

Cell-Based ELISA

Base Kit 1

Catalog Number KCB001

For the development of Cell-Based ELISAs to simultaneously measure two proteins in whole cells using rabbit and mouse antibodies.

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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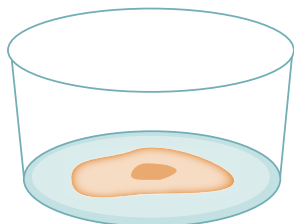
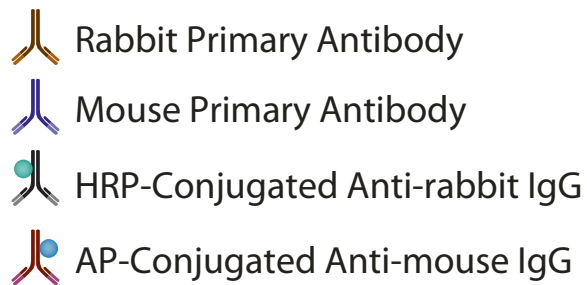
PRINCIPLE OF THE ASSAY

In combination with the rabbit and mouse primary antibodies, this Cell-Based ELISA Base Kit contains the components required to develop a Cell-Based ELISA to simultaneously measure two proteins in whole cells. Using this assay format, two target proteins can be analyzed or one protein can be used to normalize for well-to-well variations in cell numbers. This simple and efficient assay eliminates the need to prepare cell lysates and is broadly applicable for hypothesis testing and drug screening. This assay format can be used to develop an assay to assess protein phosphorylation status or effect of stimulation and/or inhibition of compounds and ligands on target molecules. These results may be normalized to the total protein, regardless of the phosphorylation state, or a house keeping protein. Cells are grown in 96-well plates and stimulated with ligands and/or incubated with inhibitors. Cells are then fixed and permeabilized in the wells. The status of target protein in response to treatment is analyzed using a double immunoenzymatic labeling procedure. The cells are simultaneously incubated with two primary antibodies: a rabbit antibody specific for the protein of interest and a mouse antibody that recognizes a normalization protein. After washing away the unbound antibodies, anti-rabbit and anti-mouse secondary antibodies labeled with horseradish-peroxidase (HRP) and alkaline phosphatase (AP), respectively, and two spectrally distinct fluorogenic substrates for either HRP or AP are used for detection. The fluorescence of the target protein is normalized to that of the normalization protein in each well for the correction of well-to-well variations. This ratiometric analysis of two wavelengths derived within the same microplate well enables reliable measurement of proteins in whole cells.

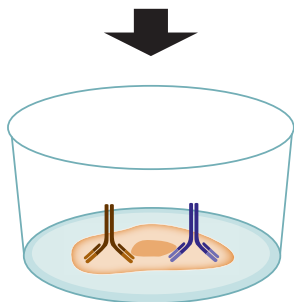
TECHNICAL HINTS AND LIMITATIONS

- FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.
- This Cell-Based ELISA Base Kit 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.
- For assay optimization, as HRP is the more robust enzyme, it is recommended that the HRP-conjugated secondary antibody be used with the primary antibody detecting the less abundant protein. The AP-conjugated secondary antibody should be used with the primary antibody detecting the more abundant protein.
- Suitability of the primary antibodies for the assay and the final concentrations used are to be determined by individual laboratories.
- 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.

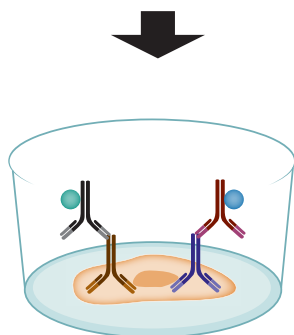
ASSAY OVERVIEW



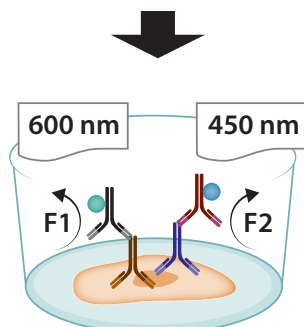
1. Seed cells in a 96-well plate. Stimulate cells with ligands. Fix, permeabilize, and block cells.



2. Add rabbit and mouse primary antibodies.



3. Add secondary antibodies (HRP-conjugated anti-rabbit IgG and AP-conjugated anti-mouse IgG).



4. Add fluorogenic substrates F1 and F2 and measure fluorescence.

MATERIALS PROVIDED

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

PART	PART #	DESCRIPTION	STORAGE OF OPENED/ RECONSTITUTED MATERIAL
HRP-conjugated anti-rabbit IgG	893230	2 vials (110 µL/vial) of HRP-conjugated goat anti-rabbit IgG secondary antibody.	May be stored at 2-8 °C for up to 2 months.*
AP-conjugated anti-mouse IgG	893231	2 vials (110 µL/vial) of AP-conjugated goat anti-mouse IgG secondary antibody.	
Substrate F1 Concentrate	893232	2 vials (50 µL/vial) of a fluorogenic substrate for horseradish-peroxidase (HRP).	
F1 Diluent	893233	2 bottles (10 mL/bottle) of a solution for diluting the Substrate F1 Concentrate.	
Substrate F2	893234	2 bottles (10 mL/bottle) of a fluorogenic substrate for alkaline phosphatase (AP).	
Blocking Buffer	893235	2 bottles (35 mL/bottle) of 10% fetal bovine serum in diluent with preservatives.	
Wash Buffer (5X)	893236	2 bottles (60 mL/bottle) of a buffered surfactant with preservatives.	Store at room temperature.
Microplate	607582	2 96-well cell culture clear-bottom black microplate and cover for use as a vessel in the assay.	
Plate Sealers	N/A	8 adhesive strips.	

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

OTHER SUPPLIES REQUIRED

- Rabbit primary antibody
- Mouse primary antibody
- 37% formaldehyde (Molecular Biology Grade, Sigma; Catalog # F8775). Refer to MSDS prior to use.
- 30% H₂O₂ (Sigma, Catalog # H1009). 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.
- Fluorescence plate reader with two channels: excitation 540 nm/emission 600 nm and excitation 360 nm/emission 450 nm.

REAGENT PREPARATION

Primary Antibody Mixture* - Optimal concentrations of the two primary antibodies used in the assay should be determined by each laboratory. Immediately before use, dilute the primary antibodies to a final concentration in Blocking Buffer. For two full plates, 20 mL of diluted primary antibody is needed. If both plates are not being assayed, adjust volumes accordingly.

Secondary Antibody Mixture* - Immediately before use, dilute the HRP-conjugated and AP-conjugated antibodies 1:100 in Blocking Buffer. For two full plates, 200 μ L of the HRP-conjugated antibody and 200 μ L of the AP-conjugated antibody are added to 19.6 mL of Blocking Buffer. If both plates are not being assayed, adjust volumes accordingly.

Substrate F1 - Add the contents of each Substrate F1 Concentrate vial (50 μ L) to each of the 10 mL bottles of F1 Diluent (brown bottles).

1X Wash Buffer - Add 120 mL of Wash Buffer (5X) to 480 mL of 1X PBS to prepare 1X Wash Buffer.

SOLUTION PREPARATION

4% Formaldehyde (for adherent cells) - Add 2.6 mL of 37% formaldehyde to 21.4 mL of 1X PBS to prepare 4% formaldehyde. **This is enough for two plates. If both plates are not being assayed, adjust volumes accordingly.**

8% Formaldehyde (for non-adherent cells) - Add 5.2 mL of 37% formaldehyde to 18.8 mL of 1X PBS to prepare 8% formaldehyde. **This is enough for two plates. If both plates are not being assayed, adjust volumes accordingly.**

Quenching Buffer - Add 400 μ L of 30% H_2O_2 to 19.6 mL of 1X Wash Buffer to prepare 0.6% H_2O_2 . **This is enough for two plates. If both plates are not being assayed, adjust volumes accordingly.**

*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 μ L of 10,000-20,000 adherent cells into each well of the black 96-well microplate provided 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 target protein. 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 μ L of 4% formaldehyde in 1X PBS. Add the plate cover and incubate for 20 minutes at room temperature. For maximum sensitivity, it is recommended that the assay be performed immediately after cell fixation. Alternatively, apply plate sealers and store the plates containing the fixed cells at 2-8° C for up to 2 weeks.

Caution: *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 μ L of 1X Wash Buffer. Each wash step should be performed for 5 minutes with gentle shaking.
5. Remove the Wash Buffer and add 100 μ L of Quenching Buffer. Add the plate cover and incubate for 20 minutes at room temperature.
6. Remove the Quenching Buffer and wash the cells 3 times with 200 μ L of 1X Wash Buffer. Each wash step should be performed for 5 minutes with gentle shaking.
7. Remove the Wash Buffer, and add 100 μ L of Blocking Buffer. Add the plate cover and incubate for 1 hour at room temperature.

B. Binding of Primary and Secondary Antibodies

Note: *Optimal concentrations for primary antibodies should be determined by each laboratory for each assay.*

1. Remove the Blocking Buffer and wash the cells 3 times with 200 μ L of 1X Wash Buffer. Each wash step should be performed for 5 minutes with gentle shaking.
2. Add 100 μ L of the Primary Antibody Mixture to each well. Cover with a plate sealer and incubate at 2-8 °C. In cells known to generate high amounts of target protein, a 2-3 hour 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 control (secondary antibody alone).*

3. Remove the Primary Antibody Mixture and wash the cells 3 times with 200 μ L of 1X Wash Buffer. Each wash step should be performed for 5 minutes with gentle shaking.
4. Add 100 μ L 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. Fluorogenic Detection

1. Remove the Secondary Antibody Mixture from each well and wash the cells 2 times with 200 μ L of 1X Wash Buffer, followed by 2 washes with 200 μ L of 1X PBS. Each wash step should be performed for 5 minutes with gentle shaking.
2. Remove the 1X PBS from the plate and add 75 μ L of Substrate F1 to each well. Incubate for 20-60 minutes at room temperature. Protect the plate from direct light. A pink or rosy color should develop in the wells.

Note: *It is critical to add Substrate F1 into each well and incubate for 20-60 minutes for fluorescence development prior to the addition of Substrate F2. Adding Substrate F1 and Substrate F2 simultaneously into the wells will result in the inhibition of fluorescence development.*

3. Add 75 μ L of Substrate F2 to each well and incubate for an additional 20-40 minutes at room temperature. Protect the plate from direct light.
4. Read the plate using a fluorescence plate reader with excitation at 540 nm and emission at 600 nm. Then read the plate with excitation at 360 nm and emission at 450 nm. The readings at 600 nm represent the amount of signal from Substrate 1, while readings at 450 nm represent the amount of signal from Substrate F2.

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 μ L 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 $0.2-1.0 \times 10^6$ 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 μ L of 5X treatment media to the wells and bring the total volume up to 125 μ L. 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 2-8 °C and remove media.
5. Fix the cells by adding 100 μ L 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 5.

CALCULATION OF RESULTS

Control wells with no primary antibody (secondary antibody alone) should be included in each experiment. The fluorescence (RFUs) from these wells is the background fluorescence and is subtracted from all sample wells.

If normalization is desired, the fluorescence at 600 nm derived from the target protein in each well is normalized to the fluorescence at 450 nm derived from normalization protein. The normalized duplicate readings for each sample are then averaged

KIT PERFORMANCE DATA

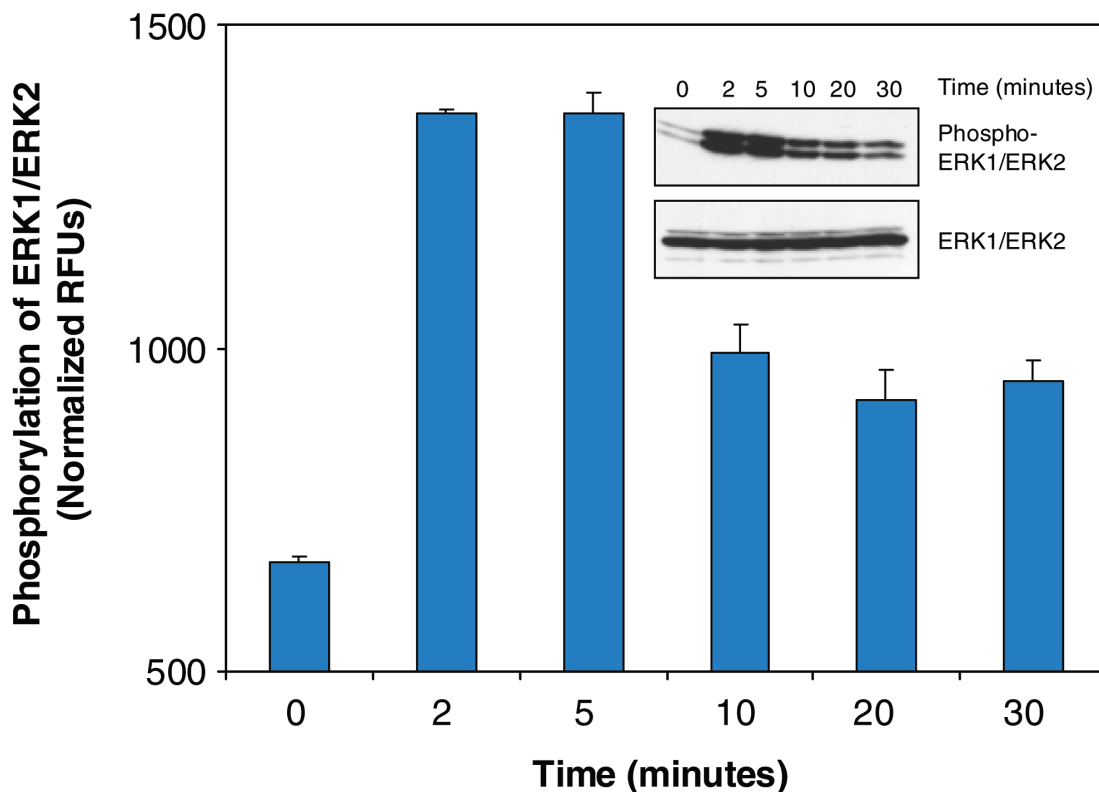


Figure 1: Time course of EGF-induced ERK1/ERK2 phosphorylation. A431 human epithelial carcinoma cells were cultured in 96-well plates and treated with 100 ng/mL of recombinant human EGF (R&D Systems, Catalog # 236-EG) for the indicated times. After fixation of cells with 4% formaldehyde, cells were incubated simultaneously with rabbit anti-phospho-ERK antibody and mouse anti-total ERK antibody. Phosphorylation of ERK was then determined using this Cell-Based ELISA Base Kit 1 and normalized to the total ERK in the same well for the correction of well-to-well variations. Values represent the mean \pm the range of duplicate determinations. Analysis of ERK1/ERK2 phosphorylation by Western blotting using the antibodies supplied in this kit is also shown (inset

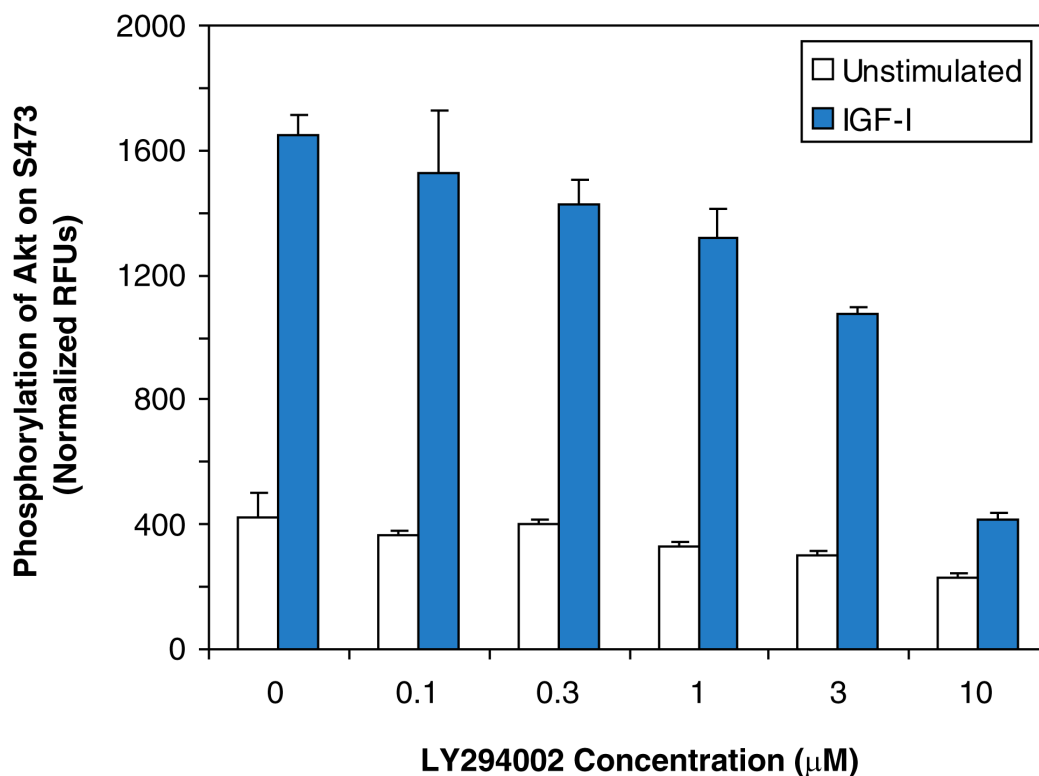


Figure 2: Effects of PI 3-kinase inhibitor (LY294002) on IGF-I induced Akt phosphorylation. MCF-7 human breast cancer cells in 96-well plates were pretreated for 10 minutes with the indicated concentrations of LY294002 and then incubated with no additions or with 25 ng/mL of recombinant human IGF-I (R&D Systems, Catalog # 291-G1) for 20 minutes. After fixation of cells with 4% formaldehyde, cells were incubated simultaneously with rabbit anti-phospho-Akt antibody (S473) and mouse anti-total Akt antibody. Phosphorylation of Akt on S473 was then determined using this Cell-Based ELISA Base Kit 1 and normalized to the total Akt in the same well for the correction of well-to-well variations. Values represent the mean \pm the range of duplicate determinations.

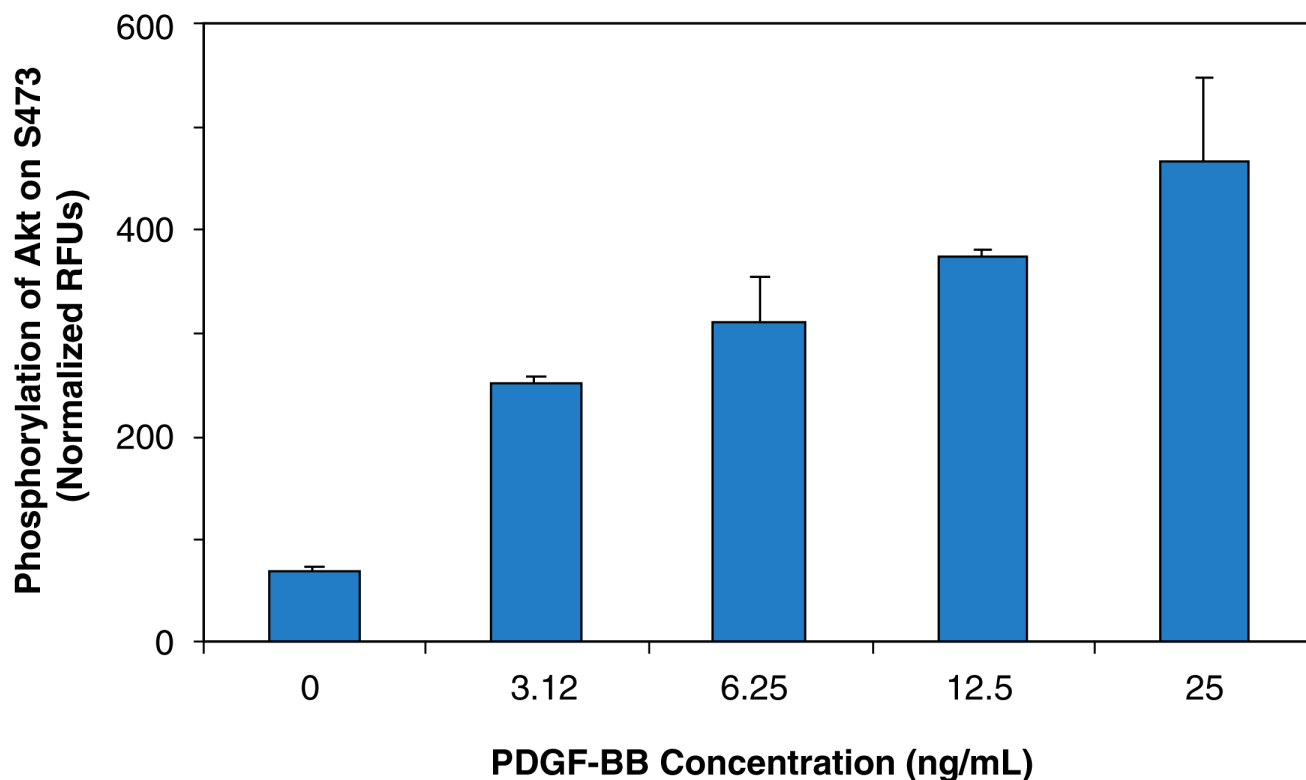


Figure 3: Dose response of PDGF-BB induced mouse Akt phosphorylation. NIH-3T3 mouse embryonic fibroblasts were cultured in 96-well plates and treated with the indicated amounts of recombinant PDGF-BB (R&D Systems, Catalog # 220-BB) for 20 minutes. After fixation of cells with 4% formaldehyde, cells were incubated simultaneously with rabbit anti-phospho-Akt antibody (S473) and mouse anti-total Akt antibody. Phosphorylation of Akt on S473 was then determined using this Cell-Based ELISA Base Kit 1 and normalized to the total Akt in the same well for the correction of well-to-well variations. Values represent the mean \pm the range of duplicate determinations.

PLATE LAYOUT

Use this plate layout as a record of samples assayed.

12								
11								
10								
9								
8								
7								
6								
5								
4								
3								
2								
1								
	A	B	C	D	E	F	G	H