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.

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).

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>
<thead>
<tr>
<th>Table 1: Pro and cons of generic and specific assays</th>
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<tr>
<td><strong>Generic assays</strong></td>
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<tr>
<td>Instantly available</td>
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<tr>
<td>No need for preparation work</td>
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<td>Availability is limited to the most common expression systems</td>
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<tr>
<td>Specificity might not be suitable, high risk of undetected HCP species</td>
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<tr>
<td>Costs – initially fairly cheap, but for an entire DSP, development costs add up quite fast</td>
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<tr>
<td>Dependency on one vendor, black box for reagents and controls</td>
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<td>Not suitable for supporting marketing authorisation applications</td>
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Figure 1: Choice of antigen for the development of a specific HCP assay

Early stage antigen
Many protein species (generic HCP; LOQ 5ppm)
Highly suitable for demonstrating reduction factors
Possibly not representative of HCP in final bulk (common practice)

Later stage antigen
Few protein species (specific HCP; 0.1-2ppm, 2.5ng/mL)
Possibly not suitable for demonstrating reduction factors
More representative of HCP in final bulk

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

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
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 pl 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-
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 (S). 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...
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 consequently 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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