Getting Specific

The usual method of controlling process-related impurities in biopharmaceuticals leads manufacturers to consider new product-specific assay development at early stages of drug development

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Host cell proteins (HCP) are an inevitable impurity of biopharmaceuticals, regardless of whether they are produced by recombinant fermentation or extracted from natural sources. Even after multiple sophisticated purification steps, HCPs remain or co-purify. They represent a heterogeneous variety of different proteins that need to be quantified in the drug substance and in intermediates from the downstream purification process. The risk for adverse effects, such as immunogenic reaction, does not necessarily correlate with the amount of certain host cell proteins, and even small traces of certain HCPs can be highly immunogenic (1-2).

Traditional protein detection methods such as HPLC and total proteins stains are not suitable due to their insufficient sensitivity and specificity. Consequently, optimised immunoassays (ELISA) have established themselves as the method of choice for the measurement of HCPs. In contrast to single contaminants or leachables such as trypsin or protein A, which can be approached by a monoclonal antibody, the heterogeneity of the HCPs always requires a polyclonal antiserum for the assay development.

Generic Versus Product-Specific Assays

Information on HCP removal is necessary in early process development. The development of a specific assay is time-consuming and consequently generic assays or commercial kits are used.

Choice of Antigen

The selection of an appropriate antigen is crucial for the development of any HCP assay. However, there are limitations that typically occur for each type of expression system. For example, a prokaryotic expression system, based on inclusion bodies, typically needs to start with a whole cell lysate as an antigen for immunisation. In the case that the protein of interest is secreted from the expression systems, the cell-free supernatant can be used as an early stage antigen. In this context, it is important to prevent the inclusion of potentially antigenic material from non-HCP origin, such as components

Table 1: Pro and cons of generic and specific assays

Generic assays	Project specific assays
Instantly available	Requires antigen preparation, including cloning of a mock cell line
No need for preparation work	Assay development, including antigen characterisation, immunisation and qualification, takes a minimum of four months
Availability is limited to the most common expression systems	Feasible for all recombinant expression systems
Specificity might not be suitable, high risk of undetected HCP species	Typically enhanced specificity; antibody coverage more customised to the specific HCP pattern
Costs – initially fairly cheap, but for an entire DSP, development costs add up quite fast	Initially costly, but the break-even point is typically at a value of 100 kits
Dependency on one vendor, black box for reagents and controls	Full control of all reagents and buffers, optimisation/adaptation is always possible
Not suitable for supporting marketing authorisation applications	Suitable from early development to final product release of authorised product

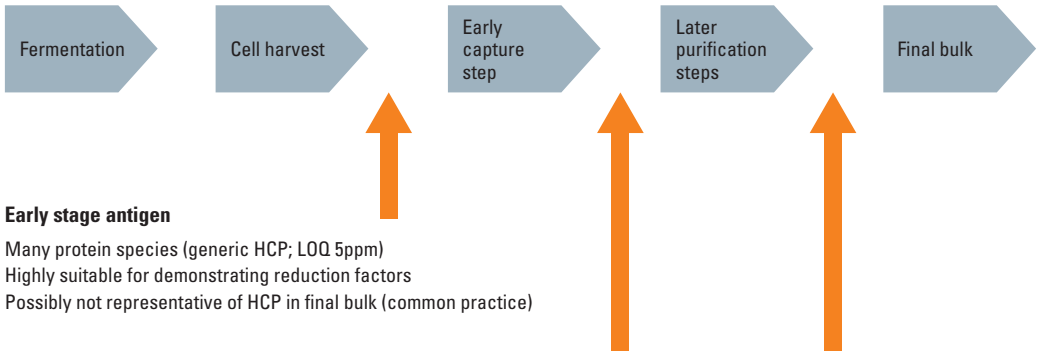


Figure 1: Choice of antigen for the development of a specific HCP assay

from the growth media. Many media additives such as hormones or serum proteins will result in non-HCP specific antibodies and significant background in the immunoassay. Growing cells in serum-free media or thorough washing of cells/ bacteria can help to eliminate such unwanted antigens.

In some cases it can make sense to start with material immediately following the first capture step, or even material collected further downstream. The later-stage antigen is more representative of the HCP in the final product, where an antigen from the early downstream purification process can help to demonstrate much better reduction factors of the HCP content. However, there are some practical considerations, such as the generation of sufficient antigen material for the immunisation and assay development, which is usually in the range of 200 to 300mg of total protein at a concentration of approximately 1mg/ml.

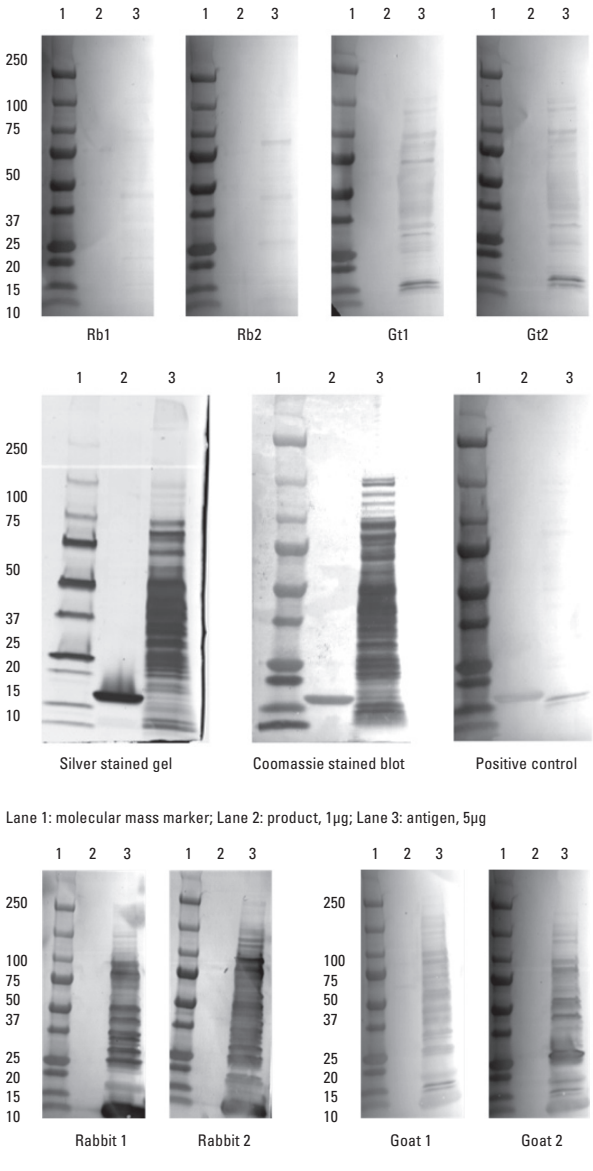
Immunisation Procedure

The immunisation procedure is the most critical and time consuming, but simultaneously the most unpredictable part of

Figure 2 (right): Testing of pre-immune sera for cross-reactivity. These Western blots demonstrate that the pre-immune sera have no cross-reactivity with the product (no band in lane 2). In lane 3, the goat-derived antisera has already shown some reactivity with the antigen (host cell proteins)

Figure 3 (right): Antigen controls. Lane 1: molecular weight marker; lane 2: product, 1µg; lane 3: HCP antigen, 5µg

Figure 4 (right): Immunoblots using antisera from the first bleeding. The results of the western blots demonstrate that the rabbit and goat antisera from the first bleed are able to detect a wide spectrum of host cell proteins. Within the limit of detection there is no cross-reactivity of the antisera against the product



assay development. Consequentially, all possible actions must be taken to limit the disturbing parameters, which include the animals' species, health conditions and nutrition, and administration of the antigen and collection and testing of antisera. The use of specific pathogen free (SPF) animals, which are kept under controlled conditions and nourished with a defined nutrition plan, helps to reduce the background, unwanted immunogenic reactions against food-borne antigens. This applies to bacterial and yeast-derived products, since many animal foods are based on yeast extracts and peptones. A typical immunisation programme utilises two species (four rabbits and two goats), which ensures sufficient flexibility to set up a sandwich ELISA, as well as sufficient quantity to supply several hundred, if not thousands, of ELISA measurements.

Typically, an immunisation period of three months with three bleedings results in the following quantities of antiserum, assuming that the individual bleedings will be equivalent and suitable for the further assay development:

- Goat: final blood volume per animal = approximately 700-1,000ml
- Rabbit: final blood volume per animal = approximately 120ml

From these crude antisera, 100ml are processed further by affinity purification and labelling, resulting in 10ml of purified anti-HCP antibody, which is sufficient for 500 ELISA plates (assuming a 1:500 dilution).

The literature describes several so-called 'cascade' immunisation protocols, named as such because they involve repeated removal of antigens, and production of further antisera directed against antigens in the remainder (3). In theory, intermolecular immune competition can be avoided by removing strong antigens from the extracts. The remaining antigens, which give no immune response in the primary immunisation, are used for further immunisation. However, this approach is quite laborious, time-consuming and costly and is therefore applied in exceptional cases only.

Purification of the Polyclonal Antibodies from Crude Serum

The optimal performance of the HCP ELISA requires the purification of the antibodies from crude serum. This is typically performed by affinity chromatography against the HCP. The goal is to improve specificity and sensitivity by the removal of non-specific antibodies and other non-relevant components which can result in artificial signals during the immunoassay. On average, when affinity-purified antibodies were used, a 100-fold increase of the sensitivity is observed, compared to the use of IgG antibody fractions only.

The concentration of the anti-HCP antibodies during the purification step helps to increase the linearity of the assay as well as its working range. Careful consideration needs to be taken for the potential loss of specific anti-HCP antibodies during this immunoaffinity chromatography procedure. A comparison of Western blots using crude serum for detection with those obtained from purified antibodies can indicate if relevant antibody fractions were lost.

Characterisation of the Antibody Population

The goal of the entire exercise is the detection of a maximum number of antigen species in process intermediates and the final product (2,4). Consequentially, the coverage of the antibody population for a relevant HCP population needs to be checked and demonstrated. The gold standard to address this coverage question is still the use of high resolution 2D gels and Western blots. The antigens are put through a first run, separated based on their isoelectric point, and a second run, separated depending on their molecular weight in a high resolution SDS-PAGE. One gel will undergo a total protein stain, whereas the second gel will be developed in a Western blot using anti-HCP antibodies. The coverage is calculated by an image analysis tool. Coverage of approximately 80 per cent in the pI range of four to seven and a molecular weight between 10 and 120kDa is achievable and meets regulatory approval. The 2D gels and immunoblots in Figure 5, 6 and 7 illustrate this characterisation procedure and show the differences between a generic and specific approach.

Assay Set-Up and Calibration

Once the antibodies are checked and qualified, the quantitative assay development can be initiated using the following guidelines. Ninety-six-

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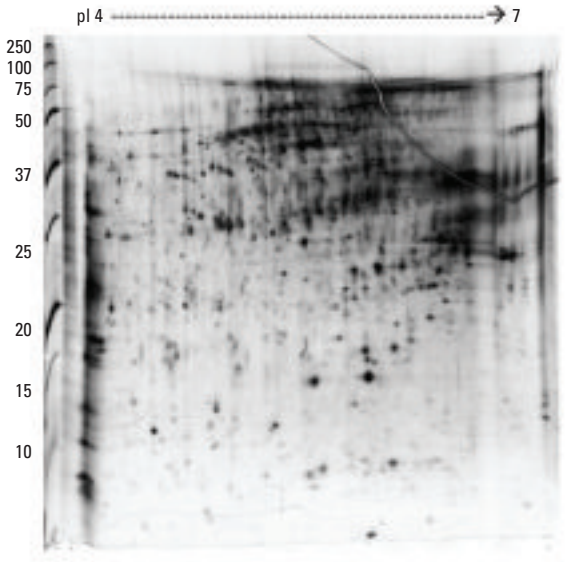

Figure 5: 2D SDS-PAGE total protein silver stain of the CHO HCP proteome. This silver-stained 2D electrophoresis shows a good separation of CHO host cell proteins in a pI range of 4 to 7 and a molecular weight distribution between 120 and less than 10kDa. The material used for this and the following blots represents CHO antigens from a mock fermentation after a cell retention step.

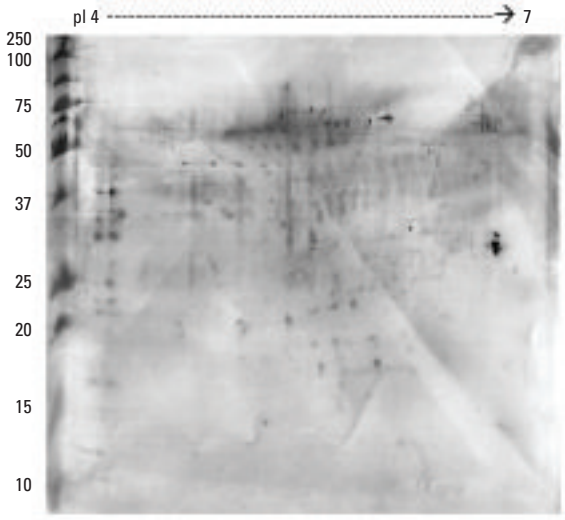

Figure 6: 2D Immunoblot Anti-CHO HCP, Cygnus, Anti-CHO HCP from F015 CHO HCP Kit (1:500). 2D Immunoblot of CHO HCPs and detection by Western blot using anti-HCP antibodies from Cygnus (F015) kit in a 1:500 dilution. The majority of signals in this Western blot experiment are between 100kDa and 20kDa. However, there are a significant number of spots, especially in the lower molecular weight area, which are clearly visible in the total protein stain but not detected by the antibodies.

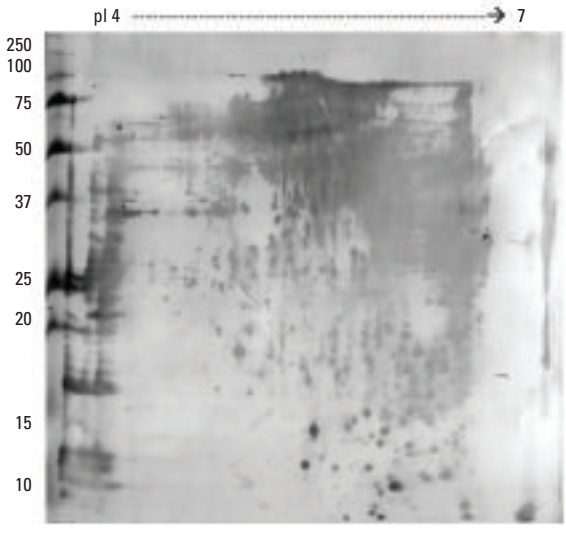

Figure 7: 2D Immunoblot, specific affinity purified antibody rabbit (1:500). This immunoblot using specific affinity purified anti-CHO-HCP antibodies shows an increased coverage compared to the pattern in Figure 5 detected by the kit antibody. Note the number of smaller protein species (more than 20kDa) is significantly increased using identical experimental conditions.

well ELISA plates are coated using the unlabelled purified anti-HCP antibodies. After a blocking step using synthetically blocking reagents, the reference standards, samples and control samples are added. Depending on the detection system, the labelled anti-HCP antibodies are added, followed by the addition of the relevant substrate (dye, fluorescence or chemiluminescence). After the incubation time, signals are determined by using a 96-well-plate reader. A calibration curve is prepared by using standard concentrations ranging from five to 5,000ng/ml, and the statistical evaluation usually employs a four parameter fit. The overall performance of each individual experiment is controlled by the following system suitability criteria:

- Recovery of the HCP spike – adding a known amount of HCP antigen to the test item in order to check for any matrix influence of the test item/matrix
- Detection limit – measuring of six background values and calculation of the detection limit where x = 3+s
- x = mean OD value of six single background signals
- s = standard deviation of the six determinations

Validation and Routine Testing

As HCP assays are used for process validation and the testing of drug substances, a full ICHQ2R1 assay validation is mandatory before the release of the product (5). The scope of the validation and typical results from more than 20 different HCP validation projects (specific and generic) are shown in Table 2 (see page 80).

The validation data, especially the working range of 1 to 1,000ng/ml, clearly demonstrated that these assays are suitable for lot release testing on drug substances, as well as the evaluation of HCP content in samples from the downstream purification process. The results for accuracy and precision are comparable for generic

Table 2: Validation results

Validation parameter	Experiment	Typical result
Precision (repeatability)	Seven-fold determination of a drug substance sample in one assay, recovery by spiking, one day, same technician	Coefficient of variation: seven to 10 per cent
Precision (intermediate precision)	Seven-fold determination of a drug substance sample in one assay, recovery by spiking, four days, four technicians	Coefficient of variation: 15 to 20 per cent
Accuracy	Three-fold determination of drug substance and process intermediates of interest, spiking with three different amounts of reference standard	Standard deviation from spike eight to 15 per cent
Recovery	Mean value of more than 20 tests	75 to 120 per cent
Linearity	Analysis of calibration curve with HCP spike	Acceptable working range from 1ng/ml to 1,000ng/ml
Detection limit	Lowest point on calibration curve which is distinct from the background	Less than 1ng/ml
Quantitation limit	Lowest amount of spike which is quantitatively recovered from sample	1ng/ml
Specificity	Assay with product protein and HCP mixture from different species	No signal above detection limit

assays and those specifically developed for a certain product (6,7).

Conclusion

Immunological methods are the only tool currently available for a quantitative analysis of residual HCPs with a sufficient sensitivity (more than 10-100ppm). The quantitative results are derived from comparisons to a reference standard. Therefore, accurate results can only be expected when the test item and reference standard are comparable. In addition to an optimised sensitivity, the coverage of the HCP population is of equal importance since, as recently described, undetected HCP species can cause severe adverse effects in patients, resulting in a setback to the entire drug development process. This does require a thorough characterisation exercise, starting from the antigen up to the final serum and antibody preparations. An optimised 2D gel-electrophoresis is the method of choice to achieve evidence on the coverage and finally the suitability of the antibodies used for the quantitation. This applies for specific individual assay development as well as for a generic approach based on commercially available sources (kits). The evidence of the final HCP results strongly depends on the quality of the immunological reagents, and consequentially unique strategies need to be considered for individual projects, balancing pressure for fast process development with the need for a precise and specific assay.

References

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