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Application Note

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

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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 assays using HTRF® technology from Cisbio.

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 can 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.

The cAMP assay is a competitive immunoassay. The Europium Cryptate donor molecule is conjugated to anti-cAMP antibody, while the d2 acceptor molecule is conjugated directly to cAMP. Binding of d2-conjugated cAMP results in a FRET reaction that emits light at 665 nm when europium cryptate is excited. The free cAMP and d2-conjugated cAMP compete with each other for the antigen recognition site of the anti-cAMP antibody. At low concentrations of free cAMP, the d2-labeled cAMP predominately 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 (Figure 1). Increasing amounts of free cAMP will lead to decreasing levels of energy transfer. The generated signal will result in a sigmoidal shaped curve when signal vs. cAMP concentration is plotted.

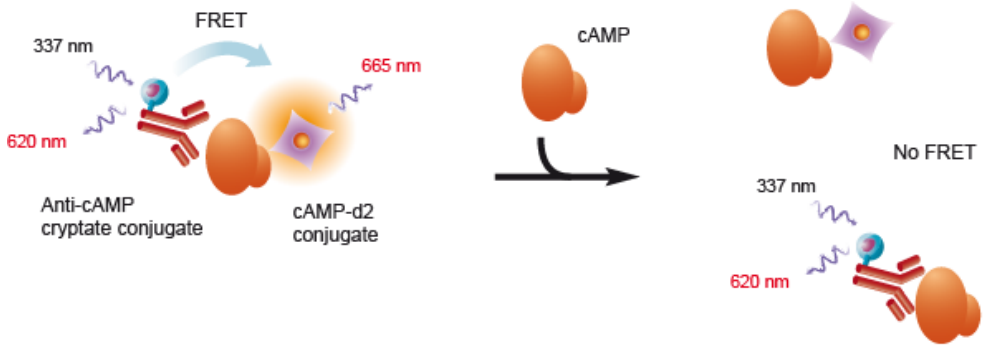

Figure 1. Schematic depicting the competitive binding of cAMP and cAMP-d2 moieties to a Europium Cryptate labeled antibody. Binding of d2-conjugated cAMP results in a FRET reaction that emits light at 665 nm when the Europium Cryptate is excited with a 320 nm light source. Competition with free cAMP results in a decrease in cAMP-d2 binding to the donor antibody and thus a decrease in FRET signal.

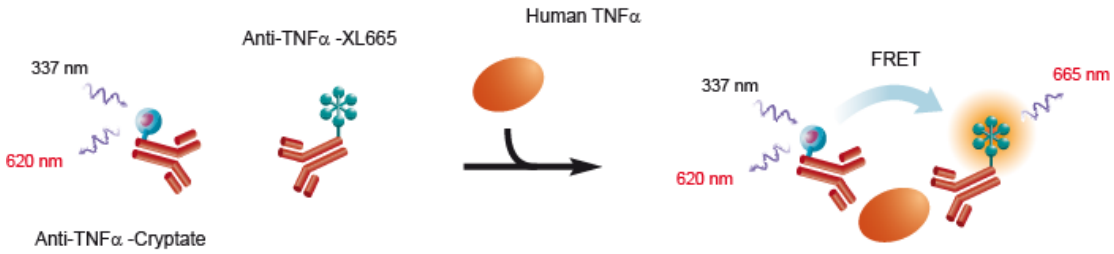

Figure 2. Schematic depicting the binding of donor and acceptor antibodies to Human TNF-α. Energy from the excitation of Europium Cryptate conjugate is transferred via FRET to the XL665 conjugated MAb. Energy is released as light emission at 665 nm. Only when both antibodies are bound to TNF-α will FRET occur. Increasing amount of TNF-α results in increasing amount of 665 nm emission signal.

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. The higher the TNF-α concentration the more donor-acceptor MAb pairs are formed, resulting in higher energy transfer (Figure 2). The signal will increase linearly with TNF-α concentration.

The Synergy™ 2 is a new type of reader that provides the combined benefit of bringing to research laboratories performance and technology usually found on high-end HTS instrumentation, while at the same time delivering flexibility and efficient cost-control to screening laboratories (Figure 3). The Synergy 2 utilizes multiple sets of optics to provide optimal performance regardless of the detection technology. 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. 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. Fluorescence polarization is accomplished with the use of polarizing filters in conjunction with label specific dichroic mirrors for wavelength specificity. For time-resolved fluorescence measurements, the Synergy 2 integrates a high-energy xenon flash lamp with excitation and emission filters and PMT detector. Luminescence measurements are made using a liquid-filled optical fiber to capture light along with a low noise PMT. The Synergy 2 is capable of reading plate formats up to 1536 wells, is robot compatible, and provides temperature control and shaking as standard features.

Figure 3. Synergy™ 2 Multi-Detection Microplate Reader.

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 µl assay volume per well. Reagents were dispensed sequentially in the following order and volumes. First, 50 µl aliquots of standard or sample were pipetted into solid white plates, followed by the addition of 25 µl of cryptate antibody. Finally 25 µl of either XL665 or d2 conjugate was 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 UV 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

Filter set 1	
Ex. Filter	330 nm (BioTek p/n 7082263)
Em. Filter	620 nm (BioTek p/n 7082265)
Mirror	UV dichroic (BioTek p/n 7138365)
Lag time	100 µsec
Integration time	300 µsec
Number of flashes	50
Sensitivity	171
Filter set 2	
Ex. Filter	330 nm (BioTek p/n 7082263)
Em. Filter	665 nm (BioTek p/n 7082266)
Mirror	UV dichroic (BioTek p/n 7138365)
Lag time	100 µsec
Integration time	300 µsec
Number of flashes	50
Sensitivity	221

Results

Figure 4 demonstrates the mathematical calculations performed to transform raw data to Delta F% values for each data point. With FRET, the acceptor signal is typically smaller than the donor emission, which can be offset by an increase in the gain on the PMT (Table 1). 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 result 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.

Figure 4. The energy transfer is calculated as follows:

$$\text{Ratio} = \left(\frac{RFU_{665}}{RFU_{620}}\right) X\ 10000$$

$$\text{Mean Ratio} = \frac{\Sigma\ \text{ratio}}{\text{No. of replicates}}$$

$$\text{Delta F\%} = \frac{Ratio_{sample} - Ratio_{neg}}{Ratio_{neg}} X\ 100$$

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

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 (nM)				
712	41721	5009	1201	9
	42914	5327	1241	12
178	41394	7896	1908	73
	42060	7494	1782	61
44.5	41670	15518	3724	237
	42180	14633	3469	214
11.125	41700	23909	5734	419
	42066	26185	6225	464
2.78	40869	31935	7814	607
	41856	35426	8464	666
0.69	40457	36016	8902	706
	40673	37237	9155	729
0.17	41135	37924	9219	735
	40837	38821	9506	761

As demonstrated in Figures 5 and 6, TNF-α and cAMP respectively can 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 shown in Figure 7, like samples return equivalent Delta F% results demonstrating good well-to-well repeatability using the Synergy 2 reader.

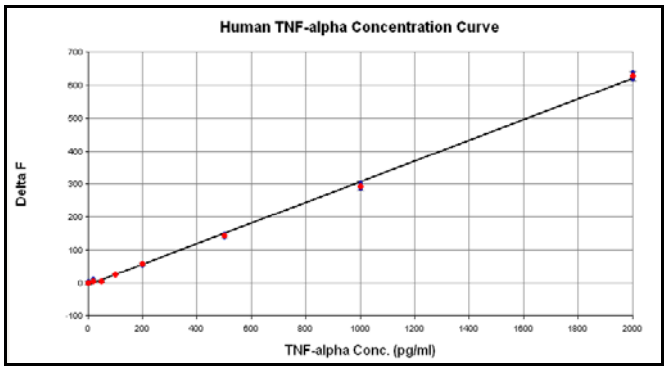

Figure 5. Human TNF-α Concentration Curve. Increasing amounts of human TNF-α were tested in duplicate and the Delta F% calculated using Gen5 Data Analysis Software. Data was plotted using a linear regression.

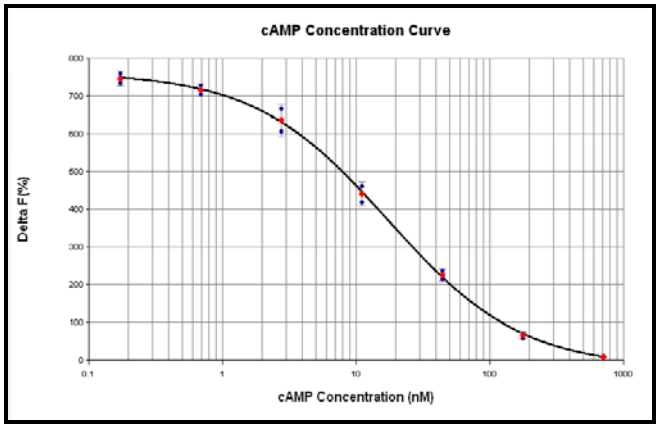

Figure 6. cAMP Concentration Curve. Increasing amounts of cAMP were assayed in duplicate. The Delta F% values were calculated from the subsequent fluorescent measurements and plotted in Gen5 Data Analysis Software using a 4-parameter logistic fit.

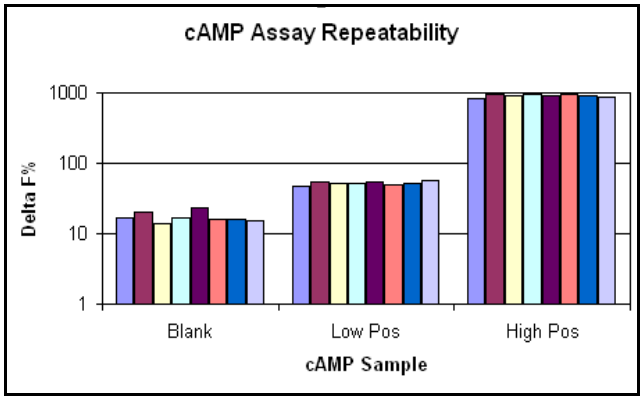

Figure 7. Repeatability of Determination. Eight determinations of a blank, a low positive, and a high positive sample were assayed and the Delta F% calculated.

Discussion

The data presented here demonstrate the utility of the Synergy™ 2 Multi-Detection Microplate Reader in conjunction with Gen5™ Data Analysis Software to perform HTRF assays. The combination of HTRF assay technology, reader performance and automated data reduction results in the correct interpretation of results. Experimental wells will often contain different treatments resulting in different baseline physical properties on a well-to-well basis. Homogeneous assay formats, by their nature, do not involve any washing or purification steps. As a result, potentially inhibitory compounds are not removed nor are the photophysical differences equalized within wells prior to the fluorescence measurements. In addition, pipetting errors, which result in different sample volumes, can take place. This can lead to misleading interpretation of the results if only the acceptor molecule fluorescence is measured. HTRF® reactions are measured at two different emission wavelengths, quantitating both the donor and the acceptor molecule's fluorescence. This allows the use of the donor fluorescence (620 nm) as an internal control, while the acceptor fluorescence (665 nm) is used as the experimental indicator. Because well-to-well differences caused by interfering agents or pipetting errors will affect both the 620 nm and 665 nm emissions, the ratio will remain the same.