

# DuoSet<sup>®</sup> IC

## Human/Mouse/Rat Total p70 S6 Kinase

Catalog Number DYC8962-2

DYC8962-5

**For the development of sandwich ELISAs to measure p70 S6 Kinase 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<sup>®</sup> IC ELISA contains the basic components required for the development of sandwich ELISAs to measure p70 ribosomal S6 kinase (p70 S6K) in cell lysates. An immobilized capture antibody specific for p70 S6K binds both phosphorylated and unphosphorylated p70 S6K. After washing away unbound material, a biotinylated detection antibody specific for p70 S6K is used to detect both phosphorylated and unphosphorylated protein, utilizing a standard Streptavidin-HRP format.

In addition, this DuoSet IC ELISA also measures p85 ribosomal S6 kinase (p85 S6K). The two S6Ks are isoforms expressed from a common transcript using alternative translation initiation sites. p85 S6K differs from p70 S6K by having an additional 23 amino acids at the N-terminus.

## MATERIALS PROVIDED

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

			Vials Provided	
Description	Part #	Storage Conditions	Cat. # DYC8962-2	Cat. # DYC8962-5
Total p70 S6 Kinase Capture Antibody	841582	2 - 8° C	1	2
Total p70 S6 Kinase Detection Antibody	841583	2 - 8° C	1	2
Total p70 S6 Kinase Standard	841584	2 - 8° C	3	5
Streptavidin-HRP	890803	2 - 8° C	1	1

DYC8962-2 contains sufficient materials to run ELISAs on at least two 96 well plates.\*

DYC8962-5 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 azide (NaN<sub>3</sub>) (Sigma # S2002)
- Sodium fluoride (NaF) (Sigma # 201154)
- Sodium orthovanadate (Na<sub>3</sub>VO<sub>4</sub>) (Sigma # S6508), activated
- Sodium pyrophosphate (Na<sub>4</sub>P<sub>2</sub>O<sub>7</sub>) (Sigma # P8010)
- Triton<sup>®</sup> 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<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.

**Wash Buffer** - 0.05% Tween<sup>®</sup> 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 (R&D Systems, Catalog # DY995).

**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 diluent 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, 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 (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), prepared as described in the DYC001 insert.

*Triton is a registered trademark of the Union Carbide Corp.  
Tween is a registered trademark of ICI Americas, Inc..*

## REAGENT PREPARATION

Bring all reagents to room temperature before use.

**Total p70 S6 Kinase Capture Antibody** (Part 841582) - Each vial contains 720  $\mu\text{g}/\text{mL}$  of mouse anti-human p70 S6 Kinase antibody when reconstituted with 200  $\mu\text{L}$  of PBS. After reconstitution, store at 2 - 8° C for up to 30 days or aliquot and store at  $\leq -20^\circ\text{C}$  in a manual defrost freezer or at  $\leq -70^\circ\text{C}$  for up to 3 months.\*

**Total p70 S6 Kinase Detection Antibody** (Part 841583) - Each vial contains 14.4  $\mu\text{g}/\text{mL}$  of biotinylated goat anti-human p70 S6 Kinase 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  $\leq -20^\circ\text{C}$  in a manual defrost freezer or at  $\leq -70^\circ\text{C}$  for up to 3 months.\*

**Total p70 S6 Kinase Standard** (Part 841584) - Each vial contains 100 ng/mL of recombinant human p70 S6 Kinase when reconstituted with 500  $\mu\text{L}$  of IC Diluent #7. **Use within 1 hour of reconstitution. A fresh standard should be used for each assay.** Immediately before use, an initial 6-fold dilution should be made in IC Diluent #8. Additional dilutions should be made in IC Diluent #3. A seven point standard curve using 2-fold serial dilutions and a high standard of 1000 pg/mL is recommended.

**Streptavidin-HRP** (Part 890803) - 1 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  $1 \times 10^7$  cells/mL in Lysis Buffer #6 and allow samples to sit on ice for 15 minutes. Assay immediately or store at  $\leq -70^\circ\text{C}$ . 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 protective gloves, clothing, eye, and face protection. Wash hands thoroughly after handling.

## **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 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.

# GENERAL ELISA PROTOCOL

A plate layout is provided to record standards and samples assayed.

## Plate Preparation

1. Dilute the Capture Antibody to the working concentration of 4.0  $\mu\text{g}/\text{mL}$  in PBS without carrier protein. Immediately coat a 96 well microplate with 100  $\mu\text{L}$  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  $\mu\text{L}$ ) 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  $\mu\text{L}$  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  $\mu\text{L}$  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 1000 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. Add 100  $\mu\text{L}$  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  $\mu\text{L}$  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  $\mu\text{L}$  of Substrate Solution to each well. Incubate for 20 minutes at room temperature. Avoid placing the plate in direct light.
8. Add 50  $\mu\text{L}$  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.

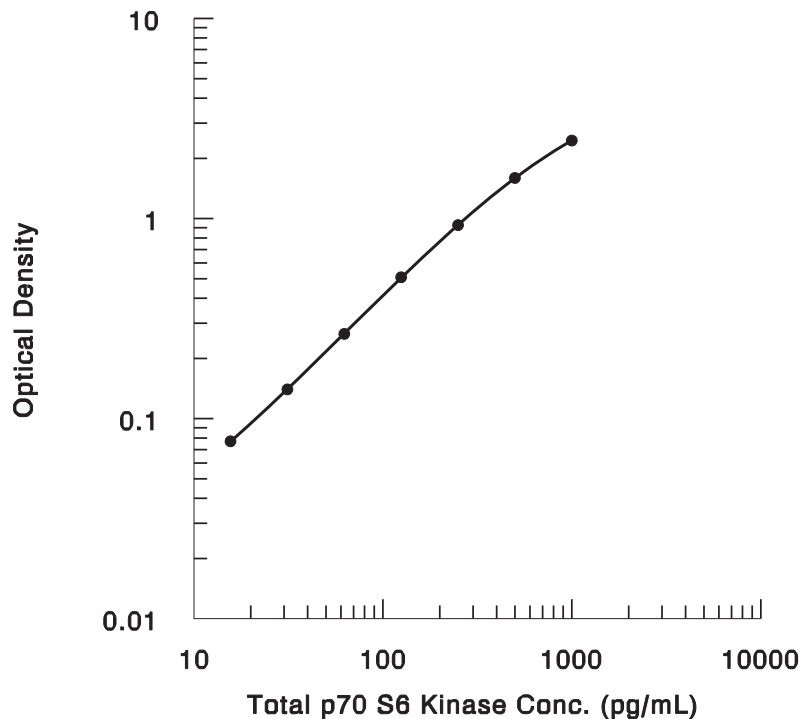
## CALCULATION OF RESULTS

Average the duplicate readings for each standard and sample, 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 p70 S6 Kinase 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 the Human/Mouse/Rat Total p70 S6 Kinase DuoSet IC ELISA. The standard curve was calculated using a computer generated 4-PL curve-fit. This standard curve is for demonstration purposes only.



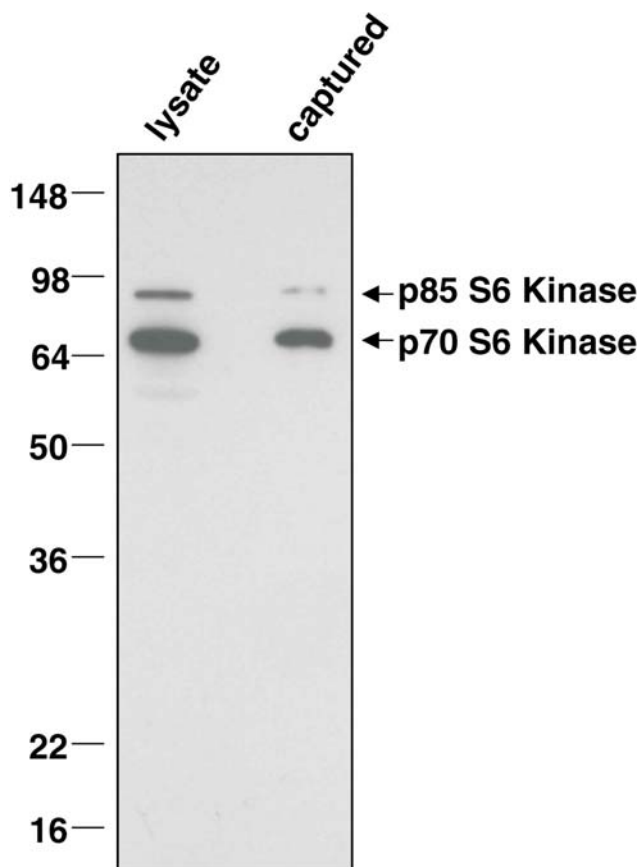


## CALIBRATION

This DuoSet IC ELISA is calibrated against a highly purified *E. coli*-expressed recombinant human p70 S6 Kinase produced at R&D Systems.

## SPECIFICITY

The Human/Mouse/Rat Total p70 S6 Kinase DuoSet IC ELISA specifically recognizes p70 S6K and p85 S6K. Specificity was demonstrated by Western blot analysis of the protein bound by the capture antibody supplied in the kit.

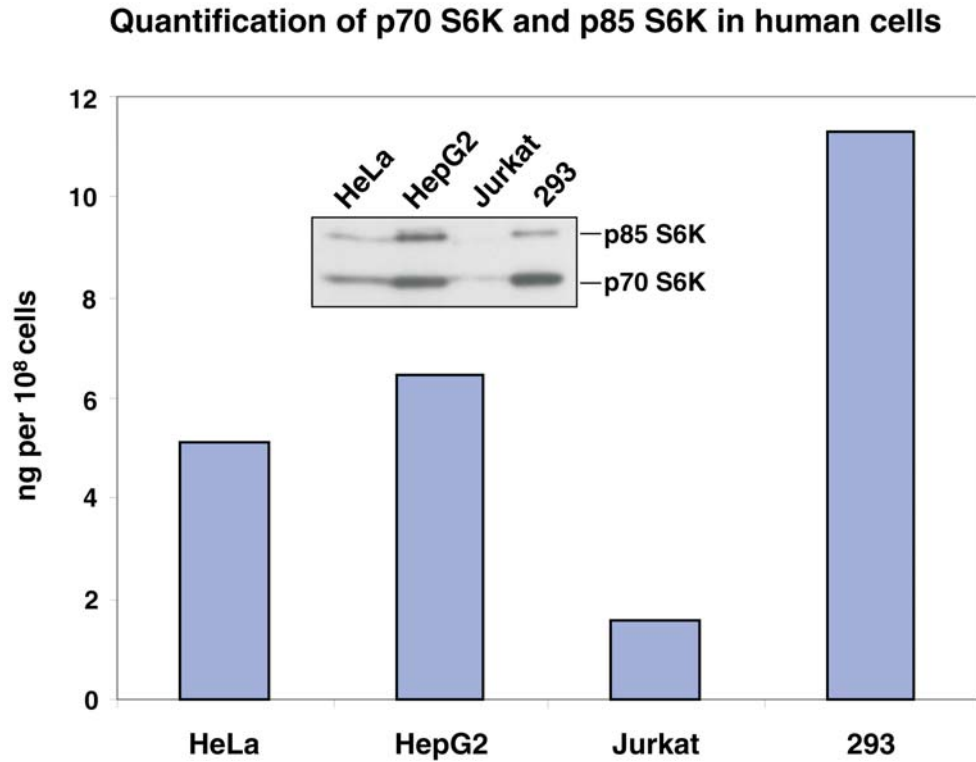


**Figure 1:** Lysates prepared from human MCF-7 cells were incubated in wells coated with Total p70 S6 Kinase DuoSet IC Capture Antibody. Unbound material was removed by washing and bound material was solubilized in SDS gel sample buffer. The same lysate and captured proteins were electrophoresed, transferred to a PVDF membrane and immunoblotted with Total p70 S6 Kinase DuoSet IC Detection Antibody. Two bands corresponding to p70 S6K and p85 S6K were detected.

To further determine specificity, recombinant human (rh) RSK1, rhRSK2, rhMSK1, and rhMSK2 were assayed at 50 ng/mL and did not cross-react or interfere in the assay.

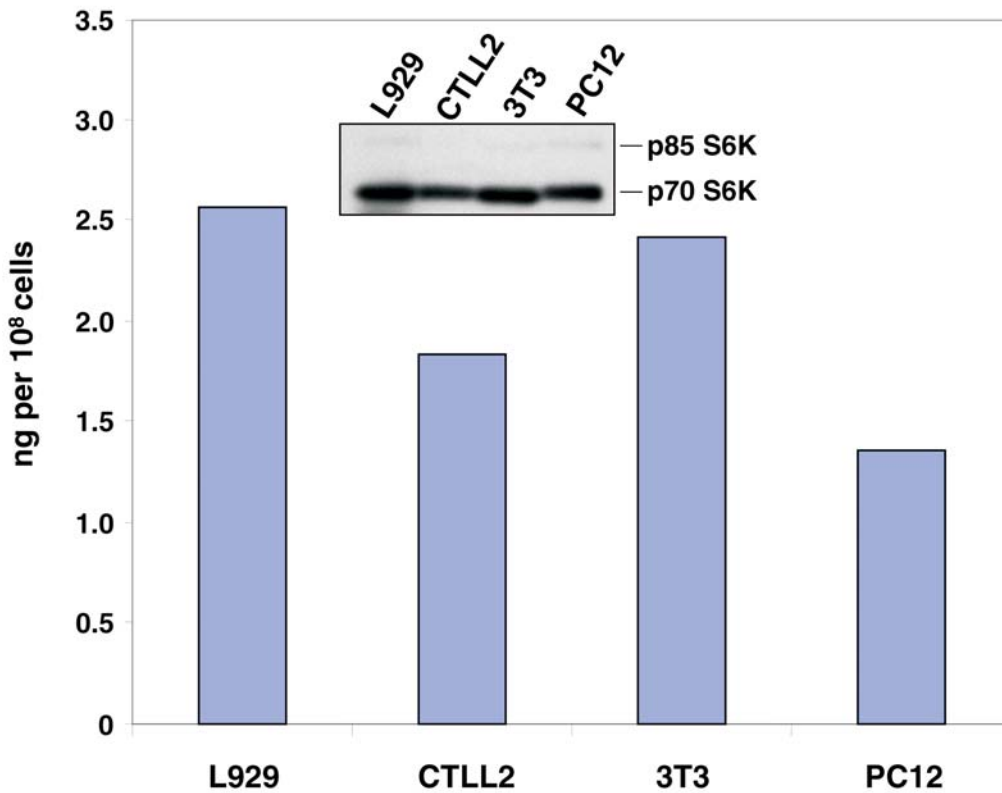
## QUANTIFICATION

Amounts of p70 S6K and p85 S6K, as quantified by the Human/Mouse/Rat Total p70 S6 Kinase DuoSet IC ELISA, are consistent with the amounts of p70 S6K and p85 S6K determined by qualitative Western blot analysis.



**Figure 2:** Lysates prepared from human HeLa, HepG2, Jurkat, and 293 cells were quantified with this DuoSet IC ELISA. The same lysates were also immunoblotted (inset) with anti-p70 S6K monoclonal antibody (R&D Systems, Catalog # MAB8962). The DuoSet IC ELISA results correlate well with the relative amounts of p70 S6K and p85 S6K detected by Western blot.

### Quantification of p70 S6K and p85 S6K in mouse and rat cells



**Figure 3:** Lysates prepared from mouse L929, CTLL2, NIH/3T3, and rat PC12 cells were quantified with this DuoSet IC ELISA. The same lysates were also immunoblotted (inset) with anti-p70 S6K monoclonal antibody. The DuoSet IC ELISA results correlate well with the relative amounts of p70 S6K and p85 S6K detected by Western blot.

# PLATE LAYOUT

Use this plate layout as a record of standards and samples assayed.

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