Antibody Affinity Extraction (AAE)

A superior alternative to 2D Western blot for determination of polyclonal anti-HCP reactivity

Summary

Antibody Affinity Extraction is a novel method developed by Cygnus Technologies to test reactivity of polyclonal anti-HCP antibodies to individual HCPs that co-purify with biopharmaceutical products. AAE is performed by immobilizing the anti-HCP antibody on a chromatography support, typically Sepharose®. The HCP sample is extracted and eluted multiple times over the column until no additional binding occurs. The eluted fractions are all pooled, buffer exchanged and concentrated back to the original sample volume. Analysis of HCPs bound to the AAE column can be performed by 2D PAGE silver stain comparison of the starting sample and the AAE sample. The spot matching coverage analysis can also be performed by Differential Gel Electrophoresis (2D-DIGE) in which both samples are labeled with differential fluors and loaded onto the same gel. Unlike 2D PAGE fractionation followed by Western blot (2D WB) where sensitivity is limited by loading capacity, AAE sensitivity can be over 100 times higher due to the ability to extract and concentrate large sample volumes. In a typical study comparing AAE to 2D WB, AAE showed coverage to 92% of the individual HCPs compared to 55% for 2D WB. The specificity of AAE is also much better than 2D WB. Non-specific binding to the AAE column is less than 0.1% as compared to 2D WB which will often react with up to 50% of all spots present in 2D PAGEs with non-immune antibodies.

Contents

Introduction

A robust and broadly reactive host cell protein (HCP) ELISA is a critical tool for monitoring purification process consistency as well as final drug substance purity. As such, regulatory agencies around the world have put measures in place to ensure the HCP ELISA used by a sponsor is fit for this purpose. It is advised to employ orthogonal methods to demonstrate antibody coverage to individual HCPs to support that the HCP ELISA is fit-for-purpose. For lack of better methods, 2D WB has been traditionally performed from large format two-dimensional gel electrophoresis (2D PAGE) gels with protein transfer to membranes for 2D WB comparison to silver stain. Due to the sensitivity limitations of 2D WB, coverage can only be estimated on upstream, harvest samples where the concentration of most HCPs are still within the sensitivity limitations of various staining methods. Because of the recognized sensitivity limitations of 2D WB, the conventional acceptance criteria are that >50% of the total HCP should be reactive and that the antibody must recognize HCP in all quadrants of a 2D PAGE gel. Due to the inherent sensitivity limitations of 2D WB, such as (1) loading capacity, (2) destruction of native epitopes by harsh sample treatment, (3) failure to transfer some HCPs out of the gel, (4) HCPs bound to the membrane such that antibody binding is sterically inhibited, (5) difficulties in aligning the dissimilar PAGE gel to a WB membrane images, and (6) poor specificity, 2D WB significantly underestimates true antibody coverage to upstream HCPs. More importantly, 2D WB does not predict how that antibody will quantitatively react to the most important HCPs which are those that co-purify with the drug substance. To overcome the analytical deficiencies of 2D WB, Cygnus developed AAE with the goals of improving the sensitivity and coverage assessment of the total HCP mixture present in cell culture harvest stream, and also to allow for detection and demonstration of reactivity to downstream, process-specific HCPs which are the most important HCPs. The most recently revised regulatory guidelines from the FDA, EMA and USP allow antibody affinity methods like AAE as an acceptable alternative to determine coverage.

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Methods

Electrophoresis

Isoelectric focusing was performed in a 3cm tube gel with carrier ampholines ranging from pI 3–10. The strips were transferred to a large format (20cm) Tris-Glycine gel with a single lane on the basic edge for standards. The gels were then run at 25mAmp/gel for 5 hours. The gels were then either transferred to PVDF membranes for Western blotting, silver stained, or imaged for the 2D-DIGE analysis.

Western Blotting Methods

The blots were stained with Coomassie Brilliant Blue R-250 and desktop scanned. The blots were blocked for two hours in 5% Nonfat Dry Milk (NFDM) in Tween-20 tris buffer saline (TTBS) and rinsed in TTBS. The blots were then incubated in primary antibody overnight then washed 3 × 10 minutes in TTBS. The blots were then placed in secondary antibody for two hours, rinsed in TTBS as above, treated with ECL reagent, and exposed to x-ray film.

2D WB Computerized Comparisons

2D WB films and duplicate silver-stained gels were obtained from the sample and scanned with a laser densitometer. The images were analyzed by automated software and checked manually. The light exposure of the 2D WB films was used to aid in spot matching for the overexposed areas of the dark exposure. Spots detected with the antibody were added to the master spot lists even if not detectable by silver staining.

Antibody Affinity Extraction

The polyclonal anti-HCP antibody is covalently immobilized on a chromatography support (see Figure AAE, top right of the page). The column is then conditioned to prevent significant leaching of the antibody and to greatly minimize any non-specific binding. The HCP sample in its native, undenatured state is passed over the column for binding and then eluted with acid. The HCP sample is again cycled over the column by binding and elution until no additional HCP is bound. All HCP elution fractions are pooled, buffer exchanged, and concentrated back to the original sample volume. The final sample is then separated by 2D SDS-PAGE and analyzed by either a comparison to a silver stain of starting, unextracted sample or by 2D-DIGE using Cy5 labeled extracted sample and Cy3 labeled starting, unextracted sample. In experiments reported below, AAE columns were prepared using Cygnus Technologies 3G goat anti-CHO HCP antibodies as employed in our CHO HCP ELISA Kit (Cat# F550).

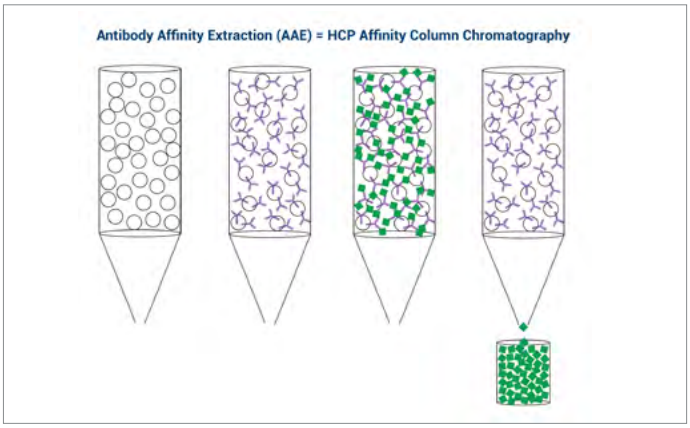

Figure AAE, Antibody Affinity Extraction (AAE).

A control column was prepared using normal goat IgG. The following conditions were tested:

1. CHO HCP passed over the 3G CHO HCP column: Test condition
2. CHO HCP passed over the normal goat IgG column: Non-specific binding control
3. *E. coli* HCP passed over the 3G CHO HCP column: Non-specific binding control

Silver Stain Analysis of Antibody Affinity Extraction

Duplicate gels were performed for the starting CHO HCP sample and the AAE eluted HCP. The initial separation was performed by IEF in tube gels prior to loading on the large format gel. The gels were run at 25mAmp/gel for 5 hours. After washing, the gels were silver stained and analyzed by computer analysis with manual checking.

2D-DIGE

The starting CHO Harvest sample and the CHO AAE eluate were each labeled with Cy3 and with Cy5 respectively. The labeled samples were mixed according to the table below and separated by 2D electrophoresis on large format gels.

Gel ID #	Sample	µl loaded	µg loaded	CyDye
LF 855 #6	CHO Harvest	75	250	Cy3
	CHO AAE Eluate	75	250	Cy5
LF 855 #7	CHO AAE Eluate	75	250	Cy3
	CHO Harvest	75	250	Cy5

Computerized Comparisons

The fluorescent CyDye images were analyzed using Progenesis SameSpots software (version 4.5, 2011, TotalLab, UK) and Progenesis PG240 software (version 2006, TotalLab, UK). The Cy3 and Cy5 images are obtained from the same 2D gel and so are superimposable. Computerized analysis includes image warping, spot outlining, background subtraction (average on boundary), and quantification in conjunction with detailed manual checking. No differences were observed in the reverse labeling configuration (data not included) indicating the proteins were not differentially labeled by Cy3 and Cy5.

Results

Conventional 2D WB Analysis, Figures 1-3

CHO Harvest Material Silver Stain versus CHO Harvest Material 2D WB Detected by 3G Goat Anti-CHO HCP Antibody:

- Figure 1 is a silver stain (LF 845 #1) of the commercial CHO harvest material. A total of 1293 unique spots were detected by either silver stain or WB.
- Figure 2 is the combined 2D WB images (LF 845 #3, 30 seconds, and 3 minutes) of the 2D WB of the commercial CHO harvest material detected by the Cygnus Technologies 3G goat anti-CHO HCP antibody.
- Figure 3 is an overlay of the silver stained gel (LF 845 #1) and the total spots from the combined 2D WB images (LF 845 #3, 30 second and 3 minute exposures). **A total of 717 WB spots were detected for a total coverage of 55%**.

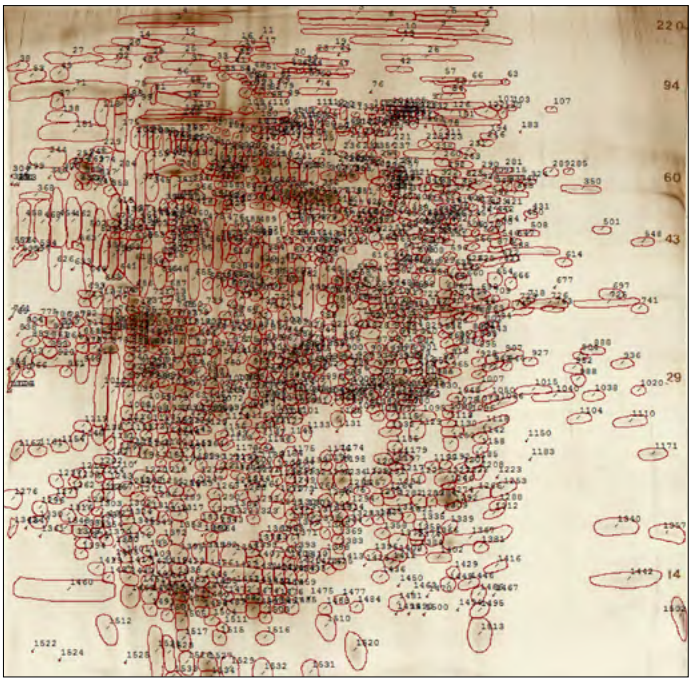

Figure 1. All spot numbering for silver-stained gel image of CHO harvest material (LF 845 #1). Spots detected with the antibody but not detectable by silver staining are indicated with small dots on the silver-stained gel.

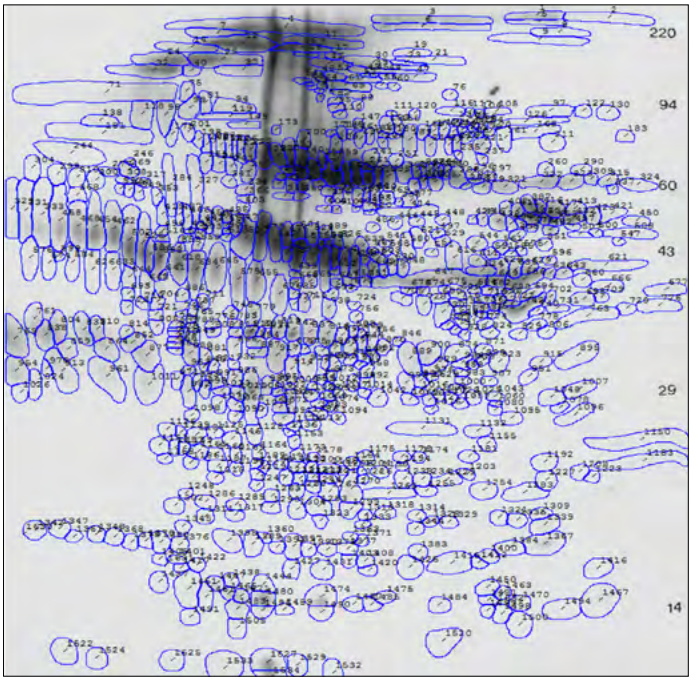

*Figure 2. Light 2D WB film image of goat anti-CHO antibody against CHO Harvest material (LF 845 #3). All spots detected on the dark and light exposures of the 2D WB are outlined in **blue**. The goat anti-CHO antibody detected **717 spots**.*

*Figure 3. Image of CHO Harvest material silver-stained gel (LF 845 #1 and LF 846 #2) showing goat anti-CHO antibody 2D WB (LF 845 #3) matches. Spots present on the silver-stained gel but missing from the 2D WB are outlined in **red**. Spots present in both the silver-stained gel and the 2D WB are outlined in **blue**. Spots detected with the antibody but not detectable by silver staining are indicated with small blue dots on the silver-stained gel and added to the total spot number. The goat anti-CHO antibody detected **717 spots out of 1293 spots** (55%) found. Spot numbering is provided in Figures 1 and 2.*

Non-Specific Binding Control 2D WB, Figures 4-6

CHO Silver Stain versus 2D WB Non-Specifically Detected by Goat Anti-*E. coli* HCP Antibody:

- Figure 4 is a silver stain (LF 845 #4) of CHO HCPs. A total of 1191 spots were detected by silver stain.
- Figure 5 is the combined 2D WB images (LF 845 #7, 3 minute and 15 minute exposures) of the 2D WB of the commercial CHO harvest material detected by the Cygnus Technologies goat anti-*E. coli* HCP antibody.
- Figure 6 is an overlay of the silver stained gel (LF 845 #4) and the total spots from the combined 2D WB images (LF 845 #7, 3 minute and 15 minute exposures). **A total 571 CHO spots were detected by 2D WB with the non-specific *E. coli* antibody for a total non-specific binding of 48% (571/1191)**.

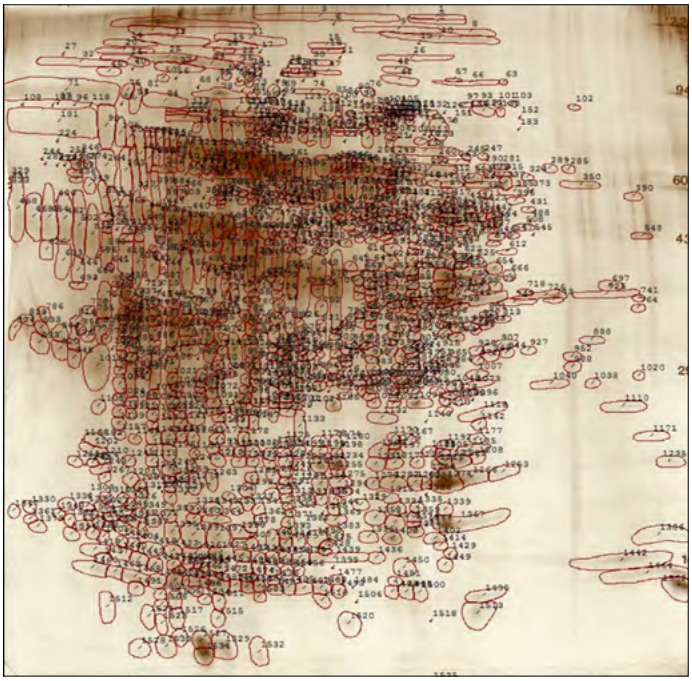

Figure 4. All spot numbering for silver-stained gel image of CHO HCPs (LF 845 #4). Spots detected with the antibody but not detectable by silver staining are indicated with small dots on the silver-stained gel.

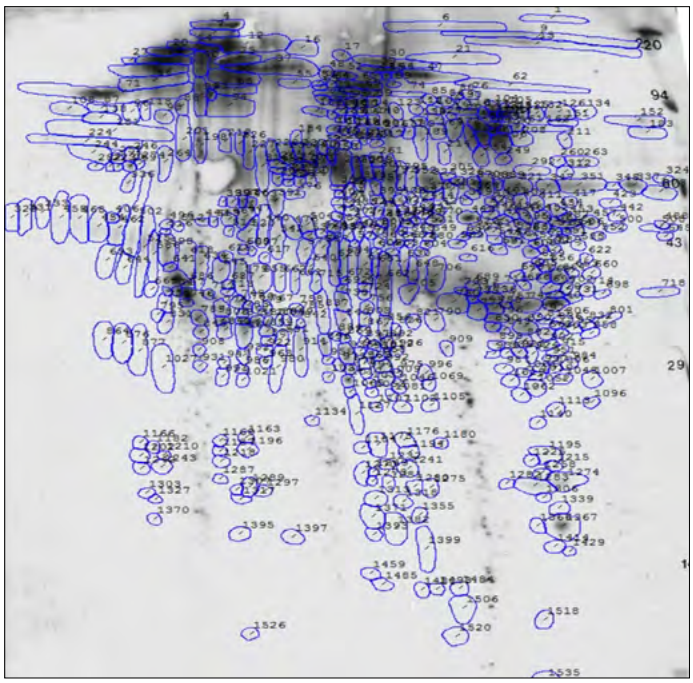

Figure 5. All spot numbering for 2D WB film image of Goat anti- E. coli antibody against affinity-stripped CHO HCP (LF 845 #7).

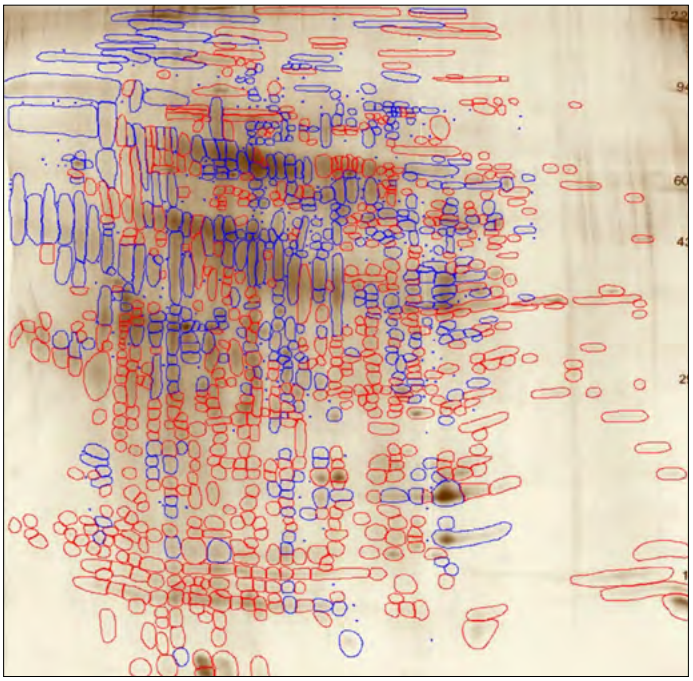

*Figure 6. Image of affinity-stripped CHO HCP silver-stained gel (LF 845 #4-5) showing Goat anti-E. coli antibody 2D WB (LF 845 #7) matches. Spots present on the silver-stained gel but missing from the 2D WB are outlined in **red**. Spots present in both the silver-stained gel and the 2D WB are outlined in **blue**. Spots detected with the antibody but not detectable by silver staining are indicated with small blue dots on the silver-stained gel and added to the total spot number. The Goat anti- E. coli antibody detected **571 spots out of 1191 spots (48%)**.*

Antibody Affinity Extraction with Silver Stain to Silver Stain Analysis, Figures Figure 7-10

Starting CHO Harvest HCP Silver Stain versus CHO Antibody Affinity Stripped HCP:

- Figure 7 is a silver stain (LF 845 #1) of the starting CHO harvest sample. A total of 1138 spots were detected by silver stain alone.
- Figure 8 is a silver stain (LF 845 #4) of antibody affinity stripped CHO HCPs. A total of 998 spots were detected by silver stain.
- Figure 9 is an overlay of the commercial CHO Harvest media silver stained gel (LF 845 #1) and the affinity stripped CHO HCP material. The affinity stripped CHO HCP material had **a total of 827 proteins in common with the harvest material out of a total of 1138 total spots detected for a total coverage of 73%.**
- Figure 10 is a spot numbering for spots present only in CHO Harvest material silver-stained gels (LF 845 #1).

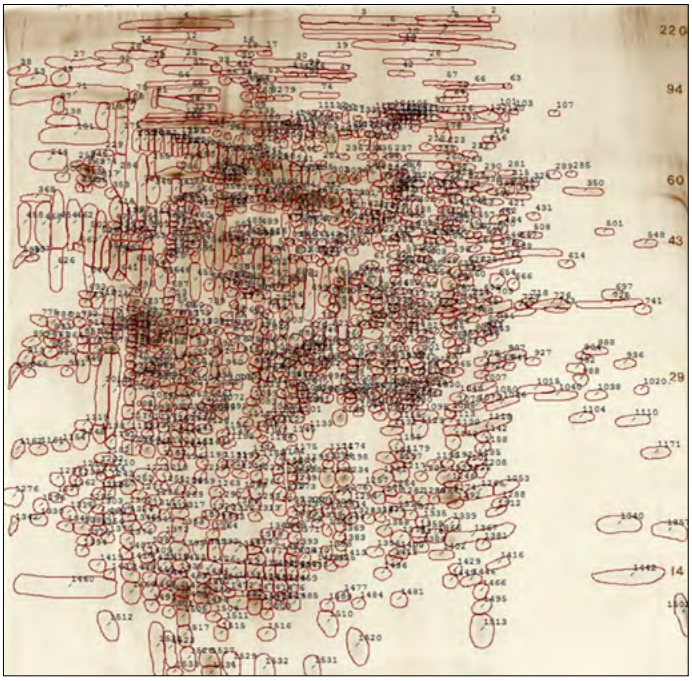

Figure 7. All spot numbering for silver-stained gel image of starting CHO host cell proteins prior to AAE (LF 845 #1).

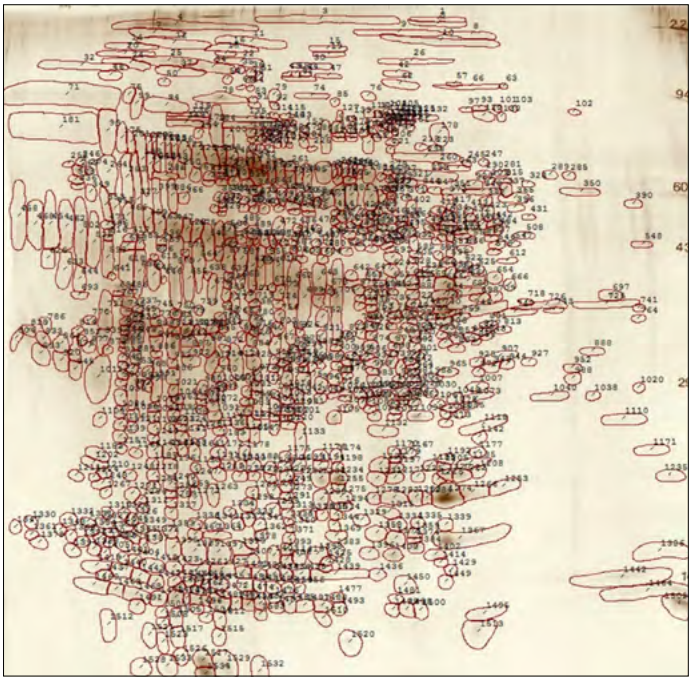

Figure 8. All spot numbering for silver-stained gel image of CHO host cell proteins recovered from AAE (LF 845 #4).

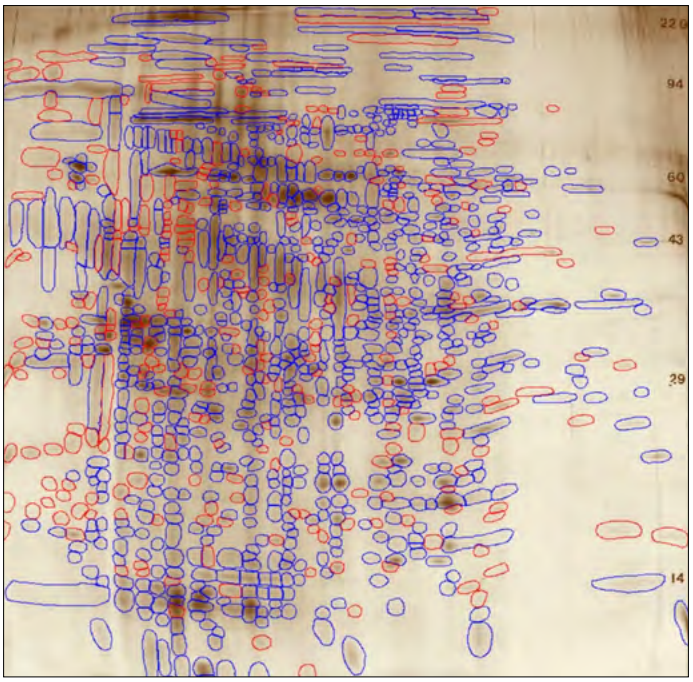

*Figure 9. 2D Gel Difference Image of silver stains for starting CHO HCP (LF 845 #1) and AAE recovered HCP (LF 845 #4). Spots present only in the starting CHO HCP sample are outlined in **red**, while spots common in both samples are outlined in **blue**. A total of 311 spots **(red)** were found to be unique to starting CHO HCP. Spots found in common between both samples totaled 827 **(blue)** out of **1138 total spots (73%)**. Spot numbering is given in Figures 7 and 8.*

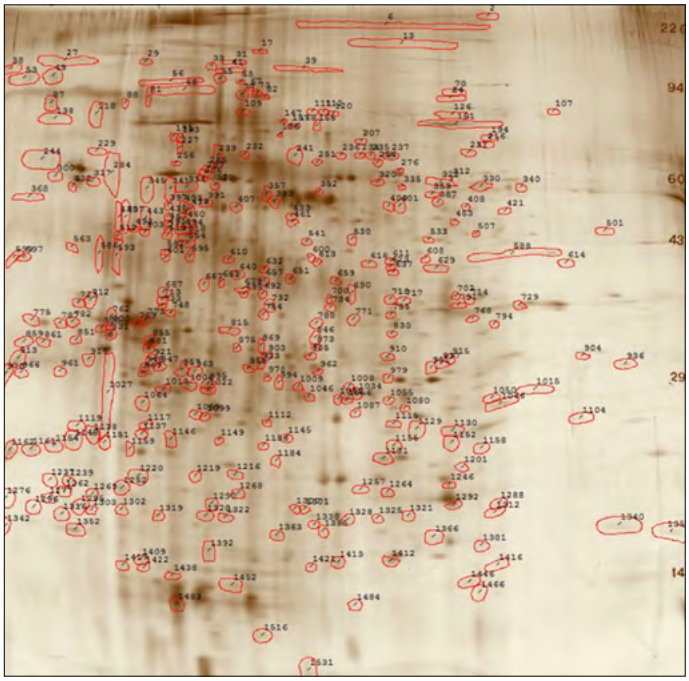

Figure 10: Spot numbering for spots present only in starting CHO HCP Antigen silver-stained gels (LF 845 #1).

Antibody Affinity Extraction with 2D Differential In-Gel Electrophoresis (2D-DIGE) Analysis

- Figure 11 is the Cy3 image (LF855 #6) of the starting CHO HCP. The red circles illustrate the 80 proteins found in the starting material that are not found in the eluate sample. The blue circles illustrate the spots that were found in the starting and eluate samples. **Based on this analysis 896 of the total 976 spots are found in both samples. This translates to a coverage of 92%.**
- Figure 12 is the Cy5 image (LF855 #6) of the CHO HCP eluate.

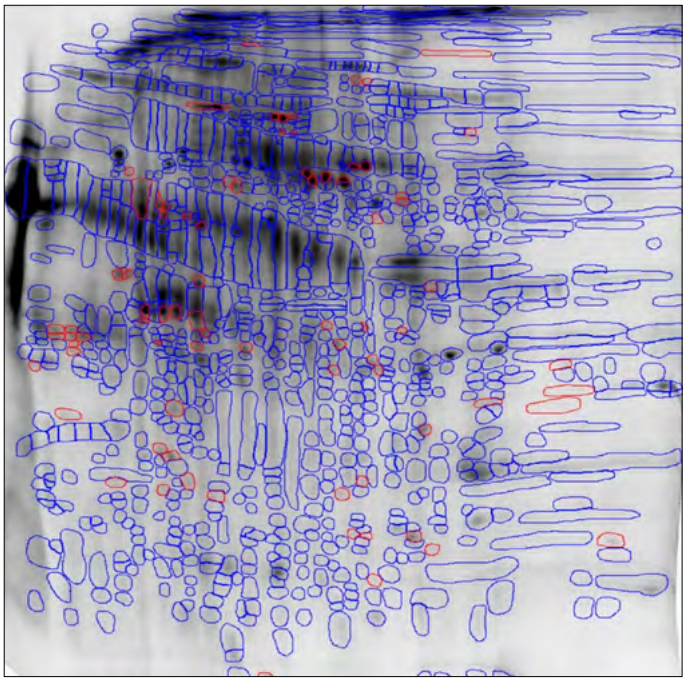

*Figure 11: 2D Image of starting CHO HCP (Cy3) pattern showing differing spots (LF855 #6). Spots present in the starting CHO HCP but missing from the AAE sample are outlined in **red**. Spots present in both samples are outlined in **blue**.*

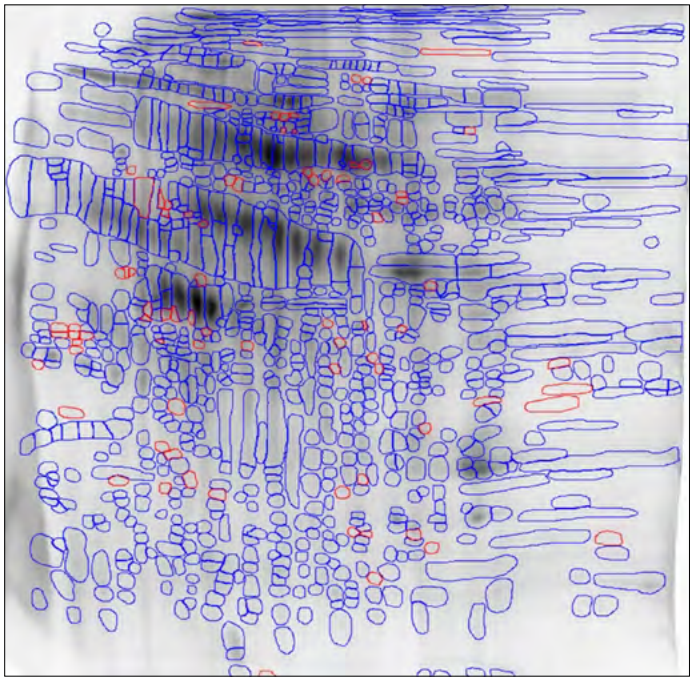

*Figure 12. 2D Image of the eluate CHO HCP (Cy5) pattern for comparison (LF855 #6). Spot outlines are identical to those in Figure 11. Spots unique to starting CHO HCP are outlined in **red**. Spots present in both samples are outlined in **blue**.*

Conclusions

This paper has evaluated three methods to analyze the coverage of polyclonal antibodies to a population of host cell proteins using two-dimensional separation. The first method was to compare duplicate gels by 2D WB and silver stain. This has been the method typically offered to regulatory agencies as part of an HCP ELISA validation or qualification package. Unfortunately, 2D WB correlation to silver stain has many limitations in both sensitivity and specificity and thus is not predictive of the antibody's performance in the ELISA.

The very poor specificity is evidenced by the 48% non-specific coverage of the goat anti-*E. coli* antibodies in the 2D WB analysis performed in Figure 6. *E. coli* HCP was subsequently tested for reactivity in the 3G CHO HCP ELISA from HCP concentrations spanning 200 ng/mL to 1 ng/mL. This testing revealed that there is no cross-reactivity between the 3G CHO HCP ELISA and *E. coli* HCP and thus the WB activity is truly non-specific. This is consistent with our experience that when WBs are designed to maximize apparent coverage to silver stain, the specificity suffers. Furthermore, WB has often been misused to make determinations as to cross-reactivity. Without carefully designed control experiments, WB should not be used in specificity determinations.

Several factors compromise the sensitivity of WB leading to much lower correlations to silver stain and unjustified concerns that the antibody might not be reactive to downstream HCPs. These include destruction of epitopes by heat and chemical treatment, poor transfer out of the gel, poor binding to the membrane, binding to the membrane such that the antibody epitopes are sterically inhibited, the difficulty aligning a

silver stained gel with a 2D WB membrane, and orthogonal differences in sensitivity between silver stain and WB. Cygnus developed the Antibody Affinity Extraction method so that the antibodies are first allowed to bind to HCPs in their natural condition as found in actual drug samples, while also over-coming the technical difficulties and subjectivity of matching silver stain spots from a gel to WB spots on a membrane.

We evaluated two methods for detection of AAE HCPs. **Comparison of the silver stain gel of unextracted, starting HCPs to another silver stain of AAE HCP yielded a 73% correlation.** This is significantly higher than 55% correlation by conventional 2D WB to silver stain comparison. AAE eliminates many of the inherent sensitivity problems of WB thus accounting for the improved coverage. Because there can still be some run-to-run variation and difficulty in accurately aligning two separate images, we have developed the alternative method of 2D-DIGE. The 2D-DIGE analysis allows for the use of a single gel by mixing the samples labeled with different fluors thus further minimizing gel and spot matching issues. **2D-DIGE analysis of AAE HCPs yielded a 92% correlation to CHO HCPs found in the starting material.**

The improved specificity of the AAE methods compared to 2D WB was demonstrated by the fact that a normal goat IgG control column non-specifically bound less than 1% of the starting CHO protein by total protein analysis. As a second demonstration of specificity, 68.4 milligrams of *E. coli* HCP was extracted multiple times using the 3G CHO antibody column. Only 1.7 micrograms of the starting 68.4 milligrams (0.002%) *E. coli* HCP was extracted using the 3G CHO AAE column as quantified by assaying in the Cygnus *E. coli* HCP ELISA. Those same *E. coli* HCPs tested in the 3G CHO ELISA gave no response.

AAE HCPs detected by the silver stain and especially 2D-DIGE demonstrate that the CHO antibody used in the Cygnus 3rd Generation CHO ELISA kit is very broadly reactive to the array of HCPs in this clarified, conditioned media. The predictably lower coverage by 2D WB compared to silver stain clearly demonstrates the superiority of AAE in terms of sensitivity. The poor specifiity of 2D WB as evidenced by 48% non-specific binding to a non-cross-reacting control antibody leads to the conclusion that 2D WB is of no predictive value in determining how the ELISA using that antibody will quantitatively react to the more limited array of HCPs in downstream samples. In addition to the focus on coverage to the entire proteome that in theory might be present in a final drug substance, orthogonal methods should determine reactivity to actual downstream HCPs. Downstream reactivity data along with qualification of the ELISA for accuracy and specificity as evidenced by dilution linearity and spike recovery analysis will determine if an assay is fit for purpose.

While coverage to the total HCP mixture present in a cell culture harvest stream by orthogonal methods has been traditionally requested by regulators, the most significant advantage of AAE is its ability to detect and demonstrate reactivity to individual, downstream HCPs. It is the HCPs that persist through a given purification process that are the most important with respect to patient safety and drug efficacy. By employing AAE analysis, biopharmaceutical companies have a method with sufficient sensitivity to evaluate individual HCPs that persist through various purification schemes. The extraction and concentration of downstream HCP by AAE will also eliminate greater than 99.9% of the drug substance. Removal of drug substance greatly improves the ability to identify individual HCPs by 2D PAGE and Mass Spectrometry analysis. Cygnus is now using AAE as a simple, lower cost liquid chromatography step for 2D-LC-MS/MS analysis of HCPs.

The question of “generic” or “platform” antibodies and assays versus “process-specific” methods has been a theoretical argument based on the assumption that generic and platform antibodies might miss certain process-specific HCPs, and thus a process-specific antibody ought to be superior. With AAE we now have the ability to look at downstream HCPs to determine objectively if a process-specific assay is necessary and superior to a well-developed generic or platform method.

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