

DuoSet[®] IC

Human/Mouse/Rat Phospho-Akt (Pan) (S473)

Catalog Number DYC887-2

DYC887-5

For the development of sandwich ELISAs to measure Akt phosphorylated at S473 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 Akt phosphorylated at S473 in cell lysates. An immobilized capture antibody specific for Akt1, Akt2, and Akt3 (also known as PKB α , PKB β , and PKB γ) binds both phosphorylated and unphosphorylated protein. After washing away unbound material, a biotinylated detection antibody specific for Akt1 phosphorylated at S473, Akt2 phosphorylated at S474, and Akt3 phosphorylated at S472, is used to detect only 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.

Description	Part #	Storage Conditions	Vials Provided	
			Cat. # DYC887-2	Cat. # DYC887-5
Phospho-Akt (Pan) (S473) Capture Antibody	841692	2 - 8° C	1	2
Phospho-Akt (Pan) (S473) Detection Antibody	841693	2 - 8° C	1	2
Phospho-Akt (Pan) (S473) Standard	841694	2 - 8° C	3	5
Streptavidin-HRP	890803	2 - 8° C	1	1

DYC887-2 contains sufficient materials to run ELISAs on at least two 96 well plates.*

DYC887-5 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_3) (Sigma # S2002)
- Sodium fluoride (NaF) (Sigma # 201154)
- Sodium orthovanadate (Na_3VO_4) (Sigma # S6508), activated
- Sodium pyrophosphate ($\text{Na}_4\text{P}_2\text{O}_7$) (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)
- Squirrt 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* in PBS (pH 7.2 - 7.4) with 0.05% NaN_3 .

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 $\mu\text{g}/\text{mL}$ Leupeptin, 10 $\mu\text{g}/\text{mL}$ Pepstatin, 100 μM PMSF, 3 $\mu\text{g}/\text{mL}$ 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 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 Union Carbide.
Tween is a registered trademark of ICI Americas.*

REAGENT PREPARATION

Bring all reagents to room temperature before use.

Phospho-Akt (Pan) (S473) Capture Antibody (Part 841692) - Each vial contains 360 µg/mL of mouse anti-human Akt (Pan) 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.*

Phospho-Akt (Pan) (S473) Detection Antibody (Part 841693) - Each vial contains 18 µg/mL of biotinylated rabbit anti-human phospho-Akt (Pan) (S473) 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.*

Phospho-Akt (Pan) (S473) Standard (Part 841694) - Each vial contains 490 ng/mL of recombinant human phospho-Akt (S473) 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 20,000 pg/mL is recommended.

Streptavidin-HRP (Part 890803) - 1.0 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 1×10^7 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 eye, hand, face, and clothing protection when using this material.

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 2.0 $\mu\text{g}/\text{mL}$ 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 2 hours at room temperature.
Note: *A seven point standard curve using 2-fold serial dilutions and a high standard of 20,000 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 0.5 $\mu\text{g}/\text{mL}$ in IC Diluent #1 before use. 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.

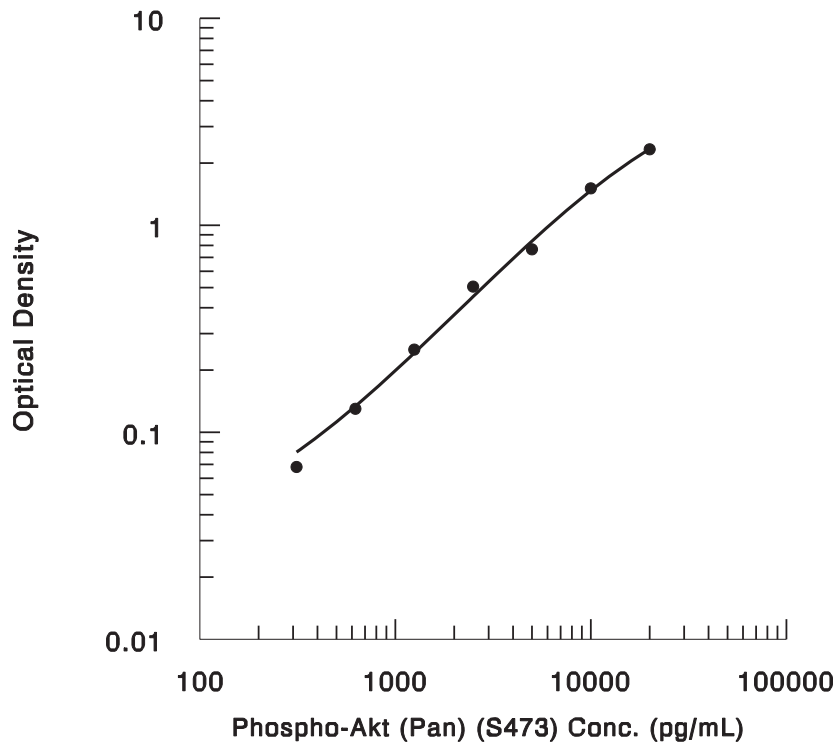
CALCULATION OF RESULTS

Average the duplicate readings for each standard and sample then subtract the average zero standard optical density. 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-Akt (Pan) (S473) 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-Akt (Pan) (S473) 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 *Sf* 21-expressed recombinant human phospho-Akt (S473) produced at R&D Systems.

SPECIFICITY

The Human/Mouse/Rat Phospho-Akt (Pan) (S473) DuoSet IC ELISA specifically recognizes Akt family members phosphorylated at sites corresponding to S473 of Akt1. Specificity was demonstrated by Western blot analysis of the protein bound by the capture antibody supplied in the ELISA.

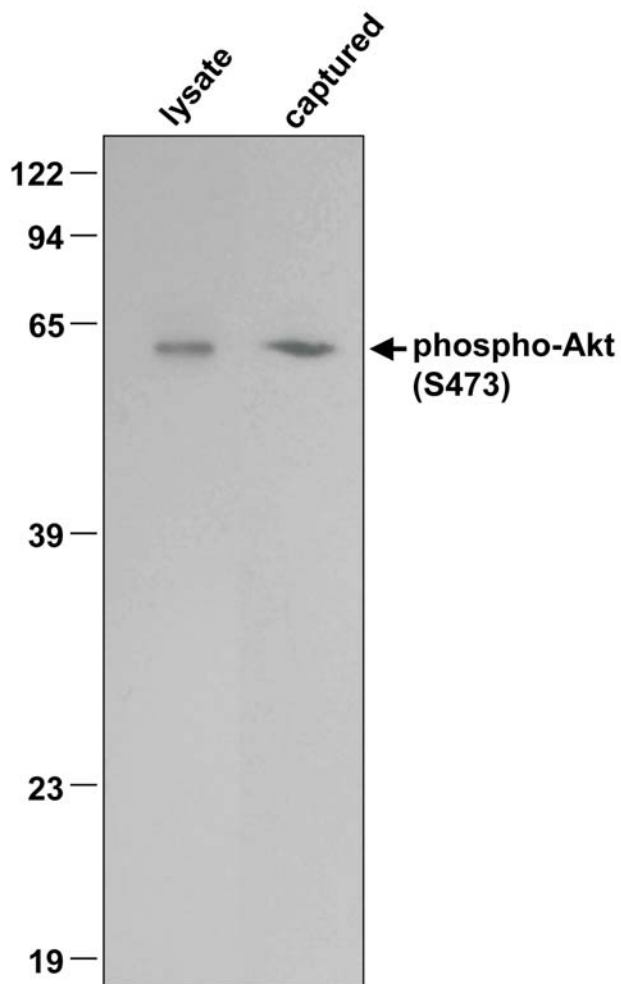


Figure 1: Lysates prepared from human MCF-7 cells treated with 100 ng/mL of human IGF-I (R&D Systems, Catalog # 291-G1) for 20 minutes were incubated in wells coated with Phospho-Akt (Pan) (S473) Capture Antibody. Unbound material was removed by washing and bound material was solubilized in SDS gel sample buffer. The same lysate and captured proteins were electrophoresed, transferred to an Immobilon-P membrane (Millipore) and immunoblotted with Phospho-Akt (Pan) (S473) Detection Antibody. Only one band corresponding to Akt phosphorylated at S473 was detected in captured material.

To further determine specificity, unphosphorylated recombinant human Akt1 and Akt3 were assayed at 200 ng/mL and read 1228 pg/mL (6% cross-reactivity) and 402 pg/mL (2% cross-reactivity), respectively. Unphosphorylated recombinant human Akt2 was assayed at 200 ng/mL and did not cross-react or interfere in the assay.

QUANTIFICATION

Amounts of human phosphorylated Akt (S473), as quantified by the Human/Mouse/Rat Phospho-Akt (Pan) (S473) DuoSet IC ELISA, are consistent with the amounts of phosphorylated Akt determined by qualitative Western blot analysis.

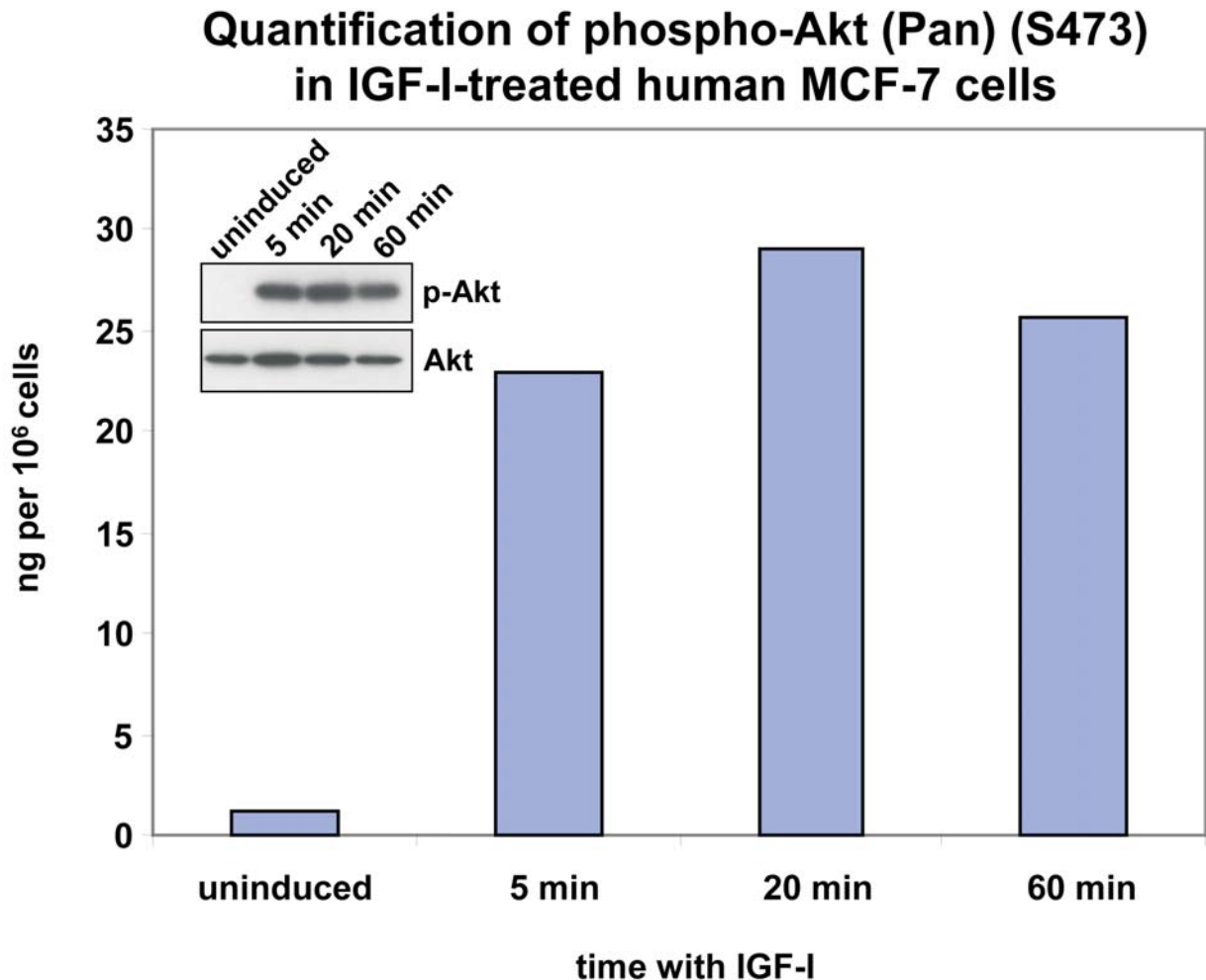


Figure 2: Lysates prepared from human MCF-7 cells induced with 100 ng/mL of IGF-I for the indicated times were quantified with this DuoSet IC ELISA. The same lysates were also immunoblotted (inset) with either anti-phospho-Akt (S473) (p-Akt) (R&D Systems, Catalog # AF887) or anti-Akt (Pan) (R&D Systems, Catalog # MAB2055) antibodies. The DuoSet IC ELISA results correlate well with the amounts of phosphorylated Akt detected by Western blot. The immunoblot with anti-Akt (Pan) antibody indicates that total levels of Akt remained constant during the induction with IGF-I.

The quantification of phosphorylated Akt (S473) with this DuoSet IC ELISA was also determined using cells pretreated with the selective phosphatidylinositol 3-kinase (PI3 K) inhibitor LY294002 (Tocris, Catalog # 1130), which indirectly blocks the phosphorylation of Akt.

Quantification of phospho-Akt (Pan) (S473) in LY294002-treated human MCF-7 cells

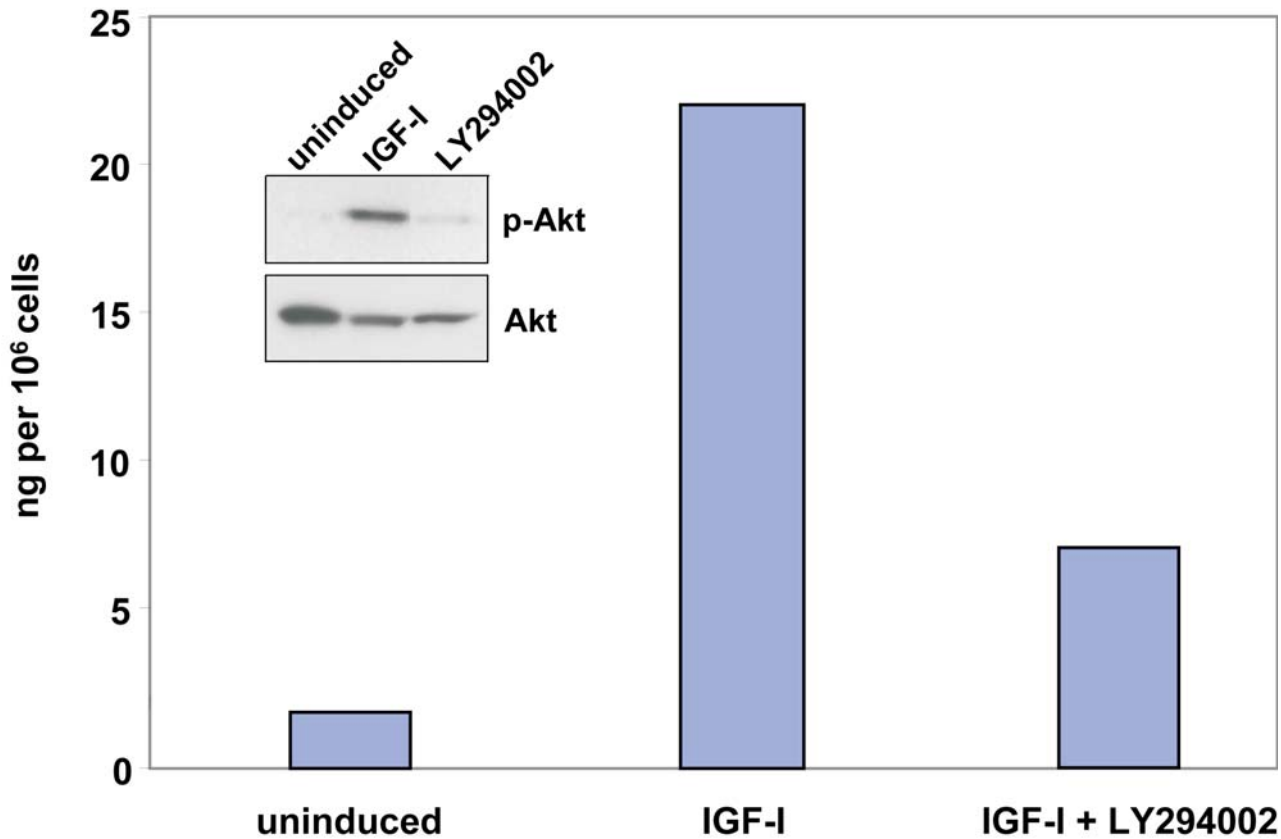


Figure 3: MCF-7 cells were incubated with no additions or with 100 ng/mL of IGF-I for 20 minutes, either with or without 100 μ M LY294002. Cells were lysed and phosphorylated Akt was quantified with this DuoSet IC ELISA. The same lysates were also immunoblotted (inset) with either anti-phospho-Akt (S473) (p-Akt) or anti-Akt (Pan) antibodies. The DuoSet IC ELISA results correlate well with the amounts of phosphorylated Akt detected by Western blot. The immunoblot with anti-Akt (Pan) antibody indicates that total levels of Akt remained constant during the various treatments.

The Human/Mouse/Rat Phospho-Akt (Pan) (S473) DuoSet IC ELISA also quantifies phosphorylated Akt levels in mouse and rat cell lysates.

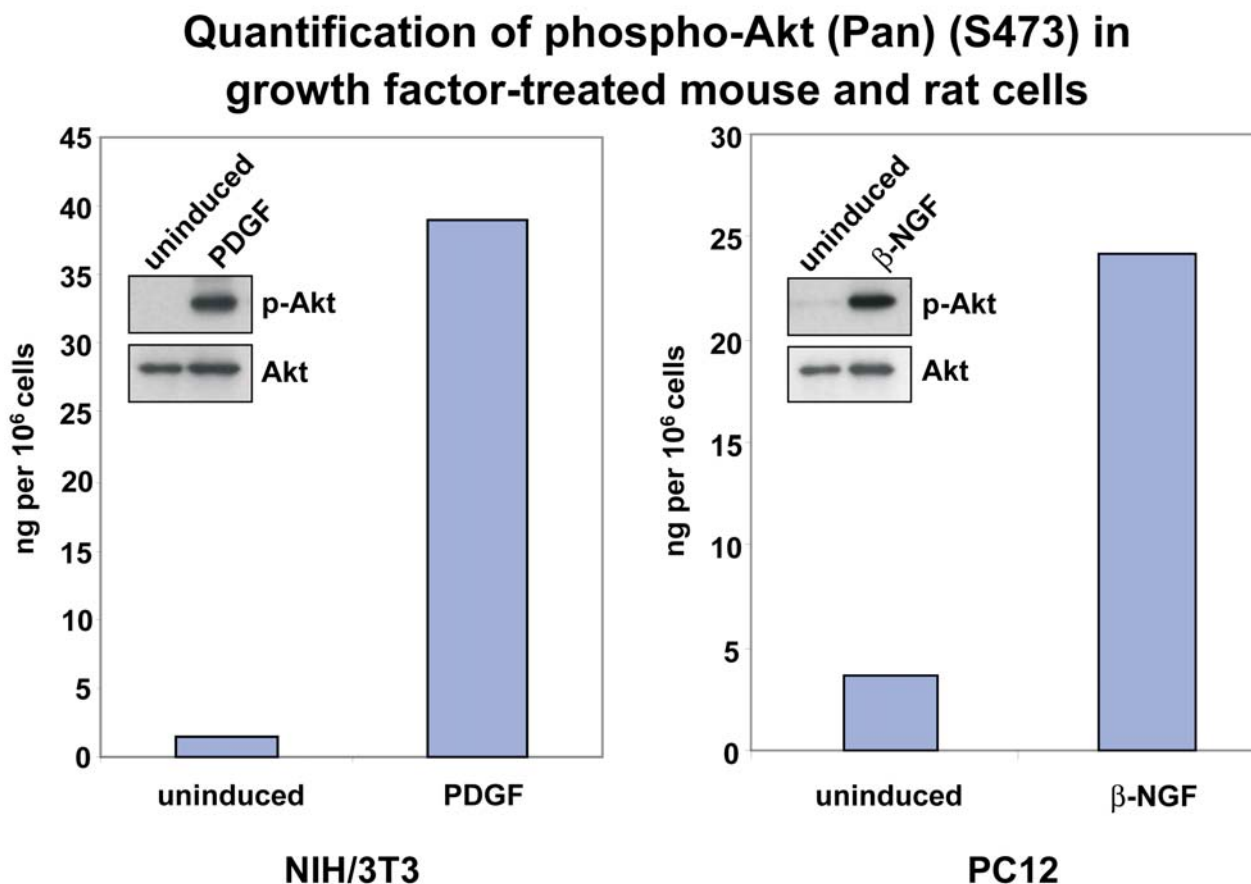


Figure 4: Lysates prepared from mouse NIH/3T3 cells either uninduced or induced with 10 ng/mL of human PDGF (R&D Systems, Catalog # 120-HD) for 5 minutes (left panel), and rat PC12 cells either uninduced or induced with 100 ng/mL of recombinant rat β-NGF (R&D Systems, Catalog # 556-NG) for 10 minutes (right panel) were quantified with this DuoSet IC ELISA. The same lysates were also immunoblotted (inset) with either anti-phospho-Akt (S473) (p-Akt) or anti-Akt (Pan) antibodies. The DuoSet IC ELISA results correlate well with the amounts of phosphorylated Akt detected by Western blot. The immunoblot with anti-Akt (Pan) antibody indicates that total levels of Akt remained constant during the growth factor inductions.