PrecisionX Cas9 SmartNuclease™ RNA System

Catalog#s CAS5xxA-1 series

User Manual

Store at -20°C upon receipt

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.

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.

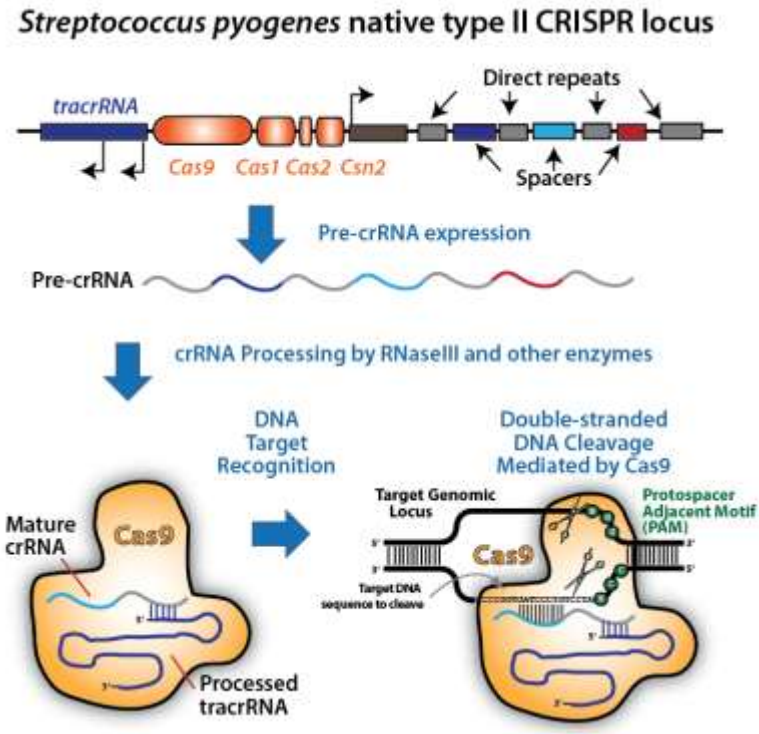


Figure 1: Overview of the CRISPR system. Figure adapted from Cong *et al*. "Multiplex Genome Engineering Using CRISPR/Cas Systems".

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 One mutant form of Cas9 nuclease (D10A) functions as a nickase (Jinek *et al.* 2012), generating a break in 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). Most 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)

An important application of the Cas9/CRISPR system is site-specific transgenesis, which allows targeted modification of embryonic stem (ES) cells for injection into a blastocyst, generating a chimeric population of cells that eventually develop into an animal with the desired genetic modifications. In such applications, the use of synthesized mRNA, as opposed to plasmid DNA, is preferred for efficient generation of transgenic organisms. mRNA, owing to their smaller size, minimal immunogenicity, and lack of genomic integration have become the modality of choice for *in vivo* delivery of Cas9 and targeting guide RNA. (Wang *et al.* 2013, Bassett *et al.* 2013, Shen *et al.* 2013). The availability of transfection-ready Cas9 mRNA and systems for the efficient synthesis of guide RNA will enable advancement of genome engineering for *in vivo* applications.

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 use of the Cas9 system more efficient, affordable, and convenient for applications requiring RNA-based versions of Cas9 and guide RNAs, SBI has developed a suite of ready-to-transfect mRNAs and T7-based systems for *in vitro* transcription of guide RNAs (Table 1).

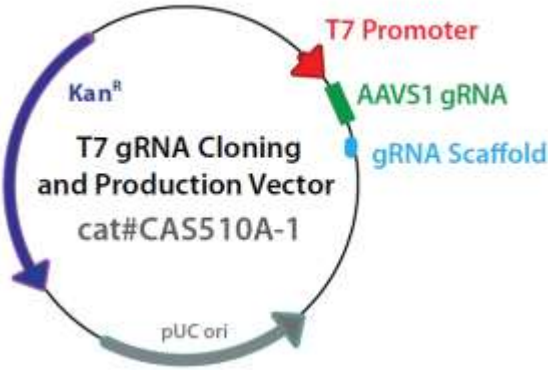


Fig. 2: Vector map of SmartNuclease™ T7 gRNA Vector (Catalog # CAS510A-1)

Table 1. List of available Cas9 ready-to-transfect mRNA and *in vitro* synthesis reagents for guide RNA production

Cat#	Description	Size
CAS500A-1	Transfection-ready hspCas9 SmartNuclease mRNA (Eukaryotic Version)	20 µg
CAS502A-1	Transfection-ready hspCas9 SmartNuclease mRNA (Prokaryotic Version)	20 µg
CAS504A-1	Transfection-ready hspCas9 (D10A) SmartNickase mRNA (Eukaryotic Nickase)	20 µg
CAS510A-1	SmartNuclease™ Linearized T7 gRNA vector	10 rxn
CAS510A-KIT	SmartNuclease™ T7 gRNA Synthesis kit (includes CAS510A-1 and T7 IVT synthesis reagents)	1 kit
CAS520A-1	Transfection-ready Cas9 SmartNuclease AAVS1 gRNA	10 µg
CAS520A-1	Transfection-ready Cas9 SmartNuclease AAVS1 gRNA	10 µg
CAS530G-1	Transfection-ready hspCas9-T2A-GFP SmartNuclease mRNA (wildtype Cas9 w/GFP marker)	10 µg
CAS531R-1	Transfection-ready hspCas9-T2A-RFP SmartNuclease mRNA (wildtype Cas9 w/RFP marker)	10 µg
CAS534G-1	Transfection-ready Cas9 Nickase-T2A-GFP SmartNickase mRNA (Cas9 Nickase w/GFP marker)	10 µg
CAS534R-1	Transfection-ready Cas9 Nickase-T2A-RFP SmartNickase mRNA (Cas9 Nickase w/RFP marker)	10 µg

Table 2. List of components in Catalog # CAS510A-1 (SmartNuclease™ Linearized T7 gRNA vector):

Reagent	Amount
SmartNuclease™ Linearized T7 gRNA Vector	10 µl
5X Ligation Buffer	10 µl
Fast Ligase	2.5 µl
Sequencing Primer (5 µM) 5' GCGGGCCTCTTCGCTATTAC 3'	20 µl

Table 3. List of components in Cat # CAS510A-KIT (SmartNuclease™ T7 gRNA Synthesis Kit):

Reagent	Amount
SmartNuclease™ Linearized T7 gRNA Vector	10 µl
5X Ligation Buffer	10 µl
Fast Ligase	2.5 µl
Sequencing Primer (5 µM) 5' GCGGGCCTCTTCGCTATTAC 3'	20 µl
2X NTP Buffer Mix	100 µl
T7 RNA Polymerase Mix	20 µl
T7 gRNA PCR primer mix (5 µM)	50 µl
DNase I (2U/µl)	10 µl

C. Validation Data for the Cas9 RNA System

We have tested the expression and functionality of ready-to-transfect mRNA for Cas9 nuclease and guide RNAs (Fig. 3) for their ability to cleave target sequences to induce HDR-directed repair in an engineered EGIP (Enhanced GFP Inhibited Protein, HEK293T parental) cell line (Fig. 4). This cell line contains a stop codon in the middle of the coding region of EGFP as well as a 53bp sequence from the human *AAVS1* gene. The results indicate that the mRNA and guide RNA combination is comparable to SBI's plasmid-based all-in-one Cas9 system (Fig. 5) with respect to HDR efficiency. The robustness of the Cas9 mRNA/gRNA system is illustrated by the fact that HDR events can be detected in as little as 18hrs post-transfection, whereas the Cas9 all-in-one plasmid system takes 24-48 hours before positive signals can be seen (data not shown). The combination of Cas9 mRNA and guide RNA presents a robust alternative to plasmid-based Cas9 systems for efficient targeting and cleavage of DNA sequences, especially suitable for *in vivo* applications.

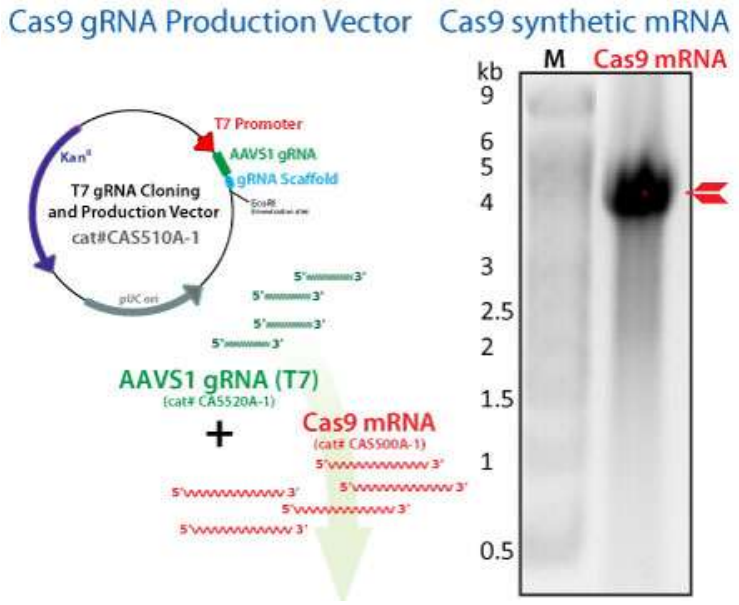


Fig. 3: Ready-to-transfect Cas9 mRNA and guide RNAs targeting human *AAVS1* prepared using the SmartNuclease™ T7 gRNA Synthesis Kit.

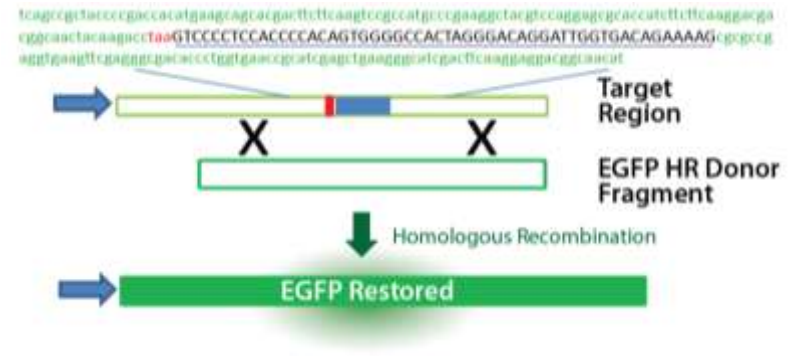


Fig. 4: EGIP cell line targeted by Cas9 mRNA and guide RNAs for monitoring HDR efficiency of donor vector bearing EGFP fragment.

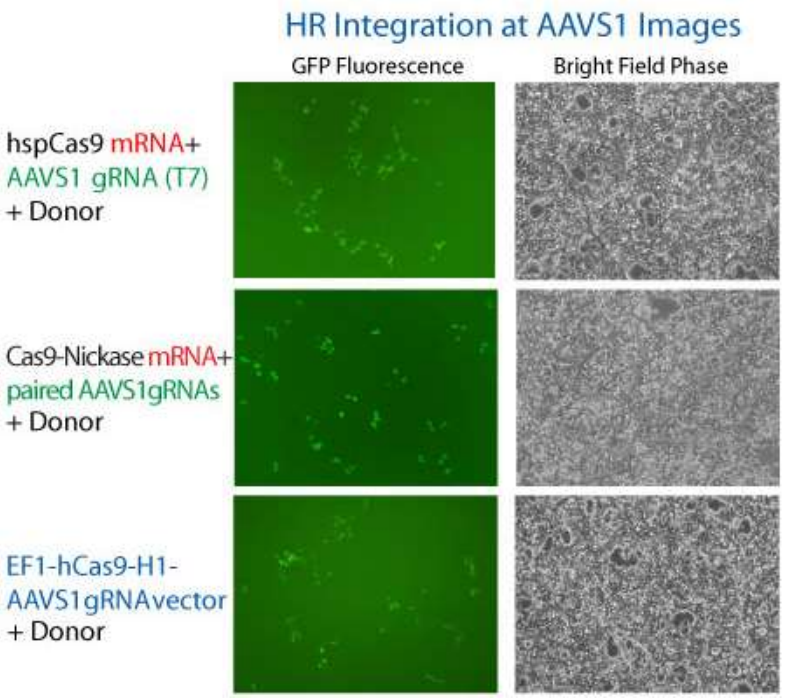


Fig. 5: HDR efficiency of donor EGFP fragment for Cas9 mRNA + gRNA system (top panel) and Cas9 SmartNickase mRNA + paired gRNAs (middle panel) compared to all-in-one Cas9 plasmid system (bottom panel) as measured by GFP positive clones at day 3 post-transfection into EGIP cell line.

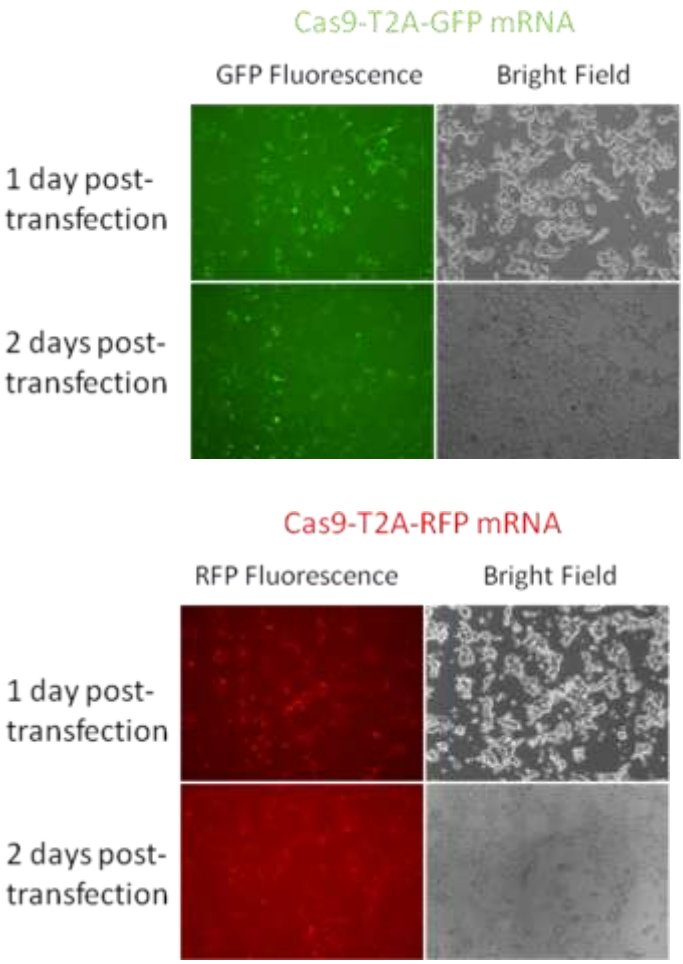


Fig. 6: Transfection efficiency of hspCas9-T2A-GFP (top panel) and hspCas9-T2A-RFP mRNA (bottom panel) in HEK293T cells measured at day 1 and day 2 post-transfection

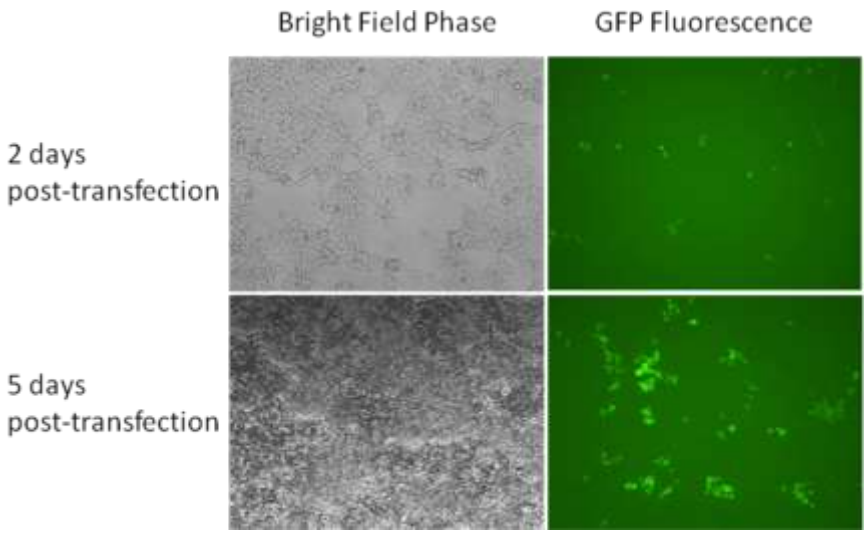


Fig. 7: Evidence for HDR of EGFP donor vector co-transfected into EGIP cell line with hspCas9-T2A-RFP mRNA and *AAVS1* gRNA. HDR was assessed at days 2 and 5 post-transfection. Only the GFP channel is shown for illustration purposes

D. Applications of the Cas9 SmartNuclease™ Expression System

The Cas9 RNA Expression System can be used by 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

E. Additional Materials Required

1) LB Agar and Broth containing 50µg/ml Kanamycin
2) Any high-transformation efficiency *E.coli* competent cells
3) Zyppy™ Plasmid MiniPrep Kit (Zymo Research, Cat. # D4019)
4) High Fidelity DNA polymerase
5) PCR purification kit
6) RNA clean up kit
7) Transfection reagent

F. Related Products

SBI offers a number of Homologous Recombination (HR) Donor Vectors, please review the selection of HR Donor vectors at http://www.systembio.com/genome-engineering-precisionx-HR-vectors/ordering.
Cas9 SmartNuclease AAVS1 Positive Control kit (CAS605A-1) would be a good option for you to be familiar with the CRISPR/Cas9 system and optimize your assay condition.

G. Shipping and Storage Conditions for Kit

PrecisionX™ Cas9 SmartNuclease RNA components are shipped on blue ice or dry ice, depending on the components.

CAS510A-1/CAS510A-KIT: These components are shipped on blue ice, and upon receiving, should be stored at -20°C. Shelf life of the product is 1 year after receipt if stored at -20°C.

All others (CAS500A-1, 502A-1, 504A-1, 520A-1, 530G-1, 531R-1, 534G-1, and 534R-1) are pre-synthesized mRNAs and will be shipped on dry ice. Upon receiving, store the components at -80°C. Shelf life of the product is 1 year after receipt if stored in -80°C.

II. Protocol for guide RNA cloning into Linearized T7 gRNA Vector

A. Quick Overview of the Protocol

The general workflow of cloning the custom guide RNA into the T7 guide RNA cloning vector (Cat # CAS510A-1) is summarized below. 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 T7 gRNA 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

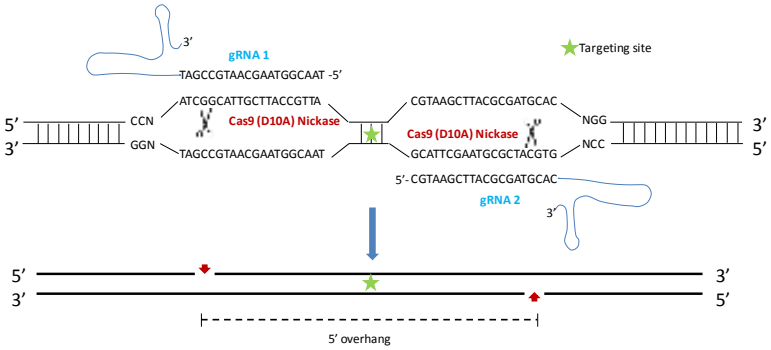
6) Linearize the positive construct with EcoRI or using PCR template for in vitro transcription (IVT) with SmartNuclease™ T7 gRNA Synthesis Kit (Cat # CAS510A-KIT)

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, hspCas9 (D10A) SmartNickase mRNA (CAS504A-1) can be used in complex with two gRNAs to generate double nicking with 5’ overhang. 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 a pair of gRNAs with hspCas9 (D10A) Nickase.

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 wide-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’ AGGGNNNNNNNNNNNNNNNNNNN 3’

3’ NNNNNNNNNNNNNNNNNNNNCAAA 5’

The top strand has an AGGG 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' – AGGGAGCGAGGCTAGCGACAGCAT - 3'

Bottom strand oligo:

5' – AAACATGCTGTCGCTAGCCTCGCT - 3'

D. Cloning into the T7 gRNA Vector

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

Dilute you 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 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. If sequence is verified, please go to Section III for *in vitro* synthesis protocol

III. Protocol for T7-based *in vitro* synthesis of guide RNA

A. Preparation of custom gRNA construct for *in vitro* transcription

There are two approaches to prepare DNA template made in Section II for *in vitro* synthesis of the guide RNA, outlined below:

1. PCR of guide RNA Template

The template for gRNA *in vitro* transcription can be generated using a PCR reaction with the primer mix provided in the SmartNuclease™ T7 gRNA Synthesis kit (Cat# CAS510A-KIT) and positive gRNA construct generated using the SmartNuclease™ T7 gRNA vector in Section II (Cat# CAS510A-1). We recommend a typical setup with Phusion enzyme (NEB) as shown below in a 50 µl reaction:

Reagent	Amount
5×HF Buffer	10 µl
dNTP Mix	1 µl
T7 gRNA PCR primer mix (5µM)	5 µl
Plasmid Template (positive T7 gRNA construct)	10-20 ng
Phusion DNA Polymerase	0.3 µl
Nuclease-Free H_2O	to 50 µl

The PCR conditions for the above setup should be:

Cycle(s)	Temperature	Time
1	98 °C	3 min
30	98 °C	30 s
	56 °C	30 s
	72 °C	10 s
1	72 °C	10 min
	4 °C	hold

Post reaction, PCR products should be examined on an agarose gel before use to verify that the products are unique, and at expected size (~130bp). The PCR product can be spin-column purified using commercial kit(s) from QIAGEN or other vendors.

2. Linearization

Plasmid DNA can be linearized with **EcoRI** downstream of the custom gRNA to be transcribed. Circular plasmid templates will generate extremely long, heterogeneous RNA transcripts because RNA polymerases are very processive. It is highly recommended to examine the linearized template DNA on a gel to confirm that cleavage is complete. Since initiation of transcription is one of the limiting steps of *in vitro* transcription reactions, even a small amount of circular plasmid in template prep will generate a large proportion of transcript. If linearizing the template, we suggest using an EcoRI restriction enzyme with no star activity, such as EcoRI-HF (New England Biolabs) for optimal results. .

a. After linearization of the template, terminate the restriction digest by adding the following in order:

• 1/20th of initial reaction volume (50 µl) of 0.5M EDTA

• 1/10th of initial reaction volume (50 µl) of 3M NaOAc or 5M NH4OAc

• 2 volumes of ice-cold 100% ethanol (~100 µl)

b. Mix well and chill at –80°C for at least 30 min.

c. Pellet the DNA for 15 min in a microcentrifuge at ~13,000rpm

d. Remove the supernatant, re-spin the tube for a few seconds, and remove the residual fluid with a very fine-tipped pipet.

e. Resuspend in nuclease-free water at a concentration of 0.5–1 µg/µl.

Note:

Occasionally, DNA from some miniprep procedures may be contaminated with residual RNase A. Also, restriction enzymes occasionally introduce RNase or other inhibitors of transcription. When transcription from a template is suboptimal, it is often helpful

to treat the template DNA with proteinase K (100–200 µg/mL) and 0.5% SDS for 30 min at 50°C, followed by phenol/chloroform extraction using 1:1 ratio and finally ethanol precipitation (100% ethanol) of the DNA (See Section III.C below)

B. *in vitro* transcription of gRNA construct

1) Thaw the frozen reagents

Place the RNA Polymerase Enzyme Mix on ice, it is stored in glycerol and will not be frozen at –20°C.

Vortex the 2X NTP Buffer Mix until they are completely in solution. ***(Keep the 2x NTP Buffer Mix at room temperature while assembling the reaction***.)

*All reagents should be microfuged briefly before opening to prevent loss and/or contamination of material that may be present around the rim of the tube.

2) Assemble the transcription reaction at room temperature in the following order

Reagent	Amount

Nuclease-free water	x μl
2x NTP Buffer Mix	10 μl
Linearized Template DNA (from Section III.A	x μl *
T7 RNA Polymerase Mix	2μl
Total reaction volume	20 μl

*Use 0.3-0.5 μg PCR-product template or ~1 μg linearized plasmid template.

3) Pipette the mixture up and down gently, and then microfuge tube briefly to collect the reaction mixture at the bottom of the tube.

4) Incubate at 37 °C, 4-6 hrs

5) Add 1 μl DNase I, mix well and incubate 10 min at 37°C. Inactivate Dnase I at 75°C for 10 min, or perform Dnase I inactivation during purification step.

C. Purification of guide RNA transcripts

Synthesized RNA can be purified by using a spin column-based method, or phenol:chloroform extraction followed by ethanol precipitation. Lithium Chloride (LiCl) precipitation is a convenient and effective way to remove unincorporated nucleotides and most proteins. However, this method may not efficiently precipitate RNAs smaller than 300 nucleotides. Therefore, we do not recommend using LiCl for gRNA transcript precipitation.

1) Spin Column Purification

Spin column-based purification will remove proteins, unincorporated nucleotides, and salts from RNA

a. Adjust the volume of the reaction mixture to 100 µl by adding nuclease-free water and mix well.

b. Purify the RNA by following the spin column manufacturer's instructions.

2) Phenol-chloroform Extraction and Ethanol Precipitation

For removal of proteins and most of the free nucleotides, phenol:chloroform extraction and ethanol precipitation of RNA transcripts is the preferred method.

a. Adjust the reaction volume to 180 µl by adding nuclease-free water. Add 20 µl of 3M sodium acetate, pH 5.2 or 20 µl of 5M ammonium acetate and mix thoroughly.

b. Extract with an equal volume of 1:1 phenol:chloroform mixture, followed by two extractions with chloroform. Collect the aqueous phase and transfer to a new tube.

c. Precipitate the RNA by adding 2 volumes of 100% ethanol. Incubate at –80°C for at least 30 minutes and collect the pellet by centrifugation.

d. Remove the supernatant and rinse the pellet with 500 µl of ice cold 70% ethanol.

e. Resuspend the RNA in 50 µl nuclease-free water with 0.1 mM EDTA. Store the RNA at –80°C.

D. Analysis of guide RNA transcripts

The size of the gRNA transcripts can be analyzed by running an aliquot of the reaction on formaldehyde-based denaturing agarose gel.

The concentration of the gRNA transcripts can be determined by reading the A_{260} of a diluted aliquot. Typically, a 1:100 dilution will give an absorbance reading in the linear range of the spectrophotometer. For single-stranded RNA, 1 A_{260} is equivalent to a RNA concentration of 40 µg/ml. The RNA concentration can be calculated as follows:

A_{260} x dilution factor x 40 = __ µg/ml RNA

IV. Protocol for Transfection of Cas9 mRNA and guide RNA

A. Overview of Transfection Conditions

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.

2) Next day, or when cells are 50-60% confluent, transfect target cells with the Cas9 mRNA and gRNA (and appropriate donor vector if HDR is desired) using a suitable transfection reagent following the manufacturer's recommended protocol for 12-well plates. The use of reduced or serum-free media containing no antibiotics to dilute the vector/transfection complex is highly recommended.

Note: We tested 8:1, 16:1, 32:1 and 64:1 ratio of Cas9 mRNA (800 ng) to AAVS1 gRNA (100ng, 50ng, 25ng, 12.5ng) with 0.5 µg HR donor vector in EGIP 293T cells for HDR application. All tested ratios achieved comparable HR efficiency in comparison to positive

control all-in-one plasmid system (0.5 µg EF1-hspCas9-H1-AAVS gRNA with 0.5 µg HR donor vector). For other cell lines, we suggest optimizing the amounts and ratios of Cas9 mRNA, gRNA, and donor vector for optimal results.

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

4) 48-72 hours after initial transfection, assay for cleavage activity using Surveyor Nuclease, PCR genotyping analysis, or HDR activity (if using donor vector in parallel)

5) If assaying for HDR of donor vector, select cells with targeted insertion of donor vector using FACS-based sorting of fluorescent marker or antibiotic selection (e.g. Puro/Neo) using a suitable concentration of antibiotics for the targeted cell line.

V. 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.

VI. References

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VII. Technical Support

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http://www.systembio.com

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VIII. Licensing and Warranty information

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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.

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