

Chapter 13

Quantitative Computerized Western Blotting

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Summary

Western blotting allows analysis of antibody reactivity against multiple antigens separated according to their molecular weights. The distinction between immune dominant and recessive antigens is often difficult and carried out by qualitative or empirical means. Quantitative computerized western blotting (QCWB) addresses this difficulty by analyzing reactivity to specific antigens and providing a statistically measurable value for each band. This allows differentiation between immunodominant and immunorecessive determinants. QCWB is appropriate for either single time point analysis or longitudinal studies where multiple time points are evaluated and the reactivities against individual bands compared. This technique can be used to study humoral responses to complex antigenic mixtures such as allergens and infectious agents, or to identify serologic markers for early diagnosis of cancer, autoimmune, or infectious diseases, or to monitor patient's clinical status.

Key words: Western blot, Quantitative computerized western blot, Immunodominant antigen, SDS-PAGE, Net intensity, Total lane intensity

1. Introduction

Western blotting is a highly sensitive technique for the detection of proteins or monitoring the presence of antibodies that react with discrete antigens in complex mixtures (1). It can be employed simultaneously against a large number of molecules to test reactivity with a panel of potential antigens (2, 3). The high sensitivity and specificity of this technique makes western blotting suitable for the detection of low concentrations of particular proteins and allows the identification of specific circulating antibodies to allergens, pathogens, tumor, and autoantigens in the sera of humans and animals. Early and sensitive disease diagnosis is a crucial factor in the effective and successful treatment and

management of various maladies or illnesses. Early diagnosis prior to the development of clinical symptoms, detection of infection in immunosuppressed patients, and the identification of asymptomatic or subclinical carriers are major diagnostic challenges which often require very sensitive and specific assays (4, 5).

Many serologic techniques used for diagnosis are based on reactivity against the whole pathogen, allergen, or tumor. These assays are commonly adjusted to detect high antibody levels, whereas early, subclinical or latent infections and relapses, or diseases are often characterized by low antibody titers against only a few specific antigens in the whole mixture (5). Western blotting allows complex mixtures to be separated into individual components based on molecular weight and posttranslational modification(s), permitting the detection of even low levels of antibody reactivity with discrete antigens present in the mixture. In general, antibody reactions against multiple components are observed when patient serum reactivity is evaluated against complex antigens by western blotting. Often bands that react strongly showing high intensities in a higher percentage of patients are termed immunodominant antigens, whereas other bands that react weakly or moderately in the same or a lower percentage of subjects are termed immunorecessive antigens (6). The distinction between these band definitions is often difficult and frequently done empirically based on single measurements or an overall qualitative impression. Quantitative Computerized Western Blot (QCWB) analysis addresses this difficulty thus allowing for numerical documentation and statistical analysis of reactivity to specific antigenic bands.

Specific immunodominant antigens identified by this method could be targets for developing rapid serodiagnostic tests, as well as effective vaccines. Two parameters can be analyzed using QCWB: first, net band intensity (NBI) that allows the evaluation of antibody reactivity to individual bands and second, total lane intensity (TLI) that measures the total serum antibody reactivity to a whole lane of separated antigens (6). The computerized quantification of band intensity allows a statistically analyzable distinction between immunodominant and immunorecessive bands.

QCWB can be very useful during a longitudinal follow up of the antibody reaction to infection, exposure to foreign proteins, or changes in clinical status when monitoring cancer or autoimmune diseases. The identification and measurement of reactivity to specific antigenic bands at multiple points over time can facilitate the identification of such bands as sensitive sentinels for early disease detection, as prominent markers of patent infection, or as markers of successful treatment. Sera collected from dogs experimentally infected with *Leishmania infantum*, a fatal protozoan parasite of humans and dogs, will be used to illustrate how QCWB can be used to evaluate and compare antibody reactivity against individual antigens in a whole parasite mixture both at a single

time point as well as over time during a longitudinal study. Other serological methods such as enzyme-linked immunosorbent assay (ELISA) are compared to QCWB using the same sera to demonstrate the advantages of the latter technique (6).

2. Materials

Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting are described here essentially as carried out by the authors (6). Other protocols with slightly different reagents can be analyzed by the quantitative computerized technique detailed in the next paragraphs.

2.1. SDS-PAGE and Western Blotting

1. Protein sample – 150 µg/lane/gel.
2. SDS-PAGE equipment.
3. Nitrocellulose paper.
4. Transfer buffer.
5. Blocking solution: Phosphate buffered saline (PBS) containing 0.05% Tween-20 and 5% dried milk.
6. Serum from test subjects and controls.
7. Washing buffer: PBS containing 0.01% Tween20 (PBST).
8. Secondary antibody at 1:1,000 dilution in washing buffer [Horseradish peroxidase (HRP)-conjugated goat anti-canine IgG (Cappel Research Reagents, ICN Biomedicals, Aurora, OH, USA)].
9. 0.0004% 3-3-diaminobenzidine (DAB), Sigma Chemical Company, Missouri, USA.
10. Molecular mass protein standard (Precision Plus Protein Standards, Bio-Rad, Hemel Hempstead, UK).
11. Digital scanner – the 6200C scanner, (Hewlett-Packard Co., Mississauga, Canada).
12. Image analyzing software such as the KODAK 1D program (Eastman Kodak Company, Scientific Imaging Systems, Rochester, NY, USA).
13. Mini-electrophoresis apparatus (Bio-Rad Laboratories, Inc., Hercules, USA).
14. Semidry or wet transfer apparatus (Bio-Rad transfer cell, Bio-Rad).
15. HRP-conjugated goat anti-canine IgG (Cappel research reagents, ICN Biomedicals, Aurora, OH, USA).
16. 3-3-diaminobenzidine (DAB, Sigma, St. Louis, MO, USA).

3. Methods

The methods below describe (1) the fundamentals of western blotting procedure in short; (2) application of an image analyzing software on a blotted membrane; (3) lane marking and individual band analysis; (4) determination of NBI; (5) defining immunodominant and immunorecessive bands; and (6) total lane intensity.

3.1. Western Blotting

While the full western blotting procedure used here was detailed at length (6), any standard procedure for blotting can be employed (*see* Chapter “Detection of La/SS-B by Western Blot Using Nanogold-Tagged Antibodies and Silver Enhancement”).

1. Crude *L. infantum* antigen (CLA, 150 µg/lane/gel) is separated on a 12% SDS-PAGE gel under nonreducing conditions using a minielectrophoresis apparatus.
2. Antigens are transferred to nitrocellulose membrane using either a semidry or wet transfer apparatus.
3. A molecular mass protein standard, preferably prestained, is also run on the same gel and transferred to the membrane.
4. The membranes are cut into individual strips and undergo three steps including blocking, incubation with dog sera, and incubation with an HRP-conjugated goat anti-canine IgG diluted 1:5,000.
5. Development is carried out with 0.0004% DAB.
6. Several rinses (3–4 times) with washing buffer are performed between each of the earlier steps listed in **steps 4** and **5**.
7. After a last wash with distilled water the blots are air dried and at the end of the western blotting procedure dry nitrocellulose membrane strips with dark bands corresponding to serum antibody reactions with protein bands of various molecular weights are obtained.

3.2. Image Formation

In order to acquire an image of highest quality and resolution, blotted membranes should be scanned using a scanner or other imaging device that permits further digital analysis. Any modification of the scanner or imaging device settings that improves the blot appearance or enhances a particular feature of the blot should be avoided. Thus the same settings for brightness, contrast, and gamma values should be strictly maintained during all analysis. Membranes should be scanned immediately following the blotting procedure in order to produce clearly resolved bands and avoid fading of the bands over time. An optimal scanning procedure is the one that keeps the lanes as straight as possible and avoids color artifacts (**Fig. 1A**).

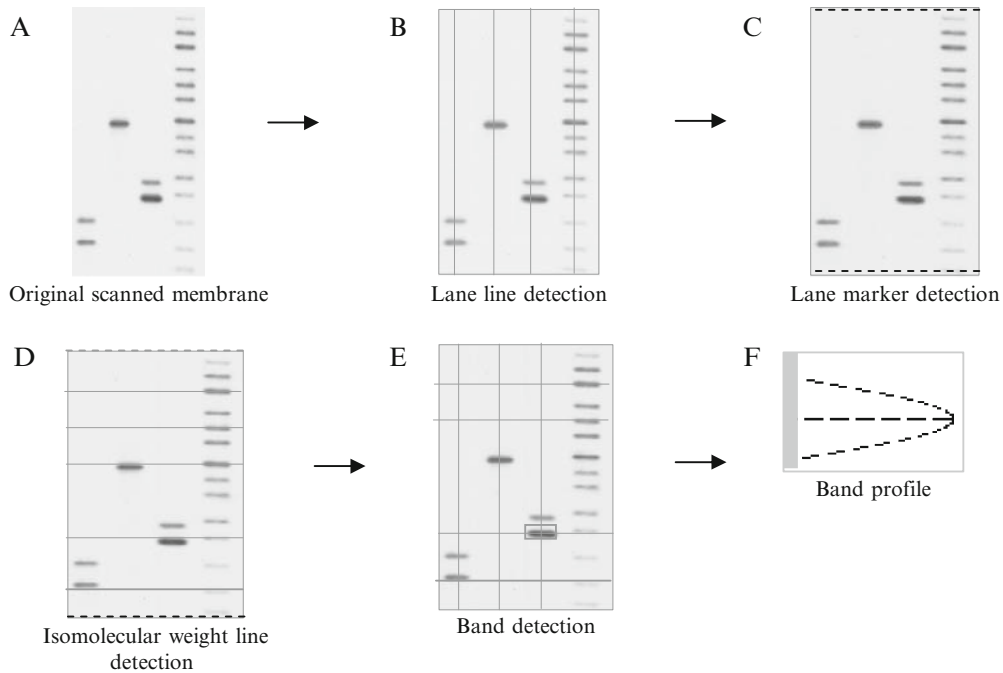


Fig. 1. Flow chart describing the different steps in image analysis of quantitative computerized western blot. Panels **(A)**: Scanning of the blot and automatic or manual lane line detection based on pixel peaks of bands. **(B)** Establish the lane markers at the top and bottom of the scanned membrane and designation of the first background reading. **(C)** Marking isomolecular weight lines enabling adjustment of individual and multiple blot membranes and determination of the molecular weight for individual bands. **(D)** Automatic software analysis and definition of rectangles around the detected bands. **(E)** Manual adjustment of the rectangle borders if needed. The software uses an optimized band sensitivity chosen according to the bands of interest. The sensitivity chosen should distinguish the bands of interest from the background while ignoring the extraneous ones. **(F)** Remodeling of profile data if needed.

3.3. Digital Analysis (see Fig. 1)

3.3.1. Defining Sample Lanes

1. Sample lanes on an image are automatically and/or manually found by the Kodak image analyzing program using the lane finding algorithm.
2. The algorithm determines the band location and calculates the spacing and the width of the different lanes from each other on a selected region of the image.
3. The program identifies the location of lanes according to the pixel peaks for bands that are adjacently localized. Therefore, if bands are not well resolved, homogeneous or the transfer is poor the lanes may need to be inserted manually.
4. Lane finding sensitivity can be set to several levels (*see also Sub-heading 3.3.5*) and corrected to fit straight, slanted, or curved lanes. When sensitivity is increased, fainter bands can be identified. However, it is advisable after adjusting the sensitivity to a desired level, to keep it constant throughout the project. It is critical to ensure that the lines defining each lane pass directly through the center of all the bands in each lane (**Fig. 1B**).

3.3.2. Lane Marker and Isomolecular Weight Lines

1. In addition to the sample lane lines, marker lines should be drawn parallel to the bands at the upper and lower edges, or top and bottom of the blotted gel (**Fig. 1C**; *see Note 1*). It is important that the marker lines accurately reflect the skew of the gel.
2. The first lane marker at the top of the gel blot designates the first background reading, so it is important not to draw lines through bands or wells.
3. The top and bottom lane markers set the region of original image selection and should be moved minimal or not at all.
4. For a better adjustment of skewed gels, isomolecular weight lines should be added (**Fig. 1D**).
5. The intersecting points between the lane lines and the isomolecular weight lines serve as control points that enable adjustment for mobility variations (*see Note 2*).

3.3.3. Labeling Lanes

The next step is to label each lane as standard or experimental. It is possible to either use the formats given by the software or create a new standard lane according to the molecular weights given by the ladder. A standard lane will define the band size in Daltons for proteins, base pairs for DNA, or bases for RNA depending on the type of gel viewed (**Fig. 1B**).

3.3.4. Receiving and Analyzing Band Data

Once the lanes have been marked, bands can be automatically located. The band finding algorithm depends on two parameters that define the band sensitivity and the profile width. These two parameters should be set at the beginning of the project and kept constant. A completely marked image can be seen in **Fig. 2**.

3.3.5. Band Sensitivity

In order to choose the correct sensitivity for a given project, resolution of the individual bands must be considered. If the bands of interest do not stand out from the background, a higher sensitivity should be chosen. Optimally the correct sensitivity should allow the program to find all the bands of interest while minimizing those bands that are irrelevant.

3.3.6. Profile Width and Band Numbering

1. Profile width defines the width of the band used for the analysis out of the total strip width, in case the band is narrower than the strip. It sets the area of the rectangles that surround each band. Optimal profile width encompasses the whole band without getting false readings from the background that surrounds it.
2. Each band is then labeled and numbered, and a horizontal marking line is stretched across it.

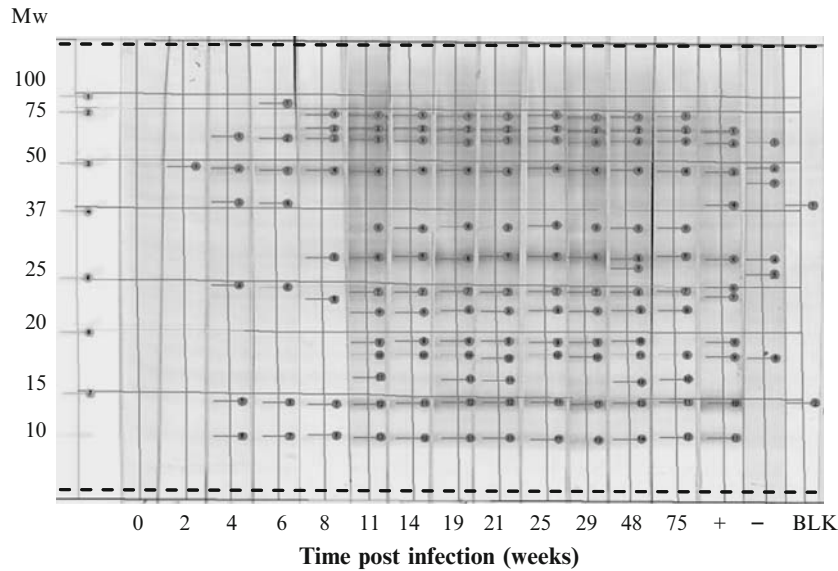


Fig. 2. An example of an image created using the Kodak 1D analyzing software on a typical western blot membrane. The image shows the reaction of sera taken from one dog experimentally infected with *Leishmania infantum* parasites on homologous parasite crude antigen at successive time points during a longitudinal follow up study. The dog was treated from weeks 32 to 45 with allopurinol. The *upper and lower long horizontal dashed lines* indicate the lane markers and the *short horizontal lines ending with a dot* mark the isomolecular weight lines. Positive and negative control sera are depicted in lanes *plus* and *minus*, respectively, and a *blank lane* depicting reactivity with diluting reagents is marked as BLK.

3. It is important to manually verify that the horizontal line is positioned exactly at the center of the band because the molecular weight is calculated based on the position of this line.
4. A rectangle is marked around the band (**Fig. 1E**).
5. As soon as the central horizontal line is drawn, it can be used to compare the band positions between the standard and experimental lanes, and it is possible to also adjust the position and boundaries of any band while examining the peak intensity viewed in the profile exhibited (**Fig. 1F**).

3.3.7. Data Modeling

After the bands are found and the band profile is created, it is important to determine whether the data needs to be modeled. This is especially important for determining the values of unresolved bands, oversaturated bands, or bands with uneven or high background.

When to Remodel Data?

1. When bands are unresolved (closely spaced) (*see Note 3*)
2. If bands are saturated (flat-peaked) (*see Note 4*)
3. When faint bands are located adjacent to strong bands (*see Note 5*)

4. If the background is uneven or variable
5. If false bands are present

In order to calculate the net intensity, the program uses the pixel values in the band rectangle. Quantitative analysis of overlapping or saturated bands is best approximated by fitting the data to a Gaussian curve (7). For saturated bands, the Gaussian technique better estimates bands that are beyond the dynamic range of the capture device.

Information received from the band analysis includes:

1. Molecular weight
2. Mobility-measured location of the band from the top lane marker
3. NBI – the band pixel value minus the background rectangle pixel values
4. Relative intensity – the percent intensity contribution of a band within a lane

3.4. Applications of Quantitative Computerized Western Blot Analysis

3.4.1. Net Band Intensity

NBI is defined as the sum of background subtracted pixels within the rectangle area formed around the band, reflecting the reaction to it. NBI enables the quantitative comparison of antibody reactivity between specific antigen bands on the same or different blots. It facilitates comparison of antibody reactivity with antigens between individuals or by the same individual at different times, thus allowing one to follow and analyze experimental or natural infections longitudinally. The ability to quantify antibody reactions in a standardized way allows the statistical assessment reactions to treatment confirming whether remission or elimination of infection and decrease of antibody responses have occurred (8). This is especially applicable in the case of diseases where infection does not correlate with clinical signs.

3.5. Immunodominant Bands

NBI can be determined individually or in comparison to other bands allowing one to determine whether specific antigens generate an immunodominant or immunorecessive serological response based on comparative intensity and prevalence (number of individuals reacting with each band).

1. In the experimental model for canine visceral leishmaniasis longitudinal quantification of serologic reactivity (NBI) to *L. infantum* antigens was recorded in six dogs over 75 weeks (6).
2. Computerized quantification was done and after taking the prevalence of the bands into consideration, statistical analysis identified the immunodominant and immunorecessive antigen bands.
3. QCWB could be applied at one time point (Table 1) or used to follow changes in seroreactivity against specific antigenic bands over time.

Table 1
Quantitative comparative Western blot analysis of antibodies against *Leishmania* antigens following experimental infection with *L. infantum*

Mean intensity	Band (kDa)	Band category
12 ^a ± 4 (5)	12	ID
35.7 ± 22.3(6)	14	ID
15.2 ± 5.2 (6)	24	ID
23.9 ± 9.7 (6)	29	ID
5.8 ± 3.0 (3)	48	ID
14.0 ± 0.1 (4)	68	ID
12.0 ± 8.4 (3)	18	ID
1.2 (1)	19	IR
2.5 ± 1.1 (3)	34–35	IR
13.1 ± 2.4 (2)	71	IR
7.3 (1)	102	IR
-----	136	IR

Comparison of the mean net band intensities ($n = 6$ dogs) at 53 weeks postinfection for each *L. infantum* antigen and categorization into immunodominant or immunorecessive bands. Bands were categorized according to both the mean net band intensity and the number of animals reacting with each band (prevalence). *ID* Immunodominant and *IR* Immunorecessive

^aMean intensity ± SEM was determined at 10⁶ pixels. Values in parentheses indicate numbers of dogs expressing bands

3.6. Total Lane Intensity

This parameter is defined as the sum of all valid band intensities for each individual lane. TLI is equated to total antibody reactivity against all the components found in a complex crude antigen and roughly parallels serologic reactivity against whole antigen by other serological methods such as ELISA.

1. The serological response against crude *L. infantum* antigen (CLA), following infection of beagles in an experimental canine visceral leishmaniasis model, was measured by TLI and ELISA at different times postinfection and compared.
2. Antibody response measured by TLI showed significantly higher folds increase.
3. At 21 weeks postinfection, TLI was 100 times greater than preinfection, whereas CLA found by ELISA was only 60 times the preinfection value ($p < 0.01$) (Fig. 3).

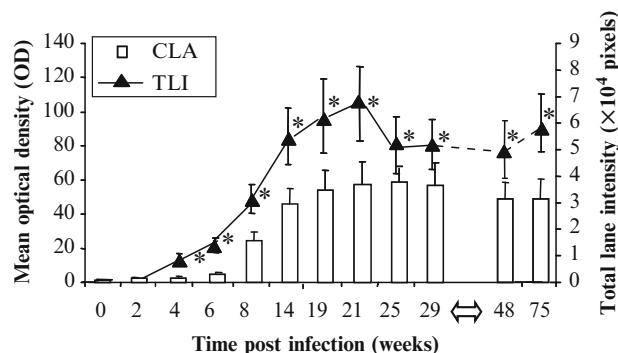


Fig. 3. Comparison of fold increase in antileishmanial antibody reaction with crude leishmanial antigen (CLA) by Quantitative Computerized Western Blotting (QCWB) and Enzyme linked immunosorbent assay (ELISA). Dogs ($n = 6$) were infected intravenously with the parasite *Leishmania infantum* and the increase in total lane intensity (TLI), determined by QCWB, and CLA by ELISA reactivity were measured over a 75-week period. The arrow on the X-axis indicates the period when the dogs were treated with (32–45 weeks postinfection). Triangular blocks and columns represent mean values standardized to time zero, with bars indicating the standard error. A significant difference ($p < 0.01$) is indicated by asterisk.

4. Notes

1. Adding lane markers is only possible at this stage and will not be permitted in later steps when using the KODAK 1D software.
2. It is important to locate these lines correctly for the accurate identification of bands with identical molecular weights that migrate differently on the same or different gels.
3. When the program identifies unresolved bands it might be difficult for it to determine the boundaries of the rectangle, thus not allowing manual insertion of a band close to the one that was found. The way to address this problem is to manually reduce the size of the rectangle confining the band, and then insert the desired neighboring band.
4. Oversaturated bands – when bands are flat peaked they indicate oversaturation, and require adjustment using the Gaussian model.
5. Very faint bands can be difficult to differentiate from the surrounding background and manual insertion may be needed.

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