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## Generating Unidirectional Deletions by *In Vitro* Transposition for Localizing and Sequencing a Cloned Gene using EZ::TN™ Transposon Tools

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Creating unidirectional deletions in cloned DNA facilitates a variety of genomics applications such as generating ordered DNA sequencing templates, producing truncated protein variants from cDNA or genomic clones and for mapping epitopes. The EZ::TN™ Plasmid-Based Deletion Machine uses the *in vitro* Tn5 transposon deletion process described by York et. al.<sup>1</sup> This process is a highly efficient, easy and reliable *in vitro* method for creating a complete population of ordered deletion

clones. In this report, we demonstrate the use of the EZ::TN Plasmid-Based Deletion Machine to sequence and identify the precise carboxy terminus of an alkaline phosphatase gene. Kits for generating unidirectional deletions in cosmid clones are also available (see the center insert).

The process for producing and identifying deletion clones is presented in Figure 1. The DNA of interest is first cloned into one of the specially constructed plasmid (Figure 3) or cosmid vectors. These vectors contain properly oriented 19-bp Tn5 transposon Mosaic End (ME) sequences

that are specifically recognized by the hyperactive EZ::TN Transposase. Random, unidirectional deletions are generated via intramolecular transposition in any DNA cloned into the multiple cloning sites of the vector by incubating the clone with the EZ::TN Transposase.

The *in vitro* transposition reaction is highly efficient; occurring in up to 70% of the DNA molecules. Subsequent transformation of *E. coli* produces a library of >10<sup>6</sup> deletion clones.

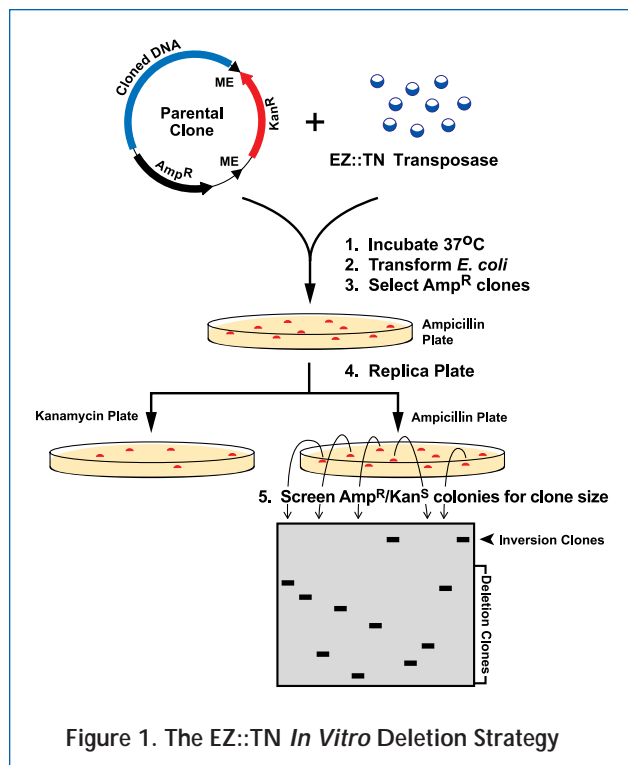
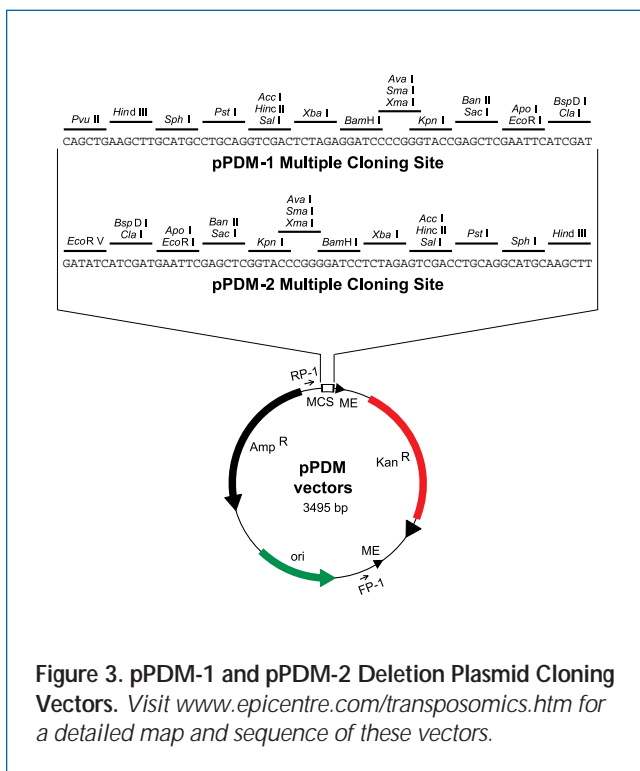
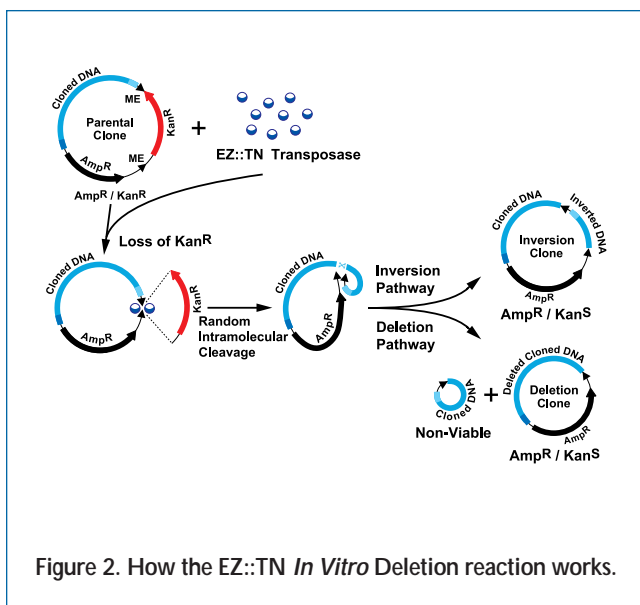


Figure 1. The EZ::TN *In Vitro* Deletion Strategy

continued

As shown in Figure 2, intramolecular transposition yields two types of products. In one case, transposition results in a random deletion from the target DNA. In the second case transposition results in an inversion of one portion of the cloned insert. Since the nature and the site of the intramolecular reaction is purely random, the result is the generation of a population of independent clones, each containing either a unidirectional deletion or inversion. Since all of the inversion clones are the same size, deletion clones can be easily distinguished from inversion clones based on size analysis by agarose gel electrophoresis.



## Materials and Methods

### Cloning of the alkaline phosphatase gene

A constitutively expressing alkaline phosphatase gene contained within a 4.7 Kb insert was directionally cloned (Xba I / Eco RI) into EPICENTRE's pPDM™-2 Deletion Plasmid Vector (Figure 3) provided in the EZ::TN Plasmid-Based Deletion Machine Kit. The resulting clone was designated pPDM-2/HKAP.

### *In situ* alkaline phosphatase activity assay

Phosphatase activity assays were performed *in situ* by covering plated cells with a thin layer of 37°C low-melting point 1% agarose solution containing 50 mM Tris-HCl (pH 9.5), 5 mM MgSO<sub>4</sub>, 1 mM CaCl<sub>2</sub>, and 0.33 µg/ml 5-bromo-4-chloro-3-indolyl phosphate (BCIP). Colonies expressing alkaline phosphatase activity turned blue and were detected in 10-15 minutes.

### The EZ::TN *in vitro* deletion reaction

The deletion reaction was performed using plasmid DNA purified from an overnight culture of a single pPDM-2/HKAP clone. The deletion reaction and the selection of deletion clones were done as described in the EZ::TN Plasmid-Based Deletion Machine Kit literature and summarized in Table 1.

**Table 1. EZ::TN Deletion Reaction and Selection Process**  
See [www.epicentre.com/transposomics.htm](http://www.epicentre.com/transposomics.htm) for details.

- Mix the following:
  - 1 µl 10X Reaction Buffer
  - x µl 0.2 µg pPDM clone DNA
  - 1 µl EZ::TN Transposase (1 U)
  - ddH<sub>2</sub>O to 10 µl.
 Incubate 2 hours to overnight at 37°C.
- Terminate the reaction by adding 1 µl 10X Stop Solution and heating at 70°C for 10 minutes.
- Transform *RecA minus E. coli* with transformation efficiency >10<sup>7</sup> cfu/µg DNA with 1 µl of the reaction mix.
- Plate cell dilutions (e.g., 1:100, 1:1000) onto LB plates containing 50 µg/ml ampicillin. Grow overnight at 37°C.
- Randomly choose Amp<sup>R</sup> colonies and replica plate onto LB plates containing either 50 µg/ml ampicillin or 25 µg/ml kanamycin. Grow overnight at 37°C.

### Identifying deletion and inversion clones

Amp<sup>R</sup>/Kan<sup>S</sup> colonies harbor either a deletion clone or an inversion clone (Figure 2). Supercoiled DNA from individual Amp<sup>R</sup>/Kan<sup>S</sup> clones was prepared and size-analyzed by 1% agarose gel electrophoresis using a modification of the colony cracking procedure described by Mantiatis, *et al.*<sup>2</sup>

**Table 2. Efficiency of the intramolecular transposition reaction**

	Clones/ $\mu\text{g}$ DNA	% Total Amp <sup>R</sup> Clones
Total Amp <sup>R</sup> Clones	$4.4 \times 10^8$	100%
Parental Clones (Amp <sup>R</sup> /Kan <sup>R</sup> )	$2.5 \times 10^8$	57.3%
Transposition Clones (Amp <sup>R</sup> /Kan <sup>S</sup> )	$1.9 \times 10^8$	42.7%
Deletion Clones (Amp <sup>R</sup> /Kan <sup>S</sup> )	$1.2 \times 10^8$	28%
Inversion Clones (Amp <sup>R</sup> /Kan <sup>S</sup> )	$5.5 \times 10^7$	12.5%

### DNA sequencing of deletion clones

DNA sequencing of all deletion clones was done using the pPDM FP-1 Forward Primer provided in the EZ::TN Plasmid-Based Deletion Machine Kit. The primer was labeled at its 5'-end with LICOR IRD-800. Sequencing reactions were performed as described in the SequiTherm EXCEL™ II DNA Sequencing Kit from EPICENTRE and sequencing results analyzed on a LICOR 400L automated DNA sequence analyzer.

## Results

### Generating deletion and inversion clones

Intramolecular transposition and subsequent ampicillin selection of transformed *E. coli* yields three types of colonies: 1) those harboring a deletion clone, 2) those harboring an inversion clone, or 3) those harboring a parental clone. Since successful transposition results in the loss of the kanamycin resistance marker from the pPDM vector (refer to Figure 2), all transposition clones (both deletion and inversion clones) will display an Amp<sup>R</sup>/Kan<sup>S</sup> phenotype. Unreacted parental clones are Amp<sup>R</sup>/Kan<sup>R</sup>. As shown

in Table 2, intramolecular transposition, generating deletion and inversion clones, is a highly efficient process.

Of 96 randomly chosen Amp<sup>R</sup> clones that were replica plated, 41 (42.7%) were determined to have undergone intramolecular transposition determined by their Amp<sup>R</sup>/Kan<sup>S</sup> phenotype. The reaction time required to achieve high intramolecular transposition appears to depend on the nature of the DNA insert (EPICENTRE, unpublished results). Although an overnight reaction was performed for this experiment, reaction times as short as 2 hours have generated comparable or higher transposition efficiencies with other DNA clones (data not shown).

### Distinguishing deletion clones from inversion clones

Deletion clones were easily distinguished from inversion clones by 1% agarose size analysis because all inversion clones are the same size (6.8 kb; pPDM-2/HKAP parental clone *minus* the 1.4 kb Kan<sup>R</sup> marker which is eliminated during the transposition reaction; refer to Figure 2). The size of the deletion clones varied from ~1.9 kb up to 6.8 kb. Representative agarose gel analysis data is shown in Figure 4.

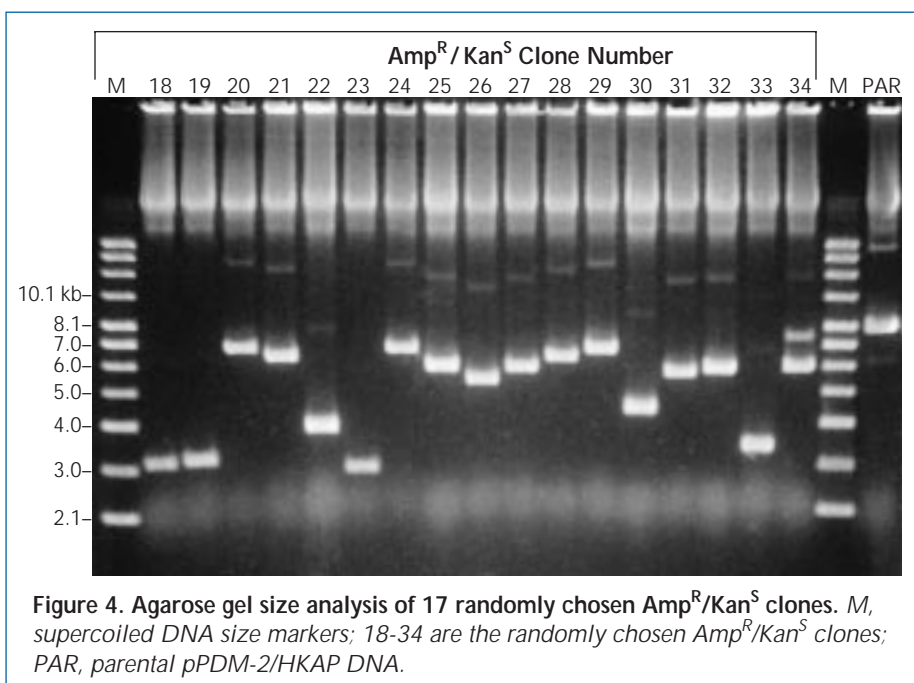
Of the 41 Amp<sup>R</sup>/Kan<sup>S</sup> colonies analyzed by agarose gel electrophoresis, 27 contained deletion clones and 14 contained inversion clones. The ratio of deletion clones to inversion clones was therefore found to be approximately 2:1 (27 : 14). Although the intramolecular transposition reaction is random and would be expected to yield equal numbers of deletion and inversion events, we typically observe a higher percentage of deletion clones.

### Construction of a deletion map

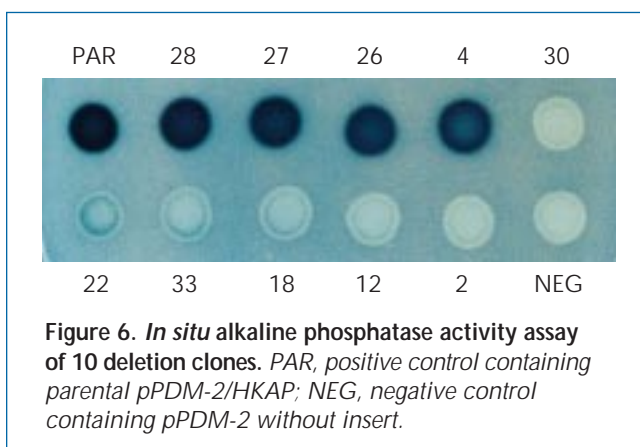
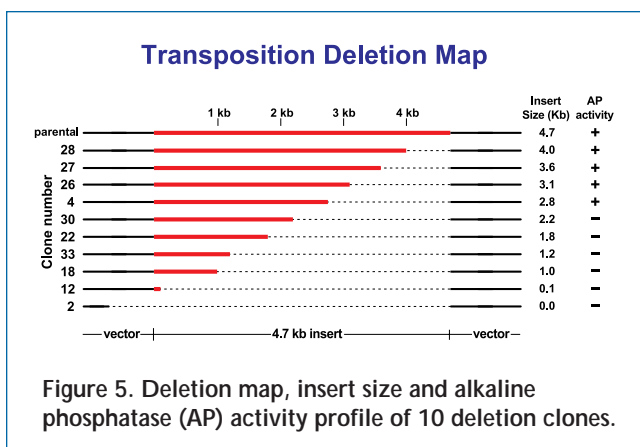
Based on agarose gel size analysis of supercoiled DNA from the 41 Amp<sup>R</sup>/Kan<sup>S</sup> colonies, 10 deletion clones, which are representative of the entire size range, were chosen for further analysis. A deletion map of these 10 clones was constructed and is shown in Figure 5, page 4.

### Identifying the precise Carboxy-terminus of the cloned alkaline phosphatase.

Previous work had established that deletion of even a small portion of the C-terminus of the alkaline phosphatase coding sequence results in loss of activity (EPICENTRE, unpublished results). When the 10 chosen deletion clones were plated and assayed for alkaline phosphatase activity *in situ* (Figure 6, p. 4) as described in Materials and Methods, the C-terminal coding region of the

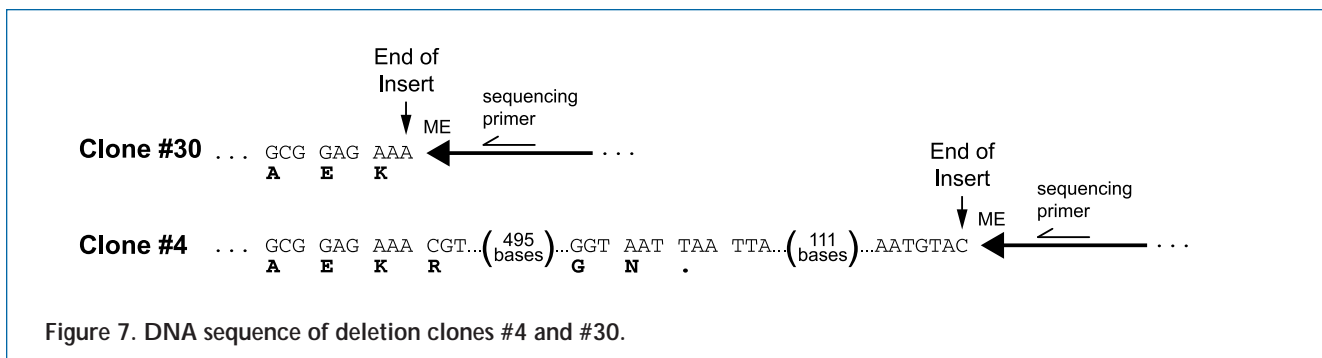
*continued*

**Figure 4. Agarose gel size analysis of 17 randomly chosen Amp<sup>R</sup>/Kan<sup>S</sup> clones.** *M*, supercoiled DNA size markers; 18-34 are the randomly chosen Amp<sup>R</sup>/Kan<sup>S</sup> clones; *PAR*, parental pPDM-2/HKAP DNA.



alkaline phosphatase gene was localized to a 0.6 Kb region of the insert falling between clone #4 and clone #30.

The precise C-terminus of the gene was identified (Figure 7) by sequencing deletion clone #4, the shortest clone that expressed alkaline phosphatase activity. Sequence analysis of deletion clone #30, the largest clone without activity, revealed that the intramolecular deletion reaction had removed 168 codons from the C-terminal end of the gene. As shown in Figure 6, removal of these codons produced a truncated protein variant that had completely lost alkaline phosphatase activity. The 19-bp Mosaic End (ME) EZ::TN Transposase recognition sequence 5'AGATGTGTATAAGAGACAG-3' occurs at the junction between insert DNA and transposon DNA of *all* deletion clones. Thus, the first base after the ME recognition site is the start of the insert sequence.



## Conclusions

The EZ::TN Plasmid-Based Deletion Machine utilizes standard molecular biology techniques such as DNA cloning, a simple enzymatic reaction, transformation and selection of *E. coli* and agarose gel electrophoresis. In this report, we demonstrate that use of the EZ::TN Plasmid-Based Deletion Machine is a fast, easy and reliable method to generate a population of random deletions. Subsequently, the deletion clones generated proved useful as sequencing template and for precisely identifying the C-terminus of a gene contained within a large DNA fragment.

## References

1. York, D. *et al.* (1998) *Nuc. Acids Res.* **26**, 1927.
2. Mantiatis, T. *et al.* (1982) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Press, Cold Spring Harbor, N.Y. p. 370.

### EZ::TN™ Plasmid-Based Deletion Machine

DPM9401-F71

Kit contains both pPDM™-1 and pPDM-2 Plasmid Deletion Cloning Vectors, Reaction Buffer, Stop Buffer, EZ::TN Transposase, unlabeled sequencing primers and Control pPDM-1 containing a 5.6 Kb insert.

To learn more about the EZ::TN Plasmid-Based Deletion Machine, pWEB::TNC™ Deletion Cosmid Kits or any of EPICENTRE's EZ::TN™ Transposon Tools, see the center insert or visit our Web site at:

 [www.epicentre.com/transposomics.htm](http://www.epicentre.com/transposomics.htm)