APPLICATION NOTE

Optimization and pharmacological validation of wild type beta-arrestin 2 plasmid transfection.

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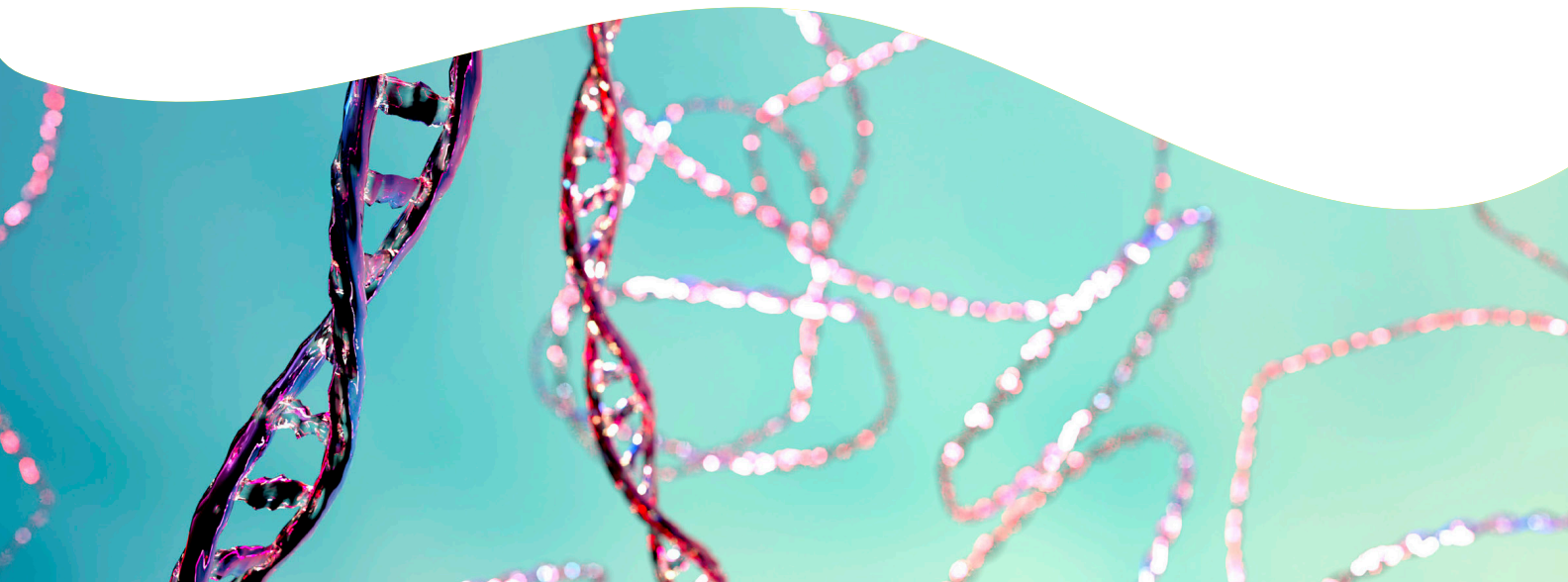
Introduction

Beta-arrestin 1 and 2 are members of a family of alternative GPCR signaling proteins, which replace G-proteins at the receptor to mediate G-protein-distinct effects in cells. While beta-arrestins were first thought to only regulate receptor desensitization and internalization, it is now clear that GPCRs can generate productive signaling cascades through the modulation of beta-arrestin functionality that are entirely separate from the usual G-protein mediators. Arrestin recruitment at the receptor is mediated by phosphorylation patterns on the intracellular GPCR tail, and is followed by AP2 binding, which then serves as an adaptor for clathrin heavy chain recruitment. The resulting network of clathrin defines an area targeted for internalization around the receptor, which ends up being degraded intracellularly or recycled at the membrane later.

The Beta-arrestin 2 Recruitment Kit (Revvity #62BDBAR2PEB/C) is a highly sensitive and specific tool that uses the interaction between beta-arrestin 2 and AP2 as a reading of beta-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/beta-arrestin interactions where beta-arrestin 2 interacts with AP2.

In a previous techical note dedicated to optimizing the Beta-arrestin 2 Recruitment Kit, we explained the endogenous expression of beta-arrestin 2 in CHO-K1 cells is very low, and generally inferior to the requirements of the Beta-arrestin 2 Recruitment Kit. We exemplified that CHO-K1 cells transiently transfected using a beta-arrestin 2 plasmid (#PWTBARR2) could yield increased beta-arrestin 2 recruitment signals, and concluded it was possible to restore the beta-arrestin 2 expression in otherwise deficient cells, to make them fit for the Beta-arrestin 2 Recruitment Kit.



In this note, we cover the best practices and elements that can be monitored and tested to achieve an effective transfection with the HTRF™ Wild Type (WT) Beta-arrestin 2 Plasmid and improve the assay window of between 10% and 100+%. Three parameters are especially important:

1. The amount of transfected DNA
2. The beta-arrestin 2 expression level and associated beta-arrestin 2 assay delta F (ΔF) of the resulting transfection
3. The levels of expression of AP2 and Alpha-tubulin

Optimizing the transfection of HEK293 and CHO cells with the WT beta-arrestin 2 plasmid

All transfection experiments described in this note were performed precisely following the protocol flowchart included with the product. All experiments were run using either Option #1 or Option #2 of the Beta-arrestin 2 Recruitment Kit, which only differ by an additional step to remove media from the wells in Option #2.

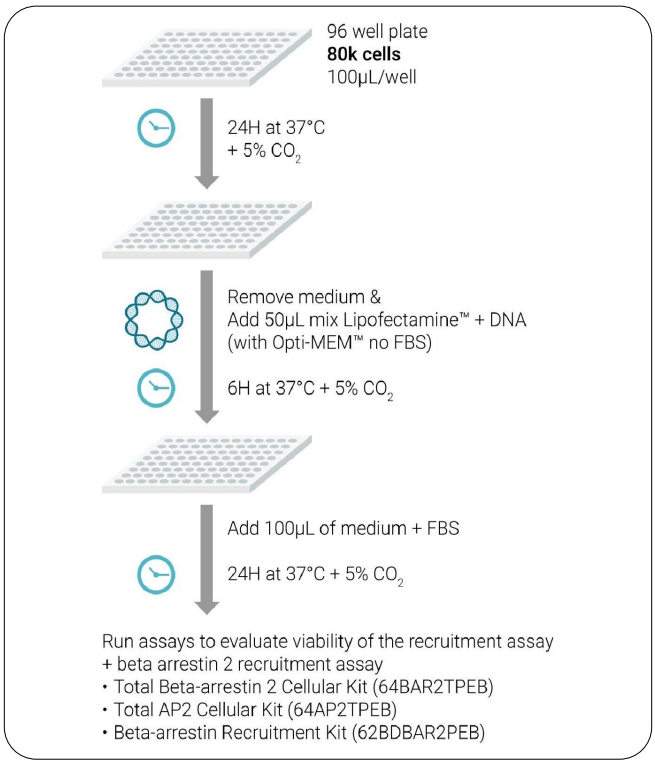


Figure 1: Three-step process of the culture and transfection of cells with the WT Beta-arrestin 2 Plasmid using CulturPlate-96 (#6005680) microplate.

Cell density

The transfection of cells with the WT Beta-arrestin 2 Plasmid is optimal at a **density of 80k cells/well**. Cell densities of 50k cells/well to 100k cells/well are acceptable and work in some cases but we recommend adhering to 80k cells/well for most applications and not using cell densities outside of the 50k-100k cells/well range.

Amount of plasmid transfected and ΔF

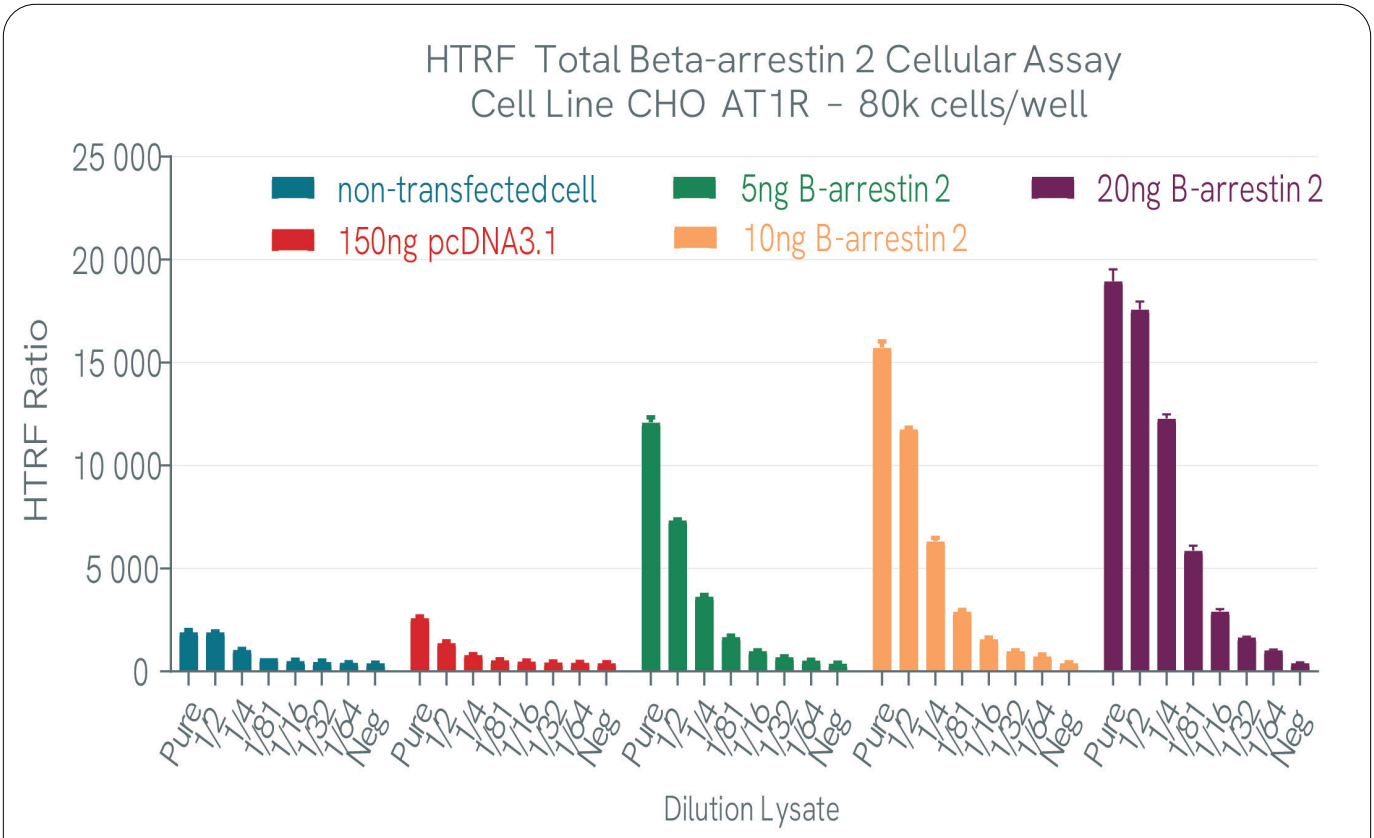
When optimizing the transfection prior to running the Beta-arrestin 2 Recruitment Kit, the key parameter to optimize is the amount of plasmid to add to the cells.

We recommend testing the transfection with three different quantities of DNA (5ng, 10ng, and 20ng), as the optimal amount is dependent on the model chosen and transfection efficiency. The effects on the assay window of each DNA amount are evaluated and compared with the resulting increase in assay windows and consistency of the EC_{50} measured with the Beta-arrestin 2 Recruitment Kit (#64BAR2TPEB) and the expression level of beta-arrestin 2 with the Total Beta-arrestin 2 Cellular Kit (#64BAR2TPEB) with ProxiPlate-384 Plus (#6008280) microplates. Our experiments indicate that operating within a certain range of ΔF for the assay is a good way to ensure the assay window is improved while also maintaining good pharmacological accuracy in the EC_{50} measurement. We recommend the acceptable **ΔF range for these assays be 1500%-4000%.**

The ΔF is a ratio usually used for the comparison of day-to-day runs of the same assay or assays run by different users. It reflects the signal-to-background of the assay. In the case of the transfected Beta-arrestin 2 Recruitment Kit, it serves to evaluate the background noise, which can become excessive due to the basal signal resulting from too high an expression of beta-arrestin 2.

$$\Delta F = \frac{Ratio_{Sample} - Ratio_{Background}}{Ratio_{Background}} \% = \frac{Signal - Background}{Background}$$

Figure 2: ΔF formula.

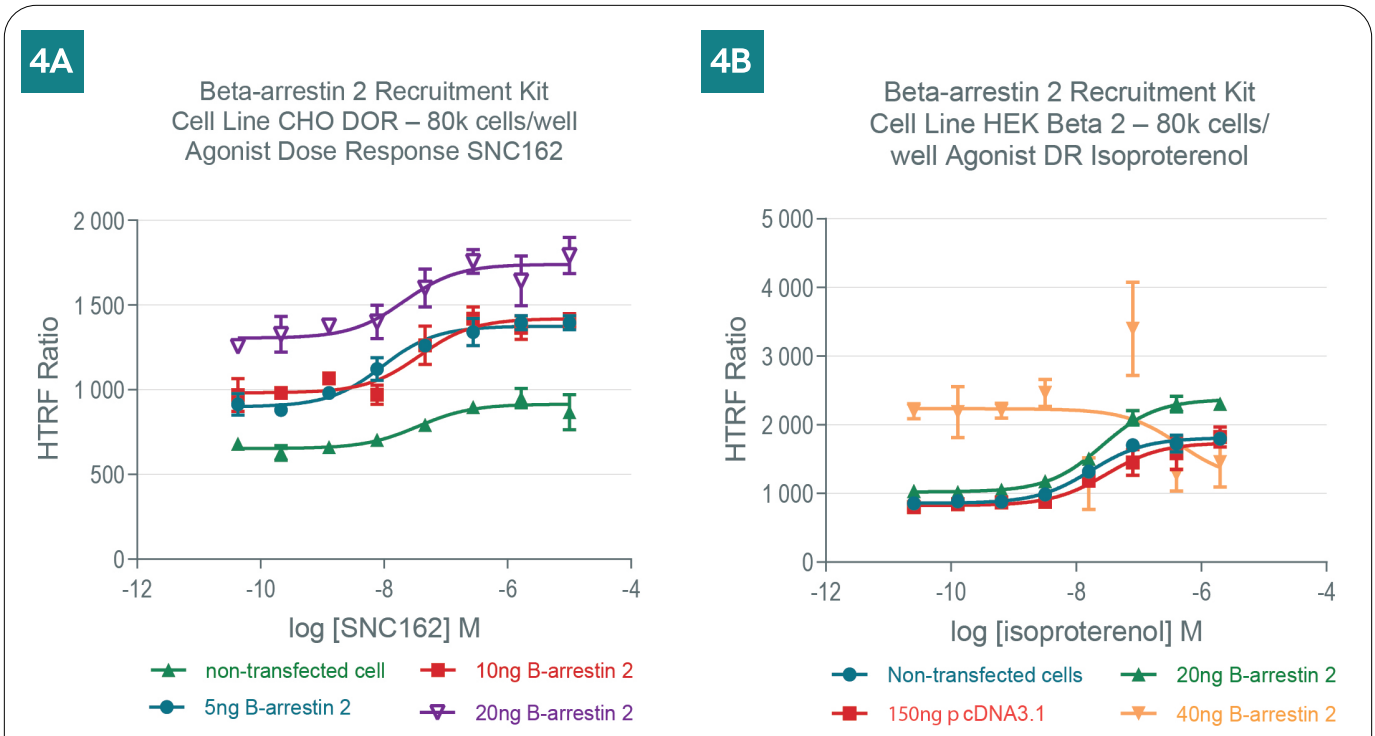


Dilution \ ΔF	NT	150ng pcDNA3.1	5ng B-arrestin 2 Plasmid	10ng B-arrestin 2 Plasmid	20ng B-arrestin 2 Plasmid
Pure	380%	501%	2737%	3727%	4291%
1/2	380%	223	1625%	2768%	3972%

Figure 3: Example of ΔF calculated for a transfection experiment. Stable CHO AT1R cells were transfected at 80k cells/well with 5ng, 10ng, or 20ng of Beta-arrestin 2 Plasmid. The levels of beta-arrestin 2 were measured using the HTRF Total Beta-arrestin 2 Assay with a ProxiPlate-384 Plus (#6008280) microplate. The assay signals were converted into ΔF for each quantity of plasmid tested, using the formula described in Figure 2.

In the example below, CHO and HEK cells were transfected with different amounts of plasmid. We notice that higher ΔF values are consistent with a wider assay window up to a point where ΔF is around 4000%. ΔF slightly above that threshold can still be acceptable (Figure 4A) but becomes rapidly worse with higher ΔF (Figure 4B) essentially due to the background increase that results from strong basal signals.

Running the transfection step with too much plasmid is not recommended as it affects the assay quality and can render the results unusable. Not adding an excess of plasmid is consistent with the ΔF guidelines, as it results in high ΔF and such values above 4000% are associated with poor results. In the example below, we see that on the one hand, 20ng of plasmid did not quite result in unusable results but took the transfection benefits to their maximum, which is consistent with the ΔF slightly above its acceptable range. On the other hand, 40ng of plasmid yielded a hook effect, where the assay cannot be used, and the ΔF value is way beyond its acceptable range.



CHO DOR	NT	5ng B-arrestin 2 Plasmid	10ng B-arrestin 2 Plasmid	20ng B-arrestin 2 Plasmid
S/B	1.4%	1.53%	1.45%	1.33
EC_{50} nM	35.6	37.6	37.6	21.2
ΔF	376%	1948%	1948%	4650%

HEK beta2	NT	15 ng pcDNA 3.1	20 ng B-arrestin 2 Plasmid	40ng B-arrestin 2 Plasmid
S/B	2.1%	2.1%	2.3%	NA
EC_{50} nM	17	30	26	
ΔF	1509%	1423%	4279%	6365%

Figure 4: Stable CHO (A) and HEK (B) cells were transfected at 80k cells/well and with varying amounts of plasmid. The resulting assay windows and EC_{50} indicate which transfected DNA amount yields the best results for each cell line, and the failure of the assay at 40ng of plasmid.

Use Alpha-tubulin and/or AP2 assay to check for transfection toxicity

The final parameter we recommend considering and testing when using the WT Beta-arrestin 2 Plasmid is the potential toxicity of the transfection. We used the HTRF Alpha-tubulin Kit (#63ADK073PEG) and AP2 Kit (#64AP2TPEB) to control the cell toxicity for different amounts of transfected DNA. Alpha-tubulin is a common marker for these applications while AP2 is the partner of beta-arrestin 2 in the recruitment assay and should not be affected by the transfection for the assay to work.

Figure 5: Stable CHO AT1R (A) cells were transfected at 80k cells/well and with 5ng, 10ng, or 20ng of plasmid. The resulting level of expression of alpha-tubulin and AP2 were measured. In the case of the CHO AT1R cells, there was no difference for any amount of DNA, indicating a non-toxic transfection.

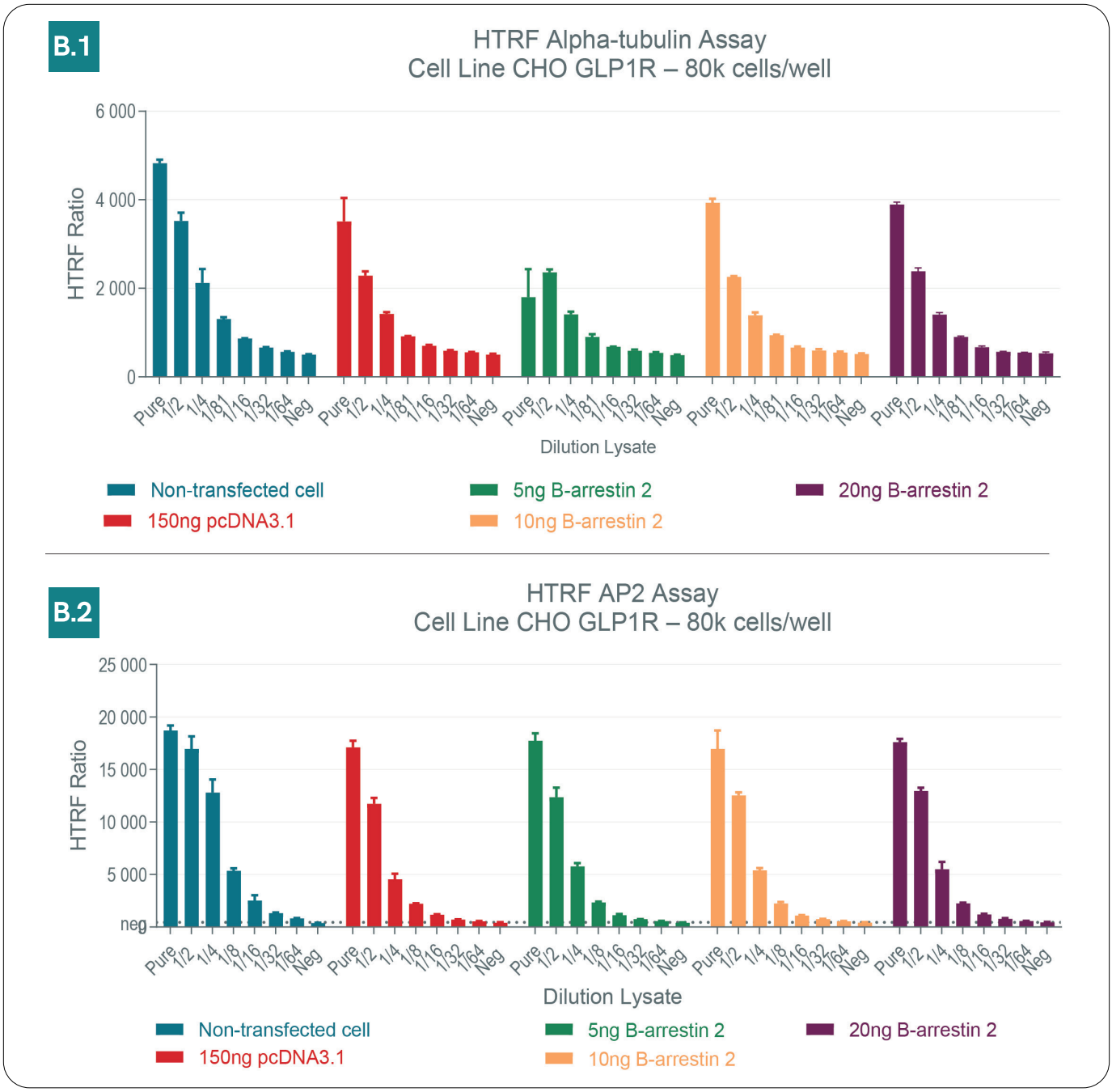


Figure 5: (Continued) Stable CHO GLP1R (B) cells were transfected at 80k cells/well and with 5ng, 10ng, or 20ng of plasmid. The resulting level of expression of alpha-tubulin and AP2 were measured. In the case of the CHO GLP1R cells, we see the levels of alpha-tubulin and AP2 are affected by the increase in plasmid, which indicates cytotoxicity.

Pharmacological study of a panel of GPCR cell lines, transfected using the WT beta-arrestin 2 plasmid

The panel of GPCR membranes chosen for this study exhibit differences in cellular backgrounds (HEK293 or CHO) and receptors. All information relative to the receptor models of this study are reported in Figure 6.

The transfection protocol was applied to each model, with the corresponding optimization parameters. The Beta-arrestin 2 Recruitment Kit was run on each model following the flowchart described in the kit package insert. In each case, the dose-response curves of reference ligands show correct characterization achieved by the assay. Thus, these data show that transfection of various models with the WT Beta-arrestin Plasmid improves the resulting assay windows while achieving pharmacological values in accordance with the literature.

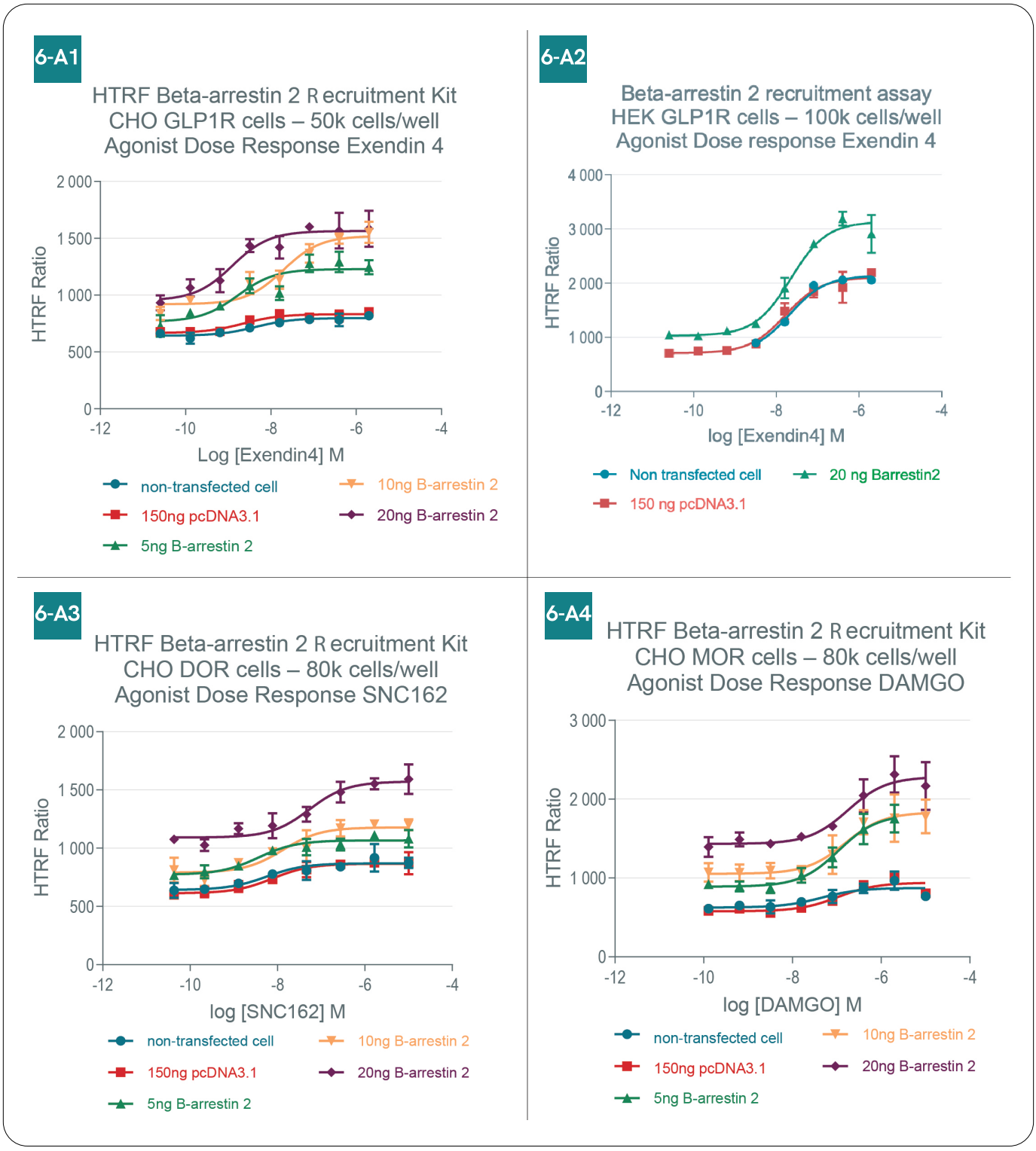


Figure 6: (A) Dose-response curves of ligands on the panel of GPCR-expressing stable cell lines transfected with WT Beta-arrestin 2 Plasmid and using the Beta-arrestin 2 Recruitment Kit.

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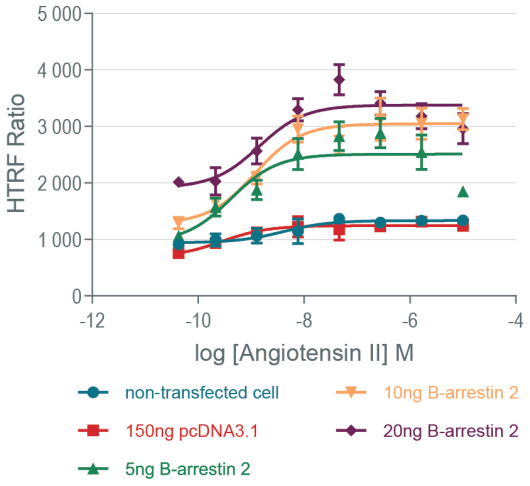

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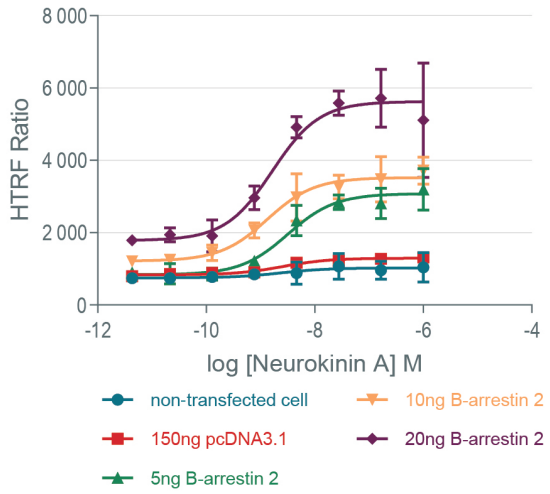

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Receptor	Ligand	Cell Line (commercial reference when available)	EC_{50} from the literature	EC_{50} assay	S/B NT	S/B best trancfection condition	% assay window gainded
GLP1R	Exendin 4	HEK	2.2 nM	22.0 nM	2.7	3	11.10%
		CHO		1.7nM	1.2	1.6	41.70%
AT1R	Angiotensin II	CHO (#ES-542-C)	1.0 nM	1.4 nM	1.54	2.95	91.60%
DOR	SINC162	CHO	12.6 nM	13.2 nM	1.36	1.49	9.60%
MOR	DAMGO	CHO (#ES-542-C)	480 nM	110 nM	1.4	2.03	45.00%
NK2	Neurokinin A	CHO (#ES-251-C)	10.0 nM	3.2 nM	1.36	3.68	170.00%

Figure 6: (Continued) (A) Dose-response curves of ligands on the panel of GPCR-expressing stable cell lines transfected with WT Beta-arrestin 2 Plasmid and using the Beta-arrestin 2 Recruitment Kit. (B) Compound's pharmacological values obtained with Beta-arrestin 2 Recruitment Kits on the studied GPCR cell lines

Conclusion

This note illustrates the application of the WT Beta-arrestin 2 Plasmid (#PWTBARR2) to transfect CHO and HEK cells expressing GPCRs and improve the assay window of a Beta-arrestin 2 Recruitment Kit on those cells. Depending on the biological model used in these experiments, different optimization conditions were selected, and a range of improved pharmacological windows were recorded. The pharmacological characterization of compounds with the Beta- arrestin 2 Recruitment Kit in transfected cells were validated using reference ligands and shown to be achieved well in all models.

The data presented here shows that the transcription described never negatively impacts the assay window and instead systematically results in improvements ranging from moderate to very large depending on the models. In the case of some cellular models tested like the CHO NK2 cells, the transfection even makes the Beta-arrestin 2 Recruitment Kit viable when it was previously not, by increasing the assay window 170% from 1.36 to 3.68.