

# GeneSuppressor™ System

For sustained knockdown of Human IKK $\gamma$

(version 02/04)

Catalog No.: IMG-812

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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 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.

<b>Target</b>	<b>Catalog No.</b>
Human p53	IMG-701
Human Lamin A/C	IMG-703
Human Caspase-1	IMG-804
Human Caspae-8	IMG-805
Human NF-κB (p65)	IMG-807
Human Syk	IMG-808
Human Chk2	IMG-809

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](http://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 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.

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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.

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, therefore 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- $\kappa$ B (nuclear factor  $\kappa$ B) is sequestered in the cytoplasm by I $\kappa$ B family of inhibitory proteins that mask the nuclear localization signal of NF- $\kappa$ B thereby preventing translocation of NF- $\kappa$ B to the nucleus. External stimuli such as tumor necrosis factor or other cytokines results in phosphorylation and degradation of I $\kappa$ B releasing NF- $\kappa$ B dimers. NF- $\kappa$ B dimer subsequently translocates to the nucleus and activates target genes. Synthesis of I $\kappa$ B $\alpha$  is autoregulated. I $\kappa$ B proteins are phosphorylated by I $\kappa$ B kinase complex consisting of at least three proteins, IKK1/ $\alpha$ , IKK2/ $\beta$ , and IKK3/ $\gamma$ . In vitro, IKK1/ $\alpha$  and IKK2/ $\beta$  can form homo- and heterodimers

that can phosphorylate IκBs at the regulatory serine residues directly. IKK3/γ preferentially interacts with IKK2/β and is required for activation of IKK complex. IKK3/γ is also known as NEMO (NF-κB Essential MOdulator). Recent data suggest that the human T-cell leukemia virus type I Tax oncoprotein that activates NF-κB binds neither to IKKα nor IKKβ, but complexes directly with IKKγ (8,9). This suggests that IKKγ may be a key molecule acting as an adapter for onco-protein specific signaling to IKKα and IKKβ.

## 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).

### IKKγ:

1. Verma, I.M., Stevenson, J.K., Schwarz, EM., Van Antwerp, D. & Miyamoto, S. *Genes. Dev.* 9: 2723-2735 (1995).
2. Verma, I. And Stevenson, J.K. *Proc. Nat. Acad. Sci. USA* 94: 11758 (1997).
3. Didonato, J.A., Hayakawa, M., Rothwarf, D.M., Zandi, E., and Karin, M. *Nature* 388: 548- (1997).
4. Regnier, C.H., Ho, Y.S., Gao, X., Goeddel, D.V., Cao, Z., and Rothe, M. *Cell* 90, 373 (1997).
5. Mercurio, F., et al. *Science* 278: 860 (1997).
6. Rothwarf, D.M. et al. *Nature* 395: 297-300 (1998).
7. Yamaoka, S. et al. *Cell* 93: 1231-1240 (1998).
8. Jin, D-Y., et al. *J. Biol. Chem.* 274: 17402-17405 (1999).

## B. Kit Components and Storage:

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

### Reagents (-20°C storage) Quantity

IMG-812-1 IKK $\gamma$ -siRNA plasmid (0.1  $\mu$ g/ $\mu$ l) ----- 20  $\mu$ l

IMG-800-6 Negative control plasmid (0.1  $\mu$ g/ $\mu$ l)----- 20  $\mu$ l

### Reagents (4°C storage)

IMG-812-2 anti-IKK $\gamma$  monoclonal antibody\* (0.5 mg/ml).... 20  $\mu$ l

### Additional required materials (not included)

ACompetent bacteria  
LB or other bacterial culture media  
Mammalian cell transfection reagent

### For permanent cell line:

G418

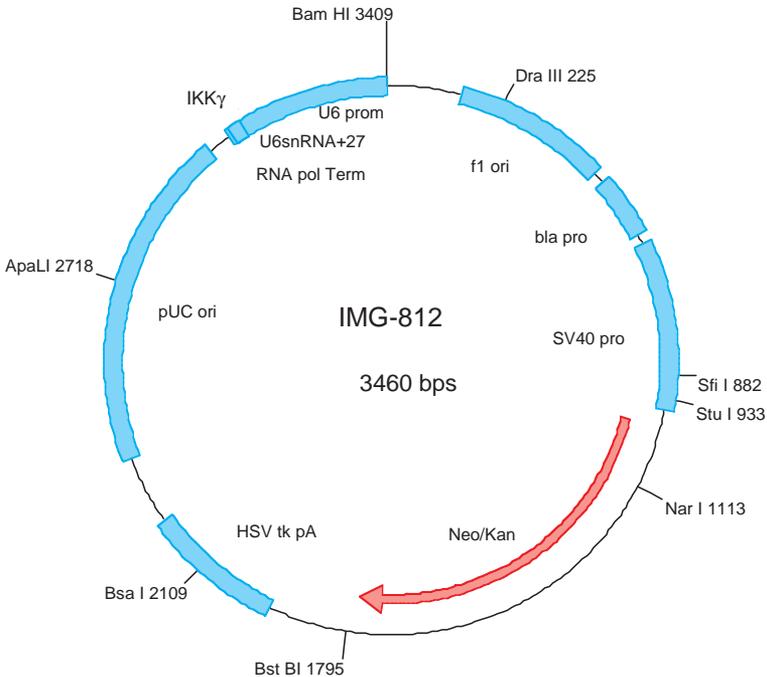
The circular control plasmid is identical to IMG-804-1 except that it contains a scrambled sequence with no significant homology with the sequences in the Genbank. Both plasmids should be amplified by transforming a suitable bacterial strain such as DH5 $\alpha$  and grown in LB medium containing 25  $\mu$ g/ml of kanamycin.

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

## 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  $\mu\text{g}$  of plasmid DNA is included in the kit. For repeat experiments, the plasmid DNA should be transformed into a suitable bacteria strain, such as DH5 $\alpha$  or HB101 and should be grown in LB or other bacterial medium containing 25  $\mu\text{g}/\text{ml}$  of kanamycin. Plasmid DNA may be prepared using any standard protocol. The IKK $\gamma$  siRNA was cloned not *Sa*I and *Xba*I sites of the vector. However, the *Sa*I site is destroyed in the plasmid after cloning the insert.



**Figure 1.** A schematic map of GeneSuppressor IKK $\gamma$  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 IKK $\gamma$  cDNA, Genbank accession no. AF074382. The target sequence was selected between nucleotides 400 and 700.

**ii) Transfection**

We use Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Harvest the cells after 48-72 hrs and proceed with an appropriate assay.

**Note:** Permanent cell lines may be created by growing the transfected cells under G418 (Invitrogen) selection. For generation of permanent cell lines, split the cells at 1: 10-20 dilution and grow the cells in medium containing 500 µg/ml of G418. Untransfected cells should die within 7-20 days. This protocol is for 293 and HeLa cells. For other cells, follow manufacturer's recommendation.

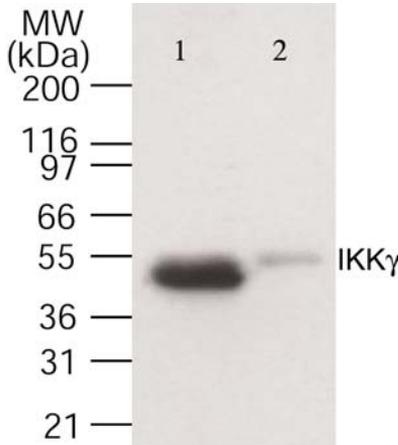
IMG-812 has been tested only on HeLa cells. Transfection efficiency may be lower for primary cells.

**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).

- 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 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.*



**Figure 2.** Western blot to demonstrate IKK $\gamma$  gene silencing using (IMG-812). Lysates from HeLa cells transfected with empty vector (Lane A) and GeneSuppressor-IKK $\gamma$  plasmid DNA (Lane B). The cells were harvested 48 hr post-transfection. The IKK $\gamma$  protein expression is significantly decreased in cells transfected with pSuppressor plasmid containing IKK $\gamma$ siRNA construct.

**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 human cell lines.
- c) Check western blotting protocol.

Gene suppressor 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.