FlashPlate® File #7

In Vitro Measurement of the Second Messenger cAMP: RIA vs. FlashPlate

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Introduction

Abnormalities in neurotransmission within the brain can lead to several disease states, including anxiety, depression or schizophrenia. Extensive research has been carried out to discover the mode of action of various transmitters, in order to understand and combat these diseases. In many cells, binding of the neurotransmitter to its receptor alters the production of the second messenger adenosine 3', 5' cyclic monophosphate (cAMP), through the enzyme adenylyl cyclase. Intracellular changes in the level of cAMP can then initiate a cascade of other cellular events.
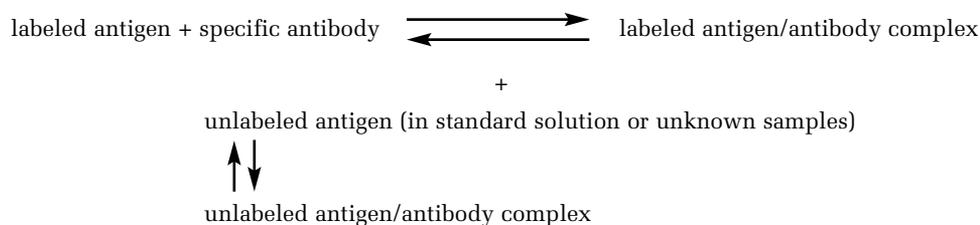
We have been investigating changes of adenylyl cyclase activity, induced by receptor stimulation in recombinant cell lines, by measuring the accumulation of cAMP. Until recently, we used the commercially available cAMP [125I] Radioimmunoassay Kit (RIA) from PerkinElmer Life Sciences. (NEK033).

This method has proved highly satisfactory, but the introduction of cAMP [125I] FlashPlate Assay (SMP001) presents a potentially more convenient system for measuring cAMP. The FlashPlate method operates on the same principle as the RIA method, but the assay is performed directly in scintillant-coated microplates where the anti-cAMP antisera has been pre-bound to the wells. We have therefore carried out a study to compare the RIA and FlashPlate methods to investigate the similarities and differences between the two.

Methods

Assay Principle
The principle of both assay methods is as follows:

labeled antigen + specific antibody $\rightleftharpoons$ labeled antigen/antibody complex

+

unlabeled antigen (in standard solution or unknown samples)

$\uparrow\downarrow$

unlabeled antigen/antibody complex

Generation of cAMP Source
The method is an adaptation from McHale, *et al.*[1]

Chinese Hamster Ovary cells expressing human $5-HT_{1D}$ receptors were seeded and grown to confluence. They were subsequently harvested and resuspended in medium containing isobutylmethylxanthine (500 µM), paroxetine (1 µM), pargyline (10 µM) and ascorbate (1 µM). After 30 minutes the reaction was started by the addition of viable cells (2×10^5) to LP4 tubes containing forskolin (10 µM) in the presence or absence of 5-HT (10^{-10} to 10^{-5} M). Basal cAMP levels were determined from cells not treated with either drug. Following a 10 minute incubation at 37°C, the reaction was stopped using 30% perchloric acid. cAMP was extracted by addition of a 1:1 mixture of trioctylamine and 1,1,2 trichlorotrifluoroethane, and after vigorous mixing the samples were centrifuged at 3,500 rpm for 15 minutes. The resulting aqueous layer was removed and the level of cAMP determined.

For the RIA method, the samples were dispensed into 1 ml tubes; for the FlashPlate method, the samples were dispensed into a 96-well microplate.

Determination of cAMP Levels using RIA
All reagents were made up as instructed in the RIA Kit (NEK033) manual. The samples were diluted three-fold in a sodium acetate buffer and pipetted manually, in duplicate, into LP4 tubes. The remaining components (i.e., standard cAMP [125I] tracer and cAMP antiserum complex) were also added manually. After overnight incubation, binding protein was added to all tubes (except totals). Following mixing, the samples were centrifuged, the supernatant discarded, and the pellets counted on a gamma counter.

Determination of cAMP Levels using FlashPlate Method

The components were made up as per the cAMP [125I] FlashPlate Assay (SMP001) manual. The samples were diluted three-fold using a Biomek 1000 Robot (Beckman), then automatically dispensed, in duplicate, into the wells of the cAMP [125I] FlashPlates. The standard curve was pipetted manually into the plates, but the cAMP [125I] tracer was added by the robot to all wells. After overnight incubation, the FlashPlates were placed onto a Packard TopCount® Microplate Scintillation Counter and the level of bound radioactivity measured.

Results

Using the RIA method, the agonist 5-HT produced a dose-dependent inhibition of forskolin-stimulated cAMP levels with a $pEC_{50} = 7.5 \pm 0.2$ and a maximum response of $84 \pm 7\%$ inhibition (Fig. 1a). The FlashPlate technique produced a comparable 5-HT inhibition curve with a $pEC_{50} = 7.7 \pm 0.1$ and a maximum response of $84 \pm 4\%$ (Fig. 1b). The two assays produced similar results between other parameters, such as absolute levels of cAMP measured, and intra-assay variation (Table 1). Inter-assay variation using the FlashPlates was minimal, with only 6% error on three separate determinations of the same sample (5.07 ± 0.33 pmol cAMP/ml). However, one obvious difference between the two methods was the time required to assay the same number of samples; the FlashPlate method saved several hours compared to the RIA method.

Figure 1a

Agonist Response on cAMP Accumulation
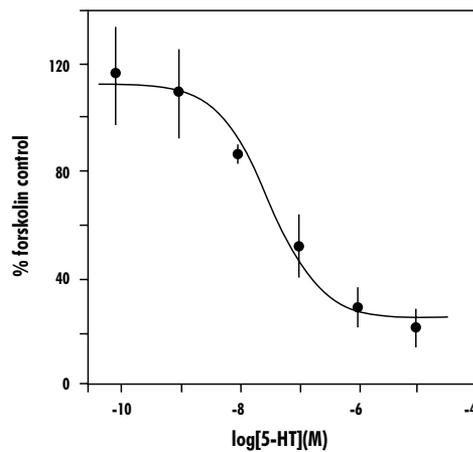
Figure 1b
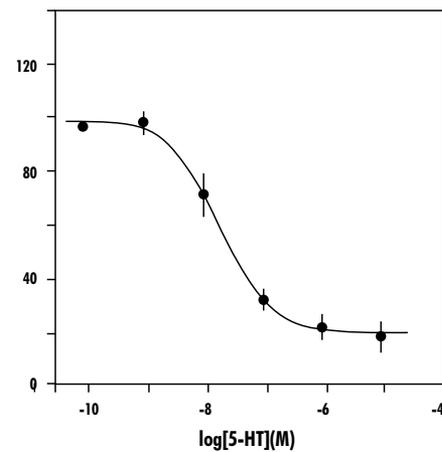
Table 1

RIA Vs. FlashPlate

Parameter	RIA	FlashPlate
Basic levels cAMP (pmol/ml)	6 ± 2	5 ± 1
Forskolin-stimulated levels (pmol/ml)	27 ± 6	39 ± 9
Intra-assay variation (error on duplicates)	11%	7%
Assay time (88 samples)	4 hours	1 hour

Discussion

To date, radioimmunoassay has been an effective method to measure cAMP within samples. It is, however, a laborious technique with few options for automation and hence not conducive for use in high throughput screening (HTS) applications. The cAMP [125I] FlashPlate Assay has overcome these drawbacks: its 96-well microplate format has proven itself highly efficacious for robotic HTS, permitting cAMP measurements to be fully automated, with improvements realized in both efficiency and accuracy. The assay kit (SMP001) is comprised of fewer components than the RIA kit, and is easier to use. Additionally, the elimination of the separation step in the FlashPlate method is a major time saving advance, and again favors full automation. The results obtained from the FlashPlates are very reproducible and comparable to those obtained from RIA, and so the former can be confidently used as an alternative to the latter. The FlashPlate method is extremely sensitive to the levels of cAMP that we are dealing with. The kit also provides the option to acetylate samples to increase sensitivity further, if required.

In conclusion, the cAMP [125I] FlashPlate Assay provides a robust and efficient method for high throughput cAMP determination. In addition, the use of this technology does not stop at second messenger assessment, but can be applied to all RIA applications as well as radioligand binding and guanine nucleotide binding protein assays, development of which is eagerly awaited.

Reference

1. McHale, M., *et al.* 1994. *FEBS Letters* 345, 147-150.

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