

## Multiplex Relative Gene Expression Analysis by Real-Time RT-PCR Using the iCycler iQ™ Detection System

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### Introduction

Reverse transcription PCR (RT-PCR) is being used more and more for gene expression analysis. Compared to northern blot and ribonuclease protection assays, it is more sensitive and allows for analysis of small tissue samples. However, because of the exponential nature of the amplification in the PCR process, even small differences in PCR efficiency will significantly affect the results and therefore conditions must be carefully chosen in order to control for even very small changes in the efficiency of reverse transcription and PCR amplification.

If absolute quantitation of gene expression is necessary, one needs to construct an RNA probe that is first reverse transcribed and then amplified together with the target gene (competitive RT-PCR). However, knowing the relative change in gene expression is often sufficient. In this situation, the ratio of the expression of the target gene to a housekeeping gene is measured and the change in this ratio is evaluated before and after an experimental manipulation. It is essential that the expression of the housekeeping gene not be changed during the experimental manipulation.

By performing multiplex RT-PCR with a target gene and a housekeeping gene in the same reaction tube, it is possible to control for tube-to-tube differences in the efficiencies of reverse transcription and PCR amplification. In addition, possible differences in RNA isolation are also controlled.

Multiplex RT-PCR is dependent upon being able to measure a specific signal from each gene, typically by using a different fluorophore for each gene. In this study a ROX-labeled Scorpions primer (Whitcombe et al. 1999) was used for the IL-8 gene (target gene) and a FAM-labeled TaqMan probe (Holland et al. 1991) for the  $\beta$ -actin gene (housekeeping gene).

### Methods

cDNA was made using a random hexamer primer kit as described by the manufacturer (GeneAmp RNA PCR kit, Applied Biosystems, Foster City, CA). A PCR master mix containing the specific primers and AmpliTaq Gold DNA polymerase was then added.

IL-8 primers: 5'-TTGGCAGCCTTCCTGATTC  
and 5'-AACTTCTCCACAACCCTCTG

$\beta$ -actin primers: 5'-TGTGCCCATCTACGAGGGGTATGC  
and 5'-GGTACATGGTGGTGCCGCCAGACA

Real-time quantitation of IL-8 mRNA relative to  $\beta$ -actin mRNA was performed with both a SYBR Green I assay and a multiplex RT-PCR assay (IL-8 Scorpions ROX-labeled primer and FAM-labeled TaqMan probe for the  $\beta$ -actin target) for comparison. Both assays were evaluated using the iCycler iQ detection system from Bio-Rad. For the SYBR Green I assay, IL-8 and  $\beta$ -actin mRNA were amplified in separate tubes using the following protocol: 95°C for 10 min, then 30 cycles of denaturation at 95°C for 30 sec, annealing at 58°C for 30 sec, and extension at 72°C for 60 sec. The increase in fluorescence was measured in real time during the extension step for the SYBR Green I assay and during the annealing step for the Scorpions and TaqMan assay.

For the multiplex RT-PCR, IL-8 and  $\beta$ -actin cDNA were amplified simultaneously in the same tube using specific primers. The IL-8 amplification was monitored in real time using a ROX-labeled Scorpions primer, whereas  $\beta$ -actin amplification was monitored using a FAM-labeled TaqMan probe.

Human adipose tissue was treated with IL-1  $\beta$  (2 ng/ml) for 4 hr to stimulate IL-8 gene expression. The threshold cycle ( $C_T$ ), which is defined as the fractional cycle number at which the fluorescence reaches 10x the standard deviation of the baseline, was determined and the relative gene expression was

quantitated as described in User Bulletin #2 for the ABI PRISM 7700 sequence detection system (Applied Biosystems). The fold change in IL-8 (target gene) relative to the  $\beta$ -actin endogenous control gene was determined by:

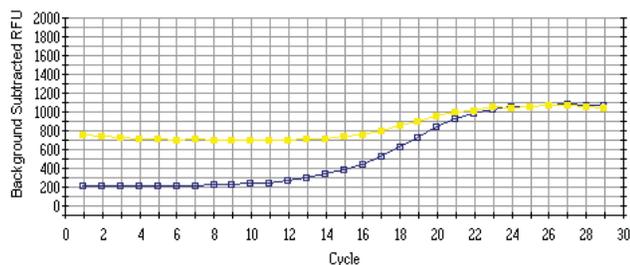
$$\text{Fold change} = 2^{-\Delta(\Delta C_T)}$$

$$\text{where } \Delta C_T = C_{T, \text{target}} - C_{T, \beta\text{-actin}}$$

$$\text{and } \Delta(\Delta C_T) = \Delta C_{T, \text{stimulated}} - \Delta C_{T, \text{control}}$$

## Results

Initially the melting properties of the Scorpions primer were investigated. The Scorpions primer is actually an ordinary primer with a blocker and a molecular beacon (Tyagi and Kramer 1996) attached at its 5' end. The primer is designed so that when *Taq* polymerase extends its 3' end, the molecular beacon unfolds and binds to the newly synthesized DNA strand (within the first 3–50 base pairs after the original primer). The Scorpions primer is closed at low temperatures due to a stem region and the fluorophore and quencher are thus positioned close to each other, so the Scorpions primer is dark. However, when incorporated into the newly formed amplicon, the Scorpions primer unfolds and becomes fluorescent. As a test of the design of the stem region, the Scorpions primer can be melted by increasing the temperature, thereby allowing the Scorpions primer to unfold, which results in attenuation of the quenching.

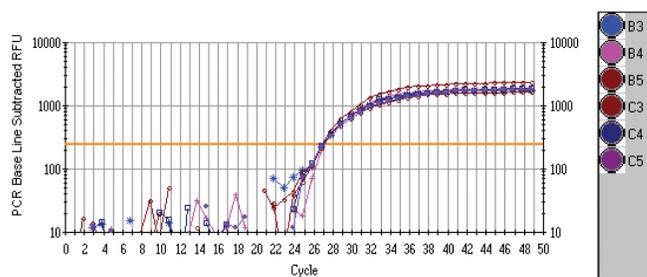


**Fig. 1.** A comparison of the fluorescence of open and closed Scorpions probes. The probes are either incorporated into the PCR product (yellow) or unbound (blue).

Figure 1 shows the fluorescence from the melt curve of two samples. One tube contained the IL-8 Scorpions primer and was subjected to 38 amplification cycles (yellow line). The other tube contained the same Scorpions primer and was also subjected to 38 cycles, but lacked *Taq* polymerase so no amplification occurred (blue line). The melt curve started at 40°C and the temperature was increased 2°C per cycle. Thus, at cycle 10 the temperature was 58°C (the temperature at which data were taken). At this temperature, the unused primer was still not melted (blue line, no increase in fluorescence) whereas the primer used for extension was highly fluorescent (yellow line). As the temperature increased further, the stem region melted and the Scorpions primer unfolded, resulting in increased fluorescence in the negative control reaction (blue line) as well as in the test reaction

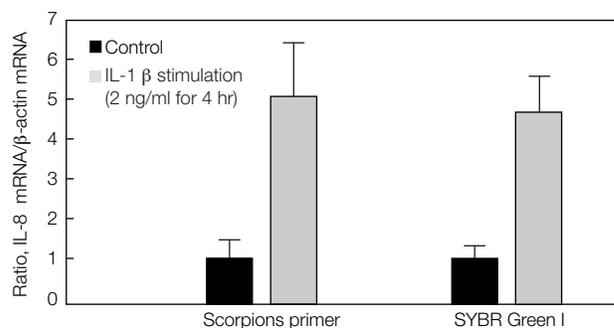
(yellow line) because any unused Scorpions primers melted and contributed fluorescence. At very high temperatures (e.g., cycle 24, 86°C; refer to Figure 1), the Scorpions primer is completely unfolded and the fluorescence of used and unused primers is the same. As shown in Figure 1, the difference in fluorescence between a quenched and an open Scorpions primer is quite large.

Next, the  $C_T$  for the IL-8 gene was determined in tubes containing only IL-8 primers (Figure 2, tubes B3, B4, and B5) and compared with the  $C_T$  for the multiplex amplification where both the IL-8 and  $\beta$ -actin genes were amplified (Figure 2, tubes C3, C4, and C5). As shown in Figure 2, the threshold cycles for the IL-8 gene were similar in both situations, indicating that the efficiency of the IL-8 amplification was not affected by simultaneous amplification of the  $\beta$ -actin gene in the same tube.



**Fig. 2.** Comparison of threshold cycles for single and multiplex detection of the IL-8 gene.

Finally, RNA from human adipocytes incubated with or without IL-1  $\beta$  was investigated. The ratio of IL-8 mRNA to  $\beta$ -actin mRNA was measured by multiplex real-time RT-PCR using a ROX-labeled IL-8 Scorpions primer and a FAM-labeled TaqMan probe for  $\beta$ -actin. Figure 3 shows that IL-1  $\beta$  stimulation increased IL-8 mRNA expression in the adipocytes approximately 5-fold. Similar results (a 4.8-fold increase in IL-8 expression) were obtained using a SYBR Green I assay where IL-8 and  $\beta$ -actin were amplified in separate tubes.



**Fig. 3.** Stimulation by IL-1  $\beta$  of IL-8 expression in human adipose tissue.

## Discussion

The iCycler iQ detection system allows multiplex real-time RT-PCR, enabling amplification of a target gene together with a housekeeping gene in the same tube. With this procedure any tube-to-tube variation in the reverse transcription step or in the PCR amplification efficiency can be controlled. The analysis presented here demonstrates the feasibility of this approach using two different probes simultaneously, the Scorpions primer and the TaqMan probe. A 5-fold increase in IL-8 expression in human adipocytes after IL-1  $\beta$  stimulation was detected with multiplex real-time RT-PCR, and a similar result (4.8-fold increase) was obtained using SYBR Green I real-time RT-PCR on targets amplified individually.

Multiplex amplification of IL-8 and  $\beta$ -actin resulted in approximately the same threshold cycle for IL-8 as when IL-8 was amplified by itself, indicating that multiplexing of two genes did not affect the amplification efficiency of the individual genes. The multiplex approach has the advantage of increased specificity compared to the SYBR Green I assay, as any nonspecific double-stranded product increases fluorescence in the SYBR Green I assay. However, if the PCR is optimized, so that no extra bands, primer-dimers, etc. are observed on gel analysis or in melting point analysis, then the SYBR Green I RT-PCR assay and the multiplex RT-PCR assay produce essentially the same results.

## References

- ABI PRISM 7700 Sequence Detection System User Bulletin #2, Relative quantitation of gene expression, Applied Biosystems (1997)
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- Whitcombe D et al., Detection of PCR products using self-probing amplicons and fluorescence, Nat Biotechnol 17, 804–807 (1999)

Practice of the patented polymerase chain reaction (PCR) process requires a license. The iCycler iQ system includes a licensed thermal cycler and may be used with PCR licenses available from Applied Biosystems. Its use with authorized reagents also provides a limited PCR license in accordance with the label rights accompanying such reagents. Some applications may require licenses from other parties.

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