

An orthogonal approach for monitoring downstream process consistency and persistent HCP impurities

Host cell proteins offer researchers a new avenue to explore when designing new therapeutics but, like with any approach, there can be a number of challenges they present. How can these be overcome?

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Challenges of host cell protein detection

Host cell proteins (HCPs) are defined as process-related impurities in drug products and thus must be monitored during the pharmaceutical process development.

The challenges of HCP detection include: low levels of residual HCPs present in large excess of product protein; the assay must measure a large number of different protein analytes; and the population of HCP species may change during process development.¹ To validate the process, a suitable method for HCP monitoring is needed.

The enzyme-linked immunosorbent assay (ELISA) is the workhorse method for HCP testing due to its unmatched throughput capacity, sensitivity and selectivity. In addition, its wide dynamic range allows for reliable results and high reproducibility.

The critical reagents for the HCP ELISA are the anti-HCP antibodies. In case of weak or non-immunoreactive HCPs, the corresponding antibodies might be underrepresented, which may cause dilution-dependent non-linearity. Another challenge is that some HCPs may not be readily detected in the ELISA, even if the antibodies for particular HCPs are

present. A reason could be the antibody-antigen binding conditions or a lack of availability of HCP epitopes in the sample.

Mixed polyclonal antibodies (PAb) might show different abundancies, affinities and avidities against an HCP immunogen preparation, which could eventually lead to an over- or underestimation of any given HCP in a drug substance.² This article reviews the strengths and limitations of the ELISA regarding HCP monitoring and will demonstrate the necessity of orthogonal methods.

Market authorisation

The US Food and Drug Administration (FDA) and European Medicines Agency (EMA), as the two major global health authorities, require that host-cell derived proteins are separated and monitored during biopharmaceutical processes. The *US Federal Regulation Title 21 on Food and Drugs, Chapter I Part 610 General biological products standards Section 610.13 on Purity (21 CFR Sec 610.13)* mandates that 'products shall be free of extraneous material except that which is unavoidable in the manufacturing process described in the approved biologics licensed application'. As commonly accepted by health authorities, the HCP levels are in the limit of 1-100ppm (or 1-100ng HCP per mg therapeutic protein). In recent

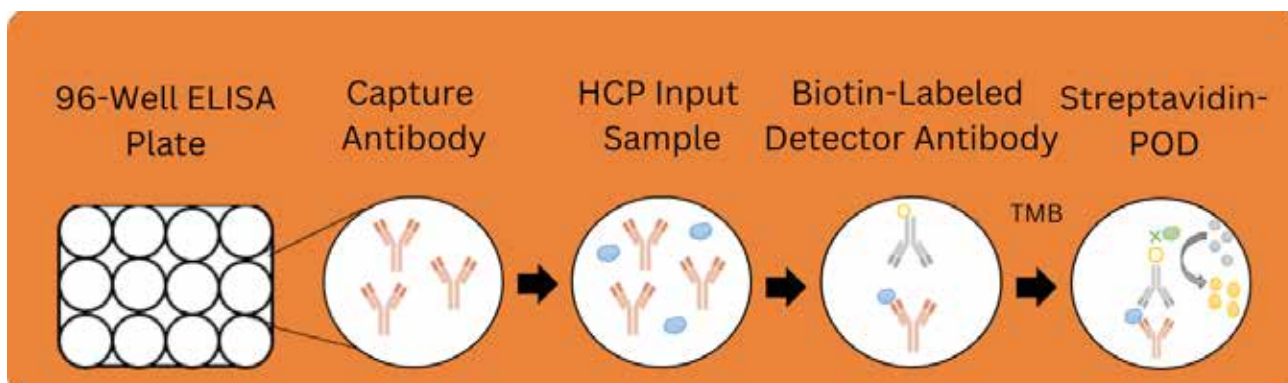


Figure 1: Schematic representation of the Sandwich ELISA set-up for HCP monitoring

years, detection methods have been improved in regard to sensitivity and volume capacity of data processing.

The rapid improvement in proteomics since creation of the guideline necessitates further levels of consideration. A new USP chapter, '1132.1 Residual Host Cell Protein Measurement in Biopharmaceuticals by Mass Spectrometry', addresses a crucial aspect of biopharmaceutical manufacturing.

ELISA as the 'gold standard' for routine quality control release testing

The sandwich format ELISA is commonly used in the biopharma industry for HCP detection and quantification using polyclonal anti-HCP antibodies for both the capture and the detection step. ELISA is a multi-analyte assay, which provides high sensitivity and a wide dynamic range. The throughput capacity can be increased by automation.² Validated assays can then be used for quality control (QC) testing in process pools. To evaluate the different steps

of a downstream process, a multi-analyte ELISA can be applied. Total immunoreactive HCP values are thereby obtained by comparing the ng/ml HCP concentration with mg/ml product concentration. The ratio is expressed in ng/ml or ppm. The highest HCP levels will be detected in the harvest cell culture fluid. During the downstream purification steps, the HCP content will decrease. To fall within the working range of the ELISA, the samples must be diluted and can then be compared with the HCP content in the final bulk/drug product. The general ease of handling of an HCP ELISA with limited requirements for equipment and personnel enables this method to be qualified and transferred into a drug manufacturing GMP environment.

The complexity of HCP populations

Therapeutics are commonly produced in Chinese hamster ovary (CHO) derived cells (eg, CHO-K1). Its genome was sequenced by Xu et al and has more than 24,000 predicted genes.⁴ Another bacterial cell line for the



	2D PAGE + WB	IAC + 2D DIGE	ELISA- or IAC-MS
Method description	Detection and direct visualisation of covered HCP on a membrane with antibody	Visualisation of covered HCP vs total HCP in an 2D DIGE approach and immunocapture on a column	Orthogonal approach for the determination of covered HCP species
Advantages	Does not require physiological buffer conditions – suitable for special sample types	Nearly native assay conditions, broad dynamic range and visual read-out	Native assay conditions and allows for simultaneous HCP quantification and identification
Limitations	Denaturing conditions, limited dynamic range	Co-detection of indirect binders and requires large amounts of antibodies	Based on database search and the comparison of the experimental raw data spectra and theoretical in silico generated MS/MS spectra

Table 1: Comparison of different methods for coverage analysis. Left: 2D SDS PAGE with Western blotting; middle: Immunoaffinity Chromatography following 2D DIGE analysis (2D DIGE-IAC); and right: LC-MS following immunocapture step (ELISA- or IAC-MS)

therapeutic production is *E. coli*, which has approximately 4,300 genes.⁵ Total lysate or cell culture supernatant is used as an antigen to generate HCP specific antibodies, typically by immunising goats or rabbits. The resulting polyclonal reagent is an assortment of IgGs and the critical factor for the functionality and suitability of the assay. It is important to ensure that the pool of ELISA antibodies covers a broad spectrum of the HCPs that potentially could persist in the final drug substance. Typically, coverage is determined by gel-based approaches.

As recommended in current guidelines, an orthogonal approach should be performed for reagent characterisation, applying methods such as 2D DIGE-IAC and IAC-MS. For problematic matrices, 2D Western Blot can be implemented, which does not require physiological buffer conditions. **Table 1** gives an overview of methods for coverage analysis.

The limitations of HCP ELISA and the usage of orthogonal methods

Naturally, an HCP ELISA only provides a total HCP signal, but does not provide information about the concentration of individual HCPs and their identities. Thus, the HCP ELISA will not be able to detect unexpected HCPs during the drug manufacturing processes that did not occur (in relevant quantities) during mock sample preparation. Another disadvantage is the dependency on polyclonal antibodies, which are time-consuming in their generation, and which can lack reactivity towards a subset of antigens, making this assay insensitive for certain HCPs. The limitations of HCP ELISAs can largely be overcome by applying

orthogonal methods to complement the HCP ELISA in detecting co-purifying HCPs.

An orthogonal method, which is unbiased towards the composition of the HCP profile in different drug manufacturing batches, is the combined usage of liquid chromatography coupled with mass spectrometry (LC-MS). In this method, an input sample undergoes pre-chromatographic treatment to help the separation of complex samples and increase the fragmentation and ionisation of peptides, which is required for the MS instrument to detect the individual fragment ions and match their sequence to database information. The LC-MS provides high precision and accuracy, and allows for identification of HCPs, independent of their immunogenic potential. A critical part is that protein identification is based on the common database search and the comparison of the experimental raw data spectra and theoretical in silico generated MS/MS spectra. Thus, it is essential to perform data processing with well-defined, consistent and documented parameters.⁶ If the database is adapted for the proteome of the selected production cell line, MS will allow for speedy implementation of novel production process monitoring due to its independence of antibodies for immunocapture. In this context, MS can be used to significantly improve risk assessment in HCP control.⁷

Further remarks and conclusion

Recent surveys among key members of the biopharmaceutical industry and participants of the BioPharmaceutical Emerging Best Practice

Association (BEBPA) and Bioprocessing Summit conferences revealed that the use of orthogonal methods constitutes a solid HCP surveillance strategy including identification and quantification of potentially high-risk proteins. The introduction of the new USP chapter 1132.1 on residual HCP measurement in biotherapeutics by MS is a significant step forward in analytical techniques. However, it's important to acknowledge that there are still some limitations associated with the use of MS for HCP analysis, particularly in terms of complete quantification.

One of the main challenges with MS-based HCP analysis is the lack of standardised calibration materials for absolute quantification. Unlike the ELISA, which typically employs protein standards for quantification, MS often relies on relative quantification methods. This makes it difficult to determine the exact concentration of individual HCPs in a sample, as there is no calibration approach generally accepted for absolute quantification.

To provide the broadest information on the total HCP content, it is recommended to use ELISA and LC-MS, the latter especially for confirming quantitative ELISA results. Quantitative LC-MS/MS methods are not typically required for routine HCP process testing for batch release, but they can provide useful information about high-risk HCPs. Once individual high-risk proteins are identified, a molecule-specific ELISA assay can be developed, which might be easier to implement. For individual HCPs with substantiated adverse clinical affects, new residual acceptance levels and tiered toxicology assessments are critical.

The BioPhorum Development Group published a comprehensive review article regarding monitoring and controlling of high-risk proteins.⁸ It is hereby declared that the assay natures of both the HCP ELISA and the LC-MS rely on completely different approaches. Thus, the HCP information by either technique is truly orthogonal, and, when combined, they provide the broadest possible image on downstream process consistency and remaining impurities in the final drug substance. The identification of problematic HCP species also allows for in-depth risk assessment of putatively remaining HCP impurities in the final drug substance, which supports the drug safety profile, increases the chances for successful Market Authorisation Application (MAA) and altogether benefits patients' safety.

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