

Short Communication

Absolute quantification of murine interleukine-4, interleukine-10 and interferon- γ gene transcripts using Real Time PCR

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ABSTRACT

The study of cytokines gene expression is quite important in various conditions of health and disease for the evaluation of clinical responses to new vaccination approaches. An absolute quantification is based on a calibration curve and production of standard controls to achieve more reliable results than in relative system. In this study we attempted to construct standard controls to evaluate the murine immune response. The number of 10 Balb/c mice immunized with hydatid cyst fluid subcutaneously with two week intervals to induce transcription of Th1 and Th2 related cytokines. Three Pairs of primers were designed to amplification of interleukine-4, interleukine-10 and interferon- γ by Oligo software. Partial sequences of three cytokine genes were cloned into pTZ577 vector. Three recombinant plasmids were purified and serial dilutions were prepared. Real time qPCR carried out using SYBR-Green I fluorescence dye and standard curves were provided by the 7500 ABI SDS software based on the exact concentration of dilutions and the amplification plots. Results showed that this method was able to evaluate the cytokines mRNA levels less than 0.01pg (~150 copy). We concluded that absolute real-time qPCR can be successfully applied to the quantification of antigen-induced cytokines.

Keywords: Real Time PCR, Cytokine, IL4, IL10, IFN- γ

INTRODUCTION

The importance of detecting transcription levels of specific genes has always been considered. The quantitative real-time polymerase chain reaction (qPCR) or kinetic RT-qPCR is a powerful and sensitive method generally used for the research and diagnostic (Bustin *et al* 2009). This method is widely and increasingly used for cytokine assay because of its high

sensitivity, good reproducibility, and broad scope of quantification (Chervoneva *et al* 2006). Among the all detection types of real time method SYBR Green I and TaqMan hydrolyzing probe assays produced comparable dynamic range and sensitivity (Peirson 2003). However, SYBR Green I detection was more accurate and produced a more linear diagram in the exponential phase of amplification than the TaqMan probe detection (Jolla 2004). Generally two approaches can be achieved in real-time qPCR method including absolute and relative. Relative quantification is based

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on the expression levels of a target gene, against a reference or control gene (Dhanasekaran *et al* 2010). It measures the target and reference gene expression only in relative condition by using a calibrator. Studies have shown that the housekeeping genes as a reference are adjustable and show a discrepancy in different experimental conditions (Pfaffl 2001). The absolute quantification method relies on a standard curve constructed from known concentrations of standards. This method provides measuring the actual copy numbers of a particular target, and is considered to be more beneficial and reliable for comparisons (Bustin 2000). However, the accurate quantification of target samples can be accomplished only by using appropriately determined standards (Bustin 2000). It has shown that the calibration curves used in absolute quantification can be based on known concentrations of DNA standard molecules (Dhanasekaran *et al* 2010). Different type of materials have been used as standards during absolute quantification, including: PCR-amplified target sequences, RT-PCR amplified product, recombinant plasmids containing the target sequence and commercially synthesized cloned genomic DNA (Lee *et al* 2006, Jolla 2004). So far it seems that design and production of reliable and long term stable standard controls for accurate determination of every desired gene is quite essential and accurate and quantitative detection of gene transcription depends completely to sensitivity of the standard curve. This necessity prompted us to construct three vectors containing three murine cytokine genes including IL-4, IL-10 and IFN- γ . This study aimed to describe the simple and cost effective SYBR Green method using the recombinant plasmids to make positive standard controls for each cytokine.

MATERIALS AND METHODS

Animals and Immunization. In this experiment the number of 10 Balb/c mice were collected from Razi institute and immunized with crude hydatid cyst fluid (HCF) emulsified in Freund's adjuvant for three sessions with an interval of 14 days subcutaneously to

induce cytokines mRNA transcription. The 6-8 weeks old mice were maintained at a stable temperature (23 ± 2 °C) and were provided with an unlimited and uninterrupted supply of food and water. All animal experiments were conducted by standard protocols of Razi vaccine and serum research institute.

RNA extraction and cDNA synthesis (RT-PCR).

Two weeks after last immunization the spleen tissues were extracted. Total RNA was extracted from the spleen tissue using High Pure RNA Isolation Kit (Roche Applied Science). Contaminating genomic DNA was eliminated by the presence of a DNase treatment step in the kit. DNase digestion takes place in a highly concentrated format allowing for the purification of DNA-free RNA. The purity of RNA was estimated by measuring optical density (OD) at 260 nm by NanoDrop. RNA was stored at -70 °C and used as template for reverse transcription in cDNA synthesis. Two step RT-PCR was carried out briefly, 1 μ g of random primer (Roche Applied Science) was added to 500 ng of RNA. Then, 4 μ l of 5X reaction mix first strand buffer, 1 μ l RNase inhibitor (10 unit), 1 μ l dNTPs (0.2 mM) (Roche Applied Science), and 200 unit Reverse Transcriptase enzyme (Thermo) was added in a total volume of 20 μ l. The reaction was incubated at 25 °C for 5 min, at 42 °C for 60 min and at 70 °C for 10 min.

PCR amplification. PCR primers 5'-agg aac att cag agc tgc ag-3' and 5'-tgt ata ggg aag cac cag gtg-3' for IFN, 5'-gag act tgc tct tgc act ac-3' and 5'-agc agt atg ttg tcc agc tg-3' for IL10, 5'-gag agt gag ctc gtc tgt ag-3' and 5'-gct tay cga tga atc cag g-3' for IL4 were designed using Oligo software, according to the known mouse mRNA sequence for IL4, IL10 and IFN- γ (accession numbers: NR_027491.1, NM_010548.2 and NM_008337.3 respectively) in GenBank. The PCR was carried out in a final volume of 50 μ l and optimized under following condition: initial denaturation at 93 °C for 3min. a 35 cycles amplification (denaturing 93 for 30 sec, annealing at 51°C for 30 sec, extension at 72 °C for 30 sec), and a final extension at 72 °C for 10 min for every three cytokines. The PCR products consisting

179bp, 256bp and 150 bp related to IFN, IL10, and IL4 respectively was checked using 2% agarose gel electrophoresis. The 340 bp sequence of β -actin reference gene was applied as a control for checking the quality of RNA and cDNA synthesis steps. The target PCR products purified using a PCR product purification Kit (Fermentas).

Cloning and Preparation of standards. External cDNA standards for each cytokine gene under study were constructed by cloning the corresponding RT-PCR products in the pTZ57T vector using the original TA cloning kit (Fermentas), using T4 DNA ligase (Fermentas), incubated at 22 °C for 16 hours and transformed into *E. coli* DH5 α competent cells and then the transformation mixture was spread on Luria Bertani (LB) agar culture medium containing ampicillin (50 μ g/ml). The transformed colonies were randomly screened performing colony PCR. The identity of the inserted genes was confirmed by gel electrophoresis and restriction digestion of PCR products with *Hind*III and *Eco*RI restriction enzymes (Fermentas). The plasmids were subsequently purified using plasmid miniprep kit (Fermentas) according to the manufacturer's instructions. The concentration (ng/ μ l) of the standards was determined by measuring the optic density at 260 nm. The serial dilutions were prepared to give final concentrations between 100 ng to 0.1 fg.

Absolute Real-time qPCR. Real-time qPCR was conducted using 2X SYBR Green I Master mix (Ampliqon). Each reaction was run in duplicate and contained 1 μ l of cDNA template along with 10 pmol primers in a final reaction volume of 20 μ l. Cycling parameters were 95 °C for 3 min, then 40 cycles of 95 °C for 15 sec and 60 °C for 1 min, with a final recording step of 68 °C for 20 sec to prevent any primer-dimer formation. To distinguish primer dimers from the specific amplicon a melting curve analysis was carried out. Melting curves were carried out using Dissociation Curves option of SDS software at 65 °C to 99 °C (Applied Biosystems) to ensure only a single product was amplified, and samples were also run on a 2% agarose gel to confirm specificity. Finally

standard curves were provided by the 7500 ABI SDS software based on the exact concentration of dilutions and the amplification plots. The copy number was calculated by the following equation (Whelan et al. 2003): Copy number = $(6.02 * 10^{23} \text{ (copy/mol)} * \text{DNA amount (g)}) / (\text{DNA length (bp)} * 600)$.

RESULTS AND DISCUSSION

Specificity Confirmation of external standards. In the present study we optimized the PCR condition for amplifying single PCR product with well-designed primers at the annealing temperature range of 51-52 °C for interleukin-4 (IL-4), interleukin-10 (IL-10) and interferon- γ (IFN- γ). In order to perform absolute quantifications, external cDNA standards, were constructed, as described in materials and methods. Specificity of RT-PCR products and the identity of the target inserts were documented with 2% gel agarose electrophoresis and resulted in a single product with the desired length; 150 bp for IL-4, 257bp for IL-10 and 179bp for IFN- γ (Figure 1).

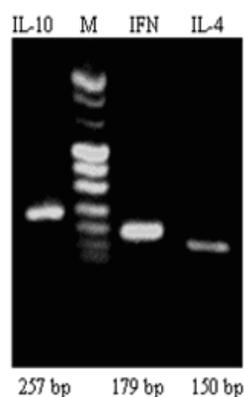


Figure 1. The confirmation of three different cytokines amplicon size in 2% agarose gel M: DNA size marker III (Roche Applied Science).

Real-time PCR amplification efficiencies and linearity. The linear range of the assay was determined by kinetic amplification of log serial plasmid dilutions from 10^{-1} to 10^{-10} . The amplification plots for dilutions are shown to demonstrate the quantitative nature of this technique (Figures 2, 3, 4 A). In addition the sensitivity of real time qPCR on concentration range of plasmids showed considerable reduction from concentration 1ng to 0.1 femto gram

(fg). Results showed that this method was able to evaluate the mRNA levels lower than 10 fg for IL-10, IFN- γ and IL-4. In order to demonstrate specificity, real-time PCR products were electrophoresed in a 2% agarose gel. The same steps were followed for each gene (Figures 2, 3, 4 D).

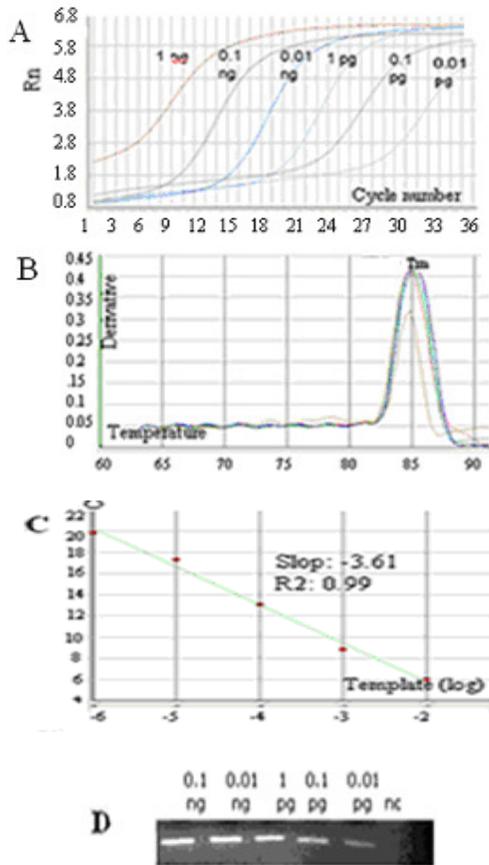


Figure 2. Optimization of Real Time PCR for IFN- γ . A: Amplification plot, B: Melting curve of IFN- γ at 86°C, C: constructed standard curve, D: electrophoresis of PCR products on 2% agarose gel.

Light Cycler melting curve analysis was carried out after the amplification process which resulted in single product specific melting temperatures as follows: IFN- γ , 86 °C; IL-10, 77 °C; and IL-4, 81 °C (Figures 2,3,4 B). Few amounts of primer-dimers were generated during the applied real-time PCR amplification cycle for IL-10. The specific amplicon T_m peak can be observed at 77 °C, which is clearly distinguishable from

the lower T_m peak of primer dimer artifacts at 67 °C in the negative control reaction (figure 3B).

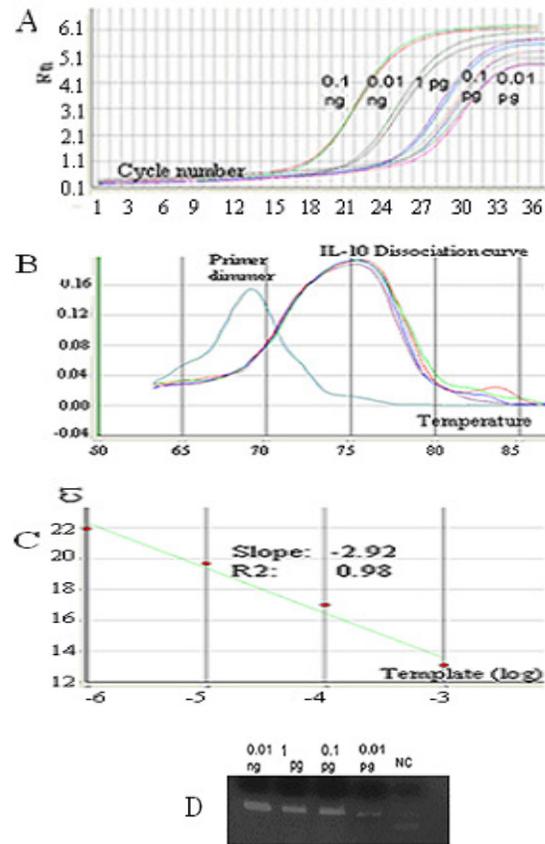


Figure 3. Optimization of Real Time PCR for IL10. A: Amplification plot, B: Melting curve of IL-10, C: Standard curve, D: electrophoresis of PCR products on 2% agarose gel.

In this study a known amount of plasmid used to construct a calibration curve. The ABI 7500 SDS software plotted the log of molecules versus the cycle of threshold (C_t) values in order to obtain a standard curve. Real-time PCR efficiencies were calculated from the given slopes in Light Cycler software during exponential phase using a linear regression according to the equation: Efficiency = 10(1/slope) ± 1. (R₂ > 0.99) Any unknown cytokine concentration sample can be calculated by simple interpolation using this curve (figures 2, 3, 4, C). In this study absolute quantification of real-time PCR was conducted using standard curves based on serially diluted concentrations of constructed

plasmid DNA. Some studies suggest that to generate standard curve the size of the used template should be very similar to the size of the amplicon to reduce the non-specific amplification (Chervoneva *et al* 2006).

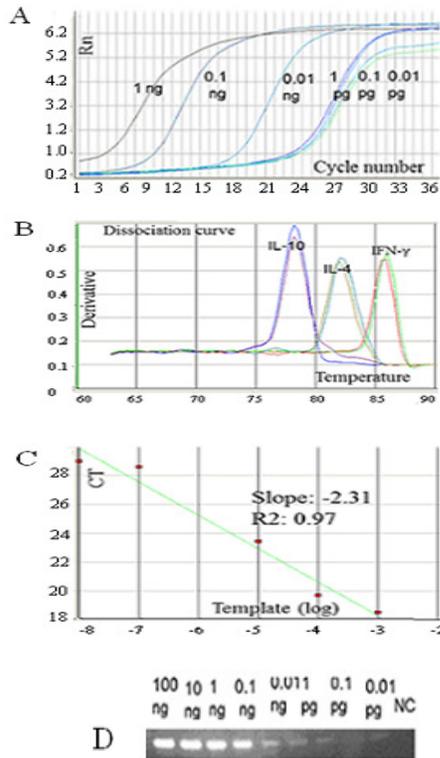


Figure 4. Optimization of Real Time PCR for IL-4. A: Amplification plot, B: Melting curve of IL-4, C: Standard curve, D: electrophoresis of PCR products on 2% agarose gel.

We verified the idea and also have shown that there is no need to clone the entire target gene or large PCR fragments, since the constructed pTZ57T plasmids contained our desired cytokines inserts and our amplicons were at the same size. We actually used the same primers for the cloning and real-time PCR so it was less time consuming and costly. Hydatid cyst fluid contains different factors that can affect dendritic cell function. Crude sheep hydatid fluid (SHF) elicits both Th1 and Th2 cell activation: the Th2 response benefits the parasite, whereas the Th1 response benefits the host (Rigano *et al* 1995a, 1995b, 1999a, 1999b, 2001, Joao & Bengamin 2006). In this study SHF as a known Th1

and Th2 inducer agent was used to provoke transcription of cytokines mRNA in mice. We successfully optimized the reverse transcriptase and real-time PCR condition to amplify partial sequences of three Th1 and Th2 related cytokines as illustrated in the results. The dissociation curves confirmed the results observed in the amplification plots and PCR products were analyzed by electrophoresis to demonstrate specificity. A distinctive melting peak at the melting temperature of the amplicon will differentiate it from amplification artifacts and primer dimer that melt at lower temperatures (Nolan *et al* 2006). The data from standard curves showed that the serially diluted plasmids can be used as a positive standard controls for transcription measurement, of course the slope and efficiencies especially about IL-4 and IL-10 requires some adjustment and modifications in practical laboratory works to obtain more efficiency. We believe generation of stable and reliable standards in absolute quantification requires high precision and hard effort but it provides accurate and trustworthy results in comparison with relative quantification. Relative real time qPCR has some potential source of errors during cDNA synthesis. Hence any potential variability caused by differences in the transcription efficiency of reference and target gene should be avoided. Since in Relative qualification calibration curve is not obligatory so it may cause wrong data (Dhanasekaran *et al* 2010). There are four additional methods using for the quantification of gene transcription: northern blotting and in situ hybridization, RNase protection assays and the cDNA arrays. All these techniques provide valuable information about mRNA. But they have low sensitivity and require high concentrations of starting template (Pfaffl 2001). There are numerous benefits to use real-time PCR over other molecular methods to quantify genes transcription or translation levels. This technique can provide an accurate dynamic range of 7 to 8 log and does not require post-PCR manipulation. Real-time PCR assays are 10,000 and 1000 fold more sensitive than RNase protection assays and dot blot hybridization, respectively. In qPCR a

single copy of a specific RNA can be detected (Wong & Medrano 2005, Ponchel *et al* 2003). Cytokine secretion has often been measured by evaluating protein expression levels in the supernatants of in vitro cell cultures by enzyme linked immunosorbent assay (ELISA), radio immunoassay and RT-PCR. Each of these assay types exhibits advantages and disadvantages (Ekerfelt *et al* 2002, Livak & Schmittgen 2001). Immunoassays are probably the most popular means of measuring cytokines due to their ease of performance (Listvanova *et al* 2003). In ELISPOT, the small amount of secreted cytokine are trapped in the surrounding cells and therefore could be more simply detectable than in ELISA and PCR which cannot distinguish between the excessive secretion activity of a few cells and the slight response of a large number of lymphocytes. (Ramos-Payán *et al* 2003). Low cytokine concentrations are often difficult to detect by ELISA, although it is well known that mRNA levels do not correlate totally with protein concentration (Schmidt *et al* 2007). Previous studies attempted to define the most suitable assay to detect cytokine responses. They compared the measurement of cytokine production by four methods. ELISA, ELISPOT, flow cytometry and real-time RT-PCR were chosen to measure cytokine production before and after vaccination. They showed that before in vivo challenge to assess immunological memory status and showed that PCR was one of the most sensitive methods but after injection at the cellular level visualizing the vaccine effects showed that intracellular cytokine detection (flow cytometry) was the most sensitive method and Real time RT-PCR method was not effective (House 2001) (Livak & Schmittgen 2001). It is, of course, difficult to popularize these findings to each antigenic vaccination system but, to ensure not to lose the cellular response; a combination of several methods should be applied. In conclusion cytokines are important factors in immune system and choosing methods for their quantification are essentially substantial. The type of assay chosen will depend on the capabilities of laboratories. It should be noted that none of the techniques are best suited for

all applications. These methods reveal different stages of T cell cytokine production including mRNA transcription, intra-cellular presence, and secretion at the individual cell level and mass of cytokine content. Therefore, one suggestion is to apply a combination of techniques (Pfaffl 2001). Our data which is provided in this article confirms that real time RT-PCR method is applicable to be used effectively to determine absolute quantification of three cytokines (IL-4, IL-10 and IFN- γ) transcription; we believe that the absolute real time qPCR technique which has been set up in this study would be routinely used for cytokine assay in research laboratories.

Ethics

I hereby declare all ethical standards have been respected in preparation of the submitted article.

Conflict of Interest

The authors declare that they have no conflict of interest.

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