Western Blotting Kit
Catalog #BHO0021

**ERK 1/2**
**[pTpY185/187]**

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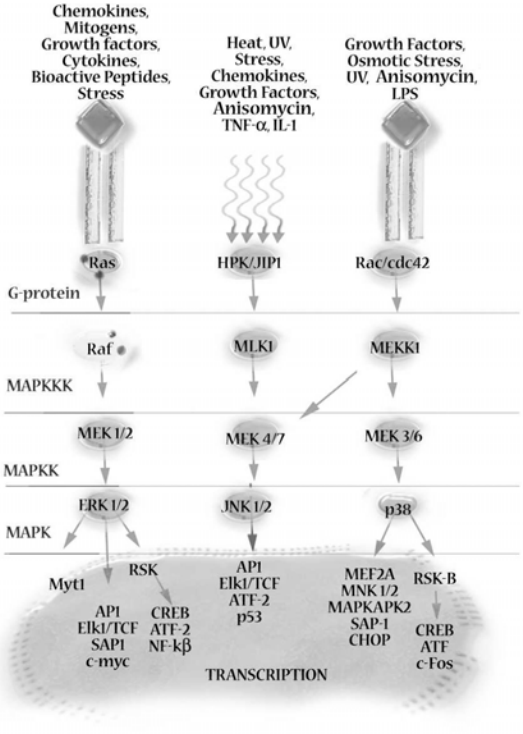
## INTRODUCTION

Extracellular Signal-Regulated Kinases 1 and 2 (ERK 1/2) are members of the Mitogen Activated Protein Kinase (MAPK) family that also includes c-Jun $NH_2$-Terminal Kinase (JNK, also called Stress-Activated Protein Kinase, SAPK) and p38 MAPK (also called SAPK2, Reactivating Kinase [RK], or Cytokine Suppressive Anti-Inflammatory Drug Binding Protein [CSBP]). These kinases define three distinct MAPK signaling modules. The MAPKs are activated by a variety of external stimuli (Figure 1). Activation of these cytosolic kinases transduces external signals into cellular responses including transcriptional activation of responsive genes. ERK 1/2 is expressed broadly in normal tissues and cell lines.

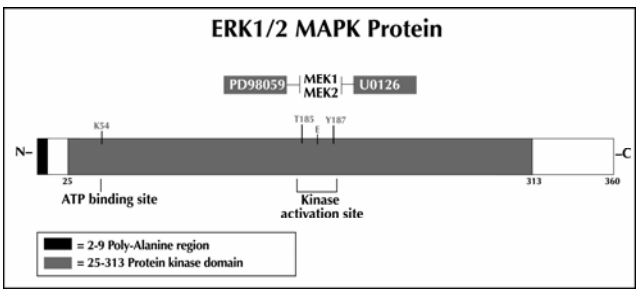
**Figure 1**


Chemokines, Mitogens, Growth factors, Cytokines, Bioactive Peptides, Stress
Heat, UV, Stress, Chemokines, Growth Factors, Anisomycin, TNF-α, IL-1
Growth Factors, Osmotic Stress, UV, Anisomycin, LPS
Ras
HPK/JIP1
Rac/cdc42
G-protein
Raf
MLK1
MEKK1
MAPKKK
MEK 1/2
MEK 4/7
MEK 3/6
MAPKK
ERK 1/2
JNK 1/2
p38
MAPK
Myt1
RSK
AP1
Elk1/TCF
SAP1
c-myc
CREB
ATF-2
NF-kβ
AP1
Elk1/TCF
ATF-2
p53
MEF2A
MNK 1/2
MAPKAPK2
SAP-1
CHOP
RSK-B
CREB
ATF
c-Fos
TRANSCRIPTION

The ERK 1/2 signal transduction pathway plays an essential role in many cellular processes, including cell proliferation, cell differentiation, and reorganization of the cytoskeleton. Activation of ERK 1/2 is part of the cell proliferative response. A variety of stimuli are observed to activate ERK 1/2, including growth factors, hormones, cytokines, and bioactive peptides. Activation of ERK 1/2 is mediated by the upstream kinases MAP Kinase Kinase 1/2 (MKK 1/2; also called MAPK/ERK Kinase 1/2, MEK 1/2). MKK 1/2 phosphorylate ERK 1/2 at threonine 185 and tyrosine 187 in the TEY motif (Figure 2). Phosphorylation of these residues activates ERK 1/2. Various targets of ERK 1/2 have been identified, including transcription factors, (e.g., Elk-1 and GATA-4), kinases (e.g., Rsk [p90 Ribosomal S6 Kinase] and GRK2 [G Protein-Coupled Receptor Kinase-2]), adaptor proteins (e.g., β-arrestin), and transporters (e.g., NHE-1 [$Na^+$-$H^+$ exchanger-1] and ENaC [amiloride-inhibitable $Na^+$ channel]).

A schematic of the ERK 1/2 protein is presented in Figure 2 below.

**Figure 2**



## ERK1/2 [pTpY187/185] WESTERN BLOTTING KIT OVERVIEW

**Antibodies.** This kit contains reagents for the detection of ERK 1/2 protein when phosphorylated at threonine 185 and tyrosine 187 and for the detection of total ERK 1/2 protein by Western blotting. Phosphorylation of ERK 1/2 is monitored with the ERK 1/2 [pTpY185/187] Phosphorylation Site-Specific Antibody (ERK 1/2 [pTpY185/187] PSSA). Total ERK 1/2 is detected with the pan antibody, which reacts with ERK 1/2 protein independent of its phosphorylation state. The ERK 1/2 [pTpY185/187] PSSA and the ERK 1/2 pan antibodies react specifically with the ERK 1/2 protein in human, mouse, and rat cells. Reactivity of the antibodies with most other mammalian species is expected based on known sequence homologies. The goat (polyclonal) anti-rabbit IgG horseradish peroxidase (HRP) conjugate binds to both the ERK 1/2 [pTpY185/187] PSSA and the ERK 1/2 pan antibody, enabling detection of the phosphorylated and total ERK 1/2 protein.

**Control Cell Extracts.** The Positive Control Cell Extracts and Negative Control Cell Extracts are provided in Laemmli sample buffer. These control cell extracts can be used as internal controls for verification of the antibody signals.

**Control Peptides.** The phosphorylated and non-phosphorylated Control Peptides are provided as lyophilized powders. These peptides are intended for use in peptide competition experiments to confirm the specificity of the reaction of the ERK 1/2 [pTpY185/187] PSSA with the phosphorylated protein. Specificity is confirmed when the phosphorylated peptide blocks the ERK 1/2 [pTpY185/187] PSSA signal, while the non-phosphorylated peptide does not impact the signal.

The ERK 1/2 [pTpY185/187] PSSA has also been validated for use in immunostaining (immunohistochemistry and immunofluorescence) and flow cytometry procedures. Representative protocols for these procedures are provided in the **Appendix**. These protocols should be regarded as starting points for optimization of the antibody reagents in each experimental system.

**This kit has been configured for research use only and is not to be used in diagnostic procedures.**

**READ ENTIRE PROTOCOL BEFORE USE**

**SAFETY**

All biological materials should be handled as potentially hazardous. Follow universal precautions as established by the Centers for Disease Control and Prevention and by the Occupational Safety and Health Administration when handling and disposing of infectious agents.

## REAGENTS PROVIDED

**Note:** *Store all reagents at -20°C.*

| Component | Form | Quantity Provided |
|---|---|---|
| ERK 1/2 [pTpY185/187] Phosphorylation Site Specific Antibody. | Purified rabbit antibody. Contains 0.05% sodium azide, and 50% glycerol. Sufficient for 10 tests. | 1 vial; 100 µL |
| ERK 1/2 Pan Antibody. | Purified rabbit antibody. Contains 0.05% sodium azide, and 50% glycerol. Sufficient for 10 tests. | 1 vial; 100 µL |
| Goat F(ab')$_2$ anti-Rabbit IgG HRP Conjugate (polyclonal). | Purified conjugated antibody. Contains 50% glycerol. Sufficient for 10 tests. | 1 vial; 100 µL |
| Positive Control Cell Extract. | Solubilized cell protein from NIH3T3 cells treated with PDGF in 1x Laemmli Sample Buffer and 0.0025% (w/v) bromophenol blue. Sufficient for 4 tests. | 1 vial; 80 µL |
| Negative Control Cell Extract. | Solubilized cell protein from untreated NIH3T3 cells in 1x Laemmli Sample Buffer and 0.0025% (w/v) bromophenol blue. Sufficient for 4 tests. | 1 vial; 80 µL |
| Phosphorylated Control Peptide. | Lyophilized. Sufficient for 10 tests. | 1 vial; 0.1 mg |
| Non-Phosphorylated Control Peptide. | Lyophilized. Sufficient for 10 tests. | 1 vial; 0.1 mg |

**Disposal Note:** This kit contains materials with small quantities of sodium azide. Sodium azide reacts with lead and copper plumbing to form explosive metal azides. Upon disposal, flush drains with a large volume of water to prevent azide accumulation.

## WESTERN BLOTTING PROTOCOL

The kit has been validated for the detection of ERK 1/2 protein phosphorylated at threonine 185 and tyrosine 187 and ERK 1/2 total protein by Western blotting using the reagents provided and the procedures and solutions detailed below. As with all experimental procedures, optimization of these procedures may be required for each experimental system.

### 1. SAMPLE PREPARATION

#### Buffer Formulations

**Cell Lysis Buffer***

10 mM Tris, pH 7.4
100 mM NaCl
0.1% SDS
0.5% sodium deoxycholate
1% Triton-X 100
10% glycerol
Phosphatase Inhibitors (1 mM EDTA, 1 mM EGTA, 1 mM NaF, 20 mM $Na_4P_2O_7$, 2 mM $Na_3VO_4$)
1 mM PMSF (from 0.3 M stock in DMSO) or 1 mM AEBSF
Protease Inhibitor Cocktail (e.g., Sigma catalog #P2714)

*This buffer is available as a formulation requiring supplementation with PMSF and protease inhibitor cocktail (Cell Extraction Buffer, Invitrogen catalog #FNN0011).

**2x Laemmli Sample Buffer**

125 mM Tris, pH 6.8
10% glycerol
10% SDS
0.006% bromophenol blue
130 mM dithiothreitol [DTT]

## Procedure

### Cell Lysate Sample Preparation

1. Lyse approximately $10^7$ cells in 0.5 mL of Cell Lysis Buffer on ice for 30 minutes. This buffer is suitable for recovery of most proteins, including membrane receptors, cytoskeletal-associated proteins, and soluble proteins.
2. Remove cellular debris by centrifuging the lysates at 14,000 x g for 10 minutes.
3. Decant the clarified cell lysates into clean tubes. Measure protein concentration using a suitable method, e.g., the Bradford assay.
4. Combine an aliquot of the lysate with an equal volume of 2x Laemmli Sample Buffer.
5. Heat the samples at 95 - 100$^o$C for 90 seconds.
6. Prior to loading onto the gel, centrifuge the samples at 14,000 x g for 5 seconds at room temperature.

**Positive and Negative Control Cell Lysate Preparation**

1. Allow the Positive and Negative Control Cell Lysates provided in the kit to thaw at room temperature.
2. Heat the samples for 90 seconds at 95 - 100°C.
3. Prior to loading, centrifuge the samples at 14,000 x g for 5 seconds at room temperature.

## 2. SDS PAGE AND ELECTROPHORETIC TRANSFER

### Buffer Formulations

**Running Buffer**
Follow manufacturer's instructions

**Transfer Buffer**
Follow manufacturer's instructions

**Tris-Buffered Saline (TBS)**
20 mM Tris, pH 7.4
154 mM NaCl

### Procedure

**Polyacrylamide Gel Electrophoresis (PAGE)**

1. Assemble single percentage or gradient minigels in the electrophoresis apparatus according to the manufacturer's instructions.

2. Load 10 - 30 µg of the prepared cell lysates into individual wells of the minigels. Electrophoresis of pre-stained molecular weight markers in adjacent wells is recommended to provide confirmation of successful protein separation and transfer to blotting membrane and to facilitate determination of the molecular weights of detected proteins.
3. If desired, load 20 µL of the Positive Control Cell Lysate and 20 µ L of the Negative Control Cell Lysate into adjacent wells.
4. Connect electrodes and resolve cell lysate proteins by SDS PAGE under appropriate running conditions, e.g., at a constant voltage of 125 V for 1.5 - 3 hours at room temperature. Length of electrophoresis is dependent on running conditions, polyacrylamide percentage, and molecular weight of protein(s) of interest.

**Electrophoretic Transfer of Proteins to Blotting Membrane**

1. Prior to the Western transfer, cut a piece of PVDF membrane slightly larger than the gels. Nitrocellulose or nylon membranes may also be used.
2. Soak the PVDF membrane in methanol for 1 minute, then rinse with dd$H_2O$ for 5 minutes.
3. Assemble and transfer proteins according to transfer apparatus manufacturer's instructions.
4. Following the transfer, rinse the membrane with Tris buffered saline for 2 minutes. No portion of the membrane should be allowed to dry during any part of the procedure.
5. Successful protein transfer can be verified by confirming transfer of the pre-stained molecular weight markers.

6. Alternatively, the PVDF membrane can be stained with **Ponceau S Solution** (0.2% Ponceau S in water) to visualize cell proteins transferred to the PVDF membrane.
    a. Place the PVDF membrane in a small plastic tray.
    b. Add 10 mL of the Ponceau S Solution (or sufficient volume to cover the PVDF membrane) and incubate for 2 minutes at room temperature.
    c. Pour off the Ponceau S Solution and rinse the PVDF membrane repeatedly with deionized water until pink bands (proteins) appear.
7. Following band visualization, rinse the PVDF membrane with TBS for 5 minutes at room temperature.
8. Rinse the PVDF membrane four additional times in TBS for 5 minutes each.

## 3. MEMBRANE BLOCKING, PROBING, AND BAND VISUALIZATION

### Procedure

**Membrane Blocking**

1. Incubate the PVDF membrane with **Blocking Buffer** (TBS plus 0.1% Tween 20 and 5% bovine serum albumin) for 30 minutes on an orbital shaker at room temperature. (The incubation can also be performed overnight at 4°C.)

**Incubation With Primary Antibody**

1. Incubate PVDF membrane with diluted primary antibody (ERK 1/2 [pTpY185/187] PSSA or ERK 1/2 pan antibody 1:1000 dilution in Blocking Buffer) for 1 hour on an orbital shaker at room temperature.
2. Pour off antibody solution.
3. Rinse PVDF membrane four times for 5 minutes each with **TBST** (TBS plus 0.1% Tween 20) on an orbital shaker at room temperature.

**Incubation With Secondary Antibody**

1. Incubate PVDF membrane with diluted secondary antibody (1:1000 dilution in Blocking Buffer) for 1 hour on an orbital shaker at room temperature.
2. Pour off secondary antibody solution.
3. Rinse PVDF membrane four times for 5 minutes each with TBST on an orbital shaker at room temperature.

**Band Visualization**

1. React the PVDF membrane with chemiluminescent HRP substrate according to the manufacturer's instructions.
2. Visualize using X-Ray film or chemiluminescent imaging equipment.

## PEPTIDE COMPETITION

The procedure presented below provides instructions for pre-incubating the ERK 1/2 [pTpY185/187] PSSA with 50-fold molar excess of phosphopeptide or non-phosphopeptide, plus a water control. The peptide pre-incubated antibody preparations can then be used as primary antibodies in Western blotting and other antibody-based detection methods to demonstrate the specificity of the PSSA.

### Peptide Reconstitution

1. Allow the *Lyophilized Control Peptides* to reach room temperature, ideally under desiccation.
2. Reconstitute each of the control peptides (supplied at 0.1 mg/vial) to a concentration of 66.7 µM with nanopure water. For a peptide with a molecular mass of 1500, reconstitution with 1 mL water yields a solution with a concentration of 66.7 µM.
3. Apportion the unused reconstituted peptide solutions into working aliquots and store at -20°C for future use.

### Antibody Dilution

1. Dilute 6.5 µL ERK 1/2 [pTpY 185/187] PSSA in 6.5 mL Blocking Buffer.

### Procedure

1. Label 3 test tubes as follows:
    - Tube 1: water only, no peptide control
    - Tube 2: phosphopeptide
    - Tube 3: non-phosphopeptide

2. Into each tube, pipette the following components.
    - Tube 1: 2 mL diluted PSSA solution plus 10 µL nanopure water.
    - Tube 2: 2 mL diluted PSSA solution plus 10 µL phosphopeptide.
    - Tube 3: 2 mL diluted PSSA solution plus 10 µL non-phosphopeptide.
3. Incubate the three tubes for 30 minutes at room temperature with gentle rocking.
4. The contents of the three tubes can then be used as the primary antibody with three replicate samples, such as three identical blocked PVDF strips onto which proteins have been transferred.

When the ERK 1/2 [pTpY185/187] PSSA is specific for the phosphorylated target protein in a particular experimental system, pre-incubation with the phosphopeptide will eliminate the antibody signal, while pre-incubatation with the corresponding non-phosphopeptide will not affect the antibody-antigen interaction.

If the phosphopeptide only partially eliminates the signal, repeat the procedure using twice the volume of water or peptide solutions listed in Step 2. If partial competition is seen following pre-incubation with the non-phosphopeptide, repeat the procedure using half the volume of water or peptide solutions listed in Step 2.

## PROTOCOL SUMMARY

| | |
|---|---|
| **Preliminary** | Resolve proteins by SDS PAGE then transfer proteins to membrane. |
| **Step 1 (30 minutes)** | Transfer the membrane to a clean tray and block membrane in blocking buffer. |
| **Step 2 (1 hour)** | Decant the blocking buffer, then react the blot with diluted primary antibody. |
| **Step 3 (20 minutes)** | Wash 4 times, 5 minutes each. |
| **Step 4 (1 hour)** | React the blot with diluted secondary antibody. |
| **Step 5 (20 minutes)** | Wash 4 times, 5 minutes each. |
| **Step 6 (5 minutes)** | Expose the membrane to chemiluminescent reagents and film. |
| **Total Time** | 3.5 hours |

## LIMITATIONS OF THE PROCEDURE

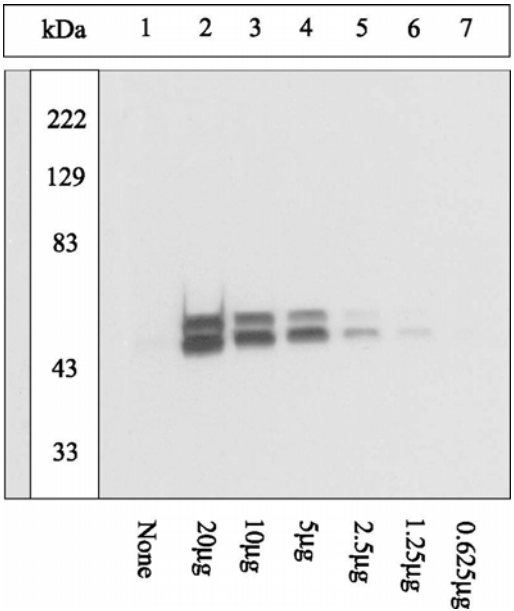
These reagents have been validated for use in Western blot analyses with cell lysates as the sample type. Additional optimization of the cell lysis procedure, as well as stimulation regimens, may be required for each specific application.

## PERFORMANCE CHARACTERISTICS

### Sensitivity

The data presented in Figure 3 show the sensitivity of the ERK 1/2 [pTpY185/187] PSSA. In this study, serial titrations of solubilized cell proteins derived from control NIH3T3 cells (Lane 1) or NIH3T3 cells treated with PDGF for five minutes (Lanes 2 - 7) were separated by SDS PAGE and transferred to PVDF membrane. Phosphorylated ERK 1/2 [pTpY185/187] was detected using the ERK 1/2 [pTpY185/187] PSSA (1:1000 dilution). Lane 1: 20 µg cell protein (control), Lane 2: 20 µg cell protein (+PDGF), Lane 3: 10 µg cell protein, Lane 4: 5 µg cell protein, Lane 5: 2.5 µg cell protein, Lane 6: 1.25 µg cell protein, Lane 7: 0.625 µg cell protein. In this exposure, signal could be detected in 1.25 µg cell protein. Signal could be detected at protein contents of less than 1 µg upon extended exposure of the film. These data demonstrate the selectivity (no or minimal signal in the absence of stimulation) and broad range of sensitivity of the ERK 1/2 [pTpY185/187] PSSA provided in this kit.

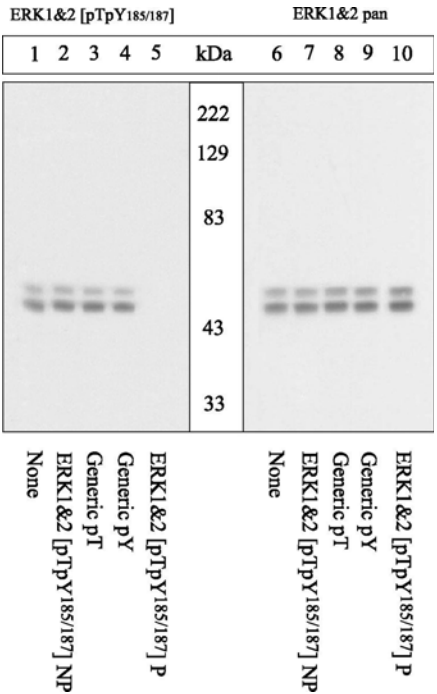
**Figure 3**


kDa
1
2
3
4
5
6
7
222
129
83
43
33
None
20µg
10µg
5µg
2.5µg
1.25µg
0.625µg

**Peptide Competition Experiments**

A study to determine the specificity of the ERK 1/2 pan antibody and ERK 1/2 [pTpY185/187] PSSA for total ERK 1/2 and ERK 1/2 when phosphorylated at threonine 185 and tyrosine 187, respectively, by peptide competition, is presented in Figure 4. In this study, solubilized cell proteins derived from NIH3T3 cells treated with PDGF for five minutes were separated by SDS PAGE and transferred to PVDF membrane. ERK 1/2 protein phosphorylated at threonine 185 and tyrosine 187 was detected using the ERK 1/2 [pTpY185/187] PSSA (1:1000 dilution; Lanes 1 - 5). Total ERK 1/2 protein was detected using the ERK 1/2 pan antibody (1:1000 dilution; Lanes 6 - 10). Antibodies were used following prior incubation with: no peptide (Lanes 1 and 6), the non-phosphopeptide corresponding to the immunogen (Lanes 2 and 7), a generic phosphothreonine-containing peptide (Lanes 3 and 8), a generic phosphotyrosine-containing peptide (Lanes 4 and 9), or the phosphopeptide immunogen (Lanes 5 and 10). These data demonstrate that the signal detected by the ERK 1/2 [pTpY185/187] PSSA was specifically inhibited by pre-incubation of the antibody with the phosphopeptide immunogen, but not with generic pT or pY phosphopeptides nor with the non-phosphopeptide corresponding to the immunogen. Signal detected by the ERK 1/2 pan antibody was not inhibited by prior incubation with any peptide, as expected. These data demonstrate the specificity of the ERK 1/2 [pTpY185/187] PSSA for ERK 1/2 protein phosphorylated at these amino acids.

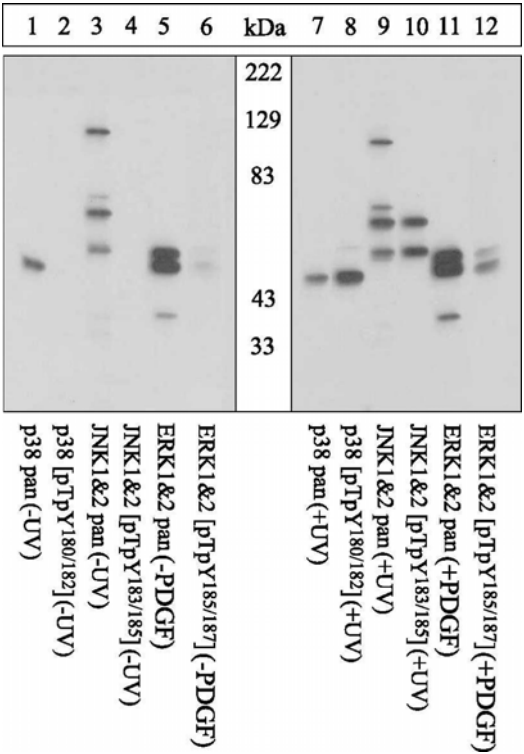
**Figure 4**


ERK1&2 [pTpY185/187]
ERK1&2 pan
1 2 3 4 5
kDa
6 7 8 9 10
222
129
83
43
33
None
ERK1&2 [pTpY185/187] NP
Generic pT
Generic pY
ERK1&2 [pTpY185/187] P
None
ERK1&2 [pTpY185/187] NP
Generic pT
Generic pY
ERK1&2 [pTpY185/187] P

**Specificity for ERK 1/2**

A study to determine the specificity of the ERK pan antibody and the ERK 1/2 [pTpY185/187] PSSA is presented in Figure 5. In this study, solubilized cell proteins from control HEK293 cells (Lanes 1 - 4), HEK293 cells irradiated with ultraviolet light for one minute (Lanes 7-10), control NIH3T3 cells (Lanes 5 and 6), or NIH3T3 cells treated with PDGF for five minutes (Lanes 11 and 12) were separated by SDS PAGE (5 μg cell protein/lane) and transferred to PVDF membrane. Total p38 MAPK protein was detected using the p38 MAPK pan antibody (1:1000 dilution; Lanes 1 and 7). Phosphorylated p38 MAPK protein was detected using the p38 MAPK [pTpY180/182] PSSA (1:1000 dilution; Lanes 2 and 8). Total JNK 1/2 protein was detected using the JNK 1/2 pan antibody (1:1000 dilution; Lanes 3 and 9). Phosphorylated JNK 1/2 protein was detected using the JNK 1/2 [pTpY183/185] PSSA (1:1000 dilution; Lanes 4 and 10). Total ERK 1/2 protein was detected using the ERK 1/2 pan antibody (1:1000 dilution; Lanes 5 and 11). Phosphorylated ERK 1/2 protein was detected using the ERK 1/2 [pTpY185/187] PSSA (1:1000 dilution; Lanes 6 and 12). These data demonstrate that the antibodies directed against each of these MAPK family members react selectively and specifically with the respective protein or phosphorylated protein. The respective antibodies do not cross-react with other MAPK family members. Thus, these antibodies can be used to probe selectively the total protein content and phosphorylation status of each individual MAPK family member.

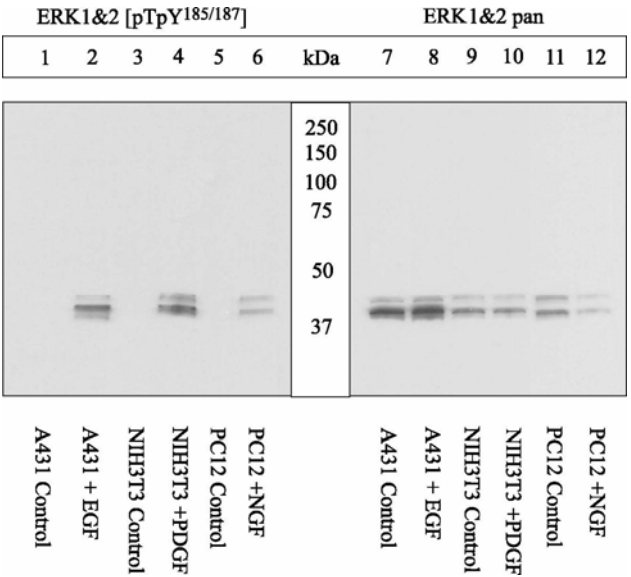
**Figure 5**


1 2 3 4 5 6 kDa 7 8 9 10 11 12
222
129
83
43
33
p38 pan (-UV)
p38 [pTpY180/182] (-UV)
JNK1&2 pan (-UV)
JNK1&2 [pTpY183/185] (-UV)
ERK1&2 pan (-PDGF)
ERK1&2 [pTpY185/187] (-PDGF)
p38 pan (+UV)
p38 [pTpY180/182] (+UV)
JNK1&2 pan (+UV)
JNK1&2 [pTpY183/185] (+UV)
ERK1&2 pan (+PDGF)
ERK1&2 [pTpY185/187] (+PDGF)

**Species Reactivity**

The reactivity of the ERK 1/2 [pTpY185/187] PSSA and the ERK 1/2 pan antibody was assessed with cell lines of human, mouse, and rat origin (Figure 6). In this study, solubilized cell proteins derived from human cells (control A431 cells [Lanes 1 and 7], A431 cells treated with EGF [Lanes 2 and 8]), mouse cells (control NIH3T3 cells [Lanes 3 and 9], NIH3T3 cells treated with PDGF [Lanes 4 and 10]), and rat cells (control PC12 cells [Lanes 5 and 11], PC12 cells treated with NGF [Lanes 6 and 12]) were separated by SDS PAGE and transferred to PVDF membrane. ERK 1/2 protein phosphorylated at threonine 185 and tyrosine 187 was detected using the ERK 1/2 [pTpY185/187] PSSA (1:1000 dilution; Lanes 1 - 6). Total ERK 1/2 protein was detected using the ERK 1/2 pan antibody (1:1000 dilution; Lanes 7 - 12). The data demonstrate that both the ERK 1/2 antibody and the ERK 1/2 [pTpY185/187] PSSA react with human, mouse, and rat ERK 1/2 protein. Thus, these antibodies are useful reagents for the analysis of ERK 1/2 protein content and phosphorylation status in cells and tissues derived from multiple species.

**Figure 6**


ERK1&2 [pTpY185/187]
ERK1&2 pan
1 2 3 4 5 6 kDa 7 8 9 10 11 12
250
150
100
75
50
37
A431 Control
A431 + EGF
NIH3T3 Control
NIH3T3 +PDGF
PC12 Control
PC12 +NGF
A431 Control
A431 + EGF
NIH3T3 Control
NIH3T3 +PDGF
PC12 Control
PC12 +NGF

## NO OR WEAK SIGNAL DETECTED

| CAUSES | SOLUTIONS |
|---|---|
| 1. Low target protein content in cell lysate sample. (Target protein content can be checked by blotting with pan antibody for target protein.) | 1.a. Increase the quantity of cell protein separated on gel.<br>1.b. Immunoprecipitate target protein prior to electrophoresis.<br>1.c. Transfect cells with target protein cDNA to increase expression level. |
| 2. Sufficient level of specific phosphorylation event is not induced by stimulation protocol. | 2.a. Repeat stimulation protocol using cells pretreated with phosphatase inhibitors.<br>2.b. Try new stimulation protocol. |
| 3. Concentration of primary and/or secondary antibody is too low. | 3.a. Increase concentrations of primary and secondary antibodies in stepwise fashion to determine optimal concentration for the specific experimental system. |
| 4. Blocking conditions interfere with antigen detection. | 4.a. Compare results obtained using milk versus BSA as blocking reagent.<br>4.b. Vary concentrations of blocking reagents (i.e., BSA/milk, Tween 20). |
| 5. Poor protein transfer to membrane. | 5.a. Increase length of transfer.<br>5.b. Transfer using higher voltage (caution – increasing voltage will increase heat generation which could interfere with transfer). |

HIGH BACKGROUND

| CAUSES | SOLUTIONS |
|---|---|
| 1. Concentration of primary and/or secondary antibody is too high. | 1.a. Titrate primary and/or secondary antibodies to determine optimal concentration for the specific experimental system. |
| 2. Blocking conditions are not sufficient to minimize background. | 2.a. Increase blocking period.<br>2.b. Increase concentration of blocking reagent(s) (i.e., BSA/milk, Tween 20).<br>2.c. Compare results obtained using milk versus BSA as blocking reagent. |
| 3. Membrane is not optimal for detection. | 3.a. Compare results obtained using PVDF versus nitrocellulose as blotting membrane. |

**APPENDIX**

IMMUNOSTAINING AND FLOW CYTOMETRY PROTOCOLS

**Immunostaining Procedure**

1. Seed cells onto sterile microscope slides or poly-L-lysine-coated microscope slides. Poly-L-lysine-coated slides should also be used for immunostaining of tissue sections.
2. Rinse slides three times with DPBS at 4°C (Invitrogen catalog #P315-500).
3. To fix cells or tissue sections add IC Fix™ (Invitrogen catalog #FB001) to entirely cover the slide surface containing cells or tissue.
4. Incubate for 15 minutes at 4°C.
5. Aspirate the fixation solution and rinse the slides three times with DPBS.
6. Block endogenous peroxidase activity (if using HRP-conjugated secondary antibody for detection) by incubation with 3% hydrogen peroxide in DPBS for 30 minutes. This step can be omitted if using fluorophore-conjugated secondary antibody.
7. Permeabilize the cells and tissue sections by incubating with 100% methanol for 30 minutes at -20°C.
8. Rinse slides three times with DPBS at room temperature.
9. Block non-specific antibody binding sites by incubating slides in DPBS containing 5% BSA for 30 minutes at room temperature.

10. Incubate slides in primary antibody (ERK 1/2 [pTpY185/187] PSSA) diluted in DPBS containing 5% BSA for 1 hour at room temperature. The suggested dilution for this application is 0.5 - 5 μL of the antibody diluted in 40 μL of IC Perm™. The optimal dilution should be determined empirically for each experimental system.
11. Rinse slides three times in DPBS at room temperature.
12. Incubate slides with appropriate conjugated secondary antibody for 1 hour at room temperature. Choices of conjugated secondary antibody include enzyme-conjugated (e.g., goat [polyclonal] anti-rabbit IgG HRP conjugate) or fluorophore-conjugated (e.g., goat [polyclonal] anti-rabbit IgG FITC conjugate).
13. Rinse slides three times with DPBS at room temperature.
14. When using an enzyme-conjugated secondary antibody, add the enzyme substrate to the cell or tissue sample and develop according to the manufacturer's instructions.
15. When using a fluorophore-conjugated secondary antibody, add an anti-fade reagent (for example, VectaShield Mounting Medium [Vector Laboratories catalog #H-1000]) to the cell or tissue sample.
16. Place a coverslip on top of the sample and seal the coverslip to the microscope slide using nail polish or another sealer.

**Flow Cytometry Procedure**

1. Stimulate cells as desired to obtain appropriate phosphorylation of the target protein.
2. Collect cells by centrifugation for 5 minutes at 300 x g at 4°C.
3. Aspirate cell culture medium.
4. Rinse cells two times with DPBS by centrifugation for 5 minutes at 300 x g at 4°C.
5. Fix cells by incubation with IC Fix™ (Invitrogen catalog #FB001) for 15 minutes at 4°C. Cells should be thoroughly resuspended in the fixative.
6. Wash the cells two times with DPBS by centrifugation at 300 x g at 4°C.
7. To permeabilize the cells, resuspend the cells in 100% ice cold methanol added dropwise while the cells are vortexing. It is crucial that the cells are resuspended thoroughly during this methanol treatment step.
8. Incubate in methanol for 15 minutes on ice.
9. Wash the cells two times with DPBS by centrifugation at 300 x g at 4°C.
10. Add approximately 1 x $10^6$ cells to flow cytometry tubes and wash one time with IC Perm™ (Invitrogen catalog #PB001) by centrifugation at 300 x g at 4°C.
11. Resuspend cells in 1 mL IC Perm™ and incubate for 30 minutes at room temperature. (This step is included to reduce the background of the signal.)

12. Dilute the ERK 1/2 [pTpY185/187] PSSA in IC Perm™. The suggested dilution for this application is 0.5 - 5 µL of the antibody diluted in 40 µL of IC Perm™. The optimal dilution should be determined empirically for each experimental system.
13. Add the diluted antibody to the cell pellet and incubate for 20 minutes at room temperature. Wash the cells two times with IC Perm™ by centrifugation at 300 x g at 4°C.
14. Resuspend the cells in 100 µL IC Perm™.
15. To prepare secondary antibody, dilute approximately 1 µg of an appropriate anti-rabbit IgG R-Phycoerythrin (RPE) conjugated secondary antibody in 100 µL IC Perm™.
16. Incubate the diluted secondary antibody with the cells for 20 minutes at room temperature.
17. Wash the cells one time with IC Perm™ followed by one time with DPBS by centrifugation at 300 x g at 4°C.
18. Resuspend the cells in 500 µL of DPBS and detect/quantitate antibody-positive cells by flow cytometry.

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**NOTES**