

# Jumping Green Genes: A Transposon-Based Approach for Rapidly Creating Functional, Fluorescent Fusion Proteins

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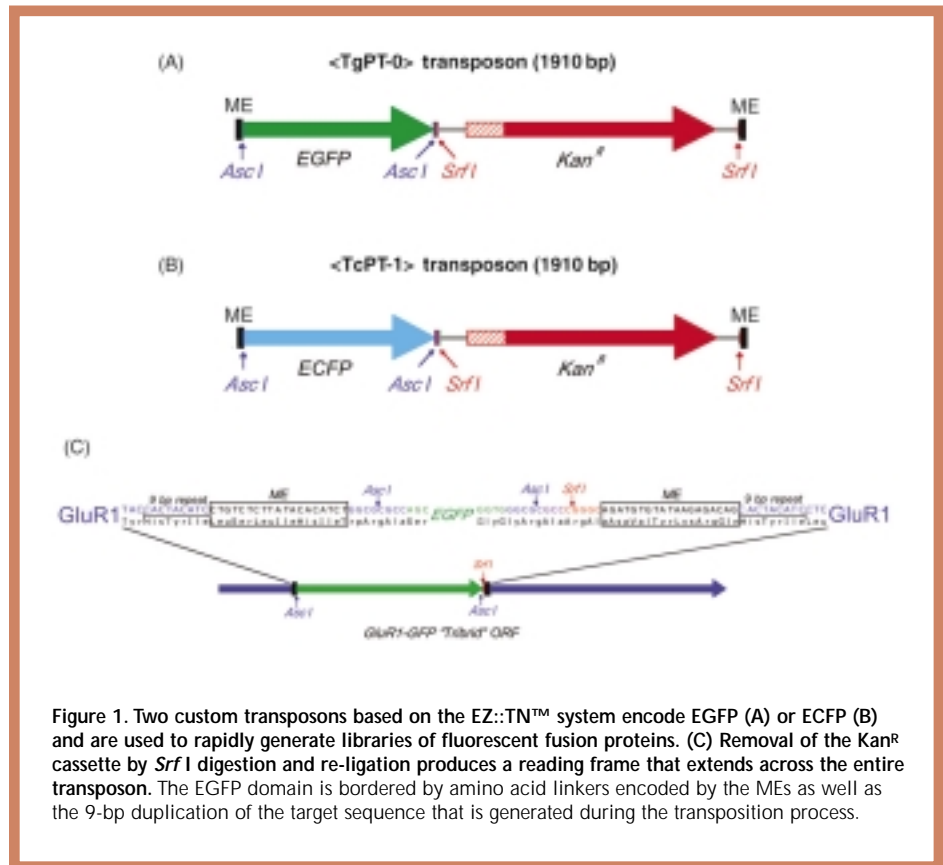
## Introduction

In 1997, Siegel and Isacoff<sup>1</sup> described the construction of a genetically encodable biosensor that could signal changes in voltage across a cell membrane. It was built by placing the green fluorescent protein (GFP) in just the right part of a Shaker potassium channel, such that conformational changes in the channel produce changes in the GFP fluorescence. Unfortunately this sensor signals fairly slowly, so Ataka and Pieribone, in an analogous approach,<sup>2</sup> placed GFP in several different regions of a sodium channel to produce a faster sensor, presumably capable of imaging events such as action potentials.

These two biosensors are just the first in a series of constructs that will undoubtedly be produced. At the moment, the single greatest impediment to building such biosensors is that it is quite difficult, often a matter of pure guesswork, to find the right place to insert the GFP. Designing and building each fusion protein usually involves weeks of work, with only a small chance of producing a functional sensor. To circumvent this bottleneck, we developed custom transposons based on the EZ::TN™ Transposon system, to rapidly create libraries of GFP fusion constructs that could then be screened for function, fluorescence and potential use as sensors.

## Methods

Transposons were designed which encode enhanced green fluorescent protein (EGFP, BD Biosciences Clontech) or enhanced cyan fluorescent protein (ECFP, BD Biosciences Clontech) based on three main criteria (Figure 1). First, to maximize flexibility, the sequences encoding the fluorescent proteins were designed as "cassettes" flanked with *Asc* I sites. Second, we designed the GFP cassettes in two different reading frames, taking advantage of 2 out of 3 possible insertions within a target. Finally, an antibiotic cassette had to be included in the transposon, so a *Kan*<sup>R</sup> cassette flanked by *Srf* I sites was added downstream of GFP. If the transposon lands in the correct orientation and reading frame, it should initially produce a trun-



**Figure 1.** Two custom transposons based on the EZ::TN™ system encode EGFP (A) or ECFP (B) and are used to rapidly generate libraries of fluorescent fusion proteins. (C) Removal of the *Kan*<sup>R</sup> cassette by *Srf* I digestion and re-ligation produces a reading frame that extends across the entire transposon. The EGFP domain is bordered by amino acid linkers encoded by the MEs as well as the 9-bp duplication of the target sequence that is generated during the transposition process.

uncated fusion protein, due to a stop codon in the *Kan*<sup>R</sup> cassette. *Srf* I digestion and re-ligation removes the *Kan*<sup>R</sup> cassette, producing a continuous reading frame across the entire transposon (Figure 1, full sequences available at: <http://momo-tion.med.yale.edu>).

Following standard cloning procedures, a primer complementary to the 19-bp mosaic end (ME) sequence was used to amplify the transposon constructs (1 cycle at 95°C for 3.50 minutes, 24 cycles of 95°C for 30 seconds, 47°C for 30 seconds, 72°C for 1 minute, and 1 cycle at 72°C for 5 minutes) with *Pfu* DNA polymerase. The PCR product was purified, concentrated, and resuspended in 1X TE buffer. Molar equivalents of transposon and target plasmid (0.4 fmoles each) were incubated with 1  $\mu$ l of EZ::TN™ Transposase in a 10  $\mu$ l *in vitro* insertion reaction according to manufacturer's recommendations.

To test the transposons, we targeted two different glutamate receptor subunits, GluR1 and GluR2.<sup>3</sup> Each subunit is capable of producing a homomeric glutamate receptor in an HEK 293 cell. Two separate transposition reactions, with two different "colored" transposons (Figure 1), were performed with each of the subunit genes. Transposed clones were then transiently expressed in HEK 293 cells and visually screened for fluorescence in a pairwise fashion (i.e., one potential green clone and one potential cyan clone per well).

Each of the clones that produced a truncated, fluorescent protein was sequenced to define the insertion site. Unique clones were digested with *Srf* I, to remove the *Kan*<sup>R</sup>, and dilutions of the restriction digestions were re-ligated. Whole-cell patch clamp recording was used to test each full length fusion protein for channel

function in transiently transfected HEK 293 cells as previously described.<sup>4</sup>

## Results & Discussion

An EZ::TN Transposon is defined as any sequence flanked by the inverted 19-bp repeats known as mosaic end (ME) sequences. EZ::TN Transposase binds these ME sequences and, in the presence of Mg<sup>2+</sup>, catalyzes the *in vitro* insertion of the transposon into target DNA. Assuming that the transposon behavior is random, the predicted frequency of insertions producing a fluorescent fusion protein in GluR1 was 7.8%. This agrees well with the observed frequency of clones that produced a fluorescent fusion protein (7.7%). It appears that GFP can be inserted virtually anywhere in another coding region and it will continue to fold and form a fluorophore.

Glutamate receptors have previously been tagged by adding the GFP to either the C-terminus<sup>5</sup> or very close to the N-terminus.<sup>6</sup> Both strategies have produced functional, fluorescent subunits. Consistent with these reports, insertions of the GFP near the N- or C-terminus produced functional channels, but there were numerous exceptions that reveal that these regions are not entirely permissive (Figure 2). There was one region in the middle half of the N-terminal domain, however, that consistently produced fluorescent receptors that continued to work. The second half of the N-terminus, and the other extracellular loop of the receptor subunit, are known to form the glutamate binding domains of the receptor.<sup>7</sup> All of the insertions into these domains destroyed receptor function.

Surprisingly, there were insertions at the very distal portions of the transmembrane domains that produced functional receptors. These are particularly exciting proteins, because the proximity of the GFP to the membrane, and the presumed channel, make them likely candidates as biosensors. These are constructs that would have been avoided in a rational design approach for fear of disrupting channel function. This is the power of a transposon approach; the fast, cheap, random nature of the process can be exploited to quickly discover fusion proteins that work and which would never have been built by hand.

## References

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A custom EZ::TN™ Transposon can be prepared quickly and easily using a Transposon Construction Vector. Clone any DNA of interest into the multiple cloning site and then generate the transposon by PCR amplification or restriction enzyme digestion. Your custom transposon will also contain primer binding sites at either end for bidirectional sequencing.

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Includes pMOD™-2<MCS> Vector and the Forward and Reverse PCR Primers.

### EZ::TN™ Transposase

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The EZ::TN™ In-Frame Linker Insertion Kit is a transposon-based protein modification system that can be used to make random, 19-amino acid in-frame insertions into genes of expressed proteins for functional analysis, protein modification or domain mapping.

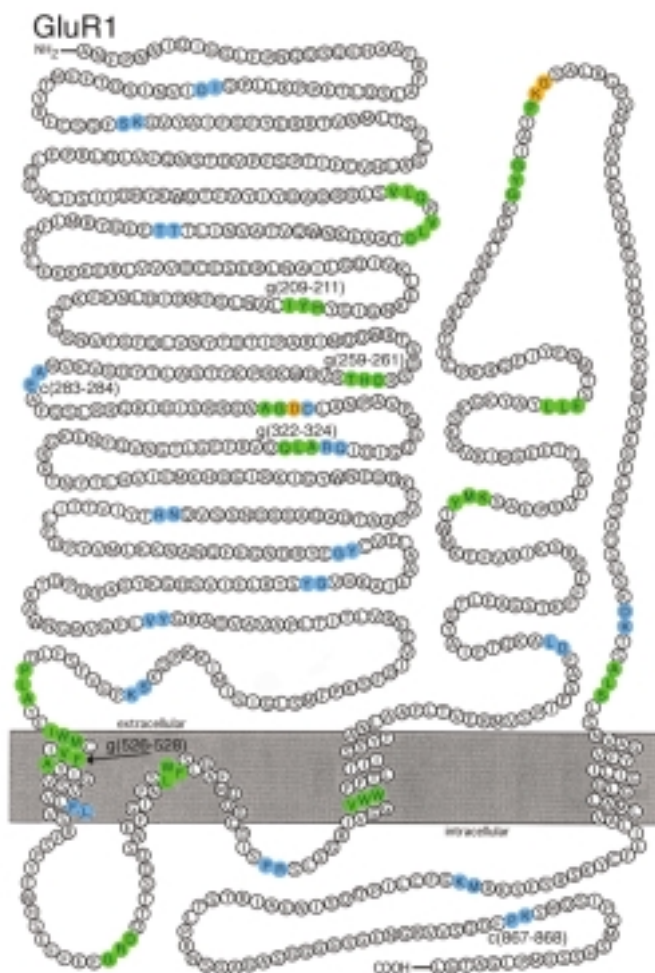
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### EZ::TN™ In-Frame Linker Insertion Kit

EZI04KN                      10 Reactions

#### Contents:

EZ::TN™ <Not I/KAN-3> Transposon, EZ::TN™ Transposase, EZ::TN™ 10X Reaction Buffer, EZ::TN™ 10X Stop Solution, Forward and Reverse Primers, Control Target DNA, and Sterile Water.



**Figure 2. Unique insertion sites and functional analysis of GluR1 fluorescent fusion proteins.** In-frame insertions of the <TgPT-O> transposon are indicated by green circles that represent the three amino acids duplicated during transposition. In-frame insertions of the <TcPT-1> transposon (cyan circles) duplicate only two amino acids. Orange circles indicate overlapping insertion sites recovered in separate clones. Insertions producing fusion proteins capable of forming functional, homomeric channels are identified by the amino acids duplicated (e.g. g209-211).