MANUAL

HTRF Kinase-6HIS Binding Discovery Kit

Part # 62KBD02PEA

Store at: ≤-16°C

Revision: #03 of September 2023

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

ASSAY PRINCIPLE

Revvity 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® technology. For additonal 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 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® 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.

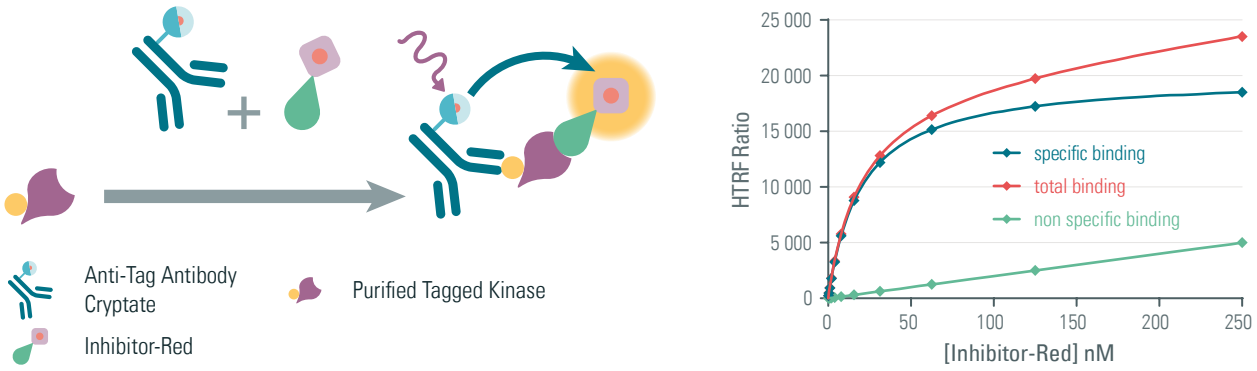
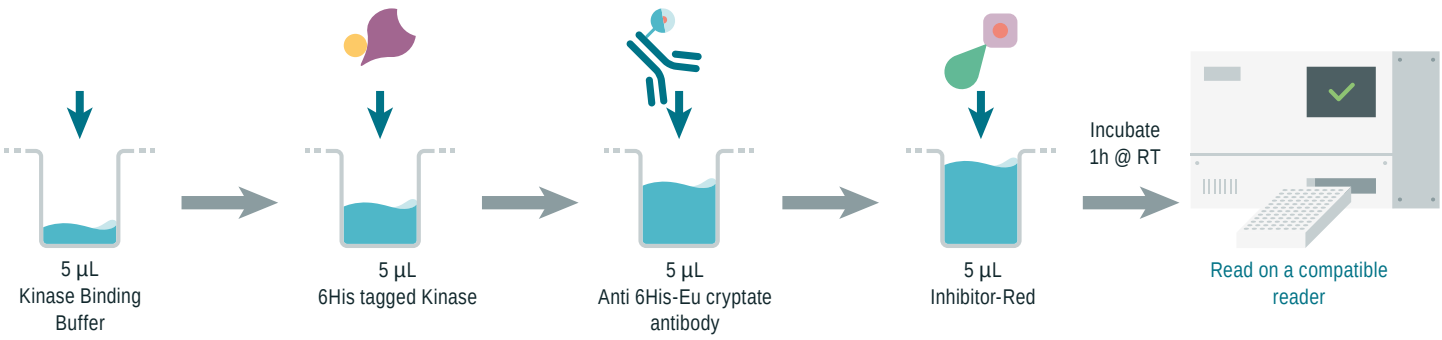


Figure 1: Principle of HTRF kinase saturation binding assay (K_D determination)

MANUAL AT A GLANCE

SATURATION BINDING ASSAY (K_D DETERMINATION



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

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 ≤-16°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 ≤-16°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^{3+} Cryptate is used.
- To perform the assay, use white plate only.

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

REAGENT PREPARATION

BEFORE YOU BEGIN:

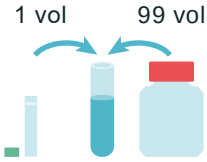
- It is very important to prepare the kit reagents using the provided Kinase Binding Buffer. The use of an incorrect diluent may affect reagent stability and assay results.
- Thaw 6His tagged Kinase on ice; other reagents can be thawed at room temperature.
- Before use, allow the buffer to warm up at room temperature and homogenize it with a vortex.
- It is recommended to filter (0.22 µm) buffer before use.

TO PREPARE REAGENT STOCK SOLUTIONS:

	MAb Anti 6His-Eu cryptate Kinase Binding Thaw the Anti 6His-Eu cryptate antibody. Homogenize with a vortex. This 100X stock solution can be frozen and stored at ≤-16°C.
	Staurosporine-Red Thaw the Staurosporine-Red vial. Homogenize with a vortex. This 25 µM stock solution in DMSO can be frozen and stored at ≤-16°C.
	Dasatinib-Red Thaw the Dasatinib-Red vial. Homogenize with a vortex. This 25 µM stock solution in DMSO can be frozen and stored at ≤-16°C.
	Sunitinib-Red Thaw the Sunitinb-Red vial. Homogenize with a vortex. This 25 µM stock solution in DMSO can be frozen and stored at ≤-16°C.
	**Kinase Binding Buffer** Thaw the Kinase binding buffer. Homogenize with a vortex. This thawed buffer can be stored at 2-8°C in your premises.

TO PREPARE BUFFER, 6HIS TAGGED KINASE, & ANTI 6HIS-EU CRYPTATE ANTIBODY WORKING SOLUTIONS:

Each well requires 5 µL of each reagent.
Prepare in separate vials.

	**MAb Anti 6His-Eu cryptate Kinase Binding** Dilute 100-fold the 100X stock solution (thawed reagent) of Anti 6His-Eu cryptate antibody with Kinase Binding Buffer. e.g. 10 µL of thawed Anti 6His-Eu cryptate antibody stock solution + 990 µL of Kinase Binding Buffer for 200 tests.
	**6His tagged Kinase** Dilute your stock solution of 6His tagged 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% DMS0 e.g. 80 µL of DMSO + 1920 µL of Kinase Binding Buffer.

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

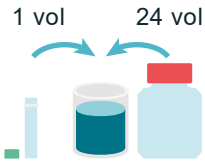
- Each well requires 5 µL of Inhibitor-Red (either Staurosporine-Red, Dastinib-Red, or Sunitinib-Red).
- In order to counteract any standard sticking, we recommend changing tips between each dilution.

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 1µM 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 µL of Inhibitor-Red stock solution and add 192 µL of Kinase Binding Buffer.

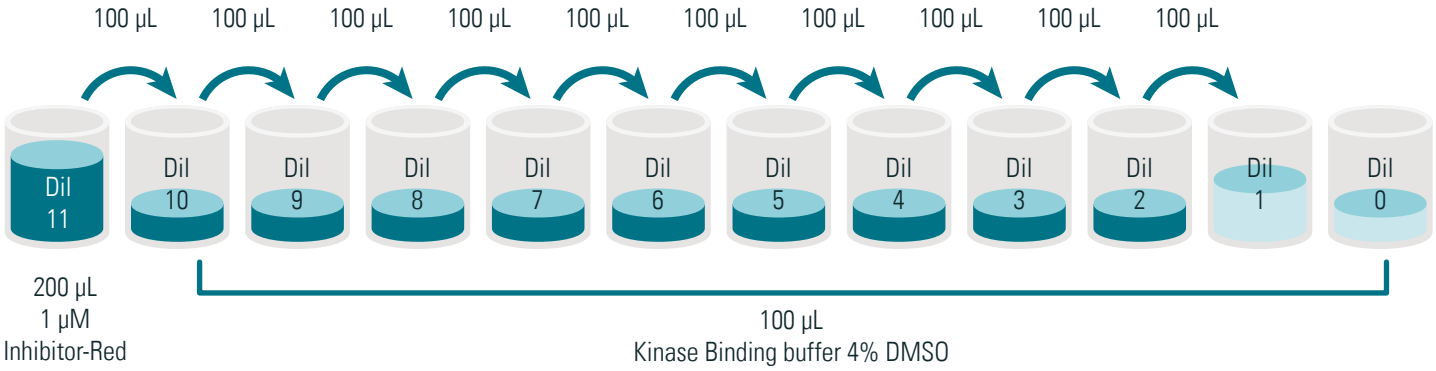


Staurosporine-Red
Dilute 25-fold the 25 µM stock solution (thawed reagent) of Staurosporine-Red with Kinase Binding Buffer (1X).
e.g. 8 µL of thawed Staurosporine-Red + 192 µL of Kinase Binding Buffer.

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.



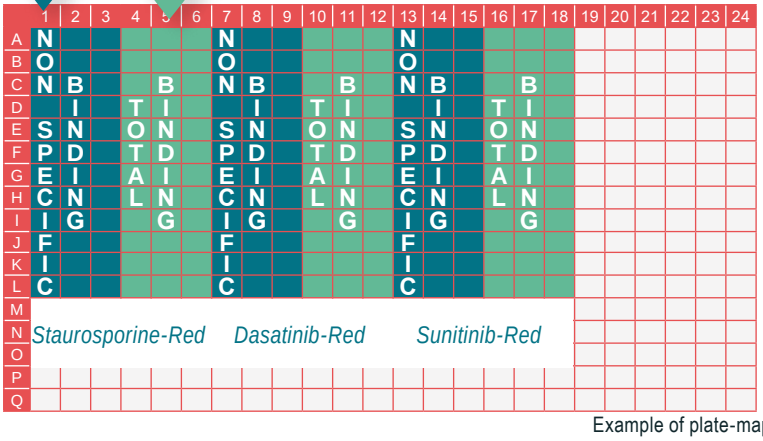
Inhibitor-Red dilutions	Dilutions	Working solutions nM	Final concentration nM
Dil 11	8 µL of stock solution (25 µM) + 192 µL Kinase Binding Buffer	1000	250
Dil 10	100 µL Dil 11 + 100 µL Kinase Binding Buffer with 4% DMSO	500	125
Dil 9	100 µL Dil 10 + 100 µL Kinase Binding Buffer with 4% DMSO	250	62.5
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 µL of Kinase Binding Buffer into each well.	
Step 2 	Dispense 5 µL of Kinase Binding Buffer into all wells.	Dispense 5 µL of 6His tagged Kinase into all wells.
Step 3 	Dispense 5 µL of Anti 6His-Eu cryptate antibody into all wells.	
Step 4 	Dispense 5 µL 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 antibodye 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 antibodye 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



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 = Bmax*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 *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).

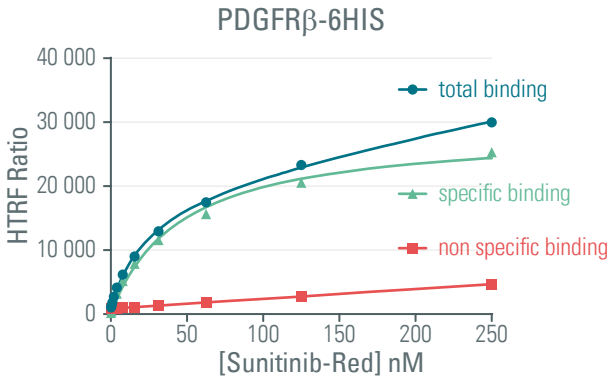
Results may vary from one HTRF® compatible reader to another.

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

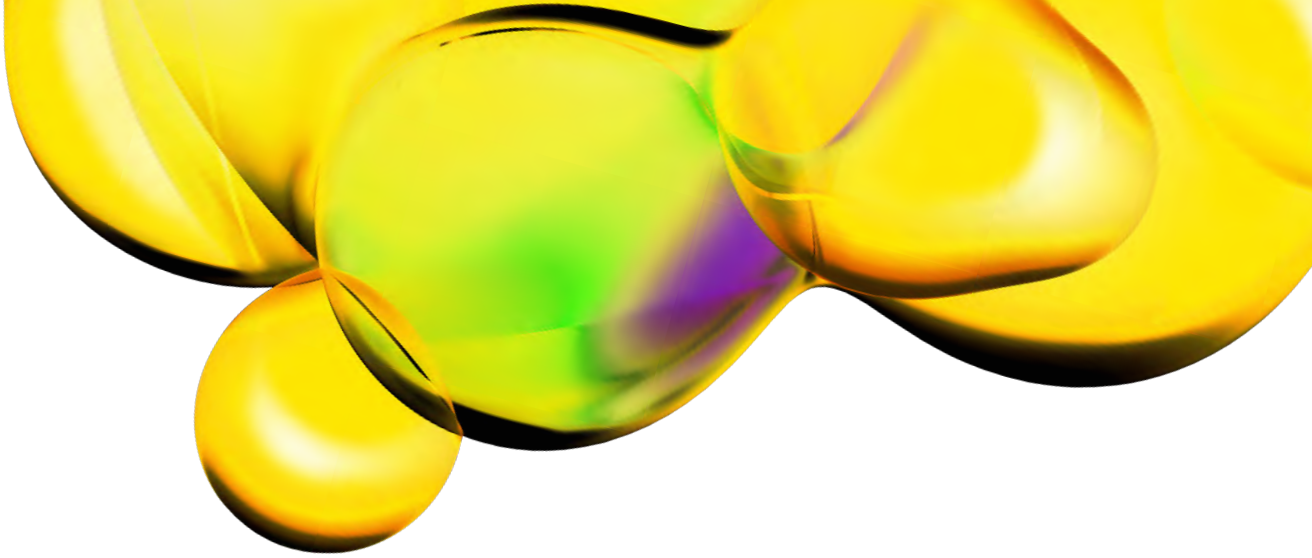
K_D DETERMINATION

Sunitinib-Red on 5 nM 6His-tagged PDGFRβ

- KD = 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 %



Manufactured by **Cisbio Bioassays** - Parc Marcel Boiteux - 30200 Codolet – FRANCE

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