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 ABIFP and HUPO initiatives. Two-dimensional gel electrophoresis (2DE) constitutes one of the most valuable protein separation techniques for proteomic analysis. Reproducibility has always been a major concern when using DIGE in comparative proteomics 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 testing and reference gels. These are related to several uncontrollable and/or modifiable factors in the image analysis process. In the last years, different methodological improvements have contributed to more robust 2DE workflows. Use of immobilized IEF strips, 10-20% DIGE gels, and Sypro Ruby have improved the reproducibility of 2DE experiments. DIGE workflows currently used in the majority of laboratories have been developed independently, based on the internal standard DIGE approach, which is more flexible and less time consuming than the other approaches, and uses an internal reference fluorophore. This is of particular importance when changes in low abundance proteins are expected. We have used the internal standard Sypro Ruby 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 stain procedures used are evaluated and compared.

Participant laboratories

Institut de Recerca Vall d’Hebron

2DE MULTI-LABORATORY ASSAY DESIGN

2DE Standard Sample Prepared and anonymized by Lab 1

2DE Standard Sample

Participant laboratories

2D Laboratories

Ag

DIGE

Ag

DIGE

2D - ready Protein extract

3 replicate gels performed at different labs, using different staining procedures.

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

20% CV

10% CV

40% CV

50% CV

60% CV

70% CV

90% CV

120% CV

Distribution of %CV

10-20% CV

20-40% CV

40-50% CV

Cyt3

Cyt5

<10% CV

<50% CV

(n=1455)

(n=2118)

(n=74)

(n=1091)

(n=2118)

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 correspond to single 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 – %CV dependence on spot volume. %CV of normalized spot volumes (single stain) or Cy3/Cy5 standardized ratios (DIGE) are plotted as a function of normalized spot volumes.

Figure 4 - Normalized frequency distribution of averaged calculated abundance ratios. For each 3 replicate gels the abundance ratios (R120, R130, R230) have been calculated based on normalized volumes (single stain) or standardized normalized volumes (ratios) 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 – Total number of matched spots (>99% CV) from 15 3-gel experiments analyzed per the staining procedure and image analysis software indicated.

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 best performance of SYPRO Ruby in the spot abundance measurements (Fig. 2) is paralleled with 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-exchanging 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 Dynamic SemiSpots results in a clear increase on well-matched spot detection, particularly evident in high abundance volumes. On the other hand, GE Decyder, performs similarly to SemiSpots 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 expected.