EPICENTRE Forum

Tools & Techniques for Genomics, Proteomics & RNA Research

Rapid, High Yield Transcription of Short Hairpin RNA (shRNA) for RNAi-Mediated Gene Silencing

Judith E. Meis, EPICENTRE

RNA interference (RNAi) is a powerful technique for elucidation of gene function.[1] Typically, the RNAi response in mammalian cells is mediated by short double-stranded RNA (dsRNA). Recently, short hairpin RNAs (shRNA), single-strand RNAs containing a high degree of secondary structure, have been shown to result in gene silencing as effectively as short dsRNA produced by methods such as chemical synthesis.[2,3,4] Here we demonstrate that EPICENTRE's new AmpliScribe™ T7-*Flash*™ Transcription Kit is ideal for rapid, high yield production of shRNA for RNAi studies.*

Methods

Design of the in vitro transcription template for shRNA production

Figure 1 provides an overview of the process for producing and using shRNA for RNAi. Two complementary 87-base DNA oligonucleotides were synthesized to serve as the *in vitro* transcription template for producing shRNA. The first oligo contained a T7 RNA polymerase promoter sequence followed by a 29-base sequence complementary to firefly luciferase mRNA, an 8-base non-complementary sequence (the "hairpin") and the complement to the 29-base firefly luciferase sequence.[3] The second oligo was the complement of the first. The 29-base sequence, complementary to firefly luciferase mRNA, was chosen to silence expression of firefly luciferase without affecting the expression of *Renilla* luciferase, which was used as a control in these experiments.

The 87-base DNA oligos were resuspended in TNE (10 mM Tris-HCl, pH 7.5; 100 mM NaCl; 1 mM EDTA), denatured at 95ºC for 5 minutes, and annealed at 65ºC for 10 minutes, followed by 37ºC for 10 minutes.

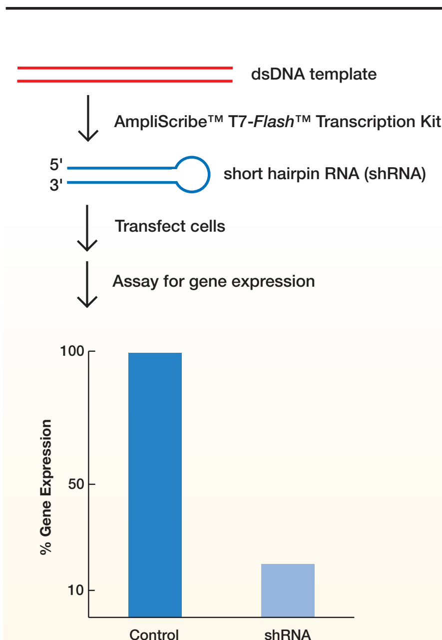


Figure 1. Overview of shRNA production, *in vitro*, and use for RNAi. Two complementary DNA oligos, containing a T7 transcription promoter and sequences of the targeted mRNA are synthesized, annealed, and transcribed *in vitro* using the AmpliScribe™ T7-*Flash*™ Transcription Kit. The short, single-stranded RNA transcript spontaneously forms a hairpin structure (shRNA). Transfection of the shRNA into cells mediates silencing of the targeted gene.

5'- pppGGA UUC CAA UUC AGC GGG AGC CAC CUG AU G A A G
3'- CCU AAG GUU AAG UCG CCC UCG GUG GAC UA G U U C

Figure 2. The 66-base, single-stranded shRNA-*luc* was transcribed from an 87-bp oligo DNA that contained a T7 transcription promoter, a 29-base sequence complementary to firefly luciferase, and an 8-base "hairpin" sequence.

In vitro transcription of shRNA using the AmpliScribe T7-Flash Transcription Kit

A 66-base shRNA (Figure 2), specific for silencing firefly luciferase and designated shRNA-*luc*, was transcribed from 1 µg of the 87-bp DNA template for 30

. . . continued on page 2

Visit us at
www.epicentre.com

In This Issue

1 Rapid, High Yield Transcription of Short Hairpin RNA (shRNA) for RNAi-Mediated Gene Silencing

3 High Quality RNA for Use with MicroArrays MasterPure™ Genomic DNA for *Bacillus anthracis* Analysis

4 DuraScript™ dsRNA Digested Using Human Dicer Enzyme Is As Effective and Specific in RNAi-Mediated Gene Silencing As Canonical "Diced" dsRNA

6 Transcribe More RNA in 30 Minutes Than Other *In Vitro* Transcription Kits Produce in 2 Hours

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

9 10 Reasons Why DNA Cloning Kits from EPICENTRE Enable the Fastest and Most Reliable Results

10 Efficient Cloning of Entire Mitochondrial Genomes in *Escherichia coli* by *In Vitro* Insertion of a Transposon

12 CopyControl™ Cloning Systems Allow Single-Copy Cloning, Then Controlled Induction for High-Copy DNA Production

14 The FailSafe™ Real-Time PCR System with SYBR® Green I Dye Provides Shorter Cycle Times and More Consistent PCR Quantitation with Every Template Every Time

minutes in a 20-µl AmpliScribe T7-*Flash* transcription reaction. Following transcription, the reaction was treated with DNase I, phenol:chloroform extracted, and the shRNA-*luc* was ethanol precipitated. Unincorporated nucleotides were removed by spin column chromatography and the yield of shRNA-*luc* was quantified by spectrophotometry.

Cell transfection and assay of shRNA-luc mediated RNAi activity

HeLa cells and Cos-7 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 100 µg/ml penicillin/ streptomycin (DMEM +10% FBS). Adherent cells were plated at 1×10^5 cells per well in 24-well plates and grown in DMEM + 10% FBS for 24 hours. Media were aspirated and replaced with 100 µl of serum-free DMEM. Cells were then transfected in triplicate by adding 2 µl of Oligofectamine™ Reagent (Invitrogen), 250 ng of pGL3-Control firefly luciferase (*luc*) expression vector (Promega), and 10 ng of pRL-SV40 *Renilla* (sea pansy) luciferase expression vector (Promega) as a control, together with 9.2 to 36.8 pmoles of shRNA-*luc*. Transfected cells were incubated for 24 hours, lysed, and assayed for both firefly and *Renilla* luciferase activities (Promega–Dual Luciferase® Reporter Assay System). The ratio of firefly luciferase to *Renilla* luciferase was normalized to replicates that were transfected with the firefly and *Renilla* luciferase expression vectors without shRNA.

Results

Typically, >2500 pmoles (>60 µg) of shRNA-*luc* were produced in a 30 minute AmpliScribe T7-*Flash* transcription reaction from 1 µg of the 87-bp DNA template. The effect of shRNA-*luc* on firefly luciferase expression in HeLa cells is presented in Figure 3A as a ratio of firefly luciferase activity to *Renilla* luciferase activity. As shown in Figure 3A, 92nM shRNA-*luc* (9.2 pmoles; ≈200 ng) suppressed expression of firefly luciferase by more than 80% without affecting expression of *Renilla* luciferase. Firefly luciferase expression was reduced by 90% by 276 nM shRNA-*luc* (27.6 pmoles; ≈600 ng). Similar results were seen using Cos-7 cells (Figure 3B).

Based on the gene silencing data of Figure 3A and 3B, a single AmpliScribe T7-*Flash* transcription reaction produced enough shRNA-*luc* for at least 100 RNAi experiments.

Discussion

Short, hairpin RNA (shRNA), prepared by *in vitro* transcription was effective in targeted gene silencing (RNAi) in HeLa and Cos-7 cells. The advantages of shRNA for RNAi studies, compared to dsRNA, include:

1. Lower cost than chemical synthesis of double-stranded RNA.
2. No need for DNA cloning of the transcription template.
3. Eliminates the need for post-transcriptional purification and annealing of sense and anti-sense RNA strands.
4. Easier design of DNA templates because shRNA is processed by the cell.[3,4]
5. No need to digest long double stranded RNA transcripts *in vitro* with RNase III or Dicer prior to transfection.

The AmpliScribe T7-*Flash* Transcription Kit produces high yields of shRNA in 30 minutes (also see the article on page 6). A single 20-µl AmpliScribe T7-*Flash* reaction produces enough shRNA for at least 100 RNAi experiments.

References

1. Fire, A. *et al.* (1998) *Nature* **391**, 806.
2. Svoboda, P. *et al.* (2001) *Biochem. Biophys. Res. Comm.* **12**(5), 287.
3. Paddison, P.J. *et. al.* (2002) *Genes & Development* **16**, 948.
4. McManus, M.T., *et. al.* (2002) *RNA* **8**, 842.

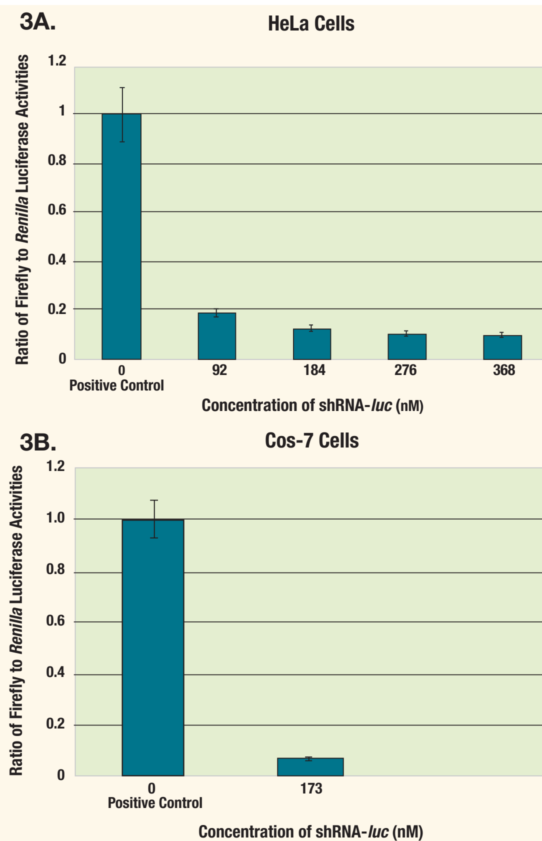


Figure 3. shRNA-*luc*, produced using the AmpliScribe™ T7-*Flash*™ Transcription Kit, effectively induced sequence-specific silencing of firefly luciferase in HeLa cells (3A) and in Cos-7 cells (3B). Transfections were performed in triplicate with firefly and *Renilla* (sea pansy) luciferase expression plasmids, as well as the indicated concentration of shRNA-*luc*, as described in the text.

www.epicentre.com/t7-flash.asp

AmpliScribe™ T7-*Flash*™ Transcription Kit

ASF3257	25 Reactions
ASF3507	50 Reactions

Contents:
AmpliScribe™ T7-*Flash*™ Enzyme Solution (with RNase inhibitor), AmpliScribe™ T7-*Flash*™ 10X Reaction Buffer, 100mM ATP, CTP, GTP, UTP Solutions, RNase-Free DNase I, DTT, RNase-Free Water, and Control DNA Template (linearized).

*EPICENTRE Technologies' products are licensed under U.S. and international patent rights owned by the Carnegie Institution of Washington that cover RNA interference. These products are accompanied by a limited non-exclusive worldwide license under the Carnegie Institution of Washington's patent rights for researchers at academic or other not-for-profit institutions to use the product for non-profit research. However, use of dsRNA for RNA interference by for-profit organizations requires a license from the Carnegie Institution of Washington. For-profit institutions should contact Gloria Brienza of the Carnegie Institution of Washington, 1530 P Street, N.W., Washington, D.C. 20005-1910.
E-mail: gbrienza@pst.ciw.edu.

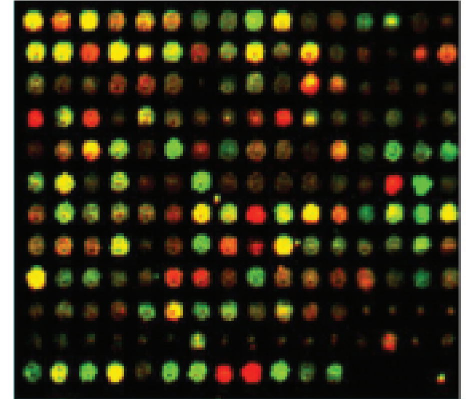
High Quality RNA for Use with Microarrays

A recent journal article[1] coauthored by scientists from the University of Washington, Affymetrix, and the Institute for Systems Biology, which is a nonprofit research institute founded by Dr. Leroy Hood, describes the use of an *E. coli*, whole-genome microarray to more completely identify all *E. coli* RNAs transcribed under different growth conditions. EPICENTRE's MasterPure™ Complete DNA and RNA Purification Kit was the method of choice for isolating total RNA for reverse transcription and cDNA synthesis.

A current challenge for genome scientists is the gap between the number of sequenced genomes and the number of completely annotated and published genomes. A major reason for the gap is the difficulty of annotating (or interpreting) raw sequence data. The bioinformatics algorithms that convert primary sequence data to predicted genes do not address untranslated regions (UTRs), including small RNAs that may have regulatory functions.

To avoid those limitations, these researchers developed a new approach to identify transcribed, intergenic regions in prokaryotes. High density oligon cleotide probe arrays (Affymetrix) covering the whole genome of *E. coli*, were hybridized with either fragmented, labeled genomic DNA or cDNA, which was prepared from RNA purified with the MasterPure Kit. The arrays assay both sense and antisense strands from intergenic regions, and sense strands from coding regions. Using this technique, they detected 4,052 coding transcripts and identified 1,102 additional transcripts from intergenic regions and 317 new transcripts of unknown function.

Thus, EPICENTRE's MasterPure™ Complete DNA and RNA Purification Kit is an effective tool in microarray technology.



Reference

1. B. Tjaden *et al.* (2002) *Nucl. Acids Res.* **30**, 3732.

For more information on MasterPure™ DNA and RNA Purification Kits, please see the center insert.

MasterPure™ Genomic DNA for *Bacillus anthracis* Analysis

A number of laboratories are working to develop quick, reliable, and sensitive assays for *Bacillus anthracis*, the causative agent of anthrax. Polymerase Chain Reaction (PCR) provides a good basis for rapid and specific analysis of genomic characteristics and is being utilized to detect and identify *Bacillus* species and strains. Performing PCR on a large number of clinical samples requires a fast, consistent method of genomic DNA purification. Here are two examples of laboratories using EPICENTRE's MasterPure™ DNA Purification Kit to prepare *Bacillus* DNA for their assays.

The international Laboratory Response Network (LRN) was established to prepare for, and respond to, potential bioterrorism threats. One of the first projects for this group was to develop rapid and highly specific assays to identify bioterrorism agents, including *B. anthracis*.[1] The LRN PCR assays for *B. anthracis* use real-time PCR and primer/probe sets against 3 genomic targets: pXO1, the 182-kb virulence plasmid; pXO2, the 96-kb virulence plasmid; and a region on the chromosome. For assay development, reaction template came from vegetative cells or from spores. The lab uses EPICENTRE's MasterPure DNA Purification Kit to purify the DNA from vegetative cells, after a lysozyme/lysostaphin pre-treatment. Spores were used directly in the assays.

In another approach to *Bacillus* identification, Radnedge et al.[2] used amplified fragment length polymorphism (AFLP) analysis, which provides a rapid method to measure phylogenetic distances. They effectively applied suppression subtractive hybridization (SSH) to develop and validate an AFLP analysis capable of generating highly specific DNA fingerprints of *Bacillus* species and strains. The lab purifies *Bacillus* genomic DNA from liquid, overnight cultures with EPICENTRE's MasterPure DNA Purification Kit.

References

1. Hoffmaster, A.R. *et al.* (2002) *Letters in Emerg. Infect. Dis.* **8**, 1178.
2. Radnedge, L. *et al.* (2003) *Appl. Environ. Microbiol.* **69**, 2755.

www.epicentre.com/purification.asp

MasterPure™ Complete DNA & RNA Purification Kit
(for isolating TNA, DNA, or RNA)

MC89010 10 Purifications
(10 TNA or 10 DNA or 5 RNA)
MC85200 200 Purifications
(200 TNA or 200 DNA or 100 RNA)

Contents:

Red Cell Lysis Solution, Tissue and Cell Lysis Solution, MPC Protein Precipitation Reagent, 2X T&C Lysis Solution, TE Buffer**, RNase A, RNase-Free DNase I, Proteinase K, and 1X DNase Buffer.

***Not provided with the 10 purification kit (cat. no. MC89010).*

MasterPure™ DNA Purification Kit (for isolating TNA or DNA)

MCD85201 200 Purifications

Contents:

Red Cell Lysis Solution, Tissue and Cell Lysis Solution, MPC Protein Precipitation Reagent, 2X T&C Lysis Solution, TE Buffer, RNase A, Proteinase K

MasterPure™ RNA Purification Kit (for isolating RNA only)

MCR85102 100 Purifications

Contents:

Red Cell Lysis Solution, Tissue and Cell Lysis Solution, MPC Protein Precipitation Reagent, 2X T&C Lysis Solution, TE Buffer, RNase-Free DNase I, Proteinase K, 1X DNase Buffer

DuraScript™ dsRNA Digested Using Human Dicer Enzyme Is As Effective and Specific in RNAi-Mediated Gene Silencing As Canonical "Diced" dsRNA

Judith E. Meis and Joanne Decker, EPICENTRE

Introduction

EPICENTRE's DuraScribe™ T7 Transcription Kit incorporates canonical GTP and ATP and 2′-fluorine-dCTP and 2′-fluorine-dUTP to yield modified RNA transcripts called "DuraScript™ RNA" (Figure 1) which are completely resistant to RNase A digestion.[1] DuraScript RNA has been shown to be stable for at least 2 hours in the presence of 10% fetal calf serum (Figure 2)[2] and short, DuraScript double-stranded RNA (dsRNA) has been used in RNAi studies to inhibit HIV replication.[3] Here we demonstrate that DuraScript dsRNA, following digestion with recombinant human Dicer enzyme to produce a mixed population of small DuraScript dsRNAs, is as effective and specific in RNAi-mediated gene silencing* as canonical dsRNA that was digested using the Dicer enzyme.

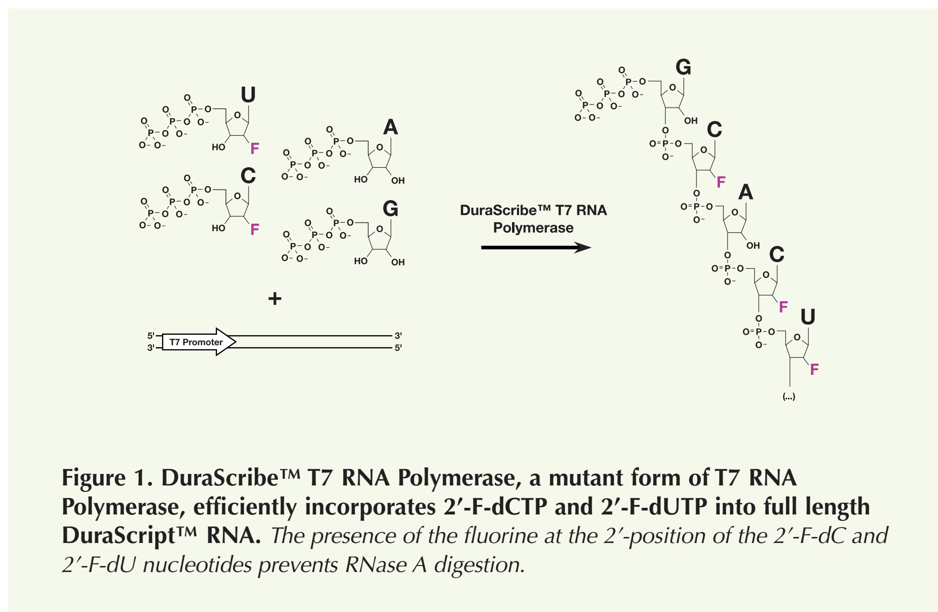


Figure 1. DuraScribe™ T7 RNA Polymerase, a mutant form of T7 RNA Polymerase, efficiently incorporates 2′-F-dCTP and 2′-F-dUTP into full length DuraScript™ RNA. *The presence of the fluorine at the 2′-position of the 2′-F-dC and 2′-F-dU nucleotides prevents RNase A digestion.*

Methods

Preparation of DuraScript dsRNA and canonical dsRNA

Sense and anti-sense RNA strands of a 1.6-kb region of the firefly luciferase gene or of an 800-bp region of human β-actin cDNA were each transcribed from 1 µg of the respective linear DNA templates. Canonical RNAs were produced using the AmpliScribe™ T7 High Yield Transcription Kit and DuraScript RNAs were produced using the DuraScribe T7 Transcription Kit, as described in the respective product's literature. Upon completion, the transcription reaction mixtures were treated with DNase I and the RNAs were purified by ammonium acetate precipitation as described in the product literature.

Complementary RNA strands were resuspended in TNE (10 mM Tris-HCl, pH 7.5; 100 mM NaCl; 1 mM EDTA), denatured for 2 minutes at 92°C, and then annealed by incubating at 75°C for 15 minutes, at 37°C for 30 minutes, and finally, at room temperature for 30 minutes. The resulting dsRNA produced was extracted with phenol/chloroform and ethanol precipitated. Digestion with *E. coli* RNase III, an enzyme known to specifically digest dsRNA, was used to verify the double-stranded nature of the RNAs produced.

Digestion of DuraScript dsRNA and canonical dsRNA using recombinant human Dicer enzyme

Thirty micrograms of each dsRNA were digested overnight at 37°C with recombinant human Dicer enzyme (Gene Therapy Systems, Inc.) using 1 Unit of Dicer enzyme per µg of dsRNA, according to the manufacturer's protocol. The "Diced" dsRNAs were purified by column chromatography and their size and purity were assayed on a non-denaturing, 12% polyacrylamide gel.

The short DuraScript dsRNAs produced by Dicer digestion of DuraScript firefly luciferase dsRNA and of DuraScript β-actin dsRNA were designated "DS-*luc*-RNAi" and "DS-actin-RNAi" respectively. The short canonical dsRNAs produced by Dicer digestion of canonical firefly luciferase dsRNA and of canonical β-actin dsRNA were designated "*luc*-RNAi" and "actin-RNAi" respectively.

Cell transfection and luciferase assay

HeLa cells and Cos-7 cells were plated at 5×10^4 cells per well in 24-well plates and grown for 24 hours in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 100 µg/ml penicillin/streptomycin before transfection. Cells were transfected by lipofection using 2 µl of Oligofectamine™ Reagent (Invitrogen), 250 ng of firefly luciferase expression vector (Promega), and 10 ng of *Renilla* (sea pansy) luciferase expression vector (Promega), together with either DS-*luc*-RNAi, DS-actin-RNAi, *luc*-RNAi or actin-RNAi.

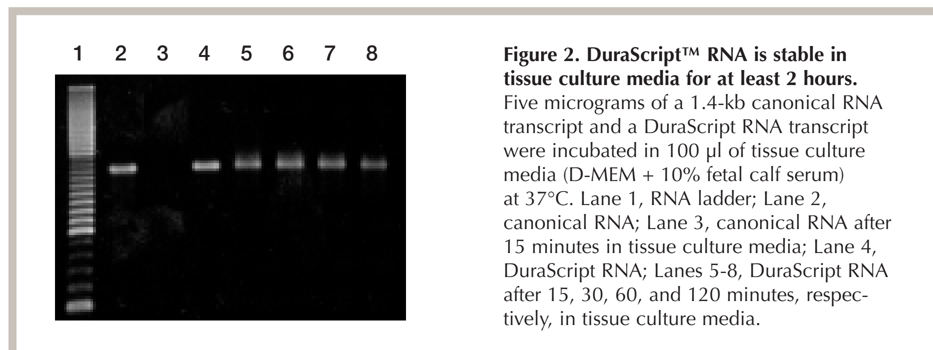


Figure 2. DuraScript™ RNA is stable in tissue culture media for at least 2 hours. Five micrograms of a 1.4-kb canonical RNA transcript and a DuraScript RNA transcript were incubated in 100 µl of tissue culture media (D-MEM + 10% fetal calf serum) at 37°C. Lane 1, RNA ladder; Lane 2, canonical RNA; Lane 3, canonical RNA after 15 minutes in tissue culture media; Lane 4, DuraScript RNA; Lanes 5-8, DuraScript RNA after 15, 30, 60, and 120 minutes, respectively, in tissue culture media.

Transfected cells were incubated for 24 hours, lysed, and assayed for both firefly and *Renilla* luciferase activities. The *Renilla* luciferase activity should not be silenced by the firefly luciferase RNAi. The DS-actin-RNAi and actin-RNAi were used to control for non-luciferase-specific RNA transfection effects.

Results

The 20-µl DuraScribe™ T7 transcription reactions produced approximately 50 µg of RNA from 1 µg of the firefly luciferase or β-actin DNA templates. The DuraScript dsRNAs were readily produced and, as shown in Figure 3, were digested with recombinant human Dicer enzyme as efficiently as canonical dsRNA.

The effects of "Diced" RNA transcripts on firefly luciferase expression are presented in Figures 4A and 4B as ratios of firefly luciferase activity to *Renilla* luciferase activity. The data shows that DS-*luc*-RNAi suppresses the expression of firefly luciferase as effectively and at the same concentration as the canonical *luc*-RNAi in both HeLa cells and Cos-7 cells. In addition, Figure 4B shows that DS-actin-RNAi, did not alter the expression of firefly luciferase, demonstrating that gene silencing by "Diced" DuraScript dsRNA is target specific.

Discussion

In this and in previous reports,[1,2] we have demonstrated several important benefits of using DuraScript RNA, produced using the DuraScribe T7 Transcription Kit, in RNAi-mediated gene silencing. These include:

1. DuraScript RNA is completely resistant to RNase A and the nucleases found on human hands permitting production and use without the need for gloves, RNase inhibitors, and DEPC-treatments.

2. A 20-µl DuraScribe T7 transcription reaction produces high yields of DuraScript RNA using DNA templates containing a standard T7 promoter.

3. DuraScript RNA is stable for at least 2 hours in the presence of cell culture media containing 10% FBS.

4. DuraScript dsRNA, digested using human Dicer enzyme, is as effective and as specific in RNAi-mediated gene silencing as canonical "Diced" dsRNA.

References

1. Meis, J.E. and Chen, F. (2002) *EPICENTRE Forum* **9**(1), 10.
2. *EPICENTRE Forum* (2003) **10**(1), 5.
3. Capodici, J. *et al.* (2002) *J. Immunol.* **169**, 5196.

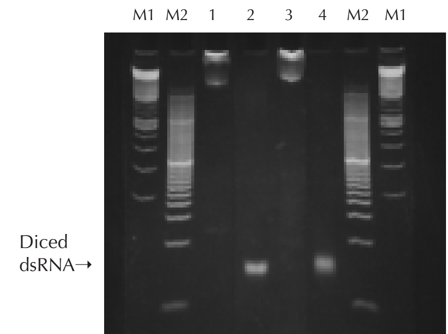
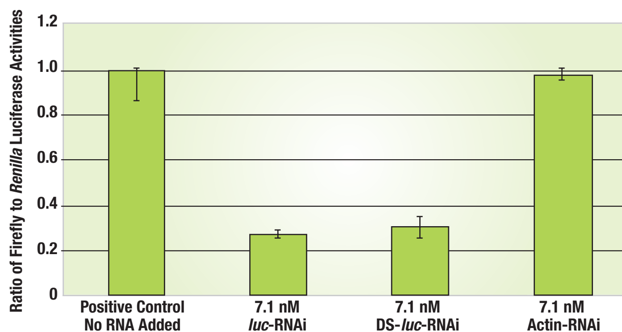


Figure 3. DuraScript™ dsRNA is digested by recombinant human Dicer enzyme as efficiently as canonical dsRNA. Lanes M1, 100-bp ladder; Lanes M2, 20-bp ladder; Lane1, 800-bp canonical β-actin dsRNA; Lane 2, canonical β-actin dsRNA digested using Dicer and purified on a column; Lane 3, 800-bp DuraScript β-actin dsRNA; Lane 4, DuraScript β-actin dsRNA digested using Dicer and purified on a column.



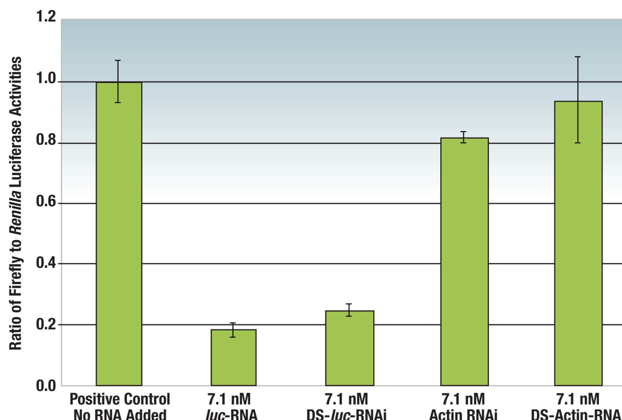


Figure 4. "Diced" DuraScript™ double-stranded RNA silences gene expression in HeLa cells (Figure 4A) and Cos-7 cells (Figure 4B) as effectively and as specifically as "Diced" canonical double-stranded RNA. Transfections were performed in triplicate with firefly and *Renilla* luciferase expression vectors and the indicated amounts of *luc*-RNAi, or DS-*luc*-RNAi. The ratios of firefly luciferase activity to *Renilla* luciferase activity are shown normalized to replicates that were transfected with the reporter expression vectors alone (positive control).

www.epicentre.com/durascribe.asp

DuraScribe™ T7 Transcription Kit

DS010910	10 Reactions
DS010925	25 Reactions

Contents:

DuraScribe™ T7 Enzyme Solution (with RNase inhibitor), DuraScribe™ T7 10X Reaction Buffer, 2'-F-dCTP, 2'-F-dUTP, ATP, GTP Solutions, DNase I, DTT, Water, and Control DNA Template (linearized).

RNase III, *E. coli*

RN02950	50 Units

Supplied at 1 U/µl.

*EPICENTRE Technologies' products are licensed under U.S. and international patent rights owned by the Carnegie Institution of Washington that cover RNA interference. These products are accompanied by a limited non-exclusive worldwide license under the Carnegie Institution of Washington's patent rights for researchers at academic or other not-for-profit institutions to use the product for non-profit research. However, use of dsRNA for RNA interference by for-profit organizations requires a license from the Carnegie Institution of Washington. For-profit institutions should contact Gloria Brienza of the Carnegie Institution of Washington, 1530 P Street, N.W., Washington, D.C. 20005-1910. E-mail: gbrienza@pst.ciw.edu.

Transcribe More RNA in 30 Minutes Than Other *In Vitro* Transcription Kits Produce in 2 Hours

Using the AmpliScribe™ T7-Flash™ Transcription Kit

Ronald Meis and Jim Pease, EPICENTRE

Introduction

The ability to transcribe large amounts of RNA, *in vitro*, is critical for the success of many RNA-dependent techniques, including gene expression, RNA processing, ribozymes, *in situ* hybridization, and microinjection studies. The AmpliScribe™ T7-*Flash*™ Transcription Kit, EPICENTRE's new T7 *in vitro* transcription kit, is specially formulated to produce the highest yield of RNA from an *in vitro* transcription reaction in the shortest amount of time. Here we report on the different parameters of an AmpliScribe T7-*Flash* reaction that affect the yield of both long and short RNA transcripts.

Methods

AmpliScribe T7-*Flash* Transcription reactions were performed as described in the product literature. Briefly, the standard 20-µl reaction contained 1 µg of linear, double-stranded DNA template and was performed at 37ºC for 30 minutes, unless otherwise indicated. After the reaction, transcripts larger than 1 kb were purified by ammonium acetate precipitation. Ammonium acetate selectively precipitates the large RNA while leaving the DNA and unincorporated nucleotides in solution. The purified RNA was resuspended in RNase-Free Water. For transcripts less than 1 kb in size, the completed reaction was digested with DNase I and the RNA was purified by column chromatography. RNA yield was quantified by absorbance at 260 nm. The integrity of the RNA was checked by electrophoresis on a denaturing, 1% agarose-formaldehyde gel.

Results

An AmpliScribe T7-Flash reaction is complete in 30 minutes and produces more RNA than other kits do in 2 hours

The AmpliScribe T7-*Flash* Transcription Kit has been formulated to transcribe large amounts of RNA in 30 minutes. In comparison, other commercial, high yield T7 *in vitro* transcription kits require at least 2 hours to maximize RNA production. As shown in Figure 1, the AmpliScribe T7-*Flash* reaction was complete in 30 minutes and produced about 3-fold more of a 1.4-kb RNA transcript than the competitive kit produced in the same amount of time, from the same template. Even after 2 hours, when the competitive kit's reaction was finally complete it had produced less RNA than the AmpliScribe T7-*Flash* reaction had in 30 minutes.

The reaction kinetics for transcribing short (<1 kb) RNAs are discussed below.

In a related series of experiments, the AmpliScribe T7-*Flash* reaction time was reduced to as little as 15 minutes by increasing the amount of DNA template in the reaction (data not shown).

AmpliScribe T7-Flash reactions produce exceptionally high yields of RNA

AmpliScribe T7-*Flash* reactions consistently produced 160 to 180 µg (8-9 mg/ml) of a 1.4-kb RNA from 1 µg of the control DNA template included with the kit. Yields of up to 200 µg of RNA were produced from 1 µg of other DNA templates that were tested (Table 1). The RNA produced was intact and full-length (Figure 2). AmpliScribe T7-*Flash* reactions can also be scaled-up to produce milligram amounts of RNA (data not shown).

RNA yield from limiting amounts of DNA template

For some *in vitro* transcription experiments, the amount of DNA template may be limiting. Therefore, we investigated the yield of RNA from AmpliScribe T7-*Flash* reactions containing less than 1 µg of the control DNA template (Figure 3). High yields of RNA were obtained in 30 minutes from as little as 100 ng of template. Increasing the reaction time or increasing the reaction temperature from 37°C to 42ºC improved the yield of RNA from reactions containing less than 1 µg

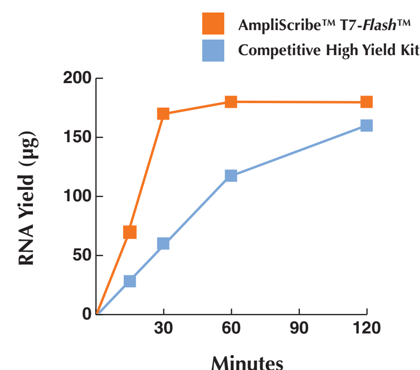
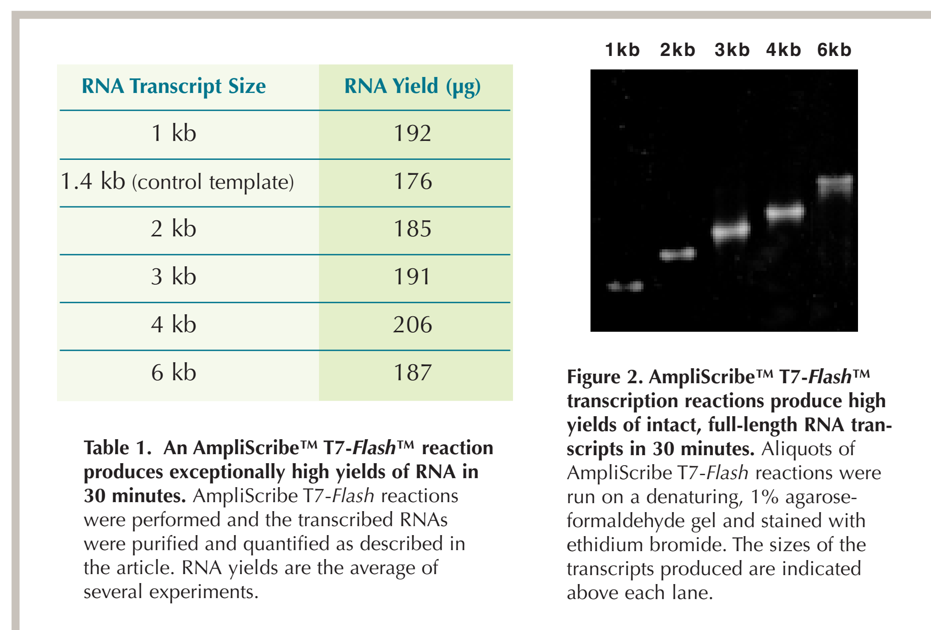


Figure 1. An AmpliScribe™ T7-*Flash*™ transcription reaction is complete in 30 minutes and produces more RNA than a competitive, high yield transcription reaction produces in 2 hours. In a comparison, 1 µg of a linear DNA template, producing a 1.4-kb RNA transcript, was transcribed in an AmpliScribe T7-*Flash* reaction and in an *in vitro* transcription reaction from a competitive kit. RNA samples were purified and quantified as described in the article.



RNA Transcript Size	RNA Yield (µg)
1 kb	192
1.4 kb (control template)	176
2 kb	185
3 kb	191
4 kb	206
6 kb	187

Table 1. An AmpliScribe™ T7-*Flash*™ reaction produces exceptionally high yields of RNA in 30 minutes. AmpliScribe T7-*Flash* reactions were performed and the transcribed RNAs were purified and quantified as described in the article. RNA yields are the average of several experiments.

Figure 2. AmpliScribe™ T7-*Flash*™ transcription reactions produce high yields of intact, full-length RNA transcripts in 30 minutes. Aliquots of AmpliScribe T7-*Flash* reactions were run on a denaturing, 1% agarose-formaldehyde gel and stained with ethidium bromide. The sizes of the transcripts produced are indicated above each lane.

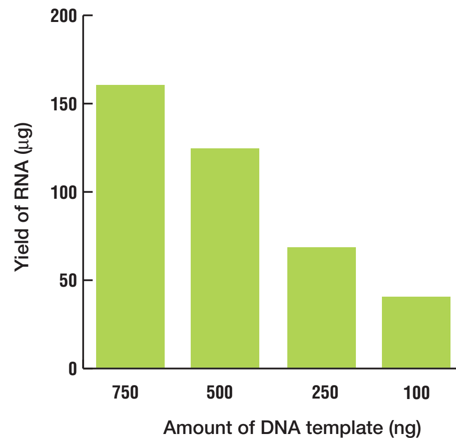


Figure 3. High yields of RNA can be obtained from limiting amounts of DNA template in an AmpliScribe™ T7-*Flash*™ transcription reaction. Decreasing amounts of a linear DNA template, producing a 1.4-kb RNA transcript, were transcribed for 30 minutes in AmpliScribe T7-*Flash* reactions. RNA samples were purified and quantified as described in the article. RNA yield from limiting amounts of template can be increased by increasing the reaction time or increasing the reaction temperature to 42ºC.

of DNA template. For example, increasing the reaction time to 2 hours produces the maximum yield (>160 µg) of RNA from 250 ng of template.

Transcription of short RNA transcripts using the AmpliScribe T7-Flash Transcription Kit

Efficient transcription of short RNAs (e.g. short hairpin RNA; see page 1) is important for many RNA interference (RNAi) studies. A variety of templates producing RNA transcripts from 26 to 335 bases were transcribed using the AmpliScribe T7-*Flash* Kit and another commercial, high yield transcription kit. In all cases tested, the AmpliScribe T7-*Flash* reaction produced more RNA than the other transcription kit. Though the microgram amounts of short transcripts produced seems low, the molar amount of short RNA produced was greater than the molar amount of long (>1 kb) transcripts generated (Table 2). Since many RNAi studies utilize on the order of 10 pmoles of RNA, a single AmpliScribe T7-*Flash* reaction produces enough short RNA for more than 100 RNAi experiments. The yield of short RNAs can be increased by:

Increasing reaction times.

Increasing the amount of DNA template.

Increasing the reaction temperature to 42ºC.

For example, when transcribing a 26-bp template, maximum yields are obtained after a 4-hour incubation at 37ºC. The reaction time can be reduced to 2 hours if done at 42ºC, or in as little as 1 hour if done at 42ºC using 3 µg of template.

Table 2. AmpliScribe™ T7-*Flash*™ reactions produce high yields of short RNA transcripts in a 30-minute reaction. Extending the reaction time, increasing the amount of template in the reaction, or increasing the reaction temperature to 42ºC will increase yields of short RNA transcripts.

RNA Transcript Size	RNA Yield (µg)	RNA Yield (pmoles)
26 bases	12	1319
47 bases	24	1459
96 bases	36	1071
335 bases	76	648
1.4 kb	176	359
6 kb	187	89

Conclusion

The AmpliScribe T7-*Flash* Transcription Kit is ideal for *in vitro* transcription of RNA because it provides the highest yields of RNA in the shortest reaction time. Transcription of long RNA transcripts was complete in 30 minutes compared to the 2-hour reaction required for other transcription systems. RNA yields as high as 200 µg were observed with some templates. Short RNA transcripts are also efficiently produced by the AmpliScribe T7-*Flash* Transcription Kit. A single 20-µl reaction produces enough picomoles of short RNA for at least 100 RNAi experiments (see article on page 1).

www.epicentre.com/t7-flash.asp

AmpliScribe™ T7-*Flash*™ Transcription Kit

ASF3257	25 Reactions
ASF3507	50 Reactions

Contents:

AmpliScribe™ T7-*Flash*™ Enzyme Solution (with RNase inhibitor), AmpliScribe™ T7-*Flash*™ 10X Reaction Buffer, 100mM ATP, CTP, GTP, UTP Solutions, RNase-Free DNase I, DTT, RNase-Free Water, and Control DNA Template (linearized).

T7 In Vitro Transcription In A FLASH!

New! AmpliScribe™ T7-*Flash*™ Transcription Kit

- 30 Minute T7 *In Vitro* Transcription Reactions
- Highest yields of RNA

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.
- 10^4 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 cm section of a mouse tail snip.
- One *E. coli* colony picked from a plate.
- A 0.5-1 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).

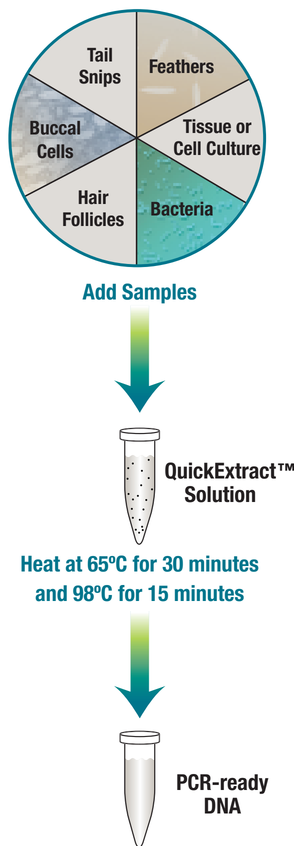


Figure 1. Procedure for obtaining PCR-ready DNA using the QuickExtract™ DNA Extraction Solution.

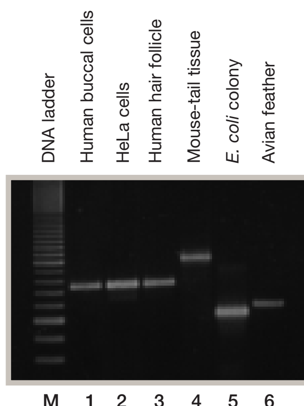


Figure 2. Genomic DNA extracted from a variety of tissues or cells using the QuickExtract™ DNA Extraction Solution were consistently amplified using the FailSafe™ PCR System. Lanes 1-3, human β-globin; Lane 4, mouse GAPDH; Lane 5, *E. coli* 16s ribosomal RNA gene; Lane 6, avian viral sequence.

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.

Catch-All™ Sample Collection Swabs

QEC091H 100 Swabs
100 individually-packaged swabs in sterile hard-pack plastic cylinders.

FailSafe™ PCR System

See the center insert for product and ordering information.

10 Reasons Why DNA Cloning Kits from EPICENTRE Enable the Fastest and Most Reliable Results

EPICENTRE's highly specialized kits enable the fastest and most efficient procedures for DNA cloning, allowing you to move forward quickly and reliably with your research.

Fast-Link™ DNA Ligation Kit

Lab Tested…Scientist Approved. Visit ***www.biowire.com*** *and search for "Fast-Link" to view users' comments.*

1. Get cohesive-end DNA ligations in 5 minutes at room temperature.
2. Ligate blunt-ended DNA in as little as 5 minutes at room temperature.
3. Perform T/A ligations in 1 hour.
4. No need to desalt the reaction mixture prior to cell transformation.

TransforMax™ EC100™ Competent Cells

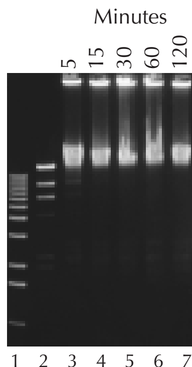
The best value in competent cells. Compare our prices and efficiencies.

5. Transformation efficiency >10^{10} cfu/µg DNA with electrocompetent cells.
6. Readily take up large DNA clones for construction of cDNA and BAC libraries.
7. Protect cells and valuable clones from destruction with Phage T1-Resistant *E. coli*.

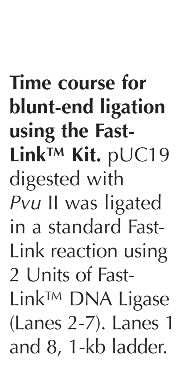
Colony Fast-Screen™ Kits

Two sensitive and easy-to-use kits for high throughput or routine screening of clones and libraries.

8. Rapidly screen the size of cloned PCR inserts and cDNA in 1 hour and BAC clones in 4 hours. Prepare PCR-ready DNA directly from clones in less than 10 minutes.
9. Use with any plasmid cloning vector and all *E. coli* host strains.
10. No need to grow cultures, isolate DNA or perform endonuclease digestions.



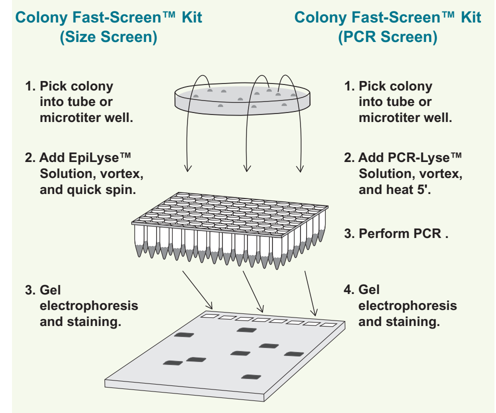
Time course for cohesive-end ligation using the Fast-Link™ Kit. Lambda *Hind* III markers were ligated in a standard Fast-Link reaction using 2 Units of Fast-Link™ DNA Ligase (Lanes 3-7). Lane 1, 1-kb ladder; Lane 2, no enzyme.



Time course for blunt-end ligation using the Fast-Link™ Kit. pUC19 digested with *Pvu* II was ligated in a standard Fast-Link reaction using 2 Units of Fast-Link™ DNA Ligase (Lanes 2-7). Lanes 1 and 8, 1-kb ladder.

DNA	TransforMax™ EC100™ Electrocompetent *E. coli*
pUC19	2.7 X 10^{10}
13.1-kb Clone	1.3 X 10^9
23.1-kb Clone	3.0 X 10^8
153-kb BAC Clone	7 X 10^7
13.1-kb clone directly from a ligation reaction	2.1 X 10^7

Comparison of the transformation efficiency of TransforMax™ EC100™ Electrocompetent *E. coli* with a variety of DNAs. Results shown are in cfu/µg of DNA and are the average transformation efficiencies obtained from several trials.



www.epicentre.com/fastlink.asp

Fast-Link™ DNA Ligation Kit

LK11025 — 25 Ligations
LK0750H — 50 Ligations
LK6201H — 100 Ligations

Contents:

Fast-Link™ DNA Ligase, Fast-Link™ 10X Ligation Buffer, and 10 mM ATP.

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.

www.epicentre.com/ec100t1r.asp

TransforMax™ EC100™-T1^R Chemically Competent *E. coli*

CCT10210 — 10 X 50 µl
Includes pUC19 control DNA.

TransforMax™ EC100™-T1^R Electrocompetent *E. coli*

EC0205T1 — 5 X 100 µl
EC0210T1 — 10 X 100 µl
Includes pUC19 control DNA.

www.epicentre.com/fastscreen.asp

Colony Fast-Screen™ Kit (Size Screen)

Rapidly screen the size of cloned inserts.
FS08250 — 1 Kit
Sufficient reagents to screen 250 colonies.

Contents:

EpiLyse™ Solution and EpiBlue™ Solution.

Colony Fast-Screen™ Kit (PCR Screen)

Rapidly prepare clones for screening by PCR.
FS0322H — 1 Kit
Sufficient reagents to screen 200 colonies. Thermostable polymerase and PCR primers are not included.

Contents:

PCRLyse™ Solution and Gel Loading Solution.

Efficient Cloning of Entire Mitochondrial Genomes in *Escherichia coli* by *In Vitro* Insertion of a Transposon

Young Geol Yoon and Michael D. Koob
Institute of Human Genetics, University of Minnesota, Minneapolis, MN

Introduction

The typical animal cell contains hundreds of mitochondria that produce the cell's ATP through oxidative phosphorylation and regulate multiple cellular processes. Even though the majority of genes needed for the biogenesis, maintenance, and regulation of this organelle are encoded in the nucleus, the mitochondrial genome is critical for normal cellular energy metabolism, and mutations in this DNA molecule are known to cause a wide range of human diseases.[1] Generally, mammalian mitochondrial genomes consist of circular double-stranded DNA (~16-17 kb) that encodes 13 polypeptide subunits of the mitochondrial ATP-generating pathway, two rRNAs, and 22 tRNAs. We have devised an efficient method for replicating and maintaining entire mitochondrial genomes in *E. coli* and demonstrate the effectiveness of this procedure by cloning individual mouse mitochondrial genomes isolated from mouse liver. The same approach has been used to clone or "rescue" plasmid DNA that would not otherwise replicate in *E. coli*.[2,3]

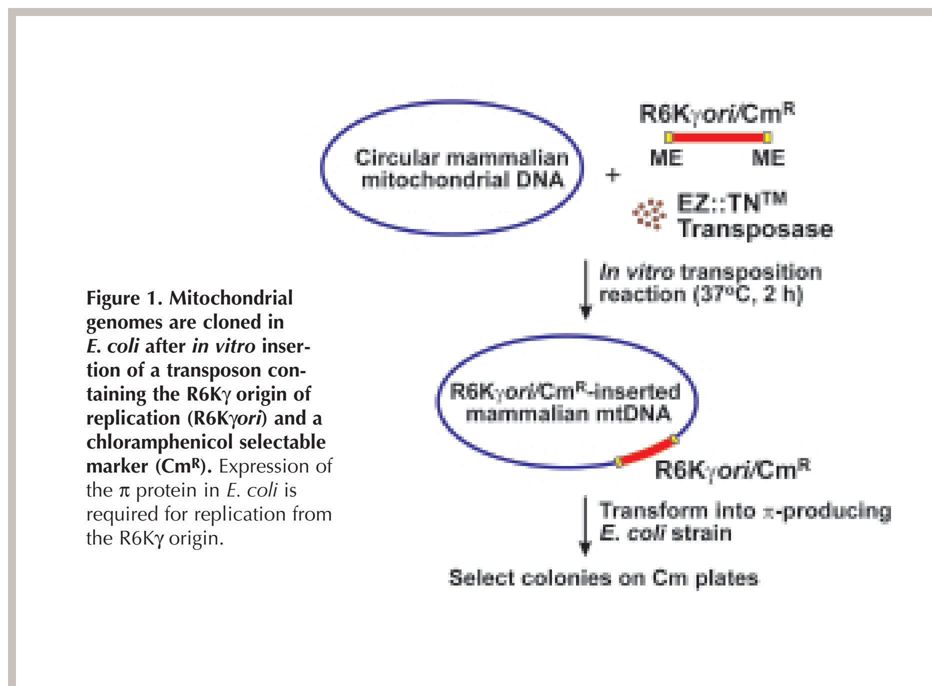


Figure 1. Mitochondrial genomes are cloned in *E. coli* after *in vitro* insertion of a transposon containing the R6Kγ origin of replication (R6Kγ*ori*) and a chloramphenicol selectable marker (Cm^R). Expression of the π protein in *E. coli* is required for replication from the R6Kγ origin.

Materials and Methods

The strategy for the EZ::TN™ transposon-mediated cloning of mitochondrial genomes is shown in Figure 1. A transposon, in which the R6Kγ origin of replication (R6Kγ*ori*) and a chloramphenicol resistant gene (Cm^R) are flanked by mosaic end (ME) sequences, was generated by a PCR reaction using a R6Kγ*ori*/Cm^R-carrying plasmid as template and the primers MESalCmR (5′-*CTG TCT CTT ATA CAC ATC T*GT CGA CAG AAG CCA CTG GAG CA-3′; ME sequence italicized, *Sal* I restriction site underlined) and MESmaγori (5′-*CTG TCT CTT ATA CAC ATC T*CC CGG GCT AAT TCT GTC AGC CGT T-3′; *Sma* I restriction site underlined). The *in vitro* transposon insertion reaction consisted of 1 Unit of EZ::TN™ Transposase (EPICENTRE), 200 ng of mouse mitochondrial DNA (mt DNA), and 10 ng of PCR-amplified transposon in the buffer provided by the supplier. After incubating the reaction mixture for 2 hours at 37ºC, the reaction was terminated as described in the product literature. A 1-µl aliquot of the transposon insertion reaction was used for electroporation of an *E. coli* strain containing the wild-type *pir* gene (*pir*+).[4] Transformants were selected on LB plates containing chloramphenicol (12.5 µg/ml).

Results and Discussion

Cloning the mouse mitochondrial genome in E. coli

For cloning complete individual mitochondrial genomes in *E. coli*, we devised a scheme that uses an *in vitro* transposition reaction[5] to insert an *E. coli* origin of DNA replication and selectable marker at random locations into circular mitochondrial DNA (Figure 1). The 1.5-kb PCR-amplified transposon consisted of a Cm^R marker and a R6Kγ*ori* flanked by the 19-bp inverted ME sequences that are specifically recognized by EZ::TN Transposase. To perform the *in vitro* transposition reaction, this linear transposon was incubated with EZ::TN Transposase and purified, circular mouse mtDNA. The products from this *in vitro* transposition reaction were electroporated into an *E. coli* strain containing a chromosomal copy of the R6Kγ *pir* gene and transformants were selected on chloramphenicol plates. The *pir* gene encodes the replication-initiator protein π needed for R6Kγ*ori* replication.

We characterized three transposon-inserted mouse mitochondrial genome clones obtained from this cloning strategy by restriction enzyme mapping and sequencing (Figure 2). This cloning procedure takes advantage of the fact that the mitochondrial genome is the only circular DNA in most eukaryotic cells and that DNA must be circular in order to replicate in *E. coli*. Since the transposition reaction does not circularize linear DNA, the genomic DNA fragments and broken mitochondrial genome fragments that invariably contaminate mitochondrial genome preparations are not cloned by this procedure and so the background was very low. The replication *ori* and selectable marker were also inserted at random locations and orientations throughout the mitochondrial genome and so many different clones were generated in the same experiment. All of the transposition reactions that generated the mouse mitochondrial genome clones described here inserted the transposon in the same orientation with respect to the mitochondrial genome and this probably reflects an inherent increased stability for this orientation versus the other. The transposon cloning approach we describe here can be used to clone completely

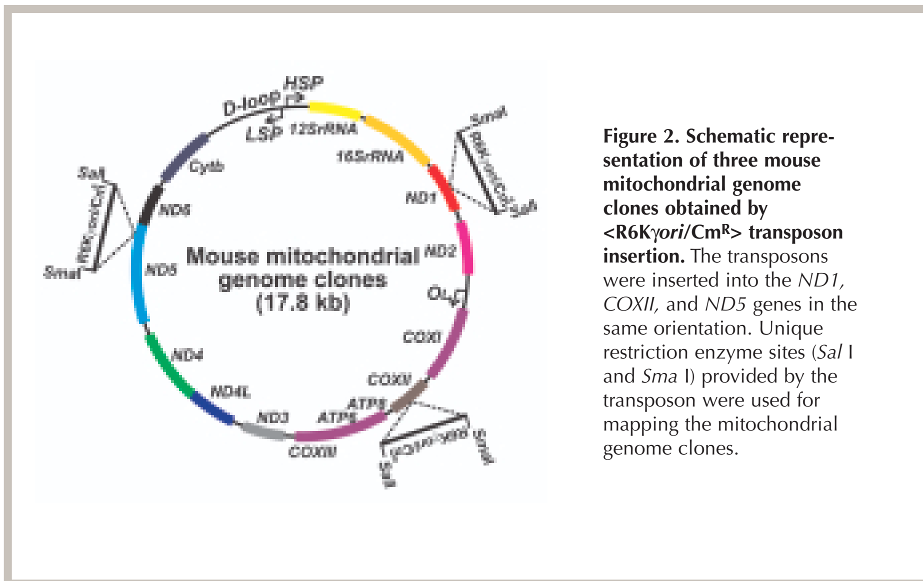


Figure 2. Schematic representation of three mouse mitochondrial genome clones obtained by <R6Kγ*ori*/Cm^R> transposon insertion. The transposons were inserted into the *ND1, COXII,* and *ND5* genes in the same orientation. Unique restriction enzyme sites (*Sal* I and *Sma* I) provided by the transposon were used for mapping the mitochondrial genome clones.

uncharacterized, circular mitochondrial genomes with no prior knowledge of either the sequence content or restriction pattern of that mitochondrial genome.

Comparison of the stability of mouse mitochondrial genome clones in E. coli at low- and high-copy number

To examine the stability of the cloned mouse mtDNA in *E. coli*, the three mapped mouse mtDNA clones were transformed into one of two *E. coli* strains. One of these strains contained the wild type *pir* gene (*pir*$^+$) in the chromosome and replicated the mtDNA clones at a moderately low number of copies/cell (10-15 copies), and the other strain contained a mutant *pir* gene (*pir*-116) that replicated the clones at a relatively high number of copies/cell (~200 copies).[4] As shown in Figure 3, lanes 2-4, no deletions or rearrangements were observed in the mouse mtDNA fragments when we used the *pir*$^+$ strain as host. When the mouse mtDNA clones were transformed into the *pir*-116 strain, however, the transformation efficiency was dramatically lower than that seen with the *pir*$^+$ strain, whereas a control plasmid without mitochondrial sequences was transformed into these two strains with equal efficiency (data not shown). Restriction analysis of the mtDNA clones from the *pir*-116 strain demonstrated that all or most of the mouse mitochondrial genome sequence was deleted (Figure 3, lanes 5-11).

The clone instability we observed in high-copy clones was probably due to the inhibition of *E. coli* cell metabolism by one or more of the 22 heterologous tRNA genes in the mouse mitochondrial genome. We identified multiple regions of the mouse mitochondrial genome that serve as transcription promoters in *E. coli* and found that the level of transcription from these sequences was dramatically higher at high-copy numbers than at low-copy numbers.[6] By using a *pir*$^+$ strain to keep the clone copy number low, however, the level of expression of the tRNA genes or other potential mtDNA inhibitory sequences was minimized and the overall mitochondrial clone stability was increased.

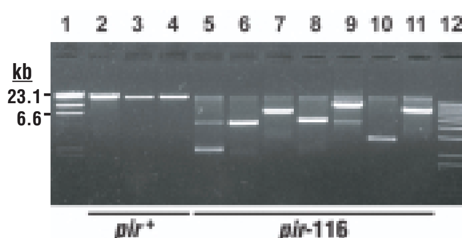


Figure 3. Effect of plasmid copy number on the stability of the cloned mouse mitochondrial genome. Restriction patterns after *Sal* I digestion of each plasmid DNA were compared on a 1% agarose gel. In the low-copy number *E.coli* strain (*pir*$^+$), the mitochondrial genome clones were stably maintained (lanes 2-4). Sequence analysis further confirmed the overall integrity of the clones. In the high-copy number *E.coli* strain (*pir*-116), all or most of the mouse mitochondrial genome sequence was deleted (lanes 5-11). Lane 1, λ/*Hind* III size marker; lane12, 1-kb plus DNA ladder.

Conclusion

We have cloned the mouse mitochondrial genome in *E. coli* by inserting an *E. coli* replication origin and selectable marker into mitochondrial DNA using an *in vitro* transposition reaction and then transforming the modified genomes into *E. coli*. The entire mouse mitochondrial genome was very stably maintained when its copy number was kept low (*pir*$^+$ strain), but was extremely unstable when it was replicated at high-copy number (*pir*-116 strain). This convenient approach should offer a number of advantages for the cloning of other mammalian mitochondrial genomes in *E. coli*.

References

1. Wallace, D.C. (1999) *Science* **283**, 1482.
2. Jendrisak, J. (2002) *EPICENTRE Forum* **9**(1), 14.
3. Kirby, C. *et al.* (2002) *Mol. Microbiol.* **43**, 173.
4. Greener, A. *et al.* (1990) *Mol. Gen. Genet.* **224**, 24.
5. Goryshin, I.Y. *et al.* (2000) *Nat. Biotechnol.* **18**, 97.
6. Yoon, Y.G. and Koob, M.D. (2003) *Nucl. Acids Res.* **31**, 1407.

Use an EZ::TN™ Insertion Kit with an EZ::TN™ Transposon containing an R6Kγori and kanamycin selectable marker to clone complete mitochondrial genomes.

www.epicentre.com/transposomics.asp

EZ::TN™ <R6Kγ*ori*/KAN-2> Insertion Kit

EZI011RK 10 Reactions

Contents:

EZ::TN™ <R6Kγ*ori*/KAN-2> Transposon, EZ::TN™ Transposase, 10X Reaction Buffer, 10X Stop Solution, Forward and Reverse Primers, Control Target DNA, and Sterile Water.

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

ECP09500 5 X 100 µl

Maintains clones at 15 copies per cell. Includes control vector containing an R6Kγ*ori*.

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

EC6P095H 5 X 100 µl

Maintains clones at 250 copies per cell. Includes control vector containing an R6Kγ*ori*.

Make a custom R6Kγori-containing EZ::TN™ Transposon with the EZ::TN™ pMOD-3 Vector.

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

MOD1503 20 µg

Includes Vector and Forward and Reverse PCR Primers.

EZ::TN™ Transposase

TNP92110 10 Units

CopyControl™ Cloning Systems Allow Single-Copy Cloning, Then Controlled Induction for High-Copy DNA Production

Featuring the Laboratories of Chris Amemiya and Eric Lafontaine

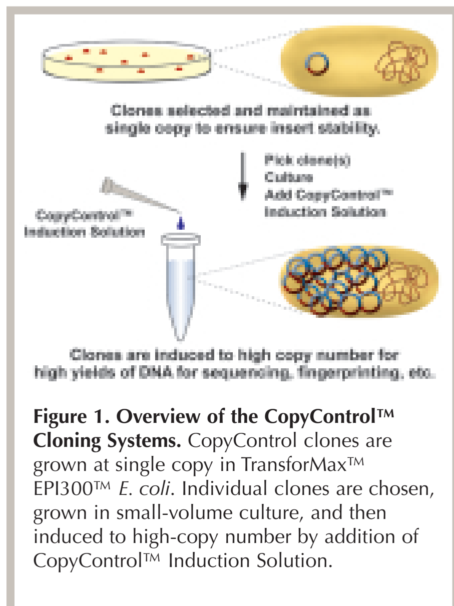


Figure 1. Overview of the CopyControl™ Cloning Systems. CopyControl clones are grown at single copy in TransforMax™ EPI300™ *E. coli*. Individual clones are chosen, grown in small-volume culture, and then induced to high-copy number by addition of CopyControl™ Induction Solution.

EPICENTRE's CopyControl™ Cloning Systems are based on a cloning technology originally developed in the laboratory of Dr. Waclaw Szybalski[1] and optimized at EPICENTRE. CopyControl Systems allow the user to maintain clones in the bacterial host at single copy, which ensures clone stability and enables cloning of toxic gene products. Then, whenever higher DNA yield and purity are required, the unique CopyControl vectors can be induced to high copy. Induction results in 10 to 200 vector copies per cell, depending upon the insert size. For an overview of the CopyControl Systems, please see http://www.epicentre.com/cc_tutorial.asp.

CopyControl vectors contain a single-copy *E. coli* F-factor replicon and a high-copy *oriV* replicon. Initiation of replication from the *oriV* requires the *trf*A gene product. Neither the CopyControl vectors, nor commonly used lab strains of *E. coli*, contain the *trf*A gene. CopyControl Systems use a specifically engineered *E. coli* host strain, TransforMax™ EPI300™, which contains a mutant *trf*A gene under tight control of an inducible promoter. When the inducer is not present, replication proceeds from the F-factor replicon, which maintains the vector at one copy per cell. Addition of the inducer to the growth medium induces expression of *trf*A protein and replication is then controlled by the *oriV* replicon, which produces 10 to 200 vector copies per cell. CopyControl Systems are available in three cloning formats:

1. CopyControl™ BAC Cloning Kits, for large inserts, typically 100 to 300 kb.
2. CopyControl™ Fosmid Library Production Kits, for inserts of about 40 kb.
3. CopyControl™ PCR Cloning Kits, for inserts less than 15 kb.

Below is information on how two labs are using the CopyControl Cloning Kits and some of the specific benefits of the CopyControl Systems.

CopyControl™ BAC Cloning

Chris Amemiya's BAC Library Production Center in Seattle

One of EPICENTRE's CopyControl BAC Cloning customers is Dr. Chris Amemiya at the Benaroya Research Institute at Virginia Mason in Seattle, Washington. Dr. Amemiya heads one of the three national BAC Library Production Centers that are funded by the National Institutes of Health (NIH). In addition to producing BAC libraries, Dr. Amemiya's lab collaborates with a number of other labs to study the organization of genes and gene families, and the effects that genomic organization has on developmental regulation and molecular evolution. These projects interconnect by their reliance on BAC (F-factor-based Bacterial Artificial Chromosome) or PAC (P1-derived Artificial Chromosome) cloning systems.

Dr. Amemiya's BAC production group has used EPICENTRE's CopyControl BAC Cloning Kit to prepare BAC libraries of *Latimeria menadoensis* (coelacanth, an ancient fish), *Alligator mississippiensis* (alligator), *Monodelphis domestica* (laboratory opossum), and *Dasypus novemcinctus* (six-banded armadillo). They have found EPICENTRE's CopyControl ligation system to be the most reliable method for cloning BAC's. (Josh Danke, technician in Dr. Amemiya's BAC production group, personal communication). For more information about this lab see (http://www.benaroyaresearch.org/bri_investigators/amemiya/default.htm)

Each CopyControl BAC Cloning Kit contains:

- Ligation-ready pCC1BAC™ vector with a choice of *Bam*H I, *Eco*R I, or *Hin*d III cohesive ends.
- Fast-Link™ DNA Ligase for rapid ligation reactions.
- A unique colony screening process for sizing BAC clones in as little as 4 hours.
- For additional information, please see http://www.epicentre.com/ccbacinfo.asp.

CopyControl™ Fosmid Library Production Kit

CopyControl™ PCR Cloning Kit

Eric Lafontaine at the Medical College of Ohio

Dr. Eric Lafontaine's lab in the Department of Microbiology and Immunology at the Medical College of Ohio in Toledo uses both the CopyControl Fosmid Library Production Kit and the CopyControl PCR Cloning Kit in their research. Dr. Lafontaine explains how they use these kits.

Our laboratory studies surface antigens expressed by the gram-negative bacterium *Moraxella catarrhalis*. This organism is a leading cause of otitis media, which is an infection of the middle ear, as well as

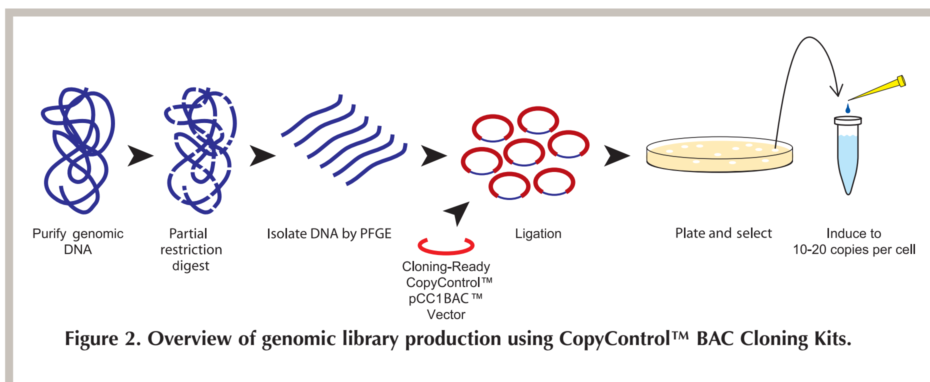


Figure 2. Overview of genomic library production using CopyControl™ BAC Cloning Kits.

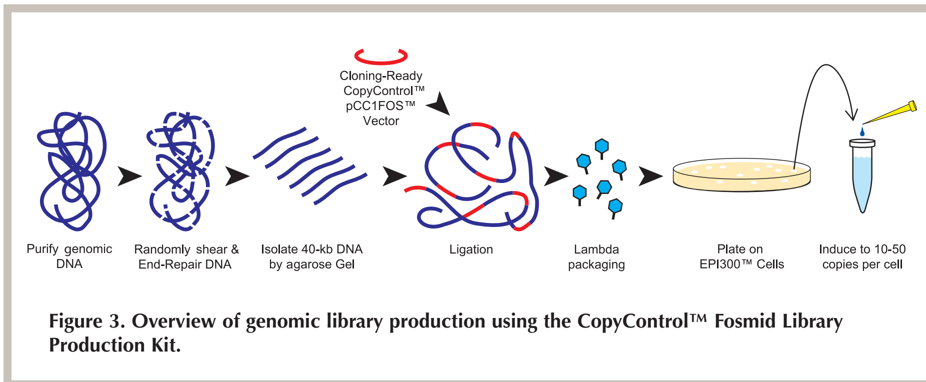


Figure 3. Overview of genomic library production using the CopyControl™ Fosmid Library Production Kit.

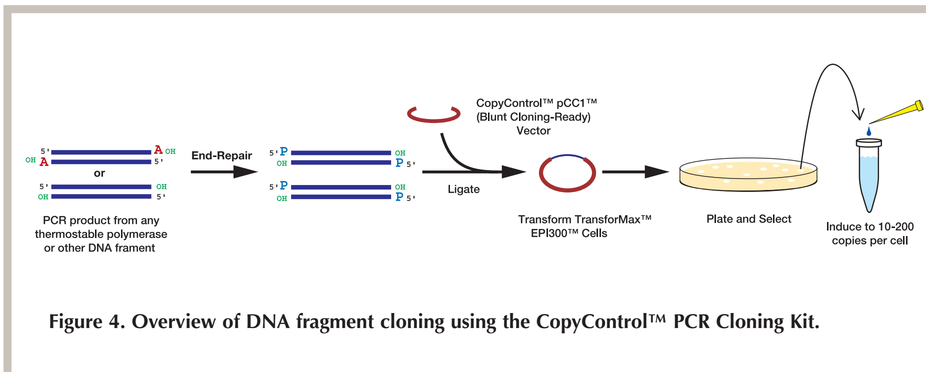


Figure 4. Overview of DNA fragment cloning using the CopyControl™ PCR Cloning Kit.

respiratory tract infections in patients with chronic obstructive pulmonary disease. We are particularly interested in identifying *M. catarrhalis* proteins that mediate adherence to cells of the human respiratory tract. It is our experience that outer membrane proteins of *M. catarrhalis*, when expressed in multiple-copy number plasmids, are toxic to *E. coli*. This is why the CopyControl technology is so wonderful! We have used EPICENTRE's CopyControl Fosmid Library Production system to generate plasmid-based libraries of *M. catarrhalis* DNA fragments. These libraries were introduced into EPICENTRE's *E. coli* EPI300 cloning strain, which does not adhere to human epithelial cells, and recombinant clones were then enriched for those that gained the ability to bind to human cells by virtue of expressing *M. catarrhalis* adhesins. Once identified, candidate adhesin open reading frames were individually cloned using the CopyControl PCR Cloning system, and recombinant clones were shown to bind to human cells. We were therefore successful in expressing recombinant functional surface antigens of *M. catarrhalis* by the use of EPICENTRE's CopyControl technology.

Editor's note: Watch for the full article on this research to be published in the August 2003 issue of *Infection and Immunity* by J. M. Timpe, M. M. Holm, S. L. Vanlerberg, V. Basrur, and E. R. Lafontaine, entitled "Identification of a *Moraxella catarrhalis* outer membrane protein exhibiting both adhesin and lipolytic activities".

Use EPICENTRE's CopyControl™ Fosmid Library Production Kit to:

- Make complete and unbiased fosmid libraries, with single-copy stability, faster and easier than BAC libraries.
- Induce CopyControl fosmid clones up to 50 copies per cell for high yields of DNA.
- For additional information, please see http://www.epicentre.com/ccfosinfo.asp.

With EPICENTRE's CopyControl™ PCR Cloning Kit:

- Clone PCR products or any double-stranded DNA fragment up to 15 kb.
- Protect clones from intermolecular recombination or segment deletions.
- Clone genes or PCR products that are toxic or detrimental to the host cell.
- Induce CopyControl PCR clones from single copy up to 200 copies per cell.
- For additional information please see http://www.epicentre.com/ccpcrinfo.asp.

Reference

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

www.epicentre.com/ccbac.asp

CopyControl™ BAC Cloning Kit (*Bam*H I)
CCBAC1B 1 Kit

CopyControl™ BAC Cloning Kit (*Eco*R I)
CCBAC1E 1 Kit

CopyControl™ BAC Cloning Kit (*Hind* III)
CCBAC1H 1 Kit

Each kit contains sufficient reagents for constructing the equivalent of one 10X human genomic library.

Note: TransforMax™ EPI300™ Electrocompetent *E. coli* or Phage T1-Resistant TransforMax™ EPI300™-T1^R Electrocompetent *E. coli*, required for inducing CopyControl BAC clones to high-copy number, are available separately.

www.epicentre.com/epi300.asp

TransforMax™ EPI300™ Electrocompetent *E. coli*
EC300105 5 X 100 µl
EC300110 10 X 100 µl

Transformation efficiency > 1 x 10^{10} cfu/µg.

Includes CopyControl™ Induction Solution and pUC19 control DNA.

Phage T1-Resistant TransforMax™ EPI300™-T1^R Electrocompetent *E. coli*
EC02T15 5 X 100 µl
EC02T110 10 X 100 µl

Transformation efficiency > 1 x 10^{10} cfu/µg.

Includes CopyControl™ Induction Solution and pUC19 control DNA.

www.epicentre.com/ccfos.asp

CopyControl™ Fosmid Library Production Kit
CCFOS110 1 Kit
Kit contains sufficient reagents to produce up to 10 CopyControl Fosmid libraries.

Phage T1-Resistant EPI300™-T1^R *E. coli* cells, required for inducing CopyControl Fosmid clones to high-copy number, are supplied with the kit.

www.epicentre.com/ccpcr.asp

CopyControl™ PCR Cloning Kit with TransforMax™ EPI300™ Electrocompetent *E. coli*
CCECPCR1 20 Reactions

CopyControl™ PCR Cloning Kit with TransforMax™ EPI300™ Chemically Competent *E. coli*
CCPCR1CC 20 Reactions

Contents:
CopyControl™ pCC1™ (Blunt Cloning-Ready) Vector, PCR Precipitation Solution, 10X Reaction Buffer, PCR End-Repair Enzyme Mix, Fast-Link™ DNA Ligase, EpiLyse™ Solution, EpiBlue™ Solution, CopyControl™ Induction Solution, Control PCR Product, Supercoiled DNA Size Marker, Water, and either TransforMax™ EPI300™ Electrocompetent *E. coli* or TransforMax™ EPI300™ Chemically Competent *E. coli*.

The FailSafe™ Real-Time PCR System with SYBR® Green I Dye Provides Shorter Cycle Times and More Consistent PCR Quantitation with Every Template Every Time

Haiying Grunenwald and Gordon S. Hunter, EPICENTRE

The new FailSafe™ Real-Time PCR System for quantitative PCR applications expands the functionality of the popular FailSafe™ PCR System. The FailSafe Real-Time PCR System incorporates SYBR® Green I dye for the detection and quantification of PCR products without the expense of labeled probes. Just like the standard FailSafe™ PCR System, this new real-time kit ensures successful quantitative PCR with every template the first time and every time.

The FailSafe Real-Time PCR System uses the FailSafe™ PCR Enzyme Mix, a unique blend of thermostable enzymes that is capable of amplifying the most difficult DNA templates with extremely high sensitivity and high fidelity. In addition, the patented FailSafe™ PCR Enhancer (with betaine)* greatly improves the specificity and consistency of PCR.

The other key components of the system are 12 unique FailSafe™ PCR PreMixes. Each FailSafe PCR PreMix contains everything else you need for a successful quantitative PCR except your own template and primers: SYBR® Green I dye, dNTPs, buffer, and varying amounts of $MgCl_2$ and FailSafe PCR Enhancer. A separate container of ROX, a fluorescent passive reference dye that is required for signal normalization in SYBR® Green I dye reactions assayed using ABI real-time PCR instruments, is also provided.

In this report, we compare the performance of the FailSafe Real-Time PCR System with real-time PCR kits from other major manufacturers. As a follow up to our report in *EPICENTRE Forum*, Volume 10, Number 1, which involved amplification of a relatively difficult DNA template (human Duchenne Muscular Dystrophy exon 43), we compare the kits from six suppliers (including EPICENTRE) for real-time amplification of a less complex DNA template (lambda *cII* gene). Comparison of data from different suppliers with an easily amplified template allows a more comparable, side-by-side analysis of real-time PCR results.

Methods and Results

FailSafe Real-Time PCR System consistently yields more specific and sensitive PCR data than all other kits tested with both difficult and routine templates

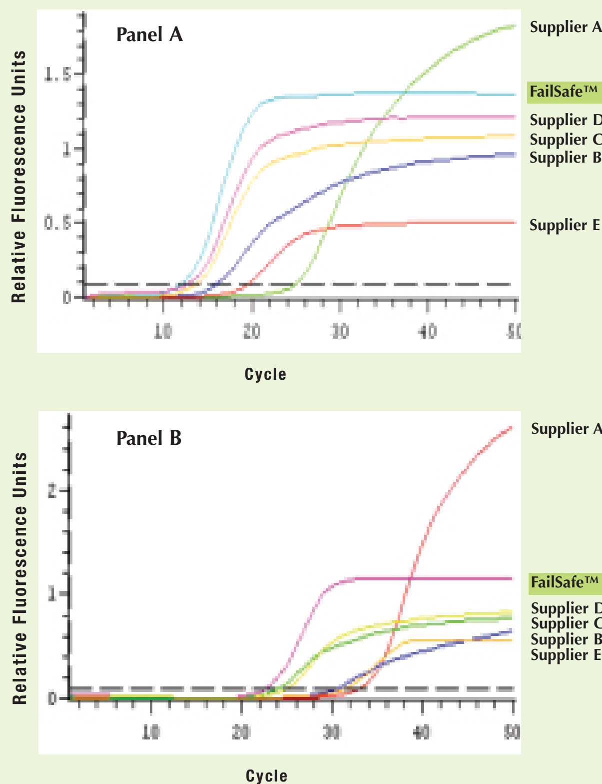


Figure 1. Real-Time PCR quantification graphs comparing FailSafe™ Real-Time PCR System to five competitors' kits. Panel A, Lambda *cII* gene amplification. Panel B, Human DMD 43 amplification. The Supplier A reaction contains a higher concentration of SYBR® Green I dye, which registers higher fluorescence signals, but not an increase in PCR products. A high concentration of SYBR® Green I dye actually inhibits the PCR reaction and results in a delayed cycle threshold.

To examine how the FailSafe Real-Time PCR System compares with 5 competitors' kits, a 460-bp fragment from the lambda *cII* gene and a 357-bp fragment of human Duchene Muscular Dystrophy exon 43 (DMD 43) were amplified using all six real-time PCR kits. The PCR reactions included: 50 pg of lambda DNA for *cII* amplification or 100 ng of human genomic DNA for DMD 43 amplification, 500 nmole each of the forward and reverse primers, and components of either the FailSafe™ Real-Time PCR System or a competitor's kit, according to the manufacturer's directions. FailSafe™ PreMix E was found to be the optimum PreMix in the first round of PCR for both amplicons and was used in the subsequent FailSafe™ Real-Time PCR reactions with these specific template/ primer sets. All reactions were set up at room temperature. Prior to thermocycling, competitors' hot-start enzymes

Table 1. Cycle Threshold (C_T) Values comparing FailSafe™ Real-Time PCR System to five competitors' kits. Panel A, Lambda *cII* gene amplification. Panel B, Human DMD 43 amplification. A lower C_T value indicates more efficient amplification.

Panel A

Supplier	C_T Value (Average of Triplicate Reactions)
FailSafe™ Real-Time PCR System	12.2
Supplier A	25.0
Supplier B	16.2
Supplier C	13.8
Supplier D	12.8
Supplier E	19.6

Panel B

Supplier	C_T Value (Average of Triplicate Reactions)
FailSafe™ Real-Time PCR System	22.6
Supplier A	33.0
Supplier B	30.6
Supplier C	24.1
Supplier D	24.7
Supplier E	31.5

were reactivated according to each manufacturer's instructions. The FailSafe™ System does not use a hot-start enzyme and required no reactivation step. PCR cycling conditions were 50 cycles of 95°C for 10 seconds, 55°C for 10 seconds, and 72°C for 30 seconds.

Figure 1 shows PCR quantification graphs for the two DNA templates and all six kit suppliers. Similarly, Figure 2 displays melt curve analyses of the same templates and kits. Table 1 shows comparative cycle threshold (C_T) values. In the Figures and Table, Panels A show real-time PCR results of lambda *cII* amplification and Panels B show results of DMD 43 amplification.

As shown in Figure 2, Panel A, all 6 real-time PCR kits worked well for lambda *cII* amplification. All graphs exhibit good peak symmetries and little or no primer-dimer formation. Upon examination of the C_T values (Figure 1, Panel A and Table 1, Panel A) the FailSafe™ Real-Time PCR System performed equally well or better than the competition for real-time amplification of the lambda *cII* template.

When comparing the DMD exon 43 real-time PCR data however, the FailSafe Real-Time Kit gave significantly better C_T values (see Figure 1, Panel B and Table 1, Panel B). The melt curve analysis graphs (Figure 2, Panel B) demonstrate a very specific, desired PCR amplicon for the FailSafe Real-Time Kit. Three of the competitors' kits only produced primer-dimers (Suppliers A, B, E). The other two had the specific PCR amplicon, but with undesirable formation of primer-dimers (Suppliers C and D).

For routine real-time PCR experiments, lambda *cII* DNA templates worked well with real-time PCR kits from most suppliers, but FailSafe™ worked better as revealed by lower C_T values. With difficult real-time PCR amplifications (e.g., human DMD exon 43), most of the competitors' kits did not perform well, while the FailSafe Real-Time System demonstrated higher sensitivity and specificity. Superior amplification performance is due to several factors including flexible optimization of reaction conditions, a unique enzyme blend, and the patented FailSafe PCR Enhancer (with betaine).

Conclusions

The FailSafe Real-Time PCR System provides consistent, highly specific and sensitive quantitative data when compared to kits from five major suppliers. Our experimental data indicate excellent results with templates that are both difficult (human DMD exon 43) and routine (lambda *cII* gene). The reported quantitative PCR analyses were performed on MJ Research's Opticon® 2 instrument. Similar results were obtained using other instruments, including BioRad's iCycler iQ and ABI's Prism 7700.

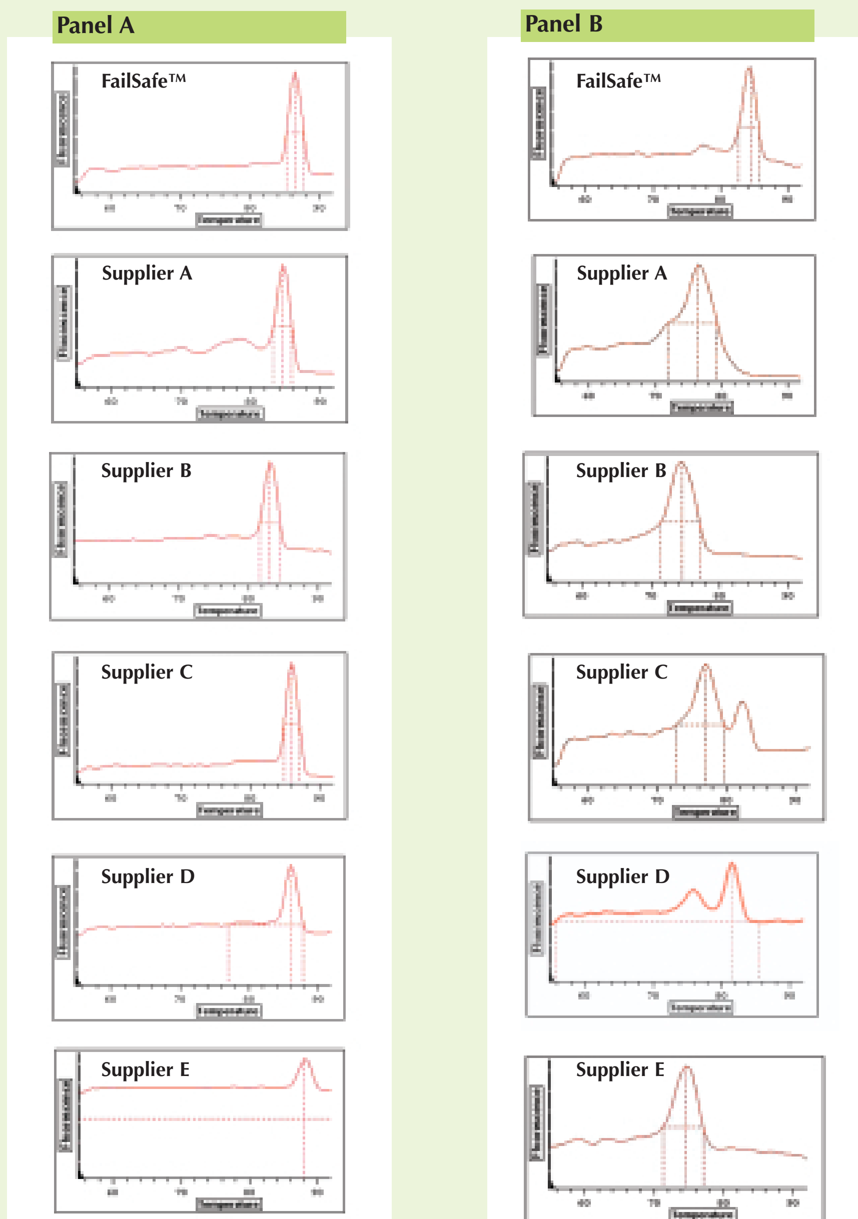


Figure 2. Melt curve analysis graphs comparing the FailSafe™ Real-Time PCR System to five competitors' kits. Panel A, Lambda *cII* gene amplification. Panel B, Human DMD 43 amplification.

www.epicentre.com/realtimepcr.asp

FailSafe™ Real-Time PCR PreMix Selection Kit

FSR0360 48 Reactions

Contents:

FailSafe™ PCR Enzyme Mix, 12 FailSafe™ Real-Time PCR 2X PreMixes, and Passive Reference Dye.

FailSafe™ Real-Time PCR System

FSR03200 200 Reactions

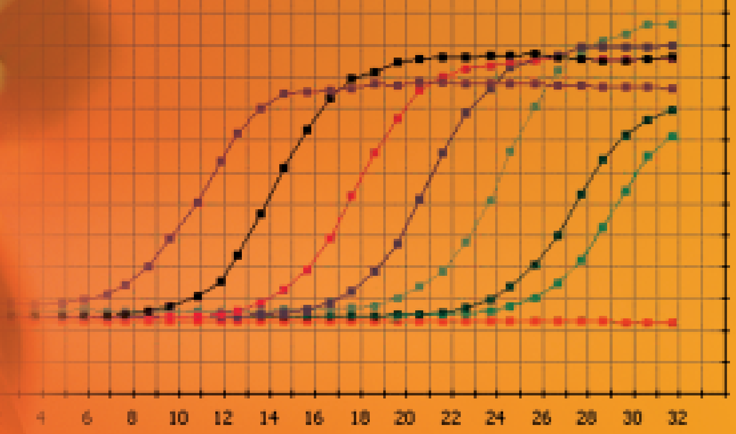
Contents:

FailSafe™ PCR Enzyme Mix, choice of two FailSafe™ Real-Time PCR 2X PreMixes, and Passive Reference Dye.

* The use of betaine in DNA or RNA polymerase reactions is covered by patent rights exclusively licensed to EPICENTRE Technologies. Purchase of EPICENTRE's products for use under these rights is accompanied by a limited non-exclusive license for the purchaser to use the purchased product solely for non-commercial life science research.

SYBR is a registered trademark of Molecular Probes, Inc. SYBR® Green I Dye is covered by patents.

This product is accompanied by a limited license to use it in the Polymerase Chain Reaction (PCR) and RT-PCR for life science research in conjunction with a thermal cycler whose use in the automated performance of the PCR process is covered by the up-front license fee, either by payment to Applied Biosystems or purchased, i.e., an authorized thermal cycler.



FailSafe PCR Results in Real-Time!

FailSafe™ Real-Time PCR System

- Extends the unsurpassed **specificity, sensitivity,** and **consistency** of the FailSafe™ PCR System to quantitative PCR applications with a **broader dynamic range.**
- Like our standard FailSafe™ PCR System, this new real-time PCR kit ensures successful quantitative PCR the first time and every time.

What makes the FailSafe™ Real-Time PCR System *"fail-safe"*?

- **FailSafe PCR Enzyme Mix:** A unique blend of thermostable enzymes that is capable of amplifying the most difficult DNA templates with extremely high sensitivity and fidelity, with no extra "hot start" step.
- **Set of 12 FailSafe PreMixes:** Include SYBR® Green I dye, dNTPs, buffer, and varying amounts of $MgCl_2$ and the FailSafe PCR Enhancer (with betaine).*

* The use of betaine in DNA or RNA polymerase reactions is covered by patent rights exclusively licensed to EPICENTRE Technologies.

EPICENTRE is a registered trademark, FailSafe is a trademark of EPICENTRE Technologies.

SYBR is a registered trademark of Molecular Probes, Inc.
SYBR® Green I Dye is covered by patents.

HOW TO CONTACT US AT EPICENTRE

Toll-free in the USA: **800-284-8474**
Tel: 608-258-3080 **Fax:** 608-258-3088
Web site: www.epicentre.com E-mail: techhelp@epicentre.com
Outside the USA: contact your local EPICENTRE Distributor.

EPICENTRE FORUM

Editor Katharine Kramer
Associate Editor Merriann Carey
Graphic Designer Julie Capadona
Additional Illustrations Ron Meis

© 2003 ***EPICENTRE*** All rights reserved. Publication date: June, 2003. Printed in USA.

EPICENTRE and Ampligase are registered trademarks and AmpliCap, BuccalAmp, Catch-All, Colony Fast-Screen, CopyControl, DuraScribe, DuraScript, EC100, EC100D, EPI300, End-It, EpiBlue, EpiFOS, EpiLyse, EZ::TN, FailSafe, Fast-Link, GELase, HK, Hybridase, HyperMu, IsoTherm, Long-Read, OmniCleave, MasterAmp, MasterPure, MaxPlax, pCC1, pCC1BAC, pCC1FOS, PCRLyse, PeriPreps, Plasmid-Safe, pEpiFOS, pMOD, pPDM, pWEB, pWEB::TNC, QuickExtract, R&DNA, Ready-Lyse, ReadyPreps, RetroAmp, RiboScribe, RiboShredder, SequiTherm, SequiTherm EXCEL, SoilMaster, Tagetin, TAQurate, TransforMax, Transposome, and Transposomics are trademarks of EPICENTRE. Product prices and specifications subject to change without notice.