*4694–4696* *Nucleic Acids Research, 1997, Vol. 25, No. 22* 

# Adaptor-tagged competitive PCR: a novel method for measuring relative gene expression

**Kikuya Kato***

Louis Pasteur Center for Medical Research and Cell Switching Project, ERATO, JST, 103-5 Tanakamonzencho, Sakyo-ku, Kyoto 606, Japan

Received July 17, 1997; Revised and Accepted September 4, 1997

## ABSTRACT

**A simple and reliable PCR-based method to quantitate gene expression is described. Following the digestion of double-stranded cDNA by a restriction enzyme, an adaptor is ligated to a cDNA from a first RNA sample, and another adaptor to a second RNA sample. The two adaptors share a common sequence at the outer region, but differ in size. Equal amounts of the ligated samples are mixed, and amplified by an adaptor-primer and a primer specific to the gene of interest. Products derived from the two sources differ in size, and can be separated by denaturing polyacrylamide gel electrophoresis. The ratio of the two products reveals the relative level of gene expression. Since the technique avoids the need to construct internal standards, it is especially useful for the analysis of many different gene transcripts.**

Northern hybridization (1) is still the method of choice for measuring the level of gene expression. The sensitivity meets routine laboratory needs, since it can detect as little as 5 pg of RNA. However, the technique requires fairly large amounts of RNA with 0.3–3 μg of mRNA required to detect very rare species. The technique is therefore not applicable when only a limited sample is available. Another weakness is that the experimental procedure is too laborious for the analysis of many different gene transcripts.

Polymerase chain reaction (PCR) is the most sensitive method for the detection of DNA or RNA (2). However, its quantitative applications (3,4) are limited. Mainly because the method requires strict control experiments for calibration, such as the construction of internal standards from the target molecule, optimization of cycle number, and construction of a calibration curve (5,6). If this step was to be simplified, quantitative PCR would be an even more useful technology. Here, I describe a novel PCR-based technique, named the adaptor-tagged competitive PCR (ATAC-PCR). The technique revealed the relative levels of gene expression in a very simple manner, and the principle is described below.

The main scientific interest in gene expression is the relative level among biological samples, not the absolute amount. Focusing on this point, the technique was invented as follows. RNA was purified from two sources, and double-stranded cDNA was synthesized using a biotinylated oligo-dT primer. Following digestion of the cDNA by a restriction enzyme, a first adaptor was ligated to the first cDNA sample, and a second adaptor to the second cDNA sample. The two adaptors shared a common sequence and had a cohesive end to restriction sites, but the

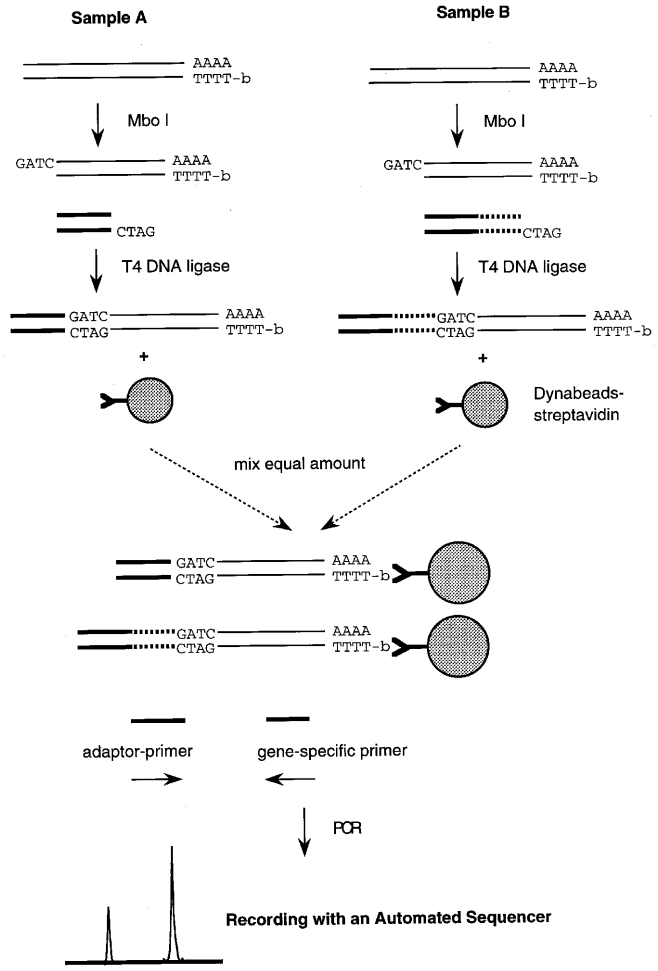


**Figure 1.** Schematic presentation of the ATAC-PCR for quantitation using *Mbo*I as a restriction enzyme.

*Correspondence should be directed to present address: Taisho Laboratory of Functional Genomics, Nara Institute of Science and Technology, 8916-5 Takayamacho, Ikoma, Nara 630-01, Japan. Tel: +81 7437 2 5583; Fax: +81 7437 2 5589; Email: kkato@bs.aist-nara.ac.jp

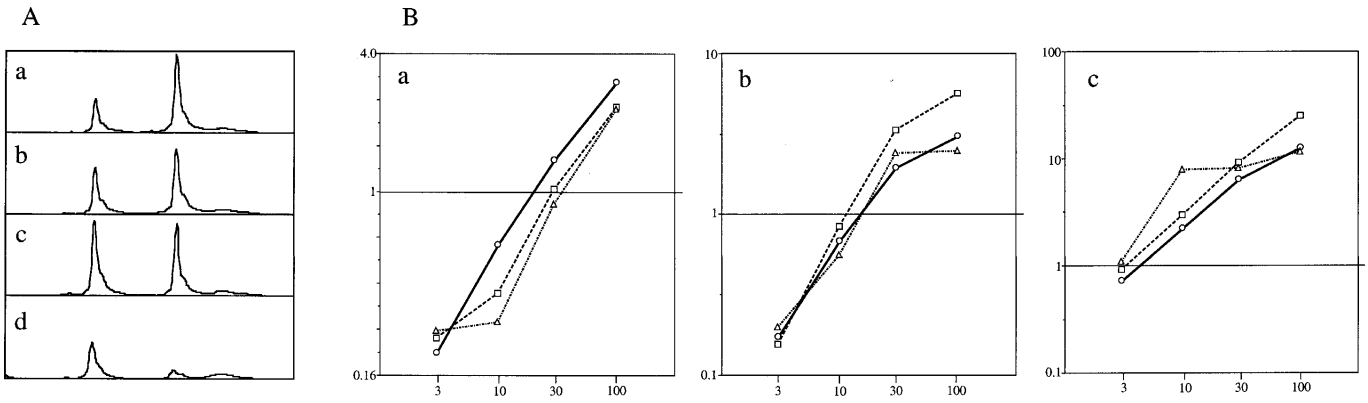


**Figure 2.** (**A**) An example of the electropherograms. *Mbo*I-digested cDNA was made from kidney RNA containing 10% liver RNA: the cDNA was ligated to the MA-1 adaptor. *Mbo*I-digested mouse liver cDNA was serially diluted with *Mbo*I-digested mouse kidney cDNA, and ligated to the MA-4 adaptor. Equal amounts of both samples were mixed and amplified with the adaptor-primer and the primer specific to apolipoprotein AI. cDNAs ligated to the MA-4 adaptor are as follows: (a) mouse liver cDNA; (b) 30% mouse liver cDNA with 70% mouse kidney cDNA; (c) 10% mouse liver cDNA with 90% mouse kidney cDNA; (d) 3% mouse liver cDNA with 97% mouse kidney cDNA. The left peak corresponds to the product with the MA-1 adaptor, and the right peak to that with the MA-4 adaptor. (**B**) Accuracy and dynamic range of ATAC-PCR. (a) Mouse kidney RNA containing 30% mouse liver RNA; (b) mouse kidney RNA containing 10% mouse liver RNA; (c) mouse kidney RNA containing 3% mouse liver RNA. Vertical axis, ratio of fluorescent intensity, calculated from the fragment derived from liver cDNA serially diluted with mouse kidney cDNA compared to that derived from mouse kidney RNA containing liver RNA. Horizontal axis, percentage of mouse liver cDNA serially diluted by mouse kidney cDNA. Genes tested were as follows. ○, apolipoprotein AI; □, prothrombin; Δ, insulin-like growth factor. (Materials and Methods) cDNA was synthesized as described (8), and cut with *Mbo*I according to the supplier's protocol. Typically, 2.5 µg of the total RNA was converted to cDNA in a 20 µl reaction mixture with 10 pmol of biotinylated $d(T)_{18}$ and 200 U SuperScript II MMTV reverse transcriptase. The cDNA was digested with 6 U *Mbo*I at 37°C for 50 min. The digested cDNA was dissolved in 1.5 ml of distilled water. 30 pmol of an adaptor was added to 10 µl of cDNA in a 15 µl reaction mixture consisting of 1× ligation buffer and 150 U T4 DNA ligase, and the sample was incubated at 16°C for >12 h. After completion of the ligation reaction, 5 µl of 5 M NaCl and 3 µl of 10 mg/ml Dynabeads–streptavidin were added to the ligation mixture and incubated at room temperature for 20 min with occasional agitation. It is important to absorb the biotinylated cDNA in each tube, because nucleic acids stick to the tubes reversibly. Following absorption, the two cDNA samples for measurement were mixed and then washed once with 1× B&W buffer and twice with distilled water. The PCR mixture consisted of 1× PCR buffer for Stoffel fragment, 2.5 mM $MgCl_2$, 0.2 mM deoxynucleotide triphosphates, 2.5 pmol Texas Red labelled-C1S, 2.5 pmol of a gene-specific primer, one third of the absorbed cDNA, and 1 U Stoffel fragment in a 10 µl reaction mixture. Therefore, ~5 ng of each total RNA was used for one assay. PCR amplification consisted of 30 cycles at 94°C for 30 s, 50°C for 1 min and 72°C for 1 min, following incubation at 72°C for 20 min. The sample was mixed with 10 µl of 10 mM Tris-Cl (pH 8.0), 10 mM $MgCl_2$, 50 mM NaCl and 0.2 mM dNTP containing 0.1 U T4 DNA polymerase and incubated at 37°C for 50 min to make sharp peaks, and then at 72°C for 10 min. After dialysis against tap water (9) to remove artifactual products of ~100 bases, 20% of the sample was applied to a denaturing polyacrylamide gel. The dialysis is not necessary for experiments using other dyes such as Cy5 and those for ABI sequencers. The sizes and fluorescent intensities of fragments were monitored by an autosequencer, SQ-5500 (Hitachi Electronic Engineering Co.) (10). In the range we routinely use, i.e., 50–5000 AFU, the fluorescent intensity is linear to the amount of DNA. Sequences of adaptors for quantitation were as follows: MA-1, 5′-GATCCGCGTTCTAACGACAATATGTAC-3′, 5′-GTACATATTGTCGTTAGAACGCG-3′; MA-4, 5′-GATCGAGCACTCTT-AGCGTTCTAACGACAATATGTAC-3′, 5′-GTACATATTGTCGTTAGAACGCTAAGAGTGCTC-3′. The sequence of the adaptor-primer (C1S) was 5′-GTACATATTG-TCGTTAGAACGC-3′. Gene-specific primers were as follows. Apolipoprotein AI, 5′-TTATTGTAAGAAAGCCAATGCG-3′; prothrombin, 5′-TTTGCAATGAGGATTC-CATAGT-3′; insulin-like growth factor AI, 5′-TTCATTGGGGGAAATGCCCATC-3′.

second adaptor had an additional 10 bases between the common sequence and the overhang. From equal amounts of the two cDNA samples, 3′ end cDNA fragments were recovered with streptavidin-coated paramagnetic beads, and then mixed. This procedure ensures quantitative recovery of cDNA. It also removed most of the adaptor molecules, a potential inhibitor of the next PCR reaction, and cDNA fragments derived from upstream regions. The PCR amplification was performed using an adaptor-primer, the sequence of which was from the common part of the adaptors, and a primer specific to the gene of interest, the sequence of which was included in the 3′ end fragment. For fluorescent detection, the adaptor-primer was labelled with fluorescent dye. Amplified fragments were separated by denaturing polyacrylamide gel electrophoresis, and the amount of fragments was measured by an automated sequencer. The same experiment is repeated with different combinations of adaptors and RNAs, the first adaptor with the second RNA, and the second adaptor with the first RNA. The geometrical mean of the ratios from the two experiments was calculated. A schematic presentation of the method is shown in Figure 1.

To demonstrate the usefulness of this method, the following experiments were performed. cDNAs for ATAC-PCR were synthesized from the following RNA preparations: kidney RNA containing 30, 10 and 3% liver RNA. Three liver-specific genes, apolipoprotein AI, prothrombin and insulin-like growth factor AI were chosen for testing. The abundance of apolipoprotein AI, prothrombin, and insulin-like growth factor AI in liver mRNA were 0.5, 0.1 and 0.01%, respectively (7). The relative expression levels of the genes in these RNA preparations were measured against liver RNA. For example, in the same amount of RNA, mRNA for apolipoprotein AI in kidney RNA containing 30% liver RNA is 30% of that in liver RNA. Each cDNA preparation was mixed with serially diluted liver cDNA, and experiments were performed to locate the point where the amount of the two fragments was equal. As an example, the amount of apolipoprotein AI mRNA in kidney RNA with 10% liver RNA should be equal to that in 10 times diluted liver RNA. Typical electropherograms results are shown in Figure 2A. The determination of the expression levels is shown in Figure 2B. The deviation from the predicted values was well within acceptable levels, and probably due to pipetting error. Sizes of fragments were also as predicted from database sequences (data not shown). The standard deviation of the value was small, being 0.14–0.25, when the amounts of two samples were nearly equal, whereas at higher concentrations of liver RNA they were relatively large, at 0.40–7.6, indicating that accurate results are obtained when both templates are at nearly equivalent concentrations (4).

ATAC-PCR eliminates the most tedious step of quantitative PCR, i.e., construction of an internal control and calibration curve. In addition, relative levels of different genes can be measured at one time using one set of cDNA preparations. Comparing this technique with northern hybridization, the amount of RNA required is reduced by >100-fold (5 ng/assay of each total RNA under the current conditions). The data can be easily obtained digitally, and the accuracy is probably similar to that of northern hybridization. Thus, the technique is especially useful for cases when the amount of RNA is limited, and it is necessary to measure expression levels of many different transcripts.

However, despite its usefulness, the technique has some weaknesses. First, the frequency of failure is higher than conventional PCR. We used the technique for >30 genes, and the success rate was ~80% (Sawada *et al*., in preparation). Often it is necessary to try several PCR primers to obtain good amplification. Secondly, the detection limit is not as good as conventional quantitative competitive PCR. The technique detects messages as rare as one in 100 000 mRNA molecules (data not shown). Finally, there are fragments due to aberrant amplification, and the fragments of interest sometimes must be identified from their sizes. These shortcomings arise from the use of a single gene-specific primer, as opposed to the two used in the conventional PCR. These weaknesses might be improved to some extent by use of nested PCR primers. However, these shortcomings are tolerable, considering the amount of RNA required, and the simplicity of the experimental procedure.

## REFERENCES

1 Thomas,P.S. (1980) *Proc. Natl. Acad. Sci. USA*, **77**, 5201–5205.
2 Mullis,K.B. and Faloona,F.A. (1987) *Methods Enzymol.*, **155**, 335–350.
3 Becker-Andre,M. and Harlbrock,K. (1989) *Nucleic Acids Res.*, **22**, 9437–9446.
4 Gilliland,G., Perrin,S. and Bunn,H.F. (1990) In Innis,M.A., Gelfand,D.H., Sninsky,J.J. and White,T.J. (eds) *PCR Protocols*. Academic Press, San Diego, pp. 60–69.
5 Celi,F.S., Zenilman,M.E. and Shuldiner,A.R. (1993) *Nucleic Acids Res.*, **21**, 1047.
6 Nagel,S., Schmidt,M., Thiede,C., Huhn,D. and Neubauer,A. (1996) *Nucleic Acids Res.*, **24**, 4102–4104.
7 Kato,K. (1995) *Nucleic Acids Res.*, **23**, 3686–3690.
8 Sambrook,J., Fritsch,E.F. and Maniatis,T. (1989) *Molecular Cloning: A Laboratory Manual*. 2nd edition. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
9 Okubo,K., Hori,N., Matoba,R., Niiyama,T. and Matsubara,K. (1991) *DNA Seq.*, **2**, 137–144.
10 Kambara,H., Nagai,K. and Kawamoto,K. (1992) *Electrophoresis*, **13**, 542–546.

## RELATED PAPERS RECENTLY PUBLISHED IN *NUCLEIC ACIDS RESEARCH*

Bernard,K., Auphan,N., Granjeaud,S., Victorero,G., Schmitt-Verhulst,A.M., Jordan,B.R. and Nguyen,C. (1996) Multiplex messenger assay: simultaneous, quantitative measurement of expression of many genes in the context of T cell activation. *Nucleic Acids Res.*, **24**, 1435–1442.

Grassi,G., Zentilin,L., Tafuro,S., Diviacco,S., Ventura,A., Falaschi,A. and Giacca,M. (1994) A rapid procedure for the quantitation of low abundance RNAs by competitive reverse transcription-polymerase chain reaction. *Nucleic Acids Res.*, **22**, 4547–4549.