



# A Novel Membrane Potential-sensitive Dye for Measuring cAMP in Live Cells

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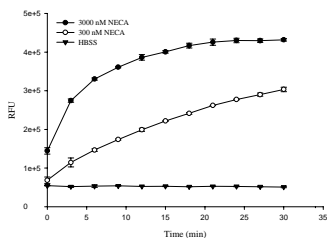
## Introduction

Nearly 2000 identified GPCRs and their signaling pathways control a variety of fundamental intracellular events and physiological processes. The disturbance of the function of these GPCRs causes many pathological changes. The agonists and antagonists of GPCRs have been widely used in search of new medicines for treating human diseases. The explosive growth of GPCR-related research and drug discovery requires high throughput screening and high content screening assays for GPCRs.

Nearly 70% of GPCRs' signals are conveyed by cyclic 3',5'-adenosine monophosphate (cAMP) to their downstream signaling cascades. Therefore, intracellular cAMP level can serve as an indicator of the function status of GPCRs. A live-cell cAMP assay has been developed to real-time record the fluctuation of intracellular cAMP. By employing a mutant cyclic-nucleotide-gated ion channel (CNG) as cAMP biosensor, the alteration of cAMP level is linked to the changes of transmembrane potential or intracellular calcium level.

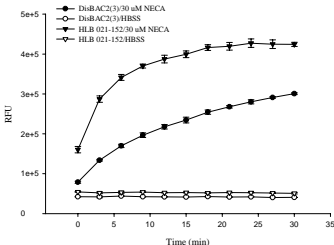
In current study, we have developed a new membrane potential dye and successfully applied it to this live-cell homogenous cAMP assay. This anionic oxonol-based membrane potential dye, HLB 021-152, has improved characteristics compared to the existing membrane potential dyes. The assay is in mix-and-read format, which is readily adapted to high throughput screening of GPCR-based drugs and GPCR functional analysis.

## Results



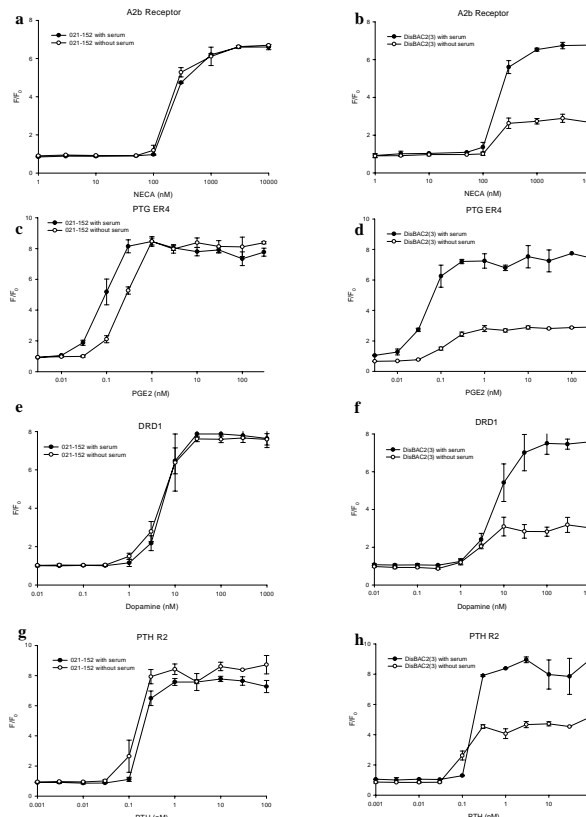
**Figure 1.** The real-time record of the increasing intracellular cAMP level by monitoring the fluorescence signal of HLB 021-152.

HEK 293 cells, stably transfected with cyclic-nucleotide-gated ion channel (CNG), were loaded with formulated HLB 021-152 solution containing phosphodiesterase inhibitor Ro 20-1724. 3000 and 300 nM of 5'-(N-ethylcarboxamido) adenosine (NECA) was added to the cells to stimulate A2b receptor. The fluorescence signal of HLB 021-152 was recorded at Ex/Em=530 nm/565 nm every 3 minutes for 30 minutes.



**Figure 2.** The comparison of fluorescence signal of HLB 021-152 and DisBAC<sub>2</sub>(3). HLB 021-152 has higher fluorescence signal in responds to the stimulation of NECA.

HEK 293 cells, stably transfected with cyclic-nucleotide-gated ion channel (CNG), were loaded with formulated HLB 021-152 or DisBAC<sub>2</sub>(3) solution containing phosphodiesterase inhibitor Ro 20-1724. 30 nM of 5'-(N-ethylcarboxamido) adenosine (NECA) was added to the cells to stimulate A2b receptor. The fluorescence signal of HLB 021-152 and DisBAC<sub>2</sub>(3) was recorded at Ex/Em=530 nm/565 nm every 3 minutes for 30 minutes.



**Figure 3.** The comparison of serum effect on the dynamic range of HLB 021-152 and DisBAC<sub>2</sub>(3). The depletion of serum reduced the dynamic range of DisBAC<sub>2</sub>(3), while it had little effect on HLB 021-152.

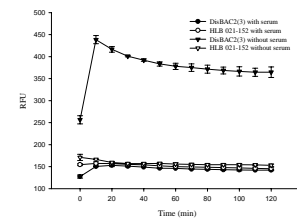
The HEK 293-derived cells were loaded with formulated HLB 021-152 or DisBAC<sub>2</sub>(3) containing phosphodiesterase inhibitor Ro 20-1724 with or without serum during the assay. Four different GPCRs were stimulated with their corresponding compounds. The fluorescence signals of HLB 021-152 and DisBAC<sub>2</sub>(3) were recorded before adding the stimulators (F<sub>0</sub>) or 30 minutes after adding the stimulators (F). The ratio of F/F<sub>0</sub> was calculated and plotted versus the concentration of stimulators.

**a,b.** The endogenous A2b receptor on CNG-channel containing HEK 293 cells were stimulated with NECA.

**c,d.** The CNG-channel-containing HEK 293 cells, stably transfected with prostaglandin E receptor 4, were stimulated with prostaglandin E2.

**e,f.** The CNG-channel-containing HEK 293 cells, stably transfected with dopamine receptor D1, were stimulated with dopamine.

**g,h.** The CNG-channel-containing HEK 293 cells, stably transfected with human parathyroid hormone receptor 2, were stimulated with PTH (1-34).



**Figure 4.** The comparison of serum effect on the baseline of HLB 021-152 and DisBAC<sub>2</sub>(3). The depletion of serum significantly increased the baseline of DisBAC<sub>2</sub>(3), while had little effect on HLB 021-152.

The CNG-channel-containing HEK 293 cells were loaded with formulated HLB 021-152 or DisBAC<sub>2</sub>(3) with or without serum during the assay. The background fluorescence of HLB 021-152 and DisBAC<sub>2</sub>(3) was monitored at Ex/Em=530 nm/565 nm for 2 hrs.

## Discussion and Conclusion

In this study, we have developed a novel anionic oxonol-based membrane potential-sensitive dye, HLB 021-152, which is chemically modified from DisBAC<sub>2</sub>(3), the gold standard dye for measuring transmembrane potentials. The new dye has improved characteristics compared to the existing DisBAC<sub>2</sub>(3) compounds. Unlike DisBAC<sub>2</sub>(3), whose dynamic range was greatly reduced in the presence of serum, HLB 021-152 maintain the similar dynamic range with or without serum. Upon stimulating the endogenous or heterogenous GPCRs on CNG-channel-cloned HEK 293 cells with agonists, the fluorescent signal of HLB 021-152 increased rapidly and can reflect the real-time fluctuation of intracellular cAMP.

In conclusion, this new membrane potential-sensitive dye can be formulated for high throughput screening of GPCRs by measuring the cAMP level in live cells.

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