Application Notes

Performing Cyclic AMP hTRF Screening Assays with the Thermo Scientific Varioskan LUX Multimode Reader

Goal

This application note describes the optimization process of a TR-FRET screening assay with Thermo Scientific™ Varioskan™ LUX multimode reader. An hTRF cyclic AMP (cAMP) assay is used as the example assay to demonstrate the measurement and calculation process during the assay setup.

Introduction

G-protein coupled receptors (GPCRs) are a major class of targets for drug discovery. Cyclic AMP (cyclic adenosine 3', 5'-monophosphate) is a second messenger and one of the most important intracellular mediators. Changes in intracellular cAMP levels correlate with GPCR activation. Measurement of cAMP levels therefore offer an assay for GPCR high-throughput screening.

The Cisbio HiRange cAMP kit is intended for the direct quantitative determination of cyclic AMP. Its principle is based on HTRF® Technology (Homogeneous Time Resolved Fluorescence). The assay is based on a competitive immunoassay between native cAMP produced by cells and the cAMP labeled with the dye D2. The specific signal (i.e., energy transfer signal) is inversely proportional to the concentration of cAMP in the standard or sample. Compared to other hTRF cAMP assays, the HiRange kit has an extended signal-to-background ratio. The kit allows the measurement of agonist and antagonist effects on Gαs- and Gαi-coupled receptors in different cell lines.

Cells are stimulated to either increase or decrease intracellular cAMP levels. For Gαi-coupled receptors, an elevation in intracellular cAMP can be stimulated using forskolin, resulting in a decrease in the energy transfer signal.

When first optimizing the assay, a suitable number of cells per well need to be chosen. The optimum cell density is the number of cells that produce the highest amplitude between the inactivated and activated state.

In this study, three different cell concentrations – 1000, 3000 and 10000 cells per well – were tested.

Thermo SCIENTIFIC

Materials and methods

- Varioskan LUX multimode reader, Thermo Scientific
- Thermo Scientific™ Nunc™ 384 Shallow Well Standard Height Plate, White, Catalog No. 264706
- cAMP HiRange kit, Cisbio assays, catalog no 62AM6PEB
- Forskolin, Sigma F6886, 10mg
- CHO-1 cells M1WT2 (ATCC® CRL-1984™)
- IBMX (3-Isobutyl-1-methylxanthine), Sigma I5879-100 mg

Two separate assays were made. In the first one, the standard curve was prepared to see the basic functionality of the system. This was followed by the cell number optimization.

Standard curve plate

The assay was performed according to kit documentation. The standards, blank and control were assayed in 6 replicates.

- Standards 1–8: 5 µl cAMP standard + 5 µl compound buffer + 5 µl cAMP-d2 + 5 µL anti cAMP-Cryptate
- Standard 0: 5 µl diluent + 5 µl compound buffer + 5 µl cAMP-d2 + 5 µL anti cAMP-Cryptate
- Negative control: 5 µl diluent + 10 µl compound buffer + 5 µL anti cAMP-Cryptate
- Buffer blank: 20 µl compound buffer (contains IBMX)

Cell assay plate

The CHO-1 cells were grown as suspension and plated 1000, 3000 or 10000 cells per well. The cells were activated with different concentrations of forskolin. Phosphodiesterase inhibitor IBMX was added to the compound buffer to prevent the cAMP degradation.

All samples were assayed as triplicates.

- Buffer blank: 20 µl compound buffer
- Cell negative control: 5 µl CHO-1 (1000/3000/10000 cells/well) + 5 µl compound buffer + 5 µl conjugate and lysis buffer + 5 µL anti cAMP-Cryptate
- Non-stimulated cells: 5 µl CHO-1 1000/3000/10000 cells /well) + compound buffer + 5 µl cAMP-d2 + 5 µL anti cAMP-Cryptate
- Stimulated cells: 5 µl CHO-1 (1000/3000/10000 cells/well) + Forskolin dilution + 5 µl cAMP-d2 + 5 µL anti cAMP-Cryptate

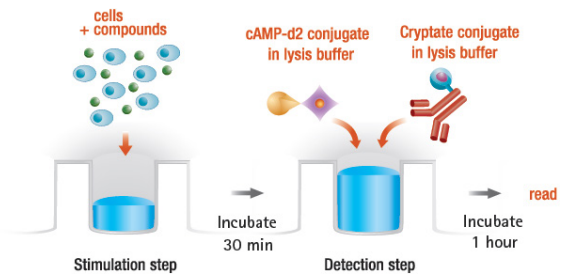

Figure 1. Description of the assay. [1]

[1]Cisbio kit insert for hiRange cAMP kit Document reference : 62AM6PEB rev 06 (Nov 2014)

The Varioskan LUX multimode reader is controlled with Thermo Scientific SkanIt™ software, which supports optimal use of the instrument features and offers effortless data analysis capabilities.

Both plates were measured using the measurement parameters listed in table 1.

Excitation wavelength	334 nm
Emission wavelength 1	620 nm
Emission wavelength 2	665 nm
Measurement time	1000 ms
Time delay	60 µs
Integration time	200 µs
Dynamic range	Autorange

Table 1 . Parameters recommended for hTRF measurements.

The Varioskan LUX multimode reader has a fixed excitation filter for TRF. Therefore, only the emission filters need to be chosen for the measurement session.

The Varioskan LUX multimode reader has a special autorange system, where the instrument automatically selects the optimal gain for each sample. This means the instrument provides high sensitivity and wide dynamic range for every run without separate gain optimization. The autorange option is explained in more detail in the Thermo Scientific Application Note "*Automatic Dynamic Range Selection – Simplified Assay Setup with the Thermo Scientific Varioskan LUX Multimode Reader.*"

Results

For all hTRF assays, the result is expressed as the ratio of the acceptor (665 nm) and donor (620 nm). This method is used to eliminate interferences.

Another parameter, Delta F, which represents the ratio *(signal - blank)/blank* is also to be determined for all samples.

$$\text{Ratio} = \frac{A_{665nm}}{B_{620nm}} \times 10^4$$

$$\text{Delta F} = \frac{\text{Calibrator or sample Ratio} - \text{Ratio}_{neg}}{\text{Ratio}_{neg}} \times 100$$

(Ratio_{neg} = negative control)

The standard curve was created with SkanIt software from the calculated Delta F values with four parameter logistics as the fitting method (Figure 2).

The standard curve shows a very wide dynamic range, and the calculated effective dose value, 24 nM, correlates well with the reported value in the kit documentation.

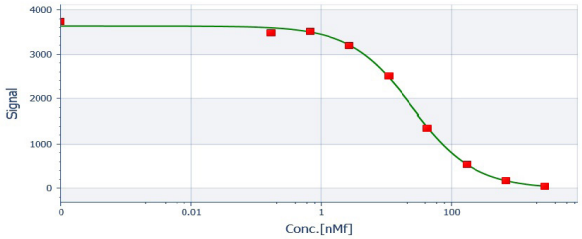

Figure 2. cAMP standard curve. On the y axis is the Delta F (%) and on the x axis is the cAMP concentration.

Figure 3 shows the effect of the forskolin on cAMP levels with different cell densities. The same data is numerically shown in table 1.

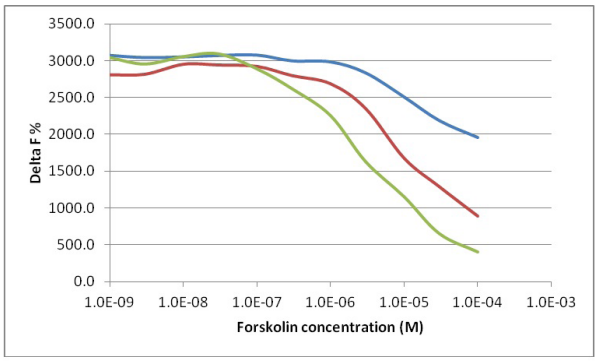

Figure 3. Delta F (%) values as a function of the forskolin concentration. Blue represents 1000 cells/well, red 3,000 cells/well, and green 10000 cells/well.

	Delta F (%)		
Forskolin (µM)	1000 cells	3000 cells	10000 cells
100	1959	888	403
30	2189	1289	652
10	2509	1676	1153
3	2837	2352	1630
1	2986	2689	2255
0.3	3000	2800	2621
0.1	3078	2925	2892
0.03	3072	2944	3095
0.01	3054	2954	3057
0.003	3044	2818	2957
0.001	3074	2810	3045

Table 2. The effect of forskolin on the Delta F (%) for the three cell densities tested.

Based on the results, 10000 cells per well provided the widest dynamic range in this study.

In addition to the hTRF calculations, the Z' prime value was also calculated for the 10000 cell assay. It is a dimensionless parameter, which describes the quality of a screening assay. The value ranges from 0-1, and assays with values above 0.5 can be considered very good.

The Z'-parameter can be calculated using the Custom Formula function of the SkanIt software. This function needs four variables:

- The average signal of the high controls
- The average signal of the low controls
- The standard deviation of the high controls
- The standard deviation of the low controls

In the case of hTRF, the ratios of the signals should be used. When the variables have been determined, the Custom Formula function can be used as a normal calculator (Figure 4).

Figure 4. Custom Formula calculation in SkanIt software.

Here the cell negative control was used as the low control, and the sample with the lowest forskolin concentration was used as the high control. An excellent Z' value, 0.94, was achieved with the system.

In addition to excellent performance, flexibility of setup and ease of use of the Varioskan LUX are also very valuable features in assay development and screening. No extra measurements are needed for instrument testing or optimization.

Summary

Varioskan LUX with SkanIt software provides both ease of use and a high-performance tool for hTRF assays.

All calculations required for this assay can be directly calculated with SkanIt software, therefore, it is not necessary to export the data to external calculation software. However, if preferred, the transfer is easy.

By using the automatic dynamic range, the system and measurement settings are always optimal, making screening assays both easy and robust.

www.thermoscientific.com/varioskanlux

North America:
USA/Canada +1 800 625 4327
Europe:
Austria +43 1 801 40 0
Belgium +32 2 482 30 30
France +33 2 28 03 20 00
Germany National Toll Free 08001-536 376
Germany International +49 6184 90 6940
Italy +39 02 95059 1
Netherlands +31 76 571 4440
Nordic/Baltic/CIS countries +358 10 329 2200
Russia +7 (812) 703 42 15
Spain/Portugal +34 93 223 3154
Switzerland +41 44 454 12 12
UK/Ireland +44 870 609 9203
Asia:
India +91 22 5542 9494
Japan +81 45 453 9220
China +86 21 6865 4588 or +86 10 5850 3588
Other Asian countries +852 2885 4613
Countries not listed:
+49 6184 90 6940 or +33 2 28 03 20 00

Thermo SCIENTIFIC
A Thermo Fisher Scientific Brand

AN_MRAMPHRTF_1015