

# The **FLEXYTE™** Platform - Expanding the Scope of Fluorescence Lifetime Assays to New Target Classes



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## Introduction

The **FLEXYTE™** assay platform is a screening technology that utilises fluorescence lifetime (FLT) as the reporting modality. As an intrinsic parameter of the fluorophore, FLT is independent of probe concentration and volume which facilitates assay miniaturisation. Furthermore, FLT is unaffected by auto-fluorescence, light scattering and inner filter effects leading to more robust assays where interference from fluorescent compound libraries can be minimised.

Key to the success of this technology has been the development and application of 9-aminoacridine (9AA) as the fluorescent reporter.<sup>1</sup> The long FLT (17 ns) of 9AA is ideally suited for assay applications, enabling discrimination from interfering species which typically have lifetimes in the 1 - 5 ns range. By employing 9AA-labelled peptide substrates, **FLEXYTE™** assays have been configured for a number of important drug targets including proteases, Ser / Thr / Tyr protein kinases<sup>2</sup> and phosphatases. In these assays the activity of the enzyme is directly reported by a change in the measured fluorescence lifetime, providing an homogeneous, non-radioactive and antibody free approach to assaying these targets.

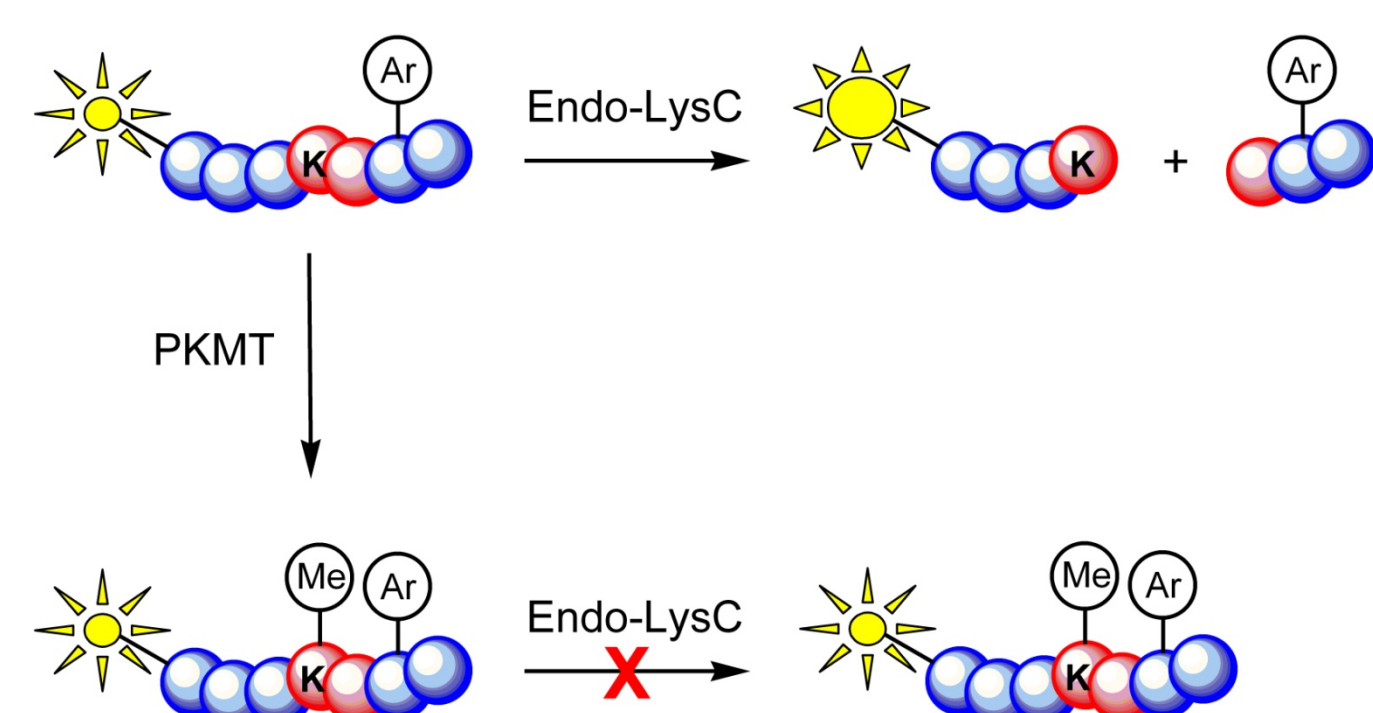
We now report on a further application of the **FLEXYTE™** platform to provide FLT assays for epigenetic enzyme targets. Homogeneous, antibody free assays for protein methyltransferases (both lysine and arginine), peptidylarginine deiminases, and histone demethylases have now been developed. Such enzymes are the subject of extensive drug discovery programmes within the pharmaceutical industry due to their role in autoimmune diseases and cancer. However, there is currently a lack of simple and effective assays for such enzymes, hence, the **FLEXYTE™** platform will offer broad utility as a robust, reliable, and cost effective solution for the screening and profiling of epigenetic targets.

We also highlight a major advantage of FLT technology – the ability to identify and correct for fluorescent compound interference.

## Protein Lysine Methyltransferases (PKMT)

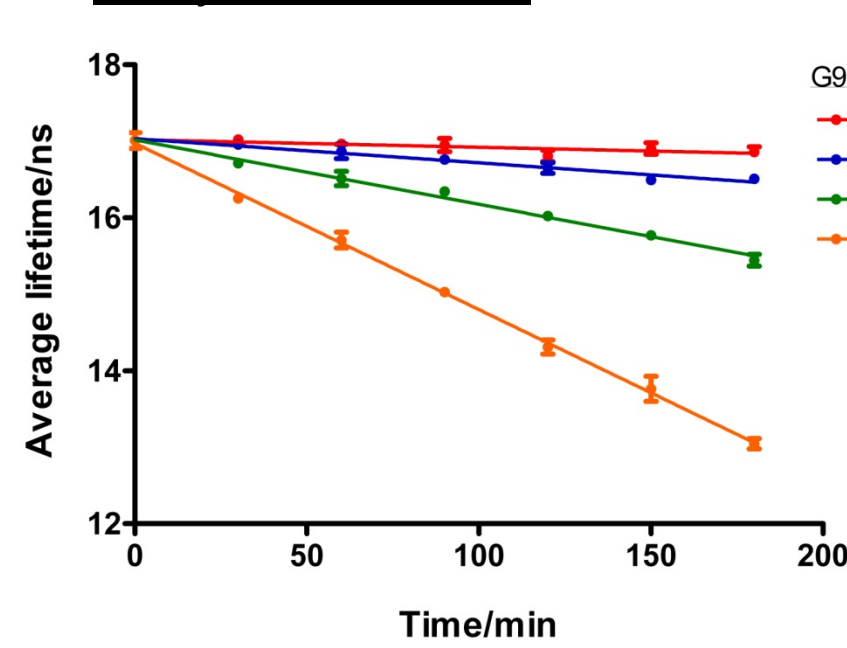
### PKMT Assay Principle

- Histone peptide substrate labelled with proprietary long lifetime 9-aminoacridine (9AA) fluorophore and incorporating an aromatic moiety which reduces the FLT of 9AA
- PKMT catalysed methylation of the histone substrate prevents cleavage by the protease, Endo-LysC, thereby maintaining modulation of the FLT of 9AA
- Hence, PKMT activity is reported by a decrease in the measured FLT of the assay

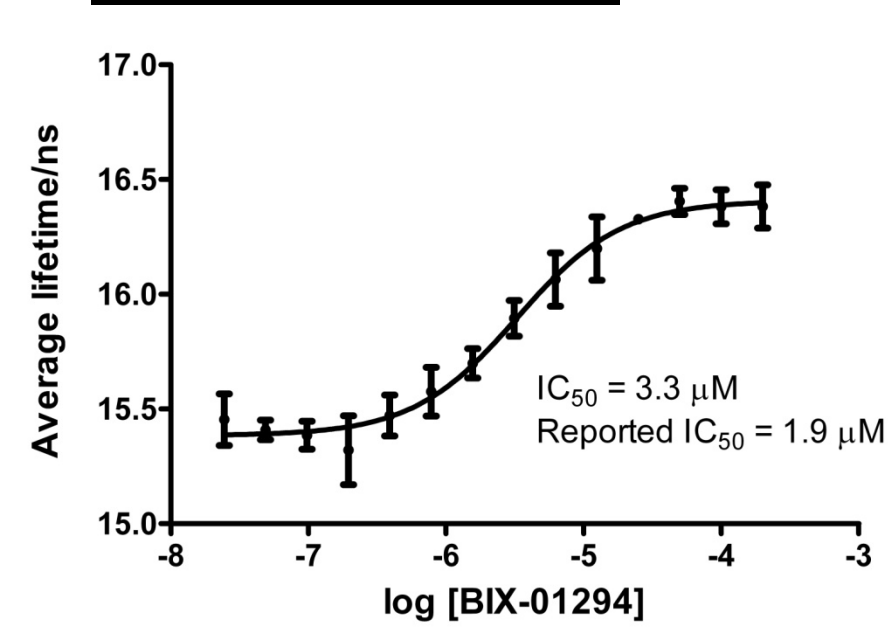


### G9a Assay – Methylation of H3K9

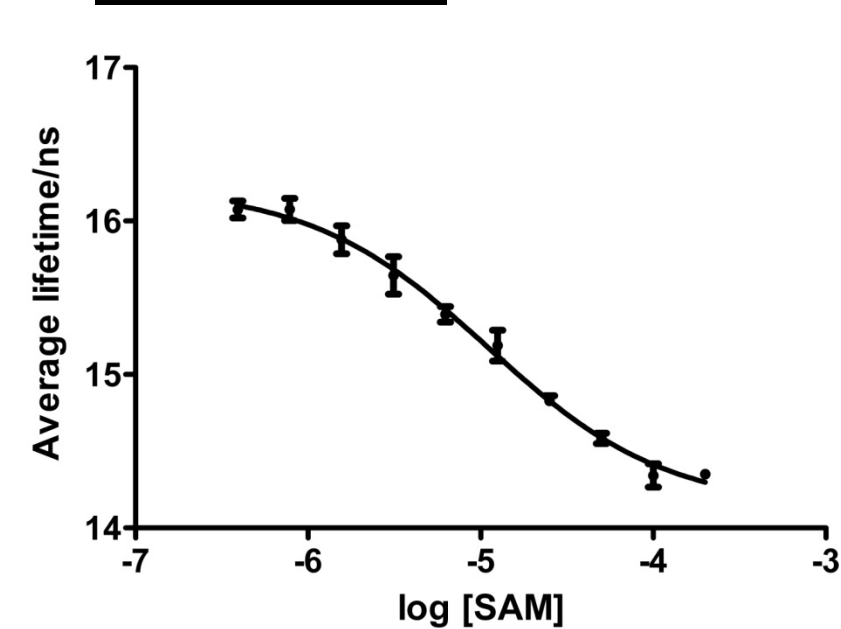
#### Enzyme Titration



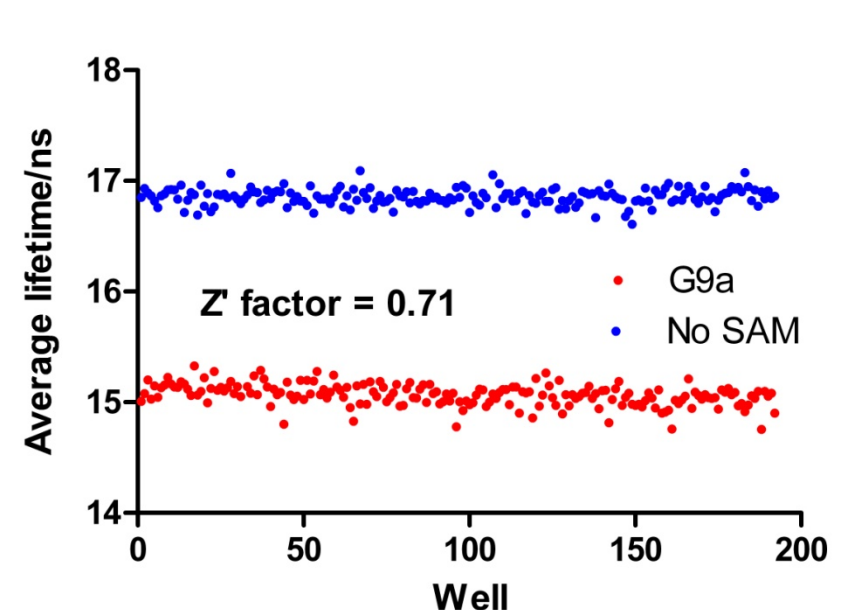
#### BIX-01294 Inhibition



#### SAM Titration



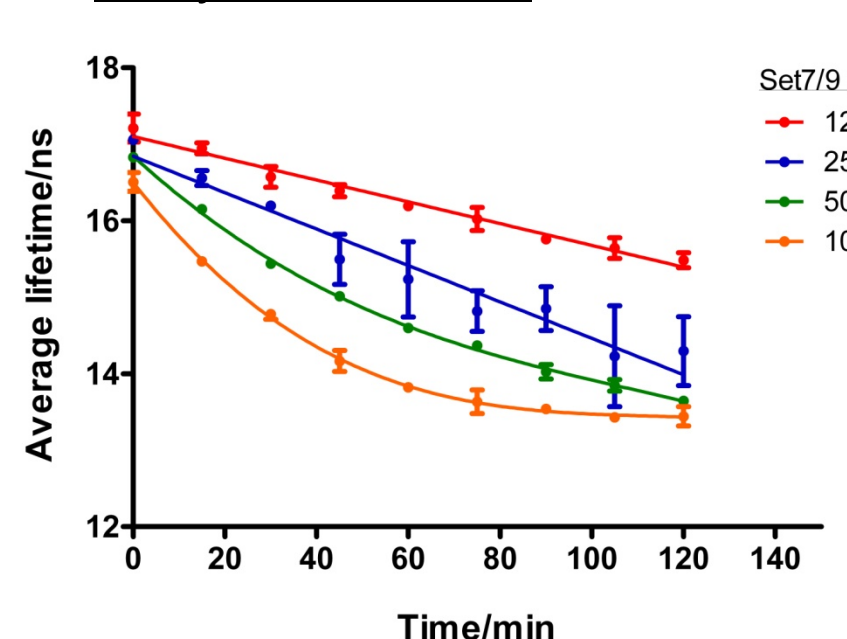
#### Z'-Factor



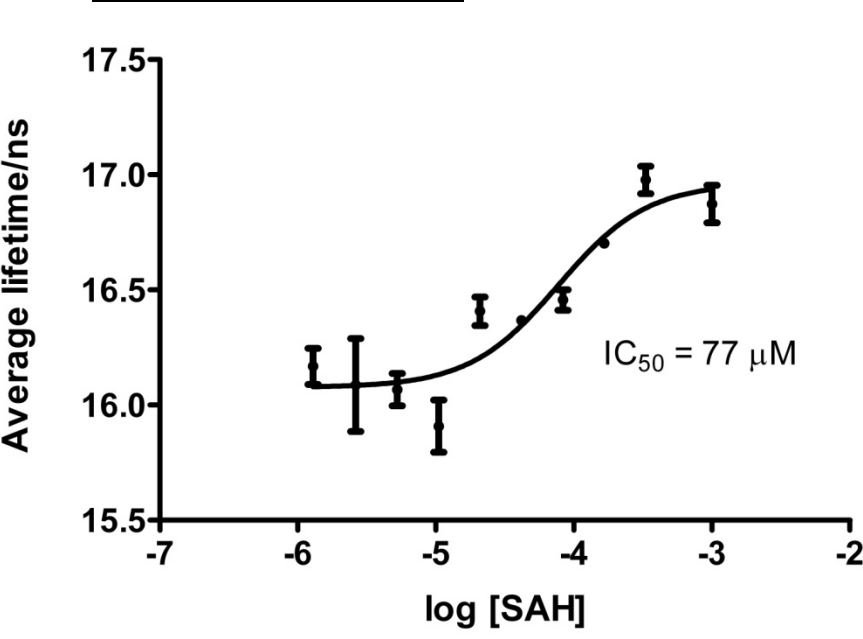
- The **FLEXYTE™** G9a assay employs a tailored histone H3 peptide substrate to efficiently report methylation at Lys9
- The assay was validated for inhibition studies using the known BIX-01294 inhibitor whose measured IC<sub>50</sub> was consistent with the literature value
- The assay was shown to be compatible with a wide range of SAM concentrations and suitable for HTS applications (Z'-factor > 0.7)

### Set 7/9 Assay – Methylation of H3K4

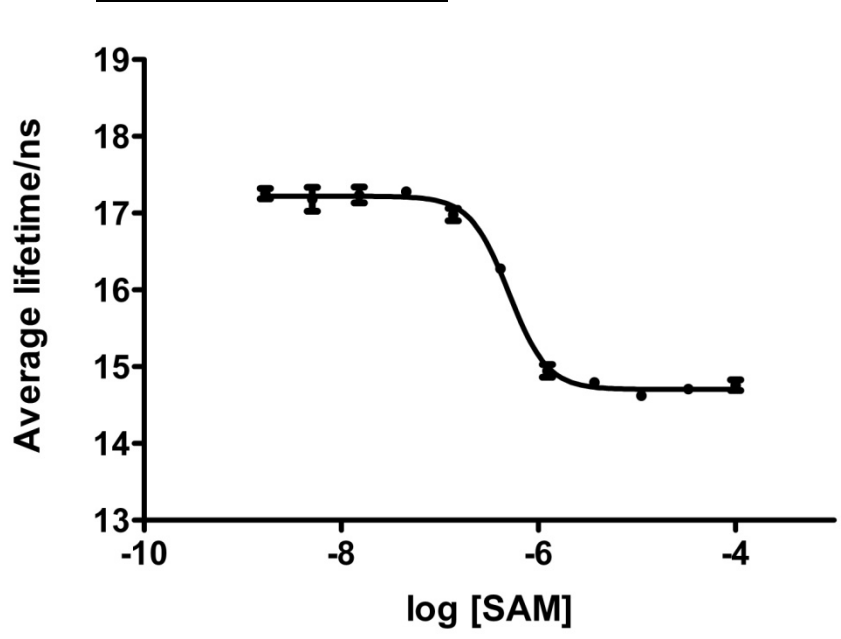
#### Enzyme Titration



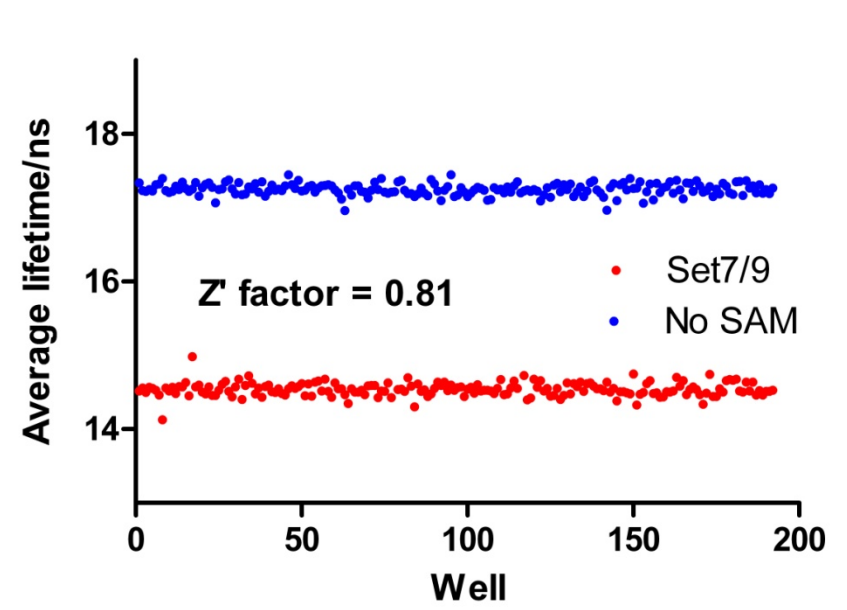
#### SAH Inhibition



#### SAM Titration



#### Z'-Factor

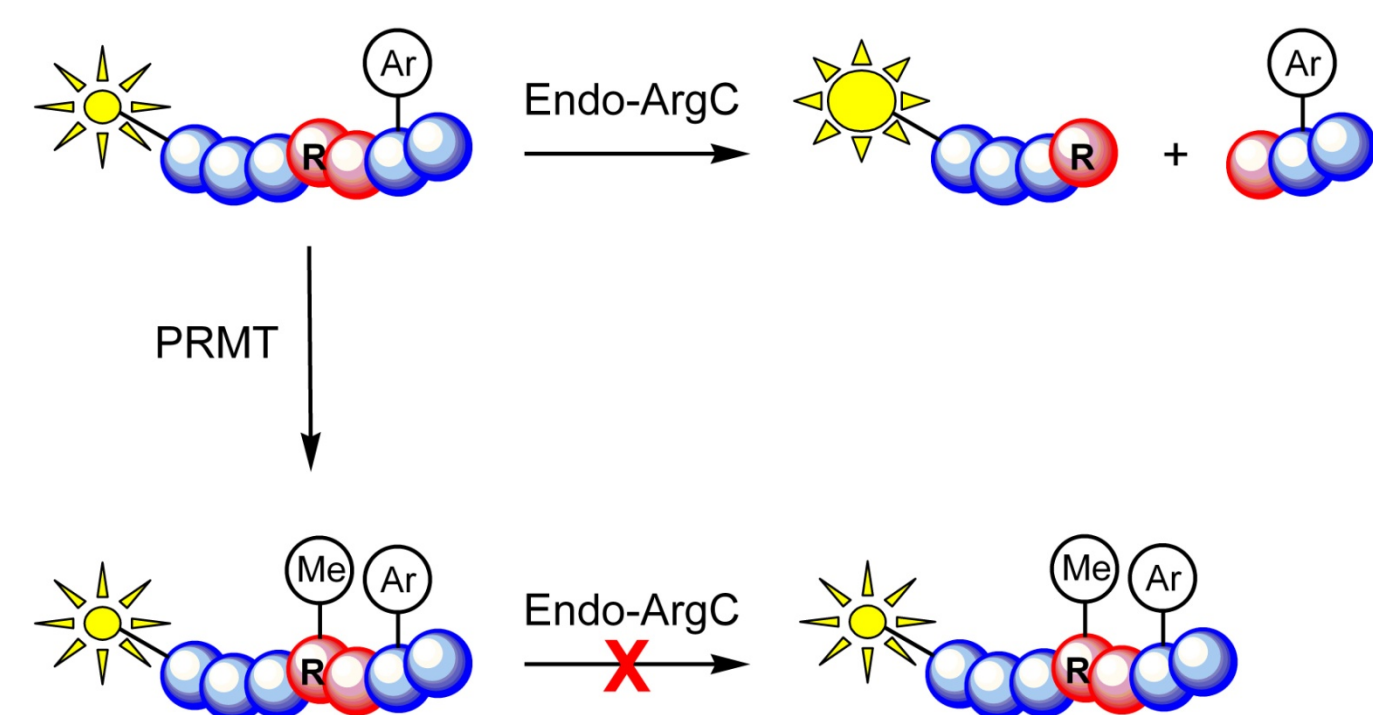


- The **FLEXYTE™** Set 7/9 assay employs a tailored histone H3 peptide substrate to efficiently report methylation at Lys4
- The assay was validated for inhibition studies using the generic SAM competitive inhibitor, S-adenosylhomocysteine (SAH)
- The assay was shown to be compatible with a wide range of SAM concentrations and suitable for HTS applications (Z'-factor > 0.8)

## Protein Arginine Methyltransferases (PRMT)

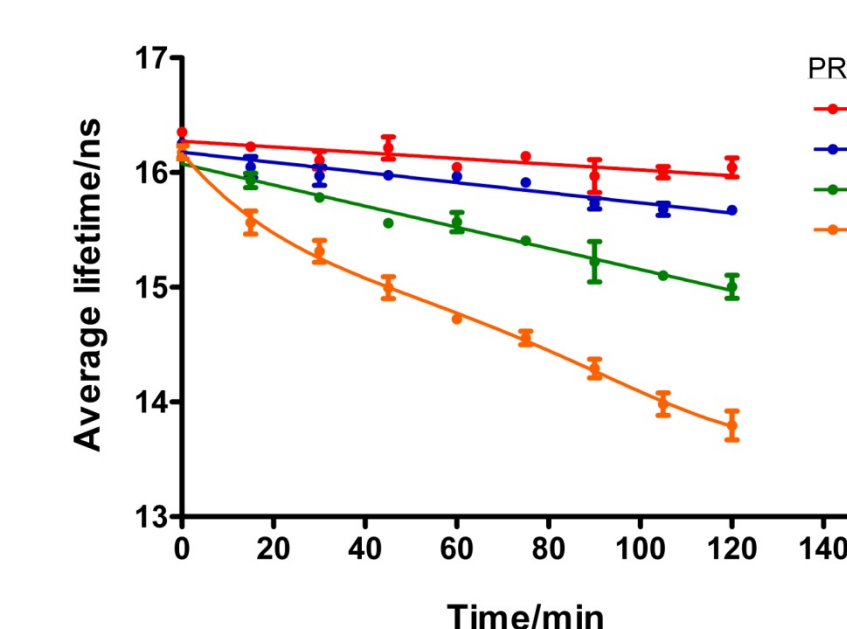
### PRMT Assay Principle

- Histone peptide substrate labelled with proprietary long lifetime 9-aminoacridine (9AA) fluorophore and incorporating an aromatic moiety which reduces the FLT of 9AA
- PRMT catalysed methylation of the histone substrate prevents cleavage by the protease, Endo-ArgC, thereby maintaining modulation of the FLT of 9AA
- Hence, PRMT activity is reported by a decrease in the measured FLT of the assay



### PRMT5 Assay – Methylation of H4R3

#### Enzyme Titration



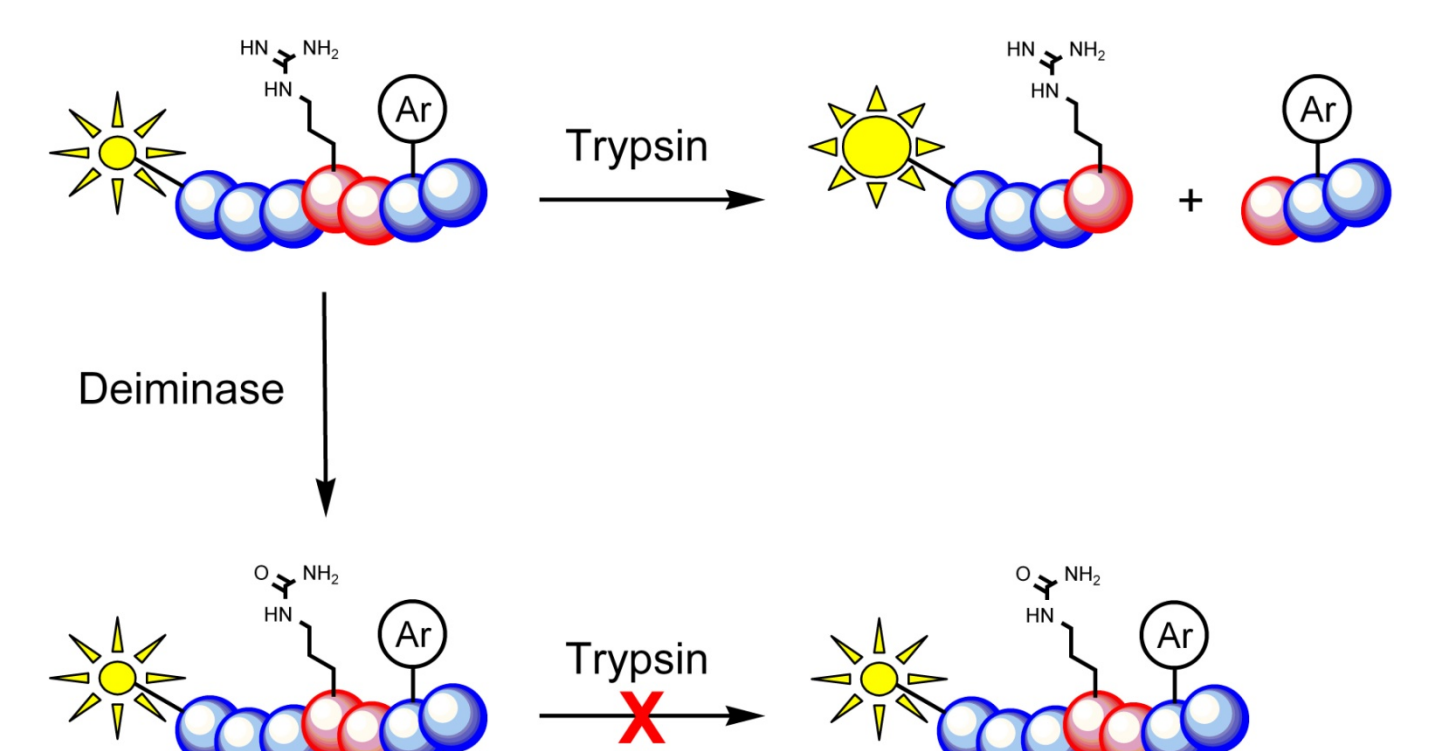
- **FLEXYTE™** methyltransferase assays have recently been extended to PRMTs, such as human PRMT5 which symmetrically methylates H4R3
- The assay employs a tailored H4 peptide substrate whose FLT is reduced until exposed to the protease, Endo-ArgC. Methylation of Arg3 by PRMT5 confers protease protection, leading to a concentration and time dependent decrease in the FLT of the peptide reporter

• A similar protease protection approach has been validated for histone demethylases, such as JHDM1A

## Peptidylarginine Deiminases (PAD)

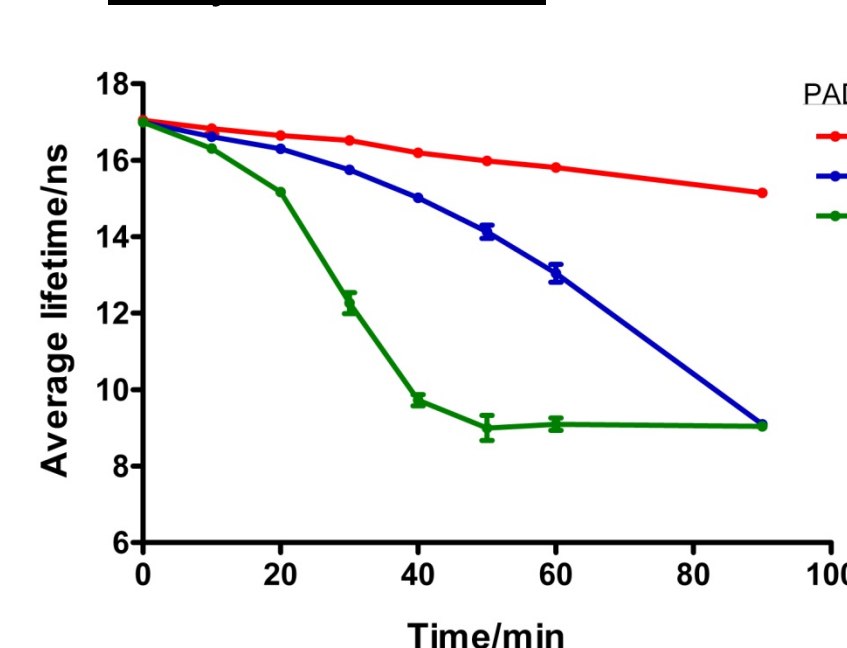
### PAD Assay Principle

- Peptide substrate labelled with proprietary long lifetime 9-aminoacridine (9AA) fluorophore and incorporating an aromatic moiety which reduces the FLT of 9AA
- Deiminase catalysed citrullination of the arginine residue prevents cleavage by the protease, trypsin, thereby maintaining modulation of the FLT of 9AA
- Hence, deiminase activity is reported by a decrease in the measured FLT of the assay

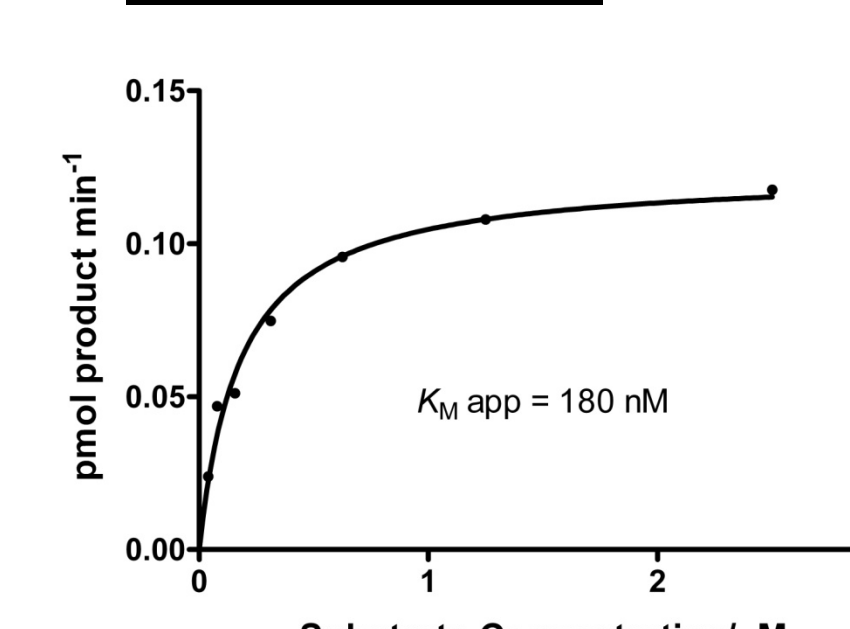


### PAD4 Assay

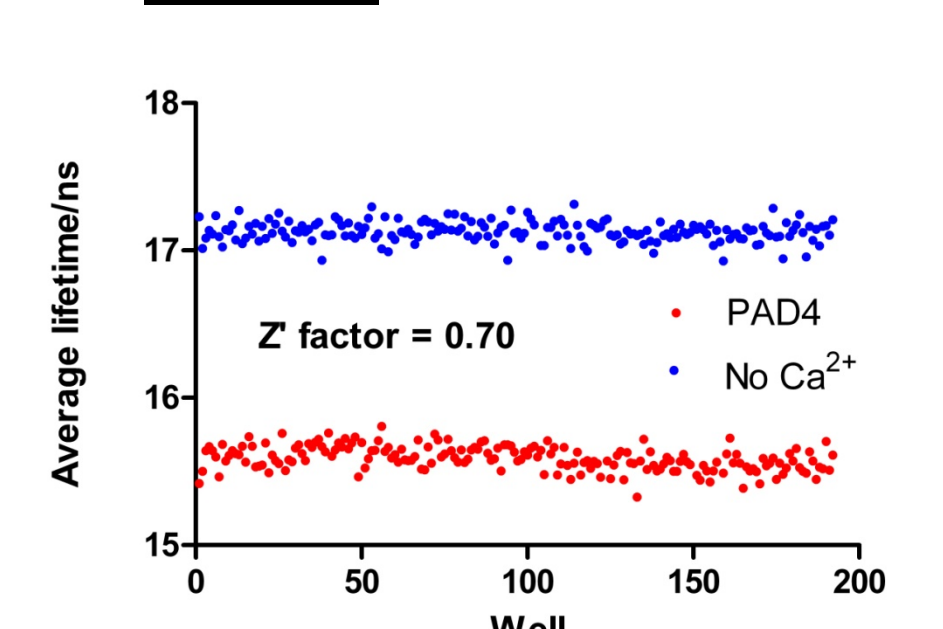
#### Enzyme Titration



#### Substrate Titration



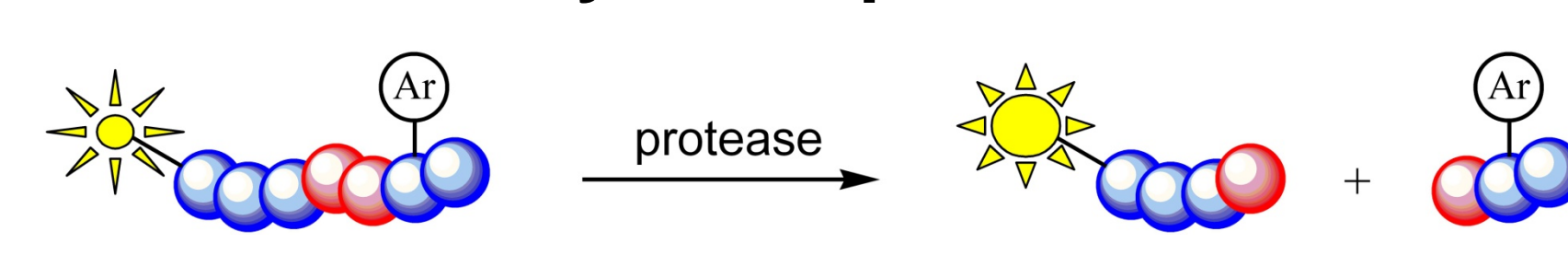
#### Z'-Factor



- A **FLEXYTE™** deiminase assay has been developed for PAD4, which is implicated in autoimmune diseases such as rheumatoid arthritis
- High assay sensitivity ensures low nM or pM enzyme requirements
- High Z'-factors confirm assay is suitable for HTS
- The protease protection approach to assaying deiminases has recently been extended to PAD2

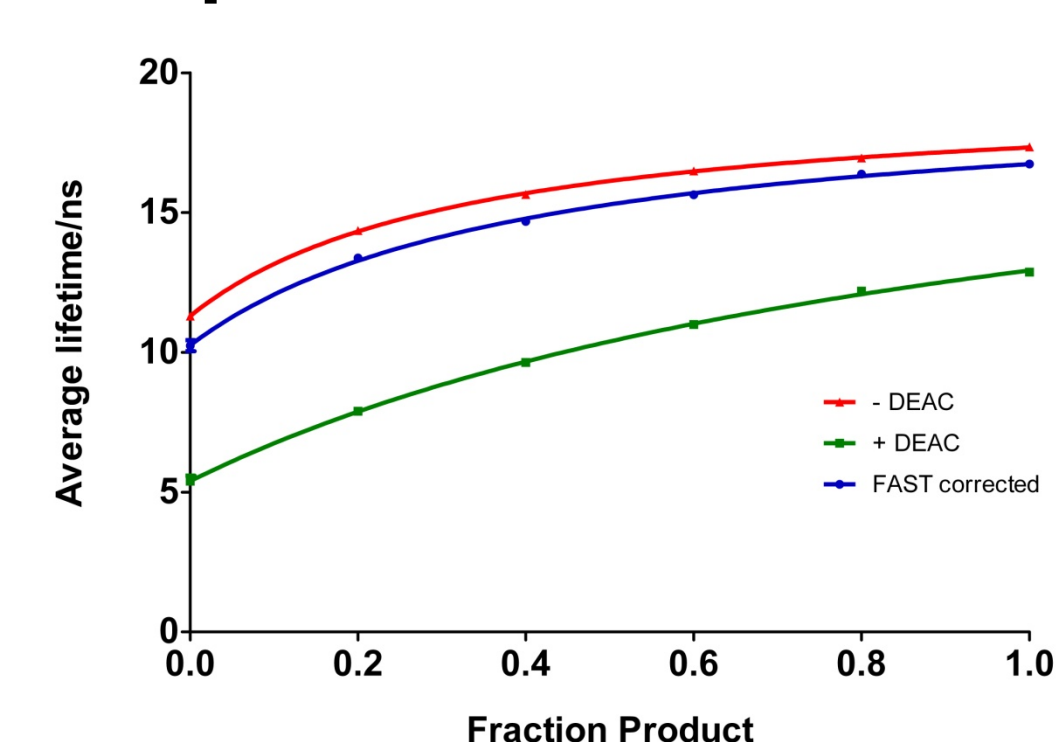
## Correcting for Interference Effects

### Protease Assay Principle



Protease cleaves substrate removing dynamic quenching of 9AA by an aromatic moiety (Ar) leading to an increase in FLT

### Caspase-3 Standard Curve



- The red line represents the correlation between average FLT and product formation for a Caspase-3 assay. Data is taken from triplicates of mixed samples of substrate and cleaved product peptides (1 µM)
- The green line is from an identical experiment but now including the interfering dye, 7-diethylaminocoumarin-3-carboxylic acid (DEAC) (10 µM). This highly fluorescent dye has a similar excitation and emission wavelength to 9AA but with a much shorter FLT (< 1 ns)

- On initial inspection, the effect of a 10-fold excess of DEAC is to reduce the observed average lifetime of the 9AA-labelled peptide, which could lead to false positives in screening situations
- However, unlike other fluorescence based assay techniques, the information rich FLT output permits analysis and correction of data using Fluorescence Analysis Software Technology (FAST) (Edinburgh Instruments Ltd.).<sup>3</sup> As the FLT of the substrate and product peptides are accurately known, the influence of an interfering short lifetime component can be easily identified and corrected for, returning the data shown in blue above
- Hence, FLT technology has the capacity to not only flag up fluorescent interfering compounds but to also correct for their effect, leading to a reduction in the number of false positives and negatives

## Summary – The **FLEXYTE™** Platform

- The **FLEXYTE™** platform has now been extended to offer assays for epigenetic targets, such as protein lysine methyltransferases, protein arginine methyltransferases, peptidylarginine deiminases and histone demethylases
- These assays, which utilise 9-aminoacridine labelled peptide substrates in a protease protection-based approach, are homogeneous and antibody free and display high sensitivity and robustness making them suitable for profiling and HTS applications
- One of the main benefits of FLT technology is the ability to mitigate compound interference. This advantage was demonstrated by mimicking a Caspase-3 protease assay using substrate and product peptides and correcting for fluorescence interference by using 'expert' analysis software (FAST)
- [1] B. A. Maltman, C. J. Dunsmore, S. C. M. Couturier, A. E. Tirnaveanu, Z. Delbederi, R. A. S. McMordie, G. Naredo, R. Ramage and G. Cotton, 9-Aminoacridine peptide derivatives as versatile reporter systems for use in fluorescence lifetime assays, *Chem. Commun.*, 2010, **46**, 6929-6931
- [2] M. J. Paterson, C. J. Dunsmore, R. Hurteaux, B. A. Maltman, G. J. Cotton and A. Gray, A fluorescence-lifetime based assay for serine and threonine kinases that is suitable for high-throughput screening, *Anal. Biochem.*, 2010, **402**, 54-64
- [3] D. M. Gakamsky, R. B. Dennis, and S. D. Smith, Use of fluorescence lifetime technology to provide efficient protection from false hits in screening applications, *Anal. Biochem.*, 2011, **409**, 89-97