Rapid and Ultra-Sensitive Automated Detection of cAMP and IP_3 Using the Homogeneous and Non-Radioactive HTS Platform AlphaScreen™

PerkinElmer™ life sciences.

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Abstract

G-protein coupled receptors (GPCRs) represent one of the most important targets in HTS. Functional responses of GPCRs include either an increase in inositol-trisphosphate (IP_3) followed by changes in intracellular calcium concentrations or a modulation (increase or decrease) of cAMP levels. Using AlphaScreen, we have developed cell-based assays to quantify both IP_3 and cAMP levels following GPCR stimulation. In contrast to many lower throughput technologies, the AlphaScreen IP_3 and cAMP assays are non-radioactive, homogeneous and fully compatible with automation. The IP_3 assay is based on the binding of a biotinylated IP_3 analog to an IP_3 binding protein. The protocol involves stimulating cells with agonist, followed by a quench step and addition of the detection reagents. Increased intracellular levels of IP_3 following GPCR activation leads to a proportional signal decrease. The cAMP assay involves the binding of a biotinylated cAMP analog to anti-cAMP antibodies. Cells are stimulated, followed by a combined cell lysis / detection step. Increasing concentrations of cAMP, as observed following forskolin or G_i coupled receptor activation, results in a concentration-dependent signal decrease. Stimulation of G_i coupled receptors results in a concentration-dependent signal increase. Here we show HTS data for both the IP_3 and cAMP assays obtained using the CCS Packard PlateTrak automated liquid handling workstation. The AlphaScreen cAMP and IP_3 assays were shown to be robust, highly sensitive and reproducible under automated conditions.

Methods (cAMP)

Standard Curves
Using a CCS PlateTrak automated liquid handling workstation, the following were added in a 96 round bottom well microplate:
- 10 µL of anti-cAMP Acceptor beads diluted in stimulation buffer at 15 µg/ml (with or without cells)
- 10 µL of cAMP dilutions made in stimulation buffer.
  (Incubate the plate 30 minutes at RT)
- 30 µL of Detection Mix.
  (Incubate the plate 1h at RT and read on the AlphaQuest analyzer)

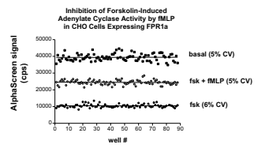
Cell-based Assay
Using a CCS PlateTrak automated liquid handling workstation, the following were added in a 96 round bottom well microplate:
- 10 µL of anti-cAMP Acceptor beads (15 µg/ml final) together with 3000 cells diluted in stimulation buffer.
- 10 µL Forskolin (10 µM) diluted in the stimulation buffer.
  (Incubate the plate 30 minutes at RT)
- 30 µL of Detection Mix.
  (Incubate the plate 1h at RT and read on the AlphaQuest analyzer)

Stimulation buffer: HBSS + 0.1 % BSA + 0.5mM IBMX.
Lysis buffer: 5mM Hepes pH 7.4, 0.1% BSA, 0.3% tween 20.
Detection Mix: Donor beads at 20 µg/ml + biotin-cAMP 10 nM, diluted in the lysis buffer

All cAMP methods presented here have been automated using either a CCS PlateTrak or Multimek automated liquid handlers

cAMP Cell-Based Assay

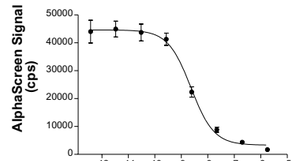
Gαi / FPR1a - (ProxiPlate-384, 15 µl)



Z' = 0.5

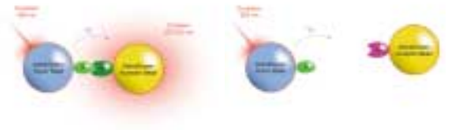
Using a Multimek automated liquid handler, the cAMP assay was miniaturized to 15 µl total volume in a 384 well ProxiPlate. Three different microplates were filled with 3000 cells per well (2.5 µl). Forskolin, used to a concentration equivalent to its EC_{75} (2.5 µl, 10 µM final), was added to 88 wells to stimulate the release of cAMP whereas assay buffer was added to 88 wells to measure the basal levels of cAMP. The agonist fMLP (2.5 µl, 100 nM final) was added to 88 wells to stimulate the FPR1a receptor and inhibit adenylate cyclase activity. Overall intraplate variability was less than 7 %. Z' value for fMLP-induced adenylate cyclase inhibition was 0.5.

IP_3 Standard Curve



Standard curves were performed by adding increasing concentrations of IP_3 (0-2 µM final) to wells containing 5000 cells/well. An IC_{50} value of 2 nM was measured and IP_3 limits of quantification ranged from 0.2 nM to 20 nM.

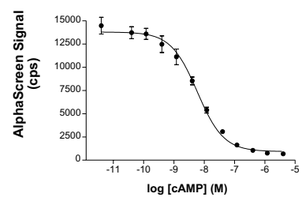
Principles of AlphaScreen



AlphaScreen is a bead based non-radioactive Amplified Luminescent Proximity Homogeneous Assay. When biological interactions bring the Donor and Acceptor beads into close proximity, reactive oxygen, generated by irradiation of the Donor beads, initiates a luminescence/fluorescence cascade in the Acceptor beads. This process leads to a highly amplified signal with light output in the 520-620 nm range.

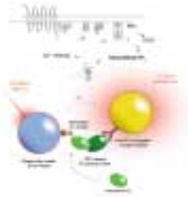
When the Acceptor and Donor beads are not in proximity, the reactive oxygen decays and only a very low background signal is generated.

cAMP Standard Curve



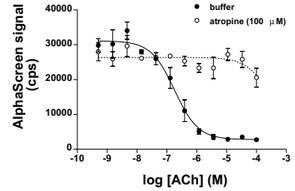
The standard curve was performed by adding increasing concentrations of cAMP (0-5 µM final) to the wells containing 3000 cells. An IC_{50} value of 5 nM was measured and the limits of cAMP quantification range from 0.5 to 50 nM.

AlphaScreen IP_3 Assay



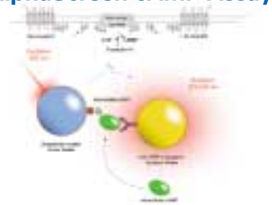
The IP_3 assay is based on the binding of a biotinylated IP_3 analog to an IP_3 binding protein, expressed as a GST fusion. In the presence of both streptavidin Donor beads and anti-GST antibody coated Acceptor beads, the AlphaScreen signal can be generated. The protocol involves stimulating cells with agonist, followed by a quench step and addition of the detection reagents. Increased intracellular levels of IP_3 following GPCR activation leads to a proportional signal decrease.

Cell-Based Assay (IP_3)



Agonist dose-response curve was performed by adding increasing concentrations of Acetylcholine manually (0 -100 µM final). An EC_{50} of 184 nM was measured. Addition of an excess of the antagonist atropine (100 µM) inhibited the acetylcholine-induced IP_3 release.

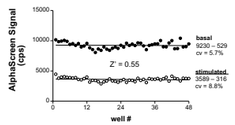
AlphaScreen cAMP Assay



The cAMP assay involves the binding of a biotinylated cAMP analog to anti-cAMP antibodies attached to Acceptor beads. The AlphaScreen signal can be generated by using streptavidin-coated Donor beads. Cells are stimulated, followed by a combined cell lysis / detection step. Increasing concentrations of cell-stimulated cAMP, following forskolin or G_s coupled receptor activation, results in a concentration-dependent signal decrease. Stimulation of G_i coupled receptors results in a concentration-dependent signal increase.

cAMP Cell-Based Assay

Forskolin stimulation of CHO cells - (96 well plates, 100 µl)



intraplate	parameter	basal	stimulated	Z'
1	average	9469	3586	0.50
	stdev	653	337	
	cv%	6.9	9.4	
2	average	9432	3382	0.55
	stdev	599	301	
	cv%	6.4	8.9	
3	average	9230	3589	0.55
	stdev	524	316	
	cv%	5.7	8.8	
interplate	average	9377	3519	0.53
	stdev	129	119	0.03
	cv%	1.4	3.4	6.3

The cAMP cell-based assay is sensitive, reproducible and highly robust. Forskolin, used to a concentration equivalent to its EC_{75} (10 µM), was added to half of the wells to stimulate the release of cAMP. The remaining wells contained assay buffer to measure the basal levels of cAMP. Intraplate variability was less than 10 % for the stimulated signal and less than 7 % for the basal signal. The slightly higher variability observed in the stimulated signal may be attributable to greater contributions from background and dark noise. Very low interplate variation was measured. Z' values were above 0.5.

Methods (IP_3)

Standard Curves
Using a CCS PlateTrak automated liquid handling workstation, were added in a 96 round bottom well microplate:
- 10 µL of stimulation buffer in presence or in the absence of cells.
- 10 µL of IP_3 dilutions diluted in PBS.
  (Incubate 30 sec.)
- 10 µL of Quench solution
  (incubate 30 min at RT)
- 60 µL of IP_3-bp (2 units per well) diluted in detection buffer
- 10 µL of Detection Mix.
  (Incubate the plate 1h at RT and read on the AlphaQuest analyzer)

Cell-based Assay
The following were added manually in a 96 round bottom well microplate:
- 10 µL of cells.
- 10 µL of Acetylcholine dilutions diluted in PBS (with or without Atropine).
  (Incubate 30 sec.)
- 10 µL of Quench solution
  (incubate 30 min at RT)
- 60 µL of IP_3-bp (2 units per well) diluted in detection buffer
- 10 µL of Detection Mix.
  (Incubate the plate 1h at RT and read on the AlphaQuest analyser)

Stimulation buffer: PBS + 15 mM Hepes pH 7.4.
Quench solution: PCA 1.05%
Detection buffer: Tris 100 mM pH 8.7, 0.1 % BSA, 0.1% Tween 20.
Detection Mix: Donor beads at 20 µg/ml + Acceptor beads 20 µg /ml + biotin-IP_3 analog 10 nM, diluted in the detection buffer

The IP_3 standard curve was automated on a CCS PlateTrak. The cell stimulation was performed manually.

Conclusions

Why AlphaScreen to quantify second messengers?

- Allows characterization of either agonists or antagonists
- Highly sensitive, detect femtomoles of cAMP or IP_3
- Non radioactive, no extraction or wash steps required
- Can be performed in 2 hours, including incubations
- Miniaturizable to 384-well format
- Compatible with automation
- Inexpensive

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