APPLICATION NOTE

Measurement of p38/MAPK activity using LANCE.

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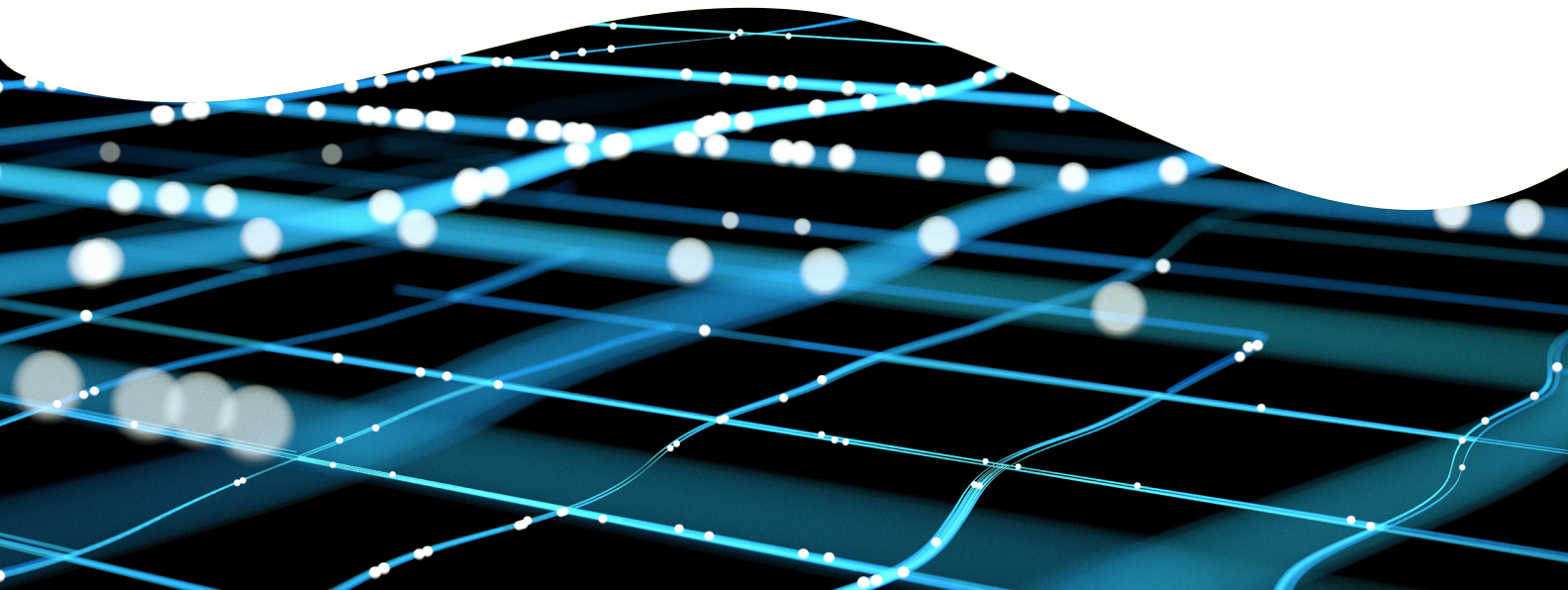
Introduction

Protein kinases regulate several important functions within cells including metabolism, cell cycle progression, angiogenesis, cell adhesion, etc. Specifically, 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 immunological disorders, including rheumatoid arthritis and cancer.

Time-resolved fluorometry (TRF) is a well-established technology that exploits the unique fluorescence properties of lanthanide chelates to provide a powerful alternative to radioisotopic assays in many HTS applications (Hemmilä and Mukkala, 2001). TRF assays exhibit low background and high signal-to-background ratios, two attributes critical for robust HTS assays. Long fluorescence decay after excitation allows time-delayed signal detection (microseconds) to virtually eliminate all natural fluorescent background caused by cells and cell debris, screening compounds, plates, and other reagents (half-life of nanoseconds). A large Stokes shift (e.g., excitation and emission wavelengths for the Eu-chelate are ~340 nM and ~615 nM, respectively) minimizes crosstalk, resulting in a high signal-to-background ratio. Therefore, because of their excellent temporal and spectral resolution, lanthanide chelate labels provide high sensitivity assays.



LANCE™ refers to homogeneous time-resolved fluorometry applications using TR-FRET (TR-fluorescence resonance energy transfer assay). Homogeneous assays particularly benefit from time-resolved fluorometry because the sample constituents present during detection cause very high background fluorescence when conventional fluorochromes are used. TR-FRET is based on the proximity of a Eu-chelate and a fluorophore that have been brought together by a specific binding reaction. The excited energy of the Eu-chelate is transferred by a resonance mechanism to an acceptor within a short distance (~ 15 nM). Fluorescent lanthanide chelates with long excited-state lifetimes are used to avoid interference caused by emission from acceptor molecules excited directly rather than by energy transfer. LANCE is widely used for a wide variety of assay types including: kinase, nuclear receptor and protein binding assays.

Herein, we present a method to evaluate inhibitors of p38 kinase activity using LANCE. This assay is similar to an assay developed using the AlphaScreen™ technology platform, however, here we are measuring energy transfer from a Eu-labeled antibody to a fluorophore-conjugated antibody binding to the GST portion of the fusion substrate. FRET will only occur when both antibodies are in close proximity and bound to the phosphorylated substrate. The results of an antibody optimization and inhibition curves with a known inhibitor of p38 kinase are presented.

Materials

OptiPlate-384	Revvity Life and Analytical Sciences	(Cat. #6007290)
SureLight™ Allophycocyanin-anti-GST	Revvity Life and Analytical Sciences	(Cat. #AD0059G)
LANCE Eu-W1024 labeled anti-rabbit IgG	Revvity Life and Analytical Sciences	(Cat. #AD0082)
p38α/SAPK2a kinase	Upstate Biotechnology	(Cat. #14-251)
phospho-ATF-2 (Thr71) Antibody	Cell Signaling Technology	(Cat. #9221L or 9221S)
ATF-2/GST fusion protein	Cell Signaling Technology	(Cat. #9224)
SB 203580 kinase inhibitor	Calbiochem	(Cat. #559389)
ATP	Sigma Chemical	(Cat. #A9187)
10X LANCE detection buffer	Revvity Life and Analytical Sciences	(Cat. #CR97-100)
HEPES (1 M) buffered solution	Invitrogen	(Cat. #15630080)
10% Tween-20	Pierce	(Cat. #28320)
0.5 M EDTA, pH 8.0 solution	Invitrogen	(Cat. #15575020)

Protocol for LANCE p38 kinase assay

Reagents:

Prepare Kinase Assay Buffer:

20 mM HEPES pH 7.0, 10 mM $MgCl_2$, 1 mM DTT, & 0.01% Tween 20

Prepare 3X (9 nM) p38α/SAPK2a kinase

Upstate Biotechnology (Cat. #14-251)
Stock concentration of 3.9 µM.

Dilute 1.15 µL of stock kinase @ 3.9 µM to final volume of 500 µL in kinase assay buffer

Prepare 3X (300 µM) ATP solution

Stock concentration is 100 mM

Dilute 3 µL to 1.0 mL in kinase assay buffer

Prepare 4.29X (429 nM) ATF-2/GST fusion protein substrate

Cell Signaling Technology (Cat. #9224)
Stock concentration of 58.8 µM

Dilute 3.65 µL of 58.8 µM stock substrate to a final volume of 500 µL in kinase assay buffer

Prepare 10X SB 203580 dilutions

Calbiochem (Cat. #559389)

Make 10 mM stock in 100 % DMSO
Prepare 10% DMSO in kinase assay buffer by adding 200 µL DMSO to 1.8 mL kinase assay buffer

Make 10X "stocks" (i.e. adding 1.5 µL to a final kinase assay volume of 15 µL)

3-fold dilutions from 10 mM stock done in 10 % DMSO in kinase assay buffer

Prepare 2.5X LANCE stop/detection mixture

Prepare stop buffer: Make 1X LANCE detection buffer by diluting 1:10 in distilled H_2O

Add:

- EDTA to a concentration of 25 mM (final concentration of 10 mM in assay)
- SureLight™ Allophycocyanin-anti-GST at a concentration of 62.5 nM APC (final concentration of 25 nM in assay)
- LANCE Eu-W1024 labeled anti-rabbit IgG at a concentration of 5 nM APC (final concentration of 2 nM in assay)
- phospho-ATF-2 (Thr71) antibody at a concentration of 7.5 nM (final concentration of 3 nM in assay)

LANCE protocol

To a 384-well OptiPlate 384 add:

- 5 µL of 9 nM p38 kinase
- 5 µL of 300 µM ATP
- 1.5 µL of SB 203580 inhibitor dilutions

Incubate for 20 minutes at RT
Add 3.5 µL of 428 nM ATF-2/GST fusion protein substrate
Incubate for 60 minutes at RT
Add 10 µL of 2.5 X LANCE stop/detection mixture
Incubate for 1 hour, in the dark, at RT.
Read plate on Victor™, EnVision™, ViewLux™ or other time-resolved fluorescence capable reader

Using the previously developed AlphaScreen assay as a reference, the LANCE assay was designed as an indirect assay; meaning that the primary antibody is not labeled with europium. Here, an anti-species, europium-labeled secondary antibody was used in conjunction with the primary antibody and the acceptor molecule to generate the LANCE FRET signal.

In this case, the p38 kinase assay was set up using an ATF-2/GST conjugated substrate. The primary antibody is a phospho-specific ATF-2 antibody recognizing the phosphorylated Thr 71 of ATF-2. This is used in coordination with either a Eu-labeled anti-rabbit or anti-mouse IgG and SureLight Allophycocyanin-anti-GST to generate the TR-FRET signal (Figure1).

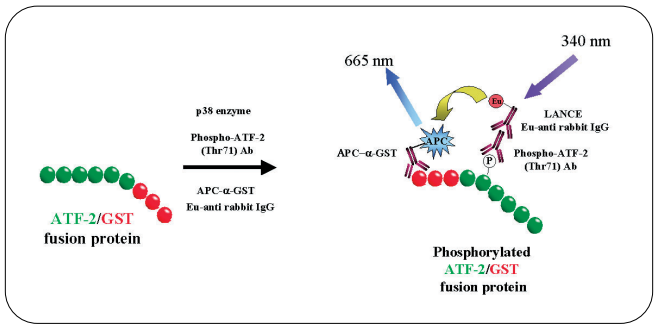


Figure 1: Schematic diagram of LANCE p38 kinase assay.

To determine the best primary antibody to use, we compared commercially available phospho-ATF-2 antibodies raised in rabbits and mice. Both antibodies were used at the same concentration and all other components were identical.

phospho-ATF-2 (Thr71) antibody comparison

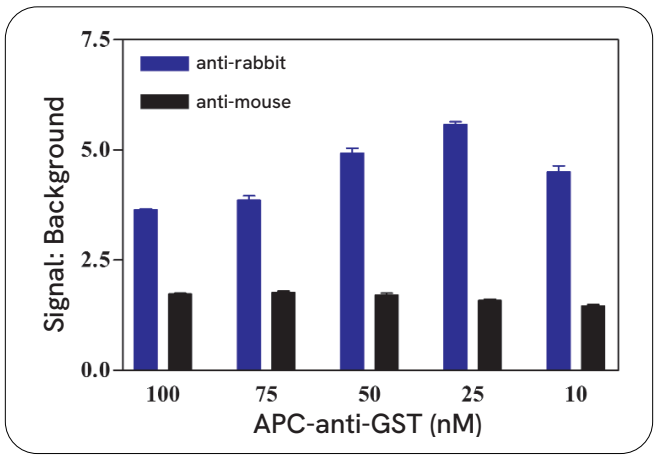


Figure 2: Comparison of phospho-ATF-2 Antibodies and Titration of APC-anti-GST. p38 enzyme (3 nM) was incubated with 100 nM ATF-2/GST substrate in kinase buffer supplemented with 100 µM ATP for 60 min. The phosphorylation of substrate was detected with 3 nM phospho-ATF-2 (Thr71) rabbit Ab or 3 nM phospho-ATF-2 (Thr71) mouse Ab, 2 nM LANCE Eu-labeled anti-rabbit IgG or 2 nM LANCE Eu-labeled anti-mouse IgG, and various amounts of SureLight APC-anti GST ranging from 10-100 nM for 60 min prior to reading with an EnVision Multilabel Plate reader using 330 nM excitation and both 665 nM and 615 nM emission filters.

As seen in Figure 2, the combination of rabbit phosphospecific ATF-2 and Eu-labeled anti-rabbit antibodies performed much better in this assay than the mouse combination at all levels of APC-anti-GST tested. In addition, the amount of SureLight APC-anti GST that gave the best results appeared to be 25 nM. These conditions were then used to test for inhibitors of p38 kinase activity using a commercially available inhibitor, SB203580.

Inhibition of p38 kinase activity

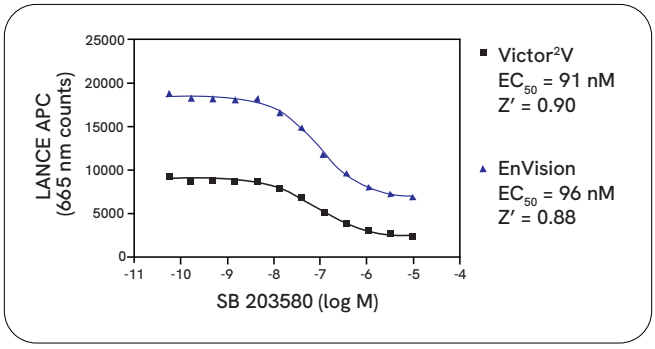


Figure 3: Inhibition of p38 kinase activity by SB 203580. p38 enzyme (3 nM) was pre-incubated for 20 min with SB 203580 dilutions prior to incubation with 100 nM ATF-2/GST substrate in kinase buffer supplemented with 100 µM ATP for 60 min. The phosphorylation of substrate was detected with 3 nM phospho-ATF-2 (Thr71) Ab + 2 nM LANCE Eu-labeled anti-rabbit IgG and 25 nM SureLight APC-anti GST for 60 min prior to reading with both Victor2V and EnVision Multilabel Plate readers.

The results of the experiments (Figure 3) reflect dose-dependent decreases in kinase activity with increasing amounts of SB 203580. The EC_{50} obtained in these experiments are consistent with literature values (34 nM). In addition, the performance of both the Victor²V and EnVision are equivalent, but the read times of the EnVision are nearly 5 times faster.

Conclusions

- The performance of different phospho-ATF-2 antibodies vary greatly and therefore testing of different antibodies is highly recommended.
- Titration of the SureLight APC-anti GST can increase your S:B
- This indirect LANCE assay provides a sensitive and homogeneous HTS platform to measure p38 kinase activity without the need to Eu-label your primary antibody.

References

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