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Comparison of the Bio Image Visage 2000® and the GELLAB-II two-dimensional electrophoresis image analysis systems

James E. Myrick[1], Peter F. Lemkin[2], Mary K. Robinson[1] & Kyle M. Upton[3]

[1]*Division of Environmental Health Laboratory Sciences, National Center for Environmental Health, Centers for Disease Control and Prevention (CDC), Public Health Service, US Department of Health and Human Services, Atlanta, GA,* [2]*Image Processing Section, Laboratory of Mathematical Biology, National Cancer Institute, Division of Cancer Biology, Diagnostics Centers, Frederick Cancer Research and Development Center, Frederick, MD and* [3]*Program Resources, Inc., Frederick Cancer Research and Development Center, Frederick, MD, USA*

To compare the Visage 2000®* analysis system (Bio Image®, Ann Arbor, MI, USA) with the GELLAB-II analysis system (National Cancer Institute, Frederick, MD, USA), we used each to perform image analysis of the same 29 silver-stained two-dimensional electrophoresis (2DE) gel image files from a study of urinary proteins in metal recovery plant workers who had confirmed body burdens of cadmium. Visage, aided by interactive analysis, detected an average of 890 ± 177.6 spots per gel, or a total of 25,800 spots, whereas GELLAB-II detected 1971 ± 198.5 spots per gel, or a total of 57,160 (a 222% increase over the Visage system), without operator intervention. Visage automatically quantified 52.5% (13,556) of the spots; 47.2% (12,173), consisting mostly of larger spots, had to be quantified interactively with an image editor, and 0.3% (71) were not quantified. GELLAB-II automatically quantified all detected spots. After we interactively assigned the maximum allowed number of landmarks (30 for Visage and 52 for GELLAB-II), we found that Visage matched 657 ± 211.2 spots per gel, and GELLAB-II matched all detected spots and also extrapolated an average of 1269 virtual spots per gel. Plots of densities from the two systems on selected spots showed excellent agreement, and both systems showed high correlation between their measurements of the β-2-microglobulin spot densities and an independent radioimmunoassay quantification of the original urine samples. By comparing the regression of the densities of all spots with urinary cadmium (UCD) levels, we found that several of the same detected spots from each system were highly correlated. The densities of four acidic proteins with relative molecular weights of approximately 112,000 Da (as quantified by GELLAB-II but not by Visage) were highly correlated with UCD concentrations. These proteins are new candidate biomarkers of cadmium toxicity. We compared the estimated labor costs of using each system to analyse a hypothetical 20-sample (60 gels) 2DE study and found that GELLAB-II was six times less expensive to use than Visage, primarily because of the operator time required to do interactive error correction with the Visage system.

Keywords: Electrophoresis, gel, two-dimensional; computer analysis; cadmium; urine; kidney; proteinuria; toxicology; beta-2-microglobulin; occupational exposure; biological markers; image analysis.

Introduction

Computer image analysis systems for two-dimensional electrophoresis (2DE) gels were first described in 1978 when Lutin published his paper on the Gaussian fitting of stained protein spots (Lutin *et al.*, 1978). During the following years, a number of complete 2DE gel analysis systems were developed by others (Bossinger *et al.*, 1979; Garrels, 1979; Anderson *et al.*, 1981; Aycock *et al.*, 1981; Lemkin & Lipkin, 1981,a–c; Vo *et al.*, 1981; Lemkin *et al.*, 1982; Miller *et al.*, 1982; Lemkin & Lipkin, 1983; Garrels *et al.*, 1984; Vincens, 1986; Vincens *et al.*, 1986; Vincens & Tarroux, 1987a,b; Olson & Miller, 1988; Garrels, 1989; Lemkin & Lester, 1989; Appel *et al.*, 1991; Hochstrasser *et al.*, 1991). During the same period, several commercial systems were developed (some based on previously cited systems), such as PDQUEST® (Protein Databases, Inc., Huntington Station, NY), which was based on the QUEST system of Garrels (1984), Visage® (Bio Image®, Millipore Corporation, Ann Arbor, MI), which was based partially on the system of Vincent *et al.* (1981), and Kepler® (Large Scale Biology Corporation, Rockville, MD), which was based on the system of Anderson *et al.* (1981).

These systems have some or all of the following capabilities: (1) A wet gel or autoradiographic film can be scanned with a digitizing camera or laser

Correspondence: Dr James E. Myrick, Centers for Disease Control and Prevention, 4770 Buford Highway NE (F19), Chamblee, GA 30341–3724, USA

*Use of trade names is for identification only and does not constitute endorsement by the Public Health Service or the US Department of Health and Human Services

Abbreviations: 2DE, two-dimensional electrophoresis; UCD, urinary cadmium; AGM, automatic global matching, a process for matching gels with the Visage 2000 software; β2M, β-2-microglobulin; Da, dalton (one atomic mass unit); EBV, error in both variables; G3PDH$_r$, molecular charge relative to glyceraldehyde-3-phosphate dehydrogenase; M_r, relative molecular weight; OD, optical density (absorbance); matchid, match identification; pI, isoelectric point; RIA, radioimmunoassay

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densitometer to create a computer file containing *x*- and *y*-coordinates of molecular charge and size and *z*-coordinates for density information. Density can be expressed either as raw (uncorrected) data or as calibrated data to show an optical density (OD) value at each *x*-, *y*-coordinate, or pixel. (2) A stained spot can be detected as a group of pixels representing the staining or autoradiographic response of a protein. (3) The response can be quantified by integrating the densities of all pixels within a spot's area. (4) The background density can be computed in the local area surrounding the spot, after which the appropriate background correction can be made to the spot's total density. (5) Other appropriate corrections (e.g. sample volume, dilution factor, and film exposure factor) can be made to the spots' density values. (6) The 2DE spots of different gels can be matched with an appropriate identifier so that the corresponding proteins in each gel can be compared with one another. (7) A database of quantified and matched proteins can be created; researchers can then query this database to find statistical associations of protein densities with other variables that are important to the study (e.g. toxicant body burden, measures of a disease process, and sample treatments).

Computer image analysis expands the capability of 2DE far beyond the high-resolution separation that the gel system can achieve. The databases that are derived from image analysis can be queried by many statistical techniques, including regression analysis of density versus another analyte's concentration, cluster analysis for detecting hierarchical associations of gels (samples) or spots according to another variable, *t*-tests or *F*-tests for hypothesis testing on the means or variances of spot densities, and missing spot tests to find new protein expression (or protein loss) in groups of samples. Only when a computer-searchable database of densities for matched proteins has been created can the ultimate potential of 2DE technology be realized.

The computational power to be derived using such databases, however, is only as strong as the image analysis system that supplies the spot-related fields, such as density, *x*-, *y*-coordinates, area, and match number. Errors in these values will yield errors in the interpretation of the study data and will likely lead the investigator to erroneous conclusions. One difficulty arising from the use of 2DE is that the protein databases produced during multiple-gel studies are enormous, because each gel resolves at least 1000 proteins (sometimes 2000–4000 proteins) depending upon the complexity of the sample and the resolving power of the gel format that is used.

Since many detected spots may be noise, researchers must use multiple samples and replicate gels per sample to gain the necessary statistical power for inferential analysis. As the number of gels and the number of spots per gel increase, especially from complicated sample matrices such as serum or urine, inaccuracies from image analysis can render the task of error correction almost unmanageable. If interactive methods must be used for error correction, labor costs can quickly exceed the perceived benefits of the study. Thus, the obvious goal of computer analysis of 2DE gels should be to automate the procedures as much as possible and to eliminate errors of spot detection, quantification, and matching.

For this report, we compared the Bio Image Visage 2000 with the GELLAB-II system to determine how well each can analyse 2DE gels*. To eliminate any camera or scanning differences, we analysed the same gel image files with each system, after first converting the files to each system's required data storage format. We compared the capability of the two systems to detect, quantify, and perform gel-to-gel matching of the silver-stained protein spots. We then analysed the resulting data sets from each system for statistical associations with urinary cadmium concentration to determine how well each system detected candidate protein biomarkers for cadmium toxicity. Finally, we estimated the costs associated with the use of each system.

Materials and methods

Sample treatment

Human urine samples were ultrafiltered, diafiltered (desalted), subjected to two-dimensional electrophoretic separation, silver-stained, and digitally scanned. These sample treatments and other analyses were done as previously described (Myrick *et al.*, 1993). Other details of the study population have been published elsewhere (Mueller *et al.*, 1989; Thun *et al.*, 1985 and 1989).

Image analysis: Visage system

The silver-stained gels were scanned and digitized with a Visage 2000® system that was fitted with a charge-coupled-device array camera. For camera calibration, grayscale-to-optical density (OD) conversions were made with a step wedge in the OD range of 0.05–3.05. The 1024- × 1024-pixel gel images with 256 gray levels per pixel (169 μm per side) were analysed with the Visage 2-D software module (version 5.1) on Sun 3 or Sun 4 computers (Sun Microsystems, Mountain View, CA). Spot detection, or segmentation, was performed with the menu selections *2-D*, *ANALYZE*, *EDITOR*, and *FIND SPOTS* with the following parameters:

- filter width = 3
- spot threshold = 1
- minimum spot width = 4 pixels
- minimum filter value = 5
- minimum spot size = 10 pixels
- window size = 151 pixels

After extensive 'titration', the above parameter values were found to be the best for accurately detecting all spots in the gels with the least amount of

*Two of the coauthors, P.F.L. and K.M.U., participated in this study only by training the other two coauthors in the use of GELLAB-II; they were not involved in the image analysis operations with the Visage system or in the data interpretation or comparison of data between the two systems. Coauthors J.E.M. and M.K.R. have had several years experience using the Visage system and occasionally obtained additional help from Bio Image technical personnel during the analysis of these gel images

error. Spots were initially quantified with the menu selection *ESTIMATE DENSITIES*, and final automated quantification was computed as a background process with the menu selection *GLOBAL QUANTIFY*. 'Integrated intensity' values, the sum of the background-corrected OD values for each spot as output by the Visage software, were used in this report. The operator interactively corrected many of the spots' centroid locations and boundaries to remove the maximum possible number of detection and quantification errors made by the software. After editing, the maximum allowed 30 'matchpoint' (landmark) spots in each gel were chosen interactively to match the same spots on the reference gel. Each gel was computer-matched by background processing to the reference gel by the program *MATCH*, and the results were interactively edited to correct any missed or false matches.

The Visage matching operation, which consisted of the *MATCH* process and interactive editing, resulted in four possible outcomes: (1) Direct matches—spots from different gels that were considered by the software or by the operator to be the same stained protein and were given the same match identification (matchid) number. (2) No matches—spots that were present only in the reference gel or only in a study gel. A matchid number was not assigned to the spot, because matchids could be assigned *only* to spots that were present in *both* the reference gel and another gel. (3) Correspondences—spots from different gels that were considered to be the same protein but were assigned different matchid numbers. Correspondences occasionally resulted from interactive error correcting. (4) Contradictions—spots from different gels that were considered to be different proteins but were given the same matchid number. Contradictions occasionally resulted from interactive error correcting. To permit statistical analysis of the Visage data, we had to correct correspondences and contradictions, that is, spots representing the same protein had to be given an identical matchid number, and spots representing different proteins had to be given different matchids. This resolution of matchids was accomplished by a SAS® (SAS Institute, Cary, NC) program written at CDC.

An automatic global matching (AGM) database within the Visage system was created as gels were matched to the reference gel. This database was accessed to dynamically query any pair of gels during the operation of the menu selections *COMPARE* or *INVESTIGATOR*, which were run to determine the accuracy of spot matching. The database was automatically updated when changes were made to spot matching. These changes included removing incorrect matches, adding new matches, or creating new correspondences or contradictions.

We were able to perform statistical analysis of cadmium exposure versus matchid data on protein response only after the Visage data was further processed, as follows: (1) A Boolean union (Girling, 1965) of the matched spots from all gels in the study was created with the menu selections *AGM REPORT* and *GENERATE COMPARISON REPORT* that were supplied with the Visage system. (2) A filter program written in C language by a CDC employee was used to strip the Boolean union file to a less complicated format containing only the gel numbers and matchids of correspondences and contradictions. (3) A concatenated spotlist (a listing of all spots in a gel) of all proteins in the study was made with the Visage program *2D_PRSPOT* and with UNIX system commands by combining individual spotlists from each gel. The spotlist contained, at least, the gel number, matchid number, x- and y-coordinates, and density value of each spot in the study. In addition, the spotlist for the reference gel contained relative molecular weight (M_r) and charge values for each spot, a result of the menu selection *STANDARDS*, which computed the M_r and charge estimates for all spots in the gel. (4) The filtered Boolean and concatenated spotlist files were processed by a CDC-written SAS program that resolved the correspondences and contradictions, performed data integrity checks, supplied other study-specific variables (sample number, urine dilution factor, urinary cadmium (UCD) concentration, and total urinary protein), and created a SAS data set that was used as a database to which other SAS queries were made to search for statistical associations. The database was also supplied with extrapolated spots, that is, with imaginary spots with zero densities and zero areas assigned to x-, y-coordinates in each image where the spots should have been, had they existed in the original gels. With the Visage system it was only possible to extrapolate spots that had been matched at least once to the reference gel. Unique spots in nonreference gels could never receive matchids (since there was no reference gel spot with which they could be matched), and they could not be extrapolated to other gels.

Image analysis: GELLAB-II system

The GELLAB-II image analysis system has been described previously (Lemkin, 1989; Lemkin & Lester, 1989; Lemkin, 1992). The current system (version 1.3.52) runs on Sun 3 and Sun 4 computers under the X-Windows (version X11R4) graphical user interface (Quercia & O'Reilly, 1989). Initially, the GELLAB-II program *PGELRC* was run to set up a project directory tree and a project 'state file.' The *MAKJOB* program was then used to create UNIX batch scripts that permit most of the GELLAB-II image processing steps to be largely unattended.

The images that were processed by GELLAB-II were the same Visage-scanned images that were converted to the GELLAB-II ppx-image format by the program *PPXCVT*. The resulting GELLAB-II images were still 1024 × 1024 × 8 bits in size, and the original grayscale-to-OD calibration was retained. These GELLAB-II images were 'accessioned' into the project by the *GETACC* program, which interactively queried the user for gel and sample information to set up or add to an accession database file that was used by all other programs. Spots were detected and segmented (quantified) by the program *SG2GII* with the following parameter values:

- lowpass averaging filter = 3 × 3 pixels
- Busse–Laplacian filter with central cone = 3 × 3 pixels
- saturated spot propagation filter = 99.7%
- split saturated spots when the central core >50 pixels
- background size filter = 64 × 64 pixels
- central core minimum size = 4 × 4 pixels

- draw boundaries and dots in the original gel image
- spot area limit = 25–10,000,000 pixels
- spot density limit = 0.0005–10,000,000
- spot OD limit = 0.0001–4.5 OD

The exaggerated upper limit of 10,000,000 for spot area and density limits was used to assure that no upper limit would be approached, even after dilution factors were applied to spot densities. Similarly, the spot OD upper limit of 4.5 was nonlimiting. The spot-splitting algorithm has been described recently by Lemkin *et al.* (1992). The resulting density values were the sum of the background-corrected OD values of all pixels within a spot boundary.

After segmentation, each gel was interactively assigned the maximum allowed number of 52 landmarks (usually spots, but sometimes x and y locations when a spot was absent) that were common to every gel with the program *LANDMARK11*. The *LANDMARK11* program operated under batch control; that is, after the operator completed the selection of landmarks for the reference gel and another gel, the batch program would automatically display the reference gel and the next gel until all gels in the project were landmarked. After landmarks were chosen for the reference gel and the other initial gel, they could not be changed—only the subsequently displayed nonreference gels could receive new landmarks, with the simultaneously displayed reference gel serving as a template. As a result, all gels were assigned the same 52 landmarks.

The gels were then matched to a reference gel (the same image as in the Visage analysis) with the *CMPGL2* program with the following parameter: ChangeThreshholds:T1 = 10 pixels, T2 = 20 pixels. The *CMPGL2* program outputs five types of spot matches, or pairs, between a study gel and the reference gel. (1) Sure Pairs—spots that met all matching criteria. By definition, landmark spots were sure pairs, and other nearby matches were usually sure pairs. (2) Possible Pairs—spots whose matching strength was somewhat weaker than sure pairs. These were often matches that were further away from landmarks than sure pairs. (3) Unresolved Spots—spots that were not found in the reference gel but were extrapolated into the reference gel's coordinate system with zero density and area. (4) Extrapolated Pairs—spots that were not found in one or more gels. Their x-, y-coordinates were extrapolated to where they should have been had they existed, and their densities were set to zero. (5) Ambiguous Pairs—multiple spots in one gel that could be matched to a single spot in another gel. These were typically spots that were far away from a landmark. For the comparisons in this study, only sure pairs, possible pairs, unresolved spots, and extrapolated pairs were used, although ambiguous pairs were retained in the composite database. These ambiguous pairs consisted either of spots that sometimes fragmented or multiple spots that were paired.

The *CGELP2* program automatically created a protein spot database from the paired spot information and other sample-associated data. Specific, matched spots from the GELLAB-II analysis that are discussed here were given the same matchid numbers as those from the Visage analysis so that the reader could more easily compare the results in this report and in that of Myrick *et al.* (1993).

All of these GELLAB-II operations were performed under batch control by unattended scripts. Because the GELLAB-II system has no image editor program, there were no opportunities for interactive editing of spot centroids, boundaries, or matches.

The *CGELP2* database program performed statistical analysis either while unattended and under batch control or under the control of an operator using a dynamic, graphical user interface to any chosen gel, usually the reference gel. In addition, *CGELP2* could be operated through the nongraphical interactive command interface. A particular spot could be queried, or the entire study database could be queried for spots that met particular conditions (prefilters), after which specific tests such as t-tests could be performed. *CGELP2* was also used to create ASCII files containing the entire GELLAB-II spotlist, including extrapolated spots (with zero density), so that regression analysis (not yet available by *CGELP2*) could be performed by the same SAS program that was used with the Visage results.

The density values for both the Visage and GELLAB-II analyses were corrected by the local background for each detected spot and by a factor that corrected the densities per ml of original urine—a dilution factor. In addition, a normalizing factor for each gel was computed to correct an image's overall density from the mean density of a group of 22 spots of rabbit-muscle glyceraldehyde-3-phosphate dehydrogenase (G3PDH, used as an internal charge calibrator in each gel), the addition of which was made in the same accurately controlled quantity to each isoelectric focusing rod. The approximately 100-fold disparity between the density values reported by the two systems resulted from the differences in the placement of the decimal point when the OD correction was made to the step wedge energy values during scanning.

Outlier determination

One sample had an abnormally large number of low molecular weight proteins in the 2DE pattern, making the gel appear radically different from others. Adequate interactive landmarking of spots in the lower half of this gel was impossible. The image analysis data for this sample was not included in the Visage or GELLAB-II databases.

Statistical methods

The methods for minimizing any confounding effects attributable to sample exposure status are described elsewhere (Myrick *et al.*, 1993). During the Visage interactive analysis, the operator was blinded to the identity of the sample for each gel and to the cadmium concentrations. This was unnecessary during the GELLAB-II analysis, since all spot detection, quantification, and matching was automated.

The corrected spot densities from each system were compared by using error in both variables (EBV) regression analysis (Cornbleet & Gochman, 1979). This regression procedure produces estimates of the intercept and slope that linearly relate the results of one method to those of the other, taking into account the measurement error in both methods. A more

general discussion of the statistical analysis of 2DE data can be found elsewhere (Caudill *et al.*, 1993). Proof of the quantification validation for the spot densities is presented elsewhere (Myrick *et al.*, 1993).

Results

Spot detection

Twenty-nine digitized images of two-dimensional electrophoresis gels with silver-stained protein spots were analysed with the Bio Image Visage 2000® and the GELLAB-II systems. The Visage system, aided by interactive analysis, detected an average of 889.7 ± 177.6 spots per gel, or a total of 25,800 spots. The GELLAB-II system, without any operator involvement, detected an average of 1971 ± 198.5 spots per gel, or a total of 57,160 spots, a 222% increase over the number found by the Visage system.

Spot quantification

The Visage system was able to accomplish correct spot segmentation only with extensive interactive control by an operator using the excellent Visage image editor. Of the 25,800 spots that were detected during the Visage analysis, 13,556 (52.5%) were detected and quantified automatically, 12,173 (47.2%) were interactively detected and quantified, and 71 (0.3%) were not quantified. After reviewing the data retrospectively, we found that the spot densities seemed to be influential in determining whether interactive quantification would be needed: spots that were quantified automatically had a mean density of 0.27 ± 0.429 (median = 0.13), and spots requiring interactive analysis had a mean density of 1.18 ± 3.07 (median = 0.44), which shows that larger spots were much more likely to be misquantified and thus require interactive analysis. Interactively quantified spots represented 79.8% of the total density of all spots in the Visage analysis. Calculations involving spot areas showed the same trend as those involving spot densities: automatically quantified spots had a mean area of 1.25 ± 0.659 mm^2 (median = 1.1), and interactively quantified spots had a higher mean area of 3.01 ± 5.38 mm^2 (median = 1.71).

Because the current version of the system has no image editor, the GELLAB-II system could not be used to interactively edit the 57,160 spots that were detected in the 29 gel images. Through visual inspection of the gel images showing spot locations and boundaries, we found that both large and small spots were quantified with little or no obvious error by the parameters that were used for the *SG2GII* program.

Figure 1 shows the results of spot quanitification and boundary detection by the two systems on the same digitized gel image. Figure 1(A and B) illustrate spot boundaries as determined by the Visage system before (A) and after (B) interactive editing by the operator. Figure 1(B) illustrates a nearly perfect segmentation of the gel region, since the operator could use the original wet gel as the definitive spot pattern to manually draw spot boundaries and locations based upon the actual stained gel viewed on a light box and the spots' slight color hues, which a human can visually discern but which the imaging systems cannot detect. Figure 1(C) shows the same region after automatic spot detection and quantification by the GELLAB-II system. The GELLAB-II results clearly show the superior automatic segmentation that is obtained without labor-intensive image editing.

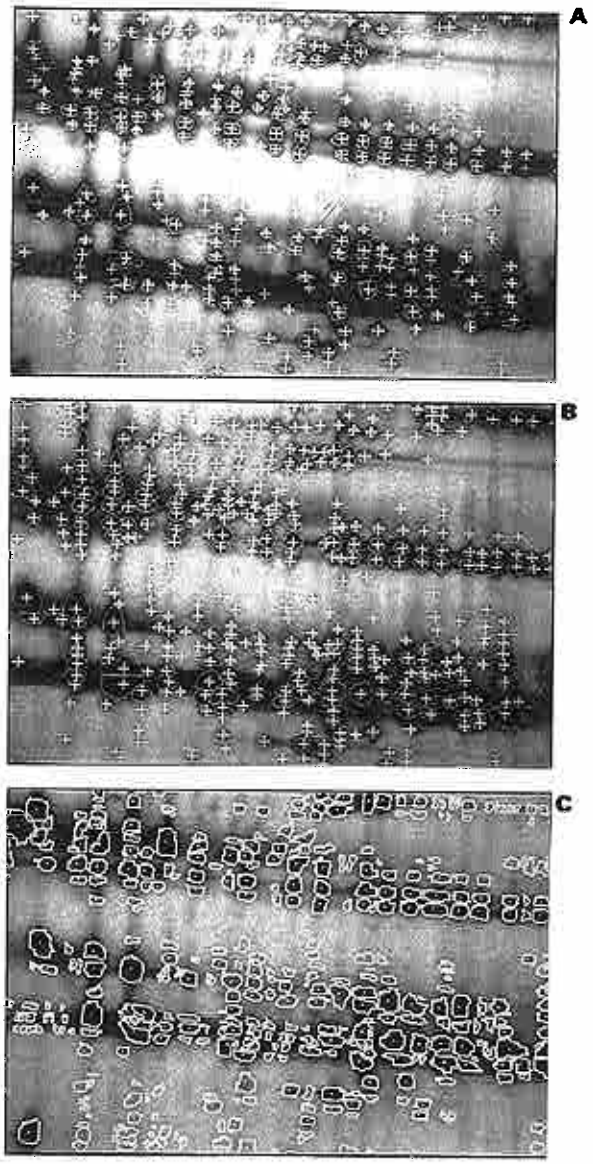

Figure 1 Photographs of the computer monitor display of the identical region of the same gel image (**A**) after automatic spot detection and quantification with the Visage system, (**B**) after operator-controlled, interactive analysis with the Visage system, and (**C**) after automatic spot detection and quantification with the GELLAB-II system

Spot landmarking

Both systems required interactive selection of spot pairs that the operator considered to be unequivocal matches between each gel and the reference gel. The Visage system allowed up to 30 'matchpoint' (landmark) spots per gel to be chosen; each pair of gels (the reference gel and a study gel) could thus receive 30 completely

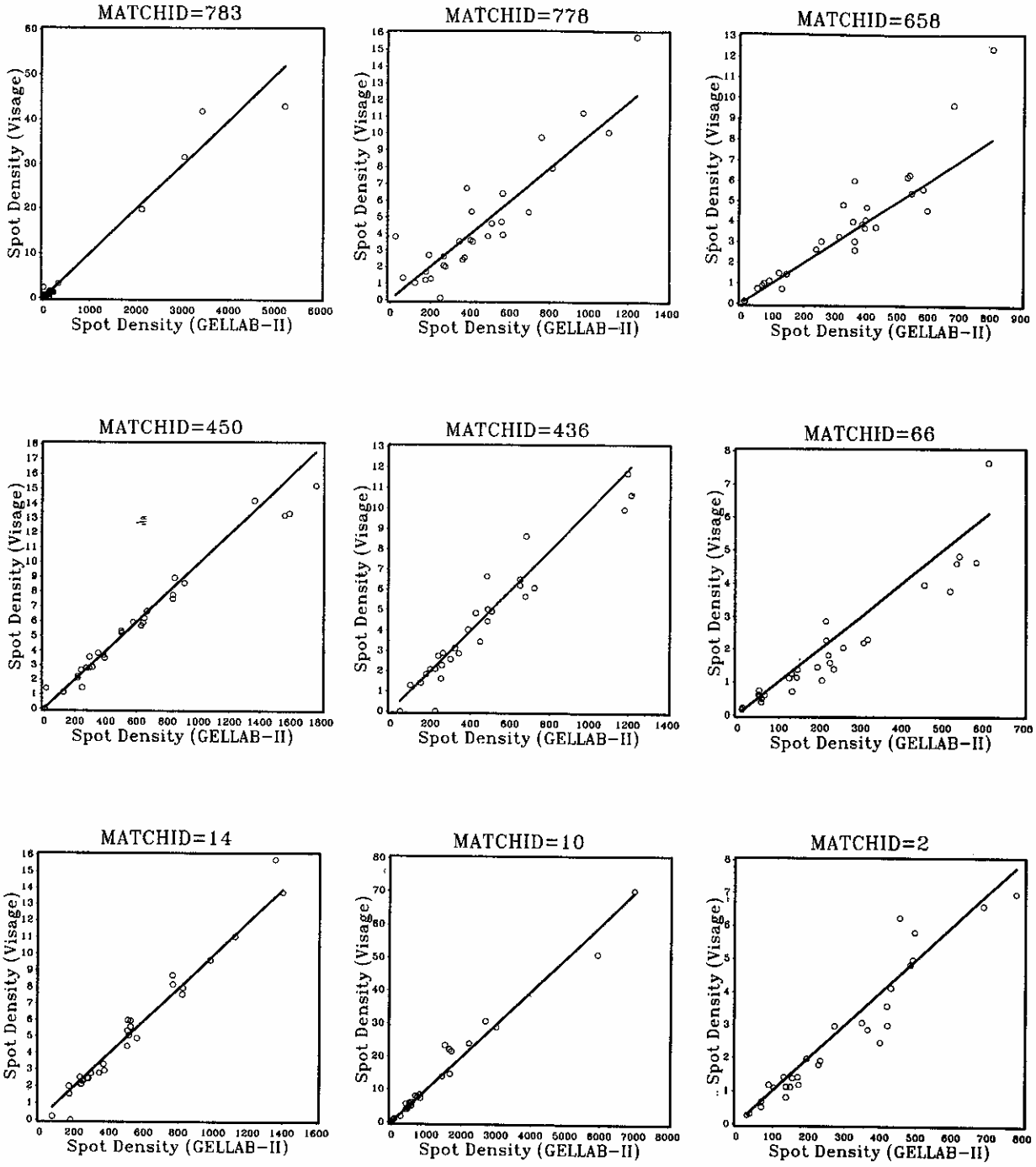

Figure 2 Comparisons of corrected densities of nine individual protein spots (matchids) that were quantified by the Bio Image Visage 2000 and GELLAB-II systems. The regression lines were determined from an error in both variables regression analysis of Visage results versus GELLAB-II results. β-2-microglobulin is matchid 783

different landmarks. A maximum of 52 landmarks per gel were allowed during the GELLAB-II analysis, but they were required to be the same 52 spots (or *x*-,*y*-coordinates) in all reference-study gel pairs.

Spot matching

After interactive analysis was performed and correspondences and contradictions were corrected with an SAS program, the Visage system matched an average of 657.2 ± 211.2 spots per gel to spots in the reference gel. This was 73.9% of the total number of detected spots and represented 85.3% of the total density of all detected spots. The Visage system created 402 correspondences and 320 contradictions.

The GELLAB-II system automatically found an average, per gel, of 3304 ± 182.1 unresolved spots, sure pairs, possible pairs, or extrapolated pairs for a total of

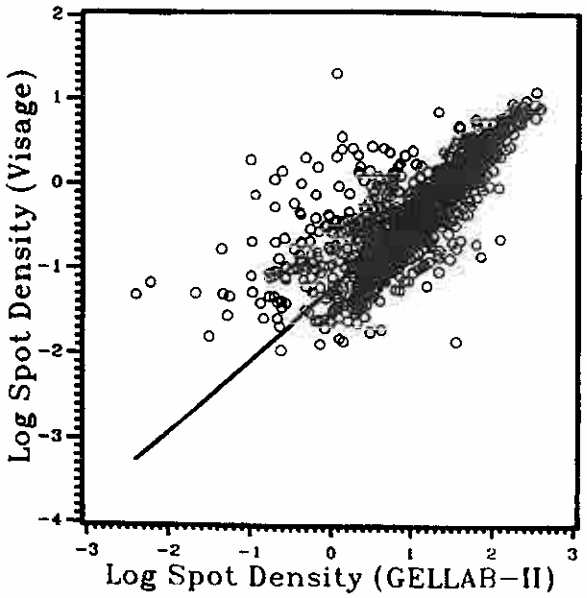

Figure 3 Comparison of the logarithms of the corrected densities of 91 matchid numbers that were quantified by the Bio Image Visage 2000 and GELLAB-II systems. The regression line was determined from an error in both variables regression analysis of Visage results versus GELLAB-II results. The computation involved 2207 spot pairs. The slope is 0.838, the *y*-intercept is –1.24, and the r^2 value is 0.664

95,356 matches. The average number of extrapolated (virtual) spots was 1269 per gel. There were 4843 (5.1%) unresolved spots, 16,727 (17.5%) sure pairs, 37,443 (39.1%) possible pairs, and 36,808 (38.4%) extrapolated pairs. Unresolved spot densities included 1.7% of the total spot densities from the GELLAB-II analysis, sure pairs included 48.2%, and possible pairs included 50.1%. By definition, extrapolated spots were assigned zero densities.

Comparisons of selected spot densities

We were able to directly compare the output from the Visage system with that from the GELLAB-II system by finding a protein's Visage matchid number and the GELLAB-II spot number and plotting the density obtained from one system versus that from the other. Several comparisons of spot densities are shown in Figure 2, indicating the general agreement of density values between the two systems. Figure 3 shows a comparison of the $\log_{10}$ densities in all 29 gels of 2207 spots representing 91 matchid numbers for which cross-system matching could be accomplished. The slope is 0.838, the *y*-intercept is –1.24, and the r^2 value is 0.664.

A comparison of the densities of a known β-2-microglobulin (β2M) spot (Edwards *et al.*, 1982) with RIA-determined concentrations of β2M in the original urine samples is shown in Figure 4. This comparison shows that spot densities from both image analysis systems also agreed with independently measured concentrations of the protein represented by that spot.

The SAS data sets containing results from the two image analysis systems were queried for spots that best correlated with UCD. The results of the EBV regression analyses of spot densities versus UCD levels are shown in the 'bubble' plots (SAS, 1990) of Figure 5. The schematic plots show the *x*-, *y*-coordinate positions of all matched spots as circles whose radii are inversely proportional to the *P*-value of the regression analysis: larger bubbles represent proteins with the

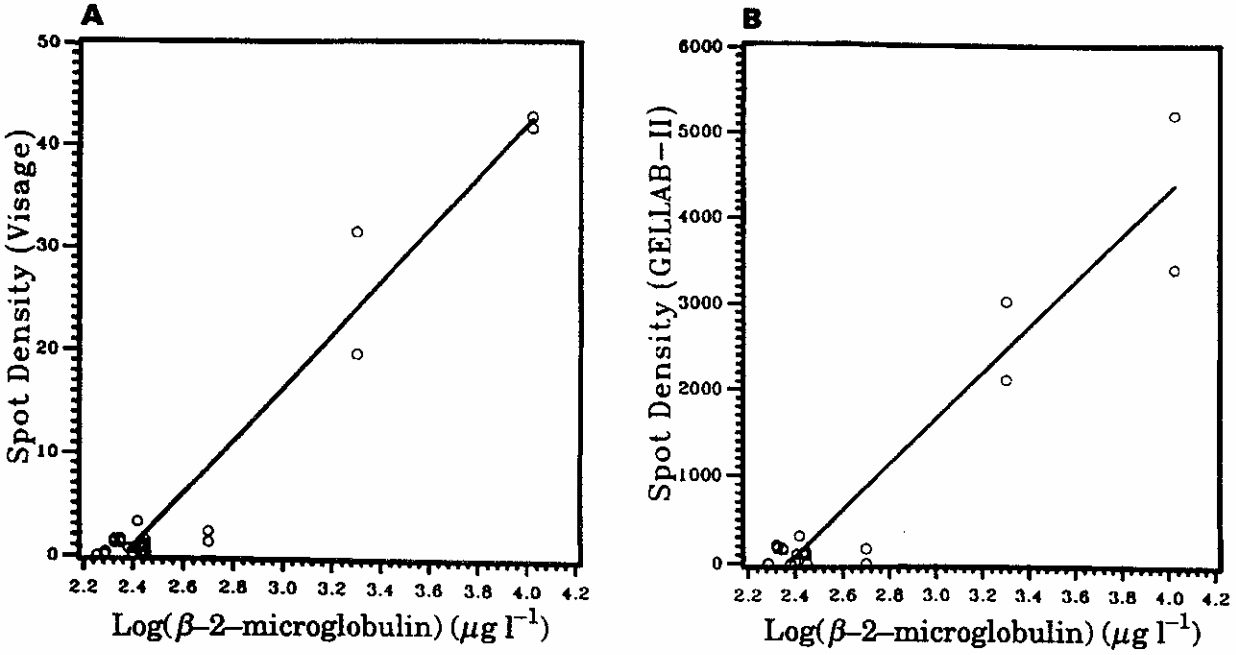

Figure 4 Methods comparison of the corrected densities of the primary β-2-microglobulin spot (matchid number 783) from the Bio Image Visage 2000 (**A**) and GELLAB-II (**B**) systems versus the $\log_{10}$ of the concentrations of β-2-microglobulin as determined by radioimmunoassay in the original urine samples. The solid lines are the regression lines obtained from an error in both variables regression analysis

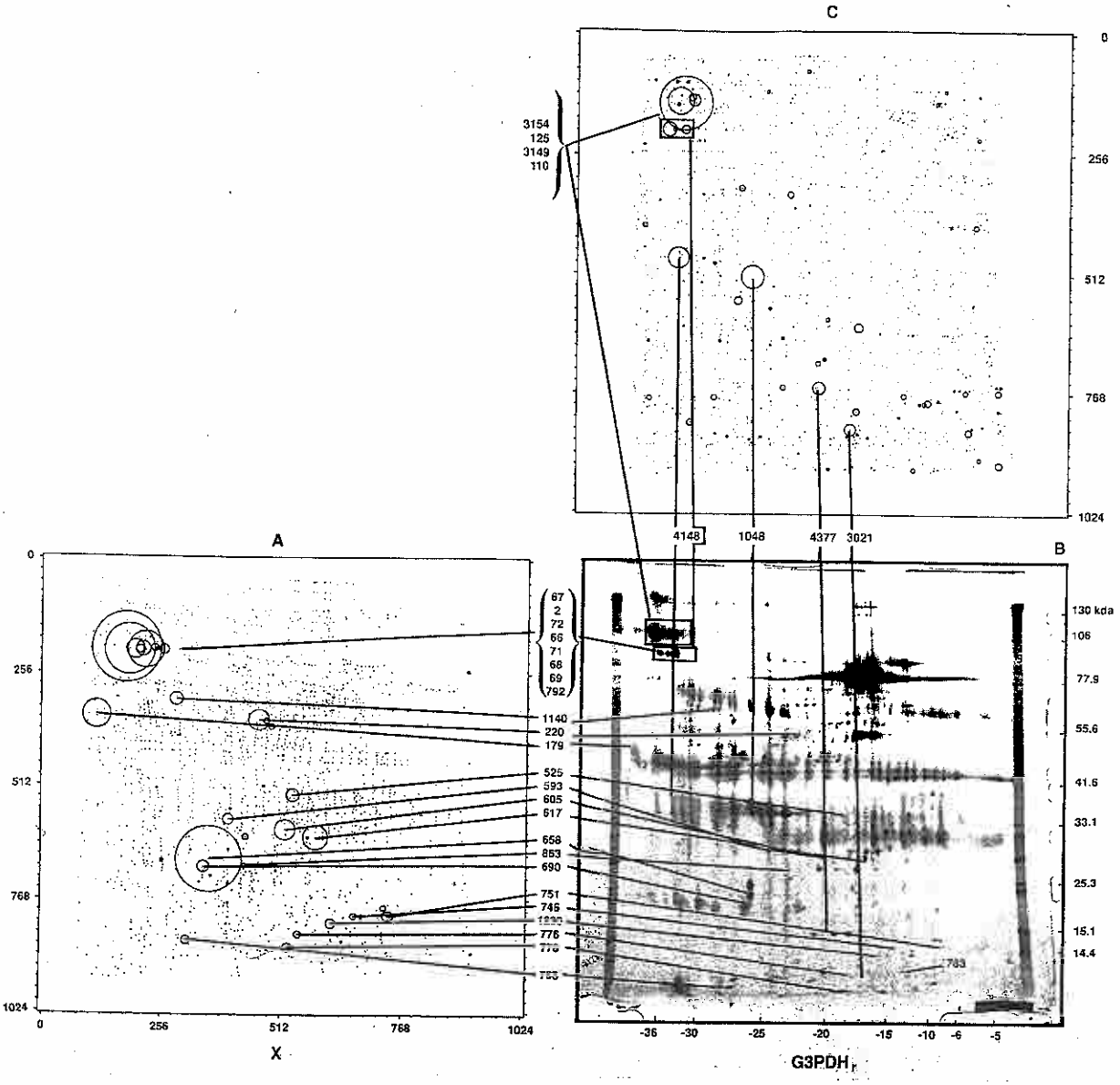

Figure 5 *P*-value bubble plots of data from the Visage 2000 system (**A**), the GELLAB-II system (**C**), and a photograph of a two-dimensional electrophoresis gel (**B**) of urinary proteins showing the locations of the protein spots that were the most significantly correlated with urinary cadmium levels. The schematics shown in parts **A** and **C** represent the *x*-, *y*-coordinates of all matched proteins in the study. The size of the circles are inversely proportional to the observed significance values (*P*-values) when the corrected spot densities are regressed against the urinary cadmium concentrations in all subjects. The indicated matchid numbers represent proteins with *P*-values from either image analysis system of less than 0.002. Matchid number 783 is shown in part **B** to illustrate the location of β-2-microglobulin

most significant correlation (lowest *P*-value) with UCD levels. There were 42 spots that exhibited high correlation ($P < 0.002$) versus UCD levels in the data from the GELLAB-II system and 19 spots that met this criteria from the Visage analysis. Eight of these 19 spots (67, 179, 1140, 220, 71, 690, 751, and 792) were manually segmented by the Visage system operator but were not automatically detected and segmented as individual spots by the GELLAB-II system. The regression analysis of the GELLAB-II data revealed 26 additional spots that were highly correlated with UCD levels, but the Visage data for these spots were unavailable for regression analysis. These 26 spots were not assigned matchid numbers by the Visage system, since they were absent in the reference gel. Table 1 lists the protein spots having the most significant correlation with UCD levels when data from both image analysis systems were analysed.

Table 2 shows an analysis of labor time and costs involved in analysing gel images. Estimates, shown for the use of both Visage and GELLAB-II systems, were made by recording the computer times for automatic analyses and the operator times for interactive analyses. The estimated time needed to complete the image analysis and create a database for a 20-sample study in which each sample is analysed in triplicate was

Table 1 Comparison of results of the Visage 2000® and GELLAB-II image analysis systems for the spots most highly correlated with urinary cadmium levels

Matchid	*Regression P-value from Visage data*	*Regression P-value from GELLAB-II data*
3154	NA	0.00012
125	NA	0.00023
1048	NA	0.00027
4148	NA	0.00029
4377	NA	0.00048
3021	NA	0.00053
3149	NA	0.00053
1368	NA	0.00072
1306	NA	0.00081
110	NA	0.00086
4389	NA	0.00092
67	0.00022	NA
658	0.00023	0.01260
2	0.00029	0.00045
72	0.00037	0.00077
66	0.00072	0.00156
179	0.00077	NA
617	0.00085	0.00072
1140	0.00087	NA
220	0.00094	NA
605	0.00099	0.00017
71	0.00101	NA
690	0.00149	NA
778	0.00155	0.00956
69	0.00177	0.00286
68	0.00181	0.00099
751	0.00183	NA
792	0.00191	NA
763	0.00196	0.00301
1857	NA	0.00130
593	0.00198	0.00207
2934	NA	0.00118
1750	NA	0.00128
4467	NA	0.00105
1793	NA	0.00096
3869	NA	0.00155
4212	NA	0.00184
602	NA	0.00162
3827	NA	0.00185
2641	NA	0.00110
3876	NA	0.00102
1000	NA	0.00135
4374	NA	0.00122
1551	0.00920	0.00128
985	0.78860	0.00048
727	0.30394	0.00118
1577	NA	0.00121
750	0.05700	0.00158
1082	0.69027	0.00097

NA = Spots were not segmented or segmented spots were not matched

Table 2 Comparison of estimated time and labor costs of analysing two-dimensional gels with the Visage 2000® and GELLAB-II systems

	Time required (per gel)		*Labor cost[a] (per gel)*	
	Visage	*GELLAB-II (h)*	*Visage*	*GELLAB-II ($)*
Image analysis & database creation	7.76	1.32	194	33
Totals for 20 samples (60 gels)	466	79	11,650	1975

[a]Labor costs at $25 per h

466 h for the Visage system and 79 h for the GELLAB-II system. At $25 per h for labor costs, the total estimated cost for the hypothetical study would be $11,650 with the Visage system and $1975 with the GELLAB-II system. The purchase cost of the Visage system or the hardware was not considered, as such expenditures are normally amortized.

Discussion

The quality of 2DE gels and their image analysis has been improving incrementally from the days of early qualitative-only analysis (O'Farrell, 1975) to today's highly sophisticated, iterative, and nearly automatic computer-processing that is able to create composite databases and to perform initial data integrity checks and subsequent unattended analyses. As gel chemistries and techniques have steadily improved to yield better gel matrices (Righetti, 1989; Patton *et al.*, 1992), better spot resolution (Hochstrasser, 1988), and even increased loading capacity (Hanash *et al.*, 1991), the need for accurate quantification of stained spots has become a necessity rather than a luxury.

2DE gels in the ISO-DALT format of 16 × 16 cm (Anderson & Anderson, 1978a; Anderson & Anderson, 1978b) can easily resolve more than 1000 proteins. Larger formats such as 20 × 25 cm (Patton *et al.*, 1990) or giant gels (Young, 1984; Levenson *et al.*, 1990) can resolve many more spots. It is virtually impossible to accomplish a thorough, qualitative analysis of these gels by visual inspection, and quantitative analysis is only feasible by using machine measurements to obtain pixel OD values.

The two image analysis systems that we compared were able to detect the individual energy values associated with an image pixel and to determine which clusters of pixels represented stained proteins. The accuracies with which the two systems detected spots and determined spot boundaries were quite different, however, especially for spots closely adjoining (overlapping) other spots (Figure 1). Repeated titration of the segmentation parameters of the Visage system could not produce a combination that would segment both large and small spots equally well. At one extreme, the segmenter would correctly quantify large spots, but would ignore smaller ones; at the other, the segmenter would break up large spots into several pieces but would correctly detect and determine the boundaries of smaller ones. We chose the parameter set that would best segment the smaller spots, leaving the larger ones for the spot editor to segment interactively. The rationale for this was that there were far more small spots than large, and the least operator time would be needed to manually draw boundaries around the large spots. One consequence of interactive editing was the increased labor time (Table 2) needed for analysis by the Visage system.

The protein spots found among the gels in a 2DE study must be matched properly if comparisons of protein concentrations with some other variable (such as case-control classification, toxicant concentration, or disease state) are to be statistically valid. When the matching is accurate, one is assured that computations involving the densities of all spots with a common matchid number are representative of a single protein

variant. Only then can researchers be assured that a statistically significant regression, *t*-test, or other measurement is valid. Both image analysis systems performed the spot matching two gels at a time; that is, the same reference gel was always matched against each of the other gels in the study.

The key to good matching performance is that the operator first choose good landmarks—spots that are unequivocal matches—usually by viewing the original wet gels on a light box. The Visage landmarking operation was preferable to that of GELLAB-II, because for each gel pair (the reference gel and another gel) a different set of 30 landmarks could be chosen that best characterized the pair. The GELLAB-II system permitted 52 landmarks to be chosen, which clearly could better represent a gel pair's common spot patterns. However, the 52 landmarks had to be the same 52 spots or *x*-,*y*-coordinates for all gels. But for a moderately sized study, such as the one reported here, it is almost impossible to pick 52 common spots by initial visual inspection. Once the 52 landmarks are chosen for the reference gel, they cannot be changed. The operator is thus forced to choose an *x*-,*y*-coordinate that best approximates where a missing landmark spot would be.

The matching accuracy of the two systems can be evaluated by several means. For Visage, one can use the menu selection *COMPARE* to visualize two gels that have been previously matched, usually the reference gel and another gel. Using an annotation option that shows match vectors (straight lines originating from a spot's centroid showing the relative direction and distance that two matched spots differ between two gels), the operator can visually detect mismatches by seeing which vectors are 'outliers' by virtue of their length or direction compared with other vectors in the immediate vicinity. The operator can then correct any mismatches with a variety of convenient editing commands. Another way to detect mismatches with Visage is with the *INVESTIGATOR* menu option, which allows the operator to choose the reference gel and 12 other gels for simultaneous display on the screen. A particular region of each gel can be displayed 'zoomed in' to see a closeup view. By clicking on a spot, the operator can mark matched spots in the other displayed gels by their matchid numbers and quickly see whether a mismatch occurred. Because the operator can display only 13 gels at one time, the utility of *INVESTIGATOR* is limited in studies with more than 13 gels. (However, once the 13 gel images are displayed, the operator can query for many matchids before displaying different images.) Another limitation to *INVESTIGATOR*'s utility is that editing is not allowed within the program; the operator has to use *COMPARE* to make changes to spot matches.

The GELLAB-II *MOSAIC* program is more automated and allows the operator to display or print individual matched spots simply by invoking the program along with a pertinent spot number. The resulting mosaic includes closeup views of the spot in every gel of the study, allowing quick visual determination of matching accuracy for any chosen spot. Although a new mosaic must be generated for each desired spot, such generation is not difficult, since a single batch file command can be invoked or a menu item selected from *CGELP2*. With the current version of GELLAB-II, mismatched spots cannot be edited to correct the database. For this report, we adjusted for any known false matches by deleting the pertinent SAS observations before conducting regression analysis.

Another difference between the two systems deserves mention, because of its importance to the interpretation of the resulting spot data. Visage spot matching only assigns matchid numbers to spots when the spot is present in the reference gel. Such an algorithm results in spots being excluded from the matchid database when they are present in study gels but missing from the reference gel. One way to avoid such exclusions is to have more than one reference gel to which all other gels are matched. However, numerous correspondences and contradictions would then be created which would have to be resolved, a time-consuming task. Further, for all spots to be included in the AGM database, each gel would have to serve as the reference gel at least once for all other gels. This requirement results in a geometric progression in the necessary number of AGM runs (and match editing procedures) that would be prohibitively labor-intensive for all but the smallest studies. One other Visage innovation was the *COMPOSITE* program, which allows the operator to combine the matched data from several gels and to save the results in a synthetic image that can be used in subsequent matches as if it were an actual gel. All spots, regardless of which gel they are found in, are put in the synthetic image. We spent some time with this program, but because of errors in the location of new spots in the synthetic gel, we had to abandon our efforts. Unless one includes these virtual spots in gels from which the real protein spots are missing, one cannot perform statistical analyses on *all* spots in the gels.

GELLAB-II handles spots that are present in study gels but absent in the reference gel by classifying them as unresolved spots and giving them estimated *x*-, *y*-coordinates and densities of zero in the reference gel. The database can also be extrapolated to add any missing spot in any gel to its estimated position in all other gels and with a density value of zero. Thus, because GELLAB-II allows researchers to correctly analyse missing proteins, they can perform satisfactory statistical analyses on the entire complement of proteins in the gels regardless of whether a protein was missing from any gel.

Because of the large differences between the two systems in detection, quantification, and matching, they produced both similar and dissimilar results in EBV regression analyses of spot densities versus UCD levels. The agreement between the two systems are worth noting first, as six out of seven of the acidic, 88–90 kDa proteins that were found to be highly significant in the Visage results (Figure 5(A) and Myrick *et al.*, 1993) were also highly significant in the GELLAB-II results (Figure 5(B) and Table 1). Eleven other spots similarly detected by both systems had regression *P*-values of less than 0.01 according to at least one database analysis. These agreements between the two systems served to confirm the analyses of these gels, as the same conclusions could be drawn from two independent sources of image analysis data.

The most significant regressions from the GELLAB-II data were for four proteins (matchids 110, 125, 3149, and 3154) with acidic isoelectric points and M_r values of approximately 112 kDa. These proteins are in the region of the gels where the Tamm–Horsfall glycoprotein migrates, but a positive identification has not been made yet. We consider these proteins to be candidate biomarkers of cadmium nephrotoxicity. Disparate results between the two systems include spots detected by Visage but not by GELLAB-II, and *vice versa*. The former included eight spots (Table 1) detected by the Visage operator through interactive editing; the coordinates and densities of these eight spots, however, were not sufficiently analogous to any found by GELLAB-II and, hence, were not regressed against UCD levels. Similarly, 26 spots were found by GELLAB-II but were not found by automatic or interactive Visage analysis. The above candidate biomarkers are included in this group.

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