Development of a Novel Cell-Based ELISA for Analysis of Intracellular Proteins or Phosphorylation of Signaling Molecules

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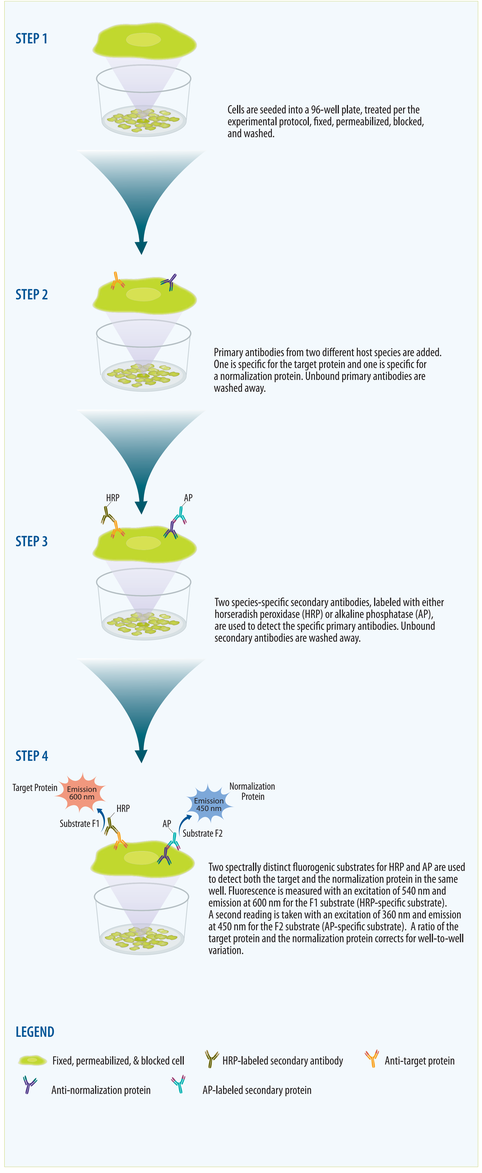
ABSTRACT

Quantification of intracellular proteins and phosphorylation events is extremely important for biomedical research. Although Western blot is the most widely used method, it is labor-intensive and time-consuming, especially when analyzing multiple samples. The plate-based immunoassay has become a popular alternative method for rapid protein detection. We have developed a fluorogenic Cell-Based ELISA that does not require lysate preparation or the multiple subsequent steps required for Western blot analysis. The Cell-Based ELISA format allows two target cellular proteins, or events, to be analyzed simultaneously in the same well, thus minimizing well-to-well variability. Cells are grown in 96-well plates and treated with the appropriate conditions, such as inhibitors or ligand stimulation. The cells are then fixed and permeabilized in the wells. This is followed by incubation with two primary antibodies derived from different species: a phospho-specific antibody and a normalization antibody that recognizes the total protein regardless of its phosphorylation status. Species-specific secondary antibodies labeled with horseradish peroxidase (HRP) and alkaline phosphatase (AP), and spectrally distinct fluorogenic substrates for each enzyme, are used for detection. The fluorescence of the phosphorylated protein is normalized to that of the total protein in each well to correct for well-to-well variations. Cell-Based ELISAs have been used to evaluate the effects of stimulators and inhibitors on cultured cells, and this has been accomplished with 10,000 cells, or less, per well. For example, phosphorylation of JNK (T183/Y185), Akt (S473), EGF R (Y1068), FRS2 (Y436), total protein levels of IκB-α induced by various stimuli, and the effects of kinase inhibitors, were assessed here using the Cell-Based ELISA method. The results were compared with Western blot and traditional sandwich ELISA. Once the cells are plated on 96-well microplates, the total hands-on time for the Cell-Based ELISA is approximately 3 hours, which is significantly less than other techniques. In addition, Cell-Based ELISAs are amenable to high-throughput applications and may prove a valuable addition to kinase inhibitor screening strategies.

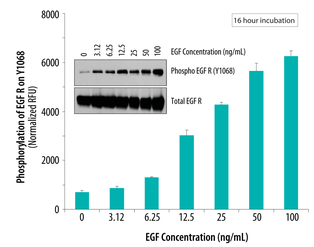
MATERIALS & METHODS

Product	Catalog #
Human/Mouse/Rat Phospho-Akt (S473) Pan Specific Cell-Based ELISA	KCB887
Human Phospho-EGF R (Y1068) Cell-Based ELISA	KCB1095
Human/Mouse/Rat Phospho-ERK1 (T202/Y204)/ ERK2 (T185/Y187) Cell-Based ELISA	KCB1018
Human/Mouse/Rat Phospho-FRS2 (Y436) Cell-Based ELISA	KCB5126
Human/Mouse/Rat Phospho-JNK (T183/Y185) Cell-Based ELISA	KCB1205
Human/Mouse Phospho-p38 MAP Kinase (T180/Y182) Cell-Based ELISA	KCB869
Human Total IκB-α Cell-Based ELISA	KCB4299

ASSAY SCHEMATIC



1A



1B

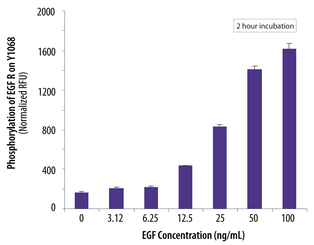
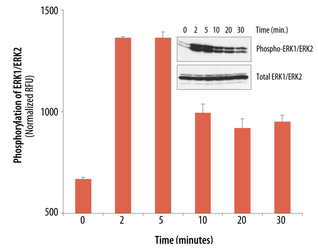


FIGURE 1: **Measurement of Total and Phosphorylated EGF R in A431 Cells Using R&D Systems Cell-Based ELISA.** A431 human epidermoid carcinoma cells were seeded at approximately 1.0 x 10^4 cells per well in 96-well plates 16 hours before treatment. The cells were treated with increasing amounts of Recombinant Human EGF (Catalog # 236-EG) for 5 minutes. After fixation of the cells, phosphorylation of EGF R on Y1068 was determined and normalized to total EGF R in the same well using the Phospho-EGF R (Y1068) Cell-Based ELISA (Catalog # KCB1095). Primary antibodies were incubated with the cells for 16 hours (**A**). The incubation time can be reduced to 2 hours for a one-day experiment with comparable results (**B**). Values represent the mean ± the range of duplicate determinations. Western blot analysis of phosphorylated and total EGF R using the antibodies supplied in this kit is shown for comparison (inset of Fig. 1A).

2A



2B

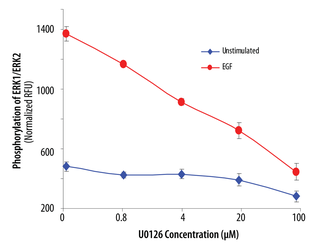


FIGURE 2: **Measurement of ERK1/ERK2 Phosphorylation in A431 Cells Using the Phospho-ERK1(T202/Y204)/ ERK2 (T185/Y187) Cell-Based ELISA.** A431 human epidermoid carcinoma cells were seeded at approximately 1.5 x 10^4 cells per well in 96-well plates 16 hours before treatment. The cells were treated with 100 ng/mL of Recombinant Human EGF (Catatog # 236-EG) for the indicated times (**A**), or were pretreated for one hour with the indicated concentrations of the MEK inhibitor U0126 and then incubated with no additions or with 1 ng/mL recombinant human EGF for 30 minutes (**B**). After fixation of the cells, phosphorylation of ERK1/ERK2 was determined and normalized to total ERK1/ ERK2 in the same well using the Phospho-ERK1 (T202/Y204)/ERK2 (T185/Y187) Cell-Based ELISA (Catalog # KCB1018). Values represent the mean ± the range of duplicate determinations. Western blot analysis of total and phosphorylated ERK1/ERK2 using the antibodies supplied in this kit is shown for comparison (inset of Fig. 2A).

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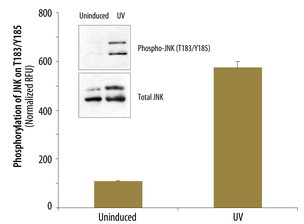

FIGURE 3: **Measurement of JNK Phosphorylation in HEK293 Cells Using the Phospho-JNK (T183/Y185) Cell-Based ELISA.** HEK293 human embryonic kidney cells were seeded at approximately 1.5 x 10^4 cells per well in 96-well plates 16 hours before treatment. The cells were uninduced or treated with UV (200 J/m^2). After fixation of the cells, phosphorylation of JNK on T183/Y185 was determined and normalized to total JNK in the same well using the Phospho-JNK (T183/Y185) Cell-Based ELISA (Catalog # KCB1205). Values represent the mean ± the range of duplicate determinations. Western blot analysis of total and phosphorylated JNK using the antibodies supplied in this kit is shown for comparison (inset).

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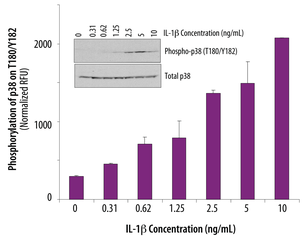
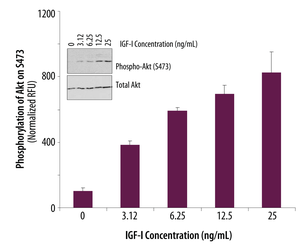


FIGURE 4: **Measurement of p38 Phosphorylation in HepG2 Cells Using the Phospho-p38 MAP Kinase (T180/ Y182) Cell-Based ELISA.** HepG2 human hepatocellular carcinoma cells were seeded at approximately 1.5 x 10^4 cells per well in 96-well plates 16 hours before treatment. The cells were treated with increasing amounts of Recombinant Human IL-1β (Catalog # 201-LB) for 25 minutes. After fixation of the cells, phosphorylation of p38 on T180/Y182 was determined and normalized to total p38 in the same well using the Phospho-p38 MAP Kinase (T180/Y182) Cell-Based ELISA (Catalog # KCB869). Values represent the mean ± the range of duplicate determinations. Western blot analysis of phosphorylated and total p38 using the antibodies supplied in this kit is shown for comparison (inset).

5A



5B

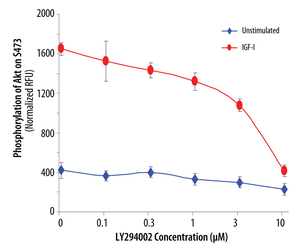
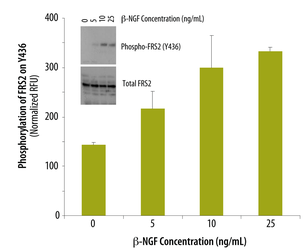


FIGURE 5: **Measurement of Akt Phosphorylation in MCF-7 Cells Using the Phospho-Akt (S473) Cell-Based ELISA.** MCF-7 human breast adenocarcinoma cells were seeded at approximately 1.5 x 10^4 cells per well in 96-well plates 16 hours before treatment. The cells were treated with increasing amounts of Recombinant Human IGF-I (Catalog # 291-G1) for 20 minutes (**A**), or were pretreated for 10 minutes with the indicated concentrations of the PI3-kinase inhibitor LY294002 and then incubated with no additions or with 25 ng/mL recombinant human IGF-I for 20 minutes (**B**). After fixation of the cells, phosphorylation of Akt on S473 was determined and normalized to total Akt in the same well using the Phospho-Akt (S473) Cell-Based ELISA (Catalog # KCB887). Values represent the mean ± the range of duplicate determinations. Western blot analysis of total and phosphorylated Akt using the antibodies supplied in this kit is shown for comparison (inset of Fig. 5A).

6A



6B

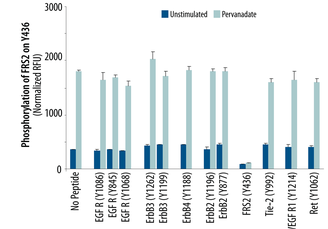


FIGURE 6: **Measurement of FRS2 Phosphorylation Using the Phospho-FRS2 (Y436) Cell-Based ELISA.** PC-12 rat adrenal pheochromocytoma cells were seeded at approximately 1.5 x 10^4 cells per well in 96-well plates 16 hours before treatment. The cells were treated with the indicated amounts of Recombinant Rat β-NGF (Catalog # 556-NG) for 10 minutes. After fixation of the cells, phosphorylation of FRS2 on Y436 was determined using the Phospho-FRS2 (Y436) Cell-Based ELISA (Catalog # KCB5126; **A**). Values represent the mean ± the range of duplicate determinations. Western blot analysis of total and phosphorylated FRS2 using the antibodies supplied in this kit is shown for comparison (inset of Fig. 6A). The specificity of this kit was further confirmed by peptide competition assay (**B**). CCD-10705K human foreskin fibroblasts in 96-well plates, untreated or treated with 100 µM Pervanadate for 10 minutes, were analyzed with this Cell-Based ELISA. The Phospho-FRS2 Antibody was either untreated (no peptide) or preincubated with the indicated phosphopeptides. Peptides were used at 5 ng/mL. A significant reduction of the phospho-FRS2 signal was observed only with the peptide containing the phospho-FRS2 (Y436) site, supporting the specificity of this Cell-Based ELISA for FRS2 phosphorylated at Y436.

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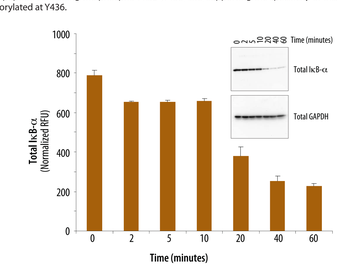

FIGURE 7: **Measurement of IκB-α Protein Levels in HeLa Cells Using the Total IκB-α Cell-Based ELISA.** HeLa human cervical cancer cells were seeded at approximately 1.5 x 10^4 cells per well in 96-well plates 16 hours before treatment. The cells were treated with 20 ng/mL of Recombinant Human TNF-α (Catalog # 210-TA) for the indicated times. After fixation of the cells, IκB-α levels were determined and normalized to total GAPDH, a housekeeping protein, in the same well using the Total IκB-α Cell-Based ELISA (Catalog # KCB4299). Values represent the mean ± the range of duplicate determinations. Western blot analysis of total IκB-α and total GAPDH using the antibodies supplied in this kit is shown for comparison (inset).

SUMMARY

Although *in vitro* biochemical kinase assays are routinely used for drug screening, they cannot replicate the intracellular environment. Cell-Based assays that measure unique events within specific signaling pathways may reflect the intracellular environment more closely. Our assay is the first dual-fluorescence Cell-Based ELISA to measure two intracellular proteins simultaneously within the same microplate well. Using this assay format, two target proteins can be analyzed or, alternatively, one protein can be used to normalize for well-to-well variations in cell number. Two types of assays have been developed. The first type of assay uses primary antibodies against phospho- and total proteins to assess the phosphorylation status of a specific signaling molecule. In the second type of assay, we employ antibodies against the protein of interest and a second housekeeping protein to determine the total protein levels of the target analyte. Both types of assays allow a reliable measurement of changes in cellular proteins in whole cells by ratiometric analysis of two wavelengths derived from the same well to minimize variability. Analyte detection using species-specific HRP- or AP-conjugated secondary antibodies in combination with spectrally distinct fluorogenic substrates allows for measurement with a standard fluorescence plate reader. Therefore, there is no need for specialized and expensive equipment. The effects of well-studied kinase inhibitors on their respective kinases were also evaluated using the Cell-Based ELISA technique, and the results were similar to those previously reported using more traditional techniques.