Normalizing between 2-D Gels with PDQuest® Software

Why Normalize 2-D Gels?

Two-dimensional gel electrophoresis methodology typically involves the comparison of at least two different samples for changes in protein expression that reflect the differences between the samples. These changes can take the form of on/off regulation, where particular proteins are uniquely expressed in either the control or the test sample. On/off regulatory changes are relatively easy to detect. However, protein expression changes can also take the form of up/down regulation. These can be much more difficult to characterize because:

- Up/down regulatory changes can be rather subtle and therefore difficult to detect
- Some degree of non-expression-related variation between gels with regard to spot intensities makes spot-to-spot comparisons difficult

The process of correction of spot quantitation values for gel-to-gel variation unrelated to expression changes is called normalization. Such variation can occur from a variety of sources that are not related to the specific expression pattern of the cell sample being studied. Some sources of such variation include:

- Inconsistent cell numbers (cell densities) used during sample preparation
- Inconsistent sample preparation efficiency due to variations in reagents or protocols, etc.
- Handling errors resulting in loss during sample preparation
- Pipetting errors during sample preparation
- Sample loss during gel loading
- Inconsistent staining times between gels
- Inconsistent labeling efficiency with radioisotopes
- Inconsistent excitation/emission efficiencies between different fluorescent tags
- Inconsistent detection between gels during image acquisition
- Inconsistent exposure times between gels during image acquisition

All of the proteins represented in a given 2-D gel are affected equally by the factors above. Their relative abundance (or representation to each other) within the gel is not changed. However, the representation of one spot when compared to its matched counterpart on another gel will be affected by these factors. To accurately compare the quantity of any spot across two or more gels, it is essential to first establish that the value reported for a spot has been corrected to account for any sample-to-sample variation.

What Is the Scientific Rationale for Performing Normalization?

In an ideal world, the same amount of a known protein loaded onto two different gels could be quantified and the two values compared. The ratio of the two values would allow calculation of a correction factor. After the value reported for the known protein spot on one gel is corrected by this factor it will be equal to the value reported for the second gel. At the same time, all the other spot data on the first gel is also corrected by this same correction factor. The data has now been normalized between the gels, and direct comparisons of the unknowns on the gels can be performed reliably. Consider the following simple example:

- A radiolabeled gel is exposed for 2 hr on a storage phosphor screen and scanned on a Bio-Rad Molecular Imager® FX system
- The same gel is then exposed for 4 hr to generate a second image*

The representation of any given spot relative to its neighbors on the second image has not changed (it's the same gel), but the integrated optical density (IOD), or absorbance data, reported for any given spot on the second gel image would be approximately two-fold larger than the same data from the first image.

- Image 1 quantity reported for a particular spot = 232 IOD
- Image 2 quantity reported for this same spot = 464 IOD

* *The time used here is for the purposes of illustration only. The actual time required to expose radiolabeled 2-D gels varies greatly depending on type and amount of isotope used.*

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Since we know that there is an identical amount of protein in the matching spots seen in both images, it is possible to derive a correction factor to normalize the two images. To correct Image 2 for the longer exposure time (normalize Image 2 against Image 1), determine the ratio of a particular spot in the images:

$$\frac{\text{Image 1 Spot Quantity}}{\text{Image 2 Same Spot Quantity}} = \frac{232 \text{ IOD}}{464 \text{ IOD}} = 0.50$$

Then apply the correction factor to all the spots on Image 2. Since all spots in a given gel are corrected by a constant, the relative representation of spots within the gel to each other is not affected. The correction factor does compensate for the difference in the image exposure times:

Image 2 Spot Quantity	x	Correction Factor	=	Normalized Data
464 IOD	x	0.50	=	232

Note that the corrected, or normalized, value for this spot in Image 2 is now equivalent to the value for this same spot in Image 1:

Image 1 Spot	Image 2 Spot (un-normalized)	Image 2 Spot (normalized)
232	464	232

Challenges of Normalization in 2-D Gels

Unfortunately, working with 2-D gels does not typically offer the simple scenario described above. The ideal scenario allows the researcher to add standards of known mass to each experiment. In applications involving configurations such as 1-D gels or arrays (blots or microplates) this is easily accomplished, since discrete lanes or positions within the array can be dedicated to standards. However, it is not possible to dedicate a discrete place within a 2-D gel to standards.

One method used to add standards involves the direct addition (or spiking) of known standards into experimental unknowns prior to separation on a 2-D gel. Many researchers are uncomfortable with this practice. They are concerned with the possibility that one of the spiked standards might obscure the view of unknown experimental proteins of similar size and isoelectric point. This concern is understandable, but the lack of standards obviously causes difficulties when trying to determine protein mass or isoelectric point. And of course, the inability to refer to a standard of known load certainly makes it difficult to normalize between gels.

How PDQuest Software Addresses the Issue of Normalization in 2-D Gels

PDQuest offers a variety of options for normalization models. Choose the normalization model that best fits the characteristics of the 2-D gel sets. Available normalization options range from corrections based on total cpm (or cell number) loaded onto a gel, to methods as sophisticated as comparing and correcting gel images based on the expression of a set of constitutive proteins across the gels. Once a normalization method is selected, researchers can elect to enable or disable normalization for reporting purposes. If enabled, the effects of normalization will be seen in histograms as well as printed and exported report formats. The normalization methods available in PDQuest are:

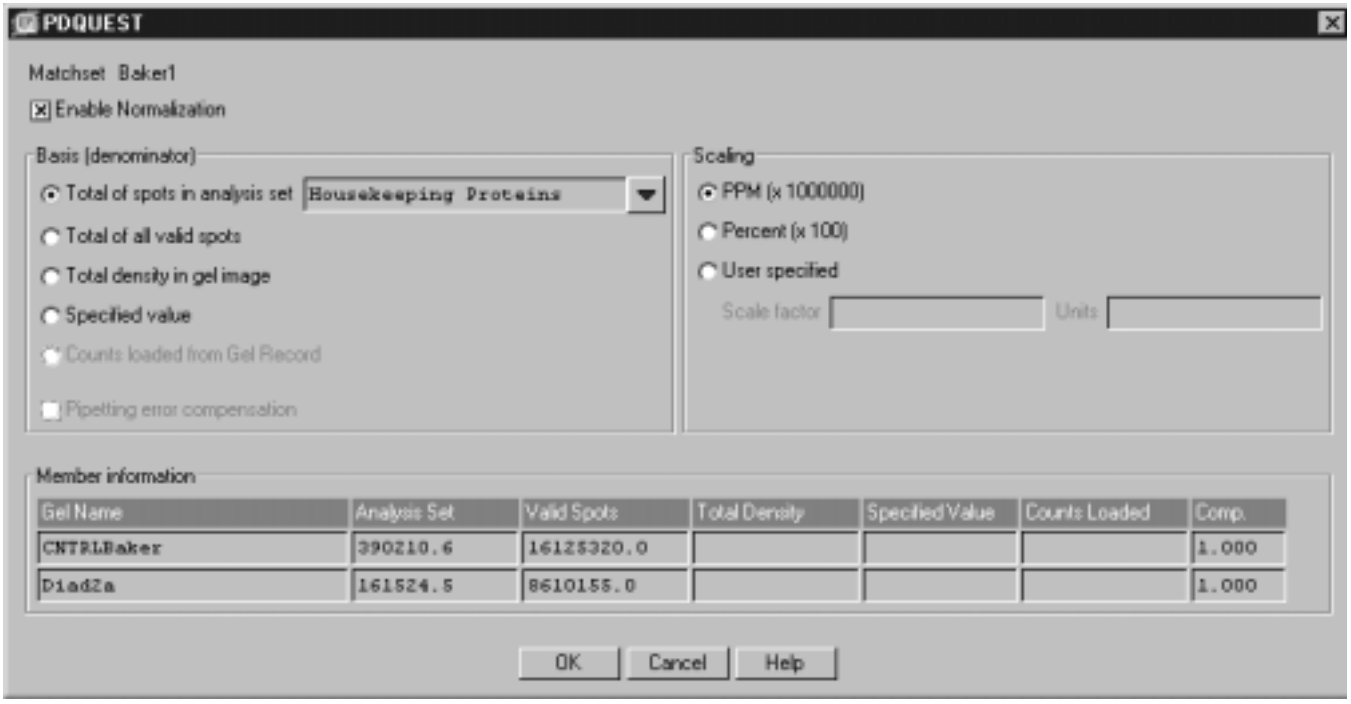
- Total of spots in analysis set
- Total of all valid spots
- Specified value
- Counts loaded from Gel Record
- Total density in gel image

Detailed Description of Normalization Methods in PDQuest

TOTAL OF SPOTS IN ANALYSIS SET

One method to determine size of proteins on 2-D gels (without adding standard markers to the sample) has been to identify common, well-characterized proteins that are typically expressed in the cell type of interest. These proteins can be considered as naturally occurring markers for mass and isoelectric point, and can be used to characterize unknowns accordingly. In some cases, subsets of these conserved proteins have been observed to be expressed in a very consistent manner at all times within a cell. These are commonly referred to as housekeeping genes or proteins. They are represented in highly consistent copy number in the cell, and expressed with little or no variation in mRNA or protein representation. Since they vary little or not at all from cell to cell, housekeeping proteins can serve as convenient normalization standards across gels.

PDQuest takes advantage of housekeeping proteins (once identified within a Reference Standard Image by the researcher) by automatically comparing all spots derived from these proteins across all the gels in a matchset. The individual gels are then normalized based on the representation of each housekeeping protein spot across the gels. PDQuest allows the identification of as many housekeeping proteins as desired; the IOD value of each will contribute to a summed total for each gel. This summed value will then be compared across gels to generate a normalization correction factor for each gel. The advantage of comparing summed multiple housekeeping proteins is that occasional minor variations in representation for a particular housekeeping protein spot will be averaged out when the group of spots is considered as a whole. This averaging will increase the accuracy of the normalization method. Another advantage of the PDQuest approach is that the housekeeping proteins are found and identified automatically by the software after initially being annotated as an Analysis Set by the user.



TOTAL OF ALL VALID SPOTS

In this model, the IOD value of all spots within a gel that have been matched to the Reference Standard Image spots are summed, and the summed values are then compared as a basis for normalization. This model assumes that few protein spots change within the experiment, and that the changes average out across the whole gel. The model can be useful if little information is known about sample variation.

SPECIFIED VALUE

In this model, each spot within an individual gel can be corrected by a user-definable normalization factor for that gel. This model can be useful when the user wishes to correct for known variations in samples across gels; for example, differences in the number of cells in an experimental sample, the amount of extract loaded, the length of time that images were detected, etc.

COUNTS LOADED FROM GEL RECORD

In this model, each gel can be corrected by a normalization factor automatically derived from the value entered by the user in the PDQuest Gel Record file for counts loaded during calibration of the gel image. This model also assumes that few protein spots change within the experiment, and that the changes average out across the whole gel. This model can be useful when the user wishes to correct for known variations in the amount of sample loaded across gels; for example, as measured with a scintillation counter prior to loading.

TOTAL DENSITY IN GEL IMAGE

In this model, the OD value of all pixels within the image are summed, and the summed values are then compared as a basis for normalization. This model assumes that all information captured in an image from both the background and the spots will be relatively consistent from gel to gel. It can be useful if little information is known about sample variation.

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