

GeneSuppressorTM System

For knockdown of Human p53

(version 07/04)

Catalog No.: IMG-701

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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.

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

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.

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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 dsDNA, 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 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.

References:

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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).

B. Kit Components and Storage:

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

Reagents (-20°C storage) Quantity

IMG-701-1 p53 plasmid (0.1 µg/µl) ----- 20 µl

Reagents (4°C storage)

GSC-1003 Deionized water----- 2 ml

GSC1010 p53 monoclonal antibody* (0.5 mg/ml) ----- 20 µl

IMG-700-6 Negative Control plasmid (0.1 µg/µl)----- 20 µl

The negative control plasmid (IMG-700-6) 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' TCGATCAGTCACGTTAATGGTCGTTtcaagagaAACGACCATTAACGTGA
CTGATTTTT -3'

AGTCAGTGCAATTACCAGCAAagttctctTTGCTGGTAATTGCACTGACTAA
AAAGATC-5'

*** 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 DH5 α or HB101. The plasmid contains ampicillin selection marker. It can be grown in LB medium containing 50-100 $\mu\text{g}/\text{ml}$ of ampicillin. Plasmid DNA may be prepared using any standard protocol. The p53 siRNA was cloned inot *Sa*/I and *Xba* I sites of the vector. However, the *Sa*/I site is destroyed in the plasmid after cloning the insert.

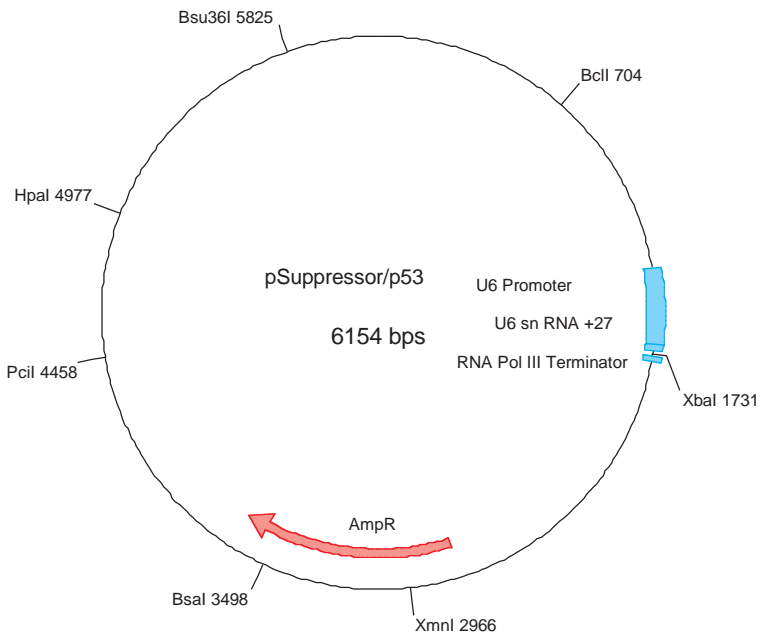
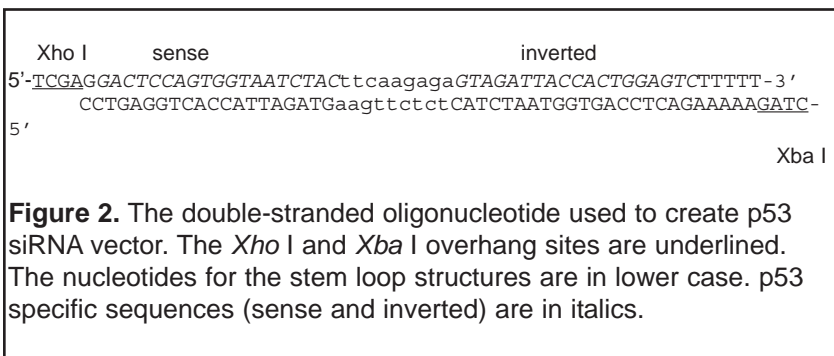


Figure 1. A schematic map of GeneSuppressor p53 plasmid DNA. In order to confirm the authenticity of the plasmid, restriction digestions can be performed using different endonucleases (shown in the map).



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.

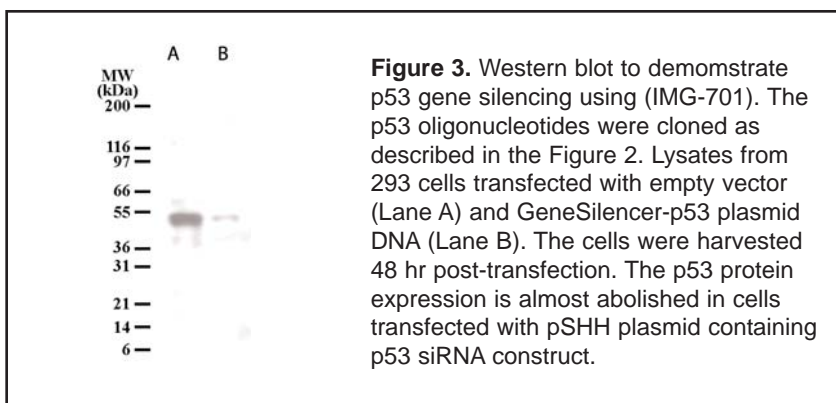
Note: Permanent cell lines may be created by growing the transfected cells under G418 (Invitrogen) selection. For generation of permanent cell lines, split the cells at 1: 10-20 dilution and grow the cells in medium containing 500 µg/ml of G418. Untransfected cells should die within 7-20 days. This protocol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

- 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 at a standard concentration of 1-2 µg/ml or as a hybridoma tissue culture supernatant diluted at 1:5 with 5% Carnation nonfat dry milk in TBST.
- 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.
- 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, IMGEX 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.*



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-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.

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 $\mu\text{g/ml}$ 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, 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 and frozen tissue sections. This protocol is provided as a guide only. It is used as Imgensex to test the product development. However, Imgensex 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 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.