AlphaScreen* cAMP Assay for the Human Epithelial Carcinoma Cell Line, A431, Using the BioRAPTR FRD™ Microfluidic Workstation and PARADIGM™ Detection Platform

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ABSTRACT

G protein-coupled receptors (GPCRs) remain the dominant target class for approved and future drugs due to their vital role in a wide variety of biological and pathological processes. Intracellular cAMP levels, as an indicator of the functional status of GPCRs, were assayed using the AlphaScreen cAMP assay from PerkinElmer Life Sciences to study the functional activity of the β 2-adrenergic receptor in response to stimuli in the human epithelial carcinoma cell line, A431. AlphaScreen is a bead-based non-radioactive luminescent homogeneous assay technology which is amenable to automation. The cAMP assay is based on the competition between endogenous cAMP and exogenously added biotin-labeled cAMP for the binding site on antibody-conjugated acceptor beads.

The protocols and data described in this poster demonstrate automatic cell, compound, and reagent dispensing for the AlphaScreen cAMP assay in a 384-well microplate format using the BioRAPTR FRD workstation. The PARADIGM Detection Platform from Beckman Coulter was used for the emission signal detection and analysis utilizing a new cartridge specifically designed for AlphaScreen applications.

Note: The BioRAPTR FRD and PARADIGM Detection Platform are for Research Use Only; not for use in diagnostic procedures.
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INTRODUCTION

BioRAPTR FRD Microfluidic Workstation

The **BioRAPTR FRD** (Flying Reagent Dispenser) Microfluidic Workstation provides non-contact liquid handling and is capable of rapidly and accurately dispensing various liquids including cells with volume ranges from 0.1 to 60 µL in 384-, 1536- or 3456-well plates. It is able to dispense the cells with a high degree of precision while maintaining cell viability during the entire process.

PARADIGM Detection Platform

The PARADIGM Detection Platform utilizes a patent-pending design that allows for real-time system configuration by the user in less than five minutes.

PARADIGM Detection Platform features:

- User-friendly integration and user-configurable modular design
- Detection of Multiple formats plates ranging from 6- to 1536 wells
- Multiple detection modes: ABS, HTRF, TRF, FP, FI, FRET and Glow LUM.
- On-the-fly detection with intelligent cartridge identification

Cartridges for Broad Array of Detections:

- Absorbance
- Multimode
- Fluorescence Intensity (fluorescein - rhodamine)
- Fluorescence Polarization (fluorescein)
- Cisbio HTRF®
- Invitrogen GeneBLAzer®
- Invitrogen® LanthaScreen®
- **PerkinElmer AlphaScreen**
- Promega MultiTox-Fluor Multiplex Cytotoxicity

The detection cartridge utilized in this poster is the AlphaScreen Cartridge which is specifically designed for AlphaScreen applications.

ASSAY PRINCIPLES

AlphaScreen is a bead-based non-radioactive amplified luminescent proximity homogeneous assay. Two types of specially coated beads are used in the assay. When a biological interaction brings the beads close enough, the chemical reactions between two beads produce a greatly amplified signal. Laser excitation of the photosensitizer in the Donor bead converts ambient oxygen to a more excited singlet oxygen molecule which reacts with a thioxene derivative in the Acceptor bead to generate chemiluminescence at 370 nm that further activates fluorophores contained in the acceptor bead. Energy transfer to fluorescent acceptors shifts the emission wavelength to 520-620 nm, as shown in Figure 1A. In the absence of a specific biological interaction, the singlet state oxygen molecules produced by the Donor bead go undetected without the close proximity of the Acceptor bead. As a result, only a very low background signal is produced (Figure 1B).

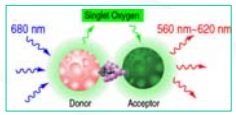

Figure 1A. When biological interactions bring the Donor and Acceptor beads into close proximity, a highly amplified signal with output in the 520-620 nm range is generated.

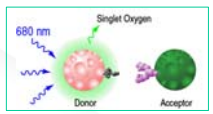

Figure 1B. When the Donor and Acceptor beads are not in close proximity, the reactive oxygen decays and there is no detectable signal generated.

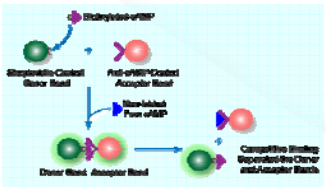

Figure 2. AlphaScreen cAMP assay principle.

AlphaScreen cAMP assay is a competitive assay. The production of cAMP by cells is detected by competing with exogenously added biotinylated cAMP that is recognized by an anti-cAMP antibody conjugated to the Acceptor bead. A decrease in signal is observed with an increase in intracellular cAMP produced. In the absence of intracellular cAMP a maximal signal is detected (Figure 2).

ASSAY PROTOCOL

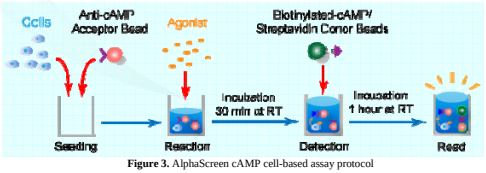

Figure 3. AlphaScreen cAMP cell-based assay protocol

As illustrated in Figure 3, A431 cells were grown in formulated EME medium, then cells were harvested and dispensed using the BioRAPTR FRD workstation into Greiner Bio-One Cellstar® white 384-well microplates at a density of 4,000 cells per well in a volume of 2.5 µL per well, followed by the dispense of 2.5 µL acceptor beads. The cells were then treated with 5 µL agonists (forskolin or isoproterenol) at varies concentrations as illustrated in Figure 4A and 4B. After 1 hour incubation at room temperature, the Biotinylated-cAMP and donor beads were dispensed into each well to start the competition reaction. After 30 min incubation at room temperature, the plate was read using PARADIGM platform to determine the cAMP level.

BIORAPTR FRD WORKSTATION SETUP

BioRAPTR 8-Tip Head Setup

Figure 4A. The AlphaScreen cAMP assay setup on the 8-tip BioRAPTR FRD workstation for standards, agonists stock solutions and assay kit reagents.

BioRAPTR Dispensing Table

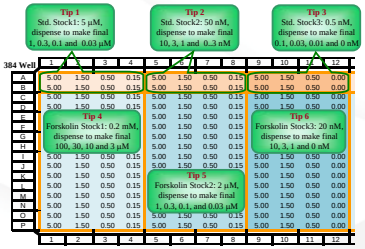

Figure 4B. The AlphaScreen cAMP assay dispensing table for the 8-tip BioRAPTR FRD workstation, for standards, agonists stock solutions and assay kit reagents to make the final reaction volume of 25 µL per well.

RESULTS

cAMP Standards and Formula

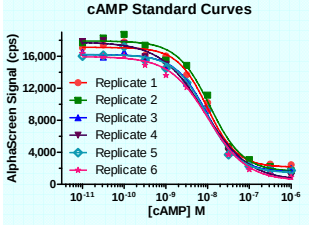

Table 1. cAMP standards dispensing table.

Std. Stocks	Dispense Vol. (µL)	[cAMP] nM
5 µM	5	1000
	1.5	300
	0.5	100
	0.15	30
0.05 µM	5	10
	1.5	3
	0.5	1
	0.15	0.3
0.5 nM	5	0.1
	1.5	0.03
	0.5	0.01
	0	0

Cell Type	Z' factor	LogEC50	CV%	Replicates
A431	0.73	-8.0 ± 0.02	0.62%	n=6

Figure 5. Six replicates of cAMP standard curves with the dispensing volumes of stocks listed in Table 1 (to final reaction volume of 25 µL).

$$z' = 1 - 3\times\frac{(\sigma_p + \sigma_n)}{(\mu_p - \mu_n)} \qquad CV = \frac{\text{Std deviation}}{\text{Mean ratio}} \times 100$$

Z' factor is the statistical parameter used to evaluate how much the dynamic range of an assay exists outside the 99% confidence intervals for the positive and negative controls. It indicates how small an effect in an assay can be reliably ascertained relative to data variation. Z-factor can be computed from the mean (µ) and standard deviation (σ) of both the positive (p) and negative (n) controls (μp, σp, μn, σn, respectively). Z' ranges from 0 to 1.0, which between 0 and 0.5 is marginal; between 0.5 and 1.0 is an excellent assay.

Forskolin Dose Responses in A431 Cells

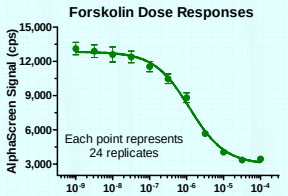

Table 2. Forskolin stocks dispensing table.

Forskolin Stocks	Dispense Vol. (µL)	[Forskolin] µM
0.2 mM	5	100
	1.5	30
	0.5	10
	0.15	3
2 µM	5	1
	1.5	0.3
	0.5	0.1
	0.15	0.03
20 nM	5	0.01
	1.5	0.003
	0.5	0.001
	0	0

Agonist	Z'	LogEC50	CV%	Replicates
Forskolin	0.74	-5.90 ± 0.02	1.92%	n=24

Figure 6. Forskolin dose responses in A431 cells.

isoproterenol Dose Responses in A431 Cells

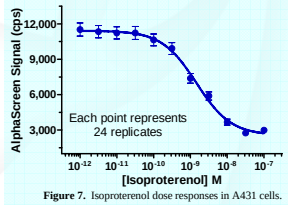

Table 3. isoproterenol stocks dispensing table.

Isoproterenol Stocks	Dispense Vol. (µL)	[Isoproterenol] nM
2 µM	5	1000
	1.5	300
	0.5	100
	0.15	30
0.02 µM	5	10
	1.5	3
	0.5	1
	0.15	0.3
0.2 nM	5	0.1
	1.5	0.03
	0.5	0.01
	0	0

Agonist	Z'	LogEC50	CV%	Replicates
isoproterenol	0.60	-8.86 ± 0.02	1.12%	n=24

Figure 7. Isoproterenol dose responses in A431 cells.

A431 cells were seeded in a white 384-well microplate (4,000 cells per well in a volume of 2.5 µL) using BioRAPTR FRD workstation. The cells were treated with agonist forskolin (Figure 6) or isoproterenol (Figure 7) for 1 hr at room temperature, followed by incubation with donor beads and Biotinylated-cAMP in the final total volume of 25 µL. After 30 min incubation in the competitive reaction reagents, the agonists-induced increases of cellular cAMP, reflected as the decreases of the AlphaScreen signals, were detected on the PARADIGM platform using AlphaScreen detection cartridge. The data suggest that forskolin, which resensitizes cell receptors by directly activating the enzyme adenylyl cyclase and increasing the intracellular levels of cAMP, can induce A431 cellular cAMP increase at the EC50 of 1.2 µM; Isoproterenol, can increase the A431 cell intracellular cAMP level by activating β2-adrenergic receptor at the EC50 of 1.4 nM after 1 hr incubation.

CONCLUSIONS

- The BioRAPTR FRD workstation and PARADIGM AlphaScreen detection cartridge were successfully used for automation and detection of cell-based cAMP assays in regular 384-well plate format.
- The data show that the combination of BioRAPTR FRD workstation and PARADIGM AlphaScreen detection cartridge is capable of rapidly and precisely performing AlphaScreen Assay with low %CV value and Z' values greater than 0.5.

Labware and Consumables

Materials	Manufacturer	Part No.
Hardware		
BioRAPTR FRD Workstation 8-tip Head	Beckman Coulter	B18492
PARADIGM AphaScreen 384 HTS Detection Cartridge PARADIGM Base Instrument	Beckman Coulter	A68247 A41574
Labware		
Laboratory Bottles, High-Density PE, Wide Mouth, 30 mL	Nalgene	2104-0001
Laboratory Bottles, High-Density PE, Wide Mouth, 60 mL	Nalgene	2104-0002
CellStar, 384W Microplate, PS, TC, ST, WHT	Greiner Bio-One	781080
Reagents		
PerkinElmer cAMP Assay kit	PerkinElmer Life Sci.	6760625D

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