Improvements in quantitative real-time RT-PCR with QIAGEN® Omniscript™ and Sensiscript™ RTs

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RT-PCR methods provide a highly sensitive and convenient way to analyze gene-expression analysis. Real-time quantitative techniques provide even more sensitivity and the ability to accurately quantify gene expression. In this review, we present some examples of highly sensitive real-time RT-PCR, with a focus on the influence of the reverse-transcription step. Sensitivity and a wide dynamic range of reverse transcription lead to improved real-time RT-PCR analysis.

Principles of real-time PCR detection

Sensitive quantification of PCR products relies on detection of a fluorescent signal proportional to the amount of product. PCR products can be measured in real time by use of a double-stranded DNA-binding dye, which binds only double-stranded but not single-stranded DNA, or labeled oligonucleotides (oligos) that specifically bind to the PCR product. The amount of PCR product can then be directly measured in real time using a fluorimeter. Quantification is based on the threshold cycle, the first PCR cycle with detectable fluorescence (Figure 1). Monitoring during the early cycles, when the PCR amplification is exponential, provides precise data for accurate quantification. Various commercial systems and chemistries are available for use in real-time PCR detection, and the most commonly used are briefly introduced below.

The **LightCycler™ System** (Idaho Technologies, Inc., licensed to Roche Diagnostics Corp.) is a rapid PCR system, capable of performing 30 PCR cycles in less than 20 minutes, with real-time fluorimetric detection of PCR products. The system can carry out continuous sampling of 32 samples, with results immediately displayed on the computer screen.

The **ABI PRISM® Sequence Detection Systems**, the ABI PRISM 5700 and 7700 (Perkin Elmer Applied Biosystems), are high-throughput systems that utilize a 96-well format. These systems have continuous fluorescent monitoring in real time, and have a typical run time of 2 hours.

Real-Time Amplification Plot

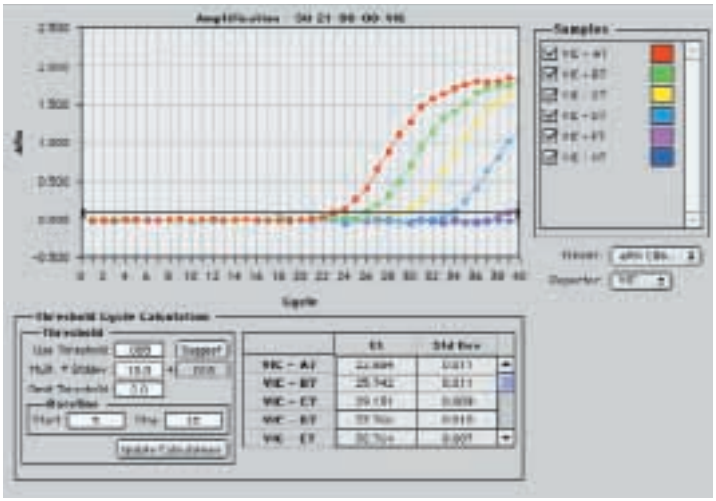

Figure 1 *Example of a PCR amplification plot monitored in real time. The threshold cycle (C_T) is the first cycle with detectable fluorescence in relation to an internal standard.*

SYBR® Green I is a double-stranded DNA-binding dye and is typically used for real-time PCR analysis on the LightCycler system. Although double-stranded DNA-binding dyes can be used with any PCR product without having to synthesize labeled sequence-specific oligonucleotides as probes, there is also the potential disadvantage that nonspecific PCR products will contribute to the signal.

Melting-curve analysis can be used to determine specificity, where the temperature is raised slowly to the melting point of the ▶

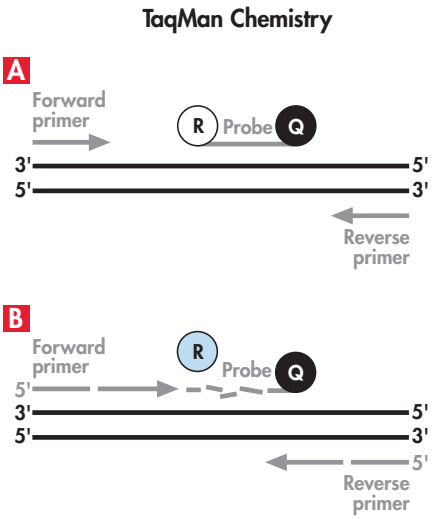

Figure 2 *Details of 5' nuclease chemistry* **A** *A fluorescent reporter dye (R) and a quencher (Q) are attached to the probe. When both R and Q are attached, the reporter-dye emission is quenched.* **B** *During each PCR extension cycle, the Taq DNA polymerase cleaves the reporter dye from the probe. The reporter dye now emits its characteristic fluorescence.*

duplex DNA and fluorescence monitored. Since SYBR Green I only binds double-stranded DNA, the fluorescent signal decreases as the T_m of the DNA duplex is reached. Analysis of the melting curve allows confirmation of PCR products as well as detection of single-base gene mutations which have small differences in the T_m.

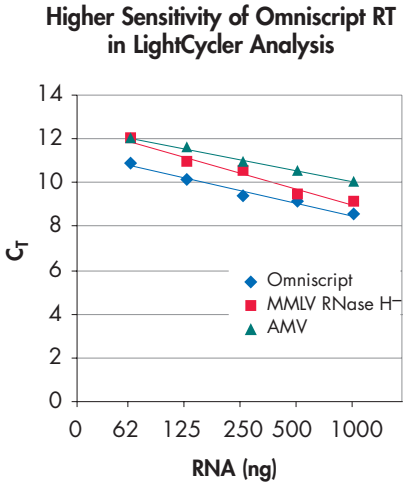

Figure 3 *Real-time RT-PCR analysis of the β-actin gene mRNA was performed using the indicated amount of total RNA purified from HeLa cells using the RNeasy Maxi Kit. Reverse transcription was carried out using Omniscript RT (QIAGEN, diamond), MMLV RNase H⁻ RT (Supplier L, square), or AMV RT (Supplier P, triangle), following manufacturers' instructions. Quantitative real-time PCR was carried out on the LightCycler system using SYBR Green I as a fluorescent label.*

TaqMan® probes and related technologies make use of labeled oligo probes and a 5' nuclease PCR assay to generate a fluorescent signal during PCR. In this system, a sequence-specific oligo probe is used with a reporter and a quencher dye attached (Figure 2A). During PCR, the probe is cleaved by the 5' nuclease activity of the *Taq* DNA polymerase, separating the reporter dye from the quencher dye (Figure 2B). This generates a sequence-specific fluorescent signal, increasing with each cycle. Multiple dyes can be used to monitor different PCR products in the same reaction. This method provides higher specificity than other methods but requires the use of expensive, specially made oligo probes. TaqMan probes are typically used on the ABI PRISM 5700 and 7700 Sequence Detection Systems.

Materials and methods

RNA was typically isolated from HeLa S3 cells using the RNeasy® Maxi Kit. Reverse trancription was carried out with the indicated amounts of total RNA using Omniscript™ (QIAGEN), Sensiscript™ (QIAGEN), MMLV RNase H⁻ (Supplier L), or AMV (Supplier P) reverse transcriptases, following manufacturers' specifications. Quantitative real-time PCR was performed on a LightCycler or ABI PRISM 7700 system, according to the suppliers' recommendations.

Results and discussion

Sensitivity in real-time RT-PCR analysis

Since RT-PCR quantification of RNA is based on amplification of cDNAs, the amount of cDNA produced by the reverse transcriptase must accurately represent original RNA amounts. The sensitivity of the reverse transcriptase, as well as the specificity of priming, can therefore greatly affect the outcome of the subsequent cDNA quantification. QIAGEN offers two reverse transcriptases to provide reverse transcription with nearly any amount of starting RNA — Omniscript Reverse Transcriptase provides highly specific and sensitive reverse transcription with any amount of RNA from 50 ng to 2 µg, and the specially developed Sensiscript RT gives extremely sensitive reverse transcription with even smaller amounts of RNA.

In comparative tests against other reverse transcriptases, QIAGEN Omniscript RT gave consistently higher sensitivity and a linear response in LightCycler real-time RT-PCR analysis (Figure 3). Similar results were obtained in a TaqMan analysis of both abundant and rare transcripts. Compared with the commonly used MMLV RNase H⁻ RT, Omniscript RT gave detection of signal 5 cycles earlier than the other RT (Figure 4).

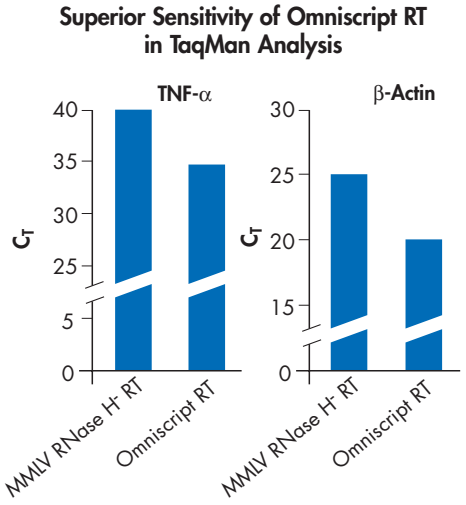

Figure 4 *Real-time RT-PCR analysis of TNF-α mRNA and, as a control, β-actin mRNA was performed using Omniscript (QIAGEN) or an MMLV RNase H⁻ (Supplier L) reverse transcriptase in two-step RT-PCR. The graph shows the number of PCR cycles needed to detect the amplicon (threshold cycle, C_T).*

Dynamic range of Omniscript and Sensiscript RT

For quantification to be meaningful, the reverse transcriptase also needs to have a correspondingly wide dynamic range in quantitative RT-PCR. To demonstrate the wide dynamic range of Omniscript and Sensiscript RT, RNA amounts from 1 µg to as little as 2 pg were used in reverse transcription, followed by detection using SYBR Green I on the LightCycler system. The results show that QIAGEN RTs provided highly accurate and linear quantification over an unprecedented 7 orders of magnitude (Figure 5).

Similar results were obtained using an ABI PRISM system. Linear quantification was accurate down to 10 pg with a rare RNA species and down to 0.1 pg with other RNAs (Figure 6).

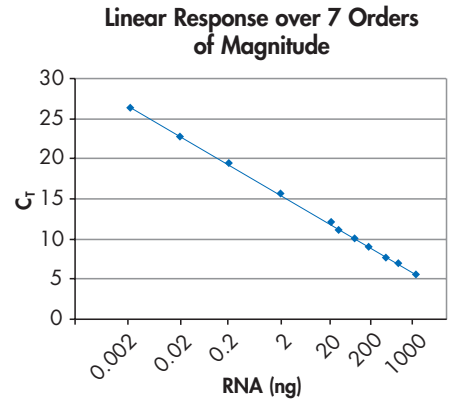

Figure 5 *Real-time RT-PCR analysis of β-actin mRNA was performed using the indicated amount of total RNA purified from HeLa cells using the RNeasy Maxi Kit. The smallest amount corresponds to 100 copies of β-actin mRNA. Reverse transcription was carried out with Omniscript RT (>50 ng RNA) or Sensiscript RT (<50 ng RNA). Quantitative real-time PCR was carried out on the LightCycler system using SYBR Green I as a fluorescent label.*

Melting curve analysis demonstrates specificity of Omniscript RT

Using Omniscript RT for quantitative RT-PCR on the LightCycler system, subsequent melting-curve analysis showed a distinct peak for the specific product with a characteristic melting point of approximately 85.5°C (Figure 7A, page 16). In comparison, a commonly used MMLV RNase H⁻ RT gave a peak that was the same magnitude as nonspecific background, indicating the correspondingly lower specificity and sensitivity of this enzyme (Figure 7B).

Conclusions

- Quantitative real-time RT-PCR analysis provides the potential for rapid and highly sensitive detection and quantification for gene-expression analysis.
- For quantitative real-time RT-PCR analyses, the reverse transcription step is critical for sensitive and accurate quantification. QIAGEN Omniscript and Sensiscript Reverse Transcriptases improved real-time RT-PCR results on both the LightCycler and ABI PRISM systems.
- Omniscript and Sensiscript Reverse Transcriptases provided high sensitivity and specificity as well as a wide dynamic range of 7 orders of magnitude in real-time RT-PCR analyses. ▶

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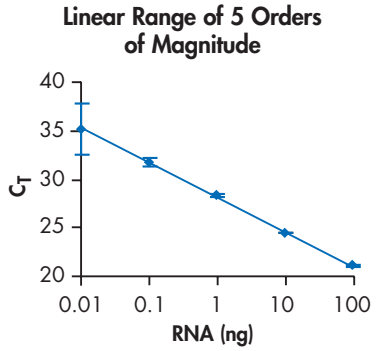

Figure 6 *Real-time RT-PCR analysis of human hypoxanthine ribosyl transferase was performed using the indicated amounts of RNA purified from HeLa S3 cells using the RNeasy Maxi Kit. Reverse transcription was carried out using Sensiscript Reverse Transcriptase. 2 µl of the RT reaction was used in a 25 µl TaqMan quantitative PCR. Standard deviation is indicated with vertical bars.*

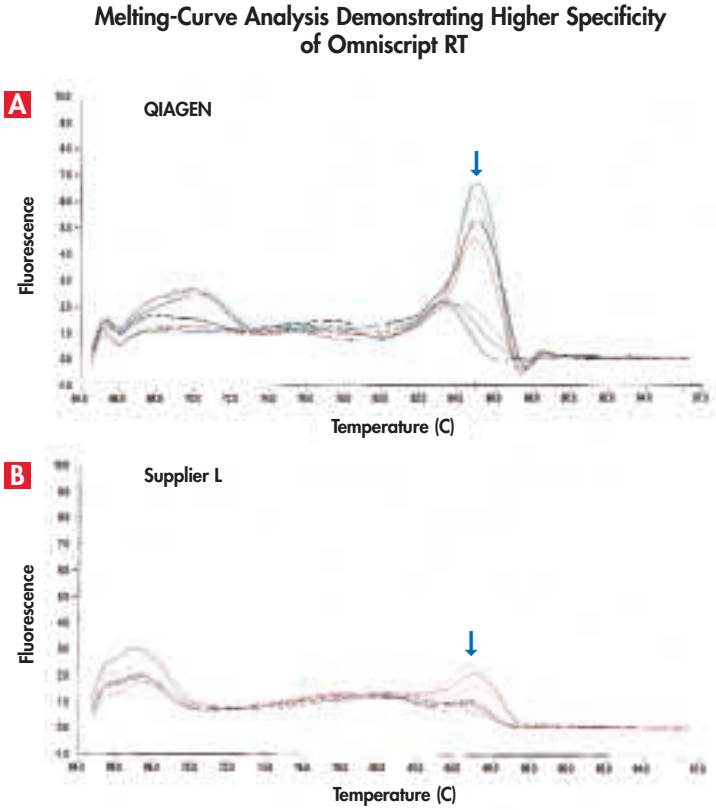

Figure 7 *Melting-curve analysis of interleukin-1α RT-PCR products was carried out on the LightCycler system using SYBR Green I as a fluorescent label. RT-PCR was carried out with serial dilutions of total RNA and* **A** *Omniscript Reverse Transcriptase (QIAGEN) in the RT step or* **B** *an MMLV RNase H– RT (Supplier L).*

Ordering Information

Product	Contents	Cat. No.
Omniscript RT Kit (10)*	For 10 reverse-transcription reactions: 40 units Omniscript Reverse Transcriptase, 10x Buffer RT, dNTP Mix,† RNase-free water	205110
Sensiscript RT Kit (50)*	For 50 reverse-transcription reactions: Sensiscript Reverse Transcriptase, 10x Buffer RT, dNTP Mix,† RNase-free water	205211
RNeasy Mini Kit (50)*	50 RNeasy Mini Spin Columns, Collection Tubes (1.5 ml and 2 ml), RNase-free Reagents and Buffers	74104
RNeasy Midi Kit (10)*	10 RNeasy Midi Spin Columns, Collection Tubes (15 ml), RNase-free Reagents and Buffers	75142
RNeasy Maxi Kit (12)	12 RNeasy Maxi Spin Columns, Collection Tubes (50 ml), RNase-free Reagents and Buffers	75162

** Larger kit sizes available; please inquire.*
† Contains 5 mM each dNTP