# Miniaturizing Protease Assays using Fluorogenic Substrates and an Optimized Fluorescence Plate Reader

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## Introduction

Members of the matrix metalloproteinase (MMP) family are responsible for the degradation of components of the extracellular matrix and play an important role in embryonic development, morphogenesis, reproduction and tissue remodelling and repair. Inappropriate MMP activity may contribute to a number of pathologies including arthritis and cardivovascular disease[1].

Fluorogenic peptide substrates provide a particularly convenient enzyme assay method and several laboratories have reported on the design of peptides for monitoring MMP enzyme activity[2,3]. Peptide Mca-RPKPVE↑(Nva)WRK-Dnp-$NH_2$ is a well characterised MMP-3 substrate[4]. Previously we labelled this peptide sequence with the donor/quencher pair Cy™3/Cy5Q, to give peptide Ac-RPK(Cy3)PVE↑(Nva)WRK(Cy5Q)-$NH_2$, and reported the development of a reliable MMP-3 activity assay for use with LEADseeker™ homogeneous imaging system or FARCyte™ fluorescence plate reader[5]. Here we show the conversion of this MMP-3 assay from a 384 to a 1536-well format using FARCyte.

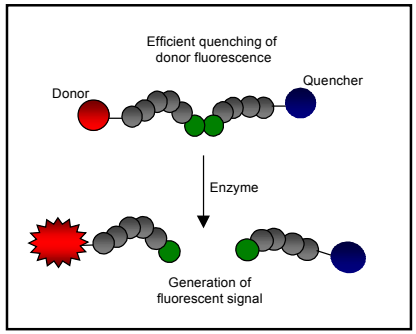



**Figure 1. Schematic of FRET Protease Assay**

## Methods

***Peptide Substrates:*** Peptide Ac-RPKPVE(Nva)WRK-$NH_2$ was synthesised and labelled with active CyDye™ esters using standard methods. Dual labelled peptide was purified by RP-HPLC, purity was assessed by mass spectrometry and material was lyophilised. Prior to use, peptide was reformulated in anhydrous DMSO and stock solutions were stored at -20°C.

***Standard Assay:*** Labelled peptide was incubated with or without recombinant human, catalytic domain MMP-3 (Calbiochem), in 50mM Tris pH7.5, containing 150mM NaCl, 10mM $CaCl_2$, 10µM $ZnCl_2$ and 0.05% (w/v) Brij™-35 at 22-25°C. Assays, 384-well format, contained 250nM peptide and 2.5ng/well enzyme in final reaction volumes of 60µl. For 1536-well assays, reaction volumes were reduced 10-fold to 6µl and peptide and enzyme were added at 125nM and 1.25ng/well respectively. Fluorescence signals were measured on FARCyte using Cy3 filters and dichroic mirrors or on LEADseeker (prototype instrument fitted with a -45°C camera) using 540/570nm excitation and emission wavelengths.

## Results

The effect of enzyme concentration on substrate hydrolysis was investigated. The results, presented in figure 2a, demonstrated a linear relationship between fluorescence signal and enzyme concentration over the range 0.02–1.25ng/well. The time course shown in figure 2b was established using 125nM peptide and 1.25ng/well MMP-3. Reactions were complete within 60 minutes at which time we measured >8-fold fluorescence signal increase. No significant signal increase was observed in no enzyme control (NEC) wells.

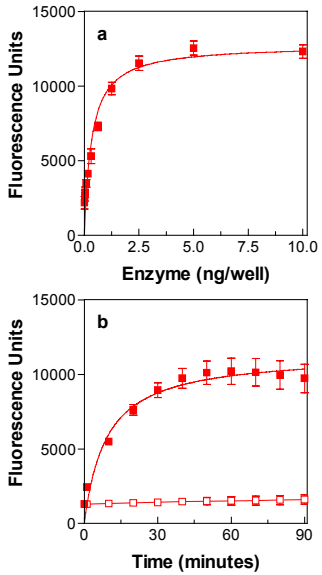
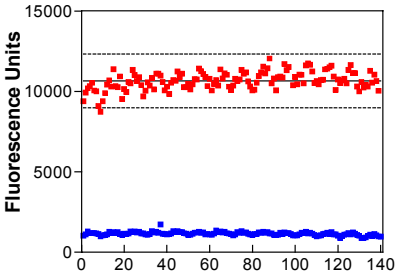



**Figure 2. Enzyme and time dependent hydrolysis of peptide substrate.** **(a)** Peptide (125nM) was incubated with MMP-3 enzyme (0.02 – 10ng/well) as described for 60 minutes. **(b)** Peptide (125nM) was incubated with 1.25ng/well MMP-3 as described. Data in this and subsequent figures is presented as mean ±SEM (n = 3).

The quality of the miniaturized MMP-3 assay was examined according to the Z'-factor analysis method recently proposed by Zhang and colleagues[6]. The Z'-factor coefficient can be any value ≤1, and assays with a Z-factor of >0.5 are considered to be reliable and reproducible. The results for the miniaturized MMP-3 assay are presented below. A Z'-factor value of 0.79 was determined.




| | | |
|---|---|---|
| **Enzyme** | **Mean Signal** | 10660 |
| | **SD** | 558.5 |
| **NEC** | **Mean Signal** | 1155 |
| | **SD** | 104.1 |
| | **S:B** | 9.2 |
| | **Z'** | **0.79** |

**Figure 3. Z' Factor analysis.** Peptide was incubated with (■) or without enzyme (■) as described for 60 minutes. The experiment has been repeated twice with consistent results. S:B, signal to background ratio.

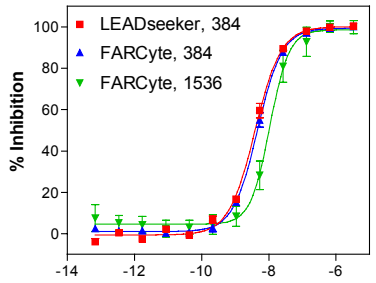



**Figure 4. Inhibitor studies.** Peptide was incubated with MMP-3 as described in the presence of 70fM to 3.3µM inhibitor reformulated in anhydrous DMSO. Fluorescence was measured on FARCyte or LEADseeker as described.

Finally, the effect of the potent, broad range MMP inhibitor N-Hydroxy-1,3-di-(4-methoxybenzenesulfonyl)-5,5-dimethyl-[1,3]-piperazine-2-carboxamide[7] on peptide hydrolysis was investigated. Figure 4 compares the results from 384 and 1536-well assays. The $IC_{50}$ values obtained using the 384-well format were 3nM and 5nM when plates were read on LEADseeker and FARCyte respectively; a value of 11nM was achieved using the 1536-well format FARCyte assay. These values are in good agreement with the reported $IC_{50}$ for this inhibitor[7].

Table 1 compares the 384 and 1536-well MMP-3 assays. Miniaturization represented a 10-fold reduction in assay volume, a 20-fold reduction in substrate usage and a 2-fold saving in enzyme costs. Overall, data generated using the different assay formats were comparable.

| | 384 Assay | 1536 Assay | Reduction |
|---|---|---|---|
| **Volume** | 60µl | 6µl | 10 |
| **Peptide** | 250nM | 125nM | 20 |
| **Enzyme** | 2.5ng/well | 1.25ng/well | 2 |
| **S:B** | 11.6:1 | 8.2:1 | - |
| **Z'** | 0.91 | 0.79 | - |
| **$IC_{50}$** | 5nM | 11nM | - |

**Table 1. Comparison of 384 and 1536 FARCyte assays.**
S:B for 384-well format was calculated in optimised assays following digestion of 250nM peptide with 2.5ng/well MMP-3 for 300 minutes.
Literature $IC_{50}$ for inhibitor is 18.4nM[7]

## CONCLUSIONS

- We have successfully miniaturized a 384-well protease assay to a 1536-well format using the FARCyte reader.
- In this case, miniaturization significantly reduced reagent consumption without compromising assay reliability. Miniaturized assay signal-to-background was >8:1 and Z'-factor values of >0.7 were achievable.
- We used MMP-3 as a model protease but the same approach could be applied to other proteases providing suitable peptide sequences are available for labelling with CyDye pairs.

## References

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