TR-FRET BASED ASSAYS – *getting better with age*

By Dr John Comley

Despite having been available for nearly 15 years, TR-FRET based assays are still the preferred fluorescent assay format for many screeners. A recent survey ranked Cisbio's TR-FRET product (HTRF®) as the most frequently used generic assay technology. GPCR second messengers and kinases remain the most popular TR-FRET applications, aided by the launch of several new assay kits addressing these popular applications over the past year. The typical screener today has familiarity with at least eight different assay technologies, and 40% of them have plan to narrow their portfolio of assay technologies. Prospects for the continued use of TR-FRET based assays, however, remain optimistic as: 1) survey respondents appear to be choosing TR-FRET over alternative assay technologies for its sensitivity and ease of use, while the main consideration for alternatives to TR-FRET was price; 2) TR-FRET assays have attained a level of maturity and acceptance in the scientific community; 3) there exists in the Pharma and Biotech a broad and highly experienced base of TR-FRET users; and 3) TR-FRET based assay technology is still actively evolving as evidenced by the increasing innovation of key vendors and suppliers. These developments include enhanced stability and performance of existing FRET pairs; increased use of red-shifted Alexa Fluor© as acceptor dyes; better assay development flexibility by direct covalent labelling of substrates or the use of antibody independent formats; the wider use of terbium complexes, including using GFP as acceptor; and a new approach using Europium chelate dyed nano-particles. Taken as a whole the diversity of TR-FRET based assay offerings looks set to continue increasing over the coming year.

The market place for assay reagent technologies is saturated with numerous alternative approaches for the assay of the common targets like kinases and GPCRs. It is therefore important to recognise what influences end users to select a particular assay reagent technology. In October 2005 HTStec undertook a market study focused on assay reagents with the particular objective of understanding and documenting current perceptions of and interest in TR-FRET (Time Resolved-Fluorescence Resonance Energy Transfer) based assays.

The TR-FRET principle

The FRET principle is based on the transfer of energy between two fluorophores, a donor and an acceptor. When the two entities come close enough to each other, excitation of the donor by a light source triggers an energy transfer towards the acceptor, which in turn emits specific fluorescence at a given wavelength. Molecular interactions between biomolecules can be assessed by coupling each partner with a fluorescent label and detecting the level of energy transfer (**Figure 1**). Time resolved FRET utilises long-lived fluorophores combined with detection on a time-resolved fluorescence basis which allows for the minimisation of background prompt fluorescence interferences (mainly compounds and proteins present in biological fluids or serum that are naturally fluorescent) which are short-lived compared to the long-lived labels used (**Figure 2**) For more information it is recommended that the reader consults the detailed technical descriptions and reviews of the technology which can be found on most TR-FRET vendor's websites, eg www.htrf-assays.com or www.invitrogen.com/LanthaScreen.

Interest in alternative TR-FRET offerings and assays

TR-FRET based assays have been around since the early 1990s and are probably the most well known of the generic fluorescent assay technologies available today. HTStec's survey found TR-FRET was still the preferred assay technology of 40% of survey respondents, which is a high level of acceptance considering the diversity and number of competitive offerings. Furthermore, Cisbio's TR-FRET product (HTRF®) was ranked the most frequently used generic assay technology, closely followed by PerkinElmer's LANCE and Molecular Devices' FLIPR assay kits. The relative use of TR-FRET and related assay technologies are presented in **Figure 3**. Of the commercial TR-FRET based assay reagent offerings available today, Cisbio HTRF and PerkinElmer LANCE have the greatest market share and were ranked as being moderately used (as opposed to being used exclusively) by respondents. In comparison, Upstate KinEASE, GE Healthcare (Amersham) TR-FRET (Eu Chelate +CyDye), Invitrogen LanthaScreen and PerkinElmer TruPoint were less popular (used infrequently) with survey respondents. The relative use of commercial TR-FRET based assay kits is summarised in **Figure 4**. Not surprisingly GPCR second messenger and kinase assays featured most highly, with greatest interest shown in cyclic AMP, followed by the various kinase offerings and then IP-one. In contrast, other available assays kits were less popular. It is of interest that about one in five respondents indicated they did not purchase commercial assay kits, and presumably developed their own TR-FRET assays using standard toolbox reagents.

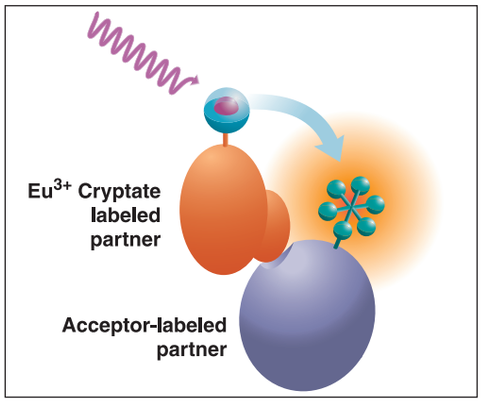


Figure 1
FRET Principle (explained in text)

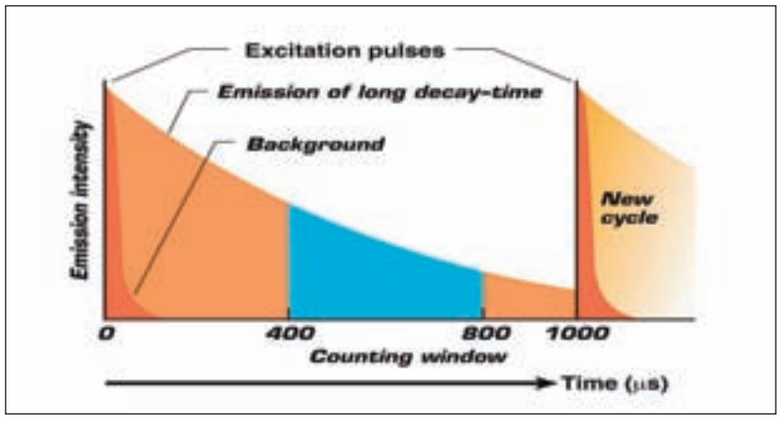


Figure 2: The fluorescence lifetime of most conventional fluorophores can be 100ns or less. Typical TR-FRET fluorophores (eg Lanathanide chelates or cryptates) exhibit a relatively long-lived fluorescence lifetime between 200µs to 1500µs. The advantage of such long lived emissions is the ability to use time-resolved fluorescence to discriminate from background emissions i.e. sample during the blue counting window

Figure 3

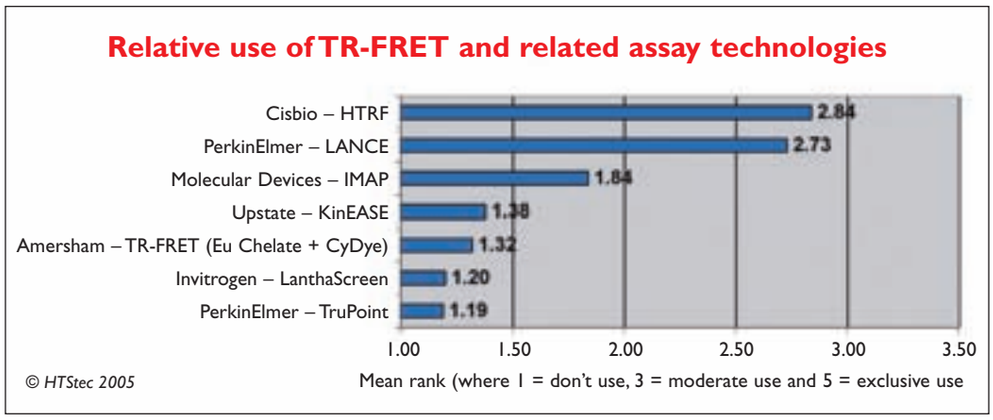


Figure 4

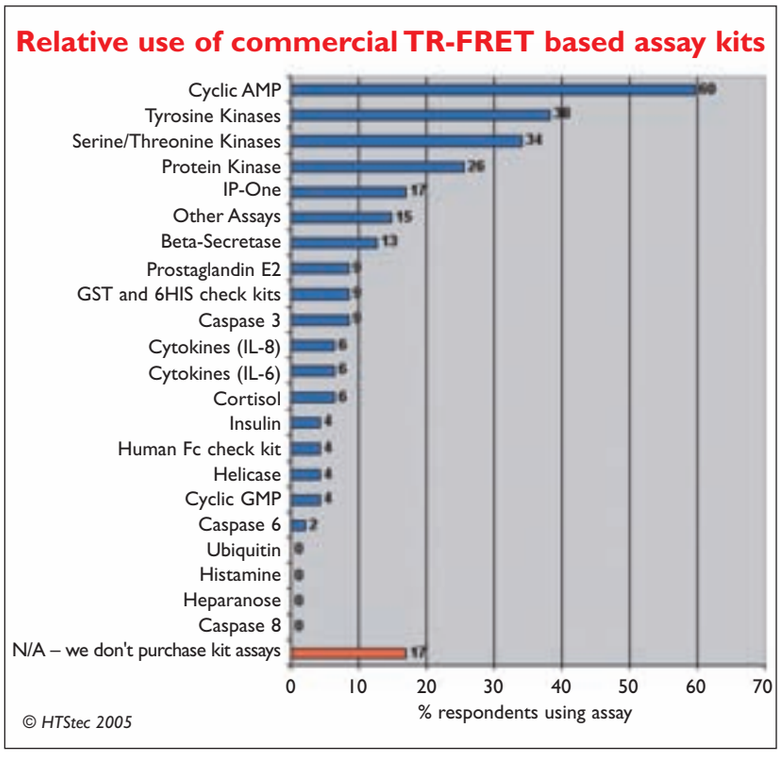
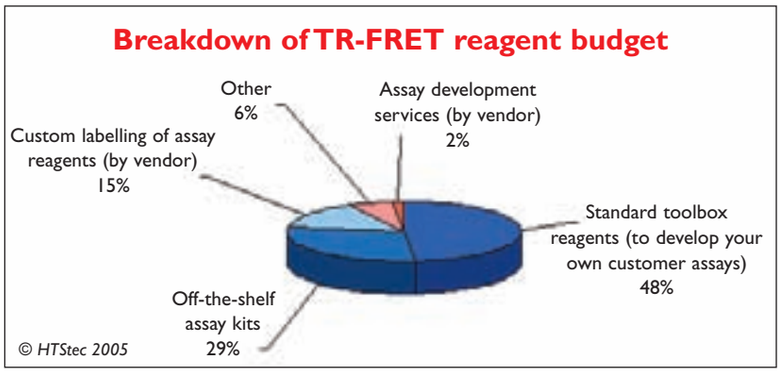


Figure 5



A typical TR-FRET screen

The survey uncovered that the typical TR-FRET screen today (2005) has an average volume of 40uL, is undertaken in a regular or low-volume 384-well plate, generates 551,000 data points and 12% are cell-based. The average annual budget for TR-FRET based assay reagents was $145,000/screening lab in 2005, and this represented 16% of respondents total assay reagent budget. The majority of the TR-FRET reagent budget was spent on standard toolbox reagents (48%) and off-the-shelf assay kits (29%) (**Figure 5**). From this data the global Pharma/Biotech market for TR-FRET based assay reagents was estimated to be around $50 million in 2005.

Why TR-FRET is preferred?

As indicated earlier, for some common target types users are spoilt for choice with respect to assay technologies. It therefore comes as no surprise that the typical assay technologist in Pharma and Biotech today is familiar with and a skilled user of at least eight different assay technologies. However, around 40% of these labs now have plans to try to limit the number of different assay technologies used. The main explanations given for planning to limit the number of different technologies used include: 1) to develop greater technology expertise in house; 2) to increase reagents consumption to get better price (volume discount); and 3) the possibility to profile a compound in various assays using the same technology. The basis for purchasing decisions between several very similar reagent offerings is not always obvious, is it therefore significant that the relative ranking of criteria by which TR-FRET based assays were chosen over alternative assay technologies (**Figure 6**) appears to suggest TR-FRET's sensitivity and ease of use are most important. In direct contrast, the main reason

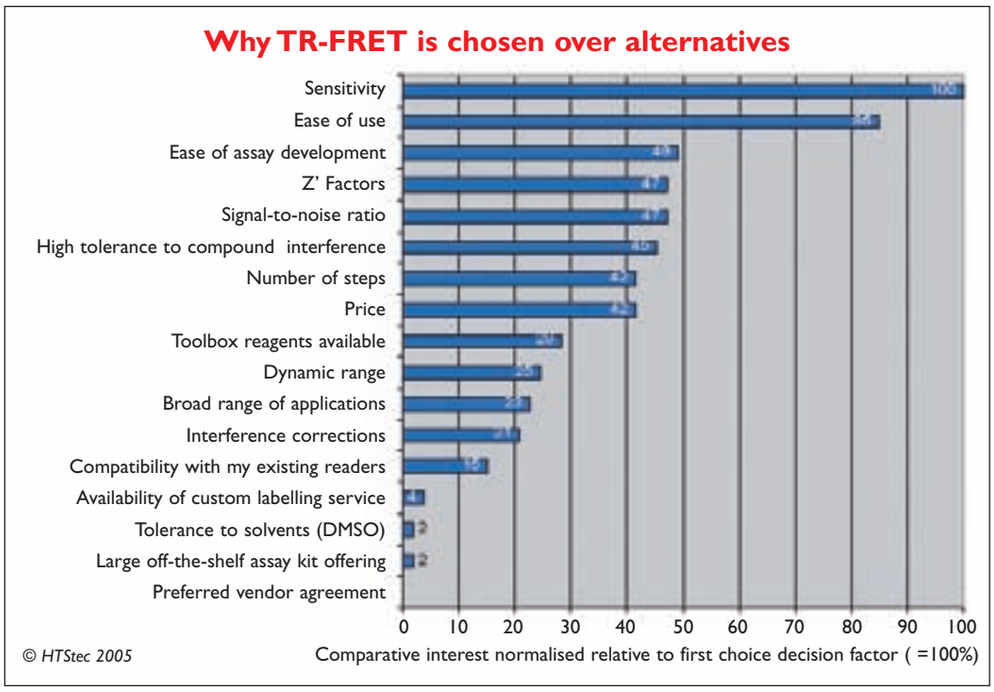


Figure 6

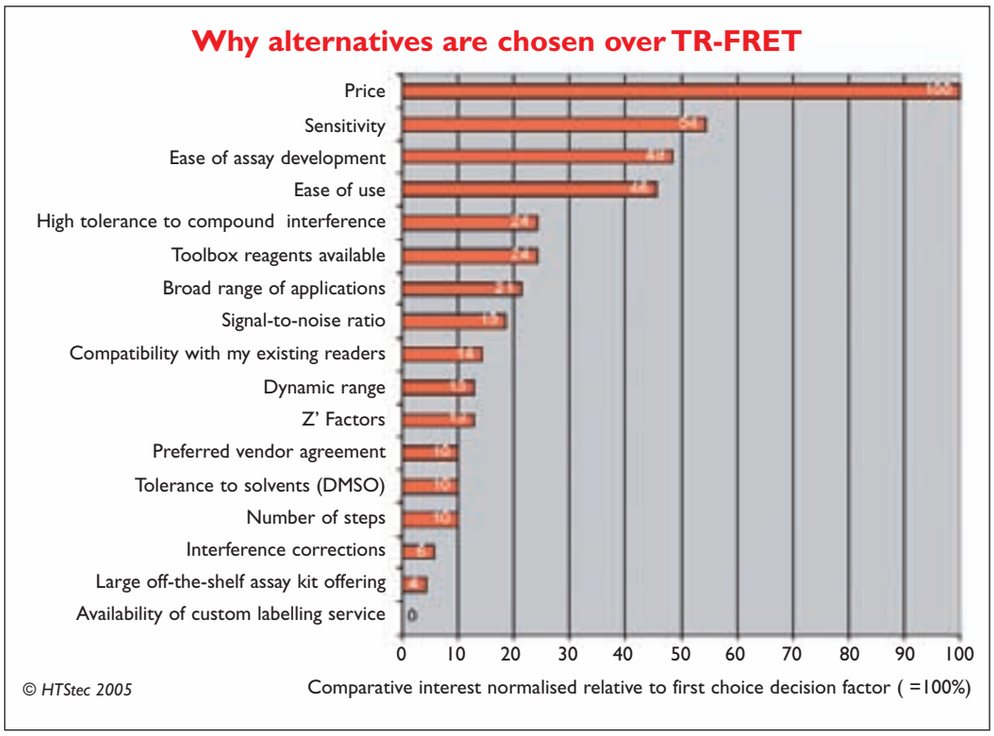
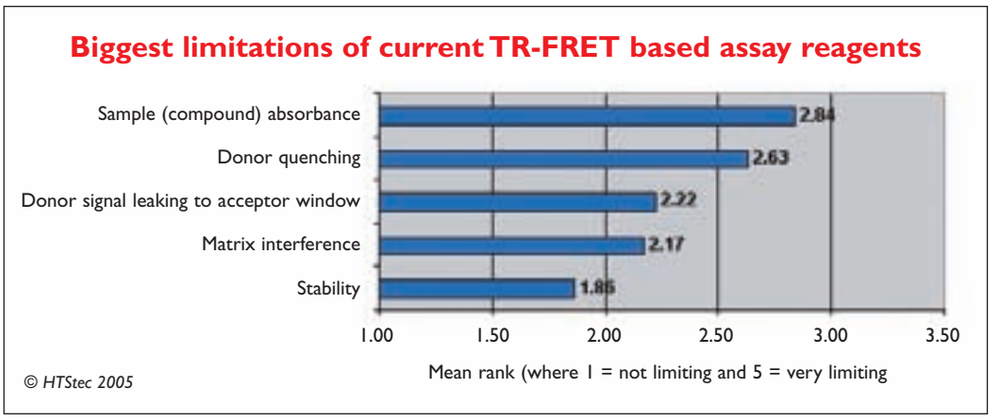


Figure 7

why alternatives assay technologies were chosen over TR-FRET based assays was predominantly price (**Figure 7**) and it is increasingly common to hear that screening groups are shopping around for the lowest quote from a shortlist of their preferred TR-FRET assay alternatives. However, like all available assay technologies TR-FRET assays are not without their limitations (**Figure 8**) and the

Figure 8



most limiting of these was perceived by survey respondents to be sample (compound) absorbance and donor quenching. The potential narrowing of the preferred assay portfolio of screening groups appears to have benefited TR-FRET by virtue of its level of maturity and acceptance in the scientific community and the broad and highly experienced user base. Together these factors may have spawned something of a resurgence of innovative activity among vendors whose products address this market. It is apparent from reading the following vendor updates that a surprisingly large number of new developments and technology improvements are ongoing.

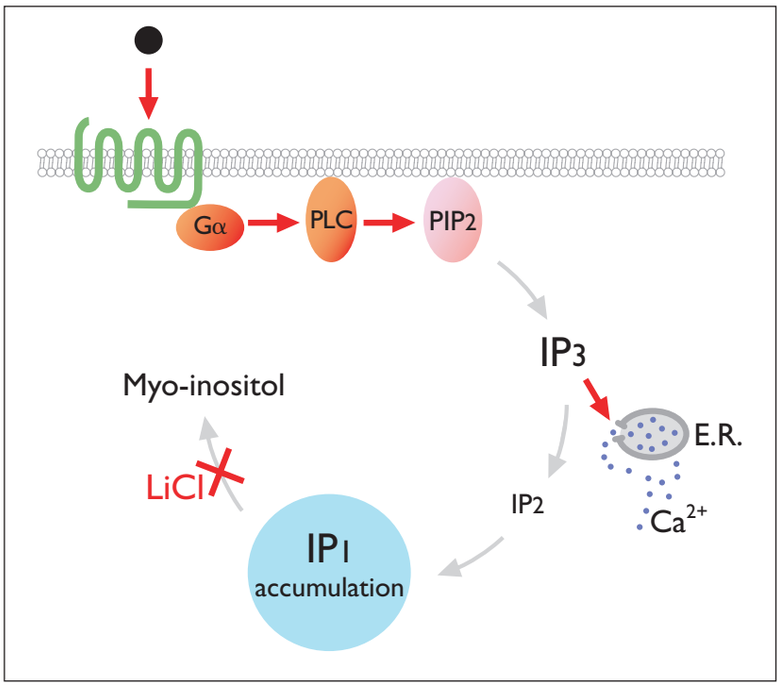


Figure 9: The IP1 pathway. Gq coupled receptor activation induces IP3 release catalysed by PLC. IP3 degradation occurs rapidly and leads ultimately to the production of IP1, while processing into myo-inositol can be prevented by the addition of LiCl

TR-FRET vendors continue to evolve the technology

Homogeneous Time Resolved Fluorescence (HTRF®), the TR-FRET technology developed by **Cisbio International** (www.htrf-assays.com) bases its principles on the photophysical properties of Eu^{3+} Cryptates when they combine to different acceptors in a FRET process. The technology has been applied to a number of biochemical assays such as enzymatic activities (kinases, proteases, ubiquitination, etc), protein-protein/nucleotide interactions (receptor dimerisation, topoisomerase etc), and immunoassays. The robustness of the technology to various assay conditions has further enabled the development of cell-based assays, allowing the transition from simple biochemical assays to high throughput functional ones, bringing more pharmacological relevance at the level of early phases of drug screening. The new GPCR screening platform illustrates this trend. Both cAMP and IP1 (inositol monophosphate) (**Figure 9**) have been successfully adapted to the same direct assessment on cells in high density plate formats. The validation of the IP-One assay on Gq-coupled receptors has already proven its relevance on more than 50 receptors, including the screening of inverse agonist activities for which few sensitive HTS methods are available. These advances in assay development are also supported by the search for technological evolutions. The new d2 acceptor – a small organic dye compatible with Eu^{3+} Cryptates – has been a key element in most recently developed HTRF® assays. As a result of these optimisations, d2 based HTRF® assays show a stability over several days allowing the number of screening runs per week to be increased

and the possible rescue of a run in the case of instrument failure. Kinases remain the main application of HTRF® after GPCRs. Most of current assays developed involve phosphospecific antibodies which detect in a sensitive and accurate manner kinase activities. This typical format is compatible with all kinds of substrates and configurations: small peptides to large proteins, multiple phosphorylation sites, auto-phosphorylations. HTRF® will soon combine with another assay concept initiated by Upstate, the KinEASE® system. This assay format consists of a universal monoclonal antibody which recognises a series of peptidic substrates of Thr/Ser kinases. So far, the new assay comprises three different substrates which have been validated on more than 70 different kinases including PKC and AKT families, and Aurora A (see Upstate's section below for additional details). For the future, Cisbio concentrates its research on technologies that can bring more relevance and reliability during drug screening phases. The goal is also to provide new HTS functional assays on living cells, adaptable to existing affordable detection devices.

For a TR-FRET assay based on HTRF® (Cisbio International), the interacting compounds are labelled with a rare earth cryptate donor and a far red acceptor (allophycocyanine, Cy5, or Dy647). This is often done by epitope tagging of the relevant proteins, (using FLAG-tag, His-tag or HA-tag), and using donor and acceptor labelled antibodies against the tags. **Covalys** (www.covalys.com) offers an alternative that enables direct covalent labelling of the proteins of interest, eliminating the antibodies and reducing assay complexity. Covalys' SNAP-tag™ is a protein tag which binds covalently to a labelled substrate forming a stably labelled fusion protein. Substrates carrying a donor cryptate (BG-TBP) or an acceptor-fluorophore (BG-647) suitable for HTRF are available (**Figure 10**). SNAP-tag labelling streamlines assay development and reduces the size of the protein complex compared to using antibodies, resulting in pronounced energy transfer. A model assay was developed using the interaction of FKBP with FRB. In the presence of rapamycin these proteins interact with high affinity (**Figure 11**). Maximum energy transfer values observed for the FKBP-FRB system were very high at above 20,000 delta F%. This indicates strong interaction between the proteins and effective energy transfer between donor and acceptor. Inhibition studies using ascomycin gave a sigmoidal displacement curve with a 50% inhibition at about 10nM, in agreement with literature values. For repetitive use of the assay a Z' value above 0.7 was found, indicating a very rugged system. SNAP-tag labelling works equally well in crude lysates, allowing the direct labelling of delicate proteins that tend to lose binding activity during purification. The ease of the

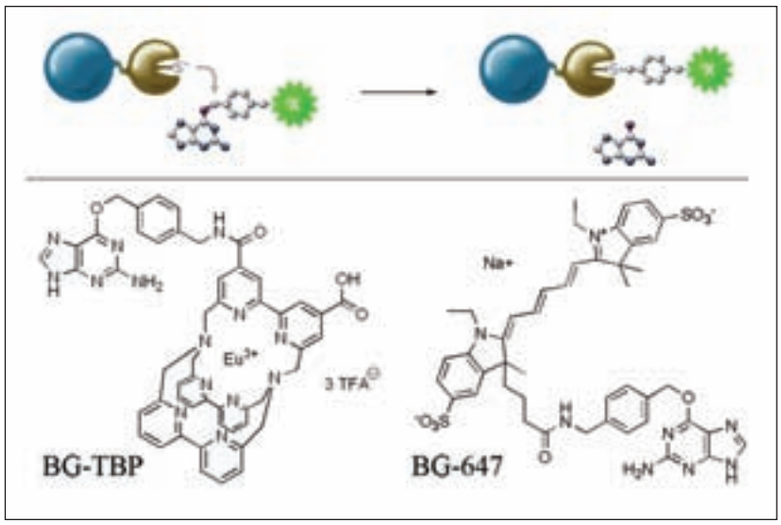


Figure 10: Top: In the SNAP-tag self-labelling reaction the benzyl-moiety carrying the label is irreversibly transferred to the SNAP-tag. Bottom: SNAP-tag substrates used for the development of HTRF® assays: BG-TBP, long lived cryptate donor, BG-647 fluorescence acceptor for BG-TBP

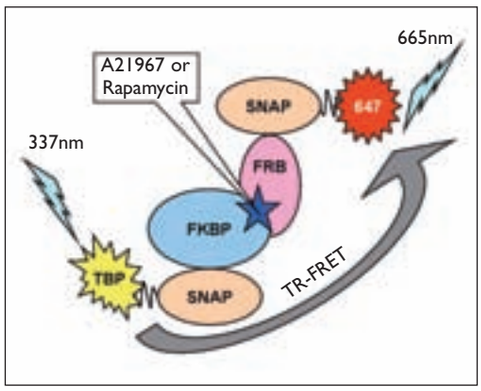


Figure 11
Schematic of the high affinity interaction of FKBP-FRB in the presence of the drug Rapamycin or analogous compounds. The limited distance between the two SNAP-tag fusion proteins allows highly efficient energy transfer from the TBP-donor to the 647-acceptor

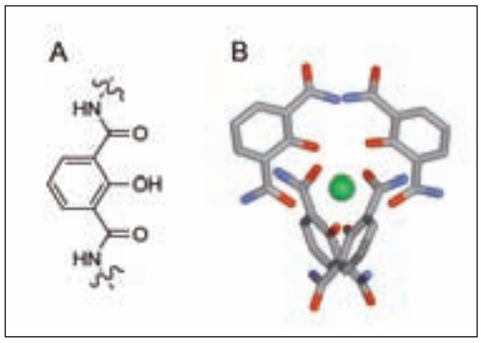


Figure 12
A: The high quantum efficiency (about 60%) and overall brightness of Lumiphore's Tb^{3+} complexes are derived from the highly efficient channelling of excitation energy from the isophthalamide chelating moiety to the coordinated Tb^{3+} ion. B: In a typical complex, there are four isophthalamide chelating groups co-ordinating the metal ion (green). These co-ordinating groups are covalently connected with a scaffold that confers high complex stability in aqueous solutions (scaffold not shown)

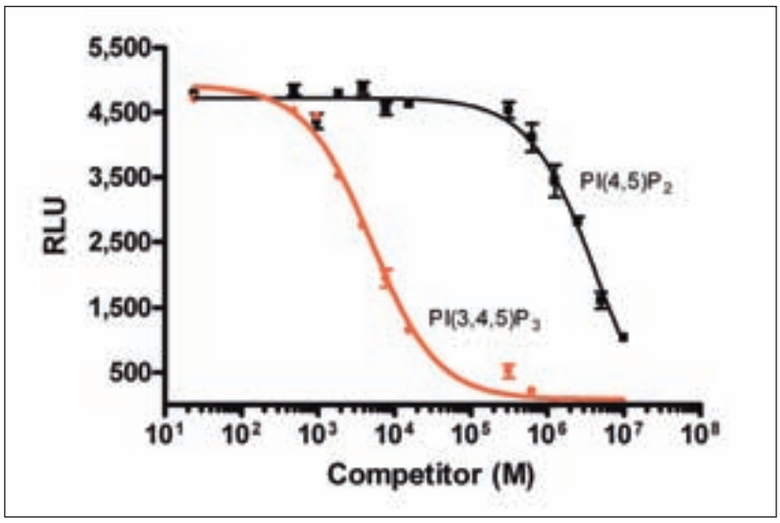


Figure 13: The PI3K TR-FRET assay is sensitive (with inhibition curves in the low nanomolar range), and specific (the PI(3,4,5)P_3 product shows greater than 70 times lower EC50 value compared to substrate)

SNAP-tag labelling approach, obviating the need to use antibodies, and the possibility to work from complex samples, make this a valuable tool for the development of HTRF assays.

Phosphoinositide kinases phosphorylate the lipid head of phosphatidylinositol at the 3', 4', or 5' position; and the lipid products of these enzymes act as cell-signalling molecules essential in multiple aspects of cell growth and survival. A particular lipid kinase, phosphatidylinositol-3-kinase (PI3K), is an established drug target for cancer therapeutics with exciting promise. The first assays for PI3K were radioactive with laborious HPLC or TLC separations. Non-radioactive assays have also been described recently, but lack of low-cost, versatile reagents has limited their commercial availability. A joint venture between **Echelon Biosciences** (www.echelon-inc.com) and **Lumiphore** (www.lumiphore.com) has resulted in a sensitive TR-FRET assay for important lipid kinases. Lumiphore has exclusive licence to specific lanthanide-complex technology from the University of California, Berkeley which it has further developed and commercialised. These highly luminescent terbium (Tb^{3+}) complexes (**Figure 12**) have a quantum yield of approximately 60%, making them the brightest lanthanide complexes available for commercial time-resolved luminescence applications in aqueous environments. A new macrocyclic Tb^{3+} complex, with two peak emission lines at 490nm and 545nm, has proven particularly useful in luminescence resonance energy transfer (LRET) applications as a donor with fluorescein and rhodamine dyes as well as phycobiliprotein and R-phycoerythrin as acceptors. Soon to be released Eu-complexes will enable multiplex assays when used in combination with Tb. Furthermore, the excitation maxima of these dyes are between 340-350nm which is sufficiently red-shifted to avoid undesirable excitation of biological samples or plastics. Echelon has incorporated Lumiphore dyes into its proprietary PI3K screening reagents and assay platforms. The initial PI3K assay is competitive with the enzymatic product, PI(3,4,5)P_3, competing with a fluorescent PI(3,4,5)P_3 tracer for binding to a specific Tb-conjugated binding protein. Exceptional signal to background binding is observed, and the assay is both sensitive and specific (**Figure 13**). Because this assay measures directly the enzymatic product of PI3K, it avoids the limitations of popular ATP depletion kinase assays and allows researchers the opportunity to screen for both active site and allosteric inhibitors. Further lipid kinase and phosphatase assays, including diabetes targets SHIP2 and PTEN, are planned for release later this year; as well as, Tb-labelled secondary antibodies and additional generic assay reagents to enable researchers flexible design of custom applications.

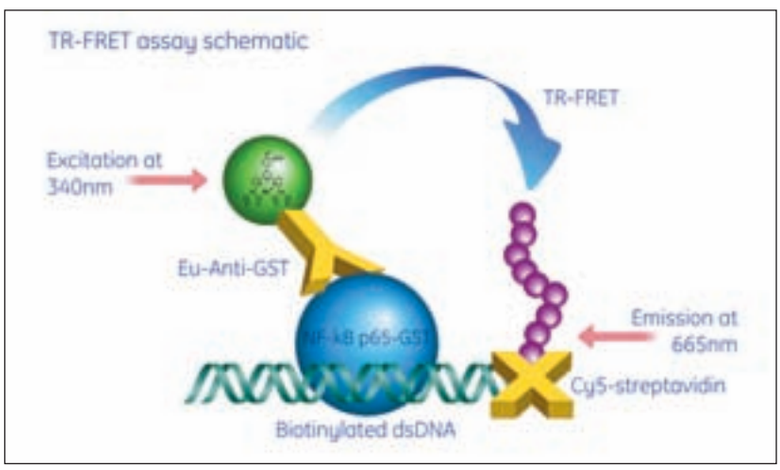


Figure 14: An example of a GE Healthcare's TR-FRET format assay

GE Healthcare (www.amershambiosciences.com) also provides a range of fluorescence assay solutions based on TR-FRET. GE's offering uses europium (TMT) chelates as donors (Eu^{3+} with terpyridine-bis(methylenamine) tetra acetic acid). The 3-amino group of the europium (TMT) chelate is readily converted to the isothiocyanate, thereby

allowing labelling of primary amine groups on a range of biomolecules. The CyDye fluor Cy5 is an excellent choice of acceptor for europium (TMT) chelates in TR-FRET format assays. This is because Cy5 is small compared to traditional acceptors such as allophycocyanin and can help reduce steric hindrance. CyDye fluors are also unaffected by amino acid residues such as tryptophan. This means there will be no change in fluorescence signal when labelling proteins or peptides with CyDye fluors. GE's offering includes a range of TRF and CyDye generic reagents (donors and acceptors) and europium (TMT) isothiocyanate (**Figure 14**).

Europium(III)-chelate dyed nanoparticles are being developed by **Hidex** (www.hidex.com) for use as labels in immunoassays. Nanoparticles have high specific activity, because several thousands of europium(III)-chelates are embedded inside a single polystyrene shell. High label content is possible, since lanthanide chelates do not suffer from self-quenching phenomenon. Nanoparticles have been used as labels in heterogeneous immunoassays and also as donors in homogeneous TR-FRET based assays. In TR-FRET based assays using europium(III)-chelate dyed nanoparticles as donors the energy is transferred from a large number of excited europium chelates via non-radiant dipole-dipole interactions to a single acceptor molecule during the long lifetime emission of the europium chelates. Thus the measured signal from a single acceptor is increased compared to traditional donor-acceptor pairs. The increased background signal caused by emission of europium(III)-chelate can be almost completely avoided by using a very small nanoparticle (eg 47nm diameter) and by spectral resolution: using an acceptor molecule, which can be measured at near-infrared area (eg at 730nm) the emission caused by europium(III)-chelates is at minimum. The polystyrene shell also protects the europium(III)-chelates from unwanted matrix effects, eg assay buffer may contain heavy metals without causing any quenching effect on the

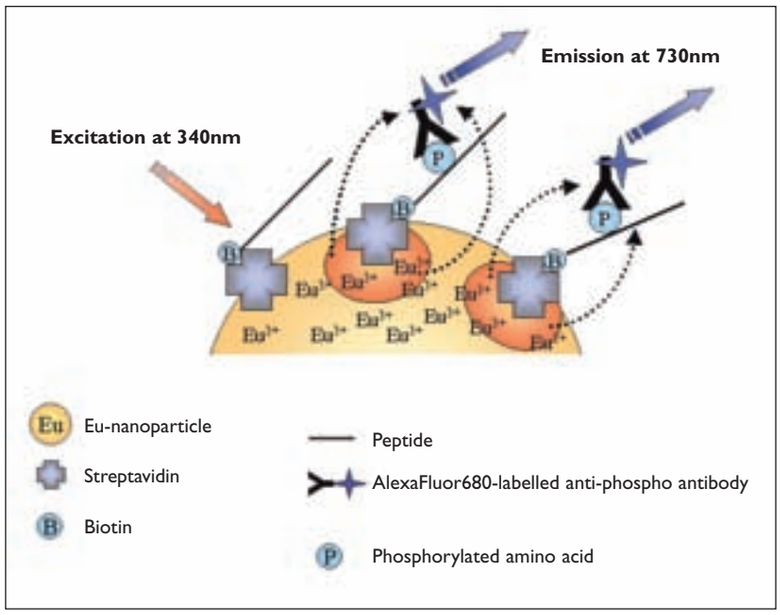


Figure 15: Principle of nanoparticle-based kinase assay. Biotinylated peptides bind to streptavidin coated nanoparticle. If the peptide is phosphorylated, acceptor-labelled antibody recognising phosporylation site binds to peptide. The europium(III)-chelates in proximity of the acceptor labels (marked with dark orange) participate to the energy transfer

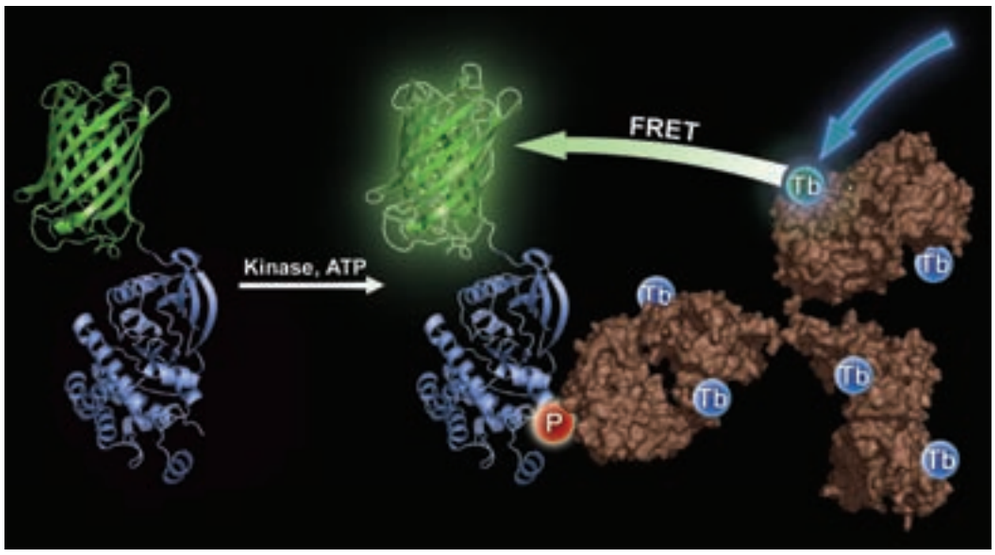


Figure 16
Invitrogen's LanthaScreen™ TR-FRET format uses terbium as the donor species, allowing acceptors such as fluorescein or GFP to be used. In kinase assays, physiologically relevant protein substrates can be prepared as GFP fusions, simplifying the assay development process

fluorescence of a particle. Homogeneous assays using europium(III)-chelated dyed nanoparticles as donors have been employed in high throughput screening. For example immunoassay for 17β-estradiol has been used to screen inhibitors for enzyme 17β-hydroxysteroid dehydrogenase type 1 (17β-HSD-1). 17β-HSD-1 catalyses a reversible reduction of estrone to 17β-estradiol and its activity is increased, eg in some breast cancer patients. Several HTS applications using the nanoparticle labels are currently under development by Hidex for example a nanoparticle-based kinase inhibitor screening assay will be commercialised this year. The assay uses streptavidin coated europium(III)-chelate dyed nanoparticles with 47nm diameter. The substrate peptide is biotinylated and antibody specific for phosphorylation is labelled with AlexaFluor 680 label (**Figure 15**). The assay has a high specific signal level, is robust and easily automated.

By using terbium rather than europium as the donor species in the FRET pair, **Invitrogen**'s LanthaScreen™ technology (www.invitrogen.com/LanthaScreen) maintains the advantages of a time-resolved, ratiometric format, but adds flexibility in terms of the types of acceptors that can be used. In addition to simplifying TR-FRET kinase assays by allowing the use of substrates that are directly labelled with fluorescein (rather than relying on biotinylated substrates that then must be developed by an addition of streptavidin-APC), physiologically relevant protein substrates can be prepared as GFP fusions, with GFP serving as the FRET acceptor for terbium (**Figure 16**). This strategy provides a facile method to assay kinases that require a docking site on the substrate for efficient phosphorylation to take place, as is the case with many MAP kinase pathway members such as p38α or RAF. Labelling kinase substrate as a GFP fusion has other practical advantages, such as batch-to-batch consistency that is not possible when a substrate protein is randomly labelled through accessible amino groups, and lower cost compared to using an acceptor-labelled antibody. The strategy of using GFP as an acceptor in a TR-FRET assay also has been applied recently to protease assays. Deubiquitinating enzymes (DUBs) are proteases that cleave ubiquitin from specific target proteins, often playing a role in cellular processes by 'rescuing' ubiquitinated proteins from proteosomal degradation. Inhibition of DUBs that are involved in the rescue of oncogenic proteins is emerging as a target for pharmaceutical intervention. Importantly, DUBs require the presence of an intact ubiquitin protein for efficient proteolysis, and

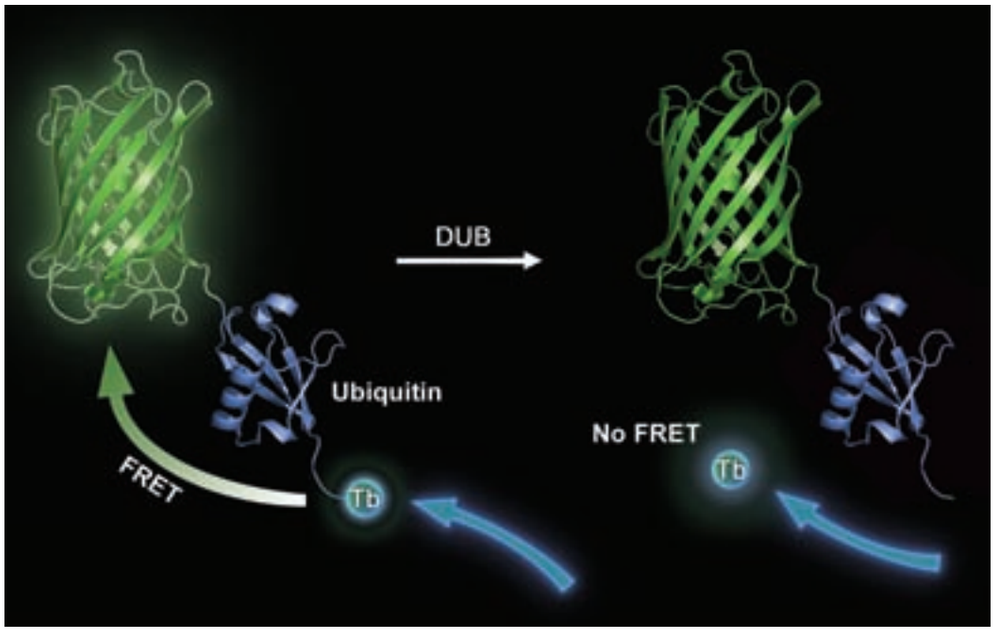


Figure 17
Invitrogen's LanthaScreen™ TR-FRET format has recently been used to incorporate GFP as an acceptor in substrates for deubiquitinating enzymes (DUBs). By genetically encoding the acceptor species and attaching the terbium-donor species downstream of the site of proteolysis, a physiologically relevant substrate that contains the full-length ubiquitin (that is required for efficient proteolysis) is prepared, and the assay can be continuously monitored to detect DUB activity or inhibition

chemically synthesised peptides have not been reported as efficient substrates for DUB activity. By expressing a DUB substrate that contains an N-terminal GFP fused to ubiquitin that contains a terbium-labelled cysteine proximal to the site of proteolysis, a substrate is prepared that shows a high degree of FRET that is reduced in the presence of DUB activity (**Figure 17**). This strategy highlights a key advantage of using GFP as the acceptor, as it would be extremely difficult to site-specifically incorporate two distinct labels using chemical labelling strategy. Again, the directly labelled format provides for specific advantages over other formats in that the reaction can be monitored in real time, providing valuable kinetic information for elucidating mechanisms of enzyme activity or inhibition. In addition to a DUB substrate, Invitrogen has also recently launched terbium- and fluorescein-labelled ubiquitin, allowing for the monitoring of mono- or polyubiquitination processes in either a real-time kinetic or an endpoint format.

The latest addition to the IMAP® platform for kinases, phosphatases and phosphodiesterases from **Molecular Devices** (www.moldev.com) is IMAP's TR-FRET detection mode. This new detection system uses all the components of the IMAP fluorescence polarisation (FP) system plus a IMAP terbium (Tb) labelled TR-FRET donor ('Tb donor', **Figure 18**) consisting of a phosphate-containing linker, a sensitiser and a Tb complex combined into one molecule. Just like IMAP FP, this assay is a simple, homogeneous 'mix and read' procedure: initiate the kinase reaction, add the Binding Solution and read. With IMAP TR-FRET, the Binding Solution includes the novel Tb-donor already bound to the binding entities. Upon addition of the Binding Solution, the phosphorylated population of the fluorescently labelled kinase substrate (FAM or TAMRA) binds as usual to the binding entities and is brought into close proximity to the Tb donor, thus producing resonance energy transfer (**Figure 19**). Due to the long lifetime of Tb fluorescence, the detection can be run in time-resolved mode, which virtually eliminates fluorescence interference from assay components

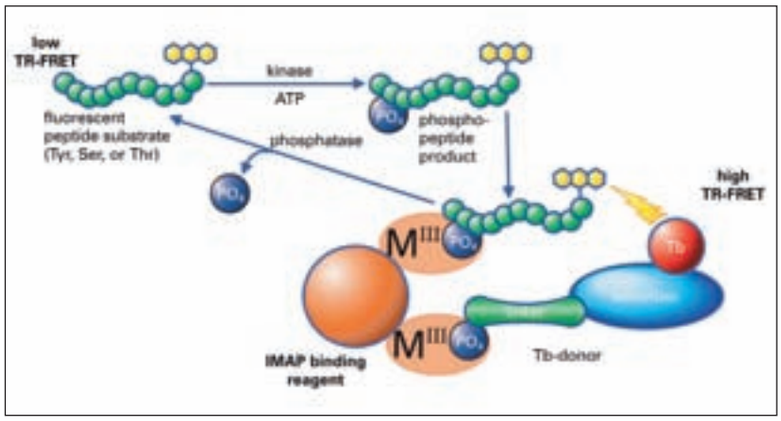


Figure 18: Principle of the IMAP TR-FRET assay. Upon phosphorylation by the target kinase, fluorescent substrate binds to the trivalent metal entities of the IMAP binding reagent. This binding brings the substrate into close proximity of the Tb donor that is pre-bound, allowing energy transfer to occur

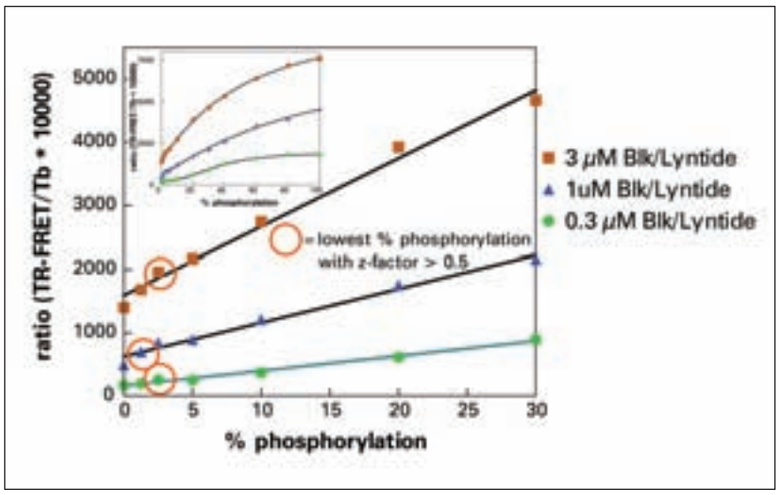


Figure 19: Calibration curve using BLK/Lyntide (5FAM-EFPIYDFLPAKKK-NH2). Unphosphorylated and phosphorylated Blk/Lyntide were diluted to 0.3µM, 1µM, and 3µM in IMAP reaction buffer. They were then mixed at varying ratios as indicated in graph by % phosphorylation and 20µL were pipetted/well (white 384 well plate). 60µL of Binding Solution with Tb-donor was added. The plate was read in TR-FRET mode after overnight incubation

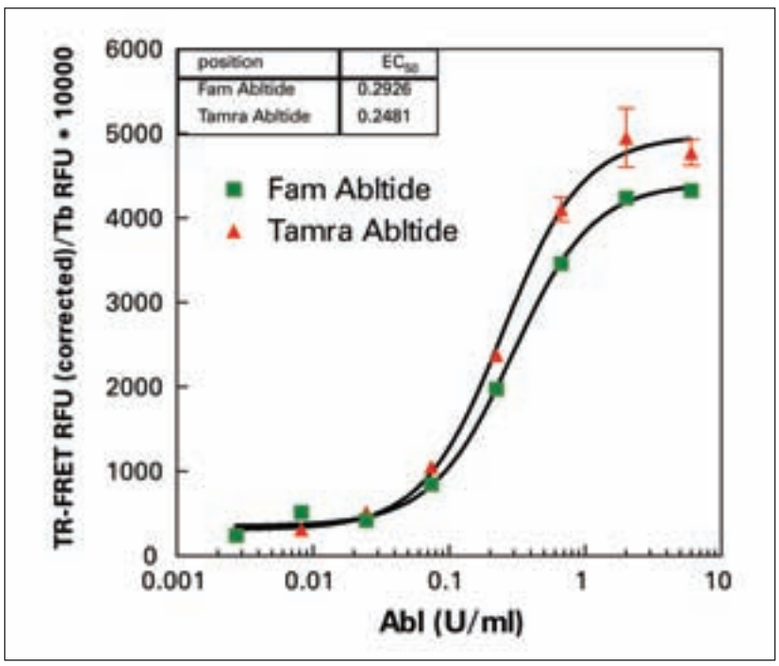


Figure 20: IMAP TR-FRET Assay for Abl kinase using TAMRA and FAM labelled Abltide (Fl-KKGEAIYAAPFA-NH2) Indicated amounts of Abl (Upstate) were incubated for 1h RT in 20µL IMAP reaction buffer in the presence of 100µM ATP and 1µM of either FAM or TAMRA labelled Abltide. 60µL of Binding Solution was added and TR-FRET was read after an incubation of 3h

or compounds in a screen. IMAP TR-FRET combines all the advantages of IMAP – an antibody-free, stable, generic, homogeneous format – with the advantages of TR-FRET for detection of phosphorylation. These advantages include 1) reduced minimum percentage substrate conversion and flexibility of substrate concentration (**Figure 20**); 2) increased substrate size, such as proteins, compared to FP detection mode; 3) low background due to time-resolved detection and 4) ratiometric read-out. These features make the assay well suited for HTS and applicable to a wide variety of kinases. IMAP TR-FRET provides further advantages over existing TR-FRET technologies. First, it is truly antibody independent. Therefore the search for a compatible antibody is eliminated, which accelerates the assay development process. Second, the signal is extremely stable. IMAP TR-FRET's novel Tb donor is remarkably stable, requiring no additives, such as fluoride, to achieve stability and signal intensity to last more than 24 hours. IMAP TR-FRET maintains the solid performance of the IMAP platform. Its signal stability allows customers to more efficiently schedule their screens compared with alternative TR-FRET detection methods. The IMAP Platform, with its flexible Progressive Binding System, various Substrate Finder plates, validated FAM or TAMRA labelled substrates (**Figure 20**), and the FP and TR-FRET detection modes accelerates the transitions from assay development to screening and hit evaluation for virtually any kinase, phosphatase and phosphodiesterase with precision and reproducibility.

In LANCE™, **PerkinElmer** (www.perkinelmer.com) offers one of the widest TR-FRET toolboxes available on the market enabling assays to be quickly developed for a vast range of drug targets especially enzymes such as kinases. TR-FRET based assays are eminently suited for the increased productivity and performance demanded today by customers, and uHTS applications can be readily adapted on to microplate imagers such as PerkinElmer's ViewLux™ enabling greatly improved precision and throughput. PerkinElmer's recently launched LANCE cAMP cell based assay for analysis and screening of GPCR drug targets is an excellent example of applying new innovation to a trusted screening platform, which is based on TR-FRET chemistry (**Figure 21**). LANCE cAMP relies on use of the proprietary Europium chelate dyes, a uniquely high performing antibody against cAMP, and was first to market with next generation higher performing small red-shifted light emission acceptor

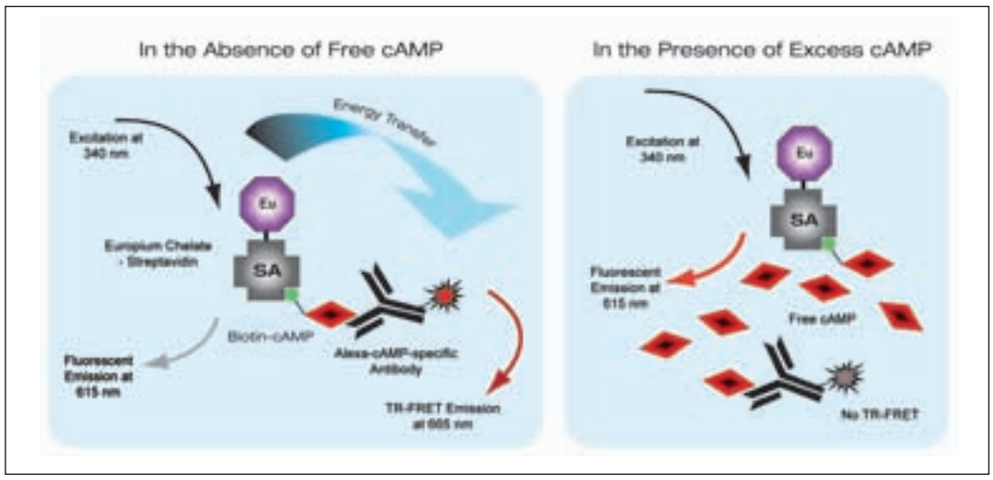


Figure 21
LANCE cAMP Assay Principle. Light pulse at 340nm excites the Europium chelate of the Eu-SA/b-cAMP tracer. The energy emitted from the chelate is transferred to the Alexa-labelled antibodies bound to the tracer, generating a TR-FRET signal at 665nm. Residual energy from the chelate will produce light at 615nm. cAMP of a sample competes with the tracer for antibody binding sites and causes signal reduction

dyes such as Alexa Fluor© 647. These new enhancements offer significant improvements in assay performance such as robustness, sensitivity, stability, ease of automation and also permit simpler assay development for tough target classes such as $G_{\alpha t}$-coupled GPCRs. Another unique TR-FRET approach PerkinElmer has taken was the development of its proprietary TruPoint™ assay system which is ideal for the analysis of many different enzyme targets such as protease and helicases. TruPoint again utilises the high energy emission Europium chelate dyes but now in conjunction with a europium dye quencher (**Figure 22**). TruPoint offers an extremely robust assay solution with enhanced sensitivity, often requiring far less enzyme than competitive technologies, can be run using cell lysates (eg caspase 3 assay), and in addition a positive signal production (increase) format yields a greatly reduced incidence of false positives. Overall PerkinElmer is committed to continue expansion in the area of high value functional biochemical and cell based assay solutions, utilising state of the art refinements to its existing TR-FRET technologies, such as seen with LANCE cAMP and TruPoint.

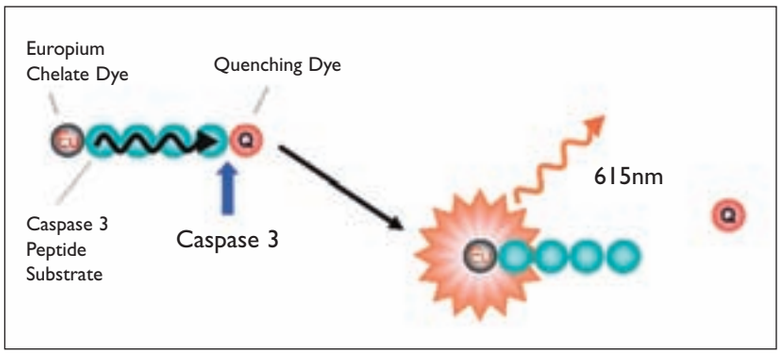


Figure 22: The TruPoint caspase-3 assay principle is based on dequenching of the Europium chelate dye signal upon cleavage of the caspase 3 peptide substrate and removal of the quenching dye from proximity to the Europium

Upstate's (www.upstate.com) original KinEASE® range of FP (fluorescence polarisation) assay formats have now been extended to include the HTRF® format in partnership with Cisbio. The platform consists of a choice of three biotinylated peptides, a proprietary monoclonal antibody labelled with Eu^{3+} Cryptate (Eu[K]) and streptavidin-XL665. A schematic version of the assay is displayed in **Figure 23**. Each peptide contains a common C-terminal phosphoserine epitope with three different N-terminal sequences allowing access to a large range of kinases. The monoclonal antibody binds to the phosphorylated serine (the epitope) in the biotinylated peptide which in turn is bound to streptavidin-XL665 allowing FRET to occur. Increasing kinase activity produces more signal and is proportional to the amount of kinase added to the well. As can be seen in **Figure 24**, MST was titrated with all three peptides, signal was observed with all three peptides; with substrate three being the optimal choice. Representative kinase/substrate pairs are shown in **Figure 24** demonstrating excellent signal/background ratios at low concentration of kinase for all three conditions. HTRF KinEASE® has been initially validated with 70 kinases and determined the optimal peptide for each kinase. It is highly likely that many more kinases will phosphorylate one or more of the three peptides. It is also possible to vary the N-terminal sequence to gain access to even more kinases, maybe in excess of 100. Hence Upstate and Cisbio bring to the marketplace a universal serine/threonine kinase assay system utilising just one labelled monoclonal antibody.

Assays

Table 1: Comparison of commercial TR-FRET offerings

SUPPLIER NAME	TR-FRET OFFERING NAME	TR-FRET PAIRS		REMARKS
		DONOR	ACCEPTOR	
Cisbio	HTRF®	Europium-Cryptate	XL665	Eu^{3+} Cryptate stability Broad product portfolio Very low compound interference
			d2	New small organic acceptor Enhanced assay stability Ultra-low miniaturisation for HTS
Echelon	TRUE FRET™	Terbium	TAMRA, Bodipy TMR, etc	Modified for increased stability and quantum yield Both assays and reagents available
			Fluorescein, Bodipy FL, etc	
GE Healthcare	TR-FRET format assays	Europium (TMT) Chelate	CyDye Cy5	Small size relative to APC reduces steric hindrance CyDye unaffected by amino acid residues like tryptophan
Hidex	Proxiscreen	Eu(III)-chelate dyed nanoparticle	AlexaFluor680	Enhanced energy transfer Decreased matrix effect due to robust donor particle
Invitrogen	LanthaScreen™	Terbium	Fluorescein, Alexa Fluor 488	520/25 emission Allows assay of substrates directly labelled with fluorescein
			GFP, YFP Rhodamine, Alexa Fluor 546	570/10 emission Allows assays using fluorescent protein fusions of native substrates
			Alexa Fluor 633 Alexa Fluor 647	665/10 emission Allows multiplexing with green acceptors with no spectral interference
Lumiphore	LumiRay Green	Terbium	FAM, TMR, RPE, etc	
Molecular Devices	IMAP TR-FRET	Terbium	5FAM, 5TAMRA	Antibody independent, select between TR-FRET or FP readout without changing IMAP technology Allows measurement of larger substrates (proteins) & higher substrate concentrations >24 hour signal stability allows for more flexible screening
PerkinElmer	LANCE™	Europium Chelate	APC	High S/B (10s) Very low compound interference
			Alexa Fluor 647	Smaller size acceptor vs APC Beneficial for competitive kinase assays Very low compound interference
	TruPoint™	Europium Chelate	QSY-7	QSY-7 is a potent quencher of europium Extremely high S/B (up to 1000) Suitable for hydrolysing enzymes & cell lysates
Upstate	HTRF® KinEASE, 4G10, PIProfiler	Europium-Cryptate	SA-XL665	Developed in collaboration with Cisbio

Summary

Despite having been available for many years TR-FRET based assay technology continues to evolve and get better, with a wide range of new developments to enhance and extend this assay formats capabilities. These developments include several new kits addressing relevant second messenger GPCR targets (eg cyclic AMP and IP-One); a new universal serine/threonine kinase assay system; the enhanced stability and performance of existing FRET pairs; increased use of red-shifted Alexa Fluor© as acceptor dyes; better assay development flexibility by direct covalent labelling of substrates or the use of antibody independent formats; the wider use of terbium complexes, including using GFP as acceptor; and a new approach using Europium chelate dyed nano-particles. Taken as a whole a surprisingly large number of TR-FRET pairings are now being promoted (see **Table 1** for a summary of donor and acceptor pairs) and the diversity of these offerings looks set to continue increasing over the coming year. In conclusion, the outlook for the sustained preferential use of TR-FRET based assays by screening groups is positive. DDW

John Comley *is Managing Director of HTStec Limited an independent market research consultancy whose focus is on assisting clients delivering novel enabling platform technologies (liquid handling, laboratory automation, detection instrumentation and assay reagent technologies) to drug discovery. Over the past 2.5 years HTStec has published 15 market reports on drug discovery technologies and Dr Comley has authored 13 review articles in* **Drug Discovery World**. *Further information on accessing the market report 'TR-FRET Based Assay Trends 2005' can be obtained by visiting www.htstec.com or e-mail john.comley@htstec.com to receive a free copy of the Report's Executive Summary and Table of Contents.*

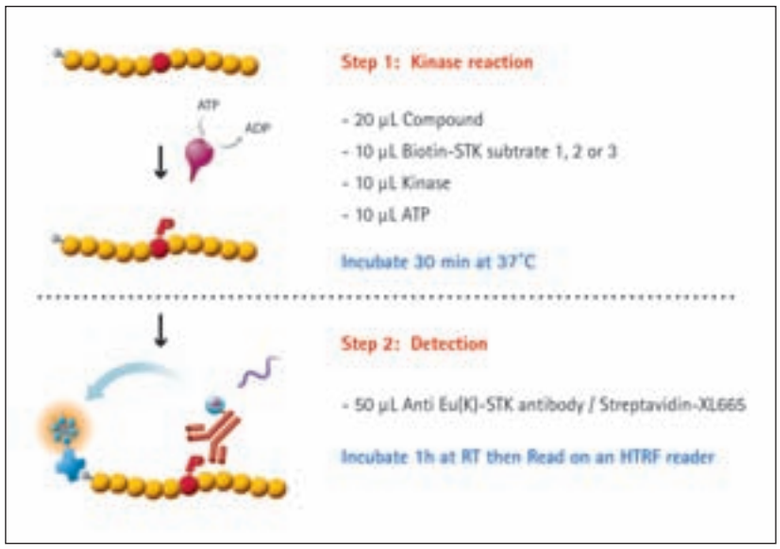
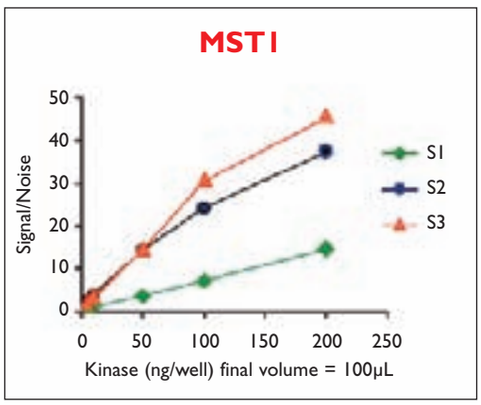


Figure 23: Schematic of the HTRF KinEASE® assay format. The kinase is incubated with compound and 1 of 3 biotinylated peptides, the reaction is started by the addition of ATP. The reaction is stopped by the addition of detection reagent containing Eu[K]-STK (monoclonal antibody) and streptavidin-XL665. Active kinase phosphorylates the peptide to which the Eu[K] labelled antibody binds, streptavidin-XL665 binds to the N-terminal biotin, hence allowing FRET. The amount of phosphorylation is proportional to the activity of the kinase. The plate may be read after 1hr and the signal is stable >24 hr. NB these reactions were performed as 50µL reaction + 50µL detection giving a total of 100µL; it is straightforward to reduce the total volume to ≦4µL



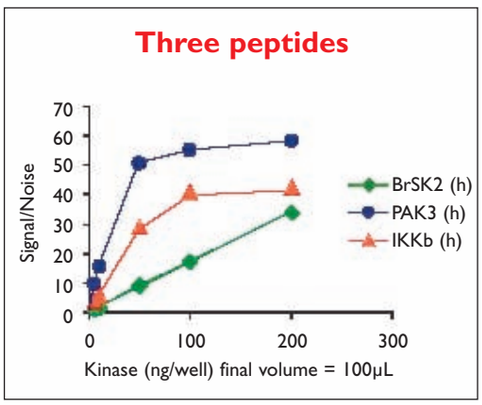


Figure 24: Examples of HTRF KinEASE® reactions. The left hand panel illustrates the use of the 3 peptides with MST 1, substrate 1, substrate 2 and substrate 3. An excellent signal/noise background ratio was obtained with substrates 2 and 3 with substrate 1 being the weakest substrate. The right hand panel shows representative examples of a kinase/substrate pairs BrSK2/substrate 1, PAK3/substrate 2 and IKKβ/substrate 3