DuoSet® IC

Human/Mouse/Rat Phospho-CREB (S133)

Catalog Number DYC2510-2
DYC2510-5

For the development of sandwich ELISAs to measure cAMP Response Element Binding Protein (CREB) phosphorylated at S133 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

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

PRINCIPLE OF THE ASSAY

This DuoSet® IC ELISA contains the basic components required for the development of sandwich ELISAs to measure cAMP Response Element Binding Protein (CREB) phosphorylated AT S133 in cell lysates. CREB, also known as CREB1 and ATF-47, is a basic region-leucine zipper (bZIP) transcription factor of the ATF/CREB family. In this DuoSet IC ELISA, an immobilized capture antibody specific for CREB binds both phosphorylated and unphosphorylated protein. After washing away unbound material, a biotinylated detection antibody recognizing CREB phosphorylated at S133 is used to detect only phosphorylated protein, utilizing a standard Streptavidin-HRP format. Using this DuoSet IC ELISA, cross-reactivity with unphosphorylated ATF/CREB family members is minimal, and peptide competition demonstrates that the detection antibody is specific for the S133 site of CREB versus other serine phosphorylation sites. This DuoSet IC ELISA works with lysates prepared from human, mouse, and rat cells.

MATERIALS PROVIDED

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

			Vials Provided	
Description	**Part #**	**Storage Conditions**	**Cat. # DYC2510-2**	**Cat. # DYC2510-5**
Phospho-CREB (S133) Capture Antibody	842088	2 - 8° C	1	2
Phospho-CREB (S133) Detection Antibody	842089	2 - 8° C	1	2
Phospho-CREB (S133) Standard	842090	2 - 8° C	3	5
Streptavidin-HRP	890803	2 - 8° C	1	1

DYC2510-2 contains sufficient materials to run ELISAs on at least two 96 well plates.*

DYC2510-5 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)
- Triton® X-100 (Sigma # T9284)
- Sodium azide (NaN_3) (Sigma # S2002)
- 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.

Tween is a registered trademark of ICI Americas.

REAGENT PREPARATION

Bring all reagents to room temperature before use.

Phospho-CREB (S133) Capture Antibody (Part 842088) - Each vial contains 720 μg/mL of goat anti-human CREB 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-CREB (S133) Detection Antibody (Part 842089) - Each vial contains 18 μg/mL of biotinylated rabbit anti-human phospho-CREB (S133) 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-CREB (S133) Standard (Part 842090) - Each vial contains 60 ng/mL of recombinant human phospho-CREB (S133) 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 2000 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. 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 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 2000 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 500 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.

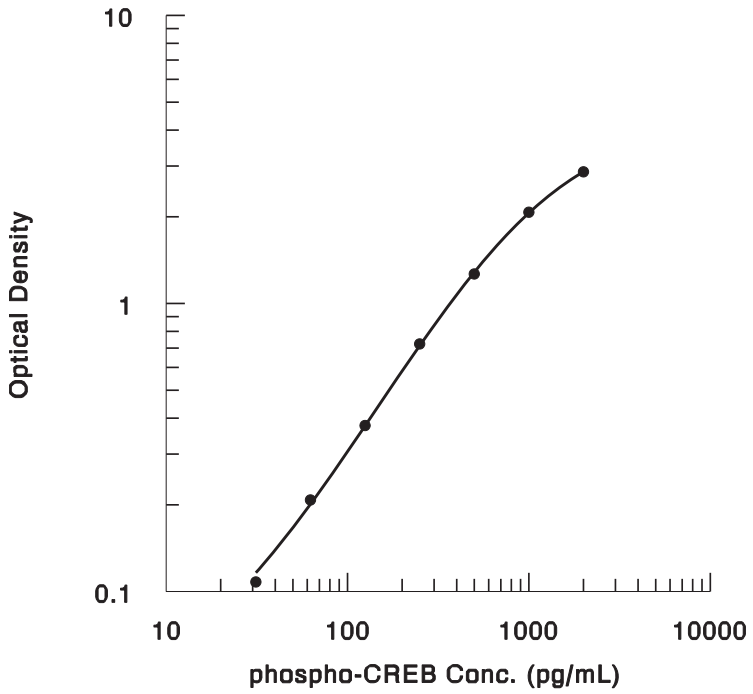
CALCULATION OF RESULTS

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-CREB (S133) 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-CREB (S133) 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-CREB (S133) produced at R&D Systems.

SPECIFICITY

The Human/Mouse/Rat Phospho-CREB (S133) DuoSet IC ELISA specifically recognizes CREB phosphorylated at S133. Specificity was demonstrated using both peptide competition and cross-reactivity analysis.

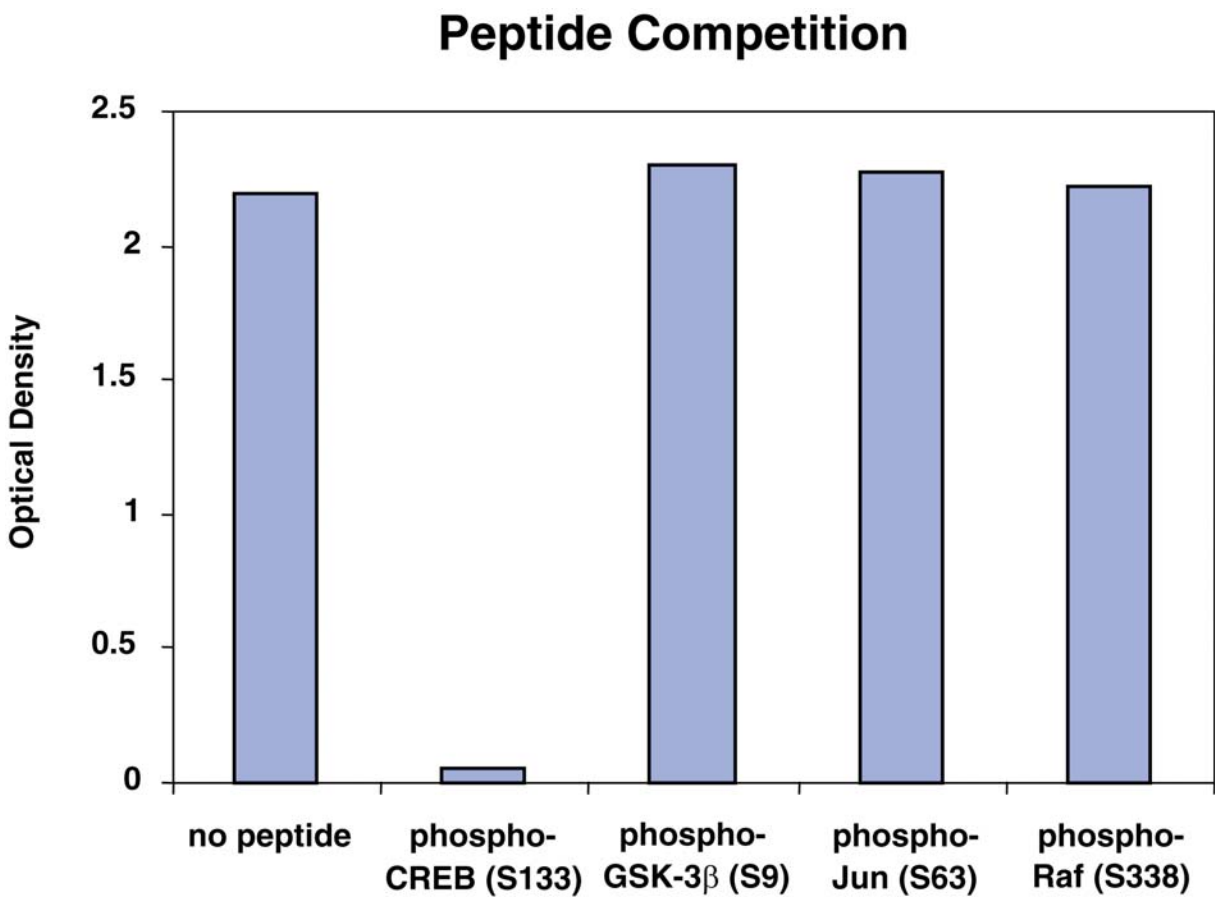


Figure 1: A lysate prepared from human HeLa cells treated with 200 nM PMA for 20 minutes was analyzed with this DuoSet IC ELISA. The Phospho-CREB (S133) DuoSet IC Detection Antibody was either untreated (no peptide) or preincubated with a phosphopeptide containing the CREB S133 phosphorylation site, a phosphopeptide containing the GSK-3β S9 phosphorylation site, a phosphopeptide containing the c-Jun S63 phosphorylation site, or a phosphopeptide containing the Raf1 S338 phosphorylation site. Peptides were used at 40 ng/mL. Only the phosphopeptide containing the CREB S133 phosphorylation site blocks the signal, indicating that the DuoSet IC ELISA is specific for CREB phosphorylation at S133.

Cross-reactivity experiments were performed with the Phospho-CREB (S133) DuoSet IC ELISA to further determine specificity. Unphosphorylated recombinant human CREB was assayed at 25 ng/mL and read as 1.65 ng/mL (6.6% cross-reactivity). Unphosphorylated recombinant human ATF-2, ATF-3, and ATF-4 were assayed at 50 ng/mL and did not cross-react or interfere in the assay.

QUANTIFICATION

Amounts of human phosphorylated CREB, as quantified by the Phospho-CREB (S133) DuoSet IC ELISA, are consistent with the relative amounts of phosphorylated CREB determined by qualitative Western blot analysis.

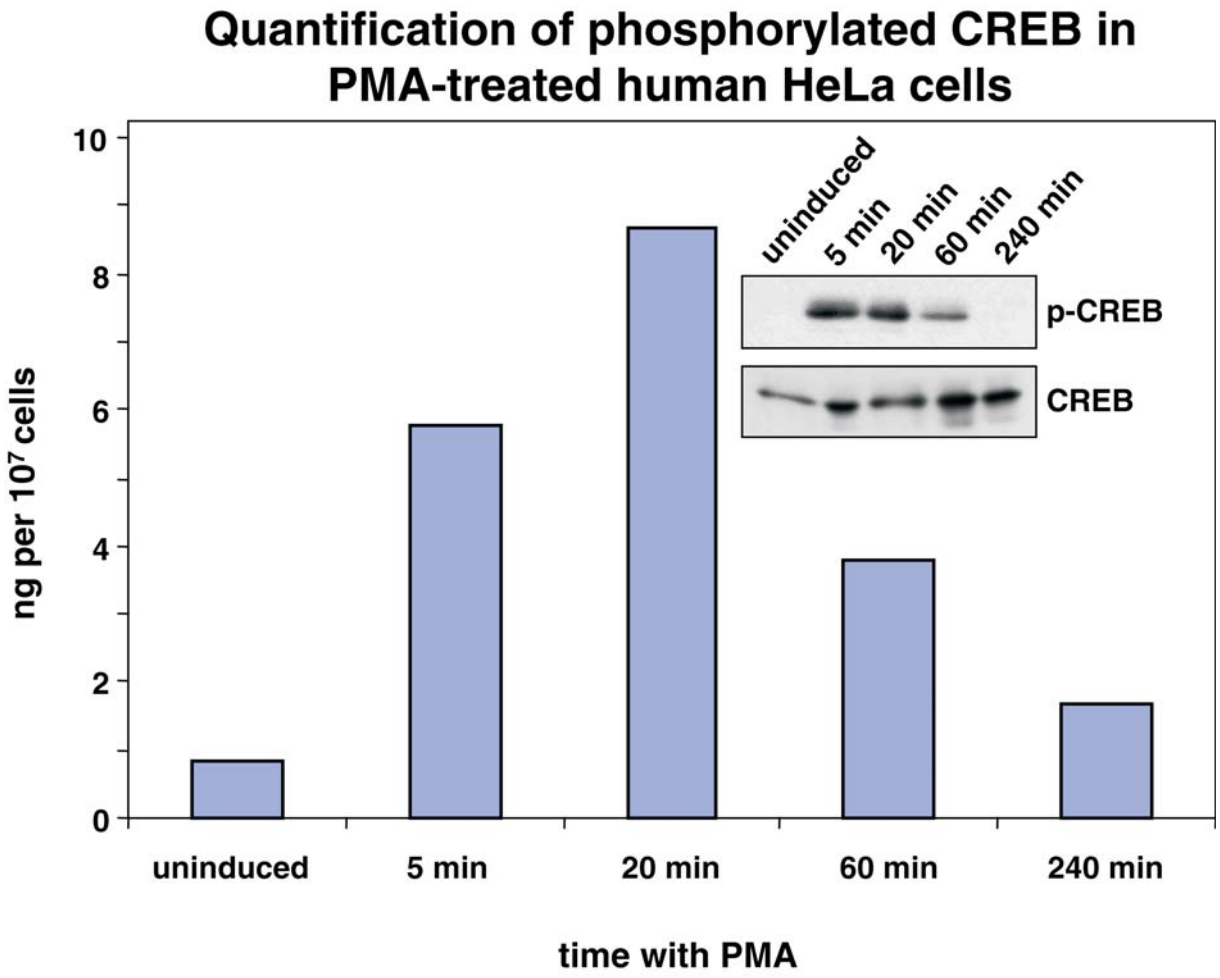


Figure 2: Lysates prepared from human HeLa cells either uninduced or induced with 200 nM PMA for the indicated times were quantified with this DuoSet IC ELISA. The same lysates were also immunoblotted (inset) with either anti-phospho-CREB (S133) (R&D Systems, Catalog # AF2510) or anti-CREB (R&D Systems, Catalog # AF2989) polyclonal antibodies. The DuoSet IC ELISA results correlate well with the amounts of phosphorylated CREB detected by Western blot. The immunoblot with anti-CREB antibody indicates that total levels of CREB remained constant during incubation with PMA.

The quantification of phosphorylated CREB with this DuoSet IC ELISA was also determined using cells pretreated with two concentrations of the MEK inhibitor U0126, which indirectly inhibits phosphorylation of CREB at S133.

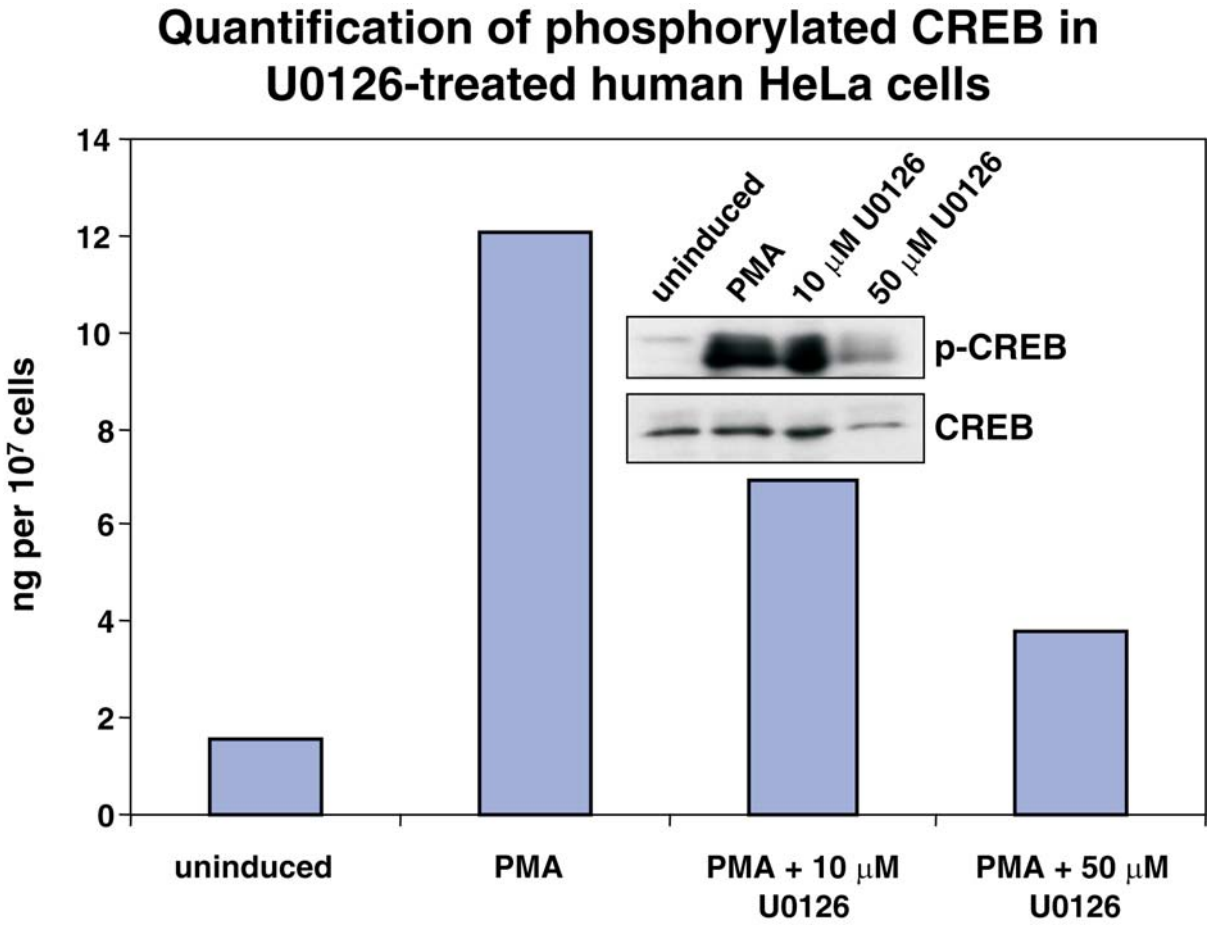


Figure 3: HeLa cells were incubated with no additions or with 200 nM PMA for 20 minutes, either with or without U0126 at the indicated concentrations. Cells were lysed and CREB phosphorylated at S133 was quantified with this DuoSet IC ELISA. The same lysates were also immunoblotted (inset) with either anti-phospho-CREB (S133) or anti-CREB antibodies. The DuoSet IC ELISA results correlate well with the amounts of phosphorylated CREB detected by Western blot. The immunoblot with anti-CREB antibody indicates that total levels of CREB remained constant during the various treatments.

The Phospho-CREB (S133) DuoSet IC ELISA also quantifies phosphorylated CREB levels in mouse and rat cell lysates.

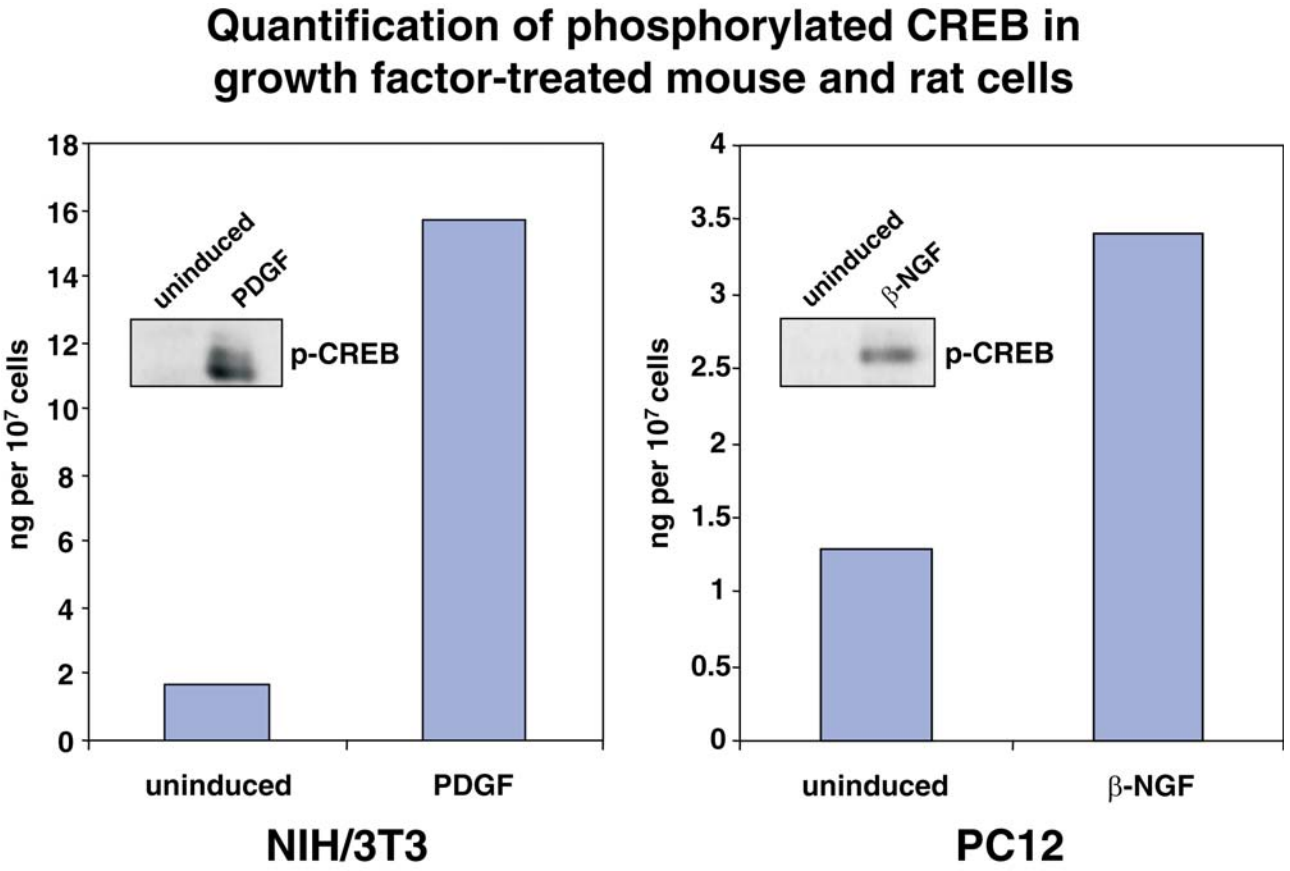


Figure 4: Lysates prepared from mouse NIH/3T3 cells either uninduced or induced with 10 ng/mL of human PDGF (R&D Systems, Catalog # 120-HD) for 5 minutes (left panel), and rat PC12 cells either uninduced or induced with 100 ng/mL of recombinant rat β-NGF (R&D Systems, Catalog # 556-NG) for 10 minutes (right panel), were quantified with this DuoSet IC ELISA. The same lysates were also immunoblotted (inset) with anti-phospho-CREB (S133) antibody. The DuoSet IC ELISA results correlate well with the amounts of phosphorylated CREB detected by Western blot.