Available online at www.sciencedirect.com
ScienceDirect
Analytical Biochemistry 375 (2008) 46–52
ANALYTICAL BIOCHEMISTRY
www.elsevier.com/locate/yabio

A real-time PCR method for the quantitative analysis of RNA editing at specific sites

Yu-Chia Chen [a,b], Shang-Ching Kao [b], Hsiao-Chin Chou [b], Wei-Hsiang Lin [b], Feng-Hua Wong [a], Wei-Yuan Chow [b,*]

[a] *Department of Life Sciences, National Yang-Ming University, Shih-Pai, Taipei 112, Taiwan*
[b] *Institute of Molecular and Cellular Biology, National Tsing Hua University, 101, Section 2, Hsinchu 30014, Taiwan*

Received 4 October 2007
Available online 9 January 2008

Abstract

In this study, a quantitative PCR (qPCR) method was developed to determine the A-to-I RNA editing frequencies at specific sites. The A-to-I RNA editing of nuclear transcripts exerts profound effects on the biological activities of gene products. RNA editing of nuclear gene transcripts have been shown to be developmentally regulated and tissue specific, and alternations of RNA editing activities have been observed under pathological conditions. Two sites of ionotropic glutamate receptor subunits, the Q/R site of zebrafish *gria2*α and the Y/C site of *grik2*α, were chosen in this study to demonstrate the applicability of the SYBR Green detection-based real-time PCR method to measure RNA editing activities during zebrafish development. The results obtained by qPCR were consistent with those obtained by the limited primer extension. However, the qPCR method has the advantages of easy handling and low cost.

Keywords: Quantitative PCR; RNA editing; Zebrafish; Ionotropic glutamate receptor; Real-time PCR

RNA editing is a site-specific modification of RNA molecules by nucleotide insertion/deletion, substitution, and modification. The RNA editing by selective deamination of adenosine (A to I) and cytosine (C to U) results in sequence discrepancies between metazoan nuclear transcripts and genomic DNA [1,2]. Many protein-coding RNAs, such as those encoding neurotransmitter receptors, ion channels, the coat protein of hepatitis delta virus, and *adar2* (one of the enzymes catalyzing A-to-I RNA editing), are modified at specific sites by the A-to-I editing [1,3,4]. Because I is recognized as G, RNA editing of the aforementioned transcripts affects the splicing of pre-messenger RNAs (mRNAs)[1] and the translated sequences. A-to-I editing of RNA usually is incomplete, resulting in the coexistence of a heterogeneous pool of RNAs with A and I (G) variations at specific sites and in protein isoforms with distinct properties [1,3]. A-to-I editing also modifies the noncoding regions and precursors of micro-RNA, affecting the biogenesis and activities of these RNAs [1,5,6]. Activity of RNA editing is under temporal and spatial regulations and is altered under pathological conditions [3,7]. For example, editing of the Q/R site, changing the glutamine codon to an arginine codon, of the AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid) receptor transcript *GluRB* usually is complete; however, Q/R editing of *GluRB* is incomplete in the white matter of adult human brain [3,8]. Reduced editing of the human *GluRB* transcript at the Q/R site has been observed in glioma and in motor neurons of sporadic amyotropic lateral sclerosis (ALS) patients [9,10]. The Q/R editing of the *GluRB* transcript alters the Ca^{2+} permeability, gating

* Corresponding author. Fax: +886 3 571 5934.
E-mail address: wychow@life.nthu.edu.tw (W.-Y. Chow).

[1] *Abbreviations used:* mRNA, messenger RNA; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid; ALS, amyotropic lateral sclerosis; qPCR, quantitative PCR; hpf, hours postfertilization; dpf, days postfertilization; cDNA, complementary DNA; dNTP, deoxynucleoside triphosphate; RT–PCR, reverse transcription PCR; C_t, threshold cycle; PAGE, polyacrylamide gel electrophoresis.

0003-2697/$ - see front matter © 2008 Elsevier Inc. All rights reserved.
doi:10.1016/j.ab.2007.12.037

kinetics, channel conductance, assembly, and trafficking of the AMPA receptor [3,11]. Lower Q/R editing levels may be responsible for the epileptic seizures in patients with malignant glioma and for the death of motor neurons in ALS patients [3,7,10]. In addition, altering RNA editing of the serotonin receptor $5HT_{2C}$ (*HTR2C*) transcript at several sites is associated with depression [7,12].

The aforementioned observations demonstrate that methods for fast and accurate quantification of variants resulting from RNA editing are invaluable for both basic research and diagnostic purposes. Sequence analysis and limited primer extension, in the presence of dideoxynucleotide, are widely used to quantitatively measure the RNA editing efficiency [12–14]. However, these methods are laborious and costly, and sometimes they require cloning. Alternatively, restriction enzyme analysis is applied when RNA editing alters the recognition sequences of enzymes [15]. In addition, a high-resolution melting technique has been shown to successfully quantify various variants resulting from editing of chloroplast transcripts [16]. Here we report an easy and cost-effective method based on SYBR Green detection-based quantitative PCR (qPCR) in quantifying the unedited variant in mixtures of edited and unedited transcripts. The essence of this method is to design variant-specific PCR primers capable of differentially amplifying two variants differing by a single nucleotide. The conditions and qPCR primers for assaying the RNA editing efficiencies of the zebrafish AMPA receptor subunit *gria2*α at the Q/R site and the kainate receptor *grik2*α at the Y/C site were established in this study. The qPCR method was demonstrated to reliably determine RNA editing frequencies between 15 and 95%, and the results obtained by the qPCR method were comparable to those obtained by primer extension.

Materials and methods

Preparation and amplification of cDNA templates for quantitative analysis

Time of development is expressed as hours postfertilization (hpf) and days postfertilization (dpf) at 28.5 °C. Zebrafish embryos (*Danio rerio*) were collected 15 min after the beginning of the light cycle and defined as 0 hpf. Total RNA, treated with DNase I, was extracted with the RNeasy kit (Qiagen). RNA (1–5 μg) was reverse transcribed by SuperScript III reverse transcriptase (Invitrogen) using oligo(dT) and random hexamer as primers. A 20-μl reaction mixture contained 1 μl of complementary DNA (cDNA), 20 pmol of primers, 200 μM each of the four deoxynucleoside triphosphates (dNTPs), 50 mM KCl, 10 mM Tris–HCl (pH 8.3), 1.5 mM $MgCl_2$, and 0.5 U of high-fidelity KOD-Plus DNA polymerase (Toyobo). The reagents and volume were reduced to half when purification of reverse transcription PCR (RT–PCR) amplicon was not required. Reactions were run for 35 cycles (10 s at 94 °C, 20 s at 55–60 °C, and 1 min at 68 °C), followed by a 10-min extension at 68 °C.

Primers A1 (5′-GGAATGGCATGGTTGGAGAACT GG-3′) and A2 (5′-ACACCACCAACTATACGGCCAG ACAA-3′), derived from the sense and antisense sequences of exons 10 and 12, respectively, were used for amplifying *gria2*α mRNA [17]. cDNA containing the Y/C site of the zebrafish kainite receptor subunit, *grik2*α, was amplified by primers K1 (5′-AGCTGATCTTGCAGTGGCGC-3′) and K2 (5′-GGCCGTGTAGGAGGAGATGATG-3′). PCR amplicons were separated by 2% agarose gel electrophoresis and purified by GENECLEAN (Bio101). Alternatively, PCR amplicons were separated by electrophoresis in a 2% low-melting-point agarose (SeaPlaque, BioWhittaker Molecular Applications), excised, washed, and used without further purification for the qPCR analysis. RT–PCR amplicons were diluted before qPCR analysis. The RNA editing frequencies determined from the purified amplicons and from the amplicons in the excised gel yielded similar results, differing by less than 2% (data not shown).

Cloning and preparation of control DNA templates

PCR amplicons were cloned to pGEMTeasy (Promega) and sequenced by the BigDye terminator system (Applied Biosystems) to identify the A and G variants of *gria2*α and *grik2*α. Plasmids of edited (G) and unedited (A) variants were linearized with appropriated restriction enzymes and cleaned by phenol/chloroform treatment. DNA concentrations were determined by spectrophotometry.

qPCR assay

The qPCR was performed using SYBR Green Master Mix following the manufacturer's suggestions (Applied Biosystems). Primer pairs were designed with the aid of Primer-Express (Applied Biosystems), the melting temperature was set at 60 °C, and the length of the amplicon was kept between 80 and 100 bp. Sequences of real-time PCR primers are listed in Table 1. Reactions contained 800 fmol of each primer in a total volume of 10 μl. The threshold cycle (C_t) values, as defined by the default setting, were measured by an ABI Prism 7500 Sequence Detection System. After an initial 2 min at 50 °C and 10 min at 95 °C, the thermal profile consisted of 45 cycles of 10 s at 95 °C and 1 min at 60 °C. Nevertheless, reactions usually reached the plateau stage before the 30th cycle, and a 35-cycle reaction was sufficient. Single-product amplification was confirmed by postreaction dissociation analysis. Duplicate samples were measured and averaged. If duplicates differed by more than 0.3 C_t value, the sample was remeasured.

Measurement of RNA editing frequency by qPCR

The amplification efficiency (standard curve) of each primer pair was established by measuring the C_t values of series dilutions of the linearized plasmid DNA templates. In