Antibody Affinity Extraction (AAE)

A Superior Alternative To 2D Western Blot

SUMMARY:

Antibody Affinity Extraction is a novel method developed at Cygnus Technologies designed to test the reactivity of HCP antibodies in a more natural state than is done with current and traditional methods. AAE is performed by construction of an affinity support composed of the HCP antibodies under study. 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 the AAE can be performed by silver stain comparison of the starting sample and the extracted sample or by 2D-DIGE in which both samples are loaded onto the same gel for analysis. AAE is shown to provide much better sensitivity and specificity than 2D WB. 2D WB correlated to silver stain for an anti-CHO antibody gave only a 55% concordance of spots. AAE correlations were 73% by comparison of silver stain to silver stain and 92% by DIGE labeling of total HCPs with Cy5 and antibody reactive HCP with Cy3. 2D WB showed very poor specificity in that 48% of all silver stain spots showed non-specific WB reactivity to a non-immune antibody. This is contrasted to AAE where non-specific binding was less than 0.1%.

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Introduction:

A robust and broadly reactive host cell protein (HCP) ELISA is critical a component of 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. The first step to demonstrate that an HCP ELISA is "fit for purpose" is to demonstrate that the antisera used in the ELISA is broadly reactive to the array of HCPs that are found in a given process. This generally means that the antibody must react to a majority (<50%) of the total HCP and that the antibody must recognize proteins in all quadrants of a gel. Traditionally the regulatory expectation for the coverage assessment has been a large format 2D Western blot comparison to silver stain. In this method a sample is run on duplicate large format (20cm) gels. One gel is transferred to a membrane detected using the anti-HCP antibodies in a Western blot. The duplicate gel is detected by silver stain. The percent coverage is determined by aligning the 2 images and determining the number spots detected by silver stain that have a corresponding Western blot reactive spot. Unfortunately, due to the severe limitations of 2D Western blot, the assessment of coverage using this method has proven to have very little predictive value in how well a HCP ELISA will perform in real world samples. These limitations include but are not limited to the destruction of conformational epitopes, harsh chemical treatment of the proteins, difficult alignment of a fixed gel and a blot, method sensitivities, poor specificity, etc.. In our experience, these serious limitations may cause a very good antibody to appear to have low coverage, and conversely, a poor antibody may appear to have very good coverage.

Due to these limitations and the poor predictive value of performance in the ELISA, Cygnus has worked to find an alternative method to 2D Western blot analysis to assess coverage of polyclonal antibodies to an HCP population. This paper will look at coverage assessment by traditional Western blot and silver stain comparison as well as with the Antibody Affinity Extraction (AAE). The AAE samples have been analyzed by silver stain/silver stain comparison as well as by DIGE.

Methods:

Electrophoresis

Isoelectric focusing was performed in a 3 cm 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 25mA 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 x 10 minutes in TTBS. The blots were then placed in secondary antibody for two hours, rinsed in TTBS as above, treated with ECL, and exposed to x-ray film.

Western Blot Computerized Comparisons

Western blot 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 western blot 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

Antibody Affinity Extraction is method devised by Cygnus Technologies to overcome the technical challenges and limitations of other orthogonal methods in assessing the coverage of a polyclonal antibody to total host cell protein (HCP). In this method the polyclonal antibody is covalently immobilized on a chromatography support. 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, un-denatured 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 a 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 Differential Gel Electrophoresis (DIGE) using Cy3 and Cy5 to label the extracted and starting, unextracted samples. In this experiment, affinity columns were prepared using Cygnus Technologies 3G goat anti-CHO HCP antibodies. 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 elution fraction. 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 CHO Harvest sample and the CHO AAE Eluate were each labeled with Cy3 and with Cy5. 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	Cy 3
	CHO AAE Eluate	75	250	Cy 5
LF 855 #7	CHO AAE Eluate	75	250	Cy 3
	CHO Harvest	75	250	Cy 5

Computerized Comparisons

The fluorescent Cy-dye images were analyzed using Progenesis Same Spots 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.

Results:

Western Blot Analysis

CHO Harvest Material Silver Stain versus CHO Harvest Material Western Blot Detected by 3G Goat Anti-CHO HCP Antibody - Figures 1-3.

- **Figure 1** is a silver stain (LF 845 #1) of the commercial CHO harvest material. Host cell proteins are found in all quadrants of the gel and span a pI range of 4.0 – 8.4 and a size range of 13.2 kDa – 216 kDa. A total of 1293 spots were detected by either silver stain or WB.
- **Figure 2** is the combined western blot images (LF 845 #3, 30 seconds, and 3 minutes) of the western blot of the commercial CHO harvest material detected by the *Cygnus Technologies* 3G goat anti-CHO HCP antibody.

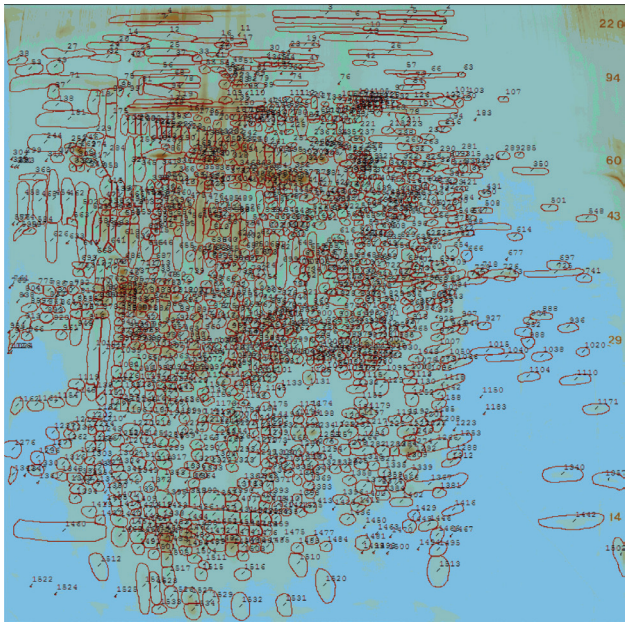

Figure 1: All spot numbering for silver-stained gel image of CHO harvest material (LF 845 #1). Spots detected with the antibody but not detectable by silver staining are indicated with small dots on the silver-stained gel.

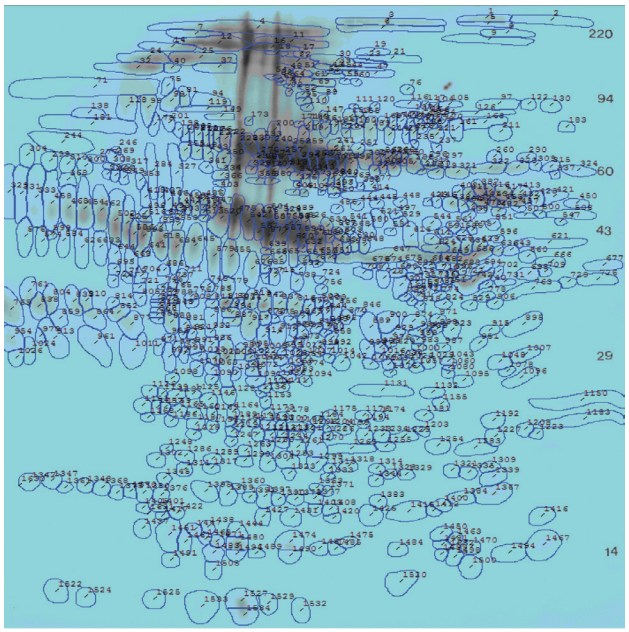

Figure 2: Light western blot film image of Goat anti-CHO antibody against CHO Harvest Material (LF 845 #3, 3 minute exposure). All spots detected on the dark and light exposures of the western blot are outlined in blue. The Goat anti-CHO antibody detected **717 spots**.

- **Figure 3** is an overlay of the silver stained gel (LF 845 #1) and the total spots from the combined Western blot images (LF 845 #3, 30 second and 3 minute exposures). **A total of 717 spots (1293 total spots detected) were detected by western blot for a total coverage of 55%.**

Non-specific binding control, CHO Harvest Material Silver Stain versus Western Blot using Goat Anti-*E.coli* HCP Antibody - Figures 4-6.

- **Figure** 4 is a silver stain (LF 845 #4) of AAE eluate. A total of 1191 spots were detected by either silver stain or WB.
- **Figure 5** is the combined western blot images (LF 845 #7, 3 minute and 15 minute exposures) of the western blot 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 Western blot images (LF 845 #7, 3 minute and 15 minute exposures). **A total of 571 (1191 total spots detected) spots were detected by western blot for a total coverage of 48%.**

Antibody Affinity Extraction with Silver Stain to Silver Stain Analysis

CHO Harvest Material Silver Stain versus Affinity Stripped CHO HCP - Figures 7-10

- **Figure 7** is a silver stain (LF 845 #1) of the commercial CHO harvest material. A total of 1138 spots were detected by silver stain alone.
- **Figure 8** is a silver stain (LF 845 #4-5) of the affinity stripped CHO material. 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%.**

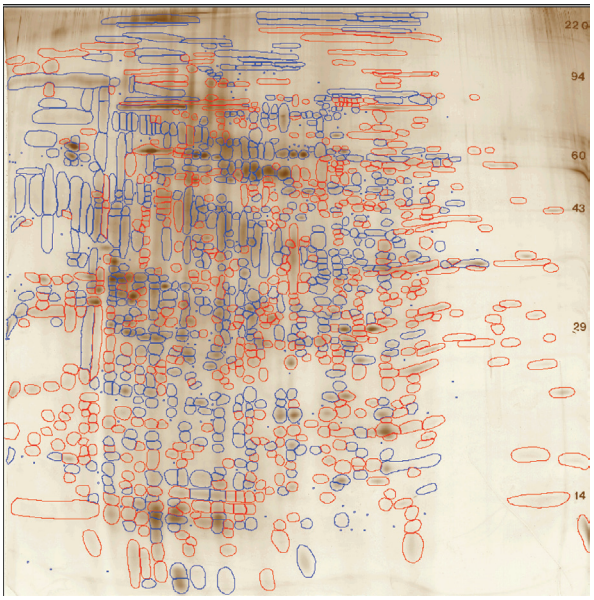

Figure 3: Image of CHO Harvest Material silver-stained gel (LF 845 #1 and LF 846 #2) showing goat anti-CHO antibody western blot (LF 845 #3) matches. Spots present on the silver-stained gel but missing from the western blot are outlined in **red**. Spots present in both the silver-stained gel and the western blot 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**.

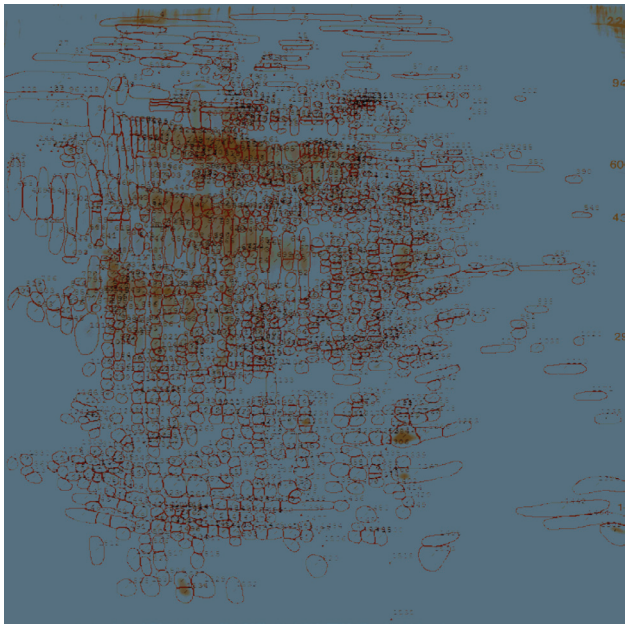

Figure 4: All spot numbering for silver-stained gel image of CHO HCP (LF 845 #4). Spots detected with the antibody but not detectable by silver staining are indicated with small dots on the silver-stained gel.

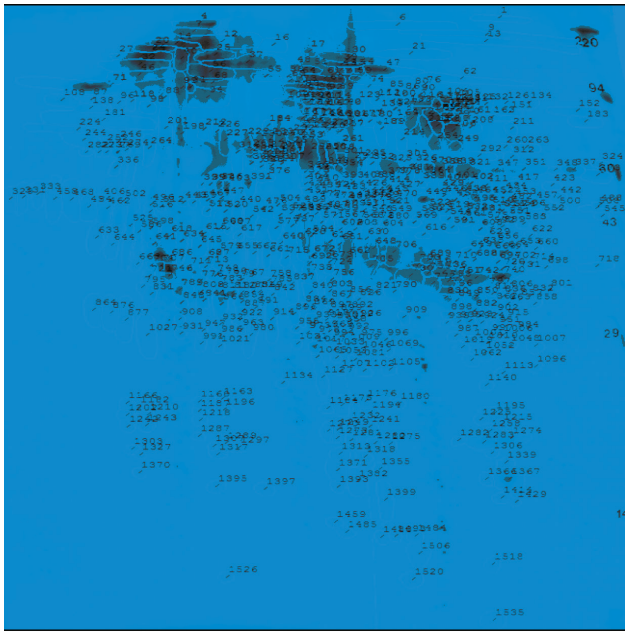

Figure 5: All spot numbering for western blot film image of anti- *E. coli* antibody against Affinity Stripped CHO HCP (LF 845 #7, 3 minute exposure).

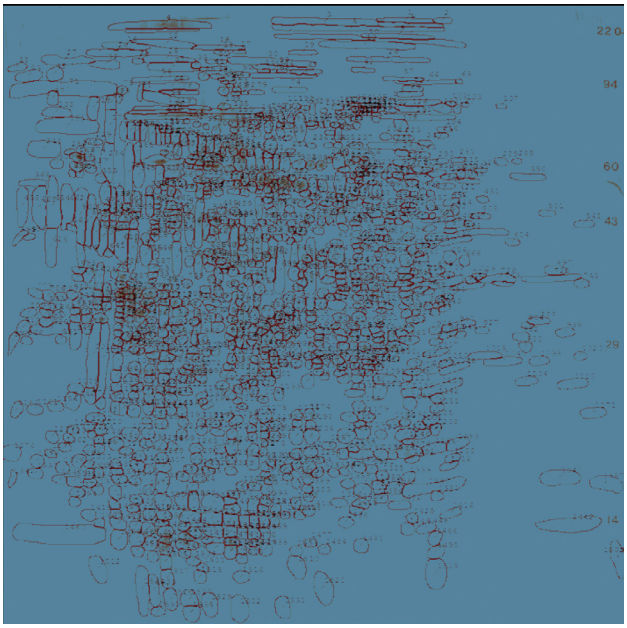

Figure 7: All spot numbering for silver-stained gel image of the starting CHO HCP prior to AAE (LF 845 #1).

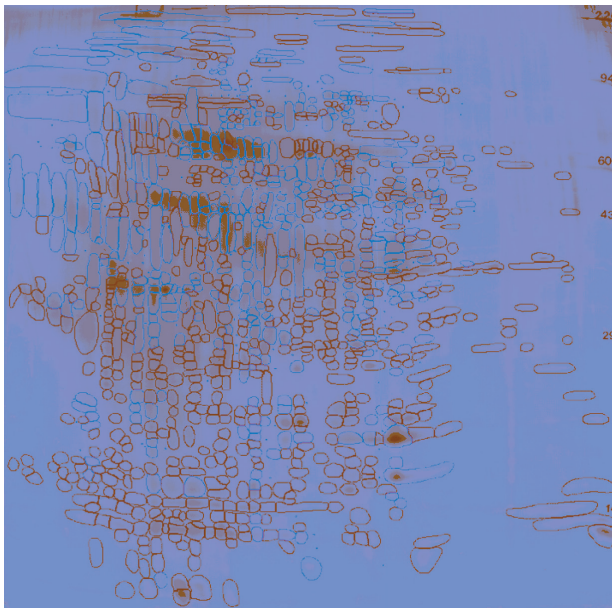

Figure 6: Image of Affinity stripped CHO HCP silver-stained gel (LF 845 #4-5) showing Goat anti-*E. coli* antibody western blot (LF 845 #7) matches. Spots present on the silver-stained gel but missing from the western blot are outlined in **red**. Spots present in both the silver-stained gel and the western blot 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%) found.

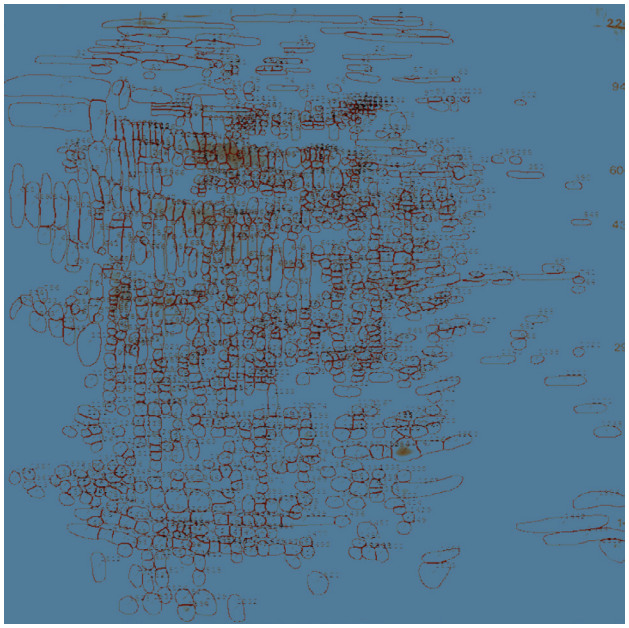

Figure 8: All spot numbering for silver-stained gel image of CHO HCP recovered from AAE (LF 845 #4).

- **Figure 10** is a Spot numbering for spots present only in CHO Harvest material silver-stained gels (LF 845 #1).

Antibody Affinity Extraction with 2D Differential In-Gel Electrophoresis 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

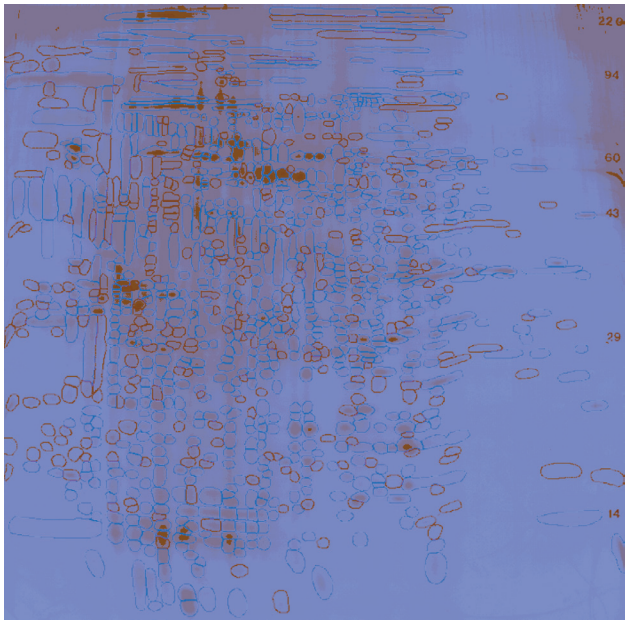

Figure 9: 2D Gel Difference Image of starting HCP and AAE recovered HCP by overlay of silver stains (LF845 #1 & #4) versus silver-stained gel (LF 845 #1). Spots present only in starting HCP are outlined in **red**, while spots common in both samples are outlined in **blue**. A total of 311 spots (**red**) were found to be unique to the starting HCP sample. 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**.

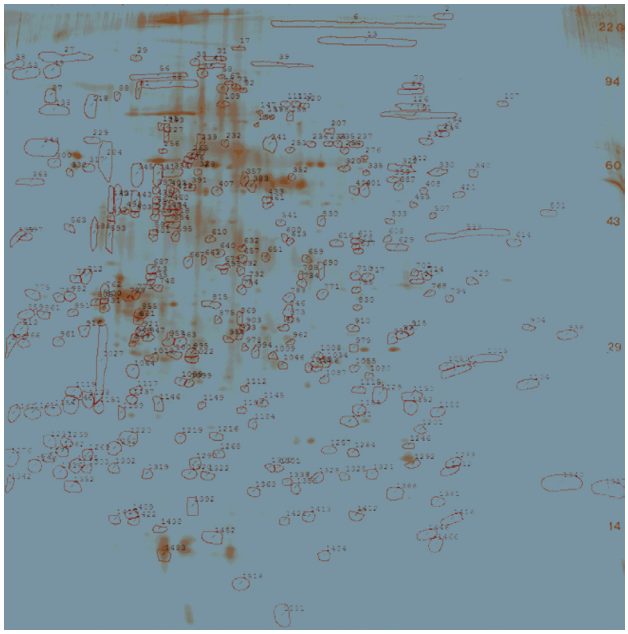

Figure 10: Spot numbering for spots present only in starting CHO HCP Antigen silver-stained gel (LF 845 #1).

Conclusions:

This paper has evaluated 3 methods to analyze the coverage of polyclonal antibodies to a population of host cell protein using 2-dimensional separation. The first method was to compare duplicate gels by Western blot and silver stain. This has been the method typically offered to and expected by 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 Western blot analysis as shown in **Figure 6**. *E.coli* HCP was subsequently tested for reactivity in the 3G CHO HCP ELISA from HCP concentrations spanning 200ng/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 the specificity

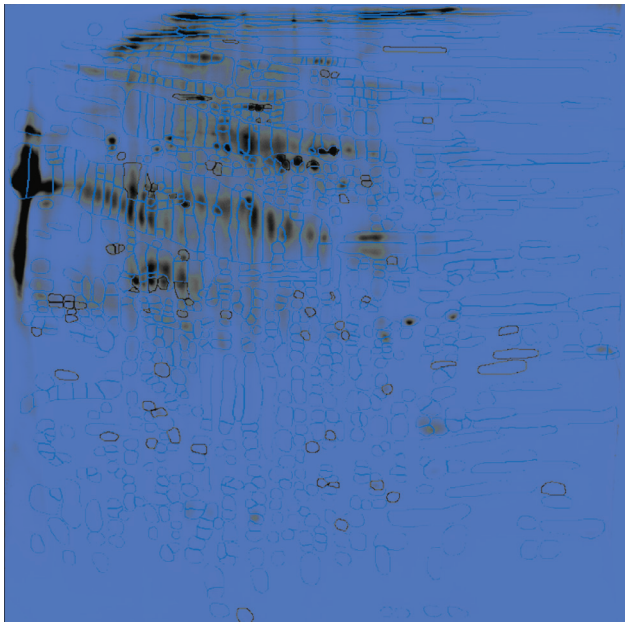

Figure 11: 2D Image of starting CHO HCP (Cy3) pattern showing differing spots (LF855 #6). Spots present in the starting CHO HCP but missing from the eluate sample are outlined in **red**. Spots present in both samples are outlined in **blue**.

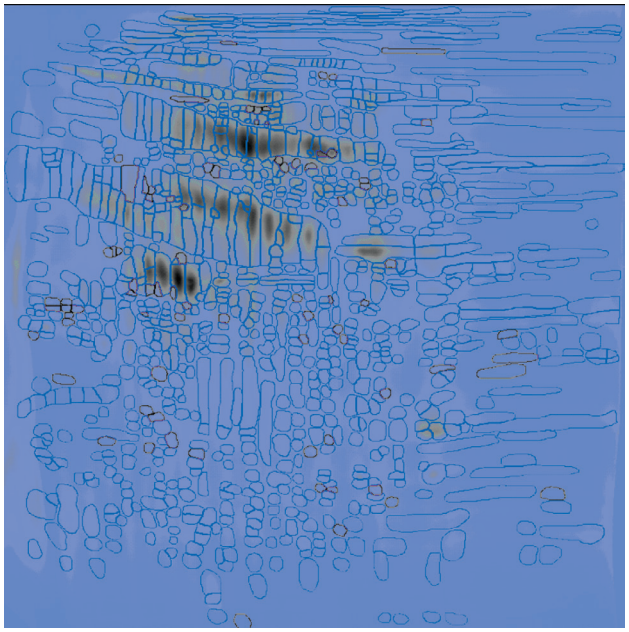

Figure 12: 2D Image of the AAE eluate CHO HCP (Cy5) pattern for comparison (LF855 #6). Spot outlines are identical to those in **Figure 1**. Spots unique to the starting HCP sample are outlined in **red**. Spots present in both samples are outlined in **blue**.

suffers. Indeed we see WB often misused to make determinations as to cross reactivity. Without carefully designed control experiments WB should not be used in specificity experiments.

Several factors compromise the sensitivity of WB leading to much lower correlations 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 Western blot 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 overcoming 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 un-extracted total HCPs to another silver stain of AAE HCP yielded a 73% correlation, versus only 55% by conventional 2D WB. AAE eliminates the problems of epitope destruction and chemical treatment and the lack of specificity for WB, accounting for the much improved coverage. Because there can still be some run-to-run variation and difficulty in accurately aligning the images, we developed an alternative method of DIGE. This allows for the use of single gel by mixing the samples labeled with different fluors and thus further minimizing gel and spot matching issues. This more direct method showed that 92% of the CHO HCP found in the starting material were also found in the AAE eluate.

The much improved specificity of the AAE methods compared to 2D WB was shown by the fact that a normal goat IgG control column removed less than 1% of the starting protein by total protein analysis. As yet another verification of specificity, 68.4 milligrams of *E.coli* HCP

Conclusions (cont.)

was extracted using the 3G CHO AAE column. Only 1.7ug was nonspecifically bound (~0.002%) as detected by the Cygnus *E.coli* HCP ELISA.

AAE HCPs detected by either silver stain or DIGE demonstrate that the 3G CHO is a very broadly reactive antibody for the array of HCPs in this clarified, conditioned media. The predictably lower coverage by 2D WB comparison to silver stain clearly demonstrates the superiority of AAE in terms of sensitivity. Combining the poorer sensitivity of 2D WB with its even poorer specificity 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 react to the more limited array of HCPs in downstream samples. Based on these findings we recommend performing the AAE followed by DIGE or silver stain to silver stain detection as a much more sensitive and specific method of determining coverage of an HCP antibody to upstream HCPs. Provided the antibody is shown to have very broad reactivity to upstream HCPs, the antibody affinity column can also be used to extract downstream HCPs while at the same time removing the drug substance itself. This very effective sample enrichment and purification step will facilitate the identification of individual HCPs that persist through a given purification process by methods such as Mass Spectrometry.