



# KINASE-6HIS BINDING DISCOVERY KIT

## PROTOCOL

Part # 62KBD02PEA

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

Revision: 02 - January 2020

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

### ASSAY PRINCIPLE

Cisbio Kinase-6His 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 6His tagged kinases, using HTRF<sup>®</sup> technology. For additional information, please refer to the [HTRF<sup>®</sup> Kinase Binding Guide](#).

The binding of the 3 red Inhibitors is detected in a sandwich assay format using a specific Anti 6His-Eu cryptate antibody labeled with Europium Cryptate (donor) which binds to 6His tagged Kinase and an inhibitor derivative labelled with d2 (acceptor). The detection principle is based on HTRF<sup>®</sup> 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 6His tagged Kinase (Fig.1).

The Kinase-6His 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.

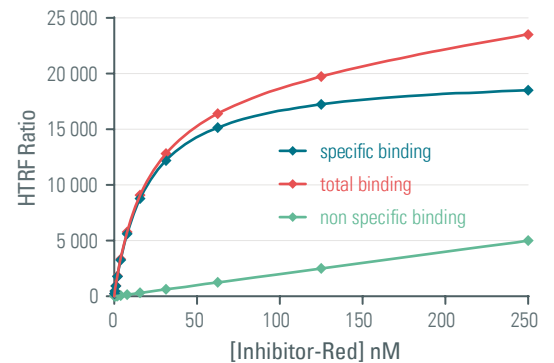
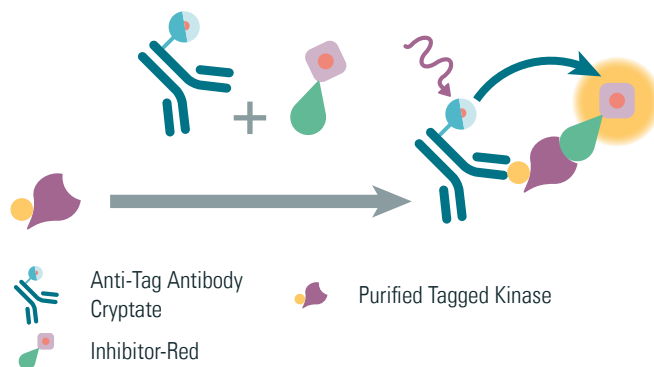
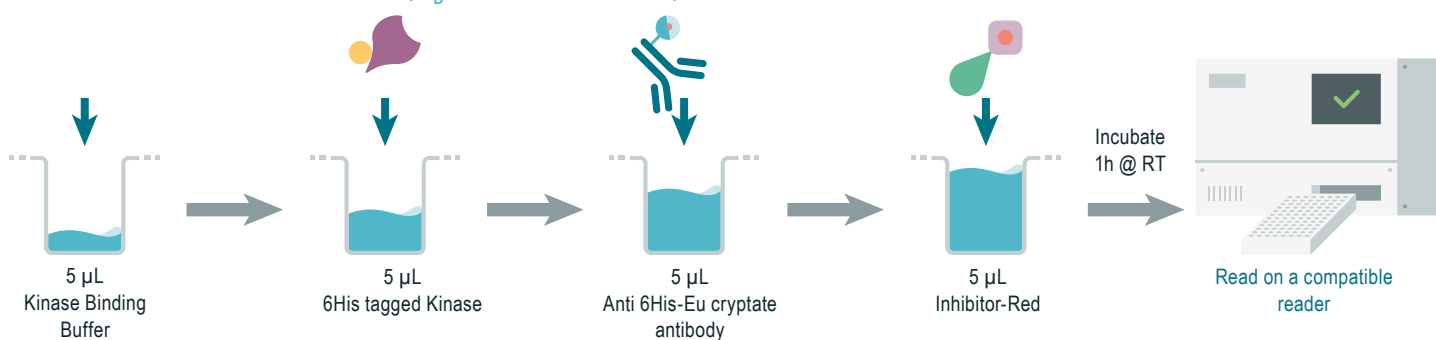


Figure 1: Principle of HTRF kinase saturation binding assay ( $K_D$  determination)

## PROTOCOL 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: <http://www.cisbio.com/compatible-readers>

### MATERIALS PROVIDED:

Kit components	Cat # 62KBD02PEA*
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
MAB Anti 6His-Eu cryptate Kinase Binding - 1,000 tests 100 X - Frozen	1 vial - 50 µL Cat# 62KBHISKAF
Kinase Binding Buffer	1 vial - 20 mL Cat# 62KBBRDD

\* 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 6His tagged-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°C on your premises.

### PURCHASE SEPARATELY:

- Purified 6His tagged 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<sup>3+</sup> 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

### 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 6His tagged 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  $\mu\text{m}$ ) buffer before use.




### TO PREPARE REAGENT STOCK SOLUTIONS:

	<b>MAB Anti 6His-Eu cryptate Kinase Binding</b> Thaw the Anti 6His-Eu cryptate antibody. Homogenize with a vortex. This 100X stock solution can be frozen and stored at $\leq -16^{\circ}\text{C}$ .
	<b>Staurosporine-Red</b> Thaw the Staurosporine-Red vial. Homogenize with a vortex. This 25 $\mu\text{M}$ stock solution in DMSO can be frozen and stored at $\leq -16^{\circ}\text{C}$ .
	<b>Dasatinib-Red</b> Thaw the Dasatinib-Red vial. Homogenize with a vortex. This 25 $\mu\text{M}$ stock solution in DMSO can be frozen and stored at $\leq -16^{\circ}\text{C}$ .
	<b>Sunitinib-Red</b> Thaw the Sunitinib-Red vial. Homogenize with a vortex. This 25 $\mu\text{M}$ stock solution in DMSO can be frozen and stored at $\leq -16^{\circ}\text{C}$ .
	<b>Kinase Binding Buffer</b> 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, 6HIS TAGGED KINASE, & ANTI 6HIS-EU CRYPTATE ANTIBODY WORKING SOLUTIONS:

Each well requires 5  $\mu\text{L}$  of each reagent.

Prepare in separate vials.

	<b>MAB Anti 6His-Eu cryptate Kinase Binding</b> Dilute 100-fold the 100X stock solution (thawed reagent) of Anti 6His-Eu cryptate antibody with Kinase Binding Buffer. e.g. 10 $\mu\text{L}$ of thawed Anti 6His-Eu cryptate antibody stock solution + 990 $\mu\text{L}$ of Kinase Binding Buffer for 200 tests.
	<b>6His tagged Kinase</b> Dilute your stock solution of 6His tagged Kinase (not provided in the kit) in Kinase Binding buffer to 20 nM (4X final concentration).
	<b>Kinase Binding Buffer with 4% DMSO</b> 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 $\mu\text{L}$ of DMSO + 1920 $\mu\text{L}$ of Kinase Binding Buffer.

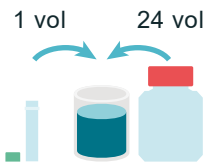
### TO PREPARE WORKING SOLUTIONS OF INHIBITOR-RED FOR $K_D$ DETERMINATION:

- Each well requires 5  $\mu\text{L}$  of Inhibitor-Red (either Staurosporine-Red, Dastinib-Red, or Sunitinib-Red).
- In order to counteract any standard sticking, we recommend changing tips between each dilution.

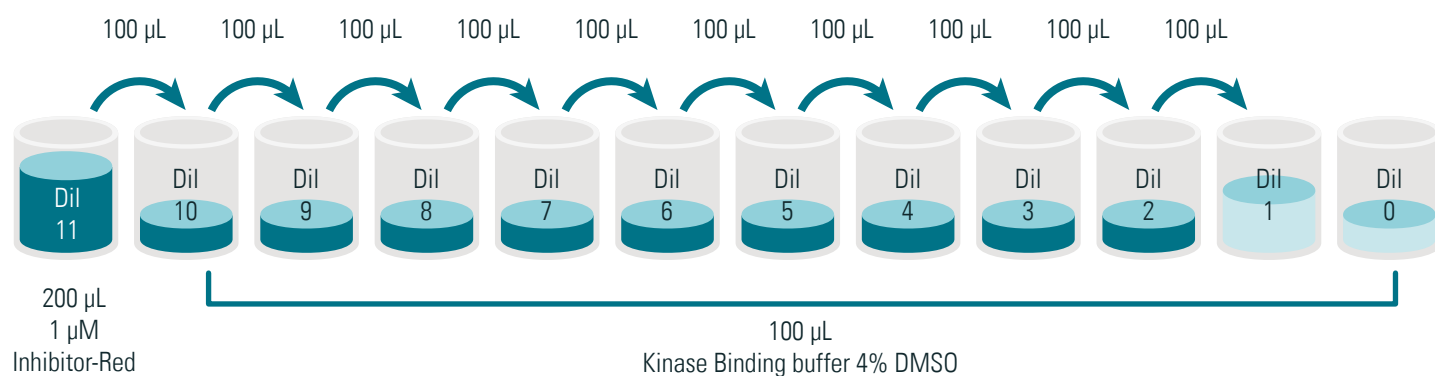
A recommended dilution procedure for Staurosporine-Red, Dastinib-Red, and Sunitinib-Red is listed and illustrated below:

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

- In a well, prepare the 1 $\mu\text{M}$  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  $\mu\text{L}$  of Inhibitor-Red stock solution and add 192  $\mu\text{L}$  of Kinase Binding Buffer.







	<p><b>Staurosporine-Red</b> Dilute 25-fold the 25 <math>\mu\text{M}</math> stock solution (thawed reagent) of Staurosporine-Red with Kinase Binding Buffer (1X). e.g. 8 <math>\mu\text{L}</math> of thawed Staurosporine-Red + 192 <math>\mu\text{L}</math> of Kinase Binding Buffer.</p>
	<p><b>Dasatinib-Red</b> Dilute 25-fold the 25 <math>\mu\text{M}</math> stock solution (thawed reagent) of Dasatinib-Red with Kinase Binding Buffer (1X). e.g. 8 <math>\mu\text{L}</math> of thawed Dasatinib-Red + 192 <math>\mu\text{L}</math> of Kinase Binding Buffer.</p>
	<p><b>Sunitinib-Red</b> Dilute 25-fold the 25 <math>\mu\text{M}</math> stock solution (thawed reagent) of Sunitinib-Red with Kinase Binding Buffer (1X). e.g. 8 <math>\mu\text{L}</math> of thawed Sunitinib-Red + 192 <math>\mu\text{L}</math> of Kinase Binding Buffer.</p>

- Starting with these 1  $\mu\text{M}$  Inhibitor-Red solutions (Dil 11), prepare 1/2 serial dilutions in Kinase Binding Buffer with 4% DMSO as follows:
  - Dispense 100  $\mu\text{L}$  of Kinase Binding Buffer with 4% DMSO into each well.
  - Add 100  $\mu\text{L}$  of Inhibitor-Red dilutions to 100  $\mu\text{L}$  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 $\mu\text{L}$ of stock solution (25 $\mu\text{M}$ ) + 192 $\mu\text{L}$ Kinase Binding Buffer	1000	250
Dil 10	100 $\mu\text{L}$ Dil 11 + 100 $\mu\text{L}$ Kinase Binding Buffer with 4% DMSO	500	125
Dil 9	100 $\mu\text{L}$ Dil 10 + 100 $\mu\text{L}$ Kinase Binding Buffer with 4% DMSO	250	62.5
Dil 8	100 $\mu\text{L}$ Dil 9 + 100 $\mu\text{L}$ Kinase Binding Buffer with 4% DMSO	125	31.25
Dil 7	100 $\mu\text{L}$ Dil 8 + 100 $\mu\text{L}$ Kinase Binding Buffer with 4% DMSO	62.5	15.62
Dil 6	100 $\mu\text{L}$ Dil 7 + 100 $\mu\text{L}$ Kinase Binding Buffer with 4% DMSO	31.25	7.81
Dil 5	100 $\mu\text{L}$ Dil 6 + 100 $\mu\text{L}$ Kinase Binding Buffer with 4% DMSO	15.62	3.91
Dil 4	100 $\mu\text{L}$ Dil 5 + 100 $\mu\text{L}$ Kinase Binding Buffer with 4% DMSO	7.81	1.95
Dil 3	100 $\mu\text{L}$ Dil 4 + 100 $\mu\text{L}$ Kinase Binding Buffer with 4% DMSO	3.91	0.98
Dil 2	100 $\mu\text{L}$ Dil 3 + 100 $\mu\text{L}$ Kinase Binding Buffer with 4% DMSO	1.95	0.49
Dil 1	100 $\mu\text{L}$ Dil 2 + 100 $\mu\text{L}$ Kinase Binding Buffer with 4% DMSO	0.98	0.25
Dil 0	100 $\mu\text{L}$ Kinase Binding Buffer with 4% DMSO	0	0

## ASSAY PROTOCOL

		Non specific binding	Total binding
Step 1		Dispense 5 $\mu$ L of Kinase Binding Buffer into each well.	
Step 2		Dispense 5 $\mu$ L of Kinase Binding Buffer into all wells.	Dispense 5 $\mu$ L of 6His tagged Kinase into all wells.
Step 3		Dispense 5 $\mu$ L of Anti 6His-Eu cryptate antibody into all wells.	
Step 4		Dispense 5 $\mu$ 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 µL Kinase Binding Buffer 5 µL Kinase Binding Buffer 5 µL Anti 6His-Eu cryptate antibody 5 µL Inhibitor-Red* dil 0	Repeat Well A1	Repeat Well A1	5 µL Kinase Binding Buffer 5 µL 6His tagged kinase 5 µL Anti 6His-Eu cryptate antibody 5 µL Inhibitor-Red* dil 0	Repeat Well A4	Repeat Well A4
B	5 µL Kinase Binding Buffer 5 µL Kinase Binding Buffer 5 µL Anti 6His-Eu cryptate antibody 5 µL Inhibitor-Red* dil 1	Repeat Well B1	Repeat Well B1	5 µL Kinase Binding Buffer 5 µL 6His tagged kinase 5 µL Anti 6His-Eu cryptate antibody 5 µL Inhibitor-Red* dil 1	Repeat Well B4	Repeat Well B4
C	5 µL Kinase Binding Buffer 5 µL Kinase Binding Buffer 5 µL Anti 6His-Eu cryptate antibody 5 µL Inhibitor-Red* dil 2	Repeat Well C1	Repeat Well C1	5 µL Kinase Binding Buffer 5 µL 6His tagged kinase 5 µL Anti 6His-Eu cryptate antibody 5 µL Inhibitor-Red* dil 2	Repeat Well C4	Repeat Well C4
D	5 µL Kinase Binding Buffer 5 µL Kinase Binding Buffer 5 µL Anti 6His-Eu cryptate antibody 5 µL Inhibitor-Red* dil 3	Repeat Well D1	Repeat Well D1	5 µL Kinase Binding Buffer 5 µL 6His tagged kinase 5 µL Anti 6His-Eu cryptate antibody 5 µL Inhibitor-Red* dil 3	Repeat Well D4	Repeat Well D4
E	5 µL Kinase Binding Buffer 5 µL Kinase Binding Buffer 5 µL Anti 6His-Eu cryptate antibody 5 µL Inhibitor-Red* dil 4	Repeat Well E1	Repeat Well E1	5 µL Kinase Binding Buffer 5 µL 6His tagged kinase 5 µL Anti 6His-Eu cryptate antibody 5 µL Inhibitor-Red* dil 4	Repeat Well E4	Repeat Well E4
F	5 µL Kinase Binding Buffer 5 µL Kinase Binding Buffer 5 µL Anti 6His-Eu cryptate antibody 5 µL Inhibitor-Red* dil 5	Repeat Well F1	Repeat Well F1	5 µL Kinase Binding Buffer 5 µL 6His tagged kinase 5 µL Anti 6His-Eu cryptate antibody 5 µL Inhibitor-Red* dil 5	Repeat Well F4	Repeat Well F4
G	5 µL Kinase Binding Buffer 5 µL Kinase Binding Buffer 5 µL Anti 6His-Eu cryptate antibody 5 µL Inhibitor-Red* dil 6	Repeat Well G1	Repeat Well G1	5 µL Kinase Binding Buffer 5 µL 6His tagged kinase 5 µL Anti 6His-Eu cryptate antibody 5 µL Inhibitor-Red* dil 6	Repeat Well G4	Repeat Well G4
H	5 µL Kinase Binding Buffer 5 µL Kinase Binding Buffer 5 µL Anti 6His-Eu cryptate antibody 5 µL Inhibitor-Red* dil 7	Repeat Well H1	Repeat Well H1	5 µL Kinase Binding Buffer 5 µL 6His tagged kinase 5 µL Anti 6His-Eu cryptate antibody 5 µL Inhibitor-Red* dil 7	Repeat Well H4	Repeat Well H4
I	5 µL Kinase Binding Buffer 5 µL Kinase Binding Buffer 5 µL Anti 6His-Eu cryptate antibody 5 µL Inhibitor-Red* dil 8	Repeat Well I1	Repeat Well I1	5 µL Kinase Binding Buffer 5 µL 6His tagged kinase 5 µL Anti 6His-Eu cryptate antibody 5 µL Inhibitor-Red* dil 8	Repeat Well I4	Repeat Well I4
J	5 µL Kinase Binding Buffer 5 µL Kinase Binding Buffer 5 µL Anti 6His-Eu cryptate antibody 5 µL Inhibitor-Red* dil 9	Repeat Well J1	Repeat Well J1	5 µL Kinase Binding Buffer 5 µL 6His tagged kinase 5 µL Anti 6His-Eu cryptate antibody 5 µL Inhibitor-Red* dil 9	Repeat Well J4	Repeat Well J4
K	5 µL Kinase Binding Buffer 5 µL Kinase Binding Buffer 5 µL Anti 6His-Eu cryptate antibody 5 µL Inhibitor-Red* dil 10	Repeat Well K1	Repeat Well K1	5 µL Kinase Binding Buffer 5 µL 6His tagged kinase 5 µL Anti 6His-Eu cryptate antibody 5 µL Inhibitor-Red* dil 10	Repeat Well K4	Repeat Well K4
L	5 µL Kinase Binding Buffer 5 µL Kinase Binding Buffer 5 µL Anti 6His-Eu cryptate antibody 5 µL Inhibitor-Red* dil 11	Repeat Well L1	Repeat Well L1	5 µL Kinase Binding Buffer 5 µL 6His tagged kinase 5 µL Anti 6His-Eu cryptate antibody 5 µL 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					O					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	T		D		D	T		D	D	T		D											
G	E	A		E		A		E	A		E	A												
H	C	N		C		N		C	N		C	N												
I	I	G		I		G		I	G		I	G												
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 6His tagged Kinase.
- Calculate the assay window for each Inhibitor-red dil = Total Binding / Non-Specific Binding.

For more information about data reduction, please visit <http://www.cisbio.com/htrf-ratio-and-data-reduction>

## 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).

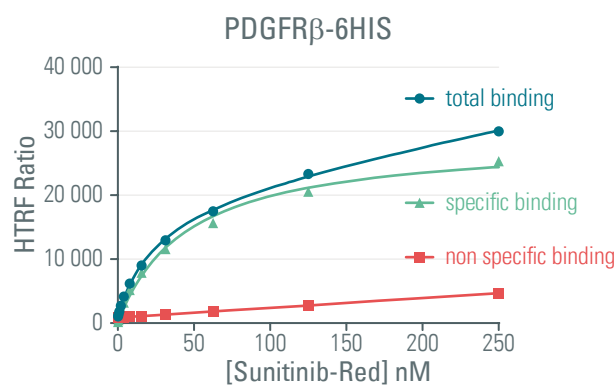
Results may vary from one HTRF® compatible reader to another.

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

### $K_D$ DETERMINATION

Sunitinib-Red on 5 nM 6His-tagged PDGFRβ

- $K_D = 45$  nM



Sunitinib-Red						
Dilutions	nM	Total Binding Ratio	Non-Specific Binding Ratio	Specific Binding Ratio	Assay window	CV
Dil 0	0	740	752		1.0	3.7 %
Dil 1	0.25	1034	859	176	1.2	0.9 %
Dil 2	0.49	1335	861	474	1.6	1.9 %
Dil 3	0.98	1820	867	953	2.1	4.5 %
Dil 4	1.95	2702	893	1809	3.0	5 %
Dil 5	3.91	4149	919	3230	4.5	2.3 %
Dil 6	7.81	6193	1021	5172	6.1	1.3 %
Dil 7	15.62	9006	1118	7887	8.1	1.3 %
Dil 8	31.25	12951	1364	11587	9.5	1.9 %
Dil 9	62.5	17480	1838	15642	9.5	0.7 %
Dil 10	125	23322	2760	20562	8.5	1.3 %
Dil 11	250	29990	4674	25316	6.4	2.4 %

This product contains material of biologic origin. Use for research purposes only. Do not use in humans or for diagnostic purposes. The purchaser assumes all risk and responsibility concerning reception, handling and storage.

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### FOR MORE INFORMATION

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