



HTRF Kinase-biotin Binding Discovery Kit

Part # 62KBD03PEA

Store at: $\leq -16^{\circ}\text{C}$

Revision: #03 of September 2023

For research use only. Not for use in diagnostic procedures.

ASSAY PRINCIPLE

Revvity Kinase-Biotin Binding Discovery Kit is intended for quantitative measurement of the dissociation constant (K_D) of three different red fluorescent derivatives of Staurosporine, Dasatinib and/or Sunitinib (Inhibitor-Red) on biotinylated kinases, using HTRF® technology. For additional information, please refer to the [HTRF® Kinase Binding Guide](#).

The binding of the 3 red Inhibitors is detected in a sandwich assay format using a specific Streptavidin-Eu cryptate labeled with Europium Cryptate (donor) which binds to biotinylated Kinase and an inhibitor derivative 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 Inhibitor-Red and will saturate depending on the dissociation constant (K_D) of the Inhibitor-Red to the biotinylated Kinase (Fig.1).

The Kinase-Biotin Binding Discovery Kit serves to determine which Inhibitor-Red might be best suited to setting up a binding assay. Depending on the kinase, each of the three Inhibitor-Red is expected to generate a different assay window and K_D . The Inhibitor-Red with the best assay properties (depending on the K_D and assay window generated) will be chosen to perform a competitive binding assay.

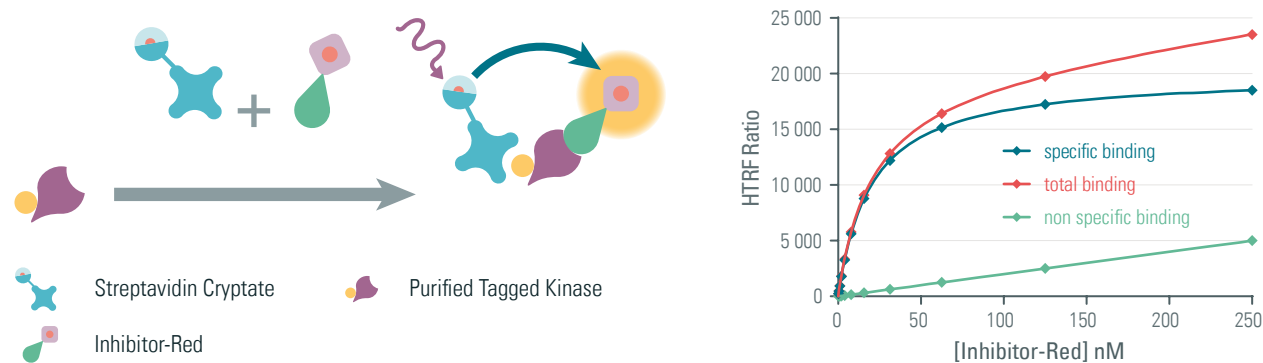
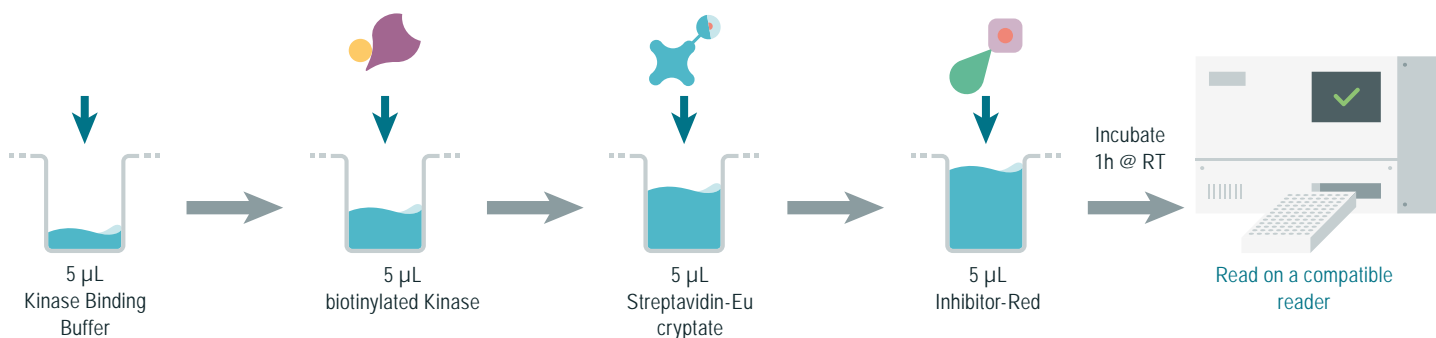


Figure 1: Principle of HTRF kinase saturation binding assay (K_D determination)

MANUAL AT A GLANCE

SATURATION BINDING ASSAY (K_D DETERMINATION)

Make sure you use the appropriate setup for Eu 3+ Cryptate. For more information about setup and HTRF® compatible readers, please visit our website at: www.revvy.com

MATERIALS PROVIDED:

Kit components	Cat # 62KBD03PEA*
Staurosporine-Red-0.5 nmol (25 µM in DMSO)	1 vial - 20 µL
Dasatinib-Red-0.5 nmol (25 µM in DMSO)	1 vial - 20 µL
Sunitinib-Red-0.5 nmol (25 µM in DMSO)	1 vial - 20 µL
Streptavidin-Eu cryptate Kinase Binding - 1,000 tests 100 X - Frozen	1 vial - 50 µL Cat# 62KBSAKAF
Kinase Binding Buffer	1 vial - 20 mL Cat# 62KBRRDD

* When used as advised, the discovery kit provides sufficient reagents to perform 2 saturation binding experiments (total and non-specific) between 0 and 250 nM with each of the 3 Inhibitor-Red, in order to determine the most suitable Inhibitor-Red for the following inhibition assays.

STORAGE AND STABILITY

Store the kit at $\leq -16^{\circ}\text{C}$. Under appropriate storage conditions, reagents are stable until the expiry date indicated on the label.

Thaw and aliquot the biotinylated-Kinase on ice.

Once thawed, other solutions can be frozen once.

To avoid freeze/thaw cycles, it is recommended to dispense remaining stock solutions into disposable plastic vials for storage at $\leq -16^{\circ}\text{C}$.

Volume of reagent aliquots should not be under 10 µL.

Thawed buffer can be stored at $2-8^{\circ}\text{C}$ on your premises.

PURCHASE SEPARATELY:

- Purified 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 Eu³⁺ Cryptate is used.
- To perform the assay, use white plate only.

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






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

REAGENT PREPARATION

BEFORE YOU BEGIN:

- It is very important to prepare the kit reagents using the provided Kinase Binding Buffer. The use of an incorrect diluent may affect reagent stability and assay results.
- Thaw biotinylated Kinase on ice; other reagents can be thawed at room temperature.
- Before use, allow the buffer to warm up at room temperature and homogenize it with a vortex.
- It is recommended to filter (0.22 μ m) buffer before use.




TO PREPARE REAGENT STOCK SOLUTIONS:

	Streptavidin-Eu cryptate Kinase Binding Thaw the Streptavidin-Eu cryptate. Homogenize with a vortex. This 100X stock solution can be frozen and stored at $\leq -16^{\circ}\text{C}$.
	Staurosporine-Red Thaw the Staurosporine-Red vial. Homogenize with a vortex. This 25 μ M stock solution in DMSO can be frozen and stored at $\leq -16^{\circ}\text{C}$.
	Dasatinib-Red Thaw the Dasatinib-Red vial. Homogenize with a vortex. This 25 μ M stock solution in DMSO can be frozen and stored at $\leq -16^{\circ}\text{C}$.
	Sunitinib-Red Thaw the Sunitinib-Red vial. Homogenize with a vortex. This 25 μ M stock solution in DMSO can be frozen and stored at $\leq -16^{\circ}\text{C}$.
	Kinase Binding Buffer Thaw the Kinase binding buffer. Homogenize with a vortex. This thawed buffer can be stored at $2-8^{\circ}\text{C}$ in your premises.

TO PREPARE BUFFER, BIOTINYLATED KINASE, & STREPTAVIDIN-EU CRYPTATE WORKING SOLUTIONS:

Each well requires 5 μ L of each reagent.

Prepare in separate vials.

	Streptavidin-Eu cryptate Kinase Binding Dilute 100-fold the 100X stock solution (thawed reagent) of Streptavidin-Eu cryptate with Kinase Binding Buffer. e.g. 10 μ L of thawed Streptavidin-Eu cryptate stock solution + 990 μ L of Kinase Binding Buffer for 200 tests.
	biotinylated Kinase Dilute your stock solution of biotinylated Kinase (not provided in the kit) in Kinase Binding buffer to 20 nM (4X final concentration).
	Kinase Binding Buffer with 4% DMSO To keep the DMSO concentration constant for the dilution series of Inhibitor-Red, it is recommended to prepare a Kinase Binding Buffer containing 4% DMSO e.g. 80 μ L of DMSO + 1920 μ L of Kinase Binding Buffer.

TO PREPARE WORKING SOLUTION OF INHIBITOR-RED FOR K_D DETERMINATION:

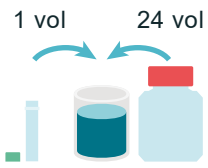
- Each well requires 5 μ L of Inhibitor-Red (either Staurosporine-Red, Dasatinib-Red, or Sunitinib-Red).
- In order to counteract any standard sticking, we recommend changing tips between each dilution.

A recommended dilution procedure for Staurosporine-Red, Dasatinib-Red, and Sunitinib-Red is listed and illustrated below:

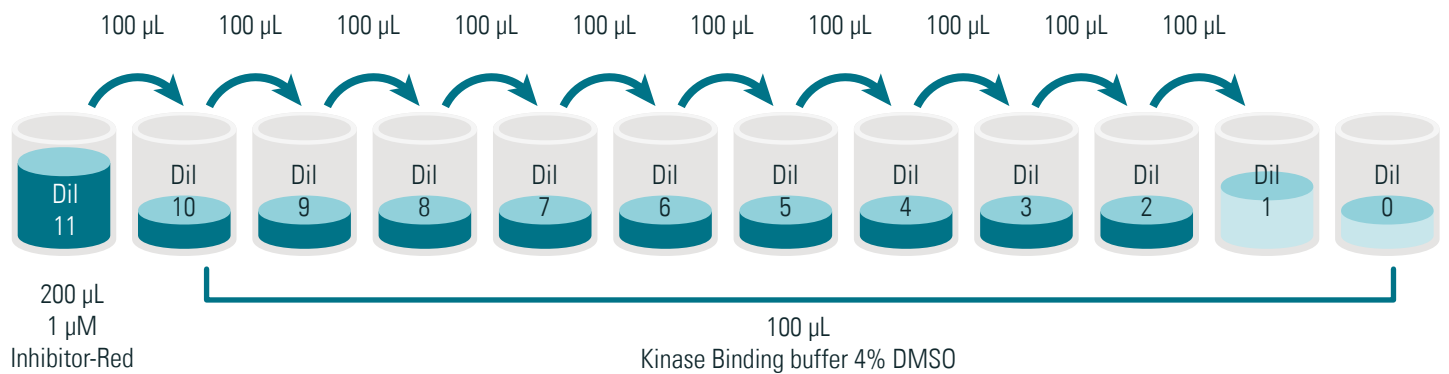
We recommend preparation of the Staurosporine-Red, Dasatinib-Red & Sunitinib-Red in a non-binding plate.

- In a well, prepare the 1 μ M Inhibitor-Red (either Staurosporine-Red, Dasatinib-Red or Sunitinib-Red) solution (Dil 11) by diluting 25-fold the Inhibitor-Red stock solution with Kinase Binding Buffer.

In practice: take 8 μ L of Inhibitor-Red stock solution and add 192 μ L of Kinase Binding Buffer.







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

- Starting with these 1 μM Inhibitor-Red solutions (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 Inhibitor-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.



Inhibitor-Red dilutions	Dilutions	Working solutions nM	Final concentration nM
Dil 11	8 μL of stock solution (25 μM) + 192 μL Kinase Binding Buffer	1000	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 MANUAL

	Non specific binding	Total binding
Step 1 	Dispense 5 μ L of Kinase Binding Buffer into each well.	
Step 2 	Dispense 5 μ L of Kinase Binding Buffer into all wells.	Dispense 5 μ L of biotinylated Kinase into all wells.
Step 3 	Dispense 5 μ L of Streptavidin-Eu cryptate into all wells.	
Step 4 	Dispense 5 μ L of each dilution of the 3 different Inhibitor-Red (Staurosporine, Dasatinib, Sunitinib) 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.	

	1	2	3	4	5	6
A	5 μ L Kinase Binding Buffer 5 μ L Kinase Binding Buffer 5 μ L Streptavidin-Eu cryptate 5 μ L Inhibitor-Red* dil 0	Repeat Well A1	Repeat Well A1	5 μ L Kinase Binding Buffer 5 μ L biotinylated kinase 5 μ L Streptavidin-Eu cryptate 5 μ L Inhibitor-Red* dil 0	Repeat Well A4	Repeat Well A4
B	5 μ L Kinase Binding Buffer 5 μ L Kinase Binding Buffer 5 μ L Streptavidin-Eu cryptate 5 μ L Inhibitor-Red* dil 1	Repeat Well B1	Repeat Well B1	5 μ L Kinase Binding Buffer 5 μ L biotinylated kinase 5 μ L Streptavidin-Eu cryptate 5 μ L Inhibitor-Red* dil 1	Repeat Well B4	Repeat Well B4
C	5 μ L Kinase Binding Buffer 5 μ L Kinase Binding Buffer 5 μ L Streptavidin-Eu cryptate 5 μ L Inhibitor-Red* dil 2	Repeat Well C1	Repeat Well C1	5 μ L Kinase Binding Buffer 5 μ L biotinylated kinase 5 μ L Streptavidin-Eu cryptate 5 μ L Inhibitor-Red* dil 2	Repeat Well C4	Repeat Well C4
D	5 μ L Kinase Binding Buffer 5 μ L Kinase Binding Buffer 5 μ L Streptavidin-Eu cryptate 5 μ L Inhibitor-Red* dil 3	Repeat Well D1	Repeat Well D1	5 μ L Kinase Binding Buffer 5 μ L biotinylated kinase 5 μ L Streptavidin-Eu cryptate 5 μ L Inhibitor-Red* dil 3	Repeat Well D4	Repeat Well D4
E	5 μ L Kinase Binding Buffer 5 μ L Kinase Binding Buffer 5 μ L Streptavidin-Eu cryptate 5 μ L Inhibitor-Red* dil 4	Repeat Well E1	Repeat Well E1	5 μ L Kinase Binding Buffer 5 μ L biotinylated kinase 5 μ L Streptavidin-Eu cryptate 5 μ L Inhibitor-Red* dil 4	Repeat Well E4	Repeat Well E4
F	5 μ L Kinase Binding Buffer 5 μ L Kinase Binding Buffer 5 μ L Streptavidin-Eu cryptate 5 μ L Inhibitor-Red* dil 5	Repeat Well F1	Repeat Well F1	5 μ L Kinase Binding Buffer 5 μ L biotinylated kinase 5 μ L Streptavidin-Eu cryptate 5 μ L Inhibitor-Red* dil 5	Repeat Well F4	Repeat Well F4
G	5 μ L Kinase Binding Buffer 5 μ L Kinase Binding Buffer 5 μ L Streptavidin-Eu cryptate 5 μ L Inhibitor-Red* dil 6	Repeat Well G1	Repeat Well G1	5 μ L Kinase Binding Buffer 5 μ L biotinylated kinase 5 μ L Streptavidin-Eu cryptate 5 μ L Inhibitor-Red* dil 6	Repeat Well G4	Repeat Well G4
H	5 μ L Kinase Binding Buffer 5 μ L Kinase Binding Buffer 5 μ L Streptavidin-Eu cryptate 5 μ L Inhibitor-Red* dil 7	Repeat Well H1	Repeat Well H1	5 μ L Kinase Binding Buffer 5 μ L biotinylated kinase 5 μ L Streptavidin-Eu cryptate 5 μ L Inhibitor-Red* dil 7	Repeat Well H4	Repeat Well H4
I	5 μ L Kinase Binding Buffer 5 μ L Kinase Binding Buffer 5 μ L Streptavidin-Eu cryptate 5 μ L Inhibitor-Red* dil 8	Repeat Well I1	Repeat Well I1	5 μ L Kinase Binding Buffer 5 μ L biotinylated kinase 5 μ L Streptavidin-Eu cryptate 5 μ L Inhibitor-Red* dil 8	Repeat Well I4	Repeat Well I4
J	5 μ L Kinase Binding Buffer 5 μ L Kinase Binding Buffer 5 μ L Streptavidin-Eu cryptate 5 μ L Inhibitor-Red* dil 9	Repeat Well J1	Repeat Well J1	5 μ L Kinase Binding Buffer 5 μ L biotinylated kinase 5 μ L Streptavidin-Eu cryptate 5 μ L Inhibitor-Red* dil 9	Repeat Well J4	Repeat Well J4
K	5 μ L Kinase Binding Buffer 5 μ L Kinase Binding Buffer 5 μ L Streptavidin-Eu cryptate 5 μ L Inhibitor-Red* dil 10	Repeat Well K1	Repeat Well K1	5 μ L Kinase Binding Buffer 5 μ L biotinylated kinase 5 μ L Streptavidin-Eu cryptate 5 μ L Inhibitor-Red* dil 10	Repeat Well K4	Repeat Well K4
L	5 μ L Kinase Binding Buffer 5 μ L Kinase Binding Buffer 5 μ L Streptavidin-Eu cryptate 5 μ L Inhibitor-Red* dil 11	Repeat Well L1	Repeat Well L1	5 μ L Kinase Binding Buffer 5 μ L biotinylated kinase 5 μ L Streptavidin-Eu cryptate 5 μ L Inhibitor-Red* dil 11	Repeat Well L4	Repeat Well L4

* Staurosporine-Red, Dasatinib-Red or Sunitinib-Red

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A	N						N						N											
B	O						B						O											
C	N	B			B		N	B				B	N	B			B							
D	S	I			T		S	I				T	S	I			T							
E	P	N			O		P	N				O	P	N			O							
F	D				D		D					D					D							
G	E	I			A		E	I				A	E	I			A							
H	C	N			L		C	N				L	C	N			L							
I	I	G			G		I	G				G	I	G			I							
J	F						F						F											
K	I						I						I											
L	C						C						C											
M																								
N																								
O																								
P																								
Q																								

Example of plate-map

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.

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

- Subtract the Non-Specific Binding ratio from the Total Binding ratio to obtain the specific binding.
- Transfer the data to GraphPad Prism™ and plot the Specific binding ratio versus the [Inhibitor-Red].
- Fit the specific binding with the 'one site - Specific Binding' equation ($Y = B_{\text{max}} * X / (K_D + X)$) and determine the dissociation constant (K_D) of the Inhibitor-Red to the biotinylated Kinase.
- Calculate the assay window for each Inhibitor-red dil = Total Binding / Non-Specific Binding.

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

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).

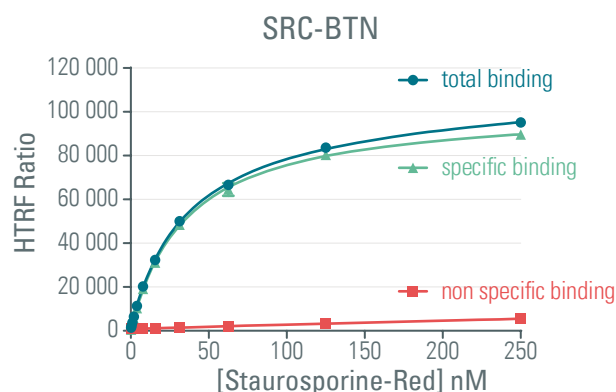
Results may vary from one HTRF® compatible reader to another.

For additional information, please refer to the [HTRF® Kinase Binding Guide](#).

K_D DETERMINATION

Staurosporine-Red pn 5 nM N-ter biotinylated SRC

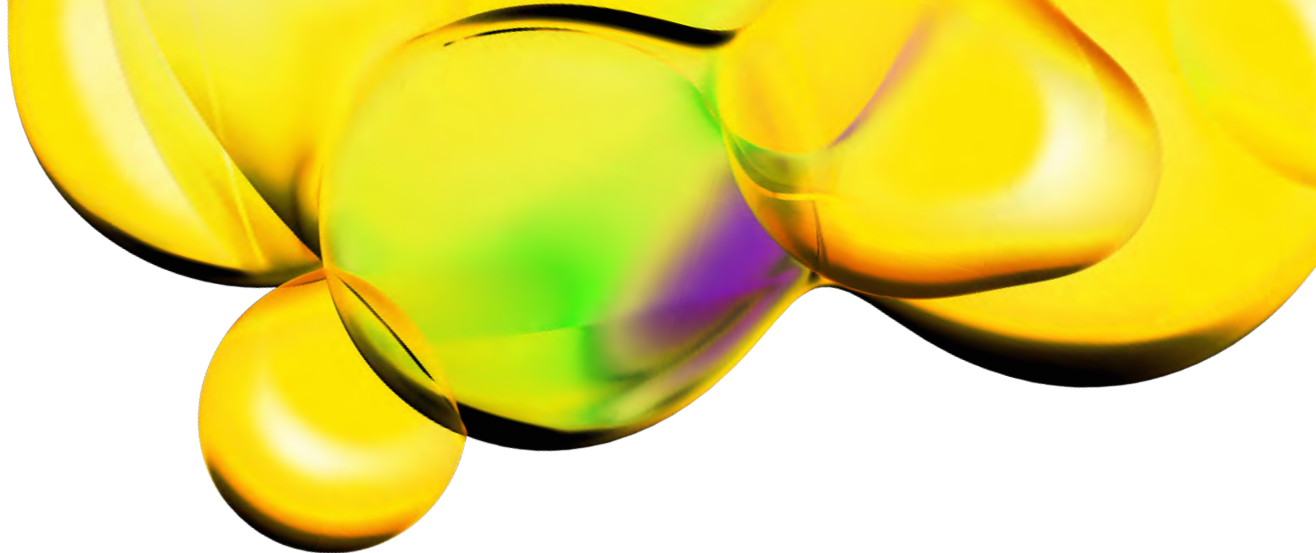
- $K_D = 35$ nM



Staurosporine-Red						
Dilutions	nM	Total Binding Ratio	Non-Specific Binding Ratio	Specific Binding Ratio	Assay window	CV
Dil 0	0	848	927		0.9	2.4 %
Dil 1	0.25	1495	900	595	1.7	4.9 %
Dil 2	0.49	2253	856	1397	2.6	3.7 %
Dil 3	0.98	3788	872	2917	4.3	4.6 %
Dil 4	1.95	6412	926	5487	6.9	2.3 %
Dil 5	3.91	11328	998	10331	11.4	4.7 %
Dil 6	7.81	20261	1135	19126	17.9	0.8 %
Dil 7	15.62	32372	1208	31164	26.8	2.8 %
Dil 8	31.25	50042	1566	48477	32.0	3.3 %
Dil 9	62.5	66677	2102	64576	31.7	4.5 %
Dil 10	125	83602	3295	80306	25.4	2.3 %
Dil 11	250	95150	5492	89658	17.3	2.0 %

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The use of the cell line will be done with appropriate safety and handling precautions to minimize health and environmental impact.



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