

EPICENTRE Forum

Tools & Techniques for Genomics, Proteomics & RNA Research

EPICENTRE Revolutionizes Cloning by Introducing CopyControl™ Cloning Systems for Single-Copy Cloning and “On-Demand” Induction of Clones to High Copy Number

EPICENTRE's new CopyControl™ Cloning Systems, with a choice of BAC, fosmid or plasmid CopyControl Vectors, allow researchers to make and maintain libraries or clones of genomic DNA, cDNA, or PCR products at a single copy and then, whenever desired, to induce the clones to high copy number (10-50+ copies per cell) (Figure 1). Thus, the CopyControl Systems combine the clone stability afforded by single copy cloning with the advantages of high yields of DNA obtained by high copy vectors.

The benefits of on-demand induction to obtain higher DNA yields from smaller amounts of bacterial culture will be obvious to genomics researchers using single-copy BAC or fosmid vectors. However, unexpectedly, single-copy vectors are also better for cloning low molecular weight DNA, such as cDNA or PCR products. For example, in one experiment, the number of cDNA clones was significantly higher using a single-copy vector compared to using a high-copy vector (personal communication). Overall, single-copy cloning results in more complete and more unbiased clone libraries.

CopyControl Cloning Systems are based on the revolutionary new technology invented in the well-known laboratory of Professor Waclaw Szybalski at the University of Wisconsin-Madison by the McArdle Laboratory team of Drs. Szybalski, Zdenka Hradecna, and Jadwiga Wild,^{1,2,3} and further developed during more than one year of intensive work by EPICENTRE's team of scientists.*

Until Now, Researchers Have Used High-Copy Vectors for Cloning Low Molecular Weight DNA and Single-Copy Vectors for Cloning High Molecular Weight Genomic DNA

Early work on molecular cloning concentrated on developing vectors with the

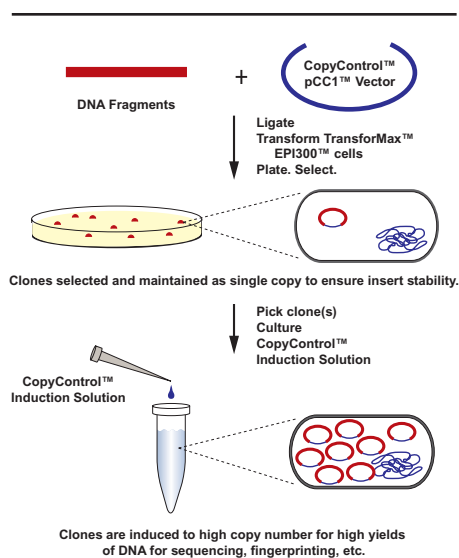


Figure 1. Overview of the CopyControl™ Cloning Systems. CopyControl BAC, Fosmid or PCR clones are initially grown as single copy clones in TransformMax™ EPI300™ *E. coli*. Individual clones are chosen, grown in small volume culture then induced to high copy by addition of the CopyControl™ Induction Solution.

highest possible copy number (e.g., pBR322, pUC18) without general concern about the not-infrequent cloning “artifacts” that likely resulted from instability of the cloned insert when clones were propagated at high copy number. The advent of genomic DNA cloning and sequencing projects has spurred the development and use of single copy Bacterial Artificial Chromosome (BAC)^{4,5} and fosmid⁶ vectors that better ensure the stability of the cloned genomic DNA and improve the probability of cloning expressed genes or DNA fragments that would be toxic or detrimental for growth of the *E. coli* host if cloned in a high-copy vector.

The disadvantage of cloning in single-copy cloning vectors is the very low

yield of cloned DNA that can be obtained for analysis. Also, since high-copy vectors yield more clone DNA relative to *E. coli* host genomic DNA than single-copy vectors, it is easier and faster to purify high-copy vector clone DNA from host DNA.

... continued on page 2

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In This Issue

- 1 EPICENTRE Revolutionizes Cloning by Introducing CopyControl™ Cloning Systems
- New!** 3 The CopyControl™ Fosmid Library Production Kit Generates More Complete and Unbiased Libraries of Stable Genomic Clones
- New!** 4 Save At Least 2 Days Constructing a CopyControl™ BAC Library Then Amplify the Clones to 10-20 Copies Per Cell
- New!** 6 The CopyControl™ PCR Cloning Kit Enables Faster, More Reliable Cloning of Any PCR Product
- New!** 7 Retrofit Existing BAC and Fosmid Clones with CopyControl™ Capability
- New!** 7 BAC-Tracker™ Supercoiled DNA Ladder
- New!** 8 TransformMax™ EPI300™ *E. coli* and Electroporation Cuvettes
- 9 Obtain PCR-Ready Genomic DNA from a Variety of Sample Types
- New!** 10 *In Vitro* Synthesis of 2'-Fluoro-Modified RNA Transcripts That Are Completely Resistant to RNase A Digestion
- 12 Transposon Mutagenesis in *Trypanosoma brucei* with the EZ::TN™ Transposome™
- 14 *In Vitro* Insertion of a Transposon Containing an *E. coli* Origin of Replication Facilitates Propagation and Sequencing of Circular DNA Molecules
- 15 Consistent High-Fidelity PCR Amplification of DNA 20 Kb and Longer
- 16 DNA Ligations in 5 Minutes

CopyControl™ Cloning Systems Provide the Advantages of Both Single-Copy and High-Copy Vectors

EPICENTRE's new CopyControl Cloning Systems combine the advantages of both single-copy vectors and high-copy vectors without the disadvantages of either. With the CopyControl Cloning Systems, the user has complete control over the clone copy number. Clones are initially grown at single copy to ensure insert stability and cloning of potentially toxic expressed DNA segments and PCR products, and then induced to high copy number whenever desired, to maximize the yield and purity of DNA for sequencing, fingerprinting and other applications.

CopyControl Systems Use a Vector with Both Single-Copy and High-Copy Origins of Replication (*ori*) and an *E. coli* Host with an Inducible Protein Required for the High-Copy *ori*

CopyControl Systems are based on CopyControl pCC1™ Vectors (Figure 2) that have two origins of replication - 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. However, the *trfA* gene is absent in both the CopyControl vectors and common lab strains of *E. coli*. CopyControl Systems utilize a specially engineered *E. coli* host strain - called TransforMax™ EPI300™ - that contains a mutant *trfA* gene under tight control of an inducible promoter. In the absence of *trfA* gene induction agent, replication of pCC1 clones is controlled

by the F-factor replicon and the vector is present at one copy per cell. Addition of a simple sugar (the CopyControl Induction Solution) to the growth medium induces expression of *trfA* and subsequent amplification of the clone to high copy number (10 – 50+ copies per cell) to facilitate purification of microgram amounts of DNA.

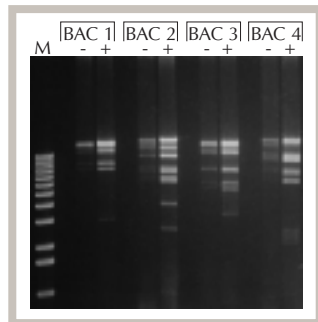


Figure 3. *Hind* III fingerprint of 1 µl of DNA isolated from an equal number of cells from induced (+) and uninduced (-) cultures of 4 CopyControl™ BAC clones. Lane M, DNA size ladder.

Existing BAC and Fosmid Clones Can Be Retrofitted with CopyControl™ Capability

BAC and fosmid clones generated in vectors that are exclusively single-copy can be easily retrofitted with CopyControl capability using the new EZ::TN™ <*oriV* /KAN-2> Insertion Kit. See page 7 to learn more about this transposon-based system for high yields of DNA and complete sequencing without primer walking or subcloning.

References

1. Hradecna, Z. *et al.* (1998) *Microbial and Comp. Genomics* **3**, 58.
2. Wild, J. *et al.* (2001) *Plasmid* **45**, 142.
3. Wild, J. *et al.* (2002) *Genomic Research* (submitted).
4. Shizuya, H. *et al.* (1992) *Proc. Natl. Acad. Sci. USA* **89**, 8794.
5. Birren, B. *et al.* (1999) *Bacterial Artificial Chromosomes in Gene Analysis: A Laboratory Manual*, Cold Spring Harbor Press.
6. Kim, U. J. *et al.* (1992) *Nucl. Acids Res.* **20**, 1083.

*CopyControl™ products are covered by U.S. Patent No. 5,874,259 licensed to EPICENTRE and by other patents pending and assigned to EPICENTRE which cover specific vectors, including, without limitation, CopyControl™ pCC1™, pCC1BAC™, and pCC1FOS™, and specific cells, including, without limitation, TransforMax™ EPI300™. By purchasing CopyControl systems or vectors, the purchaser receives the right to use the product purchased from EPICENTRE or an authorized distributor for life science research.

CopyControl™ Cloning Kits and Related Products	
CopyControl™ Fosmid Library Production Kit	Page 3
CopyControl™ BAC Cloning Kits	Page 4
CopyControl™ PCR Cloning Kits	Page 6
EZ::TN™ < <i>oriV</i> /KAN-2> Insertion Kit	Page 7
BAC-Tracker™ Supercolied DNA Ladder	Page 7
TransforMax™ EPI300™ Electrocompetent and Chemically Competent <i>E. coli</i>	Page 8

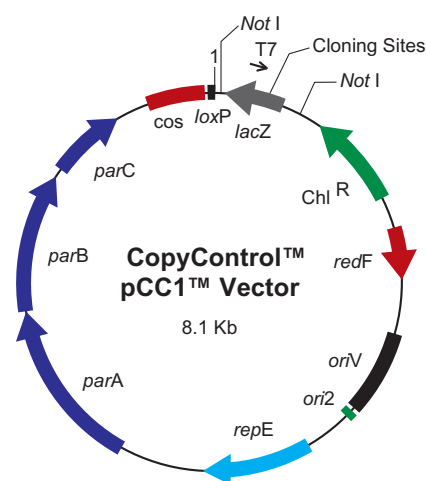


Figure 2. The CopyControl™ pCC1™ Cloning-Ready Vectors contain both a single copy and the *oriV* high copy origin of replication. The vector also contains a chloramphenicol selectable marker and T7 promoter. The CopyControl pCC1 Vector as supplied in the CopyControl Cloning Kits is linearized, completely dephosphorylated, and highly purified to ensure low background.

Benefits of the CopyControl Cloning Systems

- **Produce more complete genomic libraries and ensure successful cloning of all PCR products.**
Select and grow CopyControl clones at single copy to better ensure insert stability and cloning of encoded, expressed toxic proteins.
- **Get high yields of DNA for sequencing, fingerprinting or other applications.**
CopyControl clones, in small culture volumes, can be readily amplified from single copy to high copy number by addition of the CopyControl Induction Solution. CopyControl BAC clones are amplified to 10 – 20 copies per cell and CopyControl Fosmid and PCR clones to 10 – 50 copies per cell.
- **No need to prepare a cloning vector.**
The CopyControl Cloning Kits include a cloning-ready pCC1 vector which is linearized, dephosphorylated, highly purified and ready for ligation of the genomic DNA fragments or PCR product.
- **Rapidly screen CopyControl clones without minipreps or restriction digests.**
The size of CopyControl BAC clones can be estimated in 4 hours and CopyControl PCR clones in 1 hour without minipreps or restriction digests.
- **Retrofit existing BAC and fosmid clones with CopyControl capability.**
Existing BAC and fosmid clones can be retrofitted with the *oriV* high copy origin of replication using the transposon-based EZ::TN™ <*oriV* /KAN-2> Insertion Kit.

The CopyControl™ Fosmid Library Production Kit Generates More Complete and Unbiased (Blunt-End) Libraries of Stable, 40-Kb Genomic Clones

Fosmid vectors, containing the single copy *E. coli* F-factor replicon, were developed as an improved method for constructing libraries of cosmid-sized (approximately 40 Kb) clones. The stability of inserts cloned into fosmid vectors has been shown to be substantially greater than in high copy vectors.¹ EPICENTRE's new CopyControl™ Fosmid Library Production Kit includes the pCC1FOS™ Fosmid vector that contains both the *E. coli* F-factor replicon and the *oriV* high-copy origin of replication, thus providing the user the clone stability afforded by single-copy fosmid cloning and the high yields of DNA that can be realized from cosmid clones.

The CopyControl Fosmid Library Production Process Facilitates Construction of Complete and Unbiased Libraries

The CopyControl Fosmid Kit uses a novel strategy (Figure 1) of cloning randomly-sheared, end-repaired and 5'-phosphorylated DNA fragments. Shearing the DNA to approximately 40 Kb generates highly random DNA fragments, as opposed to more biased libraries that result from partial restriction endonuclease digestion. A CopyControl Fosmid library of >10⁷ clones can be generated in about 2 days. Thus, the CopyControl Fosmid Library Production Kit is ideal for preparing genomic libraries containing 40 Kb inserts from all sources. Even a library with 10X coverage of a genome as large as human is much faster and easier to prepare, and is more unbiased in its coverage, using the CopyControl Fosmid Vector than using a BAC vector.

At Single Copy, CopyControl Fosmid Clones Are More Stable Than Cosmids

Selection and growth of CopyControl Fosmid clones at single copy greatly improves the stability of the cloned insert by reducing the likelihood of segment deletions or rearrangements that can occur with cosmids. Additionally, single copy cloning facilitates cloning of DNA segments encoding products that can be lethal or detrimental to the host cell at high copy number. This was reported recently by researchers at the University of Maryland² who were unable to find a specific gene in a 20X cosmid library - most likely due to the toxic nature of its encoded protein. They readily identified

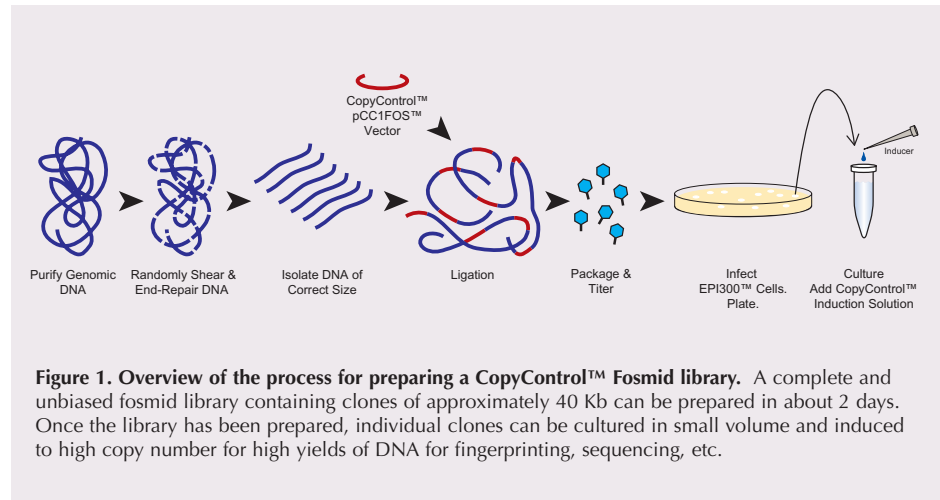


Figure 1. Overview of the process for preparing a CopyControl™ Fosmid library. A complete and unbiased fosmid library containing clones of approximately 40 Kb can be prepared in about 2 days. Once the library has been prepared, individual clones can be cultured in small volume and induced to high copy number for high yields of DNA for fingerprinting, sequencing, etc.

the gene in a fosmid library constructed using the CopyControl pCC1FOS Vector grown at single copy.

CopyControl Fosmid Clones Can Be Amplified to 10 – 50 Copies Per Cell for High Yields of DNA for Sequencing and Fingerprinting

Once the CopyControl Fosmid library has been produced, individual clones can be induced from single copy to 10 – 50 copies per cell (Figure 2) by the addition of the CopyControl Induction Solution. A small volume culture of an induced CopyControl Fosmid clone yields sufficient amounts of DNA for sequencing, fingerprinting, or other applications.

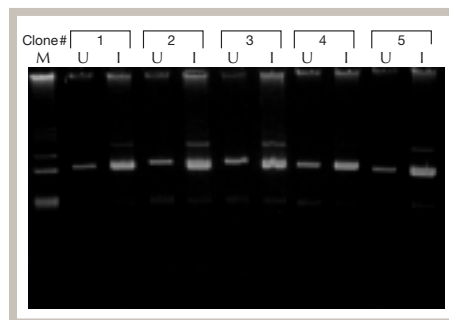


Figure 2. The copy number of CopyControl™ Fosmid clones can be induced 10 - 50 fold to greatly increase DNA yield. Five randomly chosen CopyControl Fosmid clones were grown in culture in duplicate. One sample of each was induced (I) to high copy number by addition of CopyControl™ Induction Solution. The other sample was an uninduced control (U). DNA was isolated from an equal number of cells of each and analyzed by agarose gel electrophoresis.

References

1. Kim, U.-J. *et al.* (1992) *Nucl. Acids Res.* **20**, 1083.
2. Dr. Nate Ekborg, University of Maryland, personal communication.

www.epicentre.com/ccfosmid.asp

CopyControl™ Fosmid Library Production Kit

CCFOS110 1 Kit
Kit contains sufficient reagents to produce up to 10 Fosmid Libraries.

Contents:

CopyControl™ pCC1FOS™ (Blunt) Vector, End-Repair Enzyme Mix and Buffer, Fast-Link™ DNA Ligase and Buffer, ATP, GELase™ Enzyme Preparation and Buffer, MaxPlax™ Lambda Packaging Extracts, EPI300™ *E. coli*, Control DNA, and CopyControl™ Induction Solution.

pCC1FOS™/pEpiFOS-5 Forward Sequencing Primer

F5FP010 50 µM 1 nmole

pCC1FOS™/pEpiFOS-5 Reverse Sequencing Primer

F5RP011 50 µM 1 nmole

Save At Least 2 Days Constructing a CopyControl™ BAC Library Then Amplify the Clones to 10 – 20 Copies Per Cell, for Higher DNA Yields and Purity

The CopyControl™ BAC Cloning Kits combine the advantages of the CopyControl cloning technology with an improved cloning protocol that significantly reduces the time and labor normally required to construct a BAC library. CopyControl BAC clones are initially grown in TransforMax™ EPI300™ Electrocompetent *E. coli* at single copy to ensure insert stability and cloning of potentially toxic expressed DNA segments. Once the library is produced, individual CopyControl BAC clones can be amplified, by addition of the CopyControl Induction Solution, to 10 – 20 copies per cell, to maximize the yield and purity of DNA for sequencing and fingerprinting.

Each of the CopyControl BAC Cloning Kits contain sufficient reagents to construct the equivalent of one 10X human library. The high transformation efficiency TransforMax™ EPI300™ Electrocompetent *E. coli* cells contain the inducible mutant *trfA* gene required for induction of CopyControl BAC clones to high copy number. These electrocompetent cells are available separately (see page 8).

The CopyControl BAC Cloning Kits Reduce the Time Needed to Construct a BAC Library by At Least 2 Days

In constructing a BAC library, two of the most laborious and time-consuming steps are preparing the BAC cloning vector and

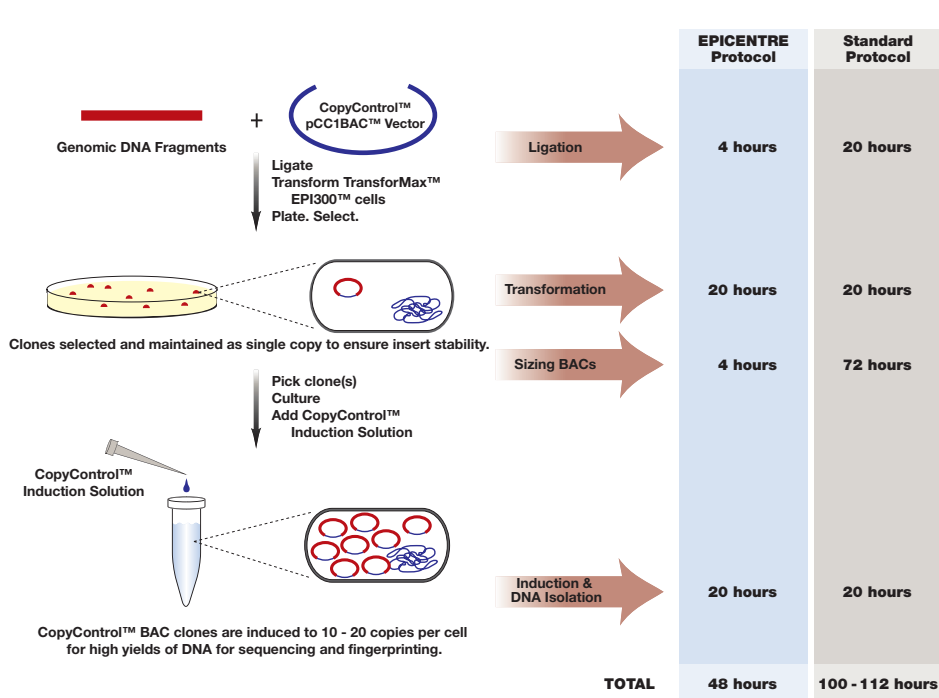


Figure 2. The CopyControl™ BAC Cloning Kits reduce the time to construct a BAC library by 2 days or more compared to standard methods.

size screening the BAC clones produced. The kits supply the cloning-ready CopyControl pCC1BAC™ Vector (Figure 1) - linearized at its *Bam*H I, *Eco*R I or *Hind* III site, dephosphorylated and then extensively purified – thus eliminating the need for and the quality concerns of vector preparation. The size of the BAC clones

produced can be estimated by agarose minigel electrophoresis in as little as 4 hours, without the need for minipreps or restriction endonuclease digestion (Figure 4B). Additionally, reaction conditions have been optimized to provide 4-hour DNA ligations. Other unique and time-saving components of the kits include the BAC-Tracker™ Supercoiled DNA Ladder, composed of supercoiled DNA gel markers of 38 Kb – 120 Kb (see page 7), a Control DNA Insert for testing ligation efficiency, and a 145 Kb CopyControl BAC clone for testing transformation efficiency and the clone amplification process.

CopyControl BAC Clones Can Be Amplified to 10 – 20 Copies Per Cell for High DNA Yields for Sequencing and Fingerprinting

Addition of the CopyControl Induction Solution to individual cultures of CopyControl BAC clones results in amplification of the clones from single copy to 10 – 20 clone copies per cell. A 1 – 2 ml culture of an induced CopyControl BAC clone yields microgram amounts of BAC DNA for fingerprinting (Figure 3) and sequencing. An additional benefit of amplifying the BAC clones is the higher

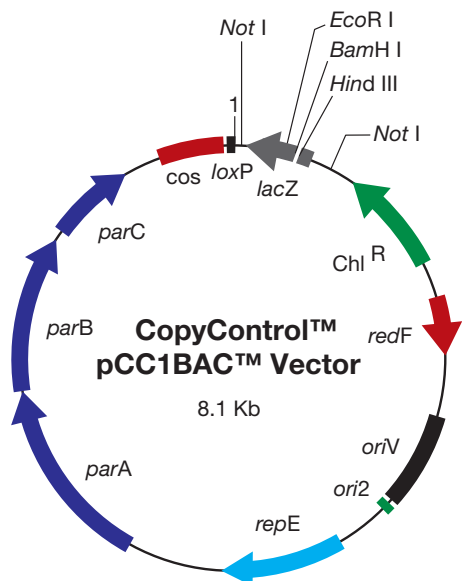
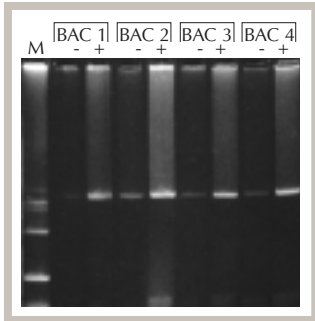


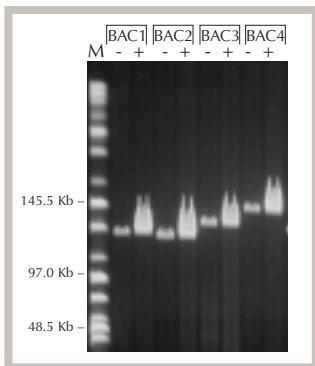
Figure 1. The CopyControl™ pCC1BAC™ vectors are supplied linearized at either the unique *Bam*H I, *Hind* III or *Eco*R I site, completely dephosphorylated and highly purified to ensure very low background. pCC1BAC is derived from pBeloBAC11 and EPICENTRE's pIndigoBAC-5.

proportion of BAC DNA relative to *E. coli* chromosomal DNA, resulting in higher BAC clone DNA purity.

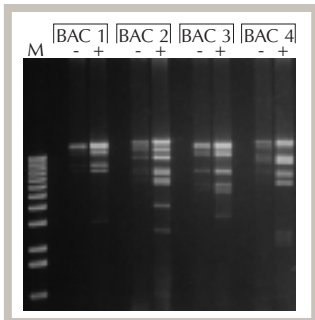
Figure 3. The copy number of CopyControl™ BAC clones can be induced 10 - 20 fold to greatly increase DNA yield. Four CopyControl BAC clones (BAC 1 = 130 Kb, BAC 2 = 125 Kb, BAC 3 = 140 Kb, BAC 4 = 145 Kb) were grown in culture in duplicate. One sample of each was induced (+) to high copy by addition of CopyControl™ Induction Solution. The other sample was an uninduced (-) control. DNA was isolated from an equal number of cells of each and analyzed by agarose gel (Panel A), *Not* I digestion (Panel B) and *Hind* III digestion (Panel C).



3A. One microliter of DNA from induced (+) and uninduced (-) cultures were resolved on a 0.8% agarose gel for 3 hours at 4°C and stained with SYBR®Gold. The induced cultures yielded 10 - 20 fold more CopyControl BAC clone DNA as determined by gel electrophoresis. M, supercoiled DNA ladder.



3B. *Not* I digests of 2 µl of DNA from induced (+) and uninduced (-) cultures resolved by PFGE and stained with SYBR®Gold. M, PFG marker.



3C. *Hind* III digests of 1 µl of DNA from induced (+) and uninduced (-) cultures. M, 1 Kb DNA ladder.

4A. A *Hind* III genomic BAC library was constructed from *E. coli* using the CopyControl™ BAC Cloning Kit (*Hind* III). A 100 µl aliquot from a 1 ml transformation of TransformMax™ EPI300™ Electrocompetent *E. coli* was plated. The kit produced > 95% white recombinant clones.

4B. The size of the CopyControl™ clones can be estimated and the quality of the library can be assessed in 4 hours. Nine randomly chosen CopyControl BAC clones were picked from the plate in Panel 3A and processed using the EpiLyse™ and EpiBlue™ screening solutions provided in the kit. The supercoiled DNA released from the cells was resolved in a 0.8% agarose gel at 4.5 V/cm for 3 hours at 4°C and stained with SYBR®Gold. M, supercoiled DNA ladder.

Cloning-Ready pCC1BAC Vectors and TransformMax EPI300 Electrocompetent *E. coli* Are Extensively Tested for Quality

The cloning-ready CopyControl pCC1BAC Vector provided in each kit is tested to ensure low background and high cloning efficiency. Each batch of the vectors is tested to produce >95% white (recombinant) colonies after ligation of the Control Insert (provided with each kit) and transformation into TransformMax™ EPI300™ Electrocompetent *E. coli*. In addition, the integrity of the *Bam*H I, *Eco*R I or *Hind* III ends is confirmed by a vector self-ligation assay after 5'-phosphorylation of the cohesive ends.

The TransformMax EPI300 Electrocompetent *E. coli* (available separately; see page 8 for additional information) are tested to give >1 X 10⁷ cfu/µg (>10²¹ cfu/mole) when transformed with a 145 Kb CopyControl BAC clone.

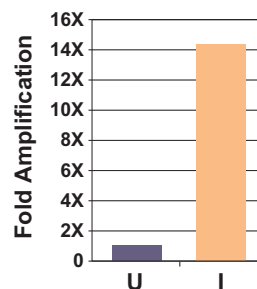


Figure 5. The yield of a BAC DNA from CopyControl™ BAC clones of 110 Kb - 145 Kb increased > 14 fold when grown in TransformMax™ EPI300™ cells and induced to high copy number by addition of the CopyControl™ Induction Solution. I, induced cells. U, uninduced control cells.

www.epicentre.com/ccbac.asp

- CopyControl™ BAC Cloning Kit (*Bam*H I)**
CCBAC1B 1 Kit
- CopyControl™ BAC Cloning Kit (*Hind* III)**
CCBAC1H 1 Kit
- CopyControl™ BAC Cloning Kit (*Eco*R I)**
CCBAC1E 1 Kit

Each Kit contains sufficient reagents to construct the equivalent of one 10X human library.

Contents:

CopyControl™ pCC1BAC™ (*Bam*H I, *Eco*R I, or *Hind* III Cloning-Ready) Vector, Fast-Link™ DNA Ligase and Buffer, ATP, BAC-Tracker™ Supercoiled DNA Ladder, EpiBlue™ Solution, EpiLyse™ Solution, Control DNA Insert, and Control CopyControl™ BAC Clone (145 Kb).

TransformMax™ EPI300™ Electrocompetent *E. coli*

(formerly called TransformMax™ EC300™ Electrocompetent *E. coli*)

- EC300105 5 x 100 µl
- EC300110 10 x 100 µl
- EC300150 50 x 100 µl

pCC1BAC™/pIndigoBAC-5 Forward Sequencing Primer

- BFP0701 50 µM 1 nmole

pCC1BAC™/pIndigoBAC-5 Reverse Sequencing Primer

- BRP0801 50 µM 1 nmole

The CopyControl™ PCR Cloning Kits Enable Faster, More Reliable Cloning of Any PCR Product Up to 15 Kb - Even If It Has High A-T, G-C or Repetitive Sequences, or Encodes a Toxic Peptide

Many researchers attempting to clone PCR products have encountered problems such as clone instability, deletions, rearrangements, mutations, and even loss of clones. Likely causes include: (1) intermolecular recombination events; (2) segment deletions, particularly if the PCR product has high A-T, or G-C content or repetitive sequences; and (3) expression of toxic or detrimental genes in PCR products cloned in high-copy vectors, either from their own promoters or by run-on transcription from vector promoters.

EPICENTRE's new CopyControl™ PCR Cloning Kits solve these problems. The unique CopyControl™ pCC1™ Vector has both a single-copy F-factor replicon and a tightly-controlled inducible high-copy origin of replication ("oriV"). The pCC1 Vector enables researchers to clone all PCR products at a single copy per cell in order to greatly reduce the possibility of sequence deletion, rearrangement, or the accumulation of lethal amounts of encoded and expressed protein. Once the CopyControl PCR clones are selected, they can be induced to high copy number (10 – 50+ copies per cell) in order to obtain high yields of DNA for sequencing, *in vitro* transcription, or other applications. Cloning of PCR products and screening of the clones can be completed in less than 24 hours.

Single-Copy Cloning Reduces the Risk of Sequence Deletions, Rearrangements and Cloning "Artifacts"

Often, the complete sequence of a PCR product is not known prior to cloning. Thus, researchers run the risk of analyzing

clones that, unknown to them, have undergone deletions or rearrangements during propagation in a high-copy vector. By cloning the PCR product in the CopyControl pCC1 Vector and growing the clones at single copy, researchers are assured of the stability of the cloned inserts and of the completeness and accuracy of sequence data.

Up to 15-Kb PCR or RT-PCR Products Obtained Using Any Thermostable Polymerase Can Be Cloned and Screened for Inserts in 24 Hours or Less

CopyControl PCR Cloning Kits employ a blunt-end cloning process (Figure 1) that converts the PCR product produced using any thermostable polymerase to blunt-ended, 5'-phosphorylated DNA for efficient ligation into the provided Blunt Cloning-Ready pCC1 Vector. The kit is ideal for cloning PCR products generated using the FailSafe™ PCR System (see center insert), but is also effective for cloning PCR products produced using proof-reading polymerases like *Pfu*, without the need to 3'-A tail as required with some PCR cloning methods. Additionally, CopyControl PCR Cloning Kits enable cloning of PCR products up to 15 Kb without the need for prior column or gel purification required by other PCR cloning procedures. Finally, the kits incorporate EPICENTRE's novel Colony Fast-Screen™ methodology for screening colonies for inserts in 1 hour or less, without minipreps or restriction digests (Figure 2).

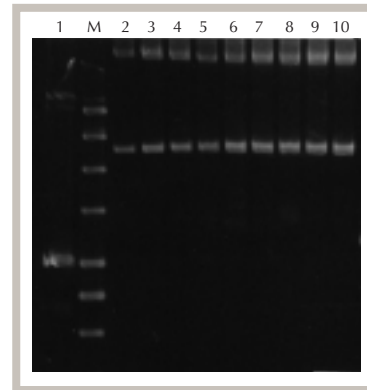


Figure 2. PCR clones can be screened in less than 1 hour without growing cultures or restriction digests. Very small portions from 9 white colonies resulting from cloning of a 5 Kb PCR product were randomly picked from a plate and processed using the EpiLyse™ and EpiBlue™ components of the CopyControl™ PCR Cloning Kit. The 5 Kb insert size of each clone was confirmed by agarose gel electrophoresis. M, DNA size ladder; Lane 1, 8 Kb supercoiled DNA marker; Lanes 2 - 10, randomly chosen clones. Total time including running the gel was approximately 60 minutes.

www.epicentre.com/ccpccr.asp

CopyControl™ PCR Cloning Kits are available with either TransformMax™ EPI300™ Electrocompetent *E. coli* or TransformMax™ EPI300™ Chemically Competent *E. coli* Cells.

CopyControl™ PCR Cloning Kit with TransformMax™ EPI300™ Chemically Competent *E. coli*

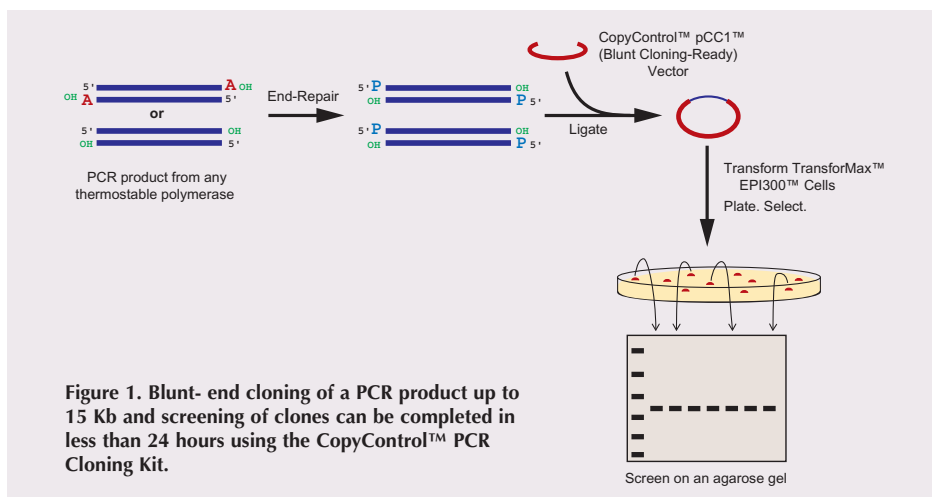
CCPCR1CC 20 Cloning Reactions

CopyControl™ PCR Cloning Kit with TransformMax™ EPI300™ Electrocompetent *E. coli*

CCEPCR1 20 Cloning Reactions

Contents:

CopyControl™ pCC1™ (Blunt Cloning-Ready) Vector, PCR Precipitation Solution, PCR End-Repair Enzyme Mix, PCR Cloning Buffer, Fast-Link™ DNA Ligase, EpiLyse™ Solution, EpiBlue™ Solution, Control PCR Product, Supercoiled DNA Marker, and Sterile Water.



Retrofit Existing BAC and Fosmid Clones with CopyControl™ Capability Using a Kit for Simple *In Vitro* Insertion of an *OriV*-containing EZ::TN™ Transposon

Many libraries have already been generated and are available in exclusively single-copy BAC or fosmid vectors. EPICENTRE's new EZ::TN™ <*oriV* /KAN-2> Insertion Kit enables researchers to integrate CopyControl™ capability into these clones. The kit features the EZ::TN™ <*oriV* /Kan-2> Transposon, which contains the *oriV* high copy origin of replication and a kanamycin selectable marker. A short, one-step *in vitro* reaction catalyzed by EZ::TN™ Transposase randomly inserts the transposon into existing BAC or fosmid clones. An aliquot of the transposition reaction is then used to transform TransforMax™ EPI300™ Electrocompetent *E. coli* (available separately, see page 8) and insertion clones are selected by growth on kanamycin (Figure 1).

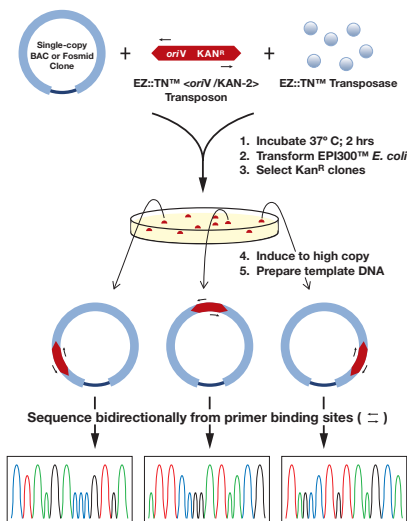


Figure 1. The process for generating EZ::TN™ <*oriV* /KAN-2> Transposon insertion clones for high yields of DNA and bidirectional sequencing.

Obtain High Yields of BAC and Fosmid DNA for Sequencing and Fingerprinting

A single, 10- μ l transposition reaction generates up to thousands of random transposon insertion clones. Each clone can be induced to 10 – 50 copies per cell by the addition of the CopyControl™ Induction Solution. Like the CopyControl™ pCC1™ Vectors, BAC and fosmid clones retrofitted with the EZ::TN <*oriV* /KAN-2> Transposon can be maintained at single copy to ensure insert stability but can then be induced to high copy number whenever desired, to maximize the yield and purity of DNA for sequencing, fingerprinting and other applications.

Sequence Bidirectionally from Randomly Distributed Primer Binding Sites

Each insertion clone not only contains *oriV* and a kanamycin marker, but unique primer binding sites near the ends of the transposon. DNA flanking the transposon can be sequenced bidirectionally from

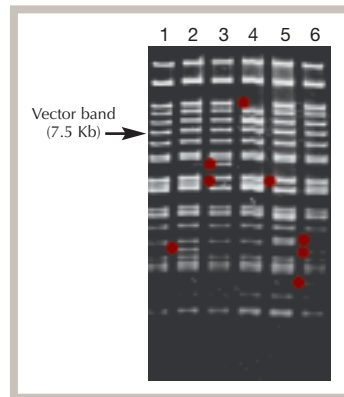


Figure 2. Unique *Hind* III fingerprints confirm that insertions of the EZ::TN™ <*oriV* /KAN-2> Transposon into a 165 Kb BAC are random.

Amplified DNA was isolated from individual insertion clones, digested with *Hind* III, and resolved on a 1% agarose gel. Lane 1, parental BAC clone; Lane 2-6, BAC insertion clones. ● indicate altered band positions resulting from a transposon insertion.

these unique sites using the primers provided in the kit. Thus, insertion of the *oriV*-containing transposon accomplishes two functions; it converts a single-copy clone to one which has CopyControl capability and it generates a library of sequencing templates with random transposon insertions (Figure 2), permitting complete sequencing of the clone with only two sequencing primers. The need for subcloning or primer walking strategies has been eliminated.

www.epicentre.com/transposomics.asp

EZ::TN™ <*oriV* /KAN-2> Insertion Kit

EZI02VK 10 Reactions

Contents:

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

TransforMax™ EPI300™ Electrocompetent *E. coli*

(formerly called TransforMax™ EC300™ Electrocompetent *E. coli*)

EC300105 5 x 100 μ l
EC300110 10 x 100 μ l
EC300150 50 x 100 μ l

BAC-Tracker™ Supercoiled DNA Ladder

EPICENTRE's new BAC-Tracker™ Supercoiled DNA Ladder is suitable for estimating the size of large supercoiled DNAs, such as BAC (Bacterial Artificial Chromosome) clones, by agarose gel electrophoresis.

The BAC-Tracker Ladder contains four discreet supercoiled DNAs of 38 Kb, 55 Kb, 95 Kb, and 120 Kb.

The Ladder is provided in a ready-to-load solution. Simply load 10 μ l of the Ladder per gel lane. Run the gel and stain using ethidium bromide or SYBR® Gold.

Applications

- Size analysis of BAC clones by agarose minigel or Pulse Field Gel Electrophoresis (e.g., CHEF, FIGE).
- Size analysis of supercoiled plasmid or extra-chromosomal DNA from any source.

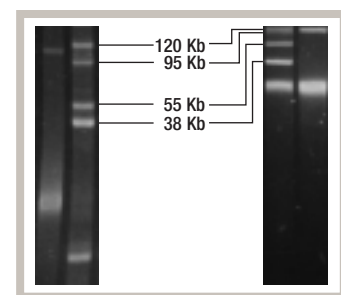


Figure 1. A purified 115 Kb BAC clone sized using the BAC-Tracker™ Supercoiled DNA Ladder by PFGE (Panel A) and by 1% agarose minigel (Panel B). Gels were stained using SYBR® Gold.

www.epicentre.com/bactrack.asp

BAC-Tracker™ Supercoiled DNA Ladder

BT010950 50 gel lanes (500 μ l)

TransforMax™ EPI300™ *E. coli*

TransforMax™ EPI300™ *E. coli* have been specially engineered for use with EPICENTRE's CopyControl™ Cloning Systems (see pages 1-2). The cells contain the mutant *trfA* gene, whose gene product is required for initiation of replication from the *oriV* origin of replication contained on the CopyControl cloning vectors or on clones retrofitted with the EZ::TN™<*oriV* /KAN-2> Transposon (see page 7). The *trfA* gene is under tight, regulated control of an inducible promoter. Addition of the CopyControl™ Induction Solution to the cells induces expression of the *trfA* gene resulting in amplification of CopyControl clones from single copy to high copy number.

TransforMax™ EPI300™ Electrocompetent *E. coli* have very high transformation efficiency, equivalent to the best competent *E. coli* cells which lack the *trfA* gene. They are qualified for use with all clone sizes up to >140 Kb clones (Table 1). With these cells, CopyControl BAC clones can be amplified to 10 – 20 copies per cell (Figure 1) and CopyControl Fosmid and PCR clones to 10 – 50+ copies per cell. Microgram amounts of DNA for sequencing, fingerprinting, sub-cloning, *in vitro* transcription, or other applications can be purified from small volume cultures of induced CopyControl clones.

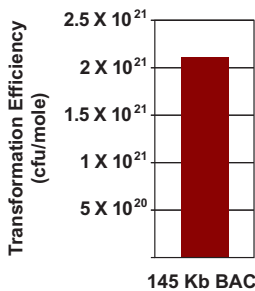


Table 1. TransforMax™ EPI300™ Electrocompetent *E. coli* are tested to ensure a transformation efficiency of >10²¹ cfu/mole (>10⁷ cfu/μg) of a 145 Kb CopyControl™ BAC clone.

Genotype

F⁻ *mcrA* Δ(*mrr-hsdRMS-mcrBC*) φ80*dlacZ*ΔM15 Δ*lacX74 recA1 endA1 araD139 Δ(ara, leu)7697 galU galK λ⁻ rpsL nupG trfA*

Important Characteristics

- High efficiency. TransforMax EPI300 Electrocompetent *E. coli* have a transformation efficiency of >5 X 10⁹ cfu/μg

Figure 1. CopyControl™ BAC clones can be induced 10 - 20 fold using TransforMax™ EPI300™ *E. coli*.

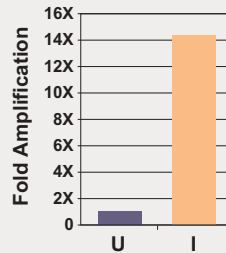


Figure 1A. The yield of BAC DNA from CopyControl BAC clones of 110 Kb - 145 Kb increased > 14 fold when grown in TransforMax EPI300 cells and induced to high copy number by addition of CopyControl™ Induction Solution. I, induced cells. U, uninduced control cells.

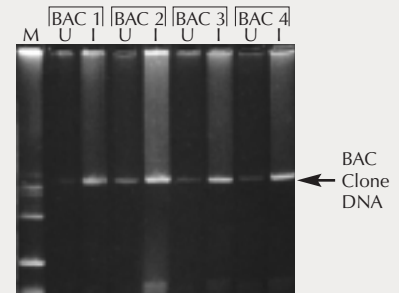


Figure 1B. Agarose gel analysis of DNA isolated from 4 CopyControl BAC clones in induced (I) or uninduced (U) TransforMax EPI300 cells. M, supercoiled DNA Marker.

using pUC19 and >1 X 10⁷ cfu/μg (10²¹ cfu/mole) using a 145 Kb BAC clone (Table 1).

- Supports blue/white screening of recombinants.
- Restriction minus for efficient cloning of methylated DNA (e.g. mammalian genomic DNA).
- RecA1* and *endA1* to ensure stability of large clones and high yields of DNA.
- Induced TransforMax EPI300 cells amplify CopyControl clones to 10 – 50+ copies per cell.

www.epicentre.com/epi300.asp

TransforMax™ EPI300™ Electrocompetent *E. coli*
(formerly called TransforMax™ EC300™ Electrocompetent *E. coli*)

EC300105	5 x 100 μl
EC300110	10 x 100 μl
EC300150	50 x 100 μl

TransforMax™ EPI300™ Chemically Competent *E. coli*
(formerly called TransforMax™ CC300™ Chemically Competent *E. coli*)

C300C105	10 x 50 μl
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Each of the above includes pUC19 control DNA and 1000X CopyControl™ Induction Solution.

Electroporation Cuvettes

EPICENTRE's Electroporation Cuvettes are manufactured to strict quality standards to ensure consistent pulse delivery and reproducible results. Electroporation Cuvettes are available in 1 mm (0.1 cm) and 2 mm (0.2 cm) gap widths.



www.epicentre.com/cuvette.asp

Electroporation Cuvettes

EC01091	1 mm	Bag of 50
	Gap Width	
EC01082	2 mm	Bag of 50
	Gap Width	

Cuvettes are individually wrapped and gamma sterilized.

- Individually wrapped and gamma sterilized to ensure sterility.
- Compatible with most electroporators including Eppendorf, BioRad, BTX Invitrogen and others.
- Available in 1 mm and 2 mm gap widths with color-coded caps for easy identification.

Obtain PCR-Ready Genomic DNA from Buccal Cells, HeLa Cells, Hair Follicles, Tail Snips, Bacterial Cells, or Feathers Using the QuickExtract™ DNA Extraction Solution

Judith E. Meis and FengLing Chen, EPICENTRE

Introduction

The QuickExtract™ DNA Extraction Solution, currently available separately or as a component of the BuccalAmp™ DNA Extraction Kit, provides an extremely efficient method for extracting PCR-ready genomic DNA from diverse samples. Extractions can be performed using the standard single-tube QuickExtract protocol on easily obtainable human and animal tissue samples for genomic, transgenic, or viral DNA screening. Here we report results for DNA extraction from buccal cells, HeLa cells, hair follicles, mouse tail snips, bacteria, and feathers. Extracted DNA was amplified using the FailSafe™ PCR System.

Methods and Results

QuickExtract DNA Extraction Protocol:

Each of the following samples was placed in 0.5 ml of QuickExtract Solution, vortex mixed, heated at 65° C for 30 minutes, vortex mixed and then heated at 98° C for 15 minutes (Figure 1):

- Human buccal (cheek) cells collected using a Catch-All™ Sample Collection Swab and rotated 5 times in the QuickExtract Solution to disperse the cells.
- 10⁴ counted human cervical carcinoma tissue culture (HeLa) cells.
- A 0.5-1 cm region of a single plucked human hair with follicle.
- A 0.5-1.0 cm section of a mouse tail snip.
- One *E. coli* colony picked from a plate.
- A 0.5-1.0 cm quill-end section of a bird breast feather that was plucked and stored at 4° C.

Amplification: Amplification of the QuickExtract DNA samples was performed using the FailSafe PCR System using 5 µl or less of each 0.5 ml sample. Reaction primers, annealing temperatures, and the FailSafe PCR 2X PreMix used for each sample varied.

Results: DNA extractions from buccal cells, tissue culture cells, hair follicles, mouse tail tissue, bacterial cells, and quill-end cells of bird feathers using the QuickExtract DNA Extraction Solution produced successful PCR amplification results with the FailSafe PCR System (Figure 2).

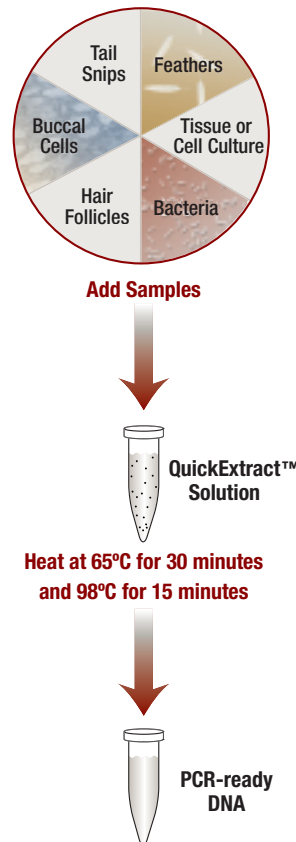


Figure 1. Procedure for obtaining PCR-ready DNA using the QuickExtract™ DNA Extraction Solution.



Figure 2. Genomic DNA extracted from a variety of tissues or cells using the QuickExtract™ DNA Extraction Solution were consistently amplified using the FailSafe™ PCR System. Lanes 1-3, human β-globin; Lane 4, mouse GAPDH; Lane 5, *E. coli* 16s ribosomal RNA gene; Lane 6, avian viral sequence.

Conclusion

Extraction of DNA using the QuickExtract DNA Extraction Solution is quick and efficient. DNA extraction, from a broad range of sample types, requires only heating. The DNA obtained is readily amplifiable by PCR, as shown here using the FailSafe PCR System.

The QuickExtract method allows for the inexpensive processing of one to hundreds of samples in less than an hour without centrifugation, spin columns, or use of toxic organic solvent. This simple process is amendable to automation but can also be easily performed manually, without expensive and troublesome robotic equipment. The QuickExtract Solution and the BuccalAmp DNA Extraction Kit also permit the use of samples obtained by non-invasive means, such as hair follicles and buccal cells rather than blood samples, thereby avoiding the health risks of needle sticks, blood storage requirements, and the expense of certified phlebotomists.

www.epicentre.com/buccalamp.asp

QuickExtract™ DNA Extraction Solution 1.0

QE09050 50 ml
Bulk solution, sufficient to perform 100 extractions.

BuccalAmp™ DNA Extraction Kits

BQ0901S 1 Kit
BQ0908S 8 Kits
BQ0916S 16 Kits

Contents:

15 tubes (1 extraction/tube) of BuccalAmp™ QuickExtract™ Solution 1.0
15 individually-packaged sterile Catch-All™ Swabs.

FailSafe™ PCR System

See the center insert for product and ordering information.

In Vitro Synthesis of 2'-Fluoro-Modified RNA Transcripts That Are Completely Resistant to RNase A Digestion Using the DuraScribe™ T7 Transcription Kit

Judith E. Meis and FengLing Chen, EPICENTRE

Introduction

In vitro transcription of DNA into RNA has become an increasingly important technique for genomic research. EPICENTRE's new DuraScribe™ T7 Transcription Kit produces about 50 µg of "DuraScript™ RNA" that is completely resistant to RNase A.

The DuraScribe T7 Transcription Kit utilizes a mutant form of T7 RNA Polymerase (DuraScribe™ T7 RNA Polymerase) that uses the same T7 transcription promoters as standard T7 RNA Polymerase. However, unlike standard T7 RNA Polymerase, DuraScribe T7 Polymerase efficiently incorporates 2'-modified-NTPs (e.g., 2'-fluoro-dNTP), as well as canonical NTPs (ATP, CTP, GTP, UTP), into full length RNA transcripts *in vitro*. DuraScript RNA is produced by completely replacing CTP and UTP with 2'-fluoro-dCTP (2'-F-dCTP) and 2'-fluoro-dUTP (2'-F-dUTP) in a DuraScribe *in vitro* transcription reaction (Figure 1). The presence of the 2'-fluoro-dC and 2'-fluoro-dU nucleotides in DuraScript RNA prevents its digestion by RNase A. However, DuraScript RNA is not resistant to other RNases that digest RNA at other nucleotides such as RNase T1 or RNase H. The sensitivity of DuraScript RNA to these RNases is beneficial in some applications.

Here we report the stability and yields of DuraScript RNA as well as its resistance to RNase A and related ribonucleases found in the lab environment.

Materials and Methods

DuraScribe T7 *in vitro* transcription reactions were performed as described in the DuraScribe kit literature. Unless otherwise noted, 20 µl DuraScribe reactions containing 1X DuraScribe™ T7 Transcription Buffer, 10 mM DTT, 1 µg of a linearized 3 Kb DNA Control Template (which produces a 1.4 Kb RNA transcript), 5 mM each ATP, GTP, 2'-F-dUTP and 2'-F-dCTP, and 2 µl DuraScribe T7 Enzyme Mix, were incubated at 37° C for 4 hours.

DuraScript RNA transcripts were precipitated from transcription reactions by addition of an equal volume (20 µl) of 5 M ammonium acetate. The tubes were held on ice for 15 minutes followed by high

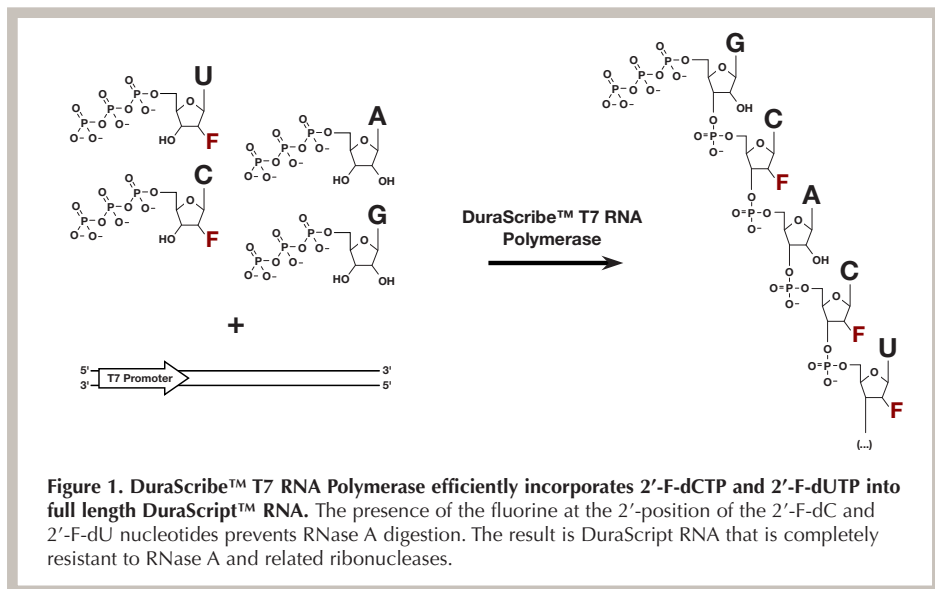


Figure 1. DuraScribe™ T7 RNA Polymerase efficiently incorporates 2'-F-dCTP and 2'-F-dUTP into full length DuraScript™ RNA. The presence of the fluorine at the 2'-position of the 2'-F-dC and 2'-F-dU nucleotides prevents RNase A digestion. The result is DuraScript RNA that is completely resistant to RNase A and related ribonucleases.

speed centrifugation for 15 minutes in a microcentrifuge. The pelleted DuraScript RNA was resuspended in water and quantitated by UV spectrophotometry.

Results

Yields of DuraScript RNA

The DuraScribe T7 Enzyme Mix is formulated to utilize very high concentrations of nucleotides in order to produce the highest possible yields of DuraScript RNA. Duplicate DuraScribe transcription reactions were incubated for 2, 4, or 6 hours at 37°C. The standard 4 hour DuraScribe transcription reaction consistently produced 40 - 60 µg of full-length DuraScript RNA from 1 µg of the control DNA template (Figure 2). Incubating the reaction for 6 hours improved the yield by approximately 10%.

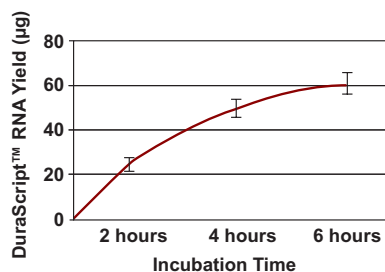


Figure 2. Forty to sixty µg of a 1.4 Kb DuraScript™ RNA is produced in a standard 4-hour DuraScribe™ T7 Transcription reaction using the control DNA template.

The standard 20 µl DuraScribe reaction was scaled-up to increase the yield of DuraScript RNA. All reaction components, including the DNA template, were scaled up 5X and 25X (to 100 µl and 500 µl reaction volumes, respectively). The 5X reaction produced >200 µg and the 25X reaction generated >600 µg of DuraScript RNA.

Frequently, the DNA template is the limiting component in an *in vitro* transcription reaction. The yield of DuraScript RNA produced from limiting amounts of template was determined by performing DuraScribe reactions, in duplicate, using decreasing amounts of the linearized Control Template DNA. As shown in Figure 3, a DuraScribe reaction produces microgram amounts of DuraScript RNA from as little as 10 ng of DNA template.

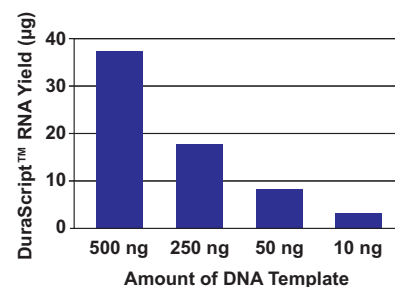


Figure 3. Limiting amounts of DNA templates are readily transcribed in a standard 20 µl DuraScribe™ reaction.

Incorporation of Labeled Nucleotides by DuraScribe T7 Polymerase

Direct incorporation of biotin-, digoxigenin-, and fluorescent-nucleotides into DuraScript RNA by DuraScribe T7 Polymerase was examined. Since DuraScribe T7 Polymerase will incorporate 2'-deoxynucleotides, and to retain the RNase A resistance of the labeled DuraScript RNA, biotin-dUTP, digoxigenin-dUTP, and fluorescent labeled-dUTP were chosen as the labeled nucleotides. By replacing a portion of the 2'-fluoro-dUTP with the respective labeled-dUTP in the reaction, full length, non-radioactively labeled DuraScript RNA was produced. Transcripts were also labeled post-transcriptionally with a Cyanine dye after incorporating amino-hexyl-ATP into the DuraScript RNA during the DuraScribe transcription reaction. Labeling protocols are provided with the DuraScribe T7 Transcription Kit.

Resistance of DuraScript RNA to RNase A and Other Ribonucleases

DuraScript RNA and standard RNA transcripts, produced by *in vitro* transcription using standard T7 RNA Polymerase, were each incubated with 1 µg/ml (final concentration) of purified RNase A (Catalog # R5250, Sigma) for 30 minutes at 37°C. An aliquot of each reaction was analyzed by gel electrophoresis to ascertain the extent of degradation by the RNase A. The standard RNA transcript was completely degraded while the DuraScript RNA remained intact (Figure 4) thus demonstrating the resistance of DuraScript RNA to even extreme levels of RNase A. It should be noted that some preparations of RNase A from nuclease-rich sources such as bovine pancreas, contain contaminating RNases that may digest DuraScript RNA. Therefore, only the highest purity RNase A should be added to DuraScript RNA preparations.

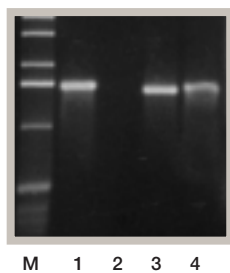


Figure 4. DuraScript™ RNA is resistant to RNase A digestion. A 1.4 Kb standard RNA transcript and a 1.4 Kb DuraScript RNA transcript were each incubated with 1 U of RNase A for 30 minutes. The standard RNA transcript was completely degraded while the DuraScript RNA transcript remained intact. M, size ladder; Lane 1, 1.4 Kb standard RNA transcript; Lane 2, standard RNA after RNase A treatment; Lane 3, 1.4 Kb DuraScript RNA; Lane 4, DuraScript RNA after RNase A treatment.

Ribonucleases present on human hands and spread throughout the lab are a major problem for those working with RNA. Because of these "fingertip" nucleases, researchers are required to wear gloves, use DEPC-treated water, bake or autoclave glassware and tubes, add RNase inhibitors to reactions and even dedicate equipment especially for RNA. Therefore, the stability of DuraScript RNA in the presence of "fingertip" nucleases was tested. DuraScribe and standard *in vitro* transcription reactions were performed in duplicate as described previously except that either RNase-free sterile water was added to one set of transcription reactions and water that had been contaminated by the hands of a test subject was added to the other set of reactions. The RNA products of each reaction were then analyzed by gel electrophoresis to ascertain the extent of degradation by "fingertip" nucleases present in the contaminated water. Figure 5 shows that DuraScript RNA was completely resistant to common fingertip nuclease degradation while standard RNA transcripts are degraded.

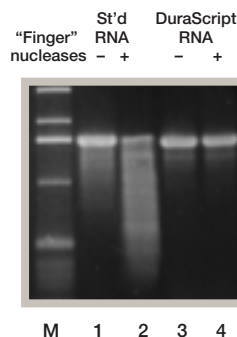


Figure 5. DuraScript™ RNA is completely resistant to "fingertip" nucleases. A 1.4 Kb standard RNA transcript and a 1.4 Kb DuraScript RNA transcript were transcribed in sterile water or water that had been contaminated by exposure to the hands of a test subject. The standard RNA shows extensive degradation from "fingertip" nucleases in the contaminated water while the DuraScript RNA remains fully intact. M, size ladder; Lane 1, standard RNA transcript; Lane 2, standard RNA after "fingertip" nuclease exposure; Lane 3, DuraScript RNA; Lane 4, DuraScript RNA after "fingertip" nuclease exposure.

Applications exist where selective sensitivity of RNA to ribonucleases is desired (e.g., RNase protection assays). We first evaluated the sensitivity of DuraScript RNA to RNase H. A DuraScript RNA transcript was annealed to two complementary DNA oligonucleotides. The DuraScript RNA/DNA hybrid was incubated with 0.1 Unit of *E. coli* RNase H for 30 minutes at 37°C. Agarose gel analysis of the resulting digestion products produced the predicted pattern of fragments from complete and specific RNase H digestion of the

DuraScript RNA/DNA hybrid. Therefore, DuraScript RNA behaves as standard RNA in an RNA/DNA hybrid and is subject to RNase H degradation.

Ribonuclease T1 digests RNA specifically at G residues. Because DuraScript RNA contains the canonical ribo-G nucleotides, it was predicted that DuraScript RNA would be susceptible to RNase T1 digestion. Agarose gel analysis of 200 ng DuraScript RNA that had been incubated 15 minutes at 37°C with 2 Units of RNase T1 showed extensive degradation of the DuraScript RNA thus confirming that DuraScript RNA is readily digested by RNase T1.

Conclusion

EPICENTRE's AmpliScribe™ High Yield Transcription Kits produce up to 150 µg of canonical RNA from a single reaction. The DuraScribe T7 Transcription Kit provides a useful new tool to efficiently prepare 2'-fluoro-modified RNAs that are resistant to RNase A. A 20 µl DuraScribe transcription reaction produces 40 – 60 µg of full-length 1.4 Kb DuraScript RNA using a linearized control template with a standard T7 polymerase promoter. The presence of the fluorine at the 2'-position of all C and U nucleotides in DuraScript RNA blocks digestion by RNase A present on human skin ("fingertip" nucleases). However, DuraScript RNA is still digested by ribonucleases that cleave at other than C or U nucleotides (e.g., RNase T1) or that cleave RNA by different reaction mechanisms (e.g., RNase H). The combination of DuraScript's resistance to RNase A and its susceptibility to RNase H and RNase T1 and other specific ribonucleases is advantageous for many applications.

References

1. Sousa, R. and Padilla, R. (1995) *EMBO J.* **14**, 4609.
2. Heidenreich, O. et al. (1994) *J. Biol. Chem.* **269**, 2131.
3. Ono, T. et al. (1997) *Nucl. Acids Res.* **25**, 4581.

www.epicentre.com/durascribe.asp

DuraScribe™ T7 Transcription Kit

DS010910	10 Reactions
DS010925	25 Reactions

Contents:

DuraScribe™ T7 Enzyme Mix, DuraScribe™ T7 10X Reaction Buffer, 2'-F-dCTP, 2'-F-dUTP, ATP, GTP, DTT, Control DNA Template, and Water.

Transposon Mutagenesis in the Parasitic Protozoa *Trypanosoma brucei* with the EZ::TN™ Transposome™

Elisabetta Ullu^{1,2}, Huafang Shi¹, Steven Wormsley¹, and Christian Tschudi^{1,3}

¹ Departments of Internal Medicine, ² Cell Biology, and

³ Epidemiology and Public Health, Yale University Medical School, New Haven, CT

Introduction

Trypanosoma brucei, a flagellate protozoa and the causative agent of African trypanosomiasis, belongs to the family *Trypanosomatidae*, which includes other important human pathogens, like the South American trypanosomes and Leishmanias. Ever since their discovery at the beginning of the last century, research on these organisms has focused primarily on their parasitic lifestyles. However, the application of recombinant DNA methodologies, as well as cell and molecular biological techniques led to the discovery of a number of unique biological properties, including trans-splicing, mitochondrial RNA editing, and polycistronic transcription units. Although first described in trypanosomes, many of these properties have subsequently been shown to be present in other members of the eukaryotic kingdom and thus have provided paradigms of eukaryotic biology. Another reason for a resurgence of interest into trypanosomes stems from the realization that many of the protozoan parasites are among the most deeply divergent eukaryotic organisms and therefore represent model systems for the study of eukaryotic evolution. The importance of trypanosomatid protozoa as model systems for unicellular pathogens is further underscored by many ongoing genome sequencing projects.

Although we have come a long way, there is still an urgent need for the development of techniques for the systematic analysis of gene function in trypanosomes. One recent advance in the functional analysis of *T. brucei* genes has been the development of a one step polymerase chain reaction (PCR)-mediated approach for the creation of chromosomal gene disruption and modification.¹ The approach is based on the PCR amplification of a reporter cassette, using two primers containing flanking sequences specific to the target gene, followed by electroporation of the PCR product into trypanosomes.

An alternative tool to study protein function, generate gene fusions and mutations, and provide physical landmarks for subsequent analysis, is the insertion of transposable elements into the genome.

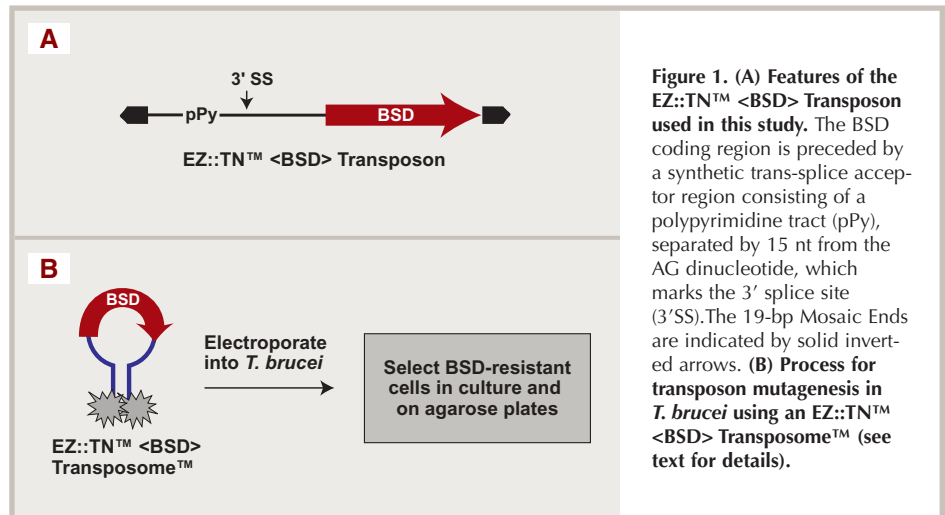


Figure 1. (A) Features of the EZ::TN™ <BSD> Transposon used in this study. The BSD coding region is preceded by a synthetic trans-splice acceptor region consisting of a polypyrimidine tract (pPy), separated by 15 nt from the AG dinucleotide, which marks the 3' splice site (3' SS). The 19-bp Mosaic End sequences are indicated by solid inverted arrows. **(B) Process for transposon mutagenesis in *T. brucei* using an EZ::TN™ <BSD> Transposome™** (see text for details).

Reznikoff and colleagues developed a technique for *in vivo* transposition using extraordinarily stable transposon-transposase complexes, referred to as EZ::TN™ Transposomes™.³ Key features of this system are: i) the transposon is defined by two 19-bp Mosaic End sequences that form an inverted repeat and the DNA between the repeat plays no role in transposition; ii) Transposomes can be assembled in the test tube and are resistant to harsh conditions, such as electroporation; and iii) there is no need to generate a transposase expression system for the organism of interest and transposon insertions are irreversible.

Electroporation of EZ::TN Transposomes has found wide applications in a variety of bacteria for the generation of insertional mutants. However, the extension of this system to eukaryotic organisms has lagged behind, barring one report of its use in *Saccharomyces cerevisiae*.³ Here we describe the adaptation of the EZ::TN Transposome system to *T. brucei*² and highlight its possible application to other parasites, like *Plasmodium*.

Methods and Results

To test the feasibility of using the EZ::TN Transposome for genetic studies in *T. brucei*, we first constructed a transposon for the expression of a drug resistance marker (blasticidin S deaminase or BSD, in our case). Generally, genes that encode housekeeping proteins in trypanosomes are transcribed into polycistronic pre-mRNAs.

Individual mature mRNAs are then generated by the addition of the spliced leader sequence at the 5' end through trans-splicing and by cleavage/polyadenylation at the 3' end. Thus, we used PCR to assemble a promoterless BSD expression cassette by attaching to the 5' end the trans-splicing signals described in Figure 1A. To avoid possible homologous recombination with trypanosome DNA, no trypanosome-derived 3' untranslated region nor sequences required for poly(A) addition were provided for the BSD gene. The BSD cassette was then cloned into the *EcoR* I and *Bam*H I sites of the EZ::TN™ pMOD™<MCS> Transposon Construction Vector. This plasmid contains a multiple cloning site flanked by the Mosaic Ends recognized by EZ::TN™ Transposase. To generate an EZ::TN <BSD> Transposome, the transposon was amplified by PCR, purified and incubated with EZ::TN Transposase in the absence of Mg⁺⁺ ions following the manufacturer's instructions (Figure 1B).

Next, we electroporated different amounts of EZ::TN <BSD> Transposomes into 10⁸ procyclic YTaT 1.1 trypanosomes using our standard conditions,⁴ except that cells were washed and electroporated in cytomix without Mg⁺⁺ ions. Blasticidin-resistant trypanosomes, which became apparent after approximately 10 days, were initially selected as populations. The genomic DNA was digested with *Kpn* I, which does not cut in the BSD cassette, and analyzed by Southern blot

hybridization with a BSD-specific gene probe (Figure 2). The hybridization pattern, with bands ranging in size from low to high molecular weight, was superimposed over a smear of hybridization and consistent with integration events at random sites in the genome.

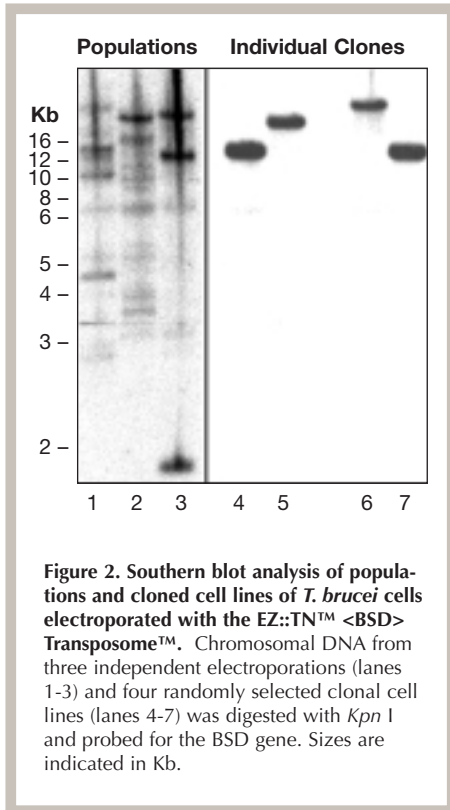


Figure 2. Southern blot analysis of populations and cloned cell lines of *T. brucei* cells electroporated with the EZ::TN™ <BSD> Transposome™. Chromosomal DNA from three independent electroporations (lanes 1-3) and four randomly selected clonal cell lines (lanes 4-7) was digested with *Kpn* I and probed for the BSD gene. Sizes are indicated in Kb.

We then generated clonal cell lines from one of the populations by plating the trypanosomes on agarose plates as described.⁵ The analysis of four such cell lines is shown in Figure 2 and revealed a single hybridizing band demonstrating that each line contained a single BSD gene insertion. Sequencing four insertion sites revealed that the transposon inserted as described for bacterial systems by a cut-and-paste mechanism and therefore generated a 9 bp duplication at the insertion site. Two insertions occurred in known genes, whereas the other two insertions were in predicted open reading frames.²

Blastocystin resistant trypanosomes arose at a frequency of about 10^{-4} to 10^{-3} using 20 to 100 ng of Transposome. This is most certainly an underestimation of the true insertion frequency since generation of a functional BSD mRNA and hence the isolation of drug resistant trypanosomes was dependent on insertion into an actively transcribed chromosomal region. Furthermore, the BSD gene had to be inserted in the same orientation as the

direction of transcription. Despite these limitations, the trypanosome insertion frequency is fairly comparable to what has been obtained in bacterial systems. Indeed, we have now generated a library of about 200,000 transposon-tagged trypanosomes, making it feasible to saturate the genome.

Discussion

Among potential applications, we envision using Transposomes as a means to identify genes and to generate fusions with the green fluorescent protein to localize protein components of different subcellular structures. Since trypanosomes are diploid, it will be necessary to generate homozygous organisms to obtain useful mutants. One possibility would be to select for loss of heterozygosity or alternatively, to chemically mutagenize cells before transposon insertion. However, for parasites with a haploid genome, like *Plasmodium* and *Toxoplasma*, this system has a great potential to provide a simple and easy way to generate mutants for functional analysis.

The EZ::TN™ pMOD™<MCS> Transposon Construction Vector used in this study has been replaced by the EZ::TN™ pMOD™-2<MCS> and pMOD™-3<R6Kγori /MCS> Transposon Construction Vectors which incorporate unique primer binding sites at each end of a custom EZ::TN Transposon for bidirectional sequencing. The latter vector as well as the EZ::TN™ <R6Kγori /KAN-2> Tnp Transposome™ also include a conditional origin of replication (R6Kγori) which can be used for "rescuing" DNA flanking the transposon insertion site. See the center insert for more information.

Ready-to-use EZ::TN Transposomes are available containing either a kanamycin selectable marker (<KAN-2> or <R6Kγori /KAN-2>) or a dihydrofolate reductase gene (<DHFR-1>) that can be selected on plates containing trimethoprim. The number of transposition clones obtained is highly dependent on the transformation efficiency of the host cell. The higher the transformation efficiency of the cell, the more clones will be produced. Electroporation of *E. coli* with a transformation efficiency for pUC19 DNA of $>10^9$ cfu/ug typically results in $>10^5$ independent insertion clones when 1 μl of EZ::TN Transposome is used.

Acknowledgements

This work was supported by grant AI28798 from the NIH.

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EZ::TN™ pMOD™-2<MCS> Transposon Construction Vector
MOD0602 20 μg

EZ::TN™ pMOD™-3<R6Kγori /MCS> Transposon Construction Vector
MOD1503 20 μg

Each includes Vector and the pMOD™<MCS> Forward and Reverse PCR Primers for amplification of transposon DNA. Primers for bidirectional sequencing of an insertion site are available separately.

EZ::TN™ Transposase
TNP92110 10 Units

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EZ::TN™<KAN-2>Tnp Transposome™ Kit
TSM99K2 10 Reactions

EZ::TN™<DHFR-1>Tnp Transposome™ Kit
TSM99D1 10 Reactions

EZ::TN™<R6Kγori /KAN-2>Tnp Transposome™ Kit
TSM08KR 10 Reactions

Each Kit contains the specific EZ::TN™ Transposome™ and forward and reverse primers for bidirectional sequencing from the transposon.

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

Jerry Jendrisak, Joanne Decker, and Ronald Meis, EPICENTRE

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 γ ori /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 γ ori /KAN-2> Transposon and 1U of EZ::TN™ Transposase in a Mg²⁺-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 γ ori.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 γ ori /KAN-2> Insertion Kit - one from a *Thermus* strain and two from *Pseudomonas*. The plasmid

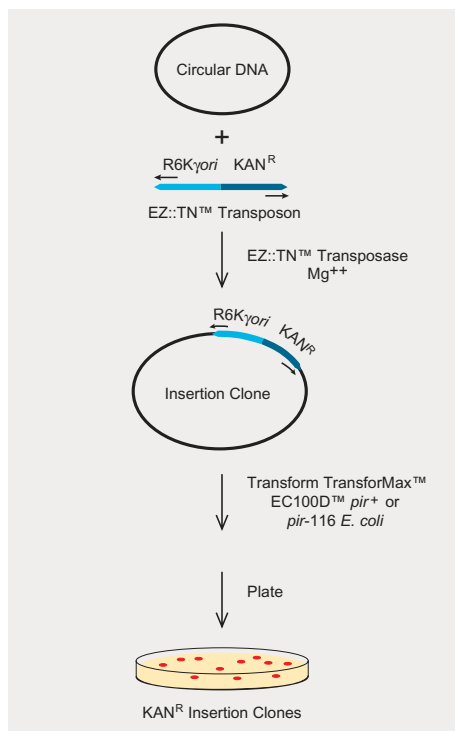


Figure 1. Circular DNA molecules, such as plasmids, are rescued in *E. coli* after *in vitro* insertion of an EZ::TN™ Transposon containing the R6K γ origin of replication (R6K γ ori) and a kanamycin selectable marker (KAN^R). Primer binding sites are located near each end of the transposon for bidirectional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

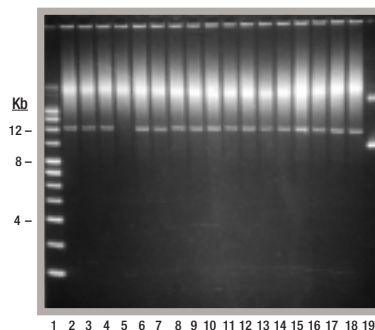


Figure 2. *T. flavus* plasmid clones containing the EZ::TN™ <R6K γ ori /KAN-2> Transposon were size selected using the Colony Fast-Screen™ Kit. Sixteen of 17 randomly chosen clones contained a 12.4 Kb plasmid which is consistent with a single 2 Kb transposon insertion into the 10.4 Kb target. One background 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

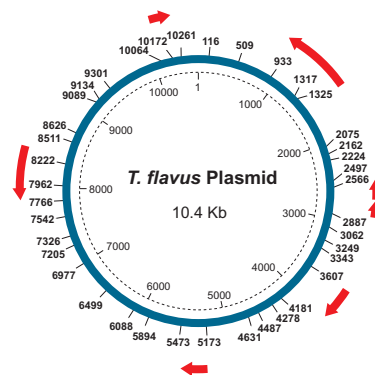


Figure 3. EZ::TN™ <R6K γ ori /KAN-2> Transposon insertions into a *T. flavus* plasmid were highly random. Hash marks indicate transposon insertion sites for 39 randomly selected clones. BLAST analysis identified seven open reading frames (red arrows) with significant homology to known gene products.

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 γ ori /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

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www.epicentre.com/transposomics.asp

EZ::TN™ <R6K γ ori /KAN-2> Insertion Kit

EZI011RK 10 Reactions

Contents:

EZ::TN™ <R6K γ ori /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 μ l
(10 Electroporations)

Maintains clones at 15 copies per cell. Includes control vector containing an R6K γ ori.

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

EC6P095H 5 X 100 μ l
(10 Electroporations)

Maintains clones at 250 copies per cell. Includes control vector containing an R6K γ ori.

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Colony Fast-Screen™ Kit

FS08250 1 Kit
Reagents sufficient for screening 250 colonies.

Contents:

EpiBlue™ and EpiLyse Solutions.

Consistent High Fidelity PCR Amplification of DNA 20 Kb and Longer Using the MasterAmp™ Extra-Long PCR System

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 MgCl₂ and MasterAmp™ PCR Enhancer (with betaine*).

Optimal Consistent Results

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.

DNA Amplification 20 Kb and Longer from Any Template

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.

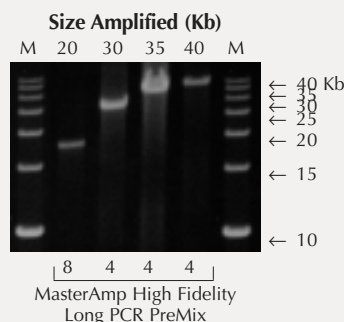


Figure 1. Amplification of 20, 30, 35, and 40 Kb sequences from lambda DNA. One nanogram of lambda DNA was used to amplify 20, 30, 35, and 40 Kb sequences. Lane M, 5 Kb DNA ladder.

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:

MasterAmp™ Extra-Long PCR 2X PreMixes 1-9
MasterAmp™ Extra-Long DNA Polymerase Mix
Control Lambda DNA/Primers

MasterAmp™ Extra-Long DNA Polymerase and individual Extra-Long PCR 2X PreMixes are also available separately. Visit www.epicentre.com/catalog/extra_long.htm

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DNA Ligation in 5 Minutes!

At room temperature

EPICENTRE's Fast-Link™ DNA Ligation kits are specially formulated to provide fast and efficient DNA ligations in as little as 5 minutes at room temperature for both routine and high throughput cloning.

Fast and Efficient DNA Ligations.

- Ligate cohesive-end DNA in 5 minutes at room temperature.
- Ligate blunt-end DNA in 15 minutes at room temperature.
- Ligate PCR product with A-overhangs in 1 hour or less at 16°C.

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Desalting the Fast-Link DNA ligation reaction prior to transformation of electrocompetent or chemically competent cells is not necessary. Aliquots (1-2 µl) of a Fast-Link ligation reaction may be used directly in electroporation.

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 x 10 ⁶
Blunt ends	15 min.	>95%	5.0 x 10 ⁵

* Cohesive-end ligation results were obtained by ligating *Hind* III-digested *E. coli* chromosomal DNA into EPICENTRE's pIndigoBAC-5 (*Hind* 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*.

Ligation Time in Minutes

5 15 30 60 120

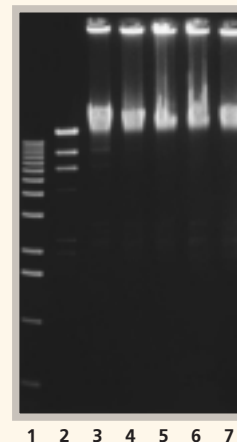


Figure 1. Time course for cohesive-end ligation using the Fast-Link™ DNA Ligation Kit. Lambda *Hind* III markers were ligated in a standard Fast-Link reaction using 2 U of Fast-Link DNA Ligase (Lanes 3-7). Lane 1, 1 Kb ladder; Lane 2, no enzyme.

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Fast-Link™ DNA Ligation Kits

LK11025	25 ligations
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EPICENTRE FORUM

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