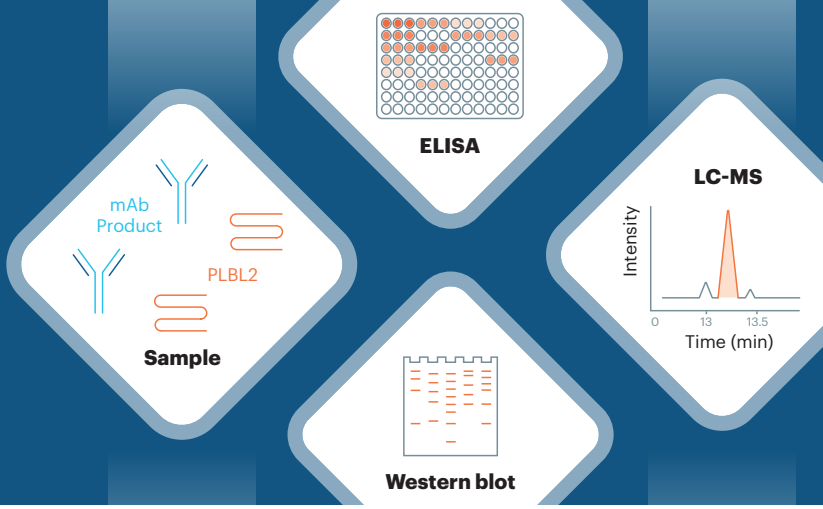
Technical Note

Purity characterization of USP PLBL2 analytical reference material



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Host cell proteins (HCPs) are a critical concern as impurities in biopharmaceutical production, posing potential risks to the final drug product quality and patient safety. These impurities originate from host cells used in the production of therapeutic proteins and other biologics that may co-purify with the desired biopharmaceuticals.[1] Among various impurities encountered during drug manufacturing, several specific HCPs present unique challenges.

One such problematic HCP commonly found in proteins manufactured by Chinese Hamster Ovary (CHO) cells is Phospholipase B- Like 2 protein (PLBL2), a 66 kDa mannose-6-phosphate glycosylated lysosomal enzyme.[2] The presence of PLBL2s in therapeutic products can initiate a potential immune response, therefore jeopardizing patient safety.[3]

PLBL2 has also been implicated in the degradation of a common excipient (polysorbate), potentially impacting the stability and quality of biotherapeutic drugs.[4]

The most common analytical method for determining the presence of HCPs during biopharmaceutical manufacturing is the enzyme-linked immunosorbent assay (ELISA), which often provides total HCP present relative to the drug product. There are only a few commercially available ELISA products capable of identifying and quantifying specific target HCPs. The preferred method for acquiring this type of specific HCP information is targeted mass spectrometry. **Figure 1** illustrates the progression in methodologies and highlights the capturable data from each.

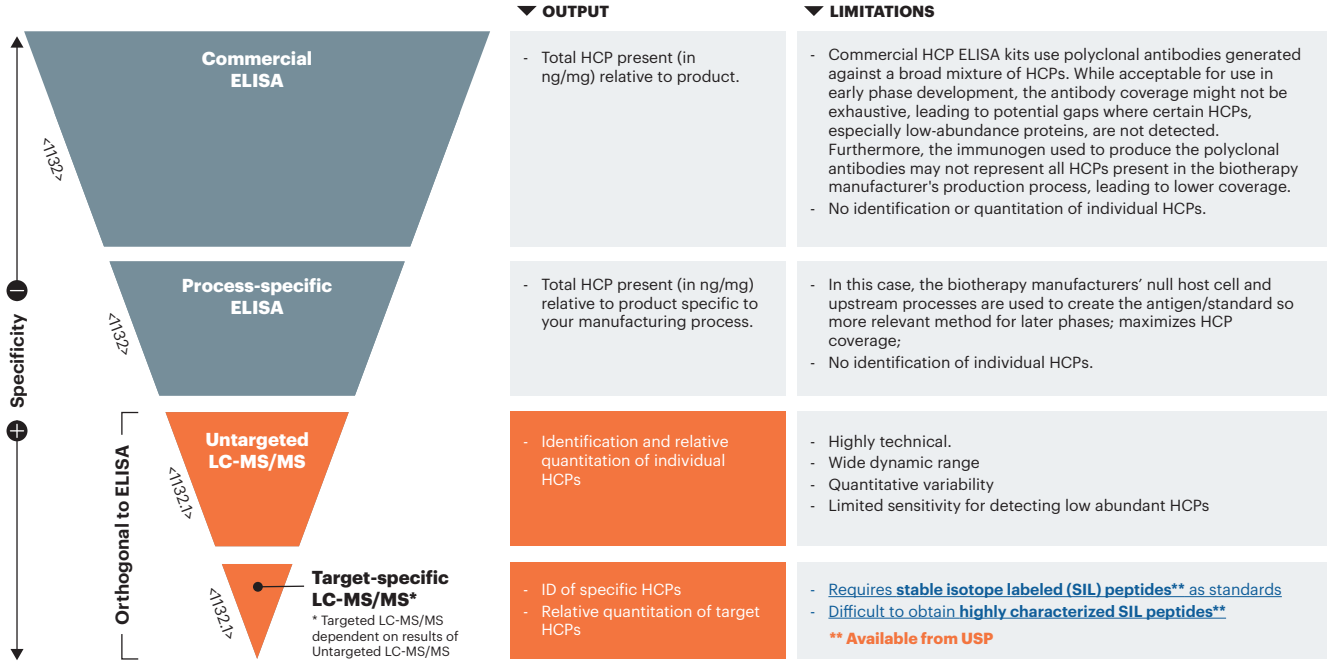


Figure 1. Overview of methods used to assess HCPs in therapeutic protein drug substances.

As a standards development organization, the United States Pharmacopeia (USP) focuses on developing documentary standards as well as physical reference standards and materials to alleviate bottlenecks in the drug development and manufacturing lifecycle/process identified by stakeholders in the biopharmaceutical industry. Physical standards to aid host cell protein analysis via ELISA and mass spectrometry (MS) methods have frequently been identified by biopharmaceutical manufacturers as important to help ensure precise and consistent detection and quantification of HCPs.

In line with this strategy, USP has introduced its first HCP Analytical Reference Material (ARM), a purified recombinant CHO PLBL2 [His], that can be used for ELISA (**Table 2**), Western blotting (**Figure 4**) and LC-MS (**Figure 5**) applications.

USP has rigorously characterized this PLBL2 ARM to maintain the reliability, consistency, and suitability of the PLBL2 standard for analytical and bioprocess applications. The analytical techniques listed in **Table 1** were used to ensure precise identification, quantification, and control of impurities. Using a less rigorously characterized standard could lead to inaccurate measurements, reduced reproducibility, and unreliable results, potentially compromising the integrity of downstream processes, product quality, and regulatory compliance.

Table 1. Analytical characterization of USP PLBL2 ARM

Characteristic	Analytical techniques
Purity and molecular weight	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) Capillary electrophoresis sodium dodecyl sulfate (CE-SDS)
Identity	Western blot
Quantification of the amount of specific protein	Targeted ELISA

Purity assessment from reduced SDS-PAGE analysis

To characterize the USP PLBL2 ARM, it was denatured and reduced by mixing with a 6x Laemmli SDS reducing sample buffer containing 2-mercaptoethanol followed by heating at 80°C for 3 minutes. Alkylation was achieved with iodoacetamide incubation at room temperature for 30 minutes. Two µg of protein were separated using a SDS-PAGE 4-12% tris-glycine gel with molecular weight standards. SimplyBlue™ SafeStain was then used for visualization.

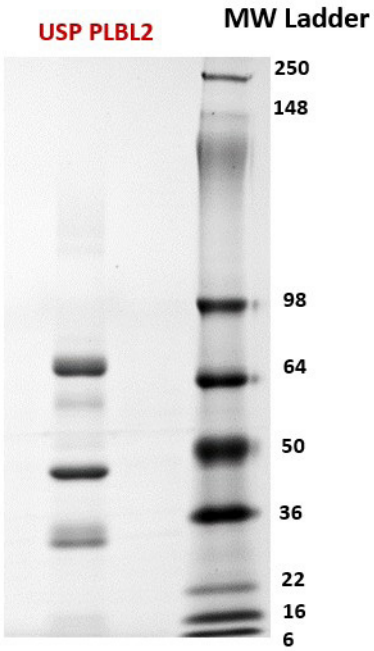


Figure 2. Reduced SDS-PAGE Gel of USP PLBL2 ARM

Reduced SDS-PAGE yielded a purity of 91.2%. The percentage is the sum of bands shown in **Figure 2** at ~66 kDa, ~60 kDa, ~42 kDa, and ~30 KDa. It has been previously documented that PLBL2 can undergo limited autolysis, resulting in the generation of two distinct fragments: a 28 kDa N-terminal prodomain and a 40 kDa C-terminal mature protein. SDS-PAGE showed high purity, with distinct bands corresponding to the expected molecular weight of PLBL2, indicating low contamination.

Size variant assessment of PLBL2 ARM through CE-SDS analysis: reducing conditions

For any protein, there can exist several forms in the final product, such as monomers, dimers, and fragments. CE-SDS provides insights into a protein's stability, confirming its appropriateness for prolonged use.

The USP PLBL2 ARM was analyzed on the Bio-Techne® ProteinSimple™ Maurice system using a 0.5 mg/mL of USP PLBL2 ARM in 1X CE-SDS PLUS Sample Buffer (50% V/V) with a 4% (V/V) CE-SDS 25X internal standard. Reduced conditions employed β-mercaptoethanol at 5% (V/V, 14.2 M). Samples were heated at 70°C for 10 minutes, cooled on ice for five minutes, and centrifuged to obtain the supernatant. The analysis conditions included injection for 20 seconds at 4600 V, followed by separation for 35 minutes at 5750 V.

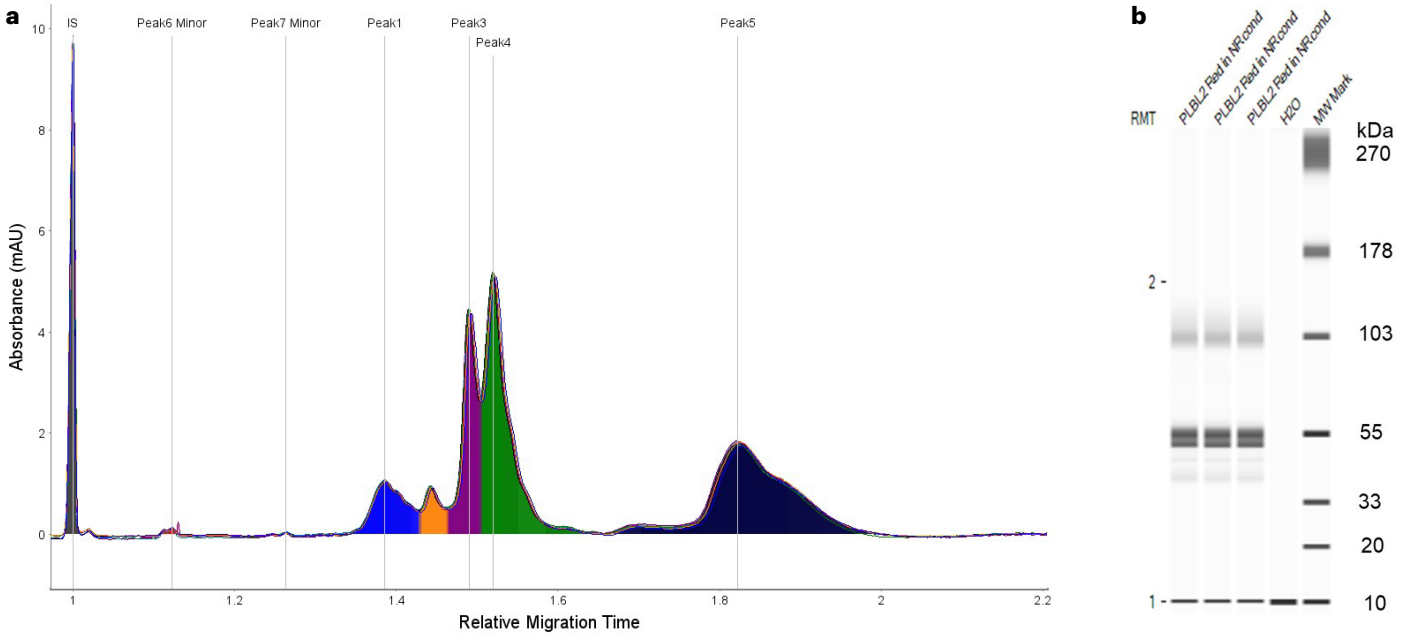


Figure 3. (a) Capillary Electrophoresis Analysis of USP PLBL2 ARM under Reducing Conditions; (b) CE-SDS Analysis of USP PLBL2 ARM: Lane View.

CE-SDS provides certain advantages over SDS-PAGE, particularly in resolving closely related protein species. In the case of PLBL2, CE-SDS demonstrated in **Figure 3b**, improved separation of the 60 kDa and 66 kDa forms, which are less distinctly resolved using SDS-PAGE (as shown in **Figure 2**). This enhanced resolution allows for a clearer differentiation between protein variants that are otherwise challenging to distinguish, making it particularly useful for detecting and characterizing closely related species in the sample.

While SDS-PAGE is effective for confirming the overall presence of PLBL2 and assessing general purity, CE-SDS excels in distinguishing between these closely related forms, providing a more precise analysis in this context. Additionally, CE-SDS offers improved throughput and ease of use, allowing for more efficient processing in a lab setting. Both techniques together offer a comprehensive view of PLBL2's protein composition, with SDS-PAGE giving a broad overview and CE-SDS providing finer resolution for protein variants such as the 60 kDa and 66 kDa forms, enhancing confidence in the sample's consistency and purity.

Detection of the USP PLBL2 ARM through Western blot analysis

Through His-tag

The USP PLBL2 ARM contains a 6X His-tag. In Western blot analysis of the USP PLBL2 ARM, mirroring SDS-PAGE procedures, 2 µg of the USP PLBL2 ARM were loaded onto a SDS-PAGE gel after reduction and alkylation. The separated proteins were then transferred to a polyvinylidene fluoride (PVDF) membrane, which was subsequently blocked with SuperBlock. Following blocking, the membrane was incubated with an anti-6xHis monoclonal antibody for 2 hours at room temperature and washed with Tris-Buffered Saline with Tween® 20 (TBST). To visualize the target protein, an anti-mouse IgG antibody conjugated with Alexa Fluor 488 was applied and incubated for 1 hour at room temperature, followed by three TBST washes. The PVDF membrane was then imaged using a Chemi-Doc system, enabling precise detection and characterization of PLBL2, including its interaction with the anti-6xHis antibody.

Consequently, in an anti-His tag Western blot analysis, both the full-length PLBL2 proenzyme (approximately 66 KDa) and the C-terminal mature protein (approximately 40 KDa) were anticipated to be detected. The USP PLBL2 ARM exhibited the expected Western blot profile with discernible bands at approximately 66 kDa and 40 kDa when probed with anti-His monoclonal antibody.

Through targeted antibody

While **Figure 4a** shows that we were able to identify the USP PLBL2 ARM due to its His tag to demonstrate the protein identified was the actual USP PLBL2 ARM, biotin conjugated goat anti-PLBL2 polyclonal Ab (pAb) was purchased from

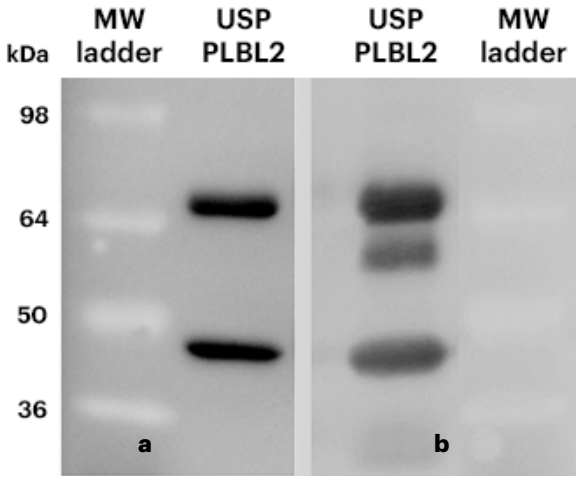


Figure 4. (a) Anti-His Tag Western Blot Image of USP PLBL2 ARM; (b) Anti-PLBL2 Western Blot of the USP PLBL2 ARM.

ICL (Immunology Consultants Laboratory, Inc: www.icllab.com) and used in a Western-blotting analysis. USP PLBL2 ARM samples were reduced and alkylated as previously described. The separated proteins were transferred to PVDF membrane. The PVDF membrane was then blocked by SuperBlock, followed by incubation with anti-PLBL2 antibody at RT for 2 hrs. After washing with TBST three times, streptavidin-Alexa 488 was added and incubated at room temperature for 15 min. The membrane was then washed by TBST three times and visualized in Chemi-Doc.

In **Figure 4b**, there is a distinct band at around 60 kDa in addition to the expected bands at approximately ~66 kDa, ~40 kDa, and ~28 kDa. Given the specificity of the anti-PLBL2 antibody used in this assay, this ~60 kDa band is likely due to post-translational modifications, splice variants, or alternative PLBL2 conformations.

Western blot analysis confirmed the identity and integrity of PLBL2, identifying both full-length and fragmented versions, which is crucial for HCP applications.

Application example: quantitation of PLBL2 through ELISA analysis

We conducted an initial evaluation on the suitability of the USP PLBL2 ARM with three commercially available PLBL2 ELISA kits. The primary objective of this study was to assess the specificity and suitability of the USP ARM as reference control material for ELISA. Data shown in **Table 2** below.

The USP PLBL2 ARM was tested, and the recovery percentages (calculated vs theoretical concentration) were found to be compatible with the reported values by the internal standards in each PLBL2 ELISA kit. The recovery percentages for USP PLBL2 ARM closely matched the predicted values, indicating high accuracy and dependability across different ELISA kits. The consistent outcomes obtained from various commercial kits demonstrate the reproducibility and accuracy of the USP PLBL2 ARM as a reference control material for PLBL2 ELISA.

ELISA experiments showed similar recovery of PLBL2 across various HCP assay kits, ensuring biopharmaceutical manufacturers can reliably use the USP PLBL2 ARM for quantification.

Table 2. ELISA analysis of USP PLBL2 ARM using different commercial kits

Kit A		Kit B		Kit C	
% Recovery	%RSD	% Recovery	%RSD	% Recovery	%RSD
101	2	124	7	101	6

Note: Percent Recovery calculated based on PLBL2 label concentration

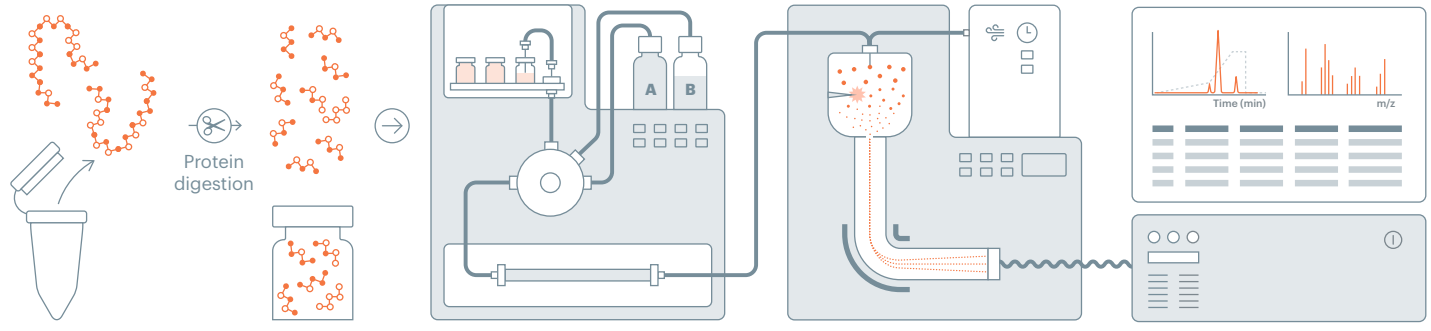


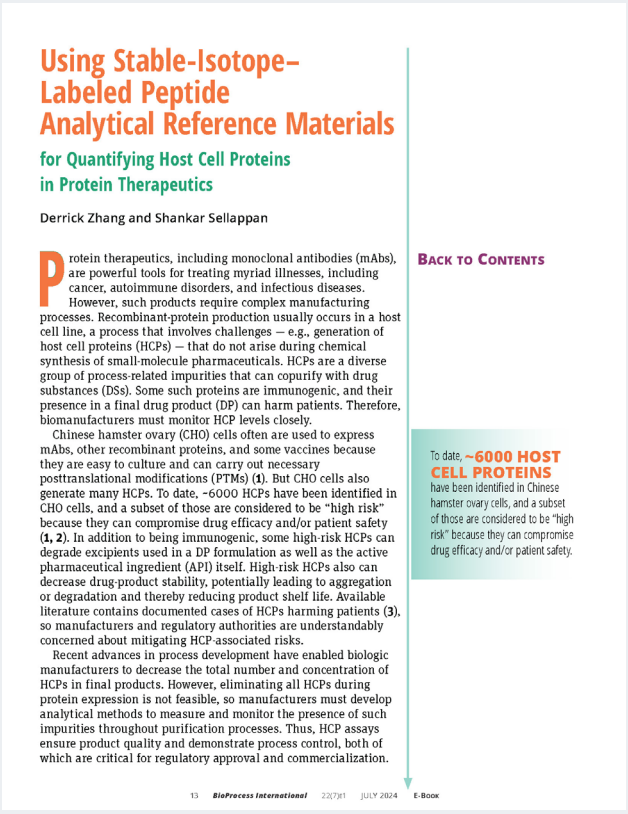
Figure 5. Host cell protein (HCP) analysis LC-MS workflow

Conclusion

The USP PLBL2 ARM has been thoroughly characterized, providing a verified resource for Host Cell Protein (HCP) analysis in both ELISA and MS methods. The ARM's identification, purity, and concentration were verified using various analytical methods. This PLBL2 ARM provides a reliable standard for biopharmaceutical businesses, enhancing accurate HCP analysis, improving product quality control, and supporting the consistency and robustness of biologics manufacture.

What's next!

When sourcing other recombinant HCPs like PLBL2 from vendors for HCP analysis, users should confirm that the material has undergone an equally rigorous characterization process as the USP standard. This ensures that the protein meets the necessary quality benchmarks for accurate and reliable HCP analysis. Without comparable characterization, variations in purity, impurity profiles, or protein stability may introduce inconsistencies in analytical results, potentially affecting the reliability of HCP quantification and compromising overall product quality and regulatory compliance. USP also offers stable-isotope labeled (SIL) peptides for problematic CHO HCPs of concern, such as Clusterin and Lipoprotein Lipase; PLBL2 SIL peptides coming soon.



Using Stable-Isotope–Labeled Peptide Analytical Reference Materials

for Quantifying Host Cell Proteins in Protein Therapeutics

Derrick Zhang and Shankar Sellappan

Protein therapeutics, including monoclonal antibodies (mAbs), are powerful tools for treating myriad illnesses, including cancer, autoimmune disorders, and infectious diseases. However, such products require complex manufacturing processes. Recombinant-protein production usually occurs in a host cell line, a process that involves challenges — e.g., generation of host cell proteins (HCPs) — that do not arise during chemical synthesis of small-molecule pharmaceuticals. HCPs are a diverse group of process-related impurities that can copurify with drug substances (DSs). Some such proteins are immunogenic, and their presence in a final drug product (DP) can harm patients. Therefore, biomanufacturers must monitor HCP levels closely.

Chinese hamster ovary (CHO) cells often are used to express mAbs, other recombinant proteins, and some vaccines because they are easy to culture and can carry out necessary posttranslational modifications (PTMs) (**1**). But CHO cells also generate many HCPs. To date, ~6000 HCPs have been identified in CHO cells, and a subset of those are considered to be "high risk" because they can compromise drug efficacy and/or patient safety (**1, 2**). In addition to being immunogenic, some high-risk HCPs can degrade excipients used in a DP formulation as well as the active pharmaceutical ingredient (API) itself. High-risk HCPs also can decrease drug-product stability, potentially leading to aggregation or degradation and thereby reducing product shelf life. Available literature contains documented cases of HCPs harming patients (**3**), so manufacturers and regulatory authorities are understandably concerned about mitigating HCP-associated risks.

Recent advances in process development have enabled biologic manufacturers to decrease the total number and concentration of HCPs in final products. However, eliminating all HCPs during protein expression is not feasible, so manufacturers must develop analytical methods to measure and monitor the presence of such impurities throughout purification processes. Thus, HCP assays ensure product quality and demonstrate process control, both of which are critical for regulatory approval and commercialization.

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To date, ~6000 HOST CELL PROTEINS have been identified in Chinese hamster ovary cells, and a subset of those are considered to be "high risk" because they can compromise drug efficacy and/or patient safety.

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