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Stable, Sensitive, Fluorescence-Based Method for Detecting cAMP

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ABSTRACT

cAMP is a universal secondary messenger that connects changes in the extracellular environment, as detected by cell surface receptors, to transcriptional changes in the nucleus. Since cAMP-mediated signal transduction plays a role in critical cell functions and human diseases, monitoring its activity can aid in understanding these responses and the process of drug discovery. This report examines the performance of a fluorescence-based competitive immunoassay in 384-well microplate format. Using purified cAMP as a competitor, the estimated detection limit was determined to be 0.1 nM and Z'-factor was greater than 0.83, which indicates that the assay is of high quality and one of the most sensitive assays currently on the market. Of note, the results obtained were similar whether the reaction was allowed to proceed for 10 min or up to 60 min. Next, HEK 293 cells were treated with the promiscuous adenylate cyclase activator, forskolin, and the β -adrenoceptor agonist,

isoproterenol. The resultant average EC_{50} values were 11 μ M and 123 nM, respectively, which correspond to those found in the literature. Together, these results demonstrate that this assay is a fast, accurate, non-radioactive method that is ideal for high-throughput screening.

INTRODUCTION

cAMP is involved in intra- and inter-cellular signaling in organisms as diverse as bacteria, slime mold, fruit flies, and humans (4,5,9). cAMP is produced from ATP by the enzyme adenylate cyclase and is hydrolyzed by cAMP phosphodiesterases. Regulation of these enzymes allows for tight control over the levels of cAMP inside the cell. In mammals, extracellular ligands, such as peptide hormones and neurotransmitters, interact with transmembrane proteins called G-coupled receptors. Ligand binding leads to a conformational change in the receptor that allows its association with a GTP-binding regulatory protein (G-protein). G-coupled receptors activate adenylate cyclase when bound to a stimulatory form of G-protein, G_s , and inhibit the enzyme when bound to an inhibitory form of G-pro-

tein, G_i . Activation of G_s -coupled receptors results in increased production of cAMP, which in turn activates cAMP-dependent protein kinase (PKA). This enzyme phosphorylates a variety of proteins, which eventually leads to the regulation of transcription factor activity and effects on gene expression. Targets for PKA include other enzymes, ion channels, and transcriptional regulators. In addition, cAMP plays a direct role in transcription by binding and augmenting the activity of the transcription factor, cAMP-responsive element-binding protein (CREB). This protein plays a role in modulating the expression of cAMP-inducible genes (6). Further complicating this pathway is the role that cAMP plays in directly regulating the activity of cation channels (10).

Depending on the cell type, this signal transduction pathway may be responsible for neural and immune responses, metabolism, mitogenesis oocyte maturation, and many other critical functions (2,5,7,8,10,11). Therefore, an accurate method for quantitative measurement of intracellular cAMP is critical for basic research and drug discovery purposes. The new fluorescence intensity-based assay described here is unique because readings

DRUG DISCOVERY

AND GENOMIC TECHNOLOGIES

Table 1. Instrument Settings for the Analyst AD

Parameter	Settings
Mode	Fluorescence intensity
Excitation filters	Rhodamine 530–25 nm
Emission filters	Rhodamine 590–20 nm
Dichroic mirror	50/50 Beam-splitter
Z-height	3 mm
Attenuator	Medium
Integration time	50 000 μ s
Lamp	Continuous
Readings/well	One
PMT Setup	Smart Read + (sensitivity = 2)
Units	cps

can be taken in as little as 10 min or as long as 24 h after substrate addition, since no termination step is needed. Also, the overall time to process the assay is approximately 3 h, and it includes only one wash step. Most importantly, the assay is one of the most sensitive assays currently available on the market. Here we illustrate these advantages and the high quality of the assay.

MATERIALS AND METHODS

Cells

HEK 293 cells (human embryonic kidney cells that stably overexpress the Adenovirus E1A protein) were grown in DMEM with 10% FBS at 37°C in 5% CO₂.

Reagents

The reagents used were Catch-Point™ cAMP fluorescent assay kit that includes solid black, 384-well microplates pre-coated with goat anti-rabbit antibody (Molecular Devices, Sunnyvale, CA, USA), DMSO, low water content (Sigma, St. Louis, MO, USA), Krebs-Ringer Bicarbonate Buffer (KRBG; Sigma), sodium bicarbonate (Sigma), cAMP agonists including

forskolin (Sigma) and isoproterenol (Sigma), phosphodiesterase inhibitor, 3-isobutyl-1-methylxanthine (IBMX; Sigma), and 30% hydrogen peroxide solution (Sigma).

Cell Preparation

HEK 293 cells were grown to approximately 80% confluence at 37°C. Cells were detached using 0.526 mM EDTA in PBS and washed once with KRBG. Cells were resuspended at a concentration of 1×10^6 cells/mL in stimulation buffer (KRBG buffer that contains 0.75 mM IBMX). Next, cells were seeded in a volume of 7.5 μ L at a concentration of 0.75×10^4 cells/well of a 384-well microplate. The microplate was incubated for 10 min at room temperature.

Cell Stimulation

To stimulate cells to produce cAMP, 15- μ L doses of activator and agonist were diluted in PBS (forskolin and isoproterenol; Sigma); for unstimulated cell samples, PBS alone was added. The range of agonist final concentrations used was 0–1 mM. In addition, 15- μ L samples of cAMP calibrator at final concentrations ranging from 0 to 3333 nM were added to the wells to generate a calibration curve. Each of the above conditions was performed in replicates of eight. The microplate was gently mixed and incubated at room temperature for 15 min. To lyse the cells, 7.5 μ L cell lysis buffer (supplied with kit) were added to each well, and the plate was agitated for 10 min at room temperature using a plate shaker. The final volume in each well was 30 μ L.

Immunoassay

The immunoassay procedure was started by transferring 20 μ L each sample to the 384-well solid black assay microplate (supplied with kit). The samples were then treated with 20 μ L rabbit anti-cAMP antibody (supplied with the kit), and the microplate was gently agitated on a plate shaker for 5 min to ensure proper mixing. Next, 20 μ L HRP-cAMP conjugate (supplied with kit) were added to all wells, and the plate was agitated and incubated for 2 h at

room temperature. The assay microplate was washed four times with 80 μ L/well of wash buffer (supplied with kit). Then, 50 μ L Stoplight Red solution (supplied with kit) was added to every well. The microplate was covered to protect it from light and incubated for up to 1 h at room temperature. The resulting fluorescence intensity was read at 10 min and 60 min using a Gemini XS and Analyst™ AD (both from Molecular Devices). The instrument settings used for the Gemini XS were excitation wavelength of 530 nm, emission wavelength of 590 nm, emission cut-off filter of 570 nm, and six readings/well with the photomultiplier tube set to automatic. Table 1 shows the instrument settings used for the Analyst AD.

RESULTS

Assay Performance

Analysis of the data obtained for samples containing cAMP calibrator resulted in the plots shown in Figure 1. These data were obtained after 10 min and 60 min incubation with Stoplight Red substrate. The coefficient C is the X-value corresponding to the Y-value that is the midpoint between the low and high values on the asymptote of the curve fit to the 4-parameter equation: $Y = (A - D) / [1 + (X/C)^B] + D$. This equation is shown below the graphs in all of the figures. The value for coefficient C is equivalent to the EC₅₀ value. In Figure 1, the value for the coefficient C (EC₅₀ value) is 2.4 nM for 10 min (Figure 1A) and 3.7 nM for 60 min (Figure 1B). The EC₅₀ values read at 10 min and 60 min were similar, which demonstrated the relative stability of the fluorescent signal over time. The signal is also stable for 24 h (data not shown). The average EC₅₀ value obtained when the assay was performed on different days was 3.55 ± 0.2 nM. This value is in agreement with the average EC₅₀ value of 3.4 ± 0.6 nM, which was obtained using the Analyst AD.

For the experiments shown in Figure 1, the signal-to-noise ratio (no cAMP calibrator) was 240, and the limit of detection (LOD, defined as three standard deviations difference from the zero cAMP calibrator control) was 0.1 nM

cAMP for both fluorescence microplate readers. The optimal performance range of the assay falls between 0.14 and 33 nM. These results are in accordance with those obtained using the Analyst AD (0.1–80 nM, data not shown). In addition, the Z'-factor for the assay was calculated. The concentration of cAMP calibrator that achieved maximal inhibition (33 nM) was used as the positive control, and the concentration of cAMP calibrator that achieved the minimal inhibition (0.14 nM) was used as the negative control. A value between 0.5 and 1.0 indicates a high-quality, robust assay appropriate for screening purposes (12).

The Z'-factors for the assay read on the Gemini XS and Analyst AD were 0.84 and 0.90, respectively, when using eight replicates. In a separate experiment, the Z'-factor was calculated using 64 replicates of 33 nM calibrator and 64 replicates of 0.14 nM calibrator and read using the Analyst AD. The resulting Z'-factor value was 0.89.

Response of HEK 293 Cells to Forskolin and Isoproterenol

Next, the response of HEK 293 cells to forskolin and isoproterenol was examined. Forskolin is a natural diter-

pene, which is a promiscuous activator of all adenylate cyclase isoforms, while isoproterenol is a β -adrenoceptor agonist (1,3). HEK 293 cells are a suitable model system with which to study the regulation of cAMP because of their low endogenous level of adenylate cyclase activity (3). The results of the experiments are shown in Figures 2 and 3. The EC₅₀ values obtained for forskolin were 12.7 nM and 9.3 nM using Gemini XS and Analyst AD, respectively (Figure 2). These results are in accordance with values obtained using other products ([FP]²™ kit from NEN® Life Science Products; Perkin Elmer Life

Figure 1. Dose response curves for cAMP calibrator using Gemini XS. Dose responses are shown for (A) 10 min and (B) 60 min after the addition of Stoplight Red substrate. Each point on the dose response curves represents the average of eight replicate samples. Error bars denote the standard deviation from the mean. R² value is 0.99 for a 4-parameter curve fit.

Figure 2. Dose response curves for forskolin-treated HEK 293 cells. Each point on dose response curves represents average RFU of eight replicate samples for one concentration of the activator. The plate was incubated with Stoplight Red substrate for 60 min before reading this data set. Error bars denote the standard deviation from the mean. R² value for the experiment was 0.99 using a 4-parameter curve fit. Curve shown in panel A was obtained using Gemini XS, and the curve shown in panel B was obtained using Analyst AD. The EC₅₀ values were (A) 12.7 nM and (B) 9.3 nM for Gemini XS and Analyst AD, respectively.

DRUG DISCOVERY

AND GENOMIC TECHNOLOGIES

Figure 3. Dose response curves for isoproterenol-treated HEK 293 cells. Each point on the dose response curves represents the average cAMP of eight replicate samples for one concentration of the agonist. The R² value for both experiments was 0.98 for a 4-parameter curve fit. The dose response curves shown were obtained after 60 min incubation of samples with Stoplight Red substrate. The curve shown in panel A was obtained using Gemini XS, and the curve shown in panel B was obtained using Analyst AD. The cAMP values shown on the Y-axis were obtained by interpolation of the original data. The EC₅₀ values were (A) 123 nM and (B) 123 nM for Gemini XS and Analyst AD, respectively.

Sciences, Gaithersburg, MD, USA).

The EC₅₀ values obtained for isoproterenol were 123 nM using both the Gemini XS and Analyst AD, respectively (Figure 3). The cAMP values shown on the Y-axis were obtained by interpolation of the original data. The graphs in Figure 3 were shown to demonstrate the ability of the software supplied with both fluorescence microplate readers to convert relative fluorescence unit (RFU) to cAMP values. The EC₅₀ values for isoproterenol are in accordance with values obtained using another cell line (1).

DISCUSSION

The CatchPoint cAMP fluorescent assay is very sensitive and has an LOD of 0.1 nM. In comparison to the Molecular Devices fluorescence polarization method for measuring cAMP (1.25 nM), Catchpoint is approximately 10-fold more sensitive. The assay kit has a short post-substrate addition incubation time required to reach a stable signal (10 min to 24 h). This makes the kit ideal for using a stacker and robotic system to transfer the microplates to a fluorescence microplate reader that may be used in screening laboratories. This point is confirmed by comparison of the results after 10 min and 60 min incubation with Stoplight Red substrate. The LOD and EC₅₀ values for the cAMP

calibrator were very similar at the two time points. Furthermore, this fluorescence assay provides a high-quality screening assay, as demonstrated by the Z'-factor and similar results obtained on different days and with different fluorescence microplate readers. In conclusion, CatchPoint cAMP fluorescent assay allows simple, convenient, and highly sensitive quantitative measurement of intracellular cAMP.

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