Louis, MO) was then added, and the mixture was shaken for 10 min at room temperature. The beads were vacuum washed on a 0.7 ml filter plate from Orochem Technologies (Gurnee, IL) with 2 ml of 25 mM ammonium bicarbonate and 2 ml of distilled water.

**MALDI-TOF MS analysis.** The beads were then resuspended in 2 µl of 10 mg/ml α-cyano-4-hydroxycinnamic acid, 0.1% trifluoroacetic acid in a 50:50 mixture of water and acetonitrile. Beads (0.5 µl) were then spotted on a MALDI plate and analyzed using a Voyager-DE Pro instrument (PerSeptive PE Biosystems, Framingham, MA). The machine was run in positive-ion reflector mode with an ion extraction delay time of 250 ns for the data shown in Figure 1, and in positive-ion linear mode with a delay time of 150 ns for the data in Figure 2. For each sample, we averaged 256 scans obtained with a laser setting of 1,800. Raw data were analyzed using PerSeptive GRAMS/32 software, and no smoothing was performed on the spectra. Predicted peptide masses were calculated using the Prowl program in monoisotopic mode (http://prowl.rockefeller.edu/cgi-bin/sequence).

***BRCA1* deletion mutation.** Codons 328–347 of *BRCA1* were amplified using the following primers: 5′-TAATACGACTCACTATAGGGAGACCACCATGGA-CTACAAGGACGACGATGACAAGTGTAATGATAGGCGGACTCCC-3′ and 5′- ATTAACCCTCACTAAAGGGATCACAGGGGATCAGCATTCAGATC-3′. As codons 328–334 and 341–347 are encoded by the primers, the true test sequence consists of codons 335–340, or 18 bases of test sequence. PCR amplification was done using primers at 500 nM in a 100 µl reaction with 300 ng of genomic DNA as template from a patient having the 1129insA or a wild-type control. Five units of Taq Gold (PE Biosystems, Foster City, CA) were used to amplify the sequence by cycling once at 95°C for 10 min, and 40× at 95°C 2 min, 55°C 2 min, and 72°C 2 min. Peptide synthesis and purification were done as described above except that 15 µl of raw PCR product was used as template.

BRCA1 **Cys-Gly amino acid substitution.** Codons 58–64 (21 bases of test sequence) of *BRCA1* were amplified from control DNA, and DNA sample NA14097 from the Coriell Cell Repositories (Camden, NJ) having the Cys61Gly mutation. Primers used for amplification were 5′-TAATACGACTCACTATAGGGAGACCACCATGGACT-ACAAGGACGACGATGACAAGCTTCTCAACCAGAAGAAAGGG-3′ and 5′-ATTAACCCTCACTAAAGGGATCACCTTTTGGTTATATCATTCTT-3′. PCR amplification, in vitro synthesis, purification, and analysis were performed as previously described for codons 328–347.

## Acknowledgments

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# Insertional transposon mutagenesis by electroporation of released Tn5 transposition complexes

**Igor Y. Goryshin[1], Jerry Jendrisak[2], Les M. Hoffman[2], Ronald Meis[2], and William S. Reznikoff[1]***

*[1]Department of Biochemistry, University of Wisconsin, 433 Babcock Dr., Madison, WI 53706. [2]Epicentre Technologies, 1202 Ann St., Madison, WI 53713. Corresponding author (reznikoff@biochem.wisc.edu).*

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**DNA transposition is an important biological phenomenon that mediates genome rearrangements, inheritance of antibiotic resistance determinants, and integration of retroviral DNA. Transposition has also become a powerful tool in genetic analysis, with applications in creating insertional knockout mutations, generating gene–operon fusions to reporter functions, providing physical or genetic landmarks for the cloning of adjacent DNAs, and locating primer binding sites for DNA sequence analysis. DNA transposition studies to date usually have involved strictly in vivo approaches, in which the transposon of choice and the gene encoding the transposase responsible for catalyzing the transposition have to be introduced into the cell to be studied (microbial systems and applications are reviewed in ref. 1). However, all in vivo systems have a number of technical limitations. For instance, the transposase must be expressed in the target host, the transposon must be introduced into the host on a suicide vector, and the transposase usually is expressed in subsequent generations, resulting in potential genetic instability. A number of in vitro transposition systems (for Tn*5*, Tn*7*, Mu, *Himar1*, and Ty*1*) have been described, which bypass many limitations of in vivo systems[2–6]. For this purpose, we have developed a technique for transposition that involves the formation in vitro of released Tn*5* transposition complexes (Transposomes™) followed by introduction of the complexes into the target cell of choice by electroporation. In this report, we show that this simple, robust technology can generate high-efficiency transposition in all tested bacterial species (*Escherichia coli*, *Salmonella typhimurium*, and *Proteus vulgaris*) We also isolated transposition events in the yeast *Saccharomyces cerevisiae.***

Tn*5* is a bacterial genetic element that transposes via a cut-and-paste mechanism[2]. The only macromolecular components required for this process are the transposase; the transposon, which can presumably be any sequence that is defined by two specific inverted 19 bp sequences; and the target DNA into which the insertions are made[2].

The Tn*5* transposition process involves the following steps: (1) binding of transposase monomers to the 19 bp end sequences; (2) oligomerization of the end-bound transposase monomers, forming a transposition synaptic complex; (3) blunt end cleavage of the transposition synaptic complex from adjoining DNA, resulting in formation of a released transposition complex or Transposome (formation of the Transposome through the normal biochemical mech-

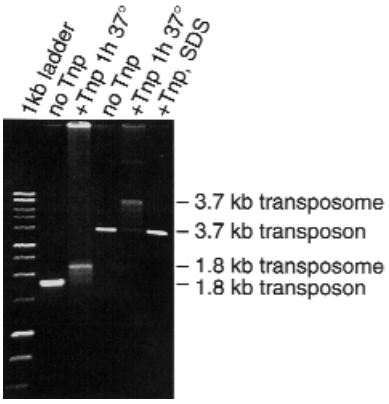


**Figure 1. Transposome formation. Agarose gel electrophoresis was used to analyze formation of transposomes using two Tn*5*–like DNAs cleaved free from adjacent donor backbone DNA through the use of *Pvu*II. The 1.8 kb Tn*5*–like transposon DNA (2.5 μg/ml) was incubated with purified hyperactive Tn*5* transposase (10 μg/ml) in 400 ml buffer for 1 h at 37°C, and the reaction mixture was then concentrated using a nitrocellulose membrane prior to electrophoretic analysis. A 3.7 kb transposon (50 μg/ml) also defined by 19 bp mosaic ends[10] was similarly incubated in 40 μl with hyperactive Tn*5* transposase (10 μg/ml), but was not concentrated prior to analysis. Performing the incubation of the 1.8 kb DNA at high concentrations resulted in the formation of Tnp-dependent multimers (data not shown). An aliquot of the 3.7 kb transposome sample was treated with 0.3% SDS to break apart the transposase-mediated synaptic complexes. The samples were analyzed by electrophoresis on a 1.2% agarose gel. The transposomes migrated at a rate expected for relaxed DNA circles, whereas with the SDS treatment, the DNA migrates similar to linear transposon DNA. The DNA remaining in the wells is a result of nonspecific transposase aggregation observed in some experiments.**

anism requires $Mg^{++}$); (4) binding to target DNA; and (5) strand transfer of the transposon 3′ ends into a staggered 9 bp target sequence (this step also requires $Mg^{++}$)[2,7,8]. Following strand transfer, host functions are thought to remove the transposase from the product DNA and repair the 9 b gaps at either end. The central DNA sequence between the 19 bp end sequences plays no mechanistic role in this process as long as it is of a size that will permit synaptic complex formation; thus, any sequence can make up this region.

It is possible to form functional Tn*5* Transposomes by incubating purified transposase with Tn*5* DNA that has been precleaved from adjacent DNA through the use of a suitable restriction enzyme (Fig. 1). No $Mg^{++}$ is necessary for the formation of these complexes because the transposition cleavage step has been bypassed. Upon addition of target DNA and $Mg^{++}$, Tn*5* Transposomes will undergo efficient transposition in vitro (J.J., unpublished results). Moreover Tn*5* Transposomes appear to be stable for over a month when stored at 4°C in the presence of 10% glycerol and for over a year at –20°C or –70°C without loss of activity (I.Y.G. and J.J., unpublished results).

A. *E. coli* K12 insertions

| Clone | Sequence | Genetic location |
|---|---|---|
| K 1 | gccatc AGTCTGGCG <Kan$^r$> AGTCTGGCG ttggaa | ORF *yahA* (331934 – 331942) |
| K 2 | tcttcg CTATTACGC <Kan$^r$> CTATTACGC cagctg | *lacZ* (365411 – 365419) |
| K 3 | acgacg GCATTCGCC <Kan$^r$> GCATTCGCC actgcc | ORF *ycdP* (1085549 – 1085557) |
| K 4 | cgatcg ACATTCATC <Kan$^r$> ACATTCATC atggcc | ORF *yceK* (1113004 – 1113012) |
| K 5 | tctcag GAACAGGAC <Kan$^r$> GAACAGGAC ggcatt | *stfR* (1429974 – 1429982) |
| K 6 | ggtaac GTTTAGCAC <Kan$^r$> GTTTAGCAC cgcctt | intergenic (1669772 – 1669780) |
| K 7 | ttggta CGTATGTAC <Kan$^r$> CGTATGTAC tgcgtg | ORF *yeaS* (1878236 – 1878244) |
| K 8 | tgaaca TGCTAATAG <Kan$^r$> TGCTAATAG tgcttc | ORF *yhiJ* (3628859 – 3628867) |
| K 9 | ggcaaa GCCCTGGAA <Kan$^r$> GCCCTGGAA taaagt | *dgoT* (3869061 – 3869069) |
| K 10 | ttatcg GCATTGACC <Kan$^r$> GCATTGACC tgagtc | ORF *yieL* (3898072 – 3898080) |
| K 11 | tgctca CCTCAGAAC <Kan$^r$> CCTCAGAAC aacaca | ORF *yi91b* (4504741 – 4504749) |

B. *S.typhimurium* insertions

| Clone | Sequence | Genetic location |
|---|---|---|
| S 1 | tgtgcc GACCAGCGC <Kan$^r$> GACCAGCGC ctaccc | *rfbD* |
| S 2 | aagtca TGGCGGTAG <Kan$^r$> TGGCGGTAG tgcgtg | *fruR* |
| S 4 | tgctaa GCTATGGAC <Kan$^r$> GCTATGGAC agccca | ? |
| S 7 | acgctt TCTTTAACC <Kan$^r$> TCTTTAACC tgcgcg | ? |
| S 8 | cgcggt GCTGGTGGC <Kan$^r$> GCTGGTGGC agtgaa | *ppsA* |

C. *P.vulgaris* insertions

| Clone | Sequence | Genetic location |
|---|---|---|
| P 1 | cattga GCCTTGATC <Kan$^r$> GCCTTGATG tgtagg | 23S ribosomal RNA |
| P 2 | ctcagt CGGTAGAGC <Kan$^r$> CGGTAGAGC agggga | tRNA phe |
| P 3 | ttaata AACCAGTTG <Kan$^r$> AACCAGTTG tatctc | fimbrial operon *atf* |
| P 4 | cgcgct GCTCAGGGA <Kan$^r$> GCTCAGGGA agaccc | *yhdG* homolog |

D. *S.cerevisae* insertions

| Clone | Sequence | Genetic location |
|---|---|---|
| 9 1 | tgaaca GCCTCTAGT <Kan$^r$> GCCTCTAGT tgatag | 25S rDNA chromosome XII |
| 9 2 | taggaa CTTCAAAGC <Kan$^r$> CTTCAAAGC gtttcc | 2 micron plasmid |
| 9 6 | aacaat ACTTGACAC <Kan$^r$> ACTTGACAC catttt | chromosome IV |
| 11 1 | aacatt CTGCTAGAC <Kan$^r$> CTGCTAGAC agattg | DUR1, 2 chromosome II |
| 11 2 | ttgcgc ACCATACAT <Kan$^r$> ACCATACAT acagga | chromosome V |
| 11 4 | gggcca CTTTCCAGC <Kan$^r$> CTTTCCAGC aaacgc | chromosome VI, near PHO4 |

**Figure 2. DNA sequence analysis of candidate inserts indicate that they represent bona fide Tn*5*-like transposition events. The DNAs were isolated and sequenced as described in the text. The location of the inserts was determined from available genome databases[12,25], or the sequences flanking the insertion site were subjected to a BLAST homology search against the complete nonredundant nucleic acid and protein databases held at GenBank as of June 1999. All inserts generated 9 bp duplications of the target DNA.**

Our initial experiments used *E. coli*K12 as a model system to study the formation of transposition events through the electroporation of premade Tn*5* Transposomes. We constructed plasmid pGRPK7876, which carries a 1.8 Kb Tn*5*–like transposon encoding kanamycin resistance ($Kan^R$) and the conditional R6Kγ origin of replication[9] with *Pvu*II sites defining the donor DNA–transposon end sequence boundaries (the 19 bp end sequences were actually hyperactive mosaic sequences[10]). The transposon, either in the context of the donor DNA or excised free of the donor DNA by prior *Pvu*II digestion, was complexed with hyperactive Tn*5* transposase. The transposase–transposon complexes were electroporated into *E. coli*K12 MC1061 (which is reported to support a high electroporation efficiency[11]) or MG1655 (the entire genome sequence of which is known[12]) and $Kan^R$ colonies were selected. Example results are presented in Table 1A.

A precleaved 1.8 kb Tn*5*-like transposon defined by mosaic ends or the 2.7 kb replication-deficient plasmid from which it was derived (pGRPK7876) were incubated for 1 h at 37°C with hyperactive Tn*5* transposase (if present) at a final DNA:transposase ratio of approximately 1:8. Indicated reactions also contained 10 mM magnesium acetate. A volume of 1 μl was electroporated into *E. coli* MC1061 and MG1655 and plated on agar containing kanamycin. The electroporation frequency for the replication-proficient KanR plasmid pGRST2 is indicated as the control plasmid result.

Transposase-dependent $Kan^R$ colonies were generated under all tested conditions, but the most efficient generation of these colonies occurred when Tn*5* Transposomes were used in the absence of $Mg^{++}$. The use of transposase-bound precut transposons bypasses two steps in transposition, and formation of the Tn*5* Transposomes in the absence of $Mg^{++}$ prevents transposition events from occurring in

**Table 1. Transposome-mediated transposon insertion in bacterial genomes following electroporation.**

**(A) c.f.u./μg**

| DNA | Tnp | $Mg^{++}$ | MC1061 | MG1655 |
|---|---|---|---|---|
| Plasmid | - | - | 0 | 0 |
| Plasmid | + | - | $7.4 \times 10^6$ | $1.7 \times 10^5$ |
| Plasmid | + | + | $8.4 \times 10^6$ | $3.2 \times 10^5$ |
| Precut transposon | + | - | $1.1 \times 10^8$ | $5.4 \times 10^6$ |
| Precut transposon | + | + | $8.3 \times 10^6$ | $3.3 \times 10^5$ |
| Control plasmid | - | - | $1.8 \times 10^9$ | $2.1 \times 10^8$ |

**(B) c.f.u./μg DNA**

| *E. coli* DH10B | *S. typhimurium* | *P. vulgaris* |
|---|---|---|
| $3.2 \times 10^6$ | $5.6 \times 10^5$ | $1.0 \times 10^5$ |
| $1.6 \times 10^6$ | | |
| $2.5 \times 10^7$ | | |
| $3.9 \times 10^6$ | | |

vitro. Thus, we have increased the quantity of active transposition complexes in the cells. Finally, the data in Table 1A indicate that transposition has occurred at 2.5–10% of the plasmid electroporation efficiency. In other experiments (using the 1.8 kb Transposomes analyzed in Fig. 1) transposition events were achieved in 8% of the total cell population surviving the electroporation protocol.

The transposon target boundaries of 11 representative Kan$^R$ products from the MG1655 electroporation experiment were sequenced in order to ensure that they represented bona fide transposition events (Fig. 2). All 11 DNAs contained unique Tn*5*-like inserts with 9 bp target duplications at the boundaries.

Similar electroporation experiments were performed with another strain of *E. coli* (DH10B), a strain of *S. typhimurium* LT-2, and a strain of *P. vulgaris* (Table 1B), with pBR322 being used as an electroporation control for the *E. coli* (DH10B) and *S. typhinurium* experiments. A mock experiment (no transposase added) failed to yield any Kan$^R$ colonies for any of the bacterial species. *Salmonella typhimurium* and *P. vulgaris* supported lower frequencies of transposition than *E. coli*, perhaps because no attempt was made to modify the transposon DNA to protect it from host restriction systems. Electroporation of pBR322 yielded 100-fold higher frequencies of antibiotic-resistant *E. coli* (DH10B) cells and twofold higher frequencies of resistant *S. yphimurium* cells than did the electroporation-transposition experiments. The transposon-target boundaries from five *S. typhimurium* and four *P. vulgaris* isolates were sequenced. All plasmids contained the predicted Tn*5* end sequences bracketed by 9 bp target duplications (data not shown).

Seven presumed transposition products in *P. vulgaris* and seven presumed transposition products in *S. typhimurium* were also studied by Southern blot analysis. As shown in Figure 3, all seven Kan$^R$ isolates of *P. vulgaris* contained single unique transposon inserts. Six of seven Kan$^R$ isolates of *S. typhimurium* also contained single unique transposon inserts; the other isolate contained three inserts (data not shown).

Tn*5* transposition is known to occur in vitro in the absence of any proteins other than transposase[2]. Thus, we assumed that electroporation of premade Tn*5* Transposomes might well give rise to transposition events in *S. cerevisiae*. This was tested by electroporating a Tn*5* Transposome into *S. cerevisiae* strain C13 ABYS-86. Following electroporation, numerous small colonies grew on G418-containing agar regardless of whether transposase was or was not present. These microcolonies did not replate on G418-containing medium. Presumably, they arise from transient expression of the G418 resistance gene from free nuclear transposon DNA. Larger, less numerous colonies were formed only when transposon + transposase complexes were electroporated into the cells. These colonies grew when replated on G418-containing media.

Genomic DNA rescue experiments were used to obtain the sequences of transposon-target boundaries. The sequences obtained from six independent transposition events indicated that a nine-nucleotide direct repeat flanked the transposable element in each case, as expected for a Tn*5* transposase-mediated transposition (data not shown).

We have shown that electroporation of preformed Tn*5* transposase–transposon complexes (Transposomes) is a simple, efficient, robust means of generating transposon insertions in *E. coli*, *Salmonella typhimurium*, and *Proteus vulgaris*. Independent experiments have demonstrated that the same technology can be used to generate transposon insertions in a Gram-positive organism, *Mycobacterium smegmatis* (personal communication, K. Derbyshire, 1999). Presumably, this technology can be used in a similar fashion for any eubacterial species that can accept DNA via electroporation. We have also shown that electroporation of Tn*5* Transposomes can be used to generate transposon insertions in the yeast *S. cerevisiae*. Other means of introducing the Transposomes into eukaryotic cells

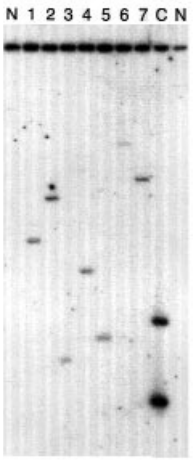


**Figure 3. Southern blot analysis of transposition clones in *P. vulgaris*. *Hpa*I/*Eco*RV digests of genomic DNAs isolated from presumed transposition clones were subjected to Southern blot analysis using as probes 23-mer and 25-mer oligonucleotides that correspond to the two ends of the transposon. The lane labeled "C" contains hybridization probe–positive controls 1,493 and 2,534 bp in length. The numbers 1–7 refer to independent transposition clones of *P. vulgaris*. The lanes designated N are negative controls containing bacterial DNA digested by restriction enzymes and not subjected to Transposome electroporation. *Hpa*I and *Eco*RV have no target sites within the transposon used; therefore, all probe targets should be >1,493 bp. The variety of target sizes is indicative of random insertion. The presence of only one target per lane indicates that each isolate had a single insertion event.**

are now being investigated. This technology avoids the need to generate species-compatible transposase expression systems and to use suicide vectors, and obviates the possibility of transposase-mediated events destabilizing the isolated inserts. Our successful results also confirm previous experimental results showing that Tn*5* transposition can occur without the aid of bacteria-specific host factors[2].

## Experimental protocol

**Media, bacterial and yeast strains, and plasmids.** Luria broth and Luria broth agar[13] (containing 20 μl/ml kanamycin where indicated) were used for all bacterial experiments, and Yeast extract, peptone, dextrose medium[14] (containing 200 μl/ml G418 where indicated) was used for the yeast experiments. The *E. coli* K12 strains used include: MC1061 (ref. 11), MG1655 (ref. 12), ECF5012 (ref. 9), and DH10B (ref. 15). *Salmonella typhimurium* LT-2 and *P. vulgaris* strains were from the American Type Culture Collection (ATCC; no. 15277 and no. 13315, respectively). The yeast strain was *S. cerevisiae* strain C13 ABYS-86 (ATCC no. 201198).

The plasmids carrying the Tn*5*-like transposons all had similar structures related to pMOD<MCS> (Epicentre Technologies, Madison, WI). In all cases, the plasmid-transposon border had the following structure: plasmid-cagCTGTCTCTTATACACATCT-transposon, in which the plasmid sequences are lowercase and the transposon 19 bp mosaic end sequence is uppercase. The *Pvu*II cleavage site used to cleave the transposon from the plasmid is underlined. The transposons used for the bacterial experiments included the R6Kγori (ref. 9) and a *kanR* gene from Tn*903* (ref. 16). Plasmid pGRPK7876, which contains the 1.8 kb transposon, has an 876 bp sequence from pUC19 as the donor backbone between the two *Pvu*II sites defining the transposon ends. The 1.5 kb transposon used for yeast studies included the p15A ori (ref. 17) and the kanMX4 cassette[18]. The sequence of all transposons used in these studies are available upon request. The control plasmids used to evaluate bacterial electroporation efficiencies were pGRST2 (ref. 19) and pBR322 (ref. 20).

Transposome formation and transposition mutagenesis. Transposomes were formed by incubating *Pvu*II (New England Biolabs, Beverly, MA) released and gel purified (QIAquick Gel Extraction Kit, Qiagen, Valencia, CA) transposon DNA (up to 50 μg/ml) with 10 μg/ml hyperactive Tn*5* transposase (Epicentre Technologies) for 10 min to 1 h at 37°C in a 20 μl reaction volume. For the experiments shown in Figure 1, the 1.8 kb Tn*5*-like transposon DNA was used at 2.5 μg/ml final concentration and incubated with purified hyperactive Tn*5* transposase in 400 μl. The reaction mixture was then concentrated 20-fold using a 0.05 micron VM nitrocellulose membrane (Millipore, Bedford, MA) prior to electrophoretic analysis. A 3.7 kb transposon also defined by 19 bp mosaic ends[10] (50 μg/ml final concentration) was similarly incubated with hyperactive Tn*5* transposase (10 μg/ml final concentration) in 40 μl of buffer, but was not concentrated prior to analysis. Various buffers have been used with equivalent results including: 25 mM TRIS acetate, pH 7.5, 100 mM potassium glutamate; and 27.5 mM TRIS-HCl, pH 7.5, 50 mM NaCl, 0.075 mM EDTA, 0.5 mM dithiothreitol, 0.05% Triton X-100, and 50% glycerol (transposomes formed in this buffer can be stored at –20°C until used).

Transposomes (1 μl) were electroporated into yeast or bacteria using standard procedures[14,15] and G418-resistant or Kan$^R$ colonies were selected.

**Sequence analysis.** Genomic DNA was purified either according to Marmur[21] or using the Master Pure DNA purification kit (Epicentre Technologies). For *E. coli*, *S. typhimurium,* and *S. cerevisiae* sequencing, the DNA was cleaved with a restriction enzyme that does not cut within the transposon (*Bam*HI, *Afl*II or *Eco*RV [New England Biolabs]), ligated using T4 DNA ligase (Epicentre Technologies), and transformed into *E. coli* DH10B or ECF5012 (ECF5012 supports R6Kγ ori functioning[9]) selecting for $Kan^R$ colonies. Both transposon borders were sequenced by standard dideoxy protocols[22] using primers complementary to sequences near the ends of the transposons. For *P. vulgaris* inserts, the chromosomal DNA (purified using the Master Pure DNA purification kit [Epicentre Technologies]) was sequenced directly by means of the BigDye Terminator cycle sequencing technology (PE Biosystems, Foster City, CA)[23].

**Southern blot analysis.** Genomic DNA purified with the Master Pure DNA purification kit was digested twice with a mixture of *Hpa*I and *Eco*RV (New England Biolabs), and electrophoresed on a 1% agarose gel. The gel was blotted and probed with $^{32}$P-end-labeled 23-mer and 25-mer oligonucleotides that corresponded to the two ends of the transposon as described[24].

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