# HTRF® KinEASE™

A universal expanded platform to address Serine/Threonine & Tyrosine kinases

APPLICATION NOTE

**ABSTRACT** Kinases are enzymes that play a central role in various signal transduction pathways involved in the control of cell growth, metabolism, differentiation and apoptosis. They exert their effect by catalysing the transfer of a phosphate group from ATP onto a target substrate (protein/peptide), which then becomes activated and performs a specific function.

HTRF KinEASE are universal tools for assessing Serine/Threonine (STK) and Tyrosine Kinase (TK) activity. A typical development for an HTRF KinEASE assay consists of five steps, and is described in this document.

Protein kinases are the second most important group of drug targets after GPCRs. The approval of two kinase-targeting drugs, Genentech's Herceptin® in 1998 and Novartis's Gleevec® in 2001, and their success in cancer therapeutics, confirm the trend towards screening new Protein Kinase Inhibitors (PKI) of various kinase targets for use in different therapeutic areas.

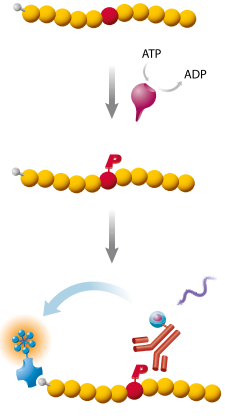
This growing interest in screening kinases has prompted the development of many assay technologies.

The new HTRF KinEASE TK kit for the measurement of Tyrosine Kinase (TK) activities combines a universal 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.

HTRF KinEASE STK is the fourth kit in the HTRF KinEASE platform, developed in collaboration with Millipore (Upstate) for profiling and HTS of Serine/Threonine and Tyrosine Kinases. The platform's other three kits are dedicated to the Ser/Thr kinases group and combine three universal biotinylated substrates, S1, S2 and S3 and a monoclonal antibody. The new HTRF KinEASE kit contains a generic substrate and a monoclonal antibody.

## HTRF KINEASE: ASSAY IMPLEMENTATION

HTRF KinEASE screenings limit assay development time and are easily miniaturizable and 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 enzymatic (kinase reaction) step followed by the detection step with HTRF reagents (Fig. 1). The assay is started by the addition of ATP (Step 1) and stopped by the addition of the HTRF detection reagents containing EDTA (Step 2).




1. ENZYMATIC STEP
- 4 µL Compound
- 2 µL Biotin-STK or TK substrate
- 2 µL Kinase
- 2 µL ATP

Incubate at 37°C

2. DETECTION STEP
- 5 µL Anti Eu(K)-STK or TK antibody
- 5 µL Streptavidin-XL665

Incubate 1h at RT then Read on an HTRF reader

Fig. 1: HTRF KinEASE assay principle: All the assays based on HTRF KinEASE involve two steps, the enzymatic step and the detection step with HTRF reagents. Step 1: During the enzymatic step the substrate-biotin is incubated with the kinase of interest. ATP is added to start the reaction. Step 2: The detection reagent catches the phosphorylated substrate and the resulting TR-FRET is proportional to the phosphorylation level.

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## A STRAIGHTFORWARD KINASE ASSAY DEVELOPMENT

A typical development for an HTRF KinEASE assay consists of five steps, and is described in a document supplied with the kit. The conditions of each step are described for MAPKAP-K2 using HTRF KinEASE STK-S1.

### 1. ENZYME TITRATION

This step gives the optimal kinase concentration, i.e.that for which the signal reaches 80% of the maximum ($EC_{80}$). Kinase is used at concentrations ranging from 0.10 ng/well to 10 ng/well, and incubated 30 min with the substrate-biotin (1 µM), and a non-limiting ATPconcentration (100 µM). The reaction is stopped by the addition of the HTRF detection reagents in EDTA. The substrate-biotin/SA-XL665 ratio of 8/1 (i.e. 62.2nM SAXL665)and the ready to use phospho specific monoclonal antibody labeled with Europium Cryptate (Eu(K)) are kept constant. The optimal enzyme concentration is chosen at $EC_{80}$ of the titration curve obtained

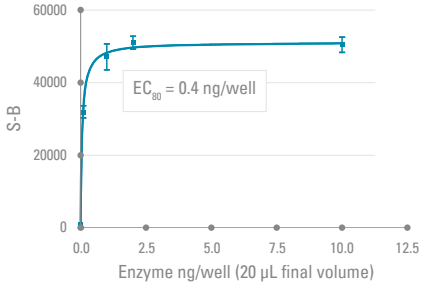



Fig. 2: MAPKAP-K2 titration

### 2- ENZYME KINETIC

Enzyme kinetic depends on the kinase and the substrate concentrations. A time course study is performed using 0.4ng of MAPKAP-K2 per well, the constant concentration of kinase determined in the previous experiment, 1 µM of substrate and a non-limiting ATP concentration (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 for MAPKAP-K2 (0.4 ng/well) to achieve maximum signal and a linear time course is chosen at 5 min, this incubation time is kept constant for the rest of the optimization .

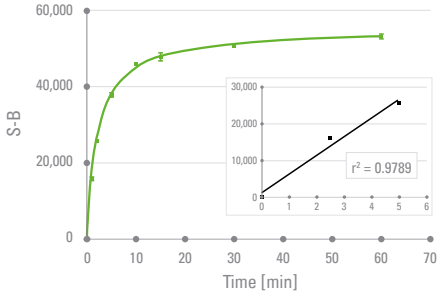



Fig 3: MAPKAP-K2 enzyme kinetic

### 3- SUBSTRATE TITRATION

In order to determine the substrate $K_M$ (app), assays are run under the conditions previously obtained (enzyme concentration: 0.4ng/well and incubation period: 5 min) using substrate concentrations ranging from 1 nM to 2 µM. During the detection step, the SA-XL665 concentration is adjusted to keep the substrate/streptavidin-XL665 ratio constant at 8/1. The signal is plotted versus substrate concentrations, and the $K_M$ (app) of 80nM is calculated using Michaelis-Menten equation.

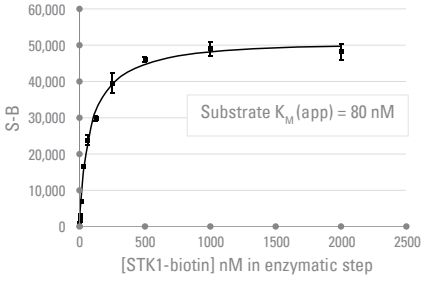



Fig 4: MAPKAP-K2 substrate titration.

### 4- ATP TITRATION

Assays are run at a non-limiting substrate concentration with ATP from 1.7 nM to 300 µM, while the enzyme quantity (0.4 ng/well) and the incubation period (5 min) are kept constant.

As in the previous step, the ATP $K_M$ (app) of 2.3 µM is calculated from the resulting plot of the signal versus ATP concentrations.

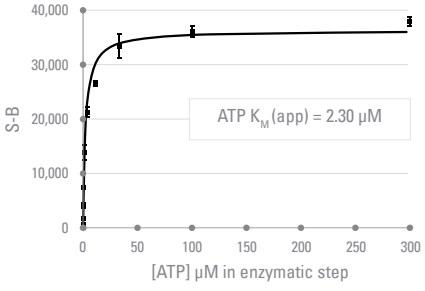


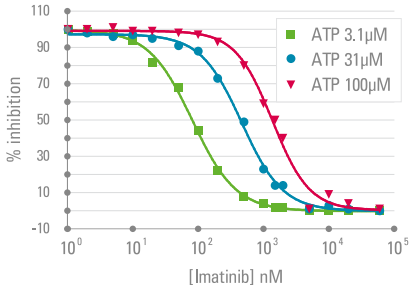

Fig 5: MAPKAP-K2 ATP titration

### 5- DETECTION STEP OPTIMIZATION

The optimization of substrate-biotin/SA-XL665 ratio is an important step which 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 obtains a good compromise between signal level and reagent consumption.

## 6- INHIBITOR TITRATION

The kinase activity is tested over a broad range of inhibitor concentrations to generate a dose-response curve. The test is generally run using the opitmal assay conditions as determined previously. Table beside shows reference inhibitor $IC_{50}$ for STK and TK kinases using HTRF KinEASE.




| [ATP] | 3.1µM | 31µM | 100µM |
|---|---|---|---|
| EC50 | 43nM | 460.7nM | 1438nM |

Fig 6: Imatinib is an ATP dependant PKI. The imatinib $IC_{50}$ is calculated with HTRF KinEASE at increasing ATP concentrations, $K_M$ (3.1µM), 10 $K_M$ (31µM), an excess of ATP (100µM), 1µM TK substrate-biotin, 1ng/well enzyme, 4/1 ratio biotin/SaXL665, [SEB]=5nM(1) and 20min incubation for the enzymatic step. The results obtained are consistent with pharmacological data already published.

| ENZYME | HTRF KINEASE | COMPOUND | $IC_{50}$ NM |
|---|---|---|---|
| Abl | TK | Staurosporine | 108 |
| | | Imatinib* | 43* |
| Csk | TK | Staurosporine | 493 |
| | | Sorafenib* | 4,220 |
| EGFR | TK | Staurosporine | 1,07 |
| | | Gefitinib* | 1* |
| JAK2 | TK | Staurosporine | 0.30 |
| | | Sunitinib* | 800 |
| JAK3 | TK | Staurosporine | 1 |
| | | Sunitinib* | 7,64 |
| PTK5 | TK | Staurosporine | 39 |
| | | Sorafenib* | >100 |
| MAPKAP-K2 | STK S1 | Staurosporine | 92 |
| CaMK IV | STK S1 | Staurosporine | 4.3 |
| PKC beta 2 | STK S1 | Staurosporine | 6 |
| Rsk3 | STK S1 | Staurosporine | 3.1 |
| Rock-II | STK S2 | Staurosporine | 10.4 |
| Pim-1 | STK S2 | Staurosporine | 8.7 |

Table 1: The $IC_{50}$ of TKIs of interest are calculated with HTRF KinEASE on a election of kinases. The values obtained are similar to the 32P incorporation method used as a reference.

*(1) SEB (Cisbio proprietary buffer) can be used for optimal kinase TK activity and is provided with KinEASE TK.*

## A PLATFORM VALIDATED ON MORE THAN 160 KINASES

Today, the platform has been validated on more than 100 Ser/Thr kinases and 60 Tyrosine kinases including both receptor and cytoplasmic kinases. Please refer to www.htrf.com/kaa for an updated list of kinases already tested with HTRF. Serine threonine kinases validated with HTRF KinEASE STK & TK:

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| HTRF® KinEASE STK S1 | AMPK | BrSK2 | CAMKI | CaMKII α | CaMKII ß | CaMKII δ | CaMKII γ | CaMKIV | CHEK1 | CHEK2 | CHK1 |
| | CHK2 | DAPK1 | DAPK2 | DAPK3 | DCAMKL2 | DRAK1 | eEF-2K | LKB1 | MAPKAP-K1 α | MAPKAPK1 β | MAPKAP-K2 |
| | MAPKAP-K3 | MAPKAPK5 | MELK | MLCK | Mnk2 | PASK | PHK γ 2 | PKC α | PKC ß I | PKC ß II | PKC δ |
| | PKC ε | PKC γ | PKC ι | PKC µ | PKC θ | PKC ζ | PKD2 | PRAK | PRK2 | PRKA | PRKD2 |
| | PRKG2 | RPS6KA2 | Rsk1 | Rsk2 | Rsk3 | STK22B | STK22D | TSSK1 | TSSK2 | ZIPK | |
| HTRF® KinEASE STK S2 | Aurora A | Aurora B | MARK2 | NEK11 | PAK2 | PAK3 | PAK4 | PAK5 | PAK6 | PAR-1B α | PKA |
| | PKG1 α | PKG1 ß | PRKACA | ROCK-I | ROCK-II | ROK α | RPS6KA6 | Rsk4 | STK6 | | |
| HTRF® KinEASE STK S3 | AKT1 | AKT2 | AKT3 | ARK5 | ASK1 | Aurora C | BrSK1 | CDC42 BPA | CDC42 BPB | CLK3 | COT |
| | DMPK | DYRK2 | DRAK2 | GRK5 | GRK6 | HIPK2 | HIPK3 | IKK α | IKK ß | LOK | MAP3K8 |
| | MARK1 | MINK | MINK1 | MRCK α | MRCK ß | MSK1 | MSK2 | MSSK1 | MST1 | MST2 | NEK2 |
| | NEK3 | NEK6 | NEK7 | NLK | p70S6K | Pim-1 | Pim-2 | PKB α | PKB ß | PKB γ | PLK3 |
| | RPS6KA4 | RPS6KA5 | RPS6KB1 | SAD1 | SGK1 | SGK2 | SGK3 | SGKL | SIK | Snk | STK23 |
| | STK3 | STK4 | TBK1 | WNK2 | WNK3 | | | | | | |
| HTRF® KinEASE TK | Abl | ABL2 | ALK | Arg | Axl | Blk | Bmx | BRK | BTK | c-Kit | CSF1R |
| | CSK | c-SRC | DDR2 | EGFR | EphA2 | EphA3 | EphA4 | EphA5 | EphA7 | EphA8 | EphB1 |
| | EphB2 | EphB3 | EphB4 | ErbB4 | FAK | Fer | Fes | FGFR1 | FGFR2 | FGFR3 | FGFR4 |
| | Fgr | Flt1 | Flt3 | Flt4 | Fms | Fps | FRK | Fyn | Hck | IGF-1R | INSRR |
| | Insulin R | IRR | ITK | JAK2 | JAK3 | KDR | Kit | LCK | Lyn | Mer | Met |
| | MST1R | MuSK | NTRK1 | NTRK2 | PDGFR α | PDGFR ß | PTK2 | PYK2 | PTK5 | PTK6 | Ret |
| | Ron | Ros | Rse | Src | Syk | TEK | Tie2 | TRKA | TRKB | TYRO3 | VEGFR1 |
| | VEGFR2 | VEGFR3 | Yes | YES1 | ZAP-70 | | | | | | |

The recommended substrates were determined for Millipore kinases.

## 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 Serine/Threonine and Tyrosine kinase activity.

HTRF KinEASE kits limit assay development time and are easily miniaturizable and 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.

## RELATED INFORMATION


HTRF® KinEASE™ TK: A new solution for Tyrosine kinases screening.
*Drexler C., SBS 13th annual conference 2007, Montreal (Canada).*

Universal Expanded Platform to address serine/threonine and Tyrosine Kinases.
*Tardieu J.L., BIOforum Europe, 42 (6/2007).*

HTRF® KinEASE™ : a new solution for screening serine-threonine kinases.
*Drexler C., Nature Methods, June 2006.*

HTRF® KinEASE™: Development of sensitive, reliable Aurora A and AMPK kinase inhibitory assays.
*Martinez S., SBS 12th annual conference. September 2006, Seattle (USA).*

HTRF® KinEASE™: A universal assay for Serine/Threonine kinases.
*Claret E., SBS 12th annual conference. September 2006, Seattle (USA).*


## ORDERING INFORMATION

HTRF® KinEASE™ for Serine / Threonine and Tyrosine kinases

| DESCRIPTION | TESTS | PART# |
|---|---|---|
| HTRF® KinEASE™-STK discovery (STK substrates 1, 2 and 3-biotin) | 1,000 tests | 62ST0PEB |
| HTRF® KinEASE™-STK S1 (STK substrate 1-biotin) | 1,000 tests<br>20,000 tests<br>100,000 tests | 62ST1PEB<br>62ST1PEC<br>62ST1PEJ |
| HTRF® KinEASE™-STK S2 (STK substrate 2-biotin) | 1,000 tests<br>20,000 tests<br>100,000 tests | 62ST2PEB<br>62ST2PEC<br>62ST2PEJ |
| HTRF® KinEASE™-STK S3 (STK substrate 3-biotin) | 1,000 tests<br>20,000 tests<br>100,000 tests | 62ST3PEB<br>62ST3PEC<br>62ST3PEJ |
| HTRF® KinEASE™-TK (TK substrate biotin) | 1,000 tests<br>20,000 tests<br>100,000 tests | 62TK0PEB<br>62TK0PEC<br>62TK0PEJ |

HTRF® KinEASE™ companion component

| DESCRIPTION | TESTS | PART# |
|---|---|---|
| STK substrate 1-biotin | 50 µg/vial<br>500 µg/vial | 61ST1BLE<br>61ST1BLC |
| STK substrate 2-biotin | 50 µg/vial<br>500 µg/vial | 61ST2BLE<br>61ST2BLC |
| STK substrate 3-biotin | 50 µg/vial<br>500 µg/vial | 61ST3BLE<br>61ST3BLC |
| TK substrate -biotin | 50 µg/vial<br>500 µg/vial | 61TK0BLE<br>61TK0BLC |
| Sa-XL665 | 250 µg<br>1 mg<br>3 mg | 610SAXLA<br>610SAXLB<br>610SAXLG |
| 5x Enzymatic buffer | 50 mL | 62EZBFDD |
| SEB buffer for HTRF KinEASE TK assays | 20,000 tests (lyoph, to be reconst. with 5 ml H2O) | 61SEBALB |
| HTRF® Detection buffer | 200 mL | 62SDBRDF |

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Europe and other countries +33(0)466 796 705 U.S. and Canada 1-888-963-4567 China +86 21 5018 9880
Japan +81 (0)43 306 8712
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