GeneSuppressor™ System

For knockdown of Human Syk

(version 02/04)

Catalog No.: IMG-808

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 are constructs are ready fro transfection into mammalian cells.

Target	**Catalog No**.
Human p53	IMG-701
Human MeCP2	IMG-802
Human Lamin A/C	IMG-703
Human IKKα	IMG-801
Human Caspase-1	IMG-804
Human Capspae-8	IMG-805
Human p65 (NF-κB)	IMG-807
Human Syk	IMG-808

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 web-site, www.imgenex.com for updates.

Table of Contents

Background 1

References. 2

Kit Components and Storage. 3

Protocols 4

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.

NF-κB (nuclear factor κB) is sequestered in the cytoplasm by IκB family of inhibitory proteins that mask the nuclear localization signal of NF-κB thereby preventing translocation of NF-κB to the nucleus. External stimuli such as tumor necrosis factor or other cytokines results in phosphorylation and degradation of IκB releasing NF-κB dimers. NF-κB dimer subsequently translocates to the nucleus and activates target genes. By using metabolic inhibitors and antioxidant enzymes, it has been well established that oxygen radicals are involved in NF-kB activation by most agents. Additionally, that H2O2 alone can

activate NF-κB has been known for almost a decade but the mechanism has remained unclear. Takada et al, 2003 (13) have demonstrated that H_2O_2 activates NF-κB without the degrading IκBα and without inducing its serine phosphorylation. Rather, it induced tyrosine phosphorylation of IκBα. H_2O_2 activated both IKKα and Syk kinase, and that this activation of Syk kinase proved to be critical for tyrosine phosphorylation of IκBκα and for NF-κB activation.

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).

NF-κB p65:

10. Nolan GO, et al. *Cell* 64: 961-969 (1991).
11. Ruben SM, et al. *Science* 251: 1490-1493 (1991).
12. Huxford T, et al. *Cell* 95 (6), 759-770 (1998).
13. Takada Y, et al. JBC Papers in Press. Published on April 23, 2003 as Manuscript M212389200.

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-808-1	Syk 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
IMG-808-4	Mouse anti-Syk monoclonal antibody* (0.5 mg/ml)-----	20 µl

The negative control plasmid (IMG-800-6) contains a scrambled sequence that does not show significant homology to rat, mouse or human gene sequences. Both plasmids should be amplified by transforming a suitable bacterial strain such as DH5α and grown in LB medium containing 25 µg/ml of kanamycin. The sequence of the insert in the control plasmid is:

```
5'TCGATCAGTCACGTTAATGGTCGTTttcaagagaAACGACCATTAACGTGACTGATTTTT -3'

AGTCAGTGCAATTACCAGCAAaagttctctTTGCTGGTAATTGCACTGACTAAAAAGATC-5
```

Permanent cell line:

It contains Neomycin resistance gene for selection in mammalian cells.

*** Mouse monoclonal antibody. Contains 0.02 % Sodium azide. Sodium azide is highly toxic.**

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 or HB101 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 Syk siRNA was cloned not *Sal* I and *Xba* I sites of the vector. However, the *Sal* I site is destroyed in the plasmid after cloning the insert. Presence of insert may verified by digesting with *Sca* I. This is an unique site present in the hairpin loop.

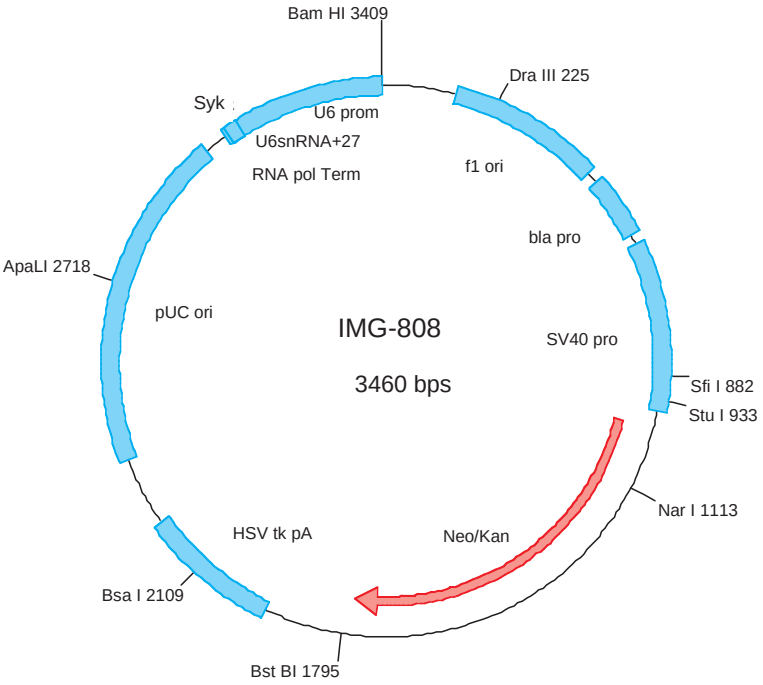

Figure 1. A schematic map of GeneSuppressor Syk plasmid DNA. In order to confirm the authenticity of the plasmid, restriction digestions can be performed using different endonucleases (shown in the map). The siRNA construct was based on human Syk cDNA, Genbank accession no. NM_003177. The target sequence was selected between nucleotides 400 and 700.

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

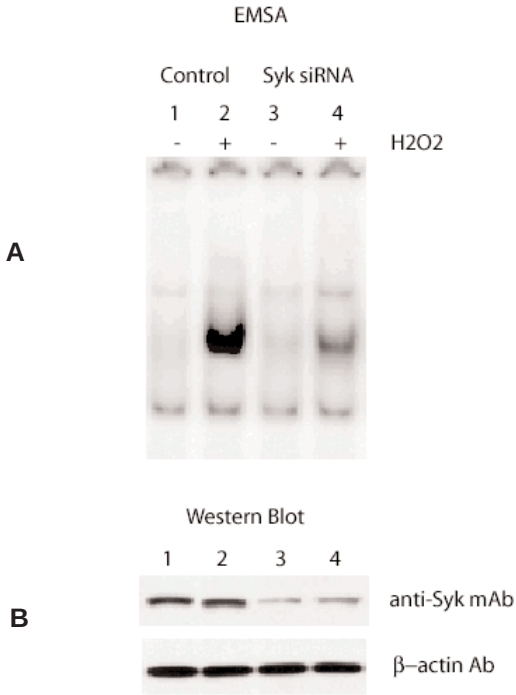

Figure 2. (A). siRNA Syk (IMG-808) transfected cells were treated with 500 mM of H_2O_2 for 5 minutes (lanes 2 and 4), and then analyzed for NF-κB activation.
(B). To determine the effect of reduced Syk expression, whole-cell extracts from H_2O_2 treated cells were subjected to SDS-PAGE for protein expression using anti-Syk antibody (1 and 2 control transfection, lanes 3 and 4 IMG-808 transfection). There is substantial reduction in Syk protein in the cells transfected with IMG-808 (lanes 3 and 4). There was no change in β-actin expression.

Picture courtesy of Dr. Bharat Aggarwal, The MD Anderson Cancer Center, Houston, TX.

iii) 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. Then 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 (1-2 µg/ml) in 1% milk/TBST overnight at 4°C.
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 HyperfilmTM -ECL films (Amersham Life Science Inc.) and expose to the blots for 10 seconds, 1 minute, 5 minutes, and 20 minutes to visualize the chemilu minescence signal corresponding to the specific antibody-antigen reaction.

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

iii) Electrophoretic mobility shift assays (EMSA). NF-κB activation may be performed by EMSA as described (13).

Briefly,

1. Prepare nuclear extracts from treated cells (2 x 10^6/ml).

2. Incubate with ^{32}P-end-labeled 45-mer doublestranded NF-κB oligonucleotide (10 μg of protein with 16 fmol of DNA) from the human immunodeficiency virus long terminal repeat, 5'-TTGTTA-CAAG**GGACTTTC**CGCTGG**GGACTTTC**CAGGGAGGCGT GG-3' (boldface indicates NF-κB binding sites) for 30 min at 37°C.

3. Separate the DNA-protein complex from free oligonucleotide on 6.6 % native polyacrylamide gels. Use a doublestranded mutated oligonucleotide, 5'-TTGTTACAA**CTCACTTTC**CGCTG**CTCACTTTC** CAGGGAGGCGTGG-3', to examine the specificity of binding of NF-κB to the DNA. The specificity of binding was also examined by competition with the unlabeled oligonucleotide. For supershift assays, nuclear extracts prepared from treated cells are incubated with antibodies against either p50 or p65 of NF-κB for 30 min at 37°C before the complex was analyzed by EMSA.

4. The dried gels are visualized, and radioactive bands quantitated by a PhosphorImager (Molecular Dynamics, Sunnyvale, CA) using Imagequant software.

Trouble shooting:

No change in the expression of gene:

a) Transfection may not be successful. Gene suppression depends on the transfection efficiency. Use a positive control plasmid, such as GFP to check for transfection efficiency. The level of gene suppression is dependent on transfection efficiency. Primary cells do not transfect well. Follow manu facturer's recommendation for transfection reagents.