APPLICATION NOTE

HTRF KinEASE a universal expanded platform to address serine/threonine & tyrosine kinases.

This application note provides you the five steps required for a typical development for an HTRF KinEASE assay.

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.

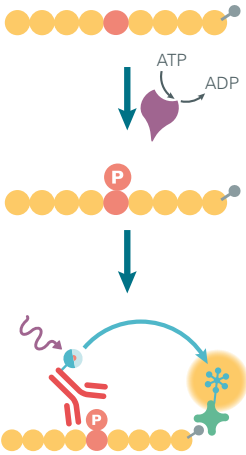
This growing interest in screening kinases has prompted the development of many assay technologies.

The HTRF KinEASE TK kit for the measurement of Tyrosine Kinase (TK) activities combines a universal peptide substrate and a single proprietary monoclonal antibody with Revvity'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 a semi-universal platform of three kits that are dedicated to the Ser/Thr kinases group. Those assays 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 and have been fully validated for over 200 kinases.

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

Figure 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.

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

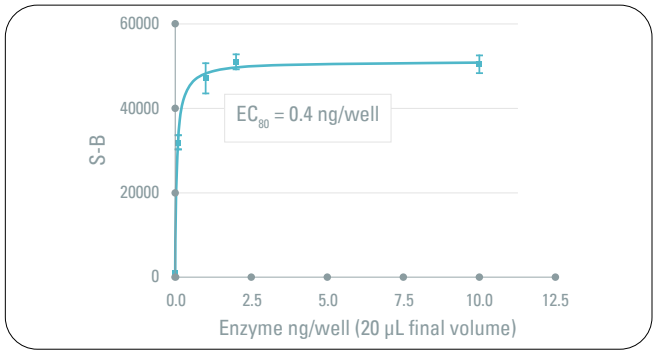


Figure 2: MAPKAP-K2 titration.

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

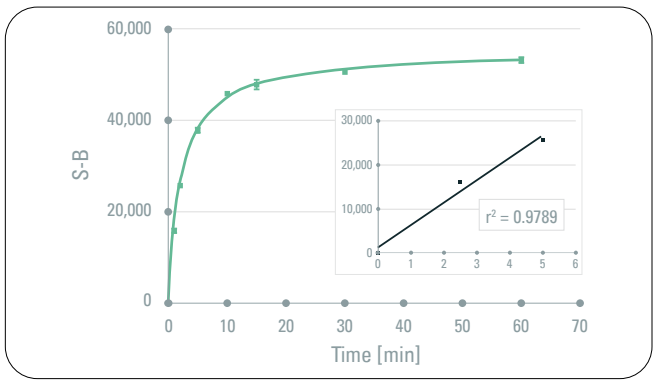


Figure 3: MAPKAP-K2 enzyme kinetic.

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.

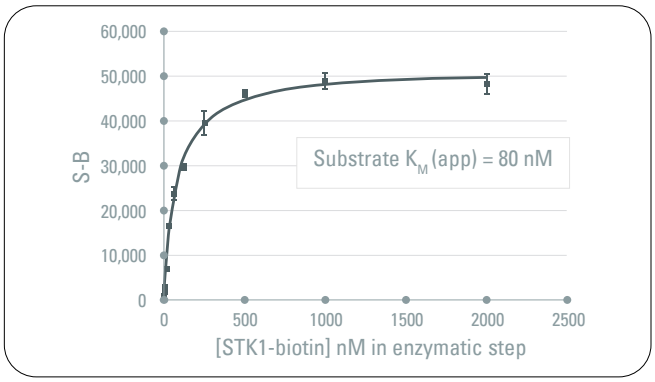


Figure 4: MAPKAP-K2 substrate titration.

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 KM (app) of 2.3 µM is calculated from the resulting plot of the signal versus ATP concentrations.

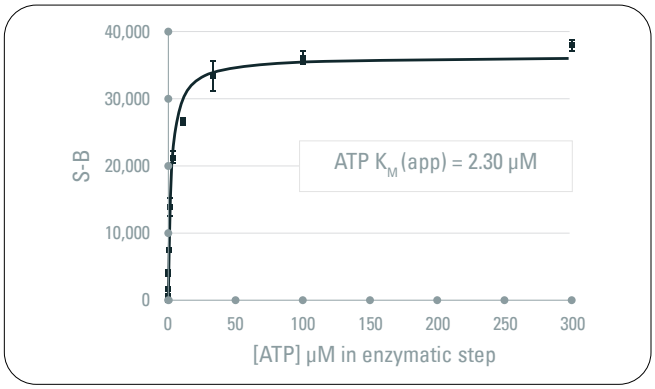


Figure 5: MAPKAP-K2 ATP titration.

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.

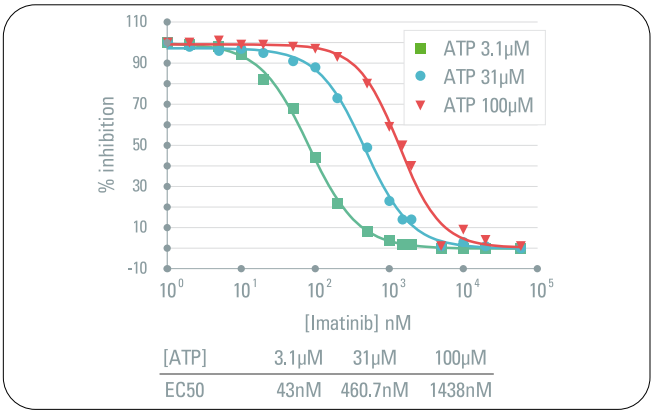


Figure 6: Imatinib is an ATP dependant PKI. The imatinib IC_{50} is calculated with HTRF KinEASE at increasing ATP concentrations, KM (3.1µM), 10 KM (31µM), an excess of ATP (100µM), 1µM TK substrate-biotin, 1ng/well enzyme, 4/1 ratio biotin/SaXL665, [SEB]=5nM(1) and 20min incubation for the enzymatic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

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.

Enzyme	HTRF KinEASE	Compound	Ic_{50} nm
Abl	TK	Staurosporine Imatinib	108 43
Csk	TK	Staurosporine Sorafenib	493 4 220
EGFR	TK	Staurosporine Gefitinib	1,07 1
JAK2	TK	Staurosporine Sunitinib	0.30 800
JAK3	TK	Staurosporine Sunitinib	1 7,64
PTK5	TK	Staurosporine Sorafenib	39 >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

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

A platform validated on more than 200 kinases

Today, the platform has been validated on more than 180 Ser/Thr kinases and 95 Tyrosine kinases including both receptor and cytoplasmic kinases.

KinEASE TK	ABL1, ABL2, ALK, Arg, AXL, BLK, BMX, BRK, BTK, c-Kit, CSF1R, CSK, DDR2, EGFR, EPHA1, 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, HER4, IGF1R, INSR, INSRR, Insulin R, IRR, ITK, JAK1, JAK2, JAK3, KDR, KIT, LCK, Lyn, LYNa, MER, MERTK, MET, MST1R, MSTIR, MUSK, NTRK1, NTRK2, NTRK3, PDGFR α, PDGFR β, PDGFRA, PDGFRB, PTK2, PTK2B, PTK5, PTK6, PYK2, RET, RON, ROS, ROS1, Rse, SRC, SYK, TEK, TIE2, TRKA, TRKB, TRKC, TYK2, TYR03, TYRO3, VEGFR1, VEGFR2, VEGFR3, YES1, ZAP70
KinEASE STK S1	AMPK α2/β1/γ1, BrSK2, CAMK1, CaMK1 α, CaMK2 α, CaMK2 δ, CaMK2 γ, CaMK2 β, CAMK2B, CAMK2D, CAMK2G, CaMK4, CHEK1, CHEK2, CHK1, CHK2, DAPK1, DAPK2, DAPK3, DCAMKL2, DRAK1, GPRK4, GRK4, GRK6, GSG2, Haspin, IRAK4, LATS1, LKB1, MAPKAP-K1α, MAPKAP-K1 β, MAPKAP-K2, MAPKAP-K3, MAPKAP-K5, MARK1, MELK, MKNK1, MKNK2, MLCK, MNK1, MNK2, MYLK, PASK, PHK γ1, PHK γ2, PKC μ, PKCα, PKC δ, PKC ε, PKC η, PKC γ, PKC ι, PKC β1, PKC β2, PKC θ, PKC ζ, PKD2, PKG2, PKN2, PLK1, PRAK, PRK2, PRKA α2/β1/γ1, PRKCA, PRKCB1, PRKCB2, PRKCD, PRKCE, PRKCG, PRKCH, PRKCI, PRKCQ, PRKCZ, PRKD2, PRKG2, RPS6KA1, RPS6KA2, RPS6KA3, RSK1, RSK2, RSK3, SIK, smMLCK, STK22B, STK22D, TBK1, TSSK1, TSSK2, ZIPK
KinEASE STK S2	AURKA, AURKB, Aurora A, Aurora B, CGK2, MARK2, NEK11, PAK2, PAK3, PAK4, PAK5, PAK6, PAK7, PAR-1B α, PKACαa, PKG1 α, PKG1 β, PLK4, PRKACA, PRKG1, PRKX, ROCK1, ROCK2, ROK α, RPS6KA6, RSK4, STK6
KinEASE STK S3	AKT1, AKT2, AKT3, ARK5, ASK1, Aurora C, BrSK1, CDC42 BPA, CDC42 BPB, CHUK, CLK3, COT, DMPK, DYRK2, EEF-2K, GRK5, GSK3 α, HIPK2, HIPK3, IKBKB, IKK α, IKK β, LOK, MAP3K8, MINK1, MRCK α, MRCK β, MSK1, MSK2, MSSK1, MST1, MST2, NEK2, NEK3, NEK6, NEK7, NLK, p70S6K, PIM1, PIM2, PKAC g, PKB α, PKB γ, PKB β, PLK3, PRKACG, RPS6KA4, RPS6KA5, RPS6KB1, SAD1, SGK1, SGK2, SGK3, SGKL, Snk, STK23, STK3, STK4, WNK2, WNK3

The recommended substrates were determined for Carna Biosciences kinases.

Conclusion

Revvity'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.

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	62ST1PEB
	20,000 tests	62ST1PEC
HTRF KinEASE-STK S2 (STK substrate 2-biotin)	1,000 tests	62ST2PEB
	20,000 tests	62ST2PEC
HTRF KinEASE-STK S3 (STK substrate 3-biotin)	1,000 tests	62ST3PEB
	20,000 tests	62ST3PEC
HTRF KinEASE-TK (TK substrate biotin)	1,000 tests	62TK0PEB
	20,000 tests	62TK0PEC

HTRF KinEASE companion components

Description	Tests	Part#
STK substrate 1-biotin	50 µg/vial	61ST1BLE
	500 µg/vial	61ST1BLC
STK substrate 2-biotin	50 µg/vial	61ST2BLE
	500 µg/vial	61ST2BLC
STK substrate 3-biotin	50 µg/vial	61ST3BLE
	500 µg/vial	61ST3BLC
TK substrate -biotin	50 µg/vial	61TK0BLE
	500 µg/vial	61TK0BLC
Sreptavidin-XL665	5 000 tests	610SAXLA
	20 000 tests	610SAXLB
5x Enzymatic buffer	10 mL	62EZBFDC
	50 mL	62EZBFDD
SEB - Suppl. Enzymatic Buffer	2.5 nmoles	61SEBALF
	12.5 nmoles	61SEBALB
HTRF Detection buffer	40 mL	62SDBRDD
	200 mL	62SDBRDF

Revvity, Inc.
940 Winter Street
Waltham, MA 02451 USA

(800) 762-4000
www.revvity.com