HTRF probes all types of protein-protein interaction, from small to very large complexes.

This application note demonstrates the suitability of HTRF® for PPI investigations whatever the size of the complexes studied.

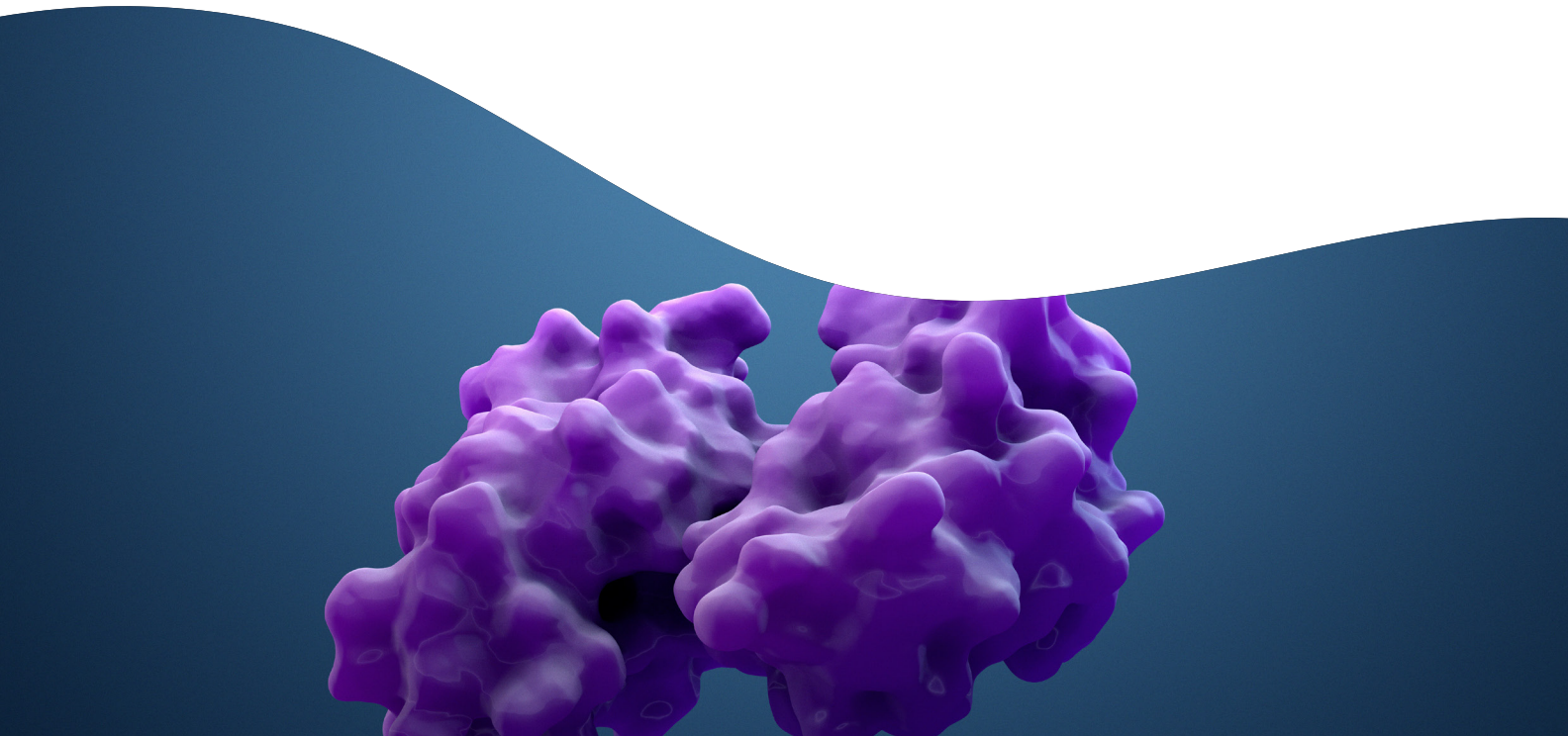
Abstract

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

The sheer number of PPIs described so far (>400,000) epitomizes the diversity found in what has been described as the human interactome. The complexes span a wide variety of sizes and molecular weight (MW), which raises doubts as to the ability of proximity-based technologies such as HTRF to investigate such a diversity.

The aim of this study is to convincingly demonstrate the applicability of HTRF to PPIs, without restrictions on the sizes or MW of the complexes probed.

Among internal case studies and various PPI models described in the literature, three different-sized PPI complexes are further exemplified: -Catenin:TCF4, TRAIL-L:TRAIL-R, and Grasp55:JamB$_{(PDZ\ domain)}$.



HTRF probes all types of protein-protein interaction, from small to very large complexes.

HTRF PPI assays to address a large panel of complex sizes

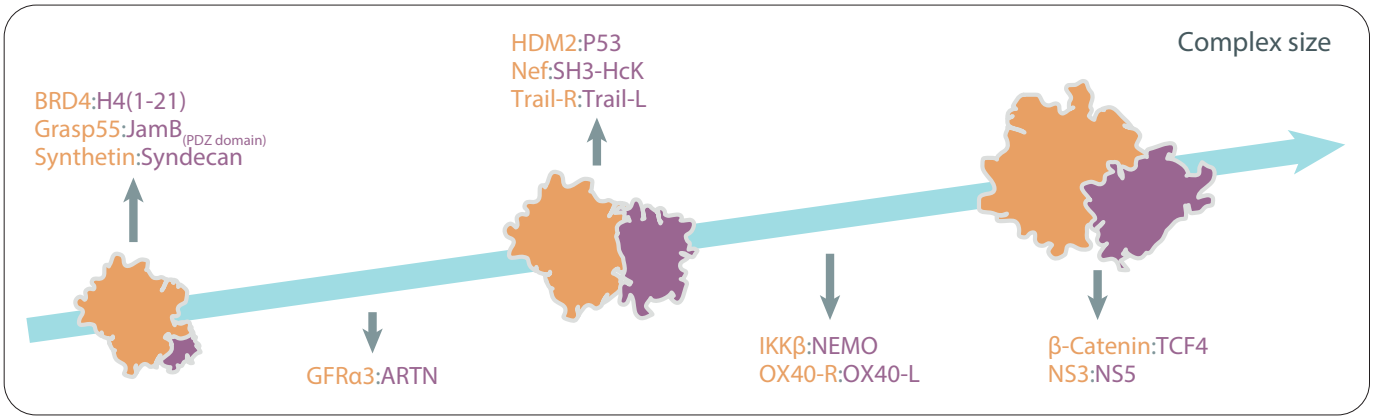


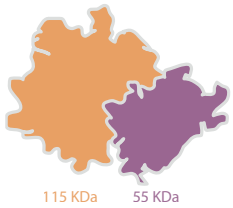
Figure 1: HTRF PPI assays to study a variety of complex sizes.

Complex	Mw recombinant protein 1	Mw recombinant protein 2	Reference
β-Catenin:TCF4	115 KDa	55 KDa	Internal case study
NS3:NS5	100 KDa	50 KDa	Milhas S, *et al.* (2016) ACS chem biol 11(8):2140-8
Ikkβ:NEMO	97 KDa	35 KDa	Gotoh, *et al.* (2010) Anal. Biochem 405:1-27
OX40-R:OX40-L	72 KDa	40 KDa	Newton P, *et al.* (2008) JBS 13(7):674-682
HDM2:P53	55 KDa	40 KDa	Kane S-A, *et al.* (2000) Anal Biochem 278:29-38
Trail-R:Trail-L	44 KDa	21 KDa	Newton P, *et al.* (2008) JBS 13(7):674-682
Nef:SH3-HcK	35 KDa	33 KDa	Milhas S, *et al.* (2016) ACS chem biol 11(8):2140-8
GFRα3:ARTN	65 KDa	12 KDa	Thornton P, *et al.* (2013) Neuroscience Letters 545:23-28
synthetin:syndecan$_{(PDZdomain)}$	60 KDa	2 KDa	Milhas S, *et al.* (2016) ACS chem biol 11(8):2140-8
Grasp55:JamB$_{(PDZdomain)}$	48 KDa	3 KDa	Milhas S, *et al.* (2016) ACS chem biol 11(8):2140-8
BRD4:H4(1-21)	42 KDa	3 KDa	Milhas S, *et al.* (2016) ACS chem biol 11(8):2140-8

Large size complex

β-CATENIN:TCF4

The formation of a complex between -Catenin and the TCF/LEF family is a key regulatory event in the wnt- signaling pathway (embryonal development, growth of colon epithelium,..). It has been reported that the complex binds reversibly in the low nM range[1].



This study was performed to show the ability of HTRF PPI reagents to address large biomolecular interactions. Recombinant human GST--Catenin (115 KDa) and 6HIS- TCF4 (55 KDa) interaction was detected using Anti GST-Eu Cryptate (150 KDa) and Anti 6HIS-d2 (150 KDa) antibodies. Inhibitors of the interaction trigger the dissociation of the complex, and thus HTRF signal extinction (Figure 2).

Titration of the 6HIS-TCF4 protein determines the optimal concentration to reach a high assay window across the dynamic detection range (Figure2).

The ITAC protein (CTNNBIP1) is a negative regulator of the wnt pathway. It binds to -Catenin, and prevents it from interacting with TCF4. ITAC and the unlabeled -Catenin proteins were characterized, showing potencies in agreement with published IC_{50} values (Figure 4).

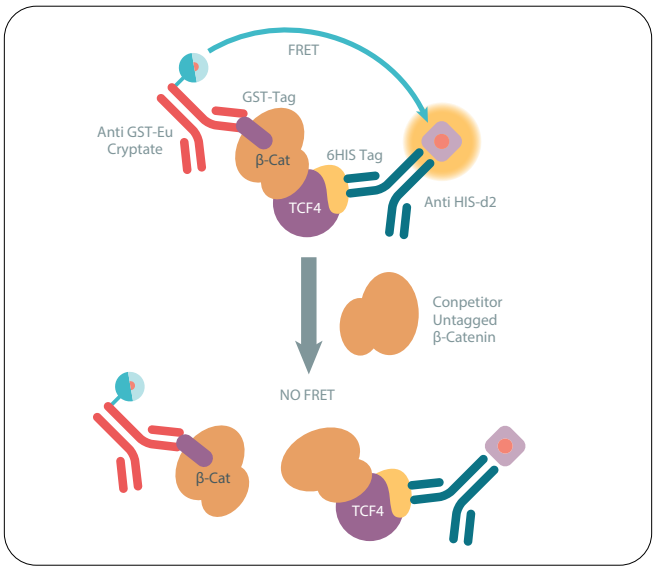


Figure 2: HTRF TCF4:β-Catenin PPI assay principle.

GST-β-Catenin and 6HIS-TCF4 interactions were detected using Anti GST-Eu Cryptate and Anti 6HIS-d2 antibodies. Introduction of an interaction competitor triggers the complex dissociation, and thus HTRF signal extinction.

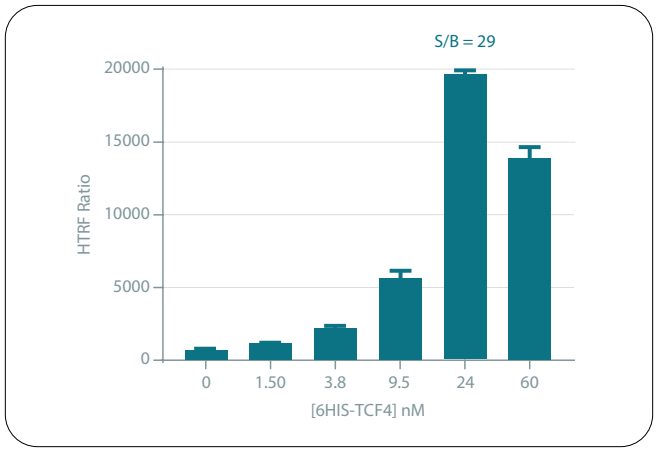


Figure 3: HTRF detection of β-Catenin and TCF4 complex.

A fixed 5 nM concentration of GST--Catenin was incubated with increasing concentrations of 6HIS-TCF4. Their interactions were detected using 0.5 nM of Anti GST-Eu cryptate and 5 nM of Anti 6HIS-d2. The assay was performed in 20 µl final volume. Signals were recorded using the PheraStar lamp reader after ON incubation. The optimal assay window of 29 was obtained for 24 nM of TCF4.

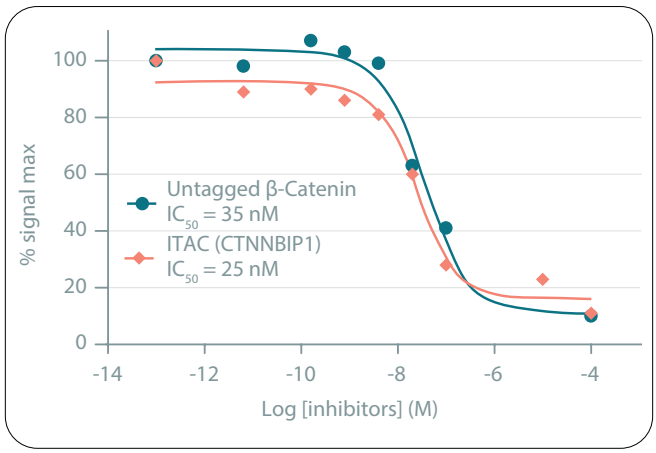


Figure 4: Inhibitors characterization in the HTRF TCF4:β-Catenin assay.

GST-β-Catenin (5nM) and 6HIS-TCF4 (20 nM) were detected in the previously described conditions. Serial dilutions of competitors enable dose-response curve generation, and thus IC_{50} calculation.

Both unlabeled β-Catenin and ITAC proteins show IC_{50} in accordance with published values 1,2,3.

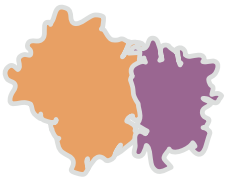
1. Knapp et al, academic press 2001
2. Lepourcelet et al, cancer cell 2004
3. Choi et al, JBC 2005

Medium size complex

TRAIL-R:TRAIL-L

Newton P, et al. (2008) JBS 13(7):674-682

TRAIL ligand (TRAIL-L) and TRAIL receptor (TRAIL-R) interaction is highly implicated in cell apoptotic signaling.



44 KDa 21 KDa

Newton *et al.* designed an HTRF assay using these two medium size recombinant proteins. The assay was validated for inhibitors screening using the unlabeled TRAIL ligand, and the relative Ki was determined. The assay principle is described in Figure 5.

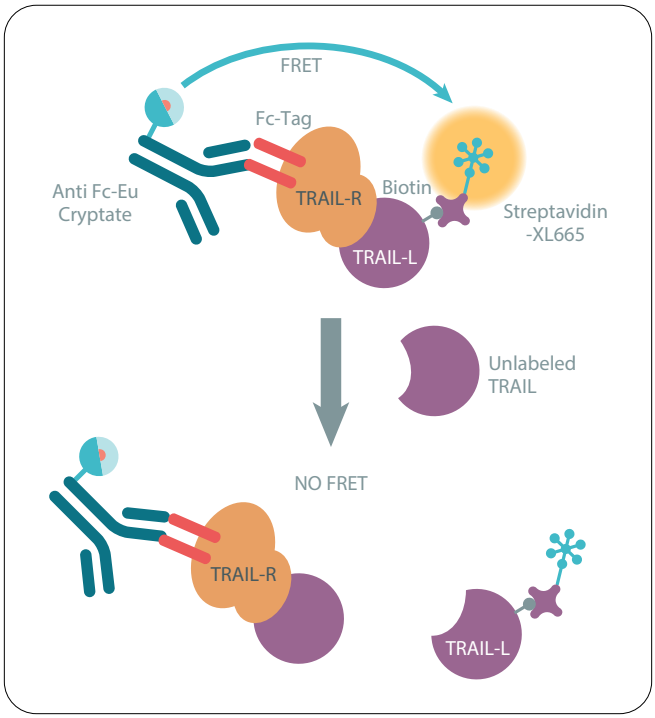


Figure 5: HTRF TRAIL-L:TRAIL-R assay principle.

Fc-tagged TRAIL-R (0.5 nM) and biotinylated TRAIL-L interaction was detected using 0.8 nM of Anti Fc-Eu Cryptate and 20 nM of Streptavidin-XL665. The addition of competitors to the interaction triggers the dissociation of the complex, and thus HTRF signal extinction.

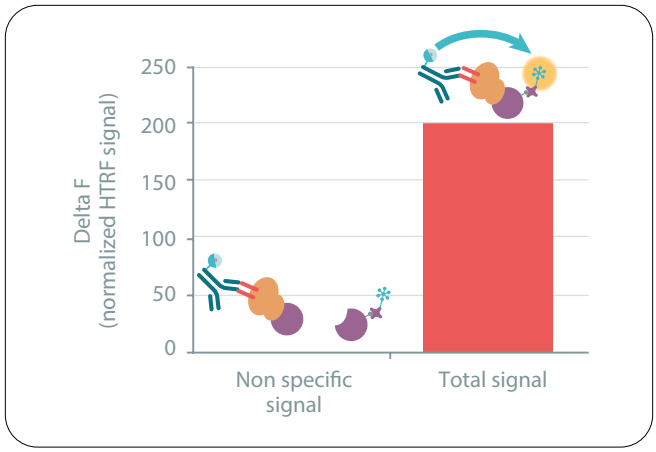


Figure 6: HTRF TRAIL-L:TRAIL-R assay performance using 20 nM of TRAIL-L.

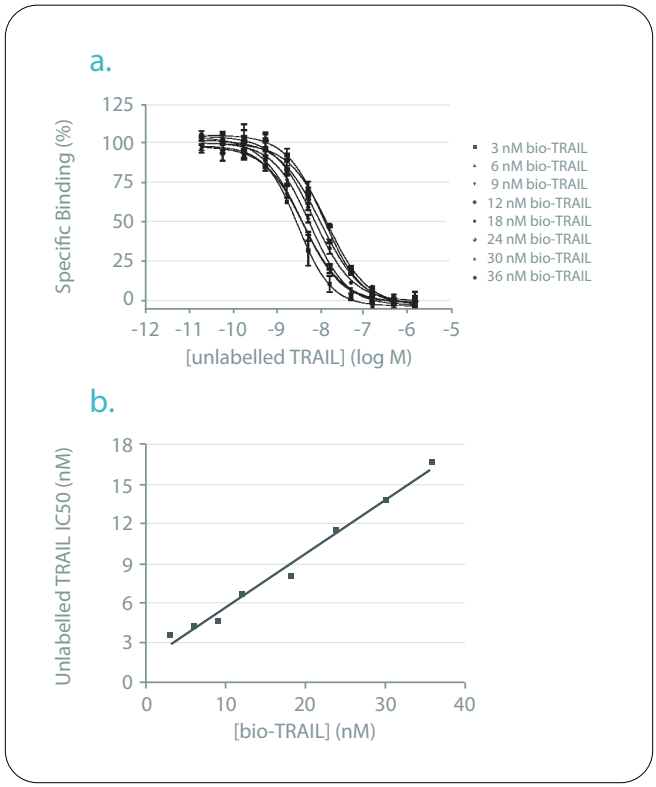


Figure 7: PPI inhibitor characterization.

Panel a shows IC_{50} plots for unlabeled TRAIL with a range of concentrations of TRAIL-biotin (3-36 nM) and a constant concentration of TRAIL-R (0.5 nM). The resulting IC_{50} plots show an expected rightward shift as the concentration of soluble TRAIL-biotin increases. Panel b shows the unlabeled TRAIL IC_{50} values from panel a plotted against concentrations of biotinylated TRAIL. Taken together, and by using the Cheng-Prusoff equation, a Ki value of 2nM was determined.

Small size complex

GRASP55:JAMB (PDZ DOMAIN)

Milhas S, et al. (2016) ACS Chem Biol 11(8): 2140-8

This PPI model was used by Milhas *et al.* to validate the 2P2I chemical library using HTRF. It features a small protein-peptide interface, which is considered as "poorly druggable".

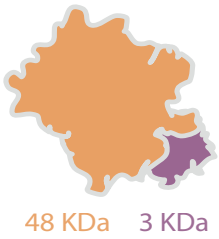


Figure 8 describes the HTRF Grasp55:JamB assay principle. It gives a comfortable assay window of 26 (Figure 9) and was validated by inhibiting Grasp55:JamB interaction with an unlabeled JamB protein. The IC_{50} resulting from this experiment was in the expected nM range (Figure 9).

In this study, the assay enables 2P2I library screening and inhibitors hit identification.

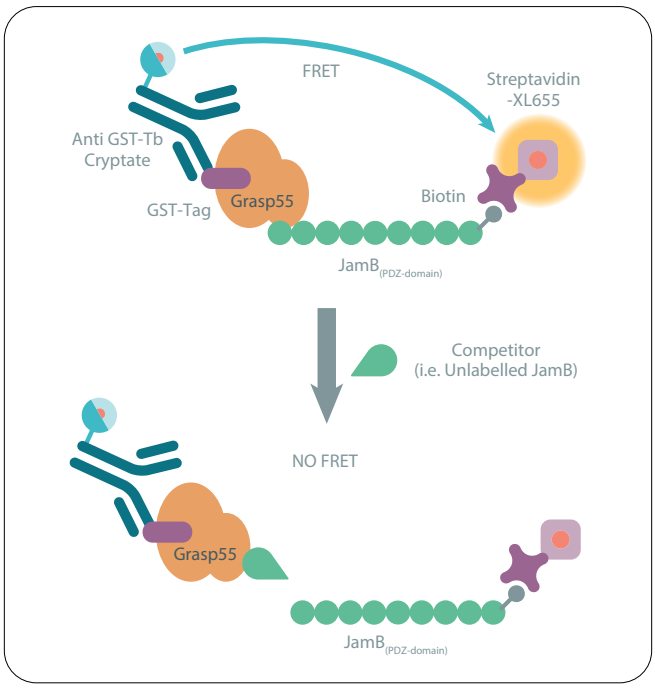


Figure 8: HTRF Grasp55:JamB (PDZ domain) assay principle.

The complex formed by the recombinant GST-Grasp55 and the biotinylated-JamB(PDZ domain) was detected using Anti GST-Tb cryptate antibody and streptavidin-d2. Inhibitors of the interaction (Unlabeled JamB, in this example) lead to HTRF signal extinction. The assay was run in a 384-well plate format, incubated ON.

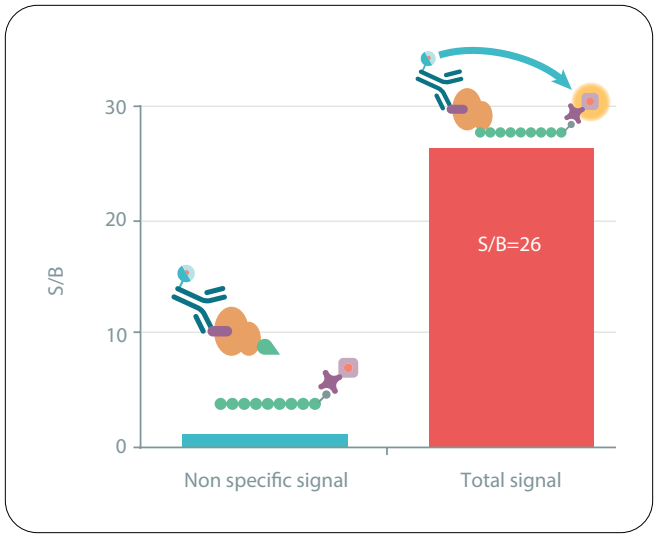


Figure 9: Assay performance.

Measurement of GST-Grasp55 (1.6 nM) and biotin- JamB peptide (5.9 nM) interaction. The S/B of 26 was obtained using 0.8 nM of Anti-GST-Tb Cryptate and 1.25 nM of Streptavidin-d2.

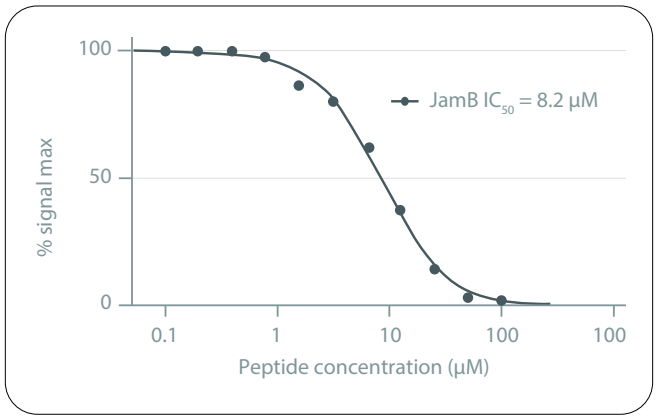


Figure 9: Assay validation for inhibitor screening.

A dose-response curve for the unlabeled JamB was performed. The IC_{50} value obtained is in good agreement with the 1 µM affinity of the complex mentioned in this paper.

Conclusion

Protein-protein interactions are remarkably complex, and depend on their composition, size, affinity, specificity and sub cellular location, among others.

A multitude of HTRF PPI assays have already been illustrated in scientific publications addressing this wide diversity. The work described above illustrates the ability of HTRF technology to specifically study biomolecular interactions for an extensive range of sizes, especially large complexes.

Here three examples, representative of a large panel of complex sizes, were selected and investigated in depth. The data obtained for β-Catenin:TCF4, TRAIL-L:TRAIL-R and Grasp55: $Jamb_{(PDZ\ domain)}$ demonstrate the suitability of HTRF for PPI investigations whatever the size of the complexes studied. Moreover, PPI modulators were characterized, including reference compounds, demonstrating the pharmacological relevance of the assays optimized.

This study demonstrates that HTRF PPI reagents are effective and relevant for robust and accurate pharmacological characterization of PPI complexes, independently of their molecular weight.

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

(800) 762-4000
www.revvity.com