A Multi-Array™ Technology Based Assay for cAMP

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Abstract

We have developed a cAMP assay on the MSD Multi-Array™ platform that provides a convenient methodology for detecting the activation of G protein coupled receptors (GPCR) in whole cells or membrane fragments. The assay is a competitive immunoassay that uses an anti-cAMP antibody and a modified cAMP carrying an electrochemiluminescent label. The protocol is simple, doesn't require a wash step and is suitable for HTS. GPCR activation studies can be performed in 60 minutes (including 30 minutes for cell or membrane stimulation) and achieve Z' scores of 0.6. Users can process 150 384-well plates in 3 hours. The assay has a sensitivity for cAMP of 8-15 nM, a dynamic range of 3 log units and displays robust performance when used to measure the activation of MC5 expressing cells with agonist: S/B of 5-10 and acceptable %CVs (10 – 15% in presence of cells, <10% in presence of membranes).

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❷ Meso Scale Discovery Multi-Array Technology

SECTOR™ Imager 6000

SECTOR Imager 6000 Features:

- Highly sensitive, ultra high-throughput
- Designed for high-throughput screening (HTS) and automated assay development
- Ideal for assay development
- Custom optics with telecentric lens design and CCD imaging detection
- High-speed motion control systems
- Electrochemiluminescence (ECL) detection

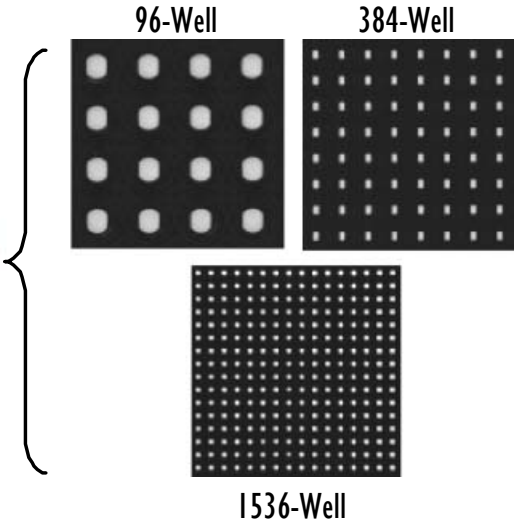

Plate Features:

- Disposable Plates
- Carbon Electrodes with high binding capacity
- Suitable electrochemistry for ECL
- Biocompatible: direct immobilization of avidin, IgG, membrane fragments, intact cells, etc.
- Functional Assays: simple binding reactions, GPCRs, enzyme cascades, post-translational modification, etc.

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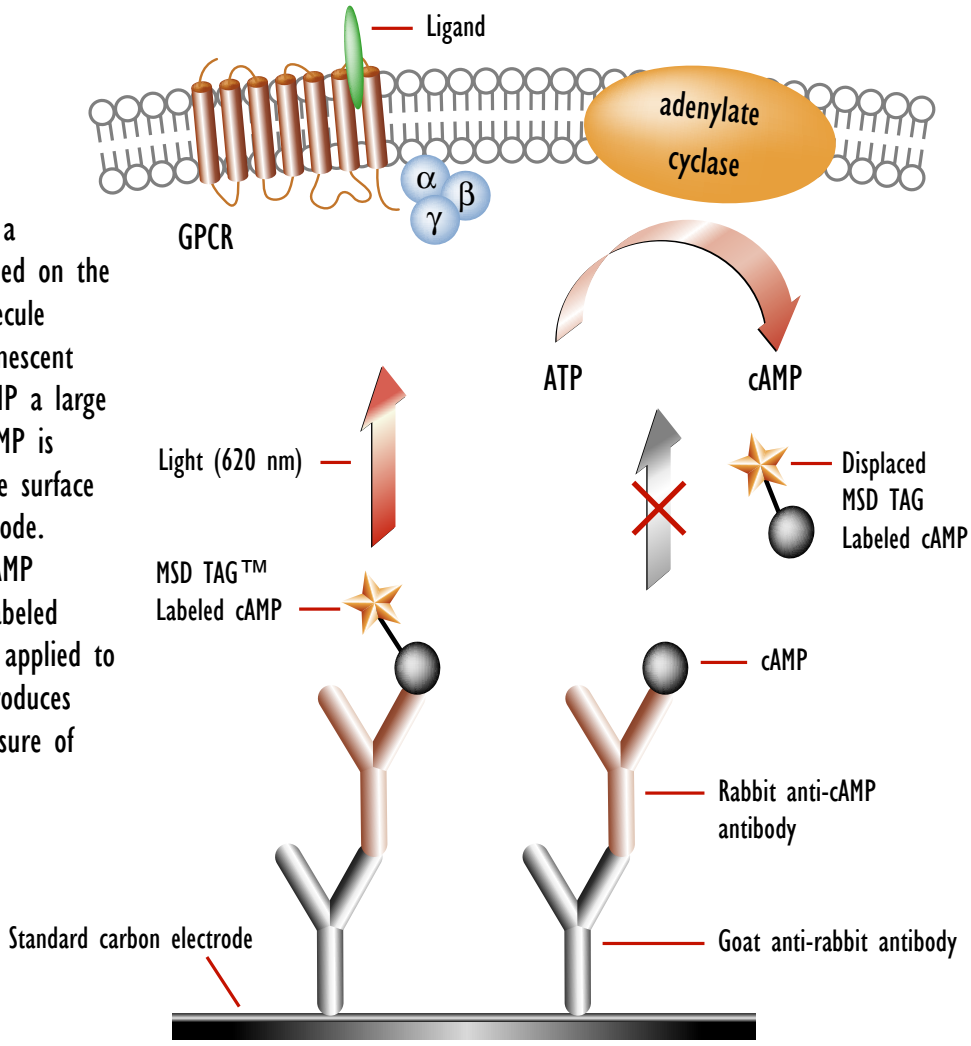
❸ Principle of the MSD Cyclic AMP Assay

The MSD cyclic AMP assay is a competitive immunoassay based on the displacement of a cAMP molecule carrying an electrochemiluminescent label. In the absence of cAMP a large proportion of the labeled cAMP is bound by an antibody on the surface of a disposable carbon electrode. Elevated concentrations of cAMP proportionally displace the labeled analog. When a potential is applied to the electrode, bound label produces light and a quantitative measure of cAMP is recorded.



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4 cAMP Assay Protocol

Begin with an MSD Multi-Array 384-well plate, coated with Goat anti-rabbit antibody

1. Add compounds of interest (e.g., cAMP standard, GPCR agonist, forskolin, GPCR antagonist, etc.) in 5 µL of solution containing up to 10% DMSO
2. Add 2,500 – 15,000 cells, 2.5 – 5.0 µg membranes, or equivalent lysate in 10 µL buffer of choice (e.g. PBS or RPMI + 10% FCS)

Incubate 30 minutes

3. Add 10 µL lysis/detection mixture containing MSD TAG-labeled cAMP and MSD anti-cAMP rabbit antiserum

Incubate 30 minutes – overnight

4. Add 10 µL 4X MSD Read Buffer T
5. Analyze plate using SECTOR Imager 6000 (~1 minute/plate)

Cells/Compounds

↓

Lysis/Detection Mixture

↓

Read Buffer

↓

Analyze with
SECTOR Imager 6000

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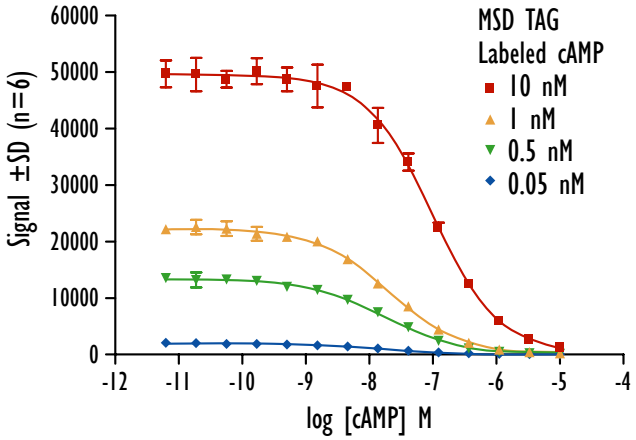
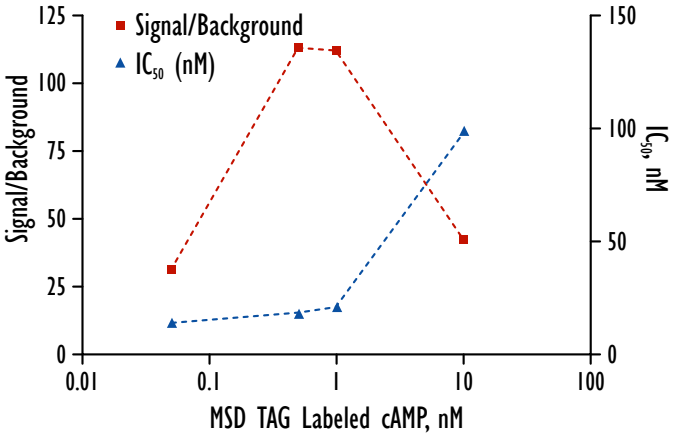
5 Optimization of the Concentration of Labeled cAMP

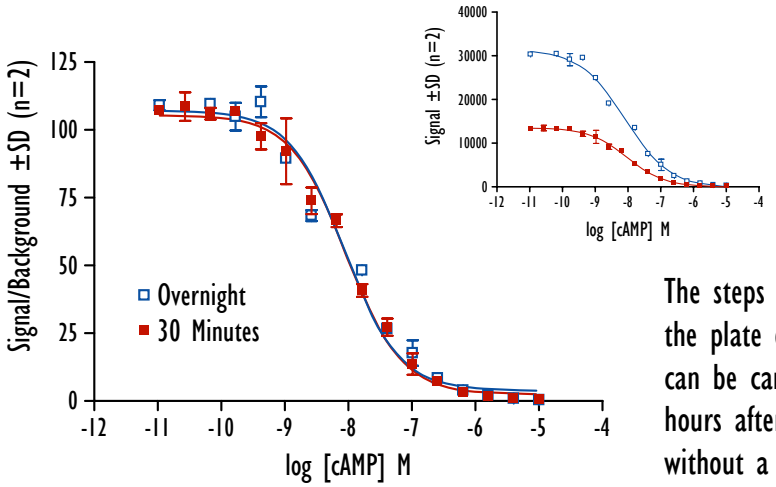




Titrations of a cAMP standard were assayed using various concentrations of MSD TAG labeled cAMP in the lysis/detection buffer. Previous experiments (not shown) determined the labeled species' dissociation constant with the anti-cAMP antibody to be 0.5 nM. Optimal sensitivity for free cAMP was achieved using a concentration of the MSD TAG labeled species near this concentration.

MSD TAG Labeled cAMP, nM	S/B	IC_{50}, nM (95% C.I.)
10	40	99 (86-110)
1	104	21 (18-23)
0.5	99	18 (16-21)
0.05	28	14 (12-18)

6 Flexibility in Scheduling Plate Read



	IC_{50}, nM (95% C.I.)
Overnight	9.4 (7.7-11.5)
30 Minutes	8.8 (6.0-12.8)

The steps of adding MSD Read Buffer T and imaging the plate on the an MSD SECTOR Imager 6000 or 2400 can be carried out as soon as 30 minutes, or up to 18 hours after the addition of lysis/detection buffer, without a change in sensitivity or signal/background.

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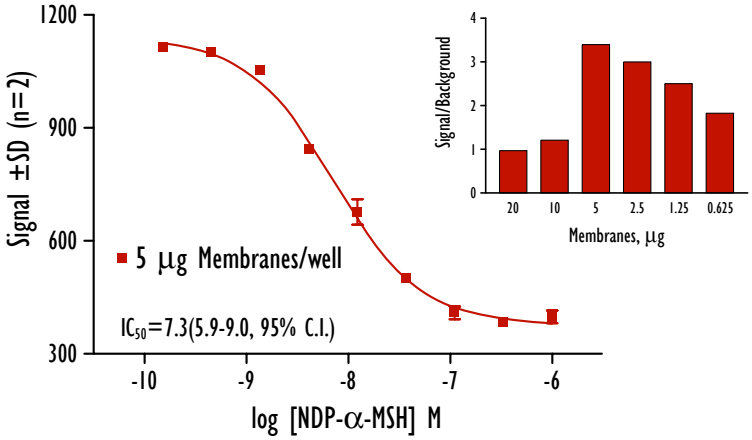
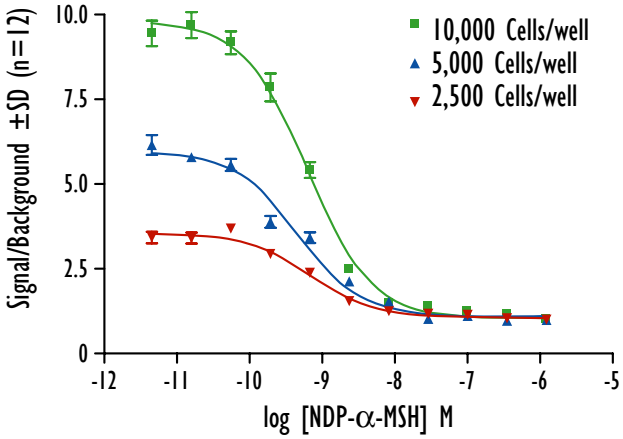
❼ cAMP Accumulation from Stimulated Membranes

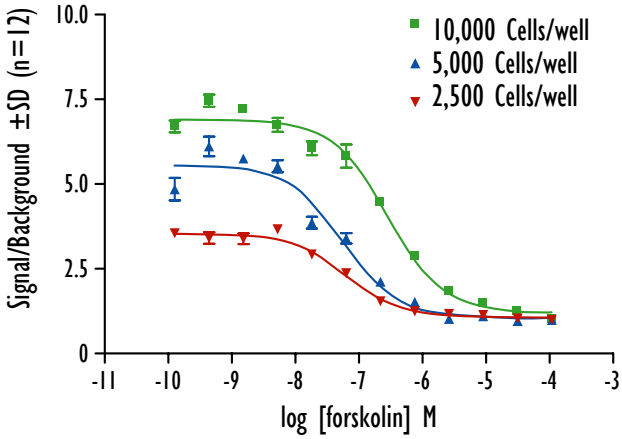
Activation of MC5 receptor in membrane fragments isolated from transfected HEK293 cells was determined using the MSD cAMP assay. A dose-response curve for stimulation with NDP-α-MSH was measured in a 384-well plate format. Optimal performance was obtained with 5 µg of membrane per well.



❽ cAMP Accumulation from Cells Stimulated with Forskolin or NDP-α-MSH



	IC_{50}, nM (95% C.I.)
10,000 cells/well	0.63 (0.52-0.77)
5,000 cells/well	0.47 (0.38-0.59)
2,500 cells/well	0.70 (0.51-0.95)



	IC_{50}, nM (95% C.I.)
10,000 cells/well	0.31 (0.25-0.38)
5,000 cells/well	0.62 (0.47-0.82)
2,500 cells/well	0.69 (0.51-0.94)

HEK293 cells transfected with the MC5 receptor were serially diluted in RPMI 1640 and then stimulated for 30 minutes with increasing concentrations of either NDP-α-MSH or forskolin. A titration of the cells shows that optimal performance was obtained with a cell density of 10,000 cells/well.

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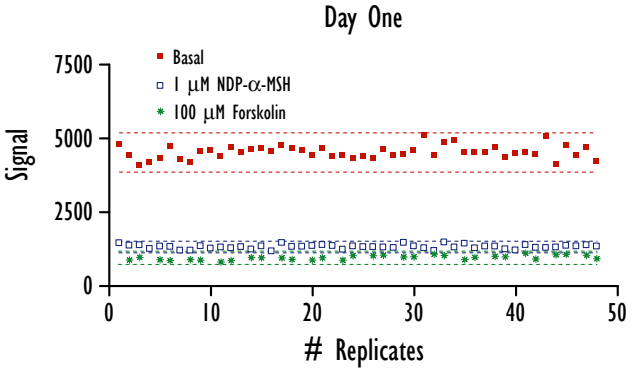
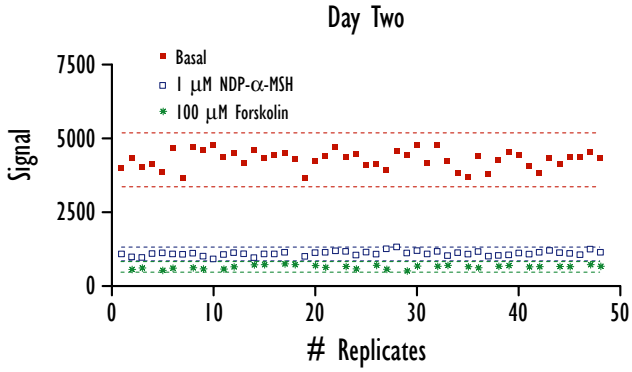
❾ HTS Compatibility



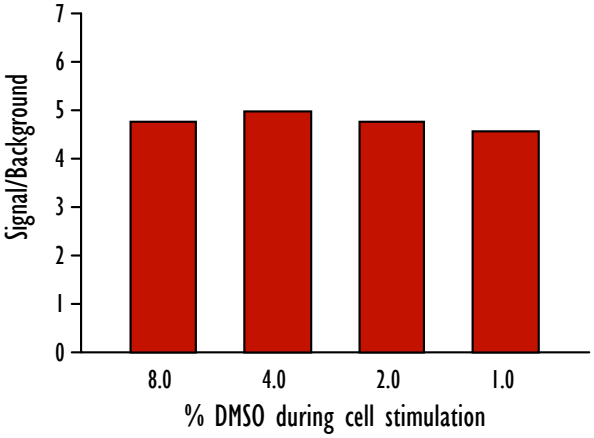
	Average	StDev	%CV	S/B	Z´
Basal	4522	223	4.9		
1 µM NDP-α-MSH	1324	71	5.4	3.4	0.72
100 µM forskolin	961	75	7.8	4.7	0.75

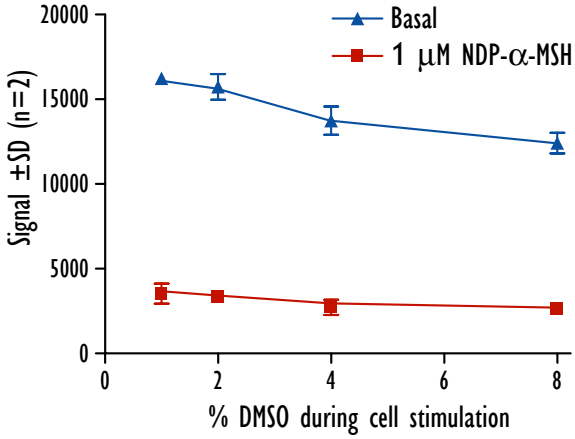


	Average	StDev	%CV	S/B	Z´
Basal	4277	304	7.1		
1 µM NDP-α-MSH	1088	78	7.2	3.9	0.64
100 µM forskolin	650	60	9.2	6.6	0.70

The accumulation of cAMP in response to NDP-α-MSH and, separately, forskolin was compared to unstimulated cells (basal) according to the protocol. The high Z' scores reveal that the assay is robust within an HTS workflow.

❿ DMSO Tolerance of the MSD cAMP Assay





Cells (5,000/well) were challenged with buffer or 1 µM NDP-α-MSH in the presence of increasing concentrations of DMSO. The assay tolerates DMSO at final concentrations of up to 8% without significant change in the signal to background ratio.

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⓫ Technology Performance Comparison

	S/B	Signal Range (counts)	IC_{50} (pmol/well)	Linear Range (pmol/well)
MSD	110	270-30,000	0.225	0.01-10
AlphaScreen™	50	50-2,500	0.358	0.03-30
HTRF™	15.5	20-320	0.302	0.02-3
HitHunter™	15	110-1,650	9.4	2-30
Fluorescence Polarization	2.4	110-240	4.9	1.0-5.0

Competitive data from: Gabriel et al., Assay and Drug Development Technologies, Vol. 1, p 291-303, 2003. Trademarks are the property of their respective owners.

⓬ Conclusions

- A cAMP assay was developed on the MSD Multi-Array platform that is simple and flexible.
- The assay boasts a maximum of 3 addition steps and protocols compatible with cells, membranes, or lysates.
- The time from cell lysis to plate read can be as little as 30 minutes.
- Quantification of cAMP accumulation in cells in response to stimulation of MC5 receptors confirms that the assay is compatible with HTS with a sensitivity of 8-15 nM (unlabeled cAMP), a S/B of 5-10 for cells, a dynamic range of 3 log units, and a Z' score > 0.6, with a throughput of 150 384-well plates per 3 hours.