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Evaluating dot and Western blots using image analysis and pixel quantification of electronic images

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Abstract. Inexpensive computer imaging technology was used to assess levels of insulin-like growth factor-I (IGF-I) on dot blots (DB) and α-Actinin on Western blots (WB). In the first procedure, known IGF-I samples were dotted on nitrocellulose membranes using a vacuum manifold. After the DB were developed and dried, the images were digitized using an HP Deskscan II® flat bed scanner, exported into Image-Pro® Plus and analyzed by taking the combined mean of 45° and 135° sample lines drawn through each dot. Dot blots corresponding to a linear concentration range from 10 to 300 ng IGF-I were assessed by this method. In the second procedure, WB were scanned with a ScanJet 3c® flat bed scanner and their backgrounds were clarified using Image-Pro® Plus. A second image analysis program, Alpha Imager™ 2000, was then used to define the boundaries of protein bands, assess pixel number and density, and to obtain final numerical data for quantifying α-Actinin on the WB. Collectively, the results of these two studies suggest that specific proteins may be evaluated by using relatively inexpensive image analysis software systems via pixel quantification of electronic images.

Key words: Dot blot, Flat bed scanner, Image analysis, Western blot

1. Introduction

Specific proteins in a biological sample are commonly evaluated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by transfer of the protein bands to a support, such as a nitrocellulose membrane, for Western blotting. After exposing the protein (antigen) to a primary antibody, a secondary antibody/enzyme substrate complex is reacted with the protein-antibody complex for quantitation by a colorimetric, chemiluminescent or radiolabeled signal. Another procedure for the rapid screening of protein in biological samples utilizes a vacuum blotting manifold to create dot blots [5]. The end result of both dot and Western blotting is a physical complex of antigen/antibody/label bound to a membrane.

Methods for quantifying proteins on blots have often used scanning densitometry or high resolution CCD (charged coupled device) cameras, in which live images [2], photographs [7] or blots [10] are translated by computer software to digitized images/values. Due to their high cost, these image analysis systems are not available to every laboratory. As an alternative method for obtaining images for computer analysis, we employed a readily accessible flat bed scanner to capture digital images from either dot or Western blots. Subsequently, these images were exported to relatively inexpensive imaging programs for further manipulation and specific quantification of proteins (Figure 1).

2. Materials

A. Supplies and reagents used to prepare dot blots
   1. Recombinant IGF-I, human (Catalog no. H-5555).[1]
   2. Nitrocellulose membranes (Catalog no. 162-0116).[2]
   3. Mouse anti-human IGF-I (Catalog no. 05-172).[3]
   4. Goat anti-mouse IgG (H&L) alkaline phosphatase conjugate (Catalog no. 172-1015).[2]
   5. Alkaline phosphatase conjugate substrate kit (Catalog no. 170-6432).[2]

B. Equipment used to prepare and analyze dot blots
   1. Refrigerated superspeed centrifuge (Sorvall model RC-5B).[4]
   2. 96-well Hybrid-dot manifold (Catalog no. R4140).[5]
   3. HP Deskscan II® flat bed scanner.[6]
   4. Image Pro® Plus software,[7] version 1.2 for Windows™.

C. Supplies used to prepare Western blots
   1. Protein Assay kit (Catalog no. 500-0002).[2]

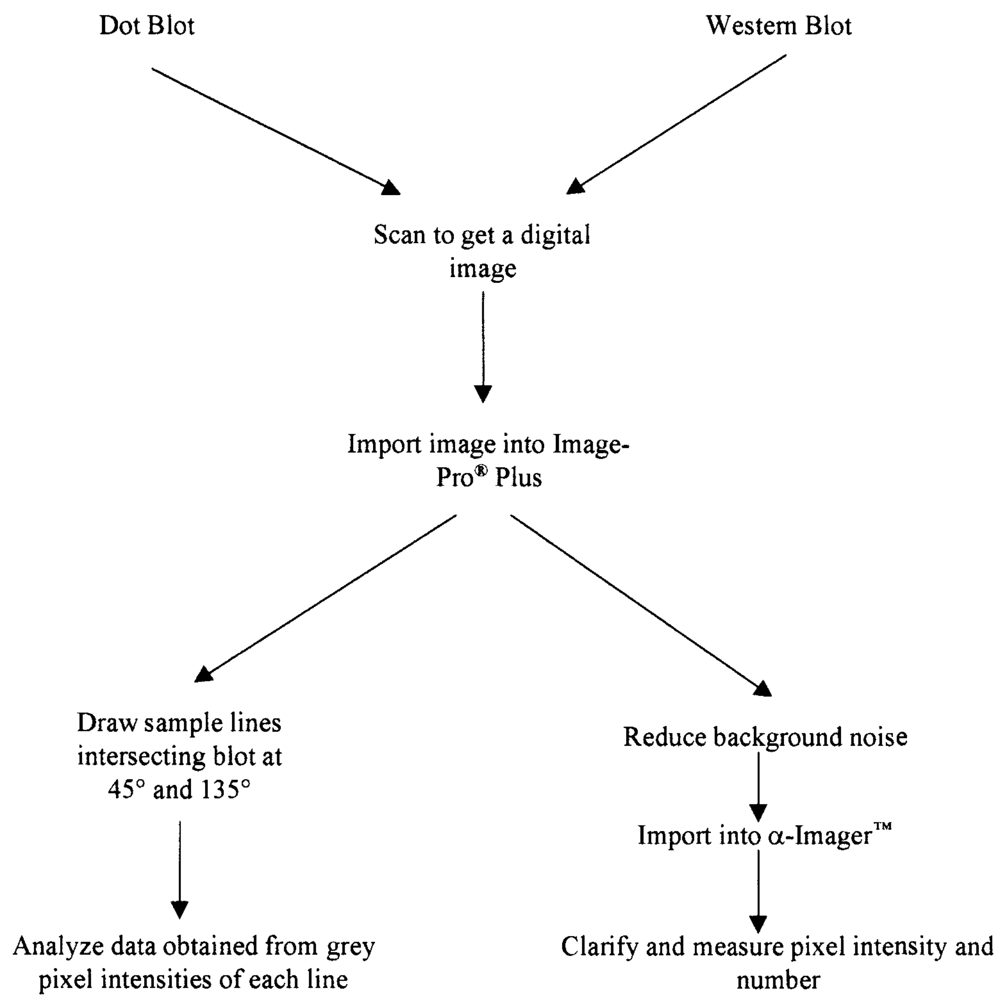

Figure 1. Flow diagram of the methods for application of scanning and image enhancement technology to assess dot and Western blots.

2. Nitrocellulose membranes (Catalog no. 162-0116).[2]

D. Reagents used to prepare Western blots
1. Tris-buffered saline (TBS), pH 7.4
   a. 4.84 g Tris base (Catalog no. T-8404).[8]
   b. 58.48 g NaCl (Catalog no. S-5886).[8]
   c. Dissolve a and b in 1.5 L of deionized, distilled (dd) H_2O.
   d. pH to 7.4.
   e. Adjust volume to 2 L with dd H_2O.
2. TTBS (Tween + TBS)
   Add 0.5 ml Tween-20 (catalog no. P-1379)[8] to 1 L of TBS.
3. Non-fat dry milk.
4. α-Actinin molecular weight standard (Catalog no. A-9776).[8]
5. Mouse anti-α-Actinin antibody (Catalog no. A-5044).[8]
6. Goat anti-mouse IgG antibody (Catalog no. 172-1015).[2]
7. Amplified alkaline phosphatase immuno-blot® assay kit (Catalog no. 170-6412-1).[2]
8. Rainbow molecular weight markers (Catalog no. RPN 756).[9]

D. Equipment used to analyze Western blots
1. Mini-PROTEAN® II electrophoresis cell (Catalog no. 165-2940).[2]
2. Trans-Blot SD® semi-dry cell (Catalog no. 170-3940).[2]
3. ScanJet 3c® flat bed scanner.[6]
4. Alpha Imager™ software.[10]

3. Procedures

A. Preparation of dot blots was as described in a previous paper [5]. In brief:
1. Samples containing the targeted protein (IGF-I) were prepared at concentrations of 10, 50, 100 and 300 ng of standard per 75 µl PBS.
2. For preparation of the dot blots, 75 µl of the diluted IGF-I samples were pipetted into individual wells of a 96-well Hybri-Dot manifold and subsequently transferred to a nitrocellulose membrane.
3. The nitrocellulose was developed by exposure to anti-human IGF-I, goat anti-mouse IgG

and alkaline phosphatase conjugate/substrate reagents.

B. Analysis of dot blot images.
   1. Immediately following a final wash, rinse and drying at room temperature, the dot blots were converted to gray-scale digital images with a HP Deskscan II® flat bed scanner (300 dpi resolution) and saved in a TIF file format (Figure 1).
   2. The images were then imported to a computer imaging software program (Image Pro® Plus) for analysis.
   3. The 8-bit images in 256 levels of gray were enlarged up to 135% to facilitate measurements.
   4. Two lines were drawn across the dot at approximately 45° and 135° by using the Line Profile command of the program, which reports gray levels for individual pixels (Figure 2).
   5. Using the guide bars provided along the initial 45° line, the edges of the dot were easily identified, and the pixel gray level for each edge was noted prior to data export to a spreadsheet program [9].
   6. Data for the dot were clipped, based on the previously noted edges.
   7. The process was repeated for the second line (135°), and the values of the two lines represented one dot.
   8. The mean of three dots was used to determine the relative gray-scale density for one treatment.

C. General SDS-PAGE and Western blot methods [1]
   1. Myofibrillar proteins were extracted from muscle following the protocols of Huff-Lonergan et al. (1994) and stored at –20 °C until used.
   2. Protein microassays were conducted on thawed extracts to determine the appropriate volume representing 30 μg of myofibrillar protein and 600 ng of α-Actinin.
   3. Subsequently 25 μl of each sample were loaded into each lane of a 10 cm, 10% discontinuous gel and separated using SDS-PAGE and a Bio-Rad Mini-PROTEAN® II Cell [6].
   4. After being separated on the gel, the proteins were transferred to a nitrocellulose membrane using a Bio-Rad Trans-Blot SD® semi-dry cell.
   5. Western blot development was as follows: to prevent non-specific antibody binding, the membrane was blocked overnight at 4 °C with 30 ml of 3% non-fat milk in Tris-buffered saline (TBS), pH 7.4, containing 0.05% Tween-20 (TTBS).
   6. Antibodies were diluted in 10 ml of 1% non-fat milk + TTBS at room temperature.

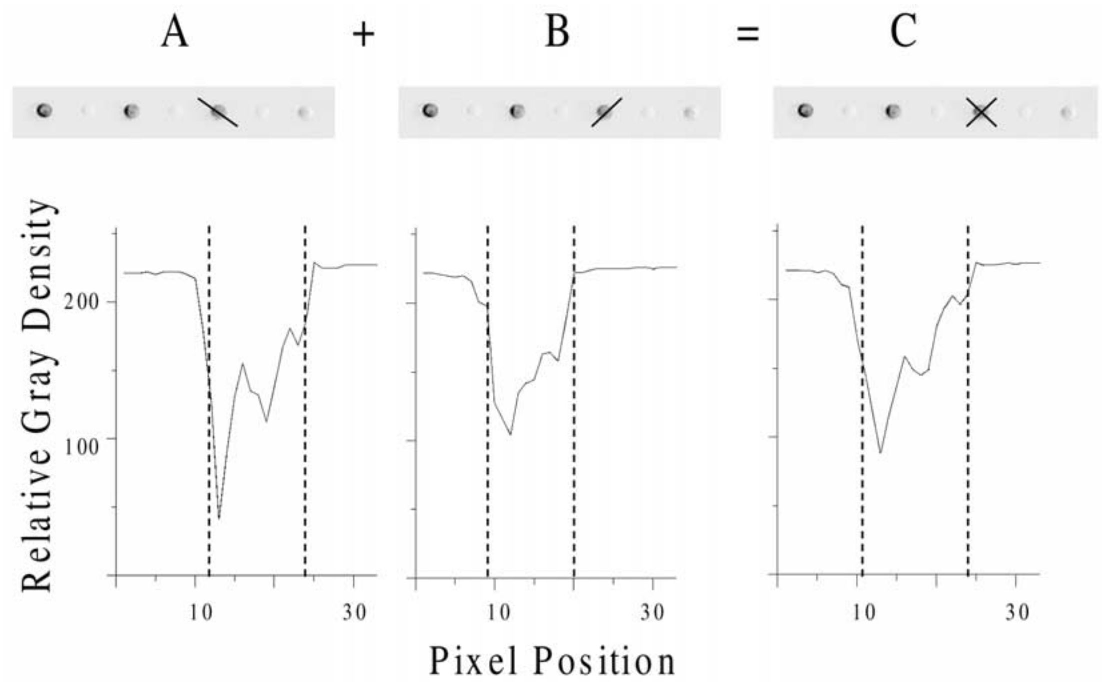

Figure 2. Measurement of developed IGF-I sample DB with image analysis. By using the Line Profile Command in Image Pro® Plus Version 1.2 for Windows™, a line was placed at approximately 45° angle across the dot (A). The program produced a densitometer-like gray density profile of the pixels in the sample pathway. Moveable guide bars allowed the user to determine the edges of the dot, which were noted prior to data export to a compatible spreadsheet program, where data was clipped. A second line (B) was positioned at approximately 135° across the dot, and the resulting data were collected. After importing all of the numeric data into Excel, the means from both lines A and B were combined to represent one sample (C). The dot blot [5] is presented with permission from Kluwer Academic Publishers.

7. The membrane was incubated with a primary antibody (mouse anti-α-Actinin, 1:400) for 90 min and washed three times with blocking buffer (20 ml/wash, 10 min/wash).
8. Next, a secondary antibody (goat anti-mouse IgG, 1:1000, Bio-Rad) was incubated with the membrane for 90 min.
9. After three washes with TTBS (20 ml and 10 min/wash), the membrane was incubated with 10 ml of streptavidin-biotinylated alkaline phosphatase complex (1:3000; Amplified Alkaline Phosphatase Immunblot® assay kit) for 90 min.
10. Following two washes with TTBS (20 ml/wash, 7.5 min/wash), antibody binding was visualized by exposure to alkaline phosphatase color development reagent.

D. Optimization of protein transfer in Western blots
1. In order to define the most effective mass amount of α-Actinin in the SDS-PAGE system, duplicate dose-response experiments were conducted.
2. Serial dilutions of α-Actinin molecular weight standard to yield mass amounts of 3000, 1500, 750, 600, 300, and 150 ng were made. Rainbow® molecular weight markers, at a mass amount of 3 μg, were used as references.
3. All samples were loaded into a 10% discontinuous gel.

E. Image Pro Plus™ and Alpha Imager™ conversion of WB analog data to digital data
1. Immediately after immunoblotting, all blots were electronically scanned with a ScanJet 3c® flat bed scanner (300 dpi resolution) and saved as 14 cm × 10 cm TIF file images.
2. Background noise was later reduced on scanned images with the Image Pro Plus™ image analysis system using brightness (78), contrast (34) and gamma (0.2) settings.
3. Before measuring the intensity of specific bands, the background of all blots was clarified using Alpha Imager™ [black (0), white (224), and gamma (0.45) settings].
4. The bands on clarified blots were measured with Alpha Imager™ with a constant measurement area around the protein bands of 960 pixels.
5. The digital numbers obtained were the integrated density (ID) values of the intensity and the size of each band.

4. Results and discussion

Dot blots. Once the dot blots were scanned and digitally transformed, methods within the confines of the imaging program were used to statistically represent various degrees of antibody reaction, as well as uneven saturation of the dot. Preliminary pilot studies were conducted to set the parameters for the final analysis protocol (data not shown). First, in order to determine the most appropriate method for sampling a dot, a single, horizontal line was drawn across the dot and analyzed with gray-scale detection to represent one sample. Three such samples from one dot were compared to a control consisting of three sets of ten lines at random angles, which bisected the center of the dot. It was determined that the mean gray-scale value of the three horizontal samples was different from that of the control ($P < 0.05$). Because the horizontal line sampling did not characterize the dot, this procedure was repeated using the mean of three vertical lines, which also failed to represent the dot ($P < 0.05$). The mean gray-scale value of three sets of lines at 90° and 180° also did not provide adequate representation of darkly saturated or marginally dark dots ($P \leq 0.05$). Finally, when the mean of three sets of crossed lines at 45° and 135° was compared to that of the controls, these lines proved to more adequately represent dark, medium and light dots ($P > 0.05$) and therefore were used for all ensuing studies.

It had been observed early on in these studies that the angle of the light source of the scanner caused secondary shadowing on the dots. Perhaps the fact that the 45° and 135° lines contained both shadowed and non-shadowed sections of the dot accounted for the closer agreement of the gray-scale values of these lines with those of the multiple-lined controls. Future sampling of a much larger number of dots would help to determine if the actual mechanics of the preparation of the dots resulted in reproducible patterns of light and dark areas. It is also possible that with a larger database of analyzed dots, the variation between different sample lines would average out.

To ensure that the initial resizing of images to aid in line placement was not altering the data, three sets of samples from original, 135% and 150% resized images were compared using 45° and 135° sampling. The resized images were not significantly different ($P > 0.05$) from the original image. In some cases however, background noise levels prevented numerical comparison between different blots. Altering brightness and contrast levels, as well as using filtering commands in order to compare different blots, seemed to skew the data. Additionally, because all dots were cup-shaped due to vacuum pressure, the scanning process itself introduced slight shadowing of the dots. Therefore, to prevent a potential problem of mistaking the shadowing for a response, sample blanks of phosphate buffered saline were loaded into nearby dots when loading normal samples. After the color reaction and imaging, blank values were subtracted from sample values prior to further processing of the data.

Dot blots representing either 10, 50, 100, and 300 ng of IGF-I per dot were assessed with this protocol (Figure 3). Inverse gray-scale values were plotted

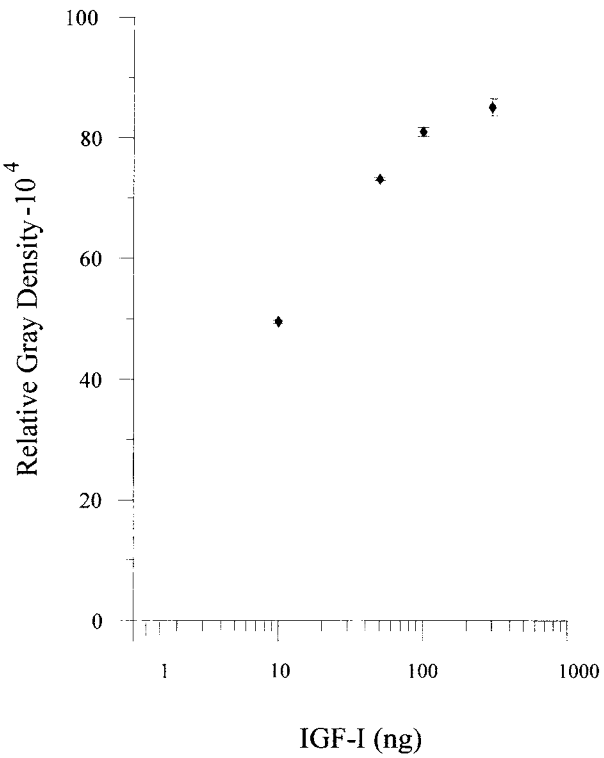

Figure 3. Data collected from a DB containing 10 ng to 300 ng IGF-I. Three crossed-line sample sets were collected from a dot blot containing 10 ng, 50 ng, 100 ng and 300 ng of IGF-I. Inverse gray-scale values were plotted against amounts of IGF-I on a semi-log scale. All of the values are significantly different (p value < 0.05), but the slight curve suggests that the blot membrane may have become saturated at levels between 100 ng and 300 ng IGF-I.

against concentrations of IGF-I on a semi-log scale. All values were significantly different ($P < 0.5$). These data suggest that this simplistic image analysis method may be capable of relating gray-scale intensities of dot blots to protein concentrations.

Western blot analysis. Standard mass levels of α-Actinin were used in a pilot study to set initial parameters of the 10% SDS-PAGE gel, WB and eventual analysis (data not shown). The maximum

Table 1. Determination of the optimal transfer time for α-Actinin detection.[a]

Transfer time (min)	Relative α-Actinin level[b]
35	306.85
45	359.72
55	203.32
65	234.6

[a] Mass amount of α-Actinin was 600 ng. α-Actinin was resolved using a BIO-RAD® mini gel system and was transferred using a BIO-RAD Trans-Blot SD® Semi-Dry Transfer Cell.
[b] Relative α-Actinin level = ID values × 10^{-2}.

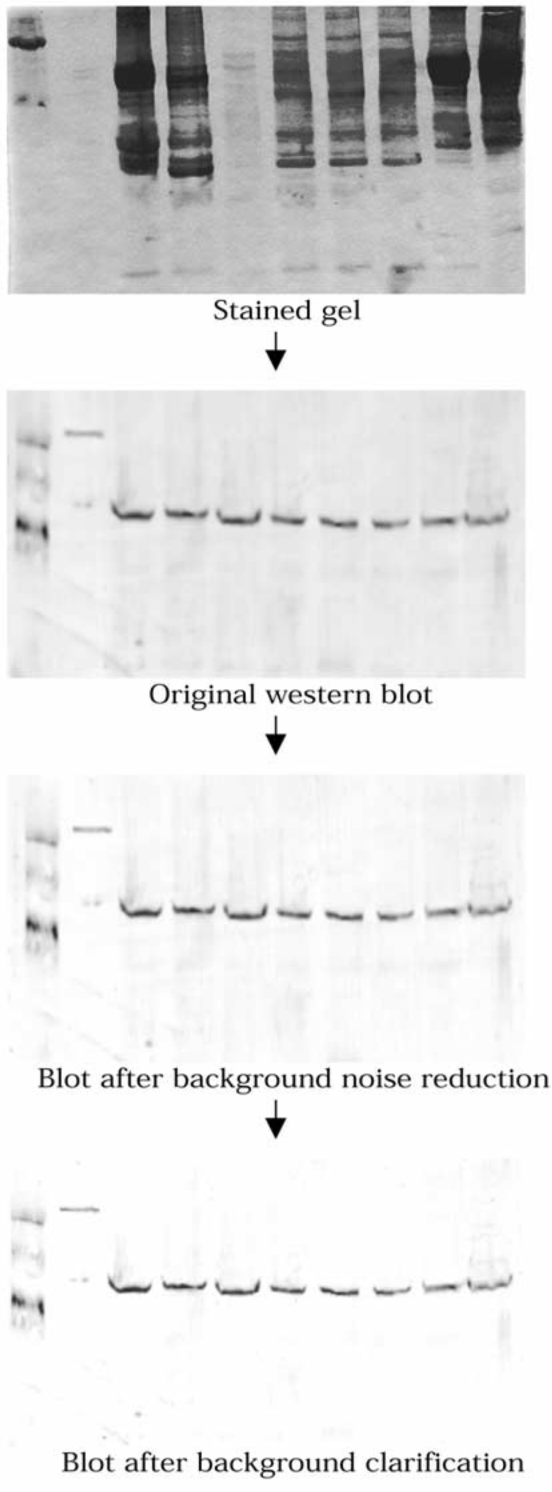

Figure 4. An SDS-PAGE gel and a Western blot of α-Actinin protein samples and standard (far left lane). After transfer of the proteins from the gel to a nitrocellulose membrane and the development of the WB, the blot was electronically scanned with a flat bed scanner and saved as TIF files. The background noise was reduced on the scanned images using the Image Pro Plus™ image analysis system. Before measuring the intensity of specific bands, the background of the blot was clarified using Alpha Imager™. Finally, the bands on the clarified blot were measured with Alpha Imager™.

efficiency of transfer of α-Actinin standard from gel to membrane occurred at 45 min (Table 1). Subsequently, complete myofibrillar protein extracts were subjected to 10% SDS-PAGE, WB and analysis (Figure 4). Rather than employing a traditional light/shadow densitometer for analysis of the WB, we used two types of image enhancement and analysis packages. Such systems can enhance the electromagnetic spectrum not normally visible to the naked eye and enable an increase in contrast and magnification of objects as small as 15–20 nm [3]. The Western blot image was saved, and elementary imaging and enhancement tools from the Image Pro Plus™ and the Alpha Imager™ software programs were used to reduce the background noise of all the Western blots and clarify the images. After image clarification, all of the Western blot bands were measured by enumerating pixel number and intensity and were converted into digital readings. These data suggest that image analysis systems may be useful for WB, also.

In summary, methods were developed in our laboratory as inexpensive alternatives for analyzing blots for proteins of interest. We incorporated a flat bed scanner with commercially available computer imaging programs to transmit and evaluate dark to lightly saturated dots containing IGF-I or to digitize and evaluate WB for α-Actinin. Biological application of computer imaging technology to different fields of study has resulted in new modes of data interpretation. The imaging program implemented here displays the dot blot 256 levels of gray, whereas the human eye can detect less than 200 different shades [8], making visual quantification of dot blots outdated. Although automated imaging programs are commercially available for gels [11], we did not find a similar product for blots. While the methods described here require manual measurements, overall they are fast and user-friendly, and the components maintain flexibility for future applications.

Notes on suppliers

1. Bachem Bioscience Inc, 3700 Horizon Drive, King of Prussia, PA 19406, USA
2. Bio-Rad Laboratories, 2000 Alfred Nobel Drive, Hercules, CA 94547, USA
3. Upstate Biotechnology Inc, 199 Saranac Avenue, Lake Placid, NY 12946, USA
4. Du Pont Instruments, BRML-Chandler Mill, Wilmington, DE 19898, USA
5. Bethesda Research Laboratories, Life Technologies, Inc, 8400 Helgerman Court, Gaithersburg, MD 20877, USA
6. Hewlett Packard Company, PO Box 10495, Palo Alto, CA 94303-0896, USA
7. Media Cybernetics, 8484 Georgia Ave, Silver Spring, MD 20910, USA
8. Sigma-Aldrich Company, PO Box 14508, St Louis, MO 63178, USA
9. Amersham Life Science, PO Box 1327, Piscataway, NJ 08855, USA
10. Alpha Innotech Corporation, 14743 Catalina St, San Leandro, CA 94577, USA

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