TECHNICAL NOTE

# Key success tips to perform B-Arr2 recruitment assays by using the B-Arr2 recruitment kit with GPCRs.


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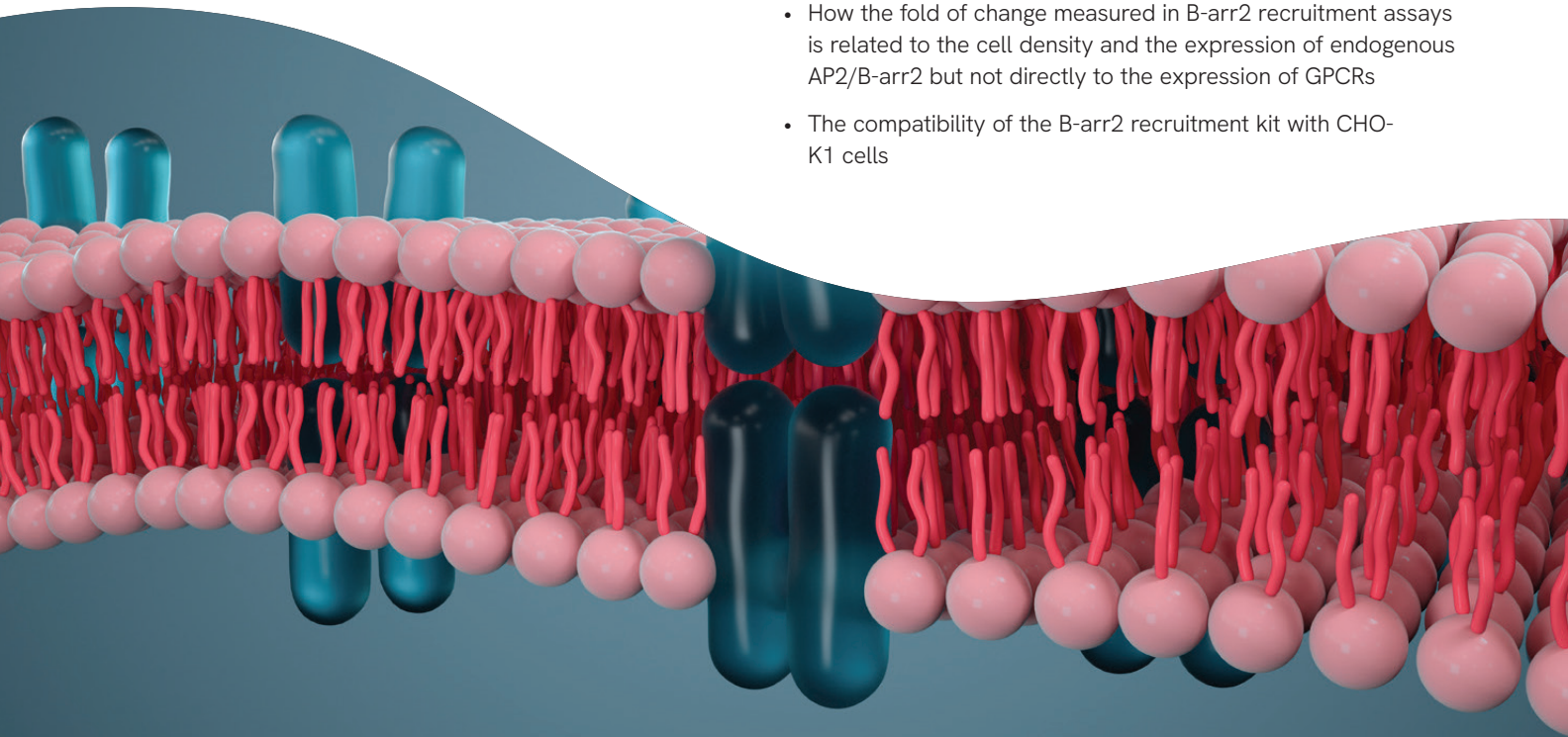
## Introduction

β-arrestin 1 and β-arrestin 2 are intracellular proteins playing central roles in GPCR signaling pathways by regulating agonist-mediated GPCR mechanisms such as internalization, where they act as endocytic adapters recruiting the GPCRs to the adapter protein complex 2 (AP-2) in clathrin-coated internalization pits. Different modes of arrestin-mediated internalization occur depending on receptors, cell types, and expression levels of β-arrestins and their partners.

The B-arr2 recruitment kit (Revvity #62BDBAR2PEB/C) is a highly sensitive and specific tool for monitoring the cellular interaction between β-arrestin 2 and AP2 as a reading of β-arrestin 2 recruitment at the GPCR. It is designed to work in cells expressing both partners and overexpressing a GPCR. The assays are compatible with all classes of GPCRs and all types of GPCR/β-arrestin interactions where β-arrestin 2 interacts with AP2.

The B-arr2 recruitment kit is easy to use following the directions for assay and data analysis described in the product insert. However, additional explanations provide a deeper understanding of the various parameters that impact the detection signal and fold of change measured that will help you obtain the best possible assay outcomes. This technical note provides valuable tips for performing B-arr2 recruitment assays on adherent cell lines overexpressing GPCRs by addressing the following points:

- How to carry-out the two-option protocol for B-arr2 recruitment assays and the purpose of both options
- How the fold of change measured in B-arr2 recruitment assays is related to the cell density and the expression of endogenous AP2/B-arr2 but not directly to the expression of GPCRs
- The compatibility of the B-arr2 recruitment kit with CHO-K1 cells



## An optimized two-option protocol to perform B-arr2 recruitment assays

**TIP | Use Option A protocol to carry-out the B-arr2 recruitment assay**

Following the directions to carry-out the B-arr2 recruitment assay with adherent cells is required to succeed. Each parameter and step of the assay protocol is optimized and fine-tuned (volumes, temperatures, buffers, and antibodies). We highly recommend following the assay flowchart precisely.

B-arr2 recruitment assays following the Option A protocol below:

In a 96-w tissue culture treated white microplate (Revvity #6005680/8/9 plates are recommended), dispense 100 µL of adherent cells diluted in the appropriate cell culture medium for the proper amount of time under suitable conditions. Cell density and culture time must be tailored to the cellular models.

Cell culture medium is then removed and 100 µL of pharmacological compounds diluted in Stimulation Buffer 4 are added (do not forget to fivefold dilute the 5X Stimulation Buffer 4 with distilled water prior to using it in the assays). Incubation time and temperature must be tailored to the GPCRs, compounds, and cells.

After incubation, the stimulation solution is removed and 30 µL of ready-to-use Stabilization Buffer 1 is added. Plates are incubated for 15 minutes at room temperature with a plate sealer.

The Stabilization Buffer is removed and cells are washed three times with 100 µL of Wash Buffer 1 (do not forget to tenfold dilute the 10X Wash Buffer 1 with distilled water prior using it in the assays). This step is needed to completely remove the Stabilization Buffer 1 components and ensure the success of the assay.

Finally, after removal of the wash buffer, 100 µL of pre-mixed antibodies (d2 and Eu-cryptate labeled) are added. Plates are incubated for at least 20 hours at room temperature with a plate sealer before reading with an HTRF®-compatible reader.

**TIP | Use Option B protocol to improve detection**

Option B (figure 2) protocol consists of an optional aspiration step that is added at the end of the Option A protocol to improve the detection signal. To perform this step, remove 80 µL from each well before reading the microplate with an HTRF-compatible reader.

Option B enables the improvement of signal amplitude by removing the unbound detection reagents from the assay, which diminishes background signal without affecting the pharmacological parameters. The reading setup is the same for the detection with 100 µL (Option A) or 20 µL (Option B) final volume. Since the Option B protocol is merely an addition to Option A, there should be no changes to the assay other than the additional aspiration step. We highly recommend doing the reading step from Option A before performing the additional step in Option B to ensure the assay is working correctly.

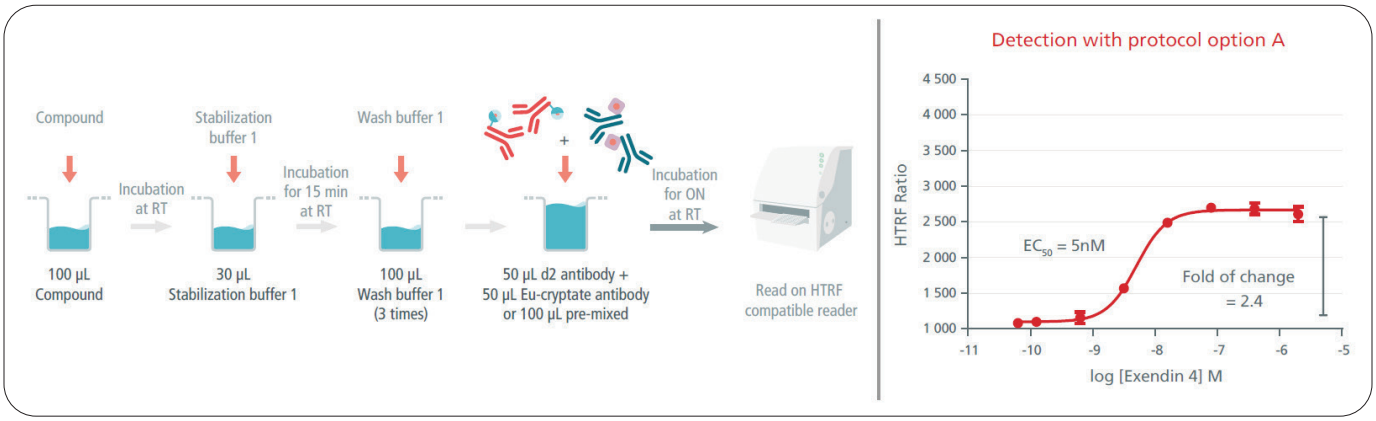



Figure 1. Left panel: Option A assay protocol . Right panel: Results obtained as an example with the HEK293 SNAP-GLP1 cell line (Revvity #C1SU1GLP1).

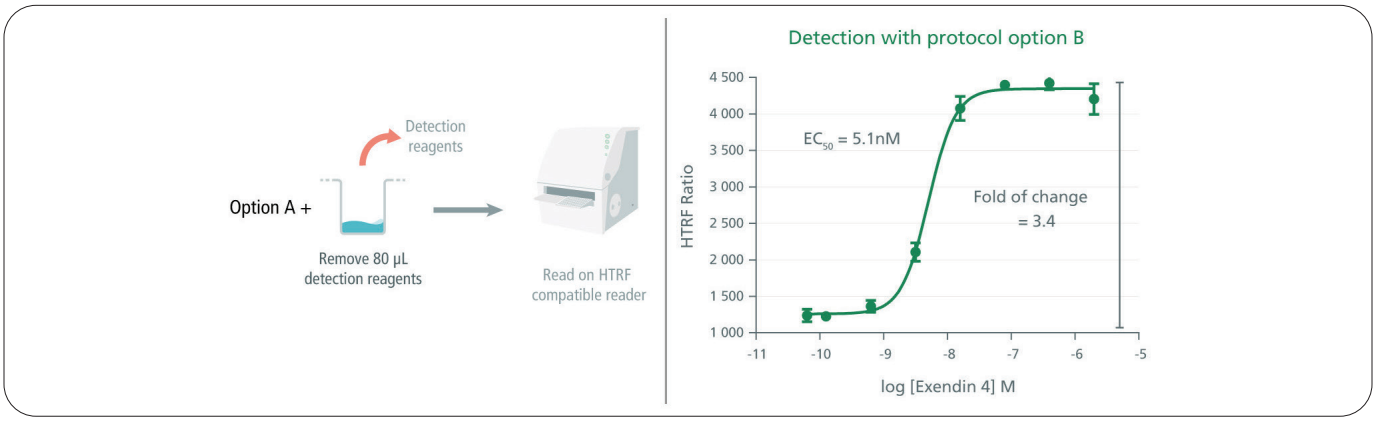



Figure 2. Left panel: Option B assay protocol. Right panel: In comparison to Figure 1, results obtained as an example with the HEK293 SNAP-GLP1 cell line (Revvity #C1SU1GLP1).

## A direct relationship between cellular parameters and signal amplitude

**TIP | Use the best cellular model expressing endogenous β-arrestin 2 and AP2**

The expression levels of GPCR, β-arrestin 2, and AP2 may vary depending on the cellular models, which results in variations of available receptors and of the amounts of B-arr2/AP2 interacting complexes that can be monitored with the B-arr2 recruitment kit.

While the experiment showed the signal amplitude to be dependent on the expression levels of both interacting partners, β-arrestin 2 and AP2, we concluded this was not the case with GPCR expression levels provided that they were higher than endogenous expression levels.

We compared the signal amplitude obtained with a large panel of overexpressed GPCRs in transient and stable HEK293 Tag-lite® cell lines. The expression level of each receptor was measured using the Tag-lite® technology indicating a relative specific expression level at 620 nm compared to non-transfected HEK293 cells as negative controls. The overview of correlated data from both expression levels of GPCRs and signal amplitude measured by the B-arr2 recruitment kit was plotted in the graph shown in Figure 3.

Results confirmed the absence of direct correlation with the signal amplitude obtained in B-arr2 recruitment assays and the expression levels of overexpressed GPCRs in HEK293 cells implicating endogenous β-arrestin 2 and AP2.

The HTRF Total β-arrestin 2 (Revvity #64BAR2TPEB/C/J) and HTRF Total AP2 (Revvity #64AP2TPEB/C/J) kits provide a convenient way of monitoring the specific expression of both proteins in cells prior to running the B-arr2 recruitment assay. In particular, this allows for the selection of the best cellular model.

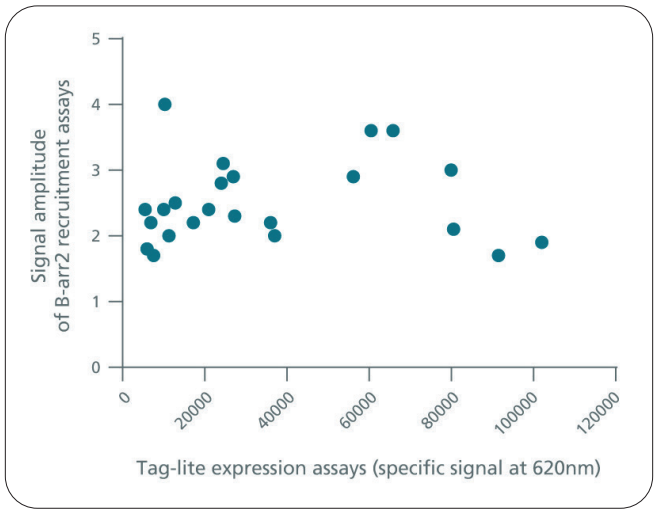



Figure 3. Correlation between signal amplitude measured by the B-arr2 recruitment and expression levels for a panel of GPCRs measured by the Tag-lite® technology.

Different types of commercialized HEK293 cells exist from various providers (e.g. ATCC, CLS) with many phenotypic profiles, compatibility to engineered modifications, and expression levels of endogenous proteins. In the following example (Figure 4), total β-arrestin 2 and AP2 assays were run in various HEK293 cells with the corresponding HTRF kits. The B-arr2 recruitment kit was then used to assess how these cells fare in relation to their β-arrestin 2 and AP2 expression levels. The results indicate that low signal amplitude may be observed if the expression of one or both partners is too low and that the best signal amplitude is obtained with the cells expressing the most β-arrestin 2 and AP2. Observing expression levels of β-arrestin 2 and AP2 is therefore instrumental in choosing the best cellular model to perform pharmacological investigations with the B-arr2 recruitment kit.

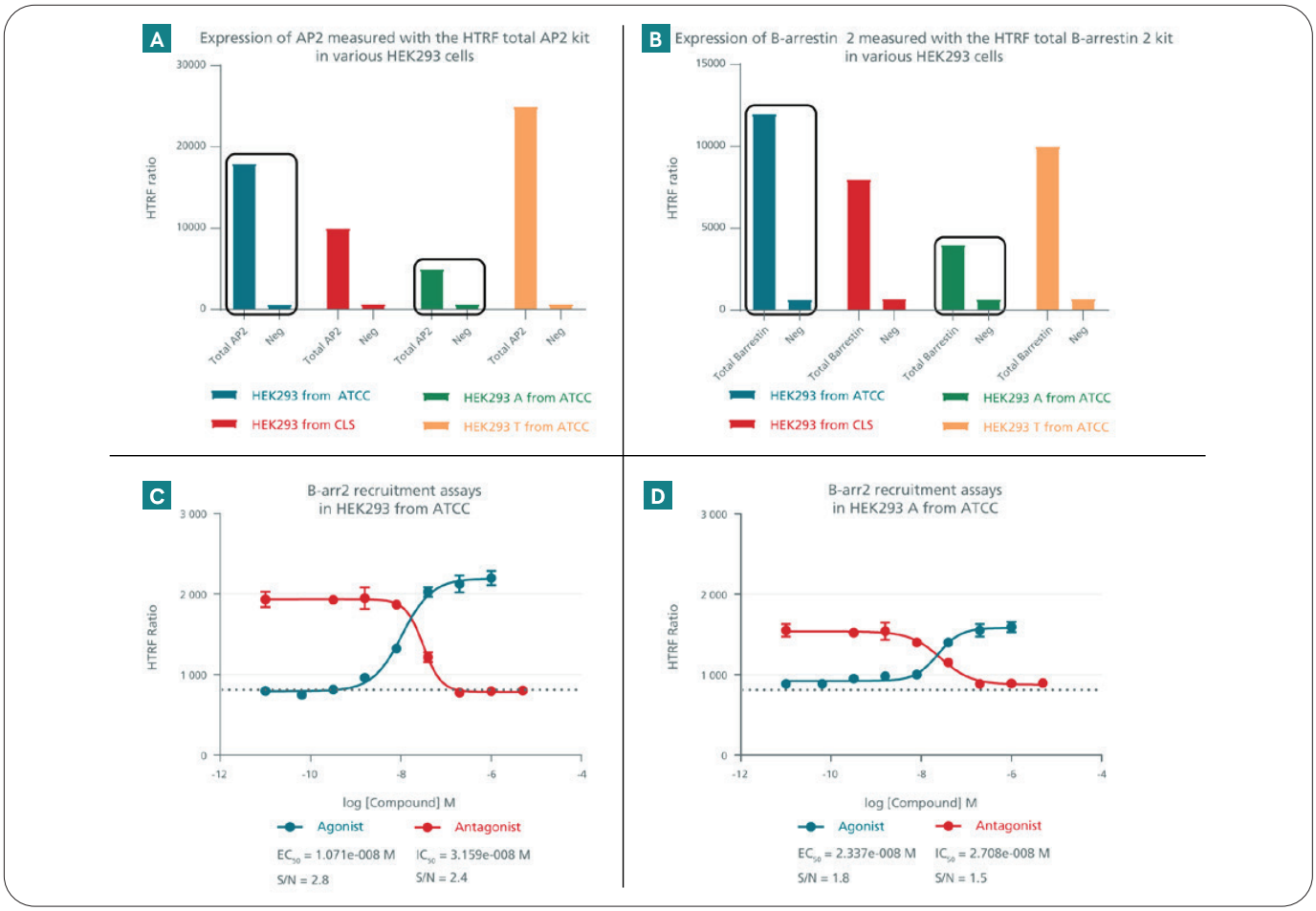



Figure 4. Example with a panel of HEK293 cells. Expression of AP2 (A) and β-arrestin 2 (B) was measured with HTRF Total β-arrestin 2 and HTRF Total AP2 kits. HEK293 from ATCC (C) and HEK293 A (D) from ATCC were used for pharmacological investigations as transient models overexpressing SNAP-Beta 2 adrenergic receptors (Revvity #PSNAPBD2) with a similar expression level.

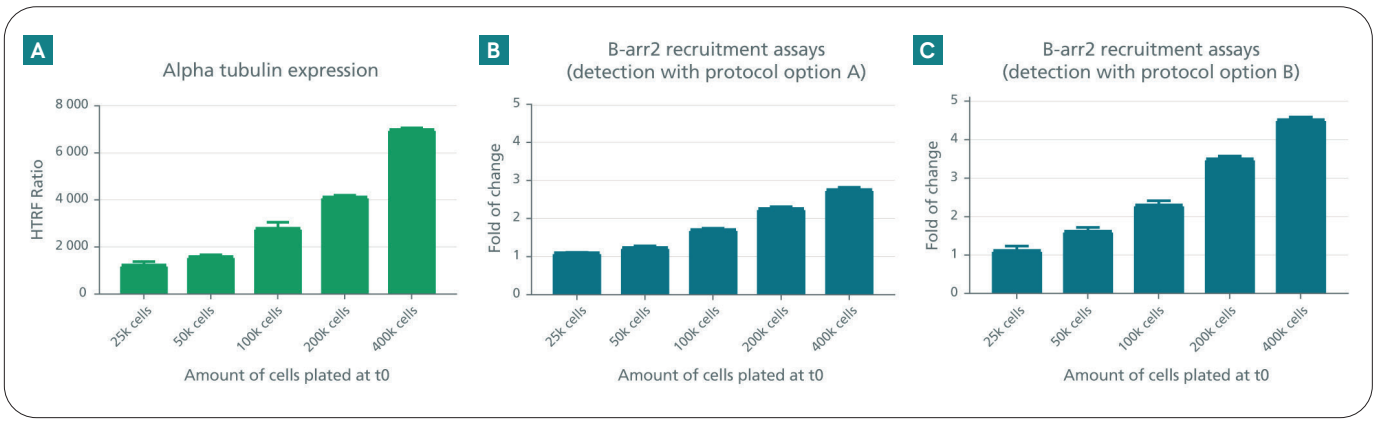



Figure 5. Illustrations with the HEK293 SNAP-GLP1 stable cell line (Revvity #C1SU1GLP1). Expression of alpha tubulin as a housekeeping gene was measured (A) with the AlphaTubulin Housekeeping Cellular Kit (Revvity # 64ATUBPEG/H), B-arr2 recruitment was monitored with the B-arr2 recruitment kit after agonist stimulation, and detection was done with the method Option A (B) and Option B (C).

In conclusion, the amount of interacting β-arrestin 2 and AP2 complexes monitored by the B-arr2 recruitment kit is directly driven by the expression levels of both partners, but indirectly by the expression levels of GPCRs. It is important to keep in mind that while different levels of GPCR overexpression result in similar signal amplitudes, there is such a thing as too low an expression, and endogenous expression levels of GPCRs are generally not sufficient to result in a successful assay.

**TIP | Optimize cellular density to get the best fold of change**

Cellular density is a relevant parameter in the signal amplitude of the B-arr2 recruitment assay. A low signal amplitude may be observed with too high of a cell density, which causes at least one of the detection reagents to be present in an insufficient amount compared to the endogenous β-arrestin 2/AP2 complexes (Hook effect). Alternatively, a low cell density may result in poor assay performance as there would not be sufficient amounts of interacting complexes to be detected.

To avoid that pitfall, we recommend evaluating cell density at 25k, 50k, 100k, 200k, and 400k cells/well to identify the best conditions for your assays. It is generally observed that signal amplitude increases with cell density in a proportional manner (Figure 5).

## Good compatibility of the B-arr2 recruitment kit with CHO-K1 cells

**TIP | Overexpress the β-arrestin 2 to perform the assays in CHO-K1 cells**

Both antibodies used in the B-arr2 recruitment kit can detect the human AP2 and β-arrestin 2 proteins as well as the hamster proteins (data not shown). Using qPCR experiments and the HTRF total β-arrestin 2 kit, we found the endogenous expression of β-arrestin 2 in CHO-K1 cells is very low (data not shown), and generally inferior to the requirements of the B-arr2 recruitment assay.

In the following example (Figure 6), we show that CHO-K1 cells transiently transfected with β-arrestin 2 result in significantly increased signals, which are on par with the performance of the assay in HEK293 cells presented in this technical note. We conclude that the restoration of β-arrestin 2 expression in CHO-K1 cells allows for the B-arr2 recruitment assay to be performed easily with satisfying results.

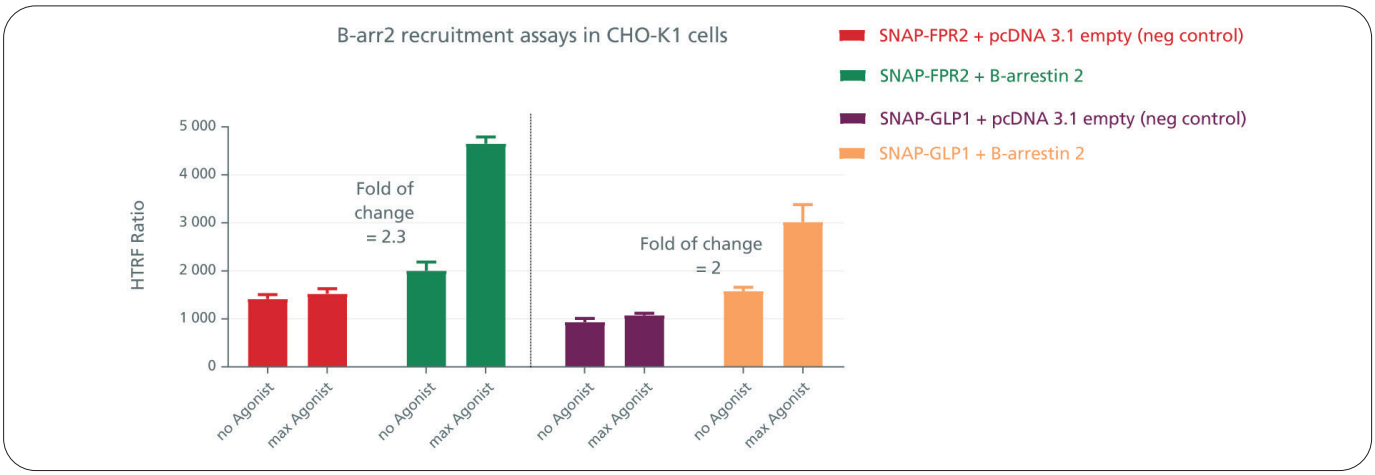



Figure 6. CHO-K1 cells were transiently co-transfected with SNAP-FPR2 and β-arrestin 2 (green bars) or pcDNA3.1 as a negative control (red bars), and with SNAP-GLP1 (Revvity #PSNAPGLP1) and β-arrestin 2 (orange bars) or pcDNA3.1 as a negative control (violet bars). Stimulations were done with the Exendin-4 (GLP-1R agonist) and WKYMVm (FPR2 agonist), then B-arr2 recruitment was monitored with the B-arr2 recruitment kit.

## Conclusions

By carefully following the directions of the product insert and applying the tips described and exemplified in this technical note, you will obtain the best possible results with the B-arr2 recruitment kit.

The key elements to watch for when running the assay are:

- The ability to extend the assay protocol by an additional aspiration step to increase the S/B signal
- The endogenous expression levels of β-arrestin 2 and AP2 are important to the signal amplitude and assessing them allows for an optimal choice of cellular model
- Cellular density affects the assay results and should be optimized for the cellular model
- CHO-K1 cells lack the endogenous expression level of β-arrestin 2 for the assay to be successful but this can be resolved by overexpressing β-arrestin 2

It is important to perform the recommended optimization steps for each new experiment since optimal assay conditions are cell, AP2, β-arrestin 2, GPCRs and pharmacological compound dependent.

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