

Protein Modification Using the New EZ::TN™ In-Frame Linker Insertion Kit

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Introduction

Consented genome sequencing projects have elucidated the coding sequence of a myriad of genes from many different organisms. An important next step is to determine the structural and functional relationships of the proteins encoded by these genes. Recently, EPICENTRE introduced the EZ::TN™ In-Frame Linker Insertion Kit to speed up and simplify protein modification and facilitate determination of key regulatory, binding and catalytic regions of cloned proteins.

The EZ::TN In-Frame Linker Insertion Kit was designed to randomly insert a 19 codon (57 nucleotide) “linker” into cloned genes. The kit features the EZ::TN <Not I/KAN-3> Transposon which contains a kanamycin-resistance (Kan^R) marker flanked by Not I restriction sites. The process for introducing random 19-codon insertions into cloned DNA is shown in Figure 1. A simple *in vitro* enzymatic reaction randomly inserts a single EZ::TN <Not I/KAN-3> Transposon into each clone and produces thousands of Kan^R insertion mutants. Insertion clones for further analysis can be identified by gene functional analysis or by restriction or DNA sequencing analysis of the transposon insertion site. Once clones are chosen, the Kan^R gene is excised from the transposon by Not I digestion (Figure 2). Each Not I digested clone is then

religated and retransformed into *E. coli*. Since the transposon mosaic ends have been modified to eliminate translational stops, the resulting clones each contain a random 19-codon insertion that can be read in all three reading frames. And, the protein will retain its original amino acid sequence on both sides of the insertion site.

In this report we demonstrate the utility of the EZ::TN™ In-Frame Linker Insertion Kit by generating and analyzing the effects of random 19-codon insertions into the tetracycline/H⁺ antiporter (tetracycline resistance, Tet^R) and β-lactamase (ampicillin resistance, Amp^R) genes of pBR322.

Materials and Methods

In vitro insertion reactions with EZ::TN <Not I/KAN-3> Transposon

The *in vitro* insertion reaction utilizes 0.2 μg of target DNA (pBR322 in this example), an equimolar amount of EZ::TN <Not I/KAN-3> Transposon, 1 U of EZ::TN Transposase, and a Mg²⁺-containing buffer. The reaction is performed for 2 hours at 37°C as described in the EZ::TN™ In-Frame Linker Insertion Kit literature.

One microliter of reaction mix was electroporated into 50 μl of TransforMax™ EC100 electrocompetent *E. coli* (EPICENTRE). After overnight selection on kanamycin (50 μg/ml) randomly chosen Kan^R colonies were replica plated onto both ampicillin- and tetracycline-containing plates. Colonies with either a Amp^R/Tet^S or Amp^S/Tet^R phenotype were selected for further analysis.

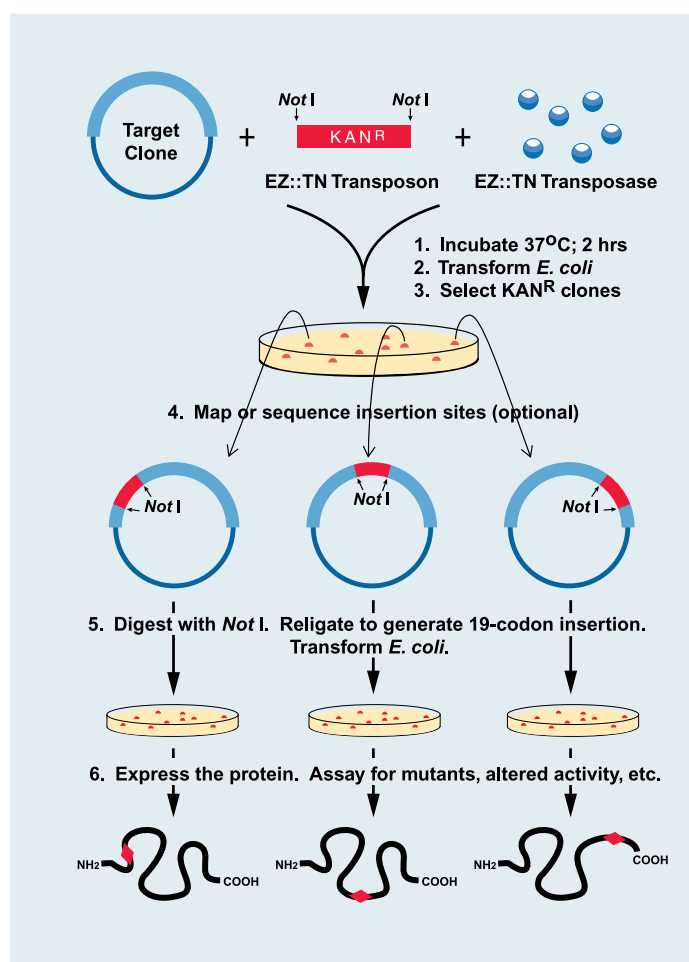


Figure 1. The EZ::TN In-Frame Linker Insertion Kit is based on the highly random Tn5 transposition system. A single *in vitro* reaction generates thousands of insertion clones- each containing a different transposon insertion.

Mapping and sequencing the EZ::TN Transposon insertion sites

The approximate EZ::TN <Not I/KAN-3> Transposon insertion site of each Amp^R/Tet^S clone or Amp^S/Tet^R clone was mapped by restriction endonuclease digestion using *Eco* RI and *Xho* I or *Eco* RI and *Xba* I, respectively. The precise transposon insertion site was determined by DNA sequencing using the primers provided in the EZ::TN In-Frame Linker Insertion Kit, which are homologous to the ends of the EZ::TN <Not I/KAN-3> Transposon.

Removal of the kanamycin resistance cassette from insertion clones

Five hundred nanograms of each pBR322 insertion clone was digested to completion with *Not* I and reaction products were electrophoresed on a 1% low melting point agarose gel. The larger fragment (i.e., plasmid minus 1.1 kb Kan^R gene) was cut from the gel, melted at 70°C for 10 minutes, and 10 microliters were placed in a microcentrifuge tube prewarmed to 42°C. The vector *Not* I ends were then religated in-gel using Fast-Link™ DNA Ligase (EPICENTRE). After heat inactivation at 70°C for 15 minutes, one microliter was used to electroporate TransforMax™ EC100 electrocompetent *E. coli* cells as described above. Transformants were plated on tetracycline or ampicillin plates as appropriate for growth and then the target gene was screened for restored activity.

Results and Discussion

The EZ::TN In-Frame Linker Insertion Kit provides several advantages for generating protein modifications. The *in vitro* EZ::TN <Not I/KAN-3> Transposon insertion reaction is very efficient (greater than 10⁶ clones per reaction) and minimizes multiple insertion events. In addition, the general location of each transposon insertion was readily determined by restriction data and more precisely defined

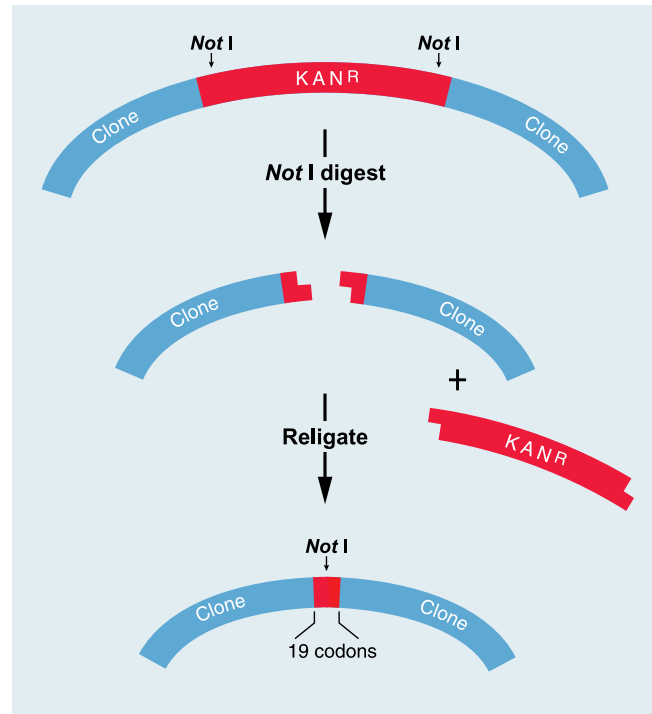


Figure 2. The EZ::TN <Not I/KAN-3> Transposon contains a kanamycin resistance gene flanked by *Not* I restriction sites. A 19-codon insertion that can be read in all three reading frames is generated following *Not* I digestion and religation.

by sequence analysis using the primers provided in the kit. Finally, as shown in Figure 3, insertions appeared to be random throughout each antibiotic resistance gene.

Excision of the kanamycin resistance gene from the EZ::TN <Not I/KAN-3> Transposon by *Not* I digestion and religation generated a 57-bp (19-codon) insertion in the gene, which could be read in all three reading frames (Figure 4). Since EZ::TN Transposons are hyperactive

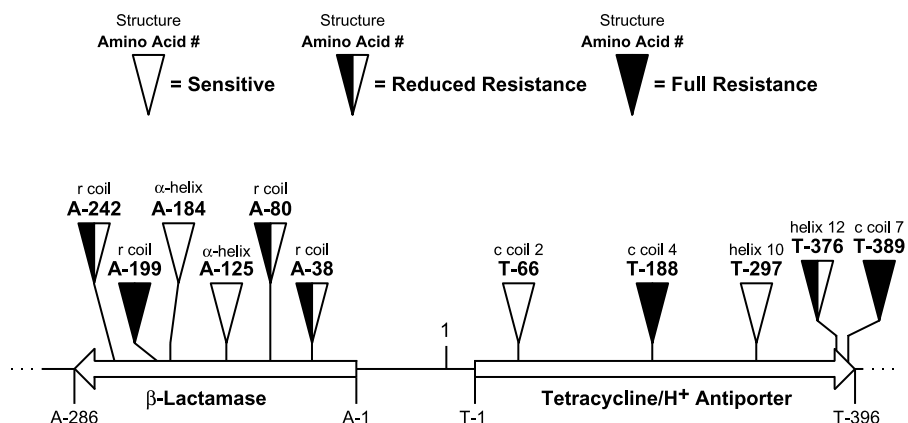


Figure 3. Random, 19-codon insertions into the tet/H⁺ antiporter and β -lactamase genes of pBR322 resulted in a range of antibiotic resistance levels. *r coil*, random coil; *c coil*, cytoplasmic coil.

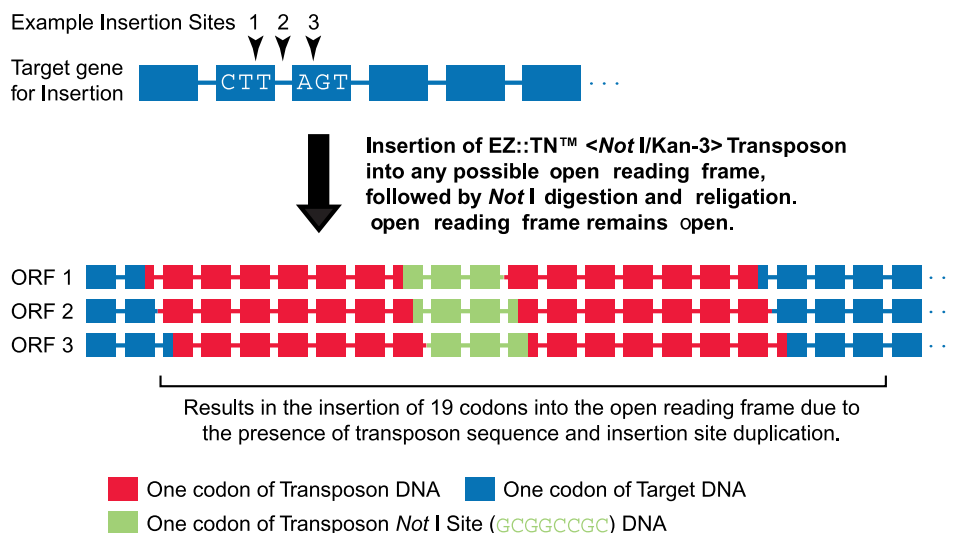


Figure 4. Excision of the kanamycin resistance gene from the EZ::TN <Not I/KAN-3> Transposon generates a 57 bp (19 codon) insertion. Insertions in each of the three reading frames remains open (i.e., does not contain a nonsense (stop) codon) and varies in composition. The amino acid sequence on both sides of the insertion remains unaltered.

forms of Tn5 transposons, 9 of the 57 bp are the result of a 9-bp duplication of target DNA that occurs during the insertion process.¹ However, the number of duplicated amino acid residues flanking transposon sequences can be two or three, depending upon the specific codon into which the transposon inserts.

The tetracycline/H⁺ antiporter (Tet^R) and β-lactamase (Amp^R) genes of pBR322 were chosen to demonstrate the utility of the EZ::TN In-Frame Linker Insertion Kit because these two antibiotic gene targets are readily detectable and the products of these genes have distinct cellular locations and modes of action.^{2,3} At this point, it is difficult to theoretically predict how the position and composition of a specific 19-amino acid insertion will affect protein activity. Nevertheless, some conclusions could be made from the data.

For example, Figure 3 shows the insertion site in 5 randomly chosen Amp^R/Tet^S clones and the effect of the 57-nucleotide insertion on each clone's ability to confer resistance to tetracycline. Because the Tet^R gene is a membrane protein with twelve putative transmembrane segments, insertions within those regions affect its topology and function as a tetracycline antiporter.² Surprisingly, in the majority of cases (clones T-188, T-376, T-389) the 19-amino acid insertions had little to no effect on the ability of the clones to grow on tetracycline.

Also, as expected, the 19-amino acid fusions in β-lactamase had varying effects. For example, the insertion in clone A-80 interrupted a random coil domain and reduced drug resistance. Similarly, the 19-amino acid insertion in clone A-184 resulted in loss of activity presumably because a crucial alpha-helical domain was interrupted. The insertion in clone A-199, however, did not appreciably affect the ampicillin resistance of bacteria harboring the plasmid and indicated a permissive region of the protein.

Conclusion

The EZ::TN In-Frame Linker Insertion Kit is a fast and efficient method for randomly inserting 19-amino acid peptides in-frame into the proteins encoded by a cloned DNA for a variety of applications. These include: 1) structure and function analysis of the protein encoded by a cloned DNA; 2) identifying permissive insertion sites for protein engineering; and 3) epitope or domain mapping of proteins.

Recently, Biery et al.⁴ described a transposon-based linker insertion method using a modified Tn7 transposon system. However, as opposed to the three open reading frames provided by the EZ::TN system described here, the Tn7 system contains a nonsense (stop) codon in one of three reading frames on each strand.

References

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EZ::TN™ In-Frame Linker Insertion Kit

EZ104KN-F73

10 Reactions

Kit includes EZ::TN <Not I/KAN-3> Transposon, EZ::TN Transposase, Reaction Buffer, Stop Solution, two unlabeled Sequencing Primers, Control DNA and Water.