revvity

HTRF

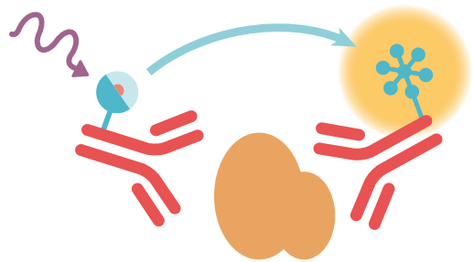
A guide to Homogeneous Time Resolved Fluorescence

Homogeneous Time-Resolved Fluorescence

HTRF®, is the premier TR-FRET (Time-Resolved Fluorescence Resonance Energy Transfer) technology on the market. TR-FRET technologies such as HTRF bring together the sensitivity of fluorescence with the homogeneous nature of FRET (Fluorescence Resonance Energy Transfer) and the low background of time resolution. HTRF uses two fluorophores, a donor and an acceptor dye, that transfer energy when in close proximity to each other. This creates a homogeneous assay format in which bound and unbound partners do not need to be separated as fluorescence emission from the acceptor is generated only upon binding.

HTRF can be used in competitive and non-competitive formats and performed as cellular or biochemical assays in 96-, 384- and 1536-well plate formats. It has been applied to a variety of applications including GPCRs, kinases, epigenetics, biotherapeutics and quantification of a range of biomarkers including cytokines.

This technical brochure reviews the general principles of HTRF and the associated Tag-lite® technology as well as discuss ways these technologies have been applied in drug discovery, biotherapeutics, and fundamental research in therapeutic areas such as Oncology, CNS, and Metabolic and Immune Disorders.



LANTHANIDE DONOR FLUOROPHORES FOR DECREASED ASSAY BACKGROUND

CRYPTATE CHEMISTRY FOR STABLE SIGNAL

HOMOGENEOUS, NO-WASH FORMAT

RATIOMETRIC MEASUREMENT FOR INTERFERENCE CORRECTION

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Homogeneous Time-Resolved Fluorescence

HTRF features

- Homogenous, add-and-read format
- Low background
- Interference correction
- Amenable to miniaturization
- Robust

Benefits

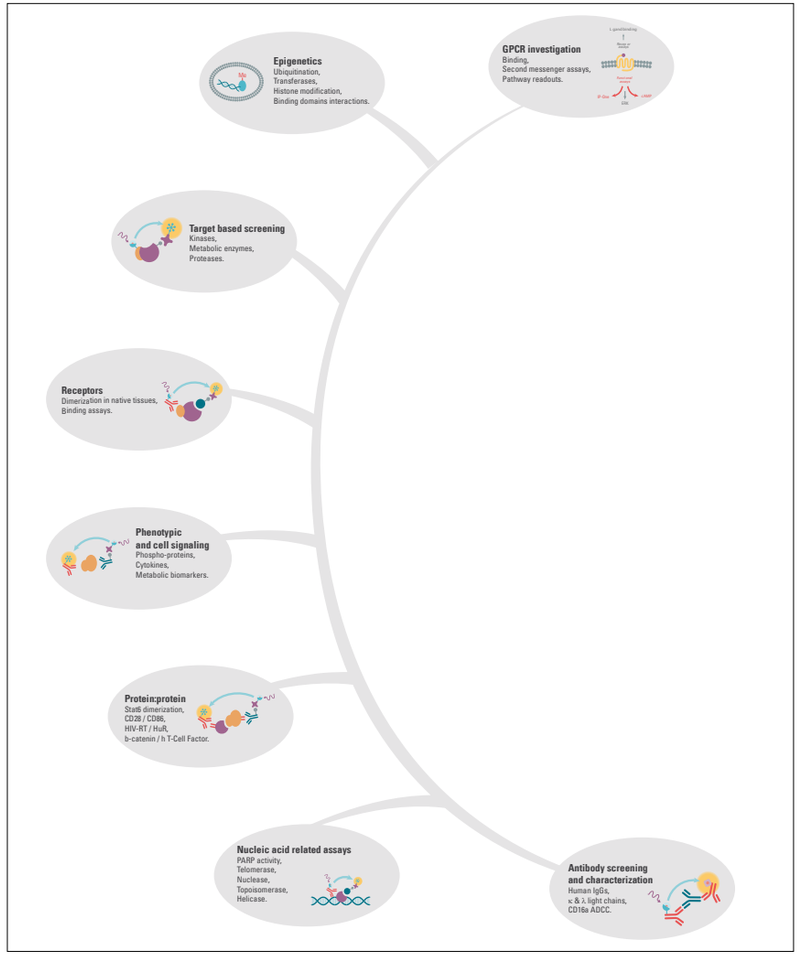
- High level of sensitivity
- Rapid, accurate results
- Application flexibility
- Easy to perform
- Time and labor savings

Targets, assays and applications

- epigenetics
- Cellular phospho-assays
- GPCRs
- Kinases
- Biomarkers
- Biotherapeutics discovery

Field of applications

- Basic research
- Target discovery and validation
- HTS and primary screening
- Secondary screening
- Lead optimization



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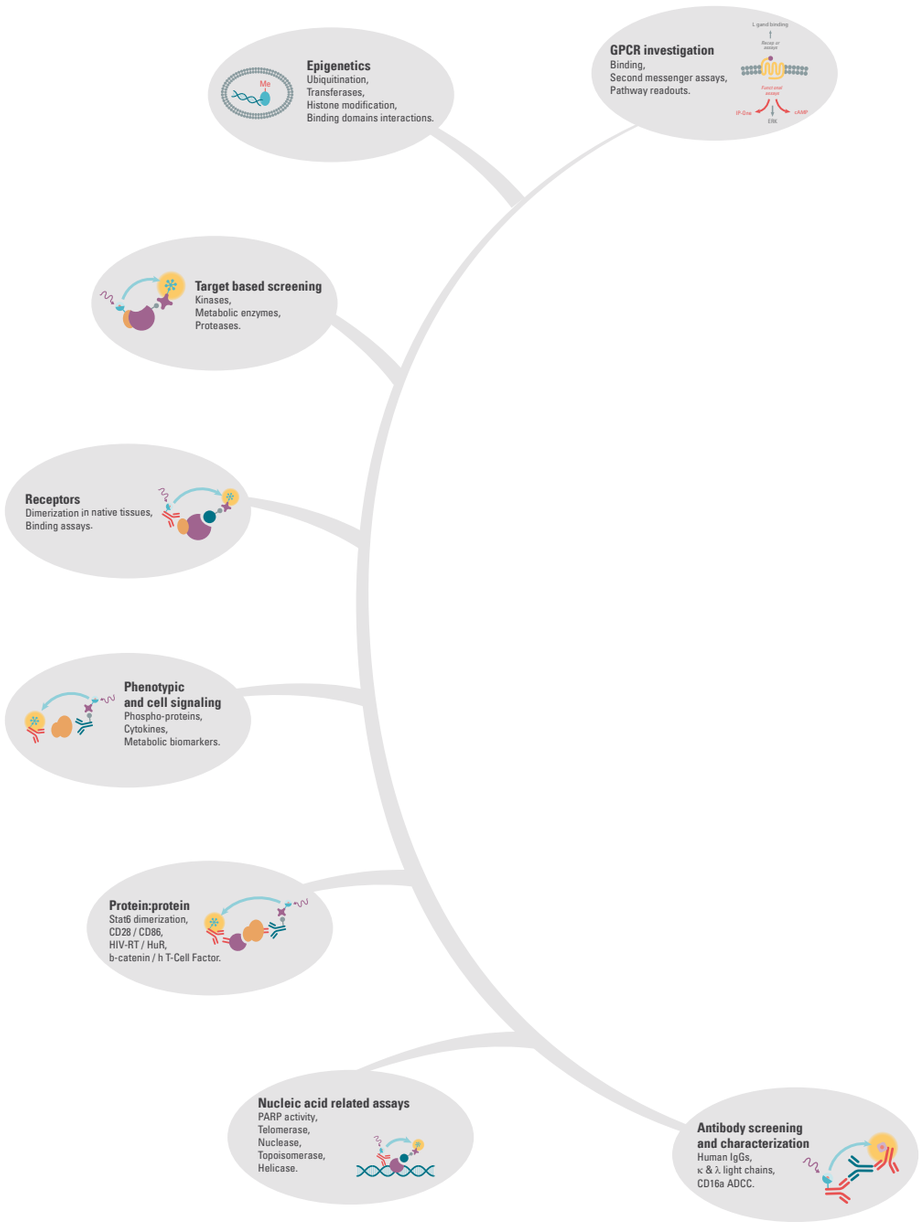

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HTRF principles

HTRF is based on several carefully selected and highly optimized donor/acceptor fluorophore pairs. Pioneered and developed in G. Mathis' group at Cisbio Bioassays, HTRF employs the properties of rare earth lanthanides to impart time resolution as well as the properties of energy transfer to create homogeneous, no-wash assays.

Key features

- Lanthanide donor fluorophores for decreased assay background
- Homogeneous, no-wash format
- Cryptate chemistry for stable signal
- Ratiometric measurement for interference correction
- Unique cellular receptor binding format (see Tag-lite section)

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Fluorescence Resonance Energy Transfer

FRET (Fluorescence Resonance Energy Transfer) is based on the transfer of energy between two fluorophores, a donor and an acceptor, which occurs only when the pair of fluorophores is in close proximity. Excitation of the donor by an energy source (e.g. a flash lamp or a laser) triggers the emission of light at donor-specific wavelengths. If within proximity, energy is transferred to the acceptor, which in turn emits light at its defined wavelengths. If the donor and acceptor fluorophores are not within proximity of each other, the donor is excited, but no energy is transferred and thus no acceptor emission is produced (Figure 2). It is this principle which allows for the homogeneous nature of HTRF assays.

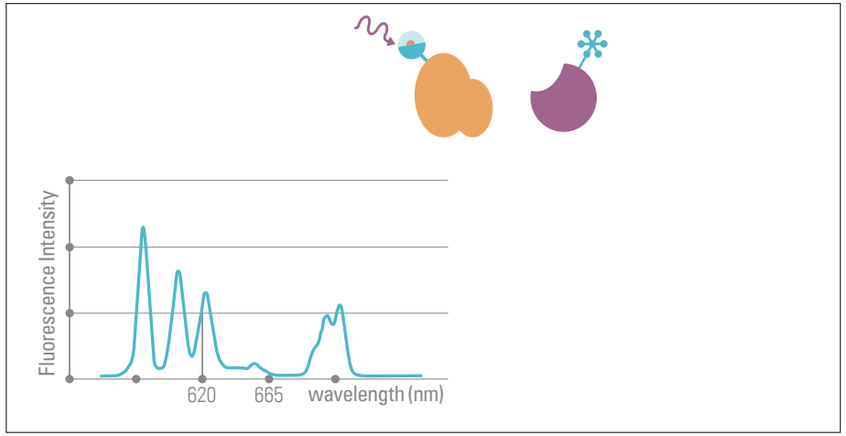

Figure 2 The detection of specific fluorescence at the acceptor's maximum emission wavelength (i.e. 665 nm, in red) is a sign that FRET has occurred, due to the proximity of the two interacting partners.

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Fluorescence Resonance Energy Transfer

In theory, FRET systems are characterized by the Förster's radius (R_0) distance at which energy transfer efficiency is 50%. For HTRF, R_0 lies between 50 and 90Å, depending on the acceptor used. R_0 does not take into account the dynamic spatial arrangements of molecular binding partners. Thus in practice, HTRF assays have been successfully implemented for a broad range of molecular complex sizes, including detection of small phosphorylated peptides, immunoassays for quantifying large glycoproteins such as thyroglobulin, and detection of protein:protein interactions via indirect sandwich assays such as the detection of Fc-tagged CD28 and CD86 binding via secondary antibodies (Figure 3).

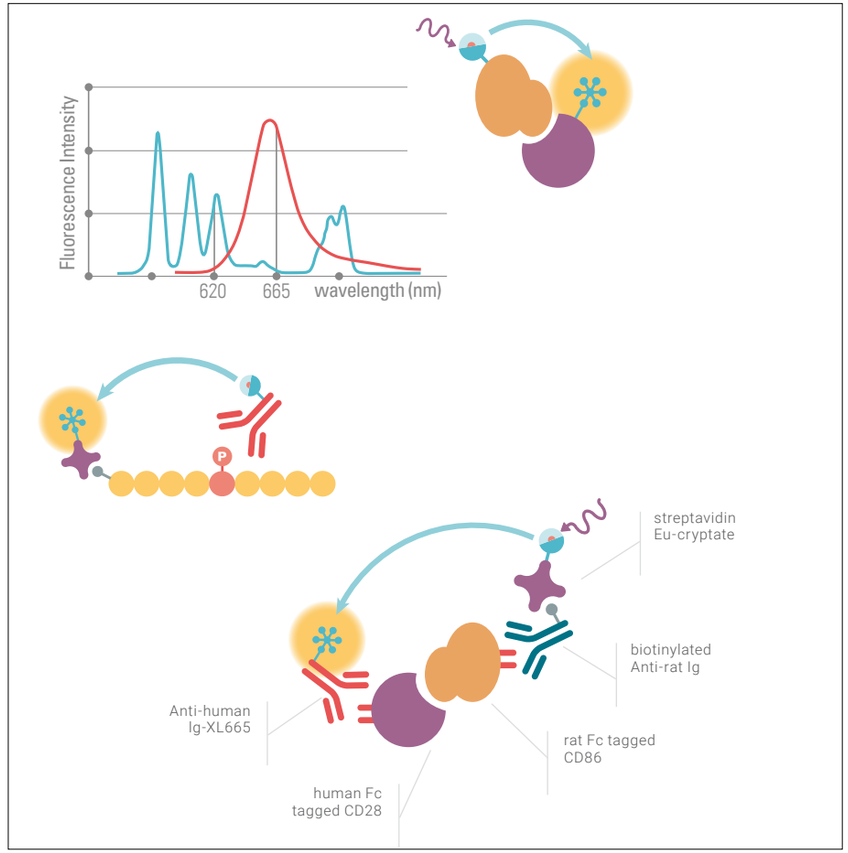

Figure 3 Two HTRF assays involving very different donor-acceptor distances. Detecting a phosphorylated biotinylated-peptide (short distance); CD28/CD86 association quantified by anti-tag conjugates (long distance)

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Fluorescence Resonance Energy Transfer

Unlike many other traditional assay methods, such as ELISAs, HTRF assays do not require the separation of bound and unbound partners typically performed using centrifugation, washing, filtration, or magnetic partitioning. HTRF assay components are often added in a single step producing a true add-and-read format that eliminates separation steps, significantly reduces assay time and is highly amenable to automation (Figure 4).

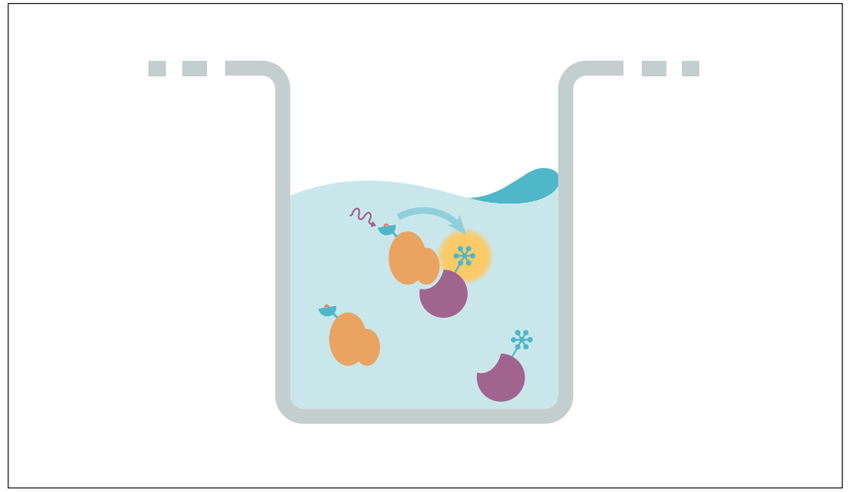

Figure 4 In a homogeneous format such as HTRF, complexed and uncomplexed moieties co-exist. Measurement of fluorescence emissions from the donor dye specifically allows for the detection of bound complexes.

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Principles of TR-FRET

Traditional fluorescence intensity and FRET chemistries employ prompt fluorophores, such as fluorescein and rhodamine, whose emissions are immediate and transient in nature. One limitation of these assays is decreased sensitivity due to background fluorescence from sample components such as buffers, proteins, chemical compounds and cell lysates which is also extremely transient in nature with a lifetime in the nanosecond range. Time-resolved methodologies such as HTRF rely on donor fluorophores with extended lifetime emission characteristics such as europium cryptate. Non-specific short-lived background fluorescence is significantly reduced by introducing a time delay (50-150 microseconds) between the initial donor excitation and fluorescence measurement.

HTRF-specific donor fluorophores emit long-lived fluorescence upon excitation while acceptors, if excited, produce prompt fluorescence. If donor and acceptor fluorophores are not within proximity, only donor emissions are detected following a time delay. Upon binding of the biomolecular assay partners (such as an antibody to a phosphorylated kinase substrate), the donor and acceptor fluorophores are brought within proximity allowing energy transfer and continued excitation of the acceptor fluorophore. This energy transfer results in prolonged acceptor fluorescence which can be detected even following a time delay (Figure 5).

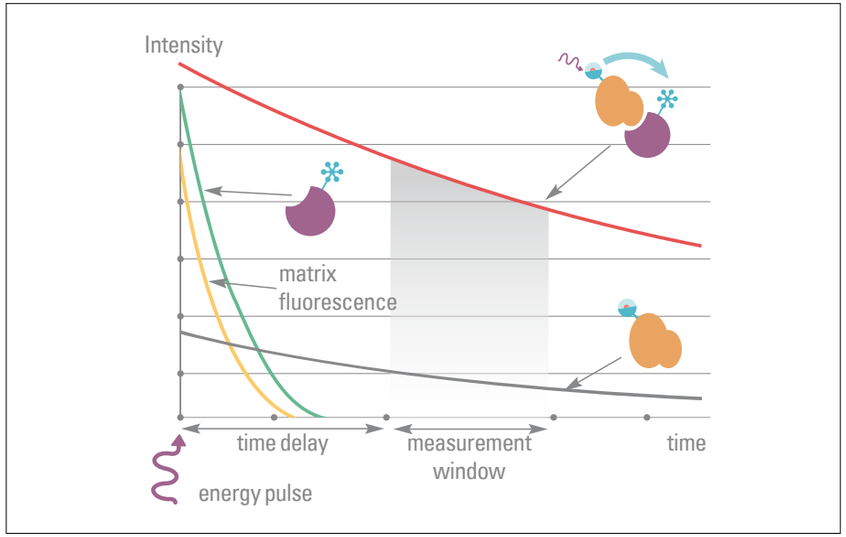

Figure 5 The energy pulse from the excitation source (flash lamp, laser) is immediately followed by a time delay, allowing interfering short-lived fluorescence (compounds, proteins, medium...) to decay.

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HTRF PRINCIPLES

HTRF donor and acceptor fluorophores

Optimum FRET (donor-acceptor) partners must fulfill multiple compatibility criteria. First, their emission spectra must have non-overlapping regions so that each partner's fluorescence can be measured individually. Second, the FRET quantum yield – i.e. efficiency of energy transfer – must be high enough. In general, the higher the donor emission yield and the larger the spectral overlap between the donor and acceptor (efficiency of donor emissions exciting the acceptor), the larger the assay window. Third, it is beneficial for fluorescence emissions to occur within a region of the spectrum remote from intrinsic fluorescence naturally produced by proteins (i.e. red-shifted emissions are favorable). HTRF uses various fluorophores, donors and acceptors, to form several TR-FRET pairs with the required FRET properties.

Two HTRF donor fluorophores:

- Europium cryptate (Eu^{3+}cryptate), the fruit of Prof. J.M. Lehn's work, at the University of Strasbourg, for which he was awarded a Nobel Prize for Chemistry in 1987 (Figure 6)
- Lumi4®-Terbium cryptate (Tb^{3+}cryptate), developed by Prof. K. Raymond's group at the University of California at Berkeley

HTRF's central element, the energy donor, called a cryptate, consists of a rare earth complex in which a lanthanide ion, such as europium or terbium, is tightly embedded in a macrocyclic motif or cage, (Figure 6). Both europium and terbium cryptates produce long-lived emissions in the range of 1 to 2 msec, a characteristic that is fundamental for time-resolved detection. Europium and terbium ions do not absorb light efficiently; they require a "light-collection" device (i.e. the cage) for excitation. Both HTRF donors use a proprietary macrocyclic cryptate structure to collect and transfer energy to the lanthanide ions. Chelate structures used in other TR-FRET system are unstable in acidic media or in the presence of divalent ions like Mn^{2+}. In contrast, the unique HTRF cryptate structure produces stable emissions under a wide range of chemical conditions and is not affected by divalent ions. These properties enhance assay window, overall sensitivity and technology robustness.

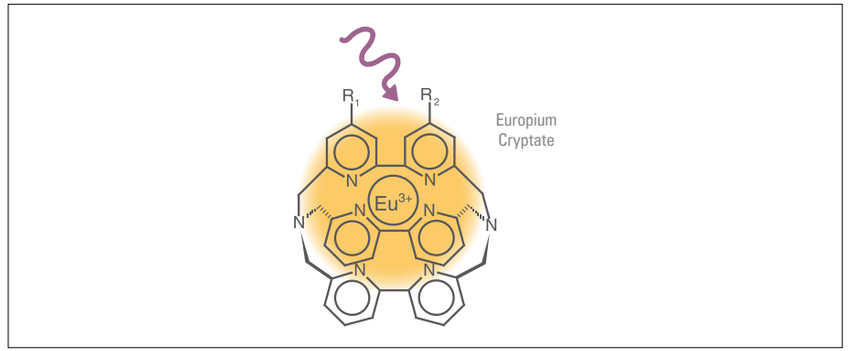

Figure 6 Europium cryptate structure consists of a tris bipyridine macrocycle in which the lanthanide ion is tightly embedded.

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HTRF PRINCIPLES

HTRF donor and acceptor fluorophores

Eu^{3+} and Tb^{3+} cryptate excitation wavelengths can be generated by most energy sources (e.g. nitrogen laser, flash lamp). Following excitation, long-lived Eu^{3+} and Tb^{3+} cryptate emissions are produced between 480 to 720 nm (Figure 7). Terbium cryptate has slightly different photophysical properties than europium cryptate, including increased quantum yield (10 to 20 times brighter) and a higher molar extinction coefficient which can significantly increase the detection sensitivity of some assays.

Two HTRF acceptor fluorophores:

- XL665
- d2

The HTRF acceptors, XL665 and d2, have been optimized to match Eu^{3+} and Tb^{3+} cryptate donor emissions. XL665, a phycobiliprotein pigment, is a large heterohexameric molecule of 105 kDa that is cross-linked after purification from red algae to provide better stability and preservation of its photophysical properties in HTRF assays. This acceptor can be excited from both Eu^{3+} and Tb^{3+} cryptates and in turn emits red-shifted wavelengths (peak emission at 665 nm). These red-shifted wavelengths are separated more easily from background autofluorescence which appears at much lower wavelengths. d2 has many photophysical properties in common with XL665 including emissions of red-shifted wavelengths, but it is an organic structure 100 times smaller than XL665. As a smaller entity, d2 minimizes the steric hindrances sometimes observed when using XL665. In many instances, d2 offers greater stability in immunocompetitive assays and in some cases has increased assay sensitivity.

Alternative acceptor fluorophores:

Terbium cryptate, unlike europium cryptate, also emits light at 490 nm upon excitation. This allows terbium donors to be paired with green-emitting (around 520 nm) acceptor fluorophores as well as the XL665 and d2 red-shifted acceptors.

Terbium cryptate's properties have led to the development of Tag-lite, a cellular HTRF-based assay platform in which a cell surface protein is labeled in situ, while the second binding partner, typically a receptor ligand or an antibody against the surface protein, is labeled with a red or a green synthetic acceptor. This allows for the investigation of cell surface receptor binding using a live cell assay format. See pages 35-42 for more information on Tag-lite technology.

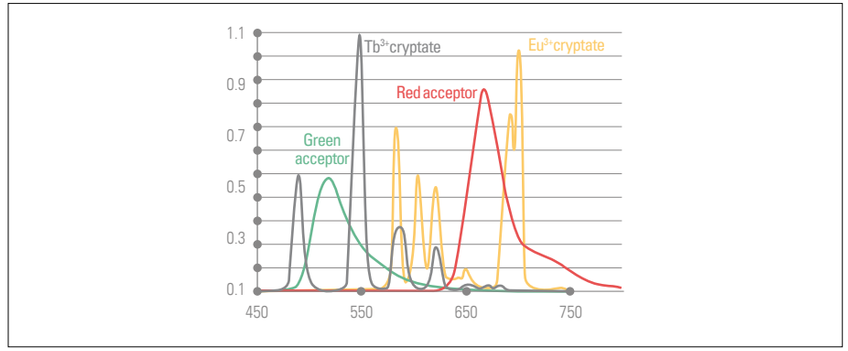

Figure 7 HTRF donor and acceptor emission spectra. Red acceptor emissions occur in a region where the donor does not produce significant emissions. Long-lived fluorescence detected at this specific wavelength is therefore characteristic of the emission of the acceptor engaged in the FRET process. The same is true for green acceptors emitting around 520 nm when combined with a Lumi4-Terbium donor.

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How close do the HTRF donor and acceptor fluorophores have to be for energy transfer?

70-100 angstroms ensures optimal FRET. However, the 3D special arrangement of molecules, often allows the fluorophores to be in close enough proximity to transfer energy for even larger complexes with multiple binding partners.

Can you label a small molecule compound with HTRF fluorophores?

Small molecules should be assessed on a case-by-case basis to develop the best labeling strategy. Proteins, peptides, oligonucleotides and other biomolecules can easily be labeled with europium or terbium-cryptate donors, the d2 red acceptor, the XL665 red acceptor, green acceptor, or the biotin tag.

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HTRF detection

Homogeneous, add-and-read platform eliminates wash steps for faster response and greater productivity

HTRF robustness and reliability are derived from both the quality of the fluorophores (Eu^{3+}/Tb^{3+}/XL665/d2) and the unique way in which signal intensities are measured and analyzed. HTRF detection and background reduction relies on two principles:

- Time-resolved readout: A time delay is introduced between excitation and measurement, allowing most background fluorescence to be eliminated (compounds, medium, cells, etc).
- Dual wavelength detection: Normalization of acceptor emissions at 665 nm (or at 520 nm when green acceptors are used) to donor emission at 620 nm. This ratiometric calculation is critical for homogeneous assays to correct for sample interference and assay medium variability.

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HTRF DETECTION
Ratiometric measurement

HTRF emissions are measured at two wavelengths, 620 nm and 665 nm (520 nm for green acceptor fluorophores). Emissions at 620 nm (donor fluorescence) are used as an internal reference, while emissions at 665 nm (acceptor fluorescence) are used as an indicator of the biological reaction being assessed. Dual-wavelength measurements are extremely advantageous and allow for compensation of well-to-well variations that are often challenging for other homogeneous technologies. In contrast to heterogeneous assays, where measurement takes place in a controlled buffer (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. Because of differences in medium additives and/or compound concentrations, each well may have different photophysical properties and degrees of signal interference, leading to varying signal intensities. This well-to-well signal variation is not due to true differences in partner binding and thus produces misleading results when only a single emission wavelength is measured. Because both the 620 nm donor emissions and 665 nm acceptor emissions are decreased to the same degree by sample interferences, the ratio remains unchanged (Figure 8) thereby compensating for well-to-well variability. The calculation of the HTRF ratio for each well is therefore critical for accurate results. This represents a significant advantage over other technologies like fluorescence intensity or luminescence for which the specific signal detected is not corrected and is therefore more likely to be influenced by medium interference partners.

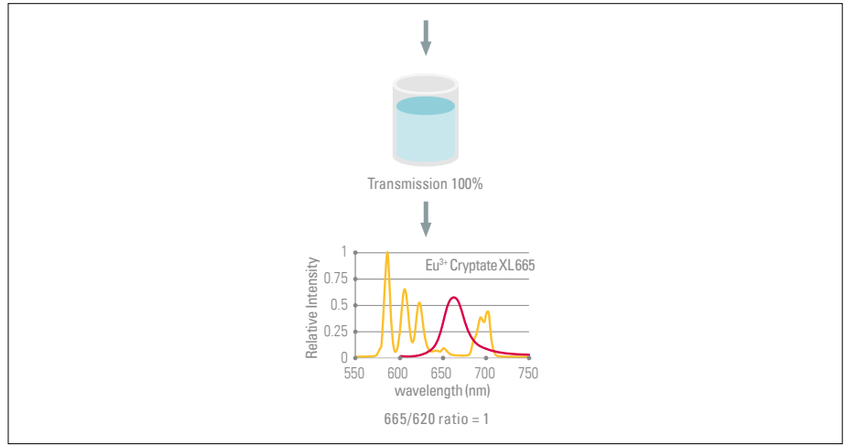
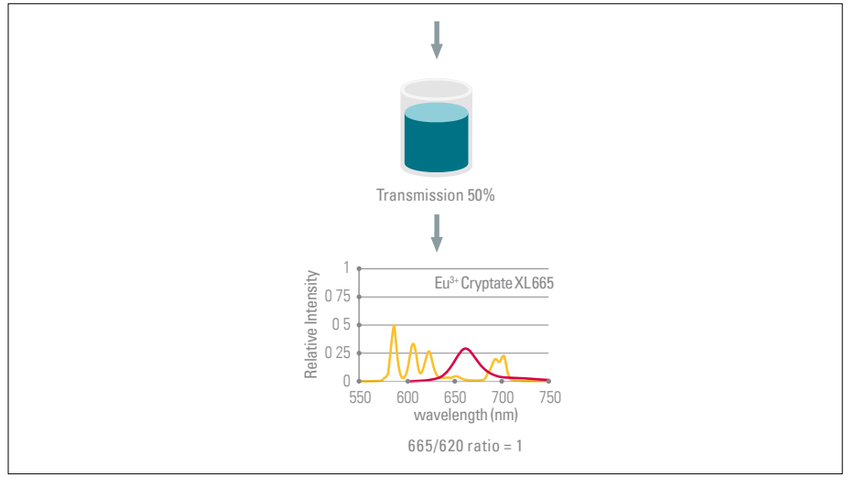


Figure 8 Filter effect may occur when highly colored compounds are used, which leads to a decrease in the excitation energy delivered. The fluorescence ratio will compensate for this phenomenon because both acceptor and donor emissions are affected in the same way.



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HTRF DETECTION
Signal stability

HTRF signal stability and robustness are derived not only from the cryptate cage of HTRF donor fluorophores, but also the addition of potassium fluoride prior to assay detection, in the case of Eu^{3+} cryptate, and inherent photophysical properties of HTRF fluorophores.

Cryptates vs. chelates:

Europium and terbium ions are tightly embedded in cryptate structure that resists harsh assay conditions and/or additives such as large quantities of challenging cations (Mg^{2+} , Mn^{2+}, etc.), chelators (EDTA), high serum concentrations (up to 50%), solvents, pH or temperature variations. In contrast, most Eu^{3+} and Tb^{3+} chelates are prone to dissociation when incubated with common concentrations of divalent cations or chelators (Figure 9).

Potassium fluoride:

Potassium fluoride (KF) is added to assay wells prior to reading which further strengthens HTRF signal stability. KF can be added at any time during the assay prior to readout. Fluoride ions act as a powerful, immediate, and irreversible fluorescence booster, preventing virtually all sample interference with Eu3+ cryptate. KF supplementation is not necessary for Lumi4-Terbium cryptate based assays although fluoride ions show some protective action under certain assay configurations (e.g. cell-based).

HTRF acceptor fluorophores:

The HTRF acceptors are carefully chosen for compatibility with a broad range of assay conditions. The introduction of small organic acceptors (d2 and green dyes) has strengthened the long signal stability even further.

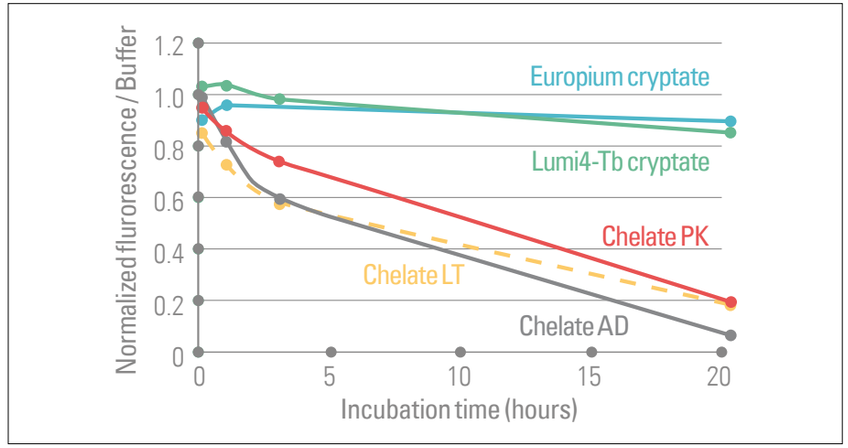

Figure 9: Lanthanide chelates are prone to dissociation in the presence of chelating agents such as EDTA 20 mM. Chelate fluorescence decreases over time while the cryptate fluorescence remains unaffected under the same conditions.

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HTRF DETECTION
Signal stability

Photobleaching:

A number of other homogeneous technologies (e.g. luminescence) are restricted by a reduction in signal intensity following assay measurement or prolonged incubations. In contrast, HTRF cryptates are not sensitive to photobleaching, a phenomenon suffered by many fluorophores, in which fluorescence emission disappears after prolonged or repeated excitation. HTRF emissions are not dampened by assay measurement, exposure to ambient light or extended incubations prior to reading (Figures 10 & 11). This extended signal stability is among the many benefits of HTRF and enables researchers to perform kinetic studies, and provides flexibility in read times during HTS campaigns, and increased sample compatibility.

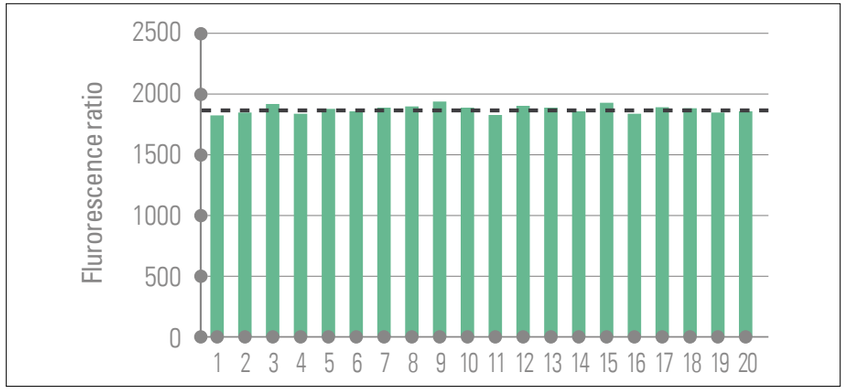

Figure 10 Twenty successive readouts of a kinase assay. Unlike luminescence, HTRF is not affected by multiple measurements, not only making kinetic studies possible, but also enabling plate re-measurement in case of a reader problem.

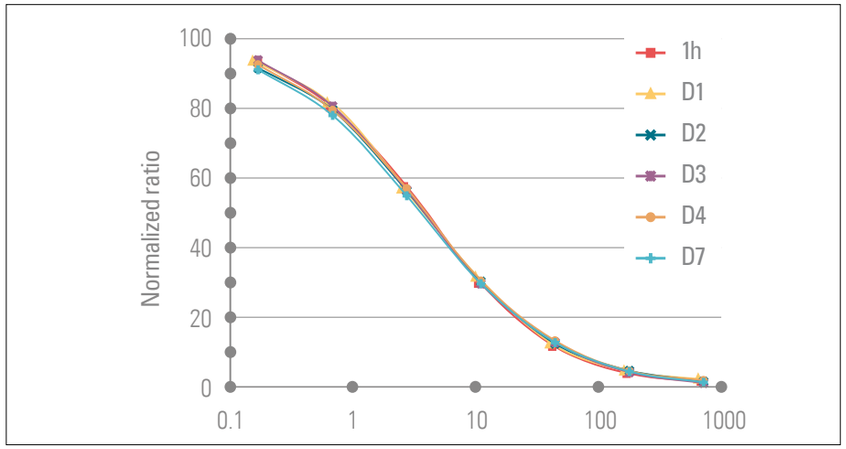

Figure 11 Seven-day signal stability of cAMP assay. Assay plates can be read multiple times over long periods of time without affecting output and reproducibility if medium evaporation is eliminated. Plates can be frozen, which is particularly convenient when the results need to be compared to another plate reader or at a second location.

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Is HTRF light-sensitive?

No. Unlike other technologies, HTRF reagents or assays do not suffer from light exposure, and do not require lab light shading during manipulation or assay setting.

Are there any compounds that typically interfere with HTRF?

HTRF is one of the most robust homogeneous technologies. Very few chemical compounds interfere with the signal itself. Interfering compounds generally behave as donors or acceptors, which can be flagged by simple signal analysis. Using the HTRF ratio measurement (using both donor and acceptor emissions), the level of true interference is typically kept below 0.05%.

If the 620 values don't change across the plate, can I just use the 665 read?

Revvity always recommends the 665/620 ratio. Using just the 665 numbers can be acceptable, but will not correct for medium or compound interference.

Why do I have to add potassium fluoride (KF) to my assay? How much KF should I add? Is it mandatory for Eu and Tb cryptates?

KF is necessary for Europium cryptate-based HTRF assays acting as a fluorescence booster. KF can be added just before readout if its presence may affect previous steps of the assay (e.g. binding). KF is generally contained in the detection buffer which is used to reconstitute HTRF conjugates. Ideally, the final KF concentration at detection should be approximately 400 mM, but can be lowered to 100 mM if high ionic strength challenges the assay. Terbium cryptate-based assays do not require the addition of KF.

Is HTRF sensitive to temperature?

HTRF chemistry itself is not irreversibly affected by temperature changes, although protein components within the assay may be temperature sensitive. Of course, as a general rule for biological assays, detection kinetics is usually influenced by incubation temperature.

Is HTRF sensitive to DMSO?

HTRF assays are generally compatible with up to 1% DMSO, final concentration.

For how long is my signal stable?

After the assay has reached equilibrium, the signal is generally stable from over several hours to days, depending on the assay. Over long periods of time, decreased signal is often due to the biology of the assay (e.g. cell or protein degradation) rather than the HTRF detection system itself.

Can I read the plate/assay several times?

Yes. This is one of HTRF's most appreciated features. The fluorescence signal is stable and is unaffected by assay detection. HTRF donor and acceptor molecules do not photobleach (i.e. they do not lose their fluorescence emission properties after multiple excitation pulses). As a consequence, HTRF can be used in a kinetic mode, or the plate read again if a plate reader issue occurs. Plates can also be sealed and frozen to be read at a later date – which is quite useful for comparing reader performance from one lab to another.

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DETECTION

Choosing the Right Plate Reader

Choosing the suitable microplate reader ensures you'll get an optimal readout. A multimodal reader gives your research the ideal flexibility and expands the field of possibilities by measuring a wide range of technologies.

Revvity offers multi mode plate readers that are certified for use with HTRF technology. Certification involves rigorous testing of plate reader with HTRF technology to ensure optimal readout. HTRF assays are also compatible with multimode readers from other providers as well.

EnVision® Nexus™

Provides lightning speed and superior sensitivity across all established detection technologies with advanced options for ultimate performance. It is the next generation of high-throughput screening, ideal for your most demanding assays. .

VICTOR® Nivo™

Packs all the latest major detection technologies into the industry's smallest benchtop footprint. The perfect microplate reader for everyday biochemical and cell-based assays.

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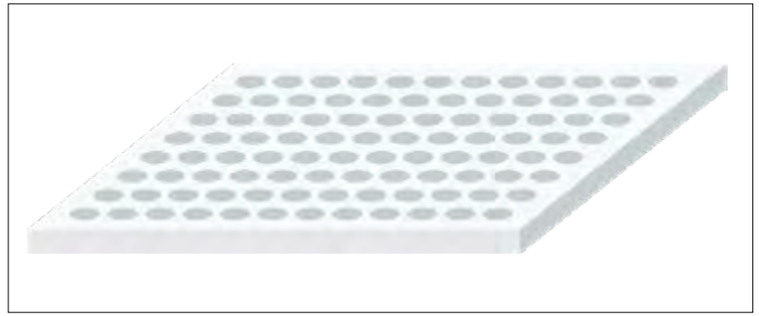
Microplate recommendations

Most brands of available microplates are compatible with HTRF technology. Nevertheless, background levels and signal-to-noise may differ among plate types. General recommendations include:

- FIA black or white plates remain the most appropriate standard.
- The signal-to-noise ratio obtained using white microplates does not generally differ substantially from black plates. Absolute counts, however, are significantly higher in white plates, thereby improving count statistics and subsequent assay reproducibility and sensitivity.
- The use of white plates is mandatory for some assays, such as IP-One, and highly recommended when detection is performed using monochromator-based plate readers.
- Tissue-culture treated, or surface-treated plates may be used.
- Glass-bottom plates are likely to produce suboptimal results and should be tested before use.
- Assay volume should match well volume so that focusing the reader excitation beam allows maximum energy delivery. For instance, when assay volumes are equal to or lower than 20 µL, user should use 384 low-volume or 1536-well plates.



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What if I don't have a 620 nm filter?

The 620 nm filter is necessary ffor detecting europium or terbium donor fluorescence. Some plate readers are also equipped with a 615 nm or a 590 nm filter, which, though not ideal, may be used instead. However the output will also depend on the actual specifications of that filter, e.g. bandwidth or % of transmission.

What reader do you recommend?

All the HTRF compatible readers have been extensively tested to meet strict performance guidelines.

What is the difference between flash lamp versus laser readers? Filter-based versus monochromatic readers?

Different sources of light for excitation: lasers allow high energy excitation at one fixed wavelength, flash lamps have less energy but a broader excitation range of wavelengths. Filters allow an optimal light transmission with limited energy loss. Monochromators offer less sensitivity but a broader choice of wavelengths.

Should I use black or white plates?

Plates must be opaque, and in general, both black and white plate can be used. Certain microplate readers require white plates for increased sensitivity. If given a choice, choose white.

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HTRF ASSAYS
Assay format overview

Cryptate chemistry provides unparalleled assay robustness and stability, delivering reliable and reproducible results

HTRF was originally created as an immunoassay technology nearly two decades ago, and continues to offer one of the most effective and flexible solutions in the diagnostics and drug discovery fields. HTRF's tremendous versatility has led to the construction of numerous assay configurations, from the most basic biochemical tests to more sophisticated cell-based assays. HTRF has become a reference technology for researchers who wish to transition conventional assays such as Western Blot, ELISA, radioimmunoassay or bead-based technologies, into simple-to-perform, cost-effective assays.

HTRF assay advantages

- Rapid, simple-to-perform assays
- Non-radioactive
- High level of application flexibility
- Biochemical and cell-based formats
- Amenable to miniaturization and automation
- Stable signal for kinetic studies

Applications include

- GPCR and cell signaling
- Kinase screening
- Epigenetic target screening
- Quantification of cytokines and other biomarkers
- Characterization of biotherapeutic antibodies
- Biomolecular binding (protein-protein, protein-peptide, protein-DNA/RNA)

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HTRF ASSAYS
Assay format overview

Competitive vs. non-competitive assay formats

HTRF immunoassays are structured using the same basic principles as conventional antibody-based detection systems. Both biochemical and cell-based assays can be structured using competitive or non-competitive formats.

Non-competitive, sandwich immunoassays

Non-competitive assays are designed to assess molecules that are large enough to encompass distinct binding sites for two antibodies or to measure the binding of two assay components each of which is recognized by a single antibody (Figure 12). HTRF sandwich assays use two antibodies coupled respectively to a cryptate donor (Eu^{3+} or Lumi4-Terbium) and an acceptor (i.e. XL665 or d2). Energy transfer, and thus signal generation, occurs if the two antibodies are brought into proximity either by binding the antigen of interest in the sample, or upon the binding of the two assay components of interest. For all non-competitive immunoassays signal intensity is proportional to the number of complexes formed, i.e. antibody-antigen or binding partners of interest.

Many HTRF ready-to-use kits use a sandwich immunoassay format including many of the biomarker kits such as cytokines, insulin and Amyloid β1-40. Additionally, the HTRF toolbox portfolio contains a variety of labeled anti-tag, anti-species and affinity system reagents. These products enable the rapid development of custom sandwich-based immunoassays.

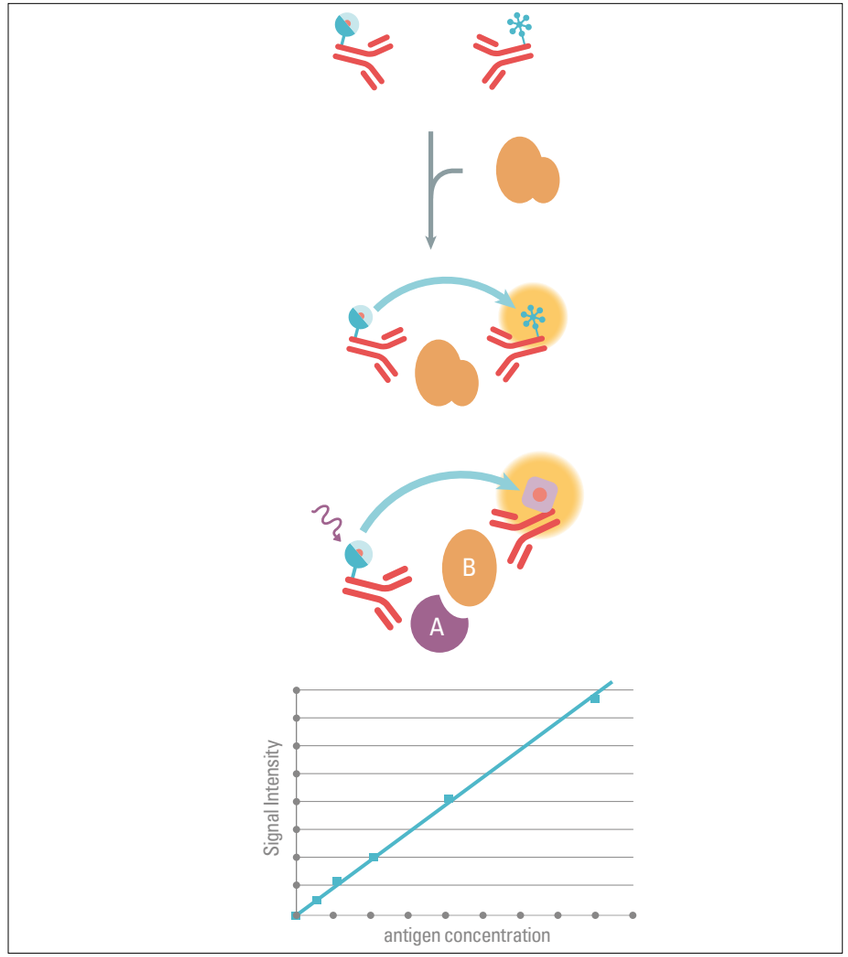

Figure 12 Sandwich assays involve two antibodies coupled to donor and acceptor dyes. They produce a signal that is proportional to the amount of analyte detected.

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HTRF ASSAYS
Assay format overview

Competitive assays

Competitive assays are generally designed to assess molecules that are too small to allow the binding of two separate antibodies. The assay components used in this format are typically the purified antigen of interest coupled to an acceptor fluorophore, and its respective antibody coupled to Eu^{3+} or Lumi4-Terbium cryptate. Unlabeled antigen present in the sample competes for binding with labeled antigen, thereby preventing FRET from occurring. FRET intensity decreases as labeled antigen/antibody complexes dissociate. Thus, assay signal is inversely proportional to antigen concentration (Figure 13).

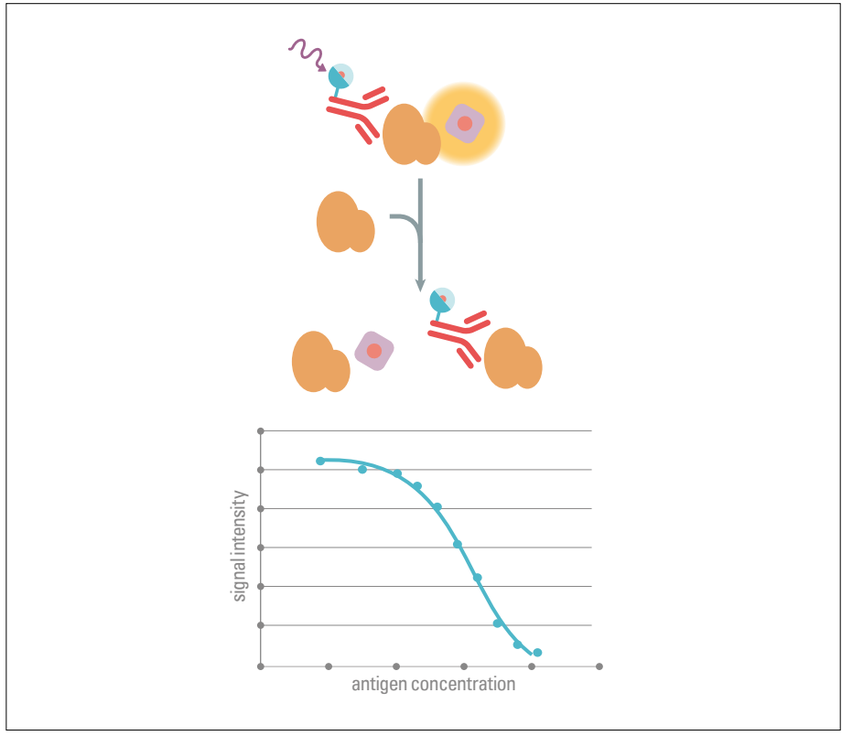

Figure 13 Immunocompetitive formats usually involve the labeling of the analyte itself, and yield a signal that is inversely proportional to the amount of free analyte detected.

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HTRF ASSAYS
Biochemical assays

Biochemical HTRF assays are performed using purified assay reagents such as antibodies, antigens, enzymes and enzyme substrates via a variety of competitive and non-competitive detection schemes. These assays can generally be divided into three categories: i) enzyme assays (kinases, epigenetic enzymes, protease or ubiquitinases); ii) biomolecular binding (protein-protein, protein-peptide, protein-DNA/RNA); and iii) protein quantification (cytokines, metabolic biomarkers, total IgG or light chains, tagged fusion proteins, etc.). Enzyme assays, unlike other biochemical assays, contain an initial step in which the enzyme reaction of interest is performed in the absence of HTRF detection reagents. Enzymatic reactions are subsequently halted and the necessary HTRF detection reagents added (Figure 14). Non-enzyme biochemical assays can generally be performed in a single step in which detection reagents are added (either pre-mixed or in two separate additions) to the sample and signal detected following a one hour incubation. A broad range of biochemical assay kits are available from Revvity or researchers can construct their own assays using HTRF-labeled toolbox reagents.

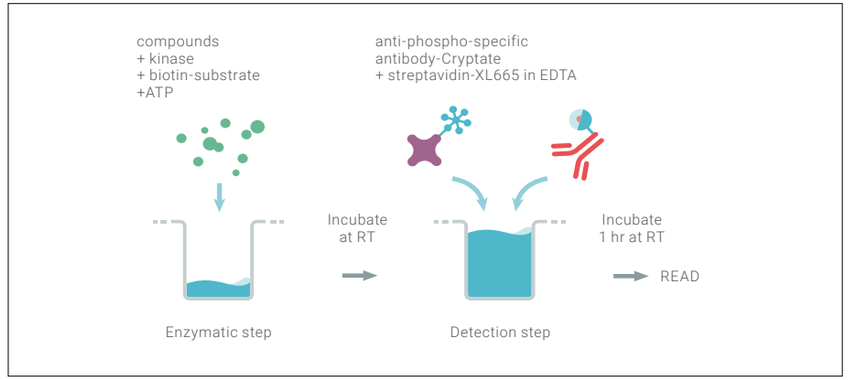

Figure 14 Assays such as kinase activity assessment are typically run under a two-step format, allowing each step to be performed in the appropriate controlled medium. Both are usually carried out in the same microplate.

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HTRF ASSAYS
Cellular assays

Most HTRF assays are compatible with both whole cells and cell supernatants. This enables functional assays to be run under more physiological conditions as the cells remain alive until the final detection step or transfer of the supernatant. In general, cell-based assays are used to quantify secreted cellular proteins or to probe various intracellular signaling or regulatory pathways (GPCRs, phospho-proteins, epigenetic enzymes, etc.), although HTRF flexibility allows for development of a range of innovative cellular assays. HTRF cell-based assay protocols can be streamlined to single-plate processes from cell culture through assay readout to simplify handling and increase throughput. Cellular assays can be competitive or non-competitive in nature, requiring either one or two HTRF reagent additions depending on the assay configuration (figure 15).

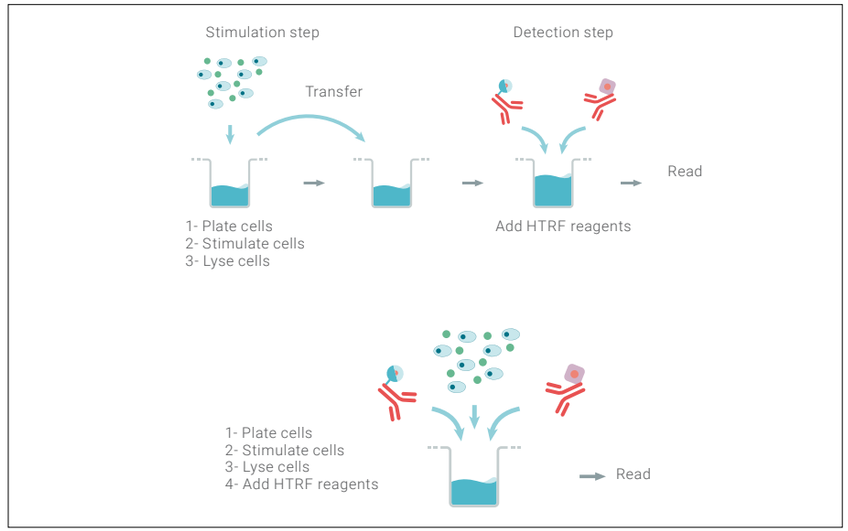

Figure 15 HTRF enables multiple assay configurations when dealing with cells. For instance, a two-plate cellular phospho-protein assay can be further streamlined to a one-plate protocol for HTS implementation.

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HTRF ASSAYS

Assay miniaturization

Not all detection technologies permit the same level of miniaturization. Assays based on absorbance or radioactivity are limited by their sensitivity when miniaturized because the assay readout is directly proportional to the quantity of the label. In contrast, fluorescence signals are concentration-dependent (i.e. at an equal concentration, the signal remains the same, whatever the volume). This property allows HTRF assays to be miniaturized easily while maintaining accuracy and reproducibility. A number of HTRF assays have been shown to perform equally well in 96-, 384- and 1536-well formats with volumes less than 5 µL, simply by proportionally decreasing the volume of the assay components. In all cases, miniaturized HTRF assays maintain excellent dynamics and sensitivity, essential for implementation of higher throughput assays.

Two general rules should be followed to achieve successful assay miniaturization:

1. HTRF emissions are dependent on fluorophores concentrations. Thus reagents should not be diluted. Instead, simply decrease the volumes used per assay. Figure 16 illustrates the miniaturization from 200 to 20 µL. Assay reagents may require concentration for maximum assay performance of ultra-miniaturized assays (lower than 5 µL). Contact our technical teams for more details.
2. The microplate reader excitation beam must be focused into the well accurately (Figure 17, case A). In case B, where the same plate format is being used with a smaller volume, unfocused excitation will lead to inferior collection of emissions. The use of a plate designed for small assay volumes, such as in case C, will restore the measurement efficacy.

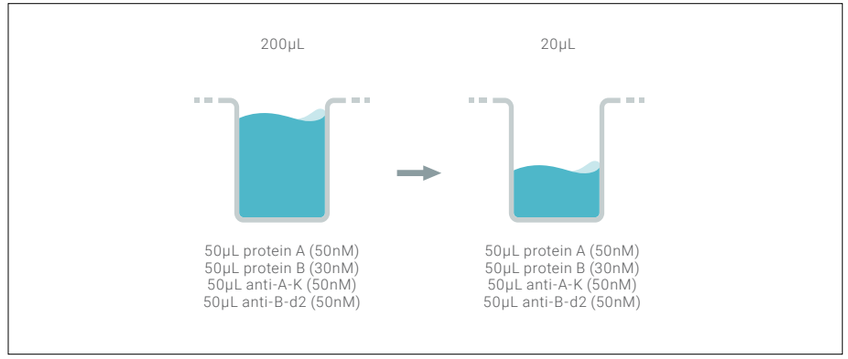

Figure 16 Proportional reagent volume downsizing offers a straightforward way to miniaturize HTRF assays to high density formats such as 384- low volume or 1,536-well plates.

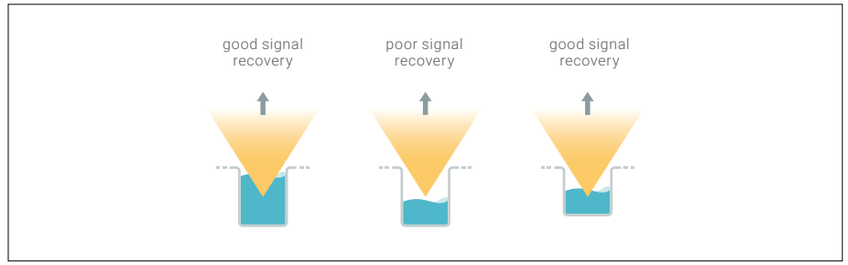

Figure 17 Plate reader optics adjustment, available on most instruments, is mandatory for high performance measurement.

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HTRF ASSAYS
FAQs

Can I pre-mix the two detection reagents before adding them to my plate for a sandwich assay?

Yes, for most sandwich assays, the donor and acceptor conjugates can be pre-mixed and dispensed at one time. Any leftover pre-mixed solution should be stored as per the conjugate storage conditions indicated in the kit instructions (i.e. frozen).

Can I pre-mix the two detection reagents before adding them to my plate for a competitive assay?

Unlike for sandwich assays, not all competitive assays can be run with a single conjugate pre-mix addition. The antibody antigen reaction will start when the two reagents are pre-mixed and this initial binding may be more difficult to revert by free antigen, or take longer. Some assays will withstand the pre-mix mode, but others will not. In general, do not implement the pre-mix protocol without first comparing it to a two-step dispensing mode to make sure assay performance is not sacrificed.

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Assay development

The flexibility to explore any target of interest with a variety of assay formats

A wide range of biochemical and cellular assays are available as HTRF kits, each containing the necessary HTRF-labeled reagents and assay protocols. Often clients wish to develop custom assays because: a) an HTRF assay kit is not available; b) they require the use of proprietary assay components; c) target-specific reagents are not available; or d) they wish to convert a current assay from an alternative read-out such as an ELISA to a higher throughout, add-and-read HTRF assay.

Options for developing an HTRF assay include:

- HTRF toolbox reagents
- Do-it-yourself labeling reagents & kits
- Custom labeling services
- Assay development services

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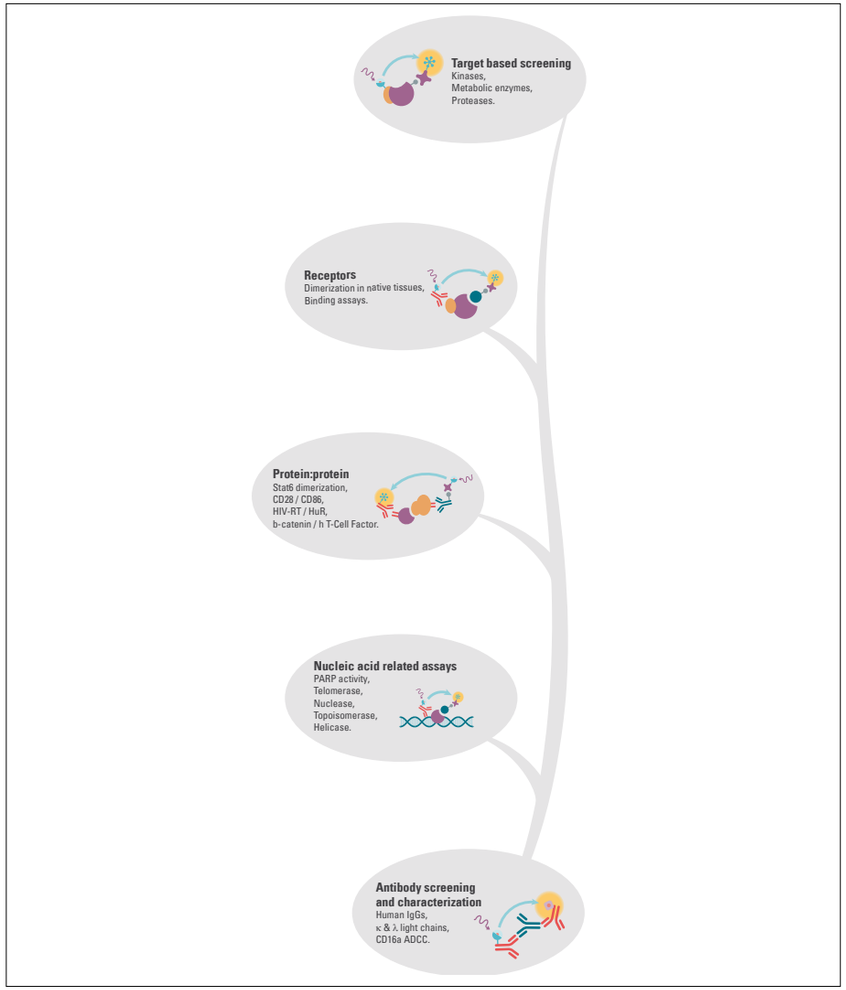
ASSAY DEVELOPMENT
HTRF toolbox reagents

The HTRF Toolbox is a line of reagents labeled with HTRF fluorophores that can be used by researchers to create custom HTRF assays. Toolbox reagents include anti-tag antibodies, streptavidins, and anti-immunoglobulin antibodies. These reagents are available as Eu^{3+} cryptate, Lumi4-Terbium cryptate, XL665, and d2 conjugates and are particularly useful in cases where reagents specific for a protein of interest are not available. The Lumi4-Terbium cryptate conjugates bring an additional level of flexibility, as they can be combined either with XL665 or d2, or with synthetic green fluorophores or GFP.

The diversity of reagents and fluorophore options provide a high level of assay design flexibility enabling multiple assay configurations. A wide range of assays has been developed with these reagents, including protein:protein interactions, nuclear receptor assays, receptor dimerization, ligand binding and enzyme assays. As with all HTRF assays, those developed using our toolbox reagents can easily be miniaturized and are amenable to automation.

Toolbox Features

- High-affinity monoclonal and polyclonal antibodies
- High-performance streptavidin reagents for assays requiring high sensitivity
- Resistant to most buffer conditions and additives (e.g. DMSO, pH, chelators, ionic strength)
- Compatible with membrane- and cell-based assays
- Lyophilized for easy handling and long-term storage
- Proven batch-to-batch reproducibility



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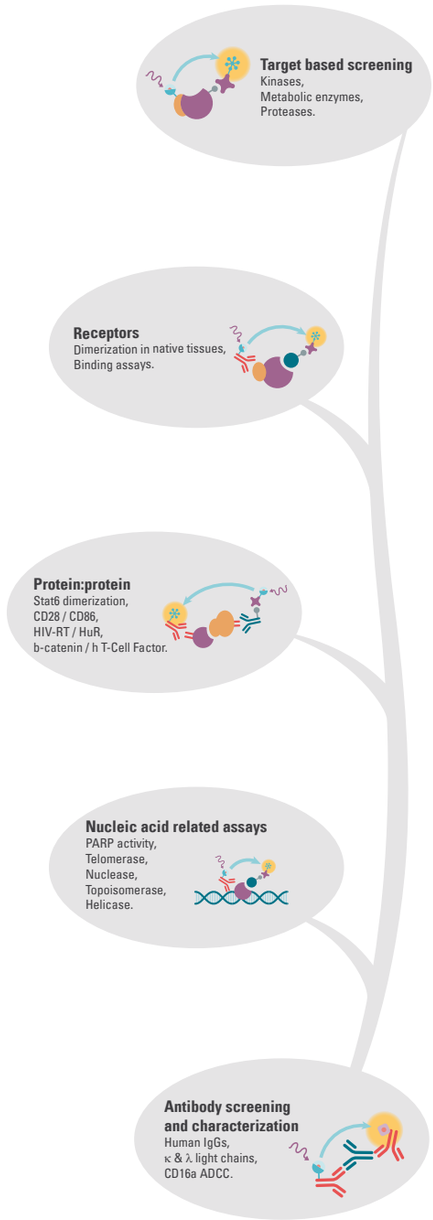

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ASSAY DEVELOPMENT
HTRF toolbox reagents

Anti-tag antibodies: The most frequently used tags for developing assays include GST, 6HIS, c-myc, FLAG and HA, maltose binding protein (MBP) or small organic motifs like dinitrophenyl (DNP). The HTRF toolbox reagents include a large selection of anti-tag antibodies labeled with europium cryptate, Lumi4-Terbium cryptate, XL665 and d2.

Anti-immunoglobulin antibodies: HTRF toolbox reagents include secondary murine, sheep, rabbit, and human antibodies, as well as protein A. These reagents are useful in developing assays for which directly labeled assay components are not available or which involve chimeric proteins. Most of our anti-immunoglobulin reagents are available conjugated to either europium-cryptate or XL665.

Affinity system reagents: The streptavidin-biotin reaction is one of the most frequently used affinity systems. HTRF toolbox reagents include a line of europium cryptate, Lumi4-Terbium-cryptate, XL665 and d2 conjugated streptavidins.

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ASSAY DEVELOPMENT
Using toolbox reagents

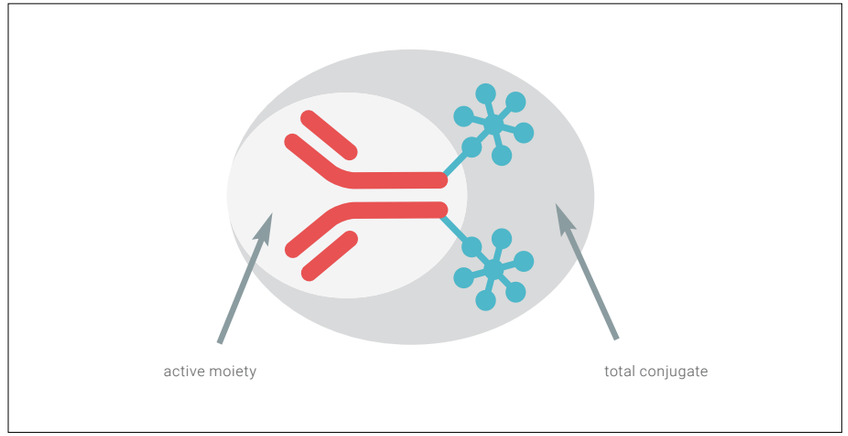
Reagents from the HTRF toolbox can be used in multiple configurations such as competitive and non-competitive assays as well as biochemical and cellular assays. Toolbox reagents are sold according to the number of tests (384-well, 20 µL reactions). The amount of active moiety per vial for each reagent is also provided. As shown beside, active moiety is defined as the active part of a toolbox reagent (i.e. antibody, or biomolecule being labeled).

How do the number of tests relate to active moiety?

The total conjugate quantity per well is information that reflects the overall total material (active moiety + HTRF fluorophore). For Eu^{3+} and Lumi4-Terbium cryptates, d2, biotin, and DNP conjugates, the total conjugate amount equals that of the active moiety, since the molecular weight of the label is negligible. For XL665 labeled entities, however, the amount of active moiety will not equal the amount of total conjugate. The quantity of total toolbox reagent supplied by Revvity will vary depending on the final molar ratio of active moiety/XL665 conjugate and therefore varies from one toolbox reagent to another. The amount of active moiety, however, is constant and based on the number of tests ordered. In practice, using the active moiety amount is generally preferred to the quantity of total conjugate as a basis for calculating reagent usage per well during assay development because the HTRF fluorophore does not influence the biological interaction being examined.

Recommended quantities of fluorophore conjugates

Most assays can be run within the nanomolar range. However, as a fluorophore, cryptate conjugates must not be in excess to prevent reader saturation and/or unacceptable background levels. In most cases, a Eu^{3+} or Lumi4-Terbium cryptate concentration of 3 to 5 nM is appropriate. Typically, for an antibody conjugated to Eu3+ cryptate, the recommended value would be close to 1 nM of antibody. The XL665 conjugate must match its assay counterpart as closely as possible so the maximum number of biomolecules can be tagged with the XL665 acceptor. Thus, to detect a GST-tagged molecule at an assay concentration of 20 nM, the concentration of anti-GST-XL665 should be equimolar or higher. The actual amount will depend on the assay configuration and the degree of miniaturization.



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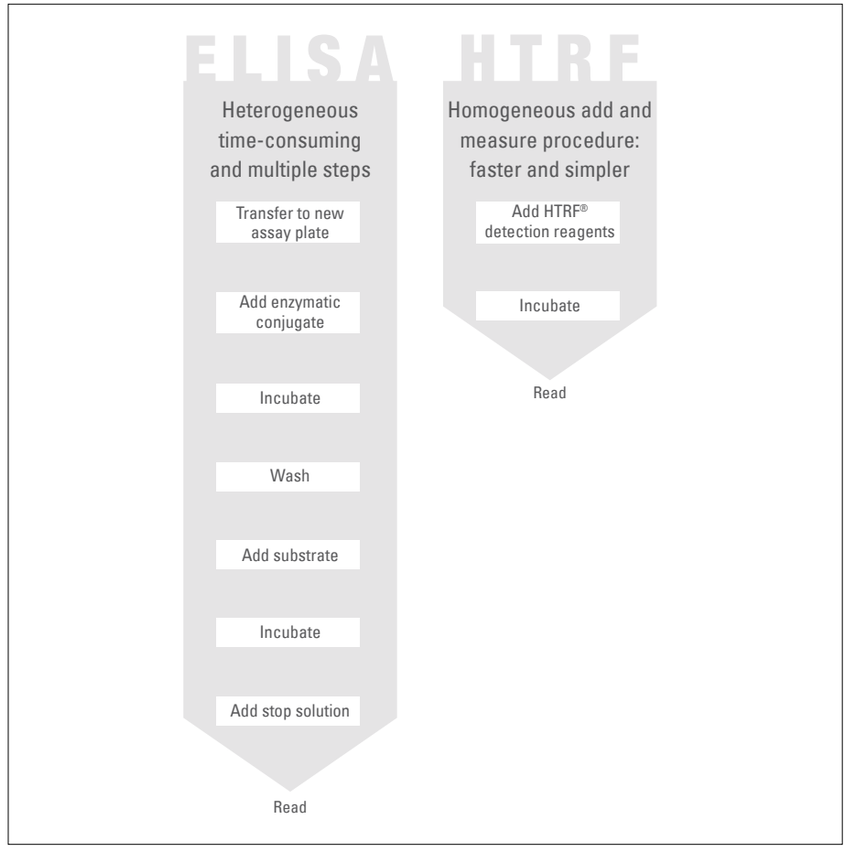
ASSAY DEVELOPMENT

ELISA conversion case study: glucagon-specific detection

Conversion of assays from ELISA to an HTRF format generally proceeds through several steps: a) identify a number of antibodies that are available for use; b) label available antibodies with HTRF fluorophores; c) test the matrix of labeled antibodies to identify pairs with high signal; d) evaluate the performance of best pairs for any other required specifications (i.e. antigen specificity, species cross-reactivity; e) perform assay with control samples to define assay sensitivity and linearity; and f) run a correlation between old and new assays.

Below is a case study in which a glucagon-specific assay was converted from an ELISA to an HTRF assay. In this case, a non-competitive sandwich immunoassay was developed to specifically measure glucagon (not related peptides produced by PC2 cleavage) in cell supernatants or plasma/serum samples.



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ASSAY DEVELOPMENT

ELISA conversion case study: glucagon-specific detection

Glucagon background

- Amino-acid sequence of glucagon is highly conserved in mammalian species (identical in human, mouse, rat, pig, cow and hamster) allowing for development of a single glucagon assay that can be used with samples from multiple species.
- Glucagon is produced from proglucagon by tissue-specific processing, mediated by the prohormone convertases (PC). As shown in Figure 18, tissue specific degradation of proglucagon leads to the release of a number peptides that may interfere with the detection of glucagon itself.
- Different peptides are produced from proglucagon cleavage in the pancreas than in the gut. Some of them (like oxyntomodulin) have a sequence very similar to Glucagon. Thus, it was very important to test the specificity of the chosen antibodies to ensure development of an HTRF assay highly specific for glucagon.

Assay development

Step 1 – Identified 7 commercially available glucagon-specific antibodies, purchased and labeled 100 µg of each antibody with cryptate (K) & d2.

Step 2 – Tested matrix of antibody pairs (matrix of 42 pairs) to determine pairs with high assay signals. Figure 19 shows that the Ab4/Ab2 antibody pairs came as the highest signal option out of those 42 possible antibody pair testing.

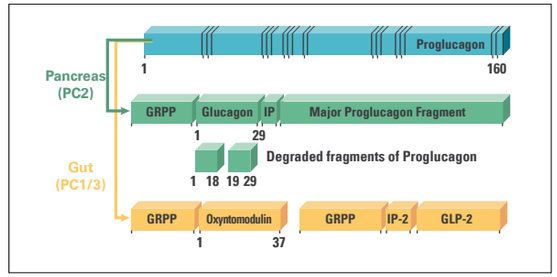

Figure 18 Tissue-specific processing of proglucagon by PC2 and PC1/3

	Ab1-K	Ab2-K	Ab3-K	Ab4-K	Ab5-K	Ab6-K	Ab7-K
Ab1-d2		3%	6%	2866%	3%	0%	1896%
Ab2-d2	2%		11%	3630%	32%	0%	1282%
Ab3-d2	469%	1526%		2229%	0%	53%	1502%
Ab4-d2	2412%	4702%	22%		52%	543%	3%
Ab5-d2	0%	320%	3%	8%		6%	0%
Ab6-d2	6%	0%	0%	2024%	0%		1628%
Ab7-d2	1155%	2478%	3%	7%	0%	379%	

Figure 19 Best antibody pair signal determination

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ASSAY DEVELOPMENT

ELISA conversion case study: glucagon-specific detection

Step3 – Tested specificity (detect glucagon but not other peptides produced by PC2 cleavage of proglucagon) of several antibody pairs identified in Step 2 to determine the best pair to be used moving forward. The Ab2/Ab4 combinations actually showed significant cross-reactivity with oxyntomodulin, one of the major degradation fragment of proglucagon. Testing of other high signal options enabled identification of the Ab2-K/Ab7-d2 pair, extremely specific for glucagon. Figure 20 displays the specificity profile of Ab2-K/Ab7-d2 pair, showing minimized cross-reactivity with proglucagon degradation fragments.

Step 4 – Performed a standard curve using best-pair and purified glucagon to determine assay sensitivity. As shown on Figure 21, we optimized and tested two different glucagon diluents to represent cell culture supernatant samples & plasma or serum samples.

Step 5 – Performed side-by-side assays to confirm correlation between ELISA and new HTRF assay. The plot on Figure 22 illustrates the tight correlation obtained between the HTRF assay developed and a commercially available reference ELISA, with similar specificity.

Outcome

A homogeneous, add-and-read HTRF assay was developed that is highly specific for Glucagon, with a dynamic range of 15.6 – 2,000 pg/mL and a sensitivity of 6 pg/mL for cell supernatant and 12 pg/mL for serum. The assay correlates strongly to the previously used ELISA assay with an r^2 value of 0.96.

	Specificity (% of recognition)
Glucagon	100%
Glucagon fragment 1-18	<1.81%
Glucagon fragment 19-29	<0.03%
Oxyntomodulin	<0.07%
GLP-1 (7-36) amide	<0.06%
GLP-1 (7-37)	<0.11%
GLP-2	<0.3%
GRPP (Glicentin-Related Pancreatic Peptide)	<0.01%

Figure 20 Glucagon assay specificity profile

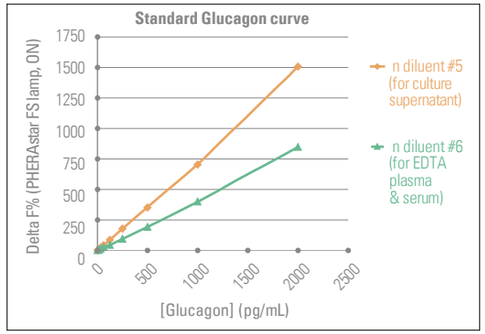

Figure 21 Glucagon assay dilution test

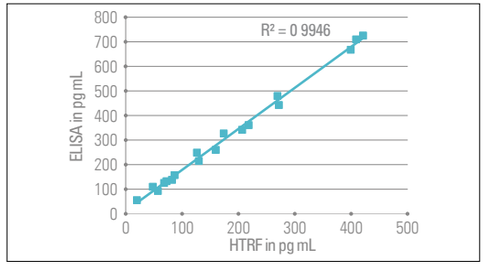

Figure 22 HTRF and ELISA glucagon assay correlation

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ASSAY DEVELOPMENT
HTRF services

Revvity services are performed by our expert team of scientific assay design specialists. A full range of services is offered from simple biomolecule labeling to complete development and implementation of HTRF or Tag-lite assays. Revvity's expertise reaches our customers through close, personal communication, enabling us to fully understand your needs and tailor a service solution to your project. Frequently, our scientific team supports researchers' efforts to convert their multi-step heterogeneous assays into streamlined and cost-effective HTRF add-and-read assays, including using a cell-based format when required.

Custom labeling – Labeling of proteins, peptides, oligonucleotides and other biomolecules with HTRF fluorophores such as cryptates (Europium and Terbium), XL665 or d2. We also provide labeling services for biotin, DNP conjugation or other fluorescent dyes. Following labeling, the sample is fully characterized to provide our customers with the appropriate working and quality control information.

Best pair identification – Labeling and testing of potential antibodies to identify the best pair for the development of a sandwich immunoassay. This is ideal for conversion of other assay formats to HTRF. For example, the conversion of a sandwich ELISA to an HTRF format may require the antibodies used in the original assay to be reassessed and combined with additional ones in order to further improve assay performance.

Assay development – Assay services ranging from feasibility studies through assay development, optimization and customer implementation. HTRF biochemical or cellular assays can be developed as well as Tag-lite assays.

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ASSAY DEVELOPMENT
FAQs

Can I label my own assay components?

Yes. Several HTRF labeling reagents are available for use in your laboratory.

Is an HTRF assay going to be as sensitive as my ELISA?

An HTRF assay can be as sensitive as an ELISA, but it is highly dependent on the antibodies chosen for use.

For custom labeling, do you supply the antibody or do I obtain it myself?

Material to be labeled can be provided by the customer or sourced from an external vendor by Revvity.

How long do assay development projects take?

Assay development is a multistep process conducted in collaboration with you. The overall process can be split into three main phases: feasibility study, assay development, and transfer. Generally, assay development is completed within 4 to 8 weeks.

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TAG-LITE
Introduction

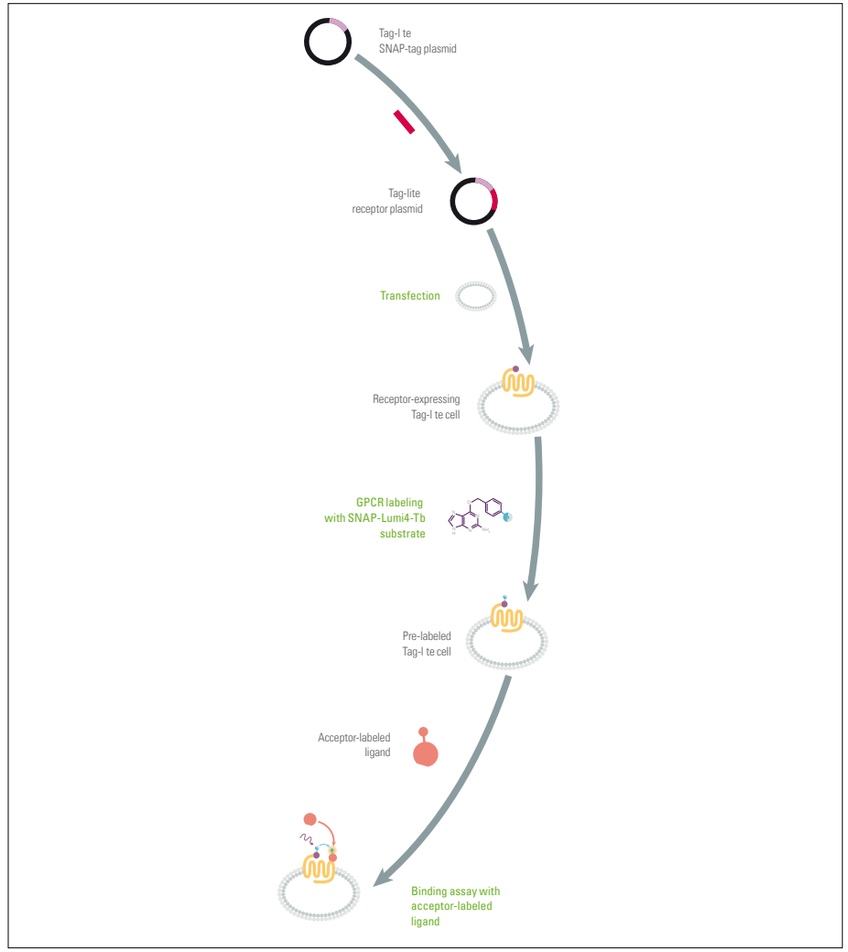
Comprehensive, non-radioactive cellular platform providing robust and sensitive assay for ligand binding studies.

Tag-lite is a non-radioactive, cellular platform that enables the investigation of natural ligand, small molecule or antibody binding to cell surface receptors which is highly advantageous in the areas of GPCR and RTK investigations, and biotherapeutic antibody development. It combines HTRF with live-cell protein labeling technologies such as SNAP-tag which provide a novel means of accurately labeling a cell surface protein of interest with an HTRF fluorophore at defined locations.

Tag-lite offers an alternative to existing cellular bioluminescence resonance energy transfer (BRET) and FRET assays. Traditional BRET and FRET technologies suffer from low signal-to-noise ratios resulting from the intrinsic background of cells, and the overlap between the emission spectra of FRET donors and acceptors. Tag-lite is based on the use of Lumi4 -Terbium cryptate which permits the use with either green or red acceptor-coupled partners, and takes advantage of the long lifetime of the terbium donor to eliminate short-lived fluorescence from cellular background or unbound acceptor conjugate.

Tag-lite features

- Cell-based assay platform
- Homogeneous, non-radioactive format
- Directly measures binding of ligands and/or soluble proteins to a specific cell surface protein
- In situ cell labeling of a specific cell surface protein at a defined location
- Available as ready-to-use cell lines or individual assay development reagents



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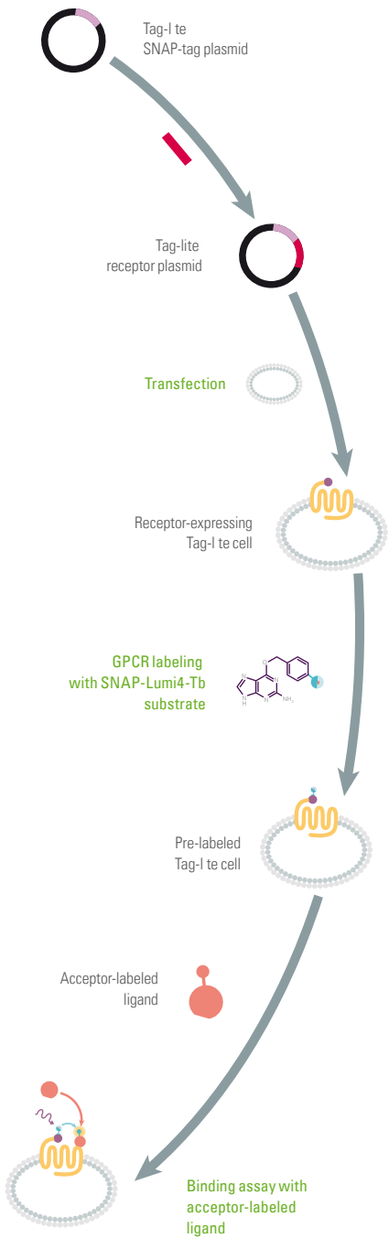

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TAG-LITE

Tag-lite tags

The use of fusion proteins for protein purification and in biomolecular assays has become widespread. Conventional fusion tags require genetic encoding for production. While traditional fluorophores such as GFP can be genetically encoded they exhibit inferior performance compared with newer synthetic fluorophores. Synthetic fluorophores, however, cannot be genetically encoded thereby limiting their use. One approach that addresses this shortcoming relies on tags that can be genetically encoded and subsequently labeled with synthetic fluorophores following cellular expression. Three in situ tag systems, SNAP-tag, CLIP-tag and HaloTag, have been combined with HTRF detection to create the Tag-lite platform.

- SNAP-tag, an NEB technology, is a 20 kDa mutant of the DNA repair protein O6-alkylguanine-DNA alkyltransferase that reacts specifically and rapidly with benzylguanine (BG) derivatives. This leads to irreversible covalent labeling of the SNAP-tag protein with an HTRF fluorophore via a SNAP-tag synthetic substrate.
- CLIP-tag, an NEB technology, is similar to SNAP-tag but reacts specifically with another substrate family, the O2-benzylcytosine (BC) derivatives. This protein tag reacts with a synthetic CLIP-tag substrate linked to an HTRF fluorophore.
- HaloTag, a Promega technology, is an orthogonal system for labeling proteins in living cells. The HaloTag protein is a 33 kDa enzyme derivatized from a prokaryotic hydrolase that reacts with a chloroalkane.

All three of these protein fusion tags form covalent bonds with specific chemical substrates upon their intrinsic enzymatic activity. By conjugating HTRF fluorophore to SNAP-tag®, CLIP-tag® or HaloTag® substrates, fusion proteins can be covalently linked to the fluorophore post expression in living cells.

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TAG-LITE

Tag-lite fluorophores and FRET

Tag-lite assays are comprised of a cell surface protein labeled in situ with an HTRF fluorophore and its binding partner of interest labeled with the corresponding HTRF fluorophore. Tag-lite technology has a high degree of assay flexibility allowing either binding partner (surface protein and soluble binding partner) to be labeled with the donor or acceptor fluorophore. Additionally, indirect assay formats in which the binding partner is detected via a labeled secondary antibody can be performed.

Once the protein of interest is expressed as a fusion protein (SNAP-tag, CLIP-tag or HaloTag), an HTRF fluorophore is covalently linked to the tag via incubation with a tag-specific substrate. Tag-lite substrates are composed of an HTRF fluorophore (Lumi4-Terbium cryptate donor, green or red acceptors) in combination with the tag-specific linker, as shown for the benzylguanine linker on Figure 23. These substrates are not cell membrane permeable. Therefore, only cell surface proteins such as GPCRs can be investigated using this technology. At the completion of the irreversible labeling reaction, 100% of the tags end up being labeled and no longer enzymatically active, and remains labeled with the HTRF fluorophore. Tag-lite substrates are chemically inert towards other proteins, and thus non-specific labeling of other cell surface proteins is not observed.

Like all HTRF assays, Tag-lite assays are homogeneous in nature and measured via dual wavelength donor and acceptor emission readouts (Figure 24). If the HTRF-labeled cell surface protein and binding partner interact, FRET occurs and HTRF emissions are produced at both the donor and acceptor wavelengths. In the absence of binding, only HTRF donor emissions are produced.

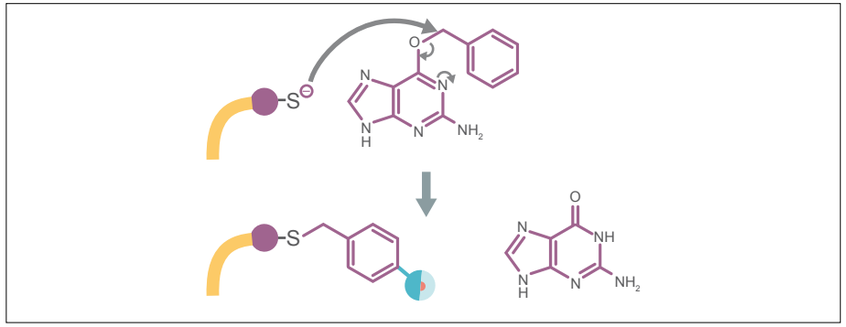

Figure 23 SNAP-tag labeling reaction. The enzyme targets the benzylguanine substrate at the level of the benzyl group bearing the fluorophore and transfers it to itself. This results in a labeled tag that no longer has enzymatic activity.

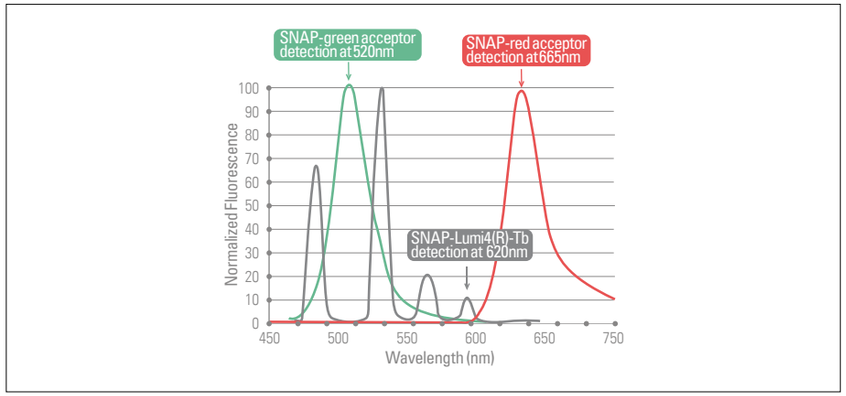

Figure 24 The use of Lumi4-Terbium cryptate enables the combination with both green and red acceptors that produce their specific fluorescence in spectrum gaps where the donor does not emit.

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TAG-LITE
Tag-lite technology workflow

1. Construction of plasmid encoding the cell surface protein of interest fused to a SNAP-tag, CLIP-tag or HaloTag (Figure 25). Revvity offers SNAP-tag, CLIP-tag, and HaloTag expression constructs for cloning your cell surface protein of interest. Additionally, Revvity has an extensive collection of pre-cloned expression constructs for tagged GPCRs and RTKs, or can develop custom ones on a fee-for-service basis.
2. Transfection of cells and expression of the tagged cell surface protein. Tag-lite is compatible with transiently or stably transfected cells. Revvity offers a variety of stable cell lines and ready-to-use frozen cells which express specific tagged-GPCRs.
3. Labeling of tagged cell surface protein using tag-specific substrates conjugated to an HTRF fluorophore. Revvity offers SNAP-tag, CLIP-tag and HaloTag specific substrates labeled with Lumi4-Terbium, green or red acceptor fluorophores.
4. Labeling of the binding partner of interest with the corresponding HTRF fluorophore. Tag-lite has been used for peptidic, non-peptidic and complex ligand binding as well as antibody binding. Revvity offers a range of HTRF-labeled ligands for immediate use, and provides custom labeling of ligands as part of its service offering.
5. Conduct biomolecular binding assays (incubation of labeled cells or cell membrane preparations with binding partners, antibodies, compounds, etc.) followed by HTRF measurement.

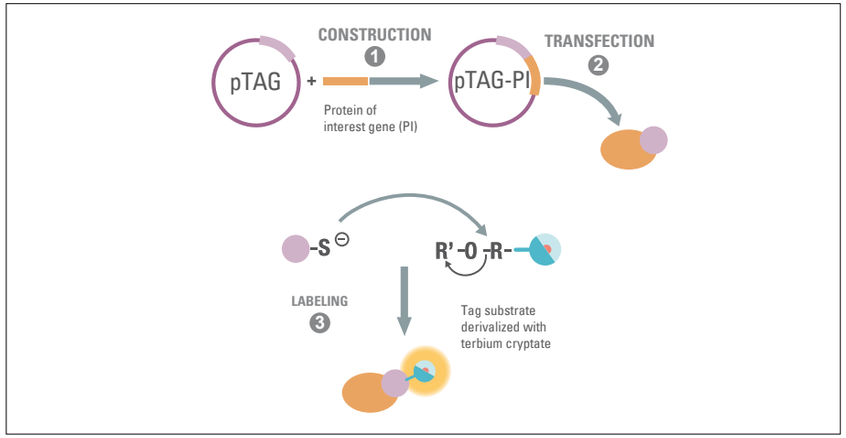

Figure 25 Generation of a Tag-lite fusion protein, from fusion protein plasmid construction to labeling with the HTRF dye.

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TAG-LITE
Tag-lite applications

Tag-lite is an innovative, highly flexible technology that provides an ideal platform for investigating cell surface protein binding interactions. Studies have confirmed that SNAP-tag, CLIP-tag and HaloTag expression and labeling on the N-terminus of receptors do not affect ligand binding or receptor signaling as determined via functional assays (Figure 26), thereby validating the physiological relevance of this platform. Tag-lite technology has been applied in a variety of fields, but has proven to be a particularly powerful tool in the fields of GPCR and receptor tyrosine kinase (RTK) research as well as biotherapeutic antibody characterization and screening.

GPCR ligand binding assays

Tag-lite ligand binding assays are homogeneous cellular assays based on: i) the expression of a tagged GPCR (which is conjugated via the tag to an HTRF fluorophore, typically Lumi4-Terbium cryptate); and ii) labeling of the cognate ligand with the corresponding HTRF fluorophore. The HTRF assay signal is proportional to the level of ligand binding. The competitive nature of these assays allows researchers to identify additional receptor agonist and antagonist. Data produced from Tag-lite ligand binding assays align with previously reported data in the literature and demonstrate the accuracy of compound pharmacology determined using this technology.

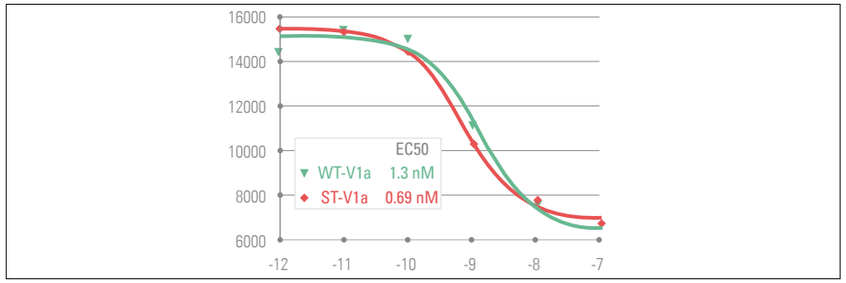

Figure 26 Tag-lite receptor tagging does not affect receptor function as shown here by the comparative assessment of IP1 in either wild type or SNAP-tagged V1a receptor expressing cells.

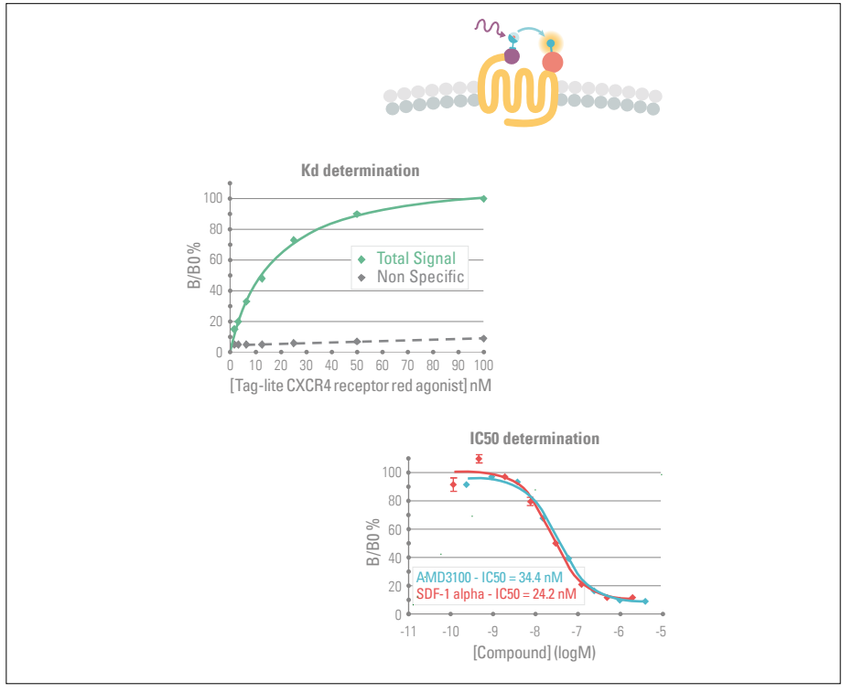

Figure 27 Tag-lite CXCR4 assay binding principle and results. The time-resolved mode enables the background signal from free or non-specifically bound ligand (SDF1α labeled with red acceptor) to be maintained at a very low level – a significant advantage when working with labeled chemokines which have a high propensity to bind in a non-specific way, often decreasing the assay window. Both the unlabeled SDF1α agonist and the AMD3100 antagonist show the expected binding inhibition.

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TAG-LITE

Tag-lite applications

Biotherapeutics

The identification of potent and selective monoclonal antibodies is a primary goal of biotherapeutic development activities. Tag-lite tools represent a unique, cell-based format for antibody characterization and screening, harnessing the robustness and flexibility of HTRF technology. Tag-lite assays can be developed with the goal of identifying antibodies that directly disrupt ligand binding or those with an increased affinity compared to reference antibodies (Figure 28). Tag-lite flexibility allows you to develop a variety of assay formats ranging from direct antibody binding assays to indirect, sandwich assay formats.

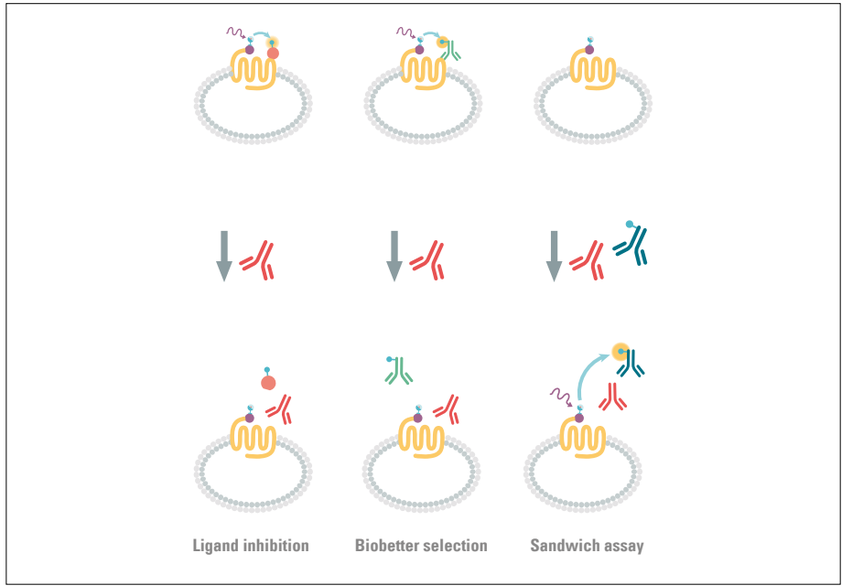

Figure 28 Three possible Tag-lite assay formats to identify antibody binding on receptors: inhibitors, biobetters, or all binders

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TAG-LITE

Tag-lite products

Tag-lite assays are available as do-it-yourself components (plasmids and substrates) or as ready-to-use solutions (pre-cloned receptors, stable cell lines and receptor/ligand binding assays).

- Tag-lite plasmids encoding the SNAP-tag, CLIP-tag, or HaloTag bordered by restriction sites for cloning a gene of interest.
- Tag-lite substrates for SNAP-tag, CLIP-tag and HaloTag labeled with HTRF fluorophores enabling labeling of the expressed fusion protein.
- Line of pre-cloned GPCR and RTK Tag-lite plasmids.
- SNAP-GPCR stable cell lines. All stable cell lines have been validated with HTRF kits and reagents for ligand binding assay and at least 2 functional assays including cAMP, IP-One, and phospho-Erk assays.
- Tag-lite ligand binding assays for a range of GPCRs which contain cells expressing the labeled GPCR of interest in conjunction with the associated labeled ligand.

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TAG-LITE
FAQs

Do I have to express my cloned receptor in a specific cell line?

Several standard cell backgrounds have been successfully used, but if possible, HEK293 cell lines are preferred.

Do I have to create a stable cell line for my cloned receptor of interest?

No. Tag-lite assays have been proven to work with both transiently transfected cells and stable cell lines.

Can I clone my own receptor as a SNAP-tag, CLIP-tag, or HaloTag fusion protein?

Yes, empty plasmids containing either tag and various antibiotic selection systems are available from Revvity. They may be used to transiently express your receptor or to generate your own stable cell line.

How do I label the receptor expressed in my cells?

Receptors can be labeled using the tag-specific Tag-lite substrate. This can be done directly in a microplate or in flask, on transiently transfected cells or a stable cell line batch. The procedure is straightforward and takes less than 3 hours to generate a full batch of labeled cells ready to be frozen or used in a Tag-lite assay.

How do I measure Tag-lite assays? Is it the same as other HTRF assays?

Tag-lite assays can be measured on all HTRF compatible readers. However, some assays work with green ligands (in combination with Lumi4-Terbium cryptate) and user should make sure that the reader has the capability to read Tb/green chemistry. Also, some monochromator-based readers may have insufficient sensitivity to detect binding assays properly. If you have such a reader, you may check this point with our technical support team.

Are the cells alive when I am performing the Tag-lite assay?

Tag-lite binding assays work with both live cells and cell membranes. It is not mandatory to have 100% cell viability to perform the assay.

Can I use Tag-lite with cytoplasmic proteins?

Not in live cells since the substrates are not cell permeable. However, fusion proteins may be labeled if the cells are lysed.

Do I need to count pre-labeled cells I purchase from Revvity before running a binding assay?

No, off-the-shelf Tag-lite pre-labeled cells must be reconstituted as advised in the instructions for use. The pre-labeled cell batch is used like a regular HTRF reagent. There is no need to re-optimize the cell density, and therefore to re-count the cells.

Can pre-labeled leftover cells be re-frozen?

No. However, cells thawed and re-suspended in Tag-lite buffer can be kept at room temperature for a day, allowing several experiments to be run.

Can I use any commercially available ligand coupled to a dye?

No. Revvity ligands have been carefully optimized to match Lumi4-Terbium cryptate labeled cells and binding conditions. Other ligands are likely to lead to different pharmacological output.

From ligand binding to downstream signaling - investigate receptor biology from different angles

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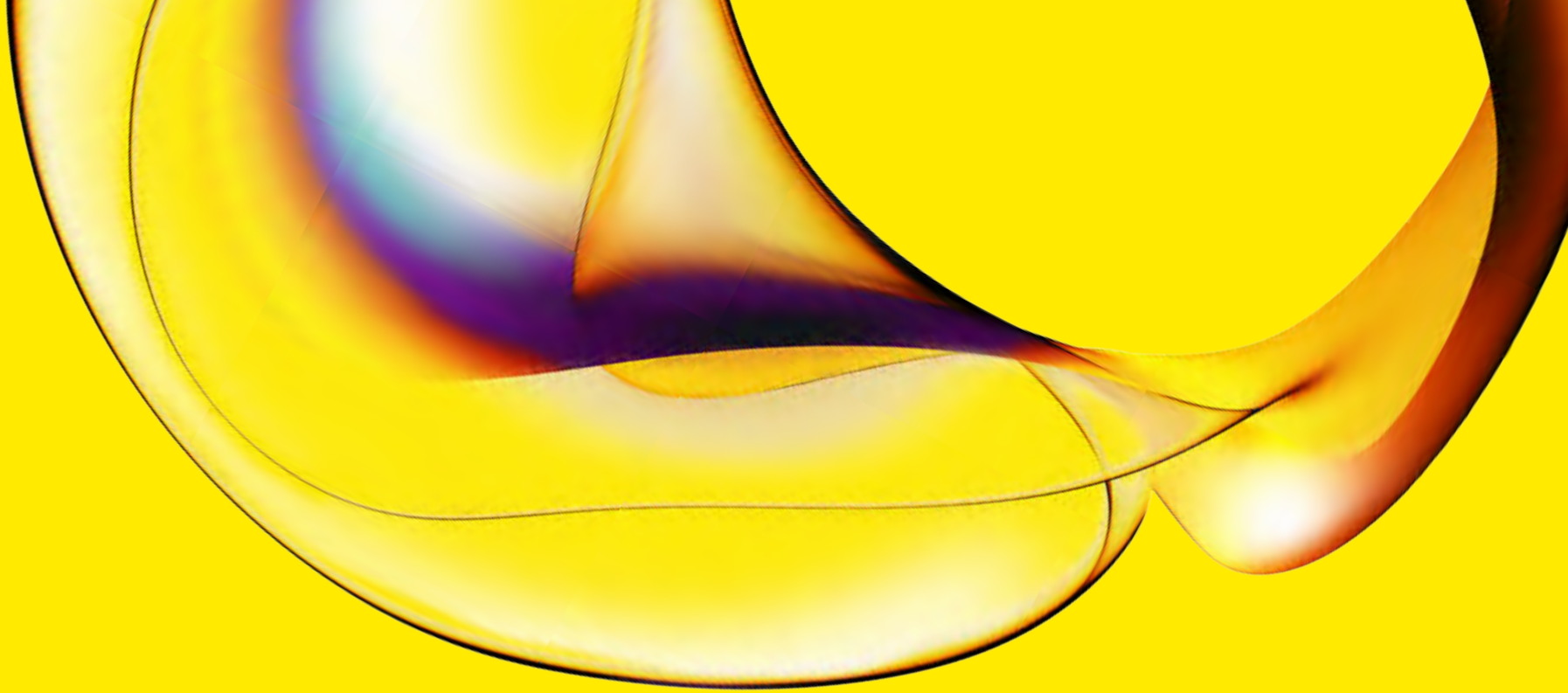

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