

Development of a miniaturised non-radioactive assay for the Ser/Thr kinase, JNK-1, using ALPHAScreen

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Introduction

The Amplified Luminescent Proximity Homogeneous Assay (ALPHAScreen, BioSignal) method relies on the transfer of singlet oxygen between donor and acceptor beads brought into proximity by biological interactions (Fig. 1). Upon excitation at 680 nm, photosensitisers in ALPHAScreen donor beads convert ambient oxygen to singlet-state oxygen, which diffuses up to 200 nm. Chemiluminescent groups in the acceptor beads transfer energy to fluorescent acceptors within the bead which then emit light at approximately 600 nm. ALPHAScreen may offer advantages for high throughput screening assays including homogeneity, suitability for miniaturisation, precision and increased energy transfer distance.

We evaluated the potential of ALPHAScreen for kinase assays by developing a screen for c-jun N-terminal kinase 1 (JNK-1), an example of Ser/Thr kinases. In the presence of ATP JNK-1 transferred a phosphate group to Thr71 of the protein substrate ATF-2, which had been biotinylated. The reaction was stopped with EDTA which was added together with the ALPHAScreen beads. Biotinylated ATF-2 was captured on streptavidin donor beads and an anti-phospho (Thr71) antibody was captured on acceptor beads coated with goat anti-rabbit IgG (Fig. 1). The higher the levels of phospho-ATF-2 present the greater the emitted light. We have characterised the assay kinetically and evaluated the reproducibility and potency of known inhibitors.

Assay Development

- Total signal and signal:noise ratio with the anti-phospho (Thr⁷¹) ATF-2 antibody were double that with the anti-phospho (Thr⁶⁹, Thr⁷¹) ATF-2 antibody. This implies the ATF-2 was not completely phosphorylated at both threonine sites or the former was a better antibody (data not shown)
- JNK-1 titration at [ATP] = 5 μM and [ATF-2] = 50 nM showed approximate linearity up to 2nM (Fig. 2) with signal:noise >10
- K_m for ATP and ATF-2 substrates were determined at 0.75 nM JNK-1
- The K_m for ATP was 700 nM (Fig. 3), which is consistent with that determined in an FP assay (Hill, S. *et al*, P3015)
- Apparent K_m for ATF-2 was determined to be approximately 20 nM (Fig. 4), but the curve 'hooked' and this value is substantially lower than in bead-free assay systems (Hill, S. *et al*, P3015). This is probably due to limited bead capacity for biotin-ATF-2 (approximately 30 nM)
- Dilution of samples to 20 nM total ATF-2 (i.e. non-phosphorylated + phosphorylated ATF-2) prior to addition of beads gave an estimated K_m of approximately 550 nM (data not shown), much closer to the values obtained in other assay systems (Hill S. *et al*, P3015)
- Time course of the enzyme reaction under the final assay conditions (0.75 nM JNK-1, 25 nM biotin-ATF-2, 1 μM ATP) was linear up to at least 120 min (Fig. 5), with signal:noise >4 and Z factor >0.8 at this time point

Materials and Method

Materials:
 Anti-phospho ATF-2 (Thr⁷¹) or (Thr⁶⁹, Thr⁷¹) antibodies (New England Biolabs)
 JNK-1 Serine/Threonine enzyme α -1 isoform (Dundee University) stored -80°C
 ATF-2 substrate (Dundee University) stored -20°C
 Streptavidin donor beads and goat anti-rabbit acceptor beads (Biosignal Inc)
 Incubation buffer: 25 mM HEPES with 0.01% Tween-20, 1 mM DTT, 10 mM MgCl₂ (pH7.4)
 Detection buffer: 50 mM HEPES containing 200 mM NaCl, 80 mM EDTA, 0.3% BSA (pH7.4)
 Other reagents were obtained from Sigma or Calbiochem
 Greiner 384 well small volume white plates

Method:
 • 1 μl compound in 10% DMSO was transferred to each well
 • 4 μl enzyme (1.9 nM) were added to each well and pre-incubated with enzyme for 20 min at room temperature
 • 5 μl biotin-ATF-2 (50 nM) / ATP (2 μM) / anti-phospho ATF-2 (0.6 nM) solution were added / well and the reaction allowed to proceed for 120 min at room temperature.
 • 5 μl EDTA (80 mM) acceptor beads (0.15 μg) / donor beads (0.3 μg) solution were added / well to stop the reaction
 • Plate incubated overnight at room temperature in the dark before reading on ALPHAScreen

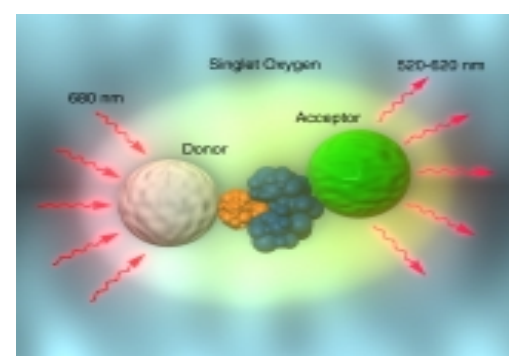


Figure 1: Principles of ALPHAScreen JNK-1 assay

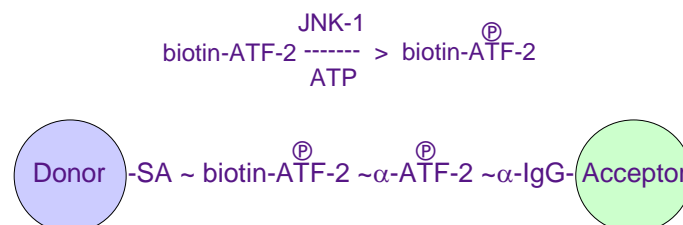


Figure 2: JNK-1 titration

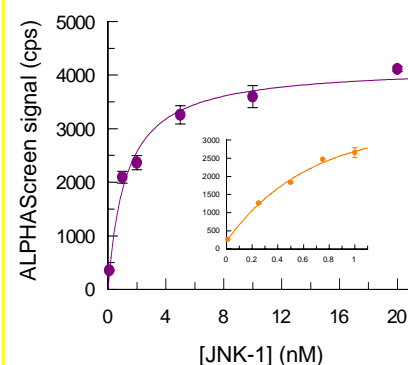


Figure 3: ATP K_m

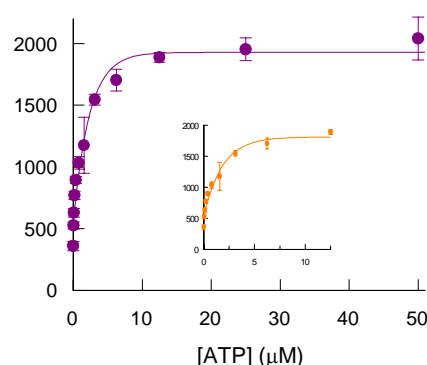


Figure 4: Apparent biotin-ATF-2 K_m

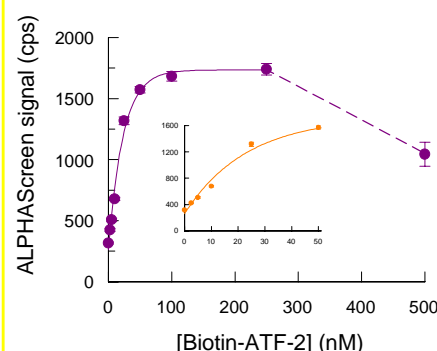
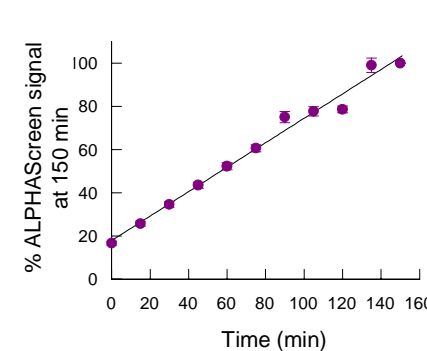


Figure 5: Time course



Data from a single experiment, mean ± sem, triplicate wells representative of at least 2 experiments

Figure 6: Reproducibility

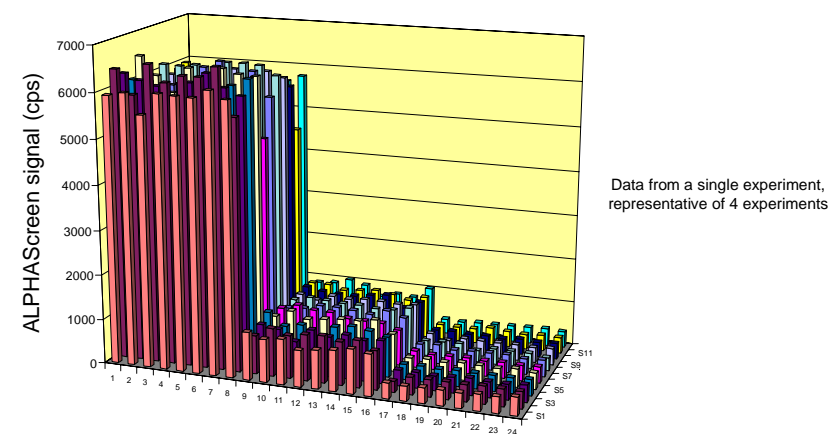
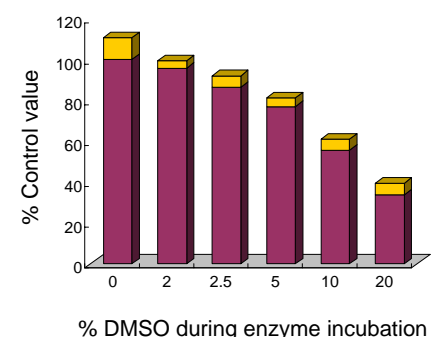
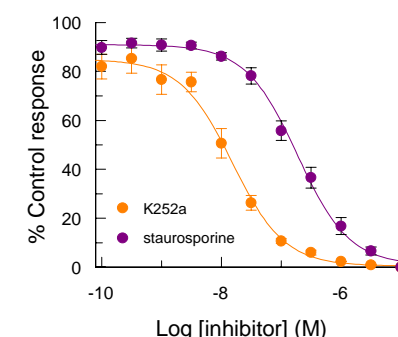


Figure 7: DMSO tolerance



Mean ± sem, from at least 3 experiments, triplicate wells in each experiment

Figure 8: Inhibitors



Reproducibility

- The fully developed assay has a total volume of 15 μl, which is amenable to 384 well small volume plates
- A Biomek 2000 and CCS Multimek-384 were used to assess reproducibility across plates
- In the example shown (Fig. 6), the left hand third of the plate contained uninhibited enzyme, the central third an IC₈₀ concentration and the right hand third a supramaximal concentration of staurosporine
- For the 384 well assay read on an ALPHAScreen with a 1 s per well read time the intra-plate CVs ranged from 8-9%
- Z factor was > 0.3 against the IC₈₀ concentration and > 0.6 against the supramaximal staurosporine data
- Same day or day-to-day inter-plate CVs ranged from 8-11%, with Z factors of 0.5-0.65 for total enzyme inhibition
- Total read time for one 384 well plate was 2.5 min

Compounds

- DMSO tolerance was tested by pre-incubating the enzyme with DMSO at varying concentrations
- There was no significant decrease in signal at concentrations up to 2.5% DMSO, but signal size decreased at higher concentrations
- Fig. 8 shows composite inhibition curves from at least three determinations for two known JNK-1 inhibitors, staurosporine and K252a
- This experiment had a window of approximately 4000 cps, giving a Z factor for the assay of >0.9
- The pIC₅₀ values determined for staurosporine and K252a were 6.7±0.1 and 7.8±0.1 respectively
- These values match closely those obtained in other assay formats (Hill, S. *et al*, P3015)
- To evaluate likely hit rates, false positive contribution and interfering compound effects, five thousand compounds were tested in the assay system
- Eighteen compounds (0.36%) gave >50% inhibition
- Three of these compounds were shown to be false positives when tested in an assay which replaced the biotin-ATF-2/ATF2 antibody component with an irrelevant (i.e. non-ATF-2) biotinylated rabbit antibody
- In addition, five compounds (0.1%) gave >50% increase in counts observed

Summary

The enzyme kinetics and sensitivity to inhibition of ser/thr kinases, exemplified here by JNK-1, in an ALPHAScreen assay are consistent with those found in other assay formats. Small volume, homogeneous, robust assays can be readily developed which are amenable to high throughput screening.

Although ALPHAScreen, filtration binding, FP- and DELFIA-based assays were feasible with this kinase (Hill S. *et al*, P3015), it did not prove possible to develop a LANCE assay. This would suggest that ALPHAScreen offers advantages in terms of energy transfer distance for dual bead systems, at least for this particular kinase / protein substrate combination. This may be useful for kinases which will not phosphorylate short peptides, or assay formats designed to look for antagonists which are substrate modifiers or which interact at the substrate binding site.