

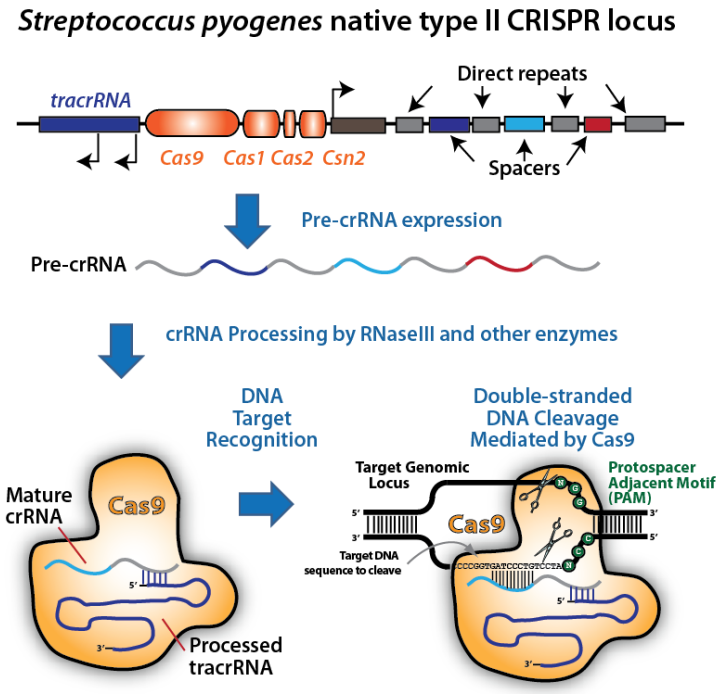


Figure 1: Overview of the CRISPR system. Figure adapted from Cong *et al*. "Multiplex Genome Engineering Using CRISPR/Cas Systems".

Recently, it has been demonstrated that the expression of a single chimeric crRNA:tracrRNA transcript, which normally is expressed as two different RNAs in the native type II CRISPR system, is sufficient to direct the Cas9 nuclease to sequence-specifically cleave target DNA sequences. By adapting the endogenous type II CRISPR/Cas system in *S. pyogenes* for utility in mammalian cells, several groups have independently shown that RNA-guided Cas9 is able to efficiently introduce precise double stranded breaks at endogenous genomic loci in mammalian cells with high efficiencies and minimal off-target effects [Cong *et al.* 2013, Mali *et al.* 2013, Cho *et al.* 2013].

In addition, several mutant forms of Cas9 nuclease have been developed to take advantage of their features for additional applications in genome engineering and transcriptional regulation Biochemical characterization of a mutant form of Cas9 nuclease (D10A) functions as a nickase (Jinek *et al.* 2012), generating a break in the complementary strand of DNA rather than both strands as with the wild-type Cas9. This allows repair of the DNA template using a high-fidelity pathway rather than NHEJ, which prevents formation of indels at the targeted locus, and possibly other locations in the genome to reduce possible off-target/toxicity effects while maintaining ability to undergo homologous recombination (Cong *et al.* 2013). Recently, paired nicking has been shown to reduce off-target activity by 50- to 1,500 fold in cell lines and to facilitate gene knockout in mouse zygote without losing on-target cleavage efficiency (Ran et al., 2013). Finally, tandem knockout of both RuvCI and HNH nuclease domains (which control cutting of the DNA strands) shows that the null-nuclease mutant (double mutant) can act as a transcriptional repressor (Qi *et al.* 2013) with minimal off-target effects, which leads to possibilities for studying site-specific transcriptional regulation.

Taken together, the RNA-guided Cas9 system defines a new class of genome engineering tools, creating new opportunities for research across basic sciences, biotechnology and biomedicine.

B. Product Information

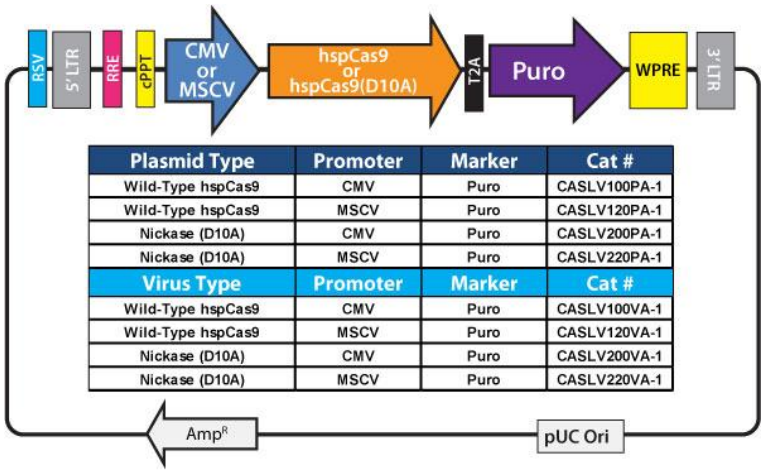
Based on our industry-leading, transfection-based, all-in-one Cas9 SmartNuclease and SmartNickase plasmid systems, we have now adapted these constructs into a lentivector format. SBI's Lenti-Cas9 SmartNuclease system is ideal for targeting cell types that are traditionally difficult to transfect with plasmids, effectively expanding the range of target cells amenable for CRISPR/Cas9-based genome engineering. The Lenti-Cas9 system also provides an easy and efficient way to generate stable Cas9 editing cell lines. SBI offers lentiviral constructs in two formats: 1) An "all-in-one" format expressing Cas9 and gRNA from a single vector and 2) a two vector system with separate Cas9 and gRNA expression vectors. All of the Cas9 lentivector constructs (Section I.C) express human-codon optimized Cas9 wild-type nuclease or mutant nickase, while the gRNA cloning/expression lentivector constructs (Section I.C) contain a pre-made tracrRNA scaffold with gRNA cloning sites driven by your choice of H1 or U6 Pol-III promoters for robust expression in a wide range of cells. The system is designed to accommodate flexible targeting of any genomic loci in the form of N_{20}NGG; however, other gRNA formats (e.g. $N_{17\text{-}18}$NGG) can be utilized as well.

SBI offers pre-packaged, ready-to-infect pseudoviral particles for expression of Cas9 wild-type nucleases and mutant nickases for generation of cell lines stably expressing Cas9. Pseudoviral particles have been packaged to exacting QC standards and comes with functional titer and in-house transduction data for each production lot of virus.

For those customers wishing to package any of the Lenti-Cas9 SmartNuclease lentivectors in their own lab, SBI offers the LentiStarter 2.0 kit, which contains all of the necessary reagents to produce high-quality virus and transduce target cells. The Kit is available as part of a bundled package with any Lenti-Cas9 vectors as well as a standalone item (SBI Cat #LV051A-1). Please refer to Section I.G for details regarding the LentiStarter 2.0 Kit.

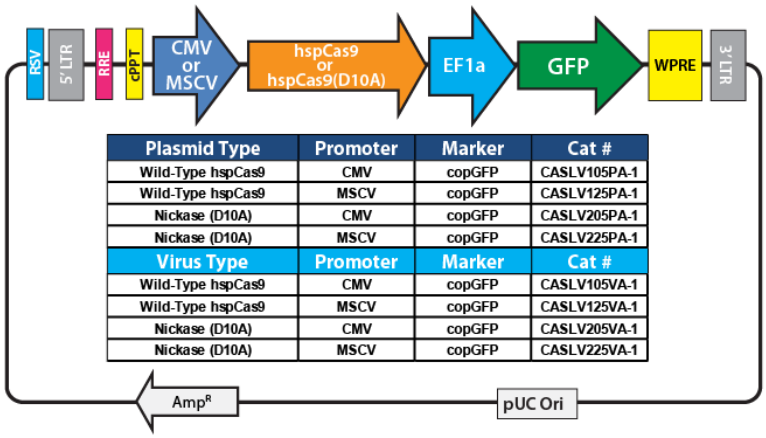
C. Vector Information

WT Cas9 and Nickase Lentivectors (T2A-Puro Format)



Plasmid Type	Promoter	Marker	Cat #
Wild-Type hspCas9	CMV	Puro	CASLV100PA-1
Wild-Type hspCas9	MSCV	Puro	CASLV120PA-1
Nickase (D10A)	CMV	Puro	CASLV200PA-1
Nickase (D10A)	MSCV	Puro	CASLV220PA-1
Virus Type	**Promoter**	**Marker**	**Cat #**
Wild-Type hspCas9	CMV	Puro	CASLV100VA-1
Wild-Type hspCas9	MSCV	Puro	CASLV120VA-1
Nickase (D10A)	CMV	Puro	CASLV200VA-1
Nickase (D10A)	MSCV	Puro	CASLV220VA-1

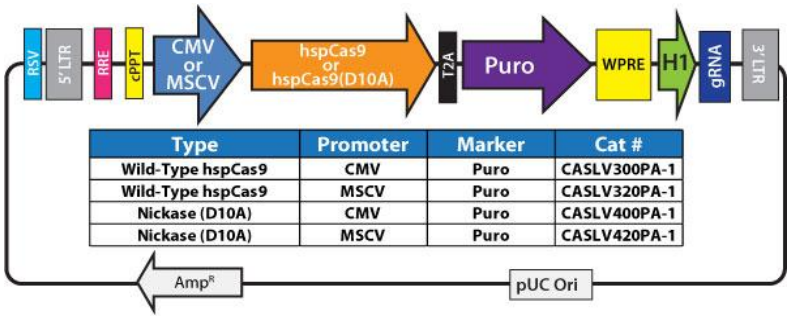
WT Cas9 and Nickase Lentivectors (Dual Promoter EF1-GFP Format)



Plasmid Type	Promoter	Marker	Cat #
Wild-Type hspCas9	CMV	copGFP	CASLV105PA-1
Wild-Type hspCas9	MSCV	copGFP	CASLV125PA-1
Nickase (D10A)	CMV	copGFP	CASLV205PA-1
Nickase (D10A)	MSCV	copGFP	CASLV225PA-1
Virus Type	**Promoter**	**Marker**	**Cat #**
Wild-Type hspCas9	CMV	copGFP	CASLV105VA-1
Wild-Type hspCas9	MSCV	copGFP	CASLV125VA-1
Nickase (D10A)	CMV	copGFP	CASLV205VA-1
Nickase (D10A)	MSCV	copGFP	CASLV225VA-1

Note: Above CASLVxxxPA-1 catalog items are provided as 10 µg of plasmid DNA. CASLVxxxVA-1 catalog items are provided as 2 x 25 ul aliquots of pre-packaged, ready-to-infect lentivirus (>10^7 IFUs/ml, >10^6 infectious units total).

WT Cas9 and Nickase Lentivectors (All-in-One Format)

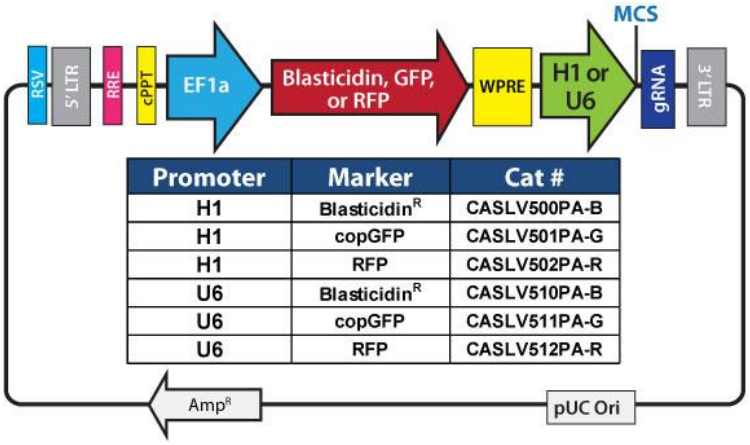


Type	Promoter	Marker	Cat #
Wild-Type hspCas9	CMV	Puro	CASLV300PA-1
Wild-Type hspCas9	MSCV	Puro	CASLV320PA-1
Nickase (D10A)	CMV	Puro	CASLV400PA-1
Nickase (D10A)	MSCV	Puro	CASLV420PA-1

Note #1: SBI also offers a positive control all-in-one construct, Cat# CASLV601A-1 (CMV-hspCas9-T2A-Puro-H1 AAVS1 gRNA vector) with a validated gRNA to cut the human *AAVS1* safe harbor locus.

Note #2: All-in-one expression vectors are provided in a pre-linearized format for ease of cloning gRNAs. These vectors cannot be propagated unless a gRNA insert has been cloned in.

gRNA Cloning and Expression Lentivectors



Promoter	Marker	Cat #
H1	Blasticidin^R	CASLV500PA-B
H1	copGFP	CASLV501PA-G
H1	RFP	CASLV502PA-R
U6	Blasticidin^R	CASLV510PA-B
U6	copGFP	CASLV511PA-G
U6	RFP	CASLV512PA-R

Note: gRNA Cloning and Expression vectors are provided in a pre-linearized format for ease of cloning gRNAs. These vectors cannot be propagated unless a gRNA insert has been cloned in.

D. Validation Data for Lenti-Cas9 SmartNuclease™ and Nickase Vectors

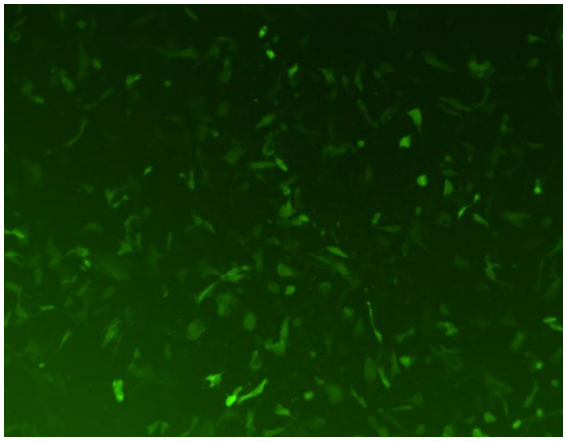


Figure 2: Fluorescence image of HT1080 cell line infected with pseudoviral particles of MSCV-hspCas9-EF1-copGFP (Cat #CASLV125VA-1) at MOI = 3. Image was taken 72hrs after virus transduction.

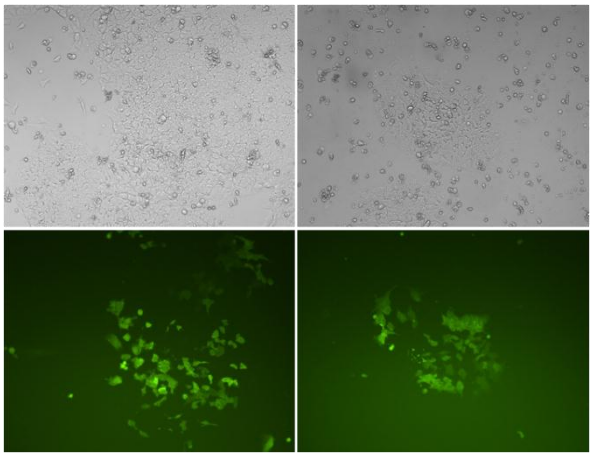


Figure 3: Fluorescence image of Human iPSC cell line infected with pseudoviral particles of MSCV-hspCas9-EF1-copGFP (Cat #CASLV125VA-1) at MOI = 60. Image was taken 6 days after virus transduction.

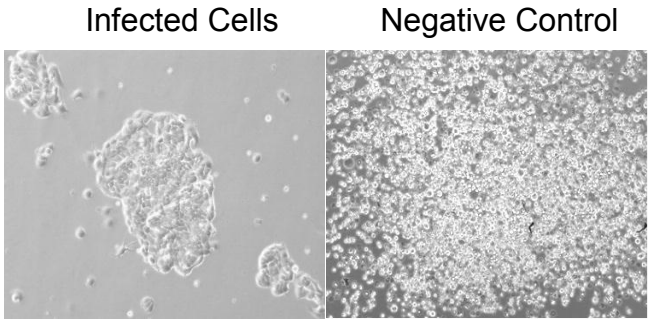


Figure 4: Phase microscopy image of MCF-7 breast cancer cells infected with pseudoviral particles of CMV-hspCas9-T2A-Puro (Cat #CASLV100VA-1) and selected with Puromycin (1 µg/ml) for 10 days in culture, showing distinct colony formation.

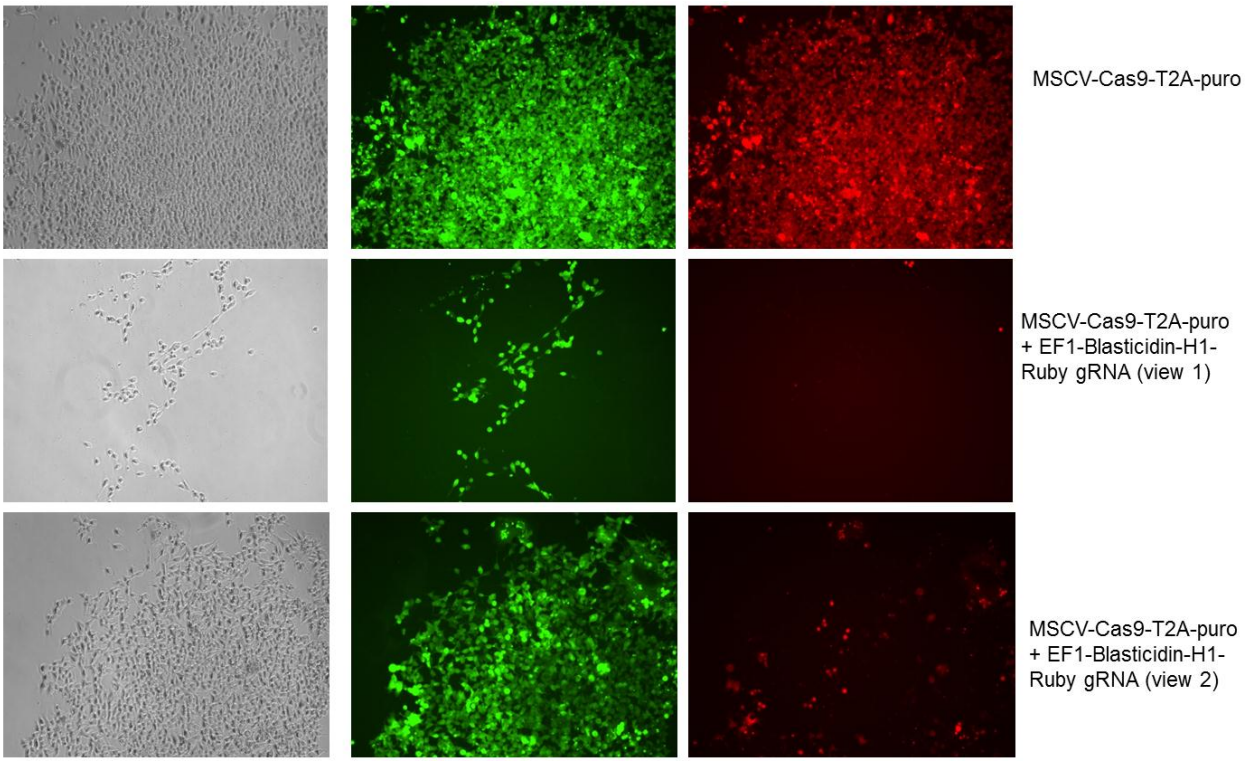


Figure 5: Phase and fluorescent images of modified HEK293T cells stably expressing RFP and GFP (top panel) which have been co-infected with Cas9-Puro (MSCV-Cas9-T2A-Puro) and gRNA virus expressing a guide RNA targeting RFP (EF1a-Blasticidin-H1-RFP gRNA) (bottom panel) at MOI = 3 for each virus. Image of cells were taken 11 days after placing the cells under selection, showing ablation of RFP expression in target cells.

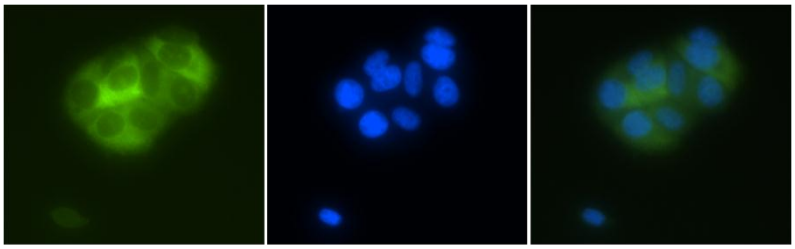


Figure 6: Immunofluorescence staining of Cas9 protein expression in MCF-7 cell lines stably transduced with Cas9 lentiviral vector, indicating punctuate nuclear and perinuclear staining.

E. Key Advantages of the Lenti-Cas9 SmartNuclease™ and SmartNickase System

- Proven 3rd-generation lentivector expression backbone containing codon-optimized hspCas9/Nickase tagged to your choice of GFP or Puro markers for generation of cell lines stably expressing Cas9;
- Deliver Cas9 to difficult-to-transfect cell lines;
- Easily make stable Cas9 cell lines for editing applications;
- gRNA cloning & expression systems contain necessary scaffolding sequences for crRNA maturation and is pre-linearized for cloning; no need to prepare or modify vector backbone;
- Precise directional cloning of the gRNA insert into vector backbone;
- Rapid, highly-efficient cloning with low background (~99% cloning efficiency);

F. Applications of the Lenti-Cas9 SmartNuclease™ and SmartNickase Expression System

We have developed the Lenti-Cas9 expression system to target a wide range of researchers who are interested in the following (but not limited to) research areas:

- Genome editing and engineering of difficult-to-transfect cell lines
- *In vivo* engineering of model organisms
- Synthetic biology applications
- Gene/Cell-based therapy
- Genome-wide functional screening

G. List of Components

1) WT Cas9 and Mutant Nickase Lentiviral Constructs (Cat #CASLV1xxPA-1, CASLV2xxPA-1)

All WT Cas9 and mutant Nickase expression lentiviral constructs are provided as 10 µg of plasmid. The plasmid can be propagated using transformation into chemically competent bacteria per standard transformation protocols. We recommend the use of Stbl2 chemically- competent cells (per manufacturer's recommended protocol) for best results.

2) All-in-One Cas9/gRNA Cloning & Expression Lentiviral Constructs (Cat #CASLV3xxPA-1, CASLV4xxPA-1, CASLV601A-1)

The all-in-one lentiviral constructs are provided pre-linearized for cloning of guide RNAs. A single sales unit contains enough reagents to perform up to 10 ligation reactions (i.e. cloning of 10 individual gRNAs).

3) gRNA cloning & expression lentiviral constructs (Cat #CASLV5xxPA-x)

The gRNA lentiviral constructs are provided pre-linearized for cloning of guide RNAs. A single sales unit contains enough reagents to perform up to 10 ligation reactions (i.e. 10 gRNA clonings).

Table 1. List of components included in all-in-one and gRNA cloning and expression vectors.

Reagent	Amount
Linearized lentiviral cloning vector	**10 µl**
5x ligation buffer	**10 µl**
Fast ligase	**2.5 µl**
H1 Fwd Sequencing primer (5 µM): **5' GCGGGCCTCTTCGCTATTAC 3'**	**20 µl**
U6 Fwd Sequencing primer (5 µM): **5'** GGACTATCATATGCTTACCG **3'**	**20 µl**

Note: The forward sequencing primer provided will depend on the vector ordered. For all-in-one vectors, only the H1 sequencing primer will be provided.

4) Pre-packaged Lenti-Cas9 SmartNuclease Lentiviral Particles (Cat #CASLV1xxVA-1, CASLV2xxVA-1, CASLV601VA-1)

WT Cas9 and mutant Nickase pre-packaged lentiviral particles (>10^7 IFUs/ml, >10^6 infectious units) are provided as 2 x 25 µl tubes with infectious titer data (IFUs/ml) for each virus lot.

5) LentiStarter 2.0 Kit (Cat #LV051A-1)

The LentiStarter 2.0 Kit can be added to any Lenti-Cas9 vector (Cat# CASLVxxxPA-KIT) or purchased separately for a complete, one-stop solution for transfection of virus producer cells, packaging of lentivectors, concentration of pseudoviral particles, and transduction of target cells. The Kit contains the following reagents as listed in the table below:

Table 2. List of components included in LentiStarter 2.0 Kit:

Reagent	Amount
pPACKH1 HIV lentiviral packaging plasmids	**100 µl**
PEG-It virus precipitation reagent	**20 ml**
PureFection Transfection Reagent	**120 µl**
TransDux virus transduction reagent	**50 µl**

There will be enough reagents provided in the Kit to produce high-quality pseudoviral particles from 2 x 150mm (or 5 x 100mm) plates of 293T producer cells (cells not included).

H. Additional Materials Required

1) LB Agar and Broth containing 50µg/ml Ampicillin or Carbenicillin.

2) Any high-transformation efficiency *E.coli* competent cells (e.g. Stbl2 cells, Life Technologies, Cat # 10268-019).

3) Zyppy™ Plasmid MiniPrep Kit (Zymo Research, Cat. # D4019).

4) Zyppy™ Plasmid MaxiPrep Kit (Zymo Research, Cat. # D4027).

5) PureFection Transfection Reagent (SBI, Cat #LV750A-1) or equivalent.

6) 293TN Producer Cell Line (SBI, LV900A-1) or equivalent cells (e.g. HEK293T or HEK293FT).

7) (Recommended) UltraRapid Global Titering Kit (SBI, Cat #LV961A-1).

I. Related Products

SBI offers a number of Homologous Recombination (HR) Donor Vectors, including the popular PrecisionX HR Targeting Vectors (Cat #HRxxxPA-1) for generating gene knockouts and knock-ins, as well as the piggyBac HR Donor for seamless excision of a selection cassette (Cat# PBHR100A-1). The full selection of HR Donor vectors may be viewed on the following webpage: http://www.systembio.com/genome-engineering-precisionx-HR-vectors/ordering.

J. Shipping and Storage Conditions

PrecisionX™ Lenti-Cas9 SmartNuclease/Nickase and gRNA cloning & expression vectors are shipped on blue ice. Upon receiving, store the kit at -20°C. Shelf life of the product is 1 year after receipt if stored in -20°C. Components in LentiStarter 2.0 Kit should be stored per the product analysis certificate (PAC) provided with the Kit.

Pre-packaged viruses are shipped on dry ice, and upon receiving please store at -80°C.Avoid excess freeze-thaw cycles for virus as it may affect performance. Shelf life of the product is 1 year after receipt if stored in -80°C.

II. Protocol for the Lenti-Cas9 Expression System

A. Overview of the Protocol

The general workflow of the cloning and infection of the Cas9 and gRNA lentiviral expression constructs into cells is listed here:

1) Design two DNA oligonucleotides that are sense and antisense sequences of the target DNA which is 20bp upstream of the PAM (5' - NGG - 3'). Other lengths (e.g. 17 or 18bp) may work as well.

2) Anneal the two oligonucleotides to generate a duplex.

3) Clone the duplex into the provided linearized gRNA cloning lentivector by ligation reaction.

4) Transform into competent cells and grow in LB/Amp plate (50 µg/ml).

5) Confirm positive clones by direct sequencing.

6) Produce lentiviral particles for gRNA and Cas9 /nickase lentivectors using the LentiStarter 2.0 Kit.

7) Infect target cells with Cas9 or nickase virus from Step 6 (please see Section “II.G” for details).

8) Establish stably transduced cells via FACS-sorting (for GFP constructs) or Puromycin selection.

9) Transduce the gRNA lentiviral particles into stable cells established in Step 8.

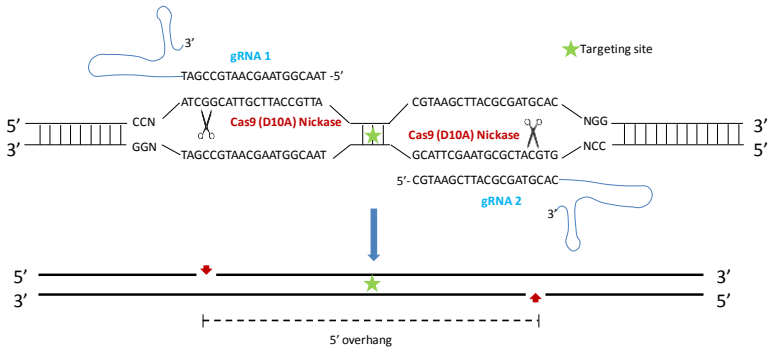
10) Assay for desired activity or phenotype 4-5 days after gRNA lentivirus transduction through Surveyor Nuclease Assay.

B. Selection of Target DNA Sequence

The selection of the target DNA sequence is not limited by any constraints, with exception of a PAM sequence in the form of 5’ -NGG - 3’ (where N = any base) immediately following the target sequence. The typical length of the target sequence is 20bp – as shown here, however, gRNA lengths of 17-18bp have been successfully utilized for genomic editing (Fu et al. 2014).

5’ NNNNNNNNNNNNNNNNNNNNNGG 3’

In order to enhance specificity, paired gRNA with Lenti-Cas9 Nickase constructs can be used to generate double nicking with 5’ overhangs. Please follow the guideline below for paired gRNA selection and design.



Choose your gRNA1 from the anti-sense strand upstream of your targeting site
Choose your gRNA2 from the sense strand downstream of your targeting site

Fig. 4: Schematic illustration of generating 5’ overhang double strand DNA breaks using paired gRNAs with Cas9 Nickase (adapted from Ran et. al. 2013).

Please note that only gRNA pairs creating 5’ overhangs with less than 8bp overlap between the guide sequences were able to mediate detectable indel formation (Ran et al. 2013). To achieve high cleavage efficiency using Lenti-Cas9 Nickase with paired gRNAs, make sure each gRNA is able to efficiently induce indels on its own when coupled with wild-type Cas9.

C. Design of Guide RNA Oligonucleotides

Design two DNA oligonucleotides (a top strand and a bottom strand) according to the following structure shown below.

For H1-based gRNA lentivectors:

5’ ATCCNNNNNNNNNNNNNNNNNNN 3’

3’ NNNNNNNNNNNNNNNNNNNNCAAA 5’

For U6-based gRNA lentivectors:

5’ ACCGNNNNNNNNNNNNNNNNNNN 3’

3’ NNNNNNNNNNNNNNNNNNNNCAAA 5’

Example:

If your target sequence is AGCGAGGCTAGCGACAGCATAGG (AGG = PAM sequence), then the oligo sequences would be the following if cloned into **H1 gRNA lentivector**:

Top strand oligo:

5’ – ATCCAGCGAGGCTAGCGACAGCAT - 3’

Bottom strand oligo:

5’ – AAACATGCTGTCGCTAGCCTCGCT - 3’

D. Cloning into the gRNA Lentivector

1) **Anneal the two single-strand DNA oligonucleotides:**

Dilute your primer at the concentration of 10μM using dH_2O and set up the annealing reaction as follows:

Materials	Amount
10μM Top strand oligo	5 μl
10μM Bottom strand oligo	5 μl
Total volume	10 μl

Incubate reaction mixture at 95°C for 5 minutes (can be done in PCR machine). Remove the tube and leave it on bench at room temperature to cool down (~10 minutes).

2) **Ligation of Oligo Duplex into Vector**

Since the tubes might be placed upside down during the shipping, and some of reagents may end up at the top of tubes, we recommend a brief spin to bring all the reagents down to the bottom of tubes before opening the tubes.

Set up the ligation reaction as follows:

Materials	Amounts
Linearized vector	1 µl
Annealed oligo mix	3 µl
5x ligation buffer	1 µl
Fast ligase	0.25 µl
Total volume	5.25 µl

Mix reaction well and incubate for 5-7 minutes at room temperature.

If you are making several constructs at the same time, we strongly recommend adding ligase and buffer separately and individually to the linearized vector (i.e., do not make and aliquot a pre-mixture of ligase and buffer to the linearized vector).

3) Transformation Reaction

a. Add a vial of competent cells to the ligation mix.
b. Place cells on ice for 15 minutes.
c. Heatshock cells at 42°C for 50 seconds, then immediately transfer cells to ice for 2 minutes.
d. Add 250 µl SOC medium and incubate at 37°C for 1 hour with shaking.
e. Spread 100 µl of cultured cells on a pre-warmed LB plate containing 50 µg/ml Ampicillin or Carbenicillin and incubate overnight at 37°C.

4) Confirmation of Positive Clones

a. Pick 1 to 2 colonies, grow in LB/Amp medium overnight at 30°C with shaking.
b. Next day, miniprep plasmid DNAs and send for sequencing using the provided sequencing primer (Note: Primer provided is ready to use, concentrated at 5 µM, simply use 1 µl per reaction).
c. Align your raw sequencing data with the top strand primer sequence. Sequence-validated clones can be used for subsequent packaging (Section E below).

E. Packaging of Lentivector Constructs

Transfection of plasmids into HEK293TN (or equivalent) producer cells

a) 18 - 24 hours prior to transfection, seed 7.0 – 8.0 x10^6 293TN cells per 150mm cell culture plate in standard growth media w/o antibiotics. Cells should be ~80% confluent by next day.

Note: The number of plates to use depends on the amount of virus desired. As a general guideline, we recommend using 2-6 150mm plates for virus production.

b) During transfection day, mix 45 µl of pPACKH1 packaging plasmid mix as provided in the LentiStarter 2.0 Kit and 4.5 µg of Cas9/nickase or gRNA lentivector in 1.6 ml of serum-free DMEM by pipetting.

c) Add 55 µl PureFection into the same tube. Vortex for 10 seconds.

Note: If using other transfection reagents (e.g. Lipofectamine 2000) please follow suggested guidelines for 150mm plates.

d) Incubate mixture at room temperature for 15 minutes.

e) Add mixture drop-wise to the dish, and swirl to disperse evenly throughout the plates.

f) Change the medium ~12 hours (or next day) after transfection.

g) At 48 hours and 72 hours after transfection, collect the medium (which now contains pseudoviral particles) into a 50-ml sterile, capped conical centrifuge tube. Centrifuge at 3000 x g for 15 minutes at room temperature to pellet cell debris. Transfer the viral supernatant into a new tube.

Caution: You are working with infectious pseudoviral particles at this stage. Please follow the recommended guidelines for working with BSL-2 biosafety agents.

F. Concentration of Pseudoviral Particles

The PEG-it™ Virus Precipitation Solution in the LentiStarter 2.0 Kit provides a simple and highly effective means to concentrate lentiviral particles. PEG-it is a formulation of polyethylene glycol optimized for the precipitation of lentiviral-based particles. The PEG-it Virus Precipitation Solution is provided as a 5x solution.

1. Transfer supernatant containing virus to a sterile vessel and add 1 volume of cold PEG-it Virus Precipitation Solution (4ºC) to every 4 volumes of virus supernatant.

(Example: 5ml PEG-it with 20ml viral supernatant).

2. Refrigerate overnight (at least 12 hours). Viral supernatants mixed with PEG-it Virus Precipitation Solution are stable for up to 4-5 days at 4°C.

3. Centrifuge supernatant/PEG-it mixture at 1500 × g for 30 minutes at 4ºC. After centrifugation, the virus particles may appear as a beige or white pellet at the bottom of the vessel.

4. Discard the supernatant into a suitable biohazard waste container. Spin down residual PEG-it solution by centrifugation at 1500 × g for 5 minutes. Remove all traces of fluid by aspiration, taking great care not to disturb the precipitated lentiviral particles in pellet.

5. Resuspend lentiviral pellets in 1/500 to 1/1000 of original volume of pooled virus supernatant using cold, sterile Phosphate Buffered Saline (PBS) or DMEM containing 25mM HEPES buffer at 4ºC.

For example, if you performed 2 collections from 2 x 150mm plates (20ml per plate), this would be approximately 80ml of media. You would resuspend the resulting pellet in 80-160 µl of 1X PBS or DMEM.

6. Aliquot in cryogenic vials and store at -80°C until ready for use.

7. The resulting pseudoviral particles can be accurately titered using SBI's UltraRapid Global Titering Kit (Cat #LV961A-1)
http://www.systembio.com/lentiviral-technology/delivery-systems/ultrarapid/overview.

G. Transduction of Pseudoviral Particles into Target Cells

For efficient transduction of target cells, the negative charges present in the virus envelope protein and the cell surface must be neutralized. SBI's TransDux reagent (provided in the LentiStarter 2.0 Kit) is a non-toxic, proprietory formulation that promotes cell-virus contact and subsequent fusion by negating these charges. The following protocol can be utilized for delivery of virus to your target cells.

Day 1

1. Plate 50,000 cells per well into 2 wells of a 24-well plate in cell culture medium. Make sure that cells are well-dispersed and are not clumped together. Include wells for negative (non-infected) cells. For suspension cells,

please plate recommended amount suitable for two wells in a 24-well plate.

Note: If infecting target cells for the first time or an optimal MOI is not known, please titrate virus at varying MOIs (1, 5, 10 and 20, etc.) to optimize transduction using a positive control virus with a fluorescent marker such as SBI's pre-packaged positive transduction control (Cat #CD511VB-1).

Day 2

2. Cells should be between 50 to 70% confluent. Aspirate medium from cells.

3. Combine culture medium with TransDux to a 1X final concentration. For example, add 2.5 µl of TransDux to 500 µl culture medium and then transfer to each well. If using other types of transduction reagents (e.g. Polybrene) please dilute the reagent to a final working concentration of 2-8 µg/ml.

4. Add **Cas9 or nickase virus** to each well and swirl to mix, for negative control wells only add media/viral transduction reagent.

FOR ALL-IN-ONE VECTORS: Due to the size of the vector, we suggest infection of target cells at higher MOIs than with Cas9/nickase vector. For example, if using MOI of 3 for a given cell type, we would suggest an MOI of 10-20 as a starting range with the all-in-one vectors.

Day 3

5. Aspirate off medium and add complete growth medium to cells.

Day 4

6. Trypsinize and pool cells from Cas9/nickase infected wells into into a single well of a 6-well plate; same for the negative control wells.

Day 5

7. Virus should be integrated into the host cell genome by this time. Begin selection procedure of transduced cells (See Section H).

H. Generation of Stable Cas9/nickase Cell Line

SBI suggests sequential infections of virus, one being the Cas9/nickase virus and the second being the gRNA virus (does not apply to all-in-one constructs). We would suggest establishing a stable Cas9/nickase cell line first in a 100mm plate to obtain ~4-6 million adherent cells to have enough cells for targeting by multiple gRNA viruses and for archival purposes. The following protocol is designed for establishment of adherent cell lines stably expressing both Cas9/nickase and gRNA.

Note: The times listed below are approximate and will depend on growth rate of cells being utilized. Please adjust timing as necessary for each step.

Days 5-6

1. Monitor growth of transduced cells and split when confluent (70-80%) into a single 100mm plate.

Days 7-8

2.

For cell transduced with Puro constructs:

Assuming cells have reached confluency in 100mm plate, add appropriate amount of Puromycin to target cells (based on results of kill curve assay). For most cell lines, a concentration of 0.5 to 1 µg/ml is sufficient.

For cells transduced with GFP constructs:

You may FACS-sort the cells and replate into single wells of 6-well plate and when confluent, split them into 100mm plates.

Days 9-10

3.

For Puro constructs:

Aspirate media containing dead, floating cells. Replace with 10 ml of fresh complete growth media + Puromycin.

For GFP-sorted cells:

Please continue to grow until they reach confluency in 100mm plates.

Days 11-12

4. When cells have reached confluency, they can be archived or re-seeded for infection in 24-well plates (see Step 1) with the gRNA virus. Expression of Cas9 protein can be detected via Western blot or immunofluorescence using a suitable anti-Cas9 antibody (e.g. Diagenode, Cat # C15200203) or equivalent.

Day 12-20

After seeding 24-well plates with Puro-resistant or GFP+ cells stably expressing Cas9, infect cells with gRNA virus at the same MOI as previously done for the Cas9/nickase virus. For gRNA viruses containing a GFP or RFP marker, successfully transduced cells can be FACS-sorted for further characterization (e.g. Surveyor Nuclease Assay or genotyping). If gRNA virus contains the Blasticidin^{TM} antibiotic selection marker, cells can be further selected (now Puro^{R} and Blast^{R}) in culture and expanded for further analysis. In either case, "double-transduced" cells can be subjected to assays to determine indel formation as soon as 72 hrs post-transduction (however indel formation may take as long as 7-10 days after infection with gRNA virus).

III. Frequently Asked Questions

Q. We prepared oligos according to the protocol, ligated the oligos to the vector, and transformed into competent cells. Very few colonies showed up in the plate. What is the reason for this?

1) Please use very high efficiency competent cells for the reaction (e.g. cells with efficiencies of >1 x 10^9 CFUs/ug of pUC18 plasmid).

2) Please make sure to not freeze-thaw stock plasmid as damage to the plasmid may result. Either store the plasmid at 4°C for short-term use (1-2 weeks) or aliquot each reaction into separate tubes for storage at -20°C

Q. How many guide RNA constructs do you have to design to target a DNA sequence of interest?

Due to the unpredictable efficacy of a particular guide RNA construct, for optimal results we suggest designing multiple (2 or more) constructs targeting a particular DNA sequence of interest.

Q. We obtained a very low virus titer after packaging Cas9/nickase construct. What might be the problems?

1. Poor Transfection Efficiency

293T Cells have too high or too low density.

Plate fewer or more cells in order to have about 50 – 80% confluency at transfection stage.

2. Inefficient Production of the Pseudovirus

293T cells are of poor quality

Optimize growth conditions. Some suggestions are:

• Check growth medium,

• 293T cells should not be grown for more than 20 passages.

• Check for mycoplasma contamination.

• Make sure the cells have not been overgrown (do not allow the cells to reach more than 90% confluency in order to keep the culture continuously in logarithmic growth phase).

3. Pseudoviral supernatant harvested too early or too late.

Harvest supernatant at least twice: 48 hours post-transfection and 72 hours after transfection, combine the volumes, concentrate virus using SBI's PEG-it reagent, and titer.

4. Cas9/nickase lenti-vector is near limits of efficient packaging.

The packaging limit for the lentiviral system is 8.5 kb from 5' LTR to 3' dLTR – the Cas9/nickase vector is ~8kb from LTR to LTR. The efficiency of packaging may drop significantly with cDNA insert sizes greater than 2 kb (Cas9/nickase is ~4kb in size). For a 3 kb insert, the titers could be 10-fold lower than for a 1 kb insert. We would suggest scaling up the number of 150mm plates for generating sufficient amounts of Cas9/nickase virus for best results.

Q. We established a Cas9/nickase stable cell line, designed a gRNA, packaged it into pseudovirus particles, and infected target cells and there is no evidence of activity. What are the possible reasons for this?

There are many possibilities of why a particular guide RNA does not show any measureable effects. Some of the possibilities include the following:

1) Poor transduction efficiency of target cells: For certain cell types (e.g. primary, stem, suspension cells), transduction may not be very efficient. In these cases, we would suggest infections using an higher MOI (30-50 may be appropriate with certain cells) or performing forced virus-cell infections ("Spin-Inoculation"). Certain cell types (e.g. T-cells) also may need to be artificially stimulated from G_0 state for efficient transduction.

2) Errors in guide RNA design: The sequences of oligo duplexes targeting the DNA should be carefully checked to follow design rules.

3) Mutation(s) in DNA sequence targeted: In certain cases, the DNA sequence targeted may contain mutations which affect recognition of the gRNA sequence, leading to failure of cleavage. It is difficult to know in advance, but if it happens repeatedly, it may be necessary to follow-up with another gRNA sequence or perhaps sequence-verifying the genomic target prior to design of gRNA constructs.

4) Length of time before assaying: We suggest a minimum of 72 hours post-infection to begin assaying for cleavage of a DNA target; however, in certain cases, it may be useful to wait up to 7-10 days to observe the full effect of cleavage.

IV. References

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V. Technical Support

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