

Antibody Affinity Extraction (AAE)

A Superior Alternative to 2D Western Blot in Establishing HCP Antibody Reactivity

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Introduction

A robust and broadly reactive host cell protein (HCP) ELISA is a critical 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 silver-stained. The percent coverage is determined by aligning the 2 images and determining the number of 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. Cygnus has developed an alternative method to 2D Western blot analysis to assess coverage of polyclonal antibodies to an HCP population – the Antibody Affinity Extraction (AAE) method.

Additionally, we are using AAE to assess reactivity to HCPs that persist through the purification process. This was not a possibility using Western blot due to the high concentration of product protein limiting the amount of HCPs that could be loaded. AAE removes the majority of the product protein and enriches HCP to levels detectable by silver stain.

Objectives

- 1) To illustrate the superiority of AAE to traditional 2D Western blot
- 2) To demonstrate the ability of AAE to assess the reactivity to HCPs that persist through the purification process

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, 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 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.

Methods and Materials

This study focuses on the Analysis of two samples. The first is a null harvest CHO sample we evaluated for coverage analysis by 2D Western blot and by AAE. The second sample is a CHO derived Protein A eluate containing human IgG for AAE analysis of reactivity in product containing samples.

Coverage Analysis:

- 1) Western blot of null CHO HCCF probed with goat anti-CHO HCP compared to silver stain of the same material.
- 2) Western blot of null CHO HCCF probed with goat anti-*E. coli* HCP compared to silver stain of the same material.
- 3) AAE analysis of the starting material compared to the AAE elution fraction by silver stain.
- 4) AAE analysis of the starting material compared to the AAE elution fraction by 2D-DIGE.

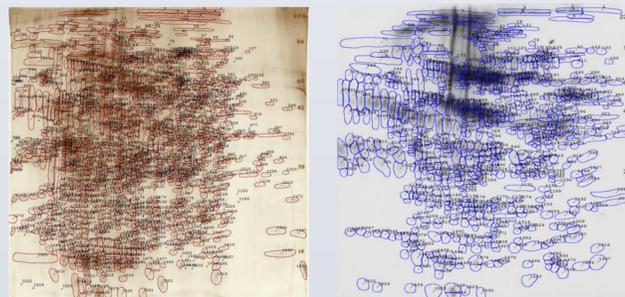
Reactivity to HCPs in Downstream Samples:

- 1) Protein A elution fraction compared to AAE eluate of the Protein A elution fraction

Results

Coverage Analysis

Western blot #1

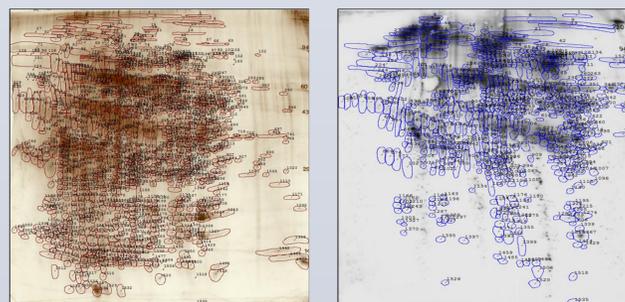


Silver stain #1

Western blot #1

The goat anti-CHO HCP antibodies reacted to 717 of the 1293 total spots for a total coverage of 55%.

Western blot #2

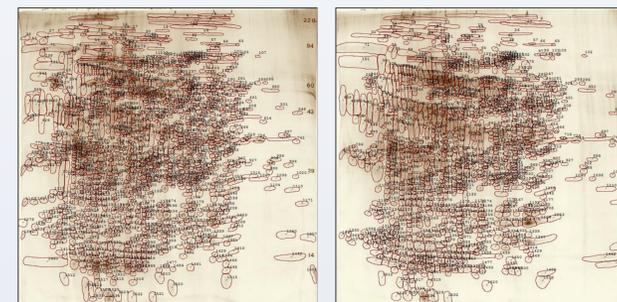


Silver stain #1

Western blot #2

The goat anti-*E. coli* HCP antibodies reacted to 571 of the 1191 total spots for a total coverage of 48%.

Antibody Affinity Extraction (Silver Stain Detection)

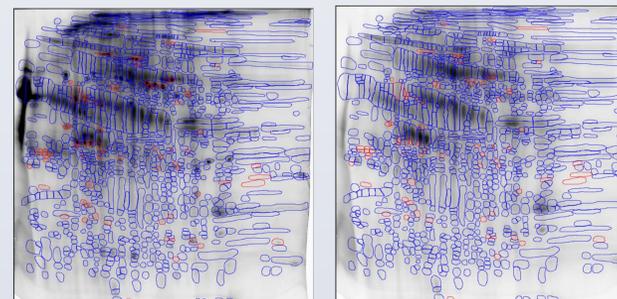


Null Harvest

AAE Eluate Fraction

The goat anti-CHO HCP antibodies reacted to 827 of the 1138 total spots for a total coverage of 73%.

Antibody Affinity Extraction (2D-DIGE Detection)

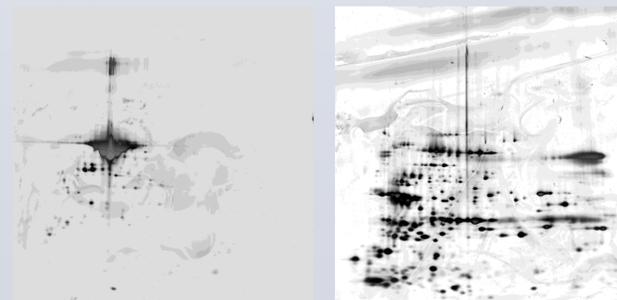


Null Harvest Labeled with Cy3

AAE Eluate Fraction Labeled with Cy5

896 of the 976 total spots were located in the AAE Eluate Fraction for a total coverage of 92%

Reactivity to HCPs in Downstream Samples



In-Process Sample Silver Stain Detection

In-Process Sample Post AAE Silver Stain Detection

This in-process sample initially had 114 spots thought to be HCPs. After AAE to deplete the product and enrich HCP 411 spots were resolved. This experiment demonstrates the power of AAE to analyze HCPs in downstream samples. In addition to visualizing the reactivity to the overall HCP population, it can also serve as a sample preparation tool for identification of individual HCPs by mass spectrometry.

Conclusions

We evaluated three 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 suffers. Indeed, we see WB often misused to make determinations as to cross-reactivity.

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 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.

(1) Comparison of the silver stain gel of un-extracted total HCPs to another silver stain of AAE HCP yielded a 73% correlation. This method eliminates the problems of epitope destruction and chemical treatment and the lack of specificity for WB.

(2) The alternative 2D-DIGE method allows for the use of single gel by mixing the samples labeled with different fluorors 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 this method 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 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. When one combines the poorer sensitivity of 2D WB with its even poorer specificity as evidenced by 48% non-specific binding (NSB) to a non-cross reacting control antibody versus less than 1% NSB by AAE, it leads to the conclusion that 2D WB is of little 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 reliable and relevant method of determining coverage of an HCP antibody to upstream HCPs. If 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 DS itself. **AAE 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.**

Moving forward Cygnus is working to develop 3-channel 2D-DIGE to more accurately identify and remove product related spots from the analysis.