Tn5 as a Molecular Genetics Tool

*In Vitro Transposition and the Coupling of In Vitro Technologies With In Vivo Transposition*

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**Summary**

The development of in vitro transposition technologies have provided many powerful tools for the molecular genetics research laboratory. In this chapter we describe some of these tools with a focus on the Tn5 transposition system. Tn5 technologies are particularly useful because the Tn5 transposition system has simple requirements, is efficient, random in target recognition, and robust. In particular we will describe the use of in vitro Tn5 transposition in transposon tagging and in the generation of nested deletions. We will also describe a unique in vitro/in vivo technology in which Tn5 inserts can be generated in a wide spectrum of bacterial species through the electroporation of preformed transposase–transposon DNA complexes.

**Key Words:** Tn5; in vitro transposition; transposon tagging; nested deletions; electroporation transposase.

**1. Introduction**

Transposition is a powerful tool for investigating and manipulating genomes. The obvious use for this technology is to create knockout mutations. However, the power of transposon mutagenesis is greatly enhanced by the fact that the DNA internal to the transposon ends can encompass a wide variety of sequences as long as the transposase can be provided by some other source. Thus the internal sequences can include, for example, selectable markers, reporter functions (such as the gene for β-galactosidase), controlling elements (such as a regulated promoter), primer binding sites, an origin of replication, an origin of gene transfer, epitope encoding sequences, and site specific recombination recognition sites.

Transposition technology is undergoing a major change as a result of the development of in vitro transposition systems. Most transposition applications
have previously been performed in vivo. There are several limitations imposed by using in vivo approaches. For instance, one must provide for the production of the transposase in the host cells, which requires the construction of specialized transposase expression systems for each target organism. In addition, it is typically desirable to limit transposase presence to a defined time frame, so that transposition events occur, but the products are stable (i.e., no subsequent transposition events occur). This outcome requires the introduction of transposase expression systems on suicide vectors that are not replicated by the target cells; the use of very tightly regulated transposase expression systems; or the transfer of the transposon products from one cell to another. Expression of active transposase in cells can be deleterious to the cells even in the absence of the desired transposition event. All of these limitations are bypassed by in vitro approaches.

A number of efficient in vitro transposition systems have been developed for practical applications. These include those derived from Tn5 (1), Ty1 (2), Tn7 (3), Mu (4), Mariner (5), and Tn502 (6). We will discuss Tn5-based technologies. The Tn5 system is simple, requiring but three macromolecular components: transposase, transposon DNA, and target DNA. This system is also efficient, random, and robust (1). In addition, one of the technologies that we will describe, electroporation of synaptic complexes (7,8) was first developed for the Tn5 system.

The basic technology used in in vitro transposition systems involves the introduction of the transposon DNA into the target DNA (see Fig. 1A). If the target (or the transposon) contains an origin of replication, then the resulting product will be a replicon that can be introduced into target cells by, for instance, electroporation, and the resulting cells containing independently replicating transposon inserts are selected. An example of this approach is the use of in vitro transposition to distribute primer binding sites along the length of a BAC clone (9). If no origin of replication is present, the single-strand gaps next to the ends of the transposon insert will need to be repaired, and then the transposon inserts can be incorporated into the target cell chromosome by homologous recombination. Examples of this approach for the generation of transposon insert mutations in Streptomyces coelicolor and Vibrio cholerae have recently been published (10,11).

Tn5 will also transpose in an intramolecular fashion (12). That is, the transposon ends will attack the transposon itself rather than a second DNA (Fig. 2). Two different groups of products are generated: One group consists of two deletion circles, and the second group consists of inversion circles. These two groups should be approximately evenly represented in the products because their respective generation is a function of the orientation of the attacking transposon ends onto the target. If the body of the transposon
Fig. 1. Steps in Tn5 transposition. (A) Standard transposition. The steps in the Tn5 transposition process are described in more detail in references (13) and (14). (B) Electroporation of synaptic complexes. The steps involved in the electroporation/transposition technology bypass some steps normally followed in Tn5 transposition. Preexcised transposon DNA is used. This allows transposase binding and synapsis without the cleavage steps. Thus these steps are performed in the absence of Mg^{2+}. The complexes are then electroporated into cells in which they encounter Mg^{2+} and target DNA and undergo strand transfer to insert the transposon.
Fig. 2. Intramolecular transposition forms nested deletions and nested inversions. The substrate plasmid contains a transposon defined by two end sequences (indicated by triangles marked with L and R) that contains between the ends an origin of replication (ori), the Amp' gene, and a target sequence encoding a protein (the N and C termini are indicated). Intramolecular transposition results in the formation of deletion circles (top) or inversions (bottom). Only one product is shown for each type of event, but in fact a library of products are made with approximately random deletion and inversion points.
substrate contains an origin of replication and a selectable marker as pictured in Fig. 2, one of the two types of deletion circles and the inversion circles can be propagated after introduction into a suitable host. Intramolecular transposition is a convenient technology for generating nested deletion families of the target gene.

We have recently developed a third technology that is a combined in vitro and in vivo approach (7). In essence this technology involves the formation of synaptic complexes in vitro, electroporation of the complexes into the cells, and selection for the products of transposition events. This technology has been successfully applied to many organisms, such as Escherichia coli K12, Salmonella typhimurium, Proteus vulgaris, Pseudomonas spp., and Mycobacterium smegmatis (7,8). It should be amenable to any organism that can be transformed via electroporation.

The basic mechanism of Tn5 transposition and how the electroporation/transposition technology has been designed around the transposition mechanism is presented in Fig. 1A and B (see refs. 7,13,14). First, the transposase binds to the 19 bp sequences that define the ends of the transposon. Second, the transposase dimerizes through the formation of protein–protein and trans protein–DNA contacts to form a synaptic complex. Third, the transposase catalyzes cleavage of the transposon DNA free of the adjoining donor DNA. These cleavage events require the presence of Mg²⁺. Fourth, the released synaptic complex binds to target DNA. Fifth, the 3'-OH ends of the transposon DNA attacks the target DNA with a 9-bp stagger, inserting the transposon DNA into the target. This insertion event also only occurs in the presence of Mg²⁺. The optimal form of the electroporation/transposition technology bypasses the third step, transposase-catalyzed cleavage of the transposon DNA from the adjoining donor DNA. Rather, precleaved transposon DNA is used in the first step, and then the end-bound transposase molecules dimerize to form precleaved synaptic complexes. These complexes are formed and are quite stable in the absence of Mg²⁺. The synaptic complexes are then electroporated into target cells. Inside the cells, the transposase is activated presumably because the cell contains Mg²⁺; transposition then occurs. Because no transposase is synthesized in the cells, no further transposition occurs.

There are several key aspects of the Tn5 transposition system that allows the electroporation/transposition technology to be used. First, in vitro experiments have shown that Tn5 transposition has simple macromolecular requirements: transposase, transposon DNA, and target DNA (1). In other words, no host functions are required, so Tn5 transposition can occur in many organisms. Second, we have generated very active forms of the transposase and the transposon end sequences so that the efficiency of transposition is quite high (15–17). Third, transposase binds to precleaved transposon end sequences in the absence
of donor or target DNA and Mg$^{2+}$. Fourth, transposase–transposon synaptic complexes are quite stable in the absence of Mg$^{2+}$. Fifth, transposase–transposon synaptic complexes are activated to transpose in the presence of Mg$^{2+}$.

Although there are limitations on the electroporation/transposition technology as we will discuss subsequently, it is an extremely powerful tool (7). In particular, it bypasses species barriers. Moreover, using an optimal E. coli K12 system and a 1.8-kb transposon, we have generated viable transposition products in as much as 8% of the cells that survived the electroporation treatment.

2. Materials

1. Transposase: The transposase is a hyperactive triple-mutant version of the Tn5 transposase. The mutations are at residues 54 (E to K), 56 (M to A) and 372 (L to P) (I). The enzyme can be purchased from Epicentre Technologies (see Note 1). N-terminal His-tagged and maltose-binding protein-fusion versions of the hyperactive transposase have also been constructed and used successfully (Yigit, H. and Reznikoff, W. S., unpublished) (18).

2. Transposon DNA: The transposon DNA can be defined by either the outside end (OE) sequence, the naturally occurring 19-bp sequence that defines the ends of Tn5 (19), or, preferably, by the mosaic end (ME) sequence that is a hyperactive mutant version of the OE (17). The ME end-defined transposon is described in Fig. 3. The DNA between the two ME sequences can carry a variety of antibiotic resistant markers or, alternatively, a multiple-cloning site for construction of the desired transposon (see Note 2). The latter is available from Epicentre Technologies.

3. Use of Precleaved Transposon: Both the in vitro transposition and the electroporation/transposition systems function with transposon DNA still embedded within plasmid DNA, and with prereleased transposon DNA. However, precleavage of the transposon DNA from adjoining sequences increases the electroporation/transposition frequency by over 10-fold. Precleaved transposons can be produced by two means: restriction digestion, or PCR amplification. As shown in Fig. 3, the ME–donor DNA sequence can be made cleavable by PvuII or PshAI (BoxI) to release the transposon from an appropriate plasmid (see Note 3).

An ME PCR primer (5’ CTGTCTTCTTATACATCT 3’) can be used for production of the “precleaved” transposon through PCR amplification. (Note: This single primer will function at both ends of the transposon. In addition, it is not necessary to employ a kinase with this primer or the resulting PCR product in order to make a functional transposon.) Since the percent of GC is low (37%), a 37°C annealing temperature is recommended for thermocycling. Taq DNA polymerase will produce untemplated 3'-A additions to a significant portion of the PCR products, which will lower the overall performance of the transposon preparation. PCR in the presence of a proofreading thermophilic DNA polymerase
Fig. 3. Basic transposon structure. The optimal transposon for the Tn5 in vitro system is defined by 19-bp mosaic end sequences. The transposon body can contain any desired sequence. The mosaic end–donor DNA junctions can be defined by Pvu II or PshA I (Box I) sites if precleavage of the transposon–donor boundaries is desired.

(e.g., Pfu or Pwo) along with Taq DNA polymerase will increase the yield of perfectly blunt-ended molecules. If Taq DNA polymerase is solely used for PCR, the transposon DNA can be end-repaired by incubation with T4 DNA polymerase and dNTPs (after purifying Taq DNA polymerase away), to yield blunt-ended DNA.

If PCR is done with primers annealing outside of the ME sequences, the PCR product (purified of Taq DNA polymerase) can be digested with Pvu II or PshAI (Box I) to yield perfectly blunt-ended DNA. If there are internal sites for these restriction enzymes within the amplified DNA, one is left with PCR using the ME primer.

The precleaved transposon DNA can be purified if desired by agarose gel electrophoresis followed by extraction using the QIAquick Gel Extraction Kit, Qiagen. Transposon DNA is stored in 10 mM Tris-HCl, pH 7.5, 1 mM EDTA.

4. Transposase Storage Buffer: The choice of an appropriate buffer for storage of the transposase is important because at high concentrations, transposase will aggregate into an inactive form at low salt conditions. For transposase concentrations equal to or less than 50 μg/mL, we typically use 0.05M Tris-HCl, pH 7.5, 0.1 mM EDTA, 0.1M NaCl, 1 mM dithiothreitol (DTT), 50% (v/v) glycerol, and 0.1% Triton X-100. For higher concentrations of transposase, the NaCl concentration is kept at 0.5 M.

5. Synaptic Complex Formation Buffer: Efficient formation of synaptic complexes has been found to occur in a variety of buffers although it is important that they not contain Mg²⁺. (Mg²⁺ is a required divalent anion for transposition catalysis [11].) We typically use 0.05M Tris-acetate, pH 7.5, 0.15M potassium acetate, 4 mM spermidine; and 1 mM EDTA. If the resulting synaptic complexes are to be stored long term at −20°C for future use, an equal volume of 100% glycerol is added after the complexes are formed. This solution does not freeze at −20°C.

6. Transposition Reaction Buffer: Efficient transposition reactions also occur in a variety of buffers. We routinely use the synaptic complex formation buffer adjusted to contain 10 mM MgCl₂.
3. Methods

3.1. Intermolecular In Vitro Transposition

The typical way to consider transposition is as an intermolecular process as pictured in Fig. 1A; that is, as the movement of a DNA sequence from a site on one replicon to a site on a second distinct replicon. It is this process that an investigator uses to tag DNA molecules with a transposon. The in vitro application of this process is primarily used in conjunction with the sequencing of large DNA stretches, by randomly inserting DNA polymerase priming sites within the target sequence (see Note 4). A typical reaction is performed in 10 μL of a solution that contains 0.1 μM transposase, 0.01 μM transposon DNA (for a transposase-to-transposon ratio of 10) and 0.01 μM target DNA. The important feature of this reaction mixture is that the molar concentration of the transposon should not exceed that of the target. If higher ratios of transposon-to-target are used, one can generate a significant number of double insertions, thus complicating the downstream analysis (see Note 5). The stated transposase concentration is well in excess and can be reduced somewhat with no significant impact on the reactions. The mixture is incubated for 2 h at 37°C, and then the reaction is terminated by adding 1 μL of 1% sodium dodecyl sulfate (SDS) and heating at 70°C for 10 min to inactivate the transposase. The mixture can be directly introduced into appropriate cells (by, for instance, electroporation) and transposition clones selected; it can be analyzed by electrophoresis; or it can be stored at −20°C for future analysis.

For experiments that involve the transposon tagging of plasmids to be introduced into E. coli K12, the products are electroporated (see Subheading 3.4. for a suggested protocol) into the appropriate host. Cells that have received tagged DNAs are selected by using an antibiotic for which resistance is encoded by the transposon. The plasmid DNAs from individual isolates can then analyzed regarding the location of the insert.

3.2. Intramolecular In Vitro Transposition
(Formation of Nested Deletions and Deletions/Inversions)

Intramolecular transposition reactions are performed exactly as described for intermolecular transposition reactions (see Subheading 3.4.) except that the second target DNA is omitted. The transposon containing clone is in essence both a transposon donor and the target.

3.3. Formation of Synaptic Complexes

Transposon DNA is incubated with hyperactive transposase typically at a 5:1 molar ratio of transposase to transposon in a 20-μL reaction volume for 1 h at 37°C. The concentrations of transposase and DNAs are typically 0.1 μM and
0.02 \mu M respectively. For smaller transposons (<2 kb) it is preferable to use larger incubation volumes to decrease the abundance of DNA intermolecular complexes (see Note 6). For these reactions a volume of 400 \mu L with a DNA concentration of 2.5 \mu g/mL can be used. The large-volume reactions need to be subsequently concentrated about 20-fold using a 0.05 \mu m Millipore VM membrane.

Following incubation a sample can be analyzed through a mobility shift analysis. Samples are electrophoresed on a 1% agarose gel in TAE buffer. A transposon-only control and a molecular-weight marker sample will aid in the identification of the desired intramolecular complexes.

3.4. Electroporation

Electroporation of the synaptic complexes can be effected using published procedures (20).

1. Electrocompetent cells are generally prepared by growing 100 mL of cells to mid-log (0.5–0.6 A_{600 nm}).
2. Cells are chilled, harvested by centrifugation, and washed with ice-cold 10% glycerol three times before suspending them in 250 \mu L of 10% glycerol. Cells are stored frozen at –70°C.
3. Electroporation is carried out by thawing cells, adding 1 \mu L of the synaptic complex reagent to 50 \mu L of thawed cells, and transferring the mixture to a 2.0-mm gap cuvet (see Note 7).
4. Cells are electroporated at 2500 volts (tau = 5 ms) using an Eppendorf multiporator. Slightly different conditions may be recommended for other brand electroporators.
5. Following electroporation, cells are diluted to 1 mL with Luria Bertani (LB) and incubated at 37°C with aeration for 1 h.

3.5. Selection of Transposition Events

Transposition events are selected by standard microbiological procedures based upon the nature of the selectable marker encoded by the transposon (see Notes 8 and 9).

3.6. Conclusion

The Tn5 transposition technology is powerful, robust and simple. The in vitro intermolecular transposition technology allows the generation of transposon insert libraries into any cloned DNA molecule. The inserts can contain virtually any desired sequence, and they will be found at essentially random locations. The in vitro intramolecular transposition technology allows the formation of random nested deletion and nested deletion/inversion libraries through a simple one-step reaction. The electroporation/transposition methodology has already allowed the expansion of transposition mutagenesis schemes to diverse microorganisms that previously were not amenable to genetic analysis.
4. Notes

1. Transposase toxicity: The hyperactive transposase is toxic to cells even in the absence of its specific DNA recognition sequences. Therefore, growth of strains producing hyperactive transposase can present problems. For instance, mutant forms of the transposase gene may arise that inactivate the protein. It is for this reason that purchase of the active enzyme is recommended.

2. DNA length limitations: There are two possible transposon-length limitations on the electroporation/transposition technology that have not been well studied. First, the ability to form intramolecular complexes should be dependent upon the relative concentration of the two ends, which is in turn dependent upon the chain length between the ends. We have found that molecules less than 5 kb long work effectively, but we have not tested longer molecules.

The second event that is length dependent is electroporation. It has been shown, for example, that smaller plasmid DNAs (e.g., 2.9 kb) are more efficiently electroporated into *E. coli* DH5α than larger DNAs (approx 50% reduction in efficiency for plasmids twice this size) (21). It is expected that larger complexes (due to the use of larger transposons) will likewise be subjected to size discrimination in many hosts, with the added complication that a nucleoprotein complex, and not naked DNA, is being electroporated into the cells. To what extent complexes are size biased has not been carefully studied.

3. PvuII star activities: It is most convenient to use prereleased transposon DNA for the electroporation/transposition technology. Frequently the prereleased transposon DNA is generated through the use of PvuII. Use of too high a concentration of PvuII and/or failure to adequately purify substrate DNA can result in extra enzyme cleavage products during the preparation of precleaved transposon DNA.

4. Target choice randomness: The randomness in target choice is an important consideration especially for the strictly in vitro technologies that we have discussed; that is, the intermolecular transposition is primarily used for introducing primer binding sites in conjunction with DNA sequencing, and the transposon must go in with sufficient randomness to permit sequence coverage. Likewise, randomness is an important feature when making nested deletions through the intramolecular transposition protocol. All transposition systems are likely to display some sequence biases in regards to target selection. A saturation transposition target-selection analysis for the Tn5 system has demonstrated some sequence preferences (22). However, even in this study, approximately 10% of the possible target sites were hit. In Fig. 4 we present example results of an in vitro intermolecular transposon experiment in which 55 independent inserts were generated in a 7775 bp target. Fifty-four separate sites were hit (one site was hit twice), and there appears to be no obvious base-composition bias for the insert sites (23).

5. Multiple insertion events following electroporation of transposition complexes: It is possible, although unlikely, that multiple transposon inserts could be generated by the electroporation of more than one complex into a cell. The number of inserts can be determined by a Southern blot assay. Using this procedure we analyzed 14 independent isolates and found only 1 isolate had more than one insert (7).
Fig. 4. Insertion data for 55 inserts generated in a 7775-bp target. Inserts were generated through the in vitro intermolecular reaction, the resulting products were introduced into cells, and the insertion sites were determined by DNA sequencing. Only two inserts (685 and 728) landed at the same site. Below the insert map is a GC content map using a 25-bp window. The insert sites demonstrated no obvious base-composition bias. The data was kindly provided by R. Meis and is similar to a figure published previously (23).
Simple ways to avoid this problem include performing the electroporation experiment at submaximal levels of complex and using more concentrated cells.

6. Excess transposon DNA and the formation of intermolecular synaptic complexes: Scientists frequently add excess reagents to increase the yield of various protocols. With regard to the electroporation/transposition technology, use of high-transposon DNA concentrations can lead to the formation of intermolecular instead of intramolecular transposase–transposon complexes, in which two ends from different transposon molecules are complexed together. While the result of electroporating such intermolecular complexes has not been deliberately studied, it is likely that they will yield double-strand breaks instead of insertion events. The abundance of intermolecular complexes can be estimated by prior agarose gel electrophoresis of the complex mixture. We have found that electroporation/transposition can be performed successfully even in the presence of some intermolecular complexes, as long as intramolecular complexes are also present and evident on gel analysis.

7. Electroporation of greater volume samples: Our recommendation for sample electroporation is to use 1 μL of sample. In order to increase the yield of cells containing transposon inserts, it may be desirable to use larger transposition-complex sample volumes, but this method will lead to arcing due to the salt content. To increase the transposition-complex sample volume we recommend dialysis of the sample versus 5 mM Tris-HCl, pH 7.9, 10% glycerol. Dialysis can be accomplished by floating a 25-mm Millipore disk (0.05 μm, VM) on the surface of 50 mL of buffer and applying a drop of up to 40 μL sample to the disk for 30 min. Glycerol is necessary if the sample is to be stored at −20°C. If the sample is to be electroporated immediately, no glycerol is necessary.

8. Use of too high a concentration of antibiotic: Many standard media contain antibiotic levels suitable for selection of multicopy antibiotic-resistant genes. The electroporation/transposition technology generates monocopy inserts, and thus standard recipes may contain excess antibiotic. This problem can be addressed by predetermining a minimum inhibitory concentration for the relevant antibiotic and the host strain, and adjusting the selection conditions accordingly.

9. Background plasmid clones after electroporation: Contamination of PvuII or PshAI digestion products with uncut plasmid DNA can result in some of the antibiotic-resistant clones arising from plasmid transformation instead of transposon integration, if the target cells support DNA replication of the plasmid from which the transposon was derived. Overdigestion with restriction enzymes followed by careful gel purification should alleviate this problem.

For PCR generated transposons, intact plasmid contamination is generally not an issue because only small amounts of plasmid template are used to amplify the transposon sequence. However, digestion of the plasmid DNA in a region outside of the transposon proper can both improve PCR performance and further lower the background. To completely eliminate any potential for background clones, the PCR reaction product can be digested with DpnI which will cleave any N6A-methylated DNA template at GATC, which would be produced in most
E. coli strains because they are Dam+. PCR products, not being methylated, are resistant to digestion.

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References