3050 Spruce Street, St. Louis, MO 63103 USA
Tel: (800) 521-8956 (314) 771-5765 Fax: (800) 325-5052 (314) 771-5757
email: techservice@sial.com sigma-aldrich.com

Product Information

TargeTron® Vector pNL9164

Catalog Number **T6701**
Storage Temperature –20 °C

TECHNICAL BULLETIN

Product Description

TargeTron Vector pNL9164 is a 9,295 bp expression vector derived from pNL9162[1]. pNL9164 is to be used in conjunction with the TargeTron Gene Knockout System, Catalog Number TA0100. This circularized vector can be used for targeted gene knockouts in gram-positive organisms such as *Staphylococcus aureus*[1]. Expression of the group II intron RNA and protein is under the control of the cadmium inducible promoter, P_{cad}-*cadC* [1,2]. To facilitate curing of the plasmid, pNL9164 has a temperature sensitive origin of replication, pT181 cop-634ts repC4 [1,2]. As supplied, the pNL9164 vector is re-targeted to the *S. aureus hsa* gene. The *hsa* gene encodes a member of the HU family of bacterial histone-like proteins[3]. For validation in a specific *S. aureus* strain, this vector can be used directly to knockout *hsa* without any further modifications using the enclosed protocol. In order to re-target this vector to knockout other genes, the *hsa* specific IBS-EBS fragment (350 bp) between the *Hin*d III and *Bsr*G I sites can be cut out and replaced with another gene specific fragment. For re-targeting protocols, substitute the restriction digest modification below when referring to the TargeTron Gene Knockout System User Guide, Catalog Number TA0100, at sigma-aldrich.com.

Digestion of 350 bp re-targeted PCR product with *Hind* III, *Bsr*G I and *Dpn* I

1. Set up a restriction digestion as follows:

8 µl	Purified PCR product (~200 ng)
2 µl	10× Restriction Enzyme Buffer
1 µl	*Hind* III (20 U/µl)
1 µl	*Bsr*G I (10 U/µl)
1 µl	*Dpn* I (20 U/µl)
7 µl	Water (molecular biology reagent)
20 µl	**Total volume**

2. Incubate the reaction for:
   40 minutes, 37 °C
   20 minutes, 60 °C
   10 minutes, 80 °C

Note: *Bsr*GI is thermophilic and has increased activity at 60 °C.

Key Features of the TargeTron Vector pNL9164
pT181 cop-634ts repC4 *ori*
Erythromycin resistance
Ampicillin resistance
ColE1 *ori*
cadC inducible promoter
5’ exon (IBS)
Intron RNA
EBS2
EBS1d
3’ exon
LtrA ORF

Reagent

Supplied at a concentration of 100 ng/µl in 10 mM Tris-HCl, pH 8.0, 1 mM EDTA.

Precautions/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

Store at –20 °C.

Protocol for Validation of *hsa* Knockout in *S. aureus* RN4220

1. Electroporate pNL9164 into *S. aureus* RN4220. **Note:** pNL9164 was propagated in *E.coli* and therefore might be sensitive to restriction-modification in certain *S. aureus* strains. RN4220 is a restriction-defective *S. aureus* strain that can be used to condition the pNL9164 vector before electroporation into other restriction-modification strains if necessary.
2. Grow cells at 32 °C in 1.0 ml BHI (brain heart infusion) medium for 1 hour.
3. Add 1.0 ml into 5.0 ml fresh BHI with erythromycin (10 μg/ml) and grow overnight at 32 °C.
4. (**Optional**) Make a 1:100 dilution of overnight culture into fresh BHI with erythromycin (10 μg/ml) and grow until early log phase (OD_{595} = 0.5). Induce the culture with10 μM $CdCl_2$ for 90 minutes. **Note:** The P*cad* promoter has low basal level transcription in the absence of cadmium. The *hsa*-24s Targetron is very efficient, routinely giving 100% knockouts even without cadmium induction in S. *aureus* RN4220[1]. For other target genes, cadmium induction may be necessary to increase knockout efficiency. Therefore, the induction step is required.
5. Plate on BHI agar plates containing 10 μg/ml erythromycin.
6. Incubate plates at 32 °C.
7. Perform colony PCR to confirm TargeTron integration using the primers listed below.
8. The PCR results should yield a 0.4 kb amplicon for wild-type RN4220 *hsa* and a 1.3 kb amplicon for the mutant *hsa* disruptant containing the intron.

***hsa* forward primer**
5'-CGGAATCAGGAGGTGAATGTCTAATGA

***hsa* reverse primer**
5'-CGGAATTCACTTAGAAGAACATTACAATTTAT

***Hsa*-24s target sequence**
TGTCTAATGAACAAAACAGATTTAATCAAT - intron
GCAGTTGCAGAGCAA

In addition to strains RN4220 and RN8098[1], the *Hsa*-24s target sequence is present exactly as presented above in all sequenced strains of *S. aureus* (per NCBI microbial BLAST), namely:

Staphylococcus aureus RF122
Staphylococcus aureus COL
Staphylococcus aureus MRSA252
Staphylococcus aureus MSSA476
Staphylococcus aureus MW2
Staphylococcus aureus Mu50
Staphylococcus aureus N315
Staphylococcus aureus NCTC 8325
Staphylococcus aureus USA300

The *hsa*-24s sequence is also present in *Bacillus halodurans* C-125 and in *Bacillus clausii* KSM-K16 with three or fewer mutations.

Temperature sensitive plasmid curing

For intron insertions in the sense direction, it is possible for the intron to splice out of mRNA with the assistance of the LtrA protein encoded on pNL9164. Thus, for accurately observing phenotypic effects of a given disruption, a re-targeted pNL9164 plasmid should be cured from the host. Conversely, the intron cannot splice out when inserted in the antisense ORF orientation and curing is still recommended, but not absolutely required. In order to cure the pNL9164 donor plasmid after a knockout has been confirmed, grow the strain overnight at 43 °C in BHI medium (without antibiotic) and screen for erythromycin sensitive colonies[1,2]. If the targeted gene is essential or partially essential for cell survival, curing of the donor plasmid could be more difficult or unattainable[1]. For example, *hsa*-24s disruptants have been shown to be conditional and can grow at 32 °C but not at 43 °C[1].

Delivery of heterologous DNA

pNL9164 has an *Mlu* I restriction site located within group II intron RNA coding sequence. Digestion at the *Mlu* I site allows for insertion of other DNA such as promoters (for mitigating polar effects), reporter genes, *loxP* recombination sequences, other antibiotic RAM-type markers, etc. The *Mlu* I site has been used to successfully deliver *tetM* and *abiD* genes[4], a trimethoprim –RAM[5], a kanamycin-RAM (plasmid pACD4K-C TA0100 kit), a removable kanamycin-RAM flanked with *loxP* sites (plasmid T2826, pACD4K-C-loxP), and a *lacZ*α gene[6]. The efficiency of the intron may be affected by insertions at the *Mlu* I site. A good starting point is to attempt to insert an intron containing heterologous DNA into an easily screenable or selectable gene. For instance, the *hsa*-24s targeting sequence in pNL9164 is very efficient and is a good control to test the effects of heterologous DNA inserted at the *Mlu* I site on intron mobility.

References:

1. Yao, J et al., *RNA*. (2006) (in press)
2. Charpentier, E., et al., *Appl Environ Microbiol*. **70**(10):6076-6085 (2004).

3. Viter S., et al., *Res. Microbiol*. **150**(4):287-290 (1999).
4. Frazier , C., et al., *Appl Environ Microbiol*. **69**(2):1121-1128 (2003).
5. Zhong, J., et al., *Nucleic Acids Res.* **31**(6):1656-1664 (2003).
6. Jones, J.P., et al., *Mol. Ther*. **11**(5):687-94 (2005).

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