

GeneSuppressor™ System

For Knockdown of Genes in Mammalian Cells

(version 07/04)

Catalog No.: IMG-700

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Advantages of GeneSuppressor kit:

GeneSuppressor is a plasmid-based system to generate siRNAs for gene knockdown. Inserts cloned into GeneSuppressor plasmids express RNAs under U6 promoter in the transfected mammalian cells. The RNAs are expressed as fold-back stem-loop structures that are processed into the siRNAs

- No worry about RNA degradation.
- Cost effective.
- Allows sustained silencing of protein production.
- Generation of permanent cell lines may be possible, where single or multiple genes are knocked down.
- Reproducible transfection efficiency.

The plasmid for cloning your gene of interest as well as some precloned GeneSuppressor plasmids are available from Imgenex. Below is a list of premade siRNA constructs. These are constructs that are ready for transfection into mammalian cells.

Target	Catalog No.
Human p53	IMG-701
Human Lamin A/C	IMG-703

Each kit contains a tested plasmid DNA construct for silencing a specific gene in mammalian cells, transfection reagents, and a specific antibody for monitoring of gene knockdown.

We are adding new products every week, please visit our website, www.imgenex.com, for updates.

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The GeneSuppressor product lines are for research use only. The products should not be used for therapeutics, diagnostics, or drug development. This product is covered by US Patent Application assigned to University of Michigan. This product is not for resale, repackaging and is non-transferable. This product is not for modification, except for cloning the target genes. Any other use requires a license from the University of Michigan (see below). Some applications in which this product may be used are covered by patents pending or issued. Because purchase of this product does not include a license to perform any patented application, users of this product may be required to obtain a patent license depending upon the particular application and country in which the product is used.

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A. Background:

Small interfering RNAs (siRNAs) have gained much attention for their powerful ability to suppress gene expression. Introduction of double-stranded RNA (dsRNA), that are homologous in sequence to a gene, has proven to suppress that gene's expression through a process known as RNA interference (RNAi); this process post-transcriptionally silences a gene through mRNA inhibition or degradation. However, in most mammalian cells dsRNA provokes a non-specific cytotoxic response. siRNAs appears to suppress gene expression without producing a non-specific cytotoxic response. It is believed that the small size of the siRNAs, as compared with dsRNA, prevents activation of the dsRNA-inducible interferon system in mammalian cells. This avoids the non-specific phenotypes normally produced by dsRNA (>30 base pairs). Similar to dsRNA, siRNAs inhibit gene expression by inducing RNAi. siRNAs are 21- to 23-nucleotide RNA particles, with characteristic 2- to 3- nucleotide 3'-overhanging ends, which are generated by ribonuclease III cleavage from longer dsRNAs.

Intracellular transcription of siRNAs can be achieved by cloning the siRNA templates into RNA Pol III transcription units, which normally encode the smaller nuclear RNA (snRNA) U6 or the human RNase P RNA H1. Two approaches have been developed for expressing siRNA. In the first, sense and antisense strands constituting the siRNA duplex are transcribed by individual promoters; in the second, siRNAs are expressed as fold-back stem-loop structures that are processed into the siRNAs. The U6 and H1 promoters are members of the type III class of Pol III promoters. U6 and H1 are different in size but contain the same conserved sequence elements or protein binding sites. The +1 nucleotide of the U6-like promoters is always guanosine, whereas the +1 for H1 promoters is adenosine. The termination signal for these promoters is defined by 5 thymidines, and the transcript is typically cleaved after the second uridine. Cleavage at this position generates a 3' UU overhang in the expressed siRNA, which is similar to the 3' overhangs of synthetic siRNAs. Any sequence <400 nucleotides can be transcribed by these promoters, therefore they are ideally suited for the expression of ~21-nucleotide siRNAs of ~50-nucleotide RNA stem-loops.

siRNA plasmid vectors appear to have an advantage over synthetic siRNAs. Cells transfected with an siRNA expression vector would experience steady, long-term mRNA inhibition, whereas cells which are transfected with exogenous synthetic siRNAs typically recover from mRNA suppression within seven days or ten rounds of cell division.

References:

1. Brummelkamp T., Bernards R., Agami R. A System for Stable Expression of Short Interfering RNAs in Mammalian Cells. *Science*, 296(5567): pp. 550-553 (2002).
2. Elbashir, SM, Harborth J, Weber K, and Tuschl T. Analysis of gene function in somatic mammalian cells using small interfering RNAs. *Methods*, 26: 199-213 (2002).
2. Hammond S.M., Boettcher S., et. al. Argonaute2, a Link Between Genetic and Biochemical Analyses of RNAi. *Science*, 293:1146-1150 (2001).
3. Lee N.S., Dohjima T., et. al. Expression of Small Interfering RNAs Targeted Against HIV-1 Rev Transcripts in Human Cells. *Nat Biotechnol* 20(5): 500-505 (2002).
4. Paddison PJ and Hannon GJ. RNA interference: the new somatic cell genetics? *Cancer Cell* 2: 17-23 (2002).
5. Paul CP. et al. Effective expression of small interfering RNA in human cells. *Nature Biotechnol*, 20 (5): 505-508 (2002).
6. Tabara H., Sarkissian M., et. al. The rde-1 gene, RNA interference, and transposon silencing in *C. elegans*. *Cell*, 99(2): 123-132 (1999).
7. Tuschl T. Expanding small RNA interference. *Nat Biotechnol* 20(5): 446-448 (2002).
8. Zamore P.D. Ancient Pathways Programmed by Small RNAs. *Science*, 296: 1265-1269 (2002).
9. Polo-like kinase (Plk)1 depletion induces apoptosis in cancer cells Xiaoqi Liu and Raymond L. Erikson. *PNAS* 100 (10): 5789-5794 (2003).(Imgenex citation)

B. Kit Components and Storage:

The components included in this kit need to be stored at different temperatures.

Reagents (-20°C storage)		Quantity
IMG-700-1	Linearized pSuppressor plasmid (50 ng/μl).....	20 μl
GSC-1005	Sequencing primer (10 pmol/μl).....	20 μl
IMG-700-6	Negative control plasmid (100 ng/μl).....	20 μl
GSC-1004	Annealing Buffer.....	50 μl

Reagents (4°C storage)

GSC-1003	Deionized water.....	2 ml
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The negative control plasmid contains a scrambled sequence that does not show significant homology to rat, mouse or human gene sequences. The sequence of the insert in the control plasmid is:

5' TCGATCAGTCACGTTAATGGTCGTTttcaagagaAACGACCATTAACGTGACTG
ATTTTT -3'

AGTCAGTGCAATTACCAGCAAaagttctctTTGCTGGTAATTGCACTGACTAAAAA
GATC-5'

It may be amplified by transforming suitable bacterial strain such as DH5α and grown in LB medium containing 100μg/ml of ampicillin.

The kit contains enough primer for 20 reactions. For more reactions, you may synthesize a primer using the following sequence:

5'-AAT. ACG. TGA. CGT. AGA. AAG. TA-3'

Additional required materials (not included)

Appropriate restriction enzymes
T4 DNA ligase and buffers
Competent bacteria
LB or other bacterial culture media

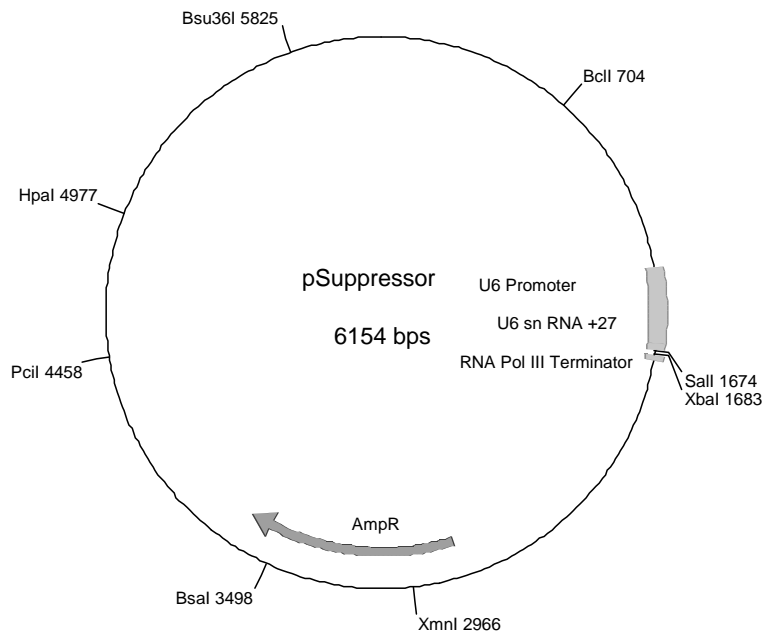


Figure 1. Schematic diagram of pSuppressor vector (based on Paul CP, et al. Effective expression of small interfering RNA in human cells. *Nature Biotechnol*, 20 (5): 505-508 (2002).

C. Protocols:

The protocols in this manual are provided as a guideline. These may need to be modified for individual experiment.

i) Cloning of the insert. Approximately 500 ng of plasmid DNA is included in the kit, which is enough for cloning 20 inserts. Below is a schematic map of the pSuppressor plasmid and description of sequences around siRNA expression cassette. The insert should be cloned into *Sa*I and *Xba*I sites. Please see next page for cloning strategy.

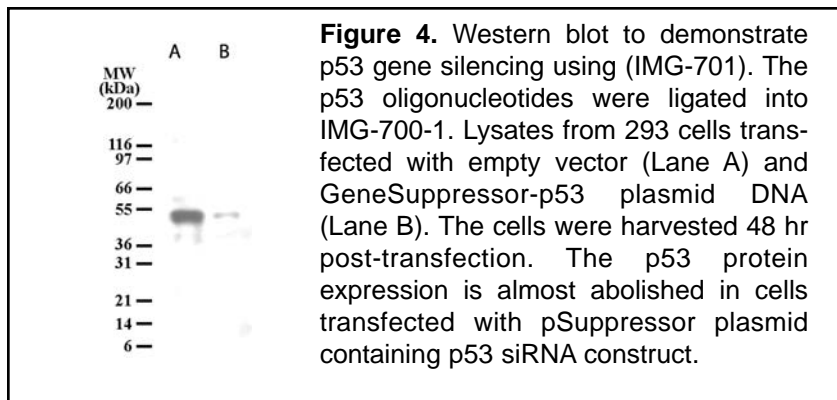
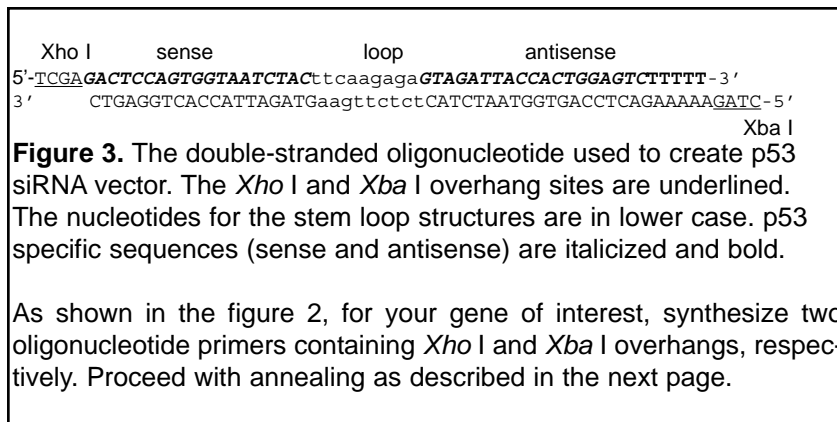
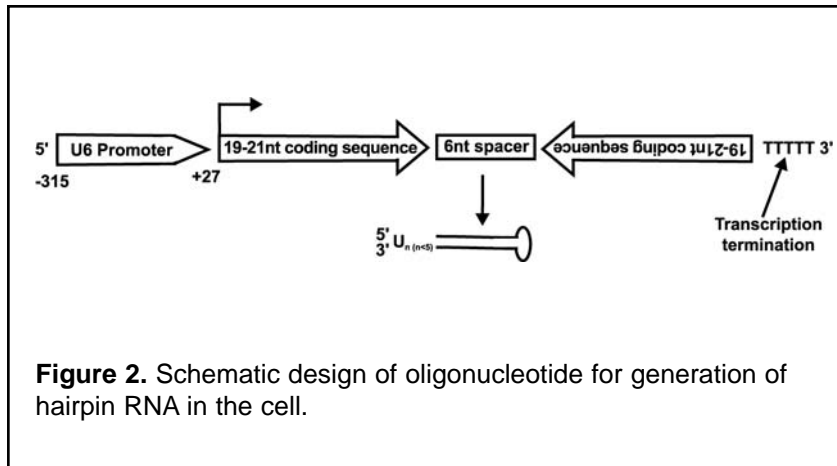
Detailed protocol- Cloning the target siRNA of interest into pSuppressor cassette.**a) Preparation of vector and insert for cloning:**

The pSuppressor vector DNA (provided in the kit) has been digested with *Sal* I and *Xba* I to generate compatible ends for cloning. To ensure that the correct restriction fragment has been cloned, add compatible restriction sites, *Xho* I at the 5' end and *Xba* I at the 3' end of the primer sequences. *Xho* I restriction site is compatible with *Sal* I site and will allow cloning into *Sal* I site. However, after cloning into *Sal* I site, both *Sal* I and *Xho* I sites are lost. This strategy allows quick screening of the recombinant plasmids. The recombinant plasmids containing the inserts will not be linearized when digested with *Sal* I, where as the wild type of plasmid DNA will be linearized with *Sal* I.

b) Design of Oligonucleotide inserts:

The most critical part of the construction of the siRNA expression plasmid is the selection of the target region of the gene, which is currently a trial-and-error process. However, Elabashir et al, 2002 have provided a guideline that may work 80% of the time. According to them, for synthesis of synthetic siRNA, a target region may be selected preferably 50 to 100 nt downstream of the start codon. 5' or 3' untranslated region and region close to the start codon should be avoided as these may be richer in regulatory protein binding sites. The ideal sequence for synthetic siRNA should be 5'-AA(N19)UU, where N is any nucleotide in the mRNA sequence and should be approximately 50% G-C content. However, for designing oligonucleotides for the expression vector, AA and UU dimer in the sequence is not needed.

For the expression vector, siRNA will be designed to produce hairpin RNAs, in which both strands of an siRNA duplex would be included within a single RNA molecule. The individual motif will be 19-21 nt long and corresponds to the coding region of the gene of interest. However, Paddison and Hannon, 2002 have suggested use of 18-28 nucleotides. The two motifs that form the inverted repeat are separated by a spacer of 4-9 nt (to form the hairpin loop) (Figure 3). The transcriptional termination signal for 5 Ts is added at the 3' end of the inverted repeat. Avoid 4-5 continuous stretches of "A" and "T" in your gene sequence, which may act as internal terminator. The insert can be prepared by synthesizing and annealing of two complementary oligonucleotides. An example of p53 siRNA sequence is given in figure 3.



c) Primer annealing (siRNA insert):

1. Add the following components to a sterile microfuge tube.

Forward primer:	1 μ l	(1 μ g)
Reverse primer:	1 μ l	(1 μ g)
Annealing buffer	2 μ l	
Deionized water:	16 μ l	
	<hr/>	
	20 μ l	

2. Incubate at 95°C for 10 minutes.

3. Gradually cool the tube to room temperature.

The annealed siRNA insert can be used immediately or stored at -20°C until needed.

d) Ligation of vector and insert:

1. Linearized vector (completely digested with *Sa*I and *Xba*I) that has been provided in the kit should be used. Set up a ligation reaction as described below. A negative control ligation should be done without insert.

Vector DNA (50 ng/ μ l)	0.5 μ l
Insert DNA (30 ng/ μ l)	1 μ l
T4 DNA ligase	1 μ l
10X ligase buffer	2 μ l
Deionized water	15.5 μ l
Final volume	20 μ l

2. Incubate the mixture at 16°C overnight.

3. Following the ligation reaction, transform the ligated plasmid DNA (10 μ l of the ligation mixture) into 90 μ l of competent cells of an appropriate host strain (DH5 α). You may also use transformation protocol routinely used in your laboratory

Note: In order to monitor the efficiency of the ligation and transformation steps, as a negative control, competent cells should also be transformed with cut vector DNA that has been ligated in the absence of an insert. Check the presence of insert by digesting with *Sa*I restriction enzyme. As mentioned earlier the recombinant plasmids containing inserts will not be linearized by *Sa*I. **Before transfecting the cells with the construct, always confirm presence of the correct insert by sequencing with the primer provided in the kit.**

e) Plasmid DNA preparation:

Any standard protocol, which provides good quality DNA can be used to prepare plasmid DNA for transfection. Most of the times plasmid DNA using Qiagen mini plasmid DNA kit would be sufficient.

i) Transfection:

The lipid based transfection reagent may be ordered separately.

1. Seed 2×10^5 cells in a well of 6-well plate. Add 1 ml of DMEM medium and let it grow overnight at 37°C in a 5% CO₂ incubator.
2. Pipette 0.5 ml of OPTI-MEM (Gibco) (serum-free medium) and add 2-5 µg of plasmid DNA in a sterile eppendorf tube. To this mixture, add 10 µl of lipid dissolved in 0.5 ml of OPTI-MEM . Mix thoroughly by pipetting several times.
3. Aspirate the DMEM medium from the well. Carefully add 1 ml of the DNA-Lipid mix (prepared in 2.).
4. Incubate the 6-well plate in a 5 % CO₂ incubator at 37°C for 4 hours.
5. Add 1 ml of DMEM medium containing 20% fetal bovine serum to each well. Incubate at 37°C for 48-72 hrs.
6. After the incubation, proceed with an appropriate assay.

Note: The NeoR gene in this vector is not functional. Permanent cell lines may be created by co-transfecting with a plasmid containing functional NeoR gene.

The plasmid has been tested successfully in CHO, NIH-3T3, and 293 cell lines. The transfection reagent has been tested on the following cell lines: A549, Calu-3, C2C12, HeLa, HeLa-S3, NIH-3T3, K562, HepG2, BHK-21, CHO-K1, CV-1, COS-1, COS-7, MDCK, HEK-293, HEK-293T, B16-F0, Jurkat, P19, Ki-Ras 267 beta1, Human Dendritic Cells, Neuro2a, MCF-7, RAW, Rat H4, Rat Eye Lens Epithelial and Fibroblast Cells.

Transfection efficiency may be lower for primary cells.

ii) Western Blotting Protocol:

1. Equal amounts (about 10-50 µg/lane) of protein samples are resolved by SDS-PAGE and electro-blotted using the Bio-Rad mini-gel transfer system (Bio-Rad Laboratories, Cambridge, MA) onto Immobilon P membranes (Millipore Corporation, MA) using a current of 0.5-0.75 A for 1 hour.
2. The blots are stained with Amido black for 1 min and destain with 10% methanol plus 10% acetic acid. Amido black helps to monitor the efficiency of transfer without interfering with subsequent immuno-reaction.
3. The blots are then blocked for 1 hour with 5% Carnation non fat dry milk in TBST (25 mM Tris-Cl, pH 8.0; 125 mM NaCl; 0.1% Tween 20).
4. The blots are incubated with primary antibody in 1% milk/TBST overnight at 4°C. The primary antibody can either be a purified mAb or polyclonal antibody, or a hybridoma tissue culture supernatant diluted at 1:5 with 5% Carnation nonfat dry milk in TBST.
5. After incubation with the primary antibody, the blots are washed five times in TBST and then incubated with a secondary antibody conjugated to horse-radish peroxidase (HRPO; 1:2000-3000 dilution; PharMingen) for 60 minutes at RT.
6. After five washes with TBST, the blots are developed for 5 minutes using a Chemiluminescence kit (SuperSignal™ CL-HRP Substrate System, Pierce, Illinois, USA).
7. X-ray films are exposed to the blots for appropriate time period. We normally use Hyperfilm™-ECL films (Amersham Life Science Inc.) and expose to the blots for 10 seconds, 1 minute, 5 minutes, and 20 minutes to visualize the chemiluminescence signal corresponding to the specific antibody-antigen reaction.

Note: *This protocol is provided as a guide only. It is used at Imgenex to test the product development. However, Imgenex does not guarantee success of a western blotting experiment using this protocol. It may be necessary for the researcher to modify the protocol according to a specific project.*

iii) Intracellular Staining (using Fluorescence):

1. Wash cells (on slides) once by immersing in PBS for 30 seconds to 1 minute.
2. Fix cells by incubating slide in 1% paraformaldehyde in PBS for 20 minutes.
3. Wash once by immersing in PBS.
4. Permeabilize the cells by incubating slide in 0.25% Triton X-100 in PBS for 20 minutes.
5. Wash once by briefly immersing in PBS.
6. Block non-specific antibody binding by incubating cells for 10 mins with 1%BSA in PBS.
7. Flick off the excess 1% BSA (do not wash off the 1% BSA) and incubate cells with anti-p53 primary antibody (we recommend diluting purified antibody at 1-2 µg/ml with 1%BSA in PBS. Incubate for 1 hr at room temperature or overnight at 4°C. Make sure that the antibody does not air dry by incubating cells in a moisture chamber.
8. Rinse slides once with PBS followed by immersing in three changes of PBS, 5 minutes each.
9. Incubate cells with FITC or PE labeled secondary antibody diluted at recommended concentration of the manufacturer using 1% BSA in PBS. Incubate for 1 hr at room temperature.
10. Rinse slides once with PBS followed by immersing in three changes of PBS, 5 minutes each.
11. Add mounting media, cover slip the slide and examine under fluorescence microscope.

iv) Intracellular Staining (using DAB staining):

1. Wash cells (on slides) once by immersing in PBS for 30 seconds to 1 minute.
2. Fix cells/tissue sections by incubating slide in 1% paraformaldehyde in PBS for 20 minutes.
3. Wash once by immersing in PBS.
4. Permeabilize the cells/tissue sections by incubating slide in 0.25% Triton X 100 in PBS for 20 minutes.
5. Wash once by briefly immersing in PBS.
6. Block non-specific antibody binding by incubating cells/tissue sections for 10 mins with 1%BSA in PBS.
7. Flick off the excess 1% BSA (do not wash off the 1% BSA) and incubate cells/tissue sections with primary antibody (we recommend diluting purified antibody at 2-5 µg/ml with 1%BSA in PBS or using undiluted hybridoma supernatant). Incubate for 1 hr at room temperature or overnight at 4°C. Make sure that the antibody does not air dry by incubating cells/tissue sections in a moisture chamber.
8. Rinse slides once with PBS followed by immersing in three changes of PBS, 5 minutes each.
9. Incubate cells with biotinylated secondary antibody diluted at 1/200 using 1% BSA in PBS.
 - i) For mouse monoclonal antibodies, we recommend using Biotin-SP-conjugated affinipure donkey anti-mouse IgG (H+L), (Jackson ImmunoResearch Laboratories Inc, Cat# 715-065-151).
 - ii) For rat monoclonal antibodies, we recommend using Biotin-SP-conjugated affinipure donkey anti-rat IgG (H+L), (Jackson ImmunoResearch Laboratories Inc, Cat# 712-065-153).
 - iii) For rabbit polyclonal antibodies, we recommend using Biotin-SP-conjugated affinipure donkey anti-rabbit IgG (H+L), (Jackson ImmunoResearch Laboratories Inc, Cat# 711 065 152).
10. Rinse slides with PBS followed by immersing in three changes of PBS, 5 minutes each.
11. Incubate cells at room temperature for 30 minutes with Peroxidase Conjugated Streptavidin reagent (LSAB 2 kit, Dako, Carpinteria, CA. This reagent comes diluted).
12. Wash off excess unbound Streptavidin conjugate by rinsing slides/tissue sections with PBS followed by immersing in three changes of PBS, 5 minutes each.

13. Incubate cells for 10 minutes with 3,3'-diaminobenzidine substrate solution (Peroxidase Substrate Kit, Vector Laboratories, Inc. Burlingame, CA).
14. Extensively wash cells/tissue with tap water.
15. Counterstain cells/tissue sections by immersing in hematoxylin I (Richard-Allan Scientific, Kalamazoo, MI), diluted 1 part hematoxylin + 4 parts water, for 30 seconds.
16. Wash in hand-warm running tap water. The warm water is necessary as it causes the hematoxylin counterstain to turn blue, which contrasts well with the brown DAB stain. Otherwise, hematoxylin is brick red and does not contrast well DAB stain.
17. Incubate cells in bluing Reagent (Richard-Allan Scientific, Kalamazoo, MI) for 30 seconds. As in step 16, this reagent causes hematoxylin to form a lighter blue counterstain for better contrast.
18. Dehydrate cells by immersing slides in 5 changes of Flex 100 (Richard-Allan Scientific, Kalamazoo, MI), 2 minutes each. Thoroughly drain the slides between each change.
19. Clear by immersing slides in 5 changes xylene (Fisher Scientific, Pittsburg, PA), 2 minutes each. Thoroughly drain the section between each change.
20. Add Cytoseal™ 60 mounting medium (Stephens Scientific/Richard-Allan Scientific, Kalamazoo, MI) and cover slip the cells/tissue sections. Examine under microscope.

Note: *This method is for immunohistochemical staining for cultured cells. This protocol is provided as a guide only. It is used at Imgenex to test the product development. However, Imgenex does not guarantee success of an immunohistochemistry or immunocytochemistry using this protocol. It may be necessary by the researcher to modify the protocol according to a specific project.*

Trouble shooting:

No change in the expression of gene:

a) Transfection may not be successful. Use a positive control plasmid, such as GFP to check for transfection efficiency.

b) Target sequence may not be appropriate for inhibition of gene expression. Choose another target sequence from a different region of the gene.

c) Always sequence your plasmid construct. The siRNAs are sequence specific. A single mutation in the insert will affect gene knockdown.

Sequencing problem:

I have difficulty in sequencing the insert. The sequence stops at the loop. How do I get a better sequencing result? Use a sequencing reaction that will allow to read through secondary structures. Addition of up to 4 % DMSO in the reaction may help. Please refer to paper published by D.C. Ducat F.J. Herrera and S.J. Triezenberg. BioTechniques 34:1140-1144 (June 2003) in order to overcome sequencing problems where secondary structures are involved.