Genome-TALER™ Human AAVS1 Safe Harbor Gene Targeting Kit

Catalog# SH-AVS-K100

User Manual

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USER MANUAL

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I. Introduction

Background of TALEN

Transcription activator-like (TAL) effectors can recognize and bind host plant promoter sequences through a central repeat domain consisting of a variable number of ~34 amino acid repeats. The residues at the 12th and 13th positions of each repeat appears to be a simple one-to-one code to each DNA base of target sequence, e.g. NI = A, HD = C, NG = T, and NN = G or A.

TAL effectors have been utilized to create site-specific gene-editing tools by fusing target sequence-specific TAL effectors to nucleases (TALENs), transcription factors (TALE-TFs) and other functional domains. These fusion proteins can recognize and bind chromosome target sequences specifically and execute their gene-editing functions, such as gene knockout, knockin (with donor plasmid), modification, activation, repression and more.

Safe gene targeting

The modification of the human genome by insertion of genes of interest and other genetic elements in unique site(s) of chromosome(s) is of great value for cell engineering. The genetically modified cells are valuable for therapeutic research, gene function study as well as lineage tracking and analysis. All these applications depend on the reliable and predictable function of the transgene without perturbing any endogenous gene and/or other regulation element. Random integration of the transgene, on the contrary, can present a threat of unpredicted insertion or mutagenesis.

The new approach recently developed is to deliver the transgene to a predetermined and safe site in a genome. AAVS1 (also known as PPP1R2C locus) in human chromosome 19, is a well-validated “safe harbor” to host the DNA fragment with expected function. It has an open chromatin structure and is transcription-competent. Most importantly, there is no known adverse effect on the cell resulting from the inserted DNA fragment of interest.

Genome-TALER™ human AAVS1 safe harbor gene targeting kit is designed to specifically transfer your gene of interest, selection marker or other genetic element from a donor plasmid into the AAVS1 safe harbor site in human chromosome 19 via TALEN-mediated homologous recombination (HR). HR is a natural DNA repair mechanism that occurs in response to DNA double-strand breaks (DSBs). The DSBs here are created by AAVS1-specific TALENs (figure 1).

Advantages

Safe integration
Designated AAVS1 human genome safe-harbor integration site ensures transcription-competency of the transgenes and presents no known adverse effect

Specific targeting
TALEN-mediated DNA DSBs at the AAVS1 site stimulate homologous recombination dramatically for transgene integration

Known copy number
Known copy number of the transgene ensures predictable expression level and simplifies phenotype interpretation

Compatible knock-in ORFs
Over 18,000 sequence-verified human ORFs are compatible for transgene donor DNA design

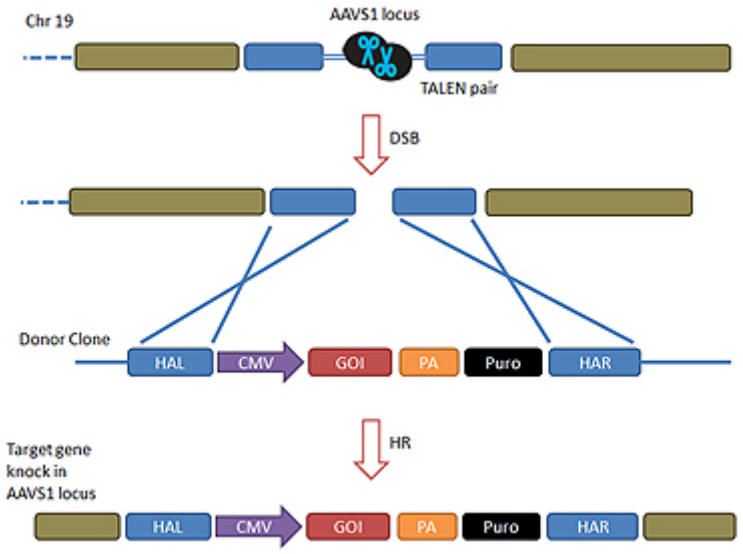


Figure 1. Illustration of TALEN-mediated transgene integration at the safe harbor AAVS1 site of human genome

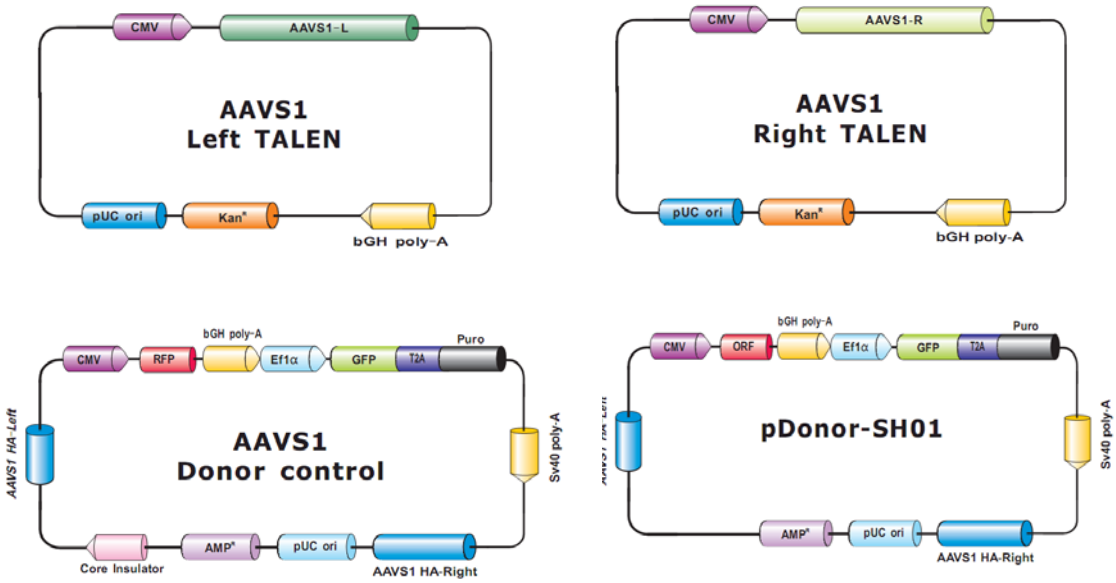
II. Content and storage

Genome-TALER human AAVS1 safe harbor gene targeting kit (Cat# SH-AVS-K100)

Cat. No.	Product name	Qty	Concentration	Shipping and Storage
TN-AAVS1-L	AAVS1 left TALEN	10 µg	500 ng/µl	Shipped at room temperature. Stored at -20°C
TN-AAVS1-R	AAVS1 right TALEN	10 µg	500 ng/µl	Shipped at room temperature. Stored at -20°C
DC-DON-SH01	AAVS1 donor vector	10 µg	500 ng/µl	Shipped at room temperature. Stored at -20°C
DC-RFP-SH01	AAVS1 RFP control	10 µg	500 ng/µl	Shipped at room temperature. Stored at -20°C
HQPAVSHR-5	5' HR primer pair	200 reactions	10 µM	Shipped at room temperature. Stored at -20°C
HQPAVSHR-3	3' HR primer pair	200 reactions	10 µM	Shipped at room temperature. Stored at -20°C

* AAVS1 donor clones can be custom-made and included in the kit instead of the empty AAVS1 donor cloning vector.

(A) TALEN and donor plasmids



(B) Knock-in verification PCR primers

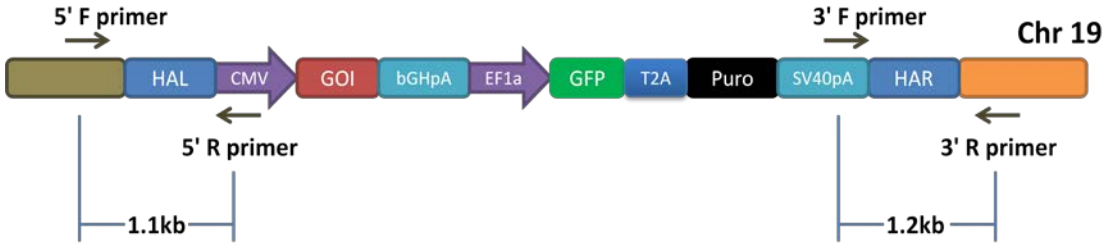


Figure 2. Safe harbor gene targeting kit components. (A) TALEN and donor plasmids (B) Knock-in verification primer pairs

Acknowledgement: Design of the AAVS1 left TALEN, AAVS1 right TALEN, and AAVS1 donor control vectors 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.

Additional materials required

1. LB Agar and broth containing 50 µg/ml kanamycin
2. 6-well tissue culture plates and related tissue culture supplies
3. Other specific media and additives specific for cell type of interest
4. Any high-transformation efficiency RecA- and EndA- E.coli competent cells
5. Dulbecco's Modified Eagle's Medium (D-MEM) high glucose with sodium pyruvate and glutamine (Invitrogen, Cat. # 11995073)
6. EndoFectin™ Plus Transfection Reagent (Genecopoeia, Cat. # EFP1003-01/02)
7. Qiagen EndoFree Plasmid Maxi Kit (Qiagen, Cat. # 12362)
8. Qiagen DNeasy Blood and Tissue Kit (Qiagen, Cat. # 69504)
9. iProof High-Fidelity DNA Polymerase (BioRad, Cat. # 172-5301)
10. Fetal Bovine Serum (Invitrogen, Cat. # 16000036)
11. Penicillin/Streptomycin (Invitrogen, Cat. # 15070063)
12. Trypsin-EDTA (Sigma, Cat. # T3924)
13. **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

III. Example

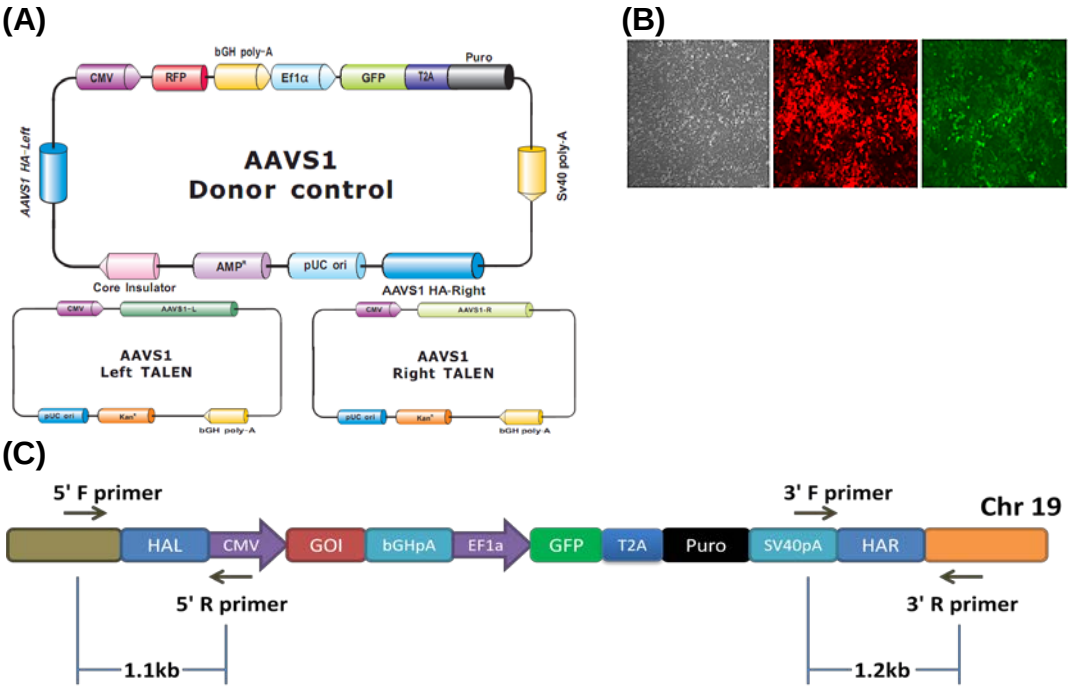


Figure 3. Human genome safe harbor AAVS1 gene targeting

(A) AAVS1 donor control plasmid pAAVS1D-RFP-copGFPpuro (800 ng) was co-transfected with AAVS1 TALEN pair(600 ng for each) or control TALEN pair into HEK293T cells in a 6-well pate.

(B) 48 hr post-transfection, the cells were split 1:10 into a new 6-well pate and selected against 1.0 µg/ml of puromycin. The images were taken after two weeks of selection. The cells transfected with pAAVS1D-RFP-copGFPpuro + control TALEN were completely killed by puromycin selection (data not shown).

(C) (D) PCR primers designed to amplify the HR junction were used to verify the successful integration.

IV. Procedure

Plasmid propagation

We recommend propagating the plasmids provided in the safe harbor kit before the gene targeting experiment. Plasmids can be transformed using standard conditions suitable in any RecA- and EndA- E.coli competent cell.

For transformation of AAVS1 TALENs, we suggest plating 50-200µl of transformed cells on fresh LB-Kanamycin plates (50µg/ml). For plasmids in pDonor-SH01 vector, use LB-Ampicillin plates (50µg/ml). Incubate the plates at 37°C overnight. Inoculate colonies from the transformation and grow them at 37°C overnight in ~200ml of LB media containing either 50µg/ml of Kanamycin (AAVS1 TALENs) or Ampicillin (pDonor-SH01 vector). Use an endotoxin-free plasmid DNA maxiprep kit to extract plasmid DNA after the overnight growth.

To confirm integrity of the amplified plasmids, we recommend restriction digestion analysis or direct sequencing.

Cloning into empty pDonor-SH01 vector

1. Ligation
   1) Digest and gel-purify the vector plasmid. Dilute it to 10ng/µl.
   2) Set up 10µl ligation reaction for each control and test sample:

Volume	Item
1.0 µl	Digested pDonor-SH01 empty 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/µl)
10.0 µl	**Total Reaction Volume**

   3) Incubate reactions at 25℃ for 1-2 hours (sticky-end ligation) or O/N at 16℃ (for blunt-end ligation)

2. Transformation
   Transform competent cells (transformation efficiency at least $1x10^9$ colonies/µg pUC19) with the whole ligation reaction (10µl) following the provided protocol of the competent cells. Plate the transformed competent cells on LB-Ampicillin/Carbencillin agar plates.

3. Screening correct clones
   1) Depending on the ratio of colony numbers for the cDNA sample vs. the negative control sample, randomly mark 5 or more well-isolated colonies.
   2) Prepare a PCR Master Mix with PCR primers flanking the insert:

1 rxn	10 rxn	Composition
0.1µl	1µl	5' PCR primer (10µM)
0.1µl	1µl	3' PCR primer (10µM)
0.2µl	2µl	50X dNTP mix (10 mM of each)
2.5µl	25µl	10X PCR Reaction Buffer
21.9µl	219µl	Nuclease-free water
0.2µl	2µl	Taq DNA polymerase (approx. 5 U/µl)
25µl	**250µl**	**Total volume**

   3) Mix the master mix very well and aliquot 24µl into each well of 96-well PCR plate or individual tubes.
   4) Pick the each marked colony from step 1) using sterilized tips and mix it to each well (or tube).

5) 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.

6) Take 5µl 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. Inoculate a positive colony containing insert in an appropriate amount of LB-Ampicillin/Carbenicillin Broth. Incubate at 37°C overnight. Extract and purify the construct using an endotoxin-free plasmid purification kit. Sequence verification of the insert is optional.

Co-transfection of AAVS1 TALEN pair and donor plasmid

1. Plate ~100,000 to 300,000 cells/well in a 6-well plate following the recommended conditions for cell type(s) being transfected. Include wells for the following: On the day before transfection, trypsinize and count the cells. The number of cells plated in each well should be determined so that they are 70-80% confluent at the time of transfection.
a) AAVS1 TALENs + positive control DC-RFP-SH01
b) Positive control DC-RFP-SH01 only
c) AAVS1 TALENs + donor in vector DC-DON-SH01
d) Donor in vector DC-DON-SH01 only

2. Next day, prepare transfection complexes of AAVS1 TALENs pair plasmids and donor plasmids using suitable transfection reagents according to the manufacturer's instructions. Leave the transfection complexes on the cells to react for >6 hours.

Example: For HEK293T cells using 6. EndoFectin™ Plus Transfection Reagent, transfect 0.5µg of each TN-AAVS1-L and TN-AAVS1-R vectors (1µg total) and 1µg of donor vector.

Tech Notes:

1) Since transfection efficiencies vary across different cell lines, we recommend optimizing the input of AAVS1 TALENs pair plasmids to donor vectors for best results. We recommend starting with a 1:1 ratio (e.g. 1µg of donor HR plasmid and 0.5µg of each TALE Nuclease plasmid).
2) For optimal results, we recommend complexing 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).
3) For hard-to-transfect cells (e.g. primary, stem, hematopoietic), it may be advisable to utilize a non-passive transfection method. Please follow recommended guidelines provided by the manufacturer for the 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 48 hours post-transfection. For 293T cells, the recommended concentration of puromycin is 1 µg/ml.

Tech Note:
Establishing a kill-curve on untransfected cells can determine the effective working puromycin concentration for a target cell line. 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.

Validation of TALEN modified and HDR recombinant cells

1. Assay for TALEN cutting and HDR of donor vectors on samples as follows:

1) AAVS1 TALENs + positive control DC-RFP-SH01:
   Select cells in Puromycin for 7-10 days, resulting colonies should be RFP & GFP positive.
2) Positive control DC-RFP-SH01 only:
   Select cells in Puromycin for 7-10 days, very few colonies (if any) should be seen comparing to positive control donor + AAVS1 TALENs (Sample a). Presence of PuroR, RFP/GFP+ colonies indicates frequency of random integration events.
3) AAVS1 TALENs + donor in vector DC-DON-SH01
   Select cells in Puromycin for 7-10 days, colonies should be GFP positive. Expression of insert may be detected by qPCR or Western blot.
4) Donor in vector DC-DON-SH01 ony
   Select cells in Puromycin for 7-10 days, very few colonies (if any) should be seen comparing to cloning donor vector + TALE-Nuclease (Sample c). Presence of PuroR, GFP+ colonies indicates frequency of random integration events.

2. To comfirm 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).

3. Protocol for Junction-PCR

1) Primer sequences

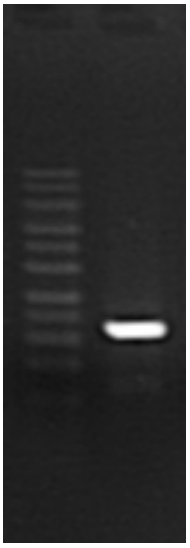
Primer description	Primer name	Primer sequence
5' AAVS1 Positive Control Donor - Forward Primer	HQPAVSHR-5F	See datasheet
5' AAVS1 Positive Control Donor - Reverse Primer	HQPAVSHR-5R	See datasheet
3' AAVS1 Positive Control Donor - Forward Primer	HQPAVSHR-3F	See datasheet
3' AAVS1 Positive Control Donor - Reverse Primer	HQPAVSHR-3R	See datasheet

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

2) Protocol details for junction-PCR assay:
   a) Isolate genomic DNA from positive control cells or test sample cells using a suitable genomic DNA miniprep kit. Please follow protocol recommended by the manufacturer.
   b) Perform junction-PCR (PCR reaction below)

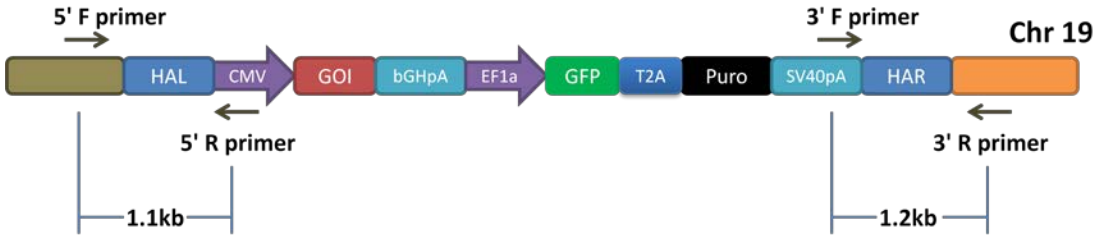
Reagent	TALEN cut+ positive control donor	Positive control donor only
Genomic DNA(60~100ng/uol)	1μl	1μl
5μM 5'AAVS1 PCR Primer Mix	1μl	1μl
5XRD Buffer	5μl	5μl
5XEnhancer	5μl	5μl
25mM dNTPs	0.2μl	0.2μl
20mM MgSO4	2.5μl	2.5μl
FL265(2mg/ml)	0.275μl	0.275μl
UltraPF(5U/μl)	0.25μl	0.25μl
PCR-grade distilled water	11.775μl	11.775μl
Total	**25μl**	**25μl**

98°C, 5min
98°C, 20sec
55°C, 30sec
72°C, 1min
35 cycles
72°C, 7min
Hold at 4~16°C



Run the PCR reaction out on the 1% Agarose/EtBr gel in 1X TAE buffer to confirm the Junction-PCR result.

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



V. Reference

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3. van Rensburg, R. et al. 2013. Chromatin structure of two genomic sites for targeted transgene integration in induced pluripotent stem cells and hepatopoietic stem cells. Gene Therapy. 2013 20(2):201-14.
4. Papapetrou, EP. et al. 2011. Genomic safe harbors permit high ß-globin transgene expression in thalassemia induced pluripotent stem cells. Nat. Biotechnol. 2011 29(1):73-8.
5. Lombardo, A. et al. 2011. Site-specific integration and tailoring of cassette design for sustainable gene transfer. Nat. Methods. 2011 8(10):861-9.

VI. Limited Use License and Warranty

Limited Use License

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