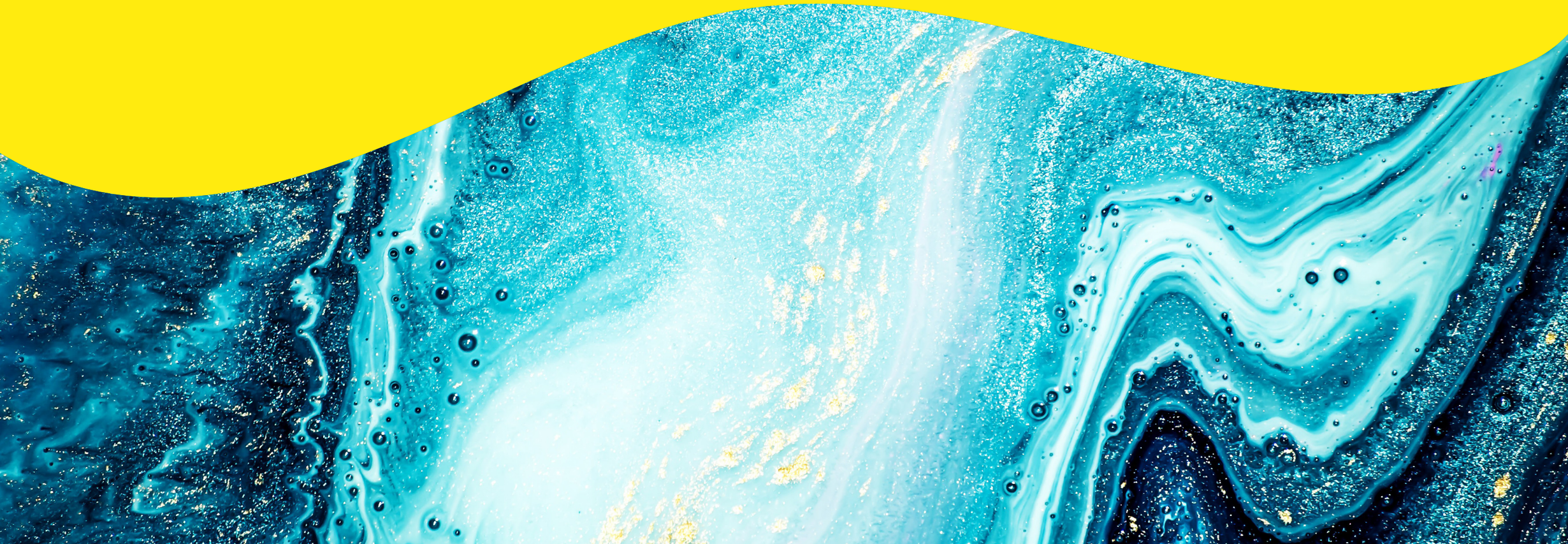
Kinase

Guide to the key to successful kinase binding assays

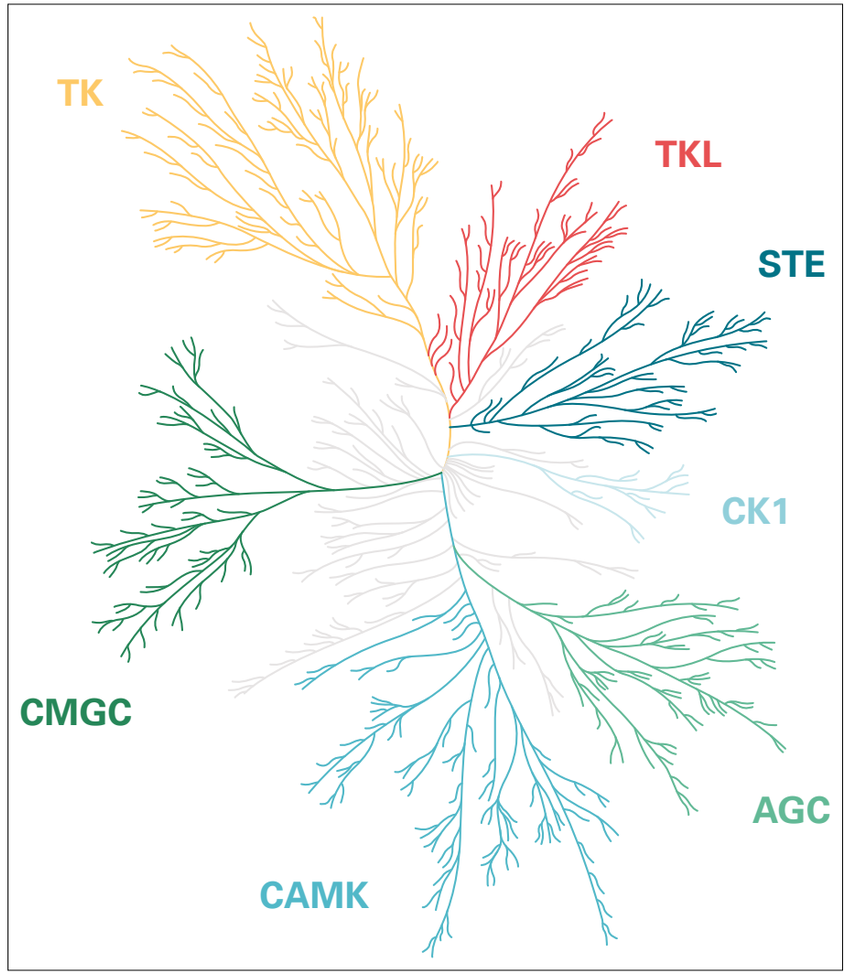


PURPOSE AND BACKGROUND

Purpose

The proper optimization of assay conditions is essential to ensure your reagent use gives the best performance. With our new HTRF® Kinase Binding format, we can guide you to properly optimize the assay tools provided in the Kinase Binding discovery kits for GST, 6-HIS or biotinylated Kinases. First, we take you through the steps to establish which fluorescent inhibitor provided in the discovery kits is best suited to your kinase of interest. Secondly, using the spare reagents provided with our offer, we show the suitable assay conditions for investigating the pharmacological characteristics of your kinase inhibitors. These guidelines are illustrated with various examples of GST, 6HIS, or biotinylated kinases.

Drawing on hundreds of data sets and cases established by Revvity's scientists over the years, our different manuals provide the Kinase community with the most up to date guidelines for the optimization of Kinase assays.



Background

Kinases

The human Kinome comprises about 518 protein kinases and 20 lipid kinases, and remains a major class of druggable targets as they have important regulatory roles in cellular signaling pathways. Their dysregulation can have major health consequences and diseases such as cancer or inflammation can result. It is estimated that the Kinome represents about 25-30% of druggable targets in humans. However the focus so far has been on only a fraction of the Kinome, and hence a large portion remains poorly characterized.
Assay technologies for kinase drug screening and characterization are therefore in high demand.

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PURPOSE AND BACKGROUND

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KINASE INHIBITORS

The interest in finding kinase inhibitors goes back to the late 1980s, when it became clear that receptor tyrosine kinases like EGFR had major implications in cancer. A breakthrough for small-molecule kinase inhibitors (SMKI) was the FDA approval and subsequent success of Gleevec® for chronic myeloid leukemia, paving the way for other SMKI to enter the market. By 2016, 28 FDA approved SMKI [1] existed, mostly targeted against cancer types, but SMKI for non-cancer indications like fibrosis (Nintedanib) and arthritis (Tofacitinib) are now also available. There are also pipelines for the central nervous system, cardiovascular diseases, and diabetes complications, showing that SMKI can be developed for a multitude of conditions.

SMKI were first directed at the ATP binding site of kinases, but because of its highly conserved nature it was soon realized that many inhibitors were not only targeted to the kinase of interest but could cross-react with others as well, leading to unwanted side effects. Although this can be convenient to develop pan-kinase inhibitors, the screening of inhibitors binding outside the main ATP binding pocket such as type III and IV allosteric inhibitors are a possible way to avoid such side effects.

KINASE BINDING ASSAY USING FLUORESCENT INHIBITORS

Kinase activity assays have long been the format of choice for inhibitor screening. Revvity's HTRF® KinEASE® platform measures the phosphorylation of either Tyrosine or Serine/threonine biotinylated peptides induced by the kinase of interest by specifically detecting the phosphorylated peptide.

With Revvity's HTRF kinase binding platform we offer a complementary method using three fluorescent inhibitors which cover roughly 80% of the Kinome. Instead of detecting compounds inhibiting phosphorylation, the binding is detected, which also gives the possibility to detect inhibitors binding to low kinases activity or non-activated kinases.

WHY SHOULD I CHOOSE THE HTRF KINASE BINDING ASSAY?

The best known kinase inhibitor, Gleevec (Imatinib), binds a non-activated form of Abl. Such inhibitors are hence more difficult to detect using activity based assays. It is therefore advantageous to set up kinase binding assays for your kinase of interest in order not to miss out on such compounds. Moreover the HTRF® kinase binding assays are easily set up, using a mix and measure detection format. ATP presence is not needed for your assay, hence you do not need to optimize your ATP concentration (as is the case for activity assays.) Direct displacement of the fluorescent-inhibitor from the ATP binding pocket is measured, enabling the affinity of your inhibitor to be determined by performing dose response curves. The Kinase concentrations used are generally 5 nM or lower, meaning a good cost reduction compared to enzymatic assays where higher Kinase concentrations are often required. Purified kinase preparation is also less critical, because measurements can also be performed on kinases which have low or no activity. This situation often occurs in cellular conditions, since concentrations of phosphorylated kinases or proteins are highly dynamic. Last but not least, while Drug-Target Residence Time is of increasing interest in the lead optimization process [2], an application derived from this kinase binding assay format permits access to kinetic parameters (Drug-Target Residence Time in particular) of the inhibitors of interest.

[1] P. Wu, T.E. Nielsen, M.H. Clausen. Drug Discovery Today 21 (2016) 5-10. [2] R.A. Copeland, D.L. Pompliano, T.D. Meek. Nat. Rev. Drug Disc. 5 (2006) 730-739

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ASSAY DESCRIPTION AND WORKFLOW

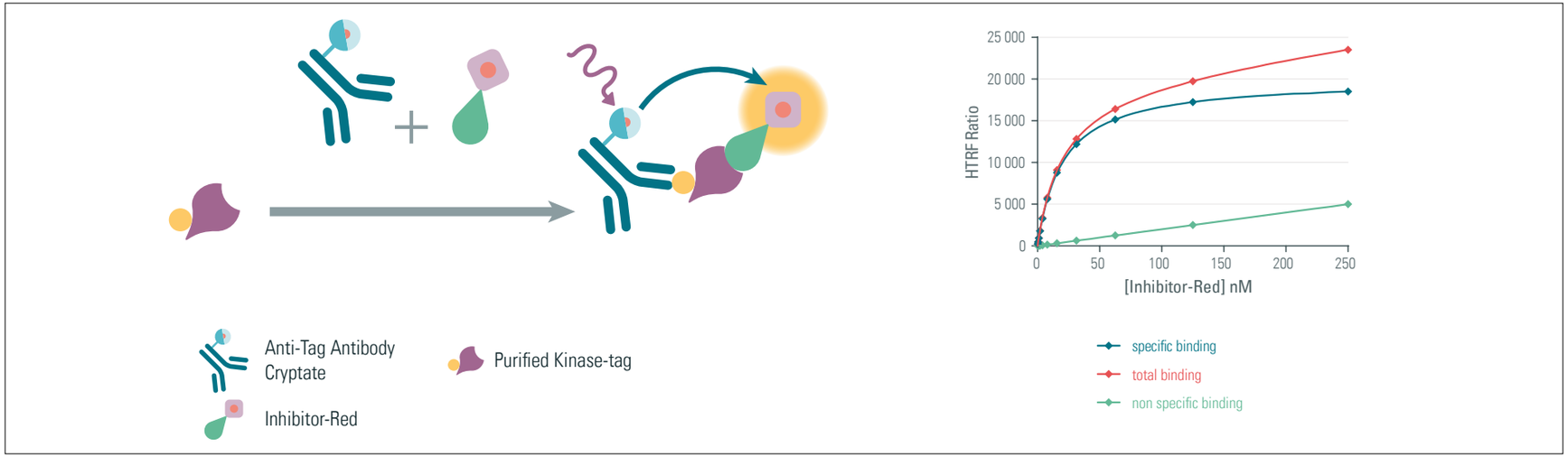
Assay principle

DETERMINATION OF THE DISSOCIATION CONSTANT (K_D) OF THE FLUORESCENT TRACER USING HTRF KINASE BINDING

The HTRF Kinase Binding assay is based on a sandwich format using either an Anti-Tag-Eu cryptate or streptavidin-Eu cryptate, and red-fluorescent derivatives of either Staurosporine, Dasatinib or Sunitinib. When a purified Kinase tagged with either a GST, 6HIS, or biotin is present, an HTRF signal is generated when the fluorescent inhibitor (tracer) is bound on the kinase.

The HTRF ratio will increase upon the addition of more tracer, and will saturate depending on the dissociation constant (K_D) of the tracer to the tagged kinase. The first step is therefore to determine which tracer might be best suited to setting up a binding assay. Depending on the kinase, each of the three tracers is expected to generate a different assay window and K_D. The tracer with the best assay properties (according to the K_D and assay window generated) will be chosen to perform competitive binding assays.

When performing the saturation binding assay, two data sets are obtained. The total binding curve is obtained in the presence of all reagents and the tracer. The non-specific binding is measured by removal of the tagged Kinase and a linear dependence on the concentration of the tracer becomes apparent. The non-specific binding can also be measured upon addition of a saturating concentration of non-labeled tracer. Subtracting the non-specific data from the total binding data gives the specific binding data, which can be analyzed by GraphPad Prism to determine the tracer K_D of the tagged-kinase of interest.



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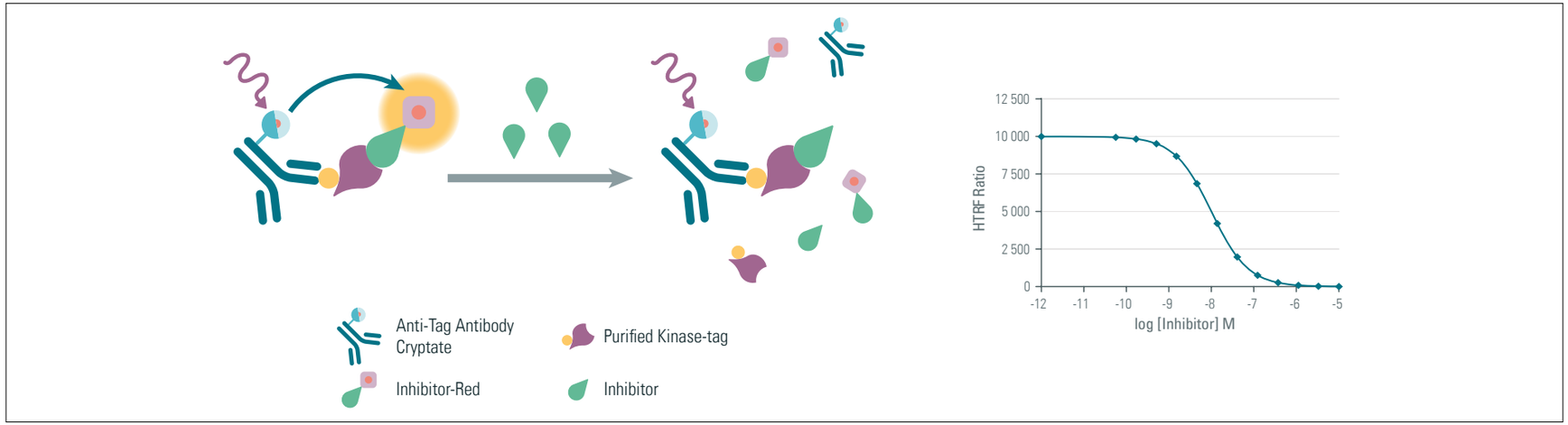
ASSAY DESCRIPTION AND WORKFLOW

Assay principle

COMPETITIVE BINDING PRINCIPLE WITH KINASE INHIBITORS USING HTRF® KINASE BINDING

After determining the tracer of choice and the K_D of the tracer to your kinase of interest as described on the previous page, the affinities of inhibitors can be tested by performing competition binding experiments and adding a fixed concentration of tracer. This concentration should preferably be at or near its K_D to maintain equilibrium conditions. By varying the concentration of the inhibitor, the fluorescent tracer will be displaced when binding of the inhibitor occurs, and the IC_{50} value of the inhibitor can be determined using GraphPad Prism. The inhibition constant (K_i) of the inhibitor to the tagged-Kinase can now be determined using the Cheng-Prusoff equation [1] , since the K_D , the tracer concentration and the IC_{50} value are known.

Cheng-Prusoff equation: $$K_i = \frac{IC_{50}}{\left(1+\left(\frac{Inhibitor\text{-}Red}{K_D}\right)\right)}$$



[1] Y.C Cheng, W.H. Prusoff., Biochem. Pharmacol. 22 (1973) 3099-3108.

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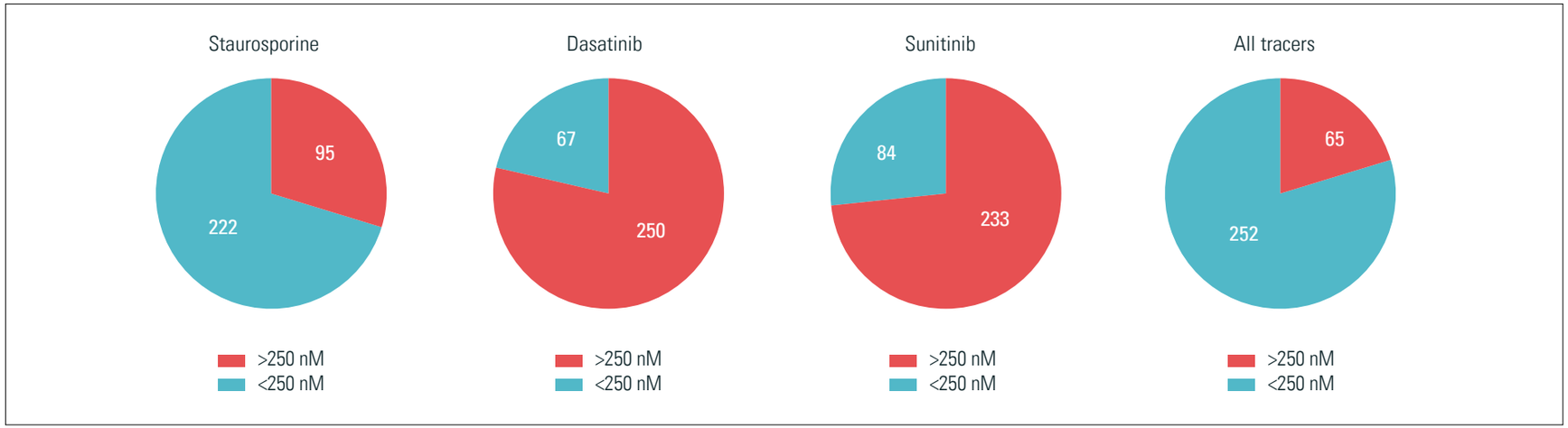
Tracers

INTRODUCTION

The fluorescent inhibitors necessary for the HTRF Kinase Binding assay were developed so that the major part of the Kinome should be addressed using the assay. Therefore broad-spectrum fluorescent inhibitors were developed, addressing both Tyrosine and Serine/Threonine protein kinases. The pan-inhibitor Staurosporine, which has shown to have affinities <100 nM to about 60% of the Kinome [1], was chosen along with Dasatinib and Sunitinib which have been shown to have promiscuous behavior as well [1]. Derivatization of these three inhibitors with Revvity's fluorescent dye 'd2' led to compounds which are able to bind to the ATP binding pocket without compromising their binding potential. With these three fluorescent inhibitors we estimate that at least 80% of the human Kinome is covered.

In the figure below we show the inhibition efficacy < 250 nM of the three inhibitors on 317 kinases, derived from data in [1], and representing a large part of the Kinome.



[1] M.Z. Karaman et al. Nat. Biotechnol. 26 (2008) 127-132.

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ASSAY DESCRIPTION AND WORKFLOW

Tracers

DASATINIB-RED

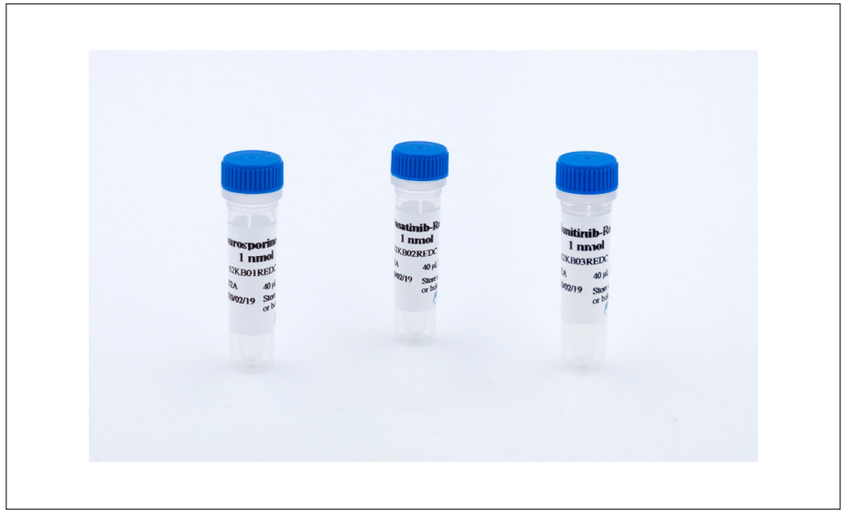
This compound is derived from Dasatinib, which is a well known inhibitor of the BCR-ABL and SRC families of Kinases. Dasatinib, which is an ATP-competitive Kinase inhibitor, is used as a drug to treat chronic myelogenous leukemia and acute lymphoblastic leukemia. It has been shown to bind with an affinity of <100 nM to about 16% of the Kinases [1], mainly to RTK, and it is therefore useful to address multiple Kinases in this assay platform. The fluorescent dye used is the 'd2' dye which absorbs with its maximum near 650 nm and fluoresces near 670 nm. It is used as an acceptor molecule in HTRF assays. Dasatinib-Red is furnished as a 25 µM solution in DMSO.

STAUROSPORINE-RED

This compound is derived from Staurosporine, which is a natural alkoid present in Streptomyces Staurosporeus. Staurosporine, a prototypical ATP-competitive Kinase inhibitor, has been shown to be highly promiscuous and therefore extremely useful for addressing multiple Kinases in this assay platform. The fluorescent dye used is the 'd2' dye which absorbs with its maximum near 650 nm and fluoresces near 670 nm. It is used as an acceptor molecule in HTRF assays. Staurosporine-Red is furnished as a 25 µM solution in DMSO.

SUNITINIB-RED

This compound is derived from Sunitinib, which is a well known inhibitor of the PDGF and VEGF RTK families. Sunitinib, which is an ATP-competitive Kinase inhibitor, is used as a drug to treat renal cell carcinoma and imatinib resistant gastrointestinal stromal tumors. It has been shown to bind with an affinity of <100 nM to about 18% of the Kinases (both RTK and Ser/Thr) [1]. It is therefore useful to address multiple Kinases in this assay platform. The fluorescent dye used is the 'd2' dye which absorbs with its maximum near 650 nm and fluoresces near 670 nm. It is used as an acceptor molecule in HTRF assays. Sunitinib-Red is furnished as a 25 µM solution in DMSO.



[1] M.Z. Karaman et al. Nat. Biotechnol. 26 (2008) 127-132.

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TAGGED KINASES

For the HTRF Kinase binding assay, a tagged kinase (either GST / 6HIS / biotin) is used. These are not furnished by Revvity and should be purchased from manufacturers such as Carna Biosciences or through your own channels. The Kinases tested by Revvity are all recombinant kinases (the whole or part of the protein), expressed and purified as either GST or 6HIS tagged kinases from either insect cells or bacterial systems. The biotinylated kinases obtained from Carna Biosciences had a single biotinylated N-terminal DYKDDDDK tag.

DISCOVERY KITS

Revvity's offer comprises three starter kits to address either GST, 6HIS, or Biotinylated Kinases. These starter kits each contain:

- Three tracers: Staurosporine-Red, Dasatinib-Red, and Sunitinib-Red - 0.5 nmol each
- Kinase binding buffer - 20 mL
- Eu-cryptate labeled anti-GST, anti-6HIS, or streptavidin - 1 000 tests

With these starter kits you can perform 2 complete saturation binding experiments (total and non-specific) with each of the three tracers, to determine which tracer is most suitable for your competitive inhibition experiments.

SPARE REAGENTS

To complement this offer, Revvity provides different quantities (1 & 20 nmol) of the three tracers, the kinase binding buffer, and the Eu-cryptate labeled streptavidin or anti-GST anti-6HIS antibodies to perform your competitive inhibition assays.

[1] M.Z. Karaman et al. Nat. Biotechnol. 26 (2008) 127-132.

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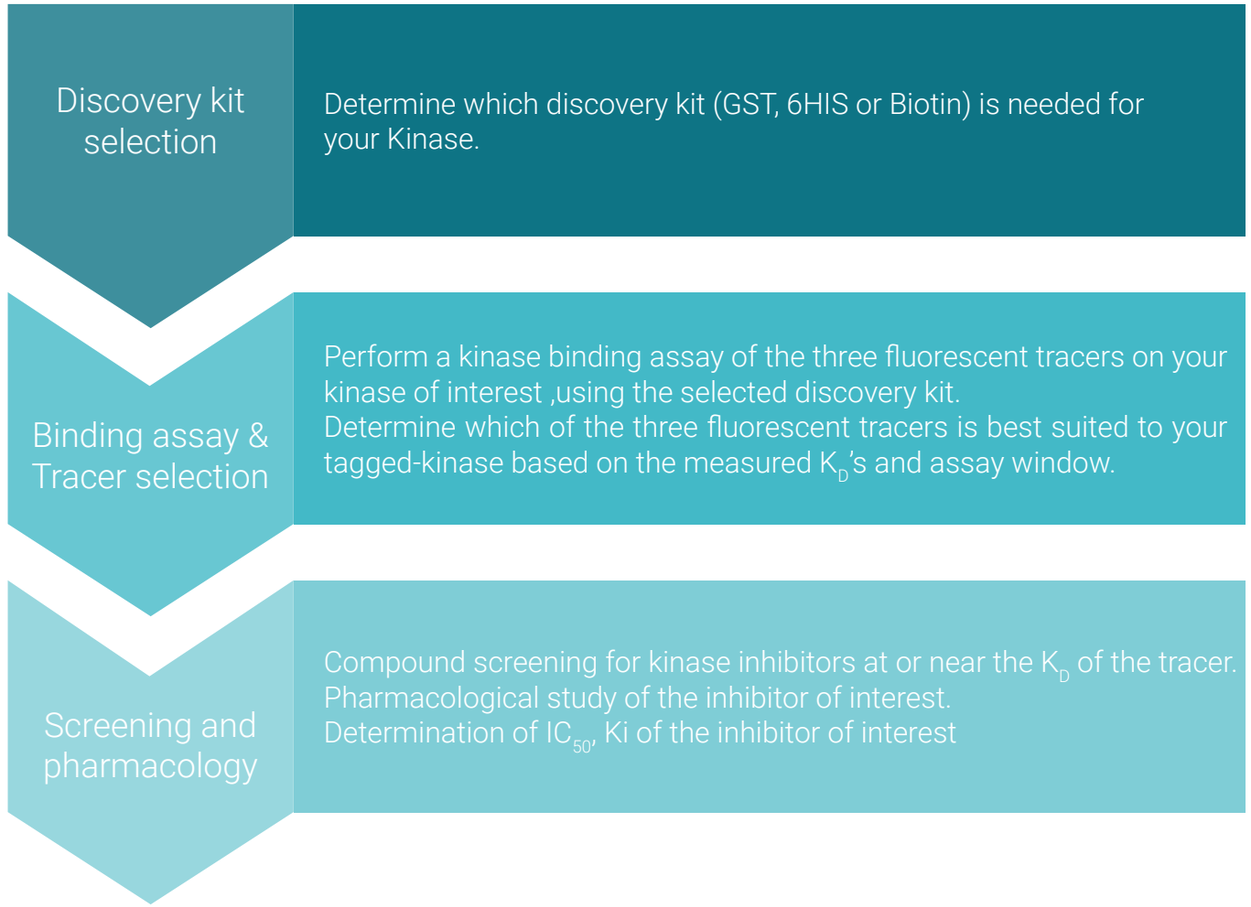

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TRACER SELECTION AND K_D DETERMINATION
Discovery kits presentation

DISCOVERY KITS, REAGENTS AND MATERIALS

Although the binding characteristics of Staurosporine, Dasatinib & Sunitinib are well documented in the literature, their affinities are not known for the whole Kinome. Moreover the inhibitor affinity may change depending on the enzyme purity, isoform activity, and proper folding, so in order to select the best tracer for your tagged kinase of interest we introduce three discovery kits.

Kinase-GST Binding Discovery kit (62KBD01PEA)	Kinase-6HIS Binding Discovery kit (62KBD02PEA)	Kinase-Biotin Binding Discovery kit (62KBD03PEA)
Staurosporine-Red 0.5 nmol (20 µL, 25 µM, green cap vial x1)	Staurosporine-Red 0.5 nmol (20 µL, 25 µM, green cap vial x1)	Staurosporine-Red 0.5 nmol (20 µL, 25 µM, green cap vial x1)
Dasatinib-Red 0.5 nmol (20 µL, 25 µM, green cap vial x1)	Dasatinib-Red 0.5 nmol (20 µL, 25 µM, green cap vial x1)	Dasatinib-Red 0.5 nmol (20 µL, 25 µM, green cap vial x1)
Sunitinib-Red 0.5 nmol (20 µL, 25 µM, green cap vial x1)	Sunitinib-Red 0.5 nmol (20 µL, 25 µM, green cap vial x1)	Sunitinib-Red 0.5 nmol (20 µL, 25 µM, green cap vial x1)
Kinase Binding Buffer 20 mL (20 mL x1, 62KBBRDD)	Kinase Binding Buffer 20 mL (20 mL x1, 62KBBRDD)	Kinase Binding Buffer 20 mL (20 mL x1, 62KBBRDD)
MAb Anti GST-Eu cryptate Kinase Binding – 1 000 tests (1 000 tests, orange cap vial (x1), 62KBGSTKAF)	MAb Anti 6HIS-Eu cryptate Kinase Binding – 1 000 tests (1 000 tests, orange cap vial (x1), 62KBHISKAF)	Streptavidin-Eu cryptate Kinase Binding – 1 000 tests (1 000 tests, orange cap vial (x1), 62KBSAKAF)

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TRACER SELECTION AND K_D DETERMINATION

Discovery kits presentation

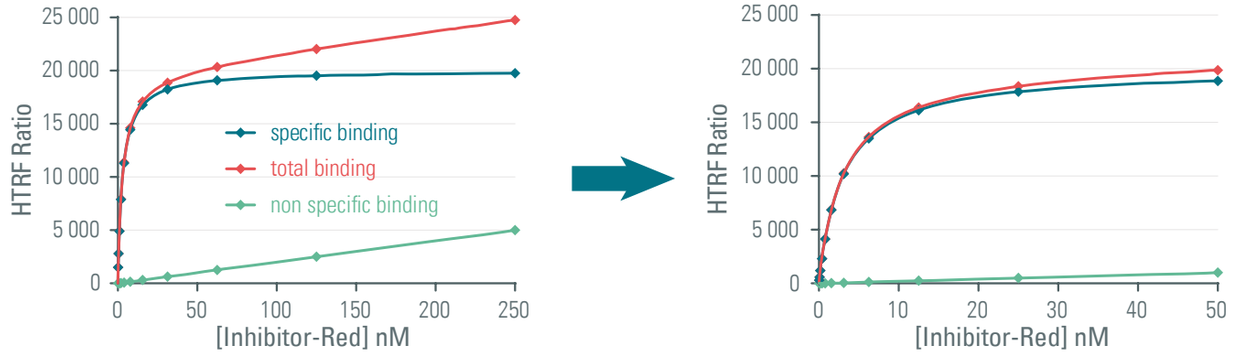
With the discovery kits you can perform two complete saturation binding assays using the three tracers, including non-specific binding signal in 20 µL 96- or 384- well plates. It comprises a two-fold dilution series for each tracer from 250 nM to zero with the Kinase Binding Buffer.

After determining the K_D's of the three tracers and their assay windows, either a second assay to confirm the results or a change in the concentration of the Kinase are both valid options.

Revvity's suggestion is to first perform the assay at 5 nM of Kinase. In all cases tested in house, good assay windows were obtained using this Kinase concentration. If the K_D's are all > 5 nM, the conditions are met for competitive inhibition studies at or near its K_D and further optimization is not necessary. If at 5 nM of Kinase the K_D's found are << 5 nM, optimal conditions are not met because of ligand depletion, and it recommended to perform the assay at lower Kinase concentrations. In these cases, on the right panel below, the full range (0-250 nM) can obviously be adapted, for instance by performing a saturation binding assay between 0 and 50 nM of tracer as is shown for a theoretical case with a K_D of 3 nM.

Reagents and Materials not included in the discovery kit:

- Purified GST-, 6HIS-, or Biotinylated Kinase
- DMSO
- 'Non-binding' black 96 well-plates for instance Greiner #655900
- Small-Volume (20 µl) 384 or 96-well plates for instance Revvity's HTRF 384 well low volume plate # 66PL384025/100) or HTRF 96 well low volume plate # 66PL96001/005/025/100)



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TRACER SELECTION AND K_D DETERMINATION
Implementation of the discovery kits

REAGENT PREPARATION

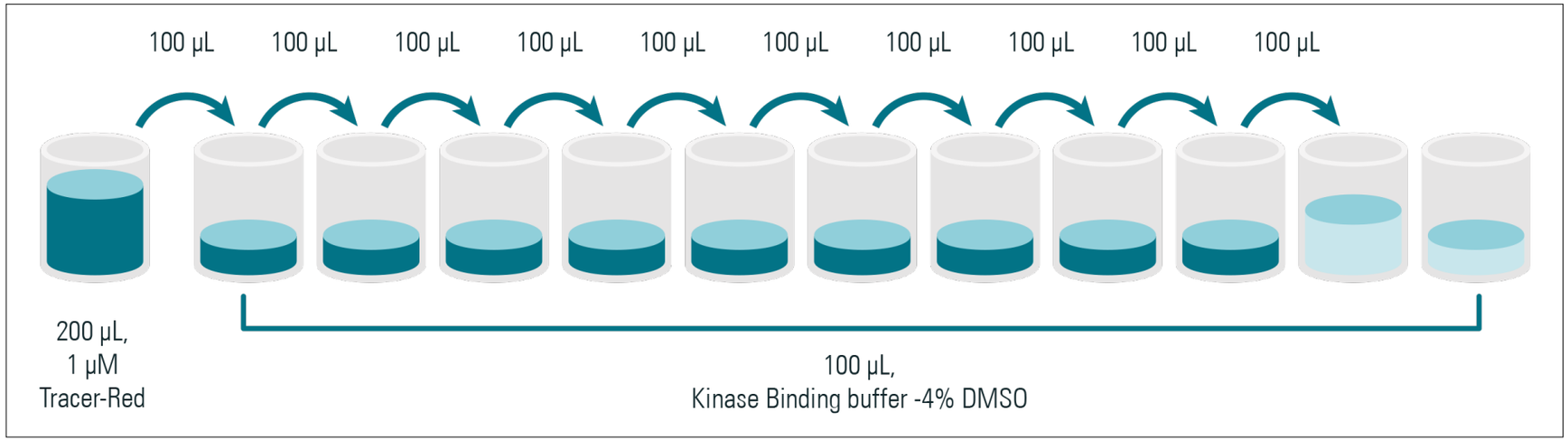
For the saturation binding assay, different stock solutions should be prepared.

First an anti-tag cryptate solution should be prepared at 4x final concentration in 'Kinase Binding Buffer'. For the exact preparation procedure, see the kit's package insert.

Secondly, a starting solution of 200 µL of 1 µM of Staurosporine-Red, Dasatinib-Red, and Sunitinib-Red in 'Kinase Binding Buffer' should be prepared from their stock solutions at 25 µM (in DMSO) delivered in the discovery kit. Note that the tracer solutions have a 4% DMSO content. In order to prepare a two-fold dilution series, it is recommended to prepare the series in a 96 well non-binding plate, starting from the 1 µM stock solution. This leads to an maximal concentration of 250 nM in the well, since a distribution format of 5/5/5/5 µL is used to give a final volume of 20 µL per well. To keep the assay at a constant concentration of 1% of DMSO, the dilution series is prepared in 'Kinase Binding Buffer' containing 4% DMSO.

We recommend preparing a dilution series to give final concentrations at 250, 125, 62.5, 31.25, 15.6, 7.8, 3.9, 2.0, 1.0, 0.5, 0.25 nM of tracer, and a negative control at 0 nM, by a serial dilution adding 100 µL of the higher concentration to 100 µL of the 'Kinase Binding Buffer' supplemented with 4% DMSO.

In order to get optimal assay conditions and reproducibility, we recommend preparing the tagged kinase (stored at -80 °C) solution as the final step. It should be diluted in the 'Kinase Binding Buffer' to an initial concentration of 20 nM (4x final concentration of 5 nM). If the kinase solution has to be prepared in advance before dispensing, we recommend storage on ice or at 4 °C.



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TRACER SELECTION AND K_D DETERMINATION
Implementation of the discovery kits

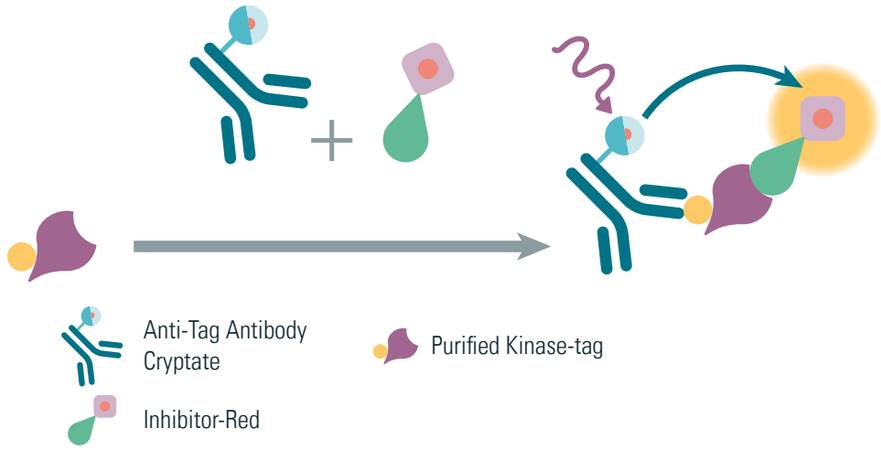
HOW TO PERFORM A KINASE BINDING ASSAY: DILUTION SERIES OF TRACERS

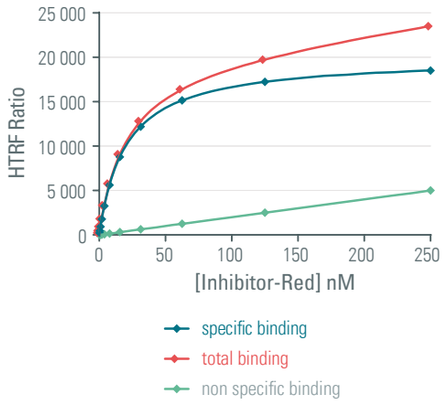
- In order to obtain the K_D of the tracer for the tagged-Kinase of interest, a saturation binding assay needs to be performed generating a total binding and a non-specific binding HTRF-ratio for the dilution series presented on the previous page. It is recommended to perform the assay in triplicate following the order presented in the table below. Non-specific binding is measured in the absence of the tagged Kinase and is due to diffusion enhanced energy transfer from the Eu-cryptate to the unbound tracer. The non-specific binding signal changes depending on the nature and concentration of the tracer and anti Tag-cryptate

K_D DETERMINATION

- By subtracting the non-specific signal from the total signal, the specific binding signal is obtained. Analyses of the specific signal by GraphPad Prism using a non-linear regression and the one-site specific binding option gives the K_D of the red-tracer to your kinase of interest.

STEP	TOTAL BINDING	NON-SPECIFIC BINDING
1	5 µL 'Kinase Binding Buffer'	10 µL 'Kinase Binding Buffer'
2	5 µL of Kinase in 'Kinase Binding buffer'	
3	5 µL of Anti-Tag Eu cryptate in 'Kinase Binding buffer'	5 µL of Anti-Tag Eu cryptate in 'Kinase Binding buffer'
4	5 µL of tracer-Red at x nM in 'Kinase Binding buffer-4% DMSO'	5 µL of tracer-Red at x nM in 'Kinase Binding buffer-4% DMSO'





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TRACER SELECTION AND K_D DETERMINATION
Implementation of the discovery kits

DMSO CONTENT

In the HTRF kinase binding assay it is best to keep the DMSO concentration constant. Adding up to 3% of DMSO did not significantly alter the binding curves, but a concentration greater than 3% may change the K_D value depending on the selected kinase. We recommend assessing the K_D value in presence of the same final DMSO percentage used for the inhibitor studies.

SIGNAL ACQUISITION

Optimal incubation time of the plate using a sealer is one hour at room temperature.

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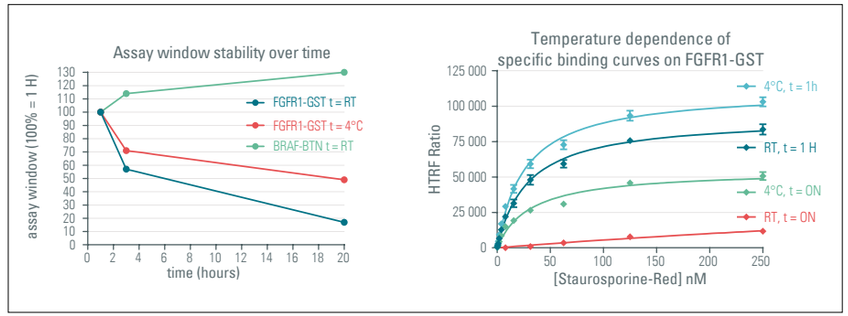
Implementation of the discovery kits

SIGNAL STABILITY

The HTRF reagents are stable in time, but we noticed a kinase dependent signal degradation if plates are kept at room temperature for extended periods, due to Kinase instability. Keeping plates at 4°C can slow down such degradation. In the graphs below the assay windows for FGFR1-GST (Staurosporine-Red) and BRAF-BTN (Dasatinib-Red) are shown depending on the incubation time after adding the reagents. No major changes in K_D values are seen, unless the specific binding curves are really low such as when FGFR1-GST was measured at RT after overnight incubation (worst case scenario).



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	FGFR1-GST : RT (STAUROSPORINE-RED)		FGFR1-GST : 4 °C (STAUROSPORINE-RED)		BRAF-BTN : RT (DASATINIB-RED)	
TIME	Max Assay window	K_D (±SD, nM)	Max Assay window	K_D (±SD, nM)	Max Assay window	K_D (±SD, nM)
1 H	9.2	27 ± 2	16	28 ± 2	9.8	46 ± 4
3 H	5.2	27 ± 2	11	28 ± 2	11.2	38 ± 4
ON	1.6	393 ± 138	8	33 ± 4	12.7	35 ± 4

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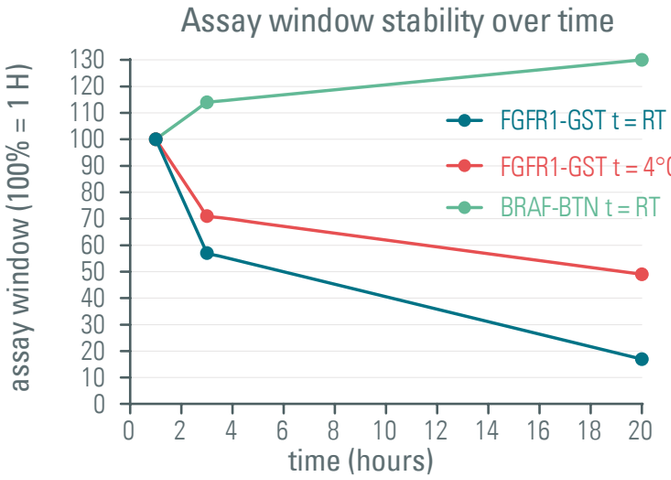

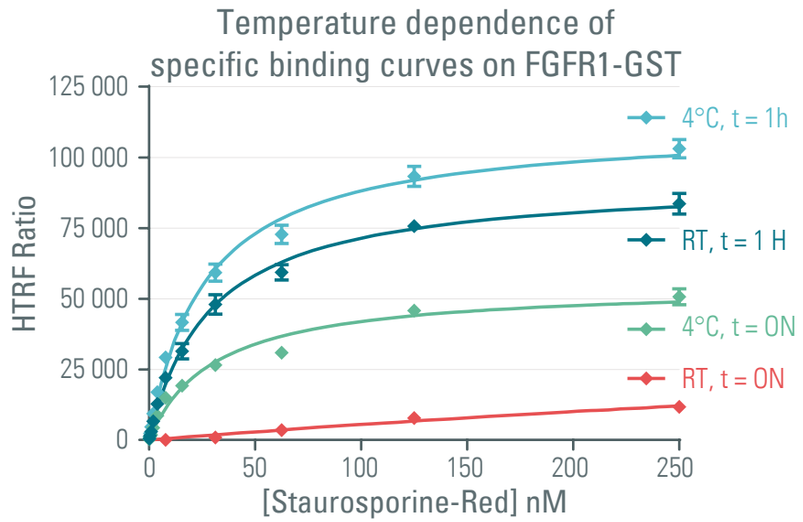

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TRACER SELECTION AND K_D DETERMINATION
Importance of kinase concentration

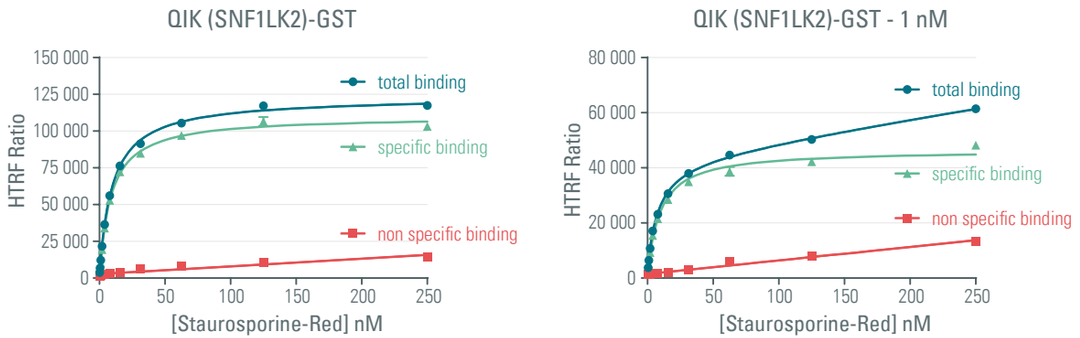
KINASE CONCENTRATION INFLUENCE ON K_D DETERMINATION

The specific binding HTRF ratio strongly depends on the concentration of the Kinase present. Usually, an increase in the enzyme concentration leads to an increase in the generated HTRF signal as well. However if the Kinase concentration is higher than the K_D value of the tracer, ligand depletion will occur that may dramatically affect the pharmacological characterization of inhibitors.

If the tracer K_D > [Kinase], most of the tracer is in solution and thus not bound to the Kinase, and hence equilibrium conditions are met. In such cases a change in concentration has no considerable consequences on the K_D measured, only on the assay window (total binding signal / non-specific signal). As an example, the binding of Staurosporine-Red to QIK-GST was measured at 1 & 5 nM of Kinase. Only a significant change in the assay window was shown, but the K_D stayed essentially the same. With a K_D of 9 nM, half of the Kinase is bound to the tracer and hence 6.5 nM (72%) is free in solution for 5 nM of Kinase. For 1 nM of Kinase, 8.5 nM (94%) of tracer is in solution. Both conditions are suitable to perform inhibitor binding studies at the K_D value.

It is worth noting that depending on the kinase studied, the HTRF Kinase binding assay platform is able to work with low kinase concentrations (down to 1 or 0.5 nM) while maintaining good assay windows and thus allowing valuable kinase savings.

QIK-GST	Tracer	KD(±SD, nM)	Assay window at KD
1 nM	Staurosporine -Red	9.0 ± 0.9	13
5 nM	Staurosporine -Red	8.6 ± 0.4	31



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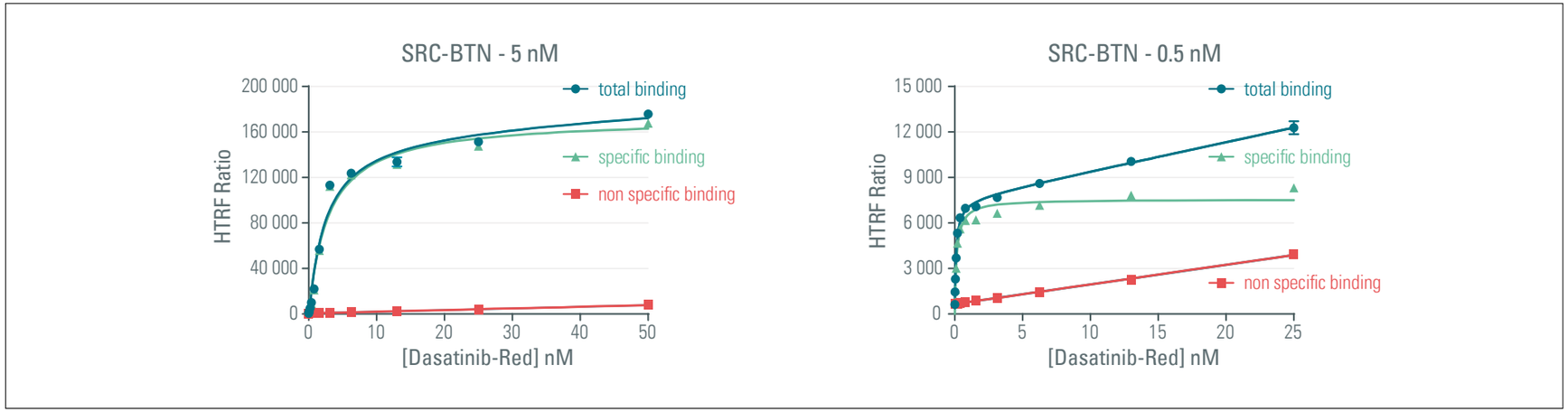
Importance of kinase concentration

Another example is shown below for the binding of Dasatinib-Red to SRC-BTN. Dasatinib is a very potent inhibitor to SRC, and as illustrated below Dasatinib-Red binds strongly to SRC-BTN with high affinity and assay window. When the concentration of SRC-BTN is reduced from 5 to 0.5 nM, we observe a different apparent K_D , indicative of very strong interaction of the fluorescent tracer with the Kinase. In fact the binding is too strong for equilibrium conditions to exist, and hence the use of Dasatinib-Red is not recommended; instead use Staurosporine-Red (see data on next slides), which has a higher K_D, for SRC-BTN.

SRC-BTN	Tracer	K_D(±SD, nM)
0.5 nM	Dasatinib - Red	0.15 ± 0.9 *
5 nM	Dasatinib - Red	2.9 ± 0.5 *

* Fitted with a one-site binding model



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TRACER SELECTION AND K_D DETERMINATION

How to select the best tracer?

PDGFRβ - GST CASE STUDY

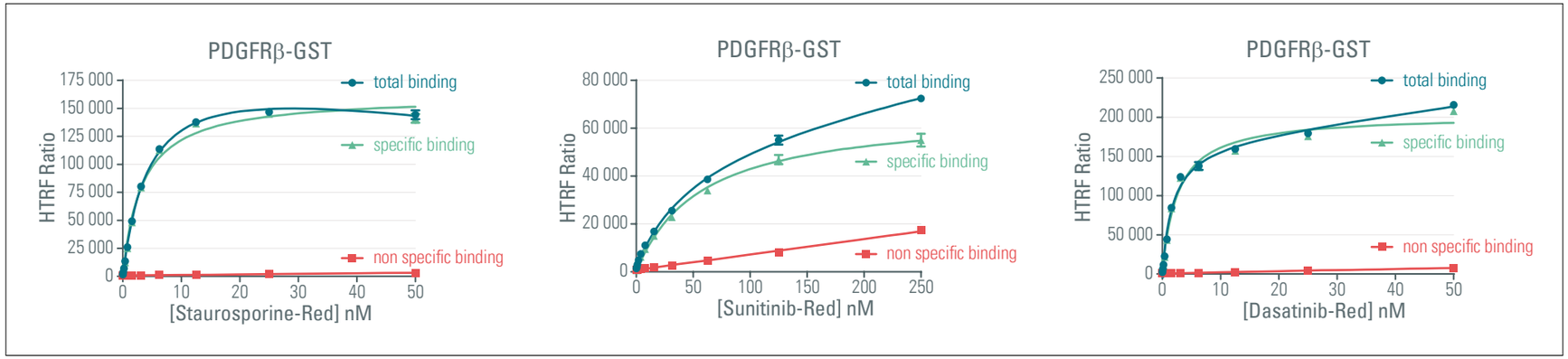
From the SRC-BTN data it becomes clear that the choice of tracer is not obvious beforehand. Low K_D tracers are not necessarily preferred to perform HTRF inhibition studies because high affinity inhibitors provoke non-optimal conditions because of ligand depletion. This is why analyzing your tagged Kinase with the HTRF® Kinase binding discovery kits is of prime importance. Below is an example testing the three tracers on 5 nM of PDGFRb-GST. PDGFRb is one of the few Kinases where staurosporine, dasatinib, and sunitinib are all potent inhibitors. The three tracers do indeed bind to PDGFRb-GST in this case, but with different affinities. Even though the affinities of Staurosporine-Red and Dasatinib-Red are superior to Sunitinib-Red, only Sunitinib-Red can be used at K_D for competitive inhibitor studies in these conditions, and it is therefore the tracer of choice.

Evaluation of Staurosporine-Red and Dasatinib-Red at lower PDGFRβ-GST concentration is another strategy to adapt.

If two or more tracers meet the optimal conditions conditions (no ligand depletion, hence K_D > kinase concentration), we recommend you select the tracer giving the best assay window (Total Signal / Non Specific Signal) at a concentration near K_D

Kinase (5 nM)	Tracer	K_D (±SD, nM)	Assay window AT K_D*
PDGFRß-GST	Staurosporine-Red	3.3 ± 0.4	83
PDGFRß-GST	Dasatinib-Red	2.6 ± 0.3	94
PDGFRß-GST	Sunitinib-Red	56.4 ± 4.5	8.3

* These were measured at Revvity's (Pherastar-FS), assay window will depend on the microplate reader used.



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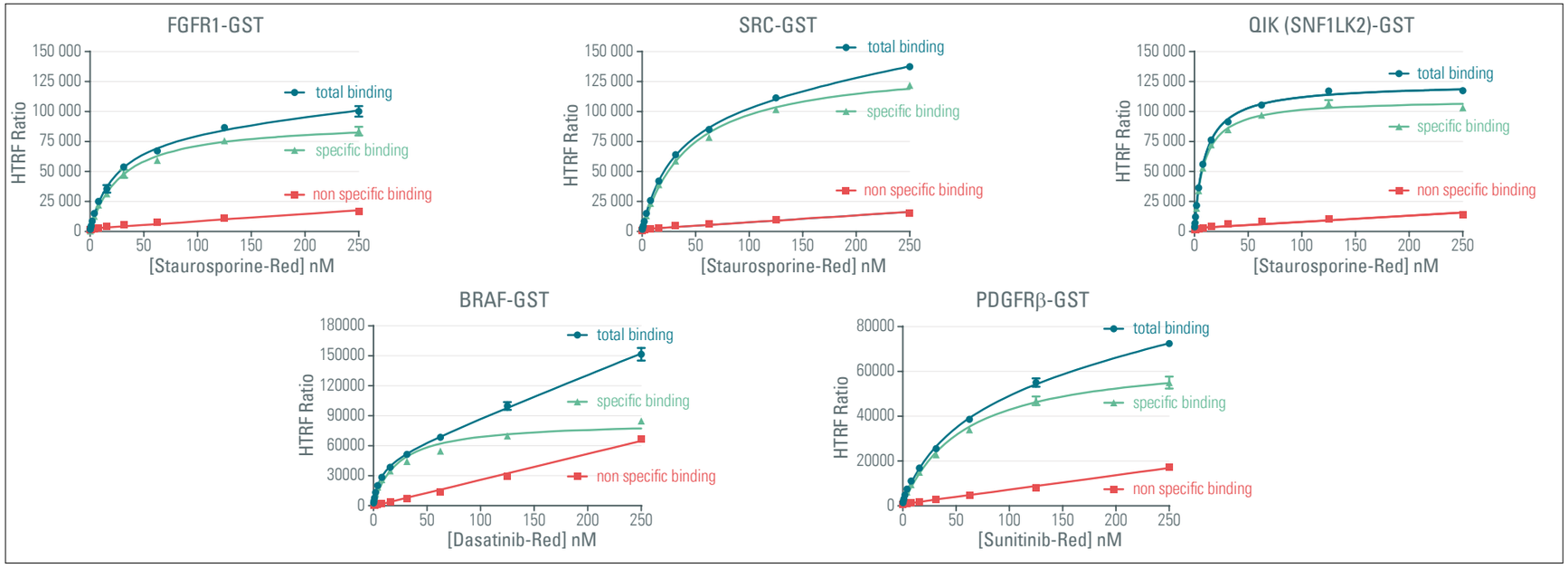
EXAMPLES ON GST TAGGED KINASES

As shown below, we have measured the binding affinity of Staurosporine-Red, Dasatinib-Red and Sunitinib-Red on several GST tagged Kinases. The data obtained were measured on a Pherastar FSX instrument after 1H of incubation using 5 nM of GST-Kinase and without Kinase for the non-specific binding signal.

One-site specific binding analyses using GraphPad Prism resulted in the K_D's reported in the table. In these

Examples, assay windows at the K_D value were all superior to 8.

Kinase	Tracer	K_D(±SD, NM)
FGFR1	Staurosporine-Red	28.9 ± 1.9
SRC	Staurosporine-Red	43.5 ± 2.7
QIK	Staurosporine-Red	8.6 ± 0.4
BRAF	Dasatinib-Red	22.2 ± 4.6
PDGFRb	Sunitinib-Red	56.4 ± 4.5



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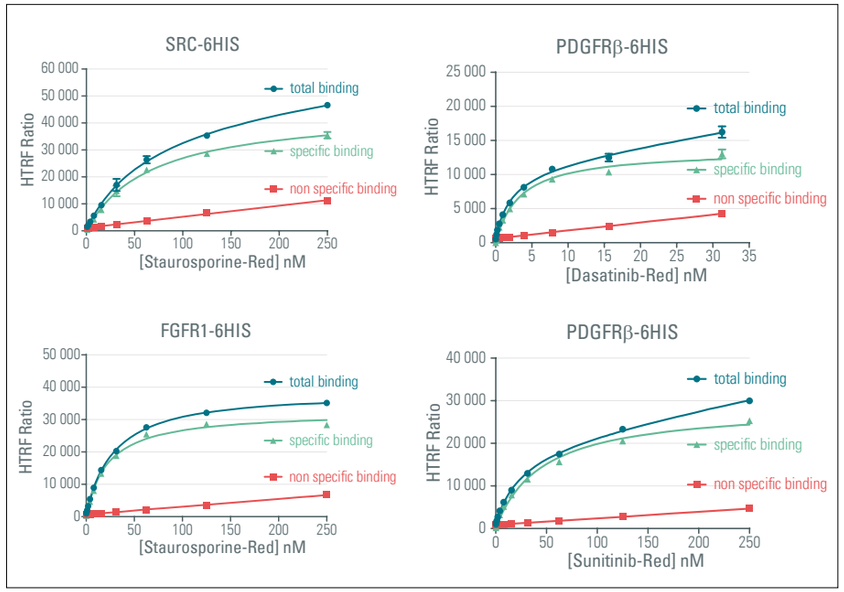
EXAMPLES ON 6HIS TAGGED KINASES

As shown below, we have measured the binding affinity of Staurosporine-Red, Dasatinib-Red, and Sunitinib-Red on several 6HIS tagged Kinases. The data obtained were measured on a Pherastar FSX instrument after 1H of incubation using 5 nM of 6HIS-Kinase (unless stated otherwise) and without Kinase for the non-specific binding signal.

Kinase	Tracer	K_D(±SD, nM)
SRC	Staurosporine-Red	66.3 ± 4.4
FGFR1	Staurosporine-Red	21.9 ± 1.8
PDGFRb (2 nM)	Dasatinib-Red	3.3 ± 0.4
PDGFRb	Sunitinib-Red	45.1 ± 4.8

One-site specific binding analyses using GraphPad Prism resulted in the K_D's reported in the table. In these

Examples, assay windows at the K_D value were all superior to 7.



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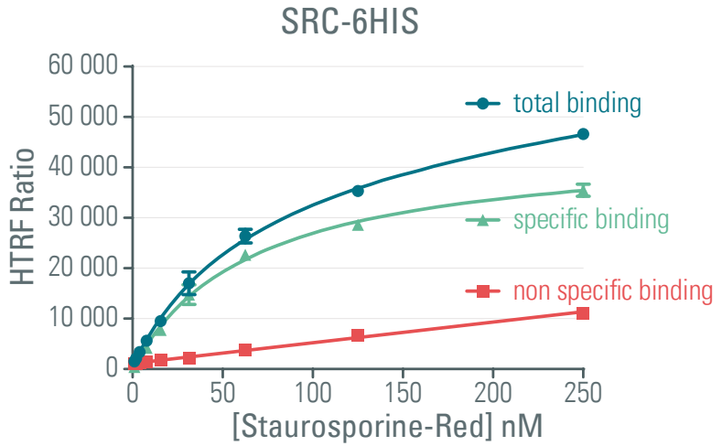

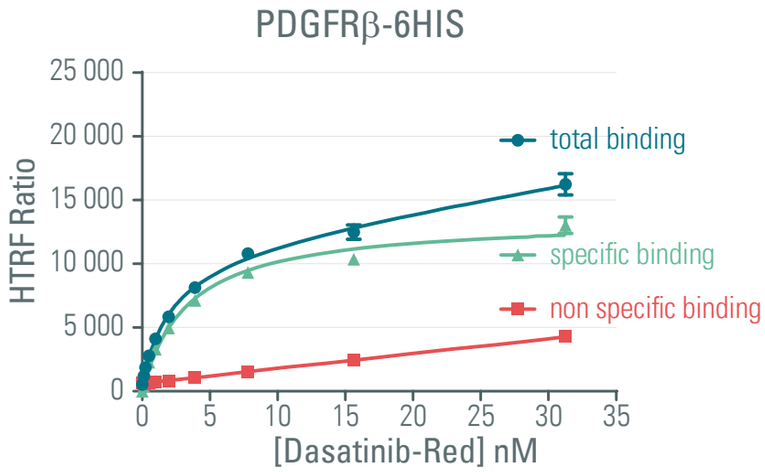

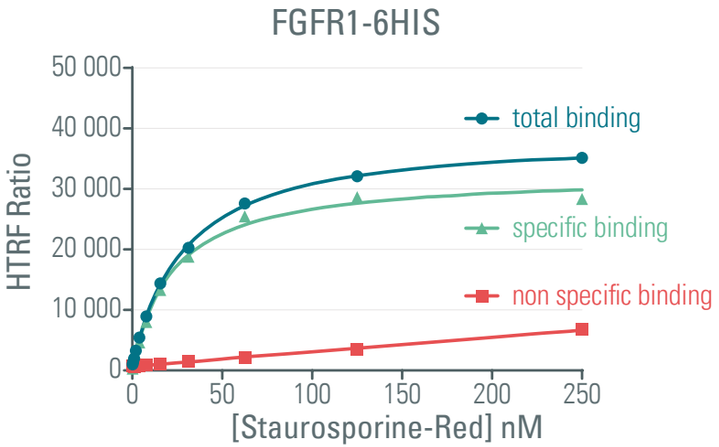

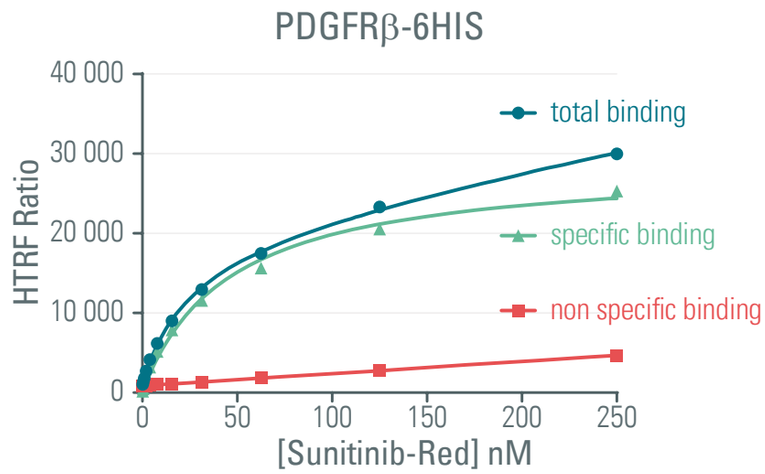

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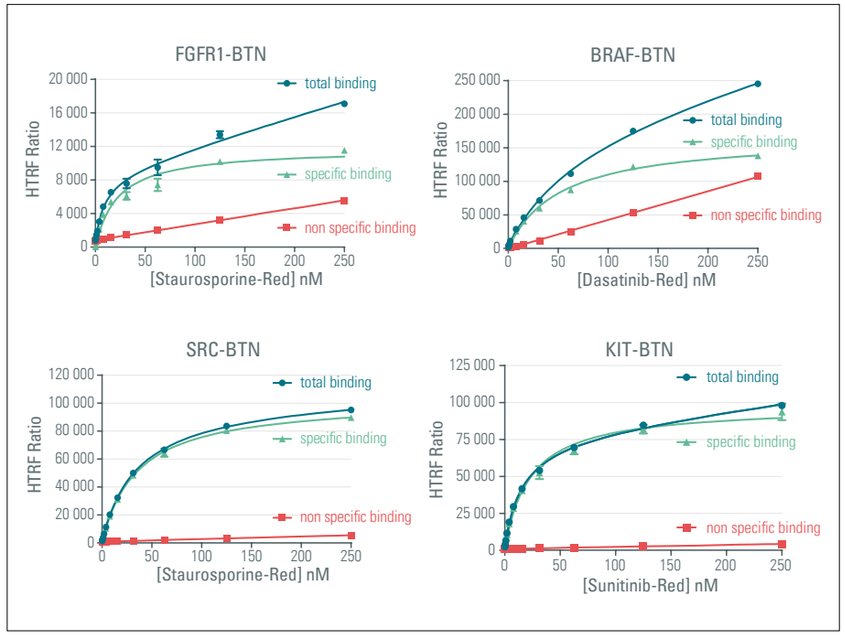
EXAMPLES ON BIOTINYLATED KINASES

As shown below, we have measured the binding affinity of Staurosporine-Red, Dasatinib-Red, and Sunitinib-Red on several N-terminal biotin tagged Kinases. The data obtained were measured on a Pherastar FSX instrument after 1H of incubation using 5 nM of Biotin-Kinase and without Kinase for the non-specific binding signal.

Kinase	Tracer	K_D(±SD, nM)
FGFR1	Staurosporine-Red	22.1 ± 4.1
SRC	Staurosporine-Red	35.2 ± 0.5
BRAF	Dasatinib-Red	52.3 ± 4.3
K_IT	Sunitinib-Red	22.5 ± 2.5

One-site specific binding analyses using GraphPad Prism resulted in the K_D's reported in the table

Examples, assay windows at the K_D value were all superior to 5.5.



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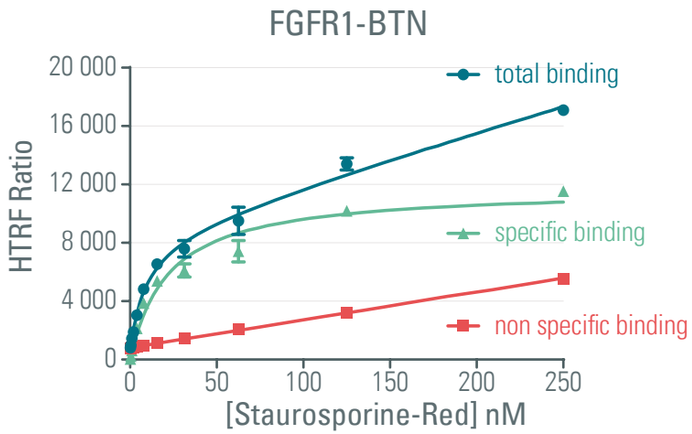

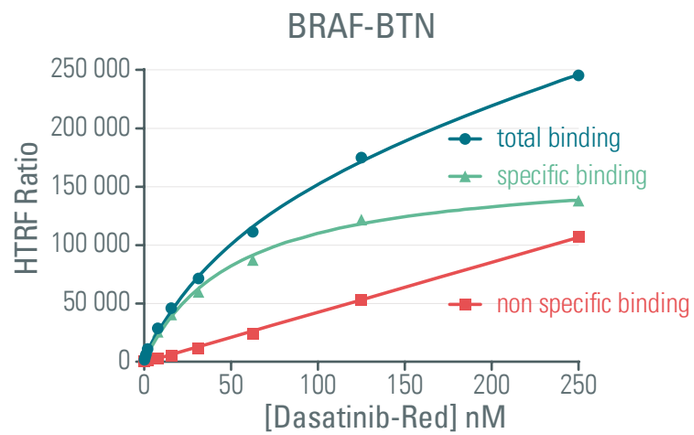

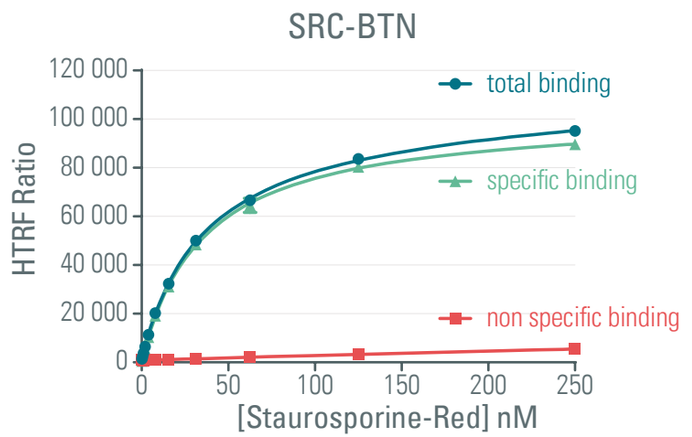

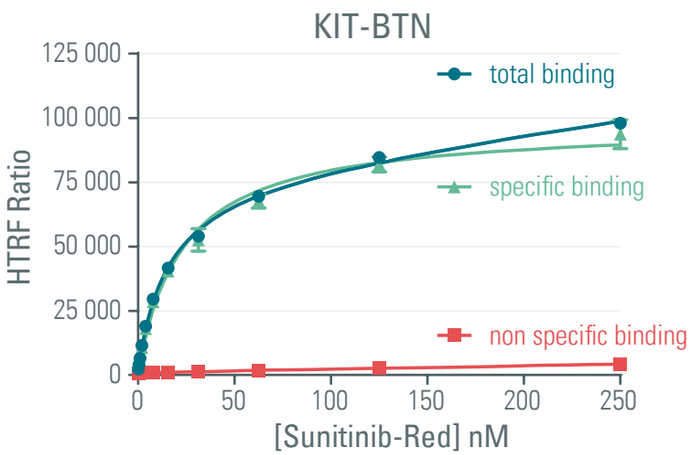

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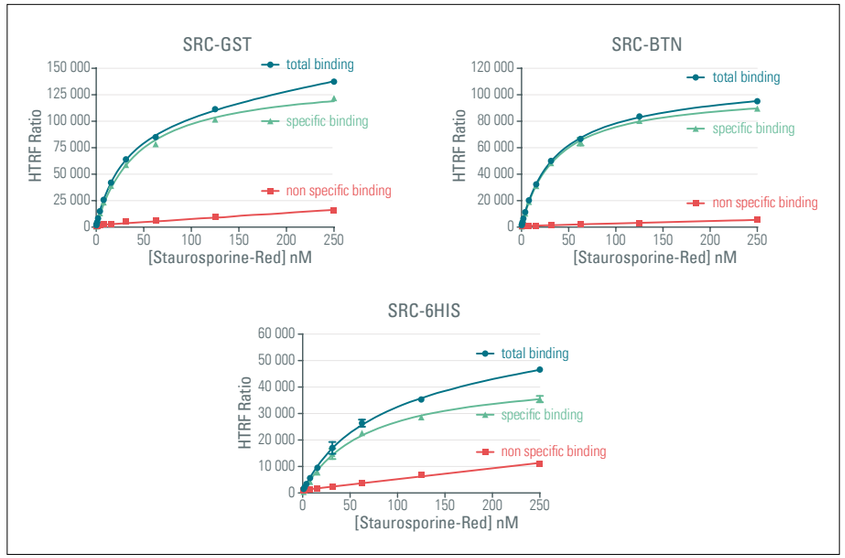
INFLUENCE OF THE TAG ON THE KINASE BINDING ASSAY

The assay also enables the evaluation of the influence of the tag on the affinity and the K_D of the Kinase. An interesting example is the case of SRC, a non-receptor tyrosine kinase which can be obtained as the complete Kinase either tagged at its N-ter with GST, Biotinylated at its N-ter, or tagged at its C-ter with a 6-HIS. As shown below, we have measured the binding affinity of Staurosporine-Red on three tagged SRC. The data obtained were measured on a Pherastar FSX instrument after 1H of incubation using 5 nM of Kinase and without Kinase for the non-specific binding signal.

Kinase	Position of tag	K_D(±SD, NM)
SRC-GST	N-ter	43.5 ± 2.7
SRC-BTN	N-ter	35.2 ± 0.5
SRC-6HIS	C-Ter	66.3 ± 4.4

One-site specific binding analyses using GraphPad Prism resulted in the K_D's reported in the table.

Using the Eu labeled GST MAb, 6-HIS MAb or Streptavidin, it is possible to perform such a comparative assay with excellent assay windows. In this example there is only a minor difference in calculated K_D's between the differently tagged SRC.



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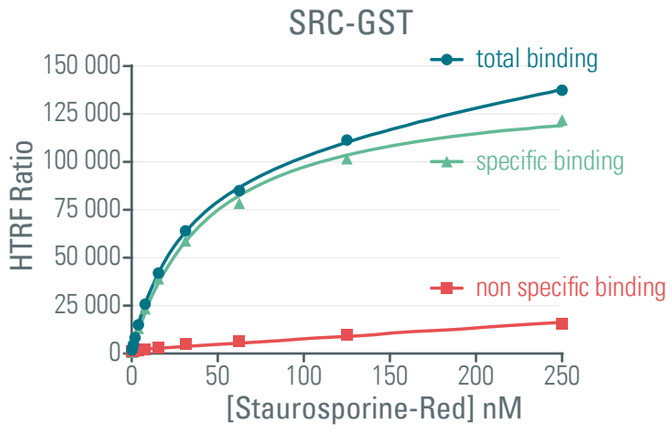

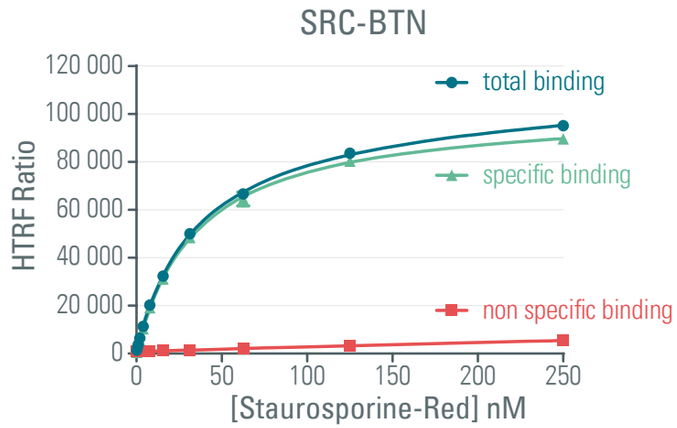

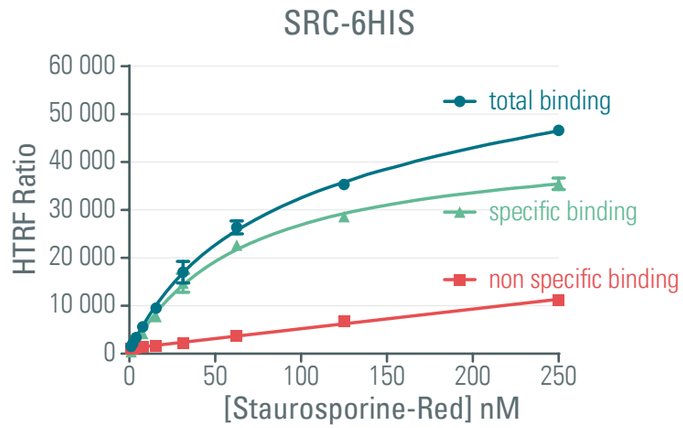

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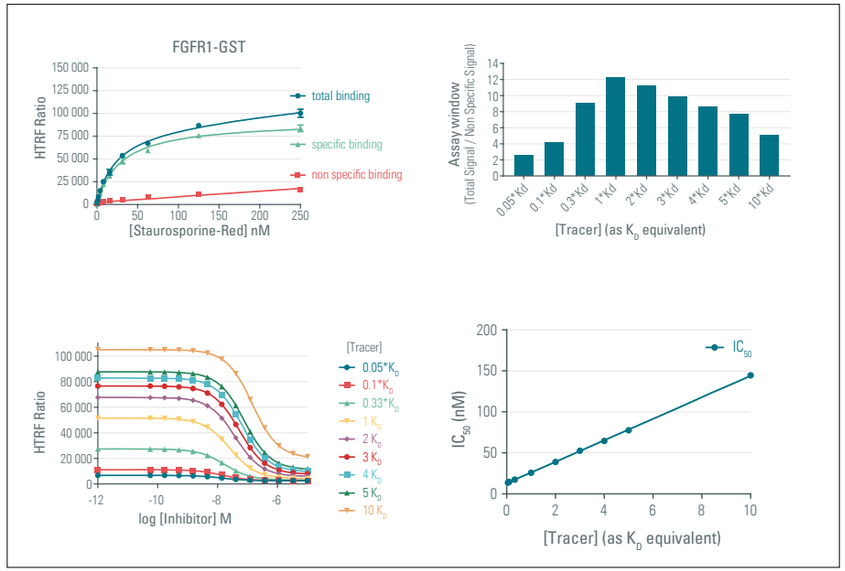
How to select the optimal tracer concentration for competition assays?

Once the optimal tracer and optimal kinase concentration have been selected, screening or pharmacological study of inhibitors can be performed. In order to do so, it is necessary to select the optimal tracer concentration. This optimal concentration is a compromise between assay window (or assay robustness) and assay sensitivity (or capability to detect inhibitor competition with low affinity). As shown below (top right panel), when titrating the tracer, the assay window (= Total Signal / Non Specific Signal) increases while tracer concentrations increase until it reaches a plateau, and then decreases (bell-shape curve)*. On the other hand, inhibitor dose-response curves performed with increasing concentrations of tracer (bottom panels) display increasing IC_{50}. We recommend selecting the optimal tracer concentration between 1 and 4 times the K_D value. If the assay window is high enough, use a concentration at K_D value. If the assay window has to be increased, increase the tracer concentration while avoiding saturating conditions (below 4 times the K_D value is ideal).



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* Note that these data are just presented as an example. The values and particularly the assay window and the Tracer concentration at maximum assay window will change depending on the kinase, tracer, anti Tag-cryptate, and microplate reader used.

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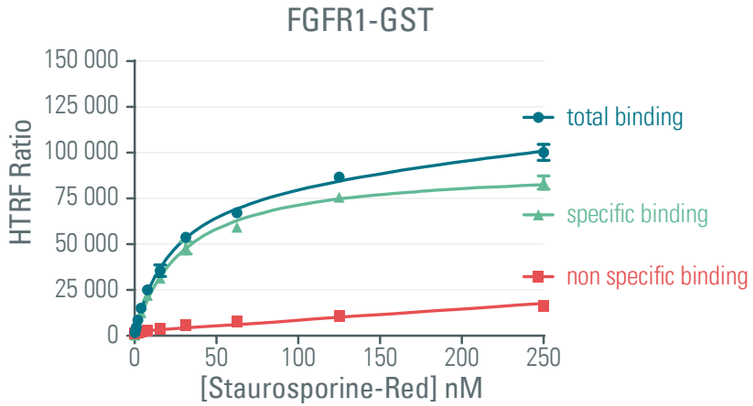

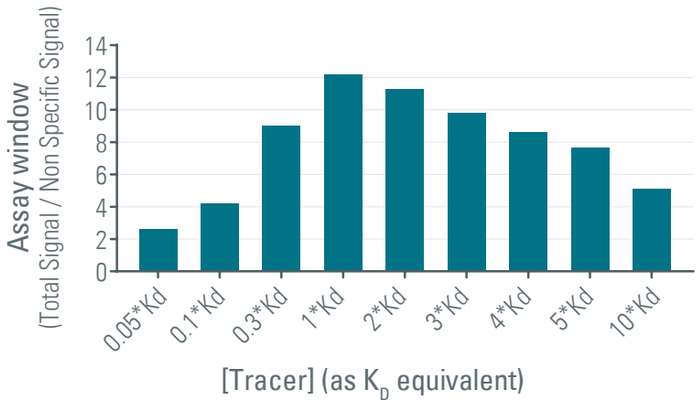

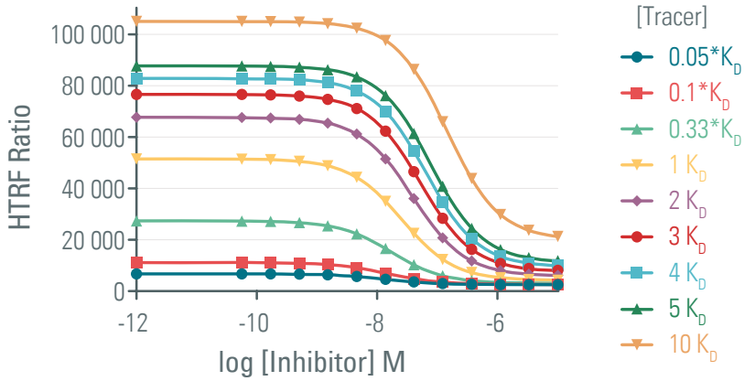

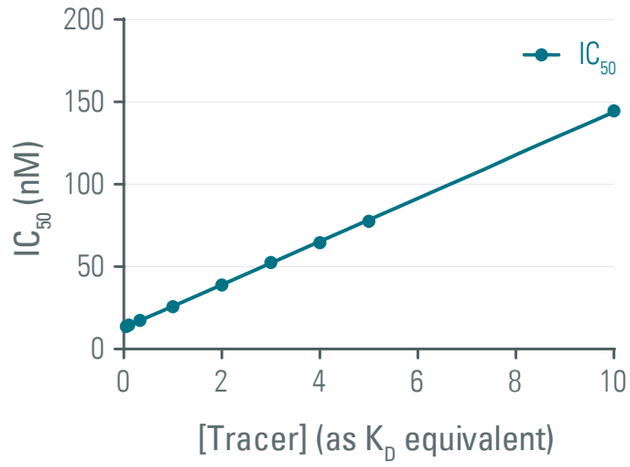

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PHARMACOLOGICAL STUDIES OF KINASE INHIBITORS

Pharmacological studies

SPARE REAGENTS

For pharmacological studies of Kinase inhibitors, the following spare reagents are available from Revvity.

SPARE REAGENTS	PRODUCT	CONTAINS	PRODUCT REFERENCE
Tracers	Staurosporine-Red 1 nmol	1x Staurosporine-Red 1 nmol (40 µL, 25 µM, blue cap vial x1)	62KB01REDC
	Staurosporine-Red 20 nmol	1x Dasatinib-Red 20 nmol (40 µL, 25 µM, purple cap vial x1)	62KB01REDE
	Dasatinib-Red 1 nmol	1x Dasatinib-Red 1 nmol (40 µL, 25 µM, blue cap vial x1)	62KB02REDC
	Dasatinib-Red 20 nmol	1x Dasatinib-Red 20 nmol (800 µL, 25 µM, purple cap vial x1)	62KB02REDE
	Sunitinib-Red 1 nmol	1x Sunitinib-Red 1 nmol (800 µL, 25 µM, blue cap vial x1)	62KB03REDC
	Sunitinib-Red 20 nmol	1x Sunitinib-Red 20 nmol (800 µL, 25 µM, purple cap vial x1)	62KB03REDE
Buffers	Kinase Binding Buffer-20 mL	1x Kinase Binding Buffer 20 mL	62KBBRDD
	Kinase Binding Buffer-200 mL	1x Kinase Binding Buffer 200 mL	62KBBRDF
Anti-Tag	MAb Anti GST-Eu cryptate Kinase Binding – 1 000 tests	1 000 tests MAb Anti-GST-Eu cryptate (orange cap vial x1)	62KBGSTKAF
	MAb Anti GST-Eu cryptate Kinase Binding – 20 000 tests	20 000 tests MAb Anti-GST-Eu cryptate (red cap vial x1)	62KBGSTKAB
	MAb Anti 6HIS-Eu cryptate Kinase Binding – 1 000 tests	1 000 tests MAb Anti 6HIS-Eu cryptate (orange cap vial x1)	62KBHISKAF
	MAb Anti 6HIS-Eu cryptate Kinase Binding – 20 000 tests	20 000 tests MAb Anti 6HIS-Eu cryptate (red cap vial x1)	62KBHISKAB
	Streptavidin-Eu cryptate Kinase Binding – 1 000 tests	1 000 tests Streptavidin-Eu cryptate (orange cap vial x1)	62KBSAKAF
	Streptavidin-Eu cryptate Kinase Binding – 20 000 tests	20 000 tests Streptavidin-Eu cryptate (red cap vial x1)	62KBSAKAB

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PERFORMING A COMPETITIVE DOSE RESPONSE BINDING ASSAY:

To determine the affinity of the inhibitor of interest, competitive binding experiments can be done using a fixed concentration of fluorescent tracer. To ensure optimal equilibrium conditions, it is advisable to work between 1-4 times the K_D concentration depending on the assay window and/or the concentration of kinase used. The assay set up needs similar steps as for the saturation binding experiments. 1 µM solutions of inhibitors in DMSO should be prepared before use.

First, an anti-tag cryptate solution should be prepared at 4x final concentration in 'Kinase Binding Buffer'. For the exact preparation procedure see the kit's package insert.

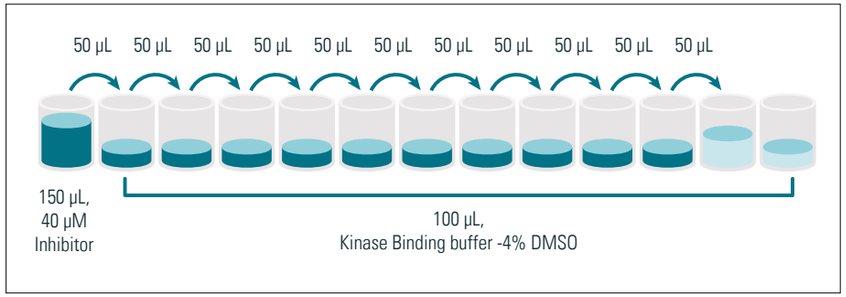
Secondly, a solution at 4x the desired concentration of either Staurosporine-Red, Dasatinib-Red, or Sunitinib-Red in 'Kinase Binding Buffer' should be prepared from their stock solutions of 25 µM (in DMSO).

STEP	INHIBITOR DOSE RESPONSE
1	5 µL dilution series of inhibitor in 'Kinase Binding buffer-4% DMSO'
2	5 µL of Kinase in 'Kinase Binding buffer'
3	5 µL of Anti-Tag Eu cryptate in 'Kinase Binding buffer'
4	5 µL of tracer-Red at x nM in 'Kinase Binding buffer'

Thirdly, for a three-fold dilution series it is recommended to prepare the series in a 96 well non-binding plate, starting from 1 µM inhibitor solution in DMSO to be diluted to an initial concentration of 40 µM in 'Kinase binding buffer'. A dispensing format of 5/5/5/5 µL into the final plate gives a final volume of 20 µL per well. To keep the assay at a constant DMSO concentration, the inhibitor dilution series is prepared in 'Kinase Binding Buffer' containing 4% DMSO.

We recommend the preparation of a 12 point dilution series in triplicate to give final concentrations between 10 µM and 56 pM of inhibitor, and a negative control at 0 nM of inhibitor, using the prepared 'Kinase binding buffer, 4% DMSO.

In order to get optimal assay conditions and reproducibility, we recommend preparing the tagged kinase (stored at -80°C) solution as the final step. It should be diluted into the 'Kinase Binding Buffer' to an initial concentration of 20 nM (4x final concentration of 5 nM). If the kinase solution has to be prepared in advance before dispensing, we recommend storage on ice or at 4 °C.



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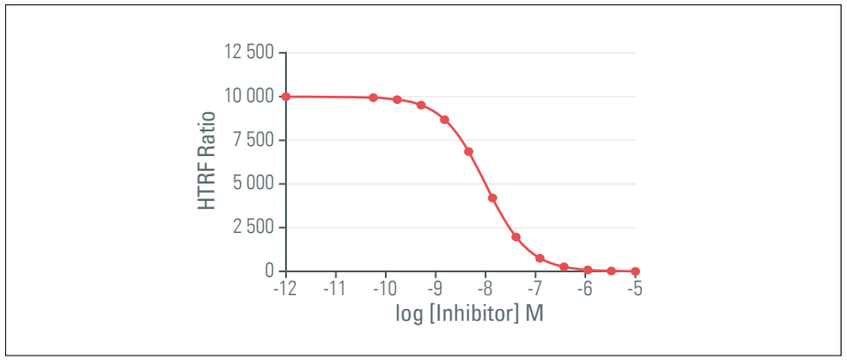
DETERMINATION OF IC_{50} AND K_I VALUES

Tracing the HTRF ratio and plotting it against the log of the inhibitor concentration gives data which can be fitted by using the non-linear regression function "log(inhibitor) vs response-variable slope (four parameters)" in GraphPad Prism. This gives sigmoidal dose-response curves such that as presented below.

IC_{50} values for the inhibitors can now be determined, and since the K_D of the tracer is known, inhibition constants (K_I) can be calculated using the Cheng-Prusoff equation [1] :

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



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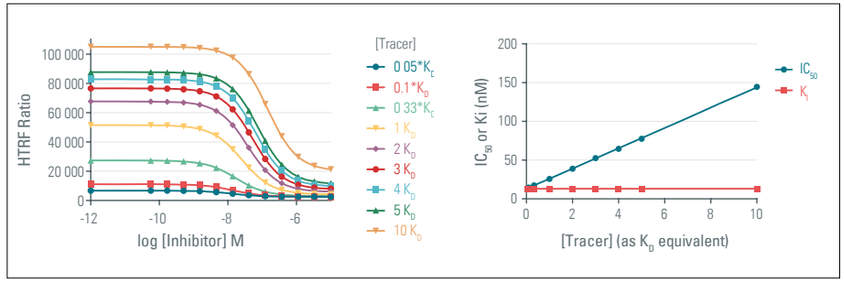
[1] Y.C Cheng, W.H. Prusoff., Biochem. Pharmacol. 22 (1973) 3099-3108.

Of note, as in classical inhibition assays, inhibitors IC_{50} depend on the tracer concentration used (and its K_D). It is worth noting that the inhibition constant (K_I) calculated using the Cheng-Prusoff equation [1], remains constant whatever the tracer concentration used (see graph above). Then we recommend the calculation of K_I values which do not rely on assay conditions in order to enable comparison with other assays, for example.



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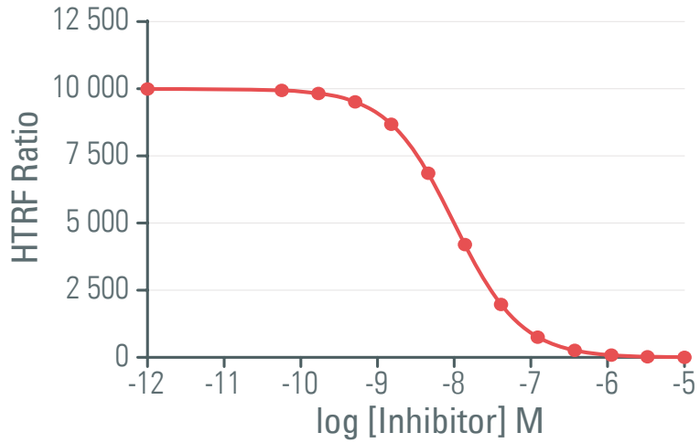

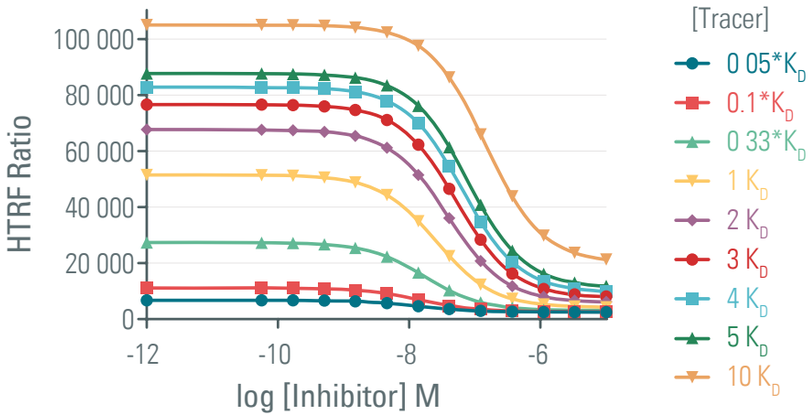

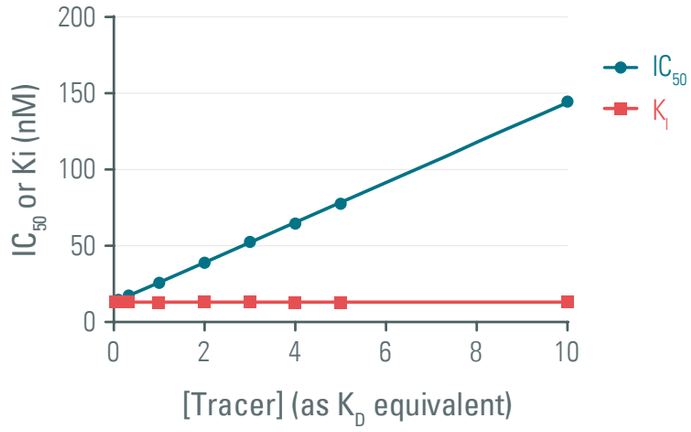

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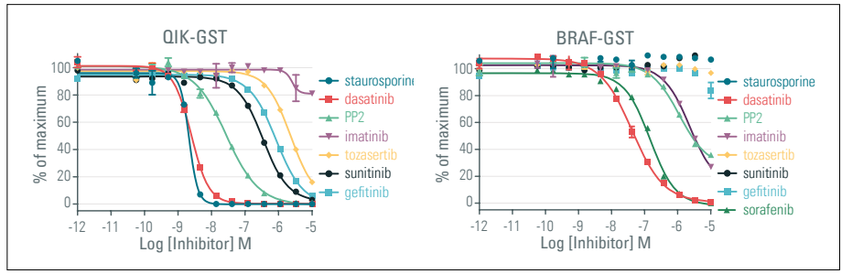
DATA EXAMPLES ON GST TAGGED ENZYMES

With the differently tagged Kinases and the three different tracers, we gathered inhibition data using the HTRF Kinase binding format and compared the outcomes with literature data. The data obtained were all measured on a Pherastar FSX instrument after 1H of incubation using 5 nM of GST-Kinase at the K_D of the tracer as indicated. For a more extensive data set, we refer to the accompanying application notes.



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INHIBITOR	QIK (SNF1LK2)-GST (STAUROSPORINE-RED)			BRAF-GST (DASATINIB-RED)		
	IC_{50} (nM)	K_i (nM)	literature	IC_{50} (nM)	K_i (nM)	literature
Staurosporine	1.7	1	1.1 [1]	>10 000	-	>10 000 [1]
Dasatinib	2.1	1.3	6.4 [2]	41	21	82 [2]
PP2	44	26	-	1 097 [3]	549	-
Imatinib (Gleevec)	>10 000	-	>10 000 [1]	2 304 [3]	1 152	4 560 [2]
Tozasertib (VX680)	2 564	1 531	1 700 [1]	>10 000	-	>10 000 [1]
Sunitinib (Sutent)	466	278	580 [1]	>10 000	-	>10 000 [1]
Gefitinib (Iressa)	924	552	2 100 [1]	>10 000	-	>10 000 [1]
Sorafenib (Nexvar)	[4]	-	>10 000 [1]	145	73	540 [1] 22 [5]

[1] M.Z. Karaman et al. Nat. Biotechnol. 26 (2008) 127-132. (K_D) [2] V. Georgi et al. J. Am. Chem Soc. 140 (2018) 15774-15782 (K_D-Eq.) [3] Incomplete inhibition. [4] Not tested.
[5] S.M. Wilhelm et al. Cancer Res. 64 (2004) 7099-7109. (IC_{50})

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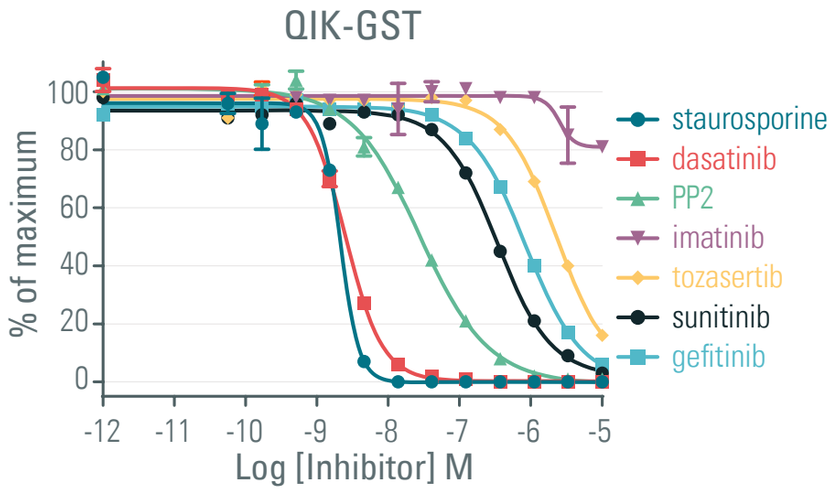

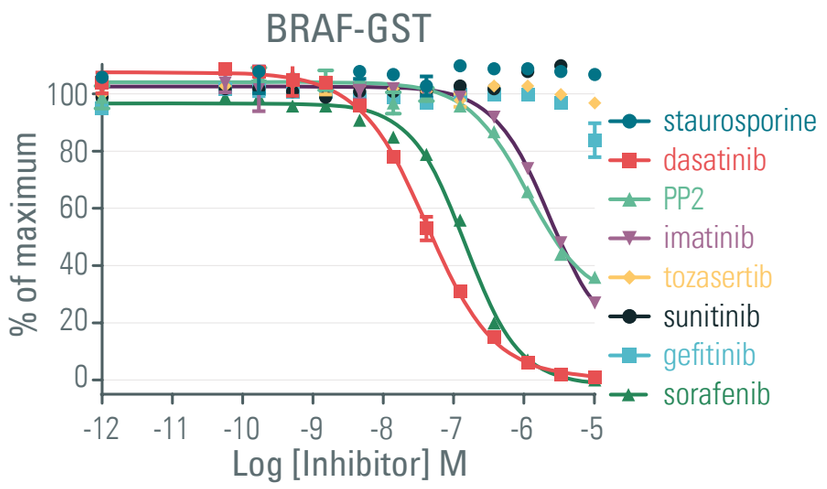

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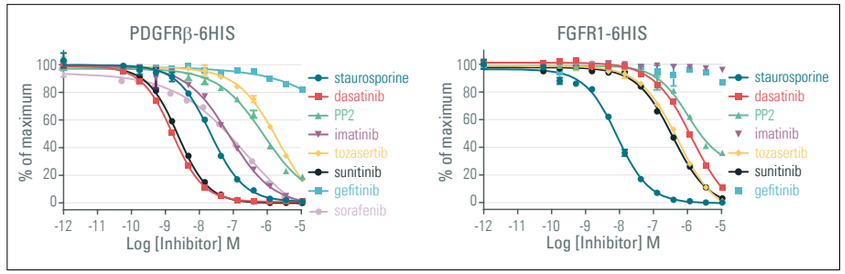
DATA EXAMPLES ON 6HIS TAGGED ENZYMES

With the differently tagged Kinases and the three different tracers, we gathered inhibition data using the HTRF Kinase binding format and compared the outcomes with literature data. The data obtained were all measured on a Pherastar FSX instrument after 1H of incubation using 5 nM of 6HIS-Kinase at the K_D of the tracer as indicated. For a more extensive data set, we refer to the accompanying application notes.



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INHIBITOR	PDGFRB-6HIS (SUNITINIB-RED)			FGFR1-6HIS (STAUROSPORINE-RED)		
	IC_{50} (nM)	K_I (nM)	literature	IC_{50} (nM)	K_I (nM)	literature
Staurosporine	20.3	10.2	1.8 [1]	8.3	4.2	9.1 [2]
Dasatinib	1.6	0.8	0.63 [1]	1 291	645	870 [2]
PP2	732	366	-	987	494	-
Imatinib (Gleevec)	73	36.5	14 [1]	>10 000		>20 000 [2]
Tozasertib (VX680)	1 964	982	310 [1]	509	255	201 [2]
Sunitinib (Sutent)	2.4	1.2	0.08[1] 5.7[4]	356	178	147 [2]
Gefitinib (Iressa)	>10 000	-	>10 000 [1]	>10 000		>20 000 [2]
Sorafenib (Nexvar)	198	99	37 [1] 57 [3]			

[1] M.Z. Karaman et al. Nat. Biotechnol. 26 (2008) 127-132. (K_D) [2] V. Georgi et al. J. Am. Chem Soc. 140 (2018) 15774-15782 (K_D-Eq.) [3] S.M. Wilhelm et al. Cancer Res. 64 (2004) 7099-7109. (IC_{50}), [4] D. Kitagawa et al. Genes Cells 18 (2013) 110-122. (IC_{50})

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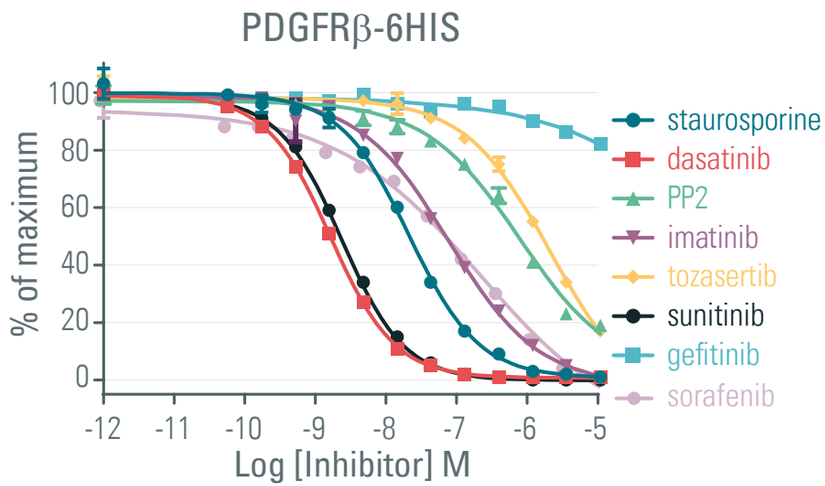

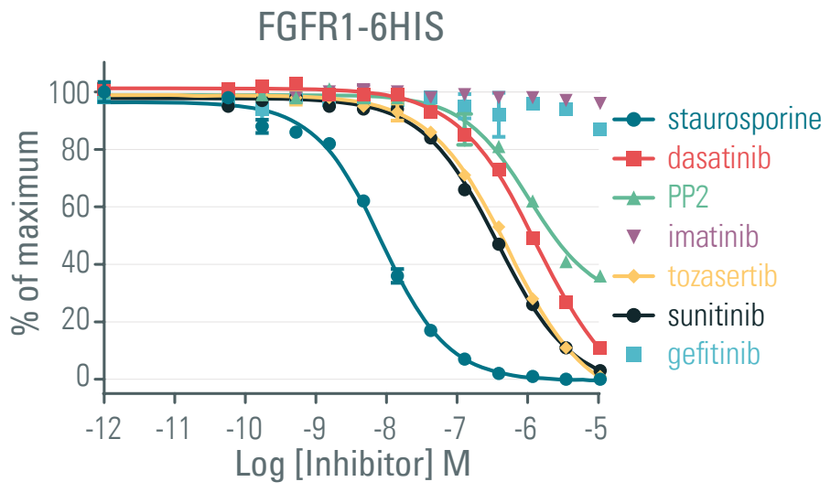

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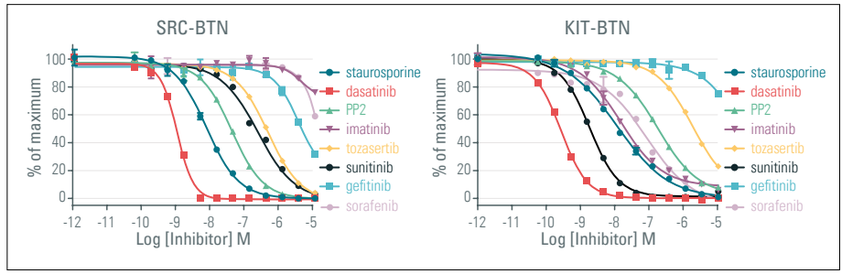
DATA EXAMPLES ON BIOTINYLATED ENZYMES

With the differently tagged Kinases and the three different tracers, we gathered inhibition data using the HTRF Kinase binding format and compared the outcomes with literature data. The data obtained were all measured on a Pherastar FSX instrument after 1H of incubation using 5 nM of GST-Kinase at the K_D of the tracer as indicated. For a more extensive data set, we refer to the accompanying application notes.



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INHIBITOR	SRC-BTN (STAUROSPORINE-RED)			K_IT-BTN (SUNITINIB-RED)		
	IC_{50}(nM)	K_I (nM)	literature	IC_{50} (nM)	K_I (nM)	literature
Staurosporine	6.9	3.5	2.6 , 1.3 [1]	11.4	5.7	19 [2] 22 [1]
Dasatinib	1	0.5	0.21 [2] 0.06 [1]	0.26	0.13	0.62 [2] 0.38 [1]
PP2	38	19	36 [3]	174	87	-
Imatinib (Gleevec)	>10 000	-	>10 000 [2]	14.6	7.3	14 [2] 26 [1]
Tozasertib (VX680)	433	216	170 [2] 118 [1]	1 823	911	240[2] 1 590 [1]
Sunitinib (Sutent)	235	118	104 [1]	1.8	0.9	0.37 [2] 0.8 [1]
Gefitinib (Iressa)	3 312 (incomplete)	1656	3 800 [2] 344 [1]	>10 000	-	10 900 [1]
Sorafenib (Nexvar)	>10 000	-	>10 000 [2]	82	41	31[2] 68 [4]

[1] V. Georgi et al. J. Am. Chem Soc. 140 (2018) 15774-15782 (K_D-Eq.) [2] M.Z. Karaman et al. Nat. Biotechnol. 26 (2008) 127-132. (K_D) [3] J. Bain et al. Biochem. J. 408 (2007) 297-315 (IC_{50}).
[4] S.M. Wilhelm et al. Cancer Res. 64 (2004) 7099-7109. (IC_{50})

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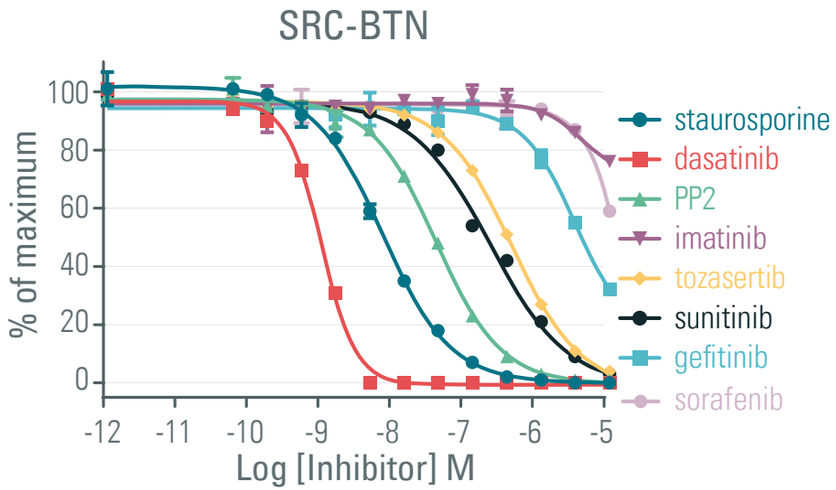

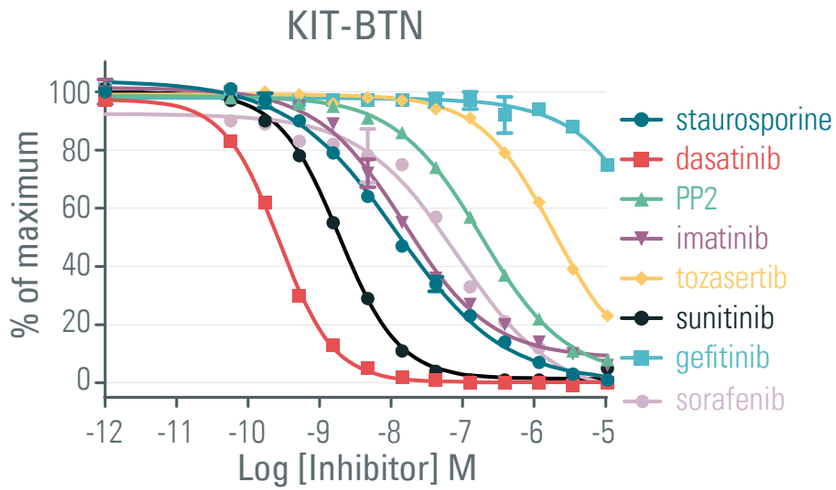

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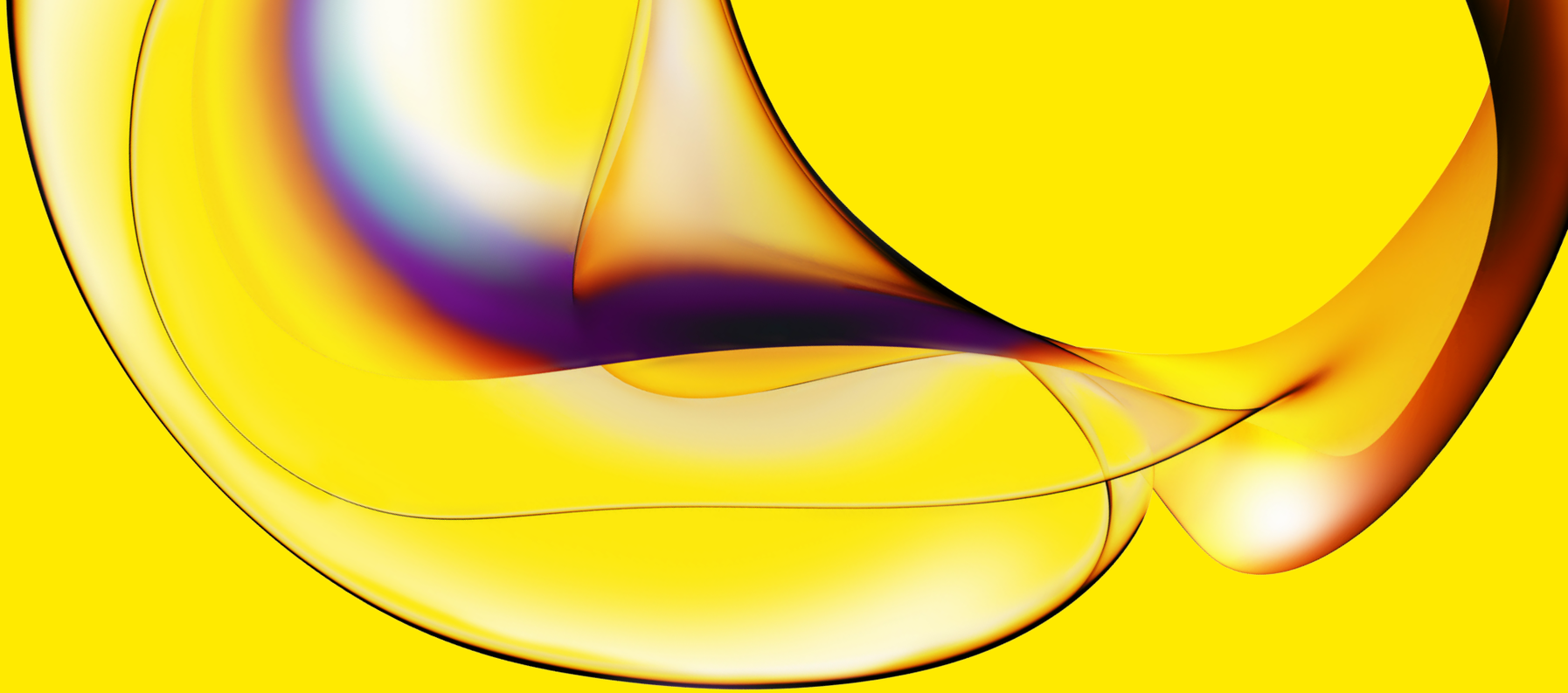

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