Molecular Devices

Product Insert

CatchPoint™ Cyclic-AMP Fluorescent Assay Kit (Bulk Kit)

Product # R8089

Quantity: 960, 120 µL reactions

Introduction

About the cAMP Assay

The CatchPoint™ cyclic-AMP Fluorescent Assay Kit measures levels of 3', 5'-cyclic guanosine monophosphate (cAMP) or guanylate 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.

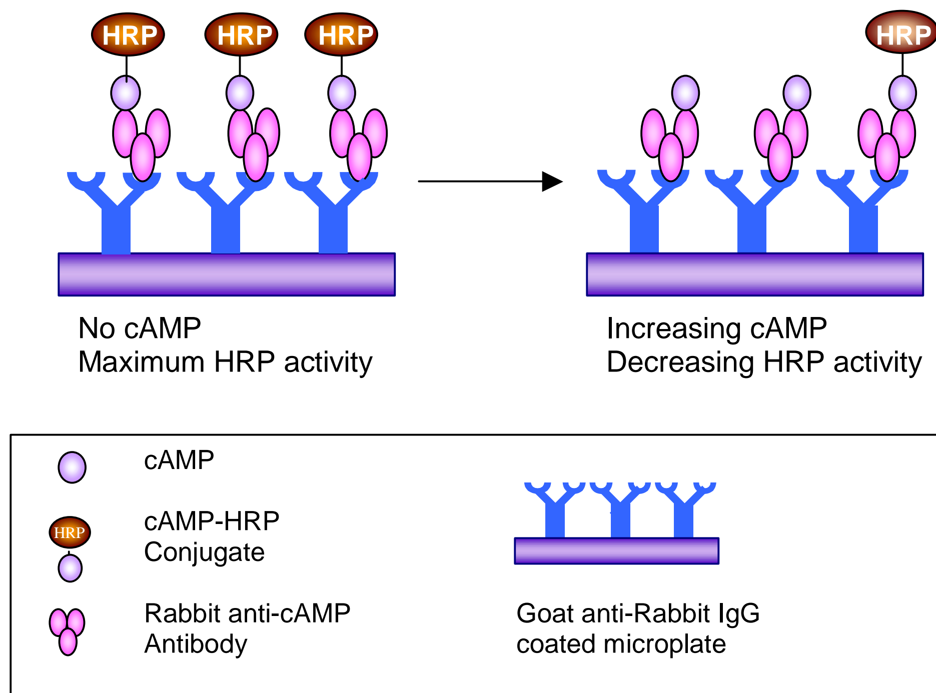

Figure 1. Principle of the CatchPoint cAMP assay system

Molecular Devices

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 Assay Kit (P/N R8089) contents

Reagent	Quantity	Description
96-well black plates (clear bottom)	10 plates	96-well plate coated with Goat anti-Rabbit IgG. Plates are pre-blocked and ready to use.
cAMP Assay Buffer	950 mL	cAMP Assay Buffer, pH 5.8
Rabbit anti-cAMP Antibody	2 vials	Antibody, lyophilized
cAMP Calibrator	2 vials	30 µM cAMP Calibrator, lyophilized. On reconstitution with 5 mL assay buffer each vial contains 30,000 pmol cAMP / mL.
HRP-cAMP Conjugate	2 vials	HRP-cAMP Conjugate, lyophilized
10X Wash Concentrate	950 mL	10X Wash Concentrate, pH 7.4
Cell Lysis Buffer	450 mL	Cell Lysis Buffer, pH 7.3
Stoplight Red ™ Substrate	2 x 900 µL	100X Substrate in DMSO
Substrate Buffer	950 mL	Substrate Buffer, pH 7.5

When the working concentrations of the above reagents are used as suggested, each kit provides sufficient reagents for ten 96-well microplates with 120 µL total assay volume.

Molecular Devices

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 is sensitive to light. To insure optimal performance we recommend preparing a fresh stock solution (keep protected from light) and adding to the assay plate within 60 minutes. 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™ AD	MDC P/N 42-000-0096
- Analyst™ HT	MDC P/N 42-000-0100
- Acquest™	MDC P/N 42-000-0102
- Screen Station™	MDC P/N 42-000-0151
Gemini XS™ System	MDC P/N 0200-3940
FlexStation™	MDC P/N 0200-4000
Embla 96/384™ plate washer	MDC P/N 0200-3948

Molecular Devices

CatchPoint cAMP Fluorescent Assay Kit Experimental Protocol

Preparing the Reagents

The following protocol provides sufficient reagents to run five 96-well plates, each with 16 calibrators, 2 controls, and up to 78 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 (pH 5.8) to one of the vials for a 10X stock. Mix well to ensure dissolution of all contents. Transfer the entire contents of the vial to a 100 mL graduated cylinder by repeated washings with assay buffer. Adjust the final volume to 100 mL with assay buffer and mix thoroughly. The diluted HRP-cAMP conjugate is ready for use in the assay. Store on ice or at 4°C.
3	Reconstitute one vial of Rabbit anti-cAMP Antibody: Add 10 mL of cAMP Assay Buffer (pH 5.8) to one of the vials for a 10X stock. Mix well to ensure dissolution of all contents. Transfer the entire contents of the vial to a 100 mL graduated cylinder by repeated washings with assay buffer. Adjust the final volume to 100 mL with assay buffer and mix thoroughly. The diluted Rabbit anti-cAMP conjugate is ready for use in the assay. Store on ice or at 4°C.
4	Dilute 10X Wash Concentrate 10-fold in deionized water, i.e. for one assay plate dilute 500 mL of 10X Wash Concentrate in 4500 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) final concentrations in the assay. 1. Add 500 µL of the 30 µM cAMP calibrator to 1000 µL of cAMP Assay Buffer. This is the 10,000 nM stock calibrator. 2. Add 20 µL of the 10,000 nM stock calibrator to 1980 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 five times to produce the 33, 11, 3.7, 1.2, and 0.4 nM cAMP stock calibrators. 40 µL of each calibrator are required per replicate. Prepare 1000 µL of each (ample for 10 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 duplicate.

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:

<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>1</td><td>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 preparing your plate.

<table>
<tr><td></td><td>Column</td><td></td><td></td></tr>
<tr><td>Row</td><td>1</td><td>2</td><td>3</td></tr>
<tr><td>A</td><td>3300 nM
(200 pmol)</td><td>3300 nM
(200 pmol)</td><td>Control 1</td></tr>
<tr><td>B</td><td>33 nM
(2 pmol)</td><td>33 nM
(2 pmol)</td><td>Control 2</td></tr>
<tr><td>C</td><td>11 nM
(0.66 pmol)</td><td>11 nM
(0.66 pmol)</td><td rowspan="6">Column 3 (C – H) and all of columns 4 - 12: Samples for analysis</td></tr>
<tr><td>D</td><td>3.7 nM
(0.22 pmol)</td><td>3.7 nM
(0.22 pmol)</td></tr>
<tr><td>E</td><td>1.2 nM
(0.074 nM)</td><td>1.2 nM
(0.074 nM)</td></tr>
<tr><td>F</td><td>0.41 nM
(0.025 pmol)</td><td>0.41 nM
(0.025 pmol)</td></tr>
<tr><td>G</td><td>0.14 nM
(0.008 pmol)</td><td>0.14 nM
(0.008 pmol)</td></tr>
<tr><td>H</td><td>0 nM
(buffer only)</td><td>0 nM
(buffer only)</td></tr>
</table></td></tr>
<tr><td>2</td><td>Add 40 µL of the appropriate concentration of Calibrator working solution (see step 5 from “Preparing the Reagents” on Table 4, page 4) to columns 1 and 2 (rows A – H). Column 3 (A & B) is designated here for optional controls. For example, one may wish to use a zero dose calibrator with no Rabbit anti-cAMP Antibody to evaluate background.</td></tr>
</table>

Table 5: To prepare the assay plate:

3	Place 40 µL of samples to be analyzed in appropriate wells [e.g. Column 3 (C – H), and columns 4 – 12]. See Appendix A for cell stimulation and lysis protocols.
4	Add 40 µL of reconstituted Rabbit anti-cAMP Antibody to all wells except any reserved for no antibody controls. If these are to be performed, add 40 µL assay buffer in place of antibody.
5	Place plate on shaker for 5 minutes or gently agitate by hand to ensure mixing.
6	Add 40 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 300 µL wash buffer for each wash.
9	Prepare Stoplight Red Substrate: Dilute 600 µL of 100X stock Stoplight Red Substrate into 60 mL of Substrate Buffer, then add 68 µL of 3% H_2O_2 (880 mM) to bring the final concentration to 0.0034% (1 mM) H_2O_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. At all times keep substrate protected from light.
10	Add 100 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. For optimal performance you may want to briefly mix the plate just prior to reading.

Analyst Parameter Settings

Reading the Plate in Analyst HTS Assay Detection System

The following instrument settings are recommended when using 96-well plates with a 100 µL volume in Analyst HTS systems:

Table 6: Fluorescence intensity settings for Analyst HTS Detection System:

Parameter	Setting
Mode	Fluorescence Intensity
Excitation filters	530-25 nm

Molecular Devices

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 Detection System

The following instrument settings are recommended when using 96-well plates with a Gemini XS system:

Table 7: Fluorescence intensity settings for GEMINI XS Detection System

Parameter	Setting
Instrument Settings	Endpoint
Mode	Fluorescence (RFUs)
Excitation	530 nm
Emission	590 nm
Cutoff	570 nm
Sensitivity	6
PMT	Auto
Auto Calibrate	ON

Data Analysis

Analyzing the Calibration Data

The average intensity values (y-axis) can be plotted against calibrator concentration (x-axis) 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 software 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™.

Molecular Devices

CatchPt cAMP Fluorescent Assay: Plate# 1841

Group: Calibrator | Edit... | Clear | Sample: Cal05 | Series | Assign

	1	2	3	4	5	6	7	8	9	10	11	12
	Calibrator		Blank					Group#1				
A	Cal01	Cal01	BL	Gr01	Gr01	Gr01	Gr01	Gr01	Gr01	Gr01	Gr01	Gr01
B	Cal02	Cal02	BL	Gr01	Gr01	Gr01	Gr01	Gr01	Gr01	Gr01	Gr01	Gr01
C	Cal03	Cal03	Gr01	Gr01	Gr01	Gr01	Gr01	Gr01	Gr01	Gr01	Gr01	Gr01
D	Cal04	Cal04	Gr01	Gr01	Gr01	Gr01	Gr01	Gr01	Gr01	Gr01	Gr01	Gr01
E	Cal05	Cal05	Gr01	Gr01	Gr01	Gr01	Gr01	Gr01	Gr01	Gr01	Gr01	Gr01
F	Cal06	Cal06	Gr01	Gr01	Gr01	Gr01	Gr01	Gr01	Gr01	Gr01	Gr01	Gr01
G	Cal07	Cal07	Gr01	Gr01	Gr01	Gr01	Gr01	Gr01	Gr01	Gr01	Gr01	Gr01
H	Cal08	Cal08	Gr01	Gr01	Gr01	Gr01	Gr01	Gr01	Gr01	Gr01	Gr01	Gr01

Print | Cancel | OK

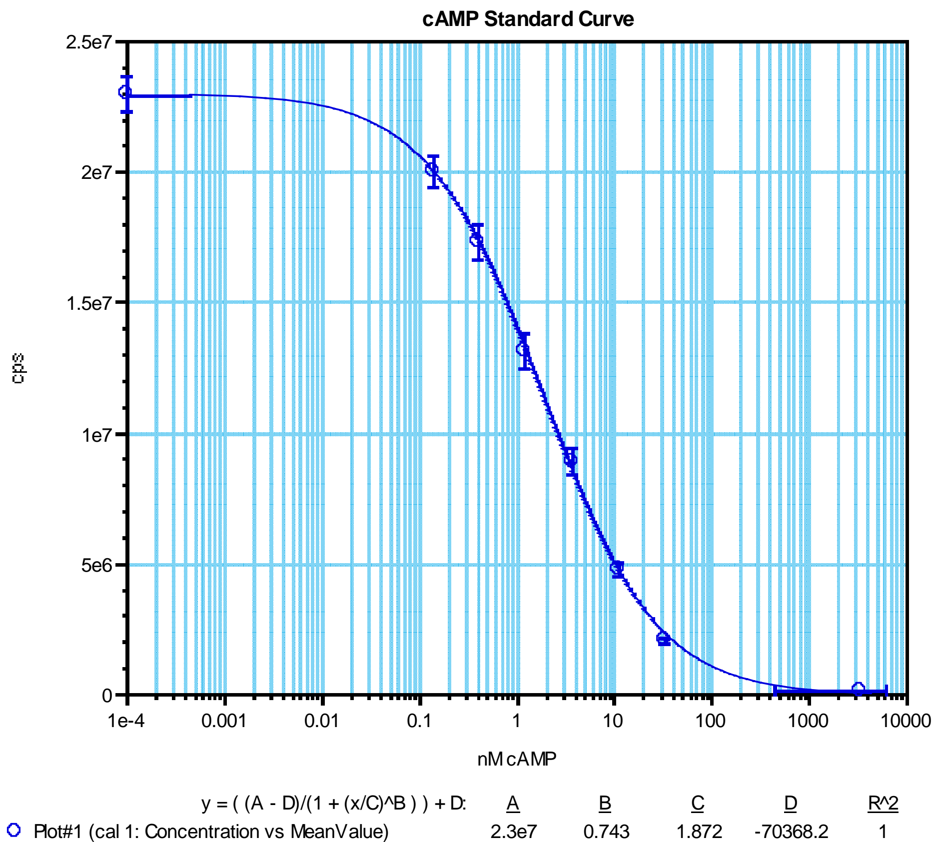

Figure 2. Calibration curve for the CatchPoint cyclic AMP Fluorescent Assay Kit, read on the Analyst system. Data was taken 60 minutes after addition of Stoplight Red substrate. Fluorescence intensity settings for Analyst HTS Detection System were as stated in Table 6. The EC_{50} value of the calibration curve, calculated as coefficient C using SoftMax Pro, was 1.9 nM (228 fmol).

Molecular Devices

ADDITIONAL INFORMATION

Sensitivity (Typical values)

Minimum Detectable Concentration = 0.2 nM (24 fmol / well)
Maximum Measurable Concentration = 120 nM (14 pmol / well)
EC_{50} = 2.8 $\pm$ 0.7 nM (340 fmol)

Intra-assay precision

Intra-assay precision was calculated from measurements of 0 and 1.2 nM calibrator. The results for a single assay are shown below:

Signal intensity / background (for 0 nM calibrator) [§] Signal $intensity_{Avg}$ / background = 224 Standard deviation (SD) = 15 %CV = 7 n = 11	**%B/B_o* precision for 1.2 nM calibrator** %B/B_o (mean) = 40 Standard deviation (SD) = 3 %CV = 7 n = 11

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

$$\%B/B_o = 100 \times (I_{1.2\,nM} - I_{\infty}) / (I_o - I_{\infty})$$

where $I_{1.2\,nM}$ = Signal intensity for 1.2 nM calibrator
I_o = Signal intensity for 0 nM calibrator
I_{∞} = Signal intensity for 3333 nM calibrator

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

Inter-assay precision was calculated from measurements of %B/B_o for 1.2 nM calibrator in 3 successive assays. The results are shown below:

Measurement	Data points	%B/B_0	SD	%CV
A	n = 11	47	3	6
B	n = 11	44	3	7
C	n = 11	43	3	8

Precision correlation

22 replicates of each of the standards were prepared and % coefficient of variation was determined for each concentration using SoftMax Pro.

Sample	Concentration	Mean Value	SD	%CV
cal 01	3300 nM	235233	6858	2.9
cal 02	33 nM	3290964	97455	3.0
cal 03	11 nM	7759904	269832	3.5
cal 04	3.7 nM	14100413	452480	3.2
cal 05	1.2 nM	20677540	887325	4.3
cal 06	0.41 nM	24928064	1250637	5.0
cal 07	0.14 nM	27540365	1658360	6.0
cal 08	0 nM	29579839	1118954	3.8

Molecular Devices

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)

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 40 µL per well neat or suitably diluted lysate in additional lysis buffer (see Step 3, page 6 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 a 96-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 40,000 cells/well if 40 µl/well is dispensed in a 96-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 40 µL volume of cell suspension to wells in the 96-well plate.

8) Add 20 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 20 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 3 on page 6 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.

Molecular Devices

Molecular Devices Corporation
1311 Orleans Drive
Sunnyvale, CA 94089 USA
Email: info@moldev.com
www.moleculardevices.com

Sales Offices

USA 800-635-5577 • **UK** +44-118-944-8000 • **Germany** +49-89-9620-2340 • **Japan** +06-6399-8211
Check our web site for a current listing of our worldwide distributors.

SOFTmax anss SPECTRAmax are registered trademarks and CatchPoint, Criterion and Analyst are trademarks of Molecular Devices Corporation.

R3399B

© 2001 Molecular Devices Corporation. Printed in U.S.A.