Rapid Measurement of IgG Using Fluorescence Polarization

Using BioTek Multi-Mode Microplate Readers to Quantitate IgG in Solution

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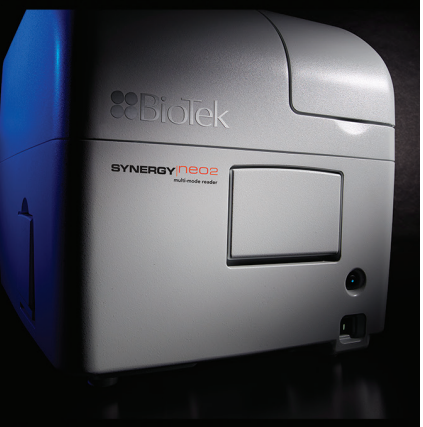

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IgG
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Abstract

The accurate, rapid and high-throughput measurement of IgG is essential in the development, and subsequent manufacture, of most therapeutic antibodies, as monoclonal antibodies are becoming increasingly dominant in biopharmaceuticals. Here we describe the use of the Synergy™ Neo2 Hybrid Multi-Mode Reader, to rapidly determine IgG concentrations using fluorescence polarization based Valita™TITER assay kit provided by Valitacell.

Introduction

Antibody production has risen sharply during the last decade as biologics, most notably monoclonal antibodies, have become a significant therapeutic platform. It has been estimated that biologics comprise 19-20% of the global market value of drugs in 2017[1]. In addition, the more traditional uses of antibodies have steadily been increasing. ELISA, first described in 1971, uses specific antibodies attached to a solid substrate to capture and quantitate analytes[2]. Protein immunoblotting, often referred to as western blots, is a widely used analytical technique to detect specific proteins in a sample that have been separated by polyacrylamide gel electrophoresis[3]. Immunohistochemistry and immuno-fluorescence use specific antibodies to detect and label specific proteins within cells and tissue slices, which can then be viewed using light or fluorescence microscopy depending on the label. All of these technologies have been tremendously assisted by the advent of monoclonal antibodies, first described in 1975, which allows for the unlimited production of specific antibodies[4].

IgG molecules (monoclonal or polyclonal) are the primary agent for all the previously described technologies. Immunoglobulin G (IgG) is the major class of the five classes of immunoglobulins (IgM, IgD, IgG, IgA, and IgE) in human beings. IgG antibodies are comprised of four different subclasses, which vary with respect to their amino acid content and immunological function[5]. In order to assess IgG antibody production or optimize cell growth conditions for IgG production it is imperative that the concentration of IgG is determined accurately.

There are several different means to quantitate antibody proteins. HPLC Protein-A column concentrated and purified antibodies have all of the media components removed, which allows for measurements using absorbance at 280 nm. Biolayer interferometry uses light wave interference to estimate the amount of material bound to the sensor[6]. The microagglutination assay uses microspheres sensitized with anti-IgG polyclonal antibodies that increasingly agglutinate with increasing amounts of IgG[7]. Proximity assays such as SPARCL[8], HTRF®[9], and FRET-PINCER®[10] are mix and read assays that employ antibody pairs specific to IgG to bring reagents in close proximity in order to elicit a measurable response without any wash steps. ELISA reactions bind IgG to a solid substrate that allows the removal of unbound and unwanted materials prior to detection[11]. While all are effective in their own way, each has deficiencies in either the necessary sample preparation, instrumentation requirements, and time required for processing that limits their usefulness with process samples from antibody production.

Here we describe a generic assay suitable to measure the concentration of any IgG or IgG variant during the discovery, cell Line development and in-process phases of the biopharmaceutical critical path. The assay is simple, rapid, cost effective and readily automatable.

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Assay Basis

The Valita™TITER assay is a mix and read assay suitable for high sample throughput operation (Figure 1). It relies on the fluorescence polarization (FP) detection of IgG Fc interactions with fluorescein labelled protein G[12]. Protein G is an immunoglobulin binding protein expressed in group G Streptococci capable of binding IgG through its Fab and Fc regions. Native Protein G (G148) has inherent binding sites for albumin, which has been removed through selective cloning[13]. Fluorescence polarization (FP) is a fluorescence technique that is based on the observation that fluorescent molecules, when excited by plane polarized light, will emit plane polarized light. In solution, proteins are free to rotate, thus the plane of polarized light emitted can change based on the fluorescence lifetime of the fluorophore in question and the extent of rotation the molecule undergoes during that time frame. The molecule's rotational speed is influenced by solution viscosity, absolute temperature, molecular volume and the gas constant. If one keeps viscosity and temperature constant, then the key variable for rotational speed differences is molecular volume or to a first approximation, molecular weight.

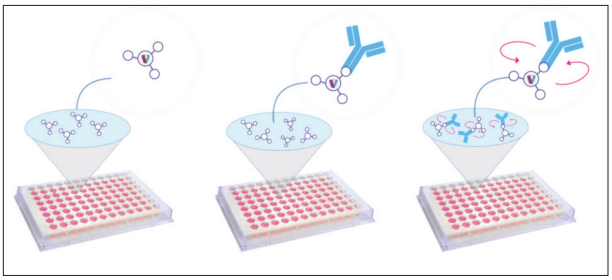

Figure 1. **Principle of the Valita™TITER Fluorescence Polarization Assay.** Fluorescein labelled Protein G (the tracer) is pre-coated in the wells of the Valita™MAb Plates. Addition of Valita™MAb Buffer resuspends the tracer. Addition of standard or sample containing IgG will be bound by the tracer: the more IgG present in the standard/sample, the greater the polarization of plane polarized excitation light.

FP effectively analyses the size of molecules/complexes by measuring how fast they rotate in solution. Consequently, when fluorescently labelled Protein G is unbound, it tumbles rapidly and depolarizes the light more than when it is bound to an IgG (which is 5x larger). This change in polarization can be used to measure the degree of Protein G binding and thus the amount of IgG in the solution. FP is measured by exciting the solution with plane polarized light and measuring the intensity of light emitted in the plane parallel to the exciting light (polarized proportion) and perpendicular to the exciting light (depolarized portion). The FP is expressed as a normalized difference of these two intensities which is typically in millipolarization units (mP).

Synergy™ Neo2 is a Hybrid Multi-Mode Reader (BioTek Instruments, Inc.) with patented Hybrid Technology™ that combines filter-based and monochromator-based detection systems in one unit. The multi-mode reader can accommodate absorbance (UV-Vis), fluorescence, luminescence, and AlphaScreen™ detection modalities. In addition, the reader possesses time resolved fluorescence (TRF) and fluorescence polarization (FP) capabilities. In the case of FP, the reader uses a filter-based system of excitation and emission filters, polarizers, and dichroic mirrors in conjunction with a high performance xenon flash lamp and dual-matched photomultiplier tubes (PMT) to simultaneously detect the parallel and perpendicular fluorescent emissions.

Materials and Methods

Purified mouse IgG1 and IgG2b samples along with corresponding supernatants from hybridoma cell lines was a generous gift from Green Mountain Antibodies (Burlington, VT). In addition, several different species of IgG, including Goat (I-5326); Rabbit (I-5006); Bovine (I-5508); Human (I-4508); Rat (I-4131); Mouse (I-5381); Porcine (I-4531); Sheep (I-5131) were obtained from Millipore-Sigma in powdered form and dissolved in RPMI media supplemented with 10% Fetal Bovine Serum (FBS), penicillin and streptomycin antibiotics. Valita™TITER assay kits were from Valitacell (Dublin, Ireland www.valitacell.com).

Standards were prepared according to the respective product instruction for use. Commercially available purified IgG from several different animal species was reconstituted with media to a concentration of 5 mg/mL. A portion of these stocks was further diluted to 100 µg/mL with media. A range of standards were prepared by making serial (1:2) dilution with media as the diluent. Valita™MAb Buffer (60 µL) was pipetted into each well of the Valita™MAb Plate, along with 60 µL of IgG standards. Contents were mixed and incubated in the dark for 30 minutes before being read on the BioTek Synergy Neo2.

The reader was controlled and the data captured using Gen5 Data Analysis software (BioTek Instruments). FP measurements were made using a deep blocking filters with an excitation of 485/20 nm and an emission of 528/20 nm in conjunction with a 510 nm cut off dichroic mirror and polarizing filters. Instrument settings used are provided in Table 1.

Parameter	Synergy Neo2	Synergy S2	Cytation 1
PMT Gain	50 / 50	65	51
Reads/data point	50	50	50
Probe height	8.00	7.00	9.75
Lamp Energy	High	N/A	High

Table 1. Plate Reading Parameters.

To minimize experimental differences in meaurements, FP data were adjusted using a G-factor to return a value of 110 mP for free unbound Protein G tracer. The data was plotted as the change in polarization from the tracer only standard and a 4-parameter logistic fit applied by the Gen5 software.

Results

The ability of the Valita™TITER assay to quantitate IgG in media was assessed using a serial dilution of a mouse monoclonal IgG1 isotype. As demonstrated in Figure 2, increasing concentration of the IgG results in an increase in the polarization value returned.

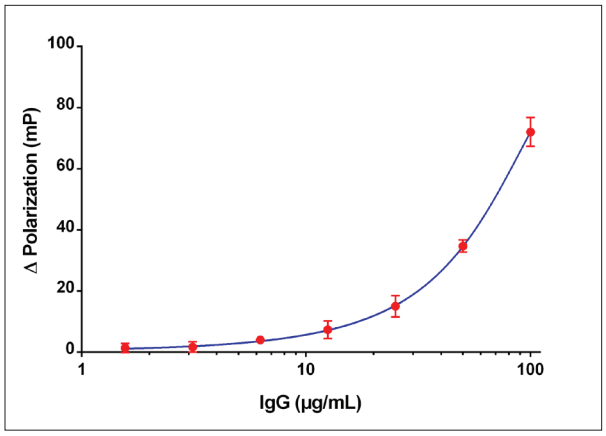

Figure 2. **Typical IgG Standard Curve.** Dilutions of purified mouse IgG previously quantitated using a colorimetric protein assay were assayed using the Valita™TITER assay. The change in polarization was plotted and a 4-parameter logistic fit applied. Data points represent the mean and standard deviation of 3 data points.

This technique compares favorably with the use of HPLC as a means to quantitate IgG molecules. When like samples are assayed using the both the Valita™TITER and HPLC methods, a linear relationship between the calculated concentrations is observed (Figure 3). HPLC is generally regarded as the gold standard method for IgG determination in the biopharmaceutical industry. Results demonstrate a high degree of correlation between Valita™TITER and HPLC (R2=0.9926).

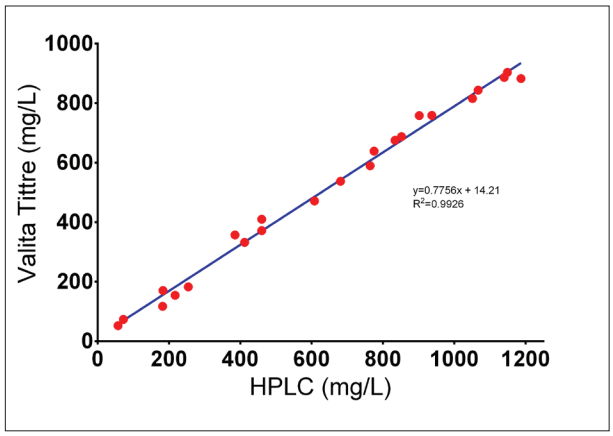

Figure 3. **Valita™TITER vs. HPLC.** A range of 'in process' IgG samples from cell culture supernatants were quantified using a 'gold standard' protein A HPLC method in parallel with the Valita™TITER assay system. The resultant concentrations of each method were plotted against each other and a linear regression performed on the data.

While the vast majority of monoclonal antibodies are produced from mouse, rat, and rabbit species, polyclonal antibodies are used from any number of mammalian donors. As demonstrated in Figure 4, the Valita™TITER assay is capable of detecting and quantitating any of the mammalian species we tested. Differences in response are likely the result of a combination of differential binding of the protein G tracer, as well as deviations in the reported IgG concentration of the commercially obtained source and solubilization of the lyophilized powder.

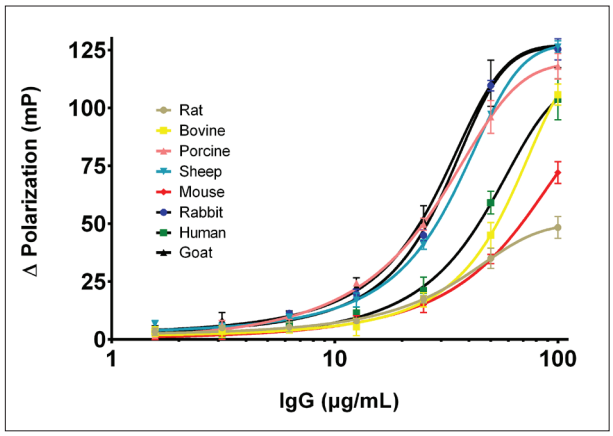

Figure 4. **Species Comparison.** Several different species of commercially available IgG were diluted and assayed using Valita™TITER kit. The change in polarization was plotted and a 4-parameter logistic fit applied. Data points represent the mean and standard deviation of 3-data points.

The specificity of the protein G tracer is demonstrated in Figure 5. Dilutions of human IgG and IgM were assayed using the Valita™TITER kit and the resultant change in polarization plotted. While human IgG had a change of over 100 mP units with increasing antibody concentration, IgM resulted in virtually no change in polarization. Because FP is in essence an assay that measures changes in size, one can infer that the assay tracer does not bind to nor detect IgM.

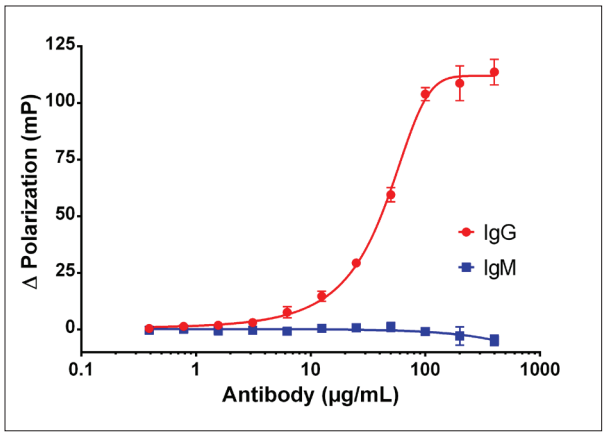

Figure 5. **Human IgG vs IgM Signal.** Human IgG and Human IgM dilutions were assayed using the Valita™TITER assay.

BioTek offers a number of different multi-mode reader options to meet the needs of different investigators. Figure 6 demonstrates the repeatability of determination between different readers, each with different fluorescence optics, to make polarization measurements. When the fluorescence polarization of samples from the same plate is determined using three different readers, substantially the same response curve is returned.

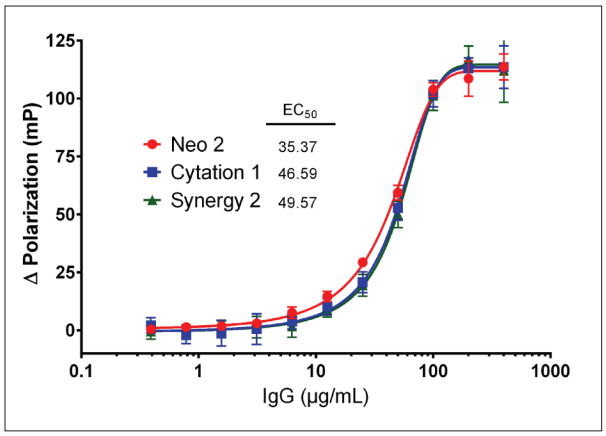

Figure 6. **Reader Comparison.** The polarization values from a titration of Human IgG was determined using three BioTek Multi-mode Microplate readers representing different optical designs. The change in polarization was plotted and a 4-parameter logistic fit applied and EC_{50} values determined. Data points represent the mean and standard deviation of 4-data points.

Discussion

These data presented demonstrate the utility of the Valita™TITER assay in conjunction with the BioTek Multi-mode readers to rapidly quantitate IgG concentrations from mixed samples. The production of IgG antibodies has become widespread in biomedical research and manufacture. The ability to quickly determine IgG concentration from a mixed sample is of particular importance during production as an assessment of production rate and a final concentration determination. Cell culture media normally contains animal serum, salts, cofactors, and amino acids necessary for cell growth, whose presence often precludes the use of traditional colorimetric or fluorometric protein assays when media supernatants are being assessed. Only with the removal of these components through purification (e.g. column chromatography) can these methods be employed. The Valita™TITER assay allows the direct quantitation of IgG from process samples without sample prep or often without dilution.

The combination of the Valita™TITER assay and the Synergy™ Neo2 Hybrid Multi-Mode Reader offers a number of features to the end user. FP calculations can be made directly using BioTek's Gen5™ Microplate Reader and Imager Software. Alternatively, parallel and perpendicular fluorescence measurements can be imported into Valita™ App software, which allows for sample results and standards FP results to be stored in a database, allowing for trend analysis.

BioTek offers a number of different multi-mode reader options depending on the end-user' s needs and budget. The Synergy Neo2 offers rapid determinations through the use of multiple PMTs for simultaneous parallel and perpendicular measurements. The Synergy 2 and Synergy H1 measure parallel and perpendicular signals in sequence, while the Cytation™ 1 offers the ability to enable digital microscopic im age-based analysis of saples in addition to its PMT-based plate reading capabilities.

All the BioTek readers are coupled with Gen5 Microplate Reader and Imager Software (BioTek Instruments), which controls reader functions, collects and stores generated data, and performs data reduction. A number of different curve fits are available to describe data or to be used as standards curve for interpolation to calculate unknown sample concentrations. For GMP environments, the software is available as a secure version that has ISO 21 CRF part 11 compliance, including password protection, user- based privileges, and electronic signature.

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