#6261 Store at –20°C

SignalSilence® NF-κB p65 siRNA (Human Specific)

☑ 10 µM in 300 µl

Cell Signaling TECHNOLOGY®

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	Species	Molecular Wt.	Assays
NF-κB p65 siRNA	H	N/A	50–100 transfections

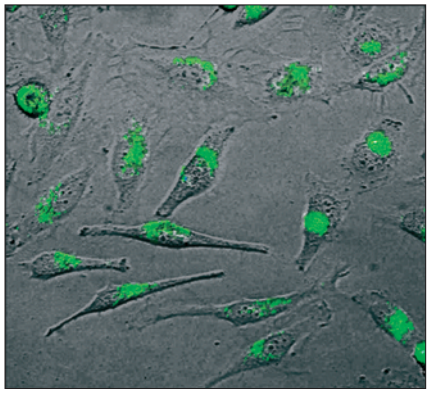
Introduction: SignalSilence® NF-κB p65 siRNA from Cell Signaling Technology allows the researcher to specifically inhibit NF-κB p65 expression using RNA interference, a method in which gene expression can be selectively silenced through the delivery of double stranded RNA molecules into the cell. All SignalSilence® siRNA s are rigorously tested in-house and have been shown to reduce protein expression.

Directions for use: CST recommends transfection with 100 nM human-specific NF-κB p65 siRNA 48 hours prior to cell lysis. See Protocol for transfection procedure.

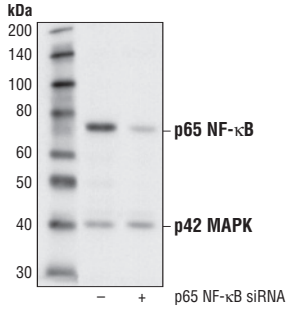
Tested cell lines: HeLa

Background: The transcriptional nuclear factor κB (NF-κB) plays a pivotal role in inflammatory and immune responses (1,2). NF-κB is composed of a heterodimer of p65 and p50 subunits in most cell types and is sequestered in the cytoplasm by its inhibitory proteins, the IκBs (3-5). NF-κB-activating agents can induce the phosphorylation of IκBs; phosphorylation targets them for rapid degradation through a ubiquitin-proteasome pathway, thereby releasing NF-κB to enter the nucleus for gene expression (6-8). It has been shown that the p65 NF-κB subunit is also phosphorylated during the phosphorylation and degradation of IκBs; the phosphorylation site is serine 536 in the carboxy-terminal transactivation domain (9).

Small Interfering RNA (siRNA) has been used to specifically silence NF-κB p65 in colon cancer cells (10).



Fluorescent detection of SignalSilence Control siRNA (Fluorescein Conjugate) #6201 in living HeLa cells 24 hours post-transfection, demonstrating nearly 100% transfection efficiency.



Western blot analysis of extracts from HeLa cells transfected with nontargeted (-) or targeted (+) siRNA. p65 was detected using NF-κB p65 Antibody #3034, and p42 was detected using p42 MAPK Antibody #9108. The NF-κB p65 Antibody confirms silencing of p65 expression, and the p42 MAPK Antibody is used to control for loading and siRNA specificity.

Storage: NF-κB p65 siRNA is supplied in RNase-free water. Aliquot and store at –20°C.

SignalSilence® NF-κB p65 siRNA (Human Specific) #6261

Final concentration 100 nM

Companion Products:

SignalSilence® NF-κB p65 siRNA Kit (Human Specific) #6260

SignalSilence® Control siRNA (Fluorescein Conjugate) #6201

Phospho-NF-κB p65 (Ser536) Antibody #3031

NF-κB p105/p50 Antibody #3035

NF-κB p65 Antibody #3034

Phototope®-HRP Western Detection System, Anti-rabbit IgG, HRP-linked Antibody #7071

Anti-rabbit IgG, HRP-linked Antibody #7074

Prestained Protein Marker, Broad Range (Premixed Format) #7720

Biotinylated Protein Ladder Detection Pack #7727

LumiGLO® Reagent and Peroxide #7003

Species Cross-Reactivity Key: **H**—human **M**—mouse **R**—rat **Hm**—hamster **Mk**—monkey **Mi**—mink **C**—chicken **X**—*Xenopus* **Z**—zebra fish **All**—all species expected
Species enclosed in parentheses are predicted to react based on 100% sequence homology.

Background References:

(1) Baeuerle, P.A. and Henkel, T. (1994) *Annu. Rev. Immunol* 12, 141-179.

(2) Baeuerle, P.A. et al. (1996) *Cell* 87, 13–20.

(3) Haskill, S. et al. (1991) *Cell* 65, 1281-1289.

(4) Thompson, J.E. et al. (1995) *Cell.* 80, 573-582.

(5) Whiteside, S.T. et al. (1997) *EMBO J.* 16, 1413-1426.

(6) Traenckner, E.B. et al. (1995) *EMBO J.* 14, 2876-2883.

(7) Scherer, D.C. et al. (1995) *Proc. Natl. Acad. Sci. USA* 92, 11259-11263.

(8) Chen, Z.J. et al. (1996) *Cell 84*, 853-862.

(9) Sakurai, H. et al. (1999) *J. Biol. Chem.* 274, 30353-30356.

(10) Verma, U.N. et al. (2003) *Clin. Cancer Res.* 9, 1291–1300.

Transfection and Western Immunoblotting Protocol

CST recommends that researchers first confirm that the protein of interest can be detected by Western blotting in lysates from the cell type of interest.

siRNA Transfection Protocol:

Use sterile technique and wear gloves to avoid cell contamination and RNA degradation.

A.) Day 1: Trypsinize and plate cells to a 12-well plate in medium containing 10% serum at a density that will allow cells to reach 50% confluence on day 2.

B.) Day 2: (Indicated values are for a 12-well plate)

1. Remove medium from cells and replace it with 500 µl fresh serum-containing medium.
2. Add 100 µl of serum-free medium to a clean, sterile microfuge tube.
3. Add 2 µl of Transfection Reagent to the tube. Mix by pipetting up and down.
4. Incubate at room temperature for 5 minutes.
5. Add the appropriate volume of siRNA (stocks are 10 µM in RNase-free water) to the tube. For example, add 6 µl of 10 µM stock siRNA to the microfuge tube to yield a final concentration of 100 nM, or 3 µl to yield a concentration of 50 nM, when the mixture is added to the well containing 500 µl. See data sheet for recommended final siRNA concentration. Mix by pipetting up and down gently.
6. Incubate for 5 minutes at room temperature.
7. Add 100 µl of the mixture to the well containing 500 µl medium all at once (not drop-wise).
8. Agitate vigorously to disperse siRNA evenly, but avoid spillage of medium from one well to another.

C.) Day 3: Replace the medium with fresh medium. Examine fluorescein-labeled non-specific siRNA-transfected cells using a fluorescence microscope to determine transfection efficiency. For a 24 hour time point, proceed to step "D".

D.) Day 4 (48 hour time point):

To prepare cell lysates for Western blot analysis, proceed to step 2 of Protein Blotting protocol. CST recommends that researchers perform a preliminary Western blot using control (non-targeted) antibody to detect protein from approximately 7 µl of each cell lysate to confirm that there is an equal concentration of cellular protein in each sample.

Solutions and Reagents

Note: Prepare solutions with Milli-Q or equivalently purified water.

Transfer Buffer:
25 mM Tris base, 0.2 M glycine, 20% methanol (pH 8.5)

SDS Sample Buffer (1X):
62.5 mM Tris-HCl (pH 6.8 at 25°C), 2% w/v SDS, 10% glycerol, 50 mM DTT, 0.01% w/v bromophenol blue or phenol red

Blocking Buffer:
1X TBS, 0.1% Tween-20 with 5% w/v nonfat dry milk; for 150 ml, add 15 ml 10X TBS to 135 ml water, mix. Add 7.5 g nonfat dry milk and mix well. While stirring, add 0.15 ml Tween-20 (100%).

10X TBS (Tris-buffered saline):
To prepare 1 liter of 10X TBS: 24.2 g Tris base, 80 g NaCl; adjust pH to 7.6 with HCl (use at 1X).

Primary Antibody Dilution Buffer:
1X TBS, 0.1% Tween-20 with 5% BSA (for polyclonal antibodies) or 5% nonfat dry milk (for monoclonal antibodies or a combination of a polyclonal and a monoclonal antibody); for 20 ml, add 2 ml 10X TBS to 18 ml water, mix. Add 1.0 g BSA or nonfat dry milk and mix well. While stirring, add 20 µl Tween-20 (100%).

Phototope®-HRP Western Blot Detection:
Biotinylated protein marker, secondary antibody conjugated to horseradish peroxidase (HRP), anti-biotin antibody conjugated to HRP, LumiGLO™ chemiluminescent reagent, peroxide

Wash Buffer TBS/T:
1X TBS, 0.1% Tween-20

Blotting Membrane

This protocol has been optimized for nitrocellulose membranes, which we recommend. PVDF membranes may also be used.

Protein Blotting

A general protocol for sample preparation is described below.

1. Treat cells by adding fresh media containing regulator for desired time.
2. Aspirate media from cultures; wash cells twice with 1X PBS; aspirate.
3. Lyse cells by adding 1X SDS Sample Buffer (50 µl per well of 12-well plate). Immediately scrape the cells off the plate and transfer the extract to a microcentrifuge tube. Keep on ice.
4. Sonicate for 10–15 seconds to shear DNA and reduce sample viscosity.
5. Heat a 20 µl sample to 95–100°C for 5 minutes; cool on ice.
6. Microcentrifuge for 5 minutes.
7. Load 20 µl onto SDS-PAGE gel (10 cm x 10 cm).

Note: CST recommends loading prestained molecular weight markers (#7720, 10 µl/lane) to verify electrotransfer and biotinylated protein markers (#7727, 10 µl/lane) to determine molecular weights.

8. Electrotransfer to nitrocellulose membrane.

For Western blots, incubate membrane with diluted antibody in 5% BSA, (for polyclonal antibodies) or 5% nonfat dry milk (for monoclonal antibodies or a combination of a polyclonal and a monoclonal antibody). 1X TBS, 0.1% Tween-20 at 4°C with gentle shaking, overnight.

Western Immunoblotting Protocol

Membrane Blocking and Antibody Incubations

Note: Volumes are for 10 cm x 10 cm (100 cm^2) of membrane; for different sized membranes, adjust volumes accordingly.

1. (Optional) After transfer, wash nitrocellulose membrane with 25 ml TBS for 5 minutes at room temperature.
2. Incubate membrane in 25 ml of Blocking Buffer for 1 hour at room temperature.
3. Wash 3 times for 5 minutes each with 15 ml of TBS/T.
4. Incubate membrane and primary antibody with loading control antibody (at the appropriate dilution) in 10 ml Primary Antibody Dilution Buffer with gentle agitation overnight at 4°C.
5. Wash 3 times for 5 minutes each with 15 ml of TBS/T.
6. Incubate membrane with HRP-conjugated secondary antibody (dilution varies with manufacturer) and HRP-conjugated anti-biotin antibody (1:1000) to detect biotinylated protein markers (if using) in 10 ml of Blocking Buffer with gentle agitation for 1 hour at room temperature.
7. Wash 3 times for 5 minutes each with 15 ml of TBS/T.

Detection of Proteins

1. Incubate membrane with 10 ml LumiGLO™ (0.5 ml 20X LumiGLO™, 0.5 ml 20X Peroxide and 9.0 ml Milli-Q water) with gentle agitation for 1 minute at room temperature.

Note: LumiGLO™ Substrate can be further diluted if signal response is too fast.

2. Drain membrane of excess developing solution, do not let dry, wrap in plastic wrap and expose to x-ray film. An initial ten-second exposure should indicate the proper exposure time.

Note: Due to the kinetics of the detection reaction, signal is most intense immediately following LumiGLO™ incubation and declines over the following 2 hours.