EPICENTRE Forum

Tools & Techniques for Genomics, Proteomics & RNA Research

Construction of Four CopyControl™ BAC Libraries by BACTROP – a BAC-Based Platform to Study Tropical Plant Species

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BIOTROP is a unit of CIRAD (Centre de Cooperation Internationale en Recherché Agronomique pour le Development) the French scientific organization specializing in development-oriented agricultural research for the tropics and sub-tropics. The BIOTROP unit develops molecular technologies to study biodiversity of tropical species with the aim to create and identify well adapted genotypes, particularly in terms of their quality and their tolerance to the biotic and abiotic constraints of their environment.

At BIOTROP, we recently created BACTROP: a platform of BAC libraries for studying the genome structure and evolution of tropical plant species. BACTROP represents a valuable tool for map-based cloning of genes involved in quality traits (e.g., fruit maturation) and disease resistance and to initiate studies of linkage disequilibrium in tropical crop plants.

In 2001, we successfully constructed five BAC libraries using the pIndigoBAC-5 cloning vector from EPICENTRE. At the Plant and Animal Genome Conference (PAG X) in January, 2002 we learned of the advantages of EPICENTRE's new CopyControl™ BAC Cloning Kits. The ability to induce the CopyControl BAC clones from single-copy to higher-copy number to improve DNA yield for analysis was very appealing. Since January we have constructed an additional four BAC libraries using the CopyControl BAC Cloning Kits. Here we report our experiences constructing the CopyControl BAC libraries and inducing the clones to higher-copy number.

Constructing the CopyControl BAC libraries

Four CopyControl BAC libraries from banana, cocoa, coffee and coconut were constructed using the CopyControl BAC Cloning Kit (*Hin*d III). For each library, high molecular weight DNA was partially digested with *Hin*d III. Ligation of the *Hin*d III-cut DNA into the CopyControl™ pCC1BAC™ (*Hin*d III) Vector was done for 3 hours at 30ºC for the coconut library and overnight for the other three libraries. Each library contained less than 1% blue (non-recombinant) colonies and very few empty white clones. The average insert size was determined by *Not* I digestion of randomly picked clones followed by 1% agarose PFGE (Figure 1). A summary of the four libraries is presented in Table 1.

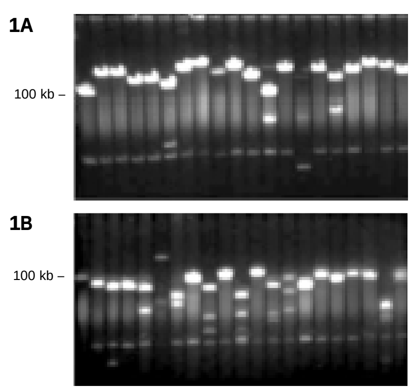

Figure 1. Analysis of the size of BAC clones produced from cocoa (Panel A) and banana (Panel B) using the CopyControl™ BAC Cloning Kits. In each example clones were randomly picked from the respective library, induced to high-copy number, digested with *Not* I, and analyzed by 1% agarose PFGE.

Species	Number of clones	Average insert size	Genome equivalents
Banana	36,864	135 kb	9
Coconut	92,160	135 kb	5
Coffee	55,296	135 kb	9
Cocoa	36,864	120 kb	11

Table 1. A summary of four CopyControl™ BAC libraries constructed by BACTROP using the CopyControl™ pCC1BAC™ (*Hin*d III) Vector.

. . . continued on page 2

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The low background and large insert sizes of the libraries demonstrated to us the high quality of the linearized and dephosphorylated pCC1BAC Vector preparation that is provided in the kits.

Induction of the CopyControl BAC clones to higher copy number

At BACTROP, like many laboratories, we have a need to rapidly analyze a large number of clones. We have developed a 96-deep well, high-throughput process for inducing the CopyControl BACs to high-copy number. We found the average induction level to be approximately 15-fold using this protocol. The large amount of DNA produced from each clone is sufficient for many applications including fingerprinting analysis for assembly of contigs, defining genomic regions around genes of interest or Quantitative Trait Loci (QTLs).

The CopyControl system has also enabled high-throughput, direct sequencing of BAC-ends using template purified after induction to high-copy number.

Conclusion

The CopyControl technology developed at EPICENTRE has enabled the rapid development of the BACTROP platform and exploitation of BAC libraries representing the genomes of tropical species. The advantages of the CopyControl system will accelerate the analysis and sequencing of BAC clones of interest. We can then rapidly identify genomic regions containing quality traits or resistance genes to phytopathogens of tropical plants.

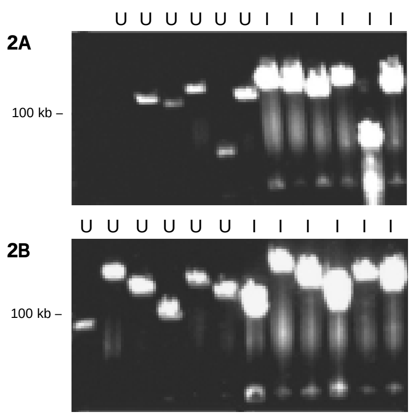


Figure 2. The CopyControl™ BAC clones were readily induced to high-copy number. DNA from an equal number of cells from uninduced (U) and induced (I) cultures of CopyControl BAC clones from coconut (Panel A) and Cocoa (Panel B) was digested with *Not* I and analyzed by 1% agarose PFGE. Induced cultures yielded about 15-fold more BAC DNA.

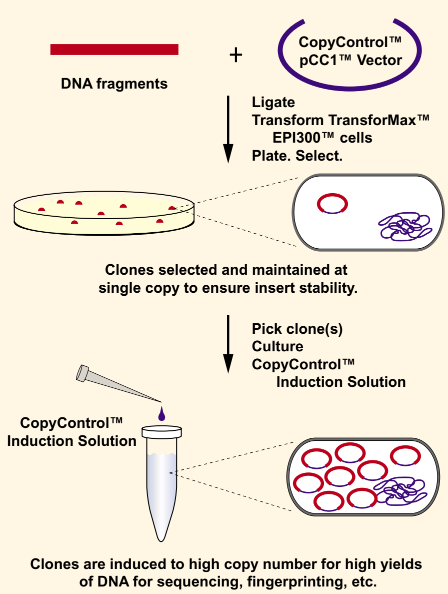
How the CopyControl™ BAC Cloning Kits Work

The CopyControl BAC Cloning Kits–based on technology developed in the laboratory of Dr. Waclaw Szybalski[1] at the University of Wisconsin-Madison –enable researchers to make and maintain BAC clones at single-copy number to ensure insert stability and then, whenever desired, to induce the clones to high-copy number for high yields of DNA for fingerprinting and DNA sequencing.

The pCC1BAC™ Vectors, provided in the kits, contain both the single-copy *E. coli* F-factor replicon and a high-copy origin of replication called "*oriV*." Initiation of replication from *oriV* requires the "*trfA*" gene product supplied by the TransforMax EPI300™ *E. coli* that contain the *trfA* gene under tight control of an inducible promoter.

In the absence of *trfA* gene induction, replication of CopyControl pCC1 clones is controlled by the F-factor replicon and the vector is present at one copy per cell. Addition of the CopyControl™ Induction Solution to CopyControl BAC clones grown in culture induces expression of the *trfA* gene resulting in initiation of replication from *oriV* and amplification of the clone to 10–20 copies per cell.

CopyControl capability can be easily introduced into existing single-copy BAC and fosmid clones (see the center insert). In addition, a CopyControl Fosmid Library Production Kit and CopyControl PCR Cloning Kits are available (see the center insert).



References

1. Wild, J. *et al.*, (2002) *Genome Research* **12**, 1434.

Unlike Other PCR Products, the FailSafe™ PCR System Amplifies a GC-Rich Template and a 20-Kb Template on the First Try

Haiying Grunenwald, EPICENTRE

Introduction

The FailSafe™ PCR System provides consistent and dependable amplifications of any template up to about 20 kb in length, even difficult templates, such as those with high-GC content or secondary structure, and multiplex PCR amplifications. The FailSafe PCR System combines a unique blend of high-fidelity thermostable enzymes with an extensively tested set of FailSafe™ PCR PreMixes that include dNTPs, buffer, $MgCl_2$, and the patented FailSafe™ PCR Enhancer Technology. In this report we compare how the FailSafe PCR System performs the first time used with GC-rich and long templates compared with results using seven other significant suppliers of PCR enzymes and systems.

First Time

Perform PCR with your template and primers using the FailSafe™ PCR PreMix Selection Kit and choose the PreMix that provides the best amplification.

and Every Time

Get the selected PreMix with the FailSafe™ PCR System and use it for consistent amplification of your template/primer pair.

Methods and Results

Amplification of a GC-rich template

A 268-bp region (GC content = 75%) of the human apoliproprotein E gene was amplified. The reactions were set up according to each of the manufacturer's standard protocol instructions. In brief, for each 50-µl PCR reaction, the following components were included: 25 pmole of each of the forward and reverse primers, 100 ng of human genomic DNA, 200 µM of dNTPs, 1X reaction buffer , and 1.25 U of appropriate DNA polymerase. The FailSafe™ PreMix Selection Kit was used with the 12 FailSafe PCR PreMixes provided. Cycling conditions were 5 minutes at 94ºC, followed by 35 cycles of 95ºC for 30 seconds, 60ºC for 30 seconds, and 72ºC for 1 minute. As shown in Figure 1, only the FailSafe PCR System using PreMix K successfully amplified the *apoE* gene.

Amplification of a 20-kb template

A 20-kb region of lambda DNA was amplified. The reactions were set up according to each of the manufacturer's standard protocol instructions. In brief, for each 50-µl PCR reaction, the following components were included: 50 pmole of each of the forward and reverse primers, 1 ng of lambda DNA, 200 µM of dNTPs, 1X reaction buffer with $MgCl_2$, and 2.5 U of appropriate DNA polymerase. For the FailSafe System, the 12 PCR PreMixes in the FailSafe PreMix Selection Kit were used as directed. Cycling conditions were 1 minute at 94ºC, followed by 20 cycles of 98ºC for 20 seconds and 68ºC for 20 minutes. As show in Figure 2, both FailSafe and Supplier "6" successfully amplified the 20-kb PCR, however about 2-fold more PCR product was obtained from the FailSafe reaction using PreMix D.

Summary

This report demonstrated how the FailSafe PCR System amplified a high-GC template and a 20-kb long template the first time on the first try. Other PCR products did not perform as well when the standard protocol was used. It is important to note that each of these other PCR products have optimization recommendations to try in case a PCR reaction does not amplify the first time. However, as demonstrated, with the FailSafe PCR System, no time or effort were needed to amplify these templates successfully the first time. The same FailSafe PCR PreMixes that amplify a given template give consistent PCR results in all subsequent amplification reactions. Due to the system's performance, reliability, and ease of use, the FailSafe™ PCR System is the method of choice.

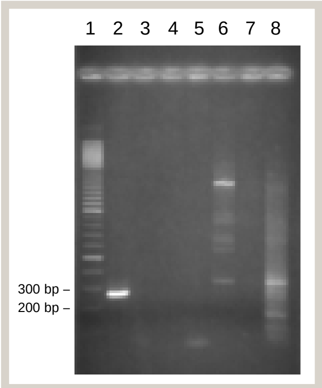


Figure 1. Amplification of a high-GC content template using the FailSafe™ PCR System and other methods. Lane 1, marker; Lane 2, FailSafe™ PCR System using PreMix K; Lane 3, Supplier 1 PCR Master Mix; Lane 4, Supplier 2 *Taq*; Lane 5, Supplier 3 *Taq*; Lane 6, Supplier 4 *Taq* (with GC-rich template amplification claims); Lane 7, Supplier 5 enzyme for use with GC-rich templates; Lane 8, Supplier 6 *Taq* for hot start PCR.

Figure 2. Amplification of a 20-kb PCR using the FailSafe™ PCR System and other methods. Lane 1, marker; Lane 2, FailSafe™ PCR System; Lane 3, Supplier 1 PCR Master Mix; Lane 4, Supplier 2 *Taq*; Lane 5, Supplier 3 *Taq*; Lane 6, Supplier 4 *Taq*; Lane 7, Suppler 5 enzyme for long PCR amplification; Lane 8, Suppler 6 enzyme for long PCR amplification; Lane 9, Supplier 7 enzyme for long PCR amplification.

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

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Interdepartmental Neuroscience Program,[1] Department of Pharmacology,[2] and Department of Ophthalmology and Visual Science,[3] Yale University School of Medicine, New Haven, CT

Introduction

In 1997, Siegel and Isacoff[1] 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,[2] 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.

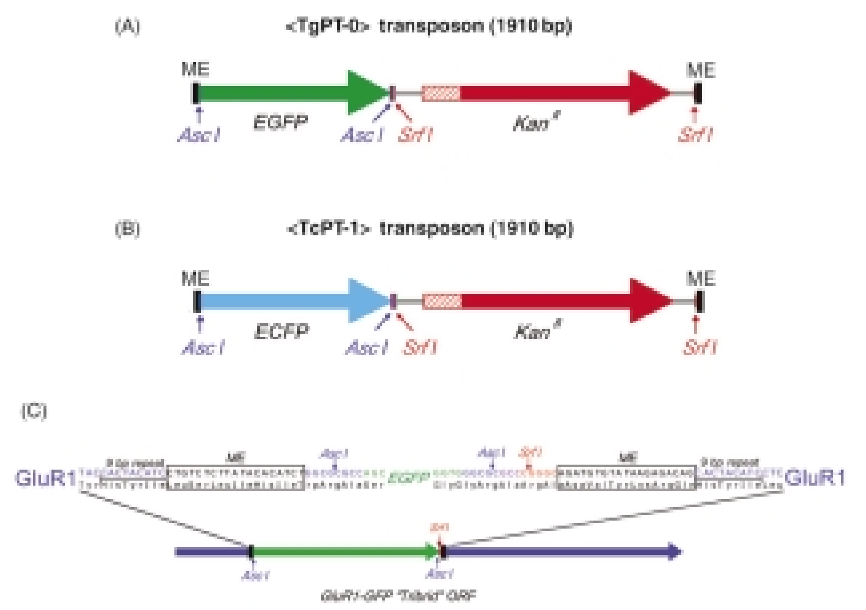

Figure 1. Two custom transposons based on the EZ::TN™ system encode EGFP (A) or ECFP (B) and are used to rapidly generate libraries of fluorescent fusion proteins. (C) Removal of the Kan^R cassette by *Srf* I digestion and re-ligation produces a reading frame that extends across the entire transposon. 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.

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^R cassette flanked by *Srf* I sites was added downstream of GFP. If the transposon lands in a coding sequence, in the correct orientation and reading frame, it should initially produce a truncated fusion protein, due to a stop codon in the Kan^R cassette. *Srf* I digestion and re-ligation removes the Kan^R cassette, producing a continuous reading frame across the entire transposon (Figure 1, full sequences available at: http://momotion.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 µl of EZ::TN™ Transposase in a 10 µl *in vitro* insertion reaction according to manufacturer's recommendations.

To test the transposons, we targeted two different glutamate receptor subunits, GluR1 and GluR2.[3] 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^R, 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.[4]

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^{2+}, 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[5] or very close to the N-terminus.[6] 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.[7] 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.

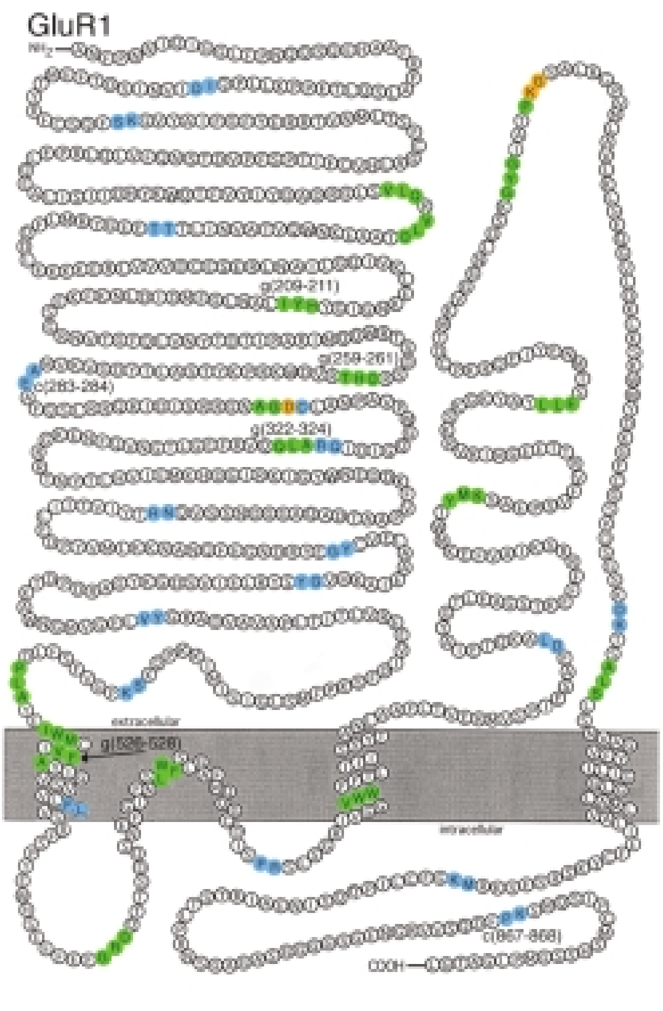

Figure 2. Unique insertion sites and functional analysis of GluR1 fluorescent fusion proteins. In-frame insertions of the <TgPT-0> transposon are indicated by green circles that represent the three amino acids duplicated during transposition. In-frame insertions of the <TcPT-1> transposon (cyan circles) 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).

References

1. Siegel, M.S. and Isacoff, E.Y. (1997) *Neuron* **19**, 735.
2. Ataka, K. and Pieribone, V.A. (2002) *Biophys. J.* **82**, 509.
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5. Marshall, J. *et al*. (1995) *Neuron* **14**, 211.
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7. Armstrong, N. and Gouaux, E. (2000) *Neuron* **28**, 165.

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.

www.epicentre.com/transposomics.asp

EZ::TN™ pMOD™-2<MCS> Transposon Construction Vector

MOD0602 20 µg

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

EZ::TN™ Transposase

TNP92110 10 Units

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.

Simple, Efficient Production of Short Double-Stranded RNA Using RNase III

Judith E. Meis, EPICENTRE

Double-stranded RNA (dsRNA) has become a powerful tool for modifying cellular gene expression levels.[1-3] Current methods used to produce dsRNA for evaluation in gene silencing experiments include *in vitro* transcription of a PCR- amplified or cloned DNA template or chemical synthesis. Recently, Yang *et al*. [4] reported that a heterogeneous population of short dsRNA can be efficiently generated by partial digestion of long dsRNA templates using *E. coli* ribonuclease III (RNase III). These researchers also demonstrated that dsRNA up to 30 bp in length effectively mediated RNA interference in cultured cells.

Here we report the rapid and simple production of short dsRNA using EPICENTRE's AmpliScribe™ High Yield Transcription Kits and RNase III.

Table 1. Reaction conditions for a combined AmpliScribe™ T7 and AmpliScribe™ T3 *in vitro* transcription reaction.

1. At room temperature, combine:

T7 template DNA (linearized)	1 µg
T3 template DNA (linearized)	1 µg
AmpliScribe™ T7 10X Reaction Buffer	4 µl
100 mM each ATP, CTP, GTP, UTP	3 µl each
AmpliScribe™ T7 Enzyme Mix	1 µl
AmpliScribe™ T3 Enzyme Mix	1 µl
100 mM DTT	4 µl
RNase-Free Water	to 40 µl total volume

2. Incubate the reaction at 37ºC for 2 hours.

3. Add 1 U of RNase-Free DNase I and incubate at 37ºC for 15 minutes to remove the DNA templates.

Preparation of DNA templates for *in vitro* transcription

A 1.4-kb DNA fragment was cloned into plasmid vectors containing either a phage T7 or phage T3 promoter. Clones were chosen so that the insert in the T7 clone would produce the "sense" 1.4-kb RNA transcript and the T3 clone would produce the complementary "anti-sense" 1.4-kb RNA transcript. The T7 and T3 transcription templates were prepared by separately digesting each with a restriction endonuclease that linearized the DNA downstream from the 1.4-kb insert. After quantitation, equal amounts of the two linear templates were mixed together and simultaneously transcribed in a combined AmpliScribe T7 and AmpliScribe T3 High Yield *in vitro* transcription reaction.

In vitro transcription of dsRNA

AmpliScribe T7 and AmpliScribe T3 transcription reactions were performed simultaneously in one tube using the reagents provided in the AmpliScribe Kits as described in Table 1. The sense and anti-sense RNA strands that were produced in the 2-hour reaction annealed spontaneously to form a 1.4-kb dsRNA. Approximately 50 µg (1.25 mg/ml) of intact, full-length 1.4-kb dsRNA was produced by the combined AmpliScribe T7 and T3 reaction as judged by agarose electrophoresis gels (Figure 1). The simultaneous AmpliScribe T7 and T3 reaction can be scaled up to produce milligram amounts of dsRNA if desired.

Combined AmpliScribe T7 and T3 reactions can be performed in a single tube because their optimal reaction buffers are very similar. However, the optimal AmpliScribe SP6 reaction buffer is significantly different from both the AmpliScribe T7 and AmpliScribe T3 buffer. Therefore, to produce dsRNA from templates with both SP6 and T7 promoters or both SP6 and T3 promoters, we recommend that the reactions be done separately and then the single-stranded RNA (ssRNA) reaction products be combined in equivalent amounts, followed by incubation for 1 hour to overnight at room temperature to allow the ssRNAs to anneal.

Production of short dsRNA fragments by partial digestion of long dsRNA with RNase III

The 1.4-kb dsRNA transcript was purified by precipitation with 2.5 M ammonium acetate and then resuspended in TA Buffer (33 mM Tris acetate (pH 7.8), 66 mM potassium acetate, 10 mM magnesium acetate, 0.5 mM dithiothreitol) to a concentration of 0.5 µg/ml. Five micrograms of the 1.4-kb dsRNA were digested

Figure 1. Synthesis of dsRNA in a single tube from simultaneous AmpliScribe™ T7 and AmpliScribe™ T3 High Yield Transcription reactions. Lane 1, 1.4-kb ssRNA produced from an AmpliScribe T7 High Yield Transcription; Lane 2, 1.4-kb ssRNA produced from an AmpliScribe T3 High Yield Transcription reaction; Lane 3, 1.4-kb dsRNA produced from single combined AmpliScribe T7 and AmpliScribe T3 reaction. All reactions were treated with RNase-Free DNase I to remove the DNA template. M, RNA size standards.

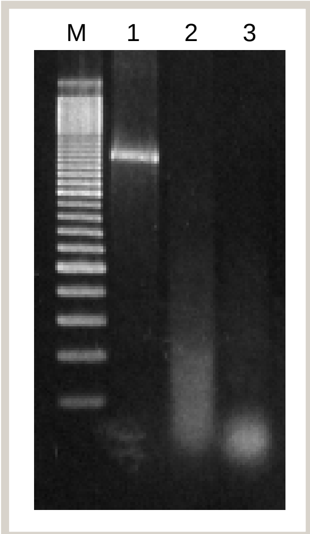

Figure 2. RNase III rapidly digests long dsRNA into short dsRNA. Five micrograms of a 1.4-kb dsRNA were digested as described in the text. Reaction aliquots were removed after 1 minute and 2 minutes of digestion and analyzed by 12% PAGE. Lane 1, 1.4-kb dsRNA; Lane 2, 1.4-kb dsRNA after 1 minute digestion with RNase III; Lane 3, 1.4-kb dsRNA after 2 minute digestion with RNase III.

with 1 Unit of RNase III (EPICENTRE) in 50 µl of TA Buffer at 37ºC. Aliquots of the RNase III digestion reaction were taken at 1 minute and 2 minutes and the RNA analyzed by electrophoresis on 12% polyacrylamide gels. Double-stranded RNA fragments of approximately 15–30 bp were produced in 1 minute and complete digestion of the 1.4-kb dsRNA to dsRNA fragments of 12–15 bp was observed after 2 minutes (Figure 2).

Conclusion

Short double-stranded RNA fragments can be rapidly and inexpensively produced by partial RNase III digestion of long dsRNA obtained from reactions using AmpliScribe T7, T3 and SP6 High Yield Transcription Kits.

References

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2. Misquitta, L. and B.M. Paterson (1999) *Proc. Natl. Acad. Sci. USA* **96**, 1451.
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Note: RNA interference is covered by patent applications owned by the Carnegie Institution of Washington. Use of RNA made for double-stranded RNA interference by for-profit organizations may require a license from the Carnegie Institution of Washington and/or other parties. For further information related to a license from the Carnegie Institution of Washington, contact the Director of Administration and Finance, Carnegie Institution of Washington, 1530 P Street, N.W., Washington, D.C. 20005-1910. Tel: 202-939-1118.

Get Consistently High Yields of Full-Length RNA from Both Long and Short DNA Templates Using AmpliScribe™ T7 High Yield Transcription Kits

Now, make double strand RNA from an AmpliScribe High Yield Reaction. **See p. 6 for details.**

Getting consistent *in vitro* transcription of full-length RNA transcripts from a variety of DNA templates is critical to the success of many gene expression experiments. For this reason, EPICENTRE's AmpliScribe™ High Yield Transcription Kits have become the preferred *in vitro* transcription kits for many labs. Recently, a competitor reported their "inability" to produce full-length transcripts >1 kb using EPICENTRE's AmpliScribe T7 High Yield Transcription Kits. We find this result quite remarkable since a number of researchers have reported to us that, in side-by-side comparisons, AmpliScribe Kits have consistently produced better results with their templates than kits from competitors.

Here, as reported previously (see *EPICENTRE Forum* 7:2) we demonstrate the yield and integrity of RNA produced using the AmpliScribe T7 High Yield Transcription Kits. In this study, we report the results of transcribing nine different DNA templates, producing transcripts ranging in size from 46 bases to 6.0 kb using AmpliScribe T7 High Yield Transcription Kits.

High yields of full-length, long RNA transcripts from an AmpliScribe T7 High Yield Transcription reaction

Four linear DNA templates, producing RNA transcripts of 3.0, 4.0, 5.0, and 6.0 kb, were individually transcribed in standard 20- µl AmpliScribe T7 High Yield Transcription reactions for 2 hours at 37ºC. As shown in Figure 1, each reaction produced a full-length RNA transcript. RNA yields ranged from 169 to 185 µg of RNA.

"The main thing I like about the AmpliScribe Kits is their consistently higher yields and lower price than the competition."

– Dr. JLE Dean, Imperial College School of Medicine, London

"I just finished a side-by-side comparison of the AmpliScribe Kit to the (competitor's kit). I wanted to let you know that the AmpliScribe Kit by far and away blows (the competitor's kit) away. In terms of both quality and yield."

– Aaron Nagel, Genome Solutions

High yields of full-length, short RNA transcripts from an AmpliScribe T7 High Yield Transcription reaction

Five linearized DNA templates, producing RNA transcripts of 46, 69, 88, 95, and 242 bases, were individually transcribed in standard 20-µl AmpliScribe T7 High Yield Transcription reactions for 2 hours at 37ºC. Figure 2 shows that each reaction produced the expected full-length transcript. RNA yield ranged from 41 to 78 µg of RNA.

AmpliScribe™ T7 High Yield Transcription reactions consistently produce high yields of RNA transcripts

Each new batch of AmpliScribe T7 High Yield Transcription Kits must meet stringent quality testing parameters for RNA yield prior to release for sales. The RNA yield from five recent batches of the AmpliScribe T7 High Yield Transcription Kits is shown in Figure 3. Each lot of kits produced >150 µg of a 1.4-kb RNA transcript.

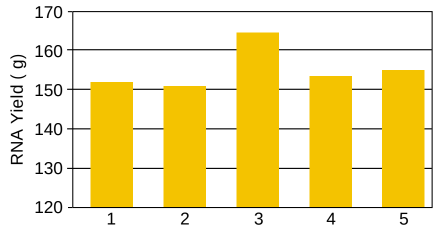

Figure 3. AmpliScribe™ T7 High Yield Transcription Kits consistently produce high yields of RNA. AmpliScribe T7 transcription reactions from five recent lots of kits each generated >150 µg of RNA as determined by EPICENTRE's Quality Control Lab using 1 µg of linearized control DNA template, producing a 1.4-kb RNA transcript, in a 2 hour reaction.

AmpliScribe™ High Yield Transcription Kits are the best value for *in vitro* transcription

EPICENTRE's competitively-priced AmpliScribe High Yield Transcription Kits consistently produce high yields of RNA transcripts from both long and short DNA templates.

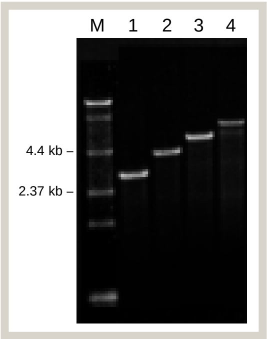

Figure 1. An AmpliScribe™ T7 High Yield Transcription reaction produces full-length, long RNA transcripts. Aliquots from standard 20-µl AmpliScribe T7 High Yield Transcription reactions were loaded onto a 1% agarose-formaldehyde gel and stained with ethidium bromide. The size of the RNA transcripts and RNA yields (in parentheses) were: Lane 1, 3.0 kb (185 µg); Lane 2, 4.0 kb (178 µg); Lane 3, 5.0 kb (176 µg); Lane 4, 6.0 kb (169 µg); M, RNA size marker.

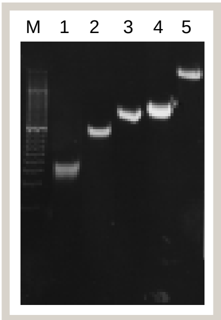

Figure 2. An AmpliScribe™ T7 High Yield Transcription reaction produces full-length, short RNA transcripts. Aliquots from standard 20-µl AmpliScribe T7 High Yield Transcription reactions were loaded onto a 12% polyacrylamide gel and stained with ethidium bromide. The size of the RNA transcripts and RNA yields (in parentheses) were: Lane 1, 46 bases (41 µg); Lane 2, 69 bases (55 µg); Lane 3, 88 bases (61 µg); Lane 4, 95 bases (65 µg); Lane 5, 242 bases (78 µg).

www.epicentre.com/ampliscribe.asp

AmpliScribe™ High Yield Transcription Kits

T7

AS2607	25 Reactions
AS3107	50 Reactions

SP6

AS2606	25 Reactions
AS3106	50 Reactions

T3

AS2603	25 Reactions
AS3103	50 Reactions

Contents:

AmpliScribe™ T7, SP6 or T3 Enzyme Mix (with added RNase Inhibitor), 100 mM ATP, CTP, GTP and UTP Solutions, AmpliScribe™ 10X Reaction Buffer, RNase-Free DNase I, RNase-Free Water, DTT, and Linearized Control DNA Template.

New TransforMax™ EC100™ Chemically Competent *E. coli* Beat the Competition in Transformation Efficiency

In side-by-side comparisons with competent cells from the leading supplier, EPICENTRE's new TransforMax™ EC100™ Chemically Competent *E. coli* cells gave better transformation efficiencies for all DNA sizes tested, even if the DNA was directly from a ligation reaction (Table 1). Thus, TransforMax EC100 Chemically Competent cells and the previously-available TransforMax™ EC100™ Electrocompetent *E. coli* are best choices of competent cells for most cloning applications, including PCR cloning and cDNA library construction.

DNA	TransforMax™ EC100™ Chemically Competent *E. coli*	Leading Competitor
pUC19	1.4 X 10^8	2.2 x 10^7
8.1-kb Clone	1.3 X 10^7	7.4 X 10^6
13.1-kb Clone	4.3 X 10^6	4.1 X 10^6
23.1-kb Clone	9.2 X 10^5	4.5 X 10^5
13.1-kb Clone direct from a ligation reaction	2.2 X 10^5	1 X 10^5

Table 1. Comparison of the transformation efficiencies of TransforMax™ EC100™ Chemically Competent *E. coli* with comparable cells from a leading competitor. Transformations were performed using purified supercoiled DNAs of the indicated sizes and a 1-µl aliquot from a standard 10-µl ligation reaction. Transformations were performed by the standard "heat-shock" procedure. Results shown are the average transformation efficiencies obtained from several trials. All values are in cfu/µg DNA.

	5-Minute Transformation	Standard "Heat-Shock" Transformation
pUC19 DNA	8.5 X 10^6	1.4 X 10^8

Table 2. Transformations can be performed in just 5 minutes using the TransforMax™ EC100™ Chemically Competent *E. coli* and clones containing an ampicillin resistance marker. The 5-minute transformation procedure and the standard heat-shock procedure are both supplied with the cells. All values are in cfu/µg DNA.

Important Phenotypes

- Supports blue/white screening of vectors.
- Restriction minus for efficient cloning of methylated DNA.
- Endonuclease minus (*endA1*) to ensure high yields of DNA.
- *RecA* minus for insert stability.

Single-Use Format…Perform Transformations Directly in the Tubes

TransforMax EC100 Chemically Competent *E. coli* cells are supplied in 50-µl aliquots so you use only the number of tubes that you need. Just add your DNA directly to the tube in which the cells are provided. This eliminates extra pipetting steps and results in more consistent transformation results.

Perform 5-Minute Transformations of TransforMax™ EC100™ Chemically Competent *E. coli*

Two transformation protocols are provided with the cells...the standard "heat-shock" procedure and a new 5-minute transformation procedure (Table 2). The 5-minute procedure does not require a "heat-shock" step or outgrowth of cells following transformation. The 5-minute tranformation procedure can be performed using any clones where an ampicillin resistance marker is used for selection.

Genotype

F⁻ *mcrA* Δ(*mrr-hsdRMS-mcrBC*) ϕ80*dlacZ*Δ*M15* Δ*lacX74 recA1 endA1 araD139* Δ(*ara, leu*)*7697 galU galK* λ- *rpsL nupGX*

The Best Value in Chemically Competent Cells

Compare our transformation efficiency. You'll find that TransforMax EC100 Chemically Competent *E. coli* are the best value.

www.epicentre.com/ec100.asp

TransforMax™ EC100™ Chemically Competent *E. coli*

CC02810 10 X 50 µl

TransforMax™ EC100™ Electrocompetent *E. coli*

EC10005 5 X 100 µl

EC10010 10 X 100 µl

Transformation efficiency >5 X 10^9 cfu/µg.

Quick and Efficient Purification of Single- or Double-Stranded RNA Transcripts Using the MasterPure™ RNA Purification Kit

Judith E. Meis, EPICENTRE

Introduction

In vitro transcription reactions are performed to produce RNA transcripts of various sizes and types including both single- and double-stranded RNA. For traditional applications, a simple precipitation step is sufficient purification of the RNA, but for many new techniques it is important to remove the DNA template, RNA polymerase, and buffer components, before proceeding. The Master-Pure™ RNA Purification Kit is a quick and effective solution to transcript purification. Designed for the efficient recovery of even the smallest amounts of RNA, this salt-based extraction method can be used to purify RNA transcripts of any type or length.

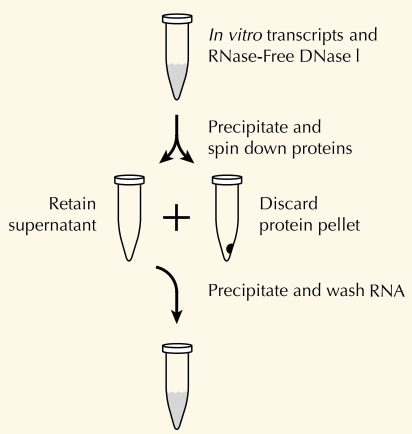

Figure 1. Quick and efficient recovery of RNA transcripts with the MasterPure™ RNA Purification Kit. Strategy for transcript purification.

The MasterPure RNA Purification protocol uses RNase-Free DNase I to completely and safely remove the transcription template. Then, the RNA polymerase and DNase I are inactivated and removed with the MasterPure Protein Precipitation Reagent, and the RNA is precipitated, removing remaining transcription reaction components (Figure 1). Here we demonstrate the purification of single-stranded and double-stranded RNA transcripts and even transcripts less than 100 bases in length using the MasterPure RNA Purification Kit.

Methods

High yield transcription reactions

Transcripts of various sizes were produced, in yields of up to 150 µg of RNA, using the AmpliScribe™ T7 or T3 High Yield Transcription Kits as per protocol. Single-stranded sense RNA transcripts were annealed to anti-sense RNA transcripts at 37°C, in AmpliScribe Reaction Buffer, to produce double-stranded RNA (dsRNA) transcripts.

RNA purification

The MasterPure RNA Purification protocol is depicted in Figure 1. To remove the DNA template, the 20-µl transcription reactions were treated with 1 Unit of RNase-Free DNase I for 15 minutes at 37°C, in AmpliScribe Reaction Buffer. Then, 280 µl of Tissue & Cell Lysis Solution were added, followed by 150 µl of MPC Protein Precipitation Solution. The reactions were vortexed and then spun for 10 minutes at full speed in a microcentrifuge. The supernatant was transferred to a new tube and the RNA was precipitated with 500 µl of isopropanol. The RNA was spun down for 10 minutes at full speed, and the pellets were washed twice with 70% ethanol. The RNA was resuspended in 100 µl of TE Buffer.

RNA transcript probe

To demonstrate the functionality of the RNA transcript, a 1.4-kb RNA probe was generated using the DuraScribe™ T7 Transcription Kit, which generates RNase A resistant transcripts. Unlike standard T7 RNA Polymerase, DuraScribe T7 RNA Polymerase efficiently incorporates 2'-deoxy-NTPs and 2'-modified-NTPs such as 2'-Fluoro-dNTPs into full length transcripts. The RNA transcript was labeled with biotin, by partial substitution of 2'-Flouro-UTP (3.5 mM) with biotin-dUTP (to 0.42 mM) in the reaction. The transcript was purified using the MasterPure RNA Purification Kit by the protocol described above and was then used to detect various amounts of DNA template blotted to a membrane.

Results

Identical amounts of 1.4-kb RNA transcripts before and after clean-up with the MasterPure RNA Purification Kit were analyzed by gel electrophoresis. Figure 2 demonstrates the efficient removal of the *in vitro* transcription template and the recovery of the single-stranded RNA transcript with MasterPure RNA Purification. The RNA recovery, as estimated by gel electrophoresis, approached a complete

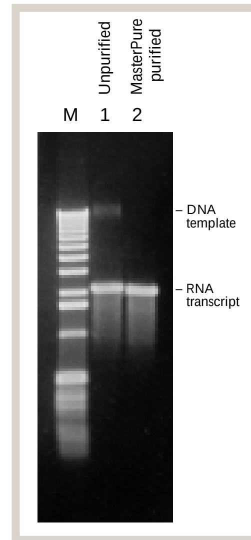

Figure 2. Complete removal of the transcription template with the MasterPure™ RNA Purification Kit. The 1.4-kb transcript, produced from a linear 3-kb DNA template, was purified using the MasterPure Purification Kit protocol. Lane M, kb ladder; Lane 1, unpurified transcript; Lane 2, purified RNA transcript.

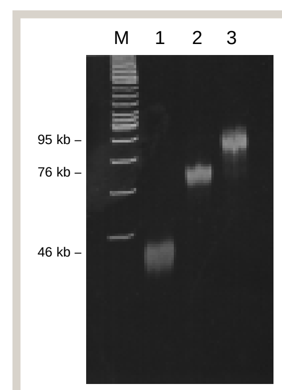

Figure 3. Efficient recovery and purification of even small RNA transcripts. Lane M, 100-bp ladder; Lane 1, 46-base transcript; Lane 2, 76-base transcript; Lane 3, 95-base purified RNA transcript.

recovery. The MasterPure RNA Purification Kit efficiently purifies even RNA transcripts of less than 100 bases in length. Three AmpliScribe transcripts of 46, 76 and 95 bases in length were purified and recovered using this procedure (Figure 3). The purified transcripts all had 260/280 absorbance ratios of 1.9 to 2.0.

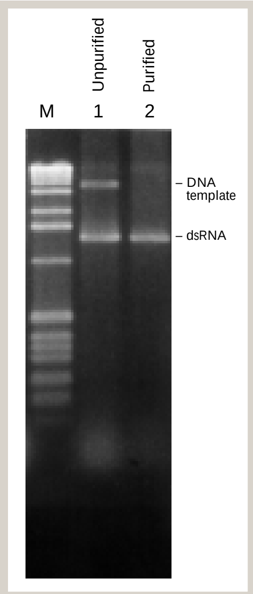

Figure 4. Double-stranded RNA is purified effectively with the MasterPure™ RNA Purification Kit. Lane M, kb ladder; Lane 1, dsRNA transcript before purification; Lane 2, dsRNA transcript purified with the MasterPure Purification Kit.

A double-stranded RNA transcript, produced by annealing two single-stranded AmpliScribe control transcripts together, was treated with RNase-Free DNase I and purified using the same protocol described above. The purified double-stranded RNA shows no remaining DNA template and resulted in only a slight loss of RNA during purification (Figure 4). Alternatively, to improve the RNA recovery, single-stranded RNA transcripts could be purified and subsequently annealed after MasterPure RNA Purification. The MasterPure purified biotin-labeled DuraScribe™ transcript detected various amounts of DNA template blotted to a membrane when used as a probe (Figure 5).

Conclusion

This simple and quick, solution-based clean-up method was designed to maximize the recovery of RNA from even the smallest amount of starting material. The easily scaleable, MasterPure RNA Purification Kit works effectively to remove proteins, including transcription enzymes and nucleases, as well as DNA templates, and transcription buffer components from single-stranded and double-stranded RNA transcripts.

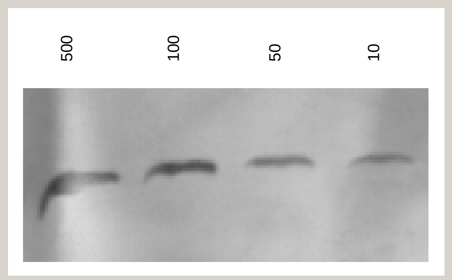

Figure 5. Detection of decreasing amounts of DNA template using a MasterPure™ purified biotin-labeled DuraScribe™ transcript as probe. Lane 1, 500 pg; Lane 2, 100 pg; Lane 3, 50 pg; Lane 4; 10 pg of DNA template.

Obtain the Highest DNA Yields from Yeast with the MasterPure™ Yeast DNA Purification Kit

The MasterPure™ Yeast DNA Purification Kit is a simple and nonenzymatic approach to yeast genomic DNA purification. High quality yeast DNA can be obtained in less than 40 minutes without columns, resins or organic extractions. Recover DNA from a wide variety of yeast species including *Candida, Saccharomyces, Pichia*, and *Schizosaccharomyces,* and filamentous fungi such as *Asperigillus* and *Penicillium*. The MasterPure Kit produces high-quality DNA that can be used directly for many applications including PCR amplification, restriction endonuclease digestion, Southern blotting, and genomic library preparation.

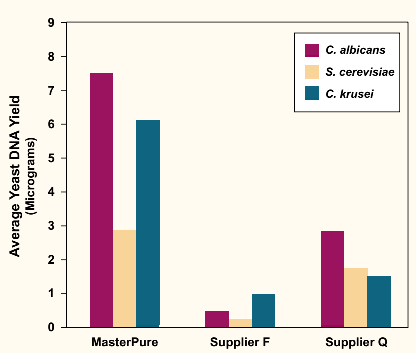

Figure 1. The MasterPure™ Yeast DNA Purification Kit gives higher yields of DNA than other kits. DNA yields were from 1.5 ml cultures and quantitated by fluorometry with Hoescht 33258 dye. The data represent the average of duplicate extractions from either one (*C. krusei*) or two (*S. cerevisiae* and *C. albicans*) experiments.

Higher yield than the competition

Using a simple, short protocol, DNA yields obtained with the MasterPure Kit were consistently above those of two competing kits (Figure1). For example, the kit from supplier F averaged 0.25 µg of DNA from 1.5 ml of *S. cerevisiae*, whereas the MasterPure Kit produced an average of 2.94 µg, almost twelve times as much. The supplier Q kit produced 1.8 µg of *S. cerevisiae* DNA from the same culture volume.

High molecular weight DNA

The MasterPure Kit yields high molecular weight yeast DNA. As determined by pulsed field gel electrophoresis, the size of the *S. cerevisiae* DNA isolated with the MasterPure Yeast DNA Purification Kit is approximately 40-50 kb, while DNA of the same yeast species purified with the kit from supplier Q was mostly degraded to fragments smaller than 40 kb (Figure 2). A similar size discrepancy was observed for *C. albicans* DNA (data not shown).

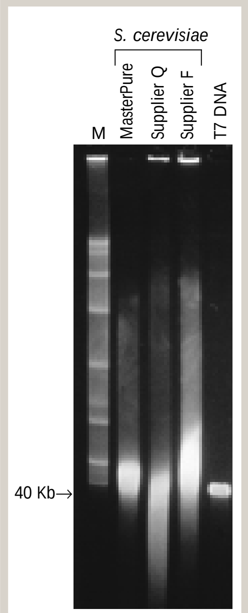

Figure 2. Yeast DNA purified using the MasterPure™ Kit has a higher molecular weight than DNA purified using other kits. 500 ng of purified yeast DNA was analyzed by pulsed field gel electrophoresis on a 1% agarose gel. Lane M, lambda DNA ladder.

Ampligase® Thermostable DNA Ligase

Ampligase® Thermostable DNA Ligase catalyzes NAD-dependent ligation of double-stranded DNA at high temperature. Derived from a thermophilc bacterium, the enzyme is stable and active at much higher temperature than conventional DNA ligases.

Features and Benefits

- Half-life of 48 hours at 65ºC and greater than 1 hour at 95ºC.
- Active for at least 500 thermal cycles (94ºC/80ºC) or 16 hours of cycling.[1]
- Enables extremely high hybridization stringency and ligation specificity.
- No activity on blunt-ended DNA, RNA, or RNA:DNA hybrids.
- One unit of Ampligase Thermostable DNA Ligase is equivalent to at least 15 "cohesive-end units" or "nick-ligation units" defined elsewhere.
- Assayed to ensure absence of blunt-end ligation activity and to be free of detectable exo- and endonucleases and RNases.

Applications

- Ligation Amplification (Ligase Chain Reaction, LCR) for detection of any defined DNA sequence by amplification of ligation products complementary to the sequence.[2]
- Repeat Expansion Detection (RED)[3] method of genetic screening that detects multiple nucleotide repeats.
- Simultaneous mutagenesis of multiple sites.[4]
- High-fidelity gene synthesis.[5]
- Ligation under high stringency conditions.

References

1. Schalling, M. *et al.* (1993) *Nature Genetics* **4**, 135.
2. Landegren, U. *et al.* (1988) *Science* **242**, 229.
3. Sirugo, G. and Kidd, K.K. (1995) *EPICENTRE Forum* **2** (3), 1.
4. Moore, D.S. and Michael, S.F. (1995) *EPICENTRE Forum* **2** (4), 4.
5. Sutton, D.W. (1995) *EPICENTRE Forum* **2** (2), 1.

Visit our website for a complete list of references.

www.epicentre.com/ampligase.asp

Ampligase® DNA Ligase Kit

A8101	5 U/µl	1,000 U
A30201	5 U/µl	5,000 U

Contains 1,000 or 5,000 units of Ampligase® DNA Ligase, Ampligase® 10X Reaction Buffer, and Ligation Control DNA.

Ampligase® Enzyme & Buffer

A0102K	100 U/µl	2,500 U
A32250	5 U/µl	250 U
A32750	5 U/µl	750 U
A3202K	5 U/µl	2,500 U

25 µl of Ampligase® 10X Reaction Buffer is supplied with each 50 units of Ampligase® DNA Ligase.

Ampligase® DNA Ligase

A0110K	100 U/µl	10,000 U
A0125K	100 U/µl	25,000 U
A3210K	5 U/µl	10,000 U
A3225K	5 U/µl	25,000 U

Supplied as enzyme only; Reaction Buffer is not included.

Ampligase® 10X Reaction Buffer

A1905B	5 ml

Ampligase® 1X Storage Buffer

A3201S	1 ml
A3205S	5 ml

Ampligase® Thermostable DNA Ligase is also available in bulk. Please inquire.

Get Cleaner Plasmid Preparations with Plasmid-Safe™ ATP-Dependent DNase

Even after spin-column or CsCl purification, cloning vector preparations are frequently contaminated with fragments of bacterial chromosomal DNA that can be ligated into the cloning vector and result in false positives and high backgrounds. Plasmid-Safe™ ATP-Dependent DNase digests these linear double-stranded chromosomal DNA contaminants to deoxynucleotides yielding cleaner vector preparations for every cloning application. The enzyme has no activity on nicked or closed circular double-stranded DNA or supercoiled DNA. Therefore, Plasmid-Safe DNase is ideal as the final purification step for plasmid, cosmid and BAC vector preparations.

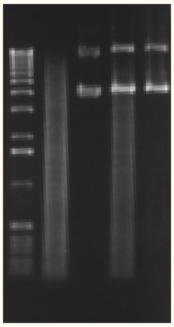

Figure 1. Use of Plasmid-Safe™ ATP-Dependent DNase to remove contaminating linear DNA from plasmids. *Lane 1, 3 µg of Sma I-digested bacterial chromosomal DNA; Lane 2, 500 ng of uncut plasmid DNA; Lane 3, mixture of 3 µg of digested bacterial chromosomal DNA and 500 ng of uncut plasmid before Plasmid-Safe DNase treatment; Lane 4, mixture of chromosomal DNA and plasmid DNA after Plasmid-Safe DNase treatment (incubated with Plasmid-Safe DNase for 30 minutes at 37°C); M, kb ladder.*

Features and Benefits

- Improves cloning results by reducing backgrounds and false positives.
- Minimizes the possibility of cloning contaminating bacterial chromosomal DNA fragments.
- Fast and easy protocols for minprep, midiprep and maxiprep purifications are provided.

www.epicentre.com/plasmid_safe.asp

Plasmid-Safe™ ATP-Dependent DNase

E3101K	10 U/µl	1,000 U
E3105K	10 U/µl	5,000 U
E3110K	10 U/µl	10,000 U

Increase Protein Yield for Proteomics, High-Throughput Screening or Preparative Purification

Ready-Lyse™ Lysozyme Solution

Gentle and efficient protein purification without denaturation.

Many protocols for the isolation of proteins for high-throughput activity assays or for preparative purification from bacteria use egg white lysozmye to aid the breakdown of cell walls. Egg white lysozyme is positively charged at neutral pH and will bind and precipitate negatively charged proteins thus reducing their yield. Ready-Lyse™ Lysozyme Solution is a non-mammalian, non-avian recombinant lysozyme preparation.

Advantages of Ready-Lyse Lysozyme:

- Specific activity 200-fold higher than that of egg white lysozyme. Much less Ready-Lyse Lysozyme is required per reaction resulting in increased yield of protein with minimal contamination by exogenously added protein.
- Readily lyses both Gram-negative and Gram-positive bacteria.
- Utilizes a gentle procedure that does not require sample agitation or heat that can result in protein denaturation.
- Stable at -20ºC, thus eliminating the need to prepare a fresh solution for each use, as is the case for egg white lysozyme.

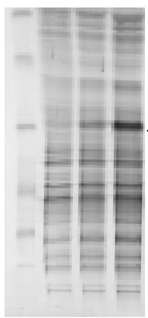

Figure 1. Use of Ready-Lyse™ Lysozyme Solution to recover recombinant proteins. One ml of induced cells from a recombinant *E. coli* clone was pelleted by microcentrifugation before induction and at 1 and 3 hours after induction. Each sample was resuspended in 50 µl of cold TEBG buffer. One µl of Ready-Lyse Solution was added to each suspension and the cells were incubated at room temperature for 30 minutes. The cell debris was pelleted and 10 µl of the supernatant were run on an SDS-PAGE gel. Lane1, molecular weight markers; Lanes 2-4, time points of induction. The induced protein is designated by an arrow.

OmniCleave™ Endonuclease

Remove traces of contaminating nucleic acids from protein preparations.

OmniCleave™ Endonuclease is a highly purified enzyme that degrades all forms of single- and double-stranded DNA and RNA to di-, tri- and tetranucleotides (Figure 1).

OmniCleave Endonuclease is active within a broad range of conditions normally used in protein preparations.

Use OmniCleave Endonuclease to:

- Improve the handling and yield of protein preparations by reducing the viscosity of cell lysates due to nucleic acids.
- Remove trace contamination by nucleic acids in protein preparations.
- Improve the electrophoretic and chromatographic separation of proteins isolated from whole cell lysates.

OmniCleave Endonuclease is tested to be free of detectable protease activity.

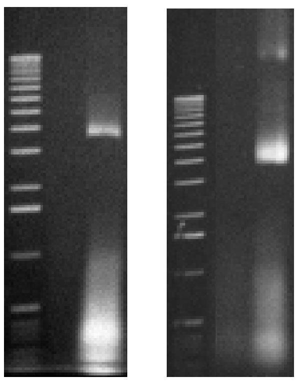

Figure 1. Removal of nucleic acids from cell lysates using OmniCleave™ Endonuclease. Cell lysates were prepared with or without OmniCleave Endonuclease from *E. coli* cells expressing human lactate dehydrogenase B (LDH) and *E. coli* alkaline phosphatase (AP). Two microliters of each of the cell lysates were separated by electrophoresis on a 1% agarose gel and the nucleic acids detected by ethidium bromide staining. Lane M, 1 kb ladder.

ReadyPreps™ Protein Preparation Kit

The ReadyPreps™ Protein Preparation Kit combines Ready-Lyse Lysozyme Solution with OmniCleave Endonuclease for routine, high-throughput and preparative scale purification of protein from bacterial cells.

The ReadyPreps Kit is:

- Rapid - most preparations can be completed in 20 - 45 minutes.
- Gentle - no heating steps that can denature proteins are required.
- Compatible with most downstream purification processes (e.g., ammonium sulfate precipitation, column chromatography, etc.).
- Consistent - get more reproducible results than by mechanical lysis methods.

RNase I is included in the kit for removing cellular RNA in the event that the plasmid DNA and protein are extracted simultaneously.

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Ready-Lyse™ Lysozyme Solution

R1802M	2×10^6 U
R1804M	4×10^6 U
R1810M	10×10^6 U

This product is available animal product-free in bulk. Please inquire.

OmniCleave™ Endonuclease

OC7810K	200U/µl	10,000 U
OC7850K	200U/µl	50,000 U

Provided with Dilution Buffer.

This product is available animal product-free in bulk. Please inquire.

ReadyPreps™ Protein Preparation Kit (for total cellular proteins)

RP78100	100 1-ml Preparations

Reagents sufficient to process 5 g of cells.

Contents:

ReadyPreps™ Lysis Buffer A, ReadyPreps™ Lysis Buffer B, OmniCleave™ Endonuclease, RNase I, 1 M $MgCl_2$, 0.5 M EDTA, and Lysis Test Reagent.

Identification of a Potential Virulence-Related Operon by Rescue Cloning EZ::TN™ Transposon Insertion Sites from a Bacterial Pathogen

Caleb W. Dorsey, Andrew P. Tomaras, John C. Berschback, and Luis A. Actis
Miami University, Oxford, Ohio

Introduction

Acinetobacter baumannii is a Gram-negative, opportunistic pathogen that causes severe infections in compromised patients. Since this pathogen resists a wide range of antimicrobial compounds these infections are a serious concern in human medicine. However, little is known about the genes and factors involved in *Acinetobacter* basic physiology and virulence properties. Here we demonstrate that electroporation of the EZ::TN™ <R6Kγ*ori*/KAN-2>Tnp Transposome™ proved to be an effective and simple approach to generate random transposon insertions in the *A. baumannii* 19606 genome. Moreover, we were able to recover or "rescue" the interrupted chromosomal regions as plasmids due to the presence of an origin of replication within the transposable element.

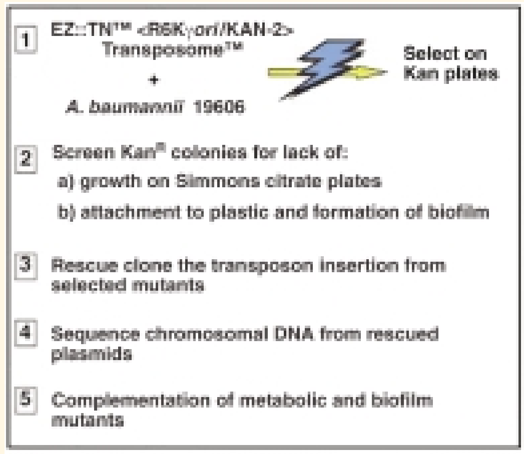


Figure 1. Overview of the strategy used to generate and screen EZ::TN™ <R6Kγ*ori*/KAN-2> Transposon insertions in *A. baumannii* 19606. The EZ::TN™ Transposome™ is the stable complex formed between an EZ::TN Transposon and EZ::TN™ Transposase that can be electroporated into bacterial cells. Once inside the cell, the transposon component is randomly inserted into the host's genomic DNA.

Materials and Methods

Insertional mutagenesis, mutant screening, and rescue of interrupted sequences

The strategy used to generate and screen *A. baumannii* 19606 insertion mutants is shown in Figure 1. The EZ::TN <R6Kγ*ori*/KAN-2>Tnp Transposome was electroporated into *A. baumannii* 19606 electrocompetent cells with the 2510 Eppendorf electroporator and 2-mm-wide cuvettes at 2.5 kV. Electroporated cells were suspended immediately in SOC broth, allowed to recover at 37ºC for 1 hour, and then plated on LB agar containing 40 µg/ml kanamycin. Growth on Simmons citrate agar and attachment to plastic tubes or microtiter plates were used to identify insertion derivatives affected in metabolic and attachment/biofilm functions, respectively.

Genomic regions harboring EZ::TN™ <R6Kγ*ori*/KAN-2> Transposon insertions were rescued as shown in Figure 2. The genomic DNA flanking an insertion was sequenced bidirectionally with primers (supplied with the kit) that anneal near the ends of the transposon. Further extension of nucleotide sequences was done using custom-designed primers and plasmid DNA as a template. Sequencher 4.1.2 (Gene Codes Corp.) was used to examine and assemble nucleotide sequences, which were analyzed with DNASTAR, BLAST, and the software available through the ExPASy Molecular Biology Server (http://www.expasy.ch).

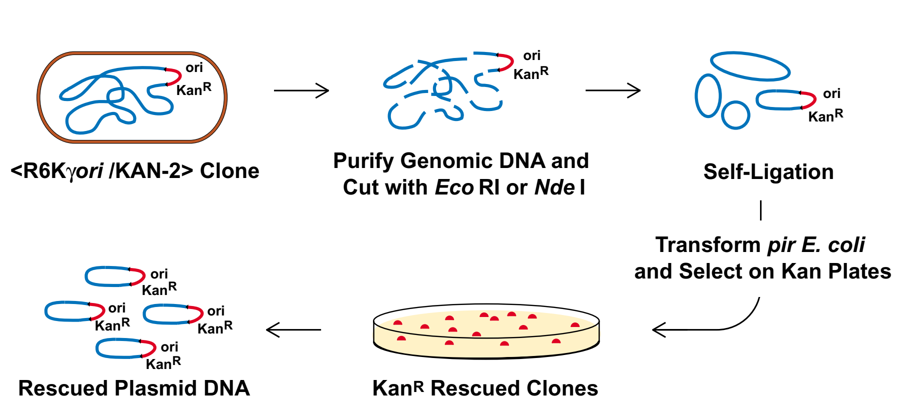


Figure 2. Rescue cloning transposon insertion sites from *A. baumannii* 19606 genomic DNA using the EZ::TN™ <R6Kγ*ori*/KAN-2>Tnp Transposome™ and TransforMax™ EC100D™ pir$^+$ Electrocompetent *E.coli*. The *pir* gene product is required for propagation of plasmids containing the R6Kγ origin of replication.

Biochemical and genetic complementations

Positive growth on supplemented Simmons citrate agar was recorded as concomitant detection of abundant bacterial growth on the streaked areas and change of color from green to blue in the surrounding areas, after overnight incubation at 37ºC. Genetic complementation of insertional mutants required PCR amplification of the uninterrupted gene of interest from the genome of the parental strain and cloning in the shuttle vector pWH1266.[1] Restoration of cell attachment/biofilm formation was determined after 24 hours stagnant incubation of LB broth cultures at 37ºC in polystyrene tubes.

Results and Discussion

Electroporation with 1 µl of the EZ::TN <R6Kγ*ori*/KAN-2>Tnp Transposome generated ~3,500 Kan^R colonies. Southern blot analysis of restricted genomic DNA isolated from 18 of these Kan^R derivatives showed that all of them harbored the kanamycin-resistance gene, which could not be detected in the genome of the parental strain (data not shown). With the exception of one, all the derivatives appeared to contain a single EZ::TN <R6Kγ*ori*/KAN-2> Transposon insertion.

The efficient rescue of the interrupted chromosomal sequences as plasmids allowed us to rapidly determine the precise EZ::TN <R6Kγ*ori*/KAN-2> Transposon insertion site in selected mutants. Sequence analysis demonstrated that mutants unable to grow on Simon citrate agar were impaired in functions required for the generation of energy or the production of precursors required for the biosynthesis of nucleic acids, proteins, and cell wall components (Table 1). For example, mutants 9, 19, and 23 carry EZ::TN <R6Kγ*ori*/KAN-2> Transposon insertions within genes required for tryptophan biosynthesis. Growth of these mutants was restored to levels similar to those of the parental strain when this chemically defined medium was supplemented with 1 mM tryptophan. In contrast, these three insertion derivatives and the parental strain showed identical growth curves when cultured in LB broth.

The attachment/biofilms assays yielded mutant 144, which has an EZ::TN <R6Kγ*ori*/KAN-2> Transposon inserted within a gene encoding a polypeptide highly similar to the *Vibrio parahaemolyticus* CsuE protein. The *csuE* gene is the last component of *csuABCDE*, a predicted polycistronic locus that potentially codes for functions required for the secretion and assembly of bacterial proteins involved in pili formation in other Gram-negative bacteria.[2] Previous work has established that this type of cell surface appendage is involved in cell attachment and biofilm formation on abiotic surfaces. Electroporation of a pWH1266 derivative harboring the uninterupted *csuE* gene restored the ability of mutant 144 to attach to and form biofilm on plastic surfaces in a fashion similar to the parental strain (Figure 3). Currently, we are determining the nucleotide sequence and genetic structure of the *A. baumannii* 19606 *csu*-like operon, whose products have the potential of playing an important role in the biology and virulence properties of this bacterium.

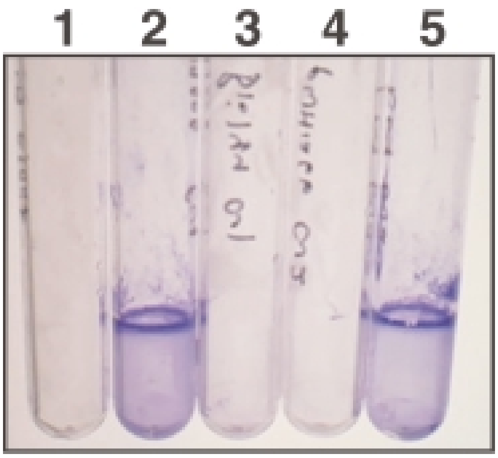


Figure 3. Genetic complementation of the *A. baumannii* biofilm-deficient mutant 144. The 19606 parental strain (sample 2), mutant 144 harboring either no plasmid (sample 3), the shuttle vector pWH1266 (sample 4), or a derivative of the latter containing the parental *csuE* gene (lane 5) were tested for attachment/biofilm formation after stagnant culture in LB broth. A tube containing sterile LB broth (sample 1) was used as negative control. Tubes were stained with 0.1% crystal violet to detect bacterial cells.

In summary, the utilization of the EZ::TN <R6Kγ*ori*/KAN-2>Tnp Transposome system proved to be an efficient tool for the generation of random *A. baumannii* mutants affected in metabolic and attachment/biofilm functions. This convenient approach should facilitate the genetic and functional analysis of this poorly characterized opportunistic human pathogen.

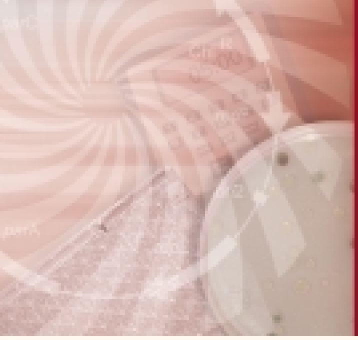
Table 1. Characterization of EZ::TN™ <R6Kγ*ori*/KAN-2> Transposon insertions in *A. baumannii* 19606.

Mutant	Insertion Site[a]	Gene - Function Disrupted[b]
1	GCCCTAAAA	*dapA* – dihydrodipicolinate synthase
9	CGCGGATAC	*trpD* – anthranilate phosphoribosyltransferase
10	GTTTATTCA	*argF* – ornithine carbamoyltransferase
12	GGGCCATAC	*argG* – argininosuccinate synthase
13	ATAGAATGG	*aceE/aceA* – pyruvate dehydrogenase E1
15	AATGGAAAC	*proA* – g-glutamyl phosphate reductase
16	AATATACGT	*cysI/nirA/sir* – sulfite reductase
19	CTACGATGC	*trpE* – anthranilate synthase
23	CTATTCACA	*trpE* – anthranilate synthase
26	CATGTGAAT	*cysI/nirA/sir* – sulfite reductase
28	CATGTGAAT	*cysI/nirA/sir* – sulfite reductase
31	CTGCAAACC	*hisH* – glutamine amidotransferase
44	GTTTTACGT	*cysI/nirA/sir* – sulfite reductase
144	GTCACAAAC	*csuE* – chaperone/usher secretion system

[a]Bases indicate a 9-bp duplication that flanks each side of the inserted transposon.
[b]Potential genes and functions were predicted by BLASTp and BLASTx searches.

References

1. Hunger, M. *et al*. (1990) *Gene* **87**, 45.
2. Thanassi, D.G. *et al*. (1998) *Curr. Opin. Microbiol*. **1**, 223.



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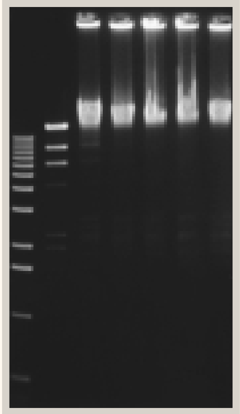

Figure 1. Time course for cohesive-end ligation using the Fast-Link™ DNA Ligation Kit. Lambda *Hin*d III markers were ligated in a standard Fast-Link reaction using 2 U of Fast-Link DNA Ligase (Lanes 3-7). Lane 1, 1 kb ladder; Lane 2, no enzyme.

Representative Transformation Results with the Fast-Link DNA Ligation Kit*

	Ligation Time	% White Colonies	Recombinants per µg DNA
Cohesive ends	5 min.	>90%	2.0×10^6
Blunt ends	15 min.	>95%	5.0×10^5

* Cohesive-end ligation results were obtained by ligating *Hin*d III-digested *E. coli* chromosomal DNA into EPICENTRE's pIndigoBAC-5 (*Hin*d III Cloning-Ready) BAC vector. Blunt-end ligation results were obtained by ligating a 1.2 Kb blunt-ended fragment into a blunt-end dephosphorylated pUC-based plasmid vector. One microliter from each ligation reaction was used to transform TransforMax™ EC100™ Electrocompetent *E. coli*.

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