# Anti-HCP antibody characterisation using SpotMap image analysis software for analysis of 2D SDS-PAGE and Western blots.

TOTALLAB Application Note

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## INTRODUCTION

This application note demonstrates the characterisation of two samples of pooled anti-*Pichia pastoris* antisera by comparison of a 2D gel and Western blots using SpotMap software. A Western blot of each antisera sample was compared to the 2D total protein gel, percentage coverage was calculated and spots unique to each image identified. Percentage coverage was also calculated for low and high molecular weight HCPs recognised by each sample and the specific spots against which each antisera sample reacted were identified.

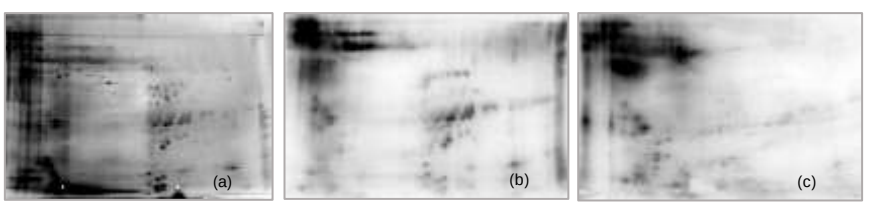
Pool 1 antisera showed broader coverage with 44% compared to pool 2 antisera with 39% coverage. Both antisera samples had higher percentage coverage in the high molecular weight region (48% and 39% coverage for pool 1 and 2 respectively) compared to the low molecular weight region (40% and 39% coverage for pool 1 and pool 2 respectively). Both antisera samples recognised the same 118 HCP spots, however pool 1 recognised 65 spots which pool 2 did not and pool 2 recognised 42 spots which pool 1 did not.

SpotMap is the only software developed specifically for analysis of HCP coverage and antibody product and process characterisation. It addresses the challenge of comparing different spot patterns seen between 2D gels and Western blots and calculates the relative percentage coverage between images. The simplicity and flexibility of SpotMap allows quick yet objective analysis of multiple images and combinations of 2D gels and Western blots.

## METHOD

### Images

A 2D gel (Gel) and two Western blot (Pool 1 and Pool 2) images [1] were uploaded to SpotMap.




The (a) original silver stained Gel image was compared to (b) original Pool 1 Western Blot image and (c) original Pool 2 Western blot to measure anti-HCP antibody coverage.

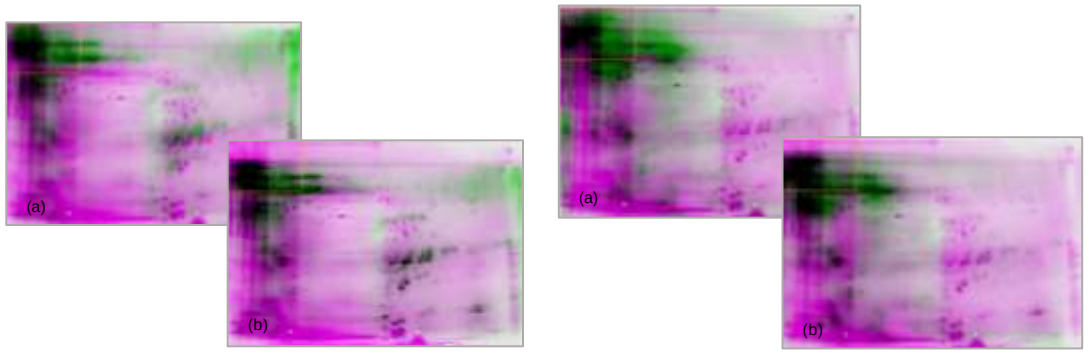
### Alignment

Alignment is a unique method that addresses the challenge of comparing two very different spot patterns commonly seen between gels and blots.

Alignment is performed at the pixel level to provide direct and accurate comparison of images. Alignment is completed automatically or manually. Manual vectors are added to assist alignment of images where large positional differences exist.

Both Western blots were aligned to the Gel.

Alignment can be performed and returned to for corrections at any point of the analysis.




Left: Overlaid Gel (purple) and Pool 1 blot (green) images (a) pre and (b) post alignment.
Right: Overlaid Gel (purple) and Pool 2 blot (green) images (a) pre and (b) post alignment.

### Spots

A single spot pattern was created which identifies the location of all spots in the analysis. Due to the alignment, corresponding spots on different images are in the same coordinate space. Each image is overlaid by the spot map and each spot outline is characterised as present or absent from each image.

Automatic spot detection was used on the Gel image to create an initial spot map. Parameters of: Peak sensitivity =0, Smoothing = 1 and within an area of interest.

Following the spot detection edits were made to split, merge or delete spots.

Once the spot map was correct overlaid on the gel the two Western blot images were aligned to the gel.

Spot presence or absence was initially categorised as any spot on either Western blot with a peak height ≤ 20. Further manual categorisation was completed and additional spots not present on the Gel were added.

Final master spot map of all images. A total of 417 spots were identified across all three images.

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## Results

Setting spots as present of absent from the map allowed the categorisation and spots on the blots as common (present on both the gel and blot), additional (present only on the blot and absent on the gel.) or missing (present on the gel image but absent on the blot.) These categories of spots were used to calculate the percentage coverage of each antisera sample. Changing the base image allows you to compare other images to one another.

$$\% \text{ coverage} = \frac{\text{Number of spots present on the blot}}{\text{total number of spots present in the comparison}} \times 100$$

In the calculation of percentage coverage. It is assumed that spots present on a blot which were not detected on the gel on the gel were present within the sample fell below the limit of detection.

## Results and Discussions

Comparison of relative coverage of each Western blot compared to the 2D gel indicates that pool 1 antisera had broader activity against P. pastoris HCPs with 44% coverage compared to 39% coverage by pool 2 antisera.

A total of 417 spots were identified on the 2D gel. Pool 1 antisera recognised 174 of the spots presented on the 2D gel and pool 2 recognised 153. Both antisera identified additional spots not represented on the 2D gel, 9 by pool 1 antisera and 7 by pool 2 antisera. Spots may be identified only on the western blots due the presence of a highly immunogenic protein which fell below levels detectable by the 2D gel stain used.

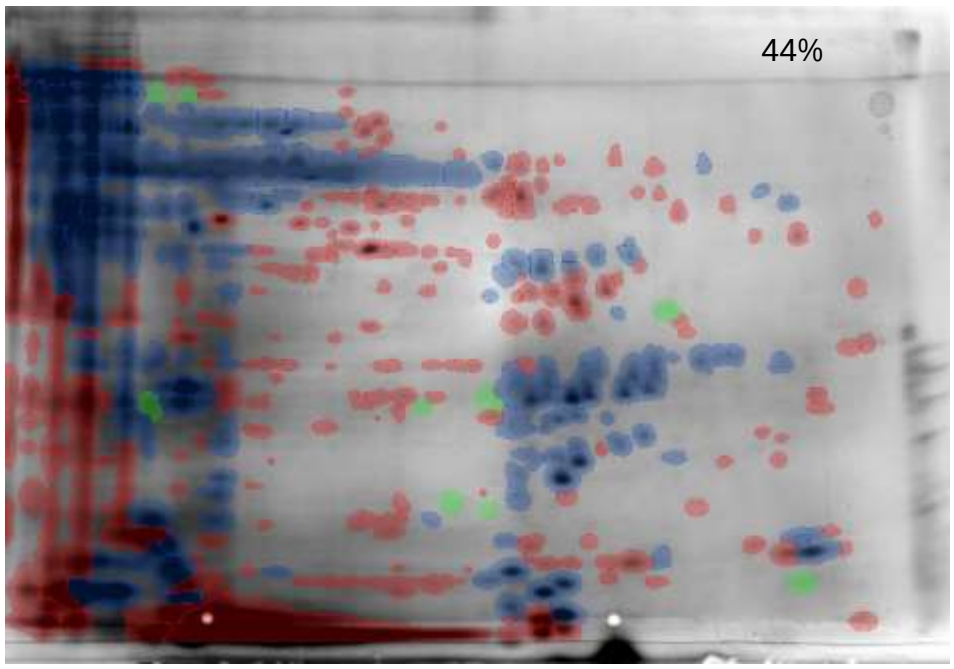
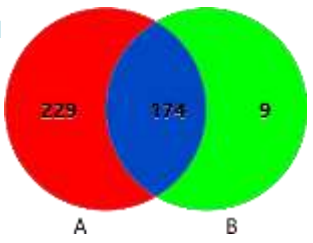



Figure 1. Colour coded spot map overlaid on the 2D gel image representing the coverage of pool 1 antisera. Number of spots within each category are represented in the Venn diagram. Red – Spots present only on the 2D gel, blue – Spots present on both the 2D gel and Western blot, green- spots present only on the Western blot.



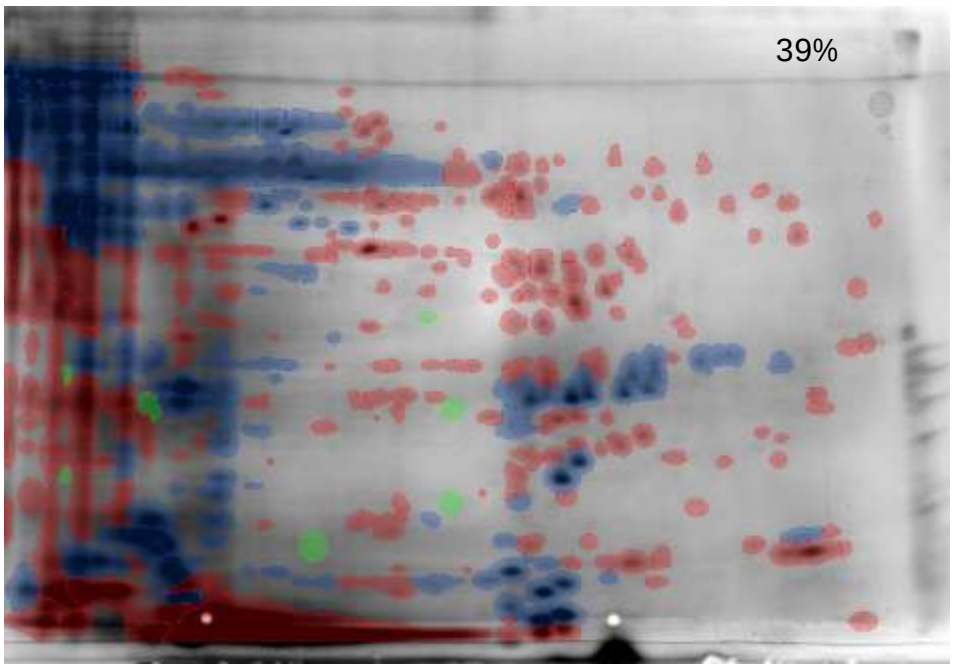
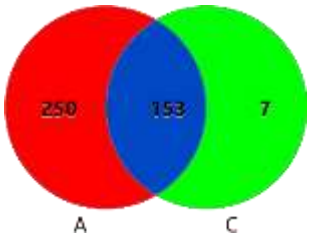




Figure 2. Colour coded spot map overlaid on the 2D gel image representing the coverage of pool 2 antisera. Number of spots within each category are represented in the Venn diagram. Red – Spots present only on the 2d gel, blue – Spots present on both the 2D gel and Western blot, green- spots present only on the Western blot.



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## Results and Discussions (continued)

Analysis of spot numbers and percentage coverage was also compared between high and low molecular weight proteins. 219 spots were within the high molecular weight (HMW) range, 48% and 39% coverage was recorded for pool 1 and pool 2 antisera respectively. Lower coverage was recorded of the 198 spots in the low molecular weight (LMW) range: 40% coverage by pool 1 antisera and 39% coverage by pool 2.
The ability to monitor the antisera coverage at different molecular weights and / or pH (not shown) allows confirmation that the antisera has reactivity to the whole proteome and identifies potential areas which should be investigated or targeted for antibody modification. Specifically, looking at LMW regions allows confirmation than antibodies have been developed against LMW HCPs which is often more challenging.

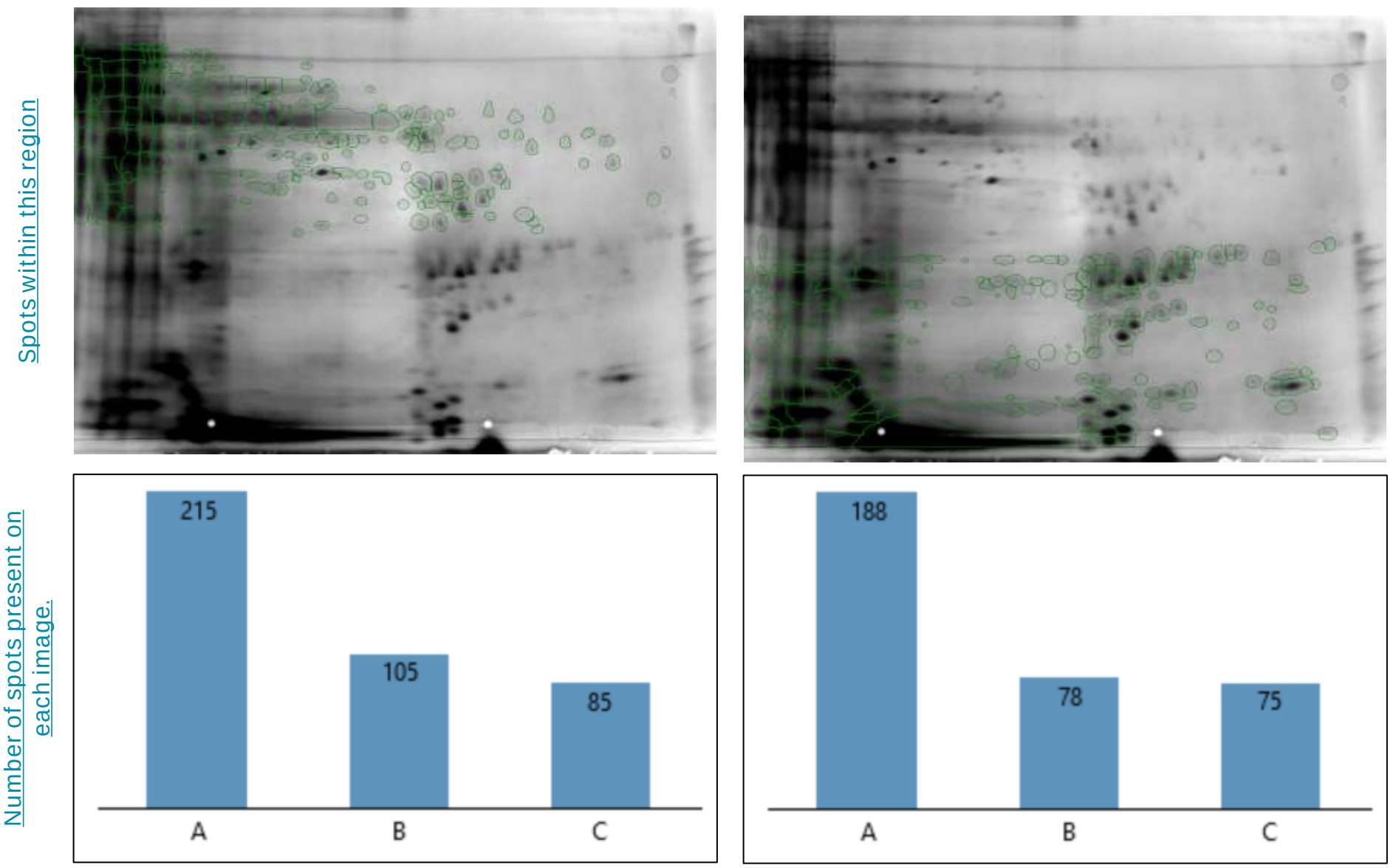


Figure 3. Analysis of antisera pools when separated by high and low molecular weight proteins. Colour coded spot maps identify spots not recognised by the antisera but present on the 2D gel – red, spots present on the 2D gel and recognised by the antisera – blue and spots recognised only by the antisera, but not present on the 2D gel in green. Graphs show the number of spots present on each image in the low / high molecular weight regions.

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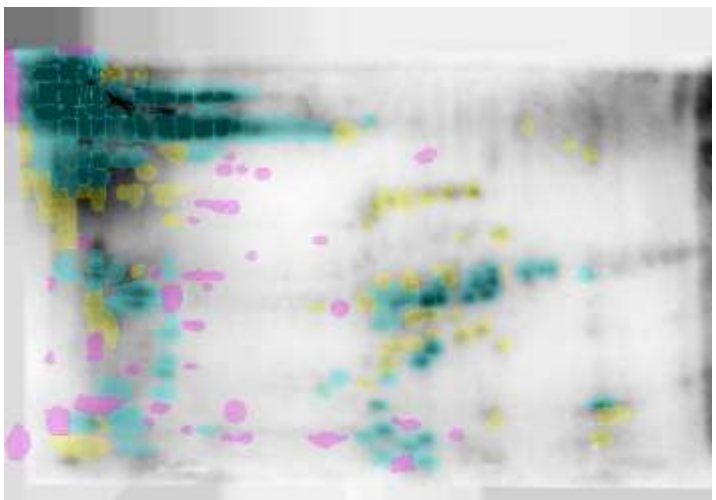
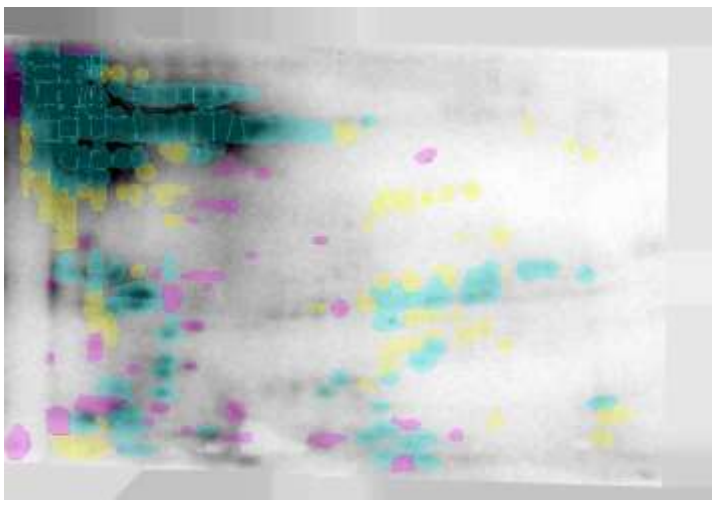
## Results and Discussions (continued)

Use of a single spot map allows comparisons to be made not only to measure the coverage of each pool of antisera to the 2D gel but also allows comparison of the specific spots against which each pool of antisera recognise. Both pool 1 and pool 2 antisera recognise 125 spots in common. 70 spots were recognised only by pool 1 antisera and 48 spots were recognised only by pool 2 antisera. 203 spots were not recognised by either pool of antisera.





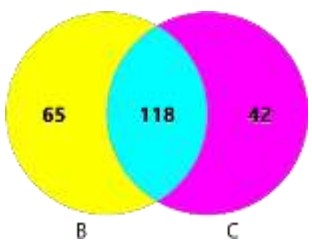



Figure 4. Colour coded spot map overlaid on the 2D gel image representing the spots recognised by both pools of antisera (turquoise), pool 1 antisera only (yellow), pool 2 antisera only (magenta) and not recognised by the antisera (red). Venn diagram represents the number of spots recognised by each pool of antisera.

## Conclusions

The comparison of 2D total protein gels and Western blots is often challenging, however it is a key orthogonal method used to measure the anti-HCP antibody coverage. SpotMap allows analysis of multiple images and may be used to characterise both antigen and antibodies by comparison of 2D gels or 2D gels and Western blots.

In this analysis a total of 439 spots were detected on the 2D silver stained gel image. Pool 1 antisera recorded a coverage of 44%, recognising 190 of the spots present on the 2D gel and an additional 5 recognised only by the antisera. Pool 2 antisera recorded a coverage of 39%, recognising 171 of the spots present on the 2D gel and an additional 2 recognised only by the antisera. Lower percentage coverage was recorded in the LMW regions by both antisera samples.

## References & Acknowledgments

[1] Images supplied by Rockland Immunochemicals Inc. Limerick, PA, USA. Thank you to Maheen Sayeed for all the information regarding this analysis.