

# A Novel Homogeneous, Fluorescence-based Technology for Dual Measure of Phosphodiesterases and Their Downstream Effector Kinase Activities in Biochemical and Cell-based Assays

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## Abstract

Measure of post translational modifications, such as the addition of phosphates, their removal or the cleavage of biological substrates provides the most meaningful assessment of a given signal transduction status of a cell. Major transducers of cellular signaling processes are the second messengers cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) via their interaction with downstream targets such as Protein Kinase A (PKA) and Protein Kinase G (PKG). The balance of cAMP and cGMP is tightly controlled by phosphodiesterases (PDEs) through their hydrolysis of the cyclic nucleotide bonds. Here we present a fluorescent sensing approach with which PDE as well as kinase activities can be monitored with one platform. The platform is based on photo-induced electron transfer quench of fluor labeled substrates by a metal ion upon its association to a phosphoryl group present on the substrate. Since the mechanism of electron transfer does not require spectral overlap between donor and acceptor molecules, fluorescence of any fluor can be quenched by one sensor. Hence the platform is ideally suited for multiplexed measurement of various substrate modifications. We show highly sensitive and simultaneous detection of cAMP and cGMP hydrolyses by PDE1C in biochemical assays as well as in lysates of rat brain. Assays are homogeneous and when measured in endpoint mode deliver Z' factors of 0.8. In addition to being suitable for robotics, the platform can be run in kinetic mode, thus greatly simplifying mode of action analysis of inhibitors. Monitoring of PDE4 and PDE5 activities within one well in the presence of specific inhibitors produced IC<sub>50</sub> values, which closely match reference values. Lastly, we demonstrate the correlation between PKA-mediated phosphorylation of fluor-labeled Kemptide with cAMP hydrolysis within lysates of rat brain in one experiment. In conclusion, the platform promises to be a cost effective new tool to gain a better understanding of a drug's action on catalytic events within interconnected cellular pathways.

## Materials and Methods

### Materials

- Fluorescence plate reader (BioTek, Winooski, VT)
- PDE Assay kit (Gyrasol Technologies, Santa Fe, NM)
- Fluorescein-cAMP (Axxora, San Diego, CA)
- TAMRA-cGMP (ABD Bioquest, Sunnyvale, CA)
- PDE enzymes (BPS Biosciences, San Diego, CA)
- PDE Inhibitors (Biomol, Plymouth Meeting, PA)
- 384-well Assay Plates (Cliniplate, Thermo Scientific, Pittsburgh, PA)

### Methods

- Substrates (2  $\mu$ M cAMP, 1  $\mu$ M cGMP), inhibitors and enzymes were combined in wells of a 384 well plate in a total volume of 15  $\mu$ L
- Reactions proceeded for 1 hour at room temperature
- For endpoint monitoring, Sensor was diluted in Sensor Dilution Buffer and 30  $\mu$ L added to wells
- For kinetic monitoring, Sensor was diluted in Assay Buffer and 30  $\mu$ L added to wells

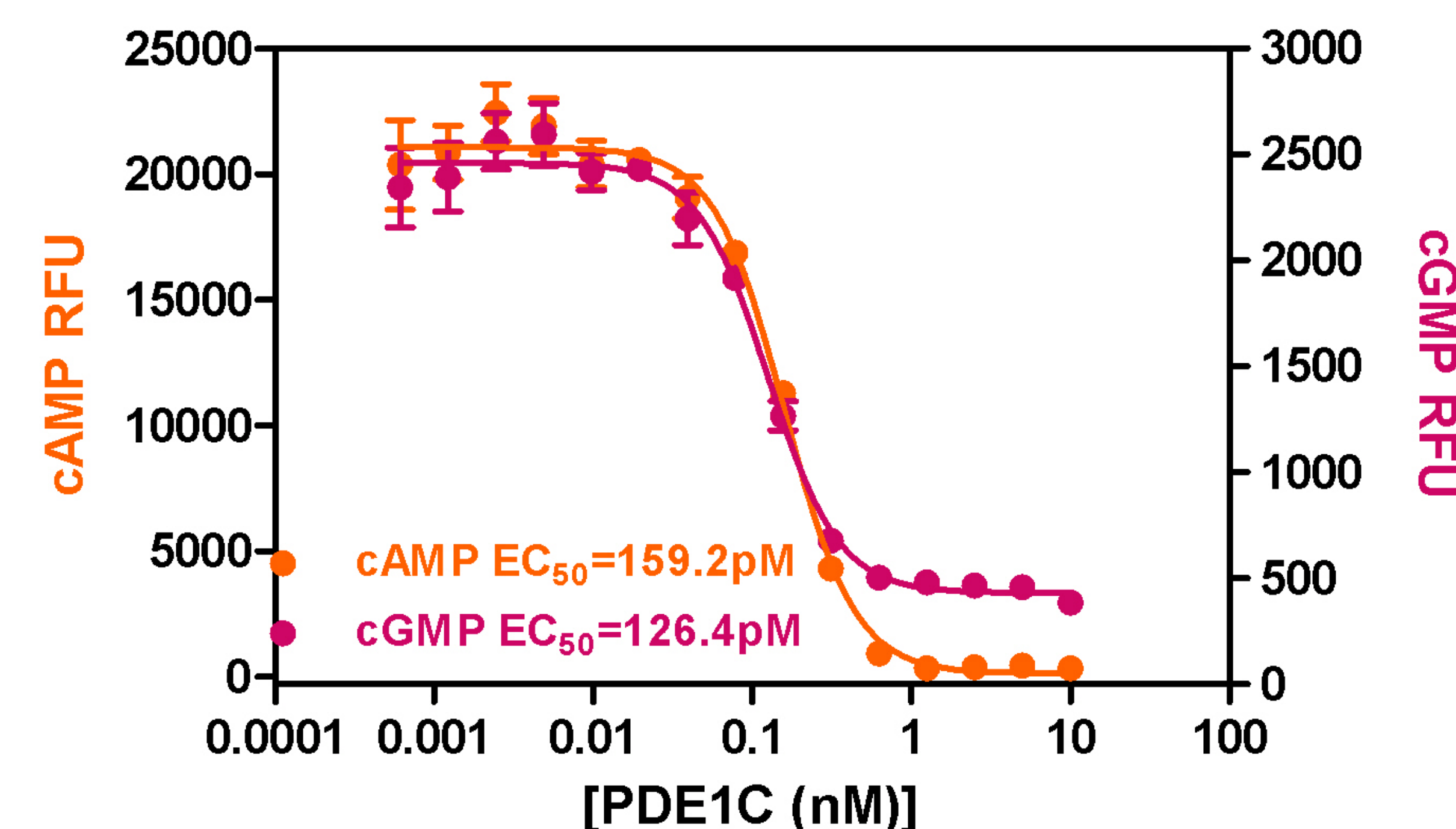
### Synergy 4 Settings

- TAMRA
- Excitation: 540 nm
  - Emission: 580 nm
  - Top probe vertical offset: 6 mm
  - Fluorescein
  - Excitation: 490 nm
  - Emission: 520 nm
  - Top probe vertical offset: 8 mm



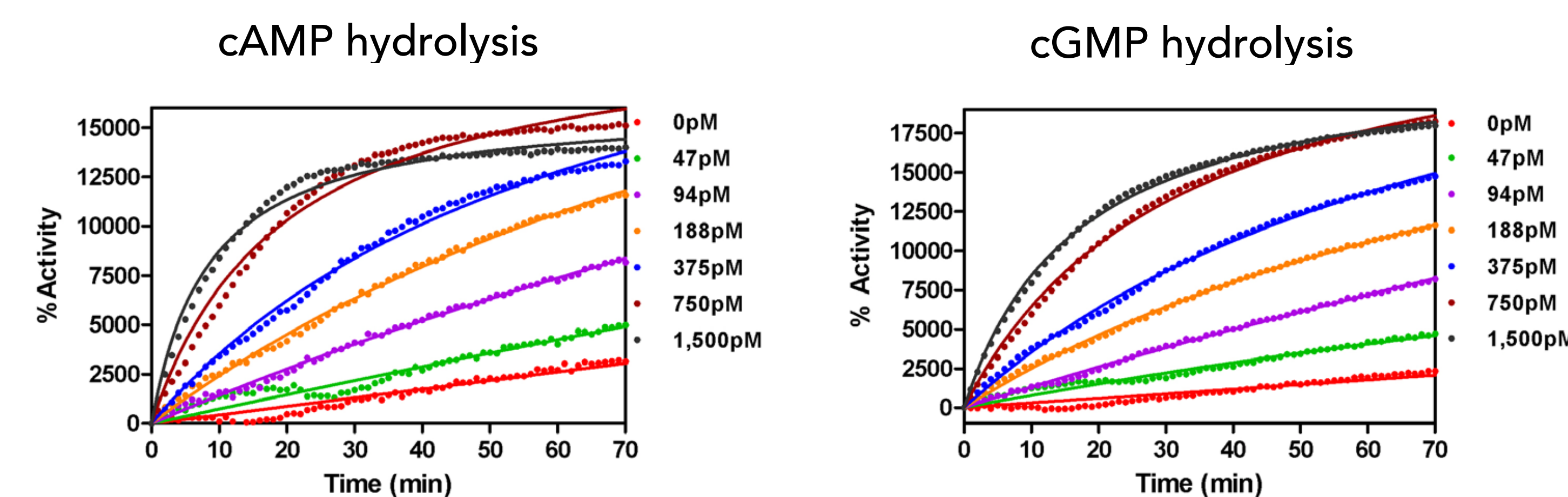
## Simultaneous Monitoring of cAMP/cGMP Hydrolysis by PDE1C

### Endpoint Mode- Biochemical Assay



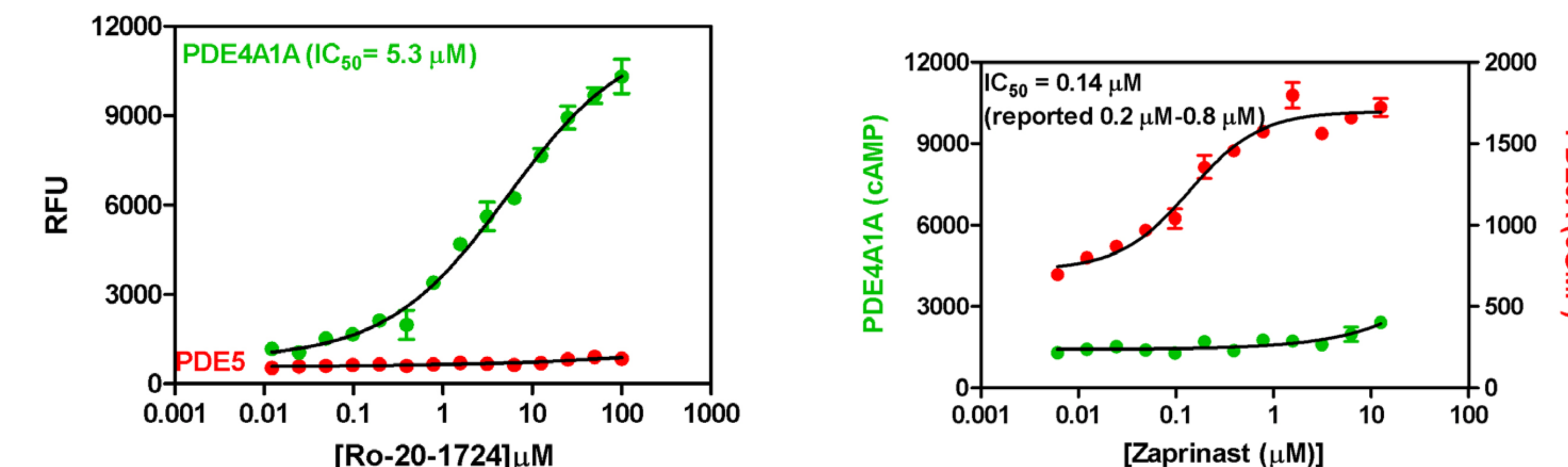
## Simultaneous Monitoring of cAMP/cGMP Hydrolysis by PDE1C in One Well

### Kinetic Mode- Biochemical Assay



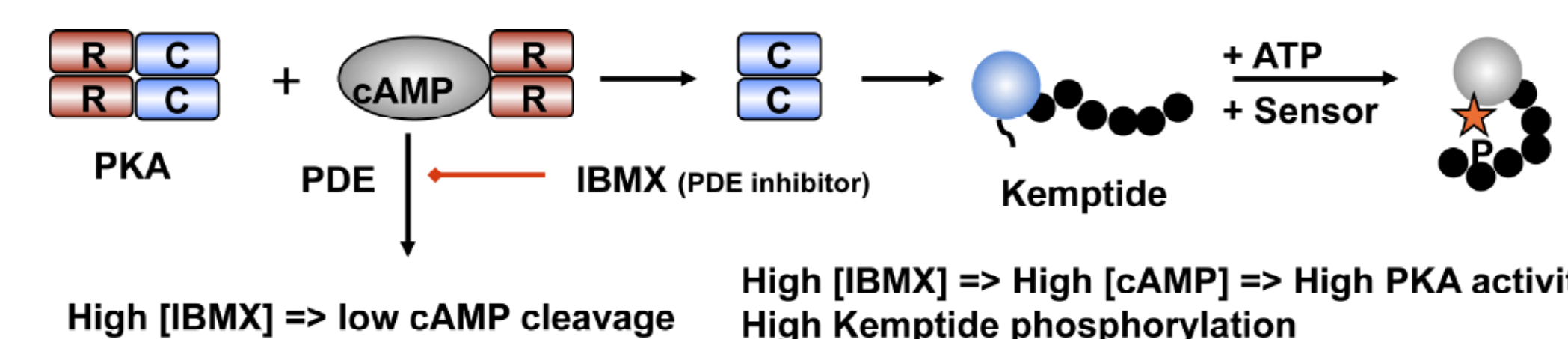
## Simultaneous Monitoring of cAMP/cGMP Inhibition of Two PDEs

### Endpoint Mode - Biochemical Assay PDE4A1A, PDE5A



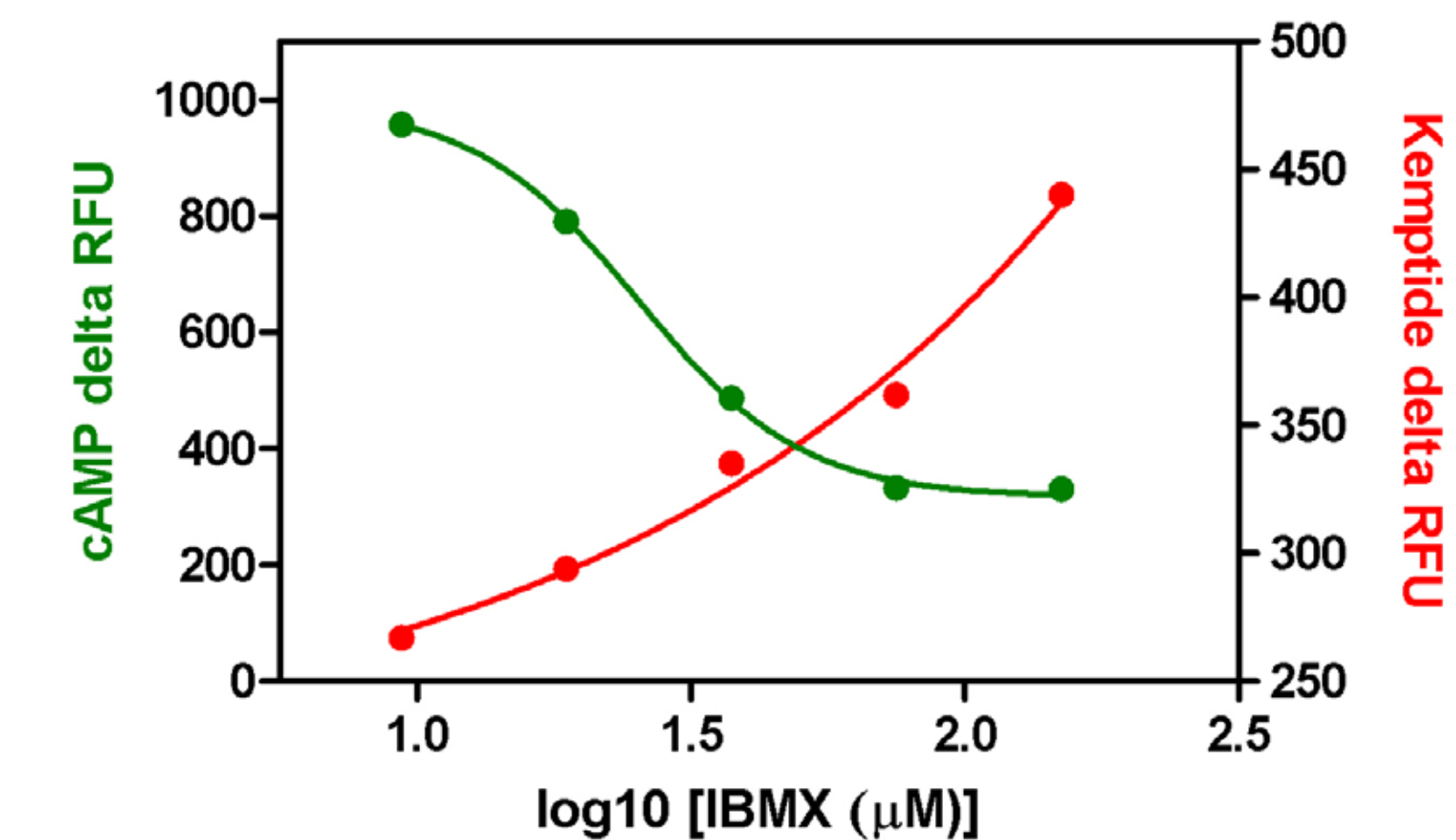
- Ro-20-1724: PDE4A1 inhibitor (IC<sub>50</sub>: 5.3  $\mu$ M; reported: 2  $\mu$ M)
- Zaprinast: PDE5A inhibitor (IC<sub>50</sub>: 0.14  $\mu$ M; reported: 0.2 – 0.8  $\mu$ M)

## Connecting PDE and PKA Activities in Rat Brain Lysates



- PKA consists of 2 Regulatory subunits (R) and 2 Catalytic subunits (C)
- cAMP dissociates C subunits which become active and phosphorylate Kemptide
- Gyrasol Sensor can be used to monitor Kemptide phosphorylation and cAMP hydrolysis
- IBMX is PDE inhibitor: with increasing concentration:
  - cAMP hydrolysis is reduced
  - Kemptide phosphorylation is increased

### Endpoint Mode- Rat Brain Lysate Assay

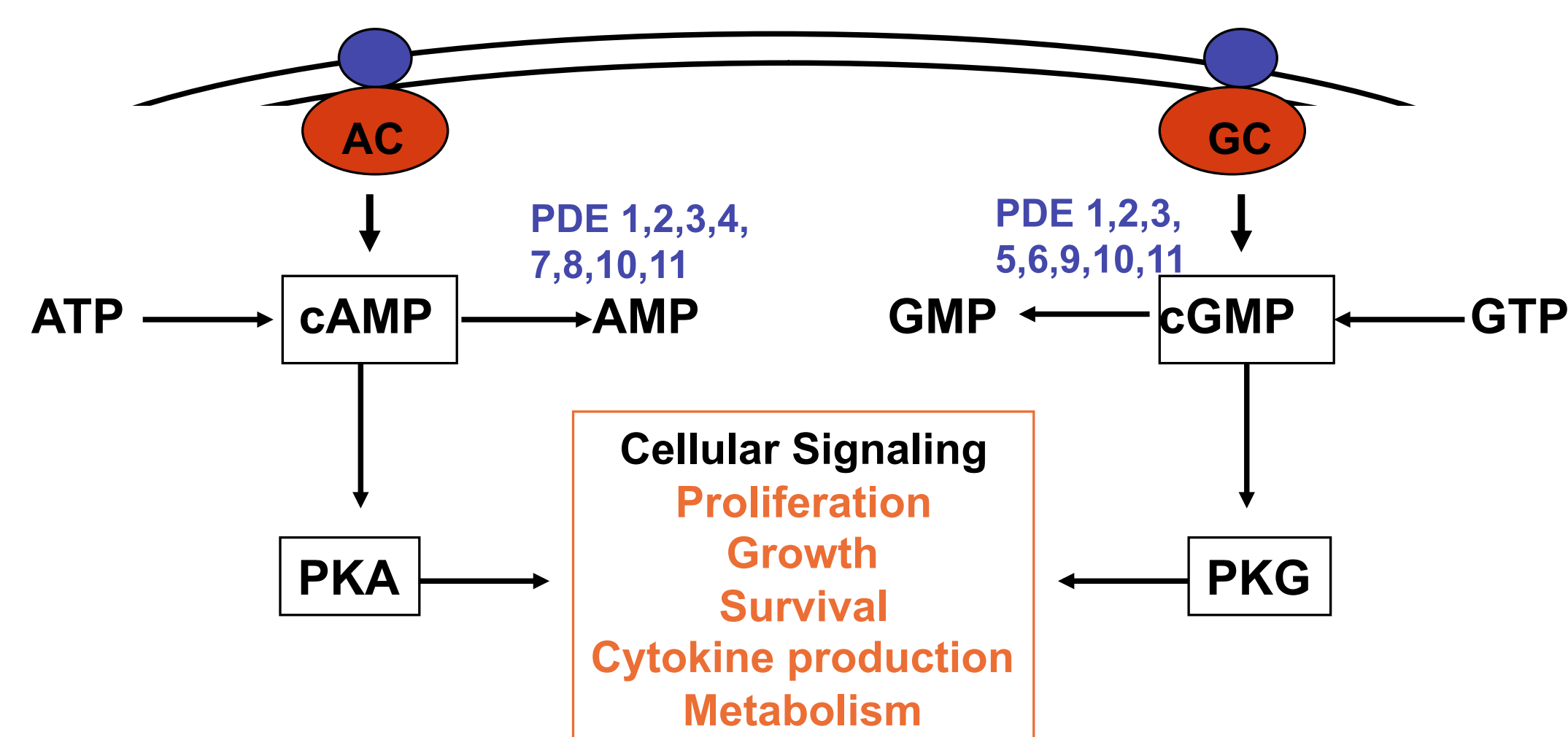


- 5  $\mu$ g rat brain lysate
- Good correlation between amount of substrate phosphorylation and inhibition of PDE activity on cAMP

## Summary: Gyrasol Sensor Advantages

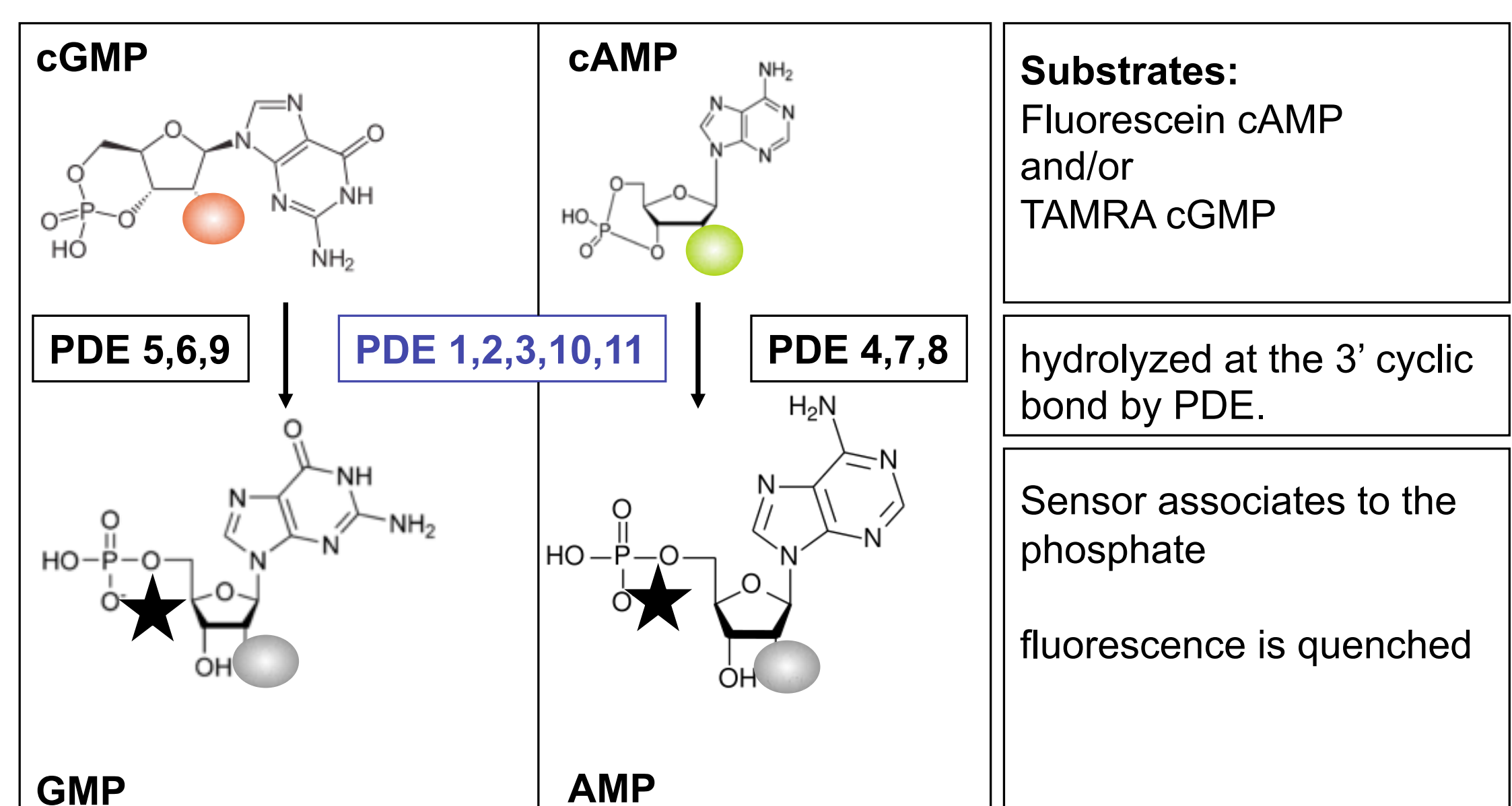
- High Substrate Tolerance: (nM-> 100  $\mu$ M):screening at relevant Km
- Kinetic monitoring: simplifies MoA
- Highly sensitive: reduces sample sizes
- High Signal stability (~24 h); adaptable to HTS
- Extremely cost-effective
- No compound interference: using red shifted fluors
- Adaptable to cell/tissue lysates
- Multiplexable: Modulation of cAMP and cGMP hydrolysis for PDEs with dual substrate specificity can be accomplished in one screen
- Detection of kinase/phosphatase and PDE activities: Connection of PDE activities with downstream targets possible

### cAMP/cGMP Second Messenger Signaling



- Cyclases (Adenylyl and Guanylyl) mediate GPCR signal transduction
- Produce cyclic nucleotides which act as 2<sup>nd</sup> messengers
- cAMP acts on Protein Kinase A; cGMP acts on Protein Kinase G
- Phosphodiesterases regulate cyclic nucleotide levels by hydrolysis to monophosphate nucleotides

### Gyrasol Sensor: Multiplexed Detection of PDE Activities



- Gyrasol Sensor: trivalent metal ion complex which binds to phosphoryl groups
- Binds to monophosphate nucleotides and quenches fluorescence
- Quench mechanism is e- transfer (not FRET) thus can multiplex using Fluorescein and TAMRA
- Monitoring PDE activities with single and dual substrate specificities in one well