Quantitative Computerized Analysis of Silver-Stained and Coomassie Blue-Stained Two-Dimensional Protein Maps

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Two-Dimensional Electrophoresis, Protein Mapping, Computerized Analysis of Proteins, Mutations, Embryological Malformations

Using the two-dimensional gel electrophoresis and two systems for computer analysis of the resulting maps, we obtained a good quantitative reproducibility of individual spots of silver-stained and Coomassie Blue-stained gels from mouse fetus liver samples. The GelScan-XL system was established for the evaluation of silver-stained gels. The Gel-Image software has shown to be capable of the analysis of Coomassie Blue-stained gels and autoradiographs. For these systems the linear relationship between defined concentrations of protein loaded on the gel and the resulting integrated intensities of spots were examined. The results show that both systems are efficient to detect quantitative changes in protein expression correlated with malformations in mouse fetuses.

Introduction

Two-dimensional gel electrophoresis (2DE) of proteins is a technique which allows separation of hundreds of gene products (O'Farrell, 1975). The usefulness of high-resolution 2DE in order to study the expression of mutations has been demonstrated by several authors. Protein mapping by means of 2DE was described as a method to test for chemically induced point mutations in mice (Klose, 1977) and to study the genetic variability on different inbred mouse strains (Klose and Putz, 1983). Baier *et al.* (1984) have examined radiation-induced chromosomal deletions at the albino locus region resulting in a lack of specific polypeptides. The results of Giometti *et al.* (1987) indicated that 2DE can be used to detect mutations expressed as quantitative changes in protein expression.

The 2DE technique was used to answer questions concerning a correlation between differences in protein expression and abnormal development of mice in utero. Thus, malformed fetuses developed from blastocysts exposed to N-methyl-nitrosourea (MNU) show alterations in protein expression related to mutations occurring after the chemical exposure to the embryos (Bossert and Iannaccone, 1985; Bossert *et al.*, 1990). Our results (Hillebrandt and Streffer, 1994) demonstrate an increased frequency of changes in protein expression in the liver of mouse fetuses with gastroschisis induced by X-rays exposure of the zygotes compared with fetuses without the malformation. Gastroschisis is a herniation of abdominal viscera without a covering membranous sac. It was assumed that the protein changes detected in late stages of prenatal development (19 days after conception) result from mutations as the radiation exposure of the embryos took place during the 1-cell stage (Pampfer and Streffer, 1988).

Several investigators (Anderson *et al.*, 1990; Cellis *et al.*, 1991) studied the quantitative analysis of 2DE protein patterns using various computer programs. In this paper we describe the computer-assisted quantitative analysis of 2DE gels of liver proteins from the day 19 mouse fetuses with the GelScan-XL software acquired from Pharmacia LKB and the Gel-Image software acquired from Pharmacia Biosystems. First, we analyzed silver-stained gels with the GelScan-XL system to examine the quantitative reproducibility of the protein patterns of the liver. Secondly, we analyzed Coomassie Blue (CB)-stained proteins of the liver and autoradiographs of phosphorylated proteins of the skin with the other system. The results reveal a

Abbreviations: 2DE, two-dimensional electrophoresis; DTT, dithiothreitol; PMSF, phenylmethylsulfonylfluoride; CHAPS, 3-[(cholamidopropyl)dimethylammonio]-1-propanesulfonate.

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good quantitative reproducibility of the analyses of the protein patterns on silver-stained gels analyzed with the GelScan-XL software. However, when these gels were analyzed with the Gel-Image system unsatisfactory results were obtained. On the other hand the usefulness of the Gel-Image system for the quantitative analysis of the CB-stained gels and autoradiographs was demonstrated.

Materials and Methods

Sample preparation

Heiligenberger mice were killed by cervical dislocation on gestation day 19 and fetuses were collected. The proteins of the livers were isolated as described previously (Hillebrandt and Streffer, 1988).

The radiolabeling of cellular proteins was performed as follows: Slices of skin were incubated for 1 h at 37 °C under 95% O_2 and 5% CO_2 in 1 ml low-phosphate Krebs-Henseleit bicarbonate buffer containing 9.25 MBq carrier-free [^{32}P]phosphate. After the incubation the tissue was homogenized in 500 µl buffer containing 20 mM Tris/HCl, pH 7.4; 3 mM $CaCl_2$; 0.25 M sucrose; 10 mM sodium fluoride; 10 mM sodium vanadate; 10 mM disodium pyrophosphate and centrifuged for 60 min at 225,000×*g* in a Beckman ultracentrifuge. The proteins in the soluble fraction were precipitated by addition of 500 µl of ice-cold acetone and further incubated for 10 min on ice. The precipitated proteins were then pelleted by centrifugation at 10,000×*g* for 10 min at 4 °C. The pellet was dissolved in 50 µl of isoelectric focusing lysis buffer consisting of 9.5 M urea, 4% CHAPS, 2% DTT, 10% glycerol and 10% of a 50:50 mixture of Ampholytes, pH 3.5–9.5 and pH 4.0–6.5. The whole sample was loaded on the isoelectric focusing gel.

Two-dimensional electrophoresis

Supernatants frozen at −80 °C were thawed and aliquots (10 µl) were mixed with 4 vol. of the isoelectric focusing lysis buffer described above. The separation of proteins by isoelectric focusing was performed using 1.5×6×180 (mm) glass tubes (Bio-Rad) at 20,000 volt hours or in the case of radiolabeled proteins at 10,000 volt hours. A 50:50 mixture of Ampholytes, pH 3.5–9.5 and pH 4.0–6.5 was used to generate the pH gradient. For electrophoretic separation (second dimension) 10% SDS polyacrylamide gels were run (2 h at 15 mA and 3 h at 30 mA for 1 gel). Proteins were visualized by the silver-based staining technique according to Blum (1987).

Alternatively, 10 µl aliquots of the lyophilized protein sample were mixed with 40 µl of isoelectric focusing lysis buffer and proteins were separated by 2DE as described before. Then the gels were stained with the CB based method, which shows a lower sensitivity than the silver-based technique. The gels were fixed for 30 min in 12% TCA solution and incubated overnight in a solution consisting of 0.1% CB G-250, 1.2% H_3PO_4, 10% ammoniumsulfate and 10% (v/v) methanol (based on the work of Neuhoff *et al.* (1985)).

After separation of radiolabeled proteins the gels were fixed, dried and exposed for 2–3 weeks to Hyperfilm-MP (Amersham) for autoradiography at −80 °C.

Computer analysis

The dry gels were scanned in sections by the UltroScan XL laser densitometer (LKB).

Quantitative computer analysis of individual spots of the silver-stained gels was made with the GelScan-XL software consisting of a program for 2D gel-imaging, quantitative spot integration and background subtraction. The program calculates the spot intensity by integrating the densitometric data with areas of spots defined by the operator by drawing a contour and selecting the background. The background absorption was then subtracted from the total absorption of the single spots. The GelScan-XL system also contains a program for overlaying sections of two different gels which is useful for examination of the patterns for new or missing protein spots.

CB-stained gels and autoradiographs were analyzed with the Gel-Image software. This software contains a program for automatic spot recognition. The operator sets a threshold above which a spot should be distinguished from the background. Each of these absorbance levels is then shown in a specific colour on the monitor. In order to calculate the "volume" of a spot from the data file of the laser densitometer the program multiplies the

area of the spot (in mm^2) with the absorbance unit (AU). The AU corresponds to the gray level of the spot under the assumption of a Gaussian distribution of the protein within the spot. Since most spots are not of an ideal Gaussian form, fitting of irregular spots to this form was performed by the computer program. Areas of spots which have not been separated by the spot recognition, can be split in the corresponding subsets of spots by an erosion algorithm. A compression algorithm of Gel-Image reduces data received from the densitometer enabling a shorter time of evaluation. Fig. 1 demonstrates a compressed section of a silver-stained gel.

In order to find quantitative protein changes a section of the gel containing a spot of interest was compared with the corresponding spots of 10 control gels. For these control gels, mean integrated intensities of several reference spots and the standard deviations were estimated. With this method an average control gel could be defined. For a section of a particular gel containing the spot of interest the ratio of the integrated intensities of the reference spots to the mean integrated intensities of these spots from the corresponding section of the control gel was calculated. The integrated intensity of the spot of interest was then divided by the average of these ratios. Such a correction of the integrated intensity of spots measured by the computer programs is needed in order to normalize various gels with regard to the overall stain intensity, which can vary because of the variability in the loading or staining of the gels. The adjustment method used by us was described by Kuick *et al.* (1987) as one of the basic methods that seeks to remove technically caused variability of spots.

The calculation of the mean integrated intensities and standard deviations of the spots of the control gels was performed with a statistic computer program.

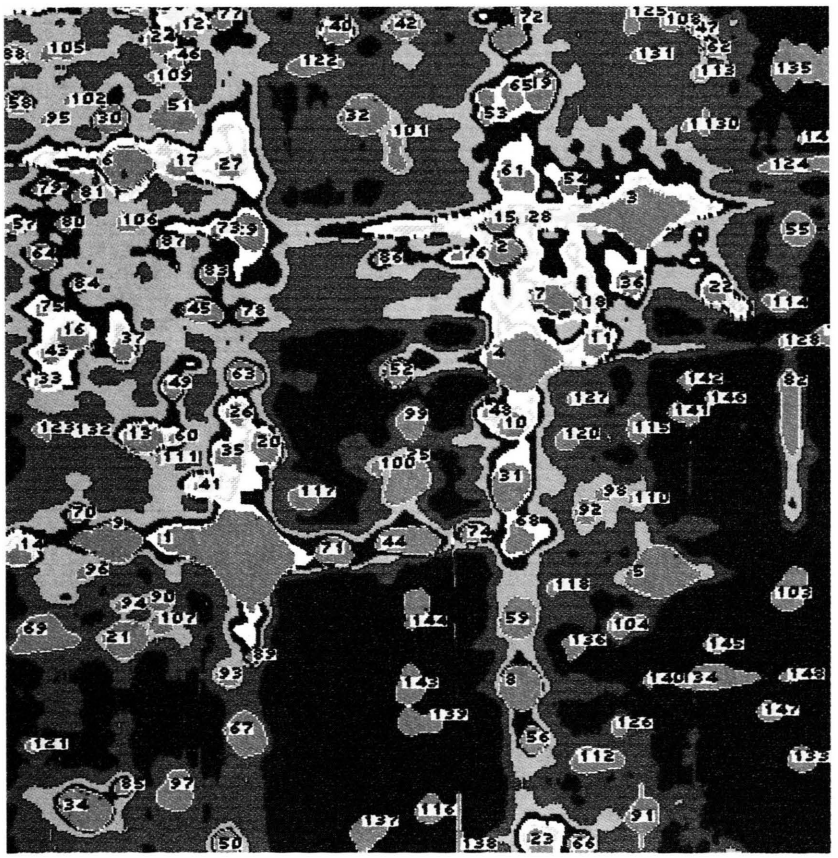

Fig. 1. Computer printout of a section of a silver-stained gel evaluated with the 2DEVA software.

Results and Discussion

Quantitative reproducibility of silver-stained protein maps analyzed with the GelScan-XL software

We ran a series of 10 20×25 cm 2D gels (Fig. 2) from 10 individual fetal liver samples and selected 40 spots present on all gels. For these spots, called reference spots, mean integrated intensities and standard deviations (SD) were estimated. The quantitative reproducibility of these spots was evaluated by examining the coefficients of variations (CV) calculated by the ratio of SD to the mean value. Our results indicate that 31 of 40 reference proteins have CV values of 0.12 to 0.15 and 2 proteins CV values of 0.16 and 0.17 respectively. Only 7 proteins show values smaller than 0.11 (CV) or larger than 0.20 (CV).

It was reported that spots with CV values of 0.15 or less have a good quantitative reproducibility (Giometti *et al.*, 1987). A major source of the large CV values appears to occur by overlapping between neighbour spots caused by the high density of spots on silver-stained gels (Fig. 2). On the other hand, when a series of 5 gels of the same liver sample was analyzed, 20 measured reference spots showed CV values not higher than 0.12. This indicates that the quantitative reproducibility of performance of 2DE gels on the same protein sample and their evaluation is very satisfying. The variation for protein patterns from malformed fetuses observed by us is therefore not the result of technical factors such as loading or staining of the gels, but is a consequence of a different protein expression in the liver of individual fetuses.

A very important question to be asked concerns the relationship between the concentration of protein loaded on the gel and the resulting integrated intensities of spots. This is extremely dependent upon the staining method selected and the conditions of staining. The standard curve for silver-stained gels established in our laboratory shows a linear relationship in the range of 0.01 μg to 1.3 μg bovine serum albumin (BSA standard grade, Fig. 4A). Care was taken for the further studies that the spot intensity measured lay within the linear range of the standard curve for silver-stained gels.

Quantitative reproducibility of CB G250-stained protein maps and autoradiographs analyzed with the Gel-Image software

Fig. 3 demonstrates the two-dimensional pattern of liver proteins after staining with CB G250. Compared to the silver-stained protein map (see

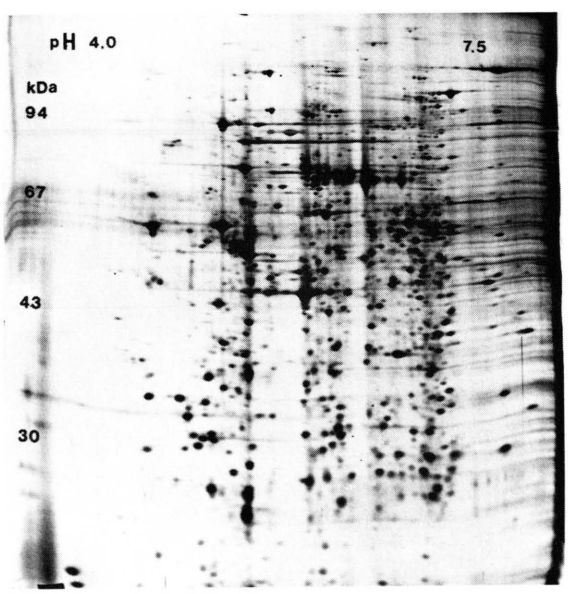

Fig. 2. A silver-stained 2-D gel of proteins from the liver of an unirradiated normal mouse fetus.

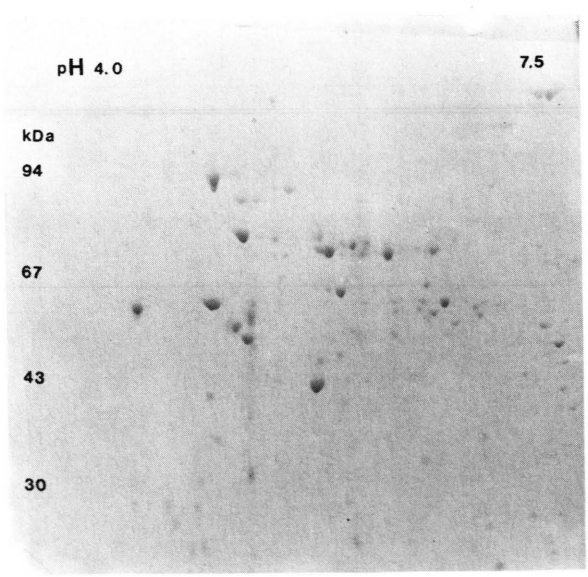

Fig. 3. A CB-stained 2-D gel of proteins from the liver of an unirradiated normal mouse fetus.

Fig. 2) a reduced background and no overlapping with surrounding spots was observed for CB G250-stained gels. On the other hand, the CB-based staining technique shows much lower sensitivity than the silver-based staining technique.

For 10 gels we have measured the CV values of a subset of 25 spots as the reference set. 21 of these spots show CV's not higher than 0.15 and 4 spots with a low amount of protein show values in the range of 0.16 to 0.17.

The colloidal nature of staining with CB was demonstrated by Neuhoff *et al.* (1985). Colloidal staining is, however, to a high degree a non-linear process with important consequences for the densitometric measurements (Pardowitz *et al.*, 1990). Such non-linearities result in a loss of information, which appears to be the main source of imprecision, and hence large CV values were calculated for spots with a low amount of protein. We have observed a linear relationship between the defined concentrations of a BSA standard and the resulting integrated intensities of spots in the range of 0.15 µg to 2.5 µg BSA (Fig. 4B). The intensity of measured protein spots are found within the linear range of the standard curve.

The two-dimensional pattern of phosphoproteins (autoradiograph) extracted from skin slices after 1 h of incubation with [^{32}P]phosphate is shown on Fig. 5. Six reference protein spots measured on 6 autoradiographs demonstrate CV values between 0.15 and 0.18. The cause of the high quantitative variation of these spots is the poor resolution of spots known for autoradiographs of electrophoretic separation of proteins labeled with ^{32}P. This is related to the comparatively long range of ionizing particles from ^{32}P decay.

We have tried to use the Gel-Image system for quantitative analysis of silver-stained gels. However, the high background and the high density of spots make the evaluation difficult. The spot-finding program very often recognizes sets of nearby spots as one very complex spot, which cannot be separated by the erosion algorithm.

Application in search for mutations

The determination of small quantitative changes in protein expression in mammalian tissues is of high importance for the detection of alterations of the genome and its expression. Quantitative changes of protein expression and qualitative changes of proteins are a highly sensitive parameter for detecting gene mutations induced by radiation or chemicals. The results of our previous studies (Hillebrandt and Streffer, 1994) demonstrate an increased frequency of alterations in protein expression in the liver of mouse fetuses with radiation-induced gastroschisis compared with fetuses without the malformation. It was assumed that the protein changes were due to mutations induced by the radiation exposure of the embryos during the one-cell stage. Apparently, quite a num-

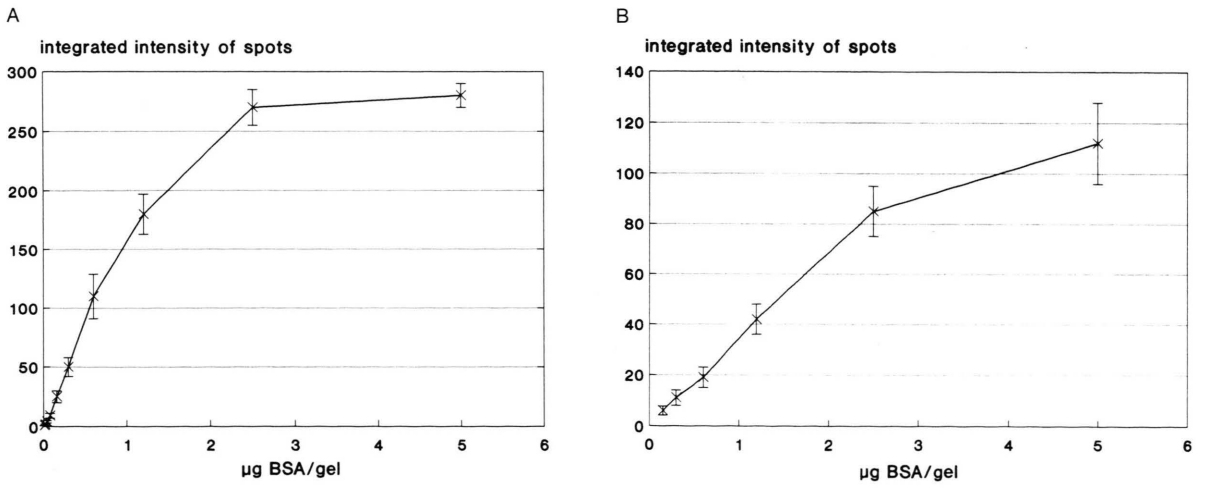

Fig. 4. A plot of integrated intensity of spots *versus* the concentration of BSA measured on silver-stained gels (A) and CB-G250-stained gels (B).

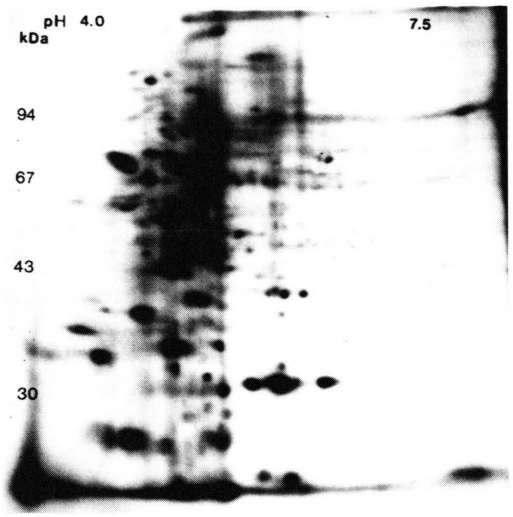

Fig. 5. Autoradiograph of 2-D electrophoretic separation of phosphoproteins extracted from skin slices of a mouse fetus.

ber of protein alterations is connected to the development of gastroschisis after irradiation.

The choice of the staining method is an important point in the attempt to search for mutations. Using silver-stained 2-D protein maps we have found several quantitative protein changes in the liver of the irradiated fetuses with gastroschisis (Hillebrandt and Streffer, 1994). However, when the gels were stained with CB only one of these alterations was detected in a fetus with gastroschisis. The other proteins showing changes on silver-stained gels could not be visualized by staining with CB.

Conclusion

We have analyzed silver-stained and CB-stained 2 D gels with 2 systems: the GelScan-XL software and the Gel-Image software. Since the first one does not contain a program for automatical spot recognition the spot areas were defined by the operator by drawing a contour and selecting the background. The usefulness of this system for the analysis of silver-stained gels was demonstrated. In contrast, the Gel-Image system consists of a program for automatic spot finding provided that the operator has selected a threshold above which a spot is distinguished from the background. This system has been established for evaluation of CB-stained gels showing a good separation of spots without fusion of adjacent spots. On the other hand the analysis of silver-stained gels with Gel-Image was difficult.

With both computer programs only sections of gels containing spots of interest and not the complete gels were evaluated. Therefore a large number of proteins (hundreds of spots) such as described by Anderson *et al.* (1985) could not be analyzed. Nevertheless, the analysis methods described in this paper should be effective to investigate the reliability of our data.

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Anderson N. L., Nance S. L., Tollaksen S. L., Giere F. A. and Anderson N. G. (1985), Quantitative reproducibility of measurements from Coomassie Blue-stained two-dimensional gels: Analysis of mouse liver protein patterns and a comparison of BALB/c and C57 strains. Electrophoresis **6,** 592–599.

Anderson N. L., Esqer-Blasco R., Hofman J.-P. and Anderson N. G. (1990), A two-dimensional gel database of rat liver proteins useful in gene regulation and drug effect studies. Electrophoresis **12,** 907–930.

Baier L. J., Hanash, S. M. and Erickson R. P. (1984), Mice homozygous for chromosomal deletions at the albino locus region lack specific polypeptides in two-dimensional gels. Proc. Natl. Acad. Sci. U.S.A. **81,** 2132–2136.

Blum H., Beier H. and Gras H. J. (1987), Improved silver staining of plant proteins, RNA and DNA in polyacrylamide gels. Electrophoresis **8,** 93–99.

Bossert N. L. and Iannaccone P. M. (1985), Midgestational abnormalities associated with *in vitro* preimplantation N-methyl-N-nitrosourea exposure with subsequent transfer to surrogate mother. Proc. Natl. Acad. Sci. U.S.A. **82,** 8757–8761.

Bossert N. L., Hitselberger M. H. and Iannaccone P. M. (1990), Protein alterations associated with N-methyl-N-nitrosourea exposure of preimplantation mouse embryos transferred to surrogate mothers. Teratology **42,** 147–156.

Cellis J. E., Gesser B., Rasmussen H. H., Madsen P., Leffers H., Dejgaard K., Honore B., Olsen E., Ratz G., Lauridsen J., Basse B., Mouridsen S., Hellerup M., Andersen A., Walbum E., Cellis A., Bauw G., Puype M., Van Damme J. and Vandekerckhove J. (1991), Comprehensive two-dimensional gel protein databases offer a global approach to the analysis of human cells: The transformed amnion cells (AMA) master database and its link to genome DNA sequence data. Electrophoresis **11,** 989–1069.

Giometti C. S., Gemmell M. A., Nance S. L., Tollaksen S. L. and Taylor J. (1987), Detection of heritable mutations as quantitative changes in protein expression. J. Biol. Chem. **262,** 12764–12767.

Hillebrandt S. and Streffer C. (1994), Protein patterns in tissues of fetuses with radiation-induced gastroschisis. Mutat. Res. **308,** 11–22.

Klose J. (1977), The protein-mapping method employed to test for chemically induced point mutations in mice. Arch. Toxicol. **38,** 53–60.

Klose J. and Putz B. (1983), Analysis of two-dimensional protein patterns from mouse embryos with different trisomies. Proc. Natl. Acad. Sci. U.S.A. **80,** 3753–3757.

Kuick R. D., Hanash S. M., Chu E. H. Y. and Strahler J. R. (1987), A comparison of some adjustment techniques for use with quantitative spot data from two-dimensional gels. Electrophoresis **8,** 199–204.

Neuhoff V., Stamm R. and Eibl H. (1985), Clear background and highly sensitive protein staining with Coomassie Blue dyes in polyacrylamide gels: A systematic analysis. Electrophoresis **6,** 427–448.

O'Farrell P. H. (1975), High resolution two-dimensional electrophoresis of proteins. J. Biol. Chem. **250,** 4007–4021.

Pampfer S. and Streffer C. (1988), Prenatal death and malformations after irradiation of mouse zygotes with neutrons or X-rays. Teratology **37,** 599–607.

Pardowitz I., Ehrhardt W. and Neuhoff V. (1990), Quantitative densitometry from the point of view of information theory. Electrophoresis **11,** 400–406.