Measuring the inter-user variation in percentage coverage of 2D gel vs 2D Western blot images using SpotMap software

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Introduction

SpotMap software has been specifically developed for analysis of HCP coverage for antibody product and process characterisation, it addresses many of the challenges faced in 2D analysis of Blot vs Gel image comparison. SpotMap enables you to produce results which are reliable and reproducible as part of a well managed process.

This report demonstrates the inter-user variation of SpotMap used by three individuals of varying experience on three data sets categorised as hard, medium and easy. Results obtained were within a range of 10% of each user, even when the most challenging images were used. Key areas of the analysis where variation may be introduced have been identified.

Method

Three data sets of a 2D gel and a Western blot were each analysed by three users. See table 1 for descriptions of the data sets. Users ranged in experience of the software from novice, experienced and expert.

Each data set was analysed using SpotMap to determine the relative coverage of the Western blot vs the 2D gel image. For each data set the percentage coverage results were compared between users.

Difficulty	Experiment	Justification for difficulty rating
Hard	Silver stained gel vs Western blot (*Plasmodium falciparum* immunoproteomic experiments)	Low quality images: 8-bit and areas of saturation. Few spots present on blot, and presence of non-spot features. Required a high amount of manual editing to obtain results.
Medium	Silver stained gel vs Western blot	Gel and Blot images significantly different sizes, Blot image was not straight, and low image quality: 8-bit. Required a high amount of manual image editing prior to analysis.
Easy	CHO Lysate Oriole™ stained gel vs anti-CHO HCP blot	No flagged image QC issues, few non-spot features, require minimal manual editing of spot pattern.

Table 1. Data sets used for analysis : Hard (1), medium(2) and easy (3)

Results

User	Data set		
	Hard	**Medium**	**Easy**
Novice	24%	52%	14%
Experienced	14%	54%	15%
Expert	18%	57%	16%
Range between users	**10%**	**5%**	**2%**
Coefficient of Variation	**27%**	**5%**	**7%**

Table 2. Percentage coverage results obtained through use of SpotMap for each data set. % Coverage = number of spots on blot / total number of spots x 100

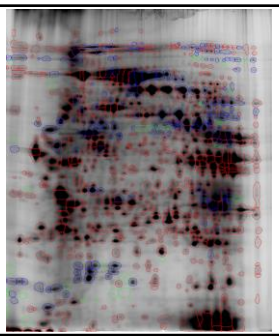
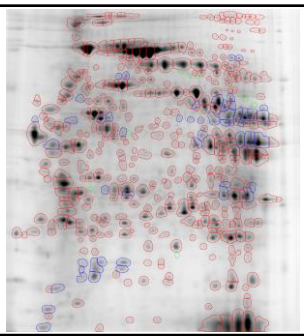
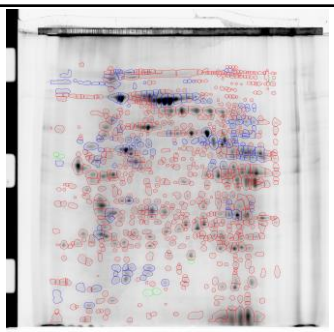
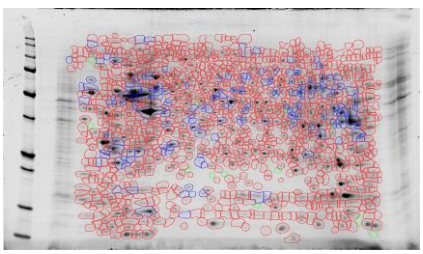
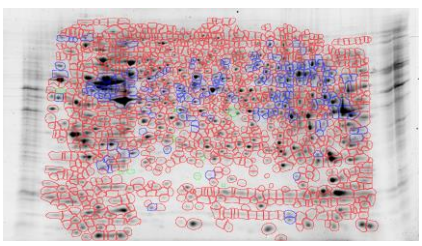
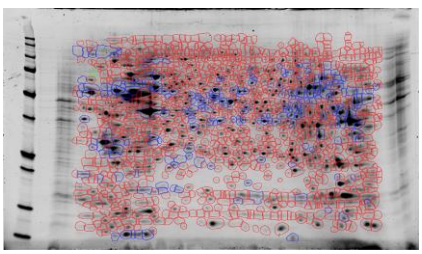
	Novice	Experienced	Expert
Difficult			
Easy			

Table 3. Difficult and Easy data sets analysed by novice, experienced and expert users. Red outlines represent spots unique to gel, Blue outlines are common to both gel and blot and Green outlines are unique to Blot. (Medium difficulty data set cannot be shown).

(1) Gathoni Kamuyu, KEMRI-Wellcome Trust Research Programme, *Plasmodium falciparum* immunoproteomic experiments, (2) Anonymised internal data set,(3) Bio-Rad, Tech note (Reliable, Streamlined 2-D Western Blot Workflow for Evaluation of Antibodies Developed for Detection of Host Cell Proteins)

Discussion

Hard

The most challenging data set delivered results within a range of 10% (14 -24% coverage). This data set showed the highest amount of variation in results due to the low quality of the images. This reduced the objectivity by increasing the requirement for subjective decisions by the user to compare the spot patterns.

Both images were flagged during the automatic image QC as 8-bit images, the gel image was flagged as saturated. There was a large amount of noise surrounding the images.

Medium

The images in the medium data set were of low quality, however a similar approach was applied to analysis by all three users, resulting in a range of 5% (52-57% coverage) between results.

The gel image was half the size of the blot image. Both images were flagged by automatic QC as having a low colour depth of 8-bit. As seen in analysis of the hard data set, use of low quality images reduces the objectivity of the software.

All three independent users edited the images prior to analysis including cropping, rotation and resizing.

Easy

The easy data set was the simplest for analysis due to the use of high quality images or large matching spot patterns. This allows the software to be used objectively for analysis of the data by reducing the subjectivity of decisions by users. Variation between users was within a range of 2% (14-16% coverage).

The images flagged no QC issues. The 16 bit images allow a significantly larger range of values to be used for each pixel in the image, compared to using 8-bit images.

A similar approach was used by all users to analyse this data set.

Quality of the images used for analysis is the largest source of variation

The quality of the images used in the analysis is the largest source of variation in the analysis. The use of high quality images increases the objectivity and reliability in your results. The automatic quality check of SpotMap assesses image quality issues, including: colour depth, saturation, image compression, dynamic range, image stretching and intensity levels. On upload of images to SpotMap you can visualise the gel and the blot side by side, this highlights any key issues with the position, size and shape of the images.

Colour Depth:
Use of 16 bit images significantly increases the objectiveness and accuracy of the software as seen in analysis of the easy images. 8-bit images, used in the hard and medium data sets, limit the value of each pixel to a scale of 0-255. 16 bit images increase the colour depth to a scale of 0-65535. Figure 3 highlights the importance of colour depth for automatic spot detection. Spots missed from the automatic detection may be added manually, however this increases the subjectivity of the analysis.

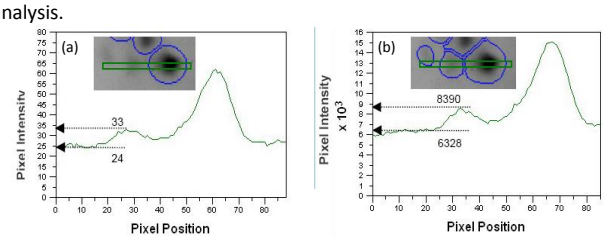


Figure 1. Automatic spot detection requires a significant difference in the intensity of the spot and the background intensity to identify spots. (a) Using 8-bit images the intensity of the spot against the background level is low, this results in the spot being missed from the automatic detections. (b) Using 16 bit images however there is a significant difference between the spot and the background, the spot detection automatically adds this spot to the map.

Saturation
Image QC in the hard data set highlighted that areas of the gel image were saturated. Saturation results in flattened peaks of spots when viewed in 3D view. The automatic spot detection cannot identify individual spots in saturated areas, reducing the accuracy. Manually splitting spots is required to identify 2 spots detected as one.

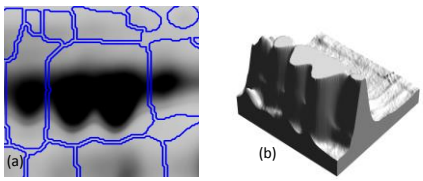


Figure 2. (a) Automatically detected spots on gel image hard dataset, 2 spots are seen visibly present, however 1 spot was detected due to saturation. (b) 3D view of saturated area, peaks of spots are flattened therefore the software cannot objectively distinguish presence of one spot or more.

Areas of SpotMap analysis to consider:

Individual decisions are required in the creation of the spot map and identification of presence or absence of spots on the blot image. Variation in these decisions will affect the coverage result. Using low quality images increases the subjectivity of the analysis, high quality images allow a more objective approach. Despite the subjective decisions required by analysis of hard images the variation between the three users was within a range of 10%.

Conclusion

Typically with 2D gels and Western blots the biggest source of variation is the image quality. The automatic image QC flagged two of the data analysis results as being potentially imprecise due to the use of 8-bit images. Smearing/steaking, saturation and presence of non-spot features also affects analysis precision.

There are areas of the analysis that require particular consideration to improve objectivity and precision. Within the software, image editing, alignment vectors added, spot detection parameters used and individual decisions on the presence of a spot will all impact the results.

However, SpotMap v2.0 gives reproducible and reliable results within a range of 10%, even using challenging images. The use of high quality images which enable a consistent analysis approach between users will reduce the variation, as seen in the easy data set where results are within a range of 2%.

Production of an SOP that addresses all aspects of the analysis, including optimised gel running and image capture would increase the objectivity of the results, reducing the subjective decisions required during analysis.