# PrecisionX™ T7 Cas9 SmartNuclease Vector System (Tagged and Untagged)

## Catalog#s CAS7xxA/R/G-1

## *User Manual*

**Store at -20°C upon receipt**

# Contents

# I. Introduction

## A. Overview of CRISPR 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.

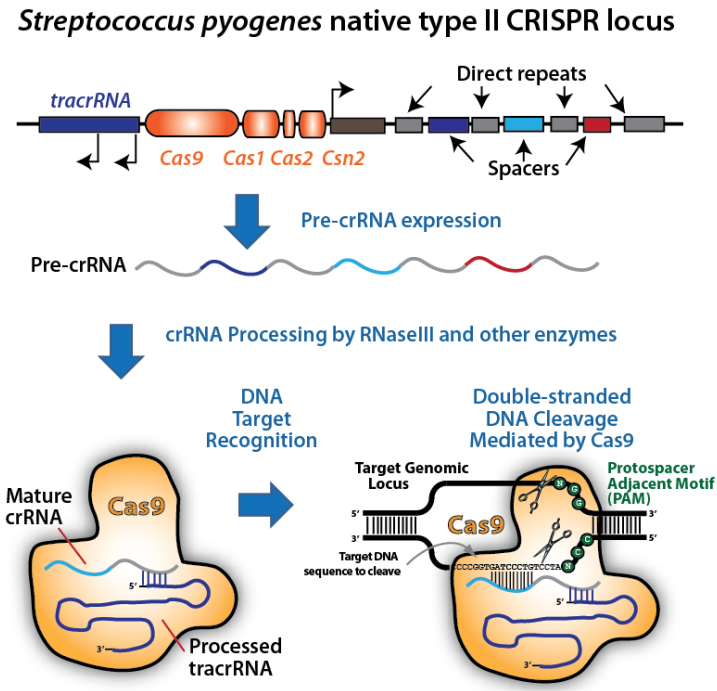



Figure 1: Overview of the CRISPR system. Figure adapted from Cong *et al*. "Multiplex Genome Engineering Using CRISPR/Cas Systems".

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 and Vector Maps

To make the RNA-directed Cas9 system more efficient, affordable, and convenient to use, SBI has developed the all-in-one, programmable PrecisionX™ Cas9 SmartNuclease and Nickase expression vectors, including a human codon optimized Cas9 (hspCas9) or Cas9 mutant (Cas9 Nickase) along with a custom guide RNA (gRNA) consisting of a chimeric crRNA-tracrRNA transcript expressed from a single construct (Fig. 2 and Fig. 3). In addition, these vectors are offered as untagged or GFP/RFP-tagged for tracking transfection efficiency into target cells as well as a T7 promoter for in vitro transcription of the Cas9 and gRNA for preparation of mRNA and IVT gRNAs for *in vivo* applications (e.g. oocyte microinjections). SBI's all-in-one SmartNuclease and Nickase expression constructs include the following core features:

1) The hspCas9 and Nickase used in this system include two nuclear localization signals (NLS) at the N-terminus and C-terminus to ensure efficient import of the hspCas9 protein into the nucleus.

2) The expression vectors also contain a Myc-tag at the N-terminus for ease of detection and purification of the recombinant Cas9 protein.

3) To facilitate diverse applications of the system, hspCas9 and Nickase may be expressed from a number of different commonly utilized promoters that are active in mammalian cells. (See Tables 1 and 2).

4) The hspCas9 and Nickase is followed by a regulatory element called WPRE (Woodchuck virus post-transcriptional regulatory element) to boost gene expression and stabilize the mRNA transcript.

To avoid reconstituting the CRISPR/Cas9 RNA processing machinery, a custom gRNA (crRNA-tracrRNA chimeric transcript) can be generated from the pre-cut, ready-to-use linearized vectors through the use of annealed oligonucleotide duplexes encoding the 20bp target sequence upstream of PAM, with the gRNA expressed under the control of a robust, full-length H1 polymerase III promoter. Our programmable, all-in-one vector format allows for highly flexible targeting of any genomic loci in the form of $N_{20}$NGG.

Table 1. List of available all-in-one **untagged** Cas9 SmartNuclease™ and Nickase Vectors with built-in T7 promoter

| Cat# | Description | Size |
|---|---|---|
| **CAS700A-1** | **EF1-T7-hspCas9-H1-gRNA linearized SmartNuclease vector** | **10 rxn** |
| **CAS720A-1** | **CAG-T7-hspCas9-H1-gRNA linearized SmartNuclease vector** | **10 rxn** |
| **CAS740A-1** | **CMV-T7-hspCas9-H1-gRNA linearized SmartNuclease vector** | **10 rxn** |
| **CAS750A-1** | **Cas9 Nickase: EF1-T7-hspCas9 nickase -H1-gRNA linearized SmartNickase vector** | **10 rxn** |
| **CAS770A-1** | **Cas9 Nickase: CAG-T7-hspCas9 nickase -H1-gRNA linearized SmartNickase vector** | **10 rxn** |
| **CAS790A-1** | **Cas9 Nickase: CMV-T7-hspCas9 nickase -H1-gRNA linearized SmartNickase vector** | **10 rxn** |

Table 2. List of available all-in-one **tagged** Cas9 SmartNuclease™ and Nickase Vectors with built-in T7 promoter

| Cat# | Description | Size |
|---|---|---|
| **CAS700G-1** | **EF1-T7-hspCas9-T2A-GFP-H1-gRNA linearized SmartNuclease vector** | **10 rxn** |

| | | |
|---|---|---|
| **CAS701R-1** | **EF1-T7-hspCas9-T2A-RFP-H1-gRNA linearized SmartNuclease vector** | **10 rxn** |
| **CAS720G-1** | **CAG-T7-hspCas9-T2A-GFP-H1-gRNA linearized SmartNuclease vector** | **10 rxn** |
| **CAS721R-1** | **CAG-T7-hspCas9-T2A-RFP-H1-gRNA linearized SmartNuclease vector** | **10 rxn** |
| **CAS740G-1** | **CMV-T7-hspCas9-T2A-GFP-H1-gRNA linearized SmartNuclease vector** | **10 rxn** |
| **CAS741R-1** | **CMV-T7-hspCas9-T2A-RFP-H1-gRNA linearized SmartNickase vector** | **10 rxn** |
| **CAS750G-1** | **Cas9 Nickase: EF1-T7-hspCas9 nickase-T2A-GFP-H1-gRNA linearized SmartNickase vector** | **10 rxn** |
| **CAS751R-1** | **Cas9 Nickase: EF1-T7-hspCas9 nickase-T2A-RFP-H1-gRNA linearized SmartNickase vector** | **10 rxn** |
| **CAS770G-1** | **Cas9 Nickase: CAG-T7-hspCas9 nickase-T2A-GFP-H1-gRNA linearized SmartNickase vector** | **10 rxn** |
| **CAS771R-1** | **Cas9 Nickase: CAG-T7-hspCas9 nickase-T2A-RFP-H1-gRNA linearized SmartNickase vector** | **10 rxn** |
| **CAS790G-1** | **Cas9 Nickase: CMV-T7-hspCas9 nickase-T2A-GFP-H1-gRNA linearized SmartNickase vector** | **10 rxn** |
| **CAS791R-1** | **Cas9 Nickase: CMV-T7-hspCas9 nickase-T2A-RFP-H1-gRNA linearized SmartNickase vector** | **10 rxn** |

In addition to the features above, both untagged and tagged versions of the CAS7xx series vectors are specially designed for use in multiple gRNA cloning applications, where 2 or more gRNAs can be expressed from the same vector under the control of Pol-III promoters such as H1 and U6. SBI's innovative Multiplex

gRNA Cloning Kit (Cat #CAS9-GRNA-KIT) allows for rapid and efficient cloning of multiple gRNAs into CAS7xx vectors, based on SBI's proven restriction enzyme and ligase-free Cold Fusion. SBI also offers a "combo" kit (Cat #CAS7xxA/R/G-KIT) which allows pairing of any CAS7xx series vector with the Multiplex gRNA Cloning Kit, providing researchers with a complete, "one-stop" solution for genome engineering applications requiring expression of multiple gRNAs. More detailed information on the combo kit can be found here: <<<<User manual link for combo kit>>>>

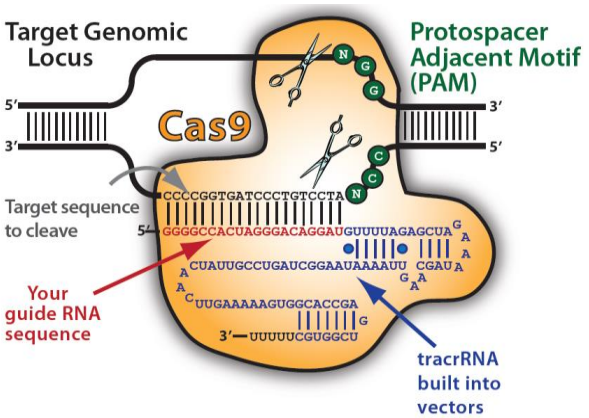
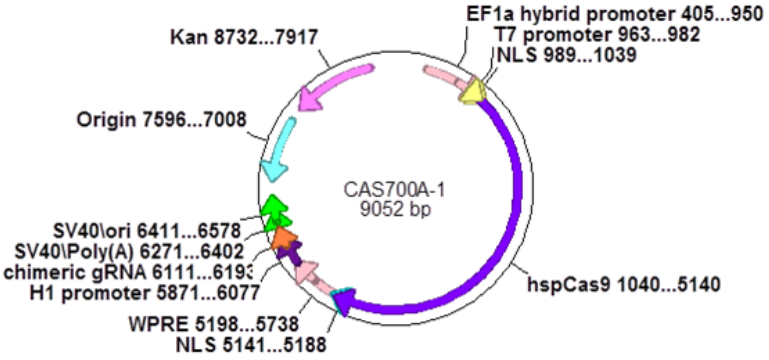
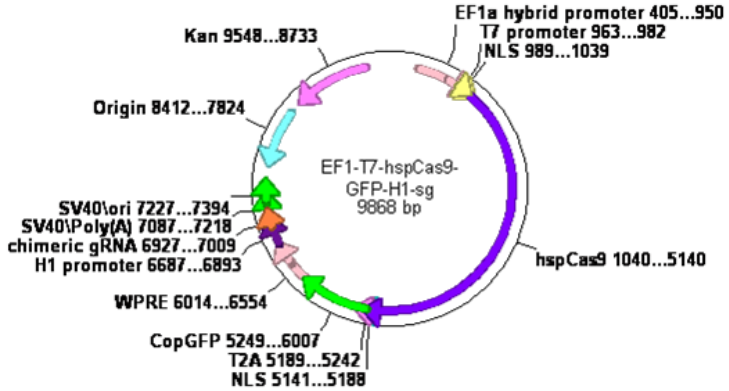
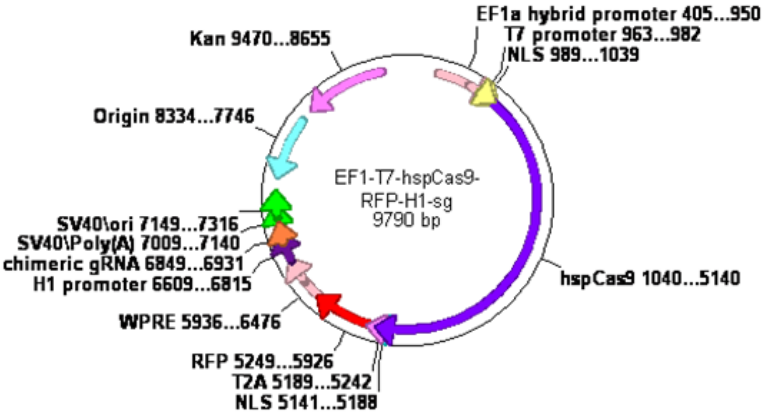



Fig. 2: Schematic Figure of Cas9 SmartNuclease/Nickase Expression System

## Selected Cas9 SmartNuclease Constructs






***In addition to EF1a, CMV and CAG versions of the Cas9 SmartNuclease and Nickase vectors are also available***

Fig. 3: Vector maps of Cas9 SmartNuclease Expression Vectors with T7 promoter

## C. Validation Data for Cas9 SmartNuclease™ Vectors

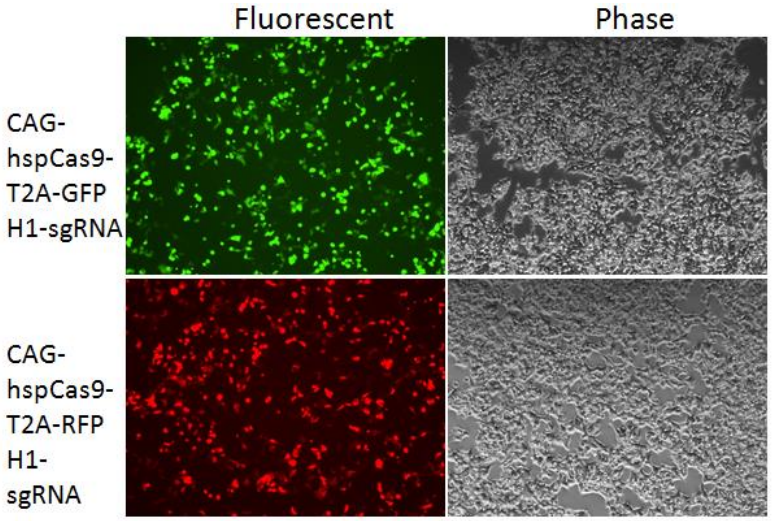



Fig. 4: GFP and RFP expression data for selected CAG-hspCas9 expression vectors (Cat #CAS720G-1 and CAS721R-1) in HEK293T cells

Representative data regarding the cleavage efficiency of selected gRNAs (e.g. human *AAVS1* and luciferase) using wild-type Cas9 expression vectors can be found here:

http://www.systembio.com/genome-engineering-cas9-crispr-smartnuclease/gene-editing
http://www.systembio.com/genome-engineering-cas9-crispr-smartnuclease/gene-knockout

Using our Cas9 SmartNuclease Expression System (EF1a-T7-

hspCas9-T2A-RFP-H1, Cat# CAS701R-1), we cloned in a guide RNA sequence targeting human *AAVS1* gene (Fig. 5A) and looked at the ability to induce homology-directed repair (HDR) using a GFP repair donor in an engineered cell line called EGIP (Enhanced Green Fluorescent Inhibited Protein). The EGIP cell line contains a stop codon in the middle of an EGFP coding region (thus truncation of full-length EGFP) as well as a 53bp sequence from the human *AAVS1* gene (Fig. 5B) for targeting by the gRNA. Our data indicates successful transfection of the vector into cells (as evidenced by RFP expression) as well as significant levels of HDR (~8-10%) as early as two days post-transfection (Fig. 6), indicating functionality of the system.

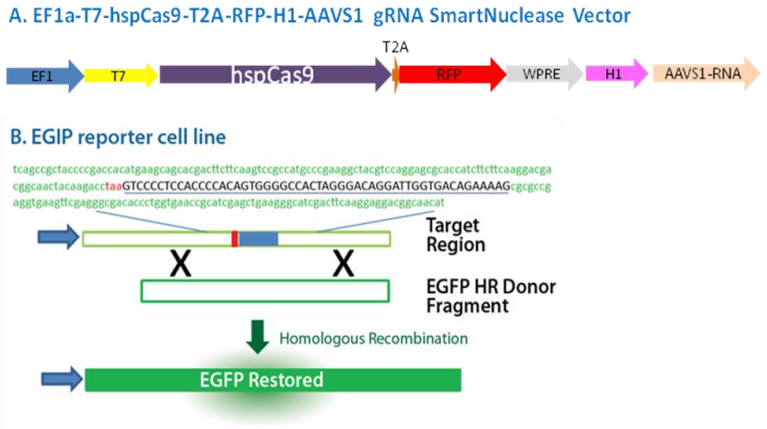



Fig. 5: A) Schematic diagram of EF1a-T7-hspCas9-T2A-RFP-AAVS1 gRNA vector. B) Diagram and strategy for HDR of GFP donor vector in EGIP cell line containing a premature stop codon and *AAVS1* gRNA target site engineered into the EGFP sequence

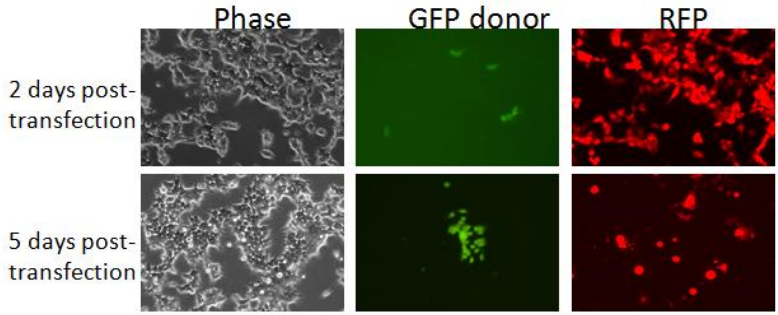



Fig. 6: Data showing transfection of the EF1a-T7-hspCas9-T2A-RFP-AAVS1 gRNA vector into EGIP (HEK293T) cells (right panes) and HDR of GFP donor vector (middle panes) at days 2 and 5 post-transfection

## D. Key Advantages of the Cas9 SmartNuclease™ System

Each kit provides enough materials for 10 reactions to generate your own Cas9 SmartNuclease/Nickase expression construct with the following features:

- All-in-one vector system combining codon-optimized hspCas9/Nickase and gRNA cloning and scaffolding sequences – no need for multiple plasmid constructs
- Pre-linearized vector is ready-to-use, no need to prepare or modify the vector backbone
- Precise directional cloning of the gRNA insert into vector backbone
- Rapid, highly-efficient cloning with low background (~99% cloning efficiency)

- Cloning compatibility – the same gRNA insert can be easily exchanged into other Cas9 linearized vectors with a one-step cloning reaction

## E. Applications of the Cas9 SmartNuclease™ Expression System

We have developed the all-in-one 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 model organisms
- Synthetic biology applications
- Gene/Cell-based therapy

## F. List of Components

The kit contains enough reagents to perform up to 10 ligation reactions in an easy-to-use format (Table 3)

Table 3. List of components included in the Cas9 SmartNuclease/Nickase Expression System

| Reagent | Amount |
|---|---|
| **Linearized SmartNuclease/Nickase Vector** | **10 µl** |
| **5x ligation buffer** | **10 µl** |
| **Fast ligase** | **2.5 µl** |
| **Fwd Sequencing primer (5 µM): 5' GTCATCAACCCGCTCCAAGG 3'** | **20 µl** |

## H. Additional Materials Required

1) LB Agar and Broth containing 50µg/ml Kanamycin

PrecisionX™ T7 Cas9 SmartNuclease System (Tagged and Untagged)
Cat. # CAS7xxA/R/G-1

2) Any high-transformation efficiency *E.coli* competent cells
3) Zyppy™ Plasmid MiniPrep Kit (Zymo Research, Cat. # D4019)
4) Qiagen EndoFree Plasmid Maxi Kit (Qiagen, Cat. # 12362)
5) PureFection Transfection Reagent (System Biosciences, Cat #LV750A-1) or equivalent

## G. Related Products

1) Multiplex gRNA Cloning kit (Cat #CAS9-GRNA-KIT)

2) Cas9 Combo Kit (Cat #CAS7xxA/R/G-KIT)

3) 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 knockins, as well as the piggyBac HR Donor for seamless excision of 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.

## H. Shipping and Storage Conditions for Kit

PrecisionX™ Cas9 SmartNuclease/Nickase Expression System components 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.

# II. Protocol for the Cas9 SmartNuclease™ Expression System

## A. Quick Overview of the Protocol

The general workflow of the cloning, validation, and transfection of the Cas9/gRNA SmartNuclease/Nickase expression constructs into cells is depicted in Fig. 7. Briefly, here are the steps involved in the process:

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')

2) Anneal the two oligonucleotides to generate a duplex

3) Clone the duplex into the provided linearized Cas9 vector by ligation reaction

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

5) Confirm positive clones by direct sequencing

6) Transfect sequence-verified all-in-one construct into mammalian cells using standard transfection protocols

7) Sort transfected cells by standard FACS sorting, and perform Surveyor Nuclease assay (or other suitable mismatch cleavage assays) to check the site-specific genome cleavage, or perform homology recombination assays for genome modification using a suitable donor vector.

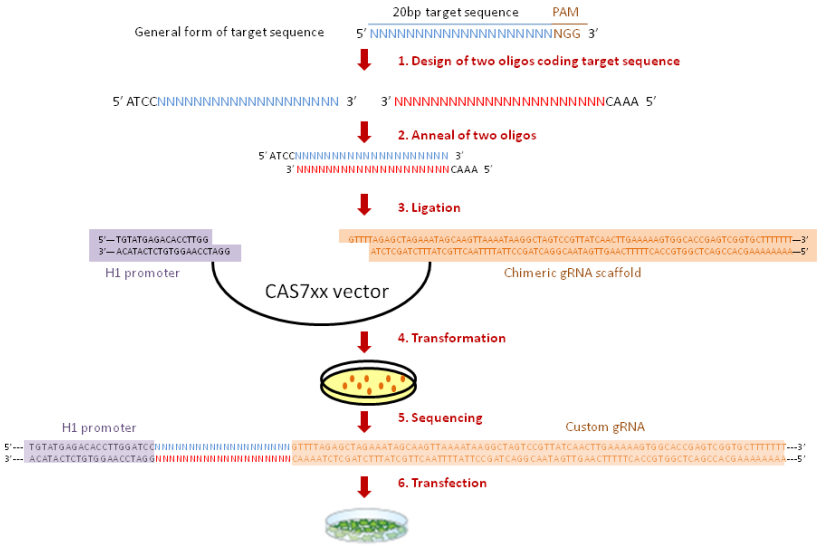


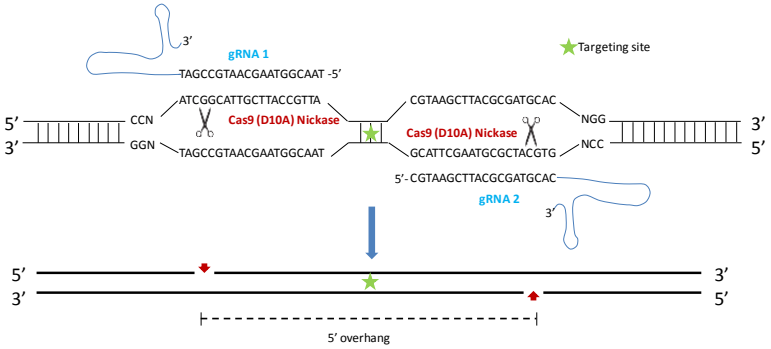

Fig. 7: General Workflow for RNA-Guided Cas9 SmartNuclease/Nickase Expression System.

## 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:

5' NNNNNNNNNNNNNNNNNNNNNGG 3'

In order to enhance genome editing specificity, paired gRNA with Cas9 Nickase constructs (CAS75x, CAS77x, and CAS79x vectors) 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. 8: 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 Cas9 Nickase with paired gRNAs, make sure each gRNA is able to efficiently induce indels 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.

5' ATCCNNNNNNNNNNNNNNNNNNN 3'
3' NNNNNNNNNNNNNNNNNNNNCAAA 5'

*** Please note that the adaptor sequences for the CAS7xx series have changed from the previous generation vectors**

**(CAS9xx series). Please check to make sure the oligo pairs contain the correct sequences!**

The top strand has an ATCC overhang at its 5'end, followed by the selected target sequence. The bottom strand has an AAAC overhang at its 5' end followed by a target sequence complementary to the top strand.

Example:

If your target sequence is AGCGAGGCTAGCGACAGCATAGG (AGG = PAM sequence), then the oligo sequences would be:

**Top strand oligo:**

5' – ATCCAGCGAGGCTAGCGACAGCAT - 3'

**Bottom strand oligo:**

5' – AAACATGCTGTCGCTAGCCTCGCT - 3'

## D. Cloning into the Cas9 SmartNuclease™ Vector

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

Dilute your stock primers to 10uM using 1x Annealing Buffer (10mM Tris pH7.5, 50mM NaCl, 1mM EDTA) and set up the annealing reaction as follows:

| Materials | Amount |
|---|---|
| ***10uM Top strand oligo*** | 5 µl |
| ***10uM 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 to RT.

Alternatively, you can set a thermocycler program to cool down the oligos at a rate of 1°C/min (will take ~40min to 60min to complete).

**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.

**Note: Due to the sensitivity of the vectors to repeated freeze- thaw cycles, please store the vectors at 4C for short-term usage or aliquot to individual tubes for long-term storage.**

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 Kanamycin and incubate overnight at 37°C

**4) Confirmation of Positive Clones**

a. Pick 1 to 2 colonies, grow in LB/Kanamycin medium overnight at 37°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.

## E. Transfection of the Cas9 SmartNuclease™ Construct into Target Cells

1) Plate 100,000 to 200,000 of target cells (e.g. 293T cells) into a single well of a 12-well plate in 1 ml of appropriate growth medium. Include a single well of cells as negative control (which can be non-relevant plasmid DNA or linearized Cas9 SmartNuclease plasmid DNA) as well as replicate wells for additional analysis (see Step 4)

2) Next day, or when cells are 50-60% confluent, transfect

target cells with the Cas9 SmartNuclease vector (and donor vector if HDR is desired) using a suitable transfection reagent following the manufacturer's recommended protocol. The use of reduced or serum-free media containing no antibiotics to dilute the vector/transfection complex is highly recommended.

**Note: For 293T cells, we transfected 0.5 µg of the Cas9 SmartNuclease vector into cells for cleavage of gene targets and used a 1:1 ratio of Cas9 SmartNuclease vector (0.5 µg) and HR donor vector (0.5 µg) for HDR applications. In general, we suggest optimizing the amounts and ratios of Cas9 SmartNuclease and donor vectors for optimal results in a target cell line.**

3) Allow at least 12 hours before changing transfection media to complete growth media

4) After 48-72 hrs post-transfection, sort GFP or RFP positive cells using a fluorescence-activated cell sorter (FACS) per standard sorting parameters. We recommend a minimum of 25,000 to 50,000 positive events for subsequent subculturing of cells. Assay for cleavage activity in sorted cells using Surveyor Nuclease Assay, mutation characterization by genotyping analysis, or HDR activity (if using donor vector in parallel) of sorted cells

**Note: If assaying for HDR of donor vector in RFP or GFP-sorted cells, please subculture the sorted cells and select for cells that have undergone HR using an antibiotic selection marker present in the HR donor vector. The optimal concentration of antibiotic for the targeted cell line must be determined empirically by a kill curve assay.**

# 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 4C for short-term use (1-2 weeks) or aliquot each reaction into separate tubes for storage at -20C

**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. By designing several constructs (following the simple design rules outlined in Section II. B and C), one has increased chances of finding a construct(s) to cleave target DNA with the highest efficiency.

**Q. We designed a guide RNA construct to transfect into 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 transfection efficiency of target cells:** For certain cell types (e.g. primary, stem, suspension cells), passive transfection may not be very efficient. In these cases, active transfection systems (e.g. NucleoFection) may provide better results.

**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 48 hours post-transfection to begin assaying for cleavage of a DNA target; however, in certain cases, it may be useful to wait up to 1 week to observe the full effect of cleavage.

**Q. We want to perform HDR applications using the Cas9 SmartNuclease system, but we do not have the corresponding donor vectors. What are our options in this case?**

There are several options for performing HDR of a donor vector into cells that have been targeted with the Cas9 SmartNuclease system.

**Option #1** – Design an HDR donor vector containing the region of DNA to be inserted or corrected into target cells. Typically, this vector contains 5' and 3' arms homologous (~800bp) to the desired insert/correction region, and may contain selection or fluorescent markers for selection of cells after HDR. In addition, single stranded oligo donor vectors can be constructed with areas of small homology (<50bp) flanking the cutting site and containing an small oligonucleotide sequence in the middle. These can be

combined with Cas9/Nickase GFP or RFP expression vectors for FACS sorting to study those cells that have been successfully transfected.

**Option #2 –** SBI provides a full suite of off-the-shelf HDR cloning vectors containing multiple MCS for cloning in of homology arms and insert sequences, as well as selectable fluorescent and antibiotic selection markers. Please inquire for availability of these vectors.

**Option #3 –** SBI can build a custom HR donor vector targeting any sequence of interest as part of our custom cloning services. Please inquire with services AT systembio.com to discuss a custom project or request a quotation.

# IV. References

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# V. Technical Support

For more information about SBI products and to download manuals in PDF format, please visit our web site:

**http://www.systembio.com**

For additional information or technical assistance, please call or email us at:

**System Biosciences (SBI)**

PrecisionX™ T7 Cas9 SmartNuclease System (Tagged and Untagged)
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# VII. Licensing and Warranty information

**Limited Use License**

Use of the PrecisionX™ Cas9 SmartNuclease Expression System (*i.e.*, the "Product") is subject to the following terms and conditions. If the terms and conditions are not acceptable, return all components of the Product to System Biosciences (SBI) within 7 calendar days. Purchase and use of any part of the Product constitutes acceptance of the above terms.

The purchaser of the Product is granted a limited license to use the Product under the following terms and conditions:

- The Product shall be used by the purchaser for internal research purposes only. The Product is expressly not designed, intended, or warranted for use in humans or for therapeutic or diagnostic use.

- The Product may not be resold, modified for resale, or used to manufacture commercial products without prior written consent of SBI.
- This Product should be used in accordance with the NIH guidelines developed for recombinant DNA and genetic research.

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PrecisionX™ T7 Cas9 SmartNuclease System (Tagged and Untagged)
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SBI is committed to providing our customers with high-quality products. If you should have any questions or concerns about any SBI products, please contact us at (888) 266-5066.