Assays for Controlling Host-Cell Impurities in Biopharmaceuticals

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One critical task during the manufacture of biopharmaceuticals is purification of the drug substance. The downstream process must remove all contaminants, including host cell material such as DNA and cellular protein. Trace amounts of host-cell DNA and proteins can be copurified along with the drug substance. Such contaminants are obviously undesirable because of possible consequences if they are injected into patients along with it. They could potentially cause allergic reactions (proteins) or even transfection of cells (DNA) resulting in tumors.

Because of those potential negative effects, regulatory authorities have released several guidance documents about what levels of impurities are acceptable. Residual DNA in final bulk products should be generally lower than 100 pg per therapeutic dose (1).

PRODUCT FOCUS: PRODUCTS OF CELL CULTURE AND FERMENTATION

PROCESS FOCUS: DOWNSTREAM (SEPARATION/PURIFICATION)

WHO SHOULD READ: ANALYTICAL AND PROCESS SCIENTISTS, QA/QC

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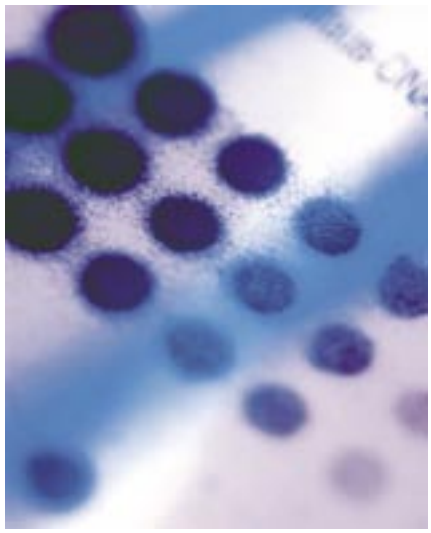
LEVEL: INTERMEDIATE

Other references suggest that higher levels may be acceptable (2). The 100-pg limit requires a purification process that is very effective and robust in removing DNA — as well as analytical methods that are extremely sensitive and reliable to prove it. Host-cell proteins in the drug substance should be "below detectable levels using a highly sensitive analytical method" (1). As a rule, that amount should not exceed a level of about 100 ppm. But no exact limit is set for proteins; therefore, the specification for proteins must be determined case by case.

According to the European Agency for the Evaluation of Medicinal Products, EMEA (3), two different strategies can be used to ensure that a drug substance is within the allowable limits of contamination with DNA or host-cell proteins. You can either validate that a process removes sufficient amounts of such contaminants or perform routine final product testing to determine whether they are present.

With the validation approach, known amounts of DNA are spiked into a downstream process. The various steps within that process are then examined to determine their capability to remove the contamination. This approach is most commonly used for DNA. Robustness and consistency in DNA removal also must be shown by measuring the amounts contaminating several batches of product. Routine testing determines host-cell DNA and proteins in each product batch as part of the lot-release data.



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No matter which approach is used, the assays and methods involved in determination of residual DNA and proteins must fulfill ICH validation requirements (4, 5). Obviously those techniques must be sensitive enough to determine very low levels of contamination (e.g., in the ppm range). Here we describe some common analytical methods used.

QUANTIFICATION OF HOST-CELL DNA

The limit of 100 pg DNA per dose set by regulatory authorities roughly equals the amount of DNA from less than 17 diploid Chinese hamster ovary (CHO) cells. To determine such small amounts of DNA, an

analytical method must be extremely sensitive and robust. In principle, three techniques have the required sensitivity: hybridization, methods based on DNA-binding protein (such as the Threshold assay from Molecular Devices, www.moleculardevices.com) (6), and quantitative PCR (q-PCR) (7). The 2004 *European Pharmacopoeia* clearly advises that residual DNA should be determined using sequence-independent techniques — and goes on to specify that methods such as hybridization or DNA-binding protein assays be used.

The basic principle of a hybridization assay is binding of DNA probes to immobilized and denatured host-cell DNA. Demands of the guidelines suggest that those probes be manufactured independent of the DNA sequence. That can be achieved, for example, by a "random priming" procedure. Probes are usually labeled with radioactive tags or fluorescent dyes, resulting in a signal proportional to the amount of DNA immobilized on a filter. Signal detection is performed by phosphor- or fluorescence-imaging systems.

By contrast, the Threshold assay is based on sequence-independent binding of two proteins specific to single-stranded DNA (ssDNA). One binding protein is an antibody, and the other is called single-stranded binding (SSB) protein. First, a reaction complex is formed when the biotinylated SSB protein and the anti-ssDNA antibody (conjugated to urease) bind to single-stranded host-cell DNA. A filtration stage follows, during which the strong affinity of streptavidin for biotin is used to capture and concentrate those reaction complexes onto a biotinylated membrane. For detection, the membrane is placed into a reader that contains the substrate urea. Inside, urea is hydrolyzed by urease to produce a pH change, which is relative to the amount of host-cell DNA in the sample.

Initial sample treatment is often the most critical factor in successful analysis, regardless which analytical technique is used. Each sample requires matrix-specific pretreatment. All assays differ in sensitivity to residuals such as organic solvents, detergents, high salt concentrations, ethanol, or residual proteins. Several DNA purification methods — such as protease treatment, organic extraction, phenol-free "Wako" extraction (Wako Chemicals, www.wako-chem.co.jp/english), and precipitation by ethanol or glycogen — should be checked for their suitability.

A few differences between DNA testing procedures should be taken into account when interpreting quantitative data. The hybridization assay randomly measures total DNA, from a few base pairs (bp) up to thousands of base pairs in length. It is specific for the source DNA but not the sequence.

DNA-specific binding during Threshold assays is performed by two DNA-binding proteins, both of which must bind to each ssDNA fragment. So this assay requires DNA fragments longer than about 600 bp (6) to form a reaction complex that will produce a signal in the reader. And this assay is not specific to the DNA source.

PCR-based assays are specific to their target sequence, and the amount of total DNA is derived from measured target copy numbers.

Figure 1: Dot-blot hybridization assay for quantifying residual genomic *E. coli* DNA. Samples are analyzed in duplicate with and without addition of reference DNA (DNA spike). A genomic *E. coli* DNA calibration row is applied from 1600 pg to 0.8 pg. In this case, the typical quantification range is 3.1–800 pg. Calibration row, controls, and test samples are applied to the filter by a vacuum-based blotting procedure. DNA is cross-linked by ultraviolet (UV) light and then incubated with a 32P-labeled, random-primed DNA probe. After it is washed, the filter is evaluated by a phosphor imaging instrument (BAS Reader from Fuji of Saitama City, Japan, www.fujimed.com). The DNA content of the samples is calculated using the calibration row signals. For each test sample, an individual recovery is calculated with the values of the spiked sample, spike controls, and the negative controls.

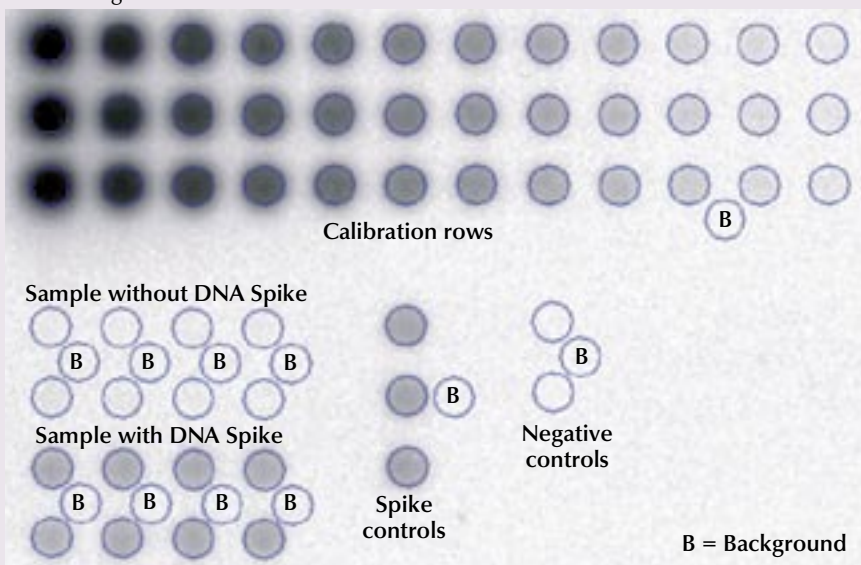


Table 1: Intraassay, interassay, and biological variability coefficients of variability (CVs)

	Hybridization	Threshold	PCR
Specific for	Random sequence, species-specific	Single-stranded DNA, not species-specific	Specific for target sequence
Estimated minimal detection length of DNA (base pairs)	50	600	150
Robustness to interfering substances	++	+	+
Time required (hours)	48	6	2
Sensitivity	6 pg	3 pg	<1 pg

Figure 2. Development steps of a quantitative host cell protein assay

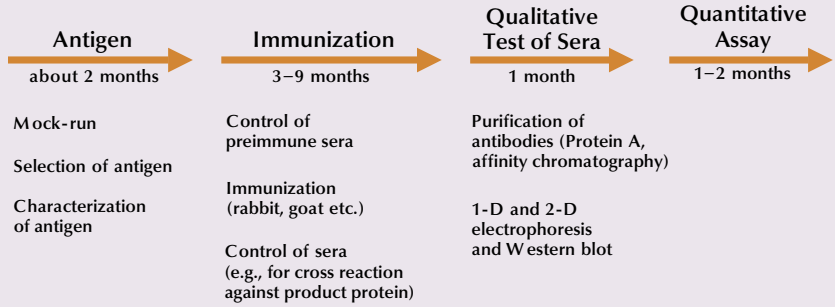
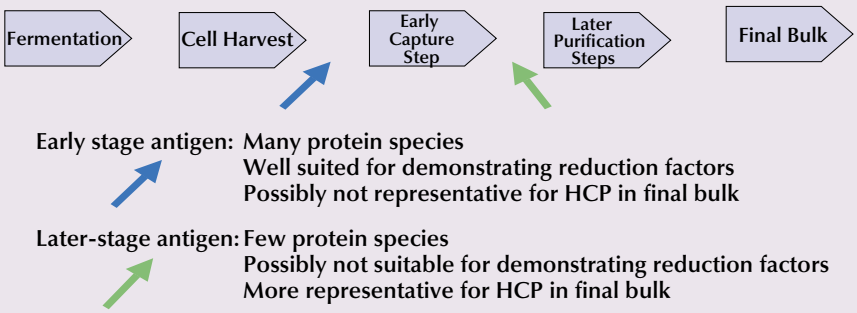


Figure 3: Selection of the antigen for a host cell protein assay



The amplified sequence must be demonstrated to represent the total amount of DNA.

All DNA quantification assays need sample pretreatment and therefore should be designed as spike-recovery assays to control the loss of material during sample preparation. Figure 1 illustrates a spike-recovery control strategy for a hybridization assay. DNA standards for the host cell and vector in use are usually commercially unavailable. Therefore, in-house standards must be produced and quantified by UV absorption. Those reference materials should be additionally quantified and qualified by agarose gel electrophoresis to confirm the UV data and control degradation of the DNA. Assay robustness is different for each assay, especially with respect to interfering substances (Table 1). Overall, hybridization assays are more robust than Threshold and PCR assays.

Quantification of Host-Cell Protein

Those designing downstream processes for biopharmaceutical drug products must also consider removal of host-cell proteins (HCPs). These can be present in significant concentrations following several purification steps — or even be copurified and concentrated along with the drug substance itself. Many purification steps may be needed within a downstream process to remove HCPs. Each step has the potential for loss of product during purification. So during process development, a suitable assay must be available for determining both product concentration and the levels of HCP contamination.

The type of assay required to determine HCP concentration depends on the phase of clinical studies being performed with the material produced. In earlier clinical phases, a generic assay may be sufficient. However, by the time a biopharmaceutical is going into phase 3 studies, regulatory authorities normally require a product-specific HCP assay. The rest of this article describes steps involved in producing, optimizing, and validating a product-specific quantitative HCP assay (Figure 2).

Antigen and Antibody Production: Development of a specific HCP assay must begin early in the development of a biopharmaceutical product because of the time required for antibody production and assay validation. Assay development, regardless of which format is used, requires about 9–12 months. That timeframe covers purification of the antigen to be used, immunization of animals, and antisera collection, through to the validation of the assay. The actual time required for antibody production can vary greatly depending on the antigenicity of the proteins used for immunization. As a rule, you should allow about six months' time for antibody production alone.

The first step in development of an HCP assay is generation and purification of an appropriate antigen to be used for immunization. This requires fermentation of the native cell line without the gene sequence of the biopharmaceutical inserted (8), commonly termed a "mock fermentation run." Supernatant from this mock run is purified using exactly the same purification scheme as for the product itself. The result is a protein-containing solution without the product that can be used as an antigen for production of polyclonal antibodies for the HCP assay.

Because there are normally multiple steps in a downstream process, however, it is necessary to determine which step will produce that antigen. A broad spectrum of HCPs would be detectable with the results of the first purification step, but that does not represent the potential HCP in your final product. If you use the results of the final purification step as your antigen, the HCP may better represent that in a final product but be too specific and thus demonstrate insufficient HCP removal. Decisions must be made case by case. Look at the spectrum of proteins present (using Western blotting, for example), and base your decision on that protein pattern. Figure 3 shows one possible approach to choosing antigens from a mock fermentation run.

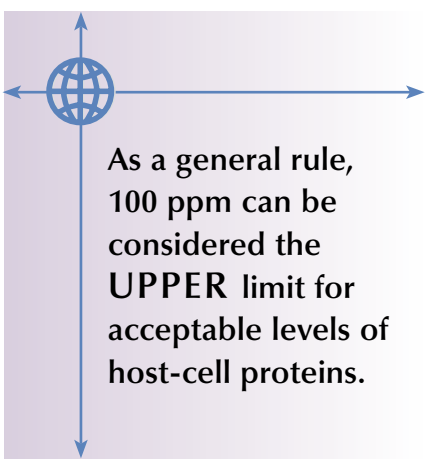
Another important aspect to consider during antigen preparation is the presence or absence of serum antigens. Serum proteins can be very antigenic and thus "mask" the antibodies to the HCPs. Here is

yet another reason why serum-free media should be used whenever possible.

Various immunization schemes can be used to increase polyclonal antibody production. A cascading immunization scheme (**9**, **10**) is commonly used because it allows less antigenic proteins to produce an immune response. That broadens the spectrum of polyclonals and prevents focusing on the most strongly antigenic proteins within the protein mixture presented to the animal.

Affinity chromatography is the ideal method of purifying those antibodies. The antigen used for immunization is immobilized on column chromatography media, and then animal sera is passed through that column to separate and purify the antibodies specific to it. The results tend to have a higher HCP affinity than those purified by a different method. Some antibodies may be lost during affinity purification, however, so they should be quantified using two-dimensional gel electrophoresis and Western blotting to ensure sufficient amounts.

Antigens and antibodies that will be used as future reference material must be stored properly. Limited amounts should be purified from the sera: The shelf life of purified polyclonals is limited, whereas raw sera can be stored for over 10 years at –80 °C. The antigen should be divided into aliquots before storage to avoid unnecessary freeze–thaw cycles. Control the quality of your reference material using electrophoresis and determination of total proteins.



Quality Control: The most important technique for quality control of polyclonal antibodies is two-dimensional gel electrophoresis followed by Western blotting (**9**). Together they allow separation and identification of the antigen protein mixture used. The electrophoresis step separates individual proteins, and the Western blot controls the specificity of antibodies to those individual proteins. Signals from the blots and silver-stained gels prepared in parallel are compared using software developed for this purpose.The results of that comparison can be used to predict the suitability of the antibodies for use in an HCP assay. Figure 4 shows the proteins of an *Escherichia coli* HCP mixture separated using two-dimensional gel electrophoresis, and Figure 5 is the corresponding Western blot.

The quality, specificity, and sensitivity of any antibody-based assay used for detection of HCP is related directly to the quality of the antibodies themselves. They are not likely to detect all the HCP proteins present. Those that were low in concentration or that cause only a weak antigenic response will go undetected. The goal of the assay is, however, to detect a variety of different proteins that represent the HCP spectrum (**11**).

Developing a Quantitative HCP Assay: Based on either the enzyme-linked immunosorbent (ELISA) or immunoligand (ILA) assay format, quantitative assay can be developed using qualified antisera for the marker and the antigen as the reference standard. The sensitivity of this assay is of great importance. As a general rule, 100 ppm can be considered the upper limit for acceptable levels of HCP (**11**). So the assay used to determine HCP concentration must be more sensitive than that. Both ELISAs and the ILAs can be developed with significantly >100-ppm sensitivities. Our experience has shown, however, that the ILA format is much more sensitive than the ELISA method. But it requires the use of the

Figure 4: Separation of *E. coli* host cell proteins on a two-dimensional gel using a pH gradient from 4 to 7

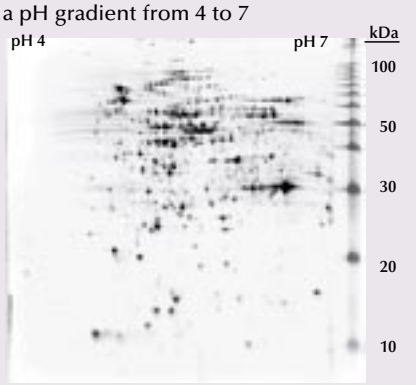
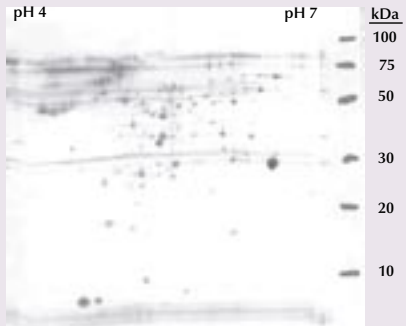


Figure 5: Western blot of proteins separated according to Figure 4 on a nylon membrane. Proteins were detected by a polyclonal goat serum against *E. coli* host cell proteins (antibodies affinity purified). Detection was performed by a rabbit antigoat antibody coupled to horseradish peroxidase.



Threshold system. Table 2 compares the two assay formats.

Validation of Analytical Methods: Any assay used for biopharmaceutical lot-release testing, including the quantitative HCP assay, should be validated as described in the ICH guidelines (**4**, **5**). Parameters to validate include precision, accuracy, linearity, and specificity. During validation, the quantitation limit is defined, which thereby determines assay sensitivity. Table 2 compares validation data for a generic HCP tested with the ILA and the ELISA methods. The same polyclonal antibodies were used in both assays.

Generic and Specific HCP Assays: Depending on the stage of development for the biopharmaceutical in question, either a generic HCP assay or a specific HCP assay can be useful. The generic assay should detect

Table 2: Validation data of host-cell protein assay (*E. coli*)

Validation Parameter	ELISA	ILA
Intermediate precision	9%	10%
Accuracy	91%	101%
Lower quantitation limit	12.5 ng/mL	5.0 ng/mL
Working range	between 12.5 ng/mL and 200 ng/mL	between 5.0 ng/mL and 40 ng/mL

any possible HCP contamination. Normally, cell lysates are used as antigens when developing antisera for such assays. For a product-specific assay, proteins derived from the mock fermentation–purification run are used to develop antisera.

A generic assay is useful in developing a downstream manufacturing process. HCP levels can be controlled following any change in that process at relatively low cost. The generic assay also can be used for lot-release testing in early clinical studies. However, once manufacturing process development has been finalized, product-specific assay development should be initiated. It should be started early because it will take up to 12 months to finish — and because the generic assay is normally unacceptable for lot-release testing material used in later clinical trials. Such studies normally require the use of a product-specific HCP assay.

The Sooner the Better

Early concerns over negative influences from cellular-based contamination in biopharmaceuticals have proven unjustified. Residual DNA, for example, has caused no cellular transformations (e.g. cancers), nor have there been serious immunological problems due to HCPs. Perhaps the danger was not as great as originally thought — or maybe the precautions taken during manufacturing to reduce those potential dangers have been effective. Special chromatographic media can be used to reduce both residual DNA and residual HCP. Sensitive assays have been developed to detect possible residual contamination at extremely low levels.

Although no serious problems with cellular-based contamination have come up yet, it is an issue that will continue to need addressing. Levels of allowable contamination may change, but the purity of biopharmaceuticals must be ensured. Regulatory authorities will continue to expect development of product-specific HCP assays — not only for cellular contaminants, but also for any potential contaminants that could come from media components (for example). Assays will also need to show that those are not present in the final product.

Assay development for possible contaminants should start as early in the drug development process as possible. Once developed, these assays are useful in developing a production process as well as in determining product purity. Because many are product specific, you cannot underestimate the time involved not only in their development, but also for their validation as required by the authorities.

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