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QUANTITATIVE PCR

Alice M. Wang and David F. Mark

PCR analysis has been used for RNA blot analysis, mRNA phenotyping, and nuclease protection analysis for the study of short-lived, low-copy-number mRNA transcripts (Kawasaki *et al.* 1988; Rappolee *et al.* 1988; Harbarth and Vosberg 1988; Lee *et al.* 1988; Price *et al.* 1988; Dobrovic *et al.* 1988; Hermans *et al.* 1988). Quantitation of the PCR analysis would provide more information for these studies. Such an approach has been used to study the relative amount of dystrophin transcript in different human tissues (Chelly *et al.* 1988). It was also used to quantitate the relative amounts of $apoB_{100}$ and $apoB_{48}$ message after thyroid hormone treatment to demonstrate that the apoB mRNA modification can be hormonally modulated (Davidson *et al.* 1988). A possible application of this approach includes the measurement of specific mRNA levels in drug-resistant and drug-sensitive human carcinoma cells. The early detection of specific mRNA patterns could lead to the development of more effective clinical protocols in cancer therapy (Kashani-Sabet *et al.* 1988).

It has been difficult to quantitate the amount of specific mRNA without an internal standard. We report here a technique that uses a synthetic AW106 cRNA as an internal standard for quantitating

PCR Protocols: A Guide to Methods and Applications

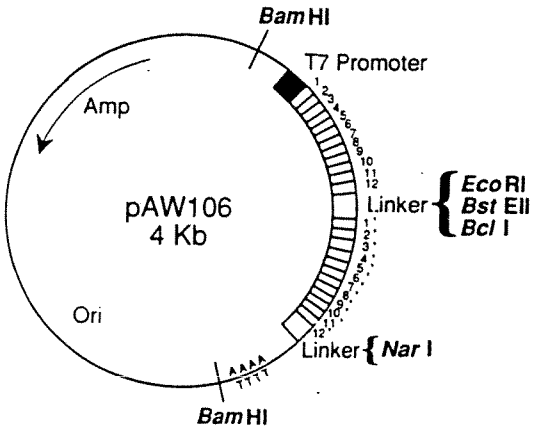

Figure 1 Structure of plasmid pAW106. Primers 1–12 are upstream primers of tumor necrosis factor (TNF), macrophage colony stimulating factor (M-CSF), platelet-derived growth factor A (PDGF-A), PDGF-B, apolipoprotein E (apo E), low-density lipoprotein receptor (LDL-R), 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG CoA-reductase), Interleukin-1α (IL-1α), IL-1β, IL-2, PDGF-R, and lipoprotein lipase (LPL), respectively, followed by the complementary sequences of their downstream primers 1′-12′ in the same order. Both upstream and downstream primers are followed by a restriction enzyme linker for easy insertion of primers for other genes.

the amount of specific mRNA by PCR. The AW106 cRNA was synthesized as a sense-strand from plasmid pAW106 by T7 polymerase (Fig. 1). This synthetic gene has twelve target genes' upstream primers connected in sequence followed by the complementary sequences of their downstream primers in the same order (Fig. 1). The PCR product synthesized from the cRNA standard by each primer set is around 300 bp and is designed not to overlap in size with the PCR products amplified by various primer pairs from the mRNA of these target genes. The AW106 cRNA contains polyadenylated sequences at the 3′ end, and it can be reverse transcribed and amplified together with the target mRNA in the same tube. The size difference in the PCR products permits easy separation of the cRNA product from the target mRNA product by gel electrophoresis. Since the same primer set is used in the PCR amplification on both templates, differences in primer efficiency are minimized. In the exponential phase of the amplification, the amount of target mRNA can be quantitated by extrapolating against the AW106 cRNA internal standard curve. In addition, the internal standard developed here contains the primer sequences for multiple genes so that the same standard can be used to quantitate a number of different mRNAs of interest.

Protocols

Reagents

1× PCR buffer	20 m*M* Tris–HCl (pH 8.3)
	50 m*M* KCl
	2.5 m*M* $MgCl_2$
	100 μg/ml of bovine serum albumin

RNA Preparation

Use standard protocols to prepare total cellular RNA isolated from cell lines or tissues (see Chapter 18). Synthesize AW106 cRNA by the transcription system of Promega. Purify the resulting cRNA product through an oligo(dT) column and quantitate it by absorbance at 260 nm.

Reverse Transcriptase Reaction

To 10 μl of a reverse transcriptase reaction, add 1 μg of total cellular RNA, 1.77×10^2 to 1.77×10^7 molecules of AW106 cRNA, 1× PCR buffer, 1 m*M* DTT, 0.5 m*M* each dNTP, 10 units of RNasin, 0.1 μg of oligo(dT) primer, and 100 units of Moloney murine leukemia virus reverse transcriptase (Bethesda Research Laboratories, Inc.). Incubate 60 minutes at 37°C. Heat inactivate the enzyme at 95°C for 5 minutes, and then quick chill on ice.

Polymerase Chain Reaction

A threefold dilution series of the reverse transcriptase product is mixed with 1× PCR buffer, 50 μM of each dNTP, 5 pmol each of the upstream and downstream primers, 1×10^6 cpm of one ^{32}P-end-labeled primer, and 1 unit of *Taq* polymerase, in a 50-μl reaction volume. The mixture is overlaid with 100 μl of mineral oil to prevent evaporation and then amplified by PCR for 25 cycles. The amplification cycle profile is denaturation for 30 seconds at 95°C, cooling for 2 minutes to 55°C, annealing of primers for 30 seconds at 55°C, heat-

ing for 30 seconds to 72°C, and extension of primers for 1 minute at 72°C.

Gel Electrophoresis

A 5- to 10-μl portion of the PCR mixture is electrophoresed in 10% polyacrylamide gel in TBE buffer. Stain the gel with ethidium bromide and photograph. Appropriate bands are excised from the gel, and the radioactivity is determined by scintillation fluorography.

Quantitation

The amount of radioactivity recovered from the excised gel bands is plotted against the template concentrations. The fact that the amplification rates of internal standards and specific mRNA are identical within the exponential phase of the PCR allows one to construct a standard curve that can be used to quantitate the actual amount of a specific mRNA species. As shown in Fig. 2, 20 ng of PMA-induced

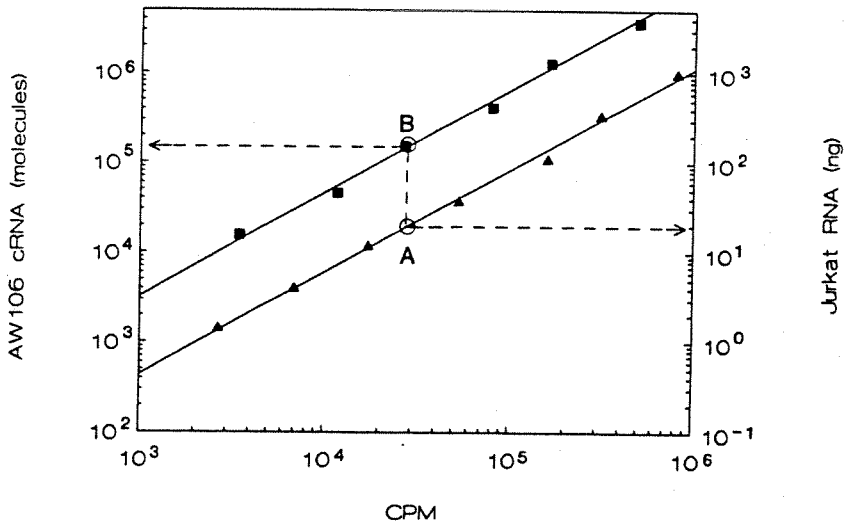

Figure 2 Quantitation of IL-2 mRNA in Jurkat cells by co-amplification with internal standard AW106 cRNA. The variable template concentrations of the internal standard AW106 cRNA and Jurkat RNA were plotted against the radioactivity of their PCR product. The PCR analysis was performed with IL-2 primers. (■) PCR product of AW106 cRNA. (▲) PCR product of Jurkat RNA.

Jurkat RNA yielded 30,000 cpm of interleukin-2 (IL-2)-specific PCR product, point A, which translated to point B on the standard curve as 1.5×10^5 molecules of AW106 cRNA. In other words, 20 ng of induced Jurkat total RNA contained 1.5×10^5 molecules of IL-2 mRNA.

Discussion

Larzul *et al.* (1988) demonstrated that the amount of amplified DNA fragment in a given sample has an influence on amplification efficiency. When a high template concentration is used, the primer concentration, *Taq* polymerase availability, amplified product snapback, etc., can be limiting factors for efficient amplification. To reliably quantitate the specific mRNA using an internal standard, the range of concentrations of both templates and the number of amplification cycles are chosen such that they stay within the exponential phase of the PCR. Therefore, it is necessary to first titrate the specific mRNA to find the range of concentrations that gives exponential amplification over a defined range of cycle numbers.

When a quantitative evaluation is required, the conditions have to be very strictly controlled in this extremely sensitive PCR technique. A negative control tube is necessary to monitor false positives and to give the amount of background counts.

Because of its high sensitivity, speed, and accuracy, the RNA/PCR quantitation method can be used to detect gene expression in a more extensive way. It can accurately quantitate the amount of specific mRNA. In many cases the detection of the differences in levels of expression of specific RNA molecules can provide useful information for the diagnosis of infectious disease or cancer.

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PCR
PROTOCOLS

A GUIDE TO METHODS AND APPLICATIONS

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Thomas J. White
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ACADEMIC PRESS, INC.
Harcourt Brace Jovanovich, Publishers
San Diego New York Berkeley Boston London Sydney Tokyo Toronto

This book is printed on acid-free paper. ∞

Academic Press, Inc., San Diego, California 92101
United Kingdom Edition published by
Academic Press Limited, 24–28 Oval Road, London NW1 7DX

Library of Congress Cataloging-in-Publication Data

PCR protocols.
Includes index.
1. Polymerase chain reaction. 2. Gene amplification.
I. Innis, Michael A. [DNLM: 1. DNA Polymerases.
2. Gene Amplification--methods. 3. Genetic Engineering
--methods. 4. RNA Polymerases. QH 442 P3479]
QP606.D46P36 1989 574.87'328 89-6938
ISBN 0-12-372180-6 (alk. paper)
ISBN 0-12-372181-4 (pbk. : alk. paper)

Printed in the United States of America
89 90 91 92 9 8 7 6 5 4 3 2 1

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