APPLICATION NOTE

Complete cAMP workflow solution using the CatchPoint cAMP Fluorescent Assay Kit

Introduction

In this study, we demonstrate how the CatchPoint® cAMP Fluorescent Assay Kit combined with the SpectraMax® i3 Multi-Mode Detection Platform can be used to monitor the response of HEK293 cells to forskolin, an activator of adenylate cyclase (Figure 1).

G-Protein Coupled Receptors (GPCRs) are important transmembrane proteins that translate extracellular signals into intracellular responses. These intracellular responses are comprised of cell-signaling cascades that initiate changes in protein activity and expression within the cell [1].

Cyclic adenosine 3', 5'-monophosphate (cAMP) is a secondary messenger that works downstream of GPCR activation. Upon ligand binding to a GPCR, a conformational change occurs, activating the receptor and in turn activating a G protein. Further signal transduction depends on the type of G protein activated. Activation of G_s leads to upregulation of cAMP by adenylate cyclase, which activates protein kinase A, causing phosphorylation of targets involved in processes such as dopamine signaling, gluconeogenesis, vasodilation, as well as mitogenesis and oocyte maturation [2–6].

The CatchPoint cAMP Fluorescent Assay Kit measures cAMP levels via a competitive immunoassay (Figure 2). The assay requires only a single washing step, and readings can be taken in as little as 10 minutes or as long as 24 hours following substrate addition.

Materials

- CatchPoint cAMP Fluorescent Assay Kit (Molecular Devices cat. #R8088)
- HEK293 cells (ATCC cat. #CRL-1573)
- Krebs-Ringer bicarbonate buffer - KRGB buffer (Sigma cat. #K4002)
  - Sodium bicarbonate (Sigma, cat. #S5761)
- Phosphate-buffered saline (PBS, Life Technologies cat. #10010)
- Phosphodiesterase inhibitor, 3-isobutyl-1-methylxanthine (IBMX, Sigma cat. #I7018)
- 3% hydrogen peroxide (H_2O_2) solution
- Minimum Essential Medium with Earle's salts and L-glutamine (Corning cat. #10-010)
  - Fetal bovine serum (Gemini Bio-Products cat. #100-106)
  - Penicillin-streptomycin (Life Technologies cat. #15070-063)
- Forskolin (Sigma cat. #F6886)
- Poly-D-Lysine Coated 96-well Microplates (Corning cat. #354413)
- SpectraMax i3 Multi-Mode Microplate Reader
- SpectraMax® MiniMax™ 300 Imaging Cytometer
- MultiWash+™ Microplate Washer

Benefits

- **Accurate measurements of GPCR activity through cAMP detection**
- **Assay requires only a single wash step**
- **High signal stability (10 minutes to 24 hours)**
- **Z' factor of 0.91**

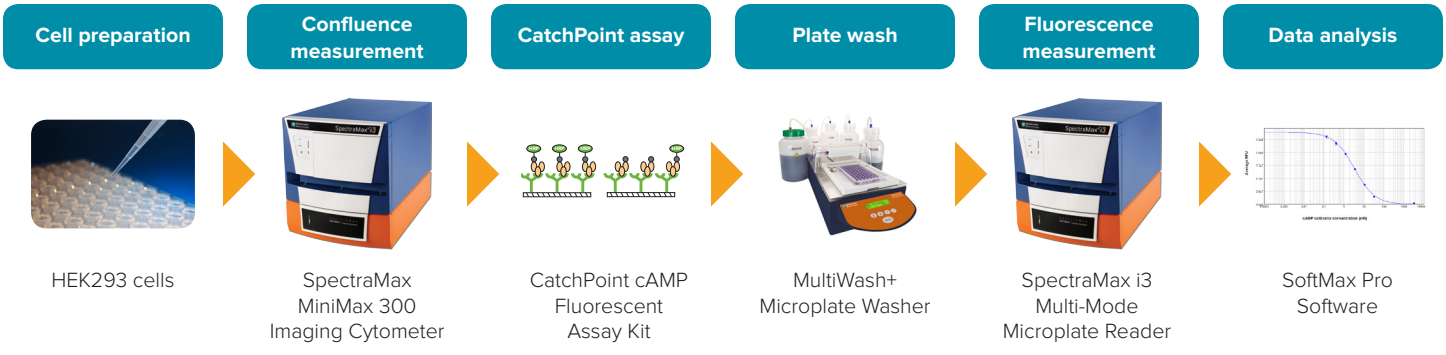


Figure 1: CatchPoint cAMP workflow.

Methods

HEK293 cells were cultured in complete growth medium (MEM + 10% FBS + 1% Pen/Strep) and grown to 80-90% confluence in T75 flasks. Cells were harvested using 0.05% trypsin, and 25,000 cells were seeded in each well of a poly-D-lysine coated, 96-well, black-wall, clear-bottom microplate (25,000-100,000 cells/well is an acceptable range). Cells were allowed to adhere for at least 18 hours in a 37°C incubator with 5% CO_2.

On the day of assay, cell confluence was measured using the transmitted light channel of the SpectraMax MiniMax 300 Imaging Cytometer and StainFree™ Cell Detection Technology, which enables accurate measurement of cell counts or percent area covered by cells (confluence) for quality control purposes (Figure 3). Rows of cells with high cell confluence and low inter-well variability were selected for the CatchPoint assay. Cells were pre-stimulated with 0.75 mM IBMX for 10 minutes at room temperature. They were then treated with a dilution series of forskolin starting at 1,000 µM with a 1:3 dilution series for 15 minutes at 37°C. Cells were then lysed according to the CatchPoint assay protocol.

The CatchPoint assay was performed as described in the product insert. A cAMP calibrator curve was performed to verify assay performance, as well as provide a means of calculating the amount of cAMP in cellular assay samples. Washing was performed on the MultiWash+ Microplate Washer. Samples were read on a SpectraMax i3 Multi-Mode Microplate Reader 30 minutes after StopLight Red substrate addition. All data analysis and curve fitting were performed with SoftMax® Pro Software. A preconfigured protocol is available in the software's protocol library.

Results

To assess cell confluence before stimulation, the MiniMax cytometer and StainFree technology were used to measure percent cell coverage in wells of the microplate (Figure 3). Rows A and C were chosen for the assay due to consistent percent confluence from well to well. Triplicate samples were taken from each treated well and assayed.

Figures 4 and 5 show results for the CatchPoint cAMP calibrator curve and

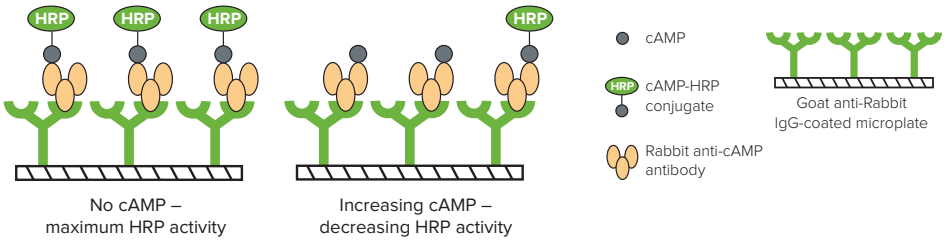


Figure 2: CatchPoint cAMP assay mechanism. Unlabeled cAMP produced by cells competes with cAMP-HRP conjugate for binding to anti-cAMP antibodies. Increasing amounts of cellular cAMP are detected through decreasing HRP activity.

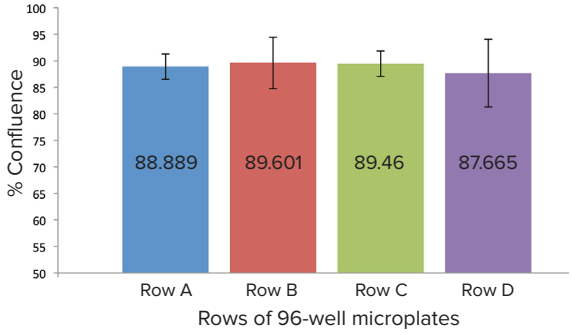


Figure 3: HEK293 cell percent confluence determined using the MiniMax cytometer and StainFree analysis. Rows A and C had similar cell confluence and the lowest variability between wells and were selected for assay.

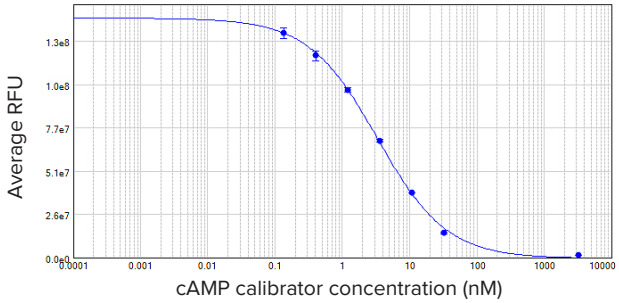


Figure 4: cAMP calibrator curve. EC_{50} was 3.3 nM, similar to previously published results. Samples were run in duplicate.

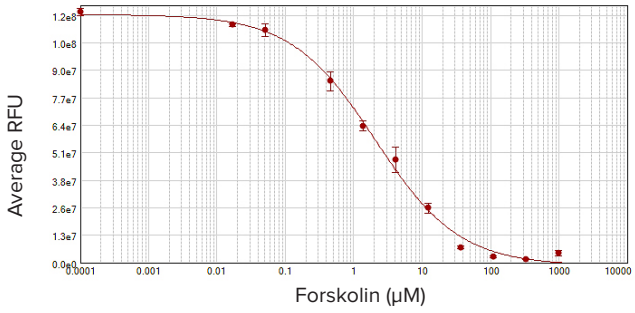


Figure 5: Cell-based assay performed with HEK293 cells. A forskolin concentration-response curve was run, starting at 1,000 µM with a 3-fold dilution series, with samples run in duplicate. EC_{50} was 2.3 µM.

cell-based assay. Both curves were generated using the 4-parameter curve fit in SoftMax Pro Software. The calibrator curve had an EC_{50} of 3.3 nM, which agreed closely with previous published data [7]. The Z' factor was 0.91.

The forskolin concentration-response curve had an EC_{50} of 2.3 µM, which is consistent with expected results for HEK293 cells assayed in an adherent format.

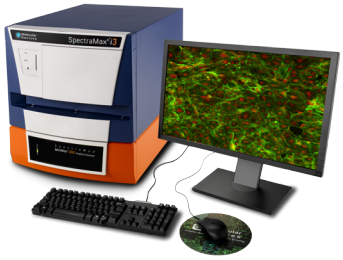
Conclusion

The CatchPoint cAMP Fluorescent Assay Kit allows for accurate measurements of GPCR activity through cAMP detection. High signal stability (10 minutes to 24 hours), excellent Z' factor, and the ability to be performed on plate readers with a fluorescence intensity detection mode make the CatchPoint assay a versatile choice for high-throughput screening.

Ordering information

Reagent	Description	Part number
CatchPoint cAMP 96-well Explorer Kit	192 reactions	R8088
CatchPoint cAMP 96-well Bulk Kit	960 reactions	R8089
CatchPoint cAMP 384-well Explorer Kit	768 reactions	R8044
CatchPoint cAMP 384-well Bulk Kit	7680 reactions	R8053

Compatible with these Molecular Devices systems



SpectraMax i3 Platform with SpectraMax MiniMax 300 Imaging Cytometer

MultiWash+ Microplate Washer

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