HTRF assays on the new SpectraMax M5e multi-detection microplate reader

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INTRODUCTION

The SpectraMax® M5e is a dual-monochromator, multi-detection plate reader capable of fluorescence, time-resolved fluorescence (TRF), fluorescence polarization, absorbance, and luminescence assays. Developed in collaboration with Cisbio, SpectraMax M5e offers the additional advantage of HTRF® certification, allowing life sciences and drug discovery researchers to increase their productivity through greater flexibility in TRF detection.

HTRF is a versatile technology developed by Cisbio for detecting molecular interactions between biomolecules.[1] Combining the features of fluorescence resonance energy transfer (FRET) and TRF, it uses Europium (Eu) Cryptate as its donor fluorophore and a modified allophycocyanin (XL665 or d2) as the acceptor fluorophore. When interacting biomolecules are labeled with Europium Cryptate and XL665, some of the excitation energy of the Cryptate is released as fluorescence emission at 620 nm, while the remaining energy is transferred to XL665, resulting in fluorescence emission at about 665 nm. Well-to-well variability is reduced by using Cisbio's unique HTRF ratio calculation, which utilizes the ratio of the 665 nm and 620 nm signals.

HTRF assay performance on the SpectraMax M5e is demonstrated here with two different HTRF assay kits, cAMP dynamic 2 and IP-One. G-protein coupled receptors signal through two major pathways, regulation of cAMP and increase in intracellular calcium levels mediated by IP3. IP1 is a downstream metabolite of IP3 that accumulates in signalling cells. The cAMP dynamic 2 kit includes a monoclonal anti-cAMP antibody labeled with Eu Cryptate and d2-labeled cAMP. Free cAMP produced by cells competes with cAMP-d2 for binding to the anti-cAMP Cryptate, thus an increase in cellular cAMP leads to a decrease in FRET, which is detectable as a decrease in the emission at 665 nm. The IP-One assay has a similar format and uses a monoclonal antibody specific to IP1. Standard curves for both assays are presented, and cell-based data are included for the cAMP assay. These HTRF data demonstrate the excellent dynamic range and Z'- factors[2] obtainable with SpectraMax M5e.

MATERIALS

- cAMP dynamic 2 kit, 1000 tests (Cisbio Cat. #62AM4PEB)
- IP-One kit, 1000 tests (Cisbio Cat. #62IP1PEB)
- Cell line: CHO-M1 (M1WT3; ATCC Cat. #CRL-1985)
- Forskolin (Sigma Cat. #F3917)
- 3-isobutyl-1-methylxanthine (IBMX; Sigma Cat. #I7018)
- White 384-well microplates (Corning Cat. #3705)
- SpectraMax M5e multi-detection microplate reader (Molecular Devices)

METHODS

Note: For optimal signal detection, it is best to use opaque, rather than clear-bottom, white microplates. For cell-based assays where it is necessary to monitor cells' growth and appearance, one may culture cells and perform the assay in a clear-bottom microplate of any color, then transfer the assay mixture to an opaque white microplate for detection.

cAMP Dynamic 2 Cell-Based Assay

CHO-M1 cells were grown in Ham's F12 medium supplemented with 10% FBS, 1% penicillin/streptomycin/L-glutamine, and 200 mg/mL G418. For cAMP assays, cells were trypsinized the day of the assay, resuspended in Ham's F12 medium without additives, and seeded at 4000 cells per well in a volume of 10 µL per well in a white 384-well plate. To stimulate cAMP production, cells were treated with forskolin at concentrations of 3 mM to 1 mM for one hour prior to addition of detection reagents. IBMX was added to assay wells at 0.5 mM to inhibit phosphodiesterase-mediated cAMP degradation.

After one hour of forskolin treatment, cAMP-d2 and anti-cAMP Cryptate were added to the cells. A cell negative control containing untreated cells received anti-cAMP Cryptate but no cAMP-d2. This control, which is negative for FRET, was necessary for calculating Delta F values as outlined in the HTRF package insert.[3]

Standards for cAMP were prepared as indicated in the cAMP dynamic 2 HTRF package insert. cAMP final concentrations ranged from 0.17 nm to 712 nM, and a positive control without cAMP (maximum FRET) was included. A cAMP control consisting of free cAMP was used to monitor assay activity, and a negative (no-FRET) control without cAMP or cAMP-d2 was used to calculate Delta F. cAMP-d2 was added to all standards and to the positive control, but not to the negative control. Anti-cAMP Cryptate was added to all standards and controls. After a one-hour incubation, the plate was read on the SpectraMax M5e. (See Table 1 for instrument settings.)

Table 1. Instrument Settings for SpectraMax M5e

Read Type	Endpoint		
Read Mode	Time-Resolved		
	Delay: 50		
	Integration: 400		
	Top Read		
Wavelengths	Ex	Em	Cutoff
	314	620	570
	314	668	630
Sensitivity	Readings: 100		
	PMT: Auto		
Autocalibrate	On		
Setting Time	Off		
Carriage Speed	Normal		

IP-One Standard Curve

IP1 calibrators ranging from 21.5 to 22,000 nM, as well as a positive control standard without IP1, were prepared by diluting reconstituted IP1 calibrator in stimulation buffer as indicated in the IP-One HTRF package insert.[4] A positive IP1 control sample and a negative control without IP1 were included. IP1-d2 conjugate was added to all standards and to the positive IP1 control, but not to the negative control. Anti-IP1 Cryptate was added to all samples. After incubating for one hour, the plate was read on the SpectraMax M5e. (See Table 1.)

Data Analysis

Data for both cAMP and IP-One assays were analyzed according to Cisbio's guidelines, using SoftMax® Pro software from Molecular

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Devices. An upcoming release of SoftMax Pro will include ready-to-use HTRF protocols designed to simplify data acquisition and analysis. For further details on assay setup and data calculations, please see the HTRF cAMP dynamic 2 and IP-One package inserts.

Instrument Settings

Wavelength, delay and integration settings have been optimized for the monochromator-based SpectraMax M5e. Most notably, using 668 nm as the emission wavelength for XL665/d2 acceptor can give better results than 665 nm.

RESULTS

For cAMP cell-based assays, best results were obtained using about 4000 CHO-M1 cells per well of a 384-well plate. Optimal cell density may vary according to cell type and specific assay conditions. A one-hour treatment of the cells with forskolin yielded a dose-response curve with an EC_{50} of 11.4 µM and Z'- factor of 0.86. (See Figure 1.) Delta F% values calculated using Cisbio's ratiometric formulas ranged from 47 to 707, similar to the range observed for the cAMP standard curve. (See Figure 2.) Using the standard curve, forskolin-induced cellular cAMP levels were calculated. (See Table 2.)

Table 2. Summary of cAMP Cell-Based Assay Data With cAMP Concentrations Calculated Using cAMP Standard Curve

Forskolin (µM)	Average Delta F%	Std Dev	CV	Calculated cAMP (nm)
1000	47	5.93	12.7	162.76
200	83	5.63	6.8	84.32
40	215	16.25	7.6	22.34
8	406	26.56	6.5	6.5
1.6	576	25.93	4.5	2.23
0.32	643	58.47	9.1	1.34
0.064	673	20.56	3.1	1.02
0.0128	707	29.68	4.2	0.72
0.003	707	25.75	3.6	0.72
0	760	39.39	5.2	0.34

Preparation of IP1 standards as directed in the IP-One HTRF package insert yielded a standard curve with Z'- factor of 0.9, and Delta F% values ranging from 46 to 719, demonstrating the excellent assay range and low standard deviations obtained with the SpectraMax M5e. (See Figure 3.)

SUMMARY

The SpectraMax M5e multi-detection microplate reader with HTRF certification expands the range of assay methods available to researchers who want the flexibility of a monochromator-based system and the versatility of multiple detection modes. IP-One and cAMP dynamic 2 HTRF assays run on the SpectraMax M5e exhibit wide dynamic ranges and Z'- factors above 0.8. Data analysis is simplified using SoftMax Pro software with preconfigured HTRF protocols.

For more information, visit our web site at www.moleculardevices.com/m5 or email *Cathy.Olsen@moldev.com*

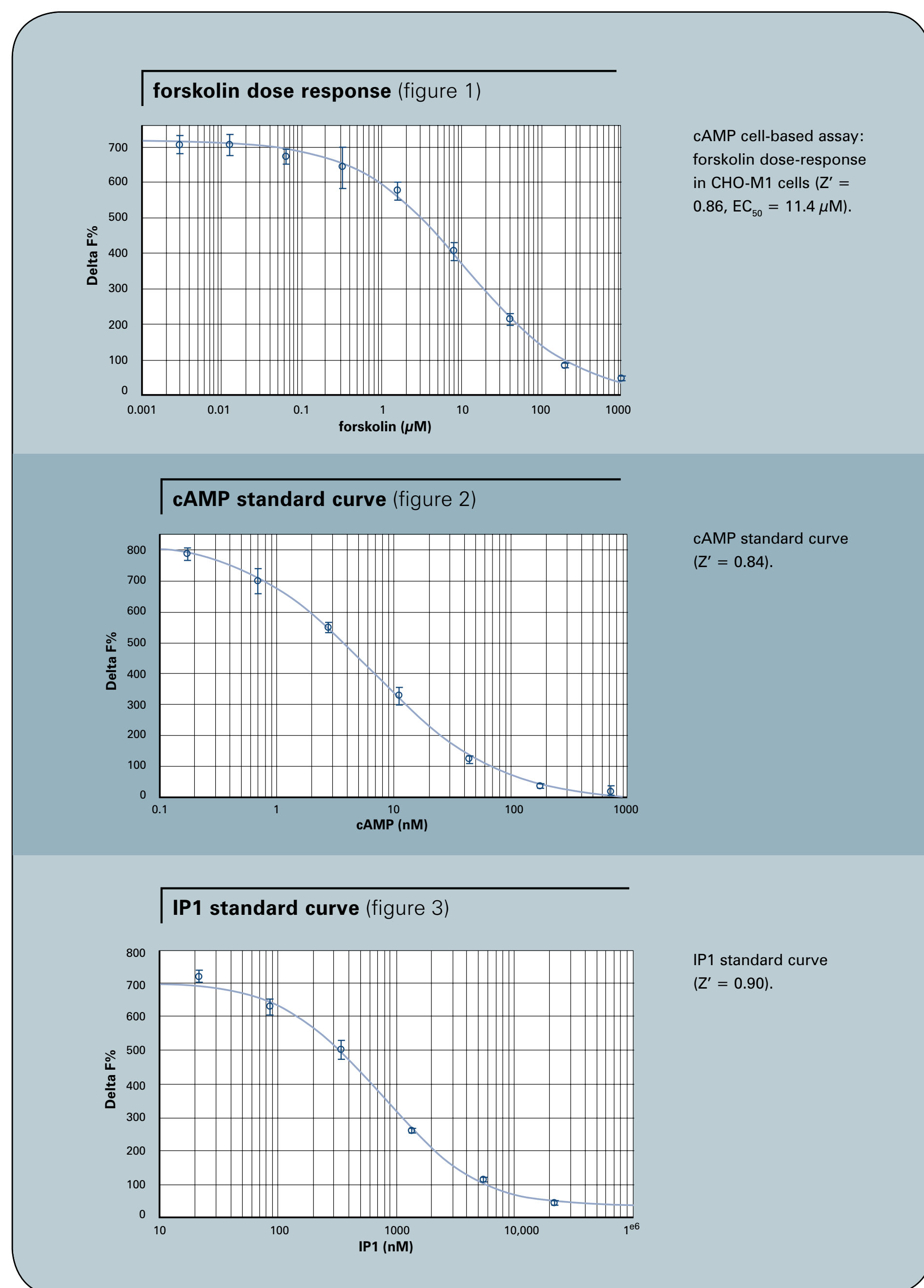
REFERENCES

1. TR-FRET Basics. www.htrf-assays.com

2. Zhang, J. H., Chung, T. D. Y., and Oldenburg, K. R. (1999). A simple statistical parameter for use in evaluation and validation of high throughput screening assays. *J. Biomolecular Screening* 4(2): 67-73.

3. cAMP dynamic 2 package insert (Cisbio international, France).

4. IP-One package insert (Cisbio international, France).



cAMP cell-based assay: forskolin dose-response in CHO-M1 cells (Z' = 0.86, EC_{50} = 11.4 µM).

cAMP standard curve (Z' = 0.84).

IP1 standard curve (Z' = 0.90).