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Case Study: Antibody Coverage Analysis of *E. coli* HCPs in the BL21(DE3) strain by AAE-MS™

Comparing reactivity of three E. coli HCP Antibodies from Cygnus Technologies' two E. coli HCP ELISA Kits, F410 and F1020, and BL21(DE3) HCP ELISA Kit, F1060, to HCPs in the BL21(DE3) HCP Antigen

Summary

To determine the suitability and similarity of Cygnus *E. coli* HCP ELISA Kits to quantify BL21(DE3) Host Cell Proteins (HCPs), antibody coverage analysis of the BL21(DE3) HCP antigen was performed using **Cygnus Technologies' Antibody Affinity Extraction and Mass Spectrometry (AAE-MS™)** approach. AAE™ was performed using Cygnus' commercially available anti-*E. coli* antibodies from three *E. coli* HCP ELISA kits: F410, F1020, and F1060. HCPs were identified in the Pre-AAE and Post-AAE samples by liquid chromatography/mass spectrometry (LC-MS). HCP coverage was determined by comparing the number of immunoreactive HCPs in the AAE elution fraction compared to the starting sample containing all BL21(DE3) HCPs. Prior to AAE extraction, 924 HCPs were detected in the BL21(DE3) HCP antigen sample, and the F410 and F1020 antibodies reacted with 836 and 829 proteins, respectively, indicating comparable performance. In contrast, the F1060 antibodies captured 923 proteins in the Post-AAE elution fraction, suggesting better coverage of the BL21(DE3) HCPs. Importantly, potentially high-risk HCPs were identified as immunoreactive with the Cygnus anti-*E. coli* antibodies. The antibody coverage of the BL21(DE3) host cell proteins was **90% for F410, 89-90% for F1020 and 99-100% for F1060**.

Contents

Introduction

Production of therapeutic recombinant proteins is often performed in *E. coli* due to ease of manipulation and reasonable cost. Several strains of *E. coli* have been successfully used for this purpose, with the BL21 strain and its derivatives being a workhorse of recombinant protein production. Although these recombinant proteins are subjected to extensive purification processes prior to therapeutic use, regulatory agencies require assessment and reporting of residual host cell protein concentrations to ensure the safety and stability of the final drug substance. A robust and broadly reactive HCP ELISA is a gold standard method for purification process monitoring and product lot release testing for HCPs. Once qualified, these assays are used to support drug manufacturing throughout clinical development and marketing post-approval. Thus, it is important that the assays used to verify acceptable levels of residual HCPs are reliable, reproducible, and available throughout the drug's lifecycle.

Cygnus Technologies developed two generations of *E. coli* HCP ELISA kits (Item # F410, first generation, and # F1020, second generation) to detect host cell proteins derived from several *E. coli* strains commonly used for therapeutic protein production. To further improve detection of BL21(DE3) HCPs, Cygnus Technologies developed a specific BL21(DE3) HCP ELISA kit, Item # F1060, which is based on an antibody generated against HCPs derived from BL21(DE3) strain. Coverage analysis of this new F1060 antibody against BL21(DE3) HCPs indicates that the new kit exhibits broader coverage compared with the original pan-*E. coli* antibodies, making it suitable for further testing of drug substances produced in BL21(DE3) and its derivative strains.

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Materials and Methods

Sample Preparation

	Product Information	Corresponding Kit
Antigen	F1063X CYG BL21 (DE3) Antigen Concentrate (Lot # 280223)	—
Antibodies	F412-AF Anti-*E. coli* HCP (Lot# 184) F1022-AF Anti-*E. coli* (Lot # 270122) F1062-AFBL21(DE3) Anti-*E. coli* (Lot # 51023)	F410 *E. coli* HCP ELISA Kit F1020 *E. coli* HCP ELISA Kit, 2G F1060 BL21(DE3) HCP ELISA Kit

The BL21(DE3) Antigen Concentrate had a pH of 7.5 and total protein concentration of 49 µg/mL by Coomassie assay. The sample was passed through a 0.2 µm filter prior to AAE.

AAE™ Column Preparation

The procedures used in the column preparation and in the AAE protocol are proprietary to Cygnus Technologies. Should regulatory agencies desire more detailed protocols, Cygnus can provide more information upon request. The affinity purified antibody pools utilized in the F410, F1020 and F1060 anti-*E. coli* HCP ELISA kits were covalently bound to separate Sepharose chromatography columns. The columns were conditioned using proprietary methods to minimize leaching of the antibody and non-specific binding.

HCP Antibody Affinity Extraction (AAE™)

The BL21(DE3) antigen sample was passed over each antibody affinity column to extract the immunoreactive HCPs using a GE Healthcare Äkta Pure 25L fast protein liquid chromatography (FPLC). The columns were extensively washed to remove all unbound HCPs prior to elution of bound HCPs with acid. The eluate was immediately neutralized to pH 7.0 using a basic buffer system. Unbound HCPs were passed back over the column under the same conditions, eluted, and combined with the first cycle. This process was repeated three (3) times, after which the eluted fractions were combined, concentrated, and prepared 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.

Custom LC-MS Method Development

2 µg of peptides from digested proteins were separated with a reversed phase C18 column and injected using a Vanquish Horizon UHPLC (Ultra high-performance liquid chromatography system) into an Orbitrap Eclipse Tribrid MS (Thermo Fisher Scientific) with a factory established limit of detection (LOD) of 0.5 parts per million (ppm). Data were acquired in data dependent acquisition (DDA) mode with survey spectrum (mass to charge ratio (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. Cygnus Technologies' proprietary curated *E. coli* HCP database containing UniProt proteomes with isoelectric point (pI) and molecular weight (MW) and common contaminants such as bovine serum albumin, keratins, and trypsin was used to identify proteins.

HCP Identification by LC-MS

The Pre- and Post-AAE samples were analyzed with the Custom 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 searched using Proteome Discoverer 2.5 (Thermo Fisher Scientific) with a false discovery rate confidence threshold of 0.01%. Data of identified HCPs were exported from Proteome Discoverer into Microsoft Excel and analyzed.

Virtual 2D Gel Graphs and Polyclonal ELISA Antibody Coverage Calculation

2D virtual gel graphs were generated from MS data using Prism 8 (GraphPad Software). pI and MW values were derived from Cygnus' proprietary *E. coli* HCP database described above. Polyclonal Enzyme-linked Immunosorbent Assay (ELISA) antibody coverage is represented by a range between the lower and upper coverage boundary calculations. The lower coverage boundary was calculated using the equation (Post-AAE proteins/Unique proteins), which includes the calculation for the number of unique proteins ((Pre-AAE + Post-AAE proteins)-Matching proteins). The upper coverage boundary was calculated by dividing the number of Post-AAE proteins by the number of Pre-AAE proteins.

Results

LC-MS assessment detected 924 HCPs present in the BL21(DE3) antigen sample prior to AAE extraction **(Table 1)**. Analysis of Post-AAE elution fractions indicated that 836 proteins were detected by F410 antibodies, 829 proteins were detected by F1020 antibodies, and 923 proteins were detected by F1060 antibodies. Whereas the vast majority of HCPs detected by these antibodies were also detected in the Pre-AAE sample, there were some proteins that were unique to each fraction (Pre-AAE or Post-AAE); the numbers of matching and unique proteins for each fraction are visually demonstrated in **Figures 1-3**. Using these numbers, the number of total unique BLD21(DE3) HCPs could be calculated, followed by the percentage antibody coverage **(Table 1)**. While the overall antibody coverage was estimated to be approximately 90% for the F410 antibody and 89-90% for the F1020 antibody, the estimated coverage of the F1060 antibody was 99-100%, indicating an overall broader coverage of BL21(DE3) HCPs compared to F410 and F1020 **(Table 1)**.

Table 1. *F410, F1020, and F410 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	**924**	94	**930**	830	90%	90%
	Post	**836**	6				
F1020	Pre	**924**	98	**927**	826	89%	90%
	Post	**829**	3				
F1060	Pre	**924**	7	**930**	917	99%	100%
	Post	**923**	6				

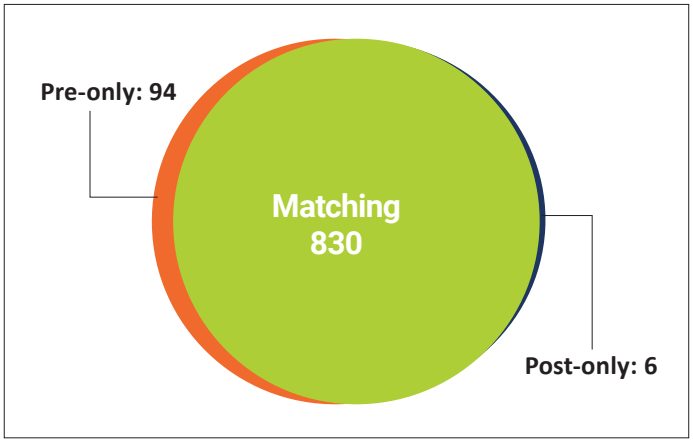

Figure 1. *F410 anti-E. coli antibody coverage of total HCPs in the BL21(DE3) sample Pre- and Post-AAE.*

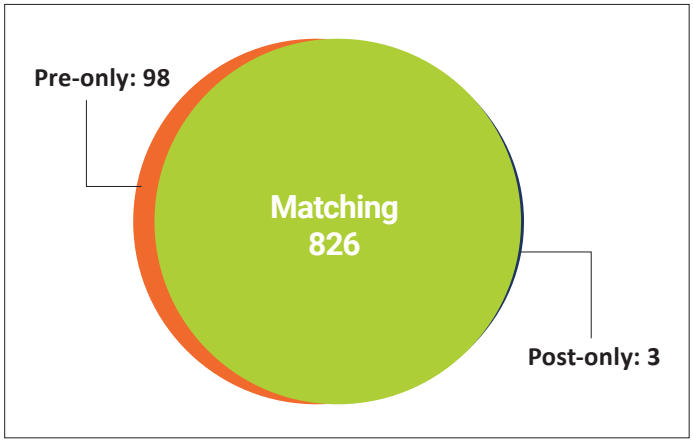

Figure 2. *F1020 anti-E. coli antibody coverage of total HCPs in the BL21(DE3) sample Pre- and Post-AAE.*

To ensure the HCPs detected by these antibodies were not limited to a particular size range or charge, two-dimensional 'virtual gel' plots were constructed to examine the range of molecular weight (MW) and isoelectric point (pI) of all detected proteins. Importantly, the HCPs identified across all Pre-AAE and Post-AAE samples were within the same MW and pI range, covering the major ranges associated with the *E. coli* proteome **(Figures 4-6)**.

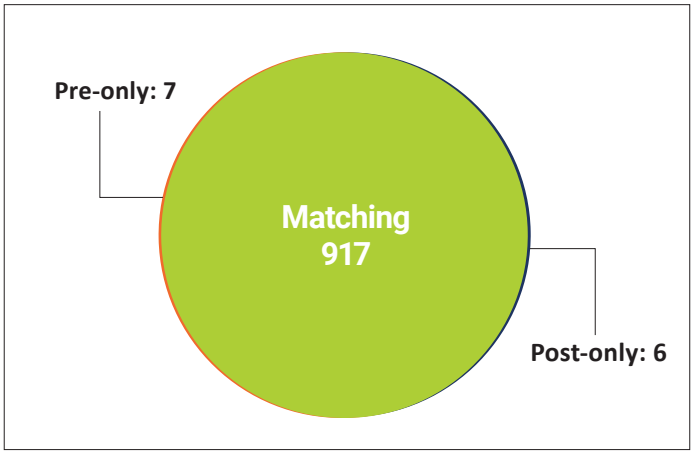

Figure 3. *F1060 anti-E. coli antibody coverage of total HCPs in the BL21(DE3) sample Pre- and Post-AAE.*

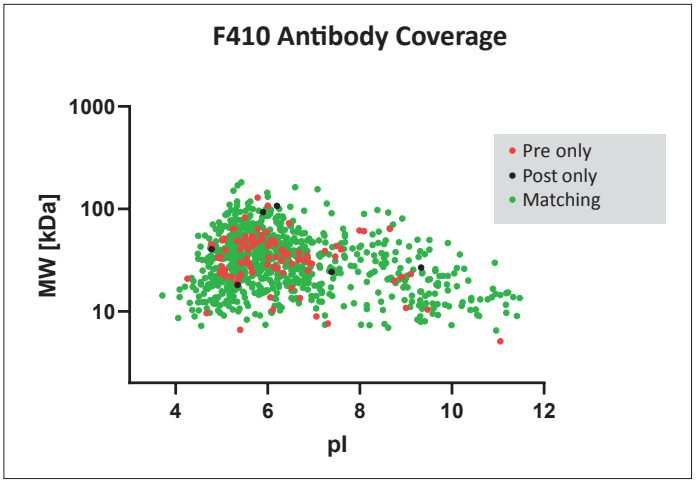

Figure 4. *Virtual 2D Gel of F410 anti-E. coli antibody coverage of total HCPs in the BL21(DE3) Antigen.*

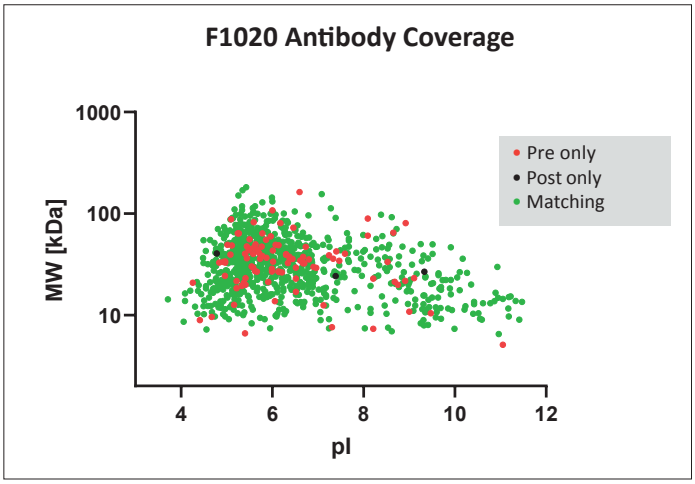

Figure 5. *Virtual 2D Gel of F1020 anti-E. coli antibody coverage of total HCPs in the BL21(DE3) Antigen.*

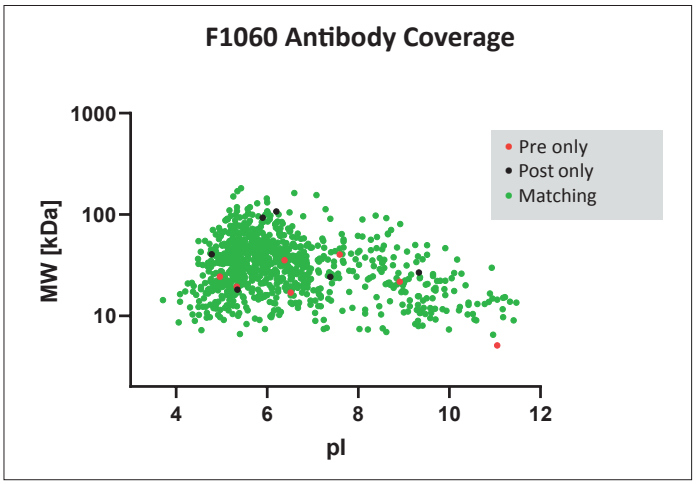

Figure 6. *Virtual 2D Gel of the F1060 anti-E. coli antibody coverage of total HCPs in the BL21(DE3) Antigen.*

Next, a similarity comparison of the proteins found in the three elution fractions for F410, F1020 and F1060 antibodies was performed **(Table 2)**. 797 BL21(DE3) HCPs were detected by all three antibodies, while F410 and F1060 shared 836 proteins, and F1020 and F1060 shared 829 proteins. This indicates that the coverage of F410 and F1020 overlaps by approximately 95%, while there is approximately 90% and 91% overlap of the total proteins detected by F1020/F1060 and F410/F1060, respectively. The lower overlap of F1060 compared to the other antibodies is due to the exclusive detection of 55 proteins by F1060 that were not detected by F410 or F1020; additionally, F410 and F1020 did not detect any unique proteins when compared with F1060. Altogether, these results indicate that F1060 detects more BL21(D3) proteins compared to F410 and F1020 antibodies.

Table 2. *Similarity comparison of BL21(DE3) HCPs immunoreactive with the F410, F1020 and the F1060 Anti-E. coli HCP antibodies.*

Antibody Comparison		AAE (number of protein IDs)				
First Antibody	Second Antibody	Unique to first antibody	Matching Proteins		% of Matching Proteins	
F410	F1020	0	797	797	95%	86%
	F1060		836		91%	
F1020	F1060	0	829		90%	
	F410		797		95%	
F1060	F1020	55	829		90%	
	F410		836		91%	

Examination of specific HCPs was next performed based on their propensity to be problematic in downstream applications. For the BL21(DE3) sample, potentially high-risk HCPs such as 50S ribosomal subunit assembly factor BipA and Pyruvate kinase were identified as immunoreactive with the F410, F1020, and the F1060 anti-*E. coli* antibodies **(Table 3)**. Deeper proteomic coverage of the F1060 BL21(DE3) HCP Antibody revealed three potentially problematic HCPs (Metalloprotease LoiP, Peptidase T, and Protease 3) not found to be covered by the F410 and F1020 *E. coli* HCP Antibodies. This finding suggests F1060's broader immunoreactivity extends to those HCPs of most interest.

The list of HCPs in **Table 3** has been curated from publicly available scientific literature [1-7] and Cygnus Technologies' years of HCP experience. Many of the *E. coli* HCPs on this list were detected by AAE-MS, indicating that the antibodies have reactivity to those identified proteins. *E. coli* HCPs identified in the Post-AAE fraction but not Pre-AAE sample 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 anti-*E. coli* antibodies are not immunoreactive with these proteins.

Table 3. *Potentially problematic E. coli HCPs and their coverage by F410, F1020 and F1060 anti-E. coli antibodies.*

Potential High-Risk HCPs	Pre	Post F410	Post F1020	Post F1060	pI	MW
50S ribosomal subunit assembly factor BipA	Y	Y	Y	Y	5.07	72.4
Aminoacyl-histidine dipeptidase	Y	Y	Y	Y	5.39	52.9
ATP-dependent protease ATPase subunit HslU	Y	Y	Y	Y	5.35	49.6
ATP-dependent zinc metalloprotease FtsH	Y	Y	Y	Y	6.24	70.6
Beta-barrel assembly-enhancing protease	Y	Y	Y	Y	7.68	53 .9
D-alanyl-D-alanine carboxypeptidase DacB	N	N	N	N	8.78	51.8
Elongation factor G	Y	Y	Y	Y	5.38	77.5
Elongation factor Ts	Y	Y	Y	Y	5.29	30.4
Enolase	Y	Y	Y	Y	5.48	45.6
Flagellin	N	N	N	N	4.73	51.3
Glutathione S transferase	N	N	N	N	5.17	23.7
Glyceraldehyde 3 phosphate dehydrogenase A	Y	Y	Y	Y	7.11	35.5
Heat shock protein HslJ	N	N	N	N	7.24	15.2
Hydrogenase 2 maturation protease	Y	Y	Y	Y	4.73	17.7
Hydrogenase 3 maturation protease	N	N	N	N	4.18	17.0
Metalloprotease LoiP	Y	N	N	Y	6.13	26.8
Metalloprotease PmbA	Y	Y	Y	Y	5.60	48.3
Metalloprotease TldD	Y	N	Y	Y	5.06	51.3
Methionine aminopeptidase	Y	Y	Y	Y	5.96	21.3
Peptidase T	Y	N	N	Y	5.59	44.9
Peptidyl prolyl cis trans isomerase B	Y	Y	Y	Y	5.80	18.1
Periplasmic pH-dependent serine endoprotease	N	N	N	N	5.95	47.2
Periplasmic serine endoprotease DegP	Y	Y	Y	Y	8.56	49.3
Peroxiredoxin OsmC	Y	Y	Y	Y	5.86	15.1
Phosphoglycerate kinase	Y	Y	Y	Y	5.22	41.1
Phospholipase A1	N	N	N	N	5.33	33.1
Protease 3	Y	N	N	Y	6.09	107.6
Protease 4	N	N	N	N	6.20	67.1
Protease HtpX	N	N	N	N	7.14	31.9
Pyruvate kinase	Y	Y	Y	Y	6.90	51.3
Serine endoprotease DegS	N	N	N	N	5.67	37.6
Serine-type D-Ala-D-Ala carboxypeptidase	N	N	N	N	8.03	43.6
Small heat shock protein IbpA	Y	Y	Y	Y	5.83	15.8
Thioesterase 1/protease 1/lysophospholipase L1	Y	Y	Y	Y	7.58	23.6
Thiol:disulfide interchange protein DsbA	Y	Y	Y	Y	6.34	23.1
Thiol:disulfide interchange protein DsbC	Y	Y	Y	Y	6.79	25.6
Thiol:disulfide interchange protein DsbD	N	N	N	N	7.20	61.8
Thiol:disulfide interchange protein DsbG	Y	Y	Y	Y	8.27	27.5
Thioredoxin 1	Y	Y	Y	Y	4.88	11.8
Thioredoxin 2	Y	Y	Y	Y	5.17	15.5

Box 1. Key Points

- The overall antibody coverage to BL21(DE3) antigen was estimated to be approximately 90% for the F410 antibody, 89-90% for the F1020 antibody, and 99-100% for the F1060 antibody.
- F1060 exclusively detected 55 BL21(DE3) proteins that were not detected by F410 or F1020. Conversely, F410 and F1020 did not detect any unique BL21(DE3) proteins when compared with F1060, indicating that F1060 detects more BL21(D3) proteins than F410 or F1020.
- F1060 reacts with all problematic HCPs detected by MS in the BL21(DE3) antigen sample.

Discussion/Conclusions

Here, the ability of two pan-*E. coli* HCP antibodies (F410 and F1020) to detect host cell proteins from the BL21(DE3) strain was compared to F1060, a new antibody generated specifically against BL21(DE3) antigen. MS analysis detected a total of 924 host cell proteins in the BL21(DE3) antigen sample ("Pre-AAE"). AAE-MS analysis indicated that the F410 HCP antibodies were immunoreactive with 836 proteins, the F1020 antibodies were immunoreactive with 829 proteins, and the F1060 antibodies were immunoreactive with 923 proteins from the BL21(DE3) antigen sample. From this data, the antibody coverage boundaries of the F410, F1020 and the F1060 anti-*E. coli* antibodies was determined for the BL21(DE3) antigen as 90% for F410, 89-90% for F1020 and 99-100% for F1060, indicating that F1060 is the most suitable antibody for further testing of in-process or drug substance samples produced in BL21-derived strains **(Box 1)**. Similarity of BL21(DE3) HCP coverage between the new antibody and each original pan-*E. coli* antibody was determined to be approximately 90%; when considering the number of HCPs detected by all three antibodies, the coverage similarity is 86%. With these coverage percentages in mind, it is important to perform a thorough "fit-for-purpose" evaluation of the F1060 kit in comparison with F1020 or F410 to assess any differences **(Box 2)**.

Box 2. Qualifying the F1060 kit in your lab

Changing the immunoreagents alters the originally validated specificity of the HCP ELISA for each drug product, regardless of whether the measured ppm levels are equivalent. As a result, immunoreactivity must be revalidated, and the HCP ELISA standard operating procedure must be updated before the new F1060 kits can be used for lot release testing of DS lots previously approved for testing with F1020 or F410. Cygnus suggests at least the following studies be performed to qualify the F1060 kit and corresponding capture antibody:

1. Establish the mean and acceptable range for your controls with the F1060 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 your current kit and F1060 kits in parallel to determine if there is a consistent and significant difference and bias (higher or lower values).
3. Perform dilution linearity and spike recovery on your samples to assure accuracy and specificity with the new antibodies.
4. Orthogonal determination of coverage is best determined using AAE. Cygnus recommends performing AAE on at least two samples:
   - An upstream harvest sample to determine coverage to the majority of the proteome;
   - A downstream sample to determine coverage of those HCPs that persist through the purification process. Cygnus can perform the AAE analysis for you.
5. If you are using the F1020 or 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.

Though western blotting has been historically used for antibody coverage analyses, the use of AAE for antibody coverage studies is superior to western blotting for several reasons, including the ability to assess the binding capacity of antibodies in a non-denaturing column environment, which better mimics the conditions in which the ELISA will ultimately be performed. Additionally, the use of columns is not limited by sample volume, offering improved sensitivity compared to gel-based methods.

MS is a very sensitive technique for the identification and quantification of proteins from the AAE eluate; however, there are some limitations to this method. As the established instrument LOD is 0.5-1.0 ppm, identifications below this LOD should be considered unreliable. However, all detections during this assessment were above the instrument LOD. Identification of proteins from MS data includes high-confidence spectra matches generated from sequence-based peptide predictions; the composition of these peptides may be impacted by a number of factors, including persistence of cell culture contaminants or bioprocessing components that could interfere with protein quantification and peptide charge, which can result in unequal sample injection, UHPLC column loading, and peptide ionization. Furthermore, LC-MS sample preparation requires concentration and injection using plastic materials that may preferentially adsorb hydrophobic proteins. Finally, detection of peptide sequences shared between isoforms could result in an underrepresentation of the number of identified proteins. Despite these limitations, the use of DDA methodology overcomes any issues due to coeluting peptides by measuring the top eluting peptides in order of abundance to ensure that all theoretical peptides are detected. Furthermore, stringent bioinformatic settings are imposed to minimize false positives, allowing for a direct calculation of antibody coverage analysis. Overall, the ability of MS to conclusively identify proteins is a significant advantage in comparison to gel-based techniques, which are reliant on subjective identification of the numbers of protein spots. While detailed HCP identification is not currently required by regulatory agencies, it is of great benefit to fully characterize the composition of in-process and drug substances to ensure safety and stability during clinical use.

This coverage assessment is the first step towards determining if the F1060 ELISA is fit-for-purpose for a given bioprocess. This data must be considered as one part of a package that includes the qualification data generated on in-process and drug substance samples in the respective HCP ELISA. This ELISA qualification data should include dilution linearity, accuracy, precision, and the lower limit of quantification (LLOQ). If the coverage assessment demonstrates that the antibody is broadly reactive and the performance in the ELISA is acceptable, only then the ELISA can be deemed fit-for-purpose.

Abbreviations

AAE = Antibody Affinity Extraction

DDA = Data Dependent Acquisition

DS = Drug Substance

ELISA = Enzyme-linked Immunosorbent Assay

FPLC = Fast Protein Liquid Chromatography

HCP = Host Cell Protein

LC-MS = Liquid Chromatography/Mass Spectrometry

LLOQ = Lower Limit of Quantification

LOD = Limit of Detection

MS = Mass Spectrometry

MW = Molecular Weight

pI = Isoelectric Point

ppm = Parts Per Million

UHPLC = Ultra High-Performance Liquid Chromatography System

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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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