HTRF® theory in brief - selected bibliography


IP-One is a functional assay for investigating several classes of pharmaceutical compounds, such as agonists, antagonists, allosteric modulators and inverse agonists on constitutively active receptors. IP-One is the first HTS compatible and non-isotopic assay for Gq Protein Coupled Receptors.

IP-One cell-based assays can be used with living or frozen cells and are compatible with a wide range of receptor types. Compatible with endogeneous receptors, transient or stable transfections.

Cisbio has developed and patented an assay for IP1, a downstream metabolite of IP3. GPCR Gq stimulation is known to induce phospholipase C (PLC) activation and trigger the inositol phosphate (IP) cascade. Several products in this pathway, including IP3, have extremely short half lives, making them difficult to use as a measure of Gq receptor activation. IP1 accumulates and is stable in the presence of LiCl and is therefore a viable and proven functional indicator of GPCR Gq activation.

**Features**
- Cell-based functional assay run in a single microplate
- Competition immunossay involving a cryptate labeled anti-IP1 MAb and IP1-d2
- 1 hr incubation at room temperature after cell stimulation
- Developed with the new d2 HTRF® acceptor
- Number of steps: 2 incubation step protocol
- EC50: 500 nM (IP1 final concentration)
- Detection limit: 15 nM
- Specificity: No cross-reactivity with 50 µM myo-inositol, PIP2, IP2, IP3, IP4 or PIP3
- S/B (calibration curve): 10
- Z’: 0.87 (20 µL, 384 wells)
- Miniaturization down to < 10 µL
- DMSO tolerance > 2%
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**THE ADVANTAGES**
- IP-One is a functional assay for investigating several classes of pharmaceutical compounds, such as agonists, antagonists, allosteric modulators and inverse agonists on constitutively active receptors.
- IP-One is the first HTS compatible and non-isotopic assay for Gq Protein Coupled Receptors.
- IP-One cell-based assays can be used with living or frozen cells and are compatible with a wide range of receptor types.
- Compatible with endogenous receptors, transient or stable transfections.

Visit www.htrf.com for the latest list of IP-One validated GPCRs.
**IP-One assay**

**Assay protocol**
The IP-One assay is a competitive immunoassay that uses cryptate-labeled anti-IP1 MAb and d2-labeled IP1. The assay protocol consists of two incubation steps: cell stimulation by the ligand or target compounds, followed by IP1 detection using HTRF®reagents. The assay can be run in a single microplate and requires only a single 1 hour incubation following cell stimulation. LiCl is added to the cell stimulation buffer causing the accumulation of IP1 upon receptor activation. The detection process involves the addition of the two conjugates (cryptate-labeled anti-IP1 and d2-labeled IP1).

** Ordering information**

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**Assay in action**

Evaluation of IP-One, a new HTRF® assay for monitoring Gq coupled GPCR response.

**Comparison in HTS conditions with FLIPR®**
Recombinant CHO-K1 cells expressing Receptor X (target X) or Receptor Y (target Y) were used for measuring IP1 production by stimulation of ligands A or B respectively. In a 384-well format, cells were dispersed at 7,500 cells/40 µL/well and Fluo 3 as the Ca2+ indicator. The assay was performed using 7,500 cells/40 µL/well and Fluo 3 as the Ca2+ indicator. The IP-One assay was performed using 7,500 cells/40 µL/well and Fluo 3 as the Ca2+ indicator.

**Table 1**

<table>
<thead>
<tr>
<th>GPCR target</th>
<th>Cell Line</th>
<th>Agonist</th>
<th>HTRF®</th>
<th>Isotopic method</th>
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<tr>
<td>Muscarinic M1 (Gq)</td>
<td>CHO-K1</td>
<td>Acetylcholine</td>
<td>72 nM</td>
<td>42 nM</td>
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<tr>
<td>Vasopressin V1a (Gq)</td>
<td>CHO-K1</td>
<td>Vasopressin**</td>
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<td>Oxytocin OT (Gq)</td>
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<td>Oxytocin**</td>
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<td>Histamin H2 (Gq)</td>
<td>CHO-K1</td>
<td>Histamin</td>
<td>13 nM</td>
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<td>Purinergic P2Y1 (Gq)</td>
<td>CHO-K1</td>
<td>2-methylthioADP*</td>
<td>8.0 nM</td>
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<td>Cholecystokinin CCK1 (Gq)</td>
<td>CHO-K1</td>
<td>CCK8 sulfated**</td>
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<td><strong>T</strong>est with adherent cells</td>
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**Key**
- **T**est with adherent cells
- n.d. Not Determined
- **T**est with adherent cells
- * T**est with adherent cells

**Notes**
- * T**est with adherent cells
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**References**
**IP-One assay**

**Assay protocol**
The IP-One assay is a competitive immunosassay that uses cryptate-labeled anti-IP1 Mab and d2-labeled IP1. The IP-One assay protocol consists of two incubation steps: cell stimulation by the ligand or target compounds, followed by PI detection using HTRF® reagents. The assay can be run in a single microplate and requires only a single 1 hour incubation following cell stimulation. Licit is added to the cell stimulation buffer causing the accumulation of IP1 upon receptor activation. The detection process involves the addition of the two conjugates (cryptate-labeled anti-IP1 and d2-labeled IP1).

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**A S S A Y  I N  A C T I O N**

Evaluation of IP-One, a new HTRF® assay for monitoring Gq coupled GPCR response. Comparison in HTS conditions with FLIPR®.

Recombinant CHO-K1 cells expressing Receptor X (target X) or Receptor Y (target Y) were used for measuring IP1 production by stimulation of ligands A or B respectively. In a 384-well format, cells were dispersed at 7,500 cells/40µL/well (target X) and 6,000 cells/20µL/well (target Y). After incubation at 37°C, the culture supernatants were completely discarded. Immediately afterwards, 4 µL (32 µL in 384-well format) of stimulation buffer containing various concentrations of ligands A or B were added. After incubation at 37°C for 1 hr, 2 µL (5 µL in 384-well format) IP1-d2 conjugate followed by 2 µL (5 µL in 384-well format) of Eu3+ cryptate labeled anti-IP1 antibody were added.

Time-resolved fluorescence at 620 nm and 665 nm was measured with ViewLux, and the signal ratios were calculated. A FLIPR® assay in a 384-well format was performed using 7,500 cells/40µL/well and Fluor 3 as the Ca2+ indicator.

IP-One ELISA

The fundamental assay for IP1 quantification and assessment of Gq coupled GPCR activation.

Description

The IP-One ELISA assay has been designed to monitor the activation of Phospholipase C (PLC) coupled receptors. Among them, the Gq coupled GPCRs represent the most important family of receptors which can activate the β subtype of the PLC family. Other receptor types, like protein tyrosine kinase receptors, antigen or immunoglobulin receptors, or collagen receptors, are known to activate another PLC subtype, PLC-γ.

Features

• Cell-based functional assay
• Monoclonal antibody based
• Highly sensitive (detection limit: 10 nM)
• Simple protocol
• EC50: 110 nM
• No cross-reactivity with 50 µM myo-inositol, PIP2, IP2, IP3, IP4 or PIP3

Cell stimulation step

1. Plate cells in appropriate cell culture plate (overnight incubation)
2. Stimulate cells with ligand of choice or the drug of interest (1 hour incubation)
3. Lyse cells (30 min incubation)

Detection with IP-One ELISA reagents

4. Transfer cell lysate to the ELISA plate supplied with the kit, add IP1-HRP conjugate and anti-IP1 MAb (3 hour incubation)
5. Add HRP substrate TMB following plate wash
6. Stop the reaction after 20 to 30 minutes and read optical density at 450 nm

THE ADVANTAGES

• The perfect tool to select and validate GPCR functionality during cell engineering processes.
• IP-One ELISA is a functional and sensitive assay developed to follow inositol 1 phosphate accumulation following Phospholipase C coupled receptors’ activation.
• High pharmacological relevance, ideal for lead optimization phases.

SELECTED BIBLIOGRAPHY


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<td>IP-One Elisa</td>
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These HTRF® kit components can be ordered separately (except for Cryptate and XL665 conjugates).
cAMP assays

A line of ready-to-use assay kits for measuring cAMP concentrations. Each kit is optimized for detection of a specific range of cAMP concentrations, to allow fine tuning of assay sensitivity. These kits enable high quality cell-based assay screening in 96-, 384- or 1536-well formats.

Description
Cyclic AMP (adenosine 3',5'-cyclic monophosphate) is a key second messenger in the G-Protein Coupled Receptor (GPCR) signaling pathway. GPCR ligand binding leads to Gi or Gs protein activation, which in turn regulates adenylate cyclase (AC), the enzyme responsible for modulating intracellular levels of cAMP. In the presence of a phosphodiesterase inhibitor (e.g. IBMX) cAMP can accumulate in the cell. Measurement of cAMP levels is widely used as an indicator of GPCR function (Figure 1). Cisbio offers three HTRF®-based kits that cover a broad range of cAMP concentrations. Figure 2 and the table below show working range expressed as the linear range between EC20 and EC80.

The cAMP femto 2 assay was designed for high sensitivity requirements and applications with low cAMP. The cAMP dynamic 2 assay covers a cAMP concentration range adapted for various cell lines and targets either Gi or Gs. The cAMP HiRange assay shows an extended signal-to-background ratio, particularly well suited to high working ranges of cAMP concentrations and monitoring of purified adenylate cyclase activity.

Features
• Developed with d2, the new HTRF® acceptor
• Competition immunoassay involving Eu®-cryptate-labeled MAb anti-cAMP and cAMP-d2
• One 30 min. incubation at room temperature following cell stimulation
• Streamlined single-plate protocol for HTS
• Cell-based assay compatible with fresh or frozen cells
• Stable EC50 from 30 minutes to 7 days
• Minititration down to <1µL
• DMSO tolerance > 5%

THE ADVANTAGES
• Monitoring Gi or Gs coupled receptors for high throughput agonist/antagonist screening in cell-based assays on living or frozen cells.
• Related applications:
  • Phosphodiesterase (PDE) follow-up by measuring the degradation of cAMP into AMP.
  • Adenylate cyclase activity.
  • Measuring GPCR activity in membrane preparations with cAMP HiRange.

Assay protocol
Our cAMP kits may be run using either a single or a two-step dispense protocol. The basic kit protocol includes stimulation by the compound followed by detection with HTRF® reagents, during which time cell lysis and detection occur simultaneously. All HTRF® cAMP kits are unaffected by culture media additions such as serum, biotin, or colored compounds and tolerant to >5% DMSO. cAMP assays have been validated using fresh suspension cells, adherent cells and frozen cells.

Protocol with two dispensing steps after cell stimulation:
- the d2-labeled cAMP and cryptate-labeled antibody are added separately after stimulation.

Protocol with only one dispensing step after cell stimulation:
- in this protocol, one of the HTRF® reagents (cAMP-d2) is distributed during stimulation.

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<td>Diluent for standard curve preparation</td>
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WWW.HTRF.COM
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Protocol with two dispensing steps after cell stimulation:

1. **d2-labeled cAMP** and cryptate-labeled antibody are added separately after stimulation.
2. **Protocol with only one dispensing step after cell stimulation:**
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</table>

These HTRF® kit components can be ordered separately (except for Cryptate and d2 conjugates).
Cisbio created the cAMP membrane kit specifically to address the use of cellular membranes in GPCR activation and adenylate cyclase activity screening. The cAMP membrane assay is highly robust and produces quality results while eliminating the need for a continuous supply of living cells.

**Description**

The localization of the adenylate cyclase enzyme within membranes allows the use of cellular membrane preparations rather than live cells when measuring Gi and Gs-coupled GPCR activation. Cisbio’s cAMP membrane assay exhibits a high signal-to-background ratio (S/B=12) and EC50 of 6.6 nM in 20 µL final assay volume (132 fmol/well). This level of assay sensitivity thus requires the use of only a small quantity of membrane per well.

**Features**

- Membrane-based assay
- Immunoassay competition between cAMP-XL665 and cAMP with a monoclonal anti-cAMP-cryptate antibody conjugate
- Number of steps: two incubation step protocols (see assay principle)
- Incubation: 90-120 min. at room temperature
- Small quantity of membrane required per assay (0.125 µg to 1.5 µg depending on receptor type)
- Amenable to automation
- S/B > 10
- EC50 = 6.6 nM

**ASSAY IN ACTION**

Thawed CHO-K1 cells that stably expressed the H3 receptor (Gi) were exposed to increasing concentrations of Methylhistamine in the presence of 30 µM of forskolin (EC80). Assays were performed in 20 µL in Greiner 384-well plates. The plates were read at several intervals following cell stimulation, from 1 hour to 7 days. The EC50 values calculated with the three kits are remarkably stable even after 7 days, which is a significant advantage during screening campaigns.


**SELECTED BIBLIOGRAPHY**


Hamon J, Martin B, Meunier E, Casamitjana O, Ouagued M, Roman F. Validation of the HTRF® technology for high throughput quantification of cAMP production mediated by activation of GPCR. Comparative with flashplate. SBS 8th Annual Conference. September 2009, Baltimore (USA).

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**THE ADVANTAGES**

A cAMP detection assay to monitor Gi/s activation and adenylate cyclase screening optimized for use with cellular membranes.
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- Amenable to automation
- S/B > 10
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**CAMP Assays**

**CAMP Membrane Assay**

A cAMP detection assay to monitor Gi/s activation and adenylate cyclase screening optimized for use with cellular membranes.

**ASSAY IN ACTION**

Thawed CHO-K1 cells that stably expressed the H3 receptor (Gi) were exposed to increasing concentrations of Methylhistamine in the presence of 30 µM of forskolin (EC\textsubscript{80}). Assays were performed in 20 µL in Greiner 384-well plates. The plates were read at several intervals following cell stimulation, from 1 hour to 7 days.

The EC\textsubscript{50} values calculated with the three kits are remarkably stable even after 7 days, which is a significant advantage during screening campaigns.


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**cAMP membrane assay**

**Assay principle**

Two dispensing protocols are available for use depending on automation constraints. Both protocols include:

**Stimulation:** membranes and compounds are dispensed followed by incubation (30 to 60 minutes at room temperature).

**Stop:** the stop reagent supplied with the kit is added.

**Detection:** addition of HTRF® detection reagents (1 hour at room temperature).

**Ordering information**

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<td>Diluent for standard curve prep.</td>
<td>62DL1DDD</td>
<td></td>
</tr>
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</table>

* on request

These HTRF® kit components can be ordered separately (except for Cryptate and XL665 conjugates).

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**SELECTED BIBLIOGRAPHY**