

Using the Synergy™ 2 Multi-Detection Microplate Reader to run cAMP and Tumor Necrosis Factor HTRF® Assays for High-Throughput Screening

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Abstract

cAMP and Tumor Necrosis Factor (TNF-α) are important mediators and indicators for a litany of cellular responses. As such, assays of these moieties are common in research and drug discovery, including high throughput screening (HTS). The ability to accurately measure changes in these compounds, using a homogeneous assay technology, saves considerable amounts of time and expense. High-throughput assays require that both the assay technology, as well as the instrumentation necessary to measure the results, be sensitive, reliable and cost effective. Assays based on Cisbio's Homogeneous Time-Resolved Fluorescence (HTRF®) technology use a combination of Time-Resolved Fluorescence (TRF) and Fluorescence Resonance Energy Transfer (FRET) to investigate biomolecular interactions. BioTek's Synergy™ 2 Multi-Detection Microplate Reader combines a high intensity xenon flash lamp with dichroic mirrors and deep blocking fluorescence filters to provide exquisite sensitivity. The combination of a high performance multi-detection reader and a robust homogeneous assay technology provides for a reliable HTS solution. Here we describe the use of the Synergy 2, in conjunction with Gen5™ Data Analysis Software, to quantitate the signal and perform the data reduction for cAMP and TNF-α assays using HTRF technology from Cisbio. Examples of typical performance of these assays when measured on a Synergy 2 will be provided along with an overview of both the assay technology and reader design.

Introduction

HTRF (Homogeneous Time-Resolved Fluorescence) is based on a technology referred to as TR-FRET. TR-FRET combines Time Resolved Fluorescence (TRF) and Fluorescence Resonance Energy Transfer (FRET) to provide exceptional specificity and sensitivity in a homogeneous assay format. TRF takes advantage of the unique fluorescent properties of compounds known as lanthanides. These compounds have large Stoke's shifts and extremely long emission half-lives when compared to most fluorescent compounds. As a result, these compounds provide much lower background fluorescence than traditional fluorescent compounds. FRET refers to the transfer of energy from one fluorescent moiety to another (donor to acceptor). Excitation of the donor by an energy source triggers an energy transfer to the acceptor if they are in close proximity. The acceptor in turn emits light at its specific emission wavelength. As a result of this energy transfer, molecular interactions between two biomolecules can be quantitated by coupling each partner with a fluorescent label (donor and acceptor) and measuring the amount of energy transfer. In addition, the acceptor emission can be detected without the need to separate bound from unbound assay constituents. This homogeneous format is extremely beneficial in terms of time and cost.



Figure 1. Synergy 2™ Multi-Detection Microplate Reader. The Synergy 2 utilizes multiple sets of optics to provide optimal performance regardless of the detection technology. Fluorescence measurements are made using either a continuous tungsten-halogen lamp or a xenon-flash lamp with bandpass filters with or without dichroic mirrors for wavelength selection and PMT for detection. For time-resolved fluorescence measurements, the Synergy 2 integrates a high-energy xenon flash lamp with excitation and emission filters and PMT detector. Fluorescence polarization is accomplished with the use of polarizing filters in conjunction with label specific dichroic mirrors for wavelength specificity. Luminescence measurements are made using a liquid-filled optical fiber to capture light along with a low noise PMT. Absorbance measurements use a xenon-flash lamp with a monochromator for wavelength selection, allowing the selection of any wavelength for endpoint or kinetic measures from 200 nm to 999 nm. The Synergy 2 is capable of reading plate formats up to 1536 wells, is robotic compatible and provides temperature control and shaking as standard features.

Materials and Methods

Human TNF-α and cAMP HTRF® reagent kits were provided by Cisbio International (France). Both assay kits were prepared as described in the kit assay instructions. Each point of the calibration curves was prepared in duplicate in 100 l assay volume per well. Reagents were dispensed sequentially in the following order and volumes. First, 50 l aliquots of standard or sample were pipetted into solid white plates, followed by the addition of 25 l of cryptate antibody. Finally, 25 l of either XL665 or d2 conjugate were added. cAMP reactions were allowed to incubate for 1 hour; while TNF-α reactions were incubated overnight. Measurements were made using a BioTek Synergy 2 Multi-Detection Microplate Reader. The Europium Cryptate donor was excited using a Xenon flash lamp with a 330 nm filter for wavelength specificity. The cryptate emission was measured at 620 nm, while the acceptor XL665 or d2 emission was measured with a 665 nm filter. In addition, a dichroic mirror was used (Table 1). From the emission data the 665/620 ratio and the relative energy transfer or Delta F% values were then calculated using Gen5 Data Analysis Software. Delta F% values were then plotted as a function of analyte concentration.

Table 1. Measurement Parameters. Reading parameters utilized on BioTek Synergy 2 Multi-Detection Microplate Reader for HTRF measurements. In addition, excitation and emission filters, dichroic mirror and their BioTek part numbers are indicated in parenthesis.

Filter Set 1		Filter Set 2	
Ex. Filter	330 nm (7082263)	Ex. Filter	330 nm (7082263)
Em. Filter	620 nm (7082265)	Em. Filter	665 nm (7082266)
Mirror	UV dichroic (7138365)	Mirror	UV dichroic (7138365)
Lag time	100 sec	Lag time	100 sec
Integration time	300 sec	Integration time	300 sec
Number of flashes	50	Number of flashes	50
Sensitivity	171	Sensitivity	221

Figure 2. The energy transfer is calculated as follows:

$$\text{Ratio} = \left(\frac{\text{RFU}_{665}}{\text{RFU}_{620}} \right) \times 10000$$
$$\text{Mean Ratio} = \frac{\sum \text{ratio}}{\text{No. of replicates}}$$
$$\text{Delta F\%} = \frac{\text{Ratio}_{\text{sample}} - \text{Ratio}_{\text{neg}}}{\text{Ratio}_{\text{neg}}} \times 100$$

****The fluorescence ratio is a correction method developed by Cisbio and is covered by US patent 5,527,684 and its foreign equivalents;****

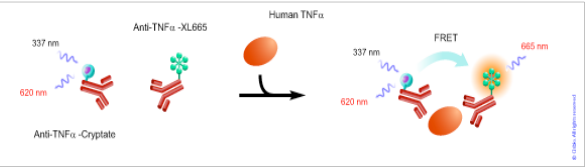


Figure 3. Schematic depicting the binding of donor and acceptor antibodies to Human TNF-α. The human tumor necrosis factor alpha (TNF-α) assay is a sandwich assay that uses two monoclonal antibodies (MAb) that recognize distinct epitopes on TNF-α. One antibody is labeled with Europium Cryptate donor, while the second antibody is labeled with cross-linked allophycocyanin (XL665) acceptor. When TNF-α is present, both MAbs bind to TNF-α and as a result are in close proximity to one another. Energy from the excitation of Europium Cryptate conjugate is transferred via FRET to the XL665 conjugated MAb. Energy is released as light emission at 665 nm. Only when both antibodies are bound to TNF-α will FRET occur. The higher the TNF-α concentration, the more donor-acceptor MAb pairs are formed, resulting in higher energy transfer. The signal will increase linearly with TNF-α concentration.

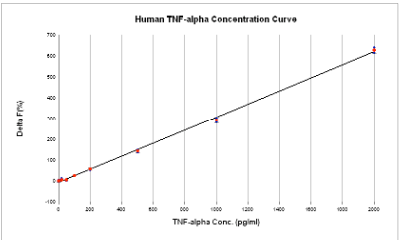


Figure 4. Human TNF-α Concentration Curve. Increasing amounts of human TNF-α were tested in duplicate and the Delta F% calculated using Gen5 Data Analysis Software. Data was plotted using a linear regression fit.

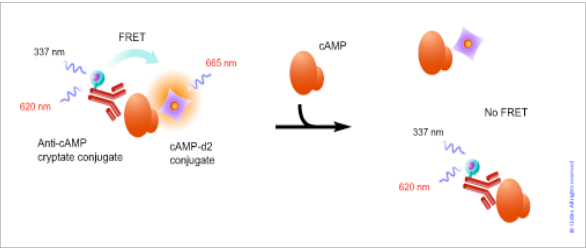


Figure 5. Schematic depicting the competitive binding of cAMP and cAMP-d2 moieties to a Europium Cryptate labeled antibody. The cAMP assay is a competitive immunoassay. Europium Cryptate donor molecule is conjugated to anti-cAMP monoclonal antibody, while the d2 acceptor molecule is conjugated directly to cAMP. The free cAMP and d2-conjugated cAMP compete with each other for the antigen recognition site of the anti-cAMP antibody. Binding of d2-conjugated cAMP results in a FRET reaction that emits light at 665 nm when the Europium Cryptate is excited. At low concentrations of free cAMP, the d2-labeled cAMP predominantly binds to the conjugated antibody, resulting in significant energy transfer from the Europium Cryptate donor to the d2 acceptor. When free cAMP levels are high, the free cAMP will predominate the binding resulting in less energy transfer. Increasing amounts of free cAMP will lead to decreasing levels of energy transfer. The generated signal will result in a sigmoidal shaped curve when one plots signal vs. cAMP concentration.

Table 2. Raw Data and Data Reduction. Typical example of raw data from a cAMP calibration curve generated using a Synergy™ 2 Multi-Detection Microplate Reader along with the prerequisite data reduction prior to plotting the curve.

Sample ID	D ₆₂₀ (RFU)	A ₆₆₅ (RFU)	Ratio	Delta F (%)
Negative Control	43265	4787	1106	
	43604	4808	1103	
cAMP Calibrator (nM)				
	712	5009	1201	9
178	42914	5327	1241	12
	41394	7896	1908	73
44.5	42060	7494	1782	61
	41670	15518	3724	237
11.125	42180	14633	3469	214
	41700	23909	5734	419
2.78	42066	26185	6225	464
	40869	31935	7814	607
0.69	41856	35426	8464	666
	40457	36016	8902	706
0.17	40673	37237	9155	729
	41135	37924	9219	735
	40837	38821	9506	761

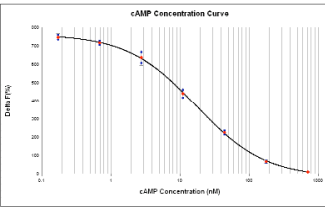


Figure 6. cAMP Concentration Curve. Increasing amounts of cAMP were assayed in triplicate. The Delta F% values were calculated from the subsequent fluorescent measurements and plotted in Gen5™ Data Analysis Software using a 4-parameter logistic fit.

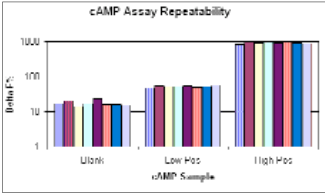


Figure 7. Repeatability of Determination. Eight determinations of a blank, a low concentration, and a high concentration cAMP sample were assayed and the Delta F% calculated.

Results

Figure 2 demonstrates the mathematical calculations performed to transform raw data to Delta F% values for each data point. The donor emission can be used as an internal control, correcting for well-to-well variations. Examples of these calculations are shown in Table 2, where the relative intensity of the donor emission remains constant over the entire cAMP concentration range, while the acceptor emission signal changes dramatically. The results of these calculations can be seen in Table 2, which depicts representative data and the calculated results. Once the ratio is calculated, the relative energy transfer rate (Delta F%) is determined. The Delta F% value is the percentage increase of the FRET signal relative to the negative control.

As demonstrated in Figures 4 and 6, TNF-α and cAMP can respectively be quantitated using HTRF® kits in conjunction with a Synergy 2 Multi-Detection Microplate Reader. The Delta F signal increases linearly over the concentration ranges tested for TNF-α. Because the cAMP assay is a competitive reaction, the signal decreases with cAMP concentration. Using a semi-logarithmic scale a sigmoid shaped curve is observed, which can be described using a 4-parameter logistic fit of the data. As demonstrated in Figure 7, when examining well-to-well repeatability values collected from the Synergy 2 Multi-Detection Microplate Reader one observes that like samples return equivalent Delta F% results.

Conclusions

1. The Synergy 2 Multi-Detection Microplate Reader was demonstrated to be an excellent detection platform in terms of sensitivity and dynamic range of both cAMP and TNF-α measurements using Cisbio's HTRF® technology.
2. Gen5™ Data Analysis Software can automatically perform the necessary calculations required for HTRF® assays.
3. Patented HTRF® ratiometric analysis minimizes well-to-well variability.