

New europium cryptates to probe molecular interactions using HTRF®



Application Note 7



Introduction

The trisbipyridine (TBP) europium cryptate currently used as energy donor in HTRF® has enabled the probing of a large number of molecular interactions, including immunoassays (1), enzymatic processes (2) or protein-protein interactions (3-4). However, there may be some limitations in the use of such europium cryptate for particular applications :

- its intrinsic fluorescence may limit the study of low kd interactions,
- the need for fluoride ions to protect the TBP europium cryptate against potential quenching agents could be troublesome in developing HTS assays in which parameters like a low ionic strength, the use of calcium ions or a cell viability are required for the detection of a biological event.

Two new europium cryptates, the trisbipyridine tetracarboxylate (TBP4COOH) europium cryptate and the pyridine bipyridine tetracarboxylate (PBP4COOH) europium cryptate (figure 1) have been designed to overcome the limitations of TBP europium cryptate.

Materials and methods

Europium cryptates' absorption spectra were recorded on a Perkin Elmer Lambda 16 spectrophotometer. Europium cryptates were diluted in a 100 mM phosphate buffer pH 7.0 at a concentration of 30 µM.

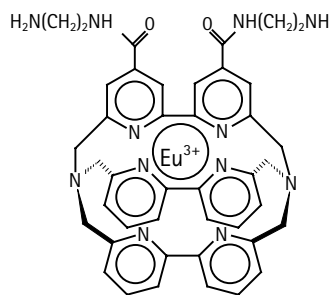
Fluorescence emission spectra and fluorescence lifetime determinations were

carried out on a Perkin Elmer LS50B spectrofluorimeter. Europium cryptates or europium cryptate conjugates were diluted in the different media at a cryptate concentration of 5µM. Fluorescence emission spectra operating conditions : excitation wavelength = 337nm (slit 10 nm), emission wavelength from 570 nm to 730 nm (slit 5 nm), delay = 10 µs, width = 10 ms. Operating conditions for lifetime determination: excitation wavelength = 305 or 320 nm (slit 10 nm), emission wavelength 620 nm (slit 5 nm), delay time from 50 µs to 600 µs, width = 1ms.

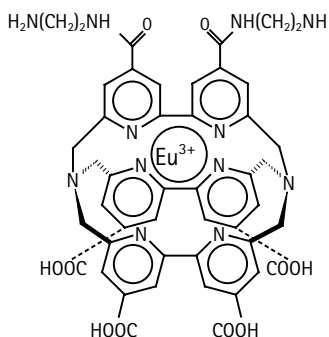
Determination of the fluorescence quenching by uric acid : streptavidin-europium cryptate conjugates (Sa-K) were diluted in a 100 mM phosphate buffer pH 7.0, 0.1% BSA, in presence or absence of 250 mM of KF (Sigma) at a final streptavidin concentration of 0.4 nM. Uric acid (Fluka) was diluted to the appropriate concentrations in a 10 mM phosphate buffer pH 7.0. Reagent solutions were distributed in a 96 well black microplate and read at 620 nm on Discovery® (Packard Biosciences).

HTRF® cAMP assay was carried out in a 96 well black microplate (Packard Biosciences). Assay buffer: 100 mM phosphate buffer pH 7.0, 0.2% BSA, 400 mM KF. Reagents concentration : [cAMP-XL665] = 0.15 nM, [cAMP] from 0 to 80 nM, europium cryptate labeled anti-cAMP antibody used at 20 ng/well or 5 ng/well. Assay was read on Discovery® after an incubation time of 1 h at 4°C.

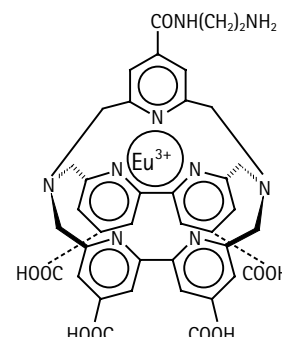
GST-protein-biotin detection assay was done in a 96 well black microplate (Packard Biosciences). Assay buffer: 100 mM phosphate buffer pH 7.0, 0.1% BSA, 400 mM KF. Reagents concentration : [streptavidin-XL665] = 20 nM, [GST-protein-biotin] from 0 to 20 nM, europium cryptate labeled anti-GST antibody used at 2 nM. Assay was read on Discovery® after an incubation time of 4 h at room temperature.



TBP europium cryptate



TBP4COOH europium cryptate



PBP4COOH europium cryptate

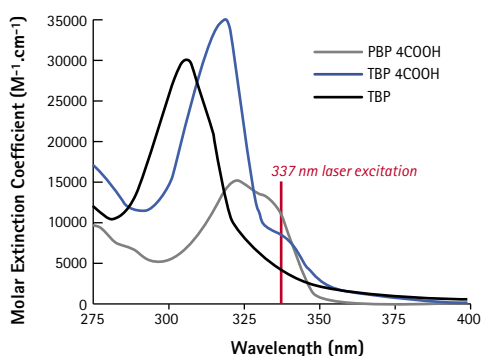


Figure 2: absorption spectra of different cryptates

The two new europium cryptates include several carboxylic groups in their cage structure which induce a shift in their maximum of absorption from 305 nm (max for TBP) to respectively 318 nm for the TBP4COOH europium cryptate and 322 nm for the PBP4COOH europium cryptate (figure 2).

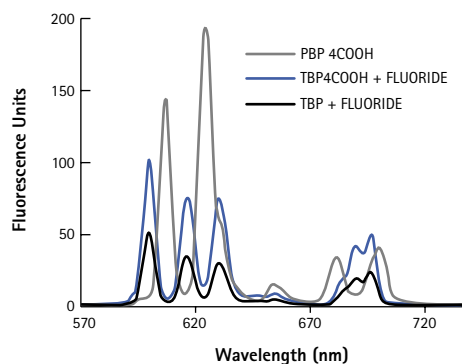


Figure 3: fluorescence emission spectra for the different cryptates (in 100 mM phosphate buffer, pH 7.0, in presence or absence of 400 mM KF). Total fluorescence areas for TBP, TBP4COOH and PBP4COOH are respectively 2910, 6328 and 8205

Upon a 337 nm nitrogen laser excitation, these new europium cryptates are therefore two to three times more fluorescent than the TBP europium cryptate currently used in HTRF® (figure 3). The yields of the different intramolecular energy transfer processes leading to europium fluorescence emission are similar for the three europium cryptates.

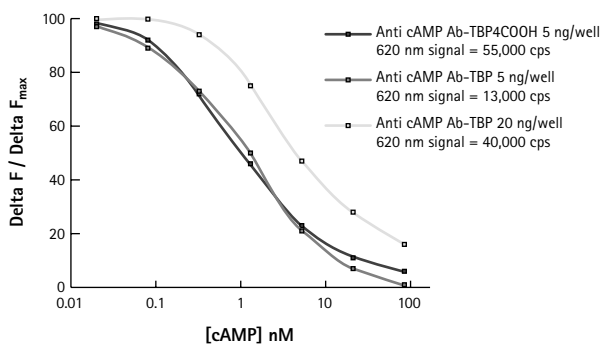


Figure 4: TBP versus TBP4COOH in the HTRF® cAMP assay

This enhancement of the fluorescence emission from the europium cryptates is of a great interest for the development of receptor ligand assays or competitive immunoassays like cyclic AMP. For instance, using the TBP4COOH europium cryptate, HTRF® cyclic AMP reached a high sensitivity with a level of signal at 620 nm around 40000 cps (figure 4). In HTS conditions, this signal level ensures a perfect correction of the HTRF® signal for medium interferences by using the signal ratio (665nm/620 nm).

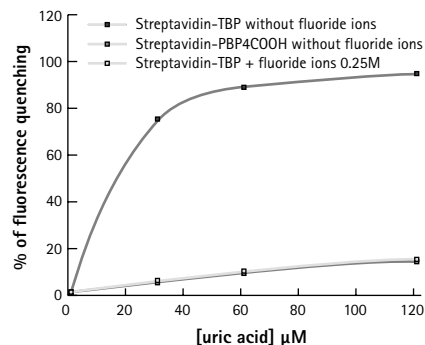


Figure 5: fluorescence quenching of europium cryptates by uric acid

The replacement of a bipyridine moiety by a pyridine moiety in the structure of the PBP4COOH europium cryptate (figure 1) enables the reduction of the size of the cryptate cage around the europium ion. With this new cage structure, both the access of potential quenching agents to the europium ion and the reduction process of the europium ion responsible for the cryptate quenching phenomenon are considerably minimized. Therefore, the PBP4COOH europium cryptate could be used without the fluoride ions which are necessary to protect both TBP or TBP4COOH europium cryptates. Figure 5 illustrates that the fluorescence at 620 nm of a streptavidin-PBP4COOH europium cryptate conjugate is not affected by large amounts of uric acid. This component of serum was shown to be a powerful quenching agent for the TBP europium cryptate in the absence of fluoride ions (figure 5).

Medium	TBP	TBP + fluoride ions (0.4 M)	PBP4COOH
10 mM phosphate buffer pH 7.0	0.55ms	1.2ms	1.04ms
50 mM Tris buffer pH 7.0	0.35ms	1.1ms	0.78ms
50 mM Hepes buffer pH 7.0	0.45ms	1.12ms	0.75ms
50% phosphate buffer 50% human serum	0.28ms	0.9ms	0.93ms

Table 1: fluorescence lifetime of the different streptavidin cryptate conjugates in various media

Table 1 also shows that the fluorescence lifetime of a streptavidin-PBP4COOH europium cryptate conjugate is greater than 0.75 ms in all the media tested.

Due to its stability properties, the PBP4COOH europium cryptate was used to probe molecular interactions without fluoride ions. Figure 6 shows, for instance, the detection of a GST-protein-biotin molecule using an anti-GST antibody labeled with europium cryptate and a streptavidin-XL665 conjugate. Performances obtained with the anti GST antibody-PBP4COOH conjugate in absence of fluoride ions are within the same range as those for the generic reagent currently used in HTRF®.

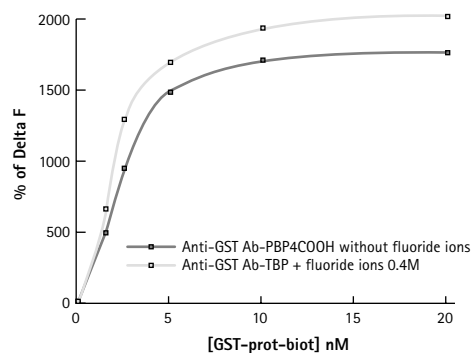


Figure 6: GST-biotinylated protein detection assay



Conclusion

The new europium cryptates, TBP4COOH and PBP4COOH, have shown interesting new photophysical properties for their use as energy donor in HTRF®.

Due to the presence of several carboxylic groups in their structure, both are significantly more fluorescent than the current europium cryptate. Moreover, the new PBP4COOH europium cryptate allows the study of molecular interaction without the need for fluoride ions.

This new generation of europium cryptates overcomes the limitations of the TBP europium cryptate and therefore, will definitely extend the application field for HTRF®.

References

Mathis G. Rare Earth Cryptates and Homogeneous Fluoroimmunoassays with Human Sera. *Clin Chem.* 1993;39:1953-9.

Kolb AJ, Kaplita PV, Hayes DJ, Park YW, Pernelle C, Major JS, Mathis G. Tyrosine kinase assays adapted to homogenous time-resolved fluorescence. *Discovery Drug Technol.* 1998;3:333-42.

Mellor GW, Burden MN, Préaudat M, Joseph Y, Cooksley SB, Ellis JH, Banks MN. Development of a CD28/CD86 (B7-2) binding assay for high throughput screening by homogenous time-resolved fluorescence. *J Biomol Screening.* 1998;3:91-9.

Zhou G, Cummings R, Li Y, Mitra S, Wilkinson HA, Eibrecht A, Hermes JD, Schaefer JM, Smith RG, Moller DE. Nuclear receptors have distinct affinities for coactivators : characterization by fluorescence resonance energy transfer. *Mol Endocrinol.* 1998; 12:1594-604..

Notes

Reagents and references

Trisbipyridine(TBP) and trisbipyridine tetracarboxylate (TBP4COOH) europium cryptates are only available for custom labeling. Please inquire for labeling modalities.

Monosubstrate derivatized TBP is now included in the cryptate labeling kit :

Designation	Part #
Eu-cryptate labeling kit (10 µg)	62EUSPEA



Copyright © 2004 Cisbio international, France. HTRF®, TRACE®, and the HTRF® logo are trademarks belonging to Cisbio international. HTRF® products are manufactured under one or more of the following patents and foreign equivalent : EP 0 180 492 / US 4,927,923 / US 5,220,012 / US 5,432,101 – EP 0 321 353 / US 5,457,185 / US 5,534,622 / US 5,346,996 / US 5,162,508 – EP 0 539 477 / US 5,512,493 – EP 0 539 435 / US 5,627,074 – EP 0 569 496 / US 5,527,684. Document reference : Application Note 7 2004 v1.0

R&D, Administration and Europe Office

In Vitro Technologies – HTRF/Bioassays
Phone : +33 (0)4 66 79 67 05
Fax : +33 (0)4 66 79 19 20
E-mail : bioassays@cisbiointernational.fr

Japan Office

Nihon Schering K.K. – Reagents Team, In Vitro Diag. & Equipment Dept.
Phone : +81-3-5715-8049
Fax : +81-3-5715-8083
E-mail : htrf-assays@schering.co.jp

USA Office

CIS-US, Inc.
Phone : 800-221-7554
Fax : 781-275-2634
E-mail : htrfsales@cisusinc.com

www.htrf-assays.com

