DuoSet® IC

Human Phospho-PRAS40 (T246)

Catalog Number DYC6890-2
DYC6890-5
DYC6890E

For the development of sandwich ELISAs to measure 40 kDa Proline-rich Akt1 Substrate (PRAS40) phosphorylated at T246 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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MANUFACTURED AND DISTRIBUTED BY:

USA & Canada | R&D Systems, Inc.
614 McKinley Place NE, Minneapolis, MN 55413, USA
TEL: (800) 343-7475 (612) 379-2956 FAX: (612) 656-4400
E-MAIL: info@RnDSystems.com

DISTRIBUTED BY:

UK & Europe | R&D Systems Europe, Ltd.
19 Barton Lane, Abingdon Science Park, Abingdon OX14 3NB, UK
TEL: +44 (0)1235 529449 FAX: +44 (0)1235 533420
E-MAIL: info@RnDSystems.co.uk

China | R&D Systems China Co., Ltd.
24A1 Hua Min Empire Plaza, 726 West Yan An Road, Shanghai PRC 200050
TEL: +86 (21) 52380373 FAX: +86 (21) 52371001
E-MAIL: info@RnDSystemsChina.com.cn

PRINCIPLE OF THE ASSAY

This DuoSet® IC ELISA contains the basic components required for the development of sandwich ELISAs to measure 40 kDa Proline-rich Akt1 Substrate (PRAS40) phosphorylated at T246 in cell lysates. An immobilized capture antibody specific for PRAS40 binds both phosphorylated and unphosphorylated protein. After washing away unbound material, a biotinylated detection antibody recognizing PRAS40 phosphorylated at T246 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 # DYC6890-2	CATALOG # DYC6890-5	STORAGE OF OPENED/ RECONSTITUTED MATERIAL
Phospho-PRAS40 (T246) Capture Antibody	843171	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-PRAS40 (T246) Detection Antibody	843172	1 vial	2 vials	
Phospho-PRAS40 (T246) Standard	843173	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.

DYC6890-2 contains sufficient materials to run ELISAs on at least two 96 well plates.†
DYC6890-5 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 # DYC6890E). Economy Packs contain sufficient materials to run ELISAs on 15 microplates. Please refer to the literature accompanying your order.

† 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 # 201154)
- 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 (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 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 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, 25 μg/mL Leupeptin, 25 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_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-PRAS40 (T246) Capture Antibody (Part 843171) - Each vial contains 1080 µg/mL of mouse anti-human PRAS40 antibody when reconstituted with 200 µL of PBS.

Phospho-PRAS40 (T246) Detection Antibody (Part 843172) - Each vial contains 18 µg/mL of biotinylated rabbit anti-human phospho-PRAS40 (T246) antibody when reconstituted with 1.0 mL of IC Diluent #1.

Phospho-PRAS40 (T246) Standard (Part 843173) - Each vial contains 750 ng/mL of recombinant human phospho-PRAS40 (T246) when reconstituted with 500 µL of IC Diluent #7. Immediately before use, 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 60,000 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 6.0 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 #3 per well. Use IC Diluent #3 as the zero standard. Cover with a plate sealer and incubate for 2 hours at room temperature.

   Note: *A seven point standard curve using 2-fold serial dilutions and a high standard of 60,000 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 500 ng/mL in IC Diluent #1 before use. Add 100 µL of the diluted Detection Antibody to each well. Cover with a new plate sealer and incubate for 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.

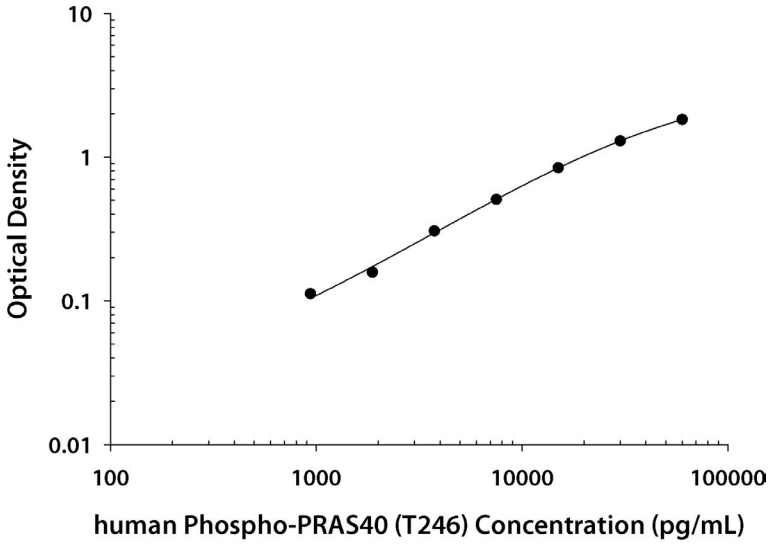
CALCULATION OF RESULTS

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-PRAS40 (T246) 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 Phospho-PRAS40 (T246) 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 Phospho-PRAS40 (T246) DuoSet IC ELISA is calibrated against a highly purified *E. coli*-expressed recombinant human phospho-PRAS40 (T246) produced at R&D Systems. Samples containing natural phospho-PRAS40 (T246) showed linear dilution parallel to the standard curve obtained using the Phospho-PRAS40 (T246) Standard. These results indicate that O.D. values from this DuoSet IC ELISA can be used to determine the relative concentration of phospho-PRAS40 (T246) in natural samples.

SPECIFICITY

The Human Phospho-PRAS40 (T246) DuoSet IC ELISA specifically recognizes PRAS40 phoshorylated at T246. 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.

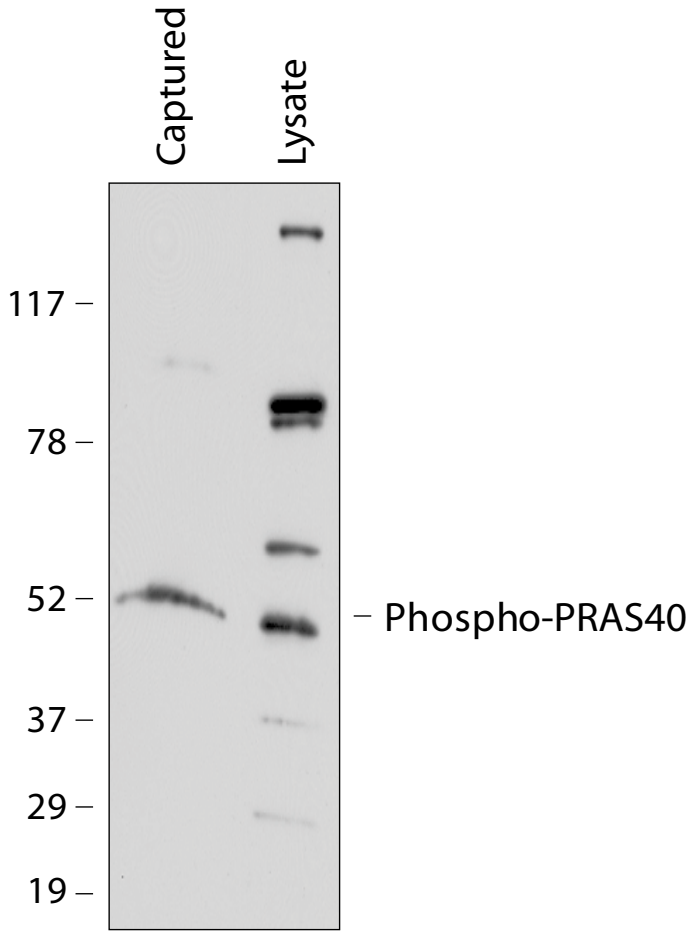


Figure 1: Lysate prepared from MCF-7 human breast cancer cells treated with 100 ng/mL of recombinant human IGF-I (R&D Systems, Catalog # 291-G1) for 20 minutes was incubated in wells coated with Phospho-PRAS40 (T246) Capture Antibody. Unbound material was removed by washing and bound material was solubilized in SDS gel sample buffer. The same lysate and captured proteins were electrophoresed, transferred to a PVDF membrane and immunoblotted with Phospho-PRAS40 (T246) Detection Antibody. Only a band corresponding to the phosphorylated PRAS40 was detected in the captured material.

Cross-reactivity experiments were performed with this DuoSet IC ELISA to further determine specificity. Recombinant human (rh) Akt phosphorylated at S473, rhp70 S6 Kinase phosphorylated at T389, and rhCREB phosphorylated at S133 were assayed at 600 ng/mL and did not cross-react or interfere in the assay. Recombinant human PRAS40 phosphorylated at S183 was assayed at 600,000 pg/mL and read 4980 pg/mL (0.83% cross-reactivity).

The specificity of this kit was also demonstrated using peptide competition.

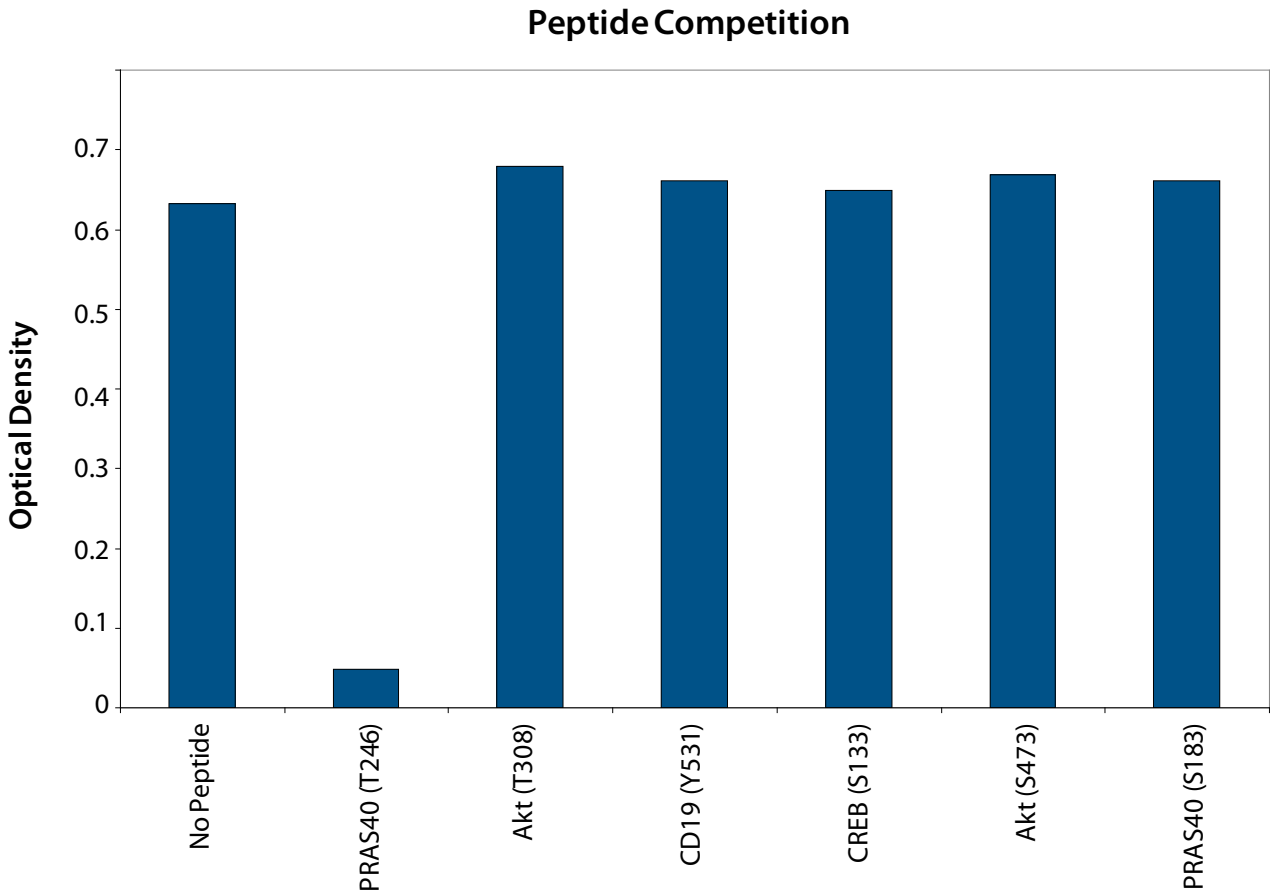


Figure 2: Lysate prepared from MCF-7 human breast cancer cells treated with 100 ng/mL of rhIGF-I for 20 minutes was analyzed with this ELISA. The Phospho-PRAS40 (T246) Detection Antibody was untreated (no peptide) or preincubated with phosphopeptide containing either the PRAS40 (T246), Akt (T308), CD19 (Y531), CREB (S133), Akt (S473), or PRAS40 (S183) phosphorylation sites. Peptides were used at 40 ng/mL. Only the phospho-peptide containing the PRAS40 T246 site blocked the signal, indicating that the DuoSet IC ELISA is specific for PRAS40 (T246) phosphorylation.

QUANTIFICATION

Amounts of phosphorylated PRAS40, as quantified by the Human Phospho-PRAS40 (T246) DuoSet IC ELISA, are consistent with the relative amounts of phosphorylated PRAS40 determined by qualitative Western blot analysis.

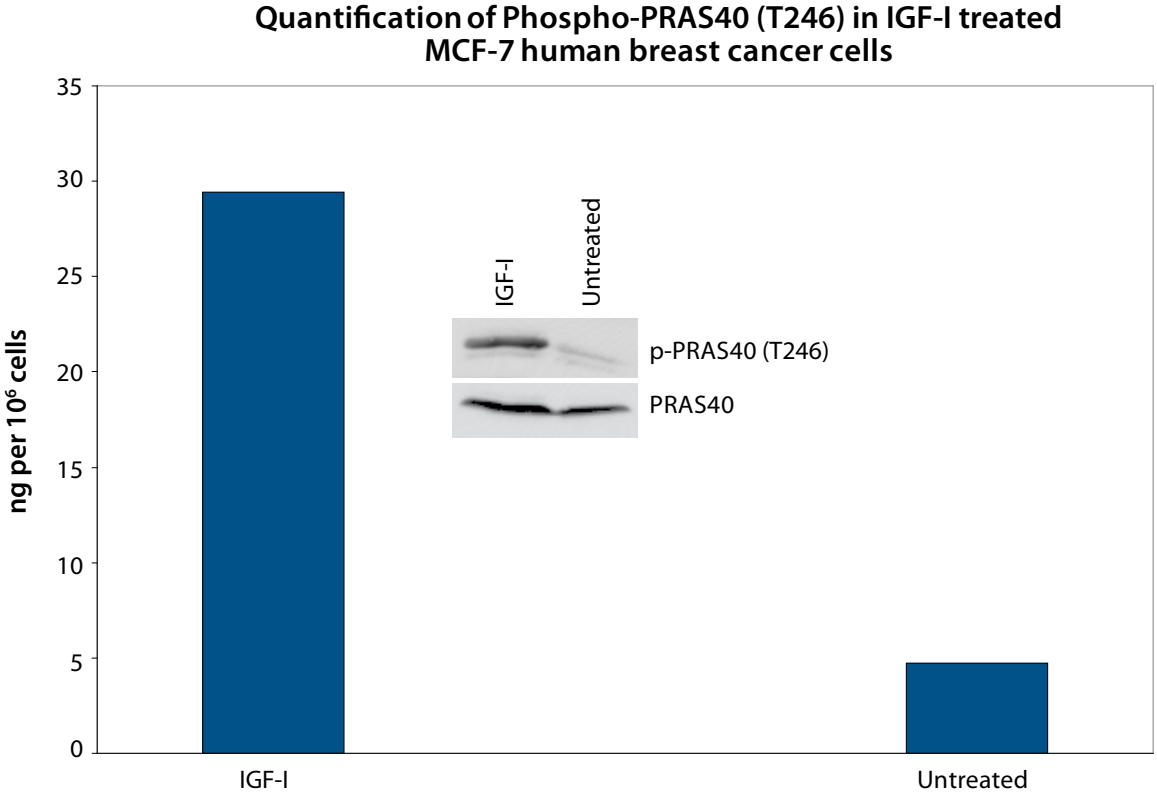


Figure 3: Lysates prepared from MCF-7 human breast cancer cells treated with 100 ng/mL of rhIGF-I for 20 minutes were quantified with this DuoSet IC ELISA. The same lysates were also immunoblotted (inset) with either anti-phospho-PRAS40 (T246) polyclonal antibody or anti-PRAS40 monoclonal antibody (R&D Systems, Catalog # MAB6408). The DuoSet IC ELISA results correlate well with the relative amounts of phosphorylated PRAS40 (T246) detected by Western blot. The immunoblot with anti-PRAS40 antibody indicates that total levels of PRAS40 remained constant during the induction with IGF-I.

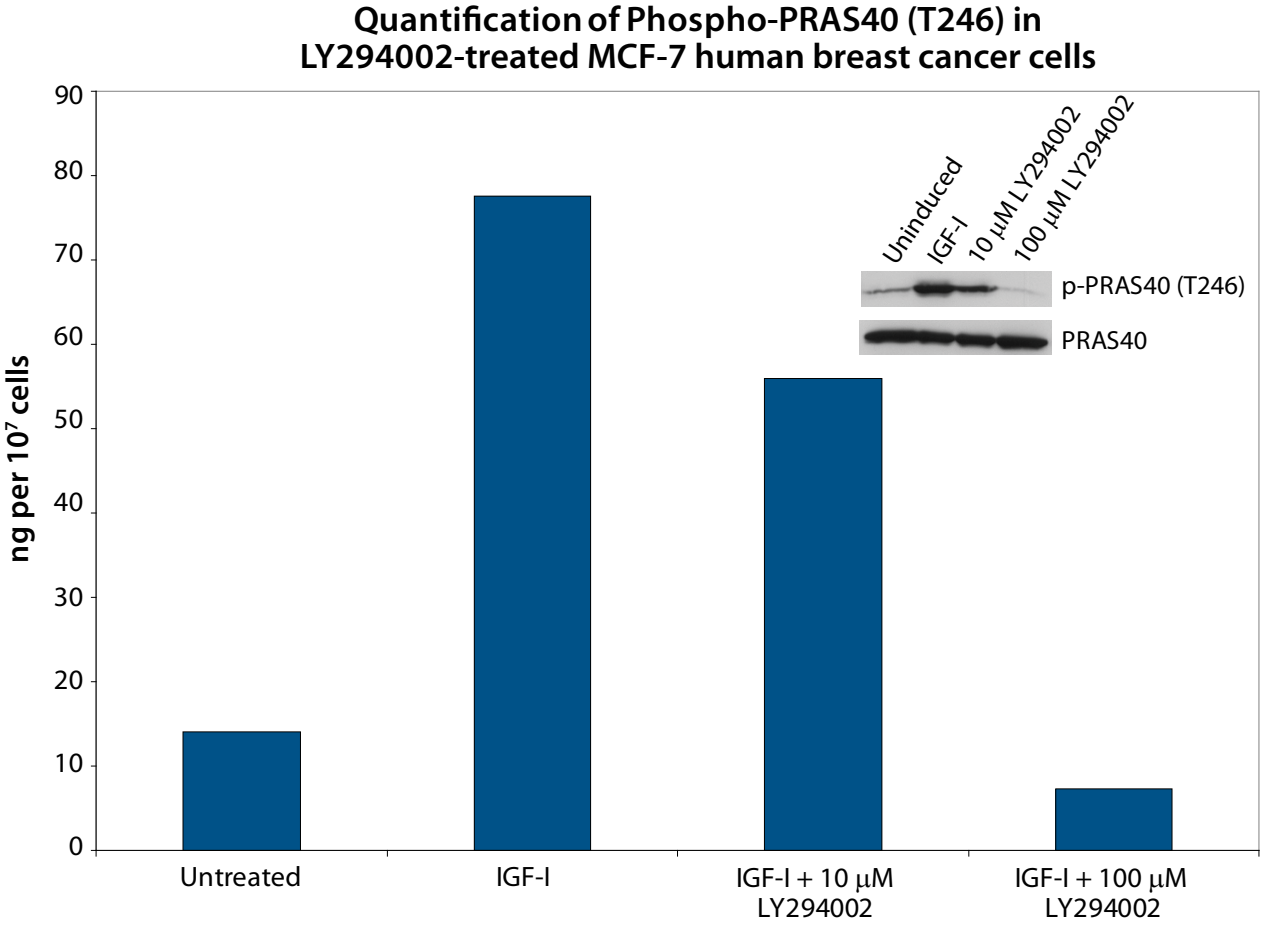


Figure 4: MCF-7 human breast cancer cells were incubated with no additions or with 100 ng/mL of IGF-I for 20 minutes, either with or without the indicated amounts of the PI3K kinase inhibitor L294002. Cells were lysed and T246 phosphorylated PRAS40 was quantified with this DuoSet IC ELISA. The same lysates were also immunoblotted (inset) with either anti-phospho-PRAS40 or anti-PRAS40 antibodies. The DuoSet IC ELISA results correlate well with the relative amounts of T246 phosphorylated PRAS40 detected by Western blot. The immunoblot with anti-PRAS40 antibody indicates that total levels of PRAS40 remained constant during the various treatments.