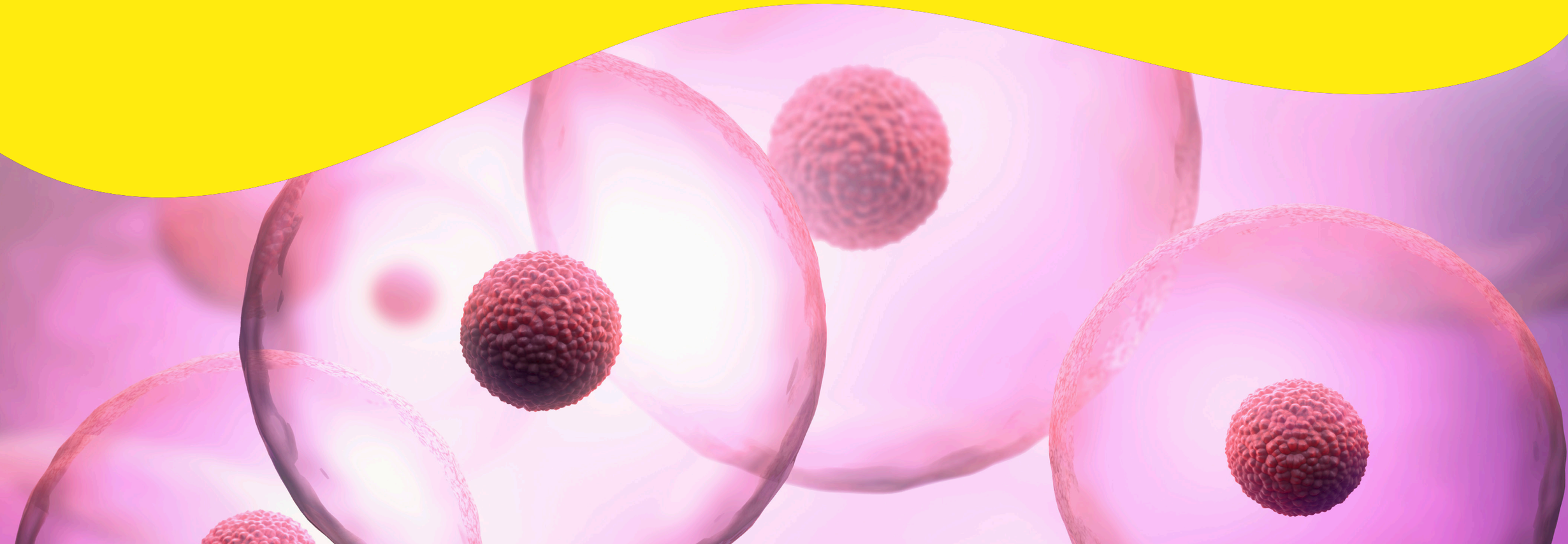
CHO Host Cell Proteins (CHO HCPs)

A guide to CHO HCP detection in biopharmaceutical development and manufacturing



What are biopharmaceuticals

Biopharmaceuticals are medicinal products sourced from living cells or organisms, and manufactured using biotechnology methods.[1] They may be extracted from natural biological sources (non-recombinant), or produced using recombinant DNA technology. While this broad definition encompasses a wide spectrum of macromolecules and other biological entities like viral vaccines and cell therapies, the vast majority of biopharmaceuticals are proteins, such as monoclonal antibodies (mAbs), growth factors, hormones and enzymes.

Naturally Sourced Products

Non-recombinant biopharmaceuticals are sourced from a wide variety of prokaryotic and eukaryotic species, including bacteria (typically E. coli), yeasts, plants, insects and mammals. Many of the earliest biopharmaceuticals were derived from animals. For example, the first commercial insulin product, commercialized by Eli Lilly in 1922, was purified from cattle and pig pancreatic extracts. Biopharmaceuticals can also be obtained from human tissues and bodily fluids such as blood, milk and urine. However, human sources are not ideal because of inherent supply limitations and the risk of disease transmission.

Among the many non-recombinant biopharmaceuticals on the market today are immune globulins sourced from animal or human donors for the treatment of immunodeficiencies, fecal microbiota used in the treatment of recurrent Clostridium Difficile Infection (rCDI), and most influenza vaccines, which are still largely produced by cultivating the parental virus in embryonic chicken eggs.[2]

Recombinant Biopharmaceuticals

The emergence of recombinant DNA technology in the late 1970s revolutionized the biopharmaceutical industry. For the first time, microbes and other living systems could be genetically engineered to serve as “factories” to express foreign proteins on demand. This meant that human proteins, in particular, could be produced at unprecedented scales.

The first recombinant biopharmaceutical was human insulin, commercialized in 1982 by Eli Lilly under the brand name Humulin. Since then, many conventional therapeutic protein products have been superseded by humanized or fully human recombinant versions. In 2002, Humira, a treatment for rheumatoid arthritis, became the first fully human recombinant antibody to be approved by the FDA.

Over 70 percent of biopharmaceuticals are produced in host cell lines derived from Chinese hamster ovary (CHO). The presence of residual host cell proteins (HCPs) in these preparations can compromise quality, delay regulatory approvals, and pose serious health risks for patients. Control and removal of HCPs during the manufacturing process is a major challenge, accounting for a significant proportion of total production costs. Here we review the challenges presented by CHO HCPs, and explore the latest strategies and tools to detect, quantify and reduce their levels during biopharmaceutical development and manufacturing.

Today, recombinant proteins—particularly mAb products—dominate the biopharmaceuticals pipeline. With the power of protein engineering technologies, it is possible to create highly targeted biomolecules that have unparalleled specificities and fewer off-target effects compared to small molecule drugs.[3] Since 1985, approximately 100 mAbs have been designated as drugs, and at least 570 therapeutic mAbs have been studied in clinical trials worldwide.[4]

Therapeutic Areas

Biopharmaceuticals on the market and in clinical development address dozens of therapeutic areas, with oncology applications leading the way by a significant margin. Cancer diseases account for about 80 percent of clinical trials involving mAbs[5] and over two-thirds of all gene therapy clinical trials worldwide.[6]

Driven by the global pandemic, COVID-19 is another area of intense investigation, with over 200 protein-based COVID-19 interventions currently in preclinical and clinical development.[7] Other key disease areas include inflammatory and infectious diseases, autoimmune disorders, metabolic diseases, hormonal disorders, cardiovascular diseases and CNS diseases.

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The Expanding Market for Biopharmaceuticals

The global biopharmaceuticals market is growing rapidly, and currently accounts for around 20% of the global pharmaceutical industry.[8] Valued at USD 265.4 billion in 2020, the market is expected to grow at an annual rate of 12.5% to reach USD 856.1 billion by 2030. Covid-19 is expected to boost the growth of the biopharmaceuticals market over the course of the pandemic. In anticipation of the rising demand, focus has shifted in recent years to scaling up manufacturing capacity and enhancing productivity. Platform technology advances, improved efficiencies in upstream and downstream processing, and increasing prevalence of infectious diseases, autoimmune diseases and cancer are among the factors driving growth of the global biopharmaceuticals market (Figure 1).

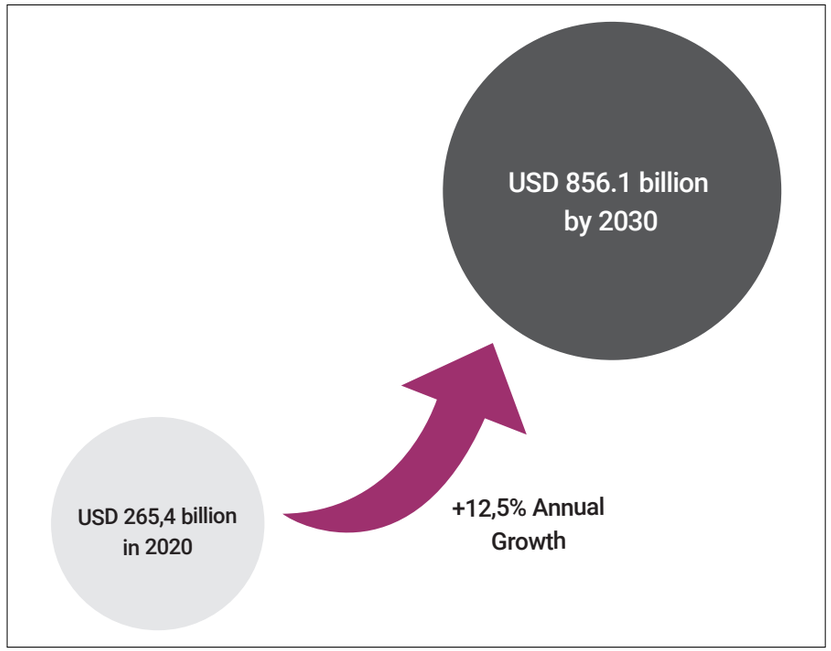

Figure 1 - Global Biopharmaceutical Growth Prevision

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Biopharmaceutical manufacturing processes are significantly more complex than chemical synthesis of small-molecule drugs. Maintaining the structural and functional integrity of living biological systems and large macromolecular products presents unique process challenges, and calls for specialized skills sets, equipment and facilities. High reproducibility and purity of the finished product are essential to ensure efficacy and avoid eliciting acute adverse responses and long-term sensitivities in patients. Today's biopharmaceutical manufacturing processes for mAb products typically achieve purities of 99.99% or greater.[9]

Overview of the Biopharmaceutical Manufacturing Process

To deliver a biopharmaceutical product that is reproducible, effective and safe for use in humans, every step of the manufacturing process must be carefully designed, optimized and controlled (Figure 2). An integrated strategy to monitor and eliminate process-related impurities is essential for success, and is therefore a key consideration during early process development. Not only can residual impurities degrade product performance and stability, even trace amounts of certain foreign substances can cause serious adverse immune responses and toxicities in some individuals.

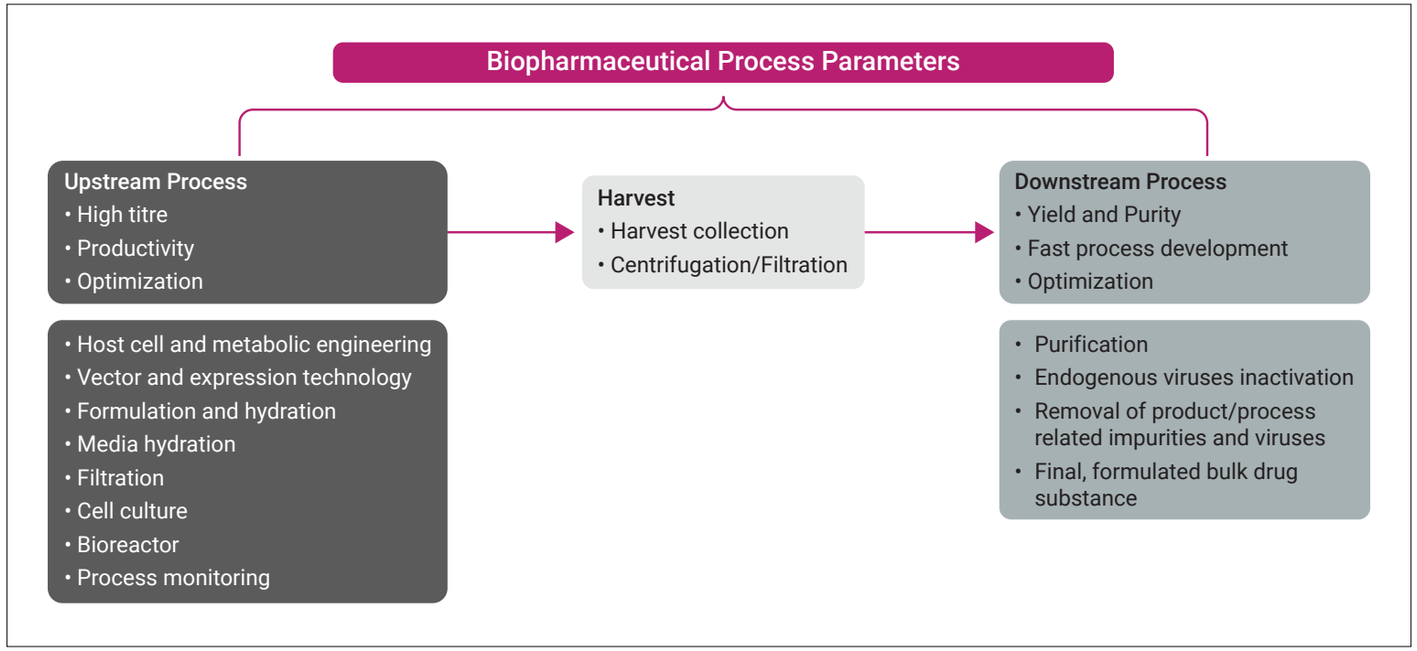

Figure 2 – Optimization areas and parameters in upstream and downstream processing

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Upstream Processing (USP)

Upstream processing starts with culture and expansion of protein-producing cell lines in a bioreactor. Although adherent cell types may be preferred for some types of products or at early stages of development, industrial-scale manufacture of recombinant protein products is usually carried out with cells grown in suspension using fed-batch methods or perfusion systems for continuous processing.

During cell expansion, optimal environmental conditions and nutrient concentrations must maintained within the bioreactor to achieve the desired growth rates. As cells reach critical mass, parameters may need to be adjusted to favor protein production.

Choice and optimization of culture media and supplements is particularly important during USP development, because it can directly impact cell growth rates, protein yields, and product quality. Fully defined, animal component-free (ACF) media are now routinely used in commercial manufacturing to avoid introducing foreign contaminants that could cause adverse immune reactions and sensitivities in patients if not removed from the final product.

Downstream Processing (DSP)

Once the desired protein titer has been reached, whole-cell lysate or cell-free supernatant is harvested for downstream processing. DSP then entails isolation, separation and purification of newly generated protein products from the host cells, cell debris, protein aggregates, waste products, media components and other undesirable substances that have accumulated in the bioreactor.

The first step in mAb purification is usually protein A or G affinity chromatography. This eliminates the vast majority of process-related impurities, yielding a product fraction that is ~98% pure.[10] Following a viral inactivation step, two to three additional chromatography “polishing” steps are typically needed to remove residual impurities that tend to co-elute with the product. The material is then put through a second viral removal step (usually nanofiltration) before ultrafiltration and diafiltration to achieve the final formulation.

Overcoming USP and DSP Process Challenges

Over the past 30 years, remarkable progress has been made in the optimization of upstream processes for antibody production. Early recombinant expression systems had low yields, on the order of tens of milligrams of protein per liter of culture media. This raised concerns about the capacity of culture systems to produce enough protein to meet high dosage requirements and satisfy rapidly expanding markets for mAb products.

As manufacturing methods for antibody production have matured, large gains in USP productivity have been achieved by overcoming biological limitations. Development of high-producing cell lines, defined ACF culture media, and improved culture platforms for large-scale manufacturing have significantly boosted USP yields. Antibody titers exceeding 10 g/L in fed-batch processes, and as high as 25 g/L with modified perfusion systems, have been reported. With bioreactor capacities as large as 25,000 L, this means that batches exceeding 100 kg of protein are now feasible.

These welcome productivity gains shifted the capacity bottleneck downstream, where there is less economy of scale in terms of production costs. The large amounts of protein being fed into DSP extraction and purification processes can easily exceed the physical limits of conventional filtration, affinity capture and column chromatography systems, which were designed to handle much lower protein inputs. These limitations in throughput and scalability translate into longer downstream processing times, greater materials consumption, and increased cost per batch. By some accounts, as much as 80% of total mAb production costs are attributable to downstream processing.

In addition to the shifting capacity bottleneck, improvements in upstream productivity have led to changes in impurity composition, including higher levels of difficult-to-remove HCP impurities.[11] Such issues with DSP capacity and selectivity have been a major driving force in the evolution of improved purification technologies, spawning many advances, including higher capacity resins with better flow rates and more robust, high-performance, chromatography membranes.

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Cell Lines for Recombinant Protein Expression

Host cell lines for industrial protein production must be stable in culture and produce a high titer of protein with the correct structure and desired therapeutic properties. Mammalian cell lines are usually preferred as expression hosts for biopharmaceuticals because they possess the machinery necessary for appropriate folding and post-translational modification of human proteins. The ability of host cells to produce glycosylation profiles similar or identical to those found in humans is especially important, because differential glycosylation can significantly alter the biological properties of recombinant therapeutics. Foreign glycan structures also have the potential to trigger serious adverse immune responses and sensitivities in patients.

A variety of mammalian cell lines have been developed for recombinant protein production. These derive from several different species, including human (CAP, HEK293, HKB-11, HT-1080, HuH-7, PER.C6®), mouse (NS0, Sp2/0), and hamster (BHK, CHO). A number of novel microbial, insect and plant-based host systems have also emerged in recent years. While these have some distinct advantages—particularly in terms of cost, ease of validation and reproducibility—the potential benefits must be weighed against their ability to deliver the desired levels of biological activity, quality and safety.

Chinese Hamster Ovary (CHO) Cells as Expression Hosts

More than 70% of biopharmaceuticals, and almost all mAb products, are currently manufactured in cell lines derived from Chinese hamster ovary (CHO). In addition to being well-characterized, with a long history of use as recombinant expression systems, CHO cell lineages have a variety of other advantages that make them the most popular choice for commercial production. These include their capacity for high yields (up to 10 g/L in fed-batch cultures), human-compatible posttranslational processing, resistance to human pathogenic viruses, and robust growth characteristics. CHO cells are readily adaptable to defined media and amenable to propagation in large industrial bioreactors in suspension formats.

CHO cells comprise a large and genetically diverse family of different cell lines.[12] Like many other immortalized cell types, their genomes are unstable, and clonal populations can become heterogenous over time in culture. This genetic instability has hampered efforts to standardize CHO expression platforms, and means that USP and DSP processes must be optimized for each recombinant cell line—particularly when it comes to ensuring removal of process-related impurities deriving from the host cells.

Over the years, the industry has invested significant effort in developing robust and reproducible CHO expression platforms for commercial manufacturing. Notable advances include the introduction of cell lines with greater genotypic and phenotypic stability, technologies for targeted gene manipulation, and better tools for high-throughput screening and identification of stable producer clones. Insights gained from proteomic, genomic, transcriptomic and other 'omics analyses are helping technologists engineer novel CHO cell lines with improved performance characteristics and enhanced functionalities.[13] Global 'omics studies are also playing a central role in elucidating the nature and dynamics of CHO-derived impurities in production processes.

CHO Host Cell Proteins

One of the central challenges in downstream processing is the removal of residual host cell proteins (HCPs), which are proteins produced by the host cells and involved in cell maintenance, growth, protein synthesis and processing functions. During culture, cells release thousands of different HCPs into the surrounding medium. These may be secreted proteins or intracellular proteins released during cell death. Certain HCP impurities that co-purify with the drug product have the potential to impact its critical quality attributes (CQAs) or safety profile in the final formulation.

Although there is significant proteomic overlap across CHO cell lineages, the HCP expression profile is unique to each recombinant host cell line and the specific conditions under which it is cultured. Proteomic and glycoproteomic analyses of CHO cells have identified over 6000 HCPs,[14] a subset of which will be present in the harvest material. For example, analysis of material harvested from a mAb-producing recombinant CHO line identified 1934 distinct HCP species in batch culture supernatants, and 2145 in fed-batch cultures.[15] The study also showed that HCP concentration profiles correlated with changes in mAb quality attributes such as aggregation, charge variants and N-glycosylation.

The first draft genomic sequence of the CHO-K1 ancestral cell line was published in 2011 by Xu et al.[16] This has since been followed by full genomic sequence analysis of six CHO cell lines derived from the three most frequently used CHO lineages in biopharmaceutical protein production (CHO-K1, DG44 and CHO-S).[17] The growing knowledge base established from these and other 'omics analyses provides an essential reference to inform strategies for HCP analysis and identification of problematic HCP impurities.

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HCP populations present at harvest exhibit diverse physicochemical properties, with a wide range of molecular weights (~5 kDa -250 kDa), isoelectric point (pI) values (~3-11), and levels of hydrophobicity. This means that several different purification technologies are usually needed to achieve the high levels of purity required for product release and regulatory approvals. Variation in HCP expression profiles during extended culture can complicate strategies for downstream clearance of difficult-to-remove impurities. For instance, if a particular HCP is strongly up- or down-regulated in response to stressful culture conditions, it may exceed the capacity of downstream purification equipment or fall below assay detection limits.

At least 118 CHO HCP species have been reported as exceptionally difficult to remove because they co-purify with mAbs during DSP.[18, 19, 20, 21, 22] Co-purification may be due to strong specific or non-specific associations with the product, chromatographic resins (e.g., protein A), or capture ligands. Levy and colleagues identified 17 HCP that exhibit strong interactions with mAbs under Protein A solution conditions.[21] In the case of high-affinity interactions, the ratio of HCP to mAb may be very low, making its detection and study even more difficult.

The BioPhorum Development Group (BPDG) recently published a https://www.biophorum.com/host cell proteins/ of HCP with high potential to impact safety, efficacy, or quality aspects of CHO-produced biologics during development and manufacturing.[23] With information gathered from extensive literature searches, surveys and pharmaceutical company experiences, the working group identified 87 CHO HCP frequently found throughout different bioprocessing steps, and classified 25 HCP as problematic or high-risk. These include a variety of enzymes (e.g.,proteases, glycosidases, and lipases), chemokines, growth factors, chaperones and proteins predicted to have high immunogenicity risk by in *silico* models such as CHOPPI and IEDB.

Understanding the Risks Posed by HCP Impurities

Among process-related contaminants, HCPs are of particular concern because as biological substances they can act in a variety of ways to reduce product quality and endanger patients, even when present at levels as low as 1-100 ppm (party per million). Demonstrating the removal and control of HCP impurities during process development and manufacturing is therefore a regulatory requirement in most countries.

In 2012, the US FDA suspended two late-stage clinical trials of IB1001, a recombinant Factor IX therapy, after patients developed antibodies to CHO HCPs at rates that were higher than expected for recombinant therapeutic product.[24] While no related adverse events were reported during these clinical studies, setbacks like these serve to illustrate the importance that regulators and the industry place on minimizing HCPs in drug preparations.

Not all residual HCPs are of equal risk, however. High-risk HCPs identified by the recent BioPhorum HCP work group are classified into four categories based on their impact to product quality, formulation, direct biological function in humans, and immunogenicity.[23]

Three of the most significant ways in which HCPs can act to compromise drug performance and safety are:

Immunogenicity

Since HCPs are foreign to the human body, their potential to induce unwanted immune responses is a major concern during pharmaceutical development and manufacturing. Immunogenic effects include acute life-threatening allergic reactions such as anaphylaxis, as well as long-term sensitivities that can affect drug efficacy and quality of life. For example, although the risk is low, HCP impurities with high similarity to human proteins can induce antibodies that cross-react with “self” proteins, potentially leading to development of a lasting autoimmune disease.

Populations of co-purifying HCPs often contain assorted binding proteins, many of which normally function in protein folding and assembly. These tend to be upregulated in high-producing host cells, and can interact non-specifically with the protein product to accelerate the formation of immunogenic aggregates. Larger aggregates, including complexes of HCP binding proteins with the therapeutic product, can also act as adjuvants to enhance immunogenicity and potentially induce anti-drug antibodies (ADA) capable of neutralizing the drug product or altering its pharmacokinetic profile.

Degradation of Product or Formulation Excipients

A study of co-purifying CHO HCPs in mAb purification processes found that over half had catalytic activity.[25] Moreover, two-thirds of HCPs listed as high-risk in BioPhorum’s HCP database are so classified because they have enzymatic activities that could impact drug quality or safety.[23]

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Host cell proteases can degrade mAbs and other therapeutic proteins, leading to impaired function and decreased solubility, both of which can eventually result in loss of drug potency during storage. Glycolytic enzymes are of concern when developing therapeutic glycoproteins because degradation of carbohydrate moieties can affect the product's pharmacodynamics, pharmacokinetics, and immunogenicity.

Formulation excipients are also susceptible to enzymatic degradation or modification. For example, various lipases, including LPL, LPA2 and PLB2L, have been shown to degrade polysorbates commonly used as stabilizing agents. Over time, even trace amounts of enzymatic activity can significantly reduce product efficacy, potency or stability.

Biological Activity in Humans

Biologically functional molecules present in HCP populations include signalling molecules, transporters, antioxidants, regulatory proteins, enzymes and chaperones. Many of these have the potential to cause deleterious effects in patients. Cytokines and chemokines involved in coordinating immune responses are of particular concern, as they can trigger histamine release and other signalling cascades that culminate in acute or even fatal reactions. Those on the BPDG list of high-risk CHO HCPs include CXCL3, MCP-1 and TGF-β1.[23]

Another consideration is that a biologically active hamster homologue similar to the biotherapeutic protein being expressed in the CHO host cell may co-purify and be administered to patients along with the product. Similarly, a mAb therapeutic product might bind the hamster homologue of the drug target with enough affinity for the complex to co-purify, again raising the risk of introducing a biologically active hamster molecule into patients.

Guidelines for HCP quantification

HCP testing is crucial to determine the composition of material harvested from bioreactors, understand how each step of the purification process contributes to HCP clearance, and demonstrate that the product is of sufficient purity for clinical release. Accurate HCP quantification is therefore essential for successful process development, validation and manufacturing control.

The International Conference on Harmonization (ICH) guideline Q11 established HCP impurities as a CQA for biopharmaceutical products. Consequently, HCP levels must be monitored and controlled to meet agreed acceptance criteria, which may be set for individual impurities or total levels. In 2016 and 2017, United States Pharmacopeia (USP) and European Pharmacopeia issued updated chapters on HCP measurement, emphasizing the importance of DSP optimization to remove as much of the residual impurities as technically feasible. Notably, the new guidelines included HCP coverage assays as best practice to mitigate risks associated with use of polyclonal antibody reagents.

Currently there are no specified thresholds or target levels for HCPs because the reactivity of each immunoassay is potentially unique, and patient responses depend on numerous clinical variables. These include the drug indication, patient population, individual patient condition, dosage, route of administration, and frequency of exposure. The level of residual HCPs permitted in final bulk material is therefore determined on a case-by-case basis. Historically, the acceptable limit for HCP impurities in biologics has been in the 1-100 ppm range, or 1-100 ng HCPs per mg of therapeutic protein. In cases where there are specific co-purifying HCPs of concern, separate assays and specifications for individual HCP impurities may be required.

During manufacture, HCP monitoring typically starts in the early phase of protein production and continues through the middle and late stages of purification, ending with release testing of the final drug product. The latest USP guideline recommends HCP testing of any lots used for preclinical toxicology assessment, all lots produced during clinical development, and any samples from the final manufacturing process that are used for process validation. Once the biopharmaceutical has been approved for use, HCP monitoring may also be required for quality control.[27]

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The central challenge in CHO HCP detection and monitoring stems from the complexity and dynamic composition of HCP populations throughout clinical development and across the manufacturing process.

To satisfy regulatory guidelines and meet criteria for clinical release, the manufacturer must be able to demonstrate clearance of HCPs across the bioprocess. The overarching goal is therefore to be able to detect the maximum number of protein species possible in process intermediates and in the final product. To achieve this, the chosen assay must combine broad proteomic coverage with high sensitivity for low-abundance proteins and a wide dynamic range.

There are a variety of HCP detection kits on the market that are generic for CHO cell lines and other expression hosts. While these may be appropriate for early stages of preclinical and clinical development, in cases where there is no available platform assay, a custom process-specific assay is usually required to demonstrate product purity in the latter stages of clinical development and post-approval.

5 Key Challenges in Reagent Development for HCP Immunoassays

To measure and control the large number of proteins present in CHO HCP populations, HCP immunoassays rely on the use of polyclonal antibodies (pAb) raised against antigen representative of the total HCP population. This means that assay quality is entirely dependent on quality of the pAb serum, which dictates the sensitivity of the assay and the degree of coverage of the HCP spectrum.

To further complicate matters, failure of particular process steps (for example, leakage of a chromatography column), or changes made during process optimization and scale-up, may lead to the appearance of new proteins that weren't present in the antigen used to generate the polyclonal antiserum. This risk needs to be mitigated when developing the strategy for polyclonal generation.

USP's general chapter on HCP measurement (USP 39, 1132) highlights five challenges specific to HCP immunoassay development for recombinant biopharmaceuticals: [27]

1. The wide variety of possible HCPs in medicinal products
2. Use of pAb reagents to detect HCPs
3. Lack of exactly matched standards for quantitation
4. Risk of considerable sample dilution effects
5. Inherent limitations to measure single HCP species

Developing Polyclonal Antibodies for CHO HCP Detection

Developing pAb reagents suitable for capture and detection of complex heterogeneous HCP populations is a pivotal step in HCP immunoassay development. The HCP antigen used for immunization, and also as a calibration control, must be representative of the CHO cell line and the manufacturing process. It should then be administered in a way that will maximize reactivity with as many different HCP species as possible. Finally, purification steps need to be designed and optimized to recover as much functional antibody as possible to support future development and manufacturing needs.

Antigen Selection and Preparation

The first step in generating anti-CHO HCP pAb is to prepare a representative HCP extract, or "antigen", by culturing null CHO cells (devoid of product-encoding genes) under the same conditions used for the protein-expressing cell line. At this stage, it is important to bear in mind that fully defined ACF culture media and supplements are recommended to mitigate the risk of non-HCP contaminants that could induce non-HCP specific antibodies, leading to elevated background signal in the immunoassay. Likewise, any purification equipment (e.g., Protein A column) upstream of the antigen collection point should be free of non-HCP proteins, including the therapeutic product.

The point in the bioprocess at which antigen is collected is crucial in determining the breadth and relative levels of HCPs represented. The most appropriate collection point depends on whether the assay is intended to be generic (appropriate for a variety of expression strains and upstream process procedures), platform-specific (suitable when the upstream process is common to multiple products), or process-specific (tailored to detect HCP impurities unique to a specific upstream or downstream process). Choice of assay type is discussed in more detail below (see *Quantifying HCP: choosing the right assay*).

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Early-stage antigens comprise the broadest range of HCPs. For this reason, harvested cell-free culture supernatant or crude cell lysate is often the most logical choice for development of commercial detection kits and platform-specific or upstream process-specific assays. Collecting antigen further downstream in the process—after the first capture step, for example—will reduce the number of HCP species present and potentially improve representation of proteins enriched in the final bulk. However, downstream process-specific assays using pAb reagents generated from null-cell antigen have a number of potential limitations, such as the risk of missing HCP that co-purify with the product, or the need to develop multiple HCP assays for slight process changes. Consequently, they are not currently recommended, except for certain products with exceptional downstream processing.[27]

Immunization Strategy and Preparation

The immunization program is considered the most critical element of pAb reagent development. Numerous variables in immunization strategy and execution can significantly impact the outcome. Since immunization programs are lengthy, resource-intensive, and unpredictable, failure to obtain antiserum of sufficient quality or quantity at the end of the process can be a major setback. Therefore, careful planning is essential, as is ensuring that quality parameters are well-controlled throughout.

Choice of host species will affect the diversity of HCP-specific IgG generated and the overall yield of antisera. Most immunization programs use from two to four host species, with rabbit, sheep, goat, donkey and chicken being the most common. Having multiple species helps compensate for the fact that not all antigens are immunogenic in all species. Including a species that is phylogenetically distant from mammals, such as chicken, may be desirable to induce antibodies that recognize highly conserved mammalian HCPs. Generation of antibodies in multiple species also provides flexibility for sandwich ELISAs, which are the most widely used assay type for HCP monitoring.

Long-term supply of pAb reagent is another key consideration when deciding on host species. Larger animal species are often favored for pAb production because of the tendency for longer life spans and higher productivity per animal. On the other hand, if the intention is to represent a greater diversity of individual immunogenic responses, then a smaller species will allow for easier management of a higher number of immunized animals.

Sourcing specific pathogen-free (SPF) animals and screening them for pre-existing anti-drug antibodies at the start of the program is essential. A well-designed nutritional plan, regular feeding schedule, careful animal handling and controlled housing conditions are also critical to avoid protocol variations or stressful conditions that could lead to variable immunogenic responses.

Immunization protocols typically start by priming with a mixture of HCP antigen and adjuvant, followed by 2-8 booster immunizations over the course of several months. Antiserum is collected before the initial priming and at 7- to 14-day intervals after each booster. To induce a more comprehensive antigenic response, various options such as size fractionation of the HCP antigen or cascade protocols (iterative depletion of immunodominant antigen) to enhance reactivity to less immunogenic species may be helpful. However, the potential benefits must be weighed against the added time, cost and complexity involved. Before pooling and purification, crude antisera are screened, typically by western blotting, to eliminate bleeds with low titers, immunodominance for a small subset of HCP, non-specific binding characteristics, or reactivity with the therapeutic product.

Antibody Purification

Antibodies in the pooled sera must be isolated from other serum components to improve sensitivity and specificity of for HCP detection. Chromatography using protein A or G (depending on the host species) is a fast and relatively robust way to isolate and concentrate the IgG fraction. However, it does not specifically enrich for anti-HCP antibodies and may yield a higher proportion of low-affinity antibodies compared to anti-HCP affinity chromatography.

HCP affinity chromatography enriches for high-affinity anti-HCP antibodies, and can increase sensitivity of the pAb 100-fold compared to IgG fractions from protein A/G columns. For this reason, it is widely used for commercial applications. Compared to protein A/G methods, however, more documentation and a high degree of skill are needed for column preparation and maintenance. For more comprehensive guidance on this topic, see USP's general chapter 39 <1132>.[27]

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Characterizing anti-HCP Antibody Pool

Each batch of antibody produced must be accurately quantified and characterized before use in HCP assays. Concentration determination using a standard UV absorbance, colorimetric or fluorometric method is a fundamental first step to ensure accuracy of any subsequent labelling or dilution steps.

Coverage analysis is then performed to estimate the percentage of HCPs that can be detected by the anti-HCP pAb pool in the matrix of interest (e.g. cell culture supernatant, process intermediates, drug substance). Among the most widely used methods for coverage assessment are two-dimensional western blotting (2D-WB), 2D difference gel electrophoresis (2D-DIGE) and 2D differential in blot electrophoresis (2D-DIBE). These methods provide high-resolution separation of the HCP population in two dimensions based on pI and molecular weight. 2D gels were then transferred to a Western Blot membrane and incubated with the anti-HCP pAb pool. Differential analysis is then performed by comparing the number of protein spots identified by immunodetection with anti-HCP pAb pool with the number of proteins detected by a total-protein stain. Greater than 50% coverage with reactivity across all the gel is acceptable by conventional criteria.[33]

An alternative method for coverage assessment is antibody affinity extraction (AAE) followed by 2D-PAGE. This approach was developed to overcome limitations of 2D-WB and 2D-DIGE with respect to sample denaturation and loading capacity. With AAE methodology, an affinity column is prepared with the HCP polyclonal antibody to be evaluated. Samples are then passed over the column repeatedly to capture native undenatured HCPs. The number of unique HCP species in the pooled concentrated eluate can then be compared to the number in the pre-AAE sample using conventional 2D gels as described above. By enriching HCPs prior to 2D analysis, AAE offers improved sensitivity, specificity and assay linearity compared to conventional methods.

Quantifying HCP: Choosing the Right Assay

The needs and requirements of HCP assays vary across the product development phases and depending on project goals. Early planning of assay development strategy is advisable to minimize the need for bridging studies, which are required whenever a new HCP assay is adopted.

Generic Versus Specific Immunoassays

HCP immunoassays can be flexible and generic (Figure 3), to accommodate changing HCP profiles during early development, or they can be tailored to detect HCP populations specific to a particular platform or bioprocess. Deciding which type to use at which stage of the development process is not easy, because it can be difficult to anticipate when downstream processes will be locked down and whether there are likely to be significant process changes during clinical trials that would necessitate development of new HCP assay.

Generic assays and commercial kits are often the most appropriate choice in the early stages of pre-clinical and clinical development, when it would be too time-consuming and expensive to develop and validate a new HCP assay each time there is a significant change in process conditions. A process-specific HCP-ELISA assay, for example, can take 1.5 years or more to develop.[34] Rather than being tailored to a specific host cell or

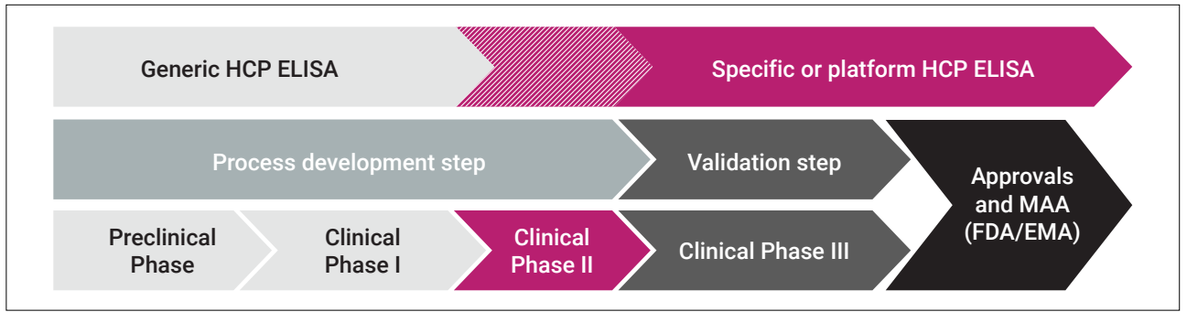

Figure 3 – Biopharmaceutical development: changing requirements for HCP ELISA

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bioprocess, generic assays are designed to give reasonable coverage of HCP populations generated by a variety of expression strains and upstream procedures. This gives maximum flexibility to test multiple cell lines and procedural variations that could impact the HCP profile. For assays used from phase III onwards, however, manufacturers must thoroughly demonstrate that the assay is still appropriate to detect specific process-related HCP. Continuity of supply and quality management are also important factors when considering commercial kits. More often than not, a custom platform or upstream process-specific assay is needed for phase III and beyond to maintain full control over the assay, avoid the need for bridging studies, and satisfy regulators.

Platform assays are designed with an HCP standard and antibody reagents specific to a company's proprietary host cell strain.[27] This assay type is thus compatible with host cells of the same type (e.g., CHO) across multiple products, provided that the upstream conditions are similar. With early planning, it may be possible to use the same platform assay from preclinical through phase III clinical development and post-approval.

Process-specific assays (also called product-specific assays) are tailored to detect HCP populations unique to specific upstream or downstream processes. Such assays are most relevant for the later stages of clinical development and post-approval, after the process has been locked down. Upstream process-specific assay reagents are usually developed using extracts collected before any purification. Like platform assays, they may be applicable to multiple products where the upstream process remains very similar. Downstream assays detect HCP subpopulations that have been enriched during purification. For reasons described earlier (see *Antigen selection and preparation*), assays specific to downstream unit operations are less commonly used, and currently not recommended except in exceptional cases.[27]

Regardless of assay type, HCP immunoassays used for process validation and drug substance testing must be thoroughly validated according to ICH Q2(R1).[35] This includes demonstrating acceptable specificity, sensitivity, accuracy and precision of the assay, as well as meeting criteria for coverage and sensitivity to both low- and high-molecular weight species.

HCP-ELISA Assays

Enzyme-linked immunosorbent assay (ELISA) is the most widely used method for HCP quantification. This flexible methodology entails direct or indirect immobilization of antigen to the surface of a microtiter plate or other solid support, followed by direct or indirect detection with an enzyme-linked antibody.[36] Assay signal is produced when the linked enzyme hydrolyzes a reporter substrate in the detection reagent mix. Antigen concentration is then determined based on a standard calibration curve generated simultaneously in the same assay.

While there are several different ELISA formats, the sandwich ELISA (Figure 4) is widely recognized as "the workhorse of HCP monitoring and quantification."[27] The key advantage of a sandwich ELISA is its high performance, even with crude HCP extracts. Sandwich assays are generally 2-5-fold more sensitive than conventional direct and indirect ELISA methods. Specificity is also enhanced through the use of two antibodies recognizing different epitopes on the same antigen.

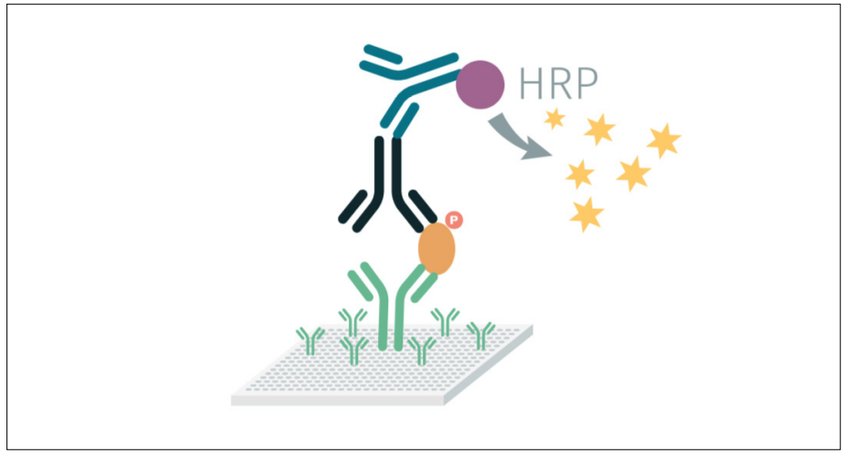

Figure 4. Sandwich ELISA principle.

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The sandwich ELISA principle is based on use of two antigen-specific antibodies: one for capture, the other for enzyme-mediated detection. The detection antibody is either linked directly to an enzyme, or carries a tag that mediates indirect detection with enzyme-linked secondary antibody. To perform the assay, test samples are added to the wells of a microtiter plate that has been pre-coated with capture antibody. After blocking and incubation to allow binding to occur, excess unbound antigen is washed away. Labeled detection antibody is then added, followed by detection reagents. The assay is read in an appropriate detector and results reported as immuno-equivalent ng HCP / mg drug substance.

Although ELISA assays provide a flexible and reliable means to quantify HCP impurities, they do not yield any information about the identities or amounts of individual HCP. Another drawback of ELISA assays is the number of wash steps and reagent additions involved, which can lead to assay variability and hamper automation. ELISA assays also tend to have a relatively narrow window for linear dynamic range (~2 logs), which is constrained by the optical density of absorbance-based readouts. This necessitates testing at multiple dilutions to ensure measurements fall within the linear portion of the curve.

HTRF™ Assays

HTRF (Homogeneous Time Resolved Fluorescence) technology offers a no-wash alternative for high-throughput HCP quantification. HTRF combines standard FRET technology with time-resolved fluorescence measurement to eliminate short-lived background fluorescence. The long fluorescence lifetimes of donor-conjugated lanthanides used for HTRF enable exceptionally high signal-to-noise ratios. With reagents that are resistant to photobleaching and a simple mix-and-read format that eliminates the need for washing, HTRF assays are faster, more robust, and easier to automate.

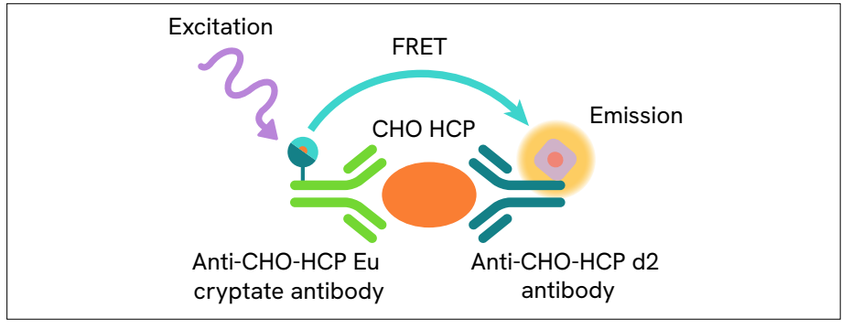

Figure 5. HTRF assay principle.

To configure a sandwich immunoassay for HTRF (Figure 5), one of the two sandwich antibodies is coupled to a FRET donor, and the other to the acceptor. Energy transfer is only possible when both antibodies bind to the same target molecule, bringing the donor and acceptor into close proximity. After pipetting a small aliquot of sample into the test well, a reaction mixture containing both donor and acceptor reagents is added. During the assay incubation period, antigen becomes sandwiched between the two antibodies. When excited, the donor emits fluorescence and transfers its energy to the nearby acceptor, resulting in specific acceptor fluorescence that is directly proportional to the target concentration. The assay readout is a ratiometric measurement of donor and acceptor fluorescence. This offers an additional benefit, since ratiometric signals are concentration-independent, making the assay easier to miniaturize for high throughput. With some commercial kit, accurate results can be obtained with as little as 10 uL sample per well. HTRF assays generally provide a larger dynamic range than standard ELISAs.

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Revvity's HTRF immunoassay kit for CHO Host Cell Protein (HCP) Detection enables the quantitative determination of CHO HCP in buffer, cell culture media, and cell supernatants. Kits are available in 96-well and 384-well formats, enabling from 500 to 10,000 assay points per kit, using as little as 16 ul of sample. Key features include:

- No wash or separation steps
- Sensitive detection
- Broad sample compatibility
- Small sample volume
- Wide range of detection
- Reduced hands-on time
- Polyclonal antibody reagents characterized by 2D-DIBE with a high percentage coverage

AlphaLISA™ Assays

AlphaLISA is a no-wash bead-based proximity assay that overcomes many limitations of standard ELISA and wash-based assays. Instead of enzymatic signal amplification, AlphaLISA technology takes advantage of luminescent oxygen-channelling chemistry[37] to greatly amplify the proximity assay signal and enable a high-sensitivity of detection.

As for ELISA sandwichs and HTRF assays, AlphaLISA assays use matched pairs of antibodies recognizing different epitopes on the same antigen. To perform the assay, antigen is sandwiched between a biotinylated anti-analyte antibody and a second anti-analyte antibody that has been coupled to an acceptor bead. Next, streptavidin-coated donor beads are added. The streptavidin-biotin interaction pulls the complex together, bringing the 2 beads into close proximity (Figure 6).

After a short incubation step to allow complex formation, the assay can be read without the need for any wash steps. Laser excitation at 680 nm releases up to 60,000 singlet oxygen molecules per donor bead, serving to amplify the assay signal to greatly enhance its sensitivity. If the singlet oxygen encounters a nearby acceptor bead, it initiates a reaction cascade inside the bead, leading to light emission at 615 nm. Signal intensity is proportional to the concentration of analyte in the microplate well. Because the wavelength of emitted light is shorter than the excitation wavelength, background interference is greatly reduced.

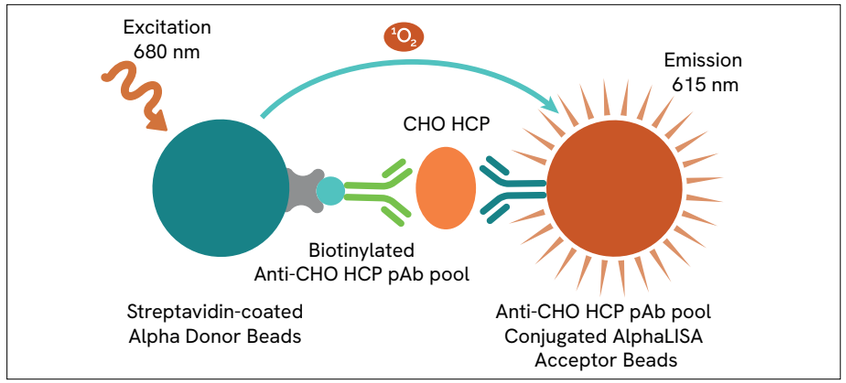

Figure 6. AlphaLISA assay principle.

Revvity's AlphaLISA immunoassay kit for CHO Host Cell Protein (HCP) Detection enables the quantitative determination of CHO HCP in buffer, cell culture media, and cell supernatants. Kits are available in 96-well and 384-well formats, enabling from 100 to 5,000 assay points per kit, using as little as 5 ul of sample. Key features include:

- No wash or separation steps
- Sensitive detection
- Broad sample compatibility
- Small sample volume
- Wide range of detection
- Results in 3.5 hours
- Reduced hands-on time
- Polyclonal antibody reagents characterized by 2D-DIBE with a high percentage coverage

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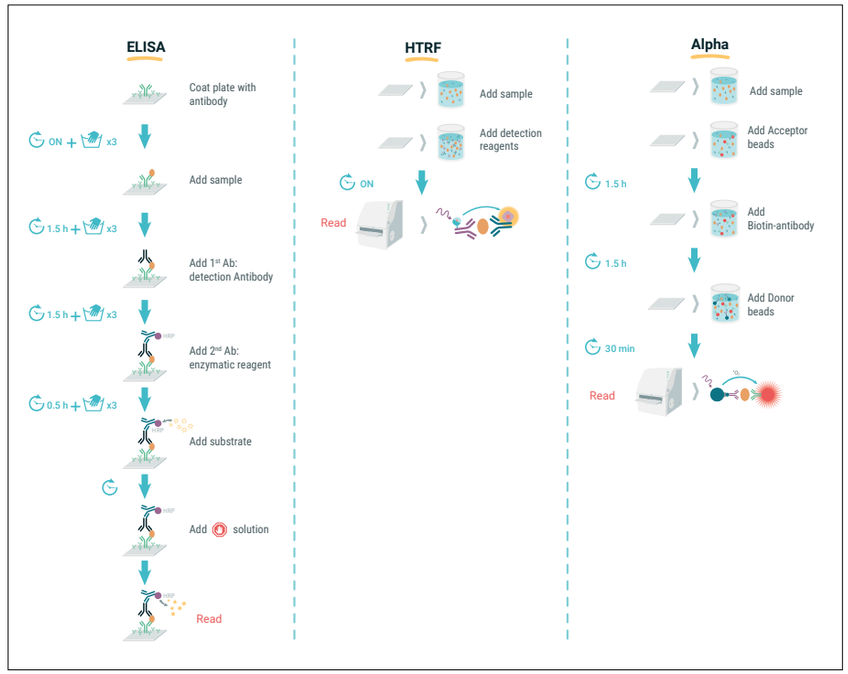
Table 1: CHO HCP kits.

	ELISA	HTRF	AlphaLISA
Wash Steps	Yes	No	No
Sample Volume	100-200 µL	16 µL	5 µL
Scalable/Miniaturization	No	Yes	Yes
Dynamic range	+	++	+++
Sensitivity	+	+	++
Hands on Time	High	Low	Low
Signal Stability	< 15 min	Hours to Days	Up to 24 hours



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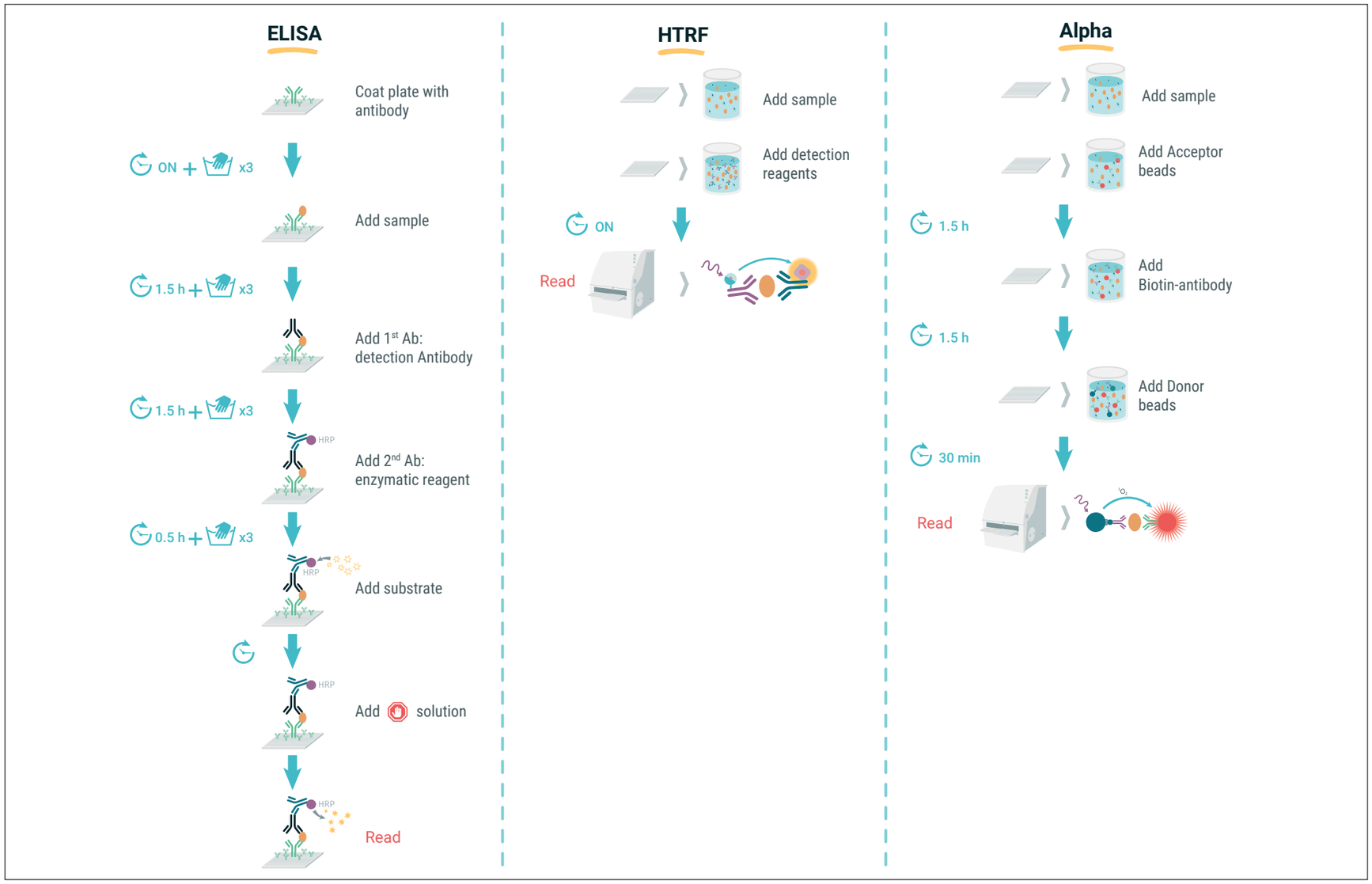

Figure 6 – Comparison of ELISA, HTRF and AlphaLISA workflows.

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Alternative and Orthogonal Methods for HCP Identification and Tracking

Reliable and robust methods for HCP quantification are fundamental to monitor, track and measure HCP impurities during bioprocess development and manufacturing, and to demonstrate purity of the final drug substance. Since each assay technology has its limitations, there is currently no universal approach that covers all needs. Consequently, the use of multiple assay technologies is highly recommended.[38,34]

While ELISA, HTRF and AlphaLISA for total HCP quantification provide a robust and rapid means of assessing impurity levels, complementary orthogonal proteomic technologies are needed to detect process-dependent changes in HCP composition and identify specific HCP species so that levels of high-risk impurities can be monitored and controlled.

Methods based on two-dimensional electrophoresis such as 2D-WB and 2D-DIGE/DIBE provide a visual snapshot of HCP populations. Differential analysis of HCP populations with these methods provides greater insight into the impact of process changes. Fluorescent staining enables quantitative analysis of individual proteins, and can be coupled with MS for identification of specific proteins. However, limitations in dynamic range can make it difficult to capture both high- and low-abundance proteins on the same gel. In addition, high levels of certain proteins, including the therapeutic product, can mask the presence of lower-abundance proteins located in the same vicinity on the gel. Gel-based methods are also labor-intensive and require a reasonable degree of skill to minimize variability.

A variety of proteomic methods involve coupling separation techniques to MS. Liquid chromatography-MS (LC-MS) in particular is used for specific, quantitative and highly sensitive identification of individual HCPs following 2D gel separation. The relatively low abundance of HCP species compared to mAb can present a significant challenge, however. The use of antibody affinity chromatography to enrich HCP populations and deplete drug substance can be an effective way to avoid downstream analytical problems, such as fragmentation bias when running LC-MS in data-dependent (DDA) acquisition mode.

Various capillary electrophoresis methods are also gaining attention as powerful and cost-effective tools for analysis of mAb preparations and associated HCP impurities.[39] Capillary zone electrophoresis (CZE) is the most widely used, largely due to its potential for miniaturization and automation.

Ultimately, combined use of these and other emerging orthogonal techniques early in upstream process development will help to drive more informed, data-driven decisions to help de-risk biopharmaceutical drug development and improve cost-efficiency.

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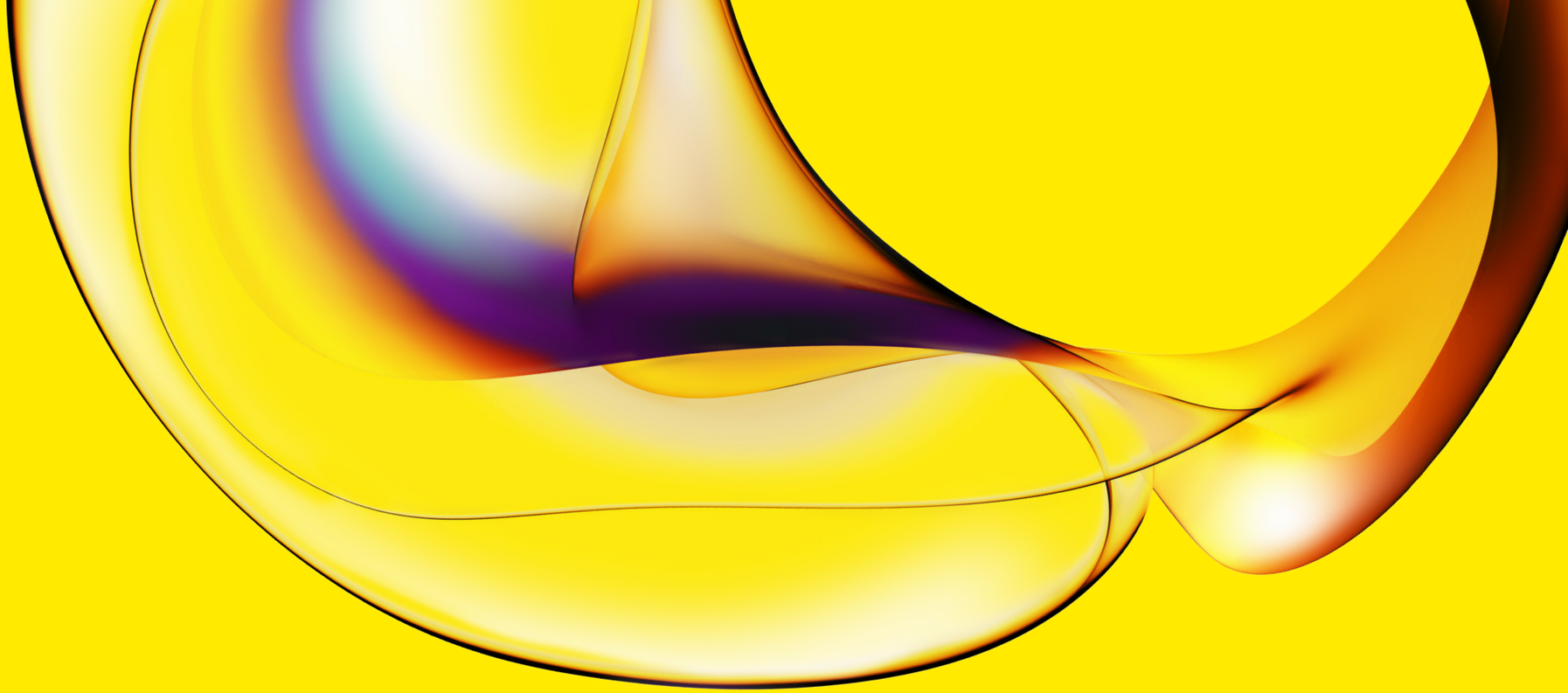

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