# Surveyor™ IC

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

Catalog Number SUV887

**For the quantitative determination of 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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## INTRODUCTION

The serine/threonine kinase Akt, also known as protein kinase B (PKB), is a central player in such diverse cellular processes as glucose uptake, cell cycle progression, and apoptosis. In mammals, three highly homologous members define the Akt family: Akt1 (PKB$\alpha$) (1), Akt2 (PKB$\beta$) (2), and Akt3 (PKB$\gamma$) (3). Akt1 is the most ubiquitously expressed member, while Akt2 is expressed predominantly in insulin target tissues such as liver, skeletal muscle, and fat. Akt3 is expressed most selectively, in brain and testis (4). All three Akts share a conserved structure: an amino-terminal pleckstrin homology domain to interact with membrane phosphoinositides; a central kinase domain; and a carboxyl-terminal regulatory domain that contains the hydrophobic motif, a hallmark of members of the AGC kinase superfamily (5).

Akt signaling is activated by receptor tyrosine kinases, B- and T-cell receptors, cytokine receptors, and other mechanisms that activate phosphoinositide 3-kinase (PI 3-K). Active PI 3-K generates the second messenger phosphatidylinositol-3,4,5-trisphosphate (PIP3), which recruits Akt to the plasma membrane via the pleckstrin homology domain for subsequent phosphorylation (6). Phosphoinositide-dependent kinase-1 (PDK-1) phosphorylates Akt in the activation loop of the kinase domain (T308 of Akt1) (7) and an unknown kinase, possibly the rictor-mTOR complex (8), phosphorylates Akt in the hydrophobic motif (S473 of Akt1) of the regulatory domain. Phosphorylation of both sites is necessary for full Akt activation (9). Downstream Akt substrates include regulators of metabolism and apoptosis, implicating Akt1 - 3 as targets for diabetes (10) and cancer therapies (11).

## PRINCIPLE OF THE ASSAY

This Surveyor IC Immunoassay employs a two-site sandwich ELISA to quantitate Akt phosphorylated at S473 in cell lysates. An antibody specific for Akt1, Akt2, and Akt3, binding both phosphorylated and unphosphorylated protein, has been pre-coated onto a microplate. Standards and samples are added and Akt1, Akt2, and Akt3 present are bound by the immobilized antibody. After washing away unbound material, a biotinylated detection antibody recognizing 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. Substrate Solution is added to the wells and color develops in proportion to the amount of Akt phosphorylated at S473 bound in the initial step. The color development is stopped and the intensity of the color is measured.

## LIMITATIONS OF THE PROCEDURE

- FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.
- This Surveyor IC Immunoassay should not be used beyond the expiration date on the kit label.
- Do not mix or substitute reagents with those from other lots or sources.
- If samples generate values higher than the highest standard, further dilute the samples with Assay Diluent and repeat the assay.
- Any variation in standard diluent, operator, pipetting technique, washing technique, incubation time or temperature, and kit age can cause variation in binding.

# MATERIALS PROVIDED

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

| Component | Part # | Quantity | Storage Conditions of Opened/Reconstituted Components |
|---|---|---|---|
| **Phospho-Akt (Pan) (S473) Microplate** - One 96 well polystyrene microplate (12 strips of 8 wells) coated with a mouse monoclonal antibody against Akt. | 841830 | 1 plate | Return unused wells to the foil pouch containing the desiccant pack, reseal along entire edge of zip-seal. May be stored for up to 1 month at 2 - 8° C.* |
| **Phospho-Akt (Pan) (S473) Standard** - Each vial contains 245 ng of recombinant human phospho-Akt2 (S473) in a buffered protein base with preservatives; lyophilized. | 841694 | 2 vials | Use within 1 hour of reconstitution. Use a fresh standard for each assay. |
| **Phospho-Akt (Pan) (S473) Detection Antibody** - 7.5 µg of a biotinylated rabbit anti-phospho-Akt (Pan) (S473) antibody; lyophilized. | 841831 | 1 vial | Store for up to 1 month at 2 - 8° C.* |
| **Lysis Buffer 6** - 21 mL of a cell lysing buffer with phosphatase inhibitors and preservatives. | 895561 | 1 vial | |
| **Sample Diluent Concentrate 1 (5X)** - 21 mL of a 5-fold concentrated buffer with preservatives. | 895562 | 1 vial | |
| **Reagent Diluent Concentrate 2 (10X)** - 21 mL of a 10-fold concentrated solution of buffered protein base with preservatives. | 841380 | 1 vial | |
| **Wash Buffer Concentrate (25X)** - 21 mL of a 25-fold concentrated solution of buffered surfactant with preservatives. | 895003 | 1 vial | |
| **Color Reagent A** - 12.5 mL of stabilized hydrogen peroxide. | 895000 | 1 vial | |
| **Color Reagent B** - 12.5 mL of stabilized chromogen (tetramethylbenzidine). | 895001 | 1 vial | |
| **Streptavidin-HRP** - 1.0 mL of streptavidin conjugated to horseradish-peroxidase. | 890803 | 1 vial | |
| **Stop Solution** - 6 mL of 2 N sulfuric acid. | 895032 | 1 vial | |
| **Plate Covers** - Adhesive strips. | 640197 | 4 strips | Store at room temperature. |

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

## OTHER SUPPLIES REQUIRED

- Microplate reader capable of measuring absorbance at 450 nm, with the correction wavelength set at 540 nm or 570 nm.
- Pipettes and pipette tips.
- Deionized or distilled water.
- Squirt bottle, manifold dispenser, or automated microplate washer.
- 100 mL and 500 mL graduated cylinders.
- Phosphate-buffered saline (PBS).
- Phenylmethylsulfonylfluoride (PMSF) (optional; Sigma, Catalog # P7626).
- Protease Inhibitor Cocktail (optional; Sigma, Catalog # P2714).
- **Polypropylene** test tubes for dilution.

## PRECAUTION

The Stop Solution provided with this kit is an acid solution. Wear protective gloves, clothing, eye, and face protection. Wash hands thoroughly after handling.

## TECHNICAL HINTS

- When mixing or reconstituting protein solutions, always avoid foaming.
- To avoid cross-contamination, change pipette tips between additions of each standard level, between sample additions, and between reagent additions. Also, use separate reservoirs for each reagent.
- To ensure accurate results, proper adhesion of plate sealers during incubation steps is necessary.
- 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 remaining Wash Buffer by inverting the plate and blotting it against clean paper towels.
- When using an automated plate washer, adding a 30 second soak period following the addition of Wash Buffer, and/or rotating the plate 180 degrees between wash steps may improve assay precision.
- Substrate Solution should remain colorless until added to the plate. Keep Substrate Solution protected from light. Substrate Solution should change from colorless to gradations of blue.
- Stop Solution should be added to the plate in the same order as the Substrate Solution. The color developed in the wells will turn from blue to yellow upon addition of the Stop Solution. Wells that are green in color indicate that the Stop Solution has not mixed thoroughly with the Substrate Solution.

## REAGENT PREPARATION

**Bring all reagents to room temperature before use.**

**Wash Buffer** - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Dilute 20 mL of Wash Buffer Concentrate into deionized or distilled water to prepare 500 mL of Wash Buffer.

**Sample Diluent 1** - Dilute 20 mL of Sample Diluent Concentrate 1 (5X) into deionized or distilled water to prepare 100 mL of Sample Diluent 1.

**Assay Diluent** - Dilute 8 mL of Lysis Buffer 6 into Sample Diluent 1 to prepare 48 mL of Assay Diluent. Prepare only enough diluent to run the assay.

**Reagent Diluent 2** - Dilute 5 mL of Reagent Diluent Concentrate 2 (10X) into deionized or distilled water to prepare 50 mL of Reagent Diluent 2.

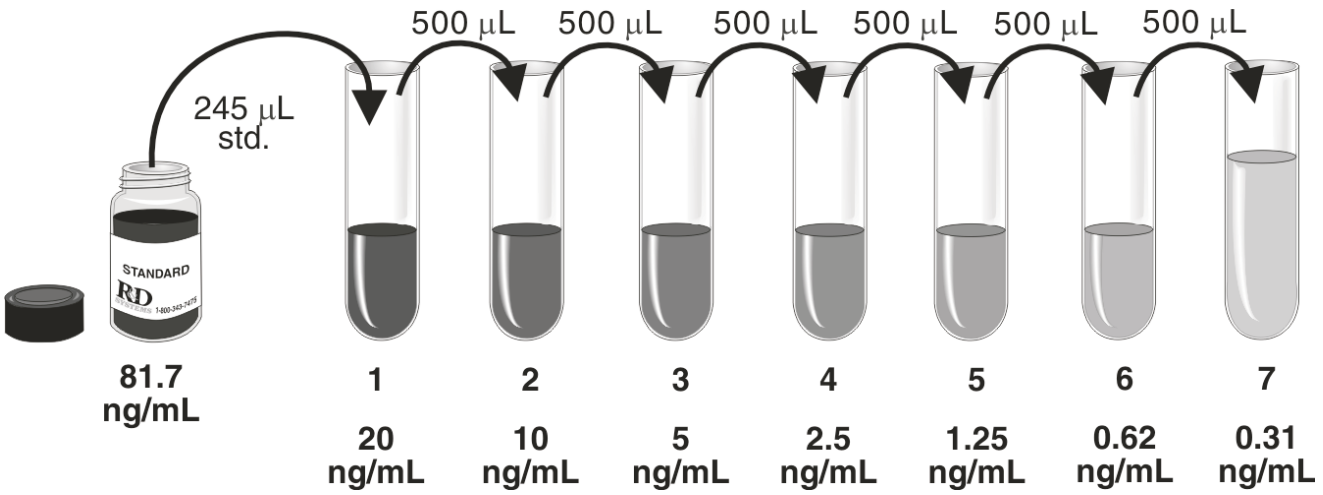
**Phospho-Akt (Pan) (S473) Detection Antibody** - Reconstitute the Phospho-Akt (Pan) (S473) Detection Antibody with 1.0 mL of Reagent Diluent 2. This reconstitution produces a stock solution of 7.5 μg/mL. Immediately before use, dilute the Detection Antibody to a working concentration of 500 ng/mL in Reagent Diluent 2.

**Streptavidin-HRP** - Immediately before use, dilute Streptavidin-HRP to the working concentration specified on the vial label using Reagent Diluent 2.

**Substrate Solution** - Color Reagents A and B should be mixed together in equal volumes within 15 minutes of use. Prepare only enough Substrate Solution to run the assay. Protect from light. 100 μL of the resultant mixture is required per well.

**Phospho-Akt (Pan) (S473) Standard** - Reconstitute the Phospho-Akt (Pan) (S473) Standard with 0.5 mL of Lysis Buffer 6. This reconstitution produces a stock solution of 490 ng/mL. Mix the standard to ensure complete reconstitution. **Allow the standard to sit for a minimum of 15 minutes.** Perform a 1:6 dilution of the Standard by adding 2.5 mL of Sample Diluent 1 to the vial. This dilution produces a solution of 81.7 ng/mL.

Label seven **polypropylene** tubes 1 through 7. Add 755 µL of Assay Diluent to tube 1 and 500 µL of Assay Diluent to tubes 2 through 7. Add 245 µL of the 81.7 ng/mL Standard to tube 1. Mix thoroughly and continue to prepare a seven point standard curve using 2-fold serial dilutions by transferring 500 µL from tube 1 into tube 2 and subsequent 500 µL transfers as shown below. Use Assay Diluent as the zero standard. **Use a fresh standard for each assay. Use within 1 hour of preparation.**




## SAMPLE PREPARATION

### Cell Lysates

**Note:** *It is recommended to supplement Lysis Buffer 6 with PMSF and the Protease Inhibitor Cocktail prior to use. Supplements should be used according to the manufacturer's instructions.*

1. Using PBS, collect non-adherent cells by centrifugation and adherent cells by scraping the culture flask.
2. Rinse cells two times with PBS, making sure to remove any remaining PBS after the second rinse.
3. Solubilize cells at 1 x $10^7$ cells/mL in Lysis Buffer 6.
4. Vortex lysates briefly and allow to sit on ice for 15 minutes or store at ≤ -20° C in a manual defrost freezer. Sample protein concentration may be quantified using a total protein assay.
5. Before use, centrifuge at 2000 x g for 5 minutes and transfer the supernates into a clean test tube.
6. For assaying, dilute lysates 6-fold with Sample Diluent 1 and make further serial dilutions in Assay Diluent.

## ASSAY PROCEDURE

**Bring all reagents and samples to room temperature before use. It is recommended that all samples and standards be assayed in duplicate.**

1. Prepare all reagents, working standards, and samples as directed in the previous sections.

2. Remove excess microplate strips from the plate frame, return them to the foil pouch containing the desiccant pack, and reseal.

3. Add 100 µL of Standard or sample* per well. Use Assay Diluent as the zero standard. Cover with the adhesive strip provided. Incubate for 2 hours at room temperature.

4. Aspirate each well and wash, repeating the process two times for a total of three 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 to good performance. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.

5. Immediately before use, prepare the Detection Antibody. Add 100 µL of diluted Phospho-Akt (Pan) (S473) Detection Antibody to each well. Cover with a new adhesive strip. Incubate for 2 hours at room temperature.

6. Repeat the aspiration/wash as in step 4.

7. Immediately before use, prepare the Streptavidin-HRP. Add 100 µL of the diluted Streptavidin-HRP to each well. Incubate for 20 minutes at room temperature.

8. Repeat the aspiration/wash as in step 4.

9. Immediately before use, prepare the Substrate Solution. Add 100 µL of Substrate Solution to each well. Incubate for 20 minutes at room temperature. **Avoid placing the plate in direct light.**

10. Add 50 µL of Stop Solution to each well. Gently tap the plate to ensure thorough mixing.

11. Determine the optical density of each well within 30 minutes, 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.

*Lysates require dilution. Refer to the Sample Preparation section.

## ASSAY PROCEDURE CHECKLIST

**1.** ☐ Bring all reagents to room temperature.
☐ Prepare reagents, samples, and standards as instructed.
☐ Return unused components to storage temperature as indicated in the instructions.

**2.** ☐ Add 100 µL Standard or sample* to each well, and incubate for 2 hours at room temperature.

**3.** ☐ Aspirate and wash each well 3 times.

**4.** ☐ Add 100 µL diluted Detection Antibody to each well, and incubate for 2 hours at room temperature.

**5.** ☐ Aspirate and wash each well 3 times.

**6.** ☐ Add 100 µL diluted Streptavidin-HRP to each well, and incubate for 20 minutes at room temperature.

**7.** ☐ Aspirate and wash each well 3 times.

**8.** ☐ Add 100 µL Substrate Solution to each well, and incubate for 20 minutes at room temperature. **Protect from light.**

**9.** ☐ Add 50 µL Stop Solution to each well. Read at 450 nm within 30 minutes (λ correction 540 nm or 570 nm).

*Lysates require dilution. Refer to Sample Preparation.

## CALCULATION OF RESULTS

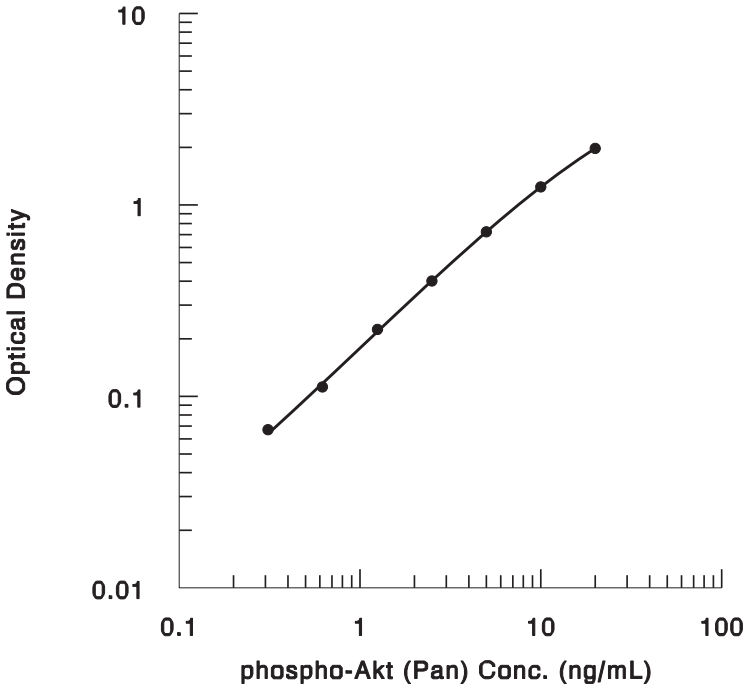
Average the duplicate readings for each standard and sample, then subtract the average zero standard optical density. Results may be normalized to total protein or cell number.

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 concentrations versus the log of the optical density and the best fit line can be determined by regression analysis. This procedure will produce an adequate, but less precise fit of the data.

Since samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

## 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) Surveyor IC Immunoassay. The standard curve was calculated using a computer generated 4-PL curve-fit. This standard curve is provided for demonstration purposes only.




| (ng/mL) | O.D. | Corrected | Average |
|---|---|---|---|
| 0 | 0.090<br>0.090 | —— | —— |
| 0.31 | 0.154<br>0.160 | 0.064<br>0.070 | 0.067 |
| 0.62 | 0.200<br>0.203 | 0.110<br>0.113 | 0.112 |
| 1.25 | 0.303<br>0.325 | 0.213<br>0.235 | 0.224 |
| 2.5 | 0.483<br>0.499 | 0.393<br>0.409 | 0.401 |
| 5 | 0.810<br>0.817 | 0.720<br>0.727 | 0.724 |
| 10 | 1.295<br>1.369 | 1.205<br>1.279 | 1.242 |
| 20 | 1.990<br>2.138 | 1.900<br>2.048 | 1.974 |

## CALIBRATION

This Surveyor IC Immunoassay is calibrated against a highly purified *E. coli*-expressed recombinant human phospho-Akt2 (S473) produced at R&D Systems.

## SPECIFICITY

The Human/Mouse/Rat Phospho-Akt (Pan) (S473) Surveyor IC Immunoassay 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.

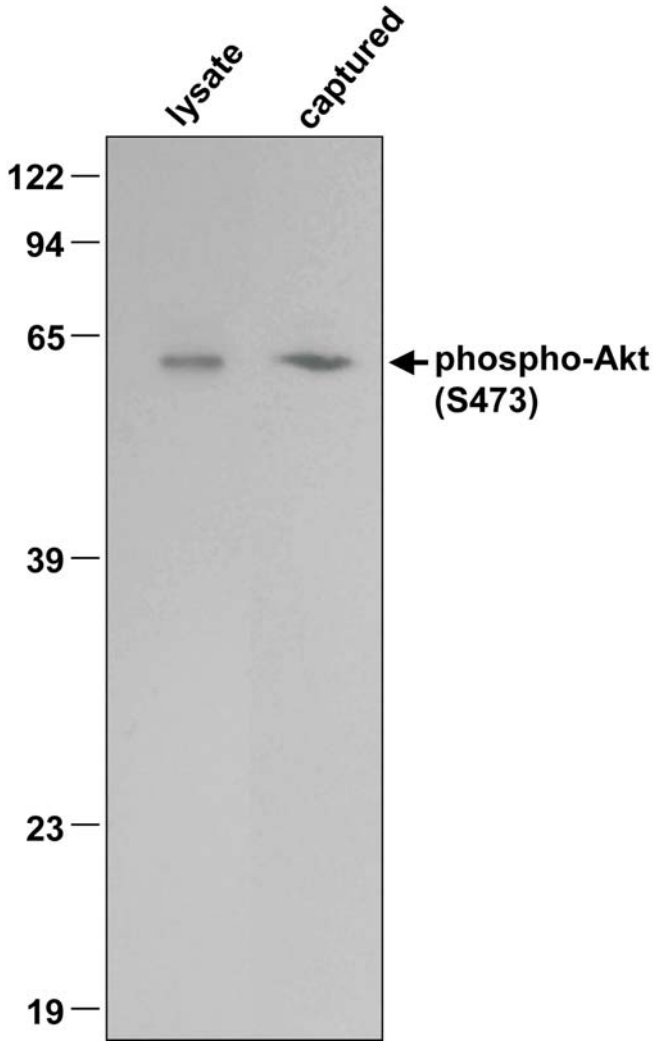



**Figure 1:** Lysates prepared from MCF-7 human breast cancer cells treated with 100 ng/mL of human IGF-I (R&D Systems, Catalog # 291-G1) for 20 minutes were incubated in wells coated with Phospho-Akt (Pan) (S473) Capture Antibody. Unbound material was removed by washing and bound material was solubilized in SDS gel sample buffer. The same lysate and captured proteins were electrophoresed, transferred to a PVDF membrane 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 in the assay.

The specificity of this kit was also demonstrated using peptide competition. Only a phosphopeptide containing the Akt (S473) phosphorylation site blocked the signal, indicating that the ELISA is both phospho-specific and specific for Akt phosphorylation at S473 versus the phosphorylation of Akt at T308, or of the related AGC kinase RSK at the hydrophobic motif site S380.

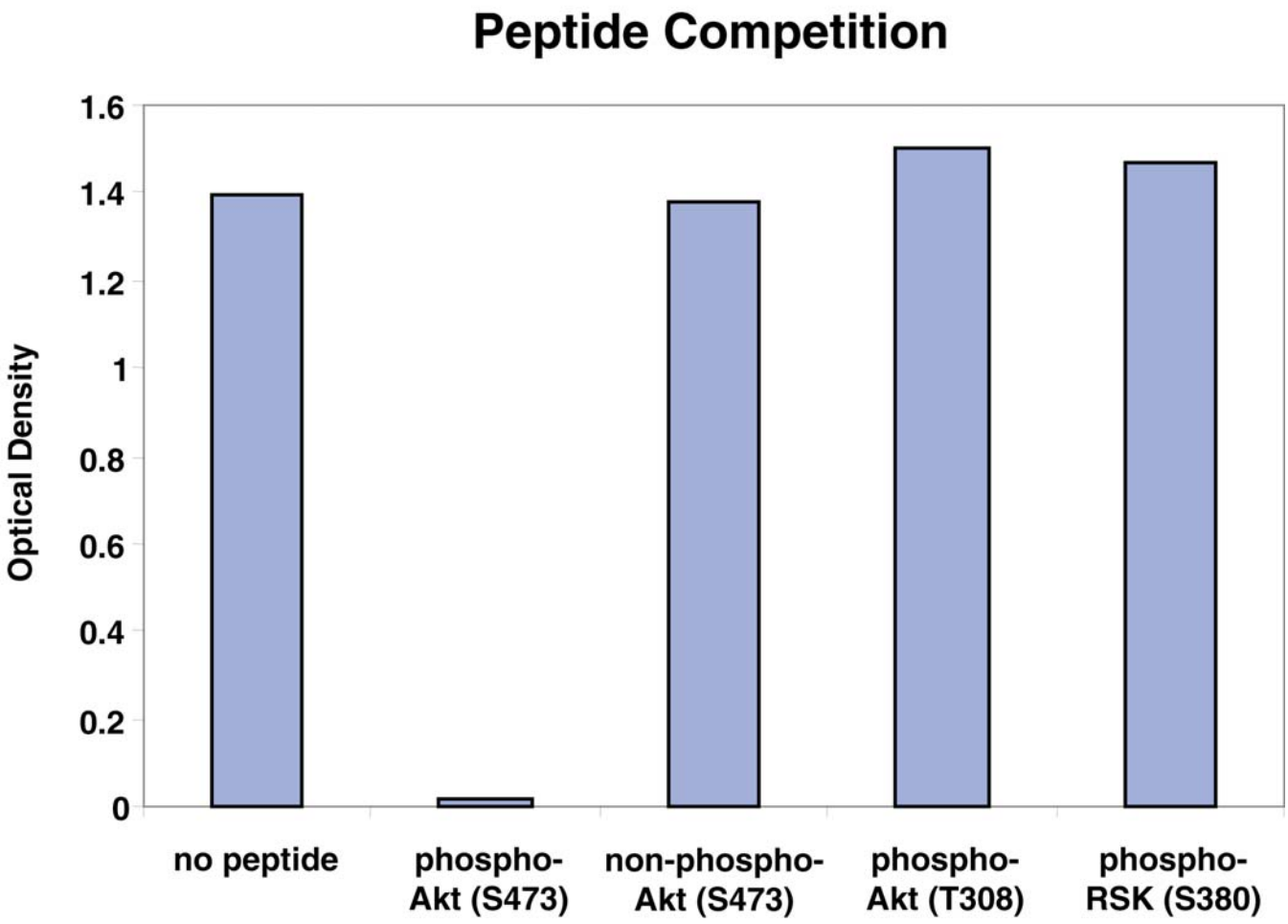



**Figure 2:** A lysate prepared from MCF-7 human breast cancer cells treated with 100 ng/mL of human IGF-I (R&D Systems, Catalog # 291-G1) was analyzed with this ELISA. The Phospho-Akt (Pan) (S473) Detection Antibody was either untreated (no peptide) or preincubated with a phosphopeptide containing the Akt S473 phosphorylation site (phospho-Akt S473), a non-phosphopeptide containing the same sequence (non-phospho-Akt S473), a phosphopeptide containing the Akt T308 phosphorylation site (phospho-Akt T308), or a phosphopeptide containing the related RSK S380 phosphorylation site (phospho-RSK S380). Peptides were used at 40 ng/mL.

## QUANTIFICATION

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

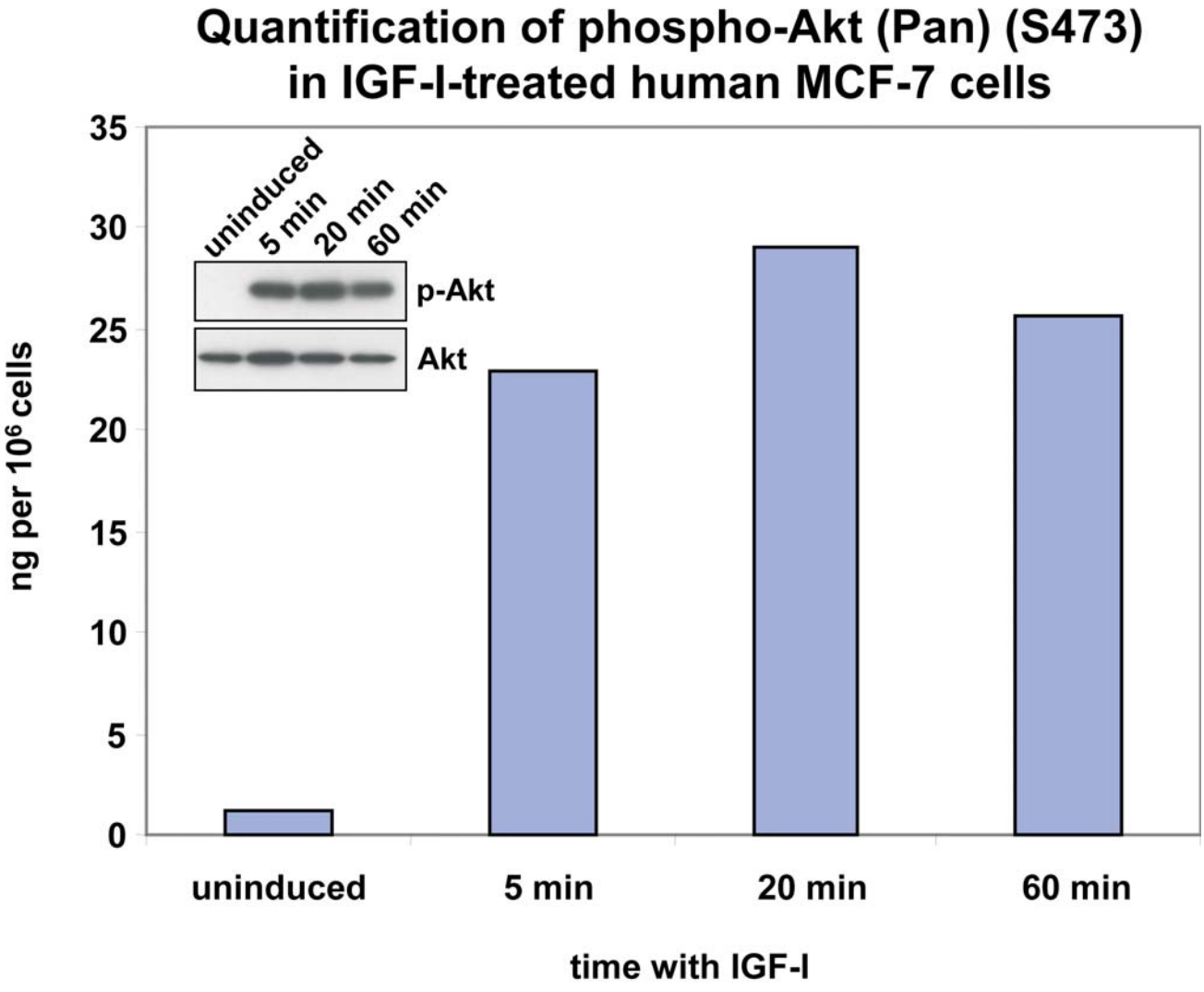



**Figure 3:** Lysates prepared from MCF-7 human breast cancer cells induced with 100 ng/mL of IGF-I for the indicated times were quantified with this Surveyor IC Immunoassay. The same lysates were also immunoblotted (inset) with either anti-phospho-Akt (S473) (p-Akt) (R&D Systems, Catalog # AF887) or anti-Akt (Pan) (R&D Systems, Catalog # MAB2055) antibodies. The Surveyor IC Immunoassay 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 Surveyor IC Immunoassay was also determined using cells pretreated with the selective phosphatidylinositol 3-kinase (PI 3-K) inhibitor LY294002 (Tocris, Catalog # 1130), which indirectly blocks the phosphorylation of Akt.

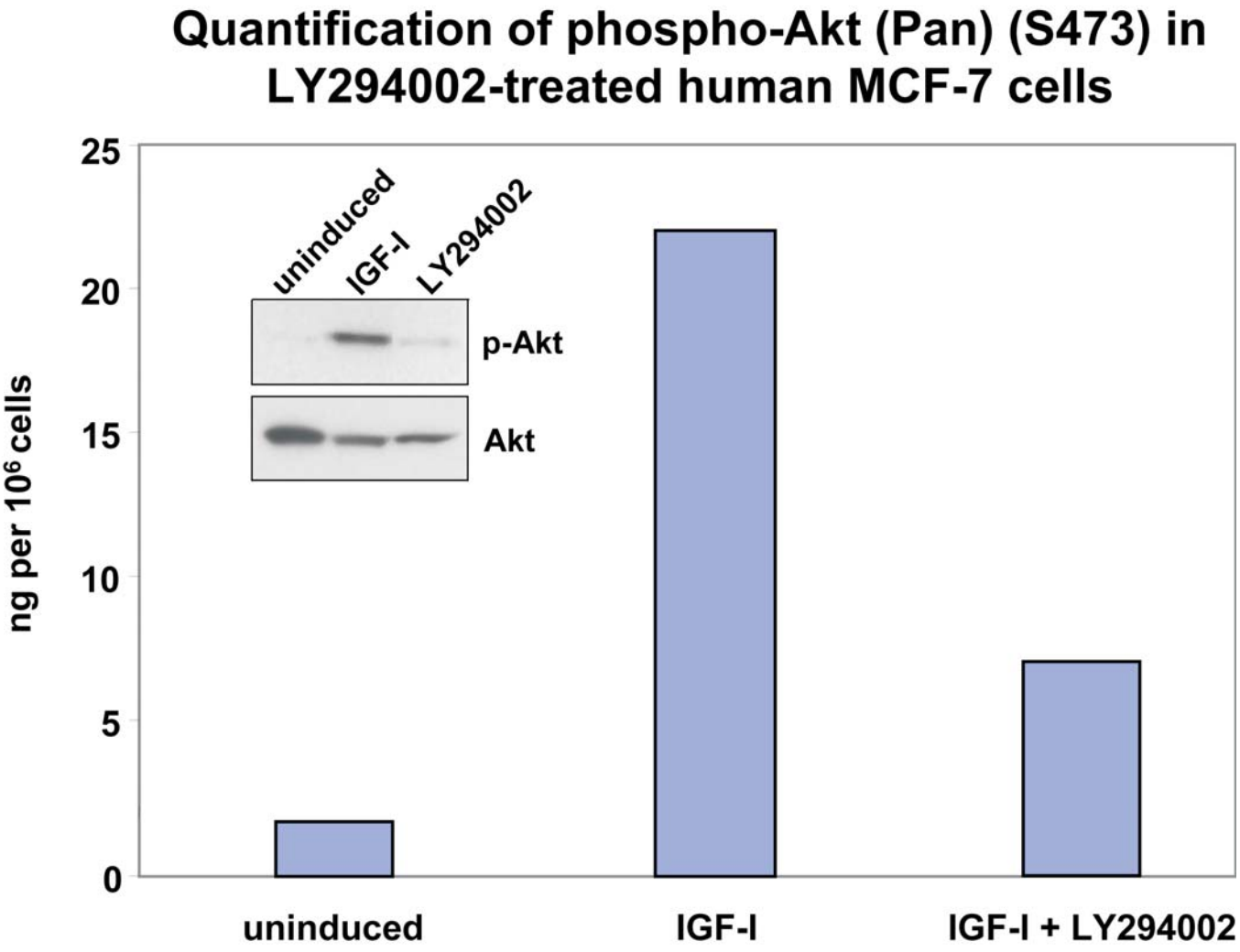



**Figure 4:** MCF-7 human breast cancer cells were incubated with no additions or with 100 ng/mL of IGF-I for 20 minutes, either with or without 100 μM LY294002. Cells were lysed and phosphorylated Akt was quantified with this Surveyor IC Immunoassay. The same lysates were also immunoblotted (inset) with either anti-phospho-Akt (S473) (p-Akt) or anti-Akt (Pan) antibodies. The Surveyor IC Immunoassay 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) Surveyor IC Immunoassay also quantifies phosphorylated Akt levels in mouse and rat cell lysates.

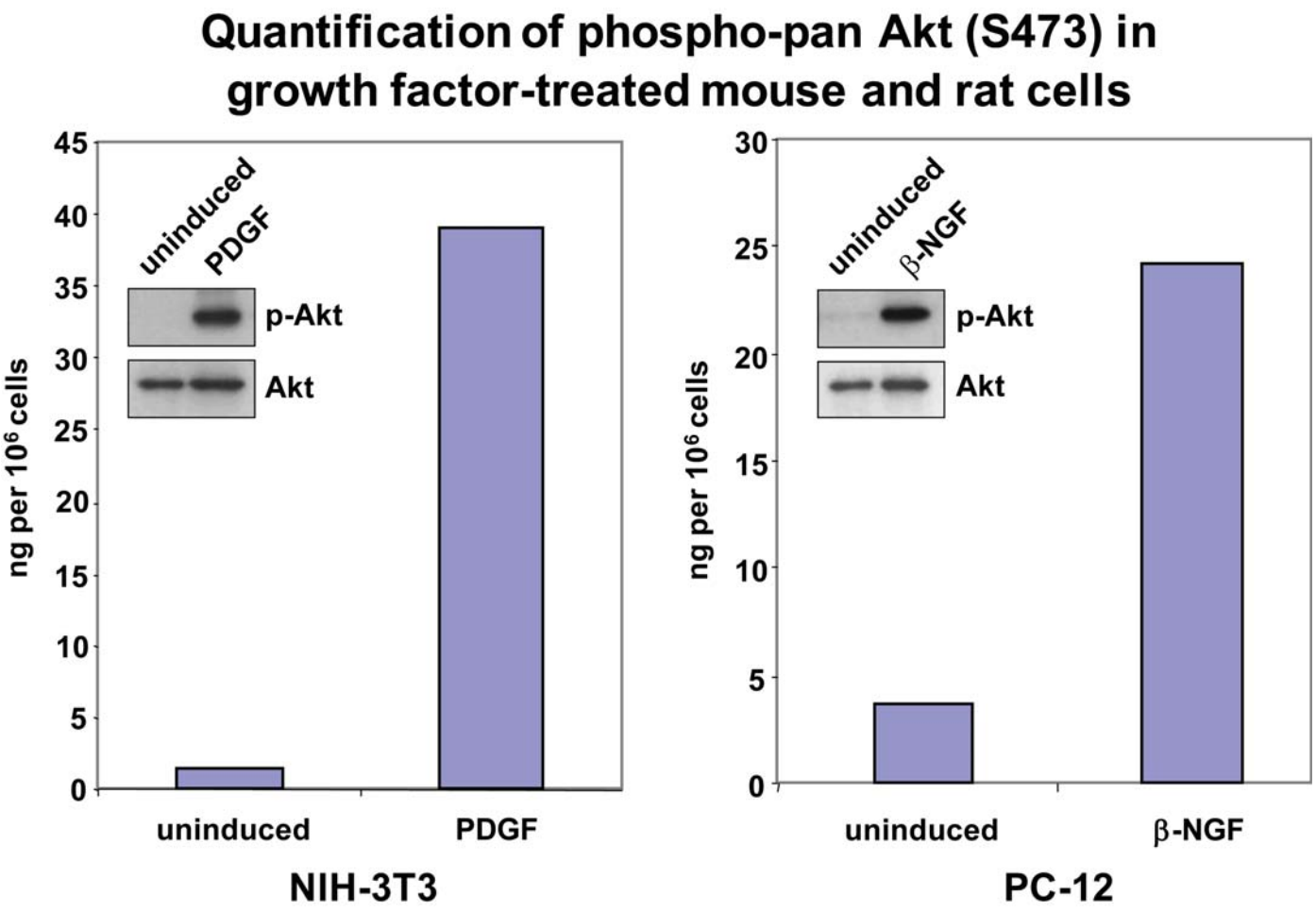



**Figure 5:** Lysates prepared from NIH-3T3 mouse embryonic fibroblast cells either uninduced or induced with 10 ng/mL of human PDGF (R&D Systems, Catalog # 120-HD) for 5 minutes (left panel), and PC-12 rat adrenal pheochromocytoma cells either uninduced or induced with 100 ng/mL of recombinant rat β-NGF (R&D Systems, Catalog # 556-NG) for 10 minutes (right panel) were quantified with this Surveyor IC Immunoassay. The same lysates were also immunoblotted (inset) with either anti-phospho-Akt (S473) (p-Akt) or anti-Akt (Pan) antibodies. The Surveyor IC Immunoassay 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.

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