HitHunter™ cAMP HS+ assay with Mithras LB 940

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Introduction

HitHunter cAMP HS+ is the next generation of EFC chemiluminescent assays designed for assessing both Gs and Gi coupled GPCR activation in cells. HitHunter cAMP HS+ features the ability to detect low levels of cAMP, such as is required for orphan or endogenous GPCR assays. Its simple, homogeneous protocol and enhanced performance make it an excellent fit for therapeutic and HTS laboratory research programs.

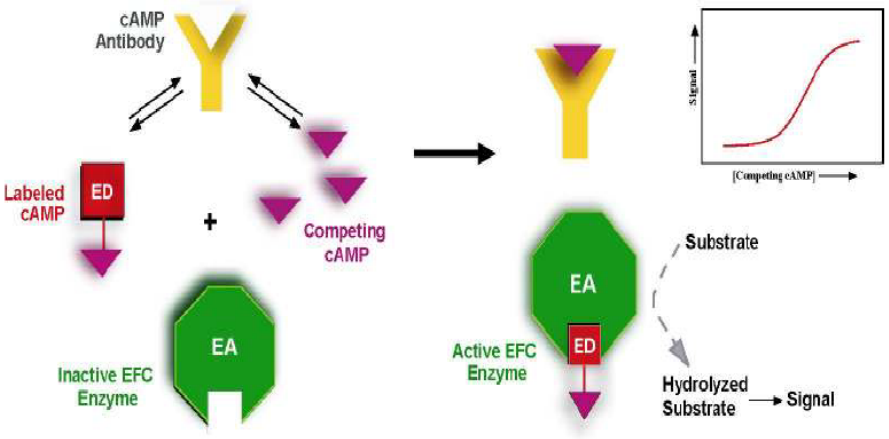

Figure 1: HitHunter™ cAMP principle

HitHunter cAMP HS+ is an in-vitro based competitive immunoassay. Free cAMP from cell lysates compete for antibody binding against labeled ED-cAMP conjugate, a small peptide fragment of ß-galactosidase (ß-gal). In the absence of free cAMP, ED-cAMP conjugates are captured by the antibody and are unavailable for complementation, resulting in low signal. In the presence of free cAMP, antibody sites are occupied, leaving ED-cAMP conjugate free to complement with EA, forming active ß-gal EFC enzyme for substrate

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hydrolysis to produce a chemiluminescent signal. A positive signal is generated in direct proportion to the amount of free cAMP bound by the antibody.
ß-galactosidase (E.C. 3.2.1.23) specifically and reproducibly hydrolyzes numerous substrates. EFC assays and EFC related solutions are preferentially developed using a chemiluminescent substrate which produces a high intensity signal with low background that is not affected by naturally fluorescent compounds.

The Mithras LB 940 is a multimode plate reader with a unique optical design (DOPS – Dedicated Optical Path System) to ensure optimized performance for the detection technologies implied. These are

- luminescence
- BRET/BRET²
- fluorescence
- UV/VIS absorbance
- fluorescence polarization
- AlphaScreen™
- TRF
- HTRF®

Figure 2: Mithras LB 940 multimode reader

In addition accessory options, e.g. reagent injectors, temperature control and cooled PMT detection units are available. The combination of an unmatched efficiency for luminescence detection including the proprietory crosstalk-reduction design with the reagent injectors make the instrument ideally suited for the HitHunter™ technology.

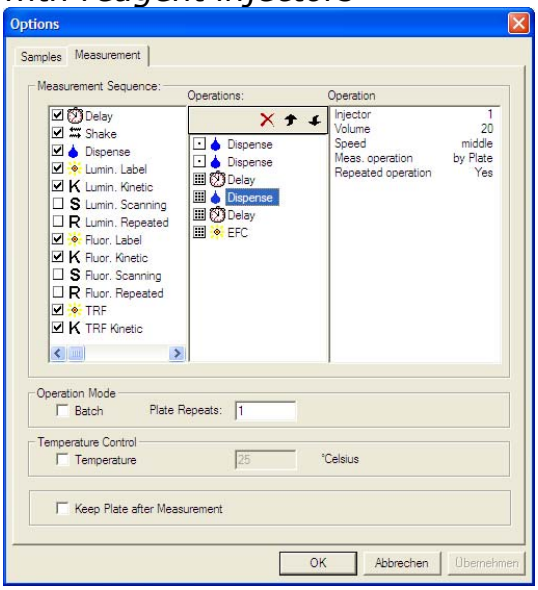
Methods

Assay Protocol:

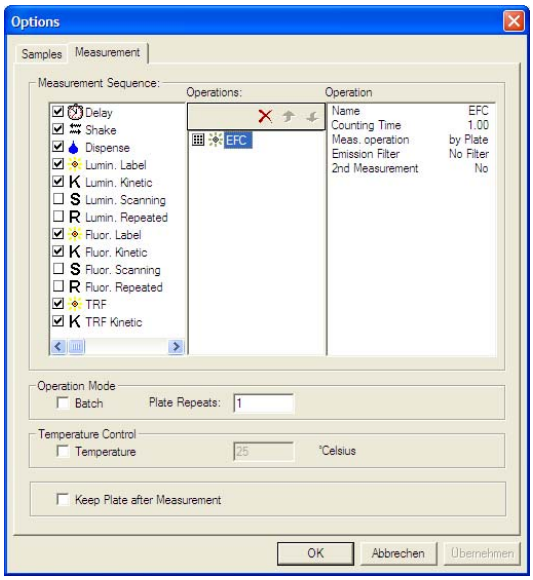
- Add 15 µL cAMP standard
  or 10 µL CHO-K1 cells plus 5 µL forskolin dilution
  or 10 µL A431 cells plus 5 µL isoproterenol dilution
- Incubate for 30 min at 37 °C
- Add 10 µL anti-cAMP Ab plus lysis reagent
- Add 10 µL cAMP-ED
- Incubate for 1 h at room temperature
- Add 20 µL EA plus chemiluminescent substrate
- Incubate for 1 hour at room temperature
- Read in luminescence mode for 1 s per well

Instrument settings:

with reagent injectors

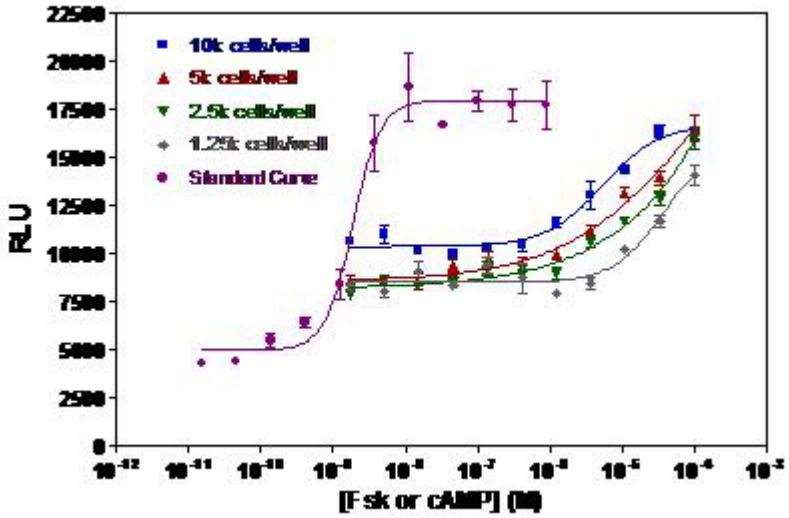


without reagent injectors



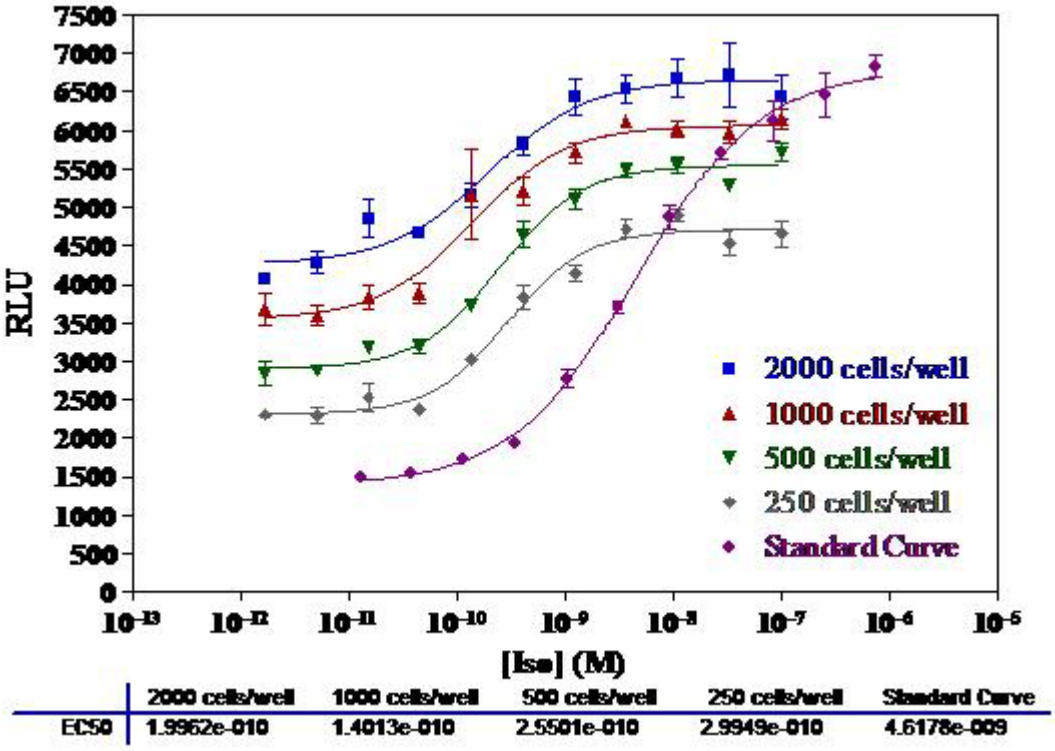
Results

The cAMP standard curve results in S/B values of 3.7 to 3.9 and EC_{50} of 1.9 to 4.6 nM cAMP respectively for the two measurements providing a convenient dynamic range for the assay. The different amounts of cells used in the experiments lead to comparable S/B values of 224 pM for the isoproterenol stimulation of the endogeneous ß2-adrenergic receptor and 19 µM for the forskolin cAMP induction in CHO-K1 cells.



	10k	5k	2.5k	1.25k	Std curve
EC_{50}	5.4 µM	1.15 µM	0.9 µM	0.34 µM	1.8 µM

Figure 3: Stimulation of CHO-K1 cells with Forskolin
CHO-K1 cells at different densities were stimulated with varying concentrations of forskolin; cAMP levels in response to forskolin stimulation was measured with the HitHunter cAMP HS+ kit
Upper plot: signal vs. cAMP resp. forskolin
Lower table: EC_{50} values



	2000 cells/well	1000 cells/well	500 cells/well	250 cells/well	Standard Curve
EC50	1.9962e-010	1.4013e-010	2.5501e-010	2.9949e-010	4.6178e-009

Figure 4: Stimulation of A431 cells in suspension with Isoproterenol CHO-K1 cells at different densities were stimulated with varying concentrations of Isoproterenol; cAMP levels in response to stimulation was measured with the HitHunter cAMP HS+ kit Upper plot: signal vs. cAMP resp. isoproterenol Lower table: EC_{50} values

Conclusion

- The Mithras shows excellent S/B values due to its sensitive luminescence detection fully matching the superb sensitivity of the HitHunter™ cAMP HS+ assay.
- We see an excellent correlation with cAMP induction in both CHO-K1 and A431 cells with forskolin and isoproterenol agonist.
- Even low number of cells (250 cells/well) can be measured due to the sensitive assay and instrument.

Materials

- Mithras LB 940, equipped with three reagent injectors (Berthold 38099)
- HitHunter™ cAMP HS+ kit (DX90009002; available through GE Healthcare)
- White microplates: 96 well (Berthold 23300) or 384 well (32505)
- additonal reagents see kit insert