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# Highly Accurate Quantification of mRNA Expression by Means of Titan™ One Tube RT-PCR and Capillary Electrophoresis

## Introduction

RT-PCR is an extremely sensitive method of mRNA analysis, sometimes exceeding traditional RNA blot techniques by a factor of 1000 (1). Despite its unique sensitivity, quantitative information can be difficult to obtain. There are several approaches for exploiting the ability of RT-PCR to measure mRNA expression quantitatively.

Of these, semi-quantitative methods with housekeeping genes as internal standards are satisfactory when comparing one transcript in different tissues (2). These methods are not as time consuming and costly for quantitative mRNA determination as other techniques (*e.g.*, competitive PCR). Apart from the determination of the RNA quality, variations in amplification efficiency are the main reason for needing housekeeping genes for normalization.

We performed the present study to demonstrate that housekeeping genes are no longer necessary for normalization, provided the quality of the template RNA is assured. For this purpose, we combined the high fidelity of the Titan™ One Tube RT-PCR System with the high sensitivity of capillary electrophoresis (CE) equipped with laser-induced fluorescence (LIF) detection.

To confirm the reliability of this combined method, we determined the mRNA of the transcription factor *c-fos* in fresh rat tissues using varying PCR cycles and concentrations.

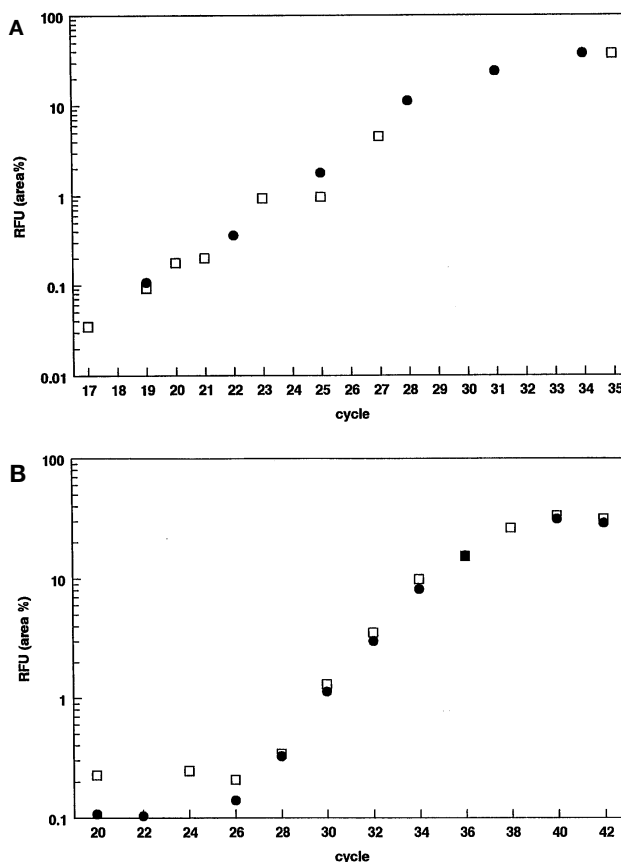
## Materials and Methods

### Tissue and RNA isolation

The liver and brain of male Wistar rats (Charles River) served as the source for total RNA extraction for *c-fos* determination. Total RNA was isolated by TriPure® Isolation Reagent (Boehringer Mannheim Biochemicals).

### Primers

Two primer pairs for rat *c-fos* mRNA were designed from the cDNA with the aid of the computer program Amplify (William Engels). Each primer set spanned an intron.



**Figure 1** *c-fos* and varying cycles.

**1A.** Template: 0.5 µg total RNA per PCR, rat liver. Linear fluorescence signal from 17 to 28 cycles.

**1B.** Template: 0.2 µg total RNA per PCR, rat brain. Linear fluorescence signal from 26 to 36 cycles.

Rat liver:

forward primer 5'-GGA GGG AGC TGA CAG ATA CG-3'.

reverse primer 5'-TCA GCC TCG GGG TAG GTG AA-3', generating a 730 bp PCR product.

Rat brain:

forward primer 5'-CCT CGA GGG GTT CCC GTA GA-3'.

reverse primer 5'-ACA GTA CGT GGA TAT AGC GA-3', generating a 497 bp PCR product.

All primers were fluorescent-labeled at the 5' end (MWG-BIOTECH).

### One tube RT-PCR

One tube RT-PCR was carried out using the Titan RT-PCR-System (Boehringer Mannheim Biochemicals). The reaction

components were set up in two master mixes. Master mix 1: dNTP (10 mM) 1  $\mu$ l; Primer 1 (20 pmol/ $\mu$ l) 0.75  $\mu$ l; Primer 2 (20 pmol/ $\mu$ l) 0.75  $\mu$ l; DTT (100 mM) 2.5  $\mu$ l; RNase inhibitor (10 U/ $\mu$ l) 1  $\mu$ l; total RNA (various concentrations) 0.5  $\mu$ l; DEPC water 18.5  $\mu$ l. Master mix 2: 5x RT-PCR buffer (contains 1.5 mM MgCl<sub>2</sub>) 10  $\mu$ l; enzyme mix (AMV and Expand™ High Fidelity PCR System) 1  $\mu$ l; DEPC water 14  $\mu$ l.

25  $\mu$ l of each master mix was added to a 0.2 ml thin-walled PCR tube and mixed properly. After centrifugation, the samples were placed in a thermocycler equilibrated at 50°C (PTC 200, MJ Research), with heated lid for 30 min.

Cycling conditions for the amplification were a single 2 min step at 94°C, followed by 10 cycles of denaturation at 94°C for 30 s, annealing at 52°C for 30 s in each case, and elongation at 68°C for 45 s. Further cycles were carried out with an additional 5 s elongation per cycle. After these cycles, a prolonged elongation of 7 min. at 68°C was used. Samples were frozen at -20°C and stored for subsequent analysis.

### Capillary electrophoresis

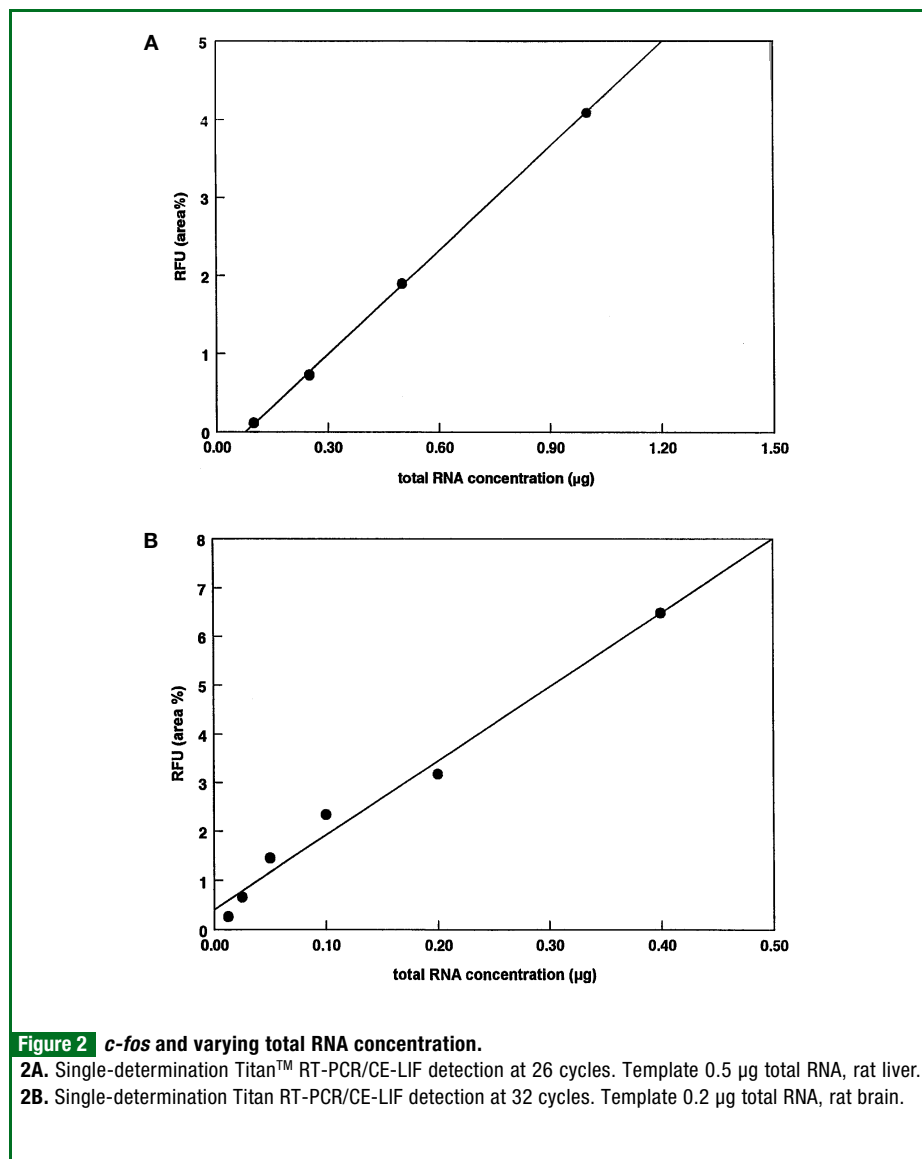
Determination of PCR products by CE was carried out in a P/ACE System 2100 (Beckmann Instruments) equipped with laser-induced fluorescence detector using an argon ion laser source. Excitation was performed at 488 nm and fluorescence emission collected through a band pass filter at 520 nm. DB-17 coated capillaries (J&W Scientific) with 100  $\mu$ m i.d. and 57 cm in length (50 cm effective length) were employed. The coating thickness was 0.2  $\mu$ m. The electrophoretic buffer consisted of 0.5% (w/w) hydroxypropylmethyl cellulose, 89 mM Tris, 89 mM boric acid, 2 mM Na-EDTA, and 100 mM NaCl, pH 8.5. The buffer was replaced after each run.

Injection was carried out under pressure for 60 s without desalting the samples. The subsequent separations were performed at a constant voltage of 175 V/cm.

*c-fos* expression was quantified by integrating the peak areas of the primers and the PCR products (Gold™ Software), and subsequent calculation of the relation between PCR product and the total measured fluorescence (= area %).

### Results and Conclusions

Titan RT-PCR was performed in the rat brain and the rat liver with two different primer pairs. First, a calibration curve for *c-fos* with increasing number of cycles was performed for each primer combination. These calibration curves were carried out as double determinations and hence display two different sets of RT-PCRs. Figures 1A and 1B show that the measured points of the different cycle numbers deviate very little from each other. These findings demonstrate the high fidelity of the Titan system and the accuracy of the detection system.



**Figure 2** *c-fos* and varying total RNA concentration.

**2A.** Single-determination Titan™ RT-PCR/CE-LIF detection at 26 cycles. Template 0.5  $\mu$ g total RNA, rat liver.

**2B.** Single-determination Titan RT-PCR/CE-LIF detection at 32 cycles. Template 0.2  $\mu$ g total RNA, rat brain.

The calibration curve for the rat brain with 0.2 µg total RNA per PCR revealed a linearity of the RT-PCR from 26 to 36 cycles, whereas the calibration curve for the rat liver with 0.5 µg total RNA per RT-PCR displayed a linearity between 17 and 28 cycles. Consequently, 32 cycles (brain) and 26 cycles (liver) were chosen for the total RNA concentration experiments.

Figures 2A and 2B show the relative fluorescence signal in relation to the increasing amount of total RNA at the chosen cycles. A strong linearity was observed in both experiments. The predicted high fidelity of the Titan system was thus confirmed.

Another factor is the technique chosen for quantification. Although several methods have been described for quantification of RT-PCR products, each involves an agarose gel separation step, coupled to either scintillation counting, radioimaging, or densitometric scanning of autoradiographs or photographs of dye-stained gels. Variations in sample load, staining time, sample preparation and strong background signal may sometimes lead to incorrect interpretation of the RT-PCR.

Capillary electrophoresis with laser-induced fluorescence detection overcomes most of these problems and is the tool of choice for visualizing the precise amplification of the Titan system.

Advantages of this method are self-evident:

- Nonradioactive, automated procedure with on-line detection.
- The columns can last for hundreds of runs.
- Detection limits are lower than those for ethidium bromide/UV detection.

The stoichiometric incorporation of the fluorescent molecules makes it easy to quantify the PCR products. It should nevertheless be borne in mind that this method can only be applied to the exponential phase of RT-PCR, which has to be determined in advance.

Overall, we believe that the combination of the Titan System with CE-LIF detection can be used to quantify any mRNA of interest with a high degree of accuracy.

### Acknowledgments<sup>‡</sup>

This work was supported by the DFG (Le 318/10-1).

### References

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2. Noonan, K.E., Beck, C., Holzmeyer, T.A., Chin, J.E., Wunder, J.S., Andrulis, I.L., Gazdar, A.F., Willman, C.L., Griffith, B., Von Hoff, D.D., and Roninson, I.B. (1990) Quantitative analysis of MDR 1 (multidrug resistance) gene expression in human tumors by polymerase chain reaction. *Proc. Natl. Acad. Sci. USA* **87**: 7160–7764.

| Product   | Cat. No.               | Pack Size                                  |
|---|------------------------|--|
| Deoxynucleotide Triphosphate Set, sodium salt solution, PCR-Grade | 1 969 064              | 4 x 25 µmol (250 µl)                       |
| dATP, sodium salt solution, PCR-Grade                             | 1 934 511<br>1 969 013 | 25 µmol<br>125 µmol                        |
| dCTP, sodium salt solution, PCR-Grade                             | 1 934 520<br>1 969 021 | 25 µmol<br>125 µmol                        |
| dGTP, sodium salt solution, PCR-Grade                             | 1 934 538<br>1 969 030 | 25 µmol<br>125 µmol                        |
| dTTP, sodium salt solution, PCR-Grade                             | 1 934 546<br>1 969 048 | 25 µmol<br>125 µmol                        |
| dUTP, sodium salt solution, PCR-Grade                             | 1 934 554<br>1 969 056 | 25 µmol<br>125 µmol                        |
| Titan™ One Tube RT-PCR Kit†                                       | 1 939 823              | 50 reactions                               |
| Titan™ One Tube RT-PCR System†                                    | 1 888 382<br>1 855 476 | 25 reactions<br>100 reactions              |
| TriPure® Isolation Reagent  | 1 667 157<br>1 667 165 | 50 ml<br>200 ml                            |
| RNase Inhibitor   | 799 017<br>799 025     | 2000 units<br>10000 units                  |
| Thin-walled PCR Tubes††   | 1 667 041<br>1 667 050 | 1000 tubes (200 µl)<br>1000 tubes (500 µl) |