Validated Dicer-Substrate siRNAs Mediate More Potent Gene Silencing

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Introduction

RNA interference (RNAi) is an intrinsic cellular mechanism that provides a powerful tool for silencing genes and determining gene function in mammalian cells. Delivery of small interfering RNAs (siRNAs) into a cell activates the RNAi pathway, and catalyzes sequence-specific degradation of a messenger RNA (mRNA), potentially reducing protein production. Successful RNAi experiments involve two necessary components: (1) an effective siRNA design that results in potent gene silencing, and (2) a successful delivery system that results in high transfection efficiency and low cytotoxicity.

siLentMer™ validated Dicer-substrate siRNA duplexes are a new class of double-stranded RNA molecules that are 27 nucleotides in length and have an altered end structure. These new siRNAs are capable of initiating an enhanced and, likely, more specific silencing response when compared to 21-mer siRNAs. Once in the cell, siLentMer siRNA duplexes are bound and cleaved by the RNA endonuclease Dicer before they enter the RNA-induced silencing complex (RISC). Specific siLentMer end modifications promote the binding of Dicer to only one of the two ends of the siLentMer molecule. This in turn leads to greater RISC incorporation of the RNA strand with complementarity to the mRNA of the gene of interest. The association with Dicer produces a highly potent siRNA effector molecule that has been shown to achieve potent gene silencing at low concentrations (≥100 pM) and sustain silencing for longer periods of time than 21-mers. Additionally, the siLentMer siRNAs do not cause certain off-target effects, such as the induction of an interferon response, and they are functionally validated by RT-qPCR, which provides some guarantee (≥85% mRNA reduction) of the siRNA performance in the cell.

siLentMer siRNA effectiveness can be enhanced when coupled with an efficient delivery method. Determining and validating the optimal delivery method for each cell line is critical to the success of RNAi experiments, because, despite continued improvements in siRNA design, experiments sometimes still fail due to poor transfection efficiency. To ensure success of an experiment, the method of siRNA delivery must be determined and validated for each cell line. Two popular methods are lipid-mediated transfection and electroporation. Validation using these methods can be accomplished by delivery of a previously validated siRNA and subsequent measurement of silencing. Alternatively, fluor-coupled siRNA can be used in conjunction with flow cytometric or microscopic measurement of siRNA incorporation.

siRNA Design

The design of the Dicer-substrate siRNA molecule is critical to producing consistent and effective gene silencing results. Reports indicate that blunt-end designs are unpredictable, yielding inconsistent dicing results that produce variable silencing performance. In contrast, the Dicer-substrate siRNAs have a unique design (Figures 1, 2), which includes end modifications and specific nucleotide length, that helps to enhance their silencing potency and achieve reproducible results (Rose et al. 2005).

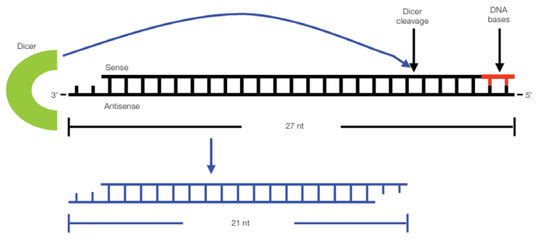


Fig. 1. Design summary of the Dicer-substrate siRNAs. The Dicer riboendonuclease (■) is blocked by the two DNA bases on the 3' end (■) and binds to the opposite 3' overhang end (■). It then cleaves the molecule in a unidirectional manner to produce a 21-nucleotide siRNA molecule (■).

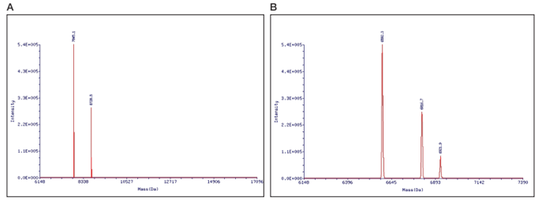


Fig. 2. A single siRNA product is created. siLentMer Dicer-substrate siRNAs induce unidirectional end-binding and cleavage (shown in Figure 1). **A**, ESI-MS peaks representing the molecular weight of each of the strands from the siRNA duplex molecule; **B**, ESI-MS peaks showing peaks that match the predicted molecular weight of a siLentMer siRNA after Dicer cleaves in the orientation shown in Figure 1. The third peak has a molecular weight equal to cleavage of the top strand to 22 nt rather than 21 nt, which is consistent with Dicer's known ability to cleave some targets at 20–22 bases. Additional data are shown in Rose et al. (2005).

Dose-Response Comparisons

Dicer-substrate siRNAs have been shown to exhibit up to 10x more potent silencing than traditional 21-mer siRNA molecules targeting the same sequence (Kim et al. 2005). Comparison of silencing examined in 5 target genes (TP53, RAF1, CDK2, ACTB, and AKT1) showed greater or comparable silencing by siLentMer siRNA duplexes (Figure 3). Additionally, this enhanced potency facilitates the use of lower siRNA concentrations per experiment. Using high siRNA concentrations can contribute to off-target effects, such as increased cell death due to toxicity. These Dicer-substrate siRNAs provide the ability to use low siRNA concentrations, which can help reduce the potential for off-target effects and increase the experimental success rate.

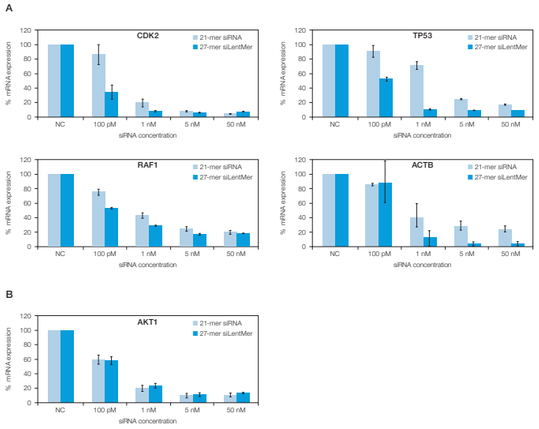


Fig. 3. Dose response to gene silencing. HeLa cells were treated with 21-mer siRNAs (■), 27-mer siLentMers (■), or nonsilencing controls (NC) over a range of siRNA concentrations and assayed for gene expression after 24 hours. **A**, overall, more potent silencing was shown by 27-mer siLentMers for the CDK2, TP53, RAF1, and ACTB gene targets; **B**, comparable silencing was shown by 21-mers and 27-mer siLentMers for the AKT1 gene target at all concentrations.

Silencing Longevity

Typically, transient transfections with 21-mers show sustained silencing only for 3–4 days. In contrast, Dicer-substrates (27-mers) can produce potent silencing results for a longer period of time, as long as 6 or more days for some gene targets (Figure 4). Previous data illustrated extended silencing for up to 9 days (Kim et al. 2005).

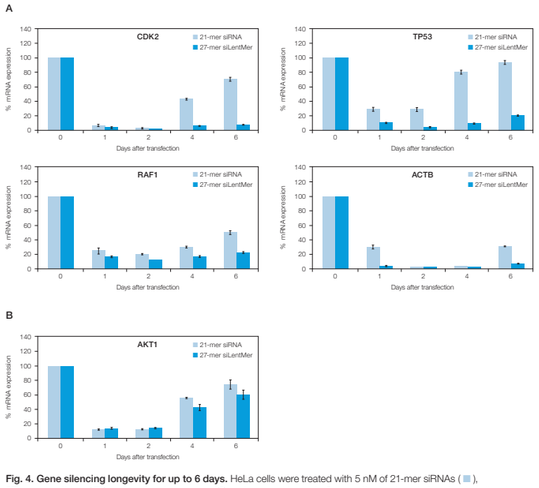
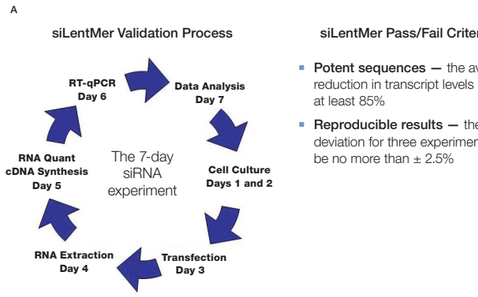


Fig. 4. Gene silencing longevity for up to 6 days. HeLa cells were treated with 5 nM of 21-mer siRNAs (■), 27-mer siLentMers (■), or nonsilencing controls (0) and assayed for up to 6 days after treatment. **A**, extended silencing was shown by 27-mer siLentMers for the CDK2, TP53, RAF1, and ACTB gene targets; **B**, similar longevity of silencing was shown by 21-mer siRNAs and 27-mer siLentMers for the AKT1 gene target.

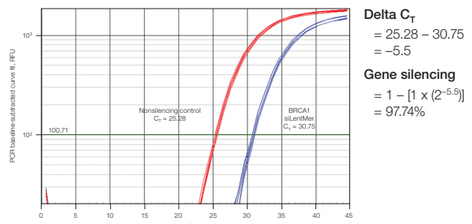
Proven Performance

Testing of numerous siRNAs is typically required to identify highly potent sequences. The siLentMer siRNAs reduce this need since they are functionally tested using 5 nM of siRNA by RT-qPCR to ensure at least an 85% reduction in target mRNA levels (Figure 5).



siLentMer Pass/Fail Criteria

- **Potent sequences —** the average reduction in transcript levels must be at least 85%
- **Reproducible results —** the standard deviation for three experiments must be no more than ± 2.5%



Delta C_T
= 25.28 – 30.75
= –5.5

Gene silencing
= 1 – [1 x ($2^{-5.5}$)]
= 97.74%

Fig. 5. siLentMer siRNA duplexes mediate effective knockdown with low concentrations of siRNA. HeLa cells were transfected with 5 nM anti-BRCA1 siRNA in a 48-well plate using 0.6 µl siLentFect™ lipid reagent for RNAi per well. At 24 hr posttransfection, total RNA was extracted and RT-qPCR was performed using the iScript™ cDNA synthesis kit and the iQ™5 real-time PCR detection system with iQ™ SYBR® Green supermix. Percent RT-qPCR was normalized to total input RNA. The level of BRCA1 transcript (■) was reduced by nearly 98% relative to a nonsilencing control (■).

Assessing Delivery Success

Delivery is one of the biggest challenges for many researchers and there are a variety of delivery methods available. It is important to assess the success of delivery when mapping out the experimental design. Measuring delivery success can be done a number of ways, such as using microscopy and flow cytometry (Figure 6). The method of choice depends on the cell lines being used. Lipid-mediated delivery is potentially the most common approach, primarily because it is effective for a variety of cells and is simple to use. Alternately, electroporation is an excellent method, particularly for cell lines that are difficult to transfect. Biolistics offer great potential for plant RNAi research using siRNAs and in vivo applications, and viral-mediated delivery is another method often used for the more difficult-to-transfect cells.

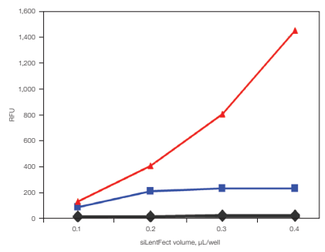


Fig. 6. Optimizing lipid and siRNA concentrations. HeLa cells were transfected with an Oregon Green dual-labeled siRNA and either no siRNA (■) or 5 nM (■) or 20 nM (■) of the fluorescently labeled siLentMer siRNA and increasing amounts of siLentFect lipid reagent for RNAi. Fluorescent measurements were made 24 hr posttransfection by flow cytometry. Results show that as you increase the amount of siRNA you might also need to increase the amount of lipid to ensure sufficient delivery. Conversely, when using low amounts of siRNA, the lipid volume requirement will reach a saturation point, and increasing the lipid amount does not offer any additional benefit.

Conclusions

- The design of an siRNA duplex — the sequence, length, and end structure — plays an important role in determining the silencing potency of the molecule
- The unique Dicer-substrate siRNA design, and its association with Dicer, enhances the potency of the siLentMer siRNA duplexes for many gene targets
- Validated siLentMer siRNA duplexes offer proven performance to help ensure a certain level of silencing and prevent excessive and unnecessary siRNA testing
- The optimal delivery method will depend on the cell line, and it is important to measure transfection efficiency to ensure successful silencing results
- The principal consideration for developing successful RNAi experiments is the combination of an effective siRNA molecule with the appropriate delivery method

References

Kim DH et al., Synthetic dsRNA Dicer substrates enhance RNAi potency and efficacy, Nat Biotechnol, 23, 222–226 (2005)

Rose SD et al., Functional polarity is introduced by Dicer processing of short substrate RNAs, Nucleic Acids Res 33, 4140–4156 (2005)

Acknowledgments

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