GenePharma

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.

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.

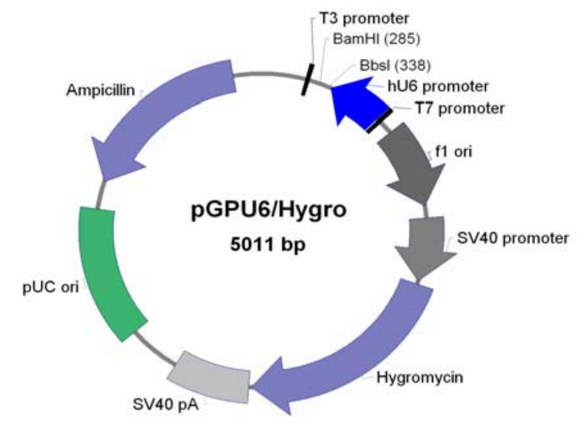
pGPU6/Hygro plasmid is supplied ligation-ready

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

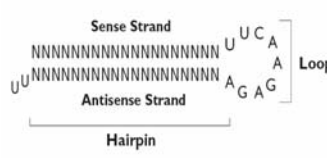
Figure 1. pGPU6/Hygro siRNA expression vector map



Feature	Nucleotide position
T3 promoter binding site	215-234
HU6 promoter	346-616
T7 promoter binding site	623-644
F1 origin	711-1125
SV40 promoter	1190-1514
Hygromycin resistance gene	1532-2555
SV40 polyA	2568-2940
pUC origin of replication	3200-3870
Ampicillin resistance gene	4015-4875

C. siRNA Template Design

Figure 2. Hairpin siRNA



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 section II.B 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)
---	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 *E. coli* 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.

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 μg	
E-05/F-05	pGPU6/Hygro	1 μg	
E-07/F-07	pGPU6/GFP/Neo	1 μg	

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

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 sequences

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 ≥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.

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.

B. Hairpin siRNA Template Oligonucleotide Design & Ordering

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.

Oligonucleotide design

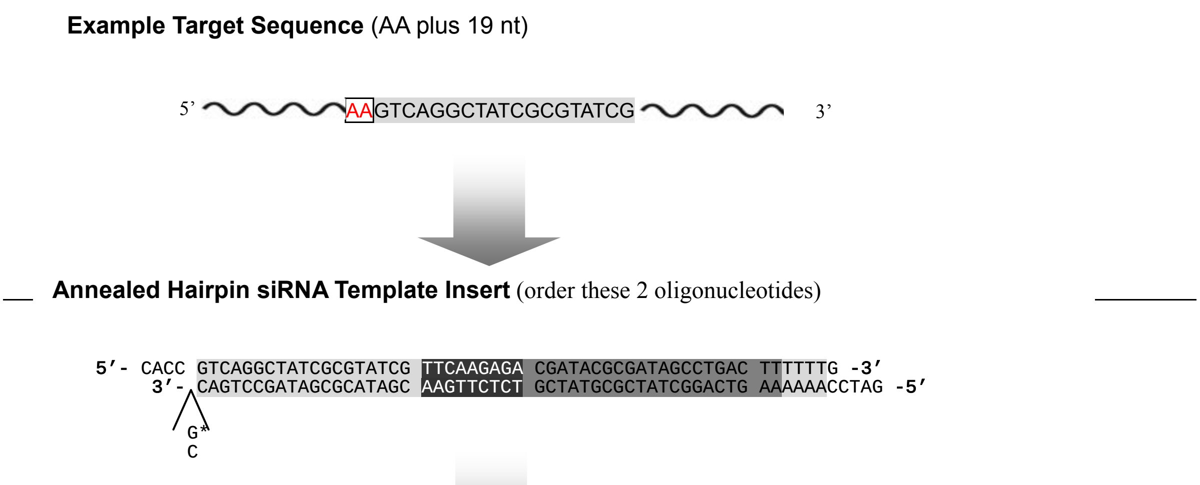
Two complementary oligonucleotides must be synthesized, annealed, and ligated into pGPU6/Hygro for each siRNA target site. Figure 3 on page 8 shows schematically how to convert siRNA target sites into oligonucleotide sequences for use in the pGPU6/Hygro vectors. The oligonucleotides encode a hairpin 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 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

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



* Include an additional GC base pair at this position ***only*** if the downstream base on the top strand (the +1 position of the siRNA) is a Tor a C; if the +1 position is a G or an A, as it is in this example sequence, do not include it. The purpose of this additional base pair is to provide a G or an A residue as the first nucleotide of the siRNA transcript because RNA pol III prefers to initiate transcription with a purine, thus it helps to facilitate efficient transcription. Note, this additional nucleotide will not be complementary to either the target mRNA or the antisense strand of the hairpin siRNA. This extra nucleotide in the sense strand appears to have no effect on the activity of the hairpin siRNA.

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 µg/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 step *2. 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 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 µl culture medium containing 25–4000 µg/ml G418.

c. 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.

2. Optimal plating density

a. Plate several different amounts of cells into separate wells of a 24 well dish 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.

III. Using the pGPU6/Hygro siRNA Expression Vector

A. Cloning Hairpin siRNA Inserts into pGPU6/Hygro

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

a. Dissolve the hairpin siRNA template oligonucleotides in approximately 100 µl of 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).

c. Dilute the oligonucleotides to approximately 100 µM.

2. Anneal the siRNA template oligonucleotides

a. Assemble the 50 µl annealing mixture as follows:

Amount	Component
5 µl	10XshDNA Annealing Solution
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.

c. The annealed siRNA template insert can either be ligated into a pGPU6/Hygro 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 µl ligation reactions; a plus-insert ligation, and the minus-insert negative control. To each tube, add the following reagents:

plus-insert	minus-insert	Component
1 µl	---	diluted annealed siRNA insert (from step 3.a.1 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)

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 µl of the ligation reaction.)

b. Plate the transformed cells on LB plates containing 50–200 µg/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 Appendix part.

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 step II.C.2 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 step II.C.1 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.

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.

3. Selecting for clones that stably express the siRNA

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.

NOTE

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.

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.

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, 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.

IV. Troubleshooting

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 III.A.3 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 µg/µl 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.

Positive Control construct containing the GAPDH Control Insert

The product of the positive control ligation (described in section IV.A 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.

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 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 ~5 µl 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.

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

The plates used for cloning should contain 50–200 µg/ml Ampicillin or carbenicillin. Carbenicillin remains active in plates for longer than ampicillin.

4. Cells were handled poorly

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 efficiency for the siRNA insert is low

See section C.2 on page 18.

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 µg/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 enough

The purity of the siRNA plasmid is vitally important for efficient transfection. Repurify plasmid preparation and transfect again.

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 ~50% of the cells are killed. Then add fresh medium lacking antibiotic and incubate the culture for 24–48 hr withoutantibiotic selection. Next add antibiotic-containing culture medium again, and culture the cells until ~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

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.

3. Non-transfected cells survive selection

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 section *II.C. 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.

V. Appendix

A. References

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B. pGPU6/Hygro siRNA Expression Kit Specifications

Kit Components and Storage

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.

Nuclease testing

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

RNase activity

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

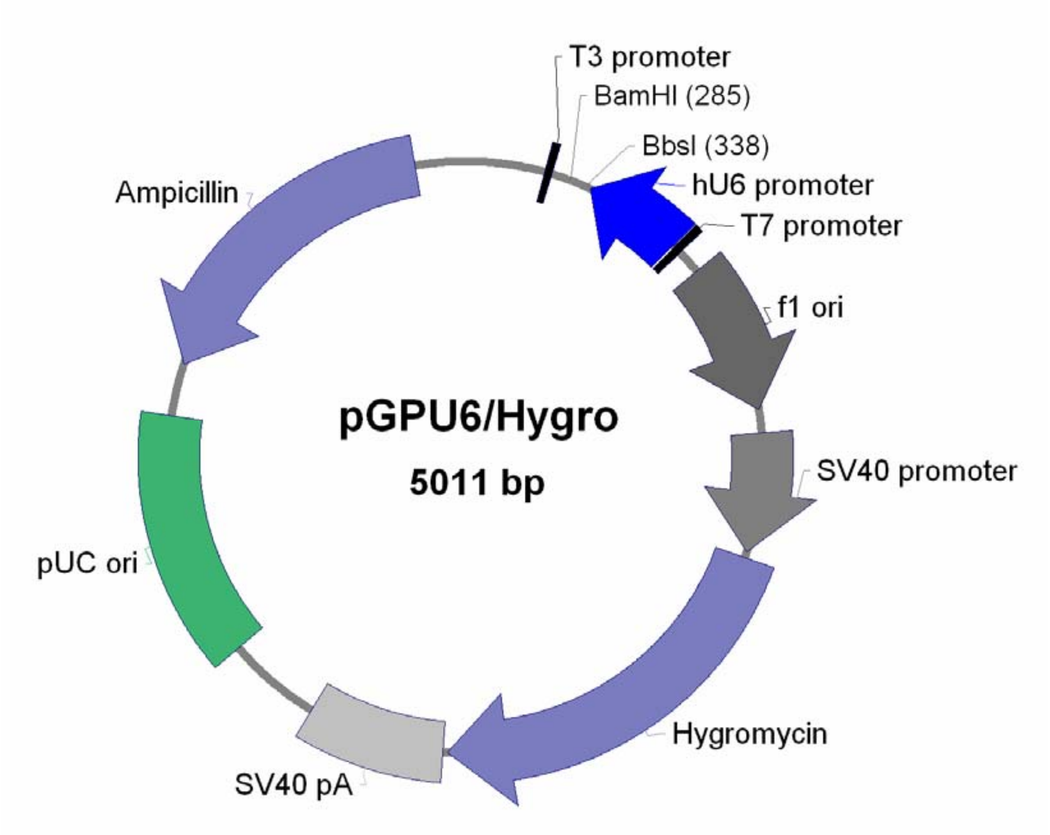
Non-specific endonuclease/nickase activity

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



Feature	Nucleotide position
T3 promoter binding site	215-234
HU6 promoter	346-616
T7 promoter binding site	623-644
F1 origin	711-1125
SV40 promoter	1190-1514
Hygromycin resistance gene	1532-2555
SV40 polyA	2568-2940
pUC origin of replication	3200-3870
Ampicillin resistance gene	4015-4875

E. pGPU6/Hygro Multiple Cloning Site Region

pGPU6/Hygro Multiple Cloning Site Region

(sequence shown 1602-2032)

GTAATACGACTCACTATAGGGCGAATTGGGTACCAAGGTCGGGCAGGAAGAGGGCCTATTTC
T7 primer binding site
CCATGATTCCTTCATATTTGCATATACGATACAAGGCTGTTAGAGAGATAATTAGAATTAATTTGACTGTAAA
CACAAAGATATTAGTACAAAATACGTGACGTAGAAAGTAATAATTTCTTGGGTAGTTTGCAGTTTTAAAAT
TATGTTTTAAAATGGACTATCATATGCTTACCGTAACTTGAAAGTATTTCGATTTCTTGGC
U6 promoter primer

*Bam*H I
TTTATATATCTTGTGGAAAGGACGAAA CACCGTGTCTTC...... G GATCCACTAG
AAATATATAGAACACCTTTCCTGCTTTGTGG CACAGAAG.......CCTAG GTGATC
Bbs I
TTCTAGAGCGGCCGCCACCGCGGTGGAGCTCCAGCTTTTGTTCCCTTTAGTGAGGGTTAATT
T3 primer binding site

F. Sequence of pGPU6/Hygro

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

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 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 CGGTTTTGGC AGTACATCAA TGGGCGTGGA 400
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 TGGGAGTTTG TTTTGGCACC AAAATCAACG GGACTTTCCA AAATGTCGTA 500
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 GTCTATATAA GCAGAGCTGG TTTAGTGAAC CGTCAGATCC GCTAGCGCTA 600
 CCGGTCGCCA CCATGGTGAG CAAGGGCGAG GAGCTGTTCA CCGGGGTGGT 650
 GCCCATCCTG GTCGAGCTGG ACGGCGACGT AAACGGCCAC AAGTTCAGCG 700
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CCCGACAACC ACTACCTGAG CACCCAGTCC GCCCTGAGCA AAGACCCCAA 1250
CGAGAAGCGC GATCACATGG TCCTGCTGGA GTTCGTGACC GCCGCCGGGA 1300
TCACTCTCGG CATGGACGAG CTGTACAAGT CCGGACTCAG ATCCACCGGA 1350
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TGCTTTAAAA AACCTCCCAC ACCTCCCCCT GAACCTGAAA CATAAAATGA 1450
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GCTTATCGAT ACCGTCGACC TCGAGGAAGA CACGGTGTTT CGTCCTTTCC 1750
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