Application Note

Drug Discovery and Development

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

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Introduction

There remains 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 many 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. Also, the formulation must be sterile insuring no viable micro-organisms 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). Also, a viral filtration (VF) step is 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 mitogenic effects.[2] Therefore, reliable, robust methods for the detection and quantification of trace amounts of PA are necessary and mandated by the FDA.

This application note demonstrates automation of an 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 10 human IgG antibodies including the active therapeutic components of Herceptin, Rituxan, and Erbitux, the antibodies trastuzumab, rituximab, and cetuximab, respectively.

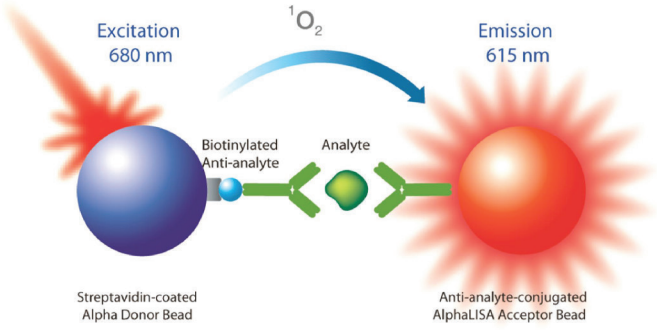

Figure 1. Assay schematic for AlphaLISA homogeneous proximity assay principle for the detection of analyte in biotherapeutic products. Upon excitation, the AlphaLISA donor bead generates singlet oxygen molecules. If the acceptor bead is in close proximity due to the creation of a sandwich immunoassay,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

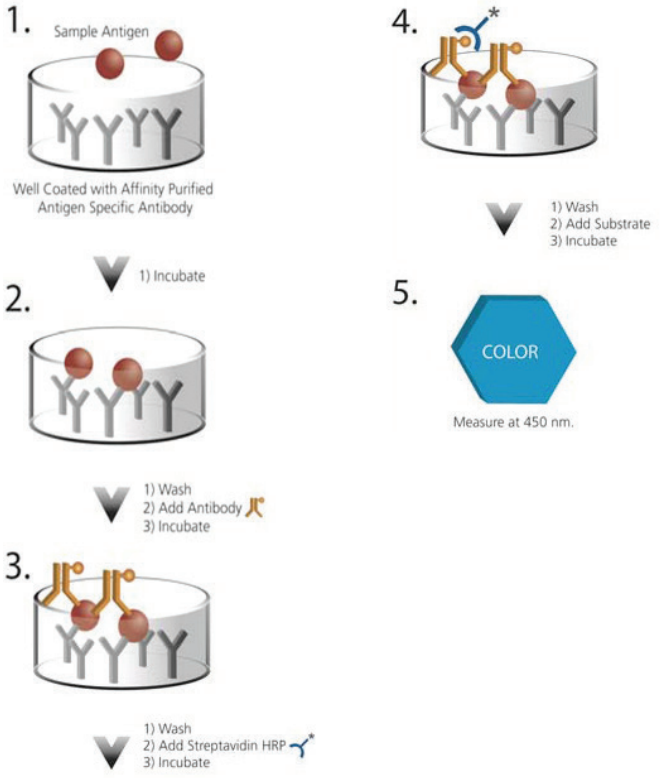

Figure 2. Assay schematic for ELISA assay principle for the detection of analyte in biotherapeutic products. Samples and standards are added to wells coated with chicken antibody specific for Protein A. The plate is washed leaving only bound Protein A. A biotinylated chicken antibody to Protein A is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

Instrumentation

Agilent BioTek Cytation 5 cell imaging multimode reader

Agilent BioTek Cytation 5 cell imaging multimode reader combines automated digital microscopy and conventional microplate detection in a configurable, upgradable platform. Agilent BioTek Cytation 5 includes both filter- and monochromator-based optics for multimode versatility and offers laser-based excitation for Alpha assays.

Agilent BioTek Synergy HTX multimode reader

Agilent BioTek Synergy HTX multimode reader is a compact, affordable system for 6- to 384-well microplates and Agilent BioTek Take 3 microvolume plates. Absorbance, fluorescence, luminescence, and AlphaScreen/AlphaLISA measurements are all made using a unique dual-optics design that provides superior performance.

Agilent BioTek MultiFlo FX multimode dispenser

Agilent BioTek MultiFlo FX multimode dispenser is an automated multimode reagent dispenser for 6- to 1536-well microplates offering unique Parallel Dispense technology by Agilent BioTek. Up to four independent reagents can be dispensed in parallel without potential carry over. The instrument was used to dispense assay specific reagents to the 384-well assay plates.

Materials and methods

Reagents

AlphaLISA Residual Protein A kit (part number AL287) was from Perkin Elmer (Waltham, MA, USA). Protein A ELISA kit (part number ADI-900-057C) was a gift from Enzo Life Sciences (Farmingdale, NY, USA).

Assay plates

AlphaPlate-384 grey, opaque 384-well microplates (part number 6005350) were from PerkinElmer (Waltham, MA). Protein A clear 384-well microplates were a gift from Enzo Life Sciences (Farmingdale, NY).

Instrument settings

The Agilent BioTek Synergy HTX was used with settings shown in Tables 1 and 2, while the Cytation 5 cell imaging multimode reader was used with Table 3 settings.

Table 1. Agilent BioTek Synergy HTX multimode reader AlphaLISA reading parameters used in Agilent BioTek Gen5 microplate reader and imager software.

Parameter	Value
Mode	Alpha
Gain	200
Filter Switching Per Well	Selected
Read Speed	Normal
Read Height	8.00 mm
Dual Filter Sets	
Filter Set 1	EX = 680/30, EM = Plug
Filter Set 2	EX = Plug, EM = 570/100

Table 2. Agilent BioTek Synergy HTX multimode reader ELISA reading parameters used in Agilent BioTek Gen5 microplate reader and imager software.

Parameter	Value
Mode	Absorbance
Wavelength	450 nm
Read Speed	Normal
Delay After Plate Movement	100 ms
Measurements Per Data Point	8

Table 3. Agilent BioTek Cytation 5 cell imaging multimode reader AlphaLISA reading parameters used in Agilent BioTek Gen5 microplate reader and imager software.

Parameter	Value
Mode	Alpha
Gain	120
Delay After Plate Movement	0 ms
Excitation Time	80 ms
Delay After Excitation	120 ms
Integration Time	160 µs
Read Height	8.00 mm

Sample preparation

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 to 7 minutes followed by centrifugation for five minutes ~200 g.

AlphaLISA assay setup

Quadruplicate samples 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 multimode dispenser. 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 samples and standards were transferred, 25 µL, to each well of the assay plate and incubated at RT for 60 minutes with shaking at ~500 rpm. The plate was washed 4x with 100 µL wash solution using the MultiFlo FX. 25 µL biotinylated antiprotein A antibody was added to the plate, except for the blanks, and incubated at RT for 60 minutes with shaking at ~500 rpm. After the plate was washed as above, 25 µL streptavidin conjugated HRP solution was added to all wells, except for the blanks, and the plate incubated at RT for 30 minutes with shaking at ~500 rpm. The plate was washed as previously 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 to 3) and determination of the sample concentration when performing the assays. As shown 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).

The AlphaLISA assay showed excellent correlation between readers as shown by the nearly parallel standard curves in Figure 3A. The Alpha laser contained in the Agilent BioTek 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 Agilent BioTek Synergy HTX, contributing to a 3-fold higher LDL; 29 pg/mL versus 8.0 pg/mL on the Agilent BioTek 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.

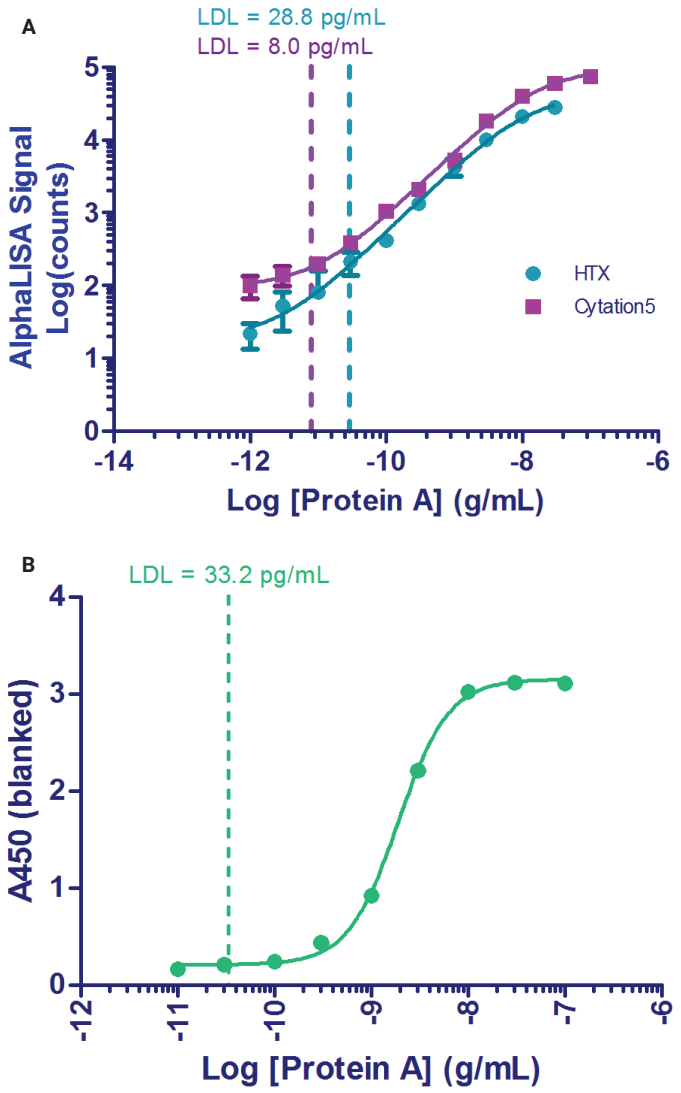

Figure 3. Protein A standard curve. (A) AlphaLISA Protein A Assay. A 12-point dilution series of the positive control was prepared ranging from 100,000 to 0.3 pg/mL was prepared. Twelve background points were used to calculate LDL. (B) Protein A ELISA assay. A 9-point dilution series of the positive control was prepared ranging from 100,000 to 10 pg/mL. Twelve background points were used to calculate LDL.

Antibody panel screen for residual Protein A

A panel of 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 high concentrations in solution, elevated concentrations above 1.0 mg/mL can interfere with assay performance therefore all samples were diluted before 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).

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.

Antibody	Stock (mg/mL)	Dilution Factor	AlphaLISA		ELISA
			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

As shown in Table 4, several of the antibodies tested in the panel showed significant residual PA including IgG4 and the therapeutic cetuximab. Rituximab and trastuzumab were also 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.

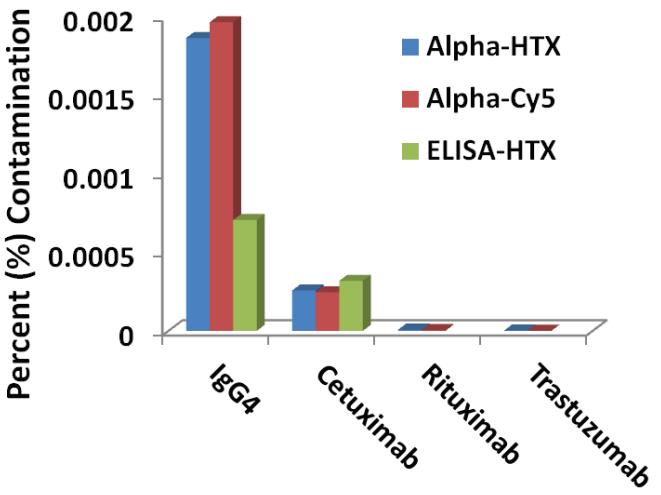

Figure 4. Residual Protein A screen of a panel of human antibodies. A panel of 10 human antibodies was screened for detectible levels of Protein A. Only those antibodies showing quantifiable [PA] are shown in the figure.

Conclusion

The assays were performed in an 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. Agilent BioTek Cytation 5 cell imaging multimode reader equipped with the Alpha-specific laser generated higher signals than the Agilent BioTek Synergy HTX multimode reader, 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. 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

1. Mehta, A. *et al.* Purifying Therapeutic Monoclonal Antibodies. CEP. SBE Special Section: Bioprocessing. S14-S20, **2007**.
2. Zhu-Shimoni, J. *et al.* Trace Level Analysis of Leached Protein A in Bioprocess Samples Without Interference From the Large Excess of rhMAb IgG. *J. Immunol. Methods* **2009**, *341(1-2)*, 59–67.

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RA44411.6009837963

Printed in the USA, May 17, 2022
5994-3362EN

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