APPLICATION NOTE

Streptavidin-Biotin: Harness avidity without compromising on pharmacology.

Abstract

Protein-Protein interactions (PPIs) play key roles in cellular signaling, making them increasingly attractive targets for the development of therapeutic drugs.

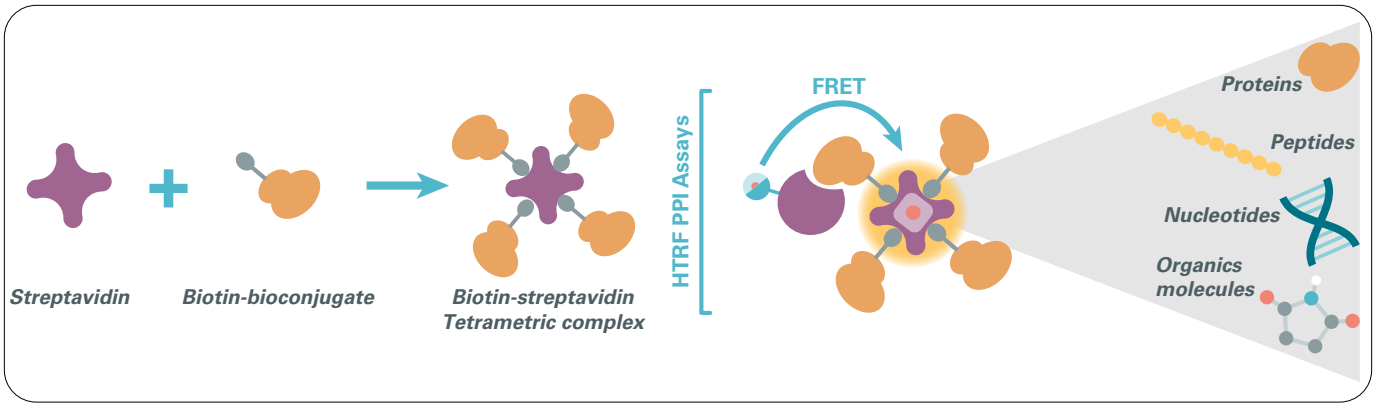
Among a large number of HTRF® PPI solutions, Revvity offers labeled streptavidin reagents to probe all kinds of biotinylated molecules. Thanks to the biotin-streptavidin (biot-SA) tetrameric complex, Protein-Protein interaction assays can benefit from enhanced apparent affinity (i.e. avidity).

Two different models, BRD4:H4 peptide and CD16a:Fc IgG, were investigated to evaluate the impact of the biot-SA interaction on the affinity determination. These case studies raised the possibility of being able to harness this detection format for compound characterization.

This study demonstrates a differential effect of the biot-SA system on the affinity gain depending on the complex, while maintaining an accurate pharmacological characterization of reference compounds.

The Biotin-Streptavidin system

The biotin-streptavidin system (biot-SA) is the strongest non- covalent biological interaction known, with a very high affinity of 10-14 M. Streptavidin is a homotetramer, which has four binding sites for biotin molecules. Once formed, this complex is known to be very stable, as it is unaffected by extreme conditions such as high temperature, denaturing agents, extreme pH, or enzymatic degradation[1]. It is therefore not surprising that the biotin-SA interaction has been used in many biological applications: protein purification, bio-imaging, drug delivery, and detection assays. It is also a popular system for protein-protein interaction assays.



Moreover, it is known that multimers can display a gain in affinity. This is because the multiple interactions can synergize to enhance affinity, a phenomenon which is commonly referred to avidity. This gain in affinity has already been related to a gain in target residence time (i.e. forced proximity)[2]. Nevertheless, the high affinity of streptavidin for free biotin could differ depending on the biotinylated partner, and various interaction affinities of streptavidin with free biotin or biotinylated molecules have been reported . Therefore, depending on parameters such as the accessibility of the biotin (which could be also related to the size of the biotinylated molecule), or the biotin linking chemistry (i.e. the presence of a bridging group), a gain in relative avidity can be observed or not[3].

Based on these interesting biotin-SA system properties, two different models have been explored in HTRF PPI assays, in order to show the advantage of this avidity system and to validate the accurate characterization of reference compounds.

Biot-SA based HTRF PPI assay with high avidity effect

BRD4(1):H4(1-21) peptide

The BRD4(1), a member of the bromodomain BET family, binds to acetylated lysine residues of histones playing an important role in gene transcription regulation. BRD4(1) associates with the acetylated Lys(5,8,12,16) H4 (1-21) peptide, displaying a low affinity of 2-3 µM [4,5]. This protein protein interaction has been widely studied in cancer therapy for inhibitor compound identification.

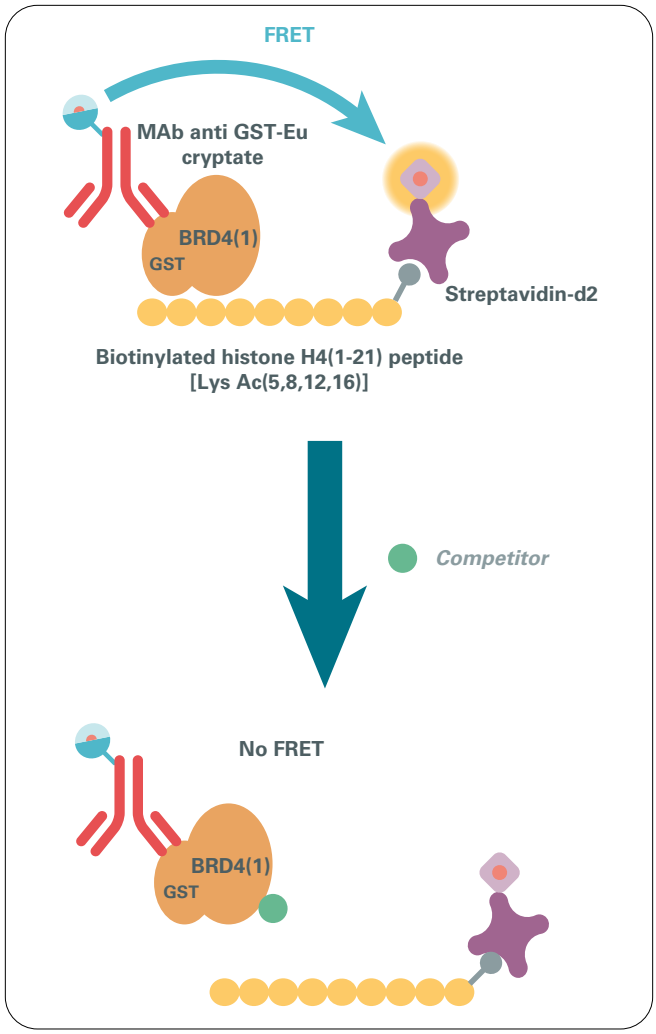


Figure 1: BRD4(1):H4 peptide-biotin interaction assay principle

Biotinylated H4 peptide was detected using d2 labeled Streptavidin. The GST-tagged BRD4 protein was detected with the MAb anti GST-Eu cryptate antibody. The HTRF signal appears once the complex is formed. Addition of competitors leads to complex dissociation and signal extinction (Figure 1).

Kd determination to evaluate enhanced affinity using the biot-SA system

In order to determine the affinity of the complex, a fixed concentration of BRD4 at 5 nM and MAb anti GST-Eu cryptate at 0.5 nM were added to a range of concentrations of biotinylated H4-peptide. The peptide-biotin/streptavidin-d2 ratio was fixed at 8/1 for all the peptide concentrations. Figure 2 shows an apparent affinity of 3 nM for the complex. These findings suggest a significant enhancement of the Kd using the biot-SA avidity system compared to the published affinity of the complex of 2 µM, assessed without the biotin-SA system (ITC reference method).

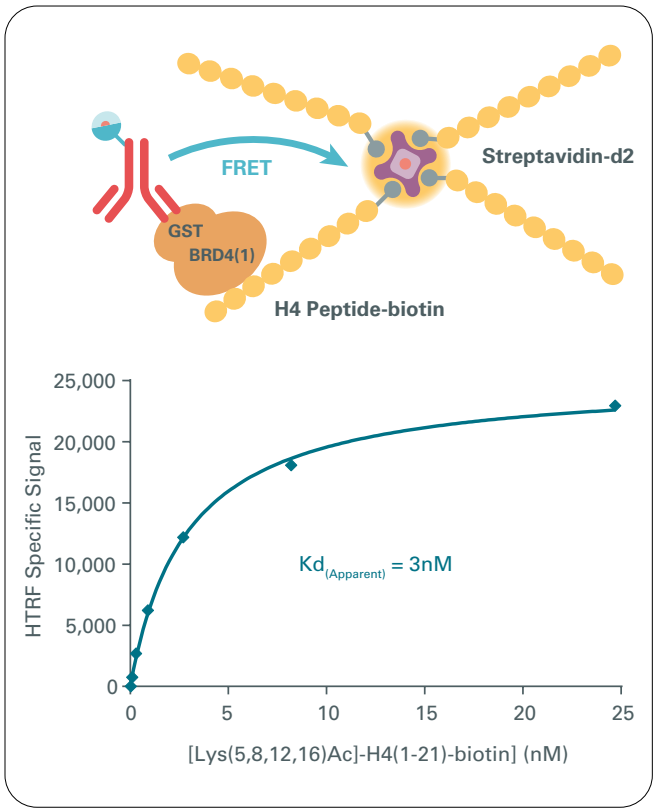


Figure 2: BRD4(1):H4 peptide-biotin apparent affinity determination.

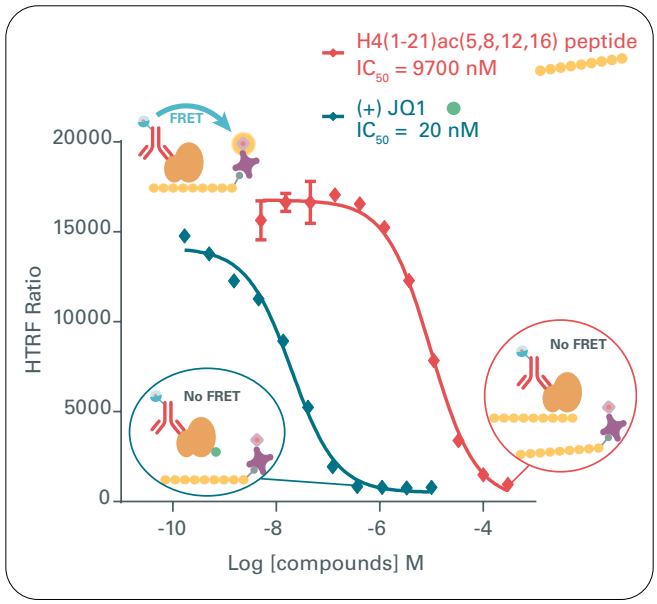
This apparent affinity gain enables a reduction in protein concentration consumption within the nM range.

Pharmacological validation

In order to ensure that the gain in apparent affinity has no effect on the pharmacological characterization of any inhibitors of the complex, two reference compounds were tested.

Dose-response curves were generated to determine the IC_{50} of the inhibitors. Using the Cheng-Prusoff equation[7], potencies (i.e. Ki values) were determined.

The unlabeled H4 peptide and the JQ1 reference compounds gave Ki values of 4.8 µM and 8 nM respectively. These values are in accordance with the published values[4,5,6].



Compound Affinities	Unlabeled H4 peptide	JQ1
Ki (nM)	4850	8
Published values (nM)	3100	27

Figure 3: BRD4(1):H4 peptide complex inhibitor characterizations.

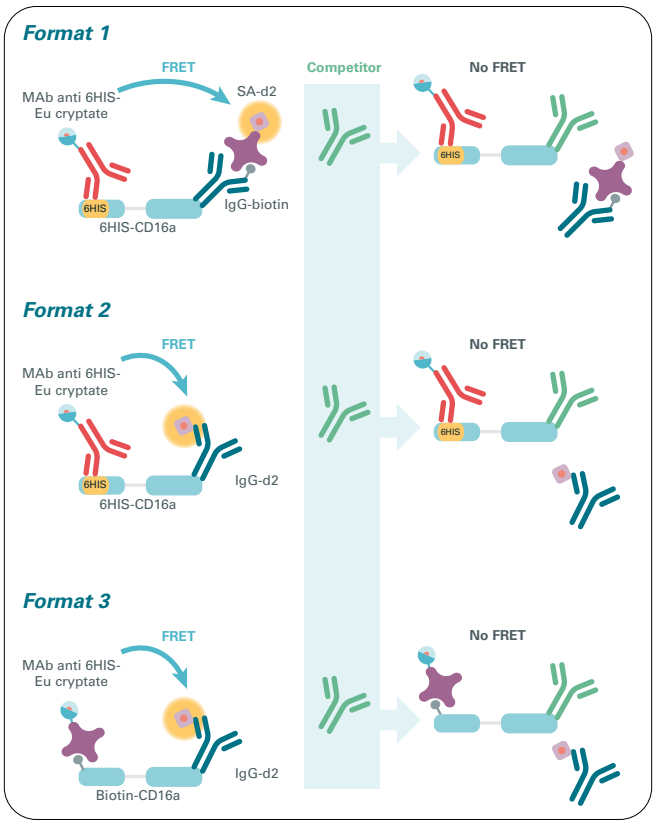
This model shows a high affinity gain using the biot-SA model, lowering the apparent Kd of the peptide-biotin:BRD4 complex from the µM to the nM range. Moreover, the Ki determined for the unlabeled H4 peptide and JQ1 are in good agreement with the literature, confirming the ability of the system to accurately characterize the pharmacology of reference compounds.

Biot-SA based HTRF PPI assay with low avidity effect

CD16a:Fc-IgG

Fc-Gamma Receptors (FCGRs) are members of the immunoglobulin superfamily, and are highly involved in the functions of therapeutic antibodies. Among different classes of FCGRs, FCGR3A (also known as CD16a) is involved in key immunological processes, such as phagocytosis or antibody-dependent cellular cytotoxicity (ADCC)[8]. Thus, IgG-Fc regions bind to CD16a to induce ADCC responses with low/intermediate variable affinities for different classes of human IgGs. This receptor is known to display a higher affinity for IgG3 and IgG1 than for IgG2 and IgG4[9][10].

To assess the biot-SA avidity system in this model, three assay formats were generated (formats 1-3). Formats 1 and 2 differ in the presence or absence of the biot-SA system. The last format, number 3, was generated to evaluate the biotin position effect compared to that of Format 1.



	System	Biotin position
Format 1	Biotin-SA	IgG tracer
Format 2	No avidity system	No biotin
Format 3	Biotin-SA	CD16a

Figure 4 : CD16a:IgG-Fc assay formats

As described in figure 4, the CD16a (158V):IgG-Fc assay uses HTRF PPI reagents for detection. In all cases, the HTRF signal appears when the complex is interacting. Inhibitor additions (such as unlabeled IgGs), lead to competition with the IgG labeled tracer and thus, to HTRF signal extinction. All the assays were performed in the PPI Europium dilution buffer.

All the assays formats were optimized to reach optimal assay windows >15.

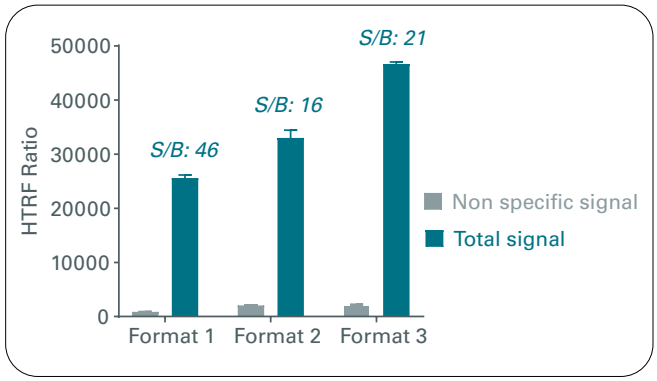


Figure 5: CD16a:IgG- Fc assay performance.

Kd determination to evaluate the enhanced affinity using the biot-SA system

Relative affinities (i.e. Kd values) were determined in order to identify the impact of the biot-SA system for this protein protein interaction model.

Dose-response curves of serial dilutions of the IgG tracers to a fixed concentration of CD16a protein were plotted for each format to determine the apparent Kd. As an example, Figure 6 shows a saturation binding curve for Format 1. The apparent Kd obtained for the 3 formats is summarized in the related table.

Kd value comparisons for Format 1 and Format 2 show that the biot-SA system slightly enhances the relative apparent affinity (from 118 nM to 22 nM). The avidity effect of the biotin-SA system in this protein protein interaction model is low compared to the previously described BRD4:H4 model, where a dramatic affinity enhancement was observed.

In order to check if avidity depends on the biotin position, Format 3 was explored and compared to Format 1 and 2. Results suggest that biotin position does not have any impact on the avidity gain.

To validate the minimal avidity impact on this model, a Ki determination assay was performed by running a dose response competition curve with the unlabeled IgG tracer in the 3 formats. The competition curve for Format 1 is shown, and all the Ki values are reported in the related table (Figure 7). Firstly, the affinity (Ki) of the unlabeled IgG tracer is similar whatever the detection format. Secondly, the Ki values of the unlabeled IgG are in the same range as the Kd values of the IgG tracers (Figures 6 & 7). Finally, Ki values of the unlabeled IgG tracer are in accordance with previously determined apparent Kd in the nM range. Taken together, these results confirm the very low impact of biot-SA on the apparent affinity of this complex.

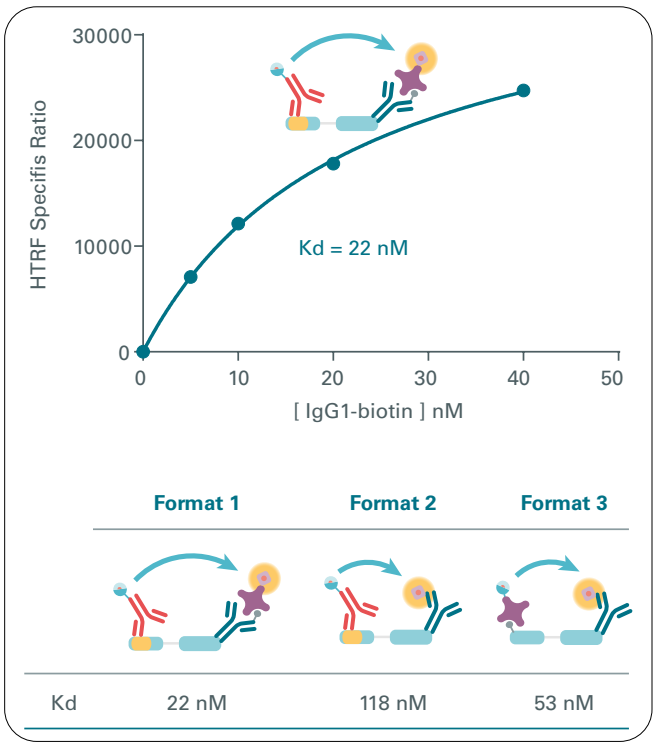


Figure 6: Apparent affinity determinations. For the three formats, the plotted IgG tracer dose-response curves enable Kd determination. The resulting binding curve for Format 1 is shown.

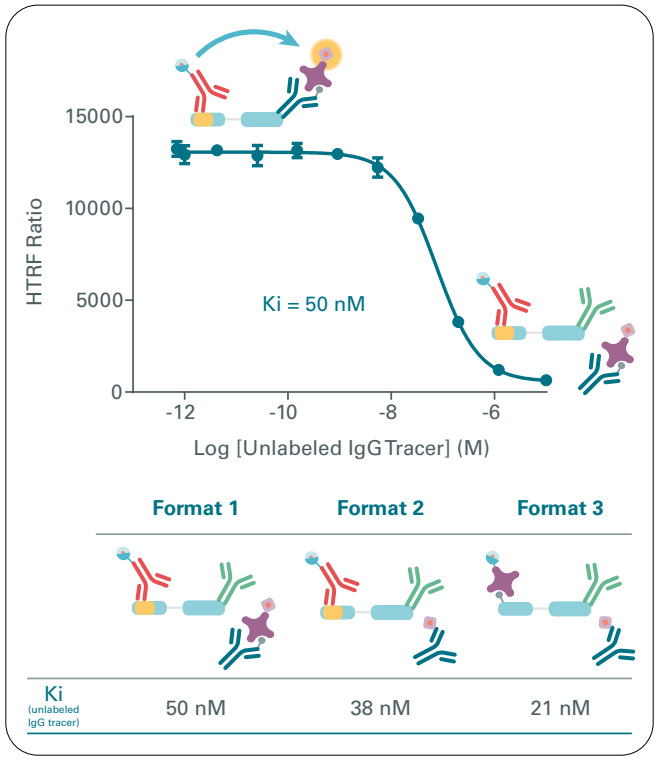


Figure 7: Unlabeled IgG Ki determination in the 3 formats

The biot-SA ratio was more deeply investigated for Format 1 to identify its impact on the apparent affinity of the complex. Different quantities of biotinylated CD16a were mixed with a fixed concentration of streptavidin at 0.5 nM in order to obtain a biot-SA ratio of 2, 4 or 8. Titration of the IgG-d2 tracer was then plotted to calculate the Kd.

Ratio biot-SA	2	4	8
Kd (nM)	53.6	53.3	63.5

In this model, the apparent affinity does not vary whatever the biot-SA ratio applied.

Given all these results, the very low enhancement of the relative affinity in this model can be explained by the large size of the IgG or the CD16a biotinylated molecules, which could hinder biotin accessibility for binding to streptavidin. These data are in agreement with published papers demonstrating that affinity enhancement using the biot-SA system can be impaired by the size of the biotinylated molecule[3].

Pharmacological validation

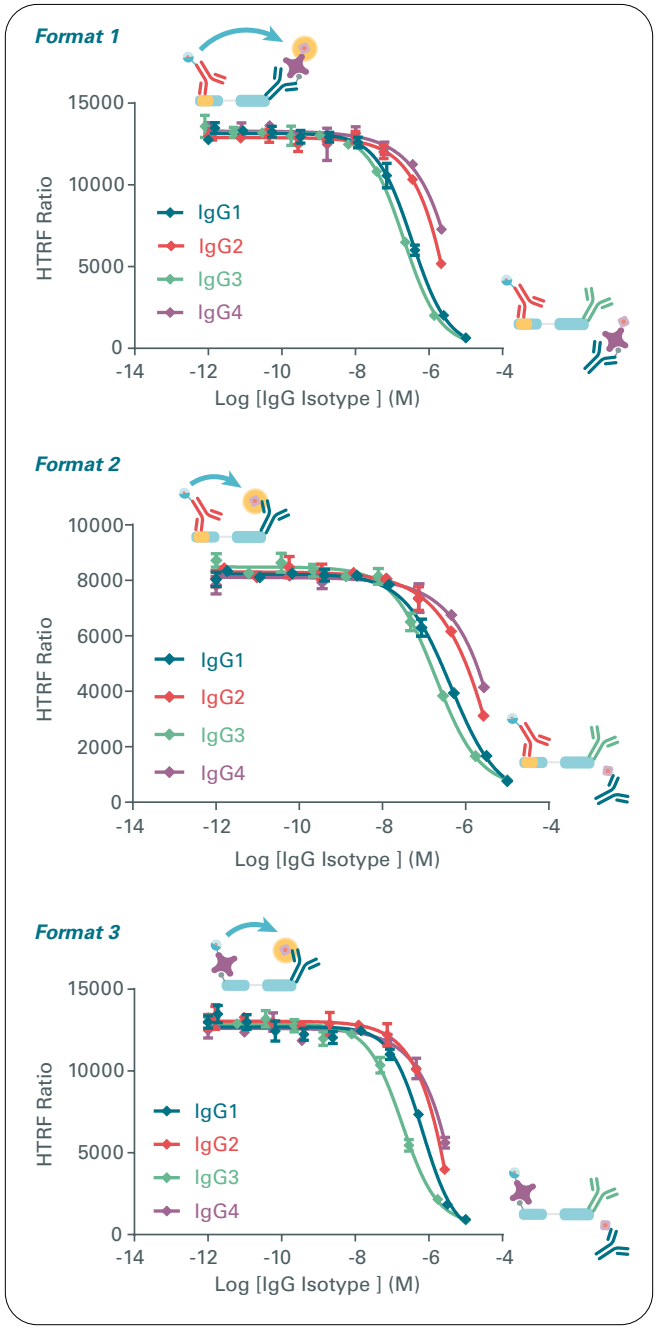
To show the ability of this assay to address inhibitor characterization, determination of the potencies of different IgGs for binding to CD16a (158V) was carried out. Interactions of the CD16a with different human IgG isotypes were largely explored. It is known that CD16a binds human IgGs with different affinities, and that it displays a higher affinity for IgG3 and IgG1 than for IgG2 and IgG4[9][10].

The three different formats were therefore assessed to demonstrate the accuracy of them all for compound characterizations.

The results are presented in Figure 9, and the Ki values (calculated from IC_{50} using the Cheng-Prusoff equation[7]) are summarized in the related table.

The three formats display the same ranking of IgG isotypes (with IgG3 and IgG1 more potent than IgG2 and IgG4). Moreover, the Ki values are very similar for all the formats, and are in accordance with published affinities concerning the SPR method. This confirms that CD16a:IgG-Fc assays, using a biot-SA system or not, enable accurate pharmacological characterization of compounds.

In this study three formats, differing in the presence or absence of the biot-SA system or in the biotin position, were explored. It was demonstrated that the biot-SA system does not significantly enhance the relative affinity in this CD16a:Fc IgG protein protein interaction model, probably due to the large size of the biotinylated biomolecules. Moreover, all the assay formats, using biot-SA system or not, enable the accurate pharmacological characterization of inhibitors as shown by Ki value determinations in agreement with the published affinities.



		IgG Subtype			
	Format	IgG1	IgG2	IgG3	IgG4
HTRF Ki values (nM)	Format 1	237	>1000	142	>1000
	Format 2	443	>1000	191	>1000
	Format 3	560	>1000	158	>1000
Published affinity by SPR (nM)		500	14000	100	4000

Figure 9: CD16a:IgG-Fc PPI inhibitor characterization in the three assay formats.

Conclusion

The biotin-streptavidin system is widely used in biological immunoassays, as it benefits from a generic method to probe biomolecules coupled with an incredibly high affinity.

This application note is intended to illustrate the potential benefit of the biot-SA format in HTRF PPI assays.

Two examples, representative of various assay formats, affinities, and biotinylated molecule sizes, were further investigated.

The results obtained with the BRD4:H4 peptide model demonstrate that the biot-SA avidity complex can dramatically enhance the apparent affinity of a PPI interaction. This apparent affinity gain enables a reduction in protein consumption and the possibility to assess PPIs displaying a low affinity range.

However, the apparent affinity improvement is not guaranteed, and will depend on the type of interaction and the nature of the biotinylated biomolecules. Involved. For example, the CD16:IgG-Fc interaction does not benefit from it, as shown in this study.

Last but not least, the pharmacological potency of inhibitors can be characterized with or without the biot-SA system. Both formats enable an equally accurate acquisition of results, in agreement with published Ki values.

(1) Dundas et al. Appl Microbiol Biotechnol (2013) 21 : 9143-53
(2) Vauquelin et al. British journal of pharmacology (2013) 168 :1771-1785
(3) Vincent et al. Journal of Immunological Methods (1993) 165 : 177-182
(4) Filippakopoulos and Knapp, FEBS Letters (2012) 586 : 2571-2799
(5) Philpott et al. Mol. BioSyst. (2011) 7(10) : 2899-908
(6) Filippakopoulos et al. Nature (2010) 468(7327) : 1067-73
(7) Refer to the application note: «Best practices for pharmacological characterization of PPI inhibitors»
(8) Ochoa et al. Immunology and cell biology (2017) 95; 347-355
(9) Bruhns et al. Blood (2009) 113; 3716-3725
(10) Rosales et al. Frontiers in Immunology (2017) 8; doi: 10.3389/ fimmu.2017.00280

Revvity, Inc.
940 Winter Street
Waltham, MA 02451 USA

(800) 762-4000
www.revvity.com