

Synergy™ Microplate Readers and HTRF® Detection

Introduction

In this Application Guide, we will discuss the principles of HTRF® detection and proximity assays in general based on time-resolved fluorescence resonance energy transfer. We will explain how the Synergy™ product line of Multi-Mode Microplate Readers has been designed specifically for high performance HTRF assays and finally describe some specific applications pertinent to modern drug discovery for both small molecule and biomolecular putative therapeutics.

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Principles of FRET

Förster (Fluorescence) resonance energy transfer (FRET) describes the energy transfer between two fluorophores: the donor absorbs energy (usually a photon) and transfers its potential energy to an acceptor which then fluoresces. The efficiency of this energy transfer is inversely proportional to the sixth power of the distance between donor and acceptor making FRET extremely sensitive to small distances. This attribute is useful for studying molecular interactions between biomolecules. If respective binding partners are conjugated with donor and acceptor fluorescent labels, FRET will only occur to any measurable extent when the binding occurs (see Figure 1). This is the basic principle of proximity assays which allow for homogeneous assay work flows characterized by sequential additions of reagents. There are no wash steps such as those typical with ELISA technology.

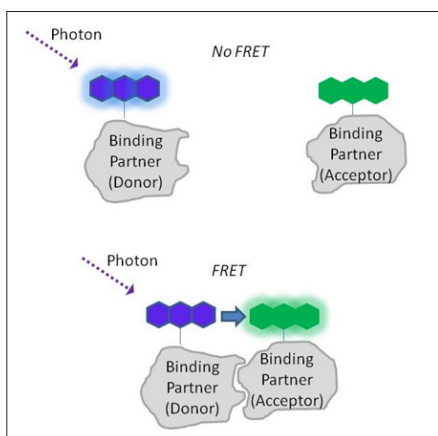


Figure 1. If there is no binding interaction between Binding Partners, the Donor fluorophore will absorb the photon and emit fluorescence (i.e. blue). If there is interaction between Binding Partners, the Donor fluorophore will transfer its energy from photon absorption to the Acceptor fluorophore resulting in Acceptor fluorescence (i.e. green). Physicochemical measurements for the binding interaction and its inhibition can be evaluated by measuring the relative extent of blue and green fluorescence.

Principles of TR-FRET

Time-resolved FRET (TR-FRET) is a variant of FRET where the main difference is that a specific Donor Fluorophore is used. In its simplest form, the Donor fluorophore consists of two parts: a chelating agent that possesses UV-absorbing chromophores and a lanthanide element, such as Europium (Eu) or Terbium (Tb). In the presence of the lanthanide, the chelating agent will bind it with extremely high affinity, essentially forming a new molecule. Donor fluorescence is generated when the chelate's chromophores absorb UV light (typically between 330 – 350 nm, depending on molecular structure). This energy is then imparted into the atomic orbitals of the lanthanide from which fluorescence can occur. There are three unique features to lanthanide-chelate fluorescence relative to that from typical organic small molecule fluorophores, such as fluorescein:

1. The fluorescence lifetime is about 10^6 times longer due to the intersystem crossing of energy between the molecular orbitals of the chelate's chromophores and the atomic orbitals of the lanthanide element.
2. The Stokes shift (wavelength difference between absorption and emission maxima) is about 10 times larger.
3. As there is no molecular structure in the lanthanide element, the fluorescence is essentially concentrated over a narrow wavelength range.

These attributes of the lanthanide chelate Donor fluorophore provide a means to develop proximity assays with far greater analytical performance than standard FRET assays.

The dramatic increase in fluorescence lifetime of the lanthanide chelates to the msec time frame makes it easy to use electronic time-gating in instruments to delay integrating fluorescence signal until all fluorescent background signals have decayed. This is best accomplished by using flash lamps or pulsed lasers to deliver a finite pulse of excitation energy. The large Stokes shift and concentrated fluorescence also serves to minimize background signals as spectral filters can be easily chosen to ensure there is no crosstalk between excitation and emission filters. Combined, these attributes provide much higher signal to background ratios relative to FRET.

HTRF®

HTRF® (Homogeneous Time-Resolved Fluorescence) is a registered trade mark of Cisbio Bioassays. It is used to describe their proprietary TR-FRET technology which is used in multiple applications in modern drug discovery and life science research. HTRF's central element, the Donor fluorophore, consists of a rare earth complex in which the lanthanide ion (Europium or Terbium) is tightly embedded in a macrocycle. This very unique type of structure gives HTRF donors their long-lived fluorescence properties, as well a robustness that enables these molecules to be used in most assay conditions.

Two donors are currently included in HTRF products. Europium cryptates (Eu(III) cryptate), the product of Prof. J.M. Lehn's work, for which he was awarded a Nobel Prize for Chemistry in 1987, are a series of rare earth complexes whose macrocycle is based on an embedded trisbipyridine motif (Figure 2). The other, Lumi4®-Tb cryptate was developed by Prof. K. Raymond's group at Berkeley, and consists of a tight association of a terbium ion with a cage of similar structure and properties.

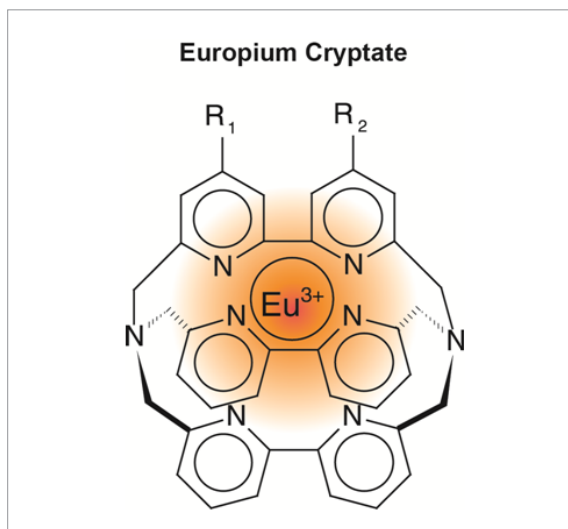


Figure 2. Europium (III) cryptate structure typically consists of a tris bipyridine macrocycle in which the lanthanide ion is tightly embedded.

HTRF acceptors have been optimized for Eu(III) and Tb(III) cryptate donors, and in particular to match their emission properties. Eu(III) cryptates are mainly compatible with near infrared acceptors showing a peak emission at 665 nm, whereas Lumi4®-Tb can be paired to the same red acceptors as well as green ones like fluorescein or GFP, emitting around 520 nm (Figure 3).

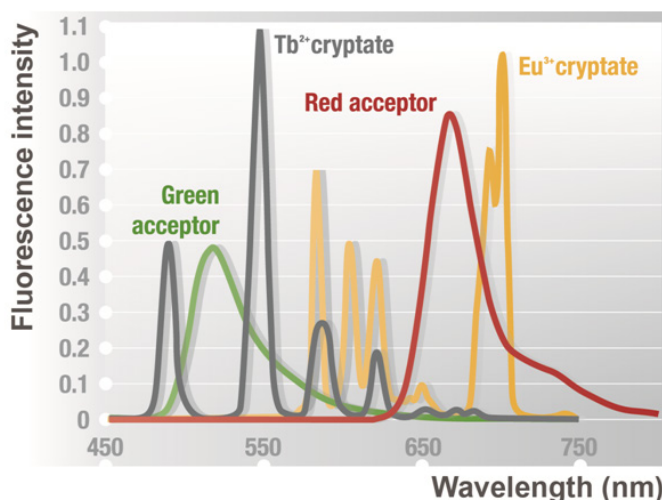


Figure 3. HTRF donor and acceptor emission spectra. Red acceptor emissions occur in a region where the donor does not emit significantly. Long-lived fluorescence detected at this specific wavelength is therefore characteristic of the emission of the acceptor engaged in the FRET process. The same is true for green acceptors emitting around 520 nm when combined with a Lumi4®-Tb donor.

The first acceptor developed for HTRF was XL665, a phycobilliprotein pigment purified from red algae. The second generation of acceptors (i.e. d2) is characterized by organic structures 100 times smaller, displaying a series of photophysical properties very close to those of XL665. These acceptors fulfill the previously-mentioned compatibility criteria. Their excitation spectra overlap those of Eu³⁺ and Lumi4®-Tb cryptate emissions, thereby allowing the donor to excite the acceptor, whose maximum emission at 665 nm spans a region where HTRF cryptates do not emit or do so only weakly.

Synergy™ Multi-Mode Microplate Readers and HTRF

The HTRF product lines provide diverse offerings for modern drug discovery that includes biochemical and cell-based assays suitable for both small molecule and biotherapeutic drug discovery and development. BioTek's Synergy™ product line of Multi-Mode Microplate Readers is ideally suited to the HTRF technology providing high performance HTRF detection. For applications requiring high sample throughput such as high throughput screening of small molecules, the Synergy NEO is the optimal choice. For applications requiring less performance, such as screeners applying much smaller small molecule libraries or biotherapeutics drug discovery, a more cost-effective choice would be the Synergy H1. Each microplate reader uses filter cubes and minimal optical components (no fiber optics or reflecting mirrors) to ensure high light transmission and high sensitivity.

Synergy™ NEO

Synergy NEO has been designed specifically for high performance detection from microplates. For HTRF detection, the unit uses a filter block design that allows for simultaneous measurement of Donor and Acceptor Fluorescence using dual PMTs, one which is a red-shifted PMT with high sensitivity for the far red spectral emissions of XL665 and d2. A schematic of the two filter cubes used for HTRF detection is shown in Figure 4.

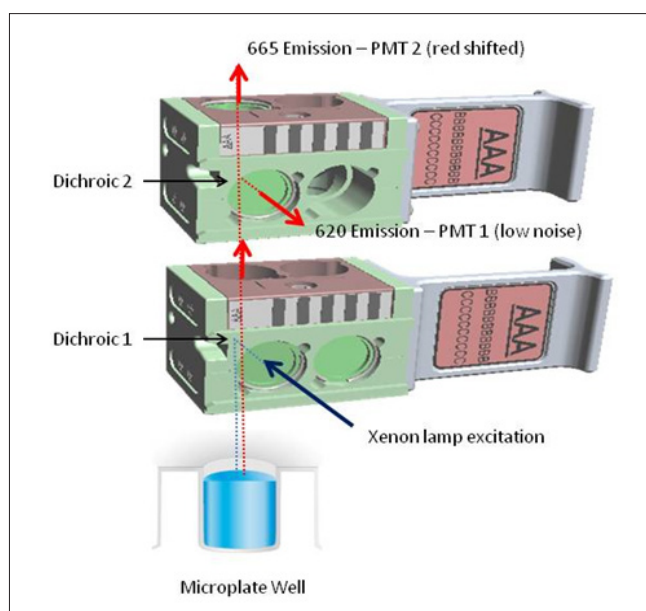


Figure 4. Schematic of the dual filter cubes in Synergy NEO used for the simultaneous detection of Donor and Acceptor fluorescence using the far-red Acceptor fluorophores XL665 and d2.

Synergy™ H1

Synergy H1 has been designed to provide high sensitivity HTRF assays, but does use a single filter block and PMT which provides sequential detection of Donor and Acceptor fluorescence. Similar to the Synergy NEO design, the optical path is devoid of components that limit light transmission, such as fiber optic cables and reflective mirrors which enables the high sensitivity.

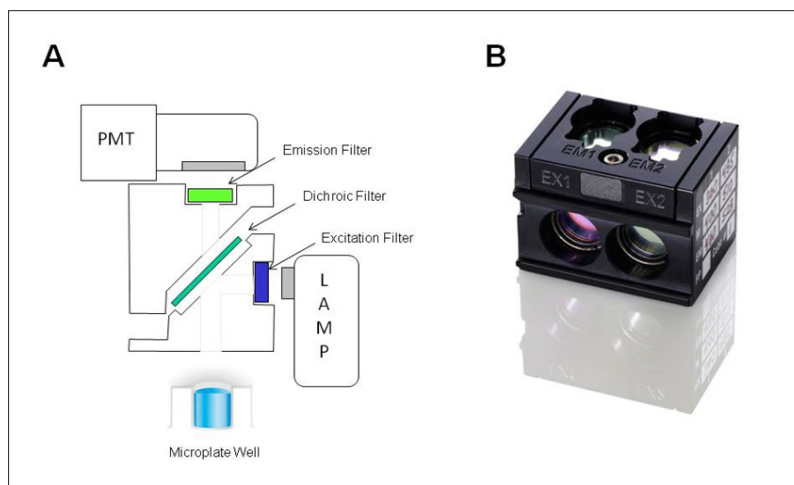


Figure 5. A. Exploded schematic of the Synergy™ H1 filter cube used for sequential detection of Donor and Acceptor fluorescence; B. Image of a Synergy H1 filter cube.

Overview of Applications

The following is a series of HTRF applications that have been detected with Synergy Readers. We will demonstrate applications in small molecule screening using the Tag-lite® technology for GPCRs; cellular kinase functional assays that can be used for both small molecule and biotherapeutics discovery and CD16 assays suitable for developing Antibody – Dependent Cell-mediated Cytotoxicity assays for biotherapeutics and biosimilars development.

Cell-based GPCR Ligand Binding Assays

Tag-lite® is a comprehensive selection of tools and reagents streamlined to specifically label fusion proteins with synthetic HTRF dyes. Tag-lite® combines HTRF with SNAP-tag® and CLIP-tag® technologies. This solution is ideal for a wide range of applications, such as GPCR ligand binding assays and cell surface receptor mechanistic studies. SNAP-tag® and CLIP-tag® are small fusion tags that covalently interact with dyes conjugated to guanine or chloropyrimidine leaving groups via a benzyl linker. SNAP-tag® and CLIP-tag® can easily be fused to either the N- or the C-terminal position on proteins of interest, and can then be specifically and covalently labeled with their defined substrates (Figure 6).

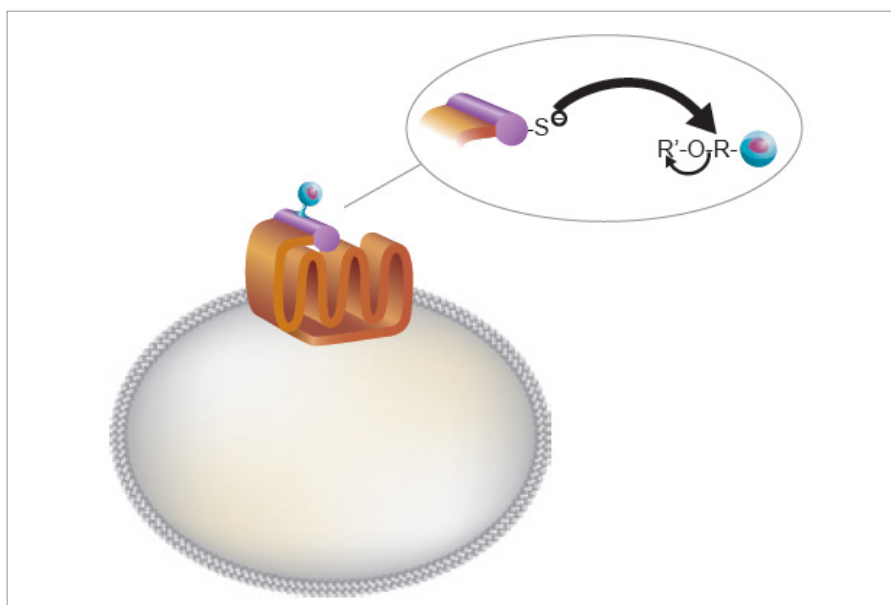


Figure 6. Tag-lite® system comprised of HTRF Donor fluorophore labeled GPCR. GPCR with SNAP-tag® was cloned into the cell line which can then be specifically labeled with Donor fluorophore.

The Tag-lite® toolbox offered by Cisbio provides a choice of reagents that include plasmids for “do-it-yourself” transfection, fluorescence substrates for labeling the cloned “tag”, Acceptor fluorophore – labeled ligands to GPCRs and developed cell lines expressing GPCRs with their tags ready to be labeled with Lumi4®-Tb.

GPCR Ligand Binding Studies

Receptor ligand binding studies are classically performed using radioisotope-labeled ligands that bind to the receptor. By removing unbound labeled ligand, typically through a wash step, the remaining bound ligand can be quantified based on the beta particle emissions from the ^{125}I - or ^3H -label used. This allows for the determination of the equilibrium dissociation constant (K_d) of the labeled ligand and also further allows for the assessment of competitive ligands or compounds to this specific binding event.

Tag-lite provides a compelling alternative since it can provide the same data, but using simpler, homogeneous work flows with non-radiometric reagents that have no safety concerns or disposal costs. We have demonstrated the ability of Tag-lite to provide GPCR ligand binding data using the chemokine CXCR4 on Synergy H1[1]. Figure 7 demonstrates the determination of the K_d for the d2-labelled CXCR4 agonist SDF1- α binding to Lumi4®-Tb-labelled CXCR4.

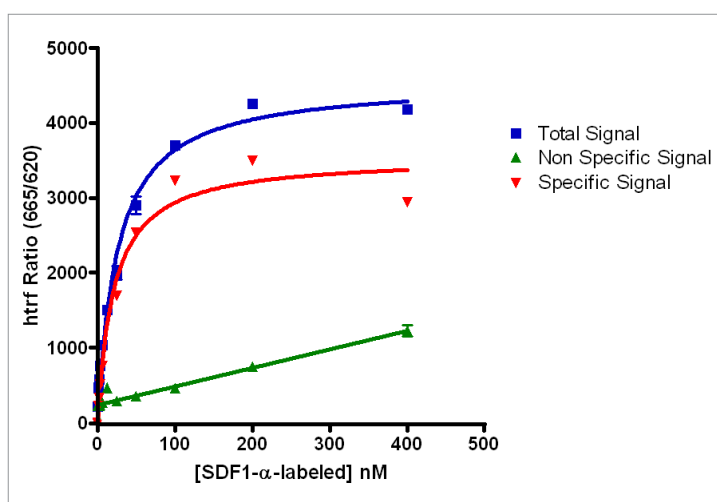


Figure 7. K_d determination for d2-SDF1- α using Synergy H1. K_d was determined to be 19.6 nM, compared to Cisbio literature of 18.7 nM.

Inhibition studies are also easily accomplished with the Tag-lite® technology. Figure 8 illustrates the potency of unlabelled SDF1- α and the small molecule AMD3100 to compete off the labeled ligand.

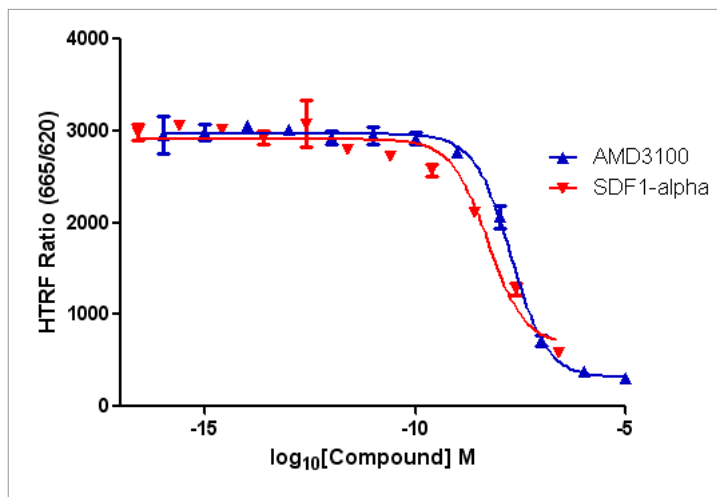


Figure 8. Competition studies using Synergy H1. IC_{50} 's were determined to be 29.4 and 29.0 nM, respectively for AMD3100 and SDF1- α .

Cell-based GPCR Small Molecule HTS

GPCRs continue to be molecular targets of interest for small molecule drug disease intervention. This gene family has proved to be one of the most “druggable” with about 50% of marketed small molecule drugs directed against them. HTS is still the method of choice for the starting process of lead generation in drug discovery. Tag-lite is well suited to HTS with its homogeneous cell-based assay format. We have performed a small screen with a 500 natural product compound library using MultiFlo™ for all reagent dispensing and Synergy NEO to read the 96-well microplates in HTS mode [2]. Figure 9 illustrates a histogram of the results from the screen.

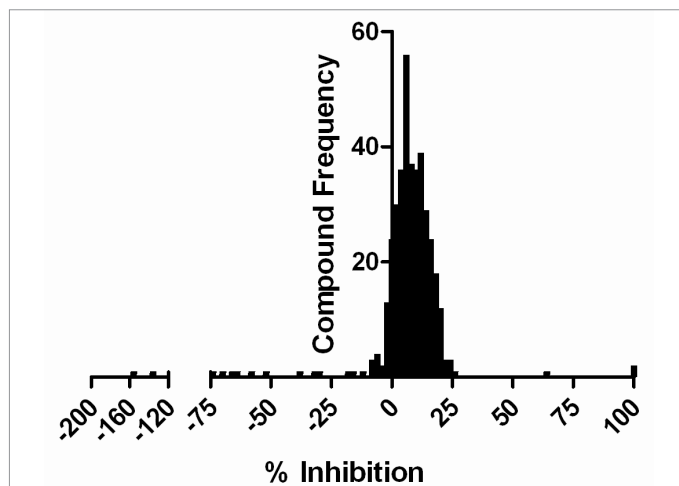


Figure 9. Natural Product Compound Library screen demonstrating percent inhibition distribution.

When plotting the compound percent inhibition data, a distribution profile similar to that seen in larger compound library primary screens was observed. Most compounds exhibited little to no inhibition, while a small percentage demonstrated high positive or negative inhibition. The Donor molecule fluorescent signal assessment can be used to ensure that compounds exhibited true positive or negative red-labeled agonist binding inhibition. This control, inherent in HTRF technologies, provided a rapid method to remove false hits. An example of a false result is Chelerythrine. This compound exhibited high negative inhibition, but also decreased the donor molecule signal by approximately 50%. In contrast, Exendin-3, Exendin-4 and GLP-1 all showed high positive inhibition with no negative effect on the donor molecule's fluorescent signal.

Synergy NEO can also be used for secondary screening purposes, where full dose response curves can be generated to determine potency of hits from the screen. This is evident in Figure 10 for the positive controls used in the screen, Exendin-3, Exendin-4 and GLP-1.

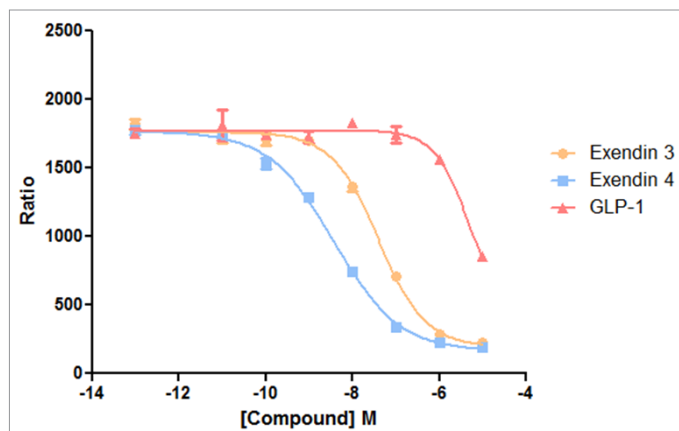


Figure 10. Dose response curves generated for positive inhibitor compounds.

Cell-based Protein Kinase Assays

HTRF® cellular kinase assays measure site-specific phosphorylation in signal transduction pathways. Upon activation of the pathway of interest and phosphorylation of the endogenous kinase substrate of interest, cell membranes are lysed releasing the phosphorylated substrate to the contents of the microplate well where it can be detected using the kit reagents. All assays are based on the same sandwich immunoassay construction, comprising two specific monoclonal antibodies coupled to HTRF Donor and Acceptor fluorophores (Figure 11).

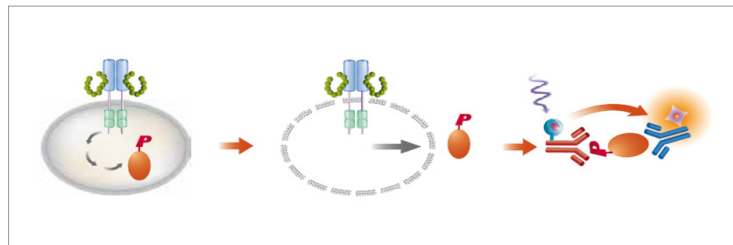


Figure 11. HTRF Cellular Kinase assay principle showing the three steps of pathway activation leading to kinase substrate phosphorylation; followed by cell membrane lysis to release of the phosphorylated substrate into the assay milieu; followed by detection after addition of sandwich immunoassay reagents.

Due to their high sensitivity, the assays are suitable for either over-expressed or endogenous kinases and their substrates, and can be used in many types of cells including primary cells. Being truly homogenous, they represent a robust, reliable and rapid alternative to more conventional technologies such as Western Blot or ELISA. These assays can be used for drug screening for both small molecule and biologic investigation.

AKT(Ser473) Phosphorylation in Human Primary Cells

Assays involving cell models are supplanting biochemical assays primarily due to a perceived increased biological relevance in the results. Primary cells represent perhaps the highest level of biological relevance that can be obtained in an assay as it most closely resembles an *in vivo* context. These assays are challenging, however primarily due to cell culture constraints, especially in volumes sufficient for primary screening campaigns. Thus sample throughput requirements are modest and Synergy H1 may be a suitable choice for microplate reader.

In previous work, we established a robust HTRF p-AKT(Ser473) assay (z' score of 0.73) using human umbilical vein endothelial cells (HUVECs) [3]. We used VEGF as the pathway stimulant through VEGFR2 endogenous expression in the HUVECs and also performed inhibition studies at the level of PI-3 kinase in the pathway. Figure 12 demonstrates PI-3 kinase inhibition with the small molecule inhibitors PI-103 and LY294002.

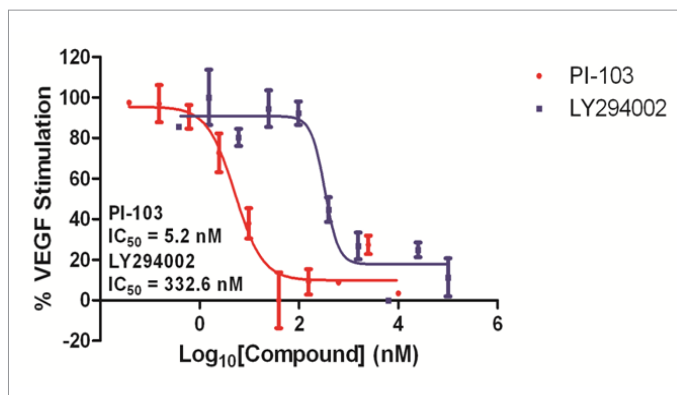


Figure 12. Inhibition of VEGF-induced AKT(Ser473) phosphorylation by small molecule PI-3 kinase inhibitors. Molecular pharmacology is consistent with other assays. Detection was performed on Synergy H4, which has a similar performance to Synergy H1, especially with regards to sample throughput.

STAT3(Tyr705) Phosphorylation in A431 Cell Line

Cell-based HTS campaigns typically rely on the use of immortalized cell lines such that sufficient quantities of cells may be cultured with consistent expression levels of the molecular target of interest. Primary screening requires the use of sensitive microplate readers with high sample throughput, such as the Synergy NEO.

We have demonstrated such an application using Synergy NEO, the HTRF® Cellular Kinase STAT3(Tyr705) kit and a human epithelial carcinoma cell line, A431 [4]. Assay performance was robust as demonstrated by a S:B > 4 in the EGF dose response and a z' score of 0.79 (Figure 13). Inhibitory studies demonstrated consistent molecular pharmacology compared to other studies (Figure 14).

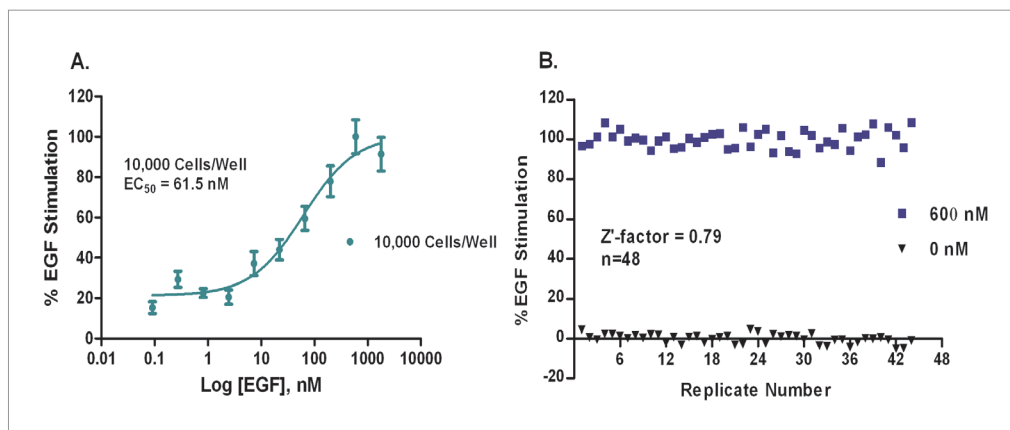


Figure 13. A. EGF stimulation of STAT3(Tyr705) phosphorylation demonstrated as a full dose response; B. z' score from 40 replicate measurements each at [EGF] of 600 nM and vehicle.

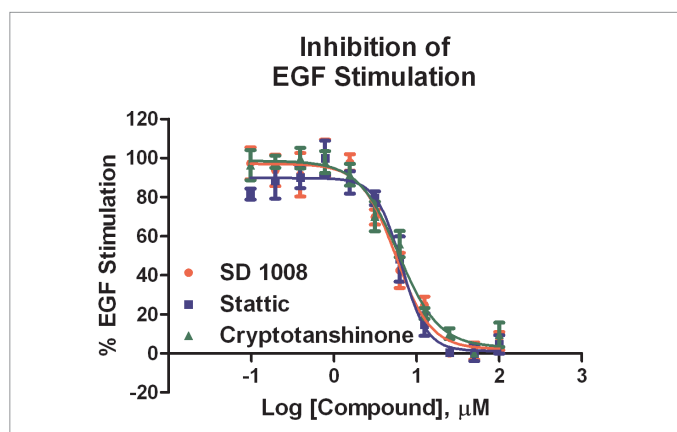


Figure 14. Inhibition of EGF-induced STAT3(Tyr705) phosphorylation using the small molecule inhibitors, SD 1008, stattic and cryptotanshinone.

Biotherapeutics Drug Discovery

Since the end of the 1990's, the pharmaceutical industry has increased their interest in biologics, especially for oncology and inflammation indications. The selection of potent and selective monoclonal antibodies for specific target receptors, such as Receptor Tyrosine Kinase (RTK) and G protein-coupled receptors (GPCRs), is the first step in successfully developing an antibody-based drug. In addition to potent binding, some antibodies have the ability to recruit immune system effector cells, a process known as Antibody-Dependent Cell-Mediated Cytotoxicity (ADCC). The ability to promote ADCC is an important attribute of successful candidates, and should be sought in the final drug design.

We recently developed two HTRF assays for the characterization and selection of potent antibody drug candidates [5]. The first assay quantifies the binding affinity of antibodies to their target antigen, on live cells. The second assay measures the affinity of the antibody Fc portion to the CD16 receptor also on live cells. As Fc-CD16 binding affinity correlates well with effector function, the combined method is a simple, yet efficient and precise way of assessing biological activity through ADCC. These assays assess and characterize immunotherapeutics and as such, do not require the level of sample throughput of HTS assays for small molecule drug discovery. Synergy™ H1 is a good choice for these types of assays.

RTK Binding Assays

An indirect cell-based assay for measuring cetuximab binding to the Receptor Tyrosine Kinase EGFR was constructed by cloning with a SNAP-tag®. This was then labeled with Lumi4®-Tb to create the RTK labeled with the Donor fluorophore. Upon cetuximab binding, an anti-hFc antibody labeled with d2 was added to generate the HTRF signal (Figure 15).

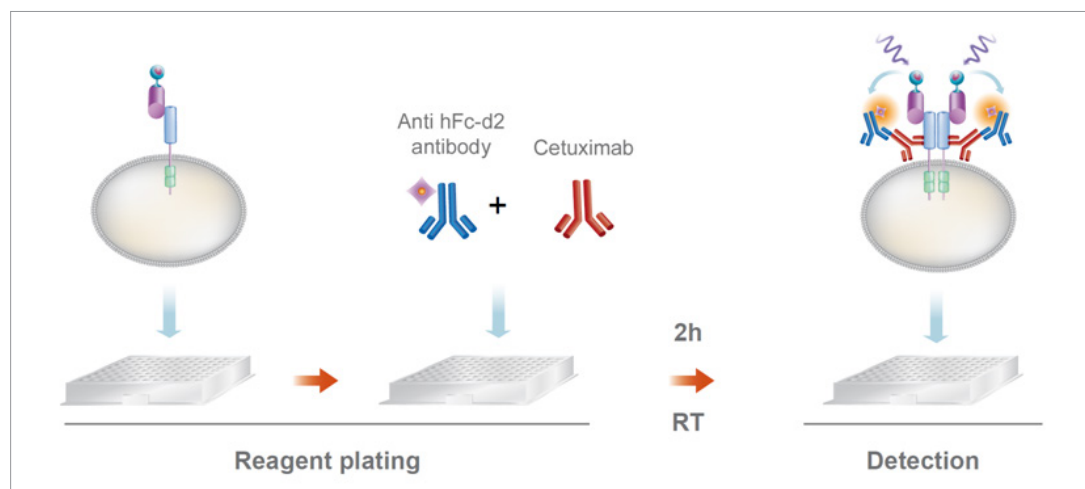


Figure 15. EGFR, a Receptor Tyrosine Kinase, is labeled with Lumi4®-Tb. Binding of antibodies to the target antigen is measured through the use of an anti-species specific Fcd2 labeled antibody. The target antibody binds to the Lumi4®-Tb labeled receptor antigen. Upon addition, the secondary labeled antibody binds to the primary, unlabelled antibody, causing an increase in HTRF signal.

Characterization of cetuximab binding can be performed with this assay, as evident in Figure 16, where a K_d of 70 pM was determined.

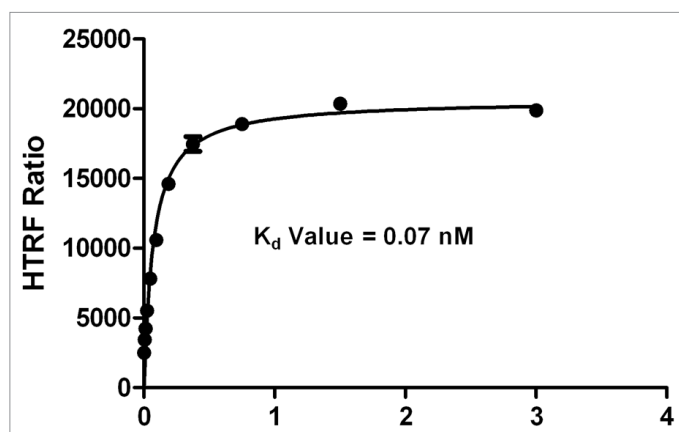


Figure 16. Cetuximab dose-response curve to compute K_d for cetuximab. Detection was performed on a Synergy H4, which has a similar performance to Synergy H1, especially with regards to sample throughput.

CD16a Cellular Binding Assay

In this assay, cells were cloned with the FcγRIIIA and gamma chain labeled with Lumi4®-Tb through its SNAP-tag®. Adding a human IgG labeled with the d2 Acceptor fluorophore generates a specific HTRF signal. Unlabeled IgG test antibodies can then be assessed for their ability to bind to the FcγRIIIA and gamma chain, which subsequently displaces the labeled IgG antibody, causing a decrease in HTRF signal (Figure 17).

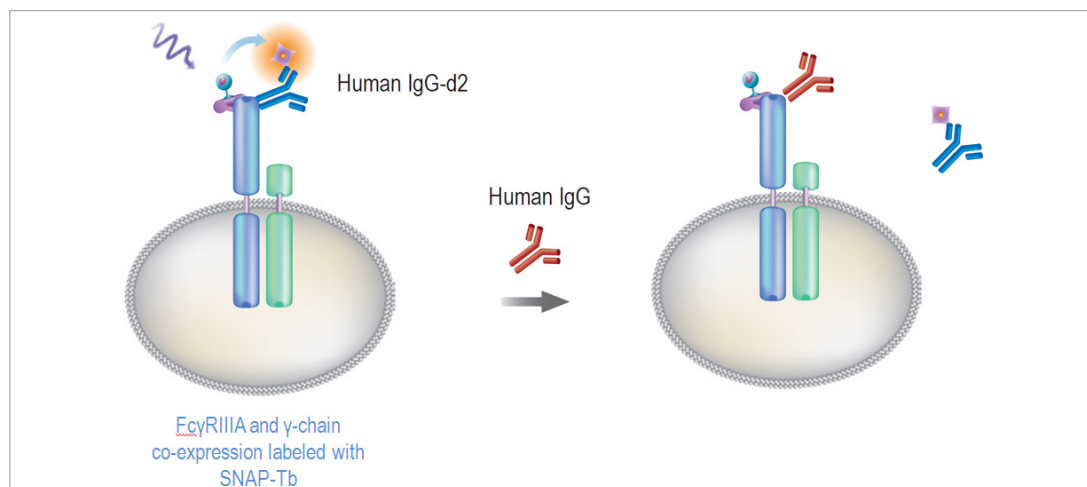


Figure 17. Competitive cell-based assay to assess immunotherapeutics for FcγRIIIA binding and suitability for ADCC assay.

The assay was used to test a human IgG1 isotype with known affinity to bind CD16a and recruit effector cells, cetuximab and a mouse 12G5 antibody, which should not compete with the human IgG labeled with the d2. In Figure 18, we verified hIgG1 binding of FcγRIIIA with high affinity and demonstrated that cetuximab could also recruit effector cells. As expected, the mouse antibody did not compete with the human IgG labeled with the d2.

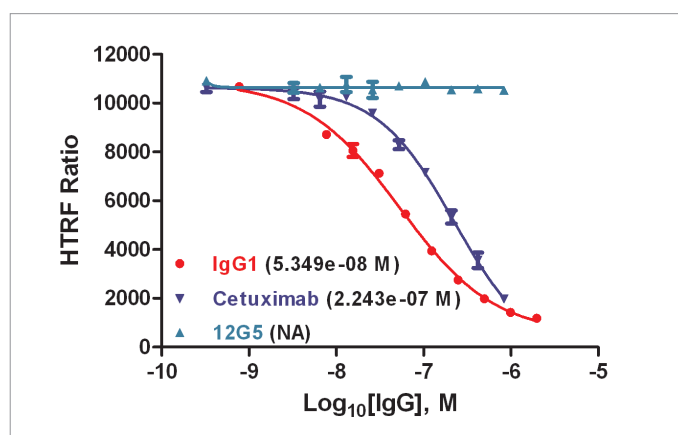


Figure 18. Determination of Therapeutic Antibody Binding Affinity to FcγRIIIA. Detection was performed on a Synergy H4, which has a similar performance to Synergy H1, especially with regards to sample throughput.

Conclusions

HTRF technology is a powerful platform for modern drug discovery, whether small molecules or biomolecular therapeutics are desired. Here we have demonstrated a series of cell-based assays, using immortalized cell lines and primary cells, for assessing the putative therapeutic's effects on drug targets such as GPCRs, protein kinases, receptor tyrosine kinases and an immunotherapeutic's ability to recruit effector cells through binding to CD16a receptors.

BioTek's microplate readers Synergy NEO and Synergy H1 are particularly suited for HTRF detection. For those applications requiring high sample throughput such as primary screening for small molecule libraries, Synergy NEO provides rapid analyses through the simultaneous detection of both Donor and Acceptor fluorescence. For applications that have a more limited throughput requirement such as biotherapeutics development, Synergy H1 may be a more cost-efficient microplate reader choice.

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