

Comparison of SYBR Green and TaqMan methods in quantitative real-time polymerase chain reaction analysis of four adenosine receptor subtypes

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Abstract

Background: Real-time polymerase chain reaction (PCR) is based on the revolutionary method of PCR. This technique is the result of PCR enormous sensitivity and real-time monitoring combination. In quantitative gene expression analysis, two methods have more popularity, SYBR Green and TaqMan, SYBR Green is relatively cost benefit and easy to use and technically based on binding the fluorescent dye to double-stranded deoxyribonucleic acid (dsDNA) where TaqMan method has more expensive and based on dual labeled oligonucleotide and exonuclease activity of Taq polymerase enzyme. Specificity is the most important concern with the usage of any non-specific dsDNA-binding Dyes such as SYBR Green while more specificity showed by labeled oligonucleotide method such as TaqMan. In this study, we compared two common RT PCR methods, TaqMan and SYBR Green in measurement gene expression profile of adenosine receptors.

Materials and Methods: Gene expression profiles of A1, A2A, A2B and A3 Adenosine receptors were analyzed by optimized TaqMan and SYBR Green quantitative RT PCR in breast cancer tissues. Primary expression data was normalizing by B. actin reference gene.

Results: Efficiencies were calculated more than 95% for TaqMan and SYBR Green methods in all genes. The correlations between means of normalized data of each gene in two methods were positive and significant ($P < 0.05$).

Conclusion: Data analysis showed that with the use of high performance primer and by use proper protocols and material we can make precise data by SYBR Green as TaqMan method. In other word by optimization of SYBR Green method, its performance and quality could be comparable to TaqMan method.

Key Words: Quantitative real-time polymerase chain reaction, SYBR Green, TaqMan

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INTRODUCTION

Polymerase chain reaction (PCR) was developed by Kary Mullis in the 1980.^[1,2] This method of genetic analysis used widely in Measuring and monitoring biological responses to various stimulants.^[3-5] Quantitative gene analysis deoxyribonucleic acid (DNA) used to determine the genome quantity of a

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particular gene like hepatitis virus.^[6] There are many methods for the quantitative analysis of nucleic acid sequences.^[7-9] PCR has a key role in quantitative nucleic acid analysis. PCR and reverse transcriptase (RT)-PCR can detect the minimal quantities of nucleic acid.^[10-12] Although PCR is a powerful genetic analysis method, it has some limitations such as inefficiency to measurement of the initial target sequence quantity.^[10,13,14] Several procedures are used for semi-quantification of PCR and real-time (RT) PCR product, Such as fluorescent labeling of PCR products followed by visualizing on agarose and acrylamide gels and other gel densitometry methods.^[10,15,16] These are successful proved methods, but each requires the post-PCR procedure that takes more times and usually lead to laboratory contamination and throughput of these methods is normally low and limited.^[10] RT PCR is the result of revolution in PCR method. Real-time enables researchers to better find the amount of starting DNA in the sample before the amplification by PCR. Real Time PCR is the result of enormous sensitivity of PCR and real-time monitoring of its products.^[1,2,17,18] Roche molecular systems and Chiron scientists, Higuchi *et al.* were unveiling RT PCR for the first time in 1993.^[19,20] They recorded PCR reaction containing ethidium bromide (EtBr) dye under ultraviolet light by video camera. Thus, RT PCR born in early 1990s.

First RT PCR machine, made commercially by applied Biosystems (ABI) in 1996, thereafter other companies such as Roche applied science, Qiagen, BioGene, Bioneer, Bio-Rad, Cepheid, Corbett Research, Idaho Technology, MJ Research and Stratagene added new instrument to the market. Exclusivity of RT PCR is the ability to monitor the progress of DNA amplification in real-time. This accomplished by specific chemistries and instrumentation. In general, chemistries consist of special fluorescent dye and probes in the PCR including DNA-binding dyes such as EtBr or SYBR Green I, hydrolysis probes, hybridization probes, molecular beacons and peptide nucleic acid light-up, sunrise and scorpion primers probes.^[1-4] SYBR Green I when binds to the minor groove of double-stranded deoxyribonucleic acid (dsDNA) emits fluorescence 1,000-fold greater than it's free in solution.^[5] Therefore, the increasing amount of dsDNA present in the reaction tube lead to the greater amount of bound dyes and increasing fluorescent signal from SYBR Green I. This method is relatively cost benefit and easy to use. Specificity is the most important concern with the usage of any of these non-specific dsDNA-binding dyes. Non-specific products reflected in dissociation curve of the amplified product as non-specific peaks.

Hydrolysis probes are sequence-specific dually fluorophore-labeled DNA oligonucleotides and most

popular in probes chemistry category that offer an alternative approach to the problem of specificity and generally called TaqMan probes.^[1,2] One fluorophore termed the quencher and the other is the reporter. The quencher and reporter are in close proximity on the same short oligonucleotides. The quencher absorbs the signal from the reporter during amplification, DNA polymerase 5'-nuclease activity broken apart the oligonucleotide and the reporter and quencher separate allowing the reporter's fluorescent signal be liberated. Hydrolysis probes have similar precision as SYBR Green I.^[3] However, they have better specificity, because only sequence-specific amplifications measured. In this study, we compared performance and efficiency of two common RT PCR methods, TaqMan and SYBR Green with measurement of same genes expression profile especially in this case adenosine receptor subtypes.

MATERIALS AND METHODS

Preparation of tissue samples

Breast cancer tissue samples were obtained from 20 women (mean age 59.9 years, 46-74 years range). All samples [Table 1] were quickly placed into liquid nitrogen and then stored at -80°C .

Ribonucleic acid (RNA) extraction

An aliquot of the frozen tissue (20-40 mg) homogenized in RLT buffer using the bead-milling method and the total RNA was then extracted with RNeasy plus mini kit (Qiagen, Germany). The quality and concentration of the RNA samples was tested by ultraviolet absorption at 260/280 nm in Nano Drop system and electrophoresis on denatured 1% agarose gel to determine the concentration.

Real-time quantitative RT PCR

To quantitatively examine adenosine receptor subtypes gene expression we performed a RT PCR assay using the rotor gene 6000 RT PCR detection system (Corbett Research, Australia). 1 μg of RNA was reverse transcribed by Quantitect Rev, transcription kit (Qiagen, Germany) and the complementary DNA (cDNA) obtained used for real-time quantitative PCR. 5-fold serial dilution of breast tumor cDNA was performed to made standard curves. For TaqMan assay primers and probes mix (assays-on-demand TM Gene expression Products) were

Table 1: Demographic characteristics of subjects with ductal carcinoma breast cancer (N=20)

Characteristics	
No. of subjects	30
Age (years)	
Mean	59.9 \pm 8.72 (SD)
Range	46-74

obtained from (ABI, USA). Reaction mixture (25 μ l) containing 2 μ l of cDNA template, 1.5 μ l each of primer and probe mix and TaqMan Universal PCR master mix (ABI, USA) amplified as follows: denaturation at 95°C for 10 min and 40 cycles at 95°C for 10 s, 60°C for 20 s. Direct detection of PCR products monitored by measuring the fluorescence produced by the result of TaqMan probe hydrolysis after every cycle.

For SYBR Green assay primers were designed by beacon designer version 8.0 so that spans exon-junctions and synthesis by TIB molbiol (TIB molbiol, Germany): A1 forward primer: 5'-TCGCCATCCTCATCAACA-3', A1 reverse primer: 5'-ACCATCTTGTACCGGAGAG-3', A2A forward primer: 5'-CTCCATCTTCAGTCTCCTGG-3', A2A reverse primer: 5'-AAGCCATTGTACCGGAGC-3', A2B forward primer: 5'-CTCCATCTTCAGCCTTCT-3', A2B reverse primer: 5'-ACCAAACCTTTTATACCTGAGC-3', A3 forward Primer: 5'-TTGCCTACTGCTTATCTT-3', A3 reverse Primer: 5'-TCTTGTATCTGACGGTAA-3'. Reaction mixture (25 μ l) containing 2 μ l of cDNA template, 1.5 μ l each of primers and Quantitect SYBR Green master mix (Qiagen, Germany) amplified based on SYBR Green method. Direct detection of PCR products was monitored by measuring the fluorescence produced due to SYBR Green dye binding to dsDNA after every cycle.

For both TaqMan and SYBR Green methods amplification efficiencies were tested for the gene of interest (GOI) and housekeeping gene. All samples were tested with the reference gene Beta Actin (ACTB) for data normalization to correct for variations in RNA quality and quantity. All samples were performed in Triplicate.

These measurements were then plotted against cycle numbers. The parameter threshold cycle (Ct) defined as the cycle number at which the first detectable fluorescence increase above the threshold observed. For fold-changes calculation in relative gene expression, equation Δ CT, where Δ Ct = Ct (GOI) – Ct (ACTB) was used.

Statistical analysis

Statistical analysis was conducted using the statistical program SPSS 18.0. The correlation between messenger RNA expressions of adenosine receptors in two methods was compared using the Pearson test. Differences considered statistically significant when $P < 0.05$ was achieved.

RESULTS

Efficiencies were calculated above 97% for all TaqMan and SYBR Green analysis [Table 2]. Standard curves

for SYBR Green and TaqMan analysis showed in [Figures 1 and 2] respectively.

The average values of normalized adenosine receptors gene expression levels were 1.44, 2.38, 3.79 and 3.55 for A1, A2A, A2B and A3 adenosine receptors for SYBR Green method and value for TaqMan method were 1.38, 2.43, 3.84 and 3.58 [Table 3] respectively.

In the case of association between data of gene expression resulting from TaqMan and SYBR Green, Pearson analysis showed a significant and positive correlation between all method-pair gene expression analysis Table 4.

DISCUSSION

Since the advent of RT PCR, some of the measuring methods are used to compute data and each one optimized for an especial goal. In quantitative gene expression analysis two methods have more popularity, TaqMan and SYBR Green. In general, working with the SYBR Green method is cheaper and easier than TaqMan because of no need to probe design and synthesis, but in many cases scientists prefer TaqMan method.^[17,21] Power of TaqMan method due to its unique design based on oligonucleotide double labeled probe and the exonuclease activity of Taq polymerase enzyme, whereas SYBR Green design

Table 2: Calculated efficiencies of two methods

Gene	Method	
	TaqMan	SYBR Green
A1	0.986	0.978
A2A	0.993	0.985
A2B	0.984	0.992
A3	0.982	0.991
B. actin	0.994	0.999

Table 3: Adenosine receptors expression levels in breast cancer

Method	Adenosine receptors expression fold ^{a,b}			
	A1	A2A	A2B	A3
SYBR Green	1.44±0.31	2.38±0.16	3.79±0.38	3.55±0.08
TaqMan	1.38±0.28	2.43±0.22	3.84±0.41	3.58±0.12

^aDifferential expression measurements were performed by real-time PCR, as described under materials and methods. ^bValues are expressed as mean \pm standard deviation

Table 4: Correlation between results of SYBR Green and TaqMan methods

Gene	Pearson correlation	
	r	P
A1	0.651	0.002
A2A	0.616	0.004
A2B	0.555	0.011
A3	0.913	0.000

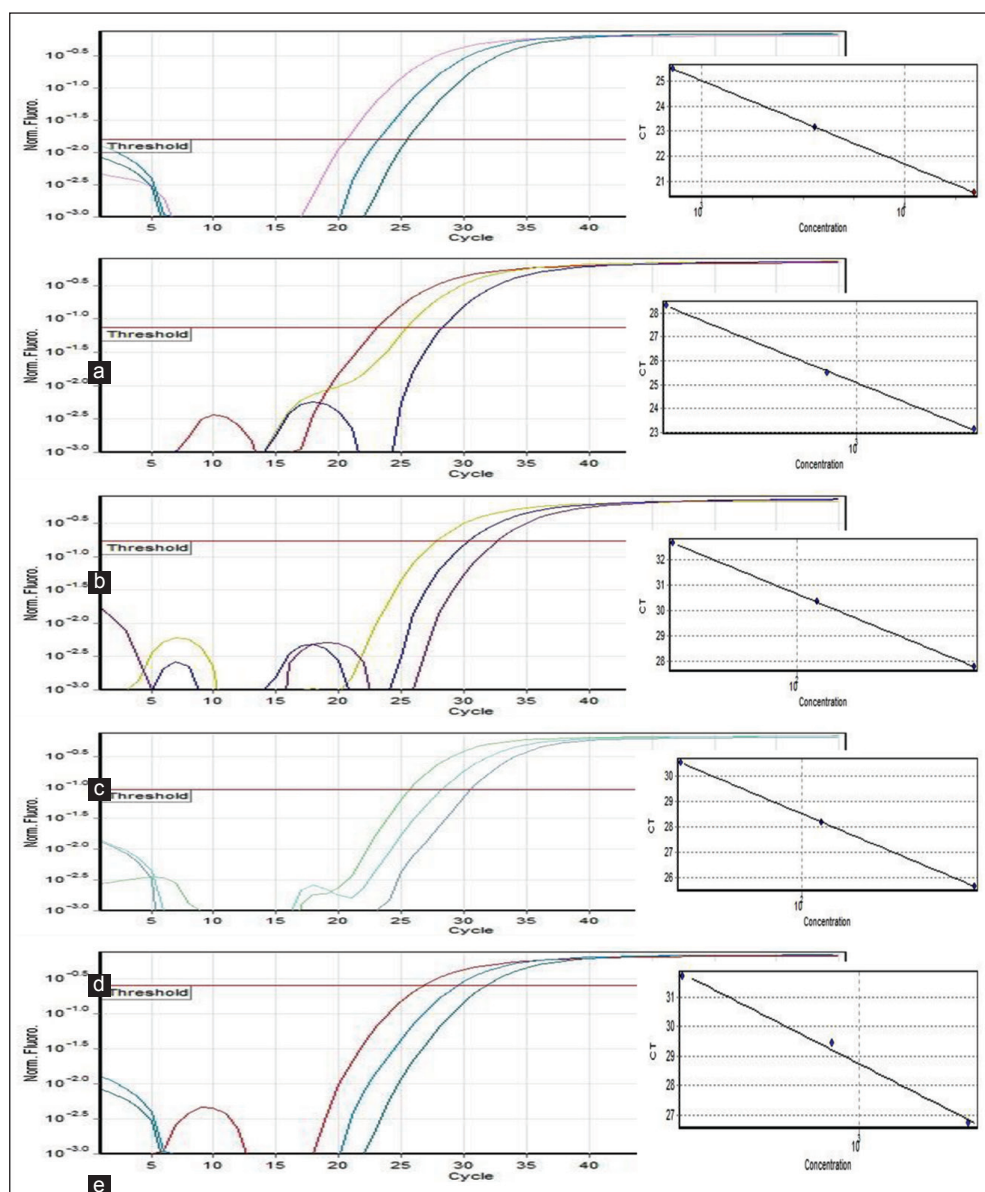


Figure 1: Standard curves of SYBR Green method, (a) A1 adenosine receptor, (b) A2A Adenosine receptor, (c) A2B adenosine receptor (d) A3 adenosine receptor, (e) B. actin

based on binding of fluorescent dye to dsDNA. Clearly in SYBR Green method, any non-specific product like primer-dimer can make false positive results and this incorrect and shifted data can finally lead to decrease the performances.^[21-25]

Undoubtedly primers were one of critical components in PCR reaction. Design of primers was not easy at the beginning of PCR and researchers had to design primers by themselves without help of designing software. Now, different softwares are available for primer and probe designing,^[26] but some of these softwares showed better performances and quality in designing. Almost more expensive softwares showed better performance and quality. Nowadays

some strategies apply to optimization the PCR. For example: design optimal primer pairs that are closely matched in T_m , calculate or estimate approximate T_m , using standard hot-start PCR mixes, using the enhancing agents and proper master mixes.

In this study, we used Beacon Designer 8.0 software to design exon-junction span primer for SYBR Green-base adenosine receptors gene expression analysis. According to our result, all gene expression profiles in each pairs of the method have positive and significant correlation, also all ten experiments performed with high efficiencies. These evidences prove optimized SYBR Green method has similar performances to TaqMan method and data analysis was show with

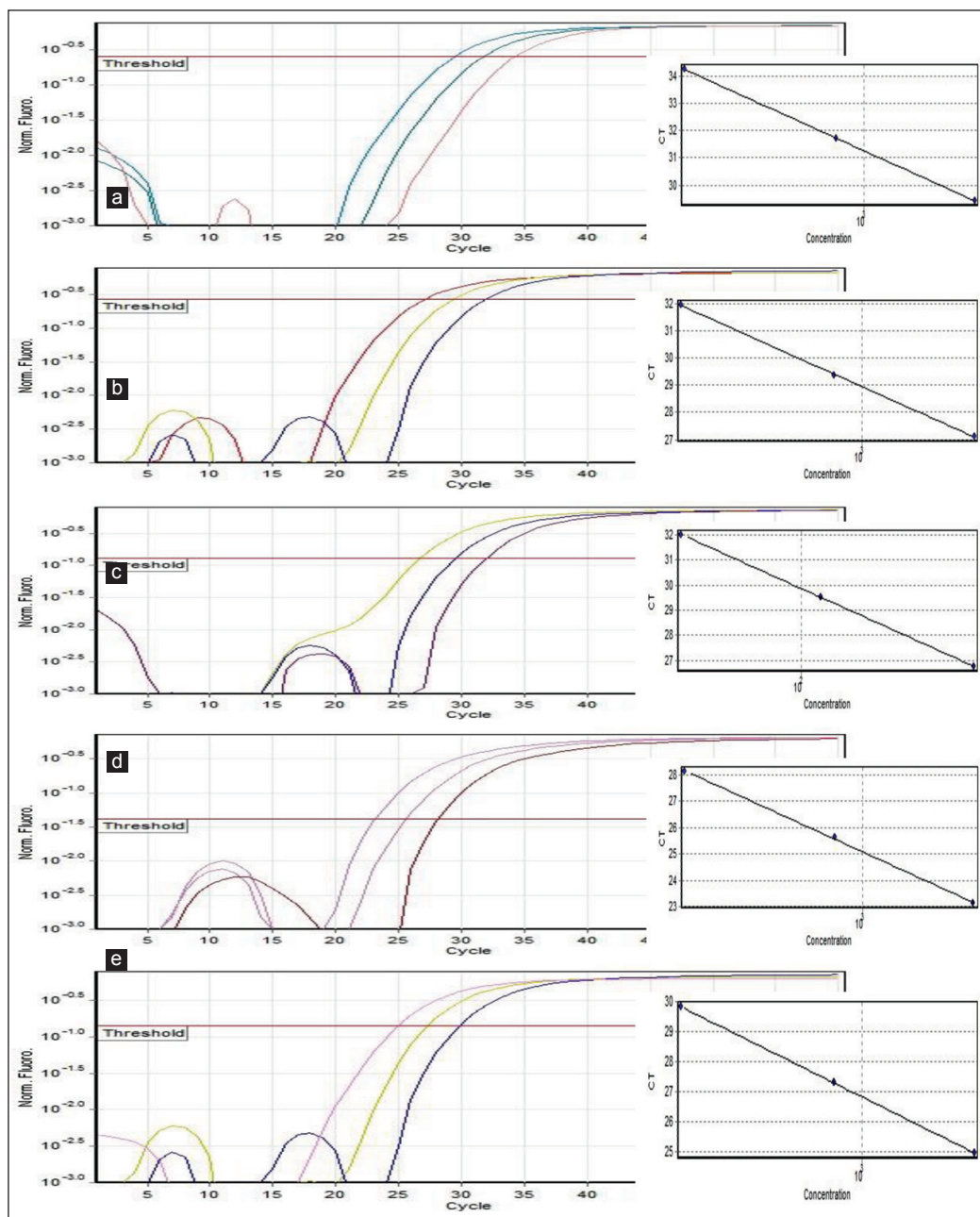


Figure 2: Standard curves of TaqMan method, (a) A1 adenosine receptor, (b) A2A Adenosine receptor, (c) A2B adenosine receptor (d) A3 adenosine receptor, (e) B. actin

the help of high performance primer designing software and by utilization proper protocols and material we can achieve high quality and precise data by SYBR Green as the TaqMan method. In other word by optimization of SYBR Green method its performance and quality could be comparable to TaqMan method.

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