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Case Study: Bridging Anti-*E. coli* HCP Antibodies and *E. coli* Antigens from Two Cygnus *E. coli* HCP ELISA Kits by AAE-MS™

Antibody coverage analysis and comparison of polyclonal antibodies to E. coli HCPs supporting qualification of the 2nd generation E. coli HCP ELISA Kit, 2G, F1020 vs the E. coli HCP ELISA Kit, F410

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Summary

Cygnus Technologies has performed an antibody coverage bridging study between the *E. coli* HCP ELISA, Item #F410, and the 2nd generation *E. coli* HCP ELISA, Item #F1020, by Antibody Affinity Extraction (AAE™) with host cell protein (HCP) identification by LC-MS (AAE-MS™). The immunoreactivity of anti-*E. coli* HCP antibody supporting the F410 kit and anti-*E. coli* HCP antibody supporting the F1020 kit to HCPs in the antigen used to generate the F1020 antibody was 82% and 89%, respectively. The overlap between HCPs immunoreactive with the F1020 and F410 *E. coli* HCP Antibodies was determined to be 83%. The F1020 *E. coli* HCP ELISA Kit, 2G, has high antibody coverage and is a suitable replacement for the F410 kit.

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Introduction

An essential function of process development and quality control groups in the biopharmaceutical industry involves monitoring of HCPs by ELISA to show clearance during drug substance (DS) purification. Data collected with in-process and DS samples are used to qualify whether an ELISA is fit for downstream process monitoring. Typically, ELISA is qualified for dilutional linearity, accuracy, precision, and lower limit of quantitation (LLOQ). 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 immunoreactive HCPs in a bioprocess that can be detected and quantified by an ELISA.

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 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 and the source antigen pool alters the originally validated specificity of the HCP ELISA for each product. This is true regardless of whether or not the measured HCP levels, typically expressed in parts per million (ppm) or nanograms of HCP per milligram of DS (ng/mg), remain the same. 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 the DS.

Qualifying the F1020 kit in your lab

We suggest at least the following studies be performed to qualify the F1020[1] kit and corresponding capture antibody:

1. Establish the mean and acceptable range for your controls with the F1020 kit. These values may be different (higher or lower) relative to the current antibody. To avoid failing runs due to 'out of specification' controls, it may be necessary to set a new range. Note that other curve parameters (e.g., ODs as an indirect specification) also may require a new range.
2. Test in-process and DS samples using the F410 and F1020 kits in parallel to determine if there is a consistent and significant difference and bias (higher or lower values) from the F410 kit.
3. Perform dilution linearity and spike recovery on your samples with the new antibodies to assure accuracy and specificity.
4. Orthogonal determination of coverage is best determined using our AAE method. We recommend performing AAE on at least two samples: 1) an upstream harvest sample to determine coverage to the majority of the proteome and 2) a downstream sample to determine coverage of those HCPs that persist through the purification process. We can perform the AAE analysis for you.
5. If you are using the F410 kit for lot release testing, determine what, if any, effect differences in control and sample values will have on your release criteria and document those changes. Changing the immunoreagents alters the originally validated specificity of the HCP ELISA for each product, regardless of whether the measured ppm levels are the same or not. That means that immunoreactivity must be revalidated and the HCP ELISA SOP must be updated before the new F1020 kits can be used for lot release testing of DS lots previously approved for testing with F410.

Qualification data packages are used as the basis for performing their ELISA assay validation by biopharmaceutical companies. Once validated ELISA assays have been approved by regulatory groups, changing them and revalidating them in a cGMP environment can be difficult and time intensive. 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.

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.

Here we describe the analyses necessary to support a transition from the *E. coli* HCP ELISA Kit (Item Number F410) to the *E. coli* HCP ELISA Kit, 2G (Item Number F1020). To enable our HCP community to seamlessly transition from the F410 kit to the F1020 kit, we quantified and compared the characteristics of the original and new HCP antigen lots of *E. coli* HCPs and performed a comparative coverage analysis study to assess the reactivity of anti-*E. coli* HCP antibodies that support these kits. Cygnus Technologies applied years of experience with *E. coli* HCP analysis to curate a proprietary database of *E. coli* HCPs. The documented *E. coli* HCPs include Flagellin, Phospholipase A1, ATPases, heat shock proteins, metalloproteases, peptidases, 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

Cygnus Technologies produced, characterized, and qualified large pools of the new capture and detection antibodies that will replace the current antibody lots. These antibodies have been generated through immunization against a new concentrated *E. coli* master antigen (EMA) lot (EMA-60521) of *E. coli* HCPs derived from the mild lysate of DH5a, BL21, JM109, TOP1 OF, K12, and MC1061 *E. coli* strains. EMA-60521 is compatible with *E. coli* HCP ELISA Kit, 2G, F1020. The immunization was conducted in the same way as was performed in generating the F410 reagents. For the reference, *E. coli* antigen lot EMA-20125, which is compatible with *E. coli* HCP ELISA Kit, F410, was included in the comparative analysis. The antibodies were affinity purified using the same procedures as the original reagents. The EMA was filtered with a 0.2 µm filter prior to AAE.

Quantification of HCPs

The total protein concentrations of the EMAs were determined by the Coomassie (Bradford) assay: EMA-60521 was 5.6 mg/mL and EMA-20125 was 8.9 mg/mL.

AAE Column Preparation

The procedures used in the column preparation and the AAE protocol are proprietary to Cygnus Technologies. Should regulatory agencies desire more detailed protocols, we can provide more specifics. The affinity-purified antibody pools of our *E. coli* HCP ELISA were covalently bound to a Sepharose

chromatography column. The column was quenched to remove any unreacted moieties, blocked to minimize non-specific binding, and further conditioned by a proprietary method to minimize the leaching of the antibody.

HCP Affinity Extraction

The Cygnus *E. coli* antigen concentrates were passed over the antibody affinity column to extract the reactive HCPs using a Akta 25L Fast Protein Liquid Chromatography (FPLC). The column was extensively washed to remove all unbound sample and then eluted with acid. The eluted material was immediately neutralized to pH 7.0 using a basic buffer system. Unbound HCP was passed back over the column under the same conditions, eluted, and combined with the first cycle. The sample was extracted in this way four times. The eluted fractions were combined and then concentrated by diafiltration at 3 kDa for LC-MS analysis.

LC-MS Sample Preparation

The Pre- and Post-AAE HCPs were precipitated, dissolved, reduced, alkylated, digested with trypsin, desalted, and concentrated.

Database Setup

Cygnus Technologies' proprietary curated *E. coli* HCP database containing UniProt *E. coli* and NCBI proteomes with an isoelectric point (pI) and molecular weight (MW) and common contaminants such as BSA, keratins, and trypsin was used to identify proteins.

Custom LC-MS Method Development

25 µg of peptides from digested proteins were separated with a reversed phase C18 column and injected using a Vanquish Horizon Ultra High-Performance Liquid Chromatography System (UHPLC) into an Orbitrap Eclipse Tribrid MS (Thermo Scientific) with a factory-established limit of detection (LOD) of 0.5 ppm. Data were acquired with a 75-minute gradient at a flow rate of 70 µL/min 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 during DDA were used for HCP identification and are referred to as the Custom LC-MS method.

HCP Identification by LC-MS

The Pre- and Post-AAE samples were analyzed with the Custom LC-MS Method independently in triplicate. 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 searched using Proteome Discoverer 2.5 (Thermo Fisher Scientific) with the following settings:

- Orbitrap Resolution: 240,000
- Modifications: Oxidation, Deamidation, Acetylation
- Max. number of missed trypsin cleavages: 2
- Precursor Mass Tolerance: 10 ppm
- Fragment Mass Tolerance: 0.6 Da
- Instrument: Orbitrap Eclipse
- Number of High Confidence peptides: 2
- False discovery rate confidence threshold of 0.01

For antibody coverage analysis, HCPs were identified by two peptides per protein to identify the most proteins above the LOD of 1 ppm. For quantification, HCPs were quantified by greater than or equal to two peptides per protein to include high-confidence protein identifications. Data of identified HCPs were exported from Proteome Discoverer into Microsoft Excel and analyzed.

HCP Quantification by LC-MS

The Cygnus Protein Standard (CPS) was spiked into all quantification samples prior to LC-MS sample preparation. Quantification of HCPs in ppm was calculated relative to CPS at 1,000 ppm. The ng/mL of HCPs were calculated by multiplying the Coomassie (Bradford) quantification in mg/mL of the sample to the ppm value. The lower limit of quantitation (LLOQ) of the CPS relative quantification is 10 ppm and the LOD is 1 ppm.

Virtual 2D Gel Graphs and Polyclonal ELISA Antibody Coverage Calculation

A 2D virtual gel graph was generated from AAE-MS data using Prism 8 (GraphPad Software). Gray spots represent proteins found only in the Pre-AAE samples. Beige spots represent proteins enriched by both the F1020 and F410 Post-AAE samples. Red spots represent proteins found only in the F1020 Post-AAE sample and black spots represent proteins found only in the F410 Post-AAE sample. 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). A 2D virtual gel graph was also generated comparing the baseline protein IDs for the EMA-60521 and EMA-20125 antigen lots. In this graph, spot colors represent proteins identified in both lots (beige), proteins only identified in EMA-60521 (red), and proteins identified only in EMA-20125 (black). To determine the percent similarity in antibody reactivity or protein content of the EMA lots, the number of matching proteins was divided by the total number of proteins.

Results

In EMA-60521 prior to AAE extraction, there were 1,462 HCPs present (Table 1 and Figure 1). In the Post-AAE samples, 1,302 proteins were identified as reactive with the F1020 antibodies, and 1,198 proteins were identified as reactive with the F410 antibodies. A comprehensive comparison of the presence/absence of HCPs Pre- and Post-AAE was performed (data not shown).

Table 1. *F410 and F1020 antibody coverage of total HCPs in EMA-60521.*

Sample		AAE (number of protein IDs)				% Antibody Coverage	
Name	AAE	**Total**	Unique to each fraction	**Total Unique**	Matching	Lower Boundary	Upper Boundary
F410	Pre	**1462**	284	**1482**	1178	81%	82%
	Post	**1198**	20				
F1020	Pre	**1462**	180	**1482**	1282	88%	89%
	Post	**1302**	20				

In EMA-20125 prior to AAE extraction, there were 676 HCPs present (Table 2 and Figure 2). In the Post-AAE samples, 761 proteins were identified as reactive with the F1020 antibodies and 780 proteins were identified as reactive with the F410 antibodies. A comprehensive comparison of the presence/absence of HCPs Pre- and Post-AAE was performed (data not shown).

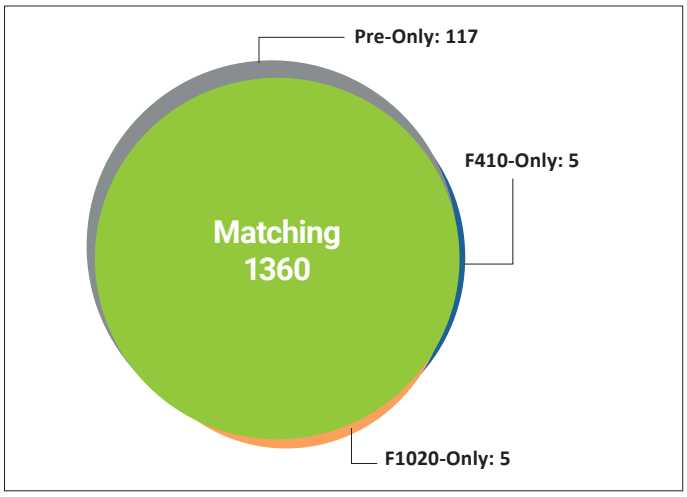

Figure 1. *Quantitative Venn diagram of F410 and F1020 antibody coverage of total HCPs in EMA-60521.*

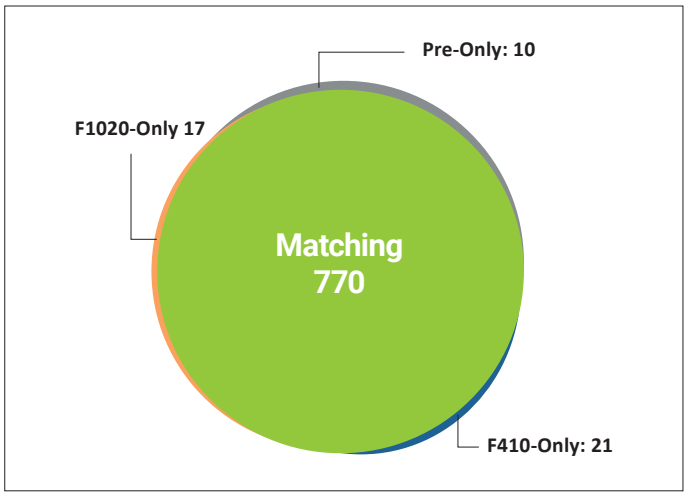

Figure 2. *Quantitative Venn diagram of F410 and F1020 antibody coverage of total HCPs in EMA-20125.*

Table 2. *F410 and F1020 antibody coverage of Total HCPs in EMA-20125.*

Sample		AAE (number of protein IDs)				% Antibody Coverage	
Name	AAE	**Total**	Unique to each fraction	**Total Unique**	Matching	Lower Boundary	Upper Boundary
F410	Pre	**676**	21	**801**	655	97%	100%
	Post	**780**	125				
F1020	Pre	**676**	36	**797**	640	95%	100%
	Post	**761**	121				

The HCPs identified in the Pre- and Post-AAE samples in the virtual two-dimensional gels of our EMAs were within the same range of MW and pI and covered the major range of the *E. coli* proteome. The 2D virtual gels (Figure 3) of the proteins identified by both antibodies and antigen lots provide a comprehensive visual aid to show there is no bias toward protein identification. In addition to comparing the coverage of the F1020 and F410 antibodies with respect to the HCPs in the EMA concentrates, their reactivity was directly compared to assess their percent similarity (Figure 3, Table 3, Table 4). Of the 1,370 total proteins identified in the EMA-60521 Post-AAE eluate, 1,130 proteins were identified with both antibodies (Table 3). The F1020 antibodies identified 172 proteins not identified with the F410 antibodies; conversely, the F410 antibodies identified 68 proteins not identified with the F1020 antibodies (Table 3). Of the 808 total proteins identified in the EMA-20125 Post-AAE eluate, 733 proteins were identified with both antibodies (Table 4). The F1020 antibodies identified 28 proteins not identified with the F410 antibodies; conversely, the F410 antibodies identified 47 proteins not identified with the F1020 antibodies.

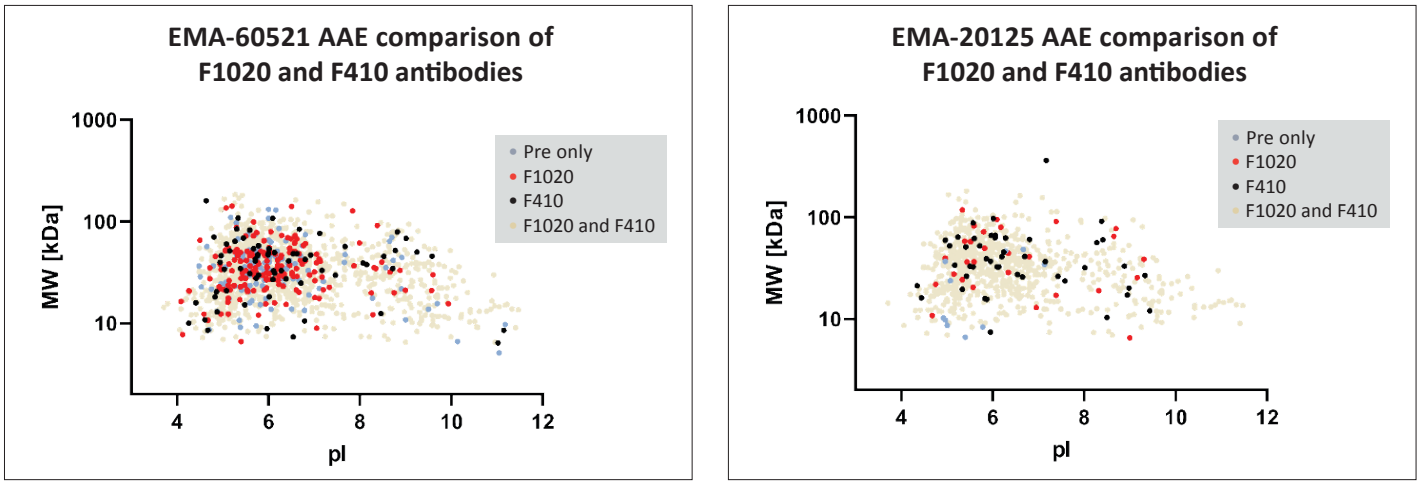

Figure 3. *Virtual two-dimensional gels of F1020 and F410 AAE studies with EMA-60521 (Left) and EMA-20125 (Right).*

Table 3. *Comparison of F1020 and F410 protein identifications for EMA-60521.*

Sample				
Antibody	Total Proteins	Matching Proteins	Unique Proteins	% Similarity
F1020	1302	1130	172	82.5%
F410	1198		68	

Table 4. *Comparison of F1020 and F410 protein identifications for EMA-20125.*

Sample				
Antibody	Total Proteins	Matching Proteins	Unique Proteins	% Similarity
F1020	761	733	28	90.7%
F410	780		47	

The EMA lots (pre-AAE samples) were also assessed for baseline similarity (Figure 4, Figure 5). Both lots covered a similar range of MW and pI of the *E. coli* proteome showing 60% similarity.

For the EMA samples, potentially high-risk *E. coli* HCPs such as ATPases, peptidases, and proteases were identified that were immunoreactive with both F1020 and F410 HCP antibodies (Table 5). Both antibodies provided a similar level of coverage against these potentially problematic HCPs. At least two prominent HCPs (Hydrogenase 2 maturation protease and DsbG) were missing from both EMA lots or were present at only very low abundance.

The representative list in Table 5 has been generated from publicly available scientific literature relating to *E. coli* HCPs (1-4) and Cygnus Technologies' years of HCP experience. *E. coli* HCPs identified in the Post-AAE fraction but not the Pre-AAE sample

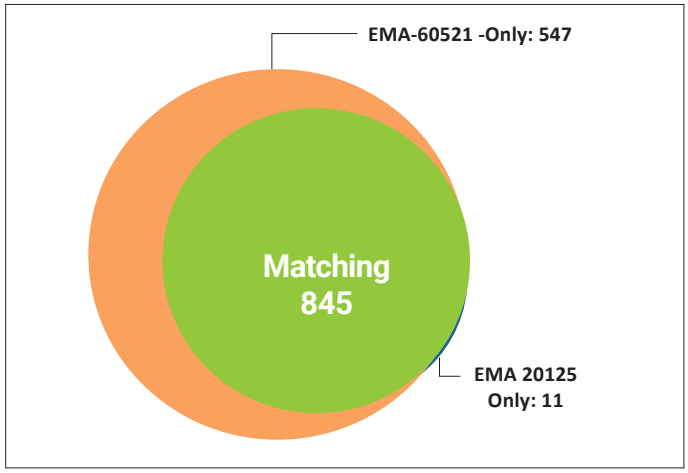

Figure 4. *Quantitative Venn diagram of total HCPs in EMA-60521 and EMA-20125.*

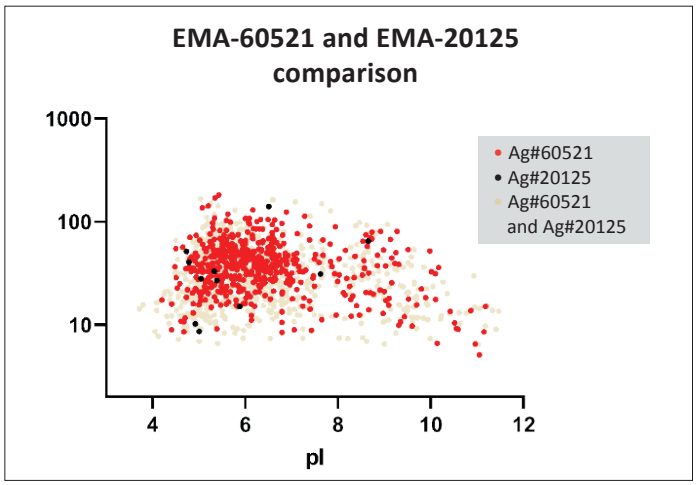

Figure 5. *Virtual two-dimensional gel comparing EMA-60521 and EMA-20125.*

indicate
that without AAE enrichment these HCPs may be below the LOD of the MS. *E. coli* HCPs identified in the Pre-AAE sample but not the Post-AAE eluate indicate that the antibodies may not have reactivity with these proteins. All identified HCPs in the antigen concentrates are available upon request.

Table 5. *Potentially problematic HCPs in the F410 and F1020 coverage of total HCPs in EMA-60521 and EMA-20125.*

Potential High Risk HCPs	EMA-60521			EMA-20125			pI	MW
	PRE	F1020	F410	PRE	F1020	F410		
ATP-dependent zinc metalloprotease FtsH	Y	Y	Y	Y	Y	Y	6.24	70.6
Enolase	Y	Y	Y	Y	Y	Y	5.48	45.6
Metalloprotease LoiP	Y	Y	Y	Y	Y	Y	6.13	26.8
Metalloprotease PmbA	Y	Y	Y	Y	Y	Y	5.60	48.3
Methionine aminopeptidase	Y	Y	Y	Y	Y	Y	5.96	21.3
Peptidase T	Y	Y	Y	Y	Y	Y	5.59	44.9
Peptidyl prolyl cis trans isomerase B	Y	Y	Y	Y	Y	Y	5.80	18.1
Periplasmic serine endoprotease DegP	Y	Y	Y	Y	Y	Y	8.56	49.3
Peroxiredoxin OsmC	Y	Y	Y	Y	Y	Y	5.86	15.1
Protease HtpX	Y	Y	Y	Y	Y	Y	7.14	31.9

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 on 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 represent 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.

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-*E. coli* HCP antibodies used in the F410 and F1020 kits were immunoreactive with 82% and 89% of HCPs in the EMA-60521, respectively. Also, AAE-MS coverage analysis showed that the anti-*E. coli* HCP antibodies used in the F410 and F1020 kits were reactive with 100% of HCPs in the EMA-20125, respectively.

AAE-MS analysis showed that the similarity of *E. coli* HCPs immunoreactive with both antibodies was 83% for EMA-60521 (antigen used to generate antibodies for the F1020 kit) and 91% for EMA-20125 (antigen used to generate antibodies for the F410 kit), 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 *E. coli* HCPs including Metalloproteases LoiP, Metalloprotease PmbA, Periplasmic serine endoprotease DegP, and Protease HtpX which were identified with the same MS2 spectra in both the F410 and F1020 *E. coli* AAE samples.

Conclusion

There were 1,462 proteins identified in EMA-60521. After AAE, 1,302 proteins were identified as immunoreactive with the F1020 antibodies and 1,198 proteins were identified as immunoreactive with the F410 antibodies. There were 676 proteins identified in the EMA-20125. After AAE, 761 proteins were identified as immunoreactive with the F1020 antibodies and 780 proteins were identified as immunoreactive with the F410 antibodies. The established instrument LOD for tandem MS is 0.5-1.0 ppm and identifications below this LOD should be considered unreliable but are included for comprehensiveness. Protein identifications include high-confidence spectra matches generated from sequence-based peptide predictions. 1,402 and 212 proteins were quantified above the established instrument LLOQ of 10 ppm for EMA-60521 and EMA-20125, respectively. For the Post-AAE fraction of EMA-60521, 1,051 and 897 proteins were quantified above the LLOQ for the F1020 and F410 antibodies, respectively. For the Post-AAE fraction of EMA-20125, 566 and 624 proteins were quantified above the LLOQ for the F1020 and F410 antibodies, respectively. All quantified and identified proteins were represented by multiple unique peptides.

MS is a very sensitive technique for the identification and quantification of proteins, with limitations. LC-MS sample preparation requires concentration and injection using plastic lines that may adsorb hydrophobic proteins. Free amino acids or detergents present in cell culture or bioprocessing fluids could interfere with protein quantification and peptide charging, which can in turn cause unequal sample injection, UHPLC column loading, and peptide ionization. Most importantly, detection of common peptides between isoforms could lower the number of identified proteins, but the number is not subjective as in differentiating between a protein spot versus a gel artifact. DDA methodology overcomes the limitation of coeluting peptides by measuring the top eluting peptides and then proceeding to the next abundant peptides to ensure that all theoretical peptides are detected. When considering that MS identifies proteins in comparison to gel-based techniques, the advantages of MS outweigh its limitations.

By using stringent bioinformatic settings to minimize false positives, a direct calculation of antibody coverage analysis is recommended. The coverage assessment data demonstrates that the F1020 antibody is broadly reactive at 88% to 89% in EMA-60521 and 95% to 100% in EMA-20125. The coverage assessment data demonstrates that the F410 antibody is broadly reactive at 81% to 82% in EMA-60521 and 97% to 100% in EMA-20125. The similarity of coverage for these antibodies was estimated in the range of 83-91%.

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.

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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