BioGenes ASSAYS

Development of a Specific Host Cell Protein Assay for the Plant-based Expression System Physcomitrella patens (Moss)

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Plant-based expression systems, such as Physcomitrella patens (Moss), have only recently emerged as an alternative to established recombinant protein production platforms (e.g. bacterial cell lines, yeast cell lines or mammalian cell lines). Generally, generic Host Cell Protein ELISAs (HCP ELISAs) are used for the detection of potential impurities during downstream process development of drug products and in early clinical phases (phase I and II). However, no generic HCP ELISAs for plant-based expression systems are available. To meet the requirements for release of drug product to be used in phase 1 clinical trial in Germany it was necessary to develop an ELISA specific for P.patens HCP.

Antibody Generation

For the generation of specific anti-Moss-HCP antibodies, five rabbits were immunized according to an immunisation schedule over 175 days. Total-HCP material, derived from the supernatant of a mock fermentation of Physcomitrella patens, was used as immunogen for injection (fermentation was performed according to standard cultivation conditions for manufacturing of most drug products at Greenovation Biotech GmbH). Animals were bled eight times including a final bleeding, resulting in a total volume of 1.1 L of pooled antiserum. 200 mL of antiserum was affinity-purified against the corresponding HCP material generating the capture antibody (anti-Moss-HCP-IgG). For the preparation of the detector antibody, an aliquot of the capture antibody was biotinylated (anti-Moss-HCP-IgG-Biotin).

ELISA Development

For ELISA development the following parameters were optimised: capture antibody concentration; detector antibody concentration; antigen incubation time; detector antibody incubation time. An acceptable intra-assay precision with CV values between 1.2% and 3.8% was obtained. The preliminary working range was set to 0.1 – 5.0 ng/mL. The corresponding standard curve is shown in Fig. 1.

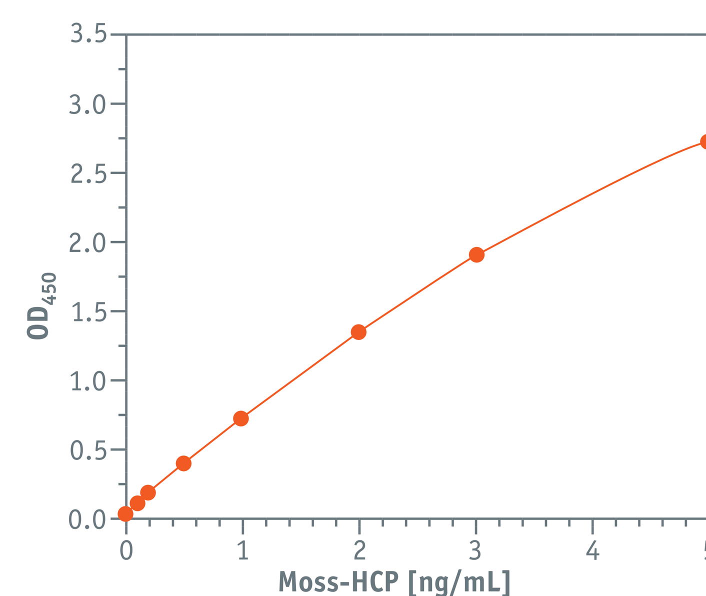

Fig. 1: Standard curve of the optimised Moss-HCP ELISA. Each standard concentration was analysed with 12 replicates.

Cross-Reactivity Testing of the Capture Antibody and ELISA Inhibition Tests

Following the successful optimisation of the Moss-HCP ELISA in a buffer matrix, different in-process samples were analysed as part of a feasibility study. Unfortunately, cross-reactivity of a small part of the HCP-specific antibodies with the recombinantly expressed drug substance (alpha-Galactosidase, α-Gal DS, MW 46 kDa) was observed, as demonstrated by 1D Western blotting and ELISA inhibition test (Fig. 2 at approx. 50 kDa and Fig. 3). In such an inhibition test, samples are pre-incubated with antibodies in excess over residual HCP in order to fully inhibit the HCP-derived signals. If any remaining ELISA reactivity is observed in the sample as compared to a suitable buffer control, this indicates product cross-reactivity with antibodies used in the assay.

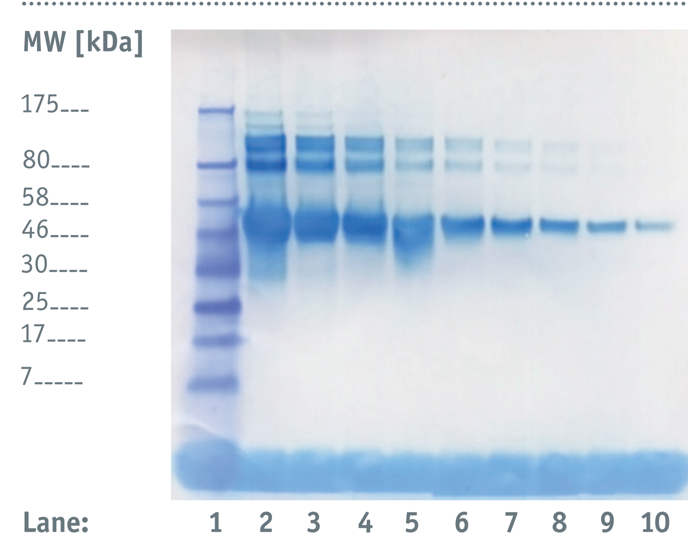

Fig. 2: Western Blot of α-Gal DS titration (10 - 0,039 µg/lane) and immunostaining with anti-Moss-HCP-IgG.

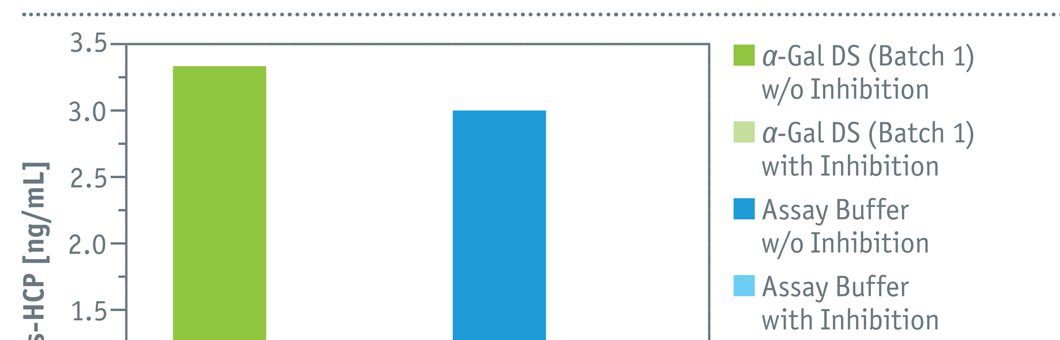

Fig. 3: Results of the ELISA inhibition test using α-Gal DS Batch 1.

Antibody Immune Adsorption Against Drug Product

As a next step, the cross-reacting capture antibody (anti-Moss-HCP-IgG) was purified on an affinity column with immobilised α-Gal DS. The immune-adsorbed antibody (anti-Moss-HCP-IgG-IA) was tested by immunostaining of Western-blotted Moss-HCP (Fig. 4) and α-Gal DS (Fig. 5).

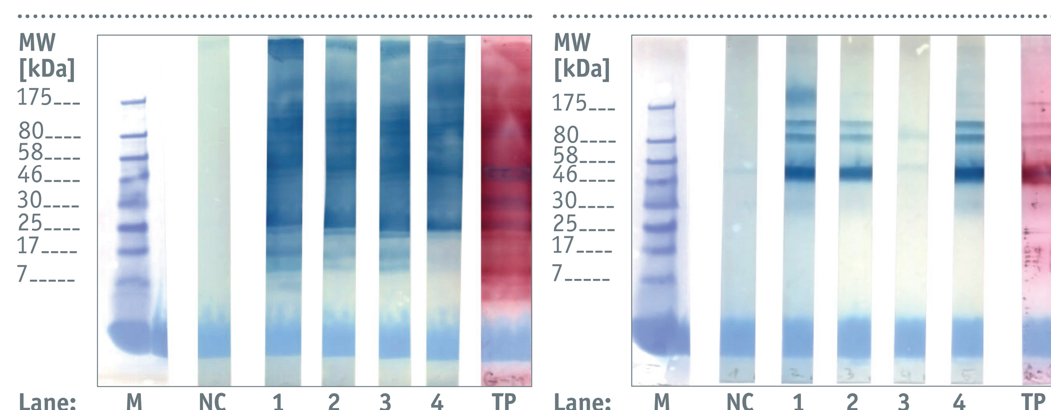

Fig. 4: HCP-specific Western blot reactivity after antibody immune adsorption: Protein amount per lane: 5 µg (Moss-HCP1); NC: negative control (preimmune serum test pool, diluted 1:500); lane 1: HCP-specific antiserum pool (diluted 1:500); Lane 2: anti-Moss-HCP-IgG (2 µg/mL); lane 3: anti-Moss-HCP-IgG-IA (2 µg/mL); lane 4: eluate of immune adsorption (2 µg/mL); TP: total protein staining with colloidal gold.

Fig. 5: Western blot cross-reactivity after antibody immune adsorption against α-Gal DS: Protein amount per lane: 500 ng (α-Gal); NC: negative control (pre-immune serum test pool, diluted 1:500); lane 1: HCP-specific antiserum pool (diluted 1:500); lane 2: anti-Moss-HCP-IgG (2 µg/mL); lane 3: anti-Moss-HCP-IgG-IA (2 µg/mL); lane 4: eluate of immune adsorption (2 µg/mL); TP: total protein staining with colloidal gold.

The results clearly demonstrate, that the capture antibody could be successfully depleted of cross-reacting antibodies by immune adsorption against α-Gal DS (faint signals migrating at approximately 50 kDa were present as well in the negative control and are likely to be non-specific; Fig. 5, NC). Different in-process samples were measured using the Moss-HCP ELISA including capture antibodies with and without immune adsorption (Table 1).

Sample ID:	HCP content (dilution-corrected) α-Gal DS (Batch 1)	α-Gal DS (Batch 2)	α-Gal DS (Batch 3)
Capture Antibody			
anti-Moss-HCP-IgG	659.0 ng/mL	1195.5 ng/mL	1250.0 ng/mL
anti-Moss-HCP-IgG-IA	687.5 ng/mL	1143.5 ng/mL	1216.5 ng/mL

Table 1: HCP determination of three α-Gal DS Batches (sample working dilution: 1:500).

Highly similar HCP concentrations were detected for each sample using both capture antibody species, demonstrating that no relevant HCP-specific antibodies were co-depleted during immune adsorption.

Additionally, ELISA inhibition tests were performed using α-Gal DS (Table 2). No residual post-inhibition reactivity related to α-Gal DS was observed for the immune-adsorbed antibody. Thus, in line with the previous Western blot data, also ELISA tests confirmed the successful depletion of cross-reacting antibody species.

Sample ID:	HCP content α-Gal DS (Batch 3)		Assay Buffer Control (5 ng/mL HCP)	
State:	w/o Inhibition	with Inhibition	w/o Inhibition	with Inhibition
Capture Antibody				
anti-Moss-HCP-IgG	2.5 ng/mL	0.16 ng/mL	4.47 ng/mL	< LOQ
anti-Moss-HCP-IgG-IA	2.43 ng/mL	< LOQ	4.58 ng/mL	< LOQ

Table 2: ELISA inhibition test using α-Gal DS Batch 3: The given HCP contents are not corrected for dilution. Sample working dilution: 1:500 (α-Gal DS concentration: 2 µg/mL).

As shown in Table 2 (anti-Moss-HCP-IgG), the remaining reactivity after inhibition was quantified to 0.16 ng/mL, which corresponds to about 6% based on the total HCP content without inhibition. This means that 2 µg/mL of α-Gal DS cross-reactivity correspond to 0.16 ng/mL HCP reactivity. Although such a low level of cross-reactivity may be considered to be negligible, it might become an issue at much higher drug substance concentrations at described below. If the HCP content reduces by a factor of ten as for example occurred during the downstream development of the project, the concentration of the drug substance would increase by a factor ten relative to the HCP concentration and thus increase the relative cross-reactivity by a factor of ten. This would then constitute a theoretical relative cross-reactivity of 60% and thus unacceptable for HCP ELISA measurement. This example clearly points out the necessity of the antibody immune adsorption approach.

Coverage Determination of the Anti-Moss-HCP-IgG-IA Antibody

In order to check the quality of the generated antibodies, HCP-coverage was determined by 2D DIGE Western blot analysis. A 2D fluorescent Western blot allows detection of high and low abundant proteins on the same membrane. For the 2D analysis, the Moss-HCP sample was separated by 2D electrophoresis; separating the proteins in two dimensions according to their isoelectric point (isoelectric focusing – IEF) and to their molecular weight (SDS-PAGE). After Western blotting and immunostaining with the anti-Moss-HCP-IgG-IA antibodies (using a Cy3 labelled secondary antibody) the images of the Cy5 HCP pattern and the Cy3 immunodetection patterns were obtained (Fig. 6).

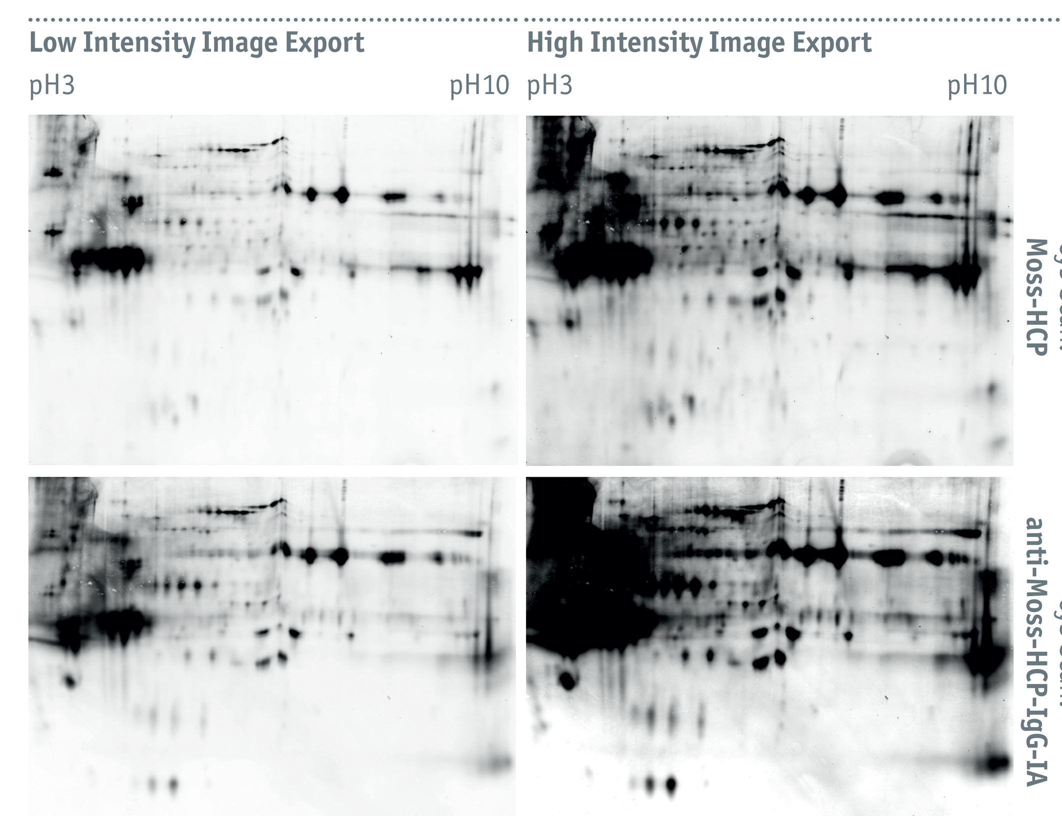

Fig. 6: Cy5- and Cy3 scan of 2D fluorescence Western blot: 2D Fluorescence Western blot of Cy5-labelled Moss-HCP (50 µg) and immunostaining using anti-Moss-HCP-IgG-IA (20 µg/mL, 2 mg) detected with anti-Rabbit-IgG-Cy3 conjugate, two different intensity exportations per dye of a single fluorescence scan (PMT Scanning settings: Cy5 525 V, Cy3 425 V).

For coverage evaluation, the electronic spot detection of the total protein pattern (Cy5, Fig. 6) with ImageMasterTM software resulted in a number of 580 spots (Fig. 7). The comparison of the anti-Moss-HCP-IgG-IA specific immunodetection pattern of the fluorescent Western blot with the electronically detected Moss-HCP spots of the Cy5 total protein pattern (Fig. 7) resulted in 527 corresponding immuno-detected spots (framed blue in Fig. 8) which is equivalent to a coverage of 90.8%.

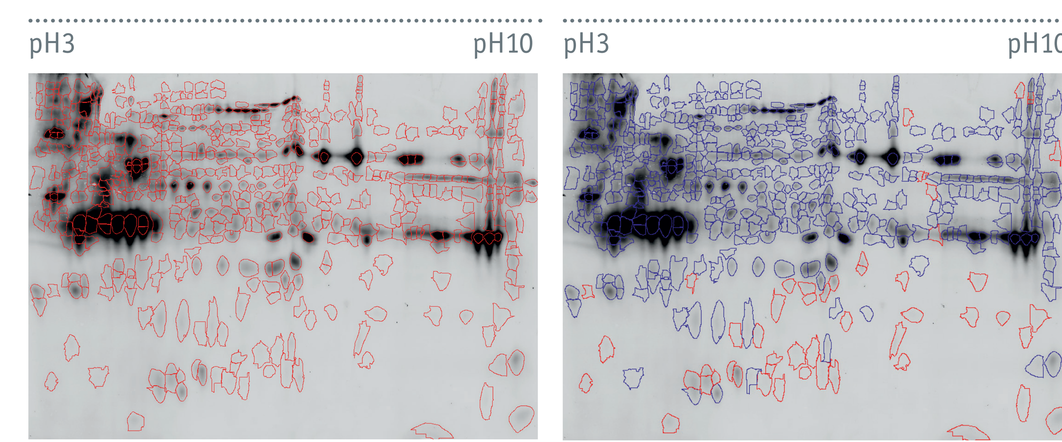

Fig. 7: Electronic spot detection of the 2D fluorescence Western blot (Moss-HCP-Cy5 Image): Electronic spot detection was performed using the program ImageMaster™ 2D Platinum 7.0 (GE Helthcare). Electronically detected protein spots are framed red; protein: 50 µg Cy5-labeled Moss-HCP; number of detected spots: 580.

Fig. 8: Electronic spot detection of Moss-HCP-Cy5 protein pattern compared with anti-Moss-HCP-IgG-IA immunodetection pattern: Electronic spot detection was performed using the program ImageMaster™ 2D Platinum 7.0 (GE Helthcare). 527 spots were detected by the antibody framed blue; undetected spots remain red-framed (total spot amount 580); coverage was calculated as 90.8%.

Summary

A high performance specific Physcomitrella patens-HCP ELISA with a pre-validated working range of 0.1 – 5.0 ng/mL was developed. Cross-reacting antibodies were successfully depleted by immune adsorption against α-Gal DS as clearly demonstrated by 1D Western blot and ELISA inhibition tests. The immune-adsorbed Moss-HCP-specific antibodies yielded 90.8% HCP-coverage in 2D DIGE Western blot.

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