

GeneSuppressorTM System

For knockdown of Human IKK α

(version 02/04)

Catalog No.: IMG-801

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Advantages of GeneSuppressor kit:

GeneSuppressor is a plasmid-based system to generate siRNAs for gene knock-down. 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 pre-made siRNA constructs. These are constructs are ready fro transfection into mammalian cells.

Target	Catalog No.
Human p53	IMG-701
Human Lamin A/C	IMG-703
Human Caspase-1	IMG-804
Human Caspase-8	IMG-805
Human NF- κ B (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 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 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- κ B (nuclear factor κ B) is sequestered in the cytoplasm by I κ B family of inhibitory proteins that mask the nuclear localization signal of NF- κ B thereby preventing translocation of NF- κ B to the nucleus. External stimuli such as tumor necrosis factor or other cytokines results in phosphorylation and degradation of I κ B releasing NF- κ B dimers. NF- κ B dimer subsequently translocates to the nucleus and activates target genes. Synthesis of I κ B α is autoregulated. I κ B proteins are phosphorylated by I κ B kinase complex consisting of at least three proteins, IKK1/ α , IKK2/ β , and IKK3/ γ . In vitro, IKK1/ α and IKK2/ β can form

homo- and heterodimers that can phosphorylate IκBs at the regulatory serine residues directly. IKK1/α and IKK2/β are phosphorylated by NF-κB-inducing kinase (NIK) and MAP kinase kinase kinase-1 (MEKK1) respectively. Targeted disruption of IKK1/α gene in mice results in skin and limb abnormalities and death of newborns.

References

siRNA:

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IKKα:

1. Didonato, J.A., et al. M., et al, M. *Nature* 388: 548- (1997).
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5. Takada, K., et al. *Science* 284: 313-316 (1999).
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Citations

1. **CXCL16 signals via G_i, PI3 kinase, Akt, IκB kinase and nuclear factor-κB, and induces cell-cell adhesion and aortic smooth muscle cell proliferation.** Bysani Chandrasekar, Sailaja Bysani, and Srinivas Mummidi. *J. Biol. Chem.*, 279: 3188-3196 (2004).
2. **Activation of Intrinsic and Extrinsic Proapoptotic Signaling Pathways in Interleukin-18-mediated Human Cardiac Endothelial Cell Death.** Bysani Chandrasekar, Kirankumar Vemula, Rama Mohan Surabhi, Min Li-Weber, Laurie B. Owen-Schaub, Liselotte E. Jensen, and Srinivas Mummidi. *J Biol Chem* Vol. 279: 20221–20233 (2004).

B. Kit Components and Storage

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

Reagents (-20°C storage)		Quantity
IMG-801-1	IKK α plasmid (0.1 μ g/ μ l) -----	20 μ l
IMG-800-6	Negative control plasmid (0.1 μ g/ μ l)-----	20 μ l
Reagents (4°C storage)		
GSC-1003	Deionized water-----	2 ml
IMG-801-4	anti-IKK α monoclonal antibody* (0.5 mg/ml)----	20 μ l

The negative control plasmid (IMG-800-6) 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' TCGATCAGTCACGTTAATGGTCGTTttcaagagaAACGACCA
TTAACGTGACTGATTTTT-3'

AGTCAGTGCAATTACCAGCAAaagttctctTTGCTGGTAATTGC
ACTGACTAAAAAGATC-5'

IMG801-1 and IMG800-6 should be amplified by transforming a suitable bacterial strain such as DH5 α and grown in LB medium containing 25 μ g/ml of kanamycin. It contains Neomycin resistance gene for selection in mammalian cells.

Additional required materials (not included)

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

*** 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 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 50-100 µg/ml of kanamycin. Plasmid DNA may be prepared using any standard protocol. The IKKα 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.

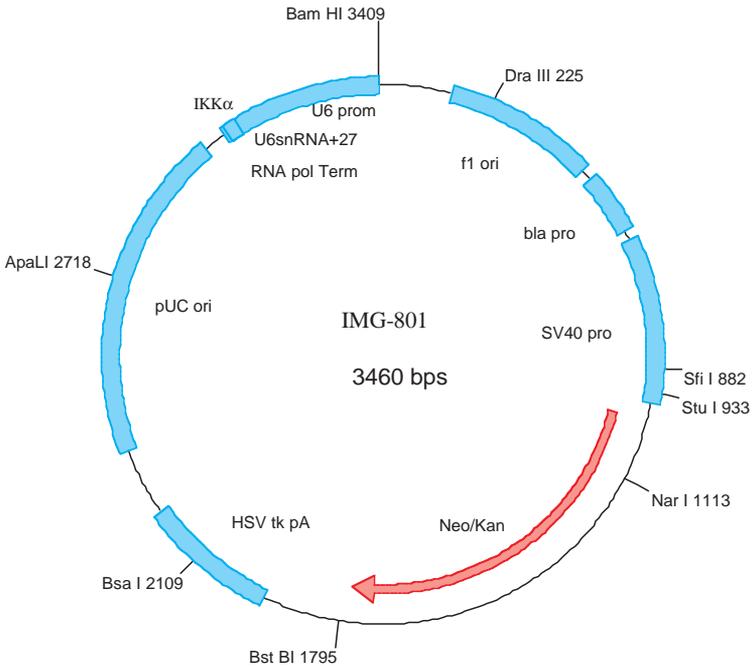


Figure 1. A schematic map of GeneSuppressor IKKα plasmid DNA. In order to confirm the authenticity of the plasmid, restriction digestions can be performed using different endonucleases (shown in the map). The siRNA construct was based on human IKKα cDNA, Genbank accession no. NM_001278. The target sequence was selected between nucleotides 200 and 300.

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.

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 horseradish 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 by the researcher to modify the protocol according to a specific project.*

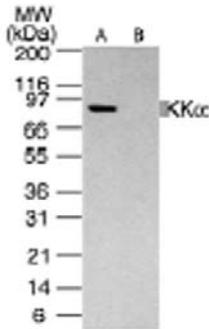


Figure 2. Western blot to demonstrate IKK α gene silencing using (IMG-801). Lysates from 293 cells transfected with empty vector (Lane A) and GeneSuppressor-IKK α plasmid DNA (Lane B). The cells were harvested 48 hr post-transfection. The IKK α protein expression is abolished in cells transfected with pSuppressor plasmid containing IKK α siRNA construct.

iv) 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-IKK α primary antibody (we recommend diluting purified antibody at 1-2 μ g/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.

v) 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/tissue sections for 10 minutes with 3,3'-diaminobenzidine substrate solution (Peroxidase Substrate Kit, Vector Laboratories, Inc. Burlingham, 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/tissue sections 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/tissue sections 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, Pittsburgh, PA), 2 minutes each. Thoroughly drain the section between each change.
20. Add Cytoseal TM 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 and frozen tissue sections. 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) 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.