

Application Note

Comparing Western Blot and 2D Gel images to characterise proteins using Progenesis SameSpots

Overview:

- Secondary staining, including Western Blotting, of 2D gels gives you the power to characterise statistically significant protein expression differences in your sample and validate discoveries¹⁻⁴
- Cell free extract from *E.Coli* P2 over-expressing a His-tagged protein was separated on duplicate 2D gels
- One was stained with Colloidal Coomassie Blue while the other was Western Blotted using Mouse Monoclonal Anti-polyHistidine primary antibody before being Coomassie stained for protein
- Analysis of 2D gel images and Western Blot image by Progenesis SameSpots enabled direct comparison of the different spot patterns, confirming the presence and location of the His-tagged protein
- In this way quantification by 2DE and simultaneous, or subsequent, characterisation using common secondary staining techniques can be performed with the same software

Introduction:

2DE is a common technique for quantifying differentially expressed proteins in discovery-type proteomics experiments. Confirming the identity of these proteins is often performed at the same time. This validation can be required to obtain resource for more extensive studies or to publish results. Western Blotting can be applied to characterise proteins separated by 2D gels¹⁻⁴. However, antibodies show >10-100-fold higher sensitivity⁴ and they produce images with far fewer points of reference. This makes it a challenge to confirm the presence and location of the corresponding spot on a 2D gel image. **Progenesis SameSpots** has a unique alignment based analysis approach that allows you to **directly compare and quantify** the same spot in **Western Blot and 2D gel images**.

Results:

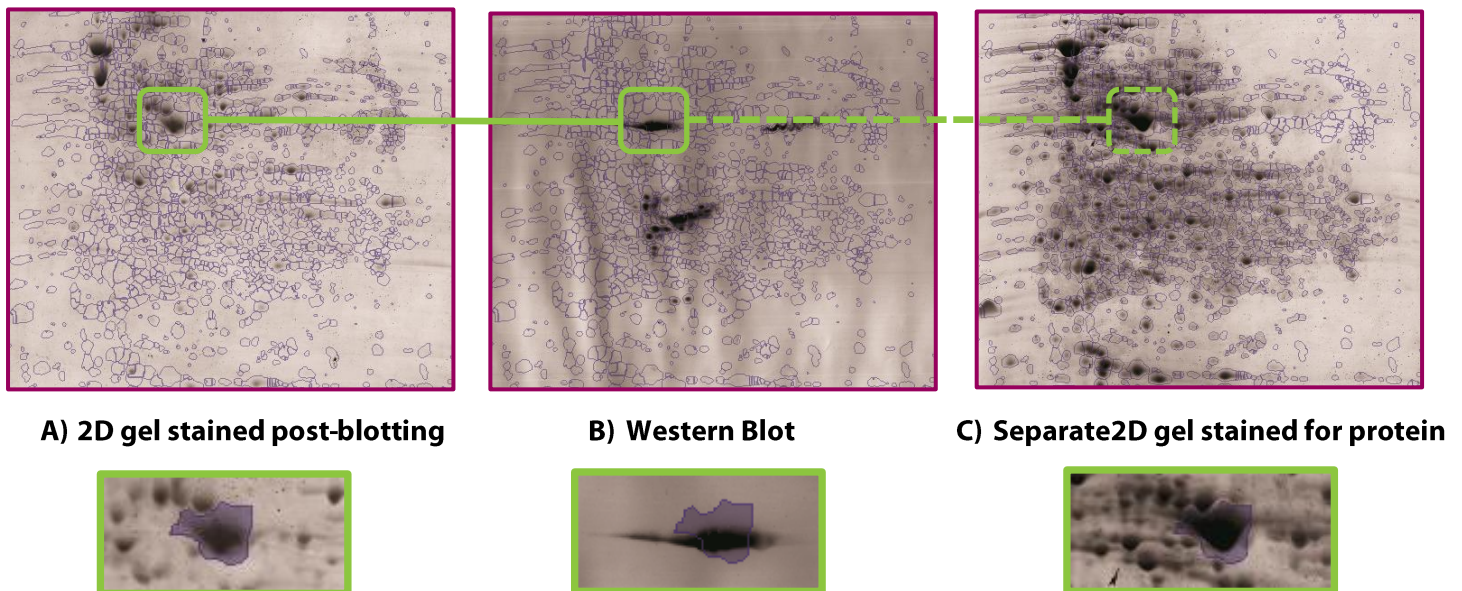
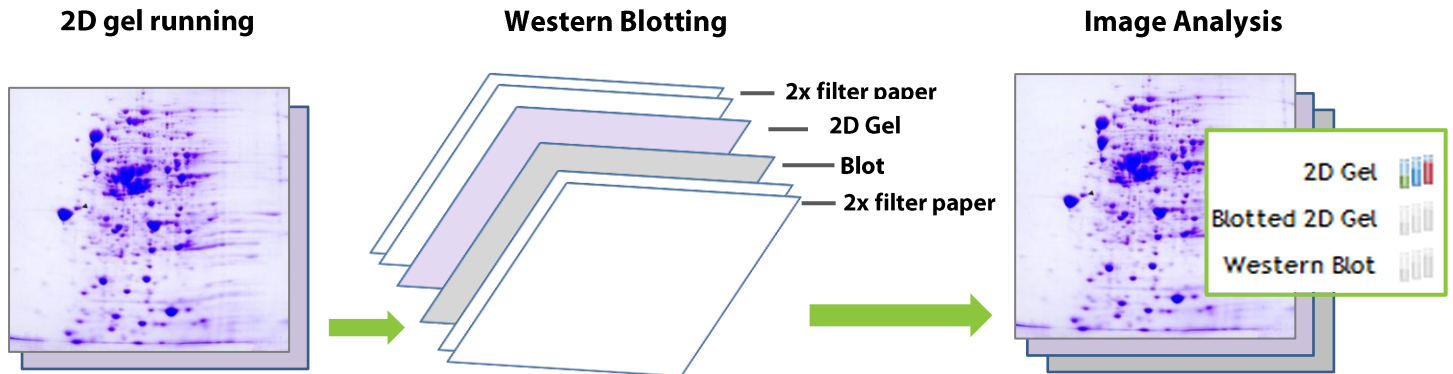


Figure 1: Comparing spot patterns generated by aligning and detecting two duplicate 2D gels with a Western Blot. Circles and a zoomed-in view from each image show the His-tagged protein of interest. (A) 2D gel run and Colloidal Coomassie Blue stained AFTER blotting to check effective protein transfer **(B)** Western Blot of 2D gel A **(C)** Replicate 2D gel run at the same time and under same conditions as 2D gel A). This was Coomassie stained first to show protein separation had worked. This replicate 2D gel image was used as the Reference Image that facilitated alignment and same spot detection of 2D gels vs. Western Blot. **Dotted green line** represents the fact that the blot image was aligned to the 2D gel stained post-blotting, which was in turn aligned to the duplicate gel stained but not blotted. This multiplex configuration provided the best results for comparative image analysis. 2DE and blotting methods are detailed overleaf.

Analysis Approach

- Duplicate 2D gels were run under identical conditions
- One Coomassie Blue stained to check separation worked
- One unstained for blotting
- Unstained 2D gel was blotted, probed using 1° & 2° antibodies and .tiff image generated
- Blotted 2D gel was Coomassie stained to check how effective protein transfer had been
- 2D gel and Western Blot .tiff images imported into **Progenesis SameSpots**
- Analysed as “multiplex image without internal standards” experiment
- Unblotted 2D gel used as Reference Image
- Align, detect and compare spot patterns



2DE and blotting methods are detailed below. For instructions on how to set up analysis of multiple images from the same, or duplicate, 2D gels look at Progenesis SameSpots “How To” documents on <http://support.nonlinear.com/kbase>

Conclusions:

Western blotting gives you the power to characterise proteins showing statistically significant differences. It can be performed using duplicate 2D gels, one which is stained and one blotted then stained, or the more expensive method of staining, de-staining and blotting the same 2D gel. In either case you must compare images that are different in terms of the number and intensity of spots visualised. You must also compensate for technical variation in replicate 2D gel and blots generated. Progenesis SameSpots has a unique alignment based analysis approach that allows you to directly compare and quantitate the same spot in replicate Western Blots and protein stained 2D gel images. **This type of validation can be easily added to your results** using Progenesis SameSpots.

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2DE & Western Blotting Methods:

2DE: *E. Coli* P2 (ZP_02929368) was expressed in 50ml, 20°C overnight. Cell free extract was generated by sonication and protein mixture was resuspended in 350ul rehydration solution prepared for IEF (7uL of IPG buffer, 1.4mg of DTT in each 350ul). pH 3-10 IPG strips were rehydrated overnight and proteins were separated for 8hs in IEF. IPG strips were equilibrated before subjecting to second dimension separation by 12% SDS-PAGE, visualised using Colloidal Coomassie Blue stain.

Western Blot: Soak SDS-PAGE gel in transfer buffer for 15min. Assemble blotting sandwich. Bottom to top: 2 layers filter paper; 1 layer nitrocellulose membrane; 2D gel; 2 layers of filter paper. Wet filter paper with transfer buffer. Transfer protein at 0.857mA/cm² for 60min. Block non-specific binding sites by incubating overnight in 5% w/v Marvel in PBS-Tween buffer (1g Marvel to 20ml). Discard solution and incubate for 2-3 hours in 1° antibody solution, Monoclonal anti-polyHistidine, Clone HIS-1 (Sigma) diluted in 5% w/v Marvel in PBS-Tween 1:1000. Wash with 5 changes of PBS-Tween, leaving the buffer on the blot for 5-10min. Incubate for 2-3 hours in 2° antibody solution, A1293-AlkPhos APA Mouse Fab ads HlgG, diluted in 5% w/v Marvel in PBS-Tween 1:1000. Wash with 5 changes of PBS-Tween. Develop with BCIP/NBT reagents @37°C. Wash with MilliQ water and dry the membrane for scanning and analysis.

References:

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