A Novel Ruthenium-Based Time-Resolved Fluorescence Assay for High-Throughput cAMP Detection

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OVERVIEW

Compared to traditional immunoassay methods which are typically time-consuming and laborious, the single-step, wash-free homogeneous immunoassays have been of high interest to researchers. In this study, we report a novel, ruthenium-based time-resolved fluorescence method for the high-throughput detection of cAMP in research and drug discovery settings. Ruthenium-based dyes provide the advantage of time-resolved detection at a nanosecond time scale. The high quantum efficiency ruthenium dyes have bright far-red signals with low background due to time resolved detection. They do not require strong UV excitation and can be excited with visible light. They are chemically robust and stable in many biological matrices. The time-resolved signal is measured using a novel detection cartridge in combination with select SpectraMax® Multi-Mode Microplate Readers. This method provides several advantages, including a homogeneous, no-wash workflow, fast reading speed, stable assay signal, and reduced background. This technology is adaptable to many applications such as cell signaling, biomarker detection and protein-protein interaction.

ASSAY PRINCIPLE

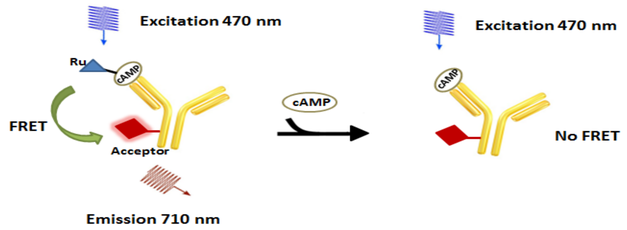
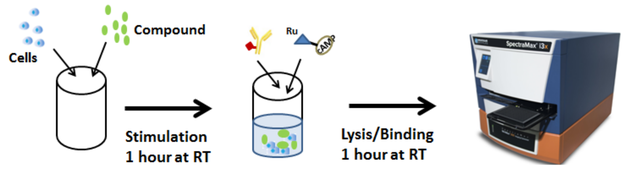


Figure 1. TR-FRET cAMP assay based on Ruthenium as donor

- As illustrated in Figure 1, the ruthenium based TR-FRET cAMP assay is a homogeneous competitive assay. The acceptor-conjugated cAMP antibody and the ruthenium (donor)-conjugated cAMP (Ru-cAMP) in the reagent solution form a complex which generates FRET signals with excitation at 470 nm. The cAMP molecules present in the test samples such as those produced during cell signaling events compete with the ruthenium-conjugated cAMP provided in the reagent solution for the antibody-antigen complex. The result is a decrease of the FRET signal with increasing concentration of cAMP.
- Unlike europium or other lanthanide complex which requires excitation at the UV range, ruthenium can be excited with visible light at 470 nm and has a short lifetime τ = 3.2 µs. These unique properties affords a few distinctive advantages of ruthenium labels for time-resolved fluorescence assay, including fast detection time and reduced background signals.

MATERIALS & METHODS

- The assay reagents consist of ruthenium-labeled cAMP and an acceptor dye-conjugate cAMP antibody. The working solution is prepared in lysis buffer.
- This new ruthenium-based homogeneous cAMP assay was compared to HTRF® (Cisbio) and AlphaScreen® (Perkin Elmer) assay platform. The experiments were carried out following manufacturers' recommended protocols.
- Cell based assays were performed as illustrated below.



- The assay signals were measured using SpectraMax i3x Multi-Mode plate reader equipped with the corresponding cartridges and pre-configured SoftMax® Pro protocols.

SpectraMax i3x Multi-Mode Platform

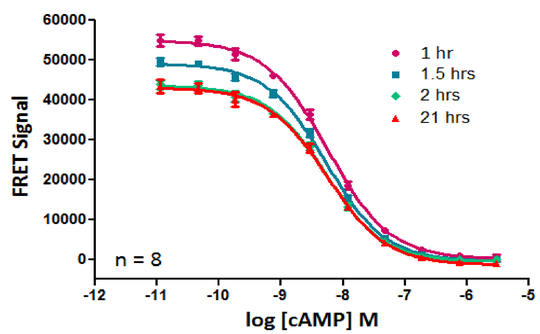
The SpectraMax® i3x Multi-Mode Detection Platform comes with standard spectral absorbance, fluorescence, and luminescence detection. Additionally, user-installable options allow the SpectraMax i3x System to address changing application needs and go beyond the standard reader applications. The MiniMax™ Imaging Cytometer option adds cellular imaging capability with fluorescence and bright-field detection to enable a wide range of cell-based assays.

Key benefits

- Users can upgrade the 3-mode base system with application cartridges for HTRF®, AlphaScreen®, and other assays, as well as cellular imaging.
- SpectraMax® MiniMax™ Imaging Cytometer simplifies complex imaging workflows.
- SoftMax® Pro Microplate Data Acquisition & Analysis Software streamlines complex data analysis with built-in protocols for many applications.

RESULTS

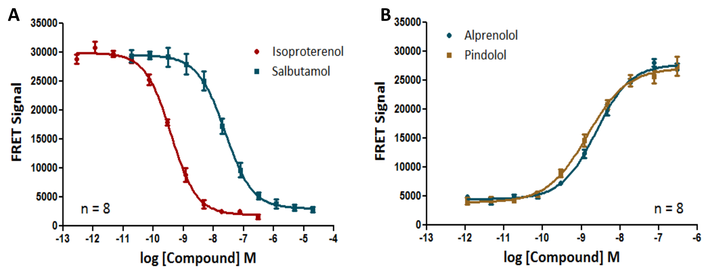
cAMP Standard Curve



Incubation time (hour)	1	1.5	2	21
IC_{50} (nM)	5.6	5.3	5.2	5.5

Figure 2. The cAMP standards were prepared by 1:4 serial dilution in phosphate buffered saline (PBS) plus 0.1% bovine serum albumin (BSA). The mixtures were incubated at room temperature and signals were measured at 1, 1.5, 2, and 21 hours after the reagents were added.

Stimulation and Inhibition of Endogenous β Adrenoceptor in A431 Cells

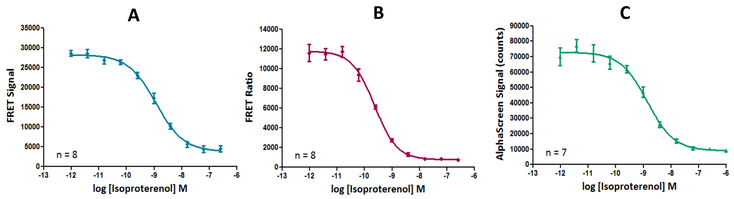


Compound	Isoproterenol	Salbutamol
EC_{50} (nM)	0.4	24
Signal/Background	15	10
Z'	0.83	0.77

Compound	Alprenolol	Pindolol
IC_{50} (nM)	2.4	1.4

Figure 3. Concentration Response Curves of (A) β Adrenoceptor stimulation by agonists Isoproterenol and Salbutamol and (B) inhibition by Alprenolol and Pindolol in the presence of 5 nM isoproterenol. A431 cells were treated with the compounds for 1 hour at room temperature. The assay reagents in lysis buffer were added and incubated for 2 hours at room temperature.

A431 Response to Isoproterenol: Comparison to HTRF and AlphaScreen Assays



Assay	Ru-based	AlphaScreen	HTRF
EC_{50} (nM)	1.2	1.4	0.23
Signal/Background	7	8	15
Z'	0.80	0.88	0.77
Read Time (384 well)	~ 3 min	~ 3 min	~ 8.5 min

Figure 4. Concentration Response Curves of β Adrenoceptor stimulation by agonist Isoproterenol. A431 cells were treated for 1 hour at room temperature. Ru-based reagents in lysis buffer were added and incubated for 1 hour at room temperature (A). The AlphaScreen (B) and HTRF (C) assays were carried out following manufacturers' protocols.

Summary

- The Ru-based TRF assay provides robust assay and an easy-to-use, homogeneous (no wash) workflow.
- Results obtained from this Ru-based assay are comparable to those obtained from other assay platforms in sensitivity, stability, and data acquisition speed.
- Upon further development and optimization, this novel assay can be applied to various targets with improved throughput and assay performance.

MOLECULAR DEVICES