# DuoSet® IC

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

Catalog Number DYC1018-2
DYC1018-5

**For the development of sandwich ELISAs to measure 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.**

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## PRINCIPLE OF THE ASSAY

This DuoSet® IC ELISA contains the basic components required for the development of sandwich ELISAs to measure phospho-ERK1 (T202/Y204) and phospho-ERK2 (T185/Y187) in cell lysates. Immobilized capture antibodies specific for ERK1 and ERK2 bind both phosphorylated and unphosphorylated protein. After washing away unbound material, a biotinylated detection antibody specific for ERK1 or ERK2 dually phosphorylated at T202/Y204 or T185/Y187, respectively, is used to detect only phosphorylated protein, utilizing a standard Streptavidin-HRP format.

## MATERIALS PROVIDED

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

| | | | Vials Provided | |
|---|---|---|---|---|
| **Description** | **Part #** | **Storage Conditions** | **Cat. # DYC1018-2** | **Cat. # DYC1018-5** |
| Phospho-ERK1/ERK2 Capture Antibody | 841326 | 2 - 8° C | 1 | 2 |
| Phospho-ERK1/ERK2 Detection Antibody | 841327 | 2 - 8° C | 1 | 2 |
| Phospho-ERK1/ERK2 Standard | 841328 | 2 - 8° C | 3 | 5 |
| Streptavidin-HRP | 890803 | 2 - 8° C | 1 | 1 |

DYC1018-2 contains sufficient materials to run ELISAs on at least two 96 well plates.*

DYC1018-5 contains sufficient materials to run ELISAs on at least five 96 well plates.*

*Provided the following conditions are met:

- The reagents are prepared as described in this package insert.
- The assay is run as described in the General ELISA Protocol on page 6.
- The recommended microplates, buffers, diluents, substrates, and solutions are used.

## OTHER MATERIALS REQUIRED

- Aprotinin (Sigma # A6279)
- Leupeptin (Sigma # L8511)
- Pepstatin (Sigma # P4265)
- Phenylmethylsulfonylfluoride (PMSF) (Sigma # P7626)
- Sodium pyrophosphate ($Na_4P_2O_7$) (Sigma # P8010)
- Sodium orthovanadate ($Na_3VO_4$) (Sigma # S6508), activated
- Sodium fluoride (NaF) (Aldrich # 201154)
- Sodium azide ($NaN_3$) (Sigma # S2002)
- Triton® X-100 (Sigma # T9284)
- Urea
- Pipettes and pipette tips
- Deionized or distilled water
- 96 well microplates [Costar EIA Plates (Catalog # 2592 or R&D Systems Catalog # DY990) are suggested]
- Plate sealers (R&D Systems, Catalog # DY992)
- Squirt bottle, manifold dispenser, or automated microplate washer.

## SOLUTIONS REQUIRED

**PBS** - 137 mM NaCl, 2.7 mM KCl, 8.1 mM $Na_2HPO_4$, 1.5 mM $KH_2PO_4$, pH 7.2 - 7.4, 0.2 μm filtered.

**Wash Buffer** - 0.05% Tween® 20 in PBS, pH 7.2 - 7.4 (R&D Systems, Catalog # WA126).

**Block Buffer** - 1% BSA,* 0.05% $NaN_3$, in PBS, pH 7.2 - 7.4.

**IC Diluent #1** - 1% BSA* in PBS, pH 7.2 - 7.4, 0.2 μm filtered.

**IC Diluent #8**** - 1 mM EDTA, 0.5% Triton X-100, 5 mM NaF in PBS, pH 7.2 - 7.4

**Note:** *IC Diluent #8 is also the base buffer for IC Diluent #3, IC Diluent #7, and Lysis Buffer #6. Approximately 50 mL of this diluent is required to run the assay on one plate.*

**IC Diluent #3**** - 1 mM EDTA, 0.5% Triton X-100, 5 mM NaF, 1 M urea in PBS, pH 7.2 - 7.4.

**IC Diluent #7**** - 1 mM EDTA, 0.5% Triton X-100, 5 mM NaF, 6 M urea in PBS, pH 7.2 - 7.4

**Lysis Buffer #6**** - 1 mM EDTA, 0.5% Triton X-100, 5 mM NaF, 6 M urea, 10 μg/mL Leupeptin, 10 μg/mL Pepstatin, 100 μM PMSF, 3 μg/mL Aprotinin in PBS, 2.5 mM sodium pyrophosphate, 1 mM activated sodium orthovanadate in PBS, pH 7.2 - 7.4.

**Substrate Solution** - 1:1 mixture of Color Reagent A ($H_2O_2$) and Color Reagent B (Tetramethylbenzidine) (R&D Systems, Catalog # DY999).

**Stop Solution** - 2 N $H_2SO_4$ (R&D Systems, Catalog DY994).

*The use of Reagent Diluent Concentrate 2 (R&D Systems, Catalog # DY995) or Millipore Bovine Serum Albumin, Fraction V, Protease free (Catalog # 82-045) is recommended. All buffers containing BSA must be stored at 2 - 8° C.

**Sample Diluent Concentrate 1 (5X) (R&D Systems, Catalog # DYC001), supplemented as per the package insert.

## REAGENT PREPARATION

**Bring all reagents to room temperature before use.**

**Phospho-ERK1/ERK2 Capture Antibody** (Part 841326) - Each vial contains 720 μg/mL of rabbit anti-human ERK1/ERK2 antibody when reconstituted with 200 μL of PBS. After reconstitution, store at 2 - 8° C for up to 30 days or aliquot and store at ≤ -20° C in a manual defrost freezer or at ≤ -70° C for up to 3 months.*

**Phospho-ERK1/ERK2 Detection Antibody** (Part 841327) - Each vial contains 14.4 μg/mL of biotinylated rabbit anti-human phospho-ERK1 (T202/Y204)/ERK2 (T185/Y187) antibody when reconstituted with 1.0 mL of IC Diluent #1. After reconstitution, store at 2 - 8° C for up to 30 days or aliquot and store at ≤ -20° C in a manual defrost freezer or at ≤ -70° C for up to 3 months.*

**Phospho-ERK1/ERK2 Standard** (Part 841328) - Each vial contains 2600 ng/mL of recombinant human phospho-ERK2 (T185/Y187) when reconstituted with 500 μL of IC Diluent #7. **Use within one hour of reconstitution. A fresh standard should be used for each assay.** An initial 6-fold dilution should be made in IC Diluent #8. Further dilutions should be made in IC Diluent #3 immediately before use. A seven point curve using 2-fold serial dilutions and a high standard of 20,000 pg/mL is recommended.

**Streptavidin-HRP** (Part 890803) - 1.0 mL of streptavidin conjugated to horseradish-peroxidase. Store at 2 - 8° C. **DO NOT FREEZE.**

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

## PREPARATION OF SAMPLES

**Cell Lysates** - Rinse cells two times with PBS, making sure to remove any remaining PBS after the second rinse. Solubilize cells at 1 x $10^7$ cells/mL in Lysis Buffer #6 and allow samples to sit on ice for 15 minutes. Assay immediately or store at ≤ -70° C. Before use, centrifuge samples at 2000 x g for 5 minutes and transfer the supernate to a clean test tube. Sample protein concentration may be quantified using a total protein assay. For assaying, dilute lysates 6-fold with IC Diluent #8 and make further serial dilutions in IC Diluent #3.

**Note:** *The final concentration of urea in all samples and standards should be 1 M prior to addition to the plate.*

## PRECAUTION

The Stop Solution suggested for use with this kit is an acidic solution. Wear eye, hand, face, and clothing protection when using this material.

## TECHNICAL HINTS AND LIMITATIONS

- This DuoSet IC ELISA should not be used beyond the expiration date on the kit label.
- Individual results may vary due to differences in technique, plasticware and water sources.
- It is important that the diluents selected for reconstitution and for dilution of the standard reflect the environment of the samples being measured. The diluent suggested in this protocol should be suitable for most cell lysates.
- The type of enzyme and substrate and the concentrations of capture/detection antibodies used can be varied to create an immunoassay with a different sensitivity and dynamic range. A basic understanding of immunoassay development is required for the successful use of these reagents in immunoassays.
- 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 any remaining Wash Buffer by inverting the plate and blotting it against clean paper towels.
- Use a fresh reagent reservoir and pipette tips for each step.
- It is recommended that all standards and samples be assayed in duplicate.
- Avoid microbial contamination of reagents and buffers. This may interfere with the sensitivity of the assay. Buffers containing protein should be made under aseptic conditions and stored at 2 - 8° C or be prepared fresh daily.

# GENERAL ELISA PROTOCOL

## Plate Preparation

1. Dilute the Capture Antibody to a working concentration of 4.0 μg/mL in PBS, without carrier protein. Immediately coat a 96 well microplate with 100 μL per well of the diluted Capture Antibody. Seal the plate and incubate overnight at room temperature.
2. Aspirate each well and wash with Wash Buffer, repeating the process two times for a total of 3 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 for good performance. After the last wash, remove any remaining Wash Buffer by aspirating or by inverting the plate and blotting it against clean paper towels.
3. Block plates by adding 300 μL of Block Buffer to each well. Incubate at room temperature for 1 - 2 hours.
4. Repeat the aspiration/wash as in step 2. The plates are now ready for sample addition.

## Assay Procedure

1. Add 100 μL of sample or standards in IC Diluent #3 per well. Use IC Diluent #3 as the zero standard. Cover with a plate sealer and incubate 2 hours at room temperature.

   **Note:** *A seven point standard curve using 2-fold serial dilutions and a high standard of 20,000 pg/mL is recommended.*
2. Repeat the aspiration/wash as in step 2 of Plate Preparation.
3. Dilute the Detection Antibody to a working concentration of 400 ng/mL in IC Diluent #1 before use. Add 100 μL of the diluted Detection Antibody to each well. Cover with a new plate sealer and incubate 2 hours at room temperature.
4. Repeat the aspiration/wash as in step 2 of Plate Preparation.
5. Immediately before use, dilute the Streptavidin-HRP to the working concentration specified on the vial label using IC Diluent #1. Add 100 μL of the diluted Streptavidin-HRP to each well. Incubate for 20 minutes at room temperature. Avoid placing the plate in direct light.
6. Repeat the aspiration/wash as in step 2 of the Plate Preparation.
7. Add 100 μL of Substrate Solution to each well. Incubate for 20 minutes at room temperature. Avoid placing the plate in direct light.
8. Add 50 μL of Stop Solution to each well. Gently tap the plate to ensure thorough mixing.
9. Determine the optical density of each well immediately, 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.

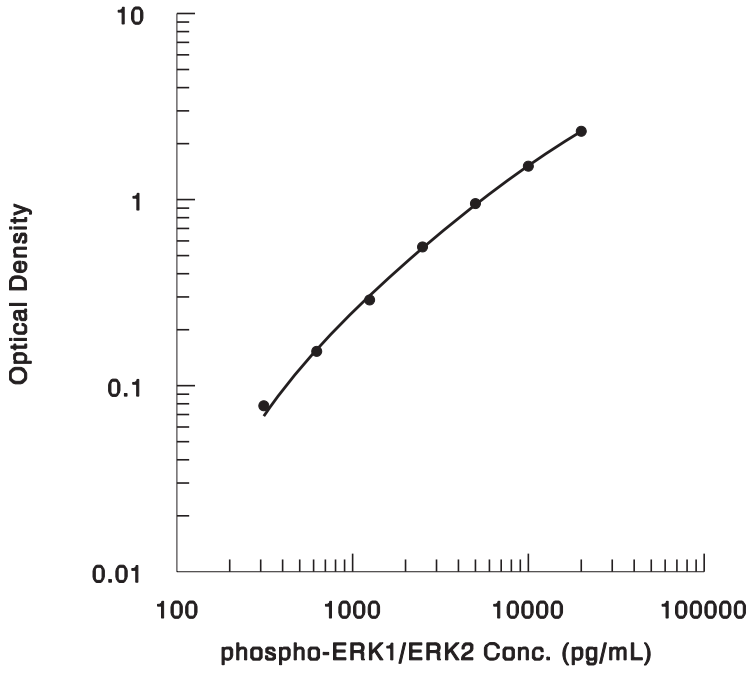
## CALCULATION OF RESULTS

Average the duplicate readings for each standard and sample and 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-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.

## 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-ERK1 (T202/Y204)/ERK2 (T185/Y187) DuoSet IC ELISA. The standard curve was calculated using a computer generated 4-PL curve-fit. This standard curve is for demonstration purposes only.




## CALIBRATION

This DuoSet IC ELISA is calibrated against a highly purified *E. coli*-expressed recombinant human phospho-ERK2 (T185/Y187) produced at R&D Systems.

## SPECIFICITY

This 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.

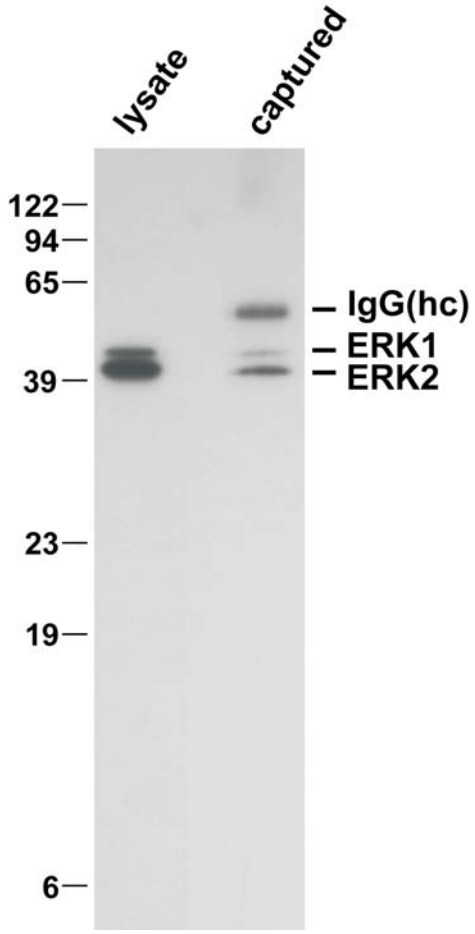



**Figure 1:** Lysates prepared from human HeLa cells treated with 200 nM phorbol 12-myristate 13-acetate (PMA) were incubated in wells coated with Phospho-ERK1/ERK2 Capture Antibody. Unbound material was removed by washing and bound material was solubilized in SDS gel sample buffer. The same lysate and captured proteins were electrophoresed, transferred to an Immobilon-P membrane (Millipore) and immunoblotted with Phospho-ERK1/ERK2 Detection Antibody. In addition to IgG heavy chain, only two bands corresponding to phosphorylated ERK1 and ERK2 were detected.

To further determine specificity, unphosphorylated recombinant human ERK1 and ERK2 were assayed at 50 ng/mL and read as 1346 pg/mL (2.7% cross-reactivity) and 387 pg/mL (0.78% cross-reactivity), respectively. Unphosphorylated recombinant human JNK1 and p38$\alpha$ were assayed at 200 ng/mL and did not cross-react or interfere in the assay.

## QUANTIFICATION

Amounts of human phosphorylated ERK1 and ERK2, as quantified by the Human/Mouse/Rat Phospho-ERK1 (T202/Y204)/ERK2 (T185/Y187) DuoSet IC ELISA, are consistent with the amounts of phosphorylated ERK1 and ERK2 determined by qualitative Western blot analysis.

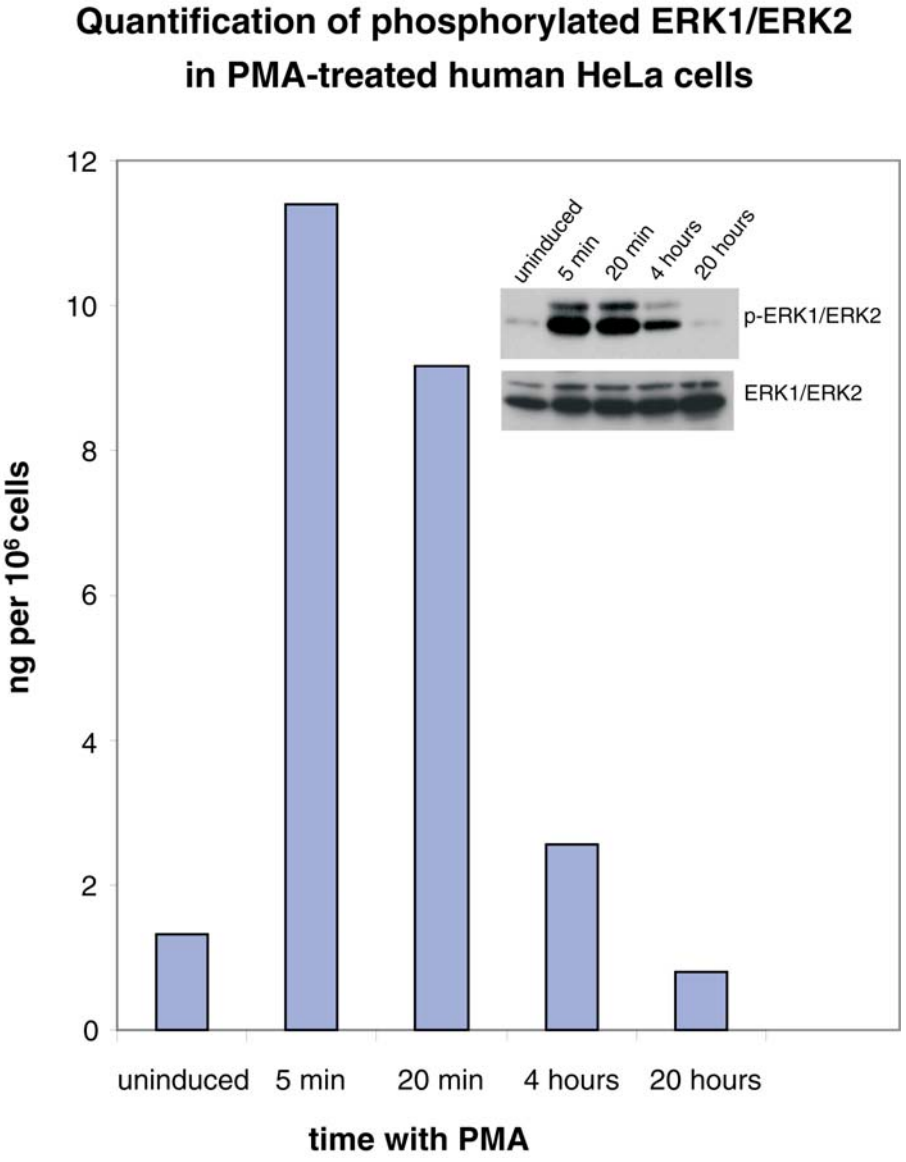



**Figure 2:** HeLa cells were treated with 200 nM PMA for the indicated times. Following cell lysis, phosphorylated ERK1 and ERK2 were quantified with the DuoSet IC ELISA. The same lysates were also immunoblotted (inset) with either anti-phospho-ERK1/ERK2 (R&D Systems, Catalog # AF1018) or anti-ERK1/ERK2 (R&D Systems, Catalog # MAB1576) antibodies. The DuoSet IC ELISA results correlate well with the total amounts of phosphorylated ERK1 and ERK2 detected by Western blot. The blot with anti-ERK1/ERK2 antibody indicates that total levels of ERK1 and ERK2 remained constant during incubations with PMA.

The quantification of phosphorylated ERK1 and ERK2 with this DuoSet IC ELISA was also determined using cells pretreated with the MEK1/2 inhibitors U0126 and PD98059, which indirectly inhibit phosphorylation of ERK1 and ERK2.

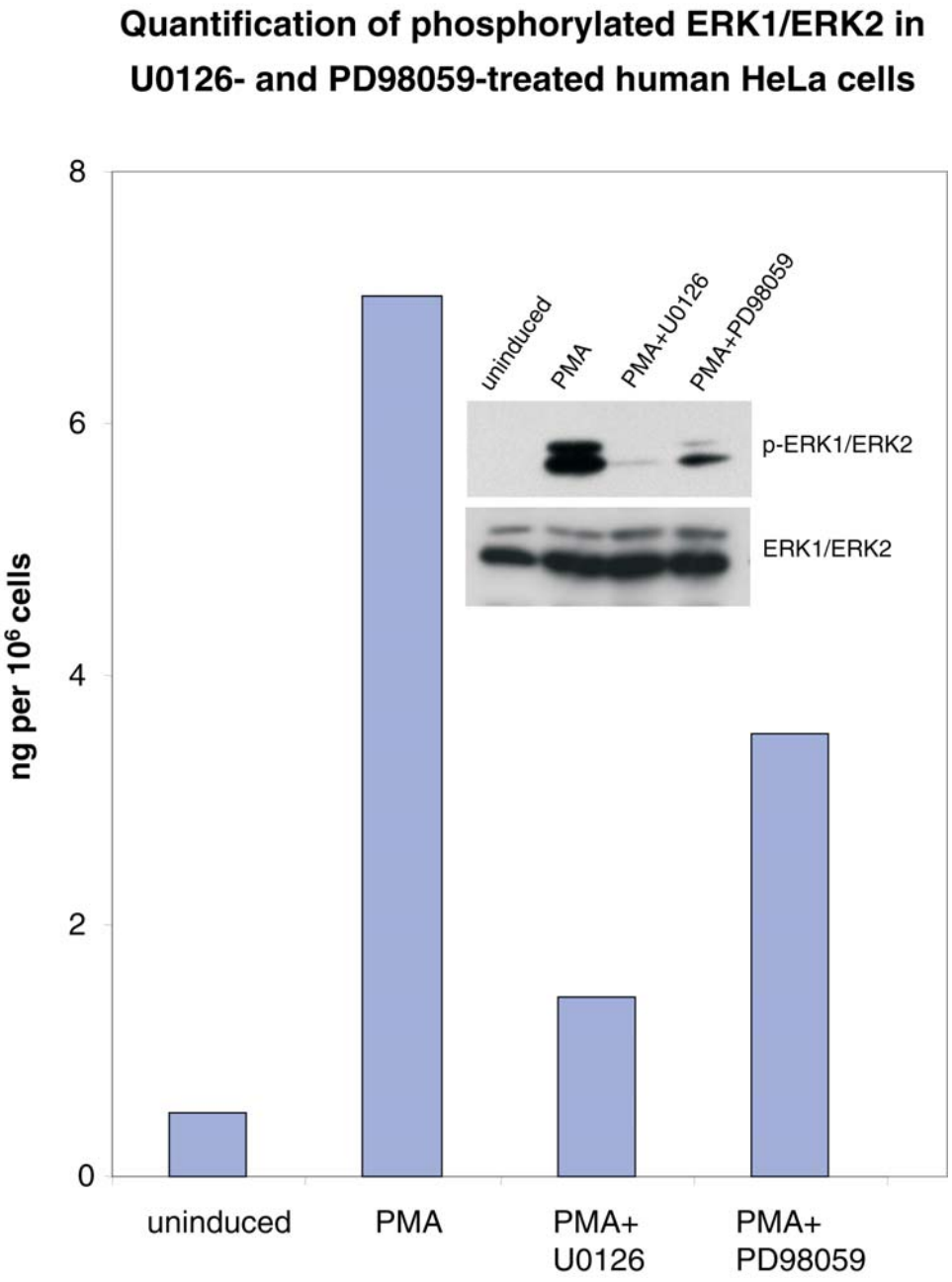



**Figure 3:** HeLa cells were incubated with no additions or with 200 nM PMA for 20 minutes, either with or without U0126 or PD98059. Cells were lysed and phosphorylated ERK1 and ERK2 were quantified with the DuoSet IC ELISA. The same lysates were also immunoblotted (inset) with either anti-phospho-ERK1/ERK2 or anti-ERK1/ERK2 antibodies. The DuoSet IC ELISA results correlate well with the total amounts of phosphorylated ERK1 and ERK2 detected by Western blot. The blot with anti-ERK1/ERK2 antibody indicates that total levels of ERK1 and ERK2 remained constant during the various treatments.

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.

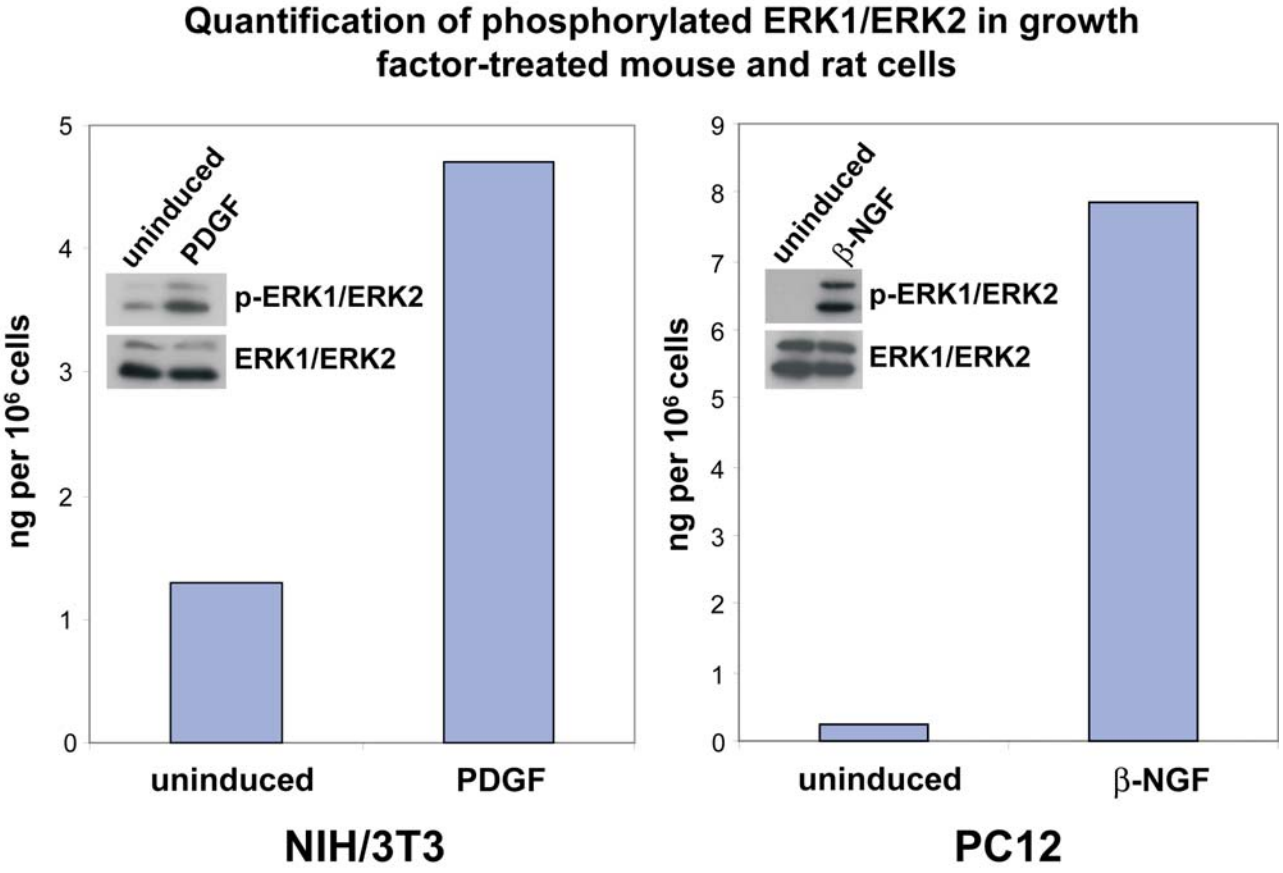



**Figure 4:** Lysates prepared from mouse NIH/3T3 cells either uninduced or induced with PDGF (R&D Systems, Catalog # 120-HD) (left panel), and rat PC12 cells either uninduced or induced with β-NGF (R&D Systems, Catalog # 556-NG) (right panel) were quantified with the DuoSet IC ELISA. The same lysates were also immunoblotted (inset) with either anti-phospho-ERK1/ERK2 or anti-ERK1/ERK2 antibodies. The DuoSet IC ELISA results correlate well with the total amounts of phosphorylated ERK1 and ERK2 detected by Western blot. The blots with anti-ERK1/ERK2 antibody indicate that total levels of ERK1 and ERK2 remained constant during the growth factor inductions.