MANUAL

HTRF KinEASE-TK kit

Part # 62TK0PEB, 62TK0PEC & 62TK0PEJ

Test size: 1,000 tests (62TK0PEB), 20,000 tests (62TK0PEC), 100,000 tests (62TK0PEJ)

Revision: #06 of September 2023

Assay volume: 20 µL

Store at: 2-8°C

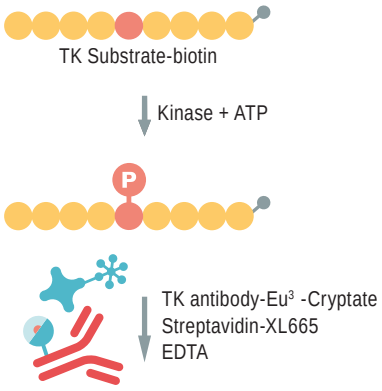
This product is intended for research purposes only. The product is not intended to be used for therapeutic or diagnostic purposes.

ASSAY DESCRIPTION

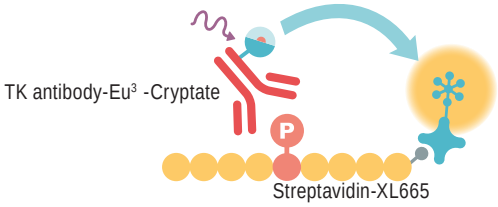
HTRF® KinEASE-TK is a generic method for measuring Tyrosine kinase activities using one substrate and a universal detection system. More than 60 kinases have been tested with this kit (visit our website at: *www.revvity.com*).

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.

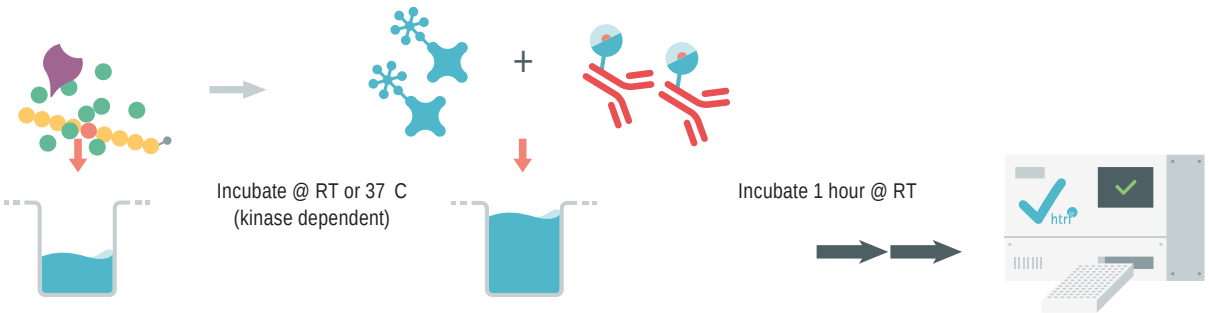


2. Detection step:
The detection reagents will catch the phosphorylated substrate. The resulting TR-FRET signal is proportional to the phosphorylation level.
The TK-Antibody labeled with Eu^{3+}-Cryptate and streptavidin-XL665 are mixed in a single addition with EDTA (used to stop the kinase activity).



MANUAL AT A GLANCE

ADD **READ & ANALYSE**



4 µL compounds (or kinase buffer)
2 µL kinase
2 µL TK Substrate-biotin
2 µL ATP

5 µL Sa-XL665 in detection buffer
5 µL TK antibody-Eu^{3} in detection buffer

MATERIALS:

Kit components	1,000 tests Cat # 62TK0PEB	20,000 tests Cat # 62TK0PEC	100,000 tests Cat # 62TK0PEJ
TK Substrate-biotin *Lyophilized*	1 vial of 50 µg # 61TK0BLE*	1 vial of 500 µg # 61TK0BLC*	3 vials of 500 µg # 61TK0BLC*
Streptavidin-XL665 MW: 60 KDa *Lyophilized*	1 vial of 250 µg # 610SAXLA*	1 vial of 3 mg # 610SAXLG*	5 vials of 3 mg #610SAXLG*
TK Antibody-Cryptate *Lyophilized*	1 vial of 1,000 tests	1 vial of 20,000 tests	5 vials of 20,000 tests
Supplemented Enzymatic Buffer (SEB) *Lyophilized*	1 vial # 61SEBALF*	1 vial # 61SEBALB*	5 vials # 61SEBALB*
Enzymatic buffer *5X*	1 vial of 10mL # 62EZBFDC*	1 vial of 50mL # 62EZBFDD*	5 vials of 50mL # 62EZBFDD*
Detection buffer (contains EDTA) *Ready to use*	1 vial of 40 mL # 62SDBRDD*	1 vial of 200 mL # 62SDBRDF*	5 vials of 200 mL # 62SDBRDF*

* These kit reagents are also available separately

ADDITIONAL MATERIAL - NOT PROVIDED

	Recommended supplier*	Stock solution to prepare
HTRF 96-well low volume plate**	Revvity # 66PL96001	
Kinase	Upstate - follow supplier's instructions	follow supplier's instructions
ATP	Sigma # A7699	5 mM in 50 mM Hepes buffer

* Suppliers' names are indicative

**For HTRF microplate recommendations, please visit *www.revvity.com.*

For reading, an HTRF®-compatible reader is needed. Make sure to use the setup for Eu^{3+} Cryptate.

For a list of HTRF®-compatible readers and setup recommendations, please visit *www.revvity.com.*

- The enzymatic buffer must be supplemented with any components required by the kinase of interest. You will find details for all the validated enzymes in the technical notes posted on our website at: *www.revvity.com*

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

* Suppliers' names are indicative

STORAGE AND STABILITY

Store the kit at 2-8°C or below. Under appropriate storage conditions, reagents are stable until the expiry date indicated on the 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. The volume of antibody aliquots should not be under 10 µL.

Enzymatic and detection buffer can be stored at 2-8°C on your premises.

REAGENT PREPARATION

BEFORE YOU BEGIN:

- It is very important to prepare the reagents in the specified buffers. The use of an incorrect diluent may affect reagent stability and assay results.
- Before use, allow all reagents to warm up at room temperature.
- Homogenize buffers with a vortex.
- It is recommended to filter buffers before use.
- Detection reagent solutions must be prepared in individual vials and can be mixed prior to dispensing.

TAKE CARE TO PREPARE STOCK AND WORKING SOLUTIONS ACCORDING TO THE DIRECTIONS FOR THE KIT SIZE YOU HAVE PURCHASED.

PREPARATION OF WORKING SOLUTIONS:

- Working solutions cannot be stored and must be used immediately, except for the TK Antibody-Cryptate which can be stored 1 week at +4°C or dispensed into single use aliquots and stored at -20°C.
- In order to avoid degradation, the enzyme working solution must be kept in an ice bath for the time of the experiment.
- The enzymatic buffer 5X does contain 0.05% BSA but is not supplemented with nonionic surfactants. The surfactant of your choice can be added in case of aggregation or sticking issue.
- DTT and SEB diluted in the enzymatic buffer are stable one day at 2-8°C.
- HTRF® detection reagent concentrations have been set for optimal assay performances. Note that any dilution or improper use of the XL665 and Cryptate-conjugates will impair the assay's quality.

Buffer preparation	
Kinase buffer = 1X supplemented enzymatic buffer	Dilute 1 volume of enzymatic buffer 5X with 4 volumes of distilled water and complement it with all the supplements required by the kinase of interest, i.e. DTT, MgCl2, MnCl2, etc. (for informations about validated kinases, see, application notes on *www.revvity.com*. For some tyrosine kinases, the addition of the SEB reagent in the kinase buffer 1X will help to catalyze the enzymatic reaction (refer to SEB titration pg. 5)
Detection buffer	Ready to use

Reagent preparation	1,000 tests	20,000 tests	100,000 tests
TK Substrate-biotin	**For 1,000 tests size kit** **1.** Reconstitute the vial of 50 µg with distilled water to obtain a 50 µM TK Substrate-biotin stock solution (refer to product label). **2.** Dilute the 50 µM TK Substrate-biotin stock solution with 1X kinase buffer to prepare the working solution which has 5X the required final concentration for the enzymatic step (10 µL). E.g. For 10 nM final concentration, prepare a 50 nM working solution: dilute 1000-fold the reconstituted 50 µM TK Substrate-biotin stock solution	**For 20,000 tests and 100,000 tests size kit** **1.** Reconstitute the vial of 500 µg with distilled water to obtain a 500 µM TK Substrate-biotin stock solution (refer to product label). **2.** Dilute the 500 µM TK Substrate-biotin stock solution with 1X kinase buffer to prepare the working solution which has 5X the required final concentration for the enzymatic step (10 µL). E.g. For 10 nM final concentration, prepare a 50 nM working solution: dilute 10000-fold the reconstituted 50 µM TK Substrate-biotin stock solution	
Streptavidin-XL665 MW: 60 Kda	**1.** Reconstitute with distilled water to obtain a 16.67 µM stock solution in streptavidin (refer to product label). **2.** Dilute the 16.67 µM Streptavidin stock solution with detection buffer to prepare a working solution which has 4X the required final concentration for the final volume assay (20 µL). E.g. For 125 nM final concentration prepare a 500 nM working solution: dilute 33.3-fold the reconstituted 16.67 µM Sreptavidin-XL665 stock solution.		
TK Antibody-Cryptate	**For 1,000 tests size kit** Reconstitute the vial of 1,000 tests with 5 mL of Detection buffer to get the ready to use TK-antibody-cryptate solution.	**For 20,000 tests and 100,000 tests size kit** **1.** Reconstitute the vial of 20,000 tests with 1 mL of distilled water to get a 100X TK-antibody-cryptate intermediate solution. **2.** To obtain the final 1X TK-antibody-cryptate working solution, dilute 100-fold the TK-antibody-cryptate intermediate solution with the detection buffer. E.g. Add 99 mL of detection buffer to 1 mL of intermediate TK-antibody-cryptate intermediate solution.	
Supplemented Enzymatic Buffer (SEB)	**For 1,000 tests size kit** Reconstitute the vial of 1,000 tests with 1 mL of distilled water to obtain a 2,500 nM SEB stock solution.	**For 20,000 tests and 100,000 tests size kit** Reconstitute each vial of 20,000 tests with 5 mL of distilled water to obtain a 2,500 nM SEB stock solution.	
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 (10 µL).		
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 (10 µL).		
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 (10 µL). E.g. For an ATP 100 µM in the enzymatic step, prepare a 500 µM ATP working solution.		

ASSAY MANUAL

		Kinase assay	Negative
		Enzymatic step - 10 µL	
1		4 µL of compounds* 2 µL of TK Substrate -biotin 2 µL of kinase 2 µL of ATP**	4 µL of compounds* 2 µL of TK Substrate-biotin 2 µL of kinase buffer 2 µL of ATP**
2		Seal the plate and incubate*** at room temperature or 37°C.	
		Detection step - 10 µL	
3		5 µL of Streptavidin-XL665 5 µL of TK Antibody-cryptate	
4		Seal the plate and incubate 1 hour at room temperature.	
5		Remove the plate sealer and read on an HTRF® compatible reader.	

- * For low volume compound addition, adjust volume to 4 µL with 1x kinase buffer. Keep DMSO ≤ 2% in the enzymatic step.
- ** The kinase reaction is started by the addition of ATP (Enzymatic step) and is stopped by the addition of the detection reagents which contain EDTA (detection step). The incubation time is dependent on the activity of the kinase tested.
- *** The incubation period for the enzymatic step is optimized depending on the kinase.

DATA REDUCTION & INTERPRETATION

1. Calculate the ratio of the acceptor and donor emission signals for each individual well.

$$\text{Ratio} = \frac{\text{Signal 665 nm}}{\text{Signal 620 nm}} \times 10^4$$

2. Calculate the delta ratio of the acceptor and donor emission signals for each individual well. The Negative control plays the role of an internal assay control.

$$\text{delta Ratio} = \text{Ratio Standard or sample} - \text{Ratio Standard 0}$$

3. Calculate the % CVs. The mean and standard deviation can then be worked out from ratio replicates.

$$\text{CV (\%)} = \frac{\text{Standard deviation}}{\text{Mean Ratio}} \times 100$$

For more information about data reduction, please visit www.revvity.com

OPTIMIZATION OF A 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 Antibody-Cryptate		Ready to use	Ready to use

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 buffer1x to reach a SEB working concentration of 2nM.
The different SEB supplemented kinase buffers are dispensed under 4µL (first dispensing step - refer to assay manual pg. 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 application notes on *www.revvity.com*).
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, 20µL final volume) and saturating concentrations of TK-Substrate-biotin (1 µM)* and ATP (100 µM)*. 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**)

* Final concentrations in the enzymatic step (10 µL).
** Final assay concentration (20 µL).

2. ENZYME TITRATION

This step enables 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 µM) and ATP (100 µM) 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).

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 µM) and substrate (1 µM). 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 $R2>0.99$) and from this section, the optimal incubation time to use for the next experiments.

4. SUBSTRATE TITRATION

This step enables the determination of substrate Km (app).
Use the optimal enzyme concentration and a saturating ATP concentration (100 µM). We recommend testing different TK Substrate-biotin concentrations ranging from 2 µM 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 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 µL)	Final assay conc. (20 µL)	Final assay conc. (20 µL)
2 µM	1 µM	0.125 µM
1 µM	0.5 µM	62.50 nM
0.5 µM	0.25 µM	31.25 nM
0.25 µM	0.125 µM	15.61 nM
0.125 µM	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 Km (app).

5. ATP TITRATION

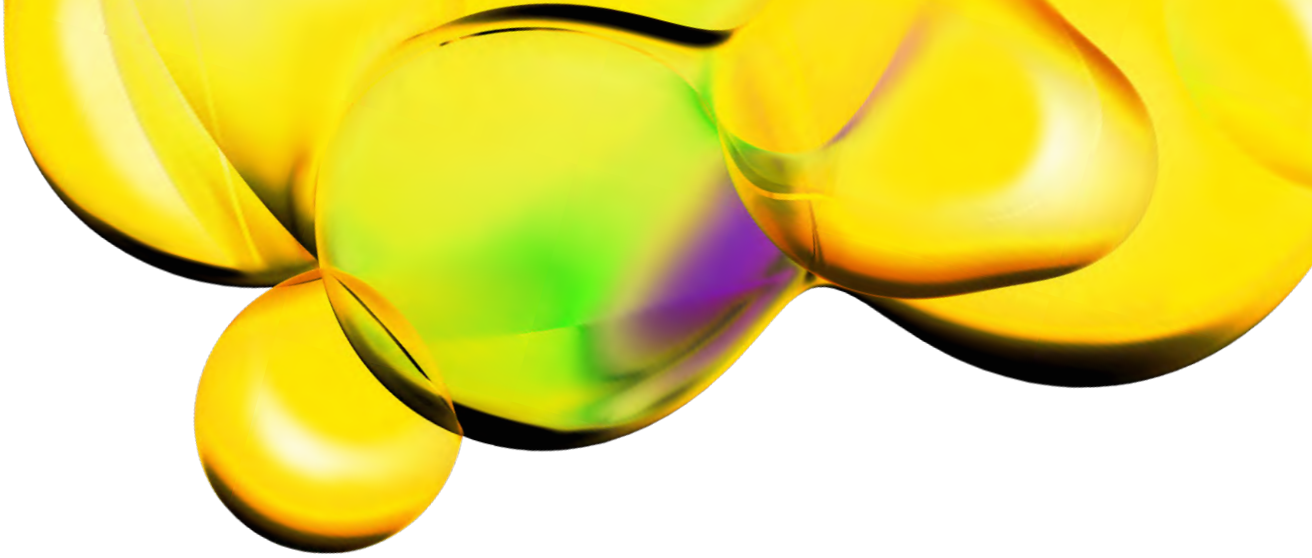
This step enables the determination of ATP Km (app).
Use the optimal enzyme concentration and a saturating TK Substrate-biotin concentration (1 µM).
We recommend testing ATP concentrations ranging from 300 µM to 1.7 nM (three-fold serial dilutions). The kinase reaction is stopped at the optimal incubation 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 Km (app) value must be determined from this experiment using either a Michaelis-Menten or a Lineweaver-Burke plot.

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

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.



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