

EPICENTRE Forum

Tools & Techniques for Genomics, Proteomics & RNA Research

Volume 11-2

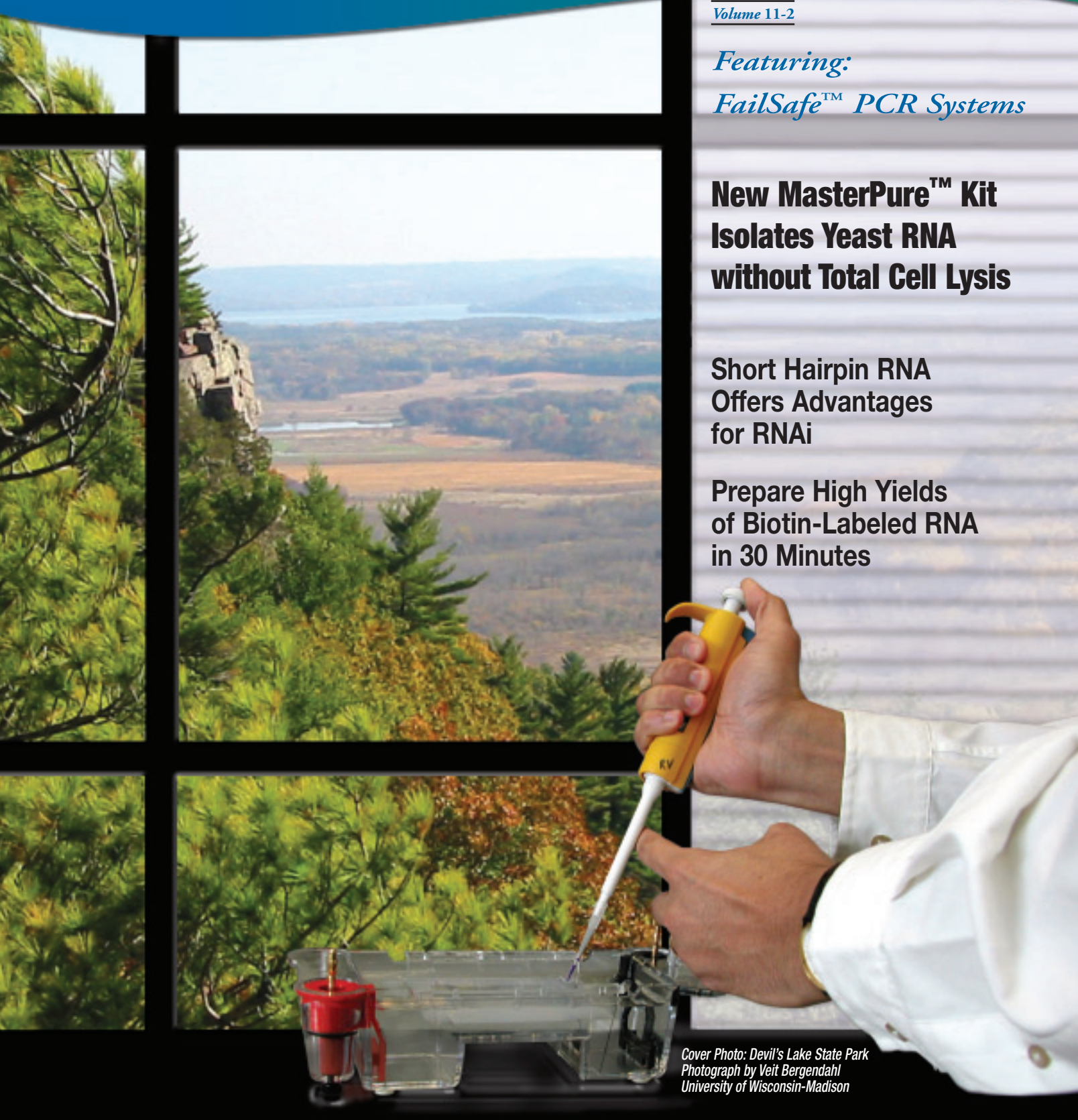
Featuring:

FailSafe™ PCR Systems

**New MasterPure™ Kit
Isolates Yeast RNA
without Total Cell Lysis**

**Short Hairpin RNA
Offers Advantages
for RNAi**

**Prepare High Yields
of Biotin-Labeled RNA
in 30 Minutes**



Cover Photo: Devil's Lake State Park
Photograph by Veit Bergendahl
University of Wisconsin-Madison

Construct Complete BAC or Fosmid Libraries That Are Inducible to High-Copy Number



with CopyControl™ Cloning Kits

from EPICENTRE

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EPICENTRE's CopyControl™ Cloning Kits enable construction of genomic libraries of single-copy clones to ensure insert stability and cloning of potentially toxic gene products. Then, whenever desired, induce the clones to high-copy number for high yields of higher purity DNA.

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- Get high cloning efficiency and low backgrounds using the Cloning-Ready CopyControl™ pCC1BAC™ Vector supplied in the kits.
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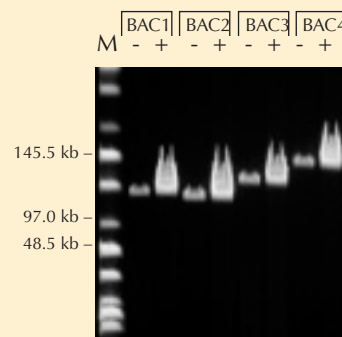
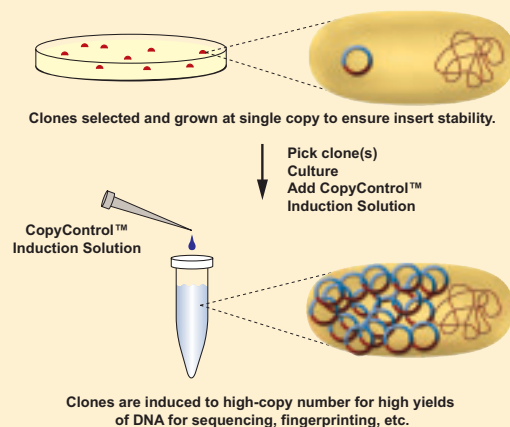
Now, Incorporate CopyControl Capability Into Existing Single-Copy BAC and Fosmid Clones.

See www.epicentre.com/transposomics.asp for information.

References

1. Wild, J. et al. (2002) *Genome Research* 12, 1434.
2. Piffanelli, P. (2002) *EPICENTRE Forum* 9 (3), 1.

www.epicentre.com/ccfos.asp
www.epicentre.com/ccbac.asp
www.epicentre.com/transposomics.asp



CopyControl™ BAC clones can be induced from single-copy to high-copy number for higher yields of DNA. DNA from an equal number of cells of induced (+) and uninduced (-) cultures of 4 CopyControl™ BAC clones was digested with *Not I* and analyzed by PFGE.



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Editor: KATHARINE KRAMER

Graphic Designer: JULIE CAPADONA

Additional Illustrations: RON MEIS

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On the Cover:

EPICENTRE thanks Veit Bergendahl for the use of his photograph taken from a cliff at Devil's Lake State Park in Wisconsin. Veit is a post doc in the laboratory of Richard Burgess at the University of Wisconsin-Madison. The Burgess Lab, in the McArdle Laboratory for Cancer Research, studies RNA polymerases and transcription factors. Transcription is a complex series of events that requires the interaction of multiple proteins, and studying transcription uses a variety of biochemical techniques. Abnormal transcriptional regulation plays an important role in neoplastic transformation. The Burgess Lab has also been instrumental in developing new protein purification methods.

Hands on the Cover:

Ramesh Vaidyanathan, EPICENTRE R&D Scientist



A Novel, Rapid Method to Release Intact Yeast RNA Using the MasterPure™ Yeast RNA Purification Kit

Les Hoffman and Bruce W. Jarvis, EPICENTRE

Introduction

Yeast RNA has traditionally been extracted using hot acid phenol.¹⁻⁴ Recently, other methods have been introduced that require either physical shear force or enzymatic lysis to break the very resilient yeast cell walls. These methods require special steps to “break open” the cells and release the contents, after which the RNA is purified away from the other cell components. However, the MasterPure™ Yeast RNA Purification Kit uses a revolutionary RNA isolation method that does not require special steps to lyse the yeast cells and that leaves the yeast cells essentially intact, based on observations using a light microscope. The MasterPure Kit protocol releases RNA that is largely free of DNA and protein, without using organic solvents or caustic reagents.

Here, we demonstrate use of the MasterPure Yeast RNA Purification Kit to isolate total RNA from three different yeast species - *Candida albicans*, *Saccharomyces cerevisiae*, and *Schizosaccharomyces pombe* - all grown under standard conditions to mid-log phase. Approximately 25 µg of total RNA is obtained from 1-ml cultures of each of these yeast species - more than enough for synthesis of cDNA. Major component rRNAs are intact, as shown by denaturing gel electrophoresis. The RNA obtained from *Saccharomyces* is shown to be suitable for a variety of other applications, including synthesis of cDNA and dye-labeling for microarray hybridizations (Marilee Morgan, personal communication). Isolated rRNAs, and small nuclear

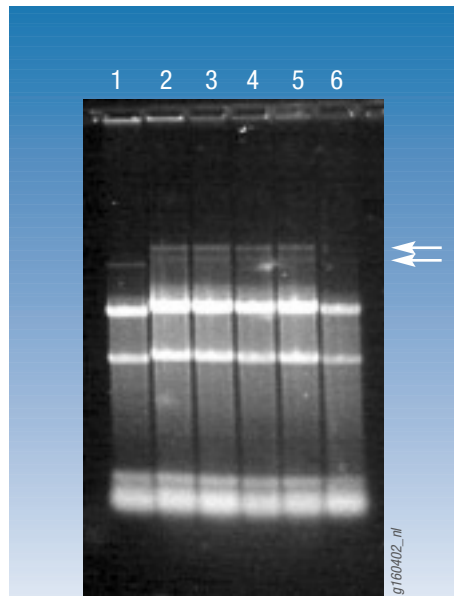


Figure 1. A SYBR® Gold-stained denaturing gel shows the size spectrum of RNA purified using the MasterPure™ Yeast RNA Purification Kit. Each lane contains 1 µg of *S. cerevisiae* (lanes 1 and 6) or *S. pombe* (lanes 2-5) total RNA. Arrows indicate the sizes expected for precursor rRNAs, 35S and 32S, which are approximately 7 kb.

RNAs (snRNAs) are undegraded, based on Northern blot analysis. RT-PCR experiments further demonstrate the quality of this mRNA.

The MasterPure Yeast RNA Purification Kit compares favorably to two other commercially available yeast RNA purification kits

with respect to yield, quality of the isolated mRNA, ease of use, and cost.

Materials and Methods can be found online at: www.epicentre.com/f11-2mmmp.asp

Results

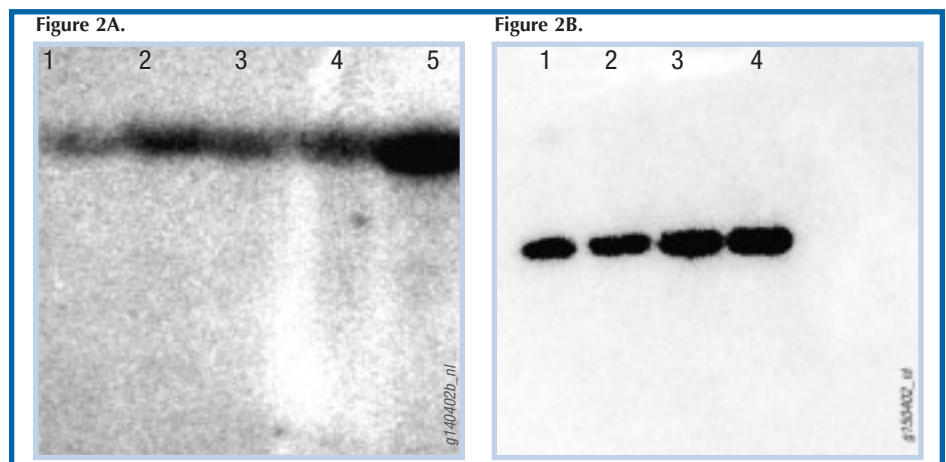
Electrophoresis and Northern blots show intact RNA

A denaturing agarose gel, stained with SYBR® Gold, shows the size spectrum of RNA isolated from *S. cerevisiae* and *S. pombe* (Figure 1). Arrows indicate the sizes expected for the precursors of rRNAs, 35S and 32S, which are approximately 7 kb.

We demonstrate the integrity of RNA from MasterPure preparations by two different methods, Northern blotting and RT-PCR. For Northern blots, 5 to 10 µg of total RNA, purified using the MasterPure Yeast RNA Purification Kit, was electrophoresed in denaturing gels, blotted to nylon membranes, and sequentially hybridized with probes for the 25S (3391 nucleotides) rRNA of *S. cerevisiae* and the 26S (3497 nucleotides) rRNA of *S. pombe*. The data indicate that the large 25S and 26S rRNAs, from both the budding and fission yeast species, are intact (Figure 2A). Based on the substantial amounts of rRNA present, we would expect to detect any significant degradation of the RNA by Northern blots.

To assess the possible degradation of another yeast RNA, we chose the U2 snRNA. The unusually long *S. cerevisiae*

Figure 2. Northern blots indicate the integrity of MasterPure™ Yeast RNA. RNA from *S. cerevisiae* or *S. pombe* (5 to 10 µg) were separated on a 1% agarose/formaldehyde gel and transferred to a nylon membrane. Probes for (A) 25S and 26S rRNA, or (B) U2 snRNA were hybridized to the blots. **2A.** *S. pombe* RNA (lanes 1-4), *S. cerevisiae* RNA (lane 5). **2B.** *S. cerevisiae* AB1380 (lanes 1 and 2), *S. cerevisiae* InvSc2 (lanes 3 and 4).



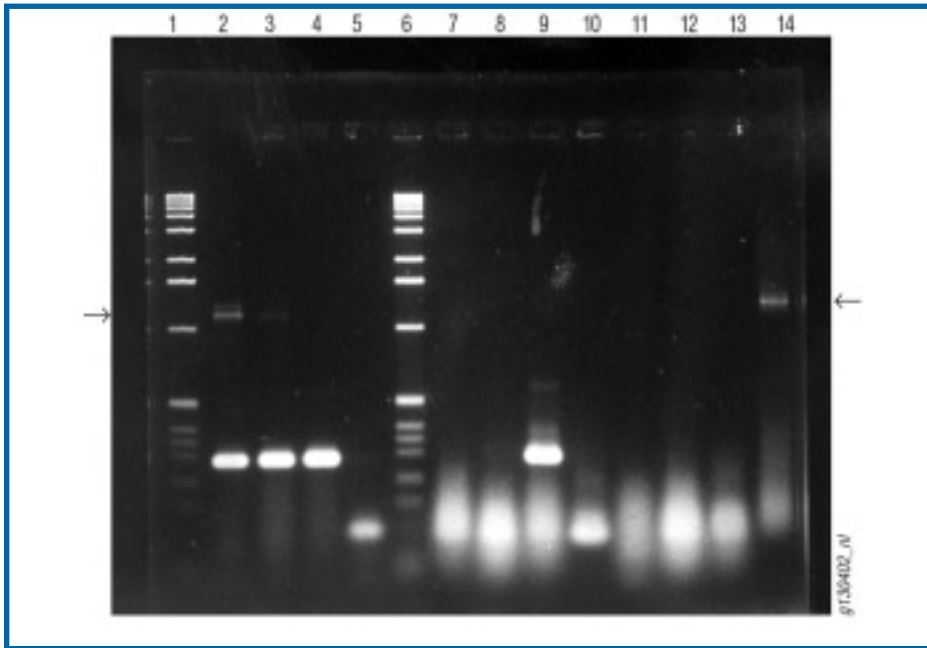


Figure 3. Results of RT-PCR, using RNA purified by the MasterPure™ Yeast RNA Purification Kit or two other yeast purification kits, show differences in the quality of purified RNA. RT-PCR of *DBP2* mRNA was performed using a two step (lanes 2-5) or one step (lanes 7-14) RT-PCR protocol. MMLV-RT was omitted from samples in lanes 11-14. Supplier A RNA is used in lanes 2, 7 and 11; Supplier Q RNA is used in lanes 3, 8, and 12; MasterPure RNA was used in lanes 4, 9, and 13. Control reactions were done with RNA-free yeast DNA in lanes 5, 10, and 14 to amplify only genomic *DBP2* sequences.

U2 snRNA (1175 nucleotides) resides principally in the nucleus, but shuttles between the nucleus and cytosol during its maturation and processing.⁵ The Northern blot in Figure 2B shows that U2 snRNAs from 2 strains of *S. cerevisiae* appear full-length after MasterPure isolation.

Another advantage of the MasterPure Yeast RNA Purification Kit is the integrity of low molecular weight RNA, such as 5.8S RNA, 5S RNA, and tRNA, that it yields. Electrophoresis in a 2% agarose gel showed very discreet low molecular weight species in MasterPure yeast RNA (data not shown).

RT-PCR amplification of mRNA

RT-PCR is a useful technique to evaluate the quality of mRNA in a total RNA sample. However, if DNA is present, gene-specific primers can produce a false-positive

amplicon. To verify that the amplicon was a product of the cDNA rather than genomic DNA, we chose a gene containing an intron and designed primers to amplify the region containing the intron in genomic DNA. The *S. cerevisiae DBP2* gene contains a 1001 bp intron and codes for an RNA helicase featuring a DEAD-box.⁶ Because the RT-PCR product from the mRNA is 1 kb shorter than the PCR product amplified from genomic DNA, it can easily be distinguished from the DNA amplification product by electrophoresis.

Using the MasterPure™ High Fidelity RT-PCR Kit, we compared RT-PCR results of *S. cerevisiae* total RNA that was purified by the MasterPure Yeast RNA Purification Kit or by kits from Suppliers Q and A. Yeast RNA from all of the kits tested amplified by the two-step RT-PCR

(Figure 3, lanes 2-4.). However, the gel also shows DNA amplification of the target gene with RNA prepared with Supplier Q and A kits (see arrow, lanes 2 and 3).

Using one-step RT-PCR conditions, *DBP2* RNA isolated by Suppliers Q and A kits gave no detectable product (Figure 3, lanes 7 and 8), while the MasterPure RNA gave a significant product band (Figure 3, lane 9). To verify that this problem was not attributable in some way to the *DBP2* gene, we also tried to amplify *S. cerevisiae ERD2* mRNA by one-step RT-PCR. Again, The MasterPure RNA gave a strong product band and the RNA from the other two kits gave no detectable product (data not shown).

In one-step RT-PCR negative control reactions, without reverse transcriptase, none of the yeast RNA, purified by any of the kits, gave a product (Figure 3, lanes 11, 12 and 13). Control yeast DNA (RNA-free) produced only the genomic-template amplicon (see arrow, lane 14).

MasterPure versus other yeast RNA isolation kits

Spin-column methods of RNA purification rely on the lysis of yeast cells by enzymatic treatment and/or mechanical shearing. RNA yields from these methods are variable, and the RNA requires further clean-up for some applications. In Table 1, we compare the MasterPure Yeast RNA Purification Kit to the two other commercially available kits for *S. cerevisiae* RNA purification. The MasterPure Yeast RNA Purification Kit easily, quickly and safely

Table 1. Comparison of the MasterPure™ Yeast RNA Purification Kit protocol with other yeast RNA kit protocols.

	MasterPure	Supplier Q	Supplier A
Method	Aqueous Extraction	Spheroplasting	CHCl ₃ /Phenol/Beads
Number of Steps	10	15	19
Time*	40 min	73 min	33 min
Extra Item to Buy	None	Zymolyase	Vortex Adapter

* Without DNase I digestion

isolated intact total RNA at a lower cost per purification than the other kits.

Discussion

The MasterPure Yeast RNA Purification protocol requires no additional enzymes or special equipment purchases. The simple procedure, with fewer steps and safer reagents, means that the MasterPure method is useful for high throughput applications. Because this method does not use spin columns, accurately calculating the starting number of cells is not necessary and scale-up does not require a different kit.

The RNA from *S. cerevisiae* and *S. pombe* was analyzed using Northern blotting. Several RNA types appeared to be intact, up to approximately 7 kb in length. Stained gels suggest that the MasterPure Yeast RNA Purification Kit extracts nuclear precursors of rRNAs. The U2 snRNA of *S. cerevisiae*, which is much longer than most snRNAs in yeast, is recovered as a full-length molecule with MasterPure. U2 is especially susceptible to degradation

during isolation (Stephanie Ruby, personal communication).

The MasterPure RNA was readily converted to cDNA by both one-step and two-step RT-PCR protocols and only produced the smaller, intronless product. RNA isolated with kits from Suppliers Q and A produced the smaller product in the two-step, but not the one-step, RT-PCR protocol, and also produced some of the larger, DNA template product.

Finally, avoiding hot phenol extraction conditions is another principal advantage of the MasterPure Yeast RNA Purification Kit. The dangers of using hot organic solvents are obvious, and this RNA isolation procedure effectively purifies RNA without those conditions.

Acknowledgments

We wish to thank Dr. Stephanie W. Ruby and Marilee Morgan at the University of New Mexico Heath Sciences Center, Albuquerque, NM for invaluable discussions and for performing cDNA synthesis, labeling, and microarray hybridizations using yeast RNA.

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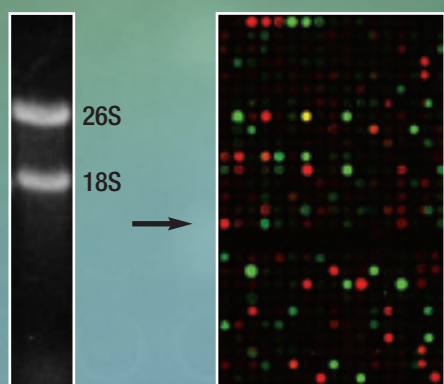
MasterPure™ Yeast RNA Purification Kit

MPY03010	10 Reactions
MPY03100	100 Reactions

Contents:

- Extraction Reagent for RNA
- MPC Protein Precipitation Reagent
- TE Buffer (in 100 rxn kit only)
- Proteinase K (50 µg/µl)
- RNase-Free DNase I (1 Unit/µl)
- 10X DNase Buffer
- 2X T & C Lysis Solution

Microarray Application Data for MasterPure™ Yeast RNA Purification Kit



S. cerevisiae RNA → Labeled cDNA from MasterPure RNA

Figure 1. RNA purified with the MasterPure™ Yeast RNA Purification Kit was reverse-transcribed, labeled, and hybridized to an *S. cerevisiae* DNA microarray. The cDNA from cells under oxidative stress was labeled with Cy5, and cDNA from control cells was labeled with Cy3.

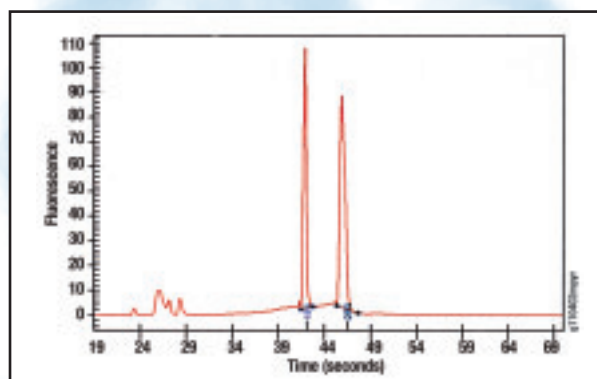


Figure 2. Agilent Technologies 2100 Bioanalyzer® electrophoretogram of the *S. cerevisiae* RNA shown in Figure 1. RNA was purified by the MasterPure™ Yeast RNA Purification Kit and stored at -20°C for 2 months before analysis. Observe the high purity, intact RNA.

High Efficiency Real-Time PCR Amplifies a 1.6-kb Template Using the FailSafe™ Real-Time PCR System

Haiying Grunenwald, EPICENTRE

Introduction

Real-time PCR monitors the accumulation of PCR products during the logarithmic phase of the reaction, when none of the components should be rate limiting. As they are produced, PCR products are detected by an increase in fluorescent signal. A variety of creative techniques have been developed to ensure an accurate relationship between the fluorescent signal and the amount of the accumulating product. The simplest and most flexible technique uses SYBR® Green I dye (Molecular Probes), which binds to the minor groove of double-stranded DNA.

To obtain valid quantitation of different templates, real-time PCR requires a highly efficient reaction. PCR efficiency is reduced when the polymerase stops or pauses at secondary structures in a DNA template. Designing PCR experiments to produce short amplicons (75 to 150 bp) lowers the probability that template secondary structure will interfere with PCR efficiency. However, selecting a short amplicon, does not guarantee reaction efficiency.¹

The FailSafe™ Real-Time PCR System was designed to eliminate DNA polymerase stops and to quickly determine the optimal real-time PCR reaction conditions of any template/primer combination.² The PreMixes in the FailSafe™ PCR Systems contain varying concentrations of the FailSafe™ PCR Enhancer (with betaine*). One of betaine's functions in the reaction is to eliminate DNA polymerase stops by decreasing the melting temperature of GC-rich regions.³ This reduces template secondary structure and increases reaction efficiency. The FailSafe PCR Systems also contain the FailSafe™ PCR Enzyme Mix, a unique blend of thermostable enzymes that is formulated to efficiently amplify DNA templates with high fidelity. Here we demonstrate the high efficiency of the FailSafe Real-Time PCR System by amplifying a relatively long (1.6 kb) template.

Materials and Methods

The FailSafe™ Real-Time PCR PreMix Selection Kit was used for the first round of PCR reactions. The kit includes 12 FailSafe™ Real-Time PCR PreMixes, which insure optimized reaction conditions for any template/primer combination.

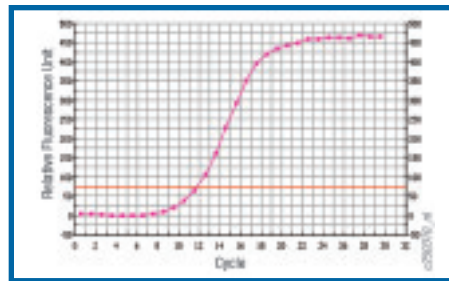


Figure 1A. PCR quantification plot for a 1.6-kb amplicon in a FailSafe™ Real-Time PCR reaction.

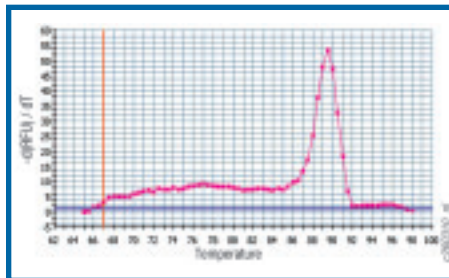


Figure 1B. Melt curve analysis for a 1.6-kb amplicon.

Real-time PCR reactions were set up with each of the 12 FailSafe PCR PreMixes and plasmid DNA template, primers, and FailSafe PCR Enzyme Mix. Real-time PCR was performed and analyzed on Bio-Rad's iCycler iQ™ Real-Time Detection System with an initial denaturation of 94°C (2 minutes) and 30 cycles of 98°C (10 seconds), 55°C (10 seconds), and 72°C (1 minute).

Of the 12 FailSafe PCR PreMixes, PreMix E gave the fastest threshold cycle (C_T), the sharpest melt curve peak and no primer/dimers or nonspecific (data not shown). Subsequent reactions with this template/primer combination were performed using PreMix E.

Results

Figure 1A shows a PCR quantification plot for the amplification of the 1.6-kb PCR product. The sharp, single peak of

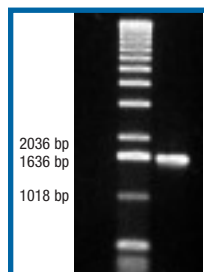


Figure 2. Agarose gel electrophoresis of the 1.6 kb amplicon from a FailSafe™ Real-Time PCR reaction. From a 50 µl reaction, 1 µl was run on an 1% agarose gel and stained with SYBR Gold. Marker is a 1-kb DNA ladder.

the melt curve shown in Figure 1B indicates that no primer/dimers or nonspecific products were formed in the reaction. To verify the melt curve data, the PCR product was assayed by gel electrophoresis (Figure 2), which shows no additional bands.

Conclusion

The quantification plot and the melt curve analysis indicate that a 1.6-kb template, which is longer than typically used for real-time PCR, was efficiently amplified using the FailSafe™ Real-Time PCR System. The FailSafe PCR Enzyme Mix and the PreMixes, with varying concentrations of betaine, combine to provide extremely efficient real-time PCR. Efficiency of the real-time PCR reaction is crucial to obtaining reliable quantitative data. The FailSafe™ Real-Time PCR Capillary System is also available for real-time PCR instruments with glass capillary tubes.⁴

References

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www.epicentre.com/pcr.asp

FailSafe™ Real-Time PCR PreMix Selection Kit

FSR0360 48 Reactions

Contents:

FailSafe™ PCR Enzyme Mix
12 different FailSafe™ Real-Time PCR 2X PreMixes

FailSafe™ Real-Time PCR System

FSR03200 200 Reactions

Contents:

FailSafe™ PCR Enzyme Mix
Choice of two FailSafe™ Real-Time PCR 2X PreMixes

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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.

Ask Frank

by Fred and Hank



FRED HYDE



HANK DAUM

Questions about FailSafe™ PCR Systems

Q: How does the FailSafe™ PCR System work?

A: The FailSafe PCR System is unique in that the optimal conditions for amplification are determined automatically by performing a single round of PCR, which is especially useful for solving tough PCR problems. The FailSafe™ PCR PreMix Selection Kit accomplishes this by using 12 different PCR PreMixes, which include buffer, deoxyribonucleotides, salt, and varying concentrations of magnesium salt and of the FailSafe™ PCR Enhancer (with betaine)*. The first time you use a specific template/primer combination, prepare PCR reactions using your template and primers with each of the 12 FailSafe™ PCR PreMixes. Analyze the PCR product from each PreMix reaction by agarose gel electrophoresis and visually determine which PreMix gives the best amplification: a single, strong PCR product of the desired length. Then use the selected PreMix for all subsequent amplifications with that template/primer combination. The product is called "FailSafe" because at least one of the 12 FailSafe PreMixes should give the desired product. Optimization is automatic.

Q: Can FailSafe be used for real-time (quantitative) PCR?

A: Yes! EPICENTRE has two unique FailSafe PCR Systems for real-time PCR. In both kits, the combination of the FailSafe™ PCR Enzyme Mix, the FailSafe PCR Enhancer and the optimized concentration of SYBR® Green I dye provide consistent and reproducible PCR efficiencies, which result in a broad dynamic range and some of the lowest possible threshold cycles. The FailSafe™ Real-Time PCR System is designed for well-based real-time PCR instruments and is very similar to the original FailSafe PCR

System (it uses 12 Real-Time PCR PreMixes, containing an optimal amount of SYBR® Green I dye). A separate tube of ROX dye is also provided as a reference standard for use in real-time PCR instruments manufactured by Applied Biosystems. The FailSafe™ Real-Time PCR Capillary System is designed specifically for capillary real-time PCR instruments and contains 8 PCR Capillary PreMixes for use with a smaller (20 µl) reaction volume. The optimization procedure for the FailSafe Real-Time PCR Systems is identical to the standard FailSafe PCR System - simply run PCR using each individual Real-Time PCR PreMix and select the best PreMix based on the lowest threshold cycle (C_T) and best melt curve.

Q: What DNA Polymerase is used in the FailSafe System?

A: The FailSafe PCR Enzyme Mix is a proprietary blend of thermostable DNA Polymerases, and includes a proofreading polymerase. This combination of enzymes provides excellent PCR efficiency and much higher fidelity than *Taq* DNA Polymerase, and allows amplification of even the toughest DNA templates. Regardless of the source or properties of the template, including those with high GC content, this enzyme mix efficiently amplifies the DNA.

Q: Can I use Topo or TA-cloning with the PCR product from the FailSafe PCR System?

A: Yes. The FailSafe PCR Enzyme Mix adds an uncoded "A" to both ends of many of the PCR products, and thus can be used in TA-cloning applications using T-vectors and also with Topo-TA cloning products.

Q: Which FailSafe PreMix should I use for my GC-rich template? I don't want to test all of the PreMixes.

A: It is nearly impossible to determine, without experimentation, which FailSafe PCR PreMix will work best with a given template/primer combination. We strongly recommend using all of the PreMixes for optimization. If only selected PreMixes are tested, you take the chance of selecting PCR conditions that may not be optimal for your reaction.

Q: I want to amplify a template of about 20 kb. Should I use the FailSafe PCR System or the MasterAmp™ Extra-Long PCR Kit?

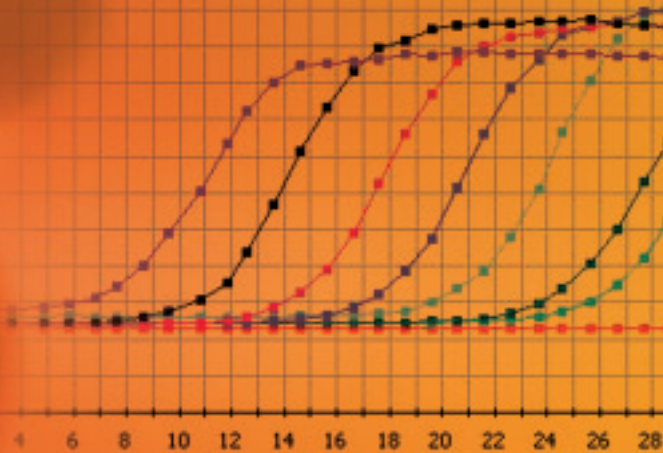
A: Both products can amplify templates around 20 kb. With FailSafe, for templates >10 kb, we recommend using 2.5 Units of the FailSafe PCR Enzyme Mix (for templates <10 kb, use 1.25 units in 50-µl reactions). For any template over 20 kb use the MasterAmp™ Extra-Long PCR System.

**Patents issued and pending.*

Dear Hank,
 Thank you very much for your time today - I greatly appreciate your help and advice. The detail on the email is wonderful! I will order the Selection Kit straight away and look forward to emailing you a photo of the results!
 Kirsten

Kirsten St. George
 Clinical Virology Laboratory
 University of Pittsburgh Medical Center

Now available for
Capillary-Based PCR
Thermal Cyclers



FailSafe PCR Results in

Real-Time!

We Promise Successful Quantitative PCR — First Time and Every 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 regardless of template length or sequence with extremely high sensitivity and fidelity, and with no extra "hot start" step.
- **A set of FailSafe PreMixes:** Include SYBR® Green I dye, dNTPs, buffer, stabilizer and varying amounts of MgCl₂ and the FailSafe PCR Enhancer (with betaine).*



EPICENTRE®
www.epicentre.com

For additional technical information, visit:
www.epicentre.com/pcr.asp

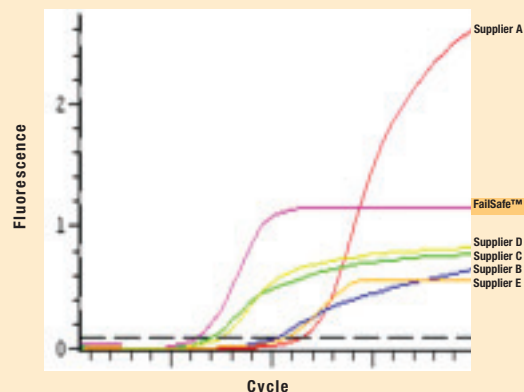
*The use of betaine in DNA or RNA polymerase reactions is covered by patent rights exclusively licensed to EPICENTRE Technologies. EPICENTRE is a registered trademark, FailSafe is a trademark of EPICENTRE Technologies. SYBR is a registered trademark of Molecular Probes, Inc. SYBR® Green I Dye is covered by patents.

The FailSafe™ Real-Time PCR Systems contain everything you need . . .
 for successful PCR, including the FailSafe™ Enzyme Mix, reaction PreMixes that contain dNTPs, buffer, MgCl₂,
 and the patented FailSafe PCR Enhancer Technology.*

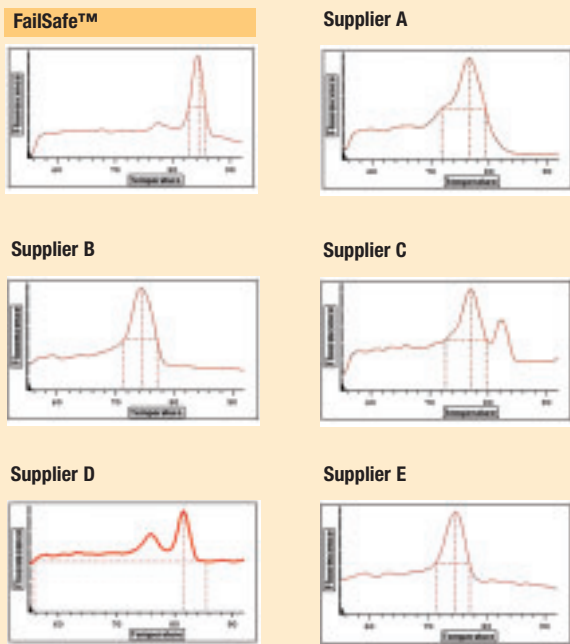
Higher Specificity

Real-time PCR amplification of a 357-bp fragment from human DMD exon 43 was performed with FailSafe™ Real-Time PCR System and 5 major hot-start real-time PCR suppliers' kits. Real-time PCR amplification was carried out on MJ Research's Opticon2™ Real-Time PCR Detection System.

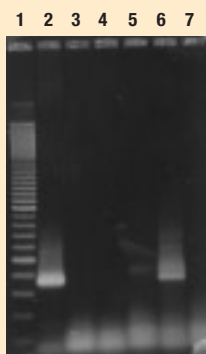
PCR quantitation graph



Melt curve analysis

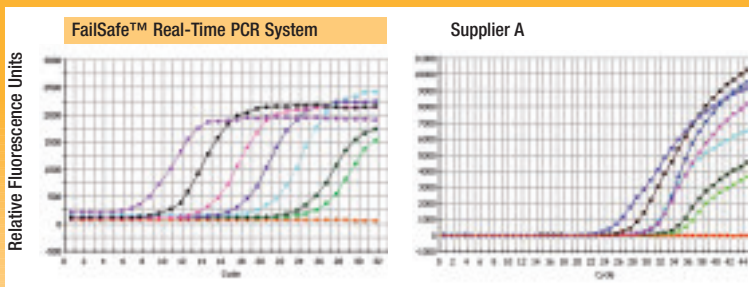


Agarose gel electrophoresis



1. 100 bp ladder
2. FailSafe™
3. Supplier A
4. Supplier B
5. Supplier C
6. Supplier D
7. Supplier E

Broader Dynamic Range



Real-time PCR amplification using 0, 1, 10, 100, 10³, 10⁴, 10⁵, and 10⁶ copies of lambda DNA comparing the FailSafe™ Real-Time PCR System to a hot-start real-time PCR from a leading competitor's kit (Supplier A). Real-time PCR amplification was carried out on Bio-Rad's iCycler iQ™ Real-Time PCR Detection System.

Higher Sensitivity and Faster C_t Value

Supplier	C _t Value (Average of Triplicate Reactions)
FailSafe™ Real-Time PCR System	24.5
Supplier A	26.1
Supplier B	30.1
Supplier C	28.0
Supplier D	27.4
Supplier E	29.8

Real-time PCR amplification of a 181-bp fragment from human DMD exon 47 was performed with 10 ng of human genomic DNA using the FailSafe™ Real-Time PCR System and 5 major hot-start real-time PCR suppliers' kits. C_t values obtained from triplicate reactions of each PCR kit were averaged. Real-time PCR amplification was carried out on Bio-Rad's iCycler iQ™ Real-Time PCR Detection System.

www.epicentre.com/pcr.asp

FailSafe™ Real-Time PCR PreMix Selection Kit FSR0360 48 Reactions <i>Contents:</i> FailSafe™ PCR Enzyme Mix 12 different FailSafe™ Real-Time PCR 2X PreMixes	FailSafe™ Real-Time PCR Capillary PreMix Selection Kit FSRC3832 32 Reactions <i>Contents:</i> FailSafe™ PCR Enzyme Mix 8 FailSafe™ Real-Time PCR Capillary 2X PreMix
FailSafe™ Real-Time PCR System FSR03200 200 Reactions <i>Contents:</i> FailSafe™ PCR Enzyme Mix Choice of two FailSafe™ Real-Time PCR 2X PreMixes	FailSafe™ Real-Time PCR Capillary System FSRC3896 96 Reactions FSRC38384 4 X 96 Reactions <i>Contents:</i> FailSafe™ PCR Enzyme Mix Choice of one FailSafe™ Real-Time PCR Capillary 2X PreMix

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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.

EPICENTRE'S Customer Focus

Scott McClain

IBT Reference Laboratory

I have been incorporating genomics into my professional and academic work since around 1996. Currently, I work for IBT Reference Laboratory in Kansas City, KS and focus on immunology, spending most of my time designing assays that can be used in allergy determination, medicinal clinical trials, and basic research. Although I work on a variety of projects, in several different species, I will focus on the continually expanding field of single nucleotide polymorphisms (SNPs) and my collaborations with EPICENTRE.

I have used EPICENTRE as a source for molecular biology reagents after becoming frustrated with large, non-specialized vendors. Recently, I used a FailSafe™ Real-Time PCR System to develop a reliable PCR protocol to amplify genomic DNA and determine SNPs within the interleukin-4 (IL-4) receptor gene.

PCR can produce poor results in sections of DNA template with high GC content. This is particularly problematic in SNP evaluation, where it is necessary to observe multiple melt-curve peaks. The multiple peaks generated in the melt-curve analyses can easily overlap if the PCR reaction is not fully optimized, making evaluation of the different alleles difficult. The current protocol for analyzing SNPs uses hybridization probes.

EPICENTRE does not currently offer a product for the capillary tube PCR system that does not contain SYBR® Green I, which is incompatible with hybridization probes. However, Fred Hyde, one of EPICENTRE's technical service scientists, has been willing to work with me and sent me a custom "beta-test" kit which is essentially the FailSafe™ Real-Time PCR Capillary PreMix Selection Kit without the SYBR® Green I. This allowed me to efficiently optimize many SNP reactions so that a panel of SNPs, within a particular gene, could be evaluated using multiplex PCR reactions.

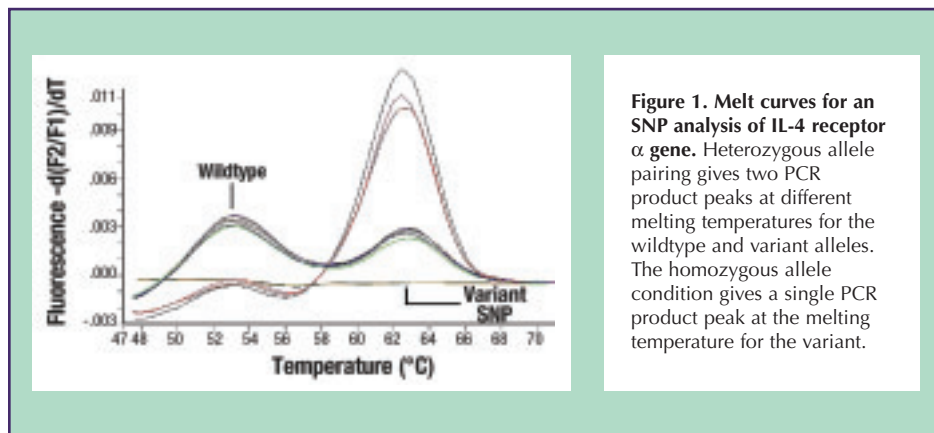


Figure 1. Melt curves for an SNP analysis of IL-4 receptor α gene. Heterozygous allele pairing gives two PCR product peaks at different melting temperatures for the wildtype and variant alleles. The homozygous allele condition gives a single PCR product peak at the melting temperature for the variant.

Figure 1 shows melt curves for an SNP analysis of the recently optimized IL-4 receptor α gene. The graph shows several DNA samples displaying either a heterozygous allele pairing (two PCR product peaks with different melting temperatures) or a homozygous allele condition (single PCR product peak) with the presence of a variant nucleotide.

In addition to SNP analyses, I am also exploring mRNA expression in allergy-mediating cells, such as basophils, in order to measure the *in vivo* expression potential for different forms of allergies. For gene expression I use non-capillary based real-time PCR and use the FailSafe™ Real-Time PCR System for primer development.

The goal of most of my assays is to transfer them to our commercial lab, which requires an easily standardized methodology. Establishing a consistent assay is important because, due to the technical complexity of our analyses, it can be difficult to train scientists and technicians who are new to PCR and gene quantification. With FailSafe I am able to quickly optimize a PCR reaction and then transfer the technology within our company, while maintaining robust, stable, and quantifiable results.

www.epicentre.com/pcr.asp

FailSafe™ Real-Time PCR Capillary PreMix Selection Kit

FSRC3832 32 Reactions

Contents:

FailSafe™ PCR Enzyme Mix
8 FailSafe™ Real-Time PCR Capillary
2X PreMixes

FailSafe™ Real-Time PCR Capillary System

FSRC3896 96 Reactions

FSRC38384 4 X 96 Reactions

Contents:

FailSafe™ PCR Enzyme Mix
Choice of one FailSafe™ Real-Time PCR
Capillary 2X PreMix

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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.



shRNA Offers Important Advantages Over siRNA for RNAi Studies: MessageMuter™ shRNAi Production Kit

Introduction

RNA interference (RNAi) is a powerful technique for elucidation of gene function in eukaryotic cells. The RNAi effect can be mediated by transfecting cultured cells with short interfering RNA (siRNA), which is short, double-stranded RNA of 21 to 23 base pairs, or, more recently demonstrated, with short hairpin RNA (shRNA)^{1,2,3,4} (Figure 1). Though siRNA and shRNA elicit comparable gene silencing results in RNAi experiments^{1,2,5}, preparation of shRNA using EPICENTRE's new MessageMuter™ shRNAi Production Kit offers significant advantages compared to either chemical synthesis or *in vitro* transcription of siRNA.

The MessageMuter shRNAi Production Kit produces transfection-ready shRNA in about 4 hours using a simple, 3-step process⁴ (see side bar on page 13). Compared to chemical synthesis of siRNA, the kit produces shRNA faster and at a significantly lower cost. Compared to *in vitro* transcription of siRNA, shRNA produced using the MessageMuter Kit is faster and easier to prepare and offers greater flexibility in target sequence selection.

Produce an shRNA from a single oligodeoxynucleotide and a single *in vitro* transcription reaction

Traditional methods of producing siRNA by *in vitro* transcription requires the user to design and synthesize at least two and, in some procedures, as many as four oligodeoxynucleotides to serve as *in vitro* transcription templates. Once the oligos

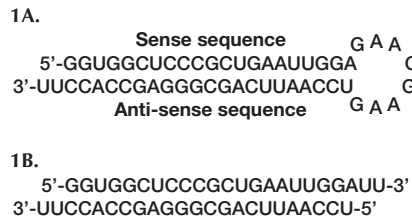


Figure 1. Structural comparison of short hairpin RNA (shRNA) and siRNA directed against the same target sequence of firefly luciferase.

1A. The shRNA produced using the MessageMuter™ shRNAi Production Kit contains a sequence homologous to the target mRNA (sense sequence), a “loop” region and a sequence complementary to the target sequence (anti-sense sequence). In the example shown, the shRNA targets a 21-base sequence of the firefly luciferase mRNA. **1B.** An siRNA targeting the same 21-base sequence of firefly luciferase.

Advantages of shRNA made with the MessageMuter Kit

- Produced from a single oligodeoxynucleotide
- No need to anneal RNA strands
- Target any sequence 21 – 29 bases long

are annealed, two separate *in vitro* transcription reactions of 2 hours or longer are needed to generate the sense and anti-sense strands of the siRNA.

By comparison, shRNA prepared using the MessageMuter Kit is produced from a single oligodeoxynucleotide. Following a 30-minute “fill-in” reaction which generates the transcription template, *in vitro* transcription of shRNA is performed using EPICENTRE's new AmpliScribe™

T7-Flash™ transcription system. The AmpliScribe T7-Flash system enables the user to generate high yields of shRNA in just 30 minutes.

Thus, compared to producing siRNA by *in vitro* transcription, producing shRNA with the MessageMuter kit procedure requires less up-front oligo design and synthesis and uses a shorter, high-yield transcription reaction.

Producing shRNA does not require annealing of two RNA strands

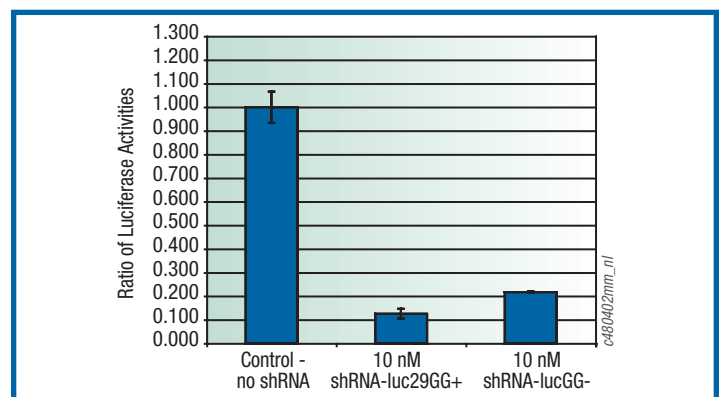
Preparation of siRNA typically requires overnight annealing of the sense and anti-sense strands of RNA produced by the *in vitro* transcription reactions. The overnight annealing adds a significant amount of time to the production procedure and increases the potential for RNase contamination, which will compromise the yield and integrity of the siRNA.

Once transcribed, shRNA spontaneously forms a hairpin structure in the transcription reaction mix and avoids the need for a long and potentially harmful annealing step. Following clean-up, the shRNA is ready for transfection into cells.

shRNA is readily produced against any target sequence

For efficient *in vitro* transcription initiation, T7 RNA polymerase requires a ‘GG’ or ‘GA’ dinucleotide⁶ at the start of the transcript. When preparing siRNA by *in vitro* transcription, this limits the selection of mRNA targets to sequences that begin with ‘GG’ or ‘GA’. Alternatively,

Figure 2. Adding a 5'-GG to the target sequence of a shRNA does not significantly affect effective gene silencing. A 29-base shRNA (shRNA-luc29GG+) was prepared against a 29-base target sequence in firefly luciferase mRNA. The targeted sequence contained a 5'-GG dinucleotide. A 27-base shRNA (shRNA-luc27GG-) was prepared against a 27-base target sequence two bases “upstream” of the shRNA-luc29GG+ target and lacking a 5'-GG or 5'-GA dinucleotide. The dinucleotide ‘GG’ was added 5' to the target sequence in the user-designed oligo used to produce shRNA-luc27GG-, so this shRNA contains a ‘GG’ that is not target specific. The shRNA-luc29GG+ and shRNA-luc27GG- were independently co-transfected into HeLa cells in triplicate with firefly and *Renilla* (control) luciferase expression vectors. Results are presented as a ratio of firefly luciferase to *Renilla* luciferase activities. Both shRNA-luc29GG+ and shRNA-luc27GG- reduced firefly luciferase expression by 80% or more.



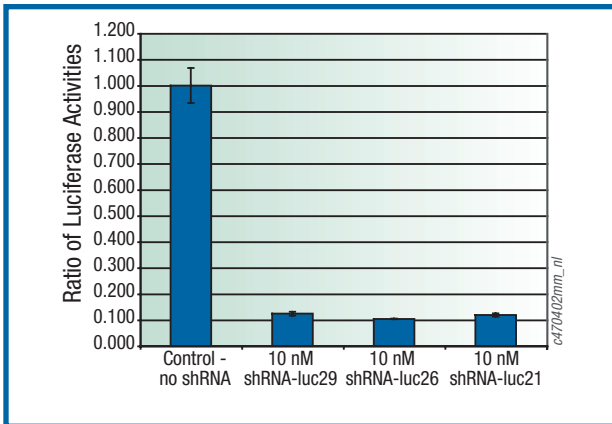


Figure 3. shRNA produced against target sequences ranging from 21 to 29 bases effectively induced silencing of firefly luciferase. The MessageMuter™ Kit was used to produce shRNAs directed against 21-base (shRNA-luc21), 26-base (shRNA-luc26), and 29-base (shRNA-luc29) target sequences in the same region of firefly luciferase mRNA. Transfection of COS-7 cells was done in triplicate with firefly and *Renilla* (control) expression vectors and 10 nM of the respective shRNA-luc. Results are presented as a ratio of firefly luciferase to *Renilla* luciferase activities.

another supplier's siRNA production kit allows the user to synthesize a transcription template that adds a 5'-GG dinucleotide to the target sequence. However, the 5'-GG of the resulting siRNA must be removed prior to cell transfection using a time-consuming and carefully controlled ribonuclease digestion. Thus, *in vitro* transcription of siRNA either limits the user's selection of target mRNA sequences or, if using the other supplier's kit, requires an additional ribonuclease treatment.

In contrast, shRNA can be easily prepared for any target sequence, even those without the 5'-GG or 5'-GA. To prepare MessageMuter shRNA to these target sequences, see the MessageMuter protocol at www.epicentre.com/shRNA.asp. shRNA targeted to these sequences does not require post-transcriptional manipulation.

Additionally, shRNA can be designed against target sequences from 21 to 29 bases long. Targeting larger sequences, in conjunction with cellular processing of the shRNA^{1,5} does not alter, and may enhance the overall silencing effect. Using the MessageMuter shRNAi Production Kit, we prepared shRNAs directed against 21-base, 26-base and 29-base target sequences in the same region of firefly luciferase mRNA. These shRNAs demonstrated virtually no difference in their ability to silence expression of firefly luciferase in COS-7 cells (Figure 3). Comparable results were seen in HeLa cells, Normal Rat Kidney (NRK) cells and mouse embryo fibroblast (NIH/3T3) cells (data not shown).

Discussion

EPICENTRE's new MessageMuter™ shRNAi Production Kit produces transfection-

ready shRNA, faster and easier than *in vitro* transcription methods for making siRNA and at significantly lower cost than chemical synthesis methods. *In vitro* transcription of shRNA using the MessageMuter Kit enables the user to:

- Eliminate annealing of RNA strands.
- Produce shRNA from a single oligodeoxynucleotide.
- Produce shRNA from a single *in vitro* transcription reaction.
- Target a sequence that does not begin with a 'GG' or 'GA' dinucleotide without the need for a post-transcriptional ribonuclease digestion.
- Target a sequence of 21 to 29 bases in length.

References

1. Paddison, P.J. *et al.* (2002) *Genes & Development* **16**, 948.
2. Yu, J-Y *et al.* (2002) *Proc. Natl. Acad. Sci. USA* **99**(9), 6047.
3. Meis, J.E. (2003) *EPICENTRE Forum* **10**(2), 1.
4. Meis, J.E. (2004) *EPICENTRE Forum* **11**(1), 1.
5. M^cManus, M.T. *et al.* (2002) *RNA* **8**, 842.
6. Milligan, J.F. *et al.* (1987) *Nuc. Acids Res.* **15**(21), 8783.

www.epicentre.com/messagemuter.asp

MessageMuter™ shRNAi Production Kit

MM031110 10 Reactions

Each kit provides reagents to prepare 10 different shRNA in sufficient quantity for 100's of transfections. User provides one oligodeoxynucleotide.

Produce transfection-ready shRNA in about 4 hours using the MessageMuter shRNAi Production Kit

The MessageMuter shRNAi Production Kit utilizes a simple and unique 3-step process that yields transfection-ready shRNA in about 4 hours. Each reaction produces enough shRNA for 100's of transfections.

1. Anneal the T7 Promoter Oligo (a short oligodeoxynucleotide containing a phage T7 transcription promoter sequence, provided in the kit) to an oligodeoxynucleotide designed and provided by the user.
2. "Fill-in" the ends of the annealed duplex using the Klenow exo-minus fragment of DNA Polymerase and dNTPs (both provided in the kit) to generate a linear double-stranded DNA template for *in vitro* transcription by T7 RNA Polymerase.
3. Transcribe shRNA from the DNA template in a rapid, high-yield *in vitro* transcription reaction using reagents that are provided in the kit. The transcribed RNA spontaneously forms a hairpin structure (shRNA) in solution. Following clean-up, the shRNA is ready for transfection into cultured cells.

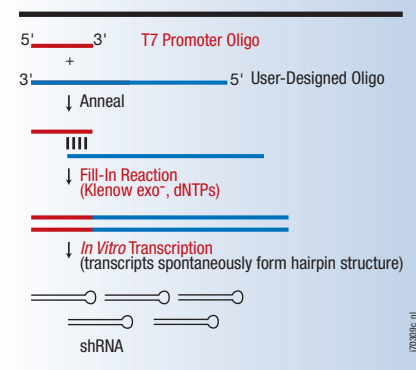


Figure 4. Overview of the method used to produce shRNA using the MessageMuter™ shRNAi Production Kit. All reagents are supplied with the kit except for the user-designed oligo – a 60- to 76-base oligodeoxynucleotide.

Generate High Yields of Biotin-Labeled RNA Using the AmpliScribe™ T7-Flash™ or the AmpliScribe™ T3-Flash™ Transcription Kits

Ronald Meis, EPICENTRE

EPICENTRE's new AmpliScribe™ T7-Flash™ and AmpliScribe™ T3-Flash™ Transcription Kits produce the highest yield of RNA transcripts from an *in vitro* transcription reaction in the shortest reaction time. Previously, we demonstrated that an AmpliScribe T7-Flash reaction produces 160 to 180 µg (8 to 9 mg/ml) of RNA in a 30 minute reaction^{1,2}...more RNA than other kits produce in 2 hours. Subsequently, we have shown that an AmpliScribe T7-Flash reaction can be scaled up to produce milligram quantities of RNA in 30 minutes³ and that AmpliScribe T7-Flash reactions produce exceptionally high yields of short (<500 bases) RNA transcripts.⁴

Here we report reaction conditions that produce high yields of biotinylated-RNA using the AmpliScribe T7-Flash or AmpliScribe T3-Flash Kits.

1. Assemble the reaction components at room temperature.

- X µl RNase-Free water, for a final reaction volume of 20 µl
- 1.0 µg linear template DNA with a T7 or T3 promoter
- 2.0 µl AmpliScribe™ T7-Flash™ or AmpliScribe™ T3-Flash™ 10X Reaction Buffer
- 1.8 µl 100 mM ATP
- 1.8 µl 100 mM CTP
- 1.8 µl 100 mM GTP
- 1.3 µl 100 mM UTP
- 5.0 µl 10 mM Biotin-16-UTP[†] (Roche)
- 2.0 µl 100 mM DTT
- 2.0 µl AmpliScribe™ T7-Flash™ or AmpliScribe™ T3-Flash™ Enzyme Solution

20 µl Total reaction volume.

2. Incubate the reaction at 37°C for 30 minutes.
3. Add 1µl of RNase-Free DNase I (included in the kits) and incubate at 37°C for 15 minutes to remove the DNA template.
4. Purify the biotin-labeled RNA as described in the AmpliScribe T7-Flash and AmpliScribe T3-Flash product literature.

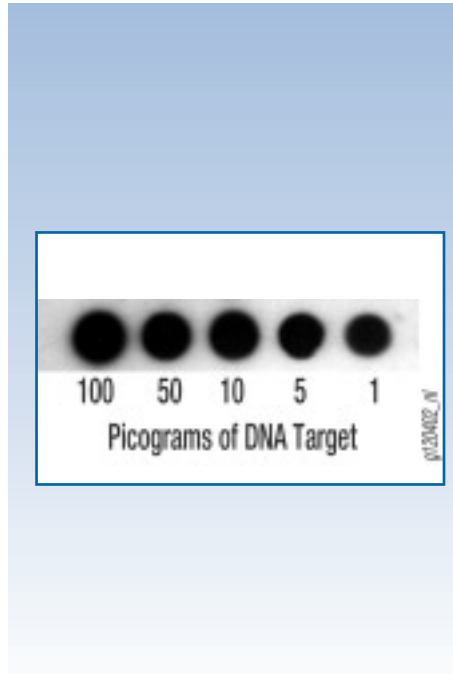


Figure 1. Dot-blot analysis of biotinylated-RNA produced from an AmpliScribe™ T7-Flash™ reaction. Serial dilutions of unlabeled, control-plasmid template DNA, supplied in the AmpliScribe T7-Flash Kit, were spotted onto a Nytran® SPC membrane (Schleicher and Schuell) using a vacuum manifold. The membrane was probed with 200 ng of biotinylated-RNA, about 0.1% of the biotinylated-RNA produced by the reaction procedure described in the text. Hybridized RNA was detected by chemiluminescence using a streptavidin-alkaline phosphatase conjugate and CDP-Star® substrate with the Southern-Star™ System (ABI/Tropix), according to the manufacturer's protocol. The biotinylated-RNA detected 1 pg of target DNA with a 15 second exposure.

Results

AmpliScribe T7-Flash or T3-Flash transcription reactions are easily modified to incorporate biotin-rNTP nucleotides. The 30-minute transcription reaction described above yields ≥160 µg (>8 mg/ml) of biotinylated-RNA using the 1.4 kb control DNA template provided in the kits. As shown in Figure 1, 200 ng of the resulting biotinylated-RNA detected as little as 1 pg of target DNA in a dot-blot assay. An AmpliScribe T7-Flash or AmpliScribe T3-Flash transcription reaction produces enough biotinylated RNA to do more than 800 comparable experiments.

† Biotin-nucleotides are not supplied with the AmpliScribe T7-Flash or AmpliScribe T3-Flash Kits. Biotin-rNTPs, other than UTP, may be used, but a final concentration of 2.5 mM biotin-rNTP and 6.5 mM unlabeled rNTP should be maintained in the transcription reaction.

References

1. Meis, R. and Pease, J. (2003) *EPICENTRE Forum* **10**(2) 6.
2. Meis, R. *et al.* (2003) *Bioscience Technology*, **28**(9) 8.
3. *EPICENTRE Forum* **10**(3) 8.
4. Meis, R. (2004) *EPICENTRE Forum* **11**(1) 7.

www.epicentre.com/flash.asp

AmpliScribe™ T7-Flash™ Transcription Kit

ASF3057-F12	5 Reactions	\$ 50
ASF3257	25 Reactions	
ASF3507	50 Reactions	

AmpliScribe™ T3-Flash™ Transcription Kit

ASF03725	25 Reactions
ASF03750	50 Reactions

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* CDP-Star is a registered trademark and Southern-Star is a trademark of Applera Corp., Norwalk, CT.

Get the Highest Yield of 5'-Capped RNA from an *In Vitro* Transcription Reaction in Just 30 Minutes Using the AmpliCap-Max™ High Yield Message Maker Kits

EPICENTRE's new AmpliCap-Max™ T7 and AmpliCap-Max™ T3 High Yield Message Maker Kits are specially formulated to produce the highest yield of 5'-capped RNA from an *in vitro* transcription reaction in the shortest reaction time.

The new AmpliCap-Max™ T7 and AmpliCap-Max™ T3 High Yield Message Maker Kits feature:

- High yields, up to 60 µg, of 5'-capped RNA per reaction.
- 30-minute reaction time.
- Up to 80% of the RNA is capped.

- An optimized AmpliCap-Max™ Cap/NTP PreMix, containing m⁷G[5']ppp[5']G Cap Analog and NTPs.
- A separate vial of GTP for efficient production of long, 5'-capped RNA.

www.epicentre.com/acmax.asp

AmpliCap-Max™ T7 High Yield Message Maker Kit

ACM04037 25 Reactions

AmpliCap-Max™ T3 High Yield Message Maker Kit

ACM04033 25 Reactions

Each kit contains either the T7 or T3 AmpliCap-Max™ Enzyme Mix (including RNase inhibitor), AmpliCap-Max™ Cap/NTP PreMix, 20 mM GTP AmpliCap-Max™ 10X Transcription Buffer, 100 mM DTT, RNase-Free DNase I, Control Template DNA, Sterile Water

Klenow Fragment Exo⁻ DNA Polymerase

Klenow Fragment Exo⁻ DNA Polymerase is a DNA-dependent DNA polymerase that lacks both the 5' → 3' and 3' → 5' exonuclease activities of *E. coli* DNA Polymerase I, from which it is derived.

Applications

- Random primer labeling of DNA.
- DNA sequencing.
- Second-strand cDNA synthesis.
- Strand displacement amplification.

Concentration 20 Units/µl

Specific Activity Greater than 1 X 10⁴ Units/mg

Unit Definition

One Unit of Klenow Fragment Exo⁻ DNA Polymerase converts 10 nmoles of dNTPs into acid-insoluble material in 30 minutes at 37°C.

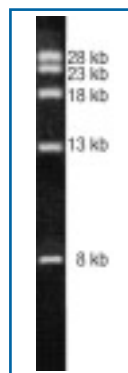
www.epicentre.com/exominus.asp

Klenow Fragment Exo⁻ DNA Polymerase

KL04011K 20 Units/µl 1000 Units

Supercoiled DNA Marker Set

The new Supercoiled DNA Marker Set produces five discrete bands of 8 kb, 13 kb, 18 kb, 23 kb, and 28 kb with approximately equal staining intensity. Use 10 µl of the ready-to-load solution per lane in a minigel. This ladder complements the BAC-Tracker™ Supercoiled DNA Ladder with bands at 38 kb, 55 kb, 95 kb, and 120 kb.



Applications

- Size analysis of supercoiled CopyControl™ pCC1™ PCR clones by agarose gel or Pulse-Field Gel Electrophoresis.
- Size analysis of any supercoiled plasmid or extrachromosomal DNAs larger than 8 kb.

www.epicentre.com/scdna.asp

Supercoiled DNA Marker Set

SCD31050 500 µl

RepliPHI™ Phi29 DNA Polymerase

- Available at 1 µg/µl and at 0.1 µg/µl
- Free of Detectable DNA Contamination
- Specific Activity of 1 X 10⁶ Units/mg

Unit Definition:

One unit of RepliPHI Phi29 DNA Polymerase converts 25 pmoles of deoxyribonucleotides into acid insoluble material in 30 minutes at 30°C.



RepliPHI™ Phi29 DNA Polymerase 1 µg/µl

RepliPHI™ Phi29 DNA Polymerase (Enzyme Only)

PP031010	10 µg (10,000 Units)	1 µg/µl (1,000 Units/µl)
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RepliPHI™ Phi29 Reagent Set (Enzyme, dNTPs, Buffer, DTT)

RH031110	10 µg (10,000 Units)	1 µg/µl (1,000 Units/µl)
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RepliPHI™ Phi29 DNA Polymerase 0.1 µg/µl

RepliPHI™ Phi29 DNA Polymerase (Enzyme Only)

PP040110	10 µg (10,000 Units)	0.1 µg/µl (100 Units/µl)
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RepliPHI™ Phi29 Reagent Set (Enzyme, dNTPs, Buffer, DTT)

RH040210	10 µg (10,000 Units)	0.1 µg/µl (100 Units/µl)
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EPI Announcements, etc.



Remove PCR Inhibitors

The SoilMaster DNA Kit contains spin columns and resin for removing PCR inhibitors from the DNA. These columns and resin are now available as stand-alone products.

Spin Columns - SC04350
Inhibitor Removal Resin - SR04350

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