

A MULTI-LABORATORY ASSAY TO ASSESS REPRODUCIBILITY IN 2D-ELECTROPHORESIS USING DIGE AND SINGLE-STAIN METHODOLOGIES.

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Introduction

Standardization and robustness evaluation of methods and procedures are becoming key issues for ABRF and HUPO initiatives. Two-dimensional gel electrophoresis (2DE) constitutes one of the most widely used protein separation technologies for proteome analysis. Reproducibility has always been a major concern when using 2DE in comparative proteomic experiments. The main issues affecting reproducibility of 2DE are the gel to gel variation of the two-dimensional separation patterns themselves, and the accuracy in comparing relative protein abundances based on the measured spot volumes. Reproducibility is not only affected by experimental conditions, but also by investigator introduced bias in the image analysis process. In the last years, different methodological improvements have contributed to more robust 2DE workflows: use of immobilized IEF strips, fluorescence based difference gel electrophoresis (DIGE), new software tools, etc. In order to assess the reproducibility of 2DE experiments using current workflows, we have set up a multi-laboratory assay, conducted at 22 laboratories of the PROTEORED network (Spanish network of proteomics facilities), plus other associated laboratories. All participating labs have received an identical protein extract, prepared from cultured A431 human carcinoma cells, to be run in triplicate gels under selected 2DE conditions in all labs. Approximately, one third of the labs have performed the experiment using one of three different workflows: DIGE, silver single-stain and fluorescent single-stain. Each laboratory has then used its own protocols and software for image-analysis, to evaluate the number of spots observed and the variability in the spot volume measurements between the three replicate gels. The information of the experiments has been then gathered, using criteria conforming to the current MIAPe guidelines developed by the HUPO-PSI, in order to test and evaluate these guidelines. Based on the collected data, gel to gel variability intra- and inter-laboratory and variability for the three different workflows assayed are evaluated and compared.

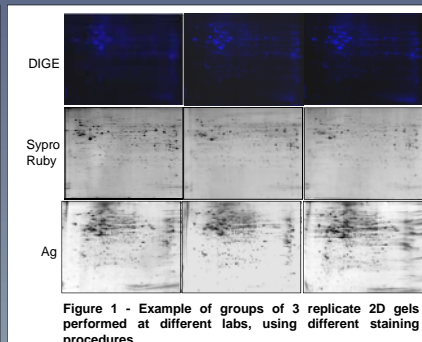
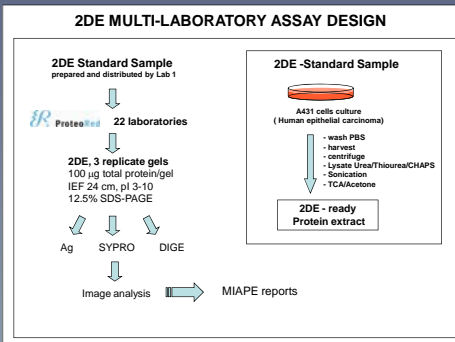


Figure 1 - Example of groups of 3 replicate 2D gels performed at different labs, using different staining procedures.

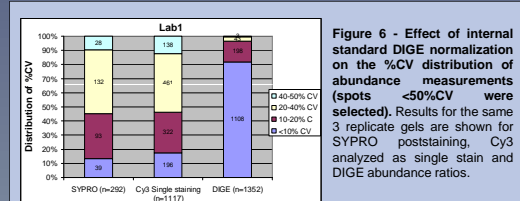


Figure 6 - Effect of internal standard DIGE normalization on the %CV distribution of abundance measurements (spots <50%CV were selected). Results for the same 3 replicate gels are shown for SYPRO poststaining, Cy3 analyzed as single stain and DIGE abundance ratios.

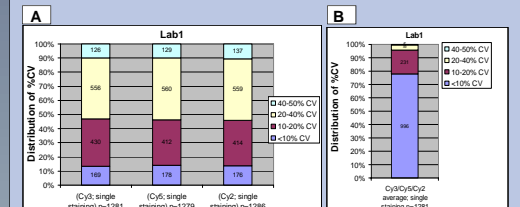


Figure 7 - Variability due to Cy dye labeling and gel-to-gel (A) vs in-gel variation (B). A) Cy3 and Cy5 samples were labeled individually for each of 3 replicate gels. A single Cy2-labeled sample was loaded on each gel. The corresponding Cy dye images of the three replicate analysis were analyzed as single stain. B) The three Cy (Cy2/Cy3/Cy5) images of a single gel were analyzed as single stain.

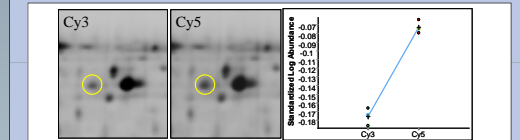


Figure 8 - Variability due to differential Cy3 / Cy5 labeling. Only 5 out of 1457 spots were differentially labeled by Cy3/Cy5 dyes with an average ratio >1.2 or <-1.2 and p < 0.05 (T-test, n=3). Spot shown: Average ratio 1.27, p= 0.00015.

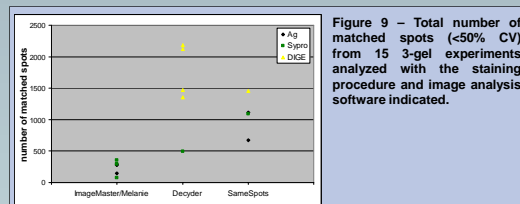


Figure 9 - Total number of matched spots (<50% CV) from 15 3-gel experiments analyzed with the staining procedure and image analysis software indicated.

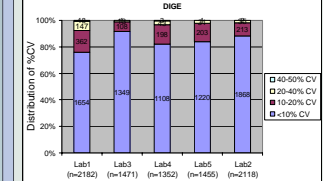
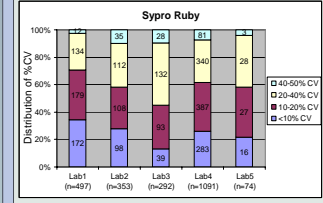
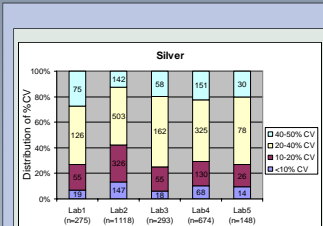


Figure 2 - Effect of staining procedure on %CV (coefficient of variation) distribution of abundance measurements. 5 labs x 3 replicate gels per staining procedure. Spots with <50% CV selected. Spots with higher %CV, which likely include most wrongly matched or unmatched spots, have been discarded from the analysis. Numbers within the bars indicate the number of spots in each range.

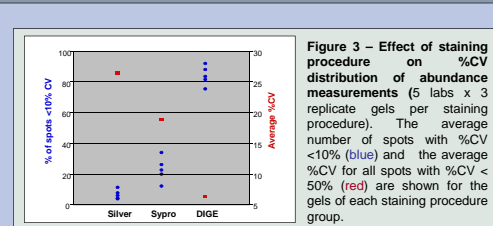


Figure 3 - Effect of staining procedure on %CV distribution of abundance measurements (5 labs x 3 replicate gels per staining procedure). The average number of spots with %CV <10% (blue) and the average %CV for all spots with %CV < 50% (red) are shown for the gels of each staining procedure group.

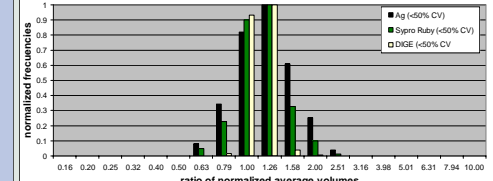
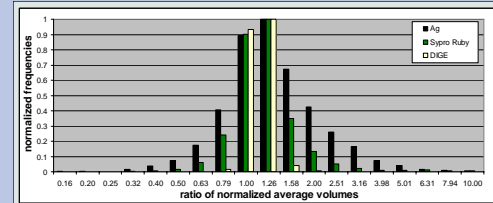


Figure 4. Normalized frequency distribution of averaged calculated abundance ratios. For each 3 replicate gels the abundance ratios (R1/R2; R1/R3; R2/R3) have been calculated based on normalized volumes (single stain) or standardized normalized volumes (ratiometric) for DIGE Cy3 images. A: all spots (5 labs x 3 replicate gels per staining procedure). B: spots with < 50%CV selected (5 labs x 3 replicate gels per staining procedure)

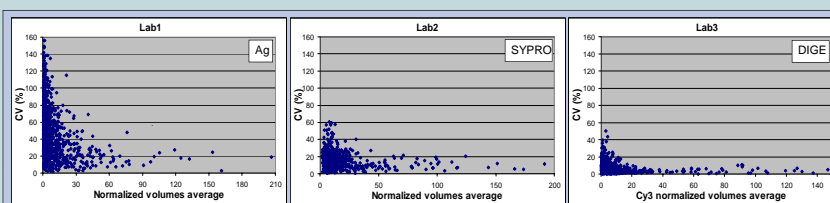


Figure 5 - %CV dependence on spot volume. %CV of normalized spot volumes (for single stain) or Cy3/Cy2 standardized ratios (DIGE) are plotted as a function of normalized spot volumes.

CONCLUSIONS

- Despite differences in protocols and image analysis software used, the variability observed between replicate gels shows a consistent pattern for each of the staining procedures. The distributions of %CV of the spot abundance measurements (Fig 2, 3 and 4) reflect a better performance of fluorescent versus silver staining, and a dramatic decrease in variability by using the internally standardized DIGE approach.

- The observed variability displays a clear inverse dependence on spot volume (Fig 5). For all three staining procedures the variability increases rapidly below a certain spot volume threshold. Again, DIGE displays the best performance.

- Analysis of the Cy dye images separately or using DIGE internal normalization allows the evaluation of different sources of variability. Fig. 6. shows that individual Cy dyes perform similarly to SYPRO Ruby when analyzed as single stain images, while the effect of normalization using the internal standard is the main factor in the reduction of variability. Fig. 7 shows that the variability introduced by performing separate Cy dye labeling reactions of the samples is negligible, and that gel-to-gel variation is the major source of variability. Fig. 8. shows that preferential Cy dye labeling is only observed for a small number of proteins. On the other hand, this source of bias can be easily avoided by dye-swapping experiment designs.

- Comparison of the performance of the image analysis software used in the different experiments have to be taken with caution, considering that essentially identical images are being compared, and that factors as time spent on the analysis have not been taken in consideration. However, it is apparent that Non-Linear Dynamics SameSpots results in a clear increase on well-matched spot detection, particularly evident in single-stain procedures. On the other hand, GE Decyder, performs similarly to SameSpots for DIGE experiments.

- Overall, the assay conducted highlights the need to adjust the number of technical replicates in 2DE proteomic experiments, to account for the expected variability depending on the procedures used. This is of particular importance when changes in low abundance proteins are compared. As clearly shown, the use of a strategy with internal standardization introduces a large improvement in accuracy.