# GeneSuppressor™ System

For Knockdown of Mouse Platelet-Derived

Growth Factor β Receptor

(version 02/04)

Catalog No.: IMG-817

**IMGENEX Corporation**
11175 Flintkote Avenue, Suite E
San Diego, CA 92121
Tel: 858-642-0978
Toll free: 888-723-4363
Fax: 858-642-0937
Email: info@imgenex.com
Web site: www.imgenex.com

**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 a single or multiple genes are knocked down.
-Reproducible transfection efficiency.

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

| **Target** | **Catalog No**. |
|---|---|
| Human p53 | IMG-803 |
| Human Lamin A/C | IMG-703 |
| Human IKKa | IMG-801* |
| Human Caspase-1 | IMG-804 |
| Human Caspase-8 | IMG-805 |
| Human p65 | IMG-807 |
| Human Syk | IMG-808 |
| Human Chk2 | IMG-809 |

* All IMG-800 series vectors may be used for generation of permanent cell lines.

Each kit contains a tested plasmid DNA construct for silencing of 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.

## Table of Contents

Background . . . . . 1

References. . . . . 2

Kit Components and Storage. . . . . 3

Protocols . . . . . 4

Trouble shooting. . . . . 7

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 resell, repackaging and is non-transferable. 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.

University of Michigan
Office of Technology Transfer
2071 Wolverine Tower
3003 S. State Street
Ann Arbor, MI 48109-1280
Phone: (734) 764-8202
Fax: (734) 936-1330

## 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.

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 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 promoter, therefor they are ideally suited for the expression of ~21-nucleotide siRNAs of ~50-nucleotide RNA stem-loops.

siRNA vectors appear to have an advantage over synthetic siRNAs. Cells transfected with a 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.

Two distinct human PDGF receptor transmembrane binding proteins have been identified, a 170 kDa alpha-receptor (PDGFR alpha) (10) and a 190 kDa beta-receptor (PDGFR beta)(11). A similar system has also been identified in mouse.Many mechanisms for stress induced p53 phosphorylation have been identified in the past (12). However, the signals responsible for the N-terminal p53 phosphorylation were not identified. Chen et al.,(2003)(13) have shown

that activation of p53 by oxidative stress involves Platelet-Derived Growth Factor-β receptor. They demonstrated that $H_2O_2$ activates p53 in endothelial cells through transactivation of the PDGF-B receptor and subsequent ATM kinase activation. In order to confirm that ATM kinase activation involves PDGF-β receptor kinase activity, an si-RNA approach was used where inhibition of PDGFR-β expression in NIH3T3 cells led to a reduced p53 phosphorylation and ATM kinase activity in response to $H_2O_2$.

**References:**

**siRNA:**

1. Brummelkamp T., Bernards R., Agami R. *Science*, 296(5567): 550-553 (2002).
2. Elbashir, SM, Harborth J, Weber K, and Tuschl T. *Methods*, 26: 199-213 (2002).
3. Hammond S.M., Boettcher S., et. al. *Science*, 293:1146-1150 (2001).
4. Lee N.S., Dohjima T., et. al. *Nat Biotechnol* 20(5): 500-505 (2002).
5. Paddison PJ and Hannon GJ. *Cancer Cell* 2: 17-23 (2002).
6. Paul CP., Good, P.D., Winer, I., and Engelke, D.R. *Nature Biotechnol*, 20 (5): 505-508 (2002).
7. Tabara H., Sarkissian M., et. al. *Cell*, 99(2): 123-132 (1999).
8. Tuschl T. *Nat Biotechnol* 20(5): 446-448 (2002).
9. Zamore P.D. *Science*, 296: 1265-1269 (2002).

**PDGF-B:**

10. Claesson-Welsh, L. et al. (1989) Proc. Natl. Acad. Sci. USA 86:4917
11. Gronwald, R.G.K. et al. (1988) Proc. Natl. Acad. Sci. USA 85:3435.
12. Shieh, S. Y.,et al. (1997) Cell 91, 325-334
13. Chen K et al., (2003) JBC 278 (41) 39527-39533


Imgenex Tel: 858-642-0978 Toll Free: 1-888-723-4363 Fax: 858-642-0937
Email: info@imgenex.com Web Site: www.imgenex.com

## B. Kit Components and Storage:

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

| Reagents (-20°C storage) | | Quantity |
|---|---|---|
| IMG-817-1 | PDGFR-β siRNA plasmid (0.1 μg/μl)---------- | 20 μl |
| IMG-800-6 | Negative control plasmid (0.1 μg/μl)---------- | 20 μl |
| **Reagents (4°C storage)** | | |
| GSC-1003 | Deionized water---------- | 2 ml |

The negative control plasmid is identical to IMG-817-1 except that it contains a scrambled sequence with no significant homology with the sequences in the Genbank. Both plasmids should be amplified by transforming a suitable bacterial strain such as DH5α and grown in LB medium containing25 μg/ml of kanamycin. The sequence of the insert in the control plasmid is

```
5'TCGATCAGTCACGTTAATGGTCGTTttcaagagaAACGACCATTAACGTGACT
GATTTTT -3'

AGTCAGTGCAATTACCAGCAAaagttctctTTGCTGGTAATTGCACTGACTAAAA
AGATC-5'
```

**Additional required materials (not included)**

Competent bacteria
LB or other bacterial culture media
Transfection reagent


Imgenex Tel: 858-642-0978 Toll Free: 1-888-723-4363 Fax: 858-642-0937
Email: info@imgenex.com Web Site: www.imgenex.com

## C. Protocols:

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

**i) Amplification of the plasmid DNA.** Approximately 2 μg of plasmid DNA is included in the kit. For repeat experiments, the plasmid DNA should be transformed into a suitable bacteria strain, such as DH5α and should be grown in LB or other bacterial medium containing 25 μg/ml of kanamycin. Plasmid DNA may be prepared using any standard protocol. The PDGFR-β siRNA was cloned into *Sal* I and *Xba* I sites of the vector. However, the *Sal* I site is destroyed in the plasmid after cloning the insert. There is a *Sca* I site in the loop for checking presence of the insert.

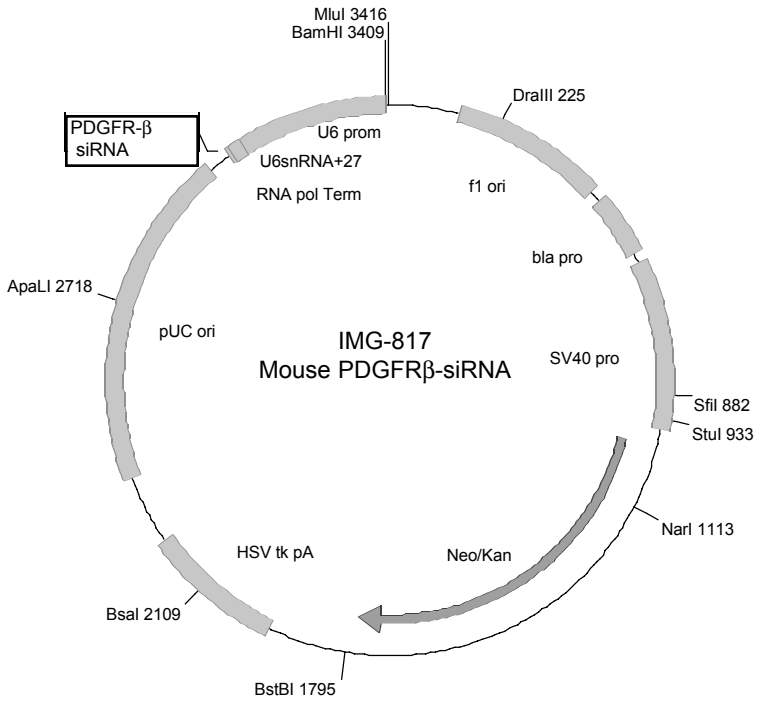


**Figure 1.** A schematic map of GeneSuppressor PDGFR-β plasmid DNA. In order to confirm the authenticity of the plasmid, restriction digestions can be performed using different endonucleases (shown in the map). The siRNA construct was based on Mouse PDGFRβ cDNA, Genbank accession no. NM_008809 . The target sequence was selected between nucleotides 3000-3200.


Imgenex Tel: 858-642-0978 Toll Free: 1-888-723-4363 Fax: 858-642-0937
Email: info@imgenex.com Web Site: www.imgenex.com

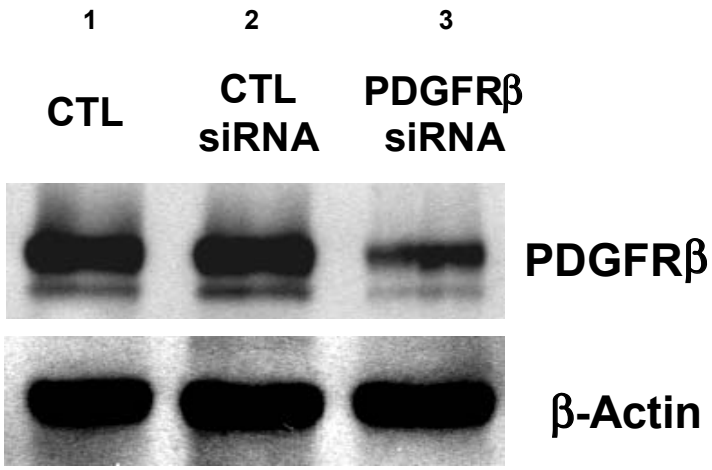


**Figure 2.** Demonstration of PDGFRβ suppression in NIH3T3 cells. Lane 1: Untransfected NIH 3T3 cells. Lanes 2 and 3, NIH 3T3 cells transfected with control (CTL) or PDGFRβ siRNA siRNA vector, respectively. Cells were exposed to $H_2O_2$ 48 hrs after transfection. PDGFR-β expression level was assessed by Western blotting. There was significant decrease in PDGFRβ protein in PDGFRβ–siRNA treated cells. However, β-actin expression was not affected.

Figure Courtesy of Kai Chen, Adam Albano, Alan Ho, and John F. Keaney Jr. Activation of p53 by Oxidative Stress Involves PDGF-beta Receptor-Mediated ATM Kinase Activation. *J Biol Chem* 278 (41) 39527-39533 (2003).


Imgenex Tel: 858-642-0978 Toll Free: 1-888-723-4363 Fax: 858-642-0937
Email: info@imgenex.com Web Site: www.imgenex.com

**ii) Western Blotting protocol:** PDGFR-β inhibition can be carried out by Western Blotting as described (13).

Briefly,

1. NIH/3T3 cells were cultured in DMEM supplemented with 10% FBS. After confluence, cells were routinely equilibrated for 30 min in HEPES-buffered physiologic salt solution containing 22 mM HEPES (pH 7.4), 124 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1.5 mM $CaCl_2$, 0.16 mM $HPO_4$, 0.4 mM $H_2PO_4$, 5 mM $NaHCO_3$, and 5.6 mM glucose prior to $H_2O_2$ exposure.

2.After treatments, cells were washed with ice-cold PBS, and incubated in lysis buffer for 30 min on ice followed by brief sonication.

3.Cell lysates were then cleared by centrifugation (13,600g) for 10 min, and the supernatants incubated with primary antibody for 16 h at 4°C .

4. Western blot analysis was performed as described by Chen, K. et al., Cell Mol.Biol (Noisy.-Le-Grand) 46, 609-617. Proteins were detected using an enhanced chemiluminescence detection kit (Cell Signaling, Beverly, MA).


Imgenex Tel: 858-642-0978 Toll Free: 1-888-723-4363 Fax: 858-642-0937
Email: info@imgenex.com Web Site: www.imgenex.com

**D. 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) Make sure you are using mouse cell lines.

c) Check western blotting protocol.

Gene suppression is dependent upon transfection efficiency. If you get only 50% transfection efficiency, you would see only 50% reduction in the western blot from total cell lysate. Normalization with 50% transfection efficiency would result in 100% gene silencing.


Imgenex Tel: 858-642-0978 Toll Free: 1-888-723-4363 Fax: 858-642-0937
Email: info@imgenex.com Web Site: www.imgenex.com