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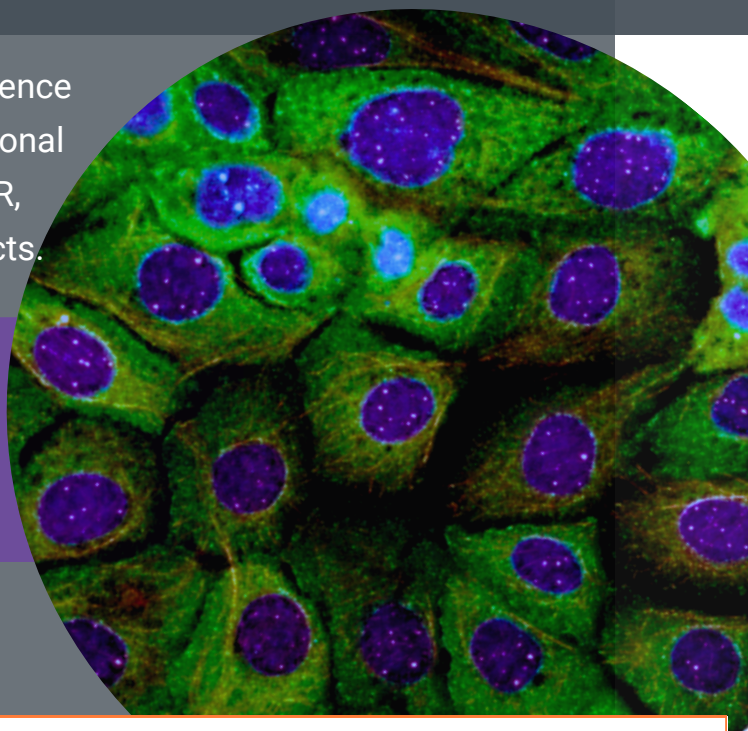
Assays for GPCR Drug Discovery

Among all FDA approved drugs, 35% target G-protein coupled receptors (GPCRs) [1]. GPCRs, are the largest and most diverse group of cell surface receptors in eukaryotic cells. They are 7-pass membrane proteins, whose activity depends on the recruitment of G-proteins, which elicit intracellular responses such as the generation of cAMP, release of intracellular calcium, activation of downstream signaling pathways and β-arrestin-triggered receptor internalization. GPCRs are involved in physiological processes ranging from vision, taste, and smell to mood regulation and immune response. This also extends to smooth muscle contraction and cardiovascular and digestive tracts regulation. Since they are central to cellular communication, GPCRs are a prime target for drug discovery.

At Paraza Pharma, our scientists have extensive experience developing and executing a range of binding and functional cellular assays, required for all different stages of GPCR, ion channel, and Receptor Tyrosine Kinase (RTK) projects.



- Radioligand binding assay
- cAMP detection
- Calcium assay
- Universal tool cell lines

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Membrane-based radioligand binding assay

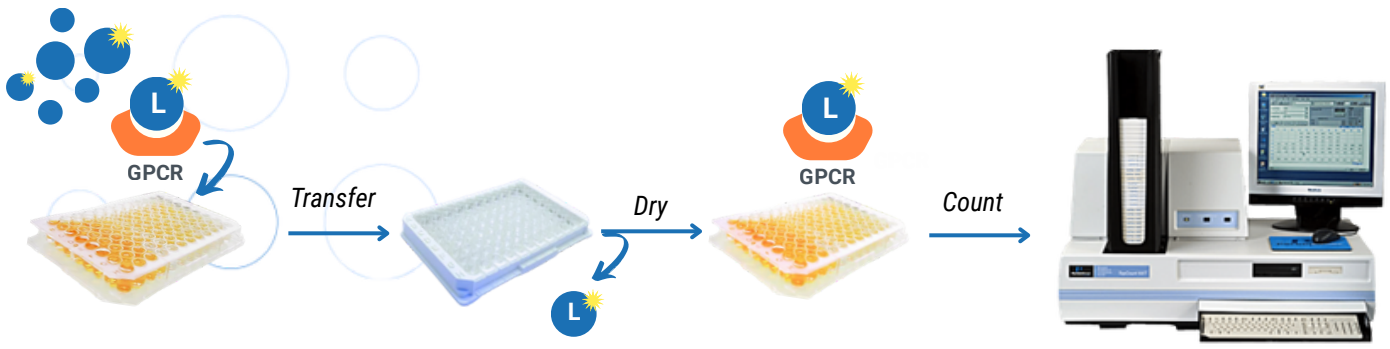
Binding assays are fundamental for drug structure-activity relationship studies and for the development and characterization of pharmaceutical compounds targeting GPCRs.
Through various protocols. such as saturation, competition, and kinetic binding, valuable quantitative information about receptor density, ligands' equilibrium dissociation and association constants/rates, can be obtained, as well as distinguish between orthosteric and allosteric receptor interactions. At Paraza, we are fully licensed to utilize radiolabeled probes to perform binding studies.

Membrane preparations

One of the challenges when setting up biochemical assays for GPCRs is having an enriched population of functional receptors, as this requires proper folding of the seven transmembrane domains within a lipid bilayer. Careful preparation of micelles, originating from the membranes of animal tissue or from cell lines overexpressing the receptor of interest is a crucial first step to the development of an accurate and reproducible assay. At Paraza, we have experience in selecting and setting up receptor preparation protocols for optimal expression level and scalability potential, including the implementation of Expi293™/ExpiCHO™ expression systems to perform bulk membrane preparations with exogenous expression of target GPCRs, suitable for HTS screening.

The gold-standard technique to evaluate drug-receptor binding affinity

HTS radioligand binding assay workflow



Receptor membrane preps are incubated with radiolabelled and unlabelled ligands.

Separation by filtration to remove excess unbound ligand.

Only receptor-bound ligands are retained on filter plate.

Measure binding level with a scintillation counter, TopCount.

Fig 1. Workflow of the radioligand binding assay.
Membrane preparations isolated from cells expressing the receptor of interest are incubated with radiolabeled ligand and competing compound in binding buffer in 96-well assay plates. Volumes are then transferred to 96-well filter plates and are filtered using a vacuum manifold to retain the bound-radiolabeled ligand. Filter plates are then washed several times to remove excess unbound radiolabeled ligand and are then incubated at 50°C until fully dry. Scintillation cocktail is then added to wells and radioactivity is measured with a TopCount scintillation counter.

*Expi-293 and Expi-CHO are registered trademarks of Life Technologies Corporation.

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Kd value determination

In-house membrane preparations from Expi293™ cells over-expressing the GPCR of interest can be used to perform saturation binding assay with radioisotope-labeled ligand. Non-specific binding (NSB) is determined in the presence of saturating concentration of cold ligand subtracted from total binding to obtain specific binding saturation curves. Data are plotted on GraphPad Prism using “one-site specific binding” model to obtain Kd and BMax values. CPM values can be converted to pMol/mg protein by considering the specific activity of the ligand, the protein concentration and the counter efficiency.

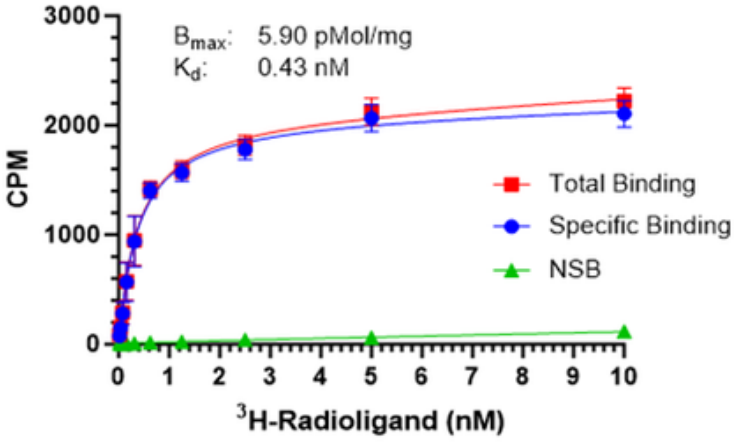
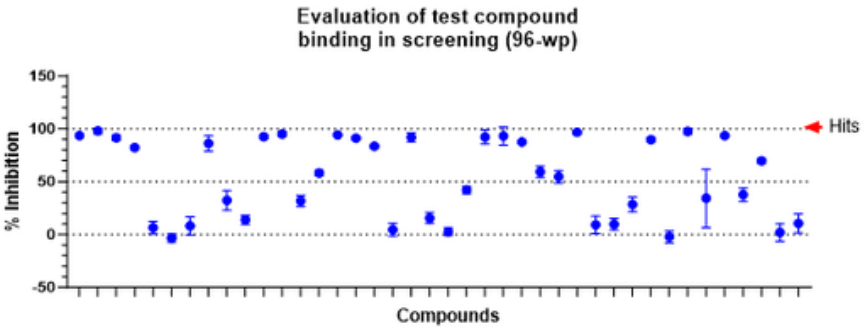


Fig 2. Kd value and receptor occupancy determination using radioligand binding assay with 3H-labelled probe.
Data points represent the corresponding ligand concentrations (X-axis), and the CPM measurements obtained (Y-axis).

Compound screening using radioligand-binding assay

Once assay conditions are established, binding assays are well-suited to medium-throughput screening or quantitative dose-response evaluation of test compounds. In this example, 8 hits were identified and were subsequently validated in dose-response competition biding to determine their relative potencies.



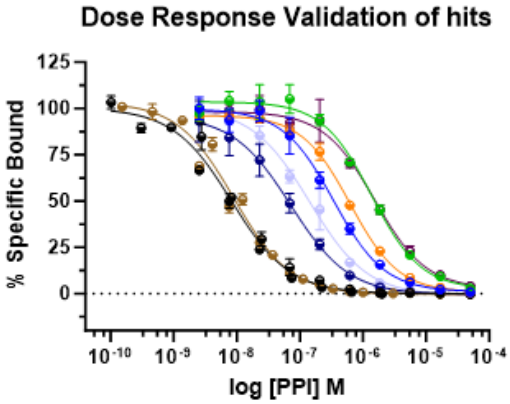
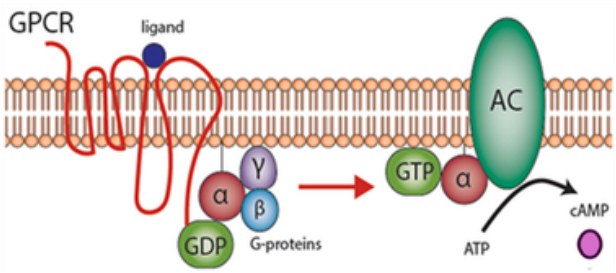


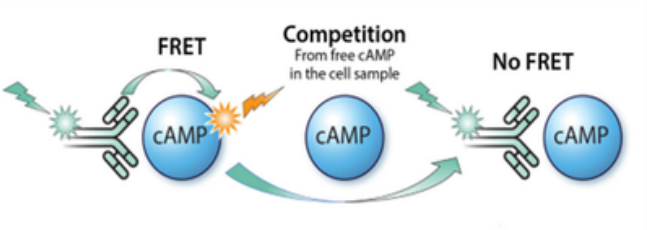
Fig 3. Single point screening and hit validation of compounds by filtration-based competition binding assay.
Competition binding assay was performed to assess the ability of 10 µM compounds to completely inhibit binding of 3H-ligand to the target GPCR. Up to 40 compounds were tested per 96-well plates with technical duplicates.

cAMP as a downstream readout of GPCR activity

GPCRs that stimulate cAMP production will activate adenylyl cyclase via stimulatory G protein (Gs); conversely, action through inhibitory G protein (Gi) can inhibit cAMP production. Monitoring intracellular cAMP levels is a robust and well-validated approach to evaluate GPCR agonists and antagonists, as well as allosteric modulators. cAMP detection assays are popular screening methods due to their high sensitivity and the availability of many quantifiable detection methods.

TR-FRET is one of the most versatile methods to quantify cAMP. In this assay, the level of cAMP is measured indirectly through competition with Eu-labelled cAMP for binding to the cAMP antibody. The higher the intracellular cAMP concentration, the lower the TR-FRET signal. This assay can be used for characterization of agonists and antagonists as well as positive or negative allosteric modulators (PAMs, NAMs).





cAMP assays afford customization and adaptability of screening workflows.

HTS Workflow

Dispense compounds

Echo Acoustic Dispenser

Tecan D300e

Plate cells

Multidrop

Apricot S3

Read signal

Envision Plate Reader with plate stacker

Fig 4. Assay workflow for cAMP assay leveraging nanodispensers and automation to increase throughput.

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Measuring GPCR activity in CHO-K1 cells

GPCR overexpression in CHO-K1 cells constitutes a well-established system to study GPCR signaling. For Gs-coupled receptors, overexpression results in a robust concentration of cAMP at basal levels that is present even in non-stimulated conditions, allowing straightforward use of the cells for compound screening.

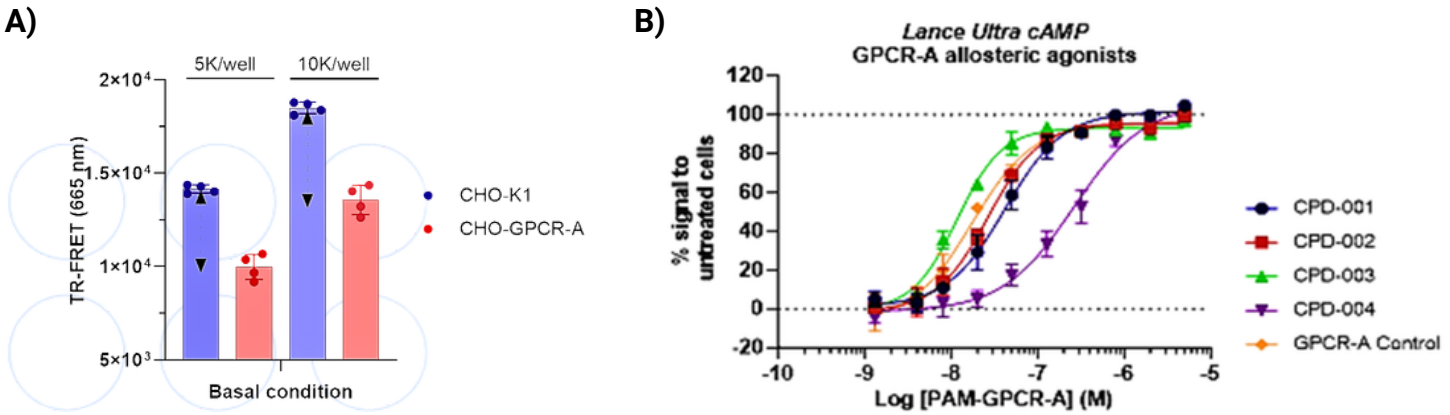


Fig 5. GPCR-A overexpression system in CHO-K1 cells used to screen agonists. *(A) Basal cAMP increase is detected in non-stimulated overexpressed cells. (B) Characterization of allosteric agonists against GPCR-A. TR-FRET signal is reproducible and robust (Z' > 0.5) for agonist assays.*

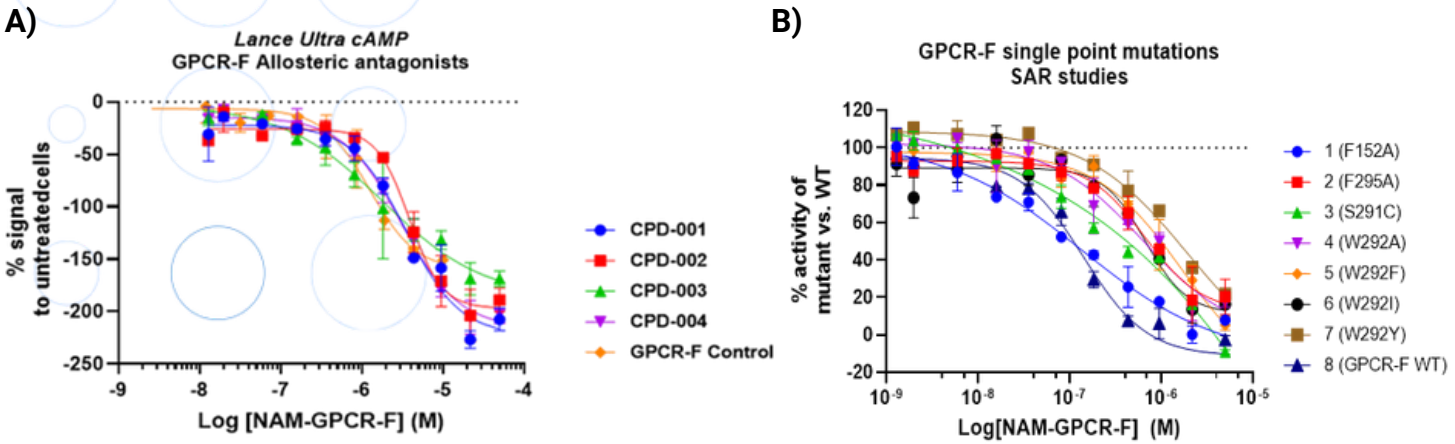
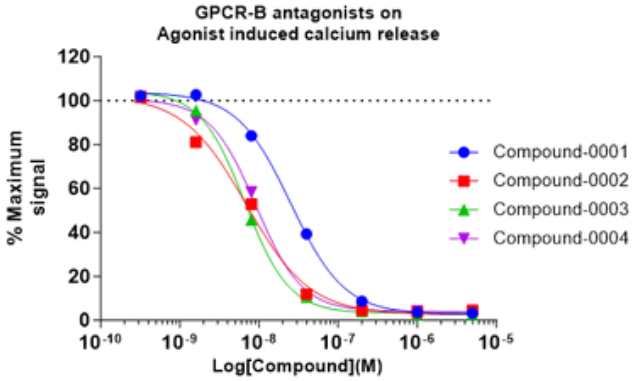


Fig 6. GPCR-F overexpression system in CHO-K1 cells used to screen antagonists and identify key residues of the predicted active site. *(A) Allosteric antagonists against GPCR-F were tested using Lance Ultra cAMP detection kit. Under optimized conditions, TR-FRET signal is reproducible and robust (Z' > 0.5) for antagonist assays. (B) A human GPCR-F homology model was predicted[3]. To validate the model, rationalize the structure-activity relationships and identify the key residues in the binding crevice for ligand recognition, we cloned several GPCR-F single point mutations and expressed in CHO-K1 cells. Activity of the mutants were compared to wild the using GPCR-F control compound.*

Measuring Calcium Release After GPCR / Ion Channel Activation

A change in the concentration of Ca2+ is an important step in signal transduction. Several types of receptors, ion channels and GPCRs convert extracellular signals into intracellular responses [3]. The calcium mobilization assay is a robust screening method due to its high sensitivity and the availability of cell-permeable calcium fluorescent dyes. Detection methods are compatible with both FlexStation® 3 and real-time fluorescence plate reader (FLIPR), allowing assay development followed by high throughput compound testing.

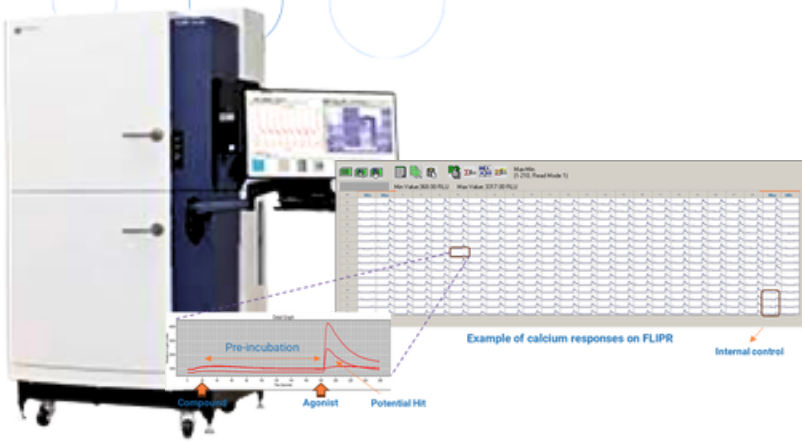
FlexStation 3 for GPCR-mediated Ca^{2+} signaling (low throughput)



Calcium signal detection is a rapid and scalable way to characterize GPCR ligands

Fig 8. Antagonist mediated calcium mobilization decrease of a Gq-coupled GPCR endogenously expressed in a kidney cell line. *Dose-response curves of antagonists pre-incubated for 2 min followed by the addition of EC80 concentration of the target receptor agonist.*

FLIPR for HTS Ca2+ assays



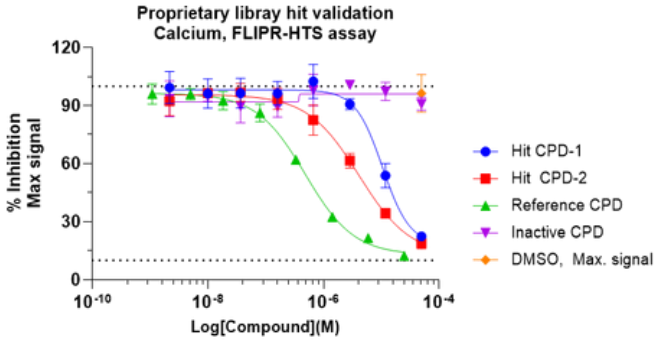


Fig 9. Hit identification from our proprietary library against a ligand-gated and calcium sensitive ion channel overexpressed in HEK293 cells. *Dose-response curves of antagonists pre-incubated for 2 min followed by the addition of EC80 concentration of the target receptor agonist.*

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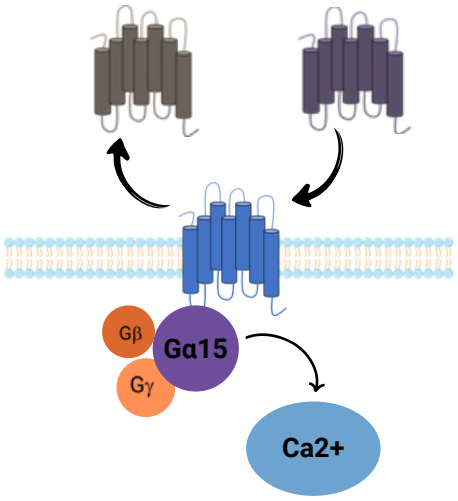
Universal Tool Cell Lines for GPCR-Screening

Cell-based functional assays for GPCR screening are often limited to a subset of Gα subunits and can only be applied for GPCRs with well-established signaling pathways. A universal HTS approach for GPCR targets provides the framework to study a broader range of GPCRs, including orphan receptors. Previous studies have identified Gα15 as a promiscuous G protein subunit that directs GPCR signaling to the calcium (Ca2+) mobilization pathway and have been used for HTS of GPCRs [4].

To enable a universal cell-based assay for the identification of compounds targeting select GPCRs a HEK293 cell line stably expressing Gα15 protein was established at Paraza. Agonist-stimulation of over-expressed Gαi-coupled receptor in these cells promotes non-canonical calcium mobilization response. Further stable cell lines expressing other promiscuous G protein subunits as Gqi5, Gqs5 are being established to broaden options for screening.



HEK293-Gα15 cell line for a universal GPCR Calcium mobilization assay

GPCR antagonist dose-response

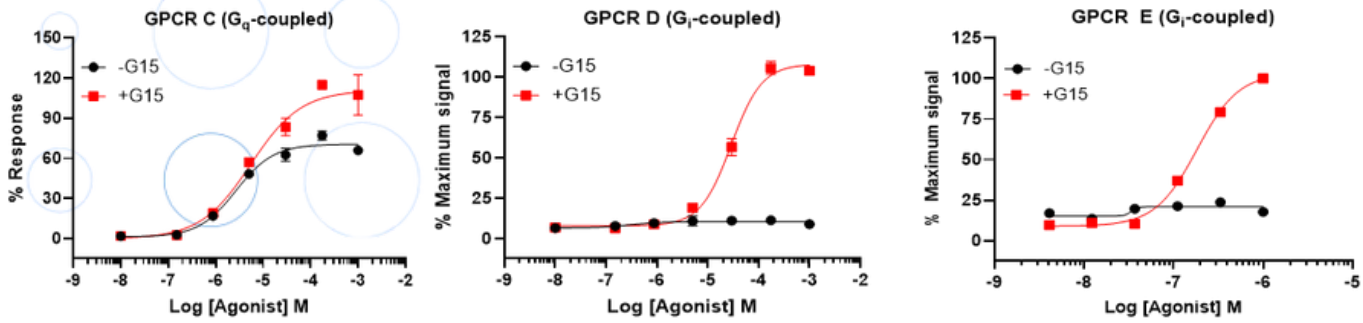


Fig 10. Agonist-mediated calcium mobilization of Gq- or Gi-coupled GPCRs transiently expressed in HEK293 cells overexpressing control plasmid or Gα15 subunit. *Using the FlexStation platform, Ca2+ response was recorded after agonist-stimulation of Gi or Gq-coupled receptor in HEK293 cells expressing the GPCR. This response was augmented in HEK293 cells only co-expressing the Gα15 subunit.*

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