SUITABILITY OF TWO-DIMENSIONAL ELECTROPHORETIC PROTEIN SEPARATIONS FOR QUANTITATIVE DETECTION OF MUTATIONS

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1 INTRODUCTION

Separation of proteins by two-dimensional electrophoresis (2DE) provides a powerful method for mutagenesis studies, since hundreds of proteins can be monitored simultaneously. In previous mutation studies in which 2DE has been used, however, only qualitative protein differences were monitored (1, 2); quantitative protein variations were not evaluated. Although significant differences in protein abundance can be detected by eye (3), the large number of protein spots present in 2DE patterns together with the large number of individual patterns required for a mutagenesis study would necessitate the use of a computerized analysis system to detect the rare quantitative protein changes indicative of gene deletions or inactivation of genes by point mutations in regulatory genes. A gene deletion in a gamete, for instance, should cause a 50% decrease in the expression of the corresponding protein in progeny (4, 5). Our laboratory is conducting a pilot study to search for heritable mutations induced by treatment of mice with either ethylnitrosourea (ENU) or gamma radiation. In addition to qualitative protein changes, we are monitoring the samples for quantitative changes that reduce the amount of protein by about 50%. An earlier paper from this laboratory (4) addressed the feasibility of this approach. We now present results from data generated during the first six months of our pilot study. In particular, we address three questions: (i) Can pattern quality and reproducibility be maintained for large experiments of long duration, involving hundreds of patterns over a period of months? (ii) Is the quality of the data sufficient to detect mutations by the reduction of a particular protein by 50%? (iii) If the data quality enables measurement of quantitative protein charges, how many spots are statistically stable enough to be monitored? The results of this analysis are discussed in terms of the feasibility and limitations of quantitative 2DE analyses used for the detection of heritable mutations.

2 MATERIALS AND METHODS

Male C57Bl/6 mice were treated with ENU and subsequently bred with untreated female BALB/c mice. Liver homogenates were prepared from the offspring of this cross and analyzed by 2DE as described in Refs. 4 and 6. A set of 159 different (i.e., no duplicate patterns of the same sample) 2DE patterns were selected from this set of data for computerized image analysis. Since the reported mutation frequency (qualitative variants) in the offspring of ENU-treated mice is low (one protein change in approximately 30 individuals [2]), it was assumed for this study that any mutations should have minimal effect on the data presented here.

The gels, stained with Coomassie Blue R250, were digitized in a tray of water with an Eikonix 785 scanner. Data were analyzed by using the Tycho II system of Argonne National Laboratory (7). Interactive examination was done with the GR42 system. Patterns were corrected by merging those spots that were multiply-detected in some patterns but not in others. Patterns typically exhibited about 500 spots. The analysis of the patterns requires the matching of corresponding spots from a master pattern with each of the individual sample (object) patterns. Interactive matches were set by using the GR42 system. Each pattern was then matched and stretched into registration according to the algorithms described in Ref. 8. Any obvious mismatches were corrected with the GR42 system.

3 RESULTS AND DISCUSSION

Spot volumes from each pattern were scaled by requiring the sum for a set of 20 spots to be the same. The resulting scale factors (plotted in Fig. 1) turned out to be relatively insensitive to the exact makeup of the scale set.

Pattern quality was assessed by monitoring the resolution and the stretching reproducibility according to the protocols described in Ref. 9. The initial goal was to keep the resolution measures above 17,000. Figure 2 shows a histogram of the these values. Most of the patterns met the goal, but at times the resolution for whole sets of gels fell to about 15,000. However, this resolution was still considered adequate considering the number of spots visualized on the pattern. Positional reproducibility is particularly important in a mutation experiment in which charge-shift variants are expected. Figure 3 shows the results of a positional reproducibility analysis for the entire set of data. The normalized misregistration distance

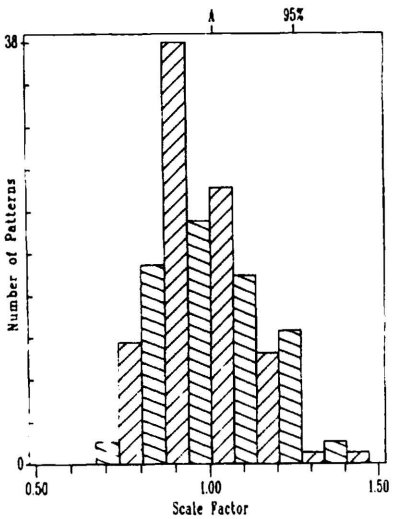

Fig. 1. Histogram of volume scale factors of the patterns.

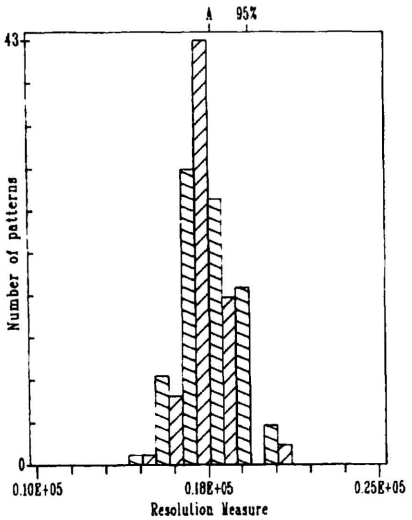

Fig. 2. Histogram of resolution measures for the patterns.

is essentially the average distance from a spot in the master pattern to its counterparts in the object patterns (after stretching) divided by the half-width of the spot (9). Thus, in this data set, 95% of the spots are stretched to within a quarter of a spot "diameter". This level of accuracy should be sufficient to ensure accurate spot identifications with only a few exceptions. Problem areas are primarily confined to the edge of the pattern, especially the basic side. These results suggest that data quality can be maintained for experiments of long duration. Positional reproducibility seems to be much less of a problem than maintenance of good resolution with minimum streaking. Monitoring the resolution is necessary in order to correct problems early.

The problem of detecting quantitative mutations is essentially a search for outliers in the spot volume. Therefore, the reproducibility of spot volumes must be good in order to detect mutations efficiently. If the spread of the spot volumes is too high, then the rate of false-positive detections will be excessive. We measured the coefficient of variation (standard deviation/mean) for all spots in the master pattern. Many of the spots are minor ones and are too close to threshold for reliable detection. We therefore restricted the analysis to those spots that were detected in 95% of the object patterns. Figure 4 shows a plot of the coefficient of variation (CV) of the spot

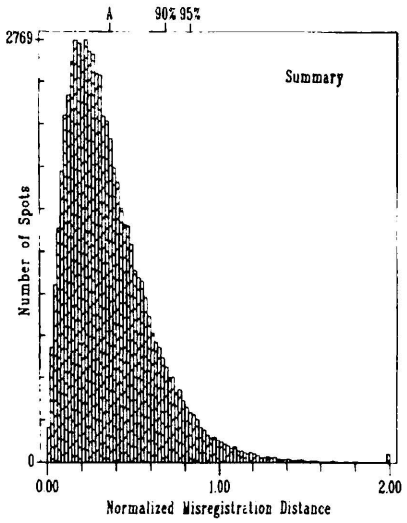

Fig. 3. Histogram of the normalized misregistration distance for all spots after stretching the patterns into registration with the master.

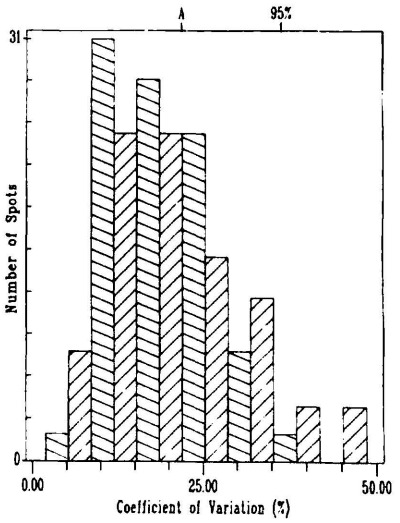

Fig. 4. Histogram of coefficients of variation for the scaled volumes of those spots which were detected in 95% of the patterns.

volumes for these spots. (It should be remembered that the spread of values represented by the CV for a spot includes biological as well as laboratory and measurement variation. The CVs reported in [1] were for repeat runs of a single sample.) Many spots show sufficiently low CVs to be useful for mutation detection. If one assumes normal distributions for the spot volumes and a 50% reduction in both volume and standard deviation for a population of identical mutants, then CVs of 15% or less may be required. Spots with CVs from 15 to 20% may be useful if multiple gels are run from each animal. The present data contain 55 spots with CVs less than 15% and 93 spots with CVs less than 20%.

A set of serial dilutions of the same sample was run to test the response of the individual measurements to variation in protein sample loading. In particular, we were interested in knowing if halving the protein amount would produce a corresponding decrease in measured volume. Of the 55 spots with CVs less than 15%, only one showed a saturation effect whereby the measured volume for half the normal loading was significantly higher than predicted. Thus, we are left with more than 50 spots that are suitable for monitoring purposes.

The results of this study indicate that the key methods to improve the application of 2DE to mutation screening are to increase the number of measurable spots (i.e., improve stain sensitivity) and to decrease the spread of values for the volume measurements. Even small improvements in these areas could greatly increase the number of monitorable spots. Efforts to improve the reproducibility of the spot volumes by optimizing the sampling and preparative techniques are under way.

4 ACKNOWLEDGMENTS

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