Surveyor™ IC

Human/Mouse/Rat Phospho-ERK1 (T202/Y204)/ ERK2 (T185/Y187) Immunoassay

Catalog Number SUV1018B

For the quantitative determination of ERK1 phosphorylated at T202/Y204 and ERK2 phosphorylated at T185/Y187 in cell lysates.

This package insert must be read in its entirety before using this product.
For research use only. Not for use in diagnostic procedures.

TABLE OF CONTENTS

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INTRODUCTION

As key components of the Raf-MEK-ERK signal transduction module, the mitogen-activated protein kinases ERK1 and ERK2 regulate cellular proliferation, differentiation, and survival. ERK1 (also known as MAPK3 and p44 MAPK) and ERK2 (also known as MAPK1 and p42 MAPK) are 44 and 42 kDa Ser/Thr kinases, respectively, with 90% sequence identity in mammals. Initially isolated and cloned as kinases activated in response to insulin and β-NGF (1, 2), these ERKs are both expressed in most, if not all, mammalian tissues. While ERK1 and ERK2 are highly homologous, differences in their activities have been noted, including their roles in mesoderm differentiation (3), MEK scaffolding (4), and thymocyte maturation (5). The MAPK kinases MEK1 and MEK2 activate both ERKs by dual threonine and tyrosine phosphorylation. These phosphorylation sites reside in a Thr-Glu-Tyr motif within the kinase activation loop, at T202/Y204 for human ERK1 and T185/Y187 for human ERK2. Full ERK activation requires phosphorylation at both sites, with Tyr phosphorylation preceding that of Thr (6).

The ERKs are proline-directed protein kinases, phosphorylating Ser or Thr residues within the motif Pro-Xxx-Ser/Thr-Pro. Docking sites present on physiological substrates confer additional specificity and team with scaffolding proteins to ensure signaling fidelity and enzymatic efficiency (7). Activated ERK1 and ERK2 regulate growth factor-responsive targets in the cytosol and also translocate to the nucleus where they phosphorylate a number of proteins regulating gene expression. Nuclear targets include the transcription factors Elk-1 (8), Myc (9), BRF-1 (10), and UBF (11). ERK1 and ERK2 also regulate transcription indirectly by phosphorylating kinases from the RSK (12) and MSK (13) families.

PRINCIPLE OF THE ASSAY

This Surveyor IC Immunoassay employs a two-site sandwich ELISA to quantitate human/mouse/rat phospho-ERK1 (T202/Y204) and phospho-ERK2 (T185/Y187) in cell lysates. Antibody specific for human/mouse/rat ERK1 and ERK2, binding both phosphorylated and unphosphorylated protein, has been pre-coated onto a microplate. Standards and samples are added and ERK1 and ERK2 present are bound by the immobilized antibody. After washing away unbound material, a biotinylated detection antibody recognizing human/mouse/rat ERK1 dually phosphorylated at T202 and Y204, and human/mouse/rat ERK2 dually phosphorylated at T185 and Y187, is used to detect only phosphorylated protein, utilizing a standard streptavidin-HRP format. Substrate Solution is added to the wells and color develops in proportion to the amount of phosphorylated ERK1 and ERK2 bound in the initial step. The color development is stopped and the intensity of the color is measured.

LIMITATIONS OF THE PROCEDURE

- FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.
- This Surveyor IC Immunoassay should not be used beyond the expiration date on the kit label.
- Do not mix or substitute reagents with those from other lots or sources.
- If samples generate values higher than the highest standard, further dilute the samples with Assay Diluent and repeat the assay.
- Any variation in standard diluent, operator, pipetting technique, washing technique, incubation time or temperature, and kit age can cause variation in binding.
- Variations in sample collection, processing, and storage may cause sample value differences.

TECHNICAL HINTS

- When mixing or reconstituting protein solutions, always avoid foaming.
- To avoid cross-contamination, change pipette tips between additions of each standard level, between sample additions, and between reagent additions. Also, use separate reservoirs for each reagent.
- To ensure accurate results, proper adhesion of plate sealers during incubation steps is necessary.
- A thorough and consistent wash technique is essential for proper assay performance. Wash Buffer should be dispensed forcefully and removed completely from the wells by aspiration or decanting. Remove remaining Wash Buffer by inverting the plate and blotting it against clean paper towels.
- When using an automated plate washer, adding a 30 second soak period following the addition of wash buffer, and/or rotating the plate 180 degrees between wash steps may improve assay precision.
- Substrate Solution should remain colorless until added to the plate. Keep Substrate Solution protected from light. Substrate Solution should change from colorless to gradations of blue.
- Stop Solution should be added to the plate in the same order as the Substrate Solution. The color developed in the wells will turn from blue to yellow upon addition of the Stop Solution. Wells that are green in color indicate that the Stop Solution has not mixed thoroughly with the Substrate Solution.

PRECAUTIONS

The Stop Solution provided with this kit is an acid solution.

Some components in this kit contain ProClin® which may cause an allergic skin reaction. Avoid breathing mist.

Color Reagent B may cause skin, eye, and respiratory irritation. Avoid breathing fumes.

Wear protective gloves, clothing, eye, and face protection. Wash hands thoroughly after handling. Please refer to the MSDS on our website prior to use.

MATERIALS PROVIDED & STORAGE CONDITIONS

Store the unopened kit at 2-8 °C. Do not use past kit expiration date.

PART	PART #	DESCRIPTION	STORAGE OF OPENED/ RECONSTITUTED MATERIAL
Human/Mouse/rat Phospho-ERK1/ERK2 Microplate	843532	96 well polystyrene microplate (12 strips of 8 wells) coated with a monoclonal antibody against ERK1/ERK2.	Return unused wells to the foil pouch containing the desiccant pack. Reseal along entire edge of the zip-seal. May be stored for up to 1 month at 2-8 °C.*
Human/Mouse/Rat Phospho-ERK1/ERK2 Standard	841328	2 vials (615 ng/vial) of recombinant human phospho-ERK2 (T185/Y187) in a buffered protein base with preservatives; lyophilized	Use within 1 hour of reconstitution. Use a fresh standard for each assay.
Human/Mouse/Rat Phospho-ERK1/ERK2 Detection Antibody	842235	6.0 µg of a biotinylated rabbit polyclonal anti-phospho-ERK1/ERK2 antibody; lyophilized.	May be stored for up to 1 month at 2-8 °C.*
Lysis Buffer 6	895561	21 mL of a cell lysing buffer with phosphatase inhibitors and preservatives.	
Sample Diluent Concentrate 1 (5X)	895562	21 mL of a 5-fold concentrated buffer with preservatives.	
Reagent Diluent Concentrate 2 (10X)	841380	21 mL of a 10-fold concentrated buffered protein base with preservatives.	
Wash Buffer Concentrate	895003	21 mL of a 25-fold concentrated buffered surfactant with preservative. *May turn yellow over time.*	
Color Reagent A	895000	12 mL of stabilized hydrogen peroxide.	
Color Reagent B	895001	12 mL of stabilized chromogen (tetramethylbenzidine).	
Streptavidin-HRP	890803	1.0 mL of streptavidin conjugated to horseradish-peroxidase.	
Stop Solution	895032	6 mL of 2 N sulfuric acid.	
Plate Sealers	N/A	4 adhesive strips.	

* Provided this is within the expiration date of the kit.

OTHER SUPPLIES REQUIRED

- Microplate reader capable of measuring absorbance at 450 nm, with the correction wavelength set at 540 nm or 570 nm.
- Pipettes and pipette tips.
- 50 mL, 100 mL and 500 mL graduated cylinders.
- Deionized or distilled water.
- Squirt bottle, manifold dispenser, or automated microplate washer.
- Phosphate-buffered saline (PBS).
- Phenylmethylsulfonylfluoride (PMSF) (optional; Sigma, Catalog # P7626).
- Protease Inhibitor Cocktail (optional; Sigma, Catalog # P2714).
- **Polypropylene** test tubes for diution of standards and samples.

REAGENT PREPARATION

Bring all reagents to room temperature before use.

Wash Buffer - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Add 20 mL of Wash Buffer Concentrate to deionized or distilled water to prepare 500 mL of Wash Buffer.

Sample Diluent 1 - Add 20 mL of Sample Diluent Concentrate 1 (5X) to deionized or distilled water to prepare 100 mL of Sample Diluent 1.

Assay Diluent - Add 8 mL of Lysis Buffer 6 to Sample Diluent 1 to prepare 48 mL of Assay Diluent. Prepare only enough diluent to run the assay.

Reagent Diluent 2 - Add 5 mL of Reagent Diluent Concentrate 2 (10X) to deionized or distilled water to prepare 50 mL of Reagent Diluent 2.

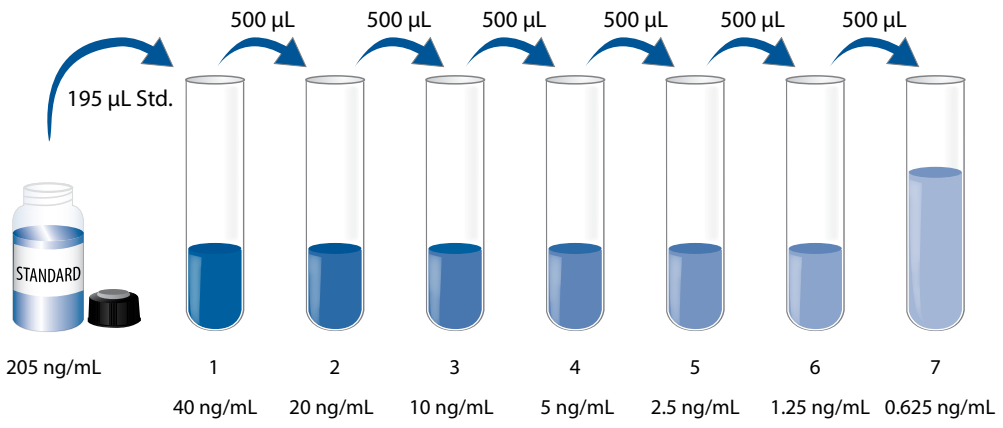
Human/Mouse/Rat Phospho-ERK1/ERK2 Detection Antibody - Reconstitute the Human/Mouse/Rat Phospho-ERK1/ERK2 Detection Antibody with 1.0 mL of Reagent Diluent 2. This reconstitution produces a stock solution of 6.0 µg/mL. Immediately before use, dilute the Detection Antibody to a working concentration of 400 ng/mL in Reagent Diluent 2.

Streptavidin-HRP - Immediately before use, dilute Streptavidin-HRP to the working concentration specified on the vial label using Reagent Diluent 2.

Substrate Solution - Color Reagents A and B should be mixed together in equal volumes within 15 minutes of use. Prepare only enough Substrate Solution as needed to run the assay. Protect from light. 100 µL of the resultant mixture is required per well.

Human/Mouse/Rat Phospho-ERK1/ERK2 Standard - Reconstitute the Human/Mouse/Rat Phospho-ERK1/ERK2 Standard with 0.5 mL of Lysis Buffer 6. This reconstitution produces a stock solution of 1230 ng/mL. Mix the standard to ensure complete reconstitution. **Allow the standard to sit for a minimum of 15 minutes.** Perform a 1:6 dilution of the Standard by adding 2.5 mL of Sample Diluent 1 to the vial. This dilution produces a stock solution of 205 ng/mL.

Label seven **polypropylene tubes** 1 through 7. Add 805 µL of Assay Diluent into tube 1 and 500 µL of Assay Diluent into tubes 2 through 7. Add 195 µL of the 205 ng/mL stock solution to tube 1. Mix thoroughly and continue to prepare a seven point standard curve using 2-fold serial dilutions by transferring 500 µL from tube 1 into tube 2 and subsequent 500 µL transfers as shown below. Use the 40 ng/mL Standard as the high standard. Use Assay Diluent as the zero standard. **Use a fresh standard for each assay. Use within 1 hour of preparation.**



CELL LYSIS PROCEDURE

Note: *It is recommended to supplement Lysis Buffer 6 with 1 mM DTT, PMSF, and the Protease Inhibitor Cocktail prior to use. Supplements should be used according to the manufacturer's instructions.*

1. Using PBS, collect non-adherent cells by centrifugation and adherent cells by scraping the culture flask.
2. Rinse cells two times with PBS, making sure to remove any remaining PBS after the second rinse.
3. Solubilize cells at 1 x 10^7 cells/mL in Lysis Buffer 6.
4. Vortex lysates briefly and allow to sit on ice for 15 minutes or store at ≤ -20 °C in a manual defrost freezer. Sample protein concentration may be quantified using a total protein assay.
5. Before use, centrifuge at 2000 x g for 5 minutes and transfer the supernates into a clean test tube.
6. For assaying, dilute lysates 6-fold with Sample Diluent 1 and make further serial dilutions in Assay Diluent.

ASSAY PROCEDURE

Bring all reagents and samples to room temperature before use. It is recommended that all standards, samples, and controls be assayed in duplicate.

1. Prepare all reagents, working standards, and samples as directed in the previous sections.
2. Remove excess microplate strips from the plate frame, return them to the foil pouch containing the desiccant pack, and reseal.
3. Add 100 µL of Standard or sample* per well. Use Assay Diluent as the zero standard. Cover with the adhesive strip provided. Incubate for 2 hours at room temperature.
4. Aspirate each well and wash, repeating the process two times for a total of three washes. Wash by filling each well with Wash Buffer (400 µL) using a squirt bottle, manifold dispenser, or autowasher. Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.
5. Immediately before use, prepare the Detection Antibody. Add 100 µL of diluted Human/Mouse/Rat Phospho-ERK1/ERK2 Detection Antibody to each well. Cover with a new adhesive strip. Incubate for 2 hours at room temperature.
6. Repeat the aspiration/wash as in step 4.
7. Immediately before use, dilute the Streptavidin-HRP to the working concentration specified on the vial label using Reagent Diluent 2. Add 100 µL of the diluted Streptavidin-HRP to each well. Incubate for 20 minutes at room temperature.
8. Repeat the aspiration/wash as in step 4.
9. Immediately before use, prepare the Substrate Solution. Add 100 µL of Substrate Solution to each well. Incubate for 20 minutes at room temperature. **Avoid placing the plate in direct light.**
10. Add 50 µL of Stop Solution to each well. Gently tap the plate to ensure thorough mixing.
11. Determine the optical density of each well within 30 minutes, using a microplate reader set to 450 nm. If wavelength correction is available, set to 540 nm or 570 nm. If wavelength correction is not available, subtract readings at 540 nm or 570 nm from the readings at 450 nm. This subtraction will correct for optical imperfections in the plate. Readings made directly at 450 nm without correction may be higher and less accurate.

*Lysates require dilution. Refer to the Cell Lysis Procedure.

CALCULATION OF RESULTS

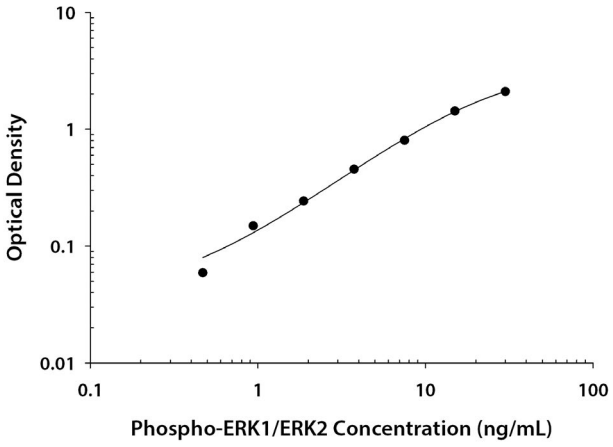
Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density (O.D.). Results may be normalized to total protein or cell number.

Create a standard curve by reducing the data using computer software capable of generating a four parameter logistic (4-PL) curve-fit. As an alternative, construct a standard curve by plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis and draw a best fit curve through the points on the graph. The data may be linearized by plotting the log of the human/mouse/rat phospho-ERK1 (T202/Y204)/ERK2 (T185/Y187) concentrations versus the log of the O.D. and the best fit line can be determined by regression analysis. This procedure will produce an adequate but less precise fit of the data.

Since samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

TYPICAL DATA

A standard curve should be generated for each set of samples assayed. The graph below represents typical data generated when using this Human/Mouse/Rat Human/Mouse/Rat Phospho-ERK1 (T202/Y204)/ERK2 (T185/Y187) Surveyor IC Immunoassay. The standard curve was calculated using a computer generated 4-PL curve-fit. This standard curve is provided for demonstration purposes only.



(ng/mL)	O.D.	Corrected	Average
0	0.160 0.160	—	—
0.625	0.219 0.236	0.059 0.076	0.068
1.25	0.294 0.302	0.134 0.142	0.138
2.5	0.400 0.417	0.240 0.257	0.248
5	0.598 0.612	0.438 0.452	0.445
10	0.987 0.997	0.827 0.837	0.832
20	1.549 1.603	1.389 1.443	1.416
40	2.215 2.265	2.055 2.105	2.080

CALIBRATION

The Human/Mouse/Rat Phospho-ERK1 (T202/Y204)/ERK2 (T185/Y187) Surveyor IC Immunoassay is calibrated against a highly purified *E. coli*-expressed recombinant human phospho-ERK2 (T185/Y187) produced at R&D Systems. Samples containing natural ERK1 phosphorylated at T202 and Y204 or ERK2 phosphorylated at T185 and Y187 showed linear dilution parallel to the standard curve obtained using the Human/Mouse/Rat Phospho-ERK1/ERK2 Standard. These results indicate that O.D. values from this Surveyor IC Immunoassay can be used to determine the concentration of ERK1 phosphorylated at T202 and Y204 or ERK2 phosphorylated at T185 and Y187 in natural samples.

SPECIFICITY

The Human/Mouse/Rat Phospho-ERK1 (T202/Y204)/ERK2 (T185/Y187) DuoSet IC ELISA specifically recognizes ERK1 dually phosphorylated at T202 and Y204, and ERK2 dually phosphorylated at T185 and Y187. Specificity was demonstrated by Western blot analysis of the protein bound by the capture antibody supplied in the kit, cross-reactivity analysis, and peptide competition.

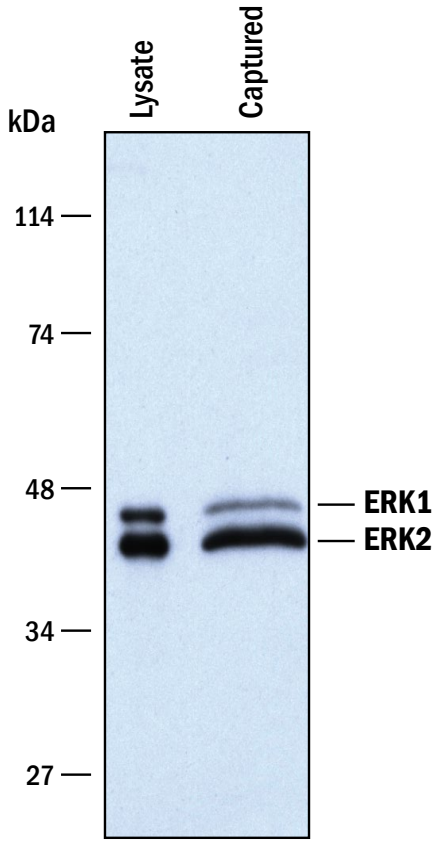


Figure 1: Lysates prepared from HeLa human cervical epithelial carcinoma cells treated with 200 nM phorbol 12-myristate 13-acetate (PMA) for 20 minutes were incubated in wells coated with Human/Mouse/Rat Phospho-ERK1/ERK2 Capture Antibody. Unbound material was removed by washing and bound material was solubilized in SDS gel sample buffer. The same lysate and captured proteins were electrophoresed, transferred to a PVDF membrane, and immunoblotted with Human/Mouse/Rat Phospho-ERK1/ERK2 Detection Antibody. Only two bands corresponding to phosphorylated ERK1 and ERK2 were detected in the captured material.

Cross reactivity experiments were performed with this DuoSet IC ELISA to further determine specificity. Unphosphorylated recombinant human (rh) ERK1, rhERK2, rhJNK1, and rhp38α were assayed at 300 ng/mL and did not cross-react or interfere in the assay.

SPECIFICITY CONTINUED

The specificity of this kit was also demonstrated using peptide competition.

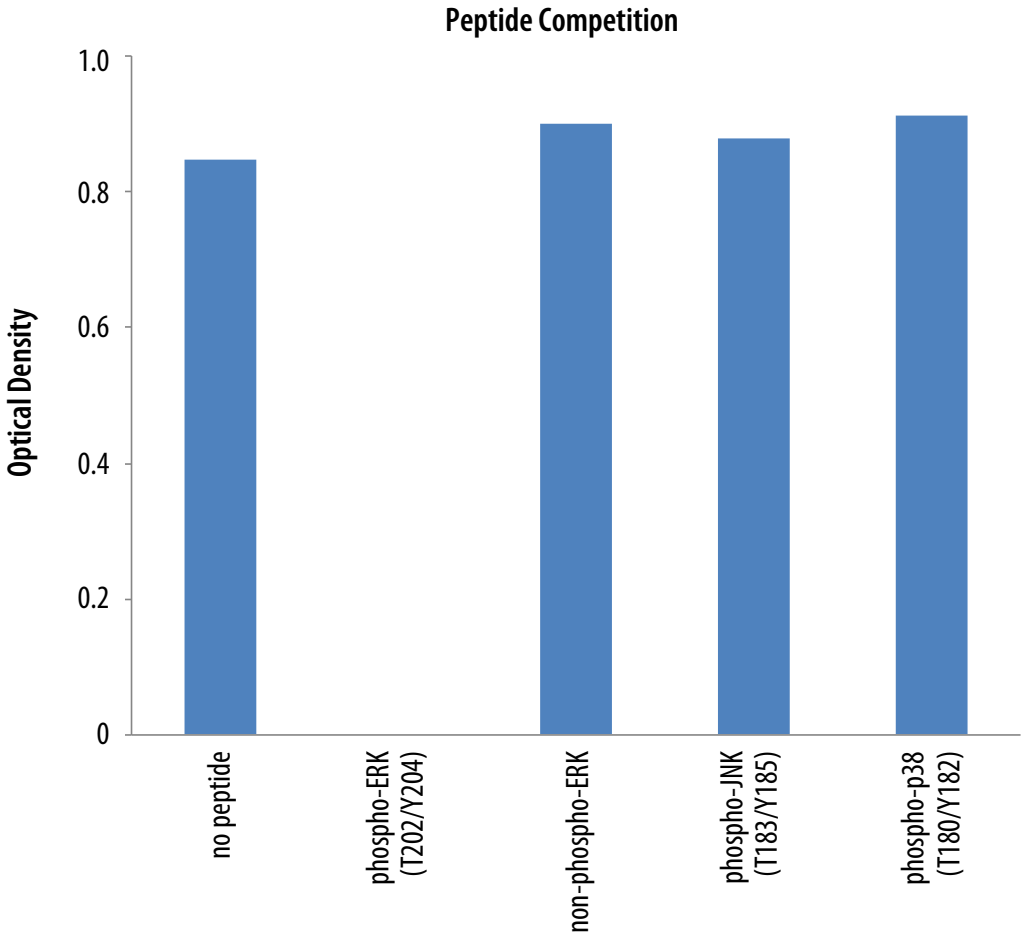


Figure 2: Lysates prepared from HeLa human cervical epithelial cells treated with 200 nM PMA for 20 minutes were analyzed with this DuoSet IC ELISA. The Human/Mouse/Rat Phospho-ERK1/ERK2 Detection Antibody was untreated (no peptide) or preincubated with peptides containing ERK (T202/Y204) dual phosphorylation site, a non-phospho-ERK, JNK (T183/Y185) dual phosphorylation site, or p38 (T180/Y182) dual phosphorylation site. Peptides were used at 40 ng/mL. Only the phosphopeptide containing the ERK (T202/Y204) phosphorylation site blocked the signal, indicating that this DuoSet IC ELISA is specific for ERK phosphorylated at T202/Y204.

QUANTIFICATION

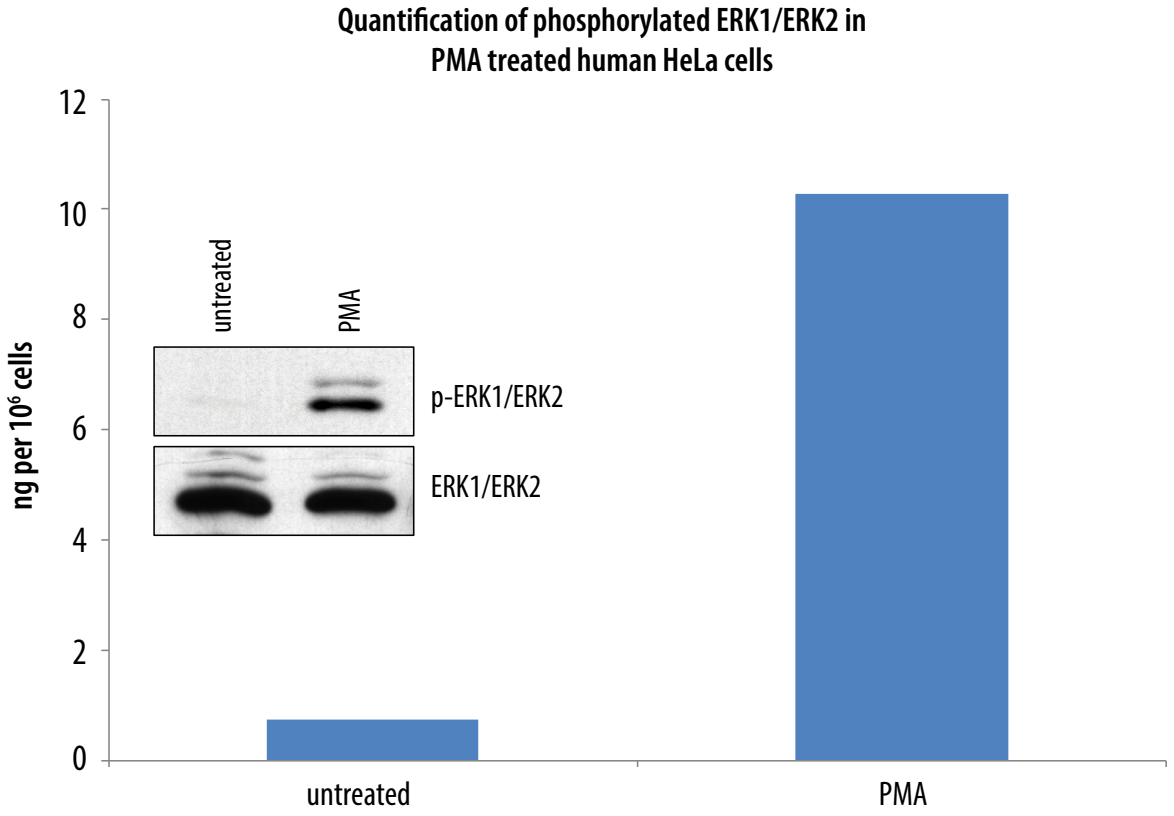


Figure 3: Lysates prepared from HeLa cells were treated with 200 nM PMA for 20 minutes. Following cell lysis, phosphorylated ERK1 and ERK2 were quantified with this DuoSet IC ELISA. The same lysates were also immunoblotted (inset) with either anti-phospho-ERK1 (T202/Y204)/ ERK2 (T185/Y187) (R&D Systems, Catalog #AF1018) or anti-ERK1/ERK2 (R&D Systems, Catalog #MAB1576) antibodies. The DuoSet IC ELISA results correlate well with the relative amounts of phosphorylated ERK1/ERK2 detected by Western blot. The immunoblot with anti-ERK1/ERK2 antibody indicated that total levels of ERK1 and ERK2 remained constant during incubations with PMA.

QUANTIFICATION *CONTINUED*

The Human/Mouse/Rat Phospho-ERK1 (T202/Y204)/ERK2 (T185/Y187) DuoSet IC ELISA also quantifies phosphorylated ERK1 and ERK2 levels in mouse and rat cell lysates.

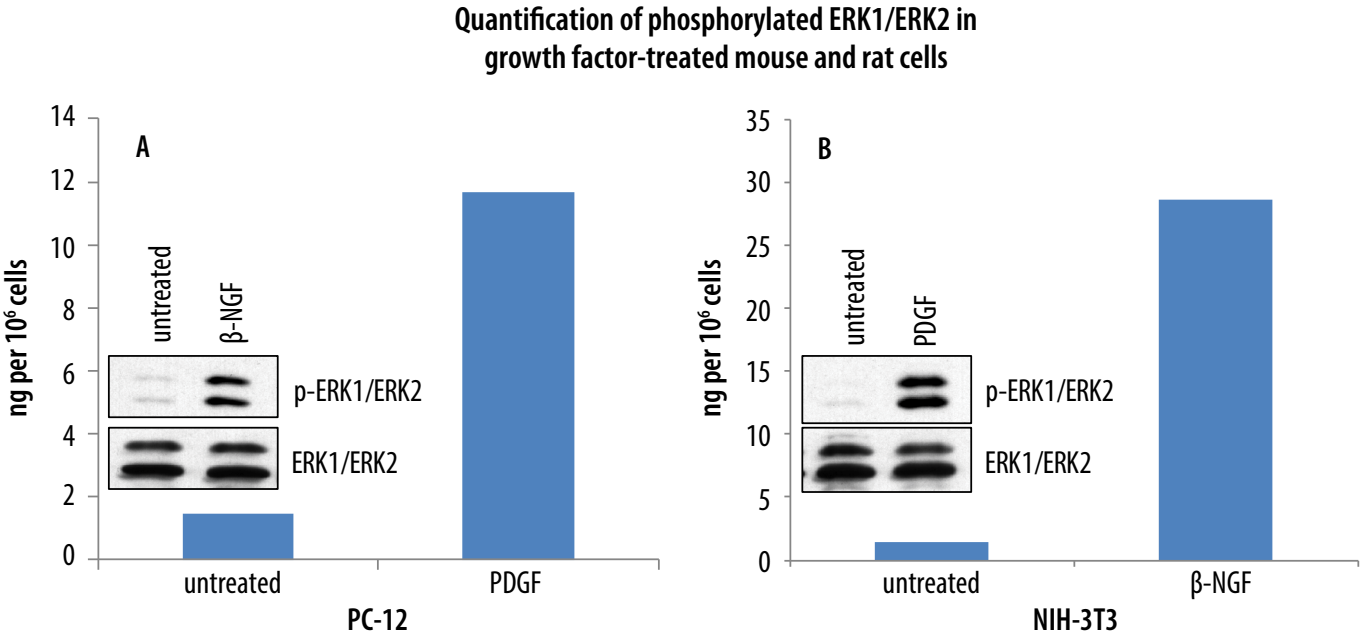


Figure 4: Lysates prepared from PC-12 rat adrenal pheochromocytoma cells were **(A)** untreated or treated with recombinant rat β-NGF (R&D Systems, Catalog #556-NG) or **(B)** NIH-3T3 mouse embryonic fibroblast cells were untreated or treated with human PDGF (R&D Systems, Catalog #120-HD) were quantified with this DuoSet IC ELISA. The same lysates were also immunoblotted (insets) with either anti-phospho-ERK1 (T202/Y204)/ERK2 (T185/Y187) or anti-ERK1/ERK2 antibodies. The DuoSet IC ELISA results correlate well with the relative amounts of phosphorylated ERK1 (T202/Y204)/ERK2 (T185/Y187) detected by Western blot. The immunoblot with anti-ERK1/ERK2 antibody indicates that total levels of ERK1 and ERK2 remained constant during incubations with PMA.

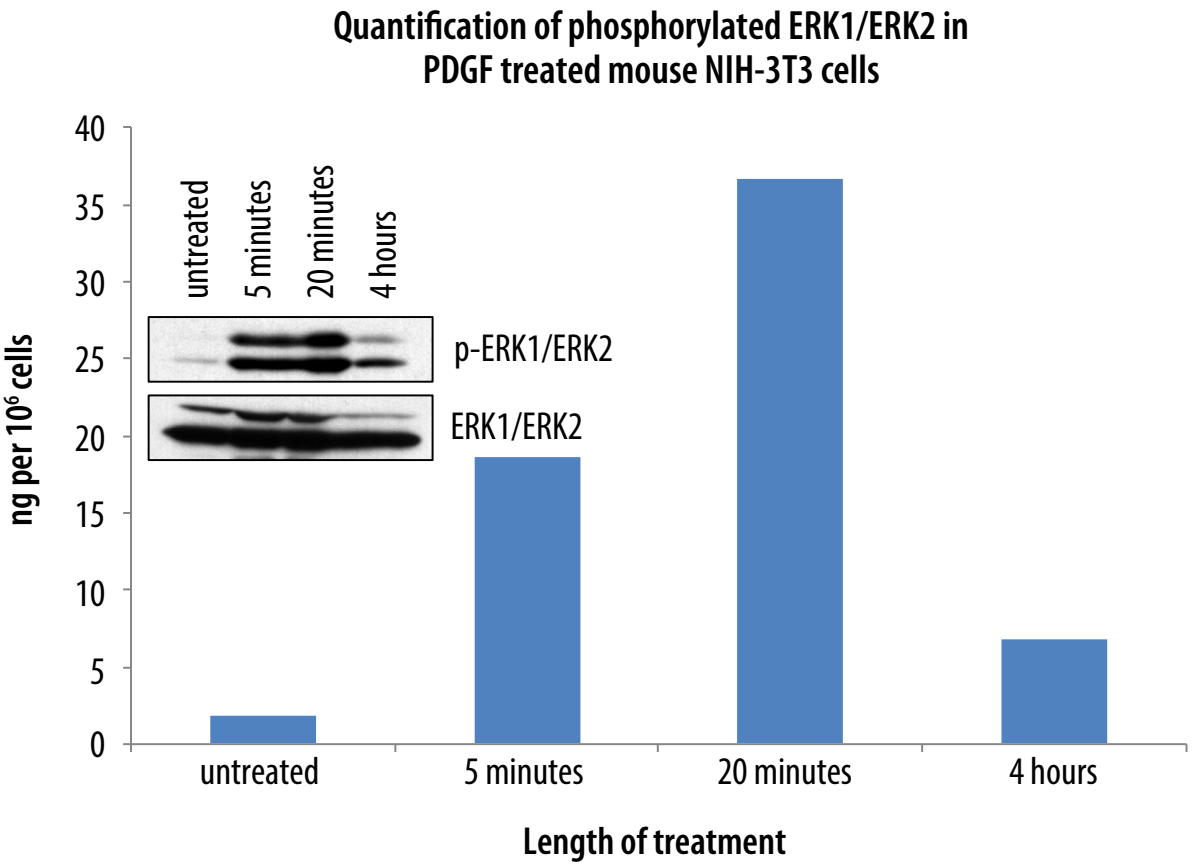


Figure 5: NIH-3T3 cells were treated with 100 ng/mL of human PDGF for the indicated times. Following cell lysis, phosphorylated ERK1 and ERK2 were quantified with the DuoSet IC ELISA. The same lysates were immunoblotted (inset) with either anti-phospho-ERK1 (T202/Y204)/ ERK2 (T185/Y187) or anti-ERK1/ERK2 antibodies. The DuoSet IC ELISA results correlate well with the relative amount of phosphorylated ERK1 and ERK2 detected by Western blot. The blot with the anti-ERK1/ERK2 antibody indicates that the total levels of ERK1 and ERK2 remained constant during incubations with PDGF.

QUANTIFICATION *CONTINUED*

The quantification of the phosphorylated ERK1 and ERK2 with this DuoSet IC ELISA was also determined using cells pretreated with varying amounts of the MEK1/2 inhibitor U0126, which indirectly inhibits the phosphorylation of ERK1 and ERK2.

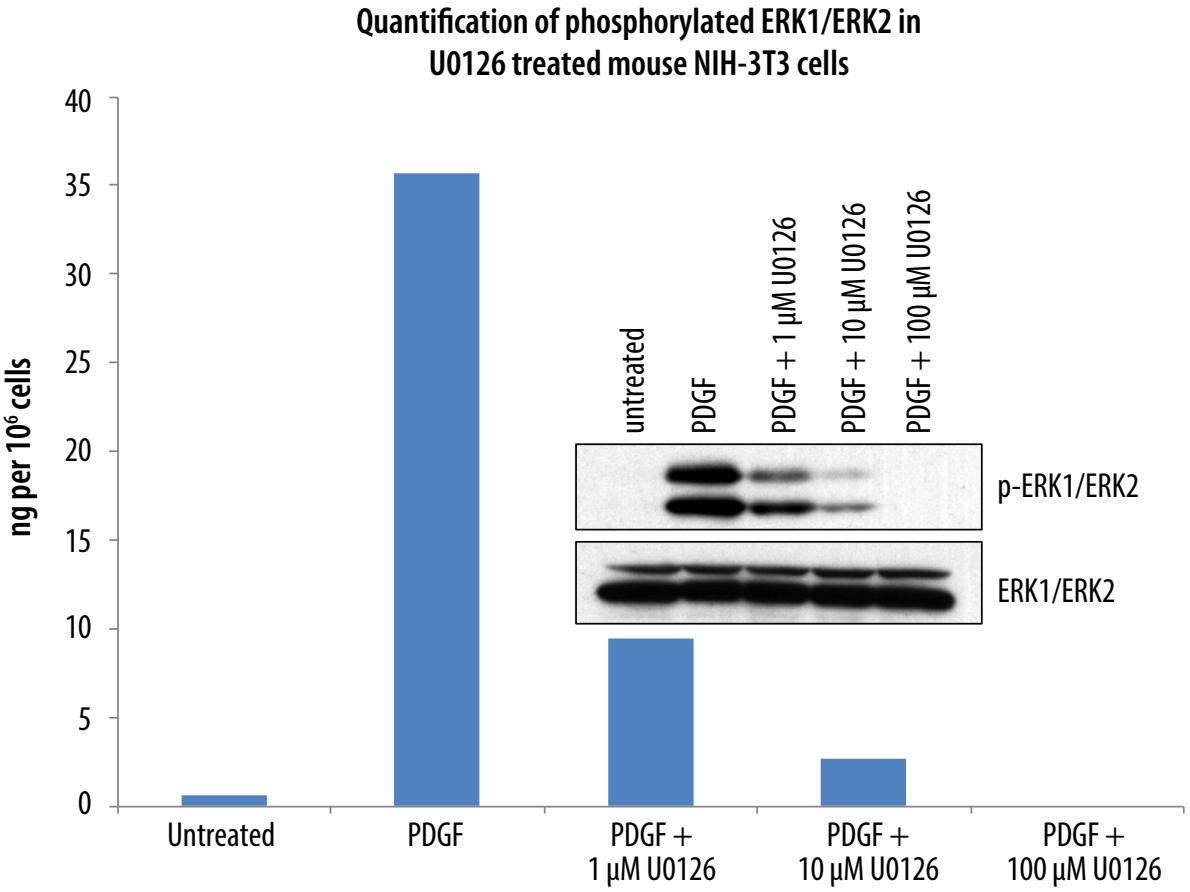


Figure 6: NIH-3T3 cells were incubated with no additions or treated with 100 ng/mL of human PDGF for 10 minutes, either with or without U0126. Following cell lysis, phosphorylated ERK1 and ERK2 were quantified with the DuoSet IC ELISA. The same lysates were immunoblotted (inset) with either anti-phospho-ERK1 (T202/Y204)/ERK2 (T185/Y187) or anti-ERK1/ERK2 antibodies. The DuoSet IC ELISA results correlate well with the relative amount of phosphorylated ERK1 and ERK2 detected by Western blot. The blot with the anti-ERK1/ERK2 antibody indicates that the total levels of ERK1 and ERK2 remained constant during the various treatments.

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