APPLICATION NOTE

KINASE ASSAYS

Development of an AlphaScreen assay to measure the activity of the serine/threonine kinase JNK3

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Introduction

Protein kinases are directly implicated in many human diseases including inflammatory diseases and cancer. Kinase inhibitors show great promise as new therapeutic drugs. Herceptin®[1] and Iressa[2] are representative examples of drugs currently used to fight breast and non-small cell lung cancers respectively.

Kinases can be divided into two main families based on the amino acid they phosphorylate on their respective targets: tyrosine or serine/threonine. Since various generic and high affinity antibodies (ex. PY20, PT66, p-Tyr-100) can detect phosphotyrosine residues, many homogeneous and non-radioactive HTS technologies can measure tyrosine kinase activity with high efficiency. This is not the case for serine/threonine kinases due to the lack of good generic anti-phosphoserine/threonine antibodies. Recognition of phosphoserine/threonine residues by antibodies is known to be sequence specific. Large peptide sequences or entire phosphorylation domains are thus needed to generate selective and high affinity antibodies for specific serine/threonine kinase substrates. Due to the constraints mentioned above, assays to measure the activity of serine/threonine kinases often work best when complete phosphorylation domains are used as substrates instead of short peptide sequences. This represents a challenge for HTS technologies such as TR-FRET, where assay partners should interact in the same plane within 7 nm proximity; or fluorescence polarization where kinase peptide substrates are limited to approximately 20 amino acids.

AlphaScreen™ is a two bead-based proximity-dependent chemical energy transfer luminescent assay platform. Unlike many other energy transfer chemistries, AlphaScreen permits partners to be up to 200 nm apart. This makes AlphaScreen ideal for serine/threonine kinase assays involving either large peptide or complete protein sequences. As an example, we have developed an assay, shown in Figure 1, to measure the activity of JNK3 based on the phosphorylation of its substrate c-Jun(1-79), a 50 kDa GST fusion protein.

The c-Jun N-terminal kinase (JNK) family are serine/threonine protein kinases that phosphory-

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late c-Jun[3], a component of the transcription factor activator protein-1 (AP-1)[4]. JNK kinases are members of the mitogen-activated protein kinase (MAPK) family including the extracellular regulated kinases (ERKs) and p38 kinases. Three JNK genes (JNK1, 2, and 3) have been identified in humans so far. JNK1 and JNK2 have a broad tissue distribution, whereas JNK3 seems primarily localized to neuronal tissues and cardiac myocytes. JNK activity appears essential for both the immune response and programmed cell death. Finding inhibitors of JNK3 activity may lead to effective drugs to treat diseases such as arthritis, stroke, myocardial infarction, ischemic injury and Parkinson's disease.

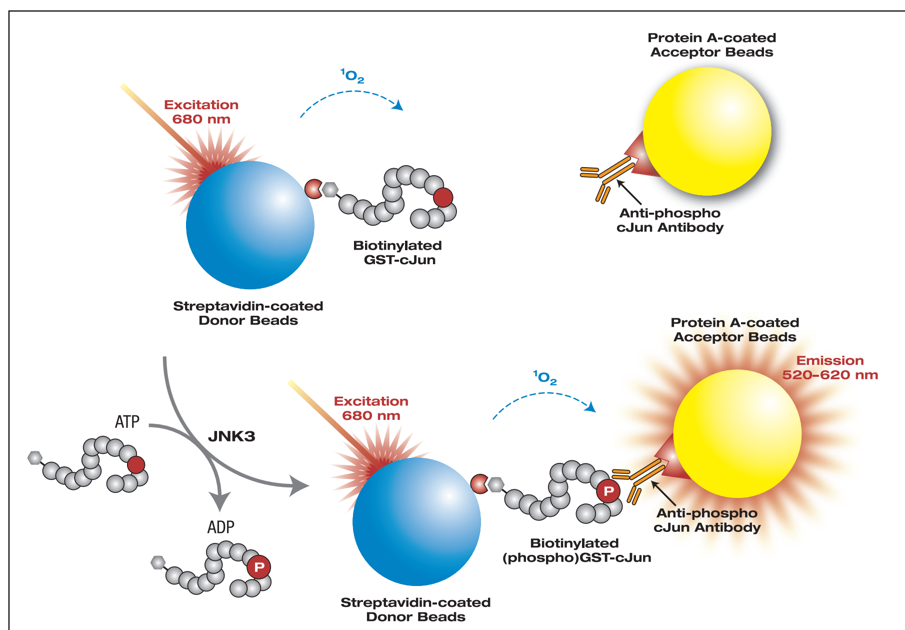

Figure 1. **AlphaScreen JNK3 assay configuration**. Biotin-GST c-Jun is phosphorylated by JNK3 to produce biotin-(phospho)GST-c-Jun. This product is then simultaneously captured by streptavidin-coated Donor beads and anti-(phospho)c-Jun antibodies bound to Protein A coated Acceptor beads. Both Donor and Acceptor beads are thus brought into proximity to generate a signal detectable between 520–620 nm after laser excitation at 680 nm.

Materials and Methods

Materials

AlphaScreen General IgG (Protein A) Detection Kit (#6760617), TruHits Kit™ (#6760627), OptiPlate™-384 white opaque 384-well microplates (#6007290) and TopSeal-A™ 384 (#6005250) were obtained from PerkinElmer Life and Analytical Sciences (Boston, MA, USA). GST-cJun (1-79) fusion protein (#205145) was ordered from Stratagene (La Jolla, CA, USA). Recombinant JNK3/SAPK1b active (#14-501) and anti-(phospho)cJun (Ser73) antibody (#06-659) were purchased from UpState (Charlottesville, VA, USA). Staurosporine (#S4400) was purchased from Sigma. EZ-Link™ Biotin-BMCC (#21900) and D-Salt Polyacrylamide Desalting Columns (#43240) were purchased from Pierce (Rockford, IL, USA). AlphaScreen assays were read on an EnVision™ HTS Multilabel Plate Reader with an AlphaScreen module (#2101-0010) from PerkinElmer (Boston, MA, USA).

GST-cJun biotinylation

GST-c-Jun fusion protein was biotinylated as follows: 500 µL of protein (1 mg/mL) were mixed with 500 µL of 0.1 M MES buffer, then 2.25 µL of a 10 mg/mL solution of biotin-BMCC were added and incubated two hours at 37 °C. GST-cJun was then desalted on a PD-10 column equilibrated in PBS containing 0.01% Tween-20. Biotinylated GST-cJun was titrated using the AlphaScreen TruHits assay to determine the concentration of biotinylated protein detectable by the streptavidin Donor beads.

Kinase assay

Kinase reagents (biotin-GST-cJun, anti-(phospho)cJun antibody and active JNK3) were first diluted in kinase buffer (20 mM Tris-HCl pH 7.6, 10 mM $MgCl_2$, 1 mM DTT, 100 µM Na_3VO_4, 0.01% Tween-20) and added to wells (15 µL). Reactions were then incubated in the presence of 10 µM of ATP for 1h at 23 °C. Detection was performed by an addition of 10 µL of bead mix (Protein A Acceptor 20 µg/mL and streptavidin Donor 20 µg/mL), diluted in detection buffer (20 mM Tris-HCl pH 7.4, 20 mM NaCl, 80 mM EDTA, 0.3% BSA), followed by another one hour incubation at 23 °C in the dark. The AlphaScreen signal was analyzed directly on the EnVision.

Results

One of the primary tasks was to optimize the kinase assay conditions using recombinant kinase. Based on previous AlphaScreen assay developments[5], fixed concentrations of biotin-GST-cJun (10 nM) anti-phospho cJun (30 nM), and enzyme (3 nM) were used to determine the enzyme K_m for ATP. As shown in Figure 2, the AlphaScreen signal increased in an ATP dose-dependent manner, reaching a maximum at 10 µM of ATP.

No signal was observed in the absence of JNK3. We then evaluated the optimal concentration of substrate using 10 µM ATP and

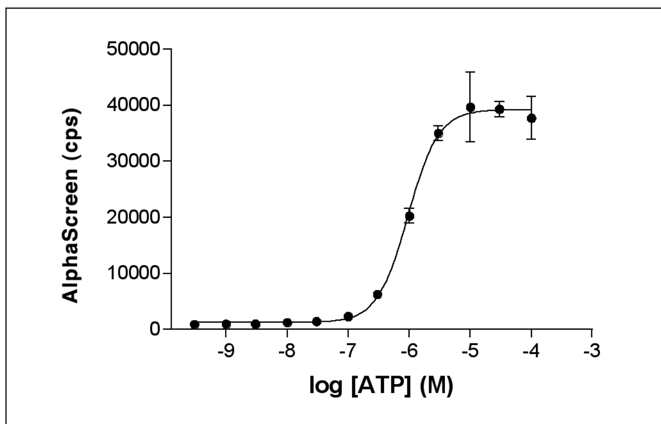

Figure 2. **ATP titration**

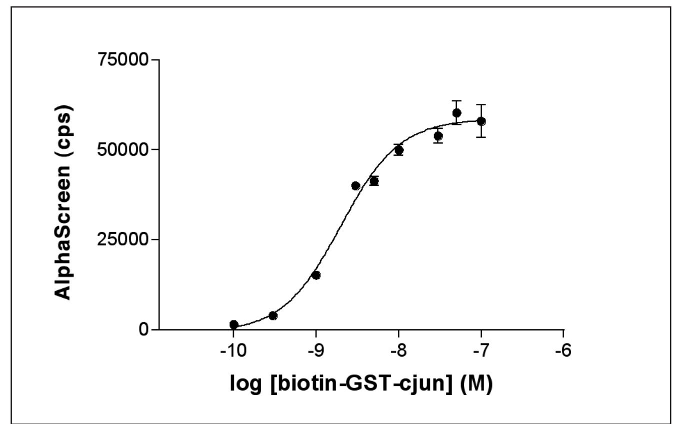

Figure 3. **Substrate titration**

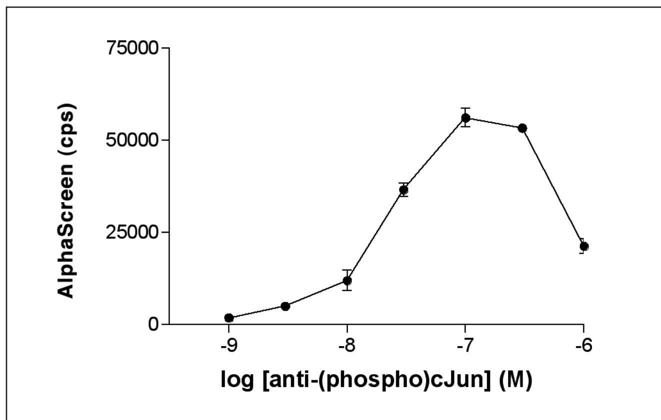

Figure 4. **Anti-(phospho)cJun IgG titration**

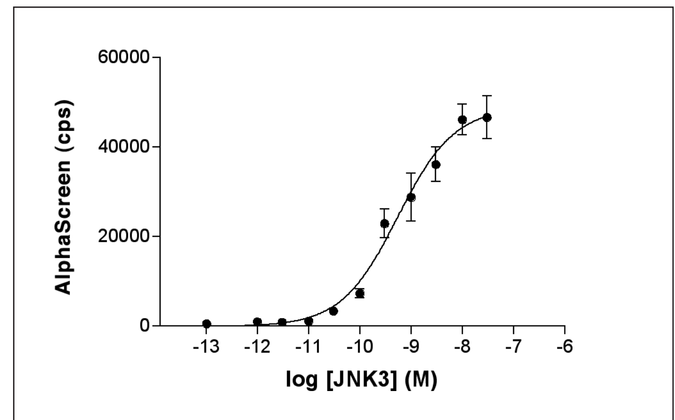

Figure 5. **Enzyme titration**

fixed concentrations of anti-phospho cJun (30 nM) and JNK3 (3 nM) (Figure 3). The emission was maximal with 10 nM of substrate (biotin-GST-cJun). We thus pursued the assay development using the latter concentration of substrate.

The same approach was followed to optimize the anti-(phospho)cJun antibody concentration. The maximal signal was obtained at 100 nM of antibody Figure 4. To develop an assay at the lowest cost possible, and supported by the fact that the signal was already excellent at 30 nM of antibody (S:B = 45), we chose to pursue the assay development using this precise concentration.

We then performed a dose-response curve using increasing JNK3 concentrations in the optimized conditions (10 nM ATP, 10 nM biotin-GST-cJun and 30 nM anti-phospho cJun antibody). As shown in Figure 5, the linearity of the response had more than two logs of dynamic range (0.1–10 nM) with a Hill slope around 1. Based on these results, 3 nM JNK3 was used for subsequent assay development steps.

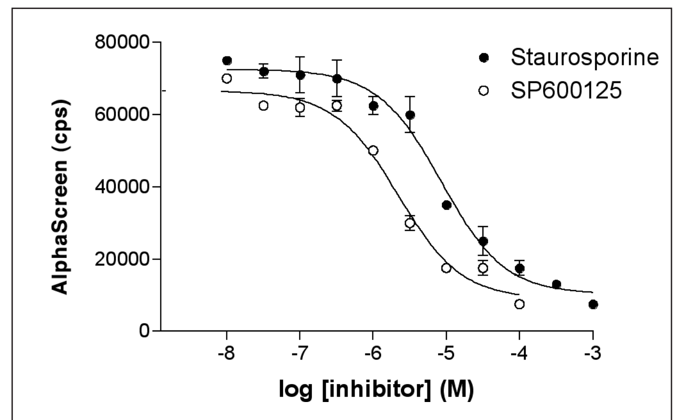

Figure 6. **Enzyme inhibition using conventional kinase inhibitors**

Using optimized assay parameters (10 µM ATP, 3 nM JNK3, 10 nM biotin-GST-cJun and 30 nM anti-(phospho)cJun IgG), we validated the assay using conventional kinase inhibitors Staurosporine and SP600125.

As observed in Figure 6, increasing concentrations of both compounds produced proportional signal decreases resulting from a progressive enzyme inhibition.

The rank order of potency and IC_{50} values for the inhibition of JNK3 activity by Staurosporine and SP600125 were consistent with values reported in the literature.

Discussion

Protein kinases show potential for becoming the major drug targets of this century given that more than 500 of these proteins have been shown to be encoded by the human genome[6]. The wide majority of these encode for serine kinases. Efficient assay platforms are therefore needed to validate, characterize and eventually perform HTS on this particular class of targets. Based on its unique capacity to handle large protein complexes, AlphaScreen represents an ideal tool to accomplish this.

Thus we used AlphaScreen to develop an assay for measuring the activity of the MAP kinase JNK3 based on the phosphorylation of the δ-domain of c-Jun(1–79) expressed as a 50 kDa GST fusion protein. Based on the price and the medium affinity of the anti-(phospho)cJun antibody for its antigen, we used an indirect assay format using Protein A coated Acceptor beads instead of conjugating the antibodies directly onto raw Acceptor beads. Capturing antibodies with Protein A-coated Acceptor beads offers the advantage of aligning the antibodies through their heavy chains so the light chains become optimally oriented to react with their specific antigens.

Straightforward assay development was conducted using reagents commercially available. Titrations of the essential assay components revealed that robust assay conditions could be obtained using nM concentrations of substrate, kinase and antibodies. Under optimized conditions, inhibition of JNK3 activity was measured using two conventional inhibitors: Staurosporine and SP600125. The rank order of potency and IC_{50} values for the inhibition of JNK3 activity by Staurosporine and SP600125 were consistent with values reported in the literature.

Conclusion

The results clearly demonstrate that AlphaScreen is highly effective in developing robust and sensitive assays that measure the activity of serine/threonine kinases involving entire phosphorylation domains. The robust assay conditions obtained using nM concentrations of reagents and the expected pharmacodynamic results obtained under these conditions, indicate that AlphaScreen is an enabling solution to produce cost-effective screens for serine/threonine kinases.

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