Molecular Devices

Product Insert

CatchPoint™ Cyclic-AMP Fluorescent Assay Kit

Product # R8044
Quantity: 768, 60 µL reactions

Introduction

About the cAMP Assay

The CatchPoint™ cyclic-AMP Fluorescent Assay Kit measures levels of 3', 5'-cyclic adenosine monophosphate (cAMP) or adenylate cyclase activity via a competitive immunoassay for cAMP. The assay requires only a single washing step, and readings can be taken in as little as 10 minutes or as long as 24 hours following substrate addition, since no termination step is needed. The cAMP Fluorescent Assay Kit is suitable for use in cell-based assays.

Principle of the Assay

The cAMP in the sample or standard competes with horseradish peroxidase (HRP)-labeled cAMP conjugate for binding sites on the anti-cAMP antibodies (Fig. 1). In the absence of cAMP, most of the HRP-cAMP conjugate is bound to the antibody. Increasing concentrations of cAMP competitively decrease the amount of bound conjugate, thus decreasing measured HRP activity.

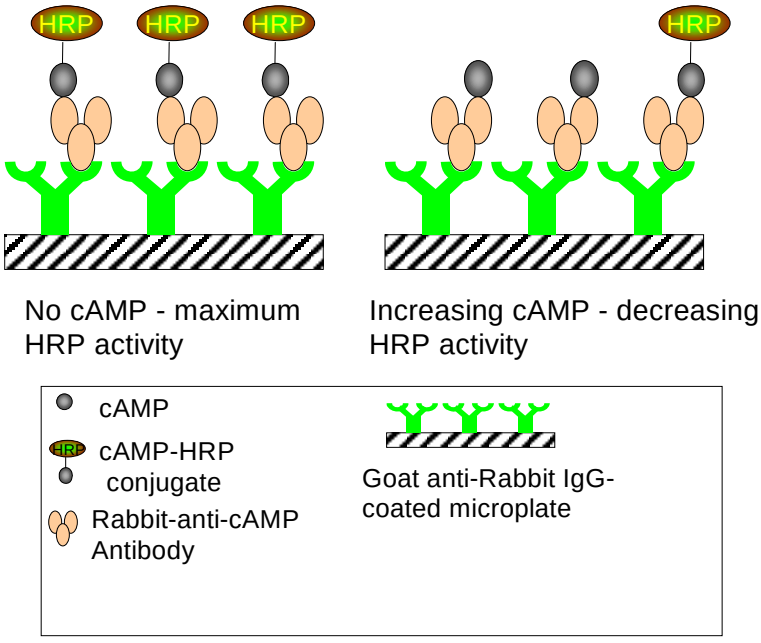

Figure 1. Principle of the CatchPoint cAMP assay system

Applications

The kit is designed for use in applications where cAMP is generated in biochemical assay systems, such as assays of adenylate cyclase activity. In addition, this kit can be used for cell-based assays to measure intracellular cAMP levels following treatment with agonists or antagonists to G-protein coupled receptor systems.

Materials and Equipment

Kit Components

The following table lists the kit components.

Table 1: The CatchPoint cAMP Fluorescent Kit (P/N R8044) contents

Reagent	Quantity	Description
384-well black plates (clear bottom)	2 plates	384-well plates coated with Goat anti-Rabbit IgG. Plates are pre-blocked and ready to use.
cAMP Assay Buffer	100 mL	cAMP Assay Buffer, pH 5.8
Rabbit anti-cAMP Antibody	2 vials	Antibody, lyophilized
cAMP Calibrator	2 vials	30 μM cAMP Calibrator, lyophilized
HRP-cAMP Conjugate	2 vials	HRP-cAMP Conjugate, lyophilized
10X Wash Concentrate	100 mL	10X Wash Concentrate, pH 7.4
Cell Lysis Buffer	25 mL	Cell Lysis Buffer, pH 7.3
Stoplight Red ™ Substrate	450 μL	One vial containing 100X Substrate
Substrate Buffer	100 mL	Substrate Buffer, pH 6.0

When the working concentrations of the above reagents are used as suggested, each kit provides sufficient reagents for two 384-well microplates with 60 μL total assay volume.

Storage and Handling

All kit components are to be stored at 4°C.

IMPORTANT: Allow all the reagents to warm to room temperature prior to use.

Note: Small volumes of product will occasionally collect in the cap of the product vial during shipment. Gently tap the vial on a hard surface or briefly centrifuge the vial to collect any liquid trapped in the vial cap.

When stored properly, the kit components are stable for six months from the date of receipt.

The reconstituted working solutions of Rabbit anti-cAMP, cAMP Calibrator, and HRP-cAMP Conjugate are stable for 3 weeks at 4°C. To insure optimal performance of the reagents do not store below 4°C once reconstituted.

If the entire plate will not be used, protect any unused wells with a plate sealer and store at 4°C in the original foil wrapper protected from light.

IMPORTANT: The reconstituted substrate solution should be protected from light. To insure optimal performance we recommend preparing a fresh stock solution and making additions within 60 min, protected from light. Following substrate addition, readings can be taken at 10 minutes or as long as 24 hours.

Materials Required but not Provided

The following tables list the materials required but not supplied.

Table 2: Reagents and supplies

Reagent Item	Source
3% wt / vol hydrogen peroxide (H_2O_2)	Major laboratory suppliers (MLS)
1.5 mL polypropylene capped centrifuge tubes	MLS
10 mL capped centrifuge tubes	MLS
Pipettors – adjustable volume	MLS
Multi-channel troughs	MLS

Table 3: Compatible Instruments available from Molecular Devices (MDC)

Equipment Item	Source
Analyst System One of the following: - Analyst™ GT - Analyst™ HT	 MDC P/N 0200-6003 or 0200-6004 MDC P/N 0200-6043 or 0200-6044
Gemini XPS	MDC P/N Gemini XPS Spectrophotometer
Gemini EM	MDC P/N Gemini EM Spectrofluorometer
SpectraMax® M2	MDC P/N M2
SpectraMax® M5	MDC P/N M5
FlexStation™	MDC P/N FlexStationII Scanning Fluorometer
Embla 96/384™ plate washer	MDC P/N 0200-3948
AquaMax DW4 with 96-well	MDC P/N AquaMax DW4 Liquid Handling System

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CatchPoint cAMP Fluorescent Assay Kit Experimental Protocol

Preparing the Reagents

The following protocol provides sufficient reagents to run one 384-well plate, including calibrators, controls, and samples.

Table 4: Preparation of reagents

Step	Action
1	Reconstitute one vial of 30 μM cAMP Calibrator: Add 5 mL of cAMP Assay Buffer (pH 5.8) to the vial. Mix well to ensure dissolution of all contents. Store on ice or at 4°C.
2	Reconstitute one vial of HRP-cAMP Conjugate: Add 10 mL of cAMP Assay Buffer to one of the vials. Mix well to ensure dissolution of all contents. Store on ice or at 4°C.
3	Reconstitute one vial of Rabbit anti-cAMP Antibody: Add 10 mL of cAMP Assay Buffer to one of the vials. Mix well to ensure dissolution of all contents. Store on ice or at 4°C.
4	Dilute 10X Wash Concentrate 10-fold in deionized water, i.e. for one assay plate dilute 50 mL of 10X Wash Concentrate in 450 mL of deionized water.*
5	Prepare calibrators: The 30 μM cAMP calibrator is diluted in cAMP Assay Buffer to prepare stock calibrators of 10000, 100, 33, 11, 3.7, 1.2, and 0.4 nM cAMP. These give 3300, 33, 11, 3.7, 1.2, 0.41, and 0.14 nM (200, 2, 0.67,0.22, 0.074, 0.025, and 0.008 pmol/well) final values in the assay. 1. Add 100 μL of the 30 μM cAMP calibrator to 200 μL of cAMP Assay Buffer. This is the 10,000 nM stock calibrator. 2. Add 10 μL of the 10,000 nM stock calibrator to 990 μL of cAMP Assay Buffer. This is the 100 nM stock calibrator. 3. In cAMP Assay Buffer, serially dilute the 100 nM stock calibrator in a 3-fold fashion 5 times to produce the 33, 11, 3.7, 1.2, and 0.4 nM cAMP stock calibrators. 20 μL of each calibrator is required per replicate. Prepare 100 μL of each (ample for 3 replicates). As a 0 nM (zero dose) calibrator, use the cAMP Assay Buffer itself.

* On dilution of the 10X Wash Buffer concentrate, the reagent contains 0.02 M Tris, 150 mM NaCl, 0.05% Tween™ 20, and 0.05% Proclin™ 200 (pH 7.4).

Preparing the Plate

For ease of analysis and calculation, the assay plate contains the calibrators, controls, and samples as outlined below. Use the suggested template (Table 5) and run the samples in triplicate.

Note: The order of addition of each component to the wells and the incubation time are important. Mix samples and all reagents gently but thoroughly before use.
All assay wells will be prepared by dispensing the assay components in the following order:

1) Calibrator, control, or sample
2) Rabbit anti-cAMP Antibody
3) HRP-cAMP Conjugate

Table 5: To prepare the assay plate:

Step	Action
1	Designing your assay plate (plate map): We suggest preparing the plate according to the template below. If you are using SoftMax® Pro to analyze your results you may set up a template before or after reading the plate. If you set up the plate before, you can print out a template to help in pipetting your plate.

	Column 1	2	3	4	5	6	7	8	9
Row A B C	control	3300 nM (200 pmol)	33 nM (2 pmol)	11 nM (0.66 pmol)	3.7 nM (0.22 pmol)	1.2 nM (0.074 pmol)	0.41 nM (0.025 pmol)	0.14 nM (0.008 pmol)	0 nM (buffer only)
	Rows D – P and all of columns 10-24: Samples for analysis								

2	Add 20 µL of the appropriate concentration of Calibrator working solution (see step 5 from "Preparing the Reagents on page 4) to columns 2 through 9 (rows A – C). Column 1 is designated here for optional controls. One may wish to use a zero dose calibrator with no Rabbit anti-cAMP Antibody, for example, to evaluate background.
3	Place 20 µL of samples to be analyzed in appropriate wells (e.g. Columns 1 – 9, rows D – P, and columns 10 – 24). See Appendix A for cell stimulation and lysis protocols.
4	Add 20 µL of reconstituted Rabbit anti-cAMP Antibody to all wells except any reserved for no antibody controls. If these are to be performed, add 20 µL assay buffer in place of antibody.

Table 5: To prepare the assay plate:

5	Place plate on shaker for 5 minutes or gently agitate by hand to ensure mixing.
6	Add 20 µL reconstituted HRP-cAMP Conjugate to every well.
7	Mix well and allow to incubate 2 hours at room temperature.
8	Aspirate plate contents and wash 4 times with wash buffer, 80 µL/well with each wash.
9	Prepare Stoplight Red Substrate: Dilute 220 µL of 100X stock Stoplight Red Substrate into 22 mL of Substrate Buffer, then add 25 µL of 3% H_20_2 (880 mM) to bring the final concentration to 0.0034% (1 mM) H_20_2. IMPORTANT: The reconstituted substrate solution is sensitive to light. To insure optimal performance we recommend preparing a fresh stock solution and adding directly to assay plate within 60 min, protected from light.
10	Add 50 µL Stoplight Red substrate to every well, minimizing the time between starting and finishing. Cover the plate and leave at room temperature for at least 10 minutes, shielded from light.
11	Read the fluorescence intensity of the plate on an appropriate instrument (see below for settings). Plates may generally be read anytime between 10 minutes and 24 hours.

Analyst Parameter Settings

Reading the Plate in Analyst HTS Assay Detection System

The following instrument settings are recommended when using 384-well plates with a 50 µL volume in Analyst HTS systems:

Table 6: Fluorescence intensity settings for Analyst HT and GT Detection System:

Parameter	Setting
Mode	Fluorescence Intensity
Excitation filters	530-25 nm
Emission filters	590-20 nm
Dichroic mirror	50 / 50 beamsplitter
Z-height	3 mm
Attenuator	Medium
Integration time	50,000 µsec
Lamp	Continuous
Readings per well	One
PMT setup	Smart Read+ (sensitivity = 2)
Units	Counts / sec

Reading the Plate in the Gemini XS, Gemini XPS, Gemini EM, SpectraMax® M2 and SpectraMax® M5 Detection System

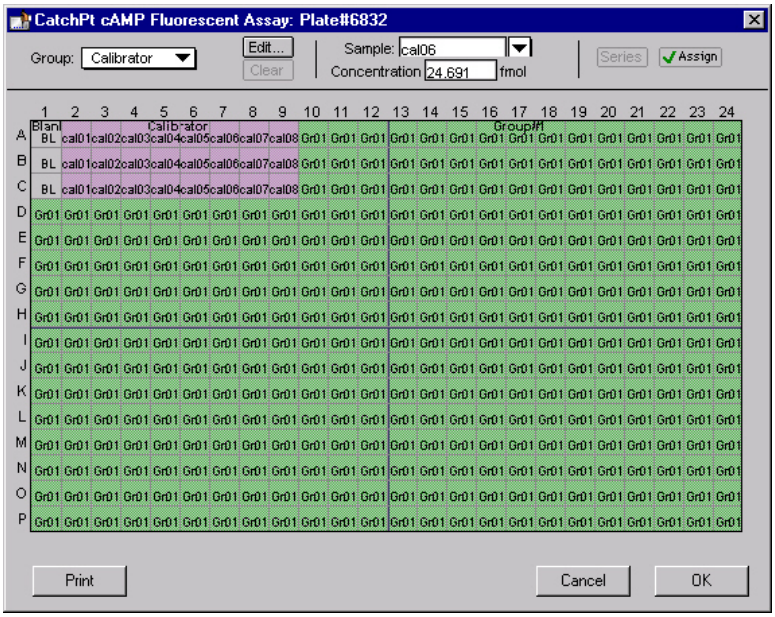
The following instrument settings are recommended. If reading from the top, be sure to insert a purple plate adaptor in the plate carriage. plates with a
Gemini XS system:

Table 7: Fluorescence intensity settings for GEMINI XS, Gemini XPS, Gemini EM, SpectraMax® M2 and SpectraMax® M5 Detection System

Parameter	Setting
Read Mode	Endpoint
Read Type	Fluorescence (RFUs)
Excitation	530 nm
Emission	590 nm
Cutoff	570 nm
Sensitivity	6
PMT	Auto
AutoCalibrate	On

Data Analysis **Analyzing the Calibration Data**

The average intensity values can be plotted vs. calibrator concentration to create a calibration or standard curve. Unknown samples can be interpolated using this curve to calculate cAMP doses in sample. We recommend using SoftMax® Pro for accurate curve fitting and interpolation of data from MDC instruments. Figure 2 shows a SoftMax® Pro template and calibration curve for the cAMP Assay when read on the Analyst™.



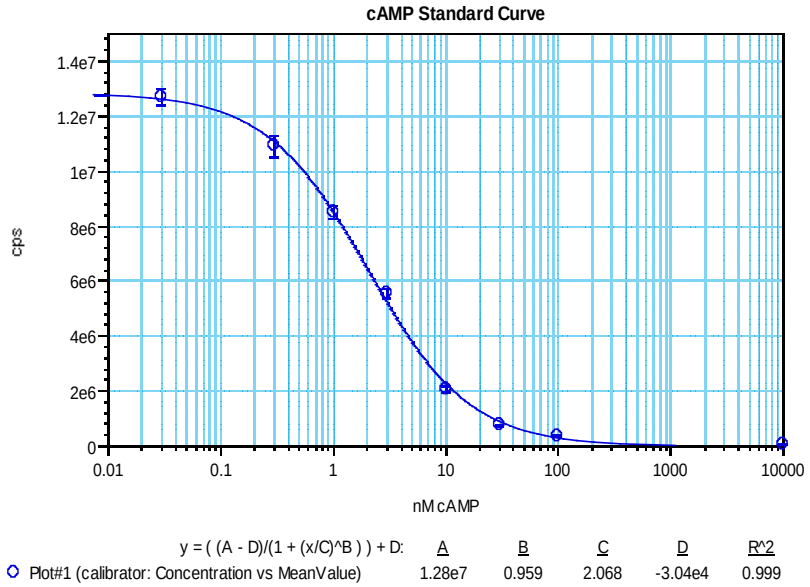


Figure 2. Calibration curve for the CatchPoint cyclic AMP Fluorescent Assay Kit, read on the Analyst stystem. Data was taken 10 minutes after addition of Stoplight Red substrate. Fluorescence intensity settings for Analyst HTS Detection System were as stated in Table 6. EC_{50} of cAMP calibration curve was 2.1 nM (126 fmol/well) in sample.

ADDITIONAL INFORMATION

The following typical information is based on data from an Analyst HT. Absolute readings may differ on other instruments, but relative values should be comparable.

Sensitivity (Typical values)

Minimum Detectable Concentration = 0.1 nM (6 fmol/well)
Maximum Measurable Concentration = 80 nM (4800 fmol/well)
EC_{50} = 3.4 $\pm$ 0.6 nM (204 fmol/well)

Intra-assay precision

Intra-assay precision was calculated from measurements of 0 and 3.7 nM calibrator. The results for a single assay are shown below:

Signal intensity / background (for 0 nM calibrator) [§]	**Precision for 3.7 nM calibrator** (expressed as $\%B/B_o$) *
$Signal\ intensity_{Avg}$ / background = 460 Standard deviation (SD) = 24 %CV = 5.4 n = 64	$\%B/B_o$ = 47 Standard deviation (SD) = 3 %CV = 6.3 n = 64

[§] Background taken as the signal intensity for wells in which no cAMP-HRP or Rabbit anti-cAMP Antibody are added

* $\%B/B_o = 100 \times (I_{3.7\ nM} - I_\infty) / (I_o - I_\infty)$

where $I_{3.7\ nM}$ = Signal intensity for 3.7 nM calibrator
I_o = Response at 0 nM calibrator
I_∞ = Response at 3333 nM calibrator

Inter-assay precision (three assays performed on separate days)

Inter-assay precision was calculated from measurements of 3.7 nM calibrator on 3 successive assays. The results are shown below:

Measurement	Data points	$\%B/B_0$	SD	%CV
A	n = 8	53	2	4.7
B	n = 8	54	3	5.2
C	n = 8	58	2	3.7

Precision correlation

64 replicates of each of the standards were prepared and % coefficient of variation was determined for each concentration.

Standard nM	%CV
3300.00	1
33.00	5
11.00	4
3.70	4
1.20	3
0.41	3
0.14	3
0.00	2

Appendix A: Preparing cell lysates for the cAMP Assay

The two protocols below illustrate an application of the CatchPoint cAMP Fluorescent Assay. The protocols have been developed for use with adherent or suspension cells stimulated with forskolin. The procedure may be modified for agonists or antagonists such as the beta-adrenergic agonist isoproterenol or antagonist propranolol.

For best results each cell line should be evaluated to determine the optimal concentration of cells per well, incubation times, and concentration of adenylate cyclase stimulators or inhibitors.

Use this protocol as a guide to help optimize the assay.

Reagents

Reagent	Stock Concentration
cAMP stimulator forskolin, dissolved in dimethyl sulfoxide (DMSO)	30 mM
Phosphodiesterase inhibitor, 3-isobutyl-1-methylxanthine (IBMX) dissolved in DMSO	800 mM

Buffers

Buffer	Source
Krebs-Ringer Bicarbonate Buffer (KRBG) containing 10 mM Glucose (pH 7.4)	Sigma Chemical Company (P/N K4002) **Note**: 15 mM sodium bicarbonate is not included with this product and must be added separately.
Lysis Buffer (pH 7.3)	Molecular Devices Corporation (included in CatchPoint cAMP Fluorescent Assay Kit)
Phosphate Buffered Saline, PBS	Invitrogen™ Life Technologies (P/N 10010-023)

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Assay Protocol

Adherent Cells

The following has been used for the adherent cell line T-47D :

1) Culture cells (100 μL/well) in standard 96-well microtiter plates (tissue culture grade) with cell concentration at 2.5 – 10 x 10^5 cells/mL (25,000 – 100,000 cells/well).

2) Incubate plated cells overnight at 37°C in a humidified atmosphere of 5% CO_2 : 95% air.

3) Gently aspirate off media and slowly add 250 μL of Krebs-Ringer Bicarbonate Buffer with glucose, pH 7.4 (KRBG Buffer); use multi-channel pipettor.

   Note: Be careful not to aspirate the cells.

4) Gently aspirate off the KRBG and add 100 μL Stimulation Buffer

   Stimulation Buffer (containing 0.75 mM IBMX in KRBG Buffer; make fresh on day of experiment).
   10 ml of KRBG Buffer (pH 7.4)
   9.4 μL of 800 mM IBMX

   Note: Once IBMX has been added to the KRBG, mix vigorously to obtain a homogeneous solution.

5) Incubate for 10 min at room temperature.

6) Add 50 μL of 3X forskolin or PBS. Gently mix and incubate at 37°C for 15 minutes.

   forskolin, 3X (20 μM final concentration)
   1500 μL PBS (pH 7.4) containing
   3.0 μL of 30 mM forskolin (60 μM stock)

7) Add 50 μL of Lysis buffer to each well. Agitate cells to facilitate cell lysis: this can be achieved by shaking the plate on a plate shaker for 10 min after adding the lysis reagent.

8) If desired, carry out microscopic evaluation using Trypan blue to check if cells have lysed (1:1). Cell membrane may still be visible after cell lysis.

9) Lysed cells are now ready for use and should be immediately processed in the cAMP Fluorescent Assay Kit. Use 20 μL per well neat or suitably diluted lysate in additional lysis buffer (see Step 3, page 5 of the Product Insert). Be sure to include any necessary controls and calibration curve. To insure accurate determination of cAMP concentrations, prepare the calibration curve with the appropriate ratio of calibrator to lysis buffer, e.g. three parts calibrator to one part lysis buffer, prior to plate addition.

Cells in Suspension

If an adherent cell line is to be assayed in suspension, cells should be treated with 0.02% EDTA (in Dulbecco's PBS without Ca^{+2} or Mg^{+2}) to gently detach cells. The following protocol has been developed for the adherent cell line HEK 293 (an adherent cell line). The assay can be performed in the 384-well plate:

1) Grow cells in T-75 or T-175 flasks to 85-90% confluence.

2) On the day of experiment, aspirate growth media and rinse cells with PBS.

3) Remove PBS and add 1 to 2 ml of 0.02% EDTA solution to detach cells (Sigma, P/N E8008). To facilitate detachment of cells, incubate for 3 to 5 minutes at 37°C.

4) Resuspend cells by adding 10 ml of growth media to cell suspension. Count cells and then centrifuge the cells at 1000 – 1500 x g for 5 min to form a pellet.

5) Wash cell pellet once with KRBG.

6) Resuspend cells in Stimulation Buffer to desired density (i.e., 1 x 10^6 cells/ml will give 5,000 cells/well if 5 µl/well is dispensed in a 384-well plate). Incubate for 10 min at room temperature.

   Stimulation Buffer (containing 0.75 mM IBMX in KRBG Buffer; make fresh on day of experiment).
   10 ml of KRBG Buffer (pH 7.4)
   9.4 µL of 800 mM IBMX

   Note: Once IBMX has been added to the KRBG, mix vigorously to obtain a homogeneous solution

7) Dispense 5 µL volume of cell suspension to wells in the 384-well plate.

8) Add 10 µL of 1.5X forskolin or PBS to the cells in suspension. Gently mix and incubate at 37°C for 15 min.

   forskolin, 1.5X (20 µM final concentration)
   3000 µL PBS (pH 7.4) containing
   3.0 µL of 30 mM forskolin (30 µM stock)

9) Add 5 µl of cell lysis buffer to the cells and incubate for 10 min to terminate the stimulation and to lyse the cells. To facilitate cell lysis, place plate on a plate shaker for 10 min after adding the lysis reagent.

10) Proceed with the cAMP Assay at step 4 on page 5 of the Product Insert. Be sure to include any necessary controls and calibration curve. To insure accurate determination of cAMP concentrations, prepare the calibration curve with the appropriate ratio of calibrator to lysis buffer, e.g. three parts calibrator to one part lysis buffer, prior to plate addition.

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