A HIGH THROUGHPUT SCREENING ASSAY FOR THIAMINE PYROPHOSPHATE KINASE ACTIVITY USING SPA

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Introduction

Thiamine pyrophosphate kinase (TPPK: E.C. 2.7.6.2) converts thiamine to thiamine pyrophosphate (TPP) which acts as the major metabolic thiamine signal within the cell [(1)].

TPP functions as a cofactor for many enzymes involved in carbohydrate metabolism, and in *Schizosaccharomyces pombe,* has been shown to be a critical metabolic signal for mating. TTP is also thought to be important in phosphate metabolism and growth in yeast [(2)]. Therefore, inhibition of TTPK may be a target for anti-fungal therapy.

Current methods for analysing TTPK activity such as HPLC [(3)] and ion exchange chromatography[(4)] are not amenable to high throughput screening; therefore, we have developed a scintillation proximity assay (SPA) to measure TPPK activity.

In this assay, TPPK from *Saccharomyces cerevisiae* was used to convert [^{3}H]thiamine to [^{3}H]TPP which was selectively captured using yttrium silicate (YSi) SPA beads. (Fig. 1).

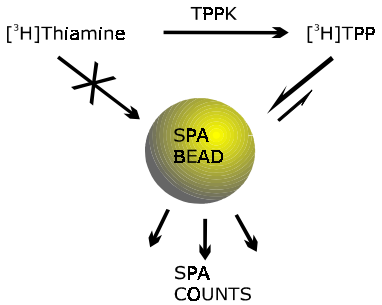

Figure 1. Diagrammatic representation of the TPPK SPA assay.

Method and Results

SPA assays contained TPPK (0.5µg), unlabelled thiamine (10nmol) and [^{3}H]thiamine (70pmol, 1µCi) in a total volume of 100µl 5mM glycine buffer, pH 8.6, containing 13µM ATP and 2mM $MnSO_4$. Assays were incubated at room temperature for 20 minutes before being stopped by the addition of 50µl de-ionized water containing 1mg YSi SPA beads. Non-specific binding was determined in the absence of TPPK.

Assays were counted for 1 minute/well using a TopCount™ microplate scintillation counter. Library compounds were added in DMSO to give a final concentration of 1% DMSO (v/v) and 10^{-5}M test compound in the assay well.

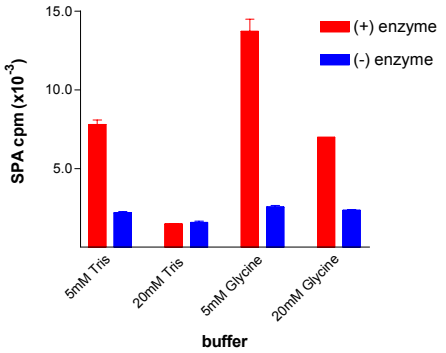

Figure 2. Effect of buffer type and concentration on SPA assay signal. Both tris and glycine buffers (containing 13µM ATP and 2mM $MnSO_4$) were used at pH 8.6. Values are means ±SEM (n=2).

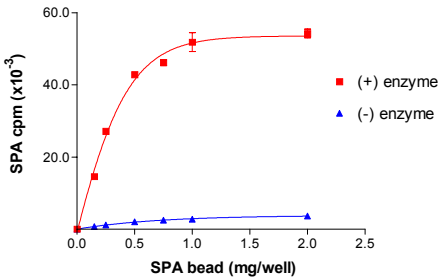

Figure 3. Effect of increasing concentrations of YSi bead on SPA assay signal. Assays contained 1.0µg enzyme/well. Values are means ±SEM (n=2).

As can be seen from Figure 2, both the type of buffer, and the buffer concentration, appeared to have a considerable effect on the SPA cpm obtained. The use of glycine buffer resulted in ~40% increase in SPA cpm compared to tris buffer. Increasing the glycine buffer concentration from 5 to 20mM appeared to reduce the SPA cpm by ~50%.

A titration of SPA bead (Fig.3) indicated that the maximum signal: background was obtained with 1mg YSi bead/well.

Experiments indicated that the assay signal was linear up to 1.25µg/well added enzyme (Fig.4).

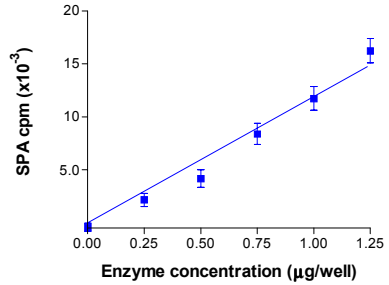

Figure 4. Effect of increasing enzyme concentration. Values are means ±SEM (n=3).

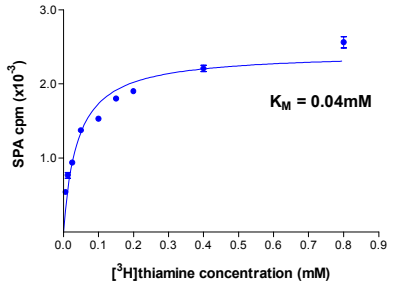

Figure 5. Michealis-Menten plot for TPPK with [^{3}H]thiamine as substrate. Values are means ±SEM (n=3).

Non-linear regression analysis of the substrate concentration curve obtained with [^{3}H]thiamine (Fig.5) gave a K_M value of 40µM (± 8µM). This is in good agreement with the previously reported K_M values for *procaryotic* TPPK (75µM for *Saccharomyces cerevisiae* TPPK[(5)], 6µM for *Saccharomyces pombe* TPPK[(2)] and 38µM for *Paracoccus denitrificans* TPPK [(6)]).

Pyrithiamine, a substrate analogue and thiamine antagonist, is thought to be a direct competitive inhibitor of TPPK. Reported K_i values for pyrithiamine of the procaryotic TPPK are 6µM[(2)] and 19µM[(6)]. As can be seen from Fig.6, inhibition studies indicate that pyrithiamine does inhibit *Saccharomyces cerevisiae* TPPK with an estimated IC_{50} value of 19µM.

The robustness of this SPA assay was tested using a high throughput screening system, screening 42 x 96-well plates of our compound library, where each plate

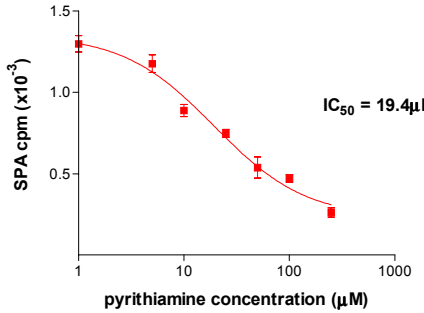

Figure 6. Inhibition of TPPK by pyrithiamine. Values are means ±SEM (n=3).

included 8 wells of positive controls (in the presence of TPPK enzyme) and 8 wells of negative controls (in the absence of TPPK), testing a total of 3360 compounds. The average of the cpm values for the positive controls was 6245 cpm with a CV of 7%. The average cpm value for the negative control was 816 cpm with a CV of 8%. To evaluate the quality of the HTS we calculated the Z' value[(7)] and found it was 0.7. The quality of the assay allows implementation in an HTS system with delivery of precise and reproducible data.

Conclusions

- We have developed a high throughput screening assay to measure the inhibition of TPPK activity.
- The assay is robust, and can be used for automated screening of a large number of compounds for discovery of hits and development of therapeutic leads.

References

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