Pharmacology and Pharmacodynamics | Methods and Cases for *In Vitro* Assay of GPCR Targets

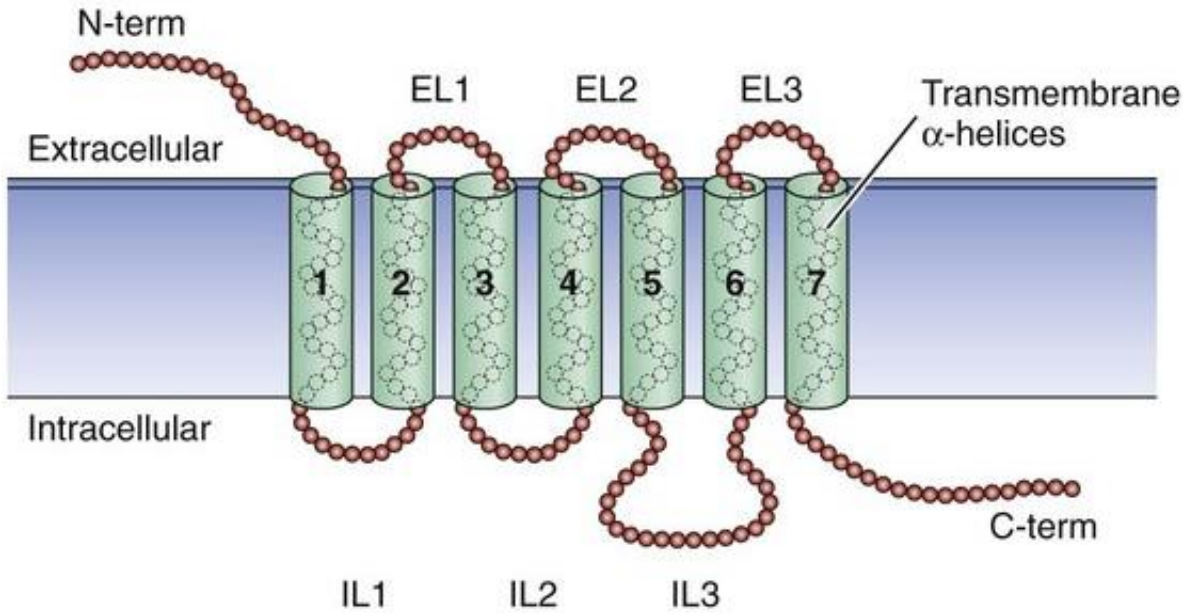
Sanyou Biopharmaceuticals Co., Ltd.

Innovation / Outstanding / Reliability

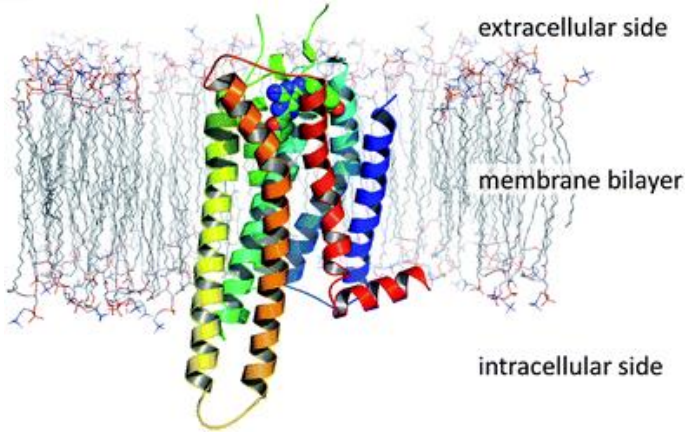
GPCR - Background

- **G protein-coupled receptor (GPCR) is a class of 7-transmembrane proteins expressed on the plasma membrane, with more than 800 members. It is the largest membrane protein family in mammalian genome.**
- **GPCR proteins are widely distributed in organs and tissues such as the central nervous system (CNS), immune system, cardiovascular system and retina, participating in the development and normal functional exercise of the body. Abnormal signal transduction and mutations are closely related to various diseases.**
- **GPCRs can respond to a diverse range of natural ligands including light, biogenic amines, amino acids, nucleosides/nucleotides, ions, pH, pheromone, odor, metabolites, lipids, glycoproteins, proteases, peptides and proteins.**

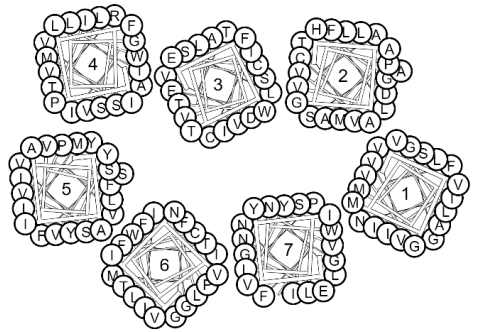
Pattern of GPCR



Stereogram of GPCR



adenosine A_{2A}



Spatial Graph of GPCR (Top View)

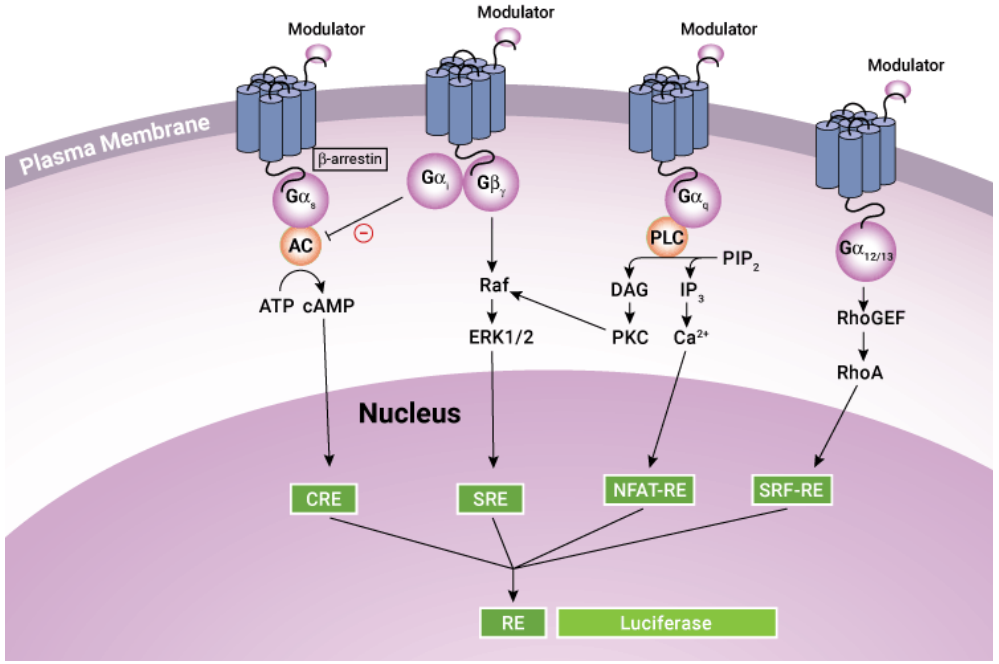
Wikimedia, GPCRdb,
Stephen P. Andrews, *et al*. Stabilised G protein-coupled receptors in structure-based drug design: a case study with adenosine A2A receptor, *Med. Chem. Commun*. 2013

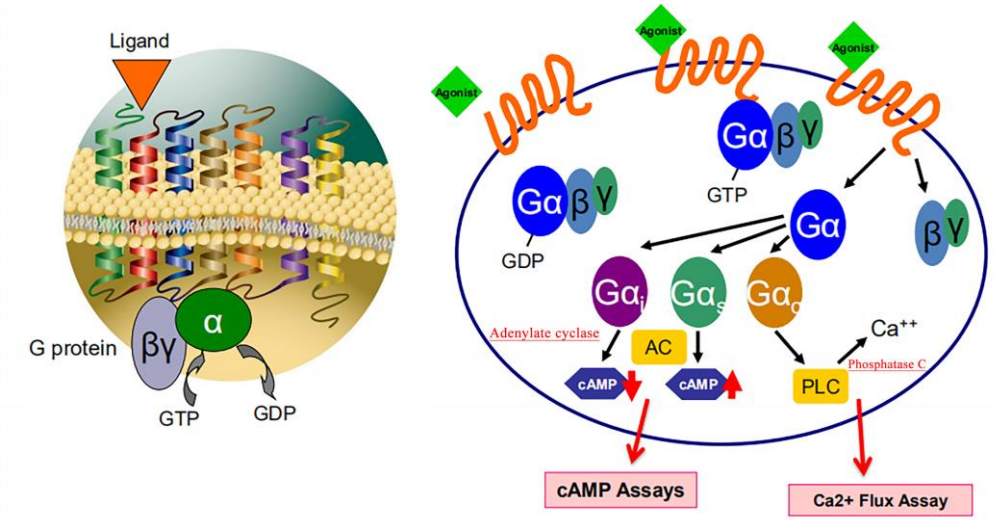
Summary of GPCR-mediated Signaling Pathways and Assay Methods

The common *in vitro* pharmacological assay methods for GPCR-mediated signaling pathways are as follows:

Number	Screening level	Receptor	Read-out system	Platform
1	**cAMP accumulation assay (Case 2)**	Gs/Gi coupled	Fluorescence/luminescence	HitHunter™
			ALPHA® (optical)	AphaScreen™
			Luminescence	cAMP Glosensor™
			HTRF	HTRF®-based cAMP
2	**Ca^{2+} (Case 3)**	Gq coupled (possible universal)	Fluorescence	FLIRP™
3	InsP3		Luminescence	AequoScreen™
4	InsP1 accumulation assay		FP	HitHunter™-IP3
			HTRF	IP-ONE™
5	β-Arrestin recruitment	Universal	Fluorescence	Transfluor™
			Luminescence	PathHunter™
6	Integrated cellular response	Universal	SPR	Biacore
				ECIS™
			Impedance	CellKey™
				XCELLigence™
			RWG	BIND™
				EPIC™
7	Ligand binding	Universal	Radiometric	Filtration assay
				FlashPlate®
				SPA
			HTRF	DELFIA™
				Tag-lite™
8	G protein activity	Gi/G0 coupled	Radiometric	Filtration assay
				FlashPlate®
				SPA

Summary of GPCR-mediated Signaling Pathways and Detection Methods - FACS Test, cAMP Assay, and Calcium Flux Assay





1. Alhosaini K, Azhar A, Alonazi A, Al-Zoghaibi F. GPCRs: The most promiscuous druggable receptor of the mankind. Saudi Pharm J 29, 539-551 (2021)
2. Martins SAM et al. Towards the miniaturization of GPCR-based live-cell screening assays. Trends in Biotechnology 30, 566-574 (2012)

Intracellular signaling after binding of GPCRs to ligands occurs through four G protein families (Gαs, Gαi/Gα0, Gαq/Gα11 and Gα12/Gα13).

cAMP: It is an important cellular signaling molecule that regulates multiple physiological processes within cells. Activated GPCRs can activate adenylate cyclase via G proteins, resulting in increased cAMP synthesis. Therefore, changes in intracellular cAMP levels can reflect the activation of GPCR receptors.

Calcium flux: Activated GPCRs can affect the concentration of intracellular calcium ions, so intracellular signaling triggered by receptor activation can be detected via calcium flux.

FACS Test Case

Background: FACS Binding refers to the binding between fluorescent dye and cell surface or intracellular molecules during Fluorescence-Activated Cell Sorting (FACS) in flow cytometry.

Purpose: To test the binding capacity of candidate antibodies to target cells.

Method: Bind the antibodies to the target cells, then bind the antibodies to the fluorescent secondary antibody, and test the mean fluorescence intensity (MFI) of the cells by flow cytometry to assess the binding activity between the antibodies and targets.

Results: Fig. 1 presents the binding activity test results of candidate antibodies on overexpression cells, and Fig. 2 presents the peak diagram of positive antibody binding capacity deviation. As shown in Fig. 1, all candidate antibodies exhibited good affinity with cells. The EC_{50} of Benchmark was 4.78 nM, with clearly defined upper and lower plateaus, indicating a wide window.

Conclusion: The binding of candidate antibodies on overexpression cells is weaker than that of the positive antibody.

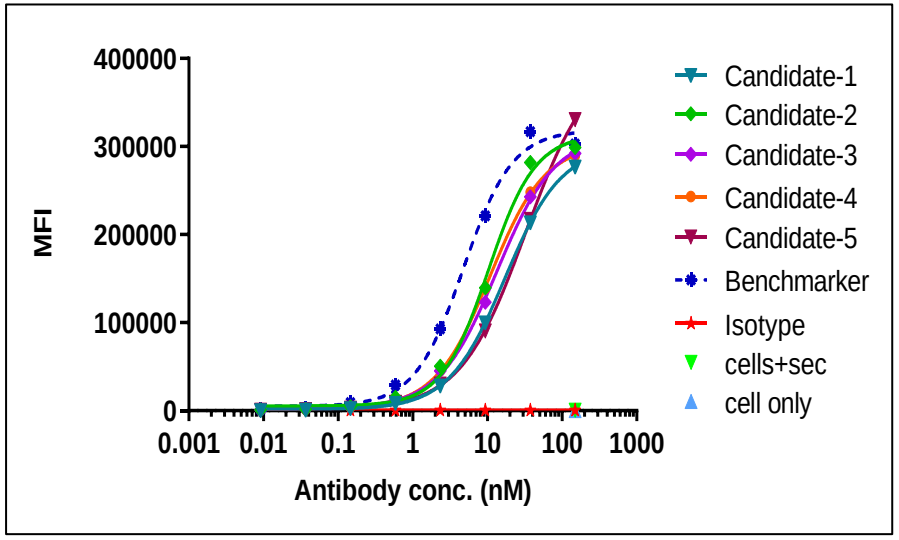


Fig. 1 Binding Activity Test of Candidate Antibodies on Overexpression Cells

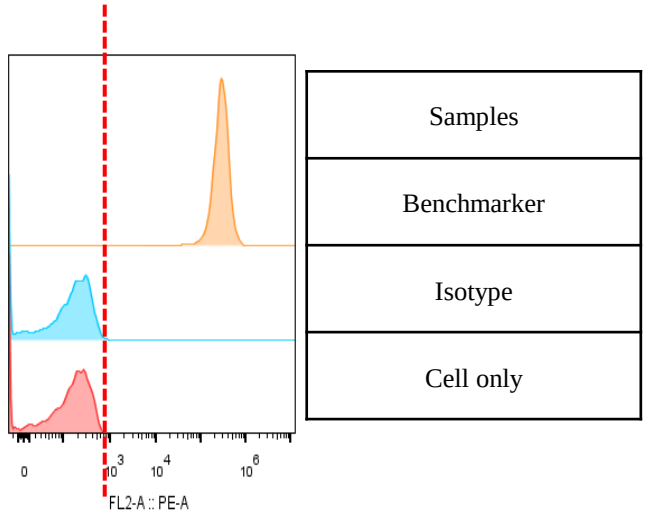


Fig. 2 Peak Diagram of Positive Antibody Binding Capacity Deviation

cAMP Assay Case - 1

Background: cAMP (cyclic adenosine monophosphate) is a ubiquitous second messenger in various organisms, playing a crucial role in regulating multiple physiological processes. Accurate assay and quantification of cAMP are of great scientific and clinical significance.

Purpose: To assess the activation of cAMP by ligands by fluorescence resonance energy transfer (FRET).

Method: Starve the cells 1 day in advance, digest them the next day, stimulate with ligands for 15 min, add cAMP assay reagent to incubate at room temperature for 1 h, and then detect the signals with a multifunctional microplate reader.

Results: Fig. 4 presents the signal values of cAMP generated by ligand-activated cells, and Fig. 5 presents the converted concentration values of cAMP. The IC_{50} value of ligand 1 was 0.036 nM, and that of ligand 2 was 40.47 nM, with clearly defined upper and lower plateaus, indicating a wide window.

Conclusion: The ligands can effectively stimulate cAMP signaling, and the activation ability of ligand 1 is better than that of ligand 2.

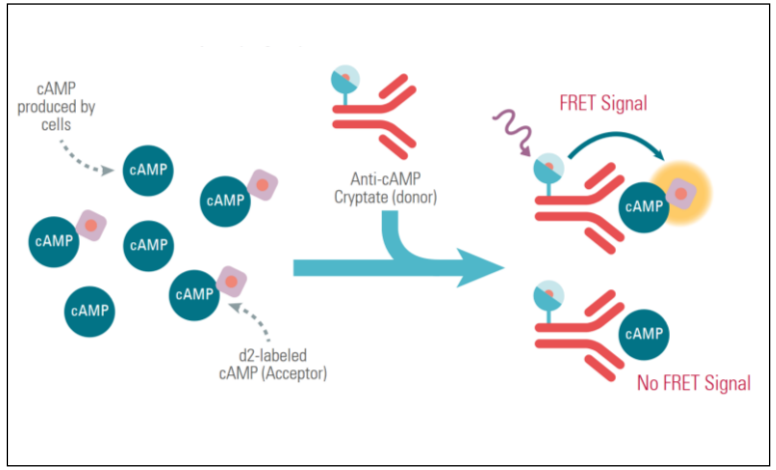


Fig. 3 Schematic Diagram of cAMP Assay

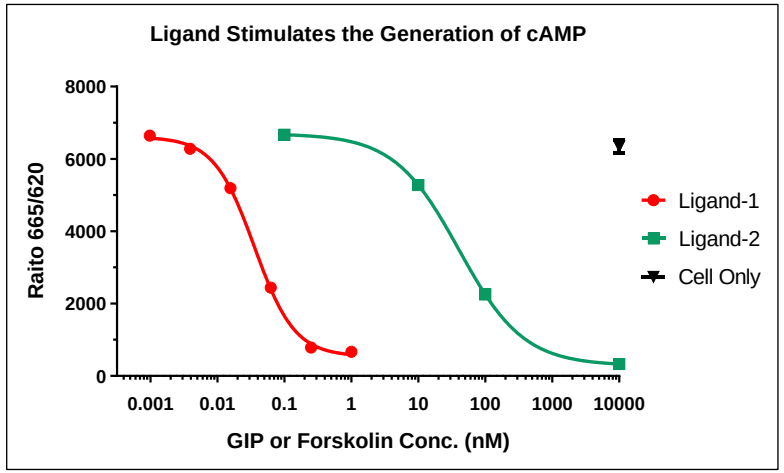


Fig. 4 Signals of cAMP Generated by Ligand-activated Cells

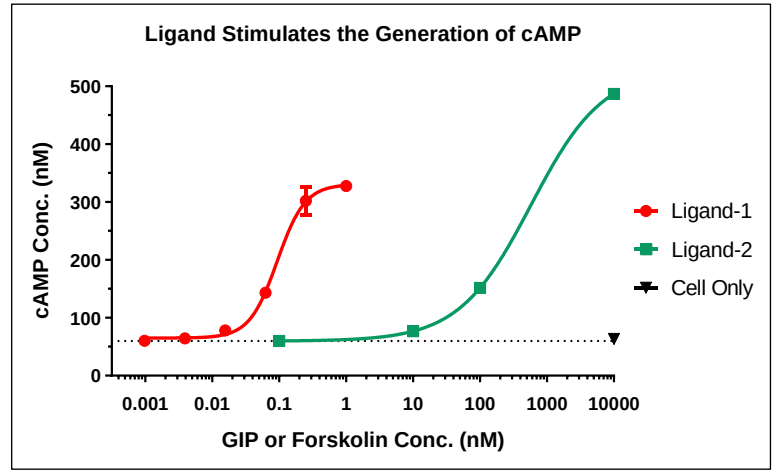


Fig. 5 Converted Concentration Values of cAMP

cAMP Assay Case - 2

Background: cAMP (cyclic adenosine monophosphate) is a ubiquitous second messenger in various organisms, playing a crucial role in regulating multiple physiological processes. Accurate assay and quantification of cAMP are of great scientific and clinical significance.

Purpose: To assess the inhibition of antibodies on cAMP activation by FRET.

Method: Starve the cells 1 day in advance, digest them the next day, incubate with diluted antibodies for 50 min, stimulate with ligands for 15 min, add cAMP assay reagent to incubate at room temperature for 1 h, and then detect the cAMP signals.

Results: Fig. 6 presents the cAMP standard curve, Fig. 7 presents the signal value of cAMP generated by Benchmarker inhibiting ligand-activated cells, and Fig. 8 presents the converted concentration values of cAMP. As shown in the figures, the IC_{50} value of the antibodies was 5.29 nM at a ligand concentration of 0.25 nM and 2.29 nM at a ligand concentration of 0.05 nM, with clearly defined upper and lower plateaus, indicating a wide window. All the candidate antibodies had clearly defined upper and lower plateaus.

Conclusion: The antibodies can effectively inhibit the activation of cAMP by ligands.

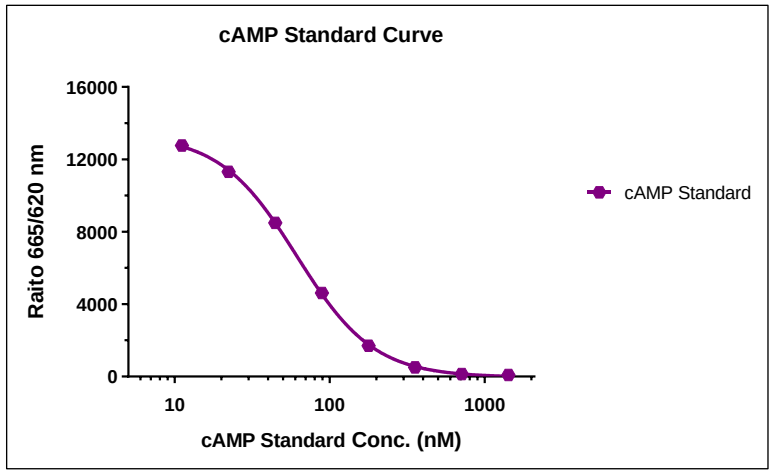


Fig. 6 Standard Conversion Curve of cAMP Concentration to 665/620 Signal Ratio

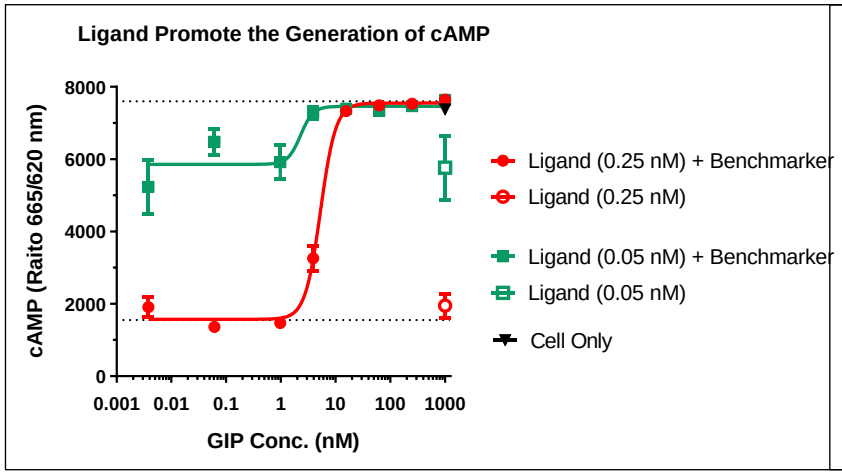


Fig. 7 Signals of cAMP Generated by Benchmarker Inhibiting Ligand-activated Cells

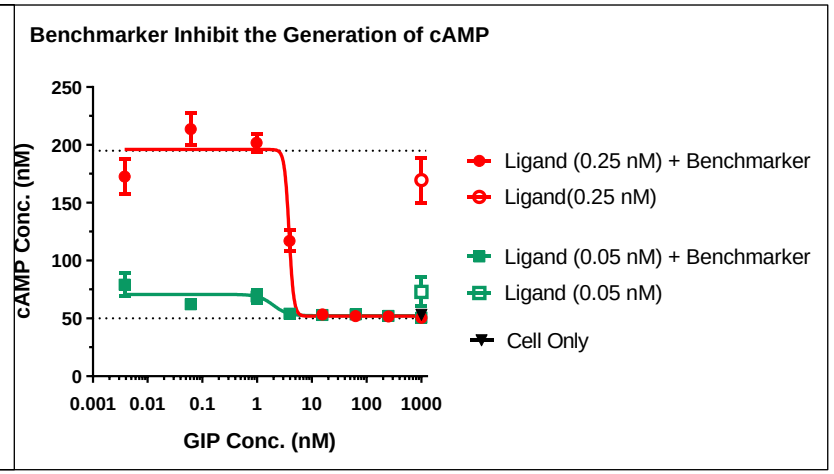


Fig. 8 Converted Concentration Values of cAMP

Calcium Flux Assay Case

Background: Calcium flux assay is an important technique in various fields such as cell signaling, neuroscience, and cardiovascular physiology. As a ubiquitous second messenger, calcium ion (Ca^{2+}) plays a crucial role in cells and regulates numerous life processes such as muscle contraction, neurotransmitter release, enzyme activity regulation, and gene expression. Therefore, it is of great significance to accurately monitor the dynamic changes of intracellular and extracellular Ca^{2+} for understanding cell function and disease mechanism.

Purpose: To assess the inhibition of antibodies on calcium flux activation by probe technique.

Method: Adjust the cell density, plate the cells, add Fluo-8 to incubate at 37° C for 30 min, add antibodies to incubate at room temperature for 30 min, stimulate the cells with ligands, and then detect the signals with a multifunctional microplate reader.

Results: Fig. 9 presents the results of calcium influx generated by Benchmarker neutralizing ligand-activated cells, suggesting that calcium influx can be effectively reduced by adding Benchmarker. Fig. 10 presents the results of calcium influx generated by candidate molecule neutralizing ligand-activated cells, suggesting that with the increase of antibody concentration, the positive antibody and candidate molecules exhibit significant inhibitory effects, with clearly defined upper and lower plateaus, indicating a wide window.

Conclusion: The candidate antibodies and positive control can effectively inhibit the activation of cAMP by ligands.

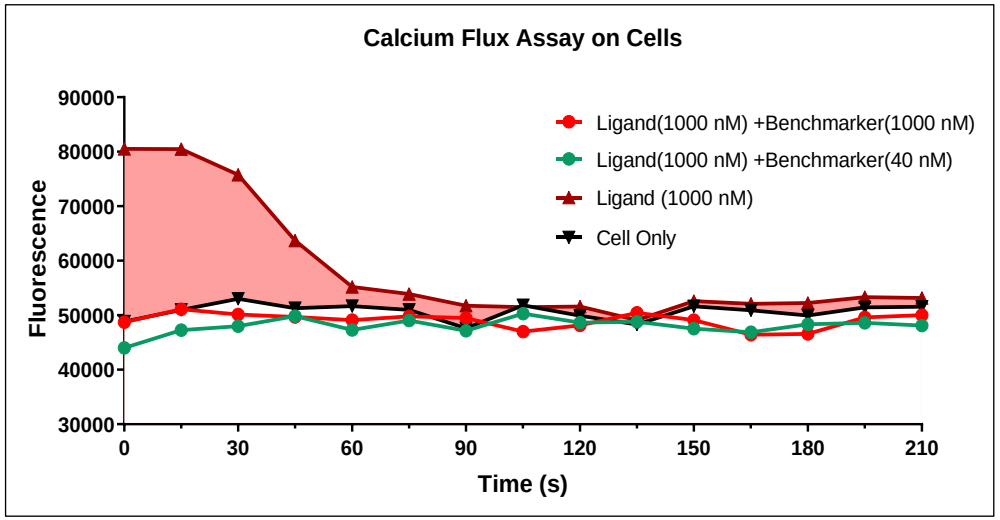


Fig. 9 Calcium Influx Generated by AMG133 Neutralizing GIP-Activated GIPR-HEK293 Cells

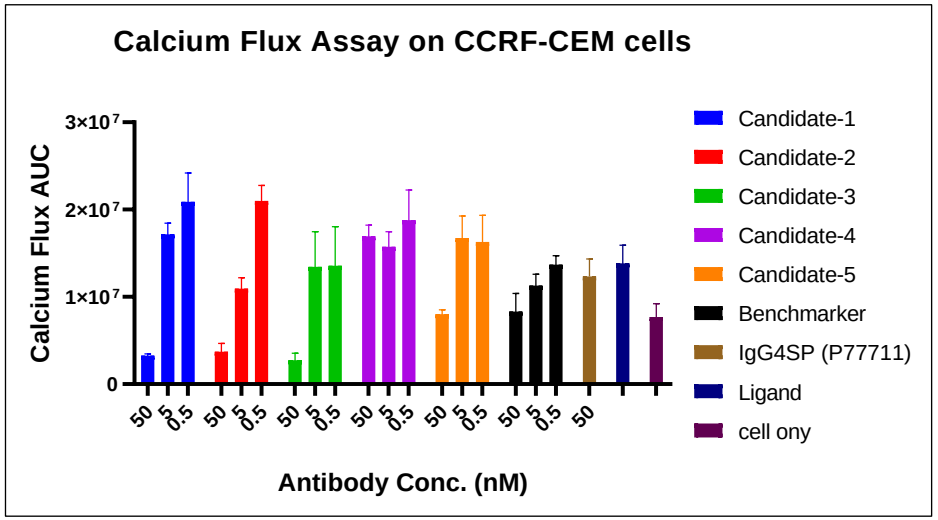


Fig. 10 Calcium Influx Generated by Candidate Molecule Neutralizing Ligand-Activated Cells

THANKS

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