Homogeneous and Non-radioactive Cellular Assay Platforms for the Characterization of Kinase Inhibitors.

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1 Abstract

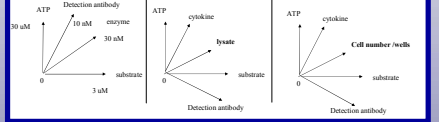
Protein kinases are directly implicated in many human diseases such as diabetes and cancer. Therefore kinase inhibitors show great promises as new therapeutic drugs. In an effort to facilitate the screening and the characterization of such inhibitors, we have developed a luminescence-based high throughput screening (HTS) method to measure their effect on kinase activity both *in vitro* and in cell based assay.

Using a protein substrate (GST-cJun), we demonstrate that before performing assay optimization, three well known technologies (AlphaScreen™, LANCE™, and FlashPlate®) were able to monitor kinase activity using purified enzyme (JNK3). However, only the LANCE and AlphaScreen technology generated a significant signal when measuring the activity of stimulated endogenous kinase (Il-1b stimulated JNK3). The exploitable signal obtained under non fully optimized assay conditions highlights the qualities of the latter two technologies.

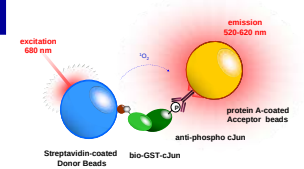
Following further optimization, AlphaScreen and LANCE allowed to monitor JNK3 activity from 1) purified kinase preparation and 2) endogenous kinase from whole cell lysates pre-activated with different cytokines (Il-1b and TNFα).

AlphaScreen only required minor tweaking to detect the activity of known JNK3 inhibitors and generated z' values of 0.5 with the cell based assay. Due to the versatility of the AlphaScreen, this cell-based JNK3 kinase assay could be adapted to other kinases and would represent a powerful tool to evaluate endogenous kinase activity and test a large number of potential inhibitors in a more physiological environment.

2 Process flow for cell-based kinase assay development

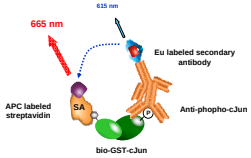


3 AlphaScreen kinase assay principle



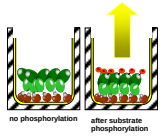
In the AlphaScreen™ assay, the phosphorylation of the biotinylated fusion protein substrate (bio-GST-cJun) is detected by a specific anti-phospho-cJun antibody. When protein A Acceptor beads are used in conjunction with the streptavidin Donor beads, only the phosphorylated substrate is able to bring the beads into close proximity resulting in the generation of an AlphaScreen signal. In the absence of phosphorylation, singlet oxygen can not reach the Acceptor beads and no signal can be observed.

4 LANCE kinase assay principle



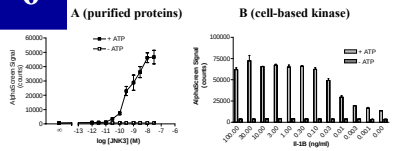
In the LANCE™ assay, phosphorylation of the same biotinylated fusion protein substrate (bio-GST-cJun) is detected by the same specific anti-phospho-cJun antibody as in the AlphaScreen assay. Upon JNK3 substrate phosphorylation, the anti-phospho antibody recognizing the phospho-substrate is captured by a secondary antibody labeled with europium (Eu). Upon phosphate addition, the phosphorylated substrate brings the APC into close proximity to the Eu, which permits the transfer of energy from Eu to APC with the resulting emission of light at 665 nm.

5 FlashPlate kinase assay principle



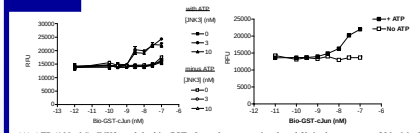
In the FlashPlate assay, streptavidin coated FlashPlates are used to capture the biotinylated substrate (bio-GST-cJun). Upon JNK3 substrate phosphorylation, incorporation of radio-labeled phosphate (P^{33} ATP), activation of the scintillant incorporated in the plates allows for the emission of light that is recorded by the TopCount (PerkinElmer LAS, Inc.).

6 AlphaScreen kinase assay performance



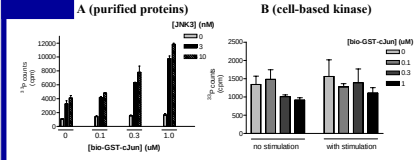
(A) In this assay, phosphorylated substrate (5 nM) is incubated in the presence of JNK3, ATP (10 uM), and the anti-phospho substrate antibody (30 nM). Following two hours incubation, protein A Acceptor beads are added to the kinase reaction with the streptavidin Donor beads, in the presence of EDTA. The detection reaction is incubated for 1h before reading. (B) Cells are plated at a concentration of 3000 cells/well and let to attach overnight. Cells are then stimulated with different concentration of Il-1b. Following 30 min stimulation, a mix of protein A Acceptor and streptavidin Donor beads is added to the kinase reaction. The reading is performed using the EnVision reader (PerkinElmer LAS, Inc.) after 1h incubation in the dark.

7 LANCE kinase assay performance



(A) ATP (100 uM), JNK3, and the bio-GST-cJun substrate are incubated 2h in the presence of 30 nM of the anti-phospho-cJun. The kinase reaction is stopped with EDTA (5 min), and APC labeled streptavidin (3 nM) is added to the reaction with the Eu labeled secondary antibody (3 nM). (B) Cells are plated at a concentration of 3000 cells/well. After overnight incubation, cells are stimulated for 30 min with 10 ng/ml of Il-1b and then incubated for 2h with ATP, anti-phospho-cJun (30 nM), and bio-GST-cJun substrate (30 nM) in the presence of Tween-20. (A) and (B), the kinase reaction is stopped with EDTA (5 min), and APC labeled streptavidin (3 nM) is added to the reaction with the Eu labeled secondary antibody (3 nM). Plates are read after 1h incubation on the EnVision Reader (PerkinElmer LAS, Inc.).

8 FlashPlate kinase assay performance

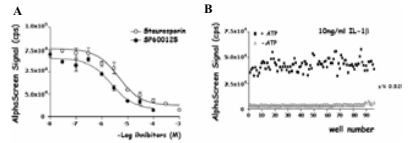


(A) In the Flashplate, bio-cJun and JNK3 are incubated 2 h at room temperature with a mix of cold and labeled ATP (100 nm vs 5 nM). (B) Cells are plated at a concentration of 3000 cells/well. After overnight incubation, medium is aspired and cells are stimulated for 30 min with 10 ng/ml of Il-1b. Following stimulation, bio-GST-cJun and a mix of cold and labeled ATP (100 nm vs 5 nM) are added to the wells and incubated for 2h. For both (A) and (B), the kinase reaction is stopped with EDTA (15 mM), and wells are washed twice with PBS before read out on TopCount reader (PerkinElmer LAS Inc.)

9 AlphaScreen Optimized assay protocols

A (purified proteins)	B (cell-based kinase)
Diluted in kinase reaction buffer:	Seed 3000 cells/well in microtiter plates
5 ul ATP	Incubate overnight
5 ul anti-phopho-antibody	Remove medium and add 5 ul of cytokine
5 ul biotinylated substrate	Incubate 30 minutes at 37C for 30 minutes
5 ul purified JNK3	Aspirate stimulating media and add:
Incubate 2 hours at 23C	5 ul of lysis Buffer
Diluted in detection buffer:	5 ul ATP diluted in Reaction Buffer
5 ul Protein A / SA Beads	5 ul Anti-phospho-cJun diluted in Reaction Buffer
Incubate 1 hour at 23C	5 ul Bio-GST-cJun diluted in Reaction Buffer
Read on EnVision Alpha	Incubate 2 hours at 23C
	5 ul Protein A / SA Beads diluted in Detection Buffer
	Incubate 1 hour at 23C
	Read on EnVision

10 AlphaScreen Optimized Cell-based kinase assay



Optimized all-in-one well measure of endogenous JNK activity in HepG2 cells. (A) Inhibition of endogenous IL-1b-induced JNK activity with kinase inhibitors [staurosporine (○) and SP600125 (●)] and (B) Agonist induced (10 ng/ml IL-1b) JNK activity measured in 384 wells/plate (n=96). Z' value of 0.52 indicates that the assay is amenable to HTS. The results presented in this figure are representative of three independent experiments.

11 Summary

We have demonstrated that **before** performing any optimization steps:

1) We were able to detect the phosphorylation of a protein kinase substrate using the three technologies presented using purified enzyme preparation.
2) AlphaScreen and LANCE were the only platforms able to generate signals using endogenous kinase from cell lysates, demonstrating the ease of use of these two technologies.

Following optimization of the AlphaScreen cell-based kinase assay, we demonstrated that:

1) We were able to confirm the specificity of the reaction by the effect of inhibitors on the Il-1b stimulated endogenous JNK3 enzyme
2) We were able to generate Z' values over 0,5 demonstrating the amenability of the AlphaScreen whole cell kinase assay to HTS protocols.

Please see presentation **P08028** by Roduit et al. entitled:
"Homogeneous and Non-radioactive HTS Platform for the Characterization of Kinase Inhibitors in Cells Lysates"