In Vitro Insertion of a Transposon Containing an *E. coli* Origin of Replication Facilitates Rapid Recovery, Propagation and Sequencing of Circular DNA Molecules from Heterologous Organisms

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Introduction

Circular DNA molecules are known to play important roles in both eukaryotic and prokaryotic cells, but their study in many systems has been hindered by a lack of appropriate, easy-to-use genetic tools. For example, although plasmids play an important role in bacterial diversity and pathogenicity, plasmids from many bacteria do not replicate in *E. coli*, lack selectable or easily screenable markers, and may be present at only low copy number in host cells. Here, we demonstrate that EPICENTRE's recently-introduced EZ::TN™ <R6K*γori* /KAN-2> Insertion Kit greatly facilitates recovery and study of such plasmids. A more complete description of this work was presented as a poster at the 2nd ASM/TIGR Conference on Microbial Genomes in Las Vegas, Nevada in February, 2002. The complete poster is available online at www.epicentre.com/tigrposter.asp

Methods

The strategy used for "rescue" cloning of circular DNA molecules is presented in Figure 1. Plasmid DNA was purified from two different strains–*Thermus flavus* AT62 and *Pseudomonas syringae* var. *tagetis* - using a standard alkaline lysis procedure. Then, 200 ng of plasmid DNA was incubated with a one-tenth molar quantity of the EZ::TN™ <R6K*γori* /KAN-2> Transposon and 1U of EZ::TN™ Transposase in a Mg^{2+}-containing buffer. The reaction was performed for 2 hours at 37°C as described in the product literature. One microliter of reaction mix was electroporated into TransforMax™ EC100D™ *pir*+ cells which express the π protein ("*pir*" gene product) required for replication from the R6K*γori*.[1] After overnight selection on plates containing kanamycin, insertion clones were screened for size on a gel using the Colony Fast-Screen™ Kit. Some of the transposon insertion clones were sequenced bidirectionally using the two primers provided in the kit that anneal near each end of the transposon.

Results and Discussion

Three different plasmids were rescued from the two bacterial strains studied using the EZ::TN <R6K*γori* /KAN-2> Insertion Kit - one from a *Thermus* strain and two from *Pseudomonas*. The plasmid

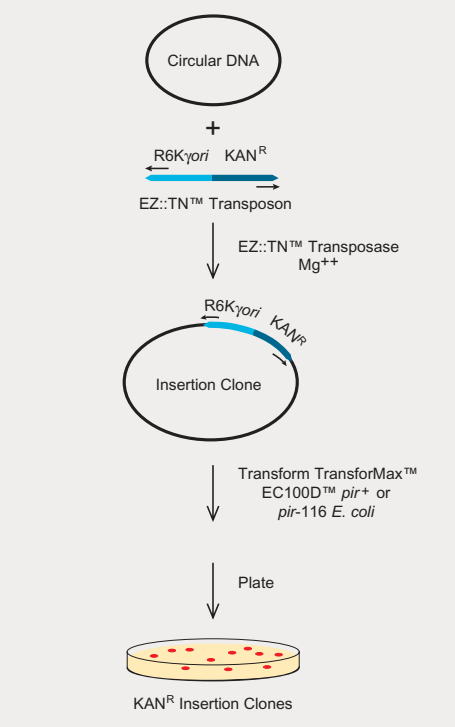

Figure 1. Circular DNA molecules, such as plasmids, are rescued in *E.coli* after *in vitro* insertion of an EZ::TN™ Transposon containing the R6K*γ* origin of replication (R6K*γori*) and a kanamycin selectable marker (KAN^R). Primer binding sites are located near each end of the transposon for bidirectional sequencing of the insertion site.

from *T. flavus* was completely sequenced quickly and easily by assembling overlapping sequences obtained by bidirectional sequencing of <50 randomly-chosen insertion clones.

A key factor in the success of this strategy for rescue and sequencing of heterologous circular DNA molecules was the fact that EZ::TN Transposon insertion frequencies were high. For example, about 26,000 transposon insertion clones were obtained for the 10.4-Kb *Thermus* plasmid from a single 10-µl *in vitro* insertion reaction using a 1-to-10 molar ratio of transposon DNA to plasmid DNA. Even more insertion clones would have been obtained if a higher molar ratio of transposon-to-plasmid DNA had been used. However, higher ratios were not used because we observed that a 1-to-1 ratio increased the frequency of double insertions and circular background clones. Background clones, which were generated via an intramolecular transposition mechanism,[2] are approximately 2 Kb in size and were easily detected by size screening on a gel (Figure 2).

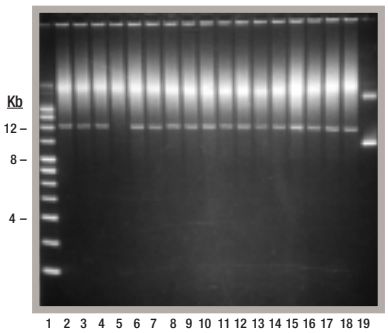

Figure 2. *T. flavus* plasmid clones containing the EZ::TN™ <R6K*γori* /KAN-2> Transposon were size selected using the Colony Fast-Screen™ Kit. Sixteen of 17 randomly chosen clones contained a 12.4 Kb plasmid which is consistent with a single 2 Kb transposon insertion into the 10.4 Kb target. One background clone contained a 2 Kb plasmid (Lane 5). Lane 1, supercoiled DNA ladder; Lane 19, purified plasmid DNA from *T. flavus*.

In addition to the observed high transposon insertion frequency, another important reason for the success of this strategy was that transposon insertion was highly random (Figure 3). The absence of insertion "hot spots" (i.e., clustered or identical insertion sites) or extensive gaps between insertions permitted easy assembly of the complete plasmid sequence using a minimal number of randomly-chosen insertion clones. It was, of course, also beneficial that the insertions sites could be sequenced in both directions using only

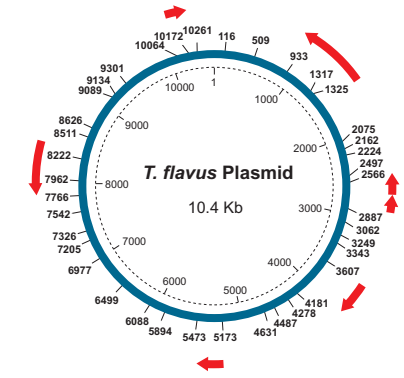

Figure 3. EZ::TN™ <R6K*γori* /KAN-2> Transposon insertions into a *T. flavus* plasmid were highly random. Hash marks indicate transposon insertion sites for 39 randomly selected clones. BLAST analysis identified seven open reading frames (red arrows) with significant homology to known gene products.

the two provided primers that anneal near each end of the transposon. This resulted in the maximum amount of sequence data per insertion clone and saved the expense and time that would have been required if primer walking or subcloning strategies had been used.

Conclusion

In vitro transposon insertion using the EZ::TN <R6K*γori* /KAN-2> Insertion Kit provides a powerful and efficient method to rescue circular DNA molecules, such as plasmids, from heterologous organisms and propagate them in *E. coli*. Once insertion clones are obtained, the complete sequence of the circular DNA molecules is easily, rapidly, and inexpensively determined by sequencing bidirectionally from randomly distributed primer binding sites that are near the ends of the inserted transposon. Moreover, many insertions will potentially create gene "knockouts", which, if expressed in *E. coli*, could be used for identification of gene function.

References

1. Metcalf, W.W. *et al.* (1994) *Gene* **138**, 1.
2. York, D. *et al.* (1998) *Nucl. Acids Res.* **26**, 1927.

www.epicentre.com/transposomics.asp

EZ::TN™ <R6K*γori* /KAN-2> Insertion Kit

EZI011RK 10 Reactions

Contents:

EZ::TN™ <R6K*γori* /KAN-2> Transposon, EZ::TN™ Transposase, 10X Reaction Buffer, 10 X Stop Solution, Forward and Reverse Primers, Control Target DNA, and Sterile Water.

TransforMax™ EC100D™ *pir*$^+$ Electrocompetent *E. coli*

ECP09500 5 X 100 µl (10 Electroporations)

Maintains clones at 15 copies per cell. Includes control vector containing an R6K*γori*.

TransforMax™ EC100D™ *pir*-116 Electrocompetent *E. coli*

EC6P095H 5 X 100 µl (10 Electroporations)

Maintains clones at 250 copies per cell. Includes control vector containing an R6K*γori*.

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Colony Fast-Screen™ Kit

FS08250 1 Kit

Reagents sufficient for screening 250 colonies.

Contents:

EpiBlue™ and EpiLyse Solutions.

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The MasterAmp™ Extra-Long PCR System enables consistent, high fidelity amplification of long DNA sequences at least 20 Kb in length. This increased consistency and fidelity is accomplished by combining the MasterAmp™ Extra-Long DNA Polymerase, a unique blend of thermostable polymerases that insure high fidelity, with an extensively tested set of 9 MasterAmp™ Extra-Long PCR 2X PreMix solutions. Each PreMix contains dNTPs, buffer, and various amounts of both $MgCl_2$ and MasterAmp™ PCR Enhancer (with betaine*).

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The MasterAmp Extra-Long PCR System gives consistent and reliable results due in part to the MasterAmp Extra-Long PCR 2X PreMixes. Optimal results are obtained by performing PCR using the MasterAmp Extra-Long PCR Kit containing the DNA Polymerase Mix and the 9 PreMixes then selecting the MasterAmp Extra-Long PreMix that provides the best amplification for your template/primer pair. The selected PreMix can then be used for all your subsequent PCR amplifications of that template/primer pair.

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As shown in Figure 1, amplifications of up to 40 Kb DNA are obtained rapidly using the MasterAmp Extra-Long PCR System from lambda DNA. Any genomic DNA template can be used and typically, "hot start" PCR is not required.

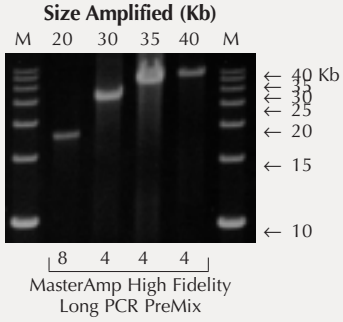

Figure 1. Amplification of 20, 30, 35, and 40 Kb sequences from lambda DNA. One nanogram of lambda DNA was used to amplify 20, 30, 35, and 40 Kb sequences. Lane M, 5 Kb DNA ladder.

High Fidelity PCR of Long DNA for Sequencing and Expression

The MasterAmp Extra-Long DNA Polymerase Mix contains a 3′ → 5′ proofreading enzyme that delivers fidelity at least 3-times higher than *Taq* DNA Polymerase.

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MasterAmp™ Extra-Long PCR Kit

MHF9220 50 Reactions

Contents:

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MasterAmp™ Extra-Long DNA Polymerase Mix
Control Lambda DNA/Primers

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