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Featured Papers on RT-PCR/PCR:

New Quantitative RT-PCR System – 3

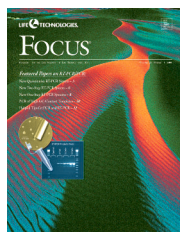
New Two-Step RT-PCR System – 6

New One-Step RT-PCR Systems – 8

PCR of High GC-Content Templates – 10

Helpful Tips for PCR and RT-PCR – 12





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 Photograph by Merle Wasson.
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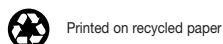
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SPECIAL WEB PROTOCOL

Single cell gene detection!
 Go to the Life Technologies web site to find out how to detect genes from a single cell using one-step RT-PCR. Find this protocol at www.lifetech.com. Click on FOCUS Journal under "Technical Resources". Simply open FOCUS 22.1 Table of Contents and look for "[Detection of Genes From a Single Cell Using One-step RT-PCR.](#)"



Essential Technologies for the Science of Life™

The Evolution of RT-PCR Products

RT-PCR and PCR are powerful techniques that have allowed researchers to answer questions not possible with classic molecular biology techniques. RT-PCR and PCR have been refined and improved as they have gained popularity.

Life Technologies has applied our expertise in reverse transcriptase engineering to RT-PCR starting with GIBCO BRL® SUPERSCRIPT™ RNase H⁻ RT and followed closely by the release of the SUPERSCRIPT Preamplification System [see *FOCUS* (1991) 13, 26]. The original two-step RT-PCR system has been updated and improved with the GIBCO BRL SUPERSCRIPT II First-Strand Synthesis System for RT-PCR to provide you with superior results (see page 6).

The convenience of the one-step RT-PCR format was captured with the introduction of the GIBCO BRL SUPERSCRIPT One-Step RT-PCR System (contains *Taq* DNA Polymerase) [see *FOCUS* (1997) 19, 39]. As new PCR enzymes have been introduced, we have now expanded our SUPERSCRIPT One-Step systems to include GIBCO BRL PLATINUM® *Taq* DNA Polymerase (for higher specificity and yield) and PLATINUM *Taq* DNA Polymerase High Fidelity (for long templates; see page 8).

The use of RNase H⁻ RTs (SUPERSCRIPT II or THERMOSCRIPT™ RT) has made more challenging RT-PCR easier due to their greatly improved sensitivity and yield of full-length cDNA. The higher thermostability of these enzymes also allows for higher reaction temperatures, which minimize problems of secondary structure in the mRNA. Xu *et al.* (page 3) describe a new system using THERMOSCRIPT RT for quantitative RT-PCR.

For some difficult RNA targets, an optimized PCR system is as critical as the appropriate RT. For example, some targets

Comparison of Two-Step and One-Step RT-PCR	
TWO-STEP PROCEDURE	ONE-STEP PROCEDURE
Prime first-strand cDNA with: Oligo(dT) Random hexamers GSP primers	Prime first-strand cDNA with: GSP primers
Provides: <ul style="list-style-type: none"> • Flexibility <ul style="list-style-type: none"> Choice of primers. Choice of amplification enzyme. • Ability to optimize for difficult RT-PCR. <ul style="list-style-type: none"> Combine with PLATINUM enzymes for higher specificity. Combine with PLATINUM <i>Pfx</i> DNA Polymerase for greater fidelity. • Ideal for detecting or quantifying several messages from a single sample. 	Provides: <ul style="list-style-type: none"> • Convenience <ul style="list-style-type: none"> Amplification enzymes premixed with reverse transcriptase. Fewer pipetting steps and reduced chances of contamination. • High sensitivity. • Ideal for analysis of large numbers of samples. • Ideal for quantitative PCR.
Choose from: <ul style="list-style-type: none"> • SUPERSCRIPT First-Strand Synthesis System for RT-PCR • THERMOSCRIPT RT-PCR Systems 	Choose from: <ul style="list-style-type: none"> • SUPERSCRIPT One-Step RT-PCR System • SUPERSCRIPT One-Step RT-PCR System with PLATINUM <i>Taq</i> DNA Polymerase • SUPERSCRIPT One-Step RT-PCR for Long Templates • PLATINUM Quantitative RT-PCR THERMOSCRIPT One-Step System
Notes on amplification enzymes and product size:	
<ul style="list-style-type: none"> • Use <i>Taq</i> DNA Polymerase or PLATINUM <i>Taq</i> DNA Polymerase for products <4 kb. • Use PLATINUM <i>Pfx</i> DNA Polymerase or PLATINUM <i>Taq</i> DNA Polymerase High Fidelity for products <12 kb. • Use ELONGASE Enzyme Mix for products >12 kb. • For one-step RT-PCR, use <i>Taq</i> DNA Polymerase or PLATINUM <i>Taq</i> DNA Polymerase for products <3.5 kb; and use PLATINUM <i>Taq</i> DNA Polymerase High Fidelity for products <9 kb. 	

PCR Enzyme Selection Guide				
Enzyme	Size	Yield	Specificity	Fidelity
<i>Taq</i> DNA Polymerase	<5 kb	•	•	•
PCR SUPERMIX	<5 kb	•	•	•
PLATINUM <i>Taq</i> DNA Polymerase	<5 kb	••	••	•
PLATINUM PCR SUPERMIX	<5 kb	••	••	•
PLATINUM <i>Taq</i> PCR _x DNA Polymerase	<5 kb	••	•• ^a	•
PLATINUM <i>Pfx</i> DNA Polymerase	<12 kb	••	•• ^a	••••
PLATINUM <i>Taq</i> DNA Polymerase High Fidelity	<12 kb	•••	••	•••
PCR SUPERMIX High Fidelity	<12 kb	••	•	•••
PLATINUM GENOTYPE <i>Tsp</i> DNA Polymerase	<0.5 kb	•	••	•
ELONGASE Enzyme Mix	<30 kb	••	•	••

^a Contains PCR_x Enhancer System, ideal for GC-rich templates or other problematic templates.

are difficult to amplify due to high-GC content. A novel co-solvent, PCR_x Enhancer Solution, helps with these difficult templates (see page 10).

As a leader in RT-PCR and PCR technology development, we are continually updating and expanding programs to share information—through TECH-ONLINESM on our web site, training courses in our Training Center (see inside front cover for schedule), and documents from Technical Services

(page 12). Above are quick reference tables to help you choose the right reverse transcription system and amplification enzyme.

Life Technologies is committed to providing you the *essential technologies for the science of life*. We hope that these new products and protocol information will improve your PCR and RT-PCR. **FOCUS**

Doreen Cupo
Editor, *FOCUS* Magazine

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Jun E. Lee, Michael Smith,
Jason Potter, Gulshan Dhariwal,
Kim Rosenthal, Margret Nathan,
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One-Step Analysis and Quantification of RNA by RT-PCR: Using High-Temperature Reverse Transcription

ABSTRACT

We have developed a one-step RT-PCR system for the convenient, sensitive, and reproducible detection and quantification of RNA molecules. This new system, the GIBCO BRL® PLATINUM® Quantitative RT-PCR THERMOSCRIPT™ One-Step System, takes advantage of high-temperature reverse transcription by THERMOSCRIPT Plus RT coupled to an automatic hot-start PCR (1,2) provided by PLATINUM Taq DNA Polymerase (3). cDNA synthesis and PCR are performed in a single tube using gene-specific primers and RNA target(s) from either total RNA or mRNA. High-temperature reverse transcription improves primer specificity in cDNA synthesis and can ease limitations imposed by secondary structure or high-GC content (4,5). Reaction conditions have been optimized for real-time quantitative RT-PCR (qRT-PCR) and are effective also for conventional end-point RT-PCR.

Real-time qRT-PCR provides a simple and highly reliable method for quantitative analysis of gene expression (6). This technique uses fluorogenic hybridization probes (6-8) or dsDNA-specific fluorescent dyes (9,10) to detect PCR product during amplification (real-time detection) without purification or separation by gel electrophoresis. The sensitivity of these probes allows measurement of PCR product during the exponential phase of amplification before critical reactants become limiting. Analysis of the resulting PCR growth curves (6,11) from real-time qRT-PCR provides highly sensitive, precise, and accurate quantification of target sequence with a linear-dose response over a wide range of target concentrations.

Accurate and reliable real-time quantification of gene expression is dependent on highly efficient and consistent reverse transcription of mRNA and amplification of the resulting cDNA. These attributes are effected by numerous factors, such as template sequence, intrinsic secondary structure (12), potential variations in RNA stability, as well as enzymatic properties of the particular reverse transcriptase. Use

Component	Amount
THERMOSCRIPT Plus RT/ PLATINUM Taq DNA Polymerase	100 µl
2X THERMOSCRIPT reaction mix (buffer with 0.4 mM of each dNTP, 6 mM MgSO ₄)	2 × 1.25 ml
50 mM magnesium sulfate	1 ml
product profile sheet	one

TABLE 1. Components of the PLATINUM Quantitative RT-PCR THERMOSCRIPT One-Step System.

of RTs with reduced RNase H activity has been shown to improve both the length and yield of first-strand product (13). Additionally, use of an elevated temperature to relax intrinsic secondary structures (4,5) can improve cDNA yield for some messages. Previously the use of THERMOSCRIPT RT has been described in two-step RT-PCR (14,15). THERMOSCRIPT RT is a genetically engineered derivative of avian retroviral reverse transcriptase that has reduced RNase H activity and increased thermostability. For high-throughput analysis, the use of the more convenient and sensitive one-step assay format is preferred. Here we describe a new one-step quantitative RT-PCR system, the PLATINUM Quantitative RT-PCR THERMOSCRIPT One-Step System.

PCR Product	Forward Primer	Reverse Primer	Fragment Size (bp)
Pol ε	AAGGC TGGCG GATTA CTGCC	GCGGG TGAGT GAGGA CGGCT CAG	606
PP2A	GTTTCG ATGTC CAGTT ACTGT CT	CACAT CTTAT TATCT GCAGT CTCTC AGAG	1,093
PP2A	GTTTCG ATGTC CAGTT ACTGT CT	AGGCA GTGAG AGGAA GATAG TC	331
RPA	CAAGA TGTGG AACAG TGGAT TC	CCATC TATCT TGATG TTGTA ACAAG C	1,092
β-actin	CCTGG CACCC AGCAC AAT	GGGCC GGACT CGTCA TAC	145
Tc ^R	GCGGA TGAAC AGGCA GACAT	GAGGA TGGCA GCGTG ATTTTC	146
18S rRNA	CGGCT ACCAC ATCCA AGGAA	GCTGG AATTA CCGCG GCT	187

Probe	Sequence	Reporter
Tc ^R	CACCG CCGAGC ACAGC ACCAC C	FAM
β-actin	AGCCG CCGAT CCACA CGGAG T	FAM
18S rRNA	TGCTG GCACC AGACT TGCCC TC	JOE

TABLE 2. Primer and probe sequences.

Quantitative Analysis continued

METHODS

Total HeLa RNA was isolated with TRIzol[®] Reagent. One-step RT-PCR was carried out with the PLATINUM Quantitative RT-PCR THERMOSCRIPT One-Step System (Cat. No. 11731, *table 1*). Reactions (50- μ l volume) were assembled on ice in 0.2-ml PCR tubes or 96-tube reaction plates using GIBCO BRL Distilled Water, DNase/RNase-Free, 2X THERMOSCRIPT reaction mix, gene-specific primers (200 nM each, *table 2*), total HeLa RNA, and THERMOSCRIPT Plus/PLATINUM *Taq* enzyme mix, and transferred to a thermal cycler equilibrated to the cDNA synthesis temperature. Sample RNA (5 to 10 μ l) was added to reactions as a final step to minimize cross-contamination. Real-time RT-PCR was performed using specific TaqMan[®] Probes (100 nM final concentration, *table 2*), the ABI PRISM[®] 7700, and SDS analysis software. First-strand synthesis was performed at 60°C for 30 min unless indicated otherwise. This was immediately followed by 95°C for 5 min and PCR cycling. Real-time qRT-PCRs were amplified for 40 to 45 cycles of 95°C for 15 s and 62°C for 1 min. End-point RT-PCRs were amplified for 35 cycles of 95°C for 15 s, 55°C for 30 s, and 72°C for 1 min.

RESULTS AND DISCUSSION

EFFECT OF RT REACTION TEMPERATURE. For some templates, higher-temperature cDNA synthesis can improve the yield of one-step RT-PCR (*figure 1*). Strongest band intensities were obtained using 60°C. The high-temperature first-strand synthesis also can increase the stringency of primer annealing and minimize primer dimer and other non-specific artifacts. Primer artifact can be especially problematic in one-step RT-PCR since most RTs can use either RNA or DNA as template, and the extended incubation at

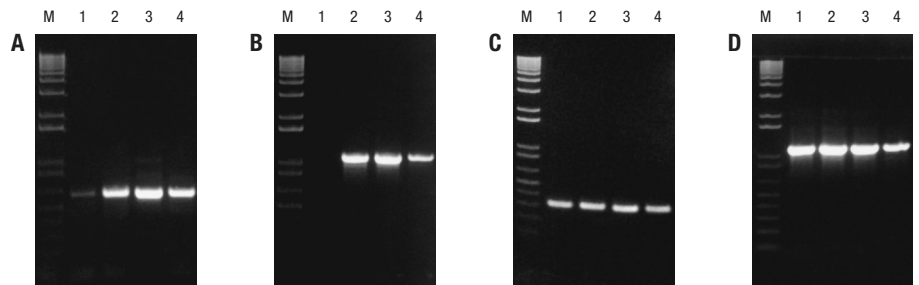


FIGURE 1. Effect of RT temperature on RT-PCR. cDNA was synthesized from 10 ng of total HeLa RNA at 50°C, 55°C, 60°C, or 65°C (lanes 1-4, respectively). One-step RT-PCR was used to amplify a 606-bp region of DNA polymerase ϵ (panel A); 1,093-bp (panel B) or 331-bp (panel C) regions of protein phosphatase 2A; and a 1,092-bp fragment of the 30-kDa subunit of replication protein A (panel D). 8 μ l of each 50- μ l reaction were analyzed by electrophoresis on a 1% agarose gel.

low temperature required for cDNA synthesis can exacerbate nonspecific annealing and extension of primers. Additionally, high temperature can relax intrinsic secondary structure to facilitate hybridization of the primer within closed structured regions, or aid procession of the RT.

REAL-TIME qRT-PCR. The PLATINUM Quantitative RT-PCR System had a large dynamic range that allowed quantitation of

β -actin message from 5 μ g to 50 fg of total HeLa RNA (*figure 2*). In contrast, quantitation was only possible for 50 ng to 5 pg of input RNA with the rTth system. Consistent and reliable reverse transcription of mRNA by THERMOSCRIPT Plus RT was evident in the high precision of quantification shown in *figure 3*. Similar results were seen using molecular beacons (8) for detection (*data not shown*).

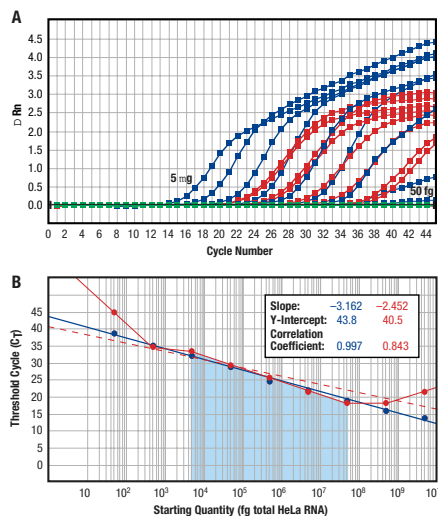


FIGURE 2. Real-time qRT-PCR. qRT-PCR of a 145-bp β -actin fragment was performed with the THERMOSCRIPT One-Step System (■) or an rTth System (■). Starting RNA varied from 5 μ g to 50 fg in 10-fold serial dilutions. Normalized relative fluorescence (ΔR_n) was collected using TAMRA as the passive reference. *Panel A.* Linear-scale amplification plots. *Panel B.* Semi-log standard curve plots. Linear regression analysis is depicted as dashed lines. The linear dynamic region for the rTth system (correlation coefficient = 0.999) is highlighted in light blue.

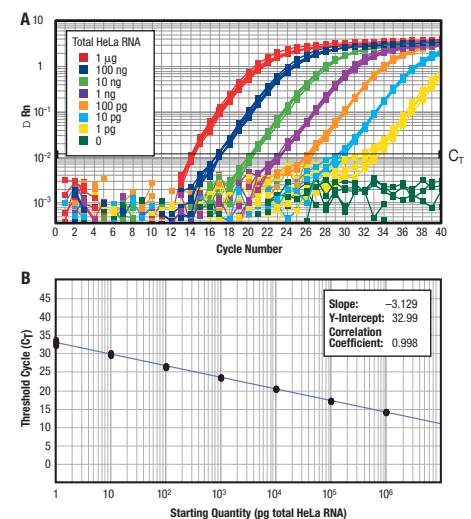


FIGURE 3. Reproducibility of one-step qRT-PCR. Amplification of a 145-bp β -actin fragment was carried out on 6 replicate RNA samples from 10-fold serial dilutions of total HeLa RNA (1 μ g to 1 pg) as described in *Methods*. *Panel A.* Log-scale amplification plots. *Panel B.* Semi-log standard curve plot.

The consistency of cDNA synthesis efficiency and broad range of starting target concentration are ideal for relative expression studies and multiplex RT-PCR. Here, the desired target gene and invariant high-copy housekeeping gene are co-amplified in the same reaction. Reference gene levels are used to normalize target gene quantification and compensate for variations in the amount or quality of RNA. A high capacity for cDNA synthesis and broad dynamic range are crucial in order to capture subtle changes in refer-

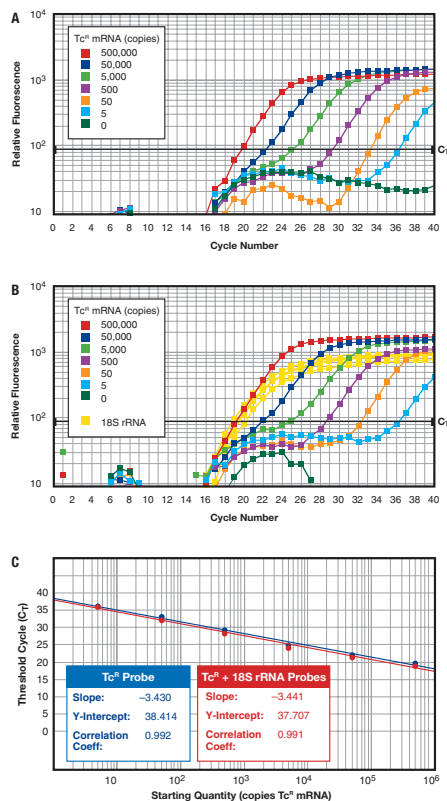


FIGURE 4. Multiplex one-step qRT-PCR. *In vitro* synthesized tetracycline-resistance (Tc^R) mRNA was added to 10 ng total HeLa RNA. One-step qRT-PCR was carried out in 25- μ l volumes essentially as described in *Methods*. Amplification of Tc^R mRNA was performed using 100 nM 6-FAM-labeled TaqMan probe and 200 nM each primer. Amplification of 18S rRNA used 50 nM JOE-labeled TaqMan probe and 100 nM each primer. The passive reference was set to "none." *Panel A.* Log-scale amplification plots for Tc^R mRNA. *Panel B.* Log-scale amplification plots for multiplexed qRT-PCR of Tc^R mRNA and 18S rRNA. *Panel C.* Composite standard curves for Tc^R mRNA performed alone (■) or co-amplified with 18S rRNA (■).

ence gene amounts while maintaining accurate and sensitive quantification of the desired target gene. With the THERMOSCRIPT One-Step system, standard curve plots and corresponding C_T values for Tc^R qRT-PCR were identical whether segregated or co-amplified with r18S (*figure 4*). Multiplex qRT-PCR did not affect quantification of Tc^R mRNA, even for very low target amounts (5 copies). RT-PCR amplification efficiency for the 18S rRNA and Tc^R mRNA was equivalent, as indicated by comparable standard curve slopes obtained for each target system when run separately (*data not shown*).

In summary, the combination of high-temperature, high-efficiency cDNA synthesis and automatic hot start simplifies and improves the reliability of qRT-PCR. The convenient one-step format is ideally suited to the demands of RNA quantification and high-throughput gene expression studies. **FOCUS**

REFERENCES

1. Chou, Q., Russell, M., Birch, D., Raymond, J., and Bloch, W. (1992) *Nucleic Acids Res.* 20, 1717.
2. Sharkey, D.J., Scalice, E.R., Christy, K.G., Atwood, S.M., and Daiss, J.L. (1994) *BioTechnology* 12, 506.
3. Westfall, B., Sitaraman, K., Hughes, J., and Rashtchian, A. (1997) *FOCUS* 19, 46.
4. Myers T.W. and Gelfand D.H. (1991) *Biochem.* 6, 7661.
5. Brooks, E.M., Sheffin, L.G., and Spaulding, S.W. (1995) *BioTechniques* 19, 806.
6. Gibson, U.E.M., Heid, C.A., and Williams, P.M. (1996) *Genome Res.* 6, 995.
7. Heid, C.A., Stevens, J., Livak, K.J., and Williams, P.M. (1996) *Genome Res.* 6, 986.
8. Tyagi, S. and Kramer, F.R. (1996) *Nature Biotechnology* 14, 303.
9. Higuchi, R., Dollinger, G., Walsh, P.S., and Griffith, R. (1992) *Biotechnology* 10, 413.
10. Wittwer, C.T., Herrmann, M.G., Moss, A.A., and Rasmussen, R.P. (1997) *Biotechniques* 22, 130.
11. Higuchi, R., Fockler, C., Dollinger, G., and Watson, R. (1993) *Biotechnology* 11, 1026.
12. Kuo, K.W., Leung, M.F., and Leung, W.C. (1997) *Mol. Cell Biochem.* 177, 1.
13. Gerard, G.F. (1998) *FOCUS* 20, 65.
14. Schwabe, W., Lee, J.E., Xu, R.H., Sitaraman, K., Smith, M., Potter, R.J., Rosenthal, K., Rashtchian, A., and Gerard, G.F. (1998) *FOCUS* 20, 30.
15. Schuster, D.M., Darfler, M., Lee, J.E., and Rashtchian, A. (1998) *FOCUS* 20, 34.

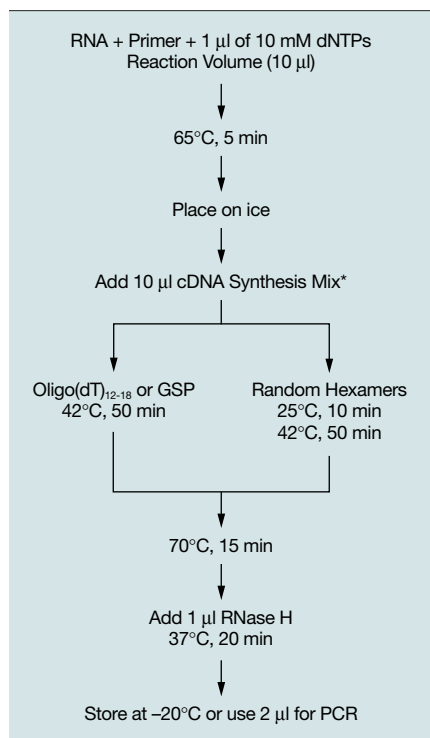


"We don't have any books, sir. This is a DNA Library."

A New Highly Sensitive Two-Step RT-PCR System

ABSTRACT

A sensitive and high-yield SUPERSCRIPT™ First-Strand Synthesis System for RT-PCR has been developed. This system can detect β -actin or GAPDH mRNA in as low as 1 pg of total HeLa RNA and can detect 100 copies of in vitro transcribed CAT mRNA. Full-length genes (up to 7 kb) were detected with <100 ng of total RNA and a 12.3-kb sequence with <2 μ g of total RNA. The SUPERSCRIPT First-Strand Synthesis System provides an effective tool for detecting gene expression and cloning full-length genes.



* cDNA Synthesis Mix consists of 2 μ l of 10X RT buffer, 4 μ l of 25 mM MgCl₂, 2 μ l of 0.1 M DTT, 1 μ l of RNASEOUT Inhibitor, and 1 μ l of SUPERSCRIPT II RT.

FIGURE 1. Schematic of SUPERSCRIPT First-Strand cDNA Synthesis System procedure.

RT-PCR is a sensitive and powerful tool for RNA analysis. As RT-PCR gained popularity, systems, such as the SUPERSCRIPT Preamplification System for First-Strand cDNA Synthesis, were designed to facilitate the technique (1). SUPERSCRIPT RT (2) was used in the system since it had been shown to yield more cDNA and more full-length cDNA compared to MMLV-RT and AMV RT (3). As the need for higher-sensitivity RT-PCR has grown, we have developed an improved two-step RT-PCR system. This paper describes this new SUPERSCRIPT First-Strand Synthesis System for RT-PCR.

METHODS

Total HeLa RNA and total rat brain RNA were isolated using TRIzol® Reagent (4). cDNA was synthesized using the SUPERSCRIPT First-Strand Synthesis System for RT-PCR (Cat. No. 11904) as outlined in figure 1. RNA was primed with 5 μ M oligo(dT). Amplification reactions consisted of 0.2 μ M primers, 200 μ M each dNTP, 1X PCR buffer, 2 μ l of the cDNA reaction, 2 units of PLATINUM® Taq DNA Polymerase (for fragments <4 kb), or 1 unit PLATINUM Taq DNA Polymerase High Fidelity (for fragments >4 kb). After an incubation at 94°C for 2 min, amplification was 35 to 40 cycles of 94°C for 15 s, 55°C–60°C for 30 s, and 68°C for 1 min/kb. The PCR products (10 μ l) were analyzed on agarose gels containing 0.5 μ g/ml ethidium bromide.

RESULTS AND DISCUSSION

Multiple factors influence the sensitivity, yield, and specificity of cDNA synthesis, including the quality of RNA, concentration of magnesium ion, reverse transcriptase, and temperature of RT reaction. To increase the sensitivity of two-step RT-PCR, we examined several factors,

including when dNTPs were added, adding additional protein, and magnesium concentration.

Most RT-PCR protocols perform the denaturation of RNA and annealing of primer before addition of any other reaction components. The addition of dNTPs to the RNA template-primer annealing step improved the yield of RT-PCR product (figure 2). Similar results were seen with RT-PCR products from β -actin, phosphatase 2A, replication protein A, and cap binding protein (data not shown). How the dNTPs enhance sensitivity is not clear, but it may help stabilize the RNA-primer hybridization.

It has been shown that acetylated BSA increased the sensitivity of RT-PCR for <50 ng total RNA. The addition of RNASEOUT™ Ribonuclease Inhibitor was examined to gain the benefits of additional protein as well as to protect the template RNA from degradation during cDNA synthesis. Our results indicate that addition of 40 units of RNASEOUT RNase Inhibitor to the RT reaction increased the sensitivity of RT-PCR ~10 times (figure 3). Different amounts of RNASEOUT Inhibitor in combination with different amounts of BSA gave similar results (data not shown).

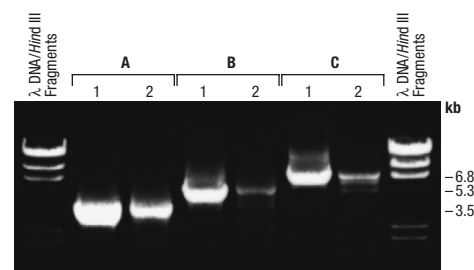


FIGURE 2. The effect of dNTP addition at the primer-annealing step. cDNA was synthesized from 100 ng total HeLa RNA. Lane 1. dNTPs were added at the RNA-primer preannealing step. Lane 2. dNTPs were added in the cDNA synthesis mix. 35 cycles of PCR were performed with primers for DNA polymerase ϵ (3.5 kb, panel A), tuberosclerosis (5.3 kb, panel B), and DNA polymerase ϵ (6.8 kb, panel C).

Magnesium concentration is a critical parameter in both RT and PCR reactions. The magnesium concentration can be affected by the amount of total RNA. While 2.5 mM magnesium ion gave good sensitivity for most RT-PCR ($\leq 1 \mu\text{g}$ RNA), it is recommended to use 5 mM of magnesium ion when $>1 \mu\text{g}$ of total RNA is used.

The optimized conditions discussed above were incorporated into the SUPERSCRIPT First-Strand Synthesis System for RT-PCR. The improved system was compared to the SUPERSCRIPT Preamplification System for several rare to moderately expressed messages from 1.6 to 12.3 kb in size. While the SUPERSCRIPT Preamplification System gave good product yield when large amounts of RNA were used, the optimized SUPERSCRIPT First-Strand Synthesis System had better product yield when less RNA was used, and for the larger fragments (figure 4). The sensitivity of the SUPERSCRIPT First-Strand Synthesis System allowed detection of messages from 1 pg of total HeLa RNA (figure 5).

For *in vitro* synthesized CAT mRNA, the new system detected 100 copies of CAT mRNA while the SUPERSCRIPT Preamplification System only detected 100,000 copies of CAT mRNA without the addition of

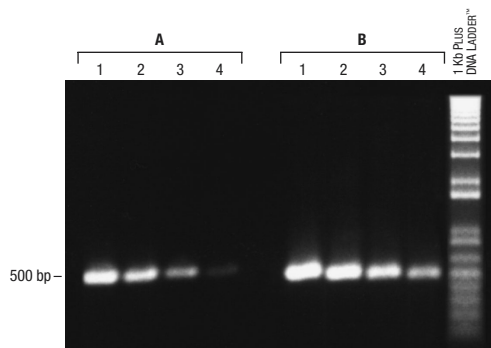


FIGURE 3. The effect of RNASEOUT Ribonuclease Inhibitor in cDNA synthesis. cDNA was synthesized from 10^5 , 10^4 , 10^3 , and 10^2 copies of CAT mRNA (lanes 1-4, respectively) in the absence (panel A) or presence (panel B) of RNASEOUT Inhibitor. PCR was 40 cycles of 94°C for 15 s, 58°C for 30 s, and 68°C for 90 s.

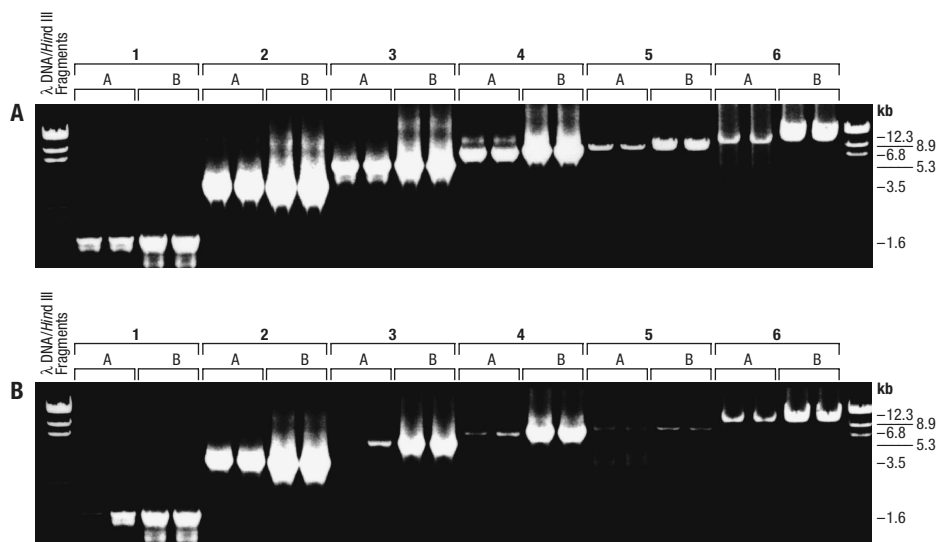


FIGURE 4. Comparison of SUPERSCRIPT Two-Step RT-PCR systems. cDNA was synthesized using the protocols from the SUPERSCRIPT First-Strand Synthesis System (panel A) and SUPERSCRIPT Preamplification System (panel B). The preamplification system uses more RT, does not add BSA or RNASEOUT inhibitor, and adds the dNTP to the cDNA synthesis mix. 35 cycles of PCR were performed for all reactions. All fragments were amplified from HeLa RNA except dynein (12.3 kb) that used rat brain RNA. Two amounts of total RNA were used for each template. Cap binding protein (lane 1), 10 or 100 ng (A and B); DNA polymerase ϵ (lanes 2 and 4), 100 ng or 1,000 ng (A and B); tuberous sclerosis (lane 3), 100 or 1,000 ng (A and B); adenomatous polyposis (lane 5), 0.5 and 1 μg (A and B); and dynein (lane 6), 2 and 5 μg (A and B).

acetylated BSA (data not shown) or 10^4 copies with BSA(3). Similar results were seen if gene-specific primers were used (data not shown).

In summary, the SUPERSCRIPT First-Strand Synthesis System for RT-PCR has been optimized to provide significantly better sensitivity in two-step RT-PCR applications.

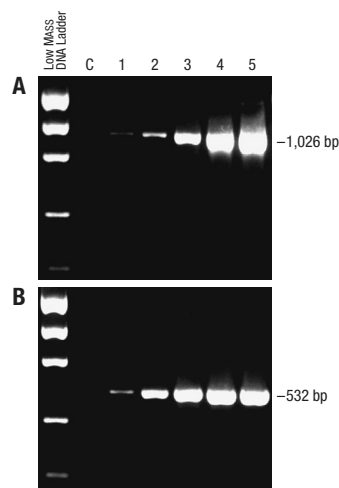


FIGURE 5. Sensitivity of the SUPERSCRIPT First-Strand Synthesis System. cDNA was synthesized from 1 pg, 10 pg, 100 pg, 1 ng, and 10 ng of total HeLa RNA (lanes 1-5, respectively). 40 cycles of PCR contained primers for β -actin (panel A) or GAPDH (panel B). Lane C. Control contained 10 ng RNA, but no RT.

ACKNOWLEDGEMENT

We thank Dr. Gary Gerard for cDNA synthesis analysis of SUPERSCRIPT II RT and great support throughout this work. We are grateful to Dr. Donna Fox and Dr. Lawrence Mertz for their helpful suggestions and discussion. **FOCUS**

REFERENCES

- Hu, A.L.W., D'Alessio, J.M., Gerard, G.F., and Kullman, J. (1991) *FOCUS* 13, 26.
- Gerard, G., Schmidt, B.J., Kotewitz, M.L., and Campbell, J.H. (1992) *FOCUS* 14, 91.
- Nathan, M., Mertz, L.M., and Fox, D.K. (1995) *FOCUS* 17, 78.
- Farrell, R.E. (ed.) (1993) *RNA Methodologies: A Laboratory Guide for Isolation and Characterization*. Academic Press.
- Nathan, M. and Fox, D.K. (1997) *FOCUS* 19, 50.

The Help Box from Your
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Team

- Q.** In a two-step RT-PCR, how much of the first-strand cDNA reaction is used for PCR?
A. Use up to 10% of the first-strand reaction.

Improved SUPERScript™ One-Step RT-PCR Systems

One-step RT-PCR is a highly sensitive and convenient tool for analysis of gene expression. The SUPERScript One-Step RT-PCR System (1) consists of an enzyme mixture of SUPERScript II RT (reverse transcriptase) and *Taq* DNA polymerase. cDNA synthesis and subsequent PCR are performed in an optimized reaction buffer (1). Here, we report improvements in the SUPERScript one-step enzyme mixture by addition of PLATINUM® *Taq* antibodies, and addition of a thermostable DNA polymerase with proofreading activity.

METHODS

One-step RT-PCR was performed with the GIBCO BRL® SUPERScript One-Step RT-PCR System (Cat. No. 10928-018), the GIBCO BRL SUPERScript One-Step RT-PCR System with PLATINUM *Taq* DNA Polymerase (Cat. No. 10928-034), or the GIBCO BRL SUPERScript One-Step RT-PCR for Long Templates (Cat. No. 11922; contains PLATINUM *Taq* DNA Polymerase High Fidelity enzyme mix). Total HeLa RNA was isolated using TRIZOL® Reagent (2). Standard aseptic techniques for RNA handling were followed. Gene-specific primers were used for one-step RT-PCR (1). Reactions were set up on ice and contained 0.2 μM each primer, 1 μl (2 units) enzyme mix, varying MgSO₄ (1.2 to 1.8 mM), and 1X buffer (which includes 0.2 mM dNTPs and 1.2 mM MgSO₄). Components were added in this order: primers, RNA, and cocktail including buffer, magnesium, enzyme mix, and water. Reactions were mixed by vortexing and placed into a 50°C thermal cycler. cDNA synthesis was at 50°C for 30 min, followed by 94°C for 2 min. The cycling profile was 40 cycles of 94°C for 15 s, 55°C–60°C for 30 s, and 68°C for 1 min/kb. 10 μl of each 50-μl reaction were analyzed.

RESULTS AND DISCUSSION

PLATINUM *Taq* DNA Polymerase inhibits *Taq* DNA polymerase activity until the PCR denaturation step at 94°C. PLATINUM *Taq* DNA Polymerase has been shown to have improved yield and specificity over *Taq* DNA polymerase (3). The increased yield of PCR product was seen also with the one-step RT-PCR system (figure 1). The use of PLATINUM *Taq* DNA Polymerase in the one-step enzyme mix resulted in RT-PCR products from <1 pg of total RNA for 2 high-copy mRNAs (figure 2). The sensitivity can vary depending

on the primers and template. The assay conditions described here were streamlined for success with a variety of templates and primers.

The addition of a proofreading polymerase to *Taq* DNA polymerase extends the length of PCR products and can improve yield (4,5). For one-step RT-PCR, we recommend using PLATINUM *Taq* DNA Polymerase High Fidelity (contains *Taq* DNA polymerase with a proofreading polymerase) for templates above 3 kb for good yield and reproducibility. Also, for best results, more total RNA is used in the

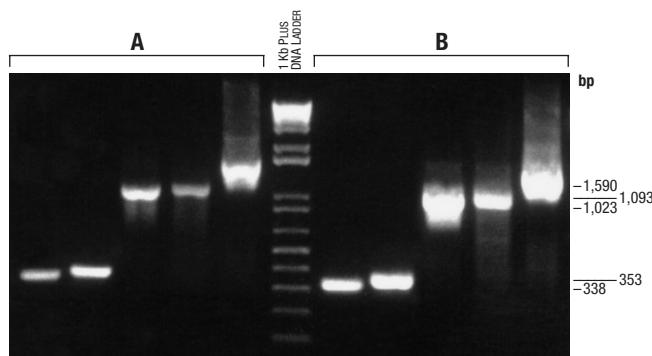


FIGURE 1. Improved product yield with PLATINUM *Taq* DNA Polymerase. cDNA was synthesized and amplified with *Taq* DNA Polymerase (panel A) or PLATINUM *Taq* DNA Polymerase (panel B) from 100 ng total HeLa RNA with gene-specific primers for replication protein A (338 bp), β-actin (353 bp), DNA polymerase ε (1,023 bp), protein phosphatase 2A (1,093 bp), and cap binding protein (1,590 bp). The magnesium concentration was 1.2 mM. The PCR cycling profile was 94°C for 15 s, 55°C for 30 s, and 68°C for 2 min. Products were analyzed on a 1.2% agarose/0.5X TBE gel stained with 0.25 μg/ml ethidium bromide.

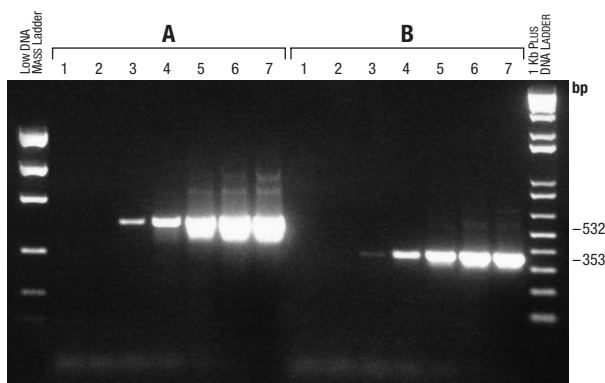


FIGURE 2. Sensitivity of SUPERScript One-Step RT-PCR with the PLATINUM *Taq* DNA Polymerase. cDNA was synthesized and amplified from 0, 0.01, 0.1, 1, 10, 100, and 1,000 pg total HeLa RNA (lanes 1-7, respectively) using primers for GAPDH (532 bp, panel A) and β-actin (353 bp, panel B). The magnesium concentration was 1.2 mM. The PCR cycling profile was 94°C for 15 s, 55°C for 30 s, and 68°C for 1 min. Products were analyzed on a 1.2% agarose/0.5X TBE gel stained with 0.25 μg/ml ethidium bromide.

reactions. This is demonstrated in *figure 3*. Using the high-fidelity enzyme mix allowed amplification of a wide range of PCR product sizes (*figure 4*).

Some optimization may be necessary for longer templates (particularly >5 kb), including magnesium concentration, annealing temperatures, primer amounts, RNA amounts, and enzyme amounts, as well as RT reaction temperatures. In *figure 5*, the effect of increased RT reaction temperature is shown. This laboratory is currently working on simplifying the optimization of long RT-PCR further.

In summary, the addition of PLATINUM *Taq* antibodies to the SUPERScript One-Step RT-PCR enzyme mix resulted in improved sensitivity and yield. When the advantages of PLATINUM *Taq* DNA Polymerase were combined with a second DNA polymerase with proofreading activity, amplification of targets up to 8.9 kb was seen. In addition, the fidelity of the PLATINUM *Taq* DNA Polymerase High Fidelity enzyme mix is over 6 times better than *Taq* DNA polymerase using a *lacZ* fidelity assay (*data not shown*). These improvements will facilitate high-throughput screening and other one-step RT-PCR applications. **FOCUS**

REFERENCES

1. Lee, E.H., Sitaraman, K., Schuster, D., and Rashtchian, A. (1997) *FOCUS* 19, 39.
2. Sewall, A. and McRae, S. (1998) *FOCUS* 20, 36.
3. Westfall, B., Sitaraman, K., Solus, J., Hughes, J., and Rashtchian, A. (1997) *FOCUS* 19, 46.
4. Westfall, B., Sitaraman, K., Berninger, M., and Mertz, L.M. (1995) *FOCUS* 17, 62.
5. Sitaraman, K., Darfler, M., and Westfall, B. (1999) *FOCUS* 21, 10.

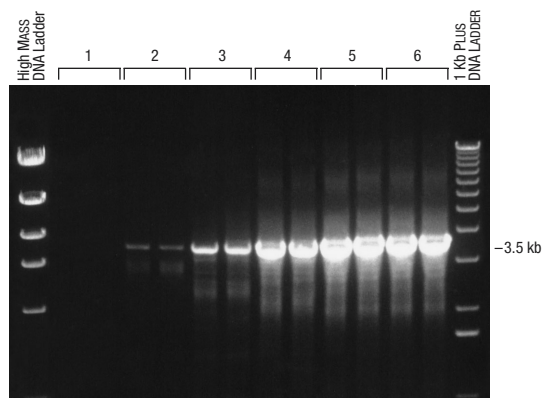


FIGURE 3. Sensitivity of one-step RT-PCR for long targets. cDNA was synthesized and amplified in duplicate from 0, 0.4, 2, 10, 50, and 250 ng total HeLa RNA (*lanes 1-6, respectively*) using the SUPERScript One-Step RT-PCR System for Long Templates (contains PLATINUM *Taq* DNA Polymerase High Fidelity enzyme mix). The optimized magnesium concentration for this DNA polymerase ϵ 3.5-kb fragment was 1.6 mM $MgSO_4$. The PCR cycling profile was 94°C for 15 s, 60°C for 30 s, and 68°C for 4.5 min. Samples were analyzed on a 1.0% agarose/1X TAE gel stained with 0.25 μ g/ml ethidium bromide.

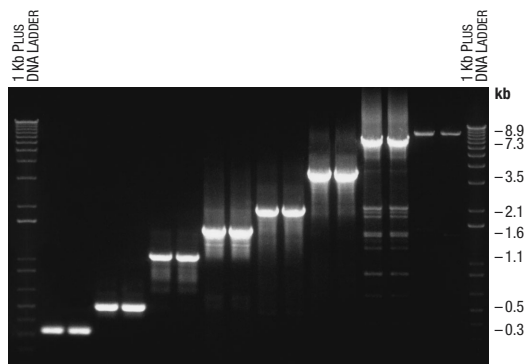


FIGURE 4. Range of product size with the SUPERScript One-Step RT-PCR System for Long Templates. cDNA was synthesized and amplified in duplicate from 100 ng of total HeLa RNA with primers for protein phosphatase 2A (331 bp), replication protein A (514, 1,092, and 2,057 bp), cap binding protein (1,590 bp), polymerase ϵ (3.5 kb), and adenomatous polyposis coli (7.3 and 8.9 kb). The optimized conditions included 1.2 to 1.8 mM $MgSO_4$. Reactions were grouped in 3 separate thermal cyclers for 1, 3.5, and 9 min extension at 68°C for 331 to 1,092 bp, 1,590 bp to 3.5 kb, and 7.3 to 8.9 kb, respectively. The annealing temperature was 55°C for fragments <3.5 kb and 60°C for fragments \geq 3.5 kb. Samples were analyzed on a 1.0% agarose/1X TAE gel stained with 0.25 μ g/ml ethidium bromide.

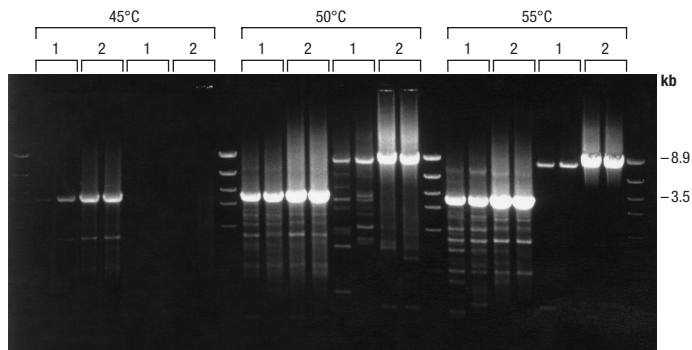


FIGURE 5. Optimization of RT temperature. The 3.5-kb DNA polymerase ϵ and 8.9-kb adenomatous polyposis coli fragments were amplified in duplicate using 10 ng (*lane 1*) and 100 ng (*lane 2*) of total HeLa RNA, 4 units of enzyme mix, and 400 nM of each primer with varying RT reaction temperatures. The PCR cycling profile was 94°C for 15 s, 60°C for 30 s, 68°C for 3.5 min (3.5 kb) or 9 min (8.9 kb). Samples were analyzed on a 1.0% agarose/1X TAE gel stained with 0.25 μ g/ml ethidium bromide.

PCR from Problematic Templates

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PCR is generally robust, amplifying specific targets under standard conditions. However, some templates require optimization of PCR conditions, including the magnesium concentration, denaturing and annealing temperatures, and cycle number, for successful amplification. The optimization of one or more of these parameters is often adequate to increase yield and specificity (amplification of only the desired product).

Templates with high-GC content are particularly difficult to amplify, due to their high melting temperatures, and may require additional measures beyond optimizing reaction conditions. Incomplete separation of DNA strands can adversely affect amplification efficiency. In addition, template secondary structure or unmelted GC-rich regions can prevent primer binding and enzymatic elongation (1-3). Cosolvents (formamide, DMSO, glycerol, and betaine) that affect DNA melting temperature provide another method for improving product specificity and yield (1-3). However, reactions containing DMSO, formamide, or glycerol require significant optimization to prevent inhibition of *Taq* DNA polymerase (2,4).

Here we introduce GIBCO BRL® PCR_x Enhancer System, a novel PCR cosolvent and buffer system for the amplification of sequences that are 50% to 90% GC. PCR_x Enhancer Solution improved the yield and specificity of PCR from difficult templates and required less optimization than other cosolvents.

METHODS

All reagents and enzymes were obtained from Life Technologies. Amplification reactions were performed in 50- μ l volumes using 2.5 units of PLATINUM® *Taq* DNA Polymerase. Reactions were assembled at room temperature. Samples

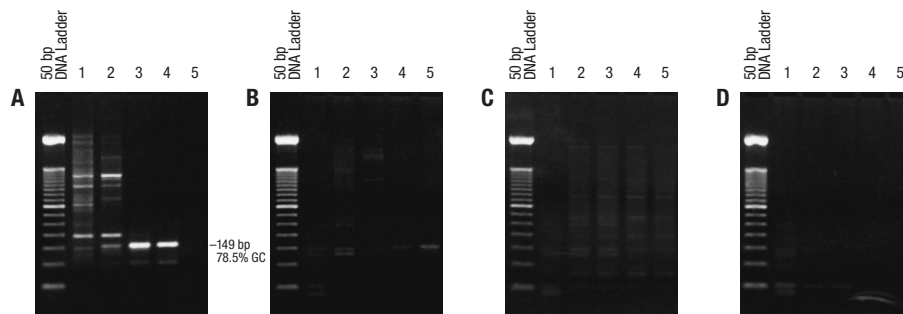


FIGURE 1. Comparison of cosolvents. A 149-bp region of the AF064849 locus (78.5% GC) was amplified from human genomic DNA in the presence of varying amounts of cosolvents. *Panel A.* PCR_x Enhancer Solution: 0, 1X, 2X, 3X, and 4X (lanes 1-5, respectively). *Panel B.* Betaine: 0, 0.5 M, 1 M, 1.5 M, and 2 M (lanes 1-5, respectively). *Panel C.* DMSO: 0, 1.25%, 2.5%, 5%, and 10% (lanes 1-5, respectively). *Panel D.* Formamide: 0, 1.25%, 2.5%, 5%, and 10% formamide (lanes 1-5, respectively). *Panels B-D* were performed in standard 1X PCR buffer with 1.5 mM MgCl₂. *Panel A* was performed in 1X PCR_x Amplification Buffer with 1.5 mM MgSO₄. The cycling profile was 95°C for 30 s, 58°C for 30 s, and 68°C 1 min.

contained 1X PCR_x Amplification Buffer or 1X PCR buffer, 1.0-2.5 mM MgSO₄, 200 μ M dNTP, 200 nM each primer (*table 1*), 100 ng K562 human genomic DNA, and PCR_x Enhancer Solution or other cosolvents as indicated. Amplification was 95°C for 1 min followed by 35 cycles of 95°C for 30 s to 45 s, 55°C to 66°C for 30 s, and 68°C for 30 s to 1 min.

RESULTS AND DISCUSSION

Several cosolvents were compared for their effectiveness in amplifying a 149-bp region of human genomic DNA with a GC-content of 78.5% and a CGG trinucleotide repeat. The specific product was obtained using PCR_x Amplification Buffer with PCR_x Enhancer Solution (*figure 1*). Simply using the PCR_x Amplification Buffer, which has lower ionic strength than standard amplification buffer, yielded several nonspecific bands. In addition, *figure 1* shows that PLATINUM *Taq* DNA Poly-

merase, which increases specificity by inhibiting *Taq* DNA polymerase activity during PCR set-up (5), was not sufficient to eliminate all the nonspecific amplification from this GC-rich template.

The optimal reaction conditions may vary depending on the GC-content of the template. The best concentration of PCR_x Enhancer Solution can be determined by performing a titration experiment similar to the one shown in *figure 1*. For targets 45% to 60% GC, a concentration between 0.5X and 1X is recommended. Targets with higher GC-content (65% to 90%) may require up to 4X concentration. For example, the amplification of the GCG repeat region of the FMR-1 gene (82% to 90% GC-rich) required 3X PCR_x Enhancer Solution (*data not shown*) (6).

It is important to use optimal annealing temperatures. The annealing temperature affects both specificity and yield. If the annealing temperature is low, multiple non-

Target	Primer	Sequence	GC Content (%)	Product Size (bp)
AF064849	Sense	ATC CCC CAC CCC CGC ACC	78.5	149
	Antisense	GGG CGC GAG ATG GGC TGC		
IGFBP-3	Sense	GCC CCG GTT GCA GGC GTC ATG	78.2	261
	Antisense	GCA CGG CTG GCC CTC GCT CAG		

TABLE 1. Primer sequences.

specific products may be amplified; if it is high, product yield may be reduced. PCR_x Enhancer Solution broadened the range of annealing temperatures that yield desired product for a 78% GC-rich template, therefore decreasing the need for temperature optimization (figure 2).

The effectiveness of PCR_x Enhancer Solution in the presence of varying concentrations of magnesium was examined. The addition of 3X PCR_x Enhancer Solution gave the expected 149-bp product (figure 3) and eliminated the amplification of other nonspecific products at all four (1.0, 1.5, 2.0, and 2.5 mM) magnesium concentrations. Note: The 3X concentration gave the desired result when a 55°C annealing temperature was used (figure 3), but a 1X concentration allowed for amplification of the correct product when annealing temperatures above 58°C were used (figure 2). This illustrates the complex interplay between annealing temperature, magnesium concentration, and cosolvent concentration.

PCR_x Enhancer Solution is also beneficial in improving RT-PCR detection. The inclusion of PCR_x Enhancer solution during amplification allowed for amplification of a 78% GC-rich template that the RT had no difficulty reverse transcribing (figure 4).

In summary, we have demonstrated that PCR_x Enhancer Solution increased the yield and specificity of products amplified from difficult, GC-rich templates. PCR_x Enhancer Solution broadened the range of annealing temperatures and magnesium concentrations, reducing the complexity of conventional PCR optimization.

ACKNOWLEDGEMENT

We thank Holly Anderson, Senior Technical Service Customer Training Team Specialist, for writing this manuscript. **FOCUS**

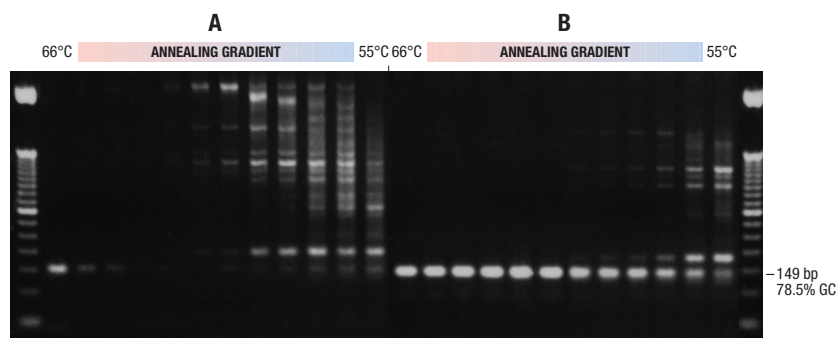


FIGURE 2. Broader effective annealing temperature. The 149-bp region of the AF064849 locus (78.5% GC) was amplified from human genomic DNA in 1X PCR_x Amplification Buffer without (Panel A) or with (Panel B) 1X PCR_x Enhancer Solution. The annealing temperature ranged from 55°C to 66°C in 1°C increments. The cycling profile was 95°C for 45 s, 55°C to 66°C for 30 s, and 68°C for 1 min.

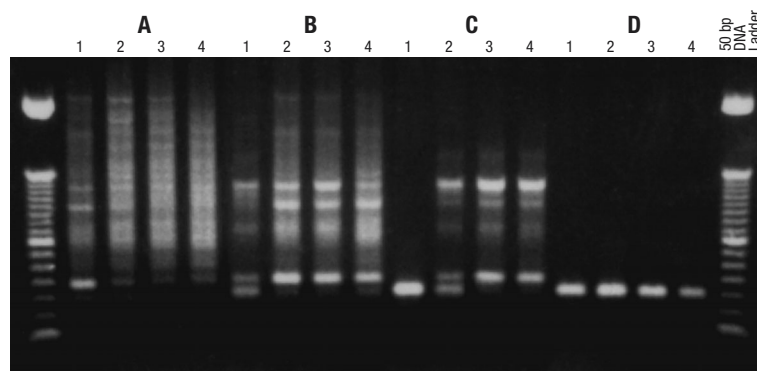


FIGURE 3. Broader magnesium optima. The 149-bp region of the AF064849 locus (78.5% GC) was amplified from human genomic DNA in 1X PCR_x Amplification Buffer with 0, 1X, 2X, or 3X PCR_x Enhancer Solution (Panels A-D) and 1.0, 1.5, 2.0, or 2.5 mM MgSO₄ (lanes 1-4, respectively). The cycling profile was 95°C for 30 s, 55°C for 30 s, and 68°C for 1 min.

REFERENCES

1. Pomp, D. and Medrano, J.F. (1991) *BioTechniques* 10, 58.
2. Varadaraj, K. and Skinner, D.M. (1994) *Gene* 140, 1.
3. Baskaran, N., Kandpal, R., Bhargava, A., Glynn, M., Bale, A., and Weissman, S. (1996) *Genome Res.* 6, 633.
4. Erlich, H.A. (1989) *PCR Technology, Principles and Applications for DNA Amplification*. Stockton Press.
5. Westfall, B., Sitaraman, K., Solus, J., Hughes, J., and Rashtchian, A. (1997) *FOCUS* 19, 46.
6. Westfall, B., Sitaraman, K., Lee, J.E., Borman, J., and Rashtchian, A. (1999) *FOCUS* 21, 46.

Editor's Note: PCR_x Enhancer Solution is a component in GIBCO BRL PLATINUM Taq PCR_x DNA Polymerase (Cat. No. 11509) and GIBCO BRL PLATINUM Pfx DNA Polymerase (Cat. No. 11708).

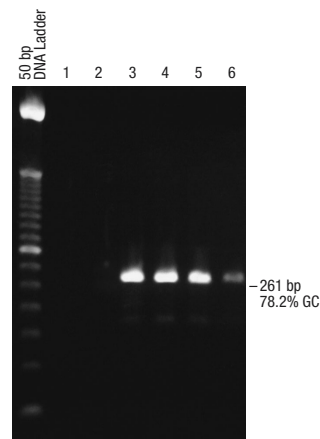


FIGURE 4. RT-PCR of a difficult template. cDNA was synthesized at 60°C for 30 min from 1 µg of total HeLa RNA with oligo(dT)₂₀ and THERMOSCRIPT™ RT. A 261-bp region of the 5'-end of IGFBP-3 (78.2% GC) was amplified for one-tenth of the first-strand reaction with PLATINUM Taq DNA Polymerase in 1X PCR_x Amplification Buffer with 0, 0.5X, 1X, 2X, 3X, or 4X (lanes 1-6, respectively). PCR was 95°C for 1 min followed by 35 cycles of 95°C for 45 s, 60°C for 30 s, and 68°C for 30 s.

Helpful Tips for PCR

When is hot start helpful for PCR?

Hot start helps circumvent nonspecific priming during the setup and start of PCR. This unwanted priming can produce nonspecific products that interfere with downstream applications. Hot start delays DNA synthesis by withholding *Taq* DNA polymerase (or another PCR component) until the thermal cycler reaches the denaturation temperature. Though effective, manual hot start is cumbersome and carries a higher risk of contamination. GIBCO BRL® PLATINUM® DNA Polymerases are convenient and highly effective enzymes for automatic hot-start PCR. They contain a DNA polymerase complexed with monoclonal antibodies to inactivate the polymerase. The antibodies and DNA polymerase dissociate during the denaturation step and the enzyme is released into the reaction with full activity [see *FOCUS* (1997) 19, 46.]

How do I determine the appropriate annealing temperature?

Start with an annealing temperature 5°C below the T_m of the amplification primers. The T_m can be determined by most computer programs used to design primers. You can use the following formula:

$$T_m = 81.5 + 16.6 \times (\log_{10} [Na^+]) + 0.41 \times (\% GC) - 675/N$$

This formula takes into account the salt concentration ($[Na^+] = Na^+$ or K^+) and N = number of bases.

How do I determine the concentration of magnesium in my PCR?

The starting concentration in typical PCR containing 200 μ M dNTPs is 1.5 mM. To determine the optimal magnesium concentration, perform a magnesium titration using from 1 mM to 3 mM in 0.5 mM

increments. To reduce the need for magnesium optimization, use PLATINUM *Taq* DNA Polymerase, which functions over a broader range of magnesium concentration than *Taq* DNA polymerase.

Why is magnesium so important in PCR?

Magnesium affects DNA polymerase activity, which can affect yield; and primer annealing, which can affect specificity. The dNTPs and template bind magnesium and reduce the amount of free magnesium available for enzyme activity. Higher concentration of free magnesium can result in greater yield but can also increase nonspecific amplification and reduce fidelity.

How can I increase the fidelity of my PCR?

Choosing the correct enzyme is critical for high-fidelity PCR. *Taq* DNA polymerase lacks 3' to 5' exonuclease (proofreading) activity. PLATINUM *Pfx* DNA Polymerase, a thermostable polymerase with 3' exonuclease activity, has significantly better fidelity than *Taq* DNA polymerase and the high yield and specificity of PLATINUM enzymes [see *FOCUS* (1999) 21, 46]. Alternatively, mixing *Taq* DNA polymerase with a second polymerase with 3' exonuclease activity provides greater fidelity than *Taq* DNA polymerase with the benefit of longer PCR products.

Besides the enzyme, other factors can improve fidelity. Decreasing the concentration of dNTPs from 200 μ M to 25-50 μ M and ensuring that the concentration of all 4 nucleotides is the same can improve fidelity. Performing fewer cycles of PCR can increase fidelity, since the product of each cycle becomes template for subsequent cycles.

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I am getting ready to try PLATINUM *Pfx* DNA Polymerase. Are there any differences compared to other proofreading polymerases I need to be aware of?

PLATINUM *Pfx* DNA Polymerase may not perform to its full potential (e.g. priming specificity or yield) when the enzyme is "dropped" into the protocol optimized for another enzyme. Key differences include the amounts of enzyme and magnesium that are optimal for PLATINUM *Pfx* DNA Polymerase.

Compared to your current protocol, you may need to:

- Lower your magnesium concentration to the range recommended for PLATINUM *Pfx* DNA Polymerase.
- Use a lower annealing temperature, since the magnesium concentration is lower.
- Lower the number of units of enzyme to the amount recommended for PLATINUM *Pfx* DNA Polymerase; more enzyme may hinder the reaction.

Note: Be sure to use the 10X *Pfx* Amplification Buffer since it is pre-optimized to work for PLATINUM *Pfx* DNA Polymerase.

With PLATINUM *Pfx* DNA Polymerase, sometimes I have seen DNA in the wells of my agarose gel. Why is this?

It is probably a combination of the following:

- PLATINUM *Pfx* DNA Polymerase has more protein per unit of enzyme than other amplification enzymes.
- PLATINUM *Pfx* DNA Polymerase may have different DNA-binding properties than other enzymes. If the protein remains bound to the DNA, migration is decreased in the gel.

If this reduces the yield of product migrating into the gel, add SDS to the loading buffer to 0.1%. **FOCUS**

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Answers to Frequently Asked Questions About RT-PCR

Reverse transcription of RNA followed by PCR amplification of the cDNA is a sensitive method to detect small amounts of specific RNA. This article addresses some common questions about RT-PCR.

How can I ensure that my RNA is of high quality?

Careful preparation and storage result in high-quality RNA. High-quality RNA is substantially full length and does not contain inhibitors of reverse transcriptases such as EDTA or SDS. One popular method for isolation of RNA is the single-step isolation protocol using guanidine isothiocyanate/acidic phenol. TRIZOL[®] Reagent is an improvement on this method and has been used to isolate high-quality RNA from various cells and tissues [see *FOCUS* (1993) 15, 99].

For added protection, store isolated RNA in pure deionized formamide at -70°C [see *FOCUS* (1998) 20, 82]. When ready to use the RNA, precipitate by adding NaCl to 0.2 M followed by 4 volumes of ethanol. Incubate 3 to 5 min at room temperature and centrifuge at $10,000 \times g$ for 5 min. Dissolve in RNase-free water.

How can I treat my RNA samples prior to RT-PCR to eliminate DNA contamination?

Combine 1 μg total RNA, 1 μl 10X DNase I buffer, 1 μl DNase I, Amplification Grade (1 unit/ μl) and bring the reaction volume to 10 μl with DEPC-treated water. Incubate at room temperature for 15 min. To inactivate the DNase I, add 1 μl of 25 mM EDTA and heat for 15 min at 65°C . Collect the reaction by brief centrifugation. This mixture can be used directly for reverse transcription.

I treat my RNA with DNase I prior to RT-PCR. Will the EDTA added to inactivate the DNase inhibit my RT reaction?

No. The EDTA added was sufficient to chelate the magnesium ion in the DNase I reaction. However, more magnesium is added to the RT reaction (EDTA chelates magnesium in a 1:1 ratio). Note: The EDTA is necessary during the inactivation of DNase I because at 65°C free divalent metal ions can promote RNA hydrolysis [see *Molekulyarnaya Biologiya* (1987) 21, 1235].

Can I isolate RNA from a small number of cells (100 to 10,000) with TRIZOL Reagent?

Yes. Add glycogen (250 $\mu\text{g}/\text{ml}$) directly to the TRIZOL Reagent and sample after homogenization. The glycogen remains in the aqueous phase and is co-precipitated with the RNA. It does not inhibit first-strand synthesis at concentrations up to 4 mg/ml and does not inhibit PCR. We obtained nanogram amounts of total RNA from as little as 100 cells [see *FOCUS* (1999) 21, 38].

My sample contains high amounts of proteoglycans and/or polysaccharides (such as rat liver, rat aorta, plants).

How can I ensure that the RNA isolated with TRIZOL Reagent is high quality?

To remove the proteoglycans and polysaccharides, modify the RNA precipitation step as follows. To the aqueous phase, add 0.25 ml of isopropanol followed by 0.25 ml of a high-salt solution [0.8 M sodium citrate and 1.2 M NaCl (no pH adjustment necessary)] per 1 ml of TRIZOL Reagent. Mix, centrifuge, and proceed with isolation as described in the protocol. In this protocol, the RNA precipitates while the proteoglycans and polysaccharides remain in the solution. To isolate pure RNA from plant material containing a very high level of

polysaccharides, combine the modified precipitation with an additional centrifugation of the initial homogenate [see Chomczynski, P. and Mackey, K. (1995) *BioTechniques* 19:6, 942].

How do I choose a format for RT-PCR?

The choice of format for RT-PCR is dependent on your experimental goals. One-step RT-PCR is easier to use when processing large numbers of samples and helps minimize carry-over contamination, but requires gene-specific priming of the RT reaction. Two-step RT-PCR is useful for detecting multiple messages from a single RNA sample and offers choice of primer, reverse transcriptase, and PCR enzyme (see page 2 in this issue).

How do higher RT incubation temperatures improve RT-PCR?

Higher RT incubation temperatures can help disrupt RNA secondary structure and increase the yield of product. In addition, higher temperatures can improve specificity, especially when using a gene-specific primer for cDNA synthesis. THERMOSCRIPT[™] RT allows RT reaction temperatures up to 65°C [see *FOCUS* (1998) 20, 30]. SUPERSOFT[™] II RT allows RT reaction temperatures up to 55°C (see *FOCUS* (1997) 19, 39).

What is the advantage of treating my first-strand product with RNase H?

RNase H degrades the RNA strand in RNA:DNA hybrids, theoretically increasing the availability of the cDNA as a target in the subsequent PCR. Though not always necessary, treating the first-strand cDNA may increase the yield of PCR products. RNase H treatment is often necessary when amplifying long or low-copy cDNA targets [see *FOCUS* (1995) 17, 78]. **FOCUS**

Improved Purification of DNA from *Entamoeba histolytica*

ABSTRACT

A previously described protocol for the isolation of amoeba DNA was modified and used for the purification of RNA-free genomic DNA from *E. histolytica* trophozoites. The method rendered high-quality preparations that were suitable for standard techniques of molecular biology.

Some organisms contain polysaccharides that copurify with DNA. For example, in the human pathogen *Entamoeba histolytica*, glycogen has been found as a major cell constituent (1) and used as a parameter for the clinical identification of the parasite (2). Polysaccharides can interfere with DNA isolation, resulting in poor-quality preparations that are not suitable for standard molecular biology techniques. Polysaccharides inhibit the activity of a wide range of DNA-modifying enzymes such as restriction endonucleases, polymerases, and ligases (3-6).

The inability to obtain high-quality DNA from amoeba has seriously limited the possibility of explaining a number of biochemical aspects of parasite metabolism at the molecular level. This knowledge would allow the design of new strategies for its control. A major interest of this laboratory is to set up a simple procedure for the routine isolation of high-quality amoeba DNA from a small number of trophozoites. Tannich *et al.* (7) described a procedure for the extraction of DNA from isolated nuclei. In our hands, this protocol did not result in a significant increase in purity, yield, and reproducibility over other procedures (8). This paper describes modifications to this procedure to obtain high-quality DNA from amoeba.

METHODS

Modifications to the Tannich protocol (7) were: a) DNA was purified from whole trophozoites instead of isolated nuclei; b) cells were harvested from mid-logarithmic phase (48 h); and c) cells were lysed in a modified lysis buffer.

Briefly, *E. histolytica* trophozoites (HM1-IMSS) were maintained and propagated in the medium in conditions described elsewhere (9) except that propagation time was reduced to 48 h. Trophozoites were

harvested, ice-chilled for 10 min, and collected by low-speed centrifugation at 4°C for 5 min. The cell pellet was frozen under liquid nitrogen and ground to a fine powder with a mortar and pestle. The powder was transferred to a 15-ml conical tube, suspended in 3 ml of lysis buffer [200 mM Tris-HCl (pH 8.5), 200 mM NaCl, 0.5% SDS, 25 mM EDTA, and 1 mg/ml GIBCO BRL® Proteinase K], and incubated at 65°C

Polysaccharides can interfere with DNA isolation, resulting in poor-quality preparations that are not suitable for standard molecular biology techniques.

for 30 min. Over 90% of the cells were broken as judged by microscopic examination. The homogenate was divided into 3 aliquots, which were centrifuged at 2,900 × g for 5 min to pellet the heavy cell fragments. The debris-free supernatant was very viscous and was carefully aspirated.

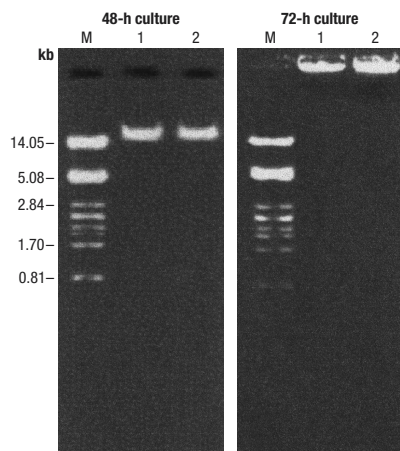


FIGURE 1. DNA purified by the modified protocol. Purified DNA was dissolved in 50 μ l of water and 1- μ g samples were electrophoresed in a 1% agarose gel. Lanes 1 and 2 are samples from two different DNA preparations purified from 48-h and 72-h cultures of trophozoites. Lane M, λ DNA/Pst I digested.

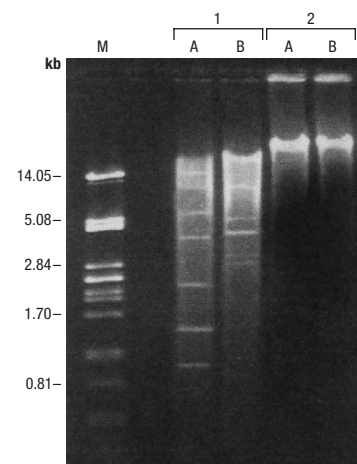


FIGURE 2. Restriction digest of purified DNA from *E. histolytica*. Purified DNA (1 μ g) isolated from 48-h (lane 1) and 72-h (lane 2) cultures of trophozoites was digested for 1 h at 37°C with 5 units of BamH I (lane A) or Pst I (lane B) and electrophoresed in a 1% agarose gel.

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A 1-ml aliquot was mixed with 0.5 ml of phenol:chloroform:isoamyl alcohol (24:24:1, by volume) in a 1.5-ml microcentrifuge tube. Following phase partition by centrifugation at $8,200 \times g$ for 10 min, the aqueous phase was carefully removed, avoiding any contamination with the interphase material, and extracted once more with 0.5 ml of the phenol:chloroform:isoamyl alcohol mix. The aqueous phase was extracted with 0.5 ml of chloroform:isoamyl alcohol (24:1, by volume). 0.45 ml of the aqueous phase were mixed with 0.045 ml of 5 M ammonium acetate and 0.9 ml of cold ethanol, and incubated at -20°C . After 1 to 2 h, precipitated DNA was pelleted by centrifugation at $11,800 \times g$ for 15 min at 4°C , washed with cold 70% ethanol, dissolved in 50 μl of deionized water, and incubated with 5 μl (50 μg) of GIBCO BRL RNase T1 for 30 min at 37°C .

RESULTS AND DISCUSSION

High-quality genomic DNA was obtained with the modified protocol. Yields

were 40 to 60 μg DNA per 10^8 trophozoites grown for 48 h. The A_{260}/A_{280} ratio was 1.9–2.0, indicating high-quality DNA. When DNA was extracted from 10^8 cells grown for 72 h, yields increased to 80 to 100 μg . This is consistent with other findings (11) that in 72-h and 96-h cultures, the number of cells with higher DNA content increased up to 45%. While more DNA was purified at 72 h, the DNA was unable to enter into an agarose gel (figure 1) and was not digested by restriction endonucleases (figure 2). The DNA purified from 48-h cultures was able to serve as a substrate for PCR (figure 3).

Two key changes were made to the Tannich protocol. Above we demonstrated that the age of the trophozoites was critical (72 h resulted in poor-quality DNA). The second change, using a modified buffer, allowed for simplifying the protocol by using whole cells directly, rather than purifying nuclei first. Note: The unmodified buffer did not work well on 72-h cells or nuclei. It was not tested on 48-h cultures (data not shown).

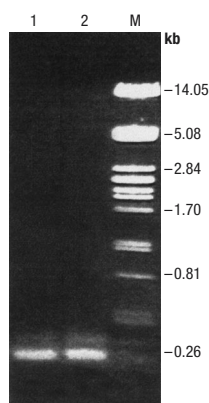


FIGURE 3. PCR of DNA purified from *E. histolytica*. The ITS1 region of the ribosomal unit of *E. histolytica* was amplified from 2 independent samples (lanes 1 and 2) of genomic DNA purified from 48-h-old trophozoites in mixtures containing 100 ng DNA, 2.5 units GIBCO BRL *Taq* DNA Polymerase, 2.5 mM MgCl_2 , 0.25 mM each dNTPs, and 100 nM each primer [ITS1 and ITS2 (10)]. After 3 min at 94°C , PCR was 30 cycles of 94°C for 1 min, 46°C for 50 s, and 72°C for 2 min. Samples were electrophoresed in a 1.4% agarose gel.

ACKNOWLEDGEMENT

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FOCUS

REFERENCES

- Bakker-Grunwald, T., Martin, J.P., and Gerard, K. (1995) *J. Euk. Microbiol.* 42, 346.
- Guerrero-Alcazar, M. and de la Hoz-Couturier, R. (1971) *Arch. Inv. Med. (México)* 2, 199.
- Aoki, Y. and Koshihara, H. (1972) *Biochim. Biophys. Acta* 272, 33.
- Do, N. and Adams, R.P. (1991) *BioTechniques* 10, 163.
- Fang, G., Hammar, S., and Grumet, R. (1993) *BioTechniques* 13, 52.
- Furukawa, K. and Bhavadna, V.P. (1983) *Biochim. Biophys. Acta* 740, 466.
- Tannich, E., Horstmann, R.D., Knobloch, J., and Arnold, H.H. (1989) *Proc. Natl. Acad. Sci. USA* 86, 5118.
- Mirelman, D., Nuchamowitz, Y., Bohm-Gloning, B., and Walderich, B. (1996) *Mol. Biochem. Parasitol.* 70, 47.
- Villagómez-Castro, J.C., Calvo-Méndez, C., Vargas-Rodríguez, L., Flores-Carreón, A., and López-Romero, E. (1998) *Exp. Parasitol.* 88, 111.
- White, T.J., Burns, T., Lee, S., and Taylor, J. (1990) *Sequencing of Fungal Ribosomal RNA Genes for Phylogenetics. PCR Protocols: A Guide to Methods and Applications*, p. 315.
- Gangopadhyay, S.S., Ray, S.S., Kennady, K., Pande, G., and Lohia, A. (1997) *Mol. Biochem. Parasitol.* 90, 9.

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SAN DIEGO

Efficient Transfection of Epstein Barr-Derived Vectors in Human Tumorigenic Cells

ABSTRACT

Many investigators use shuttle vectors, such as EBV-derived vectors, for unraveling biological jigsaws in cultured human cells. However, the level of gene transfer of EBV plasmids is low, mainly due to their large size (>10 kb). We have found GIBCO BRL® LIPOFECTAMINE™ 2000 Reagent as an efficient reagent (40% to 50% cells transfected) for transferring various large EBV vectors into human tumorigenic cell lines. With smaller plasmids (~7 kb), transfection efficiencies reached 60% to 70% in RKO, HeLa, H1299, or HCT116 cells. In HEK 293 cells, we easily obtained 80% to 90% positive cells with both plasmids. Transfections were performed with serum in the culture medium. The procedure is very simple, and in opposition to other liposomic reagents, LIPOFECTAMINE 2000 Reagent had reduced toxicity in most cell lines tested.

Transfection efficiency is dependent on the strategy of transfer, the cell type used, the size of the plasmid, and the intracellular degradation of plasmid DNA. Transfection efficiencies decrease with increasing plasmid size. Gene transfer in cultured human tumorigenic cells is poor especially with EBV shuttle vectors (1% to 5% cells transfected), except for particular cells such as HEK 293. HEK 293 cells are better than most human tumorigenic cells in their transfection ability with EBV vectors. With the usual procedures of transfection, such as calcium phosphate precipitation, and in avoiding cell toxicity, transfection efficiencies for HEK 293 cells reached 20% of cells with 10-kb plasmids. This study evaluated LIPOFECTAMINE 2000 Reagent for transferring genes in human cells. Transfection experiments were performed with 8 tumorigenic cell lines and 10 plasmids; 8 of them are EBV-derived vectors over 10 kb. The expression of the autofluorescent EGFP protein was followed, as well as the protein level of Kin17, a protein involved in DNA metabolism.

METHODS

PLASMID CONSTRUCTION. To monitor transfection, a cDNA coding for the Enhanced

Green Fluorescent Protein (EGFP gene) was used. Different desired cDNAs were introduced downstream of the CMV promoter, giving rise to different plasmids: pAdenoCMVEGFP (pB417; 7,401 bp); pEBVCMVEGFP (pB442; 10,059 bp); pEBVCMVasEGFP (pB442as; antisense orientation, used as a control); pRetroCMV_{Hs}Kin17 (pB462; 8,285 bp, from the pLNCX); pEBVCMV_{Mm}Kin17 and pEBVCMVas_{Mm}Kin17 (pB291; sense and antisense orientation; 11,546 bp); pEBVCMV_{Hs}Kin17 and pEBVCMVas_{Hs}Kin17 (pB399; sense and antisense orientation; 10,750 bp); pEBVCMV_{Hs}Kin17-G418 (pB465; 10,991 bp); and pEBVCMVas_{Hs}Kin17-G418 (pB466; 11,702 bp). The Kin17 plasmids were constructed with _{Mm}Kin17 cDNA (391 amino acids) and _{Hs}Kin17 cDNA (393 amino acids). EBV-derived shuttle vectors are described in reference 1. Plasmid DNA was purified using an anion-exchange resin before transfection.

CELL CULTURE. Human cell lines were used: HeLa cells (uterine cervical carcinoma); H1299 cells (non-small-cell lung carcinoma); HEK 293 cells (human embryonic kidney cells transformed with adenovirus 5 DNA); Hos cells (osteosarcoma); MeWo cells (melanoma); and

Cell Lines	Seeding Density (cells/cm ²)	Cells Expressing EGFP (%)	
		pAdenoCMVEGFP (7.4 kb)	pEBVCMVEGFP (10.9 kb)
HEK 293	50,000	> 90	75
RKO	30,000	60	45
H1299	10,000	60	45
HeLa	30,000	40	40
HCT116	50,000	40	40
LoVo	50,000	50	45
MeWo	20,000	20	1
Hos	20,000	10	1

TABLE 1. Transfection efficiencies. Cells were transfected 2 to 3 days after plating. Efficiencies were determined 24 h after transfection by monitoring fluorescence of EGFP. Percentages of transfected cells were calculated by comparing the number of EGFP-expressing cells to the number of cells in the same field observed by phase contrast. Data are the mean of at least 3 independent transfections.

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HCT116, RKO, and LoVo cells (3 colon carcinoma cell lines). Cells were maintained in GIBCO BRL Dulbecco's Modified Eagle Medium (D-MEM) with 10% FBS, 100 units/ml of penicillin and 100 μ g/ml of streptomycin, under 5% CO₂. To maintain plasmids, cells were cultured with 125 μ g/ml GIBCO BRL Hygromycin B for HEK 293, HCT116, and H1299 cells; 250 μ g/ml for HeLa cells; and 500 μ g/ml for RKO cells.

TRANSFECTION. Transfection was carried out at least 3 times using different DNA preparations. Cells were plated in 6-well dishes with 2 ml of D-MEM with 10% FBS. When the expected cell density was reached (2 or 3 days later), cells were transfected. The day of transfection, medium was replaced with fresh D-MEM containing 10% FBS (unless otherwise indicated). In a polypropylene tube, 2 μ g of DNA were diluted to 100 μ l in MEM (Minimum Essential Medium with Earle's salts) and then combined with 6 μ l of LIPOFECTAMINE 2000 Reagent (Cat. No. 11668) diluted in 100 μ l of MEM. The mixture was incubated 30 min at room temperature, and the complexes were added dropwise onto the cells. Living cells were monitored for EGFP expression 24 h after transfection with the ACAS 570 cytometer (1,2). At 48 h post-transfection, cells were stained with propidium iodide and then fixed with 4% paraformaldehyde for analysis of EGFP fluorescence (1,2). Propidium iodide stains nuclei of viable cells. Proteins were extracted for Western blot analysis. Low-molecular-weight DNA was recovered (by alkaline lysis) for analysis in bacteria (2).

ANALYSIS WITH THE INTERACTIVE LASER CYTOMETER. Cells were monitored simultaneously for EGFP and propidium iodide by laser cytometry using an ACAS 570 cytometer equipped with a 5-watt argon laser light source (3). After excitation at 488 nm, green fluorescence (EGFP) was detected at

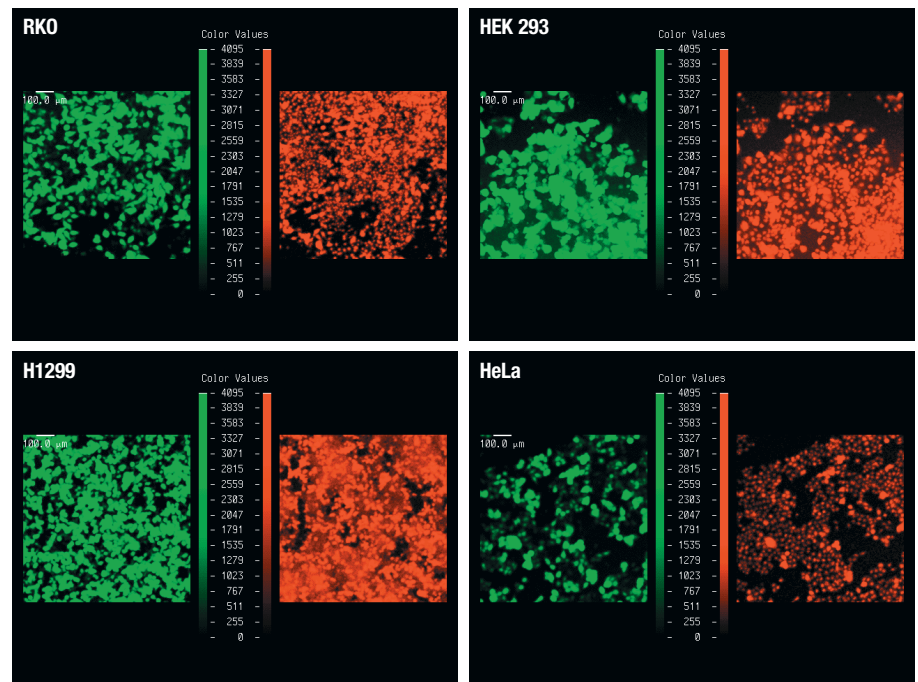


FIGURE 1. Transfection efficiency and viable cells. Cells were transfected with 2 μ g of pEBVCMV_{EGFP} (10 kb) and 6 μ l of LIPOFECTAMINE 2000 Reagent. 48 h after transfection, cells were fixed and stained with propidium iodide. The EGFP and propidium iodide fluorescence were analyzed.

530 nm with a 30-nm interference filter, while the red fluorescence of propidium iodide was detected above 605 nm. Fluorescence intensities were represented with a panel of pseudocolor digitized computer images. For brightfield examination (phase contrast), living cells were examined with the ACAS 570 in a phase mergescan routine.

RESULTS AND DISCUSSION

TRANSFECTION EFFICIENCY. Transfection efficiencies and conditions were determined for 8 cell lines (table 1). Among the tested cells, only the MeWo and Hos cells had low transfection efficiencies. In contrast to other transfection reagents (1), the maximum EGFP expression levels were detected 24 h after transfection of RKO, HEK 293, H1299, or HeLa cells (data not shown). These high levels of expression were maintained at 48 h (figure 1). Surprisingly,

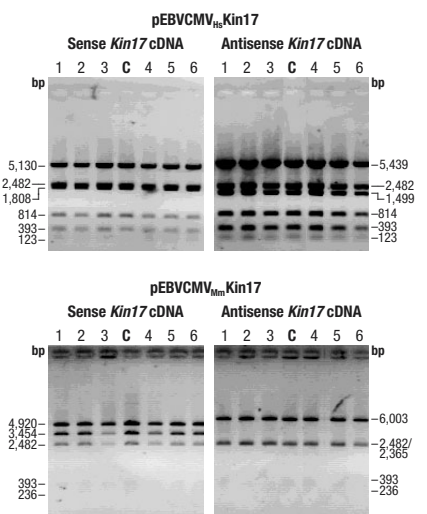


FIGURE 2. Stability of DNA. Low-molecular-weight DNA was recovered from RKO cells by alkaline lysis, and DH5 α ™ bacteria were transformed. DNA from independent clones was extracted, digested by EcoRI, and electrophoresed on a 0.6% agarose gel. The gel was stained with ethidium bromide. The photograph represents the negative image of the gel to highlight the bands. 6 clones from the sense and antisense plasmids are shown as well as digestion of purified plasmid DNA (Lane C).

Epstein Barr-Derived Vectors continued

only a small decrease in the transfection efficiency was observed for the 10-kb plasmid compared to the 7.4-kb plasmid (*data not shown*). Previously, we have observed a dramatic decrease in the number of transfected cells (with the larger plasmid) when transfection was carried out with other methods. Therefore, transfection with the LIPOFECTAMINE 2000 Reagent allowed rapid and high expression of the gene of interest.

To assess plasmid integrity, low-molecular-weight DNA was recovered from RKO cells, transformed into bacteria, and analyzed in an agarose gel after *EcoRI* digestion (*figure 2*). The transfected DNA was stable. Therefore, the transfection step did not damage DNA in these eukaryotic cells.

GENE EXPRESSION. Since EGFP protein has a long half-life not common for human proteins, we examined the expression of a short half-life protein, Kin17 (from murine or human cDNA). In RKO cells, transfection with increasing amounts of DNA led to increased $_{Hs}$ Kin17 protein (*figure 3A*). Efficient expression of mouse and human Kin17 was seen in the 5 tumorigenic cell lines tested (*figure 3B*). Transfection experiments and Western blot analysis were reproduced at least 3 times with the same results.

TRANSFECTION IN SERUM. To assess the toxicity of LIPOFECTAMINE 2000 Reagent, RKO cells were transfected twice. This double transfection with LIPOFECTAMINE 2000 Reagent did not increase cell toxicity in RKO cells (*figure 4*).

STABLE TRANSFECTION. Cells were propagated in culture with hygromycin B to evaluate their clonogenic growth. All transfected cells exhibited a normal growth, viability, and sensitivity to hygromycin B compared to what we previously observed with other transfection reagents (1). Three months after transfection, HEK 293 cells overexpressed the protein of interest, such as the EGFP protein (*data not shown*).

In summary, transfection with LIPOFECTAMINE 2000 Reagent is easy and works well in the presence of serum. Results were reproducible with 6 EBV-derived vectors using different cells and DNA preparations. High protein expression was only slightly dependent on the size of the plasmid. Stable clones were obtained. LIPOFECTAMINE 2000 Reagent allows us to study genes of biological interest in a wide range of tumorigenic cell lines using Epstein Barr-derived vectors. **FOCUS**

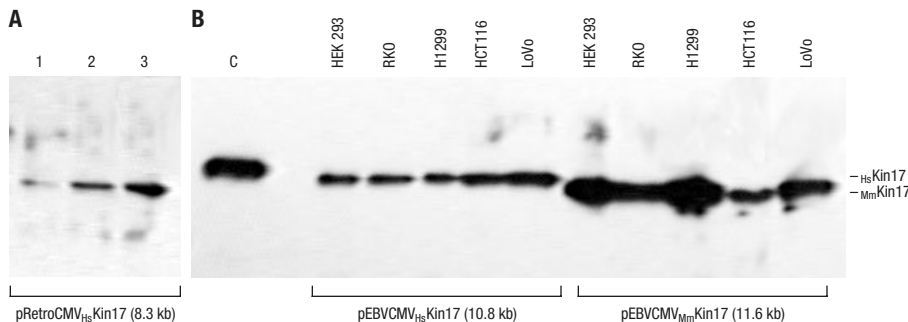


FIGURE 3. Kin17 protein expression. Cells seeded in 6-well dishes were transfected with 6 μ l of LIPOFECTAMINE 2000 Reagent. 48 h later, cells were trypsinized, lysed, and protein extracts electrophoresed on a 12% SDS polyacrylamide gel. Murine and human Kin17 proteins were identified using an affinity-purified anti-RecA antibody specific to Kin17 (3). *Panel A.* RKO cells were transfected with 1, 2, or 4 μ g (lanes 1-3, respectively) of pRetroCMV $_{Hs}$ Kin17. *Panel B.* 5 tumorigenic cells were transfected with 2 μ g of either human or murine Kin17 using EBV-derived vectors (>10 kb). *Lane C.* Human recombinant $_{Hs}$ Kin17 protein purified from baculovirus-infected Sf9 cells.

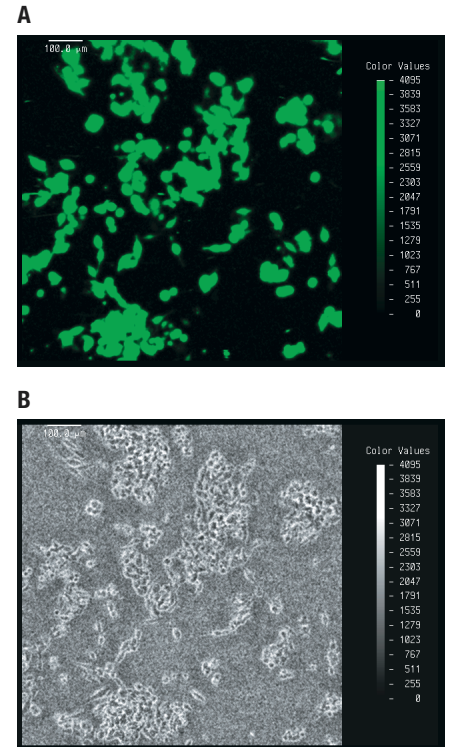


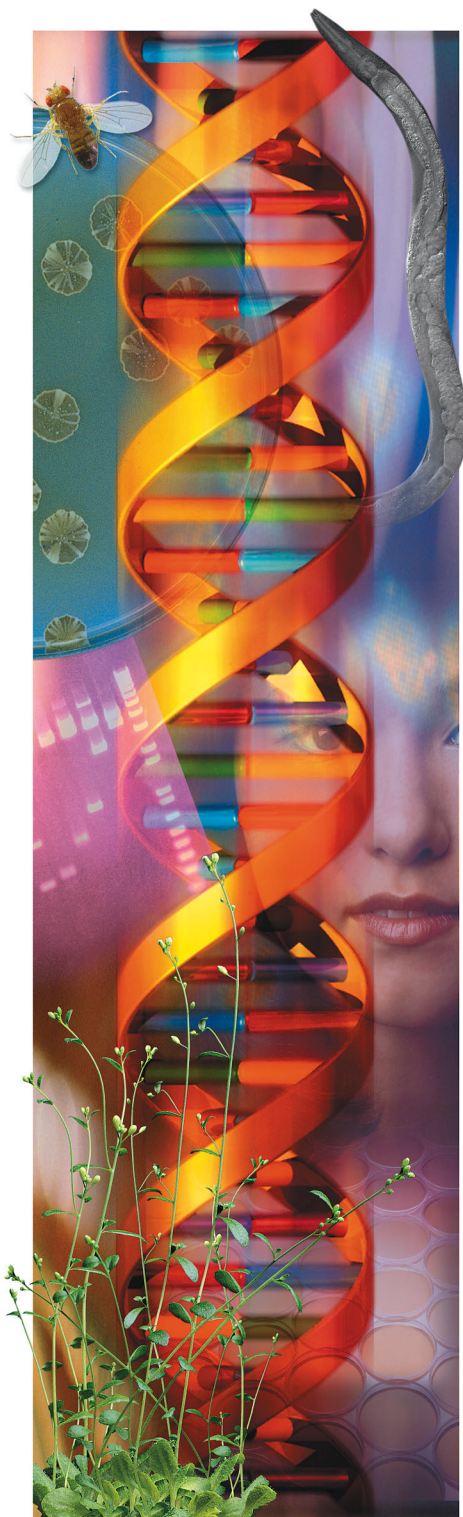
FIGURE 4. Effect of two transfections on cells. 3 days after plating, RKO cells were transfected with 2 μ g of pEBVCMV $_{EGFP}$ and 6 μ l of LIPOFECTAMINE 2000 Reagent. After 24 h, cells were transfected a second time with 2 μ g of pEBVCMV $_{EGFP}$ and 6 μ l of LIPOFECTAMINE 2000 Reagent. Living EGFP-positive cells were analyzed 24 h after the second transfection. *Panel A.* EGFP expression. *Panel B.* Phase contrast.

REFERENCES

1. Biard, D.S.F., Kannouche, P., Lannuzel-Drogou, C., Mauffrey, P., Apiou, F., and Angulo, J.F. (1999) *Exp. Cell Res.* 250, 499.
2. Biard, D.S.F., Cordier, A., and Sarasin, A. (1992) *Exp. Cell Res.* 200, 263.
3. Biard, D.S.F., Saintigny, Y., Maratrat, M., Vozenin, M.C., Martin, M., Daburon, F., and Angulo, J.F. (1997) *Arch. Dermatol. Res.* 289, 448.

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Reconstitution and Storage of Commonly Used Reagents



Sometimes molecular biologists buy reagents as a powder or in a lyophilized form to save expense or to increase the shelf life of the product. When it gets to the point of resolubilization/reconstitution, they are often left wondering what is the proper solvent and how to store the solution. This piece addresses many commonly asked questions on reconstitution and storage of reagents.

PROTEINASE K

Dissolve Proteinase K in 10 mM Tris-HCl (pH 7.5), 20 mM CaCl₂, and 50% glycerol at a concentration of 20 mg/ml. The Ca²⁺ serves as a stabilizer to suppress autolysis. Store at -20°C or 4°C. The stock solution is stable for 18 months at -20°C and 12 months at 4°C. Avoid freeze/thaw because it may cause loss of enzyme activity.

DTT

DTT is dissolved in water. To make the recommended concentration of 1 M, dissolve 3.09 g DTT in 20 ml distilled water. Dispense into 1-ml aliquots and store at -20°C. The stock solution is stable for 1 year at -20°C. After 2-3 freeze/thaws, discard the solution. At 4°C, the solution is stable <1 week.

X-GAL/BLUO-GAL

X-gal and Bluo-Gal are normally reconstituted in dimethylformamide (DMF) as a 2% stock solution.

Alternatively, they can be dissolved in 100% DMSO. Aliquot before storing since DMSO freezes at -20°C. These solutions are stable for >1 year at -20°C or for several weeks at 4°C. Solutions should be stored in a glass tube with a screw cap wrapped in foil.

Alternatively, X-gal can be dissolved in a 50:50 DMSO:water solution. Dissolve the X-gal in DMSO first, and then add an equal volume of distilled water. [See

Karlinsky, J.E. and Hughes, K.T. (1993) *BioTechniques* 15.2, 293.]

CUSTOM PRIMERS

Oligonucleotides are shipped lyophilized. Centrifuge the oligonucleotide to gather all of the DNA at the bottom of the tube. Add TE or water to reconstitute your primer for storage at -20°C. If storing at 4°C, dissolve the primer in TE. We recommend that you pipet up and down at least 10 times to ensure complete solubilization of the lyophilized oligonucleotide. Store primers at a concentration ≥10 μM.

IPTG

IPTG is dissolved in water at 100 mM (100 mM stock = 23.8 mg/ml). Store it in working aliquots at -20°C. The solution is stable for 6 months at -20°C.

ANTIBODIES

There are 2 methods to reconstitute antibodies. Procedure A is preferred because the glycerol prevents freezing at -20°C and is an effective biological inhibitor when the product is stored at 4°C.

Procedure A. Add 1 ml of 50% glycerol to the vial of antibody. Rotate the vial until the lyophilized pellet is totally dissolved. The presence of stabilizing proteins may prolong the time required to totally dissolve the pellet. The stock solution is stable for at least 1 year. At a working dilution, the concentration of glycerol is too small to affect most assays. Dilute to the desired concentration with PBS or other buffer.

Procedure B. Add 1 ml of reagent-quality water to the vial of antibody. Aliquot the solution if storing at -20°C to prevent excessive freeze/thaws of the stock. The antibody is stable for 6 to 12 months at -20°C or 4°C, depending on the antibody. Specific stability information is provided with each antibody. **FOCUS**

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