White paper

ELISA CHO HCP Antibodies – Coverage Considerations

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Abstract

Therapeutic monoclonal antibodies (mAb) are protein drugs produced by Chinese hamster ovary (CHO) host cells cultured in large-scale bioreactors. Separation of each mAb drug from contaminating CHO host cell proteins (HCP) is monitored by a robust immunological test, the ELISA (enzyme-linked immunosorbent assay). A critical component of the latter is the polyclonal antibody used for HCP detection. Narrowly reactive polyclonal Abs might give misleading ELISA results; a broadly reactive HCP Ab is desirable. Coverage assays provide an assessment of how completely a polyclonal Ab recognizes a complex population of HCPs. Here we review ELISA coverage assays, with a focus on the 2-dimensional western blotting method used at Kendrick Labs. Examples of detection of persistent HCP contaminants clusterin and phospholipase B-like 2 are shown.

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Introduction

FDA approval of one class of biologics, therapeutic monoclonal antibodies (mAb), has surged over the past five years [1]. These important drugs are typically produced by large-scale cultivation of Chinese hamster ovary (CHO) host cells engineered to secrete humanized glycosylated mAb proteins into the harvest cell culture fluid (HCCF). Depending on culture conditions, HCCF also contains an unknown amount of secreted host cell proteins (HCP) plus any released by damaged cells. HCPs that co-purify with the mAb drug substance (DS) may have deleterious effects in drug recipients [2].

There are many *potential* CHO HCP contaminants. Hefner et al. identified ~9400 unique cellular proteins in pelleted CHO-S and CHO DG44 cells by mass spectrometry (MS), of which 5500-6100 were in each cell line at any given timepoint [3]. Kumar et al. identified 1015 secreted proteins in CHO-K1 cultured cell fluid using MS in combination with bioinformatics tools [4].

Both upstream and downstream parameters affect HCP levels. Park et al. showed that HCCF from CHO cells cultured for three days had HCP concentrations of 14 µg/ml versus a whopping 128 µg/ml for cells cultured eight days when cell viability had fallen [5]. Protein A affinity chromatography, which selectively binds mAb during rinses, removes the bulk of HCPs but not necessarily all. Some mAbs carry specific HCPs along during the purification process. Sisodiya et al. demonstrated this by spiking 21 purified mAbs into the same null HCCF with known HCP content. After equilibration, the mAbs were re-purified with two different Protein A affinity resins to control for resin nonspecific binding. The HCP content of the 21 eluates, in agreement between the two resins, varied dramatically between mAbs. The majority, 14 eluates, contained <2,500 ng/mg HCP but the remaining seven contained higher amounts. Two of the latter contained *>15,000* ng/mg HCP [6]. Which HCPs might co-purify with a given mAb cannot be predicted.

The ELISA is used to monitor immunogenic HCP proteins

The enzyme-linked immunosorbent assay (ELISA) is the workhorse for monitoring HCP levels during recombinant protein production [7]. It is based on detection of proteins by a polyclonal antibody from a goat or other large mammal that has been immunized with a CHO protein mixture, the null protein lysate for example. Results are reported as a numerical value in parts per million (ppm), the ratio of nanograms HCP to milligrams DS. The value may represent a single HCP or collection of several; it may be disproportionately weighted towards highly immunogenic species. Although not perfect, ELISAs are convenient to set up and provide quantitative, sensitive comparisons of HCP purification steps.

Mammalian antibodies vary between animals and must be tested for breadth of response. An Ab that reacts narrowly, with only a few HCPs, would give misleading results. Determining polyclonal Ab selectivity for individual proteins in a mixture of several thousand is non-trivial.

Two USP coverage assays are in place to assess ELISA Ab reactivity

In 2016, the US Pharmacopeia National Formulary (USP 39 NF 34) provided guidance for coverage assays where coverage is defined as the assessment of how completely a population of polyclonal Abs recognize the population of HCPs [8]. To obtain a numerical value for percent coverage, proteins detected by an immunoaffinity method are compared to total proteins detected on

2D gels with a sensitive stain. The immunoaffinity step may be affinity purification by resin-bound Ab to detect native HCPs (AAE) or 2D western blotting (2D WB) to detect denatured HCPs [8]. It should be noted that Ab coverage is unlikely to be 100% for either method because of mammalian self-tolerance mechanisms [9]. Proteins conserved between CHO (hamster) and goat, for example, would be recognized as self by the goat and not elicit antibodies.

1. Antibody Affinity Extraction (AAE) Coverage Analysis

For AAE, the HCCF supernatant is passed over an ELISA antibody affinity column repeatedly, then bound proteins are eluted. Coverage is determined by comparing the supernatant protein content for differences before and after affinity chromatography. An advantage to AAE is that conformation epitopes recognized by ELISAs remain intact. A problem is that an unknown amount of secondary binding may occur, depending on the number of passages over the affinity resin. Native HCPs that bind indirectly to their captive binding partners rather than directly to immobilized ELISA Abs would skew coverage values to be high. Since protein-protein interactions are commonly studied by native co-immunoprecipitation [10], it seems likely that secondary binding is significant. For example, heat shock cognate 71 protein, an abundant protein chaperone and common HCP contaminant [11], would likely pull down multiple chaperoned partners.

2. 2D Western Blot (2D WB) Coverage Analysis

The 2D WB coverage method utilizes binding of ELISA antibodies to linear epitopes on denatured CHO HCPs resolved by 2D electrophoresis and transblotting. The 2D WB pattern is compared to that of a 2D silver or Sypro ruby stained pattern from the same sample for differences. Figure 1 shows an example taken from the USP/NF guidance paper [8]. In this figure, the Sypro Ruby stained pattern on the left shows total proteins, while the corresponding WB pattern showing immunogenic proteins recognized by an ELISA CHO Ab is on the right. Since the same membrane was used for both, the patterns are superimposable and thus comparable.

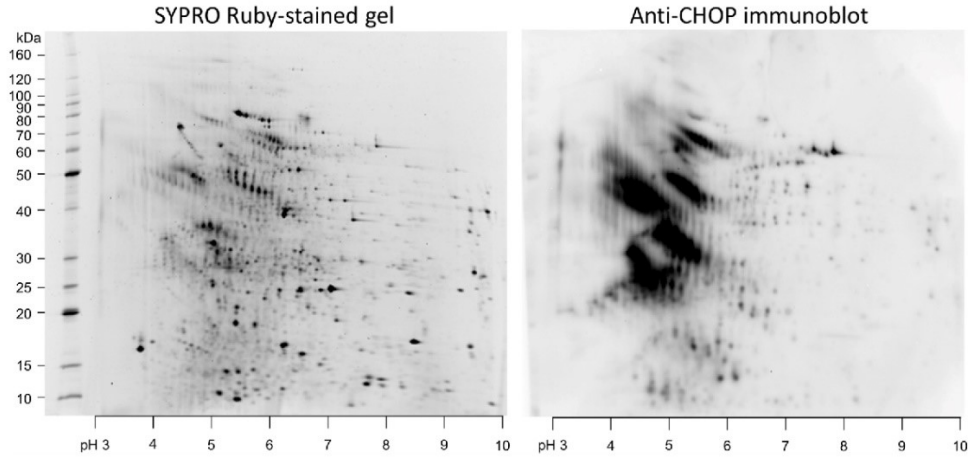

Figure 1. Example images taken from USP 39 NF 34 (ref 9, p1424). Left: CHO HCP 2D pattern obtained with a sensitive fluorescent stain versus Right: western blot image of the same gel.

Polyclonal Abs detect both linear and conformational epitopes of proteins.

It is tempting to think of a polyclonal antibody as a mixture of monoclonal antibodies with one each for every antigenic protein in the sample. In fact, a polyclonal antibody may contain several monoclonal antibodies for each antigenic protein, against different linear and conformational epitopes as shown by Forsstrom et al. for large recombinant protein fragments [12]. This group analyzed eight rabbit polyclonal antisera immunized with different recombinant protein fragments 50-150 amino acids in length (~5,000-15,000 kDa), called Protein Epitope Signature Tags (PrESTs) used for the Human Protein Atlas Project [13]. The percent of polyclonal Abs targeting linear versus conformational epitopes was determined using affinity chromatography. The polyclonal sera were passed sequentially over a series of six affinity columns with 15-mer linear epitopes and then over a final affinity column with the full PrEst sequence containing conformational epitopes. Antibody amounts in each fraction were determined by 280 nm absorbance.

For six of the target PrESTs, 70-90% of the antibodies recognized linear epitopes. For one PrEST, 80% of the antibodies bound conformational epitopes. For the eighth target, 50% of the antibody mixture bound linear epitopes and 50% conformational epitopes. As expected, antibody mixtures against linear epitopes were useful for western blotting, whereas those against conformational epitopes were not. Extrapolating from these results, ELISA polyclonal mixtures probably contain Ab species against linear epitopes reactive in western blotting for most immunogenic proteins.

Kendrick Labs uses custom 2D WB to assess ELISA Ab coverage

A sodium dodecyl sulfate (SDS)-compatible variant of 2D electrophoresis is used at Kendrick Labs that employs acrylamide tube gels for the first dimension isoelectric focusing step that separates proteins based on isoelectric point (pI). The second dimension, SDS polyacrylamide gel electrophoresis (PAGE), separates proteins on the basis of molecular weight (MW). SDS allows solubilization of membrane proteins and generally improves 2D pattern reproducibility 4.

For coverage testing, the complex HCP antigen is typically run at 250 µg/gel for WB and at 25 µg/gel on duplicate 2D gels for silver staining. Proteins in the WB gel are transblotted to PVDF, the membrane stained with Coomassie blue, and then scanned to record the total protein pattern. After removal of the blue stain with a methanol rinse, the PVDF membrane is western blotted with the ELISA antibody. Since the silver staining method [15] used at Kendrick Labs is about 10X more sensitive than Coomassie, the silver 2D pattern matches that of the 10X loaded Coomassie-stained blot, which in turn is superimposable with the WB film. Thus, the Coomassie WB pattern serves as a bridge for aligning silver and WB patterns.

Coverage Analysis example

Figure 2 shows results from a typical coverage analysis of a complex CHO-GS null sample versus the client's corresponding ELISA antibody. Western blotting, with lower limit of sensitivity of around 0.1 ng depending on the monoclonal antibody [16], is considerably more sensitive than silver staining with a lower limit of ~5 ng. Thus, the 2D WB shows many protein spots that are undetectable by staining. For this experiment a total of 1243 protein spots were found, of which 230 were detectable only by WB, 414 only by silver staining, and 599 by both methods. The ELISA Ab detected 829 by WB (599 + 230) for a coverage value of 67%.

A numerical coverage value helps assess whether the ELISA antibody detects a wide array of HCPs. Visual inspection of the patterns is useful as well. In this case, spots with blue outlines detected by the antibody are spread throughout the pattern. Strongly reacting, low abundance proteins appearing as a string of isoforms are likely secreted glycoproteins. Such proteins resolve into multiple isoforms on the western blot but are faint/undetectable by silver staining.

Unglycosylated proteins that appear as a single spot intermingle with glycosylated charge-train isoforms. Adjacent spots cannot be assumed to be isoforms of the same protein as shown by MS analysis [17]. At Kendrick Labs, charge isoforms are split to achieve consistency between Analysts. The charge isoforms are counted separately and included in the total number of protein spots, increasing the coverage value if they are only visible on the WB. Since the latter are highly immunogenic in the goat (or whatever species is used), we hypothesize that they would be highly immunogenic in humans and cause problems if they slipped through with the DS.

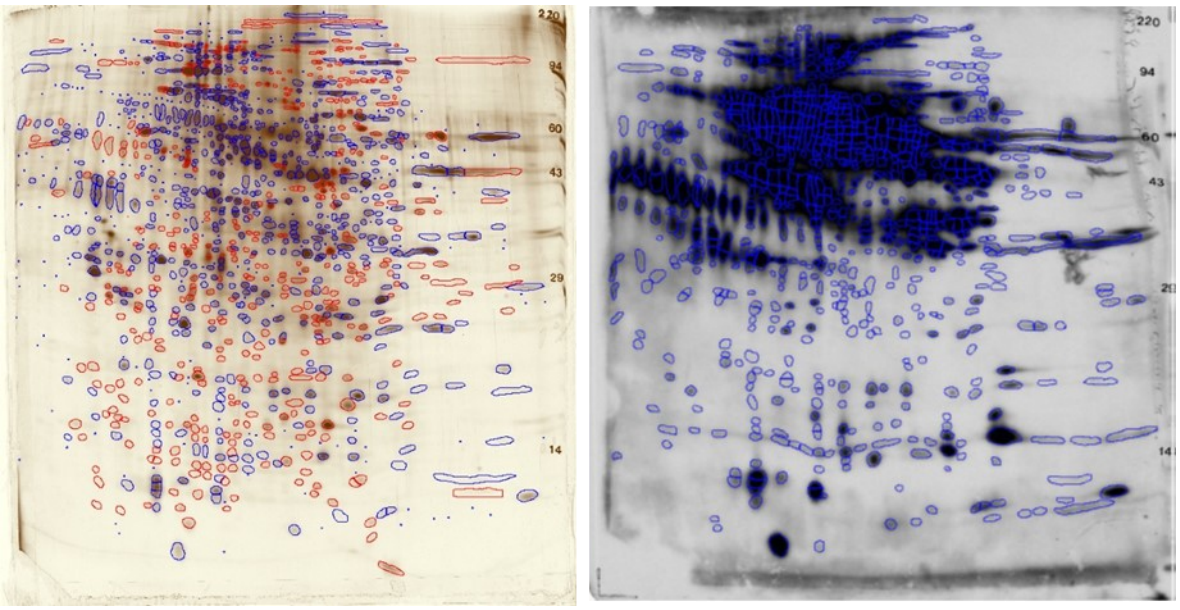

Figure 2. Images showing typical Kendrick Labs 2D Coverage patterns. Left: CHO-GS HCP pattern obtained with a sensitive silver stain (shown with client's permission) versus Right: anti-CHO western blot image from a duplicate gel run with the same sample. Spots present on the silver-stained gel but missing 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 ELISA antibody detected 829/1243 protein spots for a coverage of 67%. Light film exposures and low contrast setting were used to resolve the dark mass of immunogenic proteins in the upper middle of the WB.

2D WB Coverage assay - standardization and reproducibility

The method of 2D SDS PAGE has multiple steps and is most reproducible when standardized. At Kendrick Labs SOPs are in place for each step and training records are maintained for all personnel. Although client confidentiality precludes detailed discussion of results, we can say in two instances that HCP antibody analyses were performed in triplicate on the same sample, i.e. 1 run/day on 3 different days, with each run analyzed by a different 2D Analyst. In one case the Coefficient of Variation (SD/Mean*100, n = 3) for the % coverage result was 8.1%; in the other, the CV was 5.1%.

Persistent/troublesome contaminants in DS processing

The ELISA quantifies total HCP in a DS but cannot distinguish between proteins. For the latter, mass spectrometry (MS) is required. Valente et al. reviewed eight MS studies covering 29 mAbs to assess "persistent" HCPs that are difficult to remove from DS. Three criteria were used for persistent proteins: 1) association with the mAb during cross-interaction chromatography, 2) presence in the Protein A eluate, or 3) presence in the final DS [11]. Twenty HCPs were identified that met these criteria in ≥20% of the mAbs. Clusterin, a secreted mammalian chaperone known to bind to immunoglobulins [18, 19], was persistent in all 29 mAbs. Thirteen other proteins were persistent in over 50% including serine protease HTRA1 that might degrade DS [20] and heat shock 71 protein that might affect patient immune response [21]. Phospholipase B-Like 2 (PLBL2), detected in 4 of the 29 mAbs, is known to be troublesome. It may cause dilutional non-linearity during ELISA testing [22] and an immune response in patients [23]. MS has become increasingly sensitive through the years, and is now able to detect HCPs such as PLBL2 down to 10 ppm in mAb DS [24].

2D WB may be used to detect persistent contaminants in DS

The 2D WB method is also sensitive down to ≤10 ppm PLBL2 in faux mAb DS as shown in Figure 3. For this figure commercial IgG purified from human serum was used as a faux mAb DS sample. The maximum amount for DS load, 1.25 mg, was loaded onto triplicate gels spiked with 0, 10, and 25 ng recombinant CHO-PLBL2 protein. The top row shows the dark, diffuse 2D pattern of heavily loaded IgG. The bottom row shows anti-CHO-PLBL2 western blots from each.

PLBL2 migration on 2D gel/blot pairs shown in panel C/D and E/F is unaffected by the heavy IgG load. The 10-ng load is clearly detected in the presence of 1.25 mg IgG, i.e. sensitivity is 10 ppm or better. The 25-ng spot looks proportionally darker for the same film exposure time of 3 min. Longer exposure for HCPs loaded with 1 mg DS would likely give better sensitivity.

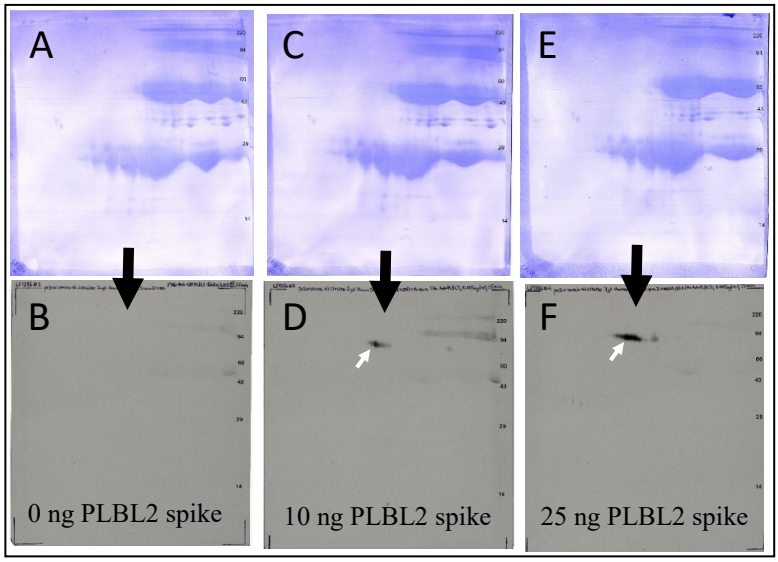

Figure 3. Detection of PLBL2 recombinant protein spiked at 0 ng (A & B), 10 ng (C & D) and 25 ng (E & F) into faux DS heavily loaded at 1.25 mg/large format (20 x 25cm) 2D gels. Panels A, C and E show the Coomassie blue patterns of the PVDF membranes. Panels B, D and F show corresponding WBs obtained with a commercial CHO-PLBL2 antibody. White arrows indicate 10 ng (D) and 25 ng (F) PLBL2.

Checking ELISA reactivity with specific HCPs

In some cases, it might be useful to verify ELISA detection of specific HPCs at the onset of a project. Figure 4 shows 2D WBs of the CHO-GS HCP null sample from Figure 2 versus commercial mAbs against clusterin (A) and CHO-PLBL2 (B). The ELISA polyclonal Ab pattern from Figure 2 is shown for comparison (C). Clusterin is clearly detected by the ELISA Ab as shown by matches of discrete spot trains (red arrows). The PLBL2 monoclonal signal aligns with a crowded area of the ELISA WB pattern and is not a definitive match.

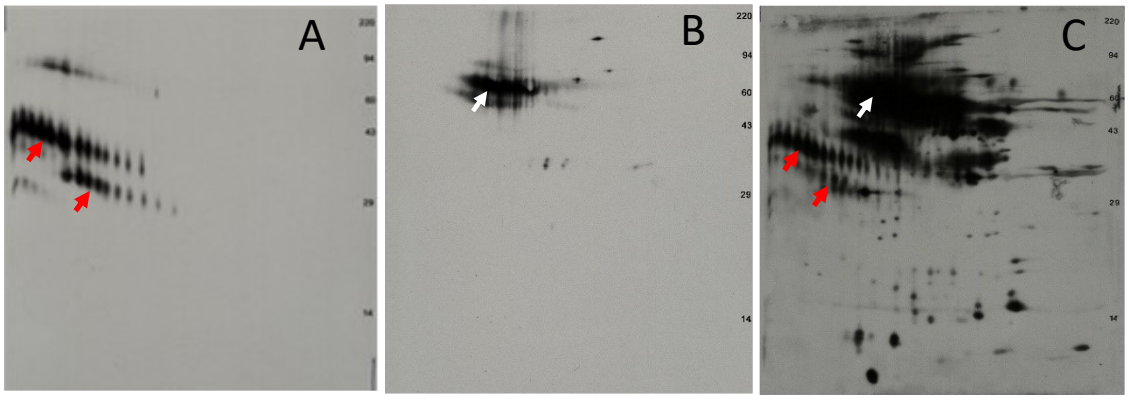

Figure 4. The same CHO-GC HCCF sample western blotted with three different antibodies. A. clusterin mAb B. PLBL2 mAb, and C. ELISA polyclonal antibody (image taken from Figure 2). Clusterin is well resolved in both mAb and polyclonal Ab patterns (red arrows). PLBL2 is well resolved with the mAb but migrates to a crowded region of the ELISA polyclonal WB pattern (white arrows).

Discussion and Conclusions

ELISAs are used to monitor HCP levels during DS purification while MS may be used to identify HCPs above acceptable levels [24, 25]. MS has the great advantage of being able to identify multiple proteins simultaneously. Yang et al. was able to detect ≤10 ppm of PLBL2, a troublesome HCP contaminant, along with six other protein standards spiked into 1 mg of mAb DS. Furthermore, they were able to identify 19/22 proteins in the Universal Proteomic Standard-1 mix spiked into DS at that level [24].

However, MS identification of proteins is based on matching two or more peptide sequences to a unique protein in the CHO database. It consists of 4 complex steps, each of which can be subject to high variability as discussed by Bittremieux et al. [26]. The steps are: 1) proteolytic digestion of proteins into peptides, 2) separation of the peptides via liquid chromatography, 3) generation of spectra via mass spectrometry, and 4) interpretation of the spectra via bioinformatics. Variability at each step can, of course, be managed by quality control measures. Even so, stochastic variability introduced in one step, an unexpected contaminant for example, can affect the next. MS identification is not always definitive and is hard to check.

The primary use of 2D WB is to verify broad coverage of the ELISA polyclonal Ab. Our experience has been that visual inspection of 2D WB patterns may provide additional useful information if a problem arises. For example, protein charge isoforms are not distinguished by MS. Peptides with glycan post-translational modifications have a mass shift and so do not match sequences from genomic databases. MS analysis of protein glycan structure remains a formidable task [27].

At least half of proteins and virtually all secreted proteins are glycosylated. Glycan sugar chains are capped with negatively charged sialic acids that impart charge microheterogeneity, causing a fuzzy beads-on-a-string appearance on 2D WBs. Glycosylation changes a protein's solubility, pI, and MW [28, 29]. Knowledge about the extent of glycosylation may be helpful in choosing a purification strategy. The method of 2D WB serves as an orthogonal check for charge isoforms of HCPs that are detected by MS.

Materials & Methods

Protein 2D electrophoresis and WB were performed essentially as described in Kendrick et al. [16]. Materials for Figures 3 and 4 were purchased as follows: IgG purified from human serum was purchased from MilliporeSigma, St. Louis, MO (cat No. I4506). Purified recombinant phospholipase B-like 2 (PLBL2) protein expressed in CHO-3E7 cells was purchased from ICL, Portland, OR (Cat # AG65-0324-ZB-Z) along with an anti-CHO_PLBL2 Ab (Cat # GPLB-65B-Z). Purified clusterin protein was purchased from R&D Systems, Minneapolis, MN (Cat # 2747-HS) along with an anti-clusterin Ab (Cat # AF2747-SP).

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