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HTRF® KinEASE™: a new solution for screening serine-threonine kinases

The generation of drugs that modulate kinase activity has become a prime focus of the pharmaceutical industry. Cisbio, in partnership with Upstate, has made available the universal HTRF KinEASE assay for serine-threonine kinases (STKs). This article describes the use of this assay for kinase assay optimization and adaptation for high-throughput screening.

There is a constant need to improve high-throughput assay formats to generate accurate and rapid results for kinase screening. One potential alternative to radiometric methods involves the use of phosphospecific antibodies that specifically recognize the phosphorylated target of a kinase. In the past, the development of assays for STKs has often been slow, owing to the limited availability of highly specific antibodies for each phosphorylated STK substrate. A new alternative, HTRF KinEASE, was designed to streamline the assessment of many STKs using a simple noncompetitive assay format, involving just one antibody and defined peptide substrates.

HTRF KinEASE combines Cisbio's proprietary homogeneous time-resolved fluorescence (HTRF) technology with proprietary monoclonal antibodies and three STK peptide substrates developed by Upstate. The antibody recognizes a phosphorylation epitope shared by the biotinylated peptides, and variation in the N-terminal sequence allows different STKs to be assessed.

In the assay described in **Figure 1**, incubation of an STK with one of the three peptides is followed by the addition of a premixed solution of europium cryptate (Eu(K))-labeled antibody and fluorophore-conjugated streptavidin (SA-XL665). A readout can be made after just 1 h of incubation, and the signal is stable at room temperature for at least 24 h.

Fluorescence resonance energy transfer (FRET) occurs when the two fluorescent tracers are brought into close proximity. At that moment, the XL665-specific signal resulting from FRET is proportional to the level of peptide phosphorylation. A ratio is calculated between the 665-nm emission from the XL665 and the emission obtained at 620-nm from the Eu(K) to make the measurement independent of any compound interference.

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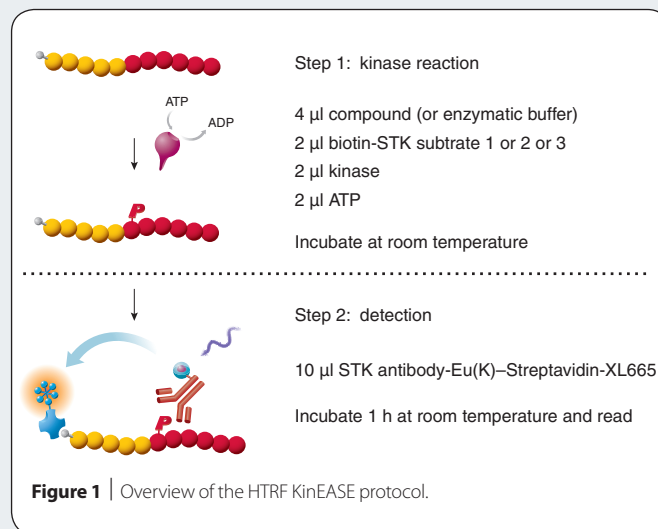
The HTRF KinEASE platform kits are available in small and bulk sizes suitable for assay development, high-throughput screening and profiling.

Each of the 72 kinases listed in **Table 1** has been validated and the most appropriate substrate has been determined. Depending on the kinase tested, HTRF KinEASE allows a signal-to-background ratio of between 5 and 30 to be obtained using 2 ng/well kinase, 1 µM substrate, 100 µM ATP, 30-min enzymatic reaction, 62.5 nM SA-XL665 and a ready-to-use solution of STK antibody-Eu(K). **Figure 2** shows the signal-to-background ratio obtained in a 20-µl final volume for PKCζ, PAK3 and IKKβ kinase assays using the optimal substrate.

Guidelines for assay optimization

Every step of the procedure has been optimized to generate rapid results.

For each new kinase, the first step involves identifying the optimal substrate with the Discovery kit, which contains all three substrates. In some cases, more than one substrate is compatible with a given STK,

Step 1: kinase reaction

ATP
ADP

4 µl compound (or enzymatic buffer)
2 µl biotin-STK subtrate 1 or 2 or 3
2 µl kinase
2 µl ATP

Incubate at room temperature

P

Step 2: detection

10 µl STK antibody-Eu(K)–Streptavidin-XL665

Incubate 1 h at room temperature and read

Figure 1 | Overview of the HTRF KinEASE protocol.

APPLICATION NOTES

Table 1 Validated Upstate kinases

Kinase	Optimal substrate
AMPK (rat), BrSK1, BrSK2, CaM KII(rat), CaM KIV, CHK1, CHK2, DRAK1, DAPK2, MAPKAPK2, MAPKAPK3, MELK, MLCK MRCKβ, PKCα, βI, βII, δ, ε, η, γ, ι, μ, θ, ζ, PKD2, PRK2, PRAK, PASK, PhKγ2, Rsk1/MAPKAPK1α, Rsk1/MAPKAPK1α (r), Rsk2/MAPKAPK1β, Rsk3, TSSK1, ZIPK	STK substrate 1–biotin
Aurora A, Aurora B, NEK11, PAK2, PAK3, PAK5, PAK6, PKA, PKG1α, PKG1β, PRKX, ROCKI, ROKα/ROCKII, Rsk4, TBK1	STK substrate 2–biotin
Akt1/PKBα, Akt2/PKBβ, Akt3/PKBγ, BrSK1, DAPK1, IKKα, IKKβ, LOK, MnK2, MRCKα, MSK1, MSK2, MST1 Pim1, Pim2, Snk, STK33, p70S6K, SGK, SGK2, SGK3	STK substrate 3–biotin

and the substrate that produces the highest signal should be selected for further investigation. The kinase is titrated using the optimal substrate to determine its optimal concentration for screening. We recommend choosing a kinase concentration at 80% efficient concentration (EC$_{80}$) of the titration curve to maximize the signal. Enzyme kinetics should be performed to determine the optimal incubation time. After fixing these parameters, ATP K_M, substrate K_M and the optimal streptavidin:biotin ratio can be determined. Finally, the assay is validated for screening using a reference inhibitor.

As an example, we evaluated inhibition of the kinase Aurora A by staurosporine using an enzyme concentration of 2 ng/well. We incubated the kinase with STK substrate 2–biotin ($K_M = 0.7$ μM) and ATP ($K_M = 70$ μM) for 10 min, then incubated with SA-XL665 (44 nM) and STK antibody–Eu(K). The 50% inhibition concentration (IC$_{50}$) for staurosporine (34.7 nM) could be reliably calculated for more than 24 h with standard deviations below 4% (experiments run in triplicate). The Z' factor, calculated between background (no enzyme) and maximum signal, was 0.91—a clear indication of the robustness of the assay.

Optimization of the screening conditions

The following additional parameters can be tested to enhance assay quality:

Figure 2 | Signal-to-background measurements obtained with HTRF KinEASE assays using three different STKs: PKCζ, PAK3 and IKKβ. Increasing kinase concentrations were tested using the optimal substrate. The concentration of substrate (1 μM), ATP (100 μM), SA-XL665 (62.5 nM) and STK antibody-Eu(K) and incubation time for the enzymatic step (30 min) were held constant.

1. With HTRF KinEASE, assays can be run at any ATP concentration. This feature is valuable to demonstrate ATP-competitiveness for lead compounds by increasing ATP concentrations up to 5 mM.
2. Enzyme and detection buffers are supplied with the kit. The enzyme buffer must be supplemented with MgCl$_2$, CaCl$_2$, dithiothreitol (DTT) or other cofactors as required by the kinase. Enzyme buffer compositions are available for STKs that have already been validated (**Table 1**). HTRF KinEASE is flexible enough to be compatible with a wide variety of enzyme buffers for further assay optimization.
3. Owing to the high stability of the HTRF detecting reagents, both are diluted into the detection buffer containing EDTA to stop the enzymatic reaction. This simplifies the procedure by eliminating an extra step for EDTA addition.
4. The choice of the peptide can affect the kinase kinetics. By simply changing the substrate, the enzymatic reaction time can be extended, thus making the assay more user-friendly and suitable for automated systems. This can be done when more than one substrate among the three available is compatible with the target STK.
5. HTRF KinEASE is easily adapted to different plate formats, from 96-well (100 μl final volume) to 1,536-well (8 μl final volume), without affecting assay performance, provided that all final reagent concentrations are maintained at the same level (that is, simply changing each volume added, keeping the same proportions).

In summary, HTRF KinEASE combines several advantages for kinase screening. By using antibody-based detection, HTRF KinEASE offers the high reliability of specific and sensitive immunodetection. In addition, the use of universal substrates greatly simplifies the assay development phase, as a common assay format is being applied. Given the high-throughput capabilities of HTRF, this new platform represents an ideal solution for kinase profiling and screening. Additional information is available on our company website (http://www.htrf.com).

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This article was submitted to *Nature Methods* by a commercial organization and has not been peer reviewed. *Nature Methods* takes no responsibility for the accuracy or othe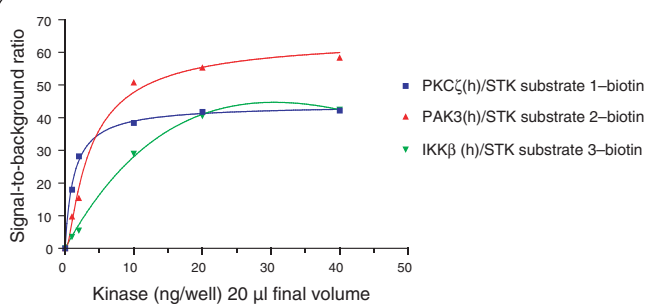rwise of the information provided.