

A New Promoter for RNAi

GeneClip™ U1 Hairpin Cloning Systems for Expression of Short Hairpin RNAs in vivo

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Abstract

The GeneClip™ U1 Hairpin Cloning Systems enable cloning and expression of short hairpin RNAs (shRNAs) for RNA interference (RNAi) experiments. The GeneClip™ Systems contain linearized vectors that facilitate cloning of complementary oligonucleotides downstream of a U1 promoter. Upon transfection with pGeneClip™ Vectors containing these cloned sequences, shRNA is produced in the cell, leading to reduction of target gene expression through RNAi. In this article we illustrate use of the GeneClip™ Systems to suppress Renilla luciferase expression in a range of cell types and to suppress p53 expression in both a transient and stable manner.

The GeneClip™ U1 Hairpin Cloning Systems are designed for rapid and efficient cloning of hairpin sequences for expression of shRNAs in vivo.

Introduction

The ability to silence gene expression can allow a researcher to determine protein function. For years researchers have sought an efficient method to suppress expression of specific target genes. The discovery of RNA interference (RNAi) as a means to achieve such suppression has been one of the most exciting innovations in cell biology, and RNAi technology has developed at remarkable speed. One of the most exciting aspects of RNAi is that it is a naturally occurring process in eukaryotic cells. Researchers can take advantage of this naturally occurring pathway to achieve targeted reduced expression of specific gene products.

During RNAi, dsRNA is recognized by an RNase III family member (e.g., DICER) and is cleaved into 21–23 nucleotide pieces. These siRNAs are incorporated into an RNA-induced silencing complex (RISC). Unwinding of the siRNA duplex occurs in the RISC complex, and the resulting single-stranded siRNA then targets the complementary mRNA for cleavage, resulting in diminished protein product. The RNAi pathway can be entered using a U1 promoter, which is recognized by RNA polymerase II (Pol II) in vivo to transcribe short hairpin RNAs (shRNAs) from a DNA template (1). Unlike other Pol II promoters, the U1 promoter does not have a TATA box but instead uses distal and proximal sequence elements to recruit RNA polymerase to the promoter. The U1 promoter also has a specific RNA termination site (Figure 1). Most other Pol II transcripts lack a definite RNA termination site. This termination allows discrete expression of the shRNA.

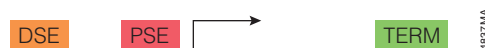


Figure 1. U1 promoter structure. The U1 promoter contains a Distal Sequence Element (DSE) and a Proximal Sequence Element (PSE) prior to the transcription start site, both of which are necessary for recruiting transcription factors to the promoter. There is also a specific termination signal (TERM) that allows cleavage of the mRNA transcript at a consistent location.

The GeneClip™ U1 Hairpin Cloning Systems

The GeneClip™ U1 Hairpin Cloning Systems^(a–f) are designed for rapid and efficient cloning of hairpin sequences for expression of shRNAs in vivo. The U1 promoter provides the benefit of production of shRNA with a defined termination site, mimicking siRNA produced in vivo when the DICER complex cleaves the loop off the hairpin. Using the GeneClip™ Systems, complementary oligonucleotides supplied by the user are annealed and cloned into the predigested vector downstream of the U1 promoter. The linearized vectors contain overhangs for increased cloning efficiency. The GeneClip™ Systems are provided in five formats. The GeneClip™ U1 Hairpin Cloning System—Basic is designed for transient suppression of the gene of interest. The GeneClip™ U1 Hairpin Cloning System—hMGFP contains the green fluorescent protein gene from *Montastrea cavernosa* and can therefore be used to determine transfection efficiency; it also allows separation of transfected cells by fluorescent activated cell sorting (FACS®). The three remaining GeneClip™ U1 Hairpin Cloning Systems provide the ability to stably select transfected cells using either neomycin, hygromycin or puromycin, so that experimental results do not depend on transfection efficiency.

Target Site Selection

The introduction of long strands of dsRNA into mammalian cells induces a strong interferon response that can lead to an overall shutdown of protein synthesis and cell death. Short dsRNA molecules (<30nt) can bypass the interferon response and still function in the RNAi pathway (2). Since the entire mRNA sequence cannot be used in mammalian cells, various target sites must be selected for use in RNAi experiments. Unfortunately not all target sequences in an mRNA will work equally well to suppress target gene expression. The siRNA Designer (www.promega.com/siRNADesigner/) is an online tool developed by Promega that is designed to assist in selection of the most likely target sites for RNAi (3,4). The user enters the sequence of interest, and the program generates a list of target sites that will most likely be

GeneClip™ U1 Hairpin Cloning Systems... continued

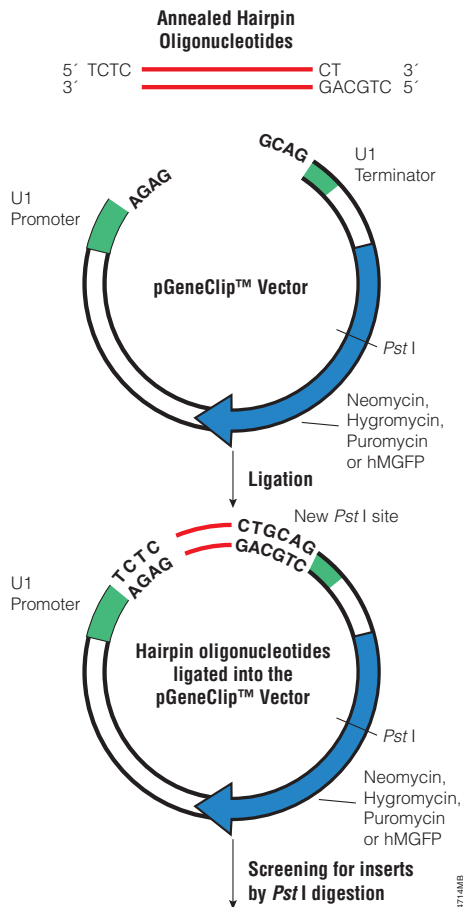


Figure 2. Overview of the GeneClip™ U1 Hairpin Cloning System protocol.

effective. The siRNA Designer uses a number of parameters, including examining the free energy of the 5' end of the antisense sequence, to evaluate siRNA candidates. Although programs such as the siRNA Designer allow selection of potentially effective siRNA sequences, they cannot guarantee that the chosen sequences will be highly effective. Experimental testing of the optimal sequences is required to confirm a high level of inhibition. The psiCHECK™ Vectors (Cat.# C8011, C8021), which enable monitoring of changes in expression of a target gene fused to a reporter gene, provide a useful screening tool to rapidly test the efficiency of siRNA sequences in suppressing expression of target genes (5).

Hairpin Cloning

Once target sites have been selected, the hairpin oligonucleotides are annealed and ligated into the pGeneClip™ Vector, which is then screened for the proper insert (Figure 2). These two oligonucleotides form a DNA insert that contains a hairpin siRNA target sequence. Standard desalting of the oligonucleotides is necessary, but gel purification and 5' phosphorylation are

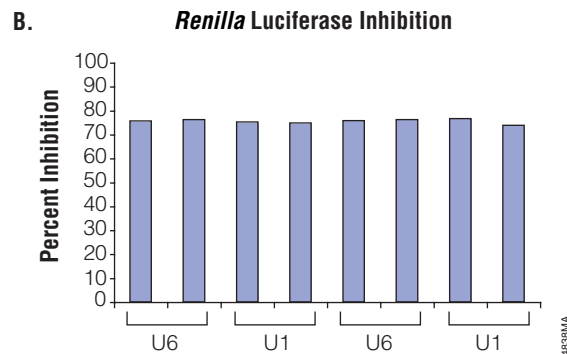
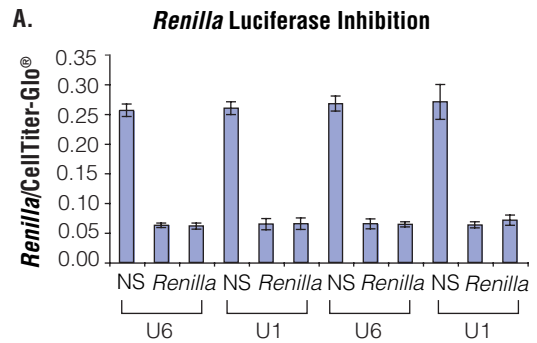


Figure 3. Transient suppression of *Renilla* luciferase with the U1-based pGeneClip™ and U6-based psiSTRIKE™ Basic Vectors. pGeneClip™ and psiSTRIKE™ Basic Vectors containing either a nonspecific shRNA (NS) or a *Renilla* luciferase-specific shRNA were transfected into HeLa cells stably expressing *Renilla* luciferase. Two independent transfections were performed with each vector system. Forty-eight hours after transfection, EnduRen™ Live Cell Substrate (Cat.# E6482) was added to each well to measure *Renilla* luciferase activity. CellTiter-Glo® Reagent (Cat.# G7571) was then added to the same well to determine cell viability. **Panel A.** *Renilla* luciferase activity was normalized to the cell viability data. **Panel B.** Luminescence in samples transfected with *Renilla* luciferase-specific shRNA was divided by the luminescence from cells transfected with vectors containing nonspecific shRNA to determine percent inhibition.

not required. Because the oligonucleotides inserted into the vector are short (~60nt), detection by standard agarose gel electrophoresis can be difficult. To make detection of inserts more convenient, a *Pst* I site is engineered into the vector backbone (Figure 2). Upon successful ligation of the annealed oligonucleotides into the vector, a second *Pst* I site is created. Digestion with *Pst* I therefore results in two easily separated bands of approximately 3kb and 1kb. If no insert is present, *Pst* I digestion will result in a single band. In our experiments, ligation of the insert typically results in over 50-fold more colonies than control ligations containing vector alone. In addition, the *Pst* I digestion showed over 90% of the colonies contained insert.

Transient Inhibition

We compared the effectiveness of the siSTRIKE™ U6 Hairpin Cloning System, which is based on the U6 promoter (6), with that of the GeneClip™ System in a transient suppression assay. The same shRNA targeting *Renilla* expression was cloned into the pGeneClip™ and psiSTRIKE™ Basic Vectors. Similar inhibition levels were

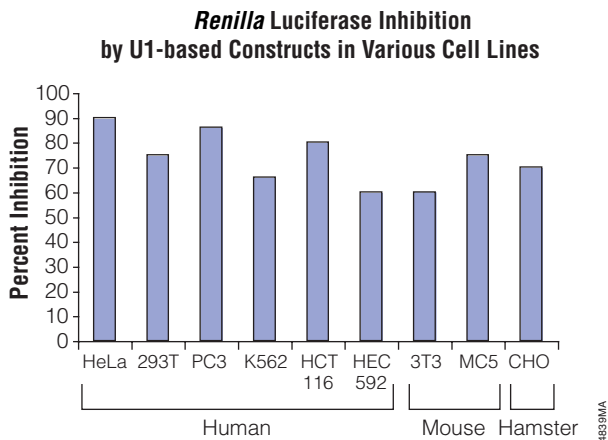


Figure 4. Effectiveness of the GeneClip™ U1 Hairpin Cloning System in various cell lines. The indicated cell lines were cotransfected with the psiCHECK™-2 Vector and a pGeneClip™ Basic Vector containing either a nonspecific or a *Renilla* luciferase-specific shRNA. *Renilla* and firefly luciferase activities were detected using the Dual-Luciferase® Reporter 1000 Assay System (Cat.# E1980). Percent inhibition of *Renilla* luciferase expression is shown. Results represent a single experiment for each cell type.

detected upon transfection of these vectors into a HeLa cell line stably expressing *Renilla* luciferase (Figure 3). Both U1- and U6-based systems resulted in more than 70% *Renilla* luciferase inhibition in many replicate experiments. These results show that the U1 promoter can function as effectively as the U6 promoter in an siRNA assay.

We tested the pGeneClip™ Basic Vector in several cell types to determine if they supported U1 activity for DNA-directed RNAi. The psiCHECK™-2 Vector, which expresses both *Renilla* and firefly luciferase activities (3), was cotransfected with a pGeneClip™ Basic Vector encoding shRNA directed against either *Renilla* luciferase or a nonspecific control sequence. shRNA expressed from the U1 promoter was able to inhibit *Renilla* luciferase expression in a number of different cell lines, including HeLa, 293T, PC3, K562, HCT116 and HEC 592 human cell lines, NIH/3T3 and MC5 mouse cell lines and the CHO hamster cell line (Figure 4).

To further test the effectiveness of the GeneClip™ U1 promoter we monitored reduction in p53 protein levels after targeting p53 mRNA. Initial testing of shRNAs targeting p53 was performed in HeLa cells using a psiCHECK™-2 Vector with the p53 gene (*TP53*) cloned 3' of the *Renilla* luciferase gene. Both *Renilla* luciferase and p53 expression were targeted using shRNAs cloned into a pGeneClip™ Basic Vector. More than 90% inhibition was observed for each protein compared to a nonspecific control (data not shown). To test p53 reduction in vivo, 293T cells, which contain elevated levels of p53, were transfected with a pGeneClip™ Basic Vector expressing p53 shRNA. Compared to nontransfected cells, the cells containing the pGeneClip™ Vector expressing p53 shRNA exhibited greater than 85% reduction in p53 protein levels (Figure 5, Panel A).

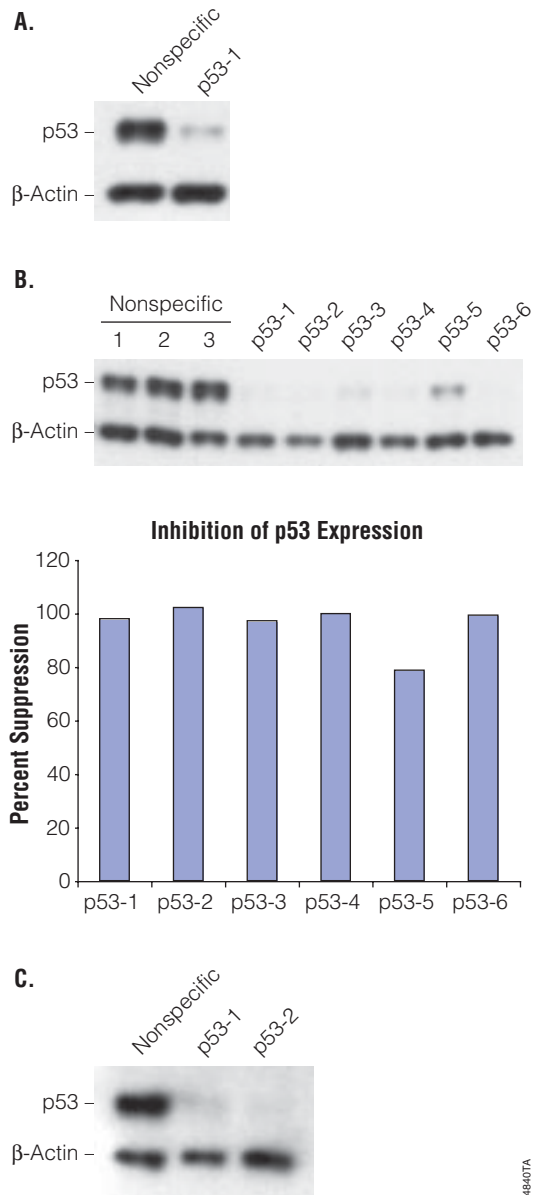


Figure 5. Inhibition of p53 expression in transient transfections and in stable clones. 293T cells were transfected with pGeneClip™ Basic Vectors containing either a hairpin target sequence directed against p53 or a nonspecific target sequence. After 48 hours, cells were collected, lysed, and protein levels quantitated by BCA assay (Pierce). For stable transfections, 293T cells were transfected with a pGeneClip™ Puromycin Vector containing either a nonspecific or a p53-specific target sequence. Cells were selected with puromycin for 7 days, and clones were assayed after passage 4 and passage 11. For Western analysis, 2µg protein per lane was loaded on an 8% Tris-Glycine gel and then transferred to nitrocellulose. Blots were probed with monoclonal antibodies to p53 (Oncogene Research Products, Ab-2, 1:1,000 dilution) and β-actin (Abcam, AC-12, 1:5,000 dilution). Detection was performed using ECL™ Plus (Amersham). Protein levels were quantitated by densitometry after exposure to film. **Panel A.** shRNA suppression of p53 expression in transiently transfected cells. **Panel B.** Stable reduction of p53 at passage 4. **Panel C.** Stable reduction of p53 at passage 11.

GeneClip™ U1 Hairpin Cloning Systems... continued

Stable Selection and Inhibition

Development of stable cell lines can allow the researcher to study the effect of silencing gene expression without the complications involved in transfection of the cells. To generate stable clones we transfected 293T cells with the pGeneClip™ Puromycin Vector containing a p53 target sequence or a nonspecific control sequence and selected for integration of the vector of interest. After transfection and selection for drug-resistant cells, a number of clones were selected and screened for p53 expression (Figure 5, Panel B). Clones containing the vector expressing a control sequence showed little reduction in p53 levels, while five of six clones expressing p53 shRNA showed >95% reduction in p53 expression. Two of the p53 clones showing reduced expression and one of the controls were cultured until passage 11 and re-examined. The level of p53 inhibition was unchanged (Figure 5, Panel C). These data show that the GeneClip™ Systems provide a useful tool to reduce or eliminate proteins of interest in a stable manner. Achieving high-level stable reduction of protein expression can help eliminate some experimental variation, providing more consistency than that achieved in repeated transient transfection assays.

Conclusion

The GeneClip™ U1 Hairpin Cloning Systems allow the researcher to perform rapid cloning and easy screening for shRNA inserts. The vectors are provided in linearized format, and a convenient *Pst* I digestion can be used to verify inserts. The U1 promoter can be used in RNAi experiments to successfully reduce expression of protein products in vivo. In addition the U1 promoter can function in a variety of cell types including human, mouse and hamster cell lines. The greatest strength of the GeneClip™ U1 Hairpin Cloning Systems may be the ability to generate stable clones for long-term gene silencing.

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Protocol

- ◆ *GeneClip™ U1 Hairpin Cloning Systems Technical Manual #TM256*, Promega Corporation.
www.promega.com/tbs/tm256/tm256.html

Ordering Information

Product	Size	Cat.#
GeneClip™ U1 Hairpin Cloning System—Basic	20 reactions	C8750
GeneClip™ U1 Hairpin Cloning System—Puromycin	20 reactions	C8760
GeneClip™ U1 Hairpin Cloning System—Hygromycin	20 reactions	C8770
GeneClip™ U1 Hairpin Cloning System—Neomycin	20 reactions	C8780
GeneClip™ U1 Hairpin Cloning System—hMGFP	20 reactions	C8790

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