HTMS Screen for Enzyme Inhibitors Utilising the Apricot ADDA for On-Line Sample Desalting

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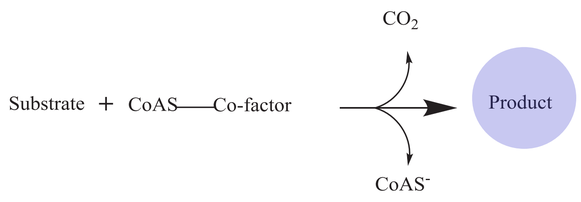
Abstract

High throughput mass spectrometry (HTMS) screening is now a well established method for providing label-free, direct measurement of reaction products produced in biochemical screens. In the main, rapid on-line solid phase extraction and injection has been reported using Agilent's proprietary RapidFire® technology. At CRL, on behalf of our client we have developed a HTMS method with a 12-second cycle time for the measurement of the highly polar amino acid product from a enzyme reaction. Here we report for the first time, the application of an alternative but analogous technology, the Apricot ADDA dual-arm system, for the conduct of a screening campaign.
The method successfully supported the analysis of >300K compounds in the primary screen. Subsequent hit confirmation and potency testing using this method identified several chemical scaffolds for the initiation of a medicinal chemistry program.

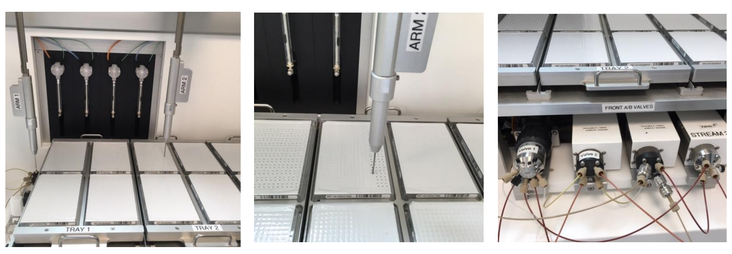
Enzyme Reaction

A biochemical assay using an isolated enzyme was required to screen library compounds in order to identify enzyme inhibitors.



Apricot ADDA

The Apricot ADDA is a dual arm, multi-injection port / switching valve robot developed on a Tecan platform and interfaces with Sound Analytics LLC ADDA software. The software facilitates excellent instrument control and can access MS/MS information stored in Sciex Analyst database following compound optimisation.



Mass Spectrometry and SPE

A sensitive and highly specific assay for the quantitative determination of the polar reaction product was achieved by the hyphenation of on-line solid phase extraction (SPE) using hydrophilic interaction chromatography (HILIC) conditions with tandem mass spectrometry (Sciex QTrap 6500).

The assay incorporated the used of a stable isotope labelled analogue of the reaction product to improve assay precision by reporting out normalised areas ratios for each sample injected.

Compound	Q1 Mass	Q3 Mass	DP	CE	CXP
Product	M	M-18	10	12	15
ISTD	M+6	(M+6)-18	10	17	15

Two Agilent 1260 isocratic pumps operating at flow rates of 1.2 mL/min were used to introduce the 'load' and 'elute' solvents onto the zirconium hydrophobic interaction chromatography (HILIC) cartridges (4 µL, 10 µM, Optimise Technologies) used for sample de-salting and elution into the mass spectrometer for analyte detection.

Pump	Solvent composition (v/v Acetonitrile: Water: Formic Acid)
Load	99: 0.5: 0.5
Elute	95: 4.5: 0.5

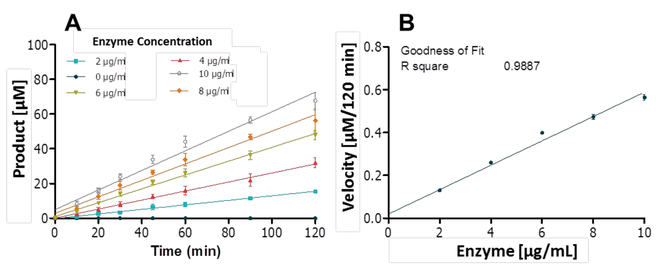
Samples were directed to the HILIC cartridge for desalting and analyte retention in a mobile phase containing a high percentage of acetonitrile, The analyte was then eluted from the cartridge and directed towards the mass spectrometry for detection in predominantly aqueous conditions.

The 'trap and elute' protocol incorporates a 6-second equilibrate and desalt phase with 12 seconds of elution solvent flow through the cartridge prior to re-equilibration as illustrated below.

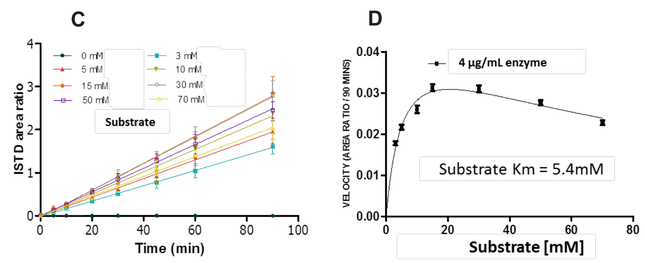
The analytical method was developed to use a 5 µL loop injection with the ADDA configured to draw and inject 10 µL to ensure the loop was overfilled. With 90 µL of sample available in each well, this allowed for the reinjection of assay plates as necessary without the need for re-picking of screening compounds and a complete re-assay should instrumental problems occur. Each 384-well plate was captured as a single data file and the individual reaction product and internal standard peak from each well integrated using Sound Review® software (Sound Analytics). The ratio of their respective AUCs was used as the primary data from which all secondary calculations were performed.

Biochemical Assay Development

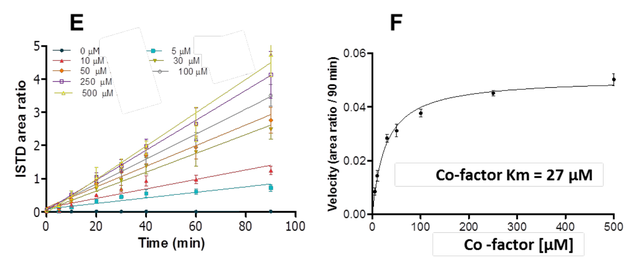
Biochemical assay development was initiated following instigation of the ADDA method as this provided the necessary readout for on-going activities. A standard suite of development assays was performed to characterise the enzyme kinetics to elucidate reagent conditions and the experiment time course.



Product formation was measured over a range of enzyme concentrations out to 120 minutes (**A**) and the rates of product formation shown to be linear (**B**). 4µg/mL of enzyme and a 90 minute incubation time were selected and further development experiments and the final screen.



Titration experiments for substrate (**C**) and co-factor (**E**) were conducted with the subsequent rates plotted to determine their respective Km values (**D** and **F** respectively). All values were within the expected range detailed by the client.

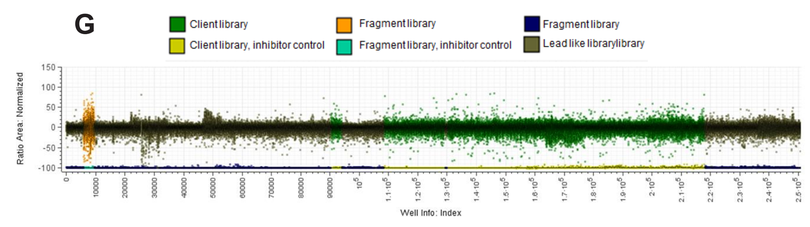


Screen

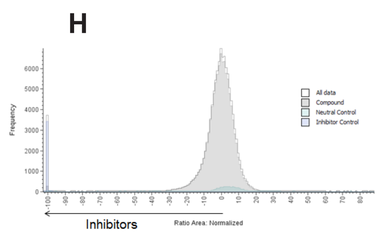
The full screen was conducted in several phases, each requiring successful completion prior to the start of the next. An initial pilot screen to assess the assay performance was followed by the primary screen, hit confirmation and finally potency testing of a selection of these hits. In total >300K unique compounds and/or fragments were tested from the CRL and the client's proprietary compound libraries.

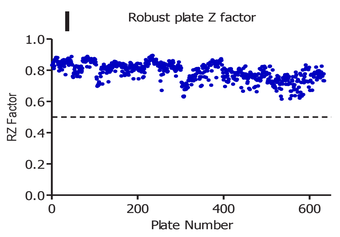
The primary area ratio data output from the ADDA-MS system was entered into Genedata Screener® (Genedata) and normalised on a plate-by-plate basis.

The full data set for all 300K compounds are shown in (G). The different libraries tested are highlighted. The fragment library was screened at 400 µM and the small molecules at 20 µM final assay concentration.

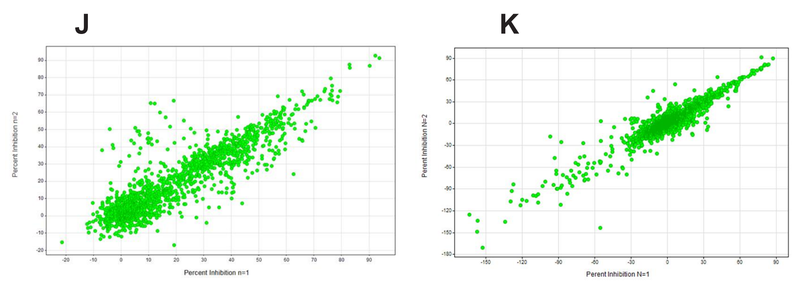


The overall HTS data was consistent with Z; values >0.5 for all plates tested (**I**) and the compound inhibition was distributed around zero (**H**).

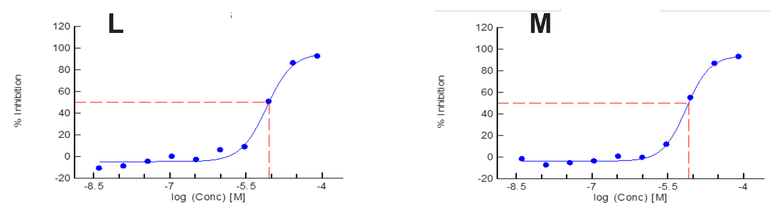




The primary screen generated two sets of hit confirmation data divided up into CRL (**J**) and client (**K**) libraries. Inhibitors and potential activators were determined within the screen.



Inhibitors were titrated from 100 µM maximum concentration over a 10-point curve in duplicate (L and M). Compounds with µM potency against the target were confirmed.



Conclusions

The HTS campaign was successfully completed using the ADDA and produced a high quality data set which facilitated both hit calling and potency determination of active compounds. Those compounds selected following hit confirmation were assessed using our frequent hitter analysis tool and any promiscuous inhibitors were removed from the project going forward.

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