Development of a Homogeneous p38 Kinase Assay using AlphaScreen™ Technology

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Abstract

Mitogen-activated protein kinases (MAPK) play a central role in the cellular response to environmental stress, growth factors, and cytokines. The serine/threonine kinase, p38, is a member of the MAPK family and has been shown to be a critical enzyme in cell proliferation and the secretion of cytokines. Intense efforts are underway to find inhibitors of this enzyme for the treatment of inflammatory diseases and cancer. AlphaScreen™ is a homogeneous, luminescent proximity assay useful for studying a wide variety of biomolecular interactions. Here, we report the development of an AlphaScreen p38 kinase assay by monitoring the phosphorylation of activating transcription factor 2 (ATF-2). A dose-response titration of the p38 inhibitor, SB203580, yielded an EC₅₀ of 100 nM with a Z' factor of 0.60 and a Signal:Background of greater than 16. These results exemplify the use of AlphaScreen technology for the screening of p38 kinase inhibitors.

Introduction

AlphaScreen is a bead based, non-radioactive, Amplified Luminescent Proximity Homogeneous Assay platform for use in a variety of drug discovery formats including enzyme assays (kinase, helicase, protease, etc.), interaction assays (ligand/receptor, protein/protein, protein/DNA), immunoassays, and GPCR functional assays (cAMP, IP₃).

AlphaScreen relies on the use of Donor and Acceptor beads. 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 to generate chemiluminescence at 370 nm that further activates fluorophores contained in the same bead. These 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. As a result, only a very low background signal is produced.

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Materials and Reagents

- AlphaScreen Protein A Detection Kit
- OptiPlate-384 NEW
- phospho-ATF-2 (Thr71) Antibody
- ATF-2/GST fusion protein
- biotin-ATF-2/GST fusion protein
- p38a/SAPK2a kinase
- SB 203580
- PerkinElmer Life Sciences (Cat. # 6760617C)
- PerkinElmer Life Sciences (Cat. # 6007290)
- Cell Signaling Technology (Cat. # 9221S or 9221L)
- Cell Signaling Technology (Cat. # 9224)
- Upstate Biotechnology (Cat. # 12-432)
- Upstate Biotechnology (Cat. # 14-251)
- Calbiochem (Cat. # 559389)

Kinase Buffer:
- 20 mM HEPES pH 7.0, 10 mM MgCl₂, 1 mM DTT, 0.01% Tween 20

Stop/Detection
- 20 mM HEPES pH 7.0, 200 mM NaCl, 80 mM EDTA, 0.3% BSA

AlphaScreen p38 Kinase Assay
Biotinylated Peptide Approach

Requirements:
- AlphaScreen Protein A Detection Kit
- biotinylated ATF-2 substrate
- p38 enzyme
- phospho-ATF-2 (Thr71) Ab
Cross-titration of Biotin ATF-2/GST Substrate and p38 Enzyme

Optimization of enzyme and substrate concentrations. p38 enzyme was titrated from 1-30 nM in conjunction with titration of biotin ATF-2/GST substrate in kinase buffer supplemented with 100 µM ATP for 60 min. The phosphorylation of substrate was detected with 3 nM phospho-ATF-2 (Thr71) Ab with 20 µg/mL Donor and Acceptor beads for 60 min prior to reading on a Fusion™-α Multilabel Reader.

Determination of Optimal phospho-ATF-2 (Thr 71) Ab Concentration

Optimization of phospho-ATF-2 (Thr71) Ab concentration. p38 enzyme (10 nM) was incubated with 30 nM biotin ATF-2/GST substrate in kinase buffer supplemented with 100 µM ATP for 60 min. The phosphorylation of substrate was detected with 0-10 nM phospho-ATF-2 (Thr71) Ab with 20 µg/mL Donor and Acceptor beads for 60 min prior to reading on a Fusion-α Multilabel Reader.
AlphaScreen p38 Kinase Assay
Biotinylated Anti-GST Ab Approach

Requirements:
- AlphaScreen Protein A Detection Kit
- ATF-2/GST substrate
- p38 enzyme
- phospho-ATF-2 (Thr71) Ab
- biotin α-GST Ab

Cross-titration of ATF-2/GST Substrate and p38 Kinase Concentrations

Optimization of enzyme and substrate concentrations. p38 enzyme (1-10 nM) was incubated with 0-30 nM ATF-2/GST substrate in kinase buffer supplemented with 100 mM ATP for 60 min. The phosphorylation of substrate was detected with 1 nM biotin anti-GST antibody + 3 nM phospho-ATF-2 (Thr71) Ab with 20 mg/mL donor and acceptor beads for 60 min prior to reading on a Fusion-α Multilabel Reader.
Determination of Optimal ATF-2/GST Substrate and Biotin Anti-GST Ab Concentrations

Optimization of antibody and substrate concentrations. p38 enzyme (3 nM) was incubated with 0-30 nM ATF-2/GST substrate in kinase buffer supplemented with 100 mM ATP for 60 min. The phosphorylation of substrate was detected with 0.3-10 nM biotin anti-GST antibody + 3 nM phospho-ATF-2 (Thr71) Ab with 20 mg/mL Donor and Acceptor beads for 60 min prior to reading on a Fusion-α Multilabel Reader.

Method

- 5 µL of p38 kinase to OptiPlate wells
- 5 µL of ATP
- 1.5 µL of SB 203580 dilutions

Incubate for 20 minutes at RT

- Add 3.5 µL of biotin ATF-2/GST substrate or ATF-2/GST substrate

Incubate for 60 minutes at RT

- Add 10 µL of Acceptor/Donor beads containing phospho-ATF-2 (Thr71) Ab or phospho-ATF-2 (Thr71) Ab + biotin anti-GST Ab

Incubate for 1 hour, in the dark, at RT

Read plate on Fusion-α or AlphaQuest-HTS
Monitoring p38 Kinase Activity by AlphaScreen
Comparison of Methodologies

Inhibition of p38 activity by SB 203580. p38 enzyme (10 nM) was pre-incubated for 20 min prior to incubation with either 30 nM biotin ATF-2/GST substrate or 10 nM ATF-2/GST substrate in kinase buffer supplemented with 100 µM ATP for 60 min. The phosphorylation of substrate was detected with 3 nM phospho-ATF-2 (Thr71) Ab +/- 3 nM biotin anti-GST Ab with 20 µg/mL Donor and Acceptor beads for 60 min prior to reading on a Fusion-α Multilabel Reader.

2 different methodologies validated and yield similar results

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<th>Advantages</th>
<th>Disadvantages</th>
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<tr>
<td>Biotinylated</td>
<td>• Single Antibody approach</td>
<td>• Smaller signal window</td>
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<tr>
<td>ATF-2/GST Substrate</td>
<td>• Can be used with any biotinylated substrate</td>
<td>• Enzyme less efficient at phosphorylating biotin substrate</td>
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<tr>
<td>ATF-2/GST Substrate +</td>
<td>• Large signal window</td>
<td>• Anti-GST Antibody may bind to Protein A and produce higher background</td>
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<tr>
<td>biotinylated anti-GST Ab</td>
<td>• Less enzyme and substrate required</td>
<td>• 2 Antibodies required</td>
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Z’ values greater than 0.5 achieved with both approaches

AlphaScreen provides a sensitive and homogeneous HTS platform to measure p38 kinase activity

Conclusions