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www.htrf.com**HTRF® package insert**

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For in vitro research use only
 Storage temperature: 2-8°C

Packaging details:

384-well low volume plate (20 µL)		
62TKOPEC	HTRF® KinEASE™-TK	20,000 tests

1. Assay description and intended use

HTRF® KinEASE™ -TK is a generic method for measuring tyrosine kinase activities using one substrate and a universal detection system. To date, more than 59 kinases have already been tested with this kit (see Appendix).

The HTRF® KinEASE™-TK assay format involves the two steps described below:

1. Enzymatic step: During this step, the kinase will phosphorylate the substrate. The TK Substrate-biotin is incubated with the kinase. ATP is added to start the enzymatic reaction.

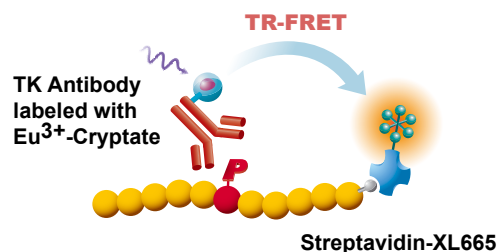
2. Detection step: The detection reagents will catch the phosphorylated substrate. The resulting TR-FRET signal is proportional to the phosphorylation level. The TK-Antibody labeled with Eu³⁺-Cryptate and streptavidin-XL665 are then added with EDTA (used to stop the kinase activity).

**TK Substrate-biotin**

↓ Kinase
ATP



↓ EDTA
Detection reagents

**Streptavidin-XL665**

2. Kit description – Stock solution preparation

The kit is designed to allow 20,000 tests to be run at 20 μ L final volume, providing that the substrate final concentration is $\leq 1 \mu$ M and streptavidin/substrate ratio $\leq 1/4$.

Components	Quantity		Stock solutions to prepare
TK Substrate -biotin	1 vial of 500 μ g Lyophilized	⇒	Reconstitute with distilled water (refer to product label) to obtain a 500 μ M stock solution
Streptavidin-XL665	1 vial of 3 mg Lyophilized	⇒	Reconstitute with distilled water (refer to product label) to obtain a 16.67 μ M stock solution in streptavidin
TK Antibody-Cryptate	1 vial Lyophilized	⇒	Reconstitute with 1 mL of distilled water (refer to product label) to obtain the TK Antibody-Cryptate stock solution *
Supplement Enzymatic buffer (SEB reagent)	1 vial Lyophilized	⇒	Reconstitute with 5 mL of distilled water to obtain a 2,500 nM SEB stock solution.
5x Enzymatic buffer HEPES 250 mM (pH7.0), NaN ₃ 0.1%, BSA 0.05%, Orthovanadate 0.5 mM	1 vial of 50 mL Liquid 5x		
HTRF® Detection buffer HEPES 50 mM (pH7.0)with additives	1 vial of 200 mL Liquid 1x		

Storage: All kit components must be stored at +4°C until the expiration date printed on the product label.

After reconstitution, the stock solutions can be stored 1 week at +4°C or dispensed into single use aliquots and stored at -20°C. Avoid repeated freezing and thawing.

*Cryptate conjugate working solution (prepared with frozen stock solution) should be filtered before use to improve assay reproducibility.

3. Additional material required (not provided)

	Recommended Supplier*	Stock solution to prepare
Kinase	Upstate (www.upstate.com)	Follow supplier's instructions
ATP	Sigma # A7699	5 mM in HEPES buffer 50 mM

Enzymatic buffer supplements: The enzymatic buffer must be supplemented with any components required by the kinase of interest (See Appendix for further details).

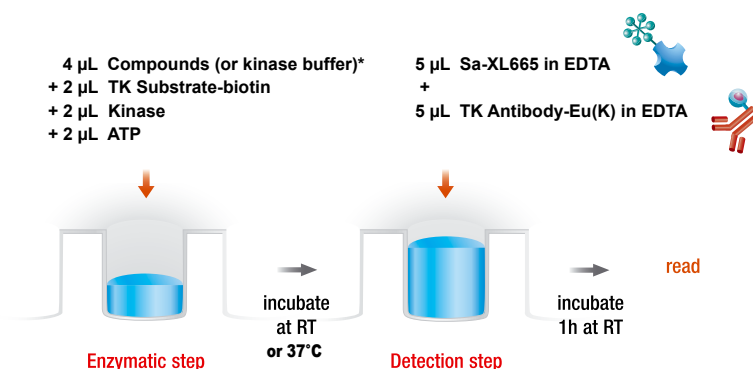
	Recommended Supplier*	Stock solution to prepare
DTT	Sigma # D0632	100 mM in distilled water
MgCl₂	Sigma # M1028	1 M (ready to use)
MnCl₂	Sigma # M1787	1 M (ready to use)

* Suppliers' names are indicative.

Storage: Stock solutions should be aliquoted and stored at -80°C for kinases and -20°C for ATP and DTT.

4. Assay protocol for 384w low volume plate (20µL)

Add assay components (working solutions) in the following order:



The kinase reaction is started by the addition of ATP (step 1) and is stopped by the addition of the detection reagents which contain EDTA (step 2). The incubation period for the enzymatic step is optimized depending on the kinase (§ 8.3).

For a 384w low volume plate, we recommend the 10 µL enzymatic step and the 10 µL detection step for a final assay volume of 20 µL. For a 96 half well plate (100 µL), each addition volume is simply multiplied by 5.

* For low volume compound addition, adjust volume to 4 µL with 1x kinase buffer. Keep DMSO ≤ 2% in the enzymatic step.

5. Preparation of the working solutions

The working solutions are prepared from stock solutions (§ 2-3) by following the instructions below:

	Buffer to prepare
Kinase buffer 1X	Prepare “enzymatic buffer 1X “ by diluting 1 volume of enzymatic buffer 5X with 4 volumes of distilled water and any supplements required by the kinase of interest, i.e. DTT, MgCl ₂ , MnCl ₂ , etc. (see appendix). For some tyrosine kinases , the addition of the SEB reagent in the kinase buffer 1X will help to catalyze the enzymatic reaction (refer to §8.1 : SEB titration).
HTRF® Detection buffer	Ready to use

	Component working solutions to prepare
Compounds	Dilute compound stock solution with kinase buffer to prepare a working solution which has 2.5X the required final concentration for the enzymatic step
TK Substrate-biotin	Dilute the substrate stock solution (500 µM) with kinase buffer to prepare a working solution which has 5X the required final concentration for the enzymatic step
Kinase	Dilute the kinase stock solution with kinase buffer to prepare a working solution which has 5X the required final concentration for the enzymatic step
ATP*	Dilute the ATP stock solution (5 mM) with kinase buffer to prepare a working solution which has 5X the required final concentration for the enzymatic step
Sa-XL665**	Dilute the SaXL665 stock solution (16.67 µM) with HTRF® detection buffer to prepare a working solution which has 4X the required final concentration for the assay (20 µL)
TK-Antibody-Cryptate	Dilute the TK Antibody-Cryptate stock solution 1:100 in HTRF® detection buffer to obtain the working solution (e.g. for 20,000 tests :1 mL of TK Antibody-Cryptate stock solution + 99 mL of HTRF® detection buffer)

* for an ATP 100 µM in the enzymatic step, prepare a 500 µM ATP working solution.

** for an Sa-XL665 125 nM final concentration, prepare a 500 nM SaXL665 working solution.

Precautions:

- It is recommended to prepare the required amount of Kinase buffer 1X (supplemented with ions, DTT required by the enzyme of interest and SEB) just before use as DTT and SEB are stable only one day at 2-8°C once diluted in the enzymatic buffer.
- Working solutions cannot be stored and must be used immediately.
- The enzyme working solution must be kept in an ice bath for the time of the experiment (to avoid degradation).
- HTRF® cryptate conjugate concentration have been set for optimal assay performances. Note that any dilution or improper use of the detection reagents will impair the assay's quality.

6. Kinase assay and controls

The kinase assay is performed as described below, using three different controls:

Negative control: used to calculate the specific signal. Appropriate Negative controls must be prepared for each Sa-XL665 concentration tested (§ 8.4).

Buffer control: used to make sure that buffers are not contaminated by Cryptate and do not generate any background fluorescence.

Cryptate control: used to check the Cryptate signal at 620 nm.

	Kinase assay		Controls	
Enzymatic step (10 µL)	Sample	Negative	Cryptate	Buffer
Compounds (or kinase buffer)	4 µL	4 µL kinase buffer	10 µL kinase buffer	10 µL kinase buffer
TK Substrate-biotin	2 µL	2 µL		
Kinase	2 µL	2 µL kinase buffer		
ATP	2 µL	2 µL		
Seal plate and incubate at RT or 37°C				
Detection step (10 µL)				
Sa-XL665	5 µL	5 µL	5 µL detection buffer	10 µL detection buffer
TK Ab-Cryptate	5 µL	5 µL	5 µL	
Seal plate and incubate 1h at RT				
Remove plate sealer and read on an HTRF® compatible reader*				
*More information at http://www.htrf.com/technology/htrfmeasurement/compatible_readers/				

Data reduction:

The fluorescence is measured at 620 nm (Cryptate) and 665 nm (XL665). A ratio is calculated (665/620) for each well. Results are expressed as follows:

Specific signal = Ratio (Sample) – Ratio (Negative control)

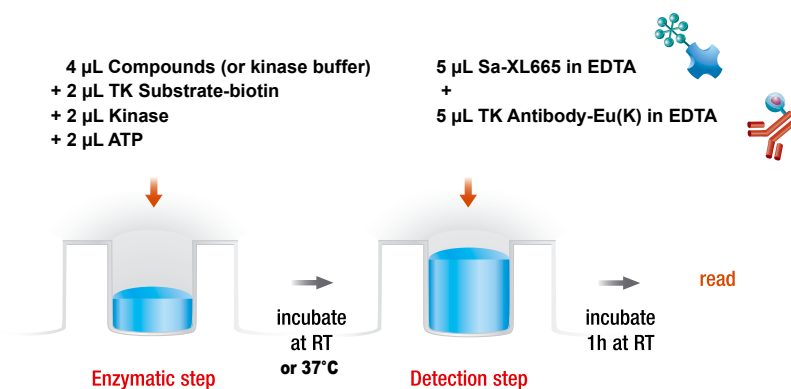
Ratio = (665 nm/620 nm) x 10⁴

Mean ratio = Σ ratio/2 (n=2)

CV% = (Std deviation/Mean ratio)*100

7. Assay miniaturization and flexibility

When used as suggested, the kit will provide sufficient reagents for 20,000 tests using a 384-well low volume plate in 20 μL final assay volume (HTRF® packaged basis):



Other plate formats (96-half-well or 1536-well) and final volumes (100 μL to less than 10 μL) can be used by simply proportionally adjusting each addition volume in order to maintain the concentrations as for the 20 μL final assay volume.

Assay format:	Miniaturization		
	1536-well (10 μL)	384-well low volume (20 μL)	96 half-well (100 μL)
Compounds/kinase/ Substrate/ATP	2 / 1 / 1 / 1 μL	4 / 2 / 2 / 2 μL	20 / 10 / 10 / 10 μL
Sa-XL665	2.5 μL	5 μL	25 μL
TK-Ab-Cryptate	2.5 μL	5 μL	25 μL
Number of test per kit	40,000 tests	20,000 tests	4,000 tests

Plate references: 96 half-well plate (Costar # 3694 or equivalent), 384-well low volume plate (Greiner # 784076), 1536-well (Greiner # 782086).

8. Optimization of the kinase assay

A typical development for an HTRF® KinEASE™-TK assay consists of the following steps:

1. SEB titration
2. Enzyme titration
3. Kinetic study
4. Substrate titration
5. ATP titration
6. Biotin/streptavidin ratio optimization
7. Inhibitor IC₅₀ determination

Final concentrations of the assay components used for kinase assay optimization are :

		Conc. Max.	Conc. Min.
TK-Substrate-biotin	Final conc. in the enzymatic step (10 μL)	2 μM	0.97 nM
Kinase		10 ng/well (1 ng/ μL)	0.1 ng/well (0.01 ng/ μL)
ATP		300 μM	1.7 nM
Sa-XL665	Final conc. in the final assay volume (20 μL)	125 nM	0.06 nM
TK-Ab-Cryptate		Ready to use	Ready to use

8.1. SEB titration

Some tyrosine kinases may require the addition of SEB reagent in the kinase buffer 1x for optimal enzymatic activity. This step enables the optimal SEB concentration, i.e. that for which the signal reaches 80% of the maximum, to be determined.

Prepare a series of Kinase buffer 1X supplemented with different concentrations of SEB ranging from 125 nM to 0 nM (Control kinase buffer 1X): dilute SEB stock solution 2500 nM 1/20 in Kinase buffer 1X to get a SEB working concentration of 125 nM. Next, make 2 fold serial dilutions in kinase buffer 1x to reach a SEB working concentration of 2nM.

The different SEB supplemented kinase buffers are dispensed under 4 μ L (first dispensing step - refer to assay protocol § 4).

To calculate SEB final concentration in the enzymatic step, divide the SEB working concentrations by 2.5.

The vial of SEB reagent enables to perform 1,000 tests using a final SEB concentration of 50 nM during the enzymatic step (maximal SEB concentration required on a selection of 59 tyrosine kinases - see Appendix for further details).

Kinase, TK-substrate-biotin and ATP must be diluted in kinase buffer 1X non supplemented with SEB.

For this step, we recommend the use of a fixed concentration of kinase (10 ng/well * in 384 half well plates, 20 μ L final volume) and saturating concentrations of TK-substrate-biotin (1 μ M)* and ATP (100 μ M)*. Allow the enzymatic reaction to run for 30 mn at RT. Add the detection reagents. The biotin/streptavidin molar ratio must be 8/1 (i.e. 62.5 nM Sa-XL665**).

The signal (ratio sample (with enzyme) – ratio negative) is plotted versus the different SEB concentrations. Determine the optimal SEB concentration for the following experiments, targeting the SEB concentration for which the signal reaches 80% of the maximum.

NB: For the next experiments, prepare the kinase buffer 1X supplemented with SEB at the desired working concentration just before use. This buffer is stable one day at 2-8°C. Enzyme, substrate, ATP and compound can be diluted directly in the SEB kinase buffer.

* Final concentrations in the enzymatic step (10 μ L).

** Final assay concentration (20 μ L).

8.2. Enzyme titration

This step allows the optimal enzyme concentration (for which the signal reaches 80% of the maximum) to be determined. A compromise may be found between a high assay signal and the enzyme consumption.

For this step, a fixed concentration of the TK-substrate-biotin (1 μ M) and ATP (100 μ M) should be tested with the following enzyme concentrations 10; 2; 1; 0.1 ng/well. Allow the enzymatic reaction to run for 30 mn.

The biotin/streptavidin ratio of 8/1 must be used (i.e. 62.5 nM Sa-XL665).

8.3. Kinetic study

Enzyme kinetic depends on the kinase and substrate concentrations.

A time course study is performed using a constant concentration of kinase (determined in the previous experiment), ATP (100 μ M) and substrate (1 μ M). The reaction is stopped at different end points by the addition of the detection reagents (1, 2, 5, 10, 15, 30, 60 min). The biotin /streptavidin ratio must remain constant and equal to 8/1 (i.e. 62.5 nM Sa-XL665).

The signal is then plotted versus the different end points. Determine the linear part of the time course (correlation coefficient $R^2 > 0.99$) and from this section, the optimal incubation time to use for the next experiments.

8.4. Substrate titration

This step allows the determination of substrate K_m (app).

Use the optimal enzyme concentration (§ 8.2) and a saturating ATP concentration (100 μ M). We recommend testing different TK substrate-biotin concentrations ranging from 2 μ M to 0.97 nM (two fold serial dilutions). The kinase reaction is stopped at the previously determined optimal incubation period.

During the detection step, it will be necessary to adjust the concentration of the SA-XL665 for each TK substrate-biotin concentration, in order to keep the biotin/streptavidin ratio constant at 8/1 as described in the following table.

Furthermore, since the background may rise with increasing XL665 concentrations, it is necessary to run a negative control (no enzyme) for each Sa-XL665 concentration.

TK Substrate-biotin		Sa-XL665
Final conc. in the enzymatic step (10 μ L)	Final assay conc. (20 μ L)	Final assay conc. (20 μ L)
2 μ M	1 μ M	0.125 μ M
1 μ M	0.5 μ M	62.50 nM
0.5 μ M	0.25 μ M	31.25 nM
0.25 μ M	0.125 μ M	15.61 nM
0.125 μ M	62.50 nM	7.81 nM
62.50 nM	31.25 nM	3.90 nM
31.25 nM	15.61 nM	1.95 nM
15.61 nM	7.81 nM	0.97 nM
7.81 nM	3.90 nM	0.48 nM
3.90 nM	1.95 nM	0.24 nM
1.95 nM	0.97 nM	0.12 nM
0.97 nM	0.485 nM	0.06 nM

The plot of the specific signal (ratio sample (with enzyme) – ratio negative) versus the substrate concentrations is then fitted to Michaelis-Menten or Lineweaver-Burke equations to calculate the substrate K_m (app).

8.5. ATP titration

This step allows the determination of ATP K_m (app).

Use the optimal enzyme concentration and a saturating TK-substrate-biotin concentration (1 μ M).

We recommend testing ATP concentrations ranging from 300 μ M to 1.7 nM (three fold serial dilutions). The kinase reaction is stopped at the optimal incubation period by adding the detection reagents.

During the detection step, the biotin/streptavidin ratio must be fixed at 8/1 (62.5 nM SA-XL665). As in the previous step, the K_m (app) value must be determined from this experiment using either a Michaelis-Menten or a Lineweaver-Burke plot.

8.6. Biotin/streptavidin ratio optimization

The optimization of the biotin/streptavidin ratio is an important step which may lead to a substantial increase in signal.

Streptavidin-XL665 solutions are prepared in order to cover 2/1, 4/1, 8/1 biotin/streptavidin ratios. The test is run using the optimal enzyme, ATP and substrate concentrations (§ 8.1-5).

Negative controls corresponding to each Sa-XL665 concentration must be used as this reagent has a direct contribution to the background level.

8.7. Inhibitor IC₅₀ determination

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 previously determined optimal assay conditions.

9. HTRF® KinEASE™ product line

The most appropriate HTRF® KinEASE™ or HTRF® KinEASE™-TK assay system can be used depending on your specific applications (see table below).

HTRF® KinEASE™ kits consist of substrate(s)-biotin, antibody labeled with Europium Cryptate (Eu(K)), Sa-XL665, enzymatic and HTRF® detection buffers. Three packaging sizes are available using a 20 μ L test format.

The kit discovery that includes the three STK substrates-biotin (1, 2 and 3) is designed to quickly test the desired Ser/Thr kinase. Once the substrate that works with the desired kinase has been identified, the kit S1, S2 or S3 including the most appropriate substrate can be used for kinase assay development.

If larger volumes are required for HTS or profiling, kits are available in Bulk or Jumbo sizes. The kit reagents like substrate-biotin, Sa-XL665 and assay buffers can also be ordered separately.

HTRF® KinEASE™ for Serine / Threonine kinases

Description	Quantity	Cat no.
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 Bulk 20,000 tests Jumbo 100,000 tests	62ST1PEB 62ST1PEC 62ST1PEJ
HTRF® KinEASE™-STK S2 (STK substrate 2-biotin)	1,000 tests Bulk 20,000 tests Jumbo 100,000 tests	62ST2PEB 62ST2PEC 62ST2PEJ
HTRF® KinEASE™-STK S3 (STK substrate 3-biotin)	1,000 tests Bulk 20,000 tests Jumbo 100,000 tests	62ST3PEB 62ST3PEC 62ST3PEJ
STK substrate 1-biotin	50 µg/vial 500 µg/vial	61ST1BLE 61ST1BLC
STK substrate 2-biotin	50 µg/vial 500 µg/vial	61ST2BLE 61ST2BLC
STK substrate 3-biotin	50 µg/vial 500 µg/vial	61ST3BLE 61ST3BLC

HTRF® KinEASE™ for Tyrosine kinases

Description	Quantity	Cat no.
HTRF® KinEASE™-TK	1,000 tests Bulk 20,000 tests Jumbo 100,000 tests	62TK0PEB 62TK0PEC 62TK0PEJ
TK substrate -biotin	50 µg/vial 500 µg/vial	61TK0BLE 61TK0BLC

Companion products

Description	Quantity	Cat no.
Sa-XL665	250 µg 1 mg 3 mg	610SAXLA 610SAXLB 610SAXLG
5x Enzymatic buffer HTRF® Detection buffer	50 mL 200 mL	62EZBFDD 62SDBRDF