〈Original Article〉

# An improved quantitative real-time PCR protocol for precisely measuring D-amino acid oxidase mRNA in rat tissues

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**Summary** Although a real-time PCR protocol for detecting D-amino acid oxidase (DAAO) mRNA in rat tissues has been previously reported, we found that the precision of the previous data set was not satisfactory. Here, we modified the real-time PCR method in three ways to achieve sufficient precision: 1) iQ SYBR Green supermix was used as fluorescence reagent to favor target DNA molecules without amplifying nonspecific DNA molecules; 2) both the RNeasy Plus Universal and QuantiTect Reverse Transcription kits were used successively to obtain genomic DNA-free RNA; and 3) a large amount of tissue sample －500 mg of wet tissue in 9.00 mL QIAzol Lysis reagent－ was used. Consequently, the modified method gave sufficient precision for assaying DAAO mRNA in rat tissues (2.30 ± 0.196 copies/$10^3$ GAPDH copies; relative standard deviation, 8.52%).

**Key words:** Real-time PCR, mRNA, D-amino acid oxidase, Precision

## 1. Introduction

Quantitative, reverse transcription-polymerase chain reaction (RT-PCR) has been widely used for estimating the amount of specific messenger RNA (mRNA) transcripts in biological samples[1-3]. The recent development of real-time PCR has offered the ability to sensitively and accurately quantify mRNA levels, which is crucial in biomedical research[4, 5]. Real-time RT-PCR can collect data during the PCR process, thus combining the amplification and detection steps. This method has three advantages: 1) an accurate range across 7 to 8 log orders of magnitude, 2) reduction of contamination with PCR amplicon molecules from the laboratory as this method requires little post-amplification processing, and 3) quantitative linearity is sufficient as reactions are characterized by the point in time when the target amplification reaches the cycle-threshold line, towards which DNAs amplify logarithmically.

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Received for Publication May 28, 2013
Accepted for Publication June 6, 2013

In real time RT-PCR, fluorescent detection techniques have encompassed various types of PCR-based detection methods. DNA-binding dyes, such as SYBR Green Ⅰ[6,7], which fluoresce brightly when bound to double-strand DNA, have been used for real-time detection during PCR. Alternatively, molecular beacons[8] and hybridization probes[9] have also been used; however, both of these methods remain more expensive. By contrast, the method using SYBR Green Ⅰ in real-time PCR is faster and can be less expensive. Nevertheless, the method with SYBR Green Ⅰ is imperfect, because it detects not only target DNA molecules, but also nonspecific DNA molecules, which results in a low precision of mRNA quantification. Therefore, to improve precision some procedural improvements that selectively favor target DNA abundance should be useful.

D-Amino acid oxidase (E.C.1.4.3.3.) is mainly expressed in the kidney, liver, and brain of higher animals[10,11]. Although real-time PCR for D-amino acid oxidase (DAAO) mRNA in rat tissues has been previously reported, we found that the precision of that data set was not satisfactory[12-14]. Thus, in this study, we aimed to investigate the use of real time RT-PCR for measuring DAAO expression by modifying previous protocols using the two methods depicted in Table 1. Here, we report an improvement in both the sample-preparation and real-time PCR steps with SYBR Green Ⅰ allowing the precise determination of DAAO mRNA levels in rat brain, liver, and kidney.

## 2. Materials and methods

1) Animals

All animal experiments were approved by the Committee of Animal Care of Toho University. Male Sprague-Dawley rats weighing 350-450 g (age, 10-11 weeks) were purchased from Charles River Japan (Kanagawa, Japan) and housed in an environmentally controlled room for at least one week before use.

2) Real time quantitative reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA molecules were extracted from rat tissues, liver, kidney, and three different brain areas (cerebellum, cerebrum, and brainstem) using the RNeasy Plus Universal kit (Qiagen, Courtaboeuf, France) or Micro-FastTrack 2.0 kit (Invitrogen, California, USA). Complementary DNAs (cDNAs) were made with the QuantiTect Reverse Transcription kit (Qiagen) or SuperScript Ⅲ First-Strand Synthesis System for RT-PCR kit (Invitrogen) after removing genomic-DNA using the RQ1 RNase-Free DNase Kit (Promega, Madison, USA).

In method Ⅰ, the Micro-FastTrack 2.0 kit, RQ1 RNase-Free DNase Kit, and SuperScript Ⅲ First-Strand Synthesis System for RT-PCR Kit were used. In method Ⅱ, the RNeasy Plus Universal kit and QuantiTect Reverse Transcription kit were used (Table 1). The gene expression of DAAO was determined by real-time RT-PCR using glyceraldehyde 3-phosphate dehydrogenase (GAPDH; accession number NM017008) as an internal control along with primers specific for DAAO (accession number NM053626) mRNA sequence. The upstream primers were 5'-CCC TTT CTG GAA AAG CAC AG-3' (DAAO), and 5'-GTG GAC CTC ATG GCC TAC AT-3' (GAPDH); the downstream primers were 5'-CTC CTC TCA CCA CCT CTT CG-3' (DAAO) and 5'-TGT GAG GGA GAT GCT CAG TG-3' (GAPDH). Expression of genes was assessed by real-time RT-PCR. Real-time RT-PCR was performed using a Chrome 4 machine and one of two reagents, an iQ SYBR Green Supermix or a SsoFast EvaGreen Supermix (Bio-Rad Laboratories, Inc., California, USA) using the following protocol: 3 min of predenaturation at 95℃, 45 consecutive cycles of 10 s denaturation at 95℃, 10 s annealing at 62℃, followed by a 1 min extension at 65℃. An amplification curve was obtained by plotting fluorescence against the PCR cycle number. The melting peak was generated by plotting the negative first derivative of fluorescence versus temperature.

The PCR products, DAAO and GAPDH, were purified after 2% agarose gel electrophoresis and subcloned into the pTZ19R vector. After the transformation of these recombinants into the *Escherichia coli* XL1-Blue using the calcium chloride method, plasmid DNAs were recovered and their nucleotide sequences were determined using an Applied

Biosystems 3730xl DNA analyzer (Applied Biosystems, Tokyo, Japan).

### 3. Results and discussion

Thus far, mRNA expression of DAAO in tissues, especially brain, of human and experimental animals has been reported by several groups, as DAAO is a susceptibility gene for schizophrenia[10, 11, 15, 16]. Thus, mRNA expression of DAAO using real-time PCR with SYBR Green I has been often reported[12, 13, 14, 17]. However, we found that the precision of the mRNA expression values in these past studies was not satisfactory.

Initially, DAAO and GAPDH DNA molecules were amplified by PCR using a 209 bp fragment and a 148 bp fragment obtained with agarose gel electrophoresis, respectively. After cloning and sequencing, those fragments were identified as a part of the genes DAAO and GAPDH, respectively. Fig. 1 shows the PCR products from rat tissues (kidney, liver, cerebellum, cerebrum, and brainstem). There were almost no differences in GAPDH expression among each tissue (Fig. 1, lower panel, 1-5). Therefore, GAPDH is suitable to use as an internal control standard for examining DAAO mRNA expression.

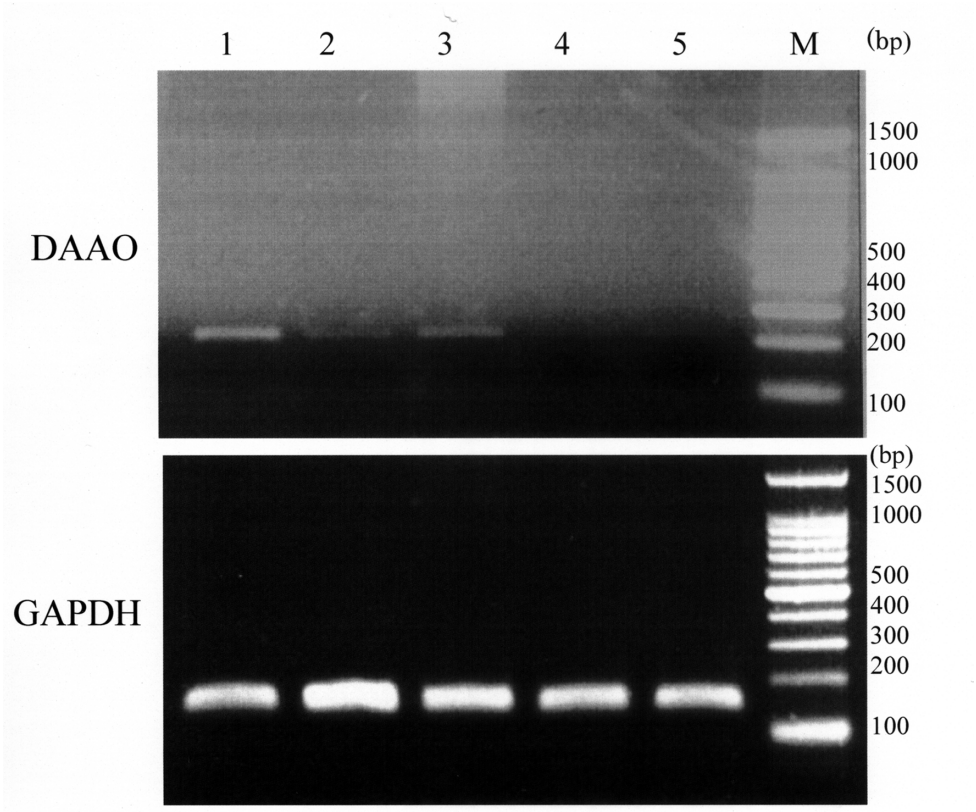


Fig. 1 Gel electrophoresis of the PCR products, D-amino acid oxidase (DAAO) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH), amplified by PCR.
PCR was carried out for each rat tissue cDNA isolate as templates and the products were electrophoresed on a 2% agarose gel. The predicted PCR product sizes were 209 bp (DAAO) and 148 bp (GAPDH). *Lane 1* kidney, *lane 2* liver, *lane 3* cerebellum, *lane 4* cerebrum, *lane 5* brainstem, and *lane M* DNA size markers.

The rat kidney appeared to show the most intense DAAO mRNA expression (Fig. 1, upper panel, 1), similar to a previous report by Konno et al. describing that the DAAO mRNA levels in kidney was remarkable10. DAAO mRNA levels in two parts of the brain, the cerebrum and brainstem, were low, but those in the cerebellum were high (Fig. 1, upper panel, 3-5). These results were also consistent with previous reports. Recently, Kapoor et al. reported that human DAAO mRNA levels in the cerebellum were significantly higher in patients with schizophrenia compared to healthy controls[15]. Therefore, a precise determination of DAAO mRNA expression is important for understanding the pathophysiology of animal models of schizophrenia.

Although the measurement of DAAO mRNA levels has been often reported, the precision was insufficient, with a standard error of 29-100%[12-14]. Therefore, we endeavored to improve the real-time PCR method for quantitating mRNA levels of DAAO

Table 1 Methods Ⅰ and Ⅱ for preparing cDNA libraries from rat tissues

| | Method Ⅰ | Method Ⅱ |
|---|---|---|
| Isolation of RNA | Micro-FastTrack 2.0 kit (Invitrogen) | RNeasy Plus Universal kit (RPU; Qiagen) |
| Cleavage of genomic-DNAs | Once in RQ1 RNase-Free DNase kit (Promega) | Twice in RPU and QRT kit (Qiagen) |
| Synthesis of cDNA from mRNA | SuperScript Ⅲ First-Strand kit Synthesis System for RT-PCR kit (Invitogen) | QuantiTect Reverse Transcription (QRT; Qiagen) |

The cleavage-step of genomic-DNAs in method Ⅰ and Ⅱ is performed once or twice, respectively.

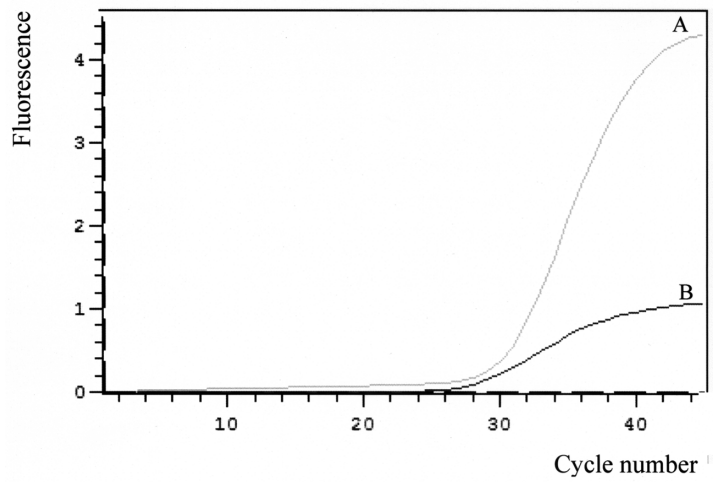


Fig. 2 A comparison of two fluorescence-reagents, SsoFast EvaGreen supermix and iQ SYBR Green supermix, in real-time PCR.
Amplification of real-time PCR products was monitored from 1 to 45 cycles. Two reagents, SsoFast EvaGreen supermix (A) and iQ SYBR Green supermix (B), were compared for detecting amplified GAPDH DNA molecules isolated from rat liver.

and GAPDH by comparing methods Ⅰ and Ⅱ (Table 1).

First, two fluorescence-reagents, SsoFast EvaGreen Supermix and iQ SYBR Green Supermix, were compared for detecting amplified DNA of GAPDH from liver prepared by method Ⅰ. The real-time PCR was carried out from 1 to 45 cycles (Fig. 2). Using SsoFast EvaGreen, the maximum intensity of the 45 cycles was high, but nonspecific DNA gradually increased from 1 to 28 cycles (Fig. 2, line A). Using iQ SYBR Green, the maximum intensity was lower than that of SsoFast EvaGreen, but the amplification of nonspecific DNA less than 28 cycles was negligible. The target DNA levels increased from 25 cycles (Fig. 2, line B). The principle of detecting DNAs on real-time PCR using either of two reagents, iQ SYBR Green and SsoFast EvaGreen, is the same, because both SYBR Green Ⅰ dye in iQ SYBR Green Supermix and the EvaGreen dye in SsoFast EvaGreen Supermix emit fluorescence only when bound to double-stranded DNA molecules. Probably, the binding ability of EvaGreen dye to DNA molecules might be higher than that of SYBR Green Ⅰ.

Therefore, we were concerned that few genomic DNA molecules would be measured when using SsoFast EvaGreen. Considering this, we believe that the iQ SYBR Green is more suitable than SsoFast EvaGreen for determining GAPDH DNA levels.

Second, the melting curve generated using two fluorescence reagents for amplifying GAPDH DNA molecules from liver was measured from 65-94℃ (Fig. 3). Using SsoFast EvaGreen, the target DNA molecule, GAPDH cDNA from mRNA, was amplified at 85℃. Two other DNA molecules, genomic GAPDH DNA and nonspecific genomic DNA, were amplified at 80-84℃ or 71-79℃, respectively (Fig. 3, line A). Using iQ SYBR Green, target DNA molecules were amplified at 85℃ and there were few nonspecific DNA molecules (Fig. 3, line B). Regarding elimination of nonspecific DNA molecules, methods Ⅰ and Ⅱ were compared (Table 1). The number of steps used for eliminating genomic DNAs in method Ⅰ and Ⅱ is once or twice, respectively. In method Ⅰ, there was insufficient elimination of genomic DNA. Method Ⅱ was used to remove almost all genomic DNAs from the extracted RNA. In Fig. 4, the melting curve visualized by iQ SYBR Green showed a sharp peak at 85℃ in the sample prepared by method Ⅱ (dotted-line). In the sample prepared by method Ⅰ, a sharp peak at 85℃ and two peaks at 71-79℃ and 80-84℃ were observed (straight-line, an enlargement of the curve shown in

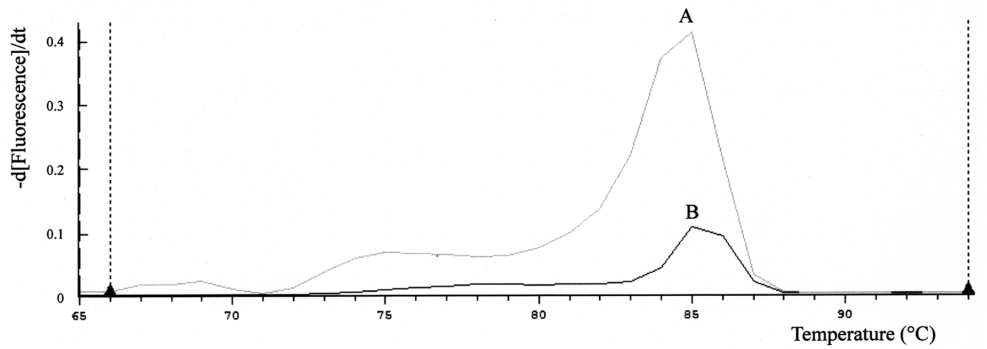


Fig. 3 The melting curve generated using one of two fluorescence-reagents, SsoFast EvaGreen supermix and iQ SYBR Green supermix, for real-time PCR.
The melting peak was obtained by plotting the negative first derivative of fluorescence against temperature, ranging 65-94℃. Two reagents, SsoFast EvaGreen supermix (A) and iQ SYBR Green supermix (B), were compared.

Fig. 3, line B). Real-time PCR is a highly sensitive method that can detect a few nonspecific DNA molecules. The samples prepared using method Ⅰ were assessed by real-time PCR for the inclusion of genomic DNA molecules. Method Ⅱ was more suitable than the method Ⅰ for eliminating genomic DNA molecules, allowing measurement of GAPDH mRNA that is more accurate. For DAAO, iQ SYBR Green and method Ⅱ were also more effective (data not shown).

To examine tissue samples by real-time PCR, two types of conditions for tissue homogenization were tested. Previously, 50 mg tissues (rat cerebellum) were homogenized in 900 $\mu$ g of QIAzol Lysis reagent (QIAGEN). QIAzol Lysis reagent is a monophasic solution of phenol and guanidine thiocyanate, designed to facilitate lysis of all types of tissues and to inhibit RNases. The homogenized samples were treated by method Ⅱ and the mRNAs of DAAO and GAPDH in the tissue sample were measured by real-time PCR with iQ SYBR Green supermix. The mRNA levels of DAAO were 2.59 ± 1.36 (n = 5, DAAO copies/$10^3$

Table 2 DAAO mRNA expression in the rat cerebellum (n = 5)
A comparison between use of 50- and 500-mg wet tissue

| | mRNA (copies/$10^3$ GAPDH mRNA copies) |
|---|---|
| 50 mg tissue | 2.59 ± 1.36 (52.5) |
| 500 mg tissue | 2.30 ± 0.196 (8.52) |

Data are presented as means ± SEM from five rats.
Numerals in parenthesis are values of the relative standard deviation (%).

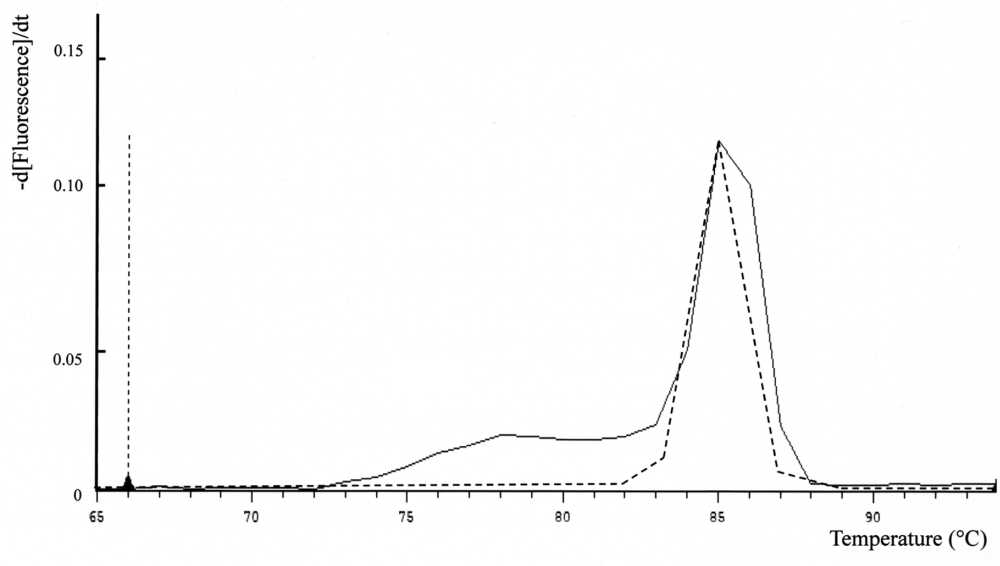


Fig. 4 A comparison of methods I and II for preparing DNAs from tissues.
Methods Ⅰ and Ⅱ (see Table 1) are represented as a straight-line and a dotted-line, respectively.

GAPDH copies) under those conditions, and the relative standard deviation was 52.5% (Table 2).

By contrast, we used 500 mg of wet tissue from the cerebellum that was homogenized in 9.00 mL QIAzol Lysis reagent. Samples were prepared using method Ⅱ and measured by real-time PCR. The mRNA levels were 2.30 ± 0.196 (n = 5, DAAO copies/$10^3$ GAPDH copies), and the relative standard deviation was 8.52% (Table 2). Using a large amount of tissue, the precision of DAAO mRNA measurement by real-time PCR was remarkably improved.

Immunohistochemical data by Horiike et al. showed that there are rich and poor DAAO-expression regions within cerebellum, pons, medulla oblongata, and spinal cord[18]. Habl et al. also reported that the expression of DAAO was not uniform in the hippocampus of a schizophrenia patient[19]. These heterogeneous expression patterns for DAAO in the same areas of tissue may be one reason why low precision of determination of DAAO mRNAs has been shown by conventional RT-PCR.

In conclusion, we improved the real-time PCR method for detecting DAAO in rat tissues in three ways. First, the iQ SYBR Green supermix in real-time PCR could detect target DNA molecules with high efficiency, but could not detect nonspecific DNA. Second, the samples were prepared using two kits (method Ⅱ), RNeasy Plus Universal Kit and QuantiTect Reverse Transcription Kit, to remove genomic DNA. Third, 500 mg tissue was used, in contrast to 50 mg used by previous investigators. These RT-PCR improvements yielded sufficient precision for measuring DAAO in rat tissues.

## Conflicts of interest

The authors report no conflicts of interest.

## Acknowledgments

This study was financially supported in part by a grant from the Ministry of Education, Culture, Sports, Science, and Technology of Japan (KAKEN 22590147).

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