NEN® Life Science Products

Adenylyl Cyclase Activation
FlashPlate® Assay*
A Direct cAMP [^{125}I]
Detection Assay

Catalog Numbers
SMP004B, 40-plate kit
SMP004C, 40-plate kit (contains stimulation buffer that does not contain IMBX)

For Laboratory Use Only.

Caution: Research chemicals for research purposes only.

* Adenylyl Cyclase Activation Assay is protected under US Patent 5,739,001 and foreign equivalents.

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PART I: ASSAY KIT INFORMATION

I. PROPRIETARY NAME

Adenylyl Cyclase Activation FlashPlate® Assay

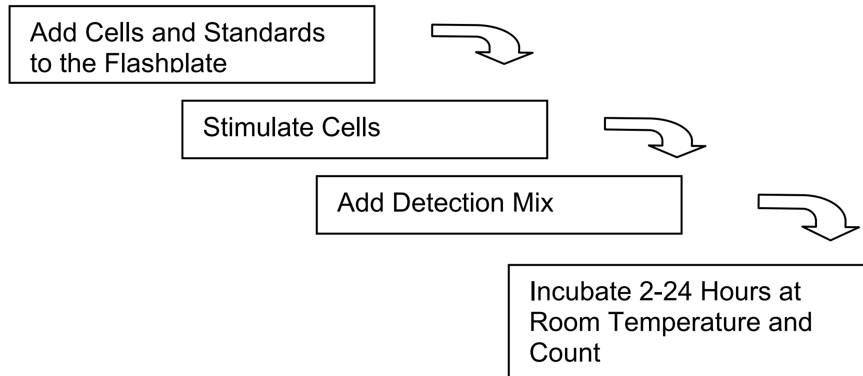
II. INTENDED USE

This product is intended to quantify levels of cAMP (adenosine 3', 5' - cyclic monophosphate) in whole cell preparations after stimulation of adenylyl cyclase. This assay is designed for use as a primary or secondary high throughput screen for G-protein coupled receptors.

III. INTRODUCTION

Adenosine 3', 5' cyclic monophosphate (cAMP) plays a critical role in the transmission of signals by functioning as a "second messenger" (1,2). The binding of a hormone to its receptor can either enhance or inhibit the rate at which cAMP is produced. This is accomplished by altering the enzymatic activity of adenylyl cyclase, the membrane associated enzyme which catalyzes the production of cAMP from ATP (3). By this mechanism, intracellular levels of cAMP are altered in response to hormonal stimulation. In turn, the intracellular level of cAMP regulates the enzymatic activity of a protein kinase, which phosphorylates other substances setting off a cascade of cellular events, which leads to the expression of the hormones (4).

The Adenylyl Cyclase Activation FlashPlate Assay allows direct measurement of receptor mediated adenylyl cyclase activation/inhibition in G-protein coupled receptor systems. The protocol is a fully homogeneous cellular assay, which measures a true second messenger response (cAMP). This relevant functional data permits faster qualification of lead compounds in high throughput screening than is possible with conventional primary screens. The assay is performed by adding live cells to the FlashPlate, stimulating (or stimulating and inhibiting), lysing and detecting without wash steps. Cyclic AMP can be measured in the range of 10 to 1000 pmol/mL. The assay is accurate over a wide range of values and has a high degree of specificity.



IV. PRINCIPLES OF THE PROCEDURE

The basic principle of the Adenylyl Cyclase Activation FlashPlate Assay is the competition between radioactive and non-radioactive cAMP for a fixed number of binding sites. This interaction is illustrated in Figure 1:

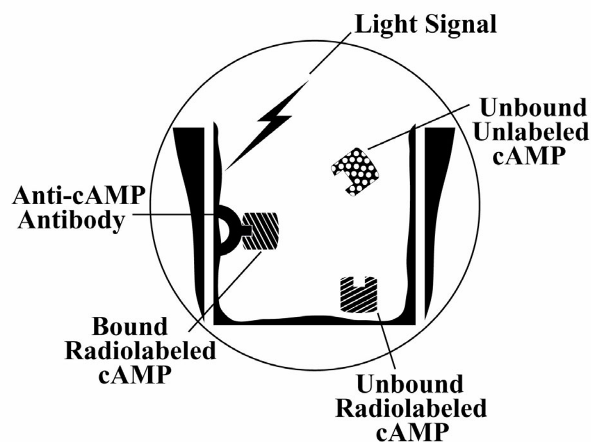


Figure 1

1. The wells of the FlashPlate are pre-coated with anti-cyclic AMP antibody.

2. A fixed amount of radiolabeled cAMP is added and binds to the anti-cAMP antibody.

3. Cells are added and stimulated. The unlabeled cAMP produced by these cells displaces the fixed amount of radiolabeled cAMP.

4. The light signal is detected. Increasing amounts of unlabeled cAMP will displace more radiolabeled cAMP, resulting in a decrease of detectable signal over time (see representative data on page 8).

5. After incubation, the FlashPlates are read on a microplate scintillation counter. The data is used to construct a standard curve from which the cAMP values produced by the stimulated cells are obtained by interpolation.

V. REAGENTS PROVIDED

All necessary reagents are supplied and are intended for LABORATORY USE ONLY. Kits are shipped at ambient temperatures and must be stored upon receipt at 2-8°C. The reagents are stable for the times indicated if the specific precautions given below are followed. Sodium azide* has been added as an antibacterial agent where appropriate.

1. **Stimulation Buffer – 1 bottle (SMP004B), 1 L**

The buffer contains a phosphodiesterase inhibitor (IBMX), protein and a stabilizer. The buffer is stable for at least two months when stored at 2-8°C. Refer to vial label for the expiration date of the reagent.

OR **Stimulation Buffer without IBMX – 1 bottle (SMP004C), 1 L**

The buffer contains protein and a stabilizer. It does not contain IBMX. The buffer is stable for at least two months when stored at 2-8°C. Refer to vial label for the expiration date of the reagent.

> NOTE: The Stimulation Buffer contains IBMX, a xanthine derivative, which may demonstrate antagonist properties with the adenosine family of receptors. As well, it may interfere with constitutively active receptors, which have higher basal levels of cAMP. It is recommended if using such receptors, to order the assay kit that includes stimulation buffer that does not contain IBMX.
>
> SMP004, 2-plate kit contains both Stimulation Buffers

SMP004A, 5-plate kit contains both Stimulation Buffers
SMP004B, 40-plate kit contains Stimulation Buffer with IMBX
SMP004C, 40-plate kit contains Stimulation Buffer without IMBX

2. **Detection Buffer – 2 bottles, 250 mL each**

   The buffer contains a permeabilizer and 0.09% sodium azide*. The buffer is stable for at least two months when stored at 2-8°C. Refer to vial label for the expiration date of the reagent.

3. **cAMP Standard, lyophilized – 4 vials, 10,000 pmol cAMP/vial**

   Reconstitute each vial with exactly 2.0 mL of distilled water. The reconstituted solution will contain: cAMP at a concentration of 5000 pmol/mL, sodium acetate buffer, 0.1% sodium azide and an inert ingredient. The cAMP Standard has been calibrated spectrophotometrically using the molar absorption coefficient, = 14.6 x 10^3 $mol^{-1}cm^{-1}$ at 259 nm, pH 6.9. The reconstituted standard is stable for at least two months when stored at 2-8°C. Refer to vial label for the expiration date of lyophilized reagent.

4. **Adenylyl Cyclase Activation FlashPlate – 40 microplates, 96-well**

   The microplates are coated with solid scintillant to which anti-cyclic AMP antibody has been bound. Plates are shrink wrapped in bags of 10 plates and should not be opened until ready to use. Refer to the label for the expiration of the plates.

5. **TopSeal ® - A Plate Cover – 40 covers**

6. **cAMP [^{125}I] Tracer (Succinyl cAMP Tyrosine Methyl Ester [^{125}I]) – two vials, 2500 µL each**

   Tracer is shipped separately upon request. Each vial contains 185 kBq (50 µCi in 2500 µL) on calibration date. Use one vial at a time as directed. The concentrate is stable for the shelf life of the kit when stored at 2-8°C. This material is radioactive and the user should follow the precautions listed below:

INSTRUCTIONS RELATING TO THE HANDLING, USE, STORAGE, AND DISPOSAL OF THIS RADIOACTIVE MATERIAL.

This radioactive material may be received, acquired, possessed, and used only by research laboratories for *in vitro* laboratory tests not involving internal or external administration of the material, or the radiation therefrom, to human beings or animals. Its receipt, acquisition, possession, use and transfer are subject to the regulations and a general license of the U.S. Nuclear Regulatory Commission or of a State with which the Commission has entered into an agreement for the exercise of regulatory authority.

1. All radioactive materials must be labeled and secured in specifically designated posted areas. Records of receipt and survey must be maintained.

2. All work with these materials must be carried out only in authorized areas.

3. Prohibit mouth pipetting of radioactive materials.

4. There must be no smoking or eating within the work area.

5. Hands must be washed after handling radioactive materials.

6. Any spilled material must be wiped up quickly and thoroughly and the contaminated substances transferred to a suitable receptacle. The surfaces involved must be washed thoroughly with an appropriate decontaminant. Monitor to ensure the area has been effectively decontaminated.

7. When use of the tracer reagent has been completed, empty and decontaminate the vial. This radioactive material can be discarded into the sanitary sewerage system, using copious amounts of water to ensure a minimal discharge concentration.

8. Prior to disposal of the empty, uncontaminated kit and tracer containers to unrestricted areas, remove or deface the radioactive material labels or otherwise clearly indicate that the containers no longer contain radioactive material.

*NOTE about Sodium Azide: The National Institute for Occupational Safety and Health has issued a bulletin citing the potentially explosive hazard due to the reaction of sodium azide with copper, lead, brass, or solder in plumbing systems. Although sodium azide is added at a minimal concentration, it is still recommended that copious amounts of water be used to flush the drain pipeline after disposal of these reagents in the plumbing system. Copper-free and lead-free discharge lines should be used whenever possible. Decontamination procedures should be followed prior to maintenance on drain lines, which have been used for azide-containing reagents. Recommended decontamination procedures are available from NEN Technical Services.

VI. ADDITIONAL MATERIALS AND EQUIPMENT REQUIRED

In addition to the reagents supplied with the kit, the following materials are required:

1. Pipettors and/or pipets that can accurately and precisely deliver the required volumes.
2. 96-Well Microplate Scintillation Counter
3. Laboratory vortex mixer
4. Test tube rack
5. Distilled water
6. Test tubes - 12 x 75 mm - polypropylene
7. A polypropylene container capable of containing the required reagent volume for the number of FlashPlates being processed
8. Radioactive waste container
9. 2 - 8°C refrigerator or equivalent
10. Centrifuge capable of 400 X g for cell processing

PART 2: ASSAY PROCEDURE

VII. CELL PREPARATION

Harvest the number of cells from tissue culture flasks as needed according to standard methods. Wash the cells by centrifugation at 400 X g (~1000 rpms) for 5 minutes to form a pellet. Aspirate the supernatant and resuspend the pellet in the Stimulation Buffer.

NOTE: Usage of a non-enzymatic cell dissociation buffer (i.e.: Sigma Cat #. C1544) is recommended for dislodging adherent cells from the culture flask.

VIII. REAGENT PREPARATION

The following is a suggested protocol for the preparation of standards. Mix each solution thoroughly before adding it to the next tube. Prepare the standards fresh each day. This is enough standard for 4 wells. Adjust reagent volumes accordingly for the number of wells required.

1. Reconstitute cAMP standard with exactly 2.0 mL of distilled water. The cAMP concentration of the reconstituted solution is 5000 pmol/mL.

2. Prepare a "zero standard" tube containing only 1 mL Stimulation Buffer.

3. Label seven standard tubes in accordance with their concentration:

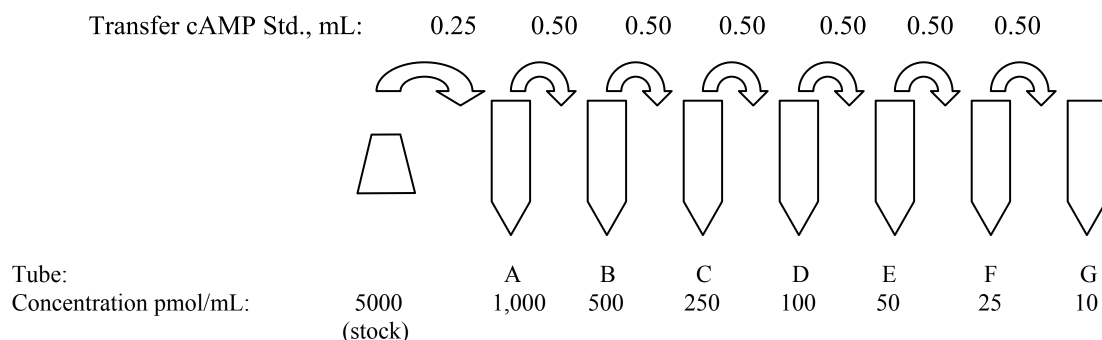
   A 1,000 pmol/mL
   B 500 pmol/mL
   C 250 pmol/mL
   D 100 pmol/mL
   E 50 pmol/mL
   F 25 pmol/mL
   G 10 pmol/mL

4. Pipette the appropriate volume of Stimulation Buffer into each of the seven tubes according to the scheme below.

Tube A:	1.0 mL
Tube B:	0.5 mL
Tube C:	0.5 mL
Tube D:	0.75 mL
Tube E:	0.5 mL
Tube F:	0.5 mL
Tube G:	0.75 mL

5. Begin serial dilutions from the stock solution of cAMP standard:

   a. Pipette 0.25 mL cAMP Stock Standard solution into Tube A. Mix well.
   b. Transfer 0.50 mL of solution A into Tube B. Mix well.
   c. Transfer 0.50 mL of solution B into Tube C. Mix well.
   d. Continue the serial dilution three more times according to the same dilution scheme.



6. Prepare the stimulator, such as Forskolin, or a ligand matched to a particular receptor of interest. Perform a serial dilution in the suggested range of 100 µM to 1 µM. **Dilution of the compounds must be done in water or PBS.**

   <u>NOTE</u>: The user must determine the optimum cell number/well and the concentration of compounds required to either stimulate or inhibit adenylyl cyclase. As a starting point, it is recommended that the user do a serial dilution of cells starting with $1x10^6$ cells/mL. For each cell concentration, several levels of stimulator should be

evaluated to determine what concentration gives approximately a 50% increase in cAMP over basal level. The chosen cell concentration selected should yield a low basal level but be adequate to respond to a relatively low stimulator concentration. If the user is evaluating inhibitory compounds, the level of stimulator should be as low as possible so that the sensitivity to the inhibitor would be high.

7. Prepare Detection Mix by adding 1.0 mL of cAMP [^{125}I]-Tracer to 110 mL of Detection Buffer. This volume is suggested for 10 FlashPlates. Prepare only the amount of Detection Mix that is required for the number of FlashPlates being assayed. Keep proportions the same.

IX. ASSAY PROTOCOL

1. Add 50 µL of standards to the appropriate wells. It is recommended that duplicate wells for both standards and samples be assayed.

2. Add 50 µL of cells to the appropriate wells.

3. Add compound/stimulator (suggested 25 µL each) to the wells containing cells and the same volume of the compound/stimulator vehicle to the wells containing standards (the final matrix for samples and standards should be the same). A non-stimulated cell control should be run for a baseline.

NOTE: Total volume should be 100 µL in the well during the stimulation step. If only one addition (no inhibitor) is made to the sample or standard, the difference is made up with water or PBS. Do not make up the difference with Stimulation Buffer. The maximum amount of Stimulation Buffer that should be present/well is 50 µL. More than 50 µL of Stimulation Buffer per well will cause a decrease in signal.

4. Incubate for an appropriate time at room temperature. Depending on the system, the concentrations and time and temperature of stimulation will need to be optimized by the user

5. Add 100 µL of Detection Mix to all wells

6. Place a plate cover on the plate and incubate 2-24 hours at room temperature.

7. After incubation, count on a 96-well microplate scintillation counter.

X. CALCULATION OF RESULTS

1. If all wells have been counted for the same period of time, use the total accumulated counts, otherwise, correct all counts to a common count rate.

2. Average the counts for each set of duplicates.

3. Express the average counts for each standard and sample as a percentage of the average counts for the zero standard. (This is termed "normalized' percent bound or % B/Bo).

$$\% \text{ B/Bo} = \frac{\text{Average Net Counts of Standard or Sample}}{\text{Average Net Counts of Zero Standard}} \times 100$$

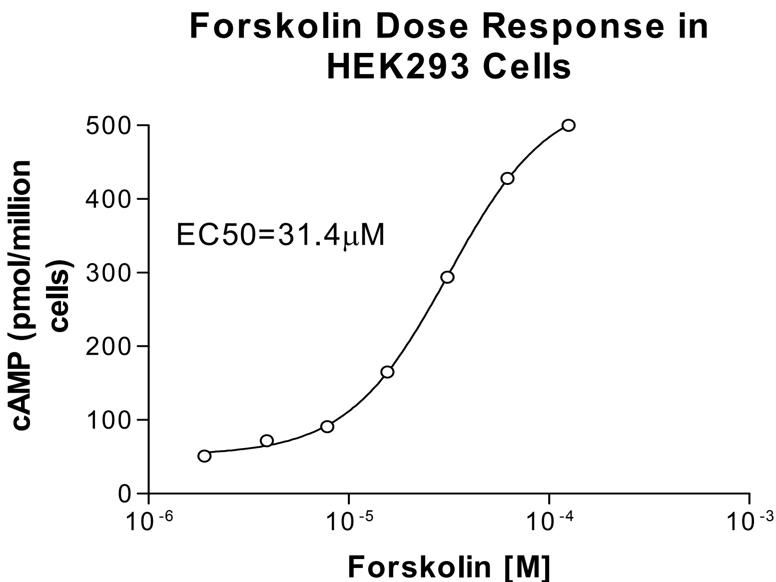
4. Using semi-logarithmic graph paper, log-logit graph paper, or a statistical software package, plot % B/Bo for each standard against the corresponding concentration of cAMP in pmol/mL or pmol/well.

5. Determine the concentration of cAMP in the samples by interpolation from the standard curve. Since identical volumes are used for standards and samples, and the standard curve is expressed as pmol/mL or pmol/well of cAMP, samples can be read directly from the standard curve as pmol/mL or pmol/well. Any samples with concentrations above the range of the standard curve must be re-assayed by decreasing the number of cells/well or the concentration of the stimulator.

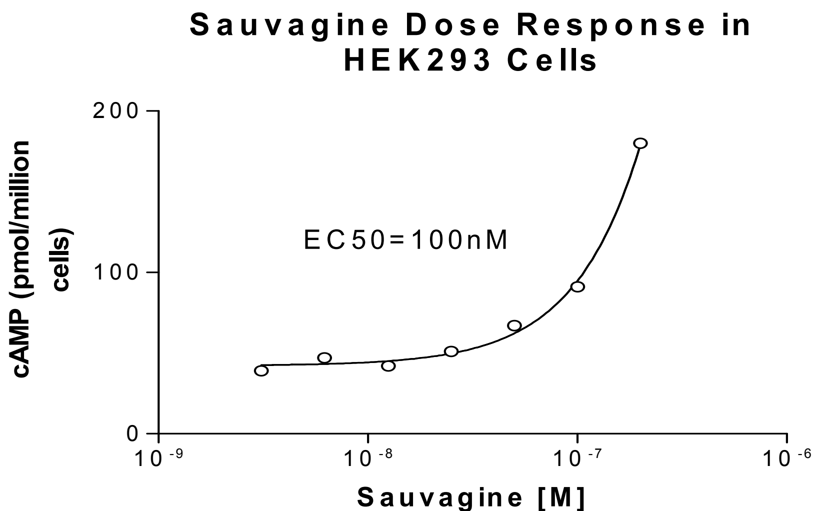
NOTE: Depending on the cell line, the baseline value of cAMP may increase with time and needs to be subtracted to obtain a more accurate result.

XI. TYPICAL DATA

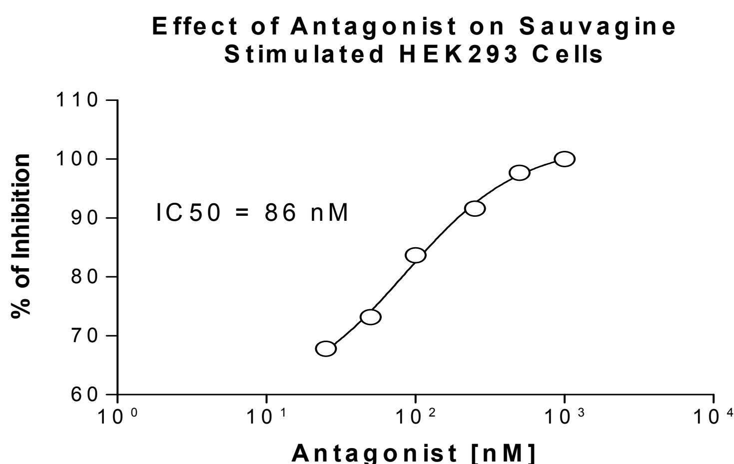
1. This graph represents a typical dose response curve for forskolin stimulation of 50,000 HEK293 cells/well. The stimulation was done for 30 minutes at room temperature.



2. This graph represents a typical dose response curve for sauvagine stimulation of 50,000 HEK293 CRF2 cells/well. The stimulation was done for 60 minutes at room temperature.



3. The adenylyl cyclase assay can also be used to study the inhibition of ligand stimulation as is done in some HTS labs. This graph represents the inhibition of ligand stimulation using a known antagonist. HEK293 cells expressing the receptor CRF2 were stimulated with 400 nM Sauvagine. The antagonist was added simultaneously at several different concentrations to determine the inhibitory effect.



XII. PRECAUTIONS

1. Incubation conditions should be standardized for proper day to day internal quality control.

2. As with all immunoassay procedures, pipetting is crucial. It is essential that pipetting be accurate and reproducible.

3. The reagents in this kit should be used as a unit. Do not mix different lots of any component within a given assay.

4. This product has not been tested for use with any methods other than those stated in this Instruction Manual.

5. WARNING: This product contains a chemical known to the state of California to cause cancer. (^{125}I Tracer)

XIII. PERFORMANCE CHARACTERISTICS

1. Reproducibility

   Within plate precision was determined by assaying multiple wells of cells at different levels. Between plate precision was determined by averaging standard curves from different plates.

   Results showed that the % C.V. for either within plate or between plate reproducibility is < 10 %.

2. Sensitivity

   The mean and standard deviation were determined for 6 duplicate measurements of the zero standard binding. The sensitivity of the method, defined as the cAMP concentration corresponding to the mean cpm minus twice the standard deviation, is 0.2 pmol/well.

3. Specificity

   The following compounds have been assayed for cross-reactivity. The percentages are calculated at the 50% B/B0 point.

   % Cross-reactivity

GMP	< 0.002 %
ATP	< 0.002 %
AMP	< 0.002 %
CGMP	< 0.04 %

XIV. REFERENCES

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3. Perkins, J. P. (1973): Adenylyl cyclase. In: Greengaard, P. and Robinson, G. A. (Eds.) Advances in Cyclic Nucleotide Research, Vol. 3:1-64. Raven Press, NY.

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PC3684-0300