tamic acid decarboxylase with nonradioactive in situ hybridization methods. J. Comp. Neurol. *331*:339-362.

2. **Esclapez, M., N.J. Tillakaratne, D.L. Kaufman, A.J. Tobin, and C.R. Houser.** 1994. Comparative localization of two forms of glutamic acid decarboxylase and their mRNAs in rat brain supports the concept of functional differences between the forms. J. Neurosci. *14*:1834-1855.

3. **Freund, T.F. and G. Buzsáki.** 1996. Interneurons of the hippocampus. Hippocampus *6*:347-470.

4. **Houser, C.R. and M. Esclapez.** 1994. Localization of mRNAs encoding two forms of glutamic acid decarboxylase in the rat hippocampal formation. Hippocampus *4*:530-545.

5. **Kourilsky, P., S. Manteuil, M.H. Zamansky, and F. Gros.** 1970. DNA-RNA hybridization at low temperature in the presence of urea. Biochem. Biophys. Res. Commun. *41*:1080-1087.

6. **Liu, Y., E.J. Oakeley, L. Sun, and J.P. Jost.** 1998. Multiple domains are involved in the targeting of the mouse DNA methyltransferase to the DNA replication foci. Nucleic Acids Res. *26*:1038-1045.

7. **Oliva, A.A., Jr, M. Jiang, T. Lam, K.L. Smith, and J.W. Swann.** 2000. Novel hippocampal interneuronal subtypes identified using transgenic mice that express green fluorescent protein in GABAergic interneurons. J. Neurosci. *20*:3354-3368.

Anthony A. Oliva, Jr. and John W. Swann[1]
Oregon Health Sciences University
Portland, OR
[1]Baylor College of Medicine
Houston, TX, USA

Increased Yield of PCR Products by Addition of T4 Gene 32 Protein to the SMART™ PCR cDNA Synthesis System

ABSTRACT

Under certain conditions, T4 gene 32 protein is known to increase the efficiency of different enzymes, such as Taq *DNA polymerase, reverse transcriptase, and telomerase. In this study, we compared the efficiency of the SMART™ PCR cDNA synthesis kit with and without the T4 gene 32 protein. The use of this cDNA synthesis procedure, in combination with T4 gene 32 protein, increases the yield of RT-PCR products from approximately 90% to 150%. This effect is even observed for long mRNA templates and low concentrations of total RNA (25 ng). Therefore, we suggest the addition of T4 gene 32 protein in the RT-PCR mixture to increase the efficiency of cDNA synthesis, particularly in cases when low amounts of tissue are used.*

INTRODUCTION

Studies of differential gene expression in different normal or tumor tissues require large amounts of mRNA. Several procedures of cDNA amplification and enrichment are available, but one of the most efficient systems is the SMART™ PCR cDNA synthesis kit (Clontech Laboratories, Palo Alto, CA, USA). Interest in these systems stems from the fact that they require very small amounts of tissue obtained from biopsies or microdissected tissue sections. The SMART kit is based on the combination of reverse transcription and PCR for the amplification of full-length mRNAs.

Substances that inhibit enzyme activity are present in several tissue samples and can limit the use of RT-PCR, especially on a very small amount of RNA (2). The identity of several inhibitors is known; however, in many cases, the cause of inhibition remains undetermined. A good approach to the problem of RT-PCR inhibition would be to coun-
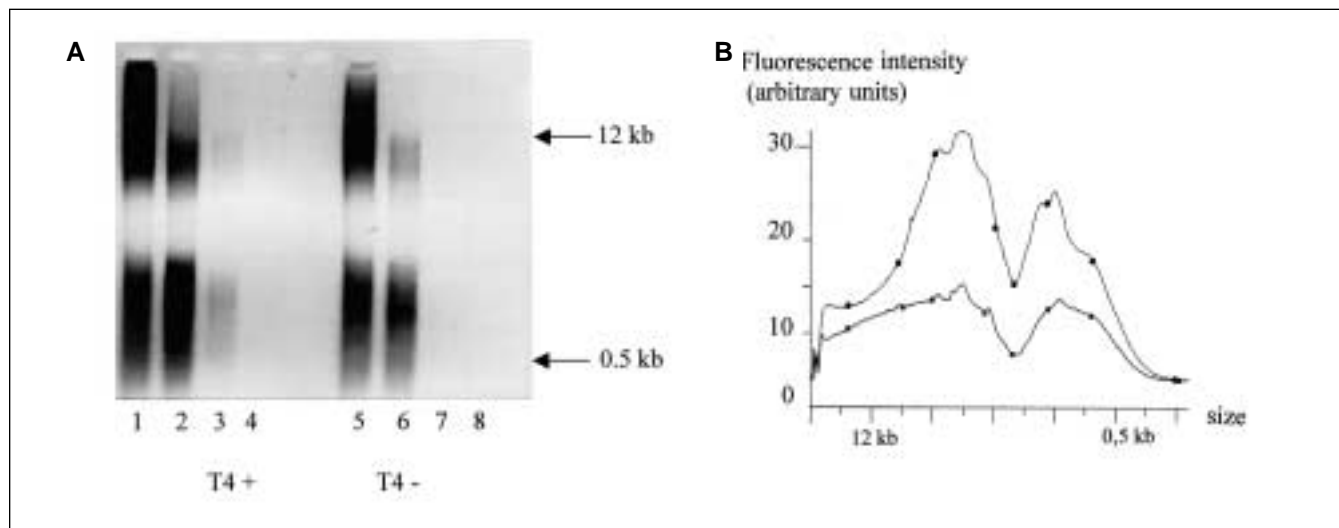
Short Technical Reports

Figure 1. Agarose electrophoresis gel and densitometric analysis of RT-PCR products formed in the absence or presence of T4 gene 32 protein with 25 ng total RNA from the COST cell line. (A) Negative picture of the gel. Effect of T4 gene 32 protein on RT-PCR amplification. Ethidium bromide-stained DNA fragments generated after reverse transcription (using the SMART PCR cDNA synthesis kit) and PCR (using a model 480 thermocycler; Applied Biosystems, Foster City, CA, USA) and fractionated by electrophoresis in 1% agarose. Lanes 1–4, PCR products obtained with T4 gene 32 protein after 24, 21, 18, and 15 cycles, respectively. Lanes 5–8, the same samples tested without T4 gene 32 protein with the same numbers of cycles. Note that reverse transcriptase reactions that contain gene 32 protein attain the optimal number of cycles ($n = 18$) earlier than negative ones. (B) Densitometric analysis profile of the RT-PCR products obtained without (squares) and with (circles) the addition of the T4 gene 32 protein. The intensity of the ethidium bromide-stained smears (fluorescence intensity) was determined by densitometric analysis of the gels with Gene Tools software as described in Materials and Methods. Note the apparent increase for longer fragments on the left part of the curves.

teract the action of offending substances. To this end, various additives (e.g., BSA, formamide, and T4 gene 32 protein) (2,4,5) have been included in the RT-PCR to relieve inhibition.

Marked secondary structures observed in DNA or RNA can hamper the fidelity and reduce the efficiency of both DNA replication and RNA reverse transcription. By interacting with DNA, RNA, and proteins, T4 gene 32 protein is known to increase the efficiency of different enzymes, such as reverse transcriptase (2,5), *Taq* DNA polymerase (10), and telomerase in the telomeric repeat amplification protocol (TRAP) assay (3). In addition, T4 gene 32 protein enhances the reverse transcriptase activity of the *Taq* DNA polymerase (2).

In this study, we demonstrated that the addition of T4 gene 32 protein to the reagents of the SMART PCR cDNA synthesis kit significantly improved the yield of cDNA products.

MATERIALS AND METHODS

Total RNA Extraction

Total RNA was extracted from three different cell sources: an anaplastic lymphoma cell line (COST), a T-cell lymphoma cell line (FEPD), and peripheral blood lymphocytes (PBL) extracted from a lymphoma patient using the RNeasy® Midi kit (Qiagen, Courtaboeuf, France), following the manufacturer's recommendations. Total RNA was quantified by measuring the absorbance at 260 nm and checked for integrity by electrophoresis on agarose gel and ethidium bromide staining.

RT-PCR Amplification of Full-Length mRNAs

Three different RNA concentrations (25 ng, 50 ng, and 1 µg) from each cell source were subjected to the SMART PCR cDNA synthesis kit, according to the manufacturer's recommendations. For all sources, each concentration was tested with and without the addition of T4 gene 32 protein. Briefly, each concentration of total RNA was tested from step 1 to 5 of the first-strand cDNA synthesis. We added 0.3 µL (150 ng/µL) T4 gene 32 protein (Roche Molecular Biochemicals, Mannheim, Germany) during step 6 of the first-strand cDNA synthesis, together with 2 µL 5× first-strand buffer, 1 µL dithiothreitol (20 mM), 1 µL 50× dNTP (10 mM), and 1 µL

Moloney murine leukemia virus reverse transcriptase (200 U/µL) in a total volume of 10 µL. Steps 6–10 were carried out according to the kit.

Amplification results were analyzed on 1% agarose gel (Tris-borate/EDTA), followed by ethidium bromide staining. The intensity of the ethidium bromide
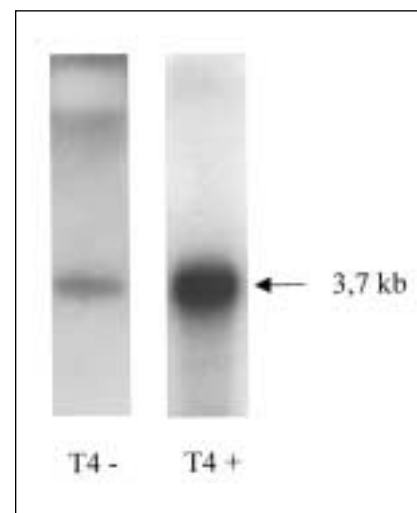

Figure 2. Virtual northern blotting with the moesin probe on the SMART product obtained with 50 ng total RNA from the COST cell line. Note the significant increase of the signal with T4 gene 32 protein.

luminescence was measured by image acquisition on a UV GeneGenius apparatus, followed by image treatment with Gene Tools software (both from Syngene, Cambridge, UK), in conditions for which the intensity of fluorescence was linear (7). The intensity of each smear was determined, and we could therefore compare relative product yield between individual RT-PCRs and measure the effects of the addition of T4 gene 32 protein.

Virtual Northern Blotting

SMART PCR-amplified cDNAs from the cell lines and PBL were electrophoresed on a 1% agarose gel, transferred onto nylon membrane (Hybond® N+; Amersham Pharmacia Biotech, Orsay, France), and hybridized with a ^{32}P-moesin cDNA probe. The probe was labeled with a multi-random priming kit (NonaPrimer®; Appligene Oncor, Illkirch, France). The filter was hybridized at 65°C overnight in 6× Denhardt's solution, 4× standard saline citrate (SSC), 0.1% SDS, and 50 μg/mL herring sperm DNA. The membrane was washed twice with 2× SSC and 0.5% SDS for 30 min and twice with 0.1% SSC and 0.1% SDS for 30 min at 68°C.

RESULTS

To determine whether the efficiency of the SMART PCR cDNA synthesis kit could be improved, T4 gene 32 protein was added to the RT-PCR mixture at the final concentration of 150 ng/μL. To verify that T4 gene 32 protein can improve reverse transcription and/or PCR, T4 gene 32 protein was added either before or after the reverse transcription step. This was performed in all samples; in each instance, we obtained the maximum cDNA yield increase when the T4 gene 32 protein was added before reverse transcription. When T4 gene 32 protein was added after the reverse transcription, no significant increase of cDNA yield was noticed.

Eighteen different experiments (i.e., nine with the addition of T4 gene 32 protein and nine without) corresponding to the three cell sources were carried out. As suggested by the manufac-turer, 15, 18, 21, and 24 cycles of PCR were performed. In each instance, the addition of T4 gene 32 protein significantly increased the efficiency of the RT-PCR of the SMART kit, regardless of the amount of total RNA used in the assay (0.025–1 μg). The reactions that contained T4 gene 32 protein attained the optimal number of cycles ($n = 18$) earlier than negative controls, even when the reaction was carried out with 25 ng total RNA, which is lower than the limit of the SMART technique (Figure 1A). The intensity of the ethidium bromide-stained smear was determined by densitometric analysis as described in Materials and Methods. The improvement of the yield of cDNA fragments varied slightly among the cell sources and ranged between 90% and 150%, irrespective of the input cDNA (25 ng, 50 ng, or 1 μg) (Figure 1B). As shown in Figure 1B, it appears that longer transcripts are more abundant with T4 gene 32 protein, suggesting that the more significant increase is obtained for longer messengers. To verify this hypothesis, virtual northern blotting was performed using the moesin probe. In each experiment, the signal intensity was clearly stronger when T4 gene 32 protein was added (Figure 2), irrespective of the cDNA concentration.

DISCUSSION

Our data provide evidence that the addition of T4 gene 32 protein to the SMART PCR cDNA synthesis kit's reagents significantly increases the sensitivity of this technique. T4 gene 32 protein has previously been used in combination with other enzymes. In particular, T4 gene 32 protein is known to increase the efficiency of reverse transcriptase, *Taq* DNA polymerase, and telomerase in the TRAP assay (2,3,5,10). The size of mRNAs is a limiting factor for reverse transcriptase and *Taq* DNA polymerase reactions (10). Secondary structures are more likely to occur in longer mRNA or DNA strands. In these particular conditions, T4 gene 32 protein has successfully improved PCR amplification of long templates (10). The mechanisms by which T4 protein functions are poorly understood. However, it is known that T4

Short Technical Reports

gene 32 protein can interact with DNA and proteins at the replication fork, thus increasing the efficiency and fidelity of DNA replication (2,8,9,12). T4 gene 32 protein is also suspected to interact with RNA, and, to date, only a few studies have demonstrated its influence on mRNA or RNA/DNA duplexes during reverse transcription (2). One such study was conducted by Chandler et al. (2), who demonstrated that the first-strand cDNA synthesis is more efficient in the presence of T4 gene 32 protein. In the previous description of the TRAP assay (3), which allows the detection of telomerase activity, T4 gene 32 protein seemed to increase the sensitivity of this technique by avoiding artifacts resulting from primer interactions. Because T4 gene 32 protein directly interacts with reverse transcriptase, it might also interact with telomerase because these enzymes share strong homologies for their catalytic subunits (6). On the other hand, several reports indicate that reverse transcriptase can interfere with PCR amplification of first-strand cDNA (1,11). The inhibitory effect of reverse transcriptase on the PCR could be mediated by the reverse transcriptase interaction(s) with the specific cDNA template. This inhibitory effect could be dependent on template concentration and removed by the addition of T4 gene 32 protein during the reverse transcription reaction.

Previous studies using T4 gene 32 protein have focused on single targets with specific primers (2,5). Here, we show that this protein is suitable for simultaneous RT-PCR amplification of full-length mRNAs of various size. As expected, transcripts of larger size, which can be missed with a standard RT-PCR assay, are efficiently amplified when T4 gene 32 protein is added. This could avoid a bias in cDNA selection during subtraction procedures.

In conclusion, we advise the use of T4 gene 32 protein in combination with the SMART PCR cDNA synthesis kit. This association significantly improves the yield of cDNA products subsequently submitted to molecular techniques, such as suppression subtractive hybridization. The SMART technique works on small amounts of total RNA (25 ng) and allows the amplification of longer messengers. The recent use of tissue microdissections is limited by a dramatic reduction of RNA amounts available for molecular techniques. The addition of T4 gene 32 protein to the SMART PCR cDNA synthesis kit would suitably overcome such difficulties.

REFERENCES

1. **Aatsinki, J.T., J.T. Lakkakorpe, E.M. Pietila, and H.J. Rajaniemi.** 1994. A coupled one-step reverse transcription PCR procedure for generation of full-length open reading frames. BioTechniques 16:282-288.
2. **Chandler, D.P., C.A. Wagnon, and H. Bolton, Jr.** 1998. Reverse transcriptase (RT) inhibition of PCR at low concentrations of template and its implications for quantitative RT-PCR. Appl. Environ. Microbiol. 64:669-677.
3. **Kim, N.W., M.A. Piatyszek, K.R. Prowse, C.B. Harley, M.D. West, P.L.C. Ho, G.M. Coviello, W.E. Wright et al.** 1994. Specific association of human telomerase activity with immortal cells and cancer. Science 266:2011-2015.
4. **Kovarova, M. and P. Draber.** 2000. New specificity and yield enhancer of polymerase chain reactions. Nucleic Acids Res. 28:e70.
5. **Kreader, C.A.** 1996. Relief of amplification inhibition in PCR with bovine serum albumin or T4 gene 32 protein. Appl. Environ. Microbiol. 62:1102-1106.
6. **Lingner, J., T.R. Hughes, A. Shevchenko, M. Mann, V. Lundblad, and T.R. Cech.** 1997. Reverse transcriptase motifs in the catalytic subunit of telomerase. Science 276:561-567.
7. **Nakayama, H., H. Yokoi, and J. Fujita.** 1992. Quantification of mRNA by non-radioactive RT-PCR and CDD imaging system. Nucleic Acids Res. 20:4939.
8. **Nossal, N.G.** 1992. Protein-protein interactions at a DNA replication fork: bacteriophage T4 as a model. FASEB J. 6:871-878.
9. **Sandhu, D.K. and P. Keohavong.** 1994. Effects of the T4 bacteriophage gene 32 product on the efficiency and fidelity of DNA amplification using T4 DNA polymerase. Gene 1444:53-58.
10. **Schwarz, K., T. Hansen-Hagge, and C. Bartram.** 1990. Improved yields of long PCR products using gene 32 protein. Nucleic Acids Res. 18:1079.
11. **Sellner, L.N., R.J. Coelen, and J.S. Mackensie.** 1992. Reverse transcriptase inhibits Taq polymerase activity. Nucleic Acids Res. 20:1487-1490.
12. **Wheeler, L.J., N.B. Ray, C. Ungermann, S.P. Hendricks, M.A. Bernard, E.S. Hanson, and C.K. Mathews.** 1996. T4 phage gene 32 protein as a candidate organizing factor for the deoxyribonucleoside triphosphate synthetase complex. J. Biol. Chem. 271:11156-11162.

C. Villalva, C. Touriol, P. Seurat, P. Trempat, G. Delsol, and P. Brousset
Centre Hospitalier Universitaire de Purpan
Toulouse, France