

siRNA Silencing of Lamin A and Quantification of the Knockdown Effect via qPCR

Effi Rees^{1*}, Bernhard Korn¹, Cornelia Falter², and Erhard Fernholz²

¹RZPD, Deutsches Ressourcenzentrum für Genomforschung GmbH, Heidelberg, Germany

²Roche Diagnostics GmbH, Penzberg, Germany

*Corresponding author: rees@hd.rzpd.de



Effi Rees

Introduction

Lamins are structural proteins which form a thin fibrous layer called the nuclear lamina. Currently, three types of lamins are known in mammalian cells: lamin A, B, and C. Lamins may be involved in DNA replication, chromatin organization, differentiation, nuclear structural support, nuclear envelope reassembly, and several other processes. Mutations of lamin A can cause muscular dystrophy [1], dilated cardiomyopathy [2], familiar partial lipodystrophy [3], and other disorders. For the detailed analysis of the different functions of this more than 70 kDa large protein, the expression should be blocked.

Post-transcriptional knockdown via siRNA has been proven to be the method of choice for studying molecular functions. This is because it is much faster and less laborious than knockouts and more reliable than antisense technologies [4].

Here we report about knockdown experiments with lamin A which can be utilized in the future to run functional studies. The effect was quantified on three different qPCR platforms.

Material and Methods

Generation of esiWay Silencing Resources[®]

We made use of the Human mRNA and EST sequences clustered by the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov) unigene project, and created consensus sequences for each cluster. These consensus sequences in turn were used to identify cross-homology between human genes. We masked repetitive and conserved sequences to an extent that was based on blast homologies limited by an expectation value of 0.001.

All remaining sequence fragments of each transcript were subjected to the rules of Elbashir *et al.* and Reynolds *et al.* to analyze the content of good siRNAs within the siRNA pool [5, 6]. The optimal regions were chosen for

PCR amplification with gene-specific primers. These PCR products of 100 bp–500 bp were tailed by strong T7 promoters at either end (= esiWay Silencing Resource[®]) and were subjected to quality control by either gel analysis or capillary electrophoresis to validate the homogeneity and the correct size of each product.

Synthesis of esiRNAs

500 ng of esiWay Silencing Resource[®] RZPDp3000C044 for lamin A were used for *in vitro* transcription reaction, employing the terminal T7 promoter sequences of the esiWay Silencing Resource[®] by using the Microarray RNA Target Synthesis Kit (T7). The RNA was purified by isopropanol precipitation, and then annealed after an initial denaturation step of 95°C, 5 minutes and a cooling step, 1 hour to reach room temperature, producing dsRNA.

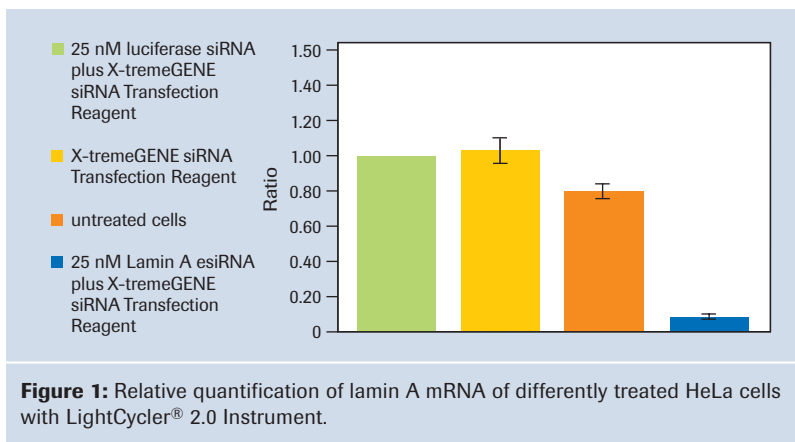
30 µg dsRNA were digested at 37°C for 16 hours, using the X-tremeGENE siRNA Dicer Kit followed by purification according to the supplier's protocol. The purified products are termed esiRNA (endonuclease digested siRNA).

Transfection of esiRNA

To achieve a required level of gene knockdown, the esiRNA has to be transfected into the target cells with high efficiency and low cytotoxicity. We used HeLa cells (DSMZ, Braunschweig, Germany, Cat.No. ACC 57). The cells were passaged 24 hours before transfection using 24-well plates. 7.4×10^4 cells per well were seeded in DMEM containing 10% fetal calf serum, 1x PEN/STREP, and 2 mM L-glutamine. The cells were transfected using 5 µl X-tremeGENE siRNA Transfection Reagent, 20 µl OPTIMEM and 25 µl OPTIMEM/esRNA mixture, according to the supplier's protocol.

RNA isolation

48 hours post-transfection the RNA was isolated using the High Pure RNA Isolation Kit, according to the protocol.



cDNA preparation

One microgram total RNA of each RNA preparation was reversely transcribed using Transcriptor First Strand cDNA Synthesis Kit, according to the instructions.

Quantification of mRNA with the LightCycler® 2.0 Instrument

Quantitative real-time PCR was performed with the LightCycler® 2.0 Instrument using the LightCycler® FastStart DNA Master HybProbe and the Universal ProbeLibrary Set, Human (probe #9). The evaluation was performed using the LightCycler® Software 4. The mRNA level was normalized versus the amount of housekeeping gene transcript HPRT1.

Quantification of mRNA with the 7500 and 7900 real-time PCR system

Alternatively, quantitative real-time PCR was performed with both a 7500 Real-Time PCR System as well as a 7900 Real-Time PCR System (both Applied Biosystems) using the FastStart Taqman® Probe Master (ROX) and again probe #9 of Universal ProbeLibrary Set, Human. For the PCR reactions in the 7900 instrument, reference dye concentration was increased to 400 nM. The mRNA level was normalized versus the amount of housekeeping gene transcript HPRT1.

Results and Discussion

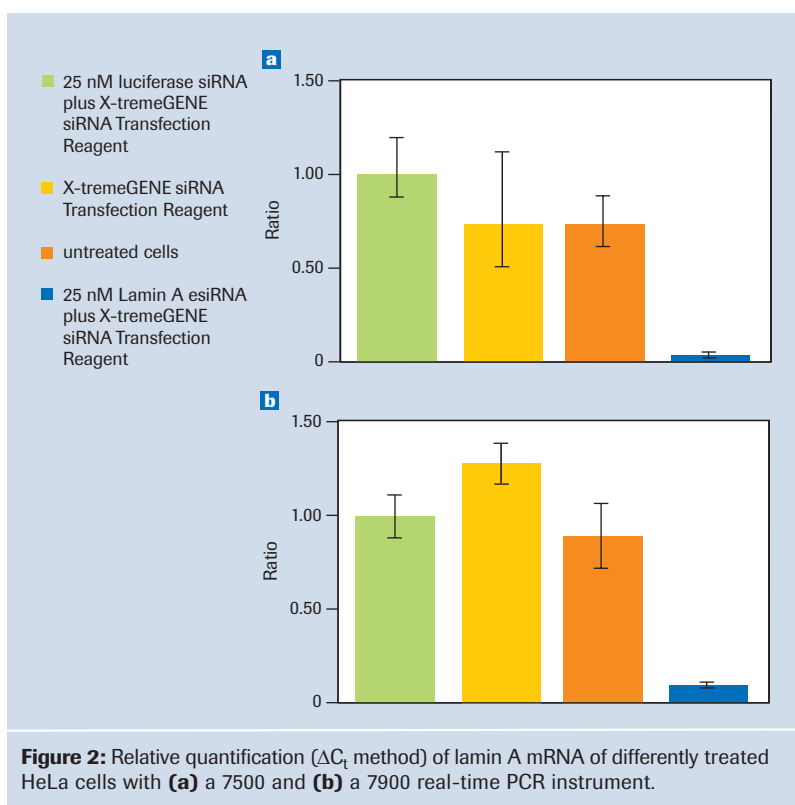
Since lamin A participates in important interactions within each cell, we designed several control experiments in order to differentiate between influences of the transfection reagent and the transfection procedure on knockdown experiments. First, siRNA against luciferase was used (Dharmacon) together with the same transfection reagent to prove the specificity. And second, the transfection reagent was used alone to differentiate from toxic side effects of the transfection reagent.

All transfection reactions were run in parallel. Visually, we did not detect significant toxicities in any experiments. Therefore, we isolated the total RNA of the treated and untreated HeLa cells and prepared the corresponding cDNA using the Transcriptor First Strand cDNA Synthesis Kit.

Quantification was carried out with three different real-time instruments using the specific LNA probe #9 of the new Universal ProbeLibrary Set. For relative quantification, the HPRT1 (housekeeping gene) specific probe #22 was used. With the LightCycler® 2.0 Instrument, a knockdown efficiency of 88% for lamin A using the esiWay Silencing Resource® RZPDp3000C044 was observed (Figure 1).

In the alternative assay, the lamin A knockdown was analyzed using 7500 and 7900 real-time PCR instruments using the new FastStart Taqman® Probe Master (ROX). The results turned out to be nearly identical to the results of the LightCycler® 2.0 Instrument, showing an effectiveness of the knock-down of about 90% (Figure 2).

Interestingly, quantification of the housekeeping standard HPRT1 mRNA isolated from the differently treated HeLa cells showed a similar C_t -value (Figure 3). This demonstrates that no toxic effects are detectable from either the transfection reagent or the lamin A knock-down.



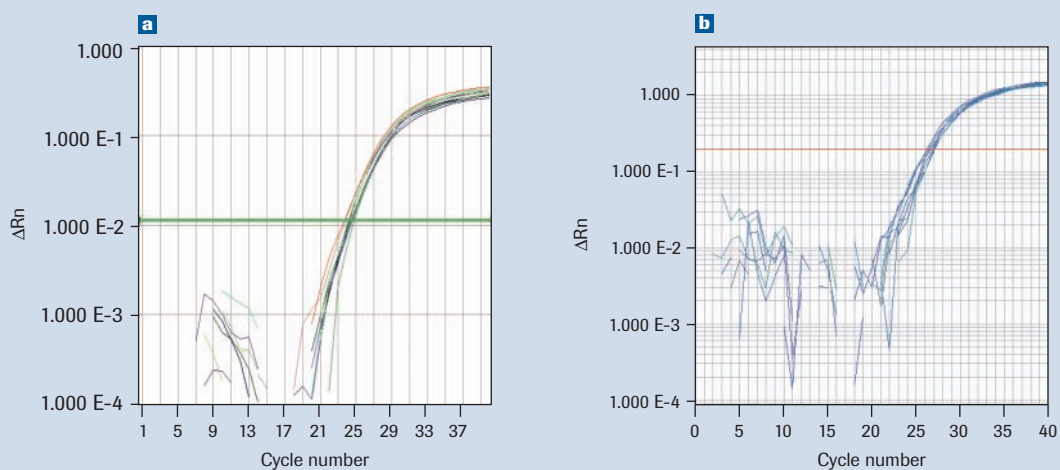


Figure 3: Amplification blot of HPRT cDNA of differently treated HeLa cells using FastStart TaqMan® Probe Master with **(a)** a 7500 and **(b)** a 7900 (b) real-time PCR System.

Conclusions

Quantification with three different real-time PCR instruments demonstrated the effectiveness of esiWay Silencing Resources®, the universal Probe Library Set and the new FastStart TaqMan® Probe Master (ROX). Usage of the alternative systems resulted in nearly identical measurements of lamin A silencing.

References

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Acknowledgements

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Product	Pack Size	Cat. No.
FastStart TaqMan® Probe Master (ROX)	2.5 ml (2 x 1.25 ml)	04 673 450 001
	12.5 ml (10 x 1.25 ml)	04 673 468 001
	50 ml (10 x 5 ml)	04 673 476 001
High Pure RNA Isolation Kit	1 kit (50 isolations)	11 828 665 001
LightCycler® FastStart DNA Master HybProbe	1 kit (96 reactions)	03 003 248 001
	1 kit (480 reactions)	12 239 272 001
Microarray RNA Target Synthesis Kit	1 kit (25 reactions)	03 266 887 001
ROX Reference Dye (1 mM)	50 µl	04 673 549 001
Transcriptor First Strand cDNA Synthesis Kit	1 kit (50 reactions)	04 379 012 001
Universal Probe Library Set, Human	1 set (90 probes)	04 683 633 001
X-tremeGENE siRNA Dicer Kit	1 kit (10 reactions)	04 579 020 001
X-tremeGENE siRNA Transfection Reagent	1 ml	04 476 093 001
	5 ml (5 x 1 ml)	04 476 115 001