Site-Specific Chromosomal Mutagenesis Using the TargeTron™ Gene Knockout System

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- Site-specific insertional mutagenesis of bacterial chromosomes and plasmids
- Minimal dependence on host factors allowing adaptation to many bacterial genera.
- Knockout genes without inserting an antibiotic selection marker[6] and expedite creation of multiple knockouts
- Ability to create knock-ins by delivering heterologous DNA to targeted chromosomal sites[5,7]

Introduction

The TargeTron™ Gene Knockout System is a novel prokaryotic functional genomics tool based on the Ll.LtrB group II intron from *Lactococcus lactis.* Like DNA transposons, mobile group II introns can inactivate genes by insertion; however, recent advances in group II intron research have enabled insertion to be site-specific.[2,6,7] At the heart of the TargeTron system is an RNA-protein complex (RNP) that can be delivered to specific DNA sequences by virtue of base pairing between the RNA component of the RNP and target site DNA (Figure 1). This allows rapid modification of the site-specificity of the RNP by PCR directed mutagenesis. The insertion of group II introns is minimally dependent on host factors, making them applicable to a broad range of bacteria. To date, the TargeTron system has successfully knocked out genes in *Escherichia coli*,[2,6,7] *Staphylococcus aureus, Clostridium perfringens, Shigella flexneri*,[2] *Salmonella typhimurium*,[2] and *Lactococcus lactis.*[1] Many re-targeted introns are so efficient that selection is not required, allowing screening for insertional mutants by colony PCR.[6] This eliminates the need to remove selection markers and expedites the creation of multiple knockouts. In addition to knockouts, knock-ins are also possible[7] since heterologous DNA can be cloned into the intron and taken to specific genomic sites by user designed introns. This feature was recently used to examine the use of the TargeTron system to introduce therapeutic sequences site specifically into mutant genes.[5]

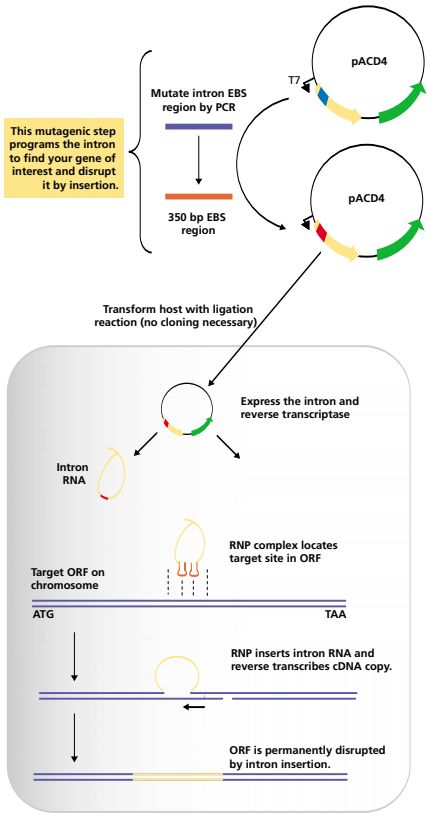

Figure 1. The DNA binding specificity of the TargeTron RNP is determined primarily by the EBS regions encoded on the intron RNA (red). *This allows rapid mutation of the EBS regions to target specific chromosomal regions for insertional mutagenesis. Sequences of IBS/EBS mutagenic primers are automatically generated by the Web-based TargeTron design tool* ***(www.sigmagenosys.com/targetron)****.*

Rapidly Mutate Entire Gene Families for Functional Genomics Studies

Recently, the targeting rules for the TargeTron intron were deciphered sufficiently to allow re-targeting to essentially any gene.[6] This allowed for the successful knockout of 27 of 28 targeted DExH/D-box genes, the only exception being an essential gene, *sec*A. In this study, 21 of the 27 knockouts were obtained without introducing antibiotic selection to the chromosome. In the six remaining cases, a novel retrotransposition activated selectable marker (RAM) was used to isolate insertional mutants by selection on trimethoprim. Recently at Sigma, the utility of the TargeTron method was used to rapidly knockout 10 randomly chosen non-essential *E. coli* genes using an alternative kanamycin RAM which is available in the current TargeTron Kit (Product Code TA0100). Table 1 lists all of the *E. coli* genes knocked out to date using the TargeTron system.

Use TargeTron Site-Specificity to Mutate Several Domains within a Gene

RNase E is known to regulate the degradation rate of thousands of mRNA transcripts encoded within the *E. coli* genome.[3] The N-terminal region has been shown to be essential, while the C-terminal region is dispensible.[4] The site-specific nature of TargeTron insertion allowed step-wise mutagenesis into the N-terminal essential region to estimate the maximum truncation the RNase E gene could tolerate and retain cell viability. An example of PCR confirmation of a targeted intron insertion, *rne*1259::intron, is shown in Figure 2. The *rne*1259::intron mutant was the smallest truncation obtained and was shown to have severely impaired growth rate (>100 min). In addition, increased levels of *rpsO* transcript were observed by qRT-PCR (Figure 3) which is consistent with previous observations of RNase E mutants.[4] Additional site-specific mutants were easily made in the C-terminal region and had no effect on transcript level as measured by qRT-PCR (data not shown).

Table 1. *E. coli* genes that have been disrupted by TargeTron insertional mutagenesis.

bcr	*fadR*	*mfd*	*recB*	*rne*	*yecE*
carB	*helD*	*nagA*	*recC*	*srmB*	*yejH*
dadA	*hepA*	*ndk*	*recD*	*thyA*	*yfjK*
dbpA	*hrpA*	*nuoE*	*recG*	*trpE*	*ygcB*
deaD	*hrpB*	*phoH*	*recQ*	*uvrB*	*yhjU*
deoR	*lacZ*	*priA*	*rep*	*uvrD*	*yoaA*
dinG	*lhr*	*proA*	*rhlB*	*ybeZ*	
endA	*malM*	*rbfa*	*rhlE*	*ycac*	

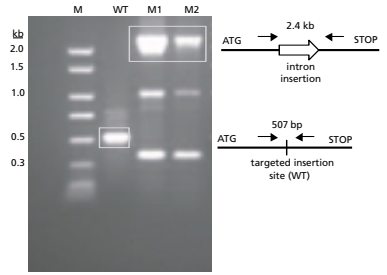

Figure 2. Targeted insertion into the RNase E ORF at nucleotide 1259. *WT: colony PCR across the intron insertion site in wild-type BL21(DE3). M1 and M2: colony PCR of two colonies showing group II intron insertion. Successful insertions were also obtained at nucleotides 2256s (enolase region), 2670s (PNPase region), and 2905a (PNPase region).*

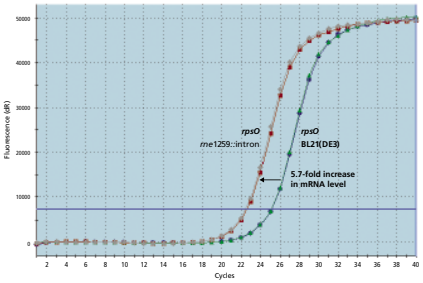

Figure 3. Relative levels of rpsO mRNA in E. coli BL21(DE3) and an rne1259::intron insertional mutant as measured by quantitative RT-PCR. *Data shown for mutant and BL21(DE3) is in duplicate. The rne1259::intron insertion resulted in a 5.7-fold increase in rpsO mRNA levels (ΔC_t = 2.5).*

References

1. Frazier, C.L., San Filippo, J., Lambowitz, A.M., Mills, D.A. Genetic manipulation of *Lactococcus lactis* by using targeted group II introns: generation of stable insertions without selection. *Appl. Environ. Microbiol.* **69**(2), 1121-1128 (2003).
2. Karberg, M., Guo, H., Zhong, J., Coon, R., Perutka, J., Lambowitz, A.M. Group II introns as controllable gene targeting vectors for genetic manipulation of bacteria. *Nat. Biotechnol.* **19**(12), 1162-1167 (2001).
3. Lee, K., Zhan, X., Gao, J., Qiu, J., Feng, Y., Meganathan, R., Cohen, S.N. and Georgiou, G. RraA: a protein inhibitor of RNase E activity that globally modulates RNA abundance in *E. coli*. *Cell.* **114**(5), 623 (2003).
4. Ow, M.C. and Kushner, S.R. Initiation of tRNA maturation by RNase E is essential for cell viability in *E. coli*. *Genes Dev.* **16**(9), 1102-1115 (2002).
5. Jones, J.P., Kierlin, M.N., Coon, R.G., Perutka, J., Lambowitz, A.M. and Sullenger, B.A. Retargeting mobile group II introns to repair mutant genes. *Mol. Ther.* **11**(5), 687-689 (2005).
6. Perutka, J., Wang, W., Goerlitz, D., Lambowitz, A.M. Use of computer-designed group II introns to disrupt *Escherichia coli* DExH/D-box protein and DNA helicase genes. *J. Mol. Biol.* **336**(2), 421-439 (2004).
7. Zhong, J., Karberg, M., Lambowitz, A.M. Targeted and random bacterial gene disruption using a group II intron (targetron) vector containing a retrotransposition-activated selectable marker. *Nucleic Acids Res.* **31**(6), 1656-1664 (2003).

Visit the TargeTron Web site **(sigma-aldrich.com/targetron)** *for product support and information.*

Ordering Information

Product	Description	Unit
TA0100	TargeTron™ Gene Knockout System	3 ea 10 ea