



GeneHero™ Mouse ROSA26 Safe Harbor Gene Knock-in Kits

Catalog# SH050

Catalog# SH054

Catalog# SH062

Catalog# SH063

Catalog# SH064

User Manual

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USER MANUAL

GeneHero™ Mouse ROSA26 Safe Harbor Gene Knock-in Kits

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I. Introduction

Safe gene targeting

Genome modification 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 studies, 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. ROSA26 (also known as ROSA β geo26 locus) in the mouse genome was first found on chromosome 6 in one particular strain of mice named ROSA β geo26-which expressed β galactosidase from a randomly inserted transgene at high levels uniformly in nearly all tissues examined. This locus expresses one coding transcript and two noncoding transcripts, and only the non-coding transcripts are disrupted by the insertion. While pups homozygous for the insertion are born at slightly lower frequency than heterozygous pups, heterozygotes appeared to develop normally and were fertile. Therefore, the “ROSA26” locus has since been used as a transgene insertion site that causes no apparent adverse effects on fitness, and permits stable gene expression.

The GeneCopoeia ROSA26-specific CRISPR-Cas9 systems can generate a DNA double-strand break (DSB) in ROSA26 on mouse chromosome 6, stimulating natural DNA repair mechanisms. In the presence of ROSA26 knockin clones, homologous recombination (HR) occurs, resulting in integration of the DNA fragment from the knockin clone into the safe harbor locus.

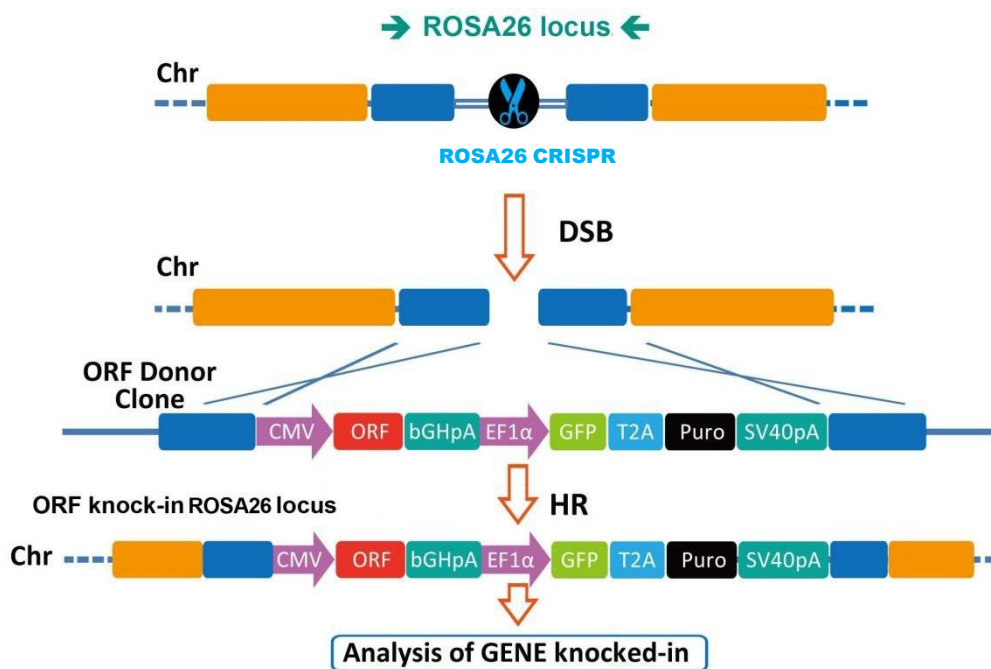


Figure 1. Illustration of genome-editing-tool-mediated transgene integration at the mouse safe harbor ROSA26 site.

Advantages

Safe integration

Safe harbor integration site ensures transcription-competency of the transgenes and presents no known adverse effects on cells.

Specific targeting

CRISPR-mediated DNA DSBs at the ROSA26 site stimulate homologous recombination dramatically for transgene integration.

Single copy number

Single copy number of the transgene ensures predictable expression levels, simplifies phenotype interpretation, and prevents transgene silencing.

Compatible knock-in ORFs

Over 20,000 sequence-verified mouse ORFs are compatible for transgene donor DNA design.

Introduction to CRISPR-Cas9

In the CRISPR-Cas9 system, the complex of a CRISPR RNA (crRNA) annealed to a trans-activating crRNA (tracrRNA) is sufficient to guide the Cas9 endonuclease to a specific genomic sequence to generate a double-strand break (DSB) in the target DNA. This system can be simplified by fusing crRNA and tracrRNA sequences to produce a synthetic chimeric single-guided RNA (sgRNA). The selected target sequence consists of a 20-bp DNA sequence complementary to the crRNA or the chimeric sgRNA, followed by the trinucleotide (5'-NGG-3') protospacer adjacent motif (PAM), which is recognized by the Cas9 and essential for cleavage. This RNA-guided DNA recognition mechanism of CRISPR-Cas9 provides a simple but powerful tool for precision genome engineering.

The GeneCopoeia GeneHero™ Mouse ROSA26 Safe Harbor Gene Knock-in Kits are designed to efficiently transfer your gene of interest, selection marker or other genetic element from a donor plasmid into the ROSA26 safe harbor site on mouse chromosome 6 via CRISPR-Cas9-mediated homologous recombination (HR). HR is a natural DNA repair mechanism that occurs in response to DNA double-strand breaks (DSB). This DSB is created by an ROSA26-specific CRISPR-Cas9 system.

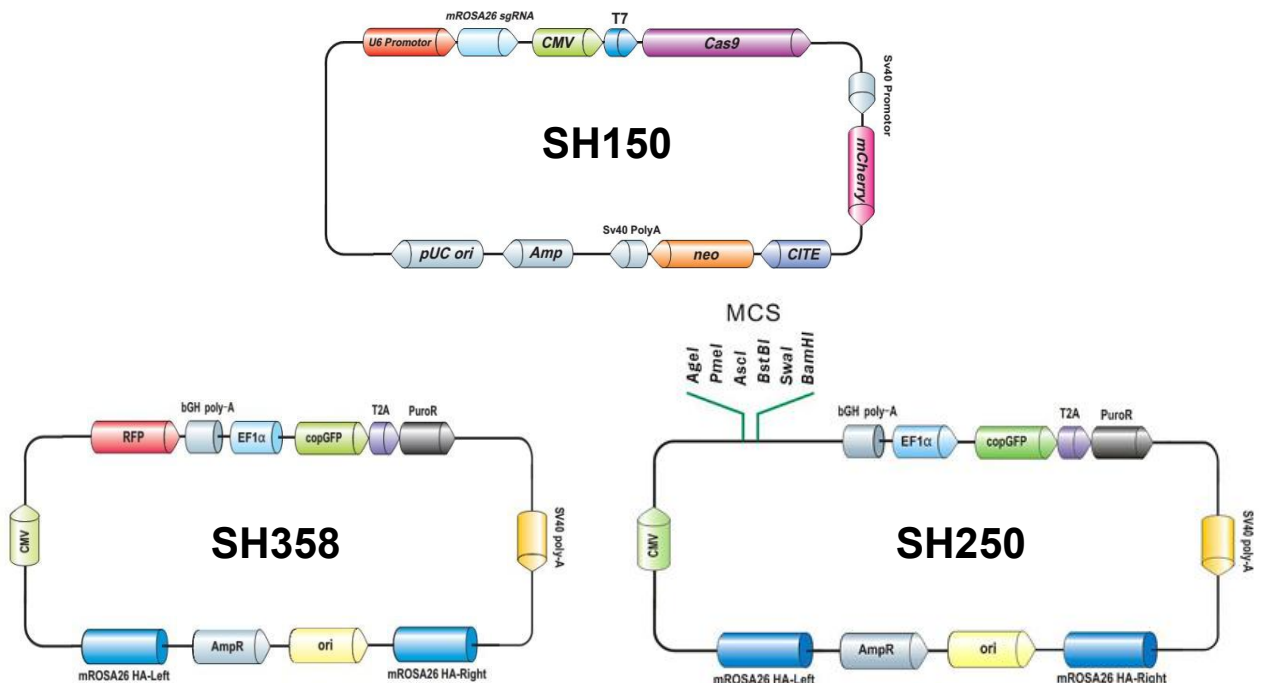
II. Contents and storage

GeneHero™ Mouse ROSA26 Safe Harbor Gene Knock-in Kit-Puro (without donor; Cat# SH050)
GeneHero™ Mouse ROSA26 Safe Harbor Gene Knock-in Kit-cloning vector-Puro (Cat# SH054)

Cat. No.	Product name	Qty	Concentration	Shipping and Storage
SH150	ROSA26 CRISPR-Cas9 clone	10 µg	500 ng/µL	Shipped at room temperature. Stored at -20°C
SH250*	ROSA26 MCS donor cloning vector-Puro	10 µg	500 ng/µL	Shipped at room temperature. Stored at -20°C
SH358	ROSA26 RFP knockin donor clone-Puro	10 µg	500 ng/µL	Shipped at room temperature. Stored at -20°C
SH450	ROSA26 5' verification PCR primers	200 reactions	10 µM	Shipped at room temperature. Stored at -20°C
SH451	ROSA26 3' verification PCR primers	200 reactions	10 µM	Shipped at room temperature. Stored at -20°C

* SH250 only comes with SH054 kit.

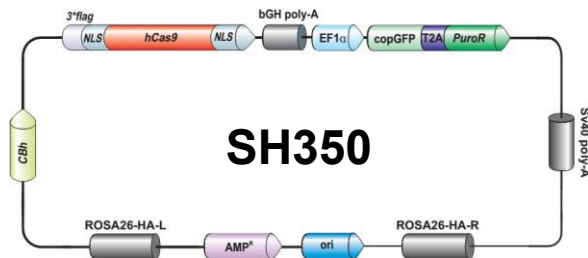
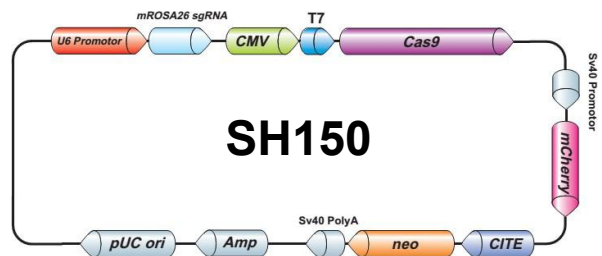
(A) CRISPR-Cas9 and donor plasmids



GeneHero™ Cas9 Mouse ROSA26 Safe Harbor Knock-in Kit-Puro (Cat# SH062)

Cat. No.	Product name	Qty	Concentration	Shipping and Storage
SH150	ROSA26 CRISPR-Cas9 clone	10 µg	500 ng/µL	Shipped at room temperature. Stored at -20°C
SH350	ROSA26 Cas9 knockin donor clone-CBh-Puro	10 µg	500 ng/µL	Shipped at room temperature. Stored at -20°C
SH453	ROSA26 5' verification PCR primers-Cas9 clones	200 reactions	10 µM	Shipped at room temperature. Stored at -20°C
SH452	ROSA26 3' verification PCR primers-Cas9 clones	200 reactions	10 µM	Shipped at room temperature. Stored at -20°C

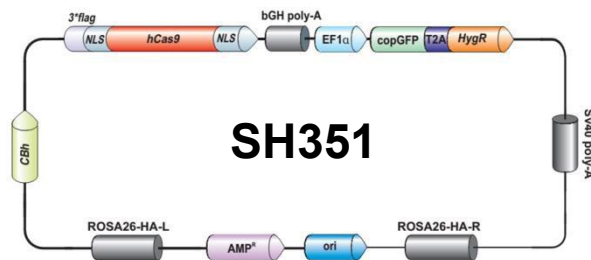
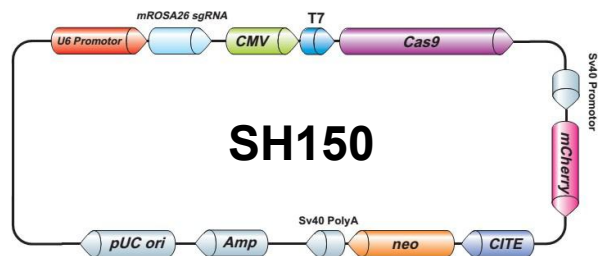
(A) CRISPR-Cas9 and donor plasmid



GeneHero™ Cas9 Mouse ROSA26 Safe Harbor Knock-in Kit-Hygro (Cat# SH063)

Cat. No.	Product name	Qty	Concentration	Shipping and Storage
SH150	ROSA26 CRISPR-Cas9 clone	10 µg	500 ng/µL	Shipped at room temperature. Stored at -20°C
SH351	ROSA26 Cas9 knockin donor clone-CBh-Hygro	10 µg	500 ng/µL	Shipped at room temperature. Stored at -20°C
SH453	ROSA26 5' verification PCR primers-Cas9 clones	200 reactions	10 µM	Shipped at room temperature. Stored at -20°C
SH452	ROSA26 3' verification PCR primers-Cas9 clones	200 reactions	10 µM	Shipped at room temperature. Stored at -20°C

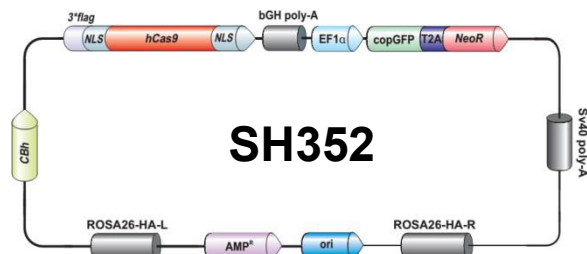
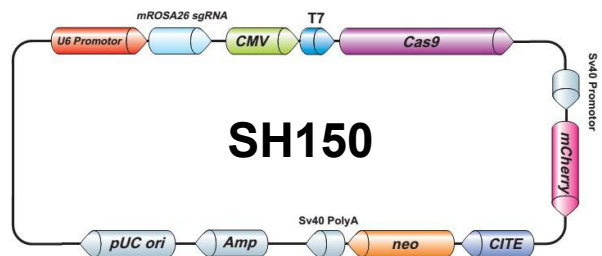
(A) CRISPR-Cas9 and donor plasmid



GeneHero™ Cas9 Mouse ROSA26 Safe Harbor Knock-in Kit-Neo (Cat# SH064)

Cat. No.	Product name	Qty	Concentration	Shipping and Storage
SH150	ROSA26 CRISPR-Cas9 clone	10 µg	500 ng/µL	Shipped at room temperature. Stored at -20°C
SH352	ROSA26 Cas9 knockin donor clone-CBh-Neo	10 µg	500 ng/µL	Shipped at room temperature. Stored at -20°C
SH453	ROSA26 5' verification PCR primers-Cas9 clones	200 reactions	10 µM	Shipped at room temperature. Stored at -20°C
SH452	ROSA26 3' verification PCR primers-Cas9 clones	200 reactions	10 µM	Shipped at room temperature. Stored at -20°C

(A) CRISPR-Cas9 and donor plasmid



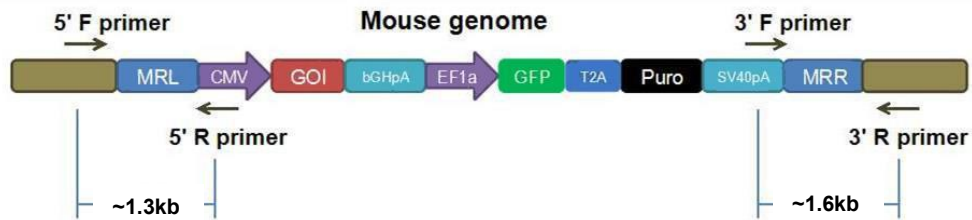
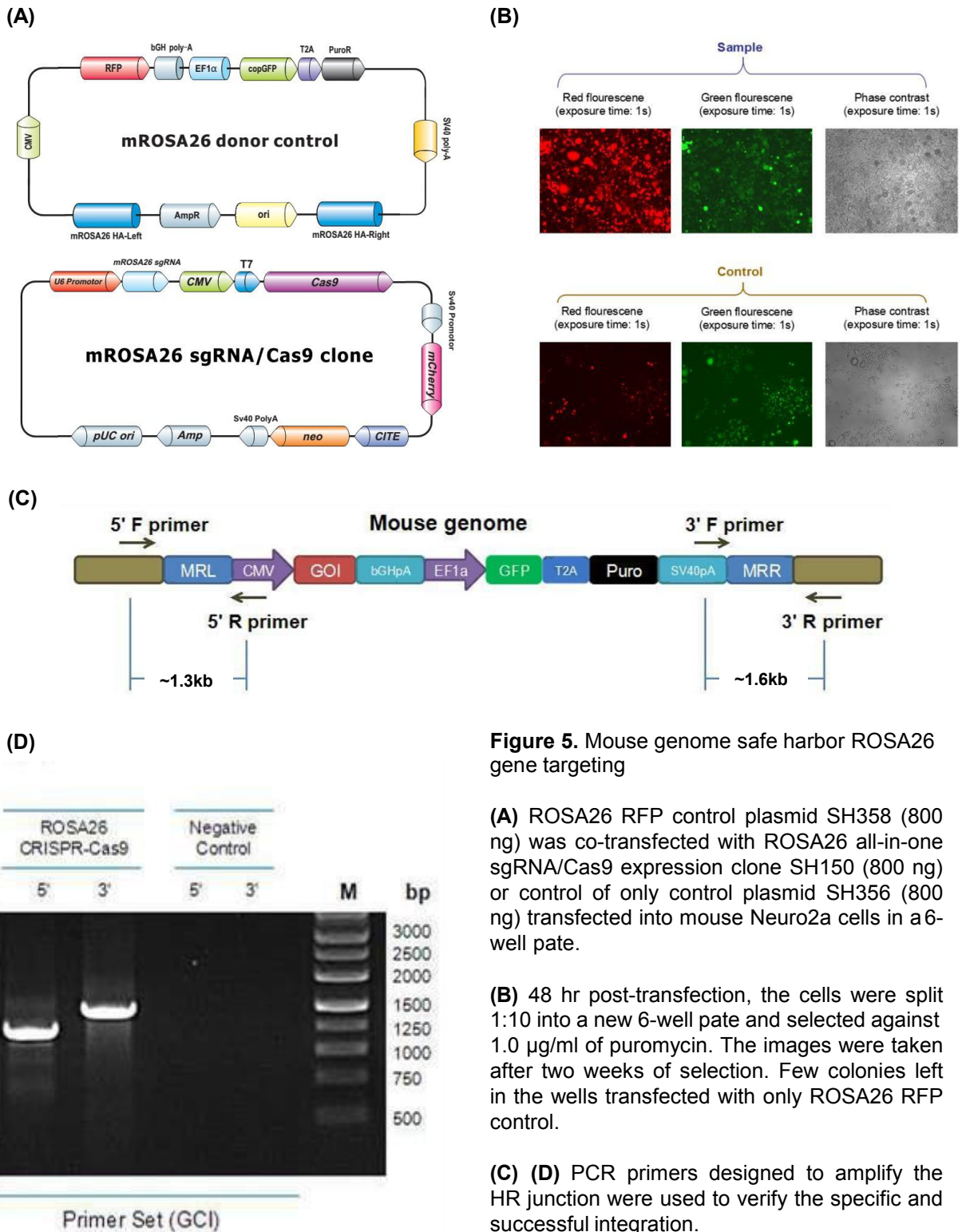
(B) Knock-in verification PCR primers

Figure 3. GeneHero™ mouse ROSA26 safe harbor gene knock-in kit components. **(A)** ROSA26 CRISPR-Cas9 and donor plasmids **(B)** Knock-in verification primer pairs.

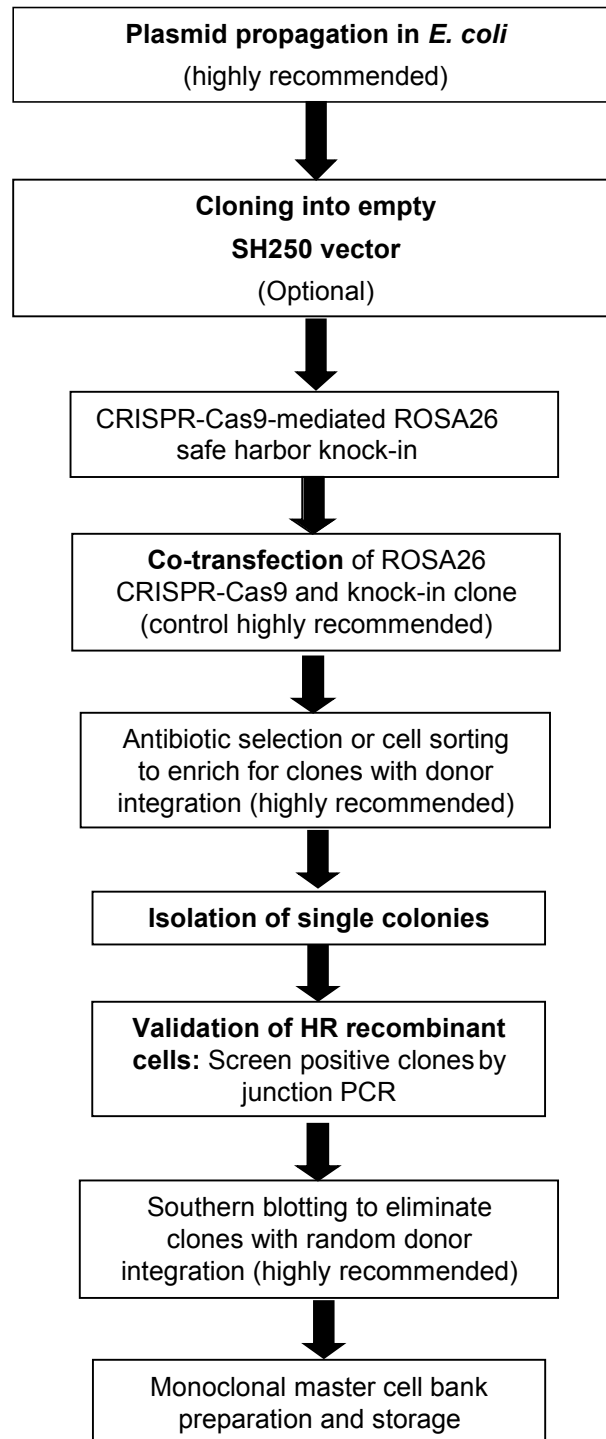
Additional materials required

1. LB Agar and broth containing 50 µg/ml Ampicillin
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 (GCI-5a chemically competent E. Coli, Cat# CC001 or CC002)
5. Dulbecco's Modified Eagle's Medium (D-MEM) high glucose with sodium pyruvate and glutamine (Invitrogen, Cat. # 11995073)
6. EndoFectin™ Max Transfection Reagent (GeneCopoeia, Cat. # EF013/014)
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 NucleoFactor or Invitrogen's Neon system) is highly recommended

III. Example



IV. Overview of Safe Harbor Integration



V. Critical Steps

A. 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 ROSA26 CRISPR-Cas9 and plasmids in the donor vector, we suggest plating 50-200µL of transformed cells on fresh 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 50µg/ml of Ampicillin. 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.

B. Cloning into empty SH250(DC-DON-SH02) 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 SH250 empty vector
7.0 µl	DNA insert (~30-50 ng) or water control
1.0 µl	10 × T4 DNA ligase buffer
1.0 µl	T4 DNA Ligase (40 U/µL)
10.0 µl	Total Reaction Volume

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

2. Transformation

Transform competent cells (transformation efficiency at least 1×10^9 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
0.1 µL	1 µL	5' PCR primer (10 µM)
0.1 µL	1 µL	3' PCR primer (10 µM)
0.2 µL	2 µL	50 × dNTP mix (10 mM of each)
2.5 µL	25 µL	10 × PCR Reaction Buffer
21.9 µL	219 µL	Nuclease-free water
0.2 µL	2 µL	Taq DNA polymerase (approx. 5 U/µL)
25 µL	250 µL	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 1 × 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.

C. Co-transfection of ROSA26 genome editing tools 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.

- SH150 + positive control donor clone
- Positive control only
- SH150 + donor clones (in vector SH250, EZ013, or Cas9 ROSA26 knockin clones)
- Donor only (in vector SH250, EZ013, or Cas9 ROSA26 knockin clones)

2. The next day, prepare transfection complexes of genome editing tool 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 Neuro2A cells using EndoFectin™ Max Transfection Reagent, transfect 1µg of SH150 plasmid and 1µg of donor vector.

Tech Notes:

1) Since transfection efficiencies vary across different cell lines, we recommend optimizing the input of genome editing tool plasmids to donor vectors for best results. We recommend starting with a 1:1 ratio, e.g. 1µg of donor plasmid, 1µg of SH150 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 (or other relative antibiotic selection) 48 hours post-transfection. For Neuro2A 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. Concentrations of other relative antibiotics can be determined by the same method after knowing their typical working range.

D. Clonal isolation of cell lines

Serial dilution is widely used to isolate single clones with desired modifications, followed by an expansion period to establish a new clonal cell line. Like most clonal isolation methods, there is no guarantee that the colonies arose from single cells. A second round is advised to increase the likelihood of clonal isolation. Also, it is worth noting that cell types can vary substantially in their responses to single-cell isolation, therefore literature specific to the cell type of interest should be consulted.

1. Fill each well of a sterile 96-well plate with 100µL of medium except for well A1, which should remain empty.

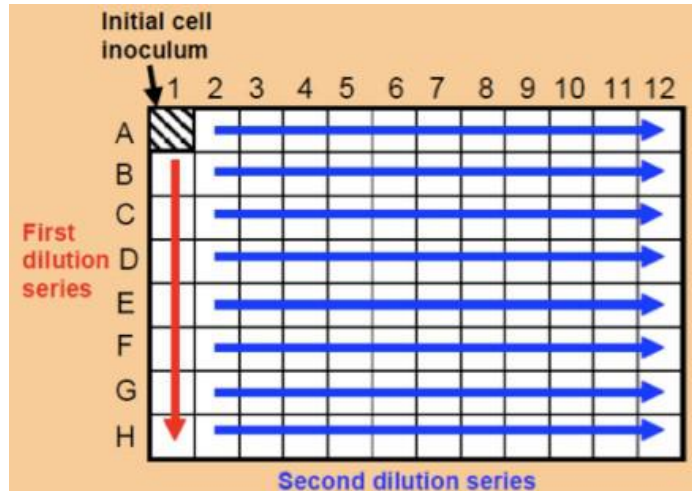


Figure 6. Illustration of serial dilution.

2. Add 200µL cell suspension to well A1. Mix 100µL from A1 with the medium in well B1. Avoid bubbles. Continue this 1:2 dilution through column 1. Add 100µL of medium back to column 1 so that wells A1 through H1 contain 200µL.
3. Mix cells and transfer 100µL of cells from column 1 into column 2. Mix by gently pipetting. Avoid bubbles. Repeat these 1:2 dilutions through the entire plate. Bring the final volume to 200µL by adding 100µL of medium to all but the last column of wells.
4. Incubate plates undisturbed at 37°C.
5. Cells will be observable via microscopy over 3 days and be ready to score in 5-8 days, depending on the growth rate of cells. Mark each well on the cover of the plate indicating which well contains a single colony. These colonies can later be subcultured from the well into larger vessels.

Tech Note:

- 1) Adding 4000 cells in well A1 (2×10^4 cells/mL) is a good starting concentration. Increase the concentration for more difficult to grow cell lines.
- 2) If the reporter gene is fluorescent, determine which of these colonies express it. If the reporter gene is not observable you will have to wait until later in the culture process.
- 3) Label each well with a single colony using a unique identification number and record this number on the plate and in your notebook.

E. Validation of HR recombinant cells

1. Assay for genome editing tools cutting and HR of donor vectors on samples as follows. For Cas9 ROSA26 knockin clones, please select with relative antibiotics:

1) SH150 + positive control SH358 :

Select cells in Puromycin for 7-10 days. The resulting colonies should be RFP & GFP positive.

2) Positive control SH358 only:

Select cells in Puromycin for 7-10 days, after which very few colonies (if any) should be seen compared with Sample a. The presence of PuroR, RFP/GFP+ colonies indicates random integration events.

3) SH150 + donor in vector SH250

Select cells in Puromycin for 7-10 days, after which colonies should be GFP positive. Expression of the insert may be detected by qPCR or Western blot.

4) Donor in vector SH250 only

Select cells in Puromycin for 7-10 days, after which very few colonies (if any) should be seen compared with Sample c. The presence of PuroR, GFP+ colonies indicates random integration events.

2. To confirm donor vector integration specifically at the ROSA26 target locus, junction-PCR can be performed using PCR primer pairs that flank the 5' ROSA26 homology arm (5' ROSA26-HA-L) and 3' ROSA26 homology arm (3'ROSA26-HA-R). Please do note that Cas9 ROSA26 knockin clones do not share verification primer sets with SH250, SH358 & EZ013. For Cas9 ROSA26 knockin use 3' universal primer set SH452 and 5' primer set 453.

3. Protocol for Junction-PCR

1) 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 the protocol recommended by the manufacturer.

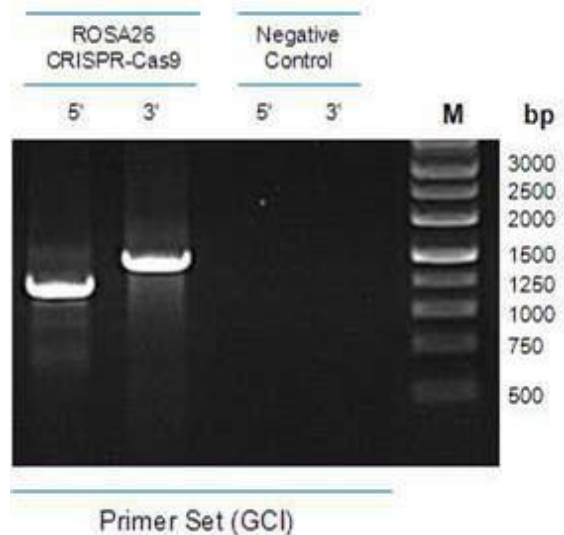
b) Perform junction-PCR (PCR reaction below)

GeneHero™ Mouse ROSA26 Safe Harbor Gene Knock-in Kits

Reagent	TALEN cut+ positive control donor	Positive control donor only
Genomic DNA(60~100ng/μL)	1 μL	1 μL
10μM 5' (or 3') ROSA26 PCR Primer Mix (or universal 3' PCR primer set for Cas9 ROSA26 knockin clones)	1 μL	1 μL
5×UltraPF™ Buffer (Mg2+ free)	5 μL	5 μL
10 mM dNTPs	0.5 μL	0.5 μL
20mM MgSO ₄	2.5 μL	2.5 μL
UltraPF(5U/μL)	0.25 μL	0.25 μL
PCR-grade distilled water	14.75 μL	14.75 μL
Total	25 μL	25 μL

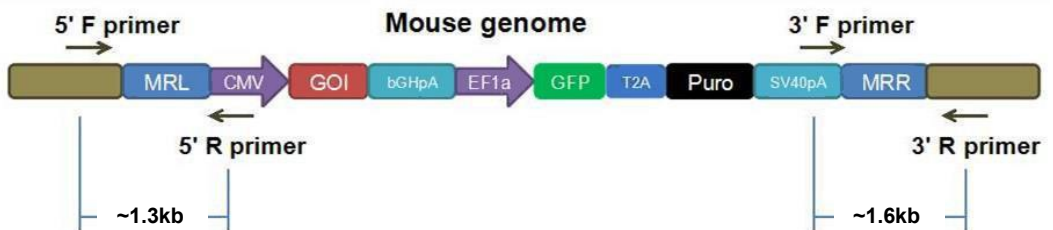
98°C, 5min
 98°C, 20sec
 55°C, 30sec
 72°C, 1min
 72°C, 7min
 Hold at 4~16°C

} 35 cycles



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

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



Tech Note:

- 1) If the 3' junction PCR band is weaker than 5' junction PCR band, it is likely that the amplification efficiency for the 3' junction region is lower due to the nature of the chromosomal structure, modification and sequence around that region.
- 2) One positive in junction PCR is sufficient to confirm the integration.
- 3) Please do note that Cas9 safe harbor knock-in clones do not share verification primer sets with SH250, SH356 & EZ013. When using junction PCR to verify integration of Cas9 safe harbor knock-in clones, use 3' universal primer set SH452 and 5' primer set SH453.
- 4) Though rare, it is possible that random integration can coexist with ROSA26-specific integration. Southern blotting can be used to detect coexisting random integration. The method is described in:
<http://www.bloodjournal.org/content/117/21/5561>

VI. References

1. Zou, J. et al. 2009. Gene targeting of a disease-related gene in human induced pluripotent stem and embryonic stem cells. *Cell Stem Cell*. 2009 Jul 2;5(1):97-110
2. Sadelain, M. et al. 2011. Safe harbours for the integration of new DNA in the human genome. *Nat Rev Cancer*. 2011 Dec 1;12(1):51-8.
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 β -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.

VII. Related Product and Services

Cas9 stable cell lines

GeneCopoeia offers stable cell lines constitutively expressing the Cas9 nuclease. These cell lines provide you with convenient means to carry out CRISPR genome editing applications with high efficiency. You can also generate your own Cas9 safe harbor stable cell lines by combining safe harbor gene knock-in kits and Cas9 safe harbor knock-in donor clones.

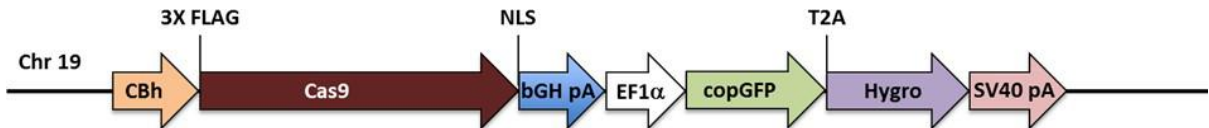


Figure 7. Example of a stably integrated CRISPR Cas9 nuclease construct.

Advantages

- Stable Cas9 integration minimizes the need for co-transfection or co-transduction of sgRNAs, ideal for high-throughput sgRNA applications.
- Safe Harbor site integration ensures stable Cas9 expression, with no adverse effects on the cells.
- Single clone isolation provides consistent, high-level Cas9 expression in a uniform genetic background.
- Compatible with GeneCopoeia GeneHero™ sgRNA clones, sgRNA libraries, and donor clones.

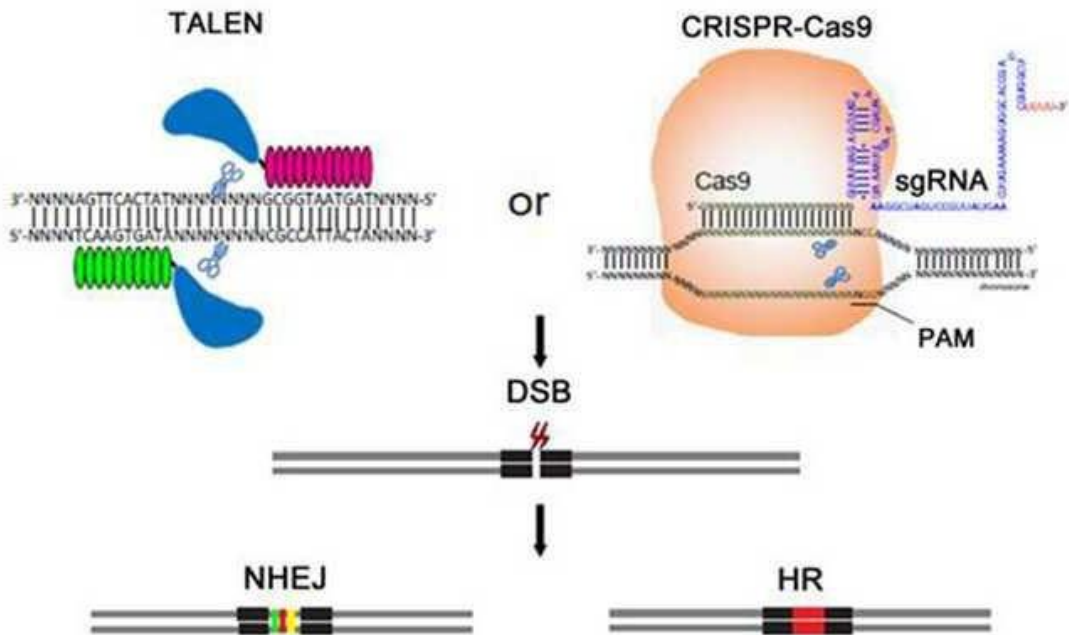
Applications

- High-throughput knockout screening with many sgRNAs, either individually or in pools. Ideal for drug target discovery.
- Convenient validation of several drug target candidates.
- Validation of sgRNA cleavage activity in a fast-growing, easy-to-transfect or transduce model cell line, either prior to transfection/transduction of your cell line, or to troubleshoot sgRNAs with little or no cleavage activity in your cell line.

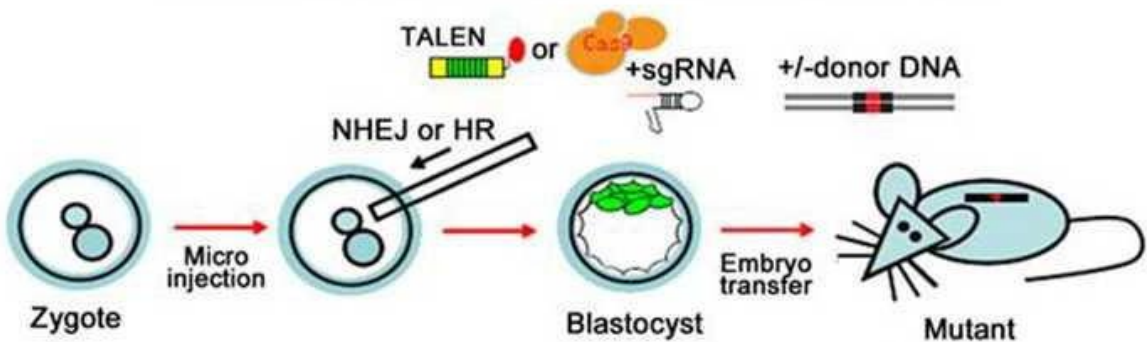
Transgenic mouse services

GeneCopoeia offers transgenic mice with customized TALEN- or CRISPR-Cas9-mediated genome modifications.

Overview of genome editing by TALEN and CRISPR-Cas9



One-step generation of mice with genome modifications



VIII. Limited Use License and Warranty

Limited Use License

The following terms and conditions apply to use of the GeneHero™ Mouse ROSA26 Safe Harbor Gene Knock-in Kits (the Product). If the terms and conditions are not acceptable, the Product in its entirety must be returned to GeneCopoeia within 5 calendar days. A limited End-User license is granted to the purchaser of the Product. 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 must not be resold, repackaged or modified for resale, or used to manufacture commercial products or deliver information obtained in service without prior written consent from GeneCopoeia. This Product should be used in accordance with the NIH guidelines developed for recombinant DNA and genetic research. Use of any part of the Product constitutes acceptance of the above terms.

Limited Warranty

GeneCopoeia warrants that the Product meets the specifications described in the accompanying Product Datasheet. If it is proven to the satisfaction of GeneCopoeia that the Product fails to meet these specifications, GeneCopoeia will replace the Product. In the event a replacement cannot be provided, GeneCopoeia will provide the purchaser with a refund. This limited warranty shall not extend to anyone other than the original purchaser of the Product. Notice of nonconforming products must be made to GeneCopoeia within 30 days of receipt of the Product. GeneCopoeia's liability is expressly limited to replacement of Product or a refund limited to the actual purchase price. GeneCopoeia'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. GeneCopoeia does not provide any other warranties of any kind, expressed or implied, including the merchantability or fitness of the Product for a particular purpose.

GeneCopoeia is committed to providing our customers with high-quality products. If you should have any questions or concerns about any GeneCopoeia products, please contact us at 301-762-0888.

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