

Improved Methods for Characterization of anti-Host Cell Protein Antibodies

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The generation of very broadly reactive polyclonal antibodies to the hundreds of different HCPs that could contaminate the final drug substance can be achieved with proper antigen processing and immunization protocols. When incorporated into a well-developed and carefully validated ELISA these antibodies yield an analytical method with the sensitivity to detect HCP levels in the final drug substance at parts per million to parts per billion. Despite this great sensitivity multiple antigen HCP ELISAs are not without their limitations. ELISA is inherently quantitative method. However, it should be understood that when applied to HCP detection ELISA is at best a semi-quantitative measure of relative HCP levels. This lack of absolute quantitation is primarily due to the fact that HCP ELISA attempts to measure hundreds of different proteins species simultaneously in the same well using standards/calibrators from some up-stream or mock purification of a null cell line source, that may not match the array and relative abundancies of actual HCP in the final drug substance.

Another potential limitation of ELISA is that it may not detect all of the hundreds to thousands of possible HCPs. Ostensibly incomplete representation of all anti-HCP species in the antibody could underestimate total HCP and even miss important HCP contaminates. Lacking sufficiently sensitive orthogonal methods it has not been possible to demonstrate the presence of antibody to all HCPs. It is probable that some HCPs in the immunogen are either too low in concentration or otherwise not immunogenic. The orthogonal standard for characterization of HCP antibodies has been Western blot (WB). We have published previously on the limitations of WB in both 1 & 2 dimensions and its complimentary orthogonal method of non-specific protein staining of the fractionated HCPs using silver stain and colloidal gold (1). It is the purpose of this article to describe a new method and strategies superior to WB in demonstrating the reactivity of the anti-HCP antibodies.

Our labs have developed and formally validated dozens of commercial and customer product specific antibodies and ELISAs to HCP from nearly all the recombinant and transgenic expression systems in use today. We have traditionally provided WB data to characterize the antibodies because it has been the accepted norm. However, we have found WB lacking in providing relevant, specific, and sufficiently sensitive information of predictive value for the final ELISA method. In the final analysis it is the formal validation of the ELISA itself that determines if the antibody is adequate in detection of final product HCP. **If the ELISA has adequate sensitivity to detect HCP in the final product and that ELISA can be “validated” by conventional FDA/ICH analytical criteria, then the ELISA is a very important analytical tool. A tool which arguably might be missing reactivity to some HCPs but in reality has the requisite sensitivity to demonstrate if one purification process is superior to another or if one lot of drug has more or less total HCP than another. If what the ELISA might fail to**

detect is a real concern and not a specious argument then we must utilize methods better than WB to answer this question.

Despite the well documented utility of semi-quantitative HCP ELISA the theoretical concern that it may be missing some HCP remains a persistent refrain throughout the industry. In a recent US forum, not attended by these authors but represented by FDA, analytical scientists, and industry consultants, a consensus was reached that 2 dimensional Western blot (2D WB) correlated to silver staining ought to be the standard for characterizing reactivity of anti-HCP antisera. This consensus went on to suggest how much orthogonal method identity should be required between 2D WB and silver stain by proposing an arbitrary number of 80% agreement. In a somewhat different approach to the same fundamental question, European regulatory agencies have manifest their concern over the potential for undetected HCPs by recommending companies develop a “process specific” assay prior to final product licensing. The assumption is that such an assay would be more specific and sensitive than a “generic” or so called “multi-use assay” (2,3) developed to HCPs derived from a process that might be somewhat different or well upstream from the drug product in question. **Our experience with process specific assays as compared to generic and multiple use HCP assays has failed to show any real benefit from process specific assays in every case where the generic methods were shown to be valid following conventional**

analytical criteria. It should be understood that a process specific assay is subject to the same limitations preventing absolute quantitation as a generic assay. Furthermore, it is not known what absolute levels of HCP will cause safety or efficacy problems. For these reasons only very arbitrary and broad ranges of “allowable” ppm HCP contamination have been espoused typically in the range of 10 to 100 parts per million (ppm). When the generic and process specific assays can be demonstrated to correlate semi-quantitatively it can be logically argued that the two assays are redundant. **Our major concern with any regulatory requirement for process specific assays is not the added and potentially unnecessary expense but rather that some companies may ignore the value of a generic assay in early drug and process development and instead wait for their process to mature before taking a critical look at HCPs. Rather than wait until Phase 3 to develop a putative process specific HCP method we believe it is much more rational that a good generic assay be applied in the earliest phase of product development.** These generic assays can enable the process development team to easily reduce HCP to the very low ppm or even ppb levels and as such, this could have a positive impact on Phase 1 safety and later clinical efficacy trials. Achieving very low HCP levels early on may avoid significant delays from re-developing the purification process should the HCP levels later be deemed too high. If the generic assay is found lacking in any significant way through comprehensive validation then the effort to develop a more process specific assay is justified.

Before describing our suggested method for HCP antibody characterization it is important to discuss the basic limitations of 2D WB and silver stain.

1. **Sensitivity:** Sensitivity, defined as limit of detection (LOD), is in general much better for ELISA as compared to WB. With a significant disparity in the LODs it should be understood that the absence of a 2D WB spot does not mean an antibody is not present

that would allow for detection of that HCP in the ELISA. While it is obvious that silver stain is orthogonal to WB one must also fully appreciate just how orthogonal WB is to ELISA even when the same antibody is utilized. Attempts to detect low abundance proteins by increasing the sample load may interfere with resolution of other higher concentration components with similar mobilities. Downstream after clearance of much of the HCP the very high concentration of drug substance itself often limits the resolution and detectability of individual proteins by WB. **For most purification schemes we see that by the first or second purification step total HCP levels fall to the low parts per thousand. At these levels WB will often be negative for HCP while the ELISA is very positive even requiring dilutions to get the signal within the analytical range of the assay.** Another sensitivity advantage of ELISA is that it measures all HCPs cumulatively whereas WB attempts to detect individual proteins.

WB sensitivity is determined not only by the amount of each HCP that can be loaded but also by the amount of antibody against that particular HCP. Just as the concentration of HCPs are widely different in an HCP mixture, the concentrations of antibodies specific to a given HCP also vary in the polyclonal antibody. Optimization of WB is often a tediously iterative process that attempts to find an optimum tradeoff between non-specific binding (background signal development) and specific binding in order to achieve the best aggregate resolution. In the presence of a very high concentration of a given HCP or a very high titer antibody to that HCP so much signal may develop as to obscure other HCP spots. By reducing the HCP load or the concentration of total antibody one may reduce background and get a WB or silver stain with better apparent resolution in some areas but at the expense of losing detectable spots to low concentration reactants.

Silver stain as a non-specific protein staining method can be more or less sensitive than WB depending on a number of factors. Not all proteins stain equally well with silver stain. Just as in WB it may be necessary to optimize gel loads to overcome over-development of certain highly staining proteins. All things equal we have seen that the LOD for silver stain is somewhat better than the LOD of WB for the majority of proteins. In addition to silver stain we routinely perform a transfer to a duplicate PVDF membrane and stain this with colloidal gold. This helps to identify if some proteins have failed to transfer well to the membrane explaining poor correlation of WB to silver stain. The colloidal gold stain also allows us to detect additional bands for proteins that silver stain very poorly.

2. **Specificity:** There are a number of explanations for how the reactivity and specificity of a given antibody can be different between WB and ELISA and why WB may fail to detect HCPs the ELISA can detect. To achieve maximum separation resolution, samples are typically exposed to harsh denaturation conditions like SDS plus DTT and then boiled for a few minutes. In you have any doubt how severely this diminishes antigenic reactivity you have only to assay HCP preparations in an ELISA before and after such treatment. WB requires that the sample be transferred to a membrane such as PVDF for the immunoblotting to take place. The transfer process will reduce some of the very components strongly inhibitory to antibody binding such as SDS, however some SDS always remains. If you wish to observe the immunological inhibition of SDS add parts per thousand of SDS to the ELISA wells. There are other issues in this transfer process that conspire to further reduce the number of WB spots as compared to silver stain spots.

PVDF membranes are reported to adsorb protein by hydrophobic interactions. Some of these same regions on the HCP may be among the most antigenic. If the antibody binding epitope is occupied or otherwise inhibited by PVDF membrane binding then WB detection will be negative even though antibody exists. That same antibody would be very reactive in the ELISA where the HCPs are in solution phase and in a more native configuration just as they are found in your real process samples. Not all proteins transfer equally well to the PVDF. The fact that they are at their isoelectric points with somewhat reduced solubility and mobility may inhibit transfer out of the gel or may actually cause some to pass through the membrane without being well-adsorbed. Again, the absence of a WB spot does not mean an antibody is not present that would allow for detection in the ELISA.

3. **Subjective Interpretation:** We find the interpretation of WB determined by counting of spots to be a very subjective endeavor. With streaks, smudges, and blurs both horizontal and vertical, the identification of real HCP versus non-specific artifacts is difficult. Optimization of the WB with the goal of demonstrating the highest correlation of spots is an iterative process that may compromise specificity of the method. By varying antigen loads and antibody concentration it is possible to get a WB to tell any story you want. If a WB is to be of analytical value it must be objectively confirmed by a negative control blot. Without a negative control to identify non-specific binding spots, a WB can be misleading.

4. **Quantitative:** WB is by today's analytical standards and capabilities a crude qualitative method subject to all the problems of specificity and sensitivity discussed above.

Perhaps a more meaningful way to look at total HCP contamination is not the question 'Does the antibody react with more than "80%" of the silver stain spots?' but rather, 'Of the HCP species your antibody does recognize, what is their contribution to the quantitative total HCP in the sample?' If a given antibody only 'sees' 70% of the possible HCP species in an upstream sample by WB correlation but in actual downstream samples sample those 70% constitute 99% of the total HCP, why would such an antibody be disqualified? A more quantitative method would clearly be of value.

The method proposed below has not only this important quantitative capability but also overcomes the significant sensitivity and specificity limitations of WB.

The method we propose to replace WB for the characterization of HCP antisera involves a 2D HPLC fractionation of HCPs followed by ELISA microtiter plate detection of the individual HCP fractions. This and similar HPLC instrumentation with versatile automated control and data analysis software are widely available with many published applications (4,5). There are perhaps other HPLC chromatography schemes using other modalities of separation that may offer further advantages but the method described below provides far superior information as to the overall reactivity of HCP antisera than can be obtained by 2D WB.

Materials & Methods:

Instrumentation & 2D Separation Methods - The HPLC system used in our study was the ProteomeLab™ PF 2D protein fractionation system from Beckman Coulter. Fractionation of our samples on this system was performed by EPROGEN with offices in Darien, IL using their ProteoVue® software analysis program. The first dimension separation employs chromatofocusing to separate proteins based on their pI range using a PS-HPCF column from Beckman. In our study the sample was resolved into 18 separate pI fractions/lanes from 3.2 up to 9.0 in lane increments of approximately 0.3 pI units. Each lane was then subjected to fractionation in the second dimension by use of Reversed-Phase HPLC using a gradient elution of acetonitrile and TFA. Reverse-Phase fractions were collected at 15 second intervals in 96 well microtiter plates for subsequent ELISA analysis at *Cygnus Technologies*. OD 214 readings were taken across the gradient elution with the resulting chromatograms linked to the corresponding microtiter plate location.

HCP Test System - *E. coli* proteins were the model HCP system used in this study. **Two samples were fractionated by 2D HPLC. The first sample was an isolate of *E. coli* obtained by the same initial step used to extract recombinant protein from periplasmic spaces in the *E. coli*.** This procedure has been shown to recover a somewhat more limited number of HCP species than might be encountered in more aggressive solubilization/lysis procedures. The periplasmic isolate was further processed by UF/DF. A load of 8.5 mg of total HCP was injected onto the CF column. **The second sample was the actual purified final drug substance after 4 further downstream purification steps. Our objective was to determine if individual HCPs fractionated from final drug substance could be detected in the very sensitive ELISA.**

ELISA Testing - All 2D HPLC fractions were then tested in a sandwich ELISA incorporating the same antibody used in the validated product release ELISA for detection of “total HCP” in final drug substance. This ELISA is a conventional 2-site immunoenzymetric method in which capture antibody is coated on to microtiter wells. Sample is added to the well along with an HRP conjugated anti-HCP antibody in a simultaneous incubation scheme. After a wash step to remove unbound reactants, the wells are reacted with the chromogenic substrate TMB. Prior to testing the real samples, experiments were performed to determine if the 2D HPLC buffers and sample treatment reagents interfered in the ELISA. In these experiments, exposure of HCPs to the reagents in the chromatofocusing step such as urea and n-octyl glucoside were evaluated. While urea inhibits ELISA if added directly to wells, most of the urea gets removed during course of the Reverse-Phase chromatography step. With the removal of urea in this way we saw no evidence of irreversible denaturation of HCP ELISA activity compared to an untreated control. The final Reverse-Phase matrix of acetonitrile and TFA concentrations as represented across the second dimension gradient fractions were similarly tested by spiking known quantities of HCP and testing in the product release ELISA. When assayed directly in this matrix without dilution there was some negative interference from acetonitrile with up to a 40% reduction in activity from control at its highest levels of 25%. However, by using a simultaneous protocol where 100µL of HRP conjugate antibody was added to the well immediately followed by the addition of 25µL of sample fraction, the strong buffering capacity of ELISA conjugate diluent buffer was capable of neutralizing and eliminating any acetonitrile and TFA inhibition.

Results:

Figure 1 is termed as a 2D Map of the periplasmic UF/DF extract of HCP. On the horizontal axis are the chromatofocusing lanes. On the vertical axis is a graphic illustration of relative (color intensity coded) OD214 absorbances as a function of retention time for the Reversed-Phase fractions within each chromatofocusing lane. This representation of the data shows a large number of bands somewhat analogous to a 2 D silver stain. The left hand side of this figure shows an inset of the Reversed-Phase chromatogram for just those HCPs found in lane 7 of the 2D Map. While OD 214 is not as sensitive as silver stain, the difference in sensitivity has been compensated by loading 8.5 mg of total protein for the 2D HPLC fractionation compared to only 50µg for the silver stain.

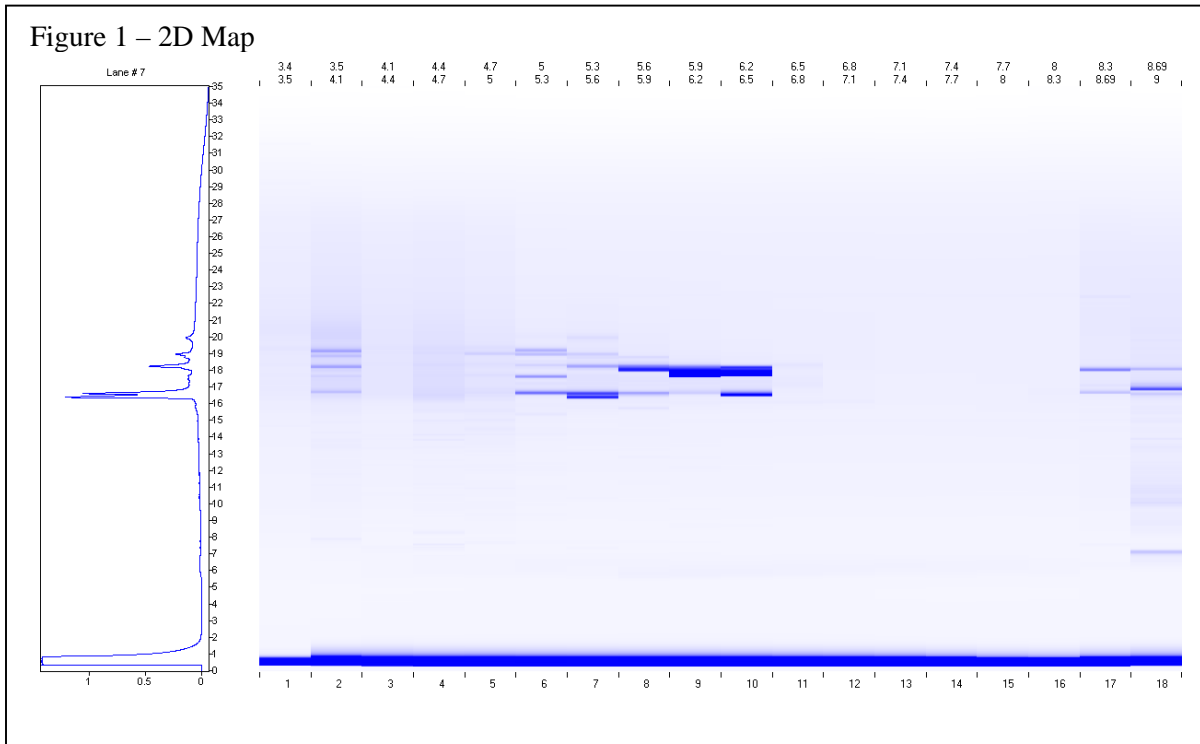
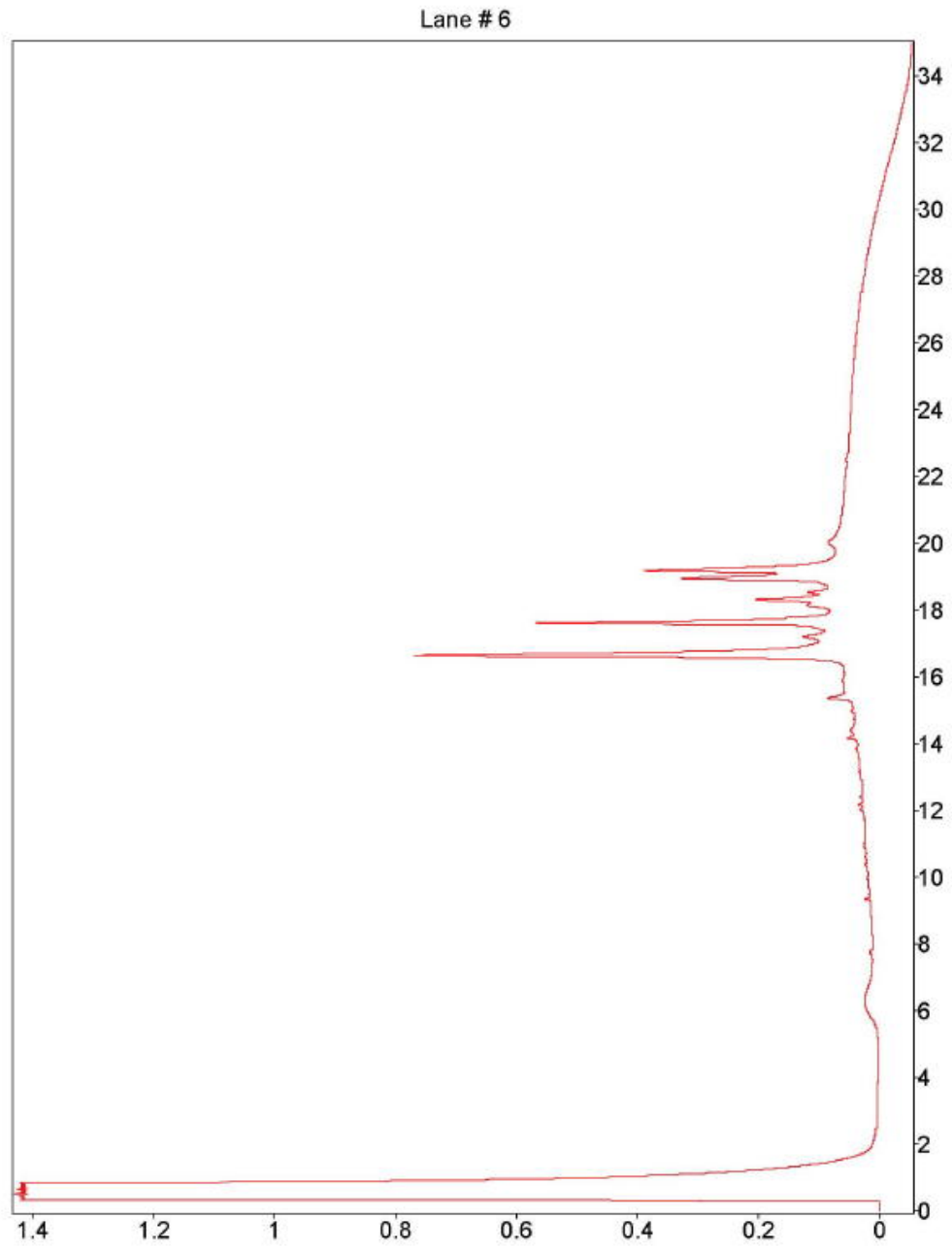


Figure 2 is a typical Reversed-Phase chromatograph for lane 6 from the fractionation of the periplasmic UF/DF extract. This is provided as an example of the scale and resolution obtainable at this step. Rather than show all 18 of the chromatograms we have instead summarized that data in **Table 1** below.

Figure 2 – OD214 Chromatogram of Lane 6 proteins resolved by Reverse Phase



Comparison of ELISA activity to OD214 detectability – The Reversed-Phase fractionation yielded 48 fractions for each of the 18 chromatofocused lanes for a total of 864 fractions. Greater resolution is possible in this system but was not deemed necessary for this study. While most fractions are likely a single protein, the possibility exists that in some cases more than one protein can be found in a given fraction and that some proteins may shoulder into adjacent fractions. Fractions were considered positive if they yielded a signal greater than the LOD of the method. **Table 1** summarizes the data collected on the periplasmic UF/DF extract by both OD214 and ELISA. Column 2 shows the number of fractions with OD214 readings above the LOD from each lane. The LOD for OD214 was stated as ~10ng/mL. A total of 144 fractions were detected from the 18 lanes. Column 3 shows those fractions with ELISA activity greater than the LOD of the assay defined as a signal corresponding to the mean of the zero standard plus two standard deviations. A total of 308 fractions were detected by the ELISA. Column 4 shows the number of OD214 fractions that did not have a corresponding ELISA activity. There were 18 such fractions. Column 5 shows the number of ELISA fractions that did not have a corresponding OD214 activity. There were 182 of these fractions.

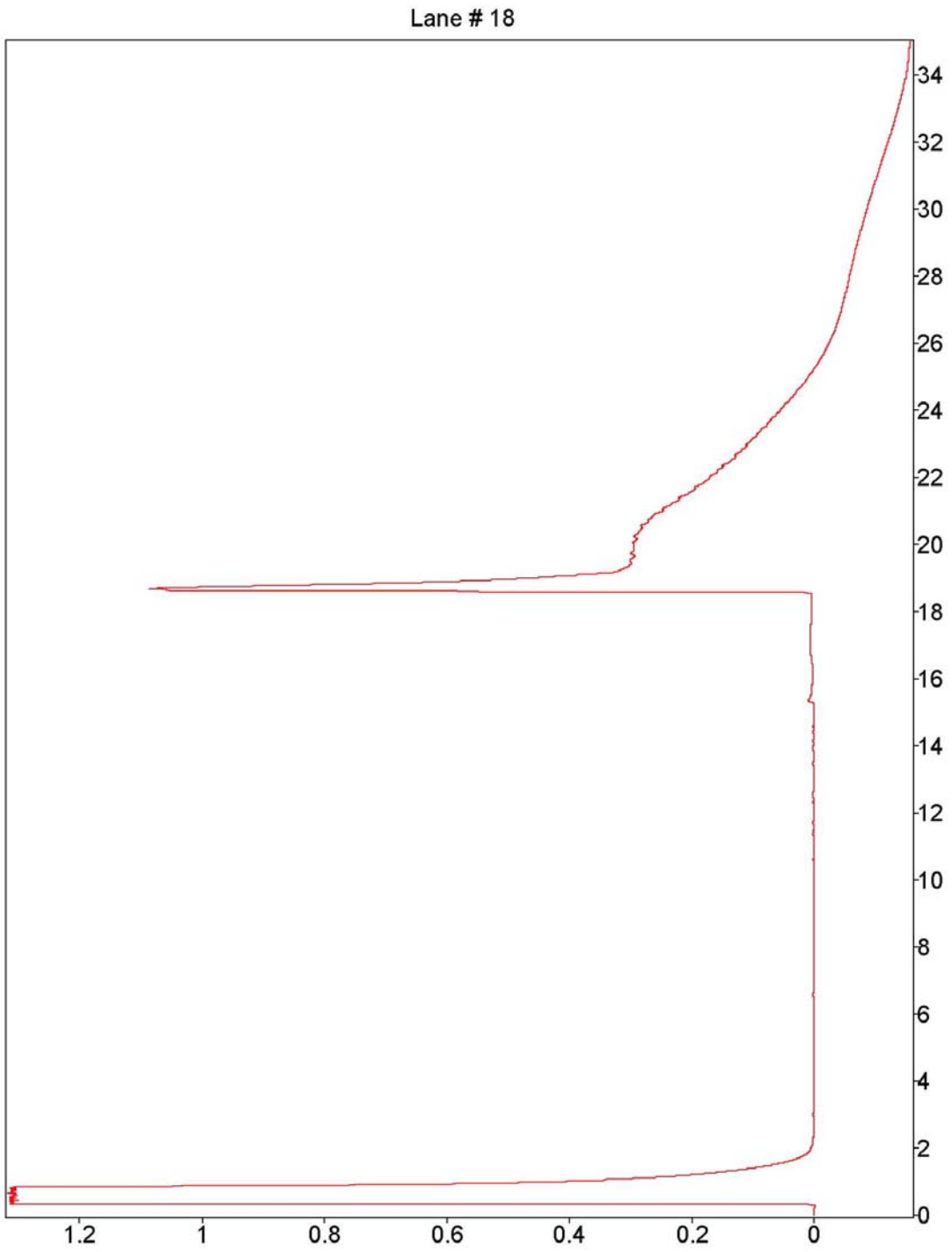
Table 1
Summary of 2D HPLC Fractionation Results
Comparison of OD214 to ELISA reactivity

Lane #, pI range	OD214 Detectable Fractions	ELISA Reactive Fractions	Fractions Detectable by OD214 only	Fractions Detectable by ELISA only
#1, 3.2 – 3.5	0	27	0	27
#2, 3.5 – 4.1	19	33	0	14
#3, 4.1 – 4.4	7	28	0	21
#4, 4.4 – 4.7	20	25	3	8
#5, 4.7 – 5.0	15	23	3	11
#6, 5.0 – 5.3	16	15	1	0
#7, 5.3 – 5.6	16	22	0	6
#8, 5.6 – 5.9	12	14	1	3
#9, 5.9 – 6.2	8	18	0	10
#10, 6.2 – 6.5	8	18	0	10
#11, 6.5 – 6.8	4	8	4	8
#12, 6.8 – 7.1	0	5	0	5
#13, 7.1 – 7.4	0	8	0	8
#14, 7.4 – 7.7	0	0	0	0
#15, 7.7 – 8.0	0	1	0	1
#16, 8.0 – 8.3	0	14	0	14
#17, 8.3 – 8.7	5	14	0	9
#18, 8.7 – 9.0	14	35	6	27
TOTALs	144	308	18	182

Further analysis of OD214 fractions unreactive by ELISA – The relative agreement of ELISA versus OD214 fractions was 87.5%. Of the 144 OD214 fractions, 126 showed ELISA activity. Of the 18 ELISA unreactive fractions, 14 gave ODs very close to the LOD as claimed by the laboratory performing the fractionation. We have less statistical confidence in this LOD and if this is justified such fractions could likely be noise in the OD214 detection system. The 4 remaining peaks also gave very low ODs of less than 0.09 but apparently above average baseline and its noise. Another way to analyze the data is to integrate the peak area under the OD214 fractions. Peak area corresponds to the protein concentration. This allows a somewhat quantitative assessment of the relative reactivities of OD214 versus ELISA. In this way we calculated that the 126 fractions with OD214 and ELISA agreement represented 96.3% of the total proteins integrated from the area under all 144 OD214 peaks.

HCP in final drug substance – 2D HPLC fractionation of the final purified drug substance detected only a single fraction by OD214 representing the drug substance itself. This peak was found in lane 18. All 864 fractions from all 18 lanes of the fractionation of the final drug substance were tested in the ELISA. The ELISA detected HCP in at least 17 separate fractions none of which had OD214 activity. **Figure 3** is the Reversed-Phase chromatogram of the final drug substance eluting in lane 18. There is a single peak attributed to the drug substance itself. The broad shoulder at retention times after about 20 minutes is not characteristic of real protein but is likely a component in the formulation buffer for this product. No other significant OD214 peaks were seen in any of the other 17 pI lanes.

Figure 3 - Lane 18 chromatogram of final drug substance



Conventional 2D WB & Silver Stain – The same materials used in the 2D HPLC and ELISA testing were also applied to conventional 2D WB & Silver Stain. **Figure 4** shows the 2D silver stain of the periplasmic UF/DF *E. coli* extract loaded at 50 μ g. **Figure 5** is the corresponding 2D WB with a load of 200 μ g. This was the first attempt at the 2D blot for these reactants and arguably it might be improved with method optimization. Consensus in our lab was that 2D silver stain showed approximately 100 spots while the 2D WB demonstrated far fewer spots due to extremely reactive areas on the film that reduced resolution requiring the operator to terminate the enzymatic photon generation perhaps before other lower reactivity spots could be visualized.

Figure 4 - Conventional 2D WB & Silver Stain of the Periplasmic Extract

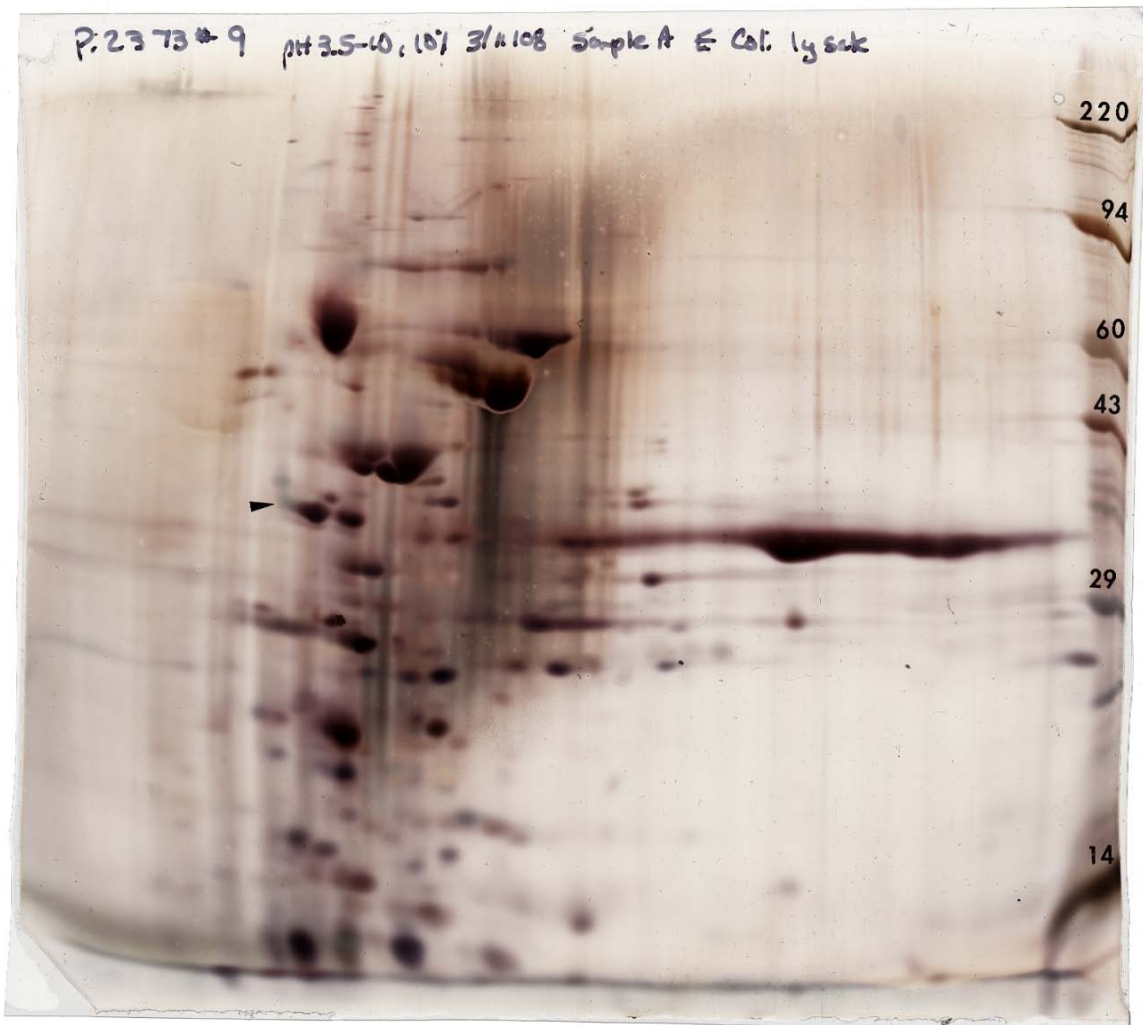


Figure 5 – 2D Western Blot of Periplasmic Extract



Table 2*E. coli* HCP detection in the upstream periplasmic extract by various methods.

Analytical method	# of HCPs resolved
2D PAGE, silver stain	~100 spots
2D Western blot	~60 spots
2D HPLC by OD214	144 protein fractions
ELISA of 2D HPLC fractions	308 protein fractions

Table 3*E. coli* HCP detection in a final drug substance by various methods

Analytical method	# of HCPs resolved
2D PAGE, silver stain	no spots other than product
2D Western blot	no spots other than product
2D HPLC by OD214	no protein fractions
ELISA of 2D HPLC fractions	17 protein fractions

Discussion:

The advantages of a 2D HPLC fractionation followed by ELISA detection are stated below and summarized in **Tables 2 & 3**.

1. Sensitivity – **A total of 308 immunoreactive HCP fractions were detected in the periplasmic UF/DF extract of *E. coli* proteins. This method was far superior to the 2D WB shown in Fig. 5 in resolving HCPs. It is also clear that ELISA of 2D HPLC proteins detects even more HCPs than can be resolved by the 2 orthogonal detection methods of 2D HPLC with OD214 detection (~144 HCPs) or 2D electrophoretically resolved silver stain proteins (~100 HCPs).** The greater load capacity of 2D HPLC as opposed to 2D WB is important in achieving much of this improved sensitivity. ELISA testing results for 2D HPLC resolved fractions from the purified final drug substance are even more dramatic in demonstrating the analytical superiority of this method. Neither 2D HPLC nor 2D silver stain alone could detect any HCP in the final drug substance. ELISA indicated at least 17 separate HCP fractions. The quantitative ELISA kit developed and validated as a release test for this drug substance gave a “total HCP” level of ~8ppm (8ng of HCP/mg of drug substance).
2. Specificity – 2D WB subjects HCPs to very harsh denaturing conditions that may destroy or alter some native antibody epitopes found in final product samples. In addition, the transfer to the blotting membrane may further block antibody access to important epitopes. The reagents used in 2D HPLC fractionation did not cause any positive or negative interference in our ELISA protocol. As previously stated, failure of WB to detect a given HCP spot does not mean there is not antibody to that HCP that would allow for detection by ELISA. Conversely, the presence of a WB spot does not guarantee the ELISA will also detect that protein. This can be due to a variety of reasons such as the requirement for at least 2 epitopes per HCP for ELISA detection. SDS/DTT/Heat denaturation may also expose epitopes that might not otherwise be available on HCPs in

- a more native configuration. So long as ELISA remains the release test for total HCP in final product owing to its high sensitivity, it is important that any method used to judge the reactivity to individual HCPs be very similar to that ELISA in terms of sensitivity and specificity.
3. Throughput – The automated 2D HPLC fractionation approach generates a large number of individual fractions for a given sample. We arbitrarily collected 864 fractions for each sample in this study but even more resolution is possible. Subjecting downstream samples to the same fractionation to resolve purification process specific HCP increases the number of fractions proportionately. With the ability to collect the fractions discretely into microtiter wells the ELISA format easily accommodates the high volume in a very cost effective way. Our analysis of this method compared to the work involved in developing, optimizing, and validating a 2D WB and silver stain method indicates they are not appreciably different in labor and cost.
 4. Quantitative – WB is not a quantitative method. The concentration of a particular HCP, the relative abundance of the specific antibody to that unique HCP in the polyclonal mixture as well as epitope accessibility and antibody affinities together determine the intensity of the 2D WB spot, i.e. a streak or blur. Although ELISA is inherently a quantitative method, as used in this study to detect 2D HPLC resolved HCPs, we made no attempt to quantitatively state the HCP concentrations. Reasonable quantitation is possible with comprehensive validation but understanding that no standards exist for individual HCP species absolute quantitation. Within its limits of sensitivity, OD214 absorption does provide at least a semi-quantitative determination of total protein. **A reasonable quantitative estimate of total HCP level can be accomplished by integrating the area under the chromatogram. By correlating ELISA reactivity to each of these OD214 fractions we can reasonably state what percentage of total HCP load has a corresponding ELISA activity. This was estimated to be greater than 96.3%. We think this method of semi-quantitative analysis is far superior to the arbitrary proposal that 2D WB on an upstream source of HCP should have 80% spot correlation with 2D silver stain.**
 5. Further analysis of purification process specific HCPs – Since samples have already been fractionated by 2D HPLC and specifically identified as HCP by ELISA, further analysis of these fractions is made possible. The high purity of these fractions should allow for a 3rd dimensional characterization and positive identification by methods like MALDI-TOF-MS if such information is deemed of value.

Conclusions:

WB analysis with its significant limitations has poor predictive value when applied to the characterization anti-HCP antibodies. In the final analysis it is those HCPs in the final drug substance and the ability of the release test ELISA to detect those HCPs that is critical. Provided the ELISA has sensitivity to detect representative HCPs throughout the purification process and can be critically validated for dilutional linearity, spike recovery, and specificity, the failure to detect all HCPs does not deny its analytical utility. The superior analytical

discrimination provided by 2D HPLC fractionation followed by ELISA detection does little to change this fundamental logic. If further characterization of HCP antibodies to individual upstream and downstream HCPs is deemed necessary or of real value beyond a successfully validated release-test ELISA, then the method must be able to provide that information. The correlation of 2D WB to silver stain is not that method.

References

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