

A Stable, Sensitive Fluorescence-based Method for Measuring Cyclic AMP Using the SPECTRAmax® GEMINI XS Spectrofluorometer

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INTRODUCTION

Adenosine 3', 5'-cyclic monophosphate (cyclic AMP, cAMP) is a universal secondary messenger that connects changes in the extracellular environment, as detected by cell surface receptors, to changes in the nucleus, which results in altered patterns of gene expression. It is involved in intra- and inter-cellular signaling in organisms as diverse as bacteria, slime mold, fruit fly and humans. Cyclic AMP is produced from ATP by the enzyme adenylate cyclase (AC) and is hydrolyzed by cyclic AMP phosphodiesterases. Activation of these enzymes allows for the tight control over the levels of cAMP inside the cell.

In mammals, extracellular ligands, such as peptide hormones or neurotransmitters, interact with transmembrane proteins called G-coupled receptors (GPCR). 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 AC when bound to the stimulatory form of G-protein, G_s, and inhibit the enzyme when bound to the inhibitory form of G-protein, G_i. Activation of G_s-coupled receptors results in increased production of cAMP, which in turn, activates cyclic AMP-dependent protein kinase or PKA. This enzyme phosphorylates a variety of proteins which eventually leads to regulation of transcription factor activity and effects on gene expression. Targets for PKA include other enzymes, ion channels and transcriptional regulators.¹ In addition, cAMP plays a direct role in transcription by binding and augmenting the activity of the transcription factor, cAMP-responsive element-binding protein or CREB, a protein that plays a role in modulating the expression of cAMP-inducible genes.²

Depending on the cell type, this signal transduction pathway may be responsible for neural and immune responses, metabolism, mitogenesis, oocyte maturation, olfaction in fruit flies, fertilization and seed formation in plants, as well as many other critical functions.¹⁻⁷ Therefore, monitoring the levels of cAMP can help in

understanding the mechanisms of action of any of the upstream components of the pathway and can be used as a screen for agents that activate or inhibit the signaling pathway.

This application note describes the use of the SPECTRAmax[®] GEMINI XS fluorescence microplate reader from Molecular Devices to study changes in levels of intracellular cAMP induced by forskolin and isoproterenol in the human embryonic kidney cell line, HEK 293. These cells represent a model system used to examine the performance of a new competitive immunoassay, the CatchPoint[™] cyclic AMP fluorescent assay kit. Two 384-well microplates are supplied with the kit, which are coated with goat anti-rabbit IgG antibody (Figure 1, step 1). Sample containing cAMP, cAMP calibrator (standard) or negative control is incubated with primary antibody and cAMP-horseradish peroxidase (cAMP-HRP) conjugate to allow for all specific binding interactions to occur (Figure 1, step 2). Cyclic AMP in the sample or standard competes with cAMP-HRP conjugate for binding to rabbit anti-cAMP antibody.

As the amount of cAMP in the sample increases, the amount of cAMP-HRP conjugate bound to the bottom of the microplate well decreases. The unbound conjugate is removed from the well using a single washing step (Figure 1, step 2). The last step in the procedure is incubation of the well contents with Stoplight Red[™] substrate. The more cAMP that is bound to the primary antibody, the lower the amount of cAMP-HRP conjugate that remains in the well and thus, the lower the Stoplight Red fluorescent signal that is detected by the SPECTRAmax GEMINI XS spectrofluorometer (Figure 1, step 3). This generates a dose response curve as seen for the cAMP calibrator in Figure 3.

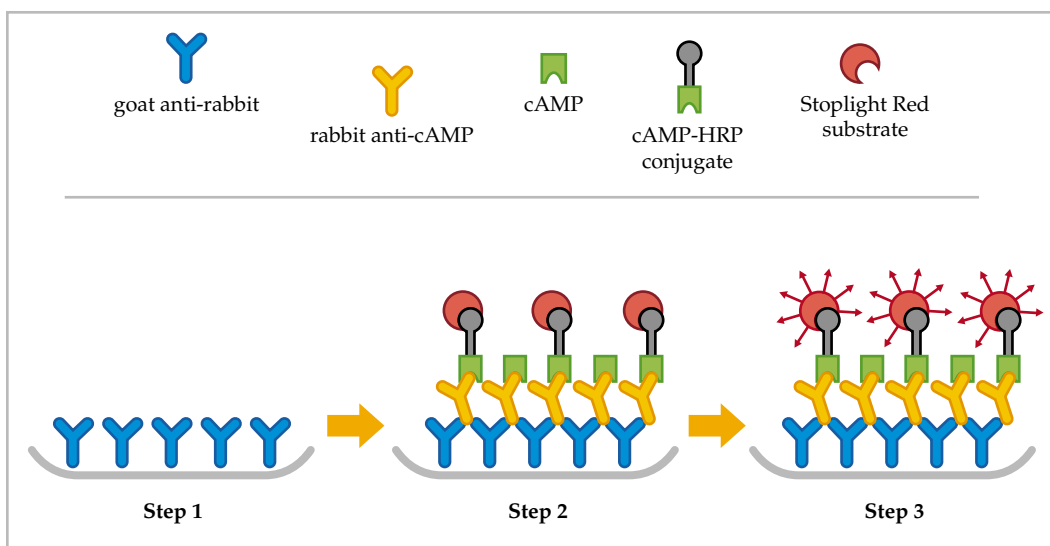


Figure 1: Principle of CatchPoint cyclic AMP fluorescent assay. The kit supplies two 384-well microplates with wells that are coated with goat anti-rabbit IgG secondary antibody (step 1). Cell lysate containing cAMP is produced and incubated with rabbit anti-cAMP antibody and cAMP-HRP conjugate in the microplate wells to allow for all binding interactions to occur in liquid phase (step 2). After one washing step, the contents of the well are incubated with the HRP substrate, Stoplight Red, which, if oxidized by hydrogen peroxide in the presence of HRP, yields a red fluorescent emission when excited at the proper wavelength (step 3).

MATERIALS

- 1 Cells:** HEK 293 cells (human embryonic kidney cells that stably overexpress the Adenovirus E1A protein).
- 2 Reagents:** CatchPoint cyclic AMP fluorescent assay kit, including lyophilized cAMP calibrator, rabbit-anti-cAMP antibody, HRP-cAMP conjugate and Stoplight Red™ substrate (Molecular Devices, cat #R8044, Tel: 1-800-635-5577). DMSO, low water content (Sigma, cat #D2650, Tel: 1-800-325-3010), sodium bicarbonate (Sigma, cat #S5761), cAMP agonists including forskolin (Sigma, cat #F6886) and isoproterenol (Sigma, cat #I5627), phosphodiesterase inhibitor, 3-isobutyl-1-methylxanthine or IBMX (Sigma, cat #I7018) and 30% hydrogen peroxide (H₂O₂) solution (Sigma, cat #H0904).
- 3 Microplates:** coated 384-well, solid black assay microplates (supplied with the kit), 384-well, clear, nonsterile polystyrene microplates (Greiner cat #78101 supplied by USA Scientific, Tel: 1-800-522-8477).
- 4 Medium and buffers:** Dulbecco's modified Eagles medium, DMEM (Irvine Scientific, cat #9031, Tel: 1-800-577-6097), 0.526 mM EDTA in PBS (Irvine Scientific, cat #9314), phosphate buffered saline, PBS (Invitrogen™ Life Technologies, cat #10010-023), Krebs-Ringer bicarbonate buffer (KRBG buffer) (Sigma, cat #K4002). Cell Lysis Buffer pH 7.3, cAMP Assay Buffer pH 5.8 and 10X Wash Solution are all supplied with the kit.
- 5 Fetal bovine serum:** (FBS) heat inactivated (Irvine Scientific cat #3003).

Reagent preparation

- 1 Cell growth medium:** DMEM with 10% FBS.
- 2 KRGB buffer:** Sigma provides detailed instructions for preparing this buffer in the product information sheet.
- 3 800 mM IBMX:** dissolve 100 mg of IBMX in 563 µL of DMSO. Store at -20 °C. IBMX blocks the breakdown of cAMP by cAMP-phosphodiesterases.
- 4 Pre-stimulation buffer:** add 9.4 µL of 800 mM IBMX to 10 mL of KRGB buffer. Vortex vigorously to ensure that IBMX is completely dissolved. This buffer should be prepared fresh on the day of the experiment.
- 5 10 mM isoproterenol:** dissolve 24.8 mg in 10 mL of distilled water. Store at -20 °C. Dilutions are prepared using PBS.
- 6 10 mM forskolin:** dissolve 10 mg in 2.4 mL of DMSO. Store dessicated at 20 °C. Dilutions are prepared using PBS.
- 7 cAMP calibrator:** reconstitute one vial of lyophilized calibrator in 1.25 mL of PBS. Further dilutions are made with cAMP Assay Buffer as shown in Table 2. Mix well to dissolve properly. Store on ice or at 4 °C for up to two weeks.
- 8 Reconstituted rabbit anti-cAMP antibody and HRP-cAMP conjugate:** reconstitute one vial of lyophilized antibody and conjugate in 10 mL of cAMP Assay Buffer, mix well to dissolve contents, store on ice or at 4 °C for up to two weeks.
- 9 3% H₂O₂:** dilute 30% H₂O₂ solution ten-fold with deionized water.

- 10 1X Wash Solution:** dilute 10X Wash Solution ten-fold with deionized water.
- 11 Stoplight Red solution:** prepare Stoplight Red solution by adding 25 μL of 3% H_2O_2 to 22 mL of Substrate Buffer. Mix well and add 220 μL 100X Stoplight Red substrate to make Stoplight Red solution. It is critical that this solution be prepared just prior to plate addition.

METHODS

Cell preparation

HEK 293 cells were grown in T-75 flasks to approximately 80% confluence in DMEM supplemented with 10% fetal bovine serum at 37 °C in 5% CO_2 .

- Step 1** Cells from one T-75 flask were rinsed once with PBS and then treated at 37 °C for 5 to 10 minutes with 3 mL of 0.526 mM EDTA in PBS.
- Step 2** The detached cells were triturated in order to form a single cell suspension and then transferred to a tube that contained 7 mL of medium containing 10% FBS. An aliquot was removed for counting of cells using a hemacytometer, and the total cell number available for the experiment was determined.
- Step 3** Cells were spun in a table top centrifuge at 1100 rpm for 5 minutes and washed once in KRBG buffer. Then the cells were resuspended in Pre-stimulation Buffer at a concentration of 1×10^6 cells/mL.
- Step 4** A set of wells of a 384-well clear polystyrene microplate was set aside for the cAMP calibrator standard curve. Seven and one half microliters of pre-stimulation buffer were added to these wells. In the other wells, cells were seeded in a volume of 7.5 μL /well (7.5×10^3 cells/well).
- Step 5** The 384-well microplate was incubated for 10 minutes at room temperature.

Assay procedure

- Step 1** Dilutions of cAMP calibrator and agonists were prepared according to Tables 1 and 2. In order to stimulate cells to produce cAMP, 15 μL doses of agonist (forskolin or isoproterenol) or PBS (negative control) were added to the wells of the microplate containing 7.5 μL cells. Cyclic AMP calibrator dilutions were added to those wells containing pre-stimulation buffer alone. Each sample was performed in replicates of eight. Samples were gently mixed and then incubated at 37 °C for 15 minutes at 5% CO_2 .

Agonist	Stock solution (pmol/mL)	Concentration (pmol/mL)¹
Forskolin or isoproterenol	1,500,000	1,000,000
	500,000	333,333
	50,000	33,333
	16,667	11,111
	5,555	3,704
	1,852	1,234
	617	411
	206	137
	20.5	13.7
	2.05	1.37
0.205	0.137	
Negative control (PBS)	1X	

Table 1: Doses of agonists used for cell-based experiments. ¹ Concentration of agonist in a 22.5 μ L volume that contains cells and agonist only.

cAMP calibrator	Stock solution (pmol/mL)	Final concentration (pmol/mL)¹
	60,000	3333
	600	33
	200	11
	66.6	3.7
	22.2	1.2
	7.3	0.41
	2.4	0.14
	0.85	0.047
	0.29	0.016
	0.091	0.005
Negative control (PBS)	1X	

Table 2: Doses of cAMP calibrator used to create the standard dose response curve. ¹ Final concentration in a 60 μ L assay volume.

- Step 2** In order to lyse cells, 7.5 μ L of Cell Lysis Buffer were added to all wells, and the microplate was agitated for 10 minutes at room temperature using a plate shaker.
- Step 3** Using a multichannel pipettor, a 20 μ L volume was removed from each well and transferred to separate wells of the 384-well solid black assay microplate. The registration of the assay plate wells was kept the same as those of the polystyrene plate.
- Step 4** Twenty microliters of reconstituted rabbit anti-cAMP antibody were added to all of the wells. The microplate was gently agitated on a plate shaker for 5 minutes to ensure proper mixing.
- Step 5** Twenty microliters of reconstituted HRP-cAMP conjugate were added to all wells of the microplate and gently mixed. Then it was incubated for 2 hours at room temperature.
- Step 6** When incubation was complete, the assay microplate was washed four times with 80 μ L/well of 1X Wash Buffer.
- Step 7** Stoplight Red solution was prepared. Fifty microliters of Stoplight Red solution were added to each well of the microplate as quickly as possible. The microplate was covered and protected from light.
- Step 8** Measurements were taken at 10 and 60 minutes by reading the fluorescence intensity at the following settings:

Mode	Fluorescence
Excitation wavelength	530 nm
Emission wavelength	590 nm
Emission cutoff filter	570 nm
Readings	6
PMT	Auto

Table 3: SPECTRAmax GEMINI XS spectrofluorometer settings for the CatchPoint cyclic AMP assay.

RESULTS

Optimization of excitation and emission wavelengths

Excitation and emission spectral scans were performed on a sample of Stoplight Red substrate diluted in PBS and compared to the fluorescent signal obtained using PBS alone. This procedure was used to determine the optimal excitation and emission wavelength and cutoff filters for the cyclic AMP fluorescent assay (Figure 2).

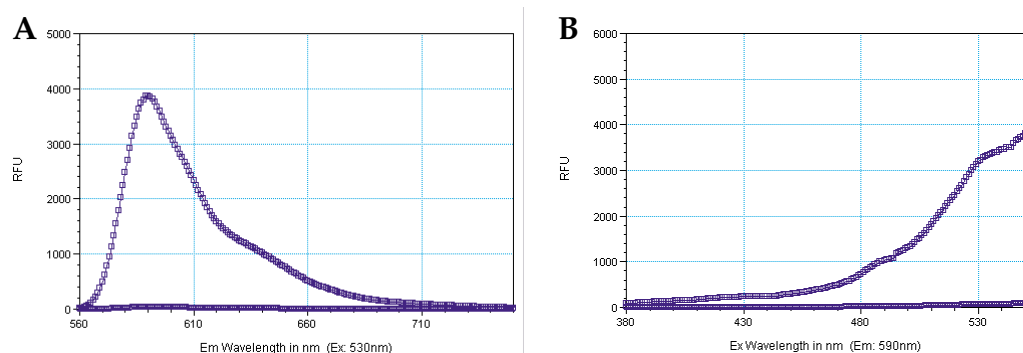


Figure 2: Excitation and emission spectral scans of Stoplight Red. Scans were performed with PMT set to “auto”, calibrate “on” and 20 reads per well. The plot of square symbols represents a solution of Stoplight Red, and the plot of circles represents PBS. (A) Excitation wavelength was fixed at 530 nm and spectral scanning for emission wavelengths was performed. The observed peak emission was 590 nm. (B) Fixing the emission wavelength to the peak of 590 nm, spectral scanning was performed for excitation wavelengths. There was a hump just before a large background peak that occurred at approximately 530 nm. Emission cutoff filters were tested and 570 nm cutoff filter was optimal and produced a signal-to-background ratio of 400.

Assay performance

Cyclic AMP calibrator curve

Analysis of the data obtained from samples containing cAMP calibrator resulted in the plots shown in Figure 3. The data was fit to a 4-parameter curve. In this case, the C value (which is 2.4 pmol/mL and 3.7 pmol/mL in Figures 3A and 3B, respectively) was the midpoint, and hence the EC_{50} value, of the curve. Of note, the EC_{50} values read at 10 minutes and 60 minutes were similar, which demonstrated the relative stability of the fluorescence signal over time. Moreover, these values were in agreement with that previously obtained with the kit using another fluorescence microplate reader, the Analyst AD (3.4 ± 0.6 pmol/mL, see product insert). When this experiment was performed on a different day, the EC_{50} value obtained was 3.4 pmol/mL, indicating again that the assay is of high quality with respect to reproducibility. The signal to background ratio (no calibrator) was 240 and the limit of detection (defined as three standard deviations difference from the zero cAMP calibrator control) was 0.1 pmol/mL cAMP. The optimal performance range of the assay falls between the maximum and minimum detectable signal. This was determined to be between 0.14–33 pmol/mL.

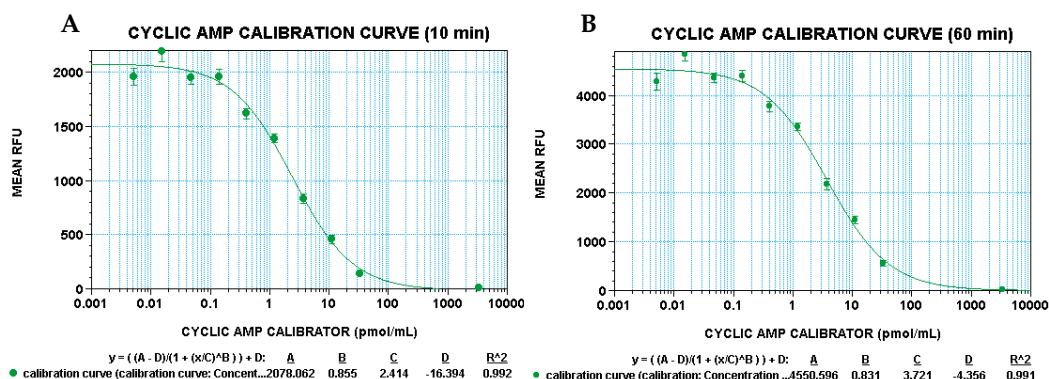


Figure 3: Dose response curves for cAMP calibrator. Dose responses are shown for (A) 10 and (B) 60 minutes after addition of Stoplight Red substrate. Each point on the dose response curve represents the average of eight replicate samples. The error bars denote the standard deviation from the mean. The R^2 value is 0.99 for a 4-parameter curve fit. In these experiment, the resulting EC_{50} value for cAMP calibrator is approximately 2.4 and 3.7 pmol/mL for 10 and 60 minutes incubation, respectively. RFU denotes relative fluorescence units.

Determination of Z' factor

Experiments were performed to determine the Z' factor value. A Z' factor value between 0.5 and 1.0 indicates a high quality, robust assay appropriate for screening purposes.⁸ The Z' factor was calculated using the formula:

$$Z' = 1 - \frac{(3\sigma_{c+} + 3\sigma_{c-})}{|\mu_{c+} - \mu_{c-}|}$$

The term σ_{c+} denotes standard deviation of the positive control and σ_{c-} denotes standard deviation of the negative control. The term $|\mu_{c+} - \mu_{c-}|$ denotes the absolute value of the difference between the mean of the positive control and the mean of the negative control. In this case, the concentration of cAMP calibrator that achieved maximal inhibition (33 pmol/mL) was used as the positive control, and the concentration of cAMP calibrator that achieved the minimal inhibition (0.14 pmol/mL) was used as the negative control. The Z' factor obtained for the CatchPoint cAMP fluorescent assay kit using the SPECTRAmax GEMINI XS spectrofluorometer was 0.84.

Response of HEK 293 cells to forskolin and isoproterenol

HEK 293 cells are a suitable model system to study regulation of cAMP because the endogenous level of adenylate cyclase activity is low.⁹ HEK 293 cells were treated with a range of 0 to 1 mM forskolin or isoproterenol (Figures 4, 5). Forskolin is a natural diterpene that is a promiscuous activator of all adenylate cyclase isoforms, while isoproterenol is a β -adrenoceptor agonist.⁹⁻¹⁰ The EC₅₀ value obtained for forskolin was 13 μ M, which is in accordance with values obtained with other products ([FP]2™ kit from NEN/Perkin Elmer, web site product information).

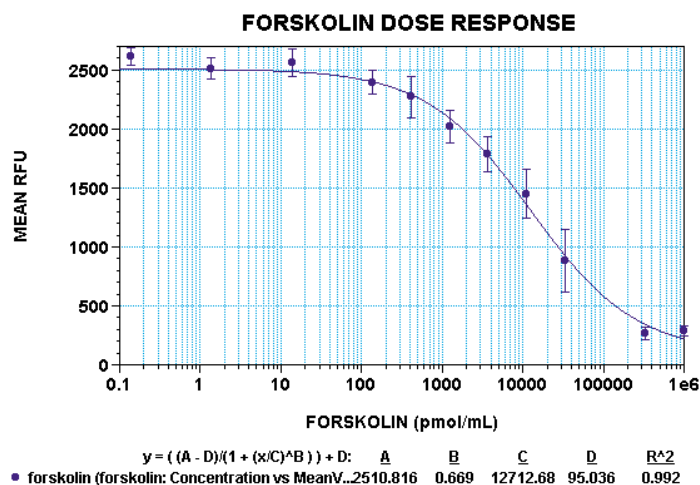


Figure 4: Dose response curves for forskolin treated HEK 293 cells. Each point on the dose response curve represents the average RFU of eight replicate samples for one concentration of the activator. The plate was incubated with Stoplight Red substrate for 60 minutes before reading this data set. The error bars denote the standard deviation from the mean. The R² value for the experiment was 0.99 using a 4-parameter curve fit. Dose response for the adenylate cyclase activator forskolin resulted in an EC₅₀ value of 13 μ M.

The EC₅₀ value obtained for isoproterenol was 82 nM, which is in accordance with values obtained with another cell line (Figure 5A¹⁰). The same EC₅₀ value was obtained after only 10 minutes incubation with Stoplight Red substrate (data

not shown). Again, this lends support for the stability of the assay's fluorescent signal. Using SOFTmax PRO[®] software, the data was interpolated against the standard curve shown in Figure 3 to obtain the graph in Figure 5B.

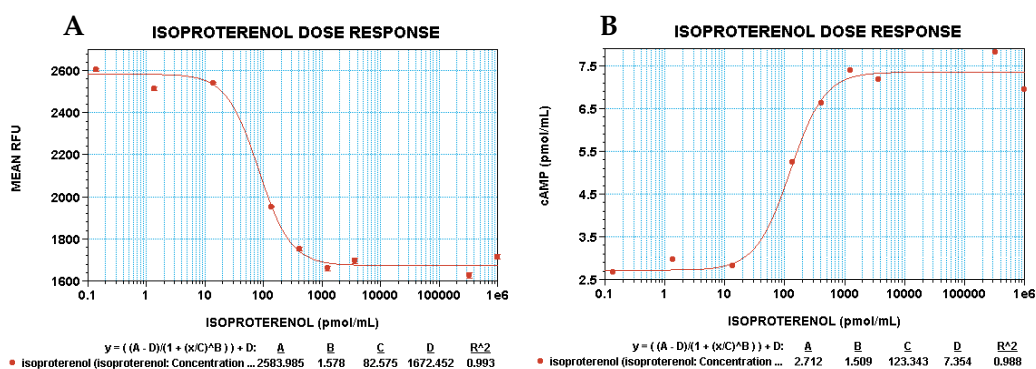


Figure 5: Dose response curves for isoproterenol treated HEK 293 cells. Each point on the dose response curves represents the average RFU of eight replicate samples for one concentration of the agonist. The error bars denote the percent coefficient of variation. The R² value for both experiments was 0.99 for a 4-parameter curve fit. The dose response curve shown in (A) was obtained after 60 minutes incubation of samples with Stoplight Red substrate. The curve shown in (B) was obtained by interpolation of the data from (A) using the standard curve shown in Figure 3. The EC₅₀ values were 82 nM and 123 nM when comparing results to RFU and cAMP concentration, respectively.

CONCLUSIONS

The CatchPoint cyclic AMP fluorescent assay kit and the SPECTRAmax GEMINI XS spectrofluorometer, both from Molecular Devices, provide a complementary set of tools for monitoring the changes in intracellular cAMP for basic research and drug development purposes. Compared to radioactive methods for cAMP detection such as scintillation proximity assays (SPA) and radioimmunoassays (RIA), this fluorescence-based assay is fast, requires no costly waste clean-up and removal and has at least 10-fold higher sensitivity. Specifically, the typical sensitivity for SPA is 2 pmol/mL for the non-acetylated form of cAMP, compared to 0.1 pmol/mL using the CatchPoint assay kit. In addition, the CatchPoint assay kit is more sensitive than fluorescence polarization methods of measuring cAMP (1.25 pmol/mL) by approximately 10-fold.

In terms of speed of assay completion and sensitivity, one luminescence-based kit is four times less sensitive than CatchPoint cyclic AMP fluorescent assay kit and also requires special instrumentation for signal detection. Finally, the short incubation time required to reach a stable signal (10 minutes to 24 hours) makes CatchPoint assay kit ideal for high throughput screening (Figure 3 and isoproterenol data). Notably, the kit provides a high quality screening assay as demonstrated by the Z' factor and reproducible results obtained for cAMP calibrator EC₅₀ values upon repeated testing. In conclusion, the CatchPoint cyclic AMP fluorescent assay kit offers a simple, convenient procedure and one of the most sensitive reagents for determination of intracellular cAMP currently in the market.

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