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Rapid Measurement of IgG Using Fluorescence Polarization

Using Agilent BioTek multimode microplate readers to quantitate IgG in solution

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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. This application note describes the use of the Agilent BioTek Synergy Neo2 hybrid multimode reader to rapidly determine IgG concentrations using the fluorescence polarization-based ValitaTiter assay 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. The market for global biologics is estimated to increase to $382.04 billion in 2022, where in 2021 25% of new molecular entities (NMEs) approved by the FDA have been biologics.[1,2] 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.[4] Immunohistochemistry and immunofluorescence 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 the unlimited production of specific antibodies.[5]

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.[6] 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 quantify IgG antibodies. HPLC protein A column concentrated, and purified antibodies have all media components removed, which allows measurements using absorbance at 280 nm. Biolayer interferometry uses light wave interference to estimate the amount of material bound to the sensor.[7] The microagglutination assay uses microspheres sensitized with anti-IgG polyclonal antibodies that increasingly agglutinate with increasing amounts of IgG.[8] Proximity assays such as SPARCL[9], HTRF[10], and FRET-PINCER[11] are mix-and-read assays that use antibody pairs specific to IgG to bring reagents in close proximity 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 before detection.[12] 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.

This application note describes 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.

ValitaTiter assay

ValitaTiter is a rapid, robust, and accurate IgG quantification assay. The ValitaTiter assay range measures IgG concentrations from 2.5 to 2,000 mg/L, with a simple add-mix-read protocol. The assay is performed in less than 15-minutes and can be incorporated into any bioprocess workflow in a 96- or 384-well plate format. The assays are high throughput and can be fully automated. Analysis can be carried out in crude cell culture media containing up to 15×10^6 cells/mL with a low-sample volume and limited test sample prepreparation. Assay detection can be performed using fluorescence polarization on a standard microplate reader (Figure 1).
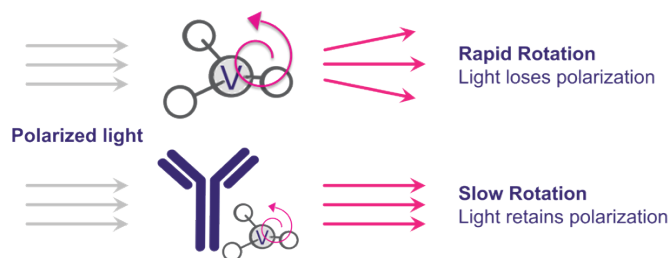
Figure 1. Principle of the ValitaTiter fluorescence polarization assay. A fluorescein-labeled protein G derivative (the probe) is precoated in the wells of the ValitaTiter microplates. Addition of cell culture media resuspends the probe. Addition of standard or sample containing IgG will be bound by the probe: the more IgG present in the standard/sample, the greater the polarization of plane polarized excitation light.

When you excite a sample with plane polarized light, the probe unbound in solution rotates rapidly leading to depolarization of emitted light. When bound to a higher molecular weight target protein the complex rotates slowly, leading to the retention of polarized light. The amount of target in solution can then be calculated, with the observed output polarization in a mixture of labeled probe and target being proportional to the fraction of bound probe in solution.

The Agilent BioTek Synergy Neo2 hybrid multimode reader with patented Hybrid Technology combines filter- and monochromator-based detection systems in one unit. The multimode 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

Human IgG (I-4506) and IgM (I-8260) were obtained from Millipore-Sigma (Burlington, MA) in powdered form and dissolved in RPMI media supplemented with 10% fetal bovine serum (FBS), penicillin, and streptomycin antibiotics. ValitaTiter microplates were from ValitaCell (Dublin, Ireland, www.valitacell.com).

Standards were prepared according to the respective product instruction for use. Commercially available purified IgG was reconstituted with media to a concentration of 5 mg/mL. A portion of the stock 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. Fresh media (60 µL) was pipetted into each well of the ValitaTiter plate, along with 60 µL of IgG standards. Contents were mixed and incubated in the dark for five minutes before being read on the Agilent BioTek Synergy Neo2.

The reader was controlled, and the data captured using Agilent BioTek Gen5 microplate reader and imager software. FP measurements were made using a deep blocking filter 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.

Table 1. Plate reading parameters.

Parameter	Agilent BioTek Synergy Neo2	Agilent BioTek Cytation 1
PMT Gain	50/50	51
Reads/Data Point	50	50
Probe Height	8.00	9.75
Lamp Energy	High	High

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

Results and discussion

ValitaTiter compares favorably with the use of HPLC as a means to quantitate IgG molecules. When like samples are assayed using both the ValitaTiter and HPLC methods, a linear relationship between the calculated concentrations is observed (Figure 2). HPLC is generally regarded as the gold standard method for IgG determination in the biopharmaceutical industry. Results demonstrate a high degree of correlation between ValitaTiter and HPLC (R^2 = 0.9926).


$y = 0.7756x + 14.21$
$R^2 = 0.9926$
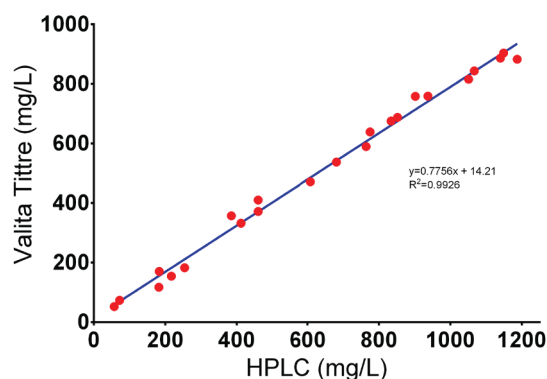
Figure 2. ValitaTiter versus HPLC. A range of in-process IgG samples from cell culture supernatants were quantified using a gold standard protein A HPLC method in parallel with the ValitaTiter assay system. The resultant concentrations of each method were plotted against each other and a linear regression performed on the data.

The specificity of the ValitaTiter probe is demonstrated in Figure 3. Dilutions of human IgG and IgM were assayed using the ValitaTiter 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 tracerprobe does not bind to nor detect IgM.
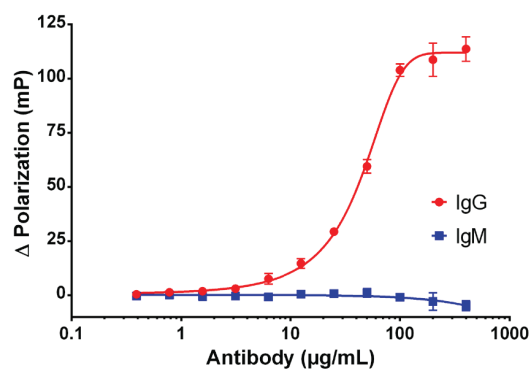
Figure 3. Human IgG versus IgM signal. Human IgG and human IgM dilutions were assayed using the ValitaTiter assay.

Agilent offers a number of different multimode reader options to meet the needs of different investigators. Figure 4 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.
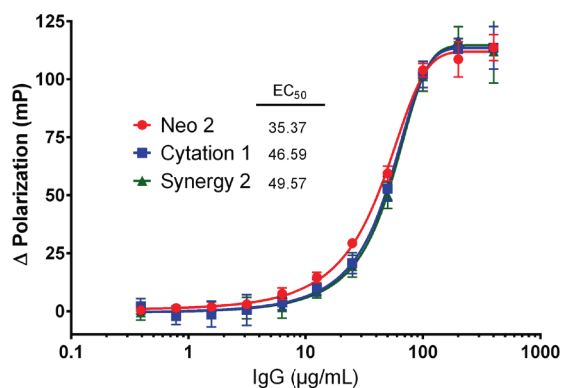
Figure 4. Reader comparison. The polarization values from a titration of human IgG was determined using three Agilent BioTek multimode microplate readers representing different optical designs. The change in polarization was plotted, a four-parameter logistic fit was applied, and EC_{50} values were determined. Data points represent the mean and standard deviation of four data points.

Conclusion

The data presented demonstrate the utility of the ValitaTiter assay in conjunction with Agilent BioTek multimode readers to rapidly measure IgG titer 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 contain 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 used.

ValitaTiter is a simple, accurate, rapid, and automation-friendly assay that enables high-throughput quantification of IgGs from crude samples. ValitaTiter is robust to cell contamination and facilitates 'straight from cell culture measurement' across a broad functional range.

The ValitaTiter assay has considerable advantages over alternative quantification assays including cost, simplicity, speed, and throughput.

The combination of the ValitaTiter assay and the Agilent BioTek Synergy Neo2 hybrid multimode reader offers a number of features to the end user. FP calculations can be made directly using Agilent BioTek Gen5 microplate reader and imager software.

Agilent offers a number of different multimode 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 H1 measures parallel and perpendicular signals in sequence, while the Cytation 1 offers the ability to enable digital microscopic image-based analysis of samples in addition to its PMT-based plate reading capabilities.

All Agilent BioTek readers are coupled with Gen5 microplate reader and imager software, 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 curves 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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