Automation of a Homogeneous Proximity Assay for Detection of Residual Protein A in Biological Therapeutics

Comparison of AlphaLISA Performance using Cytation™ 5 and Synergy™ HTX

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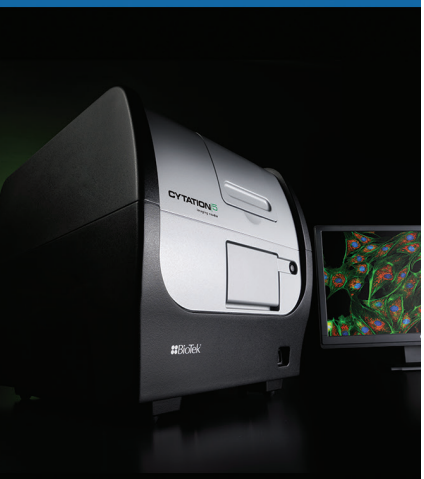

Key Words:

Protein A

Biotherapeutics

Immunogenicity

Antibody

AlphaLISA

Protein A is used in affinity chromatography for therapeutic antibody purification and has been shown to leach from columns during purification of antibodies resulting in contamination. Residual Protein A in therapeutic drugs could elicit mitogenic or immunological reactions in patients. Therefore, there are strict FDA guidelines regarding acceptable levels of Protein A in antibody preparations intended for use as therapeutics. Given the prevalence of biologics in the current drug development pipeline, highly sensitive and robust quantification methods amenable to HTS are desirable. Here we demonstrate automation of a HTS compatible homogenous proximity assay for the presence of residual Protein A in biological therapeutics using AlphaLISA technology. The demonstration includes evaluation of the lower detection limit (LDL) of Protein A as well as screening results for detection of residual Protein A in a panel of ten human IgG antibodies including the therapeutic antibodies Herceptin®, Rituxan®, and Erbitux®.

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[1]. 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); a viral filtration (VF) step is also 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 to leach from the column contaminating the antibody product. Residual PA contamination of a biotherapeutic may result in immunogenic consequences as well as toxicological and/or mitogenic effects[1]. Therefore, reliable, robust methods for the detection and quantification of trace amounts of PA are necessary and mandated in the US by the FDA.

Here we demonstrate automation of a HTS compatible homogenous proximity assay for the detection of residual PA in biological therapeutics (Figure 1). 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 therapeutic antibodies Herceptin®, Rituxan®, and Erbitux®.

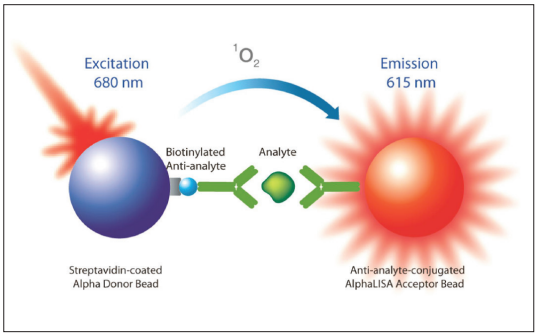

Figure 1. **Assay schematic for AlphaLISA homogeneous proximity assay principle for the detection of analyte in biotherapeutic products.** Upon excitation, the AlphaLISA donor bead generates singlet oxygen molecules. If the acceptor bead is in close proximity due to the creation of a sandwich immunoassay, 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.

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Instrumentation

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.

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 Microplate 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.

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.

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 2. **Cytation 5** AlphaLISA reading parameters used in Gen5 Data Analysis Software.

Materials and Methods

Reagents

Residual Protein A Kits (Catalog No. AL287C) were a gift from PerkinElmer (Waltham, MA, USA). The antibodies Rituxan, Herceptin and Erbitux were from a private source. Anti-hCXCR4 clone 12G5 (Catalog No. MAB170), Human VEGF R2/KDR PAb and Human TNF-α MAb (Catalog No. MAB620) was purchased from R&D Systems (Minneapolis, WI). IgG1, IgG2, IgG3 and IgG4 (Catalog Nos. I5154, I5404, I5654, and I4639) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Plates

AlphaLISA

AlphaPlate™ -384 grey, opaque 384-well microplates were a gift from PerkinElmer (Catalog No. 6005350, Waltham, MA, USA).

Instrument Setting

The Synergy HTX Multi-Mode Microplate Reader and Cytation 5 Cell Imaging Multi-Mode Reader were used with the settings shown in Tables 1 and 2, respectively.

AlphaLISA Assay Setup

The AlphaLISA assay was performed as previously described as per the manufacturer's recommendation with the following modifications. Briefly, following preparation of the Protein A analyte standard solutions and samples 80 µL of each standard was transferred to a microfuge tube and 40 µL of 3x dissociation buffer was added. The standards and samples were then heated at 98 °C for 60 minutes in a heating block. Following the incubation period the standards and samples were allowed to cool to room temperature (RT) for ~5-7 minutes followed by centrifugation for 5 minutes ≤200 g. Quadruplicate sample and standards were then transferred, 5 µL each, 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 was 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 in the dark at RT. Following the final incubation period the plate was read on the microplate readers.

Results and Discussion

Typically, residual PA is found in trace level with a large excess of IgG biotherapeutic antibody resulting in little PA found free in solution. Pretreatment of samples by heating in the presence of chaotrophic agents is therefore required to dissociate the PA-rhuMAb complex. All samples and standards were pretreated as described above. Additionally, at low concentrations of analyte adherence to vessel surfaces can occur. Therefore PA dilutions to be used for the generation of standard curves need to be prepared and used within 60 minutes for accurate quantification of residual Protein A in the samples being tested.

A 12-point standard curve with concentrations spanning ~6 decades was prepared for each experiment. 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) three background points in quadruplicate (12 data points) are also needed. The LDL is calculated by interpolating the average of the background counts (12 wells without analyte) + 3 x standard deviation value (average background counts + (3xSD)) on the standard curve (Figure 2). The concentration of analyte contained in samples is interpolated from the standard curve. If samples had been diluted, the concentration interpolated from the standard curve was multiplied by the dilution factor resulting in the corrected (corr.) residual Protein A concentration contained in the sample (Table 3).

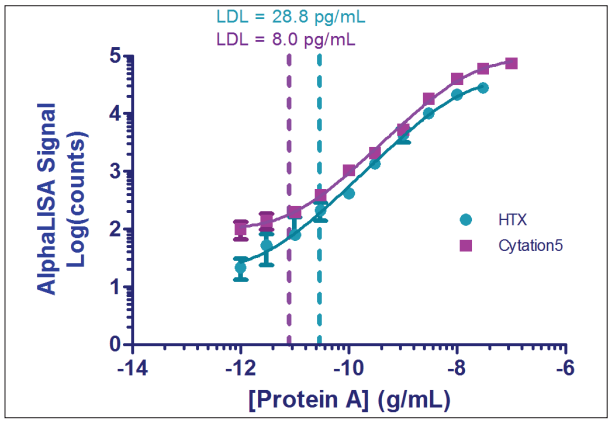

Figure 2. **Protein A Standard Curve.** A 12-point dilution series of the positive control was prepared ranging from 100,000 - 0.3 pg/mL. Twelve background points were used to calculate LDL.

As can be seen in Figure 2, there is excellent correlation between readers indicated by the nearly parallel standard curves. The Alpha laser contained in the Cytation 5 results in significantly higher signal generation, ~ 2-fold, under otherwise identical assay conditions. 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 from data. As can be seen in Figure 2, there is excellent correlation between readers as seen by the nearly parallel standard curves. Both determinants correlate well with established assay performance characteristic as per the manufacturers' specifications.

A panel of ten (10) human IgG antibodies including the therapeutic antibodies Herceptin, Rituxan and Erbitux 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. The panel of antibodies were diluted to concentration <1.0 mg/mL and pretreated as described above. Following determination of the Alpha counts 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 3).

Antibody	Stock (mg/mL)	Dilution Factor	HTX [PA] (corr.) (pg/mL)	CY5 [PA] (corr.) (pg/mL)
IgG1	1.0	2	ND	ND
IgG2	1.0	2	ND	ND
IgG3	1.0	2	ND	ND
IgG4	1.0	2	18,647	19,647
Cetuximab	5.0	20	12,850	12,306
α-VEGFR2	0.2	0	ND	ND
Rituxan	10.0	20	797	490
12G5 Mab	0.5	0	ND	ND
Herceptin	21.0	42	611	366
α-hTNF IgG	0.1	0	ND	ND

Table 3. **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 for a standard curve. Actual [PA] present in antibody stock solutions was corrected for by multiplying by the appropriate dilution factor. ND refers to Not Detected.

As seen in Table 3, several of the antibodies tested in the panel showed significant residual Protein A including IgG4 and the therapeutics Cetuximab, Rituxan and Herceptin. All other antibodies demonstrated PA levels less than the detection limit of each instrument. Figure 4 plots the antibodies showing detectable levels of PA expressed as a percentage contamination. Good correlation is apparent between both instruments. The detection of the levels of contamination present illustrates the excellent sensitivity of the AlphaLISA Protein A kit.

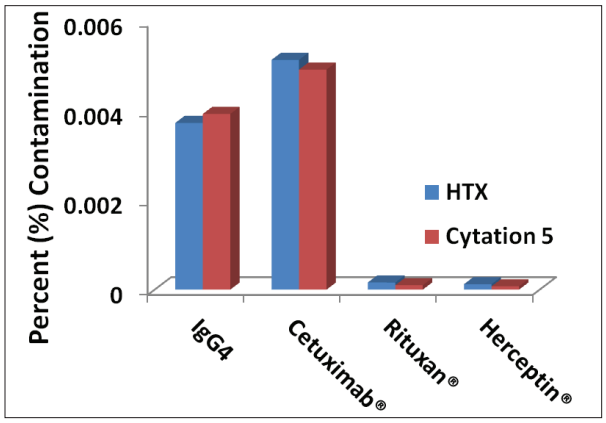

Figure 3. **Residual Protein A Screen of a panel of Human Antibodies.** A panel of ten (10) human antibodies was screened for detectible levels of Protein A. Only those antibodies showing quantifiable [PA] are shown in the figure.

Conclusion

Given the prevalence of biologics in the current drug development pipeline, highly sensitive and robust quantification methods for potential contaminants amenable to HTS are desirable. Due to the amplified nature of the AlphaLISA technology, high sensitivity detection was achieved without the need for wash or filtration steps. The data presented here show a simplified work-flow using a homogeneous assay capable of detecting pg/mL quantities of residual Protein A in a panel of human antibodies. The assay was performed in a HTS compatible 384-well microplate format using automated liquid handling for reagent dispensing. Duplicate experiments were prepared and read on two microplate readers; a multi-mode reader and a high-end, high-throughput cell imaging multi-mode reader equipped with an Alpha specific laser. Comparable results were generated between the two instruments with identification of several human antibodies containing trace levels of PA as well as several other samples containing relatively high levels of contaminant. 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 workflows.

References

1. Mehta, A., et al. 2007. Purifying Therapeutic Monoclonal Antibodies. CEP. SBE Special Section: Bioprocessing. S14-S20.

2. Zhu-Shimoni, J.; Gunawan, F.; Thomas, A.; Vanderlaan, M.; Stults, J. 2009. Trace level analysis of leached Protein A in bioprocess samples without interference from the large excess of rhMAb IgG. J. Immunol. Methods, 341 (1-2), pp. 59–67.