Development of AlphaLISA assays for Quantification and Characterization of Biotherapeutic Proteins and Process Contaminants

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1 Summary

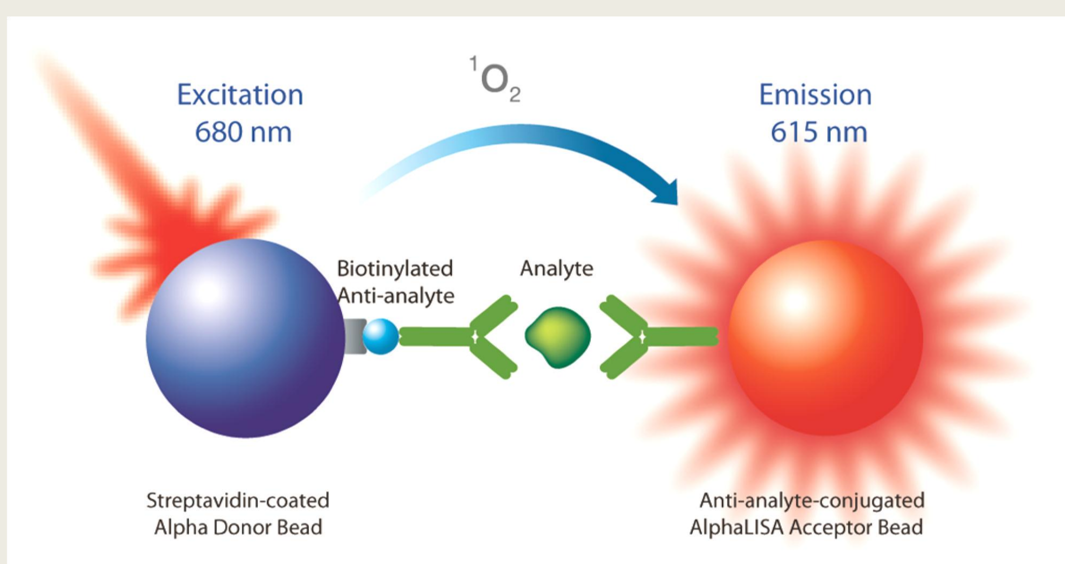
Immunoassays are commonly used in the development and characterization of therapeutic proteins and peptides, vaccines, and monoclonal antibodies (TAbs). Bioprocess groups developing lot release QC assays are subject to strict regulations and as such there is a need for novel techniques that speed drug development, while still meeting regulatory demands. An increasing need for robust, efficient and sensitive assays to detect and characterize these biologicals during various stages of development has led many researchers to AlphaLISA®. Currently, one of the most common technologies for performing immunoassays is the enzyme-linked immunosorbent assay (ELISA), which is a robust method but requires multiple, time-consuming steps that can lead to variable data. In contrast, the AlphaLISA assay format is a chemiluminescent homogeneous bead-based technology which does not require plate coating, washing or separation steps, providing significant advantages over ELISA. AlphaLISA assays are performed in 96- or 384-well plates and are run with typical sample volumes of 5 µL, conserving valuable test materials. The total assay time is less than 3 hours.

Several new AlphaLISA assays have been developed to quantify and characterize biotherapeutic proteins and Tabs purity including:

- Detection of bioprocess contaminants such as host cell proteins (CHO, E. Coli, NS0-P, PER.C6®), cell culture additives (albumin) and residual Protein A from purification.
- Detection of fucosylated IgG by use of an anti-IgG antibody and a core fucose-specific lectin.

Data provided here demonstrate that AlphaLISA technology provides an alternative and versatile assay platform to ELISA and can improve the workflow of various analytical assays in the modern biotherapeutics development laboratory.

2 AlphaLISA Technology Principle



The biotinylated anti-analyte antibody binds to the Streptavidin-coated Alpha Donor beads while another anti-analyte antibody is conjugated to AlphaLISA Acceptor beads. In the presence of the analyte, the beads come into close proximity. The 680 nm laser excitation of the Donor beads provokes the release of singlet oxygen molecules that triggers a cascade of energy transfer in the Acceptor beads resulting in a sharp peak of light emission at 615 nm.

3 Materials

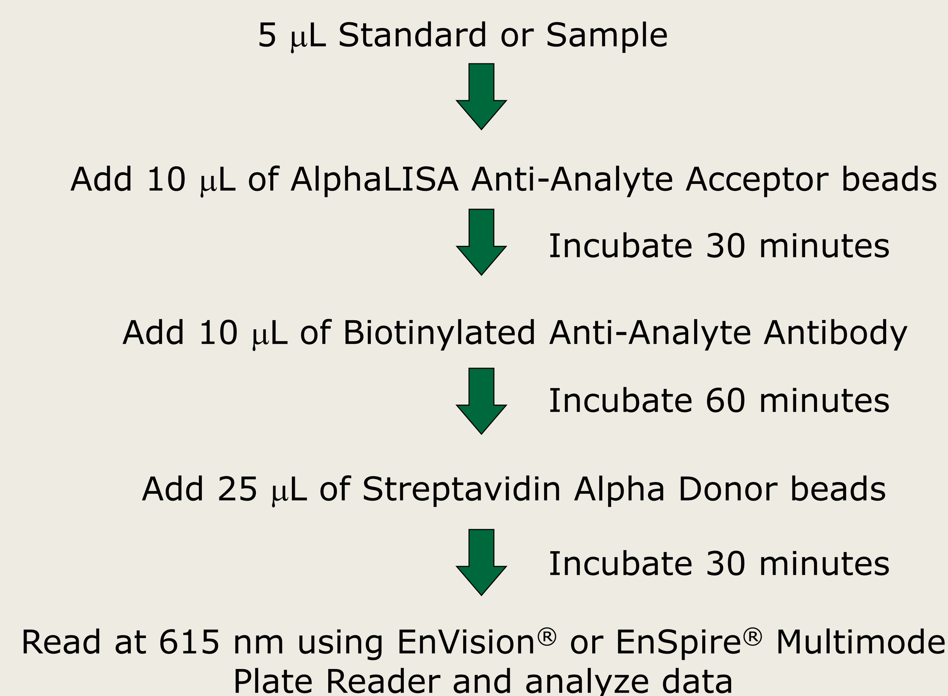
AlphaLISA products (PerkinElmer):

Standalone products:
• Alpha Donor beads: Streptavidin Donor beads (#6760002S)
• Lens culinaris agglutinin (LCA) AlphaLISA Accepter Beads (#AL140)

AlphaLISA kits:
• Chinese Hamster Ovary Cell Host Cell Proteins (CHO HCP) (#AL210)
• CHO HCP (broad reactivity) (#AL301)
•Residual Protein A (#AL287)

Other materials required for ELISA comparisons (sections 5 & 6) and fucosylated IgG4 detection assay (section 7):
• CHO HCP ELISA kit, Company X
• Protein A ELISA kit, Company X
• Human IgG4, Fitzgerald (#31-AI20)
• AffiniPure F(ab')2 Fragment Goat Anti-Human IgG, Fcγ Fragment Specific, Bethyl Laboratories (#A80-248A)

4 AlphaLISA: quick, simple, precise



Typical assay precision data

Intra-assay precision

Sample	Mean (pg/mL)	SD (pg/mL)	%CV (n=18)
A	92 819	6 955	7.5
B	10 765	641	6.0
C	1 049	92	8.8

Inter-assay precision

Sample	Mean (pg/mL)	SD (pg/mL)	%CV (n=6)
A	92 819	10 650	11.5
B	10 765	1 137	10.6
C	1 049	180	17.2

Precision of CHO HCP assay (Cat. no. AL210). Assay precision data were calculated from a total of 18 assays. Two operators performed three independent assays using three different kit lots. Each assay consisted of one standard curve and three control samples of high (A), medium (B) and low (C) concentrations, assayed in triplicate.

5 Contamination Testing Assays

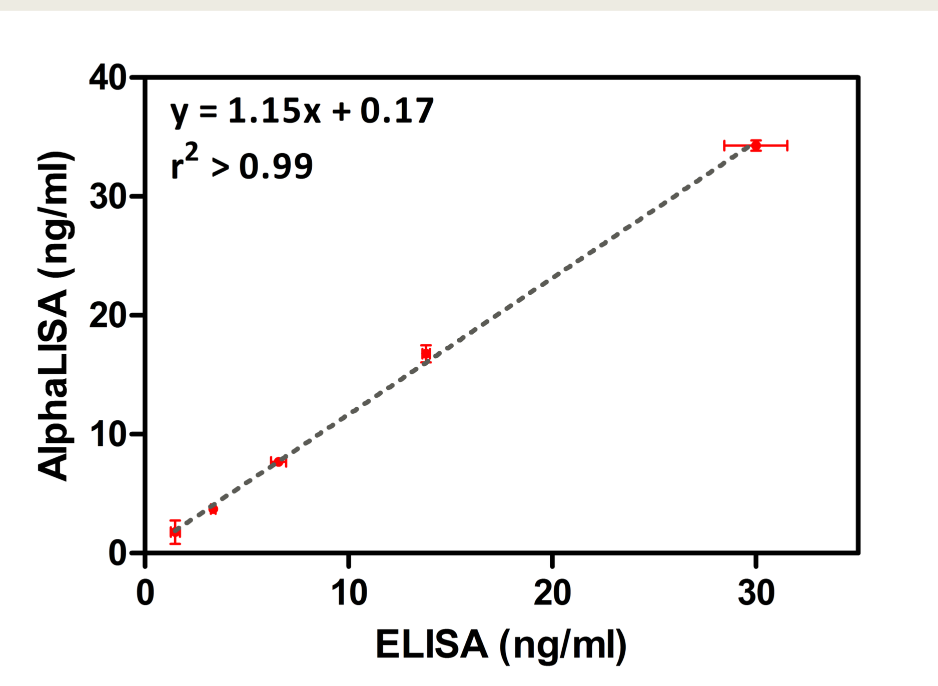
The most efficient way to produce large amounts of biotherapeutic proteins is by expression of the material in a cell line or a microorganism followed by extraction and purification. Unfortunately, even after extensive purification, unrelated material from the cells or bacteria used can remain with the protein of interest. These contaminants can have serious side effects such as toxicity, generation of immunological response or destabilization of the product. As such, regulatory bodies have imposed strict regulations on the tolerated level of contaminants. To insure quality, testing for the presence of contaminants is essential. However, such test are complicated by the complexity of such whole cell proteins mixtures and their variability from batch to batch.

We have developed AlphaLISA kits that are both of high sensitivity and possess the capacity to recognize generic pools of proteins from the most commonly used cell lines and bacteria in the industry. The lower and upper limit of detection for several contamination testing assays are provided in the table below.

Catalog number	Kit name	Lower detection limit * (ng/mL)	Upper limit ** (µg/mL)
AL210	CHO HCP	0.18	0.3
AL226	NS0-P HCP	1.6	1
AL261	E.Coli HCP	0.46	1
AL287	Resid. Protein A	0.01	0.03
AL294	Albumin	0.1	1
AL301	CHO HCP (broad)	0.45	1
AL302	PER.C6® HCP	0.78	3

* Calculated by interpolating the average background counts (12 wells without analyte) + 3 x standard deviation value on the standard curve.
** Corresponds to analyte concentration on the standard curve giving max signal.

CHO HCP Detection

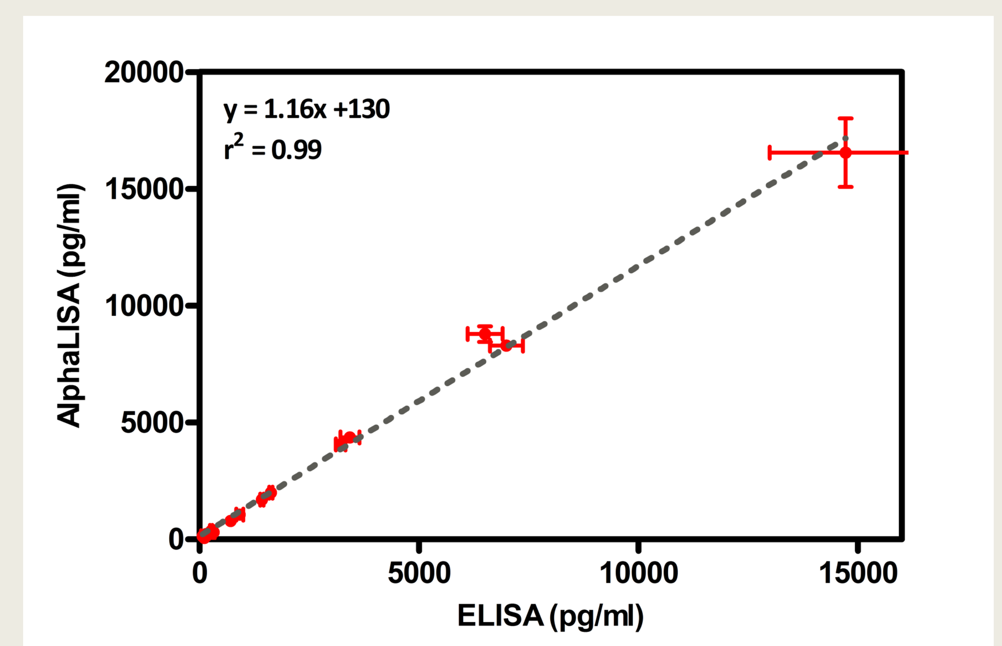


Detection of CHO HCP. HCP standards and samples were diluted in F12 media. CHO HCP was measured according to kit instructions using the high sensitivity AlphaLISA protocol or according to the manufacturer's ELISA protocol. Data was analyzed via Graphpad Prism using a 4-parameter non-linear regression fit.

6 Residual Protein A Detection

Clinical use of biotherapeutic antibodies requires the production of bulk amounts of the product. These antibodies are generally purified on Protein A chromatography systems. As protein A is a very toxic, strongly immunogenic and possibly carcinogenic substance, the final product must be free of all residual protein A that could have leeched from the column. The FDA and other regulatory organisms have thus raised strict tolerance limits for residual protein A in biotherapeutic drugs.

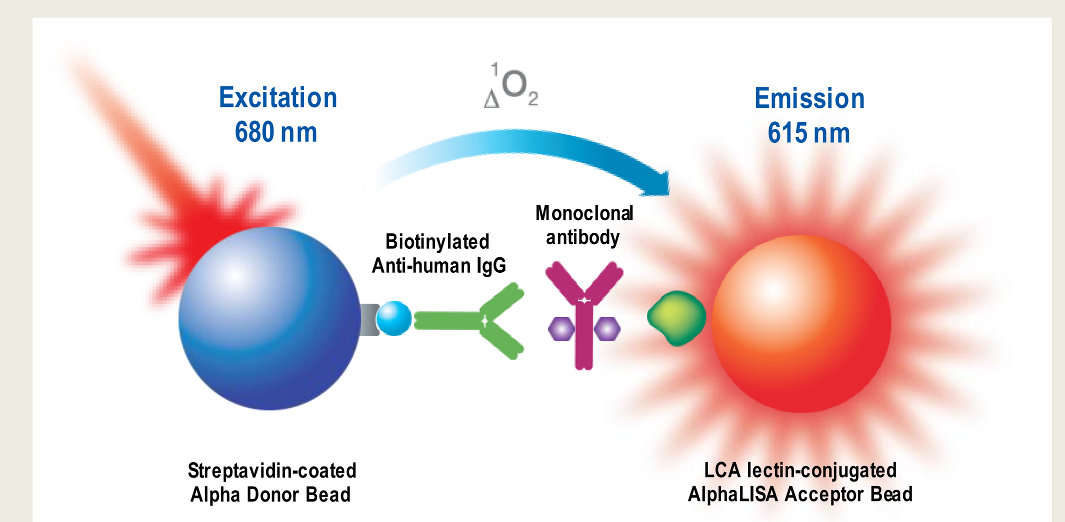
To insure such purity, an AlphaLISA residual protein A detection kit was created that allows for fast and sensitive detection of low levels of protein A in samples

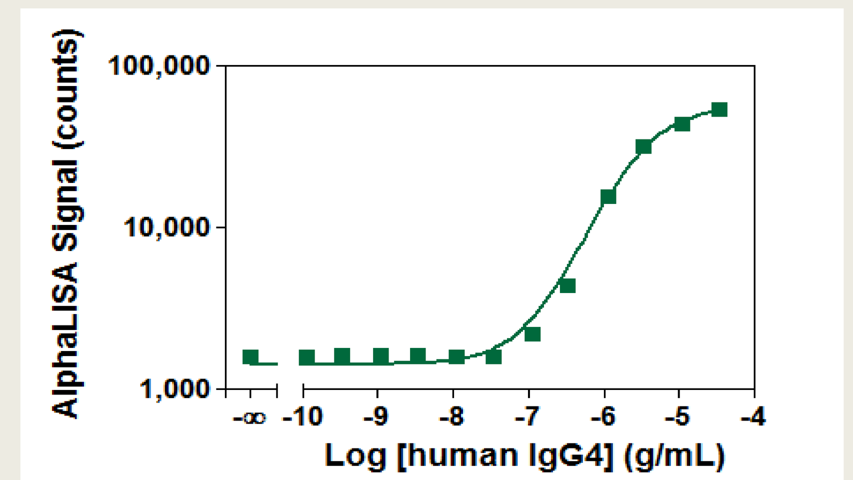


Detection of residual Protein A. Protein A was diluted in 0.1M Tris-HCl/0.1 M glycine buffer, pH 7.5. Residual Protein A standards and samples were pre-treated according to kit instructions and then Protein A was detected using the high sensitivity AlphaLISA protocol or according to the manufacturer's ELISA protocol. Data was analyzed via Graphpad Prism using a 4-parameter non-linear regression fit.

7 Fucosylated IgG Detection

The carbohydrates or glycans linked on the biotherapeutic antibody may play a major role in the mechanism of action as well as biodistribution of the drug. Detection of core fucose on monoclonal antibodies using Alpha technology can be used for antibody screening and characterization. The fucosylated IgG assay is based on the detection of core fucose by *lens culinaris* agglutinin (LCA), which has a high specificity for α1,6-linked fucose [1].





Detection of fucosylated IgG4 in serum-free cell culture medium. Human IgG4 from myeloma plasma was diluted in cell culture medium (Hybridoma-SFM Gibco #12045-084). The fucosylated IgG4 was then detected using a typical AlphaLISA protocol.

8 Summary

Sensitive and reproducible immunoassays were developed for analyses of biotherapeutics and process contaminants. These assays can be applied in different stages of biotherapeutic development, such as cloning and expression, process development and manufacturing / QC processes.

References
1. Tateno, H. *et al.* Comparative analysis of core-fucose-binding lectins from *Lens culinaris* and *Pisum sativum* using frontal affinity chromatography. Glycobiology 19, 527–536 (2009).

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