APPLICATION NOTE

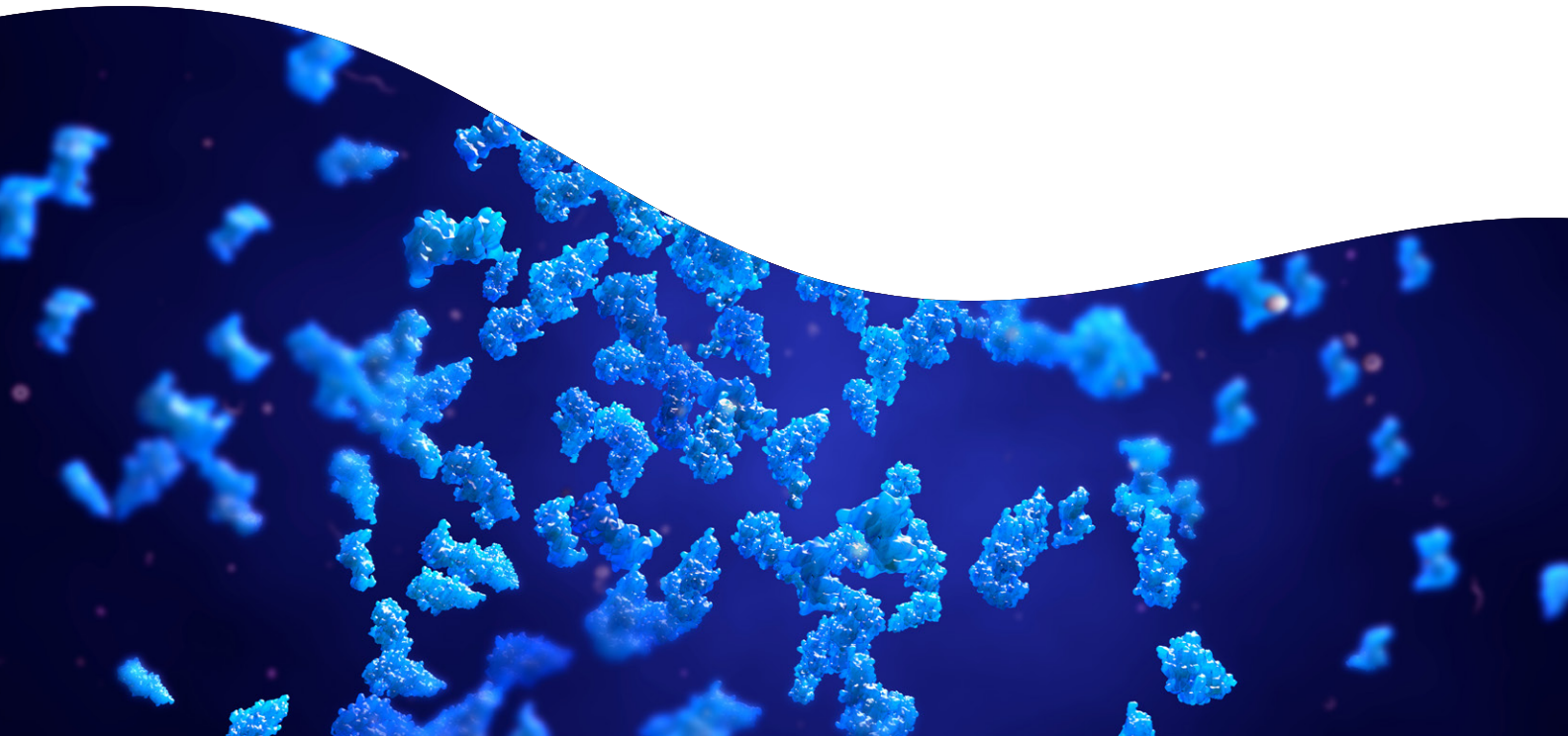
Kinetic binding of kinase inhibitors and determination of K_{On}, K_{Off} rates.

This application note presents the use of fluorescent kinase inhibitors to determine the kinetic binding properties of inhibitors of interest and demonstrates the pharmacological relevance of the kinase binding platform for both equilibrium-and kinetic profiling.

Abstract

The HTRF® kinase binding platform for GST-, 6HIS, and N-biotinylated kinases is based on measurements at equilibrium but can be used to study the binding kinetics of inhibitors. Lately, it has become clear that steady state affinities do not necessarily predict the performance of the compound in relevant biological models. Dissociation kinetics and hence the residence time of inhibitors have become key parameters to predict *in-vivo* drug-efficacy outcome [1,2]. Technologies to address these parameters are therefore in high demand [3-6].

This application note presents the use of the fluorescent kinase inhibitors to determine the kinetic binding properties of inhibitors of interest. Data obtained for FGFR1-BTN demonstrate that the fluorescent kinase inhibitors, such as Staurosporine-Red, enable accurate kinetic profiling of inhibitors, confirming the pharmacological relevance of the kinase binding platform for both equilibrium-and kinetic profiling.



Assay principle

Kinase binding assay

The assay is based on an HTRF sandwich format using Streptavidin-Eu cryptate and a fluorescent tracer. When a biotinylated kinase is present, an HTRF signal is generated.

Upon the addition of competitive inhibitors of the ATP binding site, the fluorescent tracer is displaced and the HTRF signal disappears (see below).

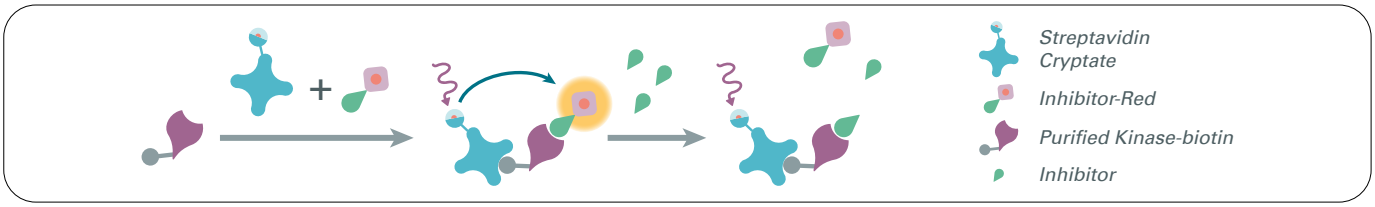


Figure 1: Kinetic binding assay schematic

Fluorescent tracer kinetic binding protocol (a)

In order to determine the kinetic parameters of the inhibitors of interest, the association (K_{on}) and the dissociation (K_{off}) rates of the tracer should be determined. To perform such an experiment, 6 different concentrations of the fluorescent tracer are first dispensed into the plate. Prepare a mix of SA-cryptate and Kinase-BTN, leave it for 15 minutes and add it to the plate. Due to the fast binding kinetics of the tracers to the biotinylated kinases, it is of interest to use a syringe-based system for the addition of the kinase-BTN/SA cryptate mix.

Determination of K_{on}, K_{off} and residence time of inhibitors (b)

Determination of the kinetic parameters of the inhibitors of interest can be measured by adding the SA-cryptate/ Kinase-BTN mixture to a plate where a 2-fold dilution series of inhibitor has been first mixed and dispensed with the red-tracer at or near its K_D. Subsequent fitting of the kinetic tracers using the Motulsky-Mahan model [7] gives the kinetic parameters for the inhibitor of interest.

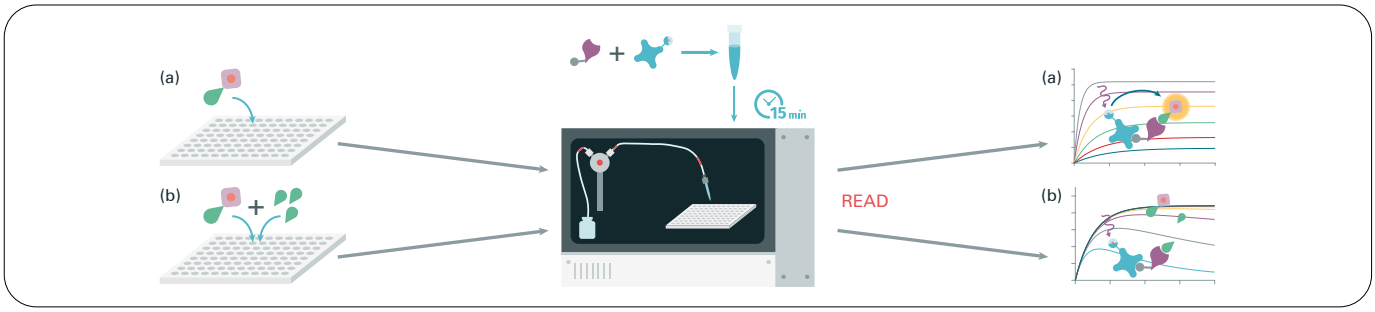


Figure 2: Assay protocol. fluorescent tracer kinetic binding protocol (a) and determination of K_{on}, K_{off} and residence time of inhibitors (b).

Kinetic binding: Kinetic parameter determination for the fluorescent tracer

Before starting your kinetic study, the first step is to determine which tracer will be the most useful for your kinase of interest. This can be tested with the HTRF kinase binding discovery kits at equilibrium conditions.

Then in order to perform the kinetic assays, it is highly recommended to have a plate reader with an injection system available. Kinetic analyses of inhibitors using the Motulsky- Mahan method [3-7] require knowledge on the K_{on} and K_{off} of the fluorescent tracer to the kinase, and hence these must be determined. The procedure to determine the kinetic parameters is described below and illustrated with examples measured inhouse.

Kinetic parameters of the fluorescent tracers

The association kinetics of the fluorescent tracers using purified biotinylated kinases are quite fast, and therefore the first 10 seconds after addition are crucial to properly determine the kinetic parameters. This means an injector system is obligatory. The data to analyze is the specific HTRF ratio, and hence a nonspecific signal (in absence of kinase for each concentration) needs to be measured and subtracted from the total signal obtained. Since the non-specific signal is not time dependent, it can be measured separately. Optimization of detection conditions (such as number of flashes or detector gain) can first be tested in equilibrium conditions. The kinetic binding of Staurosporine-Red (62KM01REDC/E) to 5 nM of FGFR1-BTN (#08-435-23N-Carna Biosciences) is shown below. The kinetic profiles of a 2-fold serial dilution series of 6 concentrations of the red-tracer is given, then analyzed by a global fit of the data using the Graphpad-Prism protocol "Association kinetics-two or more concentrations of hot".

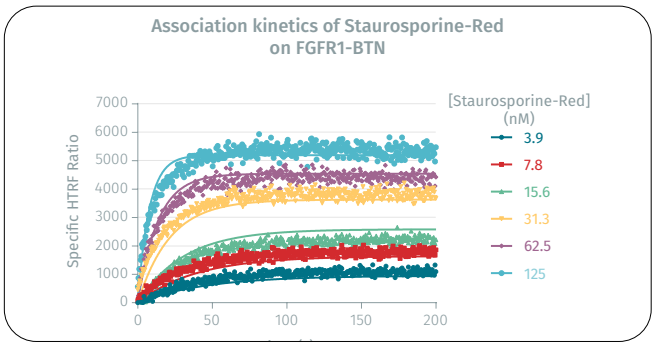


Figure 3: Association kinetics of Staurosporine-Red on 5 nM FGFR1-BTN measured in a 96-sv-well plate (66PL96005/025/100) by dispensing 5 µL of Staurosporine-Red into the plate and adding 15 µL of a mix of FGFR1-BTN and Streptavidin-Eu-cryptate (15 min of preincubation), all diluted in the 'Kinase Binding Buffer' (62KBBRDD/F). Data generated using a plate reader equipped with an injector system on 2 wells per concentration, using measurement intervals of 0.5 s and two flashes per measurement. Error bars are omitted for clarity. HTRF ratios will be dependent on the plate reader and settings used.

The results of the fit are shown in the table below. Calculating the kinetic dissociation constant (K_D) based on the K_{off} & K_{on} (KD = K_{off} / K_{on}) results in value of 21 nM, in agreement with 29 nM calculated in equilibrium conditions.

For the system FGFR1-BTN/Staurosporine-red, the experiments give solid data. However for other kinase-tracer combinations even faster kinetics can occur. Depending on the time resolution of the system, the association or dissociation kinetics might become trickier to measure. Alternatively, changing to a tracer with slower association kinetics is an option

Table 1: Association kinetics of Staurosporine-Red on FGFR1-BTM Results

Kinase			Staurosporine-Red		
FGFR1-BTN	K_{on} (10^6 $M^{-1}s^{-1}$)	K_{off} (s^{-1})	Res. Time (s)	K_D (kinetic) (nM)	K_D (equilibrium) (nM)
	0.89 ± 0.01	0.019 ± 0.01	52.6	21	29

K_{off} Tracer: Alternative method

As with traditional radioactive binding assays, the K_{off} of the tracer can also be determined by adding a large excess of fast associating unlabeled competitor to a preequilibrated kinase-tracer complex. It also verifies, independently, the K_{off} measured in the experiment described above. To demonstrate the feasibility of this approach for the HTRF kinase binding assay, 5 µL of Staurosporine (prepared at 40 µM in Kinase binding buffer) was added with an injector to a mixture of 5 µL of Staurosporine-Red (1.9-15 nM final concentration) & 10 µL of a mixture of FGFR1-BTN & Streptavidin-Eu-Cryptate. Analyses were performed fitting the specific HTRF ratio of the different concentrations by using exponential decay fitting (with the Graphpad-Prism protocol "Dissociation Kinetics-One phase exponential decay"). The resulting K_{off} was 0.018 ± 0.004 s-1 which is in agreement with the 0.019 s-1 determined in the association kinetics experiment (vide supra).

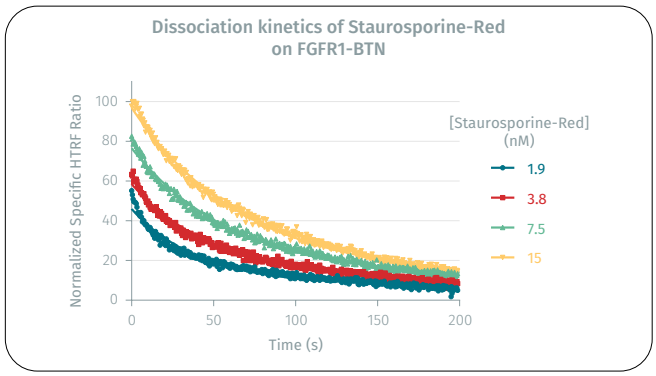


Figure 4: Dissociation kinetics of Staurosporine-Red on 5 nM FGFR1-BTN measured in a 96-sv-well plate by dispensing 5 µL of Staurosporine-Red plus 10 µL of a mixture of FGFR1-BTN and Streptavidin-Eu-cryptate (15 min of preincubation), and adding 5 µL of unlabeled Staurosporine at 10 µM final concentration. Data generated using a plate reader equipped with an injector system on 2 wells per concentration using measurement intervals of 0.5 s and two flashes per measurement. Error bars are omitted for clarity. HTRF ratios will be dependent on the plate reader and settings used.

Kinetic binding parameter determination for compound profiling

Association and dissociation kinetic analyses of unlabeled competitors can be performed using the Motulsky-Mahan method [7], which has already been shown to work for HTRF based assays [3-6]. In order to perform such analyses, the association and dissociation rates of the fluorescent tracers must be determined beforehand.

By tracing the kinetic profiles at different competitor concentrations for a fixed tracer concentration, the kinetic binding properties can be determined. In order to obtain reliable results the kinetic profiling of at least 4 concentrations of inhibitor need to be tested. Here we show data on the FGFR1- BTN/Staurosporine-Red system where we performed a fourfold serial dilution series of 8 concentrations in the presence of 21 nM of Staurosporine-Red. To initiate the kinetic experiment, a pre-equilibrated mixture of 5 nM Kinase & Streptavidin-Eucryptate was injected into the wells already containing a mixture of non-labeled inhibitor and tracer. The non-specific signal was measured by adding all reagents except the kinase.

Kinetic profiles of Staurosporine, Dasatinib, & Dabrafenib on FGFR1-BTN

The kinetic traces and global fitting of the specific HTRF ratio using the Graphpad-Prism protocol "Kinetics of competitive binding" of Staurosporine, Dasatinib & Dabrafenib in the presence of 21 nM of Staurosporine-Red are shown below.

For this proof of concept, using the K_{off}, K_{on} of Staurosporine-Red on FGFR1-BTN resulted in an excellent global fit for the three compounds. The association and dissociation rates of the inhibitors are given in the table below. Both Staurosporine and Dasatinib had similar residence times (residence time = 1 / K_{off}), but the association rate is much faster for Staurosporine, leading to its increased affinity over Dasatinib. Interestingly, the BRAFV600E clinical approved inhibitor Dabrafenib had quite a long residence time, although this needs to be confirmed using longer measurement times.

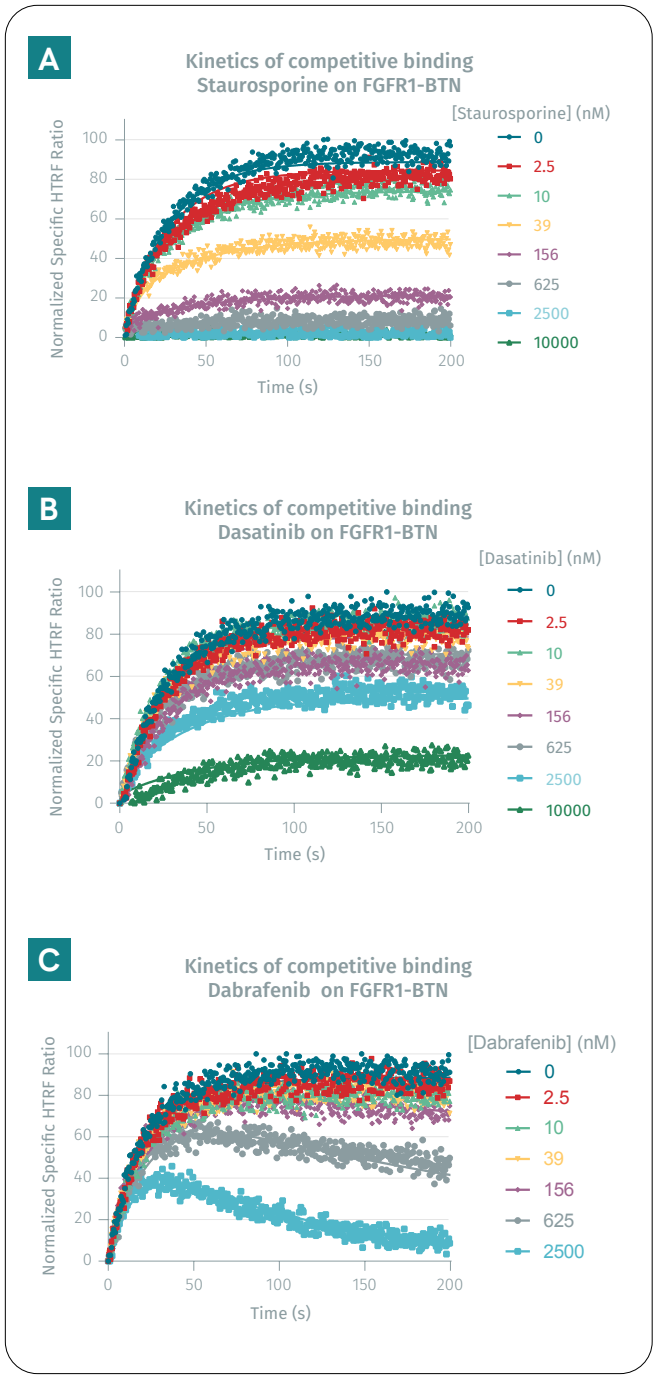


Figure 5: Inhibitor compound characterization: kinetic traces of Staurosporine, Dasatinib, & Dabrafenib on 5 nM FGFR1-BTN at different concentrations and analyzed by global fitting using the Motulsky-Mahan procedure. Data were obtained by adding 10 µL of FGFR1-BTN and Streptavidin-Eu-cryptate (15 min of preincubation) to wells containing a mixture of 5 µL Staurosporine-Red (21 nM final concentration) and 5 µL of 4x the indicated concentration of inhibitor (96-sv-well plate). Non-specific data was obtained by adding Streptavidin-Eu-cryptate without the kinase. The data was generated using a plate reader equipped with an injector system on 2 wells per concentration using measurement intervals of 0.5 s and two flashes per measurement. Error bars are omitted for clarity.

Table 2: Inhibitor kinetic data obtained from Motulsky-Mahan fitting of the above traces on FGFR1-BTN using Staurosporine-Red (*S-Red).

Kinase	Tracer	Compound	Kinetic binding parameters			
			K_{on} (10^6 $M^{-1}s^{-1}$)	K_{off} (s^{-1})	Res. Time (s)	K_D (kinetic) (nM)
FGFR1-BTN	S-Red*	Staurosporine	18.8 ± 0.7	0.048 ± 0.002	20.8	25.5
	S-Red*	Dasatinib	0.54 ± 0.08	0.11 ± 0.02	9.5	2037
	S-Red*	Dabrafenib	0.14 ± 0.01	0.0007 ± 0.002	1430	50

Conclusions and perspectives

We have demonstrated that the HTRF kinase binding platform can not only be used for equilibrium binding assays, but that kinetic binding experiments can be performed as well. This study demonstrated the accurate determination of association and dissociation constants of the Staurosporine-Red tracer for N-terminal biotinylated FGFR1. Preliminary experiments show that both Dasatinib-Red and Sunitinib-Red can be used as well (data not shown). The keys to a successful kinetic binding experiment are however a good time resolution, a precise injector system, and the use of the proper tracers, since the association kinetics of the tracers in all our experiments are fast (>10^6 $M^{-1}s^{-1}$). Preliminary experiments with Dasatinib-Red on BRAF-BTN, for instance, show even higher association rates which might be problematic to analyze if your plate reader cannot handle a speed of >1 Hz. Therefore a reader such as the Envision from Revvity is a wise choice, since it can handle measurements up to 10 Hz.

It is worth noting that the Motulsky-Mahan model requires the inhibitor and the red-tracer to be competitive (both binding on the same site), and that they are both allowed to bind on the kinase at the same time (hence the protocol described in this application note, which is slightly different from regular equilibrium experiments).

Since the affinity of biotin for streptavidin is one of the highest found in nature, using biotinylated kinases is the primary choice for these kinetic experiments. However, ongoing investigations show that using GST- or 6HIS-tagged kinases could be an alternative if biotinylated kinases are not available.

References

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7. Motulsky, H.J., and Mahan, L.C. (1984). The kinetics of competitive radioligand binding predicted by the law of mass action. Mol. Pharmacol. 25, 1–9.

Ordering Information

Table 3: Product and ordering details

Product	Size	Part#
Kinase-biotin Binding Discovery Kit	1	62KBD03PEA
Kinase-GST Binding Discovery Kit	1	62KBD01PEA
Kinase-6HIS Binding Discovery Kit	1	62KBD02PEA
Staurosporine-Red	1 nmol	62KB01REDC
	20 nmol	62KB01REDE
Dasatinib-Red	1 nmol	62KB02REDC
	20 nmol	62KB02REDE
Sunitinib-Red	1 nmol	62KB03REDC
	20 nmol	62KB03REDE
Kinase Binding Buffer	20 mL	62KBBRDD
	20 mL	62KBBRDF
Streptavidin-Eu cryptate Kinase Binding	1,000 tests	62KBSAKAF
	20,000 tests	62KBSAKAB
Mab Anti GST-Eu cryptate Kinase Binding	1,000 tests	62KBGSTKAF
	20,000 tests	62KBGSTKAB
Mab Anti 6HIS-Eu cryptate Kinase Binding	1,000 tests	62KBHISKAF
	20,000 tests	62KBHISKAB
HTRF 96-well low volume white plate	5	66PL96005
	25	66PL96025
	100	66PL96100

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