APPLICATION NOTE

A new specific kit for characterizing compounds Inducing β-arrestin2 recruitment at GPCRs.

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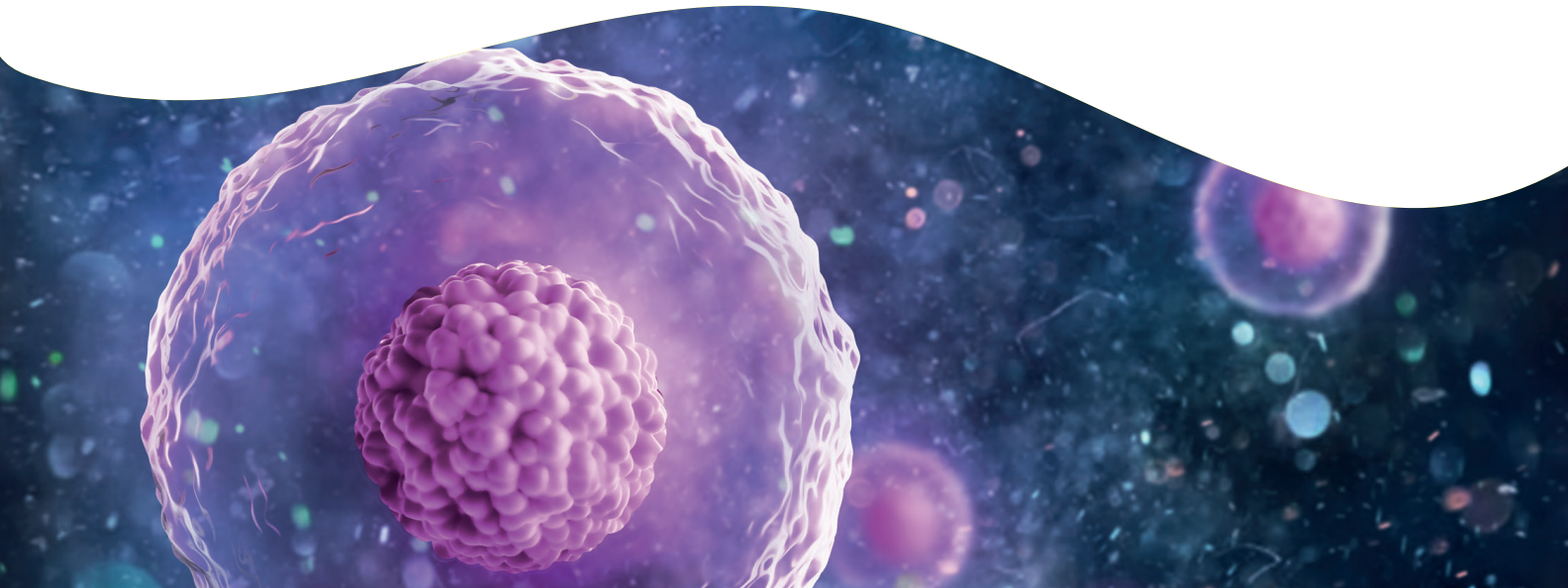
Introduction

β-arrestin1 and β-arrestin2 are intracellular proteins playing central roles in the GPCR signaling pathways by regulating agonist-mediated GPCR signaling. Although many questions remain open regarding GPCR and β-arrestin interactions, it is known that β-arrestins function as multivalent adapter proteins that can switch the GPCR from a G-protein signaling mode, which transmits short-lived signals from the plasma membrane, to a β-arrestin signaling mode that transmits a distinct set of signals. These are initiated as the receptor is internalized and transits to the intracellular compartment.

β-arrestins target many receptors for internalization by acting as endocytic adapters, recruiting GPCRs to the adapter protein 2 complex 2 (AP-2) in clathrin-coated pits. Internalized arrestin-receptor complexes traffic to intracellular endosomes for recycling or degradation. Furthermore, different modes of arrestin-mediated internalization occur depending on the receptors, cell types, and expression level of β-arrestins and their partners.

Expression of β-arrestin1, β-arrestin2, and AP2 may vary depending on the cells, and result in variations in GPCR internalization. In this context, the HTRF total β-arrestin1 (Revvity #64BAR1TPEB/C/J), β-arrestin2 (Revvity #64BAR2TPEB/C/J), and AP2 (Revvity #64AP2TPEB/C/J) kits monitor the specific expression of each protein in cells.



The β-arr2 recruitment kit (Revvity #62BDBAR2PEB/C) is based on a TR-FRET immunoassay format. The kit enables the detection of the cellular interaction between endogenous β-arrestin2 and AP2 proteins for all types of GPCR/β-arrestin interactions, and mediated by all classes of GPCR ligands.

This application note provides a convincing demonstration of the specificity of the β-arr2 recruitment kit in detecting the interaction of AP2 with β-arrestin2 vs β-arrestin1 and highlights its potential applications for a variety of compounds, GPCRs, and cellular contexts.

Assay workflow

Principle of the β-arr2 recruitment assays:

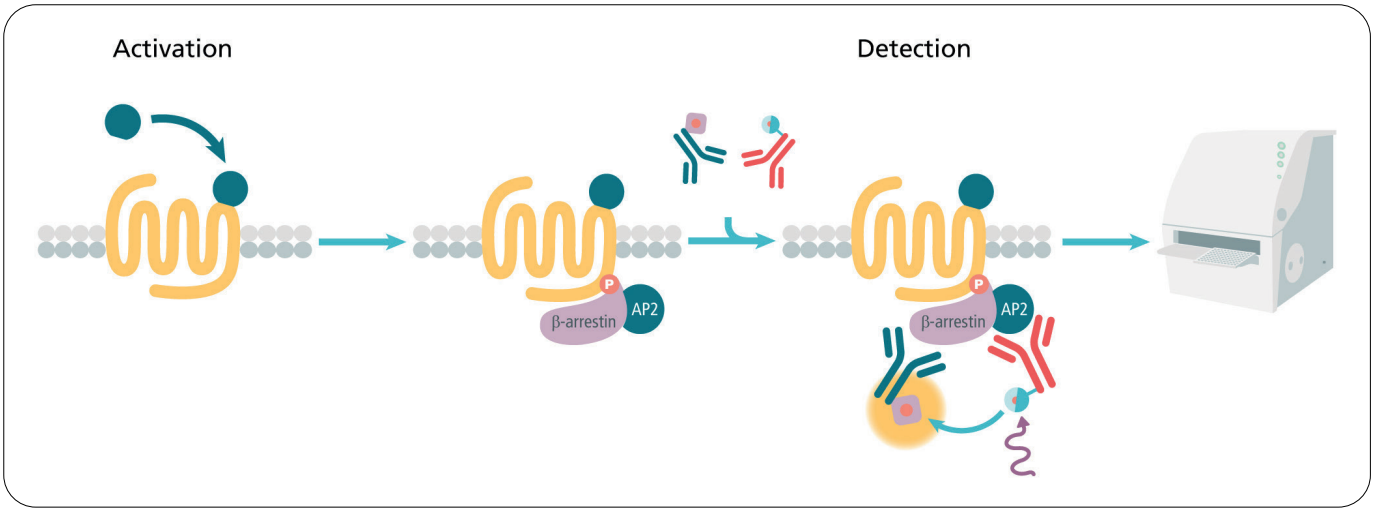


Figure 1. Principle of the β-arr2 recruitment assays.

The β-arr2 recruitment assays can be carried out on adherent cells with a two-option protocol:

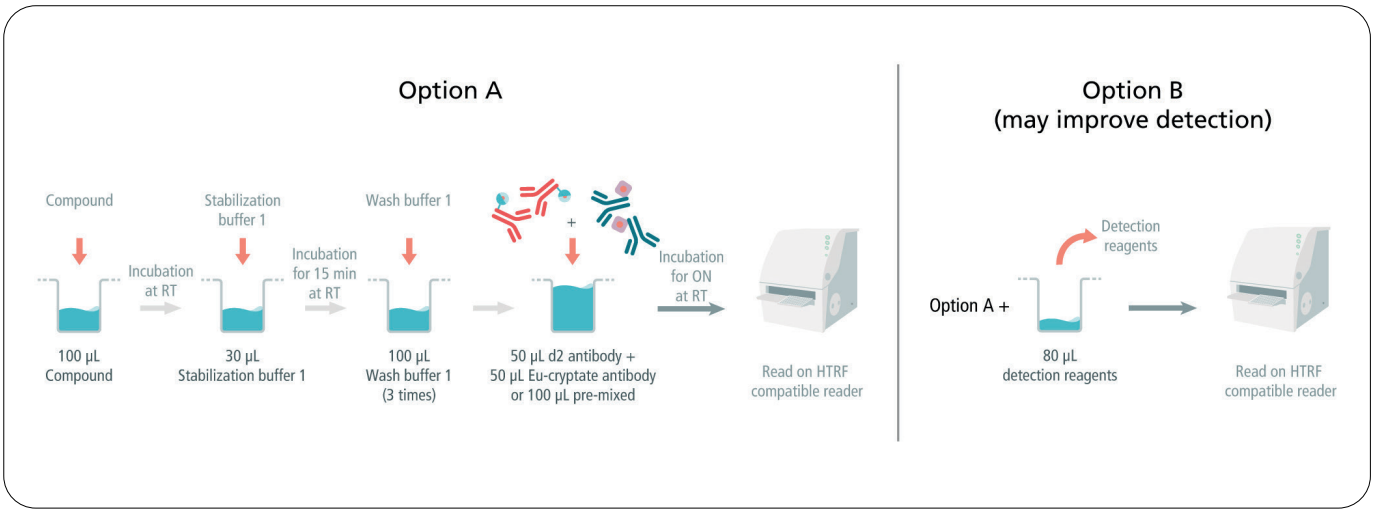


Figure 2. A two-option protocol for the β-arr2 recruitment assays.

Detection of β-arr2 recruitment with HTRF reagents can be performed in a single plate used for culturing, stimulation, stabilization, and detection. After stimulation, a stabilization step followed by three washes is required to maintain robust detection after ON incubation. This specific protocol enables detection with non-lysed cells and endogenous proteins, while maintaining robust quality at room temperature.

The antibodies anti-β-arrestin2-d2 (HTRF acceptor) and anti-AP2-Eu cryptate (HTRF donor) are added for the detection in Detection buffer 14, then incubated ON (at least 20 h) at room temperature.

Option B enables signal improvement by removing 80µL of detection reagents after ON incubation at room temperature.

A highly specific interaction of β-arrestin2 with AP2

Demonstration with double KO β-arrestin1/2 HEK 293 cells*

In order to demonstrate the specificity of the β-arr2 recruitment kit for the interaction between β-arrestin2 and AP2 (and not any interaction between β-arrestin1 and AP2), double KO β-arrestin1/2 HEK293 cells were tested.

The double KO β-arrestin1/2 HEK293 cells were plated under 100 k cells/well in a white 96-w culture treated microplate (Reevity #6005680/8/9), and incubated for 24 h at 37 °C, 5 % CO_2. Then transfections of 40 ng/well of Flag-Twin-Strep- tag®–β-arrestin1 or of Flag-Twin-Strep-tag®-β-arrestin2 with 200 ng SNAP-Beta 2 adrenergic receptor (Reevity #PSNAPBD2) were performed, with further incubation for 24 h at 37 °C, 5 % CO_2.

Finally, a 30 mn stimulation with 1 µM Isoproterenol (saturating concentration of agonist) was performed at room temperature. The results obtained with the β-arr2 recruitment kit after ON incubation at room temperature are shown in Figure 3.

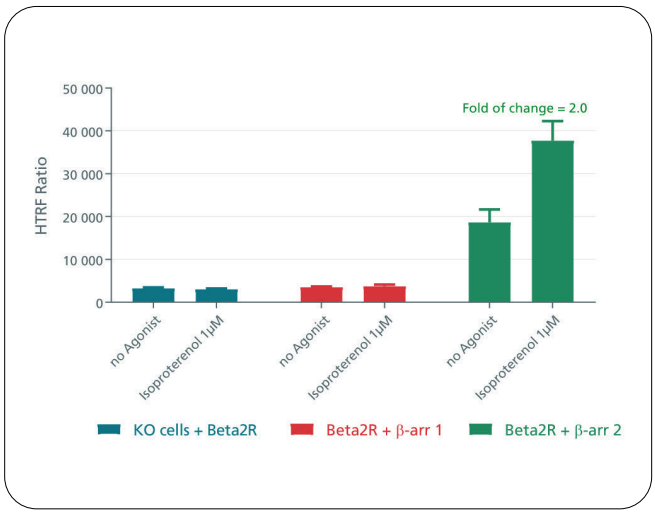


Figure 3. β-arr2 recruitment assays in double KO β-arrestin1/2 HEK293 cells.

Results show a basal and agonist-stimulated interaction with endogenous AP2 only when β-arrestin2 is overexpressed in the double KO β-arrestin1/2 HEK293 cells. This demonstrates the specificity of the β-arr2 recruitment kit for the β-arrestin2/AP2 interaction.

In parallel with these assays, validations were performed to confirm the overexpression of each partner.

First, Tag-lite® assays were done to validate the overexpression of SNAP-Beta 2 adrenergic receptor in the transfected double KO β-arrestin1/2 HEK293 cells.

The results of these validation assays are shown in Figure 4. They demonstrate a significant and similar overexpression of the Beta2 adrenergic receptors in all conditions.

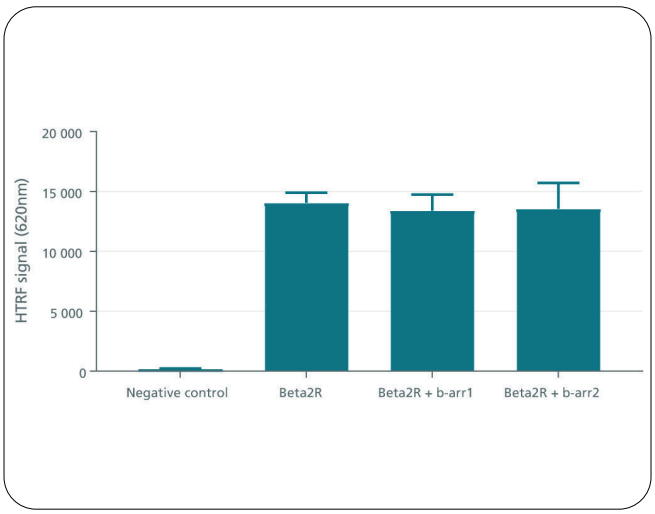


Figure 4. Tag-lite® SNAP-Beta2 adrenergic receptor expression assays.

Next, additional innovative HTRF assays were used to validate the overexpression of the two β-arrestins and compare them with each other. The detection used an antibody anti-Flag coupled to XL665 as HTRF acceptor (Reevity# 61FG2XLF/A/B) and an antibody anti-Twin-Strep-tag® coupled to Terbium cryptate as HTRF donor.

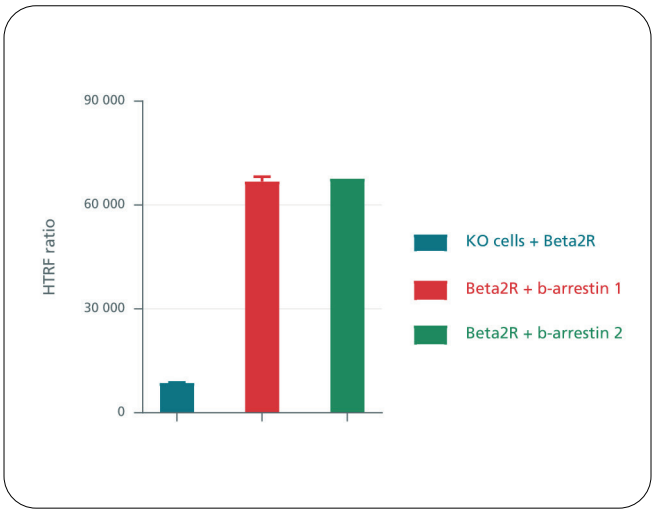


Figure 5. Monitoring of β-arrestin1 and β-arrestin2 expression in double KO β-arrestin1/2 HEK293 cells.

The results demonstrate a significant and similar overexpression of both β-arrestin1 and β-arrestin2 in the conditions tested with the β-arr2 recruitment kit. Taken together, these results demonstrate the high specificity of the β-arr2 recruitment kit for the interaction of AP2 with β-arr2 and not with β-arr1. The application of the β-arr2 recruitment kit to a variety of compound, GPCR, and cellular contexts is illustrated in the next sections.

*All results obtained with the double KO β-arrestin 1/2 HEK293 cells were obtained by the Eidos team (IGF Montpellier; Drs J-P Pin and P. Rondard's team), and were kindly provided to Revvity.

Pharmacological illustrations with various GPCRs as models

GPCRs overexpressed in HEK 293 stable cell lines

In order to illustrate the application of the β-arr2 recruitment kit to a variety of pharmacological investigations, several assays were performed with different GPCRs. The Tag-lite® SNAP-GPCRs HEK293 stable cell lines from the Revvity GPCR catalogue were used as models. In these cells, only the GPCRs are overexpressed and tagged. AP2 and β-arrestin2 are endogenously expressed.

The different Tag-lite® stable cell lines were plated at 100 k cells/well in a white 96-w culture treated microplate (Revvity #6005680/8/9), and incubated for 24 h at 37 °C, 5 % CO_2. Kinetics were then performed to optimize the stimulation time for each agonist compound at room temperature. A concentration corresponding to the EC80 of each agonist was used for antagonist compound evaluations. For the latter, antagonists were pre-incubated for 20 minutes at room temperature before adding the agonist compounds.

Results obtained with the β-arr2 recruitment kit after ON incubation at room temperature are shown in the graphs below:

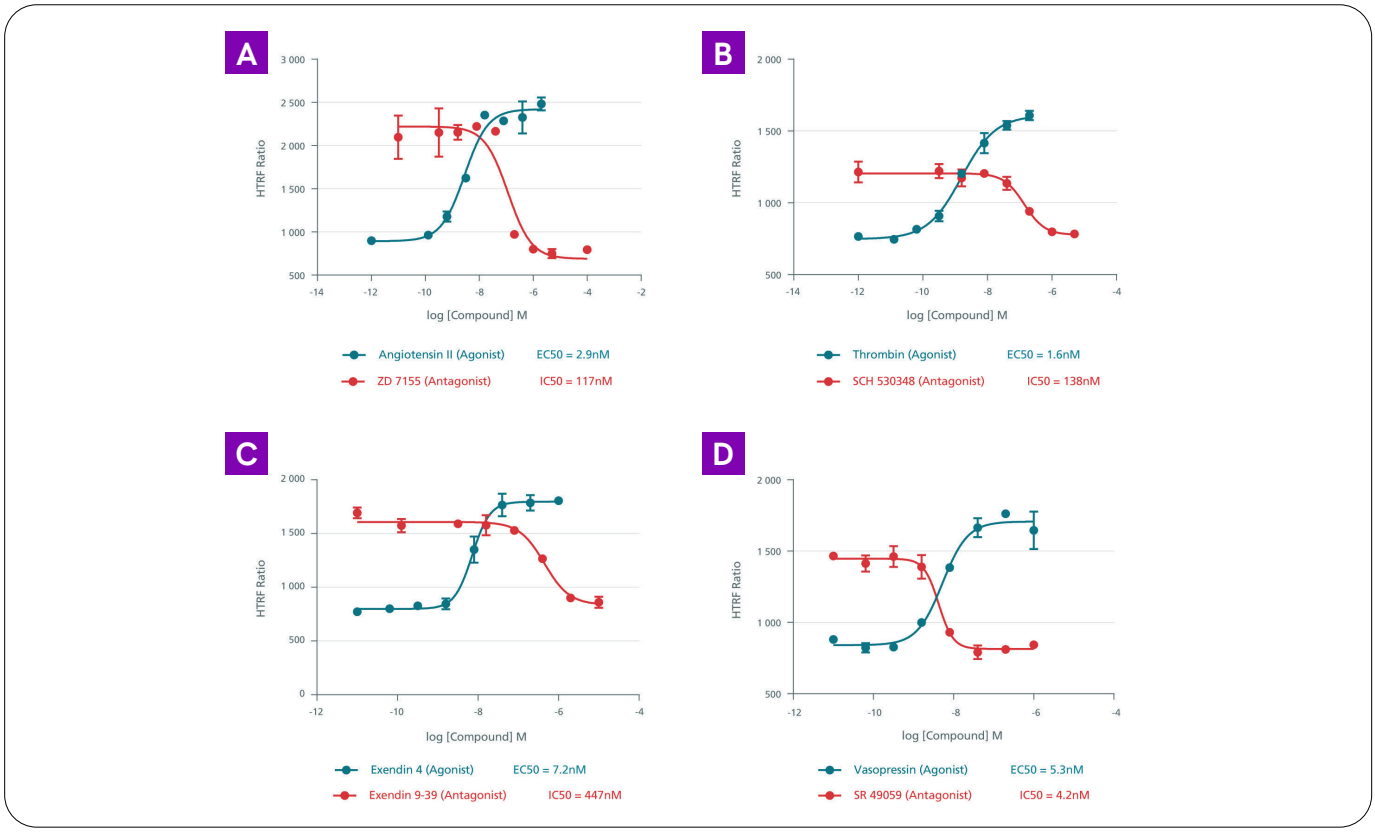


Figure 6. β-arr2 recruitment assays with various Tag-lite® SNAP-GPCR HEK293 stable cell lines.
A: Illustration with Angiotensin 1 receptor (AT1R).
B: Illustration with Proteas-activated receptor 1 (PAR1).
C: Illustration with Glucagon-like peptide-1 receptor (GLP1R).
D: Illustration with Vasopressin receptor 1A (V1AR).

As these results show, the β-arr2 recruitment kit enables consistent pharmacological characterizations with various agonist and antagonist compounds, and with potencies in good accordance with the literature. For a few compounds, some differences of potency values compared to the literature may be explained by several parameters such as the nature of those compounds and the expression level and/or conformation of AP2 and β-arrestin2. It is worth noting that the β-arr2 recruitment kit relies on endogenous AP2 and β-arrestin2, whereas all other β-arrestin assays existing in the literature are based on overexpressed and tagged proteins.

GPCRs overexpressed in transient HEK293 cells

In parallel with the investigations using stable cell lines, some experiments were also performed with transient HEK293 cells for complementary illustrations. Various GPCRs were tested: Similar receptors as with Tag-lite® stable cells, and also different receptors.

The expression level of each receptor was validated using Tag-lite® expression assays (data not shown).

Cells were plated in a white 96-w culture treated microplate (Revvity #6005680/8/9) for 24 h at 37 °C, 5 % CO2, and then transfected with Tag-lite® SNAP-plasmids (Revvity) encoding for various GPCRs as models. Stimulations with agonist and antagonist compounds were performed using the same strategy as previously described.

Results obtained with the β-arr2 recruitment kit after ON incubation at room temperature and the comparison of the two protocol options are shown in the graphs below:

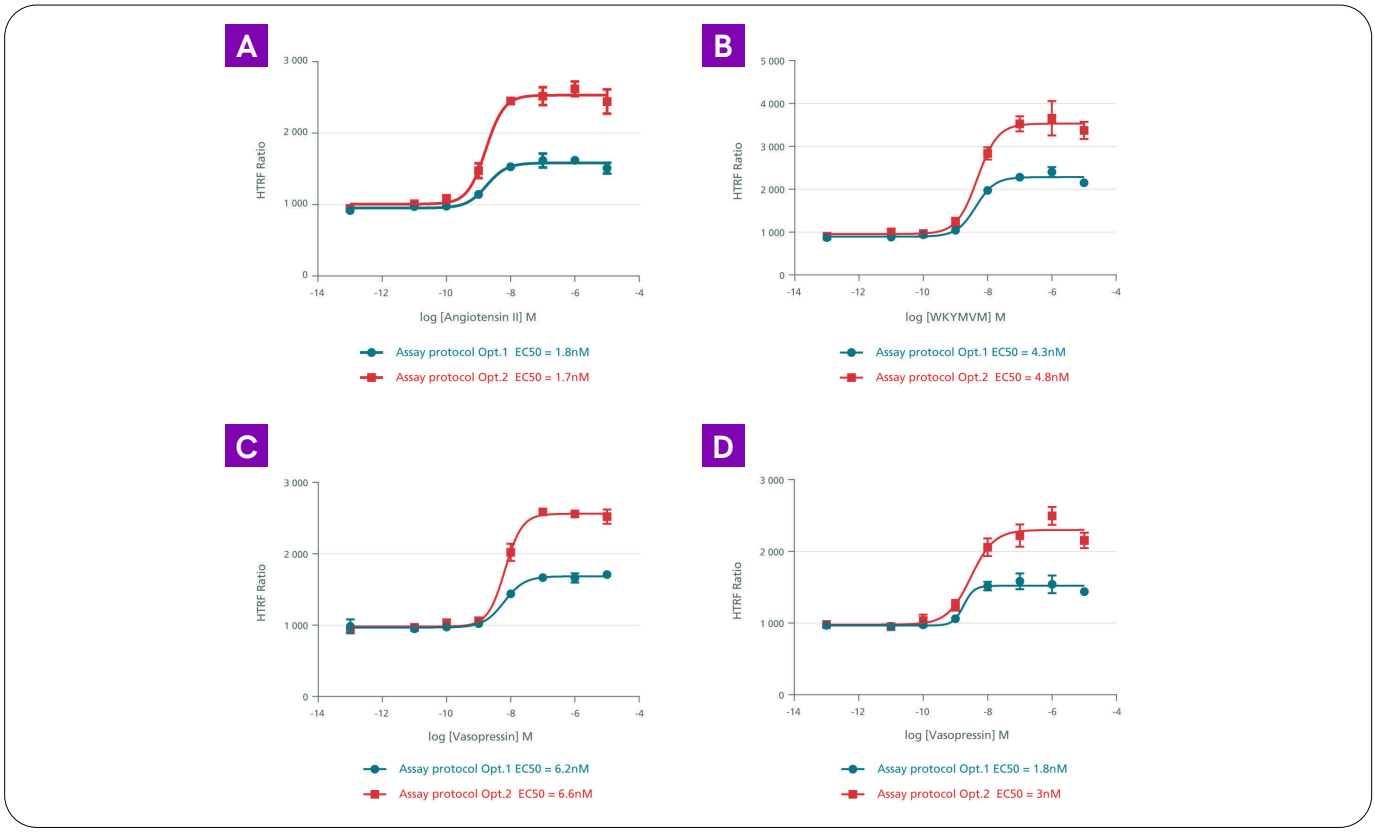


Figure 7. β-arr2 recruitment assays with various transient HEK293 cells, transfected with SNAP-GPCRs.
A: Illustration with Angiotensin 1 receptor (AT1R).
B: Illustration with N-Formyl peptide receptor 2 (FPR2).
C: Illustration with Vasopressin receptor 2 (V2R).
D: Illustration with Vasopressin receptor 1A (V1AR).

The results demonstrate the ability of the β-arr2 recruitment kit to monitor consistent agonist stimulation effects in transient cells, as well as in stable cell lines. Also, as illustrated by these results, detection protocol option 2 enables higher signal detection than option 1 without changing the potencies measured.

Pharmacological ranking of compounds on the beta 2 adrenergic receptor

Complementary investigations were performed with the Beta 2 adrenergic receptor as a model to illustrate the ability of the β-arr2 recruitment kit to rank pharmacological compounds as expected.

Illustrations with a panel of agonist compounds

The Tag-lite® Beta 2 adrenergic HEK 293 stable cell line (Revvity #C1SU1BETA2) was plated at 100 k cells/well in a white 96-w culture treated microplate (Revvity #6005680/8/9) and incubated for 24 h at 37 °C, 5 % CO_2. Then, a 30 mn stimulation with increasing concentrations of a panel of agonists was performed at room temperature.

The results obtained with the β-arr2 recruitment kit after ON incubation at room temperature are shown in Figure 8.

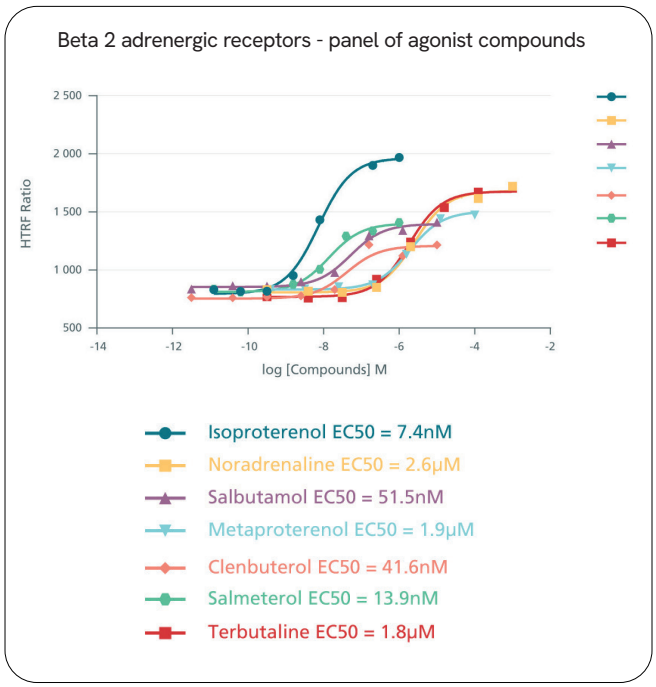


Figure 8. Pharmacological ranking of Beta2R agonists monitored by the β-arr2 recruitment kit.

These results demonstrate that the β-arr2 recruitment kit achieves consistent pharmacological ranking of various full and partial agonist compounds in good correlation with the literature.

Table 1. Comparison with the literature of agonist profi les and EC50 values measured for Isoproterenol and Clenbuterol with the β-arr2 recruitment kit.

EC50 (nM)	β-arr2 Recruitment kit	PathHunter® CHO-K1 ADRB2 β-arrestin cells**
Isoproterenol	7.4 (full)	12 (full)
Clenbuterol	40 (partial)	5 (partial)

In parallel with this study, the same Isoproterenol and Noradrenaline solutions were tested by the Arpege platform (IGF Montpellier, France) in a BRET cellular assay, as other method of reference. This assay relies on overexpressed human Beta 2 adrenergic receptor-YFP and human β-arrestin2-RLuc in HEK293 cells.

The results obtained with the Isoproterenol and Noradrenaline solutions, evaluated in parallel with the β-arr2 recruitment kit and the BRET β-arrestin2 assay, are shown in Figure 9.

As demonstrated, there is a good correlation between the two assays in ranking Isoproterenol and Noradrenaline.

Illustrations with a panel of antagonist compounds

The same Tag-lite® Beta 2 adrenergic HEK 293 stable cell line (Revvity #C1SU1BETA2) was tested with antagonist compounds. Stimulations with increasing concentrations of two antagonists were performed. The antagonists were incubated for 20 minutes at room temperature before the addition of Isoproterenol at 30 nM.

The results obtained with the β-arr2 recruitment kit after ON incubation at room temperature are shown in Figure 10.

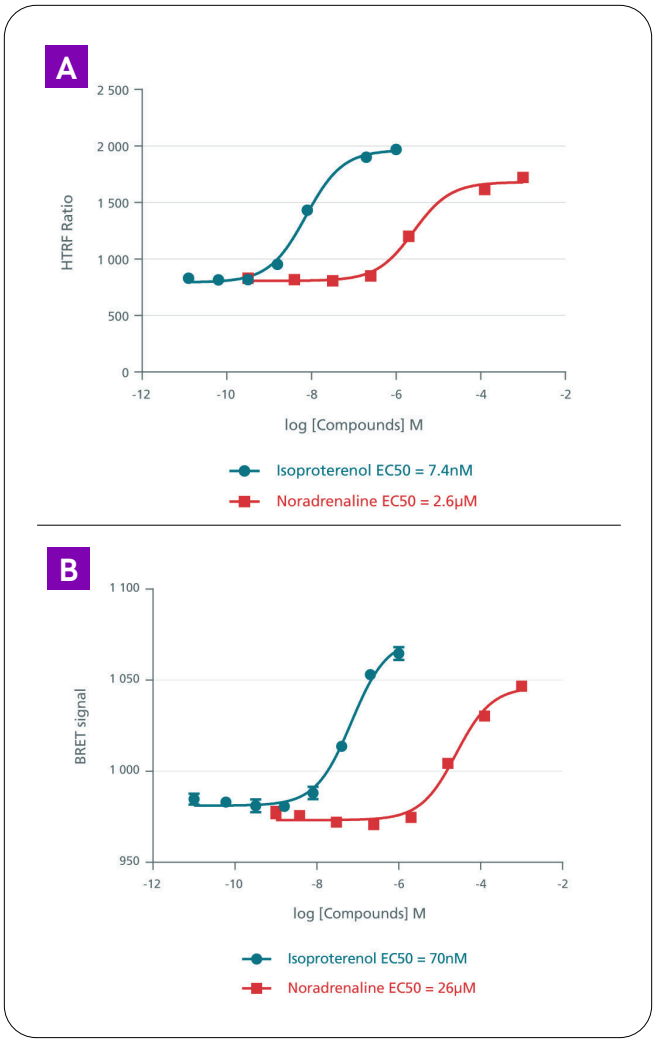


Figure 9. Pharmacological ranking of Isoproterenol and Noradrenaline compared with two β-arr2 assays.
A: Illustration with the β-arr2 recruitment kit.
B: Illustration with the BRET β-arr2 assay format.

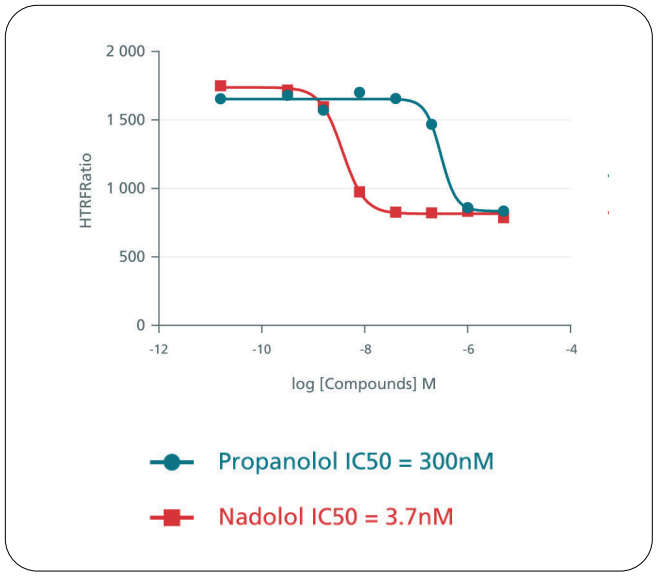


Figure 10. Pharmacological ranking of Beta2R antagonists monitored by the β-arr2 recruitment kit.

As demonstrated, the β-arr2 recruitment kit achieves the characterization and pharmacological ranking of various antagonist compounds.

For example, the potency of Nadolol was compared to the literature and showed a good correlation:

Table 2. Comparison with the literature of IC50 values measured for Nadolol with the β-arr2 recruitment kit.

IC50 (nM)	β-arr2 Recruitment kit	PathHunter® CHO-K1 ADRB2 β-arrestin cells**
Nadolol	3.7	17.3

** From website: www.discoverx.com; 210120

Conclusion

The ability of the β-arr2 recruitment kit to monitor the pharmacological effects of various compounds in different cellular contexts was demonstrated with several GPCRs in HEK293 cells implicating endogenous AP2 and β-arrestin2 proteins. The global characterization of all compounds is consistent with the literature, including β-arrestin recruitment assays in cells with β-arrestin BRET assays or PathHunter® β-arrestin assays implicating modified and overexpressed partners (GPCRs, β-arrestins...).

The β-arr2 recruitment kit is suitable for GPCR investigations in cells in which AP2 and β-arrestin2 are endogenously expressed, leading to a more physiological context.

Acknowledgements

We thank the Eidos team (IGF Montpellier; Drs. J-P Pin and P. Rondard's team) and the Arpege platform (IGF Montpellier) for their scientific contribution to this work and for the results with the double KO β-arrestin1/2 HEK293 cells.

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