revvity

APPLICATION NOTE

HTRF kinase binding assays: Staurosporine-Red validation.

This note presents a new HTRF kinase binding assay that combines the usage of a Staurosporine-Red ligand with a tagged kinase enzyme and its corresponding anti-tag-Europium labeled detection reagents.

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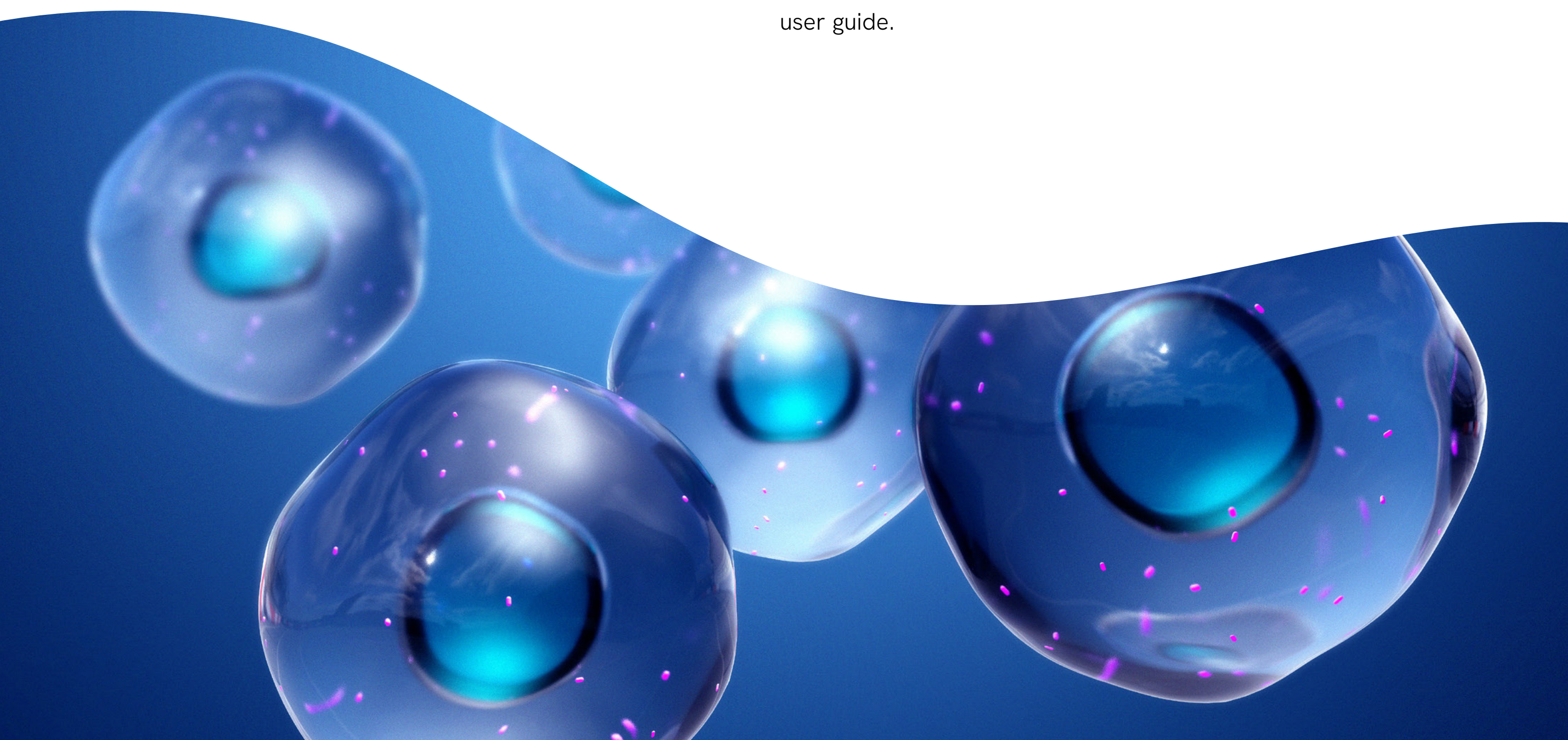
Abstract

Selection of kinase inhibitors has resulted in numerous success stories for novel drug identification, especially related to cancer treatment and metabolic diseases. Biochemical enzymatic assays such as KinEASE® are sensitive and efficient methods for drug screening, but the need for robust orthogonal assays to study or further validate the inhibitor's binding mode still remains high.

This application note presents a new HTRF® kinase binding assay that combines the usage of a Staurosporine-Red ligand with a tagged kinase enzyme (GST, 6His, or Biotin tagged) and its corresponding anti-tag-Europium labeled detection reagents. Here 6 examples, representative of the different tag formats, are investigated in depth.

The data obtained for QIK, Src, FGFR1, and MEK1 demonstrate that the Staurosporine-Red analog enables the accurate characterization of known type-I/II inhibitors independently of the enzyme detection format, confirming the pharmacological relevance of the kinase binding platform.

Revvity's comprehensive offer includes three discovery kits comprising three fluorescent tracers, Kinase Binding Buffer and either Eu cryptate labeled anti-GST, anti-6HIS Mab, or streptavidin. The individual components are also offered as spare reagents for extended pharmacological studies. With the three fluorescent tracers we estimate that 80% of the Kinome is covered. For further details, see the HTRF kinase binding user guide.



Assay principle

The assay is based on an HTRF sandwich format using either Anti-Tag-Eu cryptate MAb or Streptavidin-Eu cryptate, and a fluorescent derivative of Staurosporine (Staurosporine-Red, 62KB01REDC/E).

When a GST, 6HIS, or biotinylated kinase is present, an HTRF signal is generated. Upon the addition of competitive type I/II inhibitors of the ATP binding site or allosteric type III inhibitors, the fluorescent Staurosporine is displaced and the HTRF signal disappears (see below).

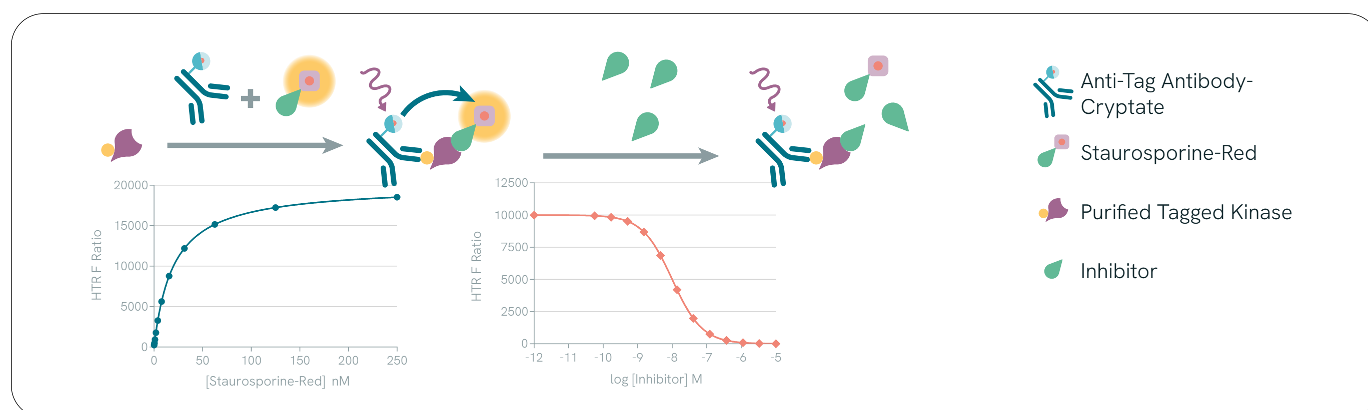


Figure 1: HTRF assay principle in a typical kinase binding assay, a purified GST-tagged Kinase at 5 nM was incubated with Anti-GST Eu cryptate MAb and a 2-fold dilution series from 0-250 nM of Staurosporine-Red, all diluted in the Kinase binding buffer. In order to determine non-specific binding, the kinase is removed. An HTRF signal is generated which is dependent on the concentration of Staurosporine-Red bound to the GST-Kinase. After determining the Kd of the tracer for your GST-Kinase of interest, the affinity of type I and II inhibitors to the ATP binding site can be assessed. A competitive binding assay is carried out by adding a fixed concentration of Staurosporine-Red at or near the Kd and a dilution series of your compound of interest.

Saturation binding assay: Kd determination on GST kinases

Determination of the Staurosporine-Red's Kd on the tagged kinase of interest

The first step in the development of the kinase inhibitor assay is to identify the optimal tracer concentration for the kinase of interest.

The dynamic range of the assay will depend on the kinase concentration. We have used kinase concentrations of either 1 or 5 nM and a concentration range from 0-250 nM of fluorescent tracer to determine the tracer Kd on several tagged kinases. Here we discuss the results using a concentration of 5 nM of kinase.

Tracer's Kds for GST-tagged kinases

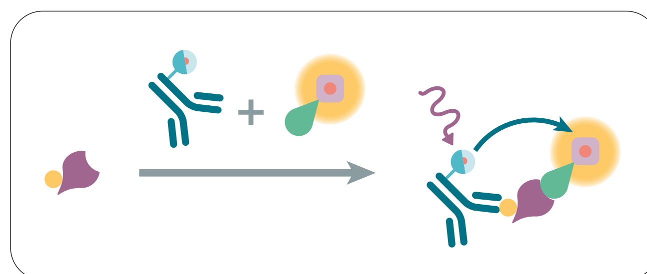


Figure 2: HTRF assay principle for GST-tagged kinases

The dissociation constant (Kd) of Staurosporine-Red (#62KB01REDC/E) on several GST-tagged kinases (5 nM) obtained from Carna Biosciences were measured using anti-GST Eu cryptate MAb (#62KBGSTKAF/B) and a dilution series of Staurosporine-Red. The results are shown below

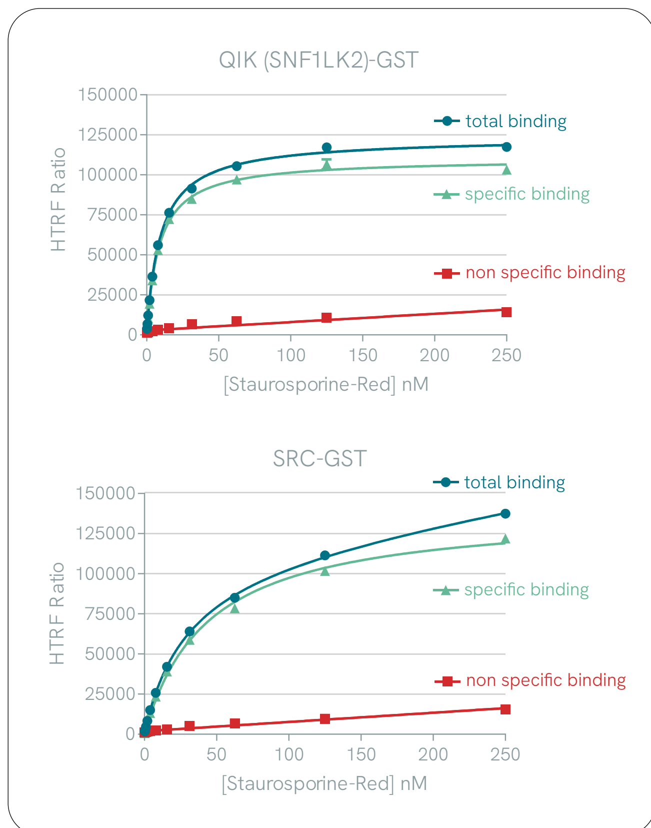


Figure 3: Analysis of QIK-GST and SRC-GST Saturation binding curves of Staurosporine-Red on 5 nM QIK-GST or SRC-GST measured in a 384-well plate by dispensing 5 µL of 'Kinase Binding Buffer', 5 µL of kinase-GST, 5 µL of anti-GST Eu cryptate Mab, and 5 mL of Staurosporine-Red from 0-250 nM, all diluted in the 'Kinase Binding Buffer'. For the non-specific binding signal, 5 µL of kinase-GST was replaced by the 'Kinase Binding Buffer'. Data generated on the Pherastar-FS reader after 1h of incubation.

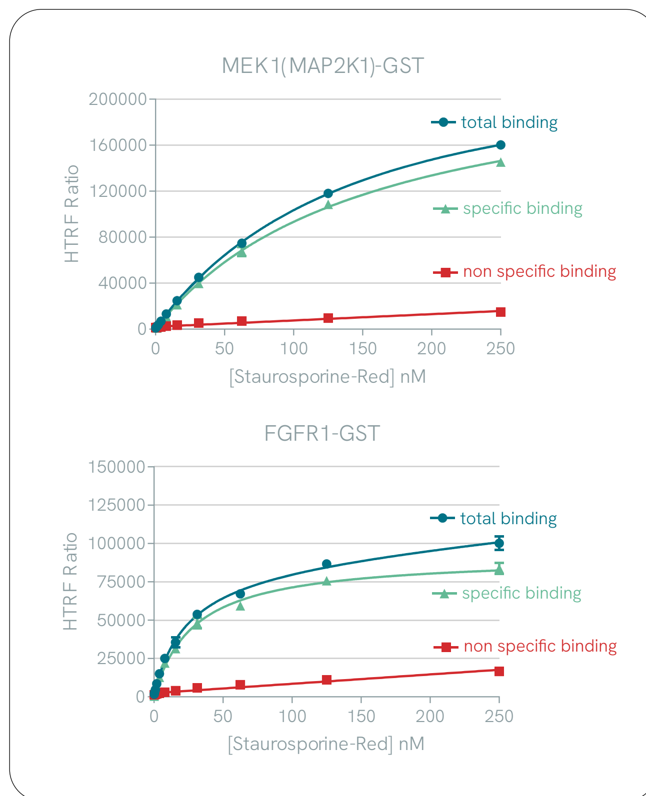


Figure 4: Analysis of MEK1-GST and FGFR1-GST Saturation binding curves of Staurosporine-Red on 5 nM MEK1-GST and FGFR1-GST. Data generated on the Pherastar-FS reader after 1h of incubation.

The Kd values of Staurosporine-Red obtained on the GST tagged kinases are shown in the table below. These results demonstrate that on these GST-tagged kinases of different natures (RTK, Ser/Thr Kinases, or Cytoplasmic) Staurosporine-Red readily binds with good affinities, giving high assay windows. Moreover, the signal is specific as shown by the difference from the signal without kinase.

Kinase	Concentration	Type	Ref carna	Kd (nM)*
FGFR1	5 nM	RTK	08-133	29
Src	5 nM	cytoplasm	08-173	43
MEK-1	5 nM	Ser/Thr	07-141	138
QIK	5 nM	Ser/Thr	02-129	8.6

*The Kd may vary depending on the source of the GST-kinase used.

Saturation binding assay: kd determination on 6HIS-Kinases and biotin-kinases

Staurosporine-Red's Kds for 6-HIS-tagged kinases

Kd measurements using Staurosporine-Red are not limited only to GST-tagged kinases. Using Revvity's Anti 6HIS Europium cryptate MAb, elevated assay windows and accurate Kd values can be obtained on 6HIS tagged kinases.

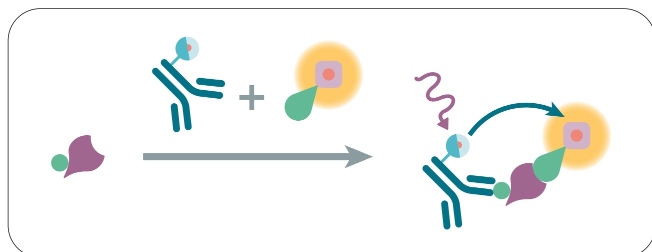


Figure 5: HTRF assay principle for 6-HIS-tagged kinases

The dissociation constant (Kd) of Staurosporine-Red on 6-HIS tagged SRC was measured using anti-6HIS Eu cryptate MAb (#62KBHISKAF/B) and a dilution series of Staurosporine-Red. The results are shown below.

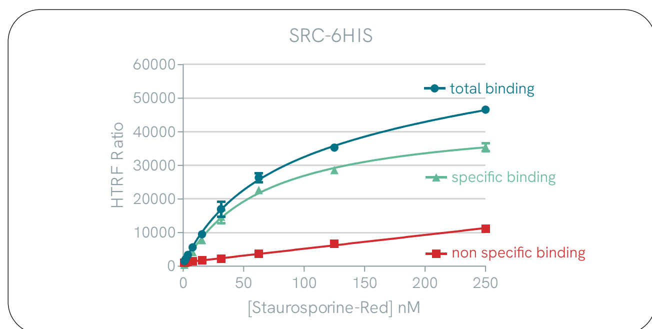


Figure 6: Analysis of SRC-6HIS

Saturation binding curves of Staurosporine-Red on 5 nM SRC-6HIS measured in a 384-well plate by dispensing 5 µL of 'Kinase Binding Buffer', 5 µL of kinase-6HIS, 5 mL of anti-6HIS Eu cryptate Mab, and 5 µL of Staurosporine-Red from 0-250 nM, all diluted in the 'Kinase Binding Buffer'. For the non-specific binding signal, 5 µL of kinase-6HIS was replaced by the 'Kinase Binding Buffer'. Data generated on the Pherastar-FS reader after 1h of incubation.

Analyses of the data revealed a Kd of 66 nM and an assay window of 7.2 at Kd. Interestingly, the Kd of the 6HIS tagged kinase is very similar to the GST-tagged kinase (43 nM), demonstrating that both tags can be used in the HTRF® kinase binding assay.

Staurosporine-Red's Kds for N-terminal biotinylated kinases

Because of the strong biotin-streptavidin interaction, the assay format will be particularly advantageous when setting up an HTRF® kinase binding assay for more advanced applications, such as measuring kinetic binding. Although this application is not described here, we were able to set up a proof-of-concept by combining 5 nM of FGFR1-BTN (#08-435-23N-Carna Biosciences) and Streptavidin-Eu cryptate (#62KBSAKAF/B).

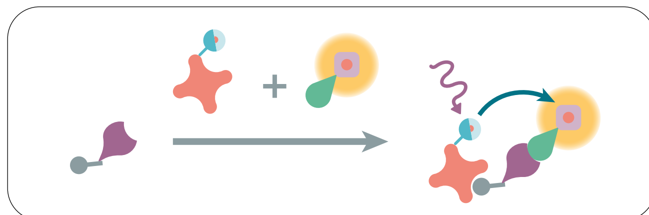


Figure 7: HTRF assay principle for N-terminal biotinylated kinases

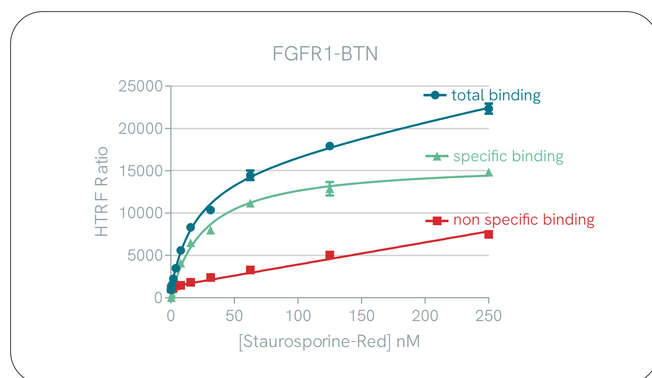


Figure 8: Analysis of FGFR1-BTN Saturation binding curves of Staurosporine-Red on 5 nM FGFR1-BTN measured in a 384-well plate by dispensing 5 µL of 'Kinase Binding Buffer', 5 µL of kinase-BTN, 5 µL of Streptavidin Eu cryptate, and 5 µL of Staurosporine-Red from 0-250 nM, all diluted in the 'Kinase Binding Buffer'. For the non-specific binding signal, 5 µL of kinase-BTN was replaced by the 'Kinase Binding Buffer'. Data generated on the Pherastar-FS reader after 1h of incubation.

Analyses of the data revealed a Kd of 25 nM and an assay window of 5.0 at Kd. The Kd of the biotinylated kinase is very similar to the GST-tagged kinase (29 nM), demonstrating that both tags can be used in the HTRF® kinase binding assay.

Competitive inhibition assay

Determination of IC_{50}/Ki of the inhibitor of interest

The simple assay format makes it easy to screen inhibitors without the need for specific substrates or the presence of ATP. After determination of the tracer Kd, inhibition assays

can be set up at tracer concentrations varying between the Kd and 4 x Kd to maintain optimal conditions. For further details about the selection of the optimum tracer concentration, please see the HTRF kinase binding user guide. Either a fixed concentration of inhibitor can be used (for high throughput studies), or a dose response curve can be determined for inhibition and pharmacological studies.

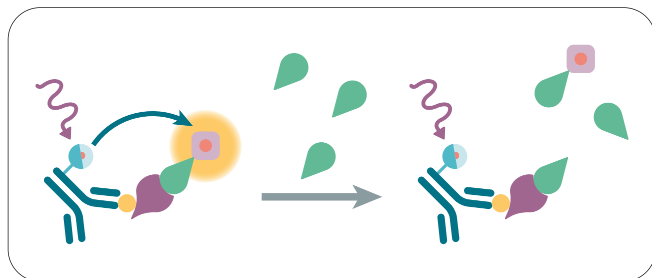


Figure 9: HTRF competitive inhibitor assay principle

Competitive inhibition assay

Inhibitor screening for GST-QIK and GST-FGFR1

A panel of well known kinase inhibitors was screened on GST-QIK and GST-FGFR1 using Staurosporine-Red at 5.4 nM (QIK) and 29 nM (FGFR1) and the results are shown below. The IC_{50} values measured were used to calculate the Ki values using the Cheng-Prusoff [1] equation below.

$$K_i = \frac{IC_{50}}{\left(1+\left(\frac{\text{Staurosporine-Red}}{K_d}\right)\right)}$$

The results were compared to data generated by Karaman et al.[2] or Georgi et al[3] and a clear correlation between these data is demonstrated proving that these assays give representative Ki values.

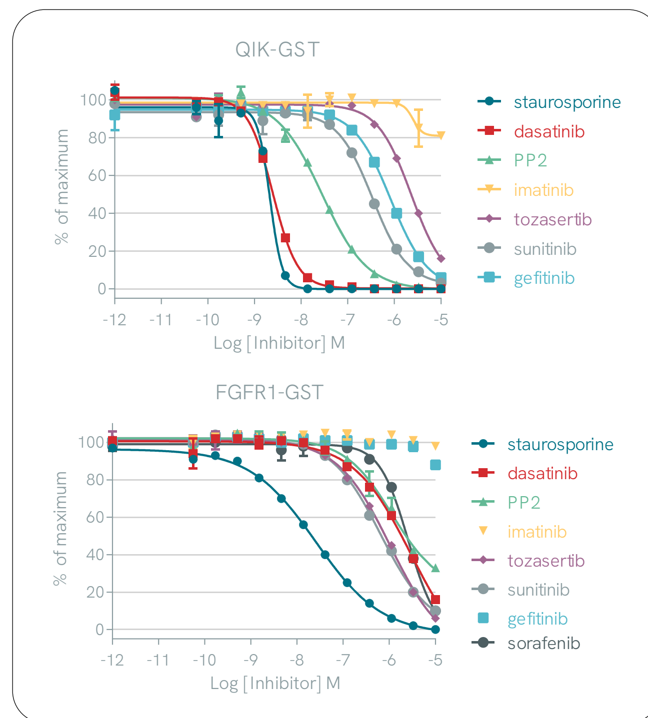


Figure 10: Analysis of Inhibitor screening for QIK-GST and FGFR1-GST Dose response curves of a panel of kinase inhibitors on 5 nM GST-QIK and GST-FGFR1 measured in a 384-well plate by dispensing 5 µL of a dilution series of inhibitor, 5 µL of kinase-GST, 5 µL of anti-GST Eu cryptate MAb, and 5 µL of Staurosporine-Red, all diluted in the 'Kinase Binding Buffer'. Data generated on the Pherastar-FS reader after 1h of incubation.

Inhibitor	GST-QIK			GST-FGFR1		
	IC_{50} (nM)	Ki (nM)	literature (Kd, nM)	IC_{50} (nM)	Ki (nM)	literature (Kd, nM)
Staurosporine	1.7	1	1.1 [2]	25.7	13	9.1 [3]
Dasatinib	2.1	1.3	6.4 [2]	2928	1464	870 [3]
PP2	43	26	nr	1 157	579	nr
Imatinib	>10 000	>10 000	>10 000 [2]	>10 000		>20 000 [3]
Tozasertib	2564	1531	1 700 [2]	1 044	522	201 [3]
Sunitinib	466	278	580 [2]	645	323	147 [3]
Gefitinib	924	552	2 100 [2]	>10 000		>20 000 [3]
Sorafinib			>10 000 [2]	2 554	1277	580 [4]

nr = not reported

Inhibitor screening for 6HIS-FGFR1 and BTN-SRC

The panel of kinase inhibitors was also screened on 6HIS-FGFR1 and BTN-SRC using Staurosporine-Red at its Kd 22 nM (FGFR1) and 35 nM (SRC). The results are shown below. A similar analysis to that described for the GST-tagged kinases was performed. Overall, a good correlation with the literature data was observed. Moreover it is worth noting that the Ki results obtained on the panel of inhibitors are in the same range whatever the tag used for FGFR1 (GST-FGFR1 and 6HIS-FGFR1).

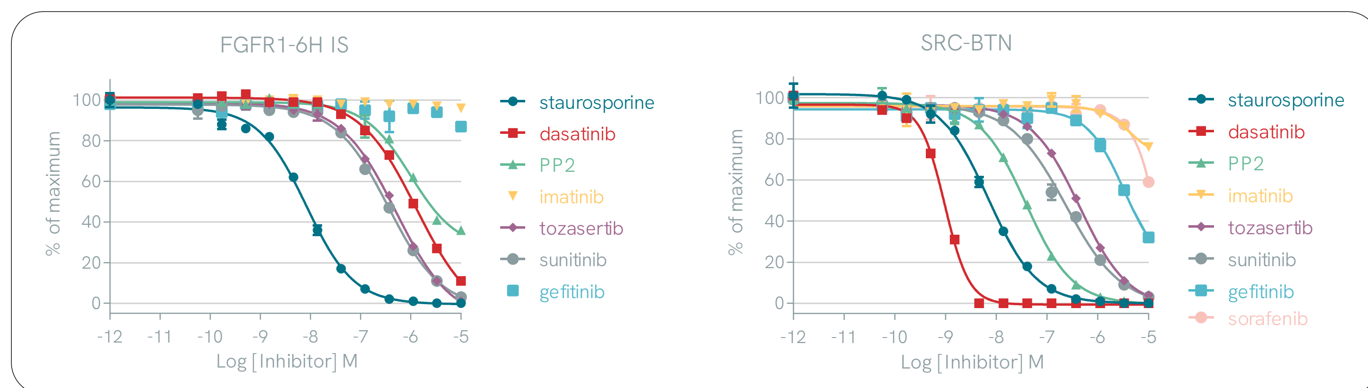


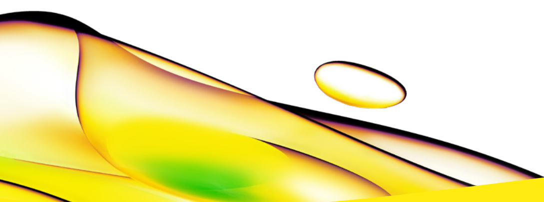
Figure 11: Analysis of Inhibitor screening for FGFR1-6HIS and SRC-BTN Dose response curves of a panel of kinase inhibitors on 5 nM 6HIS-FGFR1 and BTN-SRC measured in a 384-well plate by dispensing 5 µL of a dilution series of inhibitor, 5 µL of kinase, 5 µL of anti 6HIS-Eu cryptate MAb, or Streptavidin-Eu cryptate and 5 µL of Staurosporine-Red, all diluted in the 'Kinase Binding Buffer'. Data generated on the Pherastar-FS reader after 1h of incubation.

Inhibitor	6HIS-FGFR1			BTN-SRC		
	IC_{50} (nM)	Ki (nM)	literature (Kd, nM)	IC_{50} (nM)	Ki (nM)	literature (Kd, nM)
Staurosporine	8.3	4.2	9.1 [3]	6.9	3.45	2.6 [3]
Dasatinib	1 291	645.0	870 [3]	0.96	0.48	0.21 [2], 0.06 [3]
PP2	987	494.0	nr	38	19	36 [5]
Imatinib	>10 000		>20 000 [3]	>10 000		>10000 [2]
Tozasertib	509	255.0	201 [3]	433	216.5	170 [2], 118 [3]
Sunitinib	356	178.0	147 [3]	235	117.5	104 [3]
Gefitinib	>10 000		>20 000 [3]	3312	1656	3 800 [2], 344 [3]
Sorafinib				>10 000		>10 000 [2]

nr = not reported

References

1. Y.C Cheng, W.H. Prusoff., Biochem. Pharmacol. 22 (1973) 3099-3108.
2. M.Z. Karaman et al., Nat. Biotechnol. 26 (2008) 127-132.
3. V. Georgi et al., J. Am. Chem. Soc. 140 (2018) 15774-15782.
4. S.M. Wilhelm et al., Cancer Res. 64 (2004) 7099-7109 5] J. Bain et al., Biochem J. 408 (2007) 297-315.



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