Cell-Based Infrared ELISA

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

Catalog Number KCBIR887

A near-infrared assay using two spectrally distinct fluorophores to measure phosphorylated Akt in whole cells.

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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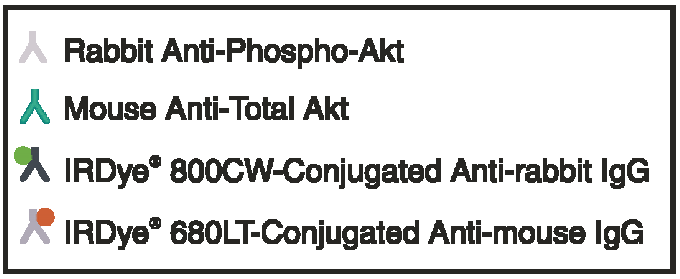
PRINCIPLE OF THE ASSAY

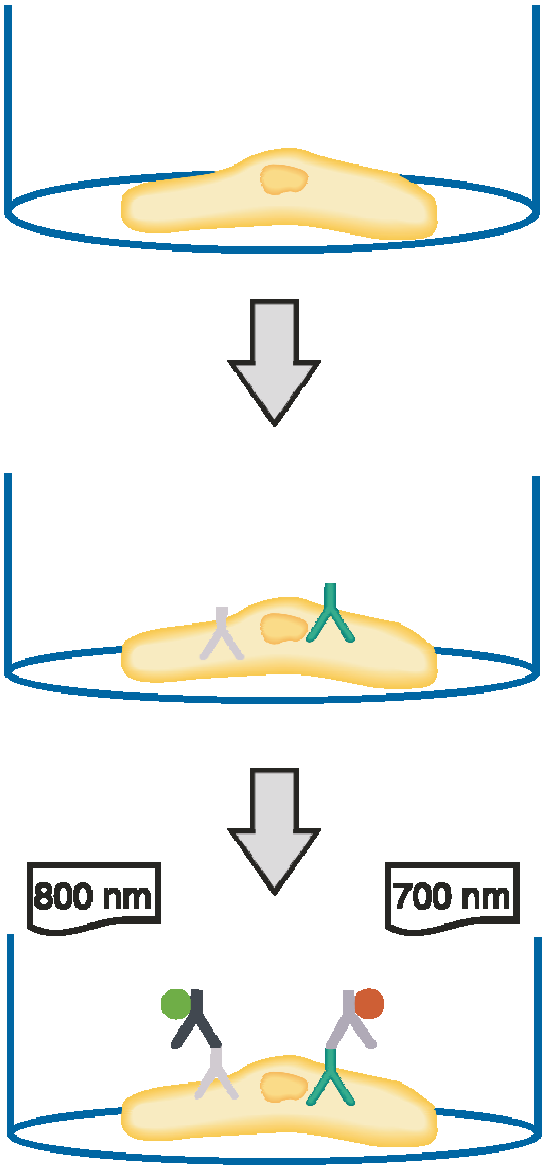
This Cell-Based Infrared ELISA contains the components required to run a near-infrared immunostaining assay to measure phosphorylated Akt (S473) in whole cells. This simple and efficient assay eliminates the need to prepare cell lysates and can be used to investigate both kinase signaling and the effects of kinase inhibitors on cells. Cells are grown in 96-well plates and stimulated with ligands. Following stimulation, cells are fixed and permeabilized in the wells. The target protein phosphorylation is measured using a double immunofluoroescent staining procedure. The cells are simultaneously incubated with two primary antibodies: a phospho-specific antibody and a normalization antibody that recognizes the pan-protein regardless of phosphorylation status. The primary antibodies are derived from different species. Two species-specific secondary antibodies labeled with two spectrally distinct near-infrared fluorophores are used for detection. The fluorescence of the phosphorylated protein is normalized to that of the pan-protein in each well for the correction of well-to-well variations. This two-wavelength assay results in precise analysis of protein phosphorylation with good reproducibility.

TECHNICAL HINTS AND LIMITATIONS

- FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.
- This Cell-Based Infrared ELISA 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.
- When mixing or reconstituting protein solutions, always avoid foaming.
- Individual results may vary due to differences in technique, plasticware, and water sources.
- A thorough and consistent wash technique is essential for proper assay performance. To minimize cell loss during the wash steps, avoid dispensing liquid directly onto the cell surface. Instead, gently dispense the liquid down the wall of the cell culture wells, always using the same side of the wells. Empty the wells by decanting and remove any remaining Wash Buffer by inverting the plate and blotting it against clean paper towels.
- To avoid cross-contamination, change pipette tips between additions of each reagent and/or sample. Also, use separate reservoirs for each reagent.
- It is recommended that all samples and controls 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.

ASSAY OVERVIEW





1. Seed cells in a 96 well plate. Stimulate cells with ligands. Fix, permeabilize, and block cells.

2. Add primary antibodies (rabbit anti-phospho-Akt (S473) and mouse anti-total Akt).

3. Add secondary antibodies (IRDye 800CW-conjugated anti-rabbit IgG and IRDye 680LT-conjugated anti-mouse IgG). The plate is imaged by scanning simultaneously at 700 and 800 nm with a LI-COR Odyssey® or Aerius™ instrument.

MATERIALS PROVIDED

Store the unopened kit at 2-8 °C. Do not use past the kit expiration date. This kit contains sufficient materials to run one 96-well plate.

Microplate (Part 607582) - One 96-well cell culture clear-bottom black microplate and cover for use as a vessel in the assay.

Phospho-Akt (S473) Antibody (Part 965564) - 1 vial of lyophilized rabbit anti-phospho-Akt (S473) antibody.

Total Akt Antibody (Part 965565) - 1 vial of lyophilized mouse anti-Akt antibody.

IRDye® 800CW-conjugated anti-rabbit IgG* (Part 894149) - 110 µL of IRDye 800CW-conjugated goat anti-rabbit IgG secondary antibody.

IRDye® 680LT-conjugated anti-mouse IgG* (Part 894151) - 110 µL of IRDye 680LT-conjugated goat anti-mouse IgG secondary antibody.

Blocking Buffer (Part 893235) - 35 mL of 10% fetal bovine serum in buffer with preservatives.

Wash Buffer (5X) (Part 893236) - 60 mL of a buffered surfactant with preservatives.

Plate Sealers - 4 adhesive strips.

OTHER SUPPLIES REQUIRED

- 37% formaldehyde (Molecular Biology Grade; Sigma, Catalog # F8775). Refer to MSDS prior to use.
- 1X PBS (Irvine Scientific, Catalog # 9240).
- Deionized or distilled water.
- Pipettes and pipette tips.
- Multi-channel pipette for washing.
- Cell culture incubator.
- Microfuge tubes.
- Orbital shaker.
- LI-COR Odyssey or Aerius Infrared Imaging System.

**This product is covered by one or more of the following US Patent numbers: US 6,995,274, US 7,504,089, PCT - WO0224815A1, and US Application 61/184,750 pending*.

REAGENT PREPARATION

Phospho-Akt (S473) Antibody - Prepare a 100X working concentration of the antibody by reconstituting with 110 µL of 1X 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 for up to 60 days.*

Total Akt Antibody - Prepare a 100X working concentration of the antibody by reconstituting with 110 µL of 1X 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 for up to 60 days.*

Primary Antibody Mixture** - Immediately before use, add 100 µL of the Phospho-Akt antibody and 100 µL of the Total Akt Antibody to 9.8 mL of Blocking Buffer. 100 µL of the primary antibody mixture is required per well. **If a full plate is not being assayed, adjust volumes accordingly.**

Secondary Antibody Mixture** - Immediately before use, centrifuge vials for 30 seconds at 1000 x g prior to removing cap. Add 20 µL of the IRDye 800CW-conjugated antibody and 20 µL of the IRDye 680LT-conjugated antibody to 10 mL of Blocking Buffer. 100 µL of the secondary antibody mixture is required per well. **If a full plate is not being assayed, adjust volumes accordingly.**

1X Wash Buffer - Add 60 mL of Wash Buffer (5X) to 240 mL of 1X PBS to prepare 1X Wash Buffer. Store at 2-8 °C for up to 60 days.

SOLUTION PREPARATION

4% Formaldehyde (for adherent cells) - Add 1.3 mL of 37% formaldehyde to 10.7 mL of 1X PBS to prepare 4% formaldehyde. **If a full plate is not being assayed, adjust volumes accordingly.**

8% Formaldehyde (for non-adherent cells) - Add 2.6 mL of 37% formaldehyde to 9.4 mL of 1X PBS to prepare 8% formaldehyde. **If a full plate is not being assayed, adjust volumes accordingly.**

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

**Once prepared, the primary and secondary antibody mixtures cannot be stored. Prepare only enough as needed to run the assay.

GENERAL ASSAY PROCEDURE

A. Culture, Stimulate, Fix, and Block Cells

1. Seed 100 µL of 10,000-20,000 adherent cells into each well of the black 96-well microplate with clear bottom and incubate overnight at 37 °C in a cell culture incubator.

   Note: *The cell number used is dependent upon the cell line and the relative amount of protein phosphorylation. Optimal cell numbers should be determined by each laboratory for each assay.*

2. Grow and treat the cells as desired.

3. Fix cells by replacing the medium with 100 µL of 4% formaldehyde in 1X PBS. Add the plate cover and incubate for 20 minutes at room temperature. Alternatively, apply a plate sealer and store the plate containing the fixed cells at 2-8 °C for up to 2 weeks.

   Warning: *Formaldehyde is highly toxic. Confine vapors to a chemical hood and wear appropriate gloves and eye protection when using this chemical. Refer to MSDS prior to use.*

4. Remove the formaldehyde solution and wash the cells 3 times with 200 µL of 1X Wash Buffer. Each wash step should be performed for 5 minutes with gentle shaking.

5. Remove the Wash Buffer, and add 100 µL of Blocking Buffer. Add the plate cover and incubate for 1 hour at room temperature.

B. Binding of Primary and Secondary Antibodies

1. Remove the Blocking Buffer and wash the cells 3 times with 200 µL of 1X Wash Buffer. Each wash step should be performed for 5 minutes with gentle shaking.

2. Add 100 µL of the Primary Antibody Mixture to each well. Cover with a plate sealer and incubate for 16 hours at 2-8 °C. In cells known to generate high amounts of phosphorylated Akt, a 2-3 hour primary antibody incubation is sufficient; however, for maximum sensitivity, an overnight incubation is recommended.

   Note: *Depending on the experimental design (refer to the Calculation of Results section), some wells should be incubated with Primary Antibody Mixture and some with only the Blocking Buffer as the negative controls (secondary antibody alone).*

3. Remove the Primary Antibody Mixture and wash the cells 3 times with 200 µL of 1X Wash Buffer. Each wash step should be performed for 5 minutes with gentle shaking.

4. Add 100 µL of the Secondary Antibody Mixture to each well. Cover with a plate sealer and incubate for 2 hours at room temperature.

   Note: *The Secondary Antibody Mixture is added into each well including the negative control wells.*

C. Near-Infrared Detection

1. Remove the Secondary Antibody Mixture from each well and wash the cells 4 times with 200 µL of 1X Wash Buffer. Each wash step should be performed for 5 minutes with gentle shaking.

2. Image the plate using a LI-COR Odyssey or Aerius instrument. The plate is scanned simultaneously at 700 and 800 nm with 169 µm resolution, medium quality, focus offset of 3.0 mm, and intensity setting of 5 for both channels.

PROCEDURE FOR NON-ADHERENT CELLS

This protocol has been validated for use with non-adherent cells by culturing and fixing cells as follows.

1. Seed 100 µL of suspension cells into each well of the black 96-well microplate in serum-free or normal growth media at the desired cell density (seeding cells at a density of 0.2-1.0 x 10^6 cells/mL is recommended).
2. Incubate the plate at 37 °C. Depending on the cell line and treatment, the typical incubation time is 0.5-16 hours.
3. Treat the cells as desired. Prepare the treatment media containing a 5X final concentration of treatment. Add 25 µL of 5X treatment media to the wells and bring the total volume up to 125 µL. Mix solution by gently agitating the plate and incubate according to your treatment protocols.
4. Centrifuge the plate at 500 x g for 3 minutes at 4 °C and remove media.
5. Fix the cells by adding 100 µL of 8% formaldehyde in 1X PBS. Apply the plate cover and incubate for 20 minutes at room temperature. Alternatively, apply a plate sealer and store the plate containing the fixed cells at 2-8 °C for up to 2 weeks.
6. Continue with section A, step 4 of the General Assay Procedure on page 6.

CALCULATION OF RESULTS

Control wells with no primary antibody (secondary antibody alone) should be included in each experiment. The fluorescence from these wells is the background fluorescence and is subtracted from all sample wells. Normalized results can be determined by dividing the phospho-Akt fluorescence at 800 nm in each well by the total Akt fluorescence at 700 nm in each well. The normalized duplicate readings for each sample are then averaged.

KIT PERFORMANCE DATA

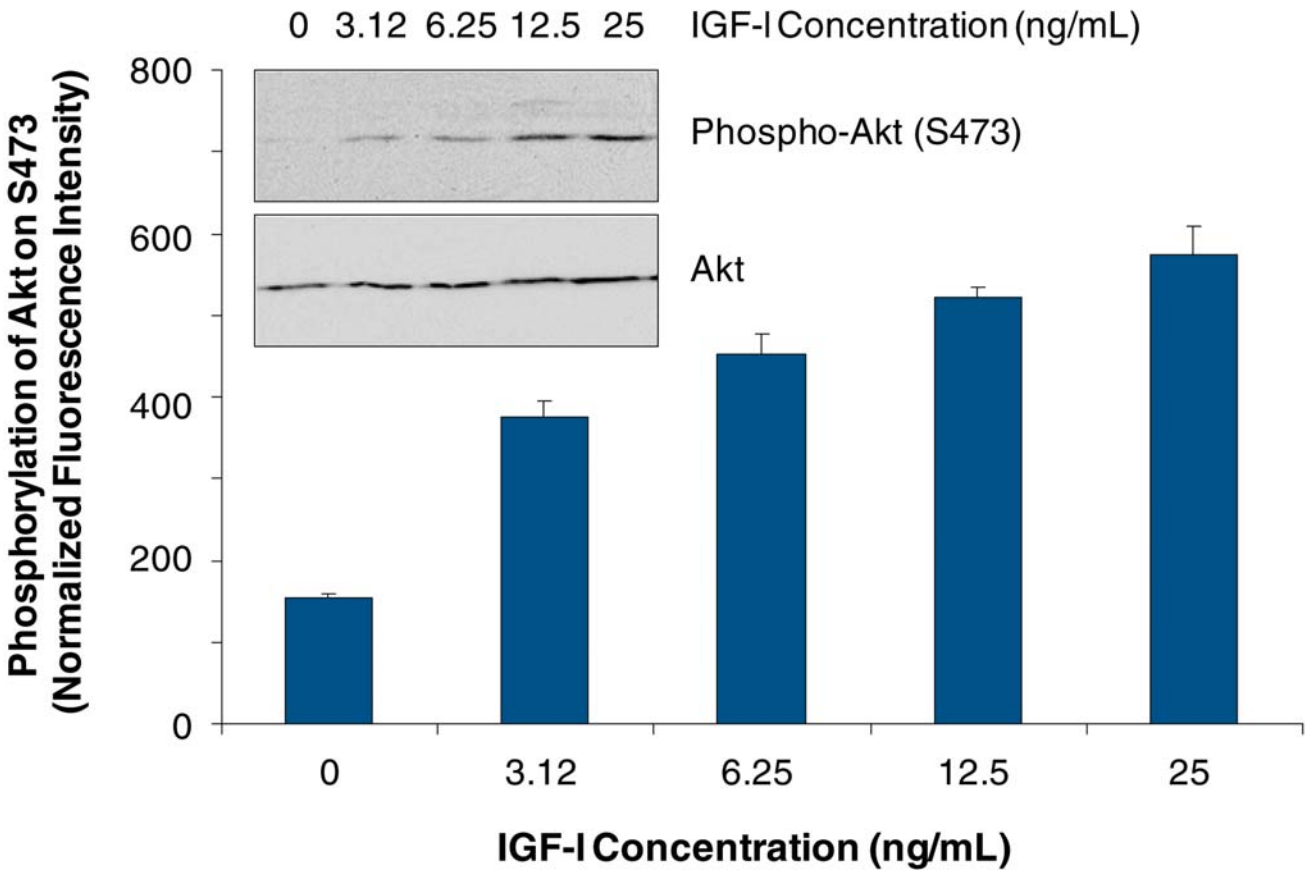


Figure 1: Dose response of IGF-I induced Akt phosphorylation. MCF-7 human breast cancer cells were cultured in 96-well plates and treated with the indicated amounts of recombinant human (rh) IGF-I (R&D Systems, Catalog # 291-G1) for 20 minutes. After fixation of cells in the wells, phosphorylation of Akt on S473 was determined using this Human/Mouse/Rat Phospho-Akt (S473) Cell-Based Infrared ELISA Kit. Values represent the mean ± the range of duplicate determinations. Analysis of Akt phosphorylation on S473 by Western blotting using the antibodies supplied in this kit is also shown (inset).

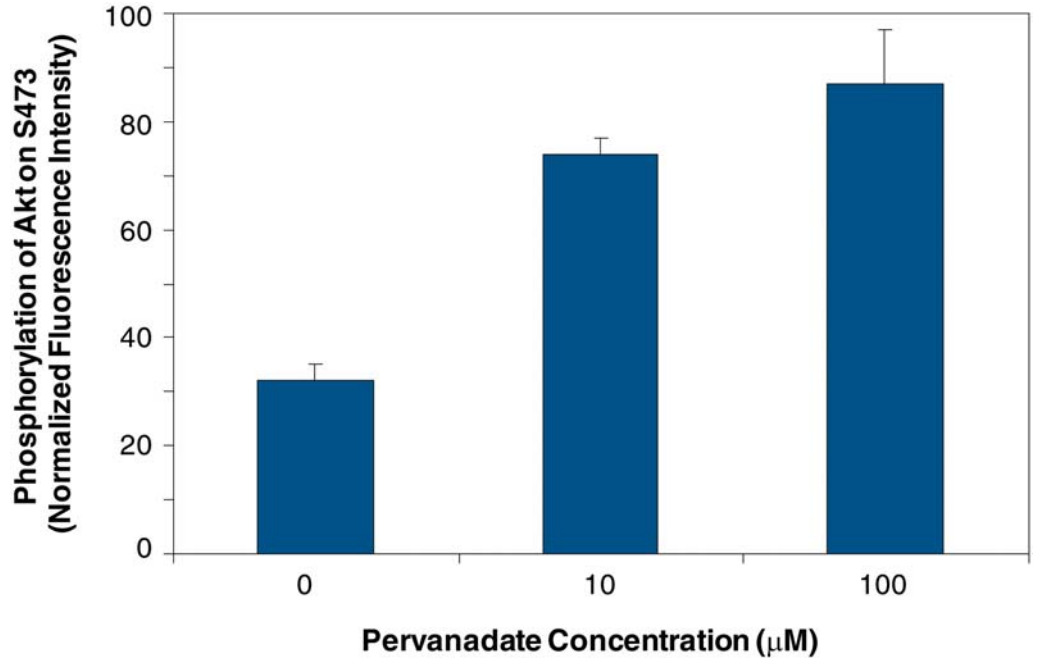


Figure 2: Dose response of pervanadate-induced Akt phosphorylation in suspension cells. Jurkat human acute T cell leukemia cells were cultured in 96-well plates and treated with the indicated amounts of pervanadate for 10 minutes. After fixation of cells in the wells, phosphorylation of Akt on S473 was determined using this Human/Mouse/Rat Phospho-Akt (S473) Cell-Based Infrared ELISA Kit. Values represent the mean ± the range of duplicate determinations.

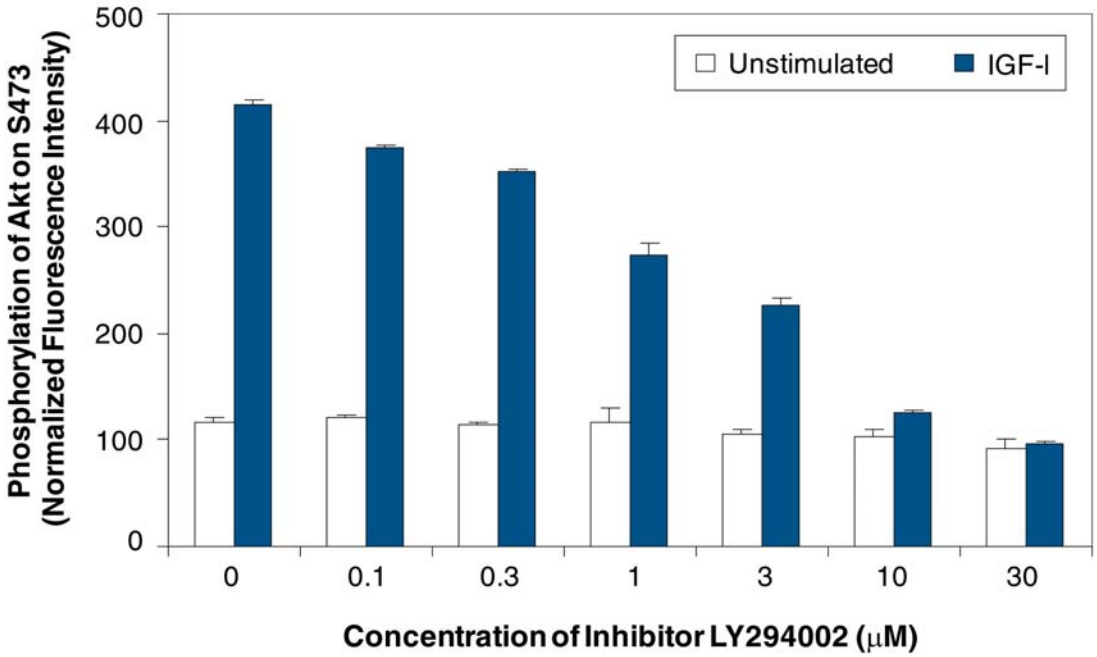


Figure 3: Effects of PI 3-kinase inhibitor (LY294002) on IGF-I induced Akt phosphorylation. MCF-7 human breast cancer cells in 96-well plates were pretreated for 10 minutes with the indicated concentrations of LY294002 then incubated with no additions or with 50 ng/mL rhIGF-I for 20 minutes. After fixation of cells in the wells, phosphorylation of Akt on S473 was determined using this Human/Mouse/Rat Phospho-Akt (S473) Cell-Based Infrared ELISA Kit. Values represent the mean ± the range of duplicate determinations.

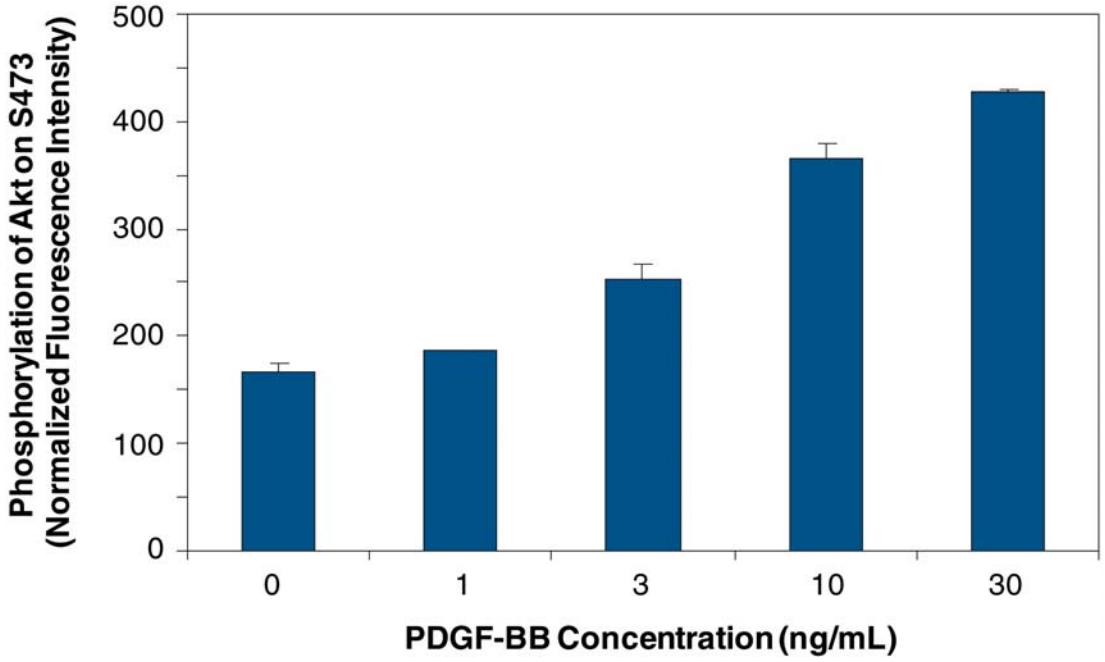


Figure 4: Dose response of PDGF-induced Akt phosphorylation in mouse cells. NIH-3T3 mouse embryonic fibroblasts were cultured in 96-well plates and treated with the indicated concentrations of recombinant human PDGF-BB (R&D Systems, Catalog # 220-BB) for 10 minutes. After fixation of cells in the wells, phosphorylation of Akt on S473 was determined using this Human/Mouse/Rat Phospho-Akt (S473) Cell-Based Infrared ELISA Kit. Values represent the mean ± the range of duplicate determinations.

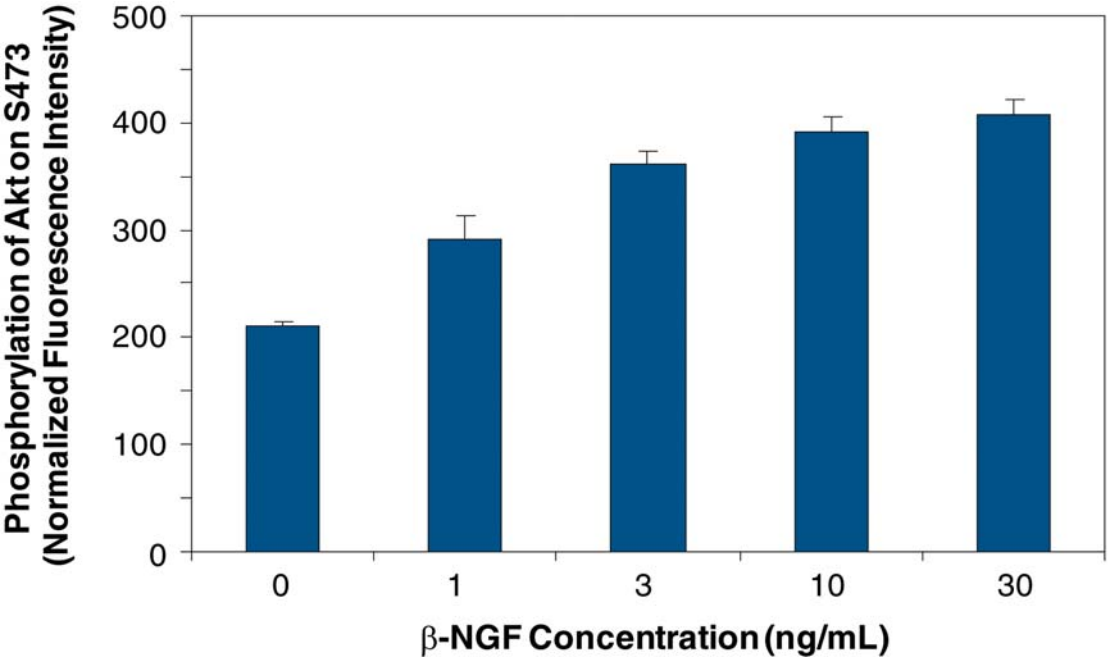


Figure 5: Dose response of β-NGF-induced Akt phosphorylation in rat cells. PC-12 rat adrenal pheochromocytoma cells were cultured in 96-well plates and treated with the indicated concentrations of recombinant rat β-NGF (R&D Systems, Catalog # 556-NG) for 10 minutes. After fixation of cells in the wells, phosphorylation of Akt on S473 was determined using this Human/Mouse/Rat Phospho-Akt (S473) Cell-Based Infrared ELISA Kit. Values represent the mean ± the range of duplicate determinations.

PLATE LAYOUT

Use this plate layout as a record of samples assayed.