Image analysis used to minimise inter-user and inter-lab variation of results from measuring HCP-antibody coverage by comparing features between 2D gel and 2D Western blots.

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Introduction

The presence of host cell proteins (HCP) must be monitored and minimised to ensure the safety, purity and potency of biologic products according to regulations (e.g. ICH Q6B, ICH Q8 and 42 USC 262). To monitor the presence of HCP requires the use of an anti-HCP antibody that is characterised by how much of the total complement of host cell proteins it reliably detects.

The greater resolving power of 2D gel electrophoresis has meant that 2D SDS-PAGE, rather than 1D SDS-PAGE, followed by Western blotting is recommended for anti-HCP antibody characterisation[1] .

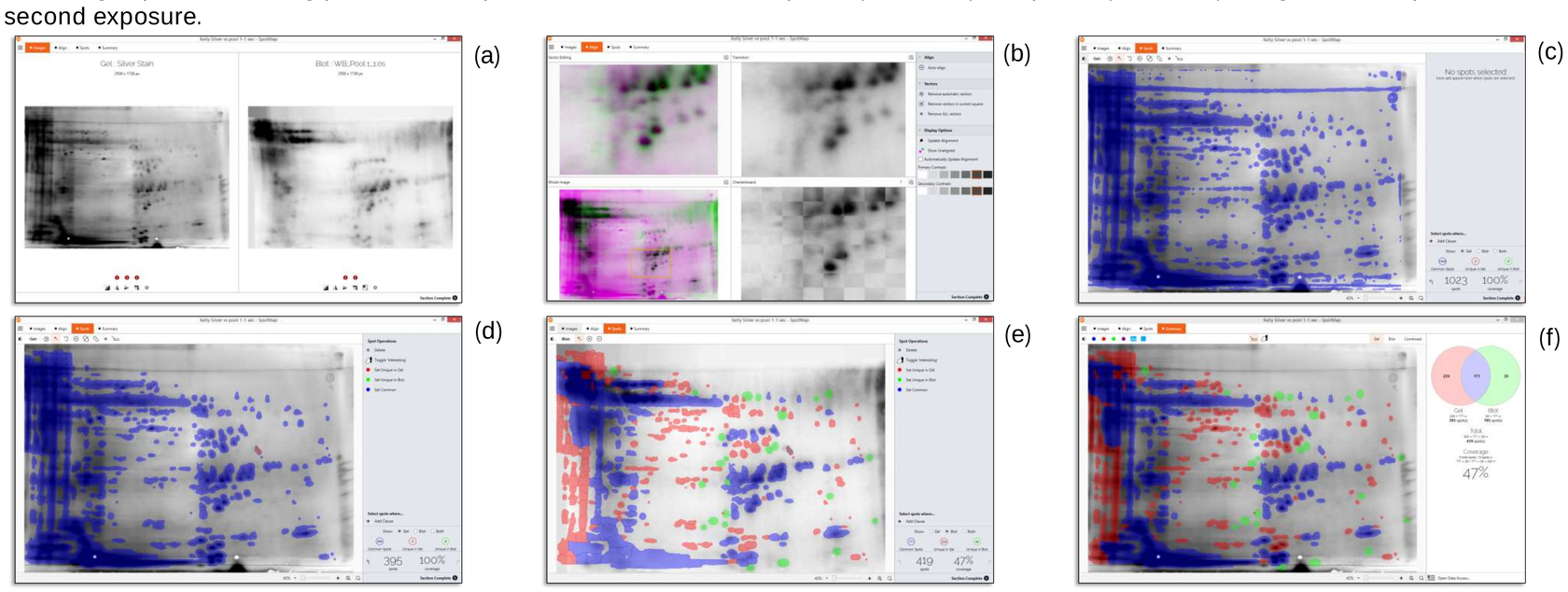
Here we evaluate the reliability and reproducibility of SpotMap software (TotalLab Ltd, Newcastle upon Tyne) to analyse significantly different spot patterns and objectively measure percentage coverage of total protein separated by a 2D gel compared to a 2D Western blot.

SpotMap addresses common challenges for characterising and validating your process and the biologic drugs it produces.

- Simple guided 4-step workflow makes the software easy to learn and quick to apply. This is advantageous when HCP characterisation is performed infrequently.
- Consistent analysis results between users regardless of expertise and between different sites.
- Unique approach to visualise analysis results and set the parameters at each step for optimal spot and blot detection.
- All parameters used, as well as high-resolution 2D gel and 2D Western blot images (analysed and unanalysed), are recorded to include in SOP documentation and reports.

Method

Two data sets were used in the analysis. One silver stained 2D gel of the fully resolved HCP profile of *Pichia pastoris* was compared to Western blot images produced using pooled anti-*P. pastoris* HCP antisera from pool 1 (5 animals) and pool 2 (4 animals). Images were captured at 1 second exposure.



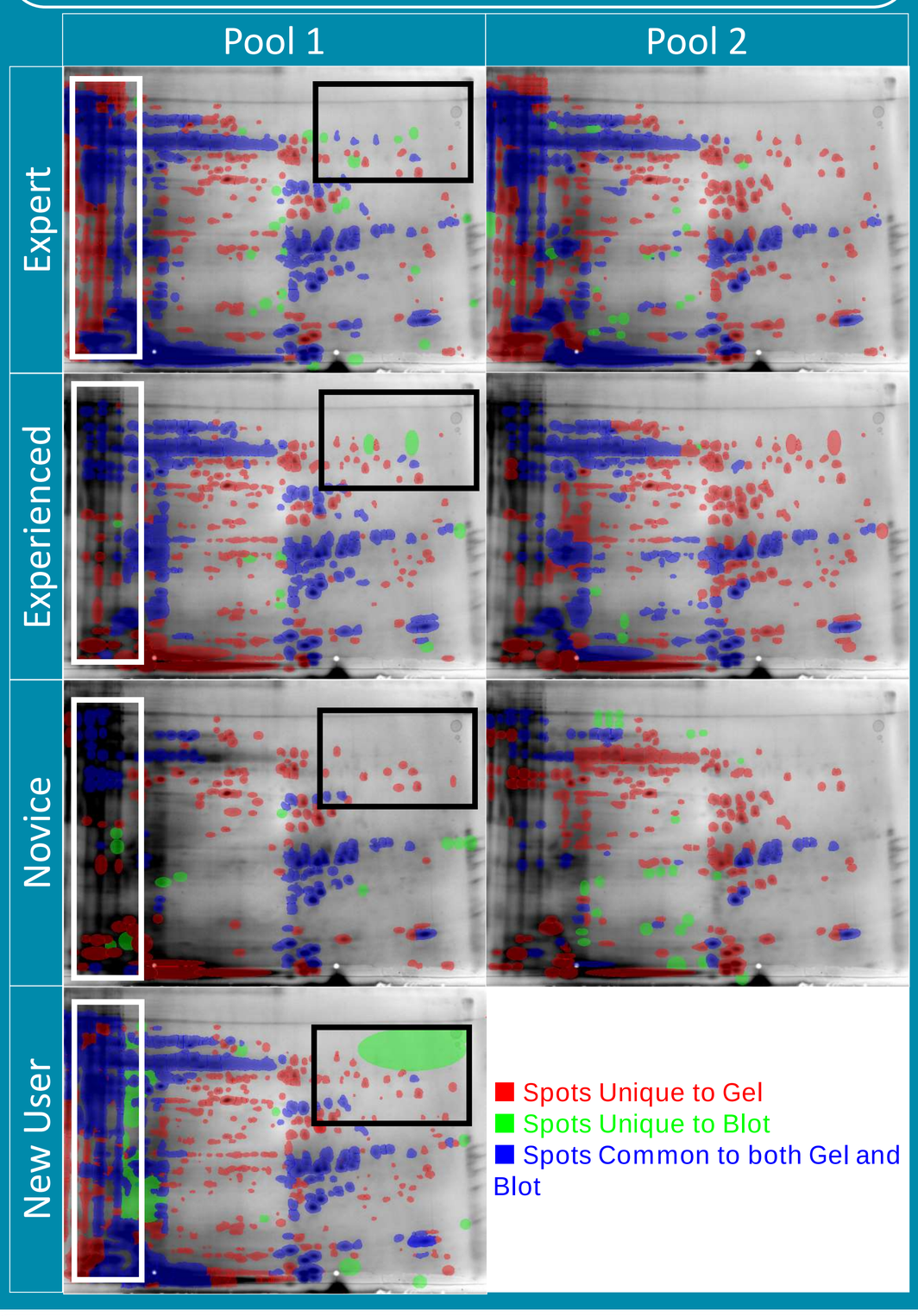
The 2D gel and Western blot images were analysed using SpotMap. **Images** (a) The images were uploaded to the software and automatically quality checked. **Alignment** (b) The blot image was aligned to the gel image to allow features common to both to be in the same coordinate space. **Spots** (c) Spots were automatically detected quickly and objectively (d) then manually refined to create a map of the proteins separated on the 2D gel. (e) This spot map was then overlaid on the blot image and presence of protein detection was set: common – blue, blot only – green, gel only – red. **Review** (f) Results screen including percentage coverage and spot numbers.

Each data set was analysed by individuals from two locations and different levels of experience using SpotMap. **Expert**: Extensive experience using SpotMap. **Experienced**: Experience using SpotMap. **Novice**: Limited experience using SpotMap. All users from TotalLab were unfamiliar with the images prior to analysis. **New User**: No prior experience of using SpotMap but experienced with the images used for analysis.

Results & Discussion

Percentage coverage results were compared between users. Despite the different experience of each user the results obtained showed variation within a range of 10% for both data sets.

Percentage coverage = total number of spots present on the blot image / total number of spots x 100



User	Location	Percentage coverage	
		Pool 1	Pool 2
Expert	TotalLab	47%	38%
Experienced	TotalLab	46%	41%
Novice	TotalLab	49%	34%
New User	Rockland Immunochemicals	39%	38%
Average percentage coverage		45%	38%
Coefficient of Variation (CV)		9.6	7.6

Spot maps created by each user from analysis of pool 1 antisera and pool 2 antisera. Spots common to both gel and blot are identified as blue, spots unique to the gel image are red and unique to blot image are green. Spot maps are presented overlaid on the gel image.

The maps visibly demonstrate significant differences between each user, however, the variation in the coverage results is low (CV = 9.6%).

Editing the spot map (White outline).

Most notably each user has edited the spot map of the left hand side of the image differently due to the challenge of identifying individual spots. Expert and New User have edited the existing detected spot pattern. Experienced and Novice users have deleted the spot pattern in this area then re-created it.

Defining a spot (Black outline).

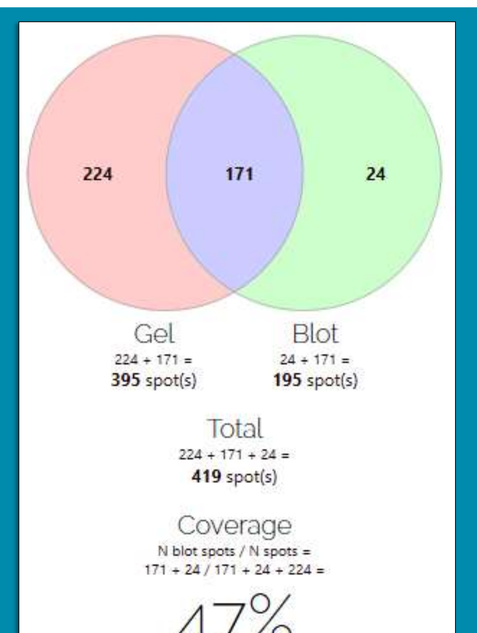
Defining a spot or blot feature was an individual decision; this can be seen most clearly in comparison of the top right corner of pool 1 analysis where the presence, size, number and shape of the spots are different between each user.

Creating a Standard Operating Procedure (SOP) for the analysis of 2D gel vs Western blot. This would further reduce the subjectivity of the analysis and variation between results.

Areas to be considered in the image analysis SOP include:

- Image cropping and resizing.
- Number and location of alignment vectors.
- Spot detection parameters used.
- Definition of a spot.
- The approach to editing the spot map, e.g. edit the detected spot pattern, or re-create a spot pattern in areas where individual spots are hard to identify.

Consideration of gel running, blotting process and image capture in the SOP. Gels and blots with well resolved proteins and high resolution image capture would provide greater sensitivity of image analysis. How, and what, data is reported downstream should also be considered. SpotMap presents coverage with the number of spots as well as recording all the parameters used and images created at each step.



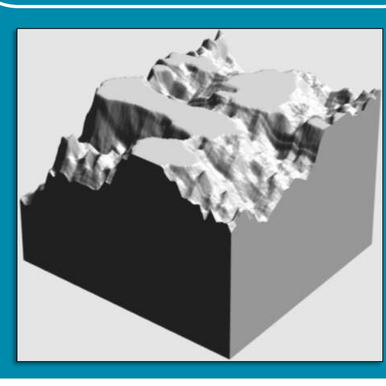
Summary of results presented by SpotMap . (Expert user, Pool 1).

Image quality checks.

The images used in this analysis triggered image quality check warnings when uploaded.

All images had an 8-bit colour depth, limiting the values of each pixel to a scale of 256 values. 16-bit images are recommended as the available scale increases to 65536 values.

The Western blot of pool 2 image was saturated in areas. This can be seen as flattened peaks when viewed in 3D view (see below). Saturation makes quantification of spots challenging, reducing the accuracy and reliability of results.



Conclusions: Comparing a 2D gel vs Western blot is part of current best practice for measuring HCP antibody reactivity. Generating reliable results from comparing a 2D gel with a Western blot requires consideration and control of three key steps. (1) the quality of images used in the analysis, (2) the image analysis itself and (3) reporting results to capture vital information. SpotMap helps to minimise and control technical variation during image analysis. The data above shows that, even without a standard operating procedure, users of varying familiarity with the data and the software can generate coverage results with less than 10% variation. The quality of your 2D gel running, Western blotting and image capture are as critical as the analysis step for reproducibility and reliability of results. Protein separation, transfer and detection should all be optimised and standardised as much as possible. When it comes to image capture then high resolution, 16-bit, grey-scale images, allow greater sensitivity and accuracy of results. Reporting is simple using SpotMap to calculate percentage coverage and spot numbers. All parameters used and results are stored as part of your analysis. These can be exported by a simple click and drag to any research or compliance documentation.

References:

[1] Rellahan, B. (2013) "Process Related Impurities and their Impact on Product Quality-An FDA Perspective and Recommendations" WCBP 2013, CASSS, Emeryville.

Acknowledgments:

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