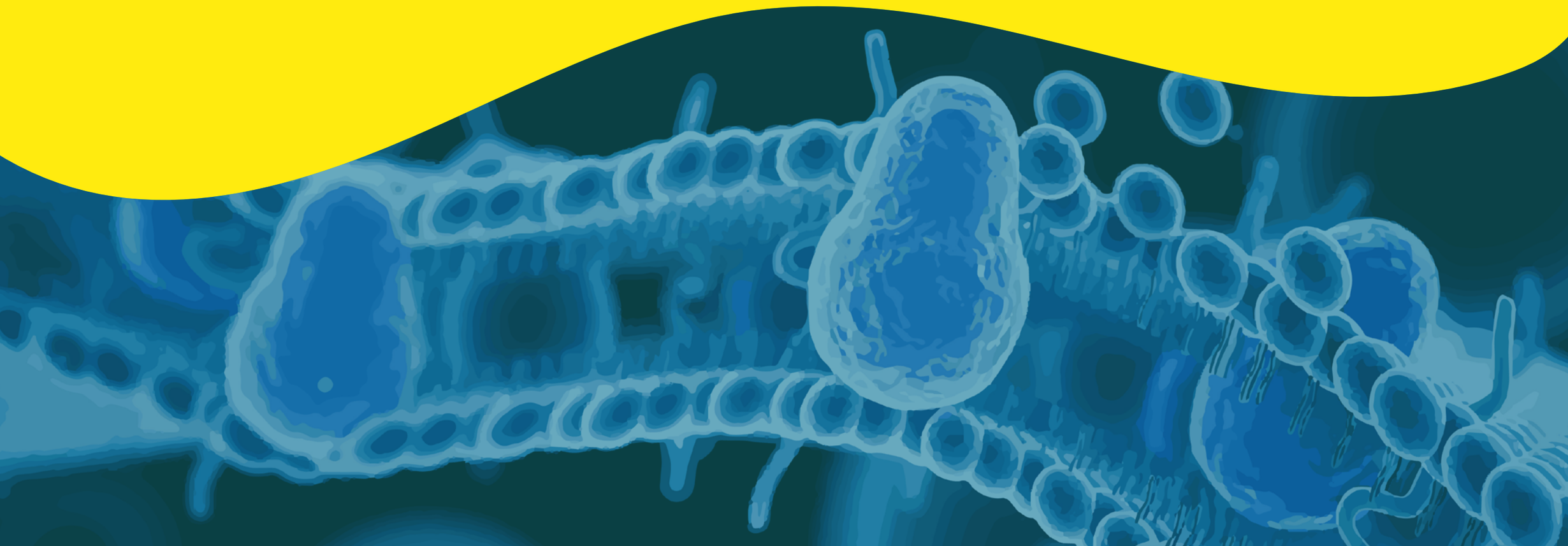
GTP binding assay

A guide to optimizing agonists of Gαi



PURPOSE & BACKGROUND

Purpose

The proper optimization of assay conditions is essential to ensure you obtain the best reagent use and performance. In the case of GPCR signaling, the most suitable assay conditions depend on the GPCR coupling efficacy as well as the pharmacological characteristics of the compound being studied (agonist or antagonist). The optimal assay conditions will also hinge on the particular membranes used for the assay. For each setup, a number of optimization steps can be implemented to ensure the best, most accurate results.

Drawing on hundreds of data sets and cases established by Revvity's scientists over the years, this manual provides the GPCR community with the most up to date guidelines for the optimization of HTRF GTP binding assays.

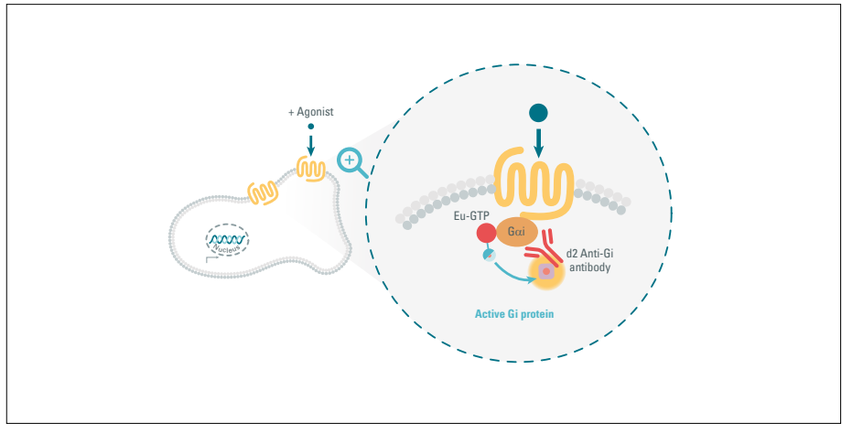
Background

The HTRF GTP binding assay measures the level of Gi protein activation following agonist stimulation of GPCRs. This assay has the advantage of studying the functional response of GPCRs at the level of one of the earliest receptor-mediated events. Following GPCR stimulation, the activation process of the heterotrimeric Gi protein leads to the dissociation of GDP from the Gα subunit, where it is replaced by the assay fluorescent GTP analog as the Gα protein goes from an inactive to an active state.

Optimization of the GTP binding assay in a membrane system is a key step, as both GPCR and G protein regulation are highly impacted by divalent cations and the presence of additives like GDP.

This non-isotopic GTP Gi binding assay developed by Revvity is specifically intended to measure Gi protein activation via the direct detection of Eu cryptate-labeled non hydrolysable GTP analog (donor) binding to GPCR-bound Gi proteins, in presence of d2-labeled anti-Gi monoclonal antibody (acceptor).

	GTP Gi binding Assay
100 tests	# 62GTPPET
500 tests	# 62GTPPEG



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GTP binding assay: An agonist binding to the GPCR induces GDP/GTP nucleotide exchange on the G alpha protein subunit, leading to the Eu-GTP analog binding. Detection is enabled by the addition of the d2 anti-Gi antibody.

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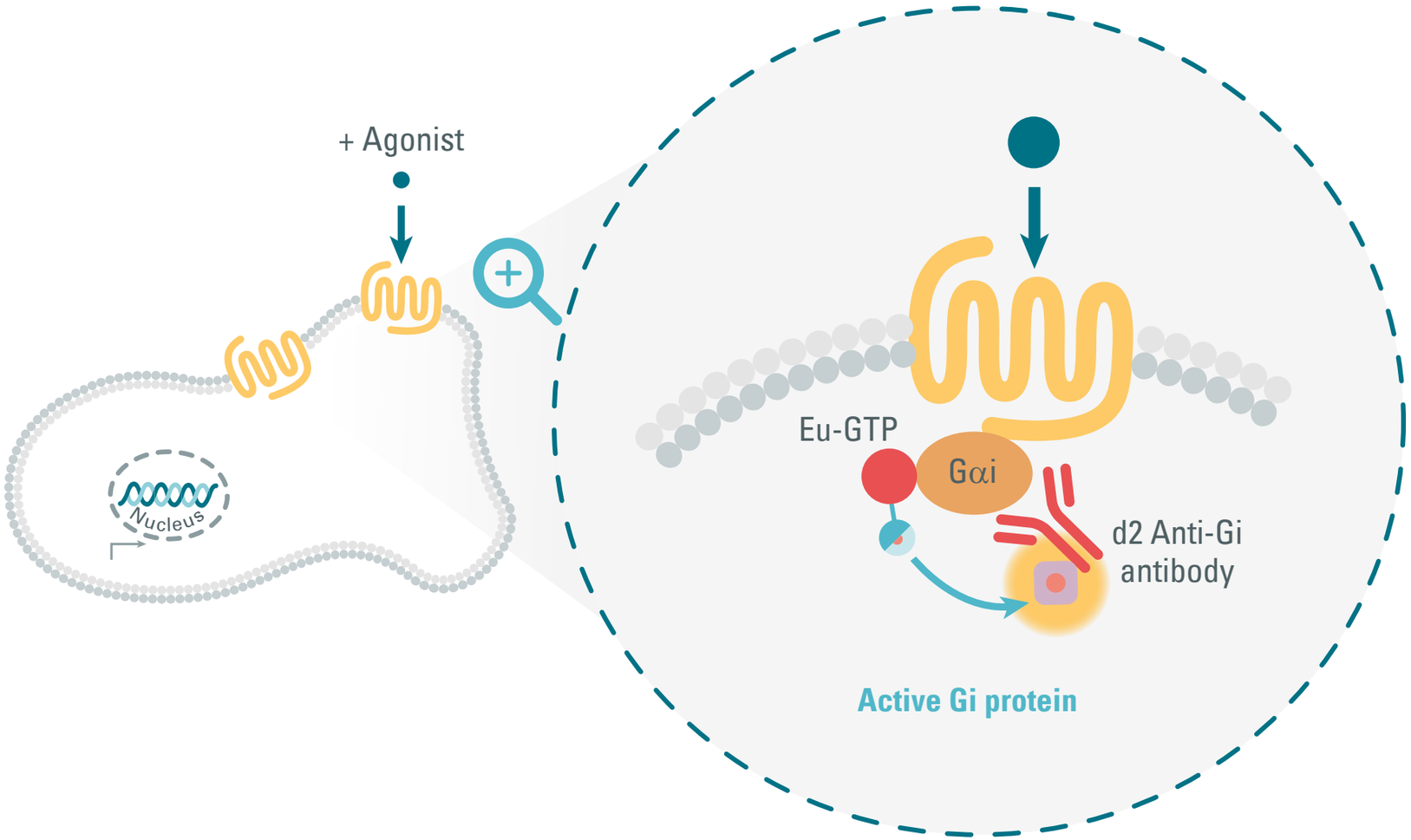

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REAGENTS & WORKFLOW OPTIMIZATION

Reagents

Stimulation Buffer #3

The stimulation buffer included in the kits is used to prepare all the reagents required for the assay: membranes, nucleotide and salt working solutions (GDP, GTPgS, and $MgCl_2$), all chemicals, compounds, and detection reagents. This buffer is ready to use for the optimization step and will be supplemented with the selected optimal GDP/$MgCl_2$ concentration for the pharmacological assay.

Detection reagents (GTP Eu Cryptate reagent & GTP d2 antibody)

Detection of Gαi protein activity is made possible by the GTP-Eu analog (donor) binding to the guanine nucleotide pocket of the protein in presence of anti-Gαi monoclonal antibody labeled with d2 acceptor. Proximity between these two reagents (donor and acceptor) allows energy transfer between them and triggers a FRET signal.

The kit contains 10X stock solutions of each detection reagent. A mixture of these two detection reagents must be prepared for one-step addition.

GTP gamma S

Guanosine 5'-0-gamma-thio-triphosphate or GTPγS is a non-hydrolysable or slowly hydrolysable GTP analog. It binds the same guanine nucleotide binding pocket of the Gα subunit as the Eu-GTP analog. When present in a saturating concentration, it displaces the Eu-GTP analog from the binding pocket, leading to the HTRF signal extinction. Thus it is used as a tool to obtain the non-specific signal of the GTP assay.

The kit contains GTPγS 10X stock solution at a concentration of 1 mM. Prepare the 100 µM working solution from stock solution diluted in stimulation buffer #3. The concentration in final assay volume is 25 µM.

Gi protein Control

The positive control of the assay is a recombinant Gαi subunit to which both the EU-GTP analog and the d2-antibody bind. It enables detection reagent control, giving a S/B > 5.

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REAGENTS & WORKFLOW OPTIMIZATION

Reagents

$MgCl_2$

Magnesium (Mg), among other cellular functions, participates in the activation of heterotrimeric G proteins. It acts as a keystone that locks G protein subunits into a conformation where the Gα unit dissociates from the Gβγ dimer. Moreover, this divalent cation is described as a positive allosteric modulator for some GPCRs, which it stabilizes in an active-like conformation (for example opioid receptors). Mg affinity for G proteins depends on the nature of the proteins, their active state (bound to GDP or GTP), and the GPCR they are associated with. Thus, Mg acts in a dose-dependent manner, where the optimal concentration differs from one system to another. Although the GTP assay has an absolute requirement for Mg^{2+} ions that promote GTP binding to the guanine nucleotide pocket of the G protein, an opposite negative impact can be observed at higher concentrations. Magnesium exhibits a biphasic effect, where the concentration limit depends on the system. This biphasic effect which we have observed is also described in the literature with the radiometric [35S]GTP assay [1]. The effects of magnesium are described at greater length in this guide, and we show the absolute requirement for this cation concentration to be optimized and adjusted from one membrane system to another. Moreover, guidelines are presented to set up a magnesium optimization matrix, in combination with the GDP.

The kit contains $MgCl_2$ stock solution at 1M.

GDP

The heterotrimeric G protein activation is mediated by a GDP / GTP exchange on the Gα subunit. This activation can either be mediated via agonist stimulation or be the result of G proteins' own constitutive activity. Therefore, to observe agonist-mediated binding of the Eu-GTP analog, it may be essential to add an optimized concentration of GDP to find the balance between constitutive activity and agonist-induced conditions. In practice, the GDP competes with the GTP-analog, and its affinity is higher for the G protein inactive state than the active one. This allows an added optimized concentration of GDP to promote agonist-mediated GTP analog binding observation by decreasing constitutive activity, while having a low impact on agonist-stimulated GPCRs.

Thus the concentration of GDP must be optimized for each biological model, depending on the nature of the GPCR. For instance, delta opioid receptors display a high constitutive activity and require GDP addition, whereas NTS1 receptors do not and are even negatively impacted by GDP addition.

GDP effects are explained at greater length in this guide, along with guidelines to set up a GDP optimization matrix in combination with $MgCl_2$.

The kit contains GDP stock solution at 100 µM.

[1] : Szekeres et.al.; J.Pharmacol.exp.theory (1997)

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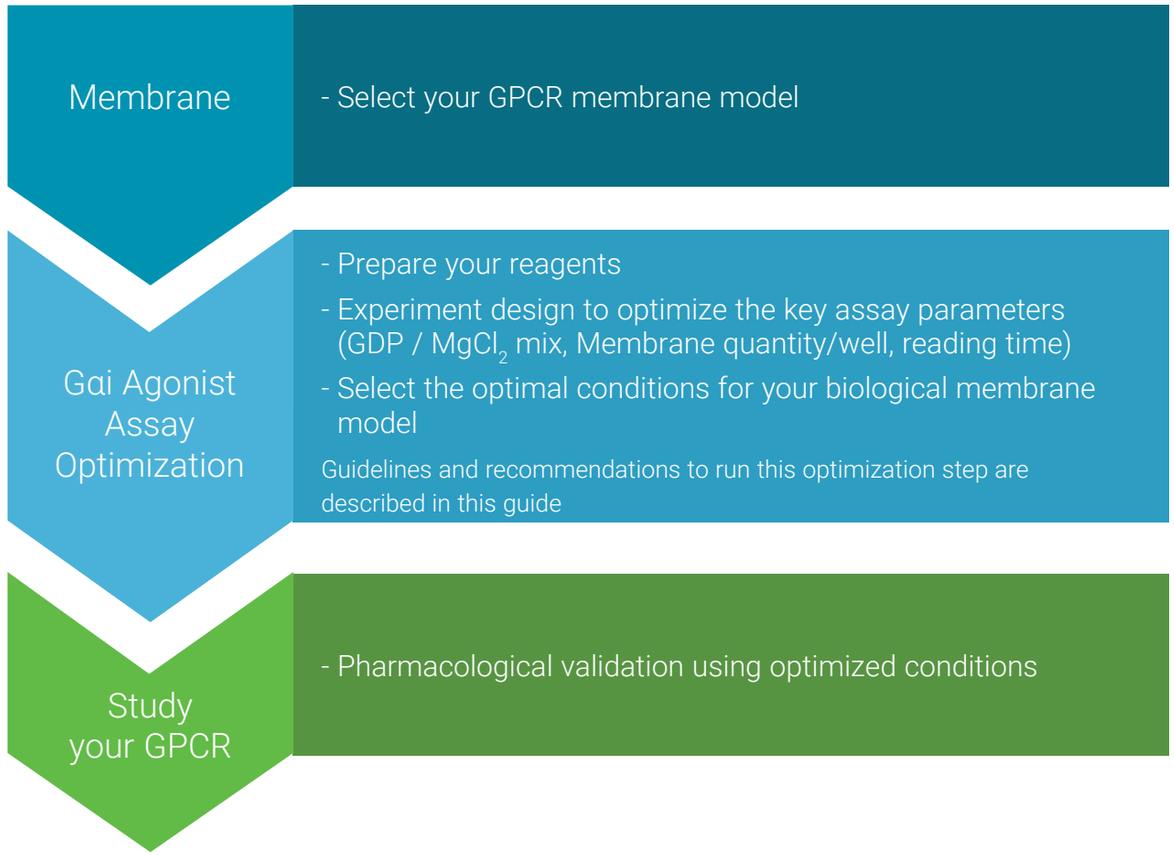
REAGENTS & WORKFLOW OPTIMIZATION

Workflow for setting up your GTP binding assay



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MEMBRANE HANDLING RECOMMENDATIONS

Membrane handling

Before you begin

Cryopreserved membrane preparations are the most frequently used biological models when performing GTP binding assays. Proper handling and care of membranes before use is a critical step, as they are very sensitive biological reagents. Some recommendations we have successfully employed to develop and run this GTP binding assay are described in this section.

Revvity offers more than 70 GPCR-expressing membrane models. See more information at https://www.revvity.com/fr/category/receptor-cell-lines-membranes.

Membranes are mainly prepared from HEK293 and CHO cells expressing GPCRs. It has been noted that the GTP binding assay generally has better performances for Gi coupled receptors and addresses CHO more easily than HEK293 cells. The stoichiometry of Gα proteins probably plays a role in these differences, as the literature describes an overall higher expression level of Gi than Gs and Gq in mammalian cells [2].

The membranes are generally used at 2.5 to 10 µg/well in GTP binding assays. This quantity is related to the total protein quantification in the membrane preparation. In addition, the GPCR expression level can be measured by performing saturation binding assays and expressed in pmol/mg of membrane protein.

Start with cryopreserved membranes:

Membrane extracts are highly sensitive to degradation. The membrane preparation must be kept on ice throughout the entire preparation procedure. Do not repeat thawing/ freezing steps. It is recommended to aliquot and to thaw once just before use.

Aliquot from stock solution (all steps performed on ice, & as quickly as possible, to avoid degradation):

- Thaw membrane stock solution on ice.
- Homogenize the membrane preparation. It is recommended to use a syringe with a 0.8mmx50mm size needle to avoid biological material damage. Aspirate and push back several times to homogenize well.
- Aliquot rapidly and store at -80°C or in liquid nitrogen.

Prepare membranes for the GTP assay (all steps performed on ice)

- Thaw the aliquot on ice (it is recommended to dispense all the reagents into the plate and to prepare the membranes just before use).
- Dilute the membrane in the stimulation buffer #3 (optimization experiment) or supplemented stimulation buffer #3 (GTP binding assay) at the desired concentration.
- Dispense into the plates. From this step on, the assay is performed at RT.

[2] Milligan et.al.; TRENDS in Pharmacological Sciences Vol.24 No.2 February 2003

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GαI AGONIST ASSAY OPTIMIZATION

Introduction

Description

Signal transduction of GPCRs, mediated through GTP binding to the Gα subunit, is controlled by many parameters. This section is intended to provide information and guidelines for GTP binding assay optimization.

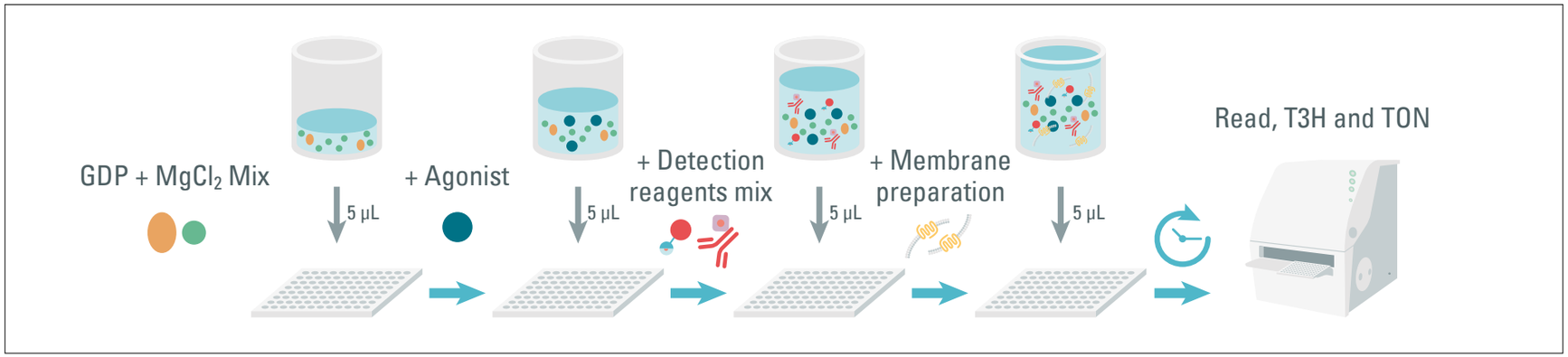
Like common radioactive assays, it requires key additives[3]. The assay is usually performed in presence of divalent cations such as Na^{2+} and Mg^{2+} and with the addition of GDP nucleotide. All these additives play critical roles in the activation process, and their contributions to the assay need to be considered. Other key parameters, such as membrane quantity, are also important. Furthermore, all the additives and conditions may produce combined effects, which require optimization for the assay to perform as well as possible.

Additionally, each GPCR membrane model has different behaviors linked to its biological functional activity, and each model requires proper optimization.

We have therefore developed an easy one-shot optimization protocol, where some parameters were fixed and others tested simultaneously through a proposed matrix model. The first part of this section is dedicated to the presentation of each key parameter to introduce their individual effects. The second part addresses the procedure to build an easy matrix for the one-shot optimization experiment, in order to achieve optimal conditions for a given studied model.

NB: To perform the optimization step properly, it is recommended to use the 500 kit size (62GTPPEG)

Protocol



[3] : Harrison et.al.; Life Sciences 74 (2003) 489–508

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Gαi AGONIST ASSAY OPTIMIZATION

Introduction

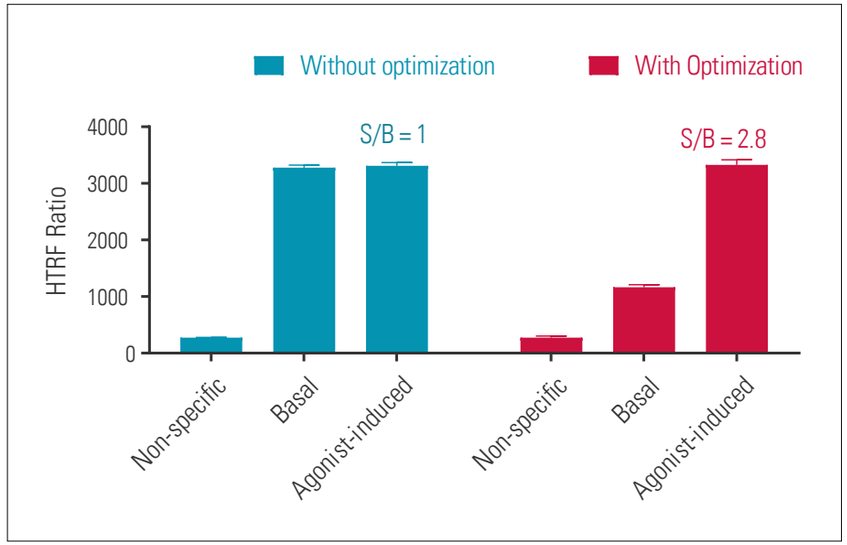
Assay conditions for the optimization step:

3 assay conditions are required for the optimization step:

Non-specific signal (NS): This is the assay background, and indicates the negative baseline to apply. A saturation concentration of 25 µM of the non-hydrolysable GTPγS is added to the assay to compete with the GTP-fluorescent analog leading to signal extinction.

Basal signal (B): This is the assay signal in the absence of compounds. It indicates the basal GPCR activity without stimulation. The level of this basal activity is calculated by dividing the HTRF Ratio of this condition by the non-specific signal (B/NS).

Agonist-Induced signal (S): This is the assay signal in presence of an agonist. It indicates the value of GTP binding to the Gα subunit in GPCR-stimulated condition. The fold of pharmacological induction is thus expressed by the signal / basal (S/B). For the optimization step, the agonist is generally at a saturation concentration in order to generate the maximal induction for a given model.



Absolute necessity of the optimization step. Case study in CHO-DOR membrane model.

5 µg of membrane were treated or not with 1 µM of the SNC-162 agonist. Plate was read after ON incubation at RT.

A high basal and no agonist-induction observed before optimization.

Optimization step led to basal decrease and consequently to detection of the agonist-induced GTP analog binding with a S/B of 2.8.

The optimized condition selection for this receptor is described in the optimization section / data reduction and analysis.

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GαI AGONIST ASSAY OPTIMIZATION

Constitutive activity of GPCRs

Agonist-unoccupied receptors can constitutively activate G proteins, which can be measured with the GTP-fluorescent analog binding. The level of constitutive activity or functional basal activity of a system can be useful to anticipate the optimization step, as Mg^{2+} and GDP concentrations are used in the assay to decrease constitutive activity signal and highlight agonist-induced signal. Constitutively active GPCRs are interesting models for some classes of pharmacological compound identification, like truly silent antagonist (neutrals) or inverse agonists (acting by stabilizing the GPCR in its G protein unbound form and inhibiting spontaneous activation of G protein).

This section shows the basal activity of a panel of GPCR membrane models and illustrates the varying nature of constitutive activity between them. The table below contains information about these membranes: Gα coupling, cell expression background, expression level of GPCRs. The data for a GTP binding assay of non-stimulated receptors is presented for these 9 models.

Receptor	Primary transduction	Cell expressing the receptor	Cat#	Expression level pmol/mg
DOR	Gi/o	CHO	RBHODM400UA	24.8
	Gi/o	HEK293	6110549400UA	10.7
OPRL1	Gi/o	CHO	6110540400UA	3.5
		HEK293	RBHORLM400UA	1.9
D2S	Gi/o	CHO	RBHD2CM400UA	11
5HT1a	Gi/o	CHO	6110501400UA	1.8
		HEK293	RBHS1AM400UA	1.7
NTS1	Gq (and Gi/o secondary transduction)	CHO	ES-690-M400UA	31
		HEK293	RBHORLM400UA	31.5

The GPCR membrane models compared for their constitutive activity in the GTP binding assay. All GPCRs presented here were overexpressed in CHO or HEK293 cells. They are mainly Gi/o coupled GPCRs (primary coupling), except NTS1, which is described to be Gq as primary coupling and Gi/o as secondary.

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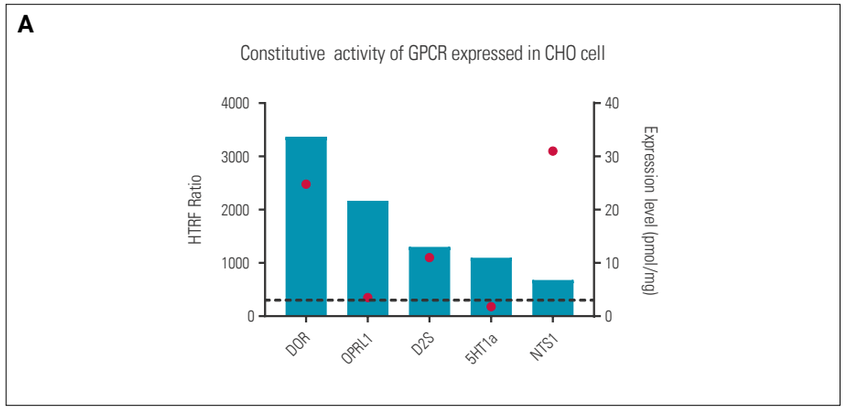
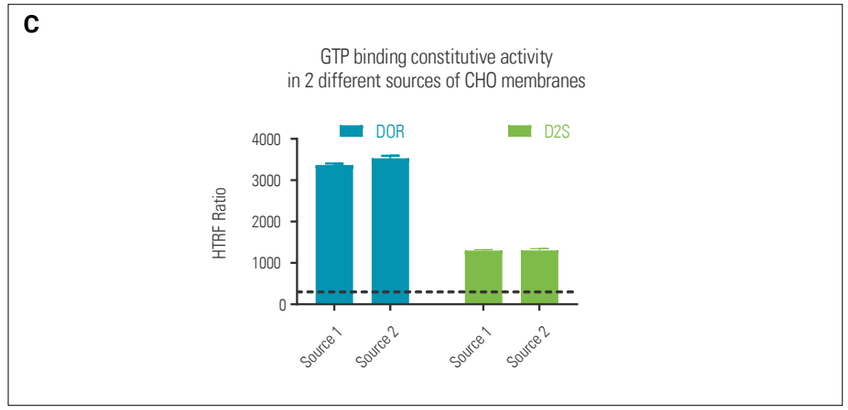
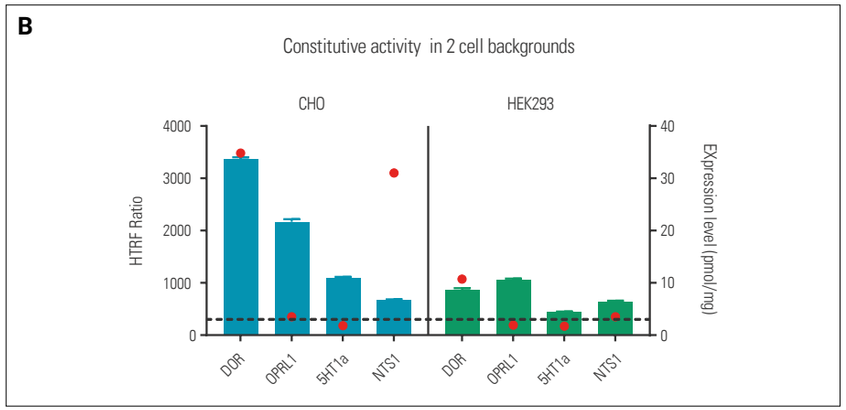
Gαi AGONIST ASSAY OPTIMIZATION

Constitutive activity of GPCRs







GTP binding constitutive activity measurement in various GPCR membrane models.

The assay was performed using 10 µg of membrane / well and 10 mM of $MgCl_2$ in stimulation buffer#3. The assay was read after ON incubation. Black dotted lines represent the non-specific signal; bars represent the measurement of basal GTP binding level; red dots represent the expression level of receptors expressed in pmol/mg of membrane proteins.

A: Comparison in the same cell background: the graph shows the functional basal activity of CHO membranes expressing a panel of GPCRs. The basal constitutive activity varies from one model to another. Moreover, it is not clearly proportional to the expression level of the GPCR, suggesting that constitutive activity is also linked to the nature of the GPCR and its own functional biology.

B: Comparison of GPCR basal activity of the same GPCRs expressed in CHO and HEK293. Some GPCRs with the same range of expression level show different basal responses in CHO and HEK293 (e.g. PRL1), while some other GPCRs display the same basal value in both cell lines, with different expression levels (e.g. NTS1).

C: Comparison of the same GPCRs from two membrane sources: Membrane extracts of DOR and D2S receptors expressed in CHO from two different sources show fairly similar basal activity behavior. DOR: source 1, Cat# RBHODM400UA; source 2: outsourced membrane extract service. D2S: source 1: Cat RBHD2CM400UA; source 2: outsourced membrane extract service.

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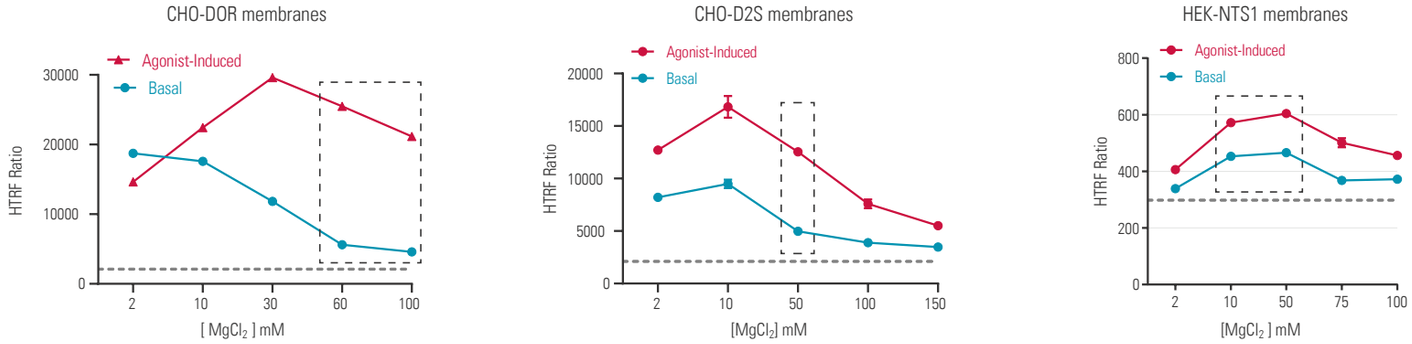
Gαi AGONIST ASSAY OPTIMIZATION
Critical parameters

Effect of Magnesium (Mg^{2+})

Mg^{2+} ions play an important role in the activation process of G proteins, and the literature describes them as influencing the receptor and/or the G protein itself. This cation is absolutely required for agonist-stimulated GTP fluorescent analog binding to be observed. It increases both basal and agonist-stimulated GTP binding to the Gα protein, with a bias for agonist-stimulated receptors. This bias enables the assay to render a more specific reading of the agonist-induced signal. However, a biphasic effect (also described by Szekeres et al., using a radioactive GTP binding assay) was observed, which calls for careful optimization of Mg^{2+} concentration [4].

DOR, D2S, and NTS1 GPCR models are presented here to illustrate magnesium's effect on basal functional activity and agonist-induced activity. Magnesium enhances the loading of fluorescent-GTP up to a certain concentration, then negatively impacts it at higher concentrations. This effect has been shown to be different depending on the activation state of the GPCR (presence of agonist or not), and it is unique to a given membrane model.

Consequently, Mg^{2+} titration is a key parameter when building the assay. The impact of the magnesium on agonists' pharmacological effects (potencies) was evaluated as well, and the data are presented below. Taking all these parameters into account, we have determined 3 key concentrations enabling assay optimization regardless of the membrane model. $MgCl_2$ at 2, 10, & 50 mM in the final assay are the recommended concentrations to build a matrix in combination with the GDP and to identify the best condition (optimization section/ procedure). The graphs below are demonstrating the Mg^{2+} titration effect on 3 membrane models in basal an agonist-induced conditions (A). Its effect on compounds pharmacological response in CHO-DOR membrane model is presented in graph B.



A. Magnesium effect on 3 different models. Membranes from cells overexpressing DOR, D2S, and NTS1 receptors were treated with an increasing dose of $MgCl_2$. The receptors were non-stimulated (basal) or stimulated with their appropriate agonist (10 µM SNC-162, 10 µM PPHT, and 10 µM neurotensin for DOR, D2S, and NTS1 respectively). Stimulation buffer contains 0.5 µM of GDP. Plates were read after ON incubation. Dotted lines represent the non-specific signal. Dotted rectangles represent the optimal working windows at a fixed 0.5 µM GDP concentration.

[4] : Szekeres et.al.; J.Pharmacol.exp.theory (1997)

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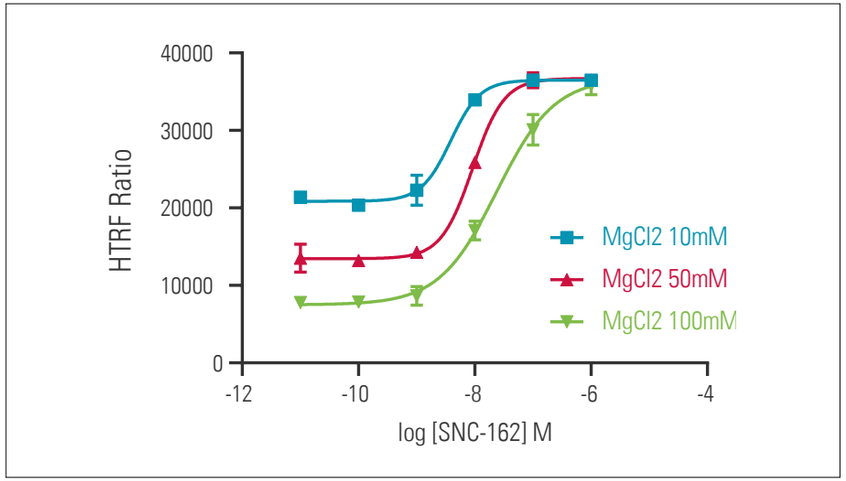

[$MgCl_2$]	SNC-162 EC50
10 mM	5 nM
50 mM	8 nM
100 mM	22 nM

B. Magnesium effect on compounds 'pharmacological effects. CHO-DOR membranes were stimulated with the SNC-162 agonist in a dose-dependent manner.

The stimulation buffer contained 0.5 µM of GDP and was supplemented with either 10, 50, or 100 mM of magnesium. Plates were read after ON incubation. The data show that the magnesium does not impact the agonist SNC-182 potency until 50 mM. A slight shift of the EC50 can be observed at 100 mM. The same effect was observed with the D2S membrane model (data not shown). These results demonstrate that high concentrations of magnesium (i.e.100 mM in this experiment) should be handled with care as they may induce a bias in compound pharmacological characterization. Thus the recommended maximal magnesium concentration is 50 mM.

Note that all GPCRs behave differently in presence of magnesium, which makes Mg^{2+} addition an unpredictable parameter that must be tested for in a given GPCR membrane model. While the recommended 50 mM fit most models we assessed, some receptors may exhibit a shift of compound potency at this concentration. In that case, we recommend testing the lower concentration of Mg^{2+} at 10 mM.

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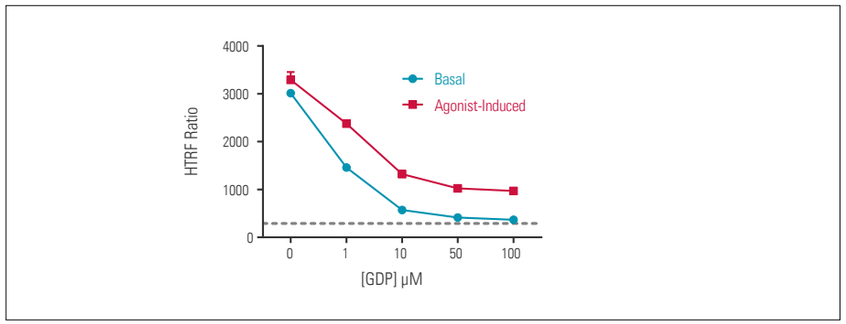
GαI AGONIST ASSAY OPTIMIZATION
Critical parameters

Effect of the GDP nucleotide

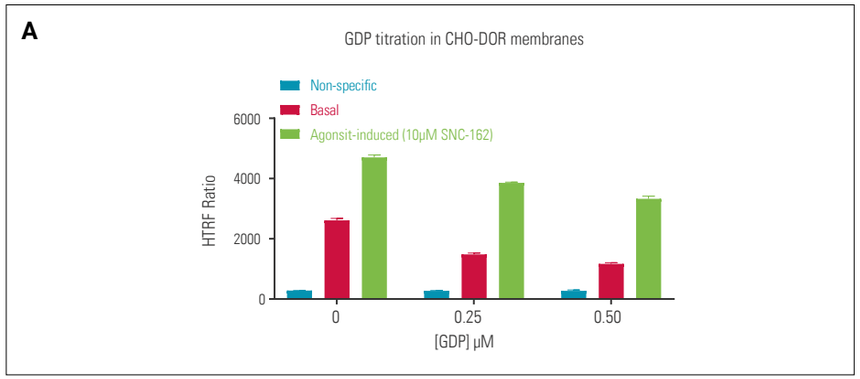
In their inactive state, G proteins exist as heterotrimeric complexes, where GDP is bound to the Gα subunit. Activation of the receptor by agonists results in the dissociation of GDP from Gα, and its replacement by GTP. As divalent ions, exogeneous GDP is mainly used to build GTP-non hydrolysable binding assays. For certain models (especially those displaying high constitutive activity), it may be necessary to add it to obtain the optimal agonist-induced GTP-fluorescent binding. More precisely, GDP competes with the GTP analog for binding to the Gα protein (preferentially to Gα associated with agonist-free receptors), thus lowering the constitutive activity detection and improving the S/B of the assay. Fine-tuning the GDP concentration is therefore necessary to find its optimal concentration in a given system.

This guide recommends the GDP concentrations to use and test at 0, 0.25, & 0.5 µM, to build a matrix in combination with the $MgCl_2$ (optimization section/ procedure).



GDP effect on the Delta opioid receptor. CHO-DOR membranes were treated with increasing concentrations of GDP. The stimulation buffer contained 10 mM of magnesium. Plates were read after ON incubation. GDP competes with the GTP-fluorescent analog, leading to HTRF signal extinction. Due to its higher affinity for the G protein inactive form (agonist-free receptors), this competition is more efficient in basal conditions.

A



B

Fluorescent-GTP agonist induced binding (S/B)			
[GDP] µM	CHO-DOR	CHO-D2S	HEK-NTS1
0	1.8	2.8	2
0.25	2.6	2.9	1.7
0.5	2.8	2.9	1.6

Recommended GDP concentrations for optimization: A. The graphs show the assay behavior in a CHO-DOR model using the three recommended GDP concentrations (0, 0.25, & 0.5 µM) under the non-specific, basal, and agonist-induction conditions. B. The table is a summary of the S/B obtained for DOR, D2S, and NTS1 membrane models with GDP titration. All the experiments were performed in a 10mM $MgCl_2$-containing stimulation buffer #3. Green boxes show the GDP concentration resulting in the best S/B. DOR, which displays the highest constitutive activity, requires more GDP than NTS1, which does not require GDP for the assay.

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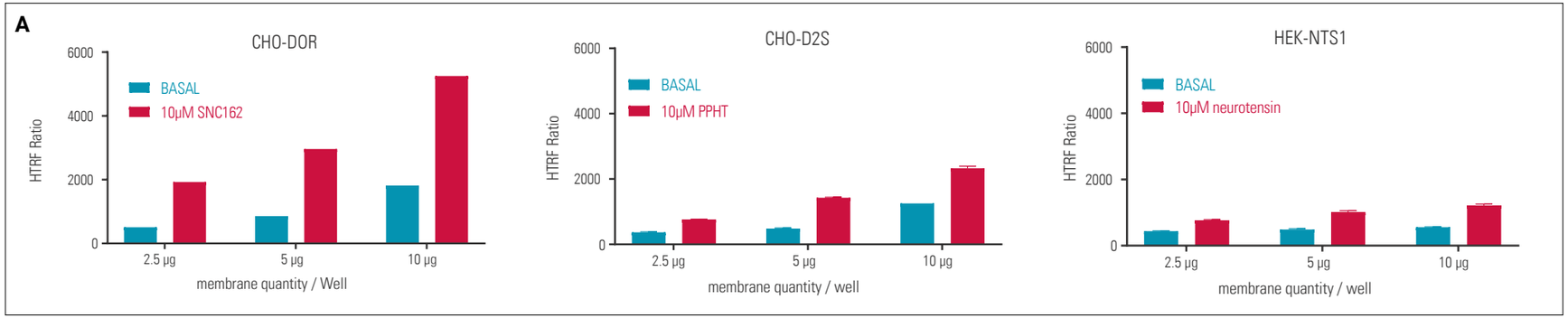
GαI AGONIST ASSAY OPTIMIZATION
Critical parameters

Membrane titration effect

IAnother parameter requiring optimization prior to running the assay is the membrane quantity / well, which should be adapted to the biological membrane model (this can be explained by the total protein concentration, the GPCR expression level or the cell background, etc.). Generally 5-10 µg/well is the most suitable amount. However, for some models it is possible to lower the membrane quantity down to 2.5 µg without impacting the biological induction detection. Three GPCRs (DOR, D2, and NTS1 membrane models) are presented here to show membrane titration impact on the GTP assay.

A



B

Pharmacological assay widow (S/B)			
Membrane	**DOR**	**D2**	**NTS1**
2.5	3.7	2	1.8
5	3.5	3	2.1
10	2.8	1.9	2.2

Membrane titration: A. Membrane titration in CHO-DOR, CHO-D2S & HEK293-NTS1 membrane models. For each model, 2.5, 5, and 10 µg of membranes / well were compared. DOR GTP assay was performed in presence of 50 mM $MgCl_2$ & 0.5 µM GDP. D2S assay was performed in presence of 50 mM $MgCl_2$ & 0.5 µM GDP . NTS1 assay was performed in presence of 50 mM $MgCl_2$ and with no GDP addition. Agonist concentration was 10 µM for the SNC-162, PPHT, and neurotensin for DOR, D2S, and NTS1 respectively. The graphs show the signal obtained in absence or presence of agonist. B. The calculated S/B is summarized in the table. Green boxes indicate the optimal range of membrane quantity. For the receptors presented here, 5 µg of membrane / well is suitable for all the models. Moreover, DOR can be performed using less membrane (2.5 µg / well).

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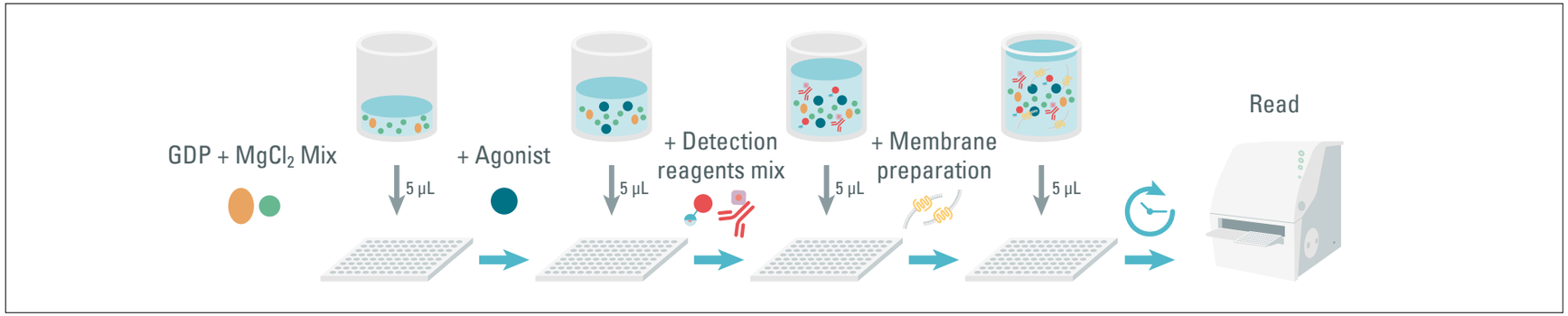
Gαi AGONIST ASSAY OPTIMIZATION

Optimization step procedure

The optimization step is used to determine the best assay conditions to detect pharmacological induction before running pharmacological studies. This section provides guidelines to efficiently build a one-shot optimization experiment: reagent dispensing, GDP/$MgCl_2$ mixture preparation, experimental plate map recommendations, and data analysis and processing. A real biological case study (CHO-DOR membranes) was taken as an example to show actual data and the strategy to select the best assay conditions.

Reagent dispensing

The reagents were dispensed as described below, and the dispensed volumes of each reagent are summarized in the table. Briefly, 5 µl of the following reagents were dispensed sequentially: GDP/$MgCl_2$ mixture, the agonist at desired concentration, the mixture of detection reagents (GTP-cryptate reagent and GTP-d2 antibody), and finally the membrane preparation. Plates were incubated at 22°C and read with an HTRF-compatible reader. Preparation of the working reagents is described in the package insert of the kit. After this step, all the reagents were diluted in the provided stimulation buffer #3.



Reagent	Stimulation buffer #3	GDP / $MgCl_2$	GTP gamma S	Gi protein control	Agonist (4X)	Detection reagent mix (4X)	Membrane preparation	Total
Basal activity	5 µl	5 µl	-	-	-	5 µl	5 µl	20 µl
Agonist-induced	-	5 µl	-	-	5 µl	5 µl	5 µl	20 µl
Non-specific signal	-	5 µl	5 µl	-	-	5 µl	5 µl	20 µl
Positive control	10 µl	-	-	5 µl	-	5 µl	-	20 µl

Positive control is optional

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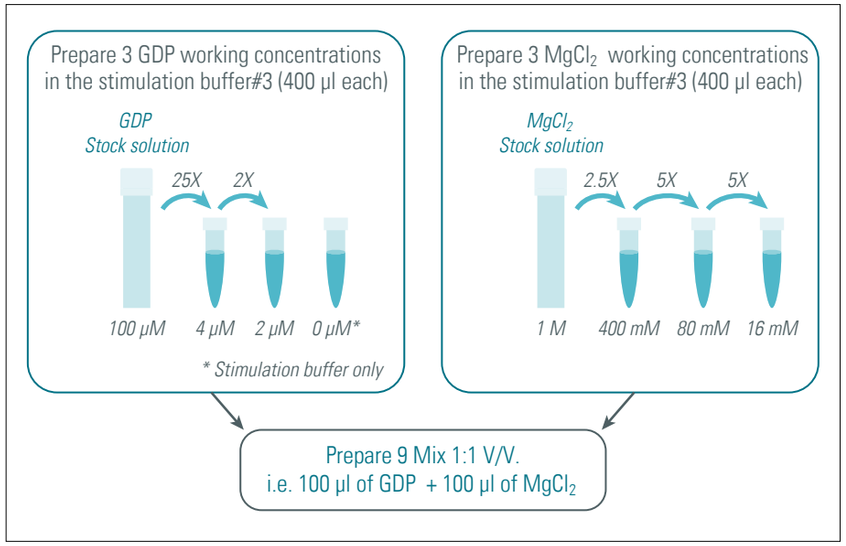
GαI AGONIST ASSAY OPTIMIZATION

Optimization step procedure

GDP / $MgCl_2$ Mix preparation

GDP and $MgCl_2$ are key additives for the GTP binding assay . The effect of each additive is described above. As they have GPCR membrane model-dependent & combined effects, a selection of the optimal $MgCl_2$/GDP mixture is required. This section describes the procedure to prepare the 9 mixtures from the stock solutions provided.

Mix of GDP & $MgCl_2$ - preparation procedure



MIX		Working [GDP]		
		0 µM	2 µM	4 µM
Working [$MgCl_2$]	16 mM	Mix 1	Mix 2	Mix 3
	80 mM	Mix 4	Mix 5	Mix 6
	400 mM	Mix 7	Mix 8	Mix 9

Working and final concentration table

MIX	Working concentration (4X)		"Final concentration in the assay"	
	GDP (µM)	$MgCl_2$ (mM)	GDP (µM)	$MgCl_2$ (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

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Optimization step procedure

Plate Map for one-shot experiment for optimization

Another parameter requiring optimization prior to running the assay is the membrane quantity / well, which should be adapted to the biological membrane model, (this can be explained by the total protein concentration, the GPCR expression level or the cell background, etc.).

Generally 5-10 µg/well is the most suitable amount. However, for some models it is possible to lower that membrane quantity down to 2.5 µg without impacting the biological induction detection. A plate map is proposed here to build an efficient matrix for the different reagents and select the best conditions for a given GPCR.

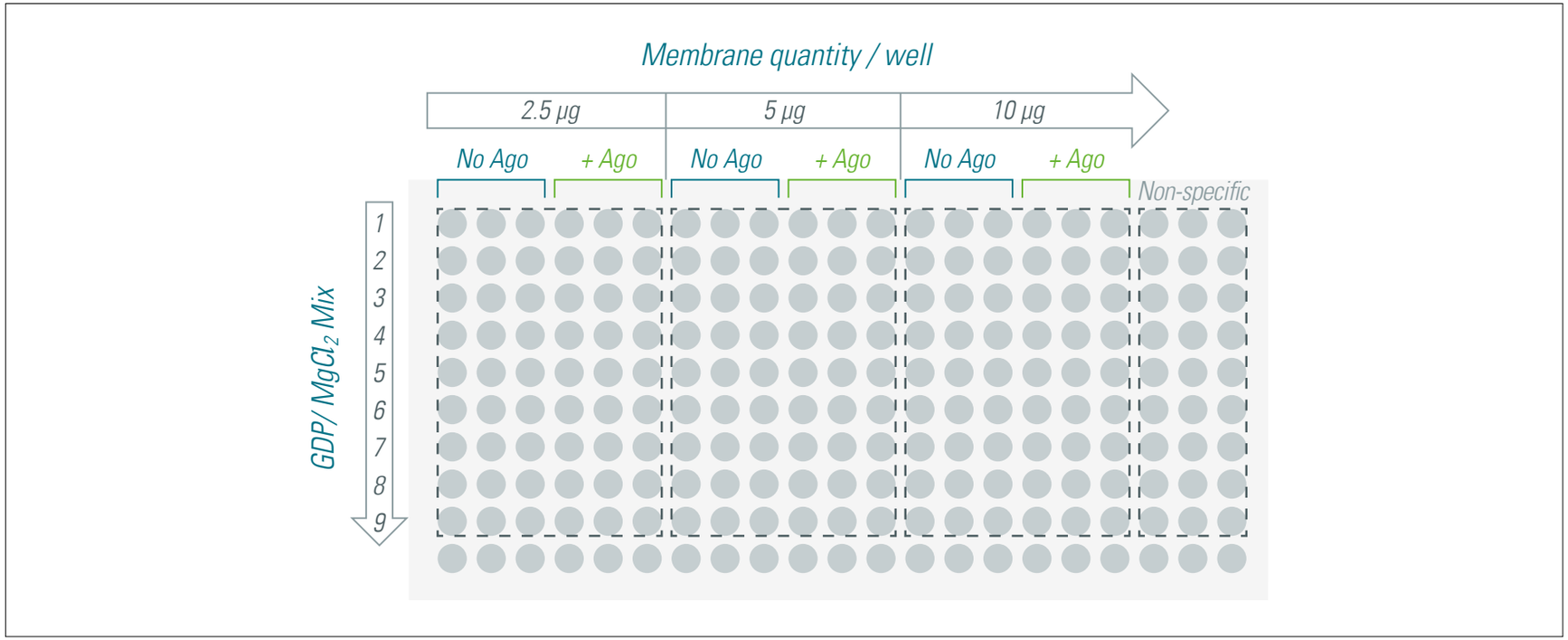

Plate Map recommendation: 9 GDP/ $Mgcl_2$ mix over the membrane quantity / well were compared. In the 3 assay conditions: Basal binding (No agonist addition); Agonist-induced binding (agonist at a saturation concentration); Non-specific binding (by addition of an excess of GTP gamma S). As the non-specific signal does not change over the different conditions, it can be done for the highest membrane and mix concentration only.

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Gαi AGONIST ASSAY OPTIMIZATION

Data reduction and analysis

For each condition tested, the HTRF Ratio data, basal and agonist-induced binding rates are calculated as follow:

Basal assay window (B/NS) = Ratio of the basal / Ratio of the non-specific signal

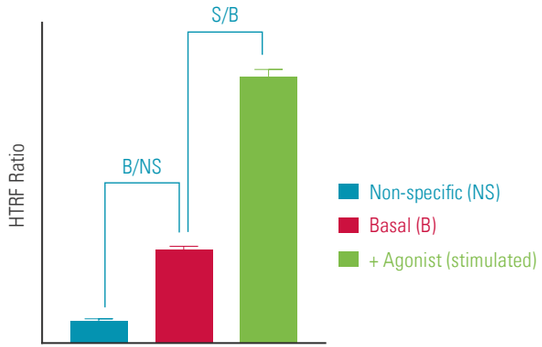
Pharmacological assay window (S/B) = Ratio of agonist condition / Ratio of the basal

The graph A shows examples of data processing to calculate the assay window.

The CHO-DOR membrane results presented here show data analysis and optimal condition selection for this model (Table B).

The chosen optimal condition in green is the one giving the best pharmacological assay window detection. Interestingly, the evolution of constitutive activity of the system (basal condition), and the non-specific signal of each condition are shown to follow detection across the matrix system (Table C and Graph D).

A



Data analysis: assay window of basal and agonist-induced GTP binding

Optimal condition selection:

B

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

Selection of the optimal assay condition in CHO–DOR membrane model: based on the optimization matrix, the table shows the results of the calculated pharmacological assay window (S/B). Optimal conditions are 5 µg of membranes / well, 0.5 µM of GDP, and 50 mM of $MgCl_2$ (Mix 9). Interestingly, 2.5 µg of membrane / well can be used for this model, with no dramatic change in the pharmacological assay window.

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Gαi AGONIST ASSAY OPTIMIZATION

Data reduction and analysis

Optimal condition selection:

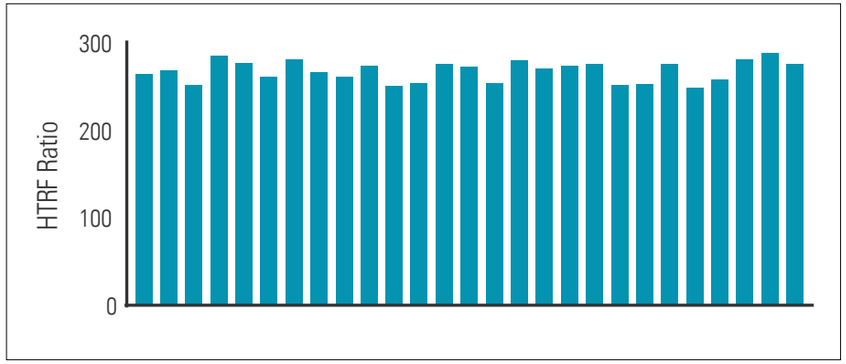
Basal evolution depending on the matrix conditions: The basal receptor functional response is dramatically impacted by the GDP/ $MgCl_2$ concentrations. This example using DOR receptor shows variations in the basal levels from Mix 2 (High basal) and Mix 9 (Low basal) .

C

Basal (Constitutive activity) (S/N)			
	Membrane µg/ / well		
	2.5	5	10
MIX 1	7.4	9.7	11.9
MIX 2	9.6	12.6	17.2
MIX 3	6.9	9.3	11.7
MIX 4	2.9	5.3	8.7
MIX 5	4.8	8.5	14.7
MIX 6	3.6	5.5	8.5
MIX 7	2.7	4.6	8.3
MIX 8	3.9	6.9	12.3
MIX 9	2.7	4.2	7.2

D

Non-specific signal recorded in all conditions



Non-specific signal evolution depending on the GDP/$MgCl_2$ matrix conditions: The non-specific signal obtained by addition of an excess of GTPgS enables the determination of the non- specific baseline of the assay. Here, we highlight the very low variability of the signal in the matrix conditions. It is thus possible to determine the non-specific signal in one condition (the highest concentration of Mix GDP/$MgCl_2$ and Membrane quantity / well).

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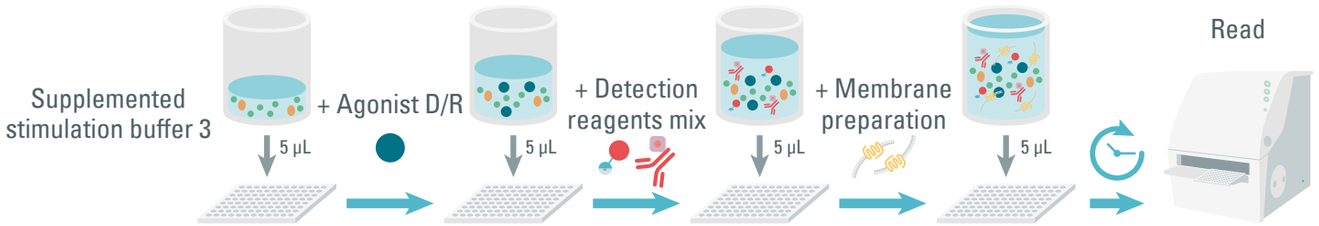
GαI AGONIST ASSAY PHARMACOLOGICAL CHARACTERIZATION

Introduction

Once the optimal conditions have been selected (refer to the optimization section), the procedure to perform an agonist pharmacological study is described in this section. The same CHO-DOR case study used for the optimization step is used here as an example to illustrate agonist studies.

Reagent dispensing

All the reagents are prepared in the stimulation buffer #3 supplemented with the optimal GDP / $MgCl_2$ concentration. A concentration range of the agonist is prepared to allow its dose-response (D/R) titration. The reagents are dispensed as described below.



Reagent	Supplemented stimulation buffer#3	Stimulation buffer #3	GTP gamma S	Gi protein control	Agonist (4X)	Detection reagent mix (4X)	Membrane preparation (4X)	Total
Agonist range	5 µl	-	-	-	5 µl	5 µl	5 µl	20 µl
Non-specific signal	5 µl	-	5 µl	-	-	5 µl	5 µl	20 µl
Positive control	-	10 µl	-	5 µl	-	5 µl	-	20 µl

Non-specific and positive control are optional.

Supplemented stimulation buffer#3 preparation:

When the GDP and $MgCl_2$ optimal concentrations have been determined, the stimulation buffer #3 is supplemented with the chosen final concentrations. All the reagents are then diluted in this supplemented stimulation buffer #3 (compounds, detection reagents, membranes), except for the positive control, which is ready to use.

Agonist concentration range preparation:

Agonist is diluted in the supplemented stimulation buffer #3 and the working concentration is prepared 4X. The concentration range depends on the compound's expected potency.

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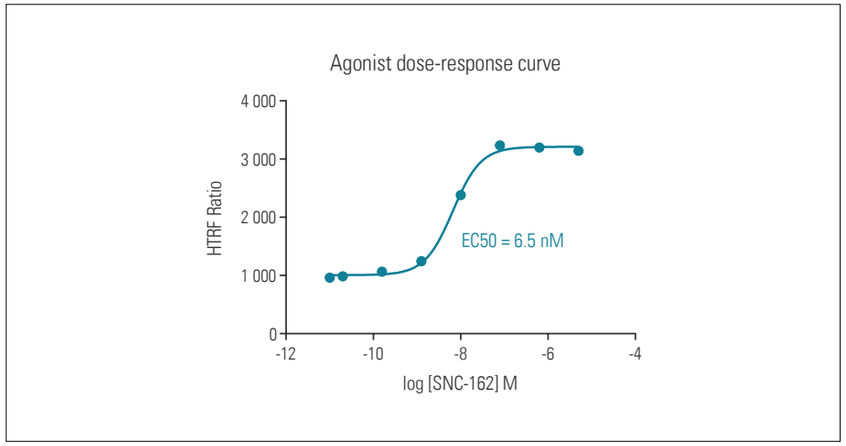
GαI AGONIST ASSAY PHARMACOLOGICAL CHARACTERIZATION

Data reduction and analysis

Agonist pharmacological characterization:

The HTRF Ratio is plotted over the concentration range of the agonist, and analyzed with a statistical software (i.e. GraphPad) to calculate the EC50 value of the compound by applying the agonist dose-response fitting curve.

The example below is that of SNC-162 agonist dose-response curve in the CHO-DOR membrane model, in the appropriate optimized conditions.

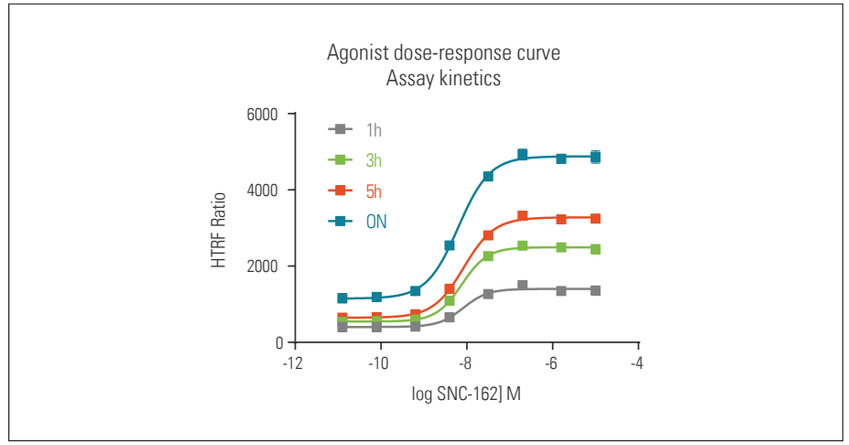


Agonist pharmacological characterization in the CHO-DOR membrane model: The assay was performed using the previously optimized conditions : 5 µg / well of membrane extract and the stimulation buffer #3 was supplemented with 0.5 µM of GDP and 50 mM of $MgCl_2$.

The SNC-162 dose-response curve fit shows an EC50 of 6.5 nM, which is in accordance with published values.

Kinetics and equilibrium:

Assay equilibrium is a key parameter for accurate pharmacological characterization. It is recommended to perform kinetics tests to ensure pharmacological equilibrium. An example of pharmacological kinetics is presented here with the CHO DOR case study..



Reading time	S/B	EC50 (nM)
T 1H	3.5	8.2
T 3H	4.6	7.7
T 5H	5.1	8.5
T ON	4.2	6.3

Assay kinetics and pharmacological equilibrium: 10 µg / well of CHO DOR membranes were stimulated with a concentration range of the SNC-162 agonist. Optimal conditions selected in the optimization step were used (0.5 µM GDP and 50 mM $MgCl_2$ in stimulation buffer #3). The plate was read at different times: 1H, 3H, 5H ,and after overnight incubation, to perform kinetics. The SNC-162 potency reached equilibrium quickly ,as the EC50 remained stable from 1h to ON.

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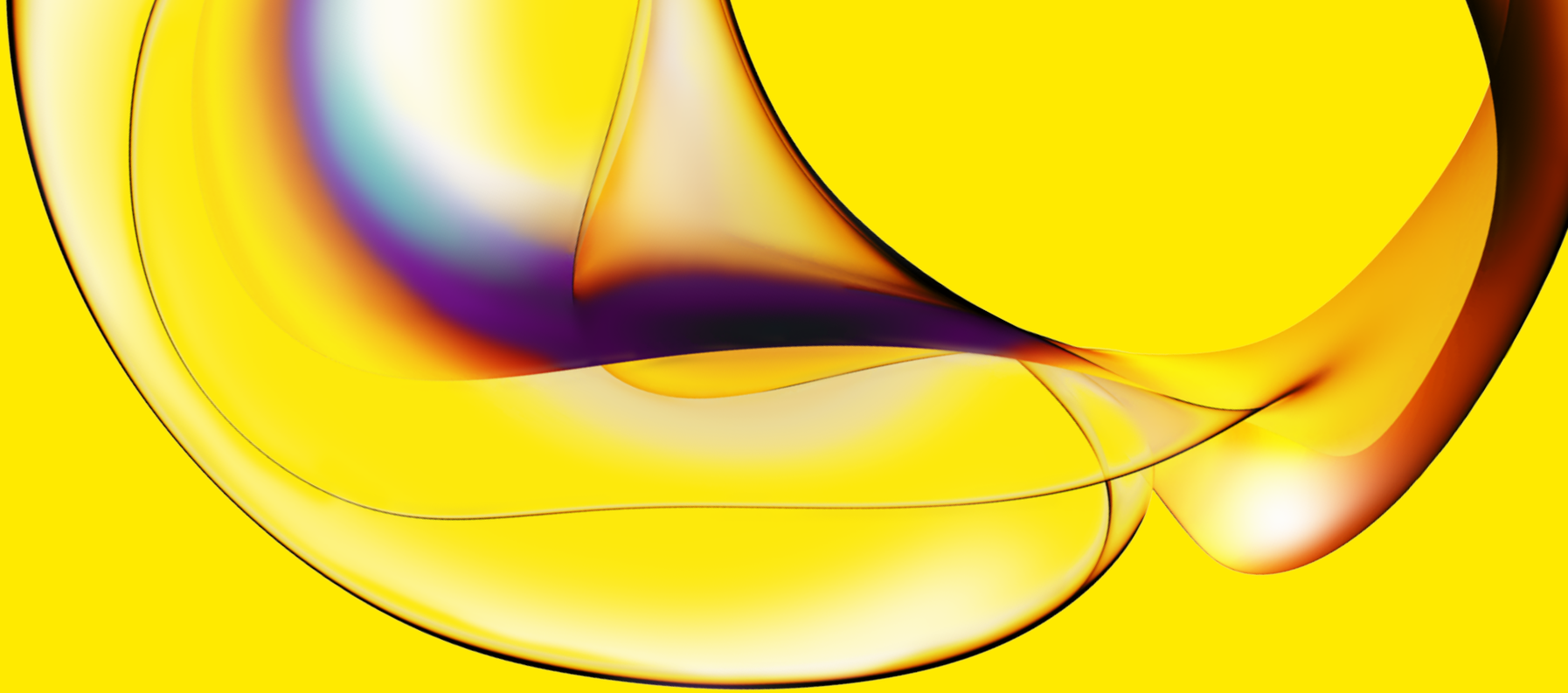

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