DuoSet® IC

Human/Mouse/Rat Phospho-RSK (S380) Pan Specific

Catalog Number DYC889B-2
DYC889B-5
DYC889BE

For the development of sandwich ELISAs to measure phosphorylated RSK (S380) 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 pan specific phospho-RSK (S380) in cell lysates. An immobilized capture antibody specific for 90 kDa ribosomal protein S6 kinase 1 (RSK1), RSK2, RSK3, and RSK4 binds both phosphorylated and unphosphorylated protein. After washing away unbound material, a biotinylated detection antibody specific for RSK1 phosphorylated at S380, RSK2 phosphorylated at S386, RSK3 phosphorylated at S377, and RSK4 phosphorylated at S389, is used to detect only phosphorylated protein, utilizing a standard Streptavidin-HRP format.

MATERIALS PROVIDED & STORAGE CONDITIONS

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

DESCRIPTION	PART #	CATALOG # DYC889B-2	CATALOG # DYC889B-5	STORAGE OF OPENED/ RECONSTITUTED MATERIAL
Phospho-RSK (S380) Pan Specific Capture Antibody	843699	1 vial	2 vials	Store for up to 1 month at 2-8 °C or aliquot and store at ≤ -20 °C for up to 3 months in a manual defrost freezer.*
Phospho-RSK (S380) Pan Specific Detection Antibody	843700	1 vial	2 vials	
Phospho-RSK (S380) Pan Specific Standard	841846	3 vials	5 vials	**Use within one hour of reconstitution. Use a fresh standard for each assay.**
Streptavidin-HRP	890803	1 vial	1 vial	Store at 2-8 °C. **DO NOT FREEZE.**

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

DYC889B-2 contains sufficient materials to run ELISAs on at least two 96 well plates.†
DYC889B-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 # DYC889BE). 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 5.
- The recommended microplates, buffers, diluents, substrates, and solutions are used.

OTHER MATERIALS REQUIRED

- Aprotinin (Sigma # A6279)
- Leupeptin (Tocris # 1167)
- Pepstatin (Tocris # 1190)
- Phenylmethylsulfonylfluoride (PMSF) (Sigma # P7626)
- Sodium Azide (NaN_3) (Sigma # S2002)
- Sodium Fluoride (NaF) (Sigma # S6521)
- Sodium Orthovanadate (Na_3VO_4) (Sigma # S6508), activated
- Sodium Pyrophosphate ($Na_4P_2O_7$) (Sigma # P8010)
- Triton™ 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_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 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, 1 mM activated sodium orthovanadate, 2.5 mM sodium pyrophosphate, 10 µg/mL Leupeptin, 10 µg/mL Pepstatin, 100 µM PMSF, 3 µg/mL Aprotinin 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.

**Sample Diluent Concentrate 1 (5X) (R&D Systems, Catalog # DYC001), prepared as described in the DYC001 insert.

REAGENT PREPARATION

Bring all reagents to room temperature before use.

Phospho-RSK (S380) Pan Specific Capture Antibody (Part 843699) - Each vial contains 720 µg/mL of mouse anti-human RSK Pan Specific antibody when reconstituted with 200 µL of PBS.

Phospho-RSK (S380) Pan Specific Detection Antibody (Part 843700) - Each vial contains 14.4 µg/mL of biotinylated mouse anti-human phospho-RSK (S380) Pan Specific antibody when reconstituted with 1.0 mL of IC Diluent #1.

Phospho-RSK (S380) Pan Specific Standard (Part 841846) - 90 ng/mL of recombinant human phospho-RSK1 (S380) when reconstituted with 500 µL of IC Diluent #7. **Use within one hour after 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 dilutions and a high standard of 5000 pg/mL is recommended.

Streptavidin-HRP (Part 890803) - 1.0 mL of streptavidin conjugated to horseradish-peroxidase.

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. 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 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

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 5000 pg/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 400 ng/mL in IC Diluent #1. Prepare only as much Detection Antibody as required to run each assay. 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.

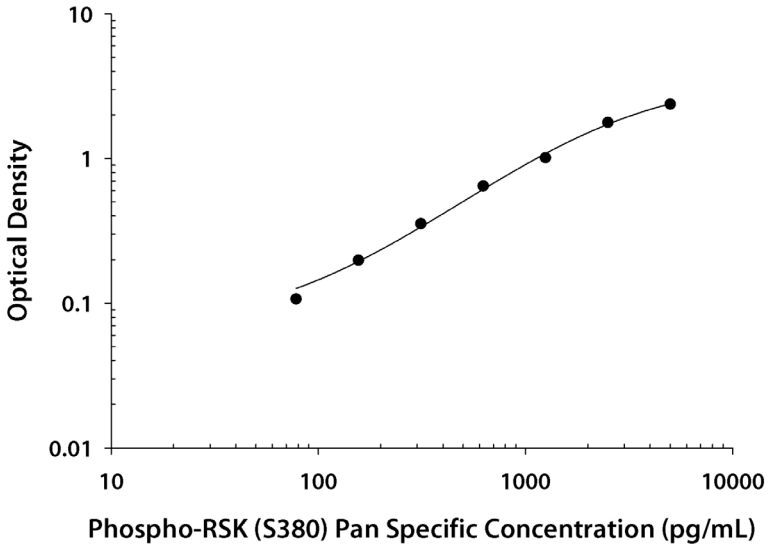
CALCULATION OF RESULTS

Average the duplicate readings for each standard and sample, 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 phospho-RSK (S380) Pan Specific 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-RSK (S380) Pan Specific 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-RSK (S380) Pan Specific DuoSet IC ELISA is calibrated against a highly purified *E. coli*-expressed recombinant human phospho-RSK1 (S380) produced at R&D Systems. Samples containing natural phospho-RSK (S380) showed linear dilution parallel to the standard curve obtained using the Phospho-RSK (S380) Pan Specific Standard. These results indicate that O.D. values from this DuoSet IC ELISA can be used to determine the relative concentration of Phospho-RSK (S380) in natural samples.

SPECIFICITY

The Phospho-RSK (S380) Pan Specific DuoSet IC ELISA specifically recognizes RSK phosphorylated at S380. Specificity was demonstrated by Western blot analysis of the protein bound by the capture antibody coated on the plate, cross-reactivity analysis, and a peptide competition.

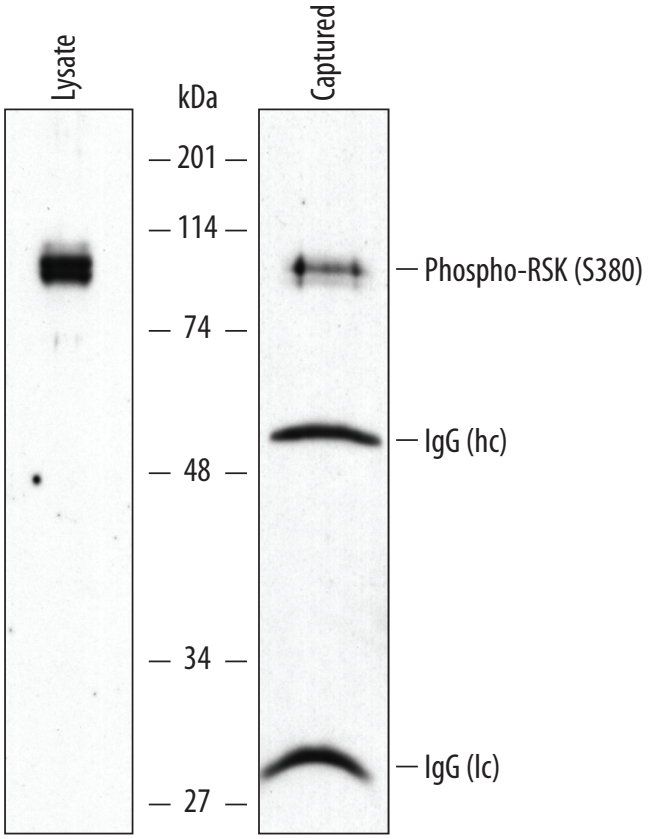


Figure 1: Lysate prepared from PC-12 rat adrenal pheochromocytoma cells treated with 100 ng/mL of rat β-NGF (R&D Systems, Catalog # 556-NG) for 10 minutes was incubated in wells coated with Phospho-RSK (S380) Pan Specific Capture Antibody. Unbound material was removed by washing and bound material was solubilized in SDS gel sample buffer. The same lysate and captured proteins were electrophoresed and transferred to a PVDF membrane. The captured protein was immunoblotted with Phospho-RSK (S380) Pan Specific Detection antibody and the lysate was immunoblotted with anti-phospho-RSK (S380) (R&D Systems, Catalog # AF889). In addition to IgG heavy chain (hc) and IgG light chain (lc), only one band corresponding to RSK phosphorylated at S380 was detected in the captured material.

Cross-reactivity experiments were performed with this DuoSet IC ELISA to further determine specificity. Unphosphorylated recombinant human (rh) RSK3 was assayed at 50 ng/mL and read 1200 pg/mL (2.4% cross-reactivity). Unphosphorylated rhRSK1, rhRSK2, rhMSK1, rhMSK2, and Phospho-RSK (S221) were assayed at 50 ng/mL and did not cross-react or interfere in the assay.

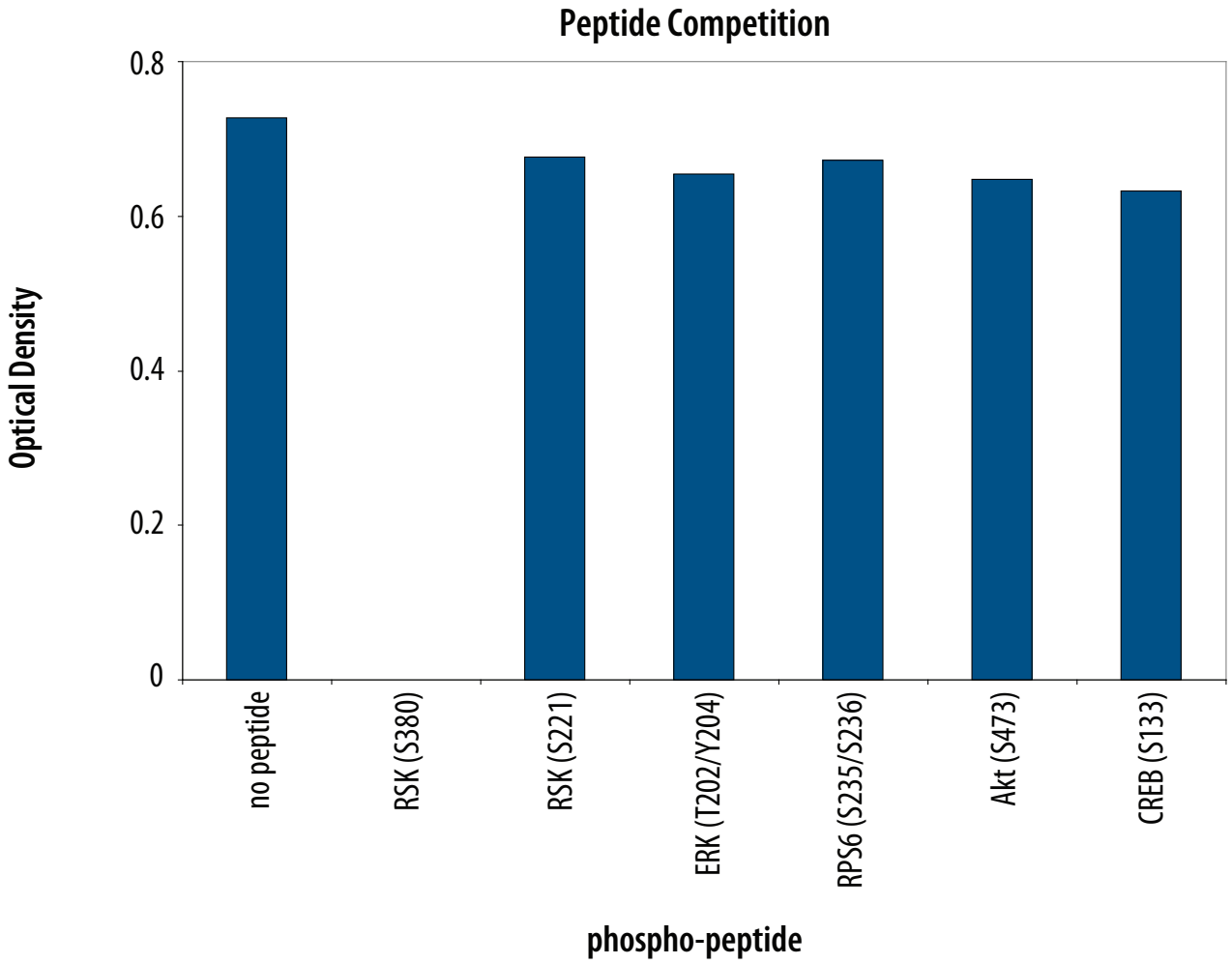


Figure 2: Lysate prepared from HeLa human cervical epithelial carcinoma cells treated with 200 nM phorbal 12-myristate 13-acetate (PMA) for 20 minutes was analyzed with this ELISA. The Phospho-RSK (S380) Pan Specific Detection Antibody was untreated (no peptide) or preincubated with phospho peptides containing RSK (S380), RSK (S221), ERK (T202/Y204), RPS6 (S235/S236), Akt (S473), or Creb (S133). Peptides were used at 40 ng/mL. Only the peptide containing the RSK (S380) phosphorylation site blocked the signal, indicating that this ELISA is specific for RSK phosphorylated at S380.

QUANTIFICATION

Amounts of phosphorylated RSK, as quantified by the Phospho-RSK (S380) Pan Specific DuoSet IC ELISA, are consistent with the relative amounts of phosphorylated RSK determined by qualitative Western blot analysis.

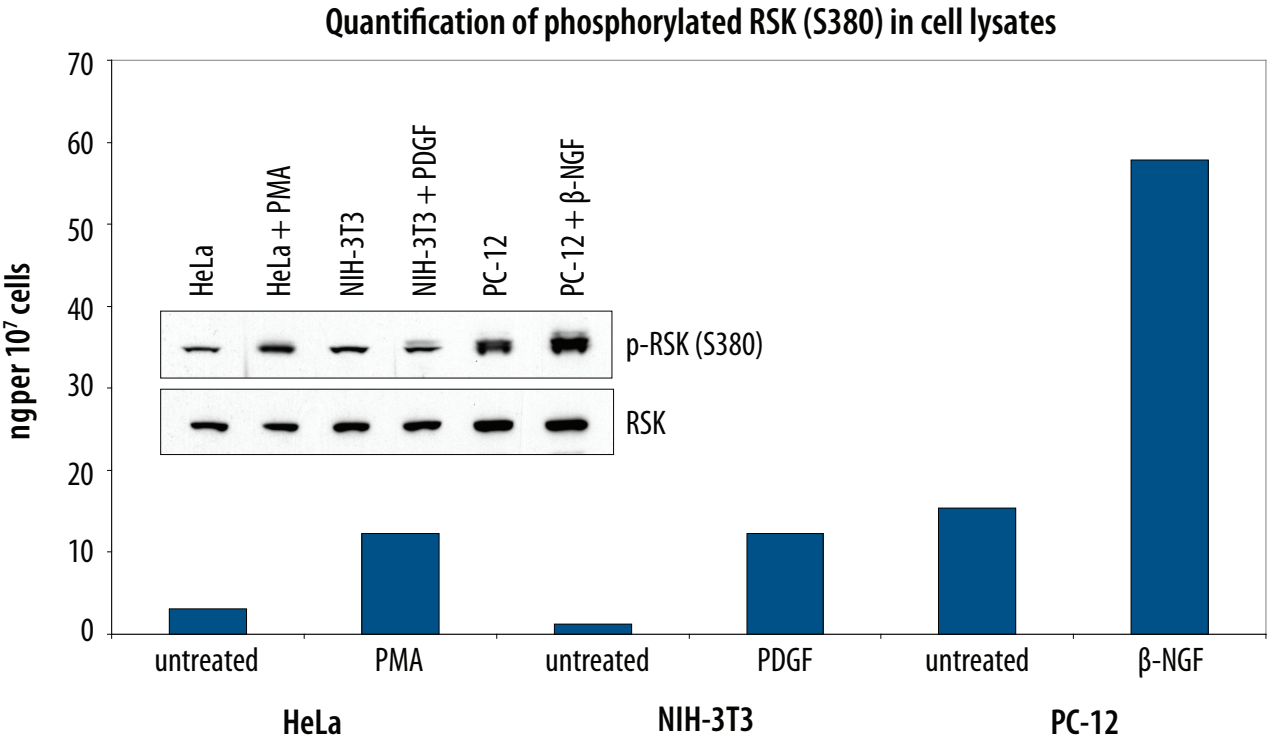


Figure 3: Lysates prepared from HeLa cells were untreated or treated with 200 nM PMA for 20 minutes, NIH-3T3 mouse embryonic fibroblast cells treated with 10 ng/mL of human PDGF (R&D Systems, Catalog # 120-HD) for 10 minutes, and PC-12 cells treated with 100 ng/mL of rat β-NGF for 10 minutes were quantified with this DuoSet IC ELISA. The same lysates were also immunoblotted (inset) with either anti-phospho-RSK (S380) (R&D Systems, Catalog # AF889) or anti-pan specific RSK (R&D Systems, Catalog # AF2056) antibodies. The DuoSet IC ELISA results correlated well with the relative amounts of phosphorylated RSK detected by Western blot. The immunoblot with anti-RSK antibody indicates that total levels of RSK remained constant.

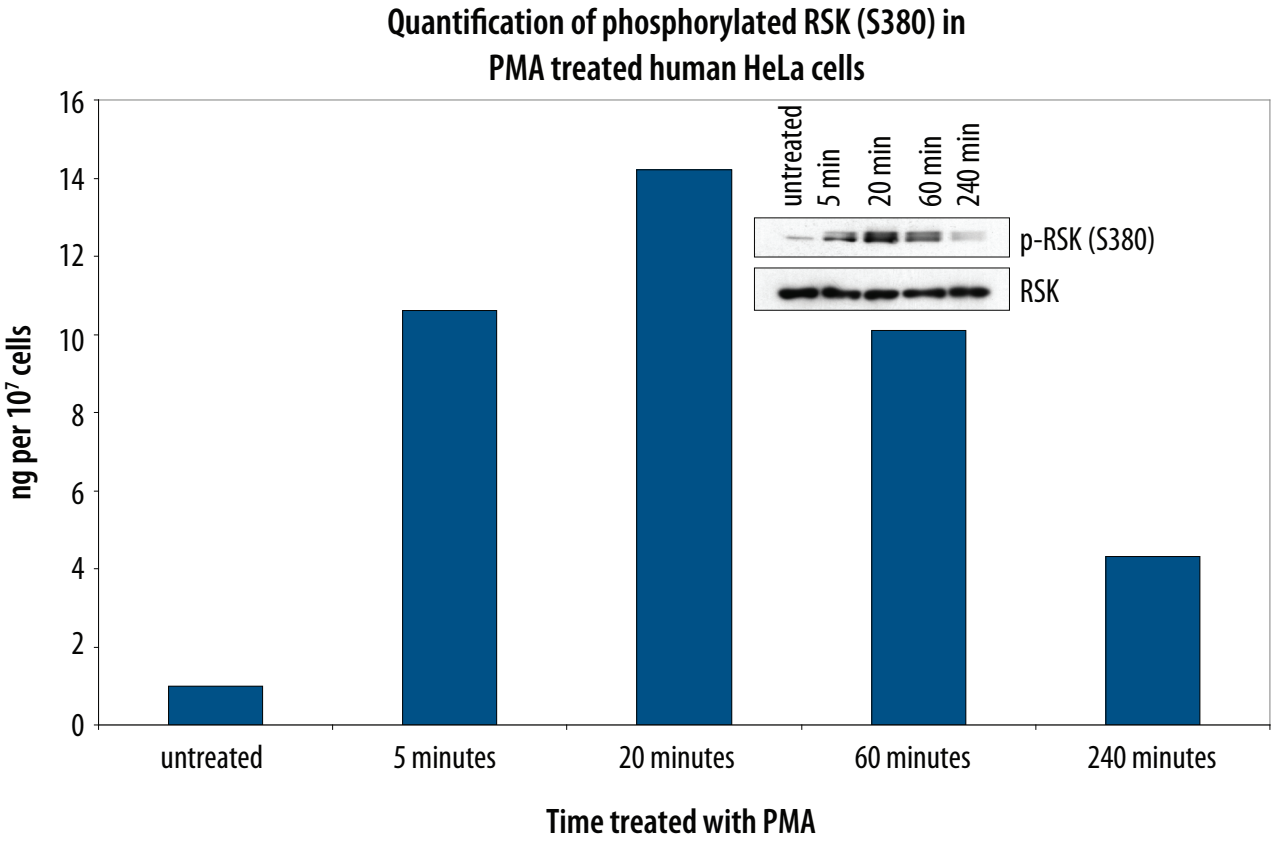


Figure 4: Lysates prepared from HeLa cells were untreated or treated with 200 mM PMA for the indicated times were quantified with this DuoSet IC ELISA. The same lyates were also immunoblotted (inset) with either anti-phospho-RSK (S380) or anti-pan RSK. The DuoSet IC ELISA results correlate well with the amounts of phosphorylated RSK detected by Western blot. The immunoblot with anti-pan RSK antibody indicates that total levels of RSK remained constant.

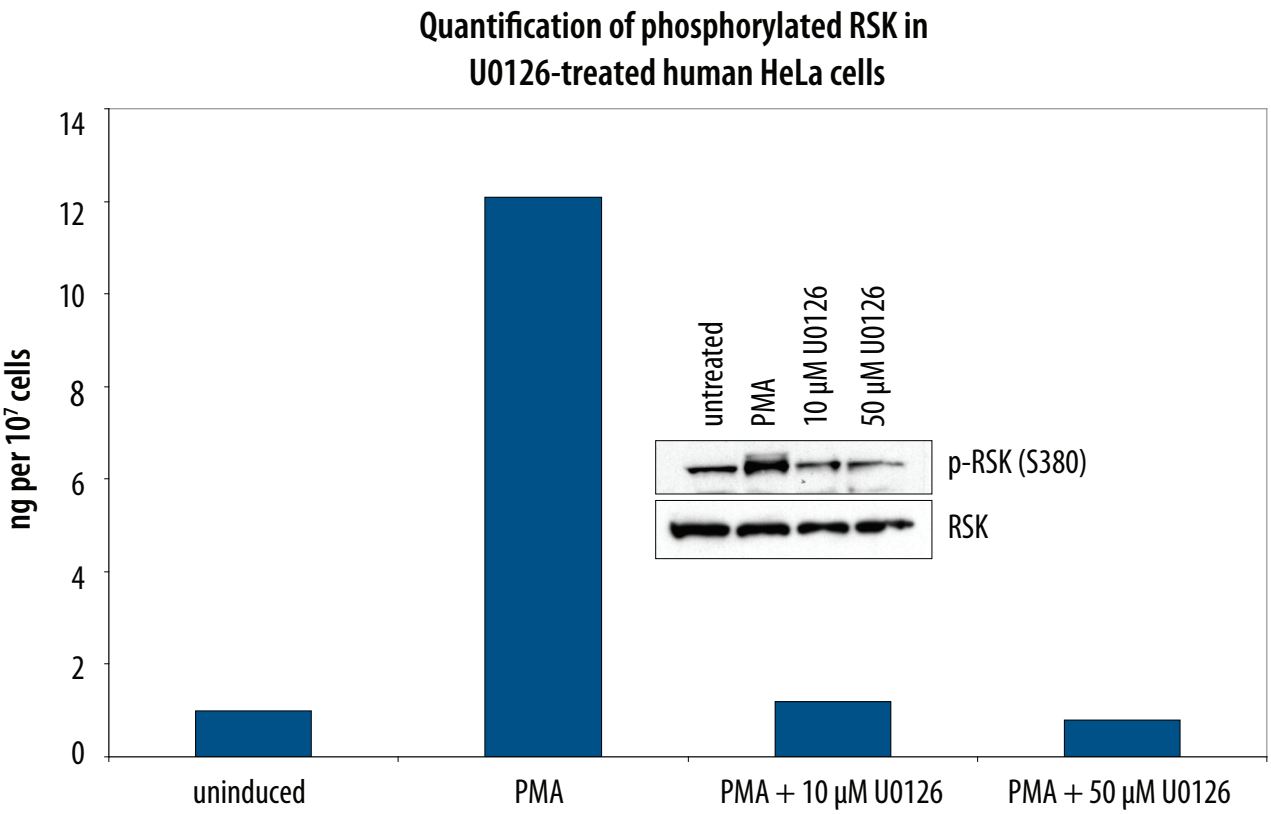
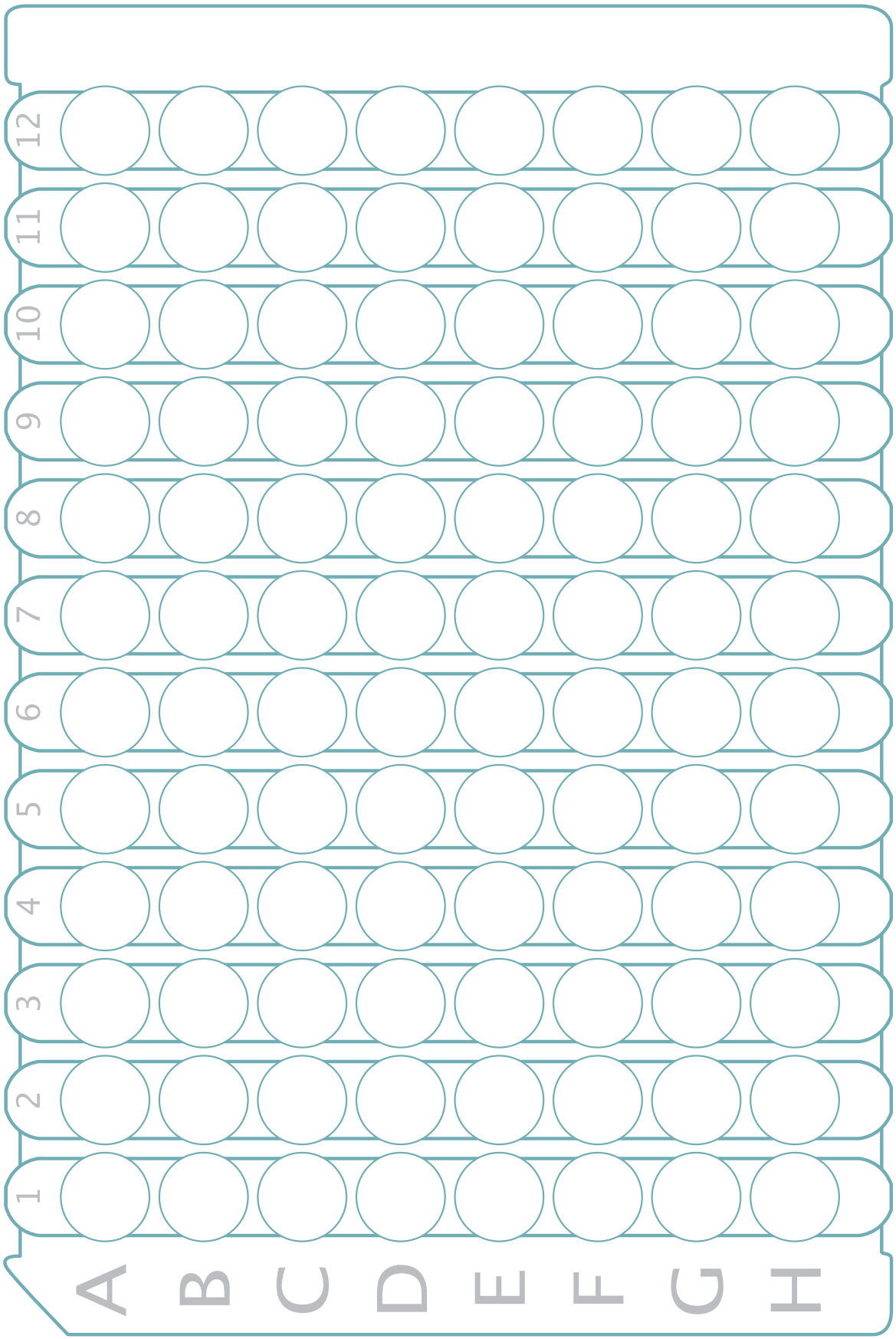


Figure 5: HeLa cells were incubated with no additions or with 200 nM PMA for 20 minutes, either with or without 10 or 50 µM U0126. Cells were lysed and RSK phosphorylated at S380 was quantified with this DuoSet IC ELISA. The same lysates were also immunoblotted (inset) with either anti-phospho-RSK (S380) or anti-pan RSK antibodies. The DuoSet IC ELISA results correlate well with the amounts of phosphorylated RSK detected by Western blot. The immunoblot with anti-pan RSK antibody indicates that total levels of RSK remained constant.

The quantification of phosphorylated RSK (S380) with this DuoSet IC ELISA was also determined in cells pretreated with the MEK1/2 inhibitor U0126 (Tocris, Catalog # 1144), which indirectly blocks the phosphorylation of RSK at S380.

PLATE LAYOUT

Use this plate layout to record standards and samples assayed.



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