Case Study: Bridging Anti-CHO HCP Antibodies from Two 3rd Generation Cygnus CHO HCP ELISA Kits by AAE-MS™

Antibody coverage comparison of polyclonal antibodies supporting the original CHO HCP ELISA Kit, 3G, F550 vs. the resupply CHO HCP ELISA Kit, 3G, F550-1

Introduction

Monitoring of host cell proteins (HCPs) by ELISA to show clearance during drug substance (DS) purification is an essential function of process development and quality control groups in the biopharmaceutical industry. To qualify whether an ELISA is fit for the purpose of downstream process monitoring, dilutional linearity, accuracy, precision, and lower limit of quantitation (LLOQ), data are collected with in-process and DS samples. A coverage assessment is performed on the antibody to demonstrate that the antibodies used in the assay are broadly reactive to the HCPs in the process to complete the qualification package. Antibody coverage assessments report the percentage of HCPs in a bioprocess that are immunoreactive, detected, and can be quantified by the ELISA.

Biopharmaceutical companies use qualification data packages as the basis for performing their ELISA assay validation. Once validated ELISA assays have been approved by regulatory groups, it can be difficult to change them and time intensive to re-validate in a cGMP environment. It is imperative to ensure a stock of validated ELISA assays from trusted suppliers can last throughout the lifetime of a program, supporting Phase I to III clinical trials and post-market product lifecycles. However, since ELISA polyclonal antibodies and kits do not have indefinite supplies, bridging studies to compare the performance of new or resupply reagents are required for continued HCP clearance monitoring. Typically, Quality Control (QC) groups will revalidate the resupply reagents and often outsource the antibody coverage assessment work.

Cygnus Technologies has transitioned the depleted CHO HCP ELISA, 3G Kit (Item Number F550) to the resupply version (Item Number F550-1). To enable the HCP community to seamlessly transition from the original F550 kit to the F550-1 CHO HCP ELISA 3G, we performed a comparative coverage analysis study to assess reactivity of anti-CHO HCP antibodies that support F550 and F550-1 kits, respectively. The study was carried out using AAE-MS for HCP detection.

Summary

Cygnus Technologies has performed an antibody coverage bridging study between the F550 and F550-1 versions of our 3rd generation CHO ELISA kits by Antibody Affinity Extraction (AAE™) with host cell protein (HCP) detection by LC-MS (AAE-MS). The immunoreactivity between F550 and F550-1 antibodies against the CHO master antigen (CMA) HCPs was 97% and 97%, respectively. F550-1 has comparable reactivity to most HCPs such as Phospholipase B Like 2 (PLBL-2), Lysosomal Phospholipase A2 (LPA2), Lipoprotein Lipase (LPL), and Serine protease (HTRA1) and other "problematic" HCPs by fold enrichment, and identical peptides were detected as compared to F550. The F550-1 CHO 3G ELISA kit has equivalent antibody coverage as the F550 kit and is a suitable replacement for the F550 HCP ELISA kit.

Contents

Note: This CHO HCP 3rd Generation ELISA Kit, Item Number F550-1 is a resupply for the depleted CHO HCP ELISA Kit, 3G, Item Number F550.

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Cygnus Technologies applied years of experience with CHO HCP analysis to curate a proprietary database of CHO HCPs. The documented CHO HCPs include PLBL2, LPA2, LPL, HTRA1, monocyte chemoattractant protein 1, serpin B6, plasminogen activator, TGF beta 1, and other proteins. The subset of HCPs that are enzymes (proteases, proteinases, peptidases, phospholipases), cytokines, and growth factors) can be especially problematic.

Materials and Methods

Sample Preparation
The CMA was filtered with a 0.2 µm filter prior to AAE.

Quantification of HCPs
Total protein levels were quantified for pre- and post-AAE samples using an extinction coefficient of 1 for UV absorbance at 280 nm, the Bradford method at 595 nm, and the BCA method (Thermo Fisher Scientific) at 562 nm. All absorbances were acquired using a SpectraMax M3 Multi-Mode Microplate Reader (Molecular Devices).

AAE
The CHO 3G polyclonal antibody from each of the F550 and F550-1 ELISA Kits (Cygnus Technologies) was covalently immobilized on a separate chromatography support (referred to as the F550 AAE column and the F550-1 AAE column, respectively). The columns were conditioned to prevent significant leaching of the antibody and to minimize non-specific binding. The HCP-containing sample was passed over the column using an ÄKTA 25L fast protein liquid chromatography (FPLC) system (GE Healthcare) for binding HCPs and collecting elution fractions. All HCP elution fractions were neutralized, pooled, buffer exchanged, and concentrated.

LC-MS
The pre- and post-AAE proteins were precipitated in greater than nine volumes of methanol overnight at-20°C. The precipitated proteins were dissolved in 8M urea, reduced, alkylated, digested with trypsin, desalted, and concentrated.

Database Setup
Cygnus Technologies' proprietary curated CHO HCP proteomic database containing UniProt CHO K1 and S secretomes with isoelectric point (pI) and molecular weight (MW) data as well as information about common contaminants such as BSA, keratins, LysC, and Trypsin was used to identify proteins.

LC-MS Method Development
Peptides from digested proteins were injected directly with a reversed phase C18 column using a Vanquish Horizon UHPLC into an Orbitrap Eclipse Tribrid MS (Thermo Scientific) with a factory established limit of detection (LOD) of 0.5-5 ppm. Data were acquired with a 60-minute gradient in data dependent acquisition (DDA) mode with survey spectrum (m/z range 350-1700) followed by MS/MS (m/z range 375-2000) of the most intense multiply charged ions using collision induced dissociation. Peptide data acquired with the LC-MS method were used for HCP identification.

HCP Identification by LC-MS
The pre-AAE and post-AAE samples were acquired with the LC-MS method independently in triplicate and in a randomized sequence. Blank washing runs were implemented in between sample injections to minimize sample carryover. HCPs were identified by two peptides per protein from triplicate runs and data were compared to the Cygnus Technologies' proprietary curated CHO HCP database using Proteome Discoverer software (Thermo Fisher Scientific) with the following settings: Orbitrap Resolution: 240,000; Modifications: Oxidation, Deamidation, and Acetylation; Max. Number of Missed Trypsin Cleavages: 2; Precursor Mass Tolerance: 10 ppm; Fragment Mass Tolerance: 0.6 Da; Number of High Confidence Peptides: 2; and a False Discovery Rate Confidence Threshold: 0.01. Data of identified HCPs were exported from Proteome Discoverer into a Microsoft Excel spreadsheet and analyzed.

Virtual 2D Gel Graphs
A Virtual 2D Gel Graph was generated from MS data. Green spots represent proteins found in both the Pre- and Post-AAE samples. Red spots represent proteins found only in the Pre-AAE sample. Black spots represent proteins found only in the Post-AAE sample.

Polyclonal ELISA Antibody Coverage Calculation
Polyclonal ELISA antibody coverage is represented by a range between the lower and upper coverage boundary calculations. The lower coverage boundary is calculated by using the lower coverage boundary equation (Post-AAE proteins/Unique proteins) which includes the calculation for the number of Unique proteins ((Pre- + Post-AAE proteins)—Matching proteins). The upper coverage boundary is calculated by the upper coverage boundary equation (Post-AAE spots ÷ Pre-AAE spots).

Results

AAE-MS was performed on the F550 CMA using separate F550 and F550-1 AAE columns. LC-MS detected 1,673 CHO HCPs in CMA; 1,649 matching CHO HCPs were detected in the F550 AAE column eluate and 1,639 CHO HCPs were detected in the F550-1 AAE column eluate **(Table 1)**. The antibody coverage of anti-CHO HCP antibodies from F550 and F550-1 were 97% and 97%, respectively **(Figures 1 and 2)**. The similarity between the immunoreactive CHO HCPs found between anti-CHO HCP antibodies from F550 and F550-1 kits was 96% **(Figure 3)**.

Table 1. *Percent Antibody Coverage and CHO HCP Numbers of F550 CHO Master Antigen (CMA) and F550 and F550-1 CHO 3G antibodies.*

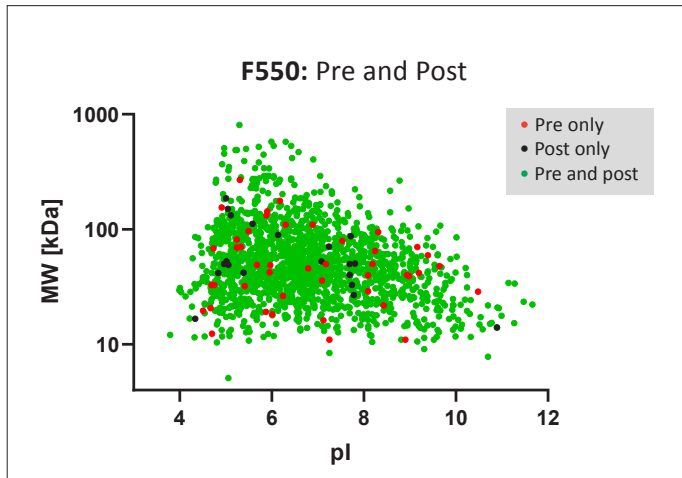| Sample		AAE (number of protein IDs)			% Antibody Coverage		
Name	AAE	Total	Unique to each fraction	Total Unique	Matching	Lower Boundary	Upper Boundary
F550	Pre	**1673**	44	**1693**	1629	97%	99%
F550	Post	**1649**	20	**1693**	1629	97%	99%
F550-1	Pre	**1673**	55	**1694**	1618	97%	98%
F550-1	Post	**1639**	21	**1694**	1618	97%	98%

Figure 1. F550 CHO 3G ELISA antibodies have 97% antibody coverage of CMA (Pre-AAE Sample).
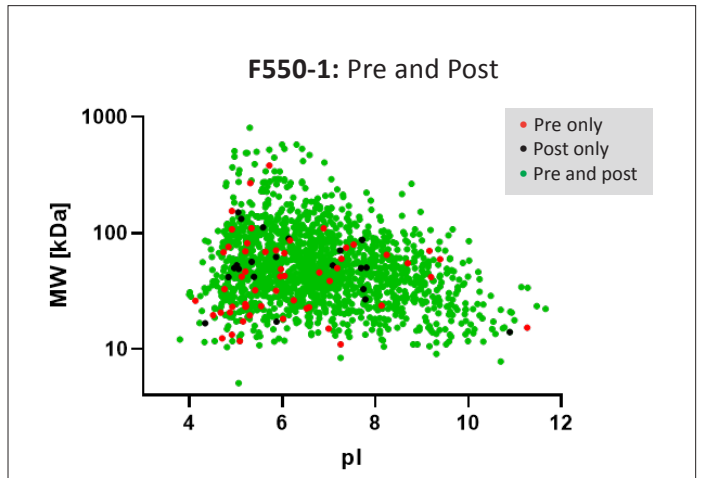
Figure 2. F550-1 CHO 3G ELISA antibodies have 97% antibody coverage of CMA (Pre-AAE Sample).

AAE			
Sample	Pre (CMA)	Post	% Antibody Coverage
No. of HCPs	1694	1649	97%

Figure 1. A Virtual 2D Gel Graph was generated from MS data. Green spots represent proteins found in both the Pre- and Post-AAE samples. Red spots represent proteins found only in the Pre-AAE sample. Black spots represent proteins found only in the Post-AAE sample.

AAE			
Sample	Pre (CMA)	Post	% Antibody Coverage
No. of HCPs	1694	1639	97%

Figure 2. A Virtual 2D Gel Graph was generated from MS data. Green spots represent proteins found in both the Pre- and Post-AAE samples. Red spots represent proteins found only in the Pre-AAE sample. Black spots represent proteins found only in the Post-AAE sample.
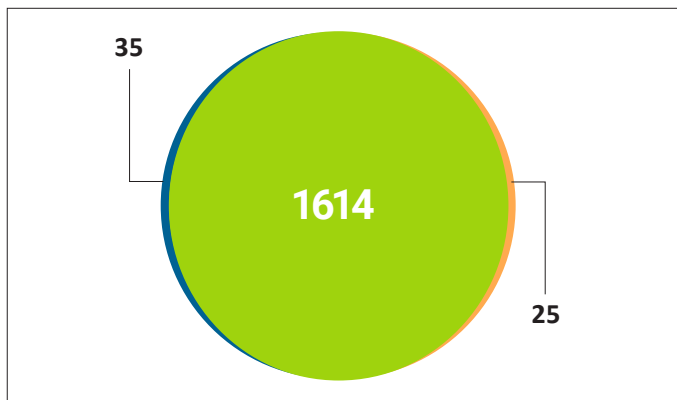
Sample				
Antibody	Total Proteins	Matching Proteins	Unique Proteins	% Simiarity
F550	1649	1614	35	96%
F550-1	1639	1614	25	96%

Figure 3. There is 96% similarity in immunoreactive CHO HCPs in F550 and F550-1 CHO 3G ELISA antibodies. The Quantitative Venn Diagram displays unique identifications of F550 (blue) and F550-1 (orange) along with proteins identified by both antigens (green).

Analysis of known immunogenic or enzymatically active PLBL-2, LPA2, LPL, and HTRA1 CHO HCPs using the Cygnus Technologies proprietary, curated CHO secretome database shows that polyclonal anti-CHO HCP antibodies used in F550 and F550-1 kits are both immunoreactive with these potentially detrimental CHO HCPs **(Table 2)**. Further analysis shows that the enrichment or detection of the same CHO HCPs in F550 and F550-1 kits by AAE-MS is equivalent **(Figure 4)**.

Table 2. Both F550 and F550-1 CHO 3G ELISA antibodies detect potentially problematic CHO HCPs PLBL-2, LPA2, LPL, and HTRA1.

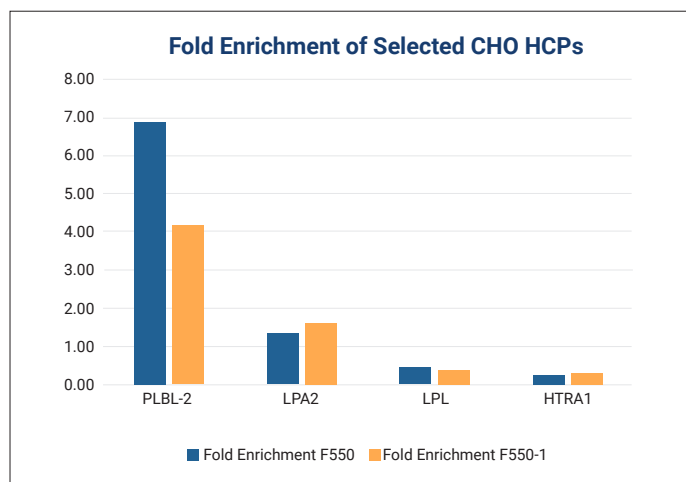|	CHO 3G Reactivity	
	F550	**F550-1**
PLBL-2	Y	Y
LPA2	Y	Y
LPL	Y	Y
HTRA1	Y	Y

Figure 4. Fold enrichment of CHO HCPs Phospholipase B Like 2 (PLBL-2), Lysosomal Phospholipase A2 (LPA2), Lipoprotein Lipase (LPL), and Serine protease (HTRA1) in CMA by F550 and F550-1 CHO 3G ELISA antibodies.

Discussion

Cygnus Technologies AAE is used for antibody coverage assessments. Western blotting (WB) is a poor choice for antibody coverage assessments, as denaturing HCPs by SDS-PAGE and embedding HCP antibody epitopes in PVDF membranes leads to low antibody coverage assessments. Because AAE has no limitations of the binding capacity of the antibodies with multiple extraction cycles in a non-denaturing environment, it is a better method than WB. AAE and 2D-PAGE with silver staining more accurately represents the sensitivity and selectivity of the polyclonal ELISA antibodies. HCPs also can be detected with fluorescent stained 2D-PAGE gels and 2D-DIGE but have limitations imposed by the emission of the fluorescent dyes. However, all SDS-PAGE-based methods of determining antibody coverage are limited due to the gel-to-gel run variability and the variability of fixing and rehydrating these gels back to the exact same size before processing.

During the development of Cygnus CHO HCP ELISA 3G assays, we have performed antibody coverage assessment by AAE with a 2D-PAGE silver stain method and established that polyclonal anti-CHO HCP antibodies from F550 and F550-1 kits were reactive with 72% and 86% of HCPs found in CHO CMA, respectively (data not shown, available upon request).

MS is a highly sensitive technique for the identification and quantification of HCPs that overcomes the limitations of gel-based methods. When combined with AAE, AAE-MS generates antibody coverage assessments to reveal the immunoreactivity of ELISA antibodies. AAE-MS coverage analysis showed that the anti-CHO HCP antibodies used in the F550 and F550-1 kits were immunoreactive with 97% and 97% of HCPs in the CMA, respectively. The slight discrepancy in percentage coverage between AAE with 2D-PAGE and AAE-MS methods can be attributed to two factors: first, coverage analysis is strongly dependent on the detection method used and MS is a more sensitive method as compared to 2D-PAGE silver stain; and second, the higher calculated percentage coverage could be attributed to MS identifying unique HCPs while the 2D-PAGE could be showing several variants of the same protein (such as glycosylated, phosphorylated, or truncated protein forms) as different spots, leading to overestimation of the total number of unique HCPs in the CMA.

AAE-MS analysis also showed that the similarity of CHO HCPs immunoreactive with both antibodies was very high (96%), thus demonstrating that these Cygnus HCP antibody generation methods deliver consistent results. Both antibodies were able to detect and enrich a subset of potentially problematic CHO HCPs, including PLBL-2, LPA2, LPL, and HTRA1 which were identified with the same MS2 spectra in both the F550 and F550-1 CHO 3G AAE samples.

Conclusion

This coverage assessment of the polyclonal anti-CHO HCP antibodies from the depleted F550 and resupply F550-1 CHO HCP ELISA 3G assays should serve as the foundation for biopharmaceutical companies to revalidate the Cygnus Technologies CHO 3G ELISA for their in-house processes. These data combined with coverage analysis of relevant process HCPs, dilution linearity, accuracy, precision, and LLOQ data show that the resupply F550-1 CHO HCP ELISA 3G antibodies are broadly reactive and the performance of the ELISA is acceptable for the purpose of bioprocess monitoring and QC release testing.

Given the comprehensive characterization performed by Cygnus, our recommendation for customers is to perform correlation studies on in-house controls and on historical retains of downstream and final drug substance samples. If that correlation is satisfactory then that data together with the Cygnus coverage analysis will provide adequate evidence that the resupply F550-1 kit is fit for use. Subsequently, AAE-MS should not only be used for coverage analysis on upstream samples but, most importantly, on in-process and DS samples to show antibody coverage to those HCPs that persist through the purification process.

The insights gained from the AAE-MS studies of in-process and DS samples can be used for further assay qualification and downstream process validation. AAE and MS can be extremely helpful in investigations of problematic HCPs. For example, enrichment of a single HCP by the purification process might typically present as a loss of dilutional linearity. With MS, it is possible to test samples that have good linearity and compare with samples that have a loss of linearity to identify the enriched protein. MS also provides information such as pI and MW of HCPs, which can be provided to the process development engineers to target the problematic protein(s) for removal.

MS also can play an important role in HCP analytics from the IND application through post-marketing, when evaluating the impacts of a process change, risk assessment, and characterizing reagent changes. And although complete characterization of downstream HCPs is not part of the current regulatory guidelines, the value of this information to biopharmaceutical companies and the importance of understanding lot-to-lot consistency to better assure safety and efficacy are recognized as value added data by proactive manufacturers and regulators the world over.

Note: This case study is a revision of the same case study originally published in January 2020.

Support and Services

Cygnus offers **AAE and Mass Spectrometry** services to help identify and quantify individual HCPs in your final drug substance or other downstream samples. Contact our technical experts at: **techsupport@cygnustechnologies.com**

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