HTRF® KinEASE™ TK: a new solution for tyrosine kinase screening

Cisbio's HTRF® KinEASE™ assay for screening serine/threonine kinases now extends to tyrosine kinases (TK). The assay, developed in partnership with Millipore, uses a unique substrate containing a single phosphorylation site recognized by europium cryptate (Eu(K))– labeled antibody to phosphotyrosine. More than 60 tyrosine kinases have already been successfully tested with KinEASE TK. HTRF KinEASE TK kit is available in small and jumbo sizes for high-throughput screening and profiling applications.

Kinases are enzymes that have a central role in various signal transduction pathways. They exert their effect by catalyzing the transfer of a phosphate group from ATP onto a target substrate (protein or peptide), which then becomes activated and performs a specific function.

The approval of two kinase-targeting drugs, Genentech's trastuzumab (Herceptin®) in 1998 and Novartis's imatinib (Gleevec®) in 2001, and their success as cancer therapeutics, validate the trend toward screening new protein kinase inhibitors of various kinases for use in different therapeutic areas.

HTRF KinEASE TK is the fourth kit in the HTRF KinEASE platform, developed in collaboration with Millipore (Upstate) for profiling and high-throughput screening of serine/threonine kinases and tyrosine kinases. The new HTRF KinEASE TK kit combines a peptide substrate and a single proprietary monoclonal antibody with Cisbio's HTRF (homogeneous time-resolved fluorescence) technology, a highly sensitive and robust technology for the detection of molecular interactions of proteins *in vitro*.

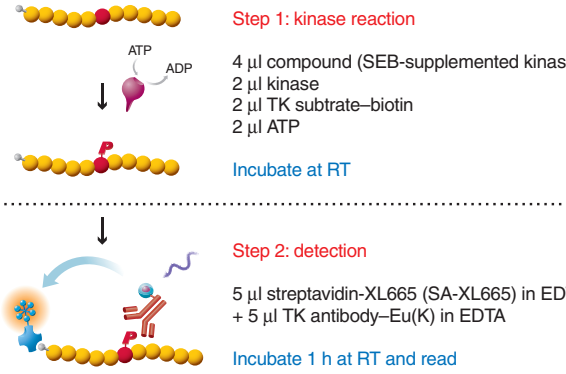

Figure 1 | All assays based on HTRF KinEASE involve two steps: the enzymatic step and the detection step with HTRF reagents. During the enzymatic step (step 1) the substrate-biotin is incubated with the kinase of interest, and then ATP is added to start the reaction. In Step 2, the detection reagent catches the phosphorylated substrate and the resulting HTRF signal is proportional to the amount of phosphorylation. RT, room temperature (18–22 °C).

A universal kinase platform

HTRF KinEASE screenings limit assay development time and are easily miniaturizable as well as flexible, meaning the assay can be performed under a wide range of kinase assay conditions, for instance, with low consumption of enzyme or with any ATP concentration.

The assay is run in two main steps, the kinase reaction step followed by the detection step with HTRF reagents (**Fig. 1**). The phosphorylation of the biotin-tagged generic peptide is initiated by the addition of ATP in the presence of the tyrosine kinase (step 1) and stopped by the addition of a mix containing the two HTRF detection reagents and EDTA (step 2). The HTRF detection reagents are an antibody to phosphotyrosine, labeled with Eu(K) (the time-resolved fluorescence resonance energy transfer (HTRF) donor), and a streptavidin-XL665 (the HTRF acceptor).

Straightforward assay development

A typical development for an HTRF KinEASE TK assay consists of six steps, outlined below and described in a document supplied with the kit.

1. SEB titration: for certain kinases, a Supplement Enzymatic Buffer (SEB), supplied with the kits, dramatically increases the specific signal of the assay (**Fig. 2**).
2. Enzyme titration: in this step the optimal kinase concentration is

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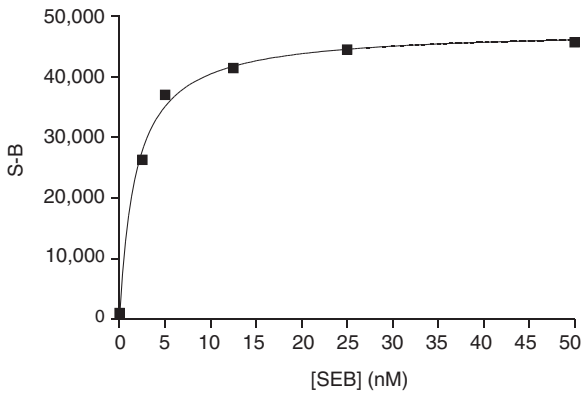

Figure 2 | SEB titration on an Abl assay using HTRF KinEASE. Abl (Millipore), used at 5 ng/well, was incubated for 30 min with substrate-biotin (1 μM), and a nonlimiting ATP concentration (100 μM). Different SEB concentrations ranging from 0 nM to 50 nM were tested in the kinase buffer. The specific signal is calculated as the signal obtained minus the background of the assay (S-B).

determined: that is, a concentration for which the signal reaches 80% of the maximum (EC_{80}).

For the titration, a tyrosine kinase is used at concentrations ranging from 0.10 to 10 ng/well (in 20 μl), and is incubated 30 min with the biotin-tagged generic substrate supplied with the kit (substrate-biotin; 1 μM) and an excess of ATP (100 μM). The substrate-biotin/streptavidin-XL665 ratio of 8/1 (62.2 nM streptavidin-XL665) and the ready-to-use Eu(K)-labeled antibody to the phosphotyrosine kinase are kept constant. The optimal enzyme concentration is chosen at EC_{80} based on the titration curve obtained.

Table 1 | IC50 for some protein kinase inhibitors on a selection of kinases

Enzyme	Kinase inhibitors	ATP (μM)	IC_{50} (nM)
Abl	Imatinib (Gleevec)	3.1	43
		31	461
		100	1,438
		IC_{50} (nM)	
		With SEB	Without SEB
Csk	Staurosporine	493	417
	Sorafenib	4,220	6,175
EGFR	Staurosporine	1,069	861
	Gefitinib	1	2
JAK2	Staurosporine	0.30	0.35
	Sunitinib	800	778
JAK3	Staurosporine	1	1
	Sunitinib	7,639	4,597
PTK5	Staurosporine	39	36
	Sorafenib	>100	>100

Imatinib is an ATP-dependent protein kinase inhibitor. The imatinib IC_{50} was calculated with HTRF KinEASE TK at increasing ATP concentrations: K_M (3.1 μM), $10K_M$ (31 μM) and an excess of ATP (100 μM). The results obtained are consistent with pharmacological data already published. The IC_{50} values of tyrosine kinases of interest were determined with HTRF KinEASE for a selection of kinases. The values obtained are similar to those obtained using the ^{32}P incorporation method used as a reference.

3. Enzyme kinetics: this depends on the kinase and the substrate concentrations, and is measured to optimize the duration of incubation.

A time-course study is performed using a constant concentration of kinase (determined in the previous step), substrate (1 μM) and an excess of ATP (100 μM).The reaction is stopped at different end points by the addition of the detection reagents (at 1, 2, 5, 10, 15, 30 and 60 min). The optimal incubation period to achieve maximal signal is chosen and kept constant for the rest of the optimization.

4. Substrate titration: to determine the substrate Michaelis-Menten constant (K_M), assays are run under the previously determined conditions (enzyme concentration and incubation period) using substrate concentrations ranging from 1 nM to 2 μM. During the detection step, the streptavidin-XL665 concentration is adjusted to keep the substrate/streptavidin-XL665 ratio constant at 8/1.
5. ATP titration: assays are run at a nonlimiting substrate concentration with 1.7 nM to 300 μM ATP, while the enzyme quantity and the incubation period are kept constant. The K_M is calculated from the resulting plot of the signal versus ATP concentration.
6. Detection optimization: the optimization of substrate-biotin/streptavidin-XL665 ratio is an important step that may lead to a substantial increase in signal. The assay is performed using the optimal enzyme, ATP and substrate concentrations. Three different molar ratios of substrate-biotin/streptavidin-XL665 are tested (2/1, 4/1, 8/1). The optimal ratio consists of a good compromise between signal and reagent consumption.

Pharmacological profiles of a selection of tyrosine kinases

To date, the platform has been validated on more than 104 serine/threonine kinases and 64 tyrosine kinases, including both receptor and cytoplasmic kinases. **Table 1** summarizes the half-maximal inhibitory concentrations (IC_{50}) of protein kinase inhibitors for a selection of tyrosine kinases. For the kinases tested, the SEB did not affect the IC_{50}.

Conclusion

Cisbio's line of HTRF KinEASE kits is based on our patented HTRF technology and can be used as a universal tool for assessing tyrosine kinase activity.

HTRF KinEASE kits limit assay development time and are easily miniaturizable as well as flexible, meaning the assay can be performed under a wide range of kinase assay conditions, for instance, with low consumption of enzyme or at any ATP concentration.

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