Quantitate In Vitro–Delivered siRNAs Using Real-Time RT-PCR

RNA interference (RNAi) is a powerful tool for targeted gene knockdown. Synthetic small interfering RNAs (siRNAs) can be introduced into a diverse range of organisms and cell types to induce degradation of mRNAs with sequence complementarity. RNAi has been used for applications that include gene function and pathway analysis, target identification and validation, and therapeutics [1]. Researchers using siRNAs for gene knockdown studies have expressed the urgent need for a simple, reliable method to quantitate siRNAs in cells and in animal tissues. The results would provide information on siRNA delivery efficiency, distribution, and stability. This article describes how Applied Biosystems Custom TaqMan® Small RNA Assays can be used for quantitation of siRNAs in cultured cells. The next article describes how these assays can be used to quantitate siRNAs in animal tissues after in vivo delivery.

Featured Products

NEW Custom TaqMan® Small RNA Assays (Early Access)

Ambion® *Silencer*® Select siRNAs

TaqMan® MicroRNA Cells-to-C$_T$™ Kit

Real-Time PCR Adapted for Tiny Targets

5' Nuclease real-time PCR using TaqMan® assays is widely used for detection and quantitation of nucleic acids because it offers gold standard specificity, sensitivity, and dynamic range. However, conventionally designed 5' nuclease real-time primer-probe sets cannot amplify very small targets, such as chemically synthesized siRNAs, microRNAs (miRNAs), or other endogenous small noncoding RNAs. With the recent explosion of interest in miRNAs, Applied Biosystems has developed TaqMan MicroRNA Assays to detect and quantitate these important regulators of gene expression. These assays employ an innovative target-specific stem-loop reverse transcription (RT) primer to address a fundamental problem in miRNA quantitation: the short length of mature miRNAs (~22 nt). The stem-loop structure provides specificity for only the mature miRNA target and produces a longer DNA template upon reverse transcription. The resulting longer RT amplicon presents a template amenable to standard 5' nuclease real-time PCR (see Figure 1). The same design principles and automated design pipeline developed to support TaqMan MicroRNA Assays can also be successfully employed to create assays for other small RNAs of interest.

New: Custom TaqMan Small RNA Assays for Customer-Defined Sequences

Applied Biosystems now offers Custom TaqMan Small RNA Assays based on the TaqMan MicroRNA Assays stem-loop primer technology. These assays are designed to detect customer-defined sequences, they can be used to analyze chemically synthesized or naturally occurring small interfering RNAs (siRNAs), miRNAs that are not yet described in the miRBase Sequence Database, and other small noncoding RNAs such as piwi-interacting RNAs (piRNAs), repeat-associated short interfering RNAs (rasiRNAs), and other uncharacterized and novel small RNAs.

In this article we demonstrate that Custom TaqMan Small RNA Assays can also be used to detect and quantitate *Silencer*® Select siRNAs. Furthermore, we show the feasibility of performing siRNA quantitation directly in cultured cell lysates without having to first isolate RNA, using the TaqMan MicroRNA Cells-to-C$_T$™ Kit (see Figure 2).

Evaluating Assay Performance in a Cell-Free System

An initial set of experiments was conducted to evaluate the efficiency and sensitivity of the Custom TaqMan Small RNA Assays for detecting *Silencer* Select siRNAs in a cell-free environment. Assays were designed to detect 106 *Silencer* Select siRNAs, each targeting a different gene. The guide strands of the siRNAs were chosen as targets for real-time PCR since they represent the "active" strand that is used by the RNA-induced silencing complex (RISC) [1].

For these experiments, *Silencer* Select siRNAs and unmodified control RNA duplexes were diluted in buffer to concentrations ranging from 1–100 pM, then reverse transcribed using the TaqMan MicroRNA Reverse Transcription Kit and the stem-loop RT primer from the corresponding Custom TaqMan Small RNA Assay. An aliquot of the resulting cDNA was used in real-time PCR with TaqMan Universal PCR Master Mix and the primer–probe mix from the Custom TaqMan Small RNA Assay.

Most assays (90 out of 106) measured the *Silencer* Select and control siRNA within

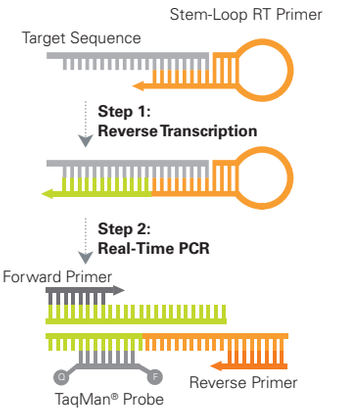


Figure 1. Custom TaqMan® Small RNA Assay Mechanism. Applied Biosystems Custom TaqMan Small RNA Assays employ the same stem-loop reverse transcription (RT) primer strategy that is used in TaqMan MicroRNA Assays. These target-specific RT primers provide specificity and serve to elongate the resulting amplicon, making it amenable to real-time PCR. This simple process brings the advantages of real-time PCR to small RNA research.

TaqMan® Small RNA Assays

- Highly specific—TaqMan Small RNA Assays designed for miRNA targets quantitate only the biologically active mature miRNAs, not precursors—with single-base discrimination of homologous family members
- Customizable—Specify your target sequence of interest and we design an assay
- Sensitive—Requires only 1–10 ng of total RNA or equivalent, enabling analysis of limited samples
- Wide dynamic range—Up to 7 logs—no sample titration needed, and you can detect high and low expressors in a single experiment
- Fast, simple, and scalable—Two-step real-time RT-PCR assay quickly provides reliable, quantitative results

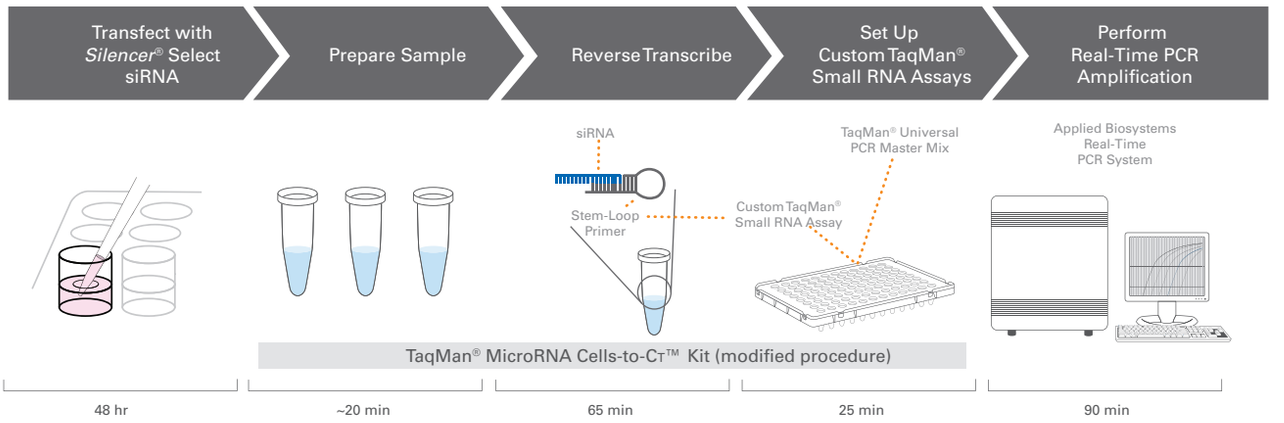


Figure 2. Workflow for Quantitation of siRNAs Directly from Cell Lysates Using Custom TaqMan® Small RNA Assays.

a difference of 1 C_T (Figure 3, Panel A). Comparison of detected fold change (ΔC_T values) between two consecutive $\log_{10}$-based concentrations showed that the majority of assays (up to 105 out of 106) demonstrated highly similar fold-change measurements ($\Delta\Delta C_T$ values between –1 and 1; Figure 3B). This indicates that the performance of Custom TaqMan® Small RNA Assays is consistent using different concentrations of *Silencer*® Select siRNAs, and is comparable to the results from the unmodified control siRNAs.

In the final set of cell-free experiments, purified RNA from human lung or HeLa cells was used as template in amplification reactions containing the 106 Custom TaqMan Small RNA Assays and TaqMan Universal PCR Master Mix. In these "no template control" reactions, most assays (99–100) exhibited C_T values greater than 35, indicating that the assays show very little nonspecific amplification (Figure 4).

In summary, all 106 assays were shown to detect their intended siRNA targets with high efficiency and low background in a cell-free system. Next, the Custom TaqMan Small RNA Assays were put to the test detecting siRNAs after transfection into cells in culture.

Quantitating siRNAs in Transfected Cell Lysates Using Custom TaqMan Small RNA Assays

Experimental Workflow for siRNA Quantitation

siRNA quantitation can potentially provide valuable information on siRNA delivery efficiency, distribution, and stability, in addition to correlation with target mRNA knockdown.

The experimental workflow shown in Figure 2 was followed to demonstrate quantitation of siRNA in transfected cells using Custom TaqMan Small RNA Assays. HeLa cells were transfected with a *Silencer* Select siRNA targeting ubiquinol-cytochrome *c* reductase (UQCR) or a nontargeting negative control siRNA and cultured for 48 hours in a 6-well plate. Reagents from the TaqMan MicroRNA Cells-to-CT Kit were used, and the protocol was extensively optimized to completely remove untransfected siRNAs that would otherwise compromise the quantitation data. To ensure removal of the untransfected siRNA, cells were washed, trypsinized, transferred to tubes, and pelleted prior to lysis using reagents from the TaqMan MicroRNA Cells-to-CT Kit. Trypsinization to transfer cells to tubes was important, since siRNA/lipid complexes tend to stick to the cell surface and culture plate. A portion of the lysate was used for real-time RT-PCR to determine target gene knockdown using an inventoried TaqMan Gene Expression Assay. The UQCR *Silencer* Select siRNA was detected and quantified from a second portion of the Cell-to-CT lysate using a Custom TaqMan Small RNA Assay. In order to

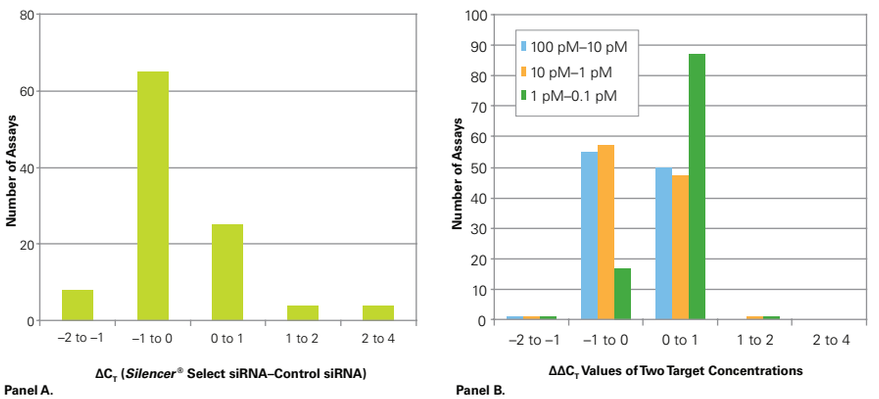


Figure 3. In a Cell-Free System, Quantitation of *Silencer*® Select siRNAs is Comparable to Unmodified Control siRNAs Using Custom TaqMan® Small RNA Assays. Panel A. ΔC_T values comparing amplification of a 100 pM solution of *Silencer* Select siRNAs or the corresponding unmodified control siRNAs. **Panel B.** The resulting $\Delta\Delta C_T$ values of two target concentrations. The assays for quantitation of siRNAs consisted of two steps: reverse transcription (RT), followed by PCR. The RT primer featured the same stem-loop design as the previously described TaqMan MicroRNA Assays. Real-time PCR was performed using standard cycling conditions on an Applied Biosystems 7900HT Fast Real-Time PCR System.

	Number of Assays		
	C_T <30	C_T = 30–35	C_T >35
Lung RNA (1 ng/µL)	1	6	99
HeLa RNA (1 ng/µL)	1	5	100

Figure 4. Custom TaqMan® Small RNA Assays Exhibit Very Little Nonspecific Amplification in a Background of Purified RNA. Purified RNA from human lung or HeLa cells was used as template in amplification reactions containing the 106 Custom TaqMan Small RNA Assays and TaqMan Universal PCR Master Mix. Real-time PCR was performed using a standard cycling conditions on an Applied Biosystems 7900HT Fast Real-Time PCR System.

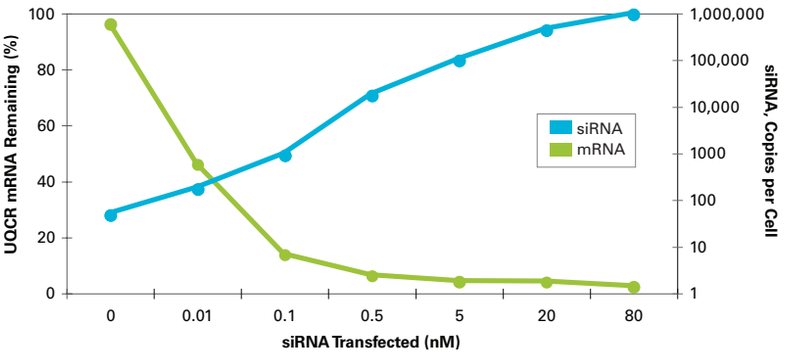


Figure 5. Relationship Between siRNA Uptake and Biological Activity. HeLa cells were transfected with 1 pm–80 nM siRNA targeting the UQCR mRNA. Cells were harvested after 48 hr, samples were prepared for real-time analysis using the TaqMan® Gene Expression Cells-to-CT™ Kit, siRNA quantitation was performed using the Custom TaqMan Small RNA Assay, and knockdown of UQCR mRNA was determined using a TaqMan Gene Expression Assay. To determine the number of siRNA copies delivered per cell, seven 5-fold serial dilutions starting from 20 pmol were separately spiked into nontransfected cell lysates harvested from the same number of cells used for the siRNA transfections. Comparison of C_T values between transfected and spiked samples enabled the calculation of siRNA copies per cell.

estimate the number of siRNA molecules in cells, siRNA detection results were compared to a standard curve generated by adding known amounts of siRNA to untreated cell lysates and then immediately processing samples using the same cell lysis/real-time RT-PCR procedure. The results are shown in Figure 5.

Sensitive siRNA Detection with Custom TaqMan® Small RNA Assays

In the Cells-to-CT lysate, the Custom TaqMan Small RNA Assay for UQCR siRNA exhibited higher nonspecific amplification than in buffer or purified RNA, with average C_T values from negative control samples at about 30 cycles. Samples transfected with siRNA targeting UQCR, however, gave C_T values of 15–20 cycles with the assay. Thus the ΔC_T value (C_T from UQCR siRNA-transfected cells – C_T from nontargeting control siRNA-transfected cells) was typically 10–15 cycles, providing sensitive detection of the *Silencer* Select siRNA.

In this set of experiments, transfection of 5 nM siRNA resulted in ~100,000 siRNA molecules detected per cell. This level of siRNA induced strong knockdown of the target mRNA, UQCR (Figure 5). Interestingly, 100,000 siRNA molecules corresponds to <1% of the 5 nM siRNA used for transfection; suggesting that 99% of the siRNA/lipid complexes did not enter cells. To confirm that this was not due to siRNA degradation inside the cell, the experiment was repeated, but quantitation was performed only 6 hours post-transfection. Similar siRNA detection results were obtained; 1–2% of siRNA used for transfection detected in the cells, but only 50% target knockdown was observed with the shortened culture time. Figure 5 also shows target knockdown and siRNA detection results with a titration of siRNA concentrations used for transfection. Using a broad range of *Silencer* Select siRNA concentrations (0.5–80 nM), similar, high levels of knockdown (~90%) were obtained. Clear differences in the number of copies of siRNA detected in cells, however, corresponded to the amount of siRNA used for transfection, varying from ~40,000 to 1,000,000 copies delivered per cell. With the lowest siRNA concentrations (<0.01 nM), fewer than 1,000 copies of siRNA per cell could be detected, and no target knockdown was observed. Similar results

continued next page

Tips from the Bench:

Avoiding siRNA Contamination in TaqMan® Small RNA Assays

When using Custom TaqMan Small RNA Assays designed to detect siRNAs, there is a risk of contamination if the siRNA transfections and TaqMan Assays are performed in the same laboratory environment. Research and Development Scientists at Applied Biosystems offer the following recommendations to minimize this risk:

- Designate an "siRNA-free" area of the laboratory that is used only for setting up real-time PCR assays
- Designate PCR pipettes that are never used for pipetting siRNAs
- Always include no-RT and no-template controls to monitor reagent contamination

TaqMan® Small RNA Assays: Other Applications

- Quantitation of siRNA delivery and persistence in animal tissues, organs, blood, or specific cells
- Investigation of naturally occurring siRNAs
- Monitoring siRNA levels expressed from plasmid or viral shRNA expression vectors
- Quantitation of novel small RNAs such as miRNAs, piwi-interacting RNAs (piRNAs), repeat-associated short interfering RNAs (rasiRNAs), and other uncharacterized small RNAs

Silencer® Select siRNAs Yield Cleaner, More Consistent Data

- Validated publication-quality data can be obtained faster, and with greater efficiency
- Novel chemical modifications reduce off-target effects by up to 90%
- Best knockdown performance in side-by-side comparisons
- Exceptional efficacy, specificity, and potency—have complete confidence in your reagents
- Cleaner, more consistent phenotypic data

were seen with other *Silencer* Select siRNAs and corresponding Custom TaqMan Small RNA Assays tested, and results from these experiments are in agreement with published reports [3].

These results demonstate that Custom TaqMan Small RNA Assays can be used to successfully determine the number of siRNA copies per cell. They also illustrate the siRNA concentration range required to induce potent mRNA knockdown.

Detect Any Small RNA Sequence with Custom TaqMan Small RNA Assays

Custom TaqMan Small RNA Assays can be designed for most target sequences. They are designed for detection and quantitation of miRNA, siRNA, shRNA, and any other small RNA species in either purified RNA samples or in cultured cell lysates prepared using Applied Biosystems Cells-to-C$_T$ products.

Results of this study corroborate earlier findings [2] that real-time RT-PCR for small RNA targets using Applied Biosystems innovative stem-loop RT primers are specific, sensitive, and easy-to-use. Furthermore, it demonstrates the utility of these Custom TaqMan Small RNA Assays for in vitro quantitation of siRNA, even with the chemical modifications used in *Silencer* Select siRNA to reduce off-target effects. This strategy can be used to evaluate siRNA delivery efficiency to different cell lines, and for studying stability of siRNAs in cells after transfection.

ORDERING INFORMATION	P/N	SIZE	PRICE
Custom TaqMan® Assays			
Custom TaqMan® Small RNA Assays	Varies	Varies	Varies
Design your own assays at **http://www4.appliedbiosystems.com/beta/smallrna.** For more information, and to obtain a quote, contact your local Applied Biosystems Sales Representative.			
***Silencer*® Select siRNAs**			
Silencer® Select Pre-designed siRNA	Varies	Varies	Varies
Silencer® Select Validated siRNA	Varies	Varies	Varies
Silencer® Select Custom Designed siRNA	Varies	Varies	Inquire*
*To search for and order *Silencer*® Select siRNAs, or to view the full list of options, visit **www.appliedbiosystems.com/geneassist**.			
TaqMan® Real-Time PCR Reagents			
TaqMan® Gene Expression Cells-to-C$_T$™ Kit	4399002	40 lysis rxns/200 PCRs	$540
	AM1728	100 lysis rxns/500 PCRs	$1,250
	AM1729	400 lysis rxns/2000 PCRs	$4,000
TaqMan® MicroRNA Cells-to-C$_T$™ Kit	4391848	100 lysis rxns/500 PCRs	$1,250
	4391996	400 lysis rxns/2000 PCRs	$4,500
TaqMan® Gene Expresssion Assays (Inventoried)	4331182§	250 rxns	$150
TaqMan® Gene Expresssion Assays (Made-to-Order)	4351372§	360 rxns	$250
§Visit **www.allgenes.com** to search for and order specific assays.			

REFERENCES

1. Dallas A and Vlassov A (2006) RNAi: a novel antisense technology and its therapeutic potential. *Med Sci Monitor* **4**, 67–74.
2. Chen C, Ridzon DA, Broomer AJ, Zhou Z, Lee DH, Nguyen JT, Barbisin M, Xu NL, Mahuvakar VR, Andersen MR et al. (2005) Real-time quantification of microRNAs by stem-loop RT-PCR. *Nucleic Acids Res* **33**, e179.
3. Overhoff M, Wünsche W, and Sczakiel G (2004) Quantitative detection of siRNA and single-stranded oligonucleotides: relationship between uptake and biological activity of siRNA. *Nucleic Acids Res* **32**, e170.

Scientific Contributors

Caifu Chen , Angie Cheng, Mu Li , Yu Liang, Susan Magdaleno, Alexander (Sasha) Vlassov, Yu Wang, Linda Wong • Applied Biosystems

Quantitate In Vivo–Delivered siRNAs Using Real-Time RT-PCR

RNA interference (RNAi) is a natural mechanism by which small interfering RNAs (siRNAs) mediate sequence-specific degradation of targeted RNA molecules. Because of their potency and specificity, siRNAs are the most widely used agents for inducing targeted gene knockdown. Quantitating siRNAs in cells and tissues can provide insight into siRNA delivery efficiency, stability, and biolocalization in gene knockdown experiments, and can also be used for the study of naturally occurring RNAi pathways in vivo. In the article *Quantitate In Vitro–Delivered siRNAs Using Real-Time RT-PCR* starting on page 25, we show that Custom TaqMan® Small RNA Assays can be used to quantitate siRNAs in cultured cells. Here we demonstrate the use of these assays for quantitating siRNAs delivered to animal tissues in vivo. We also show that resulting siRNA-induced gene knockdown can be measured by real-time RT-PCR using TaqMan Gene Expression Assays.

NEW Custom TaqMan® Small RNA Assays (Early Access)
TaqMan® Gene Expression Assays
Ambion® *Silencer*® Select In Vivo Ready siRNAs
Ambion® *mir*Vana™ PARIS™ Kit

Detect and Quantitate Any Small RNA Sequence from Any Species

As a growing number of miRNAs and other small noncoding RNAs are discovered, there is a significant need to validate and quantitate these novel sequences. With the new Custom TaqMan® Small RNA Assays from Applied Biosystems, you can now detect virtually any small RNA sequence using TaqMan MicroRNA Assay stem-loop primer technology (see Figure 1, page 25. These custom assays can be used to evaluate the expression of chemically synthesized or naturally-occurring short interfering RNAs (siRNAs), miRNAs that are not yet described in the miRBase Sequence Database or covered by our TaqMan MicroRNA Assay collection, or other small noncoding RNAs such as piwi-interacting RNAs (piRNAs), small nuclear RNAs (snRNAs), small nucleolar RNAs (snoRNAs), and repeat-associated short interfering RNAs (rasiRNAs). The previous article, *Quantitate In Vitro–Delivered siRNAs Using Real-Time RT-PCR*, describes experiments demonstrating that Custom TaqMan Small RNA Assays can be used to detect *Silencer*® Select siRNAs in cultured cells. In this article we demonstrate that Custom TaqMan Small RNA Assays can also be used to detect and quantitate *Silencer* and *Silencer* Select siRNAs from mouse tissues after in vivo delivery. Likewise, the effects of in vivo siRNA delivery are evaluated by real-time PCR, using TaqMan Gene Expression Assays, to analyze targeted gene knockdown.

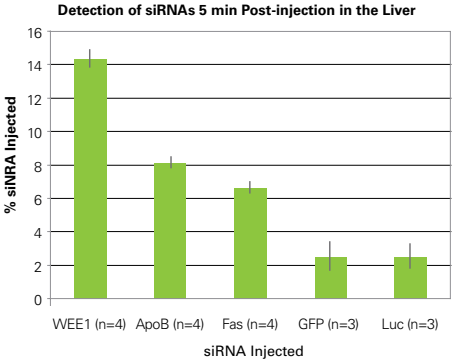


Figure 1. Quantitation of *Silencer*® and *Silencer* Select siRNAs Using Custom TaqMan® Small RNA Assays. *Silencer*® siRNAs (3 nmol) targeting mouse Fas, human WEE1, and firefly Luc, and *Silencer* Select siRNAs targeting GFP and mouse ApoB were hydrodynamically injected into mouse tail veins. Mice were sacrificed 5 minutes later, their organs were resected, and total RNA was isolated using the *mir*Vana™ PARIS™ Kit. siRNA was quantitated by real-time RT-PCR using Custom TaqMan® Small RNA Assays. To determine the number of molecules delivered to each organ, C_T values were compared to data from a standard curve generated by adding known amounts of siRNA ranging from 1 to 500 pmol to four different normal mouse whole liver lysates, and performing the same processing steps. Results are expressed as a percentage of the number of siRNA molecules injected.

In Vivo Quantitation of siRNAs and Corresponding mRNA Knockdown in Mouse Tissues

Experimental Approach

siRNAs with fluorescent labels are often used to obtain quick estimates of delivery efficiency and siRNA localization [1]. However, this approach is neither quantitative nor indicative of siRNA integrity. Furthermore, interpretation of the data is complicated because bulky conjugates may alter siRNA properties and trafficking, and fluorescent label that is not conjugated to siRNA may be present due to in vivo cleavage and/or incomplete removal after synthesis.

To demonstrate that Custom TaqMan Small RNA Assays can be used to quantitate siRNAs delivered in vivo, *Silencer* or *Silencer* Select In Vivo Ready siRNAs targeting the endogenous mouse gene Fas and the reporter genes, green fluorescent protein (GFP) and firefly luciferase (Luc), were hydrodynamically injected into the tail veins of mice (See *Silencer* Select In Vivo Ready siRNAs, page 31). Reporter plasmids encoding GFP or Luc were co-injected with their corresponding siRNAs. Nontargeting negative control siRNAs were also injected, as well as siRNAs targeting human WEE1, which does not target the mouse Wee1 homolog. At timepoints ranging from 5 minutes to 48 hours after in vivo delivery of siRNAs and reporter plasmids, mice were sacrificed, then blood and liver, lung, kidney, and spleen tissues were collected for total RNA isolation using the *mir*Vana™ PARIS™ Kit. siRNAs were quantitated from each organ using Custom TaqMan Small RNA Assays and corresponding mRNA knockdown was evaluated using the appropriate TaqMan Gene Expression Assay. For the GFP knockdown experiments, fluorescence analysis of the GFP protein in liver tissue was performed in parallel with quantitation of siRNA and mRNA levels using real-time PCR.

Injected siRNAs Were Principally Localized in the Liver

siRNAs were detected and quantitated in mouse tissues starting 5 minutes post-injection. With this delivery method and dosing level, siRNA levels rapidly decreased over time (data not shown). By 24 hours, all siRNAs were too low in quantity to be detected. In all experiments, the largest fraction (2–14%) of injected siRNAs was detected in the liver 5 minutes post-injection (Figure 1). Other tissues (kidneys, spleen, lungs, and blood) contained at least 100-fold less siRNA compared to the liver (data not shown). This distribution to the liver is consistent with published studies using hydrodynamic intravascular injection for siRNA delivery to

the liver [1,2]. Since the purpose of this study was to verify that siRNA delivered in vivo could be detected using the 5'-nuclease real-time PCR approach, known questions about the physiological relevance of in vivo delivery using the hydrodynamic intravascular injection technique were not a big concern. However, subsequent studies will take advantage of newer methodologies for in vivo delivery of siRNA, such as Invivofectamine™ Reagent from Invitrogen.

siRNA-Induced mRNA Knockdown in Mouse Liver

Since siRNAs delivered in vivo were principally detected in liver, this organ was the focus of mRNA knockdown studies. siRNA-induced knockdown of GFP and Fas in the liver was quantitated and results are shown in Figures 2 and 3, respectively. In control mice that were injected with GFP-expressing plasmids and negative control siRNAs, high levels of GFP were expressed in the liver as detected at both the mRNA and protein levels (Figure 2). When siRNAs targeting GFP were co-injected with the plasmids, a significant reduction of GFP fluorescence was visually observed and >90% knockdown was measured at the mRNA level by real-time RT-PCR.

For the endogenous mouse gene Fas, mRNA knockdown of <40% was observed in livers of animals subjected to in vivo delivery of siRNA (Figure 3). This low level of endogenous gene knockdown compared to the knockdown of exogenous targets is in agreement with published reports [2,3]. Figure 3 also demonstrates the wide variation in mRNA levels of control-treated animals, and in mRNA knockdown among biological replicates. This observation is also consistent with published reports.

TaqMan Custom Small RNA Assays enable rapid, accurate relative and absolute quantitation of siRNAs. This study represents the first published report of the use of real-time PCR to analyze siRNA biodistribution. The *Silencer* Select siRNAs that were used in this study include chemical modifications for improving specificity; however, these modifications do not improve serum stability. *Silencer* siRNAs, which were also used, are not chemically modified at all. Therefore, the siRNAs used in this study were rapidly degraded by blood and hepatocyte RNases [4, 5]. This rapid degradation explains the observation that only a low percentage of the total injected siRNA was detected in mouse

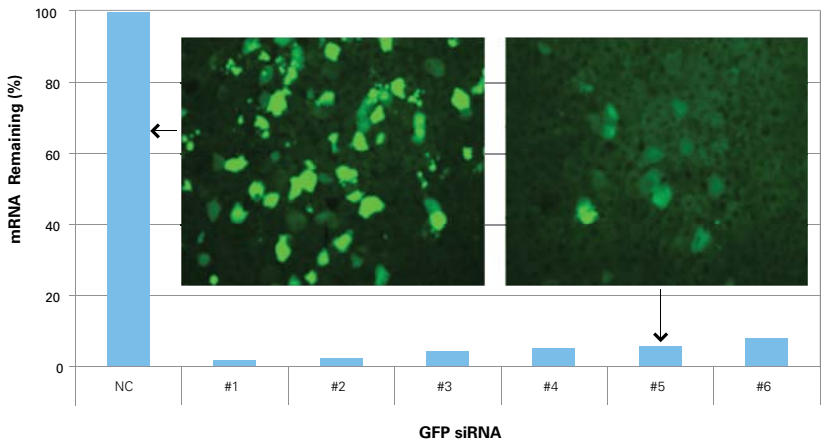
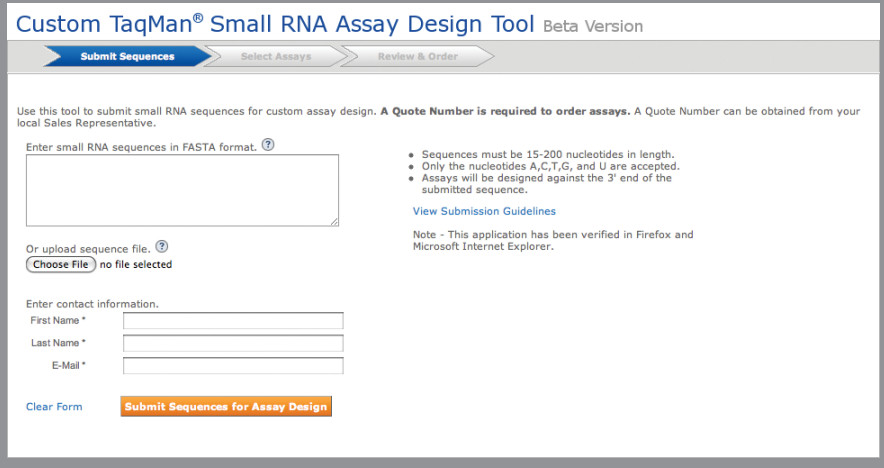


Figure 2. siRNA-Induced Knockdown of GFP in Mouse Liver. GFP-encoding plasmids and nontargeting negative control siRNA (NC, n=4) or siRNA targeting GFP (#1–6, bars represent individual animals) were hydrodynamically injected into mouse tail veins. Mice were sacrificed 24 hr later, their organs were resected, and total RNA was isolated using the *mir*Vana™ PARIS™ Kit. mRNA knockdown was quantitated by real-time PCR using the TaqMan® Gene Expression Assay for GFP. Knockdown data is expressed relative to data from mice injected with the nontargeting negative control siRNA. Representative liver sections are shown for both groups of mice. These were prepared as follows: organs were fixed in 4% formaldehyde in 1X PBS at 4°C overnight, rinsed 3 times with PBS, and immersed in 30% sucrose in PBS at 4°C overnight. The tissue was embedded with TFM™ Tissue Freezing Media (Triangle Biomedical Sciences, Inc.) and then frozen on dry ice. Cryosections (10 µm) were prepared and mounted on slides. The mice injected with GFP siRNA display a significant reduction of GFP protein levels 24 hr post-injection.

Online Design Tool for Custom TaqMan® Small RNA Assays

Don't spend your time and resources designing and optimizing primer/probe sets for real-time PCR detection of small RNAs. Applied Biosystems has just launched an easy-to-use online tool for ordering Custom TaqMan Small RNA Assays. Contact your local Applied Biosystems Sales Representative for a quote, then simply submit your sequence of interest, and the optimal small RNA-specific TaqMan Assay is designed using our proprietary small RNA assay design pipeline. When the design process is complete, you will receive an email allowing you to view and order Custom TaqMan Small RNA Assays. The tool is available at:

http://www4.appliedbiosystems.com/beta/smallrna.



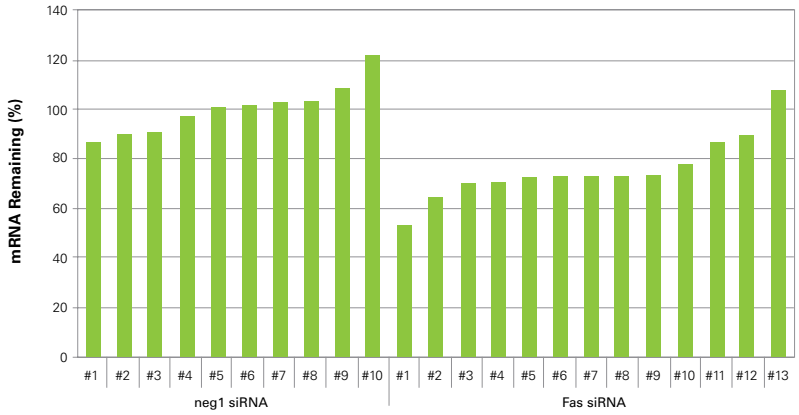


Figure 3. siRNA-Induced Knockdown of Fas in Mouse Liver. *Silencer*® siRNA targeting Fas was injected into mouse tail veins (n=13). Mice were sacrificed 48 hr later, their organs were resected, and total RNA was isolated from liver using the *mir*Vana™ PARIS™ Kit. mRNA knockdown was determined by real-time PCR using TaqMan® Gene Expression Assays. Knockdown data is expressed relative to the negative control group (n=10). Biological replicates are shown.

Silencer® Select In Vivo Ready siRNAs

Applied Biosystems *Silencer* Select In Vivo Ready siRNAs—the highest-quality siRNAs available—provide the extra level of purification and testing required for introduction into animals. Each siRNA strand is individually purified by HPLC, desalted, and annealed with its complementary strand. In Vivo Ready siRNAs are then further purified to remove excess salt. The result is a highly pure siRNA with minimal salt content, suitable for in vivo applications. In Vivo Ready siRNAs contain ≤0.6 mM Na^+, ≤2.0 mM K^+, and ≤0.1 mM Mg^{2+} when resuspended at a concentration of 50 µM in deionized water. In Vivo Ready siRNAs are then filtered through a 0.2 µm presterilized filter and endotoxin tested.

In vivo experimentation also requires larger quantities of siRNA than in vitro work; at 250 nmol, *Silencer* Select In Vivo Ready siRNAs are sized appropriately for most in vivo RNAi studies.

All *Silencer* Select Pre-designed, Validated, or Custom Select siRNA sequences can be ordered with "In Vivo Ready" purity.

ORDERING INFORMATION	P/N	SIZE	PRICE
Custom TaqMan® Small RNA Assays		150 rxns/500 rxns	Inquire
Design your own assays at **http://www4.appliedbiosystems.com/beta/smallrna.** For more information, and to obtain a quote, contact your local Applied Biosystems Sales Representative.			
***Silencer*® Select In Vivo Ready siRNAs**			
Custom Select siRNA, In Vivo	4404006	250 nmol	$1995
Silencer® Select Pre-designed siRNA, In Vivo	4404010	250 nmol	$1995
Silencer® Select Validated siRNA, In Vivo	4407267	250 nmol	$1995
Silencer® Select Negative Control #1 siRNA, In Vivo	4404020	250 nmol	$1995
Silencer® Select GAPDH siRNA, In Vivo	4404024	250 nmol	$1995
For more information or to order, visit **www.appliedbiosystems.com/geneassist**.			
TaqMan® Gene Expression Assays			
TaqMan® Gene Expression Assays (Inventoried)	4331182§	250 rxns	$150
TaqMan® Gene Expression Assays (Made-to-Order)	4351372§	360 rxns	$250
TaqMan® Gene Expression Master Mix	Varies	Varies	Varies
§Visit **www.allgenes.com** to search for and order specific assays.			

tissues. In contrast to other techniques that measure the signal intensity from radioactive or fluorescently labeled-siRNAs in crude samples, Custom TaqMan Small RNA Assays detect only full-length siRNAs. Partially degraded siRNA molecules would not be detectable using the real-time PCR approach employed in this study. Interestingly, in vivo delivery of siRNAs did result in significant knockdown of gene expression, with maximal knockdown observed 24–48 hours after in vivo delivery. This suggests that partially degraded siRNAs which were not detected using TaqMan assays contributed to biological activity. Indeed, siRNAs as short as 16 bp have been shown to induce knockdown comparable to canonical 21 bp siRNAs [6].

Sensitive siRNA Detection from Animal Tissues

Custom TaqMan Small RNA Assays give researchers the flexibility to validate newly discovered small RNAs, detect miRNAs from organisms that are not currently supported by our TaqMan MicroRNA Assay collection, and detect exogeneous, introduced small RNAs. They enable fast and sensitive detection and quantitation of any small RNA sequence including siRNA (unmodified or with limited chemical modifications), miRNA, shRNA, and piwi-interacting RNA (piRNA). In RNAi studies, the assays are valuable for analysis of siRNA delivery efficiency (for example, using Invivofectamine™ reagent—a novel reagent for in vivo delivery from Invitrogen), stability, and biodistribution in animal tissues.

REFERENCES

1. Dunne J, Drescher B, Riehle H, Hadwiger P, Young BD, Krauter J, and Heidenreich O (2003) The Apparent Uptake of Fluorescently Labeled siRNAs by Electroporated Cells Depends on the Fluorochrome. *Oligonucleotides* **13:** 375–380.
2. Lewis DL, Wolff JA (2005) Delivery of siRNA and siRNA expression constructs to adult mammals by hydrodynamic intravascular injection. *Methods Enzymol* **392:** 336–350.
3. Lewis DL, Hagstrom JE, Loomis AG, Wolff JA, and Herweijer H (2002) Efficient delivery of siRNA for inhibition of gene expression in postnatal mice. *Nat Genet* **32:** 107–108.
4. Vlassov AV (1998) Human ribonucleases. *Biochemistry (Moscow)* **63:** 1349–1360.
5. Hickerson RP, Vlassov AV, Leake D, Wang Q, Contag CH, Johnston BH, Kaspar RL (2008) Stability study of unmodified siRNA and relevance to clinical use. *Oligonucleotides* **18:** 345–354.
6. Chu CY and Rana TM (2008) Potent RNAi by short RNA triggers. *RNA* **14:** 1714–1719.

Scientific Contributors

Mu Li, Angie Cheng, Alexander (Sasha) Vlassov, Susan Magdaleno, Yu Liang, Yu Wang, Linda Wong, Caifu Chen • Applied Biosystems