







pGPU6/Hygro siRNA Expression Vector Kit

A vector designed for the cloning and stable expression of short haipin RNA (shRNA) in mammalian cells under the control of human U6 promoter

Catalog No. E-05/F-05



User Manual

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I. Product Description and Background

A. siRNA and RNA Interference

Small Interfering RNAs (siRNAs) are short, double-stranded RNA molecules that can target mRNAs with complementary sequence for degradation via a cellular process termed RNA interference (RNAi) (Elbashir 2001). Researchers in many disciplines employ RNAi to analyze gene function in mammalian cells. The siRNA used in early studies was typically prepared in vitro and transfected into cells. More recent publications feature plasmids that express functional siRNA when transfected into mammalian cells (Sui 2002, Lee 2002, Paul 2002, Paddison 2002, Brummelkamp 2002). Using siRNA expression vectors has the advantage that the expression of target genes can be reduced for weeks or even months (Brummelkamp 2002), eclipsing the 6–10 days typically observed with in vitro prepared siRNA used for transient transfection (Byrom 2002).

B. pGPU6/Hygro siRNA Expression Vector

Mammalian promoters for siRNA expression

The siRNA expression vectors employ RNA polymerase III (pol III) promoters which generate large amounts of small RNA using relatively simple promoter and terminator sequences. They also include an antibiotic resistance gene that provides a mechanism to select for transfected cells that express the introduced DNA.

GenePharma's pGPU6/Hygro siRNA Expression vector features a human U6 RNA pol III promoter, and pGPH1/Neo contains the H1 RNA pol III promoter. These promoters are well characterized (Myslinski 2001, Kunkel 1989), and they provide high levels of constitutive expression across a variety of cell types. The terminator consists of a short stretch of uridines (usually 3–4 nt); this is compatible with the original siRNA design that terminates with a two uridine 3' overhang (Elbashir 2001).



Based on comparisons of several different RNA pol III promoters, the activities of the two promoters are likely to vary from cell type to cell type (Ilves 1996). The localization of expressed RNA is also likely to vary with cell type and with RNA pol III promoter (Ilves 1996). To optimize siRNA expression, we find it beneficial to clone hairpin siRNAs into both the pGPU6/Hygro and pGPH1/GFP/Neo vectors and transfect them into the cells being targeted for gene knockdown. The promoter that is more effective for the siRNA and cell type will provide greater levels of gene silencing.

I.A. siRNA and RNA Interference 1

pGPU6/Hygro siRNA Expression Vector

Mammalian Selectable Markers



The pGPU6/Hygro siRNA expression vectors contain a hygromycin resistance gene to enable antibiotic selection in mammalian cells. Antibiotic selection can be used to enrich cultures for cells that were successfully transfected with the siRNA expression vector by killing off cells that lack the plasmid. Short term antibiotic selection is very useful for experiment systems where low transfection efficiency would otherwise preclude detection of a reduction in target gene expression. For long-term gene knockdown studies, the hygromycin resistance gene makes it possible to select cell populations, or clonal cell lines, that stably express the siRNA.



2 I.B.	pGPU6/GPF/Neo siRNA Expression Vectors	
	Product	Description and Background
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pGPU6/Hygro plasmid is supplied ligation-ready

The pGPU6/Hygro siRNA Expression vector is linearized with both *Bam* HI and *Bbs* I to facilitate directional cloning. They are purified to remove the digested insert so that it cannot re-ligate with the vector. This greatly increases the percentage of clones bearing the hairpin siRNA-coding insert after ligation, reducing the time and effort required to screen clones. Both pGPU6/Hygro and pGPH1/Neo are linearized with the same restriction enzymes, so that a given hairpin siRNA insert can be subcloned into either vector using the 5' overhangs left by restriction enzyme digestion. A basic pGPU6/Hygro vector map is shown in Figure 1 on page 3; more detailed sequence information about the pGPU6/Hygro vector is available from the Technology Support of GenePharma, Inc..

http://www.genepharma.com

support@genepharma.com

Figure 1. pGPU6/Hygro siRNA expression vector map





Feature₀	Nucleotide position
T3 promoter binding site	215-234#
HU6 promoter₽	346-616+2
T7 promoter binding site	623-644#
F1 origin₽	711-1125-
SV40 promoter₽	1190-1514-
Hvgromvcin resistance gene⊬	1532-2555₽
SV40 polvA₽	2568-2940₽
pUC origin of replication₽	3200-3870#
Ampicillin resistance genee	4015-4875-

I.B. pGPU6/Hygro siRNA Expression Vector

pGPU6/Hygro siRNA Expression Vector

C. siRNA Template Design



The prototypical siRNA comprises two hybridized 21-mer RNA molecules with 19 complementary nucleotides and 3' terminal dinucleotide overhangs. Expression vectors with dual promoters that express the two strands of the siRNA separately can be used (Lee 2002), however, a more efficient scheme is to express a single RNA that is a 19-mer hairpin with a loop and 3' terminal uridine tract (Paddison 2002) (Figure 2). When expressed in mammalian cells, the hairpin siRNA is apparently recognized by Dicer, the nuclease responsible for activating dsRNAs for the RNAi pathway, and cleaved to form a functional siRNA (Brummelkamp 2002). For cloning into an siRNA expression vector, hairpin siRNA inserts have the advantage that only a single pair of oligonucleotides and a single ligation are needed to generate plasmid for gene silencing studies. For each target gene, design complementary 55–60 mer oligonucleotides with 5' single-stranded overhangs for ligation into the pGPU6/Hygro vectors. The oligonucleotides should encode 19-mer hairpin sequences specific to the mRNA target, a loop sequence separating the two complementary domains, and a polythymidine tract to terminate transcription (this is discussed in <u>section II.B</u> on page 8).

Strategy for selection of siRNA target sites

The susceptibility of siRNA target sites to siRNA-mediated gene silencing appears to be the same for both in vitro prepared siRNAs and RNA pol III-expressed siRNAs. Thus sequences that have been successfully targeted with a chemically synthesized, in vitro transcribed, or PCR-generated siRNA should also be susceptible to down-regulation with an siRNA expressed from a pGPU6/Hygro vector. If an siRNA target site has not already been identified, then we recommend that several different siRNAs be tested per gene. This can be conveniently done using in vitro prepared or chemical synthesized siRNAs (e.g. siRNAs synthesized by Genepharma, Cat No A01005). Once an effective target site is identified, oligonucleotides encoding hairpin siRNAs can be synthesized and ligated into pGPU6/Hygro siRNA Expression Vector. This significantly reduces the time and effort required to develop an effective siRNA plasmid specific to a given gene.



D. Kit Components and Storage

Each pGPU6/Hygro siRNA Expression Vector Kit includes 4 components:

- Linearized pGPU6/Hygro siRNA Expression Vector ready for ligation
- Circular, negative control pGPU6/Hygro vector that expresses a hairpin siRNA with limited homology to any known sequences in the human, mouse, and rat genomes



• human GAPDH-specific, hairpin siRNA insert that can be used as a positive control for ligation

• 1XDNA Annealing Solution to prepare annealed DNA oligonucleotides for ligation into the pGPU6/Hygro vector

E-07	F-07	Component	
20 µl		pGPU6/Hygro (circular) (50 ng/ul)	V
	20µl	pGPU6/Hygro (linearized) (50 ng/ul)	
10 µl	10 µl	pGPU6/Hygro Negative control (0.5 µg/µl)	
10 µl	10 µl	GAPDH Control Insert (20 nM)	
0.5 ml	0.5 ml	10×shDNA Annealing Solution	

Store the pGPU6/Hygro siRNA Expression Vector Kit at -20° C (if desired the 1XDNA Annealing Solution can be stored at room temp). Properly stored kits are guaranteed for 6 months from the date received.

E. Other Required Material			
Ligation and transformation	• Two complementary oligonucleotides targeting the gene of interest for RNAi (design and ordering is discussed in section II starting on page 8)		
	• DNA ligase, ligase reaction buffer, and competent <i>E. coli</i> cells are needed to subclone the siRNA inserts.		
	• Ampicillin or carbenicillin containing plates and liquid media will also be needed to propagate the plasmids.		
Plasmid purification	For efficient transfection into mammalian cells it is crucial that preparations of pGPU6/Hygro be very pure.		
Mammalian cell transfection reagents	The optimal mammalian cell transfection conditions including transfection agent and plasmid amount must be determined empirically.		
Cell culture facility and supplies	In addition to routine cell culture media, culture media containing G418 (a Hygromycin analog) will be needed for selection of pGPU6/Hygro-transfected cells.		
	I.D. Kit Components and Storage 5		

pGPU6/Hygro siRNA Expression Vector

F.

Related Products Available from GenePharma

Cat. No.	Description	Package	Purification
A01005	Custom siRNA	5 OD	HPLC
A02005	Chemically modified siRNA	5 OD	HPLC
A03005	Fluorescent dye labeled siRNA	5 OD	HPLC
B01001	Negative Control siRNA	1 OD	HPLC
B02001	FITC negative control siRNA	1 OD	HPLC

B03001	Positive control siRNA	1 OD	HPLC
C-01	RNAi-Mate transfection Reagent	0.1 ml	
E-01/F-01	pGPU6	1 µ g	
E-05/F-05	pGPU6/Hygro	1 µg	
E-07/F-07	pGPU6/GFP/Neo	1 µg	

I.F. Related Products Available from GenePharma

Planning and Preliminary Experiments

II. Planning and Preliminary Experiments

A. siRNA Target Site Selection

Using siRNA for gene silencing is a rapidly evolving tool in molecular biology; these instructions are based on both the current literature, and on empirical observations by scientists at GenePharma.

1. Find 21 nt sequences in the target mRNA that begin with an AA dinucleotide

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Beginning with the AUG start codon of your transcript, scan for AA dinucleotide sequences. Record each AA and the 3' adjacent 19 nucleotides as potential siRNA target sites.

This strategy for choosing siRNA target sites is based on the observation by Elbashir et al. (EMBO 2001) that siRNA with 3' overhanging UU dinucleotides are the most effective. This is compatible with using RNA pol III to transcribe hairpin siRNAs because it terminates transcription at 4–6 nucleotide poly(T) tracts creating RNA molecules with a short poly(U) tail.

2. Select 2–4 target



Research at GenePharma has found that typically more than half of randomly designed siRNAs provide at least a 50% reduction in target mRNA levels and approximately 1 of 4 siRNAs provide a 75–95% reduction. Choose target sites from among the sequences identified in step 1 based on the following guidelines:

• Since a 4–6 nucleotide poly(T) tract acts as a termination signal for RNA pol III, avoid stretches of $\geq 4T$'s or A's in the target sequence.

• Since some regions of mRNA may be either highly structured or bound by regulatory proteins, we generally select siRNA target sites at different positions along the length of the gene sequence. We have not seen any correlation between the position of target sites on the mRNA and siRNA potency.

• Compare the potential target sites to the appropriate genome database (human, mouse, rat, etc.) and eliminate from consideration any target sequences with more than 16-17 contiguous base pairs of homology to other coding sequences. We suggest using BLAST, which can be found on the NCBI server at:

www.ncbi.nlm.nih.gov/BLAST.

• GenePharma researchers find that siRNAs with 30-50% G/C content are more active than those with a higher G/C content.

siRNA Target Site Selection II.A.

pGPU6/Hygro siRNA Expression Vector

3. Negative Controls A complete siRNA experiment should include a negative control siRNA with the same nucleotide composition as your siRNA but which lacks significant sequence homology to the genome. To design a negative control siRNA, scramble the nucleotide sequence of the gene-specific siRNA and conduct a search to make sure it lacks homology to any other gene.

Hairpin siRNA Template Oligonucleotide Design & Ordering Β.

To use the pGPU6/Hygro siRNAi Expression Vector Kit, you will first need to design two single-stranded DNA oligonucleotides; one encoding the target shRNA ("top strand" oligo) and the other its complement ("bottom strand" oligo). You will then anneal the top and bottom strand oligos to generate a double-stranded oligonucleotide (ds oligo) suitable for cloning into the pGPU6/Hygro vector.

The design of the single-stranded oligonucleotides (ss oligos) is critical to the success of both the cloning procedure and ultimately, the RNAi analysis. General guidelines are provided in this section to help you choose the target sequence and to design the ss oligos. Note however, that simply following these guidelines does not guarantee that the shRNA will be effective in knocking down the target gene. For a given target gene, you





may need to generate and screen multiple shRNA sequences to identify one that is active in gene knockdown studies.

Two complementary oligonucleotides must be synthesized, annealed, and ligated into pGPU6/Hygro for each siRNA target site. Figure 3 on page 8 shows schematically how to convert siRNA target sites into oligonucleotide sequences for use in the pGPU6/Hygro vectors. The oligonucleotides encode a hairpin structure with a 19-mer stem derived from the mRNA target site. The loop of the hairpin siRNA is located close to the center of the oligonucleotides; a variety of loop sequences have been successfully used by researchers (Sui 2002, Lee 2002, Paddsion 2002, Brummelkamp 2002, Paul 2002), and we have observed no particular benefit in using one or another. The loop sequence shown in Figure 3, 5'-UUCAAGAGA-3', is one possible sequence. Near the end of the hairpin siRNA template is a 5–6 nucleotide poly(T) tract recognized as a termination signal by RNA pol III that will terminate siRNA synthesis. The 5' ends of the two oligonucleotides are noncomplementary and form the *Bbs* I and *Bam*H I restriction site overhangs that facilitate efficient directional cloning into the pGPU6/Hygro vectors. Just downstream of the *Bbs* I site, it is advantageous to have a G or an A residue because RNA pol III prefers to initiate transcription with a purine.

For siRNA targets with a C or a U residue at position 1 (the first nucleotide after

II.B. Hairpin siRNA Template Oligonucleotide Design & Ordering

Planning and Preliminary Experiments

the AA in the RNA target sequence), add an additional G (shown with an asterisk in Figure 3) to facilitate transcription of the siRNA by RNA pol III.

Synthesis of hairpin siRNA template oligonucleotides for ligation into pGPU6 vectors

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Order a 1-2 OD scale synthesis of each oligonucleotide. Typically we use economical, desalted-only DNA oligonucleotides in this procedure. It is important, however, that the oligonucleotides are mostly full-length. Choose a supplier that is reliable in terms of oligonucleotide sequence, length, and purity.

Figure 3. Hairpin siRNA Template Design

Example Target Sequence (AA plus 19 nt)

5° AAGTCAGGCTATCGCGTATCG 3

Annealed Hairpin siRNA Template Insert (order these 2 oligonucleotides)

5' - CACC GTCAGGCTATCGCGTATCG TTCAAGAGA CGATACGCGATAGCCTGAC TTTTTTG -3' 3' - CAGTCCGATAGCGCATAGC AAGTTCTCT GCTATGCGCTATCGGACTG AAAAAACCTAG -5'



pGPU6/Hygro siRNA Expression Vector

C. Optimizing Antibiotic Selection Conditions

Cell type, culture medium, growth conditions, and cell metabolic rate can all affect the optimal antibiotic concentration for selection of pGPU6/Hygro- transfected cells. Identify the lowest level of G418 that kills nontransfected cells within approximately 7 days by testing antibiotic concentrations from 25–4000 μ g/ml while keeping all other culture conditions equal. See step 1. G418 titration (kill curve) below.

Using this optimum G418 concentration, optimize cell plating density. See <u>step 2</u>. *Optimal plating density* below. Plating density can have a strong impact on antibiotic selection because cells growing at higher densities are less effectively killed off than cells growing at lower densities. Also, cells that divide more rapidly typically have a lower optimal plating density than cells that double slowly.

- **1. G418 titration (kill** a. curve)
 - a. Plate 20,000 cells into each well of a 24 well dish containing 1 ml of culture medium.
 - b. After 24 hr, add 500 μ l culture medium containing 25–4000 μ g/ml G418.
 - Culture the cells for 10–14 days, replacing the antibiotic-containing medium every 3 days.
 - d. Examine the dishes for viable cells every 2 days.
 - e. Identify the lowest G418 concentration that begins to give massive cell death in



approximately 7-9 days, and kills all cells within 2 weeks. Use this G418 concentration to select cells containing the pGPU6/Hygro plasmid after transfection.

- Plate several different amounts of cells into separate wells of a 24 well dish a. containing 1 ml of culture medium.
- b. After 24 hr, add 500 µl culture medium containing G418; use the concentration identified in the previous experiment.
- c. Culture the cells for 5–14 days, replacing the antibiotic-containing medium every 3 days.
- d. Identify the cell plating density that allows the cells to reach 80% confluency before massive cell death begins; and use it to plate cells transfected with your pGPU6/Hygro clone.

10 II.C. **Optimizing Antibiotic Selecting Conditions** Using the pGPU6/Hygro siRNA Expression Vector Using the pGPU6/Hygro siRNA Expression Ш. Vector

Α. Cloning Hairpin siRNA Inserts into pGPU6/Hygro

1. Prepare a 1 µg/µl solution of each oligonucleotide

- Dissolve the hairpin siRNA template oligonucleotides in approximately 100 µl of a. nuclease-free water.
- b. Dilute 1 µl of each oligonucleotide 1:100 to 1:1000 in TE (10 mM Tris, 1 mM EDTA) and determine the absorbance at 260 nm. Calculate the concentration (in µg/ml) of the hairpin siRNA oligonucleotides by multiplying the A260 by the dilution factor and then by the extinction coefficient (~33 µg/ml).
- Dilute the oligonucleotides to approximately 100 µM. c.
- 2. Anneal the siRNA template oligonucleotides
- Assemble the 50 µl annealing mixture as follows: a.

Assemble the 5	0 μl annealing mixture as follows:	
Amount	Component	ЛА,
5 µl	10XshDNA Annealing Solution	XXX
5 µl	sense siRNA template oligonucleotide (100 uM)	
5 µl	antisense siRNA template oligonucleotide (100 uM)	
35 µl	ddH ₂ O	

- b. Heat the mixture to 95°C for 3 min, then turn off the heater and cool to room temperature slowly.
- The annealed siRNA template insert can either be ligated into a pGPU6/Hygro C. vector or stored at -20°C for future ligation.

3. Ligate annealed siRNA template insert into pGPU6/Hygro

- a. Dilute the annealed siRNA template insert with nuclease-free water for a final concentration of 20 nM.
- b. Set up two 10 μ l ligation reactions; a plus-insert ligation, and the minus-insert negative control. To each tube, add the following reagents:

			<u> </u>
plus-insert	minus-insert	Component	
1 ul		diluted annealed siRNA insert	
ιμι		(from step <u>3.a.1</u> above)	
	1 µl	1X shDNA Annealing Solution	
6.5 µl	6.5 µl	nuclease-free water	
1 µl	1 µl	10X T4 DNA Ligase Buffer	
1 µl	1 µl	pGPU6/Hygro vector	
0.5 µl	0.5 µl	T4 DNA ligase (5 U/μl)	
III.A. Cloni	ng Hairpin siR	NA Inserts into pGPU6/Hygro	11

pGPU6/Hygro siRNA Expression Vector

- c. Incubate for 1–3 hr at room temperature (the reactions can be incubated overnight at 16°C if very high ligation efficiency is required).
- d. The recommended incubation time and temperature for ligation reactions varies widely among different sources of T4 DNA ligase. Follow the recommendation provided by the manufacturer of your DNA ligase, if using other source enzymes.
- 4. Transform *E. coli* with the ligation products



- a. Transform an aliquot of cells with the plus-ligation products, and transform a second aliquot with the minus-ligation products. Use an appropriate amount of ligation product according to how the competent cells were prepared and the transformation method. (For chemically competent cells, we routinely transform with 3-10 μ l of the ligation reaction.)
- b. Plate the transformed cells on LB plates containing 50–200 μ g/ml Ampicillin or carbenicillin and grow overnight at 37°C. Generally it is a good idea to plate 2–3 different amounts of transformed cells so that at least one of the plates will have distinct colonies. *Always* include a non-transformed competent cell control: this negative control is a culture of your competent cells plated at the same density as your transformed cells.
- c. Examine each plate and evaluate the number of colonies promptly after overnight growth at 37°C (or store the plates at 4°C until they are evaluated).

5. Expected results



Non-transformed control culture:

The non-transformed control culture should yield no colonies (indicating that the Ampicillin or carbenicillin in the culture medium is effective at inhibiting the growth of *E. coli* that do not contain the pGPU6/Hygro vector)

Plus- and minus- ligation transformations

Identify the dilution of plus- and minus- ligation transformations that yield well-spaced (countable) colonies, and count the colonies on those plates. The minus- ligation will probably result in some Ampicillin or carbenicillin resistant colonies (background), but *the plus- ligation should yield 2–10 fold more colonies than the minus- ligation*. (Remember to take the dilution into account when calculating the proportion of background colonies.)

6. Identify clones with the siRNA template insert

- a. Pick clones, isolate plasmid DNA.
- b. Digest the plasmid with *Bam*H I and *Eco*R I (*Hind* III and *Pst* I may also be chosen.), the recombinant plasmid containing the shDNA insert should be linearized by *Bam*H I and uncut by *Eco*R I (or *Hind* III and *Pst* I).
- c. Sequence with the primers shown below to verify that the clone contains the insert, and that it is the desired sequence. The entire pGPU6/Hygro sequence is provided in the <u>Appendix</u> part.

12 III.A. Cloning Hairpin siRNA Inserts into pGPU6/Hygro

Using the pGPU6/Hygro siRNA Expression Vector

Primers used for pGPU6/Hygro sequence

Primer Name	Direction	Sequence
U6 sequencing primer	Forward	5'-GGA CTA TCA TAT GCT TAC CG-3'
T7 sequencing primer	Forward	5'-TAA TAC GAC TCA CTA TAG GG-3'
T3 sequencing primer	Reverse	5'-ATT AAC CCT CAC TAA AGG
		GAA-3'

7. Purify pGPU6/Hygro plasmid for transfection pGPU6/Hygro plasmid preparations must be free of salts, proteins, and other contaminants to ensure efficient transfection. We routinely purify using commercially available plasmid purification products.

B. Transfecting pGPU6/Hygro into Mammalian Cells

We recommend using GenePharma's RNAi-Mate transfection reagent (Cat.No. C-01) to deliver pGPU6/Hygro plasmids into mammalian cells with high efficiency and minimal toxicity. Follow the instructions for using RNAi-Mate provided with the product. RNAi-Mate is a proprietary formulation of polyamines that can be used in the presence or absence of serum in the culture medium. It is suitable for the transfection of a wide variety of cell types.

1. Transfect cells and culture 24 hr without selection



Transfect the purified plasmid into the desired cell line, plate transfected cells at the plating density identified in <u>step II.C.2</u> on page 10, and culture for 24 hr without selection.

It is important to include two non-transfected control cultures. One is subjected to Hygromycin or G418 selection to control for the fraction of cells that survive selection; it will help determine the effectiveness of the transfection and selection. The second control is grown without Hygromycin or G418 selection as a positive control for cell viability.

2. Add medium containing antibiotic

Add culture medium containing the concentration of antibiotic identified in <u>step II.C.1</u> on page 10.

C. Selecting Antibiotic-Resistant Transfected Cells



Once they are prepared, pGPU6/Hygro siRNA expression vectors can be used in transient siRNA expression assays, or to create cell populations or a clonal cell line that stably expresses your siRNA. Note that with normal (nontransformed) and primary cell lines, it may be difficult to obtain clones that stably express siRNA. For these types of cells, we recommend choosing the antibiotic selection strategies outlined in sections 1 and 2 below.

III.B. Transfecting pGPU6/Hygro into Mammalian Cells

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pGPU6/Hygro siRNA Expression Vector

1. Short term antibiotic selection for enrichment of cells that transiently express the siRNA In experiments where the transfection efficiency is low, a rapid antibiotic selection can be used to kill cells that were not transfected with the pGPU6/Hygro siRNA expression vector. This enrichment for transfected cells can be useful for reducing background when analyzing gene knockdown.

- a. Culture the cells for 1–3 days in the antibiotic-containing medium (added in step B.2) to enrich the culture for cells that were successfully transfected.
- b. Analyze the population for an expected phenotype and/or the expression of the target gene.



2. Selecting a population of cells that stably express the siRNA

Creating a population of cells stably expressing the siRNA involves treating cells with Hygromycin or G418 for several days to eliminate cells that were not transfected. The surviving cell population can then be maintained and assessed for reduction of target gene expression.

- a. Culture the cells in medium containing Hygromycin or G418 (added in step B.2) until all of the cells in the non-transfected control culture are killed. At this point, the selection is complete and the cells can be grown without antibiotic until they repopulate the culture vessel.
- b. Analyze expression of the target gene at any time after the cells in the non-transfected control culture have been killed.
- c. Pool and passage antibiotic-resistant cell cultures as needed. It is a good idea to periodically grow the cells with a minimal level of antibiotic selection, to prevent the accumulation of cells that no longer express antibiotic resistance. Often this "minimal level" is about half the antibiotic concentration used to kill off nontranfected cells, but this value varies widely among different cell types.

For many researchers, the goal is to create a clonal cell line that expresses the siRNA template introduced with pGPU6/Hygro. Cloning stably expressing cell lines is advantageous because strains that exhibit the desired amount of gene knockdown can be identified and maintained, and clones that are Hygromycin-resistant but which do not express the siRNA can be eliminated.

Typically the levels of siRNA expression and gene knockdown vary widely among cells. In fact pGPU6/Hygro-transfected cells that survive antibiotic selection may not have a significant reduction in expression of the target gene. Instead, they may have found a way to mitigate the effects of a reduction in the target gene expression by compensating in another fashion or by shutting down expression of the siRNA. To avoid this, it can be useful to isolate clones that can be screened to identify the cells that cause the desired reduction in target gene expression.

III.C. Selecting Antibiotic-Resistant Transfected Cells

- Using the pGPU6/Hygro siRNA Expression Vector
- a. Culture the cells in medium containing Hygromycin or G418 until all of the cells in the non-transfected control culture are killed. At this point, the selection is complete and the cells can be grown without antibiotic selection.
- b. Pick clones:
- i. To pick clones, the cells must be plated at low enough density to grow into colonies without growing into one another. Dip sterilized cloning rings into sterile grease and then place one on top of each colony. Remove the cells that are within the cloning ring and transfer them to a fresh 96 well culture dish.
- ii. When the cells have grown to confluency in a well of a 96 well culture dish,

3. Selecting for clones that stably express the siRNA



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It is often difficult to obtain a stably expressing clone from normal (nontransformed) or primary cell lines using pGPU6/Hygro siRNA expression vectors. If possible choose a transformed or immortal cell line instead.



move them to a well in a 24 well culture dish.

- iii. When the cells have grown to confluency in a well of a 24 well culture dish, split them, and grow them with a minimal level of antibiotic selection to prevent the accumulation of cells that no longer express antibiotic resistance. Often this "minimal level" is about half the antibiotic concentration used to kill off nontranfected cells, but this value varies widely among different cell types.
- c. Assay individual clones for a reduction in the expression of the targetgene.



III.C. Selecting Antibiotic-Resistant Transfected Cells

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IV. Troubleshooting

pGPU6/Hygro siRNA Expression Vector

A. Positive Control Ligation

1. Description of the GAPDH Control Insert

The GAPDH Control Insert (200 nM) is a double-stranded DNA fragment with *Bam*H I and *Bbs* I sticky ends surrounding an siRNA template that targets the GAPDH mRNA. The sequence of the GAPDH Control Insert is perfectly complementary to a region of human GAPDH mRNA. The siRNA expressed from this template sequence has been shown to effectively induce silencing of GAPDH in human cell lines. The GAPDH Control Insert is provided as a control for the ligation reaction.

2. Ligation instructions

- a. Dilute 2 μl of the GAPDH Control Insert with 18 μl nuclease-free water for a final concentration of 20 nM.
- b. Ligate 1 μl of the GAPDH Control Insert into the pGPU6/Hygro vectors using the standard protocol beginning with step <u>III.A.3</u> on page 11.

3. Expected result of the positive control ligation and *E. coli* transformation If the ligation reaction and subsequent *E. coli* transformation procedure are functioning properly, then the ligation reaction with the GAPDH Control Insert (the plus-insert reaction) should provide 2–10 times as many colonies as the minus-insert ligation reaction.

B. Using the Positive and Negative Controls

pGPU6/Hygro Negative Control



The pGPU6/Hygro Negative Control plasmid supplied with the kit is a circular plasmid encoding a hairpin siRNA whose sequence is not found in the mouse, human, or rat genome databases. It is provided ready-to-transfect at 0.5 μ g/ μ l and can be used to control for the effects of introducing the pGPU6/Hygro plasmid into cells. Cells transfected with the pGPU6/Hygro plasmid expressing your target-specific siRNA should be compared to cells transfected with the corresponding pGPU6/Hygro Negative Control.

For any RNAi experiment, it is important to include a culture that is transfected with a negative control plasmid as a basis for analysis of gene knockdown. The optimal negative control insert for expression analysis in a gene silencing experiment is the scrambled sequence of your gene specific siRNA.

16 IV.A. Positive Control Ligation

Troubleshooting

Positive Control construct containing the GAPDH Control Insert

The product of the positive control ligation (described in section <u>IV.A</u> on page 16) is a pGPU6/Hygro plasmid containing an siRNA template targeting GAPDH. This construct can be used to optimize the pGPU6 transfection procedure. Use pGPU6/Hygro-GAPDH and the pGPU6/Hygro Negative Control to transfect cells, and monitor cell viability and gene silencing of GAPDH to identify optimal transfection conditions.

When successfully transfected and expressed, the GAPDH siRNA reduces both the mRNA and protein levels of GAPDH in human cell lines. This slows the growth rate of the cells and reduces the rate of cell proliferation of most cell types. To assess whether siRNA-mediated gene silencing is occurring, levels of GAPDH RNA, levels of GAPDH protein, and/or cell proliferation can be monitored.

Any of the following assays for assessing siRNA-mediated reduction in GAPDH gene



expression can be used:

a. Quantitate mRNA levels by Northern analysis or RT-PCR.

GAPDH mRNA levels are typically reduced 50–90% 48 hr after transfection.

b. Analyze protein levels by Western blot, immunohistochemistry, or immunofluorescence.

GAPDH protein levels are typically reduced 50-90% 48 hr after transfection.

c. Look for a reduction in cell proliferation caused by GAPDH knock down.

Although it is less direct than looking at GAPDH mRNA or protein levels, a reduction in GAPDH activity can be assessed by measuring cell proliferation. Depending on cell type, there should be a >40% reduction in cell number 48–72 hours after transfection.

IV.B. Using the Positive and Negative Controls

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pGPU6/Hygro siRNA Expression Vector

C. Low E. coli Transformation Efficiency

1. Low quality competent cells

Cells could either be nonviable or exhibit low transformation competency. This can be tested by transforming a circular plasmid that has been used successfully in the past.







2. Poor ligation efficiency



If the ligation reaction (section $\underline{III.A.3}$ on page 11) is inefficient, then there will be relatively few plasmids to transform. Possible causes of poor ligation efficiency include:

a. The concentration of the annealed siRNA template insert is lower than expected.

Evaluate $\sim 5 \ \mu$ l of the insert DNA (from step III.A.3.c on page 9) using a 12% native polyacrylamide gel and compare its ethidium bromide staining to bands from a molecular weight marker or another standard of known concentration.

b. The ligase or ligase reaction buffer have become inactive.

Test your ligation components using another vector and insert or replace your ligation components and retry the siRNA insert cloning.

c. One or both of the hairpin siRNA template oligonucleotides have high levels of non-full-length products.

The size of oligonucleotides can be evaluated on an 12% native polyacrylamide gel.

d. The oligonucleotide annealing reaction was ineffective.

A low concentration of one of the oligonucleotides or incomplete denaturation of individual oligonucleotides could have reduced the relative amount of dsDNAs.

Compare the annealed siRNA template insert to each of the single-stranded oligonucleotides using native 8–12% polyacrylamide gel electrophoresis. If the annealed siRNA template insert has bands corresponding to the single-stranded oligonucleotides, then adjusting the concentrations of the single-stranded DNA molecules and heat-denaturing at a higher temperature during siRNA insert preparation (step III.A.2.b on page 9) might improve the percentage of dsDNA products. Alternatively, in some cases, gel purifying the band corresponding to annealed insert may result in better ligation.

18 IV.C. Low E. coli Transformation Efficiency

Troubleshooting

e. Ligation inhibitors in the oligonucleotide preparations

EDTA and high concentrations of salts or other small molecules can inhibit ligation efficiency. Ethanol precipitate the oligonucleotides prior to using them in the cloning procedure (either before or after annealing).

f. Incompatible ends on the insert

Verify that the sequences of the hairpin siRNA template oligonucleotides include 5' *Bbs* 1 and 3' *Bam*H I overhanging sequences for cloning (see Figure 3 on page 8).



3. Too much antibiotic or the wrong antibiotic in the media
 4. Cells were handled poorly
 The plates used for cloning should contain 50–200 μg/ml Ampicillin or carbenicillin. Carbenicillin remains active in plates for longer than ampicillin.
 Competent cells tend to be fragile, so handle them gently throughout the transformation and plating process.

D. Equal Numbers of E. coli Colonies from Minus- and Plus-insert Ligation

Transformations

1. Ligation efficiencySee section C.2 on page 18.for the siRNA insert islow

2. The concentration or activity of the Ampicillin or carbenicillin is too low or high If there are large numbers of clones derived from both ligations, then confirm that the Ampicillin or carbenicillin is active and at 50–200 μ g/ml in the medium. If there are low numbers of clones for each, try transforming a plasmid with an Ampicillin or carbenicillin resistance gene (the pGPU6/Hygro Negative Control plasmid in the kit would be ideal) and confirm that the ampicillin or carbenicillin concentration in the plates is not too high to allow the growth of transformed cells.

E. Poor Mammalian Cell Transformation Efficiency

If you suspect that pGPU6/Hygro transfection is suboptimal, consider using a mammalian expression plasmid containing a reporter gene such as GFP or β -galactosidase to troubleshoot transfection. Below are listed some general suggestions for troubleshooting mammalian cell transfection.

1. pGPU6/Hygro
plasmid is not pure
enoughThe purity of the siRNA plasmid is vitally important for efficient transfection. Repurify
plasmid preparation and transfect again.

IV.D. Equal Numbers of E. coli Colonies from Minus- and Plus-insert Ligation Transformati on

pGPU6/Hygro siRNA Expression Vector

2. Transfection protocol requires optimization The ratio of transfection agent to cells to plasmid is important. Optimize these three components of the transfection protocol.

3. Ineffective transfection reagent If you are using lipofection to facilitate transfection, then test a different transfection agent with your cells. Different cell types respond differently to different transfection reagents.

4. Ineffective siRNA

vector

If you are using siRNA-induced gene knockdown to assess transfection efficiency, consider using a different siRNA. The GAPDH positive control insert supplied with the kit can be used to prepare a vector that has been shown to reduce GAPDH mRNA and protein levels in a variety of cell types.

F. Problems with G418 Selection

1. No transfected cells, or only a few transfected cells survive antibiotic selection

a. Transfection did not work, or the transfection efficiency was poor.

Check transfection efficiency using an expression plasmid that contains a reporter such as GFP or β -galactosidase (this is not supplied with the kit, but it can be prepared using the supplied GFP Control Insert).

b. The G418 concentration is too high.

Perform a G418 dose response experiment with the cell line in your study as described in section II.C.1 on page 10. Every cell type responds differently to different antibiotics. Some cells may even be resistant to G418.

c. The siRNA target may be essential for survival.

If the siRNA target is essential for survival, cells transfected with plasmids that effectively reduce expression of the target gene may die. To test whether the target gene is essential for survival, transfect cells with the pGPU6/Hygro containing your siRNA template, and culture transformants without antibiotic selection. If significant cell death occurs, it is likely that the siRNA target is important for cell growth and metabolism.

d. Grow the cells that do survive selection (if there are any).

The cells that remain after antibiotic selection can be grown up and subsequently analyzed as a population or can be cloned using cloning rings and analyzed individually.

e. Perform a less stringent antibiotic selection.

Incubate the culture with G418 selection until only \sim 50% of the cells are killed. Then add fresh medium lacking antibiotic and incubate the culture for 24–48 hr

20 IV.F. Problems with G418 Selection

Troubleshooting

withoutantibiotic selection. Next add antibiotic-containing culture medium again, and culture the cells until \sim 50% have died a second time. Repeat this cycle until colonies are visible. Always include a control where cells that have not been transfected are grown under the same G418 selection regimen. Although it occurs at a very low frequency, cells do spontaneously become resistant to antibiotics and including a non-transfected control culture allows you to determine the effectiveness of the transfection and antibiotic selection.

f. Normal (nontransformed) and primary cell lines may not survive the transfection and/or selection process.

If possible use an immortal or transformed cell line for studies involving stable



expression of siRNA.

2. Cells become contaminated following the addition of the antibiotic

3. Non-transfected cells survive selection

The antibiotic may be contaminated. G418 solutions can be filter sterilized or purchased as sterile reagents. To prepare antibiotic solutions in the lab, use sterile reagents to resuspend antibiotics.

a. The G418 concentration is not high enough to kill cells.

A careful dose response experiment should be performed to determine the concentration that kills cells lacking a Hygromycin resistance gene. This is described in <u>section II.C.</u> *Optimizing Antibiotic Selection Conditions* on page 10. The amount of time required to completely kill the cells should also be recorded, and this concentration and time should be used for each transfection experiment.

b. Cell density is too high.

If the cells are too crowded, they may be not be killed very effectively. Split cultures that are too close to confluency for good antibiotic selection. On the other hand, low cell density cultures typically grow slowly, and may be more sensitive to antibiotics than higher cell density cultures of the same cell line.

c. The G418 may be inactive.

• At 37°C, G418 is stable for only a few days, therefore antibiotic-containing culture media must be replenished accordingly in order to apply selection pressure.

• Consider purchasing a new batch of antibiotic, or preparing a fresh solution of antibiotic.

Problems with G418 Selection

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pGPU6/Hygro siRNA Expression Vector

V. Appendix

A. References

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22 V.A. References

Appendix





E-01	F-01	Component
20 µl		pGPU6/Hygro (circular) (50 ng/ul)
	20µl	pGPU6/Hygro (linearized) (50 ng/ul)
10 µl	10 µl	pGPU6/Hygro Negative control (0.5 µg/µl)
10 µl	10 µl	GAPDH Control Insert (20 nM)
0.5 ml	0.5 ml	10×shDNA Annealing Solution

Store the pGPU6/Hygro siRNA Expression Kit at -20° C in a non-frost-free freezer (if desired the 1X DNA Annealing Solution can be stored at room temperature). Properly stored kits are guaranteed for 6 months from the date received.

C. Quality Control



Functional testing

The pGPU6/Hygro siRNA expression vector is ligated with the GAPDH Control Insert according to the instructions in this booklet. Ligation efficiency is then determined.

Each component is tested in GenePharma's rigorous nuclease assays.

RNase activity

None detected after incubation with ³²P-labeled RNA; analyzed by PAGE.

Non-specific endonuclease/nickase activity

None detected after incubation with supercoiled plasmid DNA; analyzed on 1% agarose.

Exonuclease activity

None detected after incubation with 32P-labeled *Sau*3A fragments of pUC19; analyzed by PAGE.



D. Map of pGPU6/Hygro



Appendix





V.E. pGPU6/Hygro multiple cloning site Region

pGPU6/Hygro siRNA Expression Vector

The Sequence of pGPU6/Hygro plasmid (5011bp in length)

	TAGTTATTAA	TAGTAATCAA	TTACGGGGTC	ATTAGTTCAT	AGCCCATATA	50
	TGGAGTTCCG	CGTTACATAA	CTTACGGTAA	ATGGCCCGCC	TGGCTGACCG	100
	CCCAACGACC	CCCGCCCATT	GACGTCAATA	ATGACGTATG	TTCCCATAGT	150
	AACGCCAATA	GGGACTTTCC	ATTGACGTCA	ATGGGTGGAG	TATTTACGGT	200
	AAACTGCCCA	CTTGGCAGTA	CATCAAGTGT	ATCATATGCC	AAGTACGCCC	250
	CCTATTGACG	TCAATGACGG	TAAATGGCCC	GCCTGGCATT	ATGCCCAGTA	300
	CATGACCTTA	TGGGACTTTC	CTACTTGGCA	GTACATCTAC	GTATTAGTCA	350
	TCGCTATTAC	CATGGTGATG	CGGTTTTTGGC	AGTACATCAA	TGGGCGTGGA	400
	TAGCGGTTTG	ACTCACGGGG	ATTTCCAAGT	CTCCACCCCA	TTGACGTCAA	450
	TGGGAGTTTG	TTTTGGCACC	AAAATCAACG	GGACTTTCCA	AAATGTCGTA	500
	ACAACTCCGC	CCCATTGACG	CAAATGGGCG	GTAGGCGTGT	ACGGTGGGAG	550
	GTCTATATAA	GCAGAGCTGG	TTTAGTGAAC	CGTCAGATCC	GCTAGCGCTA	600
	CCGGTCGCCA	CCATGGTGAG	CAAGGGCGAG	GAGCTGTTCA	CCGGGGTGGT	650
	GCCCATCCTG	GTCGAGCTGG	ACGGCGACGT	AAACGGCCAC	AAGTTCAGCG	700
\langle	TGTCCGGCGA	GGGCGAGGGC	GATGCCACCT	ACGGCAAGCT	GACCCTGAAG	750
	TTCATCTGCA	CCACCGGCAA	GCTGCCCGTG	CCCTGGCCCA	CCCTCGTGAC	800
	CACCCTGACC	TACGGCGTGC	AGTGCTTCAG	CCGCTACCCC	GACCACATGA	850
	AGCAGCACGA	CTTCTTCAAG	TCCGCCATGC	CCGAAGGCTA	CGTCCAGGAG	900
	CGCACCATCT	TCTTCAAGGA	CGACGGCAAC	TACAAGACCC	GCGCCGAGGT	950
	GAAGTTCGAG	GGCGACACCC	TGGTGAACCG	CATCGAGCTG	AAGGGCATCG	1000
	ACTTCAAGGA	GGACGGCAAC	ATCCTGGGGC	ACAAGCTGGA	GTACAACTAC	1050
	AACAGCCACA	ACGTCTATAT	CATGGCCGAC	AAGCAGAAGA	ACGGCATCAA	1100
	GGTGAACTTC	AAGATCCGCC	ACAACATCGA	GGACGGCAGC	GTGCAGCTCG	1150
	CCGACCACTA	CCAGCAGAAC	ACCCCCATCG	GCGACGGCCC	CGTGCTGCTG	1200
	CCCGACAACC	ACTACCTGAG	CACCCAGTCC	GCCCTGAGCA	AAGACCCCAA	1250
	CGAGAAGCGC	GATCACATGG	TCCTGCTGGA	GTTCGTGACC	GCCGCCGGGA	1300
	TCACTCTCGG	CATGGACGAG	CTGTACAAGT	CCGGACTCAG	ATCCACCGGA	1350
	TCTAGATAAC	TGATCATAAT	CAGCCATACC	ACATTTGTAG	AGGTTTTACT	1400
	TGCTTTAAAA	AACCTCCCAC	ACCTCCCCCT	GAACCTGAAA	CATAAAATGA	1450
	ATGCAATTGT	TGTTGTTAAC	TTGTTTATTG	CAGCTTATAA	TGGTTACAAA	1500
\succ	TAAAGCAATA	GCATCACAAA	TTTCACAAAT	AAAGCATTTT	TTTCACTGCA	1550
<	TTCTAGTTGT	GGTTTGTCCA	AACTCATCAA	TGTATCTTAA	CGCGCGCGCG	1600
	CAATTAACCC	TCACTAAAGG	GAACAAAAGC	TGGAGCTCCA	CCGCGGTGGC	1650
	GGCCGCTCTA	GAACTAGTGG	ATCCCCCGGG	CTGCAGGAAT	TCGATATCAA	1700
	GCTTATCGAT	ACCGTCGACC	TCGAGGAAGA	CACGGTGTTT	CGTCCTTTCC	1750
	ACAAGATATA	TAAAGCCAAG	AAATCGAAAT	ACTTTCAAGT	TACGGTAAGC	1800 🔨
	ATATGATAGT	CCATTTTAAA	ACATAATTTT	AAAAACTGCA	AACTACCCAA	1850
	GAAATTATTA	CTTTCTACGT	CACGTATTTT	GTACTAATAT	CTTTGTGTTT	1900

26 V.F. Sequence of pGPU6/Hygro

Appendix

	ACAGTCAAAT	TAATTCTAAT	TATCTCTCTA	ACAGCCTTGT	ATCGTATATG	1950
\mathcal{N}	CAAATATGAA	GGAATCATGG	GAAATAGGCC	CTCTTCCTGC	CCGACCTTGG	2000
\bigvee	TACCCAATTC	GCCCTATAGT	GAGTCGTATT	ACGCGCGTAA	ATTGTAAGCG	2050
	TTAATATTTT	GTTAAAATTC	GCGTTAAATT	TTTGTTAAAT	CAGCTCATTT	2100
	TTTAACCAAT	AGGCCGAAAT	CGGCAAAATC	CCTTATAAAT	CAAAAGAATA	2150
	GACCGAGATA	GGGTTGAGTG	TTGTTCCAGT	TTGGAACAAG	AGTCCACTAT	2200
	TAAAGAACGT	GGACTCCAAC	GTCAAAGGGC	GAAAAACCGT	CTATCAGGGC	2250
	GATGGCCCAC	TACGTGAACC	ATCACCCTAA	TCAAGTTTTT	TGGGGTCGAG	2300
	GTGCCGTAAA	GCACTAAATC	GGAACCCTAA	AGGGAGCCCC	CGATTTAGAG	2350
	CTTGACGGGG	AAAGCCGGCG	AACGTGGCGA	GAAAGGAAGG	GAAGAAAGCG	2400
	AAAGGAGCGG	GCGCTAGGGC	GCTGGCAAGT	GTAGCGGTCA	CGCTGCGCGT	2450
	AACCACCACA	CCCGCCGCGC	TTAATGCGCC	GCTACAGGGC	GCGTCAGGTG	2500
	GCACTTTTCG	GGGAAATGTG	CGCGGAACCC	CTATTTGTTT	ATTTTTCTAA	2550
	ATACATTCAA	ATATGTATCC	GCTCATGAGA	CAATAACCCT	GATAAATGCT	2600
	TCAATAATAT	TGAAAAAGGA	AGAGTCCTGA	GGCGGAAAGA	ACCAGCTGTG	2650
	GAATGTGTGT	CAGTTAGGGT	GTGGAAAGTC	CCCAGGCTCC	CCAGCAGGCA	2700
$\mathcal{N}_{\mathcal{N}}$	GAAGTATGCA	AAGCATGCAT	CTCAATTAGT	CAGCAACCAG	GTGTGGAAAG	2750
O	TCCCCAGGCT	CCCCAGCAGG	CAGAAGTATG	CAAAGCATGC	ATCTCAATTA	2800
	GTCAGCAACC	ATAGTCCCGC	CCCTAACTCC	GCCCATCCCG	CCCCTAACTC	2850
0	CGCCCAGTTC	CGCCCATTCT	CCGCCCATG	GCTGACTAAT	TTTTTTTTATT	2900
	TATGCAGAGG	CCGAGGCCGC	CTCGGCCTCT	GAGCTATTCC	AGAAGTAGTG	2950
	AGGAGGCTTT	TTTGGAGGCC	TAGGCTTTTG	CAAAGATCGA	TCAAGAGACA	3000
	GGATGAGGAT	CGTTTCGCAT	GATTGAACAA	GATGGATTGC	ACGCAGGTTC	3050
	TCCGGCCGCT	TGGGTGGAGA	GGCTATTCGG	CTATGACTGG	GCACAACAGA	3100
	CAATCGGCTG	CTCTGATGCC	GCCGTGTTCC	GGCTGTCAGC	GCAGGGGCGC	3150
	CCGGTTCTTT	TTGTCAAGAC	CGACCTGTCC	GGTGCCCTGA	ATGAACTGCA	3200
	AGACGAGGCA	GCGCGGCTAT	CGTGGCTGGC	CACGACGGGC	GTTCCTTGCG	3250
	CAGCTGTGCT	CGACGTTGTC	ACTGAAGCGG	GAAGGGACTG	GCTGCTATTG	3300
	GGCGAAGTGC	CGGGGCAGGA	TCTCCTGTCA	TCTCACCTTG	CTCCTGCCGA	3350
	GAAAGTATCC	ATCATGGCTG	ATGCAATGCG	GCGGCTGCAT	ACGCTTGATC	3400
	CGGCTACCTG	CCCATTCGAC	CACCAAGCGA	AACATCGCAT	CGAGCGAGCA	3450
	CGTACTCGGA	TGGAAGCCGG	TCTTGTCGAT	CAGGATGATC	TGGACGAAGA	3500
	GCATCAGGGG	CTCGCGCCAG	CCGAACTGTT	CGCCAGGCTC	AAGGCGAGCA	3550
\sim	TGCCCGACGG	CGAGGATCTC	GTCGTGACCC	ATGGCGATGC	CTGCTTGCCG	3600
$\langle 0 \rangle$	AATATCATGG	TGGAAAATGG	CCGCTTTTCT	GGATTCATCG	ACTGTGGCCG	3650
\sim	GCTGGGTGTG	GCGGACCGCT	ATCAGGACAT	AGCGTTGGCT	ACCCGTGATA	3700
2	TTGCTGAAGA	GCTTGGCGGC	GAATGGGCTG	ACCGCTTCCT	CGTGCTTTAC	3750
4	GGTATCGCCG	CTCCCGATTC	GCAGCGCATC	GCCTTCTATC	GCCTTCTTGA	3800
	CGAGTTCTTC	TGAGCGGGAC	TCTGGGGTTC	GAAATGACCG	ACCAAGCGAC	3850
	GCCCAACCTG	CCATCACGAG	ATTTCGATTC	CACCGCCGCC	TTCTATGAAA	3900
	GGTTGGGCTT	CGGAATCGTT	TTCCGGGACG	CCGGCTGGAT	GATCCTCCAG	3950
	CGCGGGGGATC	TCATGCTGGA	GTTCTTCGCC	CACCCTAGGG	GGAGGCTAAC	4000
	TGAAACACGG	AAGGAGACAA	TACCGGAAGG	AACCCGCGCT	ATGACGGCAA	4050

pGPU6/Hygro siRNA Expression Vector

V.F. Sequence of pGPU6/Hygro

	TAAAAAGACA	GAATAAAACG	CACGGTGTTG	GGTCGTTTGT	TCATAAACGC	4100
ЛX.	GGGGTTCGGT	CCCAGGGCTG	GCACTCTGTC	GATACCCCAC	CGAGACCCCA	4150
NX	TTGGGGCCAA	TACGCCCGCG	TTTCTTCCTT	TTCCCCACCC	CACCCCCAA	4200
	GTTCGGGTGA	AGGCCCAGGG	CTCGCAGCCA	ACGTCGGGGC	GGCAGGCCCT	4250
	GCCATAGCCT	CAGGTTACTC	ATATATACTT	TAGATTGATT	TAAAACTTCA	4300
	TTTTTAATTT	AAAAGGATCT	AGGTGAAGAT	CCTTTTTGAT	AATCTCATGA	4350
	CCAAAATCCC	TTAACGTGAG	TTTTCGTTCC	ACTGAGCGTC	AGACCCCGTA	4400
	GAAAAGATCA	AAGGATCTTC	TTGAGATCCT	TTTTTTTCTGC	GCGTAATCTG	4450
	CTGCTTGCAA	ACAAAAAAAC	CACCGCTACC	AGCGGTGGTT	TGTTTGCCGG	4500
	ATCAAGAGCT	ACCAACTCTT	TTTCCGAAGG	TAACTGGCTT	CAGCAGAGCG	4550
	CAGATACCAA	ATACTGTCCT	TCTAGTGTAG	CCGTAGTTAG	GCCACCACTT	4600
	CAAGAACTCT	GTAGCACCGC	CTACATACCT	CGCTCTGCTA	ATCCTGTTAC	4650
	CAGTGGCTGC	TGCCAGTGGC	GATAAGTCGT	GTCTTACCGG	GTTGGACTCA	4700
	AGACGATAGT	TACCGGATAA	GGCGCAGCGG	TCGGGCTGAA	CGGGGGGGTTC	4750
	GTGCACACAG	CCCAGCTTGG	AGCGAACGAC	CTACACCGAA	CTGAGATACC	4800
	TACAGCGTGA	GCTATGAGAA	AGCGCCACGC	TTCCCGAAGG	GAGAAAGGCG	4850
X	GACAGGTATC	CGGTAAGCGG	CAGGGTCGGA	ACAGGAGAGC	GCACGAGGGA	4900
\sim	GCTTCCAGGG	GGAAACGCCT	GGTATCTTTA	TAGTCCTGTC	GGGTTTCGCC	4950
	ACCTCTGACT	TGAGCGTCGA	TTTTTGTGAT	GCTCGTCAGG	GGGGCGGAGC	5000
	CTATGGAAAA	ACGCCAGCAA	CGCGGCCTTT	TTACGGTTCC	TGGCCTTTTG	5050
	CTGGCCTTTT	GCTCACATGT	TCTTTCCTGC	GTTATCCCCT	GATTCTGTGG	5100
	ATAACCGTAT	TACCGCCATG	CAT	\mathcal{V}		5150
				V		



