Biologics Process Analytics

While process-specific assays are preferable, their generic counterparts can be just as effective if developed well. An assay, regardless of the terminology used to describe it, should be comprehensively qualified to show it is fit for purpose of monitoring host cell protein clearance and lot release testing

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Over the past 15-20 years, the production of biological drugs – in particular, therapeutic antibodies – has rapidly increased. Host cell proteins (HCPs) represent a major group of process-related impurities of biological drugs produced using cell culture technologies. Even at nanogram per milligram concentrations of HCP to drug substance (DS), HCPs can elicit undesired immune response and interfere with drug efficacy. Additionally, some host cell impurities, such as proteases, have been shown to reduce the stability and efficacy of the drug substance. Given the potential adverse reactions and reduction of product activity, HCPs must be adequately removed in downstream purification processes. Sponsors are responsible to use broadly reactive HCP ELISA to track the removal of HCPs during the purification processes to ensure process consistency and final DS purity.

Biopharmaceutical manufacturers routinely use ELISA for the quantification of HCPs during downstream purification. Two types of HCP ELISA, 'process-specific' and 'generic', are commonly used in scientific publications and regulatory guidelines. The terminology distinction is based on theoretical concerns that generic assays could potentially fail to detect HCPs specific to a given culture and purification process. This concern has led to an arguable belief among many in the biopharma industry that a process-specific assay should always be developed. This belief is often contradicted by comprehensive qualification data across multiple drug products using well-developed generic assays that fulfil the analytical requirements for a final product release test. This article is intended to provide a clearer definition of the terminology and to propose how an assay, regardless of the terminology used to describe it, should be comprehensively qualified to show it is fit for purpose of HCP detection in samples throughout the purification process. When a generic assay can be comprehensively qualified across multiple cell lines and cell culture processes, companies will be able to save the cost and time of developing a redundant process-specific method.

Given the variables in antigen selection, methods employed in antibody generation and purification, and, finally, in assay development, one cannot guarantee that a process-specific antibody generated to a particular strain or growth process will be superior to antibodies generated to an essentially identical strain and process. Effective generation of antibodies to the relevant downstream HCPs must incorporate multiple elements beyond proper antigen selection. A truly process-specific assay cannot be assured by simply immunising animals with an arbitrary choice of upstream antigen. With proper methods in antigen selection, antigen preparation, and effective immunisation protocols, the generated antibodies will be reactive to those HCPs constituting more than 95% of the total mass of HCP in harvest material.

The conventional method to demonstrate antibody coverage has been two-dimensional western blot (2D WB). A 'percent coverage' is determined by attempting to match spots in a silver stain of a polyacrylamide gel to those spots seen in a 2D WB after the HCPs have been transferred to a polyvinylidene difluoride membrane and detected with the ELISA antibody. The limitations of 2D WB coverage analysis for HCPs have been well-documented. Computer-assisted spot matching between gels and membranes is qualitative, labour-intensive, highly subjective, and prone to human error. Spot matching between the two dissimilar methods is not quantitative in the sense of the relative masses of each HCP, but rather, only provides a spot-to-spot correlative percentage estimation. Low-intensity spots near the threshold of each method's sensitivity are assumed to be HCPs. In the absence of methods to determine if spots are true HCPs and not non-HCP impurities or procedural artefacts, the sensitivity and specificity limitations combine so that 2D WB will always underestimate true antibody coverage. Paradoxically, efforts to get broader coverage by 2D WB have unfortunately misguided researchers in their antigen selection and antibody generation methods, resulting in antibodies that are not optimal for detection of HCP in relevant samples. The objective qualification of an antibody and assay for coverage to downstream HCP requires orthogonal methods with greater sensitivity and specificity than can be obtained by 2D WB.

The goal of HCP analysis is not to demonstrate reactivity to every HCP in the entire proteome of a given expression organism, regardless of its relative concentration or ability to co-purify with a DS. Rather, the goal of HCP analysis is to adequately detect problematic HCPs that persist through a drug purification process. These two goals are different, conflicting, and not well-understood by many in the industry. To understand that an antibody reactive to the entire proteome may not be the best antibody for detection of the more limited array of HCPs in final DS requires knowledge of the limitations of HCP analytics. Failure to generate antibodies to low-abundance and low-immunogenic HCPs in upstream material could, in theory, lead to an under-estimation of total HCP in final DS. Such under-estimation of total HCP is very minimal in terms of 'total' HCP concentrations reported by ELISA. The most significant source of underestimation of HCP by ELISA is not the absence of antibody, but, rather, lack of antibody excess. For ELISA to be quantitative, each individual HCP must have an excess of antibody. Any major HCPs that have some affinity for the drug or its purification process can often be in excess for the amount of ELISA antibody that can be coated on the solid phase or used as the detector antibody. The qualitative observation that those major HCPs have coverage by 2D WB is of no relevance or predictive value. Without antibody excess in the ELISA, those major HCPs will not be quantitated. This stoichiometric requirement for antibody excess and related discussions of the 'Hook Effect' and the importance of demonstrating sample dilution linearity are beyond the scope of this article (1).

Definitions

The distinction in HCP assay terms, like 'process-specific' or 'generic', is more semantic than scientific, as evidenced by the fact that the objective scientific criteria required to qualify any analytical method are the same for both generic and process-specific HCP assays.

Generic

Generic is used in the biological sense whereby the antibody is intended to detect HCPs independent of the growth and purification process. The presumption for generic assays is the majority of proteins in a cell line like Chinese hamster ovaries (CHO) are highly conserved among different strains (2-3). This presumption has been supported by published genomics and proteomics studies for both CHO and *Escherichia coli* prokaryotic expression systems (4-6). Generic assays, like process-specific, typically use antigen derived from upstream, null, or mock-transfected cells. While culture processes and conditions may up- or down-regulate certain HCPs qualitatively, most of the HCPs are conserved among strains and processes (2, 7). With comprehensive qualification using orthogonal methods of antibody analysis, regulatory agencies will accept generic assays as fit for product release and process monitoring without requiring development of a redundant process-specific assay. If a biopharma company believes it has data to support the use of a generic assay for product release testing, discussing the intent with regulatory agencies involved and showing the assay data, along with orthogonal method analysis, is advisable.

Process-Specific

The term 'process-specific' is not well-defined in the literature. There are cell line-specific, growth process-specific, and downstream purification process-specific assays. The source of antigen used to generate a process-specific antibody will have a very significant impact on how an ELISA using that antibody will accurately detect HCP. How far upstream or downstream in the purification process, the use of null cells, mock-transfected, or product-expressing cells are all issues to be considered in antigen selection. To add to the confusion, some published papers claim a process-specific antibody have admittedly included non-process-specific material, such as lysates or cell debris, in their antigens. Inclusion of irrelevant antigens or a disproportionate mixture of fractionated antigens

Figure 1a: 2D PAGE showing antibody coverage by AAE. Left: 2D image of starting CHO HCP (Cy3) pattern showing differing spots. Spots present in the starting CHO HCP, but missing from the AAE sample, are outlined in red. Spots present in both samples are outlined in blue. Right: 2D image of the eluate CHO HCP (Cy5) pattern for comparison. Spot outlines are identical to those in adjacent image. Spots unique to starting CHO HCP are outlined in red. Spots present in both samples are outlined in blue

Figure 1b: Antibody coverage by 2D WB. Image of CHO harvest material silver-stained gel showing goat anti-CHO antibody 2D WB matches. Spots present on the silver-stained gel, but missing from the 2D WB, are outlined in red. Spots present in both the silver-stained gel and the 2D WB are outlined in blue. Spots detected with the antibody, but not detectable by silver staining, are indicated with small blue dots on the silver-stained gel and added to the total spot number. The goat anti-CHO antibody detected 717 spots out of 1,293 spots (55%) found

can result in a self-proclaimed process-specific antibody that is not suitable for detection of HCPs co-purifying with DS. Such illogical strategies lead to an obvious counter point. How can an antibody generated from non-process-specific antigen be labelled as process-specific?

Process-specific most commonly means using very upstream HCPs from null cells of the same strain and the growth process used to express the drug. It should be acknowledged that such an antigen is fundamentally the same as what is used for generic assays. A process-specific antigen is only distinguishable from generic by the assumption that, despite highly conserved cell lines and similar culture processes, each drug product will contain a significantly different subset of HCP that persist in significant concentration through a given purification process.

Qualification of HCP Antibodies and Assays

It should be understood that the ability of any assay to accurately and quantitatively detect HCP in relevant samples is influenced by arbitrary choices and method limitations that are more significant than just broad coverage to an upstream antigen. Any assay, regardless of what it is termed, must be subjected to comprehensive qualification to demonstrate it meets the accuracy, specificity, and sensitivity requirements to serve as a process control and quality control release test. Due to poor sensitivity and specificity, 2D WB has no predictive value in determining how that antibody, when used in an ELISA format, will quantitatively detect the most important HCPs – those that co-purify with product.

New orthogonal technologies can address the theoretical concern about what an HCP assay might be missing and answer the question of what it actually detects. A method termed antibody affinity extraction (AAE), also referred to as immunoaffinity binding, is now being widely used to show coverage to both upstream and, more importantly, downstream HCPs (8-9). AAE is over 100-fold more sensitive than 2D WB and, as such, can show reactivity to HCPs that co-purify with DS. Figure 1a shows a 2D PAGE fractionation

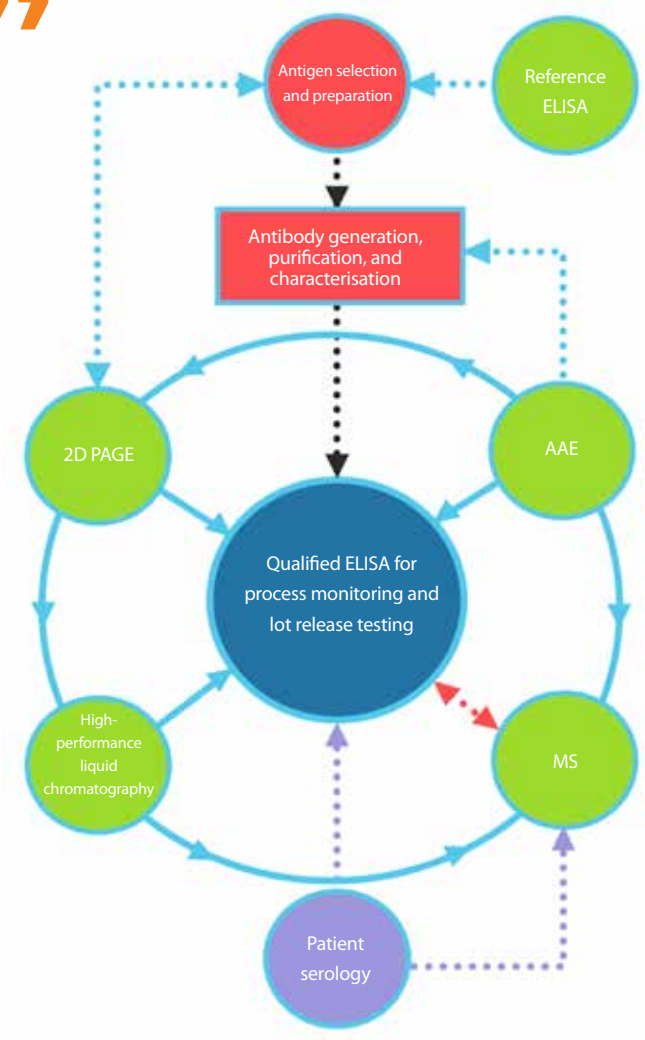
	AAE	2D WB
Sensitivity	Affinity enrichment up to milligrams of HCP (sensitivity >95%)	Coverage underestimated due to limitations of WB (sensitivity 50-70%)
Specificity	High (greater than 99.5%)	Poor (due to limitation of 2D WB ~50% to 80%)
Applications	Applicable for upstream and downstream samples	Only applicable for upstream samples

Figure 2: Comparison of AAE and 2D WB methods

"Use of these methods for comprehensive qualification of the assay may obviate the cost and time to develop a redundant process-specific assay when an acceptable generic"

of an upstream sample prior to AAE and Cy3 staining detected 976 HCP spots (blue circles). After AAE, the 2D PAGE staining with Cy5 detected 896 of the same spots for a coverage of 92%. By contrast, 2D WB only gave a coverage of 55% (see Figure 1b). The 80 spots not detectable by AAE are represented with red circles. If deemed necessary, those spots could be picked and analysed by mass spectrometry (MS) to confirm if they are HCPs, product-related, another impurity, or artefact. The table in Figure 2 summarises differences in AAE and 2D WB.

Due to significant advances in speed, resolution, and sensitivity of MS, biopharma sponsors can use MS as a valuable orthogonal method for specific identification and quantification of individual downstream HCPs (10-11). Enrichment strategies, such as 1D and 2D liquid chromatography (LC) separation, Protein A chromatography, and tryptic digestion followed by precipitation have been developed to deplete DS peptides to increase LC-MS resolving power and sensitivity (12-13). AAE is a highly efficient HCP enrichment method in offline sample preparation prior to LC-MS, reducing the cost and complexity of MS analysis. When used in conjunction with critical assay qualification criteria for accuracy and specificity by analysis of dilution linearity and spike recovery data on downstream samples, AAE and MS can demonstrate objectively if any assay is fit for purpose as a routine lot release test. The diagram in Figure 3 represents the most effective methods to develop and qualify HCP assays. Utilisation of the techniques implied in the red boxes will allow for a broadly reactive assay. The green circles are orthogonal methods that can detect individual downstream HCPs. Use of these methods for comprehensive qualification of the assay may obviate the cost and time to develop a redundant process-specific assay when an acceptable generic assay exists.



Colour key:
Green = Orthogonal methods used to support ELISA
Red = Critical decisions in the development of the ELISA
Purple = Orthogonal method not to support the ELISA development, but to support the clinical findings

Figure 3: Integration of orthogonal methods for comprehensive HCP analysis

In the absence of a process-specific assay, companies should use available generic assays for process development and early clinical trials. A qualified generic assay is a valuable purification process development tool to better assure safety and efficacy in the clinic. The suitability of a generic, or the need to develop a process-specific assay as the release test can be determined as the manufacturing and purification process, is finalised.

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Ken Hoffman, President at Cygnus Technologies, founded the company in 1996 to provide analytical solutions to the rapidly growing biopharma industry. Prior to starting Cygnus, he worked in the clinical diagnostics field for three different companies, developing automated systems and extensive menus of assays for infectious disease, metabolic disease, drugs, and hormones. Ken has his Master of Science in radiation biology/immunology from University of Iowa, US. In addition to his MS degree, he also holds a certificate in biomedical research management from The Harvard School of Public Health, US.

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