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Development of an improved standardized immunoassay for HCP detection using 2D electrophoresis and western blotting system



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ABSTRACT

Food and Drug Administration (FDA) specifications for the highest degree of purity for large molecule biopharmaceuticals require final products to be free of host cell protein (HCP) contaminants. HCPs may be left behind during the purification process from expression hosts, such as *E.coli*, insect, yeast or mammalian cells. If HCP impurities remain in products administered to patients, contaminants can result in adverse toxic or immunological reactions. To investigate the presence of residual contamination of the final biopharmaceutical product, the development of polyclonal antibodies with maximum coverage against native HCP lysate provides a valuable tool to demonstrate product purity.

Protein contaminant detection can be visualized by 1D electrophoresis followed by western blotting using antibodies raised against HCP preparations. However, the immunogenicity of individual HCPs can vary widely and highly immunogenic proteins can disguise or “mask” the effects of other, similar size proteins. The implementation of 2D SDS-PAGE assessment, by which complex protein mixtures are separated according to isoelectric point and molecular weight followed by western blotting, can deliver a more complete insight into the capability of an anti-HCP polyclonal antibody with respect to immunocoverage of protein components of the host cell lysate. This poster presents the development of a 2D SDS-PAGE and western blotting method to validate the efficacy of immunocoverage for a novel anti-HCP polyclonal antibody generated against an *E.coli* HCP lysate.

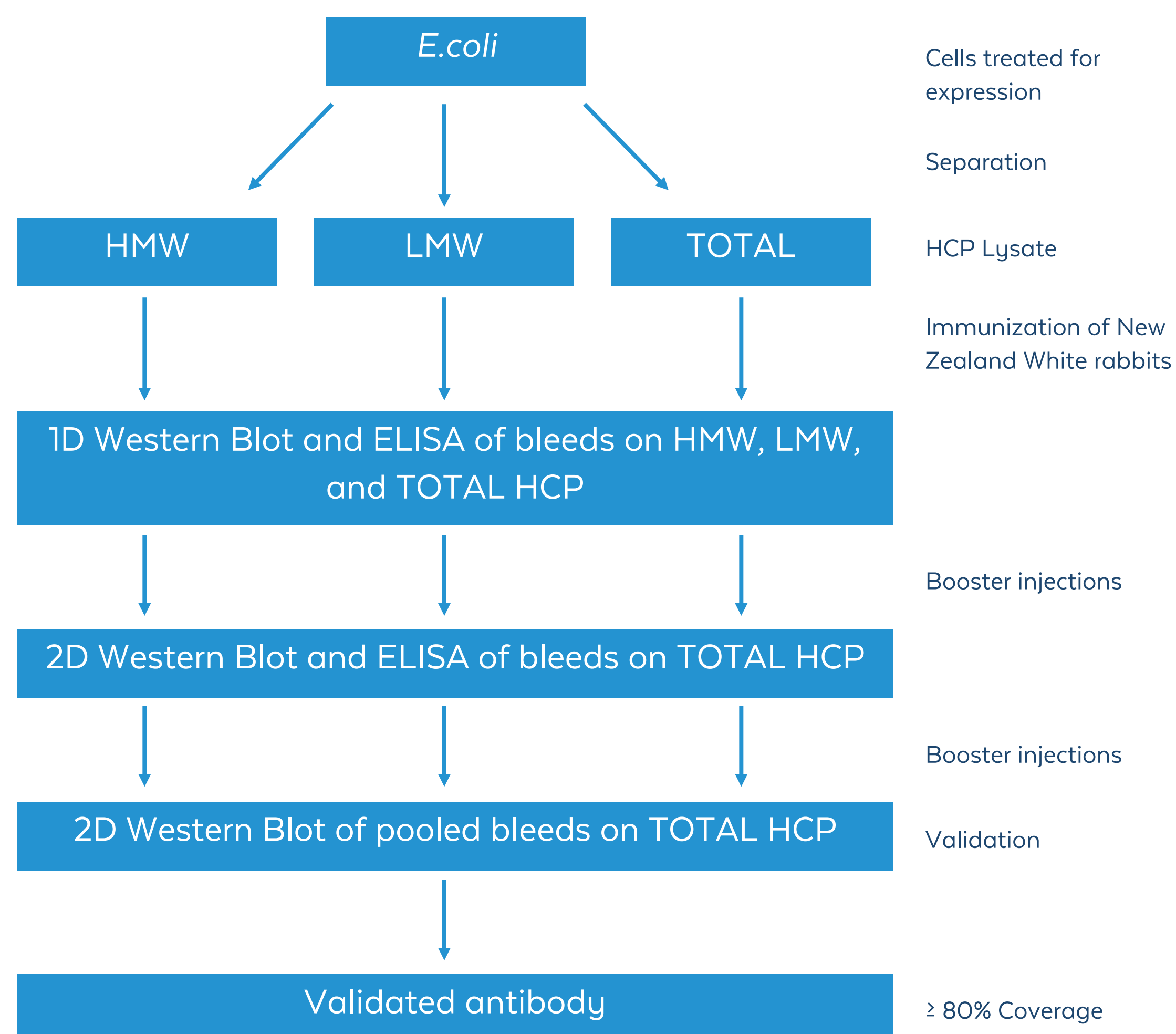
INTRODUCTION

Several effective strategies can be deployed individually or in combination when raising antibodies to HCPs including a) fractionation of HCP proteins into low molecular weight (LMW) and high molecular weight (HMW) components for separate immunizations, b) cascade immunization where an initial immunization with HCP proteins is followed by booster injections with a subset of less abundant or immunorecessive HCP proteins (prepared by subtractive immunoprecipitation using initial antibodies raised against the most abundant and immunodominant HCP proteins present in the total HCP lysate), and lastly c) the use of multiple host species to control for differences in host animal immune responses, where typically multiple mammalian and avian host animals are used.

In this study we explored HCP antibody production using antigens produced by separating total HCP protein lysates into LMW and HMW fractions by ultrafiltration. These fractions were used separately to immunize New Zealand White rabbits. The sensitivity and specificity of anti-HCP antibodies raised against these fractions was compared to anti-HCP antibodies produced by immunizing total HCP lysates directly.

We present the development of HCP antibodies that display sensitivity and specificity against a broad range of *E.coli* HCPs using a robust 2D electrophoresis assay to provide efficient immunocoverage against potential contaminants of biopharmaceutical products.

HCP LYSATE PREPARATION & HOST IMMUNIZATION



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1D WESTERN BLOT ANALYSIS OF HCP ANTIBODIES

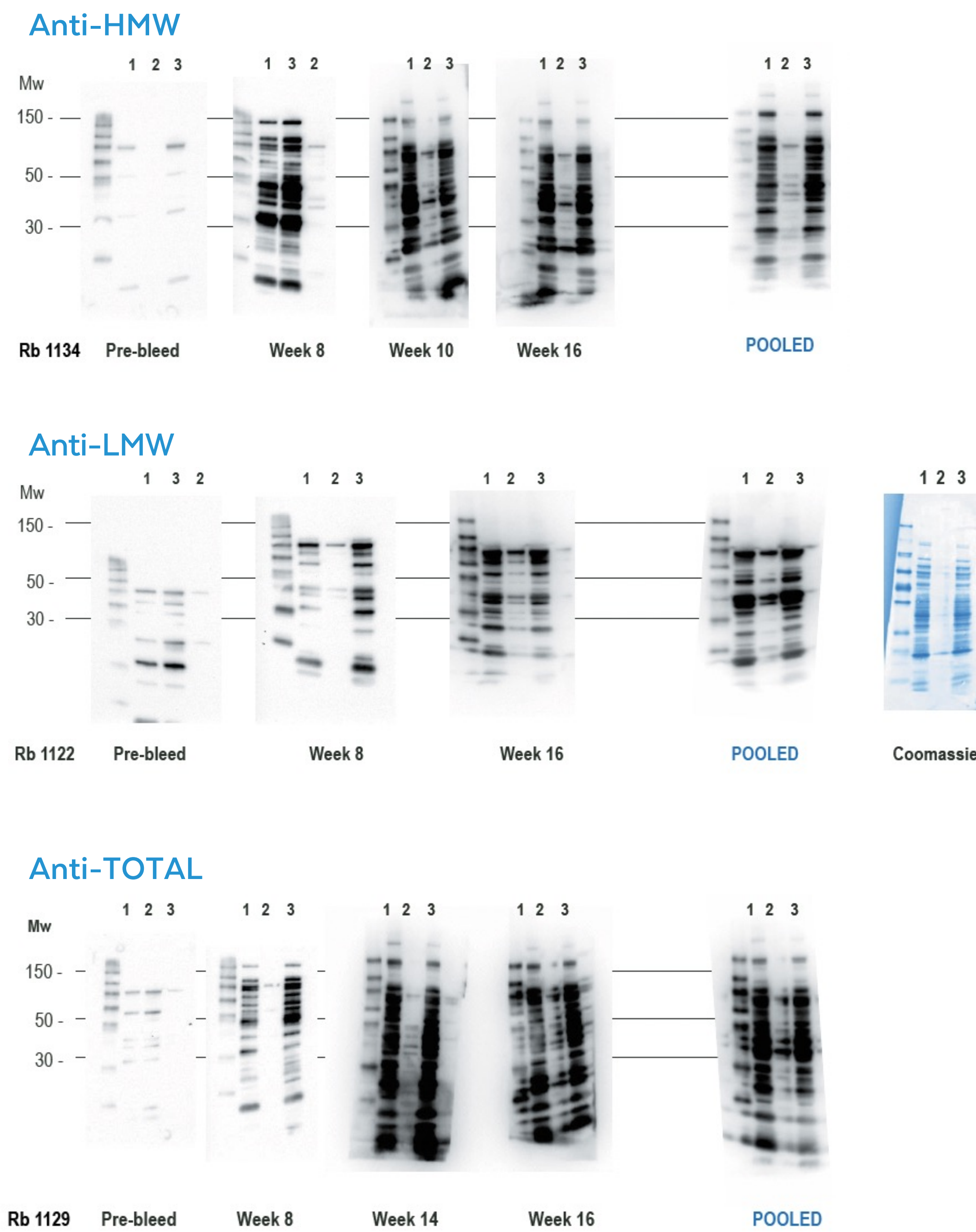


Figure 1: 1D Western blot analyses of anti-HCP antibodies harvested from respective animals immunized with HMW (>100kDa) proteins (top); LMW proteins (middle) and TOTAL (non-fractionated) HCP proteins (bottom) to investigate acceptable antigen reactivity coverage. Only blots for one animal per group are shown. The blots marked POOLED were processed using a combination of week 16 antisera from all rabbits in the immunization group. Antisera pools demonstrate the broadest range of immunocoverage when compared to proteins separated by 1D SDS-PAGE followed by Coomassie staining for visualization. Lanes are loaded as follows: 1) HMW HCP proteins, 2) LMW HCP proteins and 3) TOTAL HCP proteins.

2D SDS PAGE VALIDATION

One of the pitfalls of 2D SDS-PAGE is the resolution of all proteins in the first dimension. We developed a protocol to maximize the resolution and validate our HCP antibodies. To investigate the complexity of the total HCP lysate, the sample was resolved by 2D SDS-PAGE gel and stained using Oriole and Coomassie stains (Figure 2)

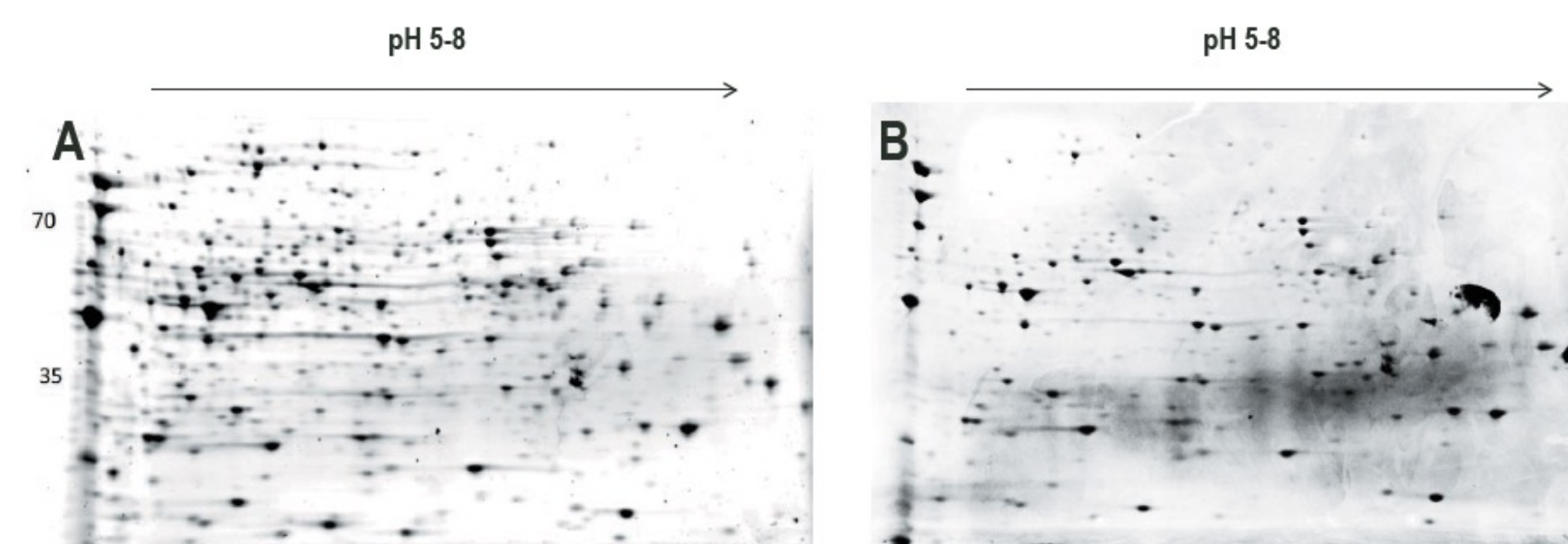


Figure 2: 2D SDS-PAGE analyses of total HCP lysate stained with Oriole (A) and Coomassie (B) stain.

- HCPs prepared for IEF with Bio-Rad's ReadyPrep kit and protocol
- IEF run on pH5-8 Ready Strip IPG with Bio-Rad's Protean i12 system
- Strips were electrophoresed on Criterion Stain-Free TGS gels, activated to visualize proteins, and transferred to PVDF with Bio-Rad's TransBlot system
- Gel was re-imaged after transfer to confirm complete protein transfer (data not shown)
- Electrophoresed proteins were visualized using Oriole and/or Coomassie stain for further antibody development experiments
- After transfer of HCPs to PVDF, membranes were blocked with Rockland's Blocking Buffer for Western Blotting (p/n MB-070) and probed with anti-LMW, anti-HMW, or anti-TOTAL sera
- Reporting was performed with Rockland's Goat anti-Rabbit IgG peroxidase antibody (p/n 611-1302)
- Image analysis was performed using Bio-Rad's PDQuest 2D Gel Analysis Software

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2D WESTERN BLOT ANALYSIS OF HCP ANTIBODIES

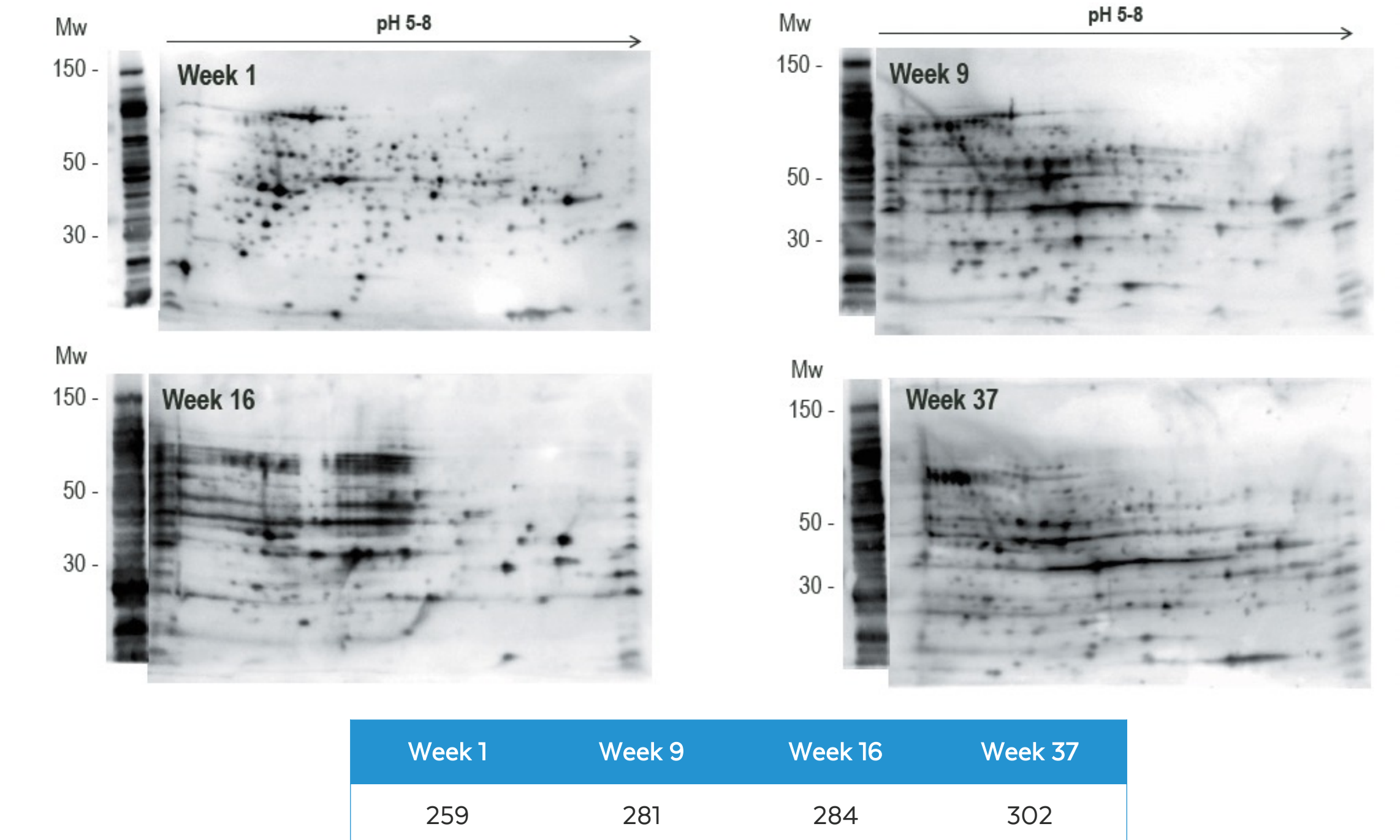


Figure 3: TOTAL HCP proteins were resolved by 2D electrophoresis and probed with sera following subsequent booster injections. As indicated by the “spot counts” presented below the blots, immunocoverage increases over time, but this increase is not apparent using solely the 1D format.

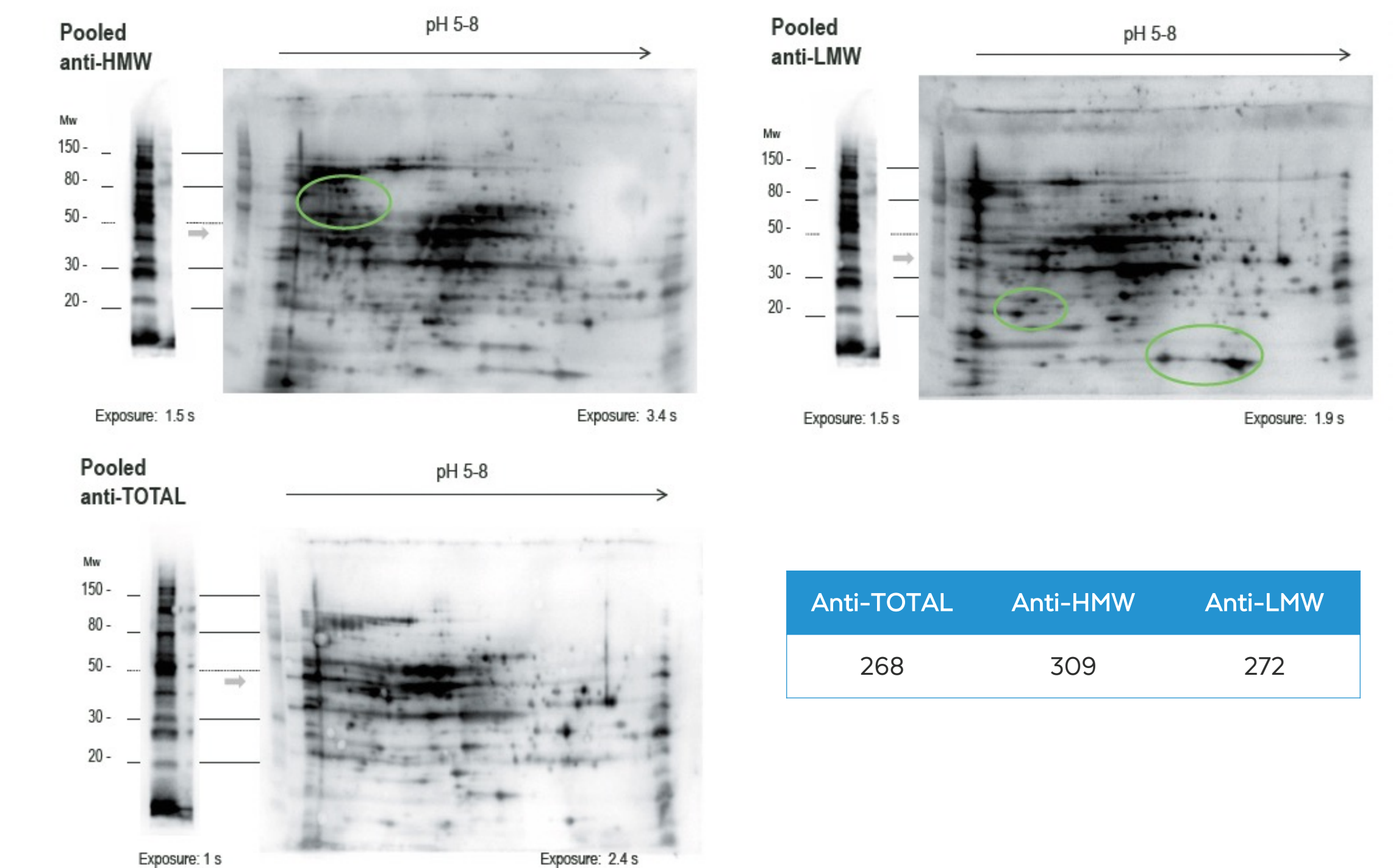


Figure 4: Total HCPs were separated in two-dimensions and probed with pools of antisera from rabbits from each immunization group (n=4, HMW, LMW, and TOTAL HCP). The distinct recognition of antigens seen as spots unique to the anti-LMW and anti-HMW blots (circled in green) which are not visualized in the anti-TOTAL blot is consistent with the LMW/HMW approach as an effective strategy to broaden specificity for HCP production. Therefore blots processed with a cocktail of anti-LMW and anti-HMW HCP antibodies should detect a greater diversity of HCP proteins when compared to blots of similar lysates probed with antibodies raised against unfractionated HCP lysates.

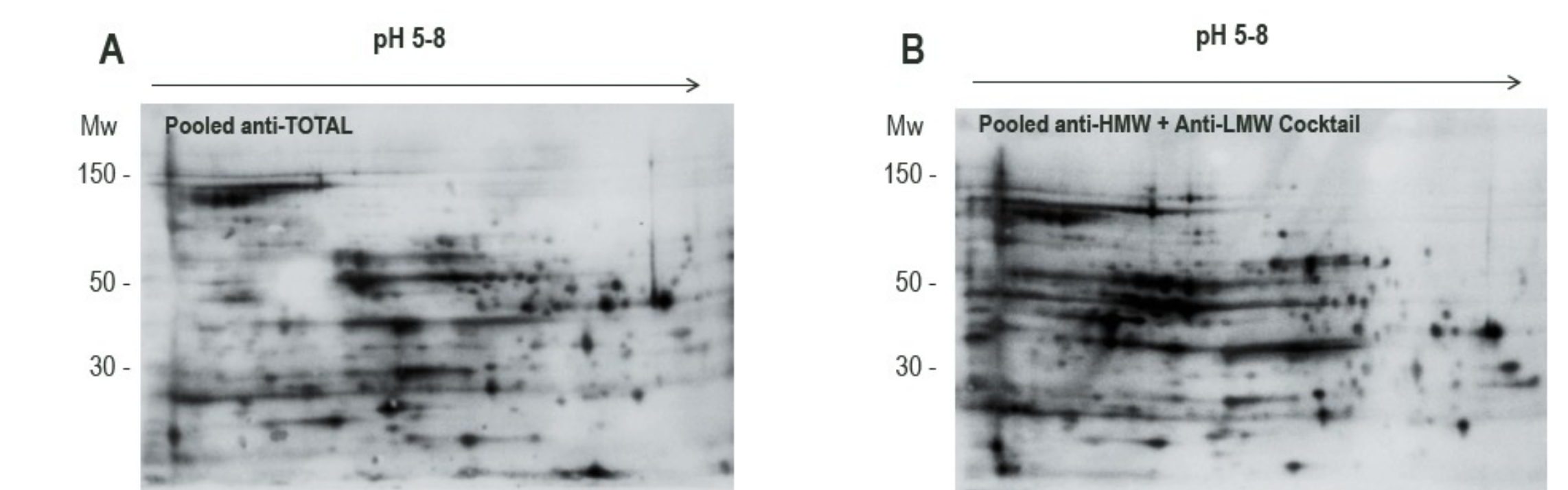


Figure 5: Two-dimensional western blotting was used to show pooled sera of rabbits from HMW and LMW immunization groups (B) detects more HCPs than anti-TOTAL HCP antibodies (A). Total HCP proteins were similarly separated and applied to both blots. By visual assessment it is evident that a greater diversity of recognized proteins occurs using the cocktail. This validates the effectiveness of the LMW/HMW method for anti-HCP development.

CONCLUSION & FUTURE DEVELOPMENT

Pooled LMW/HMW antibodies developed using fractionated HCPs provide greater sensitivity and specificity against a broad range of HCP proteins than antibodies raised against unfractionated HCP lysates.

Here we show the development of HCP antibodies validated using a robust 2D electrophoresis assay to provide efficient immunocoverage of *E.coli* HCPs. We demonstrate that 2D electrophoresis is a necessary quality assay in the development of anti-HCP antibodies. Furthermore, misleading assessments of antibody immunocoverage made solely based on one dimensional blotting are more accurately assessed utilizing a two-dimensional approach which shows greater resolution of HCPs.

Rockland offers KCA-J07, an *E.coli* HCP Western Blot kit that contains 2D validated antibodies, HRP conjugated secondary antibodies, blocking and wash solutions and *E.coli* control proteins. Rockland's 2D validated antibodies have been shown to react with more than 300 *E.coli* proteins from SDS/DTT solubilized *E.coli* cells.

Rockland is currently in the process of developing other HCP antibodies against other species that will be validated using the methods outlined here.