Chemiluminescent cAMP Detection using DiscoveRx HitHunter® Kits Compared with TR-FRET Assays

HitHunter® cAMP Benefits

- *Get large assay windows*
- *Run with high serum*
- *User fewer cells*
- *No false positives*
- *Use any luminometer*
- *Identify all compound types (agonist, allosteric, antagonist, partial, inverse)*

Introduction

cAMP detection technologies are widely used in high throughput screening (HTS), assay development or SAR groups for measuring a functional response of a GPCR consequent to ligand-binding. However, a robust and sensitive cAMP detection platform that allows differentiation of a full agonist from a partial agonist or an antagonist response from an inverse agonist response while also being able to identify allosteric compounds is preferred. DiscoveRx's HitHunter™ cAMP assay, a proven and validated platform, provides an enzyme-fragment complementation (EFC) format that detects cAMP in cell lines, primary cells, neuronal cell lines, membrane preparations and in serum samples. Large assay window, high sensitivity, ability to work with very low cell number and compatibility with any standard luminometer are hallmarks of HitHunter cAMP assays. These features offer a tremendous advantage especially for G_i-coupled GPCRs over other available kits in the market.

In this study, we compare our HitHunter cAMP assay (based on EFC technology) to assays based on time-resolved fluorescence resonance energy transfer (TR-FRET) from company B and company C. DiscoveRx's HitHunter assay while being more reflective of the actual level of cAMP in the cells. We can also tolerate higher serum levels, and can be used for antibody screening or screening several difficult to optimize G_i cell lines thus making it a superior platform. The data clearly shows the versatility, robustness and sensitivity of the HitHunter cAMP assay that provides a researcher or a high throughput screener a simple chemiluminescent gain-of-signal assay format for cAMP detection.

Materials and Methods

- cAMP Detection Reagents:
  - HitHunter® cAMP XS+ Assay
  - TR-FRET cAMP Detection Reagents
  - Forskolin
- Cell Lines
  - Glucagon like Peptide Receptor 2
  - Glutamate Metabotropic Receptor 4
  - Dopamine Receptor D2(s)
- Instrument
  - SpectraMax® L from Molecular Devices

(Please refer to Appendix A for more details)

HitHunter® cAMP Assay Principle and Protocol

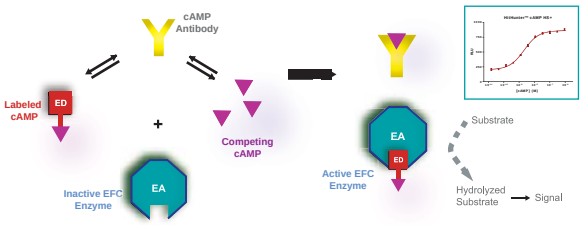


Figure 1. HitHunter® cAMP assays are competitive immunoassays. Free cAMP from cell lysates competes for antibody binding against labeled cAMP (ED-cAMP conjugate). Unbound ED-cAMP is free to complement EA to form active enzyme by EFC, which subsequently hydrolyzes substrate to produce signal. A positive signal generated is directly proportional to the amount of free cAMP bound by the binding protein.

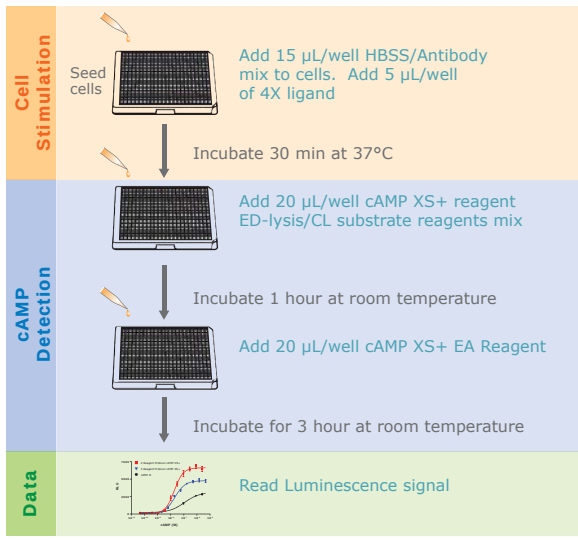


Figure 2. HitHunter® cAMP XS+ Assay protocol.

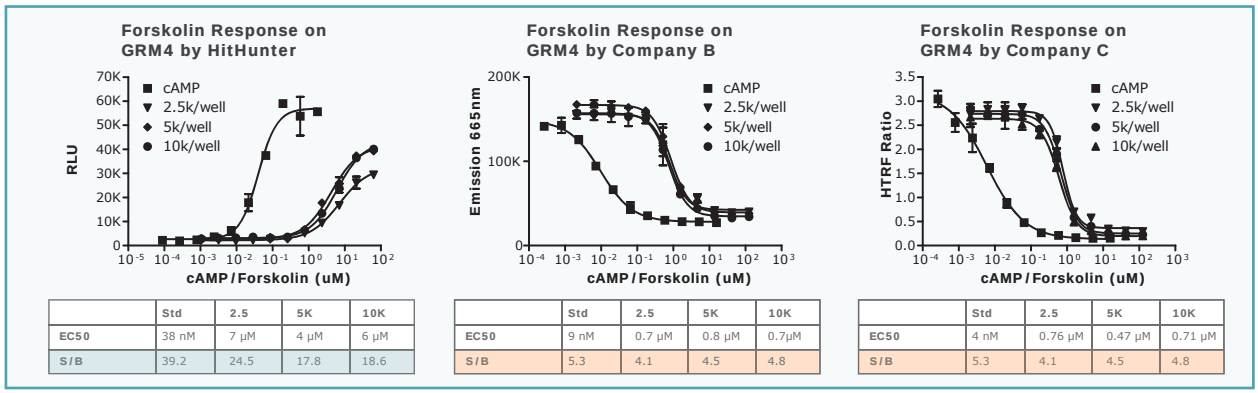
DiscoveRx

Results

HitHunter cAMP XS+ assay kits were evaluated along with two different TR-FRET assay kits. Assay conditions were optimized for the three kits by performing dose dependent responses in different cell densities.

Cell Number titration and Forskolin Stimulation: Comparison between chemiluminescent cAMP detection over ratiometric fluorescent methods

CHO-K1 cells expressing Gi coupled glutamate receptor GRM4 Cells (at 2500, 5000 or 10,000 for each well) were dispensed into a white, 384-well plate and stimulated with different concentrations of Forskolin. The resulting cAMP level was monitored with detection kits from DiscoveRx, company B and company C. Each cAMP detection kit was performed using the recommended conditions and procedures. Dose response curve was plotted and EC_{50} of each experiment was calculated using GraphPad Prism software. The cAMP standard curve is shown alongside of the forskolin curves. Enzyme-fragment complementation based HitHunter cAMP XS+ is a positive gain of signal assay allowing subtle increases in cAMP levels to be detected. Forskolin stimulation results in the production of cAMP; more cAMP in the system translates to more signal in HitHunter cAMP measurement. HitHunter cAMP XS+ kits gave the largest assay window when compared to the fluorescent loss of signal formats from Company B and Company C. Company C kit provided the lowest EC_{50} demonstrating the lowest dynamic range of the kit. Engineered cell lines that are commonly used in the screening campaigns have much higher levels of cAMP. The data generated by HitHunter cAMP is more reflective of the true levels of cAMP in the cells and not constained by the kit dynamic range. EC_{50} values obtained by HitHunter cAMP correlate with the EC_{50} obtained by forskolin stimulation of rat cortical adenylate cyclase (Seamon KB et.al, 1981). On the other hand, although the true levels of cAMP are much higher in the cells, in the HTRF and TR-FRET assays, the detection of these higher concentrations often falls outside the detection range of the kits. TR-FRET kit from Company B had the lowest dynamic range and hence lower EC_{50}s were observed.



	Std	2.5	5K	10K
EC50	38 nM	7 µM	4 µM	6 µM
S/B	39.2	24.5	17.8	18.6

	Std	2.5	5K	10K
EC50	9 nM	0.7 µM	0.8 µM	0.7µM
S/B	5.3	4.1	4.5	4.8

	Std	2.5	5K	10K
EC50	4 nM	0.76 µM	0.47 µM	0.71 µM
S/B	5.3	4.1	4.5	4.8

Figure 3. Cell Number titration & Forskolin stimulation in a Gi-coupled GPCR (Gi-coupled Glutamate Metabotropic Receptor 4)

Agonist responses in CHO-K1 cells expressing glutamate receptor GRM4 (Suspension protocol)

CHO-K1 Cells expressing GRM4 (5,000 cells/well) were tested against L-AP4 (agonist for GRM4) under the challenge of 15 µM Forskolin. EC_{50} of L-AP4 were represented as raw RLUs *(Panel A)* or cAMP levels extrapolated from the cAMP standard curve *(Panel B)*. Assay window (S/B) for each experiment was also shown. All data handling and curve fitting was by GraphPad Prism software. Figure (4) demonstrates the cAMP levels measured in response to the agonist (L-AP4). HitHunter cAMP XS+ assays provided an assay window of 11.8 as compared to 2.7 & 2.8 in kits from company B and company C. EC_{50} was consistent for HitHunter using direct cAMP measurements from the dose curve or extrapolated using the standard curve. Discrepancies were observed with the TR-FRET assays. TR-FRET assays recommend calculations of cAMP with reference to the kit standard curve. As this figure shows, there are bigger discrepancies fluorescent assays when compared to the EFC-based assay raising a question on accuracy.

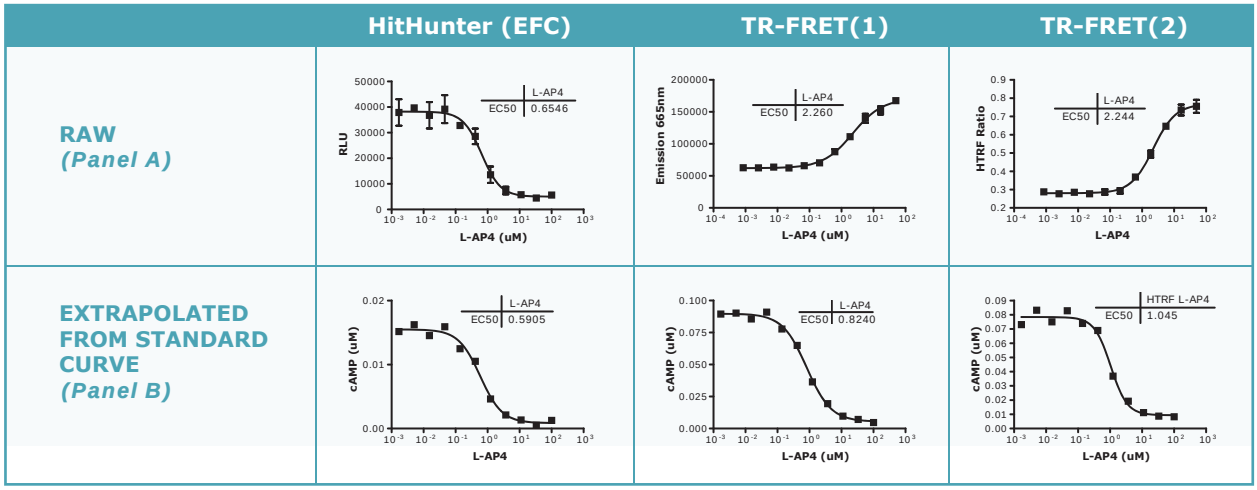


Figure 4. Agonist responses in CHO-K1 cells expressing glutamate receptor GRM4. Panel A shows the cAMP dose curves and EC_{50} from curve fitting of the raw data obtained and Panel B shows cAMP levels determined with reference to each of the standard curve and the EC_{50}s from the curve fitting of those calculated values. Data from HitHunter™ cAMP XS+ (DiscoveRx), TR-FRET (1) (Company B) and TR-FRET (2) (Company C) are represented.

Large Assay windows with HitHunter cAMP assays are ideal for G_i antagonist determination

G_i antagonist determination involves 1) Identification of EC_{80} for forskolin. 2) Identifying the EC_{50} and EC_{80} for agonist and 3) proceeding to the antagonist determination. Since studying G_i-coupled GPCRs involve an inhibition of cAMP pathway, raising the baseline levels of cAMP by forskolin is preferred. Forskolin stimulates adenylyl cyclase, which triggers cAMP production. CHO-K1 cells expressing dopamine receptor DRD2s (short variant) was used in this study. Robustness of HitHunter cAMP assays can be determined in this experiment. Hence, a higher assay window in the forskolin stimulation step is crucial to the subsequent antagonist studies. An assay window of around 15 was achieved in antagonist is very beneficial to screening and profiling applications (figure 5).

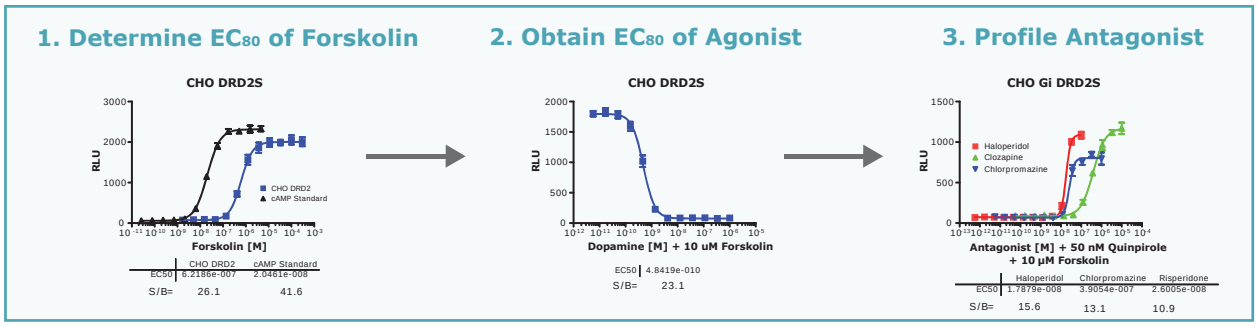
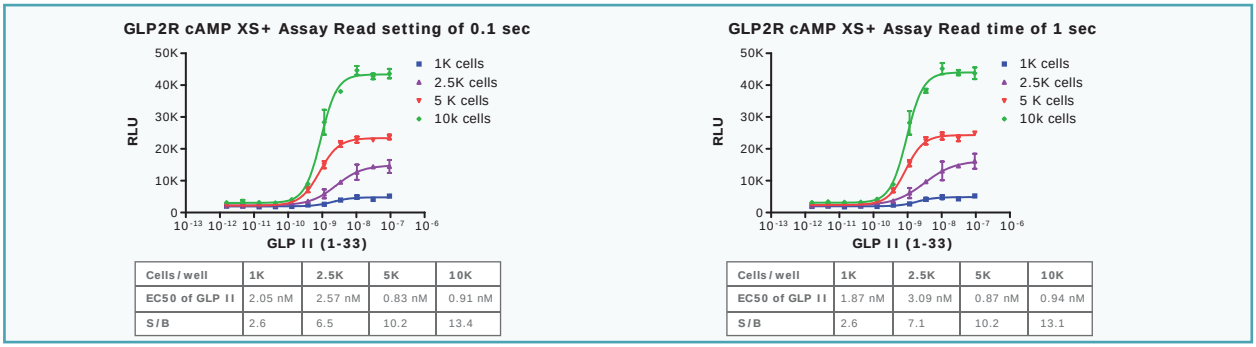


Figure 5. Large assay windows are ideal when profiling G_i antagonists

Agonist responses in Gs coupled GPCRs

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 Part # 92-1079) for 30 minutes. Signal was detected using the HitHunter cAMP XS+ Kit (90-0075) as per the assay protocol. 2,500 cells were optimal and results in a huge cost benefit and reduced tissue culture costs and resources when considering a medium to high throughput screen. Read time of 0.1 second allows batch processing of multiple plates and reduces the time required on standard instrumentation. Molecular Devices SpectraMax L was used in this experiment. Data analysis was performed on SpectraMax L.



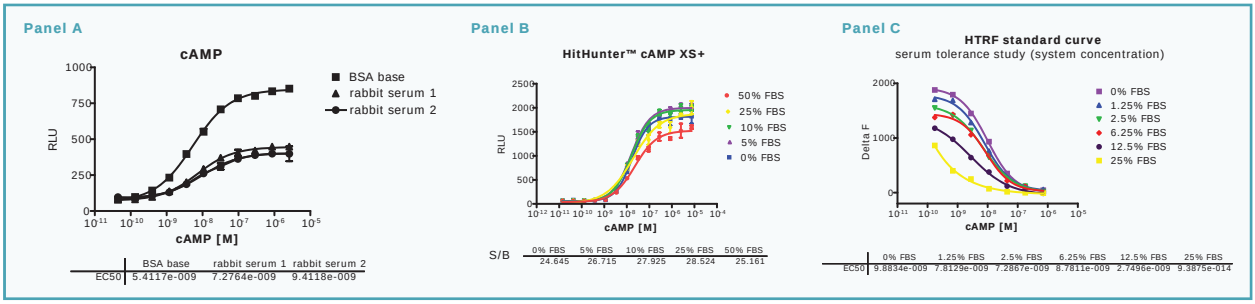
Cells / well	1K	2.5K	5K	10K
EC50 of GLP II	2.05 nM	2.57 nM	0.83 nM	0.91 nM
S/B	2.6	6.5	10.2	13.4

Cells / well	1K	2.5K	5K	10K
EC50 of GLP II	1.87 nM	3.09 nM	0.87 nM	0.94 nM
S/B	2.6	7.1	10.2	13.1

Figure 6. Agonist responses in CHO-K1 cells expressing human GLP2R receptor. 2,500 cells were optimal in HitHunter cAMP XS+ assay and a read-time of 0.1 sec/well allows a researcher to complete reading a 384-well plate in less than 40 seconds.

HitHunter cAMP reagents are useful for biomarker studies with high serum requirements

cAMP standard curve was tested in the presence of two kinds of serum. BSA and Rabbit serum were used in this assay. The antibody in the HitHunter™ reagent was diluted by 0.35X and then used as per the kit protocol. The kit is sensitive even in high amounts of serum available. Serum is often required in biomarker applications, as well as a requirement for many human and murine-derived cell lines and is often a desired feature in a cAMP detection kit. The HTRF kits have much lower tolerance to serum as suggested by figure 7.



	BSA base	rabbit serum 1	rabbit serum 2
EC50	5.4117e-009	7.2764e-009	9.4118e-009

S/B	0% FBS	5% FBS	10% FBS	25% FBS	50% FBS
	24.645	26.715	27.925	28.524	25.161

	0% FBS	1.25% FBS	2.5% FBS	6.25% FBS	12.5% FBS	25% FBS
EC50	9.8834e-009	7.8129e-009	7.2867e-009	8.7811e-009	2.7496e-009	9.3875e-014

Figure 7. The cAMP standards from HitHunter cAMP XS+ *(Panel A)* were diluted in various kinds of serum using HitHunter cAMP XS+. BSA and Rabbit serum was used in this assay. The antibody in the reagent was diluted by 0.35X and then used as per the kit protocol. The data indicates that higher amounts of serum can be tolerated by the HitHunter cAMP assay. HitHunter cAMP assay is also routinely used with plasma and serum applications (Sandrock T. et.al., 2007).

Summary

This study demonstrates that HitHunter cAMP XS+ assay kits have distinct advantages over the TR-FRET based. HitHunter kits are a gain of signal assay kits and forskolin stimulation yields in cAMP formation that does not saturate the assay range. On the other hand, the cAMP levels in TR-FRET kits easily saturate the reagents and are often outside the assay ranges resulting in inaccurate determination. TR-FRET measurement requires background subtraction, while HitHunter does not require background subtraction. This results in a discrepancy of the actual levels of cAMP measured. HitHunter calculations are truly reflective of the actual levels of cAMP in the assay system. A substantial number of compounds in screening decks are colored or fluorescent. Interference from colored compounds due to quenching, results in false positives or false negatives. Miniaturization and determining partial agonists are easier in a gain-of-signal assay. HitHunter assays are simple and do not require complex analysis. It can often be performed on standard instrument platforms.

Experimental Objectives	Results Obtained	Key Advantages
Cell Number Titration and Forskolin Optimization (Figure 3)	• 2,500 cells showed robust response. Lower cell numbers can be explored • HitHunter® cAMP XS+ showed EC_{50} of 4-7 µM TR-FRET Assay showed EC_{50} in <1 µM for forskolin • HitHunter® kit gave the largest assay window of 11.8	• Reduced cell number translates to lower tissue culture costs • Engineered cells have lot of cAMP and since HitHunter cAMP XS+ assay is a gain-of-signal format with a broader range, it is likely to help you obtain accurate cAMP measurement
Agonist Response in a Gi-coupled Receptor: Different Data Interpretation (Figure 4)	• cAMP EC_{50} values determined from raw data and extrapolated from standard curve show very little variation with HitHunter® assay but discrepancies were observed with the TR-FRET assays	• HitHunter kits are a gain-of-signal assay kits and forskolin stimulation yields in cAMP formation that does not saturate the assay range, while in the TR-FRET assays, often cAMP in the cells saturate the detection reagents
Gi Antagonist Determination (Figure 5)	• HitHunter cAMP XS+ showed a assay window of 26 for Forskolin stimulation, >20 for agonist and a >15 with the antagonists	• Large assay windows with HitHunter® cAMP assays are ideal for Gi antagonist determination
Agonist Response in Gs-coupled GPCR (Figure 6)	• 2,500 cells gave an optimal performance for this GPCR target	• 0.1 sec/well read time allows you to complete a 384-well plate under 40 seconds
Serum Tolerance (Figure 7)	• HitHunter assays can tolerate a wide variety of serum and higher amounts of serum when compared to TR-FRET assays	• HitHunter® cAMP reagents are useful for biomarker studies with high serum requirements

For more information, please visit http://www.discoverx.com/gpcrs/hithunter_camp

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For a complete list, please visit http://www.discoverx.com/gpcrs/hithunter_camp

Appendix A - Material Used:

	Vendor	Part #	Description	Intended Use
Reagents	DiscoveRx	90-0075	HitHunter® cAMP XS+	cAMP Detection
	DiscoveRx	92-0005	Forskolin	Adenylate cyclase activation
Cells	DiscoveRx	95-0112C2	cAMP Hunter® GLP2R	Screening or Profiling
	DiscoveRx	95-0117C2	cAMP Hunter® GRM4	Screening or Profiling
	DiscoveRx	95-0084C2	cAMP Hunter® DRD2(S)	Screening or Profiling
Plate	Costar	3704	TC-coated White Solid	Microtiter plater for luminescence
Instrument	Molecular Devices	0310.5132	SpectraMax	Luminometer

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