Rapid, Reproducible Real-Time Quantitative RT-PCR Using the iCycler iQ™ Real-Time PCR Detection System and iQ™ Supermix

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Introduction

We are interested in identifying genes that are differentially expressed within the central (macular) region of the human retina. Expression profiles of thousands of genes from this small, highly specialized region of the central nervous system were obtained by comparative screening of human cDNA microarrays with human macula- and mid-peripheral retina (periphery)-derived RNAs. In order to validate the macula-enriched expression of genes identified by our array analysis, we must rely on a PCR-based method of quantitation. Real-time RT-PCR quantitates the initial amount of a template with more specificity, sensitivity, and reproducibility than any other method. There are many factors that contribute to the consistent performance of a real-time quantitative RT-PCR assay, and many aspects that must be optimized when putting this powerful technology to work in a new experimental system.

Methods

Total RNA was isolated from 4 mm trephine punches of neural retina from two areas, the macula and the mid-periphery, using Trizol reagent (Invitrogen) with glycogen added as a carrier as described by Bracete et al. (1999) with the following modification: 0.9 ml Trizol reagent plus 13.5 µl glycogen (20 mg/ml, Roche Molecular Biochemicals) was added to flash-frozen tissue in a 1 ml microcentrifuge tube and vigorously homogenized for 30 sec using an Ultraturrax T8 homogenizer (Ika Laboratories). Total RNA was DNase-treated using DNA-*free* reagent (Ambion), and RNA yields were determined by fluorescence at 530 nm using RiboGreen RNA quantitation reagent (Molecular Probes) as described by the manufacturer. First-strand cDNAs were synthesized from equal amounts of total RNA (1 µg/reaction) using oligo(dT) primers and SuperScript II reverse transcriptase (Invitrogen) according to the manufacturer's instructions.

Gene-specific primers (GSPs) were designed to anneal near the 3' end of two mRNA transcripts and to generate PCR products 75–300 base pairs long. Three GSP pairs amplify different overlapping regions of a single transcript that is enriched in the macula, while one GSP pair detects a human housekeeping gene transcript, β-actin (*ACTB*), which is constitutively expressed in the neural retina. The amplified regions spanned exon-exon junctions when possible. All primers were purchased from Proligo. RT-PCR was performed using the GSP pairs in reactions amplifying across a gradient of annealing temperatures to identify optimal reaction conditions for real-time RT-PCR, and PCR product lengths were verified on a 4.5% Super AcrylAgarose gel (DNA Technologies). Real-time quantitative RT-PCR was performed using an iCycler iQ system (Bio-Rad). The rate of accumulation of amplified DNA was measured by continuous monitoring of SYBR Green I (Molecular Probes) fluorescence. Melt curves of the reaction products were generated, and fluorescence data were collected at a temperature above the melting temperature of nonspecific products (Morrison et al. 1998).

Specifically, quantitative real-time RT-PCR on the iCycler iQ was performed in duplicate or triplicate on 1 µl of template cDNA per 20 µl reaction. Mix A reactions consisted of PCR buffer (16.6 mM $(NH_4)_2SO_4$, 67 mM Tris, pH 8.8, 6.7 mM $MgCl_2$, 10 mM β-mercaptoethanol; Loging et al. 2000), 1 mM dNTPs (Invitrogen), 0.5 U of Platinum *Taq* DNA polymerase (Invitrogen), 10 nM fluorescein calibration dye (Bio-Rad), 1 µl of a 1:1,500 dilution of 10,000x SYBR Green I stock, 500 nM of each GSP, and 1 µl of cDNA. iQ supermix reactions consisted of iQ supermix (Bio-Rad) at a final concentration of 1x, 10 nM fluorescein calibration dye, 1 µl of a 1:1,500 dilution of 10,000x SYBR Green I stock, 500 nM of each GSP, and 1 µl of cDNA. To control for pipetting losses, 19 µl of each 20 µl reaction was amplified in a 96-well thin-wall PCR plate (Bio-Rad) using the following PCR parameters: 95°C for 2 min followed by 50 cycles of 95°C for 15 sec, 60°C for 15 sec, and 72°C for 15 sec. Melt-curve analysis was performed immediately following amplification by increasing the temperature in 0.4°C increments starting at 65°C for 85 cycles of 10 sec each. The presence of a single

PCR product was verified both by the presence of a single melting temperature peak representing a specific product (vs. a nonspecific primer-dimer peak) using iCycler iQ analysis software and by detection of a single band of the expected size on a 4.5% Super AcrylAgarose gel.

Real-time RT-PCR was performed in duplicate or triplicate reactions. Each GSP pair was used with each reaction mix on each of the two different cDNA templates (derived from macula or periphery). Real-time RT-PCR reactions for detection of the endogenous control gene, *ACTB,* were always run in parallel for each cDNA template in each experimental run as a reference for accuracy of sample dilution (even if not shown in figure).

Results and Discussion

Experiment 1: Performance Over Time of Mix A Protocol Optimized for Real-Time RT-PCR

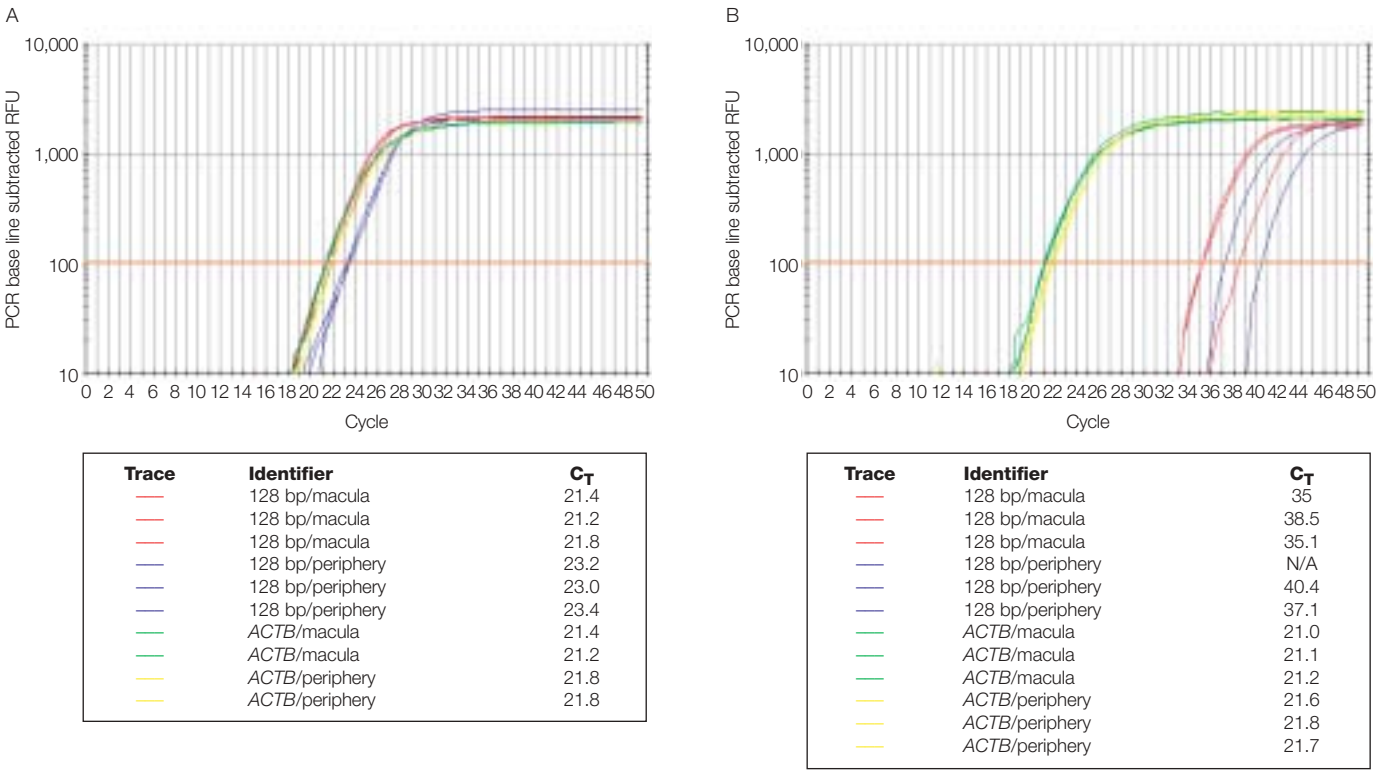
Reactions were carried out using the GSPs that amplified a 128 bp fragment of the macula-enriched transcript of interest. The amplification curve for the macula-derived sample crossed a threshold of 100 relative fluorescence units (RFU) after 21.5 cycles, and the periphery-derived sample crossed this threshold 1.7 cycles later at 23.2 cycles. These results confirmed that the transcript of interest was, indeed, enriched in the macula compared to the rest of the retina (Figure 1A). When the experiment was repeated one month later using the same reaction components, only the *ACTB*-derived PCR products were generated; none of the 128 bp target was detected (data not shown). The same set of real-time RT-PCR reactions was prepared again with previously unopened aliquots of each reagent stored at –20°C in a constant-temperature freezer. Again, only the *ACTB*-derived transcripts were amplified (Figure 1B). Traces for late amplifications (C_T > 34) of the 128 bp primer set represent primer-dimers and not specific product, as determined by melt-curve and gel analysis (not shown). These results showed that failure to amplify was not due to freeze-thaw induced deterioration of the stock reagents over time and suggested that some component of the stock reagents was unstable over time, even at –20°C.

Experiment 2: Comparison of Reactions Based on iQ Supermix vs. Mix A

Use of iQ supermix rescued the assay, resulting in accumulation of the macula-derived 128 bp products crossing the threshold of 100 RFU after 19.7 cycles (Figure 2) — almost 2 cycles earlier than in the mix A-based reactions with macula cDNAs for template (Figure 1). In the iQ supermix reactions containing periphery-derived cDNAs, the 128 bp product crossed this threshold value at 21.2 cycles (Figure 2), 1.5 cycles later than the macula reactions with the iQ supermix and almost 2 cycles earlier than with the mix A periphery reactions in experiment 1 (Figure 1). Equivalent mix A reactions run at the same time failed to amplify (Figure 2). As shown in the following experiment, the reproducibility of these results as well as the stability of the iQ supermix reagents held up over time.

Experiment 3: Performance of iQ Supermix Reactions Over Time

Real-time RT-PCR was performed as described for experiment 2, except that the iQ supermix (2x) stock used in these reactions had been stored for 4 months at –20°C. The 128 bp segment of the macula-enriched transcript was again



Trace	Identifier	C_T
	128 bp/macula	21.4
	128 bp/macula	21.2
	128 bp/macula	21.8
	128 bp/periphery	23.2
	128 bp/periphery	23.0
	128 bp/periphery	23.4
	ACTB/macula	21.4
	ACTB/macula	21.2
	ACTB/periphery	21.8
	ACTB/periphery	21.8

Trace	Identifier	C_T
	128 bp/macula	35
	128 bp/macula	38.5
	128 bp/macula	35.1
	128 bp/periphery	N/A
	128 bp/periphery	40.4
	128 bp/periphery	37.1
	ACTB/macula	21.0
	ACTB/macula	21.1
	ACTB/macula	21.2
	ACTB/periphery	21.6
	ACTB/periphery	21.8
	ACTB/periphery	21.7

Fig. 1. Real-time amplification of a 128 bp fragment of a macula-enriched target transcript and a 94 bp fragment of a constitutively expressed housekeeping gene (*ACTB*); mix A reactions containing either macula-derived or periphery-derived cDNA templates. A, traces representing amplification of the first generation of mix A reactions; B, traces representing amplification of the same target templates in mix A reactions prepared from reagent stocks that had been stored at –20°C for 1 month and freshly thawed. C_T, threshold cycle.

successfully amplified. The amplification curve for the macula sample crossed the threshold of 100 RFU after 20.1 cycles, while the periphery sample crossed the threshold 1.7 cycles later at 21.8 (Figure 3). The iQ supermix is therefore more stable over time than mix A.

Experiment 4: Amplification of a Specific Transcript Using Three Different Pairs of GSPs With iQ Supermix vs. Mix A

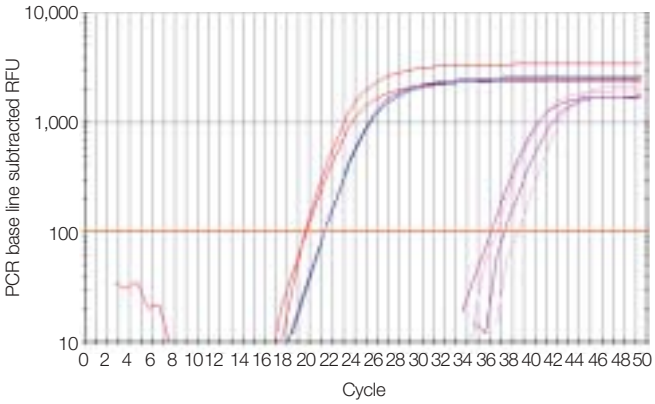
In order to test whether primer design can affect the reproducibility of amplification curves obtained for a specific transcript in a specific tissue, real-time RT-PCR was performed using three pairs of primers designed to amplify different regions of the same target transcript. The three GSP pairs generate 128 bp, 100 bp, and 99 bp products. Duplicate reactions using each primer pair with each reaction mix (A or iQ supermix) were run for each template. The performance of the iQ supermix reactions was quite consistent for each primer pair (Figure 4A), whereas the performance of the mix A-based reactions varied for each GSP (Figure 4B).

In the iQ supermix reactions, the average C_T for all six traces representing the amplification of the macula sample with three different primer pairs was 19.9 ± 0.3 cycles. The average C_T for the periphery-derived sample was 21.4 ± 0.2 cycles, resulting in an average of a 1.5 cycle difference between the two regions of human neural retina (Figure 4A). This differential expression profile for the macula-enriched gene transcript was the same as that obtained in the two previous experiments with iQ supermix reactions (Figures 2 and 3).

The traces representing the accumulation of PCR products in the mix A-based reactions varied with each GSP (Figure 4B), in contrast to the traces for the iQ supermix reactions (Figure 4A). In the mix A-based reactions, the 128 bp fragment was not amplified at all, whereas the products generated by the other two primer pairs were amplified at different rates. The amplification curves for the 100 bp fragment crossed threshold fluorescence at 25.4 cycles in the macula reactions and 25.8 in the periphery, while the curves for the 99 bp fragment from closer to the 3' end of the target mRNA crossed the threshold at 21.5 cycles in the macula reactions and 23.1 in the periphery. The average C_T values for the two GSP pairs that resulted in the expected-sized PCR products were then 23.4 ± 1.9 for the macula and 24.4 ± 1.3 for the periphery, for an average 1.0 cycle difference (Figure 4B). Clearly, the real-time RT-PCR results generated using the iQ supermix were more reliable and reproducible.

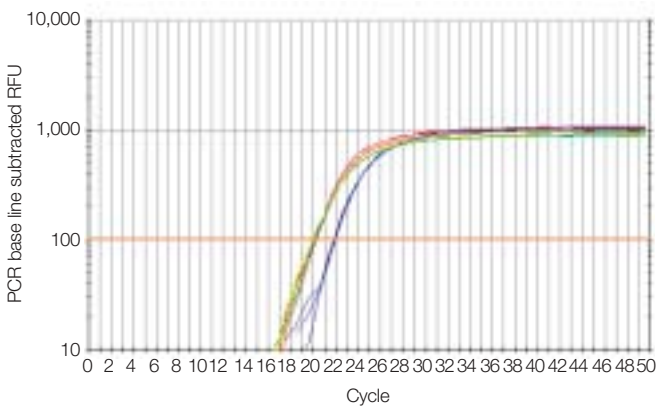
Conclusions

Although quantitative real-time RT-PCR is a powerful, sensitive, and reproducible method to quantitate differences in mRNA expression, many aspects of the reactions (i.e., primer design, annealing temperatures, and master mix reagents) must be optimized to put this powerful technology to work successfully in a new experimental system. Real-time RT-PCR is especially sensitive to product length, where longer length products and low- to medium-abundance transcripts cannot be amplified in reactions containing unstable reagents. While Bio-Rad's bulletin 2593 (Boeckman et al. 2001) for the iCycler thermal cycler recommends amplification of PCR products only within the narrow range of 75–150 bp, we have consistently been able to amplify products in excess of 300 bp using iQ supermix (data not



Trace	Identifier	C_T
	128 bp/macula (iQ supermix)	19.7
	128 bp/macula (iQ supermix)	19.6
	128 bp/macula (Mix A)	36.6
	128 bp/macula (Mix A)	38.6
	128 bp/periphery (iQ supermix)	21.2
	128 bp/periphery (iQ supermix)	21.2
	128 bp/periphery (Mix A)	36.0
	128 bp/periphery (Mix A)	37.0

Fig. 2. Amplification of a 128 bp macula-enriched target in mix A and iQ supermix reactions using cDNA templates derived from two areas of neural retina.



Trace	Identifier	C_T
	128 bp/macula	20.2
	128 bp/macula	20.0
	128 bp/macula	20.0
	128 bp/periphery	21.7
	128 bp/periphery	21.9
	128 bp/periphery	21.8
	ACTB/macula	20.0
	ACTB/macula	20.1
	ACTB/periphery	20.0
	ACTB/periphery	19.6

Fig. 3. Amplification of a 128 bp macula-enriched target alongside the constitutively expressed *ACTB* with two cDNA templates using iQ supermix after 4 months of storage at –20°C.

shown). Replicate C_T values for amplification of the control gene *ACTB* showed less variation between replicates and between experiments with either reaction mix (Figures 1 and 3). This was not simply due to the relative abundance of *ACTB* transcripts in the samples since the amount of the macula-enriched target gene was the same as *ACTB* in the macula. Instead, these results suggest that sequence-related secondary structure or transcript stability of the target gene could affect outcomes in the mix A reactions but were not a factor in the iQ supermix reactions. Finally, the iQ supermix reactions were not only more robust but also were extremely reproducible: macula-enriched target transcript C_T in the macula-derived samples was 19.9 ± 0.2 cycles (n = 11), and 21.4 ± 0.3 cycles (n = 11) in the periphery-derived samples (compare Figures 2, 3, and 4A). Here we have demonstrated that the use of iQ supermix to optimize reaction conditions allows the best consistency and reproducibility from experiment to experiment.

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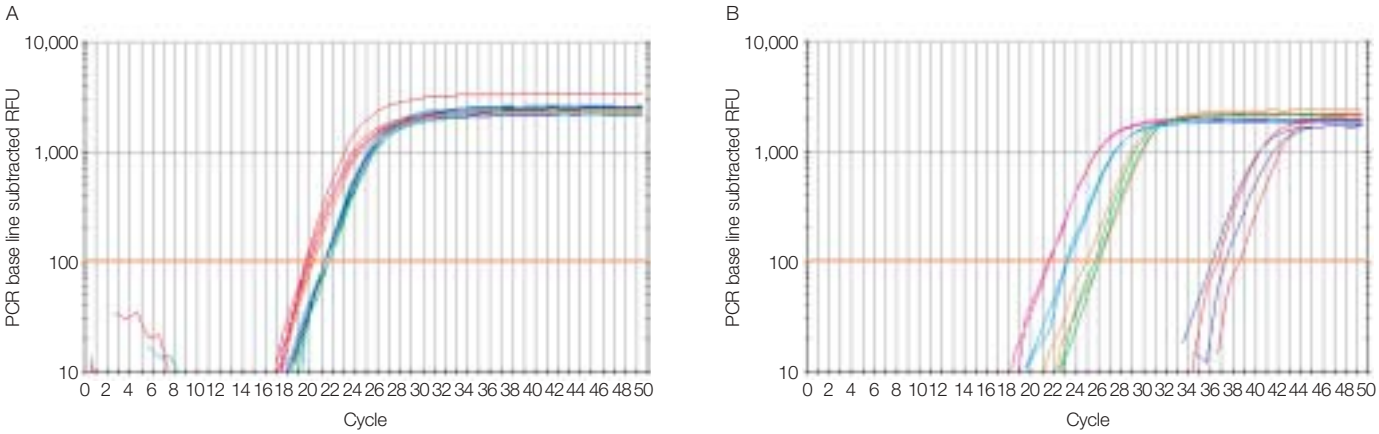

Trace	Identifier	C_T
	128 bp/macula	19.7
	128 bp/macula	19.6
	100 bp/macula	20.1
	100 bp/macula	20.2
	99 bp/macula	19.9
	99 bp/macula	19.8
	128 bp/periphery	21.2
	128 bp/periphery	21.2
	100 bp/periphery	21.6
	100 bp/periphery	21.6
	99 bp/periphery	21.3
	99 bp/periphery	21.2

Trace	Identifier	C_T
	128 bp/macula	36.6
	128 bp/macula	38.6
	100 bp/macula	24.8
	100 bp/macula	25.9
	99 bp/macula	21.6
	99 bp/macula	21.4
	128 bp/periphery	36.0
	128 bp/periphery	37.2
	100 bp/periphery	25.6
	100 bp/periphery	25.9
	99 bp/periphery	23.2
	99 bp/periphery	23.0

Fig. 4. Amplification of three different products from a macula-enriched transcript with two different cDNA templates using two mix conditions. A, traces representing the amplification of all three products using iQ supermix; B, traces representing the amplification of all three products using mix A.

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