CRISPR/Cas9 Genome Editing

Application Guide

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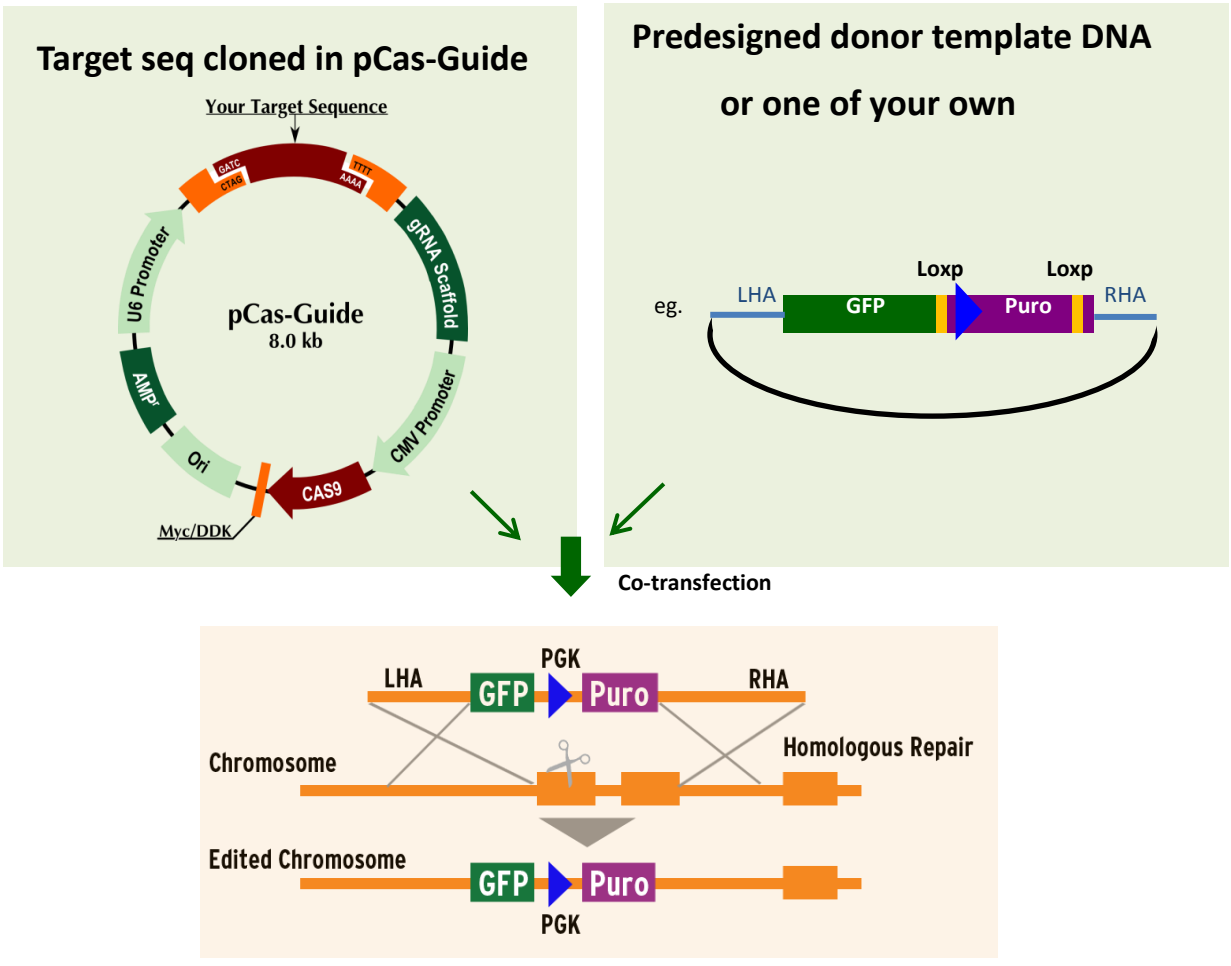
Introduction

Cas9 based genome editing has become a popular tool for targeted genome manipulation because of its simplicity and high cutting efficiency. This system requires a functional cas9 protein and a guide RNA for effective double-stranded breakage at a desired site. OriGene has developed many CRISPR vectors, including All-in-one vectors which contain both guide RNA and Cas9 expression, T7 vectors and gRNA and Cas9 separate vectors. OriGene also designed a set of donor cassettes for HDR-based (Homology Directed Repair) donor vector construction, including Luciferase-Loxp-Puro-Loxp, GFP-Loxp-Puro-Loxp, RFP-Loxp-BSD-Loxp and BFP-Loxp-Neo-Loxp. Homologous arm sequences can be cloned flanking the donor cassettes.

OriGene CRISPR products also includes CRISPR gene knockout kits, gene harbor transgene insertion via CRISPR, synthetic gRNA.

Figure 1. Flow chart of CRISPR genome editing using HDR.



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I. Genome-wide CRISPR gene knockout kit

OriGene offers genome-wide gene knockout / knockin kits via CRISPR (human and mouse); one specific kit for each gene locus. Each kit contains 2 gRNA vectors and 1 donor DNA. The gene knockout/knockin kit is a complete kit to knockout any coding gene and knockin a selection cassette.

gRNA vectors are provided in pCas-Guide vector with a target sequence cloned. Both of the target sequences are located at the 5' end of the ORF; therefore, gRNA vectors will make a precise cleavage at the 5' end of the ORF of the gene loci.

There are two types of CRISPR knockout kits: HDR mediated & KN2.0 non-homology mediated knockout kits. The difference of the two types kits is repair mechanism that is used to achieve gene knockout, Homology Directed Repair (HDR) and Non-Homology repair respectively.

Table 2. Comparison of CRISPR gene knockout kits

	HDR mediated	KN2.0 non-homology mediated
gRNA vectors	pCas-Guide	pCas-Guide
Donor	Donor cassette flanked by homologous arms	Donor cassette without homologous arms
Cell spectrum	Dividing cells	Dividing and non-dividing cells
Knockout efficiency	Medium	High

HDR-mediated CRISPR knockout kits

Package contents

- 2 vials of gRNA vectors, (SKU KNxxxxxxG1, KNxxxxxxG2), 3-5 µg DNA in TE buffer
- 1 vial of donor vector containing left and right homologous arms and a GFP-puro functional cassette (SKU KN2xxxxxD), 3-5 µg DNA in TE buffer
- 1 vial of negative scramble control vector (SKU GE100003), lyophilized. Reconstitute in 100 µL dH_2O, final concentration 100 ng/ µL.
- Certificate of Analysis
- Application Guide: https://www.origene.com/support/product-support/product-manuals

Note: The cDNA clone is shipped at room temperature, but should be kept at -20℃ for long-term storage. If properly stored, clones are guaranteed to be stable for 12 months.

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Related Optional Reagents

- LB agar plates with ampicillin, 100 μg/mL
- LB broth (10 g/L Tryptone, 5 g/L Yeast Extract, 10 g/L NaCl. Adjust pH to 7.0 with 1 N NaOH)

Related OriGene Products

- Transfection reagent: https://www.origene.com/products/others/transfection-reagents
- Reagents and supplies for immunoblots: user preferred. OriGene has a selection of antibodies and detection reagents that are available at https://www.origene.com/products/antibodies
- DNA purification reagents https://www.origene.com/products/others/nucleic-acid-purification-kits
- qPCR reagents https://www.origene.com/products/gene-expression/qpcr
- CRISPR/Cas9 products https://www.origene.com/products/gene-expression/crispr-cas9

Notice to purchaser

This product is for research use only. Use in and/or for diagnostics and therapeutics is strictly prohibited. By opening and using the product, the purchaser agrees to the following: The plasmids may not be distributed, resold, modified for resale or used to manufacture commercial products without prior written approval from OriGene Technologies, Inc. If you do not agree to the above conditions, please return the UNOPENED product to OriGene Technologies, Inc. within ten (10) days of receipt for a full refund.

Product Description

The gene knockout/knockin kit is a complete kit to knockout any coding gene and knockin a functional cassette containing a reporter and selection marker. The donor plasmid contains left homologous arm and right homologous arm flanking the donor cassette, therefore, the donor cassette will be integrated into the genome via homology-directed repair (HDR) mechanism. The reporter, such as GFP, will be under the native promoter; the puromycin resistant gene is under PGK promoter. gRNA vectors are provided in pCas-Guide vector with a target sequence cloned. Both of the target sequences are located at the 5' end of the ORF; gRNA vectors will make a precise cleavage at the 5' end of the ORF of the gene loci. A negative scramble gRNA control is also provided.

The Applications are:
1. You can knockin GFP reporter or other reporters for your promoter study.
2. Knock-out genes at the chromosomal level.

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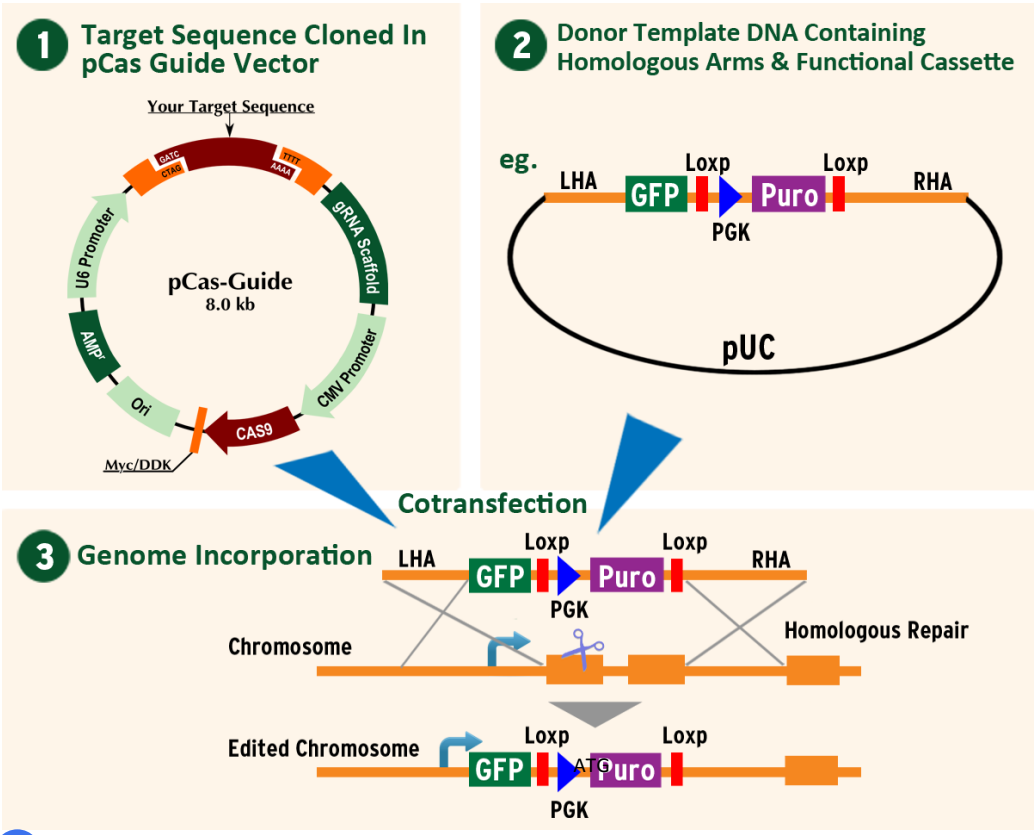
Fig. 2. Scheme of genome-editing knockout kit

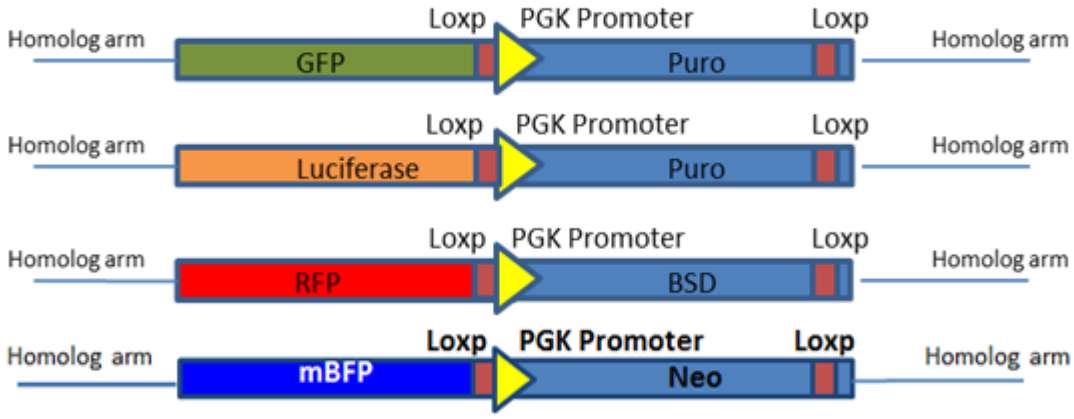


1 CRISPR/Cas cuts the double-stranded DNA at the targeting site

2 Donor template DNA provides the template for the homologous repair.

3 The functional cassette is incorporated into the genome when 1 + 2 are cotransfected.

Donor vector for each kit contains around 600 bp locus specific homologous sequence on each side of the donor selection cassette. LHA – left homologous arm, RHA – right homologous arm. Four different donor cassettes are offered for each gene:



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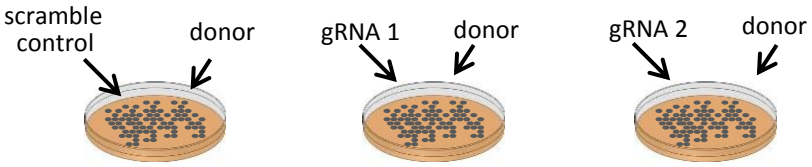
Experimental Protocol

Each kit contains two gRNA vectors, one scramble negative control and one donor vector. To ensure high efficiency of cleavage, two gRNA constructs are provided. A scrambled control vector serves as the negative control.

A sample protocol listed below is for 6-well plates and using TurboFectin (cat# TF81001) as transfection reagent. If your experiments require a different size of culture plates, just scale up or down the reagents accordingly based on the relative surface area of your plate (Table 1). Different type of cells may need a different transfection reagent; please follow the manufacturer's corresponding protocol. OriGene just launched Virus-like Viromers which are best for difficult-to-transfect cells.

1. Approximately 18-24 hours before transfection, plate ~3 X 10^5 adherent cells in 2 ml culture media into each well of a 6-well plate or ~5x10^5 suspension cells per well to obtain 50-70% confluence on the following day. The number of cells varies depending on the size of your cells.

2. Transfection in complete culture media. Three separate transfections:



In a small sterile tube, combine the following reagents in the prescribed order. The order of reagent addition is important to achieve the optimal results.

   a. Dilute 1 µg of one of the gRNA vectors (or scramble control) in 250 uL of Opti-MEM I (Life Technologies), vortex gently. Then add 1 µg of the donor DNA into the same 250 µL of Opti-MEM I. Vortex gently. Two gRNA vectors and scramble control are in three separate tubes, so the gRNA efficiency can be tested individually.
   b. Add 6 µL of Turbofectin 8.0 to the diluted DNA (not the reverse order) and pipette gently to mix completely.
   c. Incubate the mixture 15 minutes at room temperature.

   ***Note**: We recommend starting with the ratios of Turbofectin 8.0 and DNA listed in table 1; however, subsequent optimization may further increase the transfection efficiency.*

   d. Add the mixture above drop-wise to the cells plated in step 1 (no need to change the media). Gently rock the plate back-and-forth and side-to-side to distribute the complex evenly.
   e. Incubate the cells in a 5% CO_2 incubator.

3. 48hr post transfection, split cells 1:10, grow additional 3 days; then split the cells again 1:10. Split cells 7 times in total. Since puromycin resistant gene in the donor vector

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contains PGK promoter, the plasmid donor DNA before genomic integration will also provide puromycin resistance. The reason to grow cells for around 3 weeks before puromycin selection is to dilute out cells containing the donor as episomal form.

Time lines of genome editing

- ✓ CRISPR targeted gene knockout / knockin--- 1 week post transfection
- ✓ Episomal donor vector dilution with cell passaging--- 3 weeks post transfection

Note 1. *Since stable cell selection takes time, you can try to analyze the cells at P2 to detect genomic integration using genomic PCR (Fig 2). When designing primers for genomic PCR, one primer should be outside of the homologous arm region in donor DNA and one primer is in the functional cassette. Please see details in Fig. 3. The amplified PCR fragment is around 1kb. There could be some difficulties doing genomic PCR at this step before selection due to the percentage of edited cells and difficulties of genomic PCR. qPCR measuring the targeted mRNA level would not work due to the small percentage of edited cells.*

Note 2*. You might be able to use GFP to sort genomic edited cells between P2-P5 (Fig. 2). Since donor DNA contains 600bp left homologous arm sequence which is immediately upstream of ATG, Donor DNA transfected + scramble control could express weak or bright green fluorescence depending how much promoter sequence the left homologous arm sequence contains. The best-case scenario is donor DNA + scramble gives weak GFP signal while after integration the promoter is strong and constitutive, so you can sort strong GFP positive cells, thus avoiding the lengthy donor vector dilution step before puro selection.*

4. Apply puromycin selection. Split P5 or P7 cells 1:10, then grow cells directly in the puromycin containing complete media in 10 cm dishes (apply puro selection at P6 might work, but you might get more random donor integrated clones than P8). The dose of puromycin needs to be determined with a kill curve to find out the lowest dose that kills the non-transfected cells completely 4-7 days post selection; the range of puromycin is 1µg/ml to 10µg/ml). Change the media every 2-3 days.

   Note*: We recommend you keep growing or freeze some of the transfected cells without selection; just in case, you need to perform the puromycin selection again.*

5. The puromycin resistant cells are ready to be analyzed for genome editing. WB can be used to measure gene knockdown if there is a good protein-specific antibody available (better after isolating individual cell colonies). You will also need to do genomic PCR to verify the integration of the functional cassette. You can directly sequence the amplified genomic fragment using the PCR primers to verify the integration in the genome.

   Note*: Scramble control and donor vector will also give you some puromycin resistant cells as donor vector alone can randomly integrate into the genome too; however, the efficiency should be a lot lower than with a specific gRNA. Therefore, you should get more colonies with gene specific gRNA than scramble control if the gene specific gRNA cleaves efficiently.*

6. Isolate individual cell colonies.

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Two main methods, limiting dilution and cloning rings / cylinder.

1) Limiting dilution

This method is better to be used after puromycin selection. Dilute cells to seed about 1-2 cells/well in 96-well plate, after 1-2 weeks, observe under the microscope and select the wells only containing one cell colony, then further expand them to 6-well plate when they are confluent in the 96-well plate and so on.

2) Cloning rings / cylinder

This method can be used in the same time with puro selection. Seed cells at lower density, such as 5% confluency in a larger cell culture dish, such as 10cm dish, apply puromycin selection when seeding.

Note 1: How to make biallelic knockout: *If you isolate single cell colonies, in some cells gene knock-out may occur only in one allele; in some cells gene knock-out may occur in both alleles. If you only have monoallelic knockout (heterozygous) and you want to get biallelic knockout (homozygous), you can order another donor vector containing a different mammalian selection marker, such as blasticidin or neomycin resistant marker. Make sure the other allele is intact. You can confirm it with genomic PCR with a set of PCR primers amplifying the endogenous chromosome and sequence it. If this allele is targeted by Cas9/gRNA, repaired by NHEJ, introduced indels and the indels change the protein reading frame, then you have a biallelic knockout (one via HDR and one via NHEJ). If this allele is intact, you can do the knockout again. OriGene has both functional cassettes. You can do the knockout procedure again with the new donor vector to target the second allele (one allele is already targeted and replaced with GFP-puro cassette). Alternatively, you can use Cre (SKU GE100018) to flox out the puro cassette from your edited cells and use the same donor vector from the knockout kit to do the knockout again to target the second allele.*

Note 2*: If you gene is essential, you will not be able to get biallelic knockout. The solution is to do conditional knockout using LoxP system by introducing LoxP sites around the exon(s) to be knocked out.*

Table 1. Recommended starting transfection conditions for Turbofectin 8

Tissue Culture Vessel	Growth area, cm^2/well	μg of DNA	Ratio of Turbofectin:DNA
96-well plate	0.35	0.1-0.15	3:1
24-well plate	2	0.5-1	3:1
12-well plate	4	1-2.5	3:1
6-well plate	9.5	1-5	3:1
35 mm plate	8	1-5	3:1

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60 mm plate	20	2-10	3:1
100 mm plate	60	5-15	3:1

Figure 3. Diagram of cell passaging after transfection

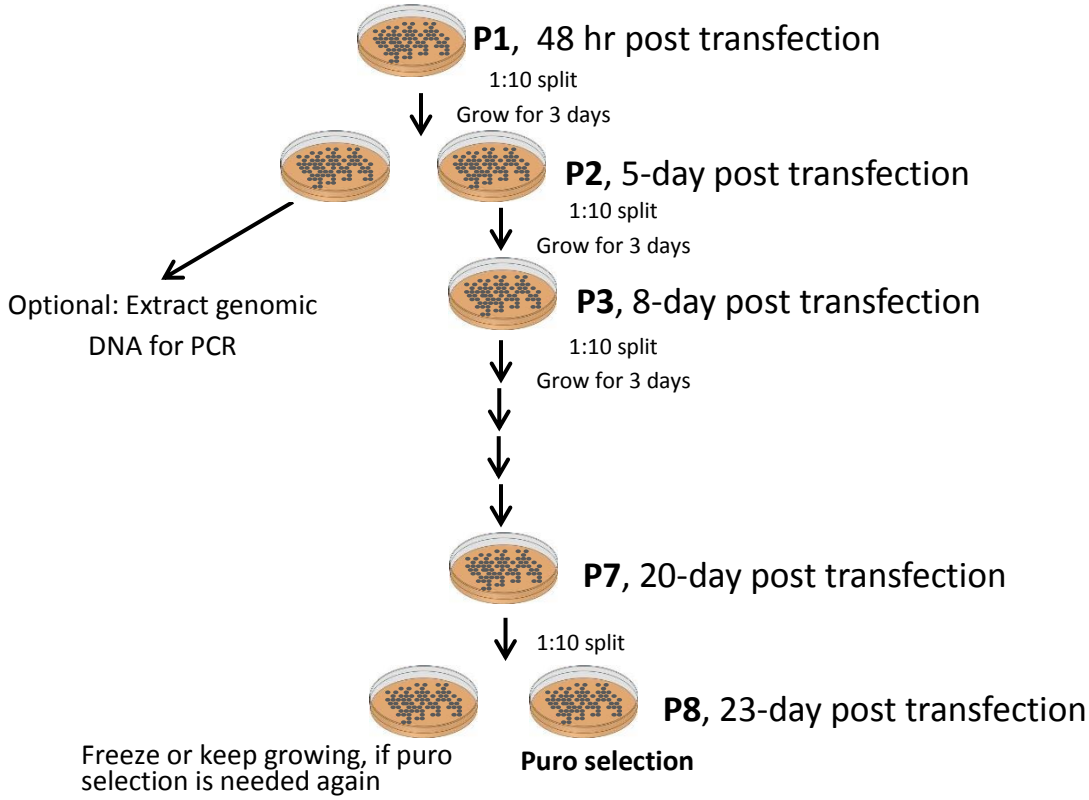
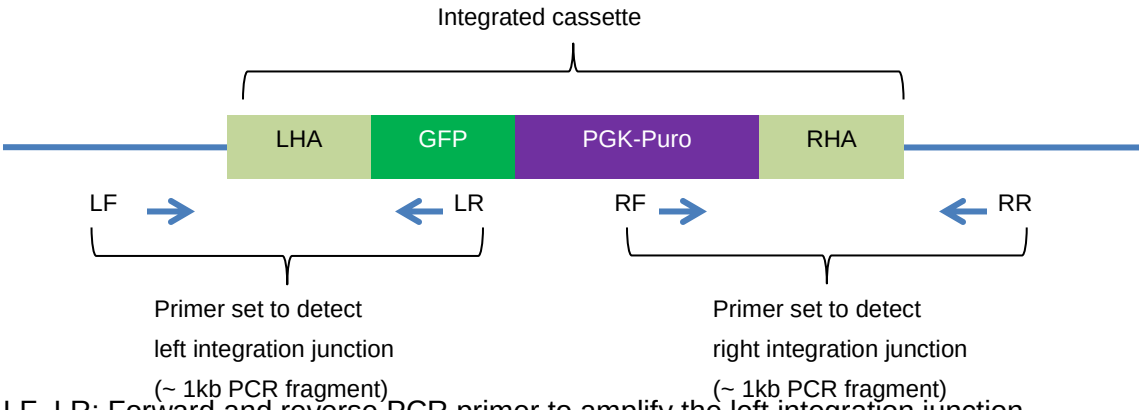


Figure 4. Diagram of genomic PCR Primer design.



LF, LR: Forward and reverse PCR primer to amplify the left integration junction
RF, RR: Forward and reverse PCR primer to amplify the right integration junction

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KN2.0, non-homology mediated CRISPR knockout kits

Package contents

- 2 vials of gRNA vectors, (SKU KNxxxxxxG1, KNxxxxxxG2), 3-5 µg DNA in TE buffer
- 1 vial of linear donor LoxP-EF1a-GFP-P2A-Puro-LoxP (SKU KNxxxxxxD), 10 ug, lyophilized.
- Certificate of Analysis
- Application Guide: https://www.origene.com/support/product-support/product-manuals

Note: The product is shipped at room temperature, but should be kept at -20℃ for long-term storage. If properly stored, it is guaranteed to be stable for 12 months.

Related Optional Reagents

- LB agar plates with ampicillin, 100 µg/mL
- LB broth (10 g/L Tryptone, 5 g/L Yeast Extract, 10 g/L NaCl. Adjust pH to 7.0 with 1 N NaOH)

Related OriGene Products

- Transfection reagent: https://www.origene.com/products/others/transfection-reagents
- Reagents and supplies for immunoblots: user preferred. OriGene has a selection of antibodies and detection reagents that are available at https://www.origene.com/products/antibodies
- DNA purification reagents https://www.origene.com/products/others/nucleic-acid-purification-kits
- qPCR reagents https://www.origene.com/products/gene-expression/qpcr
- CRISPR/Cas9 products https://www.origene.com/products/gene-expression/crispr-cas9

Notice to purchaser

This product is for research use only. Use in and/or for diagnostics and therapeutics is strictly prohibited. By opening and using the product, the purchaser agrees to the following: The plasmids may not be distributed, resold, modified for resale or used to manufacture commercial products without prior written approval from OriGene Technologies, Inc. If you do not agree to the above conditions, please return the UNOPENED product to OriGene Technologies, Inc. within ten (10) days of receipt for a full refund.

Product Description

KN2.0 gene knockout kit is non-homology mediated CRISPR kit. Target specific gRNA will cut the genome, then the linear donor DNA containing a selection cassette will be integrated at the gRNA cutting site at forward or reverse direction. The knockout efficiency is higher than HDR-mediated gene knockout. The majority gene knockout is biallelic, one allele has donor integration, the other allele has indels (insertion and deletion). Indels might affect protein coding or cause premature stop.

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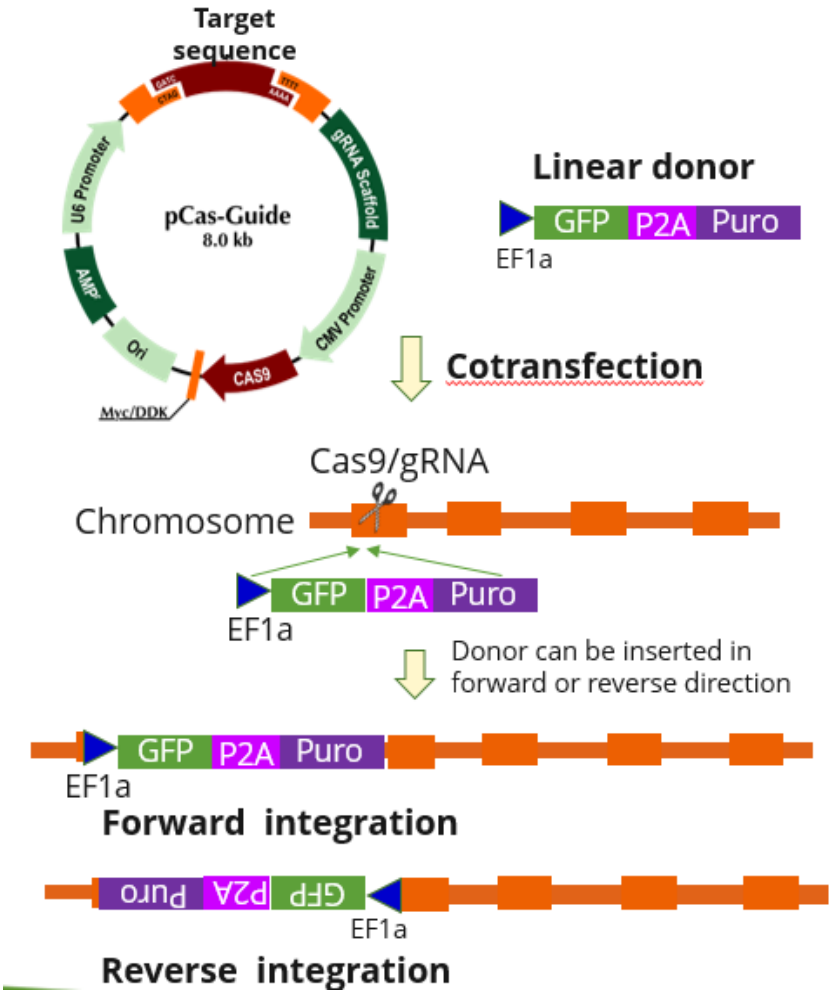
Figure 5. Diagram of KN2.0 non-homology-mediated CRISPR knockout kit



Experimental Protocol

Each kit contains two gRNA vectors, and one donor DNA. To ensure high efficiency of cleavage, two gRNA constructs are provided.

A sample protocol listed below is 6-well plates and using TurboFectin (cat# TF81001) as transfection reagent. If your experiments require a different size of culture plates, just scale up or down the reagents accordingly based on the relative surface area of your plate. Different type of cells may need a different transfection reagent; please follow the manufacturer's corresponding protocol. OriGene's Virus-like Viromers which are best for difficult-to-transfect cells.

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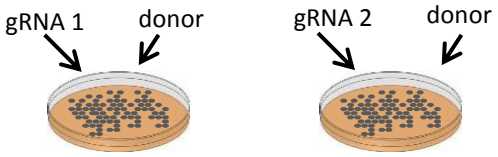
1. Approximately 18-24 hours before transfection, plate ~3 X 10^5 adherent cells in 2 ml culture media into each well of a 6-well plate or ~5x10^5 suspension cells per well to obtain 50-70% confluence on the following day. The number of cells varies depending on the size of your cells.

2. Transfection in complete culture media. Two separate transfections:



In a small sterile tube, combine the following reagents in the prescribed order. The order of reagent addition is important to achieve the optimal results.

a. Dilute 1 µg of one of the gRNA vectors in 250 uL of Opti-MEM I (Life Technologies), vortex gently. Then add 1 µg of the donor DNA into the same 250 µL of Opti-MEM I. Vortex gently.
b. Add 6 µL of Turbofectin 8.0 to the diluted DNA (not the reverse order) and pipette gently to mix completely.
c. Incubate the mixture 15 minutes at room temperature.

***Note**: 1. We recommend starting with the ratios of 3:1, Turbofectin:DNA. however, subsequent optimization may further increase the transfection efficiency.*

***Note**: 2. For your specific cells, you may need to find the best transfection method and follow the manufacturer's protocol.* Viromer *transfection reagents work better for hard-to-transfect cells. In general, transfection efficiency of linear DNA is lower than circular DNA.*

d. Add the mixture above drop-wise to the cells plated in step 1 (no need to change the media). Gently rock the plate back-and-forth and side-to-side to distribute the complex evenly.
e. Incubate the cells in a 5% CO_2 incubator.

3. 48 hrs post transfection, split cells 1:10, grow additional 3 days; then split the cells again 1:10. Split cells 2-4 times in total. Since puromycin resistant gene in the donor DNA is under EF1a-P2A, the linear donor DNA before genomic integration will also provide puromycin resistance. The reason to grow cells for around 2 weeks before puromycin selection is to dilute out cells containing non-integrated donor DNA. The shorter cell passaging time before puromycin selection, the higher false positive rate. If you passage cells for 2 weeks after transfection, the correct gene knockout rate after puromycin selection is around 50%; if you only passage cells for 1 week, the gene knockout rate is around 30%.

Time lines of genome editing

- ✓ CRISPR targeted gene knockout / knockin--- 1 week post transfection
- ✓ Non-integrated donor DNA dilution with cell passaging--- 2 weeks post transfection

***Note 1.** Since stable cell selection takes time, you can try to analyze the cells at P2 (passage 2) to detect genomic integration using genomic PCR (Fig 6). Primer pair needs to be designed to*

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amplify the donor-inserted allele due to the small percentage of edited cells. Therefore, one primer should be outside of the donor cassette and one primer is in the donor cassette. There could be some difficulties in the genomic PCR at this step before selection due to the small percentage of edited cells and difficulties of genomic PCR. qPCR measuring the targeted mRNA level would not work due to the small percentage of edited cells.

4. Apply puromycin selection or GFP sorting. Split P3 or P5 cells 1:10, then grow cells directly in the puromycin containing complete media in 10 cm dishes. The dose of puromycin needs to be determined with a kill curve to find out the lowest dose that kills the non-transfected cells completely 4-7 days post selection; the range of puromycin is 1µg/ml to 10µg/ml). Change the media every 2-3 days.

   ***Note**: We recommend you keep growing or freeze some of the transfected cells without selection; just in case, you need to perform the selection again.*

5. The puromycin resistant cells are ready to be analyzed for genome editing. WB can be used to measure gene knockdown if there is a good protein-specific antibody available (better after isolating individual cell colonies). You will also need to do genomic PCR to verify the integration of the functional cassette (figure 6). With primer pair of 5F and 3R, both alleles of donor inserted and non-edited/indel will be amplified. Please see the data in figure 7. The smaller PCR fragment could be un-edited allele or allele containing indels that could cause protein reading frame change or premature stop. You can directly sequence the amplified genomic fragment using the PCR primers to verify the sequence. It is better after isolating single cell colonies, so the sequencing data is clear.

   Figure 6. Diagram of PCR primers for genomic PCR to verify donor insertion.

To detect 5' junction, use primer pairs 5F & 5R, 3' junction, use primer pairs 3F & 3R

To detect 5' junction, use primer pairs 5F & 3F, 3' junction, use primer pairs 5R & 3R

6. Isolate individual cell colonies.

   There are two main methods to isolate single cell colonies, limiting dilution and cloning rings / cylinder.

   1) Limiting dilution.
   This method is better to be used after puromycin selection. Dilute cells to seed about 1-2 cells/well in 96-well plate, after 1-2 weeks, observe under the microscope and select the wells only containing one cell colony, then further expand them to 6-well plate when they are confluent in the 96-well plate and so on.

   2) Cloning rings / cylinder

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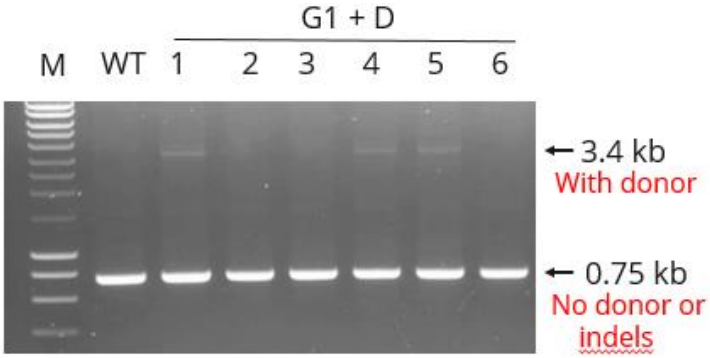
this method can be used in the same time with puro selection. Seed cells at lower density, such as 5% confluency in a larger cell culture dish, such as 10cm dish, apply puromycin selection when seeding.

***Note**. After puromycin selection, we observed some colonies are green, some colonies are not green. The efficiency of donor insertion is similar for the green and dark colonies. The reasons that the GFP expression is low in some cell colonies are not clear. It is ok to use GFP to sort out edited cells, so you will save one week comparing to puromycin selection. When you use puro selection, we recommend you analyze both green and dark cell colonies.*

Figure 7. Genomic PCR verification data using primer pair 5F and 3R to amplify both donor-inserted and non-edited/indel alleles.



Single HEK293T cell colonies were isolated after puromycin selection. Genomic DNA was extracted and PCR was performed using primer pair 5F and 3R. WT: untransfected cells.
1, 2, 3, 4, 5, 6: single colonies of G1 and donor transfected.

EF1a-GFP-P2A-Puro selection cassette sequence:

ATAACTTCGTATAATGTATGCTATACGAAGTTATCGTGAGGCTCCGGTGCCCGTCAGTGGGCAGAGC
GCACATCGCCCACAGTCCCCGAGAAGTTGGGGGGAGGGGTCGGCAATTGAACCGGTGCCTAGAGA
AGGTGGCGCGGGGTAAACTGGGAAAGTGATGTCGTGTACTGGCTCCGCCTTTTTCCCGAGGGTGG
GGGAGAACCGTATATAAGTGCAGTAGTCGCCGTGAACGTTCTTTTTCGCAACGGGTTTGCCGCCAGA
ACACAGGTAAGTGCCGTGTGTGGTTCCCGCGGGCCTGGCCTCTTTACGGGTTATGGCCCTTGCGTG
CCTTGAATTACTTCCACCTGGCTGCAGTACGTGATTCTTGATCCCGAGCTTCGGGTTGGAAGTGGGT
GGGAGAGTTCGAGGCCTTGCGCTTAAGGAGCCCCTTCGCCTCGTGCTTGAGTTGAGGCCTGGCCTG
GGCGCTGGGGCCGCCGCGTGCGAATCTGGTGGCACCTTCGCGCCTGTCTCGCTGCTTTCGATAAG
TCTCTAGCCATTTAAAATTTTTGATGACCTGCTGCGACGCTTTTTTTCTGGCAAGATAGTCTTGTAAAT
GCGGGCCAAGATCTGCACACTGGTATTTCGGTTTTTGGGGCCGCGGGCGGCGACGGGGCCCGTGC
GTCCCAGCGCACATGTTCGGCGAGGCGGGGCCTGCGAGCGCGGCCACCGAGAATCGGACGGGGG
TAGTCTCAAGCTGGCCGGCCTGCTCTGGTGCCTGGCCTCGCGCCGCCGTGTATCGCCCCGCCCTG
GGCGGCAAGGCTGGCCCGGTCGGCACCAGTTGCGTGAGCGGAAAGATGGCCGCTTCCCGGCCCT
GCTGCAGGGAGCTCAAAATGGAGGACGCGGCGCTCGGGAGAGCGGGCGGGTGAGTCACCCACAC
AAAGGAAAAGGGCCTTTCCGTCCTCAGCCGTCGCTTCATGTGACTCCACGGAGTACCGGGCGCCGT
CCAGGCACCTCGATTAGTTCTCGAGCTTTTGGAGTACGTCGTCTTTAGGTTGGGGGGAGGGGTTTTA
TGCGATGGAGTTTCCCCACACTGAGTGGGTGGAGACTGAAGTTAGGCCAGCTTGGCACTTGATGTA
ATTCTCCTTGGAATTTGCCCTTTTTGAGTTTGGATCTTGGTTCATTCTCAAGCCTCAGACAGTGGTTC
AAAGTTTTTTTCTTCCATTTCAGGTGTCGTGAATGGAGAGCGACGAGAGCGGCCTGCCCGCCATGGA

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GATCGAGTGCCGCATCACCGGCACCCTGAACGGCGTGGAGTTCGAGCTGGTGGGCGGCGGAGAG
GGCACCCCCGAGCAGGGCCGCATGACCAACAAGATGAAGAGCACCAAAGGCGCCCTGACCTTCAG
CCCCTACCTGCTGAGCCACGTGATGGGCTACGGCTTCTACCACTTCGGCACCTACCCCAGCGGCTA
CGAGAACCCCTTCCTGCACGCCATCAACAACGGCGGCTACACCAACACCCGCATCGAGAAGTACGA
GGACGGCGGCGTGCTGCACGTGAGCTTCAGCTACCGCTACGAGGCCGGCCGCGTGATCGGCGACT
TCAAGGTGATGGGCACCGGCTTCCCCGAGGACAGCGTGATCTTCACCGACAAGATCATCCGCAGCA
ACGCCACCGTGGAGCACCTGCACCCCATGGGCGATAACGATCTGGATGGCAGCTTCACCCGCACCT
TCAGCCTGCGCGACGGCGGCTACTACAGCTCCGTGGTGGACAGCCACATGCACTTCAAGAGCGCC
ATCCACCCCAGCATCCTGCAGAACGGGGGCCCCATGTTCGCCTTCCGCCGCGTGGAGGAGGATCA
CAGCAACACCGAGCTGGGCATCGTGGAGTACCAGCACGCCTTCAAGACCCCGGATGCAGATGCCG
GTGAAGAAAGAGGAAGCGGAGCTACTAACTTCAGCCTGCTGAAGCAGGCTGGAGACGTGGAGGAG
AACCCTGGACCTATGACCGAGTACAAGCCCACGGTGCGCCTCGCCACCCGCGACGACGTCCCCAG
GGCCGTACGCACCCTCGCCGCCGCGTTCGCCGACTACCCCGCCACGCGCCACACCGTCGATCCGG
ACCGCCACATCGAGCGGGTCACCGAGCTGCAAGAACTCTTCCTCACGCGCGTCGGGCTCGACATC
GGCAAGGTGTGGGTCGCGGACGACGGCGCCGCGGTGGCGGTCTGGACCACGCCGGAGAGCGTCG
AAGCGGGGGCGGTGTTCGCCGAGATCGGCCCGCGCATGGCCGAGTTGAGCGGTTCCCGGCTGGC
CGCGCAGCAACAGATGGAAGGCCTCCTGGCGCCGCACCGGCCCAAGGAGCCCGCGTGGTTCCTG
GCCACCGTCGGCGTCTCGCCCGACCACCAGGGCAAGGGTCTGGGCAGCGCCGTCGTGCTCCCCG
GAGTGGAGGCGGCCGAGCGCGCCGGGGTGCCCGCCTTCCTGGAGACCTCCGCGCCCCGCAACCT
CCCCTTCTACGAGCGGCTCGGCTTCACCGTCACCGCCGACGTCGAGGTGCCCGAAGGACCGCGCA
CCTGGTGCATGACCCGCAAGCCCGGTGCCTGAAACTTGTTTATTGCAGCTTATAATGGTTACAAATA
AAGCAATAGCATCACAAATTTCACAAATAAAGCATTTTTTTCACTGCATTCTAGTTGTGGTTTGTCCAA
ACTCATCAATGTATCTTAATAACTTCGTATAATGTATGCTATACGAAGTTAT

LoxP in cyan, EF1A promoter in blue, tGFP (699 bp) in green, Puromycin resistance gene (600 bp) purple. P2A sequence (66 bp) is in red.

II. Precut All-in-one CRISPR vector cloning kits, pCas-Guide (SKU: GE100001), pLenti-Cas-Guide (SKU:GE100009)

Package contents

The following components are included:

- One (1) vial of precut CRISPR vector DNA, lyophilized ready for ligation (10 RXNs). Reconstitute in 10 µL dH_2O, final concentration 10 ng/ µL.
- One (1) vial of forward sequencing primer, CF3 (SKU GE100008) to sequence the targeting sequence cloned into the CRISPR vector; 100 picomoles dried onto the bottom of screw cap tubes. Reconstitute the primer in 10 µL dH_2O to make a 10 µM solution.
- One (1) vial of annealing buffer (SKU GE100007) for oligo annealing (10X), 200 µL
- Certificate of Analysis
- Application Guide

** The cDNA clone is shipped at room temperature, but should be kept at -20°C for long-term storage. If properly stored, clones are guaranteed to be stable for 12 months.*

Related Optional Reagents

Nuclease free water

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T4 DNA ligase and buffer
Competent *E. coli* cells
LB agar plates with ampicillin, 100 μg/mL
LB broth (10 g/L Tryptone, 5 g/L Yeast Extract, 10 g/L NaCl. Adjust pH to 7.0 with 1 N NaOH)
DNA purification reagents

Related OriGene Products

- TrueClone™ FL cDNA clones https://www.origene.com/products/cdna-clones/trueclone
- HuSH™ shRNA Plasmids https://www.origene.com/products/rnai/shrna-plasmids
- Validated Antibodies https://www.origene.com/products/antibodies
- Purified Proteins https://www.origene.com/products/proteins
- Over-expression lysates https://www.origene.com/products/proteins/over-expression-lysates
- Transfection Reagents https://www.origene.com/products/others/transfection-reagents
- Anti-tag Antibodies https://www.origene.com/products/antibodies/tag-antibodies

Notice to purchaser

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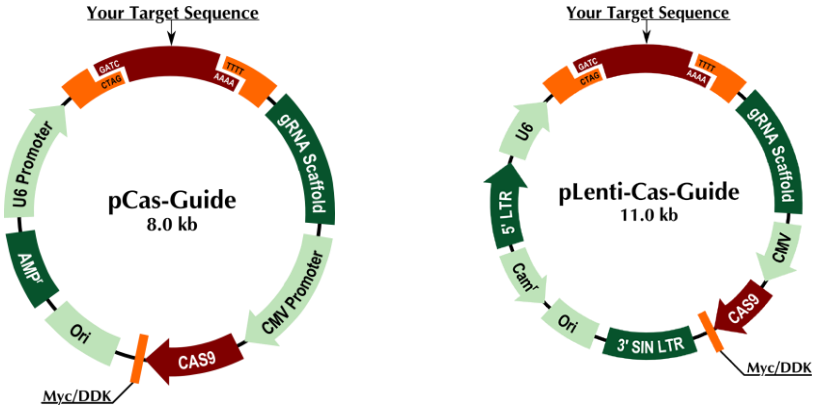
Production and Quality Assurance

The precut pCas-Guide or pLenti-Guide vectors have been tested to successfully religate to annealed oligo DNA fragments. When OriGene experimental protocol is followed (details on page 13-15), 1 μL of the ligation reaction generated with the precut vector can produce 100 colonies when transformed into 10^6 cfu/μg competent cells. The self-ligation background (vector religation to itself without an insert) is less than 5% of transformants. The amount of digested DNA provided in the kit is sufficient for ten ligation reactions.

Figure 5. The vector map of pre-cut pCas-Guide and pLenti-Cas-Guide



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Product Description

Both pCas-Guide and pLenti-Cas-Guide vectors are designed for cloning a guide RNA insert for genome editing purpose. The vectors also have a CMV-driven codon-optimized Cas9. When co-transfected with a proper donor DNA, targeted genome editing can be achieved. The vectors are supplied as precut vectors, ready for insert ligation. This system has been successfully validated in multiple cases of genome editing.

Experimental Protocols

I. Design target sequence

There are quite a few gRNA design tools available. You can find the resources on Zhang's lab: https://zlab.bio/guide-design-resources. Select 2-3 target sequences for cloning to the CRISPR vectors.

II. Addition of extra bases to the ends of the target sequence

To facilitate cloning of the 20-bp target sequence, extra bases need to be added to the ends.

1. Select a desired 20-bp sequence as a target. The following is an example sequence:

   Forward sequence: 5' ATGGGAGGTGGTATGGGAGG 3'
   Reverse complement sequence: 5' CCTCCCATACCACCTCCCAT 3'

2. Add 'gatcg' to the 5' end of the forward sequence and 'g' to its 3' end. The final sense oligo in this example will be

   5' gatcgATGGGAGGTGGTATGGGAGGg 3'

3. Add 'aaaac' to the 5' end of reverse complementary sequence and 'c' to its 3' end. The final reverse complementary sequence is

   5' aaaacCCTCCCATACCACCTCCCATc 3'

The two oligos should anneal to form the following double strand:

```
5'gatcgxxxxxxxxxxxxxxxxxxxxg 3'
    3'cxxxxxxxxxxxxxxxxxxxxcaaaa 5'
```

4. Order the two final oligos from a commercial oligo provider, such as IDT.

III. Cloning the double-stranded oligos into the pCas-Guide vector

1. Anneal the oligos to form double-stranded duplexes

   In a PCR tube, add the following:

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2 µL Forward oligo (100 µM stock)
2 µL Reverse oligo (100 µM stock)
4 µL 10X annealing buffer
32 µL dH_2O

Mix the solution and follow the steps to anneal the oligos in a PCR machine:

94^0C for 4min
75^0C for 5 min
65^0C for 15 min
25^0C for 20 min

After annealing, transfer the solution to a 1.5 mL tube and add 360 µL of dH_2O. The double-stranded oligo DNA is ready for ligation.

2. Ligation and transformation

A. Prepare the ligation according to the following protocol

Component	Volume
10x Ligation buffer	1 µL
Precut pCAS-Guide vector (10 ng/ µL)	1 µL
Annealed double-stranded oligos (diluted from step 1)	1 µL
Ligase (0.5 u/ µL, Weiss unit)	0.5 µL
dH2O	6.5 µL
Total Volume	10 µL

B. Mix the solution and incubate the tube at 22 to 37^0C or room temperature for two hours according to the manufacturer's recommendation.

C. Add 1 µL of the ligation mixture to 10 µL of competent cells (efficiency rated > 10^6 cfu/µg DNA) on ice. Do the transformation according to the manufacturer's protocol. For chemically competent cells, follow steps D-E.

D. Mix the tube gently and keep it on ice for 25 minutes.

E. Heat shock the tube for 30 seconds at 42^0C.

F. Put the tube on ice for 2 minutes, then add 500 µL LB or SOC medium.

G. Rock the tube gently at 37^0C for 1 hour.

H. Spread 50 µL of the *E. Coli* cells on an LB agar plate containing the corresponding Antibiotics (most vectors are ampicillin resistant except lenti CRISPR vectors, which is chloramphenicol).

I. Centrifuge the remaining *E. Coli* cells at 5K rpm for 5 minutes. Discard most the supernatant (around 50 µL supernatant left) and resuspend the cell pellet in the remaining liquid. Spread all the *E. Coli* cells on a separate LB-agar plate.

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J. Incubate the two plates at 37^{0}C for 16 hours to allow colony formation.

3. Screening colonies

In a typical subcloning ligation, at least 95% of the colonies should contain the desired insert. Pick 6 to 10 colonies into 5 mL LB-ampicillin culture each, and culture overnight. Perform DNA purification using a mini-prep kit from OriGene, https://www.origene.com/products/others/nucleic-acid-purification-kits/ . Sequence the purified DNA and analyze the sequencing data to identify a correct clone for proper insert identification and orientation.

Lenti-based protocols:

NOTE: Performing Lentiviral experiments REQUIRES special laboratory conditions and/or permissions (BL2). Follow the guidelines and regulations of your institution. Perform the experiments with due caution to avoid exposure to infectious materials.

A. Production of pseudo virus (10 cm plate format, the production size can be scaled up or down accordingly):

1. Day 1, plate HEK293T cells in a 10 cm dish to approximately 40% confluency the day before transfection (antibiotic-free preferred). Cells should reach 65-70% confluency within 24 hours.

2. Day 2, In sterile tube, dilute the following DNA in 1.5 mL Opti-MEM, and pipet gently to mix completely.
   a. 5 µg of lenti vector, Lenti CRISPR vector or Lenti-ORF or lenti-shRNA
   b. 6 µg of packaging plasmids, cat# TR30037 (for regular lentivirus production) or TR30036 (for integration-deficient lentivirus production).

      Add 33 µL of TurboFectin transfection reagent to the diluted DNA (not the reversed order), pipet gently to mix completely.

   c. Incubate for 15 min at room temperature.
   d. Add the transfection mixture prepared above dropwise to the cells. Gently rock the plate back-and-forth and from side-to-side to distribute the complex evenly. Incubate at 37° in a CO2 incubator.

   Note: With TurboFectin, no medium change is necessary, directly add the transfection mixture to cells in complete growth media.

3. Day 3, change the growth medium and continue to incubate the plate for 48 hours.

4. Day 5
   a. 48 hours after, transfer the cell culture supernatant to a 15 mL centrifuge tube.
   b. Centrifuge the tubes at 3K RPM for 10 mins and filter the supernatant through a syringe filter (0.45 micron) and collect the viral solution to a new sterile tube.

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5. The viral particles are ready to be used. If virus titer is needed, you can use PCR or Elisa methods and follow the corresponding protocols. Lentiviral particles can be stored at 4 °C for 2 weeks or store at -80 °C for long-term storage.

B. Transduction of lentivirus to target cells

1. Day 1, plate target cells in three 10 cm plates at a density that will produce approximately 60% confluency in 24 hours. Note: other size formats can also be used depending on the nature of your experiment. Adjust the reagent amount accordingly.

2. Day 2, Remove the growth media from the plates prepared the day before. To plate 1, add 4.5 mL of fresh growth medium and 0.5 mL of Lentiviral particles; To plate 2, add 4.0 mL of growth medium and 1 mL of Lentiviral particles; To plate 3, add 2.5 mL of growth medium and 2.5 mL of Lentiviral particles (for a low titer viral preparation, the amount of virus added can be increased to 5 mL). Mix the solution by gentle swirling.

3. Add 5 µl polybrene (1,000x, 8 mg/mL) to each plate. Mix by gentle swirling.

4. Incubate the cells at 37 °C with 5% CO2 for 4 hours. Remove the transduction medium and add 10 mL of fresh growth medium. Incubate the cells for three more days.

The transduced cells are ready for downstream analyses such as RNA and protein detection.

III. Circular All-in-one CRISPR vectors (SKU: GE100002, GE100018, GE100022, GE100010, GE100045)

Package contents

- One (1) vial of CRISPR vector DNA, 10 µg, lyophilized. Reconstitute in 100 µL dH_2O to make a final concentration of 100 ng/ µL.
- One (1) vial of CF3 sequencing primer (SKU GE100008) is provided for vectors (GE100002, GE100010) to sequence the targeting sequence cloned into CRISPR vector; 100 picomoles dried onto the bottom of screw cap tubes. Reconstitute the primer in 10 µL dH_2O to make a 10 µM solution.
- Certificate of Analysis
- Application Guide available online.

** OriGene plasmids are purified using ion-exchange columns for high-yield, low endotoxin preparations (PowerPrep® HP Midiprep Kit* https://www.origene.com/products/others/nucleic-acid-purification-kits)
The cDNA clone is shipped at room temperature, but should be kept at -20 °C for long-term storage. If properly stored, clones are guaranteed to be stable for 12 months.

Related Optional Reagents

- Oligo annealing buffer, SKU GE100007
- BamH I and BsmB I
- T4 DNA ligase and buffer
- Competent *E. coli* cells, https://www.origene.com/products/others/competent-cells

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- LB agar plates with ampicillin, 100 μg/mL
- LB broth (10 g/L Tryptone, 5 g/L Yeast Extract, 10 g/L NaCl. Adjust pH to 7.0 with 1 N NaOH)
- DNA purification reagents

Related OriGene Products

- Cloning the homologous arms in predesigned donor rescue construct harboring functional cassettes (service from www.blueheronbio.com).
  - GFP-PGK-Loxp-Puro-LoxP
  - RFP-PGK-Loxp-BSD-LoxP
  - Luciferase-PGK-Loxp-Puro-LoxP
  - mBFP-PGK-Loxp-Neo-Loxp

- TrueClone™ FL cDNA clones https://www.origene.com/products/cdna-clones/trueclone
- HuSH™ shRNA Plasmids https://www.origene.com/products/rnai/shrna-plasmids
- Validated Antibodies https://www.origene.com/products/antibodies
- Purified Proteins https://www.origene.com/products/proteins
- Over-expression lysates https://www.origene.com/products/proteins/over-expression-lysates
- Transfection Reagents https://www.origene.com/products/others/transfection-reagents
- Anti-tag Antibodies https://www.origene.com/products/antibodies/tag-antibodies

Notice to purchaser

This product is for research use only. Use in and/or for diagnostics and therapeutics is strictly prohibited. By opening and using the product, the purchaser agrees to the following: The plasmids may not be distributed, resold, modified for resale or used to manufacture commercial products without prior written approval from OriGene Technologies, Inc. If you do not agree to the above conditions, please return the UNOPENED product to OriGene Technologies, Inc. within ten (10) days of receipt for a full refund by contacting customer service at custsupport@origene.com.

Product Description

The CRISPR vectors are designed for cloning a guide RNA insert for genome editing purpose. Target sequence can be cloned into the vector via BamH I and BsmB I sites. The vectors also express a CMV-driven codon-optimized Cas9. When co-transfected with a proper donor DNA, your desired targeted genome editing can be achieved. The vectors retain the ampicillin resistance gene for the selection of *E. coli* transformants.

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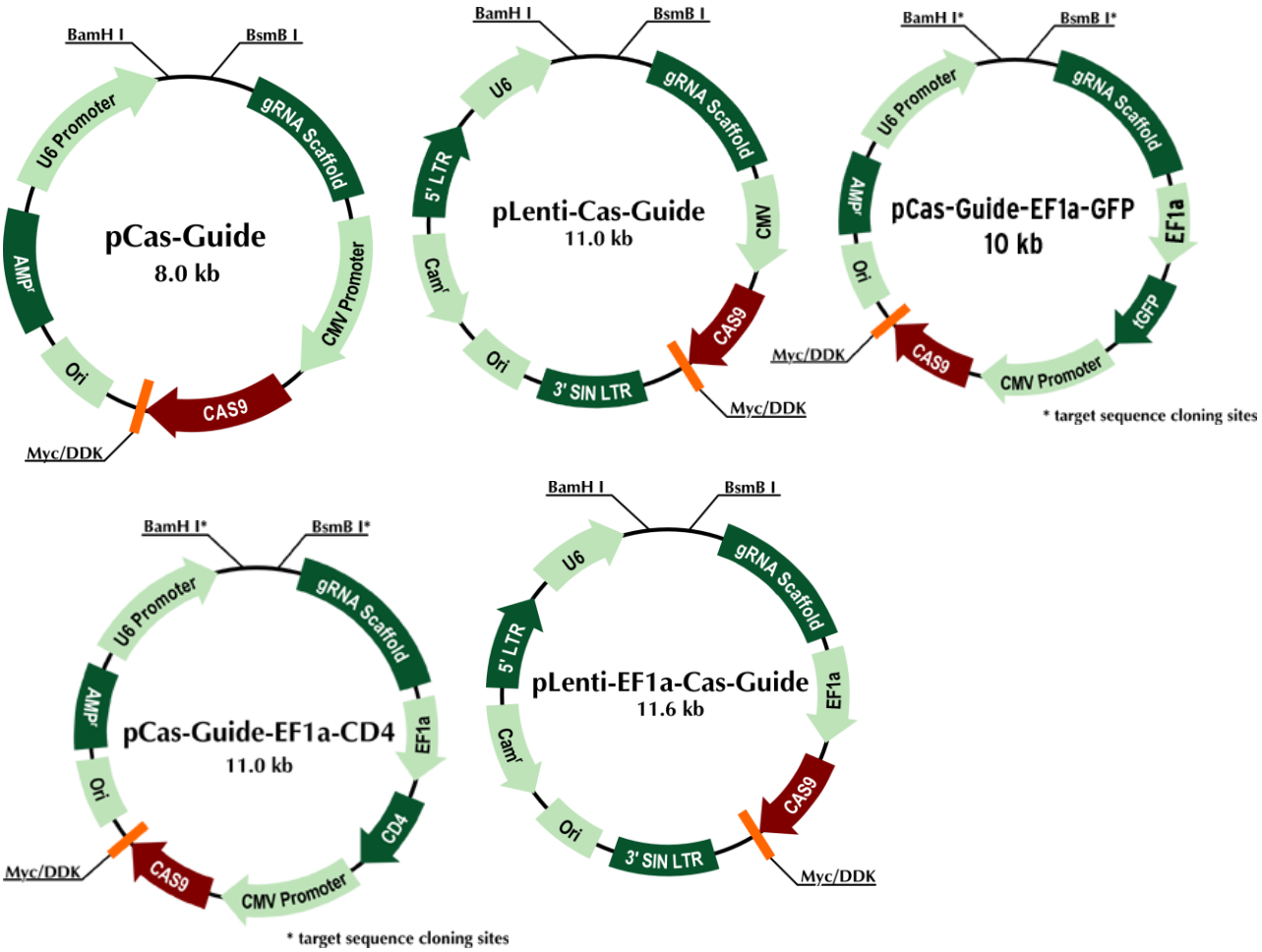
Figure 6. The vector maps of the circular CRISPR vectors.



Experimental protocol

1. Digest pCas-Guide plasmid with BamH I and BsmB I

Resuspend the 10 µg lyophilized DNA in 100 µL dH2O, and incubate for at least 30 min before use. Set up a digestion reaction as described below.

Component	Volume
10X restriction buffer	3 µL
BamH I	0.8 µL
BsmB I	0.8 µL
Nuclease free water	15.4 µL
Vector DNA	10 µL
Total volume	30 µL

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Incubate the reaction at 37ºC for 3 hrs, then add 1 µL antarctic phosphatase (units used according to the manufacturer's protocol), and continue the incubation at 37ºC for another 30 min. Dephosphorylation of the digested vector is essential to eliminate self-ligation. Purify the desired vector fragment by running the digestion reaction on an agarose gel, and isolate the appropriate band using a gel purification column. Elute the digested plasmid vector in 40 µL of 10 mM Tris buffer.

2. Target sequence designing and cloning into the precut vectors, please follow the detailed protocol from page 20-21 in this manual.

Lenti based protocol, please refer to page 16-17.

IV. gRNA only Vectors, pGuide (SKU GE100042), pGuide-EF1a-GFP (SKU GE100044), pLenti-Guide-Puro (SKU GE100032)

Package contents

- One (1) vial of circular pGuide or pLenti-Guide-Puro plasmid DNA, 10 µg, lyophilized. Reconstitute in 100 µL dH_2O to make a final concentration of 100 ng/ µL.
- Certificate of Analysis

** OriGene plasmids are purified using ion-exchange columns for high-yield, low endotoxin preparations (PowerPrep® HP Midiprep Kit* https://www.origene.com/products/others/nucleic-acid-purification-kits)
The cDNA clone is shipped at room temperature, but should be kept at -20℃ for long-term storage. If properly stored, clones are guaranteed to be stable for 12 months.

Related Optional Reagents

- Nuclease free water
- Oligo annealing buffer, SKU GE100007
- BamH I, BsmB I
- T4 DNA ligase and buffer
- Competent *E. coli* cells, https://www.origene.com/products/others/competent-cells
- LB agar plates with ampicillin, 100 µg/mL
- LB broth (10 g/L Tryptone, 5 g/L Yeast Extract, 10 g/L NaCl. Adjust pH to 7.0 with 1 N NaOH)
- DNA purification reagents

Related OriGene Products

- Cloning the homologous arms in predesigned donor rescue construct harboring functional cassettes (service from https://www.origene.com/products/gene-expression/crispr-cas9/grna-cloning-donor-construction).

  GFP-PGK-Loxp-Puro-LoxP
  RFP-PGK-Loxp-BSD-LoxP
  Luciferase-PGK-Loxp-Puro-LoxP
  mBFP-PGK-Loxp-Neo-Loxp

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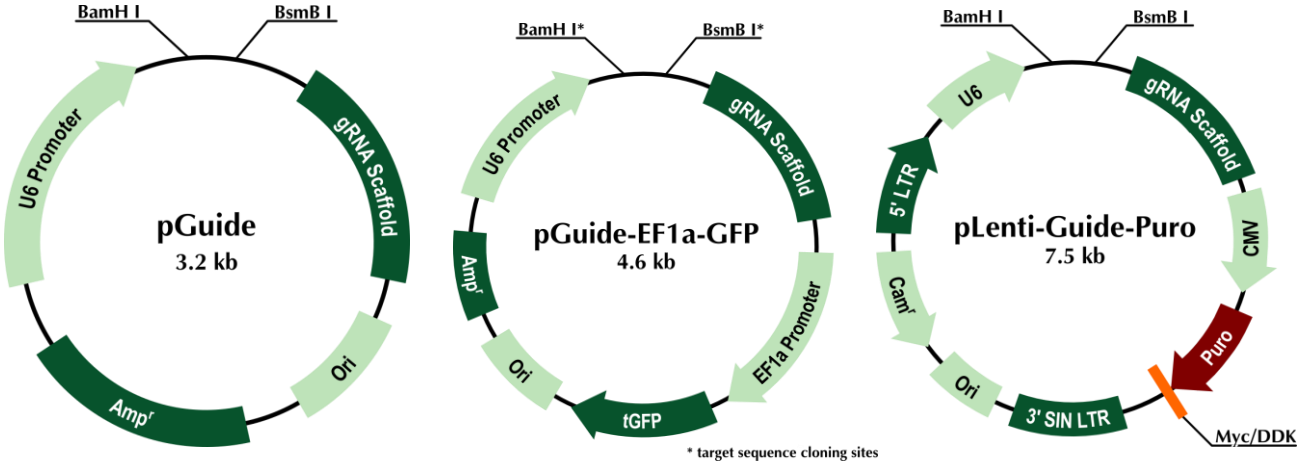
Product Description

gRNA only vectors can be used If your target cells already expressing Cas9. Two gRNA only cloning vectors are offered, pGuide, in regular mammalian expression vector, pLenti-Guide-Puro, in lenti vector backbone and contains puromycin selection marker. Target sequence can be cloned into the vector via BamH I and BsmB I sites. pGuide retains the ampicillin resistance gene for the selection of *E. coli* transformants; pLenti-Guide-Puro retains chloramphenicol resistance for E. Coli selection.

Figure 7. Plasmid maps of pGuide and pLenti-Guide-Puro.



Experimental protocol

The protocol of the gRNA only cloning is the same as the all-in-one CRISPR vector. Please see details from page 20-21in this manual.

V. Cas9 only vectors,
pLenti-Cas9 (SKU GE100028),
pLenti-Cas9-IRES-Puro (SKU GE100029),
pLenti-EF1a-Cas9-IRES-Puro (SKU GE100030),
pLenti-Cas9-P2A-tGFP (SKU GE100031)
pAAVS1-Cas9-Puro-DNR (SKU GE100037)
pAAVS1-Cas9-BSD-DNR (SKU GE100039)

Package contents

- One (1) vial of circular plasmid vector DNA, 10 μg, lyophilized. Reconstitute in 100 μL dH_2O to make a final concentration of 100 ng/ μL.
- Certificate of Analysis

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** OriGene plasmids are purified using ion-exchange columns for high-yield, low endotoxin preparations (PowerPrep® HP Midiprep Kit*
https://www.origene.com/products/others/nucleic-acid-purification-kits
The cDNA clone is shipped at room temperature, but should be kept at -20℃ for long-term storage. If properly stored, clones are guaranteed to be stable for 12 months.

Related Optional Reagents

- LB agar plates with ampicillin, 100 μg/mL
- LB broth (10 g/L Tryptone, 5 g/L Yeast Extract, 10 g/L NaCl. Adjust pH to 7.0 with 1 N NaOH)
- DNA purification reagents

Related OriGene Products

- gRNA only vectors, pGuide (GE100042), pLenti-Guide-Puro (SKU GE100032)
- Cloning the homologous arms in predesigned donor rescue construct harboring functional cassettes (service from https://www.origene.com/products/gene-expression/crispr-cas9/grna-cloning-donor-construction).
  GFP-PGK-Loxp-Puro-LoxP
  RFP-PGK-Loxp-BSD-LoxP
  Luciferase-PGK-Loxp-Puro-LoxP
  mBFP-PGK-Loxp-Neo-Loxp

Product Description

Lenti Cas9 vectors

The Lenti Cas9 only vectors express Cas9 after being transfected (using transfection reagent) or transduced (packaged into lentiviral particles) into cells, no gRNA expression cassette in the vector. The Lenti Cas9 vectors can be transfected or transduced with gRNA construct into cells or animals at the same time or develop Cas9 stable cells first, then introduce sgRNA into cells. The vectors retain the chloramphenicol resistance gene for the selection of *E. coli* transformants.

	pLenti-Cas9	pLenti-Cas9-IRES-Puro	pLenti-EF1a-Cas9-IRES-Puro	pLenti-Cas9-P2A-tGFP
Cas9 Promoter	CMV	CMV	EF1a	CMV
Mammalian selection	none	puro	puro	tGFP

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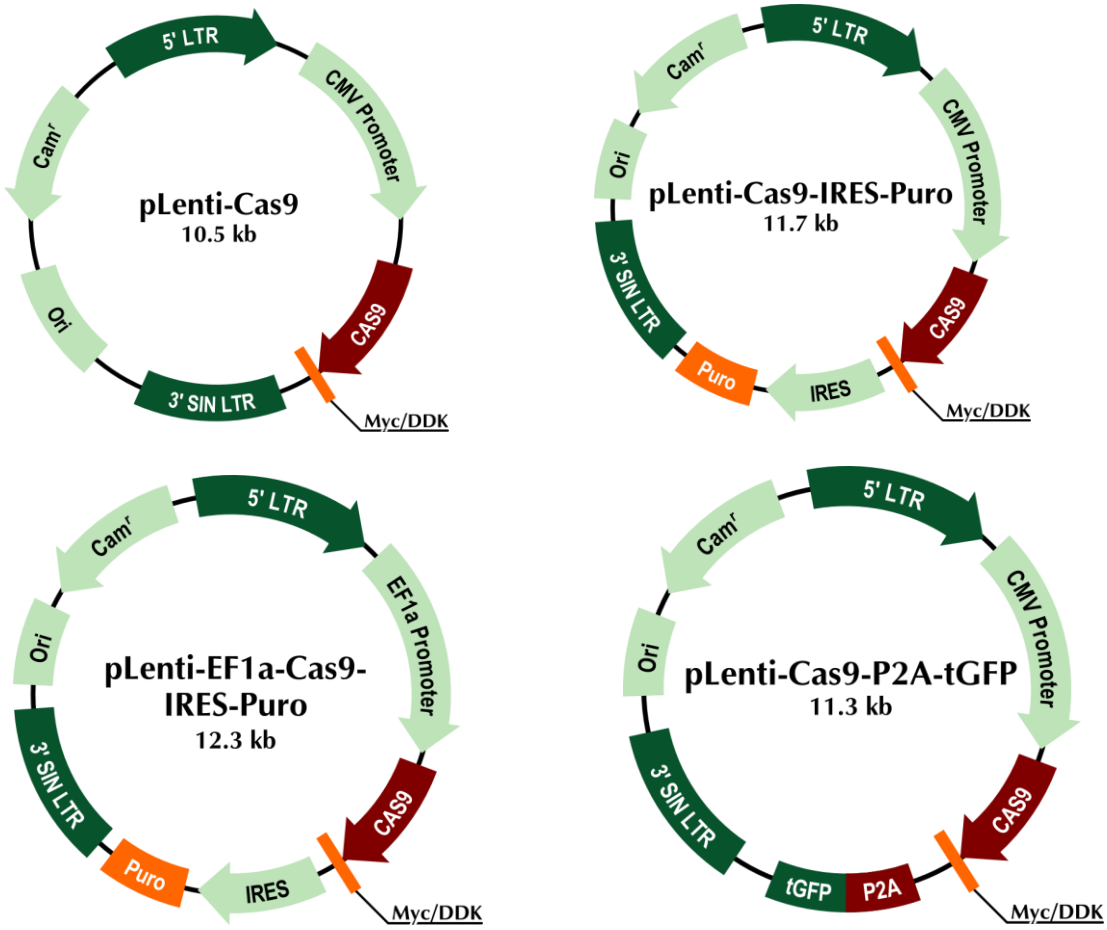
Figure 8. Plasmid maps of Lenti Cas9 vectors



pAAVS1-Cas9-Puro-DNR, pAAVS1-Cas9-BSD-DNR vectors

These two vectors can be used as regular mammalian Cas9 expression vectors when transfected into cells alone as Cas9 is driven by CMV promoter.

There are also AAVS1 homologous arm sequences in the vector, the vectors can be used as donor vectors together with pCas-guide-AAVS1 (gRNA targeting AAVS1, SKU GE100023) to insert Cas9 at AAVS1 locus in human cells. AAVS1 locus has been proven to be a safe harbor location for exogenous insertion to achieve stable and robust gene expression.

For details, please see page 44-45 in this manual.

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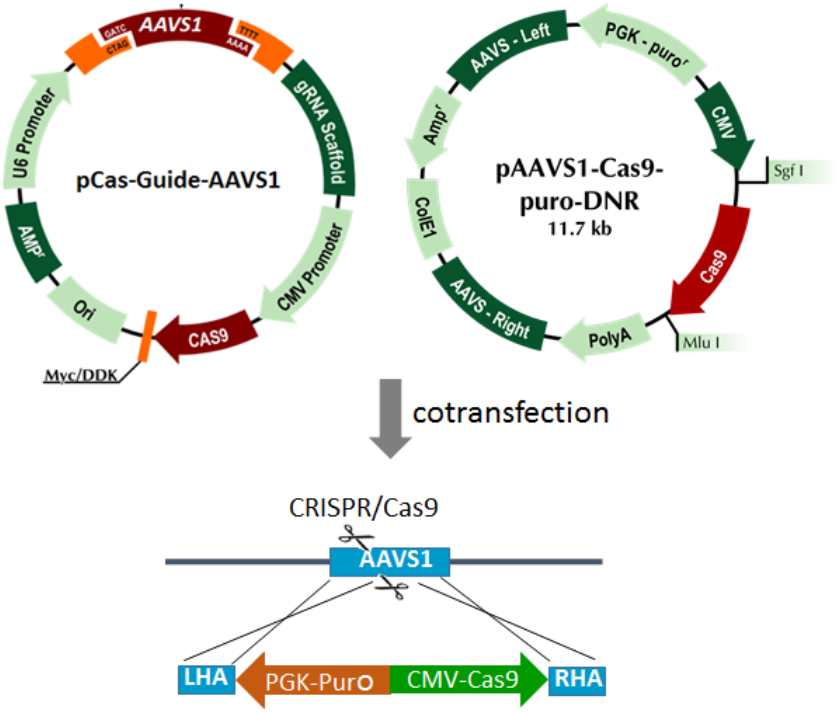
Fig. 9. Diagram of how Cas9 is inserted at AAVS1 locus



Experimental Protocol for Lenti Cas9 Vectors

1. Transient transfection with gRNA construct. Follow the manual facture's protocol of the transfection reagents.
2. Transduction with gRNA particles. Making lentiviral particles and transducing cells, follow protocol on page 16-17 in this manual.

VI. T7 driven CRISPR/Cas system-pT7-Guide-IVT (SKU: GE100025) and pT7-Cas9 (SKU: GE100014)

Package contents

- One (1) vial of circular pT7-Guide-IVT (SKU: GE100025) plasmid DNA or pT7-Cas9 (SKU: GE100014), 10 μg, lyophilized. Reconstitute in 100 μL dH_2O to make a final concentration of 100 ng/ μL.
- Certificate of Analysis

** OriGene plasmids are purified using ion-exchange columns for high-yield, low endotoxin preparations (PowerPrep® HP Midiprep Kit* https://www.origene.com/products/others/nucleic-acid-purification-kits)
The cDNA clone is shipped at room temperature, but should be kept at -20℃ for long-term storage. If properly stored, clones are guaranteed to be stable for 12 months.

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Related Optional Reagents

- Nuclease free water
- Oligo annealing buffer, SKU GE100007
- BsmB I
- T4 DNA ligase and buffer
- Competent *E. coli* cells, https://www.origene.com/products/others/competent-cells
- LB agar plates with ampicillin, 100 μg/mL
- LB broth (10 g/L Tryptone, 5 g/L Yeast Extract, 10 g/L NaCl. Adjust pH to 7.0 with 1 N NaOH)
- DNA purification reagents
- T7 *In vitro* transcription kits

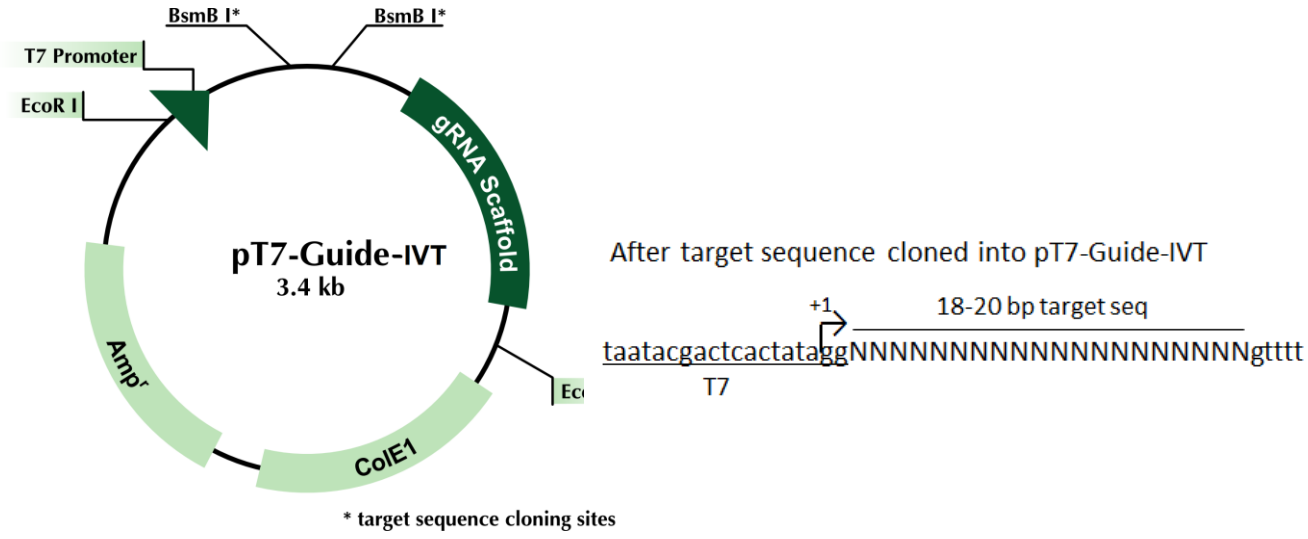
Related OriGene Products

- Cloning the homologous arms in predesigned donor rescue construct harboring functional cassettes (service from https://www.origene.com/products/gene-expression/crispr-cas9/grna-cloning-donor-construction).
  - GFP-PGK-Loxp-Puro-LoxP
  - RFP-PGK-Loxp-BSD-LoxP
  - Luciferase-PGK-Loxp-Puro-LoxP
  - mBFP-PGK-Loxp-Neo-Loxp

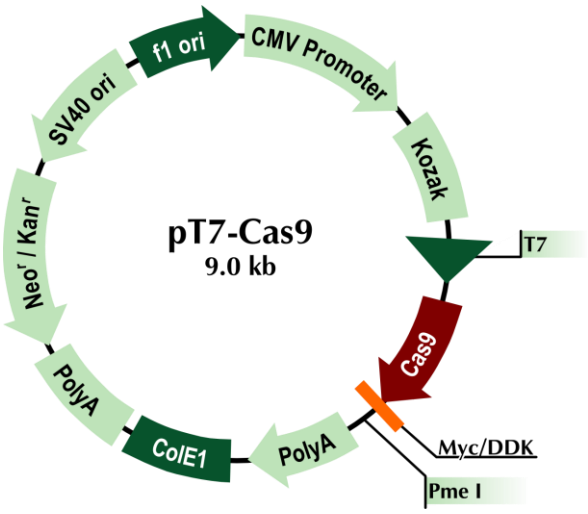
Product Description

To make gene knockout out animals, Cas9 mRNA and gRNA are often injected into the embryos; the T7 driven CRISP/Cas system will serve the purpose. pT7-Guide-IVT vector can be used to clone the target sequence; the gRNA expression is under T7 promoter. gRNA can be produced using the T7 *in vitro* transcription system. In pT7-Cas9 vector, Cas9 gene is under T7 promoter; Cas9 mRNA can be produced using the T7 *in vitro* transcription system.

Figure 10. Plasmid maps of pT7-Guide-IVT and pT7-Cas9



Only **BSmB I sites** are used to clone the target sequence into pT7-Guide-IVT (different from other pCas-Guide vectors)



Experimental protocol

I. Design genomic target sequence and cloning into pT7-Guide-IVT vector

1. Digest pT7-Guide-IVT vector with BsmB I restriction enzyme

Resuspend the 10 µg lyophilized DNA in 100 µL dH2O, and incubate for at least 30 min before use. Set up a digestion reaction as described below.

Component	Volume
10X restriction buffer	3 µL
BsmB I*	0.8 µL
Nuclease free water	16.2 µL
Vector DNA	10 µL
Total volume	30 µL

Incubate the reaction at 37°C for 3 hrs (the isoschizomer ESP3 I from Thermo Scientific can be used. Purify the desired vector fragment by running the digestion reaction on an agarose gel, and isolate the appropriate band using a gel purification column. Elute the digested plasmid vector in 40 µL of 10 mM Tris buffer.

Note: Please do not dephosphorylate the vector as oligos are not phosphorylated.

2. Target sequence designing and cloning.

OriGene has developed a proprietary Cas9 guide RNA designing tool which is free to use, http://www.blueheronbio.com/. Design a target sequence of 20 bp,

To facilitate cloning of the 20-bp target sequence, extra bases need to be added to the ends.

1). Select a desired 20-bp sequence as a target. The following is an example sequence:

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Forward sequence: 5' ATGGGAGGTGGTATGGGAGG 3'
Reverse complement sequence: 5' CCTCCCATACCACCTCCCAT 3'

Add 'atagG' to the 5' end of the forward sequence and 'G' to its 3' end.
The final sense oligo in this example will be
5' atagG ATGGGAGGTGGTATGGGAGGg 3'

2). Add 'aaaac' to the 5' end of reverse complementary sequence and 'C' to its 3' end.

The final reverse example complementary oligo:
5' aaaacCCTCCCATACCACCTCCCATc 3'

The two oligos should anneal to form the following double strand:

```
5'atagGxxxxxxxxxxxxxxxxxxxxG 3'
    3'CxxxxxxxxxxxxxxxxxxxxxxxCaaaa 5'
```

3). Order the two final oligos from a commercial oligo provider, such as IDT. The oligos are ready to be cloned into the BsmB I digested pT7-Guide-IVT vector.
Following the oligo cloning procedure on page 15-16 of this manual.

3. Sequencing the cloned target sequence in pT7-Guide-IVT can be done by the common M13 forward primer: 5' CGCCAGGGTTTTCCCAGTCACGAC 3'

II. Producing gRNA and Cas9 mRNA using T7 in vitro transcription kits

To make gRNA using pT7-Guide-IVT after the genomic target sequence cloned, we recommend using MeGAshortscript T7 kit (Life Technologies) and follow the manufacturer's protocol. pT7-Guide-IVT vector can be linearized using EcoR I. EcoR I will cut T7 and gRNA out; but you don't need to purify the fragment. You only need to clean it using a PCR purification column. Then follow the MeGAshortscript T7 kit protocol to produce gRNA. To make Cas9 mRNA using pT7-Cas9, we recommend using mMESSAGE Mmachine T7 ULTRA kit (Life Technologies) and follow the manufacturer's protocol. pT7-Cas9 vector can be linearized using Pme I which is at the 3' end of Cas9 sequence. You can then clean up the Pme I digested reaction using a PCR purification column; then follow the mMESSAGE Mmachine T7 ULTRA kit protocol to produce capped and polyadenylated Cas9 mRNA.

VII. Cre expression vector for Cre-Lox recombination, pCMV6-Entry Cre (SKU: GE100017)

Package contents

- One (1) vial of circular pCMV6-Entry-Cre (SKU: GE100017) plasmid DNA 10 μg, lyophilized. Reconstitute in 100 μL dH_2O to make a final concentration of 100 ng/ μL.
- Certificate of Analysis
- Application Guide available on line

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** OriGene plasmids are purified using ion-exchange columns for high-yield, low endotoxin preparations (PowerPrep® HP Midiprep Kit* https://www.origene.com/products/others/nucleic-acid-purification-kits)
The cDNA clone is shipped at room temperature, but should be kept at -20℃ for long-term storage. If properly stored, clones are guaranteed to be stable for 12 months.

Related Optional Reagents

- Nuclease free water
- Competent *E. coli* cells, https://www.origene.com/products/others/competent-cells
- LB agar plates with Kanamycin, 25 μg/mL
- LB broth (10 g/L Tryptone, 5 g/L Yeast Extract, 10 g/L NaCl. Adjust pH to 7.0 with 1 N NaOH)
- DNA purification reagents

Related OriGene Products

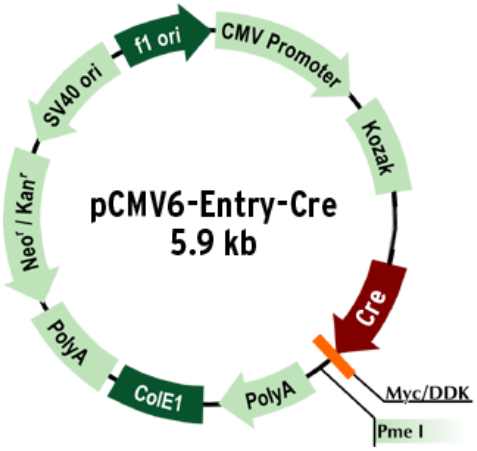
- Genome wide gene knockout/knockin kit via CRISPR, https://www.origene.com/products/gene-expression/crispr-cas9/knockout-kits
- Cloning the homologous arms in predesigned donor rescue construct harboring functional cassettes (service from https://www.origene.com/products/gene-expression/crispr-cas9/grna-cloning-donor-construction).

  GFP-PGK-Loxp-Puro-LoxP
  RFP-PGK-Loxp-BSD-LoxP
  Luciferase-PGK-Loxp-Puro-LoxP
  BFP-PGK-Loxp-Neo-Loxp

Product Description

In our predesigned donor vector cassette, the PGK-puro cassette is flanked by two LoxP sites, which can be floxed out by Cre recombinase. pCMV6-Entry-Cre is a Cre recombinase mammalian expression vector in which Cre is under CMV promoter. Expressed Cre contains a C-terminal Myc-DDK tag (DDK is the same as Flag tag).

Figure 11. Plasmid map of pCMV6-Entry-Cre



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VIII. Cas9 D10A nickase vectors, pCas-Guide-Nickase (SKU GE100019), pT7-Cas9-Nickase (SKU GE100020)

Package contents

- One (1) vial of circular nickase plasmid DNA, pCas-Guide-Nickase (SKU: GE100019) or pT7-Cas9-Nickase, 10 μg, lyophilized. Reconstitute in 100 µL dH_2O to make a final concentration of 100 ng/ µL.
- Certificate of Analysis
- Application Guide

Related Optional Reagents

- Nuclease free water
- Oligo annealing buffer, SKU GE100007
- BamH I, BsmB I
- T4 DNA ligase and buffer
- Competent E. coli cells
- LB agar plates with ampicillin, 100 μg/mL
- LB broth (10 g/L Tryptone, 5 g/L Yeast Extract, 10 g/L NaCl. Adjust pH to 7.0 with 1 N NaOH)
- DNA purification reagents

Related OriGene Products

- Cloning the homologous arms in predesigned donor rescue construct harboring functional cassettes.
  - GFP-PGK-Loxp-Puro-LoxP
  - RFP-PGK-Loxp-BSD-LoxP
  - Luciferase-PGK-Loxp-Puro-LoxP
  - mBFP-PGK-Loxp-Neo-Loxp

Product description

WT Cas9 has two active nuclease domains and it can produce double-stranded DNA breaks. D10A mutation in Cas9 disables one nuclease domain; therefore, Cas9D10A can only nick the targeted genome. pCas-Guide-Nickase (SKU GE100019) is in the same vector backbone as pCas-Guide (SKU GE100001 and GE100002) which is all-in-one vector, target sequence can be cloned and the vector express Cas9. pT7-Cas9D10A is in the same vector backbone as pT7-Cas9 (GE100014) which is used for *in vitro* production of Cas9 mRNA. Since Cas9D10A only nicks the genomic DNA, it needs two different gRNAs (one on sense strand and one on anti-sense strand) to cause double-stranded break. The off-target problem is significantly decreased as Cas9D10A needs two gRNAs.

Experimental protocol

The experimental protocol of GE100019 will be similar to pCas-Guide, page 12 on this manual. The experimental protocol of GE100019 will be similar to pT7-Cas9, page 24 on this manual. Since Cas9D10A needs two different gRNAs, you will need to validate the cleavage efficiency of each gRNA using WT Cas9 before using them together with Cas9D10A vectors.

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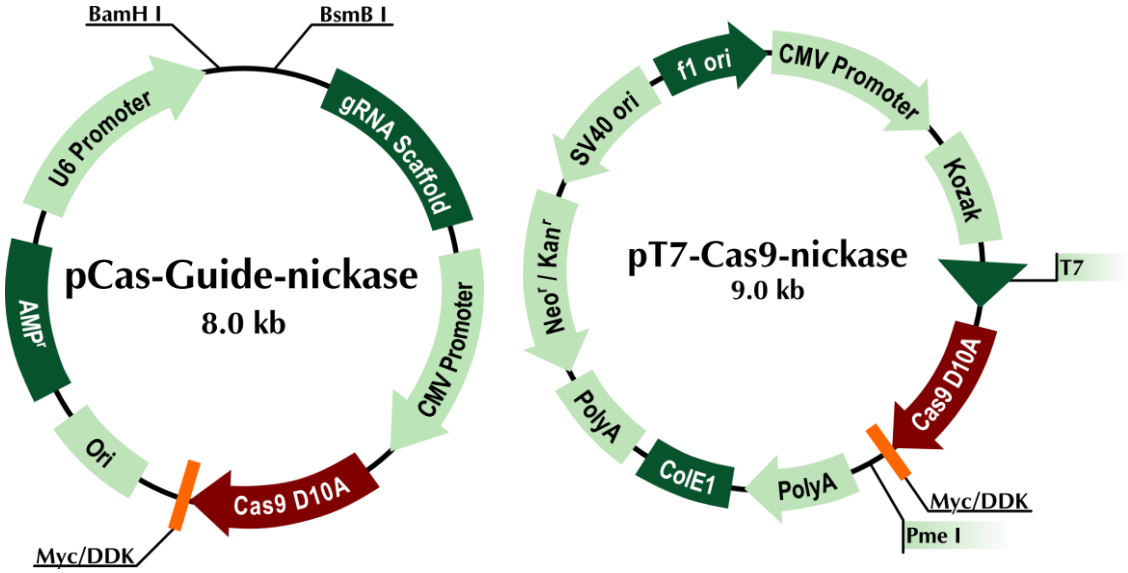
Figure 12. Plasmid maps of pCas-Guide-Nickase and pT7-Cas9-Nickase



IX. CRISPR scramble controls, pCas-Scramble (SKU GE100003) and pCas-Scramble-EF1A-GFP (SKU GE100021)

Package contents

- One (1) vial of circular plasmid DNA, pCas-Scramble (SKU: GE100003) or pCas-Scramble-EF1a-GFP (SKU GE100021), 10 μg, lyophilized. Reconstitute in 100 μL dH_2O to make a final concentration of 100 ng/ μL.
- Certificate of Analysis
- Application Guide

Product description

Using CRISPR technology for genome editing, you need a negative scrambled control, a 20bp scrambled sequence cloned in CRISPR vectors. After transfecting the CRISPR scrambled control into cells, a guide RNA containing the scrambled sequence (which does not target any sequence) will be produced.

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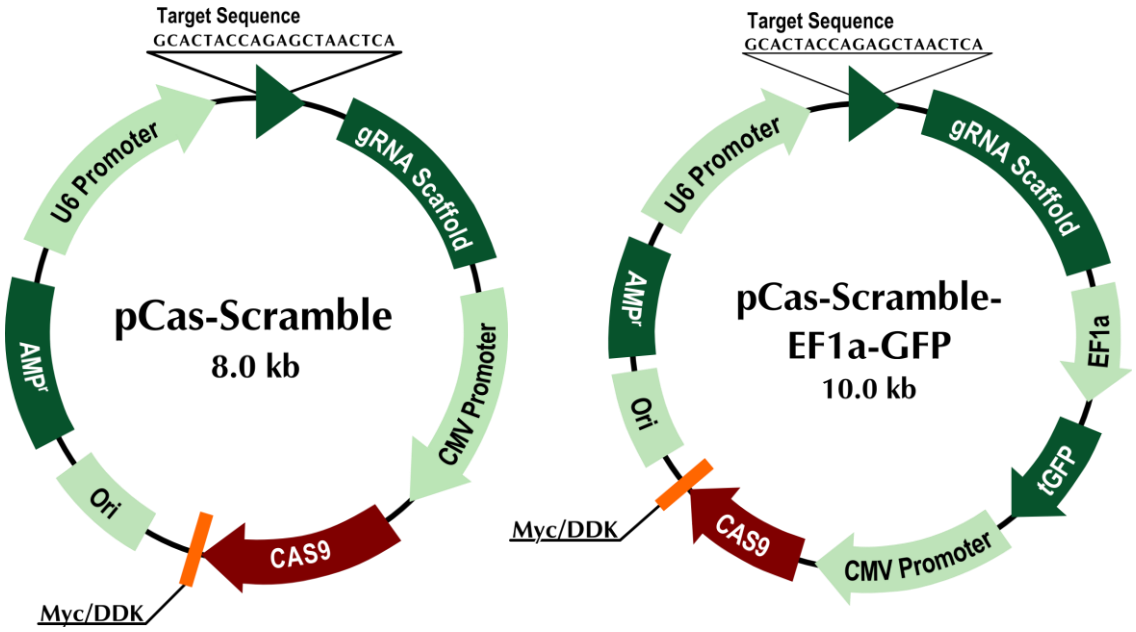
Figure 13. Plasmid maps of pCas-Scramble and pCas-Scramble-EF1a-GFP



X. Transgene Knockin via CRISPR at AAVS1 and ROSA26 Loci

Introduction

Many research projects require inserting a full-length cDNA expression cassette into a host genome for stable expression of the encoded protein. In such studies, the integration site of the transgene is very critical. An inserted gene should have the least negative effect to the host cells and the transgene should be genetically stable. Transgene expression via random integration into the genome is subjected to position effects and silencing. In addition, random gene insertion might interrupt or activate the neighboring genes. Genomic safe harbor sites are transcriptionally active and transgene insertion does not have adverse effect on the host cell genome. Adeno-associated virus (AAV) integration site (AAVS1) on human chromosome 19 has been accepted as a high gene expression and safe genomic location; for Mouse cells, ROSA26 is proved to be a genomic safe harbor.

OriGene has the largest full length cDNA collection, Human and Mouse. Development of a high efficiency transgene integration kit can facilitate the use of the cDNA collection. Taking advantage of recently discovered CRISPR technology, we developed AAVS1 and ROSA26 safe harbor knockin systems for transgene integration. The AAVS1 or ROSA26 targeting sequence is cloned in the CRISPR all-in-one vector, pCas-Guide; this gRNA/CRISPR vector will generate double stranded genomic break at AAVS1 or ROSA26 locus. The donor vector contains the AAVS1 or ROSA26 homologous arms with the gene of interest in between will be integrated at AAVS1 or ROSA26 site via homologous recombination.

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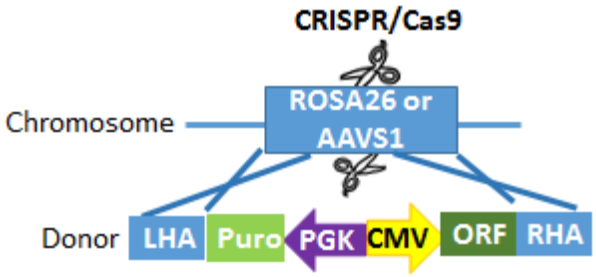
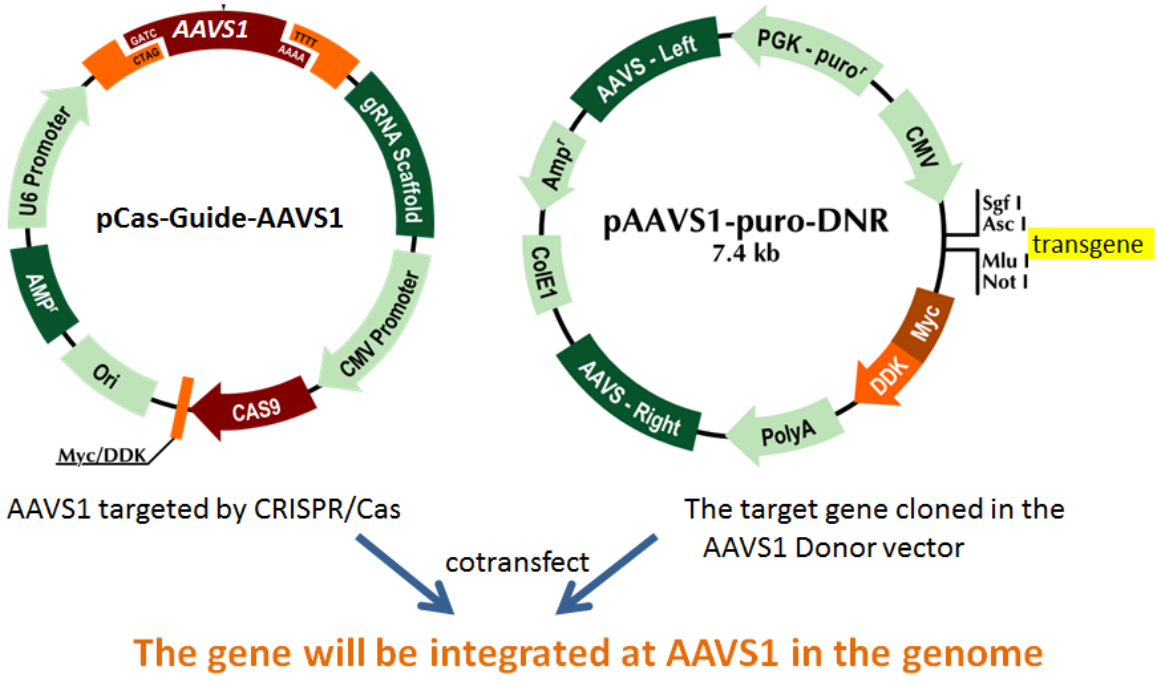
Figure 14. Diagram of transgene insertion at AAVS1 or ROSA26 via CRISPR



AAVS1 Transgene knockin via CRISPR

Figure 15. Diagram of how AAVS1 targeted insertion via CRISPR works.



AAVS1 CRISPR/gRNA vector, pCas-Guide-AAVS1 (SKU GE100023)

Package contents

- One (1) vial of pCas-Guide-AAVS1 (SKU: GE100023) plasmid DNA 10 μg, lyophilized. Reconstitute in 100 μL dH_2O to make a final concentration of 100 ng/ μL.
- Certificate of Analysis

** OriGene plasmids are purified using ion-exchange columns for high-yield, low endotoxin preparations (PowerPrep® HP Midiprep Kit)*
The cDNA clone is shipped at room temperature, but should be kept at -20℃ for long-term storage. If properly stored, clones are guaranteed to be stable for 12 months.

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Related Optional Reagents

- Competent E. coli cells
- LB agar plates with ampicillin, 100 μg/mL
- LB broth (10 g/L Tryptone, 5 g/L Yeast Extract, 10 g/L NaCl. Adjust pH to 7.0 with 1 N NaOH)
- DNA purification reagents

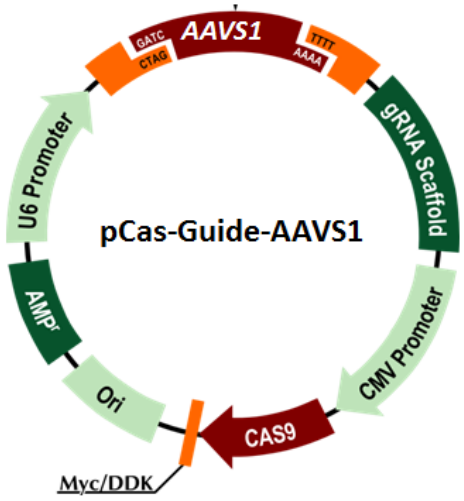
Related OriGene Products

- pAAVS1-puro-DNR (SKU GE100024)
- pAAVS1-BSD-DNR (SKU GE100035)
- pAAVS1-RFP-DNR (SKU GE100026)
- pAAVS1-Cas9-Puro-DNR (SKU GE100037)
- pAAVS1-Cas9-BSD-DNR (SKU GE100039)
- AAVS1 Transgene knockin vector kit (puro) (SKU GE100027)

Product Description

pCas-Guide-AAVS1: a plasmid DNA with Cas9 expression and gRNA targeting AAVS1 site. This all-in-one vector will generate a double strand break in human host cells at AAVS1 locus.

Figure 16. Vector map of pCas-Guide-AAVS1.



AAVS1 donor vectors

SKU	Vector Name	Promoter	Cell Selection
GE100024	pAAVS1-Puro-DNR	CMV	Puromycin
GE100035	pAAVS1-BSD-DNR	CMV	Blasticidin
GE100046	pAAVS1-EF1a-Puro-DNR	EF1a	Puromycin

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GE100048	pAAVS1-EF1a-BSD-DNR	EF1a	Blasticidin

Package contents

- One (1) vial of corresponding donor vector, plasmid DNA 10 μg, lyophilized. Reconstitute in 100 µL dH_2O to make a final concentration of 100 ng/ µL.
- One (1) Vial of dried 5' primer (100 picomoles), reconstitute in 10 µL dH_2O to make a 10 µM solution. VP1.5 for GE100024 and GE100035. EF51 for GE100046 and GE100047.
- One (1) Vial of dried 3' (XL39) primer (100 picomoles), reconstitute in 10 µL dH_2O to make a 10 µM solution.
- Certificate of Analysis

** OriGene plasmids are purified using ion-exchange columns for high-yield, low endotoxin preparations (*PowerPrep® HP Midiprep Kit*)*
The cDNA clone is shipped at room temperature, but should be kept at -20℃ for long-term storage. If properly stored, clones are guaranteed to be stable for 12 months.

Related Optional Reagents

- Competent E. coli cells
- LB agar plates with ampicillin, 100 μg/mL
- LB broth (10 g/L Tryptone, 5 g/L Yeast Extract, 10 g/L NaCl. Adjust pH to 7.0 with 1 N NaOH)
- DNA purification reagents

Related OriGene Products

- pCas-Guide-AAVS1 (SKU GE100023)
- pCas-Guide-scramble (SKU GE100003)
- pAAVS1-RFP-DNR (SKU GE100026)
- AAVS1 Transgene knockin vector kit (puro) (SKU GE100027)
- AAVS1 transgene knockin vector kit (BSD) (SKU GE100036)
- AAVS1 Transgene knockin vector kit (EF1a-puro) (SKU GE100046)
- AAVS1 transgene knockin vector kit (EF1a-BSD) (SKU GE100048)

Product Description

AAVS1 donor vectors contain multiple cloning site to clone your gene of interest. The plasmid has AAVS1 left homologous arm and right homologous arm for homologous repair to integrate the transgene cassette into AAVS1 locus. Flanked by the two arms, there is a CMV or EF1a promoter driven expression cassette for a transgene expression and a PGK driven puromycin or blasticidin resistant gene for mammalian selection (Fig. 16). A multiple cloning site downstream of the CMV/EF1a promoter is designed to be compatible to OriGene's precision shuttle vector system so that the ORF clones can be easily shuttled to this integration vector by simplely "cut and ligate".

Note: The AAVS1donor vectors don't contain your gene of interest; the gene of interest needs to be cloned.

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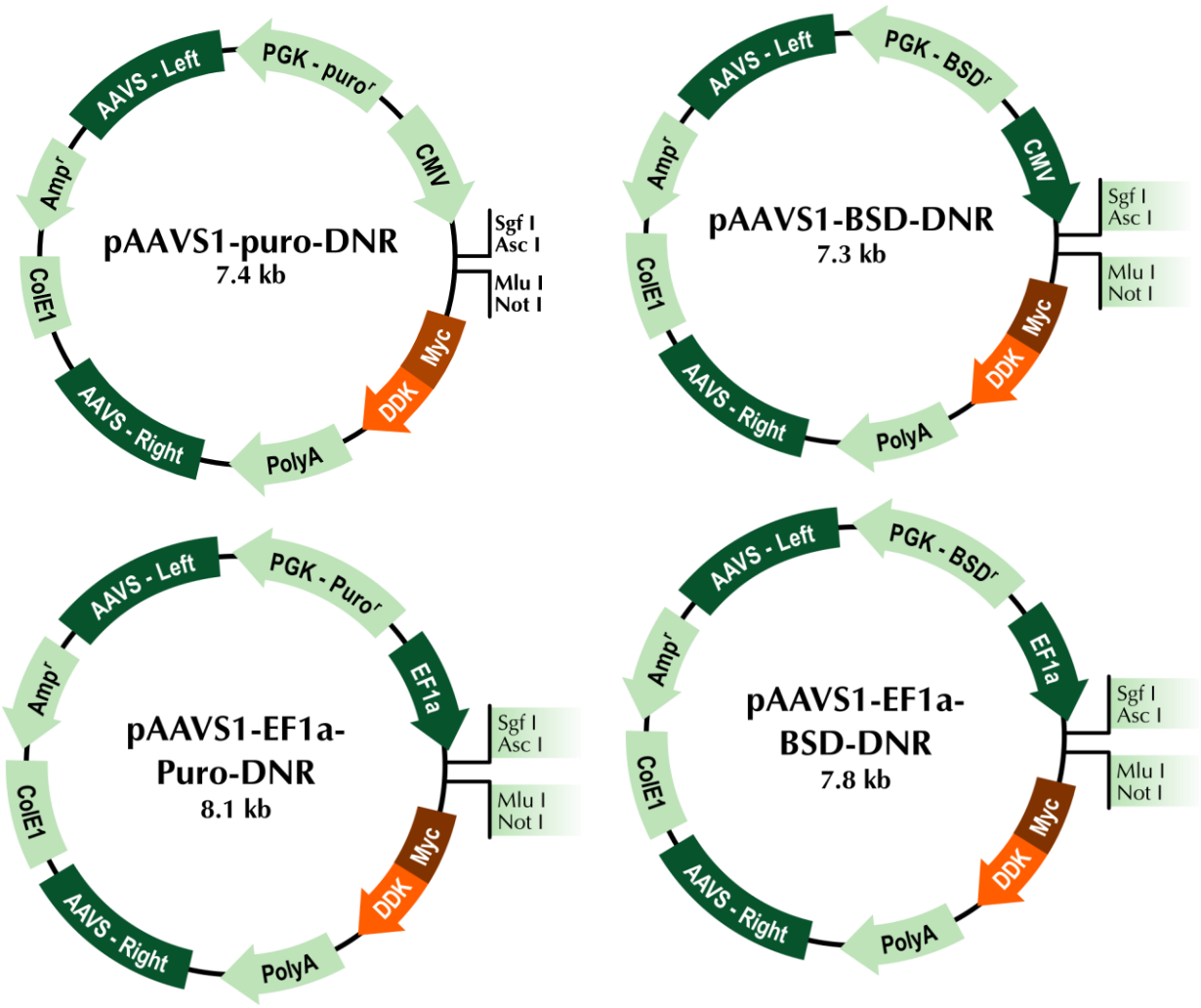
Figure 17. Vector maps of AAVS1 donor vectors



MCS of pAAVS1-Puro-DNR

Kozac Consensus

EcoR I *Sal I* *Kpn I* RBS *Sgf I* *Asc I*

```
CTATAGGGCGGCCGGGAATTCGTCGACTGGATCCGGTACCGAGGAGATCTGCCGCCGCGATCGCCGGCGCGCCAGATCT
```

Mlu I *Not I* Myc.Tag

```
CAAGCTTAACTAGCTAGCGGACCG ACG CGT ACG CGG CCG CTC GAG CAG AAA CTC ATC TCA GAA GAG
                         T   R   T   R   P   L   E   Q   K   L   I   S   E   E
```

DDK.Tag *Fse I*

```
GAT CTG GCA GCA AAT GAT ATC CTG GAT TAC AAG GAT GAC GAC GAT AAG GTT TAA ACGGCCGGCC
D   L   A   A   N   D   I   L   D   Y   K   D   D   D   D   K   V  Stop
```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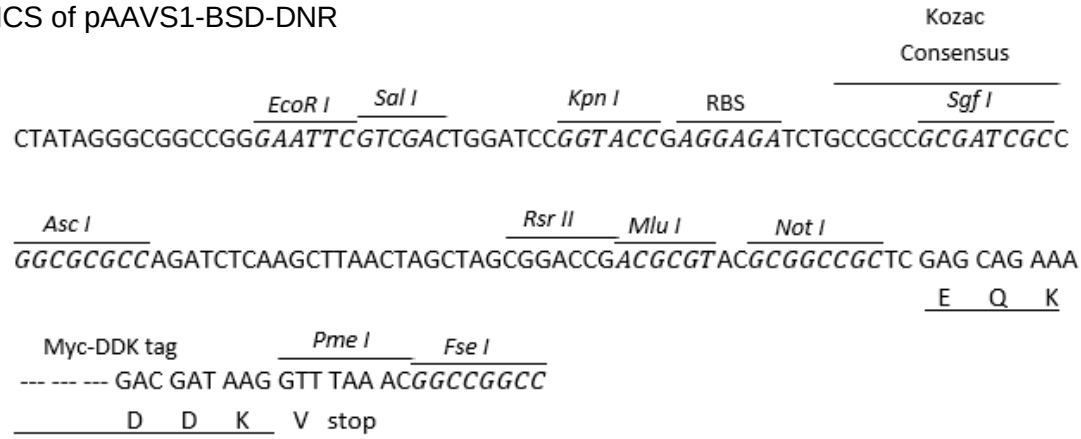
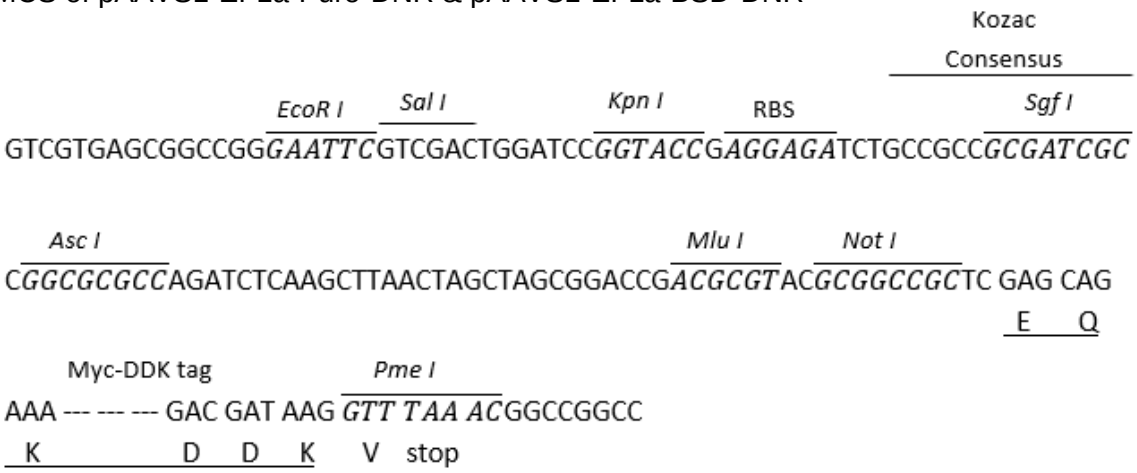
MCS of pAAVS1-BSD-DNR



MCS of pAAVS1-EF1a-Puro-DNR & pAAVS1-EF1a-BSD-DNR



Cloning a transgene to AAVS1 donor vectors

To clone an ORF insert to the AAVS1 donor vectors, the first step is to select which pair of enzymes. There are a few rare restriction enzymatic sites designed in the multiple cloning site region and c-terminal Myc-DDK tag. Four pairs of restriction enzymes can be used for cloning: SgfI/MluI, Asc/MluI, Sgf1/Not1 and Asc/NotI. Among them, SgfI/MluI is suitable for over 95% ORF insert. If the ORF inserts are from OriGene's TrueORF clones, they can be easily shuttled from pCMV6-Entry vector to the AAVS1 donor vectors using the above enzyme pairs; the insert will be in frame with c-terminal Myc-DDK tags. In case of none of the enzyme pair can be used, a single enzymatic site can be used for cloning. In such case, screening an insert with the right orientation is needed. If you want to express a native protein without a tag, a termination codon must be introduced at the 3' end of the ORF insert which can be achieved by PCR or site-direct mutagenesis. The following protocol is for shuttling an ORF insert to the donor vectors using Sgf1/MluI enzymatic pair.

Digest an ORF insert from TrueORF clone in pCMV6-Entry vector (cat# RC2xxxxxx)

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Component	Volume
10X restriction buffer	2 µl
Sgf I (10 U/µl)	0.6 µl
Mlu I (10 U/µl)	0.6 µl
nuclease-free water	13.8 µl
TrueORF clone (500 ng)	3 µl
Total volume	20 µl

Incubate at 37°C for 3 hrs.

2. Digest AAVS1 or ROSA26 donor vectors:

Component	Volume
10X restriction buffer	2 µl
Sgf I (10 U/ µl)	0.6 µl
Mlu I (10 U/ µl)	0.6 µl
nuclease-free water	14.8 µl
AAVS1 or ROSA26 donor vector (200ng)	2 µl
Total volume	20 µl

* For the 4% of the clones that have internal Sgf I or Mlu I sites, please use the appropriate combination of restriction sites as recommended by OriGene.
Incubate at 37°C for 3 hrs. Add 0.5 µl antarctic phosphatase (units used according to the manufacturer's protocol) to the digestion, and continue to incubate at 37°C for an additional 30 minutes.

3. Purify the digestion using a commercial PCR purification column and elute in 20 µl 10 mM Tris.

4. Set up a ligation reaction:

Component	Volume
10 x T4 DNA ligation buffer	1 µl
T4 DNA Ligase (4U/µl)	0.75 µl
nuclease-free water	3.25 µl
digested ORF insert (step 1)	2 µl
digested vector (Step 2)	3 µl
Total volume	10 µl

Incubate the ligation reaction at room temperature for 1 hour.

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5. Transform the ligation reaction using high-efficiency competent *E. coli* cells (≥ 1×10^8 CFU/µg DNA) following the appropriate transformation protocol. Plate the transformants on LB-agar plates supplemented with 100 µg/ml ampicillin.

6. Pick at least four colonies for subsequent DNA purification and screening. Amplify and purify the selected clone(s) by growing overnight in liquid LB containing the corresponding antibiotics (100 µg/ml ampicillin), then isolating the DNA using standard plasmid purification procedures.

Confirm the insert by restriction digestion and/or vector primer sequencing using the provided V1.5 for 5' end sequencing and XL39 for 3' end.

AAVS1 Transgene knockin vector kit, puro (SKU GE100027, GE100047) or BSD (GE100036, GE100049)

Package Contents

1. One (1) vial pCas-Guide-AAVS1 (SKU GE100023), targeting AAVS1 site, 10 µg, lyophilized. Reconstitute in 100 µL dH_2O to make a final concentration of 100 ng/ µL*.
2. One (1) vial containing pCas-Guide-scramble (SKU GE100003) negative control, 10 µg, lyophilized. Reconstitute in 100 µL dH_2O to make a final concentration of 100 ng/ µL*.
3. One (1) vial containing one of the AAVS1 donor vectors, 10 µg, lyophilized. Reconstitute in 100 µL dH_2O to make a final concentration of 100 ng/ µL*.
4. Forward and reverse (XL39) sequencing primers, 100pmols each, lyophilized. VP1.5 forward primer for GE100027 and GE100036; EF51 forward primer for GE100047 and GE100049. Reconstitute each in 10 µL dH_2O to make a 10 µM solution. *Primers are for donor vectors only, not for pCas-Guide-AAVS1 or pCas-Guide-scramble.*
5. Certificate of Analysis

** OriGene plasmids are purified using ion-exchange columns for high-yield, low endotoxin preparations (*PowerPrep® HP Midiprep Kit).
The cDNA clone is shipped at room temperature, but should be kept at -20^{0}C for long-term storage. If properly stored, clones are guaranteed to be stable for 12 months.

Related Optional Reagents

- Nuclease free water
- SgfI and Mlu I
- T4 DNA ligase and buffer
- Competent E. coli cells
- LB agar plates with ampicillin, 100 µg/mL
- LB broth (10 g/L Tryptone, 5 g/L Yeast Extract, 10 g/L NaCl. Adjust pH to 7.0 with 1 N NaOH)
- DNA purification reagents

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Related OriGene Products

- pAAVS1-RFP-DNR (SKU GE100026)
- Genome-wide ORF clones:
- Transfection reagent
- CRISPR/Cas9 products
- Reagents and supplies for immunoblots: user preferred. OriGene has a selection of antibodies and detection reagents.
- DNA purification reagents

Notice to purchaser

This product is for research use only. Use in and/or for diagnostics and therapeutics is strictly prohibited. By opening and using the product, the purchaser agrees to the following: The plasmids may not be distributed, resold, modified for resale or used to manufacture commercial products without prior written approval from OriGene Technologies, Inc. If you do not agree to the above conditions, please return the UNOPENED product to OriGene Technologies, Inc. within ten (10) days of receipt for a full refund.

Product description

The AAVS1 transgene knockin vector kits are complete vector kits to knock in your gene of interest in AAVS1 locus in human genome for robust and stable expression.

The kit contains the following three plasmids:

1. pCas-Guide-AAVS1: a plasmid DNA with Cas9 expression and gRNA targeting AAVS1 site (Fig. 15). This two-in-one vector will generate a double strand breakage in the human host cells at AAVS1 locus.
2. pCas-Guide-Scrambled control: the negative gRNA control containing a non-specific gRNA sequence in pCas-Guide vector.
3. AAVS1 donor vector: An AAVS1 repair donor vector containing multiple cloning site to clone your gene of interest. The plasmid has AAVS1 left homologous arm and right homologous arm for homologous repair to integrate the transgene cassette into AAVS1 locus. Flanked by the two arms, there is a CMV or EF1a promoter driven expression cassette for a transgene expression and a PGK driven puromycin or blasticidin resistant gene for transgene integration selection (Fig. 16). A mutiple cloning site downstream of the promoter is designed to be compatible to Origene's precision shuttle vector system so that the ORF clones can be easily shuttled to this integration vector by simplely "cut and ligate".

Note: AAVS1 donor vectors don't contain your gene of interest, it needs to be cloned.

Experimental Protocol

1. Clone your gene into the AAVS1 donor vector.
2. Follow the protocols on page: 49-52 in this manual, where pAAVS1-RFP-DNR is used. In your experiment, you can replace your pAAVS1-GOI-DNR with pAAVS1-RFP-DNR.

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pAAVS1-RFP-DNR (SKU GE100026), positive control

Package contents

- One (1) vial of pAAVS1-RFP-DNR (SKU: GE100026) plasmid DNA 10 μg, lyophilized. Reconstitute in 100 μL dH_2O to make a final concentration of 100 ng/ μL.
- Certificate of Analysis

** OriGene plasmids are purified using ion-exchange columns for high-yield, low endotoxin preparations (*PowerPrep® HP Midiprep Kit*)*
The cDNA clone is shipped at room temperature, but should be kept at -20℃ for long-term storage. If properly stored, clones are guaranteed to be stable for 12 months.

Related Optional Reagents

- Competent E. coli cells
- LB agar plates with ampicillin, 100 μg/mL
- LB broth (10 g/L Tryptone, 5 g/L Yeast Extract, 10 g/L NaCl. Adjust pH to 7.0 with 1 N NaOH)
- DNA purification reagents

Related OriGene Products

- pCas-Guide-AAVS1 (SKU GE100023)
- pCas-Guide-scramble (SKU GE100003)
- pAAVS1-puro-DNR (SKU GE100024)
- AAVS1 Transgene knockin vector kit (puro) (SKU GE100027)
- AAVS1 Transgene knockin vector kit (BSD) (SKU GE100036)
- pAAVS1-Cas9-Puro-DNR (SKU GE100037)
- pAAVS1-Cas9-BSD-DNR (SKU GE100039)

Product Description

pAAVS1-RFP-DNR is a positive donor vector with RFP cloned in pAAVS1-puro-DNR vector. This vector can be used as a positive control for CRISPR knockin system when combined with pCas-Guide-AAVS1. pCas-Guide-AAVS1 generate double strand break at AAVS1 site; then pAAVS1-RFP-DNR provides repair template for homologous recombination. The result is RFP-puro expression cassette is inserted in AAVS1 locus.

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Figure 18. Vector map of pAAVS1-RFP-DNR

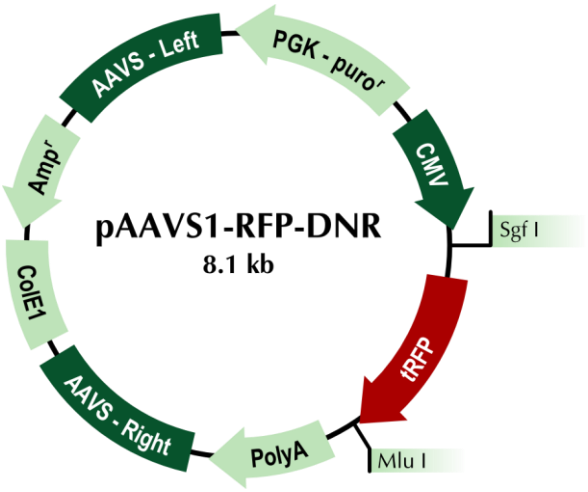
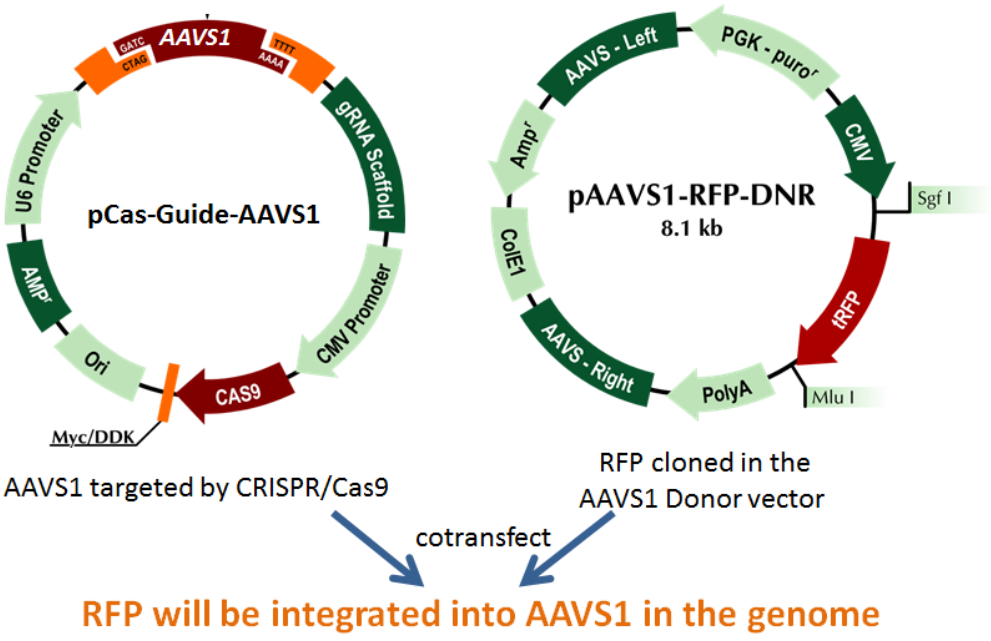


Figure 19. Diagram of CRISPR positive control to knockin RFP at AAVS1 site



Experimental Protocol

This protocol is to knockin RFP expression cassette into AAVS1 locus using pAAVS1-RFP-DNR and pCas-Guide-AAVS1; pCas-Guide-scramble is used as a negative control.
A sample protocol listed below is for experiments performed in 6-well plates and using TurboFectin (cat# TF81001) as transfection reagent. If your experiments require a different size of culture plates, just scale up or down the reagents accordingly based on the relative surface area of your plate (Table 1). Different type of cells may need a different transfection reagent;

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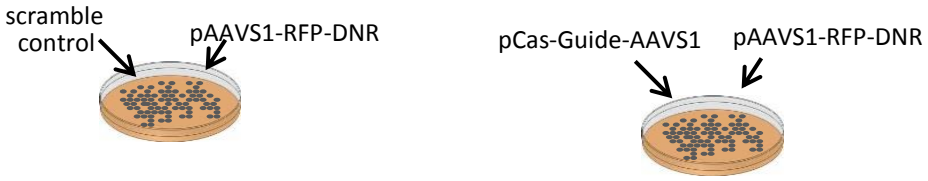
please follow the manufacturer's corresponding protocol. OriGene just launched Virus-like Viromers which are best for difficult-to-transfect cells.

1. Approximately 18-24 hours before transfection, plate ~3 X 10^5 adherent cells in 2 ml culture media into each well of a 6-well plate or ~5x10^5 suspension cells per well to obtain 50-70% confluence on the following day. The number of cells varies depending on the size of your cells.

2. Transfection in complete culture media. Two separate transfections:



In a small sterile tube, combine the following reagents in the prescribed order. The order of reagent addition is important to achieve the optimal results.

   a. Dilute 1 µg of pCas-Guide-AAVS1 (or scramble control) in 250 uL of Opti-MEM I (Life Technologies), vortex gently. Then add 1 µg of the RFP-donor DNA into the same 250 µL of Opti-MEM I. Vortex gently.
   b. Add 6 µL of Turbofectin 8.0 to the diluted DNA (not the reverse order) and pipette gently to mix completely.
   c. Incubate the mixture 15 minutes at room temperature.

***Note**: We recommend starting with the ratios of Turbofectin 8.0 and DNA listed in table 1; however, subsequent optimization may further increase the transfection efficiency.*

Note: For your specific cell lines, please choose a transfection reagent that gives you high transfection efficiency and follow the manufacture's protocol.

3. Add the mixture above drop-wise to the cells plated in step 1 (no need to change the media). Gently rock the plate back-and-forth and side-to-side to distribute the complex evenly.

4. Incubate the cells in a 5% CO_2 incubator.

5. Passage cells around 3 weeks before puro selection or RFP sorting.
   48hr post transfection, split cells 1:10, grow additional 3 days; then split the cells again 1:10. Split cells 7 times in total. Since puromycin resistant gene in the donor vector contains PGK promoter, RFP is driven by CMV promoter, the plasmid donor DNA before genomic integration will also provide puromycin resistance and express RFP. The reason to passage cells for around 3 weeks before puromycin selection is to dilute out cells containing the donor as episomal form.

Timelines of genome editing

- ✓ CRISPR targeted gene knockout / knockin--- 1 week post transfection
- ✓ Episomal donor vector dilution with cell passaging--- 3 weeks post transfection

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Note 1. *Since stable cell selection takes time, you can try to analyze the cells at P2 to detect genomic integration using genomic PCR (Fig 2). When designing primers for genomic PCR, one primer should be outside of the homologous arm region in donor DNA and one primer is in the functional cassette. Please see details in Fig. 20. The amplified PCR fragment is around 1kb. There could be some difficulties doing genomic PCR at this step before selection due to the percentage of edited cells and difficulties of genomic PCR.*

6. Apply puromycin selection or RFP sorting. Since after around 3-week cell passaging, episomal donor DNA is most in most cells, you can use RFP to do cell sorting to enrich edited cells. Another way is to use puromycin selection. Split P5 or P7 cells 1:10, then grow cells directly in the puromycin containing complete media in 10 cm dishes (apply puro selection at P6 might work, but you might get more random donor integrated clones than P8). The dose of puromycin needs to be determined with a kill curve to find out the lowest dose that kills the non-transfected cells completely 4-7 days post selection; the range of puromycin is 1µg/ml to 10µg/ml). Change the media every 2-3 days.

   Note*: We recommend you keep growing or freeze some of the transfected cells without selection; just in case, you need to perform the puromycin selection again.*

7. The puromycin resistant cells are ready to be analyzed for genome editing.
   - Use microscope to observe RFP expression.
   - WB with anti-tRFP antibody (cat# TA150061) to detect RFP expression
   - Genomic PCR to verify the integration of the functional cassette, then directly sequence the amplified genomic fragment using the PCR primers to verify the integration in the genome.

   Note*: Scramble control and donor vector will also give you some puromycin resistant cells as donor vector alone can randomly integrate into the genome too; however, the efficiency should be a lot lower than with a specific gRNA. Therefore, you should get more colonies with gene specific gRNA than scramble control if the gene specific gRNA cleaves efficiently.*

8. Isolate individual cell colonies.
   Two main methods, limiting dilution and cloning rings / cylinder.

3) Limiting dilution
This method is better to be used after puromycin selection. Dilute cells to seed about 1-2 cells/well in 96-well plate, after 1-2 weeks, observe under the microscope and select the wells only containing one cell colony, then further expand them to 6-well plate when they are confluent in the 96-well plate and so on.

4) Cloning rings / cylinder
This method can be used in the same time with puro selection. Seed cells at lower density, such as 5% confluency in a larger cell culture dish, such as 10cm dish, apply puromycin selection when seeding.

Table 1. Recommended starting transfection conditions for Turbofectin 8

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Tissue Culture Vessel	Growth area, cm^2/well	μg of DNA	Ratio of Turbofectin: DNA
96-well plate	0.35	0.1-0.15	3:1
24-well plate	2	0.5-1	3:1
12-well plate	4	1-2.5	3:1
6-well plate	9.5	1-5	3:1
35 mm plate	8	1-5	3:1
60 mm plate	20	2-10	3:1
100 mm plate	60	5-15	3:1

Figure 3. Diagram of knockin process

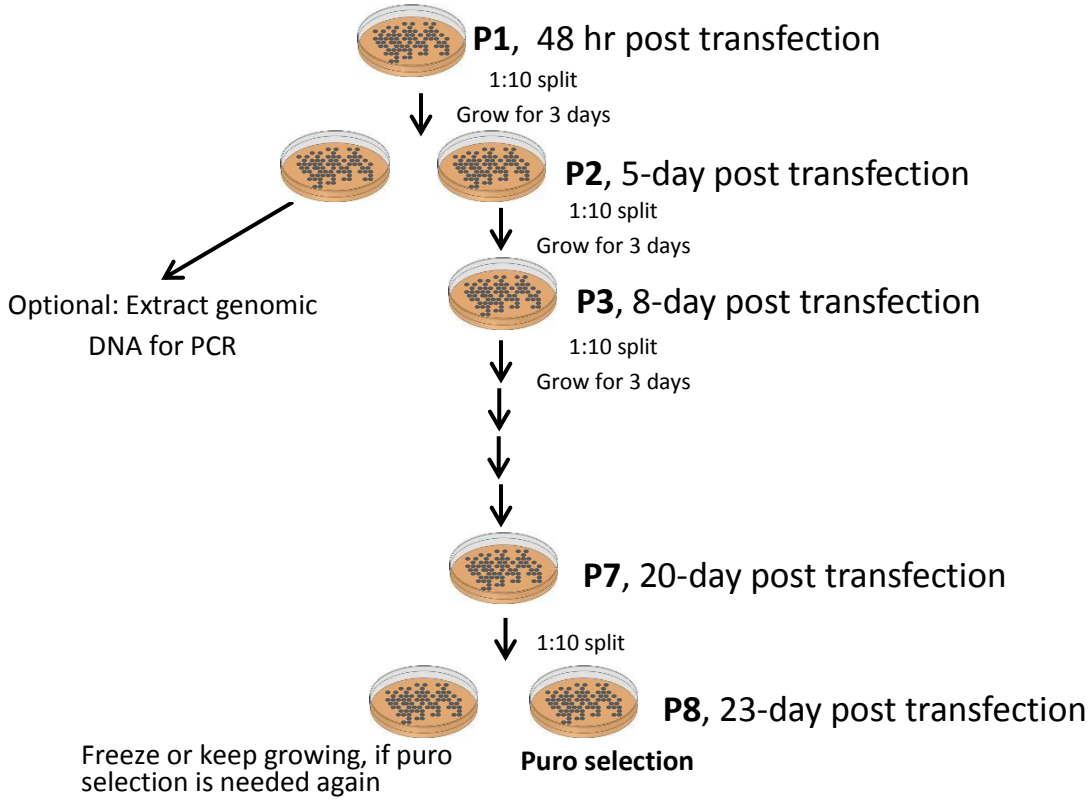

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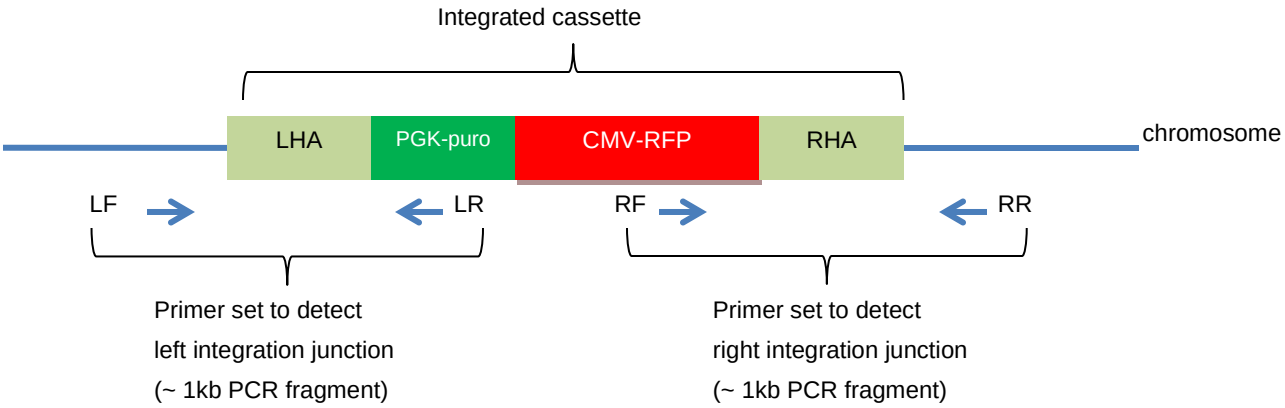
Fig. 4. Diagram of genomic PCR Primer design.



LF, LR: Forward and reverse PCR primer to amplify the left integration junction
RF, RR: Forward and reverse PCR primer to amplify the right integration junction

Cas9 insertion at AAVS1 locus

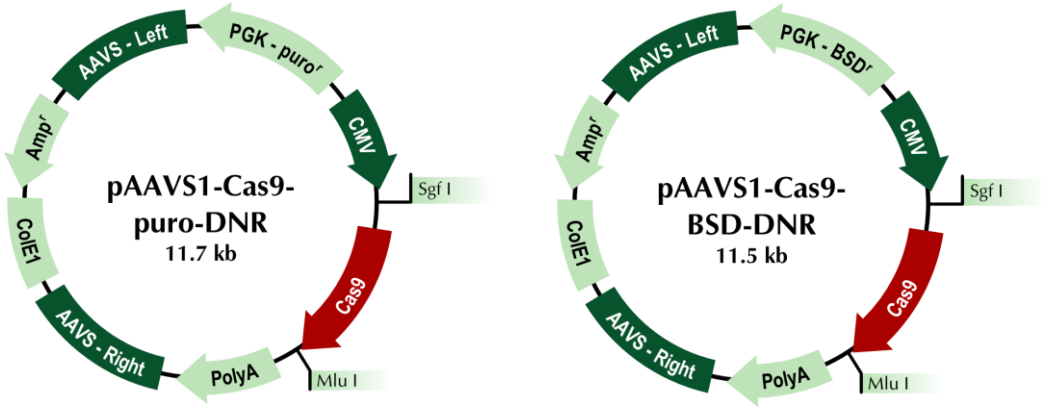
Product description

For genome editing projects, Cas9 stable cells are needed; to achieve robust and stable Cas9 expression, inserting Cas9 at AAVS1 locus in human cells is preferred. Adeno-associated virus integration site 1 (AAVS1) in human genome is a safe harbor for transgene integration. It is transcriptionally active and transgene expression from this site is robust and stable.

With AAVS1 gRNA targeting construct (GE100023, page 31 of this manual) and AAVS1-Cas9 donor vectors, Cas9 can be inserted at AAVS1 locus. AAVS1 gRNA targeting construct will lead to sequence specific double-strand break at AAVS1 locus in human cells, the AAVS1 homologous sequences in the donor vectors will lead to Cas9-selection cassette being integrated into AAVS1 site vis homologous recombination.

Cas9 AAVS1 donor vectors

Figure 20. Plasmid maps of pAAVS1-Cas9-Puro-DNR & pAAVS1-Cas9-BSD-DNR



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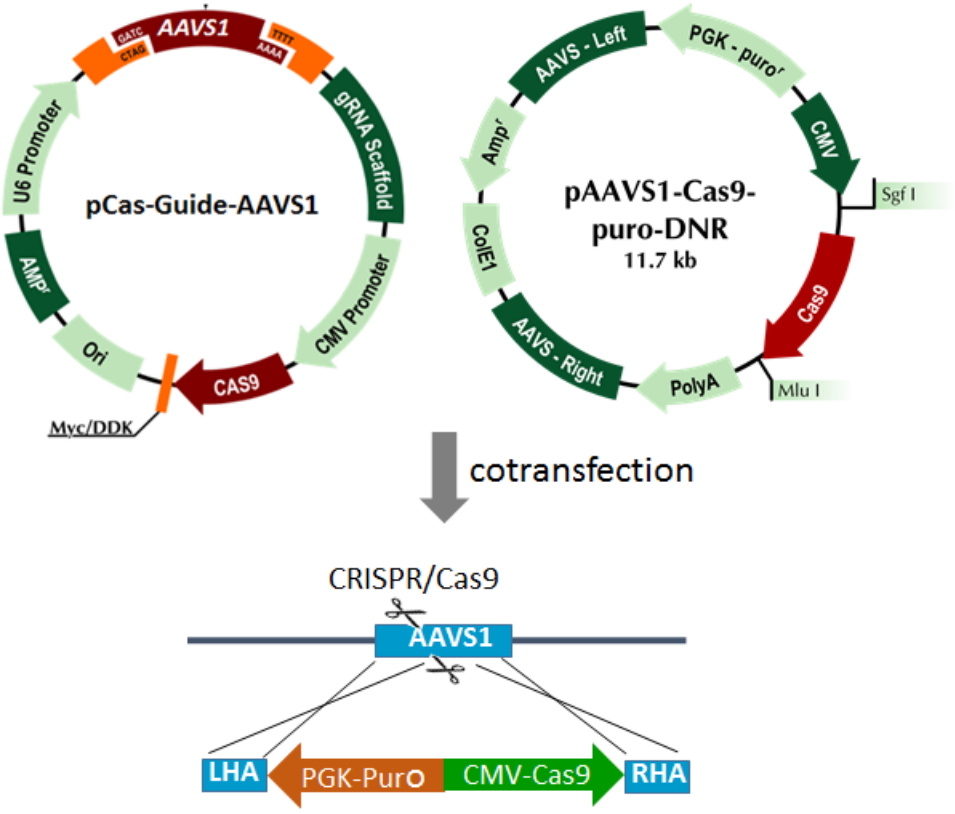
AAVS1 Cas9 insertion vector kits, Puro (SKU GE100038) and BSD (SKU GE100040)

Package Contents:

1. One (1) vial of pCas-Guide-AAVS1 (SKU GE100023), 10 μg, lyophilized. Reconstitute in 100 μL dH_2O to make a final concentration of 100 ng/ μL*.
2. One (1) vial of pCas-Guide-scramble (cat# GE100003) negative control, 10 μg, lyophilized. Reconstitute in 100 μL dH_2O to make a final concentration of 100 ng/ μL*.
3. One (1) vial of pAAVS1-Cas9-Puro-DNR (SKU GE100037) or pAAVS1-Cas9-BSD-DNR (SKU GE100039), 10 μg, lyophilized. Reconstitute in 100 μL dH_2O to make a final concentration of 100 ng/ μL*.
4. Certificate of Analysis

Figure 21. Diagram of how Cas9 is inserted at AAVS1 locus in human cells



Experimental Protocol

Inserting Cas9 into AAVS1 site is the same as inserting RFP into AAVS1 site. Please follow the protocols on page 49-52 in this manual, replacing pAAVS1-RFP-DNR with the corresponding Cas9 AAVS1 donor.

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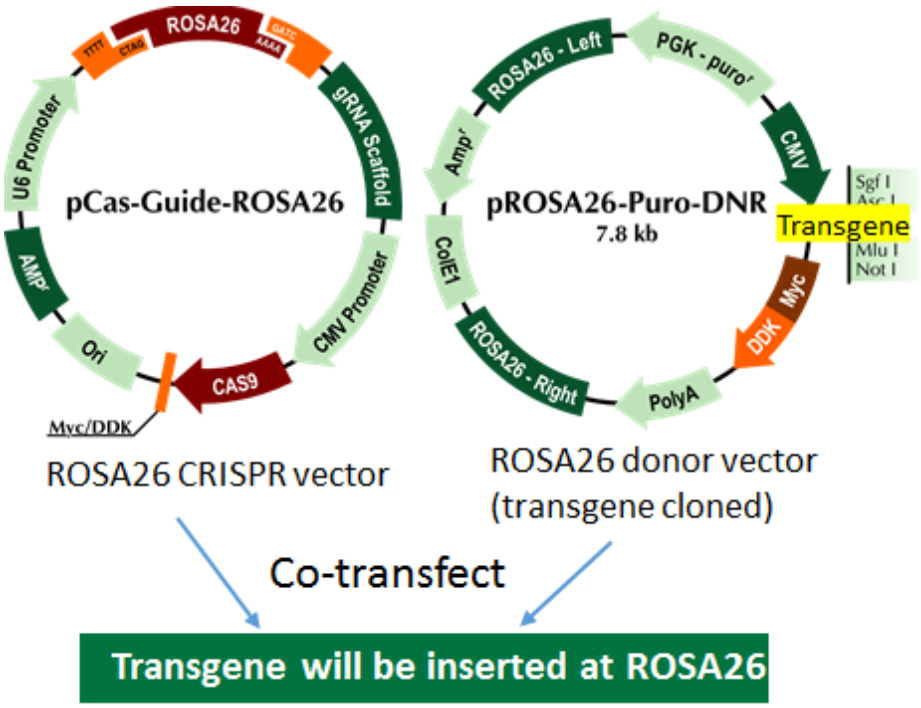
ROSA26 Transgene knockin via CRISPR

Figure 22. Diagram of ROSA26 targeted insertion via CRISPR.



ROSA26 gRNA/CRISPR construct, pCas-Guide-ROSA26 (SKU GE100050)

Package contents

- One (1) vial of pCas-Guide-ROSA26 (SKU: GE100050) plasmid DNA 10 μg, lyophilized. Reconstitute in 100 μL dH_2O to make a final concentration of 100 ng/ μL.
- Certificate of Analysis

** OriGene plasmids are purified using ion-exchange columns for high-yield, low endotoxin preparations (PowerPrep® HP Midiprep Kit). The cDNA clone is shipped at room temperature, but should be kept at -20℃ for long-term storage. If properly stored, clones are guaranteed to be stable for 12 months.*

Related Optional Reagents

- Competent E. coli cells
- LB agar plates with ampicillin, 100 μg/mL
- LB broth (10 g/L Tryptone, 5 g/L Yeast Extract, 10 g/L NaCl. Adjust pH to 7.0 with 1 N NaOH)
- DNA purification reagents

Related OriGene Products

- pROSA26-Puro-DNR (GE100051)

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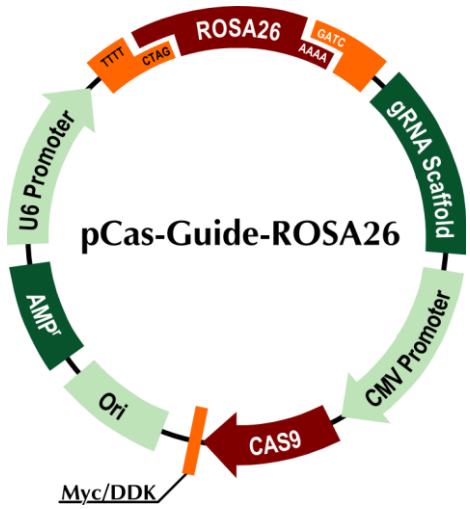
- ROSA26 transgene knockin vector kit (puro) (SKU GE100052)

Product Description

pCas-Guide-ROSA26: a plasmid DNA with Cas9 expression and gRNA targeting ROSA26 site. This all-in-one vector will generate a double strand break in human host cells at ROSA26 locus.

Figure 23. Vector map of pCas-Guide-ROSA26.



ROSA26 donor vector, pROSA26-Puro-DNR (SKU GE100051),

Package contents

- One (1) vial of pROSA26-Puro-DNR plasmid DNA 10 μg, lyophilized. Reconstitute in 100 μL dH_2O to make a final concentration of 100 ng/ μL.
- One (1) Vial of dried 5' (VP1.5) primer (100 picomoles), reconstitute in 10 μL dH_2O to make a 10 μM solution.
- One (1) Vial of dried 3' (XL39) primer (100 picomoles), reconstitute in 10 μL dH_2O to make a 10 μM solution.
- Certificate of Analysis

** OriGene plasmids are purified using ion-exchange columns for high-yield, low endotoxin preparations (PowerPrep® HP Midiprep Kit). The cDNA clone is shipped at room temperature, but should be kept at -20℃ for long-term storage. If properly stored, clones are guaranteed to be stable for 12 months.*

Related Optional Reagents

- Competent E. coli cells
- LB agar plates with ampicillin, 100 μg/mL
- LB broth (10 g/L Tryptone, 5 g/L Yeast Extract, 10 g/L NaCl. Adjust pH to 7.0 with 1 N NaOH)
- DNA purification reagents

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Related OriGene Products

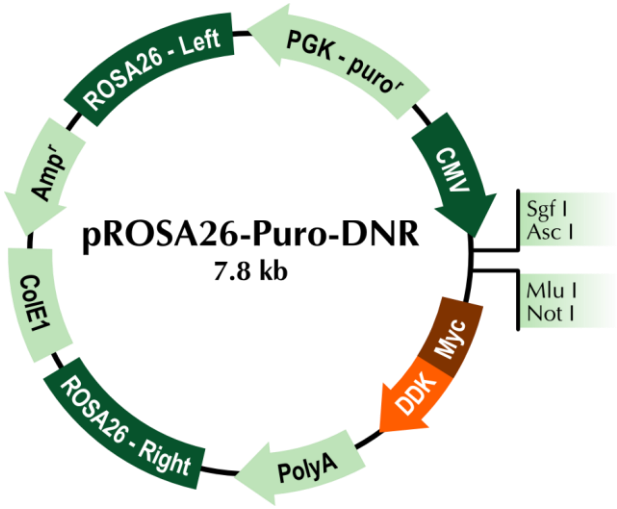
- pCas-Guide-ROSA26 (GE100050)
- pCas-Guide-AAVS1 (SKU GE100023)
- pCas-Guide-scramble (SKU GE100003)
- pAAVS1-RFP-DNR (SKU GE100026)
- AAVS1 Transgene knockin vector kit (puro) (SKU GE100027)
- AAVS1 transgene knockin vector kit (BSD) (SKU GE100036)

Product Description

ROSA26 donor vector (pROSA26-Puro-DNR): ROSA26 repair donor vector containing multiple cloning site to clone your gene of interest. The plasmid contains ROSA26 left homologous arm and right homologous arm for homologous repair to integrate the transgene cassette into ROSA26 locus. Flanked by the two arms, there is a CMV promoter driven expression cassette for a transgene expression and a PGK driven puromycin resistant gene for mammalian selection (Fig. 24). A multiple cloning site downstream of the CMV promoter is designed to be compatible to OriGene's precision shuttle vector system so that the ORF clones can be easily shuttled to this integration vector by simplely "cut and paste".

Note: pROSA26-Puro-DNR is a donor vector without your gene of interest; the gene of interest needs to be cloned.

Figure 24. Vector map of pROSA26-Puro-DNR



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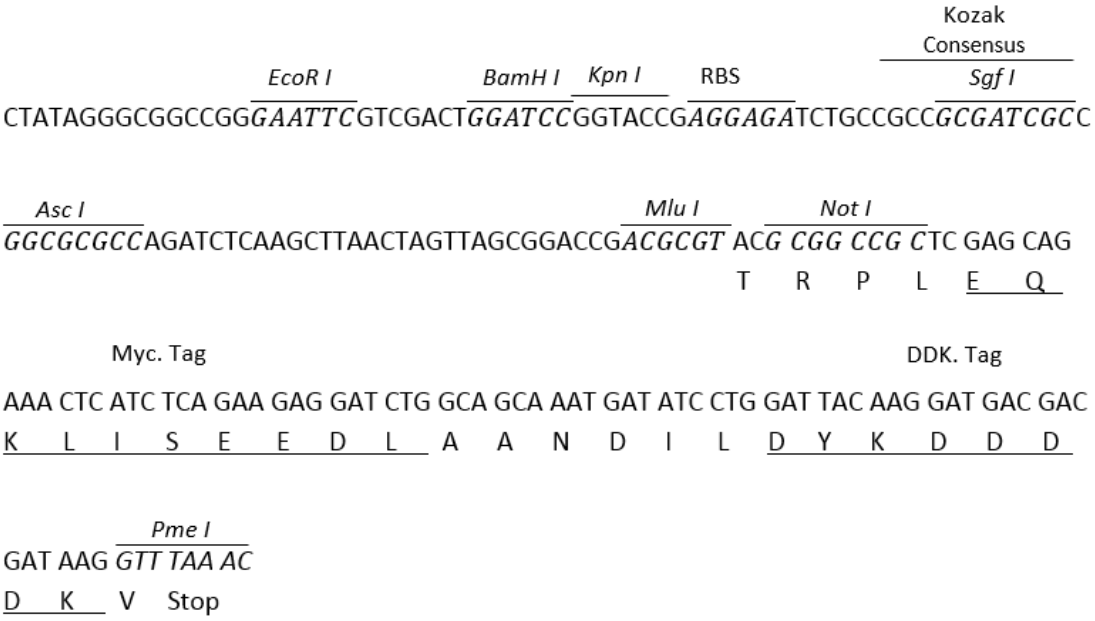
MCS of pROSA26-Puro-DNR



Cloning a transgene to ROSA26 donor vector

Please follow the protocol on page 44-45 of this manual.

ROSA26 Transgene knockin vector kit, puro (SKU GE100052)

Package Contents

1. One (1) vial pCas-Guide-ROSA26 (SKU GE100050), targeting ROSA26 site, 10 μg, lyophilized. Reconstitute in 100 µL dH_2O to make a final concentration of 100 ng/ µL*.
2. One (1) vial containing pCas-Guide-scramble (SKU GE100003) negative control, 10 μg, lyophilized. Reconstitute in 100 µL dH_2O to make a final concentration of 100 ng/ µL*.
3. One (1) vial containing pRosa-Puro-DNR (SKU GE100051), 10 μg, lyophilized. Reconstitute in 100 µL dH_2O to make a final concentration of 100 ng/ µL*.
4. Forward (VP1.5) and reverse (XL39) sequencing primers, 100pmols each, dried onto the bottom of screw cap tubes. Reconstitute each in 10 µL dH_2O to make a 10 µM solution. *Primers are for donor vector (pROSA26-Puro-DNR) only, not for pCas-Guide-ROSA26 or pCas-Guide-scramble.*
5. Certificate of Analysis

** OriGene plasmids are purified using ion-exchange columns for high-yield, low endotoxin preparations (PowerPrep® HP Midiprep Kit). The cDNA clone is shipped at room temperature, but should be kept at -20℃ for long-term storage. If properly stored, clones are guaranteed to be stable for 12 months.*

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Related Optional Reagents

- Nuclease free water
- SgfI and Mlu I
- T4 DNA ligase and buffer
- Competent E. coli cells
- LB agar plates with ampicillin, 100 μg/mL
- LB broth (10 g/L Tryptone, 5 g/L Yeast Extract, 10 g/L NaCl. Adjust pH to 7.0 with 1 N NaOH)
- DNA purification reagents

Related OriGene Products

- Genome-wide ORF clones
- Transfection reagent
- CRISPR/Cas9 products
- Reagents and supplies for immunoblots: user preferred. OriGene has a selection of antibodies and detection reagents

Notice to purchaser

This product is for research use only. Use in and/or for diagnostics and therapeutics is strictly prohibited. By opening and using the product, the purchaser agrees to the following: The plasmids may not be distributed, resold, modified for resale or used to manufacture commercial products without prior written approval from OriGene Technologies, Inc. If you do not agree to the above conditions, please return the UNOPENED product to OriGene Technologies, Inc. within ten (10) days of receipt for a full refund.

Product description

The ROSA26 transgene knockin vector kit is a complete vector kit to knockin your gene of interest in ROSA26 locus in mouse genome for robust and stable expression.

The kit contains the following three plasmids:

1. pCas-Guide-ROSA26: a plasmid DNA with Cas9 expression and gRNA targeting ROSA26 site (Fig. 23). This two-in-one vector will generate a double strand breakage in the human host cells at ROSA26 locus.
2. pCas-Guide-Scrambled control: the negative gRNA control containing a non-specific gRNA sequence in pCas-Guide vector.
3. pROSA26-Puro-DNR: A ROSA26 repair donor vector containing multiple cloning site to clone your gene of interest. The plasmid has ROSA26 left homologous arm and right homologous arm for homologous repair to integrate the transgene cassette into ROSA26 locus. Flanked by the two arms, there is a CMV promoter driven expression cassette for a transgene expression and a PGK driven puromycin resistant gene for transgene integration selection (Fig. 24). A mutiple cloning site downstream of the CMV promoter is designed to be compatible to Origene's precision shuttle vector system so that the ORF clones can be easily shuttled to this integration vector by simplely "cut and paste".

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Note: pROSA26-Puro-DNR is a donor vector, the gene of interest needs to be cloned in this donor vector.

Experimental Protocol

The protocol is similar to AAVS1 transgene insertion via CRISPR. Please follow the experimental protocol on page p49-52 of this manual, use transgene cloned pROSA-Puro-DNR instead of pAAVS1-Puro-DNR.

XI. CRISPR activation (CRISPRa) and CRISPR interference (CRISPRi)

CRISPRa SAM System

Introduction

CRISPR/Cas9 recently has been developed to modulate gene expression, CRISPR activation (CRISPRa) utilizes the enzymatically deficient Cas9 (dCas9), which contains mutations in two active endonuclease domains, losing the capability to cut DNA. However, dCas9 can bind to DNA when coupled with gRNA. When dCas9 is fused or interact with transcription activators, it can be used to activate gene expression. Guide RNA is usually designed to target promoter regions for CRISPRa. CRISPRa system can be used to activate endogenous gene expression.

CRISPRa SAM system was discovered by Dr. Feng Zhang's group. CRISPRa SAM is a robust CRISPR gene activation system to activate gene expression. CRISPRa SAM consists of dCas9-VP64, a modified gRNA containing MS2 RNA aptamers, and MS2-p65-HSF1 activation domains. VP64 has four copies of VP16, a viral protein that has been used for transcriptional activation. p65 and HSF1 are co-transactivation domains. When p65 and HSF1 are brought in proximity to dCas9-VP64 via interaction of MS2 with MS2 RNA aptamers in gRNA, the three transactivators then synergistically upregulate gene expression. CRISPRa SAM can robustly activate both coding and non-coding RNA (lincRNA).

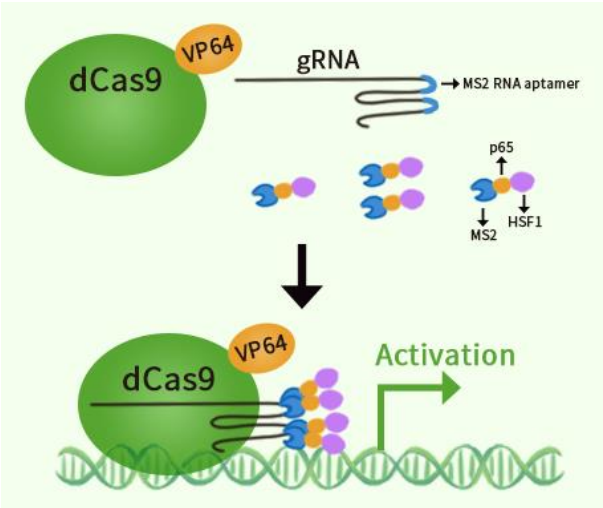

Fig. 25. Diagram of how CRISPRa SAM activates gene expression.

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CRISPRa SAM vector kit for CRISPR/Cas9 activation (SKU GE100057)

Package contents

- One (1) vial of pCas-Guide-CRISPRa vector, 10 μg, lyophilized. Reconstitute in 100 µL dH_2O to make a final concentration of 100 ng/ µL
- One (1) vial of pCas-Guide-CRISPRa-Scramble, 10μg, lyophilized. Reconstitute in 100 µL dH_2O to make a final concentration of 100 ng/ µL
- One (1) vial of pCRISPRa-Enhancer, 10μg, lyophilized. Reconstitute in 100 µL dH_2O to make a final concentration of 100 ng/ µL
- Certificate of Analysis
- Application Guide available online.

** OriGene plasmids are purified using ion-exchange columns for high-yield, low endotoxin preparations (PowerPrep® HP Midiprep Kit* https://www.origene.com/products/others/nucleic-acid-purification-kits)
The cDNA clone is shipped at room temperature, but should be kept at -20℃ for long-term storage. If properly stored, clones are guaranteed to be stable for 12 months.

Related Optional Reagents

- Nuclease free water
- BamH I, BsmB I
- T4 DNA ligase and buffer
- Competent *E. coli* cells
- LB agar plates with ampicillin, 100 μg/mL; kanamycin, 25 μg/mL
- LB broth (10 g/L Tryptone, 5 g/L Yeast Extract, 10 g/L NaCl. Adjust pH to 7.0 with 1 N NaOH)
- DNA purification reagents

Related OriGene Products

- gRNA Cloning - https://www.origene.com/crispr-cas9/grna-cloning-donor-construction.aspx
- qPCR Primer Pairs - https://www.origene.com/qpcr/primers.aspx
- Transfection Reagents - https://www.origene.com/ /cdna/transfection.mspx
- cDNA clones - https://www.origene.com/cdna/
- Validated Antibodies - https://www.origene.com/products/antibodies
- CRISPR/Cas9 products - https://www.origene.com/ /crispr-cas9

Notice to purchaser

This product is for research use only. Use in and/or for diagnostics and therapeutics is strictly prohibited. By opening and using the product, the purchaser agrees to the following: The plasmids may not be distributed, resold, modified for resale or used to manufacture commercial products without prior written approval from OriGene Technologies, Inc. If you do not agree to the above conditions, please return the UNOPENED product to OriGene Technologies, Inc. within ten (10) days of receipt for a full refund by contacting customer service at custsupport@origene.com.

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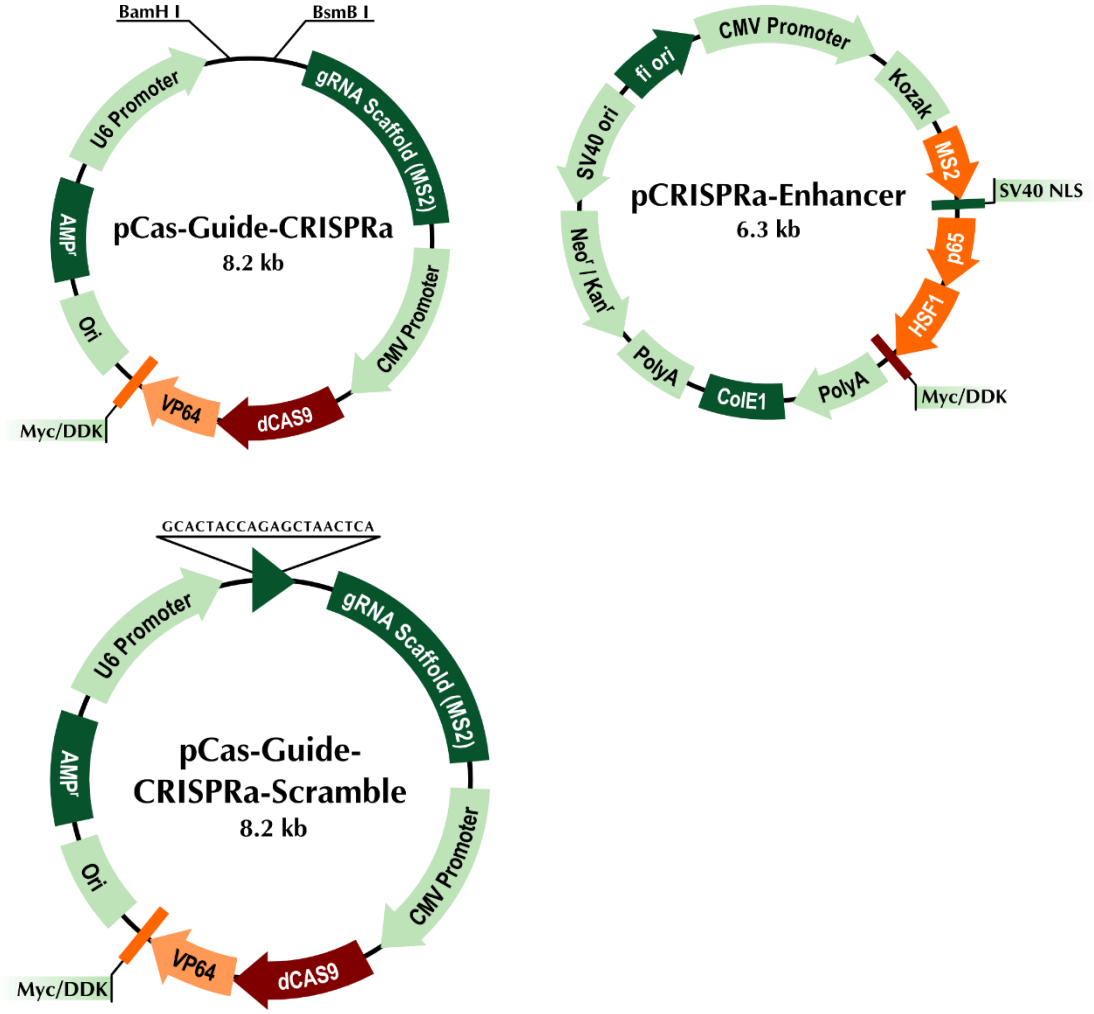
Figure 26. Plasmid maps of CRISPRa SAM Vectors.



Experimental protocol

1. Digest pCas-Guide-CRISPRa plasmid with BamH I and BsmB I

   Resuspend the 10 µg lyophilized DNA in 100 µL dH2O, and incubate for at least 30 min before use. Set up a digestion reaction as described below.

Component	Volume
10X restriction buffer	3 µL
BamH I	0.8 µL
BsmB I	0.8 µL
Nuclease free water	15.4 µL
Vector DNA	10 µL

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Total volume	30 µL

Incubate the reaction at 37°C for 3 hrs, then add 1 µL antarctic phosphatase (units used according to the manufacturer's protocol), and continue the incubation at 37ºC for another 30 min. Dephosphorylation of the digested vector is essential to eliminate self-ligation. Purify the desired vector fragment by running the digestion reaction on an agarose gel, and isolate the appropriate band using a gel purification column. Elute the digested plasmid vector in 40 µL of 10 mM Tris buffer.

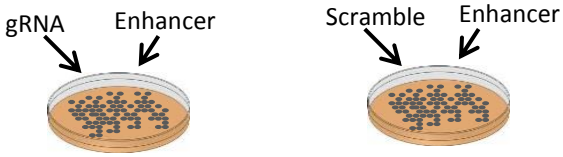
2. Target sequence designing and cloning into the precut vectors, please follow the detailed protocol from page 20-21 in this manual.

3. Transfection

A sample protocol listed below is for 6-well plates and using TurboFectin (cat# TF81001) as the transfection reagent. If your experiments require a different size of culture plates, just scale up or down the reagents accordingly based on the relative surface area of your plate. Different type of cells may need a different transfection reagent; please follow the manufacturer's corresponding protocol.

1. Approximately 18-24 hours before transfection, plate ~3 X 10^5 adherent cells in 2 ml culture media into each well of a 6-well plate or ~5x10^5 suspension cells per well to obtain 50-70% confluence on the following day. The number of cells varies depending on the size of your cells.
2. Transfection in complete culture media.

   Cotransfect your target specific pCas-Guide-CRISPRa vector together with pCRISPRa-Enhancer into your cells to activate gene expression of your gene of interest. In a separate well, cotransfect pCas-Guide-CRISPRa-Scramble with pCRISPRa-Enhancer as your negative control.



In a small sterile tube, combine the following reagents in the prescribed order. The order of reagent addition is important to achieve the optimal results.

a. Dilute 1 µg of gRNA vector and 0.3ug Enhancer vector in 250 uL of Opti-MEM I (Life Technologies), vortex gently.
b. Add 3.9 µL of Turbofectin 8.0 to the diluted DNA (not the reverse order) and pipette gently to mix completely.
c. Incubate the mixture 15 minutes at room temperature.

***Note**: 1. We recommend starting with the ratios of 3:1, Turbofectin: DNA. however, subsequent optimization may further increase the transfection efficiency.*

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***Note**: 2. For your specific cells, you may need to find the best transfection method and follow the manufacturer's protocol.* Viromer *transfection reagents work better for hard-to-transfect cells.*

3. Add the mixture above drop-wise to the cells plated in step 1 (no need to change the media). Gently rock the plate back-and-forth and side-to-side to distribute the complex evenly.

4. 48 hours post transfection, gene expression can be measured with qPCR or WB analysis. OriGene has genome-wide qPCR primer pairs and primary antibodies.

pCas-Guide-CRISPRa vector (Cat# GE100055)

pCas-Guide-CRISPRa is an all-in-one CRISPR vector containing gRNA target sequence cloning sites and expression of fusion protein dCas9-VP64. In the gRNA scaffold, MS2 RNA aptamers are inserted in the loops. dCas9-VP64 itself can activate gene expression. However, with p65-HSF1 (cat# GE100056), gene activation will be synergistically increased to thousands of folds.

Please see the plasmid map of pCas-Guide-CRISPRa on page 9.

pCRISPRa-Enhancer (Cat# GE100056)

This vector expresses CMV driven MS2-p65-HSF1 fusion proteins. MS2 interacts with gRNA via MS2 RNA aptamer, thus bringing the three transactivation domains together, VP64, p65-HSF1. This enhancer vector alone cannot activate gene expression; it needs to work with pCas-Guide-CRISPRa vector.

pCas-Guide-CRISPRa-Scramble (Cat# GE100058)

A scramble gRNA target sequence is cloned in pCas-Guide-CRISPRa vector, which serves a negative control for CRISPRa system.

CRISPRa validation data

1. Cas9-VP64 without p65-HSF1 can activate gene expression.

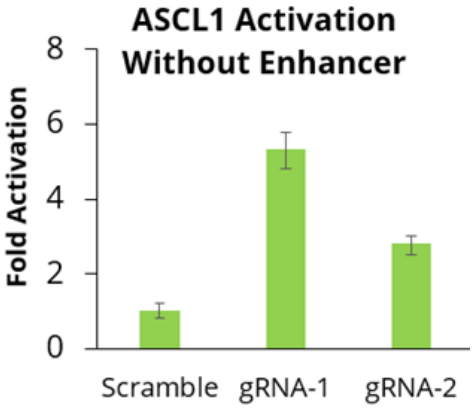

Fig. 27. dCas9-VP64 and gRNA targeting ASCL1 locus (ASCL1 target sequence cloned pCas-Guide-CRISPRa) without CRISPRa enhancer was transfected into HEK293T cells using MegaTran 2.0. Cells were harvested 48 hrs post transfection; qPCR was performed to measure gene expression.

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CRISPa SAM (VP64 with p65-HSF1) synergistically activates gene expression.

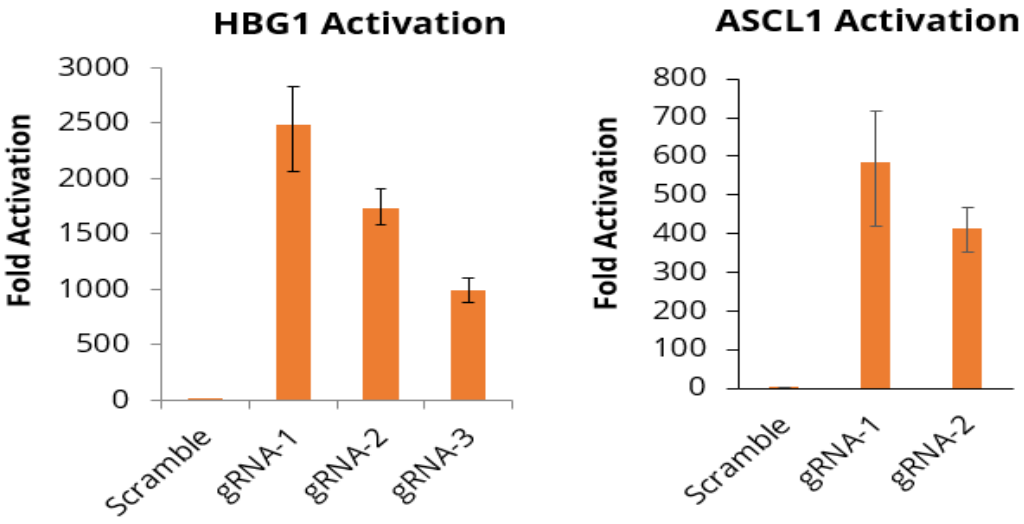

Fig. 28. Target specific pCas-Guide-CRISPRa vector (encodes dCas9-VP64 and gRNA targeting HBG1 or ASCL1 locus) and pCRISPRa-Enhancer (encodes MS2-p65-HSF1) were transfected into HEK293T cells using MegaTran 2.0. Cells were harvested 48 hrs post transfection; qPCR was performed to measure gene expression.

Genome-wide Gene Activation Kits using CRISPRa SAM

CRISPRa SAM can be used to robustly upregulate endogenous gene expression. OriGene offers genome-wide and locus specific gene activation kit using CRISPRa. Gene specific gRNA will bring dCas9-VP64 to the specific gene locus, p65 and HSF1 translovate to the gRNA by MS2, therefore robustly activate gene expression.

To activate endogenous expression, gRNAs are designed to target the promoter region. gRNA will guide dCas9-VP64 to the promoter region, the other two transactivation domains, p65-HSF1 will be recruited to the promoter region via interaction of MS2 and MS2 RNA aptamer in the gRNA.

To ensure efficient cleavage, 3 individual gRNA constructs are provided in each kit; each gRNA will be tested separately.

Package contents

- 3 vials of gene specific gRNA constructs in pCas-Guide-CRISPRa vector, SKU GAxxxxxxG1, GAxxxxxxG2, GAxxxxxxG3), 3-5 µg DNA in TE buffer
- 1 vial of pCas-Guide-CRISPRa-Scramble, 10µg, lyophilized. Reconstitute in 100 µL dH_2O to make a final concentration of 100 ng/ µL

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- One 1 vial of pCRISPRa-Enhancer, 10μg, lyophilized. Reconstitute in 100 μL dH_2O to make a final concentration of 100 ng/ μL
- Certificate of Analysis
- Application Guide available online.

** OriGene plasmids are purified using ion-exchange columns for high-yield, low endotoxin preparations (PowerPrep® HP Midiprep Kit* https://www.origene.com/products/others/nucleic-acid-purification-kits)

The cDNA clone is shipped at room temperature, but should be kept at -20℃ for long-term storage. If properly stored, clones are guaranteed to be stable for 12 months.

Related Optional Reagents

- Nuclease free water
- BamH I, BsmB I
- T4 DNA ligase and buffer
- Competent *E. coli* cells
- LB agar plates with ampicillin, 100 μg/mL; kanamycin, 25 μg/mL
- LB broth (10 g/L Tryptone, 5 g/L Yeast Extract, 10 g/L NaCl. Adjust pH to 7.0 with 1 N NaOH)
- DNA purification reagents

Related OriGene Products

- qPCR Primer Pairs - https://www.origene.com/qpcr/primers.aspx
- Transfection Reagents - https://www.origene.com/ /cdna/transfection.mspx
- cDNA clones - https://www.origene.com/cdna/
- Validated Antibodies - https://www.origene.com/products/antibodies
- CRISPR/Cas9 products - https://www.origene.com/ /crispr-cas9

Notice to purchaser

This product is for research use only. Use in and/or for diagnostics and therapeutics is strictly prohibited. By opening and using the product, the purchaser agrees to the following: The plasmids may not be distributed, resold, modified for resale or used to manufacture commercial products without prior written approval from OriGene Technologies, Inc. If you do not agree to the above conditions, please return the UNOPENED product to OriGene Technologies, Inc. within ten (10) days of receipt for a full refund by contacting customer service at custsupport@origene.com.

Experimental Protocol

A sample protocol listed below is for 6-well plates and using TurboFectin (cat# TF81001) as transfection reagent. If your experiments require a different size of culture plates, just scale up or down the reagents accordingly based on the relative surface area of your plate. Different type of cells may need a different transfection reagent; please follow the manufacturer's corresponding protocol.

1. Approximately 18-24 hours before transfection, plate ~3 X 10^5 adherent cells in 2 ml culture media into each well of a 6-well plate or ~5x10^5 suspension cells per well to obtain 50-70% confluence on the following day. The number of cells varies depending on the size of your cells.

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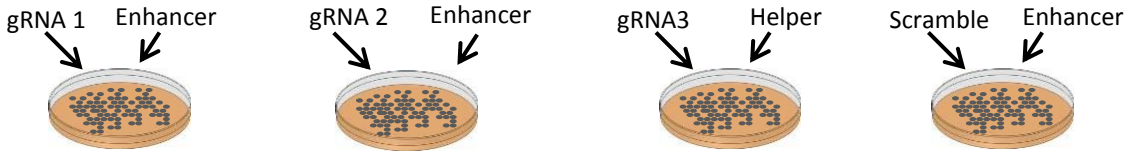
2. Transfection in complete culture media. Four separate transfections:



In a small sterile tube, combine the following reagents in the prescribed order. The order of reagent addition is important to achieve the optimal results.

d. Dilute 1 µg of one of the gRNA vectors and 0.3ug Enhancer vector in 250 uL of Opti-MEM I (Life Technologies), vortex gently.
e. Add 3.9 µL of Turbofectin 8.0 to the diluted DNA (not the reverse order) and pipette gently to mix completely.
f. Incubate the mixture 15 minutes at room temperature.

***Note**: 1. We recommend starting with the ratios of 3:1, Turbofectin: DNA. however, subsequent optimization may further increase the transfection efficiency.*

***Note**: 2. For your specific cells, you may need to find the best transfection method and follow the manufacturer's protocol.* Viromer *transfection reagents work better for hard-to-transfect cells.*

3. Add the mixture above drop-wise to the cells plated in step 1 (no need to change the media). Gently rock the plate back-and-forth and side-to-side to distribute the complex evenly.

4. Incubate the cells in a 5% CO_2 incubator.

5. 48 hrs post transfection, measure gene expression via qPCR or Western Blotting. OriGene offers qPCR primer pairs and primary antibodies.

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CRISPRi System

dCas9 can be fused with transcription repression domains to repress endogenous gene expression. In this CRISPRi system, dCas9 is fused with KRAB and MeCP2 repression domains to carry out robust gene repression. Krüppel-associated box (KRAB) is a well-known transcriptional repressor domain. MeCP2 has been shown to bind to methylated DNA.

All-in-one pCas-Guide-CRISPRi vector (Cat# GE100059)

pCas-Guide-CRISPRi vector contains gRNA target sequence cloning sites and CMV driven dCas9-KRAB-MeCP2 expression. After cloning a specific gRNA targeting sequence, transfecting this vector into cells will lead to gene repression.

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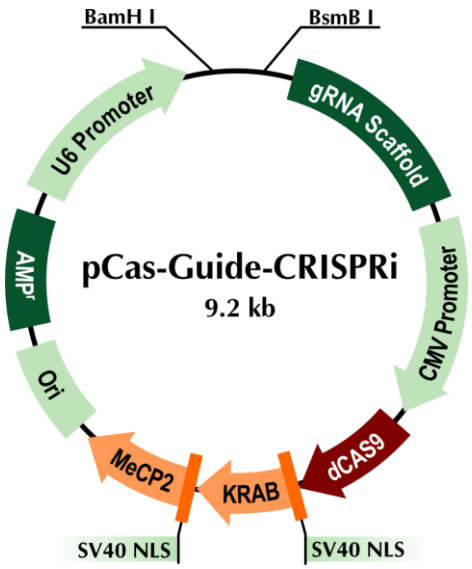
Fig. 29. Vector map of pCas-Guide-CRISPRi



Package contents

- One (1) vial of pCas-Guide-CRISPRi vector, 10 μg, lyophilized. Reconstitute in 100 µL dH_2O to make a final concentration of 100 ng/ µL
- Certificate of Analysis
- Application Guide available online.

** OriGene plasmids are purified using ion-exchange columns for high-yield, low endotoxin preparations (PowerPrep® HP Midiprep Kit* https://www.origene.com/products/others/nucleic-acid-purification-kits)

The cDNA clone is shipped at room temperature, but should be kept at -20℃ for long-term storage. If properly stored, clones are guaranteed to be stable for 12 months.

Related Optional Reagents

- Nuclease free water
- BamH I, BsmB I
- T4 DNA ligase and buffer
- Competent *E. coli* cells
- LB agar plates with ampicillin, 100 μg/mL; kanamycin, 25 μg/mL
- LB broth (10 g/L Tryptone, 5 g/L Yeast Extract, 10 g/L NaCl. Adjust pH to 7.0 with 1 N NaOH)
- DNA purification reagents

Related OriGene Products

- gRNA Cloning - https://www.origene.com/crispr-cas9/grna-cloning-donor-construction.aspx
- qPCR Primer Pairs - https://www.origene.com/qpcr/primers.aspx
- Transfection Reagents - https://www.origene.com/ /cdna/transfection.mspx
- cDNA clones - https://www.origene.com/cdna/

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- Validated Antibodies - https://www.origene.com/products/antibodies
- CRISPR/Cas9 products - https://www.origene.com/ /crispr-cas9

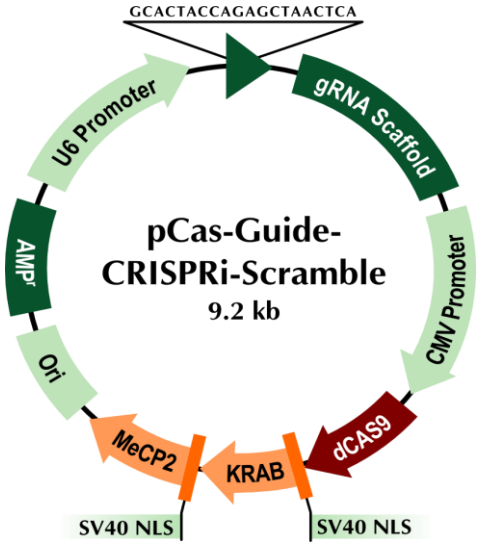
Notice to purchaser

This product is for research use only. Use in and/or for diagnostics and therapeutics is strictly prohibited. By opening and using the product, the purchaser agrees to the following: The plasmids may not be distributed, resold, modified for resale or used to manufacture commercial products without prior written approval from OriGene Technologies, Inc. If you do not agree to the above conditions, please return the UNOPENED product to OriGene Technologies, Inc. within ten (10) days of receipt for a full refund by contacting customer service at custsupport@origene.com.

pCas-Guide-CRISPRi-Scramble (Cat# GE100060)

pCas-Guide-CRISPRi-Scramble contains gRNA scrambled sequence, which serves a negative control for CRISPRi vector.

Fig. 30. Plasmid map of pCas-Guide-CRISPRi-Scramble



Experimental protocol

1. Digest pCas-Guide-CRISPRi plasmid with BamH I and BsmB I

   Resuspend the 10 µg lyophilized DNA in 100 µL dH2O, and incubate for at least 30 min before use. Set up a digestion reaction as described below.

Component	Volume
10X restriction buffer	3 µL
BamH I	0.8 µL
BsmB I	0.8 µL
Nuclease free water	15.4 µL
Vector DNA	10 µL
Total volume	30 µL

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Incubate the reaction at 37ºC for 3 hrs, then add 1 µL antarctic phosphatase (units used according to the manufacturer's protocol), and continue the incubation at 37ºC for another 30 min. Dephosphorylation of the digested vector is essential to eliminate self-ligation. Purify the desired vector fragment by running the digestion reaction on an agarose gel, and isolate the appropriate band using a gel purification column. Elute the digested plasmid vector in 40 µL of 10 mM Tris buffer.

2. Target sequence designing and cloning into the precut vectors, please follow the detailed protocol from page 20-21 in this manual.

3. Transfection

A sample protocol listed below is for 6-well plates and using TurboFectin (cat# TF81001) as transfection reagent. If your experiments require a different size of culture plates, just scale up or down the reagents accordingly based on the relative surface area of your plate. Different type of cells may need a different transfection reagent; please follow the manufacturer's corresponding protocol.

1) Approximately 18-24 hours before transfection, plate ~3 X 10^5 adherent cells in 2 ml culture media into each well of a 6-well plate or ~5x10^5 suspension cells per well to obtain 50-70% confluence on the following day. The number of cells varies depending on the size of your cells.
2) Transfection in complete culture media.

In a small sterile tube, combine the following reagents in the prescribed order. The order of reagent addition is important to achieve the optimal results.

3) Dilute 1 µg of CRISPRi vector in 250 uL of Opti-MEM I (Life Technologies), Vortex gently. pCas-Guide-CRISPRi-Scramble should be transfected separately as a negative control.
4) Add 3 µL of Turbofectin 8.0 to the diluted DNA (not the reverse order) and pipette gently to mix completely.
5) Incubate the mixture 15 minutes at room temperature.

***Note**: 1. We recommend starting with the ratios of 3:1, Turbofectin: DNA. however, subsequent optimization may further increase the transfection efficiency.*

***Note**: 2. For your specific cells, you may need to find the best transfection method and follow the manufacturer's protocol.* Viromer *transfection reagents work better for hard-to-transfect cells.*

6) Add the mixture above drop-wise to the cells plated in step 1 (no need to change the media). Gently rock the plate back-and-forth and side-to-side to distribute the complex evenly.

7) 48 hours post transfection, gene expression can be measured with qPCR or WB analysis. OriGene has genome-wide qPCR primer pairs and primary antibodies.

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FAQ

General CRISPR/Cas9

Q: A 20bp target sequence is needed with a NGG PAM seq. Shall the NGG be exactly immediately following the 3′ of this 20bp sequence?
Yes, the NGG is located immediately next to the 3′ end of the 20bp sequence in the genome. However, NGG is not included in the guide RNA sequence.

Q: How to design the 20bp target-specific sequence?
The 20bp target-specific sequence should precede NGG (PAM). Please BLAST the seed region (8-14 bp PAM-proximal) of the 20bp target sequence to make sure it's unique along the genome to guarantee its specificity.

5'-NNNNNNNNNNNNNNNNNNNN 3'-NGG
Seed-region

Q: How to avoid off target issue using CRISPR/Cas?
You can blast your target sequences. If the off-target sequences don't have the PAM (NGG), then they won't be targeted by CRISPR/Cas9. You also want to choose target sequences with mismatches in the 8-14 bp at the 3' end of the target sequences. This way, the off-target issue can be decreased dramatically. For therapeutic purpose, you can use Cas9 nickase which only cuts one strand.

Q: How many target RNA sequences should I use for a genome editing project?
Due to the un-predicable nature of gRNA, we recommend 3 and more gRNA targeting sequences to be designed to make sure that at least one targeting sequence will provide efficient cleavage.

Q: Do you know the specific cleavage site of the Cas9:gRNA complex in terms of where in the targeting sequence the cleavage occurs?
Cas9 cleaves at 3 bp away from the 3' end of the target sequence in the genome.

Q: Why I cannot find the gRNA targeting sequences in the cDNA sequence?
The targeting sequences could be in either exon or intron in the genome; the cDNA sequences only contain the exons. CRISPR/Cas9 will target the genomic sequence, then genome editing will be achieved.

Q: Why do you need T7-driven vector to express gRNA and Cas9?
For making gene knockout mice and genome editing in other organisms, such as Drosophila, some researchers do microinjection of gRNA and Cas9 mRNA into cells.

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Q: The transfection efficiency of my cell line is only 20%, how to enrich CRISPR transfected cells?
You can use pCas-Guide-EF1a-GFP to enrich transfected cells since GFP is also expressed. We also have pCas-Guide-EF1a-CD4 vector; you can use anti-CD4 antibody beads to enrich transfected cells. Alternatively, you can transfect a plasmid with a selection marker and select the cells. Lenti vector can be used and integration-deficient lentivirus can be produced using the special integration-deficient lenti packaging kit (cat# TR30036); the lenti CRISPR vectors can be delivered into hard-to-transfect cells, but not integrating into the host genome.

Q: Is there a method for isolating single cell colonies from the engineered pool of cells?
series of dilution or Isolating individual cell colonies using cloning cylinders.

Q: Do you need to linearize a donor template before transfection for efficient repair?
The donor template DNA is not preferred to be linearized as this will increase random integration.

Q: How to select edited clones if using long oligos as a donor template?
Isolate single cell colonies, do WB (for gene knockout or tagging) or genomic PCR or sequencing (for mutations) to detect the genome editing depending on the nature of the editing.

Q: How to screen the edited cells after transfecting the CRISPR/Cas9 vector?
For mutations, you can do genomic PCR and sequence it. If you do gene knockout, the selection marker in the donor template DNA will help the selection. If no donor DNA for gene knockout out, then genomic PCR and sequencing to confirm indels. If necessary, you can isolate individual cell colonies for introduction of specific mutations and other genome editing applications. You can do WB for gene knockout after isolating single cell colonies.

Q: Does CRISPR/Cas system work for non-dividing cells?
NHEJ repair works in non-dividing cells; HDR is not active in non-dividing cells.
OriGene's new KN2.0 CRISPR knockout kit works in non-dividing cells and a selection cassette can be knocked in to help screening.

Q: Using CRISPR, can you get monoallelic knockout (heterozygous) or biallelic knockout (homozygous)?
CRISPR/Cas9 double-strand cleavage is very efficient. If just using CRISPR/Cas9 vectors to introduce indels, if transfection efficiency is high, more biallelic knockout can occur. In the presence of donor DNA, since homologous recombination may be a limiting factor, some cells contain monoallelic knockout and some cells contain biallelic knock out.

Q: Do you have the cas9 antibody?
Yes, We do have Cas9 antibody (cat# TA190309). In our CRISPR/Cas9 vectors, Cas9 has a C-terminal Myc-DDK tag. DDK is the same as Flag; OriGene's anti-DDK antibody (SKU TA50011-100).

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Q: If I want to use CRISPR/Cas9 to knock down a certain gene, what kind of negative control should I use?
You can use a scramble control, pCas-Scramble, SKU GE100003, or pCas-Scramble-EF1a-GFP, SKU GE100021.

Q: For gene targeting in mice, do you recommend transfecting ES cells or pronuclei?
You can do both. You can inject mRNA (gRNA and Cas9 mRNA) or plasmid DNA (target sequence cloned pCas-Guide) into the zygotes or ES cells.

Q: What is the limit for multiple gene disruption?
You can do multiplexes using CRISPR/Cas9 system. You can co-transfect the gRNA vectors or co-inject several guide RNAs into your cells; thus, you will achieve multiple gene disruption or genome editing. The limit could be transfection efficiency.

Q: How do you make sure that Cas9 will not integrate in genome if you use lentivector?
For screening purpose, for short term, integration of Cas9 into the genome for 2 weeks does not affect cells. You can also use the integration-deficient lenti packaging kit to produce lentivirus that won't integrate into the cellular genome, acting just like plasmid.

Q: Can you introduce mutations anywhere in the genome, including in promoters or enhancers?
Yes. The 20 bp target sequences only need to precede NGG.

Q: Do you see variability in success with different cell lines?
Yes, depending on the cell line and the gRNA sequences.

Q: What is the known CRISPR/Cas9 editing efficiency relative to other genome editing approaches?
In general, the genome editing efficiency of CRISPR/Cas9 is similar or higher than TALEN. However, CRISPR/Cas9 is much more simple and easy to do. You will need to engineer the protein to recognize new DNA sequence in TALEN system, while CRISPR/Cas9 is RNA based.

Q: What is the sequence of CF3 sequencing primer?
5'-ACGATACAAGGCTGTTAGAGAG-3'
Q: What is the scrambled sequence in pCas-Scramble and pCas-Scramble-EF1a-GFP?
5' GCACTACCAGAGCTAACTCA 3'

Q: Do you provide gRNA cloning service and donor vector service?
Yes, you can order gRNA cloning service and donor vector service.

Q: Is there any safety issue with this pLenti vector?
The pLenti vector is a third-generation lentiviral vector and it is the safest lenti-viral vector because both LTRs are truncated. Please contact the biosafety office at your institution prior to

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use of the pLenti vector for permission and for further institution-specific instructions. BL2/(+) conditions should be used always when handling lentivirus. All decontamination steps should be performed using 70% ethanol/1% SDS. Gloves should be worn always when handling lentiviral preparations, transfected cells or the combined transfection reagent and lentiviral DNA.

Q: What is unique about the 3rd generation Lentiviral vectors?
The 3rd generation lentiviral vectors are safer than the 2nd generation vectors. The 3rd generation packaging systems express gag and pol from one packaging vector and rev from another vector. The 3rd generation packaging systems DO NOT express tat (Trans-Activator of Transcription).

Q: Can I use a second generation packaging system with the pLenti vectors?
Yes, a second generation packaging system should work with OriGene's third generation pLenti vectors although we have not explicitly tested this. You can use OriGene's high efficient third generation lenti-packaging kit (cat# TR30037) for pLenti-vectors.

Q: How can I sequence the target sequenced cloned in pT7-Guide vector?
M13 forward primer, 5' CGCCAGGGTTTTCCCAGTCACGAC 3'

CRISPR gene knockout

Q: For knocking down a target gene, donor plasmid is not necessary, correct?
Without donor template DNA, the double-stranded break will be repaired by NHEJ; unpredicted indels will be introduced. You will screen the deletions/insertions that cause frame shift. With donor DNA, you will get desired insertion/deletion/mutations. With donor DNA, you will have mammalian selection.

Q: How long should the LHA and RHA be?
600-1000 bp left or right homologous arms should work for HDR mediated repair.

Q: What is your validation data for your CRISPR knockout / knockin kit?
Please see the downloadable validation data at https://www.origene.com/products/gene-expression/crispr-cas9

Q: How to knockout all the splicing variants of a gene using OriGene's pre-designed donor vectors, OriGene's CRISPR knockout / knockin kit?
Different splice variants of a gene are generated from the same pre-mRNA, splicing at different locations. When we design target sequences to knockout all the splicing forms of a gene, the target sequences are located around the start codon, ATG, of the longest splice variant. The 3' end of the left homologous arm in the donor vector is right upstream of the start codon ATG. After inserting a donor selection cassette, all the splicing variants are not expressed.

Q: Do I get monoallelic knock-out or biallelic knock-out using the homology-mediated knock-out kit via CRISPR? What do I need to do to get biallelic knock-out?

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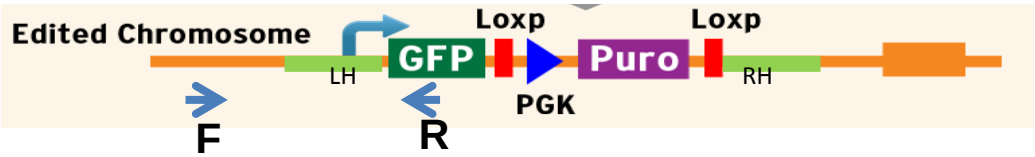
If you isolate single cell colonies, in some cells gene knock-out may occur only in one allele; in some cells gene knock-out may occur in both alleles. If you only have monoallelic knockout and you want to get biallelic knockout, you can order another donor vector containing a different mammalian selection marker, such as blasticidin or neomycin resistant marker. Make sure the other allele is intact as it can be targeted and repaired via NHEJ; confirm with genomic PCR and sequencing. OriGene has both functional cassettes. You can do the knockout procedure again with the new donor vector to target the second allele (one allele is already targeted and replaced with GFP-puro cassette). Alternatively, you can use Cre to flox out the puro cassette from your edited cells and use the same donor vector from the knockout kit and do the knockout again to target the second allele.

Q: What is the sequence of the reverse primer at the GFP region to amplify the left integration region using the homology-mediated CRISPR knockout / knockin kit

tGFP-integeration_3R TAGGTGCCGAAGTGGTAGAAGC



Q: What's the mechanism for KN2.0 CRISPR gene knockout kits mediated gene knock out and targeted donor integration?
KN2.0 is designed based on targeted genome editing technology (CRISPR-Cas9). Target specific gRNA will cut the genome, then the donor DNA containing selection cassette will be integrated at the cutting site via NHEJ (non-homologous end joining) mediated repair mechanism. The donor cassette can be integrated at forward or reverse direction. Most gene knockout are biallelic, one allele has donor integration, the other allele has indels (insertion and deletion).

Q: What are the advantages of KN2.0 CRISPR gene knockout kits?
Although homology directed recombination (HDR)-mediated gene knockout/knockin is well established, it cannot necessarily be applied in some cell types and organisms with low HDR efficiency. CRISPR KN 2.0 is specifically designed to provide a universal solution for gene knockout needs in every cell type and organism. Studies carried out in house and by collaborators show that CRISPR KN 2.0 is highly efficient and render improved knockout rate.
Q: How many cell lines have been tested for KN2.0?
KN2.0 has successfully tested in HeLa, HEK293T and MIA PaCa-2 (a human pancreatic carcinoma cell line) cells.

Q: If after puromycin selection using KN2.0 kit, I have no sells survived, what could be the reason?
Two possibilities. 1. The gene is an essential cell survival gene, so constitutive gene knockout cannot be tolerated. Conditional knockout is needed. 2. Transfection efficiency is too low. Transfection optimization or selecting different transfection method is needed, such as electroporation.

Q: Can KN2.0 be used for embryo microinjection to generate transgenic animal models?

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Theoretically, KN2.0 can be used for embryo microinjection to generate transgenic animal model. However, this has not been tested in our facility and optimization is warranted.

Q: What could be the reason that I couldn't get my gene of interest knocked out?
If your target gene is essential for cell survival, you might not be able to get constitutive gene knockout. Conditional gene knockout may be needed.

CRISPRa and CRISPRi

Q: How do CRISPRa SAM and CRISPRi work?

CRISPRa/CRISPRi is an RNA-guided genome expression regulatory tool, which was devised based on the CRISPR-Cas9 vector (Qi et al. 2013). Two mutations are introduced into Cas9 to silence the nuclease activity (called as dead Cas9 or dCas9). dCas9 can be recruited by gRNA to the target genomic loci, but don't cut the target DNA. dCas9 is used as anchor to recruit fusion partner-protein to the targeted promoter. In CRISPRa, activating factors like VP64 and VPR are fused with dCas9, while in CRISPRi, inhibitory factors like KRAB, MeCP2 are used.

Our engineered CRISPRa SAM belongs to the second generation of the CRISPRa family, which was originally reported by Dr. Feng Zhang's lab (Konermann et al. 2015). SAM stands for Synergistic Activation Mediator. The CRISPRa vector is an all-in-one vector, containing gRNA cloning sites (gRNA modified with MS2 RNA aptamers in the loop) and the expression of dCas9-Vp64 fusion protein. Another vector, called CRISPRa enhancer, expresses the co-activator MS2-P65-HSF1, which can be recruited by the MS2 RNA aptamers of the gRNA scaffold and then synergistically activates gene expression with dCas9-Vp64.

Our CRISPRi system is engineered to fuse dCas9 with dual repressive domains, KRAB domain and MeCP2 repressive domain, which has shown stronger inhibition effects compared with classical CRISPRi-KRAB system (Yeo et al. 2018).

Q: Where should CRISPRa/CRISPRi sgRNAs target?

Our CRISPRa SAM and CRISPRi system uses the same PAM (protospacer-adjacent motif) specificity as wildtype Cas9 (NGG). However, our gRNA design is optimized for CRISPRa and CRISPRi. According to the article (Horlbeck et al. 2016), CRISPRa gRNA within the region from -550bp to -25bp (distance to TSS), and CRISPRi gRNA from -25bp to +500bp (distance to TSS) shows best effects. Each gene specific CRISPRa-SAM activation kit contains 3 sgRNAs designed using our proprietary algorithm.

Q: Why doesn't my CRISPRa-SAM kit have dramatic effects?

The effects of CRISPRa are affected by multiple factors, including transfection efficiency, sgRNA targeting efficiency, and chromatin accessibility. We recommend to first optimize your transfection efficiency according to your specific cell type and reagents. CRISPRa function is also impacted by the accessibility of the target DNA (Horlbeck et al. 2016). The factors including

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nucleosome occupancy and chromatin structure can influence the reach of sgRNA and dCas9 to its target DNA. In addition, based on our experiments, the highly-expressed genes (such as MYC in HEK293T) won't show a significant response to further stimulation from exogenous CRISPRa SAM system.

Q: How to avoid off-target issues using CRISPRa/CRISPRi?

You can blast your target sequences. If the off-target sequences don't have the PAM (NGG), then they won't be targeted by CRISPR/Cas9. You also want to choose target sequences with mismatches in the 8-14 bp at the 3' end of the target sequences. It has been reported that CRISPR-fusion protein-related products can increase off-targeting issues from the nonspecific binding of the fusion proteins (Zhou et al. 2019). To offset these effects, you can use scrambled sgRNA as negative control in your experiment.

Q: How many target RNA sequences should I use for a CRISPRa/CRISPRi project?

Due to the unpredictable nature of sgRNA, we recommend designing 3 sgRNA against your target genes. In our CRISPRa activation Kit, 3 gene specific sgRNAs, a scrambled sgRNA, and CRISPRa-Enhancer are provided. In addition, it has been observed that the co-transfection of 3 sgRNA can be more potent in stimulating their target genes (Chavez et al. 2016).

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