ALPHASCREEN APPLICATION NOTE ASC-013

Application Note

MAP Kinase Assay

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Introduction

AlphaScreen™ MAP Kinase Assay was designed to measure ERK1/2 MAP kinase activity present in purified enzyme preparations or in cell extracts. The assay was developed using a very active ERK1 preparation (soluble cell extract prepared from insect cells infected with Raf1/MEK1/ERK1) made at PerkinElmer. However, it can easily be applied to ERK1 extracted from other sources and to other MAP kinases.

Mitogene-activated protein kinases (MAPKs) represent important targets for the development of therapeutic agents for a wide spectrum of diseases, including neurodegenerative disorders, inflammation, cancer and septic shock. Mammalian MAPK signaling cascades regulate important processes, including gene expression, cell proliferation, cell motility as well as cell survival and death. All cascades include a MAPK, MAPK kinase (MAPKK, MEK or MKK) and a MAPKK kinase (MAPKKK or MEKK). The archetype and best characterized of these cascades is the one leading to activation of ERK1 and ERK2, also known as p44mapk and p42mapk, respectively.

Principles of AlphaScreen Technology

AlphaScreen is a bead based non-radioactive **A**mplified **L**uminescent **P**roximity **H**omogeneous **A**ssay. When a biological interaction brings the beads together, a cascade of chemical reactions act to produce a greatly amplified signal. On laser excitation, a photosensitizer in the "Donor" bead converts ambient oxygen to a more excited singlet state. The singlet state oxygen molecules diffuse across to react with a thioxene derivative in the Acceptor bead, generating chemiluminescence at 370 nm that further activates fluorophores contained in the same bead. The fluorophores subsequently emit light at 520-620 nm.

In the absence of a specific biological interaction, the singlet state oxygen molecules produced by the Donor bead go undetected without the close proximity of the Acceptor bead. As a result, only a very low background signal is produced.

AlphaScreen provides a highly versatile, sensitive, homogeneous and miniaturizable means to efficiently perform assay development and HTS resulting in higher throughput at lower costs.

To maximize AlphaScreen signal detection, the AlphaQuest® HTS Microplate Analyzer and Fusion-Alpha™ Multilabel Reader were developed with the capability to measure assays in multi-well plates. These instruments use a highly efficient laser diode emitting at 680 nm, fiber optics and specially optimized photomultiplier tubes. For further details on AlphaScreen technology, refer to: *A Practical Guide to Working with AlphaScreen* (reference no. S4077).

AlphaScreen MAP Kinase Assay

The AlphaScreen Protein A assay kit was used to measure the activity of ERK1 MAP kinase present in an insect cell extract. In this assay, active ERK1 kinase phosphorylates a synthetic biotinylated peptide substrate derived from myelin basic protein (MBP). The resulting phosphorylated peptide is bound by streptavidin-coated Donor beads and by specific anti-phospho-MBP P12 monoclonal antibody bound to Protein A-conjugated Acceptor beads as shown in Figure 1. The Protein A approach presented here allows for substitution with a wide range of MAP kinase substrate/ antibody pairs.

Materials and Methods

The AlphaScreen Protein A Assay Kit (catalog number 6760617C) is composed of Donor-Streptavidin and Acceptor-Protein A beads.

ERK1 cell extract was prepared from insect cells infected with Raf1/MEK1/ERK1, synthetic biotinylated MBP-derived peptide (FFKNIVTPRTPPPSQGK) was from AnaSpec Inc., and anti-phospho-MBP antibody was purchased from Upstate Biotechnology Inc. (catalog no. 05-429).

The kinase buffer was composed of 8 mM Hepes (pH 7.4), 4 mM MgCl2, 0.25 mM DTT. The detection buffer (2.5X concentrated) contained 100 mM Hepes (pH 7.4), 100 mM EDTA and 0.25 % BSA.

The AlphaScreen MAP kinase assay involves the following three steps:

1. Mix ERK1 MAP kinase, biotinylated MBP-derivedpeptide substrate and ATP in a well of a 384-well plate: (ex.: PerkinElmer OptiPlate™ 384 well plates cat. No. 6007290 and 6007299); incubate for 30 minutes at room temperature (RT).

2. Quench by adding detection buffer containing EDTA, Donor-Streptavidin and Acceptor-Protein A beads/anti-phospho-MBP antibody mixture; incubate for 1 hour at RT.

3. Detect AlphaScreen signal using an AlphaQuest HTS Microplate Analyzer or a Fusion-Alpha Multilabel Reader.

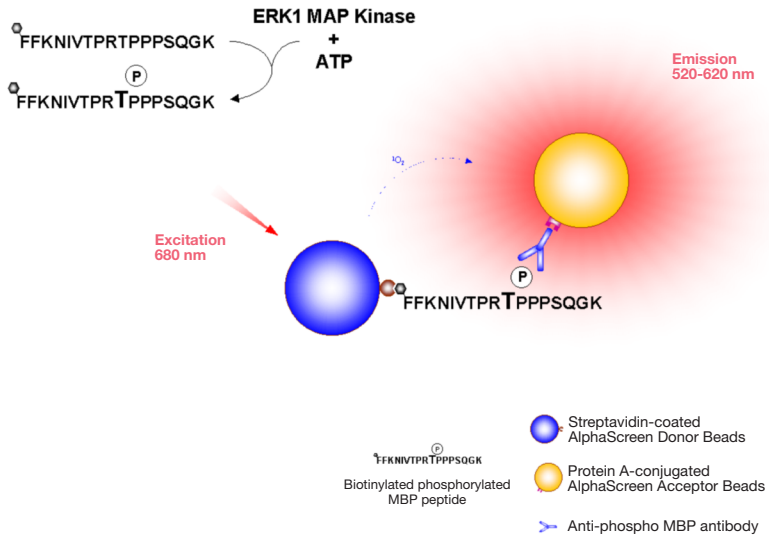

Figure 1. *Phosphorylated polypeptide bound by streptavidin-coated Donor beads and by specific anti-phospho-MBP antibodies bound to Protein A-conjugated Acceptor beads.*

Materials and Methods (continued)

All assays were performed in white, opaque 384-well plates (ex.: PerkinElmer OptiPlate 384 well plates cat. No. 6007290 and 6007299) in a final volume of 25 µL using 5 µL ERK1 extract, 10 µL biotin-peptide/ATP mix and 10 µL detection mix. For inhibition experiments, ERK1 extract was pre-incubated with 5 µL staurosporine for 15 minutes prior to adding 5 µL biotin-peptide/ATP mix. Anti-phospho-MBP antibody was used at 1 nM, and Donor-Streptavidin and Acceptor-Protein A beads were used at 20 µg/mL each.

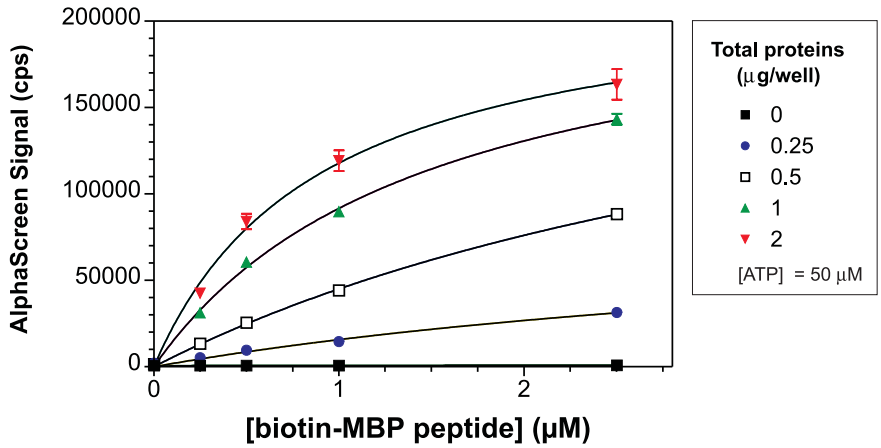

Figure 2. *Titration of Enzyme and Substrate.*

Results

Titration of Enzyme and Substrate For assay sensitivity and linearity, substrate should be used at a concentration equal to or below its K_m, and the quantity of enzyme should be as low as possible while maintaining a good S/B ratio.

The optimal amount of ERK1 extract and optimal concentrations of peptide substrate have been determined by titration of each component in the kinase assay. The apparent K_m values for MBP peptide substrate were 4.8, 4.4, 1.5 and 0.9 µM for total protein quantities of 0.25, 0.5, 1.0 and 2.0 µg/ well, respectively. As shown in Figure 2, a maximal substrate concentration of 1 µM and 1 µg or less of total protein in cell extract must be used to meet the assay requirements.

ATP Titration
The K_m for ATP, measured for the kinase reaction at 0.5 µM peptide substrate using 1 µg of ERK1 extract, was determined to be 94 µM (Figure 3). Optimal ATP concentration should range between 10 and 100 µM to run the assay near or below the K_m while maintaining a good signal-to-background ratio.

Time Course of ERK1 Phosphorylation

A time course experiment was performed using different enzyme quantities to assess the linearity of the assay. As shown in Figure 4, a quantity of ERK1 extract as low as 0.25 µg showed a linear response over 70 minutes and generated AlphaScreen counts of 55,000 after 60 minutes, corresponding to a S/B ratio of 85 (background = signal obtained in absence of enzyme). At 0.5 µg and 1 µg of ERK1 extract, activity was linear over 40 and 30 minutes, respectively. One µg of enzyme resulted in a S/B ratio of 40 after a 30-minute incubation at room temperature. These conditions were used as standard conditions for subsequent experiments.

A lag phase was observed within the first minutes, which can be attributed to either the low amount of product formed during this period (below the affinity of the antibody for this product), the slow ATP binding or the fact that the enzyme was not purified.

Inhibition by Staurosporine

Staurosporine, a general kinase inhibitor, was tested against ERK1 at two ATP concentrations, 25 and 50 µM. The IC50 value at 50 µM of ATP was determined to be 150 nM. This value was reduced by half (71 nM) when ATP concentration was decreased proportionally, as expected with a competitive inhibition by staurosporine (Figure 5).

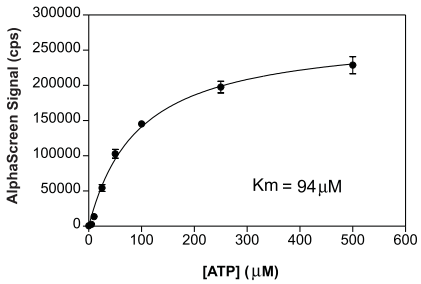

Figure 3. *ATP Titration.*

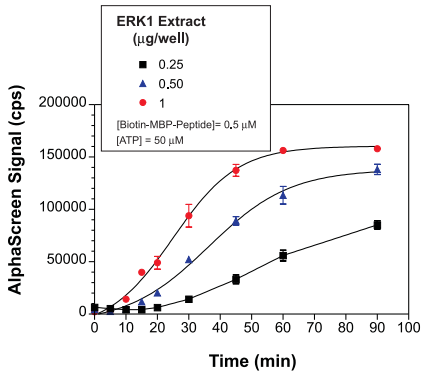

Figure 4. *Time Course of ERK1 Phosphorylation.*

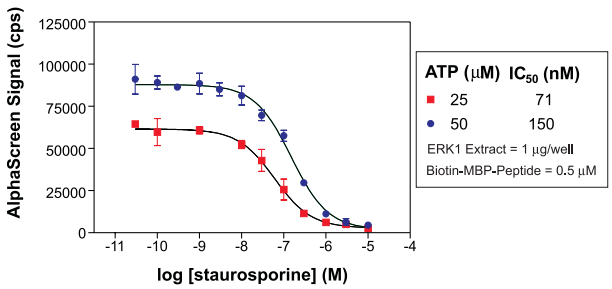

Figure 5. *Staurosporine Inhibition.*

Conclusion

AlphaScreen MAP Kinase assay is a homogenous, miniaturized and fully automatable non-radioactive screening assay for ERK1 MAP Kinase. The assay was proven to be very sensitive and reproducible in detecting phosphorylated substrates. Due to the versatility of the AlphaScreen Protein A-bound Acceptor beads used, the assay could be applied to any other MAP kinases, purified or present in soluble cell extracts, for which specific MAP Kinase substrates and antibodies are available.

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