# 2-D experimental design using Ettan DIGE system: Application of the pooled internal standard to the investigation of a model system

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# 2-D experimental design using Ettan DIGE system: Application of the pooled internal standard to the investigation of a model system

**key words:**
difference analysis • fluorescent labeling • 2-D DIGE • 2-D electrophoresis • pooled internal standard • proteomics • Ettan DIGE system

This application note describes how the multiplexing properties of 2-D DIGE using Ettan™ DIGE system can be exploited by including the same internal standard sample on each gel to be analyzed. The standard sample comprises equal quantities of each of the samples in the experiment. Each sample is compared within each gel with the same pooled standard. This largely removes experimental gel-to-gel variation leading to improved accuracy of protein quantitation between samples from different gels. The pooled standard experimental design was demonstrated by tracking known protein abundance changes across a range of spiked *E. coli* lysates.

## Introduction

Comparing different samples separated by 2-D gel electrophoresis is an established methodology for proteomic studies. The more recently introduced technique of two-dimensional fluorescence difference gel electrophoresis (2-D DIGE) is a multiplexing method enabling comparative proteomics (1). Ettan DIGE system combines labeling with CyDye™ DIGE Fluor minimal dyes and 2-D electrophoresis along with the Typhoon™ Variable Mode Imager and DeCyder™ Differential Analysis Software that enable accurate quantitation of protein differences.

This application note describes an experimental design that exploits the multiplexing capabilities of 2-D DIGE to include an internal standard sample on each gel consisting of a pool containing each sample to be compared. The internal standard sample is used to normalize protein abundance measurements between gels, which removes the gel-to-gel variation inherent with traditional comparative 2-D experiments (Fig 1).

The pooled standard experimental design was tested experimentally by spiking an *E. coli* lysate with differing, known amounts of model proteins followed by 2-D DIGE analysis. Changes in protein levels were quantitated using the pooled internal standard and compared with the empirically calculated changes. The same gels were also analyzed without incorporating the internal standard. Results from the two approaches were then compared.

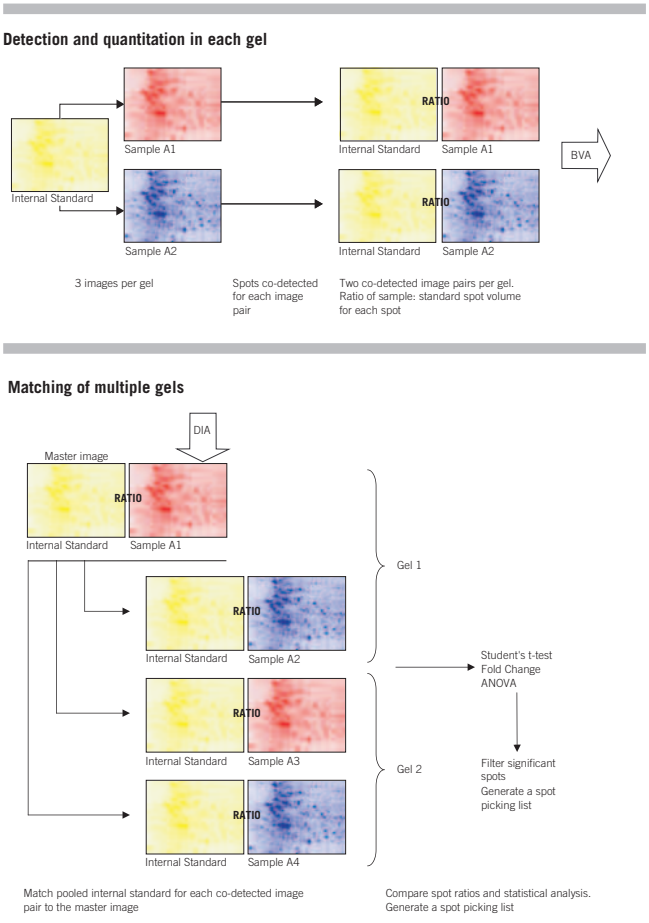


**Fig 1.** Experimental design for labeling proteins and matching gels.

## Products used

*Amersham Biosciences products used in this application note:*

| | |
|---|---|
| **Reagents for 2-D DIGE** | |
| CyDye DIGE Fluor Cy2 minimal dye, 25 nmol | RPK0272 |
| CyDye DIGE Fluor Cy3 minimal dye, 25 nmol | RPK0273 |
| CyDye DIGE Fluor Cy5 minimal dye, 25 nmol | RPK0275 |
| Pharmalyte pH 3–10 | 17-0456-01 |
| Immobiline DryStrip gels, 24 cm, pH 3–10 NL | 17-6002-45 |
| Immobiline DryStrip Reswelling Tray | 80-6371-84 |
| DryStrip Cover Fluid | 17-1335-01 |
| **Ettan sample preparation reagents** | |
| Urea | 17-1319-01 |
| CHAPS | US13361 |
| Dithiothreitol | 17-1318-02 |
| Tris | 17-1321-01 |
| **PlusOne reagents** | |
| PlusOne TEMED | 17-1312-01 |
| PlusOne Ammonium Persulfate | 17-1311-01 |
| PlusOne Agarose | 80-1130-07 |
| PlusOne Glycine | 17-1323-01 |
| PlusOne ReadySol (Acrylamide/Bis) | 17-1310-01 |
| PlusOne Sodium Dodecylsulfate | 17-1313-01 |
| PlusOne Glycerol | 17-1325-01 |
| **Equipment** | |
| Ettan IPGphor Isoelectric Focusing Unit | 80-6414-02 |
| Cup Loading Strip Holder | 80-6459-43 |
| Ettan DALT*twelve* Electrophoresis System | 80-6466-27 |
| Ettan DIGE DALT Gel Alignment Guide | 80-6496-29 |
| Typhoon 9400 Variable Mode Imager | 63-0038-54 |
| DeCyder Differential Analysis Software, Version 4.00 | 63-0055-79 |
| ImageQuant Solutions | Inquire |

## Methods

### Sample preparation and labeling

*E. coli* strain ER1647 was grown in a glucose-rich morpholinepropane sulfonic acid (MOPS) medium at 37 °C overnight followed by centrifugation for 10 min at 4 °C at 12 000 × g. The cell pellet was washed twice with wash buffer (10 mM Tris pH 8.0, 0.5 mM magnesium acetate), resuspended in lysis buffer (8 M urea, 4% [w/v] CHAPS, 10 mM Tris pH 8.0), and lysed by sonication (3 × 10 s pulses on ice). The eight different samples were prepared by adding the proteins shown in Table 1 to the *E. coli* lysate.

**Table 1.** Model proteins used

| Protein* | Accession number | $M_r$† | pI† |
|---|---|---|---|
| BSA | P02769 | 69 300 | 5.82 |
| Conalbumin | P02789 | 77 800 | 6.85 |
| Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) | P46406 | 35 700 | 8.52 |
| Trypsin inhibitor | P01005 | 22 600 | 4.75 |

* All proteins were obtained from Sigma.

† The theoretical pI values and molecular weights of the proteins were calculated based on primary structure using the ExPASy Compute pI/Mw tool (www.expasy.ch/tools/pi_tool.html).

Table 2 shows the amount of model protein in each sample. This amount was sufficient for one gel loading at 50 µg protein per CyDye DIGE Fluor minimal dye per gel. The pooled standard sample was prepared by pooling 100 µg protein from each of the samples prior to labeling. CyDye DIGE Fluor minimal dyes, reconstituted in DMF, were added to labeling reactions at a ratio of 400 pmol CyDye DIGE Fluor minimal dye to 50 µg protein followed by incubation on ice in the dark for 30 min. The reaction was quenched by the addition of 10 mM lysine (1 µl per 400 pmol CyDye DIGE Fluor minimal dye) followed by incubation on ice for a further 10 min (see Ettan DIGE User Manual for detailed protocols).

**Table 2.** Amounts of model proteins added to each sample

| Sample number* | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
|---|---|---|---|---|---|---|---|---|
| BSA (ng) | 200 | 175 | 150 | 125 | 100 | 75 | 50 | 25 |
| Conalbumin (ng) | 50 | 100 | 150 | 200 | 250 | 300 | 350 | 400 |
| GAPDH (ng) | 50 | 100 | 200 | 400 | 100 | 200 | 400 | 800 |
| Trypsin Inhibitor (ng) | 100 | 100 | 100 | 100 | 80 | 60 | 40 | 20 |

* Eight different samples were prepared containing different amounts of the proteins added to an *E. coli* lysate. **Note:** The amounts shown in the table were multiplied by 10 to provide sufficient sample for preparing the pooled standard and for gel replicates.

### 2-D electrophoresis

Labeled samples to be separated in the same gel were mixed according to the experimental design outlined in Table 3, followed by addition to an equal volume of 2× sample buffer (8 M urea, 4% [w/v] CHAPS, 130 mM DTT, 2% [v/v] Pharmalyte™ pH 3–10). Isoelectric focusing was performed using Immobiline™ DryStrip gels (pH 3–10 NL, 24 cm). Samples were applied to Immobiline DryStrip gels by cup loading near the basic end of the strips. Focusing was carried out using Cup Loading Strip Holders on an Ettan IPGphor™ Isoelectric Focusing Unit for a total of 120 kVh at 20 °C.

**Table 3.** Experimental design. Each gel contained the pooled standard and two other samples. The eight samples were analyzed in triplicate by running 12 gels

| Gel | Cy2 | Cy3 | Cy5 |
|---|---|---|---|
| 1 | Pooled Standard | Sample 1 | Sample 2 |
| 2 | Pooled Standard | Sample 1 | Sample 2 |
| 3 | Pooled Standard | Sample 1 | Sample 2 |
| 4 | Pooled Standard | Sample 3 | Sample 4 |
| 5 | Pooled Standard | Sample 3 | Sample 4 |
| 6 | Pooled Standard | Sample 3 | Sample 4 |
| 7 | Pooled Standard | Sample 5 | Sample 6 |
| 8 | Pooled Standard | Sample 5 | Sample 6 |
| 9 | Pooled Standard | Sample 5 | Sample 6 |
| 10 | Pooled Standard | Sample 7 | Sample 8 |
| 11 | Pooled Standard | Sample 7 | Sample 8 |
| 12 | Pooled Standard | Sample 7 | Sample 8 |

Following IEF, each Immobiline DryStrip gel was equilibrated with 10 ml equilibration buffer A (8 M urea, 100 mM Tris-HCl pH 6.8, 30% [v/v] glycerol, 1% [w/v] SDS, 5 mg/ml DTT) for 10 min, followed by 10 ml equilibration buffer B (8 M urea, 100 mM Tris-HCl pH 6.8, 30% [v/v] glycerol, 1% [w/v] SDS, 45 mg/ml iodoacetamide) for a further 10 min. The Immobiline DryStrip gels were loaded and run on 12.5% acrylamide isocratic gels using the Ettan DALT*twelve* Electrophoresis System. Gels were run at 5 W per gel constant power at 20 °C until the proteins had entered the resolving gel followed by 10 W per gel constant power at 20 °C until the bromophenol blue dye front had run off the bottom of the gels.

### Image acquisition

Labeled proteins were visualized using the Typhoon 9400 series Variable Mode Imager. The Cy™2 images were scanned using a 488 nm excitation wavelength and an emission filter of 520 nm with a band-pass of 40 nm (520BP40); Cy3 images using 532 nm excitation wavelength and 580BP30 emission filter; Cy5 images using 633 nm excitation wavelength and 670BP30 emission filter. All gels were scanned at 100 µm resolution. Images were cropped using ImageQuant™ Version 5.0 to remove areas extraneous to the gel image.

### Image analysis

Gel image analysis was performed using DeCyder Differential Analysis Software, Version 4.0. All pooled standard/sample gel image pairs were processed by the DeCyder DIA (Differential In-gel Analysis) software module to co-detect and differentially quantitate protein spots in the images. To reduce the hands-on time spent on data analysis, the 24 DeCyder DIA co-detection runs were defined and administered using DeCyder Batch Processor. The estimated number of spots for each co-detection procedure was set to 2500. Gel-to-gel matching of the standard spot maps from each gel, followed by statistical analysis of protein-abundance change between samples, was performed using the DeCyder BVA (Biological Variation Analysis) software module.

## Results and Discussion

Automated spot detection and quantitation were performed on overlaid Cy2 (Standard Pool)/Cy3 ($Sample_x$) and Cy2 (Standard Pool)/Cy5 ($Sample_y$) image pairs from each gel, followed by gel-to-gel matching and statistical analysis. For a given spot, the standardized abundance was expressed as a volume ratio between the pooled standard and a co-detected sample from the same gel. DeCyder Differential Analysis Software compares how the abundance ratio measurements of the same protein spot from different samples and gels relate to the standard sample, which makes further normalization unnecessary. Since each sample spot map is co-detected with a standard spot map, all of the spots are compared internally to the same pooled standard.

Following inter-gel matching, there were a total of 1166 spots matched in two or more gels. Analysis of

variance (ANOVA) was applied and the data was filtered to retain spots with ANOVA statistical significance, $p \leq 0.05$, and spots that appeared in > 75% of the spot maps. This resulted in the display of 52 spots, 43 of which occurred in a series of four clusters of spots (Fig 2a). The spots that were significant but did not occur in the clusters included artifacts such as streaks. The fact that significant spots were clustered suggests that each of the four model proteins was present as multiple isoforms in the gels. The gel positions of the clusters are shown in Figure 2. The box containing cluster 1 is consistent with the pI/$M_r$ of BSA, cluster 2 with conalbumin, cluster 3 with GAPDH, and cluster 4 with trypsin inhibitor.

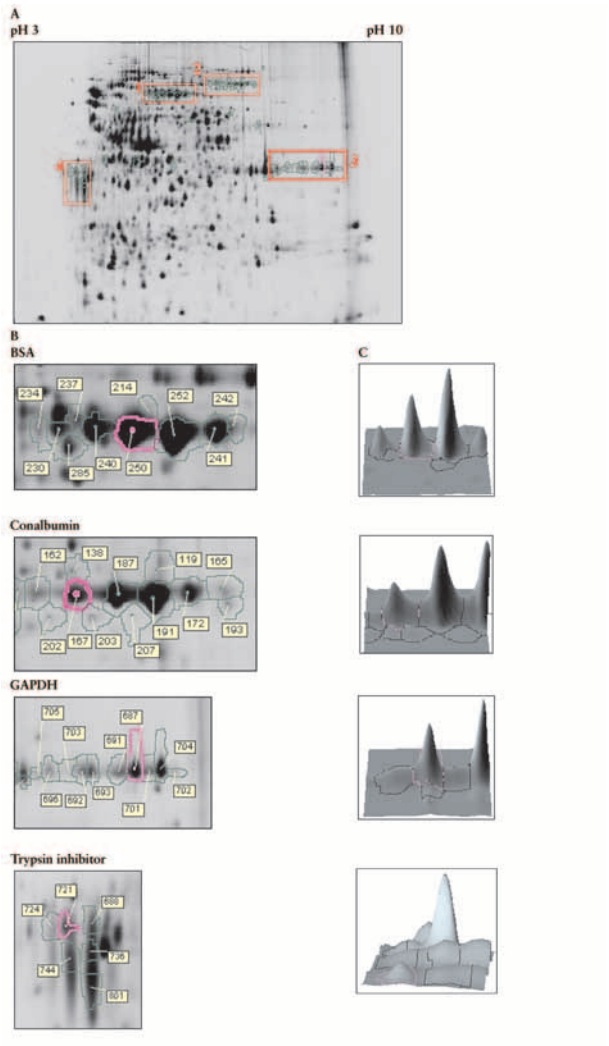


**Fig 2.** Two-dimensional analysis of *E. coli* lysate spiked with model proteins. (A) Differences with an ANOVA statistical significance, $p > 0.05$ were filtered out, (B) the four clusters (boxes 1–4) of significant differences with spot numbers, and (C) 3-D views.

Graphs of standardized abundance for a representative spot from each of the clusters generated automatically by DeCyder Differential Analysis Software (Fig 3) display abundance values relative to the measured standardized abundance. The range and overall profile of the standardized abundance graphs closely resemble the predicted abundance graphs for all four proteins.

To study the effect of the pooled standard sample on the analysis of protein abundance, comparison of the same set of gel images was carried out based on spot volume measurements rather than in-gel ratio measurements with the standard sample. Spot detection was carried out using DeCyder Differential Analysis Software on Cy3 and Cy5 image pairs followed by matching and comparison of spot volumes. The spot maps were normalized according to spot volumes. Normalized spot volumes were obtained by dividing each spot volume by the average volume in the spot map from which it was derived. The normalized volumes for a set of spots for a protein were then expressed as a ratio of the weakest spot that corresponds to the protein. This parameter is referred to as the nonstandardized abundance.

Graphs of nonstandardized abundance against sample number were generated by DeCyder Differential Analysis Software and were compared to predicted nonstandardized abundance graphs based on the actual protein loadings shown in Table 2 (Fig 4). The profiles and ranges of the measured nonstandardized abundance graphs differ greatly from the predicted graphs.

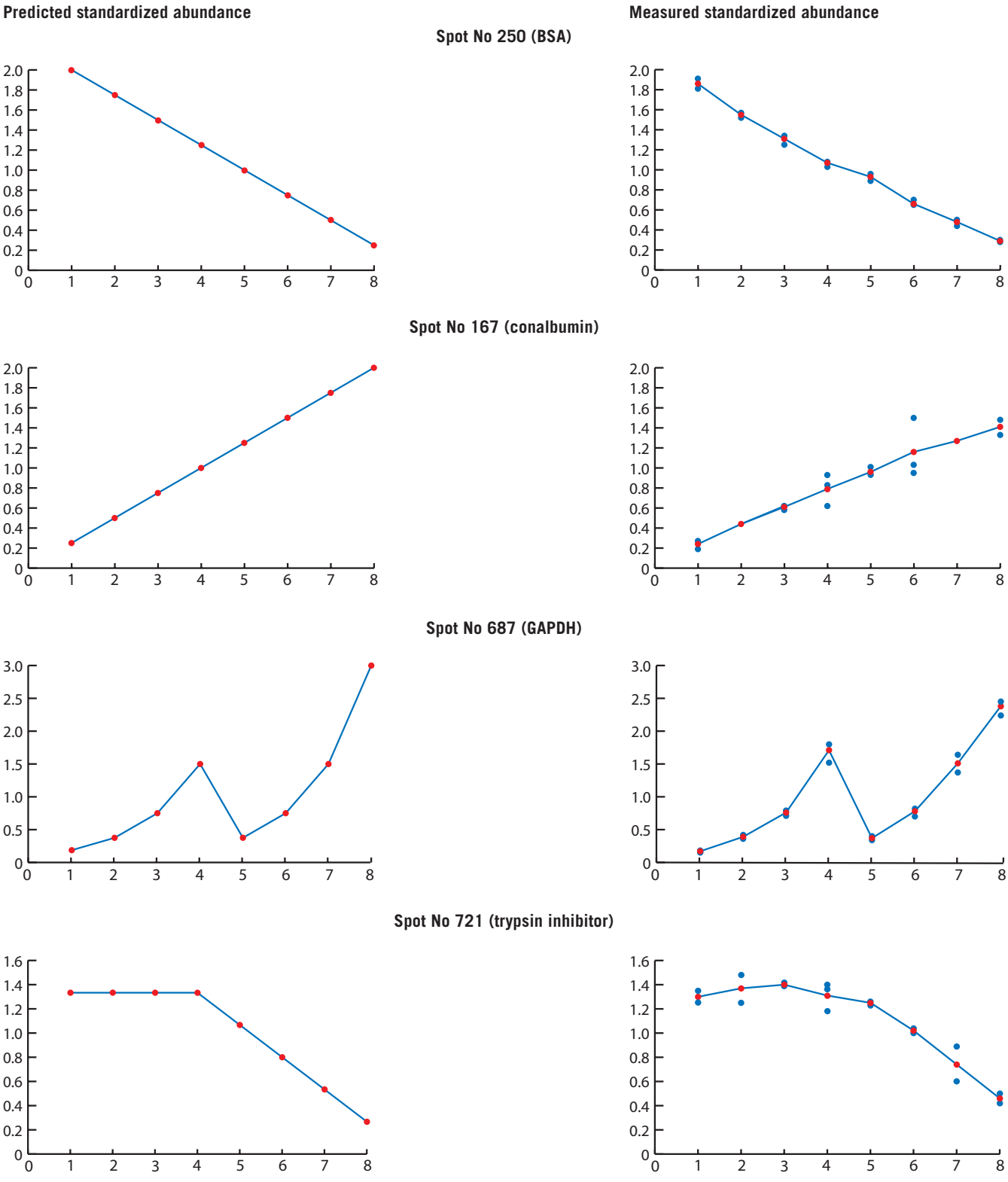


**Fig 3.** Quantitative analysis of significant differences. The predicted standardized abundance graphs (left) were generated and compared with the standardized abundance graphs generated by DeCyder Differential Analysis Software (right). The graphs were taken from the spots highlighted in magenta in Figure 2. For both the predicted and measured graphs, standardized abundance (y-axis) is plotted against sample number (x-axis).

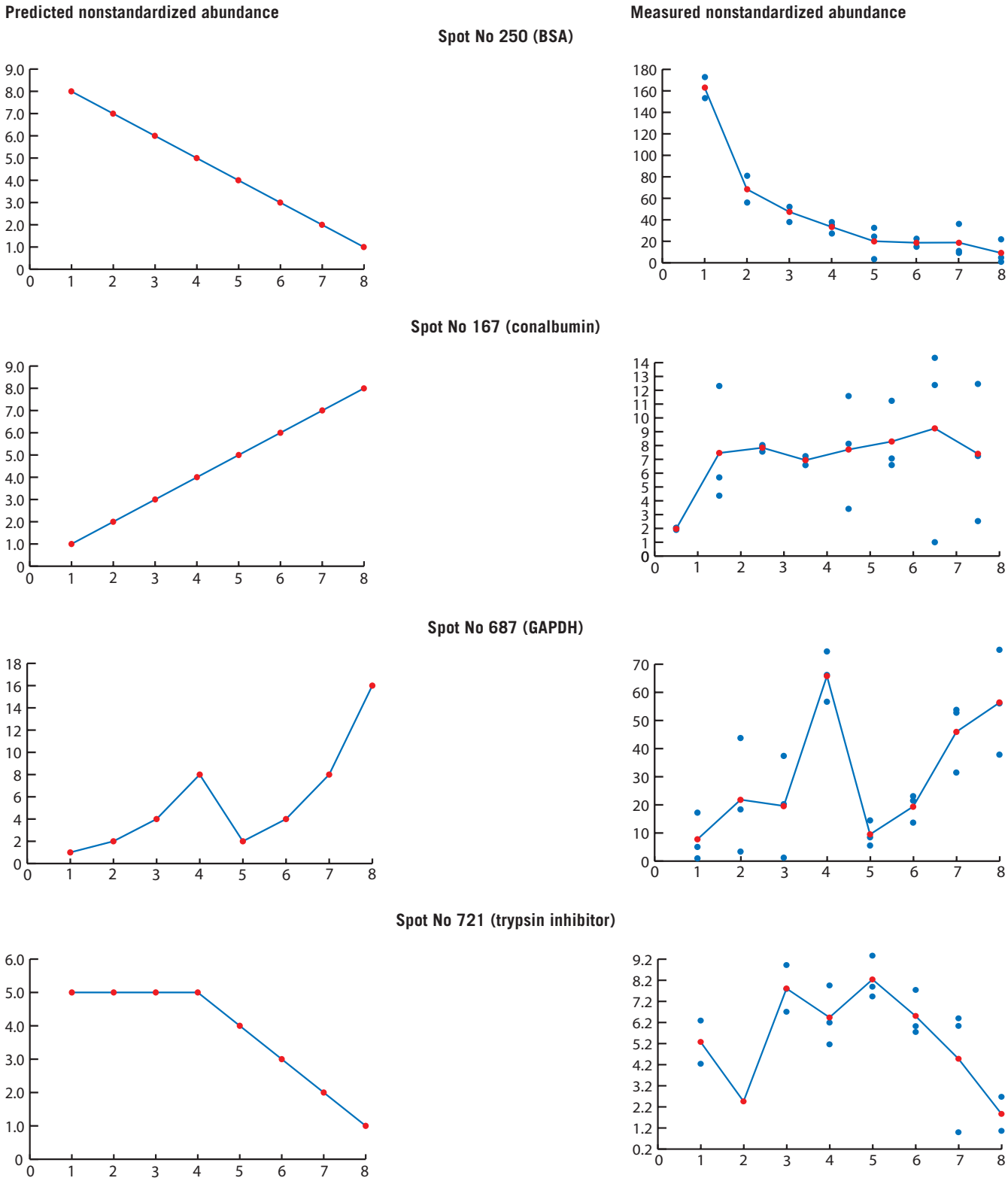


**Fig 4.** Analysis of protein abundance without the pooled standard experimental design. The predicted nonstandardized-abundance graphs (left) and measured abundance graphs (right) are shown. Nonstandardized protein abundance (y-axis) is plotted against sample number (x-axis) for all graphs. The y-axis scales are different between predicted and measured graphs as they have been adjusted to include all of the data points.

**Table 4.** Coefficient of variation (CV) analysis of standardized and nonstandardized abundance data (the data for the spots highlighted in magenta in Figure 2 were used in the analysis)

| | Spot No. 250 BSA | | Spot No. 167 Conalbumin | | Spot No. 687 GAPDH | | Spot No. 721 Trypsin inhibitor | |
|---|---|---|---|---|---|---|---|---|
| Sample number | CV standard | CV non-standard | CV standard | CV non-standard | CV standard | CV non-standard | CV standard | CV non-standard |
| 1 | 3.81 | 8.51 | 5.00 | 5.03 | 5.44 | 27.48 | 9.35 | 108.81 |
| 2 | 2.29 | 25.71 | 10.35 | 57.06 | 11.91 | 27.36 | 10.88 | 93.37 |
| 3 | 4.19 | 17.34 | 2.84 | 3.11 | 1.09 | 14.16 | 6.05 | 92.28 |
| 4 | 3.37 | 16.42 | 19.94 | 4.82 | 8.92 | 21.82 | 9.63 | 13.64 |
| 5 | 3.79 | 74.32 | 4.83 | 53.11 | 1.39 | 12.24 | 11.47 | 47.86 |
| 6 | 4.33 | 28.56 | 25.62 | 30.90 | 1.96 | 13.80 | 9.24 | 25.83 |
| 7 | 6.45 | 59.09 | 5.54 | 77.95 | 19.51 | 67.39 | 8.96 | 27.59 |
| 8 | 4.03 | 120.93 | 7.55 | 67.22 | 12.29 | 61.26 | 5.09 | 32.96 |

The coefficient of variation of the abundance and standardized abundance was calculated for each spot highlighted in Figure 2 to obtain a relative measure of the spread of data (shown in Table 4). In all but one case, the CV is larger for the nonstandardized abundance data than for the standardized abundance data indicating more experimental variation in the former than in the latter. Therefore the pooled standard experimental design greatly reduces experimental variation.

Less variation results from the inclusion of the pooled standard because the nonstandardized abundance graphs are based on comparisons of spot volumes rather than in-gel measured spot volume ratios, as used in the standardized abundance graphs. Variation in protein loading between different gels will result in variation in spot-volume measurements between gels. However, when measuring spot-volume ratios between samples in the same gel, protein loss for a particular spot will be the same for all samples. Comparing in-gel standard/sample volume ratio measurements from different gels compensates for gel-to-gel variation of spot volume and migration. Comparing in-gel spot volume ratio measurements from different gels results in less experimental variation than comparing spot volumes from different gels.

## Conclusions

The multiplexing capabilities of 2-DIGE using Ettan DIGE system allow the inclusion of an internal standard sample in each gel. Combined with the dedicated spot co-detection in DeCyder Differential Analysis Software, this facilitates automated and accurate quantitation of differences in protein levels between samples on different gels. The pooled standard experimental design reduces variation and enables more complex comparative proteomics experiments such as time course studies (2,3).

## References


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More information can be obtained in the Ettan DIGE system section at http://proteomics.amershambiosciences.com.




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