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

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ASSAY SCHEMATIC

STEP 1

Cell and antibodies are added. Incubated for 2 hours at room temperature, and primary antibodies are washed away.

STEP 2

Primary antibodies from two different host species are added. After 1-hour incubation, unbound antibodies are washed away.

STEP 3

Two species-specific secondary antibodies, labeled with either HRP or 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.

STEP 4

Fluorescence signal is measured in a plate reader, and the results are 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.

SUMMARY

Although in vitro biochemical kinase assays are routinely used for drug screening, they cannot recapitulate the intracellular environment. Cell-based assays that measure unique events within specific signaling pathways may reflect the intracellular environment more closely. Our assays are the first dual-fluorescent Cell-Based ELISAs to measure two intracellular proteins simultaneously within the same microplate well. Using this assay format, two target proteins can be analyzed or, alternatively, one target 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, primary antibodies to specific phosphorylation sites are used to assess the phosphorylation status of a specific signaling molecule.

MATERIALS & METHODS

Primary Antibodies

- Human/Mouse/Rat Phospho-4E-BP1 (Ser65) ( Catalog # XP40)
- Pan Specific Cell-based ELISA
- Human Phospho-EGF R (Y1186) Cell-based ELISA
- Human/Mouse/Rat Phospho-ERK1 (T202/Y204)/ERK2 (T185/Y187) Cell-based ELISA
- Human/Mouse/Rat Phospho-PAK (T180/Y182) Cell-based ELISA
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- Human/Mouse/Rat Phospho-PAK (T180/Y182) Cell-based ELISA
- Human Phospho-PAK (T180/Y182) Cell-based ELISA
- Human Total ERK1/2 Cell-based ELISA

Secondary Antibodies

- Anti-target protein-HRP-labeled secondary antibody
- Anti-target protein-AP-labeled secondary antibody

Procedure

STEP 1

5000 cultured human skin fibroblasts in 96-well plates, untreated or treated with 100 μM Pervanadate for 10 min, were analyzed using the Cell-Based ELISA. The cells were treated with 100 ng/mL of Recombinant Human EGF (Catalog # 236-EG) for 10 min. After treatment, the cells were fixed, permeabilized, and blocked and incubated with primary antibodies against phospho-p38 (T180/Y182) and total p38. Two species-specific secondary antibodies, labeled with either HRP or AP, and spectrally distinct fluorogenic substrates for each enzyme, were used for detection. The fluorescence of the phosphorylated protein was normalized to that of the total protein in each well.

STEP 2

Fluorescence signal is measured in a plate reader, and the results are 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.

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