How to overcome cross reactivity towards drug substances - a process-specific HCP ELISA development for the *Physcomitrium patens* (Moss) Plant-Based Expression System

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Project draft and BioGenes HCP ELISA development strategy

Developing a successful Host Cell Protein (HCP) Enzyme-Linked Immunosorbent Assay (ELISA) presents numerous technical and methodological challenges. Here, we present the tailored strategy for our valued client Eleva, which needed a new HCP ELISA development for the plant-based *Physcomitrium patens* expression system (Moss). The following aspects were covered: (1) The antibodies as crucial reagents should demonstrate a broad reactivity towards the process-related HCP composition and (2) should not show any cross reactivity towards the drug substance. Furthermore, (3) high reactivity is needed to develop a sensitive HCP ELISA with LOQ levels of a few ng/ml.

BioGenes‘ well-established general strategy for HCP monitoring includes three main phases:
(1) the HCP antigen preparation phase (enrichment of low molecular weight HCPs),
(2) the antibody generation phase (with antiserum production/ antibody purification tests and characterization) before bulk production, and
(3) the assay setup and qualification phase (ELISA optimization, prevalidation and reagent characterization).

Challenge - the detection of putative cross reactivity towards the drug substance

Generation and characterization of HCP-specific antiserum

For the generation of a broadly reactive antiserum, two groups of rabbits were immunized using either the total HCP or the low molecular weight (LMW) HCP. After several boosts and bleedings, the antisera were monitored by ELISA titer determination and 1D Western blotting (WB) with HCP antigens and the drug substance (DS) as test antigen. Considerable titers and a distinct drug-related staining signal were obtained. Additionally, the antisera were tested by 1D WB and immunostaining (*Figure 1*).

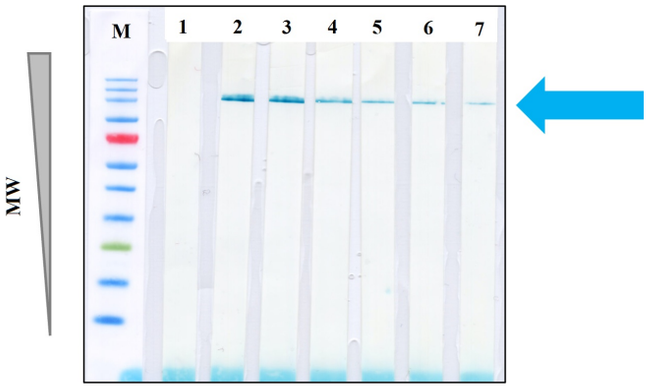

Figure 1: ***Western Blot of Moss-DS and immunostaining with rabbit antisera****. The molecular weight (MW) is indicated on the left (prestained protein standard, M). Protein amount per lane: ~0.5 µg DS; antiserum dilution of 1:250; Lane 1: Pre-immune serum test pool of rabbits; Lane 2-7: individual 4th bleeding antisera.*

As shown in Figure 1, the immunostaining of DS with rabbit antisera revealed clear signals at ~130 kDa for all rabbits.
The putative cross reactivity observed in WB correlates with the corresponding DS-related titers obtained for the rabbit antisera. In general, Western Blots are a suitable tool for assessing initial cross reactivity, but false positive signals for unpurified antisera are frequently observed. Therefore, HCP affinity purification was initiated.

Antibody purification tests and characterization

After the purification of a small-scale antiserum test pool from all rabbits‘ including LMW HCP-specific antisera using the total HCP as an affinity matrix, the antibodies were tested by 1D WB‘ including the Moss-derived DS and the human homologue (Figure not shown). Only WB staining signals for the Moss-derived DS were observed. To verify these results, a sandwich ELISA setup was carried out, where the late downstream process sample (DSP) yielded in a comparatively high HCP content (app. ~700 ppm) and also a significant dilution nonlinearity. This is indicated by a CV of more than 50% (*Figure 2*). The data observed refers to a product related reacitivity.

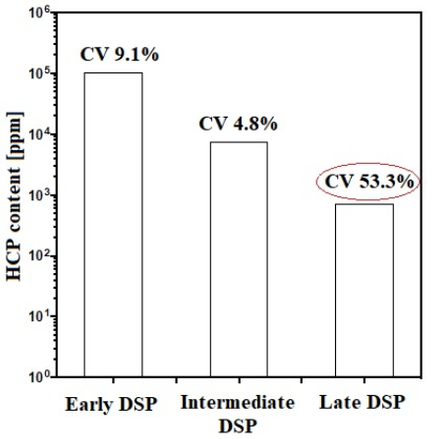

Figure 2: ***ELISA testing of process samples using anti-HCP ABs****. The HCP impuritiy level is expressed as ppm values for each successive downstream purification process. For the late process sample' the HCP impurity level was considered high and the CV value obtained was out of the acceptance range (<25%), marked with a red circle.*

Confirmation of putative cross reactivity and solution

ELISA inhibition test

To analyze the potential cross reactivity of the test antibody, an inhibition test was performed. A large amount of capture antibody was added onto the sample HCP. The DS, on the other hand, was still present in excess and would therefore not have been masked, even if a large part of the antibodies had been cross-reactive. As shown in Figure 3, a full signal inhibition for the HCP standard was observed, while the inhibited DS sample showed a remaining reactivity of about 10% (*Figure 3*). Therefore, the purification strategy had to be adjusted.

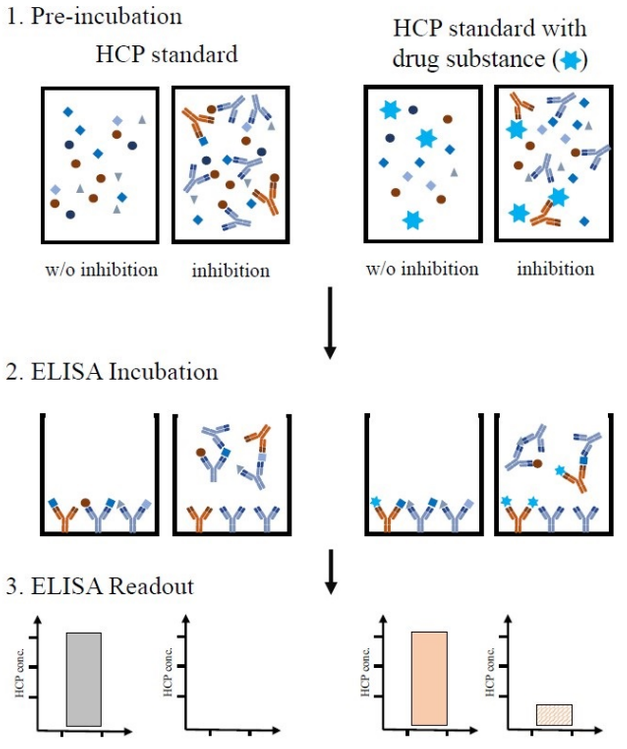

Figure 3: ***ELISA inhibition test for potential cross reactivity****. Aliquots of the four samples were diluted and pre-incubated overnight with capture antibody to block endogenous HCPs. A negative and a positive control were implemented. For the negative control' the sample dilutions were spiked with assay buffer instead of capture antibody. The upper HCP standard mixed with capture antibody served as positive control. Standards and controls were also incubated overnight in order that they are comparable. Afterwards, these samples were analyzed in the preliminary HCP-ELISA set-up.*

Final antibody purification strategy and characterization

For bulk purification of the final antibody batch, two purification steps were employed; including (1) affinity purification with the immobilized total HCP' and (2) a following affinity purification of the eluate from 1st step using the immobilized DS. The antibody pool before and after DS immune adsorption was then compared by Western Blotting (WB). While DS-related signals were observed before depletion, the signal disappeared afterwards (*Figure 4*).

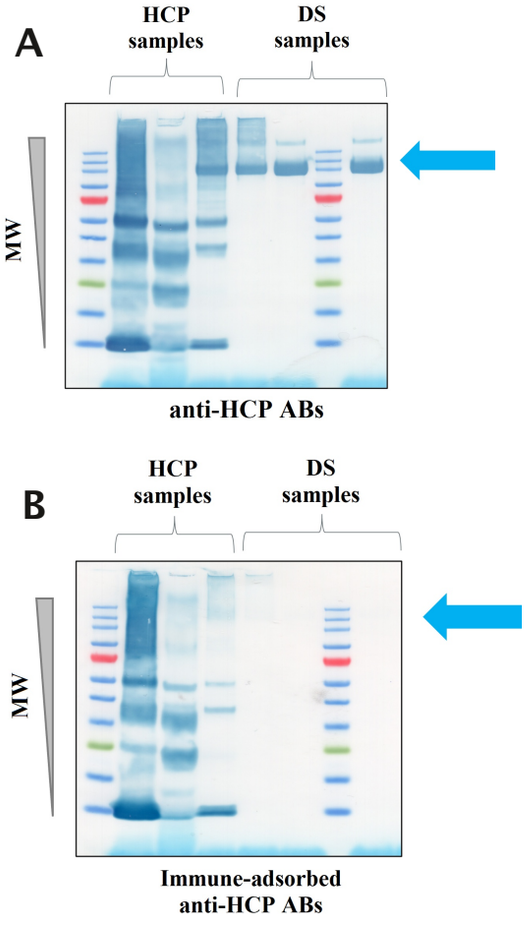

Figure 4: ***Antigen and process sample Western Blot and immunostaining with the antibody, (A) without and (B) with immune adsorption.*** *The molecular weight (MW) is indicated on the left (prestained protein standard, M). Antibody concentration 10 µg/mL; Lane*

1: Moss-HCP_Pool; Lane 2: Moss-HCP_LMW; Lane 3: DS_early; Lane 4: DS_intermediate; Lane 5: DS_late; Lane 6: DS; Protein amount per lane: ~6 µg.

The HCP-specific reactivity remained unchanged. These results indicate a successful depletion of the cross-reactive antibodies that was confirmed by the ELISA readout. The remaining DS-related ELISA signal (~10%) of the non-depleted anti-HCP ABs was successfully inhibited using the immune-adsorbed anti-HCP ABs (*Figure 5*). The absolute HCP values measured without inhibition were well comparable for both antibodies, with and without depletion. Finally, coverage evaluation was performed by immunoaffinity chromatography (IAC) and a subsequent 2D DIGE‘ generating a proper antibody coverage of 89%. Furthermore, an ELISA optimization with the immune-adsorbed final anti-HCP antibodies was performed.

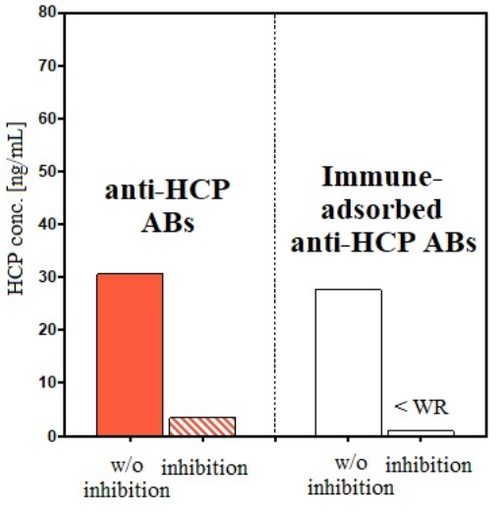

Figure 5: ***Inhibition test of anti-HCP ABs without (left) or with (right) immune adsorption.*** *HCP measurement using the anti-HCP ABs without immune adsorption yielded a remaining reactivity of ~10% when inhibited, in comparison to the ELISA signal without inhibition. Almost full signal inhibition was observed for the immune-adsorbed anti-HCP ABs, with values below the assay working range (WR).*

Conclusion

A drug substance cross reactivity was observed during characterization of the antisera and the HCP-purified test antibodies and was verified by 1D Western blot analysis and ELISA inhibition testing. This issue was addressed by purifying the final HCP-specific antibodies stepwise‘ using immobilized HCP and the DS for immune-adsorption. The absence of cross-reactive antibody species was demonstrated again by 1D Western blotting and ELISA inhibition testing. Finally, the antibody coverage was determined. BioGenes eventually developed and prevalidated a sensitive process-specific HCP ELISA applicable for the accurate and precise measurement of Moss-derived impurities.

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