

Surveyor™ IC

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

Catalog Number SUV1483

For the quantitative determination of 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

Contents	Page
INTRODUCTION	2
PRINCIPLE OF THE ASSAY	2
LIMITATIONS OF THE PROCEDURE	2
MATERIALS PROVIDED	3
OTHER SUPPLIES REQUIRED	4
PRECAUTION	4
TECHNICAL HINTS	4
REAGENT PREPARATION	5
SAMPLE PREPARATION	6
ASSAY PROCEDURE	7
ASSAY PROCEDURE CHECKLIST	8
CALCULATION OF RESULTS.	9
TYPICAL DATA	9
CALIBRATION	9
SPECIFICITY	10
QUANTIFICATION	12
REFERENCES	15

MANUFACTURED AND DISTRIBUTED BY:

R&D Systems, Inc.
614 McKinley Place NE
Minneapolis, MN 55413
United States of America

TELEPHONE: (800) 343-7475
(612) 379-2956
FAX: (612) 656-4400
E-MAIL: info@RnDSystems.com

DISTRIBUTED BY:

R&D Systems Europe, Ltd.
19 Barton Lane
Abingdon Science Park
Abingdon, OX14 3NB
United Kingdom

TELEPHONE: +44 (0)1235 529449
FAX: +44 (0)1235 533420
E-MAIL: info@RnDSystems.co.uk

R&D Systems China Co. Ltd.
24A1 Hua Min Empire Plaza
726 West Yan An Road
Shanghai PRC 200050

TELEPHONE: +86 (21) 52380373
FAX: +86 (21) 52371001
E-MAIL: info@RnDSystemsChina.com.cn

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 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 Thr202/Tyr204 for human ERK1 and Thr185/Tyr187 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 ERK2 phosphorylated at T185/Y187 in cell lysates. An antibody specific for ERK2, binding both phosphorylated and unphosphorylated protein, has been pre-coated onto a microplate. Standards and samples are added and ERK2 present is bound by the immobilized antibody. After washing away unbound material, a biotinylated detection antibody recognizing 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 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.

MATERIALS PROVIDED

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

Component	Part #	Quantity	Storage Conditions of Opened/Reconstituted Components
Phospho-ERK2 (T185/Y187) Microplate - One 96 well polystyrene microplate (12 strips of 8 wells) coated with a mouse monoclonal antibody against ERK2.	841826	1 plate	Return unused wells to the foil pouch containing the desiccant pack, reseal along entire edge of zip-seal. May be stored for up to 1 month at 2-8° C.*
Phospho-ERK2 (T185/Y187) Standard - Each vial contains 75 ng of recombinant human phospho-ERK2 (T185/Y187) in a buffered protein base with preservatives, lyophilized.	841239	2 vials	Use within 1 hour of reconstitution. Use a fresh standard for each assay.
Phospho-ERK2 (T185/Y187) Detection Antibody - 15 µg of a biotinylated rabbit polyclonal anti-phospho-ERK2 (T185/Y187) antibody, lyophilized.	841827	1 vial	Store for up to 1 month at 2-8° C.*
Lysis Buffer 6 - 21 mL of a cell lysing buffer with phosphatase inhibitors and preservatives.	895561	1 vial	
Sample Diluent Concentrate 1 (5X) - 21 mL of a 5-fold concentrated buffer with preservatives.	895562	1 vial	
Reagent Diluent Concentrate 2 (10X) - 21 mL of a 10-fold concentrated solution of buffered protein base with preservatives.	841380	1 vial	
Wash Buffer Concentrate (25X) - 21 mL of a 25-fold concentrated solution of buffered surfactant with preservatives.	895003	1 vial	
Color Reagent A - 12.5 mL of stabilized hydrogen peroxide.	895000	1 vial	
Color Reagent B - 12.5 mL of stabilized chromogen (tetramethylbenzidine).	895001	1 vial	
Streptavidin-HRP - 1.0 mL of streptavidin conjugated to horseradish-peroxidase.	890803	1 vial	
Stop Solution - 6 mL of 2 N sulfuric acid.	895032	1 vial	
Plate Covers - Adhesive strips.	640197	4 strips	Store at room temperature.

*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.
- Deionized or distilled water.
- Squirt bottle, manifold dispenser, or automated microplate washer.
- 100 mL and 500 mL graduated cylinders.
- Phosphate-buffered saline (PBS).
- Phenylmethylsulfonylfluoride (PMSF) (optional; Sigma, Catalog # P7626).
- Protease Inhibitor Cocktail (optional; Sigma, Catalog # P2714).
- **Polypropylene test tubes.**

PRECAUTION

The Stop Solution provided with this kit is an acid solution. Wear protective gloves, clothing, eye, and face protection. Wash hands thoroughly after handling.

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.

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. Dilute 20 mL of Wash Buffer Concentrate into deionized or distilled water to prepare 500 mL of Wash Buffer.

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

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

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

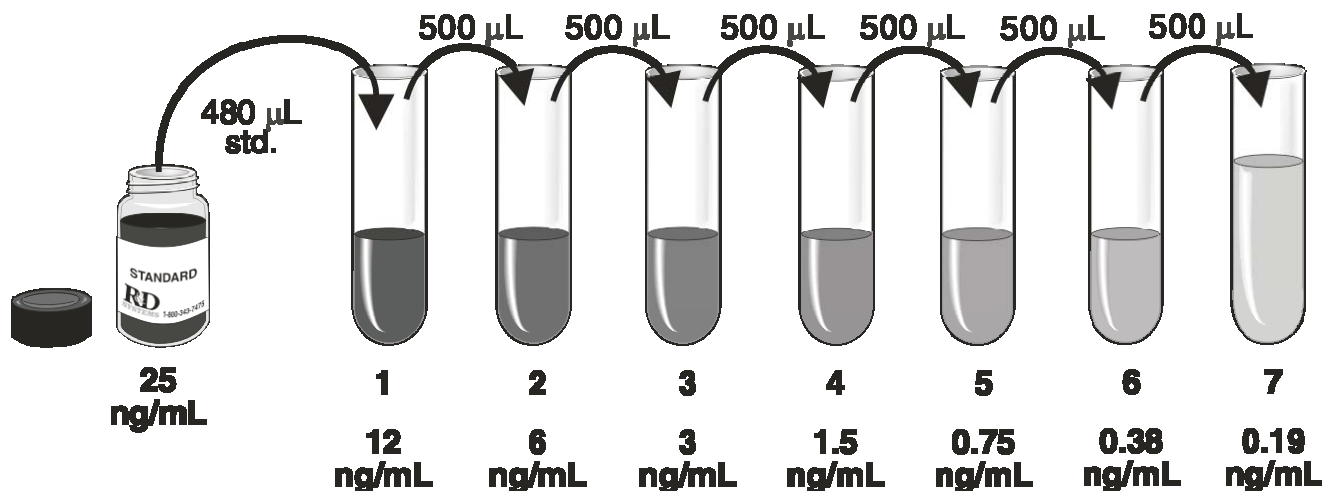
Phospho-ERK2 (T185/Y187) Detection Antibody - Reconstitute the Phospho-ERK2 (T185/Y187) Detection Antibody with 1.0 mL Reagent Diluent 2. This reconstitution produces a stock solution of 15 $\mu\text{g}/\text{mL}$. Immediately before use, dilute the Detection Antibody to a working concentration of 1.0 $\mu\text{g}/\text{mL}$ 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. Protect from light. 100 μL of the resultant mixture is required per well.

Phospho-ERK2 (T185/Y187) Standard - Reconstitute the Phospho-ERK2 (T185/Y187) Standard with 0.5 mL of Lysis Buffer 6. This reconstitution produces a stock solution of 150 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 solution of 25 ng/mL.

Label seven **polypropylene** tubes 1 through 7. Add 520 μL of Assay Diluent into tube 1 and 500 μL of Assay Diluent into tubes 2 through 7. Add 480 μL of the 25 ng/mL Standard 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 12 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.**



SAMPLE PREPARATION

Cell Lysates

Note: *It is recommended to supplement Lysis Buffer 6 with 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 1×10^7 cells/mL in Lysis Buffer 6.
4. Vortex lysates briefly and allow to sit on ice for 15 minutes or store at $\leq -20^\circ \text{C}$ in a manual defrost freezer. Sample protein concentration may be quantified using a total protein assay.
5. Before use, centrifuge at $2000 \times g$ 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 samples and standards 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 μ L 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 μ L) 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 μ L of diluted Phospho-ERK2 (T185/Y187) 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, prepare the Streptavidin-HRP. Add 100 μ L 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 μ L of Substrate Solution to each well. Incubate for 20 minutes at room temperature. **Avoid placing the plate in direct light.**
10. Add 50 μ L 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 Sample Preparation section.

ASSAY PROCEDURE CHECKLIST

1. Bring all reagents to room temperature.
 Prepare reagents, samples, and standards as instructed.
 Return unused components to storage temperature as indicated in the instructions.
2. Add 100 μ L Standard or sample* to each well, and incubate for 2 hours at room temperature.
3. Aspirate and wash each well 3 times.
4. Add 100 μ L diluted Detection Antibody to each well, and incubate for 2 hours at room temperature.
5. Aspirate and wash each well 3 times.
6. Add 100 μ L diluted Streptavidin-HRP to each well, and incubate for 20 minutes at room temperature.
7. Aspirate and wash each well 3 times.
8. Add 100 μ L Substrate Solution to each well, and incubate for 20 minutes at room temperature. **Protect from light.**
9. Add 50 μ L Stop Solution to each well. Read at 450 nm within 30 minutes (λ correction 540 nm or 570 nm).

*Lysates require dilution. Refer to Sample Preparation.

CALCULATION OF RESULTS

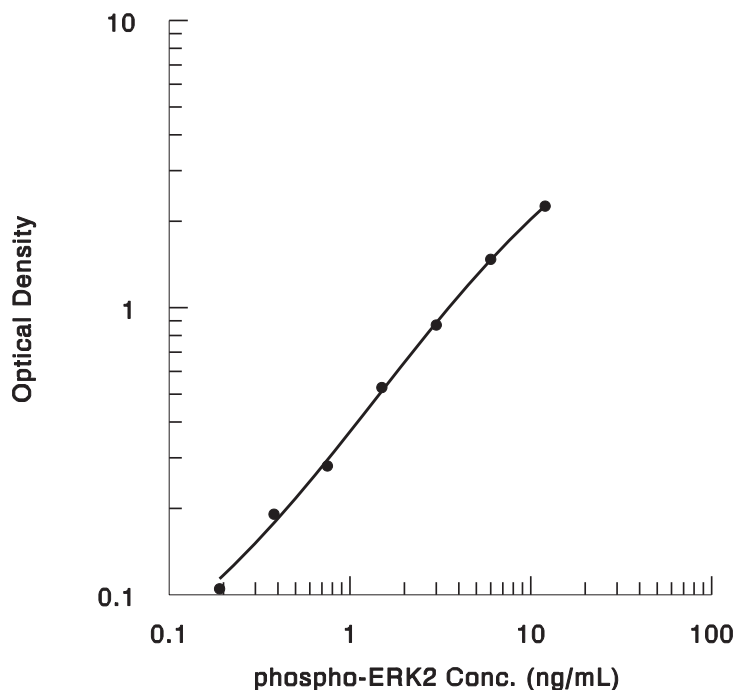
Average the duplicate readings for each standard and sample, then subtract the average zero standard optical density. Results may be normalized to total protein or cell number.

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 phospho-ERK2 concentrations versus the log of the optical density 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 Phospho-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	0.176 0.176	—	—
0.19	0.265 0.296	0.089 0.120	0.105
0.38	0.366 0.367	0.190 0.191	0.191
0.75	0.452 0.461	0.276 0.285	0.281
1.5	0.696 0.710	0.520 0.534	0.527
3	0.982 1.110	0.806 0.934	0.870
6	1.622 1.678	1.446 1.502	1.474
12	2.351 2.515	2.175 2.339	2.257

CALIBRATION

This Surveyor IC Immunoassay is calibrated against a highly purified *E. coli*-expressed recombinant human phospho-ERK2 produced at R&D Systems.

SPECIFICITY

The Human/Mouse/Rat Phospho-ERK2 (T185/Y187) Surveyor IC Immunoassay specifically recognizes ERK2 dually phosphorylated at T185 and Y187. Specificity was demonstrated by Western blot analysis of the protein bound by the capture antibody coated on the plate.

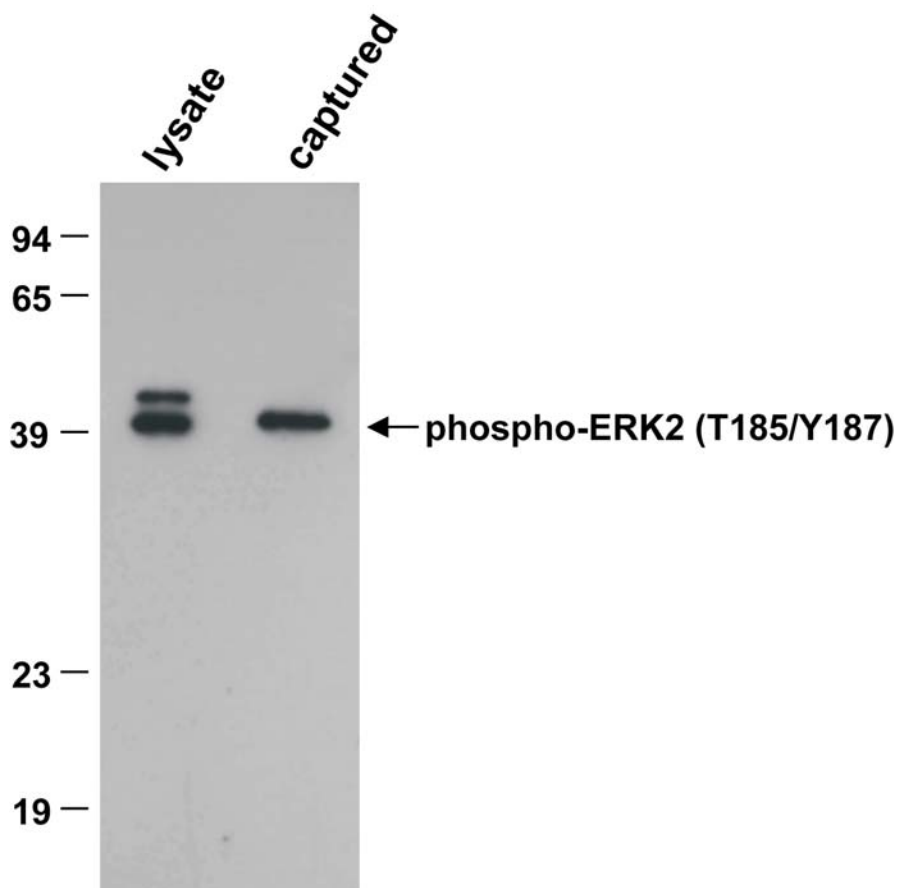


Figure 1: Lysates prepared from HeLa human cervical epithelial carcinoma cells treated with 200 nM phorbol 12-myristate 13-acetate (PMA) were incubated in wells coated with Phospho-ERK2 Surveyor IC Capture Antibody. Unbound material was removed by washing and bound material was solubilized in SDS gel sample buffer. The same lysate and captured proteins were electrophoresed, transferred to a PVDF membrane and immunoblotted with Phospho-ERK2 Surveyor IC Detection Antibody. Only a single band corresponding to phosphorylated ERK2 was detected. While present in lysates, phosphorylated ERK1 (upper band) was not present in captured material.

To further determine the specificity, unphosphorylated recombinant human ERK1 and ERK2 were assayed at 125 ng/mL and read as 4478 pg/mL (3.6% cross-reactivity) and 715 pg/mL (0.6% cross-reactivity), respectively. Unphosphorylated recombinant human JNK1 and p38 α were also assayed at 125 ng/mL and did not cross-react or interfere in the assay.

The specificity of this kit was also demonstrated using peptide competition. Only a phospho-peptide containing the ERK2 (T185/Y187) dual phosphorylation sites blocked the signal, indicating that this kit is both phospho-specific and specific for ERK phosphorylation versus the phosphorylation of related MAP kinases JNK and p38.

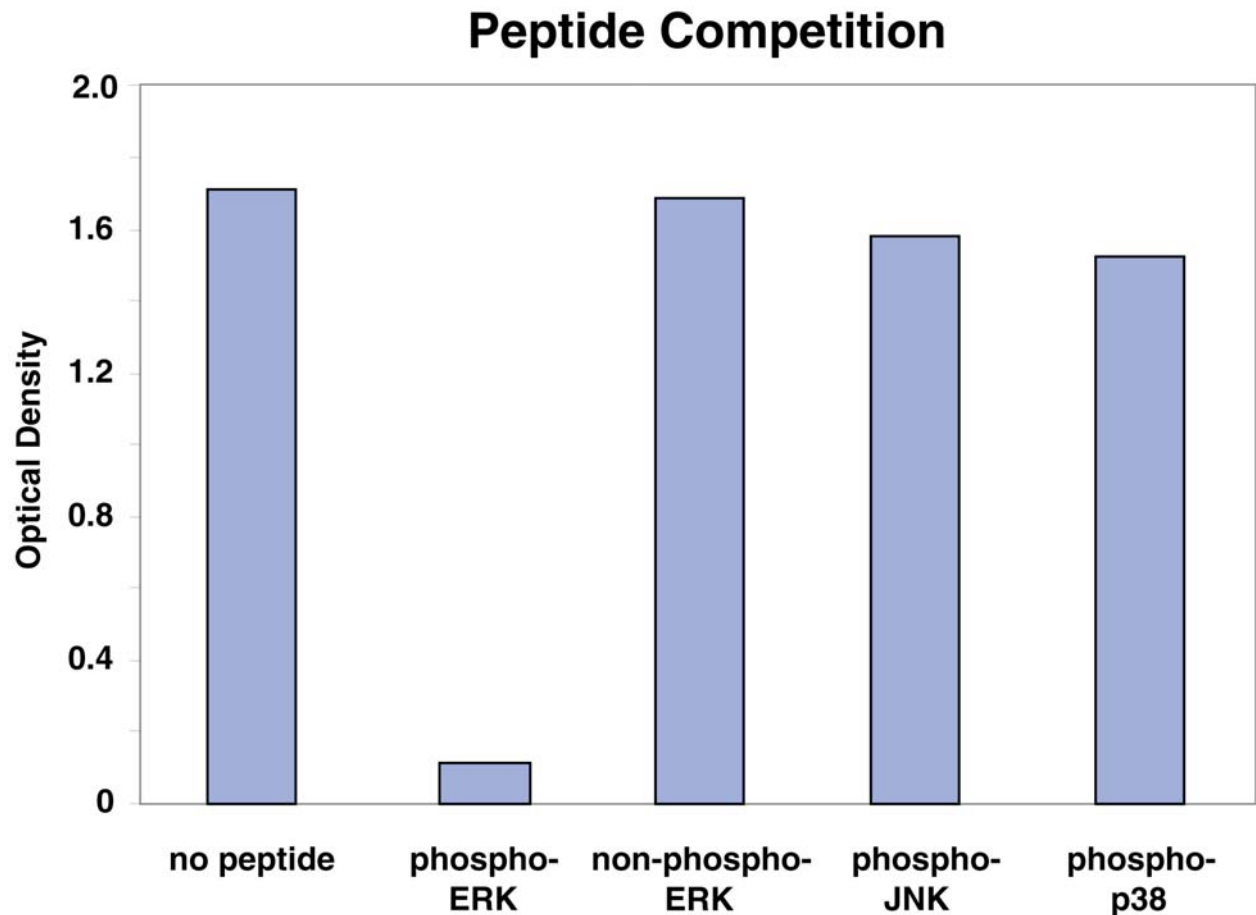


Figure 2: A lysate prepared from HeLa human cervical epithelial carcinoma cells treated with 200 nM PMA was analyzed with this kit. The Phospho-ERK2 Surveyor IC Detection Antibody was either untreated (no peptide) or preincubated with a phospho-peptide containing the ERK2 T185/Y187 dual phosphorylation sites (phospho-ERK), a non-phospho-peptide containing the same sequence (non-phospho-ERK), a phospho-peptide containing the related JNK T183/Y185 dual phosphorylation sites (phospho-JNK), or a phospho-peptide containing the related p38 MAPK T180/Y182 dual phosphorylation sites (phospho-p38). Peptides were used at 200 ng/mL.

QUANTIFICATION

The amounts of human phosphorylated ERK2, as quantified by the Human/Mouse/Rat Phospho-ERK2 (T185/Y187) Surveyor IC Immunoassay, are consistent with the relative amounts of phosphorylated ERK2 determined by qualitative Western blot analysis.

Quantification of phosphorylated ERK2 in PMA-treated human HeLa cells

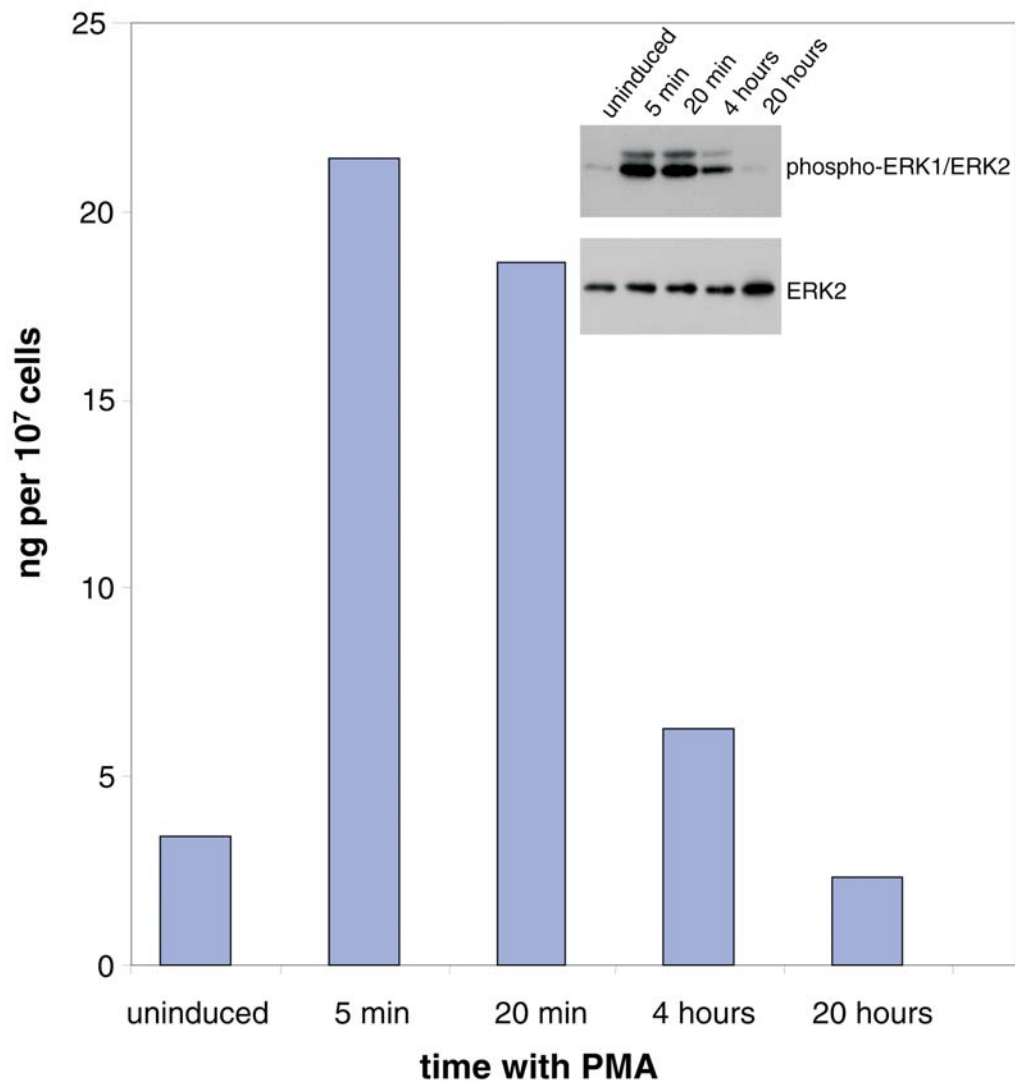


Figure 3: HeLa human cervical epithelial carcinoma cells were treated with 200 nM PMA for the indicated times. Following cell lysis, ERK2 phosphorylated at T185/Y187 was quantified with this kit. The same lysates were also immunoblotted (inset) with either anti-phospho-ERK1/ERK2 (R&D Systems, Catalog # AF1018) or anti-ERK2 (R&D Systems, Catalog # AF1230) polyclonal antibodies. The Surveyor IC Immunoassay results correlate well with the relative amounts of phosphorylated ERK2 detected by Western blot (lower molecular weight band of upper blot corresponds to phosphorylated ERK2). The lower panel with anti-ERK2 antibody indicates that total levels of ERK2 remained constant during incubations with PMA.

The quantification of phosphorylated ERK2 with this Surveyor IC Immunoassay was also determined using cells pretreated with the MEK1/2 inhibitors U0126 and PD98059, which indirectly inhibit phosphorylation of ERK2 at T185/Y187.

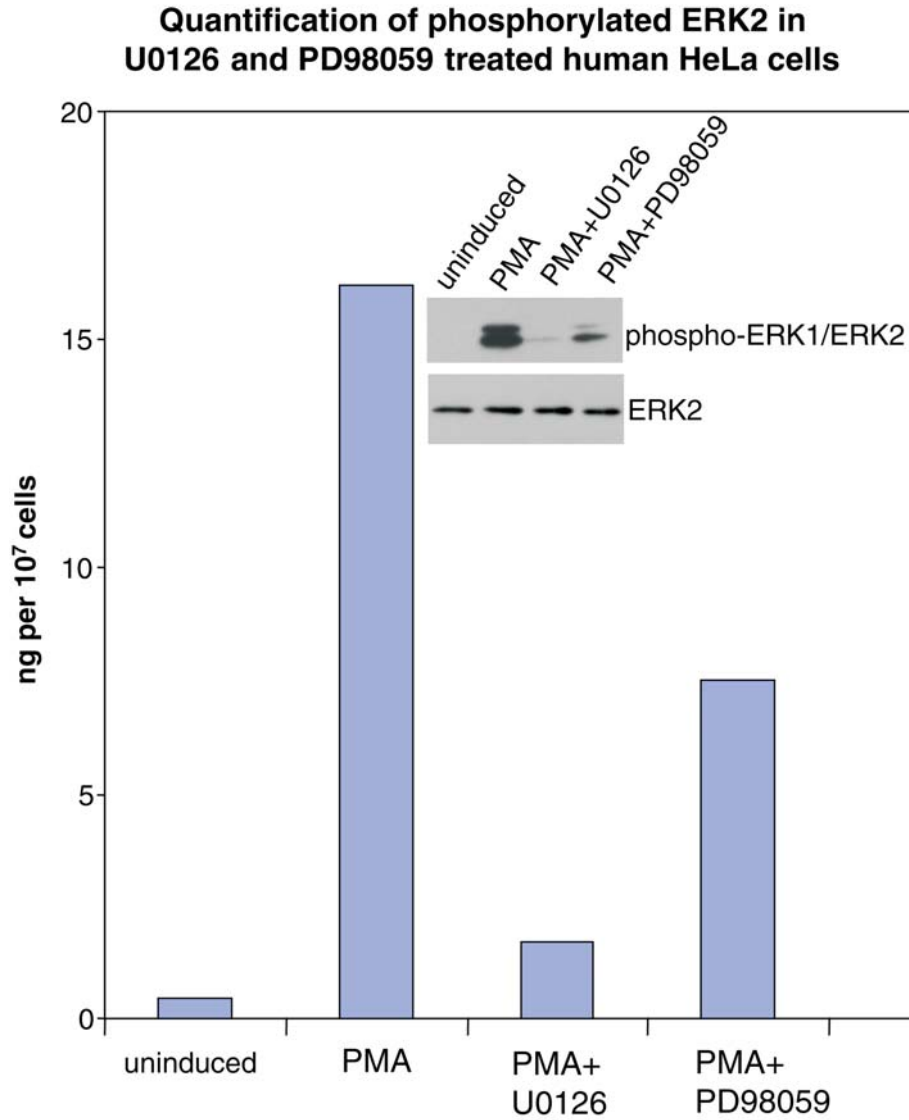


Figure 4: HeLa human cervical epithelial carcinoma cells were incubated with no additions or with 200 nM PMA for 20 minutes, either with or without U0126 or PD98059. Cells were lysed and ERK2 phosphorylated at T185/Y187 was quantified with this kit. The same lysates were also immunoblotted (inset) with either anti-phospho-ERK1/ERK2 or anti-ERK2 polyclonal antibodies. The Surveyor IC Immunoassay results correlate well with the relative amounts of phosphorylated ERK2 detected by Western blot (lower molecular weight band of upper blot corresponds to phosphorylated ERK2). The lower panel with anti-ERK2 antibody indicates that total levels of ERK2 remained constant during the various treatments.

The Human/Mouse/Rat Phospho-ERK2 (T185/Y187) Surveyor IC Immunoassay quantifies phosphorylated ERK2 levels in mouse and rat cell lysates.

Quantification of phosphorylated ERK2 in growth factor treated mouse and rat cells

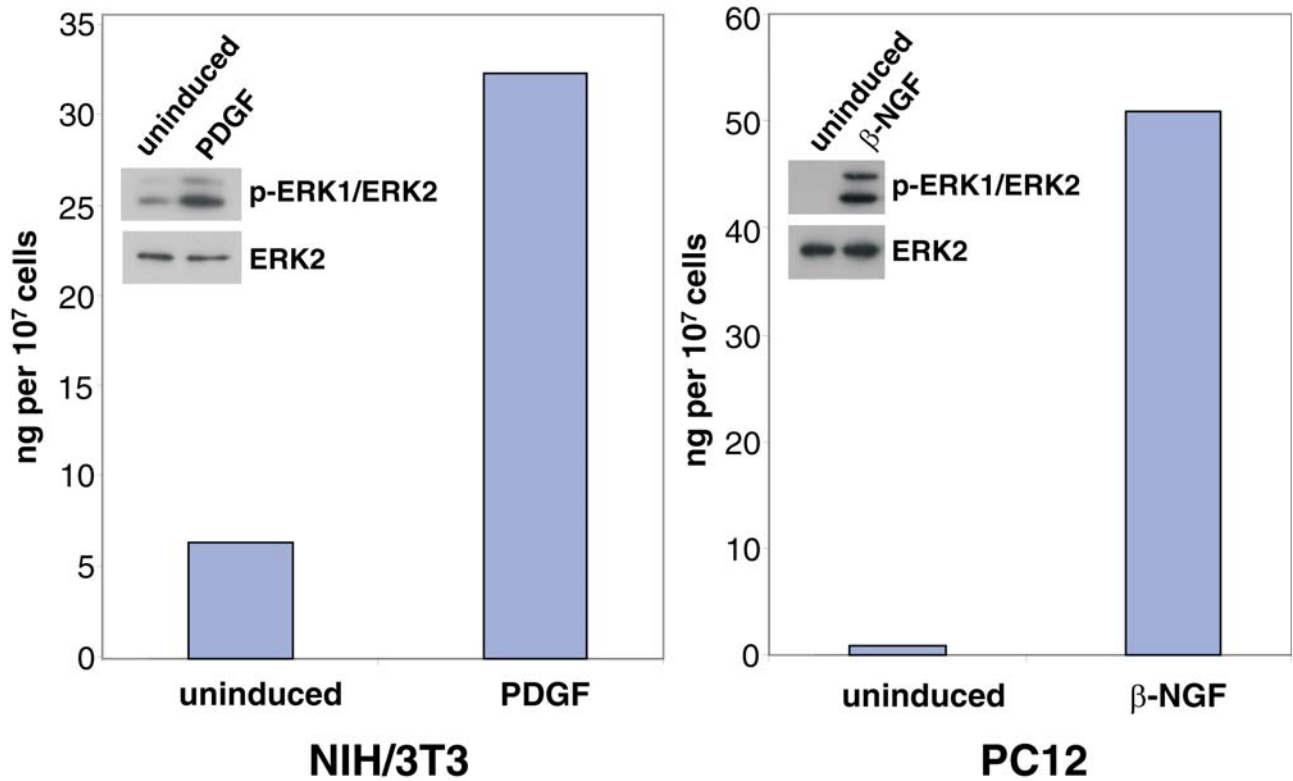


Figure 5: Lysates prepared from NIH-3T3 mouse embryonic fibroblast cells either uninduced or induced with PDGF (R&D Systems, Catalog # 120-HD) (left panel), and PC-12 rat adrenal pheochromocytoma cells either uninduced or induced with β -NGF (R&D Systems, Catalog # 556-NG) (right panel) were quantified with this kit. The same lysates were also immunoblotted (inset) with either anti-phospho-ERK1/ERK2 or anti-ERK2 polyclonal antibodies. The Surveyor IC Immunoassay results correlate well with the relative amounts of phosphorylated ERK2 detected by Western blot (lower molecular weight band of upper blots corresponds to phosphorylated ERK2). The lower panels with anti-ERK2 antibody indicate that total levels of ERK2 remained constant during the growth factor inductions.

REFERENCES

1. Boulton, T.G. *et al.* (1990) *Science* **249**:64.
2. Boulton, T.G. *et al.* (1991) *Cell* **65**:663.
3. Yao, Y. *et al.* (2003) *Proc. Natl. Acad. Sci. USA* **100**:12759.
4. Schaeffer, H.J. *et al.* (1998) *Science* **281**:1668.
5. Pages, G. *et al.* (1999) *Science* **286**:1374.
6. Ferrell, J.E. and R.R. Bhatt (1997) *J. Biol. Chem.* **272**:19008.
7. Tanoue, T. *et al.* (2000) *Nat. Cell Biol.* **2**:110.
8. Marais, R. *et al.* (1993) *Cell* **73**:381.
9. Gupta, S. *et al.* (1993) *Proc. Natl. Acad. Sci. USA* **90**:3216.
10. Felton-Edkins, Z.A. *et al.* (2003) *EMBO J.* **22**:2422.
11. Stefanovsky, V.Y. *et al.* (2001) *Mol. Cell* **8**:1063.
12. Gavin, A.C. and A.R. Nebreda (1999) *Curr. Biol.* **9**:281.
13. Deak, M. *et al.* (1998) *EMBO J.* **17**:4426.