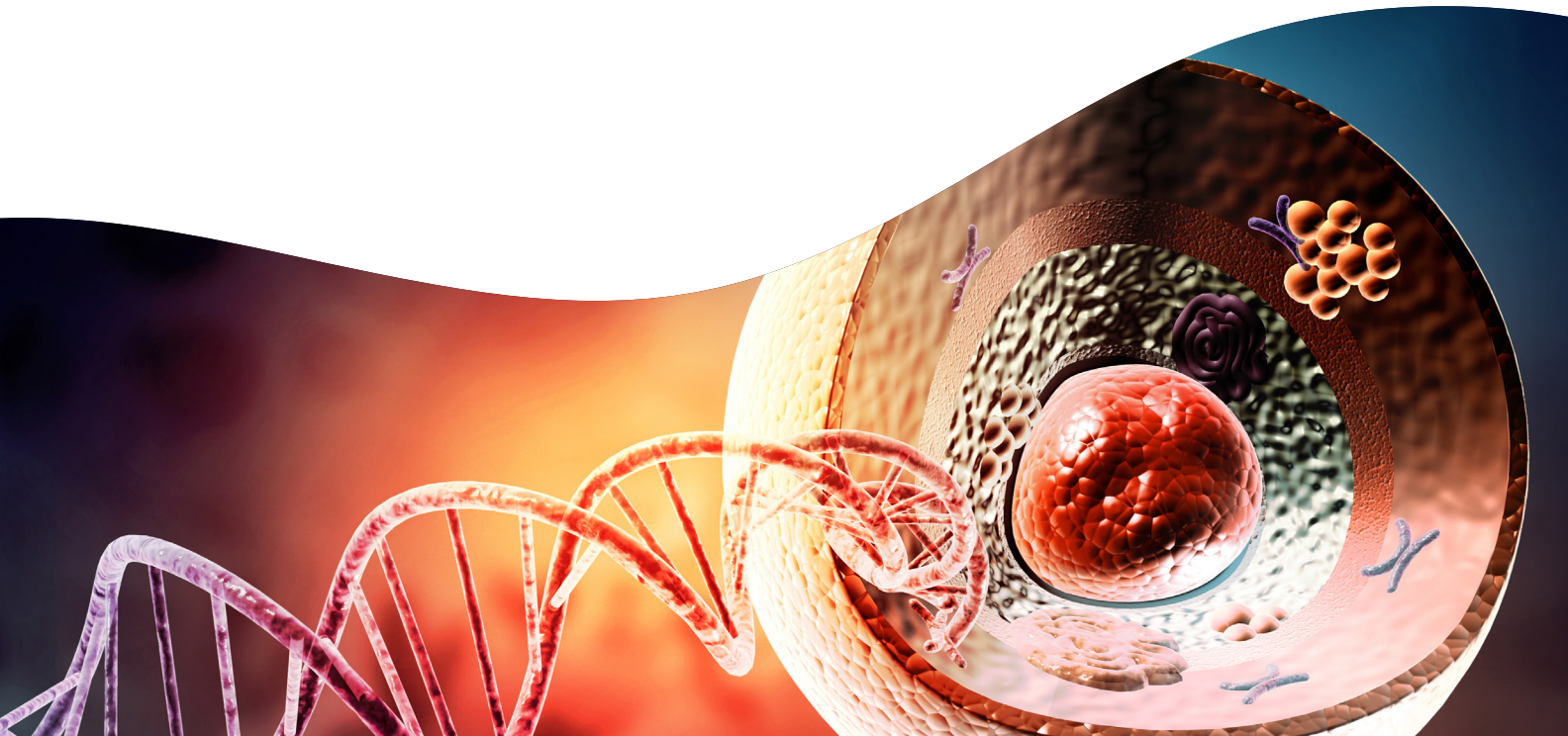
How to easily monitor nuclear receptor/ coactivator recruitment using HTRF PPI reagents.

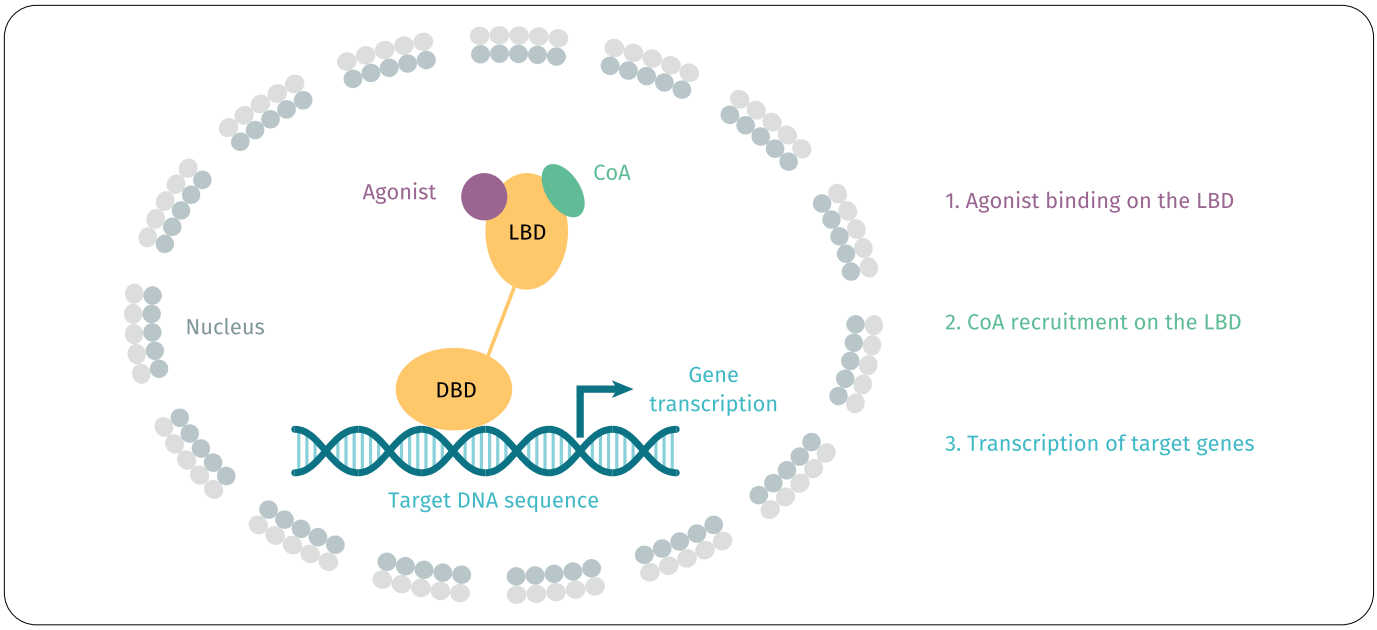
This application note illustrates the use of HTRF® PPI reagents to easily build robust and convenient biochemical NR/ CoA recruitment assays. The interactions between FXR or PPARγ and their main CoA SRC-1 or DRIP205 (also known as Trap220) have been chosen as examples. However, the general concept and the experimental design presented here can be extended to every type of NR and CoA.

Abstract

The interactome, defined as the set of protein-protein interactions (PPIs) that occur in a cell is a dynamic network essential to almost all cellular processes. As the dysregulation of PPIs is associated with multiple diseases, investigating the status of PPIs is essential in drug development. PPIs involve actors located in all cell compartments, such as cytoplasmic and nuclear proteins.

Nuclear receptors (NRs) are a family of ligand-regulated transcription factors that bind to specific DNA sequences via their DNA-binding domain (DBD) and regulate the transcription of target genes. Agonist binding on their ligand-binding domain (LBD) triggers a conformational change and the recruitment of coactivators (CoA), leading to gene transcription[1].





In the past few years, alterations in the activity of several NRs have been associated with liver diseases. More specifically, the Farnesoid X receptor (FXR) and the peroxisome proliferator-activated receptors (PPARs), which are key mediators of bile acid homeostasis and energy metabolism, have been identified as therapeutic targets in Non-Alcoholic Fatty Liver Diseases (NAFLDs). Thus, some agonists of these NRs are currently being evaluated in clinical trials for the treatment of Non-Alcoholic Steatohepatitis (NASH)[2].

This application note illustrates the use of HTRF PPI reagents to easily build robust and convenient biochemical NR/ CoA recruitment assays. The interactions between FXR or PPARγ and their main CoA SRC-1 or DRIP205 (also known as Trap220) have been chosen as examples. However, the general concept and the experimental design presented here can be extended to every type of NR and CoA.

[1] Sever, R. & Glass, C. K. Signaling by Nuclear Receptors. Cold Spring Harb. Perspect. Biol. 5, (2013).

[2] Tanaka, N., Aoyama, T., Kimura, S. & Gonzalez, F. J. Targeting nuclear receptors for the treatment of fatty liver disease. Pharmacol. Ther. 179, 142–157 (2017).

Material

HTRF PPI Reagents (Revvity)

- PPI - Europium detection buffer (#61db9rdf) or PPI - Terbium detection buffer (#61db10rdf)
- MAb Anti Gst-Eu cryptate (#61gstkla/B) or Mab Anti Gst-Tb cryptate (# 61gsttla/B)
- Streptavidin-XL665 (#610saxlf\A/B) or Streptavidin-d2 (#610sadlf/A/B)

GST-Tagged Lbds

- GST-FXR-LBD (222-472), human, expressed In E. coli (Sigma, #Srp2128)
- GST-PPARγ-LBD (204-477), human, expressed In E. Coli (Protein One, #P4036)

Biotinylated Coa Peptides

- SRC-1 peptide (676 - 700), biotinylated at the N-terminal extremity (Anaspec, #As-62152)
- DRIP205 peptide (623-641), biotinylated at the N-Terminal extremity (Polypeptide, custom Peptide)

Reference Ligands

For FXR:

- Cdca (chenodeoxycholic acid), endogenous agonist, (Sigma, #C9377)
- Int-747 (obeticholic acid, Oca or 6-Ecdca), semi-synthetic agonist (Medchem Express, #HY-12222)
- GW 4064, synthetic agonist (Tocris, #2473)

For PPARγ:

- Rosiglitazone, synthetic agonist (Tocris, #5325)
- Gw 1929, synthetic agonist (Tocris, #1664)
- Gw 9662, synthetic antagonist (Tocris, #1508)

DTT Solution

- Stock solution of Dtt (1M in H2O), stored at -20°C

Microplate

- 384- or 96-well low volume white plate, 20 µL final (e.g. Revvity #66pl384 or #66pl96)

HTRF compatible reader

- Set up for Eu- And Tb-cryptate excitation And for 620 and 665 nm emissions.
- The data presented in this document were obtained using the Pherastar FS reader (Bmg Labtech).

Experimental design

General approach

The first step of the approach consists in selecting the best format of HTRF PPI detection reagents, using a saturating dose of reference agonist. We recommend testing each anti-GST-donor antibody (Eu- or Tb-cryptate) in combination with each streptavidin-acceptor (d2 or XL665). Once the optimal format is chosen, it is possible to perform dose response experiments with your compounds in order to determine their potency in the recruitment of a specific CoA on your NR of interest.

Recommendations and guidelines

Because some recombinant LBDs require special handling precautions, it is generally recommended to thaw on ice the GST-LBD just prior to use and to perform all dilutions on ice.

After thawing, all reagents are diluted in the appropriate PPI detection buffer (Eu or Tb), freshly supplemented with 2 mM DTT.

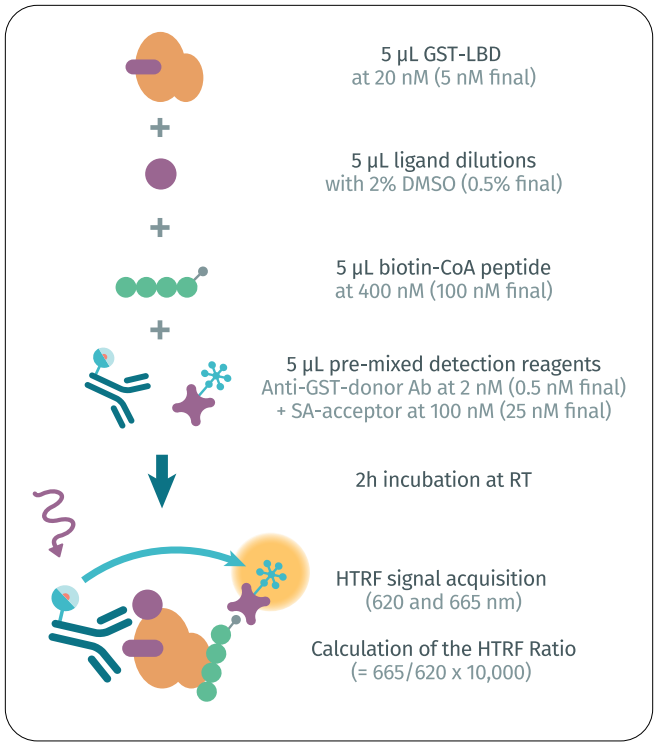
Ligand dilutions must be done with a fixed concentration of 2% DMSO to reduce sticking and compound precipitation.

Assay Protocol

Reagents are dispensed into the microplate as described below. No pre-incubation step is required.

The optimized concentrations presented here can be fine-tuned in your own assay.

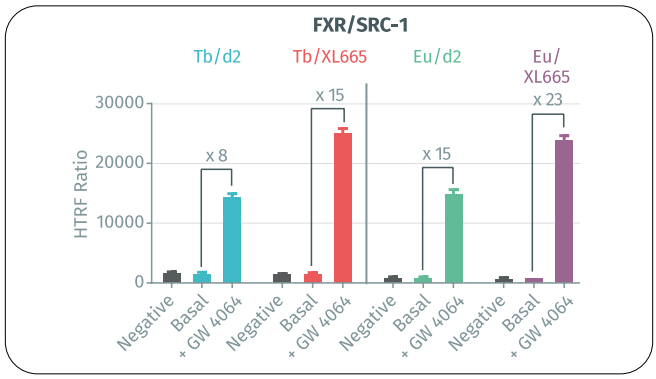
- Negative control: no GST-LBD
- Basal condition: no agonist
- Positive control: saturating dose of a reference agonist



Selection of the Best HTRF Format

Case Study on FXR/SRC-1

Each anti-GST-donor antibody (Eu- or Tb-cryptate) was tested in combination with each streptavidin-acceptor (d2 or XL665) on the FXR/SRC-1 system, using 1 µM final of the reference agonist GW 4064. The recruitment signal, corresponding to the fold between the positive condition and the basal condition, was calculated for each detection format.



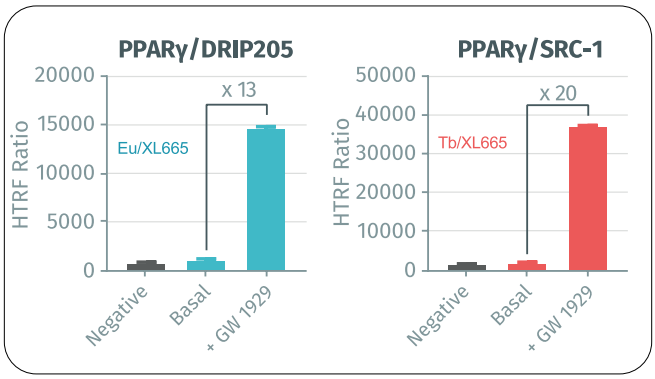
Firstly, the above results show that the signal obtained on the basal condition is similar to that of the negative control, meaning that there is no recruitment of SRC-1 on FXR in absence of agonist.

Whatever the donor antibody, the use of streptavidin-XL665 enables higher recruitment signals to be reached compared to that obtained with streptavidin-d2. The detection of the interaction of SRC-1 with FXR in presence of the agonist is even further improved when the anti-GST-donor antibody is labeled with Eu-cryptate.

The combination of the anti-GST-Eu-cryptate antibody with SA-XL665 (purple histogram), which enables a very comfortable fold activation of more than 20, was therefore selected for this recruitment assay.

Optimal Detection Format for PPARγ/DRIP205 and PPARγ/SRC-1

Following the same approach, the best HTRF detection formats were determined for the recruitment of DRIP205 and SRC-1 on the nuclear receptor PPARγ, using 1 µM final of the reference agonist GW 1929.



As in the previous case, there is no recruitment of the CoA DRIP205 and SRC-1 on PPARγ in absence of agonist.

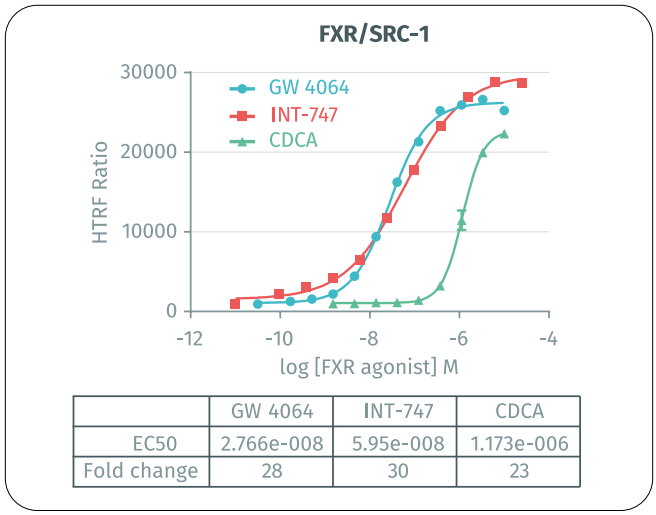
For the PPARγ/DRIP205 system, the optimal fold activation is reached using the anti-GST-Eu-cryptate antibody with SA-XL665, enabling a recruitment signal of 13. Regarding the PPARγ/SRC-1 assay, the best detection format corresponds to the combination of the anti-GST-Tb-cryptate antibody with SA-XL665 which gives a 20-fold increase in HTRF signal.

Pharmacological Validation

Dose response experiments using reference ligands (agonists or antagonists) were performed on the two best-studied recruitment assays, namely FXR/SRC-1 and PPARγ/DRIP205.

Experiments on FXR/SRC-1

Serial dilutions of the reference agonists CDCA, INT-747 and GW 4064 were assayed on the interaction of SRC-1 with FXR.



	GW 4064	INT-747	CDCA
EC50	2.766e-008	5.95e-008	1.173e-006
Fold change	28	30	23

As shown above, the three ligands trigger the recruitment of SRC-1 on FXR with robust fold activations of between 23 and 30, but with varying degrees of potency.

As expected, the synthetic agonist GW 4064 is the most potent, with an EC50 value of ~28 nM. The semi-synthetic agonist INT-747 is almost as potent, with an EC50 value of ~60 nM. Finally, higher doses of the endogenous agonist CDCA are required to induce the recruitment of SRC-1 (EC50 value of ~1.2 µM).

The EC50 values determined using the HTRF assay are well correlated to those obtained in the literature [3, 4, 5].

[3] Maloney, P. R. *et al.* Identification of a chemical tool for the orphan nuclear receptor FXR. J. Med. Chem. 43, 2971-2974 (2000)

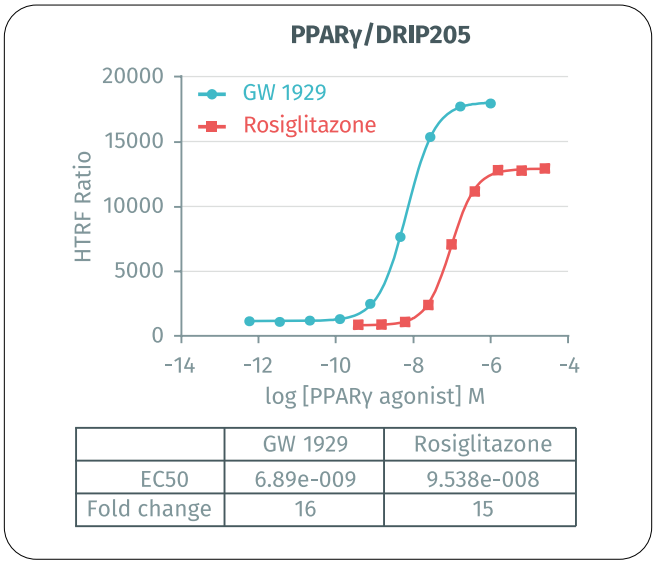
[4] Pellicciari, R. *et al.* 6alpha-ethyl-chenodeoxycholic acid (6-ECDCA), a potent and selective FXR agonist endowed with anticholestatic activity. J. Med. Chem. 45, 3569-3572 (2002)

[5] Lew, J.-L. *et al.* The farnesoid X receptor controls gene expression in a ligand- and promoter-selective fashion. J. Biol. Chem. 279, 8856-8861 (2004)

Experiments on PPARγ/DRIP205

- Dose responses with agonists

Increasing concentrations of the reference agonists Rosiglitazone and GW 1929 were tested for the recruitment of DRIP205 on PPARγ.



	GW 1929	Rosiglitazone
EC50	6.89e-009	9.538e-008
Fold change	16	15

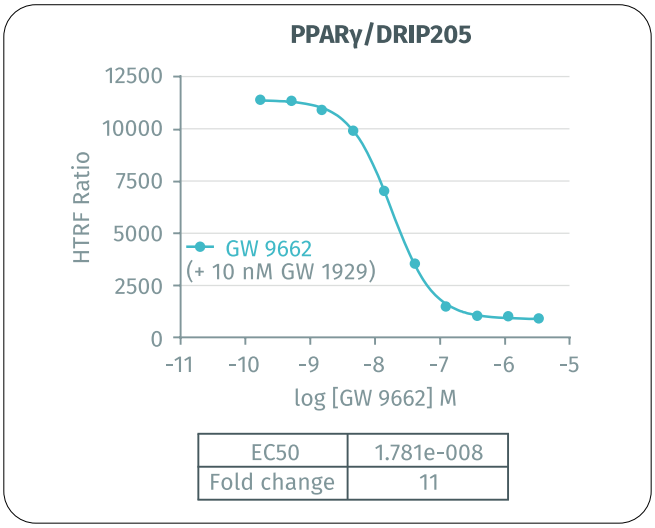
Both ligands are able to recruit the CoA DRIP205 on PPARγ with similar and comfortable signals (~15).

As expected, the synthetic agonist Rosiglitazone is the least potent with an EC50 value of ~95 nM. Regarding the synthetic agonist GW 1929, it only requires doses that are more than 10 times lower to induce the same effect (EC50 value ~7 nM).

The potency of both compounds determined by HTRF is once again consistent with data from the literature [6].

- Dose response with an antagonist

An experiment in antagonist mode was then performed using GW 1929 at a fixed concentration of 10 nM (~EC50) and various doses of the reference antagonist GW 9662.



EC50	1.781e-008
Fold change	11

The results show a complete decrease in the HTRF signal, demonstrating the ability of the antagonist GW 9662 to fully inhibit the recruitment of DRIP205 on PPARγ.

The IC50 value is similar to that mentioned in the literature [7].

[6] Hughes, T. S. *et al.* An alternate binding site for PPARγ ligands. Nat. Commun. 5, 3571 (2014)

[7] Leesnitzer, L. M. *et al.* Functional consequences of cysteine modification in the ligand binding sites of peroxisome proliferator activated receptors by GW9662. Biochemistry (Mosc.) 41, 6640- 6650 (2002)

Conclusion

This application note provides recommendations and guidelines to help you to design and set up your own biochemical NR/CoA recruitment assay. The general approach and the protocol can be easily adapted to your NR and coA of interest.

The illustrations presented here on the interaction of FXR and PPARγ with their coA peptides demonstrate the high assay robustness and quality of results, with comfortable pharmacological signals and consistent EC50/IC50 values. The convenient protocol does not require any pre-incubation step, and results can be obtained after only 2 hours of incubation.

In conclusion, HTRF PPI detection reagents represent precious tools for easily and rapidly building biochemical NR/CoA recruitment assays, and therefore facilitate the identification of potent NR ligands.

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