THE USE OF YTTRIUM SILICATE STREPTAVIDIN COATED SPA BEADS IN PROTEIN KINASE ASSAYS

M. Harvey, K. Coughlin, A. Santos, *G. Brophy and J.Anson

Amersham Biosciences, Cardiff Laboratories, Forest Farm, Whitchurch, Cardiff, CF4 7YT, UK. [Tel: (44) 1222 526417, e-mail:Cardiff_SPA_Group@amershambiosciences.com]
**Amersham Life Sciences Inc., 2636 S. Clearbrook Dr., Arlington Heights ,IL 60005. [Tel:1-888-772-4487, e-mail:spa.hits@amershambiosciences.com]*

Introduction

Scintillation Proximity Assay (SPA®) has previously been demonstrated as a high throughput method for determination of protein kinase activity[1]. The technology involves the capture of a ^{33}P-labeled peptide or protein onto streptavidin coated polyvinyl toluene (PVT) SPA beads. β-particles, emitted from the captured ^{33}P-labeled substrate in close proximity to the beads are able to excite the scintillant, resulting in the generation of light which is quantifiable. To efficiently harvest the greater energy associated with ^{33}P and to reduce non-specific signal resulting from free [^{33}P]ATP in solution, the beads are settled or centrifuged before counting. Recently, floating the beads with cesium chloride has been used to achieve the same end and substitute for the relatively long settling time associated with the PVT beads[2,3].

Recently, yttrium silicate (YSi) streptavidin coated SPA beads (RPNQ0012) have been developed which settle more rapidly than PVT beads. However, it has been noticed, when carrying out kinase assays employing [^{33}P]ATP, that optimization of conditions were required to minimise non-specific binding of radioactive tracer to the YSi bead.

Results

In-house work (data not shown) has demonstrated that the 2-5μm yttrium beads have a binding capacity of 250-290pmoles biotin/mg. The respective PVT values are 100-130pmoles biotin. For this reason, smaller mass amounts of YSi beads are required for equivalent biotin-binding capacity, compared to PVT beads.

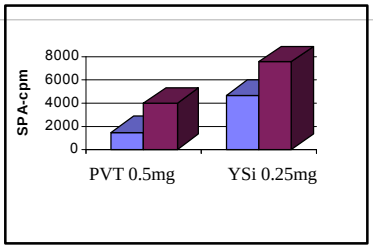

Figure 1. *Signal to noise ratios of an SPA cdc2 kinase assay, measured on PVT and YSi beads. The reaction was carried out as described[4], and stopped by the addition of the beads in 50mM ATP, 50mM EDTA, 1% (v/v) Triton™ X-100 in PBS. Following bead addition, the samples were centrifuged and counted. S/N PVT = 2.7, S/N YSi = 1.6. Values are means(n=3).*

Figure 1 demonstrates the signal to noise ratios of an SPA cdc2 kinase assay counted on both PVT and YSi beads in a standard "stop" mix. As can be seen, there is a significant increase in background (with a corresponding decrease in the S/N ratio) using the YSi beads in this buffer.

Investigations using [^{3}H] and [^{33}P]ATP (data not shown) demonstrated that the increase in counts seen with the YSi beads was largely due to non specific binding (NSB) rather than any non proximity effects (NPE). A number of strategies were investigated to reduce the NSB of ATP to these beads. It was found that increasing the amount of cold ATP in the "stop" mix from 50 to 100μM reduced background. However, by far the greatest effect was seen with pH adjustment.

High pH values (around 11.0) greatly reduce ATP binding to the bead compared to pH values in the range of the current SPA kinase "stop" buffer (pH 7.0). Using these indications, a modified "stop" buffer was developed for the cdc2 assay containing PBS, adjusted to pH11.0 with NaOH, 1% (v/v) Triton X-100 and 100μM ATP. The performance of the YSi beads in this buffer compared to that of the PVT beads in the traditional "stop" buffer is demonstrated in Figure 2 for a cdc2 SPA kinase assay. Using the modified "stop" buffer, over 95% of specific cpm were achieved with the YSi beads within four hours of stopping the reaction (data not shown). 100% of specific signal was defined to be that given by centrifuged beads.

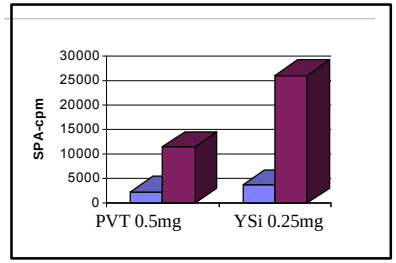

Figure 2. *Signal to noise ratios of an SPA cdc2 kinase assay as measured on PVT and YSi beads. The reactions were stopped by the addition of the PVT beads in 50mM ATP, 50mM EDTA, 1% Triton X-100 in PBS and the YSi beads in the modified "stop" solution. Following bead addition, the samples were centrifuged and counted. S/N PVT = 4.9, S/N YSi = 7.0. Values are means (n=3)*

SPA kinase reactions are run in the presence of low ATP concentrations. Experiments were designed to examine the mode of enzyme inhibition of staurosporine and poly(glu:tyr) in this ATP range using the YSi beads.

Casein kinase II (CKII) was used for these experiments. A biotinylated peptide substrate EEESEEE was used at a concentration of 2μM in 50mM MOPS, pH 7.2, 10mM $MgCl_2$, 150mM NaCl using 0.2μCi [^{33}P]ATP (Amersham AH9968) per well. Due to the relatively low pH of the reaction buffer, the beads were added in PBS, adjusted to pH 12.0.

Staurosporine has been shown to be a poor inhibitor of CKII[5]. However, it has been demonstrated that regardless of its inhibitory power, staurosporine inhibition is competitive with regard to ATP. Poly(glu:tyr) has been demonstrated to be a potent inhibitor of CKII[6]. Unlike staurosporine, inhibition is not competitive with regard to ATP but rather with the phosphate acceptor substrate.

Inhibitor profiles were constructed for the two compounds at 0.5 and 10μM. Overall SPA counts decrease with increasing ATP substrate concentration as less radioactive material is incorporated intp the peptide substrate. To reflect the degree of inhibition more accurately, the Y-axix of the profiles was converted into % activity. (Fig.3).

Table 1 shows the IC_{50} values determined for poly (glu:tyr) and staurosporine at the two different concentrations of ATP. As expected, the value for poly (glu:tyr), which is not a competitive ATP analogue, is unaffected. However, the IC_{50} value for staurosporine is increased with increasing ATP concentration.

	Poly (glu:tyr)	Staurosporine
IC_{50} @ 0.5μM	$3.03x10^{-9}$	$4.36x10^{-6}$
95% confidence intervals	$2.68 - 3.41x10^{-9}$	$3.63 - 5.26x10^{-6}$
IC_{50} @ 10μM	$3.44x10^{-9}$	$9.34x10^{-6}$
95% confidence intervals	$3.10 - 3.81x10^{-9}$	$6.62 - 13.16x10^{-6}$

Table 1. *CKII inhibition values determined from Figure 3.*

IC_{50} determinations were performed using GraphPad Prism™.

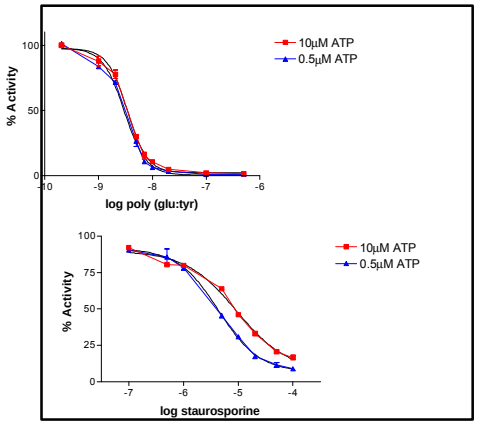

Figure 3. *CKII inhibition curves. (sigmoidal dose response curve, variable slope). Duplicate values +/-SEM (n=2).*

Discussion

YSi streptavidin coated SPA beads are an alternative to PVT beads for protein kinase assays using peptide substrates. The greater binding capacity, scintillation efficiency and density of this bead type offer advantages for HTS. However, care must be taken to reduce NSB, which is chiefly affected by the pH of the mix following the addition of the bead.

Using this bead type, inhibitor profiles for ATP and non-ATP analog kinase inhibitors have been determined. The effect of determining the profile of the ATP analog in the presence of increasing concentrations of ATP in the SPA assay is to shift the profile to the right. The results demonstrate the ability of the SPA kinase assay to distinguish competitive ATP analog inhibition from other types of inhibition.

References

1.Brophy, G *et al*., 9th International Conf. on Second Messengers and Phosphoproteins, Nashville, TN, 1995.

2.Amersham Proximity News, No. 31.

3.TopCount Topics, TCA-030.

4.Powell, D *et al*., 2nd International Conf. for the Society of Biomolecular Screening, Basel, 1996.

5.Meggio, F. *et al*., *Eur. J. Biochem*. **234**, 317-322, 1995.

6.Tellez, R. *et al*., *FEBS*. **265**, 113-116, 1990.

008908_29

PerkinElmer® For the Better