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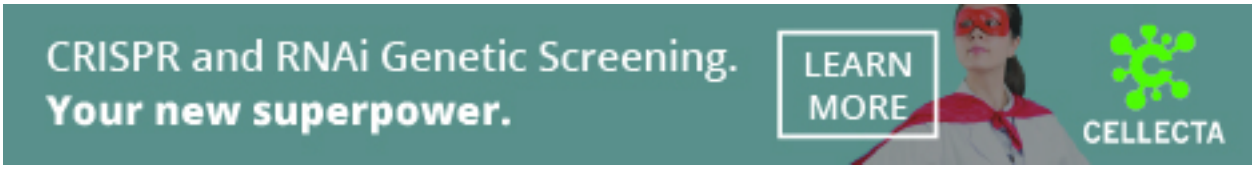

Effect of RNA Concentration on cDNA Synthesis for DNA Amplification

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DNA amplification utilizing the polymerase chain reaction (PCR) has greatly enhanced the ability to detect the presence of rare mRNA species in cells. By employing specific sense and antisense oligonucleotide primers with thermostable DNA polymerase, PCR allows amplification of target sequences by several log orders of magnitude.[1,2] This technique is being used increasingly to quantify rare mRNA species from cells.[3–6] In these studies, it is critical that the initial cDNA synthesis step be well controlled and optimized to produce a uniform substrate for amplification. We have examined RNA concentration in the initial cDNA synthesis reaction to optimize it for PCR amplification of both high- and low-abundance messages.

MATERIALS AND METHODS

Cells

Jurkat cells (kindly provided by G. Gaulton, Department of Pathology, University of Pennsylvania School of Medicine) were grown in RPMI-1640 with added penicillin/streptomycin, L-glutamine, and 10% fetal calf serum, and passaged twice weekly prior to use.

RNA Isolation and cDNA Synthesis

Cells were centrifuged (1200 rpm/10 min) and lysed in GITC-containing lysis buffer (4 M guanidine isothiocyanate, 0.5% *N*-lauroyl sarcosyl, 25 mM sodium citrate, 0.1 M β-mercaptoethanol). The lysate was extracted once with phenol, once with phenol/chloroform/isoamyl alcohol, and RNA-precipitated in 50% EtOH. The RNA pellet was resuspended in 50 μl diethylpyrocarbonate (DEP)-treated water, analyzed by agarose gel electrophoresis, and quantified spectrophotometrically (OD_{260}). Varying concentrations of RNA were utilized to synthesize cDNA with random priming in the following reaction mixture of 20 μl: 200 or 400 units of Moloney murine leukemia virus reverse transcriptase with a 1:5 dilution of 5× reverse transcriptase buffer (includes dithiothreitol), and 4 units of RNase inhibitor (all from GIBCO/BRL, Gaithersburg, MD), 0.6 or 1.2 μg of random oligonucleotides (mostly hexamers) (from Pharmacia LKB Biotechnology, Piscataway, NJ), and 0.5 or 1 mM dNTPs (equimolar in each dNTP, from Boehringer Mannheim GmbH, Germany). Following a 10-min preincubation at 25°C, the reaction was carried out for 1 hr at 42°C, then 95°C for 5 min, followed by storage at −20°C until use.

DNA Amplification

cDNA was amplified utilizing *Thermus aquaticus* DNA polymerase (*Taq* polymerase) and standard reaction conditions suggested by the manufacturer (Perkin-Elmer Cetus Corp., Norwalk, CT). If cDNA was used directly, it was diluted at least 1:20 in the PCR reaction, thereby minimizing the concentration of reagents from the cDNA synthesis step. The reaction mixture (25 μl) contained a 1:10 dilution of 10× reaction buffer, dNTPs (final concentration 200 μM in each dNTP), 1 μl of each oligonucleotide primer at 20 μM (final concentration 0.8 μM in each primer), and 1.25 μl of cDNA. This was heated to 95°C for 5 min prior to the addition of $MgCl_2$ (2.5 mM final concentration) and 1 unit of *Taq* polymerase. Primers were synthesized by the Wistar Institute oligonucleotide synthesis facility. The samples were covered with a drop of mineral oil, and amplified with a Programmable Thermal Cycler (MJ Research, Watertown, MA). This program was: melting at 94°C for 3 min; 15 cycles of melting at 94°C for 1 min, annealing at 60°C for 1 min, elongation at 72°C for 1 min; 15 cycles of melting at 94°C for 1 min, annealing at 60°C for 1.5 min, elongation at 72°C for 2 min; final elongation at 72°C for 7 min; cooling to 4°C. The reaction products were run on a 3% agarose gel, stained with ethidium bromide, and photographed under UV light.

RESULTS

Primers

The primers utilized for a low-abundance message were derived from the sequence of the human c-*myc* oncogene.[3] The sequences are:

c-*myc* 5′	1763	GCTTCTCAGAGGCTTGG	1779
c-*myc* 3′	2205	CGTCTAAGCAGCTGCAAG	2188

The primers utilized for a highly expressed message were derived from the sequences of human and murine γ-actin.[7–9] These sequences were aligned for maximal homology utilizing the programs Wordsearch and Segments.[10]

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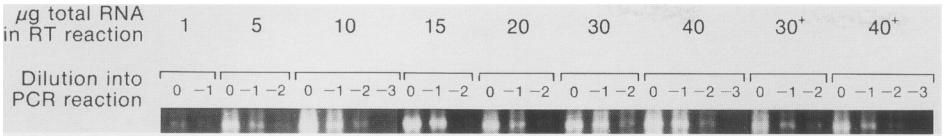


FIGURE 1 PCR with c-*myc* primers of Jurkat cell cDNA. The amount of RNA in the reverse transcriptase reaction is shown above the $\log_{10}$ of the cDNA dilution. The concentration of dNTPs was 0.5 mM except samples with a + symbol, where it was 1.0 mM in the RT reaction. Ethidium bromide-stained PCR products are shown following agarose gel electrophoresis.

The alignments and primers are shown here:

Human γ-actin
189 GACACCAGGGCGTCATGGTG 208
Murine γ-actin
155 GACACCAGGGCGTCATGGTG 174
Human γ-actin
781 GGAGAAGAGCTACGAGCTGC 800
Murine γ-actin
747 GGAGAAGAGTTACGAGCTGC 766
γ-actin 189–209 coding
GACACCAGGGCGTCATGGTG
γ-actin 804–781 noncoding
GCAGCTCGTAGCTCTTCTCC

cDNA Synthesis

RNA was extracted from log-phase growing Jurkat cells (human T-cell line), and quantified spectrophotometrically. Varying amounts were used to prime cDNA synthesis with either 0.5 or 1.0 mM dNTPs added. Serial dilutions of these cDNAs were subsequently reacted with the c-*myc* primers for 30 cycles of PCR as described in Materials and Methods. For analysis, the products were separated by agarose gel electrophoresis and visualized by ethidium bromide staining. The results are shown in Figure 1, with the amount of RNA in the reverse transcriptase (RT) reaction shown above the cDNA dilution. The samples designated with a plus sign had higher dNTP concentration (1.0 vs. 0.5 mM), as well as a higher concentration of RT (400 units vs. 200 units). The intensity of the bands at all dilutions increases with increasing RNA concentration up to ~10 μg RNA, then levels off. This is also apparent when the original gels were scanned densitometrically, and the intensity of the bands quantified (Fig. 2A,B). The addition of the higher amount of dNTPs and RT did not enhance the signal when analyzed similarly (Fig. 1).

These experiments were repeated on similar cDNAs with primers for γ-actin, a relatively high-abundance message, with multiple dilutions of the cDNAs used. When the different concentrations of RNA for RT are compared, the lowest concentrations used (1 μg) provided the least leeway for dilution. Ten micrograms of RNA was quite efficient at multiple dilutions, with PCR effective for a 10^{-3} dilution. This was also observed to be the case for 45 μg of RNA, although this higher concentration of RNA did not greatly improve the sensitivity of PCR

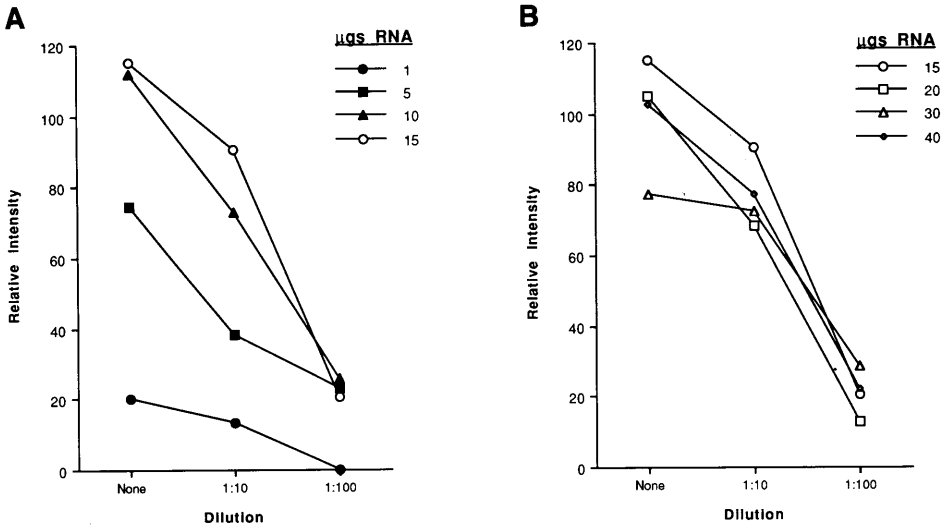
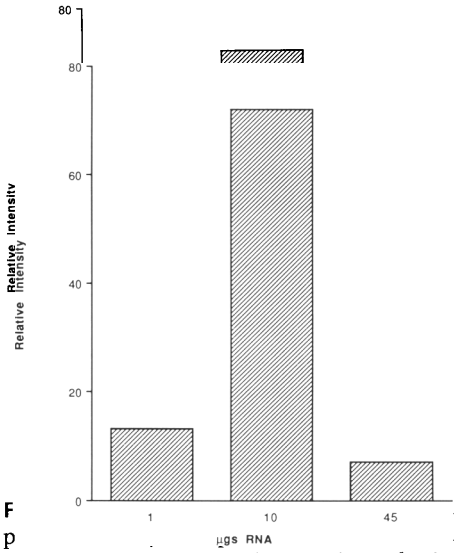


FIGURE 2 Densitometric quantitation of Fig. 1 PCR products.



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was used as a template for cDNA synthesis, and amplified for 30 cycles with γ-actin primers. Serial 10-fold dilutions of cDNA were used as substrates for PCR. Results are shown for the 10^{-2} dilution for cDNA synthesized from 1, 10, or 45 μg of RNA.

compared with 10 μg. The intensity of bands was quantitated densitometrically for the 10^{-2} dilution, with the results shown in Figure 3. While additional dilutions of RNA were not employed in these experiments, the finding of highest γ-actin signal intensity for the 10 μg concentration of RNA parallels the result for c-*myc*, suggesting that RNA concentrations in the range of 10–15 μg may provide optimal cDNA for amplification of both high- and low-abundance messages.

These studies demonstrate that cDNA synthesis can markedly affect the efficiency of PCR amplification, and should be optimized for increased PCR detection of RNA transcripts. Although the optimization protocol may vary depending on the specific transcript being analyzed, the method of cDNA priming, and other specific conditions, the initial preparation of cDNA should be carefully analyzed. Both too little or too much RNA may produce less cDNA than optimal for PCR amplification. For low concentrations of RNA, cDNA of better quality may be produced with higher amounts of dNTPs, thus enhancing detection for specific transcripts.

REFERENCES

1. Mullis, K., F. Faloona, S. Scharf, R. Saiki, G. Hprn, and H. Erlich. 1986. Specific en-

zymatic amplification of DNA in vitro: The polymerase chain reaction. *Cold Spring Harbor Symp. Quant. Biol.* **51:** 263–273.
2. Mullis, K. and L. Faloona. 1987. Specific synthesis of DNA in vitro via a polymerase catalyzed chain reaction. *Methods Enzymol.* **155:** 335–350.
3. Wang, A., M. Doyle, and D. Mark. 1989. Quantitation of mRNA by the polymerase chain reaction. *Proc. Natl. Acad. Sci.* **86:** 9717–9721.
4. Sambrook, J., E. Fritsch, and T. Maniatis. 1989. *Molecular cloning: A laboratory manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.
5. Choi, Y., B. Kotzin, L. Herron, J. Callahan, P. Marrack, and J. Kappler. 1989. Interaction of *Staphylococcus aureus* toxin "superantigens" with human T cells. *Proc. Natl. Acad. Sci.* **86:** 8941–8945.
6. Ausubel, F., R. Brent, R. Kingston, D. Moore, J. Seidman, J. Smith, and K. Struhl. 1989. *Current protocols in molecular biology*. Greene-Wiley, New York.
7. Gunning, P., P. Ponte, H. Okayama, J. Engle, H. Blau, and L. Kedes. 1983. Isolation and characterization of full-length cDNA clones for human alpha-, beta-, and gamma-actin mRNAs: Skeletal but not cytoplasmic actins have an amino-terminal cysteine that is subsequently removed. *Mol. Cell. Biol.* **3:** 787–795.
8. Erba, H.P., P. Gunning, and L. Kedes. 1986. Nucleotide sequence of the human gamma cytoskeletal actin mRNA: Anomalous evolution of vertebrate non-muscle actin genes. *Nucleic Acids Res.* **14:** 5275–5294.
9. Tokunaga, K., K. Takeda, K. Kamiyama, H. Kageyama, K. Takenaga, and S. Sakiyama. 1988. Isolation of cDNA clones for mouse cytoskeletal gamma-actin and differential expression of cytoskeletal actin mRNAs in mouse cells. *Mol. Cell. Biol.* **8:** 3929–3933.
10. Devereux, J., P. Haeberli, and O. Smithies. 1984. A comprehensive set of sequence analysis programs for the VAX. *Nucleic Acids Res.* **12:** 387–395.

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