Everything You Need for RNAi

Promega

Effective RNA interference experiments are comprised of two basic steps: 1) Finding an effective siRNA target sequence and 2) Expressing that target sequence in cells. In the three articles that follow we describe three vector systems that provide easy and fast methods for both of these steps. The psiCHECK™ Vectors (pages 2–6) provide a fast and easy method for finding an effective siRNA target sequence. The siSTRIKE™ Systems (pages 7–10) allow fast and easy ligation and expression of hairpin oligonucleotides. The siLentGene™-2 Vector Systems (pages 11–14) provide both a quick cassette method for testing target sequences and ligation of target sequences into a vector system.

Shedding Light on RNAi

The Use of Bioluminescent Reporter Genes for RNAi Optimization

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The psiCHECK™ Vectors provide a quantitative and rapid approach for the optimization of RNA interference (RNAi). The vectors enable changes in expression of a target gene to be determined by monitoring the activity of a fused reporter gene. In the psiCHECK™ Vectors, Renilla *luciferase is used as the reporter gene. A target gene of interest can be cloned into the multiple cloning region located downstream of the* Renilla *translational stop codon. Initiation of RNAi toward the target gene results in cleavage and subsequent degradation of the fusion mRNA including the* Renilla *luciferase RNA sequence. This RNA degradation results in decreased* Renilla *luciferase activity. The decrease in activity can be monitored and used as a convenient indicator of the RNAi effect on the target gene. Thus, the psiCHECK™ Vectors allow quantitative measurements of RNAi activity and can be easily adapted for use in high-throughput applications.*

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Introduction

The main functional mediators of RNAi are double-stranded, short interfering RNAs (siRNAs) 21–23 nucleotides in length. These siRNAs interact with the RNA-induced silencing complex (RISC), where they function as templates for recognition and cleavage of complementary mRNA targets. Synthetic siRNA, or siRNAs expressed as short hairpin RNAs (shRNAs), can be introduced in cells and used experimentally to induce RNAi. Not all siRNAs generated to a target gene are equally effective in suppressing target genes in mammalian cells; therefore, it is important to identify siRNA sequences that are the most effective inhibitors of target gene expression. New design rules for effective siRNAs are being proposed (1–3). Current screening technologies are based on semi-quantitative, time-consuming methods and are not easily modified for the rapid, simultaneous screening of multiple siRNA/shRNA sequences. However, as the field of RNAi advances, and more high-throughput applications are adopted, there is a growing need for rapid and quantitative screening to confirm the effectiveness of siRNAs (4,5).

Description of the psiCHECK™ Vectors

The psiCHECK™ Vectors allow selection of optimal quantitative siRNA target sites that can be adapted for use in high-throughput applications. The psiCHECK™-1[a–d] (Cat.# C8011) and -2[a–f] (Cat.# C8021) Vectors both contain a synthetic version of *Renilla* luciferase (hR*luc*) reporter gene for monitoring RNAi activity. To aid in fusion of the target gene to the synthetic *Renilla* luciferase reporter gene, several restriction sites (i.e., the multiple cloning region) were added 3´ to the *Renilla* translational stop codon. These restriction sites can be used to create genetic fusions between the gene of interest and the *Renilla* reporter gene. Because of the presence of the *Renilla* translational stop codon, no fusion protein will be produced. Consequently, there is no need to maintain functional open reading frames when inserting the target gene. Also, toxic genes or gene fragments can be analyzed using this design.

The psiCHECK™-1 Vector is recommended for monitoring RNAi effects in live cells. Changes in *Renilla* luciferase activity are measured with the EnduRen™ Live Cell Substrate[a,c] (Cat.# E6481). This approach permits continuous monitoring of intracellular luminescence. *Renilla* luciferase expression can be monitored continuously for 2 days without interfering with normal cell physiology.

The psiCHECK™-2 Vector contains a second reporter gene, a synthetic firefly luciferase gene (h*luc*+), and is designed for endpoint lytic assays. *Renilla* and firefly luciferase activity can be measured using either the Dual-Luciferase® Reporter Assay System[c,g,l,m] (Cat.# E1910) or Dual-Glo™ Luciferase Assay System[c,g,l,m] (Cat.# E2920). Inclusion of the firefly luciferase reporter gene in the psiCHECK™-2 Vector permits normalization of changes in *Renilla* luciferase expression to the expression of firefly luciferase, making the system more robust and reproducible.

How Do the psiCHECK™ Vectors Function?

Figure 1 provides a basic illustration of how the psiCHECK™ Vectors are used. The gene of interest is cloned into the multiple cloning region located 3´ to the synthetic *Renilla* gene and its translational stop codon. After cloning, the vector is transfected into a mammalian cell line, and a fusion of the *Renilla* gene and the target gene is transcribed. Functional *Renilla* luciferase is translated if the transcript is intact. Depending on your experimental design, either vectors expressing shRNA or synthetic siRNA can be co-transfected simultaneously or sequentially. If a specific shRNA/siRNA effectively initiates the RNAi process on the target RNA, the fused *Renilla* target gene mRNA sequence will be degraded, resulting in reduced *Renilla* luciferase activity.

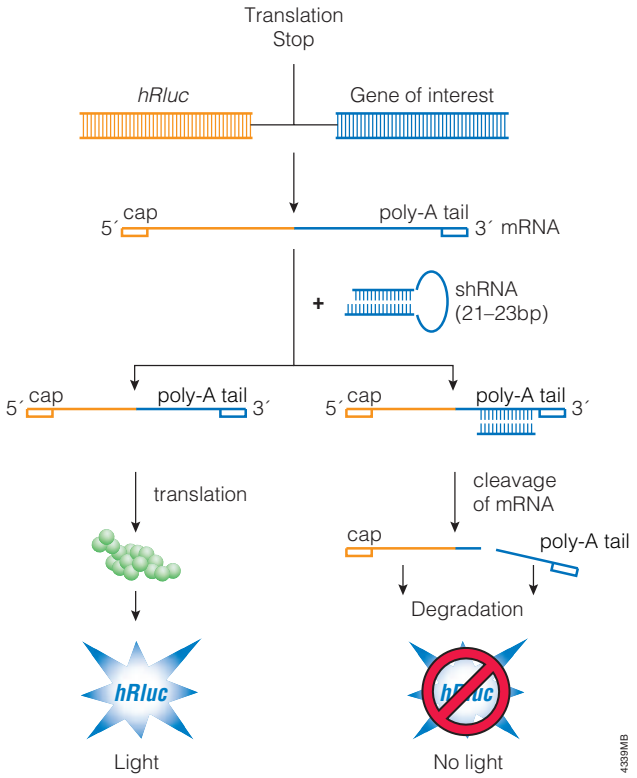

Figure 1. Mechanism of action of the psiCHECK™ Vectors.

Evaluating the Utility of the psiCHECK™ Vectors

To evaluate the usefulness of this screening method for identifying effective siRNA probes, we subcloned human p53 cDNA into the psiCHECK™-1 and -2 Vectors using the *Sgf* I and *Not* I restriction sites located in the multiple cloning region of each vector. The restriction sites are located 3´ to the *Renilla* translational stop codon. We also designed five different shRNAs of the p53 gene. To make DNA constructs containing shRNA sequences against different target sites of p53 gene, we used the siLentGene™-2 U6 Hairpin Cloning System–Basic[h–k] (Cat.# C7860). The siLentGene™-2 U6 Hairpin Cloning System permits generation of DNA cassettes containing a U6 promoter-shRNA sequence (target sequence-loop-target reverse complement sequence)-U6 termination sequence (see article on page 11) in a one-step PCR. The transcribed PCR fragments can be directly transfected into mammalian cells for efficient prescreening of shRNA target sequences, eliminating time-consuming cloning steps.

To evaluate this approach HEK293T cells were co-transfected with the psiCHECK™-2 Vector containing the p53 cDNA and linear DNA cassettes expressing either a p53 shRNA (Figure 2, sites 1–5) or the nonspecific shRNA. In addition, we used perfectly complementary, or 4-base mismatch shRNAs against *Renilla* luciferase as a positive control. The firefly and *Renilla* luciferase signals were generated using the Dual-Luciferase® Reporter 1000 Assay System[c,g,l,m] (Cat.# E1980). We determined the efficiency of different shRNA by monitoring the decrease in *Renilla* activity normalized (corrected for transfection efficiency) to the firefly luciferase signal. To determine if shRNAs expressed from linear DNA molecules show similar suppression patterns when subcloned into the vectors, we used the psiLentGene™-2 Basic Vector for our constructs. We repeated the experiments using vectors expressing shRNA against different target sites in the p53 gene. Figure 2 shows the data summary.

The data are presented as the percent suppression of normalized *Renilla* activity in cells producing shRNAs against different target sites or the nonspecific shRNA sequence. shRNAs expressed from linear DNA fragments targeting *Renilla* luciferase (generated by PCR as described in the Technical Bulletin, #TB329) showed a 43% reduction in normalized *Renilla* signal as compared to nonspecific control, and the suppression was eliminated by introduction of four mismatched nucleotides into the target sequence. All five shRNA against p53 were effective at suppressing *Renilla*/p53 expression; however, the level of suppression varied from 56% (site 2) to 74% (site 4). When linear DNA fragments were subcloned into the psiLentGene™-2 Basic Vector similar suppression patterns were observed (compare blue and red bars). In both cases, p53 site 4 showed the highest suppression level. However, the

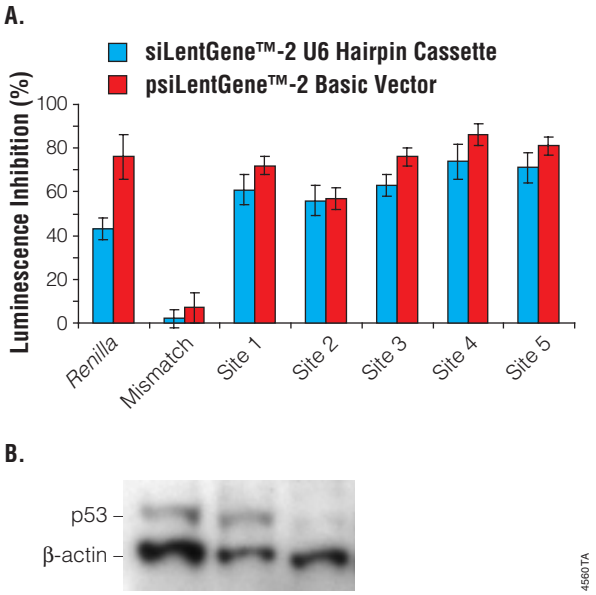

Figure 2. Target site selection for p53 gene using psiCHECK™-2 Vector. Panel A. HEK293T cells were seeded into a 96-well plate at a density of 3,000 cells/well. Human p53 cDNA was subcloned into the psiCHECK™-2 Vector using the *Sgf* I and *Not* I restriction sites. Cells were transfected with 0.02µg of psiCHECK™-2 Vector:p53 and 0.08µg of siLentGene™-2 U6 Hairpin Cassette (blue bars) or psiLentGene™-2 Basic Vector (red bars) per well. CodeBreaker™ siRNA Transfection Reagent (Cat.# E5052) was used for transfecting DNA cassette constructs, and TransFast™ Transfection Reagent[(o)] (Cat.# E2431) was used for vector DNA transfections. shRNAs were directed against 5 target sites of human p53 as controls. Perfectly complementary shRNA or a 4-base mismatch shRNA against *Renilla* luciferase was used. The data are compared to cells transfected with shRNA against nonspecific sequence. Forty-eight hours post-transfection *Renilla* and firefly luciferase activities were measured using the Dual-Luciferase® Reporter 1000 Assay System (Cat.# E1980). The data are shown as percent suppression of *Renilla* activity normalized to firefly luciferase activity in cells transfected with shRNAs against different target sequence or the nonspecific shRNA sequence. The data is the mean of 12 wells plus or minus the standard deviation. **Panel B.** Inhibition of p53 was performed in HEK293T cells and analyzed by Western blot. Cells were treated with transfection reagent alone (lane 1), with the psiLentGene™-2 Basic Vector to a nonspecific sequence (lane 2) or to a p53 target site 4 sequence (lane 3). Transient assays were examined after 72 hours.

actual levels of suppression were higher when cells were transfected with vectors expressing shRNAs. The expression of *Renilla*-p53 protein was reduced by 86% when the siLentGene™-2 Basic Vector expressing shRNA against p53 target site 4 was tested.

Several conclusions can be drawn from the data. First, both the cassette and vector system showed comparable suppression patterns. Second, DNA molecules generated by PCR can be used directly to transfect cells for rapid screening of optimal target sites. Third, it appears the suppression levels were highest when shRNA molecules were subcloned into an appropriate vector (psiLentGene™-2 Basic Vector), suggesting the optimal RNAi effect is achieved from the larger DNA vector constructs.

To validate the described approach it was important to demonstrate the ability of the psiLentGene™-2 Basic Vector expressing shRNA against p53 target site 4 to suppress the expression of endogenous p53 protein. Figure 2, Panel B, shows the Western blot analysis of HEK293T cells transfected with the psiLentGene™-2 Basic Vector expressing shRNA against nonspecific sequence (lane 2) versus shRNA against p53 target site 4 (lane 3). The Western blot analysis data indicate the greater than 80% decrease in the amount of p53 protein (normalized to β-actin) in shRNA-treated cells, confirming data obtained using the psiCHECK™ Vectors.

Monitoring RNAi Kinetics

The use of *Renilla* luciferase in the psiCHECK™ Vectors allows real-time monitoring of changes in *Renilla* expression induced by RNAi in live cells. Figure 3 demonstrates the changes in *Renilla* activity in the cells co-transfected with psiCHECK™-1 Vector containing the human p53 cDNA and a psiLentGene™-2 Basic Vector expressing shRNA against either *Renilla* luciferase, nonspecific sequence or p53. At 17 hours post-transfection nonlytic EnduRen™ Live Cell Substrate was added to the wells; relative light units were monitored for the next 26 hours until 41 hours post-transfection. We observed a dramatic increase in *Renilla* activity in cells transfected with shRNA against nonspecific sequence, while cells transfected with shRNA against *Renilla* luciferase or p53 target site 4 showed only a slight increase in *Renilla* expression. At 41 hours 80% suppression of *Renilla* luciferase was measured in cells co-transfected with functional shRNAs. Those results correspond to suppression observed in Figure 2 by using end-point analysis. CellTiter-Glo® Luminescent Cell Viability Assay Reagent[(g,n)] (Cat.# G7570) was added to cells at the end point to determine cell viability. When the *Renilla* luciferase signal was normalized to the relative number of viable cells in each well, there was no change in the suppression level, but a substantial decrease in signal variability between wells was achieved (data not shown).

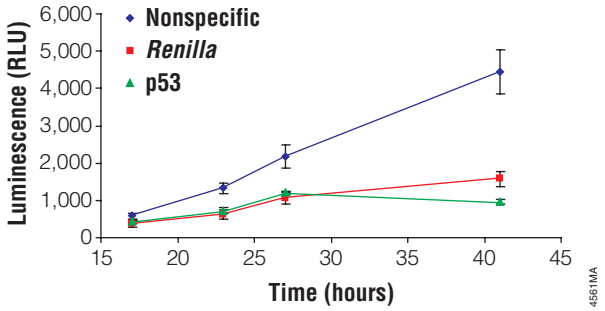

Figure 3. Measurement of changes in *Renilla* luciferase activity in live cells. HEK293T cells were plated in a 96-well plate at 3,000 cells/well. After an overnight incubation cells were treated with a transfection mixture consisting of 35µl of serum-free medium, 0.3µl of TransFast™ Transfection Reagent (Cat.# E2431), 0.02µg of psiCHECK™-1:p53 Vector and 0.08µg of psiLentGene™-2 Basic Vector per well. For this experiment the psiLentGene™-2 Basic Vector expressed shRNAs directed against human p53, *Renilla* or nonspecific sequence (negative control). After a one-hour incubation, 100µl of serum containing medium was added to the wells. At 17 hours post-transfection, EnduRen™ Live Cell Substrate (Cat.# E6481) was added to a final concentration of 60µM, and *Renilla* luciferase activity was monitored.

Screening Multiple shRNAs for Multiple Targets

We analyzed silencing by RNAi of a panel of caspase genes to determine the general applicability of the system in identifying effective RNAi sites for other genes. We generated shRNA using the rapid PCR approach offered by the siLentGene™-2 U6 Hairpin Cloning System. The generated PCR products against different target sites of caspases were co-transfected with psiCHECK™-2 Vector encoding cDNAs for caspase genes. Forty-eight hours post-transfection *Renilla* luciferase and firefly luciferase activities were measured. The cumulative data are presented in Figure 4. In Panel A, HeLa cells were co-transfected with psiCHECK™-2 Vector into which caspase 8 cDNA had been cloned and DNA cassettes expressing shRNAs against different target sites of caspase-3, caspase-7, caspase-8 and caspase-9. The data indicate that only one shRNA (site 3) containing targeted sequences against caspase-8 showed significant decrease in *Renilla* luciferase activity when co-transfected with psiCHECK™ -2:caspase-8 Vector. shRNAs against target sites of different caspases (e.g., caspase-3, -7 or 9) showed no substantial decrease in normalized *Renilla* luciferase activity.

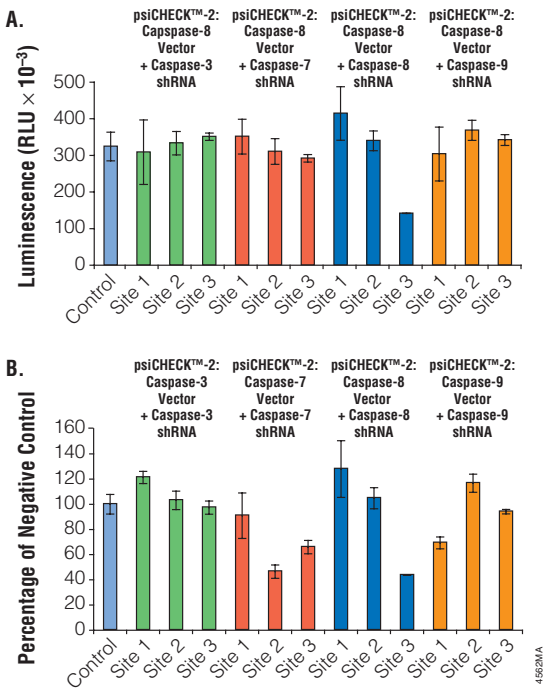

Figure 4. Screening of multiple potential shRNAs. Panel A. The cDNA for caspase-8 was cloned into the multiple cloning region of the psiCHECK™-2 Vector creating psiCHECK™-2:caspase-8 Vector. Three potential shRNA were designed to specifically inhibit caspase-3, caspase-7, caspase-8 and caspase-9. The siLentGene™-2 Cassettes expressing shRNAs were independently generated by PCR. The psiCHECK™-2:caspase-8 Vectors were co-transfected with psiLentGene™-2 DNA cassettes against different targets into HeLa cells. At 48 hours post-transfection, *Renilla* and firefly activity was measured using Dual-Luciferase® Reporter 1000 Assay System (Cat.# E1980). The *Renilla* luciferase relative light units were normalized to firefly luciferase expression. **Panel B.** psiCHECK™ Vectors into which cDNA for caspase-3, 7, 8 or 9 had been cloned were co-transfected with siLentGene™-2 Cassettes expressing shRNA to target sites designed for the corresponding caspase. At 48 hours post-transfection *Renilla* and firefly luciferase activity was measured as described in Panel A. The data are shown as percent suppression calculated based on the normalized *Renilla* activity in the cells transfected with nonspecific shRNA to cells transfected with shRNAs to different caspases and different target sites.

Data with psiCHECK™-2 Vector containing cDNA of corresponding caspase (for example caspase-3) co-transfected with DNA expressing shRNAs against several target sequences of the same caspase (in this example caspase-3) are presented in Figure 4, Panel B. The data are presented as percent suppression calculated based on the normalized *Renilla* activity in the cells transfected with nonspecific shRNA to cells transfected with shRNA to different target sites. None of the tested sites targeting caspase-3 showed a substantial decrease in *Renilla* activity. The most effective sites determined for caspase-7, -8 and -9 showed 46% to 64% suppression when expressed from DNA fragments, but based on our previous data with *Renilla* and p53 target site analysis, the level of suppression likely will increase upon subcloning DNA fragments into appropriate vectors.

Conclusions

To aid researchers in identification and quantification of potential initiators of RNAi (i.e., shRNA or siRNA) Promega developed the psiCHECK™ Vector System. After the gene of interest is cloned into the psiCHECK™ Vector of choice a quantitative and rapid approach for optimization of RNA interference can be accomplished. The psiCHECK™-1 Vector can be used with EnduRen™ Live Cell Substrate to continuously monitor live cells for a reduction in *Renilla* expression indicating RNAi. Due to the inclusion of a firefly expression cassette, *Renilla* expression resulting from the psiCHECK™-2 Vector can be normalized, thus reducing experimental and transfection variations and resulting in reliable, reproducible data.

References

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Protocol

- *psiCHECK™ Vectors Technical Bulletin* #TB329, Promega Corporation. (www.promega.com/tbs/tb329/tb329.html)

Ordering Information

Product	Size	Cat.#
psiCHECK™-1 Vector[a–d]	20µg	C8011
psiCHECK™-2 Vector[a–f]	20µg	C8021

[a] Patent Pending.

[b] Licensed under U.S. Pat. Nos. 5,292,658, 5,418,155 and other patents.

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[e] U.S. Pat. No. 5,670,356.

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[l] U.S. Pat. Nos. 5,283,179, 5,641,641, 5,650,289 and 5,814,471, Australian Pat. No. 649289 and other patents and patents pending.

[m] U.S. Pat. No. 5,744,320, Australian Pat. No. 721172, Canadian Pat. No. 2,221,522 and other patents and patents pending.

[n] U.S. Pat. No. 6,602,677, Australian Pat. No. 754312 and other patents pending.

[o] The cationic lipid component of the TransFast™ Transfection Reagent is covered by U.S. Pat. Nos. 5,824,812, 5,869,715 and 5,925,623, Australian Pat. No. 713093 and pending foreign patents.

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