AAV Cas9 SmartNuclease System

Catalog numbers: CASAAV-XXX

User Manual

Store at -20°C upon receipt

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I. Introduction

A. Overview of CRISPR/Cas9 and rAAV Systems

In the past decade, a great deal of progress has been made in the field of targeted genome engineering. 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, 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.

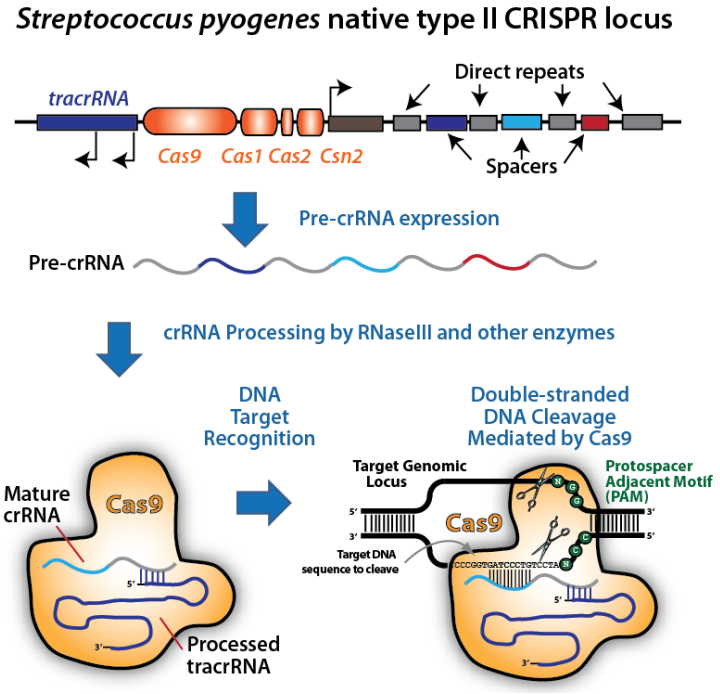


Figure 1: Overview of the CRISPR system. Figure adapted from Cong *et al*. "Multiplex Genome Engineering Using CRISPR/Cas Systems".

The development of CRISPR/Cas9 has already revolutionized what's possible when it comes to manipulating the genomes of even complex organisms. However, *in vivo* delivery has been hampered by the size of the *Streptococcus pyogenes* Cas9 gene (spCas9). To overcome this problem, Ran, *et al*,[1] characterized smaller orthologs of the Cas9 gene and found that Cas9 from *Staphylococcus aureus* (saCas9) performs as efficiently as spCas9 while being ~1 kb shorter, enabling insertion into recombinant AAV (rAAV) vectors.

rAAV vectors have been widely used for gene therapy and genome editing, mainly because of their broad tropism, the lack of disease associated with wild-type virus, ability to transduce both dividing and non-dividing cells, and long term transgene expression (Vasileva A, 2005; Petrs-Silva H, 2013). Packaging rAAV with modified capsid plasmid and adenovirus gene expression plasmid provided *in trans* makes AAV production more convenient and mitigates any biosafety risks. This makes developing a CRISPR/ Cas9 system for *in vivo* delivery via of rAAV ideal.

SBI's AAV-Cas9 expression vectors are based on the commonly used AAV2 serotype. The expression vectors contain inverted terminal repeat (ITR) sequences at both ends of the DNA strand and room for an open reading frame encoding a transgene driven by an exogenous promoter. To produce a high titer of viral particles, expression and packaging vectors are transiently co-transfected into suitable mammalian virus producer cells (*e.g.* HEK 293T cells) for subsequent isolation of rAAV virus particles. For a detailed description of SBI's rAAV isolation process from producer cell culture media, please refer to the AAVanced™ AAV Concentration Reagent user manual.

While saCas9 is just as efficient as spCas9, a few differences between the two systems will affect gRNA design[1].

- saCas9 PAM differs from spCas9 PAM
- saCas9 works most efficiently with gRNAs of 21 nt – 23 nt

saCas9 PAM sequences:

- NNGGGT
- NNGAAT
- NNGAGT

More information about the saCas9 PAM sequences and gRNA design can be found in Section II.A of this manual.

B. Product Information

The AAV-Cas9 system also provides an easy and efficient way to enable CRISPR Cas9 editing *in vivo*. SBI offers AAV-Cas9 constructs in two formats:

1) An "all-in-one" format expressing saCas9 and gRNA from a single vector:

a. **Cat# CASAAV100PA-1**: EF1a-hsaCas9-U6-gRNA(SA) linearized SmartNuclease AAV Plasmid;

2) A two vector system with separate saCas9 and gRNA expression vectors:

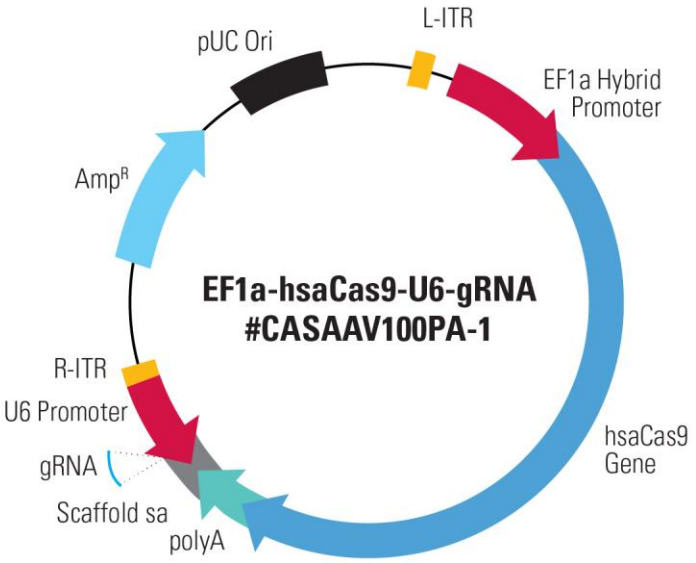
a. **Cat# CASAAV200PA-1**: EF1a-hsaCas9 SmartNuclease AAV Plasmid

b. **Cat# CASAAV300PA-1**: EF1-RFP-U6-gRNA(SA) linearized SmartNuclease AAV Plasmid

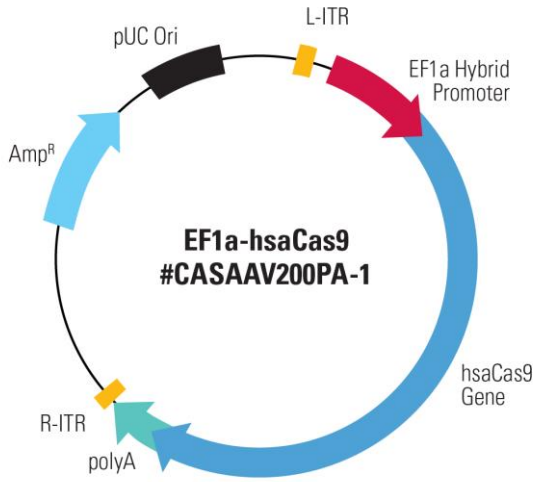
See next section (Section I.C) for Vector Maps.

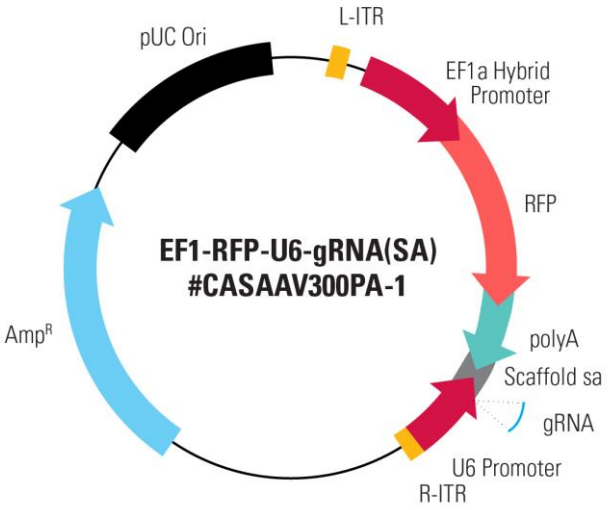
C. Vector Information

All-in-One saCas9 and gRNA AAV Vector:



AAV-Cas9 Two-vector System





D. Validation Data for AAV-Cas9 Vectors

Both SBI's all-in-one AAV-Cas9 and two-vector AAV-Cas9 systems efficiently correct a dysfunctional genomic copy of EGFP.

Starting with an Enhanced Green Fluorescent Inhibited Protein (EGIP) reporter cell line, which has an inactivated EGFP due to a premature stop codon, we designed a gRNA sequence to remove the stop codon and enable GFP expression in conjunction with a rescue HR donor. The gRNA sequence was inserted into either Cat # CASAAV300PA-1, EF1-RFP-U6-gRNA(SA) —the gRNA-expressing plasmid in the two-vector AAV-Cas9 system (top panels)—or Cat # CASAAV100PA-1, the All-in-One saCas9 & gRNA AAV vector (bottom panels). AAV virus particles isolated from both cell lysates (left panels) and packaging supernatants (right panels) were delivered to cells with the rescue HR donor and restored EGFP expression, indicating successful genome editing. Delivery of AAV plasmid(s) alone (middle panels) with HR donor also restores EGFP expression, again indicating successful genome editing.

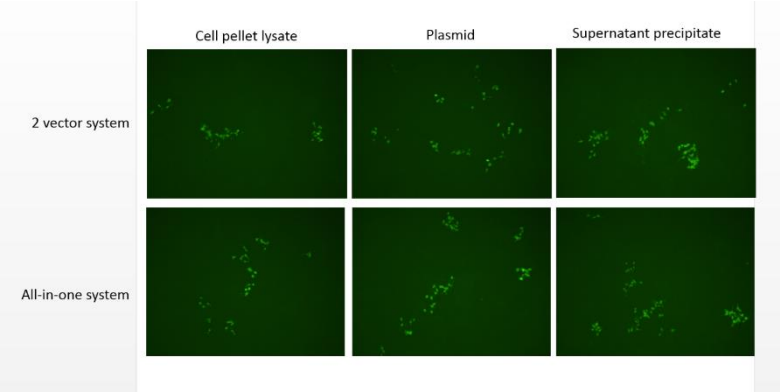


Figure caption: Approximately 1 – 1.5 x 10^6 EGIP reporter cells were seeded into a 12-well plate for infection with isolated AAV particles (left and right panels) or transfection with plasmid (middle panels), along with the rescue HR donor. The top panels were treated with the AAV-Cas9 two-vector system + donor designed to correct the defect in EGIP, restoring EGFP expression. The bottom panels

were treated with the AAV-Cas9 All-in-one system + donor. Three days post-infection/or transfection, EGFP-positive cells can clearly be seen, indicating successful genome editing.

E. Key Advantages of the AAV-Cas9 SmartNuclease™ System

Bringing together the versatile CRISPR/Cas9 genome editing system with powerful recombinant AAV (rAAV) technology, SBI's AAV-Cas9 products extend genome editing capabilities to cutting edge *in vivo* applications.

- Deliver Cas9 *in vivo*
- Edit genomes in post-natal animals
- Develop gene therapies in small animal models
- Generate novel disease models

F. List of Components

1) **Cat# CASAAV100PA-1**: EF1a-hsaCas9-U6-gRNA(SA) linearized all-in-one SmartNuclease AAV Plasmid:

   The all-in-one AAV 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).

2) **Cat# CASAAV200PA-1**: EF1a-hsaCas9 SmartNuclease AAV Plasmid

   The hsaCas9 (only) expression AAV 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 high competency competent cells (per manufacturer's recommended protocol) for best results.

3) **Cat# CASAAV300PA-1**: EF1-RFP-U6-gRNA(SA) linearized SmartNuclease AAV Plasmid

The gRNA (only) AAV 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 AAV cloning and expression vectors.

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

G. Additional Materials Required

For Cloning

- Recommended: One Shot OmniMAX 2 T1R competent cells, Cat. # C854003
- Petri plates containing LB Agar media with 50 µg/ml Ampicillin or Carbenicillin

For Screening Inserts and Sequencing

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

For Purifying cDNA Constructs after Cloning

- Plasmid purification kit
  Recommended: QIAGEN Endofree Plasmid Maxi Kit, Cat. # 12362.
  The following kit combination can be used for Midi scale (up to 200 μg of plasmid DNA) preparation of endotoxin-free DNA:
  - QIAfilter Plasmid Midi Kit, Cat. # 12243, and EndoFree Plasmid Buffer Set, Cat. # 19048

  Please visit the QIAGEN website to download the specialized protocol that is not contained in the current user manual:

  - http://www1.qiagen.com/literature/protocols/pdf/QP15.pdf

For Transfection of AAV Constructs into Target Cells

- Transfection Reagent
  (Recommended: PureFection, System Biosciences Cat. # LV750A-1)

For Packaging of AAV Constructs into Viral Particles

- In order to package your constructs into viral particles, you will need to obtain AAV Packaging Plasmids (typically consisting of plasmids containing adenovirus structural/regulatory genes and Rep-Cap protein specific for different serotypes of AAV). These packaging plasmids can be obtained from various vendors.

For Isolation of rAAV Viral Particles

- We would recommend SBI's AAVanced Concentration Reagent (Cat #AAV100A-1) for simple, one-step isolation of functional rAAV particles from producer media

H. 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.

I. Shipping and Storage Conditions

Linearized all-in-one 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.

saCas9 AAV expression vectors are shipped on blue or dry 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 AAV-Cas9 Expression System

A. Overview of the Protocol

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

1) Design two DNA oligonucleotides that are sense and antisense sequences of the target DNA which is 21bp upstream of the PAM.
   saCas9 PAM sequences:

   NNGGGT
   NNGAAT
   NNGAGT

To create gRNAs, use the following:

Fwd-5.1: ACCGNNNNNNNNNNNNNNNNNNN

(N target sequence or gRNA sequence, 21 nts)

Rev-3.1: aaacXXXXXXXXXXXXXXXXXXX

(X reverse complementary of N, 21 nts)

Example:

aavs1 gRNA for saCas9: CTGTCCCTAGTGGCCCCACTG

sa-AAVS1gRNA-5.1 ACCGCTGTCCCTAGTGGCCCCACTG

sa-AAVS1gRNA-3.1 aaacCAGTGGGGCCACTAGGGACAG

2) Anneal the two oligonucleotides to generate a duplex.

3) Clone the duplex into the provided linearized gRNA cloning rAAV vector 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 rAAV particles for gRNA and Cas9 using your standard protocol.

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

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

B. Cloning into the gRNA AAV Vector

1) Anneal the two single-strand DNA oligonucleotides:

Dissolve your primer with TE buffer at the concentration of 100μM. Then 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).

AAV-Cas9 plasmids can then be packaged into rAAV virus particles using widely available rAAV packaging kits or reagents and following standard protocols.

C. Concentration of Pseudoviral Particles

We recommend the use of SBI's AAVanced™ Concentration reagent as a simple and highly effective means to concentrate AAV particles. It is provided as a 5x solution.

1. Transfer supernatant containing virus to a sterile vessel and add 1 volume of cold AAVanced Concentration Reagent (4ºC) to every 4 volumes of virus supernatant.

(Example: 5ml AAVanced Concentration reagent with 20ml viral supernatant).

2. Refrigerate overnight (at least 12 hours). Viral supernatants mixed with AAVanced Concentration reagent are stable for up to 4-5 days at 4°C.

3. Centrifuge supernatant/ AAVanced 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 AAVanced solution by centrifugation at 1500 × g for 5 minutes. Remove all traces of fluid by aspiration, taking great care not to disturb the precipitated rAAV particles in pellet.

5. Resuspend rAAV 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.

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

D. General rAAV Transduction Protocol

A general protocol for transduction of rAAV particles is shown below. This protocol can be scaled upwards with respect to vessel size.

1. Day 1: Plate 10,000 cells per well in a 24 well plate (about 30% confluence) in culture medium.

2. Day 2: Add virus to each well at different volumes based on the experimental needs (typical amount of virus added will range from 1 – 10 ul for a single well in 24-well plate)

3. Day 3: Change to complete growth medium

4. Day 6-7: Look at the cells for reporter expression if the rAAV construct has a reporter (e.g. RFP) and/or begin screening cells for desired genome modification.

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 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 rAAV virus

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. AAV-Cas9 is near limits of efficient packaging.

The AAV-Cas9 system is near the limit of the packaging capacity of the rAAV system, resulting in low packaging efficiency. We would suggest scaling up the number of 150mm plates for generating sufficient amounts of Cas9/nickase virus for best results.

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:

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Technical Support: tech@systembio.com

Ordering Information: orders@systembio.com

VI. Licensing and Warranty information

Limited Use License

Use of the AAV-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.

SBI has pending patent applications related to the Product. For information concerning licenses for commercial use, contact SBI.

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Limited Warranty

SBI warrants that the Product meets the specifications described in this manual. If it is proven to the satisfaction of SBI that the Product fails to meet these specifications, SBI will replace the Product or provide the purchaser with a credit. This limited warranty shall not extend to anyone other than the original purchaser of the Product. Notice of nonconforming products must be made to SBI within 30 days of receipt of the Product.

SBI's liability is expressly limited to replacement of Product or a credit limited to the actual purchase price. SBI's liability does not extend to any damages arising from use or improper use of the Product, or losses associated with the use of additional materials or reagents. This limited warranty is the sole and exclusive warranty. SBI does not provide any other warranties of any kind, expressed or implied, including the merchantability or fitness of the Product for a particular purpose.

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