# Construction of a recombinant vector for site-directed mutagenesis in *Salmonella typhimurium*

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**Key words:**
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***Abstract:***

**BACKGROUND:** Among all common techniques in site-directed mutagenesis, λ Red recombinase system has been widely used to knock out chromosomal genes in bacteria. In this method, there is always the risk of DNA Linear digestion by host's restriction enzymes that leads to the low frequency of recombination. **OBJECTIVES:** To overcome this, we constructed a recombinant vector to disrupt *phoP* gene in *Salmonella typhimurium*. **METHODS:** The SOEing PCR method and restriction enzymes were used to construct the vector. **RESULTS:** The resulting plasmid, pTAAZ92, contains a Kanamycin cassette with two long homologous arms flanking of the *phoP* gene. **CONCLUSIONS:** After electrotransformation of the pTAAZ92 into the *Salmonella typhimurium* , the *phoP* gene is replaced by the Kanamycin cassette through homologous recombination. According to the high homology of the *phoP* gene in many of *Salmonella* species the pTAAZ92 can be used to disrupt the *phoP* gene in most of these species.

## Introduction

*Salmonella typhimurium* is a facultative intracellular Gram-negative pathogen (E. Garcia Vescovi et al., 2002) that belongs to the family of Enterobacteriaceae (Washington C. Winn et al., 2006). It can cause a variety of diseases in humans and farm animals ranging from self-limited gastro-enteritis to bacteremia and systemic infections (David A. Cano et al., 2001). During the infection, this organism encounters several adverse environments such as low pH of stomach, bile salts, low oxygen in the small intestine, and cationic antimicrobial peptides on epithelial cells (Cormac G.M. Gahan and Colin Hill, 1999). Different regulatory systems are used for governing these stresses during the infection process.

*phoP* in *Salmonella typhimurium* is a transcriptional regulator and a member of two-component regulatory system (*PhoP*/*PhoQ*) (Ivan Rychlik and Paul A. Barrow, 2005). The regulation of gene expression by the *PhoP*/*PhoQ* is necessary in adaptation of this bacterium to intracellular environments and for its survival within macrophages, defensin resistance, acid resistance, and murine typhoid fever pathogenesis (S.I. Miller, 1991). It controls the expression of more than 40 genes required for virulence and resistance to antimicrobial peptides, bile salts, and acid pH (William Wiley Navarre et al., 2005). Therefore deletion of *phoP* leads to the inability to survive within macrophages and increased susceptibility to such harsh conditions (E. Garcia Vescovi et al., 2002).

In this study, we constructed a recombinant vector to knock out the *phoP* gene in *Salmonella typhimurium*. Although there are several methods to knock out directed genes in bacteria and some of them have been used in *Salmonella* spp. successfully (Shi-Zhong Geng et al., 2011), developing a simple and efficient method has always been of interest to the researchers. The constructed vector, pTAAZ92, contains a Kanamycin cassette with two homologous

arms flanking of the *phoP* gene. By electroporation of the pTAAZ92 the chromosomal *phoP* sequence is replaced with the antibiotic resistance gene, Kanamycin cassette, through homologous recombination.

## Materials and Methods

**Bacteria and plasmids:** In this study, *Salmonella typhimurium* 14028 (as a positive control) and a native strain of *Salmonella typhimurium* were used. The used plasmids in this study included pKD4, a template plasmid which carries kanamycin gene flanked by FRT sites and pTZ57R/T for cloning (TA Vector, Fermentas).

**Media and chemicals:** The media used in this study was Luria-Bertani (LB agar and broth). If it were required, the media would be supplemented with Kanamycin (30µg/mL).

Taq DNA polymerase enzyme was used in this method; however, in SOEing PCR method, High Fidelity PCR Master Mix (BioNEER, Korea) was applied. Enzymes including *Bam*HI, *Pst*I, and *Hin*dIII were used for directional cloning in pTZ57R/T. The Genomic DNA of the *Salmonella* strains was extracted by MBST DNA isolation Kit (Molecular Biologtcal System Transfer). Selection of the transformants were done on LB agar medium containing Kanamycin from Sigma Aldrich Co. (Germany).

**PCR reactions:** Using PCR, upstream and downstream segments of *phoP* gene and Kanamycin resistance cassette were amplified with designed primers. To amplify the Kanamycin resistance cassette primers, $kan_1$ and $kan_2$ from pKD4 plasmid was designed. The designed primers for amplification of the upstream and downstream segments of *phoP* gene ($PhoP_{up1}$ and $PhoP_{up2}$, $PhoP_{down1}$ and $PhoP_{down2}$ in respect) were based on known *phoP* sequences data for *Salmonella typhimurium* from the Gene bank. The primer $PhoP_{down1}$ was designed in which it had a 5' tail complementary to primer $kan_2$. The $PhoP_{up1}$ and $PhoP_{up2}$ for amplification of upstream segment of the *phoP* gene were designed with cut sites for *Hin*dIII and *Bam*HI, respectively. The sequence of designed primers is displayed in Table 1.

Each PCR reaction was performed in 20µL HF PCR PreMix. The PCR amplification was performed with 35 cycles of denaturation at 94°C for 45s, annealing at 63°C for 30s, and elongation at 72°C for 90s. The initial denaturation and final extension were 94°C for 1min and 72°C for 10 min, respectively.

The corresponding bands on a %1 agarose gel were excised and purified with DNA extraction kit following the manufacturer's protocol.

**Fusion PCR reaction:** Two purified PCR products related to downstream segment of the *phoP* gene and Kanamycin resistance cassette were applied in fusion PCR as primers. The PCR amplification was performed with 10 cycles of denaturation at 94°C for 15s, annealing at 56°C for 20s, and elongation at 72°C for 1min. The initial denaturation and final extension were 94°C for 2min and 72°C for 10 min, respectively. No primer was added to the reaction mixture at first step of SOEing PCR. Second step of the PCR reaction was performed using 35 cycles of denaturation at 94°C for 15s, annealing at 62°C for 20s, and elongation at 72°C for 3 min. A total of 1000 ng of the first reaction products was used as template DNA. The initial denaturation and final extension were 94°C for 2 min and 72°C for 10 min, respectively. The corresponding band on a %1 agarose gel was excised and purified with DNA extraction kit.

**TA Vector cloning:** Purified fusion PCR product was cloned into TA vector (Figure 1). The TA vector including the insert was then transformed into competent *E. coli* DH5α. The transformed bacteria were raised on LB medium containing Kanamycin (30 µg/mL). The resulting vector was extracted by Plasmid miniprep kit (Thermo Scientific, USA).

**Enzyme digestion:** The purified PCR product of upstream segment of *phoP* gene and the resulting vector were digested with *Bam*HI and *Hin*dIII according to the manufacturer's instructions.

**Ligation:** The digested products were purified and ligated together by Ligase enzyme. This recombinant plasmid, pTAAZ92, was transformed into competent *E. coli* DH5α. After the appearance of transformed bacteria on the LB-Km-agar plates, the pTAAZ92 was extracted as described before.

**PCR confirmation:** To verify that three segments including upstream and downstream segments of the *phoP* gene and Kanamycin resistance cassette were present in the pTAAZ92, two PCRs were carried out

by $kan_1$ and $PhoP_{down2}$ , $PhoP_{up1}$, and $PhoP_{up2}$. The pTAAZ92 was also sent to GenFanAvaran Co. for sequencing to confirm the correct position of the three segments.

## Results

The primers used for PCR amplification upstream and downstream segments of *phoP* gene and kanamycin resistance cassette successfully primed the synthesis of the anticipated DNA fragments with 638bp, 1530bp and 871bp in length, respectively (Figure 2).

Then two purified PCR products related to downstream segment of the *phoP* and Kanamycin resistance cassette were joined together by SOEing PCR method. The fusion PCR product with 2401bp in length was observed on %1 agarose gel. Then, the purified product was cloned into TA.

The digestion of purified PCR product of upstream segment of the *phoP* and the resulted vector was performed with *Bam*HI and *Hin*dIII. The results of *pst*I digestion of pTZ57R/T vector containing Kanamycin resistance cassette and left junction of the *phoP* established that the fusion PCR product had an inverse position in the TA vector. After ligation of digested products, the recombinant plasmid called pTAAZ92. The two PCRs on the pTAAZ92 by $kan_1$ and $PhoP_{down2}$ , $PhoP_{up1}$ and $PhoP_{up2}$ verified the presence of three segments including upstream and downstream segments of the *phoP* gene and Kanamycin resistance cassette (Figure 3).

## Discussion

The goal of this study was to construct a recombinant vector for site-directed mutagenesis in *Salmonella typhimurium*. By the pTAAZ92, the *phoP* gene in this bacterium was disrupted and replaced by an antibiotic resistance gene, Kanamycin cassette. To perform this, the SOEing PCR method and restriction enzymes were used.

Although there are several techniques to knock out directed chromosomal genes in bacteria, developing a simple and effective method has always been of interest to researchers. *Salmonella typhimurium* has been reported to be untransformable by electroporation or transformable only at comparatively low levels (Callaghan and Alain Charbit,. 1990). Researchers have used different methods to knock out chromosomal genes in *Salmonella* sp. To disrupt the *phoP* gene in *Salmonella enterica*, researchers have applied the following methods: In a research to delete ropS and *phoP* in *Salmonella enterica* serovar Choleraesuis a 3xFLAG cassette was produced by PCR with two cut sites for *XhoI* and *NotI* enzymes. The cassette was cloned into the *XhoI* and *NotI* sites of pCMVbm2A, replacing lacZ and generating pCMV3xFLAGm2A. Then the pCMVbm2A was used as a eukaryotic expression vector in *Salmonella* (Almira Bartolome et al., 2009).

In another study to obtain mutant strains of *S. typhi*, allelic exchange method mediated by the sacB-based recombinant suicide system was used. The *S. typhi* Ty2 *phoP*::Km mutant (LF1021) was constructed by conjugation between the wild-type strain *S. typhi* Ty2 and *E. coli* w7213, which had plasmid pLL6.12 inserted into the regions flanking the *phoP* locus (Hui-Young lee et al,. 2007). In a research to generate mutants of *Salmonella enterica* serovar Typhimurium F98 and serovar Enteritidis 147, deletion of the *phoP*, *ropS* and *ompC* genes were carried out by Overlap extension PCR. The PCR

Table 1. Primers used in this study. The sequences of restriction endonuclease sites are boldface type, Underlined and the restriction endonuclease(s) are shown in parentheses after the sequence. The nucleotides in boldface type show the complementary region to primer kan2.

| Primer designation | Sequence (5 ⟶ 3) | Template plasmid /gene target |
|---|---|---|
| $PhoP_{up1}$ | AAGGCAAGCTTGGTCTGTCTAACGCAGTGTTG(HindIII) | Genomic DNA |
| $PhoP_{up2}$ | ACCTGGATCCTGGAGCTGAACCTTCAG(BamHI) | Genomic DNA |
| $PhoP_{down1}$ | CTAATTCCCATGTCAGCCGTTAAGTGTTCCTGTGTCAC--ACGATGTCATTACCACCGTAC | Genomic DNA |
| $PhoP_{down2}$ | TTGCGCACAAGGCTGGTCAGCTCACGCGTC | Genomic DNA |
| $kan_1$ | TCTTGAGCGATTGTGTAGGCTGGAGCTGCTTCGAAG | pKD4 |
| $kan_2$ | GTGACACAGGAACACTTAACGGCTGACATGGGAATTAG | pKD4 |

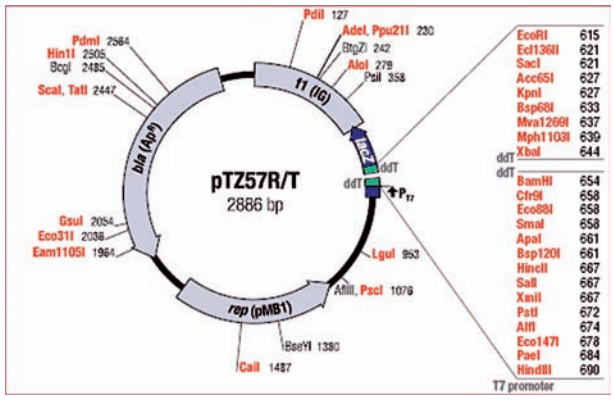


Figure 1. pTZ57R/T genetic map and cut sites of Restriction enzymes.

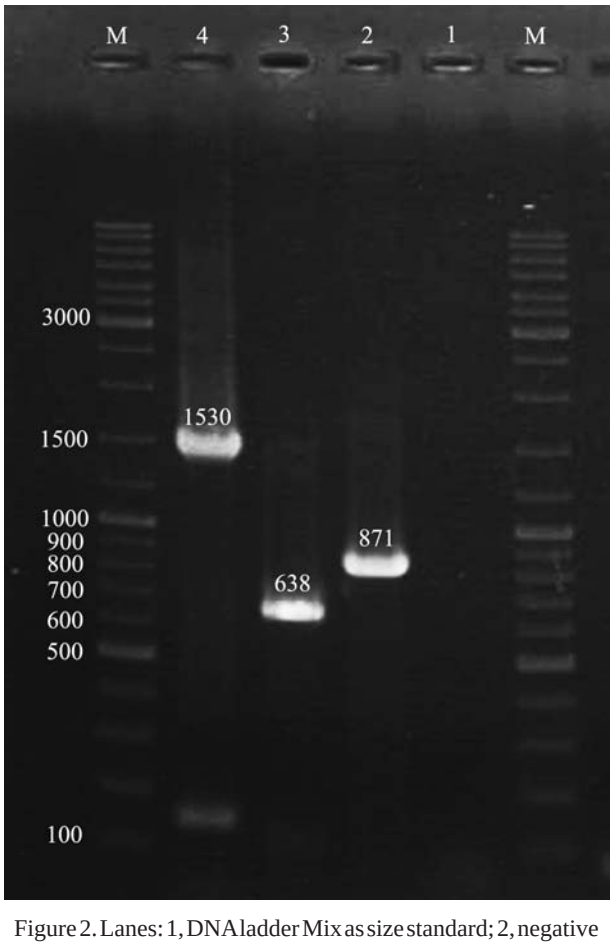


Figure 2. Lanes: 1, DNA ladder Mix as size standard; 2, negative control (no DNA); 3, Downstream segment of the phoP; 4, Upstream segment of the phoP; 5, Kanamycin cassette.

product was cloned into suicide plasmid vector pDM4, and then the plasmid was conjugated from *E. coli* 17.1 λpir into STM F98 (U. Methner et al., 2004). Such suicide vectors replicate in narrow host ranges and bacterial strains that contain suicide plasmid vectors are selected by environmental stresses, such as higher temperatures or antibiotics. Previous methods for construction of mutants in *Salmonella*, especially *S. typhimurium*, used P22 bacteriophage mediated transduction and a gene fusion protocol (Hui-Young lee et al., 2007). In a research mutation in *Salmonella typhimuium* 14028 in *smvA, acrB* and *tolC* genes were generated by the λ Red disruption system and p22 was used to transduce *tolC* to *acrB* and *smvA* mutant strains (Nicola´s A. Villagra et al., 2008). However, these methods are not adaptable to wild-type strains, because P22 is not able to infect them (Hui-Young lee et al., 2007).

In some researches mutants of the *ropS* and *phoP* regulatory genes in *Salmonella enterica* serovar Choleraesuis (ATCC 13312) were generated by Datsenko and Wanner method with minor modification. To produce PCR products, the primers were designed with 56-nt extensions homologous to region adjacent to the target gene and template plasmid (Gustavo Dominguez- Bernal et al., 2008; Kirill A. Datsenko and Barry L. Wanner, 2000). Although λ Red disruption system looked simple and was applied in *E. coli* and other Gram-negative bacteria, the performances of the Red disruption system in different bacteria can be variable due to intrinsic differences, such as Recombinase expression. According to most researchers, bacteria subjected to homologous recombination are wild types, it is probable this system is not adaptable to them (Shi-Zhong Geng et al., 2009).

In this study, primers were designed with a long flanking homology to the target gene the frequency of recombination increases, as it has been reported that for an efficient recombination in *Salmonella enteritidis* 100bp-1Kb of sequence homology is required (Lu S et al., 2003). In addition, this leads to overcome unique restriction systems especially in *Salmonella* (Mandy M Cox et al., 2007).

In this research, inserting the linear DNA into the vector leads to more stability which is an important issue, especially in wild types. From the other side, TA is a pMB1 based origin of replication plasmid; as a result, it cannot replicate in *Salmonella* sp (Helen S. Garmory et al., 2005).

Moreover, there is no need to transform a helper plasmid such as pKD46 into target bacteria to express λ Red recombinase (Shi-Zhong Geng et al., 2011). Additionally, the FRT-flanked Kanamycin gene of

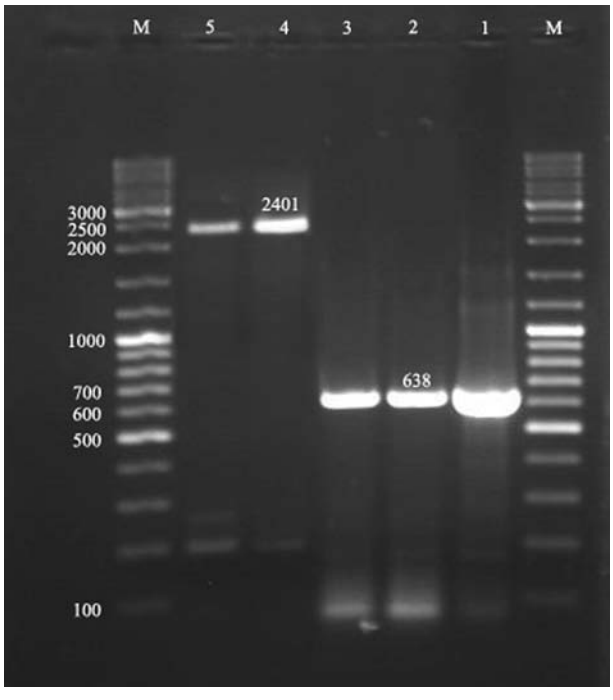


Figure 3. Lanes: 1, DNA ladder Mix as size standard; 2, Upstream segment of the phoP on genomic DNA of *S. typhimurium*; 3 and 4, Upstream segment of the phoP on pTAAZ92; 5 and 6, Downstream segment of the phoP with Kanamycin cassette;7, DNA ladder Mix as size standard.

the pKD4 plasmid of the Red disruption system can be removed by a pCP20 plasmid. This Plasmid is resistant to Ampicillin and Chloramphenicol and displays temperature-sensitive replication and thermal induction of FLP synthesis that is used to knock out the FRT-flanked resistance gene (Cherepanov PP et al., 1995, Shi-Zhong Geng et al., 2009). At the end, the pTAAZ92 can be used to delete the *phoP* gene because of its high homology within many *Salmonella* species.

In this study, we have described an improved method for gene disruption in *Salmonella typhimurium* to knock out *phoP* gene, replacing with Kanamycin gene from λ Red Disruption system based on Suicide plasmid system, which is simpler in procedures and more effective than previously reported conventional methods.

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# ساخت وکتور نوترکیب به منظور ایجاد جهش هدفمند در سالمونلا تیفی موریوم

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## چکیده

**زمینهٔ مطالعه:** در بین همه تکنیک‌های رایج برای جهش زایی هدفمند از سیستم Red recombinase λ به طور گسترده‌ای برای غیرفعال کردن ژن های کروموزومی در باکتری ها استفاده شده است. در این روش همیشه خطر تجزیه DNA خطی وارد شده توسط آنزیم های محدود الأثر وجود دارد که سبب کاهش فرکانس نوترکیبی می شود. **هدف:** برای رفع این مشکل ما یک وکتور نوترکیب برای غیرفعال کردن ژن *phoP* در سالمونلا تیفی موریوم ساختیم. **روش کار:** به منظور ساخت این وکتور از SOEing PCR وآنزیم های محدود الأثر استفاده شد. **نتایج:** پلاسمید حاصل، pTAAZ92، حاوی یک کاست کانامایسین با دو بازوی طویل هومولوگ مجاور ژن *phoP* می باشد. **نتیجه‌گیری نهایی:** با الکتروپوریشن pTAAZ92 به سالمونلا تیفی موریوم کاست کانامایسین از طریق نوترکیبی هومولوگ جایگزین ژن *phoP* می شود. با توجه به هومولوژی زیاد ژن *phoP* در بسیاری از گونه های سالمونلا می توان از این پلاسمید برای حذف ژن *phoP* در اکثر این گونه ها استفاده نمود.

**واژه های کلیدی:** اختلال در ژن، کاست کانامایسین، سالمونلاتیفی موریوم، جهش زایی هدفمند

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