Highly sensitive and specific 384 well assay for chemiluminescent detection of cyclic AMP

Richard H. Sullivan, Andrew J. Ball, Michele Hatler and Matthew Hsu

Millipore Corporation, Bioscience Division, 28820 Single Oak Drive, Temecula, CA 92590. Email: Matthew_Hsu@millipore.com

upstate · CHEMICON · Linco

The expertise of Upstate®, Chemicon® and Linco® is now a part of Millipore

Abstract

Cyclic AMP (cAMP) is a key second messenger in signal transduction, and an important modulator of metabolism, cancer, inflammation and CNS signaling. Agents which increase or decrease intracellular cAMP are of major interest in drug discovery. We describe a novel 384-well competitive immunoassay for rapid and ultra-sensitive (1.0 fmol/well) chemiluminescent quantitation of cAMP. The assay utilizes a highly sensitive anti-cAMP antibody that does not cross-react with other cyclic nucleotides, and has been validated for GPCR applications. In CHO(wt) cells seeded at 10,000 cells per well, forskolin dose-dependently increased cAMP (EC_{50} 1.5 μM). In another experiment CHO cells transfected with a Gs-coupled receptor Dopamine1(D1) were cloned, a dose response range of the natural ligand Dopamine was added, and EC_{50} values for cAMP production in 4 clones was determined. The assay we present is sensitive, highly specific for cAMP, and may be used to test a wide variety of sample types from all species.

Introduction

Cyclic cAMP acts as a key second messenger in multiple signal transduction pathways. All receptors that act via cAMP are coupled to a stimulatory G protein, which activates adenylate cyclase upon ligand binding. Many different drugs, neurotransmitters and hormones exert their cellular effects by modulating adenylate cyclase activity and thus raising or lowering intracellular cAMP concentrations. cAMP regulates many cellular functions, such as metabolism, cell growth and differentiation, gene transcription, ion transport and ion channel function. These cAMP effects, mediated primarily by cAMP-dependent protein kinase (PKA), result in cAMP being responsible for the regulation of many physiological processes, including cardiovascular, endocrine, neuronal, glandular, kidney, and immune functions, as well as general metabolism. Consequently, agents which increase or decrease intracellular cAMP levels are of major interest in drug discovery.

Millipore has developed a 384 well cAMP HTS competitive immunoassay (Catalog. No. 17-416) for *in vitro* quantitative detection of cAMP in mammalian cell lysates and supernatants. The assay has been designed and validated for use in high throughput screening applications.

Millipore and Drug Discovery

Millipore provides a broad range of products and services to enable and accelerate drug discovery

- HTS assays and profiling services: 120+ GPCRs, 252 Kinases and 29 Ion channels
- 500+ cell-based assays for stem cells, neuroscience and drug discovery
- 15,000+ antibodies, reagents and tools for HCA
- Multi-well assays and membranes: MultiScreen® and MilliCell® plates and insert
- LINCOplex® (Multiplex) and immunoassays

Assay Overview

- Fast, accurate and highly sensitive tool for cAMP detection
- Results in ~ 1.5 hours
- Each kit provides sufficient components for 768 assays

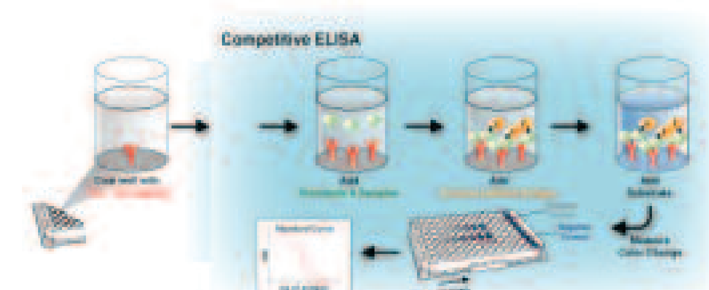
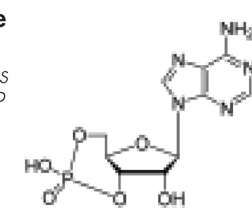


Figure 1. *Illustration of the principle of competitive immunoassays.*

Figure 2. Chemical structure of 5'-cyclic adenosine monophosphate (cAMP). *This molecule competes with the conjugated tracer for available binding sites on the highly specific anti-cAMP antibody. As cAMP availability increases, the tracer's ability to bind decreases, and thus the luminescent signal decreases.*



Protocol Summary

Add 30 μL of cAMP Standards or prepared samples

↓

Add 15 μL of diluted cAMP Alkaline Phosphatase Conjugate Tracer

↓

Add 30 μL of diluted Anti-cAMP Antibody

↓

Seal the plate with a Plate Sealer.
Incubate plate for 30 minutes at room temperature

↓

Remove fluid from wells and wash 5x with Wash Buffer

↓

Add 30 μL of diluted Alkaline Phosphatase Substrate. Seal plate with a Plate Sealer and incubate at room temperature for 30 minutes

↓

Read the plate for 1.0 second with a luminometer

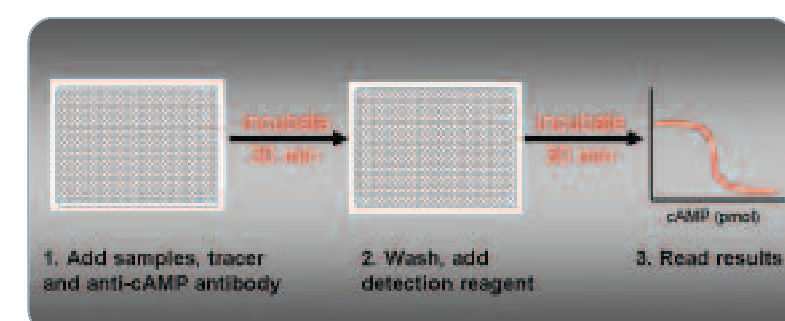


Figure 3. *Summary of the assay protocol.*

Results

Quantitation of cAMP

Figure 4. cAMP Standard Curve. *The provided cAMP standard containing 5 mM cAMP (5,000,000 pmol/mL) was used in this experiment. Seven serial dilutions were prepared in lysis buffer. 30 μL of each standard was used to generate the standard curve in a range from 0.0003 to 3,000 pmol cAMP/well. This represents a typical Standard Curve.*

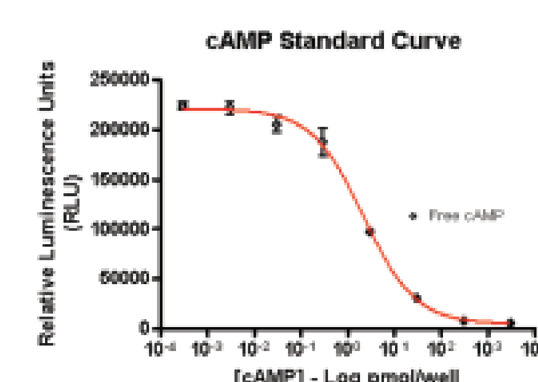


Figure 5. Forskolin dose-response *CHO wt cells were seeded at 10,000 cells per well in a 384-well plate 24 hr before the assay. Cells were pre-incubated with 1 mM IBMX for 5 minutes, then various concentrations of forskolin were added for an additional 15 minute incubation at 37°C. The reaction was terminated by the addition of cold cell lysis buffer (provided in the kit). 30 μL of the sample was used for cAMP analysis.*

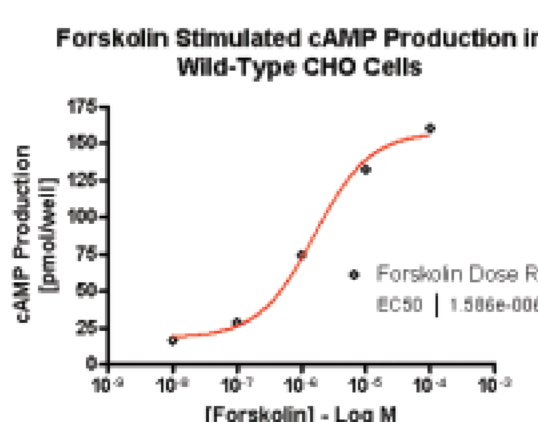
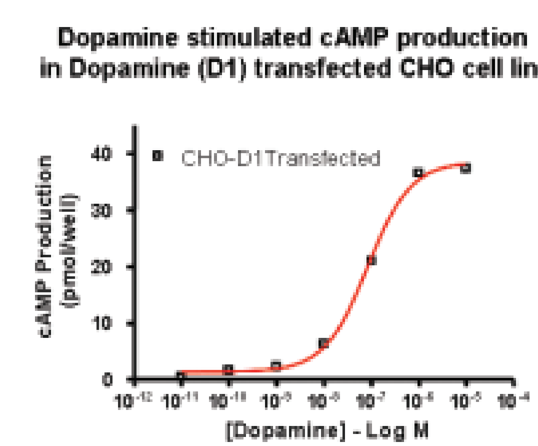


Figure 6. Validation of 17-416 assay for SAR studies *CHO cells transfected with a G_s-protein coupled receptor Dopamine1 (D1), were seeded at 10,000 cells per well in a 384-well plate 24 hr before the assay. A dose response range of the natural ligand Dopamine was added to induce the production of cAMP.*



Assay Performance and Validation

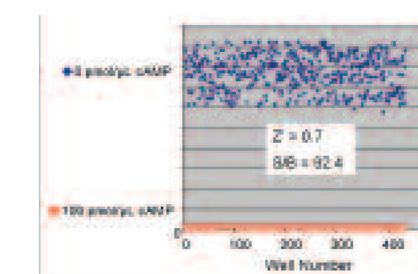


Figure 7. Validation of assay for screening studies. *Assay performance was assessed by calculation of the Z' value. A Z' value of 0.7 was obtained from multiple data points of minimum (100 pmol/μL cAMP) and maximum luminescence (0 pmol/μL cAMP). Signal/Background (SB) ratio was determined as 92.4 by calculating the ratio between the mean max signal and the mean minimum signal. This ratio describes the dynamic range of the assay.*

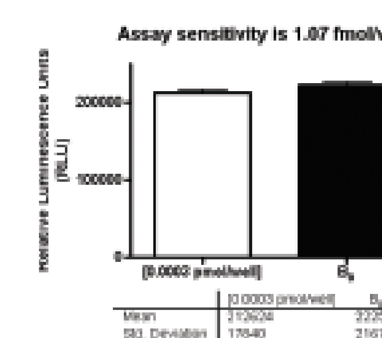


Figure 8. Calculation of Assay Sensitivity. *Sensitivity of 1.07 fmol/well was calculated by determining the average RLU for 24 wells run with the Bo, and comparing to the average RLU for 24 wells run with 0.0003 pmol/well. The detection limit was determined as the concentration of cAMP measured at two standard deviations from the zero along the standard curve.*

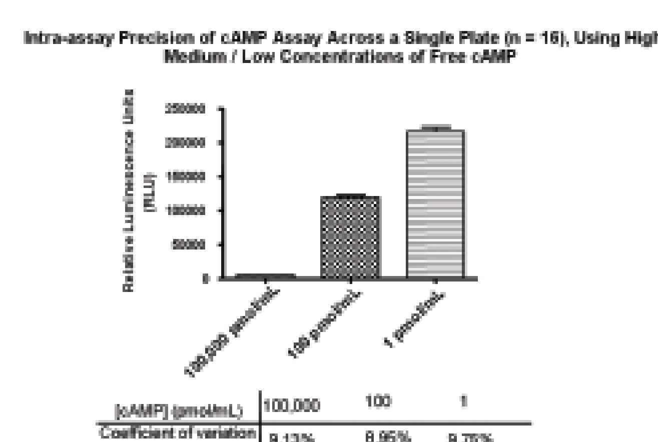


Figure 9. Calculation of intra-assay precision. *Intra-assay precision was determined by taking samples containing differing concentrations of cAMP and running these samples multiple times (n = 16) in the same assay. The precision numbers shown above represent the percent coefficient of variation for the concentrations of cAMP determined in these assays as calculated by curve fitting software.*

Cross-reactivity of anti-cAMP antibody

cAMP	100%	CTP	<0.01%
cGMP	<0.1%	AMP	<0.01%
GTP	<0.01%	ADP	<0.01%
GMP	<0.01%	ATP	<0.01%

Table 1. *Cross-reactivity of anti-cAMP antibody. The assay utilizes a rabbit polyclonal anti-cAMP antibody which shows 100% reactivity with cAMP. Potential cross reactants (cGMP, GTP, GMP, CTP, AMP, ADP and ATP) were dissolved in lysis buffer at concentrations from 2,000 to 2 pmol/mL. Samples were measured in the cAMP assay, and the measured cAMP concentration at 50% B/Bo calculated. The % cross reactivity was calculated by comparison with the actual concentration of cross reactant in the sample and expressed as a percentage.*

Summary

- Millipore's 384 well cAMP HTS Immunoassay is a highly sensitive high-throughput system for quantitation of cAMP in supernatants and lysates of cells from any species.
- Assay is rapid, with results being obtained in approx. 1.5 hrs.
- Assay is reproducible and robust, with a Z' value of 0.7 and a S/B ratio of 92.4.
- Sensitivity of the assay is 1.07 fmol/well, and the assay has a large dynamic range, detecting cAMP concentrations from 0.0003 pmol/well to 3,000 pmol/well.
- The polyclonal antibody used is highly specific for cAMP and shows minimal cross-reactivity with other nucleotides.
- Assay is free of radioactivity, using a luminescence-based readout.
- Assay has been validated for use in GPCR screening applications.

17-416 Assay Components

1. Two 384 well immuno-plates pre-coated with anti-Rabbit polyclonal antibody, sealed in a foil pouch
2. Rabbit -cAMP Antibody
3. cAMP Standard, 5 mM solution
4. cAMP Alkaline Phosphatase Conjugated Tracer
5. Assay Diluent
6. Wash Buffer
7. Lysis Buffer
8. Chemiluminescent Alkaline Phosphatase Substrate
9. Plate Sealers

Related Products

17-418	cAMP HTS Immunoassay – 96 well
17-419	cGMP HTS Immunoassay – 96 well

References

1. Antoni FA. Molecular diversity of cyclic AMP signaling. (2000). Front Neuroendocrinol. 21:103-32.
2. Maurice DH, Palmer D, Tilley DG et al. (2003). Cyclic nucleotide phosphodiesterase activity, expression, and targeting in cells of the cardiovascular system. Mol Pharmacol. 64:533-46.
3. McKnight GS. (1991). Cyclic AMP second messenger systems. Curr Opin Cell Biol. 3:213-7.
4. Montminy M. (1997). Transcriptional regulation by cyclic AMP. Annu Rev Biochem. 66:807-22.

The M logo is a trademark of Millipore Corporation.
Upstate, Chemicon, Linco, LINCOplex, MultiScreen and MilliCell are registered trademarks of Millipore Corporation.
©2007 Millipore Corporation, St. Charles, MO 63304 U.S.A. 10/07 All rights reserved.