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ProductInformation

Phospho-CREB [pSer^{133}] ELISA

Product Number **CS0570**
Storage Temperature 2-8 °C

Technical Bulletin

Product Description

Phospho-CREB [pSer^{133}] ELISA is a solid phase sandwich Enzyme Linked-Immuno-Sorbent Assay (ELISA) for the quantitative determination of phospho-CREB [pSer^{133}] in cell lysates. A monoclonal antibody specific for CREB (regardless of phosphorylation state) has been coated onto the wells of the multiwell plate strips provided. Phospho-CREB [pSer^{133}] standard dilutions, control specimens, and unknown samples are pipetted into these wells. During the first incubation, the phospho-CREB [pSer^{133}] antigen binds to the immobilized (capture) antibody. After incubation, the excess reagents are washed away and an antibody specific for CREB phosphorylated on serine 133 is added to the wells. During the second incubation, this antibody serves as a detection antibody by binding to the immobilized phospho-CREB [pSer^{133}]. After removal of excess detection antibody, horseradish peroxidase-labeled (HRP) anti-rabbit IgG is added. This binds to the detection antibody to complete the four-member sandwich. After a third incubation and washing to remove excess anti-rabbit IgG-HRP, a substrate solution is added, which is acted upon by the bound enzyme to produce color. The intensity of this colored product is directly proportional to the concentration of phospho-CREB [pSer^{133}] present in the original specimen. The optical density measured at 450 nm in the multiwell plate reader is used to calculate the concentration of phospho-CREB [pSer^{133}].

Phospho-CREB [pSer^{133}] ELISA is designed to detect and quantify the level of CREB protein phosphorylated on serine 133 in lysates of human and mouse cells. The assay also displays moderate cross-reactivity to a related family, CREM. For normalizing the phospho-CREB [pSer^{133}] content of the samples, a CREB ELISA, which is independent of phosphorylation status, is available from Sigma (Product No. CS0580).

CREB (cAMP-Response Element-Binding protein), a protein with MW of 43 kDa, is a member of the large ATF/CREM/CREB transcriptional activator family. As with other members of this family, CREB contains a highly conserved leucine zipper dimerization domain and a basic DNA binding domain at its carboxyl terminus, and a unique amino terminus. CREB is ubiquitously expressed among mammalian species, and is highly conserved evolutionarily, with numerous invertebrate, plant, and yeast homologs.

CREB activates transcription in response to stimuli that elevate cytoplasmic cAMP concentrations. The series of events leading to cAMP's activation of CREB is initiated by ligand binding to certain membrane receptors, which activate adenylyl cyclase. cAMP activates a protein kinase (PKA), which translocates to the nucleus, where it phosphorylates CREB at serine 133. This phosphorylation permits CREB to recruit CREB Binding Protein (CBP), and the CREB/CBP complex in turn stimulates gene expression by interacting directly with components of the general transcriptional machinery. In addition to fostering the formation of the CREB/CBP complex, the phosphorylation of serine 133 also enhances CREB's binding to the specific DNA sequence TGACGTCA, known as the cAMP Response Element (CRE), a sequence common to the regulatory regions of genes under the control of cAMP including Bcl-2, BDNF, the immediate early gene egr-1, and cyclin D.

In addition to stimuli that elevate cAMP levels and activate PKA, a variety of other stimuli are observed to induce CREB serine 133 phosphorylation. These include UV irradiation, cross-linking of cell membrane proteins such as surface Ig and CD28, growth factors including PDGF, NGF, EGF, FGF, and HGF, phorbol esters, serum feeding, and the Ca^{2+} flux that accompanies neuronal membrane depolarization. While PKA is considered to be the classical CREB kinase, other protein kinases are observed to directly phosphorylate CREB at serine 133, including the calcium/calmodulin-dependent protein kinases CaMK IV and CaMK II, Rsk -1, -2, and -3 (activated by the upstream kinase ERK1/2), and MAPKAP-K2 (activated by the upstream kinase, p38).

Interestingly, phosphorylation of CREB at serine 133 is found to be necessary, but not sufficient to activate

transcription in many model systems. Other events required for CREB's activation of transcription are currently being delineated. The regulation of gene expression by CREB and its role in cell growth, differentiation, and survival, as well as many areas of neuroscience, including learning and memory, regulation of mood, circadian rhythm, and drug addiction, are active areas of investigation.

Reagents

- **Phospho-CREB [pSer^{133}] Standard, Lyophilized, 2 vials, Product No. P 3372-** Full length recombinant CREB activated by PKA. Refer to vial label for quantity and reconstitution volume.
- **Standard Diluent Buffer, 25 mL, Product No. S 3943,** contains sodium azide as preservative.
- **Monoclonal-Anti-CREB-Coated 96 well plate, 1EA, Product No. C 0741 -** A plate using break-apart strips coated with monoclonal antibody specific for full-length CREB (regardless of phosphorylation state).
- **Anti-phospho-CREB [pSer^{133}], 11 mL, Product No. C 0991** A detection antibody, produced in rabbit. Contains sodium azide. Ready to use.
- **Anti-Rabbit IgG-HRP, Concentrate (100X), 1 vial, Product No. R 8153 -** contains 3.3 mM thymol and 50% glycerol, viscous. *See Reagent Preparation for handling, dilution and storage instructions.*
- **HRP Diluent, 25 mL, Product No. H 5788 -** contains 3.3 mM thymol. Ready to use.
- **Wash Buffer concentrate, 25X, 100 mL, Product No. W 2639 -** *See Reagent Preparation for handling, dilution and storage instructions*
- **Stabilized Chromogen, Tetramethylbenzidine (TMB), 25 mL, Product No. S 3318** –Avoid prolonged exposure to light. Avoid exposure to metal. Ready to use.
- **Stop Solution, 25 mL, Product No. S 2818** – Ready to use.
- **Plate Covers, Adhesive strips, 3 each, Product No. P 4870**

Reagents and Equipment required but not provided

- Multiwell plate reader capable of readings at 450 nm.
- Calibrated adjustable precision pipettes for volumes between 5 µL and 1,000 µL.
- Cell extraction buffer (see recommended extraction procedure).
- Deionized or distilled water.
- Plate washer (optional), use squirt bottle, manifold dispenser, etc.
- Glass or plastic 1.0 – 1.5 mL tubes for diluting and aliquoting standard.
- Absorbent paper towels to blot the plate.
- Calibrated beakers and graduated cylinders in various sizes.
- Vortex mixer.
- Graph paper: linear, log-log, or semi-log, as desired.

Precautions and Disclaimer

The kit is for R&D use only, not for drug, household or other uses. Please consult the Material Safety Data Sheet for information regarding hazards and safe handling practices.

Preparation Instructions

Sample Preparation

- Samples of choice – extracts of cell lysates
- Samples should be frozen if not analyzed shortly after collection.
- Avoid multiple freeze-thaw cycles of frozen samples. Thaw completely and mix well prior to analysis.

Cell Extraction Buffer

10 mM Tris, pH 7.4
100 mM NaCl
1 mM EDTA
1 mM EGTA
1 mM NaF
20 mM $Na_4P_2O_7$
2 mM Na_3VO_4
1% Triton® X-100
10% glycerol
0.1% SDS
0.5% deoxycholate
1 mM PMSF (stock is 0.3 M in DMSO) *PMSF is very unstable and must be added prior to use, even if added previously*.
Protease inhibitor cocktail (Sigma Product No. P 2714)

Add 250 µl of reconstituted cocktail per 5 mL of Cell Extraction Buffer.

Stable for 2-3 weeks at 4 °C or for up to 6 months when aliquoted (without protease inhibitors and PMSF added) and stored at –20 °C.

Thaw on ice. *Add the protease inhibitors just before use.*

Procedure for Extraction of Proteins from Cells

This protocol has been successfully applied to several cell lines of human origin. Researchers may use the procedures that work best in their hands. They will have to assay their lysates for the satisfactory extraction and/or phosphorylation.

Protocol for Cell Extraction

1. Collect cells in PBS by centrifugation (non-adherent) or scraping from culture flasks (adherent).
2. Wash twice with cold PBS.
3. Remove and discard the supernatant and collect the cell pellet. (At this point the cell pellet can be frozen at –70 °C and lysed at a later date).
4. Lyse the cell pellet in Cell Extraction Buffer for 30 minutes on ice with vortexing at 10-minute intervals.
5. The volume of Cell Extraction Buffer depends on the cell number in cell pellet and expression of phospho-CREB [pSer^{133}]. For example, 10^7 HeLa cells grown in DMEM plus 10% FBS can be extracted in 1 mL of Cell Extraction Buffer. Under these conditions, use of 1-10 µL of the clarified cell extract diluted to a volume of 100 µL/well in Standard Diluent Buffer is sufficient for the detection of phospho-CREB [pSer^{133}].
6. Transfer extracts to microcentrifuge tubes and centrifuge at 13,000 rpm for 10 minutes at 4 °C.
7. Aliquot the clear lysate to clean microcentrifuge tubes

Before assay: extracted cell lysate samples containing protein should be diluted with Standard Diluent Buffer at least 1:10. This dilution is necessary to reduce the matrix effect of the cell extraction buffer.

Reagent Preparation

Standard

Note: This CREB standard was prepared from full length, recombinant, phosphorylated CREB protein **One Unit of standard** is equivalent to the amount of phospho-CREB [pSer^{133}] derived from 80 pg of CREB that was phosphorylated by PKA.

1. Reconstitute Phospho-CREB [pSer^{133}] standard with *Standard Diluent Buffer*. Refer to standard vial label for instructions.
2. Swirl or mix gently and allow to sit for 10 minutes to ensure complete reconstitution. Label as 100 units/mL. Use standard within 1 hour of reconstitution.
3. Prepare serial standard dilutions as follows

Tube #	Standard Buffer	Standard from tube #:	Final units/mL
1	Reconstitute according to label instructions		100 units/mL
2	0.25 mL	0.25 mL (1)	50 units/mL
3	0.25 mL	0.25 mL (2)	25 units/mL
4	0.25 mL	0.25 mL (3)	12.5 units/mL
5	0.25 mL	0.25 mL (4)	6.25 units/mL
6	0.25 mL	0.25 mL (5)	3.12 units/mL
7	0.25 mL	0.25 mL (6)	1.6 units/mL
8	0.25 mL	-	0 units/mL

4. Remaining reconstituted standard should be discarded or frozen at –70 °C. for further use. Standard can be frozen and thawed one time only without loss of immunoreactivity.

Anti-rabbit IgG Horseradish Peroxidase (HRP)

Note: The *Anti-rabbit IgG-HRP* 100X concentrate is in 50% glycerol. This solution is viscous. To ensure accurate dilution:

1. Equilibrate to room temperature, mix gently, pipette slowly.
2. Remove excess concentrate solution from pipette tip with clean absorbent paper.
3. Within 1 hour of use, dilute 10 µL of this 100X concentrated solution with 1 mL of *HRP Diluent* for each 8-well strip used in the assay. Label as Anti-rabbit IgG-HRP Working Solution.
4. Return the unused concentrate to the refrigerator
5. For more strips use the following amounts:

# of 8 well strips	IgG-HRP Concentrate µL	Diluent mL
2	20	2
4	40	4
6	60	6
8	80	8
10	100	10
12	120	12

Wash Buffer

1. Equilibrate to room temperature and mix to redissolve any precipitated salts.
2. Mix 1 volume Wash Buffer Concentrate 25X + 24 volumes of deionized water
3. Label as *Working Wash Buffer.*
4. Store both the concentrate and the Working Wash Buffer in the refrigerator. Use within 14 days.

Storage/Stability
All components of this kit are stable at 2-8 °C. Any unused reconstituted standard should be discarded or frozen at -70 °C. Standard can be frozen and thawed one time only without loss of immunoreactivity.

Refer to the Certificate of Analysis for kit shelf life. To obtain C of A go to www.sigma-aldrich.com

Procedure
Precautions
- 20-30 minutes before use equilibrate kit and all reagents to room temperature (15-30 °C).
- Use only the coated 96 well capture plate provided with the kit
- Multiwell plate: equilibrate to room temperature in unopened foil bag. Remove desired number of strips, reseal the bag and refrigerate at 2-8 °C to maintain plate integrity.
- When not in use all kit components should be refrigerated.
- Assay all standards, controls and samples in duplicate.
- If particulate matter is present, centrifuge or filter prior to analysis.
- A standard curve must be run with each assay
- Maintain a consistent order of component and reagent addition from well to well. This ensures equal incubation times for all wells.
- Run in-house controls with every assay. If control values fall outside pre-established ranges, the accuracy of the assay may be suspect.
- All reagents are lot-specific. Do not mix reagents from different kit lots.
- Do not use reagents after the kit expiration date.
- Standards and samples can be made up in either glass or plastic tubes.
- Pre-rinse the pipette tip with the reagent and use fresh pipette tips for each sample, standard or reagent.
- Read absorbances within 2 hours of assay completion.

Washing directions
- The purpose of washing is to remove unbound proteins and other non-specific parts of lysate.
- Incomplete washing will adversely affect the assay and render false results.
- Use only Wash Buffer provided in kit.
- Washing may be performed using automated washer, manifold pipette or squirt bottle.
- Wash cycle four times, blotting as dry as possible after the 4th wash.
- When washing manually, fill wells with Wash Buffer, aspirate thoroughly and tap dry on absorbent tissue.
- It is recommended to use laboratory tape to hold plate strips to the plate frame while performing the plate washing and drying procedure to avoid strips coming free of the frame.

Assay Procedure

Phospho-CREB [pSer^{133}] ELISA Assay Summary

1) 100 µL of Phospho-CREB [pSer^{133}] Standards or Samples (samples diluted 1:10 or higher in Standard Diluent Buffer)
Incubate 2 hours at RT
aspirate and wash 4x

2) Add 100 µL Anti-phospho-CREB [pSer^{133}]
Incubate 1 hour at RT.
aspirate and wash 4x

3) Add 100 µL Anti-Rabbit IgG-HRP
Incubate 30 min at RT.
aspirate and wash 4x

4) Add 100 µL Stabilized Chromogen
Incubate 30 minutes at RT
***(in the dark)*.**

5) Add 100 µL of Stop Solution
Read at 450nm.

Total Assay Time - 4 hours

- Determine the number of wells for the assay run, including 2 zero wells, 2 chromogen blank wells, 14 standard dilution wells and 2 wells for each sample to be assayed.
- Remove appropriate number of multiwell strips and return the unused strips to the pouch. Reseal pouch.

1st incubation
a Add 100 µL Standard Diluent to zero wells.
b Add 100 µL standards, samples or controls to the appropriate wells.
c Samples prepared in cell extraction buffer or brain homogenate buffer must be diluted 1:10 or greater in *Standard Diluent Buffer* (for example, 10 µL sample plus 90 µL buffer). *The dilutions should be optimized for each assay.*
d Cell culture supernatants or buffered solutions; dilute 1:2 in *Standard Diluent Buffer* (50 µL buffer + 50 µL sample).
e Tap gently on the plate to mix, cover with Plate Cover and incubate 2 hours at room temperature.
f Thoroughly aspirate or decant solution from wells and discard the liquid. Wash wells for a total of 4 times following washing instructions

2nd incubation
a Add 100 µL Anti- phospho-CREB [pSer^{133}] detection antibody to all wells (except chromogen blanks).
b Tap gently on the plate to mix, cover with Plate Cover and incubate 1 hour at room temperature.
c Wash wells for a total of 4 times following washing instructions.
d After the final wash blot dry on a lint free paper towel to remove any remaining wash buffer.

3rd incubation
a Add 100 µL Anti-Rabbit IgG-HRP Working Solution to each well except the chromogen blanks.
b Cover with Plate Cover and incubate 30 minutes at room temperature.
c Wash wells for a total of 4 times following washing instructions.
d After the final wash blot dry on a lint free paper towel to remove any remaining wash buffer.

Substrate incubation
a Add 100 µL of Stabilized Chromogen into all wells. *The liquid in the wells will begin to turn blue.*
b Do not cover the plate
c Incubate approximately 30 minutes at room temperature in the dark (*place plate in a drawer or cabinet).*

Note: *If your multiwell plate reader does not register optical density (OD) above 2.0, incubate only 20-25 minutes.*

Stop reaction
a Add 100 µL of Stop Solution to each well. This stops the reaction
b Tap gently to mix. *The solution will turn yellow.*

Absorbance reading
a Any commercially available multiwell plate reader capable of reading at OD 450 nm may be used.
b Blank the plate reader against the Chromogen Blank wells (containing Chromogen and Stop Solution).

Read the absorbance of the entire plate at 450 nm within 2 hours after addition of Stop Solution

Results

1. The results may be calculated using any immunoassay software package.
2. The four-parameter algorithm provides the best curve fit.
3. If the software program is not readily available, the concentrations of Phospho-CREB [pSer^{133}] may be calculated manually.
4. Calculate the Average Net OD (average reading of 2 wells) for each standard dilution and samples as follows:
5. Average Net OD = Average Bound OD – Average Chromogen Blank OD
6. On graph paper plot the Average Net OD of standard dilutions against the standard concentration (units/mL) of phospho-CREB [pSer^{133}]. Draw the best curve through these points to construct the standard curve.
7. The Phospho-CREB [pSer^{133}] concentrations in unknown samples and controls can be determined by interpolation from the standard curve.
8. Multiply the values obtained for the samples by dilution factor of each sample.
9. Samples producing signals higher than the 100 units/mL standard should be further diluted and assayed again.

Product Profile

Typical Results

The standard curve below is for illustration only and **should not be used** to calculate results in your assay. Run standard curve in each assay.

Standard Curve

Phospho-CREB [pSer^{133}] (units/mL)	Optical density (450 nm)
100	3.30
50	1.70
25.	0.88
12.5	0.47
6.25	0.25
3.12	0.15
1.6	0.10
0	0.05

Limitations

- Do not extrapolate the standard curve beyond the 100 units/mL standard point.
- The dose response is non-linear in this region and accuracy will be compromised.
- Other buffers and matrices have not been investigated.
- The influence of various drugs, aberrant sera (hemolyzed, hyperlipidemic, jaundiced, etc.) and the use of biological fluids in place of cell extract and brain homogenate samples have not been thoroughly investigated.
- The rate of degradation of native CREB or dephosphorylation of CREB [pSer^{133}] in various matrices in various matrices has not been investigated.
- Although CREB degradation or dephosphorylation of CREB [pSer^{133}] in the Cell Extraction Buffer described in this protocol has not been seen to date, the possibility of this occurrence cannot be excluded.

Performance characteristics

Sensitivity

The analytical sensitivity of this assay is <0.09 unit/mL of phospho-CREB [pSer^{133}]. This was determined by adding two standard deviations to the mean O.D. obtained when the zero standard was assayed 30 times.

The sensitivity of this ELISA was compared to immunoblotting using known quantities of phospho-CREB [pSer^{133}]. The data presented in Figure 1 show that the sensitivity of the ELISA is approximately 4x greater than that of immunoblotting. The bands shown in the immunoblotting data were developed using rabbit anti-CREB [pSer^{133}], an alkaline phosphatase conjugated anti-rabbit IgG, followed by chemiluminescent substrate and autoradiography.

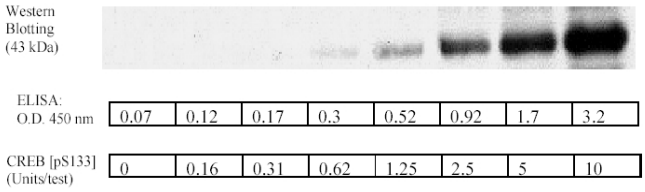

Figure 1 Detection of phospho-CREB [pSer^{133}] by ELISA vs. immunoblot

Precision

1. Intra-Assay Precision

Samples of known concentration were assayed in replicates of 16 to determine precision within an assay.

	Sample 1	Sample 2	Sample 3
Mean (units/mL)	72.8	25.8	7.9
Standard Deviation (SD)	4.0	1.0	0.2
% Coefficient of Variation	5.5	3.7	2.7

2. Inter-Assay Precision

Samples were assayed 48 times in multiple assays to determine precision between assays.

	Sample 1	Sample 2	Sample 3
Mean (units/mL)	75.6	24.4	7.9
Standard Deviation (SD)	6.1	1.4	0.3
Coefficient of Variation %	8.0	5.7	3.6

Recovery

To evaluate recovery, phospho-CREB [pSer^{133}] standard was spiked at 3 different concentrations into 10% cell extract buffer. The average recovery was 110.8%.

Parallelism

Natural CREB [pSer^{133}] from Forskolin-treated HeLa cell lysate was serially diluted in *Standard Diluent Buffer.* The optical density of each dilution was plotted against the CREB [pSer^{133}] standard curve. Parallelism demonstrated in Figure 2 indicates that the standard accurately reflects CREB [pSer^{133}] content in samples.

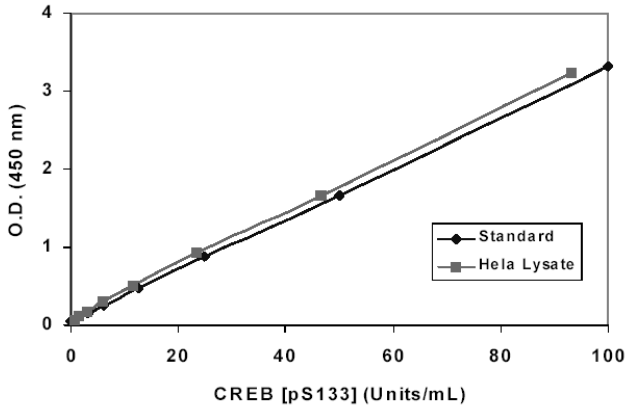

Figure 2 Parallelism: phospho-CREB [pSer^{133}]

Linearity of Dilution

HeLa cells were grown in tissue culture medium containing 10% fetal calf serum, treated with 200 µM Forskolin for 20 minutes and lysed with Cell Extraction Buffer. This lysate was diluted with *Standard Diluent Buffer* over the range of the assay and measured for CREB [pSer^{133}] content. Linear regression analysis of sample values versus the expected concentration yielded a correlation coefficient of 0.99.

Dilution	Cell Lysate		
	Measured units/mL	Expected units/mL	% Expected
Neat	93.2	93.2	100
1:2	42.1	46.6	90.4
1:4	21.1	23.3	90.4
1:8	10.7	11.6	92.3
1:16	5.2	5.8	90.0
1:32	2.8	2.9	95.7

Specificity

- The Phospho-CREB [pSer^{133}] ELISA is specific for measurement of Phospho-CREB [pSer^{133}] protein and shows moderate crossreactivity with related protein member CREM.
- To determine the specificity of this ELISA, HeLa cells were treated with 200 µM Forskolin for 20 minutes at 37 °C. Untreated HeLa cells were used as a control.
- All extracts were prepared and analyzed by the Phospho-CREB [pSer^{133}] ELISA and CREB ELISA.
- The levels of CREB protein remained comparable while the levels of Phospho-CREB [pSer^{133}] increased after Forskolin treatment.

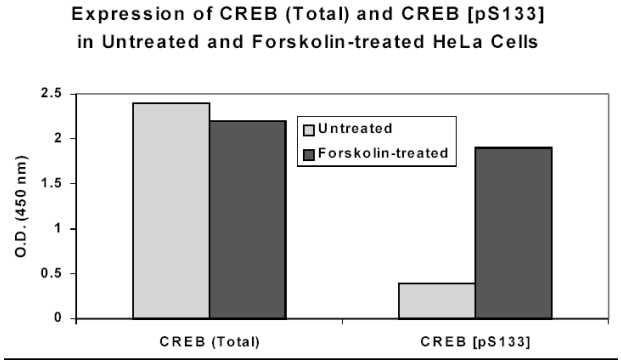

Figure 3 Expression of CREB and Phospho-CREB [pSer^{133}] in untreated and treated HeLa cells

The specificity of this assay for phosphorylated CREB [pSer^{133}] was confirmed by peptide competition. The data presented in Figure 4 show that the phospho-peptide containing the phosphorylated serine 133 blocks the ELISA signal.

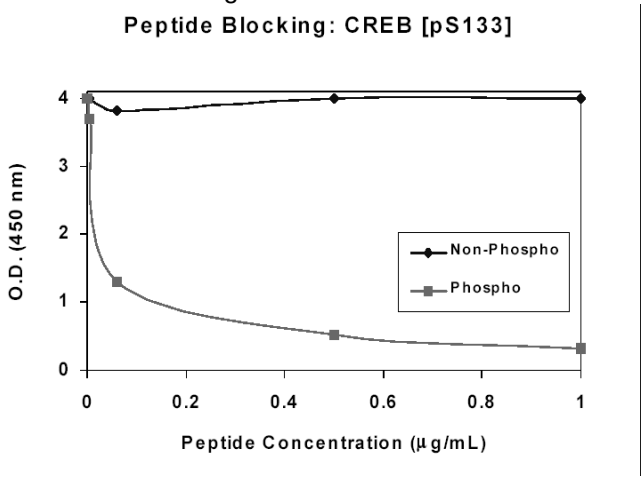

Figure 4 Peptide Blocking: Phospho-CREB [pSer^{133}]

Reference

1. Shaywitz, A.J., and. Greenberg, M.E., CREB: a stimulus-induced transcription factor activated by a diverse array of extracellular signals. Annu. Rev. Biochem., **68,** 821-861 (1999).
2. Xing, J., et al., Nerve growth factor activates extracellular signal-regulated kinase and p38 mitogen-activated protein kinase pathways to stimulate CREB serine 133 phosphorylation., Mol. Cell. Biol., **18,** 1946-1955 (1998).
3. Maldonado, R., et al., Reduction of morphine abstinence in mice with a mutation in the gene encoding CREB. Science **273,** 611-612 (1996).
4. Seternes, O.M., et al., A dominant role for the Raf-MEK pathway in forskolin, 12-O-tetradecanoylphorbol acetate, and platelet derived growth factor-induced CREB (cAMP-response element-binding protein) activation, uncoupled from serine 133 phosphorylation in NIH 3T3 cells. Mol. Endocrinol. **13,** 1071-1081, (1999).
5. Gonzales, G.A. and M.R. Montminy, Cyclic AMP stimulates somatostatin gene transcription by phosphorylation of CREB at serine 133. Cell, **59,** 675-680 (1989).

AH/PHC 09/04