

FlashPlate® File #12

Phosphorylation Assays on Nickel Chelate Coated FlashPlate PLUS

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Abstract

Molecular biology techniques have enabled the production of many therapeutic targets in the form of fusion proteins. A popular form of fusion protein expression includes a sequence of six histidine [His] residues on either terminus as a tag for immobilization of the target protein. These 6xHis proteins are often used in screening applications such as protein kinase assays, protease assays, protein-protein interactions, and protein-nucleic acid interactions. Metal chelates have been established as the best way to immobilize 6xHis fusion proteins, due to the high affinity interaction of nickel and histidine residues. A number of assays were performed to determine the effectiveness of the Nickel Chelate FlashPlate PLUS platform (SMP107) for applications involving 6xHis fusion proteins, and to compare results with alternate assay formats. Plate characterization was performed, including the determination of plate capacity for a 6xHis-tagged kemptide and a polymer comprised of histidine residues. A variety of reagents were tested to establish a tolerance profile and optimum binding conditions for the plate. Specific applications included a PKA kinase assay using [γ - ^{33}P]ATP and 6xHis kemptide.

Introduction

With the recent popularity of molecular cloning, therapeutic targets are now easily expressed in several expression systems, including fusion protein technology. This enables researchers to easily purify and immobilize a protein onto a solid phase. Fusion proteins are tagged with a specific binding domain, which can then be used as a capturing mechanism. One such mechanism in popular use is the binding interaction between a 6x-histidine (His) or a 4xHis with a nickel chelate complex.

FlashPlate is a line of white polystyrene microplates with plastic scintillant permanently coated on the side and bottom of each well. The platform has proven highly effective in numerous high throughput screening radiometric assays. The FlashPlate PLUS product line offers the FlashPlate microplate precoated with a choice of binding proteins. PerkinElmer Life Sciences has developed a nickel chelate coated FlashPlate microplate as a means of capturing histidine fusion proteins. The Nickel Chelate FlashPlate PLUS (SMP107) platform enables homogeneous assays with all isotopes commonly used in HTS, except the high energy ^{33}P isotope, which typically requires a simple wash step at the end of the assay protocol. The following data provide characteristics of the platform using a PKA and kemptide-6xHis phosphorylation system.

Materials and Methods

Peptide Substrates:

Kemptide (LRRASLG) peptide substrate for PKA (Sigma)
Kemptide-6xHis and Kemptide-GGYGG peptide substrates were synthesized (Anaspec, Inc.), and both substrates were labeled with ^{125}I (PerkinElmer Life Sciences)
Substrate inhibitor (TTYADFIASGRTGRRNAIHD) for PKA (Promega)

Enzyme:

PKA: the catalytic subunit of cAMP dependent protein kinase (Promega)

Assay Platforms:

Nickel Chelate FlashPlate PLUS: SMP107 (PerkinElmer Life Sciences)
Phosphocellulose p81 filter paper discs (Whatman)

Tracer:

[γ - ^{33}P]ATP: NEG302H (PerkinElmer Life Sciences)

Assay Buffer:

40 mM Tris-HCl, pH 7.4, 20 mM magnesium acetate, 200 μM ATP, and 2-2.5 μCi [γ - ^{33}P]ATP per reaction

Phosphorylation Reaction

Reactions were performed with PKA (units indicated in graphs) and [γ - ^{32}P]ATP in 100-200 μl per well. The assays were incubated at room temperature for the times indicated. Reactions were terminated by aspiration of well contents followed by two washes with 400 μl of 0.01% Tween-20 in PBS (Dulbecco's PBS without Ca^{++} or Mg^{++}) and a final aspiration.

Inhibition studies with a PKA substrate inhibitor were performed in the buffer described above, containing 100 $\mu\text{g}/\text{ml}$ of BSA.

Binding reactions of ^{125}I -labeled substrates on Nickel Chelate FlashPlate PLUS were performed in PBS buffer containing 0.1% BSA with an overnight incubation at room temperature. Substrate binding to the microplate was disrupted by a single 400 μl wash of 200 mM imidazole buffer. FlashPlates were read on a TopCount[®] Microplate Scintillation and Luminescence Counter (Packard Instrument Company).

Phosphorylation reactions for the p81 filter assay were analyzed by spotting 10 μl of the completed reaction mix onto filter discs, washing three times with 75 mM phosphoric acid and counting on a Liquid Scintillation Analyzer (Packard Instrument Company).

Results and Discussion

Specific binding of 6xHis-tagged substrates to the Nickel Chelate FlashPlate PLUS was demonstrated by two independent methods:

- 1) Specific binding of the histidine residues was demonstrated by examining the binding of [^{125}I]-kemptide-6xHis and [^{125}I]-kemptide-GGYGG. The [^{125}I]-kemptide-6xHis binds to Nickel Chelate FlashPlate PLUS, while [^{125}I]-kemptide-GGYGG does not. Furthermore, the binding of [^{125}I]-kemptide can be competed off the plate by increasing amounts of unlabeled kemptide-6xHis (Figure 1).
- 2) Kemptide-6xHis and kemptide were labeled by PKA with ^{32}P , then captured onto the Nickel Chelate FlashPlate PLUS. Only the kemptide-6xHis was captured onto the plate. To confirm that the capture mechanism is due to the nickel chelate complex, the plate was washed with imidazole buffer (a typical elution buffer to recover 6xHis-tagged fusion proteins from nickel chelate resins). The radiolabeled substrate was eluted off using the imidazole, but not eluted off with the detergent. When the substrate was removed, counts on the plate were reduced to background level (Figures 2 and 3). The binding capacity of the plate was determined to be 5-10 pmole/well using [^{125}I]-kemptide-6xHis (data not shown). Subsequent studies have shown that binding capacity is dependent upon the size of the 6xHis-tagged substrate, possibly due to steric hindrance. Binding of 6xHis substrate can occur within as little as 15-30 minutes of incubation at room temperature.

Simultaneous phosphorylation and the capture reactions of PKA on kemptide-6xHis substrate on Nickel Chelate FlashPlate PLUS were investigated. Furthermore, the phosphorylation of the kemptide-6xHis and kemptide were comparable, based on the filter assay (not shown). Phosphorylation of the substrate by PKA is dose dependent for both substrate and enzyme (Figures 4 and 5). PKA phosphorylation kinetics were close to completion at 30 minutes (Figure 6), and showed an acceptable signal-to-noise ratio. Inhibition assays of PKA were performed on the Nickel Chelate FlashPlate PLUS and on traditional p81 filter discs (Figure 7). The data indicate the inhibition was the same for both approaches. For the FlashPlate platform and the filter disc format, 50% inhibition of PKA occurred at 100 nM of substrate, which is comparable to the published B_{50} value for this inhibitor.

Nickel Chelate FlashPlate PLUS was characterized using [¹²⁵I]-kemptide and [³³P]-kemptide labeled by PKA. The plate showed no decrease in binding with a radiolabeled substrate after treatment with 50% DMSO, 25% ethanol or 25% methanol. Furthermore, binding of 6xHis-tagged substrates was not affected in the presence of 1% Tween-20, 1% Triton X-100, 1% NP-40 or 10% DMSO. Weak chelators are tolerable at low concentrations, which must be determined for each assay condition. We do not recommend using reducing agents such as β-mercaptoethanol, as they may interfere with binding. DTT can be used up to a concentration of 1 mM. Conditions will vary, so the optimum conditions should be determined for each particular assay.

Conclusion

As demonstrated, nickel chelate immobilized onto the surface of a FlashPlate microplate is a useful platform for the capture of histidine-tagged fusion proteins. To illustrate this, a PKA assay was successfully validated on this platform using a 6xHis-tagged Kemptide substrate. The results obtained demonstrate specific capture of 6xHis fusion proteins, and compare well to those of commonly accepted methods or models, therefore validating Nickel Chelate FlashPlate PLUS as an effective platform to measure the phosphorylation of peptide substrates. This approach can be used in a variety of assays (e.g., protein kinase, protease assays, protein-protein interaction and protein-nucleic acid interactions), and is an effective tool for high-throughput screening.

Figure 1

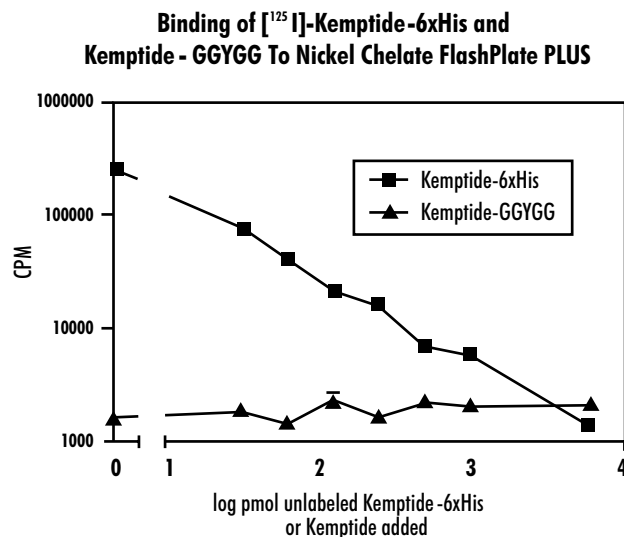


Figure 1. Two kemptide synthetic peptides (LRRASLG) were synthesized, one with a 6xHis terminal sequence and one with an irrelevant sequence which does not bind to nickel chelate coated plates, but could be iodinated with ¹²⁵I (terminal peptide sequence of GGYGG). A competitive binding experiment was performed by adding a constant amount of either [¹²⁵I]-kemptide tracers (1 μCi/well) and increasing amounts of unlabeled 6xHis kemptide or kemptide-GGYGG. The experiment was conducted in 1X Dulbecco's PBS (no Ca⁺⁺ or Mg⁺⁺), with 0.1% BSA at a total volume of 200 μl per well. The wells were aspirated and counted after an overnight incubation at room temperature. The results illustrate the specificity of the histidine interaction with the Nickel Chelate FlashPlate PLUS.

Figure 2

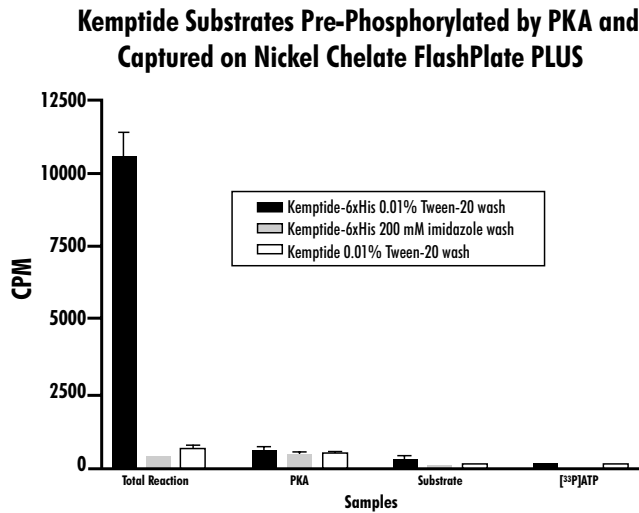


Figure 2. Kemptide-6xHis and kemptide substrates were pre-phosphorylated by cAMP dependent protein kinase (PKA) and transferred onto a Nickel Chelate FlashPlate PLUS. Binding was performed overnight at room temperature. The plate was then washed twice with 400 μ l 0.01% Tween-20 in PBS and the counts determined on a TopCount Microplate Scintillation and Luminescence Counter. To demonstrate specific binding, plates were then washed with 400 μ l of 200 mM imidazole.

Figure 3

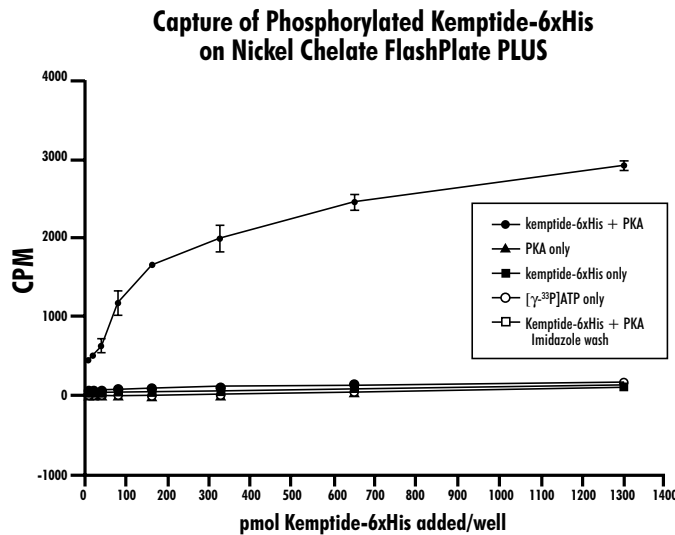


Figure 3. Various amounts of kemptide-6xHis were phosphorylated by PKA and transferred to a Nickel Chelate FlashPlate PLUS for capture. The capture of the substrate was performed overnight at room temperature. The plate was then washed twice with 400 μ l 0.01% Tween-20 in PBS and the counts determined on a TopCount. To demonstrate specific binding, the plate was then washed with 400 μ l of 200 mM imidazole.

Figure 4

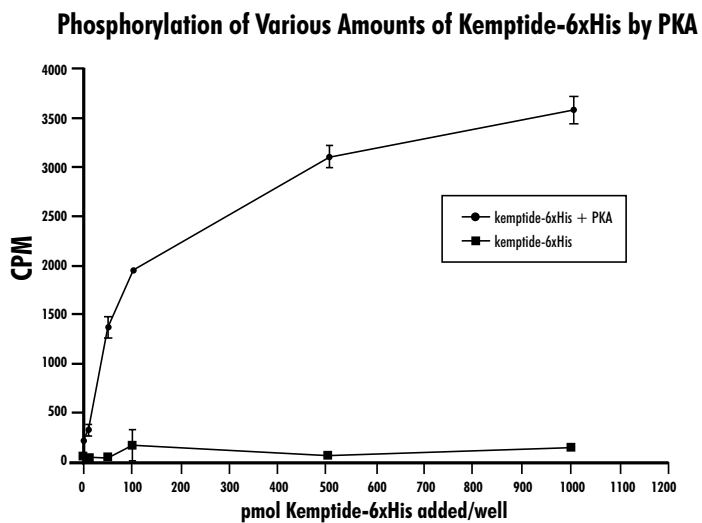


Figure 4. Various amounts of kemptide-6xHis were phosphorylated by a fixed amount of PKA (12.5 units/well) on a Nickel Chelate FlashPlate PLUS. The assay was set up using the following order of reagent additions: reaction buffer, PKA, and $[\gamma\text{-}^{32}\text{P}]\text{ATP}$. The reaction was then initiated by the addition of substrate. The assay was done at room temperature and stopped after 2 hours by aspiration and washing.

Figure 5

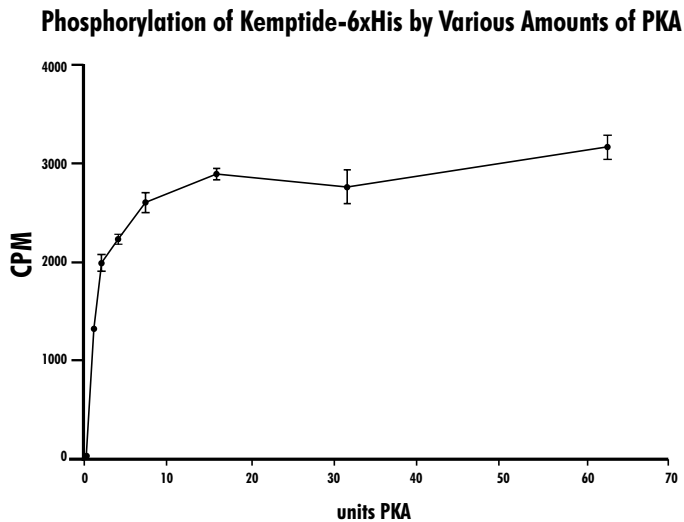


Figure 5. The PKA was titrated in a phosphorylation reaction on a Nickel Chelate FlashPlate PLUS. The assay was set up using the following order of reagent additions: reaction buffer, $[\gamma\text{-}^{32}\text{P}]\text{ATP}$, and PKA. The reaction was then initiated by the addition of the kemptide-6xHis substrate. The assay was done at room temperature and stopped after 2 hours by aspiration and washing.

Figure 6

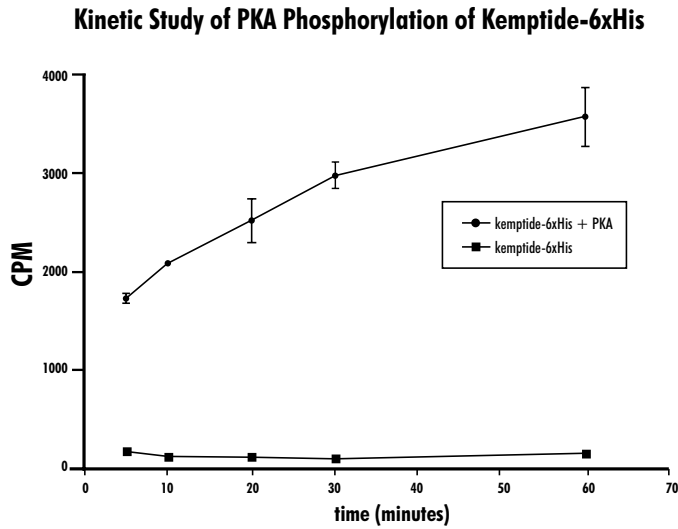


Figure 6. The kinetics of the PKA phosphorylation of kemptide-6xHis was determined on the Nickel Chelate FlashPlate PLUS. The assay was set up using the following order of reagent additions: reaction buffer, PKA (at 12.5 units/well), and $[\gamma\text{-}^{33}\text{P}]\text{ATP}$. The reaction was then initiated by the addition of 500 pmol/well substrate. The assay was incubated at room temperature for the times indicated. Overnight incubation did not result in significant increase in the cpm beyond the 60 minute time point.

Figure 7

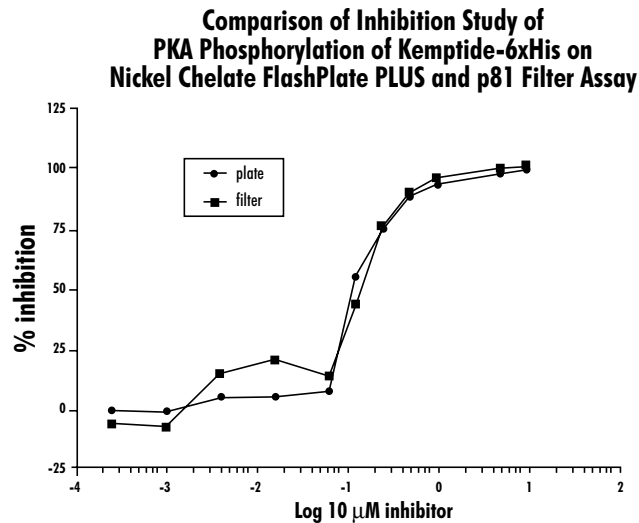


Figure 7. An inhibition study was performed on Nickel Chelate FlashPlate PLUS in comparison with a p81 filter assay. Both assays were set up by the following reagent addition order: reaction buffer (+ 100 $\mu\text{g}/\text{ml}$ BSA), PKA inhibitor, PKA (12.5 units/reaction), and $[\gamma\text{-}^{33}\text{P}]\text{ATP}$. The reactions were pre-incubated at room temperature for 10 minutes. The assay was then initiated by the addition of kemptide-6xHis. The assay was terminated after 40 minutes at room temperature.



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