Use of CyDye fluors for improved FRET protease assays on a BMG LABTECH fluorescence microplate reader

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- **Improved assay platform with BMG LABTECH readers and CyDye™ labelled peptides**
- **Enhanced and significant fluorescence increase compared to traditional fluors**
- **Development of CyDye™ labelled substrates to control MMP activity**

Introduction

Members of the matrix metalloproteinase (MMP) family play an important role in tissue remodeling and repair. However, inappropriate MMP activity has been implicated in a number of disease states including arthritis, tumour invasion and metastasis, and cardio-vascular disease. The development of agents to control MMP activity continues to be a major focus for the pharmaceutical industry.

Many synthetic MMP peptide substrates have been described which incorporate a fluorophore and a quencher moiety positioned on either side of the enzyme cleavage site. We have prepared a number of CyDye™ labelled MMP substrates. Cleavage of these substrates with specific MMP enzymes produces improved and significant fluorescence increase at donor fluorophore wavelengths (Figure 1), when compared to traditionally used fluors.

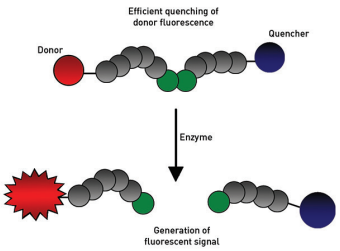

Fig. 1: FRET protease assay (schematic).

The POLARstar® Omega and PHERAstar® *FS* have 2 Photo Multiplier Tubes (PMT) and hence do not have to switch filters or polarizers to read the second channel, Simultaneous Dual Emission (SDE) makes assays like FRET faster and more precise.

Material & Experimental

Peptide Substrates:

Peptides were synthesized and labelled with active CyDye esters (Amersham Biosciences) using standard synthesis and labelling procedures. Following purification by RP-HPLC and confirmation of purity by mass spectrophotometry, dual labelled peptide was freeze-dried. Prior to use, labelled peptide substrates (Table 1) were dissolved in DMSO and stocks were stored at -20°C. Peptide Mca-PLGL-Dpa-AR-NH2, supplied as a freeze-dried powder (CN Biosciences), was dissolved in methanol as per manufacturer's instructions.

Table 1: Peptide sequences for cleavage assay

Peptide I	Cy3B-PLG↑ LAARK-Cy5Q
Peptide II	Cy3B-PLG↑ LFARK-Cy5Q
Peptide III	Mca-PLG↑ L-Dpa-AR

Mca, (7-Methoxycoumarin-4-yl)acetyl
Dpa, 3-(2,4-Dinitrophenyl)-L-2,3-diaminopropionic acid

Activation of MMP-2:

Human recombinant MMP-2 pro-enzyme (CN Bio-sciences) was activated by incubation in assay buffer containing 4-aminophenyl mercuric acetate for 2 hours at 37°C.

Standard Assay:

Labelled peptides (400 nM) were incubated at 37°C with or without activated MMP-2 enzyme in assay buffer (50 mM Tris pH 7.5, containing 150 mM NaCl, 10 mM $CaCl_2$, 10 µM $ZnCl_2$, 0.05% (w/v) Brij™-35 and 0.05% NaN_3). Assays were configured in black, opaque 384-well plates (Greiner) in final reaction volumes of 60 µL. Plates were read on BMG LABTECH fluorescence plate reader using 320/390 nm and 530/570 nm excitation and emission wavelengths for Mca and Cy™3B respectively.

Results & Discussion

We have prepared two MMP substrates that contain Cy3B and Cy5Q as a fluorophore/quencher pair. In a series of experiments, these were compared with each other and with the well characterized peptide Mca-PLGL-Dpa-AR. A time course for hydrolysis of the substrates by MMP-2 was established (Figure 2) and all three peptides were found to be effectively cleaved.

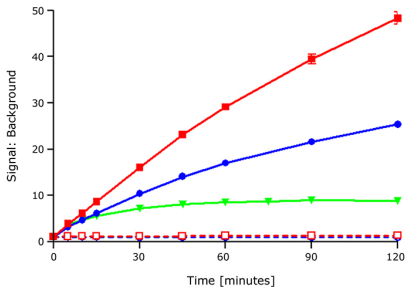

Fig. 2: Time course analysis of MMP-2 cleavage.
Peptides I (●), II (■) and III (▼) were incubated at 37°C with (solid symbols/lines) or without (open symbols/dashed lines) 4ng/well MMP-2 as described and fluorescence signals were measured over time using the BMG LABTECH reader. Values are plotted as means ± SD (n=3).

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At the assay endpoint we calculated signal/background (S/B) values for the three peptides and the recognized Z' statistical factor for peptides I and II (Table 2). Overall, results showed that peptide II was the more favourable substrate for MMP-2.

Table 2: Summary statistics for MMP-2 assay.

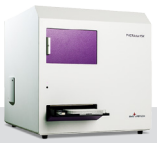
	BMG POLARstar	
	S/B	Z'
Peptide I	25:1	0.95
Peptide II	49:1	0.93
Peptide III	9:1	nd

Conclusion

We have employed CyDye fluors in a FRET protease cleavage assay for the enzyme MMP-2. Substrates combining the fluorescent donor (Cy3B) with a Cy5Q quencher in a de-quench assay format were compared with an equivalent substrate incorporating a methyl-coumarin fluor and dinitrophenyl based quencher.

All of the peptide substrates (Figure 2) were efficiently hydrolyzed by the MMP-2 enzyme. Signal increases, measured on the BMG LABTECH fluorescence microplate reader, were >25-fold following hydrolysis of the CyDye labelled substrates, compared with only a 9-fold signal increase following hydrolysis of the Mca/Dpa labelled substrate (when evaluated by time course analysis). For both of the CyDye labelled peptides, no significant signal increases were observed in control (no enzyme containing) wells. This combination of CyDye labelled peptides and detection on the BMG LABTECH reader provides an improved assay platform when compared with more traditionally used fluors (such as Mca).

The two CyDye peptides differ at the P2' position (Table 1). Other well characterized substrates contain either tryptophan or Dpa at this site. The data presented here suggest that phenylalanine is also a favourable residue in subsite P2'. This is in line with the observation that MMP enzymes favour aromatic side chains at P2'.



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