DIGE SYSTEM FOR 2D PROTEIN DETECTION

The DIGE System comprises a defined procedure for 2-D that uses an internal standard to generate the most accurate results. DIGE system is based on the technique of two-dimensional difference gel electrophoresis (2D DIGE). It is a powerful tool for separating complex mixtures of proteins by charge and size and for scanning and analyzing the resulting second dimension SDS PAGE gel images for protein differences.

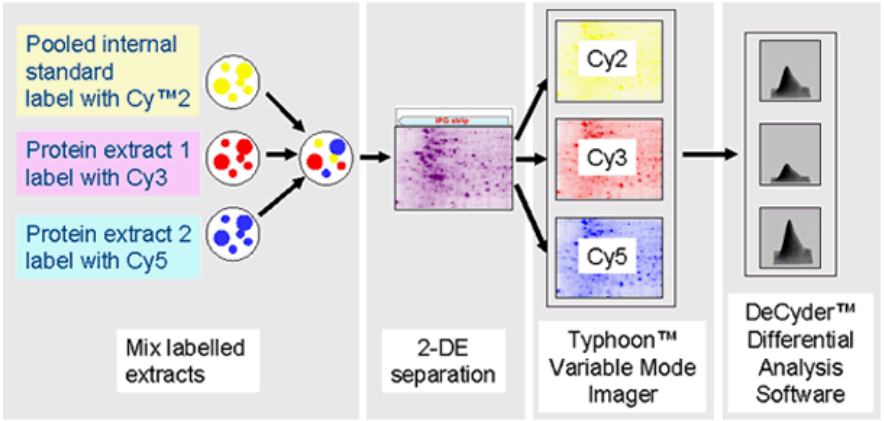
Combining novel proprietary technologies in fluorescence, sample multiplexing and image analysis, DIGE System is a fully integrated system offering significant benefits over classical second dimension SDS PAGE. The system comprises CyDye™ DIGE Fluor minimal dyes, labeling kit for scarce samples, Typhoon™ Variable Mode Imager, and DeCyder™ 2D software.

The use of CyDye DIGE Fluor minimal dyes enables multiplexing of up to three separate protein mixtures on the same second dimension SDS PAGE gel. The multiplexing capability of the 2D DIGE methodology enables the incorporation of the same internal standard on every gel and thereby eliminates gel-to-gel variation. Ensuring that each protein spot has its own internal standard is the only way to remove gel-to-gel variation, thereby significantly increasing accuracy and reproducibility. In order to increase reproducibility and statistical identity significance, it is often necessary to increase the number of biological variants in the experiment! There is a minimum of four gels needed to be able to gain statistical relevance, and this number may have to be increased dependent on the experiment.

To capitalize on this ability to multiplex, DeCyder 2D software and ImageMaster 2D Platinum software have been specifically designed for the DIGE system to accurately measure very small protein differences with high confidence. DeCyder 2D software and ImageMaster 2D Platinum software contains proprietary algorithms that perform co-detection of differently labeled samples within the same gel.

The Internal Standard and Experimental Design

The major source of error in a 2-D experiment is gel-to-gel variation. To eliminate this, an internal pooled standard should be run on all gels within an experiment. Multiplexing is the only way in which an internal standard can be used.



Labeling and internal standardization for real differences
With the DIGE System, up to three labeled protein samples can be run on the same 2–D gel simultaneously. One of these, the internal standard, results from the pooling of aliquots of all biological samples in the experiment. This internal standard is labeled with one CyDye™ DIGE Fluor minimal dyes (e.g. Cy™2) and is run together with individual samples, experimental and

control, labeled with other CyDye DIGE Fluor minimal dyes (e.g. Cy3 or Cy5). This means that every protein from all samples will be represented in the internal standard and each protein can therefore be compared to itself within the internal standard to generate a ratio of relative expression.

The same internal standard is run on all gels within an experimental series thereby creating an intrinsic link across all gels. Normalization of the internal standard across gels allows the ratio of relative expression of the same protein across gels to be compared directly, separating gel-to-gel variation from biological variation.

Even small differences in expression levels can be determined by comparing the ratio obtained from one fluorescent-labeled sample directly with another. As a result, it is possible to see < 10% differences in protein expression between samples, with > 95% statistical confidence.

Experimental Design

2D analysis experiments commonly address questions like protein level differences caused by a disease state, drug treatment, life-cycle stage etc. Some protein level differences studied are small and the results are affected by experimental variation originating both from the system and from inherent biological variation.

System related result variation

System related result variation may arise for two reasons:
- Gel-to-gel variation, which can result from differences in electrophoretic conditions between first dimension strips or second dimension gels, gel distortions, sample application variation and user-to-user variation.
- Variation due to user-specific editing and interpretation when using the data analysis software.

Inherent biological variation

Inherent biological variation arises from intrinsic differences that occur within a population. For example, differences from animal-to-animal, plant-to-plant or culture-to-culture which have been subjected to identical conditions.

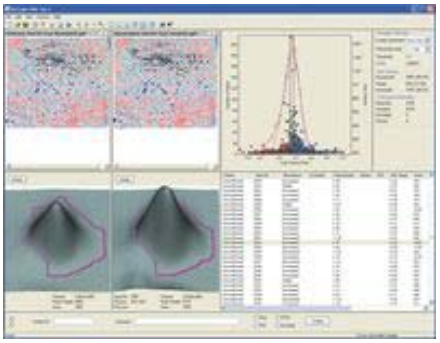
An example of a recommended experimental set-up designed to derive statistical data on differences between control and two treatment regimes A and B are outlined below. For the control and two treatment regimes, four biological replicates are included (1-4). The internal standard (a pool of equal amounts from all samples: four controls and eight treated) is labeled with CyDye DIGE Fluor Cy2 minimal dye and run on every 2-D gel. Each control and treated sample is labeled with either CyDye DIGE Fluor Cy3 or Cy5 minimal dye and loaded on gels as indicated below.

This representation is for the **minimum** 4 biological replication experiment (*please be aware that due to internal variation, additional replicates may be required for statistical significance of your samples*):

Gel	Cy2	Cy3	Cy5
1	Pooled internal standard	Control 1	Sample D
2	Pooled internal standard	Control 2	Sample C
3	Pooled internal standard	Sample B	Control 3
4	Pooled internal standard	Sample A	Control 4
5 pickgel	Pooled internal standard	Non-dye protein Control	Non-dye protein sample

A Pick Gel is prepared of a higher concentration of protein that is unlabeled and contains equal amounts of the known Control and Sample pools. The **minimum** amount of protein on a Pick Gel should be in the range of a total of 150 μg each. The Pick Gel must match the replicate gels and be within matching ranges of the software (see below) by manual adjustments.

The experiment is run with IEF focusing followed by a 1D gel electrophoresis on carefully prepared non-fluorescing glass plates. Each gel is imaged by phosphorimager lasers at all three fluorescent excitation and emission filters and are matched to each other through imaging software.



DeCyder™ 2-D Differential Analysis Software (DeCyder 2D) v7.0 is specifically designed for 2-D Fluorescence Difference Gel Electrophoresis (2-D DIGE) analysis and is a key element in the DIGE system. Unlike other 2-D electrophoresis methods, DIGE allows the option of reference to an internal standard for each spot, effectively eliminating gel-to-gel variation and delivering highly accurate, reproducible, and dependable quantitation. DeCyder 2D applies a gel comparison method that introduces zero statistical error, offering reliable data and analysis for 2-D DIGE experiments.

PROTEIN SAMPLE SUBMISSION

Please make sure that the samples are distinctly labeled as to which are considered control proteins and which are sample proteins and are submitted in good stable condition. It is required that samples submitted for 2D DIGE experiments do not contain reagents that interfere with the labeling of the protein sample with CyDye Fluor minimal dyes. These reagents include thiols used as reducing agents and polyamines used as IPG buffers (ampholytes). Samples must also be free of ionic contaminants that hamper effective isoelectric focusing of the proteins during the first dimension.

Avoid the addition of sample buffers containing coomassie dyes and glycerols as these will interfere with IEF.

Proteins can be submitted as pellets and our lab will lysis the cells in the proper buffer. Samples can also be lysed and submitted in a compatible lysis buffer (sample contaminants to avoid are listed below).

A typical DIGE experiment run in triplicates followed by a preparative gel experiment needs at least ***600 μg of each sample***.

Sample solution should contain 5-10 μg/μL of protein and the accurate protein concentration should be determined. *It is absolute to follow these guidelines for successful DIGE experiments.*

Compatible Lysis Buffers

1. 8-9 M urea
   4% CHAPS
   30 mM Tris (pH 8.5)
2. 7 M urea
   2 M Thiourea
   4% CHAPS
   30 mM Tris (pH 8.5)

Compatible Protease Inhibitors

1. Aprotinin: compatible at manufacturer's recommended concentration.
2. (4-amidino-phenyl) methane sulphonyl fluoride (APMSF): compatible at manufacturer's recommended concentration.
3. EDTA: compatible between 0.5-10 mM.
4. Phenylmethylsulphonyl fluoride (PMSF): compatible at manufacturer's recommended concentration.
5. Pepstatin A: compatible at manufacturer's recommended concentration.
6. Roche Complete Mini EDTA-free Protease Inhibitor Tablet

Compatible Phosphatase Inhibitors

1. Phosphatase inhibitor cocktail 1 (Sigma): compatible at manufacturer's recommended concentration.
2. Phosphatase inhibitor cocktail 2 (Sigma): compatible at manufacturer's recommended concentration.

Contaminants to Avoid

1. Reducing agents (e.g., DL-dithiothreitol (DTT), Tris- (2-carboxyethyl) phosphine (TCEP), tributyl phosphine (TBP), β-mercaptoethanol)
2. IPG buffers (e.g., ampholytes, pharmalytes, resolytes)
3. Ionic Detergents (e.g., SDS)
4. Buffers other than Tris (e.g., HEPES, PPA, PBS)
5. Salts (e.g., NaCl)
6. Nucleic Acids
7. Lipids
8. Polysaccharides

Note: If samples contain components not compatible with DIGE experiment, remove these contaminants by protein precipitation. A protocol for precipitation is available from our lab or a number of 2D clean-up kits are commercially available. After protein clean-up, redissolve the protein pellet with a compatible lysis buffer. Be sure to make the final protein concentration between 5-10 µg/µL. Protein precipitation and the subsequent protein quantitation can be done in our facility for additional charge.