



**TrkA**  
**siRNA/siAB™ Assay Kit**  
Upstate Catalog # 60-054  
Lot # 25904

**Kit Components**

**SMARTpool™ TrkA**

Catalog # M-003159  
One vial containing **5 nmoles** of 4 pooled SMARTselected siRNA duplexes with "UU" overhangs and a 5' phosphate on the antisense strand.

**Non-specific Control Pool  
(Negative control)**

Catalog # D-001206  
One vial containing **1 nmole** of 4 pooled non-specific siRNA duplexes with "UU" overhangs and a 5' phosphate on the antisense strand.

**1X Universal Buffer**

Catalog # B-001050  
One vial containing **1.5ml** of 20.0mM KCl, 6.0mM HEPES pH 7.5, 0.2mM MgCl<sub>2</sub>.

**Anti-TrkA  
(siAB™)**

Catalog # 06-574  
Lot # 22778

One vial containing **200µg** of protein A purified rabbit IgG in **200µl** of 0.1M Tris-glycine, pH 7.4, 0.15M NaCl containing 0.05% sodium azide. Frozen at -20°C. See enclosed Certificate of Analysis for more information.

**Non-Stimulated A431 Cell Lysate  
(Positive control)**

Catalog # 12-301  
Lot # 24927

One vial containing **100µg** of lysate in **100µl** of modified RIPA buffer diluted with non-reducing sample buffer. Frozen at -20°C. See enclosed Certificate of Analysis for more information.

Please read this product insert before use to obtain background information and a detailed protocol. See the enclosed Certificates of Analysis for more detailed information about the enclosed antibody and positive control.

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NOT FOR USE IN HUMANS OR ANIMALS**

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**Dharmacon**, the world leader in synthetic siRNA duplexes, and **Upstate**, the source for innovative cell signaling solutions, have teamed together to offer a new line of products to make your genomic functional analyses easier and more informative. We are pairing gene-specific, siRNA SMARTpool™ reagents with their respective antibodies (siAB™) to save you valuable time in obtaining reagents that work, thereby enabling you to produce data you can trust.

## RNA Interference Overview

RNA-mediated interference (RNAi) is a well-recognized pathway employed by most eukaryotes as a cellular line of defense directed against invading viral genomes or as a method to clear a cell of aberrant transcription products (1,2). While the mechanism of successful RNAi-mediated gene silencing remains to be fully elucidated, this method is proving to be an invaluable tool for analysis of gene function and target validation. With the help of Dharmacon and Upstate's growing family of siRNA and siAB™ kits and supporting resources, researchers are now more readily able to navigate the steps required to establish a successful RNAi program in their labs.

Cellular uptake of long double stranded RNA (dsRNA) has been shown to induce RNA interference in a diverse group of lower eukaryotic organisms (3-9). RNAi leads to the inhibition of protein expression by utilizing sequence-specific, dsRNA-mediated degradation of the target messenger RNA (mRNA) (10). Attempts to induce RNAi using long dsRNA in mammalian cell lines were first met with limited success, due in part to the induction of the interferon response, which results in a general inhibition of protein synthesis (11).

In 2001, Dr. Tuschl and his colleagues showed that when short RNA duplexes (19-23 bases in length) were introduced into mammalian cells in culture, sequence-specific inhibition of target mRNA was effected without inducing an interferon response (11). These short dsRNA, referred to as small interfering RNA (siRNA), act catalytically at sub-molar concentrations and can cleave up to 95% of the target mRNA in the cell. The siRNA-mediated effect has been shown to be relatively stable over time and silencing may be observed through several cell generations (Tuschl *et al.* 2002, siRNA User's Guide, <http://www.rockefeller.edu/labheads/tuschl/sirna.html>). Relative to previous antisense technology, these properties make siRNA extremely effective at inhibiting target gene expression.

The ability to assess gene function via siRNA-mediated methods represents an exciting and valuable tool that accelerates genome-wide investigations across a broad range of biomedical and biological research. The keys to siRNA-dependent gene silencing in cell culture depends on a number of critical factors:

1. Target sequence selection and siRNA design
2. Cell line and cell culture system
3. Transfection conditions
4. Abundance and turnover rate of the mRNA of interest
5. Protein half-life
6. Accuracy and ease of assaying for mRNA levels, protein levels, or phenotype.

To assist researchers in addressing and minimizing potentially confounding issues associated with siRNA-dependent gene silencing, Dharmacon has developed SMART technology™.

### **Dharmacon SMART technology™ Overview**

The selection of functional siRNAs is one of the major issues confronting the application RNA interference. To solve this issue, the Dharmacon Research and Development groups have been developing both (1) sophisticated selection criteria to identify highly active siRNAs, and (2) methods to minimize the number of assays for high-throughput siRNA studies. The results of these programs are our SMARTselection and SMARTpooling technologies. SMARTselection uses an algorithm comprised of 33 criteria and parameters that effectively eliminate non-functional siRNAs. SMARTpooling uses a sophisticated algorithm to combine 4 or more SMARTselected siRNA duplexes in a single pool, resulting in even greater probability that the siRNA pool reagent will reduce mRNA to low levels.

Dharmacon SMARTselection and SMARTpooling technologies are proving to be very useful for developing a truly viable genome-wide library of siRNA reagents. Each siRNA pool is expected to work >97% of the time with a level of effectiveness of F70 or higher, and 75% of the pools reduce mRNA levels to a level of F95 or higher. F70 and F95 are definitions for a siRNA duplex that silences or reduces mRNA levels by 70% or 95% respectively.

The other tremendous advantage we are noting is the ability to perform only one assay per gene and be guaranteed the siRNA reagent will work under appropriate cell culture conditions. We do guarantee that every pool will work as specified by the following conditions: 100nM siRNA concentration, 24 hour time point, 70% reduction or greater of mRNA level. This saves on assay cost; as well as time and uncertainty related to randomly designed siRNA duplexes.

The SMART technology™ has been tested and validated in two common cell lines: HeLa and HEK293.

### **siRNA and siAB™ Components**

Dharmacon siRNA SMARTpool™ duplexes in this kit are designed with features that are optimal for effective silencing:

1. Twenty-one-nucleotide RNA oligonucleotides forming a 19 base pair duplex core with two nucleotide 3'-overhangs
2. Symmetrical 3'-overhangs
3. 5'-phosphorylated antisense strand
4. Desalted siRNA duplex
5. Quality controlled duplex. (Duplex formation confirmed by non-denaturing gel electrophoresis and mass confirmed by MALDI-TOF mass spectrometry.)
6. Designed for use in human cell lines

In addition to the siRNA gene-specific SMARTpool™, the kit contains the following components:

1. Negative control – Designed and tested as a non-targeted negative control
2. 1X Universal Buffer – Validated and tested as a low salt buffer for most tissue culture conditions.
3. Gene specific siAB™ – Validated and batch tested for detection of protein levels
4. Cell Lysate (when available) – Validated and batch tested as a positive detection control

**Kit Contents:**

Item	Description	Quantity
SMARTpool™	4 pooled SMARTselected siRNA duplexes with “UU” overhangs and a 5' phosphate on the antisense strand.	5 nmoles
Negative Control	4 pooled non-specific siRNA duplexes with “UU” overhangs and a 5' phosphate on the antisense strand.	1 nmole
1X Universal Buffer	20.0mM KCl 6.0mM HEPES, pH 7.5 0.2mM MgCl <sub>2</sub>	1.5ml
siAB™	Antibody - Validated and batch tested	One vial
Positive Control	Cell Lysate or Recombinant protein - when available (refer to Certificate of Analysis)	One vial

Other components required but not included as part of kit:

**Reagents:**

- Cell line
- Transfection Reagent
- Reduced serum or serum-free media
- Serum-containing media
- 1 X Phosphate Buffered Saline (PBS)
- SDS-PAGE gel
- SDS-PAGE running buffer
- Transfer membrane (e.g. Nitrocellulose or PVDF)
- Transfer buffer
- Blocking buffer (PBS, 3% non-fat dry milk, 0.05% Tween-20)
- Wash buffer (PBS with 0.05% Tween-20)
- Secondary antibody of choice for primary antibody (e.g. Goat anti-mouse IgG, HRP conjugate, Upstate Catalog # 12-349 or Goat anti-rabbit IgG, HRP conjugate, Upstate Catalog # 12-348)
- Detection reagents appropriate for secondary antibody
- Autorad Orientation Markers (if using chemiluminescent detection).

**Equipment:**

- Standard cell culture capability
- Power supply
- SDS-polyacrylamide gel electrophoresis (SDS-PAGE) apparatus.
- Transfer apparatus.
- Detection equipment (e.g. autorad film processor or other device)

**Shipping and Storage:** The siRNA and siAB™ kit is shipped on dry ice. The siRNA SMARTpool™, antibody, and cell lysate are stored at **-20°C**. The 1X Universal Buffer may be stored at -20°C or room temperature.

**Precautions:** RNA oligos are susceptible to degradation by RNases which are present almost everywhere. They are also susceptible to non-specific degradation. For this reason, they should be handled and stored using RNase-free conditions and solutions. Gloves should be worn during handling and solutions should be treated to inhibit or destroy ribonucleases.

### siRNA Protocol

1. Each SMARTpool™ contains 5 nmoles of material and each non-specific control contains 1 nmole of material.
2. The siRNA SMARTpool™ may be resuspended using 250µl of the enclosed 1X siRNA Universal buffer (or another appropriately buffered RNase free solution of your choice) for a recommended concentration of 20µM (20pmol/µl).
3. The siRNA non-specific control pool may be resuspended using 50µl of the enclosed 1X siRNA Universal buffer (or another appropriately buffered RNase free solution of your choice) for a recommended concentration of 20µM (20pmol/µl). This is sufficient for the following formats:

Format (wells/plate)	~Surface area (cm <sup>2</sup> )	pmol per well	Final volume per well (ml)	siRNA final concentration (nM)	Number of wells per 1 nmole	Number of wells per 5 nmole
96	0.3	10	0.1	100	96	480
24	2.0	50	0.5	100	20	100
12	4.0	100	1.0	100	10	50
6	10	200	2.0	100	5	25

Final concentrations range from 1-200nM and should be optimized for the target of choice and assay conditions.

4. For lipid complex formation and subsequent transfection, we strongly recommend following the instructions provided by the transfection reagent manufacturer and taking measures to test and optimize the conditions best suited for the cell line or culture of choice. General recommendations include:
  - a. Cell density at ~70-90% confluent, or approximately  $1 \times 10^5$  cells/ml density, at the time of transfection (this will vary with the growth characteristics of the cells).
  - b. Standard incubation conditions for mammalian cells are 37°C in 5% CO<sub>2</sub>.
5. The lipid encapsulated SMARTpool™ and control are ready for transfection.

### Technical Support

For questions or concerns regarding the use of the SMARTpools™ in this kit, please contact Dharmacon Technical Support, 1-800-235-9880, email: [lab@dharmacon.com](mailto:lab@dharmacon.com).

For questions or concerns regarding protein detection, intracellular staining, or DAB, please contact Upstate Technical Support, 1-800-548-7853, email: [techserv@upstate.com](mailto:techserv@upstate.com).

### Licensing and Trademarks

Dharmacon is licensed to provide siRNA and RNAi products for biological and pharmaceutical research and development, excluding use in humans and use in clinical diagnostics. Dharmacon siRNA products may be used only by the purchaser and may not be resold without the express agreement of Dharmacon. The Massachusetts Institute of Technology granted one of four co-exclusive rights to Dharmacon to the claims in US Patent Applications 60/265232, 09/821832, and PCT/US01/10188, and non-US Patent Application European Serial Number 00126325. The Carnegie Institution of Washington granted rights to Dharmacon to the claims in US Patent Applications 60/068562, 09/215257, PCT/US98/27233.

Dharmacon, SMART technology, SMARTpool and SMARTselection are trademarks of Dharmacon, Inc. siAB is a trademark of Upstate USA, Inc.

All other company and product names may be trademarks of the respective companies with which they are associated.

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7. **Parrish, S., J. Fleenor, S. Xu, C. Mello, and A. Fire.** 2000. "Functional anatomy of a dsRNA trigger: differential requirement for the two trigger strands in RNA interference." *Molecular Cell*. **6**:1077-87.
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12. **Elbashir, S.M., J. Harborth, W. Lendeckel, A. Yalcin, K. Weber and T. Tuschl.** 2001. "Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells." *Nature*. **411**:494-498.

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cell signaling solutions

## Certificate of Analysis

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### Anti-TrkA

(rabbit polyclonal IgG)

Catalog # 06-574

Lot # 22778

**Immunogen:** Rabbits immunized with bacterially expressed purified protein corresponding to the entire extracellular domain of rat TrkA receptor.

**Specificity:** Recognizes TrkA, Mr 140kDa. Does not cross-react with TrkB or TrkC.

**Species Cross-reactivity:** Rat, mouse and human; other species cross-reactivity is unknown.

**Storage and Stability:** Stable for 2 years at -20°C from date of shipment. Aliquot to avoid repeated freezing and thawing. For maximum recovery of product, centrifuge the original vial after thawing and prior to removing the cap.

**Formulation:** 200µg of protein A purified rabbit IgG in 200µl of 0.1M Tris-glycine, pH 7.4, 0.15M NaCl containing 0.05% sodium azide. Frozen solution.

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NOT FOR USE IN HUMANS**

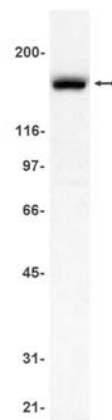
### Quality Control Testing

**Immunoblot Analysis:** 0.5-2.0µg/ml of this lot detected TrkA in RIPA lysates from human A431 cells; a previous lot detected TrkA in mouse 3T3 and rat PC-12 cell lysates.

**Included Positive Antigen Control:** Catalog # 12-301, non-stimulated A431 cell lysate. **Add 2.5µl of 2-mercaptoethanol/100µl of lysate and boil for 5 minutes to reduce the preparation.** Load 20µg of reduced lysate per lane for minigels.

**Immunoprecipitation:** 4µg of this lot immunoprecipitated TrkA from 500µg of PC-12 RIPA lysate.

**Immunocytochemistry:** 10µg/ml of a previous lot of this antibody showed positive immunostaining for TrkA in PC-12 cells fixed with 4% paraformaldehyde.



#### Immunoblot Analysis

Representative blot from a previous lot. A431 cell lysate was resolved by electrophoresis, transferred to nitrocellulose and probed with anti-TrkA (2.0µg/ml). Proteins were visualized using a goat anti-rabbit secondary antibody conjugated to HRP and a chemiluminescence detection system. Arrow indicates TrkA (140kDa).

### General References:

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Pahlman, S. and Hoehner, J.C., *Molecular Medicine Today* **2**: 432, 1996.

### Immunoblot Protocol

1. Perform SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on a cell lysate sample (cell lysis buffer: 50mM Tris-HCl, pH7.4; 1% NP-40; 0.25% sodium deoxycholate; 150mM NaCl; 1mM EDTA; 1mM PMSF; 1 $\mu$ g/ml each aprotinin, leupeptin, pepstatin; 1mM Na<sub>3</sub>VO<sub>4</sub>; 1mM NaF) and transfer the proteins to nitrocellulose. Wash the blotted nitrocellulose twice with water.
2. Block the blotted nitrocellulose in freshly prepared PBS containing 3% nonfat dry milk (Catalog # 20-200), (PBS-MLK) for 20 minutes at 20-25°C with constant agitation.
3. Incubate the nitrocellulose with **0.5-2.0 $\mu$ g/ml of anti-TrkA**, diluted in freshly prepared PBS-MLK overnight with agitation at 4°C.
4. Wash the nitrocellulose twice with water.
5. Incubate the nitrocellulose in the secondary reagent of choice (a **goat anti-rabbit** HRP conjugated IgG, Catalog # 12-348, 1:5000 dilution was used) in PBS-MLK for 1.5 hours at room temperature with agitation.
6. Wash the nitrocellulose twice with water.
7. Wash the nitrocellulose in PBS-0.05% Tween 20 for 3-5 minutes.
8. Rinse the nitrocellulose in 4-5 changes of water.
9. Use detection method of choice (enhanced chemiluminescence was used).

### Immunoprecipitation Protocol

1. Dilute the cell lysate before beginning the immunoprecipitation to roughly 1 $\mu$ g/ $\mu$ l total cell protein in a microcentrifuge tube with PBS.
2. Add **4 $\mu$ g of anti-TrkA** to 500 $\mu$ g-1mg cell lysate.
3. Gently rock the reaction mixture at 4°C overnight.
4. Capture the immunocomplex by adding 100 $\mu$ l (50 $\mu$ l packed beads) of washed Protein A agarose bead slurry, Catalog # 16-125.
5. Gently rock the reaction mixture at 4°C for 2 hours.
6. Collect the agarose beads by pulsing (5 seconds in the microcentrifuge at 14,000 x g), and drain off the supernatant. Wash the beads 3 times with either ice-cold cell lysis buffer or PBS.
7. Resuspend the agarose beads in 50 $\mu$ l 2X Laemmli sample buffer.
8. The agarose beads can either be frozen for later use or suspended in Laemmli sample buffer and boiled for 5 minutes. Collect the beads by a microcentrifuge pulse. SDS-PAGE and subsequent immunoblot analysis can be performed on a sample of the supernatant.

### Immunocytochemistry Protocol

**NOTE: Do not pour off media, blocking solution or antibodies. Draw off very carefully with pipette.**

1. Plate approximately 200 $\mu$ l of PC-12 cell suspension into each well of a slide that has been coated with ECL matrix. Incubate 24 hours in a 37°C CO<sub>2</sub> incubator.
2. Wash the cells three times for 5 minutes with PBS. Do not shake cells.
3. Add fix (4% paraformaldehyde) in PBS for 10 minutes at room temperature.
4. Wash the cells with PBS, supplemented with 1mM MgCl<sub>2</sub> and 1mM CaCl<sub>2</sub>, twice, for 15 minutes. Do not shake.
5. Add 400  $\mu$ l of 8% BSA in PBS and incubate for 1 hour at room temperature.
6. Incubate the cells with **10 $\mu$ g/ml anti-TrkA** in 1% BSA in PBS and incubate for 1 hour at room temperature.
7. Wash the cells twice with PBS, supplemented with 1mM MgCl<sub>2</sub> and 1mM ClCl<sub>2</sub>, for 5 minutes.
8. Incubate the cells with a 1:100 dilution of goat anti-rabbit IgG fluorescein conjugated secondary antibody in 1% BSA in PBS for 1 hour at room temperature.
9. Wash the cells with PBS, supplemented with 1mM MgCl<sub>2</sub> and 1mM ClCl<sub>2</sub> for 15 minutes in the dark.
10. Examine the cells under a fluorescent microscope.



**Positive Antigen Controls**

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## **Non-Stimulated A431 Cell Lysate**

**Catalog # 12-301**

**Lot # 24927**

**Product Description:** Cellular protein preparation. Cells were lysed in modified RIPA buffer (50mM Tris-HCl, pH 7.4, 1% NP40, 0.25% sodium deoxycholate, 150mM NaCl, 1mM EDTA, 1mM PMSF, 1µg/ml aprotinin, 1µg/ml leupeptin, 1µg/ml pepstatin, 1mM Na<sub>3</sub>VO<sub>4</sub>, 1mM NaF) and **diluted with non-reducing sample buffer** (31mM Tris-HCl, pH 6.8, 5% glycerol, 1% SDS, 0.002% bromphenol blue).

**Use:** Add 2.5µl of 2-mercaptoethanol/100µl of lysate and boil for 5 minutes to reduce the preparation. Load 20µg of reduced lysate per lane for immunoblot analysis. This preparation may be used as a positive control for some of Upstate's antibodies.

**Quantity and Formulation:** 100µg in 100µl of RIPA diluted with non-reducing sample buffer. Concentration: 1mg/ml. Frozen solution.

**Storage and Stability:** Stable for 6 months at -20°C from date of shipment. For maximum recovery of product, centrifuge the original vial after thawing and prior to removing the cap. Aliquot to avoid repeated freezing and thawing.

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