

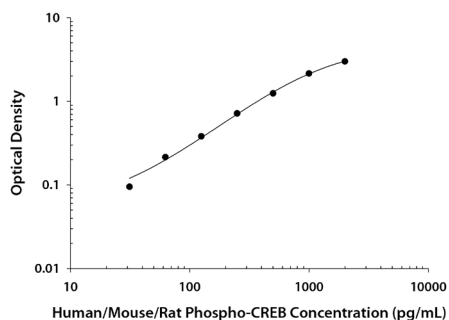
## CALCULATION OF RESULTS

Average the duplicate readings for each standard and sample, then 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-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

The Human/Mouse/Rat Phospho-CREB (S133) DuoSet® IC ELISA is calibrated against a highly purified *E. coli*-expressed recombinant human Phospho-CREB (S133) produced at R&D Systems®. Samples containing natural Phospho-CREB (S133) showed linear dilution parallel to the standard curve obtained using the Human/Mouse/Rat Phospho-CREB (S133) Standard. These results indicate that O.D. values from this DuoSet® IC ELISA can be used to determine the relative concentration of Phospho-CREB (S133) in natural samples.

## 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.

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.

## TECHNICAL HINTS & 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 samples and standard reflect the environment of the samples being measured. The diluents 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.

## PRECAUTIONS

The Stop Solution recommended for use with this kit is an acid solution.

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

Color Reagent B recommended for use with this kit may cause skin, eye, and respiratory irritation. Avoid breathing fumes.

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

**DuoSet® IC ELISA**  
INTRACELLULAR

## Human/Mouse/Rat Phospho-CREB (S133)

Catalog Number: DYC2510-2 (2 plates)

DYC2510-5 (5 plates)

## INTENDED USE

For the development of sandwich ELISAs to measure human, mouse, and rat cAMP Response Element Binding Protein (CREB) phosphorylated at S133 in cell lysates.

## 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 human/mouse/rat CREB binds both the phosphorylated and unphosphorylated protein. After washing away unbound material, a biotinylated detection antibody recognizing human/mouse/rat CREB phosphorylated at S133 is used to detect only the 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.

**Note: The reconstitution method has changed. Read this package insert in its entirety before using this product.**

**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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## MATERIALS PROVIDED & STORAGE CONDITIONS

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

DESCRIPTION	PART #	CATALOG # DYC2510-2	CATALOG # DYC2510-5	STORAGE OF OPENED/ RECONSTITUTED MATERIAL
Human/Mouse/Rat Phospho-CREB (S133) Capture Antibody	842088	1 vial	2 vials	Store for up to 1 month at 2-8 °C or aliquot and store at ≤ -20 °C or ≤ -70 °C for up to 3 months in a manual defrost freezer.*
Human/Mouse/Rat Phospho-CREB (S133) Detection Antibody	842089	1 vial	2 vials	
Streptavidin-HRP A	890803	1 vial	1 vial	Store for up to 3 months at 2-8 °C.* <b>DO NOT FREEZE.</b>
Human/Mouse/Rat Phospho-CREB (S133) Standard	842090	3 vials	5 vials	Use within one hour of reconstitution. Use a fresh standard for each assay.

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

DYC2510-2 contains sufficient materials to run ELISAs on at least two 96 well plates.<sup>†</sup>  
DYC2510-5 contains sufficient materials to run ELISAs on at least five 96 well plates.<sup>†</sup>

<sup>†</sup> 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.
- The recommended microplates, buffers, diluents, substrates, and solutions are used.

## OTHER MATERIALS REQUIRED

- Aprotinin (Tocris® # 4139).
- Leupeptin (Tocris® # 1167).
- Pepstatin (Tocris® # 1190).
- Phenylmethylsulfonyl Fluoride (PMSF) (Sigma # P7626).
- Sodium Pyrophosphate (Na<sub>4</sub>P<sub>2</sub>O<sub>7</sub>) (Sigma # P8010).
- Sodium Orthovanadate (Na<sub>3</sub>VO<sub>4</sub>) (Sigma # S6508), activated.
- Sodium Fluoride (NaF) (Aldrich # 201154).
- Triton™ X-100 (Sigma # T9284).
- Sodium Azide (NaN<sub>3</sub>) (Sigma # S2002).
- Urea.
- Pipettes and pipette tips
- Deionized or distilled water.
- 96 well microplates (R&D Systems®, Catalog # DY990)
- Plate sealers (R&D Systems®, Catalog # DY992).
- Squirt bottle, manifold dispenser, or automated microplate washer.

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## SOLUTIONS REQUIRED

**PBS** - 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.5 mM KH<sub>2</sub>PO<sub>4</sub>, pH 7.2-7.4, 0.2 µm filtered (R&D Systems®, Catalog # DY006).

**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<sub>3</sub> 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 96 well 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, 3.0 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<sub>2</sub>O<sub>2</sub>) and Color Reagent B (Tetramethylbenzidine) (R&D Systems®, Catalog # DY999).

**Stop Solution** - 2 N H<sub>2</sub>SO<sub>4</sub> (R&D Systems®, Catalog # DY994).

\*The use of R&D Systems® Reagent Diluent Concentrate 2 (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.

\*\*Alternatively, use Sample Diluent Concentrate 1 (5X) (R&D Systems®, Catalog # DYC001), prepare as described in the DYC001 insert.

## REAGENT PREPARATION

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

### Human/Mouse/Rat 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.

### Human/Mouse/Rat 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. Immediately before use, dilute the detection antibody to a working concentration of 500 ng/mL in IC Diluent #1. Prepare only as much detection antibody as required to run each assay.

**Streptavidin-HRP A** (Part 890803) - 1.0 mL of streptavidin conjugated to horseradish-peroxidase. Immediately before use, dilute the Streptavidin-HRP A to the working concentration specified on the vial label using IC Diluent #1.

**Human/Mouse/Rat Phospho-CREB (S133) Standard** (Part 842090)  
**Refer to the vial label for the stock concentration of recombinant human phospho-CREB (S133) when reconstituted with**

**500 µL of IC Diluent #7.** 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.

## 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<sup>7</sup> 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.

## 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 standard 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. 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. Add 100 µL of the diluted Streptavidin-HRP A 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 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.