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APPLICATION NOTE

GPCR compounds identification and pharmacological characterization with GTP Gi binding assay.

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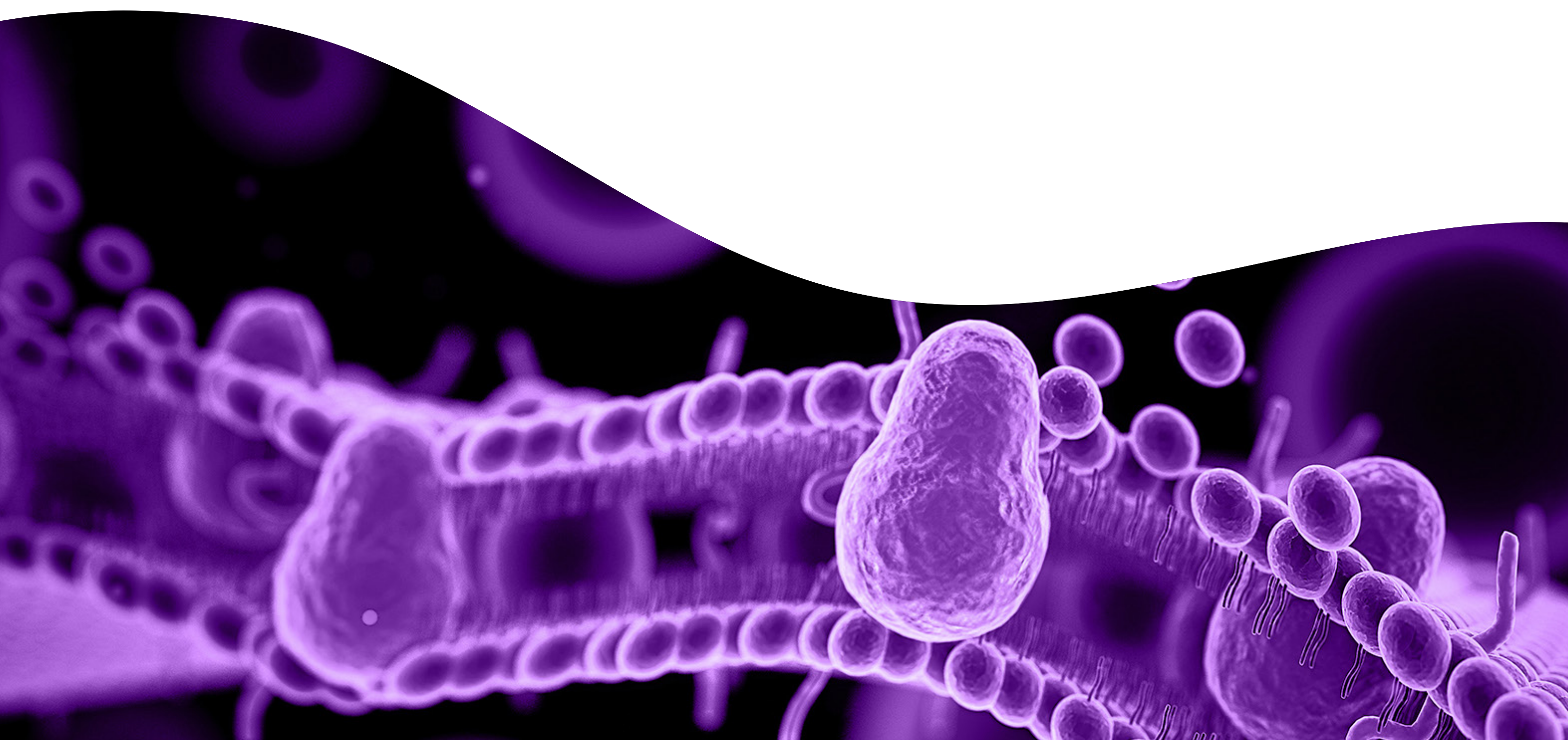
This application note discusses the characterization process for candidate molecules targeting Gα-protein activation through GPCRs with the functional HTRF® GTP binding assay. The assay measures the level of Gi protein activation and has the advantage of studying the functional response of GPCRs at the level of one of the earliest receptor-mediated events.

This non-isotopic kit (#62GTPPET/G) detects Gi protein activation via the binding of Eu-cryptate-labeled non hydrolysable GTP analog (donor) and d2-labeled anti-Gi monoclonal antibody (acceptor).

Over the years, the δ-opioid receptor (DOP) has emerged as a promising target for the development of new pain therapies. Using a CHO membrane model expressing that δ-opioid receptor (#RBHODM400UA), this application note explains how to characterize different classes of pharmacological compounds through several case studies: Full Agonists, Antagonists, Partial Agonists, and Inverse Agonists. Pharmacological data show the ability of the assay to discriminate among the different classes of pharmacological compounds, and to characterize them accurately.



Assay optimization

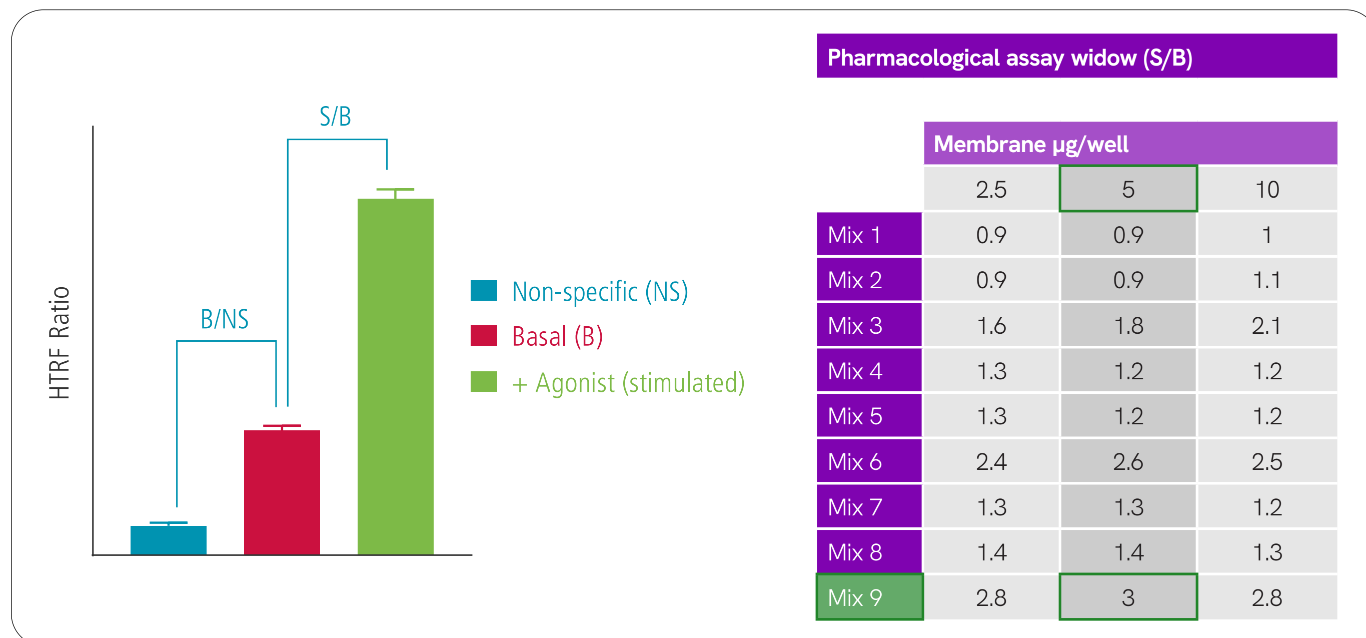
All GTP binding assays require optimization to the model involved, as the GTP binding pocket of G proteins is subject to slight conformational changes depending on the environment, in particular salt levels. This HTRF assay is no exception to the rule, and it must be remembered that a careful optimization of various parameters is generally required. Protocols, recommendations, and detailed guidelines to optimize this HTRF GTP binding assay are well described in the package insert of the kit. Here, the one-step optimization protocol was performed following the procedure described for the CHO δ-opioid receptor (DOR) membrane model, in order to select the optimal GDP and MgCl2 concentrations (Mix 1 to 9) and membrane quantity (2.5 -5- 10 µg/well). The corresponding GDP/MgCl2 Mix concentrations are reported in figure 1.

For each condition, the pharmacological assay window (S/B) was calculated by applying the ratio of membranes treated with 1 µM of SNC-162 agonist (stimulated) to the basal (unstimulated condition). The results of figure 2 show the optimal conditions for this model (highlighted in green): 5 µg/well of membrane and Mix 9 (0.5 µM of GDP and 50 mM MgCl2). These conditions were applied to all the assays featured in this note, with the exception of the inverse agonist pharmacological characterization assay, which relied on experimental conditions described in its dedicated section.

Working and final concentration table

Mix	Working concentration (4X)		"Final concentration in the assay"	
	GDP (µM)	MgCl2 (mM)	GDP (µM)	MgCl2 (mM)
Mix 1	0	8	0	2
Mix 2	0	40	0	10
Mix 3	0	200	0	50
Mix 4	1	8	0.25	2
Mix 5	1	40	0.25	10
Mix 6	1	200	0.25	50
Mix 7	2	8	0.5	2
Mix 8	2	40	0.5	10
Mix 9	2	200	0.5	50

Figure 1: GDP and MgCl2 concentrations in the different mixes.



Pharmacological assay widow (S/B)

	Membrane µg/well		
	2.5	5	10
Mix 1	0.9	0.9	1
Mix 2	0.9	0.9	1.1
Mix 3	1.6	1.8	2.1
Mix 4	1.3	1.2	1.2
Mix 5	1.3	1.2	1.2
Mix 6	2.4	2.6	2.5
Mix 7	1.3	1.3	1.2
Mix 8	1.4	1.4	1.3
Mix 9	2.8	3	2.8

Figure 2: Results of the CHO-DOR membrane optimization step to select the optimal condition (highlighted in green).

Agonist pharmacological characterization

Selective activation of the δ-opioid receptor (DOR) has great potential for the treatment of chronic pain. In particular, the resulting ancillary anxiolytic and antidepressant-like effects appear to be less adverse than for other opioid receptors, such as μ-opioid (MOR).

This section describes the method for full agonist characterization and shows data from a real case study. The effects of four well-known full agonists were measured in CHO membranes expressing the δ-opioid receptor using the functional GTP Gi binding assay. The agonist mode protocol and the pharmacological parameters thus determined were in line with the literature, with correct potencies and ranking of the different agonist compounds.

Agonist mode protocol and pharmacological parameter determination

Full agonist characterization uses the protocol described here. A range of agonist concentrations was used to stimulate the receptor in a dose-response manner. The addition of the detection reagent mix enabled detection of GTP binding to the Gi protein. The plate was read after overnight incubation at 22°C. The optimized parameters previously determined for this CHO-DOR membrane model were used, as follows: the kit stimulation buffer was supplemented with 0.5 µM of GDP and 50 mM of $MgCl_2$, and the assay was run with 5 µg/well of membrane. Dispensing order and volumes are detailed in figure 3.

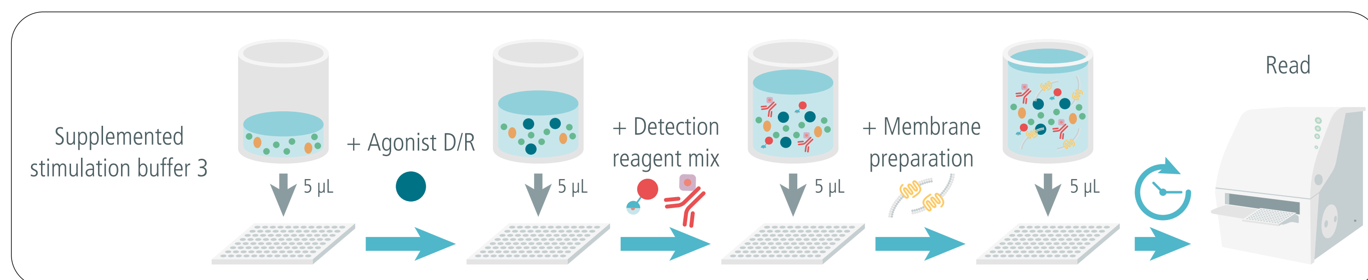


Figure 3: HTRF GTP Gi Binding assay protocol for Agonist characterization.

The compound SNC-162 (SNC-80 derivative) was taken as a case study to illustrate the determination of full agonist pharmacological parameters. The signal recorded is directly proportional to the system activation in a dose-dependent manner. The signal is plotted over the concentration range of the compound to obtain the agonist dose-response curve and to determine its potency (EC50) and efficacy (100% maximal effect for a full agonist) (Figure 4). As shown in the figure, SNC-162 displays a potency of 6.6 nM, which is accordance with published values of 8.4 nM with a cyclase functional assay[1].

To characterize and compare a range of agonists, a reference compound can be used. It is thus possible to convert the data into % of effect, where 0% is the basal (no compound addition), and 100% is the maximal sustained response (agonist saturation effect). This conversion does not change the curve behavior and EC50 value.

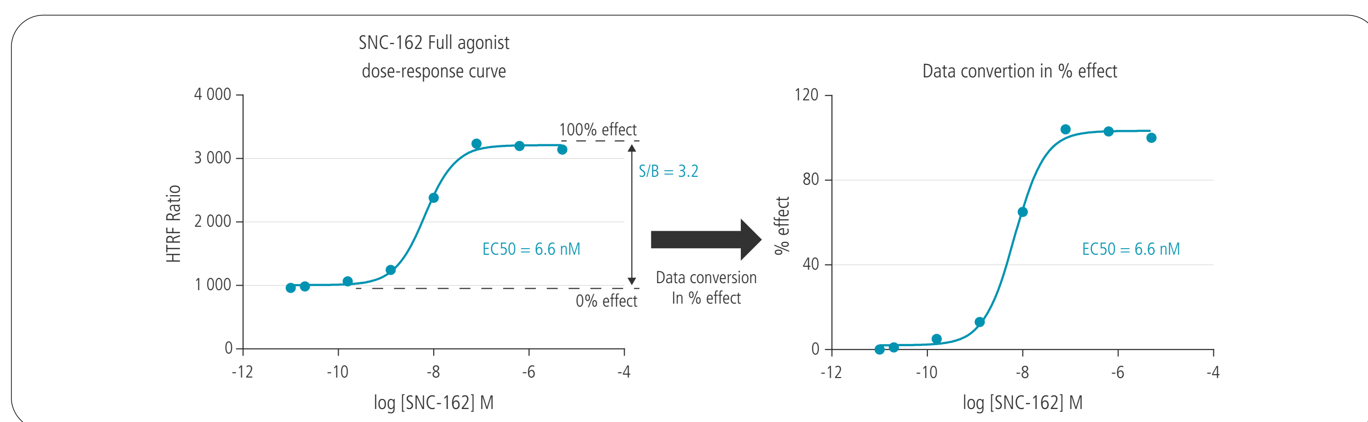


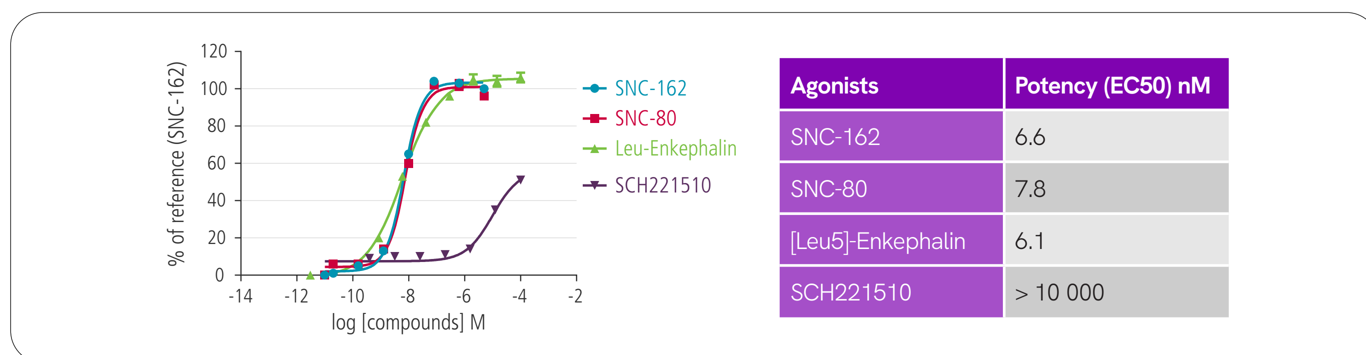
Figure 4: Full agonist pharmacological parameter determination (δ-opioid receptor case study).

Agonist characterization and ranking

Four agonists (SNC-162, SNC-80, Leu-Enkephalin, and SCH221510) were characterized in the CHO-DOR membrane model using the HTRF GTP Gi binding assay. Published functional assays (cyclase pathway with cAMP measurement) describe SNC-80, SNC-162, and Leu-Enkephalin as being in the same range of potency, with 8.2 nM, 8.4 nM, and 8.5 nM respectively[1]. The reported potency of SNC-80 in radioactive $^{[35S]}$GTPγS assay is 5-19 nM[2,3]. The SCH221510 agonist, more selective for the nociceptin receptor than for the δ-opioid one, has a potency in the μM range[4].

The titration curves of these four compounds are presented in figure 5, and the pharmacological parameters are reported in the associated table. The dose-response curves were expressed in % of reference compound (SNC-162).

The SNC-162, SNC-80, and the Leu-Enkephalin show the same potencies (nM range), while the SCH221510 is dramatically less potent (μM range). All these data are in accordance with published values[1,2,3,4].



Agonists	Potency (EC50) nM
SNC-162	6.6
SNC-80	7.8
[Leu5]-Enkephalin	6.1
SCH221510	> 10 000

Figure 5: δ-opioid receptor agonist characterization and ranking using the HTRF GTP Gi Binding assay.

Antagonist pharmacological characterization

Antagonists are a major class of experimental drugs. The method for antagonist characterization is presented in this section, which features antagonist mode protocol, pharmacological parameter determination, and antagonist characterization on the CHO-DOR receptor case study.

Antagonist mode protocol and pharmacological parameter determination

To detect antagonists and then characterize them, a dose titration curve was performed with a range of antagonist concentrations in presence of a fixed concentration of agonist with EC80-EC90 (80%-90% of maximal effect). The protocol is described in figure 6. Briefly, a concentration of antagonist is added as a first step, followed by the addition of agonist, detection reagent mix, and then membranes. CHO cell membranes expressing the δ-opioid receptor (DOR) were used as the model in this case study. The optimal assay parameters previously determined for this biological model were used, as follows: 5μg/well of membranes, 0.5 μM of GDP, and 50 mM of MgCl2. Plates were read after overnight incubation at 22°C.

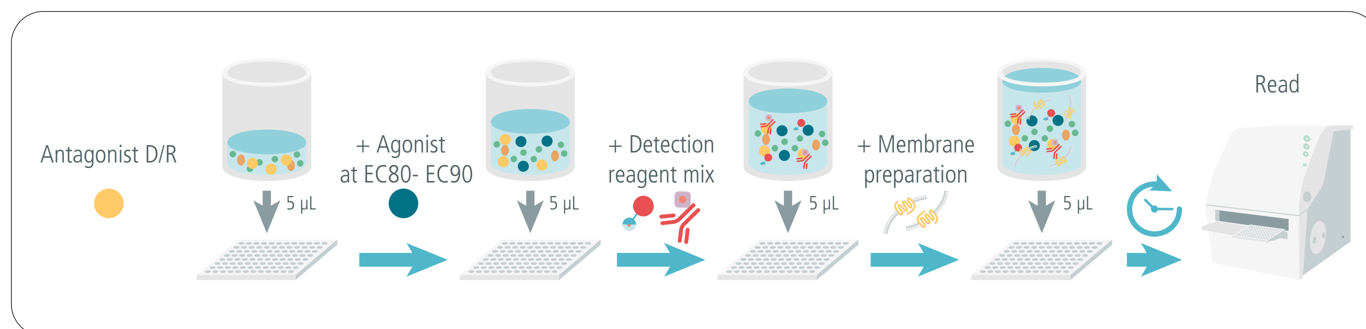
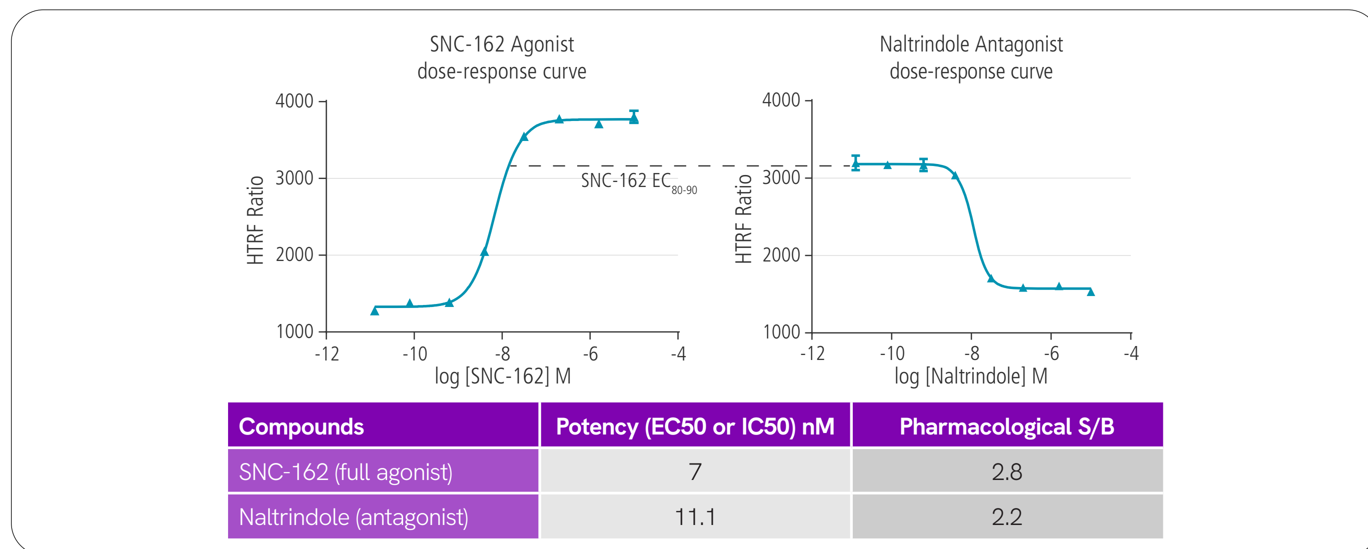


Figure 6: HTRF GTP Gi Binding assay protocol for antagonist characterization.

Antagonist characterization assays require an initial experimental step to determine the optimal agonist concentration giving 80-90% of maximal effect (Agonist EC80-90). In these conditions, the antagonist assay is performed in non-saturating conditions for accurate pharmacological characterization maintaining optimal assay window for the model studied. Figure 7 shows the optimal SNC-162 agonist concentration selected for the CHO δ-opioid membrane model corresponding to EC80-90 (15 nM). The resulting potencies and pharmacological assay windows are presented in the associated table.

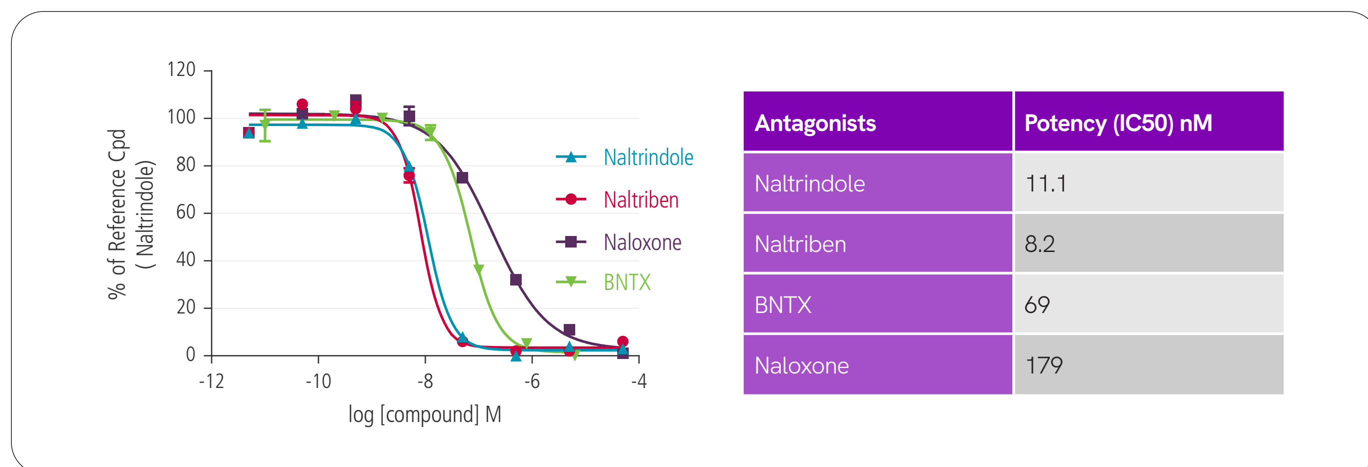


Compounds	Potency (EC50 or IC50) nM	Pharmacological S/B
SNC-162 (full agonist)	7	2.8
Naltrindole (antagonist)	11.1	2.2

Figure 7: Antagonist pharmacological parameter determination (δ-opioid receptor case study).

Antagonist characterization and ranking

Figure 8 shows a panel of antagonist compounds characterized and ranked in the CHO-DOR model using the previously described optimized conditions. The compounds were Naltrindole (considered as reference compound for this study), Naltriben, BNTX, and Naloxone. Antagonist potency ranking for the δ opioid receptor (DOR) is in accordance with the literature. Compared to Natrindole, Naltriben is reported to display the same potency in the nM range, followed by the 10-fold less potent BNTX, and the 100-fold less potent Naloxone[5].



Antagonists	Potency (IC50) nM
Naltrindole	11.1
Naltriben	8.2
BNTX	69
Naloxone	179

Figure 8: δ-opioid receptor Antagonist characterization using the HTRF GTP Gi Binding assay.

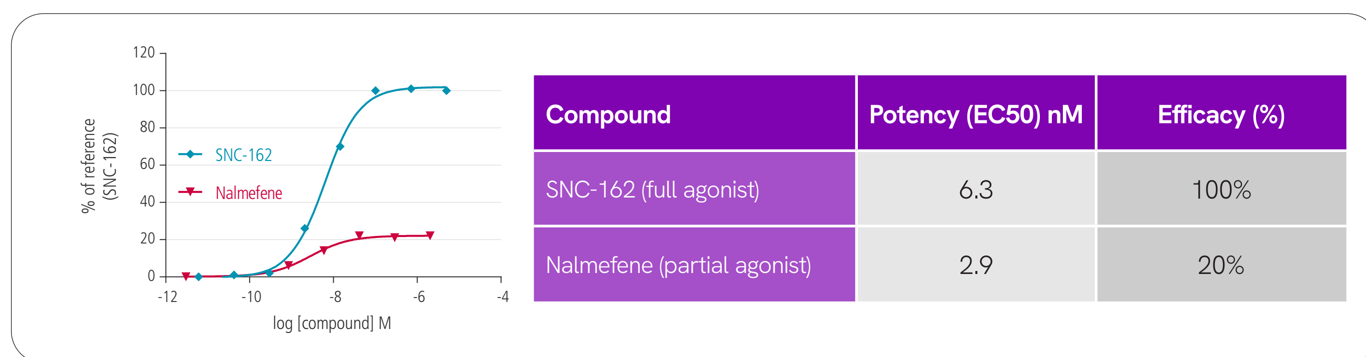
Partial agonist pharmacological characterization

Partial agonists are a class of compounds characterized by their lower efficacy compared to full agonists.

The drug Nalmefene, approved for alcohol management and other dependency treatments, is described as a modestly selective opioid antagonist. It is more selective for μ opioid and κ opioid than for δ opioid receptors, as its affinity for the latter is in the low nM range (reported Ki of 16 nM[6]). Interestingly, partial agonism of Nalmefene was described in the literature for κ and δ receptors. Toll et.al highlighted its partial agonism for δ-opioid receptors using [35S]GTPγS radioactive assays (potency of 30 nM and efficacy of 60%)[7].

Nalmefene pharmacological characterization in the CHO-DOR membrane model with the HTRF GTP Gi binding functional assay was performed using the agonist mode protocol (figure 3) and the optimized parameters described previously (figure 2).

The results of Nalmefene characterization is compared to the full agonist SNC-162 in figure 9. These results demonstrate that both SNC-162 and Nalmefene produce increasing Gi protein activation turnover, with a partial agonism activity for Nalmefene. The pharmacological parameters are reported in the associated table and show potency and efficacy values in agreement with the literature[1,6,7].

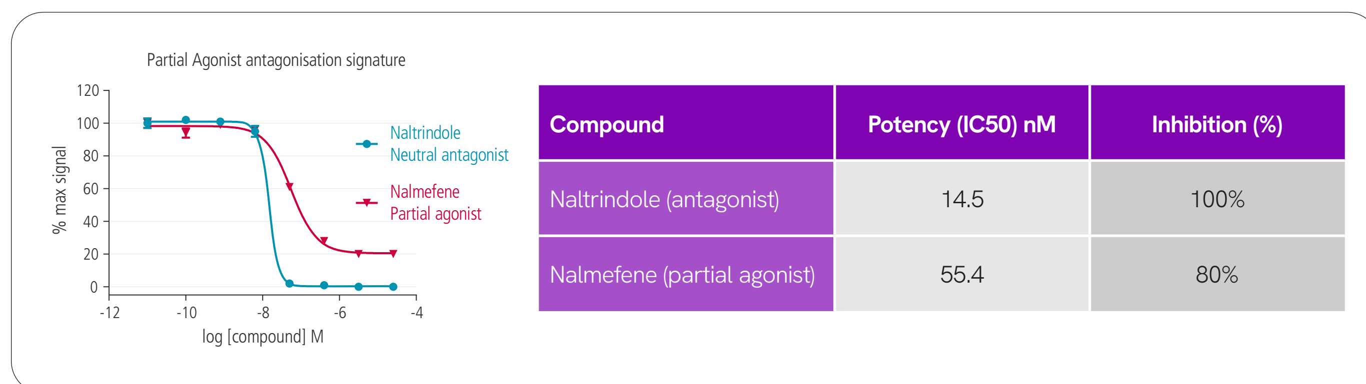


Compound	Potency (EC50) nM	Efficacy (%)
SNC-162 (full agonist)	6.3	100%
Nalmefene (partial agonist)	2.9	20%

Figure 9: δ-opioid receptor Partial agonist characterization using the HTRF GTP Binding assay.

In the presence of full agonists, partial agonists (orthosteric) are known to display antagonist-like effects due to the low-potency competition they introduce[8]. The typical signature of this antagonism is an inhibition dose-response curve with partial sustained inhibition at high concentrations (Orthosteric competition and agonism effect at high concentration).

This partial agonist typical behavior is demonstrated for Nalmefene in figure 10. The antagonist mode protocol (figure 6) was used to perform the experiment, using the previously described optimized parameters and with the SNC-162 agonist concentration at EC80 value. The neutral antagonist Naltrindole, which gives 100% sustained inhibition, was taken as a control. The data show potencies in agreement with published values[6]. Moreover, the partial sustained inhibition at a rate of 80% is consistent with the Nalfemene efficacy of 20% obtained previously (figure 10).



Compound	Potency (IC50) nM	Inhibition (%)
Naltrindole (antagonist)	14.5	100%
Nalmefene (partial agonist)	55.4	80%

Figure 10: Antagonism signature of the Nalmefene δ-opioid Partial agonist.

Inverse agonist pharmacological characterization

The existence of spontaneously active receptors leads to a constitutively active system. Ligands that block this effect are called inverse agonists because of their ability to revert active receptors to an inactive state, which gives them a negative efficacy (selective affinity for the inactive state receptor). The identification of an antagonist as an inverse agonist or neutral antagonist appears to depend on the state of the receptor. Following agonist treatment, many neutral antagonists and weak partial agonists have been reported to actually be inverse agonists.

The inverse agonism of ICI174864 for the δ opioid receptor is described in the literature, which reports it as behaving as an inverse agonist in the cyclase pathway[9]. This response was evaluated in the CHO-DOR membrane model using the functional HTRF GTP Gi binding assay.

To highlight the negative efficacy of an inverse agonist, detection of the receptor constitutive activity (basal response) is first required. The δ opioid receptor shows constitutive activity, and like many GPCRs, this activity was demonstrated to be dependent on Mg^{2+} ions (MgCl2). Figure 11 shows the effect of the magnesium concentration on the basal (data point with no-agonist addition, highlighted in yellow) and the agonist SNC-162 induced dose-response curve (highlighted in blue). MgCl2 has a dramatic effect on DOR constitutive activity in a dose-dependent manner, which warrants a specific optimization of its concentration when running inverse agonist assays.

The optimization step was performed for inverse agonist characterization in order to select the optimal assay conditions (constitutive activity detection, to show the negative efficacy of the compound). Figure 12 presents the results obtained with Mix 9 of MgCl2/GDP (figure 1), and membrane titration. Two conditions were selected to perform the assay: 5 µg of membrane/well, with mix 2 (no GDP/10 mM MgCl2) and mix 3 (No GDP/ 50 mM MgCl2).

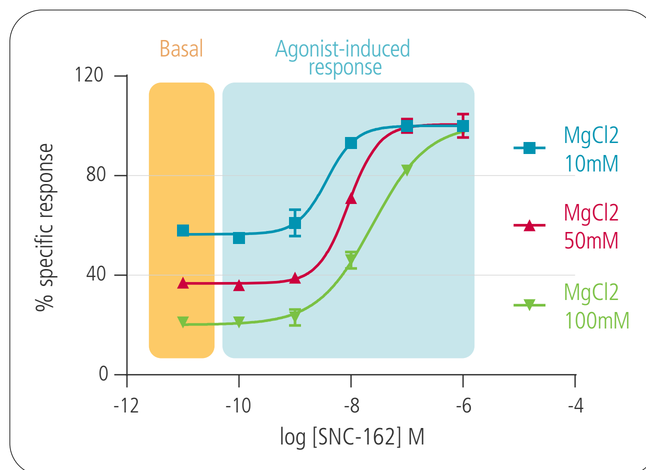


Figure 11: Basal and agonist-induced response in the CHO-DOR membrane model depending on MgCl2 concentrations.

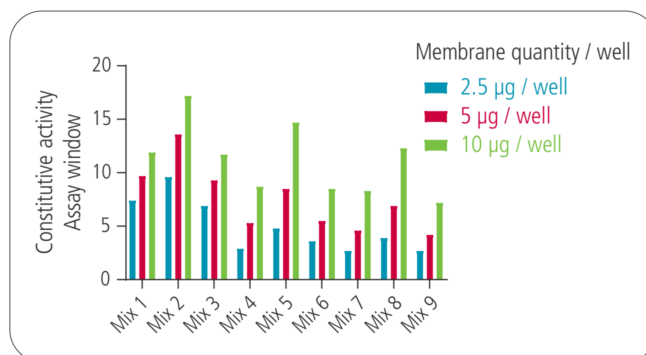


Figure 12: δ-opioid receptor optimization experiment for basal response detection.

Titration of the ICI174864 compound was performed as follows, in the same way as the agonist mode protocol (figure 13).

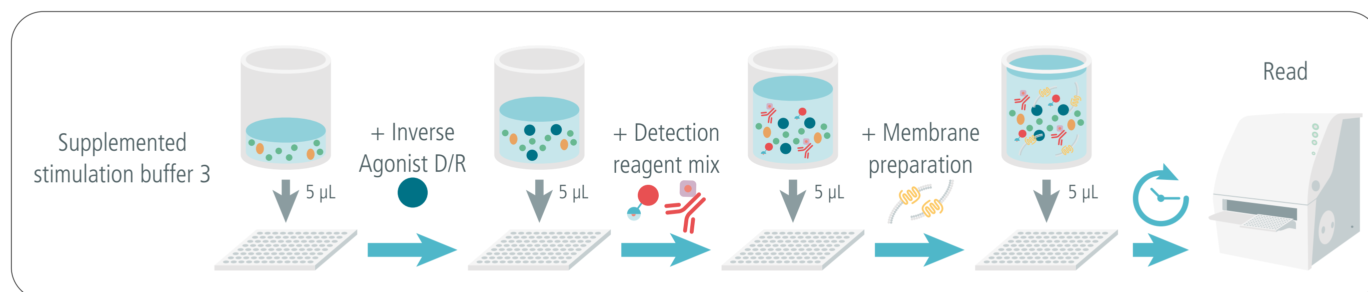
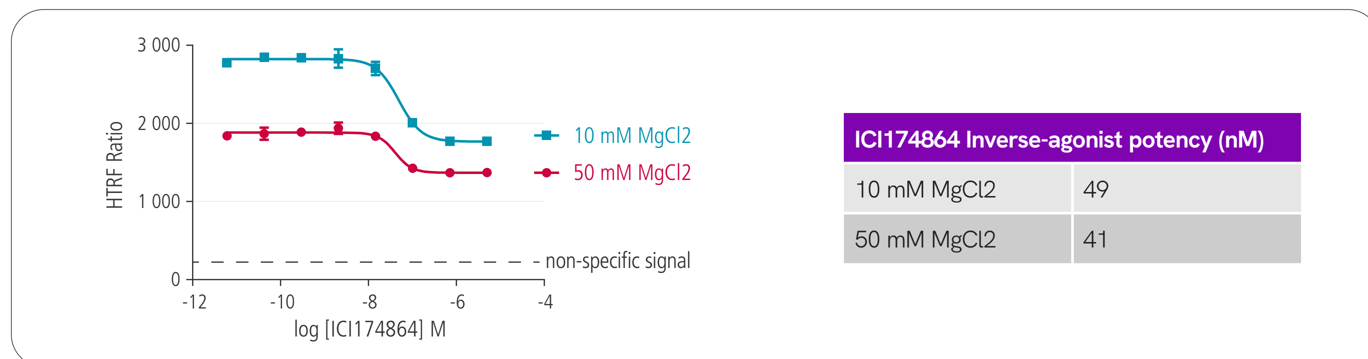


Figure 13: HTRF GTP Gi Binding assay protocol for Inverse agonist characterization.

The results clearly show the negative efficacy of ICI174864 as a consequence of δ-opioid receptor constitutive activity inhibition (in comparison with the positive efficacy of the SNC-162 agonist in figure 4). The dose-response curves of the ICI174864 using 10 mM or 50 mM of MgCl2 (Mix 2 and Mix 3) enable ICI174864 potency determination, which remains the same at both MgCl2 concentrations.



ICI174864 Inverse-agonist potency (nM)	
10 mM MgCl2	49
50 mM MgCl2	41

Figure 14: δ-opioid receptor inverse agonist characterization using the HTRF GTP Binding assay.

Conclusion

This application note demonstrates the HTRF GTP Gi binding assay's ability to characterize different classes of pharmacological compounds accurately. The δ opioid receptor (DOR) expressed in CHO membranes was used as a case study for full agonist, antagonist, partial agonist, and inverse agonist reference compound characterization. The results show the correct identification, pharmacological characterization, and ranking of all the compounds tested. Moreover, this note highlights the necessity for a preliminary optimization step to ensure the assay is run within the optimal conditions to detect each pharmacological class of compound. Accurate characterization is essential in evaluating the *in vivo* efficacy of candidate therapeutics.

References

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