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*Communication*

# Quantification of thioredoxin mRNA expression in the rat hippocampus by real-time PCR following oxidative stress[✪]

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**Thioredoxin (Trx) is a multifunctional protein with a redox-active disulfide/dithiol in the active site. Thioredoxin, with its redox-regulating and reactive oxygen species (ROS) scavenging activities, plays several important biologic roles both in intracellular and extracellular compartments. The purpose of this report was to quantify the relative expression of Trx in rat hippocampus following an oxidative stress-involving treatment such as kainic acid (KA) using real-time PCR and the $2^{-\Delta\Delta C_T}$ method. The relative changes in expression of Trx mRNA in KA-treated and control animals were significantly different as 2.02 ± 0.77 and 1.0 ± 0.26, respectively ($P < 0.05$). Minimum and maximum n-fold changes in Trx expression in KA-treated and control animals were determined as (1.4–5.2) and (0.8–1.3), respectively. Thus, real-time PCR and the $2^{-\Delta\Delta C_T}$ method for data analysis from real-time PCR were found to be an accurate and sensitive method for quantifying Trx mRNA levels.**

Thioredoxin or adult T-cell leukemia-derived factor (ADF) is a well-known antioxidant ubiquitiously present in all mammalian cells that catalyses disulfide/dithiol exchange reactions in association with thioredoxin reductase (TrxR) (Yodoi & Tursz, 1991; Mitsui *et al.*, 1992; Hori *et al.*, 1994; Holmgren, 1995; Arner & Holmgren, 2000). Trx is a stress-inducible protein whose expression is enhanced by stressors including viral infection, exposure to UV light, X-ray radiation, and hydrogen peroxide (Abate *et al.*,

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**Abbreviations**: ADF, adult T-cell leukemia-derived factor; $C_T$, threshold cycle; f, polymerase chain reaction efficiency; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; KA, kainic acid; ROS, reactive oxygen species; Trx, thioredoxin; TrxR, thioredoxin reductase; $t_m$, melting temperature.

1990; Sachi *et al.*, 1995; Nakamura *et al.*, 1994; Ohira *et al.*, 1994). Recent investigations have also shown the role and importance of Trx in the nervous system. Various types of physical, chemical or other stressful stimuli including direct trauma (Lippoldt *et al.*, 1995), nerve injury (Mansur *et al.*, 1998) and ischaemic-reperfusion insult (Takagi *et al.*, 1998a; 1998b) can up-regulate the expression of Trx transcripts in the astroglial and/or neuronal cells in and around the injured site, which suggests some sort of protective effect on the surrounding neuronal population.

Kainic acid (KA) is an agonist of ionotropic glutamate receptors. It is well known that excessive or persistent activation of glutamate-receptor gated ion channels (excitotoxicity) contributes to neuronal degeneration. The cellular and molecular mechanisms underlying the neurotoxicity induced by KA were attributed to overproduction of free radicals and activation of an apoptotic pathway (Coyle & Puttfarcken, 1993; Reynolds & Hastings, 1995; Schulz *et al.*, 1995). Excitotoxicity is considered to be a contributing factor in a number of central nervous system disorders such as stroke, epileptic seizures and Huntington's disease (Choi, 1987). Recently it has been shown that Trx attenuates hippocampal damage induced by KA in transgenic mice (Takagi *et al.*, 2000).

Reverse transcription followed by real-time-PCR is the technique of choice to analyse mRNA expression in various sources. Real-time PCR is highly sensitive and allows quantification of rare transcripts and small changes in gene expression. The simplest detection technique for newly synthesised PCR products in real-time PCR uses the SYBR Green I fluorescence dye that binds specifically to the minor groove of double-stranded DNA (Morrison *et al.*, 1998). Relative quantification describes the change in expression of the target gene relative to some reference group such as an untreated control or a sample at time zero in a time-course study. The $2^{-\Delta\Delta C_T}$ method may be used to calculate relative changes in gene expression determined from real-time experiments. Derivation of $2^{-\Delta\Delta C_T}$ equation, including assumptions, experimental design, and validation tests, have been described in Applied Biosystems User Bulletin No. 2 (P/N 4303859). Analyses of gene expression data using the $2^{-\Delta\Delta C_T}$ method have appeared in the literature (Winer *et al.*, 1999; Schmittengen *et al.*, 2000; Chang *et al.*, 2002). Additionally, Livak Schmittengen (2001) presented the derivation and application of two variations of the $2^{-\Delta\Delta C_T}$ method that may be useful in the analysis of real-time PCR data. The purpose of this report was to quantify the relative expression of Trx in the rat hippocampus following an oxidative stress-involving treatment such as KA injection using real-time PCR and the $2^{-\Delta\Delta C_T}$ method.

## METHODS

Male Sprague-Dawley rats were used for the present study. The animals were housed under controlled environmental conditions with a 12-h light-dark cycle and were fed standard laboratory food and tap water available *ad libitum*. All animals were handled in accordance with the Institutional Animal Care and Use Guidelines. The animals were divided into two groups: KA treatment (15 mg/kg, n = 4) and controls (saline, n = 4). Kainic acid was dissolved in phosphate-buffered saline and the pH was adjusted to 7.4 with NaOH. Kainic acid and saline were administered to rats intraperitoneally (i.p.). Only those rats which exhibited full limbic seizures, i.e. forelimb clonus with rearing, were used in this study. Seizures occurred approximately 45 min after injection, typically lasted 2–3 h, and were not observed to recur. The animals were decapitated under diethylether anaestesia and the hippocampus was dissected on an ice-cold plate. All samples were stored at –80°C until use.

Total RNA was extracted from the hippocampus using Trizol reagent (Gibco BRL Life Technologies, Grand Island, NY, U.S.A.) followed by phenol/chloroform extraction and isopropanol precipitation (Chomczynski, 1993). Prior to the reverse transcription reaction, potentially contaminating residual genomic DNA was eliminated with DNase I (Fermentas MBI). cDNA synthesis was carried out using random hexamer primers and MuMMLV reverse transcriptase (Fermentas MBI) using 1 $\mu$g RNA. Real-time quantitation of Trx mRNA was performed with an iCycler iQ detection system (BIO-RAD, Hercules, CA, U.S.A.) according to Yalcin *et al.* (2004).

The forward and reverse primers for Trx cDNA were derived from a previous study (Mansur *et al.*, 1998). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA primers were designed newly to achieve better sensitivity and assay repeatability. Conditions for PCRs were optimized in a gradient cycler with regard to *Taq* DNA polymerase (Perkin-Elmer), primers (TIBMOLBIOL, Berlin, Germany), $MgCl_2$ concentration and annealing temperatures. The optimized settings were transferred to real-time PCR protocols on a BIORAD iCycler iQ detection system and SYBR Green I assay was used for quantification. Primer sequences and PCR conditions are shown in Table 1.

Amplification of 4 $\mu$l reverse transcription mixture was carried out using 5 $\mu$l 10 × SYBR® Green PCR buffer, 4.0 $\mu$l (4–25 mM) $MgCl_2$, 1.0 $\mu$l 12.5 mM dNTP mix with dUTP, 1 $\mu$l of forward and reverse primers (0.1 $\mu$g/$\mu$l) and 2.5 U *AmpliTaq* DNA polymerase in a total volume of 50 $\mu$l. Cycling parameters were: 3 min at 94°C, 30 s at 94°C followed by 40 cycles of 30 s at 60°C and 45 s at 72°C.

Although it may be difficult to determine the absolute amount of a cDNA present in different samples, quantitation of test mRNA transcripts is often normalized to a reference gene such as GAPDH gene presumed to be invariant.

***Internal control and calibrator for the $2^{-\Delta\Delta C_T}$ method.*** The choice of calibrator for the $2^{-\Delta\Delta C_T}$ method depends on the type of the gene expression experiment. The simplest design is to use the untreated control as the calibrator. Using the $2^{-\Delta\Delta C_T}$ method, the data are presented as the fold change in Trx gene expression normalized to the GAPDH gene (endogenous control) and relative to the untreated control. For the untreated control sample, $\Delta\Delta C_T$ equals zero and $2^0$ equals one, so the fold change in gene expression relative in untreated control equals one, by definition. For the treated samples, evaluation of $2^{-\Delta\Delta C_T}$ indicates the fold change in gene expression relative to the untreated control.

***Data analysis using $2^{-\Delta\Delta C_T}$ method.*** Real-time PCR was performed on the corresponding cDNA synthesized from each sample. The data were analysed using the equation described by Livak *et al.* (2001), as follows:

Amount of target = $2^{-\Delta\Delta C_T}$. The threshold cycle ($C_T$) indicates the fractional number at which the amount of amplified target reaches a fixed threshold.

$\Delta C_T$ = (average Trx $C_T$ – average GAPDH $C_T$)

$\Delta\Delta C_T$ = (average $\Delta C_T$ untreated sample – average $\Delta C_T$ treated sample)

Because PCR is an exponential process, the relative copy number difference (n) is equal to the PCR efficiency (f) raised to the power of $\Delta C_T$, that is, $n = f^{(\Delta C_T)}$. The efficiency equals to 2 in an optimal reaction. Finally, the normalized target mRNA amount is equal and relative to untreated sample, that is, amount of Trx mRNA = $2^{-\Delta\Delta C_T}$.

Validation of the method. cDNA was synthesized from 10-fold serially diluted RNA samples and amplified by real-time PCR using target gene specific primers. The initial RNA concentration was 200 ng/ml. The CT (y-axis) was plotted against log of total RNA (x-axis), and the equation was calculated by linear regression analysis. To confirm the amplification specificity of the PCR, products from

each primer pair were subjected to melting curve analysis and subsequent agarose gel electrophoresis. A 15 $\mu$l sample was resolved on a 1.2% agarose gel containing ethidium bromide (final concentration 0.125 $\mu$g/ml).

***Statistical analysis.*** Data are presented as means ±S.D. The statistical significance is determined using the Fisher's protected least significance difference (PLSD) method following analysis of variance (ANOVA).

## RESULTS

### Specific amplification

Melting curve analysis demonstrated that each of the primer pairs described (Table 1) amplified a single predominant product with a distinct melting temperature ($t_m$) as shown for Trx cDNA in Fig. 1. The $t_m$ of Trx cDNA product can be seen clearly as a peak in a first derivative plot. The rapid fall of at 89.5°C indicates the presence of a specific product of Trx cDNA which melts at this temperature. The $t_m$ for GAPDH cDNA prouct was 90.5°C. Product purity for Trx and GAPDH is shown in Fig. 2. Agarose gel electrophoresis demonstrates that the Trx product is a single band of the predicted size, 339 bp.

### Correlation of RNA concentrations and CT values

$C_T$ values and log of RNA concentrations of samples show a reverse linear correlation (R = 0.99) as predicted (Fig. 3).

**Table1. Primer sequences of the genes studied and PCR conditions.**

| Gene | Primer sequences (5′–3′) Forward and reverse | Annealing temp. (°C) | Product bp and $t_m$ (°C) |
|---|---|---|---|
| GAPDH | AAGGTCATCCCAGAGCTGAA<br>ATGTAGGCCATGAGGTCCAC | 57 | 338, 90.5 |
| Trx | CCGCAACAGCCAAAATGGTGA<br>AGCATGATTAGGCAAACTCCGTAA | 55 | 339, 89.5 |

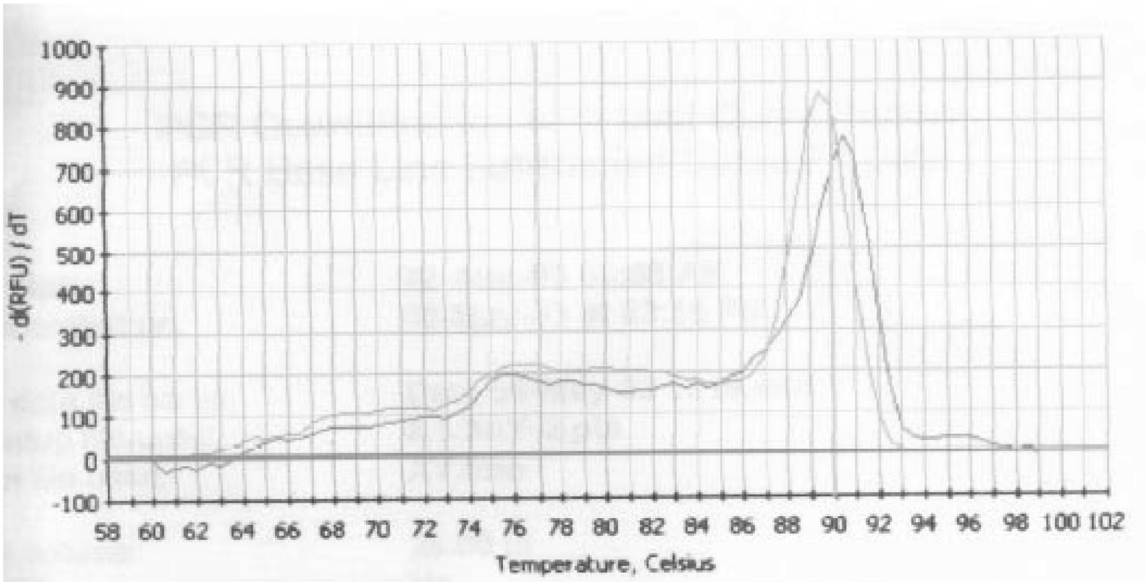


**Figure 1. Melting curve analysis of Trx cDNA and GAPDH cDNA amplification products.**

The rapid fall off at 89.5°C indicates the presence of a specific product of Trx cDNA that melts at this temperature (light gray). The melting temparature for GAPDH cDNA product was 90.5°C (dark gray).

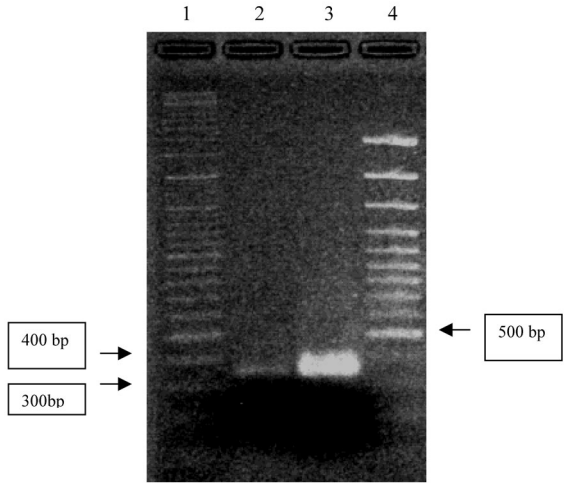


**Figure 2. Agarose gel electrophoresis of Trx cDNA and GAPDH cDNA amplification products.**

Lane 1. Marker 100 bp DNA ladder; Lane 2. Trx (339 bp); Lane 3. GAPDH (338bp); Lane 4. Lambda DNA *Eco*RI + *Hin*d III.

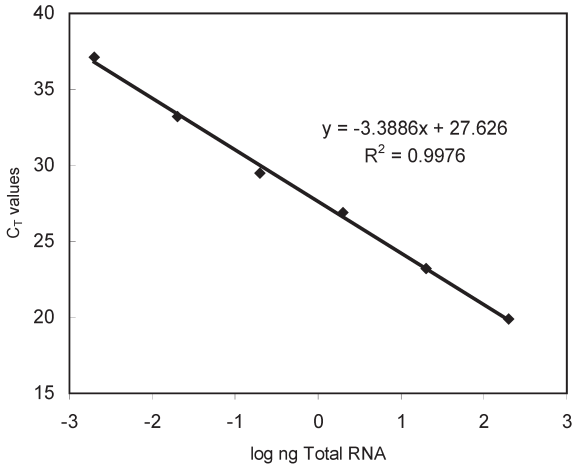


**Figure 3. Correlation between RNA concentrations and $C_T$ values of real-time PCR for Trx.**

### Expression of Trx in hippocampus

The relative changes in the level of Trx mRNA in KA-treated and saline-treated control rats are significantly different, 2.02 ± 0.77 and 1.0 ± 0.26, respectively ($P < 0.05$). Minimum and maximum n-fold changes in the expression of Trx in KA-treated and control rats are (1.4–5.2) and (0.8–1.3), respectively. Examples of analysis of the data from real-time PCR experiments for Trx mRNA are presented in Table 2.

## DISCUSSION

### Validation of the real-time quantitative PCR experiments

The present study is the first to use real-time quantitative PCR to evaluate changes in mRNA expression for Trx in the hippocampus using $2^{-\Delta\Delta C_T}$ method for quantification. In each PCR, unknown samples were simultaneously amplified with the same standard samples and corresponding negative control samples (non-reverse transcription samples). This process corrected for a potential difference in PCR efficacy between PCR assays, and also confirmed that neither genomic DNA contamination nor non-specific amplification occured. The correlation coefficient between log total RNA concentrations and $C_T$ was 0.99, indicating that quantification was accurate.

In order to perform real-time PCR analysis, GAPDH was used as a specific endogenous control, since it has been demonstrated that the RNA encoding GAPDH is a ubiquitously expressed, moderately abundant message. During real-time PCR analysis, the expression of GAPDH remained constant over time and after experimental manipulations (Edwards *et al.*, 1985; Winer *et al.*, 1999). The purity of PCR products of Trx and GAPDH was succesfully confirmed both by $t_m$ analysis and agarose gel electrophoresis.

Using the $2^{-\Delta\Delta C_T}$ method, the fold change in gene expression of Trx mRNA in KA-treated hippocampus was normalized to GAPDH mRNA. These results demonstrate that quantitative real-time PCR is an accurate and sensitive method for determining Trx mRNA levels in the rat hippocampus. Also, the $2^{-\Delta\Delta C_T}$ method for analysis data from real-time PCR is applicable to assess the changes in gene expression of Trx following any oxidative stress involving treatment such as KA.

**Table 2. Fold change in expression of Trx gene relative to the reference gene GAPDH in rat hippocampus treated with kainic acid.**

The results represent three different experiments from each rat.

| Sample | GAPDH $C_T$ | Trx $C_T$ | $\Delta C_T$ (Avg. Trx–Avg. GAPDH) | $\Delta\Delta C_T$ (Avg. $\Delta C_T$–Avg. $\Delta C_T$ control) | Normalized Trx mRNA expression relative to control $2^{-\Delta\Delta C_T}$ |
|---|---|---|---|---|---|
| KA | 16 | 20.5 | 4.5 | | |
| | 16.4 | 21.1 | 4.7 | | |
| | 16.3 | 20.2 | 3.9 | | |
| | 16 | 21.7 | 5.7 | | |
| Avg. | 16.1 | 20.8 | 4.7 | 1.02 ± 0.77 | 2.02 (1.4–5.2) |
| Control | 17.2 | 20.9 | 3.7 | | |
| | 17.4 | 21.1 | 4.1 | | |
| | 17.7 | 20.2 | 3.4 | | |
| | 17.2 | 21.7 | 3.5 | | |
| Avg. | 17.3 | 20.9 | 3.67 | 0.00 ± 0.26 | 1.0 (0.8–1.3) |

### Effect of KA on the expression of Trx mRNA in the rat hippocampus

Using *in situ* hybridization, rat Trx mRNA was observed spread in nerve cells including CA3/CA4 region of hippocampus. The highest expression of mRNA was found in regions with high-energy demand or high activity involving redox-active metabolites (Lippoldt *et al.*, 1995). Thioredoxin may play a role in keeping enzymes active in the presence of oxidizing substrates or more directly as a ROS scavenger (Mitsui *et al.*, 1992; Nakamura, 1997). Thioredoxin also regulates intracellular molecules *via* thiol redox control of apoptosis and transcription factors such as NF-$\kappa$B, AP-1 and p53 (Schenk *et al.*, 1994; Hirota *et al.*, 1997; Saitoh *et al.*, 1998). Thioredoxin also enhances DNA binding of Jun and Fos (Abate *et al.*, 1990). Trx has a general antioxidant activity and when up-regulated or overexpressed, protects against oxidative stress (Nakamura *et al.*, 1994; Ohira *et al.*, 1994). Therefore the present data, which shows up-regulation of Trx by KA treatment, may be explained with the potential protective role of Trx in defence against oxidative stress.

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