BioGenes ASSAYS

Common Challenges of Host Cell Protein ELISA Development

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The technical and methodological challenges of a successful Host Cell Protein (HCP) ELISA development are manifold.The selection of a suitable antibody for the immunodetection of a given HCP mixture is crucial. First of all, an important decision must be made regarding the use of affinity-purified anti-HCP antibodies versus the use of a Protein A-purified total IgG fraction. Furthermore, the authorities request a sufficient coverage of the selected antibody, which is usually determined by 2D electrophoresis/Western Blot. The E.coli case studies presented here address two different aspects: (1) We discuss the common advantages and disadvantages of an affinity-purified antibody and its corresponding total IgG fraction for HCP ELISA measurement of process samples. (2) We discuss the impact of the technical equipment on HCP coverage determination using 2D electrophoresis/Western Blot.

Antibody Generation, Purification and Modification

E.coli-HCP-specific antisera were raised by immunization of rabbits with the process-related E.coli mock HCP preparation (referred to as mock HCP) as the immunogen. Individual rabbit antisera were pooled (referred to as antiserum pool) and used as starting material for the preparation of either affinity-purified antibodies (referred to as specific IgG) or the corresponding Protein A-purified total IgG fraction (referred to as total IgG).

Subsequently, a part of both capture antibody preparations was separated and conjugated to biotin to prepare suitable detector antibodies for ELISA testing in a common sandwich setup.

Antibody Testing by 1D Western Blotting

The performance of both antibody preparations was qualitatively analyzed by 1D Western blotting. The respective process-related mock HCP, as well as a panel of process samples, was subjected to electrophoresis and subsequently transferred onto nitrocellulose membrane. The total protein pattern was visualized using reversible Ponceau S staining (Figure 1) prior to HCP-specific immunostaining (Figures 2 to 4). The staining results of the different antibodies and the unpurified antiserum pool were compared. Detection was enabled using a suitable anti-Rabbit IgG Peroxidase conjugate followed by incubation with a colorimetric substrate.

Significantly different immunostaining patterns, particularly at low molecular weights (approx. <58 kDa), were observed for the process-related mock HCP and the early process sample when comparing the unpurified antiserum and the derived antibodies (Figures 2 to 4, lanes 5 and 6). While the total IgG mimicked coverage performance of the unpurified antiserum pool well, immunostaining with the specific IgG revealed fewer and less intense signals in the low molecular weight region (highlighted in Figures 1 to 4).

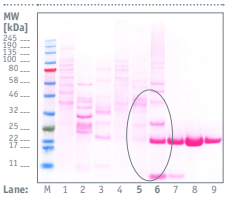

Figure 1: Western Blot of E.coli mock and process samples with Ponceau S staining for total protein
*The molecular weight (MW) is indicated on the left (prestained protein standard, **M**). Protein amount per lane: 5 µg each; **Lanes 1–4:** irrelevant mock HCPs I, II, III, IV; **Lane 5:** process-related mock HCP; **Lane 6:** early process sample; **Lane 7:** intermediate process sample I; **Lane 8:** intermediate process sample II; **Lane 9:** bulk drug substance*

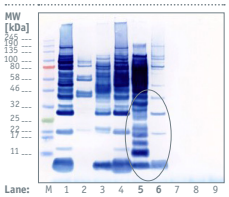

Figure 2: Western Blot of E.coli mock and process samples immunostained with anti-serum pool
*The molecular weight (MW) is indicated on the left (prestained protein standard, **M**). Protein amount per lane: 5 µg each; Anti-serum dilution of 1:250; **Lanes 1–4:** irrelevant mock HCPs I, II, III, IV; **Lane 5:** process-related mock HCP; **Lane 6:** early process sample; **Lane 7:** intermediate process sample I; **Lane 8:** intermediate process sample II; **Lane 9:** bulk drug substance*

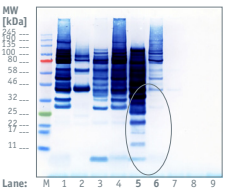

Figure 3: Western Blot of E.coli mock and process samples immunostained using specific IgG
*The molecular weight (MW) is indicated on the left (prestained protein standard, M). Protein amount per lane: 5 µg each; Antibody concentration of 20 µg/mL; **Lanes 1–4:** irrelevant mock HCPs I, II, III, IV; **Lane 5:** process-related mock HCP; **Lane 6:** early process sample; **Lane 7:** intermediate process sample I; **Lane 8:** intermediate process sample II; **Lane 9:** bulk drug substance*

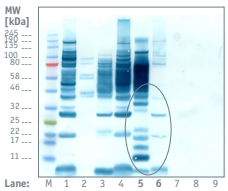

Figure 4: Western Blot of E.coli mock and process samples immunostained using total IgG
*The molecular weight (MW) is indicated on the left (prestained protein standard, M). Protein amount per lane: 5 µg each; Antibody concentration of 20 µg/mL; **Lanes 1–4:** irrelevant mock HCPs I, II, III, IV; **Lane 5:** process-related mock HCP; **Lane 6:** early process sample; **Lane 7:** intermediate process sample I; **Lane 8:** intermediate process sample II; **Lane 9:** bulk drug substance*

HCP ELISA Measurement

For a comprehensive assessment of antibody performance, both antibody preparations were further tested by ELISA as the default system in a more quantitative manner.

ELISA sample determination was performed for the early process sample and the bulk drug substance. For quantification, unknown sample measurement was carried out alongside a standard curve consisting of different predefined concentrations of the process-related mock HCP. The detection of captured HCP was enabled by the biotin-conjugated detector antibody, in combination with streptavidin-peroxidase and a colorimetric substrate.

The slope of the HCP standard curves derived from both antibodies indicated much higher assay sensitivity for the specific IgG (Figure 5). This was further supported by the dilution curves obtained for the bulk drug substance (Figure 6), clearly showing lower absolute assay reactivity in case of total IgG.

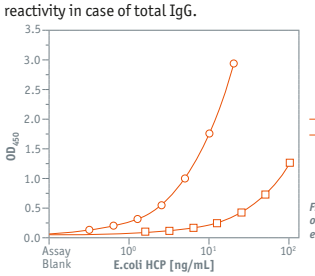

Figure 5: HCP standard curves observed when using different antibodies

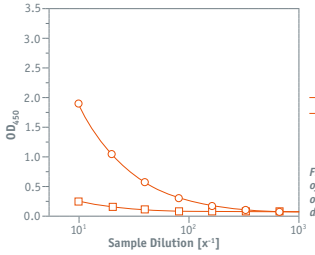

Figure 6: Dilution curves of the bulk drug substance observed when using different antibodies

ELISA determination of the early process sample resulted in a higher HCP amount of 5036.8 ppm using the total IgG when compared with specific IgG (3426.8 ppm). In contrast, highly comparable impurity levels (19.0 ppm vs. 15.1 ppm) were determined in the bulk drug substance.

	Relative E.coli HCP Impurity Content* (Early Process Sample)	
Antibody Preparation:	**total IgG**	**specific IgG**
Sample Dilution		
1:100	4821.5 ppm	>ULOQ
1:200	4883.5 ppm	3399.5 ppm
1:400	5280.3 ppm	3411.7 ppm
1:800	5285.3 ppm	3516.0 ppm
1600	4913.3 ppm	3462.7 ppm
1:3200	<LLOQ	3421.3 ppm
1:6400	<LLOQ	3349.3 ppm
1:12,800	<LLOQ	<LLOQ
Mean:	**5036.8 ppm**	**3426.8 ppm**
SD:	**227.0 ppm**	**57.0 ppm**
CV:	**4.5%**	**1.7%**

Table 1: HCP ELISA determination of the early process sample using different antibodies
***CV:** coefficient of variation; **SD:** standard deviation; ***:** calculated as the ratio [ng HCP/mg total protein] (corrected for dilution); **LLOQ:** lower limit of quantitation; **ULOQ:** upper limit of quantitation*

	Relative E.coli HCP Impurity Content* (Bulk Drug Substance)	
Antibody Preparation:	**total IgG**	**specific IgG**
Sample Dilution		
1:10	19.3 ppm	15.3 ppm
1:20	18.5 ppm	14.8 ppm
1:40	19.3 ppm	14.9 ppm
1:80	18.8 ppm	14.9 ppm
1:160	<LLOQ	15.1 ppm
1:320	<LLOQ	15.8 ppm
1:640	<LLOQ	<LLOQ
1:1280	<LLOQ	<LLOQ
Mean:	**19.0 ppm**	**15.1 ppm**
SD:	**0.4 ppm**	**0.4 ppm**
CV:	**2.0%**	**2.6%**

Table 2: HCP ELISA determination of the bulk drug substance sample using different antibodies
***CV:** coefficient of variation; **SD:** standard deviation; ***:** calculated as the ratio [ng HCP/mg total protein] (corrected for dilution); **LLOQ:** lower limit of quantitation*

Conclusion

Even though 1D Western blot analysis indicated a loss of certain antibody species during antigen-specific affinity purification, we recommend selecting the specific IgG for final ELISA development for the following reasons:

- → HCP ELISA is the default system for drug substance batch release testing
- → Highly similar HCP impurity levels were measured by ELISA in the crucial bulk drug substance indicating loss of only 'irrelevant' antibodies species during affinity purification
- → The use of specific IgG enabled clearly higher assay sensitivity which is crucial for reliable and robust measurement of HCP trace amounts

Impact of 2D Electrophoresis Instrumentation on Sample Resolution

Coverage determination by means of 2D Western blotting is widely performed for proving HCP antibody suitability for a specific process. Technological progress has been achieved regarding 2D electrophoresis instrumentation by setting up horizontal flatbed electrophoresis for large 2D gels. Spot resolution of complex HCP mixtures greatly improves when applying High Performance Electrophoresis (HPE), when compared to traditional electrophoresis systems, which involve a vertical setup for the second dimension. In particular, low molecular weight proteins are visualized well using HPE and take part in subsequent analyses (Figure 1).

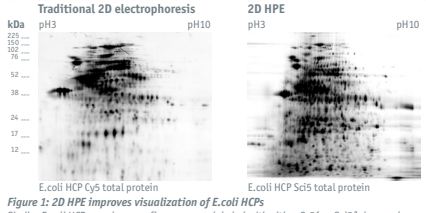

Figure 1: 2D HPE improves visualization of E.coli HCPs
*Similar E.coli HCP samples were fluorescence- labeled with either Cy5[1] or Sci5[2] dyes and analyzed by 2D electrophoresis involving: **A:** traditional (vertical) instrumentation[3] or **B:** horizontal HPE[4] for running the second dimension. The visualization and resolution of protein spots in B is greatly improved including low molecular weight proteins.*

[1] CyDye DIGE Fluor minimal dye (GE Healthcare)
[2] SERVA Lightning Sci5 (SERVA)
[3] Second dimension gel and apparatus: DALT Gel 12.5 and Ettan DALTsix Electrophoresis Unit (GE Healthcare)
[4] Second dimension gel and apparatus: HPE™ large gel 12.5% and HPE™ BlueTower (SERVA)

Sample Resolution Impacts 2D Western Blot Based Coverage Values

Up-to-date 2D electrophoresis instrumentation enables more detailed visualization of sample HCPs including the low molecular weight (LMW) proteins. Naturally, LMW proteins are less immunogenic than larger protein species, and tend to be difficult to detect simultaneously on the same 2D Western blot. There is an increased challenge of achieving sufficient coverage of the highly diverse HCP population, which differs not only in abundance but also in immunogenicity. Consequently, 2D Western blot based coverage values tend to be lower as compared to past years.

The case study presented here demonstrates how the representation of the same E.coli derived HCP mock sample changes upon improving the second dimension. Anti-HCP antibody coverage consequently dropped from 89% to 69% (Figure 2).

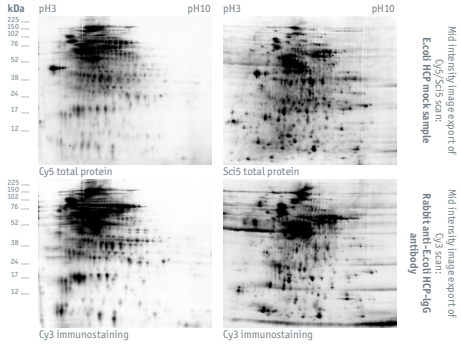

Figure 2: Comparative 2D Western blot based coverage analysis on an E.coli HCP mock sample involving traditional 2D electrophoresis or 2D HPE
Fluorescent 2D Western blot of a Cy5/Sci5-dye labeled E.coli HCP mock sample followed by immunostaining with rabbit anti-E.coli HCP-IgG[5] antibody (detected by anti-rabbit-Cy3 secondary antibody) Left panel: 2D electrophoresis instrumentation included a traditional (vertical) apparatus for running the second dimension; coverage was determined to be 89% in a semi-electronic manner involving ImageMaster 2D Platinum 7.0 software[6]
Right panel: 2D electrophoresis instrumentation included a horizontal HPE apparatus for running the second dimension; coverage was determined to be 69% in a semi-electronic manner involving Delta2D version 4.7 software[7]

[5] generated by BioGenes
[6] ImageMaster™ 2D Platinum 7.0 (GE Healthcare)
[7] Delta2D Version 4.7 (DECODON)

Conclusion

The technological progress in visualizing total protein populations of mock and process samples by 2D electrophoresis causes new challenges in coverage determination by means of 2D Western blotting. Realistic Western blot-based coverage values tend to be lower than previously expected for suitable antibody preparations.

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