Faster Chemiluminescent cAMP detection by HitHunter cAMP XS+ on the SpectraMax L Luminescence Microplate Reader

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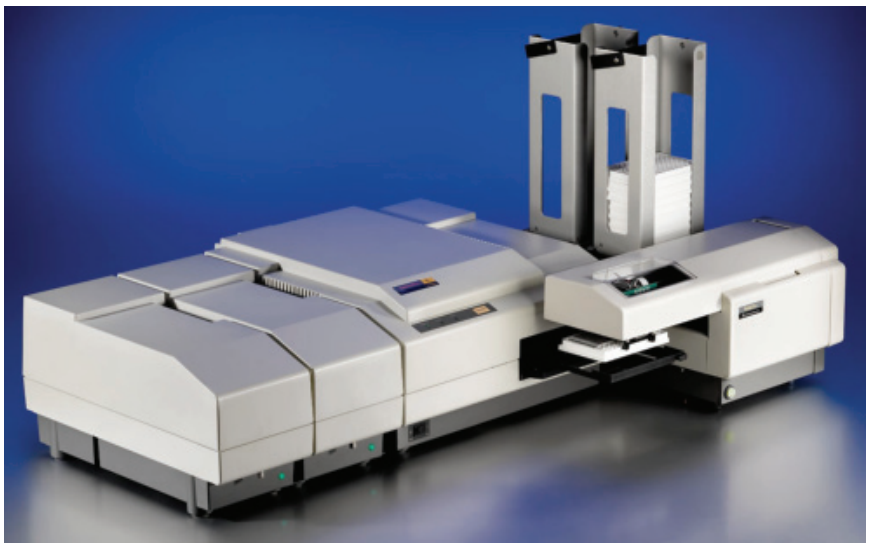
Introduction

Cyclic AMP is a widely studied second messenger in multiple signaling pathways. Measurement of cAMP levels is cruicial in drug discovery research involving $G_{\alpha s}$- and $G_{\alpha i}$-coupled receptors. DiscoveRx's HitHunter® cAMP assays provide a robust and sensitive cAMP detection platform to detect cAMP in engineered cells, primary neuronal cells, platelets, PDEs, bacteria and others. The technology is versatile and can differentiate different compound types such as full agonist, partial, antagonist, inverse agonist response or allosteric compounds.

The cAMP XS+ kit offers a large assay window and works with a low number of cells. Chemiluminescent assays have the distinct advantages of low background and fewer false positives associated with autofluorescent properties of compounds. The ready-to-assay, homogenous kit is HTS-friendly, as the assay can be miniaturized up to 3456-well format.

SpectraMax L Microplate Reader and StakMax Microplate Handling System (Figure 1)

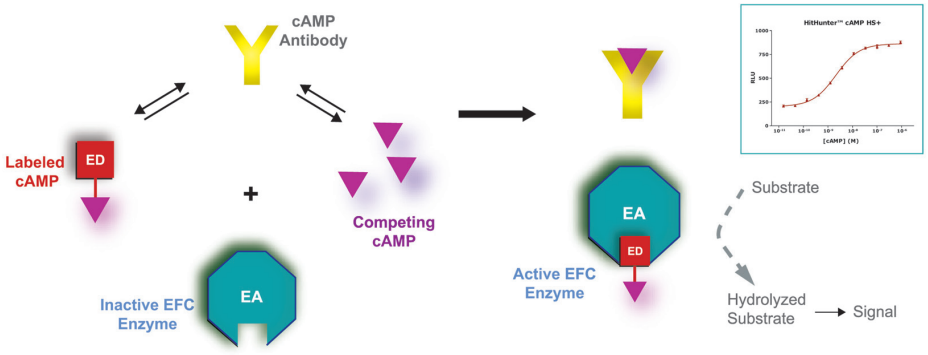


SpectraMax L Reader and StakMax Microplate Handling System handles a batch of up to 50 plates.

Molecular Devices' SpectraMax® L Luminescence Microplate Reader is a dedicated reader with an extended dynamic range of more than 8 orders of magnitude. A highly sensitive instrument with reduced noise and low cross talk, it is also a configurable system with up to 6 channels (photomultiplier tubes) and 12 injectors. Together with Molecular Devices' StakMax® Microplate Handling System, the SpectraMax L Microplate Reader provides medium to high throughput.

SoftMax® Pro Microplate Data Acquisition and Analysis Software serves as the user interface to the microplate reader. SoftMax Pro Software combines reader control and data analysis and provides sophisticated curve fitting and statistics for more in-depth bioassay analysis.

Principle of the HitHunter cAMP assays (Figure 1)



HitHunter cAMP assays are competitive immunoassays. Free cAMP from cell lysates competes for antibody binding against labeled cAMP (ED-cAMP conjugate). Unbound ED-cAMP is free to complement EA to form active enzyme by EFC, which subsequently hydrolyzes substrate to produce signal. A positive signal generated is directly proportional to the amount of free cAMP bound by the binding protein.

Materials

cAMP detection reagents

- HitHunter cAMP XS+ (DiscoveRx Cat. #90-0075)
- Forskolin (DiscoveRx Cat. #92-0005)
- GLP II (DiscoveRx Cat. #92-1079)
- CCL20 (DiscoveRx Cat. #32-1005)

Cell lines

- cAMP Hunter GLP2R (G_s) cell line
- cAMP Hunter MTNR1B (G_i) cell line
- cAMP Hunter CCR6 (G_i) cell line

Instrument and accessories

- SpectraMax L Luminescence Microplate Reader (contact Molecular Devices for ordering information)

- White tissue culture treated 384-well microplates for chemiluminescence detection (Costar Cat. #3903 or equivalent)
- Phosphate Buffered Saline

Methods

Standard curve for cAMP

Cyclic AMP standard (250 µM) provided in the kit was diluted 1 part to 8 parts with PBS to get a working standard. This dilution yielded a concentration of 4.63 µM in a 60 µL final assay volume in a 384 well plate. A 3-fold dilution series was prepared in PBS to get a standard curve ranging from 4.63 µM to 0.235 nM. PBS was added to wells as a zero standard. A standard curve was obtained by following the protocol in Table 1.

Table 1. 384-Well Microplate cAMP XS+ Protocol

Steps	384-Well Microplate cAMP XS+ Standard Curve Protocol
Standard Addition	Add 10 µL/well of diluted standard.
Buffer Addition	Add 15 µL/well HBSS/Antibody mix to cells.
Incubation	Incubate 30 min @ 37°C.
HitHunter Reagent Addition 1	Add 20 µL/well cAMP XS+ reagent ED-lysis/CL substrate reagents mix.
Incubation	Incubate 1 hour at room temperature.
HitHunter Reagent Addition 2	Add 20 µL/well of cAMP XS+ EA Reagent (no reagent preparation required).
Incubate and Read	Incubate for 3 hours to overnight at room temperature and then read luminescence signal.

Cell-Based Assay

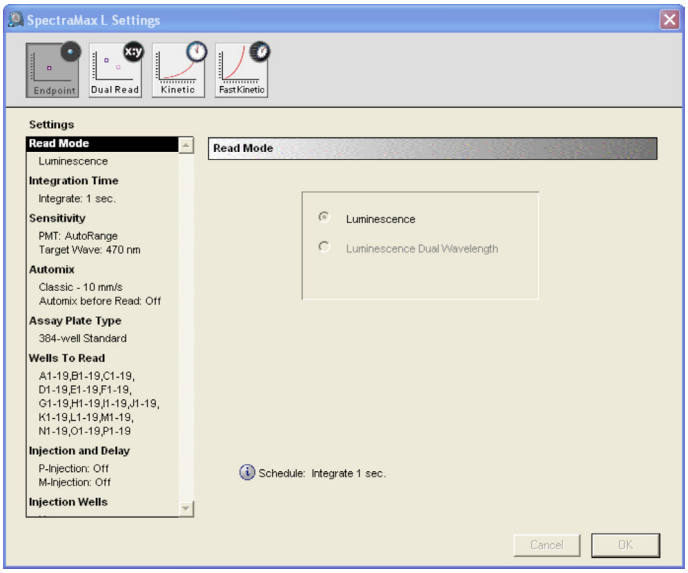
Cells were plated at densities of 1000, 2000, 5000, and 10,000 cells/well in a 384-well plate and incubated overnight in a 37°C incubator. A dose-response curve was obtained by following the protocol in Table 2.

Table 2. Cell-Based Assay Procedure	
Steps	**384-Well Microplate cAMP XS+ Protocol**
Preparation	Aspirate media from seeded cells (optional: wash cells).
Reagent Preparation	Premix HBSS/10 mM HEPES and cAMP XS+ antibody (available in the HitHunter cAMP XS+ kit) in the ration of 2:1.
Buffer Addition	Add 15 µL/well HBSS/antibody mix to cells.
Ligand Addition	Add 5 µL/well of 4X ligand.
Incubation	Incubate 30 minutes at 37oC.
HitHunter Reagent Addition 1	Add 20 µL/well cAMP XS+ reagent ED-lysis/CL substrate reagents mix. (Reagent preparation: STEP A: CL Substrate Working Solution was prepared by gently mixing 1 part of Galacton-Star, Emerald-II to 5 parts of the Lysis buffer. STEP B: ED/Lysis/CL Substrate Working Solution was generated by mixing 1 part cAMP XS+ ED reagent and 1 part CL Substrate/Lysis solution)
Incubation	Incubate 1 hour at room temperature.
HitHunter Reagent Addition 2	20 µL/well of cAMP XS+ EA Reagent (no reagent preparation required)
Incubate and Read	Incubate for 3 hours to overnight at room temperature and then read luminescent signal.

Instrument Setup

The SpectraMax L Microplate Reader was set up for endpoint reading with an integration time of 1 second or 0.1 second. PMT sensitivity was set to AutoRange with a target calibration wavelength of 470 nm, and the plate was dark adapted for 1 minute prior to reading. Instrument settings are shown in Figure 3.

Instrument settings (Figure 3)



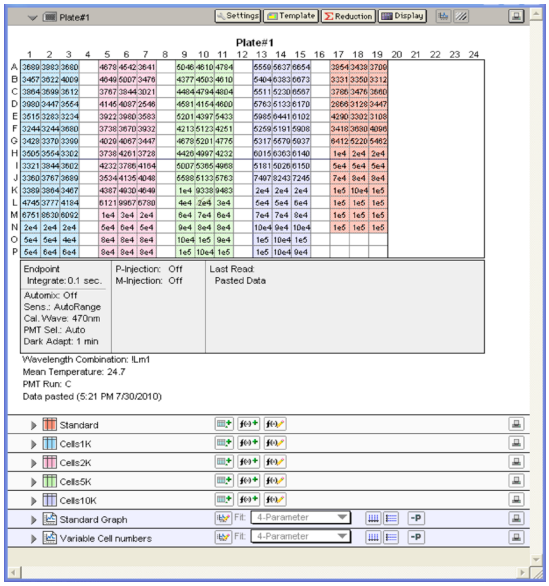
Instrument settings for detecting cAMP with HitHunter cAMP XS+ kit on the SpectraMax L Microplate Reader.

Data Analysis

The entire data analysis was done using SoftMax Pro Software. Templates were set up in SoftMax Pro Software to assign wells for the standard curve and agonist dose response for cell samples. For all template groups assigned, the software automatically created group sections with complete data analysis. Graph sections in the software were used to plot standard and dose-response curves with four parameter (4-P) curve fitting. Parameter C obtained from the 4-P curve fit provided an EC_{50} value, while signal to background ratio was calculated by dividing the upper asymptote by the lower asymptote.

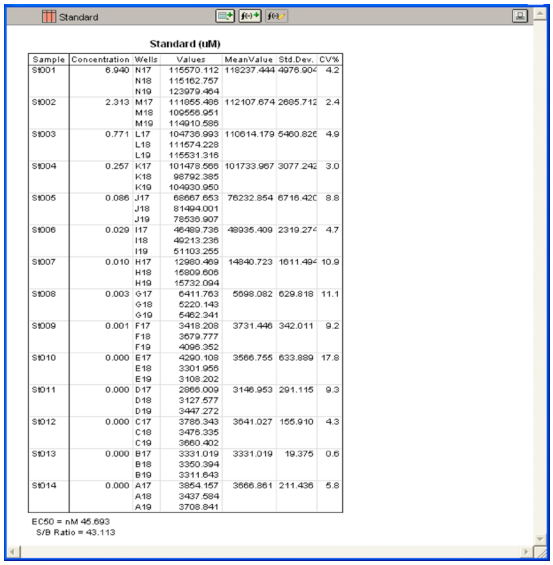
Data analysis (Figure 4)

A.



Data Analysis with SoftMax Pro Software

B.



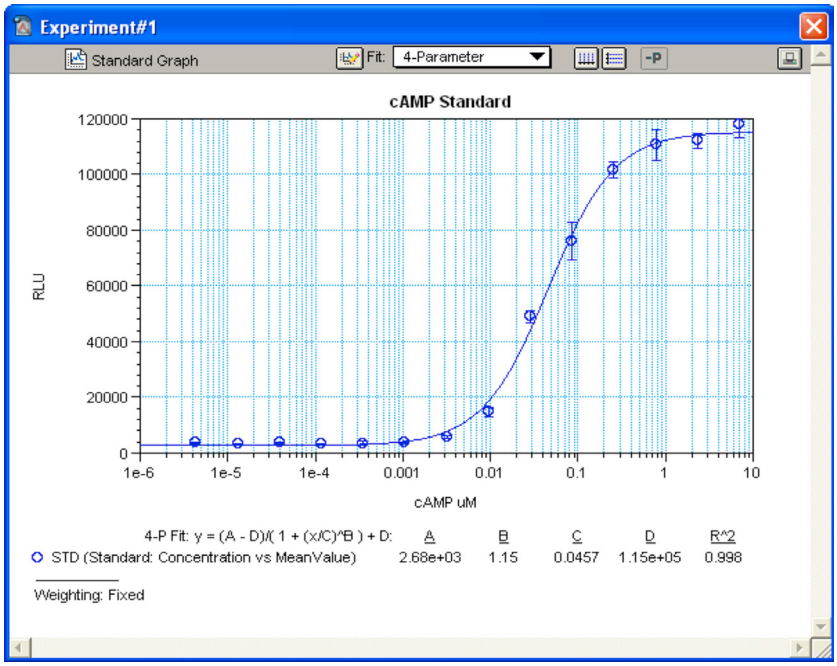
SoftMax Pro Software group table with complete data analysis

Results

Standard curve for cAMP

Figure 5 shows the standard curve for cAMP. The EC_{50} was calculated to be 45.693 nM and the signal to background ratio was 43.11.

cAMP standard Curve (Figure 5)



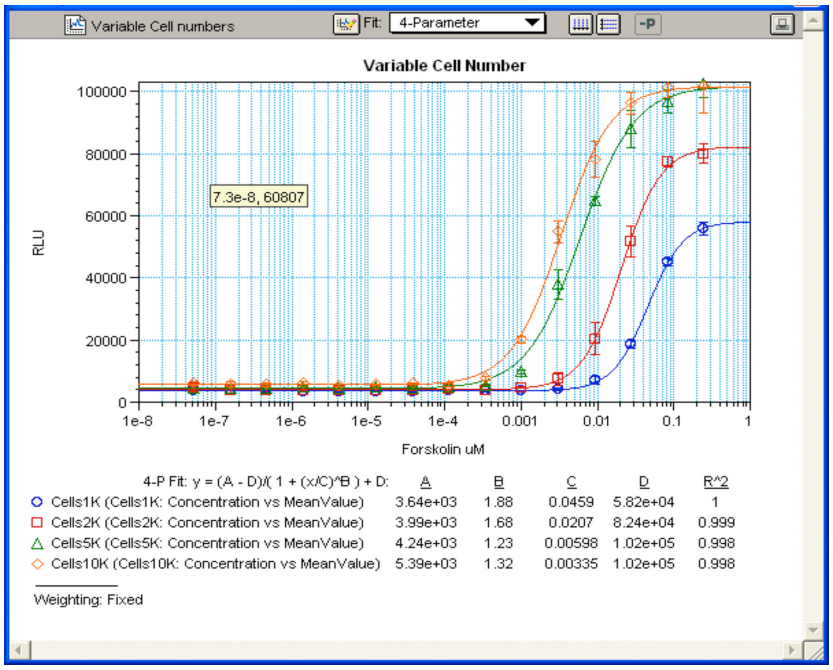
Performance of HitHunter cAMP XS+ on SpectraMax L Microplate Reader with different cell densities

cAMP Hunter CHO-K1 cells expressing the $G_{\alpha i}$-coupled melatonin receptor (MTNR 1B) were plated at cell densities of 1000, 2000, 5000, and 10,000 cells/well in a 384 well plate as per the adherent protocol and incubated overnight in a 37°C incubator. The next morning, after aspirating the media, cells were re-suspended in Hanks/10 mM HEPES and stimulated with varying concentrations of Forskolin at 37°C for 30 minutes. cAMP levels were detected using the HitHunter cAMP XS+ Kit (90-0075) as per assay protocol using SpectraMax L Microplate Reader. Results obtained with integration time of 0.1 seconds (Figure 6) and 1 second (Figure 7) were comparable (Table 3). A read time of 0.1 second allows batch processing of multiple plates and reduces the time required for plate reading.

Table 3. Comparison of HitHunter cAMP XS+ on SpectraMax L Microplate Reader with Different Cell Densities in 384-Well Microplate with Integration Time of 0.1 and 1 Second

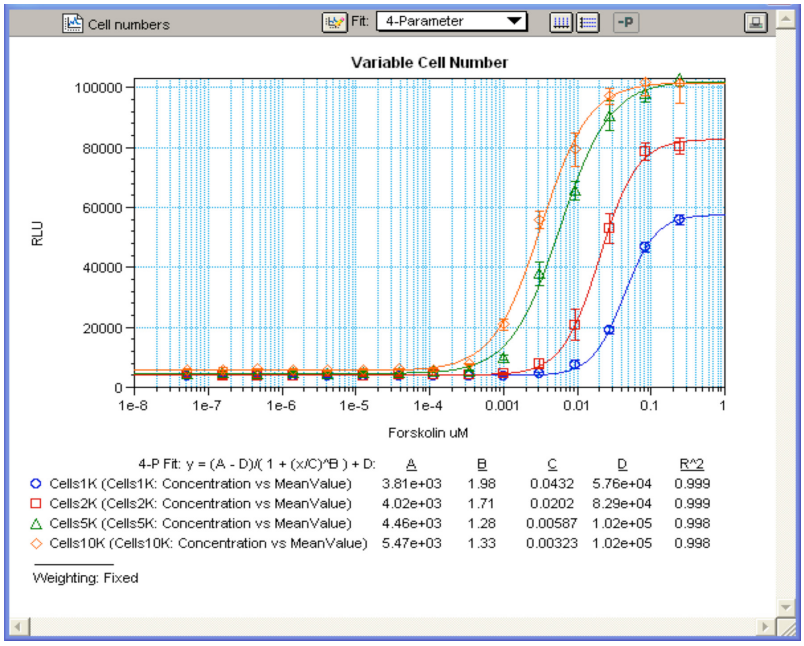
	Integration Time = 0.1 Second		Integration Time = 1 Second	
	EC_{50} (nM)	Signal-to-Background Ratio	EC_{50} (nM)	Signal-to-Background Ratio
1000 Cells/Well	45.9	15.99	43.2	15.11
2000 Cells/Well	20.7	20.66	20.2	20.61
5000 Cells/Well	5.98	24.01	5.87	22.91
10000 Cells/Well	3.35	18.88	3.23	18.62

Results obtained with integration time of 0.1 second (Figure 6)



HitHunter cAMP XS+ on SpectraMax L Microplate Reader with different cell densities in 384-well microplate with integration time of 0.1 second.

Results obtained with integration time of 1 second (Figure 7)



HitHunter cAMP XS+ on SpectraMax L Microplate Reader with different cell densities in 384-well microplate with integration time of 1 second.

Agonist-induced cAMP response in a $G\alpha_s$-coupled GPCR

Signal transduction downstream of the glucagon-like peptide 2 receptor is mediated by $G_{\alpha s}$ proteins that activate adenylyl cyclase to increase levels of cAMP. Different cell densities of cAMP Hunter CHO-K1 GLp2R cells were plated in a 384-well plate as per adherent protocol and stimulated with GLP II (DiscoveRx Cat. #92-1079) for 30 minutes. cAMP levels were detected using the HitHunter cAMP XS+ Kit (90-0075) as per assay protocol using SpectraMax L Microplate Reader. High assay windows were achieved as seen from the signal to background ratio reported in Table 3. EC_{50} values for agonist are listed in Table 4. The ideal cell density was found to be 2500 cells per well. This low cell density would result in a two-fold reduction in tissue culture costs and resources when considering a medium- to high-throughput screen. A read time of 0.1 second allows batch processing of multiple plates and reduces the time required for plate reading.

Agonist-induced cAMP response in a $G_{\alpha S}$-coupled GPCR (Figure 8)

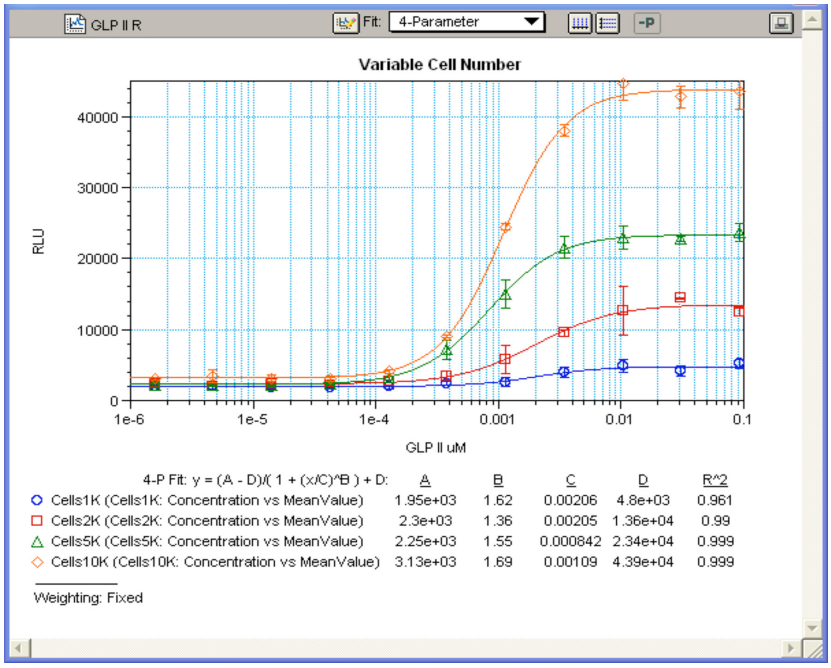


Table 4. Agonist-Induced cAMP Response in a $G_{\alpha S}$-Coupled GPCR

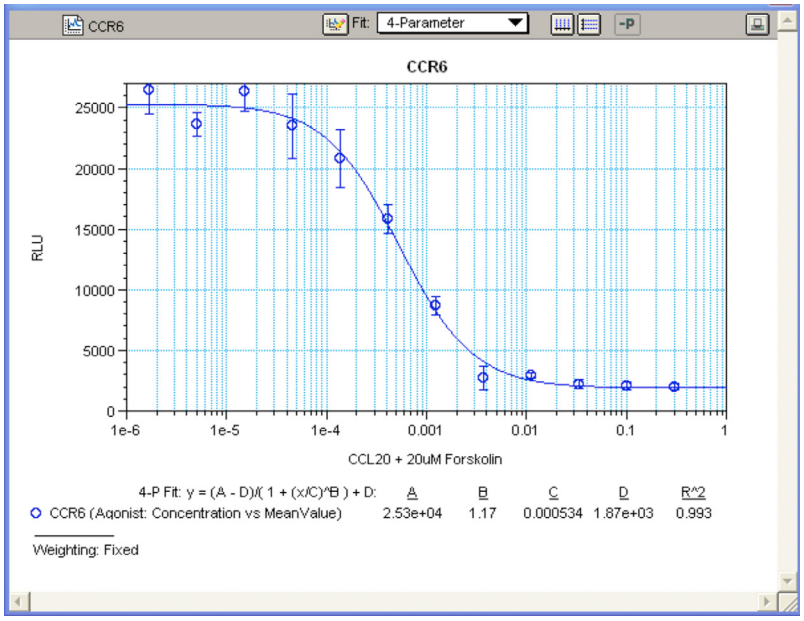
	EC_{50} (nM)	Signal-to-Background Ratio
1000 cells/well	2.06	2.464
2000 cells/well	2.05	5.899
5000 cells/well	0.84	10.380
10000 cells/well	1.09	14.027

Agonist-induced cAMP response in a Gαi-coupled GPCR

Signal transduction downstream of the CCL6 receptor is mediated by $G_{\alpha i}$ proteins that lower intracellular cAMP levels. Chemokine receptor 6 (also known as CCR6) is a CC chemokine receptor. Chemokine (C-C motif) ligand 20 (CCL20) or liver activation regulated chemokine (LARC) or Macrophage Inflammatory Protein-3 (MIP3A) activates the CCR6 receptor. Figure 9 demonstrates cAMP response when the $G_{\alpha i}$-coupled chemokine receptor CCR6 is stimulated with its ligand CCL20. Forskolin at 20 µM was used to increase the initial intracellular level of cAMP.

CHO-K1 cells expressing chemokine receptor were plated in a 384-well plate as per adherent protocol and stimulated with CCL20 (DiscoveRx Cat. #32-1005) for 30 minutes. Signal was detected using the HitHunter cAMP XS+ Kit (90-0075) as per assay protocol. EC_{50} was found to be 53.4 pM and signal-to-background ratio was found to be 13.52.

Agonist induced cAMP response in a Gαi-coupled GPCR (CCR6) (Figure 9)



Discussion

In this study, we evaluated the performance of HitHunter cAMP assays on Molecular Devices' SpectraMax L Luminescence Microplate Reader. EC_{50} values and assay windows with the SpectraMax L Reader were very similar to published values for the ligands and receptors tested.

Cyclic AMP is an important secondary messenger for $G_{\alpha s}$- and $G_{\alpha i}$-coupled receptors. We studied agonist induced cAMP response in cell line expressing GLP 2R, a G_s Coupled GPCR and agonist induced cAMP response in cell line expressing CCL6 a G_i-coupled GPCR. The data showed very high signal-to-background ratio and EC_{50} comparable to that reported in literature.

The assay was carried out in 384-well plates in a total assay volume of 60 µL. Experimental results with varying cell numbers (Figure 6, 7, and 8) showed that the assay detects measureable cAMP levels even with a low number of cells per well, resulting in reduced cost of tissue culture and resources.

Microplates were read using integration times of 1 sec/well and 0.1 sec/well. The results were comparable (Figures 5 and 6). A read time of 0.1 sec/well allows an entire 384-well plate to be read in less than 40 seconds. The kit comes a in ready to use, no-wash format. These characteristics, combined with the low cross talk and high sensitivity of the instrument, make the HitHunter cAMP assays on the SpectraMax L Microplate Reader a desirable choice for medium-throughput screening.

Conclusion

- HitHunter cAMP assays on Molecular Devices' SpectraMax L Microplate Reader are suitable for medium to high throughput screening. With up to six detectors, the SpectraMax L Microplate Reader offers up to 6-fold higher throughput than traditional single-channel luminescence microplate readers and an extended dynamic range of more than 8 orders of magnitude.
- Our data demonstrates that HitHunter technology can be readily used with the bench-top SpectraMax L Luminescence Microplate Reader, making assay development easy and convenient.
- The combination provides a highly sensitive platform and large assay window for G_i- and G_s-coupled receptors.

Additional solutions from Molecular Devices

For increased throughput requirements, Molecular Devices' StakMax Microplate Handling System integrates with SpectraMax Readers and enables automated processing of batches of 20, 40, or 50 microplates.

Molecular Devices also offers the SpectraTest® LM1 Luminescence Validation Plate—a valuable tool for all users to verify their luminescence-capable microplate readers are operating correctly."

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