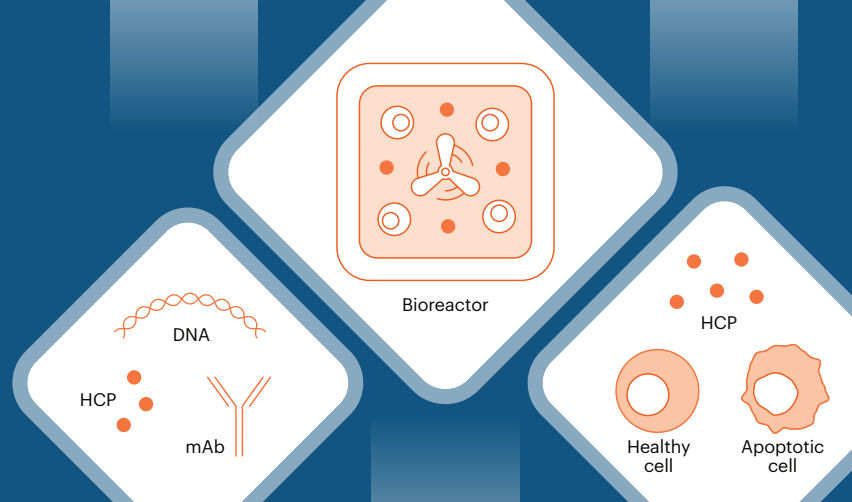


Characterization of a Pooled CHO Null Cell Harvest Cell Culture Fluid Analytical Reference Material



Introduction

The characterization of host cell proteins (HCPs) is a critical consideration in ensuring the safety, quality, and efficacy of biopharmaceutical products. Chinese Hamster Ovary (CHO) cells are the primary production hosts in the biopharmaceutical industry due to their capacity to generate high-quality therapeutic proteins, with yields up to 10g/L in fed batch monoclonal antibody (mAb) production. However, CHO cells also produce endogenous HCPs that may persist as impurities during downstream purification, posing potential immunogenic and quality risks¹.

It is therefore imperative to identify and quantify these impurities and then adjust manufacturing processes to minimize them. Despite advancements in HCP analytical methods (both immunoassay and liquid chromatography-mass spectrometry (LC-MS)), the industry continues to face challenges due to the absence of standardized, well-characterized reference materials that can adequately reflect the diversity and complexity of HCP populations encountered across different CHO production processes. Existing methods often rely heavily on process-specific samples or commercial antibodies, which may lack comprehensive coverage and comparability, creating gaps in HCP detection, assay development, and regulatory submissions.

The [USP CHO Null Cell Harvest Cell Culture Fluid \(HCCF\) Analytical Reference Material \(ARM\)](#) is a complex mixture generated by combining three distinct CHO HCCF batches in equal proportions. This approach of combining the different CHO cell lines and different growth conditions was used to generate a diverse CHO HCP population which is essential for comprehensive antibody coverage, robust assay development, and meaningful representation of CHO HCP species typically encountered across varied bioprocessing conditions. These sources include:

1. Null Cell CHOK1SV: grown under standard growth conditions and transfected with empty (null) plasmid.
2. Null Cell CHOK1: pooled replicates grown under standard growth conditions, and non-transfected.
3. Null Cell CHOK1: pooled replicates grown under variable (pH, temperature, osmolality, dissolved oxygen) growth conditions, and non-transfected.

To comprehensively characterize the HCP profile of our pooled HCCF, the following analyses were conducted:

1. Reduced sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)
2. 2D Western Coverage Analysis
3. HCP LC-MS/MS Analysis

The combined extended characterization ensures a robust understanding of the CHO null cell HCCF HCP composition. Below are details of each method used, and the results obtained.

Characterization methods used for the USP CHO Null Cell HCCF ARM

Reduced SDS PAGE

The CHO Null Cell HCCF sample was prepared under reducing conditions and separated on a gradient SDS-PAGE gel to visualize the overall protein content. Staining revealed a diverse distribution of protein bands, reflecting the complexity typical of host cell-derived mixtures

As described in [USP <1132> Residual Host Cell Protein Measurement in Biopharmaceuticals](#), SDS-PAGE serves as a critical supporting tool in HCP analysis, ideal for large-scale screening to confirm sample consistency, detect non-product proteins, and monitor manufacturing reproducibility.

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Results

The CHO Null Cell HCCF exhibits the typical SDS-PAGE profile characteristic of a crude protein sample: multiple bands of different sizes, reflecting the presence of various proteins within the sample. This means that in the SDS-PAGE gel, instead of seeing individual, well-defined bands that represent single, purified proteins, you observe diffuse or smeared regions. These smears suggest that the sample contains a complex mixture of many proteins with overlapping molecular weights rather than a few distinct species (**Figure 1**).

2D Western Coverage Analysis

Two-dimensional gel electrophoresis followed by Western blotting was conducted using three commercially available anti-CHO HCP polyclonal antibodies. This orthogonal method, as discussed in [USP <1132> Residual Host Cell Protein Measurement in Biopharmaceuticals](#) provides a semi-quantitative assessment of immunoreagent coverage across diverse HCP populations and supports fit-for-use qualification of immunoassay reagents.

Western blotting begins with the separation by two-dimensional (2D) gel electrophoresis, a powerful analytical technique used to separate complex mixtures of proteins based on two distinct physical properties: isoelectric point (pI) and molecular weight (MW).

1. In the first dimension, proteins are separated by isoelectric focusing (IEF), where proteins migrate through a pH gradient until they reach a point where their net charge is zero.
2. In the second dimension, the proteins are further separated by SDS-PAGE, which sorts proteins based on their molecular weight.

Result

This separation results in a 2D gel with distinct spots, each representing a different protein in the sample (**Figure 2**). Proteins are then visualized using a protein-specific stain (e.g. fluorescent, silver).

Next, the proteins resolved in 2D gels were transferred onto membranes and probed with three distinct anti-CHO HCP antibodies:

1. Cygnus® Anti-CHO HCP 3G Resupply Antibody
2. BioGenes Anti-CHO HCP Kit D Antibody
3. Cytiva Anti-CHO HCP Antibody

Coverage analysis determines the extent to which polyclonal antibodies detect individual HCPs within a sample. As per USP <1132>, this is crucial because not all antibodies

Figure 1. SDS-PAGE of CHO Null Cell HCCF ARM.

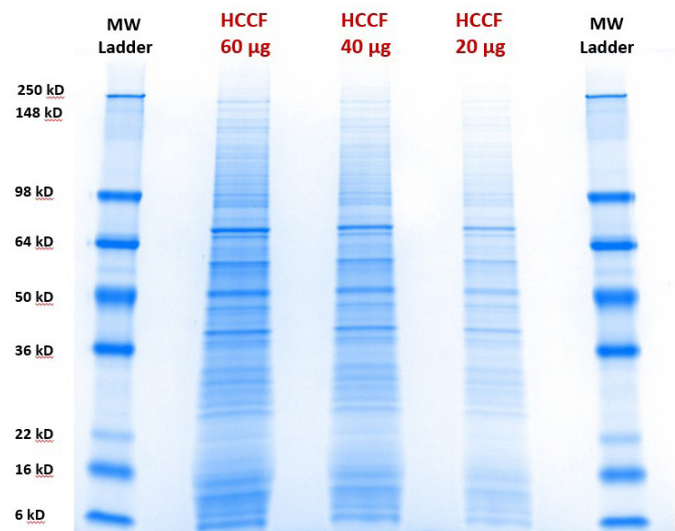
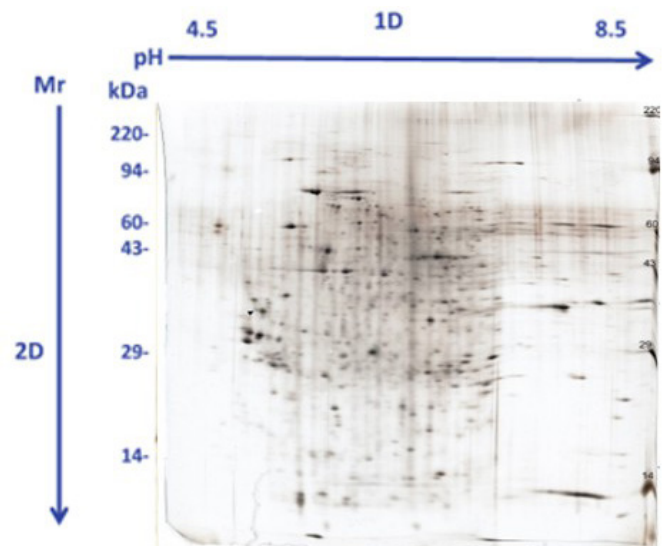


Figure 2. 2D Silver Staining of CHO Null Cell HCCF ARM.



will recognize all HCPs uniformly due to differences in immunogenicity. 2D Western blotting thus reveals antibody binding across a broad protein population and provides essential context for interpreting total HCP values obtained from ELISA assays.

Result

An example of the coverage analysis can be seen in **Figure 3**. All of the antibodies tested provided detection of high and low MW as well as high and low pI proteins with similar levels of overall coverage. A summary of coverage analysis for the three antibodies is provided in **Table 1**.

Table 1. Summary of 2D Western Coverage Analysis.

	Cygnus	BioGenes	Cytiva
Total Protein Spots	1685	1911	1764
Spots Detected by Antibody	1089	1450	1165
Coverage	65%	76%	66%

LC-MS/MS analysis for CHO Null Cell HCCF

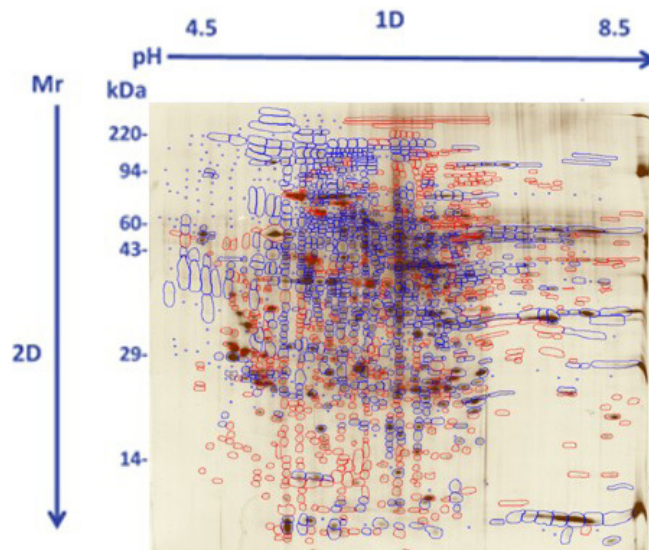
In alignment with [USP <1132.1> Residual Host Cell Protein Measurement in Biopharmaceuticals by Liquid Chromatography-Mass Spectrometry](#), which outlines best practices for LC-MS based HCP analysis, we analyzed the CHO Null HCCF using sequential window acquisition of all theoretical mass spectra (SWATH) LC-MS/MS. Relative quantification of HCPs was performed using a non-recombinant, naturally produced internal standard protein. Following the guidance in USP <1132.1> helps ensure the method is suitable for comprehensive impurity profiling and supports orthogonal assessment of residual HCPs.

This type of analysis facilitated the deeper understanding of the potentially problematic HCPs, their removal trends during downstream processing/process development, and help define a suitable risk assessment and control strategy.

Methodology

- Sample preparation:
 - HCCF Null Cell ARM was reduced with dithiothreitol (DTT), alkylated with iodoacetamide, and digested using Lys-C and trypsin.
 - A nonrecombinant natural unrelated protein was spiked in as internal standards for quantification.
- Mass spectrometry analysis:
 - Peptides were separated on a C18 column using a 40-minute gradient.
 - SWATH acquisition captured MS/MS spectra across 350–1106 m/z ranges for comprehensive peptide analysis.
- Data processing:
 - Peptides were queried against a CHO-specific protein database.
 - Quantification utilized ion libraries and response factors derived from internal standards.

Figure 3. 2D Western Coverage Analysis with Cygnus Anti-CHO HCP 3G Resupply Antibody.



Results of LC-MS/MS analysis

The LC-MS/MS analysis identified and provided relative quantities for 1138 HCPs. The total HCP concentration was 993,331 ppm. The proteins that were discovered exhibited a broad spectrum of molecular weights and pI, offering a thorough depiction of the HCPs present. Many high-risk HCPs have been identified and appear in the BioPhorum High Risk HCP database². A summary of the top 100 most abundant proteins identified in the analysis is provided in [Annex](#).

Conclusion

Utilization of the rigorously characterized [CHO Null Cell HCCF ARM](#) offers several critical advantages in the analysis and control of HCPs in biopharmaceutical development:

- Benchmarking and consistency of HCP detection methods:** By offering a well characterized source of CHO HCPs, this ARM serves as a common benchmark for developing, qualifying, and comparing host cell protein analytical methods. It helps ensure consistency across platforms and laboratories, facilitating internal harmonization.
- Supports use of orthogonal methods:** The complexity and diverse protein composition of this material supports comprehensive evaluations across ELISA, 2D Western blotting, and LC-MS analytical techniques, enabling thorough characterization and method validation aligned with USP informational chapters.

- 3. Monitoring of assay performance and stability:** The [CHO HCCF ARM](#) serves as a reliable control material for continuous monitoring of assay performance, stability, and consistency over extended periods. Regular utilization of this standardized material in routine assay validation and quality control activities allows laboratories to confidently assess and ensure assay robustness and reproducibility over time.
- 4. Facilitating technology transfer activities:** During technology transfers, the CHO Null Cell HCCF ARM can function as a standardized analytical sample, enabling clear assessment of assay equivalency between originating and receiving sites. By employing a well-characterized reference material, laboratories can objectively verify assay accuracy, sensitivity, and consistency, helping to reduce risks associated with method transfer and ensuring seamless analytical continuity.
- 5. Bridging studies for process and reagent changes:** In the context of implementing method modifications or new analytical reagents, this ARM provides a consistent reference material to demonstrate equivalency and assay robustness, supporting smooth transitions and helping to mitigate potential regulatory concerns.
- 6. Supporting regulatory submissions:** Regulatory agencies may expect a detailed understanding of HCP content beyond ELISA total values. A well-characterized HCCF supports orthogonal method development aligned with USP <1132> and <1132.1>, allowing manufacturers to demonstrate robust impurity control.



References

1. "'High-risk' Host Cell Proteins (HCPs): A Multi-company Collaborative View." *Biotechnology and Bioengineering*, vol. 118, no. 8, Apr. 2021, pp. 2870–85. doi.org/10.1002/bit.27808
2. BioPhorum. "Host Cell Proteins - BioPhorum." *BioPhorum*, 7 Aug. 2024, www.biophorum.com/workstream/host-cell-proteins
3. "UniProt." *UniProt*, www.uniprot.org/proteomes/UP000001075t



More information

www.usp.org/biologics/mabs

Questions: uspbiologics@usp.org

Annex

Table A.1. Top 100 CHO HCPs Identified in CHO Null Cell HCCF.

UniProt ²	pI	Mw	Protein name
Q9JKY1	8.21	22131	Peroxiredoxin-1
G3IAQ0	5.85	46698	Phosphopyruvate hydratase
AOA8C2LMS2	6.88	57894	Pyruvate kinase
G3I8R9	5.01	70479	Endoplasmic reticulum chaperone BiP
G3HNJ3	5.51	49473	Clusterin
AOA8C2MY60	7.87	48948	HtrA serine peptidase 1
AOA8C2MUK8	4.96	83267	Heat shock protein HSP 90-beta
G3HQM6	4.72	90244	Endoplasmic
AOA8C2MT86	7.64	23638	Glutathione S-transferase
AOA3L7H3U8	5.83	26830	Peroxiredoxin 4
G3I6T1	5.63	61824	Phospholipase B-like Protein
P19378	5.23	70674	Heat shock cognate 71 kDa protein
G3H533	9.59	23634	Peptidyl-prolyl cis-trans isomerase
G3GTT2	9.39	13275	C-C motif chemokine
P14851	8.44	17899	Peptidyl-prolyl cis-trans isomerase A
AOA8C2QG32	7.55	38661	Annexin
G3H6V7	7.95	50522	Lipoprotein lipase
AOA8C2QHX8	4.89	82493	Heat shock protein 90, alpha (cytosolic), class A member 1
G3I5L3	6.57	38862	Annexin
G3H0L9	5.73	35647	Cathepsin B
G3HC31	5.3	10051	Protein S100
G3IKC3	8.46	86560	Glutathione transferase
AOA8C2LPG3	5.78	54339	Protein disulfide-isomerase
G3H8V5	5.61	51731	Carboxypeptidase
AOA3L7H9R3	4.74	55159	Protein disulfide-isomerase
AOA8C2QN41	6.08	81803	Procollagen-lysine 5-dioxygenase
Q9EPP7	6.68	31506	Cathepsin X
AOA8C2LBB3	6.33	25005	Peroxiredoxin-6
G3I4W7	6.59	42216	Cathepsin D
G3IDT6	4.95	70225	Protein disulfide-isomerase A4
AOA8C2M7Z4	8.59	25983	Glutathione transferase
Q8K3U7	5.35	21682	Peroxiredoxin-2
AOA3L7HEZO	7.89	11951	Peptidylprolyl isomerase
G3GT45	5.22	25690	Interferon-alpha/beta receptor beta chain
AOA8C2LNQ1	6.41	49718	Heat shock 70 kDa protein 13

UniProt²	pI	Mw	Protein name
AOA8C2QEK9	6.94	20567	Peptidyl-prolyl cis-trans isomerase
G3IIB1	8.3	59090	Sialate O-acetyltransferase
G3I664	8.33	52713	Procollagen C-endopeptidase enhancer 1
G3HQY6	7.43	43748	Lipase
G3I5A4	4.93	36065	Annexin
G3HUI4	6.06	53704	Lysosomal Pro-X carboxypeptidase
G3H1D5	5.3	48265	Carboxypeptidase
G3HYJ8	9.52	11032	10 kDa heat shock protein, mitochondrial
AOA8C2QII0	6.23	23419	Heat shock protein beta-1
G3IFJ6	5.63	27662	Glutathione transferase
G3HRK9	7.81	56823	Matrix metalloproteinase 9
G3HXN7	6.72	57591	Beta-hexosaminidase
G3HUU6	6.56	11241	Protein S100
AOA8C2MV34	5.38	51588	Peptidylprolyl isomerase
AOA8C2MYU6	9.29	25204	Peptidylprolyl isomerase
G3HEV3	4.99	130049	Latent-transforming growth factor beta-binding protein 1
AOA8C2N1L9	5.12	92549	Heat shock protein 4
G3IN86	6.03	52881	Dipeptidyl peptidase 2
G3I881	8.94	13071	Peptidylprolyl isomerase
G3H638	5.42	38129	Activator of 90 kDa heat shock protein ATPase homolog 1 isoform X1
G3ILF1	6.34	26967	Glutathione S-transferase
P18687	5.3	57959	60 kDa heat shock protein, mitochondrial
G3HUU7	6.27	11247	Protein S100-A10
AOA098KXE1	6.74	56296	Cytosol aminopeptidase
AOA8C2N2G7	5.61	78065	Thimet oligopeptidase 1
G3HD97	5.25	42996	Protein disulfide-isomerase
AOA8C2MTB5	6.36	32720	Isoaspartyl peptidase/L-asparaginase
O35501	5.44	68747	Stress-70 protein, mitochondrial
G3HKV9	5.86	43551	Group XV phospholipase A2
AOA8C2L9Y0	5.54	49045	Peptidase D
G3IAGO	5.34	69858	Xaa-Pro aminopeptidase 1
G3ILF3	6.85	25878	Glutathione S-transferase
G3HBJ9	5.56	52892	Methionine aminopeptidase 2
G3HMQ0	4.84	60357	Peptidylprolyl isomerase
G3H1Z4	9.04	29841	DnaJ heat shock protein family (Hsp40) member C8

UniProt³	pI	Mw	Protein name
G3I3Y7	8.46	24155	Glutathione S-transferase
Q60446	5.37	96020	Heat shock protein 105 kDa
G3HCU8	6.21	47685	26S protease regulatory subunit 8
G3I692	5.63	52842	Carnosine dipeptidase 2
G3I887	9.35	22560	Peroxiredoxin-5
G3I2H0	5.12	82971	Dipeptidyl peptidase 3
G3HC25	5.28	11241	Protein S100-A13
AOA8C2N474	5.48	80544	Prolyl endopeptidase
G3H7R4	6.79	28125	Peroxiredoxin 3
AOA3L7IHP4	6.72	40836	Peptidyl-prolyl cis-trans isomerase D
AOA3L7HI87	5.87	49185	26S proteasome regulatory subunit 4
G3I5H1	6.36	25758	Glutathione transferase
AOA8C2LS71	5.33	75873	Annexin
AOA8C2LKI6	5.51	103523	Aminopeptidase
AOA8C2LTB5	5.18	43062	Interleukin enhancer-binding factor 2
G3GRY7	5	14432	Heat shock factor-binding protein 1
G3H4Z8	5.5	69669	Heat shock protein family A (Hsp70) member 2
G3HLV1	5.22	61264	Peptidylprolyl isomerase
G3INF7	5.98	25517	Glutathione transferase
G3I620	6.66	51947	Aspartyl aminopeptidase
G3H6B5	9.03	97551	Interleukin enhancer-binding factor 3
G3HH63	5.79	87183	Phospholipase A-2-activating protein
AOA3L7IAQO	4.47	36086	Protein disulfide-isomerase
G3H8V1	5.53	76898	Matrix metalloproteinase 9
G3HN89	7.73	32355	Palmitoyl-protein thioesterase 1
G3H748	5.79	103894	Aminopeptidase
G3GTT3	9.08	8550	C-C motif chemokine
G3HZE5	5.59	69918	Heat shock 70 kDa protein 1A
G3HXL6	5.44	89187	Annexin
G3HX09	5.66	22016	Peptidylprolyl isomerase
G3HN81	5.41	33505	Peptidyl-prolyl cis-trans isomerase E
G3HAC4	5.7	22825	Peptidylprolyl isomerase
G3HJ24	5.5	58085	Procollagen-proline 4-dioxygenase
G3I7U9	9.66	47799	HtrA serine peptidase 2