STAUROSPORINE-RED

PROTOCOL

Part # 62KB01REDC & 62KB01REDE
Amount: 1 nmol (62KB01REDC) & 20 nmol (62KB01REDE)
Concentration: 25 µM in DMSO
Form: Frozen
Store at: -16°C or below
Revision: 01 - March 2019
For research use only. Not for use in diagnostic procedures.

ASSAY PRINCIPLE

Cisbio Staurosporine-Red is intended for both quantitative measurement of the dissociation constant (K_D) and inhibitor evaluation (IC_{50}/K_I) on GST-tagged, 6His-tagged, and N-terminal biotinylated kinases using HTRF® technology. For additonal information, please refer to the *HTRF® Kinase Binding Guide*.

The binding of Staurosporine-Red is detected in a sandwich assay format using a specific anti GST , anti-6His, or Streptavidin labeled with Europium Cryptate (donor) which binds to the tagged-kinase, and a red fluorescent derivative of Staurosporine labelled with d2 (acceptor). The detection principle is based on HTRF technology. When the dyes are in close proximity, the excitation of the donor with a light source (laser or flash lamp) triggers a Fluorescence Resonance Energy Transfer (FRET) towards the acceptor, which in turn fluoresces at a specific wavelength (665 nm). The HTRF ratio (665/620) will increase upon the addition of more of the Staurosporine-Red, and will saturate depending on the dissociation constant (K_D) of the Staurosporine-Red to the tagged kinase (Fig.1) The various HTRF Kinase Binding Discovery Kits serve to determine which of the three tracers (e.g. Staurosporine-Red, Dasatinib-Red, or Sunitinib-Red) is best suited to setting up an inhibitor assay on the kinase to be studied.

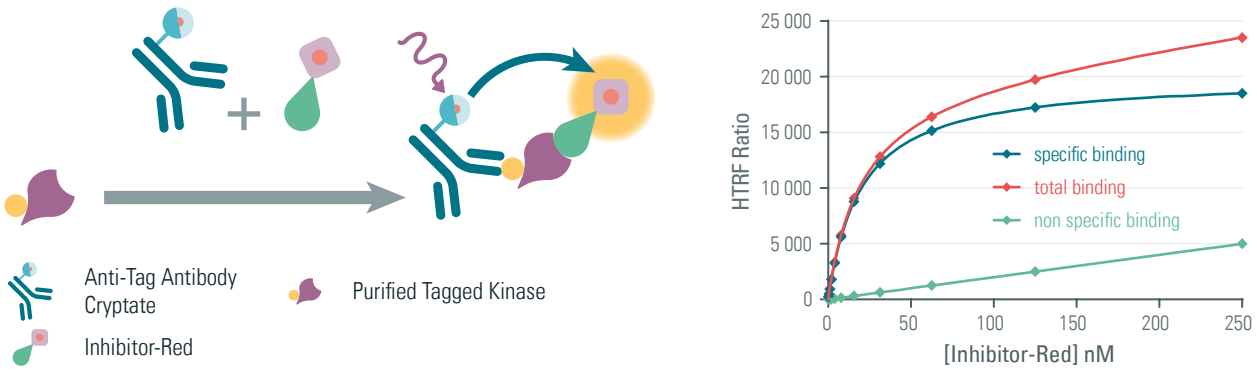


Figure 1: Principle of HTRF kinase saturation binding assay (K_D determination)

If Staurosporine-Red has a good K_D and assay window for the tagged-Kinase of interest, competitive binding assays can be set up for screening or pharmacological study, using a concentration of between 1 and 4 K_D of Staurosporine-Red (Fig.2).

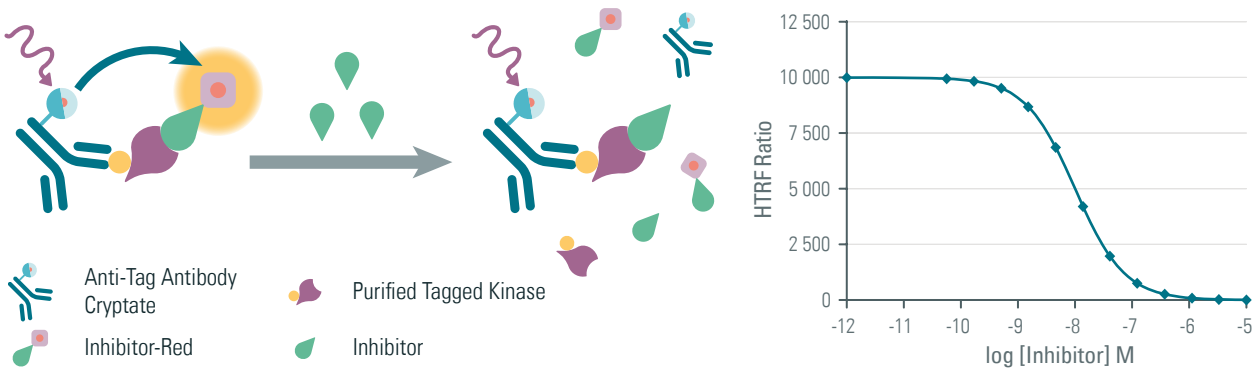
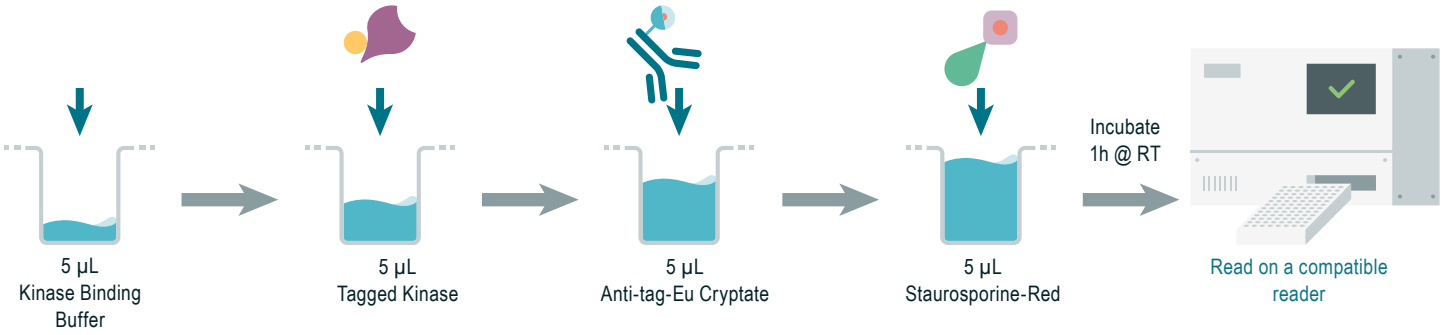


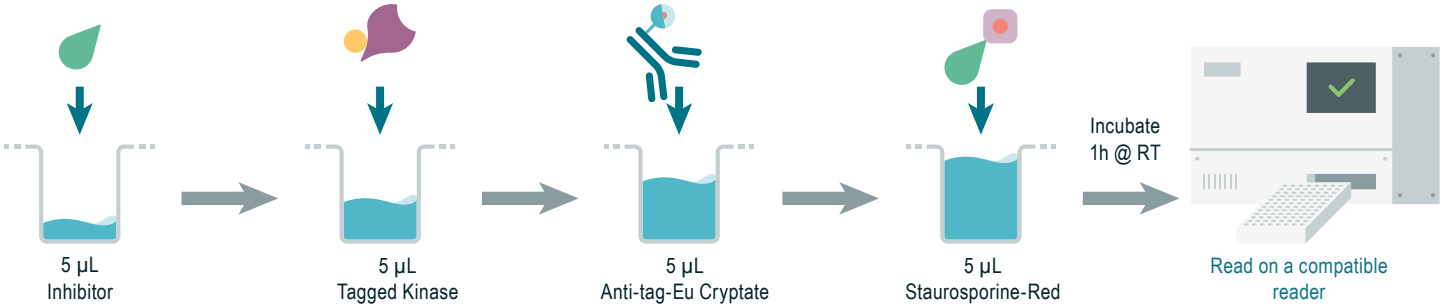
Figure 2: Principle of HTRF kinase competition binding assay (IC_{50} - KI determination)

PROTOCOL AT A GLANCE

SATURATION BINDING ASSAY (K_D DETERMINATION)



COMPETITION BINDING ASSAY (IC_{50} - K_i DETERMINATION)



MATERIALS PROVIDED:

	1 nmol Cat # 62KB01REDC	20 nmol Cat # 62KB01REDE
Staurosporine-Red (25 µM in DMSO)	1 vial - 40 µL	1 vial - 800 µL

STORAGE

Store the Staurosporine-Red at -16°C or below.

To avoid freeze/thaw cycles, it is recommended to dispense remaining stock solutions into disposable plastic vials for storage at -16°C or below.

PURCHASE SEPARATELY:

- Kinase Binding Buffer (# 62KBBRDD, # 62KBBRDF)
- Anti-Tag Cryptate Kinase Binding
  - MAb Anti-GST-Eu cryptate Kinase Binding (# 62KBGSTKAF, # 62KBGSTKAB)
  - MAb Anti-6HIS-Eu cryptate Kinase Binding (# 62KBHISKAF, # 62KBHISKAB)
  - Streptavidin-Eu cryptate Kinase Binding (# 62KBSAKAF, # 62KBSAKAB)
- Purified tagged or biotinylated-Kinase (e.g. Carna Biosciences)
- DMSO
- HTRF 96-well low volume plate Ref# 66PL96001 *
- HTRF 384-well low volume plate Ref 66PL384025 *
- Non-binding 96-well black plate
- HTRF-Certified Reader **. Make sure the setup for Eu^{3+} Cryptate is used.
- To perform the assay, use white plate only.

* For HTRF microplate recommendations, please visit http://www.cisbio.com/drug-discovery/htrf-microplate-recommendations
** For a list of HTRF-compatible readers and setup recommendations, please visit http://www.cisbio.com/compatible-readers

REAGENT PREPARATION FOR K_D DETERMINATION

- It is very important to prepare Staurosporine-Red solution in the HTRF Kinase Binding Buffer (we recommend filtering (0.22 µm) the buffer before use). The use of an incorrect diluent may affect reagent stability and assay results.
- Thaw Staurosporine-Red and homogenize it with a vortex.

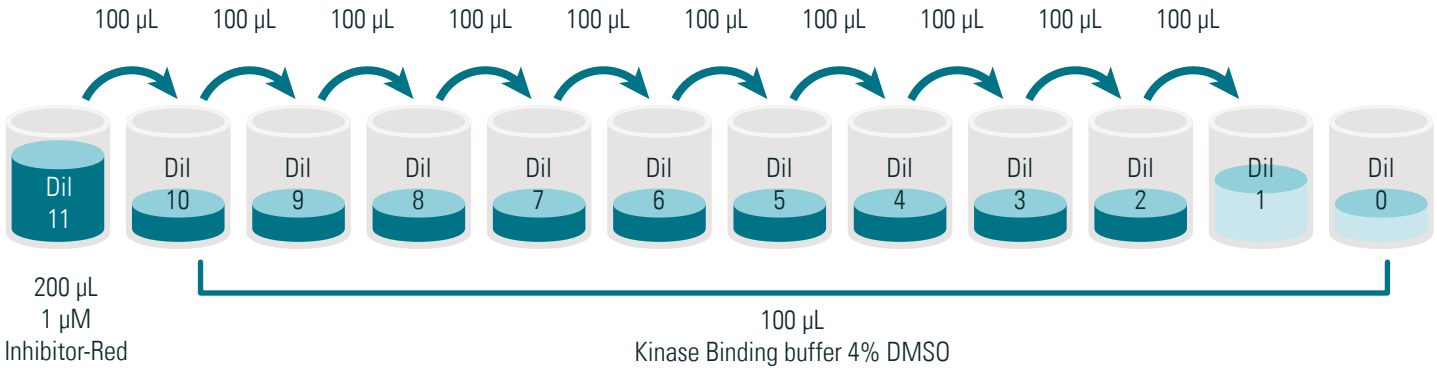
A RECOMMENDED DILUTION PROCEDURE FOR STAUROSPORINE-RED IS LISTED AND ILLUSTRATED BELOW:

We recommand preparation of the Staurosporine-Red in a non-binding plate.

- In a well, prepare the 1µM Staurosporine-Red solution (Dil 11) by diluting 25-fold the Staurosporine-Red stock solution with Kinase Binding Buffer.
  In practice: take 8 µL of Staurosporine-Red stock solution and add 192 µL of Kinase Binding Buffer.

1 vol 24 vol	**Staurosporine-Red**
	Dilute 25-fold the 25 µM stock solution (thawed reagent) of Staurosporine-Red with Kinase Binding Buffer (1X). e.g. 8 µL of thawed Staurosporine-Red + 192 µL of Kinase Binding Buffer.

- Starting with this 1 µM Staurosporine-Red solution (Dil 11), prepare 1/2 serial dilutions in Kinase Binding Buffer with 4% DMSO as follows:
  - Dispense 100 µL of Kinase Binding Buffer with 4% DMSO into each well.
  - Add 100 µL of Staurosporine-Red dilutions to 100 µL of Kinase Binding Buffer, mix gently, and repeat the 1/2 serial dilution to make the following solutions: Dil 10, Dil 9, Dil 8, Dil 7, Dil 6, Dil 5, Dil 4, Dil 3, Dil 2, Dil 1.
  - Dil 0 (Negative control) is Kinase Binding Buffer with 4% DMSO alone.



Staurosporine-Red dilutions	Dilutions	Working solutions nM	final concentration nM
Dil 11	8 µL of stock solution (25 µM) + 192 µL Kinase Binding Buffer	1 000	250
Dil 10	100 µL Dil 11 + 100 µL Kinase Binding Buffer with 4% DMSO	500	125
Dil 9	100 µL Dil 10 + 100 µL Kinase Binding Buffer with 4% DMSO	250	62.5
Dil 8	100 µL Dil 9 + 100 µL Kinase Binding Buffer with 4% DMSO	125	31.25
Dil 7	100 µL Dil 8+ 100 µL Kinase Binding Buffer with 4% DMSO	62.5	15.62
Dil 6	100 µL Dil 7 + 100 µL Kinase Binding Buffer with 4% DMSO	31.25	7.81
Dil 5	100 µL Dil 6 + 100 µL Kinase Binding Buffer with 4% DMSO	15.62	3.91
Dil 4	100 µL Dil 5 + 100 µL Kinase Binding Buffer with 4% DMSO	7.81	1.95
Dil 3	100 µL Dil 4 + 100 µL Kinase Binding Buffer with 4% DMSO	3.91	0.98
Dil 2	100 µL Dil 3 + 100 µL Kinase Binding Buffer with 4% DMSO	1.95	0.49
Dil 1	100 µL Dil 2 + 100 µL Kinase Binding Buffer with 4% DMSO	0.98	0.25
Dil 0	100 µL Kinase Binding Buffer with 4% DMSO	0	0

ASSAY PROTOCOL FOR K_D DETERMINATION

	Non specific binding	Total binding
Step 1 	Dispense 5 µL of Kinase Binding Buffer into each well.	
Step 2 	Dispense 5 µL of Kinase Binding Buffer into all wells.	Dispense 5 µL of Tagged Kinase into all wells.
Step 3 	Dispense 5 µL of Anti-tag*-Eu cryptate into all wells.	
Step 4 	Dispense 5 µL of each dilution of the 3 different Inhibitor-Red into the corresponding wells.	
Step 5 	Seal the plate and incubate 1 hour at RT.	
Step 6 	Remove the plate sealer and read on an HTRF compatible reader	

* depending on the Enzyme selected

REAGENT PREPARATION FOR K_I (IC_{50}) DETERMINATION

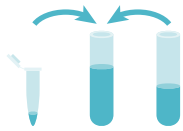
- It is very important to prepare Staurosporine-Red solution in the HTRF Kinase Binding Buffer (we recommend to filtering the buffer before use (0.22 µm) The use of an incorrect diluent may affect reagent stability and assay results.
- Thaw Staurosporine-Red and homogenize it with a vortex.

TO PREPARE STAUROSPORINE-RED, BUFFER, GST-KINASE, AND MAB ANTI-GST EU-CRYPTATE WORKING SOLUTIONS:

Anti tag-Eu cryptate	Staurosporine-Red	Tagged-Kinase	Kinase Binding Buffer
Prepare 1X anti tag-Eu cryptate	Prepare a 4X Staurosporine-Red final concentration. The final concentration can be Staurosporine-Red K_D determined in the step before.	Prepare a 20 nM = 4 X Tagged kinase final concentration.	Prepare Kinase Binding Buffer containing 4% DMSO (to keep the DMSO concentration constant).
Dilute 100-fold the 100X stock solution with Kinase Binding Buffer. e.g. 10 µL of thawed Eu cryptate reagent stock solution + 990 µL of Kinase Binding Buffer (this will provide enough Eu-cryptate for 200 tests).	Dilute Staurosporine-Red in the Kinase Binding Buffer.	Dilute your stock solution of Tagged Kinase in Kinase Binding buffer.	e.g. 80 µL of DMSO + 1920 µL of Kinase Binding Buffer.

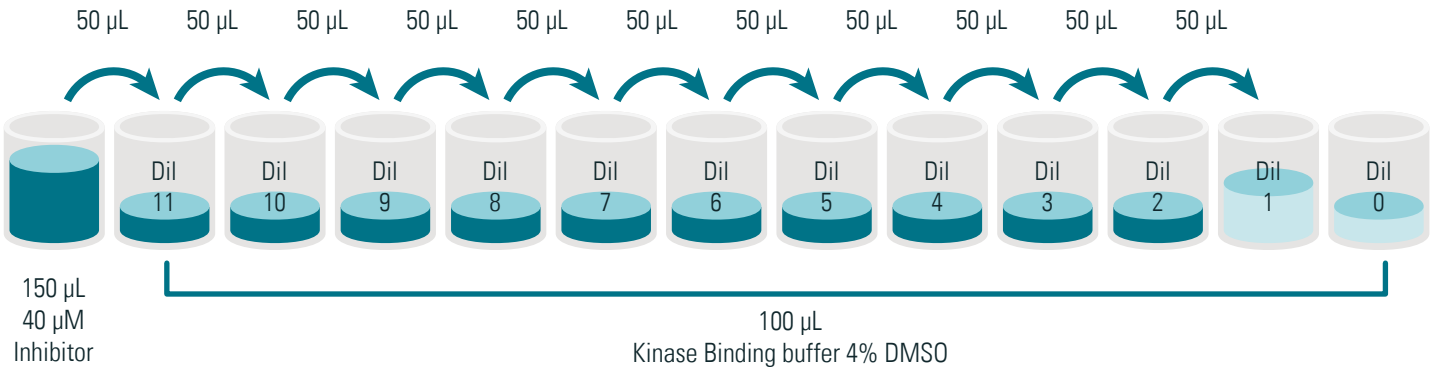
A RECOMMENDED DILUTION PROCEDURE FOR INHIBITOR PREPARATION IS LISTED AND ILLUSTRATED BELOW:

- Prepare 1 mM Inhibitor solutions in DMSO.
- Dilute the 1 mM inhibitor solutions 25-fold with Kinase Binding Buffer to obtain 40 µM inhibitor intermediate solution: take 6 µL of 1 mM Inhibitor solution and add 144 µL of Kinase Binding Buffer.



Inhibitor
Dilute 25-fold each 1 mM Inhibitor stock solution with Kinase Binding Buffer (1X). e.g. 6 µL of 1 mM Inhibitor in DMSO + 144 µL of Kinase Binding Buffer.

- Use these 1 mM Inhibitor intermediate solutions to prepare the Inhibitor dilution curve using 1/3 serial dilutions as follows:
  - Dispense 100 µL of Kinase Binding Buffer with 4% DMSO into each well.
  - Add 100 µL of Staurosporine-Red dilutions to 50 µL of Kinase Binding Buffer, mix gently, and repeat the 1/3 serial dilution to make the following solutions: Dil 11, Dil 10, Dil 9, Dil 8, Dil 7, Dil 6, Dil 5, Dil 4, Dil 3, Dil 2, Dil 1.
  - Dil 0 (Positive control) is Kinase Binding Buffer with 4% DMSO alone.



Inhibitor dilutions	Dilutions	Working solutions nM	Final concentration nM
Intermediate stock solution	6 µL of stock solution (1 mM) + 144 µL Kinase Binding Buffer	40 000	10 000
Dil 11	50 µL Intermediate stock solution + 100 µL Kinase Binding Buffer with 4% DMSO	13 333	3 333
Dil 10	50 µL Dil 11 + 100 µL Kinase Binding Buffer with 4% DMSO	4 444	1 111
Dil 9	50 µL Dil 10 + 100 µL Kinase Binding Buffer with 4% DMSO	1 481	370
Dil 8	50 µL Dil 9 + 100 µL Kinase Binding Buffer with 4% DMSO	494	123
Dil 7	50 µL Dil 8+ 100 µL Kinase Binding Buffer with 4% DMSO	165	41
Dil 6	50 µL Dil 7 + 100 µL Kinase Binding Buffer with 4% DMSO	55	13.7
Dil 5	50 µL Dil 6 + 100 µL Kinase Binding Buffer with 4% DMSO	18.3	4.6
Dil 4	50 µL Dil 5 + 100 µL Kinase Binding Buffer with 4% DMSO	6.1	1.5
Dil 3	50 µL Dil 4 + 100 µL Kinase Binding Buffer with 4% DMSO	2	0.51
Dil 2	50 µL Dil 3 + 100 µL Kinase Binding Buffer with 4% DMSO	0.68	0.17
Dil 1	50 µL Dil 2 + 100 µL Kinase Binding Buffer with 4% DMSO	0.23	0.056
Dil 0	100 µL Kinase Binding Buffer with 4% DMSO	0	0

ASSAY PROTOCOL FOR COMPETITION BINDING - K_i / IC_{50} DETERMINATION

Step 1 	Dispense 5 µL of Inhibitor from the dilution series to the corresponding wells. We recommend working in triplicates.
Step 2 	Dispense 5 µL of Tagged Kinase into all wells.
Step 3 	Dispense 5 µL of Anti-tag*-Eu cryptate into all wells.
Step 4 	Dispense 5 µL of Staurosporine working solution into the corresponding wells.
Step 5 	Seal the plate and incubate 1 hour at RT.
Step 6 	Remove the plate sealer and read on an HTRF compatible reader.

DATA REDUCTION AND 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 % 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 *http://www.cisbio.com/htrf-ratio-and-data-reduction*

FOR K_D DETERMINATION

- Subtract the Non-specific Binding Ratio from the Total Binding Ratio to obtain the Specific Binding Ratio.
- Transfer the data to GraphPad Prism™ and plot the Specific Binding Ratio versus the [Staurosporine-Red].
- Fit the specific binding with the 'one site - Specific Binding' equation (Y = Bmax*X/ (K_D+X) and determine the dissociation constant (K_D) of the Staurosporine-Red to the tagged or biotinylated Kinase.

FOR K_I DETERMINATION

- Transfer the data to GraphPad Prism™ and plot the HTRF ratio versus the log [inhibitor].
- Fit the dose-response curve using non-linear regression with the 'log (inhibitor) vs response-variable slope (four parameters).
Equation: Y=Bottom + (Top-Bottom)/(1+10^((Log IC_{50}-X)*Hill Slope) and determine the IC_{50} of the inhibitor to the tagged Kinase.
- When under equilibrium conditions, inhibition constants (K_I) can now be determined from the IC_{50} obtained using the Cheng-Prusoff equation [1] and the K_D of the tracer to the tagged Kinase.

$$K_I = \frac{IC_{50}}{(1+(\text{Staurosporine-Red} / K_D)}$$

[1] Y.C Cheng, W.H. Prusoff., Biochem. Pharmacol. 22 (1973) 3099-3108.

RESULTS

This data must not be substituted for the data obtained in the laboratory and should be considered only as an example (readouts on Pherastar FS with a flash lamp).

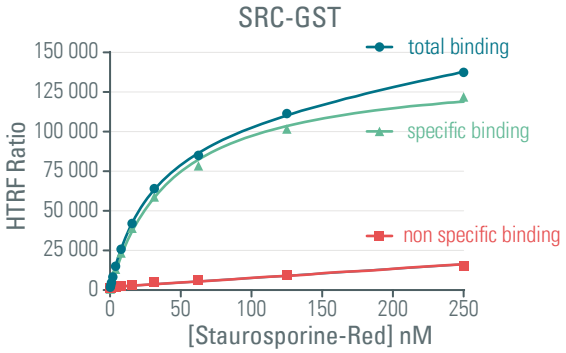
Results may vary from one HTRF compatible reader to another.

For additonal information, please refer to the *HTRF® Kinase Binding Guide*.

K_D DETERMINATION

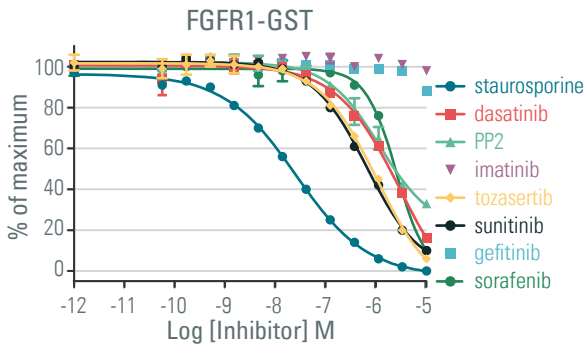
Staurosporine-Red on 5 nM GST-tagged SRC

- K_D = 43 nM



IC_{50} - K_I DETERMINATION

Data from a competitive binding experiment with 8 known kinase inhibitors using 29 nM Staurosporine-Red (K_D) on GST tagged FGFR1 is shown here. HTRF ratios were normalized and K_I values compared to data from the literature.



Inhibitor	IC_{50} (nM)	K_I (nM)	K_I (nM) literature
Staurosporine	25.7	13	9.1 [2]
Dasatinib	2928	1464	870 [2]
PP2	1157	579	not reported
Imatinib	>10 000	>10000	>20 000 [2]
Tozasertib	1044	522	201 [2]
Sunitinib	645	323	147 [2]
Gefitinib	>10 000	>10000	>20 000 [2]
Sorafinib	2554	1277	580 [3]

[2] V. Georgi et al., J. Am. Chem. Soc. 140 (2018) 15774-15782.
[3] S.M. Wilhelm et al., Cancer Res. 64 (2004) 7099-7109

FOR MORE INFORMATION
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