

Contaminant host cell-derived protein assay

INTRODUCTION

Polypeptides other than the biological product of interest that are produced by the host cell during cell culture or fermentation (“host cell-derived protein” or “HCP”) can cause an immune response in patients at levels as low as 100 parts per million (ppm)¹. Prior to the approval of a biological product for therapeutic use, the level of residual HCP in the product must be quantitatively measured, according to the “Points to Consider” documents issued by the U.S. Food and Drug Administration² or the European Commission's “Notes for Guidance”^{3,4}. Thus, HCP elimination must be demonstrated during downstream processing²⁻⁶.

Current analytical methods to assay for the presence of contaminant HCPs in recombinant biological products include SDS-PAGE, immunoblotting techniques and ELISA⁷⁻⁹. SDS-PAGE and immunoblotting techniques generally are labor intensive and provide mainly qualitative results with sensitivity in the high ppm range⁷. Better sensitivity can be achieved with ELISAs^{8,9} but they may be more labor intensive and less precise than desired.

This application note is based on the use of proprietary anti-HCP antibodies and, as examples, shows performance data for an *E. coli* HCP and a Chinese Hamster Ovary (CHO) HCP assay.

MATERIALS

- 1 **Threshold[®] System** from Molecular Devices Corporation (catalog #0200-0500), 1311 Orleans Drive, Sunnyvale, CA 94089, tel: 408-747-1700 or 800-635-5577.
- 2 **Immuno-Ligand Assay Labeling Kit** from Molecular Devices Corporation (catalog #R9002).
- 3 **Immuno-Ligand Assay Detection Kit** from Molecular Devices Corporation (catalog #R9003).
- 4 **Immunogens:** The choice of protein mixture (immunogens) used to produce antisera should reflect the specific cell line and purification process used for a certain product^{10,11}. Immunogens are generally purified by a procedure which is identical to the product purification, but using a comparable cell line and vector without the gene for the product (null-cell). This mock purification should be comparable in scale and should proceed to a theoretical product

purity of 95%-99%. An adequate amount of HCPs will be required for use as an immunogen and other purposes, e.g. analytical characterization, immunoaffinity chromatography, assay standard, etc.¹⁰.

- 5 ***Antibodies (immunoreagents):*** One of the challenges is the generation of a polyclonal antibody which is highly specific and sensitive for each of the antigenic proteins in the complex mixture of proteins used as an immunogen. An animal's immune response should be stimulated both against the strong and major antigens, as well as against the weaker and less predominant ones. This can be accomplished by either of two methods or a combination thereof. In *passive immunization*^{12,14} the immunogen is mixed with purified IgGs from a previous bleed (which recognize major antigens) and then injected into the animals. This will decrease the immunogenic reaction against the blocked major antigens and allow a stronger immunogenic reaction against weaker antigens. In the *cascade immunization*^{13,14}, IgGs from previous bleeds are used in immunoaffinity chromatography to eliminate the dominant antigens from the immunogen mixture and enrich the concentration of less antigenic proteins. This preparation is used as an immunogen in the next boost.

Characterization of antibodies

In addition to using the antibodies as an efficient tool to obtain quantitative information, the FDA requires a characterization of their specificity and anti-HCP stoichiometry by alternative technologies. Ideally, a silver staining of a 1-D or 2-D SDS-PAGE should generate the same protein pattern as the corresponding immunoblot^{10,14}.

METHODS

- 1 ***Purification of anti-HCP antibodies:*** The antibodies used should be Protein A or Protein G purified. To achieve higher sensitivity, antibodies may also be antigen affinity purified⁸. However, possible concerns related to antigen affinity purification are the potential loss of very high affinity antibodies which may bind irreversibly to the column, loss of very low affinity antibodies that do not bind to the column or the denaturation of antibodies during elution from the column.
- 2 ***Labeling and storage of antibodies:*** Aliquots of the polyclonal anti-HCP antibody are labeled with biotin or fluorescein, according to the procedure described in the ILA section of the *Threshold System Operator's Manual*. For long-term storage, labeled antibodies should be aliquoted and frozen at -20°C. However, we recommend comparing the performance of a frozen aliquot of labeled antibody to a non-frozen aliquot to determine if a freezing/thawing cycle is harmful before freezing an entire batch of antibody.
- 3 ***Assay development and optimization:*** We recommend developing and optimizing anti-HCP Immuno-Ligand Assays as described in the ILA section of the *Threshold System Operator's Manual*. Here, protocols and procedures are suggested to determine the optimal amount of labeled antibodies (loading study), the standard curve range, the optimal incubation time and temperature and the limit of detection.

APPLICATIONS

For a variety of mammalian, bacterial and yeast derived cell lines¹⁵ HCP immunoassays have been developed using the Threshold Immuno-Ligand Assay. The sensitivity and dynamic range will be dependent on the quality of the

antibodies. Their common target, however, is to fulfill the requirements of regulatory agencies: to accurately and reproducibly quantify low concentrations of host cell proteins in the presence of the product^{3,4}.

The following data are derived from an *E. coli* and a CHO HCP Immuno-Ligand Assay. Both assays were developed by Threshold customers following protocols described in the ILA section of the *Threshold System Operator's Manual*. Briefly, in the *sequential sandwich assay*, antigen and labeled antibodies form complexes in an initial liquid-phase incubation, which are filtration captured on the biotin-coated nitrocellulose membrane. The optimum incubation time is dependent on the specific requirements for sensitivity and the quality of the antibody mixture and may differ from one assay to another. The anti-fluorescein:urease conjugate (enzyme reagent) is then bound to the complexes on the membrane in a subsequent slow filtration process. This protocol has the advantage of allowing higher concentrations of antibodies to be used in the incubation, which is necessary if the detection antibody is not immunoaffinity purified. A *simultaneous* assay protocol incubates the enzyme reagent with the sample and antibodies. This protocol is **not** applicable for antibodies purified by only Protein A or G because the concentration of required fluoresceinated antibody would exceed the amount of anti-fluorescein:urease conjugate (see *Threshold System Operator's Manual*).

Table 1 presents general assay parameters for both the *E. coli* ILA and the CHO-ILA.

	<i>E. coli</i>	CHO
Standard curve range	0.20 - 16.0 ng / test	2.5 - 200 ng / test
Amount of antibody	200 ng / test	200 ng / test
Sample volume	100 μ L	100 μ L
Total assay time	3.5 hours	2.5 hours
Limit of quantitation	4 ppm	3 ppm

Table 1: General assay parameters

Assay precision

Six replicates of a sample were assayed for a within assay precision study for the *E. coli* HCP assay. In the day-to-day reproducibility study, sample measurements were repeated on 69 days for the *E. coli* HCP assay or 5 days for the CHO HCP assay.

	<i>E. coli</i>
n	6
Mean	41 ng / mL
Std. Dev.	1.2 ng / mL
C.V.	3.0%

Table 2: Within assay repeatability of the *E. coli* HCP assay

	<i>E. coli</i>	CHO
n	69	5
Mean	39 ng/mL	319 ng/mL
Std. Dev.	3.3 ng/mL	16 ng/mL
C.V.	8.4%	4.8%

Table 3: Day-to-day reproducibility of the HCP assay

DISCUSSION

Multi-antigen HCP immunoassays are challenging for several reasons. A heterogeneous mixture of host cell proteins is difficult to analyze accurately¹⁰. Since the spectrum of potential contaminating proteins depends highly on the specific cell line and specific purification process used, proprietary polyclonal anti-HCP antibodies are required for sensitive, specific and stoichiometric recognition of the antigen mixture potentially present in the final bulk product. A generic anti-HCP antibody can only be used to support the optimization of early purification steps. Assay and process development have to be closely coordinated because any modification of the purification process can alter the spectrum of potential contaminating proteins. Changes in the purification procedure upstream of the immunogen selection point should therefore be avoided. As a consequence, a well-considered timing for assay development is highly recommended. Process-specific HCP assays, in general, are targeted to be in place prior to the initiation of Phase III Clinical Trials¹⁶.

Since HCP assays use very complex mixtures of proteins as a reference standard, differential stabilities of the individual components during storage of the standard may cause inconsistencies in long-term reproducibility studies. In addition, individual host cell proteins may bind to surfaces or even denature during the assay. As one of the major advantages of ILA, antigens and antibodies can bind in *liquid phase*, which strongly favors the preservation of their native conformations. Consequently, background signals are reduced and binding kinetics are enhanced compared to solid phase binding systems such as ELISAs.

SUMMARY

This application note describes general aspects of and strategies for HCP Immuno-Ligand Assay development. HCP contaminant assays can be the most difficult assays required by regulatory agencies in terms of preparation of immunogens and antibodies, timing of assay development and coordination of process and assay development teams. Additionally, the very stringent requirements regarding sensitivity, accuracy and reproducibility add to the difficulty. Liquid phase binding reactions, the specific capture during filtration and prequalified reagents are advantages which allow the ILA format to be extremely effective and efficient for development and validation of HCP assays.

As an example, two assays were presented which were developed by Threshold customers with their proprietary, purification-process-specific antibodies. With a 2 log dynamic range, 200 ng/test of labeled antibody and incubation times

between 2-3 hours, the corresponding assays allow quantitation in the very low ppm range. Intra-day and inter-day precisions were very good with C.V.s less than 10%.

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