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Methodological Challenges of Host Cell Protein Antibody Characterization

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The reliable assessment of Host Cell Protein (HCP) antibody performance is challenging. This case study presents the development and characterization of HCP ELISA antibody reagents for quantification of process-related impurities derived from a Chinese hamster ovary production cell line (CHO).

The selection of a suitable capture antibody mixture for HCP ELISA development not only depends on its ELISA characteristics but also on the antibody coverage. Coverage is conventionally determined by 2D Western blotting (2D WB). However, 2D WB based coverage results strongly depend on the methodological/technical details of the analysis performed. The informative value of 2D WB analysis can further be limited by denaturing assay conditions, thereby potentially underestimating coverage performance. For comprehensive and reliable immunological reagent characterization under almost native assay conditions (similar to ELISA), orthogonal coverage assessment can additionally be performed employing Immuno Affinity Chromatography (IAC) followed by 2D difference gel electrophoresis (2D DIGE).

In this case study, the HCP population was fractionated and used for individual immunizations in order to improve immune response towards weakly immunogenic low-molecular weight (LMW) HCPs. The reactivity of the resulting capture antibody mixtures were tested in ELISA, by 2D WB and IAC/ 2D DIGE revealing significant differences depending on the antibody composition itself, as well as the analytical method used.

Antibody Generation, Purification and Modification

CHO HCP specific antisera were generated by rabbit immunization using a suitable process-related mock material as the antigen. One group was immunized with the unfractionated Total HCP representing a mixture of almost all the proteins present in the respective mock cell line. An additional group was immunized using the corresponding LMW HCP fraction mainly containing small proteins (<50 kDa). Different small-scale antiserum test pools were prepared and subjected to affinity chromatography yielding a set of purified antibody mixtures (Table 1), which were compared in ELISA, by 2D Western blotting and IAC/ 2D DIGE. For ELISA measurement, the corresponding detector antibodies were each prepared by conjugation to biotin.

Antiserum Pool	Antigen for Immunisation	Antigen for Affinity Purification	Antibody Mixture	Homologous Antigen/ Antigen Mixture for ELISA Testing
Overall Antiserum Pool (Total HCP/ LMW HCP)	Total HCP/ LMW HCP	Total HCP	HCP Antibody T+L	Total HCP
Antiserum Pool (Total HCP)	Total HCP	Total HCP	HCP Antibody T	Total HCP (data not shown)
Antiserum Pool (LMW HCP)	LMW HCP	LMW HCP	HCP Antibody L	LMW HCP (data not shown)
			HCP Antibody 80T:20L (mixture of 80% HCP Antibody T and 20% HCP Antibody L)	80T:20L (mixture of 80% Total HCP and 20% LMW HCP)
			HCP Antibody 50T:50L (mixture of 50% HCP Antibody T and 50% HCP Antibody L)	50T:50L (mixture of 50% Total HCP and 50% LMW HCP)

Table 1: Overview about the antibodies and corresponding antigens

HCP ELISA Measurement

The antibody mixtures (Table 1) were tested by ELISA, as this is the default system for HCP quantification.

For quantification, sample measurements were carried out alongside a standard curve consisting of different predefined concentrations of the process-related HCP. Detection was enabled by the corresponding biotin-conjugated detector antibody in combination with streptavidin-conjugated peroxidase and a colorimetric substrate.

Comparable HCP standard curves were obtained when applying different antibody mixtures to Total HCP (Figure 1) and their homologous antigen mixtures 80T:20L or 50T:50L (Figure 2) as assay standards.

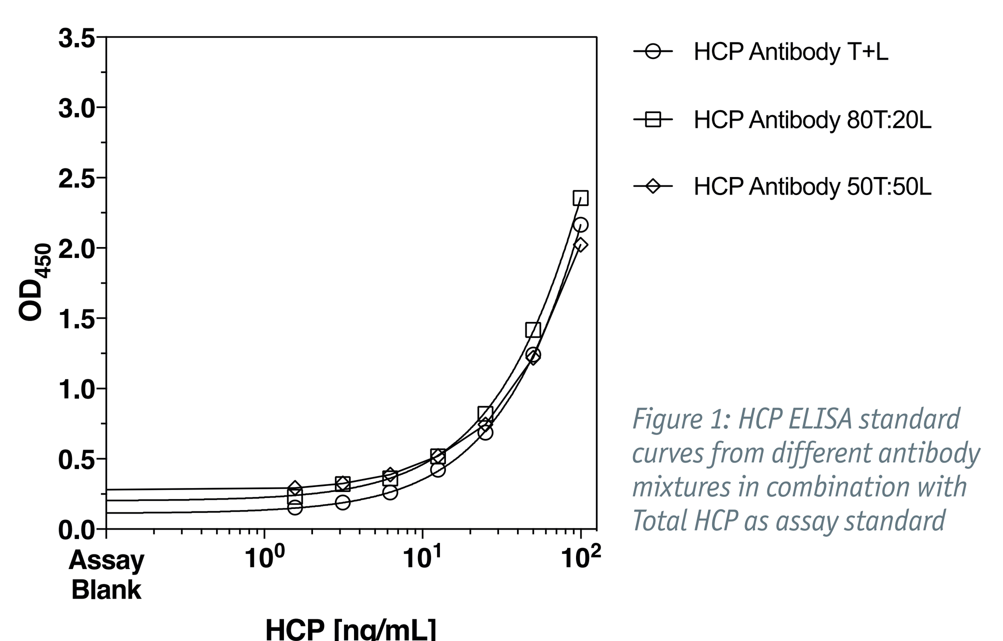

Figure 1: HCP ELISA standard curves from different antibody mixtures in combination with Total HCP as assay standard

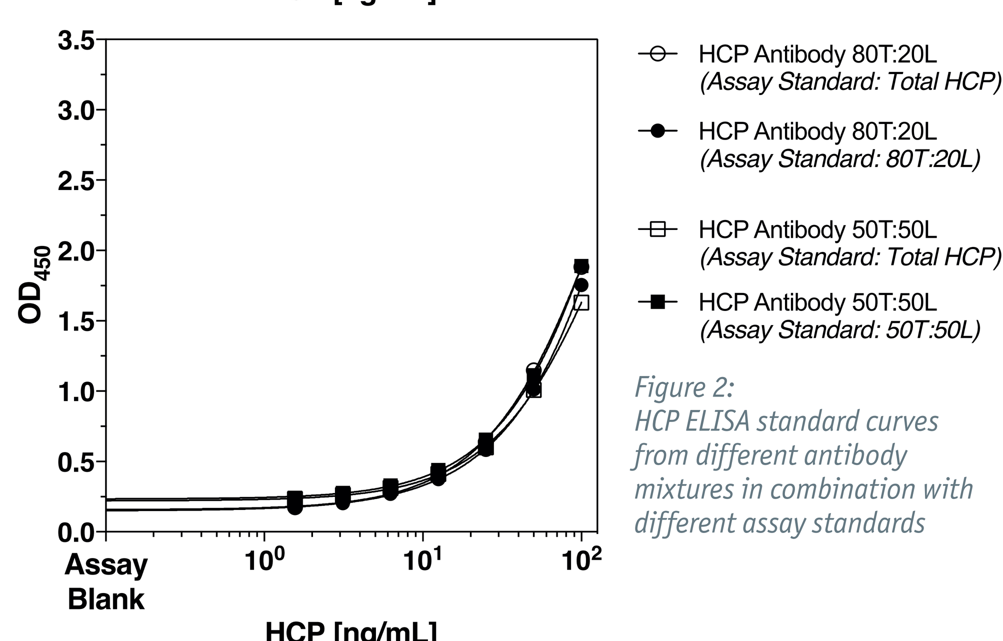

Figure 2: HCP ELISA standard curves from different antibody mixtures in combination with different assay standards

The HCP concentration measured in the drug substance clearly increased between HCP Antibody T+L (3.3 ppm), HCP Antibody 80T:20L (7.1 ppm) and HCP Antibody 50T:50L (11.2 ppm) when using Total HCP material as the assay standard. In contrast, comparable HCP levels were obtained for both T:L mixtures (8.7 ppm and 10.3 ppm) when using their homologous antigen mixtures, indicating that absolute standard curve reactivity does not bias the ELISA results while the specific antibody composition clearly impacts on assay sensitivity (Table 2). This is further supported by a comparative standard curve analysis (Figure 2).

HCP Impurity Content				
		Assay Standard		
		Total HCP	80T:20L	50T:50L
Antibody Mixture	HCP Antibody T+L	3.3 ppm ($CV_{n=3}$: 3.0%)	n/a	n/a
	HCP Antibody 80T:20L	7.1 ppm ($CV_{n=3}$: 9.2%)	8.7 ppm (n=1)	n/a
	HCP Antibody 50T:50L	11.2 ppm ($CV_{n=3}$: 10.1%)	n/a	10.3 ppm (n=1)

Table 2: HCP ELISA measurement of the drug substance using different antibody mixtures in combination with different assay standards
n: Number of independent ELISA runs performed to calculate the mean value; n/a: not applicable

Coverage Analysis

HCP antibody coverage was assessed using two orthogonal methods:

1. by fluorescent 2D WB

2. by IAC/2D DIGE

1. For fluorescent 2D WB analysis, the Total HCP sample was subjected to fluorescent minimal labeling prior to 2D electrophoresis. The total protein pattern was visualized by fluorescence scanning from the blot membrane. HCP-specific immunostaining was performed on the same membrane involving a suitable fluorescently labeled secondary antibody. The results from the 2D WB analysis are presented for HCP Antibody T+L (Figure 3) and HCP Antibody 50T:50L (Figure 4) as an example.

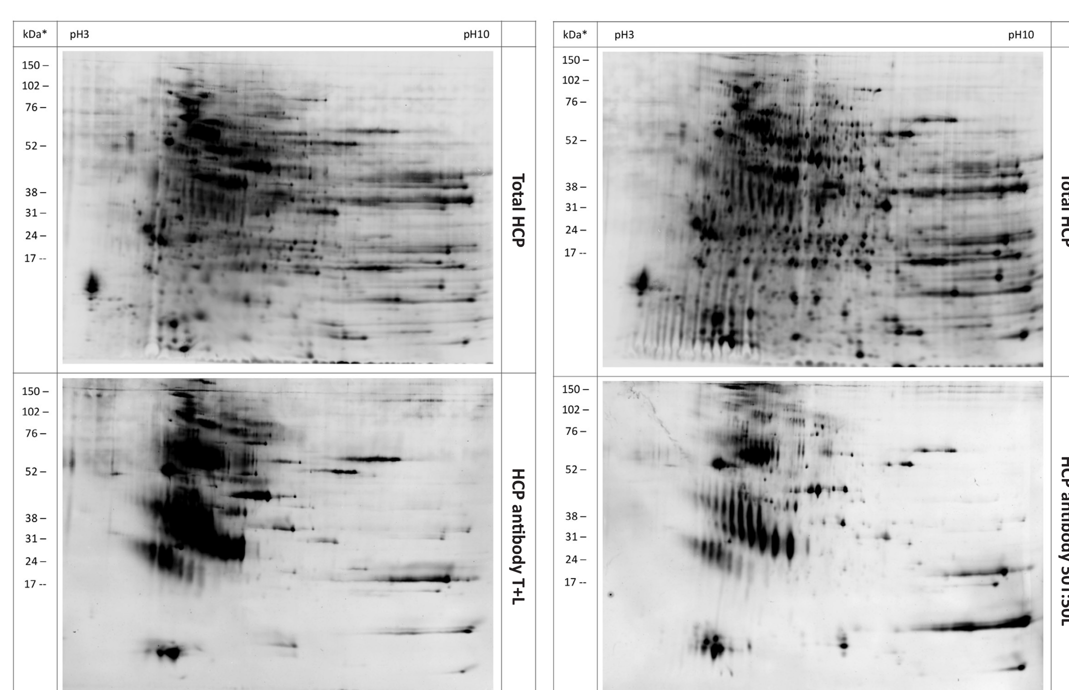

Figure 3 *Figure 4*
Fluorescently labeled Total HCP was subjected to 2D High Performance Electrophoresis (HPE) and subsequent Western blotting. Immunostaining was performed with HCP antibody T+L (Figure 3) and HCP Antibody 50T:50L (Figure 4). Corresponding total protein and immunostaining signals were obtained from the same membranes.

Fluorescent 2D WB based coverage determination revealed the successful detection of most high molecular weight (MW) proteins while coverage of medium to low MW proteins seemed much more limited.

Due to denaturing assay conditions in a Western blot approach and lower overall immunogenicity in the low MW range, HCP antibody performance is typically underestimated.

2. Method-related underestimation was addressed by applying IAC/2D DIGE as an orthogonal approach to evaluate HCP antibody coverage performance. The different HCP antibodies were immobilized on an affinity column and incubated with Total HCP. Bound HCPs were extracted and comparatively analyzed with the original Total HCP sample by 2D DIGE. The results are presented for HCP Antibody T+L and HCP Antibody 50T:50L (Figure 5) as an example.

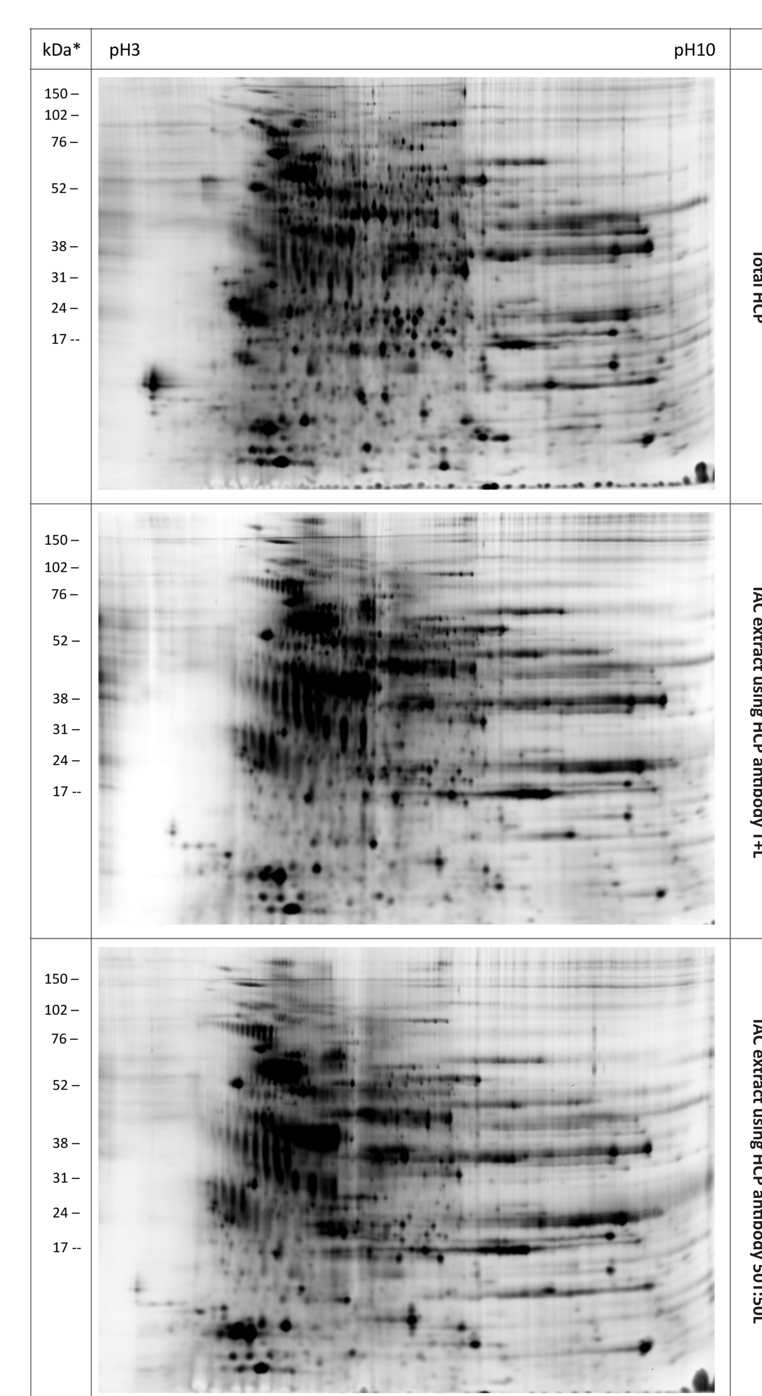

Figure 5: IAC was performed using immobilized HCP Antibody T+L and HCP Antibody 50T:50L and the Total HCP sample. The original Total HCP sample and the IAC extracts were subjected to fluorescent minimal labeling and 2D DIGE for qualitative comparative analysis.

2D DIGE revealed highly comparable HCP patterns from a qualitative point of view when analyzing the Total HCP sample and the respective IAC extracts. The results indicate successful coverage of a broad HCP spectrum from the Total HCP sample for the antibodies tested.

The detailed evaluation of fluorescent 2D WB and IAC/2D DIGE data sets involved Delta2D analysis software. The collective results are presented in Table 3.

HCP Antibody	Fluorescent 2D WB Coverage	IAC/2D DIGE Coverage
HCP Antibody T+L	49%	79%
HCP Antibody 50T:50L	50%	82%
HCP Antibody 80T:20L	47%	84%

Table 3: Collective coverage results from fluorescent 2D WB and IAC/2D DIGE

Data evaluation confirmed the findings from visual data inspection and revealed considerable discrepancies between coverage values determined by two orthogonal strategies due to the specific advantages and disadvantages of each analytical method.

Conclusions

Based on the results of this comparative study, HCP Antibody 50T:50L was selected for final HCP ELISA development including Total HCP as the assay standard for the following reasons:

- HCP ELISA is the default system for drug substance batch release testing
  » Significantly higher HCP impurity levels were measured by ELISA in highly purified drug substance
- HCP Antibody 50T:50L successfully covers a broad HCP spectrum of the Total HCP sample which serves as ELISA assay standard

The present case study demonstrates the importance of considering methodological details (such as the applied technical equipment) and method-related limitations when judging coverage values. For comprehensive performance testing, the use of orthogonal analytical strategies is highly recommended.

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