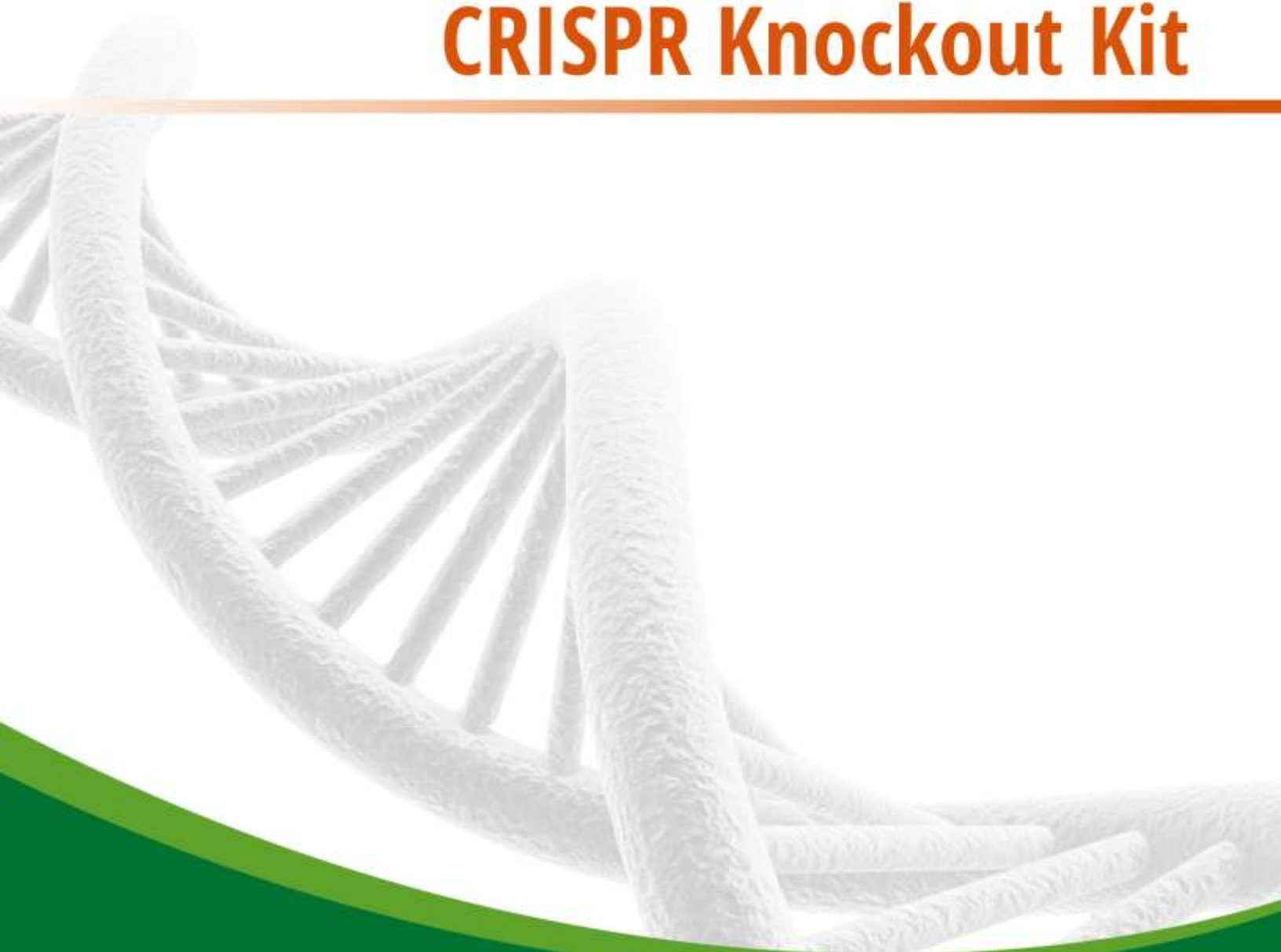


CRISPR Knockout Kit



CRISPR Knockout Kit

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Introduction

CRISPR/Cas9 is a simple and efficient genome editing tool. Although gene knockout cell lines can be generated by gRNAs without donor vector, the screening process can be very tedious. OriGene offers genome-wide CRISPR gene knockout kits for every human and mouse gene. Each kit contains 2 gRNA vectors and donor DNA. The selection marker in the donor cassette greatly facilitates the screening process.

Two types of CRISPR knockout kits are offered, differing in repair mechanism used for gene knockout. **H**omology **D**irected **R**epair (HDR)-mediated knockout kit is our first generation gene knockout kit. Gene knockout is achieved via homology directed repair (HDR). KN2.0 gene knockout kit is an improved version CRISPR knockout kit. Gene knockout is achieved via non-homology based repair. KN2.0 CRISPR knockout kit is more efficient comparing to the HDR based knockout kit, and works in both dividing and non-dividing cells.

OriGene also designed a set of donor cassettes for HDR-based donor vector construction, including Luciferase-Loxp-Puro-Loxp, GFP-Loxp-Puro-Loxp, RFP-Loxp-BSD-Loxp and BFP-Loxp-Neo-Loxp.

Table 1. 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 dH₂O, final concentration 100 ng/ µL.
- Certificate of Analysis
- Application Guide

Note: The plasmid DNA is shipped at room temperature, but should be kept at -20°C for long-term storage. If properly stored, DNA 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>
- Antibodies: <https://www.origene.com/products/antibodies>
- 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

HDR-mediated CRISPR knockout kit is a complete kit to knockout the target gene and knockin a functional cassette with one reporter gene (without promoter for the promoter study) and one drug selection marker (with PGK promoter for the selection of knockout cells).

Each kit contains two gene specific gRNA vectors, one scramble negative control and one donor vector.

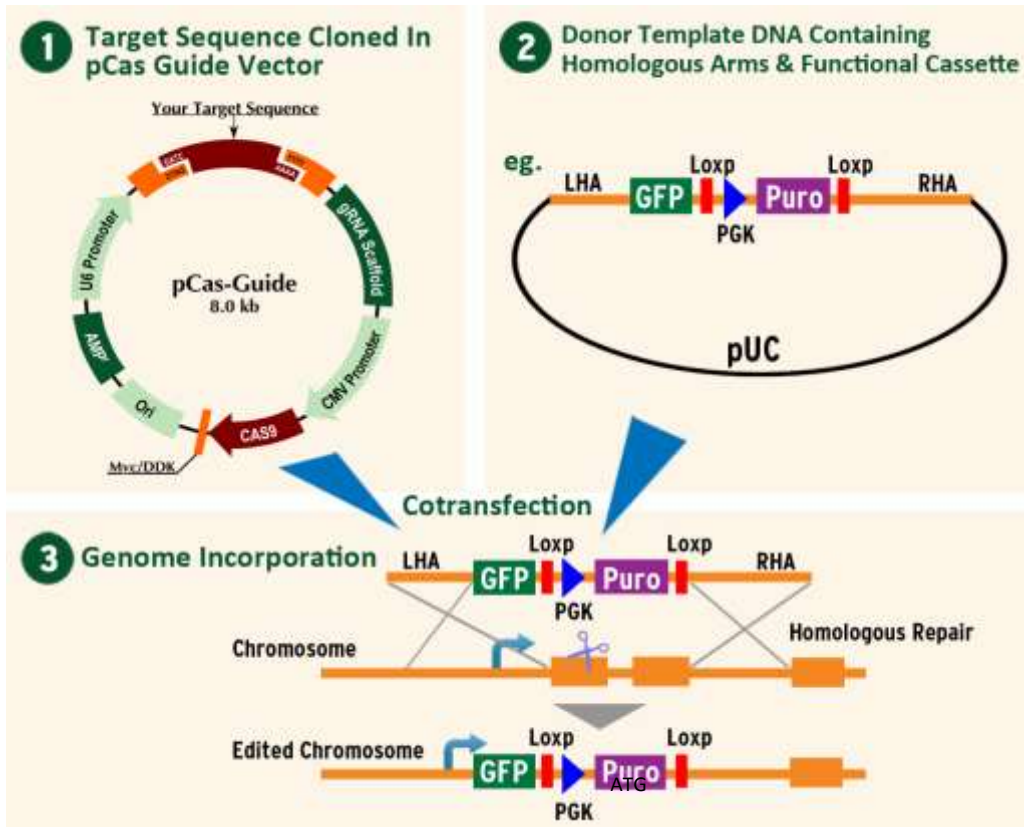
The two gene specific gRNAs are designed around 5' end of the coding sequence (ORF). After transfection, the gRNA construct can make a precise cleavage at 5' end of the ORF, and lead to an early disruption of protein coding activity. A negative scramble control in the same pCas-Guide vector is also provided in each kit to study the off target effect.

In addition to the gRNA constructs, HDR-mediated CRISPR knockout kit also includes one donor plasmid, containing left and right homologous arms (LHA and RHA) flanking a functional cassette. The cassette can be integrated into the target genome via homology-directed repair (HDR) mechanism, and encode a fluorescent reporter protein using the native promoter of target gene. More details can be found from the scheme of HDR-mediated CRISPR knockout kit (Figure 1).

The applications of HDR-mediated CRISPR knockout kit include:

1. Knockin GFP reporter or other reporters for promoter study
2. Knock-out gene expression at the genome level

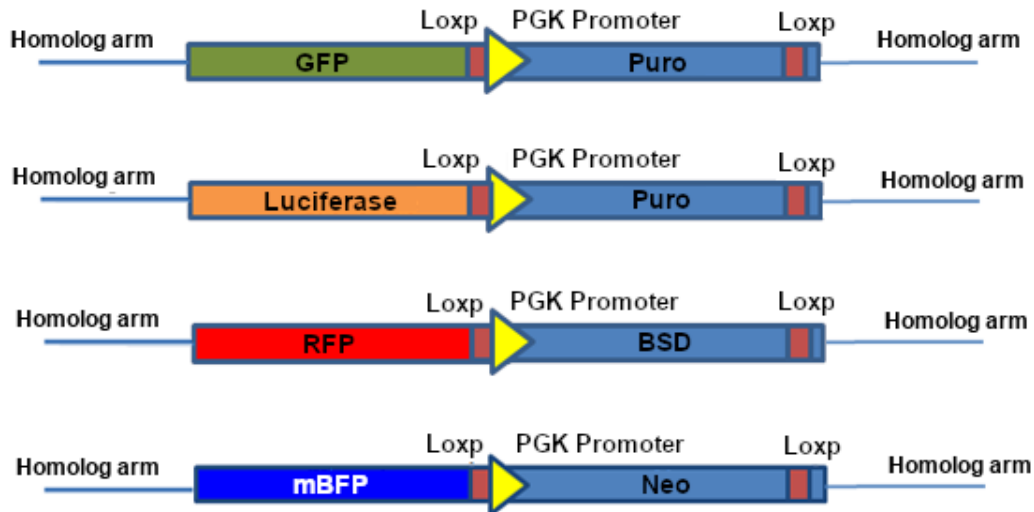
Figure 1. Scheme of HDR-mediated CRISPR knockout kit



- ① CRISPR/Cas9 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 functional cassette. There are four different predesigned functional cassettes that you can choose for your donor DNA (Figure 2).

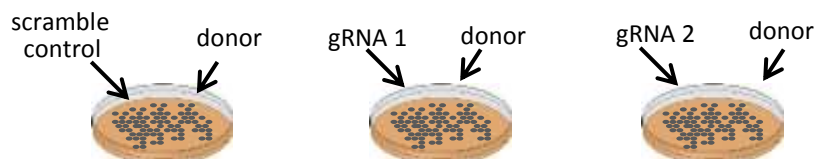
Figure 2. Four predesigned functional cassettes for the CRISPR knockout donor DNA



Experimental Protocol

A sample protocol listed below is for 6-well plates and uses [TurboFectin](#) (cat# TF81001) as transfection reagent. Please scale up or down the reagents based on the relative surface area of your plate (Table 2). Different types of cells require different transfection reagents. Please follow the manufacturer's protocol for your transfection.

1. Approximately 18-24 hours before transfection, plate $\sim 3 \times 10^5$ adherent cells in 2 ml culture media into each well of a 6-well plate, or $\sim 5 \times 10^5$ 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. Set up three transfections 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.

- a. Dilute 1 μ g of one of the gRNA vectors (or scramble control) in 250 μ L of Opti-MEM I (Life Technologies), vortex gently. Then add 1 μ g of the donor DNA into the same 250 μ L 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 μ L 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 2; 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% CO₂ 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 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.

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

Note: the "µg of DNA" is the total DNA amount used per well.

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 (Figure 3). 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 Figure 4. The amplified PCR fragment is around 1kb. There could be some difficulties doing genomic PCR or measuring mRNA via qPCR at this step before selection 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. 3). Since donor DNA contains 600bp left homologous arm sequence which is immediately upstream of ATG, Donor DNA transfected cells could express weak or bright green fluorescence depending how much promoter sequence the left homologous arm sequence contains.

Note 3. Co-transfect gRNA1, gRNA2 and donor DNA may generate better knockout efficiency.

Figure 3. Diagram of cell passaging after transfection

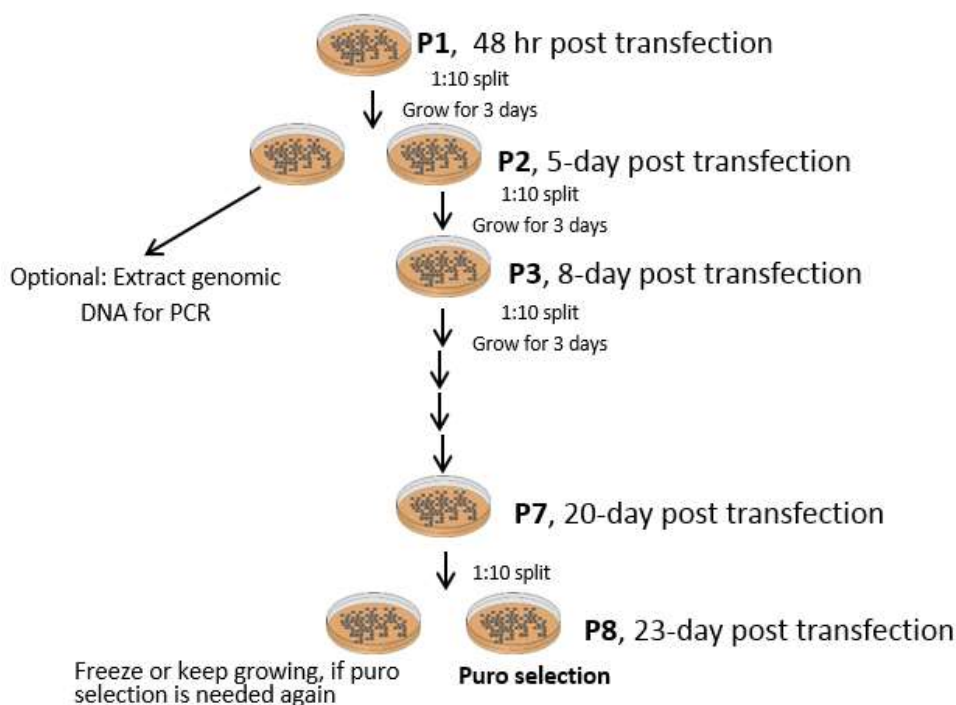
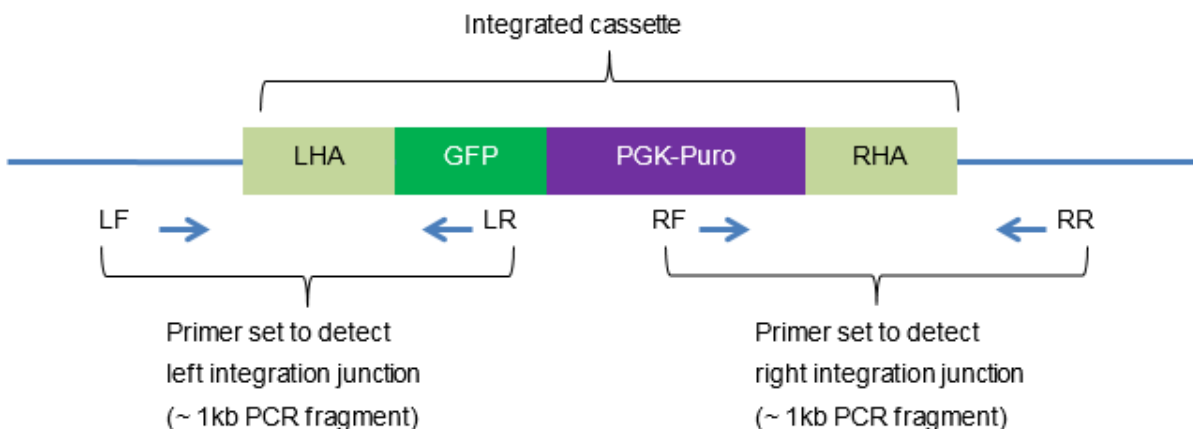


Figure 4. Diagram of genomic PCR Primer design



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

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 do this 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; however, the efficiency should be a lot lower than with a specific gRNA. You should get more colonies with gene specific gRNA than scramble control if the gene specific gRNA cleaves efficiently.

6. Isolate individual cell colonies.
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 around 1-2 cells/well in 96-well plate, after 1-2 weeks, observe under the microscope, select the wells only containing one cell colony, and expand the cells in 6-well plates when they are confluent in the 96-well plate.

- 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, apply puromycin selection when seeding.

Note 1: How to make biallelic knockout: If you only have monoallelic knockout (heterozygous) and 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. OriGene has both functional cassettes. You can do the knockout procedure again with the new donor vector to target the second allele since 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 to target the second allele.

Note 2: If your 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.

KN2.0, non-homology mediated CRISPR knockout kit

Package contents

- 2 vials of gRNA vectors, (SKU KNxxxxxxG1, KNxxxxxxG2), 3-5 µg DNA (in TE buffer or lyophilized)
- 1 vial of linear donor LoxP-EF1a-GFP-P2A-Puro-LoxP (SKU KNxxxxxD), 10 µg, lyophilized. Reconstitute in 100 µL dH₂O, final concentration 100 ng/ µL.
- Certificate of Analysis
- Application Guide

Note: The product is shipped at room temperature, but should be kept at -20°C 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>
- Antibodies: <https://www.origene.com/products/antibodies>
- 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

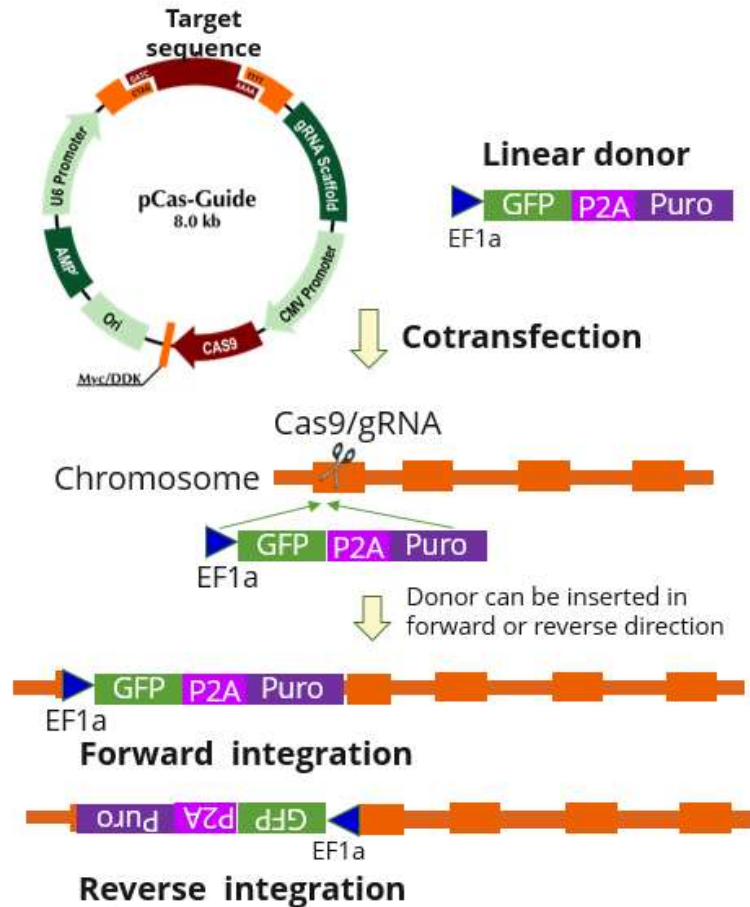
This product is for research use only. Use in and/or for diagnostics and therapeutics is strictly prohibited. By opening and using the product, the purchaser agrees to the following: The plasmids may not be distributed, resold, modified for resale or used to manufacture commercial products without prior 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. Each kit contains two gene specific gRNA vectors and one linear donor DNA. The gene specific gRNA will lead Cas9 to cut the target genome, and the cutting site will be repaired by the integration of predesigned linear donor containing selection marker and reporter gene. The integration can happen at both forward and reverse direction.

The knockout efficiency of KN2.0 gene knockout kit is much higher than HDR-mediated gene knockout kit. Most knockout cells are bi-allelic knockout, with one allele containing donor integration, and the other allele containing indels (insertion and deletion), which affects protein coding or causes premature stop. Check the following diagram to find more about KN2.0 CRISPR knockout kit (Figure 5).

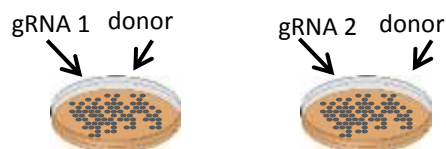
Figure 5. Diagram of KN2.0 non-homology-mediated CRISPR knockout kit



Experimental Protocol

A sample protocol listed below is for 6-well plates and using [TurboFectin](#) (cat# TF81001) as transfection reagent. Please scale up or down the reagents based on the relative surface area of your plate (Table 2). Different types of cells require different transfection reagents. Please follow the manufacturer's protocol for your transfection.

1. Approximately 18-24 hours before transfection, plate $\sim 3 \times 10^5$ adherent cells in 2 ml culture media into each well of a 6-well plate or $\sim 5 \times 10^5$ 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. Set up two separate transfections 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.

- a. Dilute 1 µg of one of the gRNA vectors in 250 µL 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 for Turbofectin:DNA. However, subsequent optimization may further increase the transfection efficiency.

Note: 2. Please find the best transfection method for your specific cells, and follow the corresponding transfection protocol. 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% CO₂ 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%.

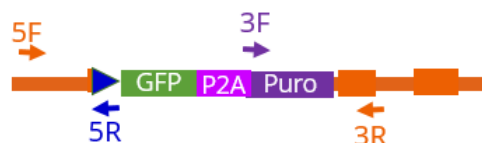
Time lines of genome editing

- ✓ 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 (Figure 6). Primer pair needs to be designed to 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. qPCR measuring the targeted mRNA level would not work due to the small percentage of edited cells.

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

Forward integration



Reverse integration

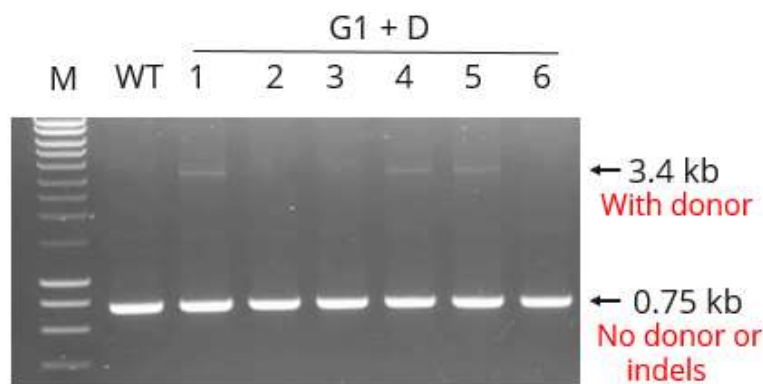


- Apply puromycin selection or GFP sorting. Split P3 or P5 cells 1:10, and then grow cells directly in the puromycin containing complete media in 10 cm dishes. The dose of puromycin needs to be determined with a kill curve to find out the lowest dose that kills the non-transfected cells completely 4-7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

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

- 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, which could also lead to protein reading frame change or premature stop. You can directly sequence the amplified genomic fragment to verify the sequence.

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 gRNA1 and donor transfected.

- Isolate individual cell colonies.

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

- 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.

- 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. 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 not-green 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 not-green cell colonies.

EF1a-GFP-P2A-Puro selection cassette sequence:

ATAACTTCGTATAATGTATGCTATACGAAGTTATCGTGAGGCTCCGGTGCCCGTCAGTGGGCAGAGCGCACATCGC
 CCACAGTCCCCGAGAAGTTGGGGGGAGGGGTCGGCAATTGAACCGGTGCCTAGAGAAGGTGGCGCGGGGTAAC
 TGGGAAAGTGATGTCGTGTACTGGCTCCGCTTTTTCCCAGGGTGGGGGAGAACCCTATATAAGTGCAGTAGTC
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 ACAAATTTACAAATAAAGCATTTTTTTCACTGCATTCTAGTTGTGGTTTGTCCAAACTCATCAATGTATCTTAATA
 ACTTCGTATAATGTATGCTATACGAAGTTAT

Features:

LoxP	cyan
EF1A promoter	blue
tGFP (699 bp)	green
Puromycin resistance (600 bp)	purple
P2A sequence (66 bp)	red

FAQ

Q1: Is donor plasmid necessary for knocking out a target gene?

Without donor template DNA, the double-stranded break will be repaired by NHEJ, which tends to introduce unpredicted indels. You will need to screen the deletions/insertions that cause frame shift of target gene. With donor DNA, you can get desired insertion/deletion/mutations. You can also add mammalian selection in donor DNA, which can greatly simplify your downstream screening procedures.

Q2: 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/knockout-kits>

Q3: 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.

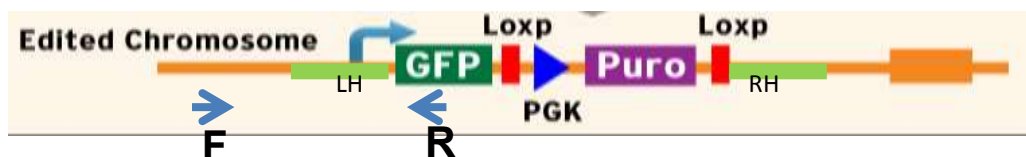
Q4: Do I get monoallelic knock-out or biallelic knock-out using the homology-mediated knock-out kit via CRISPR? What do I need to do to get biallelic knock-out?

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.

Q5: 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-integration_3R

TAGGTGCCGAAGTGGTAGAAGC



Q6: 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 knockouts are biallelic, one allele has donor integration, and the other allele has indels (insertion and deletion).

Q7: 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 also show CRISPR KN 2.0 is highly efficient and render improved knockout rate.

Q8: 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).

Q9: If after puromycin selection using KN2.0 kit, I have no cells survived, what could be the reason?

There are two possible reasons: 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, such as electroporation, is needed.

Q10: Can KN2.0 be used for embryo microinjection to generate transgenic animal models?

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 required.