APPLICATION NOTE

HTRF kinase binding assays: Dasatinib-Red validation.

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

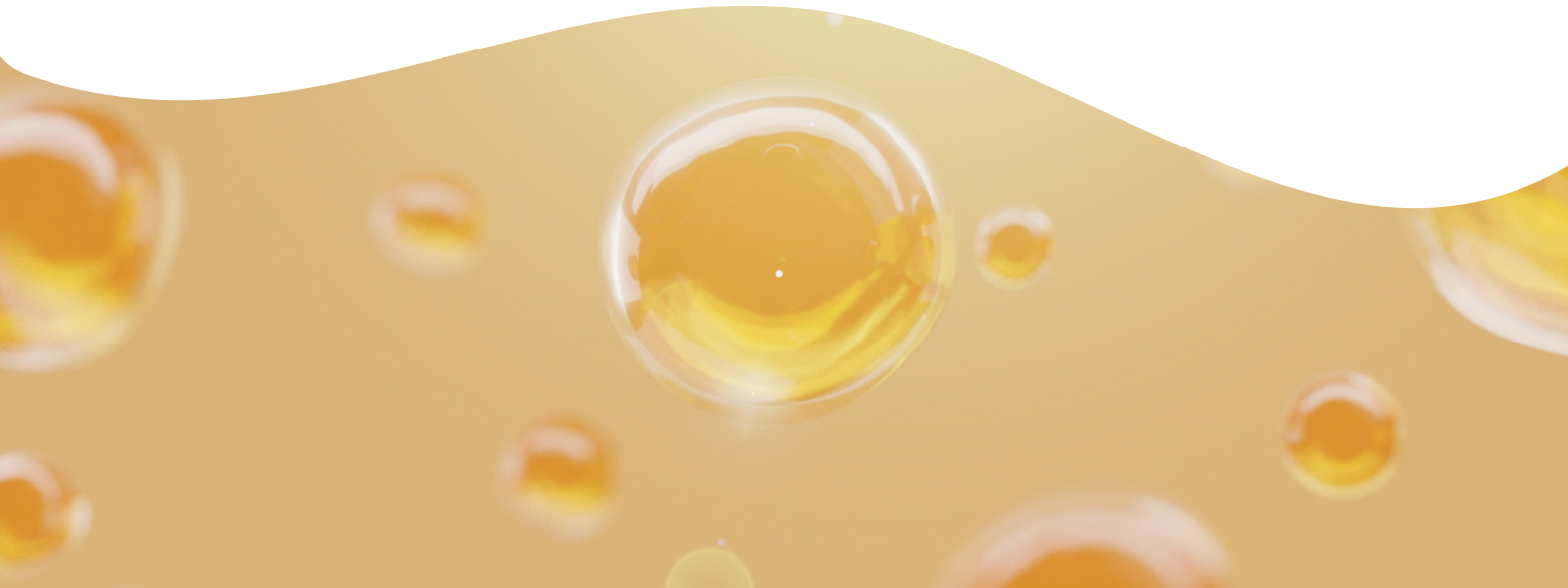
Abstract

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 inhibitors' binding mode still remains high.

This application note presents a new HTRF® kinase binding assay that combines the usage of a Dasatinib-Red ligand with a tagged kinase enzyme (GST, 6His, or biotin tagged) and its corresponding anti-tag-europium labeled detection reagents. This note highlights examples that are representative of the different tag formats.

The data obtained for BRAF, TGFBR1, and PDGFRb demonstrate that the Dasatinib-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 Dasatinib (Dasatinib-Red, 62KB02REDC/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 Dasatinib is displaced and the HTRF signal disappears (see below).

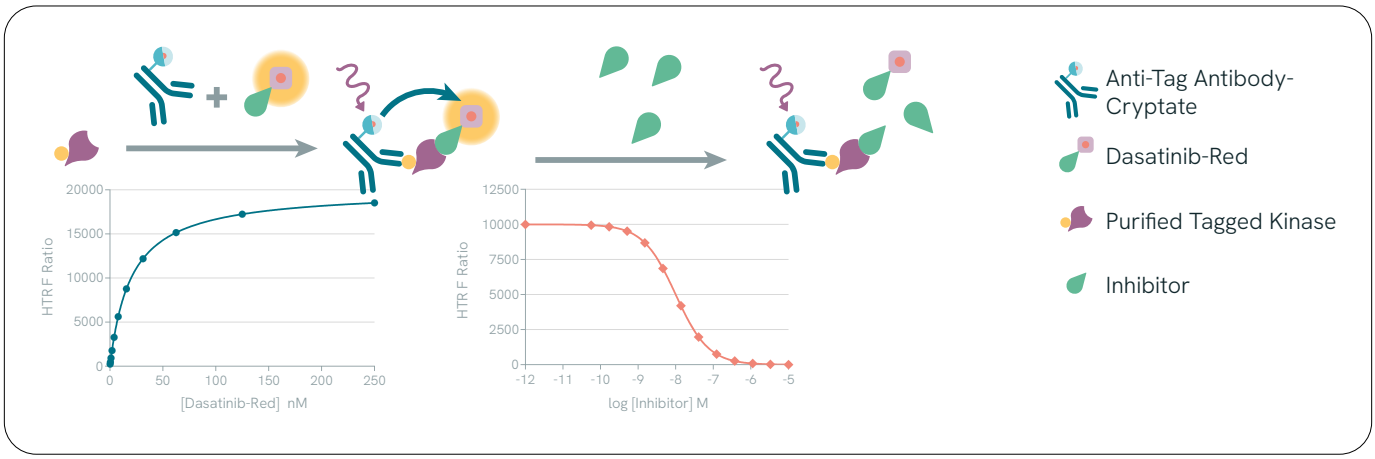


Figure 1: HTRF assay principle In a typical kinase binding assay, a purified GST-tagged Kinase at 5 nM was incubated with Anti-GST Eu cryptate MAb and a 2-fold dilution series from 0-250 nM of Dasatinib-Red, all diluted in the Kinase binding buffer. In order to determine non-specific binding, the kinase was removed. An HTRF signal is generated which is dependent on the concentration of Dasatinib-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 Dasatinib-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 Dasatinib-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 5 nM and a concentration range from 0-250 nM of fluorescent tracer to determine the tracer Kd on several tagged kinases.

Tracer Kds for GST-tagged kinases

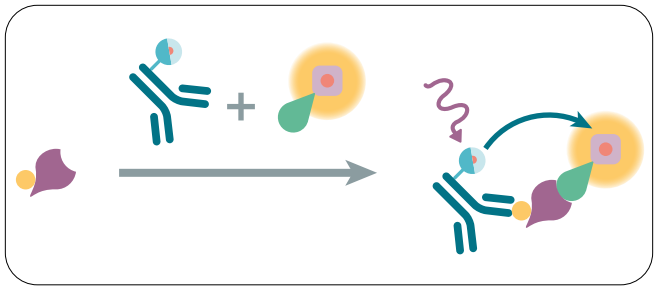


Figure 2: HTRF assay principle for GST-tagged kinases

The dissociation constants (Kd) of Dasatinib-Red (#62KB02REDC/E) on two GST-tagged kinases (5 nM) obtained from Carna Biosciences were measured using anti-GST Eu cryptate MAb (#62KBGSTKAF/B) and a dilution series of Dasatinib-Red. The results are shown below.

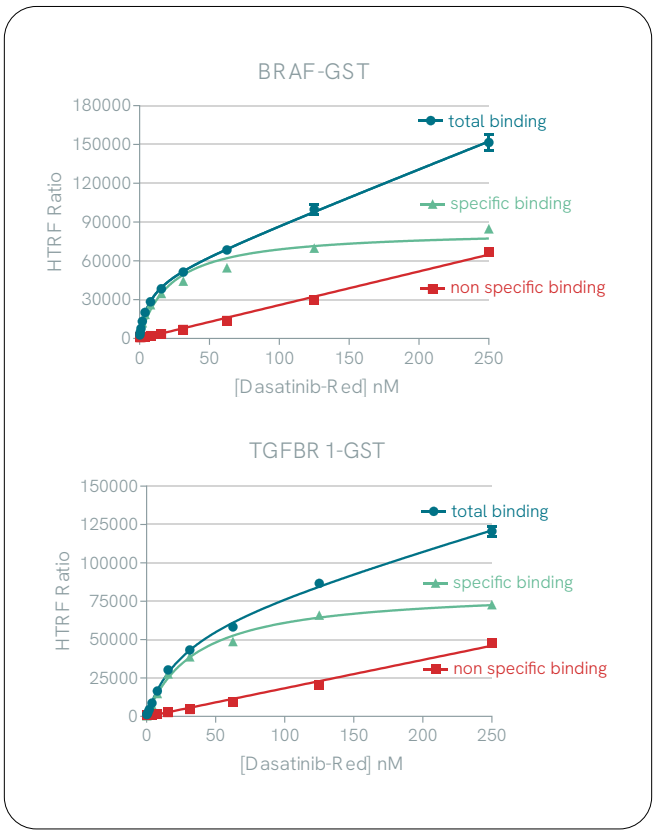


Figure 3: Analysis of BRAF-GST and TGFBR1-GST Saturation binding curves of Dasatinib-Red on 5 nM BRAF-GST or TGFBR1-GST measured in a 384-well plate by dispensing 5 µL of 'Kinase Binding Buffer', 5 µL of kinase-GST, 5 µL of anti-GST Eu cryptate Mab, and 5 mL of Dasatinib-Red from 0-250 nM, all diluted 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.

The Kd values of Dasatinib-Red obtained on the GST tagged kinases are shown in the table below. These results demonstrate that on these GST-tagged kinases, Dasatinib-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)
BRAF	5 nM	Ser/Thr	08-156	22
TGFBR1	5 nM	Ser/Thr	08-158	36

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

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

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

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

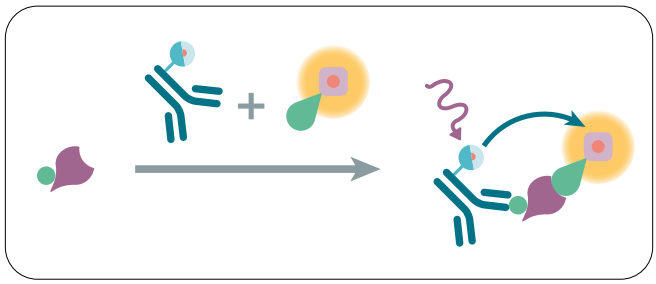


Figure 4: HTRF assay principle for 6-HIS-tagged kinases

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

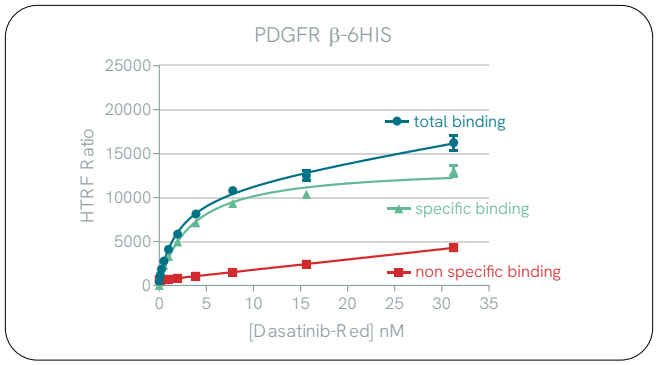


Figure 5: Analysis of PDGFRβ-6HIS Saturation binding curves of Dasatinib-Red on 2 nM PDGFRb-6HIS measured in a 384-well plate by dispensing 5 µL of 'Kinase Binding Buffer', 5 µL of kinase-6HIS, 5 mL of anti-6HIS Eu cryptate MAb, and 5 µL of Dasatinib-Red from 0-250 nM, all diluted in the 'Kinase Binding 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.

Analyses of the data revealed a Kd of 3.3 nM and an assay window of 8 at Kd.

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

Dasatinib-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 KIT-BTN (#08-456-21N-Carna Biosciences) and Streptavidin-Eu cryptate (#62KBSAKAF/B).

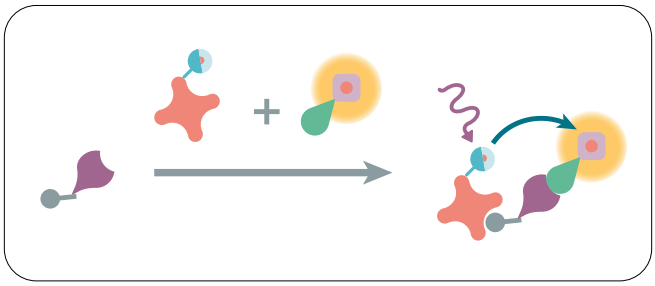


Figure 6: HTRF assay principle for N-terminal biotinylated kinases

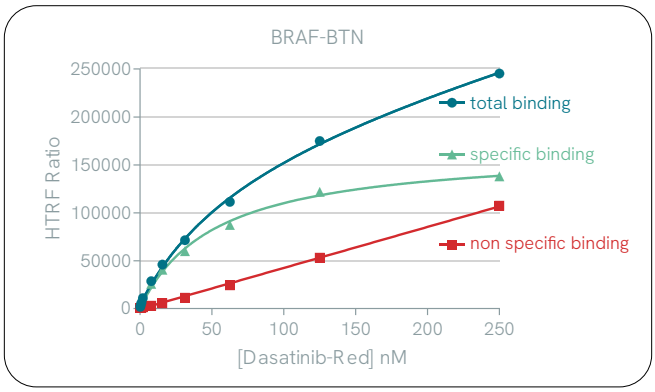


Figure 7: Analysis of BRAF-BTN Saturation binding curves of Dasatinib-Red on 5 nM BRAF-BTN measured in a 384-well plate by dispensing 5 µL of 'Kinase Binding Buffer', 5 µL of kinase-BTN, 5 µL of Streptavidin Eu cryptate, and 5 µL of Dasatinib-Red from 0-250 nM, all diluted in the 'Kinase Binding Buffer '. 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 52 nM and an assay window of 5 at Kd.

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 4x Kd to maintain optimal conditions. The optimal tracer concentration depends on the dynamic range observed for the kinase of interest.

For further details about the selection of optimum tracer concentration please see the HTRF® Kinase Binding User Guide. Either a fixed concentration of inhibitor (for high throughput studies) can be used or a dose response curve can be determined for inhibition and pharmacological studies.

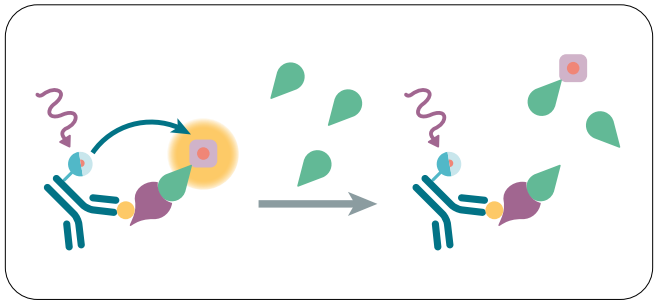


Figure 8: HTRF competitive inhibitor assay principle

Inhibitor screening for GST-BRAF

A panel of well known kinase inhibitors was screened on GST-BRAF using Dasatinib-Red at 22 nM and the results are shown below. The IC_{50} values measured were used to calculate the Ki potency using the Cheng-Prusoff [1] equation below.

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

The results were compared to data generated in the literature and a clear correlation between these data is demonstrated proving that these assays give representative Ki values.

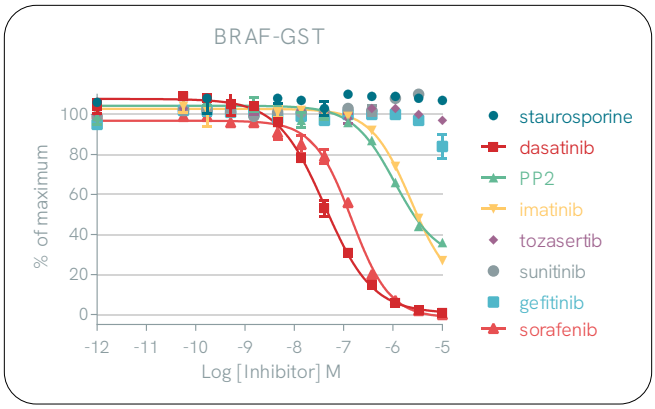


Figure 9: Analysis of Inhibitor screening for BRAF-GST Dose response curves of a panel of kinase inhibitors on 5 nM GST-BRAF measured in a 384-well plate by dispensing 5 µL of a dilution series of inhibitor, 5 µL of kinase-GST, 5 µL of anti-GST Eu cryptate Mab, and 5 µL of Dasatinib-Red, all diluted in the 'Kinase Binding Buffer'. Data generated on the Pherastar-FS reader after 1h of incubation.

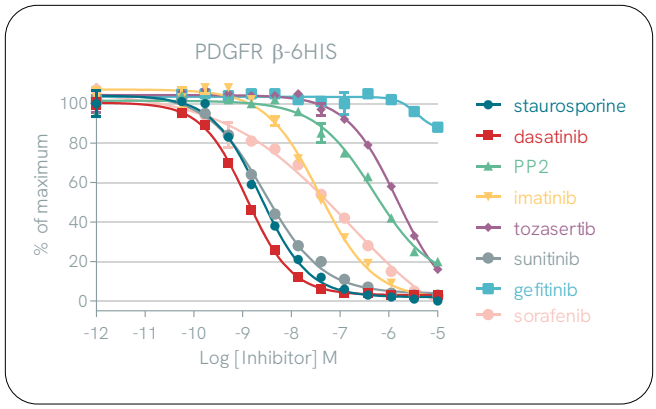


Figure 10: Analysis of Inhibitor screening for PDGFRβ-6HIS Dose response curves of a panel of kinase inhibitors on 2 nM 6HIS-PDGFRb measured in a 384-well plate by dispensing 5 µL of a dilution series of inhibitor, 5 µL of kinase, 5 µL of anti 6HIS-Eu cryptate MAb, and 5 µL of Dasatinib-Red, all diluted in the 'Kinase Binding Buffer'. Data generated on the Pherastar-FS reader after 1h of incubation.

Competitive inhibition assay

Inhibitor	GST-BRAF		
	IC_{50} (nM)	Ki (nM)	literature (Kd, nM)
Staurosporine	>10 000	>10 000	>10 000 [2]
Dasatinib	41	21	82 [3]
PP2	1 097*	549	nr
Imatinib	2 304*	1 152	4 560 [3]
Tozasertib	>10 000	>10 000	>10 000 [2]
Sunitinib	>10 000	>10 000	>10 000 [2]
Gefitinib	>10 000	>10 000	>10 000 [2]
Sorafinib	145	73	540 [2], 22 [4]

* = incomplete inhibition, nr = not reported

Inhibitor	6HIS-PDGFRb		
	IC_{50} (nM)	Ki (nM)	literature (Kd, nM)
Staurosporine	2.3	1.2	1.8 [2]
Dasatinib	1.2	0.6	0.63 [2]
PP2	484	242	nr
Imatinib	38	19	14 [2]
Tozasertib	1493	747	310 [2]
Sunitinib	2.9	1.5	0.08 [2], 5.7 [5]
Gefitinib	>10 000	>10 000	>10 000 [2]
Sorafinib	142	71	37 [2], 57 [4]

nr = not reported

Inhibitor screening for 6HIS-PDGFRb and BTN-BRAF

The panel of kinase inhibitors was also screened on 6HIS-PDGFRb and BTN-BRAF using Dasatinib-Red at its Kd (3 nM (PDGFRb) and 52 nM (BRAF)). The results are shown below. A similar analysis to that described for the GST-tagged kinases was performed . Overall, a good correlation between these and the literature data was observed, confirming the suitability of Dasatinib-Red tracer to perform pharmacological characterization of inhibitors. 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 BRAF (GST-BRAF and BTN-BRAF).

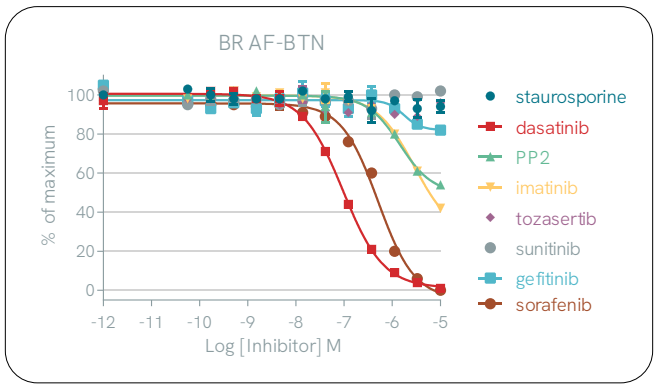


Figure 11: Analysis of Inhibitor screening for BRAF-BTN Dose response curves of a panel of kinase inhibitors on 5 nM BTN-BRAF measured in a 384-well plate by dispensing 5 µL of a dilution series of inhibitor, 5 µL of kinase, 5 µL of Streptavidin-Eu cryptate, and 5 µL of Dasatinib-Red, all diluted in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

Inhibitor	BTN-BRAF		
	IC_{50} (nM)	Ki (nM)	literature (Kd, nM)
Staurosporine	>10 000	>10 000	>10 000 [2]
Dasatinib	95	48	82 [3]
PP2	1 446*	723	nr
Imatinib	2 857*	1 429	4 560 [3]
Tozasertib	>10 000	>10 000	>10 000 [2]
Sunitinib	>10 000	>10 000	>10 000 [2]
Gefitinib	>10 000	>10 000	>10 000 [2]
Sorafinib	506	253	540 [2], 22 [4]

* = incomplete inhibition, 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
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