



Quantitation of Human TNF- α with a QuantiGlo Assay from R&D Systems Using BioTek's Synergy™ 2 Multi-Mode Microplate Reader

Luminescent ELISA Measurements

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The peptide TNF- α is a cytokine that plays a key role in inflammation and apoptosis. As such, quantitation of this cytokine from tissue culture lysates and from human serum and/or plasma samples under experimental and environmental conditions can provide important information regarding the regulation of these phenomena. Here we describe the quantitation of TNF- α using a Synergy™ 2 Multi-Mode Microplate Reader from BioTek Instruments to measure the luminescence signal of a QuantiGlo kit from R&D Systems.

Introduction

The cytokine TNF- α plays a key role in inflammation and apoptosis [1]. TNF- α is synthesized as a 26 kD transmembrane protein [2], which is assembled as a non-covalently linked trimeric protein. A soluble 157 amino acid residue from the C-terminus of the protein is released by the activity of the metalloproteinase, TNF- α Converting Enzyme (TACE) [3]. A number of different cell types including B-cells, T-cells, macrophages, monocytes, mast cells, neutrophils and adipocytes are known to express TNF- α .

The human tumor necrosis factor alpha (TNF- α) assay is a sandwich ELISA. Microplates are coated using a monoclonal antibody (Mab) that recognizes an epitope on TNF- α . Standards and samples are pipetted into wells and any TNF- α present is captured and bound by the immobilized antibody. After washing away any unbound materials, an enzyme-linked polyclonal antibody conjugate specific for TNF- α is added to the wells. Following a second wash step to remove unbound antibody-enzyme conjugate, a luminol/peroxide substrate solution is added to the wells and light is produced in proportion to the amount of TNF- α present (Figure 1). A microplate reader capable of measuring luminescence is used to measure the intensity of the light produced.

The modular Synergy 2 Multi-Mode Microplate Reader uses several dedicated optical systems to provide optimal performance depending on the read mode required. The absorbance module includes a dedicated xenon-flash lamp light source with a diffraction grating monochromator for wavelength selection and a silicon photodiode for detection.

The fluorescence module has both a tungsten halogen continuous lamp as well as a xenon flash lamp as light sources. In addition to standard fluorescence intensity determinations, the use of the flash lamp allows for timed resolved measurements, while the tungsten lamp in conjunction with polarizing filters allows for fluorescence polarization measurements. Regardless of the type of fluorescence measurement, band-pass filters provide excitation and emission wavelength specificity and a photomultiplier tube (PMT) for detection. A dedicated high efficiency liquid light guide captures the emitted light signal and channels it directly to PMT provides superior luminescence capabilities.

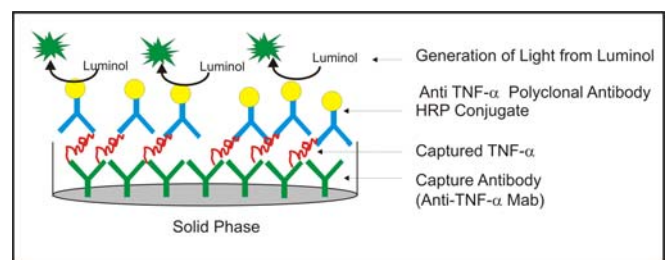


Figure 1. Illustration depicting the components of an ELISA.

Materials and Methods

A QuantiGlo Human TNF- α /TNFSF1A kit, catalog number QTA00B, was obtained from R&D Systems (Minneapolis, MN). The TNF- α assay was performed according to the assay kit instructions. The supplied lyophilized standard was reconstituted with distilled water producing a 70 ng/mL stock solution.

Using the stock solution a series of working standards ranging from 0 to 7000 pg/mL were made using either the RD5P cell culture supernatant diluent or the RD6N serum/plasma sample diluent. In replicates of 6, 100 μ L aliquots of each concentration were pipetted into the microplate (solid white) supplied with the kit.

The plate was then covered with an adhesive plate seal and allowed to incubate at room temperature for 3 hours. After incubation, the plate was washed 4 times with 400 μ L of 1X wash buffer with an ELx50 Automated Strip Washer (BioTek Instruments). The wash buffer had been previously prepared by diluting the supplied 10X concentrate with deionized water. After washing, 200 μ L of TNF- α conjugate was pipetted into all the wells of the microplate. The plate was resealed and allowed to incubate for 2 hours at room temperature. After incubation, the plate was washed as previously described and then 100 μ L of Working Glo substrate was added. Working Glo substrate was made by mixing 1 part of Glo Reagent A with 2 parts of Glo Reagent B immediately prior to use. After the addition of Working Glo reagent to the microplate the plate was placed into a Synergy 2 Multi-Mode Microplate Reader and the luminescence determined kinetically for 20 minutes.

Results

When the stability of the signal was examined, one observes a slow decrease in signal intensity over time (Figure 2). The change in signal is very consistent, losing approximately 1.5% per minute. Subsequent experiments used a 10-minute incubation for all samples.

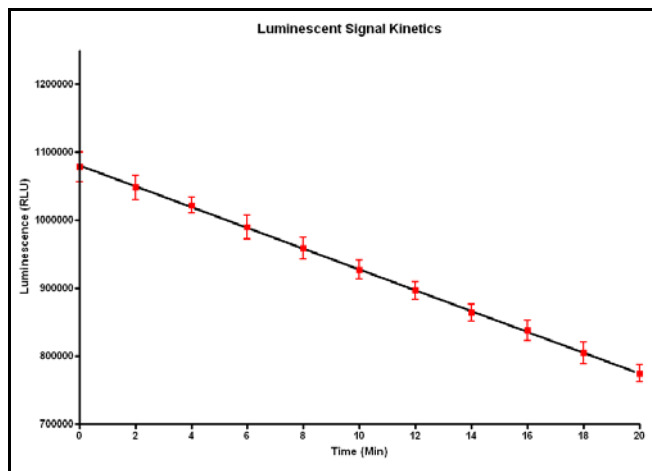


Figure 2. Luminescent Signal Kinetics. The luminescent signal of the 1400 pg/mL standards diluted in RD5P Diluent were monitored every 2 minutes for 20 minutes. Data points represent the mean of 6 determinations.

Using either of the dilution buffers provided by the assay kit, a significant relationship between the concentration of human TNF- α present and the luminescent detected was observed (Figures 3-4). In either case the lowest concentration tested (0.448 pg/ml) resulted in a signal of approximately 100 RLUs after blank correction. The highest concentration tested (7000 pg/mL) resulted in a luminescent signal of nearly 5,000,000 RLUs. When a limit of detection was calculated interpolating the regression curve with 3 times the standard

deviation of the blank wells from the data a concentration of 0.284 pg/mL and 0.152 pg/mL was determined for the RD5P and RD6N dilution buffers respectively.

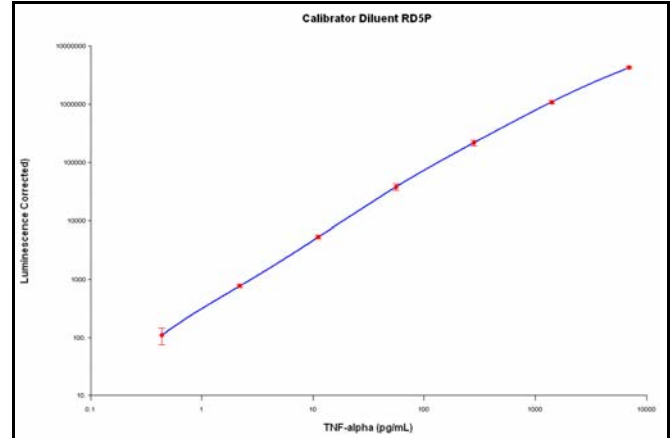


Figure 3. Calibration curve using Cell Culture Supernatant Diluent

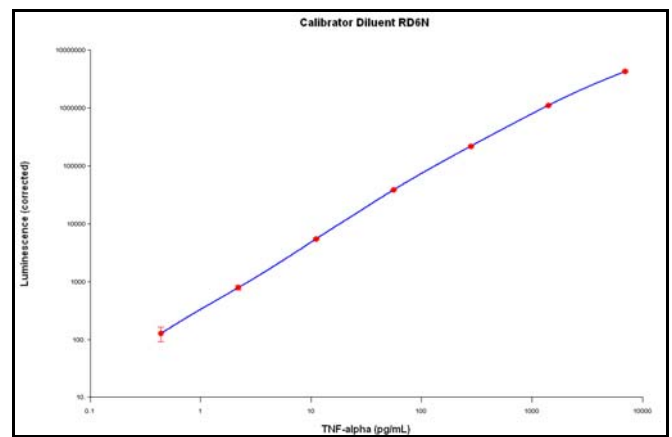


Figure 4. Calibration Curve Using Serum and Plasma Sample Diluent.

Discussion

These data indicate that the Synergy 2 Multi-Mode Microplate Reader has very sensitive luminescent capabilities in regards to the QuantiGlo TNF- α kit from R&D Systems. This ELISA based assay is easy to run, requiring only a microplate reader capable of measuring luminescence and an automated microplate washer to consistently wash the unbound materials from the well. In turn results are obtained in approximately 5 hours from the initiation of the assay. In the past TNF- α was quantitated using a bioassay that was based on the cytolytic effects of TNF- α on responsive cell lines. These assays are both tedious and prone to errors as a result of poor specificity. The advent of a rapid 5-hour ELISA that is both specific, as well as easy to run, is a significant benefit to researchers.

The kit suggests an incubation time of 5-20 minutes after the addition of the Working Glo Reagent.

The steady decrease in luminescent signal over time indicates that for maximum sensitivity, using the shortest incubation time necessary would be better. The constant change in signal over time make it imperative that the same incubation time be used for all samples. Two different diluents for the calibrators are provided as part of the assay kit, one to be used with samples from cell culture supernatants and the other to be used with serum or plasma samples. Regardless of the diluent used, very similar results were obtained in regards to the raw data from the calibrators.

Gen5™ Data Analysis Software was used to control the reader functions, as well as perform the data analysis such as plotting the standard curve, statistics etc. While the TNF- α kit suggested a cubic spline curve fit with log axis, other curve fit algorithms are available within the software. Unknown concentrations are automatically calculation by interpolation of the standard curve by the Gen5 Software.

References

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[2] Pennica, D., Nedwin, G.E., Hayflick, J.S., Seeburg, P.H., Derynck, R., Palladino, M.A., Kohr, W.J., Aggarwal, B.B., and Goeddel, D.V. (1984) Human tumour necrosis factor: precursor structure, expression and homology to lymphotoxin *Nature* 312:724-729

[3] Kriegler, M., Perez, C. DeFay, K., and Lu, S.D. (1988) A novel form of TNF/cachectin is a cell surface cytotoxic transmembrane protein: Ramifications for the complex physiology of TNF. *Cell* 53:45.-53.

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