APPLICATION NOTE

Pharmacological validation of a panel of GPCRs with the HTRF GTP Gi binding assay.

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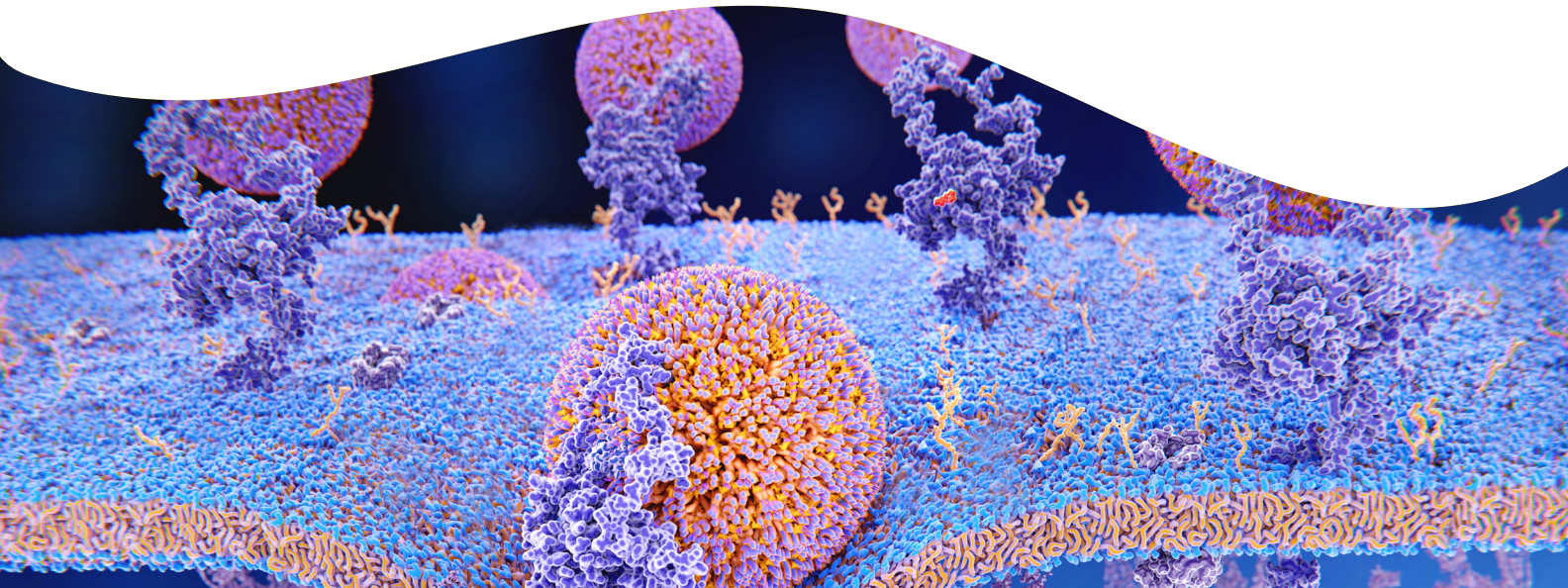
Introduction

The HTRF® GTP Gi binding assay measures the level of Gi protein activation and has the advantage of studying the functional response of GPCRs at one of the earliest receptor-mediated events. This non-isotopic kit (#62GTPPET/G/C) detects Gi protein activation via the binding of Eu-cryptate-labeled non hydrolysable GTP analog (donor) and d2-labeled anti-Gi monoclonal antibody (acceptor). This application note demonstrates the implementation of the HTRF GTP Gi Binding kit to a panel of receptor models that differ by their biological roles, basal activity behaviors, cellular expression backgrounds, and GPCR expression levels. The pharmacological characterization data presented validate the assay in all models tested using reference agonists and antagonists.

The GTP Gi binding assay principle

G-Protein-Coupled Receptors (GPCRs) are highly studied drug targets due to their involvement in human pathologies. Their contribution to therapeutic research is unmatched as drugs targeting GPCRs represent over 30% of FDA-approved drugs across all therapeutic areas. The HTRF GTP Gi Binding assay (#62GTPPET/G/C) is intended to measure the level of Gαi protein subunit activation upon the GPCR activation process. This upstream signaling activation event is characterized by the exchange of Guanidine Diphosphate (GDP) for Guanidine Triphosphate (GTP) at the α-subunit, which in turn engages downstream signaling to activate phenotypic cellular responses. The non-isotopic assay detects Gi protein activation with an Eu-cryptate-labeled non hydrolysable GTP analog (donor) that binds the Gαi protein and a d2-labeled anti-Gi monoclonal antibody (acceptor) (Figure 1).



This study uses a panel of GPCR membrane models to exemplify the GTP Gi Binding assay. The optimized assay conditions, response level (S/B), and pharmacological validation using reference agonists and antagonists are presented for each GPCR membrane model.

Membrane model characteristics

The panel of GPCR membranes chosen for this study exhibit differences in cellular backgrounds (HEK293 or CHO) and GPCR expression levels as determined by radioactive binding assays. Interestingly, a case of Gi secondary-coupled receptor model is also presented (NTS1). NTS1 is a GPCR that is primarily coupled to Gq proteins rather than Gi. All information relative to the receptor models of this study are reported in Table 1.

Optimized assay conditions

GTP binding assays call for a preliminary optimization step and it must be remembered that careful optimization of various parameters is generally required as the detection of GTP recruitment at the G protein is strongly influenced by the environment. In particular, salts and GDP concentrations are key elements that drive the ability of G proteins to undergo the conformational changes that allow for the GDP/GTP exchange. In the HTRF GTP Gi Binding assay, the optimization step is a rapid and simple protocol where membrane quantity, GDP, and MgCl2 concentrations are titrated. The effects of each parameter as well as protocols and detailed guidelines to perform this step are available and described at length in the kit's package insert and the associated guides (https://learn.cisbio.com/lpgtpγs-guides-to-optimizing-agonists-antagonists-of-gαi). The optimal conditions for each GPCR membrane model used can be seen in Table 2.

The results of the optimization step illustrate the need for assay conditions to be tailored to each GPCR membrane and highlight the importance of this step. It was observed that the same receptor models used in different GDP or MgCL2 conditions do not result in a measurable agonist-induced specific response (data not shown). Furthermore, the strength of the biological response in the presence of saturating doses of agonists may be different from one model to another (recorded S/B from 1.5 to 3.5 depending on the GPCR which are expressed in different cellular backgrounds and display different expression levels). For a given receptor, it was generally observed that CHO cells result in higher assay windows than HEK293 cells, as illustrated in this study by the delta opioid receptor.

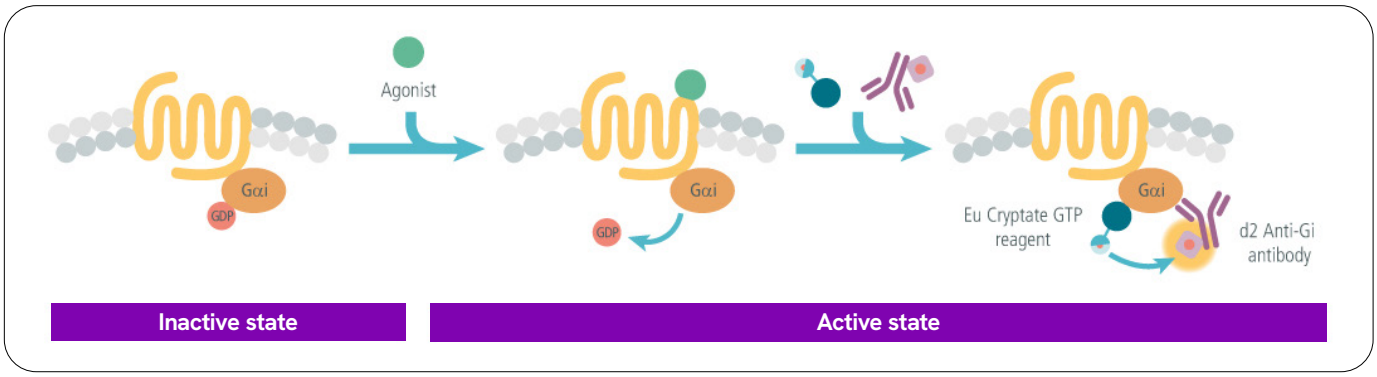


Figure 1. HTRF GTP Gi binding assay principle.

Table 1. Receptor membrane model information.

GPCR	G Signaling	Cellular background	Membrane #Cat	Receptor expression level
Delta Opioid (DOR)	Gi primary	CHO	RBHODM400UA	24.8 pmol / mg
Delta Opioid (DOR)	Gi primary	HEK 293	6110549400UA	10.6 pmol / mg
Mu Opioid (MOR)	Gi primary	CHO	ES-542-M400UA	8 pmol / mg
Nociceptin (OPRL1)	Gi primary	HEK 293	RBHORLM400UA	1.7 pmol / mg
Adenosine A3	Gi primary	CHO	ES-012-M400UA	2.3 pmol / mg
Dopamine D2S	Gi primary	CHO	RBHD2CM400UA	11 pmol / mg
Neurotensin type 1 (NTS1)	Gq primary Gi secondary	HEK 293	RBXNT1M400UA	3.45 pmol / mg
Serotonin 1A (5-HT1A)	Gi primary	HEK 293	RBHS1AM400UA	1.7 pmol / mg

Table 2. Optimal conditions for each GPCR membrane model.

Membrane model	Membrane quantity / well	Agonist (Dose µM)	Supplemented stimulation buffer 3	Incubation time	Optimal (S/B) stimulated / basal
CHO-Delta Opioid (DOR)	5 µg	SNC-162 (10 µM)	50 mM MgCl2 + 0.5 µM GDP	ON	3.5
HEK293-Delta Opioid (DOR)	5 µg	SNC-162 (10 µM)	50 mM MgCl2 + 0.5 µM GDP	ON	1.5
HEK293-Mu Opioid (MOR)	5 µg	DAMGO (10 µM)	50 mM MgCl2 + 0.25 µM GDP	ON	2.2
HEK293-Nociceptin (OPRL1)	5 µg	Nociceptin (10 µM)	50 mM MgCl2 + 0.25 µM GDP	ON	1.8
CHO-Adenosine A3	5 µg	NECA (10 µM)	10 mM MgCl2 + 0.25 µM GDP	ON	1.8
CHO-Dopamine D2S	5 µg	PPHT (10 µM)	50 mM MgCl2 + 0.25 µM GDP	ON	3.0
HEK293-Neurotensin type 1 (NTS1)	5 µg	Neurotensin (10 µM)	10 mM MgCl2 (No GDP)	ON	2.0
HEK293-Serotonin 1A (5-HT1A)	5 µg	Serotonin 5-HT (10 µM)	50 mM MgCl2 + 0.25 µM GDP	ON	1.7

Pharmacological study of a panel of GPCRs using the GTP Gi binding assay

The HTRF GTP Gi Binding assay was used on each of the previously described models (Table 1), with the corresponding optimization parameters (Table 2). In each case, the dose-response curves of reference agonists and antagonists show correct characterization achieved by the assay. Despite the range of pharmacological response strength, the assay's robustness allows correct potency calculations (Figure 2). Thus, these data show pharmacological values in accordance with the literature. All pharmacological values are reported in Table 3.

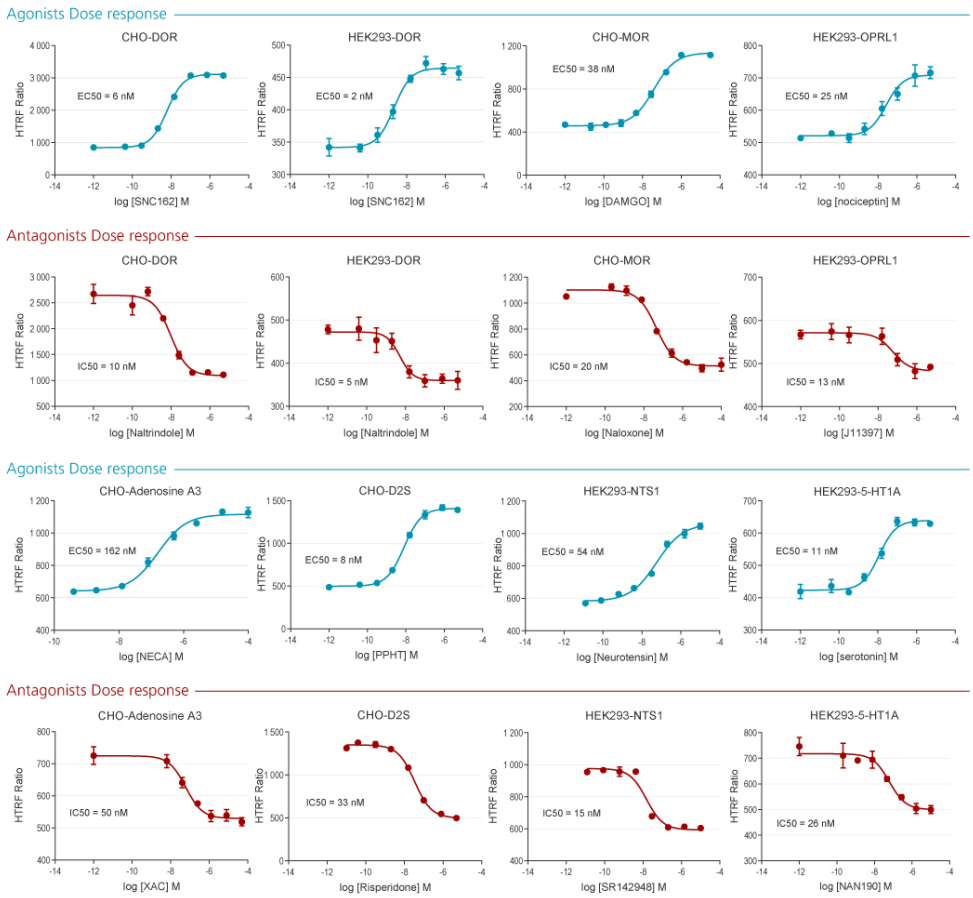


Figure 2. Dose-response curves of agonists and antagonists on the panel of receptor membrane models using the GTP Gi binding assay.

Table 3. Compound's pharmacological values obtained with HTRF GTP binding assays on the studied GPCR membrane models.

GPCR family	Receptor	Expressing model	Agonists		Antagonists	
			Compound	EC_{50} (nM)	Compound	IC_{50} (nM)
Adenosine	Adenosine A3	CHO	NECA	162	XAC	50
Opioids	Delta Opioid (DOR)	CHO	SNC-162	2	Naltrindole	5.2
		HEK293	SNC-162	6	Naltrindole	10
	Mu Opioid (MOR)	HEK293	DAMGO	38	Naloxone	20
	Nociceptin (OPRL1)	HEK293	Nociceptin	25	J11397	13
Neurotensin	Dopamine D2S	CHO	PPHT	2	Risperidone	33
Serotonin	Neurotensin type 1 (NTS1)	HEK293	Neurotensin	54	RS142948	15
Serotonin	Serotonin 1A (5-HT1A)	HEK293	Serotonin 5-HT	11	NAN-190	26

Conclusion

This study illustrates the application of the HTRF Gi Binding for Gi-coupled GPCR assay using a panel of GPCR models. Depending on the biological model used in this study, different optimization conditions were selected and a range of optimal pharmacological windows were recorded. The pharmacological characterization of compounds with the HTRF Gi Binding assay were validated using reference agonists and antagonists, and shown to be achieved regardless of the individual differences between all receptor models (biological roles, basal activity behaviors, cellular expression backgrounds, and GPCR expression levels).

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