MULTIPLEXING ENZYME REACTIONS USING RAPIDFIRE MASS SPECTROMETRY

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Introduction

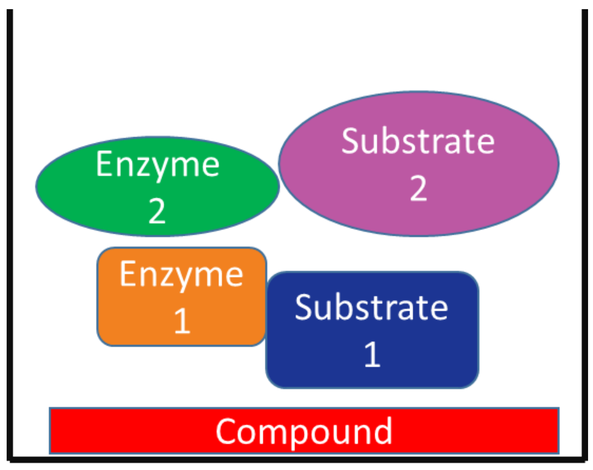
The RapidFire™ is a high throughput autosampler connected to a mass spectrometer. The Drug Discovery Unit (DDU) makes extensive use of our RapidFire and ways to increase throughput and decrease costs are sought.

A potential technique that could achieve both of these aims is multiplexing.

This involves adding multiple enzymes and their target substrates into a single well along with a compound of interest and measuring its effect on both simultaneously.

Mass-spectrometry is well suited to multiplexing, as several products can be measured as long their mass to charge ratio (m/z) is different.

We therefore set out to determine if multiplexing produced comparable data to individual experiments.



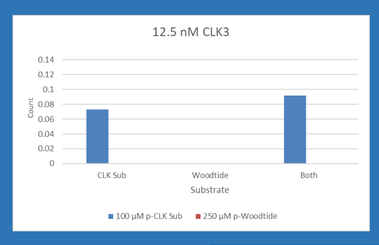
Determining Cross-reactivity

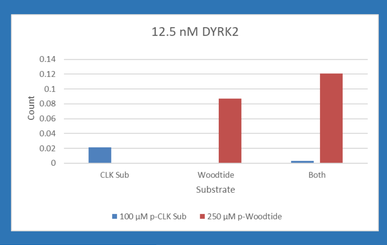
An important step of establishing a multiplex protocol is determining how much an enzyme acts on the other's substrate.

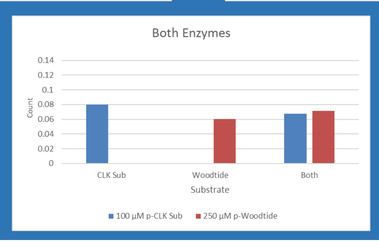
Our first experiments involved two kinases, Dual-specificity Tyrosine-Regulated Kinase 2 (DYRK2) and CDC-like kinase 3 (CLK3), so this was a particular concern.

We measured the phosphorylation of each of the kinases' substrates, (CLK Sub for CLK3,Woodtide for DYRK2), in the presence of each enzyme alone, and when multiplexed.

While DYRK2 showed some activity on CLK Sub this reduced to negligible levels when multiplexed.





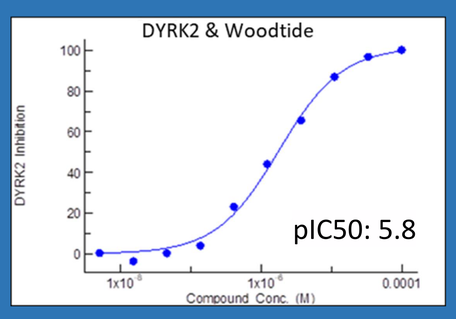


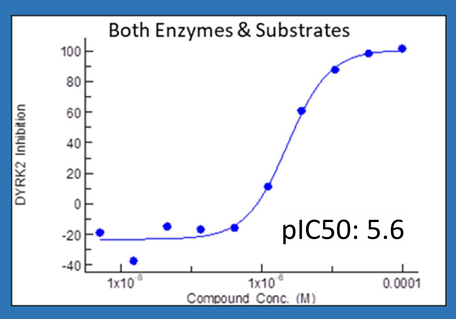
Does Multiple Enzymes Alter Inhibition?

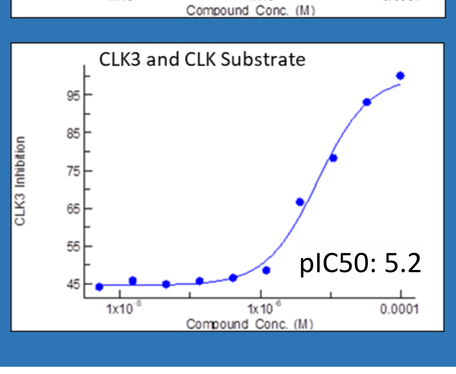
Having found cross-reactivity levels to be acceptable, we then looked to see the effects of adding a compound to the reaction

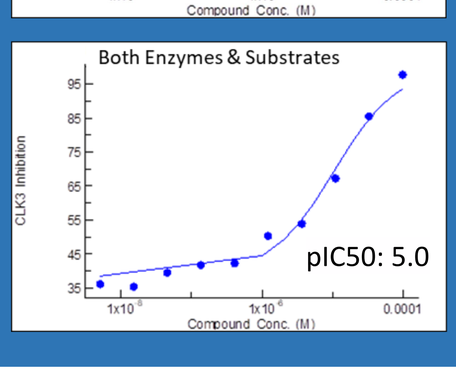
Using Harmine, a known inhibitor of DYRK1A that we determined inhibited both CLK3 and DYRK2, we looked at inhibition with and without multiplexing

Percent inhibition and IC50s were comparable for both enzymes when comparing individual data to multiplexed.









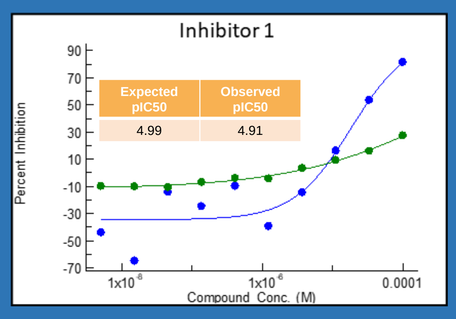
Using More Varied Enzymes

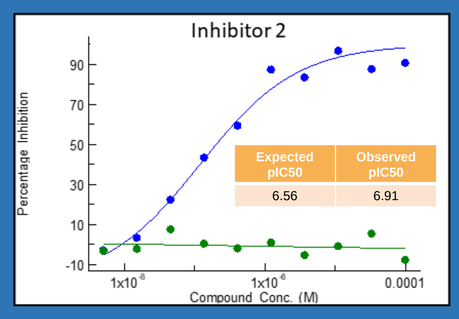
Having had success with two kinases, we then broadened our scope to look at more varied enzymes.

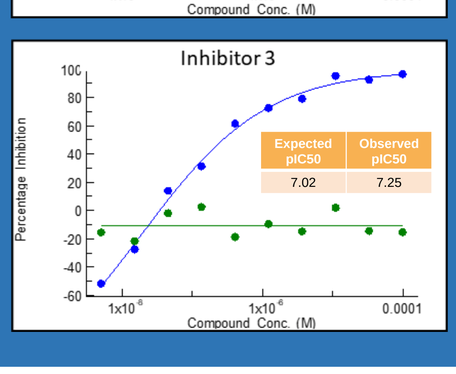
A non-kinase enzyme the DDU has worked on extensively was chosen as we had a range inhibitors with various pIC50s to test

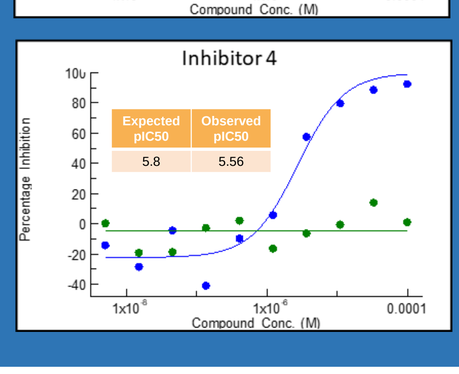
When multiplexed with DYRK2, the pIC50s against the non-kinase were comparable to the known values

DYRK2's activity was mostly unaffected by these compounds, particularly those more potent against the non-kinase enzyme









Assay Requirements

There are additional assay considerations for multiplexing to be successful

- The assay buffer must be suitable for all reactions
- RapidFire parameters must be suitable for all reactions
- The measured products cannot have the same m/z
- Enzymes should have little to no effect on the other's target substrate

Conclusions

Multiplexing is a promising tool for increasing throughput and decreasing assay costs

Exclusion assays can also be coupled to primary screens, providing key decision-making data early

Effective multiplexing can also extend the lifespan of precious compound libraries, and allow them to be screened against more targets