Production and Characterization of High Coverage, Process Specific Pichia P. HCP Antibodies

Camilo A. Moncada[1], Jonathan Birabaharan[1], Sammie McIllwain[1], Indranil Gupta[2], Raju KVN[2], Meetsharad Shah[2], Monalisa Chatterji[2], Carl Ascoli[1]

[1]Rockland Immunochemicals Inc., Limerick, PA, 19468; [2]Biocon Research Limited, Bangalore, India

Contact | cmoncada@rockland-inc.com | 484-791-3823

ABSTRACT

The production of recombinant protein therapeutics in a number of different organisms including bacteria, yeast and mammalian cells is a rapid growing field in the pharmaceutical industry. A key requirement in this process constitutes the thorough analysis of host cell proteins (HCPs) that specifically result from growth and fermentation conditions as well as downstream purification procedures. Current guidelines call for minimum levels of HCP contaminants that may be left behind during the purification process from the expression hosts. To investigate the presence of residual contamination during the bioprocessing of a final biopharmaceutical product, a common approach is the development of polyclonal antibody reagents with maximum coverage against native HCP extracts.

Here, we describe the overall process of developing a high coverage, process-specific Pichia pastoris HCP antibodies that can be used as an analytical tool for the determination of HCPs in the production of a recombinant human protein. The overall process included characterization of HCPs by one (1D) and two dimensional (2D) SDS-PAGE at distinct early steps of bioprocessing, preparation of cascade immunization antigen to improve reactivity for low abundance/non-immunogenic proteins, continuous monitoring of immune response by 1D and 2D western blot and ELISA as well as final coverage assessment by 2D western blot analysis of purified antibody.

Extensive characterization of the resulting purified HCP antibodies by 2D western blot and ELISA guarantees comprehensive reactivity toward different HCP species that makes a reagent suitable for implementation on a high throughput enzyme-linked immunosorbent assay (ELISA) for HCP testing.

INTRODUCTION

The use of recombinant proteins for effective treatment of a wide range of human diseases constitutes an increasingly adopted approach in the biopharmaceutical industry. Such therapeutic proteins include among others peptides, enzymes, monoclonal antibodies, growing factors, hormones that are produced using biological processes involving large-scale cultivation of cells engineered to express genes encoding the protein(s) of interest[1]. Cell types commonly used as hosts for this process include bacteria, yeast, insect cells or mammalian cells. Endogenous host cell proteins (HCPs) co-expressed with the protein of interest by the hosts used for biologics production constitute a complex mixture of process related impurities that have to be closely monitored and adequately removed to prevent adverse efficacy and toxicity effects[2,3].

Several analytical methods for detecting and monitoring HCPs are available including mass spectroscopy and the use of process-specific polyclonal antibodies. HCP assays greatly rely on the ability of an anti-HCP antiserum to detect HCP impurities, so the performance of the assay is tightly linked to the quality of this reagent[1]. HCP antibodies are commonly used for HCP testing using multi-analyte ELISA because it provides wide dynamic range, selectivity and sensitivity that allow detection of low levels of residual HCPs present in large excess of product protein as well as high throughput capacity[2]. Additionally, HCP antibodies can also be used in Western blot analysis combined with two dimensional (2D) electrophoresis providing complementary information like changes in HCP composition under different conditions and properties (pI, MW) of individual species[3]. Validated assays are used for supporting process development and quality control testing[2].

Here, we report the development of a process specific Pichia p. HCP antibody using protein species present at two different downstream bioprocessing steps of a null cell culture harvest. HCPs were characterized using (1D) and two dimensional (2D) SDS-PAGE at the two distinct early steps of bioprocessing before used for immunization. Reactivity for low abundance/non-immunogenic proteins was improved by cascade immunization and confirmed by 1D and 2D western blot as well as ELISA. The resulting polyclonal antibodies were finally obtained by affinity fractionation for IgG and confirmed to be a mixture that recognizes multiple Pichia p. protein species suitable for HCP testing by ELISA and/or Western blot.

HCP ANTIBODY DEVELOPMENT

Pichia pastoris Null Cell Line HCP sampling

Two distinct, early steps of bioprocessing

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Evaluation of potential HCP immunogen(s)

SDS-PAGE | Two Dimensional Electrophoresis (2-DE)

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Immunization of selected immunogen

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Assessment of immune response

Western Blot | 2-DE Western Blot | ELISA

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Cascade Immunization

Enrichment of low immunogenic proteins by immuno-depletion

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Anti-sera Validation

Western Blot | 2-DE Western Blot | ELISA

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Preparation and processing of selected anti-sera pool

Antibody Purification | Antibody Modification

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Antibody Validation

Coverage analysis by 2-DE | ELISA

EVALUATION OF POTENTIAL HCP IMMUNOGEN(S)

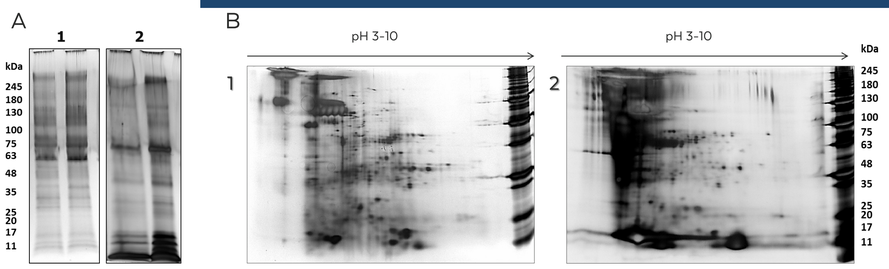
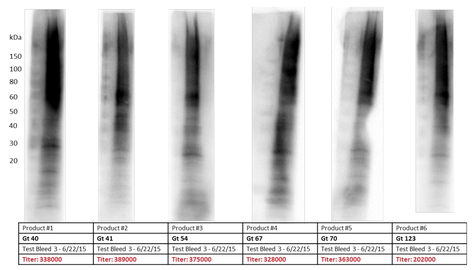


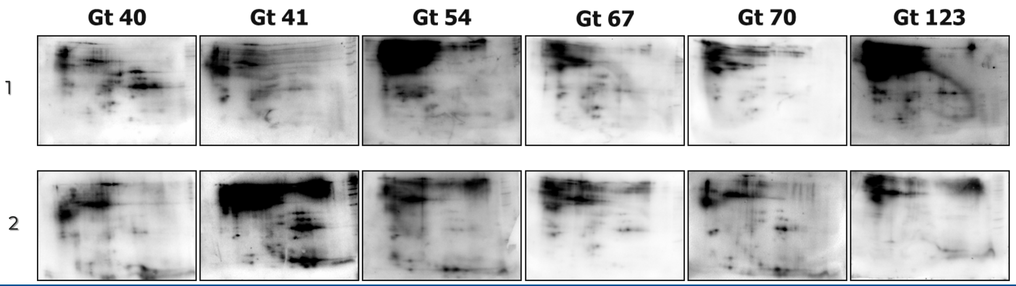
Figure 1: In order to identify best antigen for antibody development, HCP composition from different bioprocessing steps are characterized by both SDS-PAGE and 2-DE. Silver stained of Pichia p. HCP preparations at two different steps (1 and 2) of early bioprocessing by: (A) SDS-PAGE: ~10 ug/lane of material before (left) and after (right) sample prep for 2-DE. (B) 2-DE:~100 ug /strip of samples

ASSESSMENT OF IMMUNE RESPONSE



Product #1	Product #2	Product #3	Product #4	Product #5	Product #6
Gt 40	Gt 41	Gt 54	Gt 67	Gt 70	Gt 123
Test Bleed 3 - 6/22/15	Test Bleed 3 - 6/22/15	Test Bleed 3 - 6/22/15	Test Bleed 3 - 6/22/15	Test Bleed 3 - 6/22/15	Test Bleed 3 - 6/22/15
Titer: 338000	Titer: 389000	Titer:375000	Titer: 328000	Titer: 363000	Titer:202000

Figure 2: Pichia p. HCP preparations of bioprocessing steps 1 and 2 were tested either as a pool by standard Western blot (left) or separately by 2-DE Western blot (below) using anti-sera from goats immunized and boosted with the pooled preparation. Blots were developed by chemiluminescence using exposition times ~5-20s for (WB) or ~1-2s (2-DE WB). ELISA titers for the same anti-sera are also shown in red (left).



CASCADE IMMUNIZATION

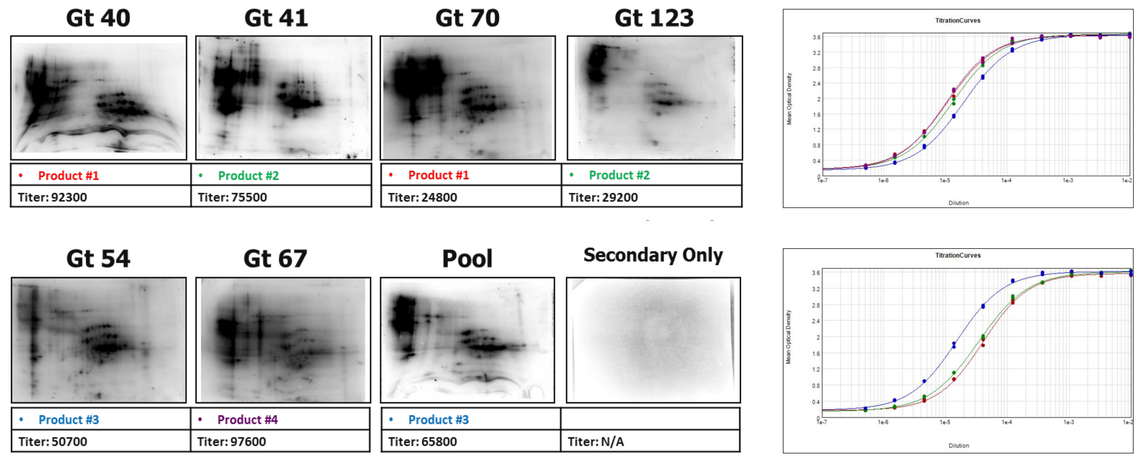
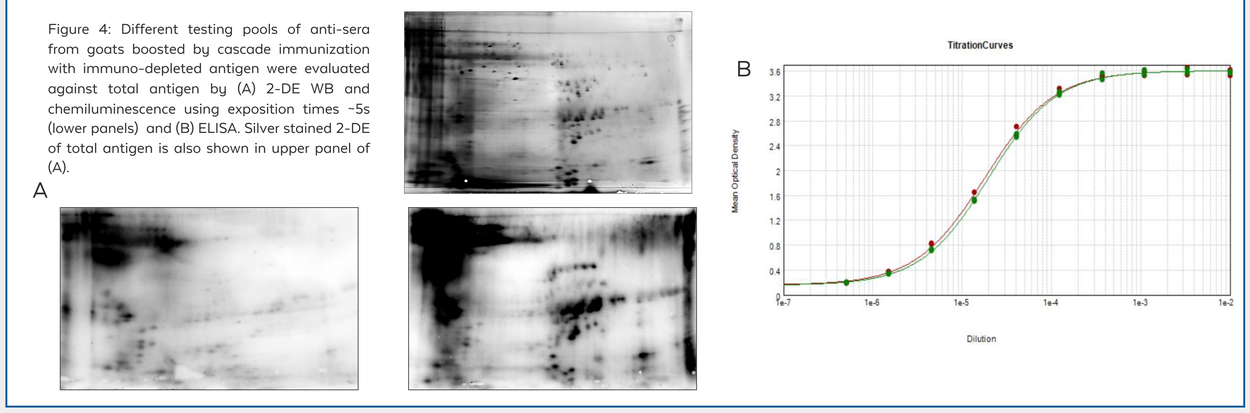


Figure 3: Anti-sera pools of individual goats boosted by cascade immunization with immuno-depleted antigen were evaluated against total antigen by 2-DE WB and chemiluminescence using exposition times ~20s (130s for incubation with secondary antibody only) and ELISA.

ANTI-SERA VALIDATION

Figure 4: Different testing pools of anti-sera from goats boosted by cascade immunization with immuno-depleted antigen were evaluated against total antigen by (A) 2-DE WB and chemiluminescence using exposition times ~5s (lower panels) and (B) ELISA. Silver stained 2-DE of total antigen is also shown in upper panel of (A).



COVERAGE ANALYSIS BY 2-DE

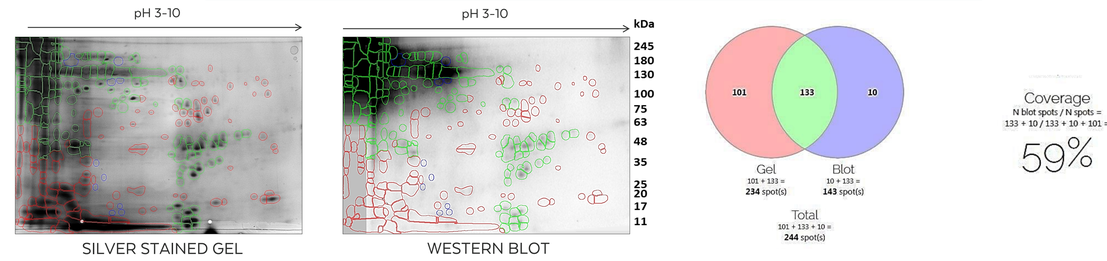


Figure 5: Pichia p. HCP antibody coverage was determined by 2-DE analysis comparing a western blot against a silver stained gel of the HCPs. Coverage analysis was using SpotMap v2 software from (TotalLab Ltd, U.K.). SDS-PAGE: ~10 ug/lane of material before (left) and after (right) sample prep for 2-DE. (B) 2-DE:~100 ug /strip of samples. HCP antibody is a Protein A/G purified IgG fraction.

CONCLUSIONS

- Evaluation of Pichia p. HCPs by two dimensional electrophoresis provided useful information in determining variations in protein composition and abundance at two different steps of bio-processing and allowed the confirmation of immunization material suitable for generation of process-specific HCP antibodies.
- Combination Rockland's optimized immunization methods along with cascade immunization resulted in the generation of goat anti-sera capable to detect a wide range of Pichia p. HCP species.
- Protein A/G purified IgG exhibited around 59% coverage by Western blot of both high and low molecular weight proteins by two dimensional electrophoresis, confirming the successful generation of a multi-analyte anti-Pichia HCP antibody.
- Western blot and ELISA data indicate the generated anti-Pichia HCP antibody suitable to support process development, process validation, and control system testing.

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