

TargeTron® Gene Knockout System

USER GUIDE

Catalog Number
TA0100

 **TargeTron.**

SIGMA-ALDRICH®

Ordering Information

Catalog No.	Product Description	Pkg Size
TA0100	TargeTron® Gene Knockout System	3 ea 10 ea

Note: Reagents in the 3 ea kit are sufficient for 3 designs, 12 reactions. Reagents in the 10 ea kit are sufficient for 10 designs, 40 reactions.

Visit our Web site at sigma-aldrich.com/targetron for updates on new TargeTron products and protocols. Future releases may include vectors requiring no selection (convenient for multiple knockouts), alternative promoters, and broad host range plasmids.

To reorder product, call 1-800-325-3010, visit our Web site at sigma-aldrich.com, or contact your local sales representative.

TargeTron® Gene Knockout System

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Product Description

The TargeTron Gene Knockout System provides optimized reagents and protocols for the rapid and specific disruption of bacterial genes by insertion of group II introns. Unlike conventional DNA transposon mutagenesis, the TargeTron system is site-specific, not random. Recent advances in group II intron research have enabled re-targeting of introns to insert efficiently into virtually any desired DNA target.^{1,2,3,4,5,6} Features of the TargeTron retrohoming mechanism make it attractive for genetic manipulation. First, retrohoming is highly efficient and specific. Retrohoming frequencies can approach 100% and are six orders of magnitude higher than the frequency of ectopic integration. Second, the introns are minimally dependent on host factors, making them applicable to a broad range of bacteria. To date, the TargeTron system has been validated in *Escherichia coli*,³ *Staphylococcus aureus*,⁵ *Clostridium perfringens*,⁶ *Shigella flexneri*,² *Salmonella typhimurium*,² and *Lactococcus lactis*.¹

Group II introns insert themselves via the activity of an RNA-protein complex (RNP) expressed from a single plasmid provided in the kit. The RNA portion of the RNP is easily mutated to re-target insertion into a user-specified chromosomal gene. A somewhat accurate analogy is that group II introns are like programmable restriction enzymes, with the added activity of inserting RNA into a cleaved DNA sequence.

Figure 1 outlines the process of using TargeTron system to knockout bacterial genes. First, a computer algorithm is used to identify target sites in your gene of interest. A typical 1 kb gene can be expected to contain 5 to 11 group II intron insertion sites. Second, the computer algorithm outputs primer sequences, which are used to mutate (re-target) the intron by PCR. Next, the mutated 350 bp PCR fragment is ligated into a linearized vector that contains the remaining intron components. The ligation reaction is transformed into the host followed by expression of the re-targeted intron. Knockouts are then selected using a kanamycin marker that is activated upon chromosomal insertion. Using gene specific primers, kanamycin resistant colonies are PCR screened to confirm insertion.

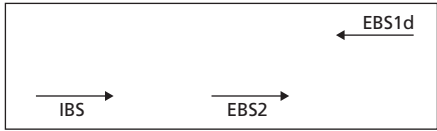
Before attempting to use TargeTron Vectors in your bacterial strain, carefully consider the plasmid replication requirements of your host. There might be host-specific requirements for the origin of replication, antibiotic resistance, and promoter.

References

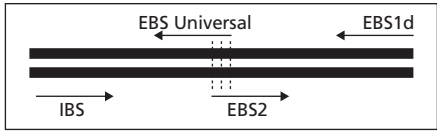
1. Frazier, C. L.; et al., Genetic manipulation of *Lactococcus lactis* by using targeted group II introns: generation of stable insertions without selection. *Appl. Environ. Microbiol.*, **2000**, *69*, 1121–1128.
2. Karberg, M.; et al. Group II introns as controllable gene targeting vectors for genetic manipulation of bacteria. *Nat. Biotechnol.*, **2001**, *19*, 1162–1167.
3. Perutka, J.; et al. Use of computer-designed group II introns to disrupt *Escherichia coli* DExH/D-box protein and DNA helicase genes. *J. Mol. Biol.*, **2004**, *336*, 421–439.
4. Zhong, J.; et al. Targeted and random bacterial gene disruption using a group II intron (targetron) vector containing a retrotransposition-activated selectable marker. *Nucleic Acids Res.*, **2003**, *31*, 1656–1664.
5. Yao, J.; et al. Use of targetrons to disrupt essential and nonessential genes in *Staphylococcus aureus* reveals temperature sensitivity of Ll.LtrB group II intron splicing. *RNA*. **2006**, *12*, 1271–81.
6. Chen, Y.; et al. Construction of an alpha toxin gene knockout mutant of *Clostridium perfringens* type A by use of a mobile group II intron. *Appl. Environ. Microbiol.*, **2005**, *71*, 7542–7547.

Figure 1: TargeTron® Gene Knockout System

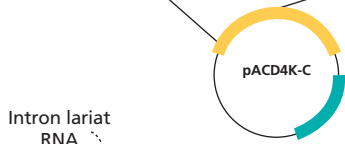
Select DNA target site from TargeTron Design Web site and order 3 primers/target site.



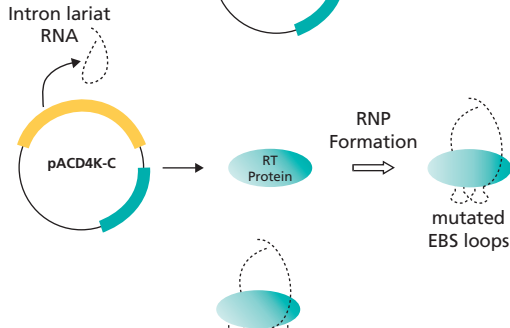
1-step assembly PCR to mutate (re-target) intron RNA (350 bp PCR product).



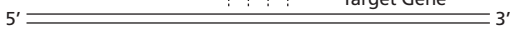
Clone mutated PCR fragment into intron expression vector.



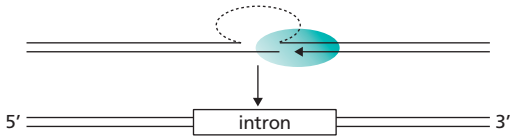
Transform host and express RNA-protein complex (RNP).



Re-targeted RNP locates genomic target.



RNP inserts RNA, reverse transcribes cDNA, and host enzymes repair to create a permanent insertion.



Precautions and Disclaimer

This product is for R&D use only, not for drug, household, or other uses. Please consult the Material Safety Data Sheet for information regarding hazards and safe handling practices.

Storage and Stability

This product ships on wet ice and storage at -20°C is recommended.

Reagents Provided

- TargeTron Access Card (provides password for online target site selection)
- Intron PCR Template
- EBS Universal Primer
- *lacZ* Control Primer Mix
- pACD4K-C Linear Vector (20 ng/mL)
- JumpStart™ REDTaq® ReadyMix™
- *Hind* III 20 U/ μL
- *Bsr*G I 10 U/ μL
- 10 \times Restriction Enzyme Buffer

Additional Reagents Recommended, Not Supplied

- Chloramphenicol, Catalog No. **C1863**
- GC5™ Competent Cells, Catalog No. **G3169**
- Gene specific primers to detect chromosomal insertions by PCR
- GenElute™ PCR Clean-up Kit, Catalog No. **NA1020**
- IPTG (Isopropyl β -D-1-thiogalactopyranosid)Isopropyl β -D-1-thiogalactopyranosid), Catalog No. **I6758**
- Kanamycin sulfate, Catalog No. **K3763**
- LB Agar EZMix™ Powder, Catalog No. **L7533**
- LB Agar plates with kanamycin (25 $\mu\text{g}/\text{mL}$)
- LB Broth with and without chloramphenicol (25 $\mu\text{g}/\text{mL}$) and 1% glucose (used with DE3 strains)
- LB Broth, liquid, Catalog No. **L2542**
- Pre-cast Agarose Gels, 1% (8-well) , Catalog No. **P5472**
- Pre-cast Agarose Gels, 1% (24-well) , Catalog No. **P5972**
- Pre-cast Agarose Gels, 4% (24-well), Catalog No. **P6097**
- Quick-Link™ DNA Ligation Kit, Catalog No. **LIG2**, or equivalent T4 DNA Ligase and buffer
- SOC Medium, Catalog No. **S1797**
- Target site primers
- Vectors
 - pACD4 TargeTron Vector Set, Catalog No. **TV0010**
 - TargeTron Vector pACD4K-C-loxP, Catalog No. **T2826**
 - TargeTron Vector pAR1219, Catalog No. **T2076**
 - TargeTron Vector pNL9164, Catalog No. **T6701**
 - TargeTron Vector pJIR750ai, Catalog No. **T7701**
- Water, Molecular Biology Reagent, Catalog No. **W4502**
- X-Gal (5-Bromo-4-chloro-3-indolyl β -D-galactopyranoside), Catalog No. **B9146**

Procedure

1. Protocol overview

Target Site Selection

- Cut and paste your gene sequence into the algorithm field on the TargeTron Design Site.
- Submit your sequence to generate potential insertion sites (usually 5 to 11 sites are found per 1 kb gene) and the primer designs to re-target the intron.
- Once the sequence is submitted, one access has been used. Please download and save the output/primer designs. Once you leave the output screen, you will not be able to view the output/primer designs again without using another access.
- Order the 3 primers required to re-target the intron to your target site.

Lab Day 1

- Perform PCR reaction to mutate (re-target) the RNA portion of intron.
- Cut the PCR fragment to produce cohesive ends (*Hind* III and *Bsr*G I).
- Ligate the mutated PCR fragment to a linearized vector containing the remaining portion of the intron.
- For strains with high transformation efficiency (e.g. $>10^7$ cfu/ug), directly transform the ligation reaction into the knockout host. For low efficiency strains, it is best to isolate a plasmid clone in an *E. coli* cloning strain, then transform the isolated re-targeted plasmid into the knockout strain.

Lab Day 2

- Grow the cells to log-phase and induce intron expression and insertion.
- Plate the cells on kanamycin-containing agar medium to select for insertions.

Lab Day 3

- Confirm the presence of gene specific insertions by colony PCR.

2. Detailed Protocol

A. Target site selection and primer ordering

Note: A set of control primers provided in the kit can be used to knockout the *lacZ* gene in *E. coli* (see page 13 for the *lacZ* target site sequence for these primers).

1. Cut and paste your target DNA sequence into the algorithm field on the TargeTron Design Site: sigma-aldrich.com/targetronaccess. Once you have input your target DNA sequence, click submit once. Submitting the target DNA sequence is considered one access.
2. From the output design options, locate the region of the gene you want to disrupt and pick the target site with the lowest E-value.

Note: Please download and save the output design options. Once you leave the output screen, you will not be able to view the output design options again without using another access.

3. Order the primer sets (IBS, EBS2, and EBS1d) corresponding to chosen design options.

Note: The TargeTron algorithm identifies potential insertion sites, each rated with an E-value. Lower E-values correspond to higher predicted intron insertion efficiency. Target sites with an E-value <0.5 are predicted to be efficient introns. The target sites are named by their insertion point within the DNA sequence and are noted as sense (s) or anti-sense (a).

A 1 kb gene should have roughly 5 to 11 target sites from which to choose. For DNA sequences <400 bp, optimal target sites may not be present. It is recommended that at least 2 target sites be selected for a gene of interest. Several factors may affect the success or failure of a given design option, including:

- Accuracy of the known genomic sequence (especially important when testing alternative bacterial strains that might possess genomic deletions or rearrangements relative to a known genomic sequence).
- Essentiality of the target gene under given growth conditions.
- Polar effects (e.g., insertion affecting expression of adjacent genes).
- The actual efficiency of the re-targeted intron.

Three unique primers, IBS, EBS2, and EBS1d, are required to re-target the intron to insert at a specific site. The sequence of these primers will be generated automatically for each target site. De-salt purity primers are sufficient in most cases; however, optimal PCR specificity and yield is obtained with primers of HPLC purity.

B. PCR for intron re-targeting

Note 1: In this step, a PCR reaction is performed which re-targets the intron by primer-mediated mutation. The PCR uses four primers in a single-tube reaction to mutate the intron at several positions spanning a 350 bp region.

1. Upon receipt of the primers, dilute (using water-molecular biology reagent) the IBS and EBS1d primers to 100 μM , and the EBS2 primer to 20 μM .
2. Make a four-primer master mix as follows:

2 μL	IBS primer (100 μM)
2 μL	EBS1d primer (100 μM)
2 μL	EBS2 primer (20 μM)
2 μL	EBS Universal Primer (20 μM)
12 μL	Water (molecular biology reagent)
20 μL	Total volume

Note 2: The *LacZ* Control Primer Mix included with the kit is a ready-to-use four-primer set. These primers will re-target the intron to knockout the *E. coli lacZ* gene and can be used to test the performance of the kit from PCR through final knockout.

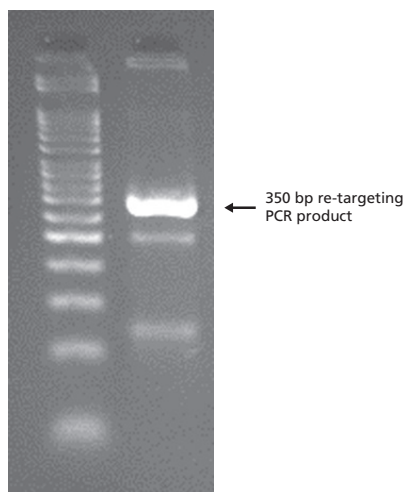
3. Set up the PCR reaction as follows:

23 μL	Water (molecular biology reagent)
1 μL	Four-primer mix (<i>lacZ</i> control or specific primers for your target site)
1 μL	Intron PCR Template
25 μL	JumpStart REDTaq ReadyMix
50 μL	Total volume

4. Cycle the PCR reaction as follows:

Initial Denaturation	94 °C, 30 seconds
30 Cycles:	
Denaturation	94 °C, 15 seconds
Annealing	55 °C, 30 seconds
Annealing/Extension	72 °C, 30 seconds
Final Extension	72 °C, 2 minutes
Soak	4 °C

5. Typical PCR yields are 10–30 ng/μL. When run on a 4% agarose gel, three bands should be visible. The uppermost 350 bp band is the desired product and should be the predominant band. Even though lower bands are visible, they do not adversely affect the cloning reaction, and do not need to be removed by agarose gel purification.



6. Purify the DNA from the reaction using the GenElute PCR Clean-up Kit, Catalog No. **NA1020**.

C. Double digestion with *Hind* III and *Bsr*G I

Note 1: Following PCR of the 350 bp fragment, a modification to the digestion reaction may be required. Please refer to the table of TargeTron vectors below in order to determine whether *Dpn* I should be added to the *Hind* III and *Bsr*G I digestion reaction.

Vector	<i>Dpn</i> I Required	<i>Dpn</i> I Not Required
pACD4K-C		X
TargeTron Vector pACD4K-C-loxP, Cat. No. T2826		X
pACD4 TargeTron Vector Set, Cat. No. TV0010		X
TargeTron Vector pNL9164, Cat. No. T6701	X	
TargeTron Vector pJIR750ai, Cat. No. T7701		X

Note 2: Following purification of the PCR product, cohesive ends must be generated for ligation into the pACD4K-C Linear Vector.

1. Set up a restriction digestion as follows:

8 μ L	Purified PCR product (~200 ng)
2 μ L	10 \times Restriction Enzyme Buffer
1 μ L	<i>Hind</i> III (20 U/ μ L)
1 μ L	<i>BsrG</i> I (10 U/ μ L)
8 μ L	Water (molecular biology reagent)
20 μ L	Total volume

2. Incubate the reaction for:

30 minutes, 37 °C

30 minutes, 60 °C

10 minutes, 80 °C

Note 3: *BsrG* I is thermophilic and has increased activity at 60 °C.

D. Ligation and transformation

Note 1: The following ligation example uses buffers and volumes recommended in the Quick-Link T4 DNA Ligation Kit Catalog No. **LIG2**.

1. Mix the following:

2 μ L	pACD4K-C Linear Vector (40 ng)
6 μ L	<i>Hind</i> III/ <i>BsrG</i> I-digested intron PCR product (350 bp)
10 μ L	Quick-Link Buffer A
2 μ L	Quick-Link Buffer B
20 μ L	Total volume

2. Mix, heat to 60 °C for 30 seconds.
3. Cool on ice for 1 minute.
4. Add 1 μ L of Quick-Link Ligase, mix.
5. Incubate at room temperature for 30 minutes.
6. Transform into cloning strain or desired knockout strain according to organism-specific protocols.

Optional: The efficiency of the control ligation reaction can be assessed by plating the transformation on LB agar-chloramphenicol (25 μ g/mL)-X-Gal-IPTG (30 mL each of 20 mg/mL X-Gal and 100 mM IPTG spread on plates) and observing the ratio of white (successfully re-targeted) to blue (non-mutated/parental) colonies. This screen must be performed in a *lacZ α* complementing strain of *E. coli* such as GC5TM or DH5aTM.

The linearized pACD4K-C plasmid sold in this kit contains a 769 bp *lacZ α* fragment between the *Hind* III and *BsrG* I sites. Upon linearization this was removed, ready for replacement with a 350 bp re-targeting fragment. Any blue colonies observed during re-targeting/cloning steps are not re-targeted and will not produce successful knockouts.

E. Induction of gene disruption

Note 1: The pACD4K-C vector contains a T7 promoter to express the intron. Thus, a DE3 strain of *E. coli* (or a strain expressing T7 polymerase) is necessary for induction of TargetTron intron-mediated gene disruption. See Appendix for guidelines on using TargetTron in non-DE3 strains.

Note 2: If the host strain is low competency ($< 10^7$ cfu/ug), it may be necessary to first transform a high competency *E. coli* strain with the ligation reaction, isolate a re-targeted clone (e.g. white colonies on plates with IPTG and X-Gal), then transform the host strain with the clonal plasmid.

Note 3: pACD4K-C is chloramphenicol resistant for general selection and propagation of the plasmid. When integrated into the chromosomal target site, a kanamycin marker is activated. The kanamycin marker located within the group II intron on pACD4K-C is interrupted by a td group I intron. When the group II intron is transcribed and spliced, the td group I intron is excised, activating the kanamycin marker. After insertion of the group II intron into the host genome, kanamycin resistance can be used to select for mutants containing genes disrupted by the group II intron.

Some vectors, such as the pACD4-A,C,G,T, Cat. No. **TV0010**, do not contain a selectable antibiotic marker in the intron. In many cases a selectable marker is not required to isolate an insertional mutant, which can often be identified simply by colony PCR screening. This can expedite sequential knockouts in multiple genes by not having to remove the selection marker.

Note 4: When culturing pACD4K plasmids in DE3 strains, 1% glucose is added to repress the lacUV5 promoter of T7 RNA polymerase on the DE3 *E. coli* chromosome. This will prevent premature group II intron transcription before IPTG induction that may have an adverse effect on viability.

Note 5: The following procedure is a general guideline. For specific procedures using *E. coli*-like organisms, refer to the next section outlining gene knockout of *lacZ* in BL21 (DE3). For other organisms, alter the *lacZ* example procedure to use growth conditions optimal for the particular strain.

1. Continue from transformation of the desired knockout strain.
2. Add part of the transformation reaction to medium containing chloramphenicol and glucose (if DE3 strain). Grow overnight or as appropriate.
3. Dilute an overnight (saturated) culture in fresh medium containing chloramphenicol and glucose (if DE3 strain) and grow to an OD_{600} of ~ 0.2 .
4. Add IPTG (or other promoter-specific induction reagent) and incubate.
5. Centrifuge the cells and resuspend in medium containing glucose (if DE3 strain).
6. Incubate cells with shaking.
7. Plate cells on nutrient agar containing kanamycin.
8. Screen kanamycin resistant colonies for gene disruption by colony PCR.

F. Example for knockout of *lacZ* in *Escherichia coli* BL21(DE3)

Note 1: The procedure below starts with the transformation of the ligation reaction or the transformation of an isolated (e.g. clonal) re-targeted plasmid. If the transformation efficiency of your BL21(DE3) strain is $> 10^7$ cfu/mg, then the ligation reaction can be used directly for knockout. If the transformation efficiency $< 10^7$ cfu/mg, isolate a clonal plasmid, then transform BL21(DE3).

1. On ice, add 1 μ L of the ligation reaction (or clonal plasmid) to 50 μ L of chemically competent *E. coli* BL21(DE3). Incubate on ice for 10 minutes.

- Heat shock the cells for 40 seconds at 42 °C. Place cells on ice for 2 minutes. Add 450 μL of room temperature SOC Medium.
- Grow at 37 °C with shaking for 1 hour. Add 100 μL of this transformation reaction to 3 mL of LB, containing 25 $\mu\text{g}/\text{mL}$ chloramphenicol, 1% glucose. Incubate overnight at 37 °C with shaking.
- The following morning, add 40 μL of the overnight culture to 2 mL of LB broth containing 25 $\mu\text{g}/\text{mL}$ chloramphenicol, 1% glucose and grow at 37 °C to an OD_{600} of ~ 0.2 .
- When the OD_{600} is ~ 0.2 , cool the incubator to 30 °C. Add 10 μL of 100 mM stock IPTG to the 2 mL culture and incubate at 30 °C for 30 minutes with shaking.

Note 2: This IPTG step induces group II intron transcription. The intron encoded protein is then translated and forms an RNP complex with the group II intron RNA transcript. This RNP scans the chromosome for the specific site of insertion and disrupts the gene of interest.

- After the 30-minute induction step, immediately centrifuge the cells at maximum speed in a microcentrifuge for 1 minute.
- Resuspend in 1.0 mL of LB broth containing 1% glucose (no chloramphenicol).
- Incubate at 30 °C for 1 hour with shaking.
- Plate 100 μL of culture on an LB agar plate containing 25 $\mu\text{g}/\text{mL}$ kanamycin.

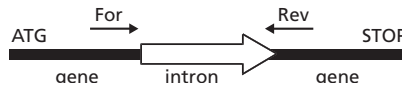
Optional: Spread 30 μL each of 20 mg/mL X-Gal and 100 mM IPTG onto the plate in order to confirm *lacZ* knockout in the control reaction (white colonies). When testing a re-targeted intron for the first time, plating the entire culture is recommended. Do this by centrifuging the 1.0 mL culture, resuspending in 100 μL of LB broth, and plating on LB agar plates containing 25 $\mu\text{g}/\text{mL}$ kanamycin.

- Grow overnight at 30 °C or on the bench top at room temperature for several days (Friday afternoon to Monday morning is sufficient at room temperature).
- Pick colonies and perform colony PCR across the intron-gene junction to verify the correct target site insertion.

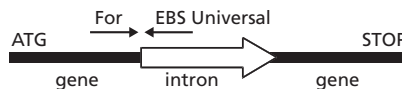
G. Confirmation of knockouts by colony PCR

Two options for PCR detection of intron insertions are shown below:

- Use gene specific primers that flank the insertion site and amplify the entire inserted intron (~ 2 kb). Here, intron insertion is shown in the sense direction.



- Use a gene specific primer and an intron specific primer to amplify across gene-intron junctions.



OR

