

Development of an In-House, Process-Specific ELISA for Detecting HCP in a Therapeutic Antibody, Part 2

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During biopharmaceutical manufacturing, final drug products can get contaminated with host-cell proteins (HCPs) derived from a production cell line. HCPs can elicit adverse immune responses, so regulatory authorities require accurate monitoring of their presence and concentration in final drug products. Because they are robust and offer good throughput, enzyme-linked immunosorbent assays (ELISAs) are the first choice for HCP detection to monitor product quality. Generic ELISA kits are commercially available for HCP detection with a number of commonly used biopharmaceutical production cell lines. Use of such kits could obviate the necessity of generating new HCP-reactive antibodies for each new manufacturing process. Although use of a universal kit could save time and money, universal HCP reactive

antibodies may not adequately react with all HCPs potentially present in every biopharmaceutical product and unique process.

Centocor's recently approved Simponi IgG product for treatment of rheumatoid arthritis (RA) is generated using the common production cell line known as Sp 2/0. Here we conclude our report on development of an in-house, process-specific HCP detection ELISA for measuring HCP content in purification process intermediates and final Simponi drug product. This is the first report on an in-house-developed process-specific HCP detection ELISA supporting late-stage development of a therapeutic antibody for market approval.

We compared the specificity and sensitivity of our in-house ELISA with a commercially available generic Sp 2/0 HCP detection ELISA to determine suitability of the latter for HCP detection in Simponi products. Our in-house ELISA detected as little as 3.9 ng/mL HCPs or 0.78 ng HCPs/mg IgG product (0.78 ppm) and exhibited no cross-reactivity with our IgG drug product. The commercial kit, however, showed very poor HCP detection sensitivity for our in-house Sp 2/0 lysate and cross-reacted with human IgG, giving a false readout of high HCP concentration in our IgG drug product. We concluded that our in-house, process-specific ELISA was



more accurate and reliable than the generic kit for detecting HCP in our IgG drug product. The principles and methods described here could be applied to other manufacturing processes (e.g., fed-batch) with different production cell lines.

Part 1 of this report (*BioProcess International*, March 2011) describes materials and methods for generation of antibodies and lysates; electrophoresis, staining, and blotting; ELISA development; and data analysis. Part 1 also presented results of lysate and antibody characterization and ELISA development. Here, we describe results of comparing our in-house ELISA's performance with that of our chosen commercial ELISA kit: the Sp 2/0 HCP Western blot detection kit from Cygnus Technologies (www.cygnustechnologies.com).

PRODUCT FOCUS: PROTEIN BIOLOGICS

PROCESS FOCUS: MANUFACTURING

WHO SHOULD READ: PRODUCT AND PROCESS DEVELOPMENT, ANALYTICAL, FORMULATIONS, AND QA/QC PERSONNEL

KEYWORDS: HOST-CELL PROTEIN, Sp 2/0 CELLS, IMMUNOASSAYS, DATA ANALYSIS SDS-PAGE, STAINING, WESTERN BLOTTING

LEVEL: ADVANCED

FURTHER RESULTS

Characterization of HCP Levels in Four

Consistency Lots: Regulations require that all biopharmaceutical manufacturing processes be validated to demonstrate their capability to consistently remove HCPs (as a potential process-related impurity) to an acceptable level (1).

Centocor manufactured four consistency lots of formulated Simponi bulk products during late-phase development. As Figure 4 shows, two protein bands (~50 kD and ~25 kD) representing the product's heavy chain and light chain are present in all four consistency lots comparable to its primary reference standard (PRS) determined by Coomassie Blue staining.

We evaluated the potential presence of residual HCPs in all four consistency lots by Western blotting and in-house ELISAs. Samples from those lots were analyzed in reduced SDS-PAGE gels with 10 µg of protein loading per sample in each lane along with the in-house HCP lysates as a positive control at different loading masses. After conducting electrophoresis, we transferred the proteins to PVDF membranes and then performed immunoblotting analysis with a rabbit biotinylated anti-HCP antibody. We used a commercial biotinylated rabbit IgG as a negative-control antibody with a matched antibody concentration for any nonspecific cross-reactivity.

When performed with a commercial biotinylated rabbit control IgG, the immunoblot showed no background signal (Figure 5). In immunoblots performed with the rabbit anti-HCP antibodies (Figure 6), no HCP-specific protein bands appeared in any of the consistency lots or the PRS at 10-µg sample loading. The rabbit anti-HCP antibodies did react positively with HCP-positive control proteins up to 0.01 µg (10 ng). Because 2D gel electrophoresis showed the anti-HCP antibodies to react with nearly all HCP proteins, and they reacted strongly and broadly in the 1D gel at 1-µg HCP load (Figure 6), some HCPs detected in the 1D gel may have been present at an average level of 2.4 ng (1-µg total

Figure 4: Coomassie Blue staining of four consistency lots and primary reference standard (PRS = primary reference standard; MW = molecular weight marker)

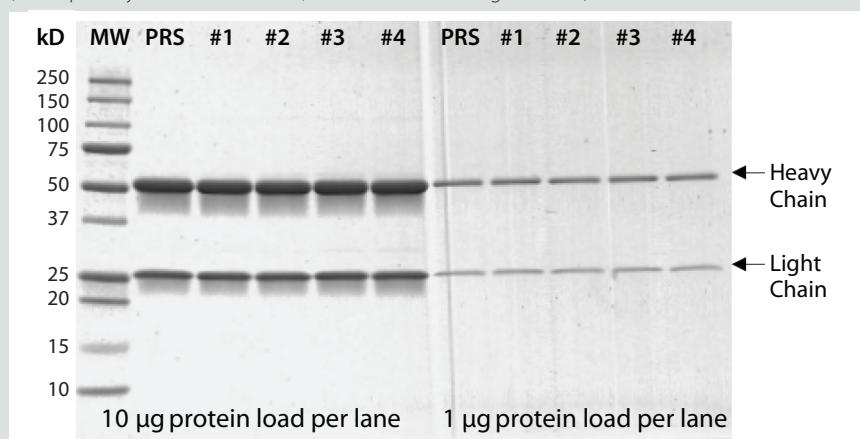


Figure 5: Detection of residual HCPs in Four Simponi consistency lots and primary reference standard using immunoblot with a biotinylated rabbit control antibody (PRS = primary reference standard; MW = molecular weight marker)

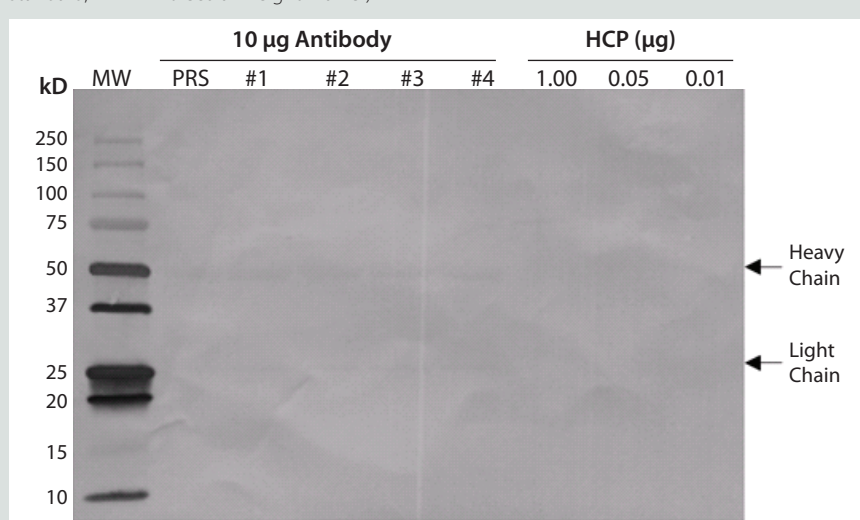
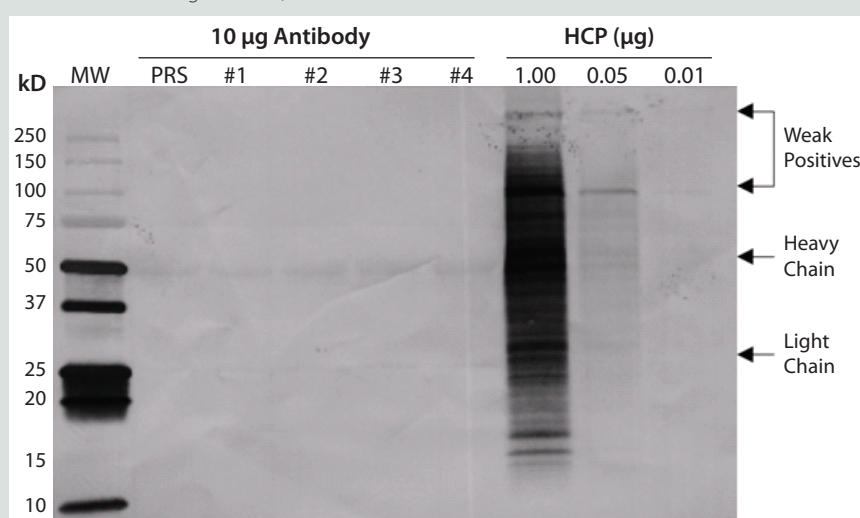


Figure 6: Detection of HCP impurities in four Simponi consistency lots and primary reference standard using immunoblot with a rabbit anti-HCP antibody (PRS = primary reference standard; MW = molecular weight marker)



HCP load divided by 423 total protein species, identified with 2D gel). Taken as a percentage of the PRS loaded at 10 µg, 2.4 ng represents a sensitivity of 0.024% for the loaded PRS. Because the anti-HCP antibodies reacted just as well with at least some HCPs at the lower loads of 0.05 and 0.01 µg in the 1D gel, sensitivity of the immunoblot is probably <0.024% of the loaded PRS.

We used the same HCP antibody preparation in our validated ELISA method for detecting HCPs under native, nondenaturing conditions. HCP levels in all four Simponi consistency lots were below the quantification limit (QL), which is <3.9 ng/mL or 0.78 ng HCPs/mg IgG (ppm) product.

Comparing Performance of In-House and Commercial Methods: Several commercially available generic HCP ELISA kits are designed to detect HCP in biopharmaceutical products. Significant time could be saved if such assays could be used for all manufacturing processes. After establishing our in-house ELISA, we sought to determine whether it performed comparably to a generic ELISA kit from Cygnus Technologies that was designed to specifically recognize HCPs from the Sp 2/0 cell line (catalog #F180).

For this analysis, we monitored the ability of each ELISA method to accurately quantify the HCP content in both Symponi PRS (representing formulated bulk) and eluates from the direct product capture (DPC) step of the manufacturing process. We performed the Cygnus ELISA according to its manufacturer's protocol. For each ELISA, we generated a four-parameter fitted standard curve using data from the

Table 6: HCP concentration in Simponi primary reference standard (2.5 mg/mL) as determined using each ELISA

	ELISA Kit	In-House ELISA
HCP (ng/mL)	84	<QL
Standard Deviation	3	NA
% CV	4	NA

QL = quantification limit

We monitored the ability of **EACH** ELISA method to accurately quantify HCP content in **BOTH** Symponi PRS and eluates from the direct product capture step.

lysate that corresponded with each method. Although the in-house ELISA detected no HCP above the QL of the assay, the Cygnus ELISA detected significant HCP levels in both PRS and DPC eluates (Tables 6 and 7). We confirmed the proficiency of each ELISA in detecting HCP in a Simponi matrix by spike–recovery experiments using both in-house and Cygnus HCP lysates (data not shown). The discrepancy in perceived HCP concentration for the identical samples could be attributed either to a “false positive” measurement from the ELISA kit or “low sensitivity” of the in-house assay. So we further investigated both possibilities.

Table 7: HCP concentration in Simponi purification column eluate as determined using each ELISA

Simponi DPC Eluate (IgG mg/mL)	ELISA Kit			In-House ELISA		
	HCP (ng/mL)	% CV	HCP (IgG ng/mL)	HCP (ng/mL)	% CV	HCP (IgG ng/mL)
20	1,227	5	61	4.0	3	0.2
10	645	6	65	2.7	7	0.3
5	254	2	51	2.2	31	0.4
2.5	137	3	55	1.2	2	0.5
1.25	79	7	63	ND	ND	ND

ND = not determined

Kit Anti-HCP Antibodies Exhibited Limited HCP Immunoreactivity and Recognized Human IgG: A false-positive result can arise if ELISA antibodies detect something other than their intended target. In this case, promiscuous detection of human IgG in our Simponi reference standard could be misinterpreted as HCP detection. To investigate this possibility, we examined the composition of the Cygnus HCP lysate by silver staining reduced polyacrylamide gels and the anti-HCP immunoreactivity of the Cygnus antibodies with Western blotting. As Figure 7 shows, the Cygnus HCP lysate contained two prominent bands ~30 and 55 kDa in size, corresponding to the light (30 kDa) and heavy (50 kDa) chains of a mammalian IgG (which Cygnus Technologies intentionally includes in the lysate as a protein stabilizer) at a concentration of 4 mg/mL.

The in-house lysates showed a great deal of complexity in protein compositions compared with Cygnus lysates. Western blot analysis of the Cygnus lysate revealed that the Cygnus antibodies demonstrated low immunoreactivity to any HCP bands of the Cygnus HCP lysate visualized by silver stain but significant immunoreactivity toward the IgG stabilizer (Figure 8, lane 6). By contrast, the in-house antibodies again demonstrated strong immunoreactivity to many distinct HCPs in the in-house HCP lysate (Figure 8, Lane 3). The Cygnus antibodies detected several bands in the in-house HCP lysate (Figure 8, Lane 4) but far fewer than the in-house antibodies recognized. This explained why the Cygnus antibodies did not detect HCP in the in-house HCP lysates relative to the in-house antibodies.

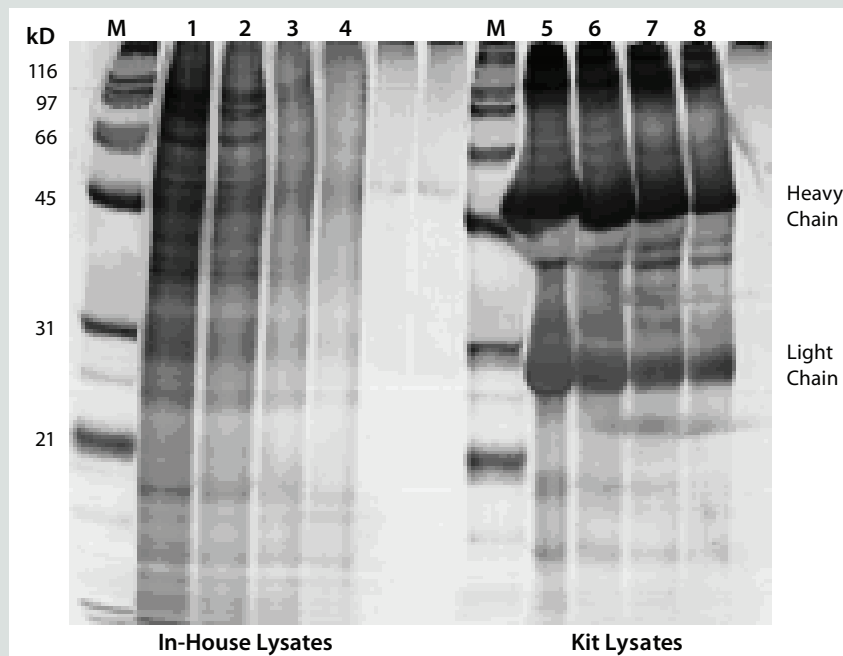
Considering the strong immunoreactivity of the Cygnus antibodies to the IgG stabilizer in relation to HCP, we subjected our Simponi reference standard and DPC eluates to Western blot with the Cygnus antibodies. Again, those antibodies detected several HCP bands in the in-house lysate and the

corresponding HCP bands in HCP lysates from the Cygnus Sp 2/0 HCP Western blot kit (Figure 9). However, the only bands detected in the Simponi samples were IgGs, which suggests that those IgG bands were responsible for the observed signal when Simponi samples were measured using the Cygnus ELISA.

DISCUSSION

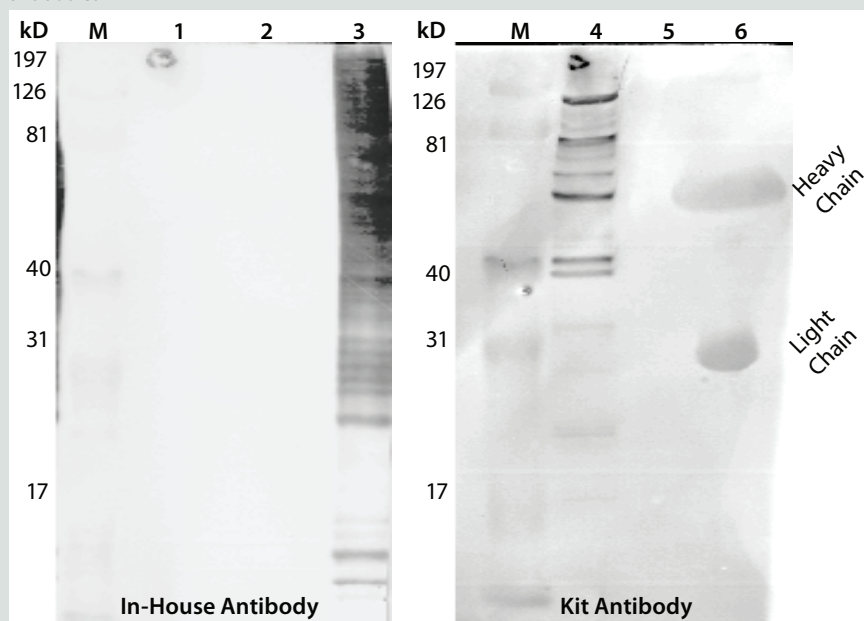
Protein therapeutics such as monoclonal antibodies are typically generated using engineered cell culture systems, in which a protein of interest is expressed at high levels. HCPs are process-related impurities generated during biopharmaceutical manufacturing, so they must be

Figure 7: Protein content of Cygnus and in-house HCP lysates (SDS-PAGE and silver staining of Cygnus and in-house lysates)



Lane 1 = 1,000 ng; Lane 2 = 500 ng; Lane 3 = 250 ng; Lane 4 = 125 ng in-house HCP lysate; Lane 5 = 1,000 pg; Lane 6 = 500 pg; Lane 7 = 250 pg; Lane 8 = 125 pg Cygnus HCP lysate; M = molecular weight marker

Figure 8: Western blot analysis of Cygnus and in-house HCP lysates with Cygnus and in-house antibodies



Lane 1 = 1 ng Cygnus lysate; Lane 2 = 1 ng in-house lysate; Lane 3 = 1 µg in-house lysate; Lane 4 = 1 µg in-house lysate; Lane 5 = 1 ng in-house lysate; Lane 6 = 1 ng Cygnus lysate; M = molecular weight marker



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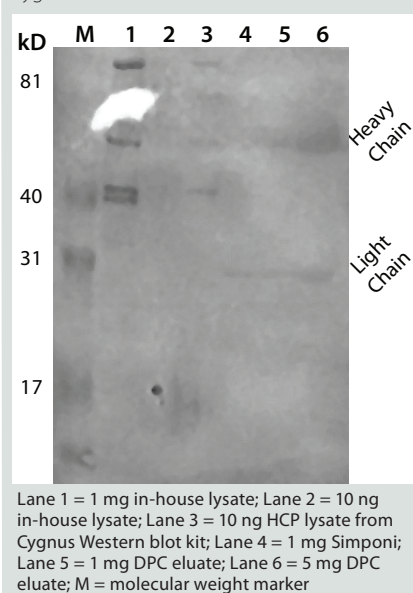
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monitored with sensitive analytical methods. In the interest of patient safety, regulatory authorities require that host-cell contaminants be quantitatively measured in final drug products (2,3). Furthermore, consistent removal of HCPs must be demonstrated and validated for all manufacturing processes (1). Different types of processes (e.g., perfusion and fed-batch cultures) can involve different levels and different species of HCP contaminants. Among all analytical methods used to detect HCP levels, immunoassays are the preferred format because of their high sensitivity, specificity, robustness, and throughput.

Here, we have described our development of an in-house, process specific ELISA assay for quantification of HCPs in a biological product made using the Sp 2/0 mammalian production cell line. The assay demonstrated high accuracy and sensitivity, with a QL of 3.9 ng/mL, or 0.79 ng HCP/mg (ppm) IgG product. We validated utility of this assay by monitoring sequential removal of HCP from Simponi purification process intermediates. When applied to determine the HCP concentration in our Simponi primary reference standard, the assay could detect no HCP above its QL.

During characterization of the anti-HCP antibody preparation and Sp 2/0 cell lysates by 2D gel electrophoresis and Western blotting, the anti-HCP antibody demonstrated strong immunoreactivity and broad recognition to >87% of the HCP protein species on Western blot compared with silver stain. This result provided assurance that critical reagents used in the in-house ELISA are highly specific and detect sufficient amounts of protein species. We used the subsequent validated ELISA assay to successfully monitor removal of HCPs during purification by sampling from different product intermediates from five different phase 3 batches and two process validation lots. Consistency of HCP removal by our manufacturing process demonstrated no detectable level of HCPs present in all four subsequent consistency lots.

Figure 9: Western blot analysis of HCP lysates, Simponi PRS, and DPC eluates with Cygnus anti-HCP antibodies



We next sought to compare our HCP detection ELISA with a commercially available assay kit. Similar performance between the two would have afforded us the luxury of using the commercial assay for future sample analyses. Use of a commercial kit would be very convenient and relieve us from continually generating and qualifying new batches of anti-HCP antibody preparations. To this end, we evaluated an ELISA for Sp 2/0 cell line HCP detection from Cygnus Technologies by directly comparing it with our in-house assay. But the commercial kit exhibited poor sensitivity in detecting HCPs from our in-house generated Sp 2/0 lysates, and our in-house ELISA detected no HCPs above the QL in HCP control lysates supplied with the kit.

Despite poor sensitivity, however, the commercial ELISA suggested the presence of significantly higher HCP levels in our drug product than was indicated by the in-house ELISA, which detected no HCPs at concentrations above the assay QL. The Cygnus ELISA product data sheet clearly outlined potential limitations of the assay, including the possibility that anti-HCP antibodies would not comprehensively detect every protein present in an end-user's samples and that certain sample components (including human IgGs) could cause

either positive or negative interference. We investigated the possibility that the product itself was causing positive interference (a false-positive signal) in the Cygnus ELISA by performing Western blot analysis of Simponi samples using the Cygnus anti-HCP antibodies. Indeed, those antibodies detected bands corresponding in molecular weight to the IgG heavy and light chains (Figures 8 and 9), which strongly suggests IgG positive interference and erroneous HCP quantification in the Cygnus ELISA.

We also sought to explain the differential sensitivity of these ELISAs in detecting HCPs in different Sp 2/0 cell lysates. We analyzed both in-house and kit lysates and HCP detection antibodies using SDS-PAGE with silver staining and by Western blotting to ascertain whether obvious differences in protein content or immunoreactivity existed. The in-house lysate contained many more protein species and appeared to be considerably less concentrated than the Cygnus lysate (Figure 7). In Western blotting, the in-house antibodies detected a vast array of proteins in the in-house HCP lysate, whereas the Cygnus antibodies detected far fewer protein species in the same lysate (Figure 8).

Limited immunoreactivity of the Cygnus antibodies to such a small subset of protein species in the in-house HCP lysate could explain the poor sensitivity of the ELISA kit when measuring HCP content of our in-house lysate. Limited immunoreactivity of the Cygnus antibodies could possibly be explained by the limited protein diversity of the cell lysate that Cygnus may have used as the immunogen for antibody generation and to affinity purify those antibodies. In addition, protein concentration of the Cygnus HCP lysate was dramatically underestimated, as evidenced by comparison with our in-house HCP lysate, which we carefully quantified with the Bradford assay. So the Cygnus antibodies may have been incorrectly "calibrated" when their capacity for HCP detection was measured, which could further explain the kit's lack of sensitivity for HCP

detection in our in-house lysate, which was much less concentrated than the Cygnus lysate.

Furthermore, affinity purification had optimized the Cygnus antibodies for a specific subset of HCPs, and the lack of ELISA sensitivity would be exacerbated if that subset was a minor constituent of our in-house lysate — or of unclarified Sp 2/0 whole-cell lysates in general. The in-house antibodies had much greater diversity because of the greater diversity of the in-house lysate, which did not undergo clarification after generation of whole-cell extracts before use as an immunogen for antibody generation. This would explain why the in-house antibodies had greater immunoreactivity than the Cygnus antibodies to HCPs in the in-house lysates. Furthermore, the limited number of distinct proteins in the Cygnus HCP lysate could explain the poor sensitivity of our in-house ELISA in accurately measuring the HCP content of the Cygnus lysate.

AN ADAPTABLE METHOD

This is the first report of an in-house, process-specific ELISA for detecting HCPs in an IgG product manufactured with a mammalian expression system, which has been subsequently approved by regulatory authorities from the United States, Europe, Japan, and other countries for treating rheumatoid arthritis. With antibodies raised against the complete pool of Sp 2/0 HCPs, our in-house HCP detection ELISA had better sensitivity in detecting HCP in a crude whole-cell extract than did a “generic,” commercial ELISA kit optimized to detect a subset of the HCP pool. Also, the in-house-generated antibodies exhibited no cross-reactivity to human IgG, which demonstrates the appropriateness of using these antibodies for detecting HCP in human IgG drug products.

Developing a universal ELISA for HCP detection in finished biopharmaceutical drug products is certainly a daunting task, considering the molecular complexity of the target and the uniqueness of individual drug production and purification processes.

The process-specific HCP detection ELISA described here can certainly be used for other IgG products produced using same host cells and perfusion bioreactor processes. The process of establishing an in-house HCP assay can also be applied to other common mammalian expression systems and different manufacturing processes.

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Find a detailed discussion of assay development and commercial ELISA kits from Cygnus Technologies online at www.bioprocessintl.com/bpiextra.

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