Using TaqMan® Gene Expression Assays to Validate siRNA-directed Gene Knockdown

Introduction

Gene silencing by siRNA-targeted transcript degradation has rapidly become an important tool in molecular and cell biology.[1,2] It is being used to knock down specific RNA transcript levels and thereby induce specific protein profile changes in a variety of different cell types. For instance, siRNAs can be used for specific knockdown of certain over-expressed genes in cancer cells, such as kinases or multi-drug resistance genes, that alter tumor development.[3] Real-time PCR measurement of mRNA levels has proven to be a simple and efficient way to measure the level of knockdown achieved with varying transfection conditions, siRNA designs, cell types, and target genes.

Applied Biosystems has evaluated siRNA knockdown by real-time PCR using TaqMan® Gene Expression Assays. The main goal of this effort was to determine whether the target location of the TaqMan Gene Expression Assay needed to correlate with the target location of the siRNA.

Abstract

We used TaqMan Gene Expression Assays (for product information, visit **www.allgenes.com**) to measure the mRNA knockdown level achieved with a subset of commercially available siRNAs designed for kinase transcripts. A total of 52 kinase transcripts were studied in siRNA knockdown experiments. No effort was made to correlate the locations of the two assays, rather, we chose an inventoried TaqMan Gene Expression Assay for the validation step. Overall results showed 37 transcripts (more than 71%) resulted in 80% or more knockdown with one or more siRNAs designed for the same transcript; eleven transcripts (21%) resulted in 50–80% knockdown; and only three transcripts (less than 8%) resulted in less than 50% knockdown. Overall, an extremely high percentage of siRNAs (more than 92%) produced significant levels of transcript reduction. Furthermore, no significant importance was observed in the location of the TaqMan Gene Expression Assay relative to the location of the siRNA.

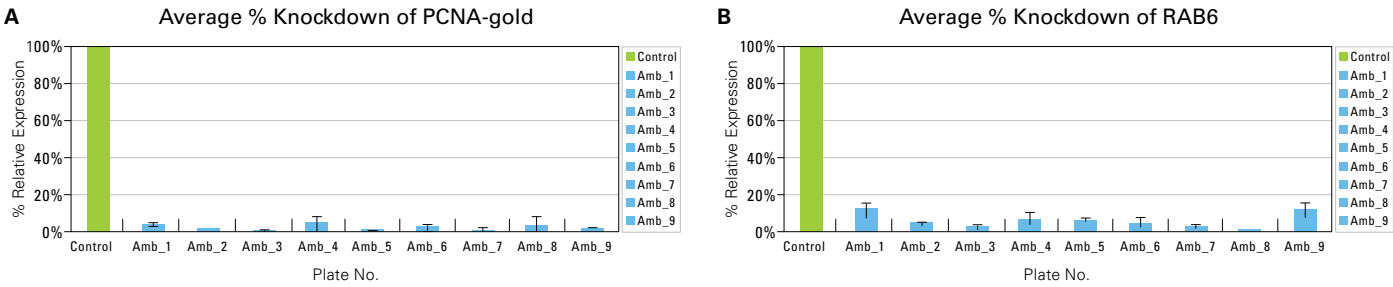

Figure 1. Performance of control PCNA (Panel A) and RAB6 (Panel B) siRNAs in an experiment-to-experiment comparison.

Methods

HeLa cells were transfected with individual siRNAs at 100 nM in 96-well plates. Forty-eight hours after transfection, total RNA was isolated and converted to cDNA. Two previously selected positive siRNA controls—targeting PCNA and RAB6—and a non-targeting negative control siRNA were included in each 96-well plate. Three different siRNAs designed to the same transcript, as well as a mixture of those same three siRNAs, were each tested in individual wells. Each siRNA was transfected in triplicate wells on the same plate to ensure reproducible results and to allow for elimination of outliers.

To measure levels of mRNA transcripts after siRNA treatment, we used real-time PCR with Applied Biosystems TaqMan Gene Expression Assays. In all, 57 assays for specific transcripts were used including five assays that were used as second assays for transcripts C8FW, LAK, SCYL, TLK1, and ZAK (Table 1, panels A and C). Additionally, detection of 18S rRNA was used to normalize total RNA purified from all cell fractions.

Transcript levels for five genes were measured using two different TaqMan® Assays. PCR reactions were performed using Applied Biosystems 2X TaqMan® Universal PCR Master Mix. The Applied Biosystems 7900HT Fast Real-Time PCR System was used to measure both 18S rRNA and specific transcript levels in each siRNA-treated cell fraction. Based on the high efficiency of TaqMan Gene Expression Assays (100%, +/- 10%), we applied the $\Delta\Delta C_T$ method for quantitation of specific (siRNA-targeted) transcript levels.[3, 4]

Results and Discussion

The study was performed to demonstrate how efficiently the siRNA knockdown effect can be measured using TaqMan Gene Expression Assays, as well as to demonstrate the effect of assay transcript location on the results. A set of pre-designed siRNAs targeting 51 human kinase transcripts was obtained from a third party. These siRNAs were transfected into HeLa cells using a 96-well plate transfection format. To ensure that all transfection experiments across the nine plates were performed uniformly, every plate had a portion of HeLa cells treated with non-targeting siRNA (negative control). Two different, previously pre-selected siRNAs that efficiently targeted PCNA and RAB6 transcripts, were used as positive controls. After siRNA treatment, total RNA purification, and obtaining cDNA from each sample, we determined transcript levels by using TaqMan Gene Expression Assays. Figure 1 demonstrates the performance of the PCNA siRNA (Panel A) and RAB6 siRNA (Panel B) controls. All plates show a high level of knockdown induced by these two controls, indicating that the efficiency of transfection for each plate was high.

Relatively small differences in knockdown efficiency were observed, which we attributed to minor plate-to-plate variation. All cDNA preparations were tested with the 18S TaqMan® Endogenous Control Assay to normalize RNA concentrations across all the samples (obtaining ΔC_T for each transcript in siRNA-treated samples and negative control siRNA-treated samples). The fold change in transcript levels from cells treated with specific siRNA and negative control siRNA (or % of remaining transcript) was calculated using the $\Delta\Delta C_T$ method as described earlier[4] (see Appendix A for an explanation of the relationship of $\Delta\Delta C_T$, fold change, and % mRNA knockdown).

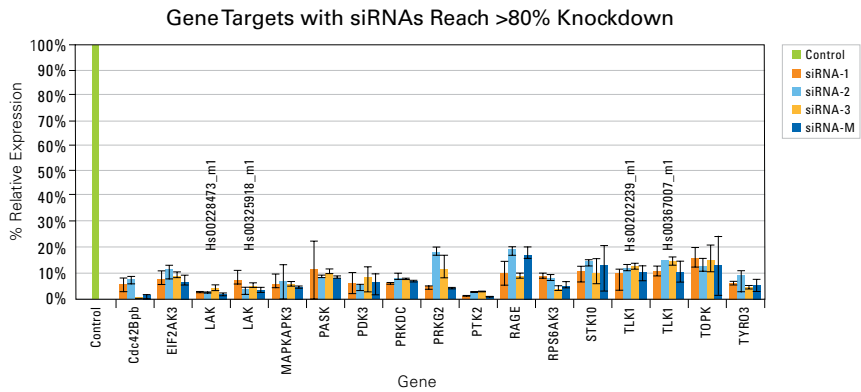

Figure 2. siRNA-induced mRNA knockdown measured by Applied Biosystems TaqMan® Gene Expression Assays. Transcripts were knocked down by specific siRNA with more than 80% efficiency with all siRNA applied.

A. AT LEAST ONE siRNA REACHES >80% KNOCKDOWN

		% Relative expression			
Gene name	**Assay ID**	**siRNA-1**	**siRNA-2**	**siRNA-3**	**siRNA-pool**
C20orf97	Hs00221754_m1	9%	13%	5%	31%
C8FW	Hs00179769_m1	4%	18%	34%	17%
CABC1	Hs00220382_m1	75%	12%	44%	11%
Cdc42Bpb	Hs00178787_m1	5%	7%	0%	2%
CKLiK	Hs00220668_m1	33%	7%	7%	13%
DAPK1	Hs00234480_m1	20%	16%	27%	25%
EIF2AK3	Hs00178128_m1	7%	12%	8%	6%
FLJ12229	Hs00388243_m1	13%	66%	18%	19%
ICK	Hs00248170_m1	52%	9%	42%	9%
LAK	Hs00228473_m1	3%	2%	4%	1%
LAK	Hs00325918_m1	8%	4%	5%	3%
LATS2	Hs00324396_m1	11%	17%	64%	41%
MAP3K8	Hs00178297_m1	66%	1%	16%	10%
MAPKAPK3	Hs00177957_m1	7%	7%	6%	4%
MAST205	Hs00248380_m1	64%	17%	44%	21%
MELK	Hs00207681_m1	32%	26%	9%	9%
NRBP	Hs00183833_m1	1%	15%	4%	3%
OSR	Hs00178247_m1	6%	8%	22%	4%
PASK	Hs00209470_m1	12%	7%	10%	8%
PDK3	Hs00178440_m1	6%	5%	8%	7%
PRKCD	Hs00178914_m1	25%	11%	6%	16%
PRKCH	Hs00178933_m1	10%	20%	7%	3%
PRKDC	Hs00179161_m1	6%	8%	7%	6%
PRKG2	Hs00178963_m1	4%	17%	11%	3%
PTK2	Hs00178587_m1	1%	2%	2%	1%
RAGE	Hs00179504_m1	10%	18%	8%	17%
RPS6KA2	Hs00179731_m1	18%	21%	6%	25%
RPS6KA3	Hs00177936_m1	7%	7%	4%	5%
SCYL1	Hs00221117_m1	7%	20%	14%	45%
SCYL1	Hs00418602_m1	6%	22%	16%	32%
SGK	Hs00178612_m1	46%	25%	17%	14%
STK10	Hs00178756_m1	10%	13%	9%	12%
STK4	Hs00178979_m1	36%	21%	22%	19%
TGFBR1	Hs00610319_m1	30%	1%	17%	1%
TLK1	Hs00202239_m1	10%	11%	12%	9%
TLK1	Hs00367007_m1	11%	19%	15%	10%
TOPK	Hs00218544_m1	16%	12%	14%	13%
TYRO3	Hs00170723_m1	6%	8%	4%	6%
VRK2	Hs00272190_m1	24%	27%	10%	6%
ZAK	Hs00213441_m1	10%	21%	5%	8%
ZAK	Hs00370447_m1	9%	29%	26%	10%

B. AT LEAST ONE siRNA REACHES 50%–80% KNOCKDOWN

		% Relative expression			
Gene name	**Assay ID**	**siRNA-1**	**siRNA-2**	**siRNA-3**	**siRNA-pool**
AKT1	Hs00178289_m1	65%	42%	69%	43%
ARK5	Hs00208057_m1	59%	24%	28%	22%
EEF2K	Hs00179434_m1	26%	36%	26%	28%
EGFR	Hs00193306_m1	44%	44%	64%	27%
GS3955	Hs00222224_m1	38%	100%	59%	70%
MAP3K12	Hs00178982_m1	30%	100%	86%	94%
MARKL1	Hs00230039_m1	100%	100%	28%	100%
SRC	Hs00178494_m1	65%	23%	70%	24%
STK3	Hs00169491_m1	29%	53%	54%	30%
TRIO	Hs00179276_m1	55%	41%	27%	35%
YES1	Hs00736972_m1	28%	43%	64%	22%

C. AT LEAST ONE siRNA REACHES <50% KNOCKDOWN

		% Relative expression			
Gene name	**Assay ID**	**siRNA-1**	**siRNA-2**	**siRNA-3**	**siRNA-pool**
C8FW	Hs00921832_m1	69%	100%	100%	100%
HIPK3	Hs00178628_m1	100%	100%	80%	90%
SNARK	Hs00388292_m1	61%	100%	100%	100%
MAP2K2	Hs00360961_m1	100%	100%	100%	100%
CAMK1G	Hs00252714_m1	No expression with all siRNA			

Assay breakdown		**Knockdown**	**Gene breakdown**		**Knockdown**
N		**group**	**%**	**N**	**group**
41		>80%	71	37	>80%
11		50–80%	21	11	50–80%
5		<50%	8	4	<50%

52 total genes
57 total assays tested

Table 1. Remaining percent of expression for 52 kinase genes after siRNA treatment.

The analysis of knockdown results showed that the transcripts studied could be sub-divided into three major groups (Table 1). Panel A shows 37 transcripts that were successfully knocked down (with efficiency greater than 80 percent) in HeLa cells by at least one transcript-specific siRNA or by the mixture of three. These kinase transcripts are listed in Table 1; Panel A. Figure 2 demonstrates levels of transcript knockdown for 15 kinase genes where percentage of remaining transcript was less than 20% after treatment with all siRNAs tested. Panel B shows the transcripts where efficiency of knockdown was in the range of 50–80%. Panel C summarizes transcripts where the efficiency of knockdown was less than 50%. This data demonstrates that for more than 71% of all targeted transcripts, there was at least one (or more) siRNA designed that efficiently (>80%) decreased the transcript level. Another 21% of transcripts demonstrated more than 50% knockdown by at least one (or more) specific siRNA.

Overall, siRNA treatment followed by real-time PCR detection produced accurate results. Standard deviation between replicated experiments was no more than +/- 0.15 C_T, or no more than +/- 10% knockdown. Four of the transcripts (Table 1, and LAK and TLK presented in Figure 2) were measured by using two different TaqMan assays. Different assays (located on different exon-exon junctions) applied to measure the same transcript degradation produced the same results with median variation of three percent and maximum variation of ten percent.

An important question was how the distance between siRNA-targeted location on a transcript and TaqMan Assay location impacted the accuracy of gene silencing detection. Because siRNA designs and corresponding real-time PCR assays were developed independently, one can assume that their co-localization is random. In order

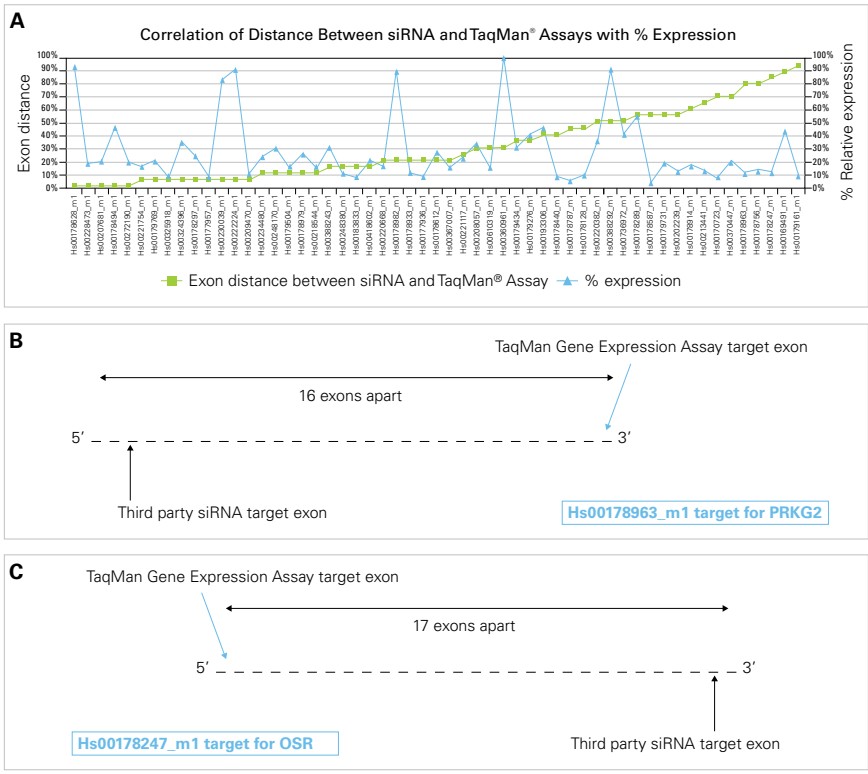

Figure 3. Correlation of percent knockdown and distance between siRNA localization and corresponding real-time PCR assays on their specific transcripts. **Panel A.** Comparison: exon distance between siRNA and TaqMan® Assay vs. remaining expression measured. **Panel B.** Example 1: PRKG2 transcript and corresponding TaqMan Assay Hs00178963_m1. **Panel C.** Example 2: OSR transcript and corresponding TaqMan Assay Hs00178247_m1.

to investigate the impact of the distance between the siRNA and the real-time PCR assay within the same transcripts on the measurements of gene silencing, we decided to look at possible correlation between these two factors.

Figure 3 clearly shows that such correlation was not observed. The graph demonstrates a large number of cases where the real-time PCR assay was very distant from the siRNA-targeted region, and at the same time, the efficiency of transcript knockdown was very high. For example, the TaqMan Assay used to measure the mRNA level of PRKG2 targets the transcript in a location 3′ of the siRNA used to knock down the gene (16 exons apart). Conversely, the TaqMan Assay used to measure the mRNA for the OSR gene targets the transcript in a location 5′ of the siRNA (17 exons apart). In both cases, the knockdown efficiency measured by the TaqMan Assays was more than 80 percent.

Conclusion

Real-time PCR measurement of mRNA levels using TaqMan Gene Expression Assays is a fast and simple method for confirming siRNA-mediated gene knockdown. Our data demonstrates that TaqMan Gene Expression Assays offer an easily accessible solution to the problem of finding high quality TaqMan® reagents for measuring gene knockdown. These assays are highly sensitive in the detection of changes in expression profiles due to siRNA-based gene silencing of multi-exon genes. The location of the quantification assay relative to the siRNA cleavage site does not influence the measurement of knockdown.

References

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Appendix A

Relationship of % Gene Knockdown and C_T Measurement

Quantitative real-time PCR uses C_T (cycle threshold) values to quantify the amount of starting template (for more detail on C_T measurement, see Applied Biosystems Chemistry Guide P/N 4348358, Rev. E).

To compare the C_T values among samples, you must first calculate the delta C_T (ΔC_T) value for each sample. ΔC_T is the difference in the C_T value of the targeted mRNA vs. the C_T of the endogenous control mRNA (any gene whose mRNA values do not change under the experimental parameters can be used as an endogenous control). Calculating the ΔC_T normalizes the data, removing variances arising from sample preparation or instrument runs.

To measure the fold change in two samples, you must first calculate the delta delta C_T ($\Delta\Delta C_T$). For RNAi knockdown, you would compare the ΔC_T of the negative control siRNA-treated sample vs. the ΔC_T of the targeted siRNA-treated sample to obtain the $\Delta\Delta C_T$. Fold change is calculated as a function of the $\Delta\Delta C_T$:

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

The following example shows the use of these calculations.

ΔC_T and Calculations Example

A. Your target siRNA-treated sample has a C_T value of 20

B. Your negative control siRNA sample has a C_T value of 18

C. Your endogenous control (using 18S) has a C_T value of 11

	siRNA-treated sample	Negative control	Endogenous control (18S)
C_T	20	18	11
ΔC_T formula	= 20 - 11	= 18 - 11	
ΔC_T	9	7	

$\Delta\Delta C_T$ Calculation:

= ΔC_T sample - ΔC_T negative control

= 9 - 7

= 2

Fold change:

= $2^{-\Delta\Delta C_T}$

= 2^{-2}

= 0.25

% Knockdown:

= 100 * (1-fold change)

= 100 * (1-0.25)

= 75%

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