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Brief note

RNA interference: PCR strategies for the quantification of stable degradation-fragments derived from siRNA-targeted mRNAs

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Abstract

mRNA targeted by siRNA is endogeneously cleaved into a 5′- and a 3′-fragment and finally degraded in cells. Little is known about the relative stability and degradation kinetics of these 5′- and 3′-fragments after the siRNA mediated first cut. We present a qRT-PCR protocol which allows the determination of the optimal time point for mRNA analyses, helping to avoid the generation of false positive effects in downstream experiments, such as microarray analysis, which may be caused by undegraded fragments of a siRNA-targeted mRNA.

Keywords: RNAi; siRNA; mRNA; Degradation; PCR; Transfection

The discovery of sequence-specific gene silencing, triggered by double stranded RNA molecules (dsRNA) has attracted enormous attention in the last few years (for review see [1]), The excitement about this phenomenon is due to the preferential specificity of the interfering dsRNA for the degradation of mRNAs containing homologous sequences. The actual mediators of RNAi are short dsRNA molecules, typically 21–25 nucleotides in length. Naturally, these short interfering RNAs (siRNAs) are a result of endonucleolytic processing of a larger precursor RNA by the RNaselll-like enzyme Dicer [2–4]. Experimentally, RNAi can be triggered in mammalian cells after transfection of synthetic siRNA using suitable transfection reagents. siRNA transfection serves therefore as a very powerful experimental tool to study the biological functions of genes in mammalian cells by post-transcriptional gene silencing [5]. siRNAs transfected into mammalian cells bind and activate a resident multi-protein complex called RISC (RNA-induced silencing complex) which guides the siRNA to endogenous mRNAs containing homologous sequences, mediating the annealing and subsequent endonucleolytic cleavage of the mRNA within the region spanned by the siRNA [6]. The two resulting mRNA fragments are then subject to further degradation, but although the mRNAs targeted by functional siRNAs will be ultimately destroyed, little is known about the kinetics and mechanism of these secondary degradation steps. Especially when the siRNA is directed against the coding region, it is conceivable that parts of the targeted mRNA remain temporarily stable in the cell, even after the primary siRNA-mediated cut has already occurred and the mRNA itself is no longer functional. Revealed by Northern Blot analysis, there is indeed evidence that intermediates of siRNA-targeted mRNAs accumulate to some extent [7]. It is therefore crucial to understand and study these RNA degradation processes, not least to avoid the generation of false positive results caused by only partially degraded mRNA fragments in downstream microarray or qRT-PCR. In addition, the abundance of individual mRNA species as well as the variable amount of siRNA-saturable RISC complexes in individual cell types may influence the efficiency of mRNA degradation and have to be considered for the design of an optimal silencing experiment. To support the investigation of individual mRNA degradation kinetics during RNAi experiments, we demonstrate the application of a quantitative RT-PCR approach, which can be used to determine the expression level of a particular mRNA and the stability of its cleavage products. In order to exemplify an appropriate qRT-PCR

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Table 1
siRNA and primer sequences

Name of gene	siRNA sequence	PCR primer 3′ of siRNA binding site	PCR primer flanking siRNA binding site	PCR primer 3′ of siRNA binding site
β-actin	AAUGAAGAUCAAGAUCAUUGC [9]	β-actin 75 fwd GCCGCCAGCTCACCA-TGGATGAT	β-actin 877 fwd CCTTCCTTCCTGGG-CATGGA	β-actin 1541 fwd GGGCATGGAGCCCAGTC
		β-actin 179 rev CCCACATAGGAATCC-TTCTGACC	β-actin 1042 rev CACACGGAGTACTTG-CGCTCA	β-actin 1679 rev GGGCACGAAGGCTCATCATTC
lamin A/C	AACTGGACTTCCAGAAGAACA [8]	Lam 573 fwd CAAGAAGGAGGGTGA-CCTGA	Lam 747 fwd CAAGAAGCAACTTC-AGGATGAGATG	Lam 2171 fwd GAACCCAGAGCCCCCA
		Lam 751 rev GCATCTCATCCTGA-AGTTGCTT	Lam 840 rev TCTCACGCAGCTCCT-CACTGTA	Lam 2262 rev AGGTGAGGAGGACGCAGGAA
nucleoporin 153	AAGGCAGACTCTACCAAATGT [9]	NUP 1042 fwd CACCTTATCAGGCAC-CAGTTAG	NUP 2708 fwd CTCTGCCTTCTGGAG-GCTCTCTA	NUP 4536 fwd TACAGTGGGGTCAAA
		NUP 1181 rev TCTTTTTGCATCCGC-TAAAGGG	NUP 2880 rev GATGAGGAAGATGTGT-CAAAGCCT	NUP 4687 rev CCCCAGCACAAAGTACAATCCA

Overview of the siRNA and PCR primer sequences used. siRNA sequence information was from the publications indicated. PCR primer pair sequences flanking the siRNA binding sites for the amplification of the 5′ or 3′ regions of each mRNA are shown.

approach addressing the question of mRNA stability during RNAi, we have examined the timeframe and kinetics of the degradation of *lamin A/C*, *β-actin*, and *nucleoporin 153* mRNAs in mammalian cells following transfections of synthetic siRNAs. These three mRNAs are not only of distinct lengths, but are also significantly different in their abundance (see below). Using the approach described here, the stability of the individual 5′- or 3′-cleavage products, derived from a siRNA-targeted mRNA, can be analysed and compared, depending on the localization of specifically designed PCR primers.

The siRNAs used in this study, were manufactured based on previously published sequence information. Briefly, the lyophylized siRNAs duplexes (HPP grade, QIAGEN) targeting *lamin A/C* [8], *β-actin*, and *nucleoporin 153* mRNA [9], were dissolved in siRNA suspension buffer (l00 mM potassium acetate, 30 mM HEPES-KOH, 2 mM magnesium acetate, pH 7.4) to a final concentration of 20 μM and stored at −20 °C until use (see Table 1).

Human HeLa S3 cells (ATCC number CCL-2.2) were cultivated at 37 °C and 5% CO_2 in Dulbecco's modified Eagle

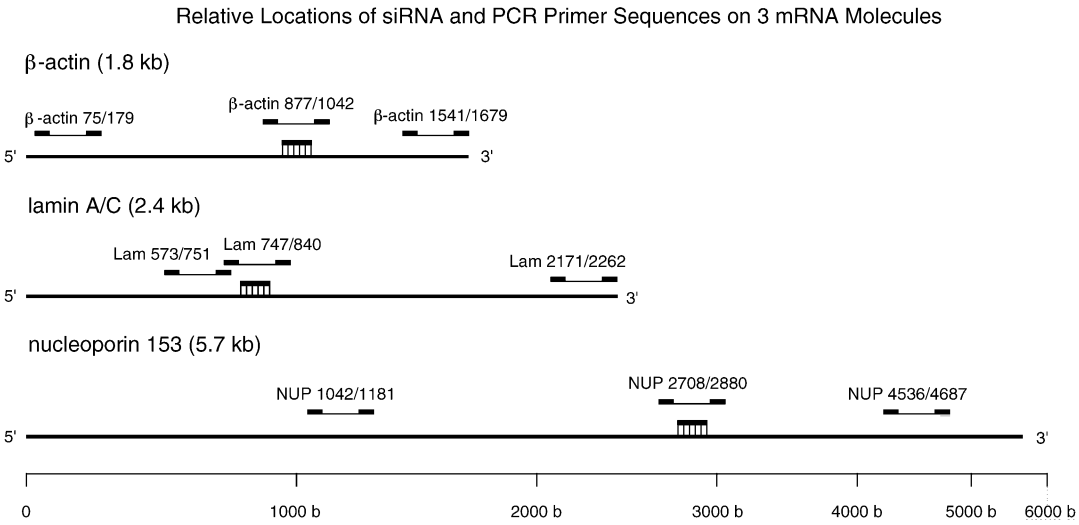

Fig. 1. The relative locations of siRNAs (striped bars) and PCR primers with their amplicons (lines) used to target the three mRNAs investigated (*β-actin*, *lamin A/C*, and *nucleoporin 153*) are indicated. PCR amplicons are located either 5′ or 3′ of the siRNA binding site or overlapping the siRNA binding site. The co-ordinates of the first base of each PCR primer is indicated.

medium (DMEM, Invitrogen) supplemented with 10% fetal bovine serum (Biochrom), 1% non-essential amino acids, and 1% penicillin–streptomycin solution (Sigma). Cells were regularly subcultured, but a passage number of 40 was not exceeded, as at higher passage numbers transfection efficiencies decreased. Twenty four hours before transfection, 6×10^4 cells per well of a 24-well plate were seeded in 500 μl culture medium. On the day of transfection, 1 μg siRNA from the stock solution was diluted in a final volume of 100 μl complete culture medium, 6 μl RNAiFect transfection reagent (QIAGEN) was added and the mixture was incubated for 15 min for complex formation. The medium of the seeded cells was replaced with 300 μl fresh complete culture medium per well and the complexed siRNA was added drop wise onto the culture. The cells were incubated at 37 °C, 5% CO_2 until analysis. All transfections were performed in triplicate and similar results were obtained in independent experiments.

At the indicated time points, cells were washed with PBS, lysed, and total RNA was isolated using the RNeasy Mini Kit (QIAGEN) according to the protocol. Total RNA (250 ng) was reverse transcribed using the Omniscript RT Kit (QIAGEN) with either oligo dT or random hexamer primers, in order to ensure complete cDNA synthesis from all mRNA template regions, especially of those located 5′ of the respec-

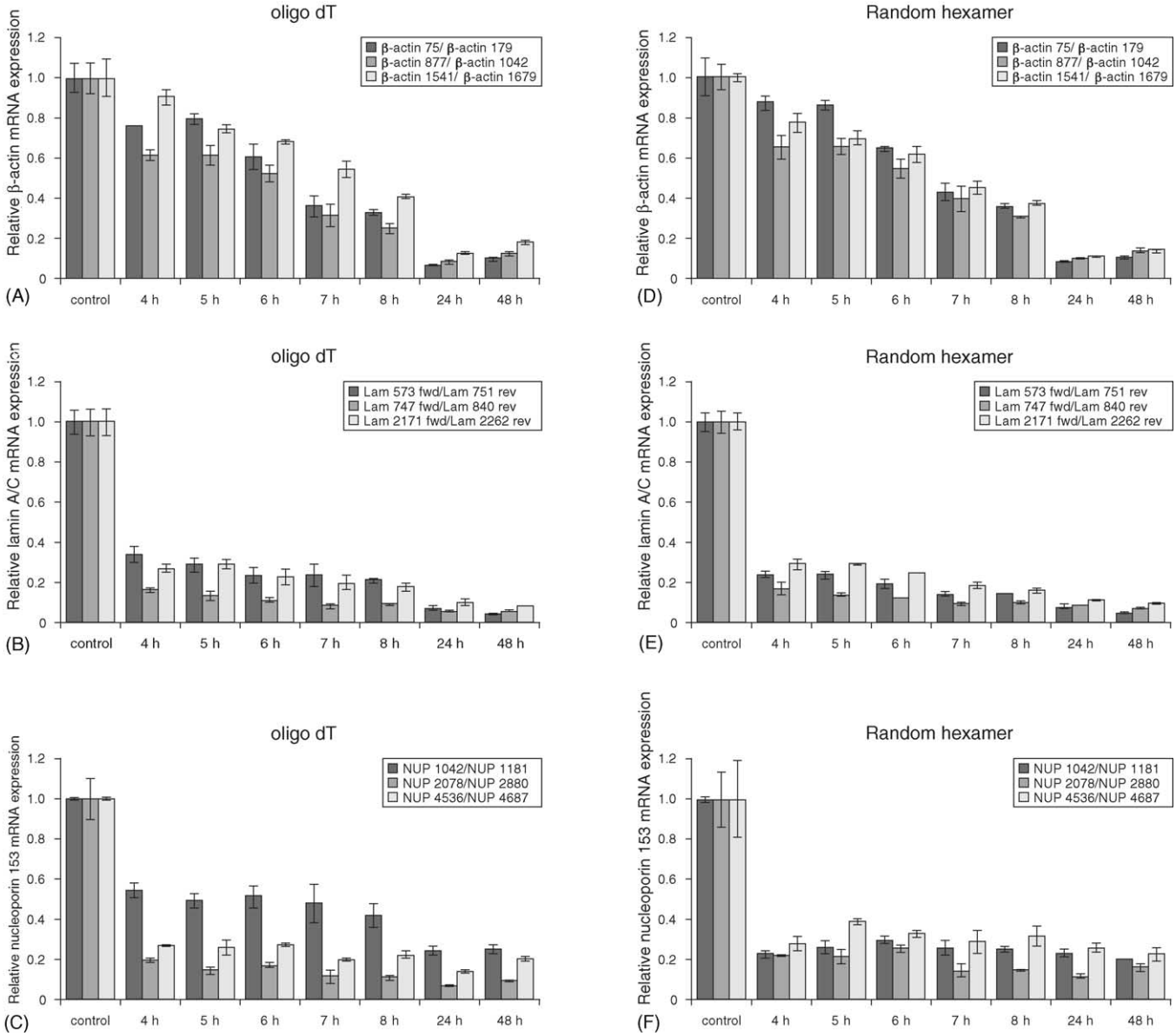

Fig. 2. HeLa S3 cells were treated with specific, functional siRNAs for the indicated times before harvesting for qRT-PCR analysis. Cells were transfected with siRNAs directed against (A, D) *β-actin*; (B, E) *lamin A/C*; or (C, F) *nucleoporin 153*. Control cells were harvested at the time point of transfection. Total cellular RNA was purified and reverse transcribed, and the cDNA was used for qRT-PCR analysis in triplicates. Values derived from qRT-PCR results from cells transfected with control siRNA are set as 1 and the relative expression of cells transfected with the experimental siRNAs and detected using each primer pair are indicated.

tive siRNA binding sites. We have used three primer pairs for each cDNA, derived from siRNA-targeted mRNA: the first pairs of primers flank the putative siRNA binding site, the second and third primer pairs are located 5′ or 3′ of the siRNA target site, respectively. Fig. 1 indicates the different combinations and relative locations of primers used for reverse transcription and qRT-PCR.

The cDNA specific PCR primers used for amplification were designed according to the following guidelines: predicted amplicon sizes were between 90 and 150 bp and the average Tm of the individual primers was between 62 and 64 °C. The primers were also designed to avoid primer dimers, using the QIAGEN oligo analysis and plotting tool. Generally, primers were chosen to target either exon–exon boundaries or being located on two adjacent exons in order to eliminate the risk of genomic DNA amplification. In the case of β-actin, where the 3′ primer combination (β-actin 1541fwd, β-actin 1679rev) was located on the large exon 6, appropriate controls such as non-reverse transcribed samples were included in all experiments, in order to rule out the amplification of genomic DNA. Additional analysis of the melting curves and agarose gel electrophoresis indicated the presence of only one specific cDNA-derived amplicon after all PCR reactions. For each of the three genes analysed here, the PCR systems had comparable sensitivity as seen by the slopes of the individual dilution curves (data not shown).

qRT-PCR was performed in triplicates using the QuantiTect SYBR Green PCR Kit (QIAGEN) with gene specific PCR primers on an ABI GeneAmp 5700 real time PCR cycler using the ABI GeneAmp 5700 SDS software with the following PCR conditions: 15 min 95 °C; 40 cycles 1 min 95 °C, 1 min 60 °C, 1 min 72 °C. CT values of the individual PCR samples (*lamin A/C*, *β-actin*, and *nucleoporin 153*) and GAPDH (internal control) were measured. In parallel, the PCR values for a standard curve, based on a dilution series derived from the reverse transcription reaction of untransfected cells (1:20; 1:200; 1:2000; 1:20000), were analysed. This was carried out for the RT-PCR values from the sequences of interest, and the internal GAPDH control. A linear regression was performed for the standard curve and for all samples, the RNA amount relative to the standard curve was calculated. The *lamin A/C*, *β-actin*, or *nucleoporin 153* expression level data were normalized to the expression level of GAPDH and the normalized values of untransfected cells were set as 1. For each of the three PCR set-ups, extracts of cells transfected with the alternative two siRNAs were used as negative controls.

Cells were analysed during a 48 h time course at the time points indicated in Fig. 2. In general, the decrease in PCR template during the time course follows a similar pattern, independent of the primer pair used. (Fig. 2A–F). The pattern differs somewhat for the *nucleoporin* mRNA (Fig. 2C). In this case the mRNA degradation pattern differs for oligo dT primed cDNA used with PCR primers located 5′ of the siRNA binding site. This may reflect the fact that a stretch of seven adenosine residues 5′ of the siRNA binding site allow additional priming from that site, thereby enhancing the amount of cDNA template for the NUP1042/NUP1181 PCR primers.

Interestingly, although it was the shortest of the mRNA molecules investigated, *β-actin* degradation after siRNA transfection follows a slower pattern compared with the kinetics of *lamin A/C* or *nucleoporin* mRNA degradation (Fig. 2 A, D). Whereas an already strong reduction of *lamin A/C* or *nucleoporin* mRNA was detected within 8 h after transfection, maximal degradation of all *β-actin* mRNA domains was only seen after 24 h. According to the comparison of relative CT values derived from untreated cellular samples, *β-actin* ap-

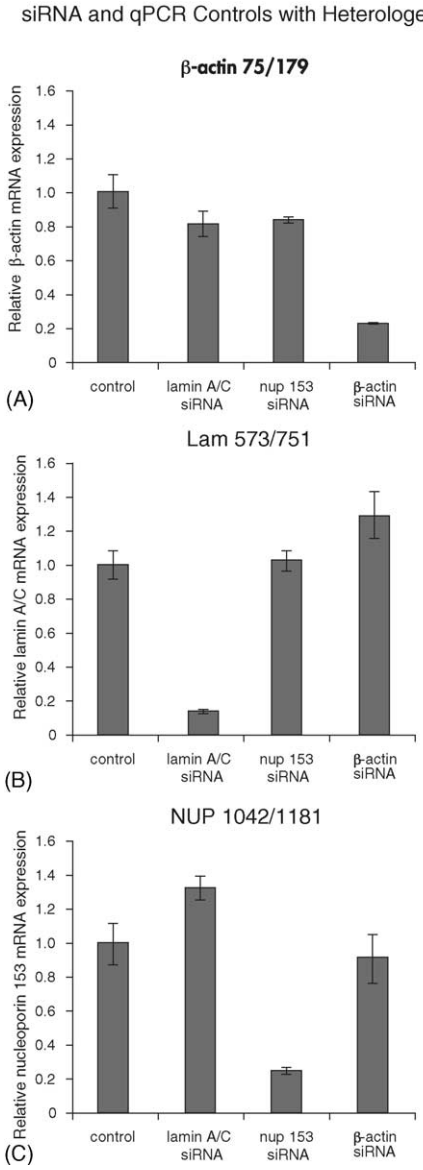

Fig. 3. Control experiments showed the specificity of siRNA-mediated knockdown. Cells were transfected with siRNAs targeting *β-actin*, *lamin A/C*, or *nucleoporin 153* (nup 153) as indicated. After 48 h, mRNA was analysed in qRT-PCR experiments for the relative expression of (A) *β-actin*; (B) *lamin A/C*; or (C) *nucleoporin 153* using the primer pairs indicated above each graph. Arbitrary expression values shown are relative to PCRs of cDNA from control cells harvested at the time point of transfection.

peared to be clearly the most abundant mRNA investigated in this study (relative CT of approximately 16). In comparison, the average relative CT value of *lamin A/C* was 20.3 and the CT of *nucleoporin* was 23.8. This fact may explain the different rates of mRNA degradation, reflecting the time to destroy an accumulated stable mRNA. Once the accumulated mRNA is destroyed, the rate of RISC mediated cleavage may be able to keep up with the rate of newly synthesized mRNA. Between 24 and 48 h after transfection, optimal silencing of the mRNAs could be detected in all cases. This was observed when both oligo dT and random hexamer primer reverse-transcribed RNA was used with all PCR primer combinations.

The control experiments shown in Fig. 3 indicate the specific knock-down effects of the individual siRNAs under investigation. HeLa S3 cells were transfected with siRNAs directed against *β-actin*, *lamin A/C* or *nucleoporin 153* mRNA. After 48 h, cells were harvested, cDNA was prepared, and qRT-PCR was performed using the indicated primers specific for each gene (Fig. 3 A–C). Values obtained with control, untransfected cells were set as one and the relative abundance of the individual PCR fragments is indicated. Prominent silencing of each gene was only detected where the gene-specific siRNA was used.

In conclusion, our results show significant degradation of all investigated target mRNAs, 24–48 h after siRNA transfection, largely independent of the primer combinations in use. This implies, that mRNA extracts from siRNA treated cells harvested 24–48 h post transfection should be usually optimal for downstream analysis of the knock down effect at mRNA level, whereas shorter time points may be critical, especially for very abundant mRNA species. The overall degradation patterns were largely similar, especially when random hexamers were used for reverse transcription. The speed of degradation is not necessarily related to the size of the mRNA molecules (with *β-actin* mRNA being the shortest), but may rather be related to the relative abundance of the individual mRNAs. In summary, this protocol allows the determination of overall mRNA degradation patterns during RNAi driven experiments, with emphasis on the detection of potentially variable stabilities of mRNA fragments cleaved by RISC.

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