PerkinElmer™
life sciences.

Development of a Homogeneous p38 Kinase Assay using AlphaScreen™ Technology

Gregory Warner[1], Robert Mercuri[2], Christopher Bunker[3], and Roger Bossé[2]

[1]PerkinElmer Life Sciences, Boston, MA 02118, USA,
[2]PerkinElmer Life Sciences, Montreal, Quebec, H3J 1R4 , Canada,
[3]Cell Signaling Technology, Beverly, MA 01915, USA

1 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 EC_{50} 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.

2 Introduction

AlphaScreen is a bead based, non-radioactive, **A**mplified **L**uminescent **P**roximity **H**omogeneous **A**ssay 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, IP_3).

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.

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. Herein, two different assay approaches were evaluated to measure p38 kinase activity. Both assays utilize ATF-2/GST fusion protein as a substrate for p38 kinase. However they differ by the way in which the substrate is coupled to the Donor bead. In one, the substrate is biotinylated to provide a direct coupling to the Donor bead. The other approach utilizes a biotin anti-GST antibody to bind the GST moiety of the substrate and couple it to the Donor bead. The results of both assay methodologies are presented.

3 Materials and Reagents

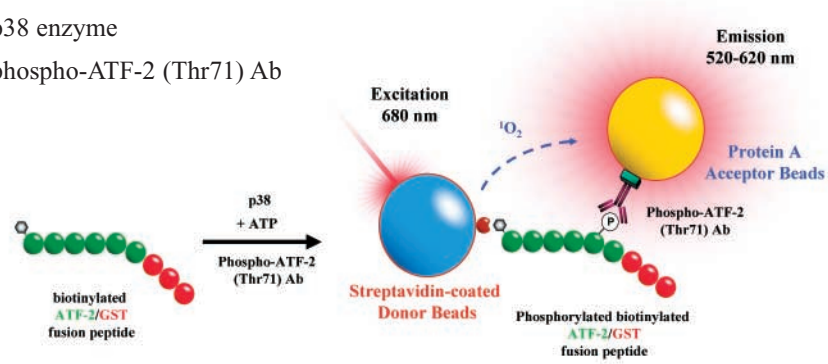
• AlphaScreen Protein A Detection Kit	› PerkinElmer Life Sciences	(Cat. # 6760617C)
• OptiPlate-384 NEW	› PerkinElmer Life Sciences	(Cat. # 6007290)
• phospho-ATF-2 (Thr71) Antibody	› Cell Signaling Technology	(Cat. # 9221S or 9221L)
• ATF-2/GST fusion protein	› Cell Signaling Technology	(Cat. # 9224)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

- Kinase Buffer:
  - › 20 mM HEPES pH 7.0, 10 mM $MgCl_2$, 1 mM DTT, 0.01% Tween 20
- Stop/Detection
  - › 20 mM HEPES pH 7.0, 200 mM NaCl, 80 mM EDTA, 0.3% BSA

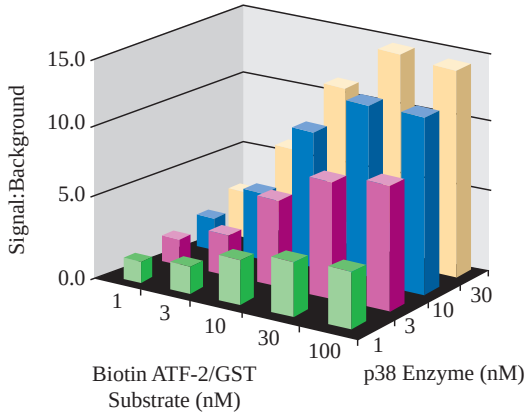
4 AlphaScreen p38 Kinase Assay Biotinylated Peptide Approach

Requirements:

- AlphaScreen Protein A Detection Kit
- biotinylated ATF-2 substrate
- p38 enzyme
- phospho-ATF-2 (Thr71) Ab

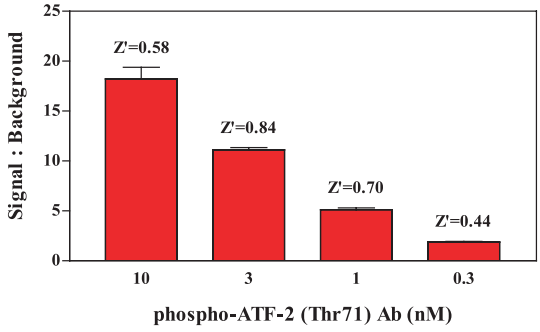


5 Cross-titration of Biotin ATF-2/GST Substrate and p38 Enzyme



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.

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



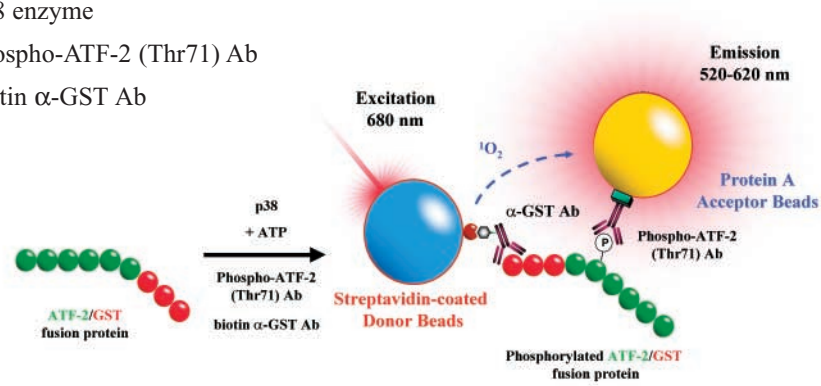
Optimization of phospho-ATF-2 (Thr71) Ab concentration. p38 enzyme (10 nM) was incubated with 30 nM biotin ATF-2/GST substrate in kinase buffer supplemented with 100 µM ATP for 60 min. The phosphorylation of substrate was detected with 0-10 nM phospho-ATF-2 (Thr71) Ab with 20 µg/mL Donor and Acceptor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

7

AlphaScreen p38 Kinase Assay Biotinylated Anti-GST Ab Approach

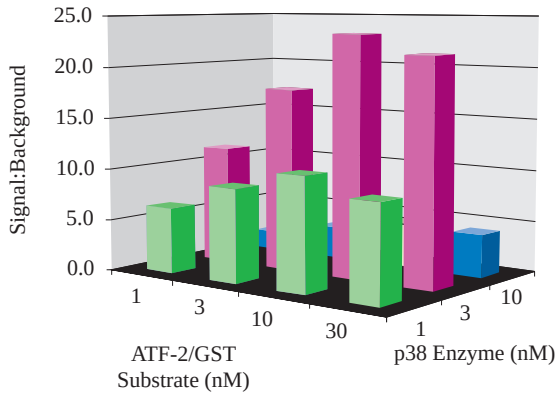
Requirements:

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



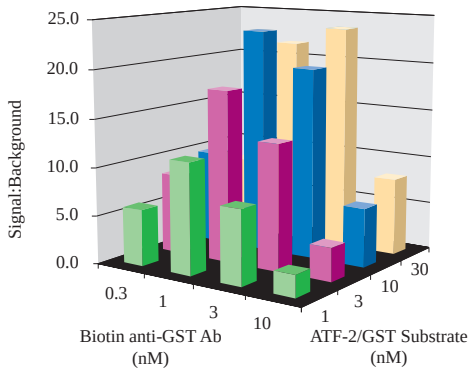
8

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



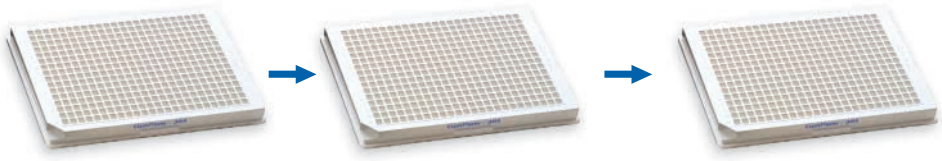
Optimization of enzyme and substrate concentrations. p38 enzyme (1-10 nM) was incubated with 0-30 nM ATF-2/GST substrate in kinase buffer supplemented with 100 mM ATP for 60 min. The phosphorylation of substrate was detected with 1 nM biotin anti-GST antibody + 3 nM phospho-ATF-2 (Thr71) Ab with 20 mg/mL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

9 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.

10 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

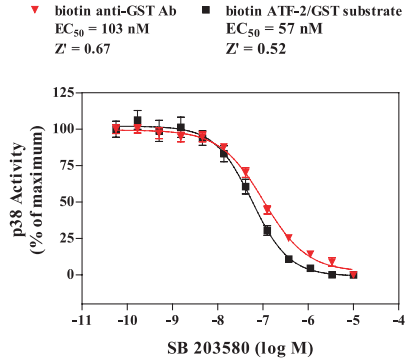
Incubate for 1 hour, in the dark, at RT

Read plate on Fusion-α or AlphaQuest-HTS

11

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.

12

Conclusions

- **2 different methodologies validated and yield similar results**

	Advantages	Disadvantages
Biotinylated ATF-2/GST Substrate	• Single Antibody approach • Can be used with any biotinylated substrate	• Smaller signal window • Enzyme less efficient at phosphorylating biotin substrate
ATF-2/GST Substrate + biotinylated anti-GST Ab	• Large signal window • Less enzyme and substrate required	• Anti-GST Antibody may bind to Protein A and produce higher background • 2 Antibodies required

- **Z' values greater than 0.5 achieved with both approaches**
- **AlphaScreen provides a sensitive and homogeneous HTS platform to measure p38 kinase activity**

Worldwide Headquarters: PerkinElmer Life Sciences, Inc., 549 Albany Street, Boston, MA 02118-2512 USA (800) 551-2121

European Headquarters: PerkinElmer Life Sciences, Inc., Imperiastraat 8, BE-1930 Zaventem Belgium

Technical Support: in Europe: techsupport.europe@perkinelmer.com in US and Rest of World: techsupport@perkinelmer.com

Belgium: Tel: 0800 94 540 • **France:** Tel: 0800 90 77 62 • **Netherlands:** Tel: 0800 02 23 042 • **Germany:** Tel: 0800 1 81 00 32 • **United Kingdom:** Tel: 0800 89 60 46

Switzerland: Tel: 0800 55 50 27 • **Italy:** Tel: 800 79 03 10 • **Sweden:** Tel: 020 79 07 35 • **Norway:** Tel: 800 11 947 • **Denmark:** Tel: 80 88 3477 • **Spain:** Tel: 900 973 255

S4062 09/02

www.perkinelmer.com/lifesciences