

# FLEXYTE Protease and Protein Kinase Assays: Realising the Benefits of Fluorescence Lifetime for Drug Discovery Applications

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## Introduction

Fluorescence lifetime (FLT) is defined as the average time a fluorescent molecule spends in the excited state before returning to the ground state. The application of this intrinsic fluorescence property as the reporting modality in biochemical and cell based assays is attractive, as it is independent of probe concentration and volume, and is unaffected by auto-fluorescence, light scattering and inner filter effects. In addition, FLT enables background interference from fluorescent compound libraries to be minimised, leading to fewer false positives and negatives in drug screening applications.

Many commercially available fluorescent dyes have lifetimes of the order of a few nanoseconds, making them unsuitable as reporting systems in FLT based assays. To address this need, Almac Sciences have recently developed fluorescence reporters based on 9-aminoacridine (9AA) possessing lifetimes up to 17 ns. Using these new long lifetime fluorophores, we have recently reported the development of a universal, FLT-based Ser / Thr protein kinase assay.<sup>1</sup> Further assay development, including substrate engineering, has resulted in a non-radioactive, antibody-free technology platform (FLEXYTE protein kinase assays) that enables the benefits of FLT to be realised for this target class.

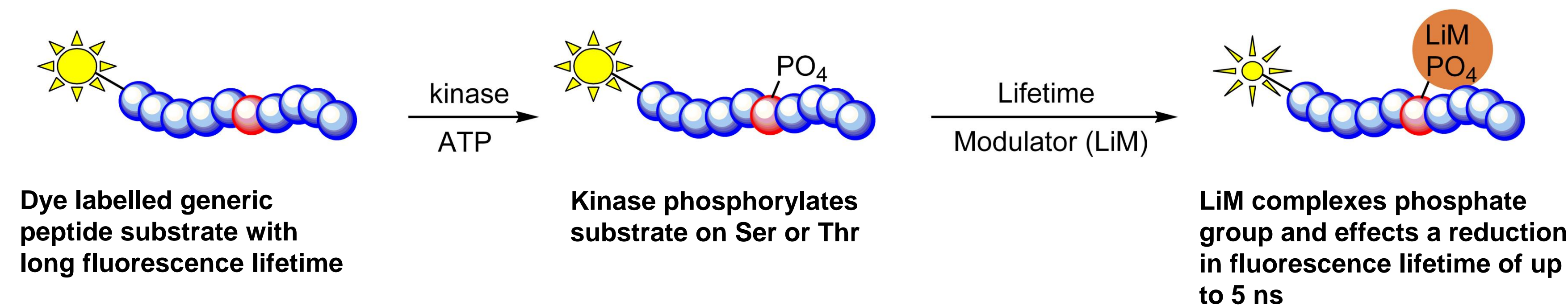
Here we report the application of our FLEXYTE assay platform to the protease family of enzymes. Proteases are an important drug target due to their role in cancer, inflammation, and cardiovascular diseases. However, to date, biochemical assays to assess protease function have been hindered by interference effects (fluorescence intensity) or the cost of complex dye-labelled substrates (TR-FRET).

Our FLT-based assay employs bespoke 9AA-labelled peptide substrates incorporating a modulator moiety, which reduces the fluorescence lifetime of the fluorophore until released by the action of the protease.

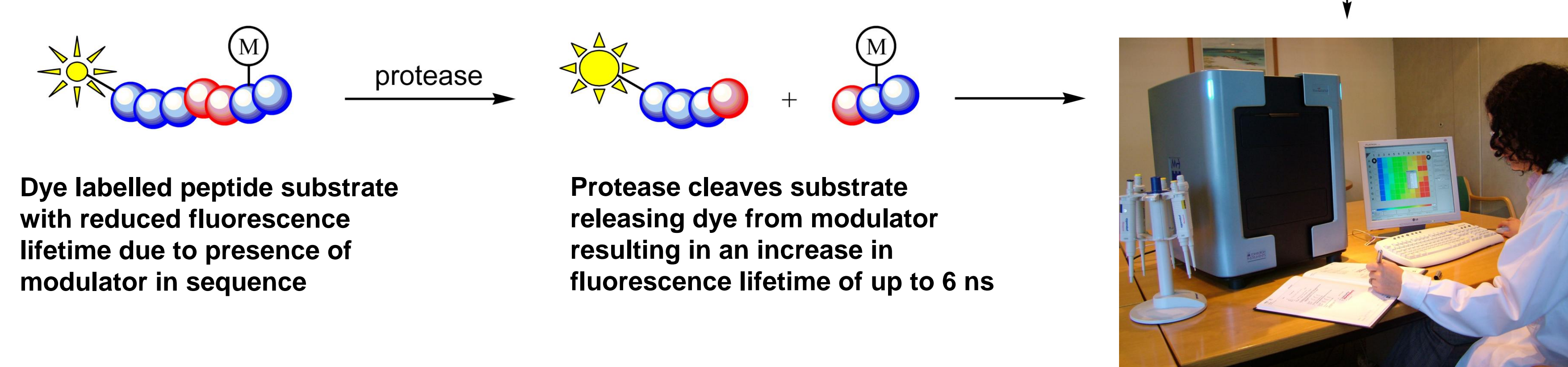
This homogeneous real-time protease assay technology shows excellent promise as a generic, cost-effective, and robust approach for screening this therapeutically important class of proteins.

## FLEXYTE Assay Principle

### Protein Kinase Assays

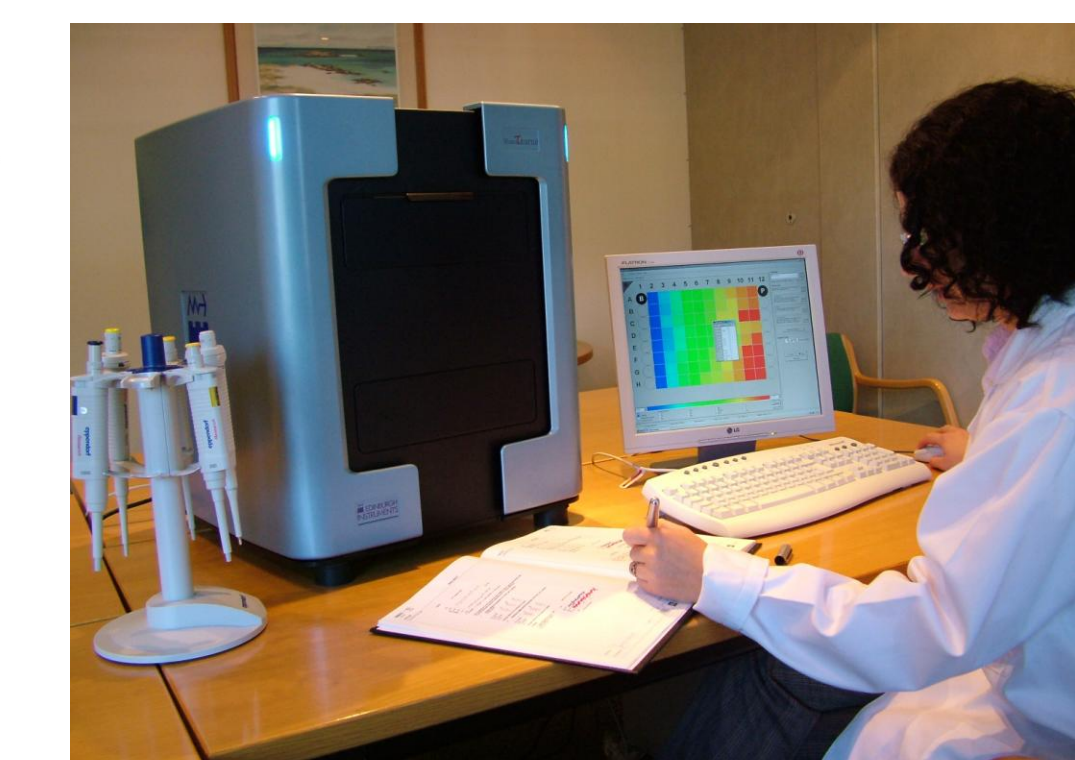


### Protease Assays



FLEXYTE protein kinase assays are performed using a simple 'mix and read' protocol with the lifetime modulator used to stop the reaction at the appropriate time point

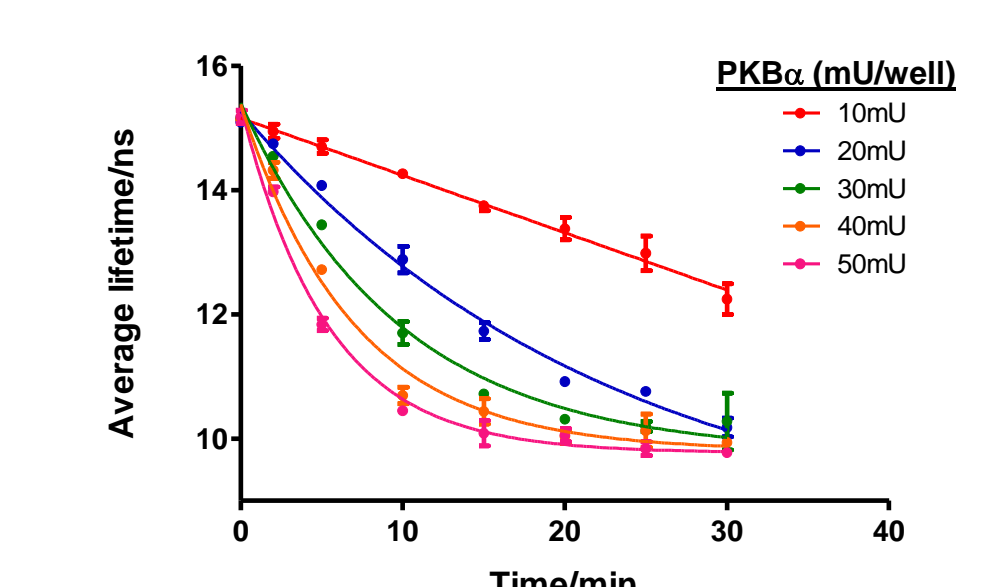
FLEXYTE protease assays can be monitored in real-time and allow kinetic measurements to be performed. Alternatively, reactions can be stopped by the addition of an appropriate reagent to facilitate HTS applications



Change in fluorescence lifetime measured directly using plate reader (Nanotaurus, Edinburgh Instruments)

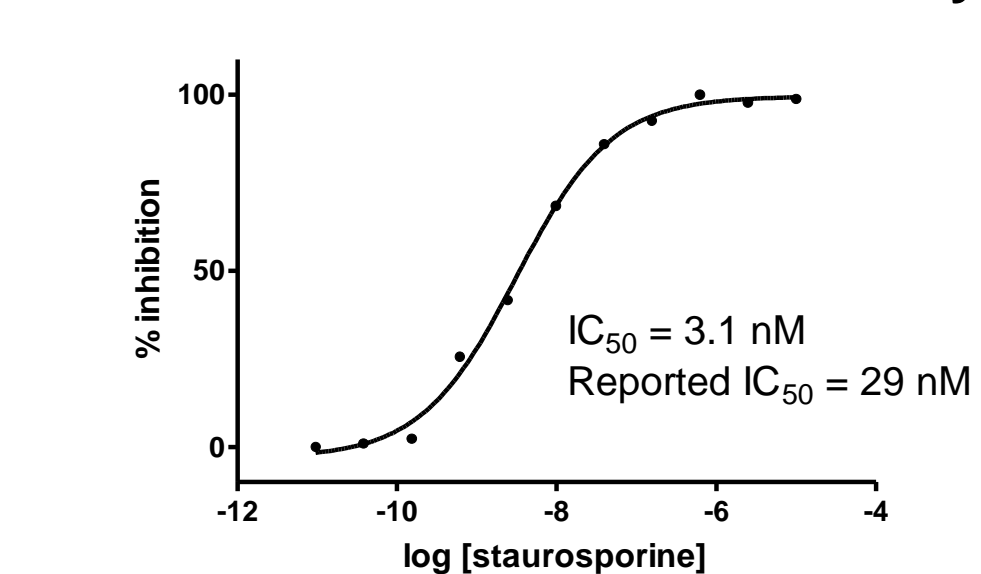
## Universal Ser / Thr Protein Kinase Assay

### PKB $\alpha$ assay – Enzyme Titration

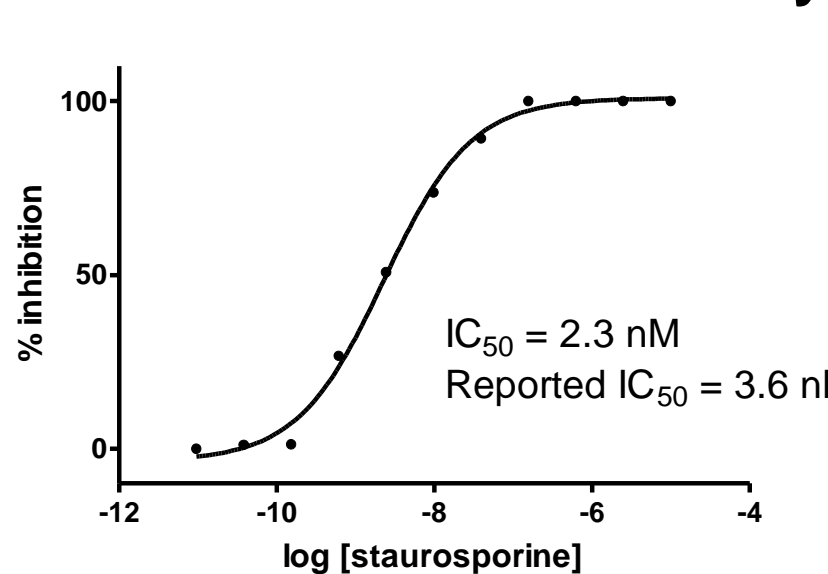


- Generic substrates (KS 1-3) configured for assaying a broad panel of Ser / Thr protein kinases (>100)
- FLEXYTE protein kinase assays give excellent Z'-factors (>0.9) indicating suitability for HTS applications
- Compatible with a wide range of ATP and substrate concentrations
- Assays are easily miniaturised to low volumes

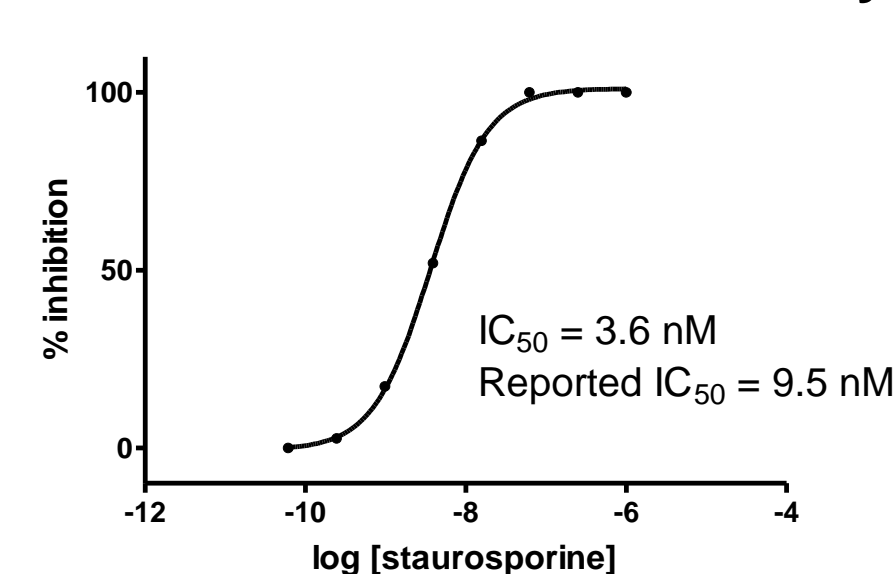
### FLEXYTE KS-1: PKB $\alpha$ assay



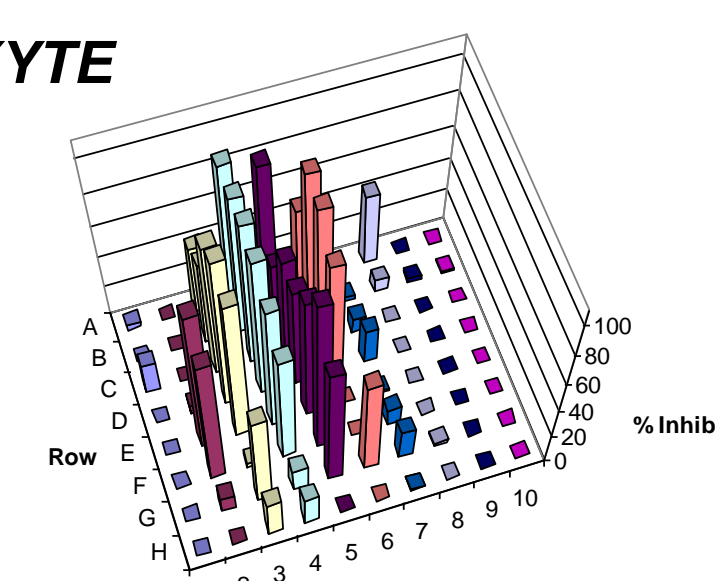
### FLEXYTE KS-2: PKA assay



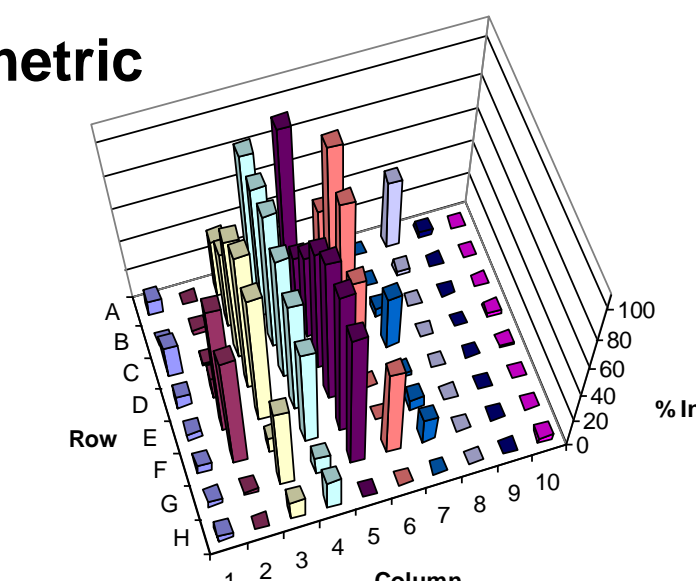
### FLEXYTE KS-3: CHK1 assay



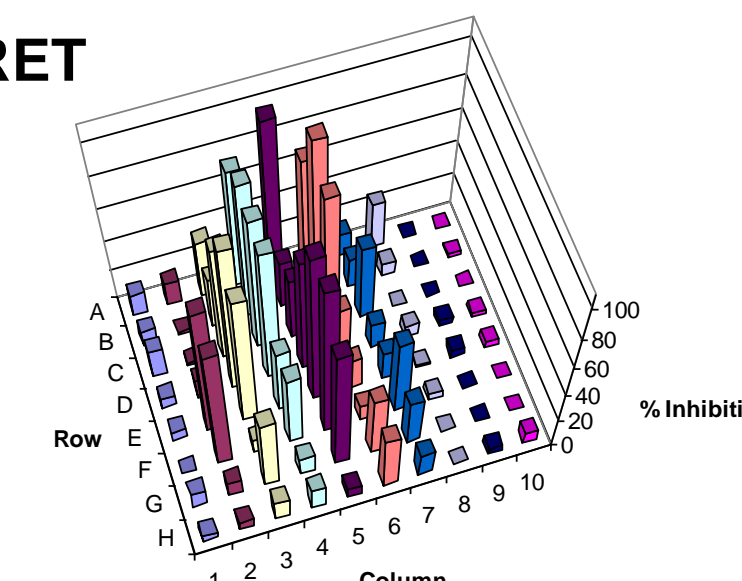
### FLEXYTE



### Radiometric



### TR-FRET

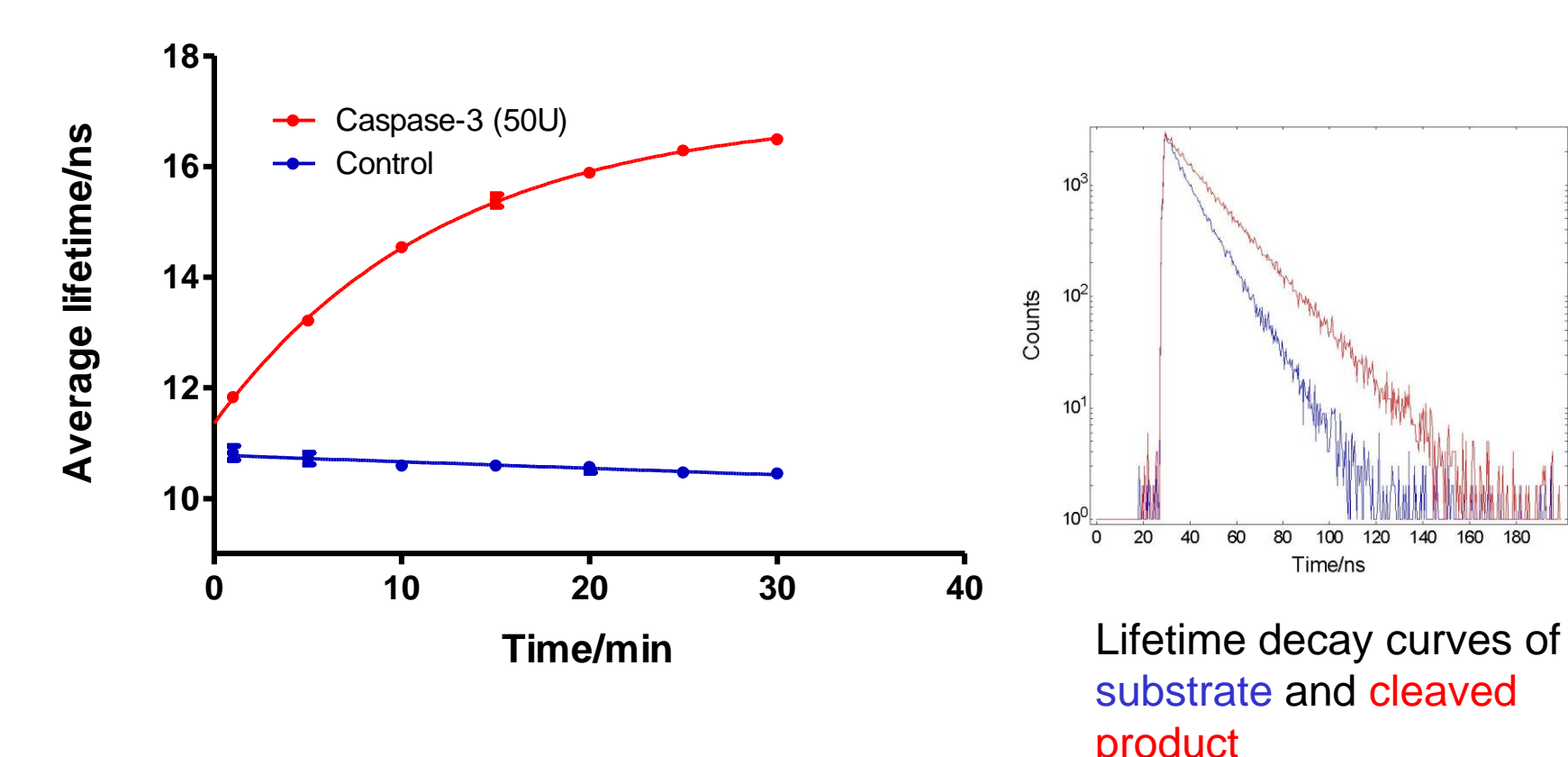


FLEXYTE PKB $\alpha$  assay shows excellent comparison with gold-standard radiometric assay and TR-FRET for hit finding (Pearson correlations: 0.98 and 0.93 respectively)

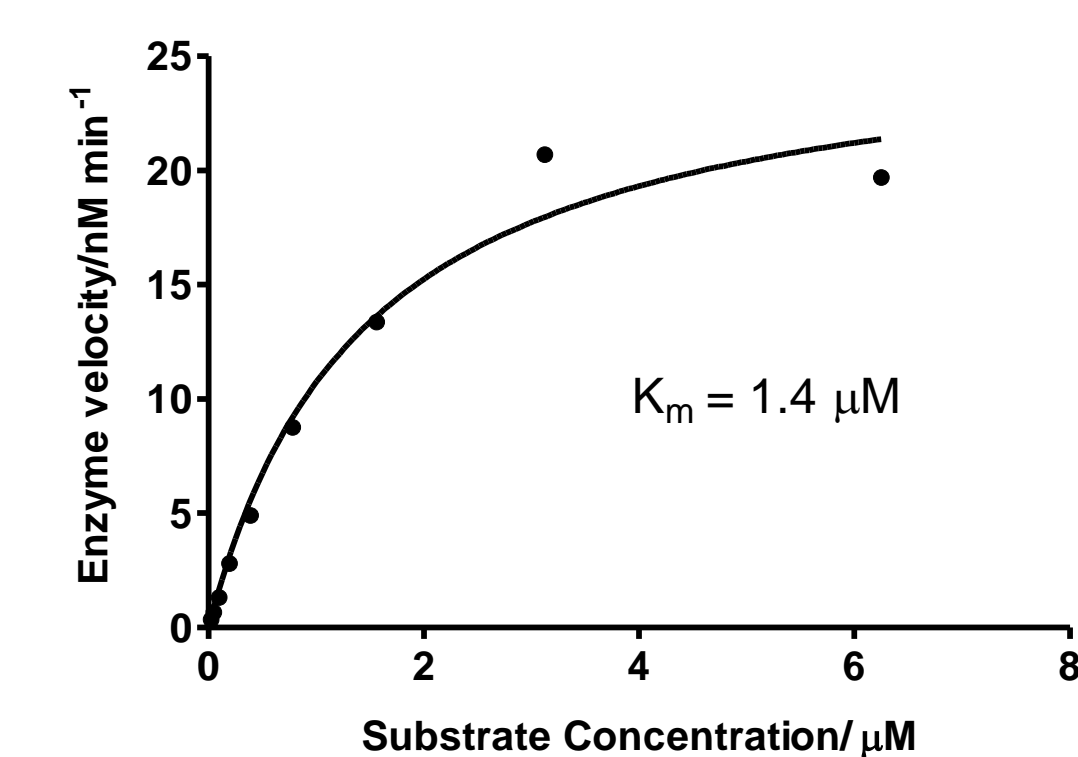
## FLEXYTE Protease Assay

Data shown below is from Caspase-3 assays employing 9AA-DEVDSX-NH<sub>2</sub> as the peptide substrate, where X = modulator.

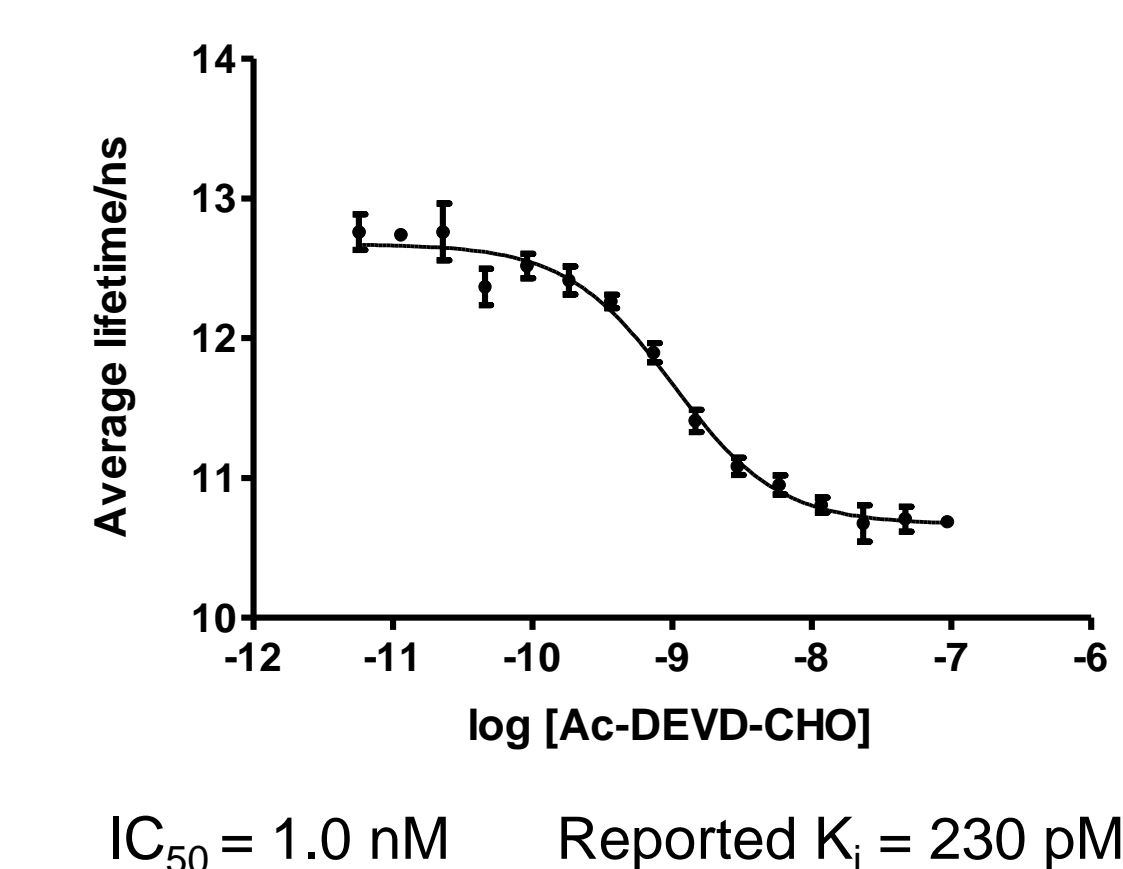
### 1. Real-Time Assay



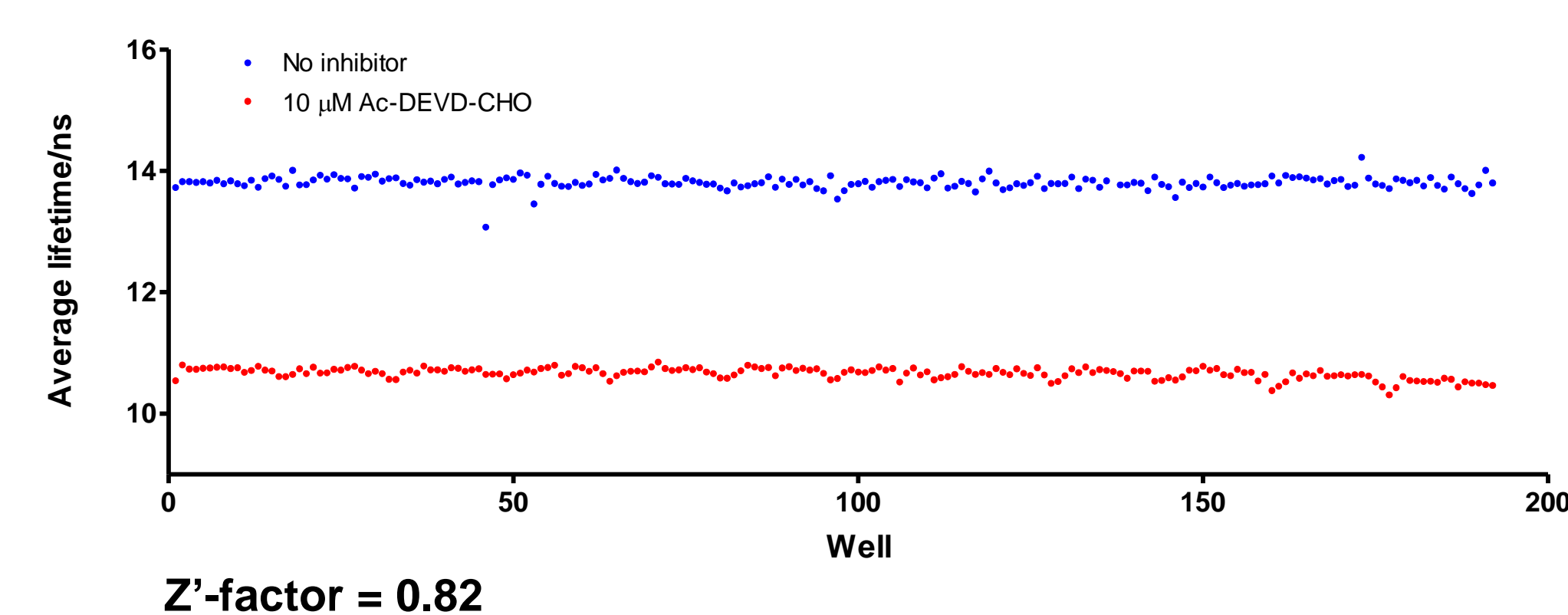
### 2. Substrate Titration



### 3. Inhibitor Titration



### 4. Z'-Factor (384-well)

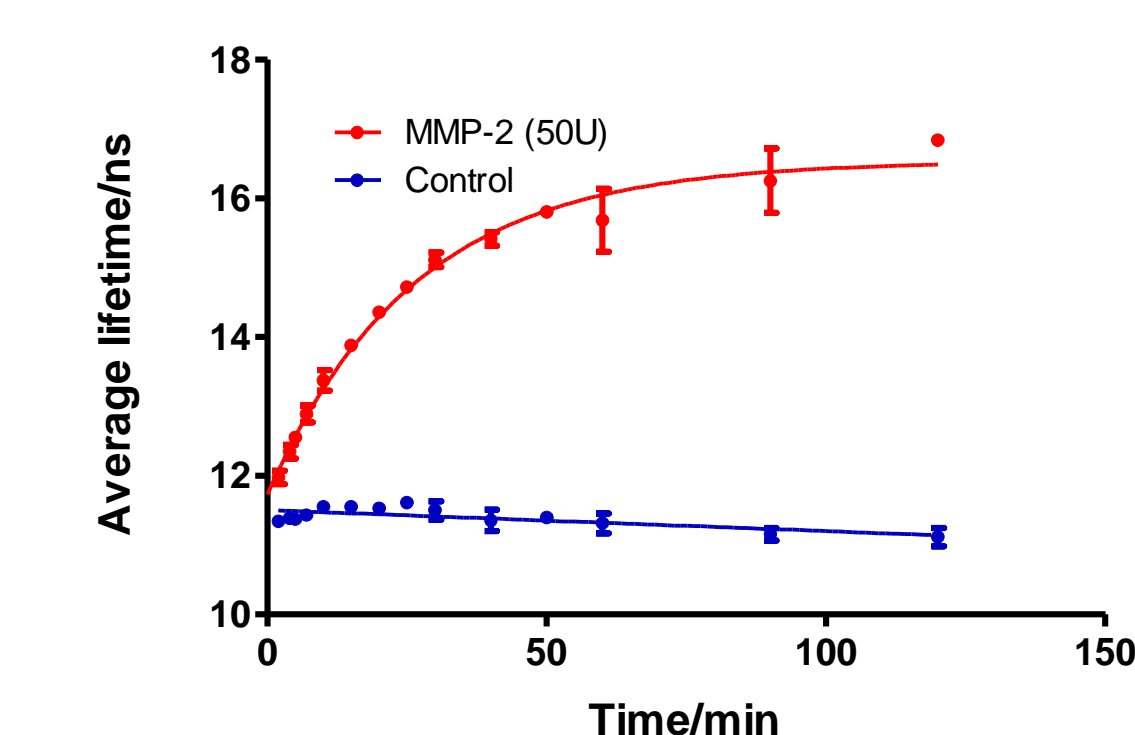


- FLEXYTE protease assays are homogeneous real-time assays that utilise a product dependent increase in fluorescence lifetime as the reporting modality
- The assay delivers a reliable response with a large dynamic range that provides for high assay sensitivity
- Excellent Z'-factors (>0.8) demonstrate that FLEXYTE protease assays are suitable for HTS and profiling applications
- Bespoke labelled peptides are shown to be excellent substrates for proteases

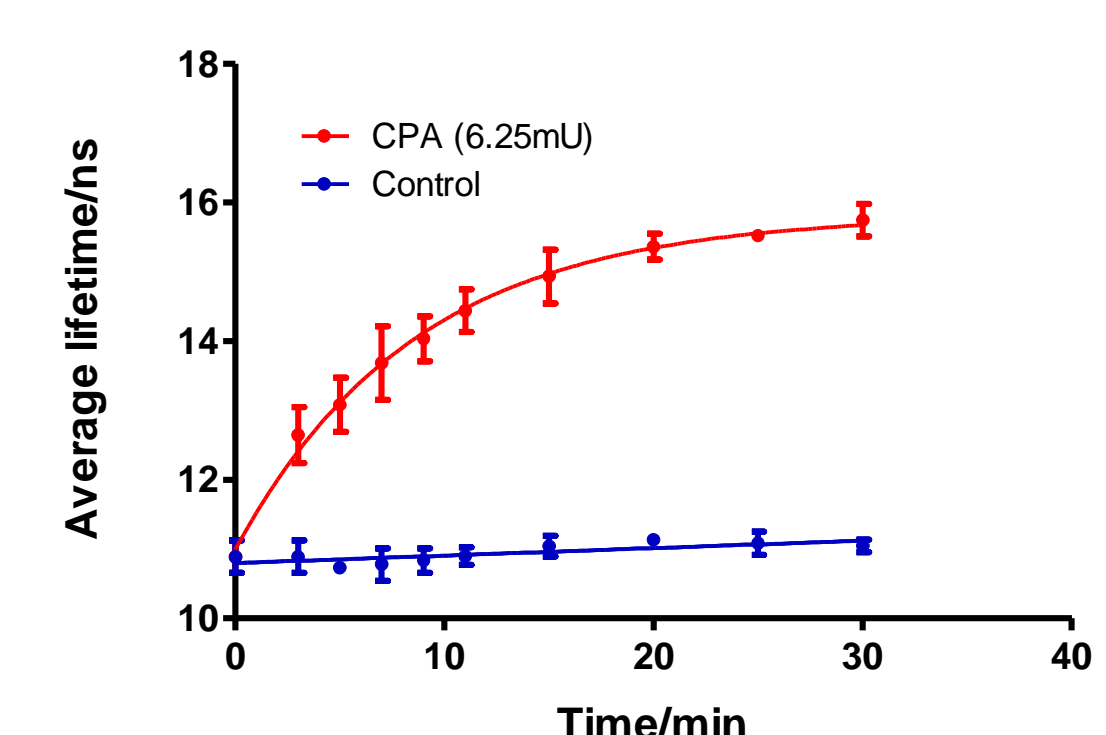
## Universal Protease Platform

Due to the fundamental principles of the technology, the FLEXYTE protease platform offers a generic solution for assaying this important class of enzymes. For example, assays for matrix metalloproteinase-2 (MMP-2) and carboxypeptidase A are shown below using peptide substrates 9AA-PLGLXAR-NH<sub>2</sub> and 9AA-GGX-NH<sub>2</sub> respectively (where X = modulator).

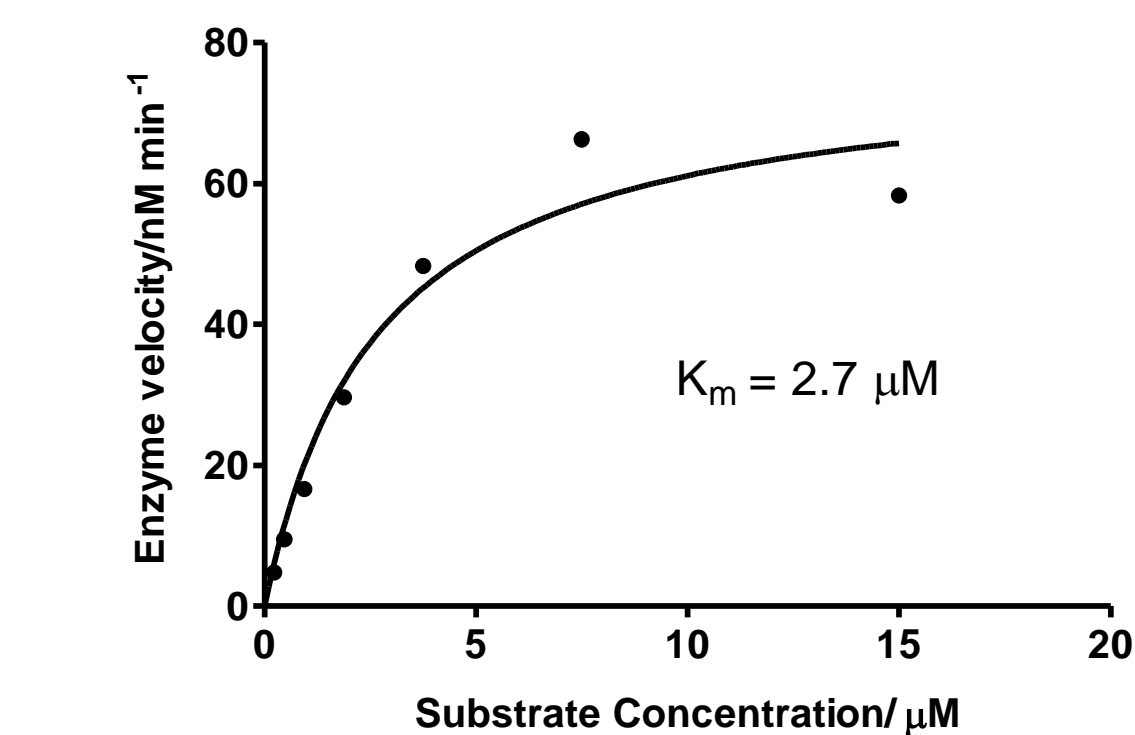
### MMP-2 Assay



### Carboxypeptidase A Assay

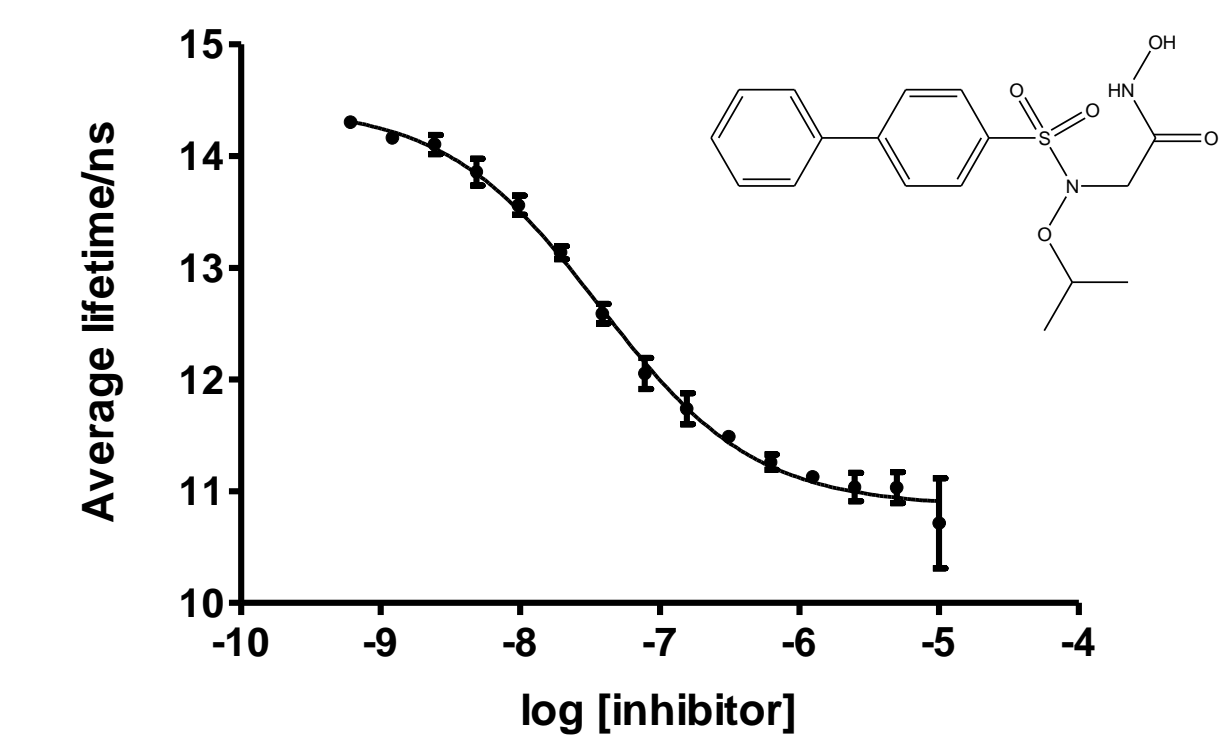


### MMP-2 Substrate Km



Z'-factor (MMP-2) = 0.88

### MMP-2 Inhibitor Assay



IC<sub>50</sub> = 37 nM Reported IC<sub>50</sub> = 12 nM

## Conclusion

### FLEXYTE Protein Kinase Assays

- A universal FLEXYTE protein kinase assay has been developed for Ser / Thr kinases based on fluorescence lifetime as the reporting modality
- This platform technology provides an antibody free, non-radioactive, and cost effective solution for protein kinase screening
- Generic substrates enable FLEXYTE assays to be configured for a broad panel of Ser / Thr protein kinases in a rapid fashion (covering >100 different kinases)
- Applicable, in principle, to any Ser / Thr kinase with an appropriate peptide substrate

### FLEXYTE Protease Assays

- FLEXYTE assays have now been developed for proteases, with enzyme activity reported directly by an increase in fluorescence lifetime
- This universal platform technology provides for homogeneous, reliable, real-time monitoring of protease activity
- FLEXYTE assays based on labelled peptide substrates have been developed for a variety of protease targets
- Generic peptide approach enables FLEXYTE assays to be configured for a broad range of proteases

[1] M.J. Paterson, C.J. Dunsmore, R. Hurteaux, B.A. Maltman, G.J. Cotton, A. Gray, A fluorescence-lifetime based assay for serine and threonine kinases that is suitable for high-throughput screening, *Anal. Biochem.* (2010), in press.