APPLICATION NOTE

HTRF PPI reagents to address low to high affinity complexes.

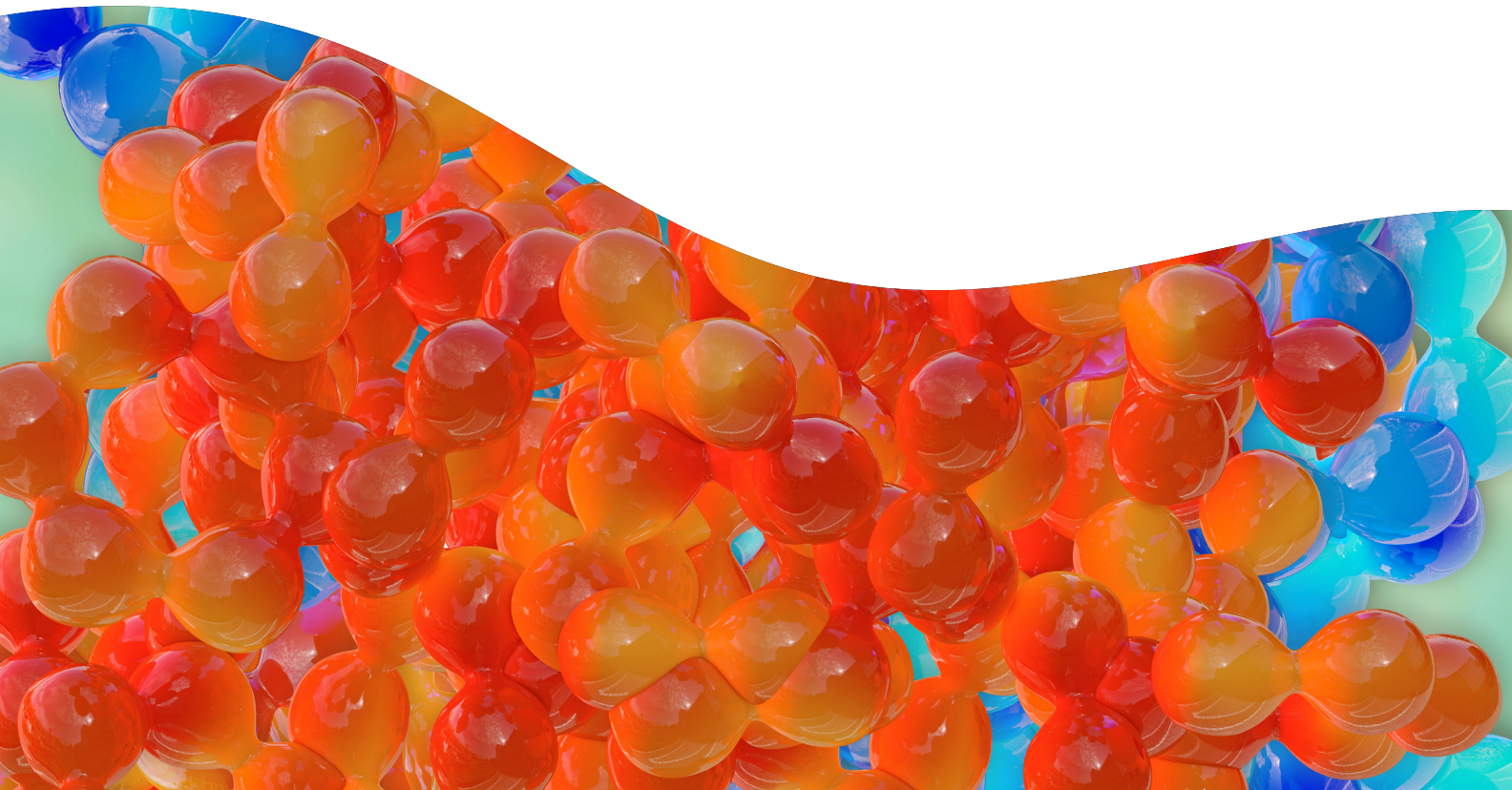
This application note demonstrated that HTRF PPI reagents are successfully used combining high detection performance, accurate modulators screening, and pharmacological characterization

Abstract

Protein-protein interaction (PPI) are involved in most biological processes, providing a multitude of opportunities for therapeutic solutions. To date, PPI inhibitors, and on a smaller scale, PPI stabilization strategies are being thoroughly explored. Thus, they are widely considered to be hot targets for the design of new compounds.

This application note, based on published papers, is intended to reflect HTRF® PPI assays' suitability to address a wide range of PPI affinities, enabling accurate pharmacological studies whatever their range of potency. Among various biochemical HTRF PPI models described in the literature, five case studies displaying different PPI affinities are further exemplified : Ox40R:OX40L, GCK:GKRP, P53:HDM2, NS3:NS5, and BRD4:Histone H4.

Revvity offers HTRF solutions to design add & read PPI assays, including anti-tags, anti-species, streptavidin, affinity tools, and PPI buffers.



HTRF PPI Assays Taken from Published Papers Displaying a Large Panel of Affinities

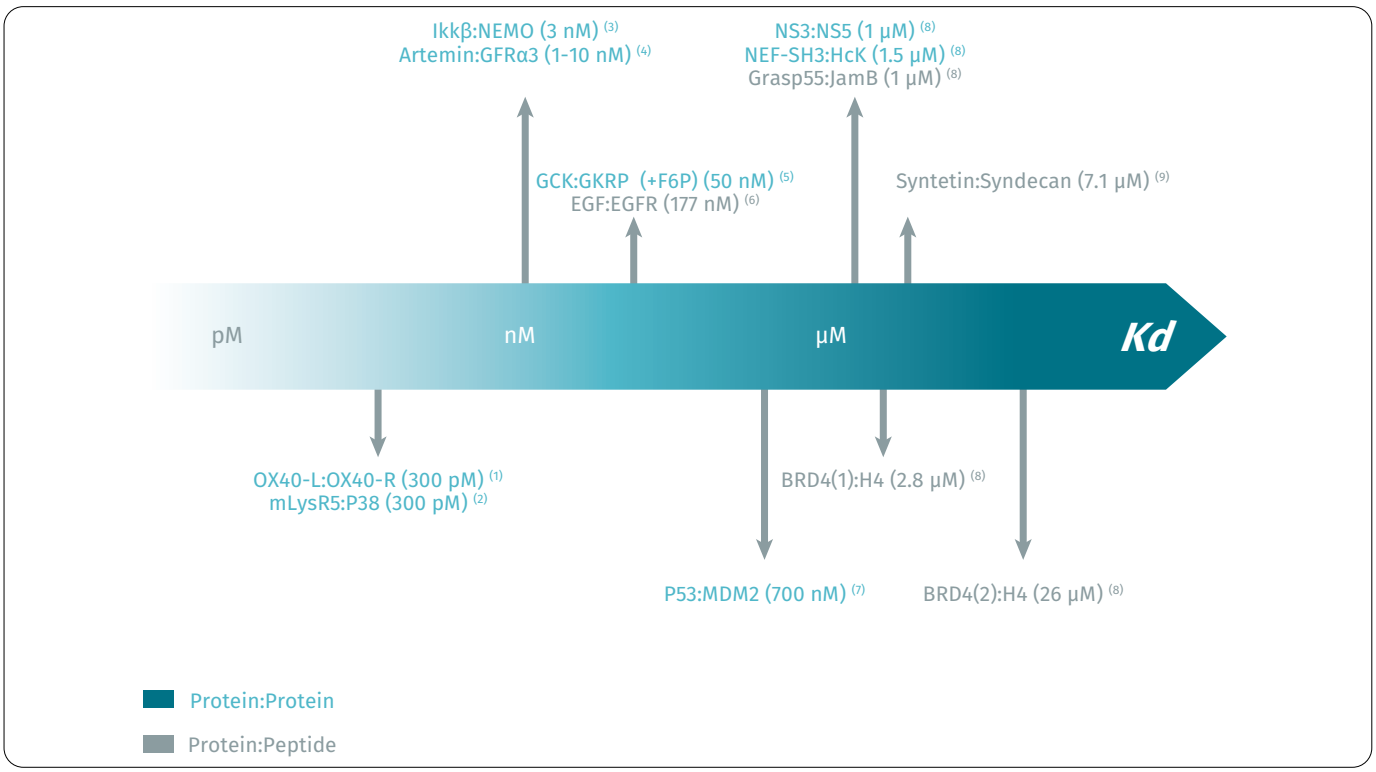


Figure 1: HTRF PPI assays taken from published papers displaying a large panel of affinities from the pM to the µM.

High Affinity PPI (Kd of 300 pM)

OX40-L:OX40-R : costimulatory molecules for immune cell activation

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

OX40 ligand (OX40-L) binds to the OX40 receptor (OX40-R), a member of the Tumor Necrosis Factor (TNF) superfamily. These costimulatory molecules play an important role in mediating T-cell activation upon antigen stimulation. Therefore, it is not surprising that modulators of OX40-R and OX40-L binding have emerged as novel therapeutic targets for the treatment of autoimmunity and Cancer. It was reported in the literature that OX40-L binds to OX40-R with a high affinity, displaying a Kd of 300 pM (*).

The HTRF OX40-L:OX40-R PPI assay described in the original paper was assessed for the pharmacological characterization of modulators. The assay format is described in Figure 2. The OX40-L:OX40-R protein interaction was specifically detected displaying an assay window of 6 (Figure 3). Moreover, competitive dose response curves of the mAb 10541 were performed. Thus, accurate determination of its high potency was determined, showing a Ki value of 560 pM (Figure 4).

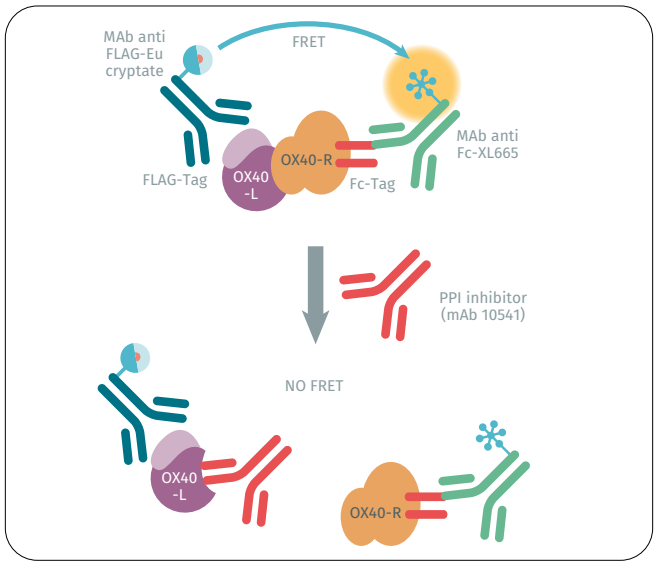


Figure 2. Illustration of the HTRF OX40-L:OX40-R PPI assay. Recombinant proteins OX40L-FLAG and OX40R-Fc were detected using the mAb Anti Flag-Eu cryptate antibody and the Anti Fc-XL665 antibody. A specific HTRF signal is detected when the two proteins are interacting. PPI inhibitor assay was performed using competitors of the interaction, leading to Protein:Protein dissociation and HTRF signal extinction. In this assay, mAb1541, a neutralizing antibody, was characterized as an Inhibitor of the interaction. The assay was run in a 384-well plate format and incubated 4H at RT.

* Kd from published data : El-Shamkhani A, et al. (1997) J Biol Chem 272(8): 5275-5282

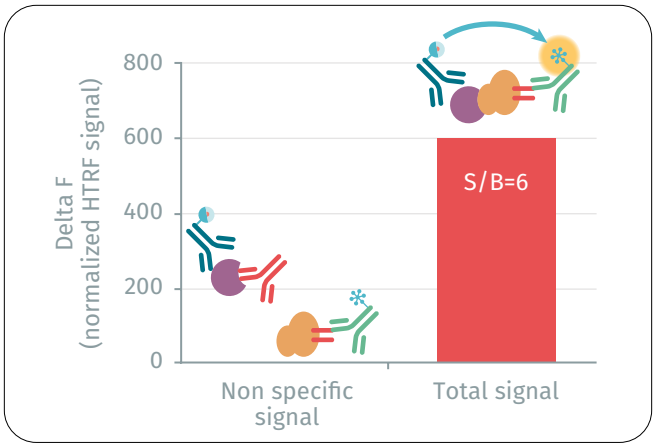


Figure 3. OX40-R PPI assay performance. Measurement of soluble OX40L-FLAG (0.5 nM) binding to OX40R-Fc (3 nM) using the mAb anti Flag-Eu cryptate (0.4 nM) and Anti-Fc-XL665 (10 nM).

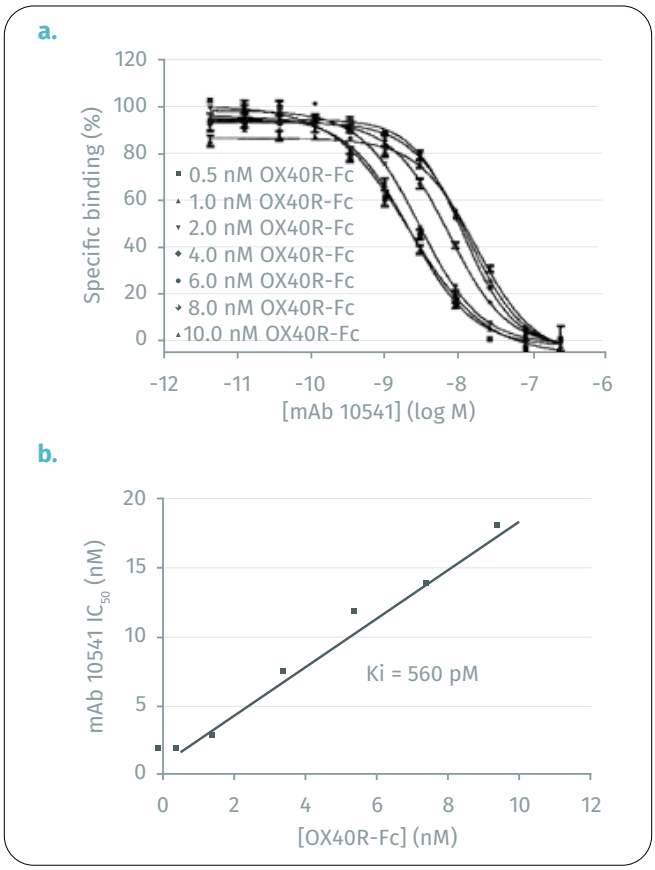


Figure 4. PPI inhibitor characterization. Panel a. shows IC50 plots for mAb 10541 with a range of concentrations of OX40R-Fc (0.5-10 nM) and a constant concentration of OX40L-FLAG (0.5 nM). Resultant IC50 plots show an expected rightward shift as the concentration of soluble OX40R-Fc is increased.
Panel b. shows the mAb 10541 IC50 values from panel a. plotted against the concentration of OX40R-Fc. Taken together, and using the Cheng-Prusoff equation, a Ki value of 560 pM was determined.

Medium Affinity PPI (Kd of 50 nM)

GCK:GKRP : A PPI target for type 2 diabetes drug development

Rees G, et al. (2014) PLoS ONE 9(2):e89335

The GKRP:GCK complex is highly implicated in the pathogenesis of type 2 diabetes since it shows a key role in the over glucose metabolism control. Perturbation of the interaction represents a potential therapeutic target for pharmacological regulation. The affinity of GKRP is enhanced 20-fold in the presence of a saturating dose of fructose 6-phosphate (F6P), lowering the Kd from 1µM to 50 nM (**).

Rees et al. have developed an HTRF GCK:GKRP PPI assay. The assay format is described in Figure 5. It enables the specific detection of the GST-GCK:FLAG-GKRP interaction displaying an assay window of 9.2 (Figure 6). Modulators of the interaction were characterized and the corresponding EC50 in the case of PPI stabilization, or IC50 in the case of inhibitors of the interaction were determined (Figure 7).

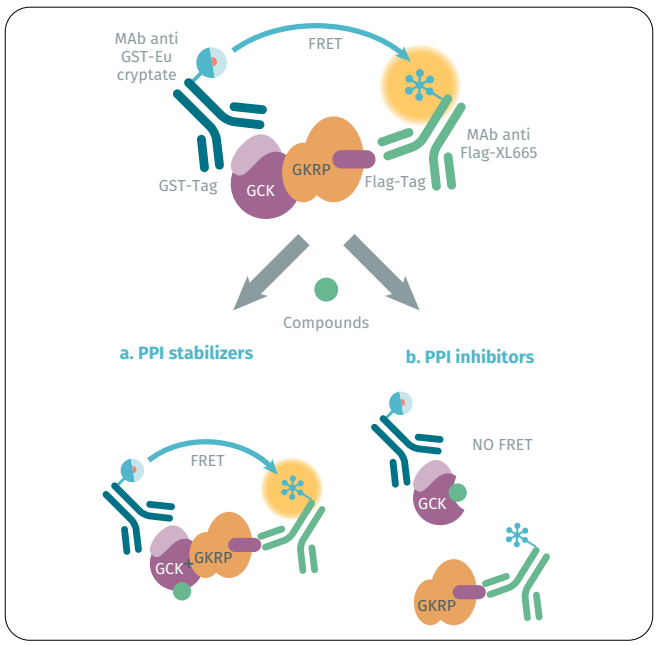


Figure 5. Illustration of the HTRF GCK:GKRP PPI assay. Recombinant GCK-FLAG and FLAG-GKRP protein interaction were detected using the MAb Anti GST-Eu cryptate and the MAb Anti FLAG-XL665 antibodies respectively. A specific HTRF signal is detected when the two proteins are interacting. PPI modulator screening assay was performed by adding compounds leading to the Protein complex stabilization (a) and consequently an increase in the HTRF signal, or on the contrary, protein complex dissociation (b) leading to the HTRF signal extinction.

(**) Kd from published data : Beck T, et al. (2013) Biochemistry 52(36): 6232-6239

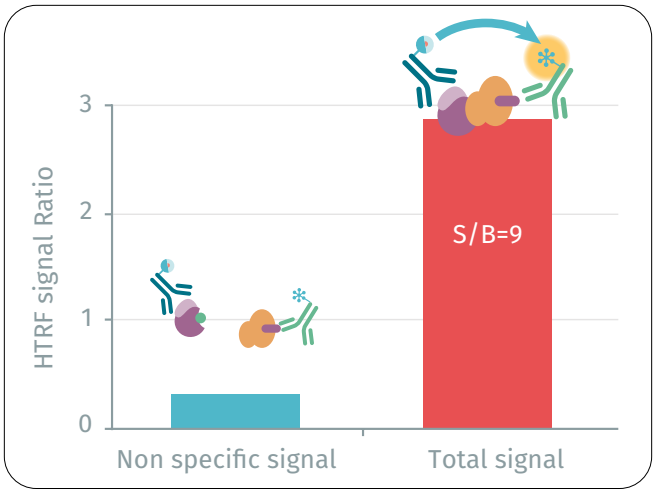


Figure 6. HTRF GCK:GKRP PPI assay performance. Measurement of GST-GCK (5 nM) and Flag-GKRP (5 nM) protein interaction in presence of the S6P (F6P analog), using a mixture of the mAb Anti GST-Eu cryptate (0.9 nM) and mAb Anti FLAG-XL665 (6.7 nM).

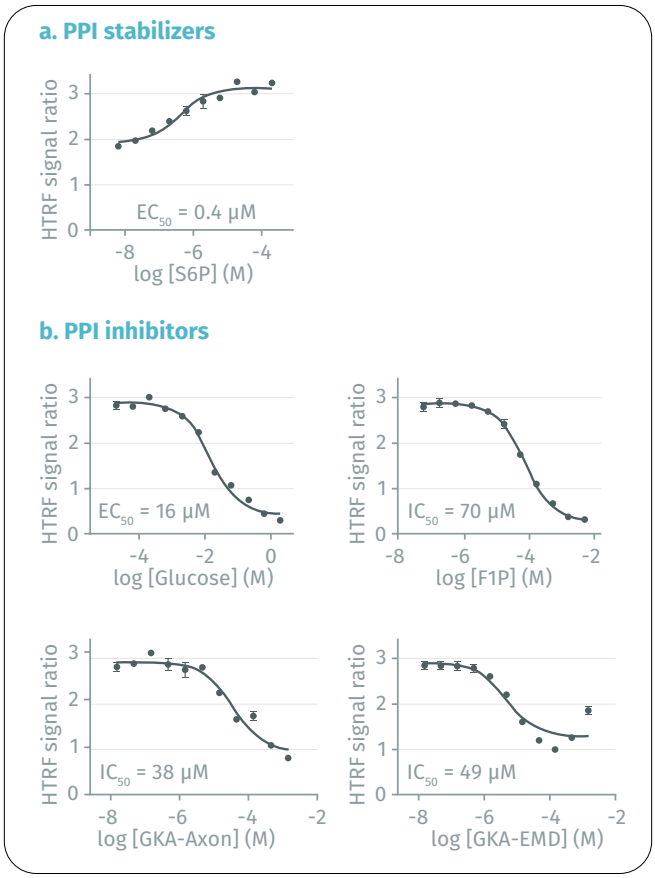


Figure 7. GCK:GKRP PPI modulator characterization. Panel a. shows dose-response curve of the SP6, an open-chain analogue of F6P, which enhances the interaction of GCK:GKRP with a potency (EC50) of 0.4 µM.Panel b. shows GCK:GKRP PPI inhibitor dose-response curves displaying potencies from the µM to the mM range.

Source: Rees G, et al. (2014) PLoS ONE 9(2):e89335

Medium Affinity PPI (Kd of 700nM)

P53:HDM2 : Targeting complex disruption for oncology applications.

Kane,S et al. (2000) Analytical Biochemistry 278 : 29-38

The P53 protein plays a key role in response to DNA damage resulting in cell cycle arrest or apoptosis. It is negatively regulated when it binds to HDM2 protein. The Kd constant of the interaction was published at a value of 700 nM (***).

Kane et al. have developed a P53:HDM2 HTRF assay to characterize HDM2 protein binders. The assay format described below (Figure 8) enables specific detection with an assay window of 8 (Figure 9). It was validated using protein controls such as unlabeled p53 protein (Figure 10). The study was assessed to determine the ability of phosphorylated P53 peptides to interact with HDM2 as well as p73 peptide, derived from the p53 homologue, (IC50 in the µM range).

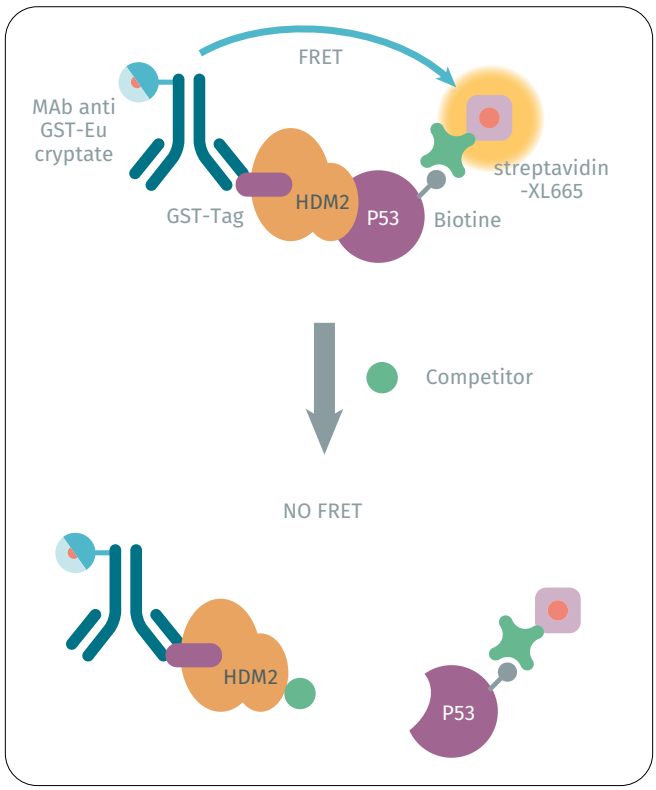


Figure 8. Illustration of the HTRF P53:HDM2 PPI assay. Recombinant GST-HDM2 and biotinylated p53 proteins were detected using the Anti GST-Eu cryptate and the streptavidin-XL665. A specific HTRF signal is detected when the two proteins are interacting. PPI modulator screening assay was performed by adding competitors leading to the protein complex dissociation and HTRF signal extinction.

(***) Kd from published data : Chene P (2004) Molecular Cancer Research 2:20-28

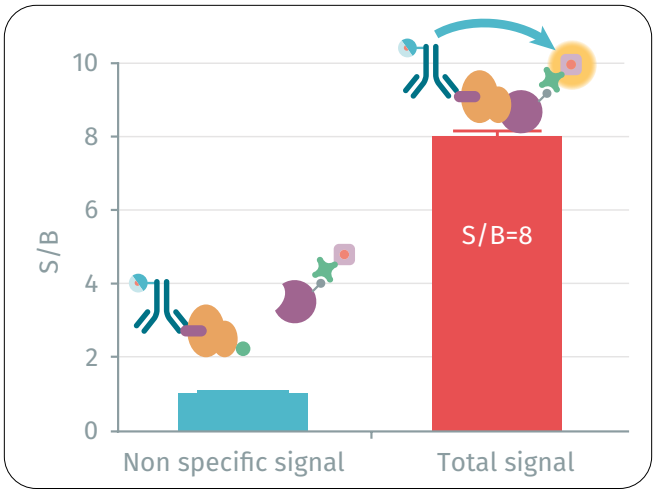


Figure 9. HTRF P:HDM2 PPI assay performance. Measurement of GST-HDM2 (5 nM) and biotin-p53 (10 nM) protein interaction, using a mixture of the Anti GST-Eu cryptate and the Streptavidin-XL665 .

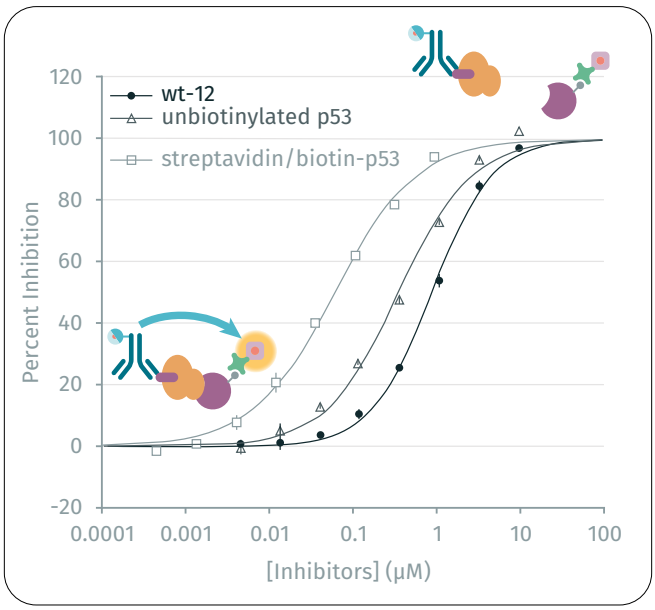


Figure 10. P53:HDM2 PPI modulator characterization. Dose-response curves of the unbiotinylated P53 show an IC50 of 350 nM which is in agreement with the published complex affinity. The IC50 of wt-12 (p53 peptide containing the essential residues for HDM2 recognition), and the preformed complex of p53 peptide and SA-XL665 were also determined (860 nM and 59 nM respectively).

Low Affinity PPI (Kd of 1 µM)

NS3:NS5 for Protein-Protein interaction inhibition (2P2I)-oriented library validation.

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

Dengue virus NS3 and NS5Mtase proteins are the major enzymatic components of the viral replication complex, which promotes efficient viral replication in close association with cellular host factors. This PPI model has been used by Milhas et al. to validate the 2P2I chemical library using HTRF technology. An affinity of 1 µM for the NS3:NS5 interaction has been reported in the literature(£) .

This PPI assay was built using two recombinant proteins: GST-NS3 and NS5Mtase-SNAP. Detection was made possible by using the following reagents: mAb Anti GST-Tb cryptate antibody and the SNAP-Red acceptor respectively (figure 11).

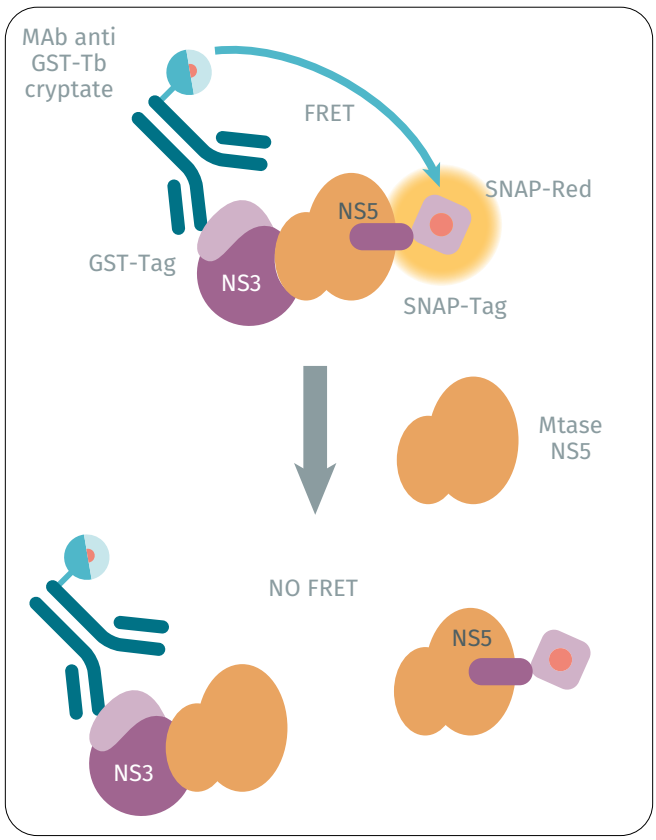


Figure 11. Illustration of the HTRF GST-NS3:SNAP-NS5MTase assay: The HTRF assay uses a GST-NS3, SNAP-NS5, and two detection reagents : the mAb Anti GST-Tb cryptate antibody and the SNAP-Red acceptor. Inhibitors of the interaction (unlabeled NS5Mtase in this example) lead to HTRF signal extinction by competing with the NS5-SNAP for its binding to GST-NS3. The assay was run in a 384 plate format, incubated 4H at RT.
(£) Kd from published data : Zhou G, et al. (2011) J Biol Chem 286(6): 14362-14372

The assay does not use an avidity system to detect this PPI model with a µM affinity range. Nevertheless, because of HTRF's high sensitivity, the interaction was efficiently measured with an assay window of 6 (Figure 12). The assay was validated for inhibitor screening using the unlabeled NS5Mtase as a control molecule. The IC50 of 0.5 µM obtained is in agreement with previously published interaction affinity (Figure 13).

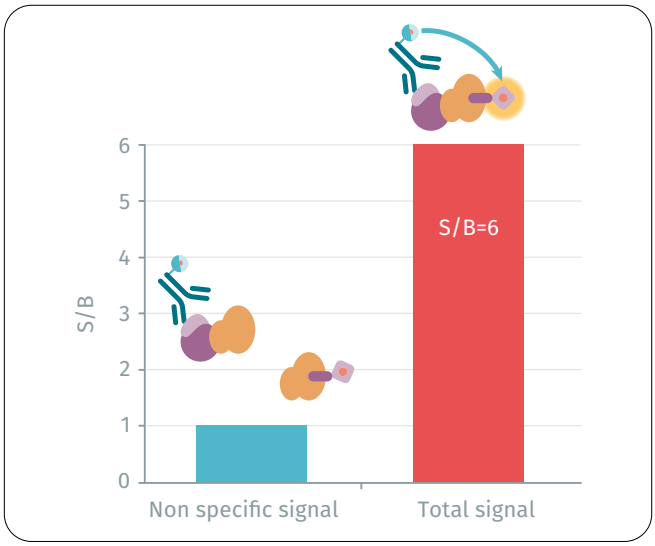


Figure 12. HTRF GST-NS3:SNAP-NS5 PPI assay performance. Measurement of the GST-NS3 at (5 nM) and the NS5Mtase-SNAP (15 nM) interaction, using the mAb Anti-GST Tb cryptate (0.8 nM) and the SNAP-Red acceptor.

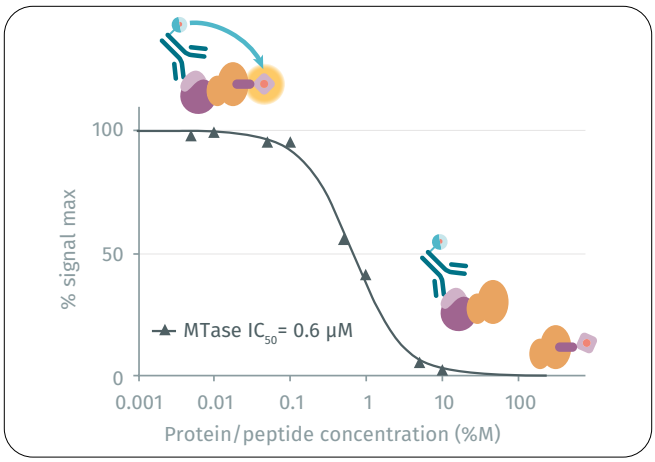


Figure 13. NS3:NS5Mtase inhibitor screening assay validation. A dose-response curve of the unlabeled NS5Mtase was performed. Thec IC50 value of 0.6 µM obtained is in good agreement with the published affinity of 1 µM for NS3:NS5 interaction.
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Low Affinity PPI (Kd of 2 µM)

BRD4:H4 for Protein-Protein interaction inhibition (2P2I)-oriented library validation.

Milhas S, et al. (2016) ACS chem biol 11(8):2140-8

The BRD4 bromodomain, a member of the BET family, binds to acetylated lysine residues of histones which play an important role in gene transcription regulation. The BRD4:H4 PPI complex display an affinity of 2.8 µM (ff). Due to the broad role of BRD4 in cancer, it has been a major focus of small-molecule development inhibitors. The JQ1 compound is the most studied BET family selective inhibitor. It competitively binds to BRD4 inhibiting gene transcription. This PPI model has been used by Milhas et al. to validate the 2P2I chemical library. The assay format is illustrated in Figure 14. It shows a high assay performance displaying an assay window of 18 (Figure 15). In addition, it was validated using two reference molecules: Unlabeled H4 peptide, and the well-known JQ1 inhibitor. According to previously published data, the unlabeled

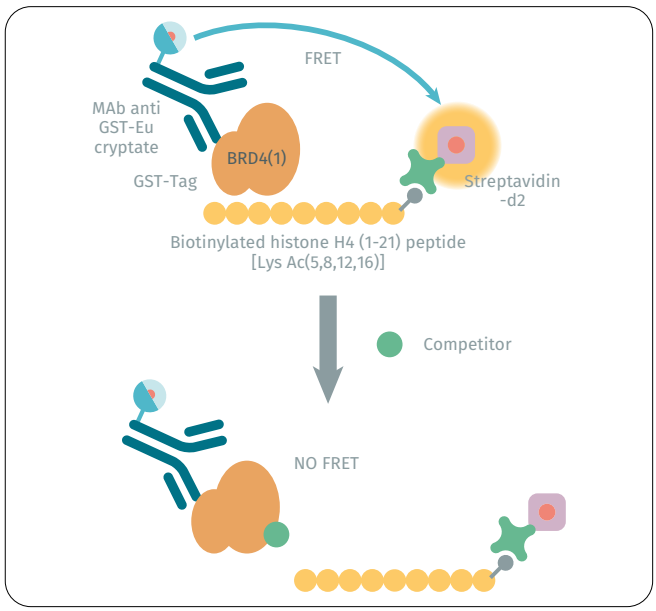

Figure 14. Illustration of BRD4 (1):H4 PPI assay . This PPI assay uses a BRD4(1) GST-Tag bromodomain protein, a Lys ac(5,8,12,16) H4 (1-21) biotinylated peptide, and two detection reagents: the mAb Anti GST-Eu cryptate and the streptavidin-d2. The HTRF signal proportionally increases with the BRD4:H4 interaction. Inhibitors of the interaction lead to HTRF signal extinction by competing with the H4-biotin peptide for it's binding to BRD4. The assay was run in a 384 plate format, incubated 3H at RT.

(ff) Kd from published data : Filippakopoulos P, et al. (2012) Cell 149(1):214-231

H4 peptide display a Ki of 2 µM, and the JQ1 an IC50 of 100 nM (Figure 16). Furthermore, two interesting hits from the 2P2I chemical library were identified (Cpd1 & Cpd2), showing IC50 ranging from 3 to 25 µM for the BET family bromodomains.

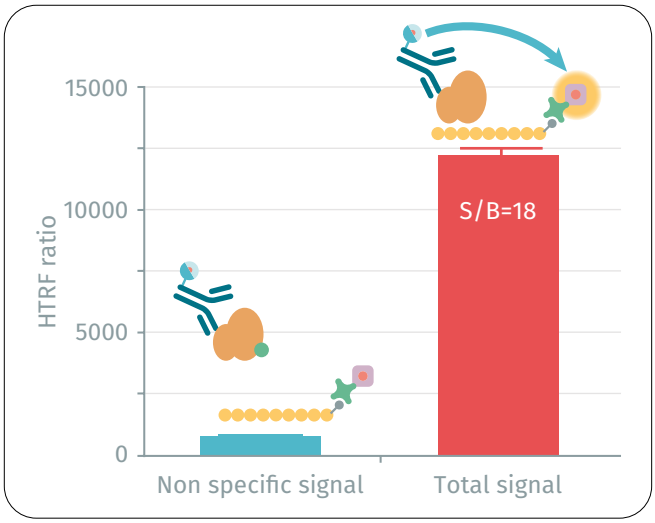

Figure 15. HTRF BRD4(1):H4 PPI assay performance. Measurement of the GST-BRD4(1) (5nM) and the biotinylated-H4(1-21) Kac(5,8,12,16) peptide (100 nM) interaction, using the mAb Anti GST-Eu cryptate (0.5 nM) and the streptavidin-d2 (12.5 nM).

Conclusion

PPIs play key roles in cellular signaling networks, making them more and more attractive for the development of new therapeutic drugs. Protein complexes vary widely depending on their composition and their affinity, with dissociation constant (Kd) ranging from the picomolar to high micromolar and whether the interaction is transient or permanent.

A large number of HTRF PPI assays have been illustrated in scientific publications that cover a wide range of affinities from the pM to the µM. Among them, five assays have been selected, addressing low, medium or high PPI complex affinities, and further exemplified. PPI modulators with different potencies (from the pM to the mM range) were characterized, including reference compounds allowing assay validation.

Thus it has been demonstrated that HTRF PPI reagents are successfully used combining high detection performance, accurate modulators screening, and pharmacological characterization.

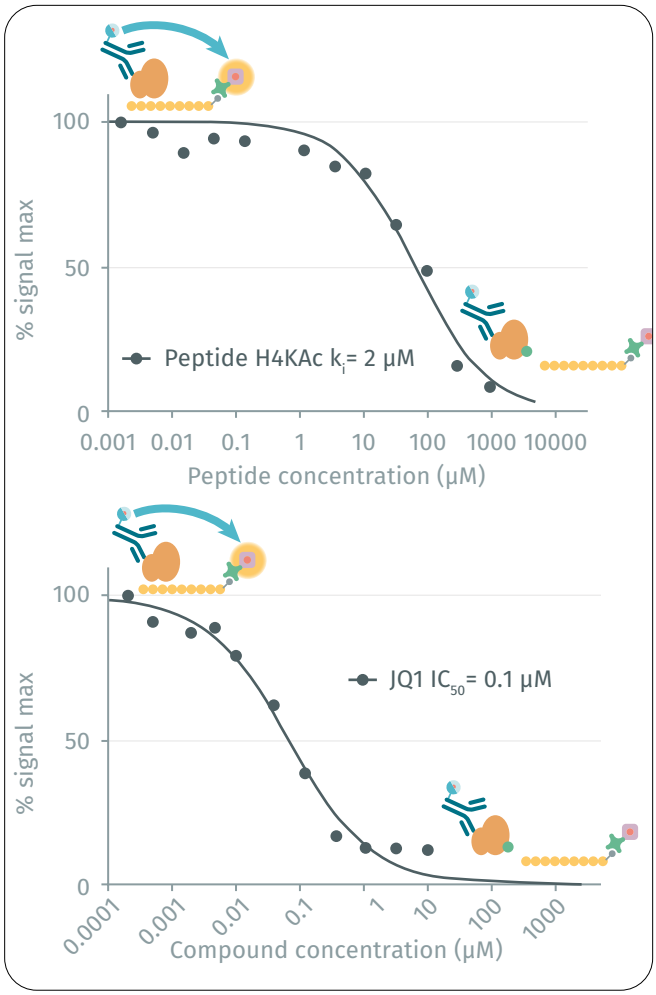

Figure 16. BRD4(1):H4 interaction inhibitors screening assay validation. Panel a. shows a dose-response curve of the unlabeled H4 Kac(5,8,12,16) peptide. The Ki value of 2 µM obtained agrees with the published affinity of 2.8 µM for BRD4:H4 interaction (18). Panel b. shows the well-known JQ1 inhibitor dose response curve displaying an IC50 of 100 nM.

References

PPI model	Ref of the HTRF PPI assays
OX40-L : OX40-R	(1) Newton P, et al.(2008) JBS 13(7):674-682
mLysR5:P38	(2) Khoder-Agha F, et al. (2018) BMC biochemistry19:2
EGF-EGFR	(3) https://www.cisbio.com/
Ikkβ:NEM0	(4) Gotoh, et al.(2010) Anal. Biochem 405:1-27
Artemin :GFRα3	(5) Thornton P, et al. (2013) Neuroscience Letters 545:23-28
GCK:GKRP	(6) Rees G, et al. (2014) PLoS ONE 9(2): e89335
P53:MDM2	(7) Kane S-A, et al. (2000) Anal Biochem 278:29-38
NS3:NS5	(8) Milhas S, et al. (2016) ACS chem biol 11(8):2140-8
NEF-SH3:HcK	(8) Milhas S, et al. (2016) ACS chem biol 11(8):2140-8
GraspS5:JamB	(8) Milhas S, et al. (2016) ACS chem biol 11(8):2140-8
BRD4(1):H4 Kac(5,8,12,16)	(8) Milhas S, et al. (2016) ACS chem biol 11(8):2140-8
Syntetin:syndecan	(9) Baietti MF, et al. (2012) Nat Cell Biol 14(7):677–685.
BRD4(2):H4 Kac(5,8,12,16)	(8) Milhas S, et al. (2016) ACS chem biol 11(8):2140-8

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Anti-human Ig	61HFCKLA	61HFCKLB	61HFCTAF	61HFCTAA	61HFCTAB	61HFCXLF	61HFCXLA	61HFCXLB	61HFCDAF	61HFCDAA	61PARDAB
Protein A	61PRAKLA	61PRAKLB	-	-	-	-	61PRAXLA	61PRAXLB	-	-	-
Streptavidin	610SAKLA	610SAKLB	610SATLF	610SATLA	610SATLB	610SAXLF	610SAXLA	610SAXLB	610SADLF	610SADLA	610SADLB
Streptavidin Xlen	-	-	-	-	-	-	611SAXLA	611SAXLB	-	-	-

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