DuoSet® IC

Human/Mouse/Rat Phospho-RPS6 (S235/S236)

Catalog Number DYC3918-2
DYC3918-5
DYC3918E

For the development of sandwich ELISAs to measure Ribosomal Protein S6 (RPS6) phosphorylated at S235 and S236 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 human/mouse/rat Ribosomal Protein S6 (RPS6) phosphorylated at S235 and S236 in cell lysates. An immobilized capture antibody specific for human/mouse/rat RPS6 binds both phosphorylated and unphosphorylated protein. After washing away unbound material, a biotinylated detection antibody specific for human/mouse/rat RPS6 dually phosphorylated at S235 and S236 is used to detect only the 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. # DYC3918-2**	**Cat. # DYC3918-5**
Human/Mouse/Rat Phospho-RPS6 (S235/S236) Capture Antibody	842936	2-8° C	1	2
Human/Mouse/Rat Phospho-RPS6 (S235/S236) Detection Antibody	842937	2-8° C	1	2
Human/Mouse/Rat Phospho-RPS6 (S235/S236) Standard	842938	2-8° C	3	5
Streptavidin-HRP	890803	2-8° C	1	1

DYC3918-2 contains sufficient materials to run ELISAs on at least two 96 well plates.*
DYC3918-5 contains sufficient materials to run ELISAs on at least five 96 well plates.*

This kit is also available in an Economy Pack (R&D Systems, Catalog # DYC3918E). Economy Packs contain sufficient materials to run ELISAs on 15 microplates.* Specific vial counts of each component may vary. Please refer to the literature accompanying your order for specific vial counts.

*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 (Tocris # 1167)
- Pepstatin (Tocris # 1190)
- NP-40 Alternative (EMD/Calbiochem # 492016)
- Phenylmethylsulfonylfluoride (PMSF) (Sigma # P7626)
- Sodium Azide (NaN_3) (Sigma # S2002)
- Sodium Deoxycholate (Sigma # D6750)
- Sodium Dodecyl Sulfate (SDS) (Sigma # L4509)
- Sodium Fluoride (NaF) (Sigma # 201154)
- Sodium Orthovanadate (Na_3VO_4) (Sigma # S6508), activated
- Sodium Pyrophosphate ($Na_4P_2O_7$) (Sigma # P8010)
- 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.

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 (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_3, 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 #15 - 50 mM Tris (pH 7.4), 150 mM NaCl, 1% NP-40 Alternative, 0.5% sodium deoxycholate, 0.1% SDS.

Lysis Buffer #10 - 50 mM Tris, 1% NP-40 Alternative, 0.25% sodium deoxycholate, 5 mM EDTA, 150 mM NaCl, 5 mM NaF, 10 μg/mL Leupeptin, 10 μg/mL Pepstatin, 100 μM PMSF, 3 μg/mL Aprotinin, 2 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 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.

REAGENT PREPARATION

Bring all reagents to room temperature before use.

Human/Mouse/Rat Phospho-RPS6 (S235/S236) Capture Antibody (Part 842936) Each vial contains 720 μg/mL of mouse anti-human RPS6 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.*

Human/Mouse/Rat Phospho-RPS6 (S235/S236) Detection Antibody (Part 842937) Each vial contains 9 μg/mL of biotinylated rabbit anti-human phospho-RPS6 (S235/S236) 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.*

Human/Mouse/Rat Phospho-RPS6 (S235/S236) Standard (Part 842938) - Each vial contains 170 ng/mL of recombinant human phospho-RPS6 (S235/S236) when reconstituted with 500 μL of IC Diluent #15. **Use within one hour of reconstitution. A fresh standard should be used for each assay.** A seven point curve using 2-fold serial dilutions and a high standard of 20 ng/mL is recommended.

Streptavidin-HRP (Part 890803) - 1.0 mL of Streptavidin conjugated to horseradish-peroxidase. Immediately before use, dilute the Streptavidin-HRP to the working concentration specified on the vial label using IC Diluent #1. 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 #10 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. If needed, further dilutions should be made in IC Diluent #15.

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. Please refer to the MSDS on our website prior to use.

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

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

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 #15 per well. Use IC Diluent #15 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 ng/mL is recommended.*
2. Repeat the aspiration/wash as in step 2 of Plate Preparation.
3. Immediately before use, dilute the Detection Antibody to a working concentration of 250 ng/mL in IC Diluent #1. 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 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.

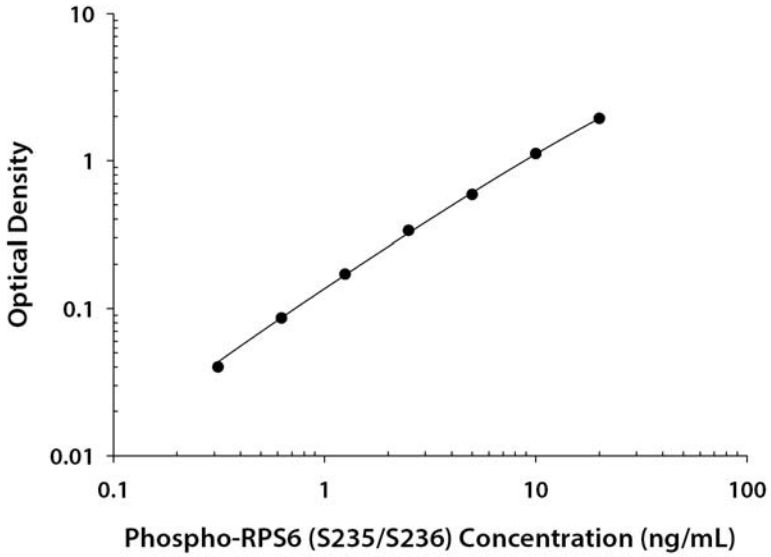
CALCULATION OF RESULTS

Average the duplicate readings for each standard and sample, 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-RPS6 (S235/S236) 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-RPS6 (S235/S236) 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-RPS6 (S235/S236) DuoSet® IC ELISA is calibrated against a highly purified *E. coli*-expressed recombinant human phospho-RPS6 (S235/S236) produced at R&D Systems. Samples containing natural phospho-RPS6 (S235/S236) showed linear dilution parallel to the standard curve obtained using the Human/Mouse/Rat Phospho-RPS6 (S235/S236) Standard. These results indicate that O.D. values from this DuoSet® IC ELISA can be used to determine the relative concentration of phospho-RPS6 (S235/S236) in natural samples.

SPECIFICITY

The Human/Mouse/Rat Phospho-RPS6 (S235/S236) DuoSet® IC ELISA specifically recognizes RPS6 dually phosphorylated at S235 and S236. Specificity was demonstrated using both peptide competition and cross-reactivity analysis.

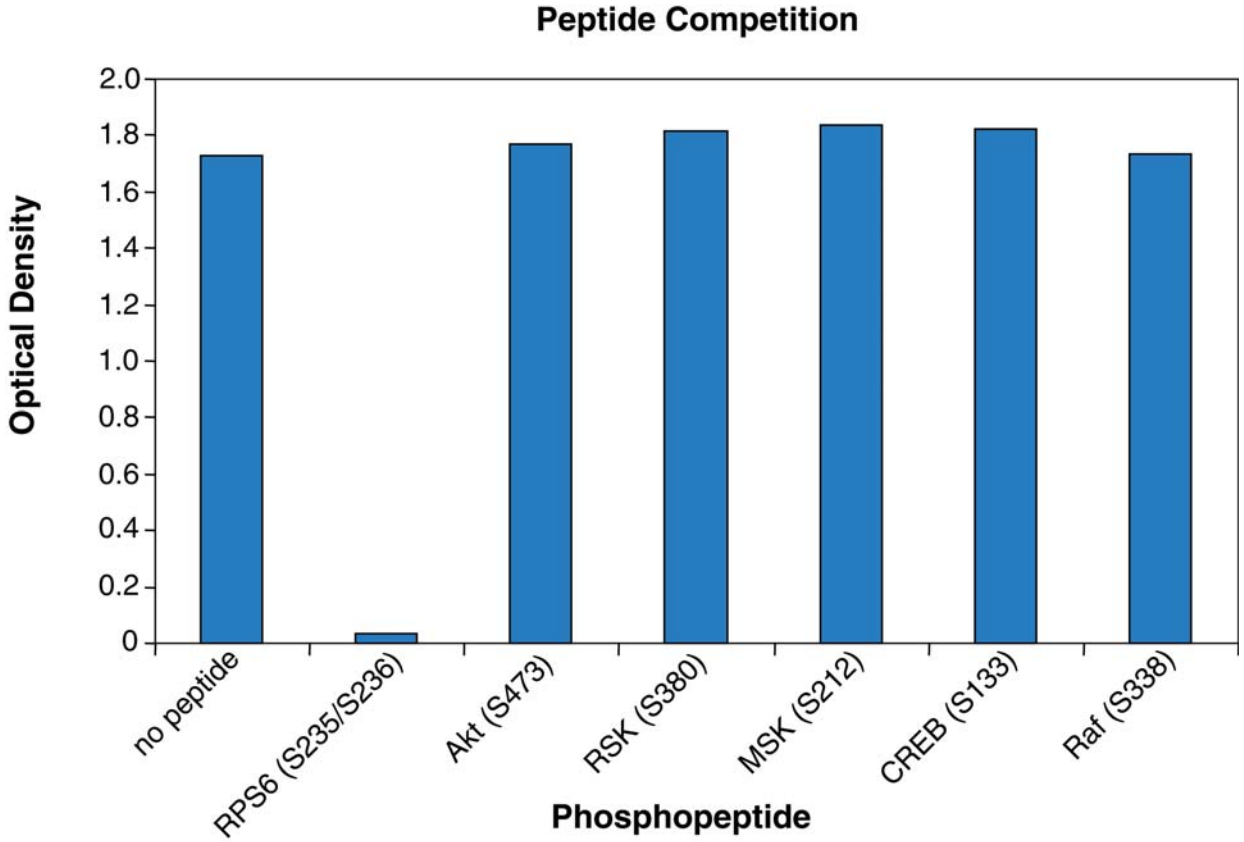


Figure 1: A lysate prepared from MCF-7 human breast cancer cells treated with 100 ng/mL of recombinant human IGF-I (R&D Systems, Catalog # 291-G1) for 20 minutes was analyzed with this DuoSet® IC ELISA. The Human/Mouse/Rat Phospho-RPS6 Detection Antibody was untreated (no peptide) or preincubated with phosphopeptide containing either the RPS6 (S235/S236), Akt (S473), RSK (S380), MSK (S212), CREB (S133), or Raf (S338) phosphorylation sites. Peptides were used at 40 ng/mL. Only the phosphopeptide containing the RPS6 S235/S236 phosphorylation sites blocked the signal, indicating that the DuoSet® IC ELISA is specific for RPS6 (S235/S236) phosphorylation.

Cross-reactivity experiments were performed with the Human/Mouse/Rat Phospho-RPS6 (S235/S236) DuoSet® IC ELISA to further determine specificity. Unphosphorylated recombinant human RPS6 was tested at 200 ng/mL and did not cross-react in the assay.

QUANTIFICATION

Amounts of phosphorylated human RPS6, as quantified by the Phospho-RPS6 (S235/S236) DuoSet® IC ELISA, are consistent with the relative amounts of phosphorylated RPS6 determined by qualitative Western blot analysis.

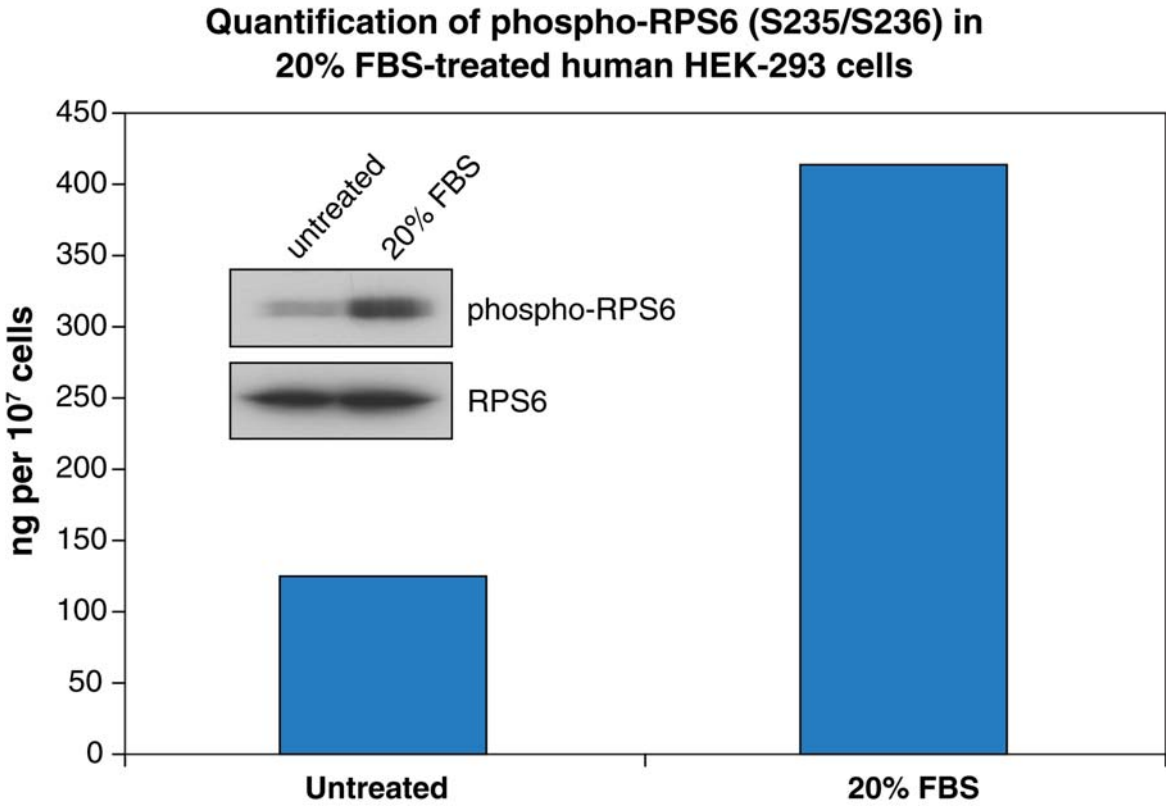


Figure 2: Lysates prepared from HEK-293 human embryonic kidney cells either untreated or treated with 20% FBS for 20 minutes were quantified with this DuoSet® IC ELISA. The same lysates were also immunoblotted (inset) with either anti-phospho-RPS6 (S235/S236) (phospho-RPS6) polyclonal antibody (R&D Systems, Catalog # AF3918) or anti-RPS6 monoclonal antibody (R&D Systems, Catalog # MAB5436). The DuoSet® IC ELISA results correlate well with the relative amounts of phosphorylated RPS6 detected by Western blot.

The Phospho-RPS6 (S235/S236) DuoSet® IC ELISA also quantifies phosphorylated RPS6 levels in mouse and rat cell lysates.

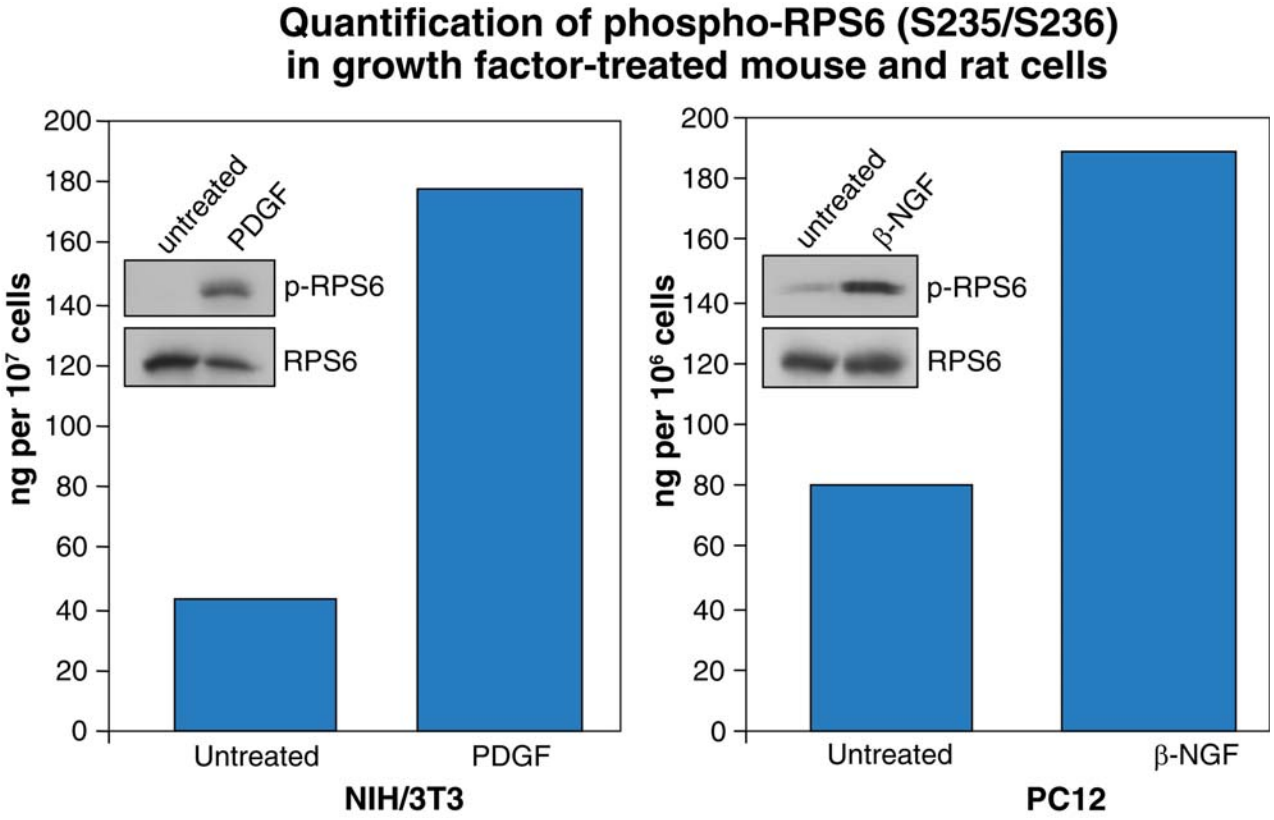


Figure 3: Lysates prepared from NIH-3T3 mouse embryonic fibroblast cells either untreated or treated with 10 ng/mL of human PDGF (R&D Systems, Catalog # 120-HD) for 5 minutes (left panel), and PC-12 rat adrenal pheochromocytoma cells either untreated or treated with 100 ng/mL of recombinant rat β-NGF (R&D Systems, Catalog # 556-NG) for 20 minutes (right panel) were quantified with this DuoSet® IC ELISA. The same lysates were also immunoblotted (inset) with either anti-phospho-RPS6 (S235/S236) (p-RPS6) or anti-RPS6 antibodies. The DuoSet® IC ELISA results correlate well with the relative amounts of phosphorylated RPS6 detected by Western blot.

PLATE LAYOUT

Use this plate layout to record standards and samples assayed.