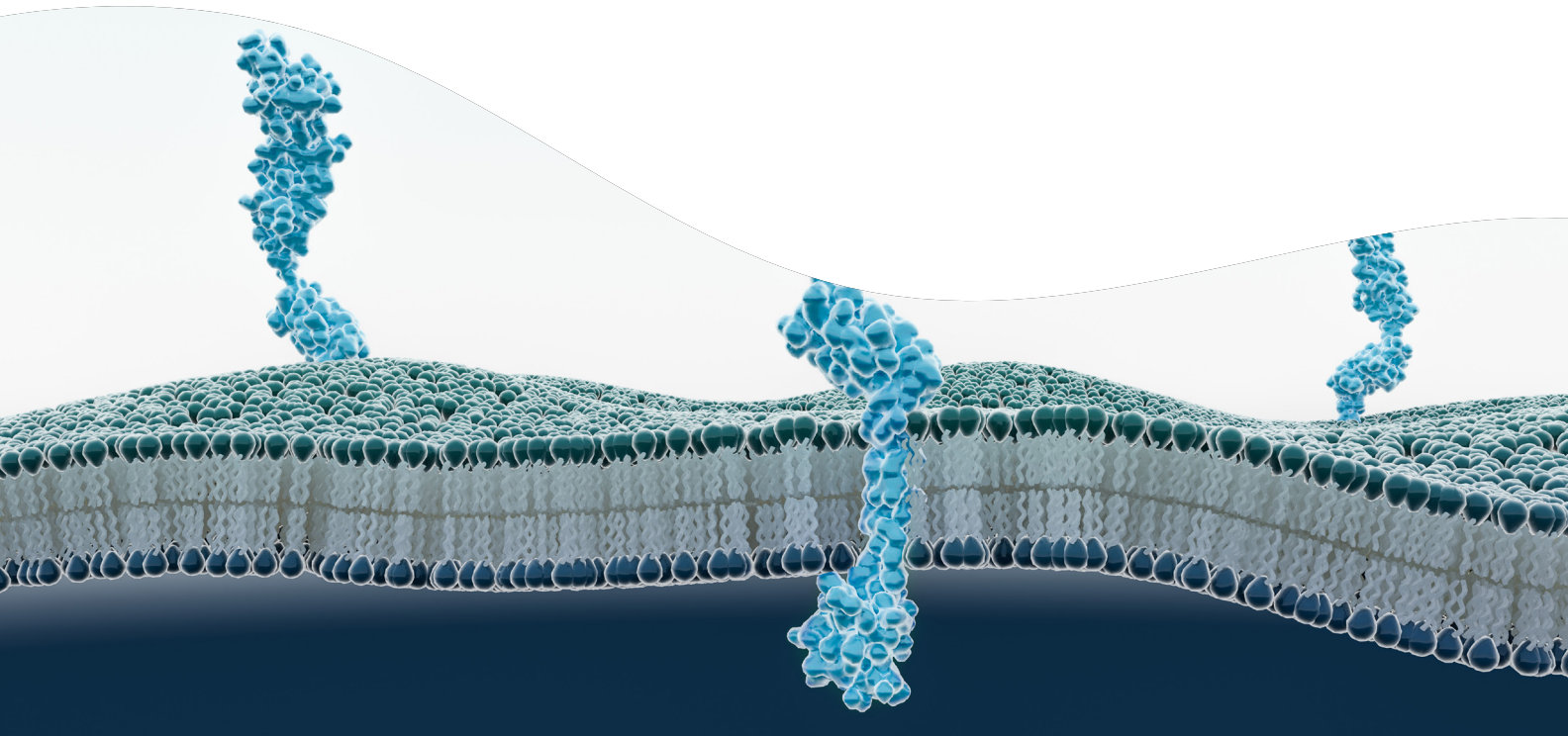
A simple method for preparing GPCR membrane model extracts from stable cell lines for use with the HTRF GTP Gi binding assay.

Introduction

G-protein coupled receptors (GPCRs) are a large family of highly studied transmembrane proteins that localize to the plasma membrane of cells. They play a critical role in the transduction of extracellular signals (such as hormones, neurotransmitters, ions, and photons) and are responsible for the majority of cellular responses.[1] GPCRs have been implicated in several areas such as cardiovascular, metabolic (obesity), neurodegenerative (Alzheimer's disease, Parkinson's disease), psychiatric (depression, dementia), cancer, and infectious diseases. In line with this prevalence in disease pathogenesis, GPCRs molecular targets make up more than 30% of all FDA-approved therapeutics in the current clinical landscape.[2]

For a large majority of GPCRs, agonist binding to the receptor's extracellular domain promotes conformational changes on the intracellular side, enabling the activation of distinct subtypes of heterotrimeric G proteins (consisting of Gα, Gβ and Gγ subunits). Concretely, the activation of G proteins results in its conformational changes that stimulates guanine nucleotide exchange in the Gα subunit, exchanging GDP for GTP. The GTP-bound Gα subunit then dissociates from the GPCR, and in turn, initiates signaling cascades by interacting with downstream effectors.[3] Conversely, GPCR antagonists block agonist-driven and/or basal GPCR activity thus inhibiting downstream signaling events.[4]



The HTRF™ GTP Gi binding assay (62GTPPET, 62GTPPEG - Revvity) is a TR-FRET-based, no-wash immunoassay technology that measures Gαi activation upon agonist stimulation of GPCRs coupled to Gi. The assay is based on the binding of an Eu-cryptate-labeled non-hydrolysable GTP analog (donor) and a d2-labeled anti-Gi monoclonal antibody (acceptor). This assay has the advantage of studying the functional response of GPCRs at the level of one of the earliest receptor-mediated events. This kit can be used to screen for the effect of a compound on Gαi activity by using commercially available model membranes (Revvity) derived from stable CHO or HEK293 cell lines overexpressing a GPCR of interest. However, the expense of purchasing model membranes, in addition to the HTRF GTP Gi binding assay, may be cost-prohibitive for some laboratories.

This technical note provides a detailed method for the preparation of model membranes from stable CHO or HEK293 cell lines overexpressing GPCRs of interest and their subsequent characterization using the HTRF GTP Gi binding assay. Laboratory-prepared CHO membranes overexpressing the Mu Opioid Receptor (CHO-MOR) were validated by a third-party (Euroscreen) using a radioligand (^{3}H-DAMGO) binding experiment, with measured Bmax and K_D values similar to commercially available CHO-MOR membranes. Lab-made CHO membranes overexpressing the neurotensin receptor 1 (CHO-NTS1) were used to titrate GDP and $MgCl_2$ concentrations, as an example of a preliminary optimization step for GTP binding assays. The impact of $MgCl_2$ on dose-response curves generated using the HTRF GTP Gi binding assay was examined using lab-made CHO-MOR membranes and the MOR-agonist, DAMGO. Additional dose-response experiments set up using CHO-NTS1, CHO-MOR, and CHO-DOR (Delta Opioid Receptor) lab-made membranes with respective GPCR agonists and antagonists resulted in curves with EC50 and IC50 values close to previously published data on commercially available model membranes. This demonstrates that lab-made GPCR membrane models work just as well as commercially available membranes for use with the HTRF GTP Gi binding assay.

Methods

Preparation of cell culture

Stable CHO and HEK293 cell lines overexpressing GPCRs of interest were cultured in T175 flasks at 37 °C under 5% CO_2 until fully confluent. The medium from each flask was removed and discarded. The adherent cells were washed with 10 mL of phosphate-buffered saline (PBS), and 5 mL of Cell Dissociation Buffer (Gibco) was added to each T175 flask. Flasks containing HEK293 cells were incubated at 37 °C for 10 – 15 minutes and cells were collected with a pipette. Flasks containing CHO cells were incubated at 37 °C for 1 hour, and the remaining adherent cells were removed using a cell scraper. A 5 mL volume of PBS was added to each flask and used to resuspend the cells. The cells were removed from the flask and transferred to a 50 mL conical tube. The cells were enumerated and centrifuged at 300 x g for 5 minutes to get rid of organelles (ER, golgi, nucleus). The supernatant was discarded, and the cell pellet was stored on ice for further processing.

Cell lysis and membrane preparation

Refer to **Figure 1** for an illustration of the steps involved in the membrane preparation process. A bulleted procedure is provided for these cell culture, lysis, and membrane preparation methods in **Appendix I**. All prepared buffers were filtered before use and stored at 4 °C. Protease inhibitors were added to the Lysis Buffer (10 mM Tris-HCl, pH 7.4, 5 mM EDTA, complete ULTRA Protease inhibitor tablets, EDTA-free – 1 per 50 mL of buffer) immediately before use.

<u>All membrane preparation steps were conducted on ice or at 4 °C:</u>

Each cell pellet was resuspended in 25 mL of Lysis Buffer and incubated at 4 °C under constant agitation (Mini LabRoller™) for 45 minutes. The cells were mechanically lysed using a handheld homogenizer (Ultra Turrax® T 25) with four cycles of 20,000 rpm for 15 seconds/cycle. The lysate was centrifuged at 300 x g for 10 minutes at 4 °C and the supernatant was collected in polycarbonate tubes, taking care to not disturb the pellet. The supernatants were centrifuged again at 43,000 x g for 20 minutes at 4 °C. This time, the supernatants were discarded, and the pellet was resuspended in 500 µL of Storage Buffer (50 mM Tris-HCl, pH 7.4, 0.5 mM EDTA, 10 mM $MgCl_2$, 10% sucrose) by carefully pipetting up and down until homogeneous. The membranes were aliquoted into 50 µL aliquots, and one aliquot was retained for use in a BCA assay to determine the total protein concentration of the membrane preparation. The remaining aliquots were snap-frozen in liquid nitrogen and stored at -80 °C until used.

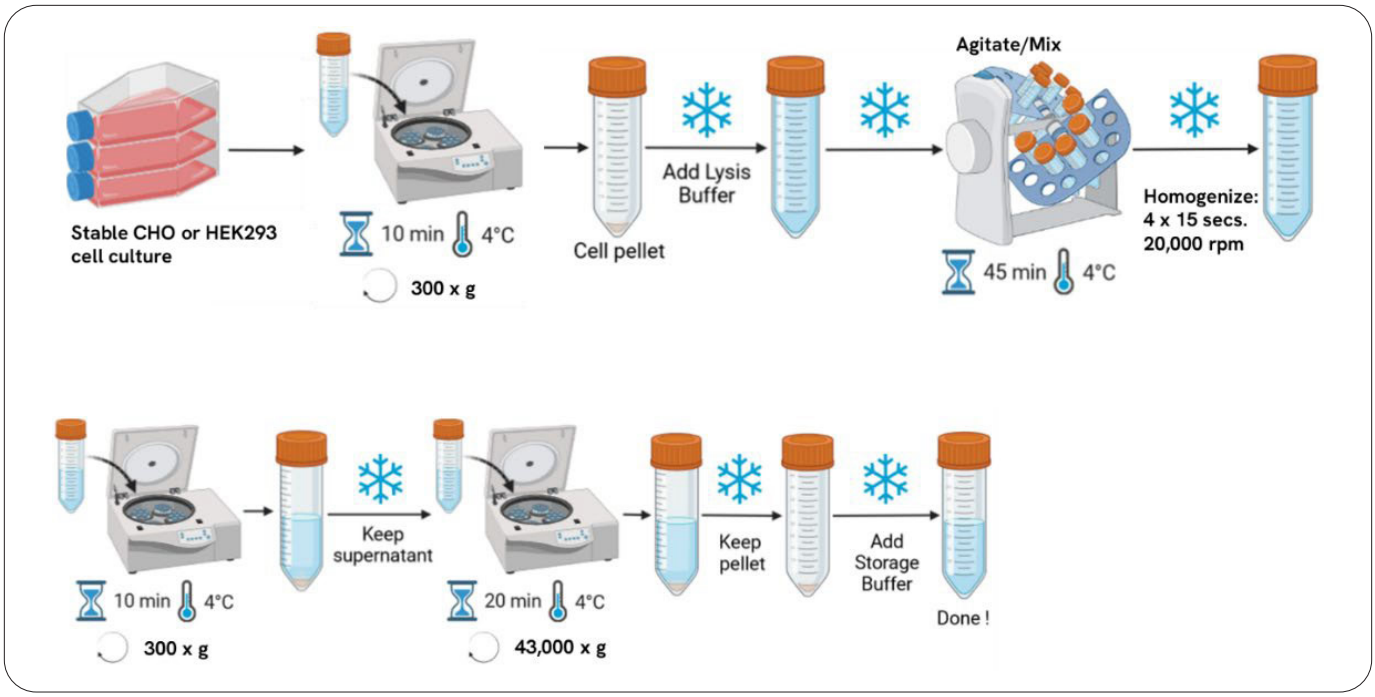
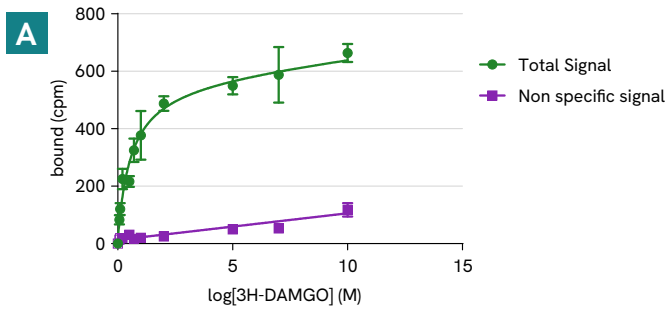


Figure 1: An Illustration depicting the steps involved in the preparation of GPCR membrane models from stable cell culture.

Third-party validation of laboratory-made GPCR membrane models

A radioligand binding assay using ^{3}H-DAMGO and lab-made CHO-MOR membranes was conducted by Euroscreen (Gosselies, Belgium) to evaluate the performances of the GPCR membrane models. The optimal membrane concentration was determined by measuring the total and non-specific binding of ^{3}H-DAMGO at a fixed radioligand concentration against varying concentrations of lab-made CHO-MOR membranes from 2 different membrane preparations in triplicate. The concentration of membranes with the highest signal-to-noise ratio at ≤ 10% bound radioligand was chosen to set up separate radioligand saturation assays to determine the dissociation constant (K_D) and maximum binding capacity (Bmax). These values were compared to values for commercially available CHO-MOR membranes and are provided along with ^{3}H-DAMGO binding curves for lab-made membranes in **Figure 2**. Commercially available membranes had a slightly lower density (Bmax) and slightly tighter binding (K_D) than lab-made membranes. The overall performances of the lab-made membranes were found to be consistent with commercially available membranes.



B

Membrane	KD(nM)	Bmax (pmol/mg)
Lab-made CHO-MOR	0.6	4.1
Commercial CHO-MOR	2.9	3.6

Figure 2: Third-party quality assessment of laboratory-prepared CHO-MOR model membranes. A. ^{3}H-DAMGO saturation of MOR (OP3) receptors in lab-made membrane preparations (1) green vs non-specific binding (purple). The signal is plotted as counts per minute (cpm) of bound radioligand vs the concentration of radioligand in nM. B. Calculated K_D and Bmax values for lab-made and commercially available CHO-MOR model membranes.

HTRF GTP Gi binding assay

The GTP Gi binding assay is intended for the simple, rapid, and direct detection of Gαi protein activation in GPCR membrane preparations. GPCR activation leads to GDP/GTP nucleotide exchange into the Gα subunit, and as such, GTP Gi binding is an upstream readout of Gi protein-coupled receptor activation. The principle of this assay is based on HTRF technology: It uses a non-hydrolysable GTP analog coupled to the fluorescent Europium cryptate donor. In practice, agonist-induced GPCR stimulation leads to Gα protein conformation change and the replacement of Gα-bound GDP by the fluorescent GTP analog in the corresponding binding pocket. Detection is made possible by the addition of d2-labeled anti-Gαi monoclonal antibody (red acceptor) **Figure 3**. When Europium cryptate and d2 are brought into proximity, the energy transfer between them triggers a FRET signal at d2. This specific signal is proportional to the Gαi activation state. The assay enables the direct pharmacological characterization of compounds acting on Gαi-coupled receptors in membrane preparations.

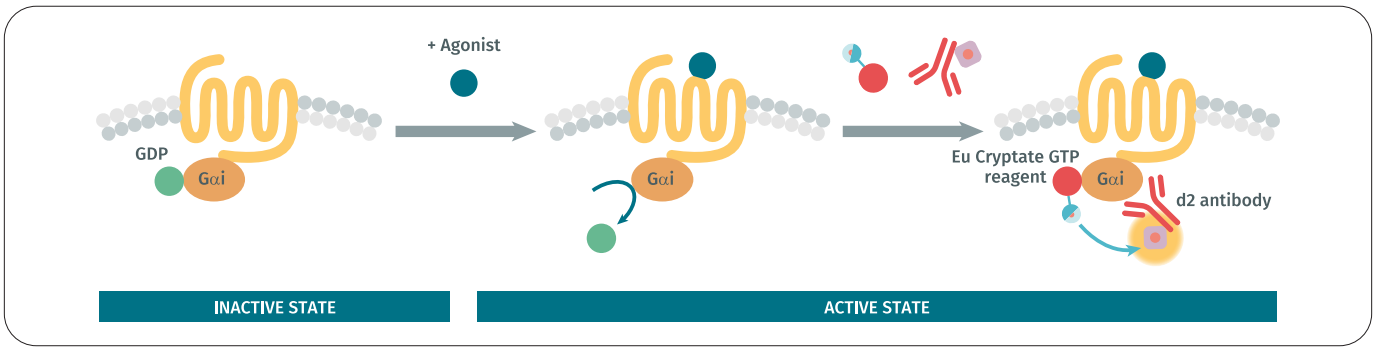


Figure 3: Principle of the HTRF GTP Gi binding assay: GPCR stimulation by agonist induces GDP/GTP nucleotide exchange at the Gαi subunit, leading to the Eu-GTP analog binding to the G protein. Detection is made possible by the addition of the d2-labelled anti- Gαi antibody.

Results

HTRF GTP Gi binding assay optimization using a GDP/$MgCl_2$ matrix and lab-made CHO-NTS1 membranes

Each membrane preparation may have slightly different characteristics, specifically in the expression level of receptors which can vary from one batch to another. For that reason, it is recommended to run an optimization step for each new membrane sample introduced to the assay. The optimization method previously established and described in the app note "GPCR compounds identification and pharmacological characterization with GTP Gi binding assay" was used here (https://www.revvity.com/fr-en/content/pharmacological-validation-panel-gpcrs-htrf-gtp-gi-binding-assay). Consequently, when generating membranes for a project and to avoid having to run the optimization step several times, it is recommended that all the required material be made in one batch and to freeze the resulting membranes.

As shown in the App Note, GDP and $MgCl_2$ are necessary additives for the HTRF GTP Gi binding assay, however, their concentration and molar ratio can influence the signal-to-background (S/B) and their optimal concentration are specific to each GPCR membrane model used with the kit. Therefore, the GDP and $MgCl_2$ concentrations must be titrated using a pre-established loading matrix to find the optimal assay conditions that result in the highest S/B ratio. As an example of this, three different quantity of lab-made CHO-NTS1 membranes (2.5 µg, 5 µg, and 10 µg/well) were mixed with increasing concentrations of GDP and $MgCl_2$ using the matrix listed in **Figure 4**. Each sample was stimulated with either 10 µM of neurotensin (high concentration of NTS1 agonist) or assay buffer (basal). The relative S/B was determined by plating each sample in an HTRF-compatible microplate in triplicate and reading the

HTRF ratio (signal at 665 nm/620 nm x 10^4) on a compatible plate reader. The signal of the neurotensin-treated samples was divided by the signal of the buffer-treated samples to calculate the relative S/B ratio and is listed for each sample in the histograms in **Figure 4**. Here, for this example, stimulation of mix 6 (0.25 µM GDP/50 mM $MgCl_2$) using 10 µg of CHO-NTS1 membranes resulted in the highest S/B ratio at 3.9 and represents the optimal assay conditions for this particular lab-made membrane extract. On the other hand, Mix 6 condition using only 2.5 µg resulted is a good alternative with lower material with S/B ratio at 3.6. A mini-guide for determining the optimal GDP/$MgCl_2$ concentration is provided in the product insert for the HTRF GTP Gi binding assay. Additional kit-specific product inserts, literature, and guides can be found on the Revvity website (www.revvity.com).

	Final Concentration per well								
	Mix 1	Mix 2	Mix 3	Mix 4	Mix 5	Mix 6	Mix 7	Mix 8	Mix 9
[GDP] µM	0	0	0	0.25	0.25	0.25	0.50	0.50	0.50
[$MgCl_2$] µM	2	10	50	2	10	50	2	10	50

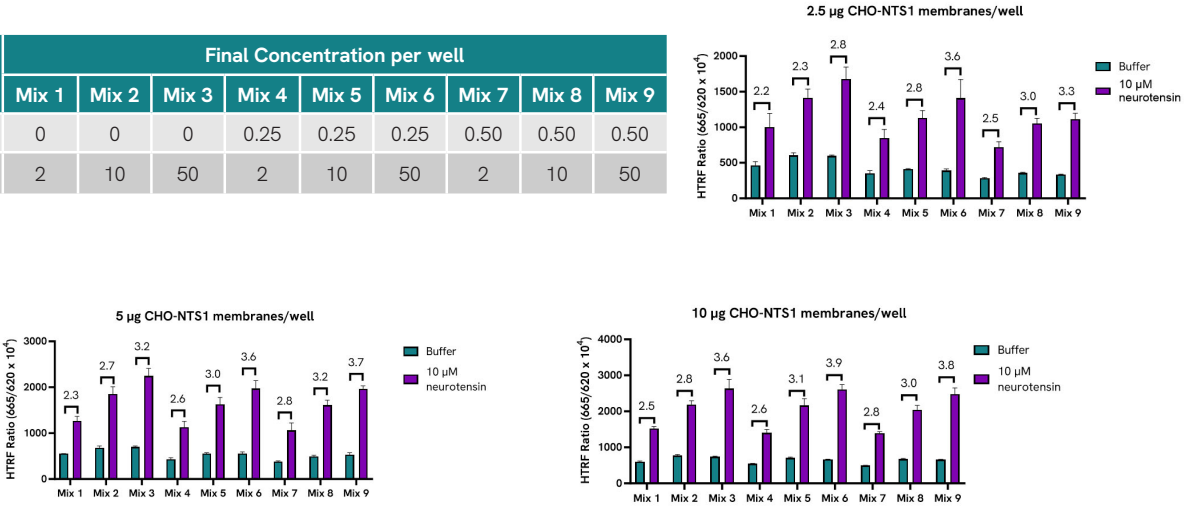
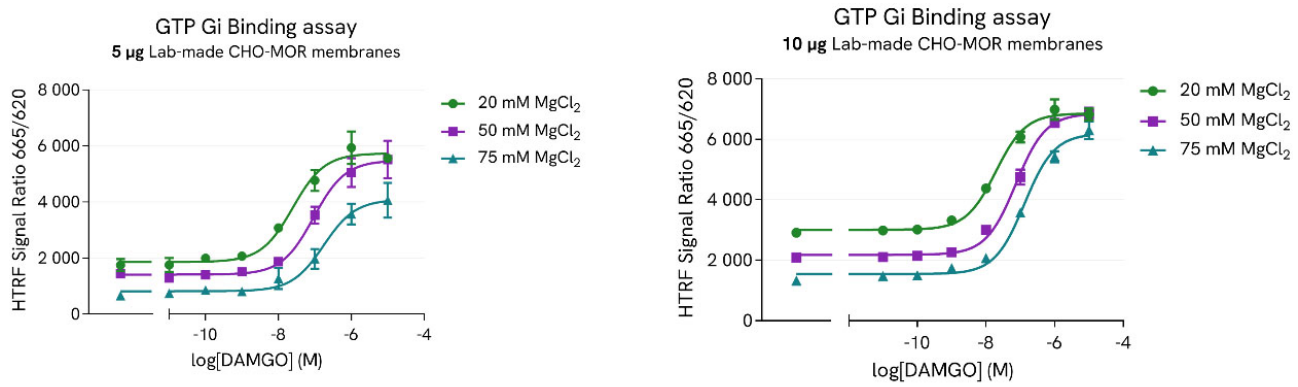


Figure 4: Optimization of GDP/$MgCl_2$ levels for the HTRF GTP Gi binding assay using lab-made CHO-NTS1 membranes. The final concentration of GDP and $MgCl_2$ are listed in the table (top-left). The histograms show the relative HTRF Ratio (signal at 660/620 nm x 10^4) for each mix of GDP/$MgCl_2$ and lab-made CHO-NTS1 membranes stimulated with 10 µM neurotensin and HTRF assay buffer (background). The ratio between the neurotensin-stimulated sample and background is listed above the bars for each mix.

Impact of $MgCl_2$ on dose-response of lab-made CHO-MOR membranes stimulated with DAMGO

Two different amounts of lab-made CHO-MOR membranes (5 µg and 10 µg) were stimulated with increasing concentrations of DAMGO in the presence of 0.25 µM GDP and three different concentrations of $MgCl_2$ (20 mM, 50 mM, and 75 mM) to determine the specific impact of $MgCl_2$ on the dose-response of the membrane extract using the HTRF GTP Gi binding assay. The curves in **Figure 5** were made by plotting the HTRF ratio against the concentration of DAMGO using a four-parameter logistic equation (4PL) with $1/Y^2$ data-weighting in GraphPad Prism. The EC50 and Assay Window (max/min average HTRF ratio) were calculated from the dose-response curves and are provided in **Figure 5**. Increasing the concentration of $MgCl_2$ resulted in an increase in both the EC50 and Assay Window. This $MgCl_2$-dependent shift in the dose-response curves is consistent across all experimental conditions where the GDP concentration is constant. We report no effects from the amount or the concentration of membranes used.



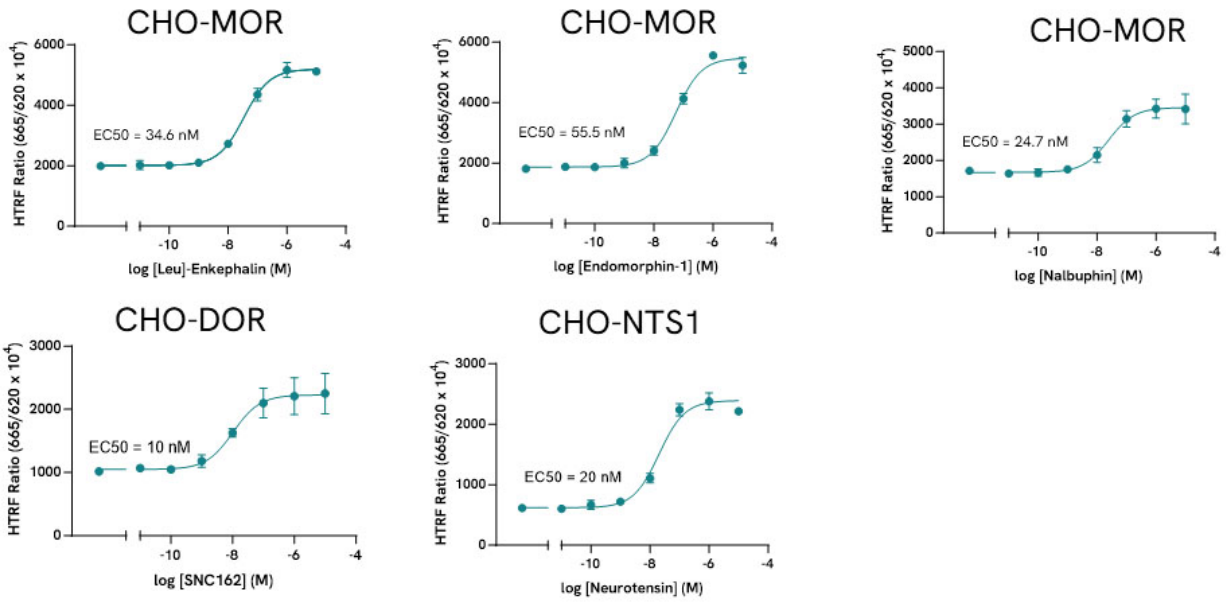
[$MgCl_2$] mM	EC50 (nM)		Assay Window (S/B)	
	5 µg	10 µg	5 µg	10 µg
20	24.5	19.2	3.1	2.3
50	92.1	73.2	4.1	3.1
75	174.7	132	5.0	4.0

Figure 5: Impact of $MgCl_2$ on the dose-response of lab-made CHO-MOR membranes stimulated with DAMGO. Dose-response curves (left) are shown for samples containing 5 µg or 10 µg of CHO-MOR membrane extracts treated with increasing concentrations of the MOR-agonist, DAMGO (with a final concentration of 0.25 µM GDP), and $MgCl_2$ at the listed concentrations. The EC50 and Assay Window for each of the dose-response curves are listed for CHO-MOR membranes at 5 µg and 10 µg respectively.

Evaluating the effect of receptor agonists and antagonists on lab-made GPCR membrane models

The effects of treating lab-made GPCR membrane models overexpressing MOR, DOR, and NTS1 receptors against GPCR agonist and antagonist compounds were determined using the HTRF GTP Gi binding assay. Membrane and compound-specific dose-response curves are provided in **Figure 6** along with the EC50 or IC50 calculated from each curve. The amount of GPCR membrane model per well, optimal GDP and $MgCl_2$ concentrations, and Assay Window for each curve are listed in **Appendix II**. CHO-MOR membranes were treated DAMGO, CHO-DOR membranes were treated with SNC162, and CHO-NTS1 membranes were treated with neurotensin at the EC80 concentration for each agonist prior to setting up the antagonist dose-response curves. CHO-NTS1 membranes were treated with an agonist or antagonist for 3 hours. All other membranes were compound-treated overnight. Notably, lab-made CHO-DOR and CHO-NTS1 membranes had an antagonist dose response that was consistent with the literature and with previously characterized commercially available membranes. These results indicate the GPCR membrane models prepared from stable cell lines overexpressing different receptors using the protocol in this application note are suitable for use with the HTRF GTP Gi binding assay.

Agonist Dose response



Antagonist Dose response

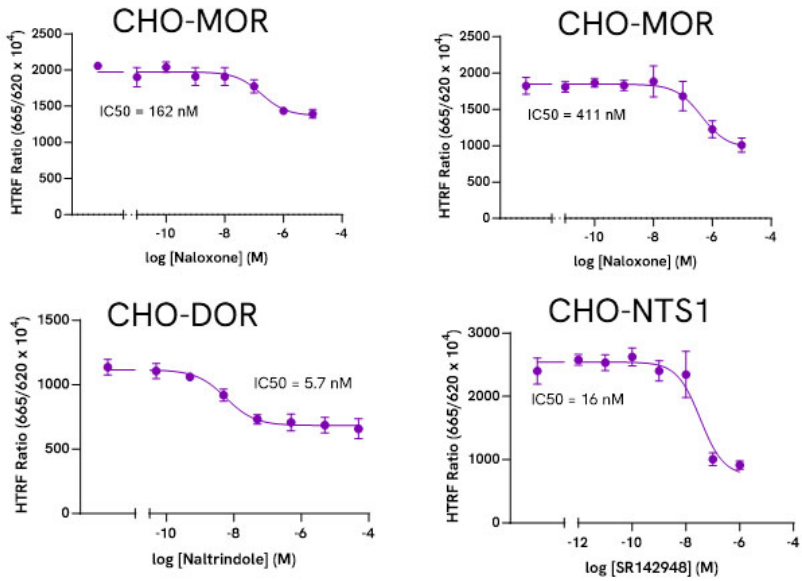


Figure 6: Effects of GPCR agonist and antagonist compounds on lab-made membrane models. Membranes were treated with an EC80 concentration of agonist prior to treatment with an antagonist. CHO-NTS1 membranes were treated for 3 hours, and all other membranes were treated overnight.

Conclusion

GPCR membrane models can be prepared from stable cell lines overexpressing receptors of interest using the protocol included in this app note. Lab-made CHO-MOR membranes were shown to have similar agonist binding parameters compared to commercially available membranes when assessed in a third-party radioligand binding assay. The optimal GDP and $MgCl_2$ concentrations should be determined for a particular membrane preparation using the matrix provided in **Figure 4** or the guide available on the Revvity website to find the conditions resulting in the highest S/B ratio prior to setting up a dose-response experiment. It is important to keep in mind that increasing the concentration of $MgCl_2$ in a sample can increase both the EC50 and Assay Window of dose-response curves set up using the HTRF GTP Gi binding assay. Both agonist and antagonist treatment of lab-made GPCR membrane models resulted in dose-response curves with EC50 or IC50 values that were consistent with previously published values and from commercially available membranes, demonstrating the quality of the lab-made membranes for the use of the HTRF GTP Gi binding assay.

References

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Appendix I.

GPCR membrane model Preparation Protocol

Buffer Recipes

Note: all buffers should be filtered before use and stored at 4 °C. Add protease inhibitors to Lysis Buffer just prior to use.

Lysis Buffer

10 mM Tris-HCl, pH 7.4

5 mM EDTA

1 x complete *ULTRA* Protease Inhibitor Tablet per 50 mL of buffer

Storage Buffer

50 mM Tris-HCl, pH 7.4

0.5 mM EDTA

10 mM $MgCl_2$

10% sucrose

Preparation of cell culture for lysis

1. Remove the cell culture medium from the T175 flask
2. Wash cells with 10 mL of 1X PBS
3. Remove the PBS and add 5 mL of Cell Dissociation Buffer to each flask
4. Incubate HEK293 cells for 10 – 15 minutes at 37 °C
5. Incubate CHO cells for 1 hour at 37 °C and use a cell scraper to remove any remaining adhered cells
6. Add 5 mL of PBS to each flask and gently resuspend the cells by pipetting before transferring to a 50 mL conical tube
7. Enumerate the cells
8. Centrifuge the cell culture at 300 x g for 5 minutes
9. Remove the supernatant and store the cell pellet on ice for lysis and membrane extraction

Cell lysis and membrane extraction

Caution: it is critical that all steps be conducted on ice or at 4 °C.

1. Resuspend the cell pellet in 25 mL of ice-cold lysis buffer
2. Incubate for 45 minutes at 4 °C under constant agitation (e.g. Mini LabRoller/tube rotator)
3. Lyse cells with a cell homogenizer (e.g. Ultra Turrax T25) on ice using four 15 second cycles at 20,000 rpm/cycle
4. Centrifuge the lysate at 300 x g for 10 minutes at 4 °C
5. Collect the supernatant in polycarbonate tubes taking care not to disturb the pellet
6. Centrifuge the supernatant at 43,000 x g for 20 minutes at 4 °C
7. Remove and discard the supernatant to collect the pellet
8. Resuspend the pellet in 500 µL of Storage Buffer and homogenize by gently pipetting up and down
9. Make 50 µL aliquots and retain a single aliquot to be used in a BCA assay to quantify the total protein concentration from each membrane preparation
10. Snap freeze the remaining aliquots in liquid nitrogen and store at -80 °C until needed

Appendix II.

GPCR	Receptor	GPC expression cell line	Membrane/ well (µg)	Optimal condition		Agonist		
				[GDP] µM	[$MgCl_2$] µM	Compound	EC50 (nM)	Assay Window
Opioid	Mu Opioid (MOR)	CHO	5	0.25	20	[Leu5]-Enkephalin	34.6	2.6
						Endomorphin-1	55.5	2.9
						Nalbuphin	24.7	2.0
	Delta Opioid (DOR)		5	0.5	50	SNC162	10	2.2
Neurotensin	Neurotension type 1 (NTS1)		10	0.25	50	Neurotensin	20	3.6

GPCR	Receptor	GPC expression cell line	Membrane/ well (µg)	Optimal condition		Agonist		
				[GDP] µM	[$MgCl_2$] µM	Compound	EC50 (nM)	Assay Window
Opioid	Mu Opioid (MOR)	CHO	5	0.25	20	Naloxone	162	1.5
						Naltrindole	411	1.8
	Delta Opioid (DOR)		5	0.5	50	Naltrindole	5.7	1.7
Neurotensin	Neurotension type 1 (NTS1)		10	0.25	50	SR142948	16	2.6

Supplemental information for lab-made GPCR membranes. The membrane per well concentration, optimal GDP and $MgCl_2$ concentration, EC50, and Assay Window are listed for each model membrane and compound used to generate the dose-response curves shown in **Figure 6**.