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SPECIAL REPORT

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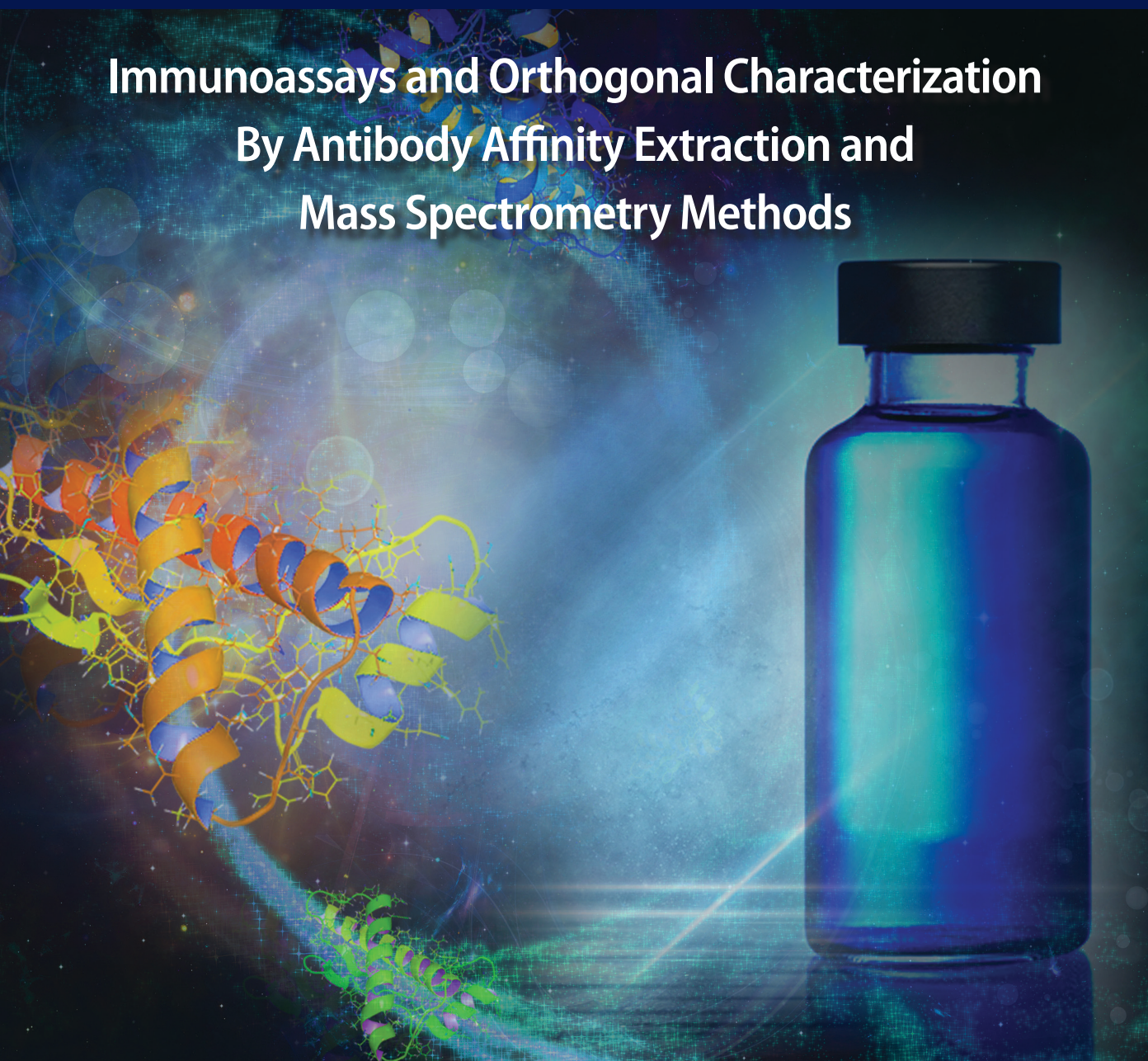
Host Cell Protein Analysis

Immunoassays and Orthogonal Characterization By Antibody Affinity Extraction and Mass Spectrometry Methods



Host Cell Protein Analysis

Immunoassays and Orthogonal Characterization By Antibody Affinity Extraction and Mass Spectrometry Methods

Alla Zilberman, Kerry Wooding, Jacob Stubbs, Jared Isaac, and Eric Bishop

Host cell proteins (HCPs) constitute a major group of process-related impurities in biological drugs produced using cell culture technology. HCPs are produced inadvertently during the expression of recombinant biopharmaceuticals and secreted from host cells in response to cell stress and/or cell lysis over the course of bioprocessing. Many HCPs are benign, but some are immunogenic. Some may interact with a drug substance, and others (e.g., proteases and lipases) can reduce effective product dosage through direct action on the drug or its stability by interfering with formulation buffers. Because HCPs can pose a risk to patients and affect the efficacy and stability of biological drugs, the quantity and nature of residual HCPs in a drug substance generally are considered to be critical quality attributes (CQAs). As such, they must be understood, monitored, and controlled as significant components of a biopharmaceutical drug developer's overall risk-management strategy (**1–3**).

HCP Immunoassays

A robust and broadly reactive HCP enzyme-linked immunosorbent assay (ELISA) is an important tool for monitoring purification process consistency and final drug substance purity. It is the gold standard method for process monitoring and product release testing for HCPs (Figure 1). Crucially, developers must ensure that selected HCP ELISA methods are fit for their intended use. That is important both from a regulatory perspective and for process development and manufacturing (**1, 2**).

Regulatory agencies around the world have put measures in place to ensure that the HCP ELISA used by a sponsor will be fit for the purpose of monitoring purification process consistency and product lot release (**1–3**). A well-developed and qualified HCP ELISA will help ensure that HCPs have been reduced to safe levels and that a purification process is consistent from batch to batch. It is advised to use orthogonal methods that work together to demonstrate antibody coverage for individual process-specific HCPs. Thus they can support a company's use of its particular HCP immunoassay. HCP coverage evaluations help developers assess the ability of HCP ELISA antibodies to recognize a broad range of HCPs — both those of a calibration standard and those that are present as part of in-process and drug substance samples. It is equally important to qualify such assays for dilution linearity, accuracy, and precision (**3**).

Figure 1: Cygnus HCP ELISA method

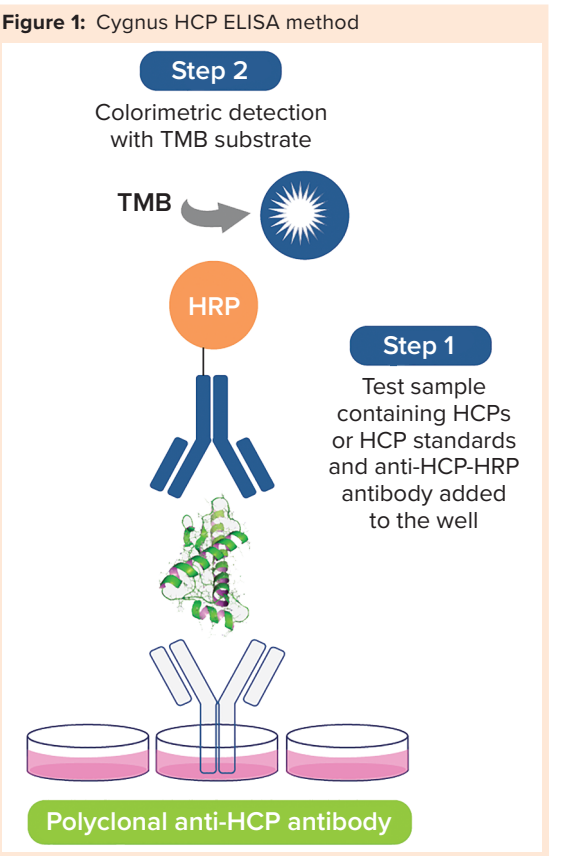
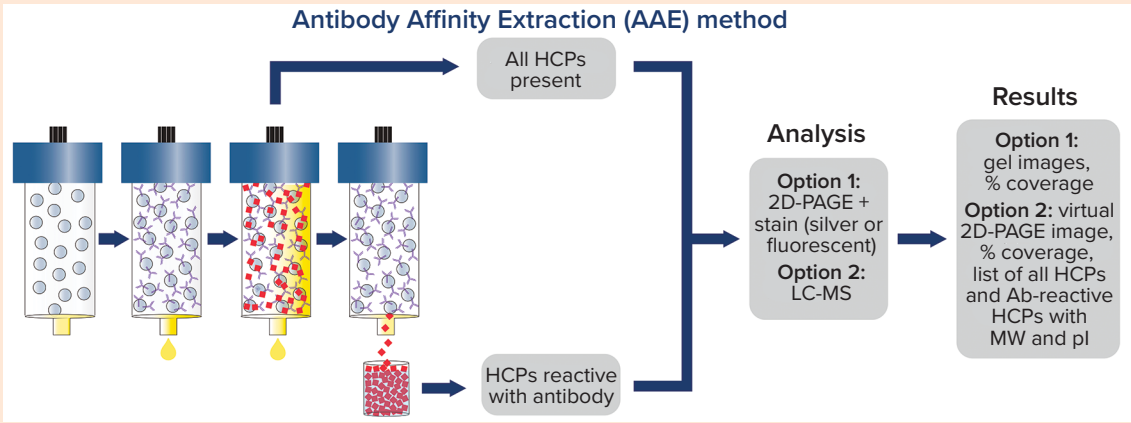


Figure 2: Antibody coverage by AAE chromatography with two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) or mass spectrometric (MS) analysis options; HCPs = host cell proteins; LC-MS = liquid chromatography with mass spectrometry; Ab = antibody

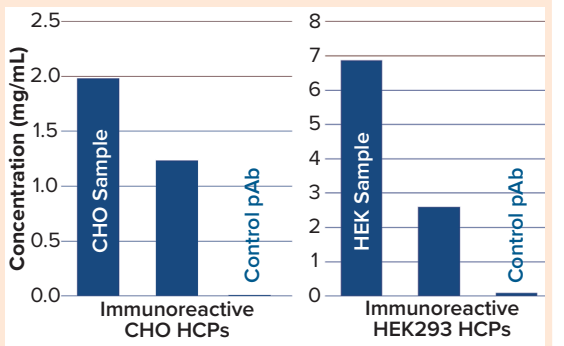


With a historical lack of better coverage analysis methods, two-dimensional Western blotting (2D-WB) traditionally has been performed using large-format, two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) gels, with protein transfer to membranes for 2D-WB comparison using silver stain. Because of recognized sensitivity limitations of 2D-WB, the conventional acceptance criteria have been that >50% of total HCPs should be reactive and that the detection antibody must recognize HCPs in all four quadrants of a 2D-PAGE gel. With 2D-WB, coverage only can be estimated on upstream harvest samples in which the concentrations of most HCPs are within the sensitivity limitations of available staining methods. In addition, the poor sensitivity and specificity of 2D-WB comes from limitations in 2D-PAGE loading capacity, destruction of native epitopes by harsh sample treatments, inefficiencies in transfer procedures, and potential steric hinderance of HCP antibody binding epitopes. For all these reasons, 2D-WB is known to underestimate true antibody coverage to upstream HCPs significantly. And more important, it cannot predict how an anti-HCP antibody will react quantitatively to the most important HCPs, which are those that copurify with a drug substance.

Antibody Affinity Extraction for Antibody Coverage Analysis

Antibody affinity extraction (AAE) immunoaffinity chromatography is an advanced orthogonal method designed to assess coverage of a polyclonal antibody for an array of HCPs present in a given process stream — as well as its reactivity to downstream, process-specific HCPs that can copurify with a drug substance. Cygnus Technologies developed this analytical method in 2013 to address the deficiencies of both traditional 2D-WB and two-dimensional differential-in-blot electrophoresis (2D-DIBE), both orthogonal methods that were used to assess coverage of polyclonal antibodies to total HCPs.

Figure 3: To assess specificity of the AAE method, (LEFT) a Chinese hamster ovary (CHO) HCP sample was passed over an F550-1 3G CHO HCP antibody AAE column and over a nonimmune goat IgG negative control column for fast protein liquid chromatography (FPLC), and (RIGHT) a HEK293 HCP sample was passed over an F650S HEK293 HCP antibody column and over nonimmune rabbit IgG negative control column for FPLC. Immunoreactive HCPs were eluted from the columns and quantified by mass spectrometry (LC-MS). Total CHO and HEK293 HCPs also were quantified using LC-MS.



In the AAE method, a polyclonal antibody is immobilized covalently onto a chromatography support. Then the column is conditioned to prevent significant antibody leaching and to minimize the potential for nonspecific binding. An HCP sample in its native, undenatured state is passed over the column for binding and then eluted with acid. Next, the HCP sample is cycled repeatedly over the column by binding and elution until no additional HCP is bound. All HCP elution fractions are pooled, then buffer is exchanged and concentrated back to the original sample volume. The final "post-AAE" sample

Figure 4A: AAE method with 2D-PAGE/silver stain detection — CHO harvest sample was loaded on an AAE column with anti-CHO HCP antibody (from F550-1 CHO HCP ELISA kit, 3G). Of the 1,176 total spots (gel image on the left), 1,005 spots (gel image on the right) were found in the AAE elution fraction, for a coverage of 86%.

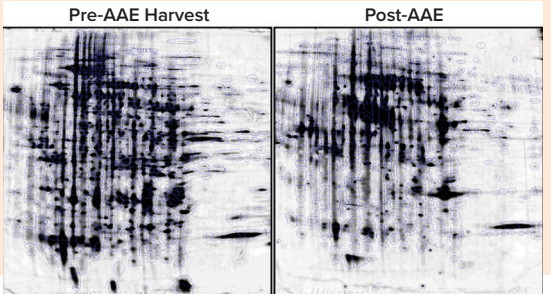


Figure 4B: AAE method with 2D-PAGE/silver stain detection — HEK293 mild cell lysate sample was loaded on an AAE column with anti-HEK293 HCP antibody (from resupplied F650S HEK293 HCP ELISA kit, 3G). Of the 1,687 total spots (gel image on the left), 1,604 spots (gel image on the right) were found in the AAE elution fraction for a coverage of 95%.

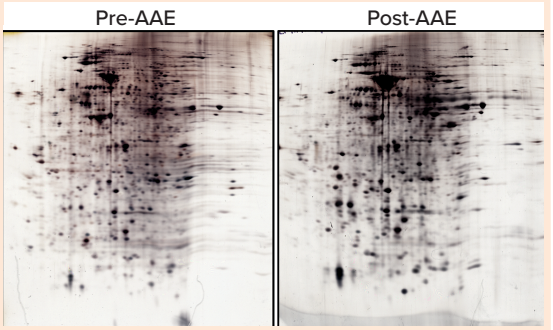
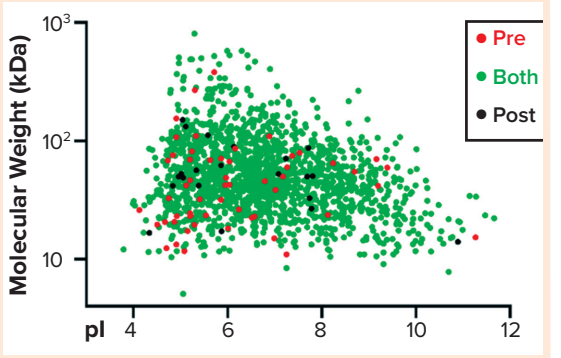


Figure 5A: Virtual two-dimensional gel of a harvest sample represents molecular weight and isoelectric points of host cell proteins (HCPs). Green spots represent proteins found in both the pre- and post-AAE samples; red spots represent proteins found only in the pre-AAE sample; and black spots represent proteins found only in the post-AAE sample. Here, anti-CHO HCP antibodies (from F550-1 CHO HCP ELISA kit, 3G) have 97% antibody coverage of HCPs found in CHO harvest (pre-AAE sample).



Number of CHO HCPs		
Pre-AAE	Post-AAE	Antibody Coverage
1,694	1,639	97%

can be analyzed with mass spectrometry (MS) or separated with 2D-PAGE and analyzed either by comparison with a silver stain of starting, unextracted "pre-AAE" sample or by differential gel electrophoresis (DIGE) using Cy3 and Cy5 dye to label the extracted and the starting (unextracted) samples, respectively (Figures 2 and 3). Antibody coverage is assessed based on comparing the number of HCPs in the silver-stain or Cy3-labeled AAE elution fraction with those seen in a silver-stain or Cy5-labeled 2D-PAGE of the starting antigen sample (Figure 4).

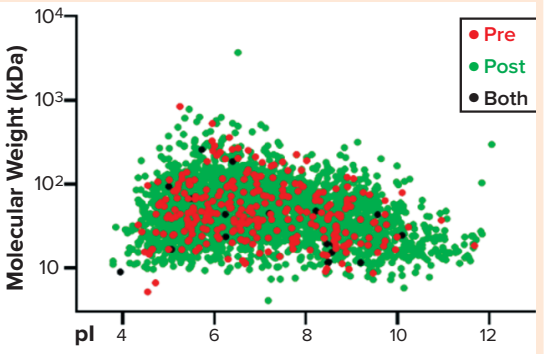
Combining AAE chromatography with mass spectrometry (AAE-MS) for HCP antibody coverage analysis creates a powerful method that also identifies all HCPs in harvest material and specifically those that are reactive with the antibody while providing molecular weight (MW) and pI (isoelectric point) information for those proteins (Figure 5). This is the only method that can be used reliably to assess HCP antibody coverage when the only available sample is harvest material containing a product (drug substance) — rather than clarified culture fluid (CCF) derived from a null cell line.

It is important to note that for the same anti-HCP antibody and process, the achievable coverage percentage depends on the assessment method chosen and can differ significantly between various methods. Numerical coverage comparisons should be used with caution because of the many method variables and the skill and expertise required to reproduce results — even with the same reagents in the same laboratory. Because of that variability, results are best evaluated qualitatively (Figure 6).

The main purpose of HCP antibody coverage analysis is to demonstrate that an anti-HCP antibody is broadly reactive to an array of HCPs that might copurify with a drug substance in a given downstream process. To demonstrate that a given HCP ELISA is fit for purpose, that assay must be qualified for dilution linearity, accuracy, and precision.

Comparative Coverage Analysis of HCP Antibodies from Two Different Serum or Plasma Pools: Ideally, once a developer has selected, qualified, and validated an HCP ELISA method as fit for its intended use of process monitoring and product lot release, the company must ensure that critical reagents supporting that validated assay will last throughout the lifetime of a program — from supporting DS manufacturing for phase 1–3 clinical trials to

Figure 5B: Virtual two-dimensional gel of a harvest sample represents molecular weight and isoelectric points of host cell proteins (HCPs). Green spots represent proteins found in both the pre- and post-AAE samples; red spots represent proteins found only in the pre-AAE sample; and black spots represent proteins found only in the post-AAE sample. Here, anti–HEK293 HCP antibodies (from a resupplied F650S HEK293 HCP ELISA kit, 3G) have 91% antibody coverage of HCPs found in HEK293 null cell lysate (pre-AAE sample).



Number of HEK-293 HCPs		
Pre-AAE	Post-AAE	Antibody Coverage
3,075	2,812	91%

commercial manufacturing throughout a drug product's life cycle. Polyclonal HCP antibodies for ELISAs cannot be supplied indefinitely, however, and sometimes such critical reagents must be resupplied. Changing an anti-HCP antibody changes the originally validated specificity of the HCP ELISA for each product regardless of whether or not the measured HCP levels are the same. Those levels typically are expressed in parts per million (ppm) or nanograms of HCP per milligram of drug substance (ng/mg). This effort requires revalidation of immunoreactivity and updating of the HCP ELISA standard operating procedure (SOP) before the new HCP ELISA can be used for lot release testing of drug substance lots that were approved for testing with the previously validated HCP ELISA.

In a recent example, Cygnus Technologies transitioned a depleted Chinese hamster ovary (CHO) HCP antibody and its corresponding CHO HCP ELISA from the original F550 kit to our F550-1 kit. A new pool of anti-CHO HCP antibody was produced. AAE-MS was used to assess immunoreactivity of both anti-CHO HCP antibodies to CHO HCPs. Results showed that the anti-CHO HCP antibodies used in the F550 and F550-1 kits both were immunoreactive with 97% of HCPs in CHO HCP samples (4). The similarity of CHO HCPs that were immunoreactive with the anti-CHO HCP antibodies from both the F550 and F550-1 kits was 96% (Figure 7).

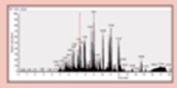
Figure 6: Coverage by evaluation method

2D Western blotting 50–60%

AAE 2D PAGE

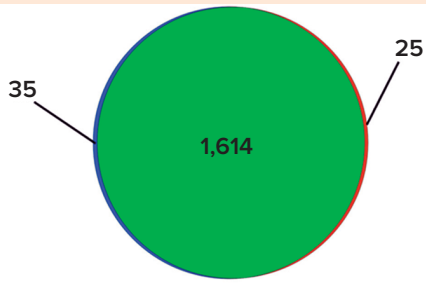
Silver 65–80%

Fluorescent 70–80%



AAE mass spectrometry 70–90%

Figure 7: Similarity between immunoreactive proteins is 96% for CHO HCPs with F550 and F550-1 CHO 3G ELISA antibodies. The quantitative Venn diagram displays unique identifications of F550 (blue) and F550-1 (red) along with proteins identified by both antigens (green).



Antibody	Total Proteins	Matching Proteins	Unique Proteins	Similarity
F550	1,649	1,614	35	96.42%
F550-1	1,639		25	

The company's proprietary, curated CHO secretome database was used to analyze known immunogenic or enzymatically active CHO HCPs: phospholipase B–like 2 (PLBL-2), lysosomal phospholipase A2 (LPA2), lipoprotein lipase (LPL), and serine protease (HTRA1). Results showed that the polyclonal anti-CHO HCP antibodies used in both kits can detect those potentially problematic CHO HCPs. Further analysis revealed that the enrichment or detection of the same CHO HCPs in F550 and F550-1 kits by AAE-MS is equivalent (Figure 8).

Identification of HCPs in Drug Substances

Coverage for the total HCP mixture present in a cell culture harvest stream traditionally has been requested by regulatory agencies. But it is the HCPs that persist through a given purification process that are the most important with respect to patient safety, drug efficacy, and product stability. Complete

Figure 8: Fold enrichment of selected CHO HCPs

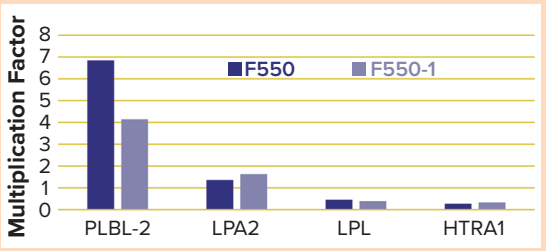
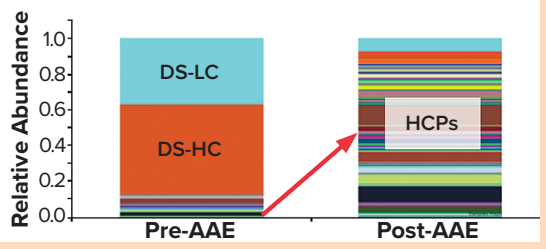


Figure 9: AAE immunoaffinity chromatography both enriches HCPs and depletes DS.



characterization of downstream HCPs is not part of the current regulatory guidelines. However, the potential for such information to help biopharmaceutical companies ensure the safety and efficacy of their products makes it recognized as value-added data by proactive manufacturers and regulators.

Identification and quantification of HCPs by MS provides a powerful complementary method to ELISA. However, drug products often mask HCPs by a factor of 10^4–10^6. The AAE technique is highly effective for enriching HCPs and depleting drug substance. Figure 9 shows the relative abundance of both according to extracted ion chromatograms that were normalized to one and graphed in a stacked bar chart. Pre-AAE results show the drug-substance heavy chain (DS-HC) in orange, light chain (DS-LC) in light blue, and HCPs in assorted colors beneath — demonstrating that most peptides in those samples belong to the drug substance. Following AAE enrichment (post-AAE results), the relative abundance of the HCPs dramatically increased while that of DS-HC and DS-LC decreased. Thus, AAE sample preparation before LC-MS increases the resolution of the method and allows for in-depth assessment of a drug substance's HCP profile, enabling true risk assessment.

MS identification of HCPs in drug substance also provides predictive MW and pI data that can be graphed into a "virtual 2D gel." Such visual representations of the HCPs present in drug-substance samples can empower strategies for downstream process development groups. By focusing on "potentially problematic HCPs" in one quadrant of a virtual gel, chromatography groups

Figure 10: Virtual two-dimensional gel of CHO HCPs in a drug substance sample represents molecular weight (MW) and isoelectric points (pI).

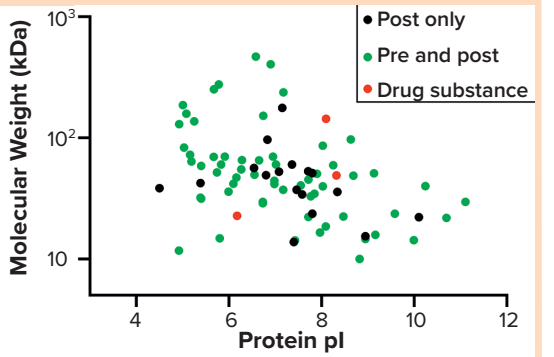


Table 1: Number of HCPs identified in four independent drug substances before and after AAE enrichment. All drug substances were produced in a Chinese hamster ovary (CHO) cell line and processed through an AAE column with anti-CHO HCP antibody from the Cygnus F550-1 kit.

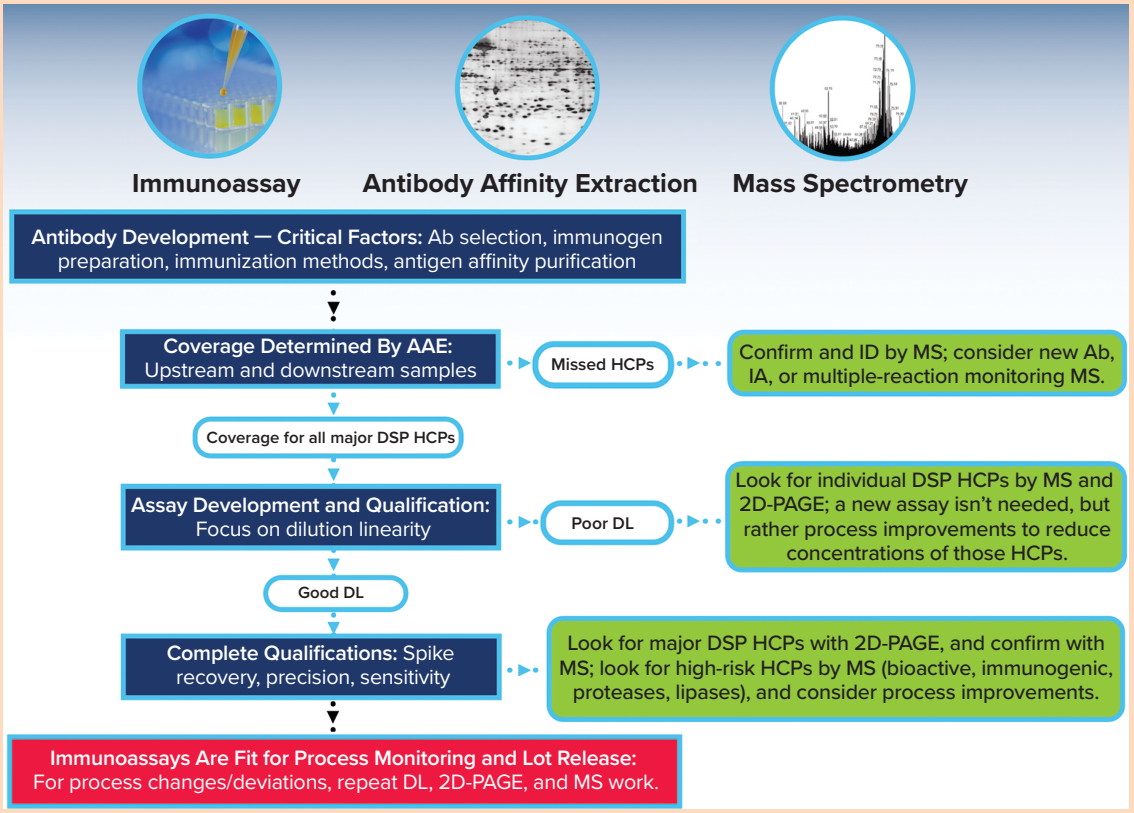
	Pre-AAE	Post-AAE	Coverage
Drug substance 1	13	28	100%
Drug substance 2	70	174	100%
Drug substance 3	62	79	100%
Drug substance 4	15	45	100%

can evaluate different column chemistries to eliminate those HCPs of interest (Figure 10, Table 1).

Integrating Methods for HCP Characterization

Sensitive and specific orthogonal methods are necessary to identify individual HCP impurities that persist through DS purification processes. These complementary technologies are an integral part of how biopharmaceutical manufacturers can ensure product purity and consistency (**3**). As discussed herein, such methods include AAE chromatography with 2D-PAGE or MS for HCP antibody coverage analysis and AAE-MS analysis for identification and quantitation of HCPs from in-process samples and final drug substances. Together with ELISA results, the integration of data from these orthogonal methods provides a comprehensive analysis of HCP content that will meet regulatory expectations. Integration of such advanced, orthogonal technologies also contributes to a data-driven approach for understanding whether a generic HCP ELISA will be fit for your process monitoring and product lot release needs or a process-specific assay must be developed. The decision tree in Figure 11 outlines a comprehensive approach to HCP analytics.

Figure 11: Integration of advanced orthogonal methods for HCP analytical development



About Cygnus Technologies

Part of Maravai LifeSciences, Cygnus Technologies (www.cygnustechnologies.com) offers generic HCP ELISA kits for 23 different expression platforms along with advanced orthogonal antibody coverage analysis services, AAE-MS identification of HCPs in process samples and drug substances, generic assay qualification services, and expert process-specific antibody and assay development services. The company's reputation for quality is recognized throughout the biopharmaceutical industry and among global regulatory agencies, with several of its generic HCP ELISA kits supporting numerous marketed biologics. Proprietary Cygnus technologies have been used to develop more than 100 process-specific antibodies and immunoassays for many global biopharmaceutical companies. Email techsupport@cygnustechnologies.com to discuss your current and future HCP analytics projects.

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*Corresponding author **Alla Zilberman,** PhD, is vice president for technical marketing at Cygnus Technologies, 4332 Southport-Supply Road SE, Southport, NC 28461; alla@cygnustechnologies.com; techsupport@cygnustechnologies.com; https://www.cygnustechnologies.com. **Kerry Wooding** is a mass-spectrometry scientist; **Jacob Stubbs** is a senior associate scientist in R&D; **Jared Isaac,** PhD, is associate director of chromatography and mass spectrometry; and **Eric Bishop** is vice president of R&D — all at Cygnus Technologies. AAE and AAE-MS are registered trademarks of Cygnus Technologies.*

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