

A Comprehensive Strategy for Host Cell Protein Analysis in Biopharmaceuticals



Current methods of HCP determination have proven to be useful tools to improve purification processes and to monitor HCP removal lot-to-lot. While low levels of HCP have rarely shown significant impact on drug efficacy or patient safety, theoretical concerns remain that drive us to consider better analytical methods. New and improved methods are being applied many of which can detect and identify individual HCPs. It is the purpose of this article to review all the methods that can be applied to HCP analysis and to propose how they can be used together in a comprehensive strategy to better assure process consistency and patient safety.

ELISA and related immunoassay methodologies is the most important tool in optimizing the purification process, monitoring that process, and providing a lot release test for licensed drugs. ELISA has excellent sensitivity and specificity. ELISA is robust, high throughput, rapid, low cost and relatively easy to perform while also being amenable to automation. ELISA is not without limitations. ELISA is only as good as the antibody and the expertise of the developer. Selection of the most appropriate HCP antigen to generate the antibody, the methods to enhance the host animal's response and then the purification and characterization of that antibody are very complex methodologies. Furthermore, when applied to the simultaneous detection of up to hundreds of individual HCPs, ELISA is at best a semi-quantitative method that can systematically under-estimate or over-estimate HCP content in final drug substance. For these reasons the industry has utilized orthogonal methods to detect HCPs and to determine the reactivity of antibodies used in the ELISA release test. HCP analysis can be divided into six analytical methods discussed in detail below:

1. **2 Dimensional PAGE analysis**
2. **2Dimensional Western blot (2D WB) or Antibody Affinity Extraction (AAE)**
3. **HPLC**
4. **ELISA**
5. **Mass Spectrometry (MS) and related methods to identify individual HCPs**
6. **Serological analysis of patient immune response to HCPs**

Written by: **Ken Hoffman** Cygnus Technologies, Inc.

2D PAGE analysis provides for very good fractionation and resolution of complex samples such as harvest material that can contain up to thousands of proteins. The detection methods of silver stain, coomassie stain or fluorescent labeling can yield sensitivities as low as 1 ng for some individual proteins. However, as you look downstream in the process the reduced levels of individual HCP in well purified products fall below the sensitivity limits of PAGE. The other major limitation is that PAGE is not specific in discriminating HCPs from product heterogeneity and other non-HCP contaminants. Despite these limitations 2D PAGE remains a valuable orthogonal method when used together with the methods discussed below.

2D Western blot has traditionally been used as an orthogonal method to assess coverage of the antibody used in the ELISA. Of the six analytical methods listed above, 2D WB is the least useful and one that should be replaced by methods more sensitive and specific. For a critical discussion on the limits of 2D WB analysis when applied to HCPs detection, the reader is referred to our Web Site www.cygnustechnologies.com under the heading of "Publications". The most significant shortcoming of 2D WB is that it lacks the sensitivity and specificity to be of any real predictive value in determining how the ELISA using that antibody will quantitatively react to the more limited array of HCPs in final drug substance. The poor sensitivity and specificity coupled with the fact that the 2D WB data is correlated to 2D silver stain which has different limitations in sensitivity and specificity, means that a % coverage calculation is rationally and statistically meaningless. Compounding this problem further, our review of several peer reviewed papers shows that assay developers driven by the naïve belief that a high correlation to silver stain for upstream HCPs ought to insure the ELISA validity, have made inappropriate choices in antigen and antibody selection and interpretation of ELISA results that cause significant underestimations of real downstream HCPs.

A sensitive and specific method capable of detecting antibody reactivity to individual to real, process-derived HCPs would be important in qualifying and characterizing the ELISA antibody. 2D WB is not that method. We have developed a new method termed Antibody

Affinity Extraction (AAE). A description of the AAE method and data showing the advantages in both sensitivity and specificity can be found in a technical paper on our Web Site www.cygnustechnologies.com under the heading of “Publications”. One of the other advantages of AAE is that it can be used to extract and identify downstream, real process-derived HCPs.

HPLC data can be used analogous to 2D PAGE. While HPLC has similar sensitivity and specificity limitations it has one significant advantage over 2D PAGE in that the fractionation of HCPs is done under conditions that do not denature or otherwise alter the native structure of the HCP. Because of this HPLC fractionated proteins can be tested directly in the ELISA to determine if the antibody is reactive. We have developed a method we call 2D HPLC/ELISA (see the technical paper on our Web Site www.cygnustechnologies.com under the heading of “Publications”). While the 2D HPLC fractionation is not as good in resolving upstream individual HCPs as 2D PAGE, it is well suited to fractionating and identifying the more limited array of HCPs in downstream samples.

ELISA to include related “sandwich” assay formats is used as the release test in almost all approved biopharmaceuticals. Despite ELISA’s proven utility there are theoretical concerns that dictate the use of orthogonal methods to comprehensively validate the ELISA to insure it is not missing certain HCPs. While there are fundamental limitations to ELISA, our observation as evidenced by some published articles on HCP is that many ELISAs are not being well-developed to their full potential. Several naïve myths and mistakes continue to be perpetuated when it comes to HCP analysis. These mistakes discussed below are often a greater source of error than are the fundamental limitations of the method. By understanding and eliminating common mistakes the industry should be able to develop even more accurate and specific ELISAs.

Mistake #1 – Use of 2D WB studies to establish ELISA validity.

As discussed above, 2D WB can be misleading. Holding to the notion that 2D WB is critical to acceptance of the antibody can result in ELISA development decisions that cause a systematic under detection of downstream, process-derived HCPs. 2D WB should be replaced by superior methods such as the AAE that have sufficient sensitivity and specificity to be of predictive value for the ELISA.

Mistake #2 – Selection of antigen to generate the antibody that is not process specific - We see many developers making the critical mistake of choosing an inappropriate or less than ideal source of antigen to generate the anti-HCP antibody. For example, when the drug product is secreted into the media the obvious choice for antigen is clarified conditioned media yet some developers will make the decision to add non-process specific antigens to the immunogen such as lysate or cell debris. Their naïve intention by increasing the number of HCPs is to make an even more broadly antibody. This misguided motivation is based on the myth that a broadly reactive 2D WB assures the antibody will make a good downstream assay. Certainly cell debris makes a potent immunogen as do lysates that often contain the most abundant structural components of the cell. Because they are very immunogenic and represent the majority of the limited amount of protein that can be loaded into the 2D method, these sources of HCP will typically yield a good 2D WB to silver correlation. However, many of those components are not a significant source of contamination in actual product harvest material. Our response to such an inappropriate strategy is “how can one call an antibody process-specific when it uses antigens that are not process specific?” If one has a therapeutic antibody that is secreted into media one does not first clarify the media and then add back lysate or debris prior to passing it over the Protein A column. Similarly, “cascade immunization” schemes where one modifies the natural mixture of antigens and boosts animals with low concentration, low molecular weight or poorly immunogenic proteins can be contrary to obtaining an antibody response that is truly process specific. If the enrichment in titer of antibody to low concentration and poorly immunogenic HCP is accomplished in a way that reduces titer to the more abundant HCPs, then the resulting blended antibody while broadly reactive on 2D WB may not be sufficiently process specific. The proponents of these strategies fail to consider the fundamental differences between WB & ELISA. The question we get back is what harm is there in adding irrelevant HCPs to the antigen mixture? The harm is that such an antibody when used in an ELISA format can cause a significant under-estimation of true process-derived HCP. The discussion in Mistake # 3 below explains the mechanism causing this under-estimation.

Mistake # 3 –Failure to understand the “stoichiometry of ELISA” - This is the most fundamental naiveté by many who develop and validate HCPs assays. The greatest source of ELISA error is not the failure to make antibody to every upstream HCP but rather the lack of excess antibody to certain downstream HCPs. The above referenced technical paper on Limitations of 2D WB gives an overview of the importance of antibody excess for ELISA to be quantitative. In addition, our web site under Technical Support/FAQs provides an in depth discussion of the manifestations of lack of antibody excess under the topics of “poor dilutional linearity” and “hook affect”. To summarize those discussions, ELISA is only quantitative when it has an excess of both capture and detector antibody for the antigen being detected. In this case the concentration response curve will be positively sloped (signal increasing with increasing analyte concentration). When the antigen is in excess the response curve but will either be flat or at very high concentrations, will become negatively sloped in a phenomenon called “high dose hook affect”. This phenomenon is even more complex in HCP assays where some HCPs will be less than the concentration of antibody while others may exceed the amount of antibody. The concentration response curve for an HCP ELISA is the cumulative response for all HCPs present. Any ELISA and especially HCP ELISA, is practically limited in the amount of antibody that can be utilized. Optimal assay development dictates that we only use relevant antibody to occupy the limited capture sites. In order to get low background signal necessary for optimal sensitivity it is best to remove irrelevant antibody in the assay. Use of antibodies that have activity to HCPs that are not process specific or failure to affinity purify the antibody to remove irrelevant IgG can cause a systematic under-estimation of downstream HCPs. Antibody excess can be determined by performing dilutional linearity studies through the entire analytical range of the assay as discussed in Mistake # 4 below.

Mistake # 4 –Failure to perform complete dilutional linearity studies and to correctly interpret that data - Most labs perform at least some dilution linearity analysis as part of assay qualification. The data is analyzed against an arbitrary Pass/Fail specification. There can be more value and qualitative information in dilutional linearity data than is appreciated by most HCP analysts. Within the typical Pass/Fail specification is qualitative information that is often not being considered or correctly interpreted. Dilutional linearity allows us to distinguish sample matrix interference from lack of antibody excess. In addition, we have seen many reports of alleged cross reactivity of an ELISA to the drug substance that were in fact not

cross reactivity but rather true HCPs . These erroneous conclusions as to cross reactivity typically result when a sensitive ELISA was not used to develop the purification process or when the assay qualification efforts did not include or correctly interpret dilutional linearity data. Our web site under the title of FAQs has a technical article discussing cross reactivity. Be advised WB is not the method to determine if there is cross reactivity and how that cross reactivity might manifest in the ELISA. By performing a full dilutional linearity analysis of your drug substance it should be possible to discriminate cross reactivity from true HCPs. While ELISA does not identify each individual HCP, informed analysis of dilutional linearity data can often detect the presence of a very limited array of HCPs that have some affinity for either the drug product or the purification process and thus are in much higher concentration than the larger array of upstream HCPs that are systematically cleared through the purification process. Analysis of dilutional linearity data provides guidance for implementation of other orthogonal methods such as HPLC and MS that in turn may guide modification of the ELISA.

Mass Spectrometry & related Methods –

We continue to identify individual HCPs in final drug substance samples particularly in CHO derived products. Some of these proteins begin as highly expressed HCPs in the harvest material or they have affinity for the drug substance or otherwise are selected along with the product in the purification process. While the ELISA may not accurately quantitate these due to lack of antibody excess, dilutional linearity analysis will often indicate their presence and alert us to potential under-quantitation. Once we have an indication of a problematic individual HCP, MS can be used to identify it and determine its structure and function. The major factor limiting the application of MS in the detection of downstream HCPs is sensitivity and specificity. In most well purified products HCP content will be composed of numerous individual HCPs that in total are less than 100ppm. MS discrimination of the drug substances at mg/mL from individual HCP at low ppm levels is problematic. Sample extraction steps to remove the bulk of the drug while concentrating HCP would be useful. The AAE and 2D HPLC methods discussed above can serve as the sample preparation methods for analysis of downstream HCPs by MS. Integration of the information provided by MS, ELISA, HPLC, and AAE should facilitate optimization of the purification process to address individual, major contaminants. In cases where it is not practical to reduce these HCPs further, then specific ELISAs to individual HCPs could be developed and used along with the total HCP ELISA. Alternatively, the data

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provided by MS may guide cell biologists to genetically re-engineer the expression system to eliminate or minimize a problematic HCP.

Serological analysis of patient immune response to HCPs

has been used to determine if residual HCPs represent safety or efficacy problems. Retrospective analysis of patient immune response could provide additional information to make further process improvements. At the very least such patient monitoring can be used as orthogonal evidence that the process as guided by other 5 analytical methods has reduced HCP to inconsequential levels. Samples collected for postmarket launch monitoring of patient anti-drug antibodies could be used to detect anti-HCP antibodies.

Integrated Approach to HCP Analytics

Understanding the strengths and limitations of the various analytical methods allows for the comprehensive detection and removal of HCP. Because regulatory guidelines are slow in coming, slow to change, and somewhat different from country to country, biopharmaceutical companies should seek the opinions and agreement of the agencies where they file for drug licenses. If your method differs somewhat from conventional methods but you can demonstrate its superiority you should be able to persuade the agency of its advantages.

The **Diagram A** outlines a logical approach based on the methods available, their strengths and limitations. Subject to proper generation and a full characterization of the antibody followed by a comprehensive qualification/validation of the assay supported by the various orthogonal methods, ELISA due to its ease of use, robustness, relatively low cost, high sensitivity, and specificity can remain as the release test.

The process begins with the rational choice of antigen for generating the antibody. To be termed process-specific the antigen should be representative of the material found at least at the harvest step. Do not include non-process specific HCP or significantly alter the relative abundance of certain HCPs in your antigen in order to get a broadly reactive 2D WB to silver stain correlation. 2D WB of upstream and non-process-specific HCPs does not predict how the antibody in the ELISA reacts quantitatively to downstream HCP. If some other antigen source such as a lysate must be used due to the presence of other antigenic non-HCP proteins added to the culture media, then that antigen should be evaluated for relevance to

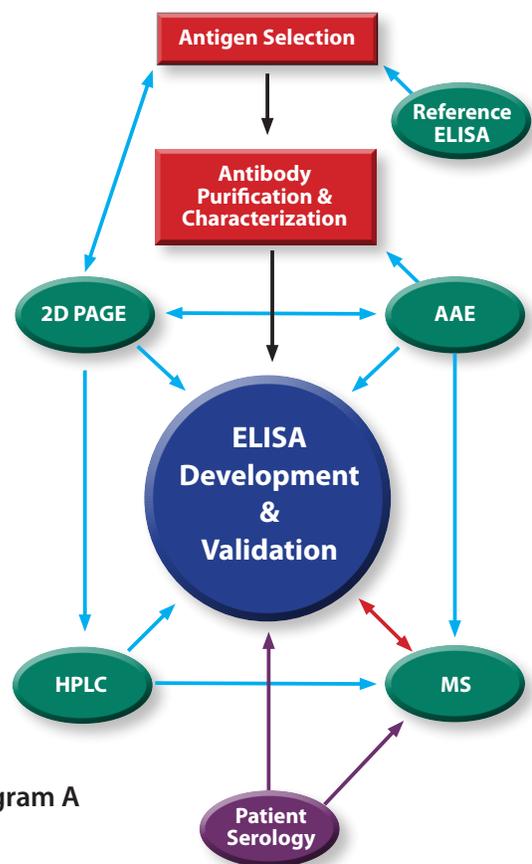


Diagram A

harvest material using methods such as 2D PAGE and a broadly reactive generic, reference assay if available. Affinity purification of the antibody is advisable to insure the assay in which it is used will be robust, specific, and sensitive. ELISA development and validation should focus on dilutional linearity analysis of downstream and final drug substance samples. Lack of dilutional linearity or the appearance of biphasic dilution responses will give insight into the array of HCPs and their relative abundance while guiding the use of the other analytical methods. The orthogonal methods of characterization such as AAE, HPLC, and MS facilitate the identification of individual HCPs. These methods complement each other and guide improvements in the purification process and the analytics. HPLC and AAE can be used as sample preparation methods for MS. Once identified by MS, any major contaminants in final drug substance can be addressed rationally by modification of the purification strategy to remove that component or by improvements in the “total” HCP ELISA or by use of mono-specific assays for individual, major HCP contaminants. Screening of patients at Phase III or later for development of anti-HCP antibodies can be used retrospectively to determine if any residual HCPs are causing safety or efficacy problems.