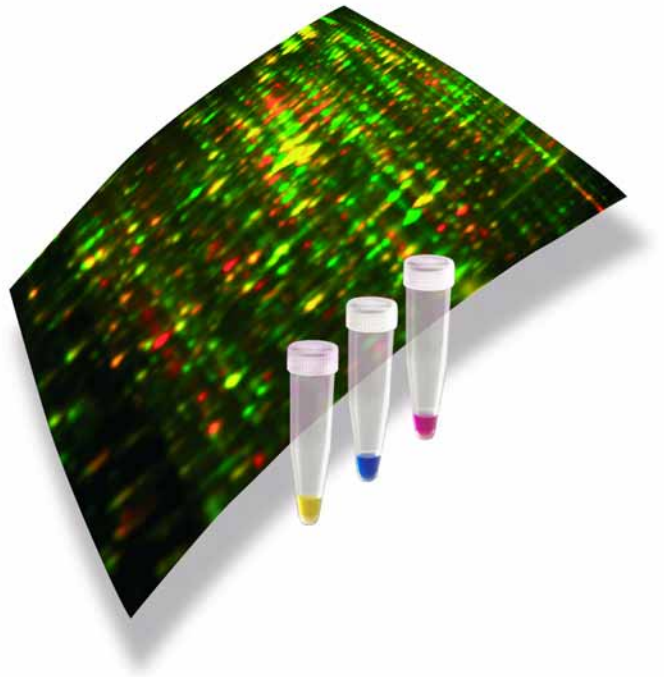


GE Healthcare

# Ettan DIGE System

User Manual



**Ettan**



## Important user information

All users must read this entire user manual to fully understand the safe use of Ettan DIGE System.

For safe use of Ettan DIGE System related products described in this user manual, see corresponding manuals.

### WARNING!



The WARNING! sign highlights instructions that must be followed to avoid personal injury. It is important not to proceed until all stated conditions are met and clearly understood.

### CAUTION!

The Caution! sign highlights instructions that must be followed to avoid damage to the product or other equipment. It is important not to proceed until all stated conditions are met and clearly understood.

### Note:

The Note sign is used to indicate information important for trouble-free and optimal use of the product.



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# Contents



# 1 Introduction

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## 1.1 Ettan DIGE System overview

Ettan™ 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, Ettan 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. Alternatively, Ettan DIGE Imager™ can be used instead of Typhoon Variable Mode Imager, and ImageMaster™ 2D Platinum software can be used instead of 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.

To capitalize on this ability to multiplex, DeCyder 2D software and ImageMaster 2D Platinum software have been specifically designed for the Ettan 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.

## 1.2 Workflows in 2D analysis

Two main workflows using Ettan DIGE System are presented:

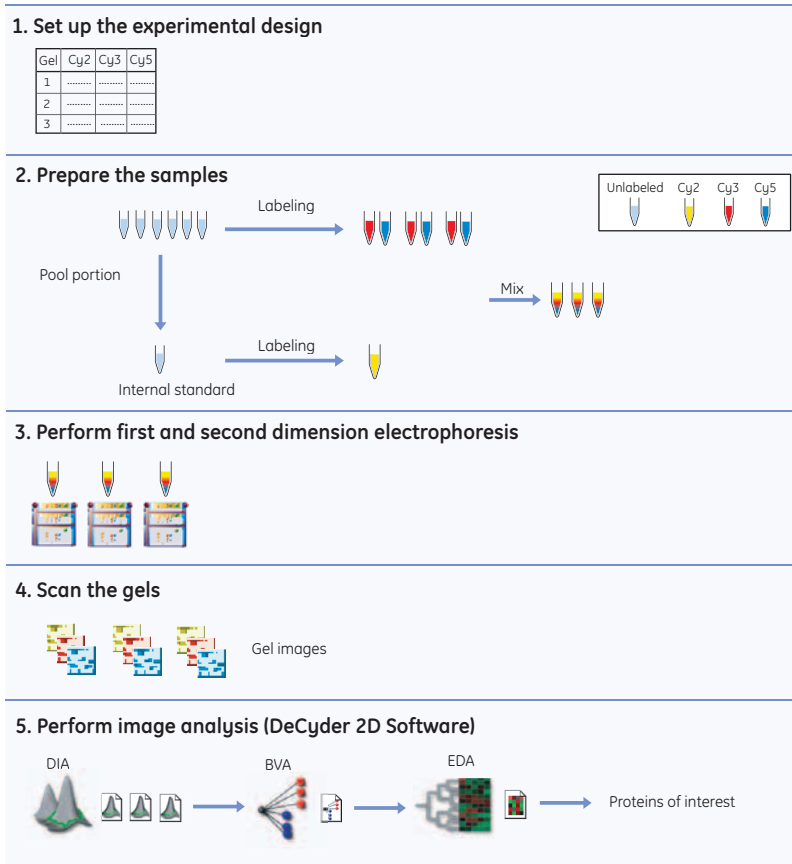
- Analytical workflow
- Preparative workflow

# 1 Introduction

## 1.2 Workflows in 2D analysis

### 1.2.1 Analytical workflow

The analytical workflow is used to find proteins of interest in an experiment. It includes defining the experimental design, preparing the samples, performing 2D separation of the proteins, scanning the gels and analyzing the gel images to find proteins of interest. Fig 1-1 outlines an overview of the Ettan DIGE System analytical workflow. See Chapters 4-8 for detailed information about the different steps in the analytical workflow.



**Fig 1-1.** Analytical workflow in Ettan DIGE System. Labeling is performed using CyDye DIGE Fluor minimal dyes. First dimension and second dimension SDS PAGE are performed using Ettan IPGphor™ 3 and Ettan DALTwelve or Ettan DALTsix. Scanning is performed using Typhoon Variable Mode. Imager and image analysis is performed in DeCyder 2D software.

### **1.2.2 Preparative workflow**

The preparative workflow is used to perform spot picking and protein identification of the proteins of interest found in the analytical workflow. To perform this, a pick list containing the coordinates for the proteins of interest to pick is created and a pick gel (preparative gel) is prepared. See Chapter 9 for detailed information about the different steps in the preparative workflow.

## **1.3 *Ettan DIGE System User Manual***

The scope of this user manual is to explain the central concept of the DIGE technology, to give an understanding of the entire experimental workflow and an overview of the Ettan DIGE related products available. Chapter 2 and 3 give a background to the DIGE system and to experimental design, chapters 4 to 8 cover the DIGE system analytical workflow, and chapter 9 contains special protocols for DIGE system preparative workflow. The four appendices cover other important information.

# 1 Introduction

## 1.4 Ettan DIGE System related products manuals

### 1.4 *Ettan DIGE System related products manuals*

Because Ettan DIGE System comprises many different products, references to the appropriate manuals for further details are made when necessary, see Table 1-1 . The manuals are also available on the web ([www.ettandige.com](http://www.ettandige.com)).

**Table 1-1.** Ettan DIGE System related products manuals.

<b>Manual</b>	<b>Code no.</b>
Ettan IPGphor 3 Control Software User Manual	11-0034-59
Ettan IPGphor 3 Instrument User Manual	11-0034-58
Ettan IPGphor Cup Loading Manifold User Manual	11-0034-60
2D Electrophoresis Principles and Methods	80-6429-60
Ettan DALTwelve system User Manual	80-6476-53
Ettan DALTsix system User Manual	80-6492-49
Typhoon User's Guide	63-0028-31
Ettan DIGE Imager User Manual	11-0036-59
ImageQuant TL User Manual	63-0050-82
DeCyder 2D Software User Manual	28-4010-06
DeCyder Extended Data Analysis Module User Manual	28-4010-07
ImageMaster 2D Platinum 6.0 User Manual	11-0034-38
Deep Purple Total Protein Stain Instructions	RPN6305PL
Ettan Spot Picker User Manual	18-1147-61
Ettan Digester User Manual	18-1167-31
Ettan Spotter User Manual	11-0003-41
Ettan Spot Handling Workstation User Manual	18-1153-55

## 2 DIGE concepts

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### 2.1 Introduction

#### 2.1.1 2D analysis result variation

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:

- 1 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.
- 2 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.

#### 2.1.2 Improvement of results by use of Ettan DIGE system

There are three main factors enabling Ettan DIGE System to provide greater accuracy than conventional 2D analysis:

- 1 Multiplexing, that is to run multiple samples on the same gel
- 2 Use of an internal standard for all proteins which can be run on all gels in a set of experiments
- 3 Experimental designs unique to this technique, see chapter 3.

##### *Compensation for system related result variation*

System variation cannot be overcome when using conventional 1-color 2D electrophoresis but by using Ettan DIGE System it is possible to minimize the gel-to-gel variation effects on results.

- In small experiments it is possible, by multiplexing, to run all samples on the same gel and thereby completely eliminate gel-to-gel variation.

## 2 DIGE concepts

### 2.2 CyDye DIGE Fluor dyes

- In larger experiments a combination of multiplexing of samples and inclusion of an internal standard within each gel all system related result variation can be adjusted for. Any protein spot can be compared and normalised to any other corresponding protein spot in the same or any other gel, in which the same internal standard has been used.

#### ***Differentiation of inherent biological variation from induced biological change***

Biological variation cannot be removed from any 2D electrophoresis experiments. However, Ettan DIGE system allows the inherent biological variation to be effectively differentiated from induced biological changes using highly accurate measurement of protein abundance changes, an appropriate experimental design and advanced statistical analysis.

The use of biological replicates in the experimental design ensures a true measurement of induced biological differences above the background of inherent biological variation. Ettan DIGE system is capable of detecting and quantifying differences as small as 10% between samples (above system variation) with greater than 95% statistical confidence.

### **2.2 CyDye DIGE Fluor dyes**

Ettan DIGE system is based upon the specific properties of the CyDye DIGE Fluor dyes. There are two different CyDye DIGE Fluor dyes available, CyDye DIGE Fluor minimal dyes and CyDye DIGE Fluor saturation dyes. The saturation dyes are also known as *labeling kit for scarce samples*. The key differences between CyDye DIGE Fluor minimal and saturation dyes are summarized in Table 2-1 .

- Use CyDye DIGE Fluor minimal dyes for normal applications. Multiplexing up to three samples and labeling of 50 µg protein is possible.
- Use CyDye DIGE Fluor saturation dyes when samples are precious or available in very small amounts. Multiplexing up to two samples and labeling of 5 µg protein is possible.

**Table 2-1.** Summary of key differences between CyDye DIGE Fluor minimal and saturation dyes.

	<b>Saturation labeling</b>	<b>Minimal labeling</b>
Sample preparation	Cell lysis buffer is at pH 8.0	Cell lysis buffer is at pH 8.5
Dyes	Maleimide dyes Label cysteine residues Two dyes available Reconstituted stock at 2 mM (analytical gels) or 20 mM (preparative gels) No dilution of stock solution	NHS ester dyes Label lysine residues Three dyes available Reconstituted stock at 1 mM Working concentration 0.4 mM
Reducing step	TCEP prior to labeling	No reduction step required
Protein labeling	Labeling reaction at 37°C Labeling reaction quenched with 2x sample buffer Labeling optimization by titrating TCEP and dye, analysis on 1D (SDS PAGE) gel Labeled proteins stable 1 month at -70°C	Labeling reaction on ice Labeling reaction quenched with 10 mM lysine Labeling optimization by comparing labeled samples, analysis on 1D (SDS PAGE) gel Labeled proteins as stable as unlabeled proteins at -70°C
Protein separation and analysis	No iodoacetamide equilibration step prior to second dimension electrophoresis A Cy™3 labeled sample is used on preparative gel for spot picking.	Iodoacetamide equilibration step required prior to second dimension electrophoresis An unlabeled sample is used on preparative gel for spot picking. Post-staining is required for matching to analytical gels

### 2.2.1 CyDye DIGE Fluor minimal dyes

#### **Chemical description**

CyDye DIGE Fluor minimal dyes are three spectrally resolvable dyes (Cy™2, Cy3 and Cy5) matched for mass and charge. Each CyDye DIGE Fluor minimal dye, when coupled to a protein, will add 450 Da to the mass of the protein. This mass shift does not effect the pattern visible on a second dimension SDS PAGE gel. A protein labeled with any of the CyDye DIGE Fluor minimal dyes will migrate to the same position on the second dimension SDS PAGE gel, thus making multiplexing possible.

#### **Sensitivity**

The dyes afford great sensitivity down to 25 pg of a single protein, and a linear response to protein concentration up to five orders of magnitude ( $10^5$ ). In

## 2 DIGE concepts

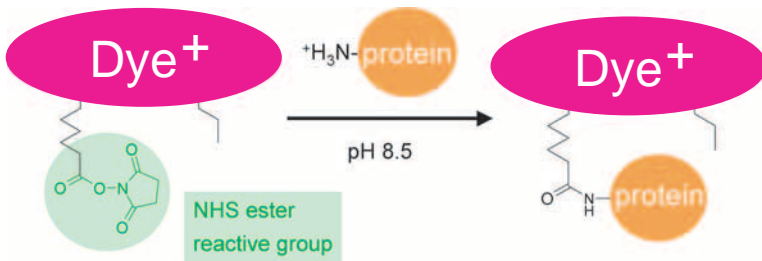
### 2.2 CyDye DIGE Fluor dyes

comparison, silver stain detects 1–60 ng of protein with a dynamic range of less than two orders of magnitude.

#### **Protein labeling**

CyDye DIGE Fluor minimal dyes have an NHS ester reactive group, and are designed to form a covalent bond with the epsilon amino group of lysine residues in proteins via an amide linkage. The dye is added to the protein such that the amount of dye is limiting within the labeling reaction. The lysine amino acid in proteins carries an intrinsic +1 charge at neutral or acidic pH. CyDye DIGE Fluor minimal dyes also carry a +1 charge which, when coupled to the lysine, replaces the lysine's +1 charge with its own, ensuring that the pI of the protein does not significantly alter.

With CyDye DIGE Fluor minimal dyes 50 µg protein is labeled in each reaction. The ratio used ensures that the dyes label approximately 1–2% of lysine residues so each labeled protein carries only one dye label and is visualised as a single protein spot. The CyDye DIGE Fluor minimal dyes therefore only label a small proportion of the total protein in a sample. For that reason, this type of labeling has been called *minimal* labeling.



**Fig 2-1.** Schematic of the minimal labeling reaction. CyDye DIGE Fluor minimal dye containing NHS ester active group covalently binds to the lysine residue of a protein via an amide linkage.



## 2.2.2 CyDye DIGE Fluor saturation dyes

### Chemical description

CyDye DIGE Fluor saturation dyes (also known as *labeling kit for scarce samples*) are two spectrally resolvable dyes (Cy3 and Cy5) matched for mass and charge. Each CyDye DIGE Fluor saturation dye, when coupled to a protein, will add approximately 677 Da to the mass of the protein. A protein labeled with any of the CyDye DIGE Fluor saturation dyes will migrate to the same position on the second dimension SDS PAGE gel, in this way making multiplexing possible.

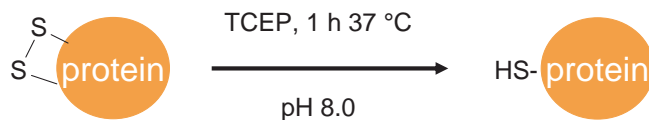
### Sensitivity

The dyes afford great sensitivity with detection lower than 25 pg of a single protein, and a linear response to protein concentration up to five orders of magnitude ( $10^5$ ).

### Protein labeling

CyDye DIGE Fluor saturation dyes have a maleimide reactive group which is designed to form a covalent bond with the thiol group of cysteine residues in proteins via a thioether linkage. CyDye DIGE Fluor saturation dyes have a neutral charge and will not affect the pI of the labeled protein.

## Reduction step



## Coupling step

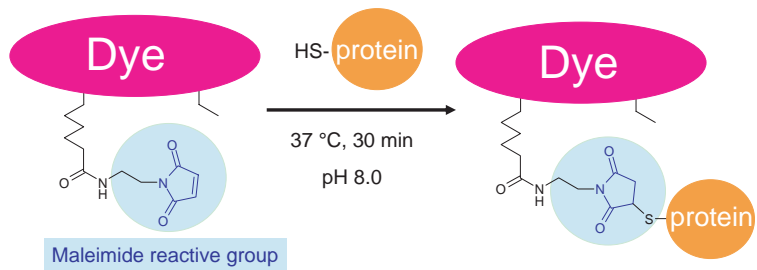


Fig 2-2. Schematic of the saturation labeling reaction.

## 2 DIGE concepts

### 2.3 The internal standard

With CyDye DIGE Fluor saturation dyes 5 µg protein is labeled in each reaction. The dye is added to the protein under such conditions that all available cysteine residues of the proteins are labeled. For that reason, this type of labeling has been called *saturation* labeling. To achieve optimum labeling a high dye-to-protein ratio is required.

### 2.3 The internal standard

The multiplexing capability of the 2D DIGE methodology enables the incorporation of the same internal standard on every second dimension SDS PAGE gel. The internal standard is a pool of all the samples within the experiment, and therefore contains every protein from every sample.

The internal standard is used to match and normalize the protein patterns across different gels thereby negating the problem of inter-gel variation, a common problem in standard “one sample per gel” 2D electrophoresis experiments.

The internal standard allows accurate quantitation of differences between samples, with an associated statistical significance. Quantitative comparisons of protein between samples are made on the relative change of each protein spot to its own in-gel internal standard. It enables accurate, statistical quantification of induced biological change between samples. The 2D DIGE methodology is the only technique to enable accurate standardized quantitation.

#### 2.3.1 Advantages of using an internal standard

The recommended protocol for experiments with more than three samples includes an internal standard that is run on all gels within an experiment together with up to two different labeled protein samples.

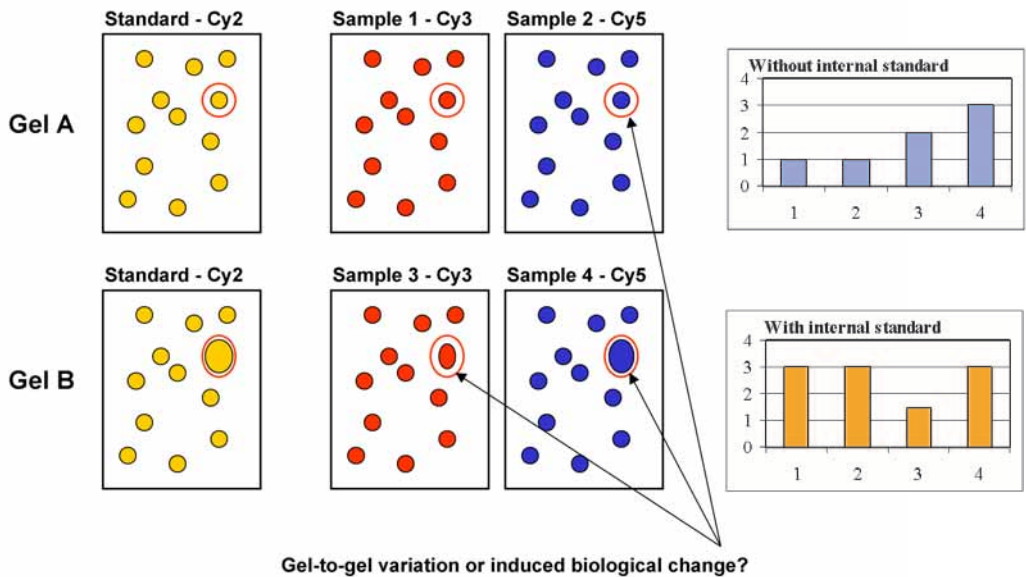
Linking every sample in all gels to a common internal standard offers a number of advantages:

- Accurate quantification and accurate spot statistics between gels
- Increased confidence in matching between gels
- Flexibility of statistical analysis depending on the relationship between samples
- Separation of induced biological change from system variation and inherent biological variation

### 2.3.2 Examples of benefits using an internal standard

The examples below illustrate the benefits of an internal standard. Fig 2-3 shows the theoretical scan results of two gels. Each gel contained two protein samples labeled with CyDye DIGE Fluor Cy3 or Cy5 minimal dyes and the same pooled internal standard sample labeled with CyDye DIGE Fluor Cy2 minimal dye.

If the gels illustrated in Fig 2-3 were analyzed without an internal standard, the conclusion would be that the volume of the highlighted protein spot in samples 1 and 2 has remained the same but is increased slightly in sample 3 and further in sample 4. However, reference to the internal standard shows that gel-to-gel variation has resulted in an increased spot volume in gel B compared to gel A. This means that instead of an increasing trend in spot volume from samples 1 to 4, the relative volume of the protein spot in sample 3 is reduced in comparison to samples 1, 2 and 4 where the spot volume ratios are identical.



**Gel-to-gel variation or induced biological change?**

Gel	Cy2 (Standard)	Cy3	Cy5
A	Pool samples 1-4	Sample 1 - untreated	Sample 2 - treated
B	Pool samples 1-4	Sample 3 - treated	Sample 4 - untreated

**Fig 2-3.** Example to illustrate the benefits of an internal standard in correctly identifying differences between samples 1, 2, 3 and 4. The right panel shows the different results achieved of the volumes of the protein spots without and with an internal standard.

## **2.4 Co-detection and matching using DeCyder 2D**

To capitalize on the ability to multiplex and use an internal standard, DeCyder 2D software has been specifically designed for the Ettan DIGE system. DeCyder 2D contains proprietary algorithms that perform co-detection of differently labeled samples within the same gel. DeCyder 2D also permits automated detection, background subtraction, quantitation, normalization, internal standardization and inter-gel matching. The benefits are low user interaction, high throughput and low experimental variation. For an introduction of DeCyder 2D, see Chapter 8. For a detailed guide, see DeCyder 2D Software User Manual.

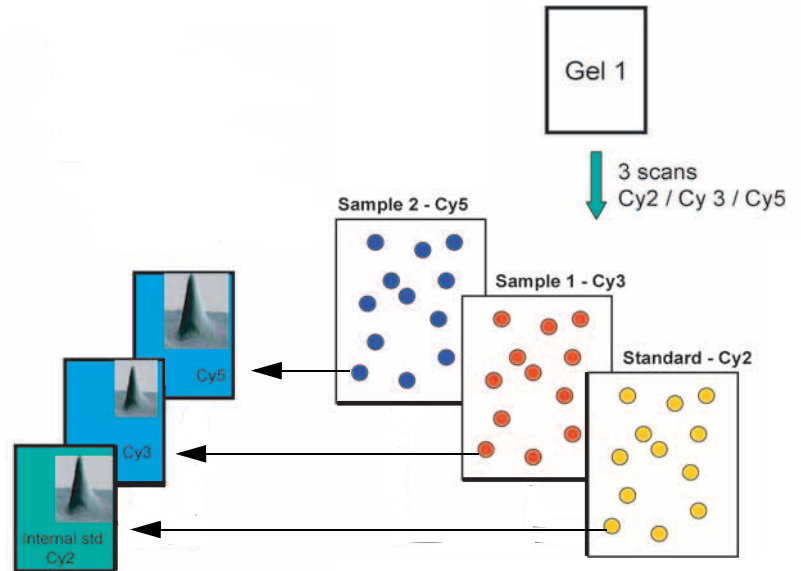
To compare protein spot volumes across a range of experimental samples and gels, two distinct steps are required:

- Intra-gel co-detection of sample and internal standard protein spots
- Inter-gel matching of internal standard samples across all gels within the experiment

Both of these analysis steps can be performed with minimal user intervention by DeCyder 2D.

### **2.4.1 Intra-gel co-detection**

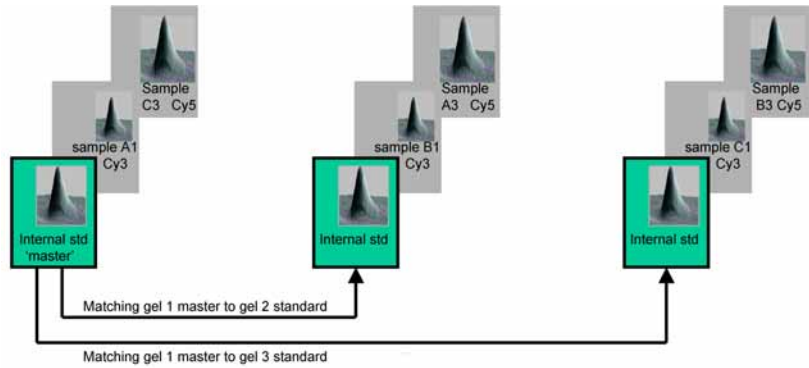
Three scans will be made of each gel, Cy2, Cy3 and Cy5 scans. Scanned images of each sample and the internal standard are overlaid in DeCyder 2D. The algorithms within the software co-detect the spots present in each scan, effectively identifying the position of each spot within the gel (Fig 2-4). The spot boundaries that result are identical for each image in the gel. This minimizes variation from detection and background subtraction, with the added benefit that every protein in the sample is intrinsically linked to the corresponding protein spot in the internal standard sample.



**Fig 2-4.** Intra-gel co-detection - All samples are co-detected with the internal standard.

### 2.4.2 Inter-gel matching

Experimental design ensures that each gel contains the same internal standard. This enables inter-gel comparisons of spot abundance. Before this can be done, it is important to ensure that the same protein spots are compared between gels. DeCyder 2D achieves this using the internal standard to match the position of each protein across all gels within the experiment. The internal standard image with the most detected spots is assigned as the 'Master'. Following co-detection, each image has a spot map species. The spot map species for the internal standard assigned as the Master, is used as a template to which all remaining spot map species for the other internal standards (intrinsically linked to their co-detected sample images) are matched (Fig 2-5).



**Fig 2-5.** Inter-gel matching - only the internal standards need to be matched. These are derived from the same sample which aids matching.

## 2.5 Protein abundance

Once the protein spots have been matched, the ratio of protein abundance between samples can be determined.

The use of an identical internal standard within all the experimental gels enables a comparison of protein abundance between samples on different gels. This is performed by comparison of the ratios of sample:standard, rather than direct comparison of raw spot volumes.

In this way differences in spot intensity that may arise due to experimental factors during the process of 2D electrophoresis, such as protein loss during sample transfer, will be the same for each sample within a single gel, including the internal standard. This means that the relative ratio of sample:standard will not be affected by such variation due to experimental factors.

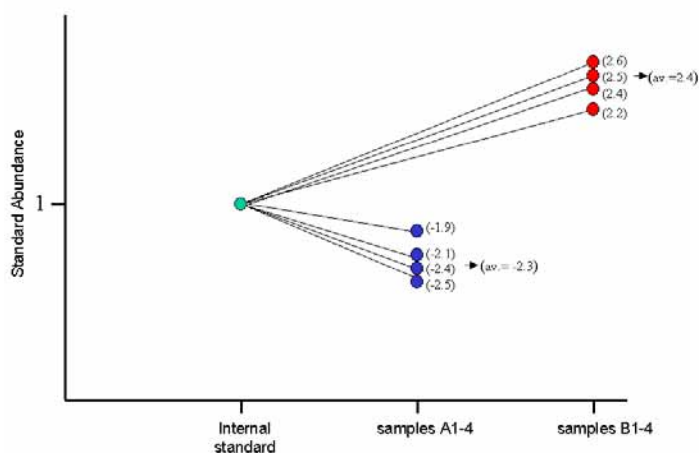
Spot volume (*i.e.* the sum of the pixel values within a spot minus background) for each experimental sample is compared directly to the internal standard by DeCyder 2D. Spot ratios are calculated ( $\text{volume of secondary image spot} / \text{volume of primary image spot}$ ) indicating the change in spot volume between the two images. The protein abundance for each spot in each sample is then expressed as a (normalized) ratio relative to the internal standard (the primary image) e.g. [Cy3 sample 1: Cy2 standard] and [Cy5 sample 2: Cy2 standard]. From this analysis, cross-sample comparisons can be made, see Table 2-2 .

**Table 2-2.** Ratio of (sample):(internal standard) for a single protein of interest.

Sample	Ratio of (sample):(internal standard) for a single protein of interest
A1	-2.1
A2	-2.4
A3	-1.9
A4	-2.5
B1	2.6
B2	2.5
B3	2.2
B4	2.4

**Note:** Down regulation of protein abundance relative to the internal standard is denoted by a negative prefix, for example, a two-fold decrease, or a conventional ratio of 0.5 is displayed as -2.0.

DeCyder 2D software can graphically display the relative abundance of each protein against the normalized internal standard, see Fig 2-6.

**Fig 2-6.** Plot of sample ratios relative to normalized internal standards.

## 2 DIGE concepts

### 2.6 Statistical tests of protein abundance in DeCyder 2D

It is possible to compare the protein abundance for a protein of interest in different samples. The ratio of (sample A):(sample B) for the protein of interest shown in Table 2-2 and Fig 2-6 can be calculated, see Table 2-3. The result shows that the protein of interest is down-regulated approximately five-fold in sample A compared to sample B.

**Table 2-3.** Ratio of (sample A):(sample B) calculated from (sample):(standard) ratios shown in Fig 2-6.

Sample	Ratio of (sample A):(sample B)
A1:B1	-5.4
A2:B2	-6.0
A3:B3	-4.2
A4:B4	-6.0

### **2.6 Statistical tests of protein abundance in DeCyder 2D**

Statistical tests are important and give the user a level of confidence by taking into account the inherent biological variation within a group compared to the induced difference between groups and assigning a confidence rating as to whether this change is above the biological variation.

Statistical tests can then be applied to the data in DeCyder 2D, for example, Student's T-test and ANOVA. The statistical tests compare the average ratio and variation within each group to the average ratio and variation in the other groups to see if any change between the groups is significant. If using the Extended Data Analysis (EDA) module of DeCyder 2D, additional multivariate statistical analyses such as Principal Component Analysis (PCA), Pattern Analysis and Discriminant Analysis can be performed.



## 3 Experimental design

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### 3.1 Introduction

Prior to commencing practical work, experimental design needs to be carefully considered. This chapter addresses experimental design unique to 2D DIGE analysis for optimal data analysis using DeCyder 2D software.

### 3.2 Designing 2D DIGE experiments

When designing 2D DIGE experiments, the following recommendations should be considered:

- 1 Inclusion of an internal standard sample on each gel
- 2 The requirement for biological replicates such as multiple cultures, tissue etc.
- 3 Randomization of samples to produce unbiased results, thus conforming with best experimental practice
- 4 No gel replicates of the same sample is needed

#### 3.2.1 Internal standard sample on each gel

It is recommended that an internal standard is run on all gels within an experiment as it is then possible to minimize effects of system related result variation, see section 2.3. The analysis of results then allows the inherent biological variation to be effectively differentiated from induced biological changes using appropriate experimental design and statistical analysis.

Quantitative comparisons of protein between samples are made on the relative change of each protein spot to its own in-gel internal standard. This removes gel-to-gel (system) variation, a common problem with conventional one sample per gel 2D studies. It also enables accurate, statistical quantification of induced biological change between samples. Ettan DIGE System is the only protein difference analysis technique that utilises this approach.

#### 3.2.2 Biological replicates

It is strongly advised that biological replicates are included in every group. By increasing the number of biological replicates it is possible to get an accurate measurement of the change due to a treatment or disease that is significant above a baseline of inherent biological variation. Gel replicates of the same biological sample will not deliver this information. Without biological replicates, results may not be biologically relevant and it is often only possible to conclude that differences in results are above system variation.

## 3 Experimental design

### 3.3 Examples of experimental design

#### 3.2.3 Randomization of samples

Randomization of samples across gels removes any bias from the experiments such as experimental conditions, sample handling and labeling, ensuring that results from 2D DIGE experiments are accepted by peers. Even if the system related result variation is low using Ettan DIGE System it is good laboratory practice to distribute individual experimental samples evenly between different CyDye DIGE Fluor dyes and different gels to avoid for example systematic errors. See Section 3.3 for examples.

#### 3.2.4 No gel replicates of the same sample is needed

As the result variation using Ettan DIGE System is so low due to the internal standard and method of analysis, any system variation will by far be outweighed by the inherent biological variation. However, gel replicates can be included if desired.

### 3.3 Examples of experimental design

In order to maximize the value of CyDye DIGE Fluor dyes and DeCyder 2D software, it is important to carefully consider the experimental testing regime. Two examples using CyDye DIGE Fluor minimal dyes and CyDye DIGE Fluor saturation dyes (sections 3.3.1 and 3.3.2, respectively), are presented below to illustrate examples of experimental design.

#### 3.3.1 CyDye DIGE Fluor minimal dyes

Comparison of protein abundance between three differently treated samples (A-C) each with four biological replicates using:

- CyDye DIGE Fluor Cy2, Cy3, and Cy 5 minimal dyes
- Internal standard labeled with CyDye DIGE Fluor Cy2 minimal dye
- A design with randomized sample labeling of either CyDye DIGE Fluor Cy3 or Cy5 minimal dyes is strongly recommended. Samples to be evenly distributed between the CyDye DIGE Fluors Cy3 and Cy5 and between gels

#### *Experimental set up*

- Mix 50 µg of each of the 12 samples (A1-A4, B1-B4 and C1-C4) together to create 600 µg of the internal standard, and label with CyDye DIGE Fluor Cy2 minimal dye
- Individually label 50µg of samples A1 - A4, B1 - B4 and C1 - C4 with either CyDye DIGE Fluor Cy3 or Cy5 minimal dye in a randomized design
- Six gels are required, loaded as described in Table 3-1

**Table 3-1.** Gels to be run for Case study 1: Samples labeled with CyDye DIGE Fluor minimal dyes.

Gel	Cy2 (Internal standard)	Cy3	Cy5
1	50 µg (4.17µg each of samples A1-4, B1-4, C1-4)	50 µg sample B2	50 µg sample C1
2	50 µg (4.17µg each of samples A1-4, B1-4, C1-4)	50 µg sample A1	50 µg sample B3
3	50 µg (4.17µg each of samples A1-4, B1-4, C1-4)	50 µg sample C3	50 µg sample A4
4	50 µg (4.17µg each of samples A1-4, B1-4, C1-4)	50 µg sample A3	50 µg sample C2
5	50 µg (4.17µg each of samples A1-4, B1-4, C1-4)	50 µg sample B4	50 µg sample A2
6	50 µg (4.17µg each of samples A1-4, B1-4, C1-4)	50 µg sample C4	50 µg sample B1
<b>Total gels = 6</b>			

**Note:** *By using three dyes instead of two the number of gels required is halved. The amount of material required is also reduced as half the amount of internal standard is used (six gels instead of twelve gels).*

### 3.3.2 CyDye DIGE Fluor saturation dyes

Comparison of protein abundance between three differently treated samples (A-C) each with four biological replicates using:

- CyDye DIGE Fluor Cy3 and Cy5 saturation dyes
- Internal standard labeled with CyDye DIGE Fluor Cy3 saturation dye
- All experimental samples labeled with the same dye (CyDye DIGE Fluor Cy5 saturation dye)
- Twelve gels are required, loaded as described in Table 3-1

### 3 Experimental design

#### 3.3 Examples of experimental design

##### **Experimental set up**

- Mix 5 µg of each of the 12 samples (A1-A4, B1-B4 and C1-C4) together to create 60 µg of the internal standard
- Label the internal standard with CyDye DIGE Fluor Cy3 saturation dye
- Individually label 5 µg of samples A1 - A4, B1 - B4 and C1 - C4 with CyDye DIGE Fluor Cy5 saturation dye
- Twelve gels are required, loaded as described in Table 3-2

**Table 3-2.** Gels to be run for Case study 2: Samples labeled with CyDye DIGE Fluor saturation dyes.

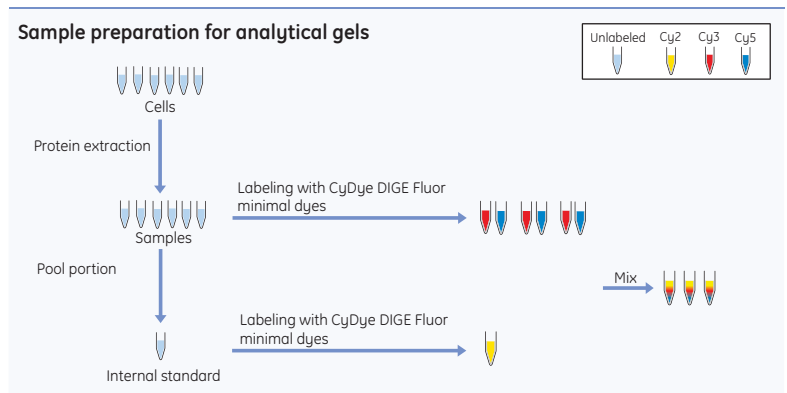
<b>Gel</b>	<b>Cy3 Standard</b>	<b>Cy5</b>
1	5 µg (4.17µg each of A1-4, B1-4, C1-4)	5 µg sample B4
2	5 µg (4.17µg each of A1-4, B1-4, C1-4)	5 µg sample C2
3	5 µg (4.17µg each of A1-4, B1-4, C1-4)	5 µg sample A4
4	5 µg (4.17µg each of A1-4, B1-4, C1-4)	5 µg sample A2
5	5 µg (4.17µg each of A1-4, B1-4, C1-4)	5 µg sample C4
6	5 µg (4.17µg each of A1-4, B1-4, C1-4)	5 µg sample B2
7	5 µg (4.17µg each of A1-4, B1-4, C1-4)	5 µg sample A1
8	5 µg (4.17µg each of A1-4, B1-4, C1-4)	5 µg sample C3
9	5 µg (4.17µg each of A1-4, B1-4, C1-4)	5 µg sample B1
10	5 µg (4.17µg each of A1-4, B1-4, C1-4)	5 µg sample A3
11	5 µg (4.17µg each of A1-4, B1-4, C1-4)	5 µg sample C1
12	5 µg (4.17µg each of A1-4, B1-4, C1-4)	5 µg sample B3
<b>Total gels = 12</b>		

**Note:** *It is possible to use CyDye DIGE Fluor minimal dyes in the same manner labeling only with two dyes.*

## 4 Sample preparation and labeling

### 4.1 Introduction

The preparation of a cell lysate and subsequent labeling with CyDye DIGE Fluor minimal dyes or CyDye DIGE Fluor saturation dyes is detailed in this chapter. The reagents and conditions stated here are those which have been found to be the most consistently useful across many sample types. However, there will be cases where some individual optimization of lysis conditions is required. See Section 2.2 for information of CyDye DIGE Fluor minimal dyes and CyDye DIGE Fluor saturation dyes and Chapter 9 for special changes in protocols when running preparative second dimension SDS PAGE for spot picking of proteins of interest.



**Fig 4-1.** Sample preparation and labeling with CyDye DIGE Fluor minimal dyes.

Recommended protocols for preparation of a cell lysate and labeling are presented in sections 4.3 to 4.8 and recipes for recommended buffers and solutions are given in section 4.9.

**Note:** Some standard methods for preparation of protein samples for conventional 2D electrophoresis may not be compatible with Ettan DIGE system.

## 4.2 Workflow

Workflow for sample preparation and labeling:

- 1 Sample preparation: Prepare cell lysates compatible with CyDye DIGE Fluor minimal dye or CyDye DIGE Fluor saturation dye labeling.
- 2 Adjust the pH of the cell lysates.
- 3 Determine cell lysates protein concentration.
- 4 Prepare an internal standard.
- 5 Label protein samples using CyDye DIGE Fluor minimal dyes or CyDye DIGE Fluor saturation dyes.
- 6 Prepare labeled protein samples for first dimension isoelectric focusing.

## 4.3 Sample preparation

The sample preparation protocol in this section is designed to use cell cultures as starting material.

For sample preparation protocols for other starting material, see [www.ettandige.com](http://www.ettandige.com).

### 4.3.1 Solution recommendations

#### *Cell wash solution*

It is recommended to use the cell wash solution. As an alternative 0.5 X phosphate buffered saline (PBS) can be used. However, if PBS is used it must be completely removed since it may cause issues with high salt load during electrophoresis.

Any other wash solutions should be tested for compatibility with the labeling step in controlled experiments. The cell wash solution used should not lyse the cells, but it should dilute and remove any growth media, or reagents that might affect CyDye DIGE Fluor dye labeling process.

#### *Cell lysis solution*

It is recommended to use the cell lysis solution. Alternatively, buffers such as Tris or Bicarbonate can be used in the protein solution. The solution should be at a concentration of approximately 30 mM. Higher concentration may affect isoelectric focusing.

**Note:** *Ensure that the pH remains between pH 8.0–9.0 in the cell lysate, by adding the cell lysis solution. Failure to include a suitable buffer will mean that the pH of the solution may fall below pH 8.0 resulting in little or no protein labeling.*

**Note:** The cell lysis solution should not contain any primary amines, such as ampholytes or thiols (e.g. DTT) as these will compete with the proteins for CyDye DIGE Fluor dyes resulting in fewer dye labeled proteins. This decrease in labeling efficiency may affect the data after scanning and spot detection.

### 4.3.2 Protocol

The example of protocol presented is adapted to *Escherichia coli* cell culture. Other wash solution might be more appropriate for different cell types. Approximately  $4 \times 10^{10}$  *E. coli* cells will result in 5-10 mg of protein in a total volume of 1 ml of standard cell lysis solution.

- 1 Pellet the cells in a suitable centrifuge at +4°C.
- 2 Pour off all growth media, taking care not to disturb the cell pellet.
- 3 Re-suspend the cell pellet in 1 ml of standard cell wash solution in a microfuge tube.
- 4 Pellet the cells in a bench-top microfuge at 12 000 × g for 4 min at +4°C. Remove and discard the supernatant.
- 5 Repeat steps 3 and 4 at least three times. Ensure all the cell wash solution has been removed.
- 6 Re-suspend the washed cell pellet in 1 ml of standard cell lysis solution and leave on ice for 10 min.

**Note:** If the protein concentration is less than 5 mg/ml after protein quantitation, see section 4.5, re-suspend cells in a smaller volume of lysis solution in subsequent experiments. Alternatively, precipitate proteins using Ettan 2D Clean-Up Kit (code no. 80-6484-51), and re-suspend in a smaller volume of cell lysis solution.

- 7 Keep the cells on ice and sonicate intermittently until the cells are lysed.
- 8 Centrifuge the cell lysate at +4°C for 10 min at 12 000 × g in a microcentrifuge.
- 9 Transfer supernatant to a labeled tube. This is the cell lysate. Discard the pellet.
- 10 Remove contaminating substances with Ettan 2D Clean-Up Kit. This procedure improves the labeling efficiency by removal of endogenous small molecules. It also may improve spot resolution and increase the number of spots detected.

## 4 Sample preparation and labeling

### 4.4 pH adjustment

- 11 Check the pH of the cell lysate and adjust if necessary, see section 4.4. The cell lysate can now be stored in aliquots, at  $-70^{\circ}\text{C}$  until protein yield is to be determined.

**Note:** When using Ettan 2D Clean-Up Kit the pH drops considerably and adjustment of pH to 8-9 is required.

### 4.4 pH adjustment

- 1 Check the pH of the cell lysate (protein sample) by spotting a small volume (1-3  $\mu\text{l}$ ) on a pH indicator strip.



- 2 Optimal cell lysate pH is 8.5 for CyDye DIGE Fluor minimal dyes and 8.0 for CyDye DIGE Fluor saturation dyes.

**Note:** The use of cell lysates with optimal pH as described above is very important. A lower pH than optimal will make labeling ineffective and a higher pH than optimal will make the labeling unspecific.

- 3 Adjust the pH if the pH of the cell lysate is outside the desired range. Normally, the pH needs to be increased.
- 4 To increase the pH in the cell lysate, make some more cell lysis solution with tris base (without the protein) with pH 9.5 or higher.
- 5 Add aliquots of the new lysis solution to the cell lysate. This will gradually increase the pH of the cell lysate. Stop when the pH of the protein sample is at pH 8.5 (for minimal labeling).

Alternatively, the pH of the lysate can be increased to pH 8.5 by careful addition of dilute sodium hydroxide (50 mM or higher).



## 4.5 Protein concentration determination

The concentration of protein in all lysates should be determined using a suitable protein assay, compatible with detergents and thiourea if these are present in the cell lysate.

Protein Determination Reagent (USB, code no. 30098) or Ettan 2D Quant Kit (code no. 80-6483-56) are both suitable for this activity.

## 4.6 Internal standard preparation

The internal standard is created by pooling an aliquot of all biological samples in the experiment and labeling it with one of the CyDye DIGE Fluor dyes (usually Cy2 when using CyDye DIGE Fluor minimal dyes and Cy3 when using CyDye DIGE Fluor saturation dyes).

The internal standard is then run on every single gel along with each individual sample. This means that every protein from all samples will be represented in the internal standard, which is present on all gels. In this way every protein spot on all gels will have an internal standard.

**Note:** *Sufficient internal standard must be prepared to allow enough to be included on every gel in the experiment.*

## 4.7 Labeling

The protocols for labeling cell lysate samples with CyDye DIGE Fluor minimal and saturation dyes are different. However, the preparation of dyes, including reconstituting, and making working dye solutions are similar. Follow protocols in sections 4.7.1 and 4.7.2, for both types of dyes but use the appropriate section of 4.7.3 and 4.7.4.

**Note:** *It is recommended that all new cell lysates or samples containing chemical components that has not been approved for DIGE use are checked for successful labeling, see Appendix A.*

### 4.7.1 Preparation of CyDye DIGE Fluor dyes for labeling

The reconstitution and storage of CyDye DIGE Fluor minimal and saturation dyes is important to the success of sample labeling. If reagents, such as dimethylformamide (DMF) are of a low quality, or the CyDye DIGE Fluor minimal dyes are incorrectly stored, protein labeling will not be efficient.

## 4 Sample preparation and labeling

### 4.7 Labeling

#### **Reconstituting CyDye DIGE Fluor dye in dimethylformamide**

CyDye DIGE Fluor minimal and saturation dyes are supplied as a solid powder and are reconstituted in dimethylformamide (DMF) giving a concentration of 1 nmol/ $\mu$ l. After reconstitution in DMF the dye will give a deep color; Cy2-yellow, Cy3-red, and Cy5-blue.

**Note:** *It is recommended that a new bottle of DMF is opened every 3 months. The DMF must be high quality anhydrous (Specification: <0.005% H<sub>2</sub>O,  $\geq$ 99.8% pure, Sigma Aldrich 22,705-6), and every effort should be taken to ensure it is not contaminated with water.*

**Note:** *DMF, once opened, will start to degrade generating amine compounds, which will react with the CyDye DIGE Fluor minimal dyes reducing the concentration of dye available for protein labeling.*

- 1 Take the CyDye DIGE Fluor minimal dye from the  $-20$  °C freezer, spin briefly to ensure that the powder is at the bottom of the tube, and leave to warm for 5 min at room temperature without opening. This will prevent exposure of the dye to condensation which may cause hydrolysis.
- 2 Take a small volume of DMF from its original container and dispense into a microfuge tube.
- 3 From this tube remove the specified volume of the aliquoted DMF (see specification sheet supplied with the CyDye DIGE Fluor dye) and add to each new vial of dye. Recommendations for:
  - CyDye DIGE Fluor minimal dyes: 25  $\mu$ l DMF to 25 nmol of dye (1 mM)
  - CyDye DIGE Fluor saturation dyes: 50  $\mu$ l DMF to 100 nmol of dye (2 mM)
- 4 Replace the cap on the microfuge tube containing the dye and vortex vigorously for 30 seconds to dissolve the dye.
- 5 Centrifuge the microfuge tube for 30 seconds at 12 000  $\times$  g in a benchtop microfuge.

CyDye DIGE Fluor saturation dye stock solution (2 mM) can be used with no further dilutions. Once reconstituted, the saturation dye stock solution is stable for three months.

CyDye DIGE Fluor minimal dye stock solution (1 nmol/ $\mu$ l) is prepared but should be diluted before use. Once reconstituted, the minimal dye stock solution is stable for three months or until the expiry date on the container, whichever is sooner.

**Note:** Check that the dye solution has an intense color. During transport, the dye powder may spread around the inside surface of the tube (including the lid). If the dye has not an intense color, then pipette the solution around the tube (and lid) to ensure resuspension of dye. Vortex and spin down.

For dilution of the CyDye DIGE Fluor minimal dye stock solution:

- 1 Dilute the minimal dye stock solution to working dye solution concentration, see section 4.7.2.
- 2 The dye stock solution should be stored in a light excluding container at  $-20^{\circ}\text{C}$ . Return to freezer as soon as possible after use. The dye stock solution is stable for up to three months at  $-20^{\circ}\text{C}$ .

#### 4.7.2 Preparation of working dye solution

##### **Amount of dye required for labeling**

It is recommended that 400 pmol of dye is used to label 50  $\mu\text{g}$  of protein. If labeling more than 50  $\mu\text{g}$  of protein then the dye:protein ratio must be maintained for all samples in the same experiment. Other dye:protein ratios can be used but must be optimized for the sample by testing the labeling on an SDS PAGE gel, see Appendix A.

##### **Dilution of dye stock solution**

Prior to labeling, the dye stock solution is diluted with DMF (see above) to a working dye solution. A concentration of 400 pmol/ $\mu\text{l}$  is recommended.

**Note:** The working dye solution is only stable for one week at  $-20^{\circ}\text{C}$ .

- 1 Briefly spin down the dye stock solution in a microcentrifuge.
- 2 Add 2  $\mu\text{l}$  dye stock solution to 3  $\mu\text{l}$  DMF.
- 3 Ensure all dye is removed from the pipette tip by pipetting up and down the working dye solution several times.

## 4 Sample preparation and labeling

### 4.7 Labeling

#### 4.7.3 CyDye DIGE Fluor minimal dye labeling

The dye labeling reaction is designed to be simple and should take about 45 min to perform.

Recommended conditions:

- It is very important that the cell lysate (protein sample) has pH 8.5 in order to achieve an efficient labeling, see section 4.4.
- Dye to protein ratio of 400 pmol dye:50 µg protein. If the ratio of dye:protein is too low, sensitivity may be decreased and if the ratio of dye:protein is too high, there is a possibility of multiple dye molecules per protein and this could lead to multiple spots per protein on the gel.
- Protein concentration of 5-10 mg/ml in the cell lysate. However, samples containing 1 mg/ml protein have been successfully labeled using the protocol below.

#### **Protocol**

The protocol illustrates labeling of a cell lysate using 400 pmol of dye to label 50 µg of protein.

- 1 Add a volume of protein sample equivalent to 50 µg to a microfuge tube. Bulk labeling reactions can be performed by scaling up as required.
- 2 Add 1 µl of working dye solution (400 pmol) to the microfuge tube containing the protein sample.
- 3 Mix dye and protein sample thoroughly by pipetting and vortexing.
- 4 Centrifuge briefly in a microcentrifuge to collect the solution at the bottom of the tube. Leave on ice for 30 min in the dark.
- 5 Add 1 µl of 10 mM lysine to stop the reaction. Mix and spin briefly in a microcentrifuge. Leave for 10 min on ice, in the dark.

Labeling is now finished. The labeled samples can be processed immediately or stored for up to 3 months at -70°C in the dark.

#### 4.7.4 CyDye DIGE Fluor saturation dye labeling

The dye labeling reaction is designed to be simple and should take about 2 h to perform.

Recommended conditions:

- It is very important that the cell lysate (protein sample) has pH 8.0 in order to achieve an efficient labeling, see section 4.4.
- TCEP:dye concentration ratio should be kept at a 1:2 ratio to ensure efficient labeling.
- Protein concentration of 0.55-10 mg/ml in the cell lysate.
- For samples containing proteins of interest with high cysteine content, more TCEP (reduces disulphide bonds) and more dye (for labeling thiol groups) are required.

#### Protocol

- 1 Add a volume of protein sample equivalent to 5 µg to a microfuge tube.
- 2 Make up to 9 µl with cell lysis solution.
- 3 Add 1 µl 2 mM TCEP.
- 4 Mix vigorously by pipetting and spin.  
**Note:** *Since cell lysates are viscous it is important to mix samples thoroughly in this and all following mixing steps to avoid non-uniform labeling.*
- 5 Incubate at 37°C for 1h in the dark.
- 6 Add 2 µl 2 mM CyDye DIGE Fluor saturation dye solution.  
**Note:** *Label the pooled protein internal standard sample with Cy3 and the experimental protein sample with Cy5.*
- 7 Mix vigorously by pipetting and spin.
- 8 Incubate at 37°C for 30 min in the dark.
- 9 Stop the reaction by adding an equal volume of 2x sample buffer.
- 10 Mix vigorously by pipetting and spin.

Labeling is now finished. The labeled samples can be processed immediately or stored for 1 month at -70°C in the dark.

## 4 Sample preparation and labeling

### 4.8 First dimension sample preparation

#### 4.8 First dimension sample preparation

The main difference between conventional 2D electrophoresis and Ettan DIGE system is that the latter will enable up to three different protein samples to be run on a single 2D gel. To achieve this you need to mix the differently labeled protein samples *BEFORE* the first dimension run.

##### Protocol

It is recommended that 50 µg (for CyDye DIGE Fluor minimal dyes) or 5 µg (for CyDye DIGE Fluor saturation dyes) of each labeled protein sample is combined for each gel.

- 1 Combine the two (for CyDye DIGE Fluor saturation dyes) or three (for CyDye DIGE Fluor minimal dyes) labeled samples into a single microfuge tube and mix. One of these samples should be the pooled internal standard.
- 2 **For CyDye DIGE Fluor minimal dye labeled samples:** Add an equal volume of 2× sample buffer to the labeled protein samples and leave on ice for 15 minutes.

The samples are now ready for the first dimension isoelectric focusing step.

**Note:** *After adding 2× sample buffer and incubating on ice it is recommended that the sample is run immediately on Immobiline™ DryStrips. Proceed to Chapter 5, First dimension isoelectric focusing (IEF).*

#### 4.9 Recipes

##### Cell wash solution

Reagent	Quantity	Final concentration
Tris (100 mM, pH 8.0)	5.0 ml	10 mM
Magnesium acetate (1 M)	0.25 ml	5 mM
Make up to 50 ml with distilled water		

Store at 4 °C. Stable for 1 month.

**Cell lysis solution**

- 1 Decide whether to use cell lysis solution 1 or 2. The cell lysis solution option 1 is very similar to option 2 except that thiourea is added, which has been shown to solubilize many more proteins, especially membrane proteins.
- 2 Adjust the whole solution to pH 8.5 using dilute HCl. Confirm the pH of your cell lysis solution by spotting 5 µl on a pH indicator strip.
- 3 Make the volume of the cell lysis solution up to 100 ml.
- 4 The cell lysis solution can now be aliquoted and stored at -20 °C.

**Cell lysis solution (option 1) - contains thiourea.**

Reagent	Quantity	Final concentration
Urea (MW 60.06)	42.0 g	7 M
Thiourea (MW 76.12)	15.22 g	2 M
Tris (1M not pH'd)	3.0 ml	30 mM
CHAPS (MW 614.89)	4 g	4% (w/v)
Make up to 100 ml with distilled water		

Small aliquots can be stored at -20°C for up to three months.

**Cell lysis solution (option 2)**

Reagent	Quantity	Final concentration
Urea (MW 60.06)	48.0 g	8 M
Tris (1 M, not pH'd)	3.0 ml	30 mM
CHAPS (MW 614.89)	4 g	4% (w/v)
Make up to 100 ml with distilled water		

Small aliquots can be stored at -20°C for up to three months.

**2× Sample buffer**

For recipes see section 5.5.

**2 mM Tris-2-carboxyethyl phosphine hydrochloride (TCEP)**

2.8 mg TCEP is dissolved in 5 ml distilled water.

Prepare fresh solution on day of use. TCEP solution is unstable and is to be used immediately.

## 4 Sample preparation and labeling

### 4.9 Recipes



## 5 First dimension isoelectric focusing (IEF)

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### 5.1 Ettan IPGphor 3 Isoelectric Focusing System

Ettan IPGphor 3 Isoelectric Focusing System and Immobiline DryStrips are recommended for first dimension electrophoresis, isoelectric focusing (IEF).

A user friendly software, Ettan IPGphor 3 Control software, is provided with the Ettan IPGphor 3 Isoelectric Focusing System. The software comprises several features for automatic protocol selection and protocol editing for experimental monitoring. For documentation purposes it is possible to save log files and experimental parameters.

The use of a combination of Ettan IPGphor 3 Isoelectric Focusing System and Immobiline DryStrips generate highly reproducible first dimension isoelectric focusing results. Up to twelve Immobiline DryStrips can be run at the same time.



**Fig 5-1.** IPGphor 3 isoelectric focusing apparatus and IPGphor 3 Control software.

## 5 First dimension isoelectric focusing (IEF)

### 5.2 Workflow



**WARNING!** The use of Ettan IPGphor Isoelectric Focusing (IEF) System includes use of high voltage. Read 2D Electrophoresis Principles and Methods, Ettan IPGphor 3 Instrument User Manual and Ettan IPGphor 3 Safety Handbook prior to operation of the Ettan IPGphor (IEF) System for detailed instructions and safety information.

#### 5.1.1 General precautions for good results

2D analysis of protein samples is highly sensitive for contaminations and in order to achieve good and reproducible results some general precautions must be considered:

- Wear gloves to minimize protein contamination.
- Clean all components with IPGphor Strip Holder cleaning solution (code no. 80-6452-78) and Milli-Q water to remove traces of protein before and after use.
- Ensure the Immobiline DryStrips do not dry out.

## 5.2 Workflow

- 1 Rehydration of Immobiline DryStrips
- 2 Sample application to Immobiline DryStrips: Two options described:
  - Cup loading protocol
  - Rehydration loading protocol
- 3 IEF on Ettan IPGphor 3 apparatus

#### 5.2.1 Sample application protocol selection

The protocol to choose is dependent of the purpose of the experiment, the desired pH range of the Immobiline DryStrips and sample protein amount and concentration, see Table 5-1 . For other types of sample application protocols, see *2D Electrophoresis Principles and Methods* handbook.

**Table 5-1.** Sample application protocol selection criteria.

<b>Experimental conditions or sample type</b>	<b>Recommended protocol</b>
Samples sensitive for e.g. proteolysis, protocol minimizes the time the sample is exposed to room temperature	Cup loading protocol
Samples with volumes up to 150 µl or with a protein amount under 150 µg. Large sample loads increase the risk for protein precipitation at the point of application	Cup loading protocol
Samples with volumes larger than 100 µl, e.g. diluted samples	Rehydration loading protocol
Experiments using basic Immobiline DryStrips (pH 6-9, pH 6-11, pH 7-11 NL)	Cup loading protocol or Paper-bridge loading protocol (see <i>2D Electrophoresis Principles and Methods</i> handbook for protocol)
Samples for preparative electrophoresis and for large sample loads (above 150 µg protein)	Rehydration loading protocol or Paper-bridge loading protocol (see <i>2D Electrophoresis Principles and Methods</i> handbook for protocol)

### 5.3 Rehydration and sample application

There are two protocols available for rehydration of Immobiline DryStrips and sample application: *Cup loading protocol* and *Rehydration loading protocol*. The protocols are rather similar. The main difference is that with *Cup loading protocol* the samples are added to the strips after rehydration and with *Rehydration loading protocol* the samples are added to the strips during rehydration.

**Note:** Some steps in the protocol below contain two options, one to be used with the *Cup loading protocol* and the other with the *Rehydration loading protocol*.

#### 5.3.1 Rehydration of Immobiline DryStrips

Immobiline DryStrips must be rehydrated before use. For rehydration an Immobiline DryStrips Reswelling Tray is used. The Immobiline DryStrips Reswelling Tray has 12 independent reservoir slots that each hold a single Immobiline DryStrip. Separate slots allow the rehydration of individual Immobiline DryStrips in the correct volume of solution.

5 First dimension isoelectric focusing (IEF)  
5.3 Rehydration and sample application



**Fig 5-2.** The Immobiline DryStrips Reswelling Tray.

Immobiline DryStrips holders can also be used. Information on using Immobiline DryStrips holders can be found in the *2D Electrophoresis Principles and Methods* handbook.

**Protocol**

- 1 Slide the protective lid completely off the Immobiline DryStrips Reswelling tray. Ensure that the tray is clean and dry. Level the tray by turning the levelling feet until the bubble in the spirit level is centred.
- 2 **Cup loading protocol:** Pipette the appropriate volume of rehydration solution or DeStreak Rehydration solution into each slot to be used, see Table 5-2. Deliver the solution slowly along the slot. Remove any large bubbles. For complete sample uptake, do not apply excess rehydration solution.

**Note:** Use DeStreak Rehydration Solution to reduce streaking, especially in the pH range 7-11. DeStreak Rehydration Solution contains DeStreak Reagent that prevents unspecific oxidation of protein thiol groups during electrophoresis.

**Rehydration loading protocol:** Use labeled protein samples prepared as described in section, 4.8. The total volume must not exceed the stated values in Table 5-2. If the volume is larger the sample must be split or concentrated. Pipette the appropriate volume of sample into each slot to be used. Deliver the solution slowly along the slot. Remove any large bubbles.

**Table 5-2.** Rehydration volumes of Immobiline DryStrips.

Immobiline DryStrips length (cm)	Total volume per strip (µl) including sample
7	125
11	200
13	250
18	340
24	450



- 3 Remove the protective cover foil from the Immobiline DryStrips gel. Use forceps to position the strip with the gel side down. To help coat the entire strip, gently lift and lower the strip and slide it back and forth along the surface of the solution. Be careful not to trap bubbles under the Immobiline DryStrips.
- 4 Overlay each Immobiline DryStrips with PlusOne™ DryStrips Cover Fluid to prevent evaporation and urea crystallization.
- 5 Slide the lid onto the Immobiline DryStrips Reswelling Tray and allow the Immobiline DryStrips to rehydrate at room temperature. A minimum of 10 h is required for rehydration; overnight is recommended.

**Tip!** Strips can be rehydrated under low voltage (30 - 50V) when using the rehydration loading protocol.

### 5.3.2 Preparations for first dimension run including Cup loading

After rehydration Immobiline DryStrips are prepared for first dimension isoelectric focusing. Samples are loaded according to the cup loading protocol in the Manifold.

5 First dimension isoelectric focusing (IEF)  
5.3 Rehydration and sample application

**Protocol**

- 1 Place precut electrode papers on a clean dry surface such as a glass plate and soak with deionized water. Remove excess water by blotting with filter paper.  
**Note:** *It is important that the paper electrodes are damp and not wet. Excess water may cause streaking.*
- 2 Transfer the Immobiline DryStrips from the Reswelling Tray to the Manifold by using a pair of forceps.
- 3 Place the Immobiline DryStrips with gel side up and with the acidic end of the strips oriented toward the anodic side of the instrument.
- 4 Place a damp paper electrode (from step 1) onto the acidic and basic ends of the gel.
- 5 Clip down the electrodes firmly onto the electrode papers. Ensure that there is good contact between the paper electrodes and the metal.
- 6 **Cup loading protocol only:** Clip a loading cup onto the acidic end of the strip so it is positioned between the two electrodes. The cup should form a good seal with the Immobiline DryStrips.  
**Note:** *To check for a good seal fill the cup to the top with PlusOne DryStrips Cover Fluid. Observe the level of the fluid to check if it is decreasing. If a leak is detected remove the PlusOne DryStrips Cover Fluid and reposition the sample cup.*
- 7 Apply 108 ml of PlusOne DryStrips Cover Fluid allowing the oil to spread so it completely covers the Immobiline DryStrips (even if fewer than twelve strips will be loaded in the Manifold).
- 8 **Cup loading protocol only:** Add samples prepared as described in section 4.8. Up to 100  $\mu$ l of a protein sample can be loaded into the bottom of the sample cup.
- 9 Close the lid of Ettan IPGphor 3 instrument.

Now it is possible to start the first dimension isoelectric focusing, see section 5.4.

## 5.4 First dimension isoelectric focusing (IEF)

First dimension isoelectric focusing using Ettan IPGphor 3 instrument can be controlled by either using a PC software (Ettan IPGphor 3 Control software) or the embedded instrument software.

Use of Ettan IPGphor 3 Control software is recommended for easy and advanced protocol handling. The software provides a number of optimized protocols for running first dimension isoelectric focusing. It is also possible to create new protocols, import existing protocols, and edit protocols. During the run, current and voltage can be monitored, displayed in a graph, and a log file created. Up to four different instruments can be monitored from one PC software. See *Ettan IPGphor 3 Control Software User Manual* for details.

### Protocol (using Ettan IPGphor 3 Control software)

- 1 Start the Ettan IPGphor 3 Control software and turn on the Ettan IPGphor 3 instrument.
- 2 Connect the software with the IPGphor 3 instrument on which the run is to be made.
- 3 Select protocol for first dimension isoelectric focusing. For selection of optimized protocols choose **Fast mode** protocol selection and enter data of experimental parameters. Alternatively, set protocol using the **Advanced mode** protocol selection,

**Note:** Do not programme the Ettan IPGphor IEF unit to deliver more than 75  $\mu\text{A}$  per Immobiline DryStrips.

- 4 Start run by clicking the start button in the Ettan IPGphor 3 Control software. The selected protocol is now downloaded to the selected instrument and the run is started.
- 5 If the Immobiline DryStrips are not run immediately on the second dimension gel, they can be stored at  $-70^{\circ}\text{C}$  in a sealed container. The container has to be rigid because frozen Immobiline DryStrips are very brittle and can easily be damaged.

**Note:** Do not equilibrate Immobiline DryStrips prior to storage. This must be carried out immediately before the second dimension separation.

## 5.5 Recipes

### 2× Sample buffer/rehydration solution stock (option 1)

Reagent	Quantity	Final concentration
Urea (MW 60.06)	10.5 g	7 M
Thiourea (MW 76.12)	3.8 g	2 M
CHAPS (MW 614.89)	1 g	2% (w/v)
Make up to 25 ml with distilled water		

Small aliquots (e.g. 2.5 ml) can be stored at -20 °C. Stable for 6 months.

### 2× Sample buffer/rehydration solution stock (option 2)

Reagent	Quantity	Final concentration
Urea (MW 60.06)	12 g	8 M
CHAPS (MW 614.89)	1 g	2% (w/v)
Make up to 25 ml with distilled water		

Small aliquots (e.g. 2.5 ml) can be stored at -20 °C. Stable for 6 months.

### Rehydration solution

Reagent	Quantity	Final concentration
2× Sample buffer/ rehydration solution stock 1 or 2	2.5 ml	
IPG Buffer, same pH interval as the Immobiline DryStrips being rehydrated.	50 µl	2%
DTT	7 mg	



**DeStreak Rehydration Solution**

Reagent	Quantity	Final concentration
DeStreak Rehydration Solution (71-5025-42)	3 ml	
IPG Buffer, same pH interval as the Immobiline DryStrips being rehydrated	60 µl	2%

DeStreak Rehydration Solution contains DeStreak reagent and optimized concentrations of Urea, Thiourea and CHAPS.

**Note:** If IPGphor standard StripHolder or Immobiline DryStrips 7-11NL and 3-11NL are used, use 0.5% IPGphor buffer instead of 2% IPGphor buffer.

**2× Sample buffer for CyDye DIGE Fluor minimal dyes**

Reagent	Quantity	Final concentration
2× Sample buffer/ rehydration solution stock 1 or 2*	2.5 ml	
IPG Buffer, pH 3-10	50 µl	2% (v/v)
DTT (MW 154.2)	50 mg	2% (w/v) (20mg/ml, 130 mM)

Do not store, prepare fresh before use.

\*Use stock option 1 or 2, depending on the rehydration buffer required.

**Note:** If using DeStreak Rehydration solution, the buffer may contain up to 10 mM DTT.

**2× Sample buffer for CyDye DIGE Fluor saturation dyes**

Reagent	Quantity	Final concentration
2× Sample buffer/ rehydration solution stock *	2.5 ml	
IPG Buffer, pH 3-10	25 µl	1% (v/v)
DTT (MW 154.2)	5 mg	0.2% (w/v) (2 mg/ml, 13 mM)

Do not store, prepare fresh before use.

\*Use stock option 1 or 2, depending on the rehydration buffer required.

5 First dimension isoelectric focusing (IEF)  
5.5 Recipes

## 6 Second dimension SDS PAGE

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### 6.1 Ettan DALT electrophoresis system

Ettan DALT electrophoresis systems (Ettan DALTsix and Ettan DALTtwelve) are recommended for second dimension separation using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS PAGE). Both systems are designed for simple assembly and rapid electrophoresis. Ettan DALTsix accepts up to six large second dimension SDS PAGE gels and Ettan DALTtwelve accepts up to twelve large second dimension SDS PAGE gels.

The Ettan DALT electrophoresis systems are designed to handle multiple large format second dimension SDS PAGE gels in a simple, efficient, and reproducible manner. Ettan DALT gels are large enough to accommodate the longest Immobiline DryStrips (24 cm).

For Ettan DIGE system applications, low fluorescent glass plates must be used. These glass plates reduce background signal and will therefore improve the quality of results.

Ettan DALT gels are poured using a gel caster. Up to 14 Ettan DALT gels can be prepared in a single batch.

For detailed description of Ettan DALT electrophoresis systems (Ettan DALTsix and Ettan DALTtwelve), components for preparation of Ettan DALT gels, and for detailed protocols for loading gels into the caster, and casting Ettan DALT gels consult the Ettan DALTtwelve system user manual and the Ettan DALTsix system user manual.



**Fig 6-1.** Ettan DALTsix (left) and Ettan DALTtwelve (right) electrophoresis systems.

## 6.2 Workflow

- 1 Cast homogeneous second dimension SDS PAGE gels.
- 2 Equilibration of focused Immobiline DryStrips.
- 3 Loading of focused Immobiline DryStrips.
- 4 Second dimension SDS PAGE.

## 6.3 Casting homogeneous gels

**Note:** Prepare gels at least one day before use to ensure reproducible results.

- 1 Use low fluorescence glass plates without scratches in order to achieve high quality images with low background.
- 2 Ensure the entire gel casting system is clean, dry, and free of any polymerized acrylamide.
- 3 Prepare 100 ml of displacing solution.
- 4 Prepare acrylamide gel stock solution. For a full 14-gel set, make up 900 ml of solution. Add TEMED but do not add APS. This amount of gel solution will provide sufficient volume to cast gels using either a funnel or a peristaltic pump.

**Tip!** Use chilled gel solutions. This will increase reproducibility since temperature effects are minimized.



**WARNING!** Acrylamide is a neurotoxin. Never pipette by mouth and always wear protective gloves when working with acrylamide solutions, Immobiline DryStrips, or surfaces that come into contact with acrylamide solutions.

- 5 Remove dust by filtering the acrylamide gel stock solution with a standard bottle top filter apparatus. Dust may create fluorescent artefactic dots on images.
- 6 Degas the acrylamide gel stock solution using a vacuum pump.
- 7 Assemble the gel caster, as described in the Ettan DALT electrophoresis unit user manuals. The caster should be placed on a level bench or on a levelling table so that the gel tops are level.

**Note:** Do not apply excess of filter or separator sheets. Leave 1-2 mm space from the edge uncovered. Too much pressure will cause gel distortions.

- 8 Add appropriate volumes of fresh APS to the acrylamide gel stock solution when ready to pour the gels, and mix thoroughly. Once the APS is added, polymerization begins.
- 9 Pour the gel solution until it is about 1-2 cm below the final desired gel height. Fill the balance chamber with 100 ml of the displacing solution. The dense displacing solution flows down the connecting tube, filling the V-well and sloped trough at the bottom of the caster. The remaining acrylamide solution is forced into the cassettes to the final gel height.
- 10 Fill the balance chamber with 100 ml of the displacing solution.
- 11 Spray (do not pipette) overlay solution on the edges of the cassettes, with for example a plant sprayer, so the edges are covered with a few mm thick liquid layer using a 0.1% (w/v) spray overlay solution. By doing this curved edges on the gels are prevented.  
  
*Tip! Alternatively, 30% isopropanol can be used to overlay gels.*
- 12 Allow the homogeneous gels to polymerize for at least 3 h before disassembling the caster. Best polymerization is achieved by letting the gel polymerize overnight at room temperature.
- 13 Once gels are completely polymerized cover the top of the gel with SDS electrophoresis running buffer for the Ettan DALT.

Freshly made gels are best to use. However, gels can be stored in an airtight container at +4 °C for up to one week provided they are submersed in 375 mM Tris-HCl to keep the gels from drying out.

### 6.4 Equilibration of focused Immobiline DryStrips

**Note:** *Equilibrate focused strips immediately before the second dimension SDS PAGE run.*

- 1 Prepare SDS equilibration solutions 1 and 2. Allow 10 ml per strip for each equilibration solution.
- 2 With forceps carefully remove the Immobiline DryStrips from the IPGphor Cup Loading Strip Holder. If the Immobiline DryStrips have been focused and stored frozen, allow the strips to defrost completely beforehand.
- 3 Place the Immobiline DryStrips in individual equilibration tubes (code no. 80-6467-79) with the support film toward the wall.
- 4 Add 10 ml of the DTT-containing equilibration solution 1 to each tube.
- 5 Incubate the strips for 15 min with gentle agitation. Do not over-equilibrate, as proteins can diffuse out of the strip during this step.
- 6 During equilibration, prepare the gel cassettes for loading by rinsing the top of the gels with running buffer. Place the glass plates in the rack upside down.
- 7 **For samples labeled with CyDye DIGE fluor minimal dyes:** Pour off the equilibration solution 1 and add 10 ml of equilibration solution 2. Incubate the strips for 15 min with gentle agitation. Pour off the solution and drain thoroughly.
- 8 **For samples labeled with CyDye DIGE fluor saturation dyes:** Do NOT use equilibration solution 2 which is containing iodoacetamide! Use equilibration solution 1, which contains DTT, instead.

## 6.5 Loading of focused Immobiline DryStrips

- 1 Place the gels in the Ettan DALT cassette rack.
- 2 Briefly rinse the Immobiline DryStrips by submerging them in a measuring cylinder containing SDS electrophoresis running buffer for Ettan DALT.
- 3 Holding one end of the Immobiline DryStrip with forceps, carefully place the Immobiline DryStrip in-between the two glass plates of the gel. Using a thin plastic spacer, push against the plastic backing of the Immobiline DryStrip (not the gel itself) and slide the strip between the two glass plates until it comes into contact with the surface of the gel.

**Note:** *The strip should just rest on the surface of the gel. Avoid trapping air bubbles between strip and the gel and avoid piercing the second-dimension gel with the strip.*

**Note:** *The acidic end of the Immobiline DryStrip should be on the right side of the gel when the shorter of the two plates is facing the user.*

**Note:** *The gel face of the strip must not touch the opposite glass plate.*



- 4 Melt an aliquot of agarose overlay solution in a heating block or boiling water bath for each Immobiline DryStrip. Allow the agarose to cool slightly and slowly pipette the molten agarose solution, along the upper surface of the gel, up to the top of the glass plate. Take care not to introduce bubbles. Do not allow the agarose to solidify.
- 5 Once the agarose solution has completely set the gel should be run in the second dimension as soon as practically possible.

## 6.6 Second dimension SDS PAGE

- 1 Fill the lower buffer tank with SDS electrophoresis running buffer for Ettan DALT. Turn on the control unit, switch on the pump and set the temperature to 20°C.  
**Note:** *To achieve good results it is important to use the recommended electrophoresis running buffer with 0.2% (w/v) SDS.*
- 2 When the running buffer has reached the desired temperature, insert the loaded gel cassettes with the Immobiline DryStrips in place. Load the unit from back to front. When all 12 slots are filled, the buffer level should be slightly below the level of the buffer seal gaskets.  
**Note:** *Blank cassette must be inserted into any unoccupied slots. Alternate gel cassettes with blank cassette inserts to facilitate handling.*  
**Tip!** *Gel cassettes and blank cassette inserts slide much more easily into the unit if they are wet. Use SDS electrophoresis running buffer for Ettan DALT from a wash bottle to wet the cassettes and inserts as they are being loaded.*
- 3 Pour SDS electrophoresis running buffer for Ettan DALT into the top of the buffer tank to the fill line.
- 4 Program the desired run parameters into the control unit, see Table 6-1 for recommendations.

**Table 6-1.** Recommended running conditions.

Step	Run duration	Effect per gel	Temperature
1	45 min	2 W	20°C
2	4 h	17 W	20°C

**Note:** *The recommended running conditions are for runs including twelve gels and should only be used as guidelines. In runs with fewer gels the electric effect can be increased (up to a maximum of 20 W per gel) which will reduce run times.*

- 5 Close the lid of the buffer tank and press start/stop to begin electrophoresis.
- 6 Run the gel until the bromophenol blue dye front reaches the bottom of the gel.



- 7 Scan the gels as soon as possible after the second dimension SDS PAGE is finished in order to minimize protein diffusion, see chapter 7, Image acquisition. Keep the gels between the glass plates.

**Note:** *Do not fix the gels before the gels are scanned as this may affect DeCyder 2D software quantitation of CyDye DIGE Fluor minimal dye labeled proteins.*

- 8 If you cannot scan the gels immediately, store the gels in SDS electrophoresis running buffer for Ettan DALT at +4°C in the dark and keep the gels moist. However, allow the gels to reach room temperature before scanning. Gels scanned more than a day after running are likely to show significant diffusion of the protein spots.

## 6.7 Recipes

### Displacing solution

Reagents	Quantity	Final concentration
Tris (1.5 M, pH 8.8)	25 ml	375 mM
Glycerol (87% (v/v))	57.5 ml	50% (v/v)
1% Bromophenol blue stock solution	200 µl	0.002% (w/v)
Make up to 100 ml with distilled water		

Prepare fresh and use immediately. Do not store.

### 12.5% Second dimension SDS PAGE gel composition for Ettan DALT

Reagents	Quantity for 900 ml of a 12.5% gel
Acrylamide/PAGE 40% (w/v)	281.25 ml
PlusOne Methylenebisacrylamide 2% (w/v)	150.3 ml
Tris (1.5 M, pH 8.8)	225 ml
10% (w/v) SDS	9.0 ml
10% (v/v) TEMED	1.24 ml
10% (w/v) APS	9.0 ml
Make up to 900 ml with distilled water	

- Use chilled solutions
- Prior to addition of APS, filter the solution through a 0.2 micron filter into a clean bottle and then degas the solution
- Prepare fresh APS solution on day of use
- Add the APS solution immediately prior to use to prevent polymerization in the bottle

**SDS electrophoresis running buffer for Ettan DALT**

Reagents	Quantity	Final concentration
Tris (MW 121.14)	60.5 g	25 mM
Glycine (MW 75.07)	288 g	192 mM
SDS (MW 288.38)	40 g	0.2% (w/v)
Make up to 20 l with distilled water		

Store at room temperature. Stable for 3 months.

**SDS Equilibration stock solution**

Reagents	Quantity	Final concentration
Tris (1.5 M, pH 8.8)	10 ml	75 mM
Urea (MW 60.06)	72.07 g	6 M
Glycerol (87% [v/v], MW 92.09)	69 ml	30% (v/v)
SDS (MW 288.33)	4 g	2% (w/v)
Make up to 200 ml with distilled water		

This stock solution can be stored at room temperature. Stable for 6 months. Add DTT or Iodoacetamide for equilibration solution 1 or 2.

**Equilibration solution 1**

Reagent	Quantity	Final concentration
SDS equilibration buffer stock solution	100 ml	-
DTT (MW 154.2)	0.5 g	0.5% (w/v)

Solution should be used immediately. Do not store.

**Equilibration solution 2**

**Note:** Iodoacetamide should not be used in combination with saturation labeling. Iodoacetamide allows effective alkylation of thiols while minimizing reoxidation of the competing thiol pairs in protein samples.

Reagent	Quantity	Final concentration
SDS equilibration buffer stock solution	100 ml	-
Iodoacetamide (MW 185.0)	4.5 g	4.5% (w/v)

Solution should be used immediately. Do not store.

**0.5% (w/v) Agarose overlay solution**

Reagent	Quantity	Final concentration
SDS electrophoresis running buffer for Ettan DALT	100 ml	-
Low melting point agarose prep	0.5 g	0.5% (w/v)
1% Bromophenol blue stock solution	200 $\mu$ l	0.002% (w/v)

Mix components in a 250 ml conical flask and heat on a low setting in the microwave for 1 minute. Ensure all the agarose has melted. Allow the solution to cool slightly before use. Store at room temperature. Do not keep for more than 1 month.

**1% (w/v) Bromophenol blue stock solution**

Reagent	Quantity	Final concentration
Bromophenol blue	100 mg	1%
Tris-base	60 mg	50 mM
Make up to 10 ml with double distilled water		

## 7 Image acquisition

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Typhoon Variable Mode Imager is recommended for scanning of DIGE second dimension SDS PAGE gels. It is also possible to use Ettan DIGE Imager, see section 7.7.

### 7.1 Typhoon Variable Mode Imager



**WARNING!** It is important that you read the safety instructions in the Typhoon User Guide before you start work.

Typhoon Variable Mode Imager is fully optimized as part of Ettan DIGE System (Fig 7-1). It is optimized to image the CyDye DIGE Fluor dyes characteristics. Several models of the Typhoon Variable Mode Imager system are available. For Ettan DIGE system use, Typhoon Variable Mode Imager Trio, 9400 and 9410 are recommended, as all three CyDye DIGE Fluor minimal dyes can be detected. However, seamless integration with DeCyder 2D software is ensured for all models.



**Fig 7-1.** Typhoon 9410 Variable Mode Imager.

For a detailed guide on Typhoon Variable Mode Imager, see *Typhoon User Guide*.

## 7.2 Workflow

The workflow for scanning gels with Typhoon Variable Mode Imager and cropping gel image files consist of following steps:

- 1 Cleaning Typhoon
- 2 Placing the gels in Typhoon
- 3 Selecting scan parameters
- 4 Scanning of the gels
- 5 Cropping of image files

## 7.3 Cleaning Typhoon

The following cleaning procedure has been shown to be compatible with the Typhoon Variable Mode Imager to remove contamination caused by fluorescent products.

- 1 Wipe the glass platen with 10% H<sub>2</sub>O<sub>2</sub> (hydrogen peroxide) using lint free paper, such as Crew Wipers.
- 2 Rinse the platen with high purity water. A pre-scan can be done to check for contaminants that may affect results of scans.

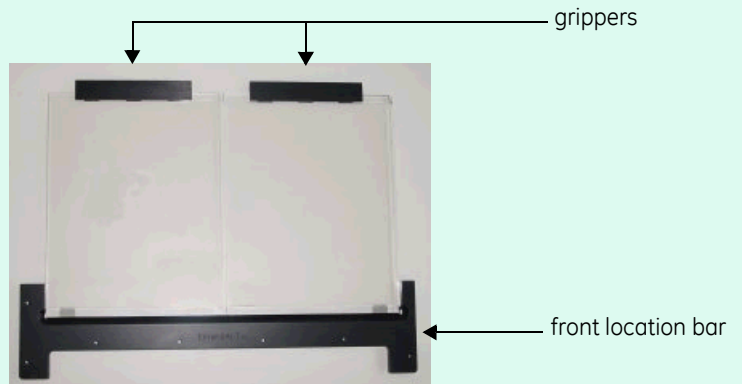
## 7.4 Placing gels in Typhoon

**Note:** *Wear powder free gloves. The powder used in laboratory gloves can fluoresce and may also scatter light affecting image quality.*

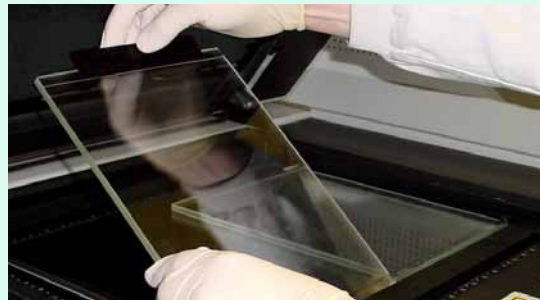
- 1 Turn on the Typhoon Variable Mode Imager and leave the instrument to warm up for at least 30 min prior to scanning. Once the instrument is warmed up it will display **READY** status.
- 2 Ensure that the gel glass plates are clean, dry and free from lint.

**Tip!** *For applications using Ettan DIGE system, the recommended glass plates have low fluorescence characteristics. The gel can then be scanned still assembled within the plates. Manipulation will be easier and there will be less risk of damage to the gel.*

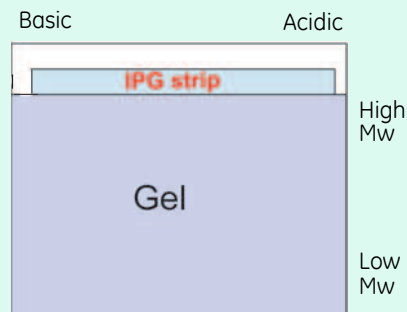
- 3 Position the main bar of the Gel Alignment Guide onto the platen.



- 4 Using the grippers, position the dried glass plate assembly with one edge on the spacer and against the front location bar and gently lower the assembled gel onto the platen.



- 5 Position the gel by using the **Gel orientation guide**. The physical gel orientation should be noted by the user, the gel orientation option in the software determines the file output orientation.



**Tip!** Using Ettan DALT trays, it is recommended that Ettan DALT gels are scanned with the short glass plate facing down to the platen.

## 7 Image acquisition

### 7.5 Scan parameters and scanning

- 6 Close the instrument lid.

### 7.5 Scan parameters and scanning

Start the Typhoon Scanner Control software. The *Typhoon Scanner Control - Multiple Sample* scan window is opened (Fig 7-2).

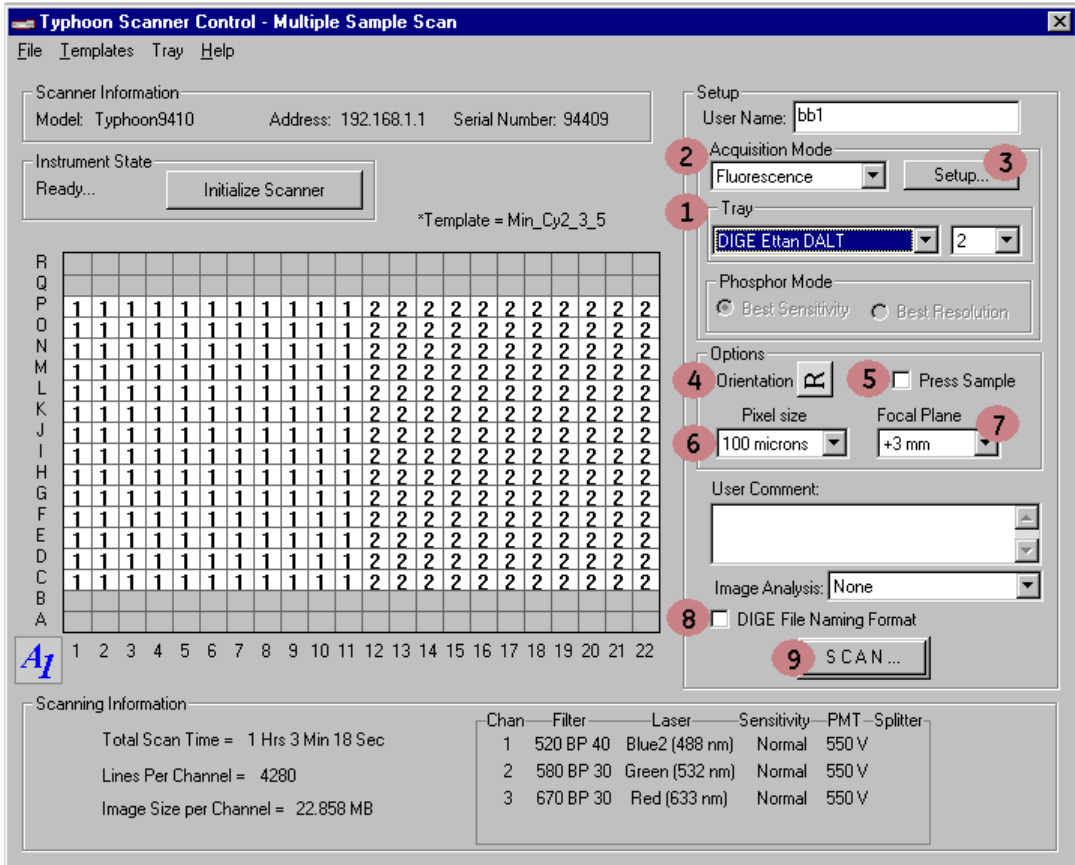


Fig 7-2. The Typhoon Scanner Control - Multiple Sample scan window.



- 1 Select scan area (pre-defined tray area) within the *Tray set-up area* (Labeled 1 in Fig 7-2), use the drop down window to select the appropriate predefined tray, i.e., the *DIGE Ettan DALT*. In this mode, the scan area is pre-defined and the software is able to recognize where individual gels will be located, resulting in separate file outputs for each gel.

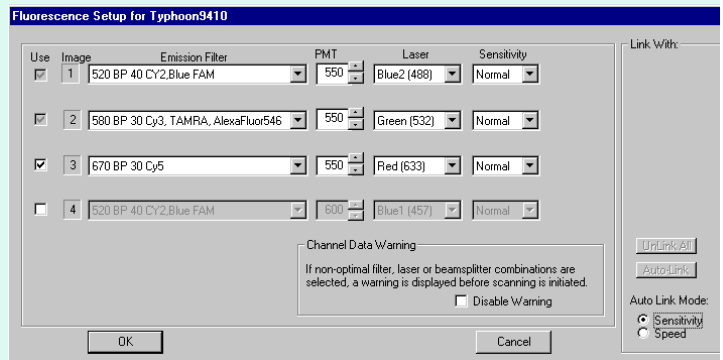
**Tip!** *The Tray Editor can be used to create tray areas for gel types other than Ettan DALT. In User Select mode, only the scan area is pre-defined. The software is unable to recognize where individual gels will be located, therefore a single file output will be generated. Manual cropping is required following the scan.*

Define the number of gels to be scanned. Use the drop down menu in the *Tray set-up area* (labeled 1 in Fig 7-2). In the DIGE Ettan DALT mode up to two gels can be selected.

- 2 Use the drop down menu under the *Acquisition Mode* heading (labeled 2 in Fig 7-2) to select *fluorescence* as acquisition mode.

**Note:** *Scan modes other than fluorescence are available, these are covered in the Typhoon User Guide.*

- 3 Set up fluorescence scan parameters:
  - a Click the **Setup** button (Labeled 3 in Fig 7-2) to activate the *Fluorescence Setup* window.



- b Select the number of scan channels to be programmed for the sample on the platen. Between one and four channels can be programmed. To select or deselect scan channels, click the **Use** check box.

## 7 Image acquisition

### 7.5 Scan parameters and scanning

- c Select appropriate emission filters from the *Emission Filter* list. This list displays the filters that are installed on the Typhoon Variable Mode Imager along with a description of the typical filter use. The Scanner Control software automatically suggests the laser to use with the emission filter selected. See Table 7-1 for recommended emission filters and laser combinations. The CyDye DIGE Fluor dye filter and laser combinations are selected to give the optimum results with minimal cross-talk.

**Table 7-1.** Recommended emission filters and laser combinations.

Dye	Emission Filter (nm)	Laser
Cy2	520 BP 40	Blue2 (488)
Cy3	580 BP 30	Green (532)
Cy5	670 BP 30	Red (633)
Deep Purple™	560 LP	Green (532)

**Tip!** For Deep Purple™-post-stained analytical gels it is recommended to use a 457 nm laser excitation in conjunction with a 610 nm band pass emission filter (or equivalent if not using a Typhoon scanner). This will minimize any potential cross-talk between Deep Purple and the CyDye DIGE fluor dyes.

- d Set the *PMT* voltage for each scan wavelength. A quick pre-scan at 500 or 1 000 µm pixel resolution should be performed initially to identify a suitable voltage. For further details, see section 7.5.1.
- e Select normal as *Sensitivity* scan setting for each scan wavelength (usually sufficiently sensitive for 2D DIGE applications).
- f Select the **Sensitivity** check box. It is essential that this is selected for analytical gels to ensure that all scans are carried out as individual scans.
- g Click **OK**.

- 4 Select orientation (orienting options button labeled 4 in Fig 7-2), see section 7.4 step 5.



**Tip!** To aid selection of the correct character the Gel Orientation Guide can be overlaid above the gel once it is in position on the Typhoon platen. The appearance of the overlaid letter "R" on the Gel Orientation Guide indicates which character to select on the Typhoon Scanner Control software screen.

**Note:** ImageQuant™ TL Tools can be used to flip or rotate images which were scanned in wrong orientation.

- 5 Select the **Press Sample** option (labeled 5 in Fig 7-2) if scanning gels between glass plates.

**CAUTION!** The **Press Sample** feature should not be used with naked or backed gels as it can damage the instrument.

- 6 Select **Pixel size** from the list (labeled 6 in Fig 7-2). A quick pre-scan at 1000  $\mu\text{m}$  pixel resolution should be performed initially to identify a suitable PMT voltage.
- Select 100  $\mu\text{m}$  as pixel size for DALT gels.
  - Select 25 or 50  $\mu\text{m}$  as pixel size for minigels.
- 7 Select **Focal Plane** from the list (labeled 7 in Fig 7-2). The **+3 mm** setting is used for most applications of the Ettan DIGE system (assembled gels or samples held on a glass plate).

## 7 Image acquisition

### 7.5 Scan parameters and scanning

- 8 Make sure the instrument lid is shut. Select DIGE file naming format. Check the **DIGE File Naming Format** box (labeled 8 in Fig 7-2) in the Scanner Control window to ensure that unique filenames can be generated for each scan channel.

**Tip!** Using the **DIGE File Naming Format** option results in all files having user defined filenames. All scan images from a given experiment can be saved into a single user defined folder. This method of file naming and folder selection results in structures that can be directly used to crop image for subsequent DeCyder 2D analysis.

- 9 Start the scan by clicking the **Scan** button (labeled 9 in Fig 7-2) in the Scanner Control window. The DIGE File Naming Format window appears.

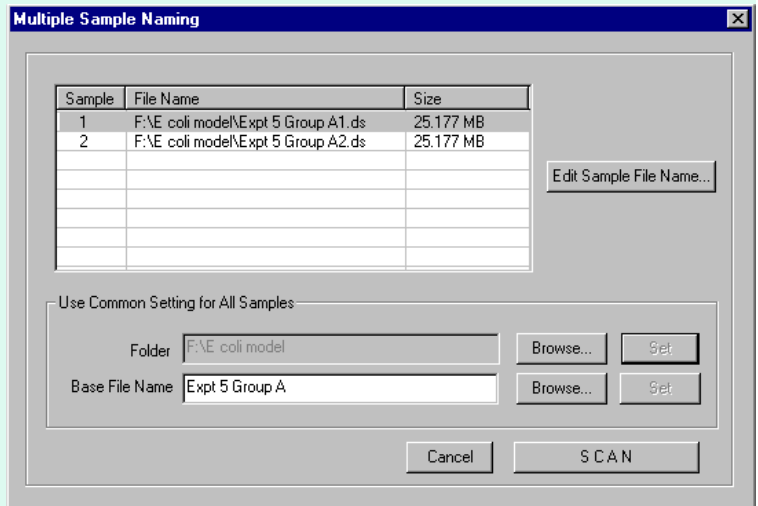
The screenshot shows the 'DIGE File Naming Format' dialog box. The 'Image Group Name' is 'F:\E-Coli model\Gel F.ds'. The 'Image 1' field contains 'Gel F STANDARD CY2', 'Image 2' contains 'Gel F Cy3', and 'Image 3' contains 'Gel F Cy5'. The 'Image 4' field is empty. The 'Standard' column has radio buttons for 'STANDARD', 'Cy2', 'Cy3', 'Cy5', and 'NONE'. The 'Scan Dye' column has text boxes for 'CY2', 'Cy3', 'Cy5', and an empty box. The 'OK' and 'Cancel' buttons are at the bottom right. A note at the bottom states 'All images have ".gel" extension'.

- If a single scan setting has been chosen, e.g. for a Deep Purple stained gel, then the resulting output on scan completion will be a *filename.gel* file in the selected folder.
  - If two or more scan parameter settings were chosen, e.g. for a Cy2/Cy3/Cy5 gel, then the resulting output upon scan completion will be a *filename.ds* file in the selected folder and a new folder called *filename.dir*. In this new folder will be the *user named.gel* files. The *filename.ds* file allows the scanned images to be overlaid in ImageQuant TL whilst the *user named.gel* files are the individual scan channel outputs and can be viewed as separate files.
- 10 The terms STANDARD, Cy2, Cy3 and Cy5 are automatically appended to the file. These terms are also automatically picked up during image analysis so reducing the requirement for user input.

- 11 The term STANDARD can be assigned to the file name of the image which contains the internal standard by clicking the relevant check box.

If not required the STANDARD name can be removed by clicking the NONE check box. The filenames can also be manually edited.

- 12 Click the **OK** button. The Multiple Sample Name dialog is displayed.



- 13 Save the images by choosing the folder and filename for each gel individually. Click on **Edit Sample File Name...**
- 14 Entering details using *Folder* or *Base File Name*, allows the user to **Set** common details for all gels in a single operation. The **Browse** option in both cases allows the user to select existing folders or file name structures. A number of gels can be set up for a single scan run and obtain unique filenames for each gel image.
- 15 Click **SCAN** to start the scan. Once the scan has started, the preview window appears.
- For unlinked scans a single image channel appears for each scan programmed, the images appearing one at a time.
  - For linked scans, two image channels appear simultaneously.
  - Where more than one gel is scanned using the DIGE Ettan DALT tray settings, a drop down numerical menu appears allowing the user to monitor each of the gels as the images are generated.

## 7 Image acquisition

### 7.5 Scan parameters and scanning

- 16 Evaluate the scan progress. Saturated data is displayed in red in the preview window. If saturated data is seen the user can cancel the scan and re-set the PMT voltage without having to complete the whole scan.
- 17 Handle image file output. The image files created are labeled as *filename.gel*. This uses a modified 16 bit TIF format. An additional text file, *filename.ds*, also exists and this links image file data together for image overlays in ImageQuant TL.

#### 7.5.1 Pre-scanning to identify a suitable PMT voltage

The PMT voltage can be set from 300 to 1 000 V although it is recommended that, where possible, work is performed between 400 and 900 V. The voltage chosen depends on the type and quantity of dye or stain present. A quick prescan at 500 or 1 000  $\mu\text{m}$  pixel resolution should be performed to identify a suitable voltage. This allows a rapid scan at a relatively low resolution that should not be used for quantitative analysis. It does however give an approximation of expected signal values which will aid determination of the PMT voltage required. The prescan can be opened in ImageQuant TL software. Spots showing the most intense signal should be selected using one of the **Object tools** such as the rectangle.

Higher resolution 100  $\mu\text{m}$  scans must be used to collect quantitative data. This resolution is required for subsequent data analysis using DeCyder 2D.



Using the **Volume Review** tool button displays the information associated with the selected area in the format.

Inspector

Volume Quantitation Statistics:

Object Name: RECT- 1  
Volume: 135208879.36  
Background: 0.000 (None)  
Average: 5671.513  
Std. Dev.: 9779.156  
Sum: 135208879.36  
Median: 2205.112  
Area: 23840  
Perimeter: 618  
Centroid: ( 923.15, 647.31 )  
Width, Height: ( 160, 149 )  
Max. Valpos: 75295.36 ( 934, 654 )  
Min. Valpos: 787.48 ( 854, 693 )  
Sum Above BG: 135208879.357  
# Pixels Above BG: 23840

Maximum pixel value and X,Y co-ordinates

**Note:** *The maximum pixel value should not exceed 100,000 as this indicates signal saturation has been reached and this will prevent quantitative analysis being achieved.*

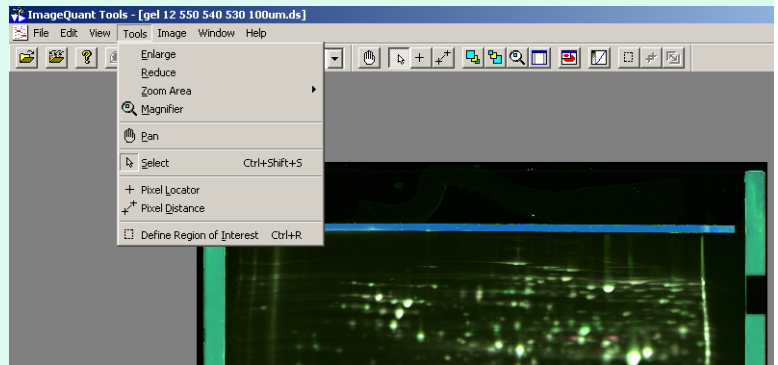
A target maximum pixel value of 50 000 to 80 000 is usually suitable. When adjusting the voltage, relatively small increments of 20 to 50 volts are recommended. If only one or two spots show saturation then only slight downward adjustments to the PMT voltage setting are normally required. Once the voltage has been optimized for one gel in an experiment, these settings can be used for all similar gels within the same experiment. The maximum pixel value should be within the specified range for all gels, to enable accurate quantitation of spot volumes.

## 7.6 Cropping using ImageQuant TL

Prior to image analysis the image files should be cropped to exclude nonessential information from the image files. To exclude the nonessential information, ImageQuant TL Tools should be used to crop the images prior to image analysis. Further cropping of the individual gel areas is normally required to remove supplementary data and can be performed in ImageQuant TL Tools.



- 1 Define an area of interest within ImageQuant TL Tools using the dashed square button or use the “Tools” menu.



- 2 Crop using **Edit:Crop Dataset** or the crop current dataset button on the toolbar.
- 3 For image analysis, save the cropped images by selecting **File:Export Gel Files from Dataset to Folder**. This method only saves the *name.gel* files and allows images from multiple gels to be saved in a common folder.
- 4 To retain dataset functionality, save the cropped images by selecting: **File:Save As....**

## 7 Image acquisition

### 7.6 Cropping using ImageQuant TL

#### 7.6.1 ImageQuant TL trouble shooting

If *out of memory* messages appear while using ImageQuant TL, causing the software to crash, this may be corrected by *decreasing* the virtual memory under 1Gb. Remember to change the setting again when using the image analysis softwares, which need a large virtual memory to function correctly.

To set the size of the virtual memory:

- 1 Right-click on **My computer** and select **Properties**.
- 2 Select the **Advanced** tab.
- 3 In the **Performance** area, click the **Settings...** button.
- 4 In the **Performance** area, click the **Settings...** button.
- 5 Click **Change...** to change the virtual memory to the appropriate value and click **OK** to change the setting.
- 6 Restart the computer to apply the new setting.



## 7.7 Ettan DIGE Imager

Ettan DIGE Imager is a scanning CCD camera-based instrument designed for high resolution images of 2D DIGE applications (Fig 7-3). In particular it can create high quality images of 2D DIGE gels. By combining very high resolution with precise motion control, the Ettan DIGE Imager produces accurate multi-channel images of Cy2, Cy3 and Cy5 labeled gels. The system has also been designed to image a wide range of other fluorescent gel and membrane applications.



**Fig 7-3.** Ettan DIGE Imager.

The imager is controlled using Ettan DIGE Imager software, and can be set up for a variety of gel and membrane formats. Data produced by Ettan DIGE Imager is directly compatible with ImageQuant TL, ImageMaster 2D Platinum and DeCyder 2D. For information about how to use the imager, refer to *Ettan DIGE Imager User Manual*.

7 Image acquisition  
7.7 Ettan DIGE Imager

## 8 Image analysis

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Two different softwares have been specially designed for image analysis in Ettan DIGE System: DeCyder 2D and ImageMaster 2D Platinum software. DeCyder 2D is recommended in complex experiments when more than two groups or conditions are used in an experiment. ImageMaster 2D Platinum software is suitable for basic experiments containing control vs. treated or healthy vs. non-healthy conditions.

These dedicated 2D software products use the internal standard to minimize gel-to-gel result variation. A detection of less than 10% difference between samples can be made with more than 95% statistical confidence.

**Tip!** Ensure DIGE images are cropped before being loaded into DeCyder 2D.

### 8.1 DeCyder 2D software

DeCyder 2D software is specially designed for Ettan DIGE system use. It enables the production of quantitative data of unparalleled accuracy, supported by statistical tests. This gives confidence that the results achieved reflect true biological outcomes and are not due to the system. DeCyder 2D is a fully automated image analysis software suite for detection, quantitation, positional matching and differential protein abundance analysis. An optional add-on module Extended Data Analysis (EDA) can handle up to 1000 spot maps. The raw data (gel images) are linked to EDA and multivariate analysis of data can be opened for display via the BVA module.

This section briefly outlines the features and capabilities of the software. For a detailed guide, please refer to the DeCyder 2D Software User Manual and DeCyder Extended Data Analysis module User Manual which both include a series of tutorials designed to provide new users with the means to gain a rapid understanding of the software's capabilities. Online helps are also integrated with the software, providing help for the different parts of the software.

#### 8.1.1 Modules

DeCyder 2D version 6.5 software comprises six modules (where the EDA module is optional):



##### **Image Loader**

Import of scanned gel images into a project within the DeCyder database, making them accessible for other modules. The import must be performed before analyses can be performed in DeCyder 2D software.



### **Batch Processor**

Fully automated image detection and matching of multiple gels without user intervention. Proteins of interest can be automatically filtered and picking list generated.



### **DIA (Differential In-gel Analysis)**

Protein spot detection and quantitation on a set of images, from the same gel. Features include background subtraction, in-gel normalization and gel artefact removal. Images must be processed in the DIA interface prior to data analysis in BVA.



### **BVA (Biological Variation Analysis)**

Matching of multiple images from different gels to provide statistical data on differential protein abundance levels between multiple groups.



### **XML Toolbox**

Extraction of user specific data from XML files generated in either the Batch, DIA or BVA modules. This data can be saved in either text or html format enabling users to access data from DeCyder 2D workspaces in other applications.



### **EDA (Extended Data Analysis)**

Multivariate analysis of data from several BVA workspaces. EDA is an add-on module for the DeCyder 2D software and can handle up to 1000 spot maps. The raw data (gel images) are linked to EDA and can be opened for display via the BVA module. In addition to the univariate analyses that can be performed in the BVA module, it is also possible to perform the following analyses in EDA:

- **Principal Component Analysis:** Produces an overview of the data. Can be used to find outliers in the data.
- **Pattern analysis:** Finds patterns in expression data (e.g. proteins and spot maps with similar expression profiles).
- **Discriminant analysis:** Finds proteins that discriminate between different samples (to find biological markers), creates classifiers and assigns samples to known classes depending on expression profiles (e.g. tumor typing).
- **Interpretation:** Finds the biological context of proteins by integrating biological information and context from in-house or public databases. It can be used to determine in what pathways and processes a protein is involved, the function of the protein etc.

## 8.2 ImageMaster 2D Platinum software

### 8.2.1 Structure

ImageMaster 2D Platinum ensures fast and reliable image comparisons. It easily manages multiple image analyses and offers the possibility to automate detection and matching steps with numerous interactive tools for optimizing and manipulating data. Furthermore, it is possible to attain a higher level of quantitative and qualitative analysis using the robust and sophisticated techniques provided in the application. ImageMaster integrates filtering, querying, reporting, and statistical and graphing options so that you can easily view, compare, analyze and present your results.

The powerful suite of features that normally apply to 2D gels in ImageMaster can now be used in conjunction with DIGE gels by using the ImageMaster 2D Platinum 6.0 DIGE module of the software.

This section briefly outlines the features and capabilities of the software. For a detailed guide, please refer to the ImageMaster 2D Platinum User Manual which also includes a series of tutorials designed to provide new users with the means to gain a rapid understanding of the software's capabilities. An online help is also integrated with the software.

### 8.2.2 Image analysis workflow

A typical image analysis using ImageMaster 2D Platinum software for DIGE gels would consist of the steps below. For detailed information, see the ImageMaster 2D Platinum User Manual.

#### 1 Acquiring data

Gel images must first be digitized using an image capture device. This will generally be done with a separate software. Open gels from TWAIN compatible scanners with ImageMaster.

#### 2 Setting up a workspace

Set up a workspace to open and work on gel images. The workspace allows to organize gels into projects, to define match sets and classes and to keep accompanying data, such as reports and image documents in project related folders. Preferred ImageMaster settings are also saved in the workspace file.

#### 3 Visualizing gels

This step is to handle the gel files (open, save, print, close), manipulate the gel images (select, move, zoom, stack, align), possibly transform the gels (rotate, crop, scale) and view the signal intensity (adjust contrast, profile, 3D view).

#### 4 **Detecting and quantifying spots**

Perform automatic spot detection. It is also possible to select, display and edit spots, as well as view spot properties and quantification values.

#### 5 **Annotating spots and pixels**

Individual pixels and spots in a gel image may be labeled with annotations. These annotations can be used in functionalities such as calibration, alignment and matching, or be utilized to mark spots with their particular characteristics. Create, use, select and display labels, categories and annotations. It is also possible to create links to external databases or data sources of any format (text, file, html, etc.).

#### 6 **Matching gels**

After spots were detected and match sets defined gel images can be matched.

#### 7 **Analyzing data**

Data analysis and classifications tools to study the variations in protein expression among gels or classes of gels can be performed. The data analysis step may be carried out at two different levels. The intra-class statistics tools include scatter plots, descriptive statistics, and factor analysis. For inter-class analyses, the so-called overlapping measures and various statistical tests can be used. Heuristic clustering can help finding classes.

#### 8 **Integrating data**

Exports spot coordinates to a spot excision robot, exports gel data to a database (for example, via XML format), or imports experimental information to be included in annotations.

#### 9 **Reporting results**

Display information on specific gels and gel components (spots, matches, classes, annotations) at any moment during the analysis. It is possible to display, use, save, customize and edit reports. Several specific report types are available.

#### 10 **Controlling and automating gel analyses**

As with reporting results, operations that were carried out on gels can be checked at any time using the *History* function. It is also possible to create Scripts for automating parts of your analysis. A multiple undo/redo feature is also available.

## 9 Preparative workflow

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### 9.1 Introduction

When proteins of interest have been identified in 2D DIGE experiments the corresponding spots can be picked and analyzed in a mass spectrometer for protein identification. Although spots of interest can be picked directly from post-stained analytical SDS PAGE gels, spot picking from preparative-scale gels provide more material for analysis by mass spectrometry.

Ettan DIGE system is fully compatible with mass spectrometry analysis:

- 1 A preparative gel can be matched to previously run analytical gels by DeCyder 2D software.
- 2 DeCyder 2D software will generate a pick list of spots of interest that can be directly exported to Ettan Spot Picker or Ettan Spot Handling Workstation.
- 3 Protein spots of interest are then automatically picked from the gel by Ettan Spot Picker or Ettan Spot Handling Workstation.

This chapter provides information of changes to the protocols described in chapters 4-8 one must make to ensure an efficient preparative workflow. The workflow is outlined in Fig 9-1.

#### 9.1.1 Staining of preparative gels

Depending on which CyDye DIGE Fluor dye has been used for sample labeling there are two options for visualization of preparative gels:

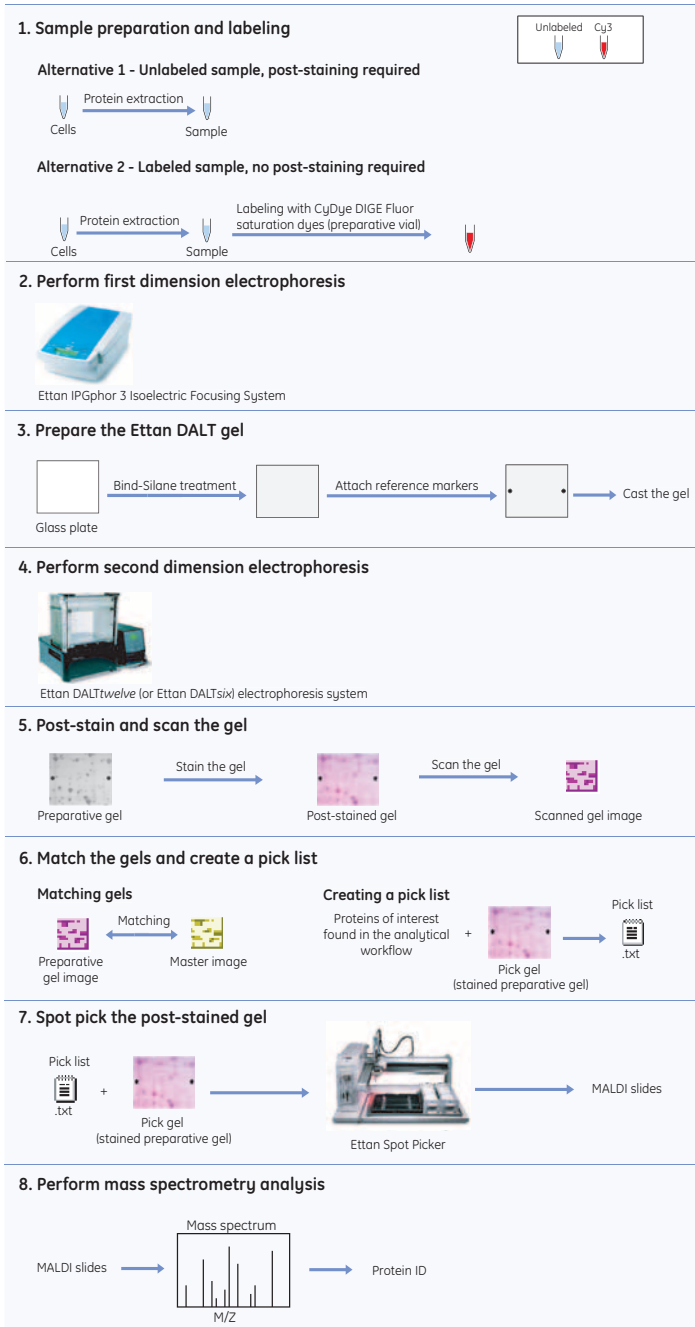
- For CyDye DIGE Fluor minimal dyes, the second dimension SDS PAGE preparative gels must be post stained. Use fluorescent protein stains, such as Deep Purple, for post-staining.

**Note:** *CyDye labeled proteins are approximately 450 Da larger in molecular weight compared to non-labeled proteins.*

- For CyDye DIGE Fluor saturation dyes no post-staining is required. Use the preparative dye vial provided in the kit instead. This vial provides amounts of CyDye DIGE Fluor saturation dyes enough for labeling preparative samples.

# 9 Preparative workflow

## 9.1 Introduction



**Fig 9-1.** Preparative gel workflow in Ettan DIGE System.



## 9.2 Sample preparation

### **Amount of sample**

Prepare samples as described in chapter 4. However, on a preparative gel larger amounts of protein sample are usually loaded, typically 500 µg or more.

### **Protein labeling**

Preparative gel samples do not require labeling with CyDye DIGE Fluor minimal dyes. It is recommended to use Deep Purple for post-staining instead, see section 9.5.

However, if samples have been labeled with CyDye DIGE Fluor saturation dyes they can be used directly in preparative gels. In *CyDye DIGE Fluor Labeling Kit for Scarce Samples and Preparative Gel Labeling* a vial of Cy3 dye is included which allows labeling of up to 500 µg of protein following the protocol 25-8009-83PL.

## 9.3 First dimension isoelectric focusing (IEF)

For preparative workflow first dimension isoelectric focusing follow protocols in chapter 5 considering:

- Use rehydration loading (section 5.3) or paper-bridge loading (see *2D Electrophoresis Principles and Methods* handbook) protocols for sample application.
- The loading should be optimized for different strip lengths and pH ranges. For a pH 3-10 NL 24 cm Immobiline DryStrips, up to 600 µg of protein can be loaded on a preparative gel.

## 9.4 Second dimension SDS PAGE

For preparative workflow second dimension SDS PAGE electrophoresis please consider:

- Clean and Bind-Silane treat the glass plates to be used
- Attach reference markers to the treated glass plates
- Ensure correct orientation of Immobiline DryStrips and the preparative second dimension SDS PAGE gel

### 9.4.1 Clean and Bind-Silane treat the glass plates

The Bind-Silane treatment of the glass plates is performed to immobilize the gel onto the glass plate and to prevent the gel from deforming during the staining, imaging and picking processes.

The following protocol for treatment of glass plates was optimized for PlusOne Bind-Silane.

**Note:** Use the shorter glass plate without reference markers.

- 1 Thoroughly scrape off any residual bound gel with a plastic scraper and wash the plate in 1% Decon™ (v/v) (branded Contrad™ in the USA) with a soft sponge to further remove the gel.
- 2 Leave the plate to soak in 1% Decon (v/v) overnight. On the following day, wash the plate with a soft sponge.
- 3 Rinse the plate with water and leave the plate to soak in 1% HCl (v/v) for 1 h.
- 4 Wash the plate in 1% Decon (v/v) with a soft sponge, then rinse with double distilled water.
- 5 Dry the plate using a lint-free tissue or leave to air dry in a dust free environment. If not to be used immediately, please store in a dust free environment.
- 6 Prepare the *Bind-Silane working solution*.
- 7 Pipette 2-4 ml (depending on plate size) of the above solution over the whole surface of the plate and wipe it over with a lint-free tissue until it is dry. Cover the plate with a lint-free tissue to prevent dust contamination and leave on the bench for 1.5 h (minimum one hour) for excess Bind-Silane to evaporate.

**Note:** *If the Bind-Silane is not left to dry sufficiently before the glass plates are assembled for casting, the solution will evaporate off the treated plate and coat the facing glass surface. This will cause the gel to stick to both plates when it sets.*

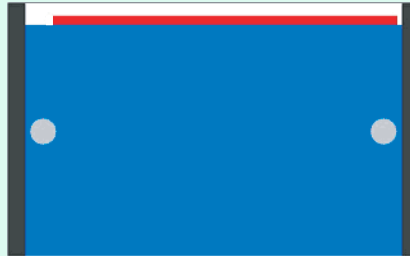
**Note:** *The gels will stay attached to Bind-Silane treated glass during electrophoresis, staining procedures, scanning and storage.*

### 9.4.2 Attach reference markers

Reference markers are used to ensure that the correct protein spots detected during image analysis are picked.

Reference markers must be attached to the short treated glass plate *before* gel pouring. It is important that the markers are appropriately placed on the treated surface of the *Bind-Silanized* plate. Take care not to place the markers where they will interfere with the pattern of protein spots in the gel. The markers should be placed according to the following protocol.

- 1 Measure the length of the treated plate edge.
- 2 Place the marker approximately half-way along this edge, away from the spacer, but not so far as to interfere with the protein spot pattern. The marker should not touch the spacer. Make sure that the markers are firmly stuck to the plate by pressing down with a lint free tissue or powder free glove.
- 3 Repeat steps 1 and 2 for the other edge of the treated backing plate.
- 4 When finished, the markers should be in positions similar to those shown below.



**Fig 9-2.** Diagram showing the preferred position of reference markers on the gel backing (short glass plate).

## 9 Preparative workflow

### 9.5 Post-staining with Deep Purple Total Protein Stain

#### 9.4.3 Second dimension SDS PAGE

We recommend using the Ettan DALT electrophoresis system.

The orientations of the Immobiline DryStrips and the reference markers are critical to ensure that the picking gel is easily matched to the analytical gels during image analysis, and that the correct spots are picked from the gel when the pick list is exported to the Ettan Spot Picker.

- 1 After a picking gel has been poured containing suitable reference markers, position the gel so the front of the reference markers are facing you.
- 2 Equilibrate the Immobiline DryStrips as described in Section 6.4, Equilibration of focused Immobiline DryStrips.
- 3 Load the equilibrated Immobiline DryStrips (see Section 6.5, Loading of focused Immobiline DryStrips), ensuring that the acidic (pointed end) of the Immobiline DryStrips is on the left hand side of the gel as shown in Fig 9-2.
- 4 To run the gel, see 6.6 for further information.

#### 9.5 Post-staining with Deep Purple Total Protein Stain

**Note:** *If the sample used has been labeled with CyDye DIGE Fluor saturation dye post-staining of the second dimension SDS PAGE gel is not required.*

The fluorescent Deep Purple Total Protein Stain fits into the standard 2D gel electrophoresis workflow and is particularly suitable for use with the Ettan DIGE system. The recommended workflow involves the matching of Deep Purple post-stained preparative gels with CyDye labeled analytical gels. Deep Purple has been shown to be compatible with the image analysis softwares and the stain is compatible with manual or automated spot picking and mass spectrometry for protein identification applications.

- 1 Place an appropriate volume of *fixation solution* into the containers that will be used to process gels. The recommended volume of fixation solution required is ~20 fold excess of the gel volume (1000 ml for Ettan DALT gels).
- 2 Dismantle the electrophoresis apparatus. Remove one glass plate and place the gel attached to the glass plate into fix solution.

**Note:** *Place only one gel in each container. The Immobiline DryStrip can be left attached to help with gel orientation.*

- 3 Incubate in the fixation solution overnight at room temperature with gentle agitation.
- 4 Take the stain out of the -15°C to -30°C freezer and allow to stand at room temperature for 5-10 minutes.
- 5 Pour off the fixation solution and replace with the *wash solution* (1000 ml for Ettan DALT gels). Wash with gentle agitation for 30 minutes.
- 6 Pour off the wash solution and replace with 500 ml water. To make up the *working stain solution*, briefly shake the stain concentrate and add 2.5 ml Deep Purple to make a 1:200 dilution. Cover the container to create a dark environment and incubate for 1 hour at room temperature with gentle agitation.

**Note:** *The solution is light sensitive and should be kept out of bright light.*

**Note:** *Containers can be wrapped in foil or covered with black plastic. It is not necessary to eliminate light completely, only to ensure that bright light is significantly reduced. Alternatively, containers with lids, that are a solid colored plastic, may be used.*

- 7 Pour off the stain and replace with 7.5% (v/v) acetic acid. Cover the container to create a dark environment and incubate with gentle agitation for at least 15 minutes.
- 8 Repeat the acetic acid step once. The gel can be imaged at this stage.

**Note:** *If speed is more important than background levels, the gel can be imaged after one acetic acid step. Further washes in acetic acid can be performed to reduce the background still further if necessary. After imaging, the gels can be stored in the dark in 7.5% (v/v) acetic acid at 2–8 °C for several weeks. This allows for further imaging at a later date if required.*

## 9.6 Gel scanning

We recommend using Typhoon Variable Mode Imager or Ettan DIGE Imager. Please refer to Chapter 7 for more details.

- 1 Place the gel (glass side down) onto a clean, dust-free platen surface.
- 2 Image the gel with the appropriate filter set and exposure times. It is recommended that the image resolution for the analytical and preparative gels are set at the same level and are at least 100 µm.

## 9 Preparative workflow

### 9.7 Matching gels and creating a pick list

- 3 Ensure that both reference markers can be clearly seen and that they appear as circles when the gel image is checked. If the markers cannot be seen, then re-scan the gel, adjusting the area to be scanned appropriately.
- 4 When scanning is finished, place the gel into the *storage solution* (7.5% acetic acid) at 4°C in the dark.
- 5 Clean the scanner platen after use to remove any fluorescent residue. If the platen is not thoroughly cleaned, this residue can interfere with subsequent scans producing high background levels. See 7.3 for information of how to clean the scanner platen.

### 9.7 Matching gels and creating a pick list

The matching of gels and creating a pick list is performed in DeCyder 2D. For information on the different steps in the workflow below, see *DeCyder 2D User Manual* and *DeCyder Extended Analysis module User Manual*.

### 9.8 Spot picking the gel

After a pick list has been created from the image analysis software, spot picking can be performed. The procedure of picking and digesting spots can be performed by manual transfer of gels and microplates between the Ettan Spot Picker, Ettan Digester and Ettan Spotter, or fully automatically in the integrated Ettan Spot Handling Workstation.

Processing is done in three main steps:

- 1 **Spot picking from second dimension SDS PAGE gels**  
Selected protein spots from stained gels are automatically picked by Ettan Spot Picker using a pick list created from the image analysis software, and the gel plugs are transferred into microplates. See *Ettan Spot Picker User Manual*.
- 2 **Digestion of the picked proteins**  
The gel plugs are first trypsin digested in Ettan Digester and the resulting peptides extracted from the gel plugs. See *Ettan Digester User Manual*.
- 3 **Spotting of samples onto MALDI targets**  
The extracted peptides are mixed with matrix solution and spotted on MALDI targets using Ettan Spotter. See *Ettan Spotter User Manual*.

Ettan Spot Handling Workstation automatically processes and transfers biomolecules from polyacrylamide gels to MS targets. A computer running proprietary software controls the whole process. For detailed instructions, see *Ettan Spot Handling Workstation User Manual*.

## 9.9 Mass spectrometry analysis

MALDI targets are analyzed by MALDI-ToF MS for protein identification. Time-of-flight mass spectrometry is a technique for analyzing molecular weights based on the motion of ionized samples in an electrical field. In MALDI-ToF, a matrix-bound sample is bombarded with a pulsed laser beam to generate ions for subsequent detection.

Protein identification by mass spectrometry is usually performed on spot-picked unlabeled protein, visualized on the second dimension SDS PAGE gel with a post-electrophoresis stain, such as Deep Purple. Some applications may require direct spot picking from a second dimension SDS PAGE gel containing protein labeled with CyDye DIGE Fluor minimal dye. The nature of the minimal labeling approach results in the majority of the protein (and peptide) population remaining unlabeled.

The results can be imported into DeCyder 2D software modules BVA and EDA for further analysis in the EDA module.

## 9.10 Recipes

Recipes for post-staining of gels:

### *Bind-Silane working solution*

Reagent	Quantity
Ethanol	16 ml
Glacial acetic acid	400 µl
Bind-Silane	20 µl
Double distilled H <sub>2</sub> O	3.6 ml

**Fixation solution**

Reagent	Quantity	Final concentration
Ethanol	100 ml	10% (v/v)
Acetic acid	75 ml	7.5% (v/v)

Make up to 1 000 ml with distilled water.  
~ 20-fold excess of the gel volume should be used.

**Wash solution (small, usually free-floating, gels)**

Reagent	Quantity	Final concentration
Na <sub>2</sub> CO <sub>3</sub>	21.2 g	200 mM

Dissolve Na<sub>2</sub>CO<sub>3</sub> in 750 ml water.  
Make up to 1 000 ml with distilled water.  
The pH of the solution should be at least 11 and should be verified.  
~ 20-fold excess of the gel volume should be used.

This solution can be stored for up to 2 weeks.

**Wash solution (large, usually backed, gels)**

Reagent	Quantity	Final concentration
Na <sub>2</sub> HCO <sub>3</sub>	2.94 g	35 mM
Na <sub>2</sub> CO <sub>3</sub>	31.8 g	300 mM

Dissolve Na<sub>2</sub>HCO<sub>3</sub> and Na<sub>2</sub>CO<sub>3</sub> in 750 ml water.  
Make up to 1 000 ml with distilled water.  
The pH of the solution should be pH 10-11 and should be verified.  
~ 20-fold excess of the gel volume should be used.

This solution can be stored for up to 2 weeks.



**Working stain solution**

Reagent	Quantity	Final concentration
Deep Purple	2.5 ml	1:200 dilution

Make up to 500 ml with distilled water.

~ 10-fold excess of the gel volume should be used.

This solution should be made fresh at the time of use by adding an appropriate aliquot of Deep Purple to water in the gel staining tank. If necessary it is possible to store this solution, protected from exposure to light, for up to 1 week at 2-8°C or 24 h at room temperature.

**Storage solution**

Reagent	Quantity	Final concentration
Glacial acetic acid	75 ml	7.5% (v/v)

Make up to 1 000 ml with distilled water

9 Preparative workflow  
9.10 Recipes

## Appendix A Testing cell lysates for successful labeling

---

### A.1 Testing new cell lysate for successful labeling

It is important to check that labeling of the proteins is optimized before the samples are taken through the 2D electrophoresis process.

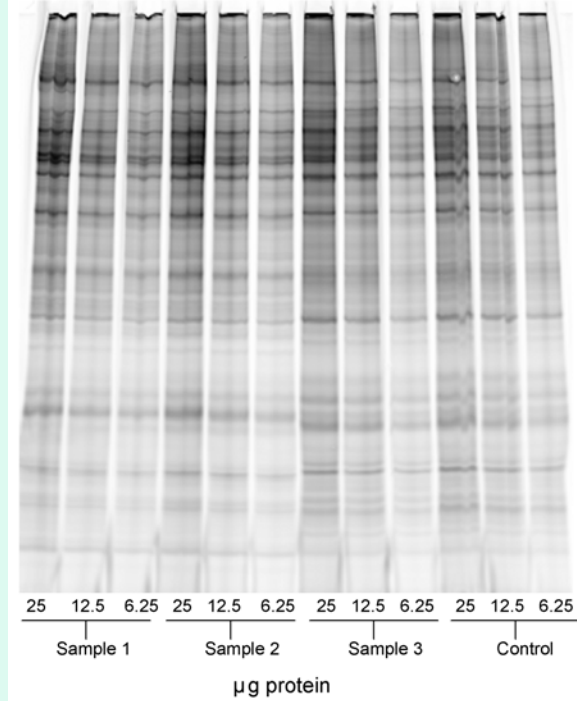
The method involves running a small sample of the freshly labeled lysate on a 1D SDS PAGE gel along with a control lysate already known to label successfully. The gel is then scanned at the appropriate wavelength for the relevant CyDye DIGE Fluor dyes. The total fluorescence of each labeled sample is then compared. The method should also be used to test protein lysates that contain previously untested chemical components.

#### Protocol

- 1 Label 50 µg of the new protein sample with 400 pmol of CyDye DIGE Fluor Cy5 minimal dye.
- 2 Add a volume of each labeled protein lysate equivalent to 50 µg, to a microfuge tube.
- 3 Add an equal volume of the 2× *gel loading buffer* to the labeled protein lysate.
- 4 Heat the samples at 95 °C for 5 min to ensure full reduction of the proteins.
- 5 Make a serial dilution of each of the lysates in the 2× *gel loading buffer*, e.g. 25 µg, 12.5 µg and 6.25 µg.
- 6 Make a 12.5% SDS PAGE gel using low fluorescence glass plates. The gel should be made with wells into which the samples will be loaded.
- 7 Load each protein serial dilution in successive lanes on the gel.
- 8 Run the samples until the Bromophenol Blue dye front has nearly reached the bottom of the gel.
- 9 Thoroughly clean the outside of the glass plates with double distilled water.
- 10 Scan the gel at the appropriate wavelength with Typhoon Variable Mode Imager or Ettan DIGE Imager.

A Testing cell lysates for successful labeling  
A.1 Testing new cell lysate for successful labeling

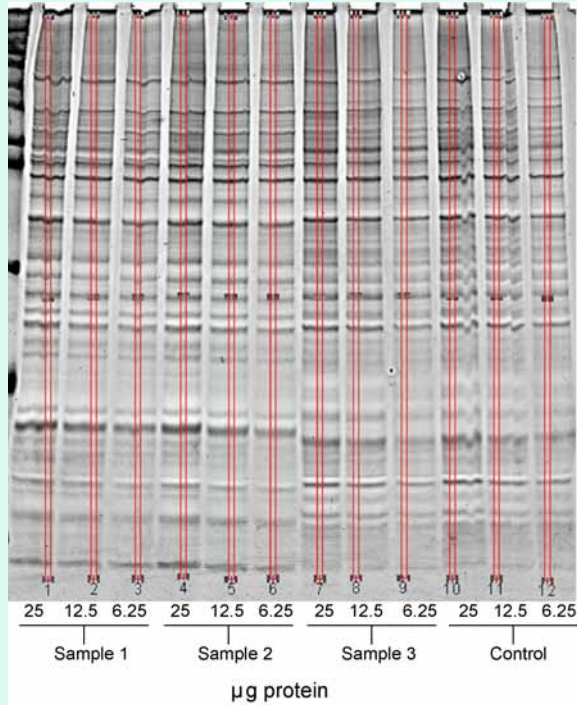
- 11 Insert the gel into the scanner in the correct orientation, see Chapter 7, Image acquisition.



**Fig A-1.** CyDye DIGE Fluor Cy5 minimal dye scanned image

- 12 Quantify the labeling of each protein sample using ImageQuant TL software.

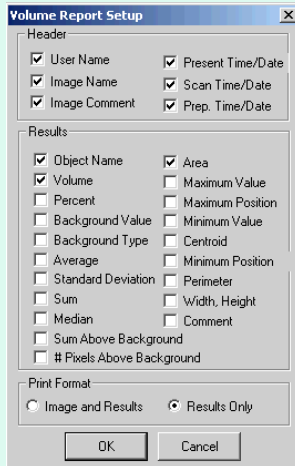
- 13 Carry out the statistics by opening the image in ImageQuant TL software. Draw a single box over the first lane using the **Object:Rectangle** Tool. *Copy* and *Paste* the rectangle for all of the samples that need to be tested in the remaining lanes.



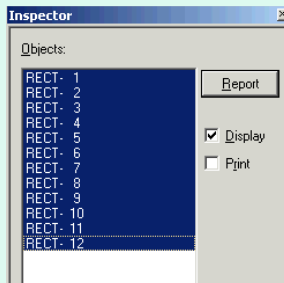
**Fig A-2.** CyDye DIGE Fluor Cy5 minimal dye scanned image. Lanes are overlaid with identical boxes to give a volume report in ImageQuant TL Toolbox.

A Testing cell lysates for successful labeling  
A.1 Testing new cell lysate for successful labeling

- 14 In **Analysis:Volume Report Setup** highlight the boxes **Object Name**, **Volume**, **Area** and select **Results Only** in the **Print Format** section.



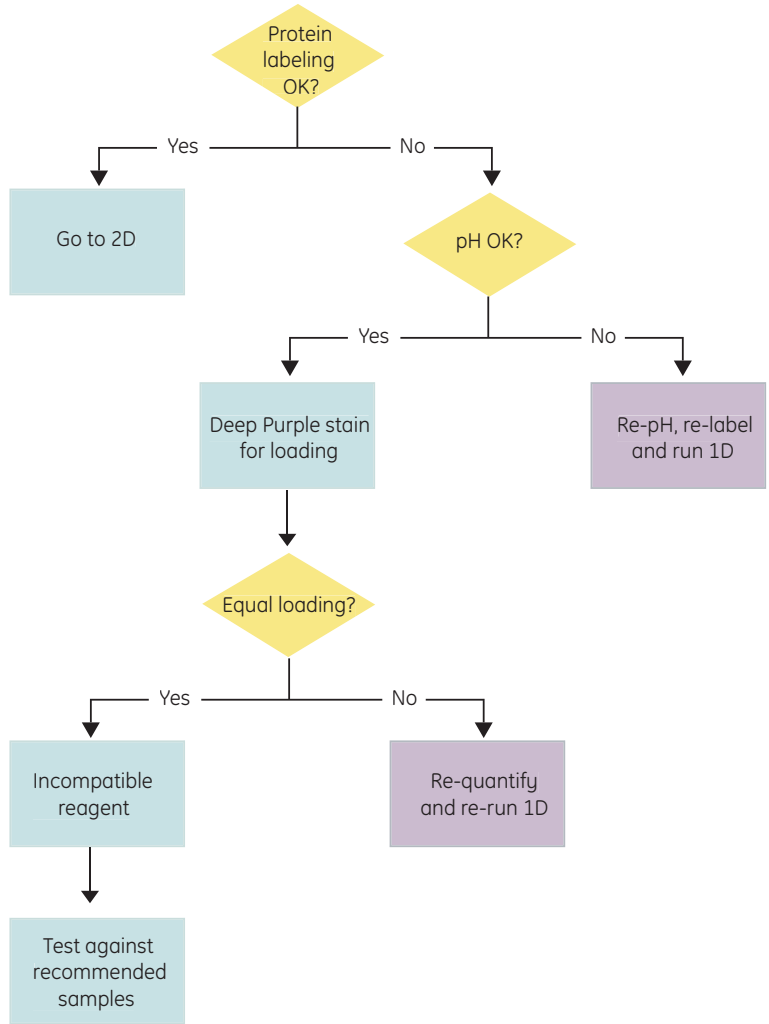
- 15 Generate a volume report by clicking **Analysis:Volume Report...** in the drop down menu.
- 16 Select all the relevant RECT in the *Inspector* window so that they are highlighted blue.



- 17 Determine the labeling efficiency by comparing the volume of the new protein samples and the control sample, which are on the same gel. The labeling efficiency of these should be equivalent.

If labeling is comparable between the control and the new protein lysates tested then samples can now be run on 2D gels. See the decision tree in Fig A-3.

To investigate the cause of the problem, post-stain the gel with Deep Purple Protein Total Stain. See Section 9.5, for information.



**Fig A-3.** Decision tree for troubleshooting labeling using 1D gels.

## A.2 Recipes

### 2× Gel loading buffer

Reagent	Quantity	Final concentration
Tris (1 M, pH 6.8)	12 ml	120 mM
Glycerol (87% [v/v])	23 ml	20% (v/v)
SDS (MW 288.38)	4 g	4% (w/v)
DTT (MW 154.2)	3 g	200 mM
Bromophenol Blue	A few grains	trace
Make up to 100 ml with distilled water		

### 12.5% 1D SDS PAGE gel composition

Reagent	Quantity for 100 ml of a 12.5% gel
Acrylamide/Bis 40% (w/v)	32.0 ml
Tris (1.5M, pH 8.8)	25.0 ml
10% (w/v) SDS	1.0 ml
10% (w/v) APS	1.0 ml
(undiluted) TEMED *add immediately prior to use*	40 µl
Make up to 100 ml with distilled water	

### 1× SDS electrophoresis running buffer

Reagent	Quantity	Final concentration
Tris (MW 121.14)	60.5 g	25 mM
Glycine (MW 75.07)	288 g	192 mM
SDS (MW 288.38)	20 g	0.1% (w/v)
Make up to 20 l with distilled water		

Store at room temperature for up to 3 months.



## Appendix B Labeling of cell surface proteins

---

### ***B.1 Selective labeling of cell surface proteins***

Cell surface proteins can be difficult to detect in a second dimension SDS PAGE gel without fractionation or some other type of enrichment partly due to their low abundance (1-2% of total cellular protein contents). They are often poorly represented in second dimension SDS PAGE gels due to their hydrophobic nature and high molecular weight.

The new protocol presented in this appendix for selective labeling of cell surface proteins using CyDye DIGE Fluor minimal dyes makes it possible to visually enrich cell-surface proteins without performing sample fractionation. This protocol is fast, simple to use and all three CyDye DIGE Fluor minimal dyes can be used to label cell surface proteins.

These features allow for multiplexing using Ettan DIGE technology and analysis of protein expression using DeCyder 2D software. In this way, the level of surface proteins can be studied in different disease states or when responding to different treatments. Small changes in abundance can be detected with high accuracy, and results are supported by defined statistical methods.

#### ***Protocol***

- 1 Grow the cells of interest until a minimal cell number of  $5-10 \times 10^6$  cells (confluent or cells in suspension) is reached. For cells growing in suspension, proceed to step 3.
- 2 Carefully detach adherent cells non-enzymatically (cell dissociation media, enzyme free PVS-based).
- 3 Count the cells and pellet them by centrifugation. From now on, keep the cells on ice.
- 4 Wash the cells by resuspending the pellet in 1 ml cold HBSS pH 8.5 buffer and transfer to 1.5 ml eppendorff tube. Centrifuge the suspension at  $800 \times g$  and  $+4^\circ\text{C}$  for 2 minutes.
- 5 Remove the supernatant and resuspend a cell pellet containing  $5-10 \times 10^6$  cells in 200  $\mu\text{l}$  cold labeling buffer (HBSS pH 8.5, 1M Urea). Add 1.5  $\mu\text{l}$  CyDye working solution (600 pmoles). Mix briefly by vortexing at slow speed.
- 6 Label the cell surface for 20 minutes on ice in darkness. Then add 20  $\mu\text{l}$  of 20 mM lysine and incubate another 10 minutes.

## B Labeling of cell surface proteins

### B.1 Selective labeling of cell surface proteins

- 7 After labeling and quenching, wash the cells twice in 500  $\mu$ l cold HBSS pH 7.4 by centrifugation at 800  $\times$  g and +4  $^{\circ}$ C for two minutes.
- 8 Resuspend the pellets directly in 150  $\mu$ l cold lysis buffer (7M Urea, 2M Thiourea, 4% CHAPS, 30 mM Tris, 5 mM MgAc pH 8.5) and leave on ice for at least 1 hour with occasional vortexing. Centrifuge the lysate at 10 000  $\times$  g and +4 $^{\circ}$ C for 5 minutes. Transfer the supernatant to a new tube.

**Note:** *Fractionation of the sample may be desired. It is not necessary for improved detection of cell surface proteins but could be used to verify lack of labeling of cytosolic proteins. Then, resuspend the cell pellet in 150  $\mu$ l cell lysis buffer included in the membrane fractionation kit (2D Sample Prep for Membrane Proteins, Pierce). After fractionation the sample can be directly applied on 2D electrophoresis.*

- 9 Add 2X Sample buffer to the lysed cell surface labeled sample. Now the sample is ready for 2D electrophoresis. Proceed with the recommended protocols for 2D DIGE electrophoresis.

## Appendix C Reagents tested for compatibility with Ettan DIGE system

This section contains examples of reagents commonly used in 2D electrophoresis experiments which have been tested for their compatibility with labeling using CyDye DIGE Fluor dyes. They all have the *DIGE approved* seal attached.

This is not a complete list of reagents; if unlisted reagents or a combination of these reagents are required in the cell lysis buffer it is recommended that the labeling efficiency is checked following the instructions in Appendix A. These examples are only intended as a guide.

### C.1 List of reagents

#### Reducing agents

<b>DL-dithiothreitol (DTT)</b>	2 mg/ml - slight reduction in labeling 5 mg/ml - 2× reduction in labeling 10 mg/ml - 10× reduction in labeling CyDye DIGE Fluor minimal dyes will react with thiols at high concentration.
<b>Tris-(2-carboxyethyl) phosphine (TCEP)</b>	0.5 to 1 mM - slight reduction in labeling 2 mM - significant reduction in labeling
<b>β-mercaptoethanol</b>	Significantly reduces labeling

#### Detergents

<b>Triton™ X-100</b>	use at 1%	17% reduction in labeling
<b>NP40</b>	up to 1%	No effect on labeling
<b>SDS</b>	up to 1%	No effect on labeling

#### Salts

Application of sample during rehydration	<10 mM recommended
Application of sample via cup-loading	<50 mM recommended

## C Reagents tested for compatibility with Ettan DIGE system

### C.1 List of reagents

#### **Buffers**

<b>Tris</b>	Recommend 10-40 mM, pH 8.0 - 9.0 pH is very important. pH 8.5 is optimal.
<b>HEPES</b>	Can cause focusing problems therefore not recommended.
<b>Bicarbonate</b>	5 mM, pH 8.5 is acceptable
<b>CHES</b>	5 mM, pH 9–9.5 is acceptable
<b>PBS</b>	150 mM phosphate buffered saline
<b>Tris sucrose</b>	250 mM sucrose, 10 mM Tris

#### **Protease Inhibitors**

For all protease inhibitors: Mix compatible proteases at recommended concentrations.

<b>4-(2-aminoethyl) benzenesulphonyl fluoride (AEBSF) (Pefabloc™)</b>	Causes charge trains unless protector reagent is used
<b>Complete™ protease inhibitor cocktail</b>	This product contains AEBSF, so the same restrictions as above apply
<b>Aprotinin</b>	Compatible at recommended concentrations
<b>(4-amidino-phenyl) methane sulphonyl fluoride (APMSF)</b>	Compatible at manufacturer's recommended concentrations
<b>EDTA</b>	Compatible between 0.5-10 mM
<b>Phenylmethylsulphonyl fluoride (PMSF)</b>	Compatible at manufacturer's recommended concentrations
<b>Pepstatin A</b>	Compatible at manufacturer's recommended concentrations
<b>Protease inhibitor mix</b>	Compatible at recommended concentrations

***Phosphatase inhibitors***

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<b>Phosphatase inhibitor cocktail 1 (Sigma)</b>	Compatible at manufacturer's recommended concentrations
<b>Phosphatase inhibitor cocktail 2 (Sigma)</b>	Compatible at manufacturer's recommended concentrations

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***Sample preparation kits***

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<b>2D Clean up kit</b>	Compatible at manufacturer's recommended concentrations
<b>SDS PAGE Clean up kit</b>	Compatible at manufacturer's recommended concentrations
<b>2D Quant kit</b>	Compatible at manufacturer's recommended concentrations
<b>Sample grinding kit</b>	Compatible at manufacturer's recommended concentrations

---

## C Reagents tested for compatibility with Ettan DIGE system

### C.1 List of reagents

## Appendix D Trouble shooting guide

The aim of this Appendix is to provide a help guide for problems that might be encountered when running experiments. For general 2D troubleshooting problems please refer to *2D Electrophoresis Principles and Methods* handbook.

### D.1 Sample preparation and labeling

Problem	Cause	Remedy
Low protein yields from the cell lysate	Lysis procedure	Ensure cell culture densities were optimal for cell lysis using sonication
		Ensure cell pellet was not lost after centrifugation
		Ensure sonication was carried out for long enough
	Insufficient protein in sample (protein concentration <1 mg/ml)	Remake protein lysate or concentrate sample by precipitation with Ettan 2D Clean-Up Kit (code no. 80-6484-51)
Horizontal streaking	Low pH prior to labeling	Check that pH is within range 8-9 immediately prior to labeling
Unexpected protein spots present in the gel	Contaminant proteins have been introduced into the sample prior to the labeling reaction	Check that gloves are used throughout the procedure
Protein spots detected more strongly with one dye	Proteins not denatured or solubilized sufficiently	Use combination of chaotrope in lysis buffer, such as 7 M urea/2 M thiourea

## D Trouble shooting guide

### D.1 Sample preparation and labeling

<b>Problem</b>	<b>Cause</b>	<b>Remedy</b>
Weak fluorescent signal on 2D gel image	Insufficient sample buffering	Use 30 mM Tris to give sufficient buffering capacity
	Thiol agents present in the sample competing for dye. Check if concentration of DTT is >2 mg/ml in protein sample preparation method	Dilute protein lysate with DTT-free lysis buffer. Clean sample by precipitation with Ettan 2D Clean-Up Kit, or increase the amount of dye in the labeling reaction
	Primary amines such as Pharmalytes or ampholytes are present in sample during labeling, competing for CyDye DIGE Fluor minimal dye	Dilute protein lysate with amine-free lysis buffer. Clean sample by precipitation with Ettan 2D Clean-Up Kit, or increase the amount of dye in the labeling reaction
	Incorrect concentration of protein in lysate	Use a detergent or thiourea compatible protein assay kit, e.g. Protein Determination Reagent or Ettan 2D Quant Kit
	Check quality of DMF	Should be >99.8% anhydrous DMF from a bottle that has not been open for longer than 3 months
	Degraded dye due to hydrolysis of NHS-ester or photodegradation of fluorophore	Ensure that the appropriate storage conditions have been used for the CyDye DIGE Fluor minimal dye (in dark at -20°C). Check specific batch expiry date and reconstitution date
	Incorrect dye:protein ratio used	400 pmol of dye per 50 µg of protein is recommended. If there is a large concentration of other components which can react with the dye, then more dye (up to 2 nmol per 50 µg of protein) can be used
Low pH prior to labeling	Check pH is 8.5 immediately prior to labeling. If necessary, increase pH using higher pH lysis buffer containing 30 mM Tris (pH 9.0-10.0) or use 50 mM NaOH	



## D.2 First dimension electrophoresis

Problem	Cause	Remedy
Current is zero or too low	External electrode contacts are poor	Ensure that the electrodes at the bottom of the strip holder (one at each end) make metal-to-metal contact with the appropriate electrode area
	Internal electrode contacts are poor	Ensure that the gel makes contact with both electrodes in the strip holder
	Immobiline DryStrips not fully rehydrated	Check that the Immobiline DryStrips are fully rehydrated along their entire length. Electrical contact at the electrodes is reduced by incomplete rehydration
	No conduction through electrode wicks	Check that the electrode wicks (if used) were moistened prior to use
Voltage too low or does not reach the maximum set value	Current limit setting is incorrect	Check that the current limit is properly set
	Incorrect number of Immobiline DryStrips	Check that the correct number of strips in place is set on the IPGphor program
	No Pharmalytes added to sample	The recommended Pharmalyte concentration is 1.0% (v/v)
Sparking or burning in strips	High current	Do not exceed the recommended setting of 50 $\mu$ A per Immobiline DryStrip
	Strips not rehydrated	Check that Immobiline DryStrips are fully rehydrated along their entire length
	Presence of bubbles	Check that any large bubbles trapped under the Immobiline DryStrips after wetting with rehydration solution are removed prior to focusing
	Strips drying out during focusing	Ensure that sufficient Immobiline DryStrips Cover Fluid has been applied
	High salt concentration	Clean sample to remove excess salts
Proteins have not focused	Poor electrical contact in first dimension	Check that the strips are in contact with the strip holder electrodes

### D.3 Second dimension electrophoresis

Problem	Cause	Remedy
No protein spots are visible on the gel	Incorrect labeling protocol	Check the sample preparation and labeling protocol
	Inefficient sample solubilization	Increase concentration of solubilizing components in the sample solution. The upper concentration limits for common reagents are: Urea 9.8 M, Thiourea 2 M, zwitterionic detergent CHAPS 4%, Pharmalyte 1%, DTT 2-3%
	No or insufficient SDS in electrophoresis running buffer	Ensure electrophoresis running buffer is correctly formulated
Individual spots appear as multiple bands or are missing, unclear, or in the wrong position	Immobiline DryStrips placement	Ensure that the plastic backing of the Immobiline DryStrips are against the glass plate on the second dimension gel, directly onto the top of the acrylamide gel
	Protein oxidation during electrophoresis	Prevent oxidation of oxygen-sensitive proteins in the gel. Check the correct equilibration conditions are used prior to the second dimension separation. DTT reduction, then treatment with iodoacetamide alkylates the sulphhydryl groups and thus prevents the reduced proteins from re-oxidizing
Formation of charge trains	Protein carbamylation	Check that all solutions containing urea were prepared freshly and ensure that all solutions containing urea were not heated above 37° C at any time
Horizontal streaking or incompletely focused spots	High sample load	Reduce sample load by adding less sample to the rehydration solution
	Insufficient focusing time	Increase total Vh for focusing

<b>Problem</b>	<b>Cause</b>	<b>Remedy</b>
Vertical streaking or incompletely focused spots	SDS depletion during second dimension electrophoresis	Use 0.2% SDS in the running buffer for both top and bottom buffer tanks. Ensure that the gel has been prepared with the correct concentration of SDS
	Over-alkylated proteins	Use lower pH, higher DTT concentration or lower iodoacetamide concentration when equilibrating Immobiline DryStrips
Sample insolubility and particulates	Insufficient chaotrope or detergent	Ensure the correct solubilization solution has been used
	Cloudy samples	Remove insoluble material from the sample using ultracentrifugation
Poor first dimension focusing	Ionic detergent concentration too high in lysis buffer	If SDS is used in sample preparation, the final concentration must not exceed 0.25% after dilution into the rehydration solution. Also ensure that the nonionic detergent is present in a concentration at least 8 times higher than the concentration of any ionic detergent to ensure complete removal of SDS from the proteins
Streaking or smearing	Sample rich in nucleic acids	Add DNase and RNase, or sonicate to hydrolyze nucleic acids
Sample aggregation or precipitation	Focusing conditions not optimized	Program a low initial voltage that increases gradually, and/or increase time at maximum voltage. Extended focusing may result in electroendosmosis where water and protein movement can produce horizontal streaking. Minimize water transport by employing a maximum pH range Immobiline DryStrips and apply electrode pads

### D.4 Typhoon Variable Mode Imager results

For a complete guide to troubleshooting Typhoon Imager results, please refer to Typhoon User Guide.

Problem	Cause	Remedy
Protein spots do not show up on the gel image	The wrong laser and emission filters have been selected for the CyDye DIGE Fluor minimal dye used	Select correct laser and filter for each CyDye DIGE Fluor minimal dye
	The labeling reaction has not been performed correctly	Reconstitute stock dye or make fresh working dye solution in fresh DMF. Repeat the labeling
	The PMT voltage is too low	Rescan with higher PMT voltage
The gel image appears black	The PMT voltage is too high	Rescan with lower PMT voltage
Appearance of nonspecific background on gel image	Platen contaminated with dye from scanning other gels	Clean platen. Rescan gel on different part of platen to confirm that background does not move with the gel
	Bacterial/mycoplasma contamination in gel running equipment	Bacterial/mycoplasma contamination in gel running equipment
Appearance of small spots (sharp peaks in gel image)	Dirt and dust on gel plates or platen	Clean platen or gel plates. Rescan gel on different part of platen to confirm that background does not move with gel
	Dirt in gel	Filter acrylamide
DeCyder 2D software gives poor spot boundaries	Incorrect scan resolution	Rescan gels with resolution set to 100 $\mu\text{m}$
Faint, ill-defined gel images, possibly with high background	Incorrect focal plane selected	Re-scan with correct focal plane selected. Setting gel orientation and scan resolution

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GE Healthcare has patent applications pending relating to its DeCyder software technology, European patent application number EP1,234,280.

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