FlashPlate® File #1

A Novel Adenylyl Cyclase Activation Assay on FlashPlate

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Introduction

Adenylyl Cyclase is a cell membrane-associated G protein-coupled enzyme which, when activated, catalyzes the production of Adenosine 3', 5' cyclic monophosphate (cyclic AMP, or cAMP) from ATP. Cyclic AMP then functions as a "second messenger," initiating a cascade of cellular signaling events that ultimately lead to a specific cellular response. The binding of a G protein-coupled receptor (GPCR) ligand can either stimulate or inhibit the rate at which cAMP is produced. Therefore, a direct measurement of cAMP produced by cells in response to receptor-ligand activation serves to determine the function of putative drug candidates. Adenylyl Cyclase is linked to a subset of the superfamily of GPCRs: the Gs and Gi/o linked receptor families. These families encompass a wide range and number of known GPCRs today, making the measurement of Adenylyl Cyclase enzyme activation applicable as a generic functional screen. This is particularly useful for "orphan" receptors for which there is no known (characterized) ligand, and thus no capability to run a receptor binding screen.

The Adenylyl Cyclase Activation FlashPlate Assay (SMP004, SMP004A) is a fully homogeneous assay kit, optimized to allow measurement of receptor-mediated Adenylyl Cyclase activation by direct measurement of cAMP production from whole cells. The assay is formatted to facilitate its use in high throughput screening, allowing functional testing to be performed at the primary screening stage rather than later. Its benefits include:

- All steps occur in one well.
- There is no cAMP extraction step.
- It may be used as a generic functional screen for a large part of the superfamily of GPCRs to determine agonist and antagonist activity.
- No washing or separation steps are required.
- Results are easily obtained on the day of the assay.
- Cells need not be grown in microplates prior to stimulation.
- There are no sample transfer steps.
- It exhibits excellent reproducibility and performance.
- It is readily convertible to a 384-well format.

Product Description and Applications

FlashPlate is a line of white polystyrene microplates with plastic scintillant-coated wells designed for high volume, in-plate radiometric assays. The Adenylyl Cyclase Activation FlashPlate Assay consists of:

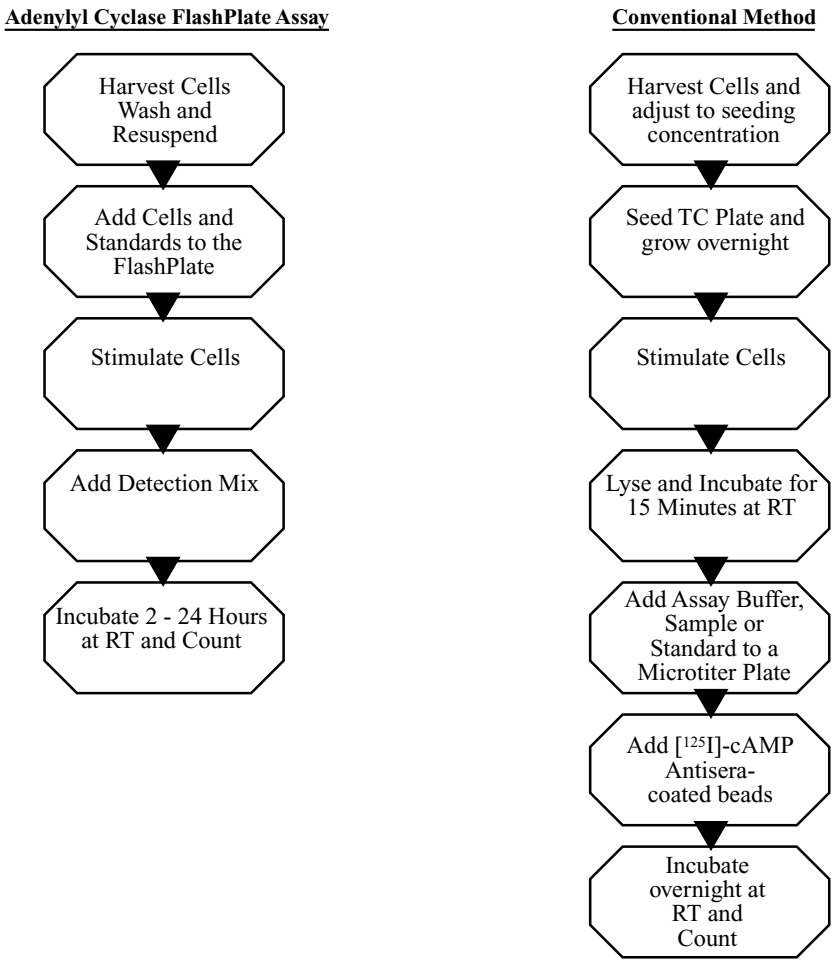
- FlashPlate microplate coated with a cAMP antibody and blocked to avoid non-specific binding
- Standard
- Stimulation buffer
- Detection buffer
- [^{125}I]-cAMP

The Adenylyl Cyclase Activation FlashPlate Assay is a homogeneous assay designed to increase productivity and reproducibility in high throughput screening, compared to conventional assay methods.

Cells from a tissue culture flask are placed directly into the FlashPlate wells. Because the cells are added as a single sample, each well receives approximately the same number of cells, and reproducibility between wells is very high. In contrast, conventional assays require that cells be grown overnight or longer in a separate tissue culture plate before lysing, incubation, and transfer to a microtiter plate. Well-to-well differences in growth rate may result in different numbers of cells per well.

The FlashPlate assay also allows for the stimulation of Adenylyl Cyclase to be performed in the same well as the detection step, and eliminates the need for a separate lysing step to release the intracellular cAMP. This significant reduction in protocol complexity makes cell assays easier to automate, which in turn makes the Adenylyl Cyclase Activation FlashPlate Assay more cost-effective for large screens. See Figure 1.

Figure 1



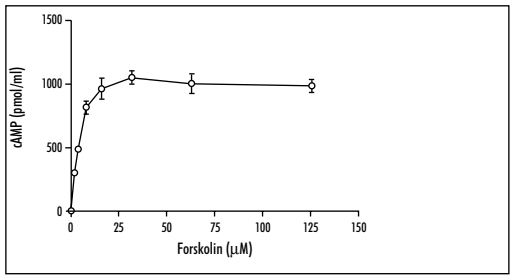
The following adherent and suspension cell lines have been evaluated in the Adenylyl Cyclase Activation FlashPlate Assay, with acceptable results:

Adherent Lines	Suspension Lines
CHO-ET_A	HL60
CHO-ET_B	CHO-ET_A
T47D	
293 CRF1	
293 CRF2	

In each case, cell lines were stimulated with Forskolin, a direct stimulator of Adenylyl Cyclase. In our experiments, we found that unstimulated cells typically maintain a very low basal production of cAMP. For every new cell line, it is important to determine the optimal incubation time for stimulation by ligand or Forskolin. It is also important to determine the number of cells per well, and the optimal concentration of the stimulator.

Figure 2 shows a typical dose response curve for Forskolin stimulation of T47D cells.

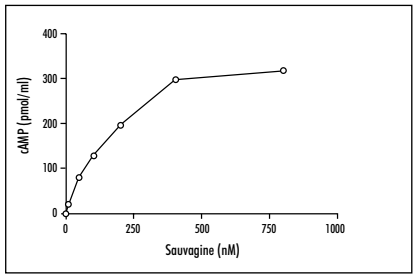
Figure 2



A typical dose response curve for Forskolin stimulation of T47D cells (50,000 cells per well). Cells were stimulated for 30 minutes at room temperature.

Figure 3 shows a typical dose response curve for a ligand stimulation of 293 cells.

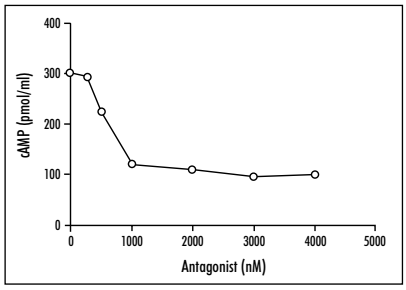
Figure 3



A typical dose response curve for Sauvagine stimulation of 293 CRF2 cells (50,000 cells per well). Cells were stimulated for 60 minutes at room temperature.

The Adenylyl Cyclase Activation FlashPlate Assay can also be used to study the inhibition of ligand stimulation, as is done in some HTS labs. Figure 4 shows the inhibition of a ligand stimulation using a known antagonist.

Figure 4



293 CRF2 cells were stimulated with 400 nM Sauvagine. The antagonist was added simultaneously at several different concentrations to determine the inhibitory effect.

Assay Optimization

In order to achieve the best results possible, it is essential to determine the optimal number of cells per well, and the amount of compound needed to either stimulate or inhibit Adenylyl Cyclase. A serial dilution of cells, starting with 1 x 10^6 cells/ml, provides a good initial test. For each cell concentration, several levels of stimulator should be evaluated to determine what concentration generates an approximately 50% increase in cAMP over basal level. The cell concentration selected should produce a low basal level, which will permit good response to relatively low concentrations of stimulator. When evaluating inhibitory compounds, the level of stimulator should be as low as possible, in order to maximize sensitivity to the inhibitor.

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