Take Aim and Score a Knock Down

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siLentGene™ U6 Cassette RNA Interference System: Rapid Screening of siRNA Targets

By Jolanta Vidugiriene, Ph.D.[1], Thomas Yeager, Ph.D.[1], Lin Wang, Ph.D.[2], Jiwu Wang, Ph.D.[2], Zhaohui Dong, Ph.D.[2], Cynthia Sprecher, B.S.[1], and Doug Storts, Ph.D.[1] [1]Promega Corporation, [2]Allele Biotechnology and Pharmaceuticals, Inc.

Abstract

We have developed an optimized reagent kit for inducing RNA interference (RNAi) effects in mammalian cells with siRNA-expressing DNA cassettes. These cassettes feature an eukaryotic RNA polymerase III U6 promoter for high efficiency and precision. The expression cassettes are designed to be synthesized by PCR and transfected into the desired cell line, making this method suitable for quick screening of multiple target sites.

The siLentGene™ System is fast and inexpensive and provides a convenient approach for rapidly screening and determining the efficacy of different siRNAs.

Introduction

Silencing gene expression using double-stranded RNA (dsRNA), known as RNA Interference or RNAi, provides a powerful tool for analyzing gene function. Selectively downregulating (or "knocking down") the expression of a particular gene allows researchers to determine its function in many cellular processes. Gene knockdown is useful for researchers who want to understand the function of a single gene. Furthermore, knockdown methods benefit the drug development process if they are sufficiently simple and predictable. Gene knockdown experiments are useful for many biological studies including: mapping cellular pathways, selecting suitable targets for pharmaceutical intervention, and developing gene therapies. The siLentGene™ U6 Cassette RNA Interference System[(a,b,c,d,e)] provides a convenient tool for researchers using RNAi to analyze gene function.

RNAi Phenomenon

RNAi refers to the cellular process induced by dsRNA that specifically degrades the mRNA that is homologous to the dsDNA. In some systems, a few copies of the dsRNA can cause total degradation of cognate transcripts in a cell (1). A number of gene products have been identified that are involved in this process, including the type III RNase "Dicer" from *Drosophila* (2). Dicer processes long dsRNAs or hairpin RNAs (3) into double-stranded, small interfering RNAs (siRNA), which are typically 21–23 nucleotides (nt) long. Once present, siRNAs trigger the formation of RNA-induced silencing complexes (RISC). Helicases in the complex unwind the dsRNA, and the resulting single-stranded RNA (ssRNA) is used as a guide for substrate selection. Once the ssRNA is base-paired with the target mRNA, a nuclease activity, presumably within the complex, degrades the mRNA (Figure 1).

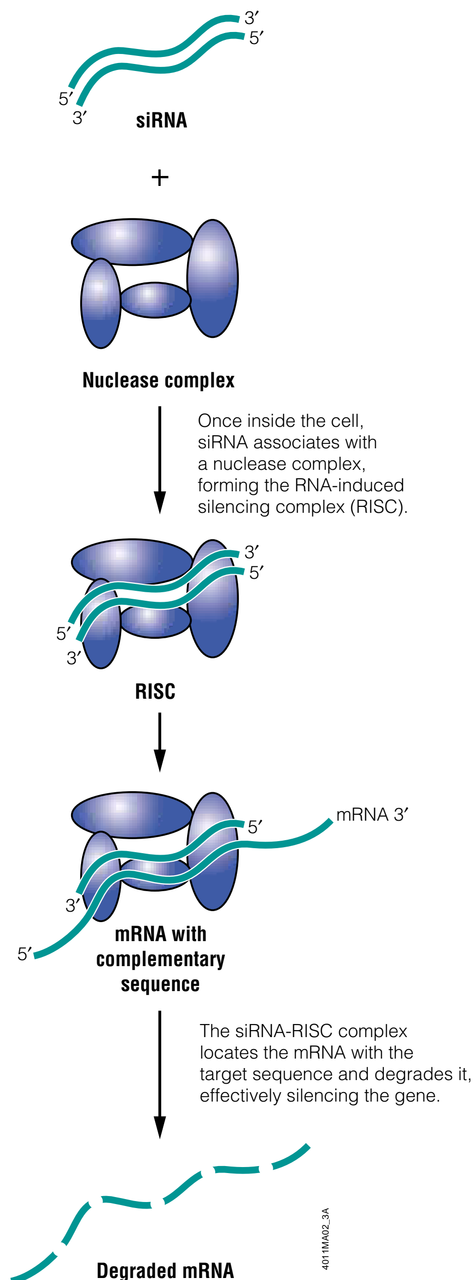

Figure 1. Proposed mechanism of RNA interference.

Methods for Inducing RNAi in Mammalian Cells

Soon after the discovery of the RNAi phenomenon by Fire *et al*. in 1998 (1), dsRNAs were used successfully as tools to analyze gene functon in nematodes, fruit flies and plants (4,5). In mammalian cells, long dsRNAs induce the sequence-specific silencing of genes in mouse embryonal carcinoma cells and embryonic stem cells (6,7). However, introducing long dsRNAs into mammalian somatic cells activates antiviral defense systems, resulting in nonspecific degradation of RNA transcripts (8) and general loss of host cell protein synthesis (9,10). These two mechanisms effectively shut down mammalian cells and thus override the ability of long dsRNAs to have specific RNAi effects. Using short (21–23nt) dsRNAs with 2-nucleotide 3′ overhangs, however, does not trigger these antiviral mechanisms and can mediate gene-specific suppression in mammalian cells. Short dsRNAs can be synthesized in vitro and introduced into mammalian cells. Alternatively DNA vector-based strategies can be used to deliver siRNA into mammalian cells, further expanding the utility of RNAi in mammals.

However, the success of these new approaches depends on selecting a specific region that gives optimal expression within a target mRNA. Although some algorithms are designed to predict how effective a given siRNA will be at suppressing the expression of its target, selecting optimal regions still remains largely a trial-and-error process. Therefore, multiple regions are generally screened for each target. Screening can be performed using synthetic RNAs, which are expensive, or with cloned DNA sequences, which is time consuming. The siLentGene™ U6 Cassette RNA Interference System avoids the expense of RNA synthesis and the time and effort of DNA cloning and allows for relatively fast and easy screening of different siRNA sequences. Effective target sequences can yield robust siRNA-mediated gene-specific inhibition, making the RNAi approach a powerful tool for gene function analysis in mammalian cells.

siLentGene™ U6 Cassette RNA Interference System

The siLentGene™ U6 Cassette RNA Interference System is a DNA cassette-based approach for creating siRNA expression constructs for direct delivery into cells in a rapid and cost-effective manner. The primer-dependent, PCR-based system places selected siRNA sequences under the control of an engineered U6 promoter and terminator. The PCR products are directly transfected into cells, eliminating the need to laboriously clone each one (Figure 2).

Relatively simple promoter and terminator sequences direct the production of large amounts of siRNA in mamalian cells by the endogenous RNA polymerase III of the transfected cells. The terminator consists of a short stretch of uridines; this is compatible with the original siRNA design that terminates with a two-uridine,

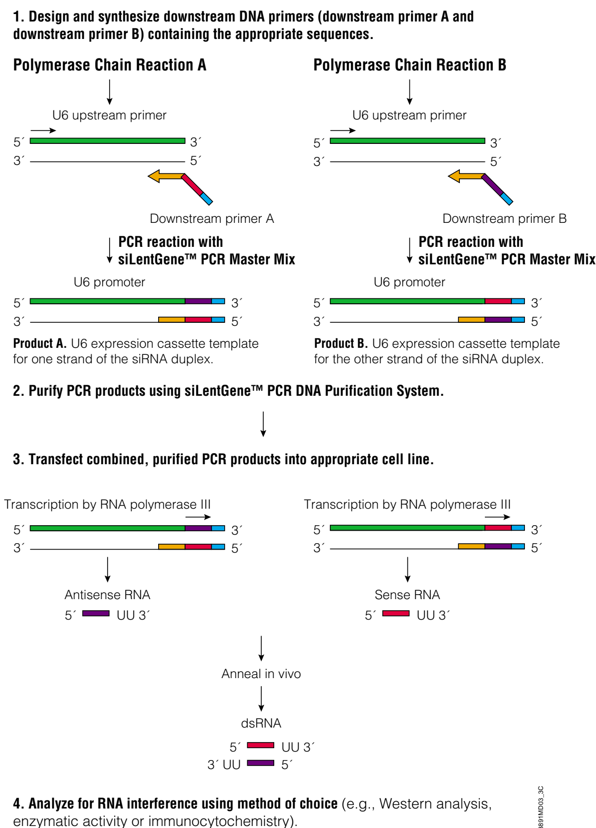

Figure 2. Overview of the siLentGene™ U6 Cassette RNA Interference System protocol.

3′ overhang (9). The siLentGene™ System is fast, inexpensive and provides a convenient approach for rapidly screening and determining the efficacy of different siRNAs.

The siLentGene™ System provides an upstream primer and a U6 cassette DNA that will act as a PCR template. Downstream primers are provided by the user. The downstream primers include three parts: a region that anneals with the U6 cassette, a region that has the sequences that will be transcribed into the siRNA, and a U6 terminator sequence. The resulting PCR products, the U6 expression cassettes, include the U6 promoter, the sense or antisense mRNA target sequence, and a terminator sequence.

Two expression cassettes, one expressing the antisense RNA and one expressing the sense RNA will be made. After column purification, these cassettes are combined and transfected into the appropriate cell line using the siLentGene™ Transfection Reagent (Figure 2).

After transfection, the endogenous RNA polymerase III in the transfected cells transcribes a short RNA from each of the expression cassettes. The resulting complementary siRNAs anneal, creating the siRNA duplexes. These duplexes trigger the specfic degradation of the target mRNA inside the transfected cells.

Screening for an Optimal Target Sequence

Determining an optimal target sequence is critical to the success of RNA interference experiments. Because predicting the optimal sequence of RNA for a given target is not possible, the siLentGene™ technology offers an ideal format to rapidly and inexpensively evaluate multiple targets. This utility is illustrated in the following experiment. Five different target sequences in the coding region of the humanized *Renilla* luciferase gene were selected according to the general guidelines described in the *siLentGene™ U6 Cassette RNA Interference System Technical Manual*, #TM061. Two DNA cassettes, one containing the sense sequence and one containing the antisense sequence, were generated for each target. PCR products were column-purified from the primers and were transfected into a CHO cell line stably expressing hR*luc*. The RNAi suppression effect was conveniently monitored by the decrease in *Renilla* luciferase activity. All five selected target sequences reduced hR*luc* activity compared to nonspecific sequences (Figure 3). However, the level of suppression varied from 20–70%, indicating variability in the ability of the different regions to direct knockdown effects. Site 3 most likely would provide the most significant RNAi effects for further experiments. On the contrary, Site 4 showed the poorest inhibition, and would not be a desirable target for any functional studies. Choosing the most efficient sequence for RNAi experiments is critical for studies in which the goal is understanding the effects of genetic loss of function, and the siLentGene™ System provides a useful tool for quickly evaluating multiple target sites.

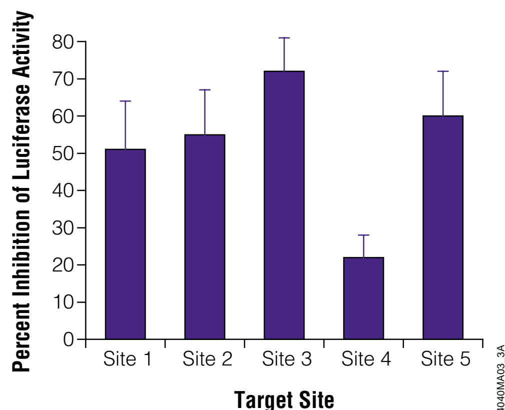

Figure 3. Suppression of humanized *Renilla* luciferase expression in a stable CHO cell line. CHO cells stably expressing hR*luc* were plated in a 96-well plate at a density of 3×10^3 cells/well (50% confluency) one day before transfection. The next day cells were transfected with 0.05µg of one of the DNA cassettes derived from the five hR*luc* sites [site 1 (75–93 from ATG); site 2 (297–315 from ATG); site 3 (375–393 from ATG); site 4 (508–526 from ATG); site 5 (756–776 from ATG)], or with a nonspecific negative control sequence. Other wells were transfected with 0.1µg of a vector expressing GFP (Cat.# E6421) for determining transfection efficiency. Ten wells per sample were plated, with five wells for determining *Renilla* luciferase activity and five wells used for determining cell number/viability. *Renilla* luciferase activity was measured using the *Renilla* Luciferase Assay System[a,f] (Cat.# E2810). Cell number/viability was determined using the CellTiter-Glo® Luminescent Cell Viability Assay[a] (Cat.# G7570). The data are shown as percent reduction in *Renilla* luciferase activity per cell number in the cells transfected with specific targeting sequence in comparison to nonspecific control. The results are shown as the average of four experiments.

Determining Optimal Suppression Conditions

Cell lines stably expressing reporter genes (e.g., humanized *Renilla* luciferase) provide a convenient system for assay optimization. We have generated several cell lines that stably express humanized *Renilla* luciferase. These cell lines were used to optimize critical suppression parameters. The cells were plated at different densities and were transfected with the sense and antisense U6 cassette containing target sequence against site 3 (described in Figure 3). The suppression level was determined after 96 hours by monitoring hR*luc* activity.

In parallel, the change in cell number was also monitored from 48–96 hours. Cells transfected at high cell density showed little or no suppression of hR*luc* activity, but cells transfected at lower cell densities showed substantial suppression of hR*luc* (up to 60%, Figure 4).

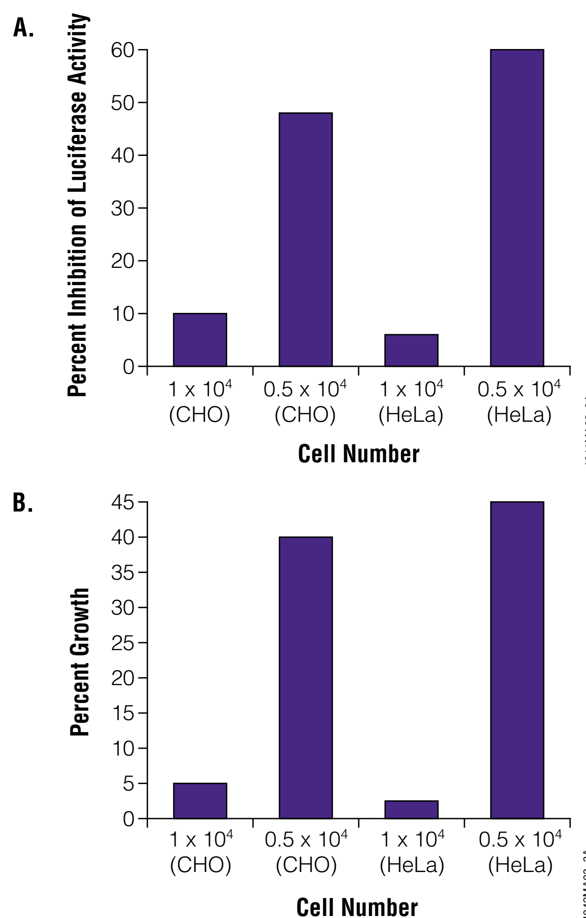

Figure 4. Correlation of suppression of hR*luc* expression with cell growth. The experiment was performed in a 96-well plate. Ten wells per sample were transfected, with five wells for determining *Renilla* luciferase activity (**Panel A**) and five wells for determining cell number/viability (**Panel B**). The suppression of *Renilla* luciferase expression was determined 96 hours after transfection. Percent growth was calculated based on the increase in cell numbers between 48 and 96 hours after transfection. Luciferase activity in cells transfected with the site 3 cassettes described in Figure 3 were compared to activity from cells transfected with cassettes containing nonspecific sequences.

These observations may result from the fact that confluent cells synthesize considerably less protein, leading to reduced RNAi effects. Cell proliferation data show that cells plated at higher density had much slower growth rates when compared to cells plated at lower densities. This indicates that maintaining a healthy cell culture is essential for successful application of the siLentGene™ U6 Cassette RNA Interference System.

Inhibiting Endogenous Gene Expression: A p53 Case Study

The transcription factor p53 is the most commonly mutated tumor suppressor gene in human cancers (11). The p53 protein has a short half-life but can be stabilized by either point mutation of the gene or interaction with certain DNA tumor viruses, such as the SV40 Large T antigen.

To test for inhibition of the p53 protein, we used the 293T cell line, because it contains the SV40 T antigen that stabilizes the p53 protein and allows for a high level of p53 accumulation. Twenty-four hours after plating, cells were transfected with U6 cassettes for p53 or a nonspecific control. Cells were also transfected with a GFP vector. Forty-eight hours after transfection the percentage of GFP-positive cells was determined in order to calculate transfection efficiency. The cells remaining were grown for 72 hours, lysed and the protein quantitated. An equal amount of protein per sample was run on an acrylamide gel and transferred to a nitrocellulose membrane. Detection was performed with a p53 monoclonal antibody. An actin antibody was used to determine equal loading between the lanes (Figure 5). The bands were then quantitated by densitometry, and the reduction in the p53 was determined and corrected using the actin loading control.

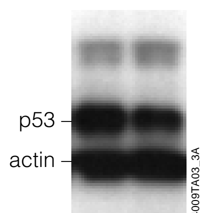

Figure 5. Suppression of endogenous p53 protein. 293T cells were transfected in a 12-well plate with 1µg/well of U6 cassette (0.5µg of each PCR product) and 6µl/well of siLentGene™ Transfection Reagent. The cells were then incubated for 72 hours, lysed and the protein quantitated. Five micrograms of protein/lane was run on a 10% tris-glycine gel and transferred to a nitrocellulose membrane. The membrane was probed with both a p53 (1:1,000 Calbiochem Ab-2) antibody and a β-actin (1:5,000 Abcam, Ab 6276) antibody to serve as a loading control. A goat anti-mouse, horseradish peroxidase-conjugated secondary antibody was used followed by chemiluminescent detection. In the first lane a nonspecific U6 cassette was tested as a negative control. The second lane was transfected with a p53 U6 cassette. Experiments were performed in duplicate.

The p53 experiment showed an average inhibition of 53%. At the same time point the GFP transfection efficiency was determined to be 40%. Inhibition levels with the U6 cassette can be slightly higher than the transfection efficiency of the plasmid marker.

Inhibition of Lamin A/C Expression

Reduction of targeted gene expression is typically analyzed by Western blotting when studying a population of cells or by immunocytochemistry to analyze individual cells. The human nuclear protein, Lamin A/C, which has been effectively inhibited in cell culture by synthesized siRNAs (12,13), was chosen as a target. HeLa cells were transfected with siLentGene™ U6 cassettes containing sense and antisense target sequences (Figure 6, Panels B and D) or with U6 cassettes containing nonspecific sequences (Figure 6, Panels A and C). Cells were fixed and stained with antibodies to Lamin A/C (red) or Lamin B1 (green) at 72 hours post transfection. Figure 6, Panel B, shows the reduction of Lamin A/C protein when HeLa cells were transiently transfected with U6 cassettes to produce siRNA directed toward the Lamin A/C target sequence. Reduction was not observed after transfection of U6 cassettes containing nonspecific sequences (Figure 6, Panel A). Additionally, the Lamin A/C PCR cassettes did not affect the expression of Lamin B1, suggesting that the observed effect is specific (Figure 6, Panels C and D).

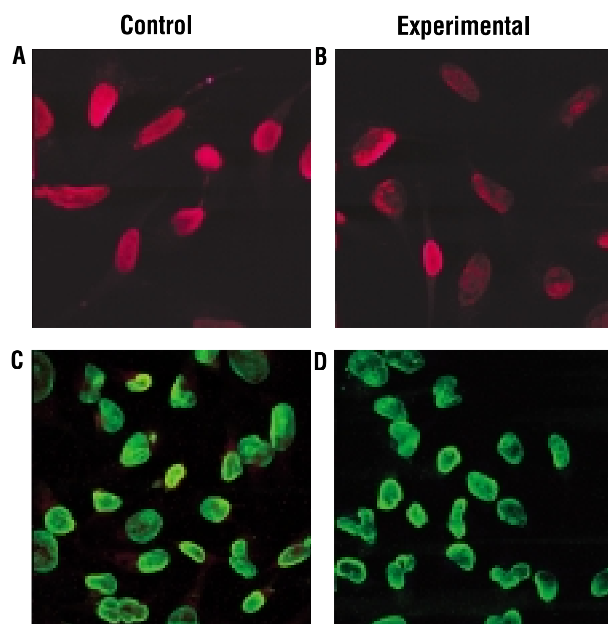

Figure 6. Inhibition of Lamin A/C expression using siRNA-containing PCR cassettes transfected in HeLa cells. HeLa cells were transfected with Lamin A/C U6 expression cassettes (**Panels B, D**) or with U6 cassettes expressing nonspecific target sequence (**Panels A, C**). Cells were stained with Lamin A/C antibodies (**Panels A, B**) or Lamin B1 antibodies (**Panels C, D**) and were analyzed with confocal microscopy.

Conclusion

The siLentGene™ U6 Cassette RNA Interference System is a rapid and inexpensive PCR-based approach for intracellular expression of siRNAs. It provides a useful experimental tool for identifying optimal siRNA-target sequences and subsequent testing for suppression of a wide range of genes.

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Protocol

- *siLentGene™ U6 Cassette RNA Interference System Technical Manual*, #TM061, Promega Corporation. (www.promega.com/tbs/tm061/tm061.html)

Doug Storts, Ph.D.
Platform Manager

Thomas Yeager, Ph.D.
R&D Scientist

Cynthia Sprecher, B.S.
R&D Scientist

Not Pictured
Jolanta Vidugiriene, Ph.D.
R&D Scientist

Not Pictured
Lin Wang, Ph.D.
Allele Biotechnology and Pharmaceuticals, Inc

Not Pictured
Jiwu Wang, Ph.D.
Allele Biotechnology and Pharmaceuticals, Inc

Not Pictured
Zhaohui Dong, Ph.D.
Allele Biotechnology and Pharmaceuticals, Inc

Ordering Information

Product	Size	Cat.#
siLentGene™ U6 Cassette RNA Interference System[a,b,c,d,e]	1 system	C7800

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