

Direct, quantitative, intracellular measurement of cyclic AMP in cells: A LEADseeker assay utilising Protein A polystyrene SPA Imaging beads

* Elizabeth Price, Jeffrey Horton, Molly Price-Jones & Pierre Graves

Amersham Biosciences UK Limited, The Maynard Centre, Forest Farm, Whitchurch, Cardiff, UK [Tel. 44 29 20526188, Fax 44 29 20526230, E-Mail.elizabeth.price@amersham.com]

Introduction

Adrenergic receptors are linked to adenylate cyclase via either inhibitory or stimulatory G-proteins. These receptors, when activated by agonist or antagonist occupancy of ligand binding sites, affect the intracellular generation of cAMP. Established methods for preparing cellular extracts for cAMP measurements involve tedious processes, such as acid or solvent extraction, cell lysis and removal of extracting agent in order to obtain samples in a suitable form for subsequent assay. These steps must be carried out sequentially, thereby adding to the time and cost of each assay. Furthermore, none of these methods are very suitable for high throughput screening where large numbers of samples are required to be processed.

A direct screening 384 well assay has been optimised for the Amersham Biosciences LEADseeker™ Multimodality Imaging system. This direct technique eliminates sample preparation. The procedure enables culture of cells, followed by the direct extraction and assay of cAMP. This process is achieved with a combination of cell lysis reagents and utilising Protein A coated polystyrene (PS) SPA imaging beads to generate signal in a simple, homogeneous format. LEADseeker Multimodality Imaging System coupled with the novel reagents of the direct assay system, simplifies existing methodology whilst increasing throughput. A single 384 well plate can be imaged in as little as 5 minutes, compared to 40 minutes when using a conventional PMT based counting instrument.

Methods

The assay method may be carried out in one of three ways (Figure 1):-

- 1) A one-stage method, where extraction and measurement are carried out in the microplate used for culturing cells.
- 2) A one-stage, total cellular cAMP method where combined intracellular and cell supernatant cAMP are measured.
- 3) A two-stage method, where the cells are cultured and lysed in a separate vessel. An aliquot of lysate is transferred to a second plate for assay.

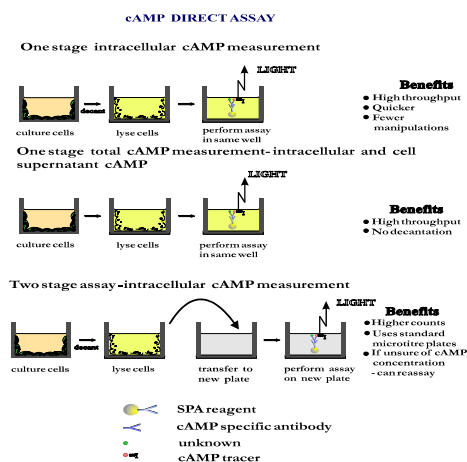
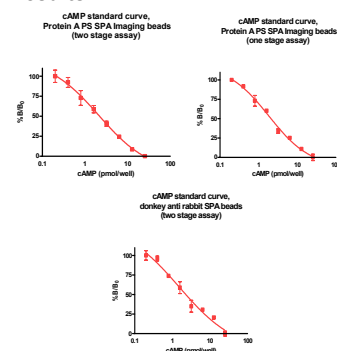


Figure 1. Principle of the direct screening assay system.

Chinese hamster ovary (CHO) or A431 cells were seeded into 96- or 384-well cluster plates suitable for both cell culture and imaging. Cells were stimulated (20 mins, 37 °C, 5% CO₂, 95% humidity) with forskolin (1-1000µM), or with one of the β adrenoreceptor agonists isoproterenol, epinephrine, salbutamol or noradrenaline (1.25-1000nM), or pre-incubated for 30 minutes with the non-selective adrenoreceptor antagonist propranolol (0.001-1250nM) before stimulation with isoproterenol (1000nM). The culture supernatant was gently aspirated and the cells lysed with 0.25% (w/v) dodecyltrimethyl ammonium bromide. Working standards (0.2-25.6pmol/well) were pipetted into empty wells of the plate used to culture the cells. Equal volumes of antisera (20µl/well), [¹²⁵I] cAMP (~35,000cpm/well) and Protein A PS SPA Imaging bead (20µl,0.4mg/well) were added to the microplate wells containing standards and samples. The plates were sealed and incubated at room temperature for 15-20 hours, without agitation. The amount of [¹²⁵I] cAMP bound to the SPA Imaging beads was determined by imaging on the LEADseeker Multimodality Imaging system for 5 minutes.

Results



NOTE: The standard curves generated in the one and two stage assay are comparable.

Figure 2. Typical cAMP standard curves. Values are means ± SEM (n=3)

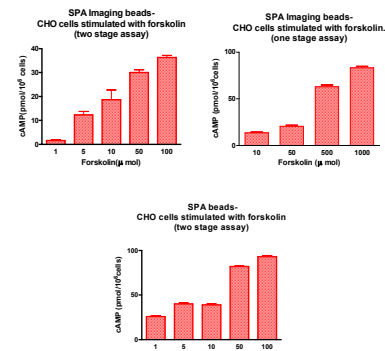


Figure 3. Comparison of data obtained from forskolin-stimulated CHO Cells. cAMP was measured using SPA and SPA Imaging beads in one and two stage assays. Values are means ± SEM (n=3)

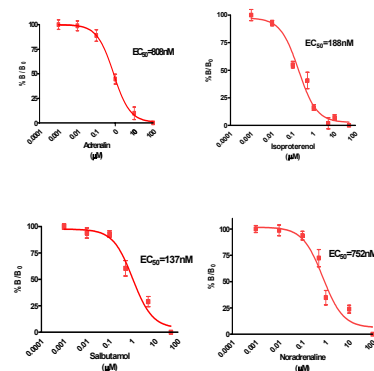


Figure 4. Agonist-induced cAMP generation from A431 Cells. Values are means ± SEM (n=3)

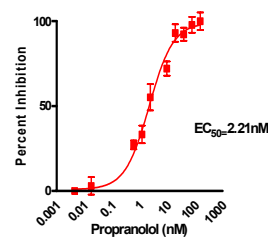


Figure 5. Effect of propranolol on isoproterenol induced cAMP generation from A431 cells. Values are means ± SEM (n=3)

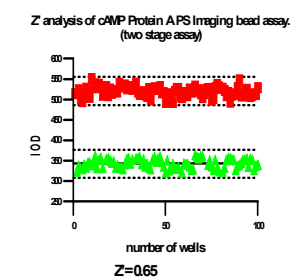


Figure 6. Z' factor data analysis was obtained from forskolin stimulated CHO cells.

CONCLUSION

- The throughput of samples for the direct measurement of cAMP in cell cultures can be significantly improved using LEADseeker Imaging technology.
- The standard curve generated in the one-stage assay and two-stage assay are comparable.
- This method has been validated with known pharmacological reagents.
- Z' factor analysis has been conducted. The value of 0.65 was obtained