Real-time Detection and Quantitation of siRNAs in vivo

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ABSTRACT

RNA interference (RNAi) is a mechanism in which the introduction of double-stranded RNA into a diverse range of organisms and cell types causes degradation of the complementary mRNA. Small interfering RNAs (siRNAs) have become a powerful tool for down-regulating gene expression within cells and animals. Applications of RNAi include gene function and pathway analysis, target validation, and therapeutics. For animal studies, a sensitive and specific assay is needed to accurately evaluate the delivery efficiency into various tissues, perform pharmacokinetics, pharmacodynamics studies, measure siRNA stability, and to follow the adsorption and distribution of siRNAs throughout the animal. We have developed TaqMan® based RT-PCR assays to detect and quantitate siRNAs in vivo. After mouse tail vein injections, *Silencer*® Select siRNAs were readily detected in several organs but only minimal signal was detected when a non-related siRNA was injected. Based on these studies, we conclude our assays can be a useful tool for quantitation of siRNAs in animals.

Keywords: siRNA, RNAi, detection, in vivo, real-time PCR

1 INTRODUCTION

RNA interference (RNAi) is a mechanism in which a double-stranded RNA causes degradation of the complementary mRNA so as to silence expression of the target gene. Small interfering RNAs (siRNAs) enter the RNAi mechanism to suppress target gene expression and have become a promising application to investigate gene functions, target validation, and therapeutic development [1, 2]. We have designed 21-nucleotide double-stranded siRNAs that demonstrate superior gene-silencing activities in cell culture. To further evaluate the siRNAs in vivo, a sensitive and specific assay is needed to accurately monitor the delivery efficiency into animals, and to follow their body distribution. We developed TaqMan® based RT-PCR assays and verified their performance in vitro, and also determined the feasibility of using these assays in a mouse model.

2 MATERIALS AND METHODS

2.1 Quantitation of siRNAs

Our assays for quantitation of siRNAs require a two-step reaction: reverse transcription (RT), followed by PCR. The RT primer uses the same stem-loop design as our previously described TaqMan® microRNA Assays (Applied Biosystems) [3]. In a typical 10µl RT reaction, 1ng/µl of total RNA and 50nM of RT primer are used, denatured first at 85°C for 5 min, 60°C for 5 min, then annealed at 4°C. After adding enzyme mix (at final concentrations, 0.25mM each of the dNTPs. 3.33 units/µl of MultiScribe™ reverse transcriptase, 1X RT buffer, 0.25 units/µl of RNase inhibitor), the reaction mixture is incubated at 16°C for 30 min, 42°C for 30 min, 85°C for 5 min, and then 4°C. Real-time PCR is performed using a standard TaqMan PCR protocol on an Applied Biosystems 7900HT Sequence Detection System. The 10µl PCR reaction mixture includes 0.67µl RT product, 1X TaqMan Universal PCR Master Mix, 0.2µM TaqMan probe, 1.5µM forward primer, and 0.7mM reverse primer. The reaction is incubated at 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min. Evaluation of the assay performance uses either the chemically modified *Silencer*® Select siRNAs (Applied Biosystems) or corresponding non-modified siRNAs for comparison of linearity and C_T difference at two concentrations (ddC_T) from 100pM to 0.1pM with one log intervals.

2.2 Animal Injections

We used hydrodynamic (high-pressure) tail vein injection to deliver siRNA [4, 5]. Purified siRNA (1000 or 3000 pmol) diluted in PBS (2.5 ml=10% body weight) was intravenously injected within 5-8 seconds. Four mice were injected per siRNA per time point. Two mice were injected with negative control siRNA, and 2 normal mice served as additional negative controls. At various time points, mice were sacrificed and dissections were performed. Whole organs (liver, lung, kidney and blood) and sliced fragments were weighed so that we could quantify how much siRNA was delivered into each organ. Total RNA was isolated from different organs using *mir*Vana™ PARIS™ Kit (Ambion). siRNA contained in these samples was quantitated by subsequent real-time PCR as described above.

3 RESULTS

3.1 Optimization of Protocol

The protocol for the two-step RT-PCR generally follows the one we used for quantitating mature miRNAs [3] with the addition of a denaturing step for the template/RT primer mix. The denaturing step was significant for the detection of *Silencer*® Select siRNAs, although it was not as significant with the unmodified control siRNAs (Figure 1). These effects are presumably explained by chemical modifications in the *Silencer*® Select siRNAs introduced to enhance its specificity.

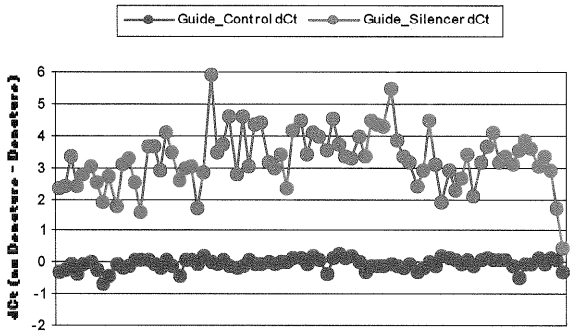

Figure 1: The denaturing step before the RT reaction is required to efficiently detect the guide strand of the *Silencer*® Select but not control (unmodified) siRNAs.

3.2 Evaluation of Specificity and Linearity of Assays to Quantitate siRNAs

The performance of our assays was mainly determined by specificity and linearity. We demonstrated the assay specificity using both no template control and tissue/cell line total RNA with no transfected siRNA, whereas the linearity of each assay was examined using 3 logs of concentration of *Silencer*® Select siRNA and its non-modified counterpart.

We focused on evaluation of only the assays that quantitate the guide strand of the double-strand siRNAs, the strand that is complementary to the target mRNA. Over 100 assays were performed, and all but one had C_T values equal to or greater than 30 when 1ng/µl either tissue or cell line total RNA were used. The one assay that didn't, had a C_T =27, apparently due to non-specific primer interactions. All assays had R-square values of at least 0.99 over 3 logs of input siRNA. ddC_T represents the consistency of dC_T between *Silencer*® Select and control siRNAs. At different concentrations for all assays tested, the ddC_T values were within the range of +1.5 and –1.5 (Table 1).

Linearity				
R-square	>0.99	0.9-0.99	0.8-0.9	
Control	**107**	**0**	**0**	
Silencer® Select	**106**	**0**	**0**	
***Silencer*® Select - Control**				
ddC_T	-1.5 to -1	-1 to 0	0 to 1	1 to 1.5
100-10pM	**1**	**55**	**50**	**0**
10-1pM	**1**	**57**	**47**	**1**
1-0.1pM	**1**	**17**	**87**	**1**
Background				
C_T	<30	30 to 35	>35	
Lung 1ng/µl	**1**	**6**	**100**	
HeLa 1ng/µl	**1**	**5**	**101**	

Table 1: Summary of the performance of the assays. Numbers represent the number of assays that fall into individual specifications.

3.3 In vivo Calibration

In order to quantify how much siRNA was delivered into each organ of the mice upon injection, we weighed liver, lung, kidney and blood and then each fragment taken to calculate the percent of the organ analyzed. Two mice were used in this experiment, and from each organ 3 fragments were taken for replicates. We spiked in 0.1, 0.5, 2 and 10pmol siRNA. After total RNA isolations, RT reactions were set up with 10ng RNA input, which was then followed by real-time PCR using TaqMan® assays. This experiment allowed us to build the "calibration curve" from which the amount (in pmol) of siRNA delivered to any of these organs upon injection could be determined, based on Ct values.

3.4 In vivo Injections

The hydrodynamic (high-pressure) tail vein injection was used to deliver siRNA in vivo [4, 5]. siRNA diluted in a large volume of PBS (2.5 ml=10% body weight) was injected within 5-8 seconds, and due to the created high pressure, siRNA should be specifically delivered to liver cells. Mice were sacrificed at 3 different time points, 5min, 2hr and 6hrs. Results of the experiment for one siRNA are shown in Table 2. We detected a large fraction of the siRNA in the liver right after hydrodynamic injections, as expected. Other organs contained at least 100x less siRNA. As time post-injection increased, less siRNA was detected in the liver, presumably due to RNase degradation of the excessive amounts of RNA (not bound by RISC). When low-pressure control injections were performed, significantly less siRNA was detected in the liver and other organs.

Interestingly, we could clearly see the difference in kinetics of the cellular levels of the guide and passenger strands of siRNA. The guide strand was detected in the liver at high levels immediately after injection, and at low levels, up to 6h post-injection. At the same time, the passenger strand was detected in comparable amounts to the guide strand 5 min post-injection, but is barely detectable in the liver at the 2hr and 6hr time points. This is in agreement with current understanding of the RNAi mechanism. The guide strand of the siRNA is preferentially taken up by the RISC complex and stays bound to it for the target mRNA search and degradation, while the passenger strand is immediately cleaved and ejected.

Organ	**siRNA Guide strand (antisense)**			**siRNA Passenger strand (sense)**		
	5 min	**2 h**	**6 h**	**5 min**	**2 h**	**6 h**
liver	430+/-80	60+/-20	40+/-10	210+/-60	<1	<1
blood	15+/-5	<1	<1	<1	<1	<1
lung	<1	<1	<1	<1	<1	<1
kidney	<1	<1	<1	<1	<1	<1

Table 2: Quantification of the Antisense and Sense Strand of siRNA in Different Organs and Tissues (in pmols). Average of 4 mice per time point shown.

4 CONCLUSIONS

Based on our studies, we conclude the newly developed TaqMan-based assays are useful for siRNA detection and quantitation in animals. After hydrodynamic tail vein injections siRNAs were found to localize mostly in the mouse liver. Five minutes post-injection, both siRNA strands were detected in comparable amounts indicating the duplex is intact. At later time points, the passenger strand levels decreased compared to the guide strand, presumably due to preferential loading of the latter in the RISC complex where it is protected from nucleases.

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