Removal of positional variation introduced during the experimental procedure to accurately compare 2D gel and Western blot using SpotMap software.

TOTALLAB Technical Note

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ABSTRACT

Positional variation in the location of protein spots is commonly introduced during 2D gel electrophoresis, Western blotting or image capture procedures, leading to challenges in direct comparison of 2D gels to Western blots. Alignment is a key feature within SpotMap image analysis software which allows removal of this positional variation, providing easy comparison of spot patterns between images. Alignment of Western blots to 2D gels has additional challenges in situations where few spots are immunodetected.

Presented is the comparison of two analyses of the same images, one analysis was completed by direct alignment of the Western blot to the 2D gel, the other completed by indirect alignment – using a stained membrane to assist the alignment. In both analyses the percentage coverage is 14% of the 2D gel by the Western blot.

INTRODUCTION

Why is comparison of 2D gels and Western blots important?

Development of biologic drug products has increased rapidly in recent years, in 2016 the FDA approved 13 new biologic licence applications [1]. The development of biologics brings with it a need to monitor impurities. Host cell proteins (HCPs) are amongst the impurities which must be monitored and reduced to the lowest possible levels within the final product to ensure the safety, purity and potency of the product [2-4]. Monitoring HCPs throughout production requires development of process specific assays with characterisation of the antigen and antibody. Orthogonal methods including comparison of 2D SDS-PAGE and Western blots are commonly used for antibody characterisation [5-6].

SpotMap

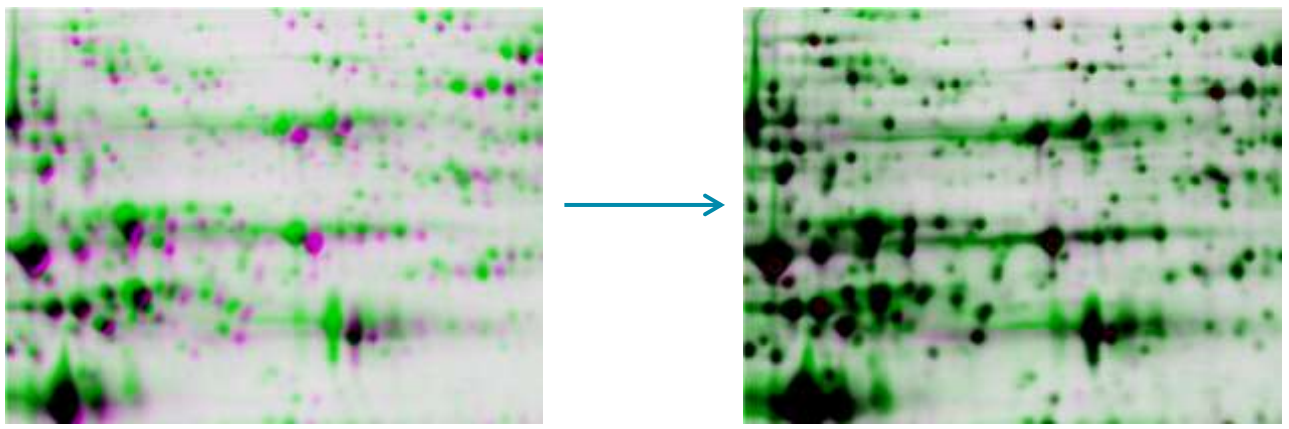
SpotMap image analysis software has a flexible workflow which allows accurate comparison of 2D gels and Western blots. Alignment is a key feature of SpotMap which removes positional variation introduced through the gel running, Western blotting and image capture processes. It places common spots in the same coordinate space for analysis, the single spot map created of the project allows direct comparison of spots present or absent on each image to identify common or unique spots and measure percentage coverage.

Challenges

When there are a low number of spots present on the Western blot, direct alignment of the images can be challenging. In these situations, the images can be aligned by an indirect method: using an external alignment target such as the stained membrane. The stained membrane is an ideal alignment target for both gels and Western blots. The spot pattern of the 2D gel and the stained membrane identifying those which were transferred will be very similar, if not the same. The spots of the Western blot will be physically in the same location as those on the stained membrane however they may not be in the same location on the image due to positional variation in the image capture processes.

Other Solutions

Other solutions to remove positional variation can include the use of Differential in Gel Electrophoresis (DIGE) techniques. Electrophoresis and transfer of a labelled sample, followed by Western blotting with a differently labelled sample would allow direct comparison of the transferred protein to the immunodetected spots without a need for alignment. SpotMap can be used to analyse images which have been detected using any stain including CyDyes for DIGE.



Alignment removes positional variation by overlaying images and identifying features common to both. Vectors are used to position common features in the same coordinate space, allowing direct comparison of spot patterns

This report demonstrates the comparison of an Oriole™ fluorescent stained 2D gel of Chinese hamster ovary (CHO) HCP with a Western blot of generic anti-CHO HCP antibodies using SpotMap. One analysis was completed by direct alignment of the Western blot to the 2D gel, the other analysis was completed using an image of the PVDF membrane stained by SYPRO Ruby prior to immunodetection as an external alignment target. All other steps of the analysis were completed in the same way. The percentage coverage results between the analyses were compared.

METHOD

Images

Images [7] of 2D gel electrophoresis separated CHO lysate and a Western blot of anti-CHO antibodies were compared to measure anti-HCP antibody coverage. An additional image of the PVDF blot membrane stained using SYPRO ruby was used for indirect alignment of the 2D gel and Western blot (figure 1).

Images were uploaded in to SpotMap, they were then aligned prior to starting the analysis.

Figure 1. Original (a) 2D gel, (b) Western blot and (c) stained membrane images.

Alignment

Alignment of the 2D gel and the Western blot was completed in one of two ways: a) by direct alignment of the Western blot to the 2D gel, or b) indirect alignment of the 2D gel and Western blot to the stained PVDF membrane.

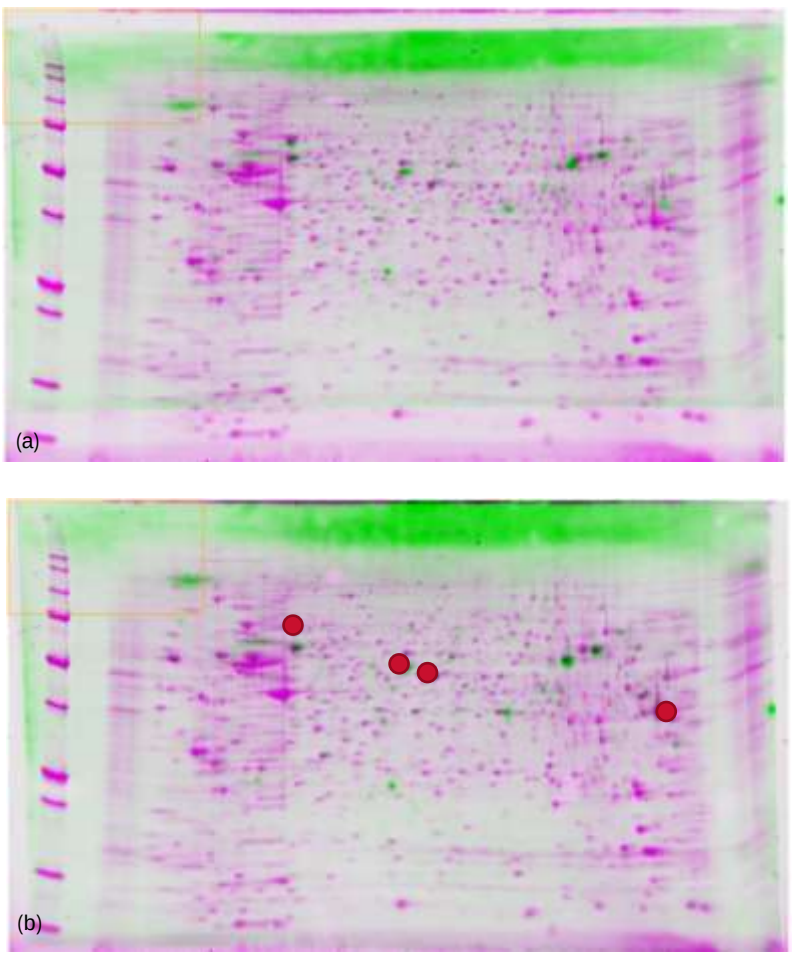


Figure 2. Overlaid images of the 2D gel (magenta) and the Western blot (green), (a) pre alignment, (b) post alignment, red dots indicate the location of manual vectors added.

a) Direct Alignment

The 2D gel was used as the alignment target. Manual vectors were added to directly align the Western blot to the 2D gel, see figure 2 (b).

b) Indirect Alignment

The stained PVDF membrane was used as the alignment target., it was added to the project within the alignment screen by selecting the "Browse for external image..." option. The benefits of using a stained membrane as the alignment target are discussed below. The 2D gel was aligned to the stained membrane using automatic alignment (figure 3). The Western blot was aligned to the stained membrane by manual vectors only (figure 4). By aligning each image to the stained membrane they are therefore indirectly aligned to one another. The stained membrane was not used further in the analysis.

Alignment Continued

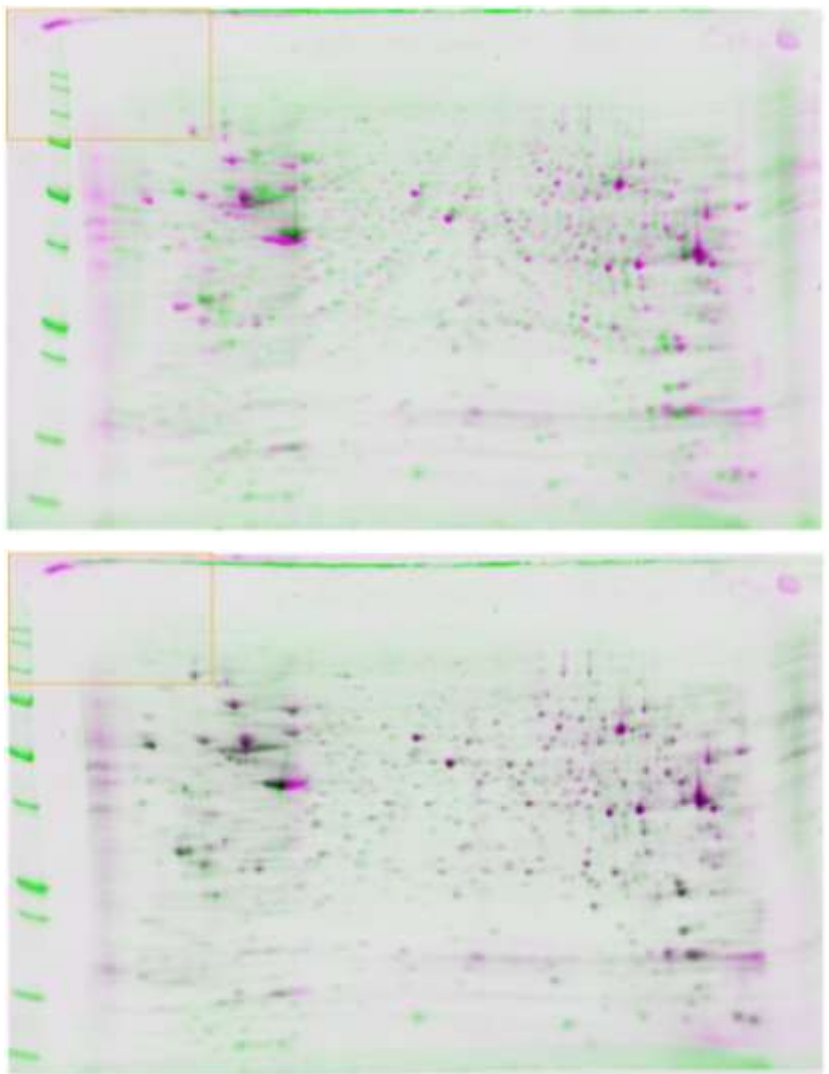


Figure 3. Overlaid images of the stained membrane (magenta) and the 2D gel (green), (a) pre alignment, (b) post alignment. No manual vectors were added, only automatic alignment was used.

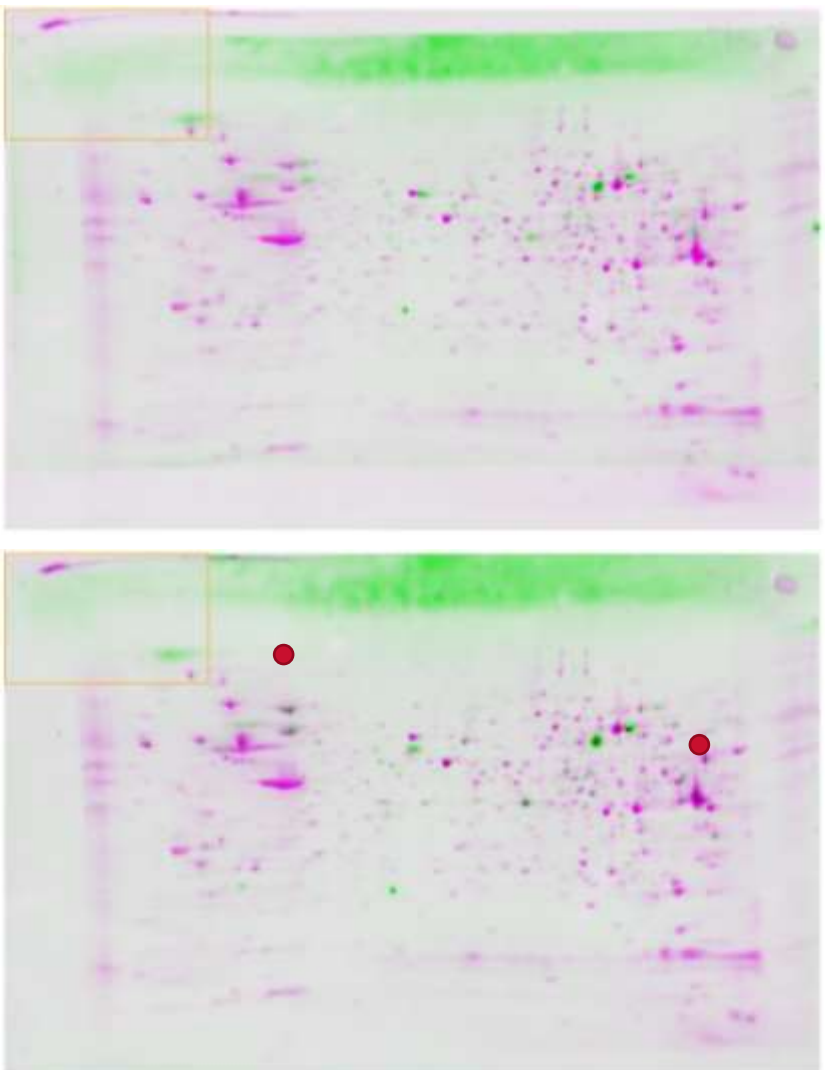


Figure 4. Overlaid images of the stained membrane (magenta) and the Western blot (green), (a) pre alignment, (b) post alignment, red dots indicate the location of manual vectors added.

Analysis

A single spot map was created to identify the location of all spots in the project. Both images were overlaid by the spot map and each spot outline was characterised as present or absent on each image.

The initial spot map was detected on the gel image using the parameters of Smoothing = 1 and Peak sensitivity = 0 within a rectangular region of interest. The map was manually refined to remove edge effects and correct any errors using the available tools.

Spots on the Western blot were filtered to identify any outline with a peak height less than 3500 and set as absent from the map. This was manually checked with the aid of 3D view and changing the contrast of the images. Any spots which were added to the Western blot were done so with the new spot assignment settings as "Present on only the active image".

Results

With the gel set as the base image, percentage coverage and number of spots common (present on both images), additional (present only on the Western blot) and missing (present only on the 2D gel) are updated in real-time throughout the analysis.

$$Percentage\ coverage\ = \frac{Common + Additional\ spots}{Common + Additional + Missing\ Spots} \times 100$$

The results screen presents the top line results of the analysis. Data Access allows easy export of all data from the project into reports and presentations.

Results

Relative coverage of the 2D gel by the Western blot following direct alignment was 14%. A total of 1273 spots were identified: 170 were common to both the 2D gel and Western blot, 1099 present only on the 2D gel and 4 present only on the Western blot, see Figure 5 and Table 1.

Following indirect alignment, 14% relative coverage of the 2D gel by the Western blot was recorded. A total of 1283 spots were identified: 174 were present on both the 2D gel and Western blot, 1108 present only on the 2D gel and 1 present only on the Western blot, see Figure 6 and Table 1.

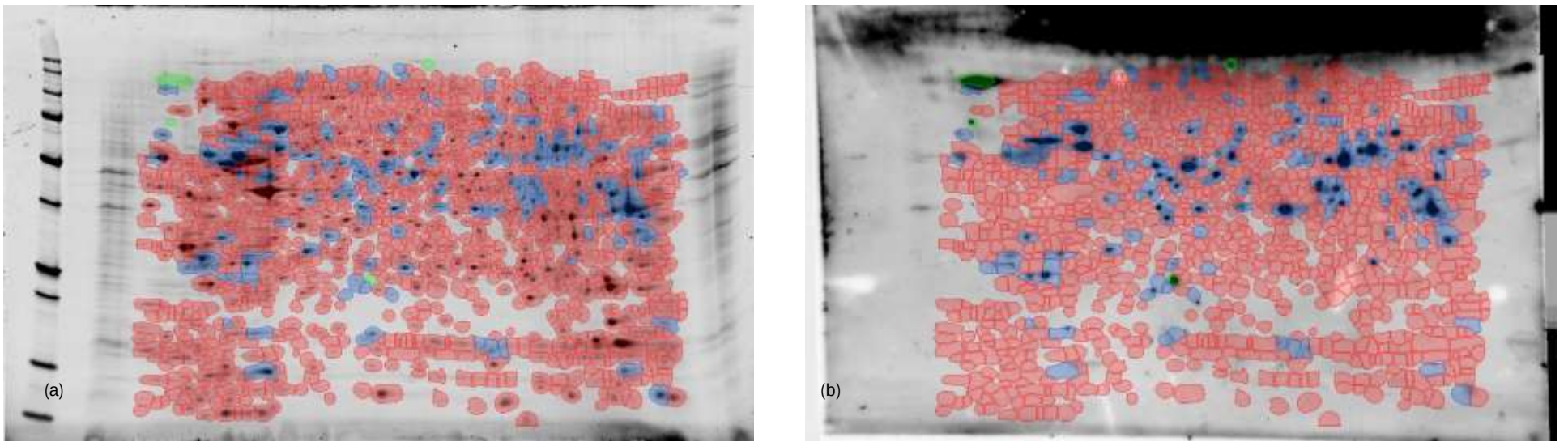


Figure 5. Colour coded spot map of results obtained by direct alignment of the Western blot to the 2D gel, overlaid on the (a) 2D gel and (b) Western blot. Blue spots are common to both the 2D gel and Western blot, red are unique to the 2D gel and green spots are unique to the Western blot.

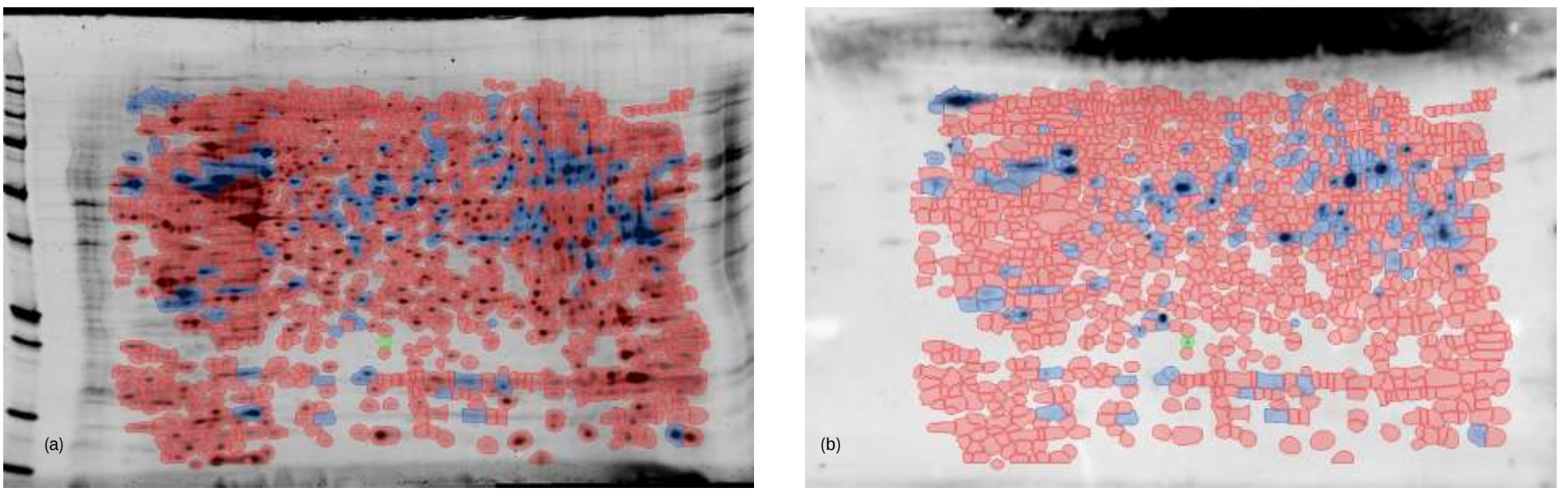


Figure 6. Colour coded spot map of results obtained by indirect alignment, overlaid on the (a) 2D gel and (b) Western blot. Blue spots are common to 2D gel and Western blot, red are unique to the 2D gel and green spots are unique to the Western blot.

Table 1. Comparison of results obtained using an external alignment image and by direct alignment of the Western blot to the 2D gel.

	Direct alignment of the 2D gel and Western blot.	Indirect alignment of the 2D gel and Western blot.
% coverage	14%	14%
Number of common spots	170	174
Number of spots unique to Gel	1099	1108
Number of spots unique to Blot	4	1
Total number of spots	1273	1283

DISCUSSION

Alignment is a key feature of SpotMap which addresses the challenge of comparing the different spot patterns commonly seen between 2D gels and Western blots. Direct alignment of 2D gels and Western blots can be challenging due to comparatively few spots present on the Western blot or the potential for spots to bloom due to the detection method. Use of an external image of the stained blot membrane as the alignment target can minimise this challenge in some cases.

The key benefit of using an external alignment target is to simplify the alignment process. The stained membrane is the ideal alignment target for both the 2D gel and Western blot. The spot pattern of both the membrane and 2D gel will be very similar as the stained membrane will show all protein spots which were successfully transferred from the gel in the same pattern. The Western blot identifies only those spots which were recognised by the antibodies, which can be easily matched back to the membrane as the only variation in spot position is introduced at the image capture process only.

The stained membrane, as well as being used as the alignment target, could also be used within the analysis. Including the stained membrane at the start of the analysis would allow it to be used to determine transfer efficiency from the 2D gel or used as the base to calculate percentage coverage based upon only those proteins which were transferred.

The use of an external alignment target is not a requirement to complete the analysis. As seen above, results obtained from direct or indirect alignment give the same percentage coverage values. Previous analysis of these images by three users, using the direct alignment method, showed percentage coverage results within a range of 2% (results of 14%, 15% and 16%) [8].

Although the percentage coverage results are the same, there is a small amount of variation in the number of spots. An additional 10 spots were identified (+4 common, +9 missing and -3 additional) on the spot map of the indirect alignment compared to direct alignment spot map. Minor differences in the shape of spots due to image warping following alignment and differences in the size and location of the regions of interest when detecting spots resulted in the different numbers of spots detected following automatic spot detection. 1455 spots following automatic detection on the directly aligned image compared to 1558 spots following indirect alignment.

CONCLUSIONS

Comparisons of 2D gels and Western blots can be challenging. Using SpotMap software for image analysis can help to make the analysis simpler. The use of additional images to be used as the alignment target can simplify the alignment process in some cases, however, it is not a necessity to complete the analysis. Direct and indirect alignment provide the same percentage coverage result. Total spot numbers varied between the two projects due to the different alignment processes. When completing an analysis, the approach used should be similar or the same each time a new analysis is used, either direct alignment or use of an external image as the alignment target.

REFERENCES

[1] FDA (2016) 2016 Biological License Application Approvals. US Food and Drug Administration. Available at: http://www.fda.gov/BiologicsBloodVaccines/DevelopmentApprovalProcess/BiologicalApprovalsbyYear/ucm482397.htm [last accessed 07/02/2017]

[2] ICH (2009) ICH Q8 Pharmaceutical Development. Available at: http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Quality/Q8_R1/Step4/Q8_R2_Guideline.pdf

[3] ICH (1999) ICH Q6B Specifications: Test Procedures and Acceptance Criteria for Biotechnological/Biological products. Available: http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Quality/Q6B/Step4/Q6B_Guideline.pdf

[4] 42 USC 262- Regulation of biological products: Available: http://www.fda.gov/RegulatoryInformation/Legislation/ucm149278.htm

[5] Pharmeuropa 2.6.34 Host cell protein assays. Draft available at: http://www.e-gmp.pl/wp-content/uploads/2015/05/2.6.34.-Host-cell-protein-assays-draft.pdf

[6] Graham, L. (2015) Risk Assessments for Host Cell Protein Control Strategies: CDER Experiences. http://c.ymcdn.com/sites/casss.site-ym.com/resource/resmgr/CMC_No_Am_Jan_Spkr_Slds/2015_CMCJ_GrahamLaurie.pdf

[7] Berkelman, T., Harbers, A. and Bandhakavi, S. (2013) Reliable, Streamlined 2-D Western Blot Workflow for Evaluation of Antibodies Developed for Detection of Host Cell Proteins – Tech Note. Bio-Rad Laboratories, USA. (Images provided by Bio-Rad and previously used in cited Tech Note).

[8] Parkin, K (2015) Measuring the inter-user variation in percentage coverage of 2D gel vs 2D Western blot images using SpotMap software – Technical note. TotalLab Ltd., UK . Available: http://totallab.com/cms/wp-content/uploads/2015/12/SpotMap-Inter-User-Variation.pdf