



AAVS1 Safe Harbor Targeting System

Cat#:s: GE6xx, CAS6xx

User Manual

See PAC for storage conditions

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I. Introduction

A. Genome targeting using CRISPR-Cas9

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, the CRISPR-Cas9 system has the potential to be a disruptive technology in the field of genome-engineering.

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 expression system, including a human codon optimized Cas9 (hspCas9) and custom guide RNA (gRNA) consisting of a chimeric crRNA-tracrRNA transcript expressed from a single construct.

B. AAVS1 Safe Harbor Site Targeting

A crucial application of CRISPR-Cas9 technology has been targeted genome engineering of human pluripotent cells such as hESCs and iPSCs, as these cells have the capacity for many broad-based applications in studying human disease. Traditional viral-mediated gene transfer into these cells has often led to variable and sometimes unpredictable results, namely transgene silencing and/or acquisition of tumorigenic phenotypes through insertional mutagenesis. To address these shortcomings, guide RNAs have been designed and validated to target the human *AAVS1* (also known as *PPP1R12C*) locus, a well-validated “safe harbor” in the human genome (Sadelain *et al.* 2011). A safe harbor is defined as a region of the genome that is considered to be both transcriptionally active and its disruption does not lead to discernable phenotypic effects. The targeting of the *AAVS1* locus by site-specific nucleases and homologous recombination of donor plasmids bearing GFP or antibiotic selection markers have been previously demonstrated in human ESC and iPS cells. The targeting efficiencies within these two cell types has been reported as ranging between 40-80% (Hockemeyer *et al.* 2011). Notably, all successfully targeted cells showed expression of GFP as well as several pluripotency markers (e.g. Oct4, Sox2, TRA-1-60). These cells retained the ability to differentiate into tissue representing all three germ layers, suggesting that targeting and modification of the *AAVS1* locus results in stable transgene expression and no discernible “off-target” phenotypes.

C. Detailed information and vector choices for AAVS1 Safe Harbor Targeting System

Delivering consistent, robust transgene expression, the *AAVS1* safe harbor site is a preferred target for gene knock-ins. Insertion at the site has been shown to be safe with no

phenotypic effects reported, and the surrounding DNA appears to be kept in an open confirmation, enabling stable expression of a variety of transgenes.

System Biosciences (SBI) now offers a wide range of tools to help researchers take advantage of the power of the AAVS1 safe harbor site, from elegantly designed vectors and kits that simplify knock-in of any gene while minimizing off-target effects, to a cell line that stably expresses Cas9 for streamlined genome-wide screening efforts. SBI's line of AAVS1 safe harbor site tools is just another way SBI transforms the latest technological advances into robust tools that accelerate your research.

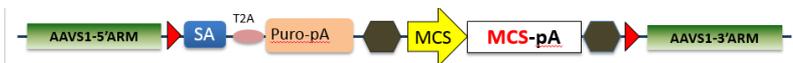
AAVS1 Donor Vectors:

SBI offers a new collection of AAVS1 HR donor vectors which limit off-target integration for highly-specific targeting of the AAVS1 site. Taking advantage of the AAVS1's location within an intron, the puromycin selection marker has only a splice acceptor site and no promoter. Expression of puromycin can only occur when the construct integrates within an intron, reducing the probability of recovering off-target integrants in the presence of puromycin selection.

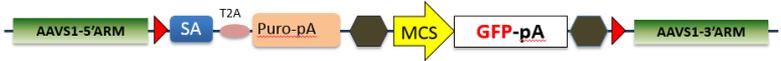
For constitutive transgene expression, we offer a vector with a multiple cloning site (MCS) downstream of the EF-1 promoter and upstream of a poly-A tail (AAVS1-SA-puro-EF1-MCS donor, Cat # GE622A-1).



Or, for more cloning freedom, we also have the same donor vector without any promoter, simply an MCS upstream of a poly-A tail (AAVS1-MCS donor-SA-puro, Cat # GE620A-1).



For reporter cell line creation, we offer an AAVS1 donor vector with an MCS upstream of GFP—just add your promoter of choice for tissue-specific or other type of conditional GFP expression (AAVS1-MCS-GFP-donor-SA-puro, Cat# GE624A-1).



Our donor vectors are designed to work in tandem with our AAVS1 gRNA/Cas9 expression vector (Cat #CAS601A-1), which encodes a pre-validated gRNA specific for AAVS1 and the PrecisionX™ Cas9 SmartNuclease. Simply co-transfect cells with the AAVS1 gRNA/Cas9 expression vector and your AAVS1 donor construct of choice for targeted integration.

AAVS1 donor vectors can be purchased alone or as part of a AAVS1 targeting kit (Cat #s GE620A-KIT, GE622A-KIT or GE624A-KIT), which comes with any of our three AAVS1 donor vectors, AAVS1 gRNA/Cas9-expression construct, and junction PCR primers to confirm the correct integration site (Cat # GE640PR-1).

Additional donor vector options include positive control pAAVS1D-CMV-RFP-EF1a-copGFP-T2A-Puro, Cat#GE603A-1, which constitutively expresses RFP, GFP and Puro markers, and another empty donor vector, pAAVS1D-PGK-MCS-EF1a-copGFP-T2A-Puro, Cat#GE602A-1, with EF1a driven GFP-Puro dual markers. All donor vectors contain homologous sequences to ~800bp upstream and downstream of the AAVS1 targeting sites.

Stable Knock-In of Cas9 into the AAVS1 Safe Harbor Site:

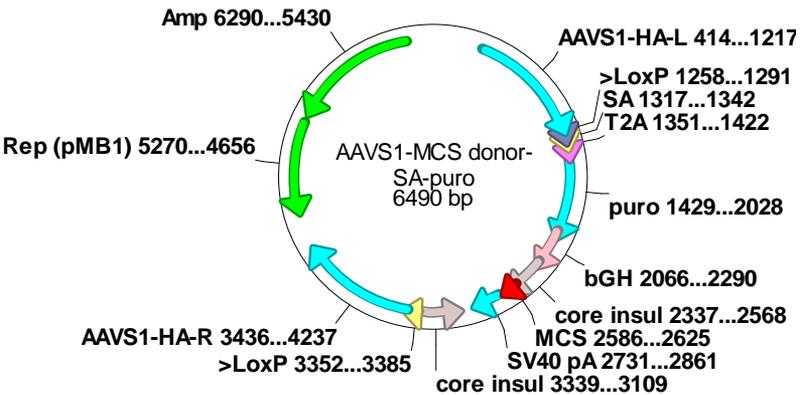
Combining the power of the AAVS1 safe harbor site with the ease and precision of CRISPR/Cas9, we offer both an AAVS1 Cas9-gene donor vector (**Cat# CAS620A-1**) and puro resistant HEK293 cells with Cas9 already integrated and expressed from the AAVS1 site (**Cat# CAS630A-1**).



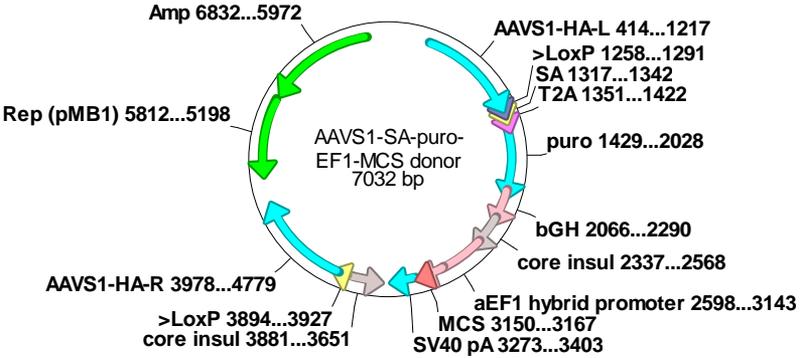
With our puro resistant Cas9/HEK293 cells, you can go directly to transfection of your gRNAs and assessment of the biology - no need to spend time cloning Cas9 into the genome. And when you need Cas9 expression from other cell types, you can turn to our AAVS1 Cas9-gene donor vector and integrate Cas9 into the AAVS1 safe harbor site in the cells of your choice.

D. Detailed Vector Maps

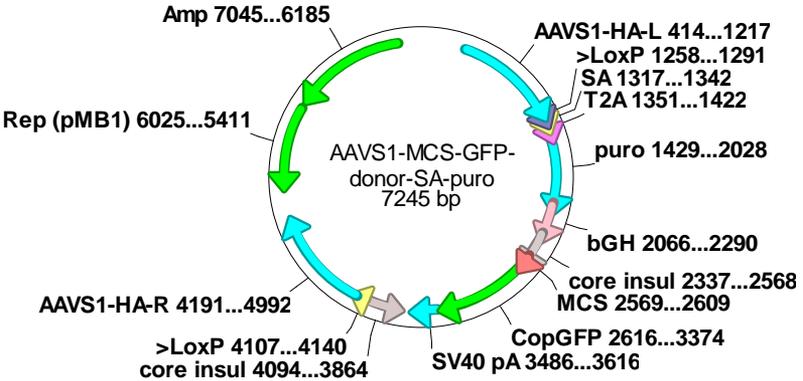
GE620A-1:



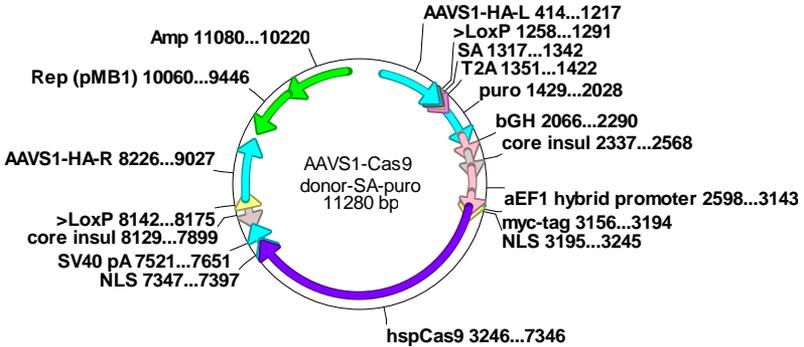
GE622A-1:



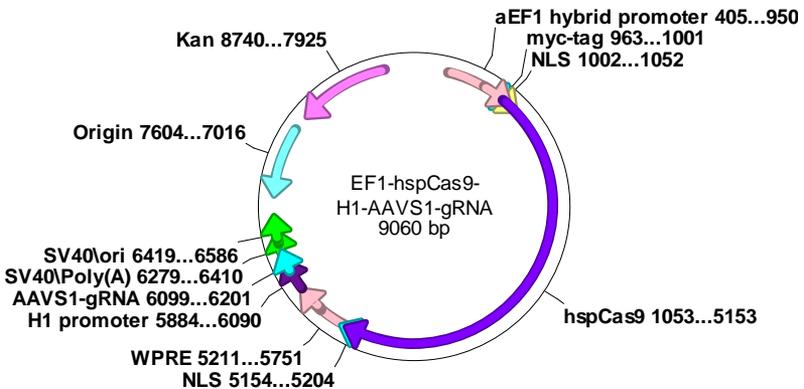
GE624A-1:



CAS620A-1:



CAS601A-1:



AAVS1-gRNA sequence:

```
ggggccactagggacaggatGTTTTAGAGCTAGAAATAGCAAGT
TAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGC
ACCGAGTCGGTGCTTTTTTTT
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AAVS1 target sequence: gggggccactagggacaggat

E. List of Components

List of Components for Cat # GE62xA-KIT (AAVS1 Targeting Kits)

CAT. NO	DESCRIPTION	QTY
GE62xA-1	AAVS1 Safe Harbor HR Donor Vector (Amp ^r)	10 µg
CAS601A-1	AAVS1 gRNA/Cas9 SmartNuclease Targeting Vector (Kan ^r)	10 µg
GE640PR-1	Primer Mix for Junction PCR to Confirm AAVS1 Integration Site	50 µl each

List of Components for Cat # CAS620A-KIT (Cas9 Knock-In Kit)

CAT. NO	DESCRIPTION	QTY
CAS620A-1	AAVS1 Cas9 Knock-in HR Donor Vector (Amp ^r)	10 µg
CAS601A-1	AAVS1 gRNA/Cas9 SmartNuclease Targeting Vector (Kan ^r)	10 µg
GE640PR-1	Primer Mix for Junction PCR to Confirm AAVS1 Integration Site	50 µl each

F. Additional Materials Required

- a) LB Agar and Broth containing 50 µg/ml carbenicillin or kanamycin
- b) Any high-transformation efficiency RecA- and EndA- *E.coli* competent cells
- c) Dulbecco's Modified Eagle's Medium (D-MEM) high glucose with sodium pyruvate and glutamine (Invitrogen, Cat. # 11995073)
- d) Lipofectamine 2000 transfection reagent (Invitrogen, Cat. # 11668019)
- e) Qiagen EndoFree Plasmid Maxi Kit (Qiagen, Cat. # 12362)

- f) Qiagen DNeasy Blood and Tissue Kit (Qiagen, Cat. # 69504)
- g) iProof High-Fidelity DNA Polymerase (BioRad, Cat. # 172-5301)
- h) Fetal Bovine Serum (Invitrogen, Cat. # 16000036)
- i) Penicillin/Streptomycin (Invitrogen, Cat. # 15070063)
- j) Trypsin-EDTA (Sigma, Cat. # T3924)
- h) 6-well Tissue Culture Plates and Related Tissue Culture Supplies
- i) Other specific media and additives specific for cell type of interest
- j) ****Optional**** - For difficult-to-transfect cells, the use of an electroporation system (e.g. Lonza's NucleoFector or Invitrogen's Neon system) is highly recommended

II. Validation Data for Stable Cas9 Knock-In to AAVS1 Safe Harbor

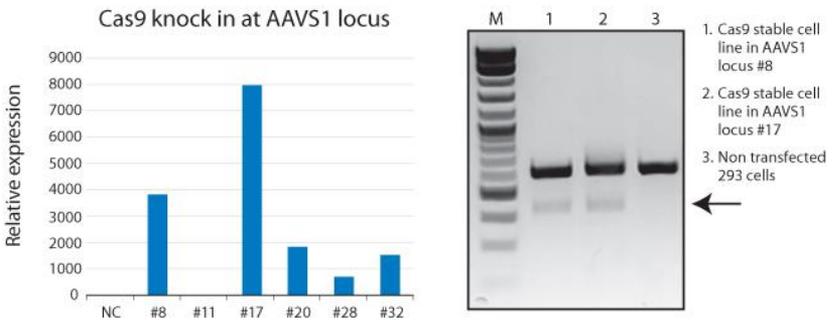


Fig. 1. High-expression of the Cas9 gene at the AAVS1 locus in HEK293 cells, as determined by qPCR (left panel). A Surveyor Assay of high expressing clones #8 and #17 shows that Cas9 is functional - arrow corresponds to band indicative of Cas9-

mediated cleavage event, which is not present in untransfected cells.

III. Protocol for Genome Engineering of Target Cells using AAVS1 Safe Harbor Targeting Kits

A. General Comments

We recommend propagation of the plasmids provided in the AAVS1 Safe Harbor Targeting kits prior to starting the experiments. The plasmids can be transformed using standard conditions suitable in any RecA- and EndA- *E.coli* competent cell.

Cells with the AAVS1 Donor vectors should be grown on LB-Carbenicillin plates (50µg/ml). Cells with the AAVS1 gRNA/Cas9 targeting vectors should be grown on LB-Kanamycin plates (50µg/ml). Incubate the plates at 37°C overnight. Colonies picked from the transformation can be grown at 37°C overnight in ~200ml of LB media containing carbenicillin. After overnight growth, plasmid DNA can be harvested from culture using an endotoxin-free DNA plasmid maxiprep kit.

For confirmation of the plasmid, we recommend performing restriction digestion analysis or direct sequencing to confirm integrity of the amplified plasmids.

B. Cloning into AAVS1 donor vectors

For rapid and efficient cloning of any insert into the donor vector, we recommend SBI's **Cold Fusion Cloning Kit** as

a ligase and restriction enzyme-free cloning method.

More details can be found here:

(<http://www.systembio.com/molecular-tools/cold-fusion-cloning/overview>)

For standard cloning strategies, use the following protocol:

1. Ligation of insert into vector

- a) Dilute gel-purified, digested vector to 10ng/ μ l
- b) Set up 10 μ l ligation reactions for each control and test samples as below:

<u>Volume</u>	<u>Item</u>
1.0 μl	AAVS1 HR cloning vector
7.0 μl	DNA insert (~30-50 ng) or water control
1.0 μl	10X T4 DNA ligase buffer
1.0 μl	T4 DNA Ligase (40 U/ μ l)
10.0 μl	Total Reaction Volume

- c) Incubate reactions at 25 $^{\circ}$ C for 1-2 hours (sticky-end ligation) or O/N at 16 $^{\circ}$ C (for blunt-end ligation)

2. Transform *E. coli* with the ligation product

Transform competent cells (with a transformation efficiency of at least 1×10^9 colonies/ μ g pUC19) with the whole ligation reaction (10 μ l) following the protocol provided with the competent cells. Plate the transformed bacteria on LB-Ampicillin/Carbencillin agar plates.

3. Identify clones with the correct insert

- a) Depending on the ratio of colony numbers for the cDNA sample vs. the negative control sample, randomly pick 5 or more well-isolated colonies and grow each clone in 100

μl of LB Broth with 75 μg/ml ampicillin/carbenicillin at 37°C for 2 hours with shaking.

b) Use 1 μl of each bacterial culture for screening DNA inserts by PCR and continue to grow the culture for another 4 hours. Store the culture at 4°C.

c) Prepare a PCR Master Mix with PCR primers flanking the insert:

<u>1 rxn</u>		<u>10 rxn</u>		<u>Composition</u>
0.5	μl	5	μl	PCR primer 1 (10 μM)
0.5	μl	5	μl	PCR primer 2 (10 μM)
0.5	μl	5	μl	50X dNTP mix (10 mM of each)
2.5	μl	25	μl	10X PCR Reaction Buffer
19.5	μl	195	μl	Nuclease-free water
0.5	μl	5	μl	Taq DNA polymerase (approx. 5 U/μl)
24.0	μl	240	μl	Total volume

d) Mix the master mix very well and aliquot 24 μl into each well of 96-well PCR plate or individual tubes.

e) Add 1 μl of each bacterial culture from step (b) into each well (or tube).

f) Proceed with PCR using the following program:

94°C, 4 min	1 cycle
94°C, 0.5 min, then 68°C, 1 min/1 kb*	25 cycles
68°C, 3 min	1 cycle

* Depending on the size of final PCR product, use a shorter or longer time.

g) Take 5 μl of the PCR reaction and run it on a 1.2% agarose/EtBr gel in 1X TAE buffer to identify clones with correct insert.

4. Grow a positive clone containing insert in an appropriate amount of LB-Ampicillin/Carbenicillin Broth, and purify the construct using an endotoxin-free plasmid purification kit. Sequence verification of the insert is optional.

C. Co-transfection of AAVS1 gRNA/Cas9 vector and HR donor plasmids

- 1) Plate ~100,000 to 300,000 cells/well in a 6-well plate according to established recommended conditions for cell type(s) being transfected. Include wells for the following:
 - a) AAVS1 gRNA/Cas9 vector + HR donor vector
 - b) HR donor vector only
- 2) Next day, prepare transfection complexes of AAVS1 gRNA/Cas9 vector and HR donor plasmids using a suitable transfection reagent such as SBI's PureFection or Lipofectamine 2000+Plus reagent according to the manufacturer's recommended instructions. Leave the transfection complex on the cells for >6 hours.

Example: For HEK293T cells using Lipofectamine 2000 reagent, transfect 1 μ g of CAS601A-1 gRNA/Cas9 vector and 1 μ g of HR donor vector.

Tech Notes:

- a) Since transfection efficiencies vary across different cell lines, we recommend optimizing the input of AAVS1 gRNA/Cas9 plasmid to HR donor plasmid for best results. We recommend starting with a 1:1 ratio (e.g. 1 μ g of donor HR plasmid and 1 μ g of the AAVS1 gRNA/Cas9 plasmid).
- b) For optimal results, we recommend complexing of DNA with transfection reagent in serum- and

antibiotic-free media and cells growing in complete media (e.g. DMEM/F12+10% FBS w/o antibiotics).

- c) For hard-to-transfect cells (e.g. primary, stem, hematopoietic), it may be advisable to utilize a non-passive transfection method such as NucleoFection (Lonza) or Neon system (Life Technologies). Please follow recommended transfection guidelines provided by the manufacturer for specific cell type(s) being transfected.
- 3) 24 hours post-transfection, remove transfection media and split the cells 1:10 and 1:20 in complete growth media w/antibiotics. Plate cells into 6-well plates and save a set of plate(s) for characterization of samples by junction-PCR assay (see below). Allow cells to recover for 24 hours.
- 4) Begin puromycin selection 1 week post-transfection. For 293T cells, the recommended concentration of puromycin is 0.5-1 µg/ml.

Tech Note:

The effective working puromycin concentration for a target cell line can be determined by establishing a kill-curve on untransfected cells. The concentration of puromycin (typical working range of 0.5µg-5µg/ml) that kills >90% of cells after 48hours of selection is the correct dose for the cells being selected.

D. Characterization of AAVS1 targeted recombinant cells

- 1) Assay for Cas9 cutting and HDR of donor vectors on samples as follows:
 - a) AAVS1 gRNA/Cas9 vector + HR donor:
Select cells in Puromycin for 7-10 days, resulting colonies should be Puro resistant.
 - b) HR donor vector only
Select cells in Puromycin for 7-10 days, very few colonies (if any) should be seen relative to cloning donor vector + gRNA/Cas9 vector (Sample a). Presence of Puro^R colonies indicates frequency of random integration events.
- 2) For confirmation of donor vector integration specifically at the *AAVS1* target locus, junction-PCR can be performed using PCR primer pairs that flank the 5' *AAVS1* homology arm (5' *AAVS1*-HA-L) and 3' *AAVS1* homology arm (3' *AAVS1*-HA-R). Junction-PCR primers are provided in the *AAVS1* Targeted Integration Kits or sold separately as GE640PR-1.
- 3) If using CAS620A-1 or CAS620A-KIT to stably integrate Cas9 into the *AAVS1* target locus, Cas9 expression can be assayed using the CAS9-PR-1 PCR primers, or a suitable Cas9 antibody to confirm protein expression.
- 4) Donor Vector Junction-PCR
 - a) Primer Sequences

5' junction PCR primers:

5' junction PCR-fwd: TCCTGAGTCCGGACCACTTT

5' junction PCR-rev: CACCGCATGTTAGAAGACTTCC

5' junction PCR amplicon: 1062bp

3' junction PCR primers:

3' junction PCR-fwd: CAGTGGCAGCCAGGTTTAGC

3' junction PCR-rev: CCTGGGATACCCCGAAGAGT

3' junction PCR amplicon: 1431bp

The primers are provided as mixes (F/R primers) at 10 μ M. Validation of either the 5' or 3' homology arms for donor integration is usually sufficient; however, both arms can be done for additional confirmation.

- b) Protocol Details for 5' AAVS1 Positive Control Donor Junction-PCR Assay:
 - i. Isolate genomic DNA from positive control cells using a suitable genomic DNA miniprep kit for extraction of gDNA from cultured cells. Please follow manufacturer's recommended protocol.
 - ii. Perform Junction-PCR (PCR reaction below)

Reagent	Cas9 cut + positive control donor	Positive control donor only
Cas9 cut + Donor plasmid DNA (200 ng/ μ l)	1 μ l	N/A
Donor plasmid only DNA (200 ng/ μ l)	N/A	1 μ l
5X PCR Buffer	5 μ l	5 μ l
10 mM dNTPs	0.5 μ l	0.5 μ l
10 μ M 5' junction PCR Primer Mix	1.25 μ l	1.25 μ l
iProof DNA Polymerase (2 U/ μ l)	0.25 μ l	0.25 μ l
PCR-grade water	17 μ l	17 μ l
Total Volume	25 μ l	25 μ l

- iii. Proceed with Touchdown PCR protocol below:

98°C, 30 sec
 98°C, 15 sec
 65 to 60°C (-0.5°/cycle), 30 sec
 72°C, 40 sec

} 10 cycles

98°C, 15 sec
 60°C, 30 sec
 72°C, 40 sec
 72°C, 1 min

} 30 cycles

Hold at 4°C

- iv. Run the PCR reaction out on a 1.5% agarose/EtBr gel in 1X TBE buffer to confirm the Junction-PCR results. Sample data can be found below the 3' Junction-PCR protocol (next section).

- c) Protocol Details for 3' AAVS1 Positive Control Donor Junction-PCR Assay :

- i. Perform Junction-PCR (PCR reaction below)

Reagent	Cas9 cut + positive control donor	Positive control donor only
Cas9 cut + Donor plasmid DNA (200 ng/μl)	1 μl	N/A
Donor plasmid only DNA (200 ng/μl)	N/A	1 μl
5X PCR Buffer	5 μl	5 μl
10 mM dNTPs	0.5 μl	0.5 μl
10 μM 3' junction PCR Primer Mix	1.25 μl	1.25 μl
iProof DNA Polymerase (2 U/μl)	0.25 μl	0.25 μl
PCR-grade water	17 μl	17 μl
Total Volume	25 μl	25 μl

ii. Proceed with a Touchdown PCR protocol below:

98°C, 1 min
 98°C, 20 sec
 66 to 61°C (-0.5°/cycle), 30 sec
 72°C, 2 min*

} 10 cycles

98°C, 20 sec
 61°C, 30 sec
 72°C, 2 min*
 72°C, 5 min

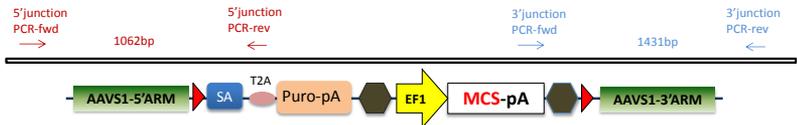
} 30 cycles

Hold at 4°C

*Indicates that long extension is needed for PCR product to be made.

iii. Run the PCR reaction out on a 1.5% agarose/EtBr gel in 1X TBE buffer to confirm Junction-PCR results. Expected size of the amplicon will be ~1.3kb.

Sample results for 5' and 3' Junction-PCR Assay shown below:



IV. References

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V. Technical Support

For more information about SBI products or to download manuals in PDF format, please visit our website:

<http://www.systembio.com>

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

tech@systembio.com

VI. Licensing and Warranty Statement

Acknowledgement:

Design of the pAAVS1D-CMV-RFP-EF1 α -copGFP-T2A-Puro vector was performed by Dr. Jizhong Zou of the NIH Center for Regenerative Medicine, a Common Fund initiative of the U.S. National Institutes of Health.

Limited Use License

Use of the AAVS1 Safe Harbor Targeting 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:

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- This Product should be used in accordance with the NIH guidelines developed for stem cell research.

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