Optimization of semi-quantitative RT PCR analysis for CPT I gene expression in Rainbow trout (*Oncorhynchus mykiss*)

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A key enzyme in mitochondrial β-oxidation, carnitine palmitoyltransferase (CPT) I, is transcriptionally regulated in mammals, but this enzyme also experiences allosteric modulations (Harano et al., 1985; Murthy and Pande, 1987; Bezaire et al., 2004). CPT I is located on the inner side of the outer mitochondrial membrane and catalyses the conversion of acyl-CoA to fatty acylcarnitine (Kerner and Hoppel, 2000; Price et al., 2000). Quantitative RT-PCR is a reliable technique for measuring transcripts in small amounts of tissue (Spriewald et al., 2000). With this technique, multiple mRNAs can be assayed simultaneously in a relatively short period of time. Here we describe the standard procedure, optimized in our laboratory, to assess CPT I levels with β-actin as an internal control in rainbow trout, and all the necessary controls to ensure a quantitative analysis.

RNA Extraction and Reverse Transcription

Total cellular RNA was isolated from liver of rainbow trout using RNX reagent (Cinnagen-Iran). To obtain cDNA, 1 µg of total RNA was subjected to reverse transcription polymerase chain reaction (RT-PCR) with MuLV reverse transcriptase using the RevertAid^{TM} M-MuLV Reverse Transcriptase Kit (Fermentase Life Science, Germany) and random hexamer primer. Reaction conditions in the reverse transcription step are mostly dependent on the enzyme and the primers of choice. Whereas other protocols to require the use of specific primers, we prefer to reverse transcribe the total RNA population with random hexamers so that different PCR analyses could be performed on the same cDNA sample.

PCR

Five µl of cDNA products were amplified with 1 unit of Taq polymerase (Cinnagen-Iran), and in the presence of the specific primers for CPT I gene together with the β-actin gene, used as an internal control as described below. The amount of dNTPs used was 0.1 mM that was sufficient for amplification. A first cycle of 3 minutes at 95 °C, was followed by 45 seconds at 95 °C, 60 seconds at 56 °C and 2 minutes at

72 °C for 35 cycles and continued with 5 minutes at 72 °C for one cycle. Each set of reactions always included a no-sample negative control. To determine specificity, all sequences were compared with the GenBank sequences using the program Blast available at the National Center for Biotechnology Information website (www.ncbi.nlm.nih.gov). The following primers were used: CPT I F- 5'-TGAAGATGCTCTCTGGGCGC -3' (melting temperature (Tm)=60.7°C), and R- 5'-GTGTGGGAGTCACGTACAGC-3' (Tm=59°C); β-actin, F- 5'-GTACCCTGGCATTGCTGA -3' (Tm=57.4°C) and, R- 5'-TTAGAAGCATTTGCGGTGGACA-3' (Tm=58°C).

PCR products were loaded on 1 % agarose gels in TAE buffer (1X) and pictures were captured by Sony XC-75 CE camera (Vilber Lourant Inc. Cedex, France) and quantification of the bands was performed by Photo-Capt v.99 Image software (Vilber Lourant Inc. Cedex, France). Length of CPT I gene was 335 bp, which was different from β-Actin (207 bp). Reliable internal quality control of cDNA synthesis is essential. Controls are generally performed by PCR amplification of reference genes, mostly common housekeeping genes (GAPDH, albumin, actins, Elongation factor-1-α (EF-1-α), tubulins, cyclophilin, microglobulins, 18S ribosomal RNA (rRNA) or 28S (rRNA). In our experiment before selecting β-actin as an internal control in liver samples of rainbow trout, we chose Elongation factor-1-α (EF-1-α) gene for internal control (Gutieres et al., 2003), but didn't see any band on the gel. This problem showed that the chosen reference genes used as well as the expression levels vary between different laboratories, and only few of them have been critically evaluated. In our conditions β-Actin content did not vary significantly within the same type of sample (i.e. the same tissue type). In conventional PCR, each primer was used in a concentration range of 0.05 to 0.4 μM. We optimized CPT I and β-actin with 0.2 μM of each of the two specific primers (Fig. 1). We usually tested the following $MgCl_2$ concentrations: 1, 2, 3, 4 and 5 mM. As shown in Fig. 2, CPT I worked best at 1, 2 mM $MgCl_2$ and β-Actin worked best at 1, 2 and 3 mM $MgCl_2$ (Fig. 2). Another parameter to be analyzed thoroughly is the number of amplification cycles to perform. It is not sufficient to visualize the amplification product on a gel. It is well known that amplification is initially exponential but reaches a plateau when the activity of the enzyme declines and when any of the reagents become limiting in the reaction. At the plateau, RNAs initial present at high levels may give products of equal intensity to low abundant RNAs. An appropriate number of cycles was determined by testing the different cycles of 20, 22, 24, 26, 28, 30, 32 and 34 for both CPT I and β-Actin amplifications in one tube, whereas CPT I and β-Actin intensity increased up to 30 cycles, no increase could be seen in both at more than 32 cycles, i.e. it had already reached a plateau (Fig. 3). To determine whether the selected conditions were suitable for semi quantitative RT PCR with both primer sets (CPT I and β-Actin) at the same time, we performed a competition control, by amplifying the same sample at the same time in the presence of the specific primers for CPT I, the internal control β-Actin, and both sets together. Furthermore, different $MgCl_2$ concentrations for each reaction were

tested. We have shown in Fig. 4 how CPT I and β-Actin did not compete when combined at 30 cycles in three mM $MgCl_2$. Different cDNA of rainbow trout liver were co-amplified with CPT I and β-actin specific primers in one tube PCR reaction to confirm the conditions being optimized (Fig. 5). According to results obtained from different conditions 30 cycles and three mM $MgCl_2$ were selected for semi-quantitative PCR.

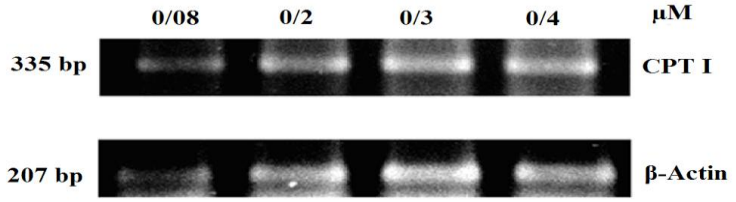

Figure 1: Effect of different primer concentrations on the PCR reaction

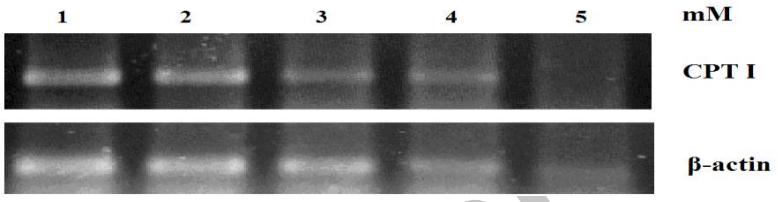

Figure 2: Effect of different $MgCl_2$ concentrations on PCR reaction

CPT I amplification was optimal at 1 and 2 mM $MgCl_2$ and decreased gradually at higher concentrations. Also, β-actin was optimal at 1, 2, and 3 mM $MgCl_2$ and decreased gradually at higher concentrations.

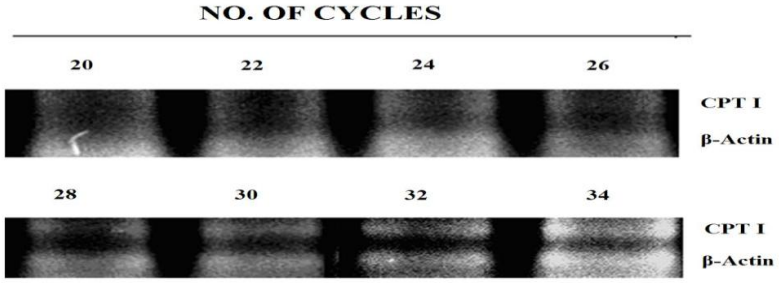

Figure 3: Determination of the exponential range of amplification for CPT I and β-Actin

The analysis was performed in 3 mM $MgCl_2$ at 63°C. The intensity of the CPT I amplification product increased up to 34 cycles, whereas β-Actin and CPT I reached a plateau at 32 cycles.

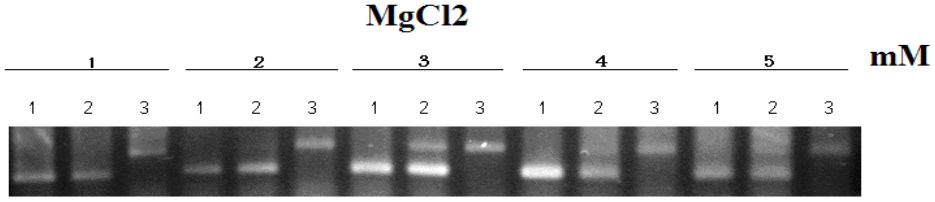

Figure 4: Control for competition between different primer sets

Reactions were performed in the same conditions for lanes 1, 2 and 3. Lane 1 contained the β-Actin primer set only, lane 3 the specific primers for the target RNA, and lane 2 both primer sets. In the selected conditions (3 mM $MgCl_2$, 63 °C, 30 cycles) CPT I and β-Actin did not compete (panel 3). The panel 1, 2, 4 and 5 show an example in which the specific primers for the CPT I competed with the β-Actin primers.

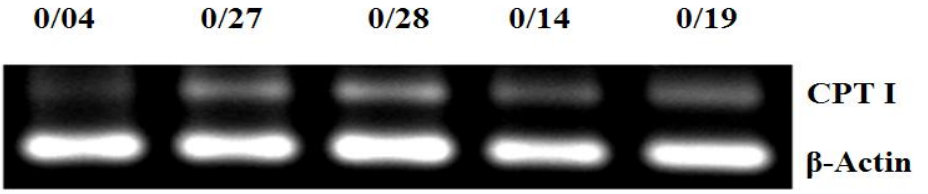

Figure 5: Representative of analyses performed on different concentration of CPT I relative to the β-Actin concentration.

(Above Numbers: The ratios **of corresponding** CPT **I**/β-actin **absorption** density **of bands on a gel).**

Our experiments confirmed that semi-quantitative RT PCR provides reliable information as long as the proper controls are all performed correctly.We have described the protocols to have been reproduced on different types of rainbow trout samples.

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