OF HCP ELISA COVERAGE METHODS

ALPHALYSE™

Index

Intro 2
SDS-PAGE based methods 5
ELISA-MS method (Immunocapture & LC-MS/MS) 10
Summary 12
References 13

Intro

Product impurities in biopharmaceuticals

Most biopharmaceutical products are expressed in host cells, such as bacteria, yeast or mammalian cell lines.

During manufacturing, a wide range of impurities reside along with the product itself, including host cell proteins (HCPs). Since HCPs might affect product quality, safety and efficacy, they are classified as one of the most critical process-related impurities in biologics.

Therefore, the HCP quantity must be determined by HCP assays before regulatory agencies can allow the product to move through clinical trials and reach patient administration.

More specifically, guidelines state that:

"*HCP assays are an essential part of purification process development and help ensure manufacturing consistency.*"

and " *… reproducible and reliable HCP assays may be required to measure residual HCPs remaining in the drug substance…"* [1].

Intro

Methods for evaluation of product impurities / Host Cell Proteins

The most common approach for HCP analysis is ELISA using anti-HCP antibodies. The ELISA provides a semi-quantitative HCP value, can be automated and can be performed with high throughput. However, it is crucial to evaluate the ELISA antibodies' ability to recognize the population of HCPs.

The evaluation is often performed to confirm antibody sensitivity for a broad range of HCPs throughout the purification steps and in the drug substance, as well as fit the calibration standard.

On the next pages, we compare the ELISA coverage evaluation methods based on 2D SDS-PAGE with the new ELISA-MS method.

SDS-PAGE
based methods

Measuring ELISA coverage by 2D SDS-PAGE and Western blot

When combining SDS-PAGE and Western blot, proteins are separated in two dimensions based on their isoelectric points and molecular weights. They are then transferred to a Western Blot membrane and detected by immunostaining using the ELISA antibodies.

The coverage percent is thus calculated from the number of spots detected by the HCP ELISA antibodies versus the number of spots from fluorescent/silver dye binding to all proteins in the gel (total protein stain).

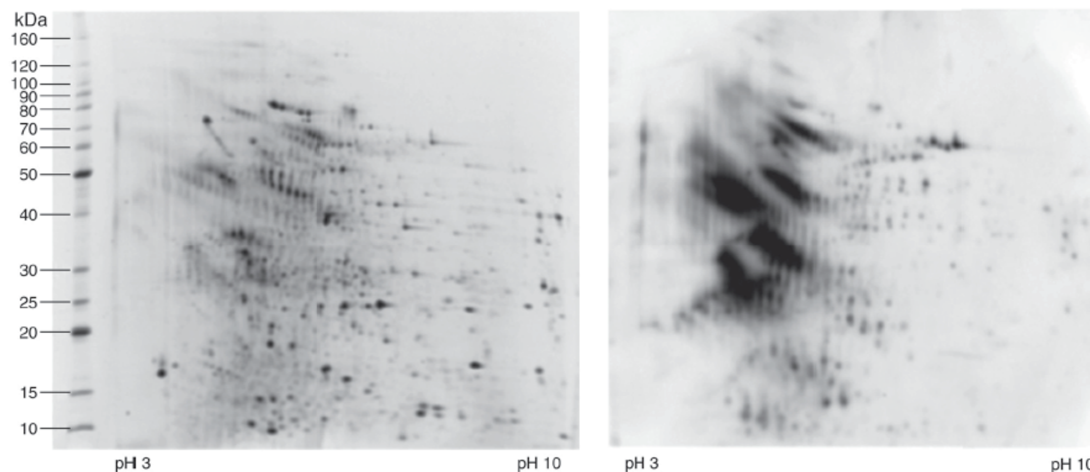

Figure 1. Left: A representative 2D SDS-PAGE gel stained with a fluorescent stain.
Right: Western blot analysis of the gel shown to the left.

SDS-PAGE based methods

Pros and cons of 2D SDS-PAGE and Western blot approach

The advantage of this method is that individual spots can be counted if a good separation of individual HCPs is achieved, but it also has several limitations:

- Since HCP antigens are denatured during the process, there's a risk that the HCP might not be recognized by the antibody.
- Furthermore, it is challenging to optimize the efficiency of transfer from gels to blotting membranes, and to match blot spots to spots on duplicate gel/blot stained for total protein.

	Pros	Cons
2D SDS-PAGE/ Western blot	Good separation of individual HCPs allows for individual spot counting.	HCP antigens are denatured, may not represent what is seen in the immunological assays (e.g. ELISA).
		High variability – numerical "percent coverage" values vary widely with the same material tested within a single lab and between different laboratories.
		Transfer efficiency of a broad range of HCPs difficult to optimize, leading to underestimates due to over-transfer through the membrane or failure to transfer from gel, dependent on molecular weight.
		Matching spots between blots and gels are difficult and not standardized.

Table 1. USP 39 Published General Chapter. Table 3, <1132>,
Residual Host Cell Protein Measurement in Biopharmaceuticals. May 1, 2016

SDS-PAGE
based methods

Measuring ELISA coverage by immunoaffinity and SDS-PAGE

When combining immunoaffinity and SDS-PAGE, a mock is loaded onto a resin with covalently immobilized HCP ELISA antibodies.

Nonspecifically bound HCPs are then washed off the resin and HCPs captured by the antibodies are eluted from the resin.

HCPs that were captured by antibodies and eluted are then run on an SDS-PAGE gel with the total HCPs running concurrently. Finally, the coverage percent is determined by comparing the two SDS-PAGE gels.

SDS-PAGE based methods

Pros and cons of immunoaffinity and SDS-PAGE approach

The clear advantage of this approach is that the HCPs bind to the anti-HCP antibodies in conditions that mimics those of the ELISA.

Also, the protein spots can be analyzed directly without blotting to a PVDF membrane, which might not transfer all HCPs.

However, by relying on antibodies to bind HCPs, there is a risk that coverage is underestimated if some HCPs are bound to tightly and do not elute from the resin.

The preparation of the resin is highly important and requires 10-15 mg of antibody. Results depend on loading and elution conditions, leading to high method variability.

	Pros	**Cons**
Immunoaffinity binding/1- or 2D SDS-PAGE	HCPs bind to antibody resin in solution under native conditions similar to the sandwich immunoassay.	May underrepresent some HCPs if they are bound too tightly and do not elute, resulting in an underestimation of coverage.
	Analysis of spots in gels does not require immunoblotting and is simpler because the problems of transfer are avoided.	Preparation of anti-HCP resin must be done carefully and may be difficult to reproduce. Results can be dependent on resin loading and elution conditions.
		Proteins at low concentration that only shows up in Western blots will not be detected.

Table 2. USP 39 Published General Chapter. Table 3, <1132>,
Residual Host Cell Protein Measurement in Biopharmaceuticals. May 1, 2016

ALPHALYSE™

SDS-PAGE
based methods

Further considerations for SDS -PAGE based methods

Both SDS-PAGE based methods have the limitation that they tend to underestimate the true antigen binding in immunoassays, thus also underestimating the HCP coverage.

Despite their high variability, they can estimate the HCP Coverage percentage for platform ELISA as well as commercial kits.

Since both use reducing 2D gel electrophoresis to separate HCPs, numerical coverage comparisons should be used with caution because of the many variables influencing the results, even with the same reagents in the same laboratory.

Finally, the uncertainty of spot counting and matching blot spots to total -protein stained gels cannot be stressed enough. Software applications are ~~now~~ available, but this way of counting proteins remains difficult and non-standardized.

ALPHALYSE™

ELISA-MS method (Immunocapture & LC-MS/MS)

Measuring HCP antibody coverage by ELISA-MS method

In this novel method, HCP antibodies are immobilized in ELISA 96-well plate and incubated with either a product-specific mock cell lysate or an early process sample.

Unbound antigens are removed by a washing step. Bound antigens are then digested in the plate by adding proteases and followed by mass spectrometry analysis using LC-MS/MS. In this way, HCPs bind to antibodies in their native state, as in the sandwich ELISA, and each protein is identified by name.

The coverage percentage is determined by comparing with LC-MS/MS results for the non-immunocaptured sample. Unspecific binding is evaluated by a parallel analysis of antigen binding to negative control antibodies in a separate 96-well plate.

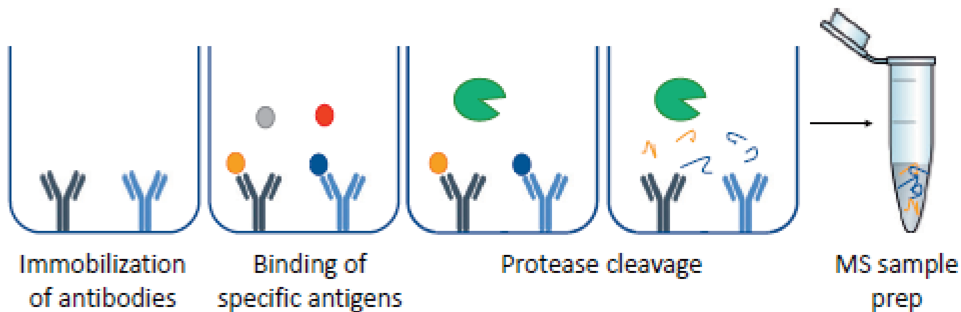

Figure 2. Illustration of the ELISA-MS method

ELISA-MS method (Immunocapture & LC-MS/MS)

Pros and cons of ELISA-MS method

The list of benefits from applying mass spectrometry as an alternative to 2D SDS-PAGE dependent methods is quite extensive:

- By using a SCIEX TripleTOF 6600 system, a high repeatability is achieved along with high sensitivity down to low ppm level.
- A complete list of individual HCPs recognized by the ELISA antibodies is obtained and compared to the list of total proteins in the mock or early process sample.
- The coverage percent is calculated with high accuracy and low variability.

ELISA-MS provides significant more information and higher resolution than SDS-PAGE methods. However, several aspects are important to perform the analysis:

- Skilled staff with mass spectrometry expertise
- Expensive LC-MS instrumentation

Summary

SDS-PAGE dependent methods vs. ELISA-MS

In summary, commercial ELISA assays are a valuable tool in process development of biologics, if their coverage of early process and final drug HCPs have been evaluated.

The new coverage analysis approach, ELISA-MS, offers various advantages compared to older methods that depend on 2D SDS-PAGE.

Most important, ELISA-MS has

- low variability
- high repeatability
- provides a list of individual proteins recognized by the anti-HCP antibodies
- and a list of HCPs in mock cell lysate or early process sample

References

[1] European Medicines Agency, 2005
GUIDELINE ON TEST PROCEDURES AND ACCEPTANCE CRITERIA FOR NEW BIOTECHNOLOGICAL/BIOLOGICAL VETERINARY MEDICINAL PRODUCTS

U.S. Department of Health and Human Services – Food and Drug Administration, **2019**
Immunogenicity Testing of Therapeutic Protein Products — Developing and Validating Assays for Anti-Drug Antibody Detection: Guidance for Industry

The United States Pharmacopeia, *USP 39 – NF 34,* **2016**
Residual Host Cell Protein Measurement in Biopharmaceuticals

Pilely *et al:* A novel approach to evaluate ELISA antibody coverage of host cell proteins—combining ELISA-based immunocapture and mass spectrometry (ELISA-MS), *Biotechnology Progress*, **2020**

https://www.alphalyse.com/coverage