

HTRF[®] theory in brief

Principles of FRET.

FRET (Fluorescence Resonance Energy Transfer) is based on the transfer of energy between two fluorophores, a donor and an acceptor, when in close proximity. Molecular interactions between biomolecules can be assessed by coupling each partner with a fluorescent label and detecting the level of energy transfer.

Specific signal emission is caused by the proximity of two fluorophores

When two entities come close enough to each other, excitation of the donor by an energy source (e.g. flash lamp or fluorometer laser) triggers an energy transfer towards

the acceptor, which in turn emits specific fluorescence at a given wavelength. Because of these spectral properties, FRET – a donor-acceptor complex – can be detected without the need for physical separation from the unbound partners. Fully homogeneous assays do not require separation steps such as centrifuging, washing, filtration, or magnetic partitioning.

The donor and acceptor can be grafted covalently onto multiple partners that can associate, among others, two dimerizing proteins, two DNA strands, an antigen and an antibody, or a ligand and its receptor. Traditional FRET chemistries are hampered by background fluorescence from sample components such as buffers, proteins, chemical compounds and cell lysate. This type of background fluorescence is extremely transient (with a lifetime in the nanosecond range) and can therefore be eliminated using time-resolved methodologies.

HTRF[®]: the reference for time-resolved FRET

HTRF[®] combines standard FRET technology with time-resolved measurement of

Time-resolved measurement allows the signal to be cleared of background fluorescence

fluorescence, allowing elimination of short-lived background fluorescence. As shown in Figure 2, introducing a time delay – approximately 50 to 150

µseconds – between the system excitation and fluorescence measurement allows the signal to be cleared of all non-specific short-lived emissions. In contrast, HTRF[®] fluorophores emit long-lived fluorescence when engaged in a FRET process. Therefore, long-lived emissions signify energy transfer through proximity of the labeled biomolecules.

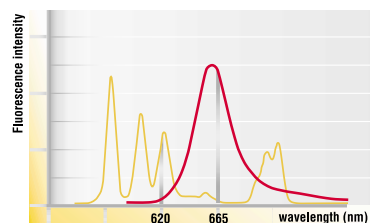
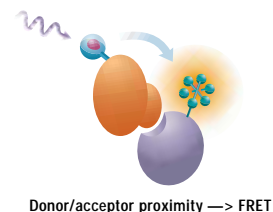
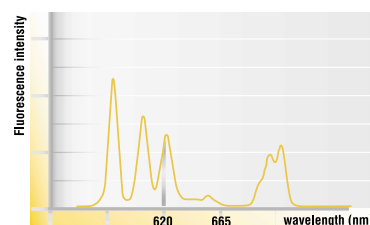
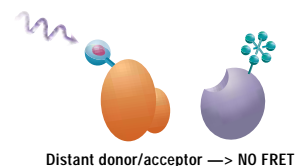


Figure 1: The detection of specific fluorescence is a sign that a FRET process has taken place, caused by the proximity of the two interacting partners.

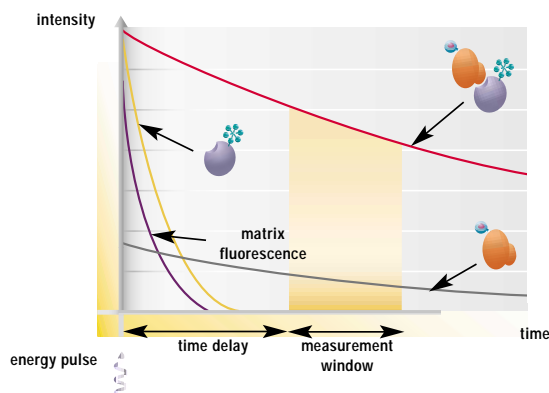
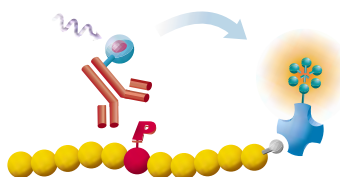


Figure 2: The energy pulse from the excitation source (flash lamp, laser) is immediately followed by a time delay, allowing interfering short-lived fluorescence (compounds, proteins, medium...) to decay.

HTRF® theory in brief



Of course, FRET is governed by the physics of molecular proximity, which only allows this phenomenon to occur when the distance between the donor and the acceptor is short enough. In practice, FRET systems are characterized by the Förster's radius (R_0) distance at which FRET efficiency is 50%. For HTRF®, R_0 lies between 70 and 90 Å, depending on the acceptor used. Nevertheless, this distance is theoretical, as it does not take into account spatial molecular rearrangements. In fact, a variety of HTRF® assays involving molecular complexes of different sizes have been implemented. This includes assessment of small phosphorylated peptides, immunoassays for quantifying large glycoproteins such as thyroglobulin, and indirect detection (via secondary antibodies) of tagged complexes such as CD28/CD86 (Figure 3).

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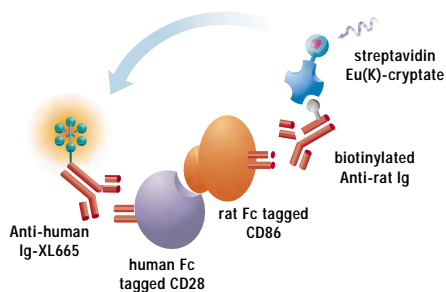


Figure 3: Two HTRF® assays theoretically involving very different donor-acceptor distances. Detecting a phosphorylated biotinylated-peptide (short distance); CD28/CD86 association quantified by anti-tag conjugates (long distance).

HTRF® fluorescent partners

HTRF® involves several carefully selected fluorophores. Obviously, FRET partners must fulfill multiple compatibility criteria. First, their emission spectra must show non-overlapping regions in order to be able to measure each partner's fluorescence individually. Second, the FRET quantum yield – i.e. its efficacy – must be as high as possible. Third, fluorescence emission must occur within a region of the spectrum remote from that naturally produced by proteins; in other words, a red-shifted emission is better for avoiding medium-intrinsic fluorescence.

HTRF® uses three specific fluorophores forming two TR-FRET systems. The central element, the energy donor, is europium cryptate (Eu^{3+} cryptate). The fruit of Prof. J.M. Lehn's work, for which he was awarded a Nobel Prize for Chemistry in 1987, these rare earth complexes consist of a macrocycle within which a Eu^{3+} ion is tightly embedded. This cage allows both energy collection and transfer to the Eu^{3+} ion, which ultimately releases this energy with a specific fluorescent pattern (Figure 4). Moreover, this type of structure confers long-lived fluorescence, one of Eu^{3+} cryptate's fundamental properties. The first acceptor developed for HTRF® is XL665, a phycobilliprotein pigment purified from red algae. XL665 is a large heterohexameric edifice of 105 kDa, cross-linked after isolation for better stability and preservation of its photophysical properties in HTRF® assays.

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This acceptor fulfils the compatibility criteria mentioned above. Its excitation spectrum overlaps that of Eu^{3+} cryptate emission, therefore allowing the donor to excite the XL665, and its maximum emission at 665 nm spans a region where Eu^{3+} cryptate does not emit or only does so weakly. In the end, energy transfer with the donor occurs with a high quantum yield.

The second generation of acceptors is characterized by organic structures 100 times smaller, displaying a series of photophysical properties very close to those of XL665. The

The new d2 acceptor, an organic motif of approximately 1,000 Da, is highly compatible with Eu^{3+} cryptate

comparison of d2 to XL665 was achieved by screening 14,700 compounds on an assay for quantifying a phosphorylated peptide.

As shown in Figure 5, the correlation between the two systems was extremely close, and validated the integration of d2 in a number of different HTRF® assays, notably cAMP and IP-One. As a much smaller entity, d2 limits the steric hindrance problems sometimes suspected in XL665-based systems. Evaluation also showed that the new acceptor contributed to significantly greater stability of immuno-competitive assays, and in some cases to better assay sensitivity.

Incomparable miniaturization capabilities

Miniaturization capabilities are an essential criteria for selecting an assay format. They contribute greatly to a screen's success, in particular because of cost savings on specific

Learn more about miniaturization with HTRF® on page 80

reagents, compounds, cells, and consumables. However, technologies do not all permit the same level of miniaturization. Assays based on

absorbance or radioactivity are quickly limited by their sensitivity when miniaturized. In contrast, fluorescence signals are strictly proportional to fluorophore concentration (i.e. at an equal concentration, the signal remains the same, whatever the volume).

A number of HTRF® assays have been shown to perform equally efficiently from 96 to 1536 well formats down to volumes lower than 5 μL simply by decreasing the various assay components proportionally. In all cases, miniaturized HTRF® assays keep excellent dynamics and sensitivity, essential to their implementation in high throughput screening.

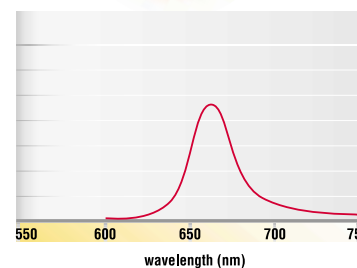
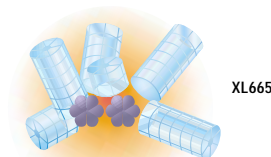
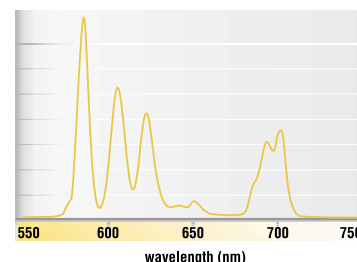
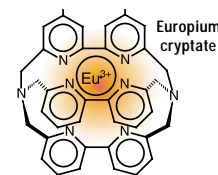


Figure 4: Specific XL665 emission occurs in a region where the donor does not emit significantly. Long-lived fluorescence at 665 nm is therefore characteristic of the emission of the acceptor engaged in a FRET process.

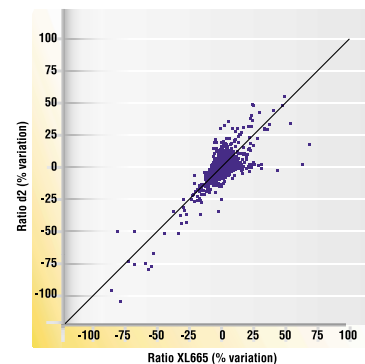


Figure 5: XL665 and d2 were compared in an HTRF® assay involving an anti-phosphotyrosine antibody conjugated to Eu^{3+} cryptate (PT66), a phosphorylated biotinylated substrate, and streptavidin alternatively labeled with XL665 and d2. Each system was tested with a library of 14,700 compounds (Schering AG). Comparison shows that the two acceptors behave very similarly and that d2 represents a promising alternative to XL665.

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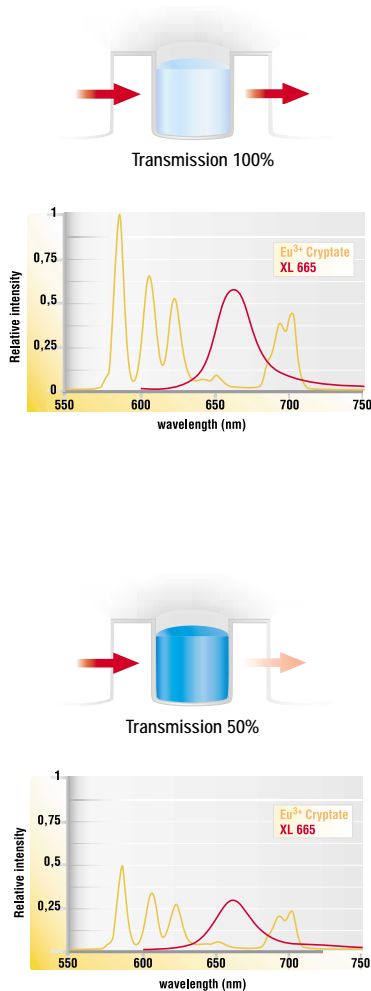


Figure 6: inner filter effect

Proven assay stability

The very nature of the fluorophores used makes HTRF® an extremely robust technology. Unlike luminescent methods, for example, detection (i.e. donor excitation) is not destructive and can be repeated as many times as required without impairing quantification. In fact, Eu³⁺cryptate is not subject to the photobleaching that affects a number of more conventional fluorophores.

Barely sensitive to quenching, the Eu³⁺ ion is almost inseparable from its macrocycle: the presence of challenging divalent cations or chelators, solvents, or high protein concentrations (e.g. serum) do not affect the fluorescent properties of Eu³⁺cryptate.

The addition of potassium fluoride (KF) further strengthens this stability. Added at any convenient time during the assay run or just before readout, fluoride ions act as a powerful, immediate, and irreversible fluorescence booster, preventing virtually any interference on Eu³⁺cryptate. As an example, the combination of several stabilizing factors (fluoride ions, d2) showed that the results obtained with the HTRF® cAMP assay were identical even one week after testing was started, thus offering unique flexibility and an incomparable guarantee during large screening runs.

HTRF® ratio optimization: an indisputable asset for measuring a homogeneous technology

In contrast to heterogeneous assays where measurement takes place in controlled media – i.e. readout is preceded by a washing step and the dispensing of an appropriate buffer – all the components of a homogeneous assay remain in the well at the time of readout and may all interfere to various extents. During screening phases for instance, chemical compounds from libraries differ in every well and it is therefore extremely important to avoid potential interference. Cisbio has developed a correction method for its HTRF® technology that can effectively deal with medium variability.

Eu³⁺cryptate is barely sensitive to quenching and has shown remarkable stability in assays

Figure 6 represents the same assay run under two different medium conditions. In the first case, the assay medium is perfectly clear, light transmission (excitation) is not impaired, and FRET is detected at the expected level. On the other hand, the assay in the second configuration is run in presence of a strongly colored medium that induces a decrease in the transmission by a factor of 2.

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Figure 6 also shows that the emission spectrum of the Eu^{3+} cryptate is affected in the same proportions as the acceptor's. Calculating the ratio of the fluorescence of the acceptor over that of the donor – i.e. 665 nm signal / 620 nm signal – is actually equivalent in both cases, and therefore exactly reflects the interaction studied.

The HTRF® ratio represents the only pertinent way to deal with medium variability

In Figure 7, the assay set up by Mellor et al. was run in presence of

an increasing concentration of comassie blue. Both measuring channels are affected in the same way and calculating the ratio demonstrates that concentrations up to 50 $\mu\text{g/ml}$ (60 μM) could still be compensated for. If only the signal intensity at 665 nm was considered, the sample would be falsely identified as an inhibitor of the biological reaction being tested. The calculation of the ratio for each well is therefore critical in decreasing false positives. This ratio (Cisbio patent US 5,527,684) is an essential tool for preventing interference due to medium variability or chemical compounds.

HTRF® signal measurement: a broad choice of microplate readers

Measuring HTRF® signals requires specific instruments capable of i) detecting fluorescence in time-resolved mode, and ii) quantifying this fluorescence selectively and efficiently at both 620 and 665 nm.

For many years, Cisbio has worked diligently to establish close partnerships with industrial experts in the field of detection, such as BMG Labtech, Tecan, and Molecular Devices. These joint efforts have made it possible to release a growing number of HTRF®

Read more about HTRF® compatible readers page 84

compatible readers, each one featuring all the technical specifications for use in HTS

(throughput, support of miniaturized formats, robotic integration). Equipped with lasers or high-energy flash lamps, optical devices and software that is either specific or compatible with HTRF®, all these readers have undergone rigorous certification testing and very selective control (HTRF® reader control kit) and bear the "HTRF® approved" label.

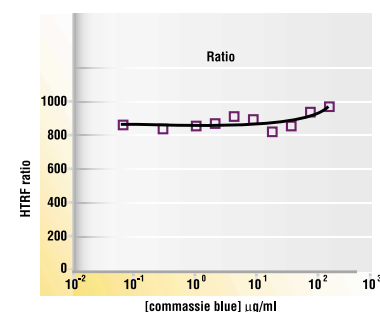
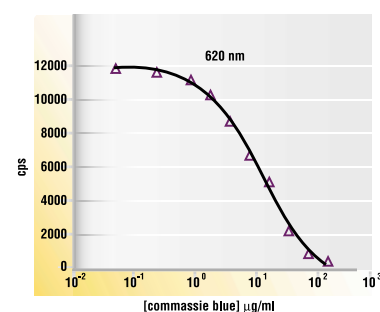
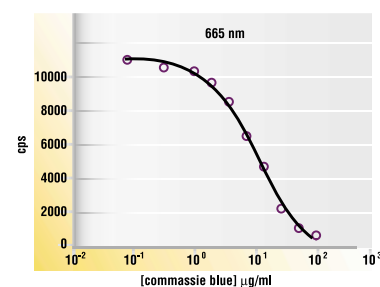


Figure 7: Effect of comassie blue in increasing concentrations on fluorescence emissions and ratio.

HTRF® theory in brief - selected bibliography

Bazin H, Trinquet E, Mathis G. Time-resolved amplification of cryptate emission: a versatile technology to trace biomolecular interactions. *J Biotechnol.* 2002;82:233-50.

Bazin H, Préaudat M., Trinquet E, Mathis G. Homogeneous time resolved fluorescence resonance energy transfer using rare earth cryptates as a tool for probing molecular interactions in biology. *Spectrochim Acta A Mol Biomol Spectrosc.* 2001;57:2197-211.

Mathis G. Bioassays: Luminescent materials in *Encyclopedia of materials. Science and technology.* Elsevier Science. 2001 p538-542

Trinquet E, Maurin F, Préaudat M, Mathis G. Allophycocyanin 1 as a near-infrared fluorescent tracer: isolation, characterization, chemical modification, and use in homogeneous fluorescence resonance energy transfer. *Anal Biochem.* 2001;296:232-44.

Galaup C, Picard C, Cathala B, Cazaux L, Tisnès P, Autié H, Aspe D. Mono(di)nuclear Europium(III) complexes of macrobi(tri)cyclic cryptands derived from diazatetralactams as luminophores in aqueous solution. *Helv Chim Acta.* 1999;82:543-60.

Mathis G. Homogeneous time resolved fluorescence, Point-counterpoint. *J. Biomol Screening.* 1999;4:309-13.

Kolb A, Burke J, Mathis G. A Homogeneous, Time-Resolved Fluorescence Method for Drug Discovery. In: Devlin JP, Ed. *High Throughput Screening, the Discovery of Bioactive Substances.* New York: Marcel Dekker, Inc. 1997. 345-60.

Mathis G. Homogeneous immunoassay and other applications of a novel fluorescence energy transfer technology using rare earth cryptates. *J Clin Ligand Assay* 1997;20:141-7.



Sittampalam GS, Kahi SD, Janzen WP. High-throughput screening: advances in assay technologies. *Curr Opin Chem Biol.* 1997;1:384-91.

Kolb J, Yamanaka G, Manly S. Use of a novel homogenous fluorescent technology in high throughput screening. *J Biomol Screening.* 1996;1:203-10.

Delain E, Barbin-Arbogast A, Bourgeois CA, Mathis G, Mory C, Favard C, Vigny P, Niveleau A. The limits in electron microscopy of macromolecular interactions. The use of new labels based on lanthanide cryptates an interdisciplinary approach. *J Trace Microprobe Tech.* 1995;13:371-81.

Mathis G. Probing molecular interactions with homogeneous techniques based on rare earth cryptates and fluorescence energy transfer. *Clin Chem.* 1995;41:1391-7.

Mathis G. Rare earth cryptates and homogeneous fluoroimmunoassays with human sera. *Clin Chem.* 1993;39:1953-9.

Sabbatini N, Guardigli M, Lehn JM, Mathis G. Luminescence of lanthanide cryptates: effects of phosphate and iodine anions. *J Alloys Comp.* 1992;180:363-7.

Alpha B, Lehn JM, Mathis G. Energy Transfer Luminescence of Europium (III) and Terbium (III) Cryptates of Macrobicyclic Polypyridine Ligands. *Angew Chem Int Ed Engl.* 1987;26:266-7.