

HTRF Technology

This application note demonstrates how a selection of cAMP kits assay covering different ranges is a significant advantage for researchers.

Cyclic-AMP: A Functional Second Messenger for Assaying GPCR Target Activation

Abstract

G-protein coupled receptors (GPCR) are one of the most popular target classes investigated in modern drug discovery. Drugs targeting GPCRs represent the core of modern medicine. They account for the majority of the best selling drugs and about 40% of all prescription pharmaceuticals in the marketplace.

With a selection of kits (cAMP femto, cAMP dynamic and cAMP HiRange), PerkinElmer allows detection of a broad range of cyclic-AMP (cAMP) concentration, a second messenger of GPCR activation.

GPCR Signaling Pathway

Following activation, GPCRs transmit information within cells via two signaling pathways: changes in the level of secondary messenger cAMP, or changes in the level of intracellular Ca^{2+} , which is liberated by secondary messenger inositol (1,4,5) triphosphate (IP3).

Traditionally, the GPCR signaling pathway is considered as a three-component system that involves:

- The receptor: 7TM (7 transmembrane domains)
- The trimeric G protein complex: $G\alpha$, $G\beta$, $G\gamma$
- The effector

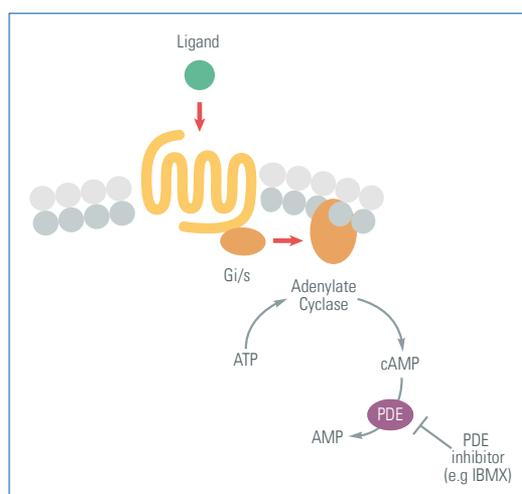


Figure 1: Gi/s Activation Pathway

When activated, the receptor (7TM) associates with the G-protein complex causing the $G\alpha$ subunit to exchange bound GDP for GTP, followed by dissociation of the $G\alpha$ -GTP complex.

The activated $G\alpha$ subunit can couple to downstream effectors to regulate the level of second messengers within the cell.

There are three main types of $G\alpha$ subunit, $G\alpha_i$, $G\alpha_s$, and $G\alpha_q$. Typically, GPCRs preferentially activate only one type of G-protein.

Table 1: GPCRs Can Mainly be Sub-Classified by G-Protein Type, i.e. $G\alpha_s$, $G\alpha_i$, $G\alpha_q$.

$G\alpha$ Subunit Activation	Effector & Signal Transduction	Second Messenger
$G\alpha_i$ (Gi)	The $G\alpha_i$ activation induces the inhibition of the effector activity (Adenylate Cyclase - AC), resulting in a decrease of the intracellular cAMP concentration*.	cAMP
$G\alpha_q$ (Gs)	$G\alpha_s$ activation leads to stimulation of the effector activity of Adenylate Cyclase (AC), resulting in an increase in intracellular cAMP concentration*.	cAMP
$G\alpha_q$ (Gq)	$G\alpha_q$ activation leads to Phospholipase C (PLC) induction of the inositol phosphate cascade, resulting in a transient increase in intracellular IP3 concentration and subsequent calcium release from the endoplasmic reticulum (ER).	IP3, IP1, Ca ²⁺

* The amount of decrease in cAMP levels by Gi-coupled receptor activation depends on the basal level of cAMP present within the cells. Often, the effects of this inhibition is more easily observed when a specific agent, such as forskolin, is used to activate adenylate cyclase.

The molecular tracers commonly used to assess GPCR activation are the second messengers cAMP, calcium and IP3.

Nevertheless, IP3 is unstable within cells ($t_{1/2} \approx 20$ seconds) and rapidly enters the metabolic inositol phosphate cascade. In the presence of lithium chloride, however, IP1, a metabolic product of IP3, accumulates within cells, and offers a viable metabolite biomarker for visualization of Gq-coupled receptor activity.

The assessment of cAMP and IP1 using HTRF technology allows researchers to implement functional assays for the screening new therapeutic candidates in HTS-compatible conditions. This application note will focus on the use of cAMP assays.

Functional Assays: cAMP Accumulation

Determination of intracellular cAMP levels allows direct pharmacological characterization of compounds acting on Gi or Gs coupled receptors.

The basal cAMP level (i.e. in the absence of compound) is compared with the cAMP level reached after stimulation with a compound of interest.

In a direct homogeneous assay compatible with HTS conditions, cAMP detection can be directly visualized using HTRF technology (Fig. 2).

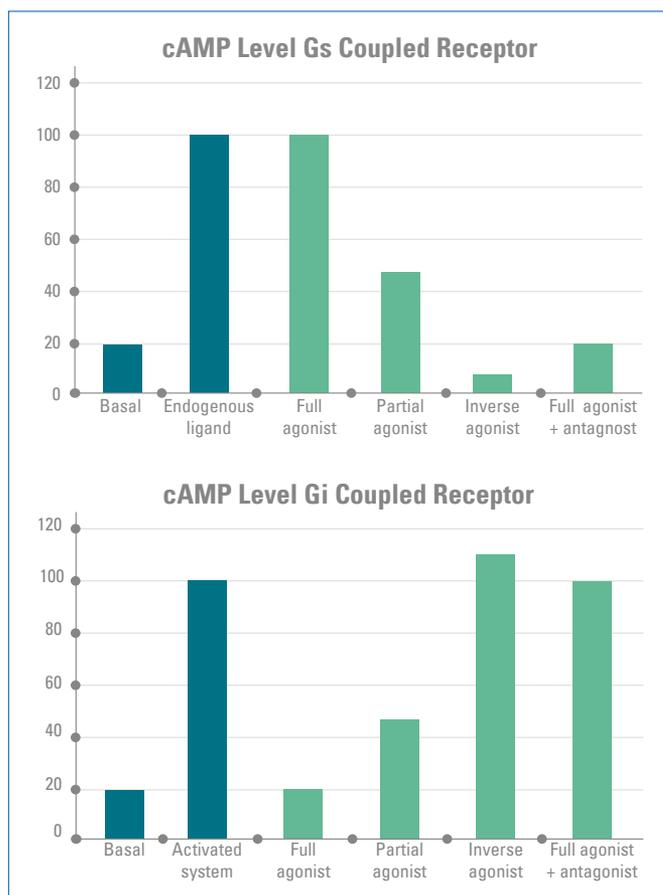


Figure 2: These Graphs Represent the cAMP Levels for Gi/s Coupled Receptor Activity in Response to Various Modulation Schemes.

GPCR Glossary the Different Forms of Receptor Modulation

Constitutive Activity (Basal Activity): The basal activity is the spontaneous activity of a receptor and second messenger production, in the absence of external modulation.

Full Agonist: A full agonist is a drug that produces the same maximal effect as the endogenous ligand.

Partial Agonist: The intracellular signal induced by the binding of the partial agonist is lower than that produced by a full agonist. A partial agonist can demonstrate an antagonist action versus a full agonist.

Inverse Agonist: Like a partial agonist, the binding of an inverse agonist to the receptor blocks the effect of a full agonist but the unique property of this group of ligands is to induce an opposite effect on the same GPCR. An inverse agonist reduces the basal activity of native or overexpressed receptors.

Neutral Antagonist: A neutral antagonist is a ligand that blocks receptor response to agonists or inverse agonists via general occupation of the receptor binding pocket.

Competitive Antagonist: A competitive antagonist is a ligand which binds to the same domain on the receptor as the agonist.

Allosteric Modulator: In contrast to a competitive antagonist, an allosteric modulator binds to its own binding site on the receptor and produces an effect on the receptor response to agonist stimulation by inducing a change in receptor conformation. Allosteric modulators can effect the efficacy of the receptor's affinity for the agonist.

Three Kit Groups to Cover a Broad Range of Assay Conditions

Cisbio offers three kit groups covering a broad range of working cAMP concentrations. The kits are intended for the direct quantitative determination of cAMP and allow the direct pharmacological characterization of compounds acting on Gi or Gs coupled receptors.

	Cryptate	S/B	IC20 NM	IC50 NM	IC80 NM
cAMP femto 2	Eu ³⁺	10	0.33	1.76	9.4
cAMP femto Tb	Tb ²⁺	18	0.91	4.07	18.1
cAMP HiRange	Eu ³⁺	41	4.27	22.8	121

The principle is based on HTRF technology. The method is a competitive immunoassay between native cAMP produced by cells and the cAMP labeled with the acceptor dye d2. The two entities then compete for binding to a monoclonal anti-cAMP antibody labeled with cryptate (Fig. 3).

The specific signal (i.e. energy transfer) is inversely proportional to the concentration of cAMP in the standard or sample.

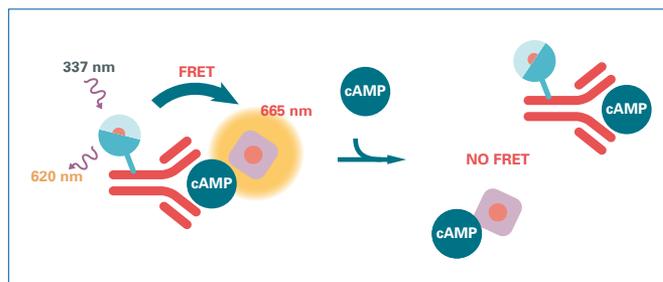


Figure 3: Assay Principle

Technical Criteria to Consider When Selecting a cAMP Assay

- Sensitivity and Resolution
- Assay Signal-to-Noise ratio (S/N)
- Working range, defined as the cAMP concentration between the IC20 and IC80. In this range the signal variation is generally linear in proportional to the cAMP concentration.
- Assay robustness and Z' factor
- Low compound interference rate
- Ease of assay development and implementation
- Applicability to high-throughput screening
- Number of steps

How to Select a Cyclic AMP Assay

Biological Criteria Influencing the Selection of a cAMP Assay

In a cell-based assay, the intracellular cAMP level depends on the agonist or antagonist activity but also on the nature of the cellular model used.

For recombinant cells, the receptor expression level and coupling efficiency directly affect cAMP level regulation. Measurement of Gi coupled receptor stimulation requires a cAMP assay able to detect a slight variation of cAMP concentration while giving a better signal-to-noise ratio. The pharmaceutical class of the drug being investigated also has a bearing on the specifications required for the cAMP assay. Finally, for antagonism assays, sensitivity is often required to detect a slight variation of cAMP concentration.

The maximal cell density with adherent cells depends also on the surface of the wells, which often becomes a limiting parameter in HTS.

In a cell-based assay, all these imposed parameters directly affect the intracellular cAMP level, and the assay window.

For a certain amount of intracellular cAMP this assay window depends on the assay sensitivity and S/B (see Fig. 4 & 5)

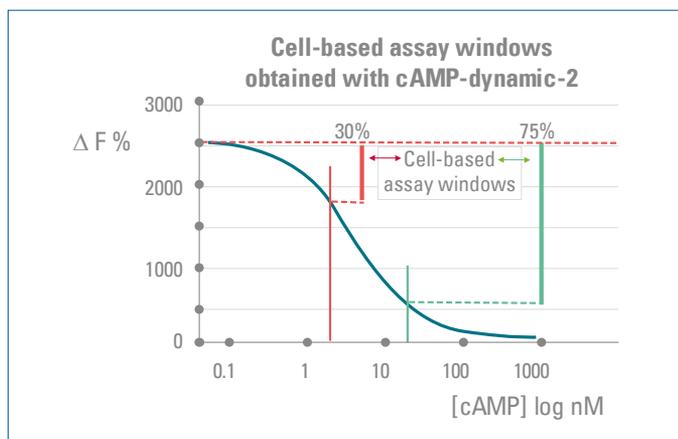


Figure 4: The assay window depends on the intracellular cAMP level. This figure illustrates, for the cAMP dynamic kit, the relation between the standard curve and the expected assay window in a cell-based assay. In a first assay producing 2 nM intracellular cAMP, the assay window is 30% of the maximal signal amplitude of the standard curve (700% Delta F). In a second assay where the cAMP level reaches 20nM the assay window covers 75% of the standard curve (1875% Delta F).

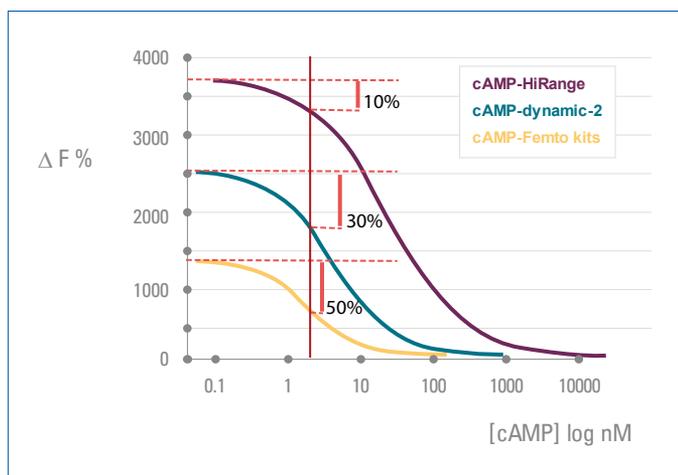


Figure 5: The standard curves of the 3 cAMP kits are presented on the same graph. When the intracellular cAMP is 2 nM, the maximal assay windows with the 3 kits is 10% for the cAMP HiRange kit, 30% for cAMP dynamic 2 kit and 50% for the cAMP femto kits.

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Conclusion

Because of the variability of the biological material and the pharmacology of compounds acting on Gi or Gs coupled receptors, a selection of cAMP kits assay covering different ranges is a significant advantage for researchers.

All of the homogeneous cAMP assay kits supplied by PerkinElmer are compatible with high-throughput screening in 96-, 384-, or 1,536 wells microplates.

These cAMP accumulation assays have been applied to the investigation of Gs and Gi coupled GPCRs, and can be used with recombinant or native receptors, using over-expressed or endogenous receptor levels, and can be applied to fresh or frozen cells with excellent performance metrics.