A Cell-Based, Microplate Format, DELFIA Assay for Determination of the Activation of MAP Kinase

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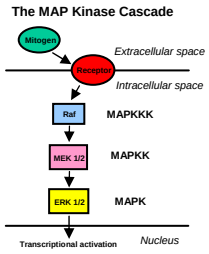
1 Introduction

Mitogen-activated protein kinases (MAPK) are activated in response to extracellular signals or cellular stress. We have set up a cell-based DELFIA® Time-Resolved Fluorescence assay to test compounds or conditions for their ability to modulate cellular stress by the activation of MAPK. The cell-based DELFIA® assay is performed in 96 well microplate format and quantitatively detects phosphorylation of MAPK. The assay replaces classical gel-electrophoresis and Western blotting techniques by the utilization of cells as the target of the assay instead of isolated proteins. The assay quantitatively detects changes in the phosphorylation level using labeled antibodies against MAPK. We take advantage of the ability to multiplex the assay by using antibodies labeled with different lanthanide chelates for time-resolved fluorescence measurements. This allows for detection of different cellular events simultaneously, in the same sample. Additionally, a fluorescent label is used as a control of equal number of cells in the wells.

2 Mitogen-activated protein kinases

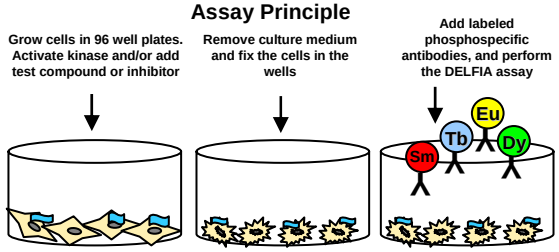
MAP kinases are serine/threonine protein kinases that are rapidly activated in response to various extracellular signals, such as growth factors, cytokines, and different types of cellular stress. Because phosphorylation of MAP kinase leads to its activation, the phosphorylation reflects its activity. The stress-induced phosphorylation of MAP kinase occurs rapidly; therefore, this event might be used as a marker for cellular stress. Considering this, it would be of interest to develop an assay in which the phosphorylation of MAP kinase is used as a tool for screening the effectivity of compounds and conditions that induce, or reduce cellular stress. In this study, we have developed a cell-based DELFIA® Time-Resolved Fluorescence assay for the phosphorylation of the p44^{MAPK} and p42^{MAPK} isoforms, ERK1 and ERK2, respectively.



3 Materials & Methods

CHO cells were used exclusively for all the experiments. The cells were cultured in RPMI medium with 10% serum and passaged routinely. For the experiments, the cells were plated in 96-well Viewplates™ (PerkinElmer 6005181) at 10 000 - 20 000 cells/ well, and grown for 1 – 2 days. Prior to addition of the test substance, the cells were serum-starved for 20 hours. The reaction was terminated by removal of the test substance and addition of a fixation solution consisting of 4% formaldehyde and 0.1% Triton in PBS. Unspecific binding was reduced by blocking with 1% BSA for 1 hour, prior to addition of 500ng/mL antibodies and 1µM SYTO24® (Molecular Probes) in DELFIA® Assay Buffer (1244-111). The antibodies were labeled with the DELFIA Eu-N1 ITC chelate (AD0001) or with the DELFIA Sm-N1 ITC chelate (AD0005). In this study we used the phospho-p44/42 MAP Kinase (Thr202/Tyr204), and the phospho-IκB-α (Ser32/36) antibodies (Cell Signaling Technology # 9101 and # 5205 respectively). The cells were incubated with the antibodies for 1 – 24 hours. The plates were washed 4 times with DELFIA® Wash Solution (1244-114) using an automated plate washer. The lanthanides were dissociated using DELFIA® Enhancement Solution (1244-105), 5 minutes incubation on shake. The SYTO24® fluorescence was measured in the Enhancement Solution (485/535nm). The plates were measured on the VICTOR^{2}V™ or on the EnVision™ plate readers.

4 Assay Principle



5 Time- and dose-dependent activation of MAPK

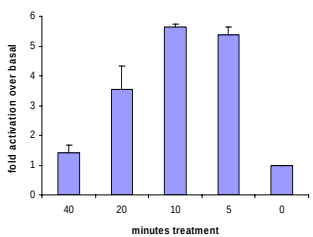


Figure 1. H_2O_2-stimulated activation of MAPK (phospho-p44/42) in CHO cells. The cells were treated with 0.1% H_2O_2 for the indicated periods of time. Data represents an average of 3 separate experiments performed on different days.

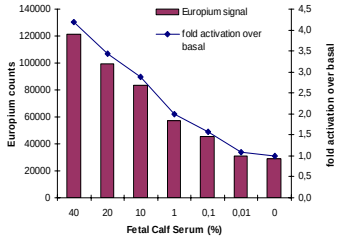


Figure 2. CHO cells were serum-starved for 20 hours, and then stimulated with an increasing concentration of serum for 5 minutes for phosphorylation of MAPK. The data is shown as Europium counts, and as fold activation of stimulated cells to untreated cells.

6 Inhibition of MAPK

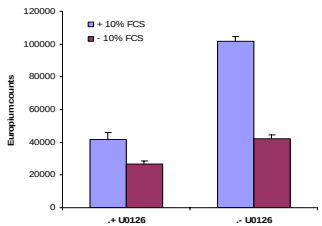


Figure 3. U0126 inhibition of MAPK activation in CHO cells stimulated with serum. The data is shown as Europium counts from cells treated with the MEK 1/2 inhibitor U0126, compared to uninhibited cells. The cells were pretreated with 10µM of U0126 for 1 hour prior to activation of MAPK by addition of 10% serum.

7 Stability

For convenience, the incubation with the labeled antibody can be performed as an overnight incubation. The europium signal remains stable after incubation overnight at +4°C.

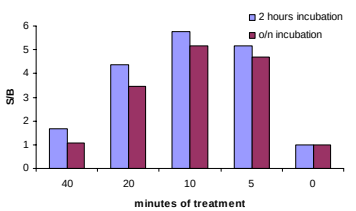


Figure 4. Peroxide-induced activation of MAPK. The signal remains stable also after an overnight antibody-incubation at +4°C. Data is shown as signal (treated cells) to background (untreated cells).

8 Multiplexing with two kinases

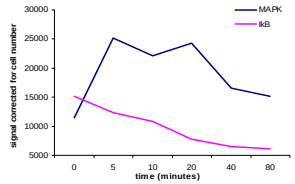


Figure 5. Phosphorylation, and dephosphorylation of the two kinases was induced by treating the cells with peroxide. The phosporylated MAPK was detected using an antibody labeled with samarium, while the phosphorylated IkB was detected using an europium-labeled antibody.

9 Normalisation

A control for equal number of cells in each well need to be included when performing cell-based assays with whole, fixed cells. Live cells grown in microtiter plates will dislodge easily during the different assay steps, especially when the cells are treated with various, potentially toxic compounds. We have included the SYTO24® nucleic acid stain to serve this purpose. The fluorescent label is not interfering with time-resolved fluorescence. The cell-permeant nucleic acid stain exhibits bright, green fluorescence upon binding to nucleic acids.

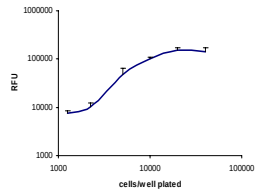


Figure 6. CHO cells were plated at increasing density and grown overnight. The cells were fixed and labeled with SYTO24® nucleic acid stain. Data represents an average of 6 parallel samples.

10 Conclusions

We have shown detection of the activation of MAPK in CHO cells using a method with whole cells and lanthanide-labeled antibodies. This assay is faster and more applicable to screening, compared to traditional Western blotting or ELISA assays with transfer of cell lysate. The advantage with this type of assay is that of using whole cells in high content screening, it enables the researcher to determine the effect of compounds in the presence of all the other associated proteins within the kinase cascade. Multi-component pathways will be present in a whole cell environment, and assays to determine kinase activation in such a system take account of these factors. Optimizations have to be done for each cell line and each antibody separately.