Using the Experion™ Automated Electrophoresis System to Assess RNA Quality and Quantity in siRNA-Induced Gene Silencing Experiments

William Strong and Teresa Rubio, Bio-Rad Laboratories, Inc.,
6000 James Watson Drive, Hercules, CA 94547 USA

Introduction

RNA interference (RNAi) has become a widely used method for evaluating and understanding gene function in biological systems, for elucidating biological pathways, and for identifying and validating potential drug targets (Hannon and Rossi 2004). RNAi is an evolutionarily conserved process of sequence-specific posttranscriptional gene silencing that uses double-stranded RNA (dsRNA) to trigger the degradation of the cognate mRNA. The mechanism by which dsRNA induces gene silencing involves multiple steps. First, long dsRNAs are recognized by Dicer, a ribonuclease (RNase) III-like enzyme that cleaves dsRNA into smaller RNAs (siRNAs) of 21–23 nucleotides. These siRNAs are then incorporated into a multicomponent nuclease-containing complex known as the RNA-induced silencing complex (RISC). The siRNA contained in this complex interacts with and mediates cleavage of mRNAs containing identical or nearly identical sequences (reviewed in Meister and Tuschl 2004).

There are several approaches to generating siRNAs for gene silencing studies, such as chemical synthesis, in vitro transcription, digestion of long siRNAs by RNase III or similar enzymes, expression in cells from an expression or viral vector, or expression in cells from an siRNA expression cassette. Traditional lipid-mediated transfection is the most common method of directly introducing siRNAs prepared extracellularly, as well as those generated in vivo from plasmids or expression cassettes containing an RNA polymerase III promoter.

Once the siRNAs have been delivered to the cell, the extent of message-specific silencing is often validated at the protein level by western blotting — or, at the message level, by one or more RNA-based analysis methods. Changes in the levels of target mRNA expression are most commonly analyzed using reverse transcriptase-quantitative polymerase chain reaction (RT-qPCR) approaches, although other methods, such as northern blots or more extensive analyses using microarrays, can also be used. For these latter methods, the isolation and quantitation of intact total RNA are prerequisites for successfully measuring the level of reduction of the mRNA transcript associated with the siRNA treatment. In this article, we discuss how the Experion automated electrophoresis system, which uses microfluidics technology, can be incorporated into an RNAi experimental workflow. Further, we show that the qualitative and quantitative RNA sample information that is obtained using this system is beneficial to the successful evaluation and validation of the efficacy of siRNA-mediated gene silencing.

Methods

siRNA Transfection

HeLa cells, plated in 12-well plates and grown to 50–60% confluence, were transfected using 0.6 µl siLentFect™ lipid reagent for RNAi and 10 nM siRNA specific to glyceraldehyde-3-phosphate dehydrogenase (GAPDH), hypoxanthine phosphoribosyltransferase (HPRT), or β-actin. A nonspecific scrambled siRNA was used as a negative control.

RNA Preparation and Analysis

Total RNA was extracted from the HeLa cells 24 hr posttransfection using the Aurum™ total RNA mini kit. RNA quality and quantity were assessed with the Experion automated electrophoresis system using the Experion RNA StdSens analysis kit. RNA quantity was also confirmed by measuring absorbance at 260 nm using a NanoDrop ND-1000 UV-Vis spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

siRNA Delivery

siLentFect Reagent

RNA Sample Preparation

Aurum Total RNA Kits

Quantitation and Quality Assessment

Experion System

RT-qPCR

iScript cDNA Synthesis Kit

iCycler iQ System

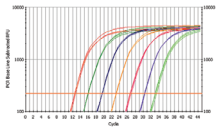

Fig. 1. Workflow from siRNA delivery to RT-qPCR.

RNA Sample Degradation

Samples of total RNA (100 ng/µl) extracted from HeLa cells transfected with either GAPDH siRNA or a scrambled control siRNA were subjected to controlled partial degradation by heating at 90°C in RNase-free TE buffer, pH 7 (Ambion, Austin, TX, USA) for 4 hr. The extent of RNA degradation was monitored using the Experion system.

Validation of Gene Silencing

From each of the various HeLa cell transfections, 500 ng total RNA was used to direct cDNA synthesis using the iScript™ cDNA synthesis kit. The cDNA product of each reaction served as template for real-time qPCR using iQ™ SYBR® Green supermix, gene-specific primers, and the iCycler iQ® real-time PCR detection system. The percentage of expression remaining after siRNA-targeted degradation of each mRNA versus the scrambled control was calculated from the difference in the threshold cycles (ΔC_T) using the formula $2^{-(\Delta C_T)}$ x 100%.

Results and Discussion

Role of Microfluidics-Based RNA Analysis in RNAi Protocols

A successful RNAi experiment involves several steps, including transfection of siRNA into cells, extraction and purification of RNA from transfected cells, quantitation and quality assessment of the RNA, and ultimate confirmation (for example, by RT-qPCR) that the levels of the targeted transcript have been reduced (Figure 1). To demonstrate how the Experion system can be integrated into this workflow, we performed an RNAi experiment using siRNAs specific to three housekeeping genes: GAPDH, β-actin, and HPRT. Total RNA was isolated 24 hr posttransfection and then analyzed using the Experion system to determine RNA concentration and to evaluate the integrity of the RNA sample.

Figure 2 shows the electropherogram and simulated gel view for the RNA prepared from HPRT siRNA-transfected HeLa cells. Figure 2A shows the pattern typically associated with good-quality RNA samples: a 28S/18S rRNA ratio of 1.9 and a relatively flat baseline, especially in the regions below the 18S rRNA and above the lower marker, which is used for well-to-well alignment.

The other RNA samples were of similar quality, with 28S/18S rRNA ratios of 1.9–2.1 (data not shown). Although UV spectroscopic analysis also indicated that the RNA samples were of high purity (A_{260}/A_{280} ratios of 2.1–2.2), the Experion microfluidic chip-based separation allowed a more direct visual evaluation of RNA quality. Experion software also reports the RNA quantity for each sample, and all these values were in agreement (<10% difference) with the concentrations measured by the absorbance at 260 nm, confirming the accuracy of the measurements made using the Experion system (data not shown).

To validate that the specific siRNAs could effectively silence the targeted messages, we used the total RNA preparations as templates to generate cDNA and then performed real-time qPCR. Figure 3 shows the relative expression levels from each of the targeted siRNA transfections compared to the scrambled control siRNA transfection, which is represented as 100% expression. The data show that, in all three cases, treatment with siRNA specific to targeted transcripts resulted in a ≥97% reduction in mRNA levels.

Validating siRNA Effectiveness — Importance of RNA Quality

The influence of RNA quality on downstream applications (such as RT-qPCR and microarrays) that measure and compare levels of gene expression is well documented (Auer et al. 2003, Bustin and Nolan 2004, Imbeaud et al. 2005). Since RT-qPCR is often used to validate RNAi experiments, the integrity of the RNA sample is an equally significant parameter when verifying the extent of siRNA-induced gene silencing.

To demonstrate how RNA quality can impact the interpretation of siRNA results, and hence the perceived effectiveness of a particular siRNA, we degraded a portion of the RNA samples from cells transfected with scrambled control and GAPDH siRNA by heating the RNA at 90°C in TE buffer. The original high-quality samples and resulting poor-quality RNA for each were then subjected to RT-qPCR.

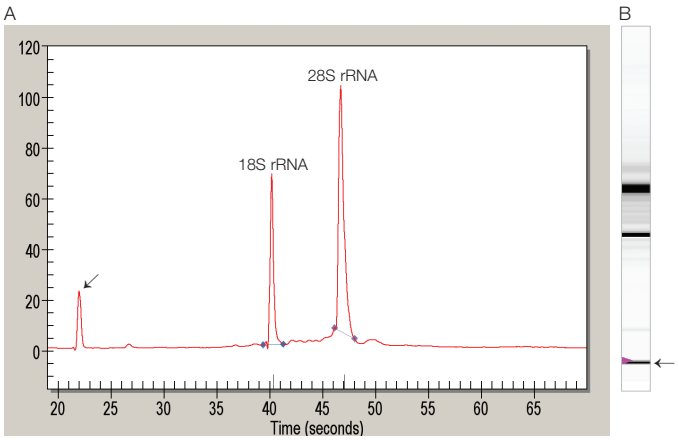

Fig. 2. Profiles of total RNA isolated from HPRT siRNA-transfected HeLa cells. Samples were separated with the Experion RNA StdSens analysis kit to generate an electropherogram (A) and simulated gel view (B). The 18S and 28S rRNA peaks are indicated. The peak at the far left of the electropherogram (A) corresponds to the lower marker (indicated by arrow, B) used for alignment of the sample wells.

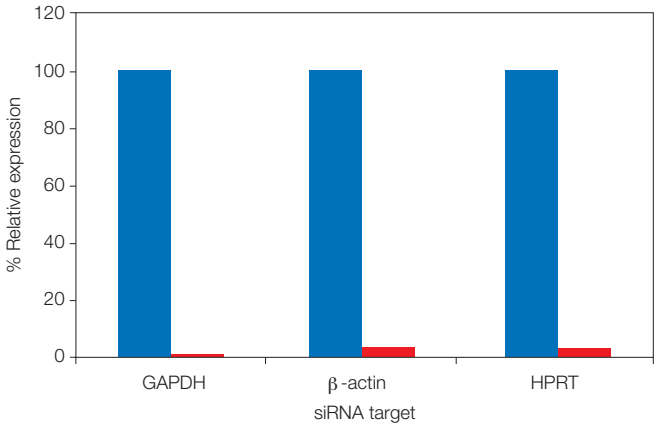

Fig. 3. siRNA-mediated silencing of three housekeeping genes. Relative expression levels for GAPDH, β-actin, and HPRT siRNA-targeted mRNAs (▬) compared to the transcript level measured in scrambled control siRNA-transfected cells (▬). Levels of expression in the controls were set at 100%.

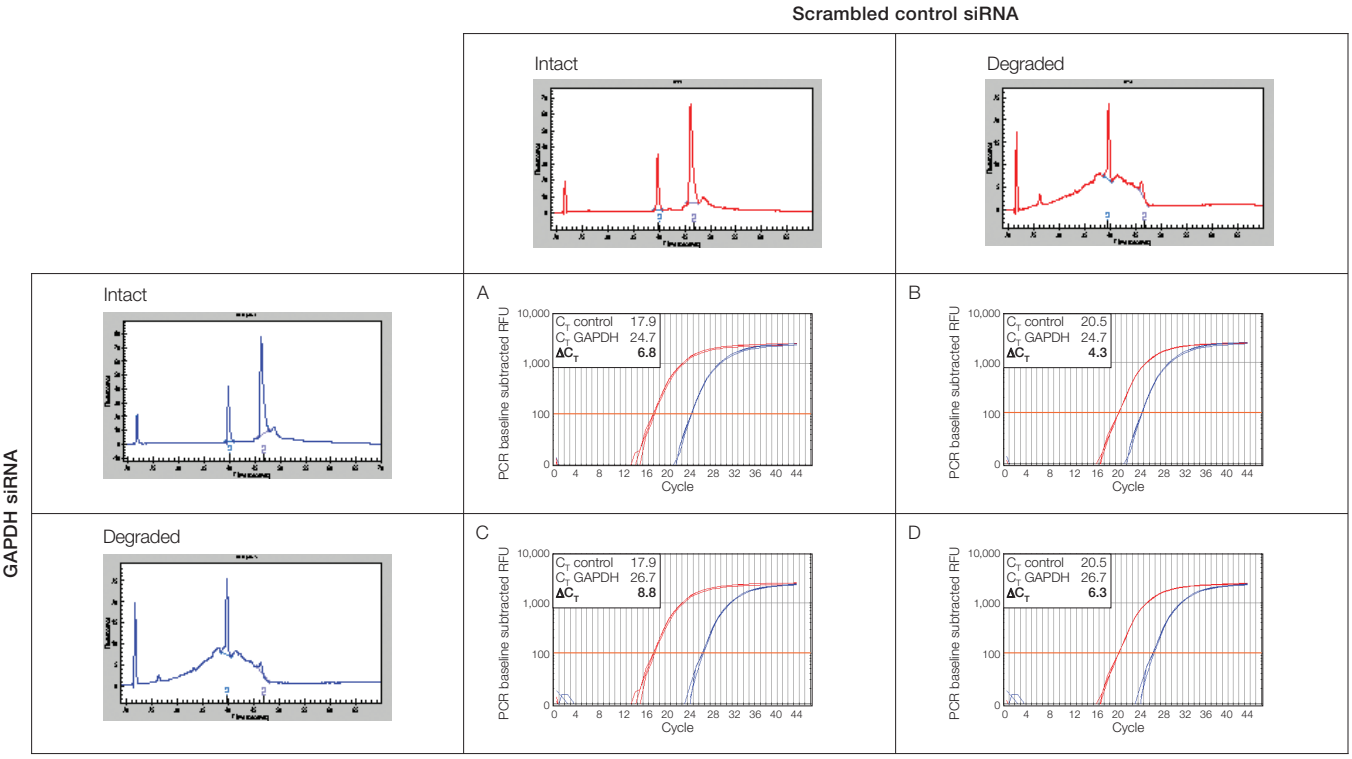

Fig. 4. Impact of siRNA-targeted RNA sample quality on RT-qPCR. Experion electropherograms of intact or partially degraded total RNA from transfections with scrambled control siRNA and GAPDH-targeted siRNA are shown. Panels A–D show RT-qPCR traces obtained using the combination of scrambled control (red) and GAPDH-targeted (blue) RNA sample indicated in the two electropherograms positioned above and beside the trace. Also shown are the average measured C_T values for each sample and the overall level of gene silencing, which is represented by the ΔC_T value.

Figure 4 shows the RT-qPCR trace derived when each possible combination of intact and degraded total RNA from scrambled control and GAPDH siRNA-transfected cells was analyzed. In each instance, electropherograms of the two RNA preparations being compared are shown beside or above the qPCR graphs. In the ideal case, where both the scrambled control and siRNA-targeted RNA preparations were of good quality, the ΔC_T for the level of the GAPDH transcript was 6.8 (Figure 4A). If both RNAs were similarly degraded, the overall level of transcript was drastically reduced, as evidenced by the increase in the C_T value for both samples (Figure 4D); however, the ΔC_T was not significantly affected since the GAPDH mRNA appears to have been degraded to a similar extent in both RNA populations. As expected, a highly degraded control RNA sample yielded an increase in the C_T value (from 17.9 to 20.5) and concomitant decrease in the ΔC_T (Figure 4B), leading to an underestimation

of specific gene silencing. In the reverse case, where the GAPDH siRNA-treated RNA sample was highly degraded and the scrambled control RNA was intact, the ΔC_T was found to widen (Figure 4C), leading to an overestimation of the effectiveness of siRNA-induced suppression.

These data illustrate that RNA quality is critical to downstream results, and poor or variable sample quality may skew results, potentially leading one to disregard siRNAs that are effective at gene silencing, or to keep siRNAs that exert only weak mRNA suppression. Although measurement of the absorbance of the sample at 260 nm would have provided adequate quantitative information for performing subsequent RT reactions, it would have failed to detect degraded RNA samples. These results demonstrate the utility of a microfluidics-based approach, such as that offered by the Experion system, for evaluating RNA quality.

Conclusions

An awareness of the quality of an RNA sample is essential to the success of experiments designed to study gene expression. Failure to evaluate the state of RNA samples before their use in downstream applications, such as validation of siRNA gene silencing using RT-qPCR, can yield faulty conclusions. The Experion system has been shown to be a useful tool in this regard, accurately quantitating RNA as well as providing information on the overall quality and integrity of the RNA that will ultimately be analyzed in various downstream applications.

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Information in this tech note was current as of the date of writing (2005) and not necessarily the date this version (Rev A, 2005) was published.

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