Cell-Based ELISA

Base Kit 2

Catalog Number KCB002

For the development of Cell-Based ELISAs to simultaneously measure two proteins in whole cells using mouse and goat 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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MANUFACTURED AND DISTRIBUTED BY:

R&D Systems, Inc.
614 McKinley Place NE
Minneapolis, MN 55413
United States of America

TELEPHONE: (800) 343-7475
(612) 379-2956
FAX: (612) 656-4400
E-MAIL: info@RnDSystems.com

DISTRIBUTED BY:

R&D Systems Europe, Ltd.
19 Barton Lane
Abingdon Science Park
Abingdon, OX14 3NB
United Kingdom

TELEPHONE: +44 (0)1235 529449
FAX: +44 (0)1235 533420
E-MAIL: info@RnDSystems.co.uk

R&D Systems China Co. Ltd.
24A1 Hua Min Empire Plaza
726 West Yan An Road
Shanghai PRC 200050

TELEPHONE: +86 (21) 52380373
FAX: +86 (21) 52371001
E-MAIL: info@RnDSystemsChina.com.cn

PRINCIPLE OF THE ASSAY

In combination with the mouse and goat 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 housekeeping 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 mouse antibody specific for the protein of interest and a goat antibody that recognizes a normalization protein. After washing away the unbound antibodies, anti-mouse and anti-goat 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.

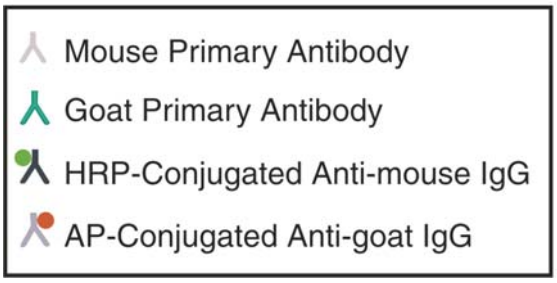
LIMITATIONS OF THE PROCEDURE

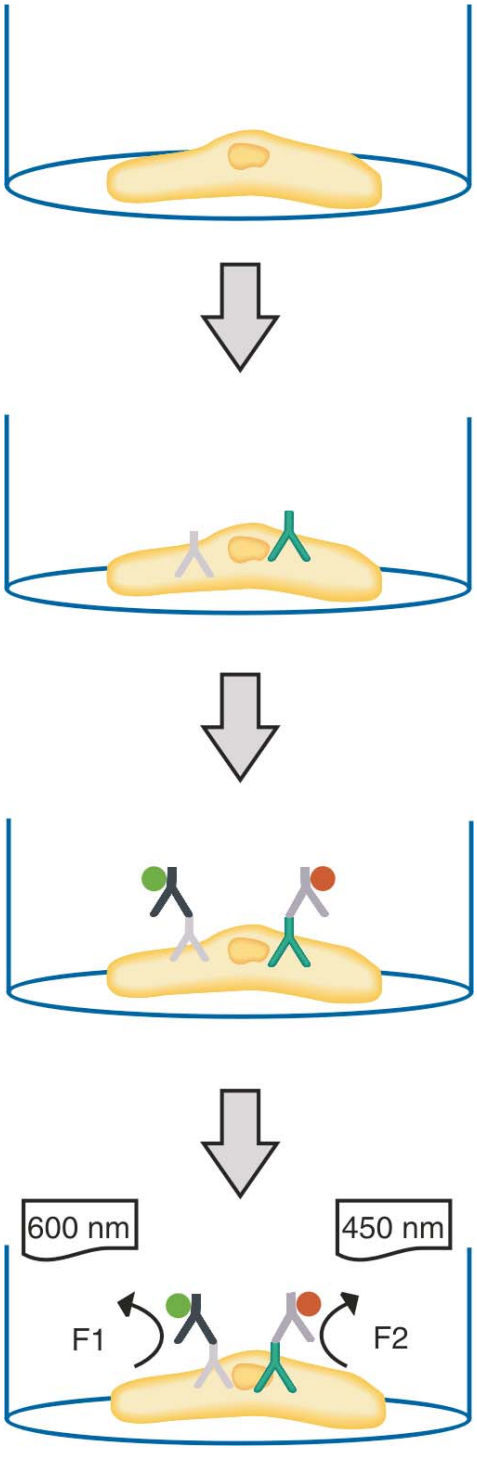
- FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.
- This Cell-Based ELISA Base Kit should not be used beyond the expiration date on the kit label.
- Do not mix or substitute reagents with those from other lots or sources.
- For assay optimization, as HRP is the more robust enzyme, it is 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.

TECHNICAL HINTS

- 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 mouse and goat primary antibodies.

3. Add secondary antibodies (HRP-conjugated anti-mouse IgG and AP-conjugated anti-goat 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 the kit expiration date. This kit contains sufficient materials to run ELISAs on two 96-well plates.

Microplates (Part 607582) - Two 96-well cell culture clear-bottom black microplates and covers for use as vessels in the assay.

HRP-conjugated anti-mouse IgG (Part 893228) - Two vials (110 μL/vial) of HRP-conjugated donkey anti-mouse IgG secondary antibody.

AP-conjugated anti-goat IgG (Part 893229) - Two vials (110 μL/vial) of AP-conjugated donkey anti-goat IgG secondary antibody.

Substrate F1 Concentrate (Part 893232) - Two vials (50 μL/vial) of a fluorogenic substrate for horseradish-peroxidase (HRP).

F1 Diluent (Part 893233) - Two bottles (10 mL/bottle) of a solution for diluting the Substrate F1 Concentrate.

Substrate F2 (Part 893234) - Two bottles (10 mL/bottle) of a fluorogenic substrate for alkaline phosphatase (AP).

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

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

Plate Sealers - 8 adhesive strips.

OTHER SUPPLIES REQUIRED

- Mouse primary antibody
- Goat primary antibody
- 37% formaldehyde (Molecular Biology Grade; Sigma, Catalog # F8775). Refer to MSDS prior to use.
- 30% H_2O_2 (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 µL of the HRP-conjugated antibody and 200 µL 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 µL) to each of the 10 mL bottles of F1 Diluent (brown bottles). Store Substrate F1 at 2-8° C for up to 60 days.*

1X Wash Buffer - Add 120 mL of Wash Buffer (5X) to 480 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 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 µL 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.**

*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 microplates 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 µL of 4% formaldehyde in 1X PBS. Add the plate covers 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.

   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 Quenching Buffer. Add the plate covers and incubate for 20 minutes at room temperature.

6. Remove the Quenching 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.

7. Remove the Wash Buffer, and add 100 µL of Blocking Buffer. Add the plate covers 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 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 plate sealers 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 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 plate sealers 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 µL of 1X Wash Buffer, followed by 2 washes with 200 µL 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 µL of Substrate F1 to each well. Incubate for 20-60 minutes at room temperature. Protect the plates 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 µL of Substrate F2 to each well and incubate for an additional 20-40 minutes at room temperature. Protect the plates from direct light.

4. Read the plates using a fluorescence plate reader with excitation at 540 nm and emission at 600 nm. Then read the plates with excitation at 360 nm and emission at 450 nm. The readings at 600 nm represent the amount of signal from Substrate F1, 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 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 (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 the normalization protein. The normalized duplicate readings for each sample are then averaged.

EXAMPLE DATA

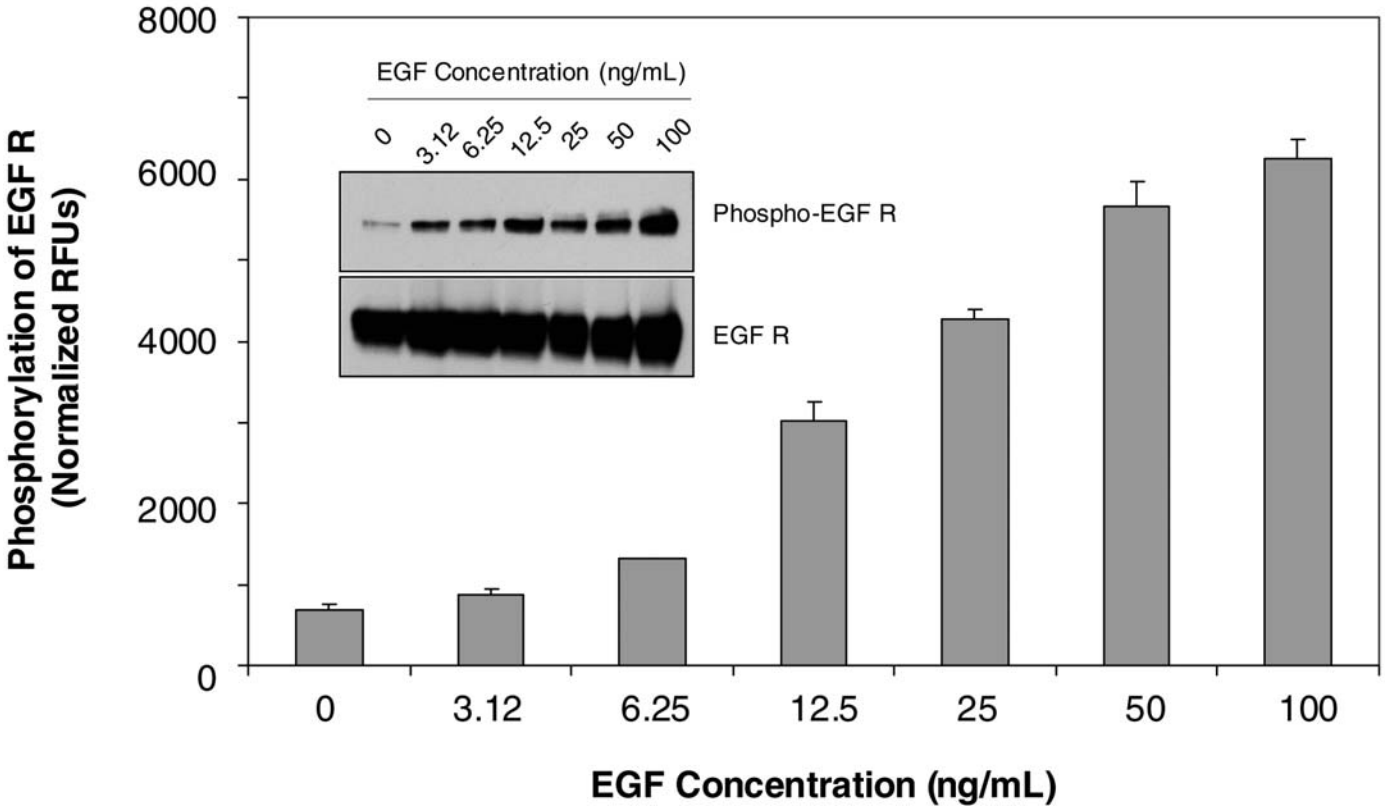


Figure 1: Dose response of EGF-induced EGF R phosphorylation. A431 human epithelial carcinoma cells were cultured in 96-well plates and treated with the indicated amounts of recombinant human EGF (R&D Systems, Catalog # 236-EG) for 5 minutes. After fixation of cells with 4% formaldehyde, cells were incubated simultaneously with mouse anti-phospho-EGF R antibody and goat anti-total EGF R antibody. Phosphorylation of EGF R was then determined using this Cell-Based ELISA Base Kit and normalized to the total EGF R in the same well for the correction of well to well variations. Values represent the mean ± the range of duplicate determinations. Analysis of EGF R phosphorylation on Y1068 by Western blotting using the antibodies supplied in this kit is also shown (inset).

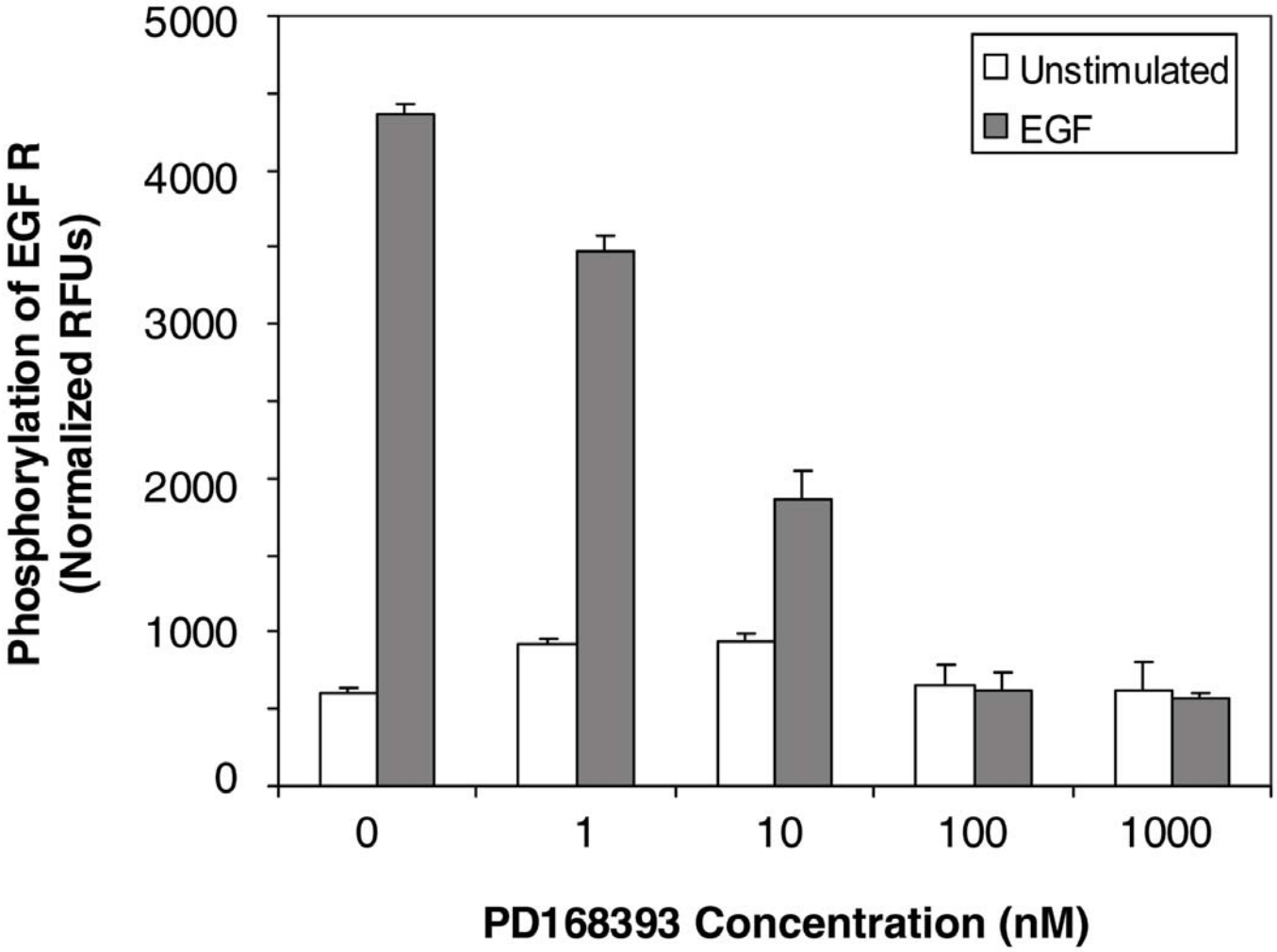
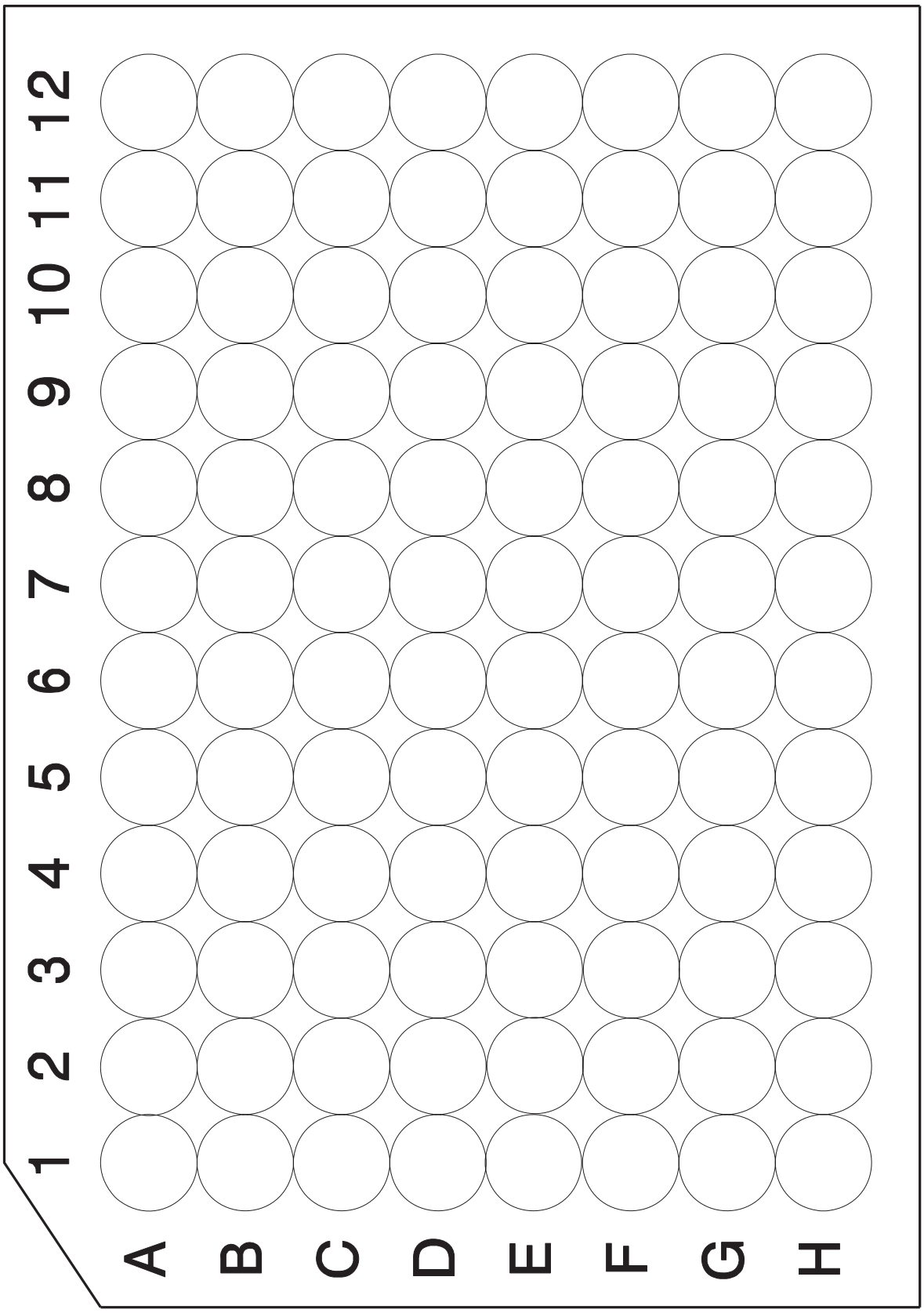


Figure 2: Effects of EGF R tyrosine kinase inhibitor (PD168393) on EGF-induced EGF R phosphorylation. A431 human epithelial carcinoma cells in 96-well plates were pretreated for 30 minutes with the indicated concentrations of PD168393 then incubated with no additions or with 50 ng/mL EGF for 5 minutes. After fixation of cells with 4% formaldehyde, cells were incubated simultaneously with mouse anti-phospho-EGF R antibody and goat anti-total EGF R antibody. Phosphorylation of EGF R on Y1068 was then determined using this Cell-Based ELISA Base Kit and normalized to the total EGF R in the same well for the correction of well to well variations. Values represent the mean ± the range of duplicate determinations.

PLATE LAYOUT

Use this plate layout as a record of samples assayed.



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