Western Blot Assessment of Polyclonal Anti-Host Cell Protein Antibody Production

Tobey Gooding[1], Murty Chengalvala[2], Patrick Dunlap[1], Jeff Patrick[1] and Shiqian Zhu[1]
[1]Covance BioCMC Solutions, Greenfield, IN; [2]Covance Research Products Inc., Denver, PA

Abstract
The most critical part of developing an ELISA for measuring Host Cell Protein (HCP) impurities in Biotherapeutics is generation of specific antibodies with appropriate recognition of the total population of HCPs potentially present in the product. The generation of the antiserum is dependent upon the sum of the individual biological responses (i.e., antibodies) of multiple animals to the antigen. Response is monitored throughout the program qualitatively by 1D (one dimension) Western Blot and adjustments are made to the antigen (immunogen) as needed to yield the broadest antigen recognition. Ultimately, the highest quality antisera from multiple animals are pooled. The quality of the final product is demonstrated through evaluation of coverage which is typically by 2D (two dimension) Western Blot or more recently by mass spectrometry. The BioCMC Group at Covance was tasked with development of a process capable of supporting this type of assessment. This capability will be illustrated through discussion of a typical antibody reagent production program.

Background
Western Blot (WB) is a technique used to visualize antibody binding to target proteins. Multiple loads of antigen are separated by gel electrophoresis. For 1D testing, one set of loads is cut from the gel and stained to visualize total protein detected. The remaining replicate loads are transferred to a membrane for immunoblotting. For 2D testing, the antigen is separated in replicate gels with one gel being stained and the others being transferred to a polyvinylidine (PVDF) membrane. The membrane is blocked, then probed with antisera or purified antibody product. After washing, the membrane is re-probed with an appropriate anti-species antibody conjugated to a fluorophore and then washed to prepare for imaging.

Table 1. Equipment and Software

Gels	Bio-Rad Criterion™ 10% Tris-HCl
Imaging System	Bio-Rad ChemiDoc™ MP
Isoelectric Focusing	Protean i12 IEF System, Bio-Rad
Imaging Software (1D)	Image Lab, Bio-Rad
Imaging Software (2D)	SpotMap, TotalLab

Pre-Study Considerations
Western Blot (WB) is a technique used to visualize antibody binding to target proteins. Multiple loads of antigen are separated by gel electrophoresis. For 1D testing, one set of loads is cut from the gel and stained to visualize total protein detected. The remaining replicate loads are transferred to a membrane for immunoblotting. For 2D testing, the antigen is separated in replicate gels with one gel being stained and the others being transferred to a polyvinylidine (PVDF) membrane. The membrane is blocked, then probed with antisera or purified antibody product. After washing, the membrane is re-probed with an appropriate anti-species antibody conjugated to a fluorophore and then washed to prepare for imaging.

Presented at BEBPA Host Cell Protein Conference 2019

Titer and 1D Western Blot Assessments
Animals continue to be monitored throughout the program through ELISA titer response as well as 1D WB. Adjustments to immunization paradigm are made as needed. For example:
- Immunogen may be fractionated by size or affinity purified to enrich for less abundant or less immunogenic proteins in the immunogen.
- May adjust dosage or adjuvant used.
- Multiple loads of the HCP antigen are loaded as paired lanes with Protein Standards.
- Comparison is made between stained gel illustrating all detectable proteins in gel vs bands visualized in WB (e.g., Fluorescence). See Figure 1 for example images.

See Table 2 for examples of titer data and Figure 1 for example WB data.

Table 2. Example Titer Data (EC50) for Four Rabbits on Immunization Schedule

Animal ID	Day 0	Day 30	Day 72	Day 114
Rabbit 1	39	16,400	15,300	40,500
Rabbit 2	13	8,660	15,600	22,900
Rabbit 3	34	6,650	5,540	16,900
Rabbit 4	12	15,600	8,420	12,800

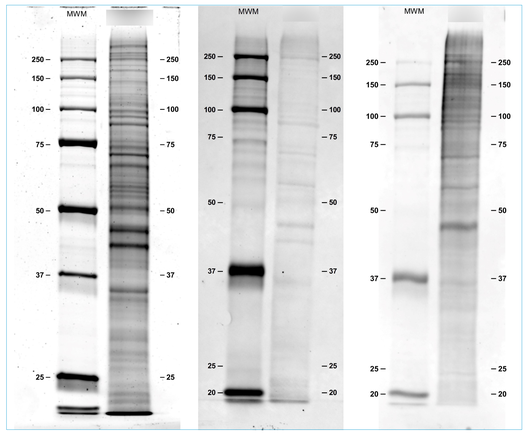

Figure 1. Sypro ruby-stained 1D gel (left) with WB of antiserum in early stages of a program (center) and in middle to late stages of a program (right).

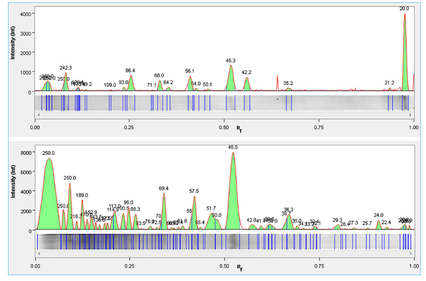

Figure 2. Changes in protein band recognition between stages of program illustrated by densitometry (top image corresponds to center image from Figure 1, while the bottom image corresponds to the right image from Figure 1).

Final Antibody Product and 2D Western
Antisera from each animal throughout the program are evaluated. The following may be factors that influence the final antibody product (including which antisera are pooled):
- Overall titer based on animal as well as stage of production
- 1D WB response, i.e. coverage at various stages of the program
- Cost

Once the pool is established, it's advisable to assess a test pool by 2D WB prior to blending antisera for purification:
- Replicate 2D gel separations are performed
- Multiple images are made at various exposure levels to ensure maximal resolution across the range of signal given off by detected proteins

Shorter exposure levels are required to discern details in recognition for proteins that are highly abundant/immunoreactive, while longer exposures are required for faint protein detection (Figures 3-5). In addition to being a shorter exposure, Figure 5 also illustrates a completed spot map.

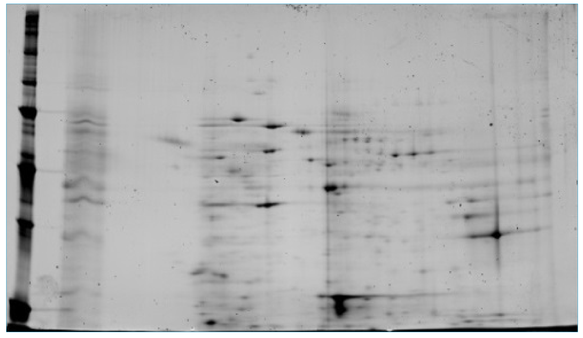

Figure 3. Example image of Sypro ruby-stained 1D gel showing total protein detected.

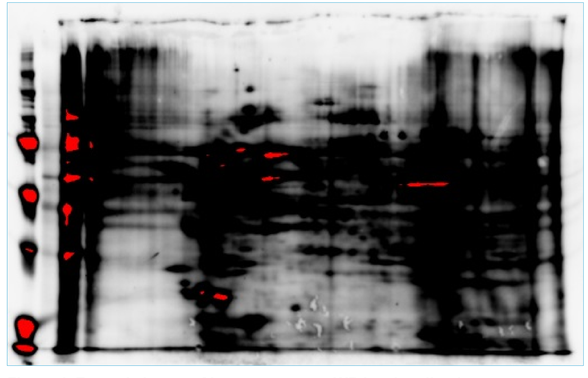

Figure 4. Example image of longer exposure WB by chemiluminescence illustrating detection of less abundant/immunoreactive host cell proteins.

The images obtained are then loaded into the SpotMap Software. These images are aligned and spots are detected and matched. See Table 3 for SpotMap results.

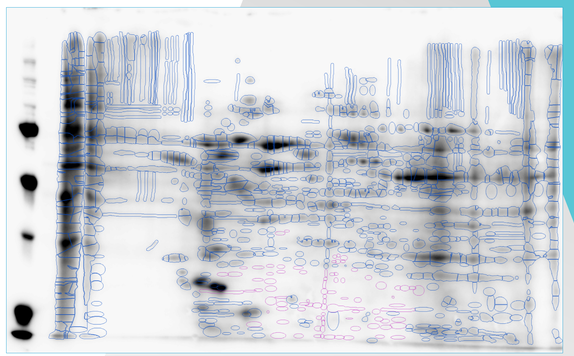

Figure 5. Example image of WB of near-final antiserum preparation following analysis with SpotMap Software.

Table 3. Example Coverage Results from Spot Map of 2D Western Blot

Total Coverage	91%
Total Number of Spots	1015
Spots in Common (Recognized by Antibodies)	922

Beyond the Final Antibody Product
With proper planning, the final product is an antibody preparation that not only demonstrates broad recognition of proteins present in the immunogen, but yields response that aligns with HCPs present in production. At this stage the product antibodies are suitable to use for ELISA development. Covance BioCMC can deliver a basic/functional assay, a fully validated assay or anything in between with capability to support routine testing upon completion.

Conclusions
- Covance BioCMC Solutions has developed a streamlined process to deliver quality Western Blot images for assessment of antibody product quality attributes.
- In partnering with CRP, antibody production is able to be fully supported within Covance. This offering is a full-service solution for generation of not only anti-HCP antibodies but also fully validated assays support.

Acknowledgements
The authors would like to acknowledge the following persons: Sian Estdale[1] Bryant Wulfkuhle[1], Derrick Gish[1], Michael Lazorchak[1], Erin Kunkel[1], Jim Mellon[1], Steve Freeby[2] and Michael Hutchins[3]
[1]Covance Inc.; [2]Bio-Rad, Hercules, CA, [3]TotalLab, Newcastle upon Tyne, UK

Additional Information available upon request.

TOTALLAB

COVANCE
SOLUTIONS MADE REAL®