MANUAL

GTP Gi Binding assay Detection Kit

Part # 62GTPPET, 62GTPPEG & 62GTPPEC

Test Size#: 100 TESTS (62GTPPET), 500 TESTS (62GTPPEG) & 20.000 TESTS (62GTPPEC)

Revision: #03 of September 2023 **Store at:** ≤-16°C

For research use only. Not for use in diagnostic procedures.

ASSAY PRINCIPLE

The GTP Gi binding assay is intended for the simple, rapid, and direct detection of Gαi protein activation in membrane preparation. It is an upstream readout of Gi protein coupled receptor activation. GPCR activation leads to GDP/ GTP nucleotide exchange into the Gα subunit. 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). When Europium cryptate and d2 are brought into close 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. (Fig. 1).

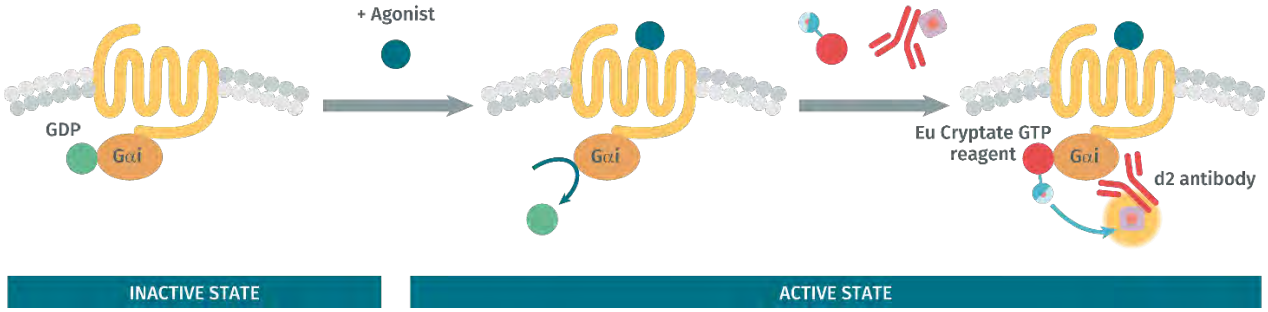


Figure 1: Principle of the HTRF GTP Gi binding assay: GPCR stimulation by agonist induces GDP/GTP nucleotide exchange at the Gαi subunit, leading to the Eu-GTP analog binding to the G protein. Detection is made possible by the addition of the d2-labelled anti- Gαi antibody.

As for all HTRF assays, the calculation of the fluorescence ratio (665 nm/620 nm) removes all possible photophysical interference and means the assay is unaffected by the experimental medium conditions. This homogeneous assay is performed in a single plate, with no washing steps required. This manual is performed in 20 µl total volume, which allows for miniaturization while maintaining the assay quality.
To set the assay parameters, please refer to these extensive guides: “GTP binding assay: a guide to optimizing Agonists of Gαi” and “GTP binding assay: a guide to optimizing Antagonists of Gαi” on www.revvity.com

For additional support, our Technical Support Team can help you to set up this assay. Please contact us at www.revvity.com

REAGENT PREPARATION

BEFORE YOU BEGIN:

1. It is very important to prepare reagents in the specified buffer. Use of an incorrect diluent may affect reagent stability and assay results.
2. Allow the lyophilized reagents to warm up to room temperature for at least 30 mins before reconstitution.
3. Working solutions must be prepared just before use.
4. Use the ready-to-use Stimulation Buffer #3 for the optimization step. This step enables the determination of GDP and MgCl2 optimal concentrations for the membrane model of interest (see corresponding guides at www.revvity.com)
5. Supplemented stimulation buffer #3: the GTP binding assay is performed with supplemented stimulation buffer #3, containing the GDP and MgCl2 optimal concentrations determined in the optimization step.
6. The mix of detection reagents solutions is prepared before use.
7. Gi protein control must be prepared in Stimulation Buffer #3 (not supplemented with GDP and MgCl2).
8. Take care to prepare stock and working solutions according to the directions below for the kit size you have purchased.
9. Be sure to set up your reader for Eu3+ Cryptate and read the fluorescence emission at two different wavelengths (665nm and 620nm) on an HTRF® compatible reader. (more information about HTRF® compatible readers and set-up recommendations available at www.revvity.com

MATERIALS & EQUIPMENT

MATERIALS PROVIDED:

KIT COMPONENTS	STORAGE	100 TESTS CAT# 62GTPPET		500 TESTS CAT# 62GTPPEG		20.000 TESTS CAT# 62GTPPEC	
GTP Eu Cryptate reagent (Stock solution 10X)	≤-16°C	Red cap	1 vial – 50 µL	Blue cap	1 vial – 250 µL	 Red cap	1 vial – 10 mL
GTP d2 antibody (Stock solution 10X)	≤-16°C	Blue cap	1 vial – 50 µL	Red cap	1 vial – 250 µL	 green cap	1 vial – 10 mL
GDP (Stock solution, 100 µM)	≤-16°C	Orange cap	1 vial – 80 µL	Orange cap	1 vial – 80 µL	White cap	1 vial – 3.2 mL
MgCl2 (Stock solution, 1M)	≤-16°C	White cap	1 vial – 500 µL	White cap	1 vial – 500 µL	White cap	1 vial – 20 mL
GTP Gamma S (Stock solution 10X, 1mM)	≤-16°C	Purple cap	1 vial – 50 µL	Purple cap	1 vial – 50 µL	Blue cap	2 vial – 1 mL
Gi protein control (lyophilized, see label for resuspension)	≤-16°C	 Green cap	1 vial	 Green cap	1 vial	 Green cap	2 vials
Stimulation Buffer #3* (Ready-to-use)**	≤-16°C	 Red cap	1 vial – 10 mL	 Red cap	2 vial – 10 mL	 Red cap	2 vials – 200 mL

* Stimulation Buffer #3 is used to prepare working d2 & Eu detection reagents, MgCl2, GDP, GTP Gamma S, Gi protein control, membranes, and compound solutions.
** The stimulation buffer is ready to use. Note it should be supplemented with the desired GDP and MgCl2 concentrations before use when optimized concentrations are known for the GPCR of interest. Once supplemented, it is therefore used to dilute working d2 & Eu detection reagents, GTP Gamma S, membranes, & compound solutions.
Note: Reagent preparation instructions are included below.

For guidelines and recommendations to set up the GTP binding assay, please refer to these guides: “GTP Gi Binding assay: a guide to optimizing Agonists of Gαi coupled receptors” and “GTP Gi Binding assay: a guide to optimizing Antagonists of Gαi coupled receptors” on www.revvity.com.

Please contact us at www.revvity.com

► PURCHASE SEPARATELY

- Membranes expressing GPCR of interest(1)
- Low volume white microplates(2)
- HTRF®-Certified Reader(3)

(1) Revvity offers more than 70 GPCR-expressing membrane models. More information at www.revvity.com
(2) Use microplates adapted for a final assay volume of 20 µl, such as HTRF 96-well low volume cat # 66PL96100 or Revvity Proxiplate Plus 384 white plates cat# 6008289. For more information about microplate recommendations, please visit our website at: www.revvity.com
(3) For a list of HTRF-compatible readers and set-up recommendations, visit www.revvity.com

STORAGE AND STABILITY

Store the kit at **-16°C or below** until the expiration date indicated on the package.

If the kit is not going to be used at once, please consider aliquoting the stock solutions before freezing as stock solutions may be stored frozen and thawed only once. Freeze in aliquots to avoid multiple freeze/thaw cycles. Stored at **-16°C or below**, they are stable for **3 months**.

Once reconstituted, it is recommanded to aliquot and store Gi protein control at -70°C or below. As the Gi protein is sensitive to degradation, freeze rapidely for storage.

Working solutions must be prepared just before use.

Membranes are very sensitive reagents and rapid degradation can be observed once thawed. Therefore, membrane working solution must be prepared on ice just before use. Refer to the GTP binding assay dedicated guides in membrane handling recommendation section. Guides available here.

REAGENT PREPARATION

Allow all reagents to thaw before use. We recommend centrifuging the vials gently after thawing, before pipetting the stock solutions.

Prepare the working solutions from stock solutions by following the instructions below.

TO PREPARE STIMULATION BUFFER:

► *Optimization step:*

The Stimulation Buffer #3 is ready to use in the optimization step to determine optimal GDP and MgCl2 concentrations as well as the membrane quantities to use. It is used to prepare compounds, GTP Gamma S (GTPγS), MgCl2, GDP, membranes, and Gi protein control working solutions. It is stable for 3 days at 2-8°C.

► *GTP binding assay:*

The Stimulation Buffer #3 should be supplemented with the determined optimal concentrations of GDP and MgCl2. Prepare the required amount of supplemented stimulation buffer before running the assay.

DETERMINE THE AMOUNT OF STIMULATION BUFFER NEEDED FOR THE EXPERIMENT. IT IS USED TO PREPARE COMPOUNDS, GTP GAMMA S, MGCL2, GDP, AND MEMBRANE WORKING SOLUTIONS.TO PREPARE POSITIVE CONTROL WORKING SOLUTION:

The Gi protein positive control is only provided as an internal assay control to check the signal quality of the results obtained. The window between the Gi protein control signal and the non-specific signal (No Gi protein control addition, which is replaced by stimulation buffer #3) should be greater than 5.

Reconstitute with Stimulation Buffer #3 (see label for reconstitution volume). Mix gently. The Gi positive control is now ready to use.

TO PREPARE GDP AND MGCL2 REAGENT WORKING SOLUTIONS:

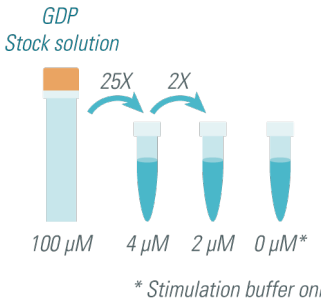
Stock solution of MgCl2 is provided at 1M, and GDP at 100 µM. GDP and MgCl2 concentrations should be optimized to the GPCR membrane model. Recommendations and the step-by-step procedure to perform the optimization step are described in the associated guides. Once optimal concentrations have been determined, GDP and MgCl2 stock solutions are directly added to the stimulation buffer to perform the GTP binding assay.

Thaw GDP and MgCl2 stock solutions. Mix gently. Dilute at the desired concentrations in stimulation buffer #3.

100 TESTS KIT #62GTPPET, 500 TESTS KIT #62GTPPEG & 20.000 TEST KIT #62GTPPEC*

GDP and MgCl2 working and Mix solution preparation for optimization step

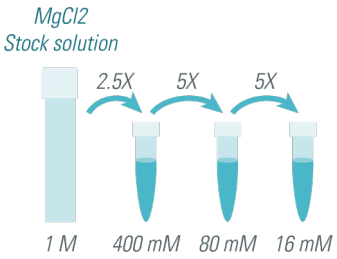
GDP working solution preparation



To prepare 400 µl of GDP working solutions:

Working GDP	4 µM	24 µl stock solution	576 µl stimulation buffer #3
	2 µM	200 µl working at 4 µM	200 µl stimulation buffer #3
	0 µM	-	400 µl stimulation buffer #3

MgCl2 working solution preparation



To prepare 400 µl of MgCl2 working solutions:

Working MgCl2	400 mM	160 µl stock solution	240 µl stimulation buffer #3
	80 mM	80 µl working at 400 mM	320 µl stimulation buffer #3
	16 mM	80 µl working at 80 mM	320 µl stimulation buffer #3

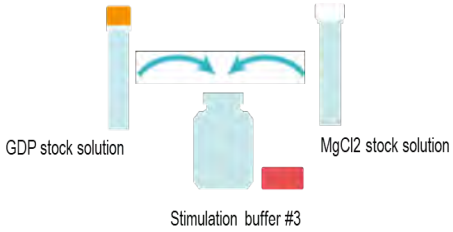
GDP /MgCl2 Mix preparation

Pre-mix each ready-to-use working solution by adding 1 volume of GDP working solution to 1 volume of MgCl2 working solution. (e.g. add 200 µl of GDP working solution to 200 µl of MgCl2 working solution). 9 different mixes of GDP/MgCl2 can be prepared at the following concentrations:

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

For descriptive indications to set up the optimization step, please refer to these guides: “GTP binding assay a guide to optimizing Agonists of Gαi” and “GTP binding assay a guide to optimizing Antagonists of Gαi” on www.revvity.com

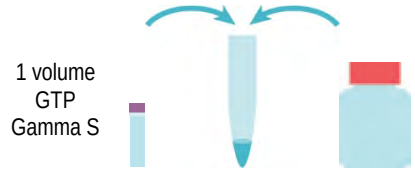
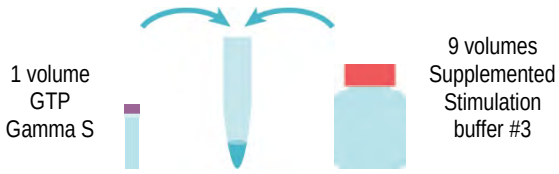
GDP and MgCl2 working preparation for GTP binding assay
Once optimal GDP and MgCl2 concentrations are known for the GPCR model of choice, they are directly added to the Stimulation Buffer #3 to dilute all the reagents required for the assay.    Add the corresponding volumes of GDP and MgCl2 stock solutions to the ready-to-use Stimulation Buffer #3 to reach the desired final concentrations (e.g. to prepare for 0.25 µM of GDP and 10 mM of MgCl2 final concentrations, add 10 µl of GDP stock solution (400 fold dilution) and 40 µl of MgCl2 stock solution (100 fold dilution) to 3,950 ml of stimulation buffer #3).

* Vial and Cap sizes and colors are representative of the 100 and 500 kit sizes. Follow the same procedure for the 20.000 kits size.

TO PREPARE GTP GAMMA S (GTPγS) WORKING SOLUTION

The GTP Gamma S (GTPγS) is provided for non-specific signal determination. This non-hydrolysable GTP is used at a saturation concentration of 25 µM. It binds Gα proteins and competes with the nucleotides present in the assay, in particular the GTP fluorescent analog. The measured signal in presence of saturating concentration of GTPγS is a measurement of the assay background.

Thaw GTPγS stock solution. Mix gently.

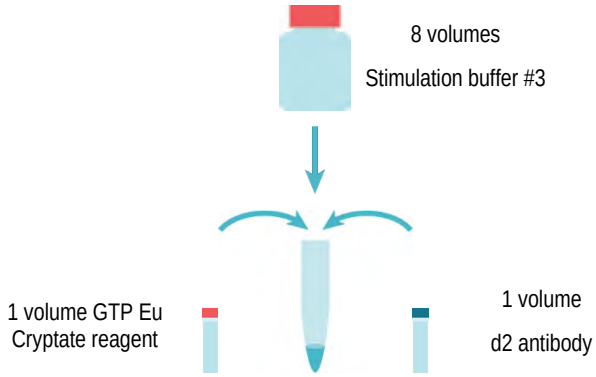
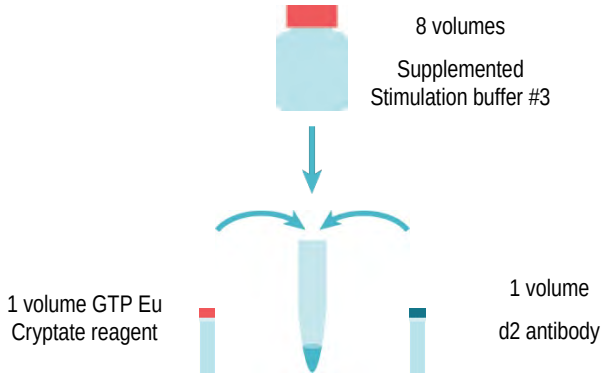
100 TESTS KIT #62GTPPET, 500 TESTS KIT #62GTPPEG & 20.000 TEST KIT #62GTPPEC*	
GTPγS working solution	
Optimization assay	GTP binding assay
 	
Dilute the GTP Gamma S stock solution 10-fold with Stimulation Buffer #3 or supplemented Stimulation buffer#3 (e.g. take 15 µl of GTP Gamma S stock solution and add it to 135 µl of Stimulation buffer). Mix gently. Working solution is at 100 µM and 25 µM in the final assay volume.	

* Vial and Cap sizes and colors are representative of the 100 and 500 kit sizes. Follow the same procedure for the 20.000 kit size.

TO PREPARE DETECTION REAGENT WORKING SOLUTIONS:

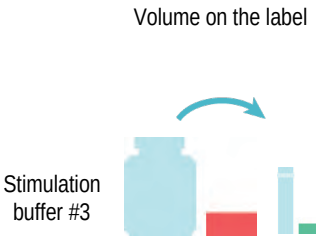
HTRF® reagent concentrations have been set for optimal assay performances. Note that any dilution or improper use of the d2-antibody and Eu Cryptate reagents will impair the assay's quality. Be careful, as working solution preparation for detection reagents may differ between the 100 and 500 tests data point kit. Working solutions are stable for 1 day at 2-8°C. Aliquot and freeze for long term storage.

Dilute the detection reagents in Stimulation Buffer #3 (optimization step) or supplemented Stimulation Buffer #3 (GTP binding assay).

100 TESTS KIT #62GTPPET, 500 TESTS KIT #62GTPPEG & 20.000 TEST KIT #62GTPPEC*	
Detection reagent mix	
Optimization assay	GTP binding assay
	
Dilute 10-fold the frozen stock solutions of GTP Eu Cryptate and d2 antibody in Stimulation Buffer #3 or supplemented Stimulation Buffer #3 (e.g. add 50 µl of GTP Eu Cryptate reagent and 50 µl of d2 antibody to 400 µl of Stimulation buffer).	

* Vial and Cap sizes and colors are representative of the 100 and 500 kit sizes. Follow the same procedure for the 20.000 kit size.

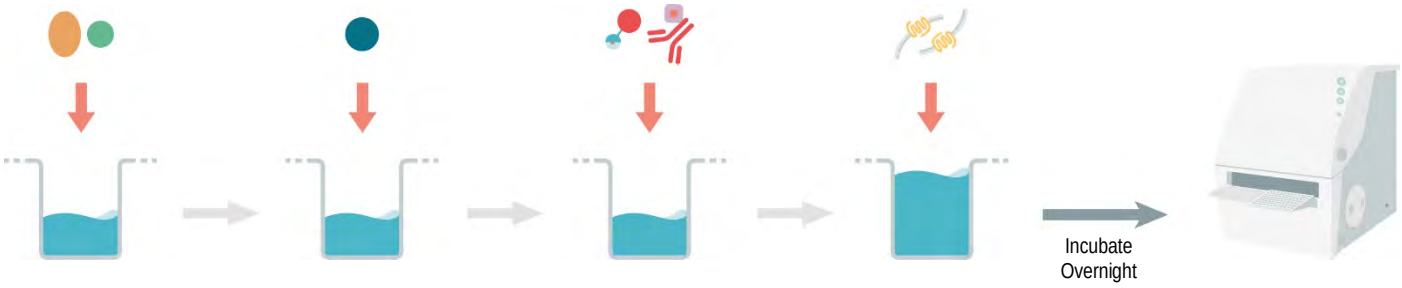
TO PREPARE GI PROTEIN POSITIVE CONTROL WORKING SOLUTION:

100 TESTS KIT #62GTPPET, 500 TESTS KIT #62GTPPEG & 20.000 TEST KIT #62GTPPEC*
Gi protein positive control working solution

Carefully open the vial cap and reconstitute the lyophilizate Gi protein control in Stimulation Buffer #3 (volume indicated on the label). The working solution is ready to use.

* Vial and Cap sizes and colors are representative of the 100 and 500 kit sizes. Follow the same procedure for the 20.000 kit size.

OPTIMIZATION ASSAY MANUAL

The 500 tests kit size is adapted to the optimization step performed before running the GTP binding assay (determination of optimal GDP/ MgCl2 Mix and membrane titration).



Incubation time can be adapted to the membrane model. Recommended reading times are at T3H and after overnight incubation.

STANDARD MANUAL FOR OPTIMIZATION STEP MANUAL IN 20 µL FINAL VOLUME

Negative control	Treated membranes	Non treated membranes	Positive control (optional)
Dispense 5 µl of GDP/MgCl2 Mix (4X)			Dispense 5 µl of stimulation buffer #3
Dispense 5 µl of GTP gamma S (4X)	Dispense 5 µl of Agonist at saturation concentration (4X)	Dispense 5 µl of stimulation buffer #3	
Dispense 5 µl of Detection reagents Mix			
Dispense 5 µl of Membrane			Dispense 5 µl of Gi positive Control

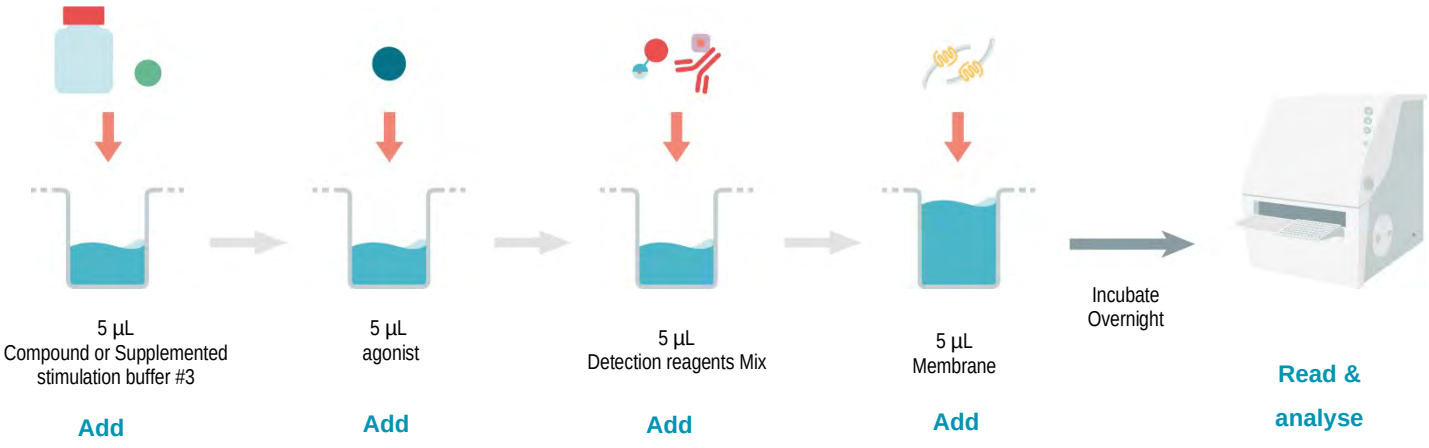
This optimization manual allows optimization of [GDP], [MgCl2] and membrane quantities required for the assay (depending on the membrane model of choice).

Recommendations and detailed guidelines to this optimization step are described in the guide www.revvity.com.

The negative control is used to determine the non-specific signal.

The Gi positive control is used to check detection reagents. The ratio between positive control / non-specific signal should be greater than 5 to ensure reagent integrity and proper use of the assay.

GTP BINDING ASSAY MANUAL



All the reagents are diluted in Stimulation Buffer #3 supplemented with optimal GDP and MgCl2 concentrations.

Manuals to study agonists or antagonists are described here. Other classes of compounds can be assessed using this assay. The manual strategy (i.e. compound dual incubation and optimal fixed agonist concentration to be used) depends on the nature of the compounds studied.

Recommendations to set up and analyze experiment for agonist and antagonist characterization are described in the guides www.revvity.com.

STANDARD MANUAL FOR GTP BINDING ASSAY MANUAL IN 20 µL FINAL VOLUME:

► *Manual for Agonist study:*

Negative control	Non treated membranes	Treated membranes
Dispense 5 µl of supplemented stimulation buffer #3	Dispense 5 µl of supplemented stimulation buffer #3	Dispense 5 µl of supplemented stimulation buffer #3
Dispense 5 µl of GTP gamma S (4X)	Dispense 5 µl of supplemented stimulation buffer #3	Dispense 5 µl of Agonist (4X)
Dispense 5 µl of Detection reagents Mix		
Dispense 5 µl of Membrane		

► *Manual for Antagonist study:*

Negative control	Non treated membranes	Treated membranes
Dispense 5 µl of supplemented stimulation buffer #3	Dispense 5 µl of supplemented stimulation buffer #3	Dispense 5 µl of Antagonist
Dispense 5 µl of GTP gamma S (4X)	Dispense 5 µl of Agonist at optimal concentration	Dispense 5 µl of Agonist at optimal concentration
Dispense 5 µl of Detection reagents Mix		
Dispense 5 µl of Membrane		

DATA REDUCTION & INTERPRETATION

1. Calculate the ratio of the acceptor and donor emission signals for each well.

$$\text{Ratio} = \frac{\text{Signal 665 nm}}{\text{Signal 620 nm}} \times 10^4$$

2. Calculate the % CVs. The mean and standard deviation can then be worked out from ratio replicates.

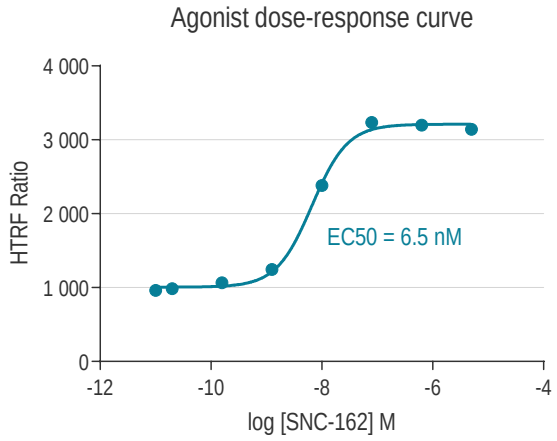
$$\text{CV (\%)} = \frac{\text{Standard deviation}}{\text{Mean Ratio}} \times 100$$

For more information about data reduction, please visit www.revvity.com

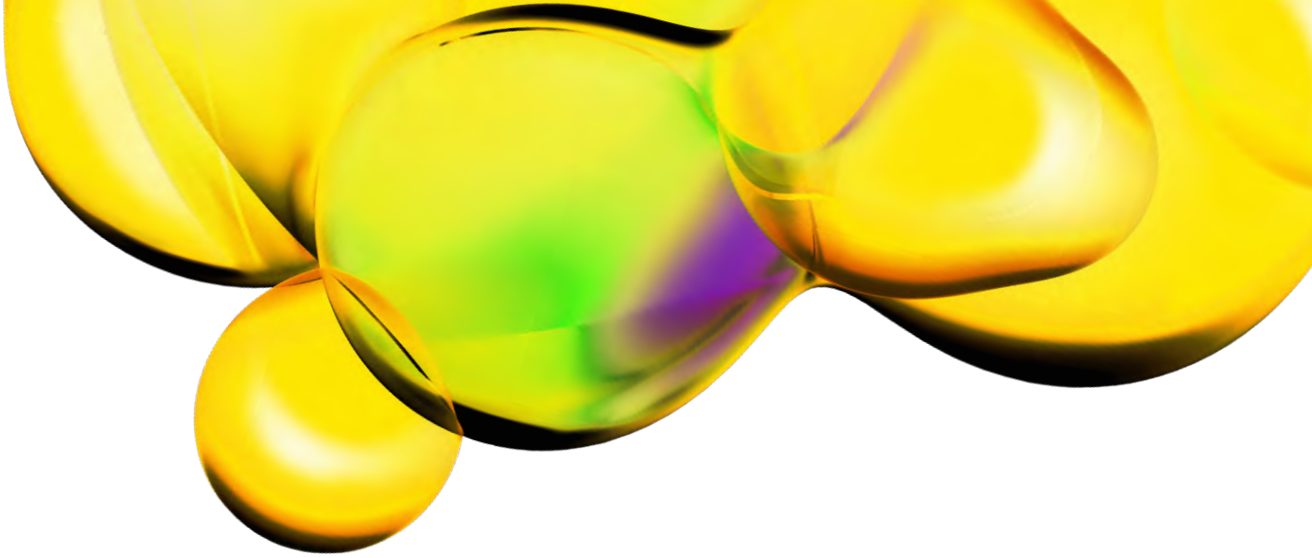
RESULTS

These data should be considered only as an example (readings on HTRF® compatible readers). Results may vary from one HTRF® compatible reader to another and depending on the membrane model.

For complete and detailed guidelines to process and analyze your GTP Gi binding assay results, please refer to our Technical Guides: GTP binding assay: a guide to optimizing Agonists of Gαi" and "GTP binding assay: a guide to optimizing Antagonists of Gαi"



Pharmacological response in CHO-Delta Opioid membrane extract: The assay was performed using the previously optimized conditions for this membrane model. Stimulation Buffer #3 was supplemented with 0.5 µM of GDP, 50 mM of MgCl2. The assay was performed using 5 µg of membranes / well. Reading was after overnight incubation time. The SNC-162 dose-response curve fit shows an EC50 of 6.5 nM, which is in accordance with published values.



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