

## Image FlashPlate

# Tyrosine and Serine/Threonine Kinase Assays formatted on the Streptavidin Coated Image FlashPlate, for the ViewLux™ ultraHTS Microplate Imager

## Introduction

Protein kinases play an integral role in numerous signal transduction pathways that are involved in the regulation of cell growth, in cell differentiation and in response to changes in the extracellular environment. Kinases are therefore considered major targets for novel drug development to treat diseases such as cancer and various inflammatory disorders. Two protein kinases of interest are tyrosine and serine/phosphothreonine kinases, which phosphorylate hydroxyl containing amino acid residues in target. The recent introduction and clinical success of Gleevec, an inhibitor of tyrosine kinase Bcr-Abl for the treatment of chronic myelogenous leukemia, validates this class of biomolecules as viable therapeutic targets.

PerkinElmer Life and Analytical Sciences (PKLAS) has developed assays for both tyrosine (c-Src) and serine/threonine kinases (Akt1) using the Streptavidin Coated Image FlashPlate platform. An Image FlashPlate is a scintillant coated 384-well shallow well microplate that is designed to be used in non-separation, high throughput screening assays. A biotinylated peptide kinase substrate is captured on a Streptavidin Coated Image Flashplate and is phosphorylated

using [ $\gamma$ - $^{33}\text{P}$ ]-ATP as the phosphate donor. Following an aspiration and wash step to reduce the background signal, the kinase activity is quantitated by imaging the plates on the ViewLux™ ultraHTS Microplate Imager. Offering a significant advantage of being able to image whole plates simultaneously, regardless of density, in turn increases screening throughput to as many as 10,000 compounds per hour. Furthermore, the current assay format does not require the use of antibodies in the detection process. This offers a clear advantage for serine/threonine kinases such as Akt, since there are no commercially available non-selective anti-phosphoserine and anti-phosphothreonine antibodies.

A key to effective transition from assay development to a HTS environment, assays must possess suitable precision, as measured by the Z' value. Optimized conditions were determined by varying the amounts of kinase, [ $\gamma$ - $^{33}\text{P}$ ]-ATP, cold ATP, peptide substrate and incubation times. Under confirmed optimized conditions both assays gave Z' values greater than 0.5, indicating excellent performance as potential uHTS assays.

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## Materials and Methods

Src kinase (Catalog #14-177), Akt1 kinase (Catalog #14-276) and staurosporine (Catalog #19-123) were obtained from Upstate Biotechnology. The biotinylated protein tyrosine kinase substrate (PKS1, biotin-KVEKIGRGTYGVVYK-amide) was purchased from Promega (Catalog #V6480). The Akt1 biotinylated substrate (biotin-spacer-RPRAASF), corresponding to the sequence flanking the phosphorylation site in GSK-3, was prepared by SynPep Corporation as previously described.

Assays were carried out in 384-well Streptavidin Coated Image FlashPlates (PKLAS, Catalog # RMP110). A 15 mL solution was added to each well consisting of a reaction buffer (25 mM Tris-HCl, pH 7.5, 2 mM DTT, 10 mM MgCl<sub>2</sub>, and 0.1% BSA), the appropriate peptide substrate, [ $\gamma$ -<sup>33</sup>P]-ATP (PKLAS, Catalog #NEG302H), cold ATP (Sigma-Aldrich, Catalog #A7669) and if needed staurosporine. The kinase reaction was then initiated by adding 5uL of kinase solution, which was prepared by a pre-dilution in reaction buffer. The plate was then incubated for 3 hours at 37°C. The

contents of the wells were aspirated and the plate was washed three times with 1x PBS.

The plates were then imaged on the ViewLux at 4x4 binning for 3 minutes.

Data was analyzed with GraphPad Prism<sup>®</sup> software (GraphPad Software, Inc.) using non-linear regression analysis. The data points shown are the mean  $\pm$  1SD.

Z' values were used as a measure of assay precision and were calculated according to a published procedure.

## RESULTS

The amount of [ $\gamma$ -<sup>33</sup>P]-ATP tracer was titrated in Src and Akt kinase assays and the results are shown in Figure 1. The Src assay utilized 1 unit of enzyme, 0.33 mM cold ATP and 0.3 mM peptide substrate in each well. The Akt assay was performed with 250 ng of enzyme, 0.33 mM cold ATP and 0.3 mM peptide substrate per well. Results indicate that as little as 0.025-.05 mCi of [ $\gamma$ -<sup>33</sup>P]-ATP per well is suitable for the Src kinase assay. These conditions yield a signal to noise

ratio  $\geq$  18. Even though the Akt kinase assay requires more [ $\gamma$ -<sup>33</sup>P]-ATP per well, as little as 0.05-0.1 mCi is needed to produce a sufficient signal:noise ratio.

In order to determine the minimum amount of enzyme required to generate a sufficient signal, the Src and Akt kinases were titrated as shown in Figure 2. The Src kinase is supplied by the manufacturer in units and Figure 2 indicates that for this particular application 1 unit is necessary to generate a suitable signal. The Akt kinase is, however, supplied in nanograms and it was determined that 200 ng would be the ideal amount for this assay.

Kinase substrates were titrated along with the amount of cold ATP per well, while keeping the proportion of cold ATP:substrate constant. Maximal signal was obtained at 0.33mM substrate (6 pmol/well) for Src versus 0.66mM substrate (12 pMol/well) for Akt. The difference in binding capacity is most likely due to the fact that the Akt substrate is a significantly smaller peptide than the Src substrate. These assay conditions also dictate that 0.33mM cold ATP is needed for the Src assay and 0.66 mM ATP for the Akt assay.

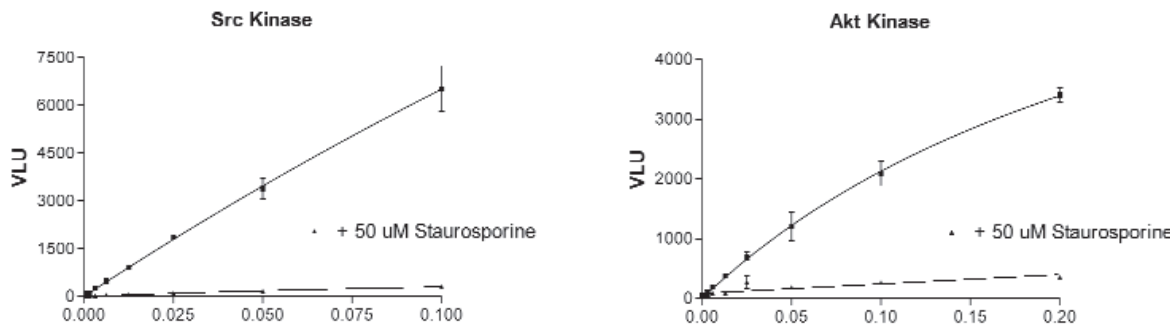


Figure 1. [ $\gamma$ -<sup>33</sup>P]-ATP tracer titration in Src and Akt kinase assays formatted on the Streptavidin Coated Image FlashPlate.

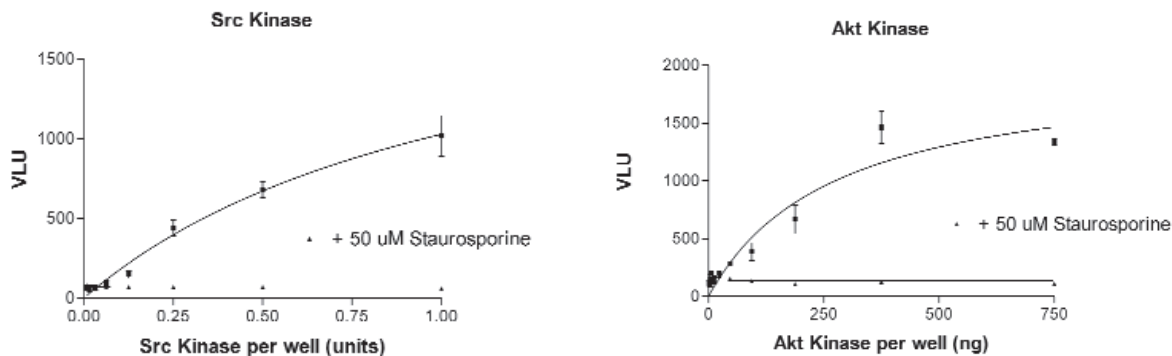


Figure 2. Enzyme titration in Src and Akt kinase assays formatted on the Streptavidin Coated Image FlashPlate. Src assay performed using 0.05 mCi of [ $\gamma$ -<sup>33</sup>P]-ATP per well; Akt assay performed with 0.1 mCi of [ $\gamma$ -<sup>33</sup>P]-ATP per well.

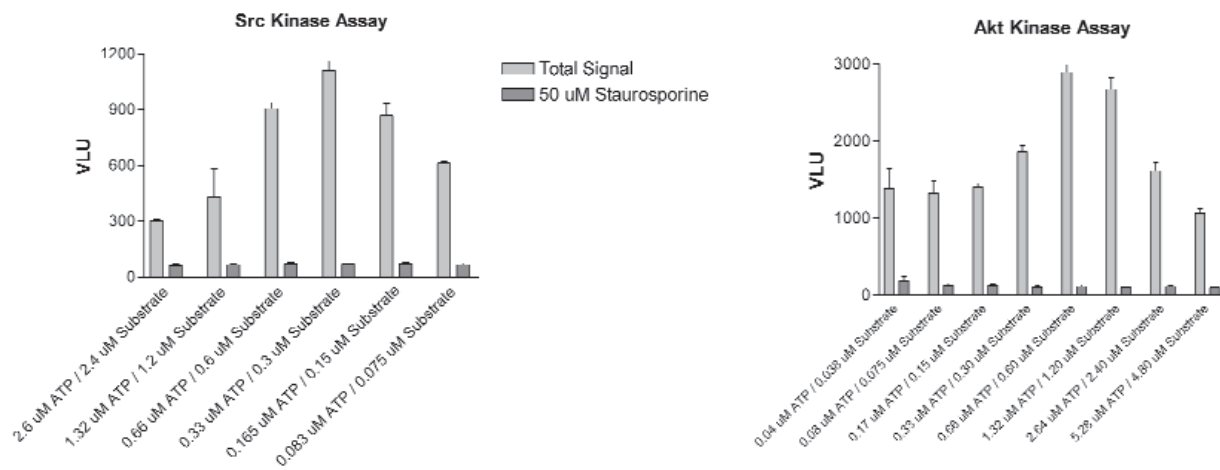


Figure 3. Enzyme titration in Src and Akt kinase assays formatted on the Streptavidin Coated Image FlashPlate.

Once optimum assay conditions were determined, inhibitor profiles and precision characteristics were examined. The activities of Src and Akt kinases are potently inhibited by staurosporine as shown in Figure 4. IC<sub>50</sub> values were within the expected range for such a broad spectrum kinase inhibitor. Z' values were calculated for both the Src kinase (1 unit of enzyme, 0.025 uCi [ $\gamma$ -<sup>33</sup>P]-ATP) and the Akt kinase (200 ng of enzyme, 0.05 uCi [ $\gamma$ -<sup>33</sup>P]-ATP). As seen in Figure 5, both assays gave excellent precision for HTS assay requirements. Optimum assay conditions for both enzymes and precision characteristics are summarized in Table 1.

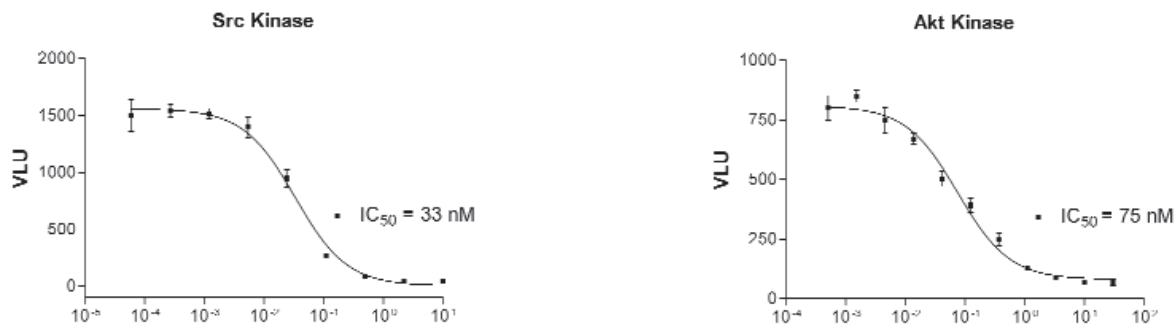


Figure 4. Inhibitor titration in Src and Akt kinase assays formatted on the Streptavidin Coated Image FlashPlate.

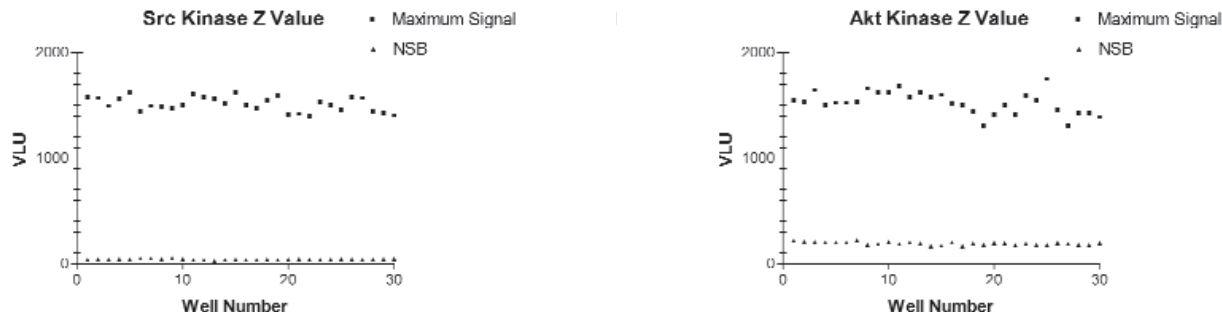


Figure 5. Precision characteristics of Src and Akt kinase assays formatted on the Streptavidin Coated Image FlashPlate.

Table 1. Assay conditions and specifications for both Src and Akt kinase assays formatted on the Streptavidin Coated Image FlashPlate.

Assay	Src kinase (Upstate cat# 14-177)	Akt1 kinase (Upstate cat# 14-276)
Enzyme	1 unit	200 ng
[ $\gamma$ - <sup>33</sup> P]-ATP (mCi)	0.025	0.050
PKS1,biotin KVEKIGRGTYGVVYK-amide substrate (mM)	0.33	0.66
Cold ATP (mM) 0.33	1.32	
Staurosporin IC50 (nM)	33	75

## Discussion

Streptavidin Coated Image FlashPlate is a suitable platform for formatting both protein tyrosine and serine/threonine kinase assays for HTS. The general format outlined in the contents of this application note can also be applied to a wide range of kinases. This is due to the fact that a peptide kinase substrate is captured on a Streptavidin Coated Image FlashPlate and enzymatically phosphorylated using [ $\gamma$ -<sup>33</sup>P]-ATP as the phosphate donor. Optimum assay conditions for two particular kinase enzymes are given herein, however, it is recommended that critical reagents be re-titrated for any new applications.

The Image FlashPlate platform has a number of attractive features making it a suitable HTS platform. For example:

- Excellent precision can be obtained as measured by the Z' value
- Imaging on the ViewLux ultraHTS Microplate Imager
- An entire 384-well plate can be read in less than three minutes, giving a significant increase in throughput compared to conventional microplate readers
- Low assay volumes conserve compound and reduce waste
- Easily automated one plate format

## References

- <sup>1</sup>Capdeville, R., Buchdunger, E., Zimmermann, J., and Matter, A. Nature Reviews/ Drug Discovery 1 493-502 (2002).
- <sup>2</sup>Turek, T.C., Small, E.C., Bryant, R.W., and Hill, W.A.G. Anal. Biochem. 299 45-53 (2001).
- <sup>3</sup>Zhang, J-H., Chung, T.D.Y., and Oldenburg, K.R. J. Biomol. Screen. 4 67-73 (1999).