

FlashPlate® File #13

A Homogeneous Protein Kinase
Assay Performed on 384-Well
Streptavidin-Coated FlashPlate
HTS PLUS

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A Homogeneous Protein Kinase Assay Performed on 384-Well Streptavidin-Coated FlashPlate® HTS PLUS

Abstract

Existing protein kinase assays on 96-well FlashPlates have used [³²P]-ATP for enzymatic phosphorylation of a substrate. In order to produce an adequate signal-to-noise ratio, these assays require a post reaction wash step to reduce background. This paper describes a new assay format on 384-well Streptavidin FlashPlate HTS PLUS, utilizing an anti-phosphotyrosine monoclonal antibody, directly or indirectly labeled with [¹²⁵I], to detect the phosphorylated substrate of a specific tyrosine kinase. This method requires no wash step — resulting in a homogeneous assay that is accurate, easy and fast. It is well suited to automation in a high throughput screening environment.

Introduction

Cellular signal transduction pathways are a focus for the research of therapeutic targets at most pharmaceutical companies. These intracellular communication systems elicit biological responses such as cell growth and cell death. Researchers are therefore interested in identifying chemical compounds that enhance or inhibit these signaling pathways.

One integral part of these pathways is the process of protein phosphorylation by specific protein kinases. Protein kinases are either tyrosine specific, serine/threonine specific, or dual specific. The classic method for the study of protein phosphorylation is to monitor the incorporation of [³²P] or [γ -³²P]ATP onto the substrate by its specific enzyme.

Existing protein kinase assays on 96-well FlashPlates use [³²P]-ATP for enzymatic phosphorylation of a substrate. In order to produce an adequate signal-to-noise ratio, these assays require a post reaction wash step to reduce background. The wash step prevents this format from being perfectly homogeneous.

With the recent availability of antibodies that recognize the phosphorylated form of the substrate (i.e., phosphotyrosine antibodies), an alternate, miniaturized FlashPlate-based assay was developed, in which an anti-phosphotyrosine monoclonal antibody is used to detect the phosphorylated substrate of a specific tyrosine kinase. Biotinylated tyrosine kinase substrates are bound to streptavidin-coated FlashPlate HTS PLUS (a 384-well microplate), then phosphorylated with human Src protein tyrosine kinase. This application can make use of either of two detection systems: a directly labeled [¹²⁵I] anti-phosphotyrosine antibody for detection of phosphorylated substrates; or a [¹²⁵I]-labeled goat anti-mouse (GAM) secondary antibody with anti-phosphotyrosine primary antibody detection. Phosphorylation of substrates is shown to be enzyme dose dependent.

The [¹²⁵I] system of detection reduces background due to the lower energy of reaction with the FlashPlate. This allows the wash step to be eliminated, converting the assay into a homogenous and easily automated format. This assay can be used for high throughput screening of protein kinase inhibitors, as shown by an inhibition profile with Staurosporine, which binds to the ATP binding domain.

Materials and Methods

Peptide Substrates

PKS 1 (Biotin-KVEKIGEGTYGVVYK-amide), a biotinylated kinase substrate (Boehringer Mannheim)
PKS 2 (Biotin-EGPWLEEEEEAYGWMDF-amide), a biotinylated kinase substrate (Boehringer Mannheim)
Biotinylated phosphopeptide control (Boehringer Mannheim)
Staurosporine, a tyrosine kinase inhibitor (BIOMOL)

Enzyme

Human c-Src Kinase (p60c-Src), (recombinant) (Upstate Biotechnology)

Antibodies and Tracers

PY20 phosphotyrosine antibody (Transduction Laboratories), labeled with [¹²⁵I] (PerkinElmer Life Sciences), 31.3 μCi/μg [¹²⁵I]-goat anti-mouse IgG (NEX159), 8.12 μCi/μg

Assay Platform

Streptavidin-coated 384-well FlashPlate HTS PLUS microplates (SMP410)

Assay Buffers

1. Assay buffer: 8 mM Imidazole-HCl, pH 7.5
8 mM glycerophosphate, 200 μM EGTA, 20 mM MgCl₂
1 mM MnCl₂, 1 mg/ml BSA
2. 100 μM ATP in assay buffer
3. 0.03% NP-40 in assay buffer

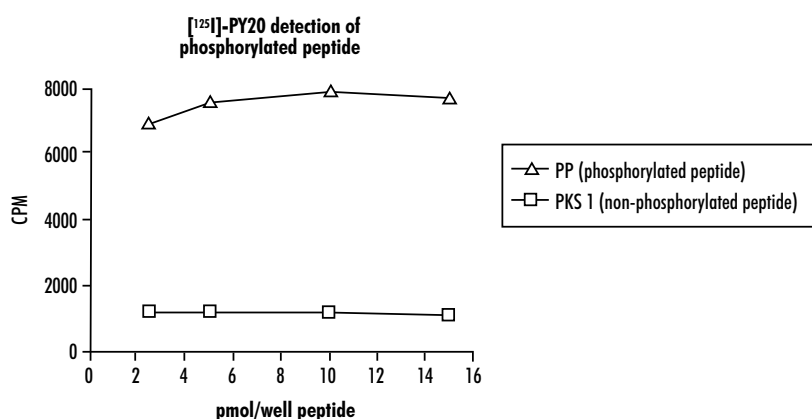
Phosphorylation Reaction

Reactions were performed with biotinylated peptide substrates diluted in assay buffer as indicated, and added 10 μl per well. 100 μM ATP in assay buffer was added 10 μl per well. Human c-Src Kinase (units as indicated in graphs) diluted in assay buffer and added 10 μl per well. The assays were incubated at 30°C for 90 minutes with plate covers. Detection antibodies were diluted to 50,000 cpm/10 μl in 0.03% NP-40 in assay buffer. Single antibody detection was performed by addition of 10 μl of assay buffer plus 10 μl of [¹²⁵I]-PY20. Dual antibody detection was performed by addition of 10 μl PY20 followed by 10 μl [¹²⁵I]-goat anti-mouse IgG. Total assay volume per well is 50 μl. FlashPlates were covered with plate covers and incubated overnight, 4°C. FlashPlates were read on a TopCount® HTS Microplate Scintillation and Luminescence Counter (Packard Instrument, CT).

Results and Discussion

Specific detection of the phosphopeptide by the PY20 antibody was demonstrated. A comparison was shown between the binding of the biotinylated phosphopeptide versus the non-phosphorylated biotinylated peptide substrate to [¹²⁵I]-PY20 in the absence of tyrosine kinase. Phosphorylated peptide generated approximately 8,000 CPM of signal, as compared with approximately 1,000 CPM of signal with non-phosphorylated peptide, equivalent to background in the absence of substrate for 50,000 CPM added per well (Figure 1).

Figure 1



Biotinylated phosphopeptide and biotinylated peptide were titrated on Streptavidin FlashPlate HTS PLUS. The assay was set up in the following reagent addition order: biotinylated peptide in assay buffer, [¹²⁵I]-PY20 at 50,000 CPM/well in assay buffer. Assay was incubated overnight, RT in the dark.

The efficiency of phosphorylation of tyrosine-containing substrates is shown to be substrate dependent. The dual antibody system is shown to be more effective in phosphorylating both the PKS 1 and PKS 2 substrates (Figure 2a). A greater differential is seen with the use of [¹²⁵I]-PY20 alone (Figure 2b).

Figure 2a

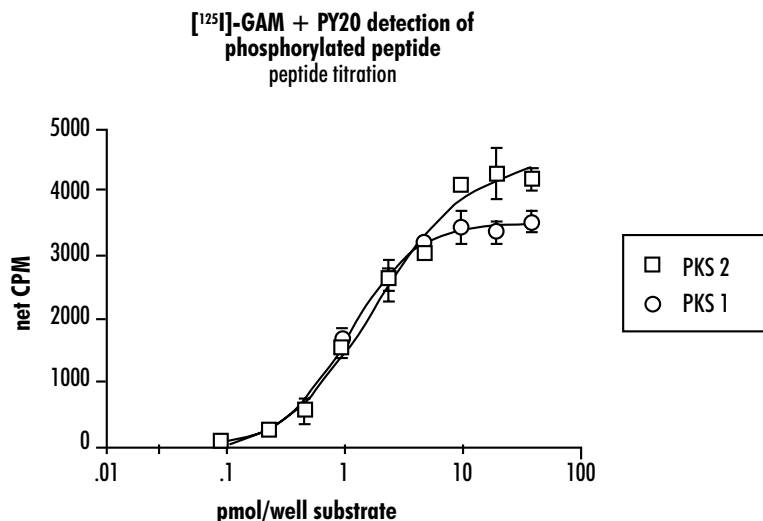
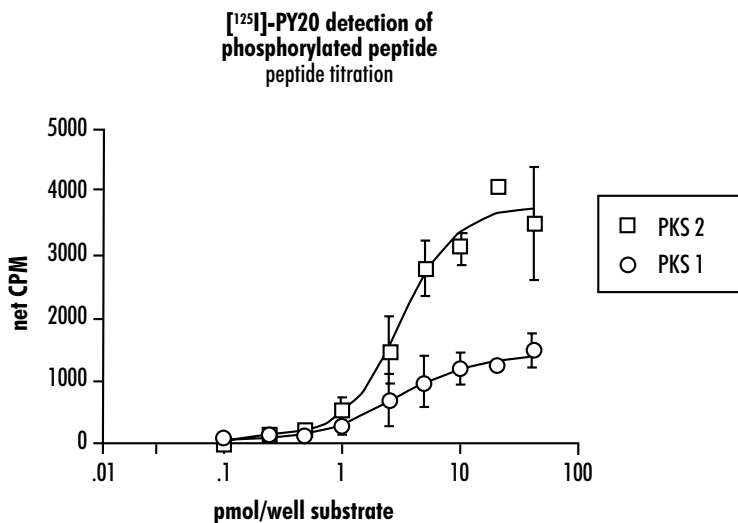


Figure 2b



The peptide substrates PKS 1 and PKS 2 were titrated in a phosphorylation reaction on Streptavidin FlashPlate HTS PLUS. The assay was set up by the following reagent addition order: titrated biotinylated peptide in assay buffer, ATP. The reaction was initiated by the addition of 0.25 units/well of Src kinase. The assay was incubated 90 minutes at 30°C. For dual antibody detection, 8 ng/well of PY20, then 50,000 CPM/well of [¹²⁵I]-GAM with NP-40 were added after the enzyme reaction. For single antibody detection, assay buffer, then 50,000 CPM/well of [¹²⁵I]-PY20 with NP-40 were added. Plates were incubated overnight, 4°C.

Both of the detection systems are shown to be enzyme dose dependent (Figures 3a and 3b).

Figure 3a

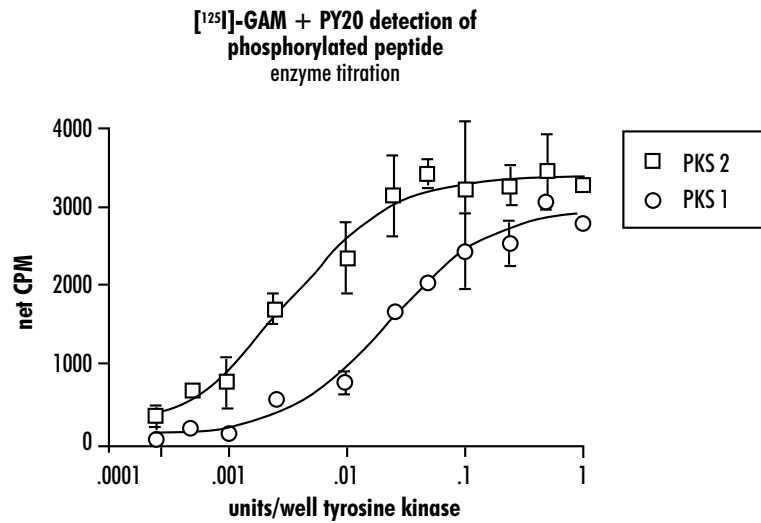
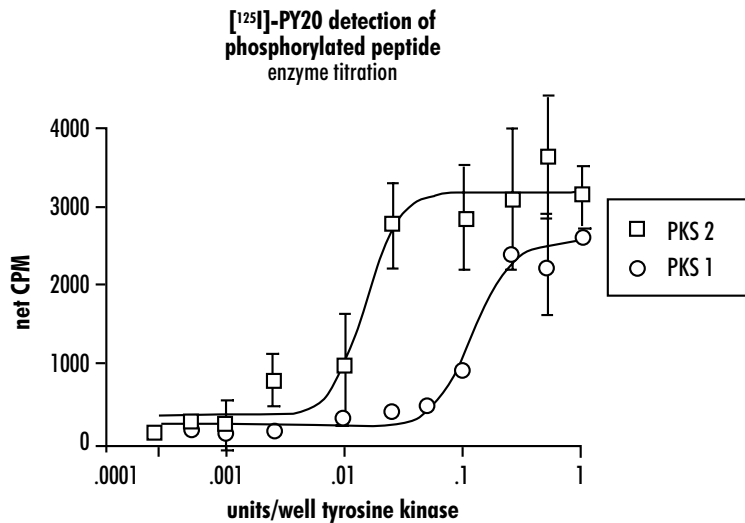


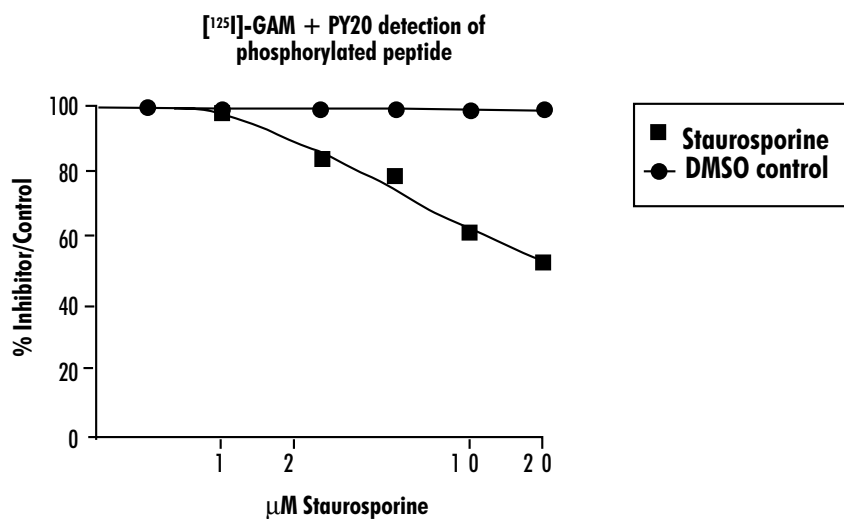
Figure 3b



The Src kinase enzyme was titrated for a phosphorylation reaction on Streptavidin 384-well FlashPlate HTS PLUS. The assay was set up with the following reagent addition order: 10 pmole/well of biotinylated peptide, ATP. The reaction was initiated by the addition of titrated amounts of enzyme. The assay was incubated at 30°C for 90 minutes. For dual antibody detection, 8 ng/well of PY20, then 50,000 CPM/well of [¹²⁵I]-GAM with NP-40 were added after the enzyme reaction. For single antibody detection, assay buffer, then 50,000 CPM/well of [¹²⁵I]-PY20 with NP-40 were added. Plates were incubated overnight, 4°C.

An inhibition assay was performed with Staurosporine. Staurosporine binds to the ATP binding domain of tyrosine kinase. Our data gives an EC_{50} of tyrosine kinase at a concentration = 4.9 μM

Figure 4



The tyrosine kinase inhibitor was titrated in a phosphorylation reaction on Streptavidin 384-well FlashPlate HTS PLUS. The assay was set up by the following reagent addition order: 10 pmole/well of biotinylated peptide PKS 2, ATP, titrated inhibitor. The reaction was initiated by the addition of 0.2 units/well of Src enzyme in a total reaction volume of 30 μl . The assay was incubated at 30°C for 90 minutes. After enzyme reaction, 8 ng/well of PY20 and then 50,000 CPM/well of [¹²⁵I]-GAM with NP-40 were added. Plates were incubated overnight, 4°C. EC_{50} = 4.9 μM .

Conclusion

This assay represents a homogeneous format which does not require a wash step prior to the addition of the detection system, nor prior to reading assay results. This is particularly useful in the 384-well miniaturized format. This assay eliminates aspiration and plate washing, therefore saving assay steps and the need for a 384-well plate washer. The platform can be used in other protein kinase systems, provided an antibody exists that recognizes the phosphorylated form of the substrate. Thus, this assay approach can be applicable to other kinase assays, such as the serine/threonine-specific protein kinase assay. This homogeneous format also adapts well to automation and high throughput screening, yielding time and labor savings.



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