

New HitHunter™ cAMP XS+ Assay for GPCR Screening Using the PHERAstar

Lindy Kauffman¹, Sherrylyn De La Llera¹ and Marjan Orban²
¹DiscoverX Corporation, Fremont, CA 94538, USA; ²BMG LABTECH GmbH, Offenburg, Germany

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- Directly assess either Gi- or Gs- coupled GPCRs in cells
- Robust assay and large S/B ratio
- Chemiluminescence detection reduces interference from fluorescent compounds
- Assay miniaturization up to 1536-well plate format

Introduction

G protein-coupled receptors (GPCRs) make up a major family of cell surface receptors which mediate intercellular communication and GPCRs are a rich source of “druggable” targets. It has been estimated that 50% of prescription drugs interact with GPCRs. With the increasing popularity of functional assays for high throughput screening, an increasing need arises for robust second messenger assays that reflect GPCR activation and that are readily amenable for miniaturization. GPCRs that modulate adenylyl cyclase activity upon agonist stimulation and, consequently, cellular cyclic adenosine monophosphate (cAMP) levels, via the G protein Gs or Gi, form a subset of therapeutic targets. cAMP acts at several downstream targets including ion channels, kinases that modulate gene transcription, and cell metabolism. Changes in the intracellular cAMP levels correlate with GPCR activation and therefore measurement of cAMP is a simple functional assay frequently utilized in the HTS laboratory. DiscoverX supplies several homogenous cAMP assays based on chemiluminescence to fit the various needs of the HTS customer. The newest product, HitHunter™ cAMP XS+, is specifically targeted to provide enhanced performance and usability. In this application note we describe a cAMP assay based on the enzyme fragmentation complementation (EFC) of β -galactosidase (β -gal) measured on BMG LABTECH's PHERAstar multimode HTS plate reader (figure 1).



Fig. 1: BMG LABTECH's high-end multimode plate reader PHERAstar was used for the HitHunter cAMP XS+ kit.

The DiscoverX EFC technology is based on two fragments of *E. coli* β -galactosidase (β -gal), a large protein fragment (enzyme acceptor, EA) and a small peptide fragment (enzyme donor, ED). Separately these fragments are inactive, but in solution they rapidly complement (recombine) to form active β -gal enzyme which can hydrolyze substrate to produce luminescence.

In this assay cAMP from cell lysates and ED-labeled cAMP (ED-cAMP) compete for antibody binding sites. Steric hindrance of EFC occurs when the antibody binds the ED-conjugated cAMP such that little or no active β -gal is formed. However, free cAMP generated by the cell can competitively displace the ED-conjugated cAMP from the antibody and allows the ED-conjugate to freely complement EA and thus generate a signal directly proportional to the amount of cAMP present in the cell (figure 2).

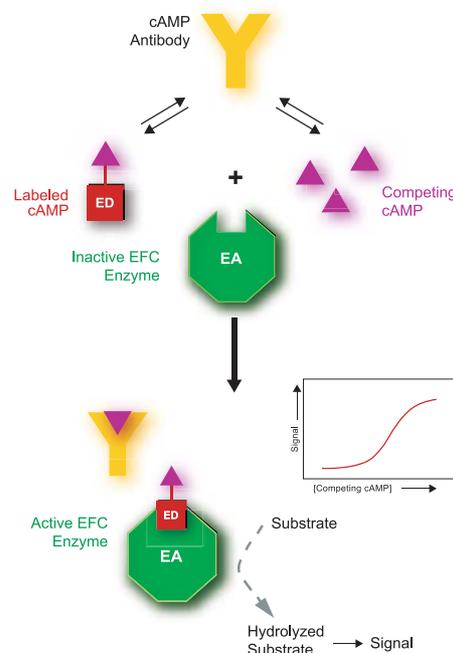


Fig. 2: Schematic principle of the HitHunter cAMP XS+ assay.

Materials and Methods

BMG LABTECH's PHERAstar combines rapid plate reading necessary for HTS with the enhanced performance and sensitivity needed to read small liquid volumes. This new multimode microplate reader has the flexibility to excel with the most demanding assays and is designed to read all leading HTS detection modes (fluorescence intensity, time-resolved fluorescence, fluorescence polarization, luminescence and absorbance) in all formats up to 1536.

The PHERAstar was run in luminescence mode for the monitoring of the HitHunter cAMP XS+ demo kit (DiscoverX Corporation, cat#90-0075) containing following reagents:

- 1) cAMP XS+ Lysis Buffer
- 2) cAMP XS+ EA Reagent
- 3) cAMP XS+ ED Reagent
- 4) cAMP XS+ Antibody Reagent
- 5) cAMP XS+ Standard (250 μ M)
- 6a) Galacton-Star[®]
- 6b) Emerald-II™

HitHunter cAMP XS+ is a single set of reagents which can be used in two different protocols, depending on the needs of the end user.

- In the **three reagent addition protocol** cells are plated and induced in PBS or media, and cell induction is followed by three reagent additions and two incubation steps.
- In the **two reagent addition protocol**, cells are plated and induced in a diluted antibody solution, and cell induction is followed by two reagent additions and two incubation steps.

Kit users must select the protocol which best fits their needs and prepare reagents and cells according to the guidelines for the selected protocol. The table below outlines the volumes and procedure for the cAMP XS+ **three reagent addition protocol** standard curve in a 384-well plate format.

Table 1: cAMP XS+ Three Reagent Addition Protocol: Standard curve

384-well Plate	cAMP XS+ Three Reagent Addition Protocol: Standard Curve
Step 1	10 µL of diluted standard 5 µL PBS
Step 2	5 µL Antibody Reagent
Step 3	20 µL ED/Lysis/CL Working Solution
Step 4	Incubate 1 hour at room temperature
Step 5	20 µL EA Reagent
Step 6	Incubate at least 1 hour at room temperature, then read luminescence signal on PHERAstar

The standard curve for DiscoverRx's cAMP XS+ kit was run according to the package insert protocol in white 384-well plates (COSTAR cat# 3711384) with 60 µL total assay volume. Chemiluminescence was read on the PHERAstar two hours after the addition of the last reagent using following protocol for plate reader setup (figure 3), which can be downloaded from BMG LABTECH's web site as well.

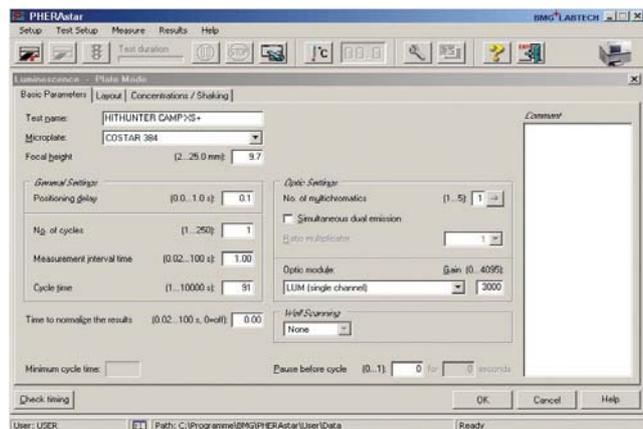


Fig. 3: This screenshot shows the HitHunter cAMP XS+ assay setup window from the PHERAstar. An optical module for HitHunter is directly available from BMG LABTECH.

Results and Discussion

DiscoverRx's HitHunter cAMP XS+ assay was prepared in 384-well format and standard curves were run on BMG LABTECH's PHERAstar in luminescence mode. Cyclic AMP standard detection reagents were added according to the assay protocols (two and three reagent addition protocols) and chemiluminescence for both was read two hours after the addition of the last reagent (figure 4). EC₅₀ values were calculated using Graphpad Prism software.

The HitHunter cAMP XS+ kit is a robust (S/B > 45) homogeneous mix and read assay that is ideally suited for robotic HTS for cAMP detection. The chemiluminescent nature of the assay reduces the compound interference issues in the screening environment. The combination of HitHunter on the PHERAstar with its unique hardware and software features allows a user-friendly and convenient approach for conducting GPCR screening.

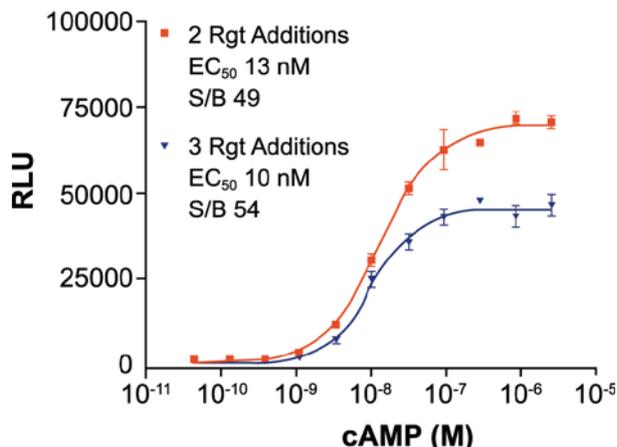


Fig. 4: Standard curve data for HitHunter cAMP+ run in the two and three reagent addition protocol in 384-well plate format.

Conclusion

GPCRs are important targets in the HTS drug discovery approach and GPCR signalling can be examined by direct quantitation of cAMP by applying the HitHunter cAMP XS+ kit. Excellent results were achieved on the PHERAstar multimode microplate reader which is designed to read all leading HTS detection modes in all formats up to 1536. The high degree of sensitivity, easy-to-use software, robust hardware, and optimized detection systems make the PHERAstar ideal for GPCR analyses in the high-throughput assay environment.

The PHERAstar was run in luminescence mode for the HitHunter cAMP assay which uses enzyme fragment complementation (EFC) for sensitive detection and fewer false positives. This assay is a useful tool for those customers screening difficult targets with low basal levels of cAMP (cell lysate, tissue, or serum), or low levels of cAMP response. The chemiluminescence signal is robust and assures minimal interference from library compounds. The stable signal allows flexibility in read time, i.e. the assay can be read the same day or after an overnight incubation.

While there are several cAMP assays currently available, most of them are not scalable for miniaturization into the 1,536-well format employed for automated high throughput screening of large chemical libraries. DiscoverRx offers several different configurations of this assay based on different applications, readouts, sample and cell types. For more information on DiscoverRx assays please refer to the web site:

www.discoverx.com

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Germany:	BMG LABTECH GmbH	Tel: +49 781 96968-0
Australia:	BMG LABTECH Pty. Ltd.	Tel: +61 3 59734744
China:	BMG LABTECH Co. Ltd.	Tel: +86 10 84110632
France:	BMG LABTECH SARL	Tel: +33 1 48 86 20 20
Japan:	BMG LABTECH JAPAN Ltd.	Tel: +81 48 647 7217
UK:	BMG LABTECH Ltd.	Tel: +44 1296 336650
USA:	BMG LABTECH Inc.	Tel: +1 919 806 1735

Internet: www.bmg-labtech.com info@bmg-labtech.com