Comparison of Assay Methods for the Detection of Residual Protein A in Biological Therapeutics

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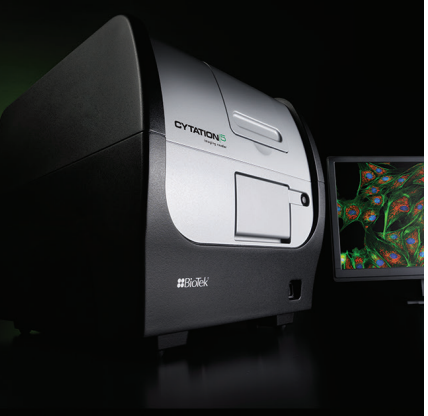

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Introduction

There continues to be a focus on the development of recombinant human monoclonal antibodies (rhuMAb IgGs) for therapeutic use with dozens reaching the market in the last decade. Therapeutic proteins of the scale needed for treatment of even a small population require industrial scale production using a variety of bioprocessing methods including recombinant cell line expression systems, chromatographic purification methods and stringent purity assessment. Purity requirements include minimizing the concentration of host cell proteins and DNA ranging in the parts per million or lower relative to the product. Additionally, the formulation must be sterile insuring no viable microorganisms exist in the final product and void of any residual contaminants from the purification process itself.

A recombinant human monoclonal antibody is commonly produced in a mammalian cell line such as Chinese Hamster Ovary (CHO) cells during large scale manufacturing. Purification typically relies on the use of a three-column chromatography process to meet the stringent purification requirements: 1) Protein A affinity chromatography, 2) Cation exchange (CEX), and Anion Exchange (AEX). Additionally, a viral filtration (VF) step is generally used during the final stages of production[1]. Resin with immobilized Staphylococcal Protein A (PA) has a high affinity for the crystallizable fragment (Fc) region present in rhuMAb IgGs allowing capture from the culture media or crude cell lysate of the host cell line. While these resins provide a high capacity and selectivity for the target protein, trace amounts of the PA ligand has been found contaminating the antibody product. Residual PA contamination of a biotherapeutic may result in immunogenic consequences as well as toxicological and/or mitogenic effects[2]. Therefore, reliable, robust methods for the detection and quantification of trace amounts of PA are necessary and mandated by the FDA.

Here we demonstrate automation of a HTS compatible homogenous proximity assay (Figure 1) and conventional ELISA method (Figure 2) for the detection of residual PA in biological therapeutics. The demonstration includes evaluation of the lower detection limit (LDL) as well as screening results for detection of residual PA in a panel of ten (10) human IgG antibodies including the active therapeutic components of Herceptin®, Rituxan®, and Erbitux®, the antibodies trastuzumab, rituximab, and cetuximab, respectively.

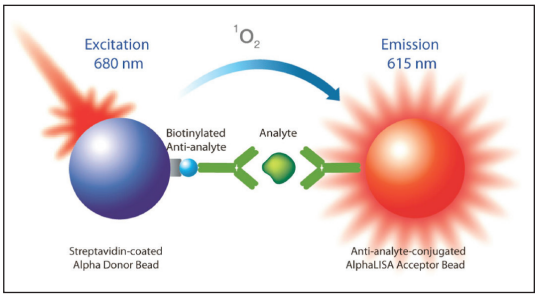

Figure 1. **Assay schematic for AlphaLISA homogeneous proximity assay principle for the detection of analyte in biotherapeutic products.** Upon excitation, the AlphaLISA donor bead generates singlet oxygen molecules. If the acceptor bead is in close proximity due to the creation of a sandwich immunoassay, the singlet oxygen molecules will trigger a cascade of energy transfer in the acceptor bead, resulting in a sharp peak of light emission at 615 nm.

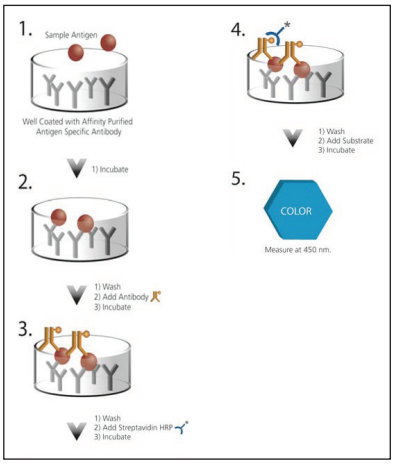

Figure 2. **Assay schematic for ELISA assay principle for the detection of analyte in biotherapeutic products.** 1. Samples and standards are added to wells coated with chicken antibody specific for Protein A. The plate is washed leaving only bound protein A. 2. A biotinylated chicken antibody to protein A is added and binds the captured Protein A. 3. Streptavidin-HRP conjugate is added which binds to the biotinylated antibody. 4. TMB substrate is added that generates a blue color when catalyzed by HRP.

Instrumentation

Cytation 5 Cell Imaging Multi-Mode Reader

Cytation™ 5 combines automated digital microscopy and conventional microplate detection in a configurable, upgradable platform. Cytation 5 includes both filter-based and monochromator-based optics for multi-mode versatility and offers laser-based excitation for Alpha assays.

Synergy HTX Multi-Mode Reader

Synergy™ HTX Multi-Mode Microplate Reader is a compact, affordable system for 6- to 384-well microplates and Take3™ Micro-Volume Plates. Absorbance, fluorescence, luminescence and AlphaScreen®/AlphaLISA® measurements are all made using a unique dual-optics design that provides superior performance.

MultiFlo FX Multi-Mode Dispenser

MultiFlo™ FX is an automated multi-mode reagent dispenser for 6- to 1536-well microplates offering BioTek's unique Parallel Dispense™ technology. Up to four independent reagents can be dispensed in parallel without potential carryover. The instrument was used to dispense assay specific reagents to the 384-well assay plates.

Materials and Methods

Reagents

AlphaLISA Residual Protein A Kit was from Perkin Elmer (No. AL287, Waltham, MA, USA). Protein A ELISA Kit was a gift from Enzo Life Sciences (No.ADI-900-057C, Farmingdale, NY, USA).

Assay Plates

AlphaPlate™ -384 grey, opaque 384-well (No.6005350) microplates were from PerkinElmer (Waltham, MA, USA). Protein A clear 384-well microplates were a gift from Enzo Life Sciences (Farmingdale, NY, USA).

Instrument Settings

The Synergy HTX was used with settings shown in Tables 1 and 2, while the Cytation 5 was used with Table 3 settings.

Synergy™ HTX Read Parameters (AlphaLISA)	
Mode	Alpha
Dual filter sets	
Filter set 1	EX=680/30, EM=Plug
Filter set 2	EX=Plug, EM=570/100
Gain	200
Filter switching per well	Selected
Read speed	Normal
Read height	8.00 mm

Table 1. Synergy HTX AlphaLISA reading parameters used in Gen5™ Data Analysis Software.

Synergy™ HTX Read Parameters (ELISA)	
Mode	Absorbance
Wavelength	450 nm
Read speed	Normal
Delay after plate movement	100 msec
Measurements per data point	8

Table 2. Synergy HTX ELISA reading parameters used in Gen5 Data Analysis Software.

Cytation™ 5 Read Parameters (AlphaLISA)	
Mode	Alpha
Gain	120
Delay after plate movement	0 msec
Excitation time	80 msec
Delay after excitation	120 msec
Integration time	160 µsec
Read height	8.00 mm

Table 3. Cytation 5 AlphaLISA reading parameters used in Gen5 Data Analysis Software.

Sample Prep

Protein A analyte standard solutions were prepared as per the manufacturer's recommendation. Samples were diluted to ensure total IgG concentration ≤1 mg/mL. 80 µL of each standard was transferred to a microfuge tube and 40 µL of 3x dissociation buffer was added. Standards and samples were then heated at 98 °C for 60 minutes in a heating block. Following incubation standards and samples were allowed to cool to room temperature (RT) for ~5-7 minutes followed by centrifugation for 5 minutes ~200 g.

AlphaLISA® Assay Setup

Quadruplicate sample and standards were transferred, 5 µL to each well, to a 384-well assay plate. A 2.5x mixture of AlphaLISA Anti-Protein A acceptor beads and biotinylated antibody anti-analyte was prepared and 20 µL added to each assay well using the MultiFlo™ FX. The plate was placed on an orbital shaker for 10 minutes then incubated at RT for a total of 60 minutes. A 2x SA-donor bead mix was prepared fresh and 25 µL added to each assay well using the MultiFlo FX followed by a 30 minute incubation at RT protected from direct light. Following the final incubation period the plate was read on the microplate readers.

ELISA Assay Setup

Quadruplicate sample and standards were transferred, 25 µL, to each well of the assay plate and incubated at RT for 60 min. with shaking at ~500 rpm. The plate was washed 4x with 100 µL wash solution using the MultiFlo FX. 25 µL biotinylated anti-protein A antibody was added to the plate, with the exception of the blanks, and incubated at RT for 60 min. with shaking at ~500 rpm. After the plate was washed as above, 25 µL streptavidin conjugated HRP solution was added to all wells, with the exception of the blanks, and the plate incubated at RT for 30 min. with shaking at ~500 rpm. The plate was washed as above and 25 µL substrate solution was added. The plate was incubated at RT for 15 min. with shaking. The reaction was halted by the addition of 15 µL of stop solution to each well and read on a microplate reader.

Results and Discussion

Protein A Standard Curve

Standard curves spanning ~6 decades were prepared for each experiment using Protein A standard provided from the kit manufacturers. These were used for optimization of Cytation™ 5 and Synergy™ HTX reader parameters (Tables 1-3) and determination of the sample concentration when performing the assays. As can be seen in Figure 3, the data can be fit using a 4-parameter logistic equation and a $1/Y^2$ data weighting. For determination of the lower detection limit (LDL) either three background points in quadruplicate (12 data points) or 16 replicates were required for AlphaLISA or ELISA respectively. The LDL is calculated by interpolating the average of the background counts (12 or 16 wells without analyte) + 3 x standard deviation value (average background counts + (3xSD)) on the standard curve (Figures 3a and 3b).

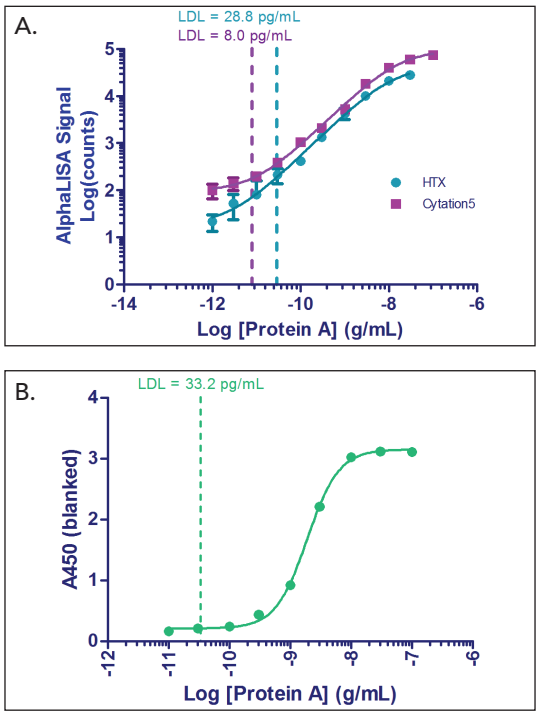

Figure 3. **Protein A Standard Curve. A)** AlphaLISA Protein A Assay. A12-point dilution series of the positive control was prepared ranging from 100,000 - 0.3 pg/mL was prepared. Twelve background points were used to calculate LDL. **B)** Protein A ELISA Assay. A 9-point dilution series of the positive control was prepared ranging from 100,000 – 10 pg/mL. Twelve background points were used to calculate LDL.

The AlphaLISA assay showed excellent correlation between readers as seen by the nearly parallel standard curves in Figure 3a. The Alpha laser contained in the Cytation 5 results in significantly higher signal generation, ~ 2-fold, under otherwise identical assay conditions (Figure 3a). There appears to be increased variability in replicate data at the lower range of concentrations when read on the Synergy HTX contributing to a >3-fold higher LDL; 29 pg/mL versus 8.0 pg/mL on the Cytation 5 (Figure 3b). The ELISA assay provided excellent correlation between replicates with a calculated LDL of ~ 33 pg/mL (Figure 3b). All determinants correlate well with established assay performance characteristic as per the manufacturers' specifications.

Antibody Panel Screen for Residual Protein A

A panel of ten (10) human IgG antibodies including the therapeutic antibodies trastuzumab, rituximab and cetuximab were assayed for the presence of residual PA. While most antibodies are stable at very high concentrations in solution, elevated concentrations above 1.0 mg/mL can interfere with assay performance therefore all samples were diluted prior to analysis. Following determination of the signal for each sample the standard curve was used to interpolate the concentration of residual PA. The actual concentration present in the stock solution of antibody was then determined using the appropriate dilution factor (Table 4).

			AlphaLISA		ELISA
Antibody	Stock (mg/mL)	Dilution Factor	HTX [PA] (corr.) (pg/mL)	Cy5 [PA] (corr.) (pg./mL)	HTX [PA] (corr.) (pg./mL)
IgG1	1.0	2	ND	ND	ND
IgG2	1.0	2	ND	ND	ND
IgG3	1.0	2	<LOQ	ND	ND
IgG4	1.0	2	18,647	19,647	7,059
Cetuximab	5.0	20	12,850	12,306	15,922
α-VEGFR2	0.2	0	ND	ND	ND
Rituximab	10.0	20	797	490	ND
12G5 Mab	0.5	0	ND	ND	ND
Trastuzumab	21.0	42	611	366	ND
α-hTNF IgG	0.1	0	ND	ND	ND

Table 4. **Panel of antibodies screened for residual Protein A.** Antibodies were diluted from stock to <1 mg/mL and screened in quadruplicate. Residual PA was determined by interpolation from a standard curve. Actual [PA] present in antibody stock solutions was corrected for by multiplying by the appropriate dilution factor.

As seen in Table 4, several of the antibodies tested in the panel showed significant residual PA including IgG4 and the therapeutic Cetuximab. Rituximab and trastuzumab also were found to contain detectible levels of PA when analyzed using the AlphaLISA assay but were undetectable using the ELISA assay method. All other antibodies demonstrated PA levels less than the limit of quantification of each instrument and assay. Figure 4 plots the antibodies showing detectable levels of PA expressed as a percentage contamination.

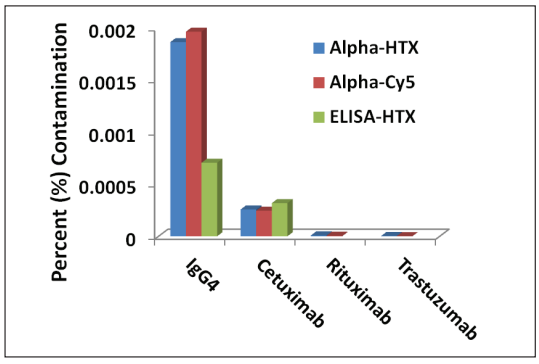

Figure 4. **Residual Protein A Screen of a panel of Human Antibodies.** A panel of ten (10) human antibodies was screened for detectible levels of Protein A. Only those antibodies showing quantifiable [PA] are shown in the figure.

Conclusion

The assays were performed in a HTS compatible 384-well microplate format using automated liquid handling for reagent dispensing. The detection of the low levels of contamination present illustrates the excellent sensitivity of both assay methods. Good correlation is apparent between both instruments for detection of residual PA using the AlphaLISA Residual Protein A and ELISA kits. Cytation 5 equipped with the Alpha-specific laser generated higher signals than the Synergy HTX, more rapid analysis times as well as an improved LDL suitable for HTS operation. Conversely, the Synergy HTX is an affordable option for non-HTS work flows. The Synergy HTX in conjunction with the higher-density, 384-well ELISA format provides for a lower-cost, higher-throughput assay platform versus a standard 96-well ELISA format.

References

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