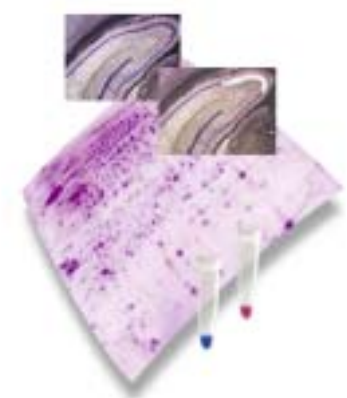
instructions

product code

25-8009-83
25-8009-84

CyDye DIGE Fluor Labelling Kit for Scarce Samples

Reagents for labelling protein with CyDye™ DIGE Fluor Cy™3 and Cy5 saturation dyes, before 2-dimensional electrophoresis



Warning

For research use only.

Not recommended or intended for diagnosis of disease in humans or animals.

Do not use internally or externally in humans or animals.

ⓘ 25800983PL Rev-B, 2003

Amersham Biosciences

Handling

Storage

Store at -15 °C to -30 °C. Avoid exposure to light, store in the dark.

Expiry

For expiry date see outer packaging.
Note: After reconstitution, CyDye DIGE Fluor saturation dyes are only stable and useable until the expiry date detailed on the tube or for 8 weeks, whichever is sooner.

Page finder

Components

25-8009-83: CyDye DIGE Fluor Labelling Kit for Scarce Samples containing:

- 100 nmol CyDye DIGE Fluor Cy3 saturation dye for analytical labelling;
- 100 nmol CyDye DIGE Fluor Cy5 saturation dye for analytical labelling.

25-8009-84: CyDye DIGE Fluor Labelling Kit for Scarce Samples plus Preparative Gel Labelling containing:

- 100 nmol CyDye DIGE Fluor Cy3 saturation dye for analytical labelling;
- 100 nmol CyDye DIGE Fluor Cy5 saturation dye for analytical labelling;
- 400 nmol CyDye DIGE Fluor Cy3 saturation dye for preparative labelling.

Safety warnings and precautions

Warning: For research use only.
Not recommended or intended for diagnosis of disease in humans or animals. Do not use internally or externally in humans or animals.

All chemicals should be considered as potentially hazardous. We therefore recommend that this product is handled only by those persons who have been trained in laboratory techniques and that it is used in accordance with the principles of good laboratory practice. Wear suitable protective clothing such as laboratory overalls, safety glasses and gloves. Care should be taken to avoid contact with skin or eyes. In the case of contact with skin or eyes wash immediately with water. See material safety data sheet(s) and/or safety statement(s) for specific advice.

CAUTION: These dyes are intensely colored and very reactive. Care should be exercized when handling the dyes to avoid staining clothing, skin, and other items. The toxicity of CyDye DIGE Fluor Cy3 and Cy5 saturation dyes has not yet been evaluated.

Other materials required

Sample preparation

• Microcentrifuge tubes:	1.5 ml
• Standard cell wash buffer:	10 mM Tris (pH 8.0), 5 mM magnesium acetate. Store at 2-8 °C. Stable for 1 month
• Milli-Q grade water	
• Cell lysis buffer:	30 mM Tris, 7 M urea, 2 M thiourea, 4% (w/v) CHAPS. Adjust to pH 8.0 with 1.0 M HCl. Aliquot and store at -15 °C to -30 °C. Stable for 3 months
• pH indicator strips :	(Sigma™ pH test strips pH 4.5-10.0 P-4536)
• 50 mM sodium hydroxide (NaOH)	
• Detergent compatible reagent for protein quantification:	We recommend Protein Determination Reagent (USB, code 30098)

Reconstitution of dye and protein labelling

• 99.8% anhydrous dimethylformamide (DMF)	Must be less than 3 months old from day of opening (Aldrich 22,705-6)
• Tris-(2-carboxyethyl) phosphine hydrochloride (TCEP)	(Molecular Probes, T-2556)
• 1x sample buffer (DTT/Pharmalyte-free):	7 M Urea, 2 M Thiourea, 4% (w/v) CHAPS. Dispense in aliquots and store at -15 °C to -30 °C. Stable for 6 months.
• 2x sample buffer:	7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 2% (v/v) Pharmalyte™, broad range pH 3-10, 130 mM DTT. Prepare fresh by adding DTT (from a fresh 1 mg/µl DTT stock solution) and Pharmalytes to 1× sample buffer. Use immediately and discard any unused material.

Isoelectric focusing

- Rehydration buffer: 7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 1% (v/v) Pharmalyte, broad range pH 3-10, 13 mM DTT. Prepare fresh by adding DTT (from a fresh 1 mg/µl DTT stock solution) and Pharmalytes to 1× sample buffer. Use immediately and discard any unused material.
- Immobiline™ DryStrip immobilized pH gradient strips
- Immobiline DryStrip Reswelling Tray
- Ettan™ IPGphor™ Cup Loading Strip Holders
- PlusOne™ Immobiline DryStrip Cover Fluid
- Ettan IPGphor IEF system
- IEF Electrode Strips

SDS-PAGE separation

- Equilibration tubes or 9 cm diameter petri dishes
- Equilibration buffer: 6 M urea, 0.1 M Tris, pH 8.0, 30% (v/v) glycerol, 2% (w/v) SDS, 0.5% (w/v) DTT. A DTT-free stock can be prepared and is stable at room temperature for 6 months. DTT should be added immediately prior to use and any unused material discarded.
- 12.5% acrylamide gel (for Ettan DALT): 281 ml acrylamide/bis 40% (v/v), 225 ml Tris (1.5 M pH 8.8), 9 ml 10% (w/v) SDS, 9 ml 10% (w/v) ammonium persulfate (freshly prepared on day of use), 1.24 ml 10% (v/v) TEMED. Make up to 900 ml with distilled water.
  900 ml is sufficient solution to prepare a complete

• Low fluorescence glass plates	set of 14 Ettan DALT gels.
• Ettan DALT gel caster	
• Displacement solution:	375 mM Tris (pH 8.8), 50% (v/v) glycerol, bromophenol blue (2 mg/100 ml). Prepare fresh and use immediately. Do not store.
• Agarose overlay solution:	0.5% LMP agarose prep, 0.1% (w/v) bromophenol blue in 1x SDS electrophoresis running buffer (see below). Stable for 1 month at room temperature.
• Water saturated butanol:	Add 50 ml water to 50 ml butan-2-ol until two layers are visible. Stable for 6 months at room temperature.
• 1x SDS electrophoresis running buffer:	25 mM Tris, 192 mM Glycine, 0.2 % (w/v) SDS. Store at room temperature.
• Suitable electrophoresis system:	Hoefer™ SE 600 Ruby gel system, Ettan DALT*twelve* gel system, Ettan DALT*six* gel system or equivalent electrophoresis system.
• Bind Silane solution:	100 µl PlusOne Bind-Silane (code 17-1330-01) added to 80 ml ethanol, 2 ml glacial acetic acid and 18 ml water.

Imaging and Image analysis

- Typhoon™ 9000 series Variable Mode Imager
- Ettan DALT Gel Alignment Guides
- Ettan DALT Gel Orientation Guide
- DeCyder™ Differential Analysis Software

Introduction

Description

2–Dimensional Fluorescence Difference Gel Electrophoresis (2–D DIGE) is a method for pre-labelling protein samples prior to 2–D electrophoresis for difference analysis (1) that enables multiplexing within the same 2–D gel.

The protocol described here is designed to work using CyDye DIGE Fluor saturation dyes that have been developed specifically for use with scarce protein samples. CyDye DIGE Fluor saturation dyes enable a full 2–D analysis of samples which under normal circumstances may be more challenging due to limiting sample quantities.

The technology is based upon the specific properties of CyDye DIGE Fluor Cy3 and Cy5 saturation dyes. The dyes are spectrally resolvable so they can be detected independently using dye-specific imaging parameters. CyDye DIGE Fluor Cy3 and Cy5 saturation dyes are also migration-matched so identical proteins labelled with each of the two CyDye DIGE Fluor saturation dyes will migrate to the same position on a 2–D gel. These combined properties allow two different protein samples to be labelled, one with each dye, separated on the same gel and co-detected. The ability to multiplex permits the inclusion of both sample and internal standard (internal reference) in every gel. The use of an internal standard within each gel, helps to limit system variation, which ultimately provides more accurate quantitation of relative protein abundance.

Ettan DIGE system has capitalized on the ability to multiplex by combining CyDye DIGE Fluor saturation dyes with DeCyder Differential Analysis Software. This software has been designed specifically for 2–D DIGE applications and utilizes a proprietary co-detection algorithm that permits automatic detection, background subtraction, quantitation, normalization, and inter-gel matching of

multiplexed fluorescent images. The major benefit of this approach is the ability to produce quantitative data of unparalleled accuracy, supported by statistical analysis. The use of an in-gel standard increases confidence that the results reflect true induced biological changes (i.e. due to a disease state or drug treatment) and are not due to system variation.

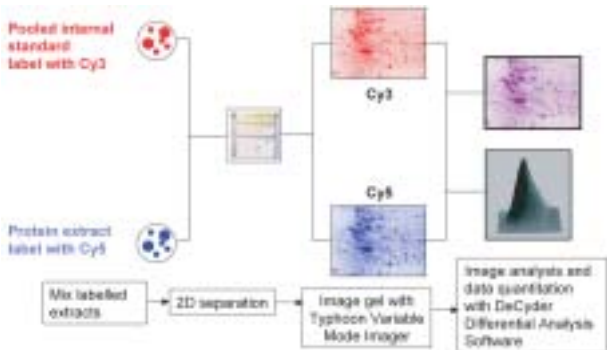


Figure 1. Outline of the basic Ettan DIGE system for saturation labelling

The system comprises CyDye DIGE Fluor saturation dyes for protein labelling; a choice of Ettan IPGphor Isoelectric Focusing System or Multiphor™ II IEF System for first-dimension separation; Hoefer SE 600 Ruby, Ettan DALT*twelve*, or Ettan DALT*six* vertical electrophoresis systems for second-dimension separation; Typhoon 9000 series Variable Mode Imager for advanced imaging; and DeCyder Differential Analysis Software for quantitation and statistical analysis of protein differences.

More detailed information and protocols for working with Ettan DIGE system can be found in the Ettan DIGE System User Manual (code 18-1173-17). This manual is also available on the Amersham Biosciences website (www.amershambiosciences.com/DIGE).

Dye characteristics

CyDye DIGE Fluor Cy3 saturation dye

Molecular formula	$C_{37}H_{44}N_4O_6S$
Formula weight	672.85
Absorption max (in DMF)	548 ± 3 nm
Emission max (in DMF)	560 ± 5 nm

Structure confirmed by NMR

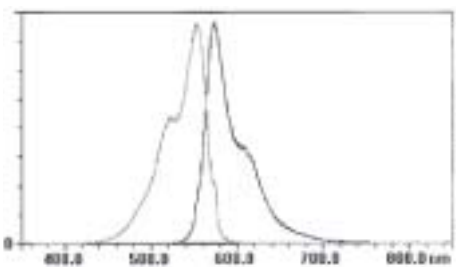


Figure 2. Absorption and emission spectra for CyDye DIGE Fluor Cy3 saturation dye in DMF

CyDye DIGE Fluor Cy5 saturation dye

Molecular formula	$C_{38}H_{44}N_4O_6S$
Formula weight	684.86
Absorption max (in DMF)	641 ± 3 nm
Emission max (in DMF)	660 ± 5 nm

Structure confirmed by NMR

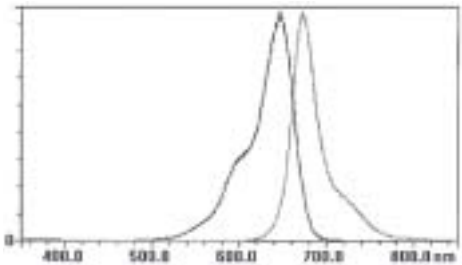


Figure 3. Absorption and emission spectra for CyDye DIGE Fluor Cy5 saturation dye in DMF

Labelling with CyDye DIGE Fluor saturation dyes

Two dyes are available for saturation labelling, CyDye DIGE Fluor, Cy3 and Cy5 saturation dyes. 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 on proteins via a thioether linkage. To achieve maximal labelling of cysteine residues, a high dye-to-protein labelling ratio is required. This type of labelling method aims to label all available cysteines on each protein under the conditions used, resulting in the majority of protein in a sample being labelled. For this reason, this method has been called "saturation" labelling

The dyes offer great sensitivity with detection over 5 orders of magnitude. Narrow excitation and emission bands mean that the dyes are spectrally distinct, which makes them ideal for multicolor detection. Most importantly, the dyes are migration-matched so that the same protein labelled with either of the CyDye DIGE Fluor saturation dyes

will migrate to the same position within a 2–D gel. The novel properties of the CyDye DIGE Fluor saturation dyes make them ideal for multiplexing different protein samples within the same 2–D gel. This permits inclusion of an internal standard within each gel which limits experimental variation and ensures accurate inter-gel matching.

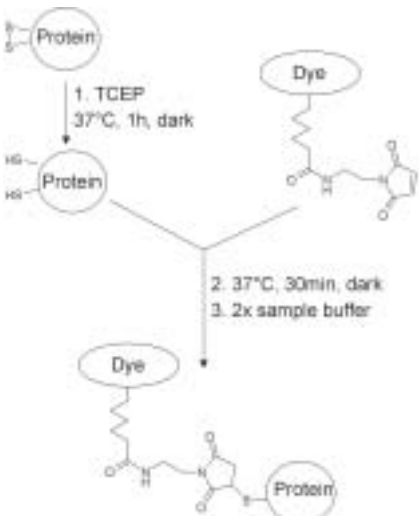


Figure 4. Schematic of labelling reaction between CyDye DIGE Fluor saturation dye and the cysteine residue of a protein.

Many thiol groups on the cysteine residues in proteins exist as disulphide bonds. In order to label these groups the protein must be unfolded and the disulphide bonds broken. This can be achieved under

denaturing conditions with a reducing agent such as tris-(2-carboxyethyl) phosphine hydrochloride (TCEP) and by increasing the temperature of labelling. In some proteins, cysteine residues are buried within the protein such that they cannot be reduced and are not available for labelling. Thus the extent of labelling of cysteine residues will depend on the accessibility of cysteines within the protein under the reaction conditions used.

The cysteine amino acid in proteins has neutral charge at neutral or acidic pH. CyDye DIGE Fluor saturation dyes are net neutral, ensuring that the pI of the protein does not significantly alter on labelling. The extent of the mass shift of a labelled protein depends on the cysteine content of the protein and the accessibility of the cysteine residues to dye in the labelling reaction.

Spot picking

Samples prepared using the saturation labelling approach can be picked directly from a preparative gel. This eliminates the need for post-staining.

Protein identification

Labelling of proteins with CyDye DIGE Fluor saturation dyes does not affect identification by mass spectrometry. Labelling on cysteine residues does not reduce the efficiency or specificity of enzymatic digestion. Cysteine-labelled proteins generate equivalent levels of peptide mass fingerprint (PMF) and sequence data to unlabelled proteins.

Ettan DIGE system workflow

The main steps in the Ettan DIGE system workflow for saturation labelling are outlined below.

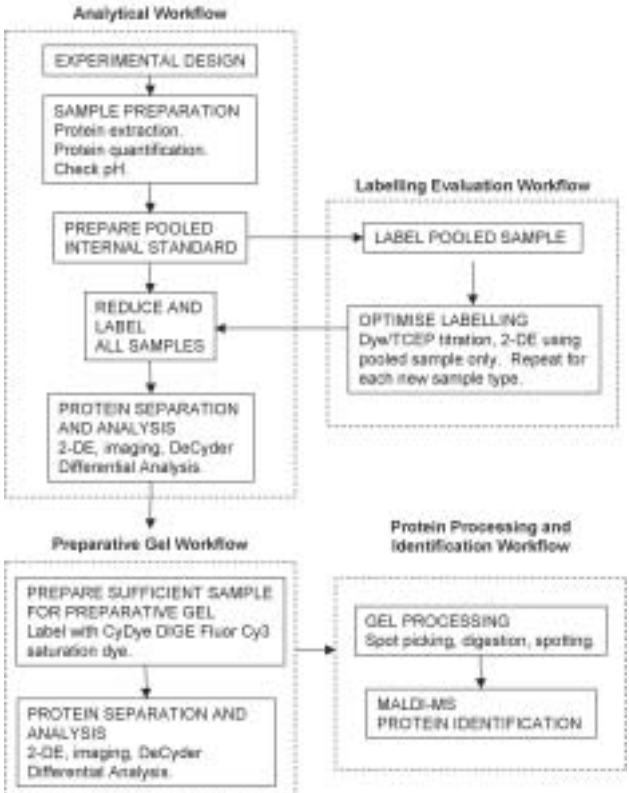


Figure 5. Ettan DIGE system workflow for saturation labelling

Measurement of variation

2–D electrophoresis experiments experience variation that arises from two main sources.

System variation may arise from two areas.
Firstly, gel-to-gel variation can result from differences in IEF and electrophoretic running conditions between different gels, gel distortions and user-to-user variation. The second source of system variation is due to user-specific editing and interpretation when using data analysis software.

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

If **induced biological changes**, (the differences that are caused by a disease state/drug treatment/life-cycle stage etc.) are to be identified, it is important to be able to differentiate them from both system variation and inherent biological variation.

System variation cannot be overcome when using conventional "one sample per gel" 2-D electrophoresis. Ettan DIGE system controls system variation by the inclusion of an internal standard within each gel, enabled by the multiplexing capability of 2-D DIGE methodology. Software-originated variation is minimized using DeCyder Differential Analysis Software. This provides automated co-detection, background subtraction, quantitation, normalization and inter-gel matching, which limits user intervention and subjective editing, generating consistent data.

To account for inherent biological variation, it is strongly advised that

biological replicates, such as multiple cultures, should be incorporated into the experimental design. The more biological replicates included in the experiment, the greater the chance that inherent biological variation will be taken into account, enabling a reliable measure of induced biological change.

Since Ettan DIGE system variation is low by virtue of the inclusion of an internal standard and the analysis method, biological variation will far exceed the system variation. As a consequence, gel replicates are no longer necessary.

Experimental design

To maximize the benefits of Ettan DIGE system, an internal standard should be incorporated within each gel. The ideal internal standard should comprise an aliquot from each biological sample within the experiment. Thus, the internal standard is a pooled sample created from all of the experimental samples. The internal standard is labelled with one CyDye DIGE Fluor saturation dye (Cy3) and is run on every gel together with experimental samples labelled with the other CyDye DIGE Fluor saturation dye (Cy5) (see Table 1). This ensures that every spot on every gel is represented within the common internal standard. Using DeCyder Differential Analysis Software each protein spot in a sample can be compared to its representative within the internal standard to generate a ratio of relative abundance (Fig 6).

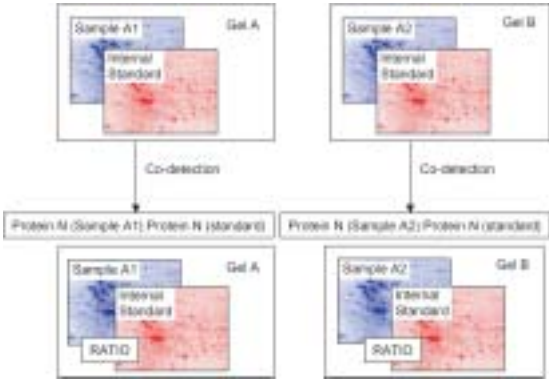


Figure 6. Quantitation of protein abundance using co-detection algorithms in DeCyder Differential Analysis Software. From each gel, two scan images are generated, Cy3 (red) for the internal standard, and Cy5 (blue) for test samples. The protein abundance of each spot in each sample is expressed as a normalized ratio relative to spots from the in-gel internal standard

The same internal standard is run on all gels within the experimental series. Matching of these internal standards creates an intrinsic link between the samples on each of the different gels (Fig. 7). Quantitative comparisons of samples between gels are made based on the relative change of sample to its in-gel internal standard. This removes inter-gel (system) variation, a common problem associated with traditional 2–D electrophoresis studies, enabling accurate, statistical quantitation of induced biological change between samples. For 2-D electrophoresis, Ettan DIGE system is the only protein difference analysis technique that utilizes this approach (2).

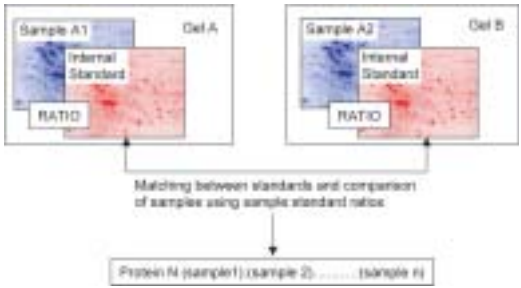


Figure 7. Matching and comparison of samples across gels. In DeCyder Differential Analysis Software the internal standard sample, present on every gel, is used to aid matching of spot patterns across all gels. The relative ratios of individual sample spots to their internal standards is used to accurately compare protein abundance between samples on different gels.

Linking every sample in-gel to a common standard offers many advantages:

- accurate quantitation and spot statistics for changes in protein abundance;
- increased confidence in matching between gels;
- flexibility of statistical analysis depending on the relationship between samples;
- separation of induced biological variation from system variation.

For a more detailed guide to the benefits of using an internal standard, see the Ettan DIGE System User Manual (code 18-1173-17. Also available on the Amersham Biosciences website, www.amershambiosciences.com/DIGE).

Table 1 shows an example of a recommended experimental set-up designed to derive statistical data on differences between control and two treatment regimens A and B. For the control and two treatment regimens, three biological replicates are included (1-3). The internal standard (a pool of equal amounts from all samples: three control and six treated) is labelled with CyDye DIGE Fluor Cy3 saturation dye and run on every gel. Care should be taken to ensure that there is sufficient sample to allow for preparation of the internal standard.

Each control and treated test sample is labelled with CyDye DIGE Fluor Cy5 saturation dye and loaded on gels as indicated below.

Gel	Cy3	Cy5
1	Pooled standard	Control 1
2	Pooled standard	Control 2
3	Pooled standard	Control 3
4	Pooled standard	Sample A1
5	Pooled standard	Sample A2
6	Pooled standard	Sample A3
7	Pooled standard	Sample B1
8	Pooled standard	Sample B2
9	Pooled standard	Sample B3

Table 1. Recommended experimental design for a 2–D DIGE saturation labelling experiment, incorporating an internal standard. Each gel contains a CyDye DIGE Fluor Cy3 saturation labelled standard which is a pool of aliquots taken from each sample. Three biological replicates (1–3) have been included for control and treated (A and B) samples which are each labelled with a CyDye DIGE Fluor Cy5 saturation dye. For further information relating to experimental design, please refer to the Ettan DIGE System User Manual (code 18-1173-17).

It is strongly advised that biological replicates are included in every experimental group. This will enable accurate measurement of the change due to a treatment/disease that is significant above a baseline of inherent biological variation. The more biological replicates, the more inherent biological variation is accounted for and therefore, the more meaningful the results. Without biological replicates, results are not biologically relevant and it is only possible to conclude that differences are above system variation. Ettan DIGE system variation is so low due to the internal standard and method of analysis, that gel replicates are not needed - any system variation should be far outweighed by the inherent biological variation. Gel replicates can be included if the user wishes.

Protocol

For users familiar with CyDye DIGE Fluor minimal labelling, please note the following key differences between minimal labelling and saturation labelling experiments before starting.

	Saturation labelling	Minimal labelling
Sample preparation	Cell lysis buffer is at pH 8.0. (For a complete recipe see page 5, "Other materials required").	Cell lysis buffer is at pH 8.5.
Dyes	Maleimide dyes. Label cysteine residues. 2 dyes available.	NHS ester dyes. Label lysine residues. 3 dyes available.
	CyDye DIGE Fluor saturation dyes are reconstituted at 2 mM (analytical gels) or 20 mM (preparative gels) Once reconstituted, the dyes are stable for up to 8 weeks at -15 °C to -30 °C.	Once reconstituted, the concentrated stock (1 mM) of CyDye DIGE Fluor minimal dyes is stable for up to 2 months at -15 °C to -30 °C.
	Once reconstituted, dyes do not need to be diluted further.	The working concentration of the dyes is 0.4 mM and is stable for 1 week.
Protein labelling	Proteins must be reduced using TCEP prior to labelling.	No reduction step required.

	Saturation labelling	Minimal labelling
Protein labelling	Labelling reaction performed at 37 °C.	Labelling reaction performed at 4 °C.
	Labelling reaction quenched using 2x sample buffer.	Labelling reaction quenched with 10 mM lysine.
	Labelling is optimized by titrating TCEP and dye (Cy3 and Cy5) then analyzing on a 2–D gel.	Labelling is optimized by comparing labelled samples on a 1-D gel.
	Labelled proteins are stable for 1 month at -70 °C.	Labelled proteins have stability equivalent to unlabelled protein at -70 °C.
Protein separation and analysis	No iodoacetamide equilibration step prior to 2–DE.	Iodoacetamide equilibration step required.
	A Cy3 labelled sample is used to prepare a preparative gel for spot picking. No staining is required.	An unlabelled sample is used to prepare a preparative gel for spot picking. The gel must be stained using a fluorescent post-stain to allow matching to analytical gels for picking.

Table 2. Key differences between minimal labelling and saturation labelling experiments

❶ Introduction

This protocol provides all the information required for the use of CyDye DIGE Fluor saturation dyes to label proteins prior to 2–D electrophoresis. It is recommended that the protocol is read thoroughly before using the system and that it is followed precisely.

For recommended materials and recipes required for saturation labelling and 2–D electrophoresis, please refer to page 5, "Other materials required".

In the standard labelling protocol, proteins are first solubilized in a cell lysis buffer. The protein concentration should then be determined using a standard protein quantitation method. Cysteine residues in the extracted proteins are reduced by incubating with TCEP, at 37 °C for 1 h. CyDye DIGE Fluor saturation dye is added to the protein lysate and the reaction incubated at 37 °C in the dark for a further 30 minutes. Finally, the reaction is quenched by the addition of 2x sample buffer.

Plastic tubes should be used when handling samples, as many proteins will adhere to glassware. The fluorescent properties of CyDye DIGE Fluor Cy3 and Cy5 saturation dyes can be adversely affected by exposure to light, so it is recommended that the exposure of dye or labelled protein to all light sources is kept to a minimum.

❷ Preparation of a cell lysate compatible with saturation labelling

Samples should be lysed in the recommended cell lysis buffer (refer to page 5, "Other materials required"). Care should be taken to exclude compounds that may interfere with labelling. These include primary amines (e.g. Pharmalytes or ampholytes) or thiols (e.g. DTT) which will compete with the protein for dye.

The concentration of the lysate before labelling should be between 0.55-10 mg/ml for running analytical gels or 1.2 -10 mg/ml for running preparative gels (concentrations >1.2 mg/ml may be required for lysates using high levels of TCEP and dye).

After lysis, the pH of the sample should be measured to check that it has not deviated from pH 8.0 (refer to page 58, "Adjustment of protein sample pH").

Extraction protocol suitable for most tissue samples.

The extraction method given below is a general method suitable for most tissue samples. Recommendations for extraction of different cell and tissue types are listed on pages 58–62.

1. Wash the tissue in 0.9% saline solution
2. Add a small volume of cell lysis buffer (30 mM Tris, 7 M Urea, 2 M Thiourea, 4% (w/v) CHAPS, pH 8.0).

Note: if the protein concentration is less than 0.55 mg/ml (for analytical samples) or 1.2 mg/ml (for preparative samples) after protein quantitation, resuspend cells in a correspondingly smaller volume of cell lysis buffer in subsequent experiments.

3. Mechanically homogenize the sample.
4. Keep the sample on ice and sonicate intermittently until the sample is

lysed. This may be performed using the sample held in a small vessel within a water bath sonicator, for extraction of samples for analytical gels. For larger amounts of sample (e.g. for preparative gels) use a probe sonicator, see page 56, “Protein lysate sonication”.

Note: the cell suspension must be kept cool at all times.

5. Pellet the tissue in a microcentrifuge at 12 000 x g for 10 min at 4 °C.

6. Transfer supernatant to a labelled tube. This is the cell lysate to be used for dye labelling. Discard the pellet. Check that the pH of the cell lysate is still at pH 8.0 by spotting 1 µl on a pH indicator strip. If the pH of the cell lysate has fallen below pH 8.0 then the pH of the lysate will need to be adjusted before labelling. See page 58, “Adjustment of protein sample pH”.

Store cell lysates in aliquots at -70 °C until protein concentration is to be determined.

Note: for determination of protein concentration, a detergent compatible assay is recommended. We recommend Protein Determination Reagent (USB, code 30098).

Extraction protocol suitable for laser capture microdissected (LCM) samples.

The extraction method given below was used to prepare a laser-capture micro-dissected mouse hippocampus lysate.

1. Capture the section(s) directly into a small volume of cell lysis buffer (30 mM Tris, 7 M Urea, 2 M Thiourea, 4% (w/v) CHAPS, 5 mM magnesium acetate, pH 8.0). The section may be still attached to the mount.

Note: if the protein concentration is less than 0.55 mg/ml (for

analytical samples) or 1.2 mg/ml (for preparative samples) after protein quantitation, resuspend cells in a correspondingly smaller volume of cell lysis buffer in subsequent experiments.

2. Keep the sample on ice and sonicate intermittently until the sample is lysed. This may be performed using the sample held in a small vessel within a water bath sonicator, for extraction of samples for analytical gels. For larger amounts of sample (e.g. for preparative gels) use a probe sonicator, see page 56, “Protein lysate sonication”.

Note: the cell suspension must be kept cool at all times.

3. Pellet the tissue in a microcentrifuge at 9 000 x g for 30 sec at

4 °C.

4. Transfer supernatant to a labelled tube. This is the cell lysate to be used for dye labelling. Discard the pellet. Check that the pH of the cell lysate is still at pH 8.0 by spotting 1 µl on a pH indicator strip. If the pH of the cell lysate has fallen below pH 8.0 then the pH of the lysate will need to be adjusted before labelling. See page 58, “Adjustment of protein sample pH”.

Store cell lysates in aliquots at -70 °C until protein concentration is to be determined.

Note: for determination of protein concentration, a detergent compatible assay is recommended. We recommend Protein Determination Reagent (USB, code 30098).

❸ Determining the optimum amount of TCEP/dye required to label a protein lysate

The amount of TCEP and CyDye DIGE Fluor saturation dye used in the labelling reaction needs to be determined individually for each protein sample type being analysed or when a non-standard cell lysis buffer is being used. A labelling optimization experiment should always be performed when:

- a new sample type is being used;
- the cell lysis buffer contains a reagent which hasn't been tested for compatibility with CyDye DIGE Fluor saturation labelling (see pages 63–65);
- the cell lysis buffer contains a reagent which has been tested for compatibility with CyDye DIGE Fluor saturation labelling, but is being used in a range known to affect labelling efficiency or is being used outside the recommended concentration range;
- the cell lysis buffer contains a combination of reagents that may or may not have been tested for compatibility with CyDye DIGE Fluor saturation labelling. The effect of different reagents on labelling efficiency is additive and may lead to unexpectedly poor labelling when one or more interfering reagents are used together.

The molar ratio of TCEP:dye should always be kept at 1:2 to ensure efficient labelling. Samples with higher cysteine content will require more TCEP to reduce the disulphide bonds and more dye to label the thiol groups. Typically, 5 µg of protein lysate requires 2 nmol TCEP and 4 nmol dye for the labelling reaction (assuming an average cysteine content of 2%). Mammalian samples, with a higher glutathione content (e.g. liver tissue) may require more TCEP (e.g. 3 nmol) for the reduction step and therefore require more dye (e.g. 6 nmol).

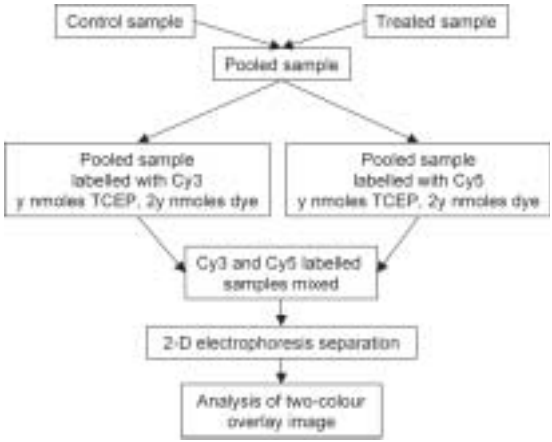


Figure 8. Scheme showing the workflow for labelling optimization. This should be repeated for 6 different TCEP/dye concentrations (see Table 3).

To determine the optimum amount of TCEP and dye required for the protein extract being used, a simple titration should be performed for each dye before proceeding with any analytical experiments (Figure 8). The protocol described below should be followed using the pooled protein extract.

To prepare a pooled protein extract, mix equal amounts of each experimental sample together. This extract is labelled using different amounts of TCEP and CyDye DIGE Fluor Cy3 or Cy5 saturation dye, as shown in Table 3. Cy3 or Cy5 labelled samples for the same TCEP:dye concentration should be run on the same gel so a total of 6 gels are required.

Note: The molar ratio of TCEP:dye should always be kept at 1:2 to ensure efficient labelling.

Gel	2 mM TCEP (µl)	TCEP (nmol)	2 mM Dye (µl)	Dye (nmol)
1	0.5	1	1	2
2	0.75	1.5	1.5	3
3	1	2	2	4
4	1.25	2.5	2.5	5
5	1.5	3	3	6
6	2	4	4	8

Table 3. Recommended amounts of TCEP and dye required for labelling optimization, prior to analytical experiments. Amounts shown in this table are for optimizing the labelling of 5 µg protein. Typical TCEP/dye quantities required for 5 µg of protein are highlighted. The recommended times for reduction and labelling reactions allow different volumes of TCEP/dye to be used without any adverse effects on reduction/labelling kinetics.

For each gel, create a red/blue Cy3/Cy5 image overlay e.g. using ImageQuant™ or Paint Shop Pro™ (a product of Jasc Software). Compare gel overlays along the titration series to decide which gel gives the best labelling results.

The criteria for optimal labelling conditions are:

- all spots overlaid;
- no significant mass trains or vertical streaks;
- no significant charge trains or horizontal streaks.

If the amount of TCEP/dye is too low available thiol groups on some proteins will not be labelled. When the maleimide dye labels a thiol group, the mass of the protein is increased but the charge is unaffected.

Thus, under-labelled samples will show MW trains and/or streaking in the vertical direction (see Figure 9b). Differential migration of Cy3 and Cy5 labelled spots for the same protein can also occur when the amount of TCEP/dye is too low.

If the amount of TCEP/dye is too high non-specific labelling of the amine groups on lysine residues can occur. When the maleimide dye labels a lysine group, the mass of the protein is increased and the charge is also reduced by 1. Thus, over-labelled samples will show pI charge trains and/or streaking in the horizontal direction.

When separating by SDS-PAGE, the migration of proteins in the range 20-30 kDa is particularly sensitive to the fine structure of the dyes attached to labelled proteins. This effect may result in a small number of spots (typically less than 1% of all spots on a gel) which, although labelled to the same extent, do not overlay on the dual-color image. These proteins should be identified during the labelling optimization experiments and spots merged when using DeCyder Differential Analysis Software version 5.0, prior to performing statistical analysis on analytical gels.

Note: If the overlays are visualized using the DeCyder DIA software, some proteins will exhibit differential detection between the two dyes. It is possible that this may be due to Cy5 quenching effects with some highly labelled proteins (3). Use of the recommended experimental design incorporating an internal standard (see Table 1) will compensate for this phenomenon.

The method of protocol optimization described above, for the determination of dye/TCEP levels can also be used to test the effect (or optimize the concentration) of additional components the user may wish to add to the cell lysis buffer.

Figures 9a-d show gel images taken from a labelling optimization experiment using rat liver, spiked with glutathione. The gels show characteristic changes in the spot pattern when progressing from non-optimal to optimal labelling conditions.

- Gel (b) shows vertical streaking, a characteristic typical of underlabelling.
- The proteins highlighted in boxes show multiple spots for lower TCEP/dye levels, each moving to a single spot using the optimum labelling conditions, gel (d). This phenomenon is characteristic of proteins which are incompletely labelled at lower TCEP/dye levels but become fully saturated when optimum conditions are reached.
- In this example, the optimal labelling conditions are 5 µg protein: 4 nmol TCEP:8 nmol dye.

This example illustrates that samples containing glutathione may require higher levels of TCEP and dye for optimal labelling to be achieved.

Once the optimal labelling conditions have been established for a particular sample, only those conditions should be used for further work. If the sample type or sample preparation method changes the labelling conditions will require re-optimization.

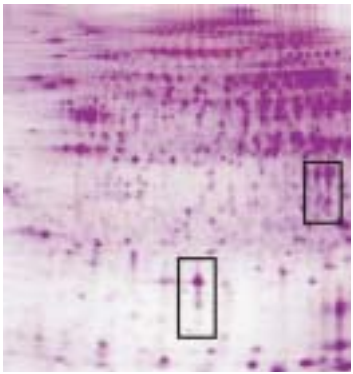


Figure 9a. Control rat liver (no glutathione) labelled with 2 nmol TCEP and 4 nmol dye. Protein spots that were markedly changed when glutathione was added to this sample (see below) are boxed.

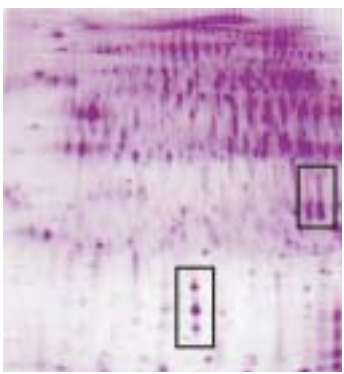


Figure 9b. Rat liver (spiked with 3 nmol glutathione) labelled with 2 nmol TCEP and 4 nmol dye. The image shows vertical streaking as a consequence of underlabelling

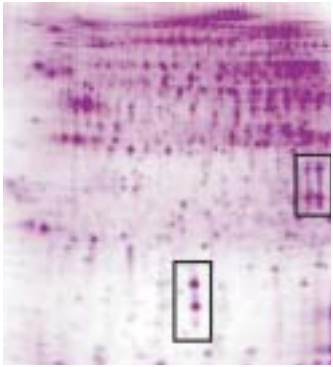


Figure 9c. Rat liver (spiked with 3 nmol glutathione) labelled with 3 nmol TCEP and 6 nmol dye. This image shows a reduction in vertical streaking and increase in mass of some underlabelled proteins, as labelling becomes more optimal.

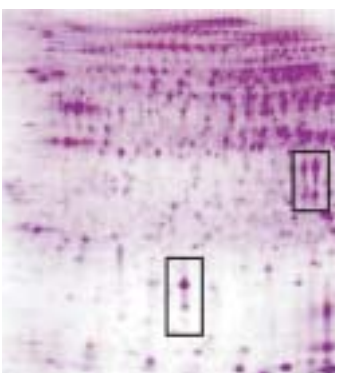


Figure 9d. Rat liver (spiked with 3 nmol glutathione) labelled with 4 nmol TCEP and 8 nmol dye. This image shows optimal labelling comparable to the glutathione-free liver sample shown in Figure 9a.

❹ Reconstitution of CyDye DIGE Fluor saturation dyes in dimethylformamide (DMF)

Each vial of CyDye DIGE Fluor saturation dye powder must be reconstituted in high quality anhydrous DMF (specification: ≤ 0.005% H_2O, ≥ 99.8% pure) open for less than 3 months. On reconstitution in DMF the CyDye DIGE Fluor will give a deep color; Cy3-red, Cy5-blue.

The quality of the DMF used in all experiments is critical to ensure that protein labelling is successful. The DMF must be anhydrous and every effort should be taken to ensure it is not contaminated with water. DMF after opening, over a period of time, will degrade with amine compounds being produced. Amines will react with the maleimide dye, reducing the concentration available for protein labelling. If in doubt use an unopened batch of DMF for reconstituting the dye.

For analytical labelling reactions, the working dye solution should be at a concentration of 2 mM. The volume of reconstituted dye added depends on the amount of dye required, as determined in the labelling optimization experiments.

For preparative labelling reactions, the working dye solution should be at a concentration of 20 mM. The volume of reconstituted dye added depends on the amount of dye required, as determined in the labelling optimization experiments.

1. Take a small volume of DMF from its original container and dispense into a fresh microfuge tube.

2. Remove the CyDye DIGE Fluor saturation dye from the -15 °C to -30 °C freezer and leave unopened for 5 min, to warm to room temperature.

3. Once at ambient temperature, add the required volume of DMF to each new vial of CyDye DIGE Fluor saturation dye.

For 5 µg analytical labelling reactions, reconstitute 100 nmol dye in 50 µl DMF to give a 2 mM working dye solution.

For preparative labelling reactions, reconstitute 400 nmol dye in 20 µl DMF to give a 20 mM working dye solution.

4. Replace the cap on the dye microfuge tube and vortex vigorously for 30 seconds.

5. Centrifuge for 30 seconds at 12 000 × g in a microcentrifuge.

6. The dye can now be used. Check that the dye solution is an intense color. During transport, the dye powder may spread around the inside surface of the tube (including the lid). If the dye is not an intense color, then pipette the solution around the tube (and lid) to ensure complete resuspension of dye. Vortex and spin down.

Note: Unused dye stock solution should be returned to the -15 °C to -30 °C freezer as soon as possible and stored in the dark.

After reconstitution CyDye DIGE Fluor saturation dyes are stable and useable until the expiry date detailed on the tube, or for 8 weeks, whichever is sooner.

❺ Saturation labelling of a protein sample

The minimum requirement for protein concentration in the lysate is DIFFERENT for analytical and preparative labelling reactions. Protein lysates used for analytical labelling reactions must contain >0.55 mg/ml protein to ensure the final volume for cup-loading does not exceed 100 µl. The recommended concentration of protein lysates used for preparative labelling is >1.2 mg/ml, when using 24 cm Immobiline DryStrips. This ensures that the volume used to rehydrate the strip does not exceed the maximum recommended rehydration volume (Table 4). Smaller strips require lower rehydration volumes and will therefore require a higher starting concentration of protein lysate. Higher protein concentrations may be required for preparative gels if large amounts of TCEP and dye are used.

Immobiline DryStrip length (cm)	Total volume per strip (µl)
7	125
11	200
13	250
18	350
24	450

Table 4. Rehydration volumes of Immobiline DryStrips

Check that the pH of the cell lysate is still at pH 8.0 by spotting 1 µl onto a pH indicator strip. If the pH of the cell lysate has deviated from pH 8.0 then the pH of the lysate will need to be adjusted before labelling (refer to page 58, "Adjustment of protein sample pH").

Prepare a pooled internal standard by mixing equal amounts of each experimental protein sample together, ensuring that there is enough of

the resulting pooled standard sample to include on each gel within the experiment.

The labelling protocol required will depend on whether samples are being labelled for analytical or preparative gels. Both methods are given below. If you wish to scale up analytical or preparative reactions for bulk labellings, remember to increase the amount of TCEP and dye accordingly. Do this by adjusting the volume added (not the reagent concentration) to reflect the amount of protein, maintaining the same ratio of protein:TCEP:dye.

Labelling samples for analytical gels

For analytical labelling reactions, the dye and TCEP should both be used at a concentration of 2 mM. The volume added for each component depends on the amount of TCEP and dye required, as determined in the labelling optimization experiment.

1. Add a volume of protein lysate equivalent to 5 µg protein to a sterile microfuge tube.

2. Make up the volume to 9 µl with cell lysis buffer.

3. Prepare 2 mM TCEP solution by dissolving 2.8 mg TCEP in 5 ml water. TCEP solution is unstable and should be used immediately. Discard any unused material.

4. Add the required volume of 2 mM TCEP appropriate for 5 µg protein (as determined in the labelling optimization experiment, see page 27). For example add 1 µl TCEP (2 nmol).

5. Mix vigorously by pipetting.

6. Spin down the sample in a microcentrifuge and incubate at 37 °C for 1 h, in the dark.

7. Add the required volume of resuspended 2 mM CyDye DIGE Fluor saturation dye appropriate for 5 µg protein (as determined in the

labelling optimization experiment, see page 27). For example add 2 µl dye (4 nmol).

Label the pooled protein extract with CyDye DIGE Fluor Cy3 saturation dye.

Label experimental protein extracts (e.g. control, treated) with CyDye DIGE Fluor Cy5 saturation dye.

8. Mix vigorously by pipetting.

9. Spin down the sample in a microcentrifuge and incubate at 37 °C for 30 min, in the dark.

10. Whilst the sample is incubating, prepare 2x sample buffer by adding Pharmalytes (2% final) and DTT (130 mM final) to 1x sample buffer (7 M Urea, 2 M Thiourea, 4% CHAPS).

11. To stop the reaction, calculate the total volume of the labelling reaction and add an equal volume of 2x sample buffer.

12. Mix vigorously by pipetting.

13. Spin down the sample in a microcentrifuge.

14. Samples are ready for use and can be stored on ice or frozen for up to one month, at -70 °C, in the dark.

Note: Protein lysates are viscous so failure to mix thoroughly at steps 5, 8 and 12 can cause non-uniform labelling. This can result in poor spot overlays due to pI differences and mass shifts between Cy3 and Cy5 labelled protein spots. Vortexing is not recommended as mixing is not adequate using this technique.

Labelling samples for preparative gels

For preparative gels, it is recommended that 500 µg of the pooled internal standard sample is labelled using CyDye DIGE Fluor Cy3 saturation dye. For applications where specific material is very scarce (e.g. sections of brain prepared by laser capture microdissection),

material from surrounding tissue can be used for the preparative gel. This approach assumes that the protein profile will be similar for both localized and surrounding tissues.

For analytical labelling reactions in a small volume, Pharmalytes and DTT are added as part of the 2x sample buffer. However, for preparative labelling, a larger volume of protein is required and in-gel rehydration sample loading must be used. The maximum volume that can be loaded by in-gel rehydration is 450 µl (for 24 cm strips). This means that 1x sample buffer, Pharmalytes and DTT must be added separately.

For preparative labelling reactions, TCEP is added in a constant volume of 10 µl and the dye is added at a constant concentration of 20 mM. The concentration of TCEP and volume of dye used depend on the amount of TCEP and dye required, as determined in the labelling optimization experiments and should be scaled up accordingly.

1. Add a volume of protein lysate (preferably the pooled internal standard for a preparative gel) appropriate for 500 µg protein to a sterile microfuge tube. For example, add 250 µl for a 2 mg/ml protein lysate. If the volume of lysate is below 250 µl, then make up the volume to 250 µl with lysis buffer before labelling.

2. Prepare TCEP solution at the required concentration appropriate for 500 µg protein (as determined in the labelling optimization experiment, see page 27).

For example, dissolve 2.8 mg TCEP in 500 µl water to give a

20 mM solution. TCEP solution is unstable and should be used immediately. Discard any unused material.

3. Add 10 µl of TCEP to the protein lysate. For example add 10 µl TCEP at 20 mM (200 nmol).

4. Mix vigorously by pipetting.

5. Spin down the sample in a microcentrifuge and incubate at 37 °C for 1 h, in the dark.

6. Add the required volume appropriate for 500 µg protein of 20 mM CyDye DIGE Fluor Cy3 saturation dye (as determined in the labelling optimization experiment, see page 27).
For example add 20 µl dye at 20 mM (400 nmol).

7. Mix vigorously by pipetting.

8. Spin down the sample in a microcentrifuge and incubate at

37 °C for 30 min, in the dark.

9. To stop the reaction, add a volume of 1x sample buffer (DTT/Pharmalyte-free) to take the total volume up to 445.5 µl. For example if your unlabelled protein started at a concentration of

2 mg/ml, you will need to add (445.5 - 250 - 10 - 20) = 165.5 µl of 1x sample buffer.

10. Mix vigorously by pipetting.

11. Add 4.5 µl Pharmalytes pH 3-10 for IEF. The total volume should now be 450 µl.

12. Mix vigorously by pipetting.

13. Add 4.5 mg DTT (final DTT concentration of 130 mM).

14. Mix vigorously by pipetting.

15. Spin down the sample in a microcentrifuge.

16. Samples are ready for use and can be stored on ice or frozen for up to one month, at -70 °C, in the dark.

Note: Protein lysates are viscous so failure to mix thoroughly after reagent addition can cause non-uniform labelling. This can result in poor preparative/analytical gel matching due to pI differences and mass shifts between labelled protein spots. Vortexing is not recommended.

❻ First dimension isoelectric focusing of labelled proteins

We recommend that labelled protein samples are loaded onto strips using the cup-loading approach for analytical gels (5 µg of protein per strip), or in-gel rehydration for preparative gels (up to 500 µg protein per strip). The methods below describe sample application using both of these techniques followed by focusing using Ettan IPGphor IEF system. For loadings above 5 µg, labelling reactions should be scaled up, maintaining the ratio of protein:TCEP:dye determined in the labelling optimization experiment.

For general information and protocols for the use of Ettan IPGphor IEF system and Multiphor II IEF system in 2–D DIGE experiments, please refer to the Ettan DIGE System User Manual (code 18-1173-17). For more detailed information on Ettan IPGphor IEF system and Multiphor II IEF system please refer to the accompanying User Manuals (code nos. 80-6415-35 and 18-1103-43 respectively).

Immobiline DryStrip rehydration for cup loading (analytical gels)

1. Pipette the appropriate volume of rehydration buffer into each of the required number of slots in an Immobiline DryStrip Reswelling Tray. The volume should not exceed the maximum volume determined for each Immobiline DryStrip size, shown in Table 5.

Immobiline DryStrip length (cm)	Total volume per strip (µl)
7	125
11	200
13	250
18	350
24	450

Table 5. Rehydration volumes of Immobiline DryStrips

2. Deliver the buffer slowly along the slot. Remove any large bubbles.

3. Remove the protective cover from the Immobiline DryStrip.

4. Position the Immobiline DryStrip with the gel side down and the pointed (acidic) end of the strip against the end of the slot closest to the spirit level.

5. Lower the Immobiline DryStrip onto the buffer. To help coat the entire Immobiline DryStrip and avoid air bubbles, gently lift and lower the strip along the surface of the buffer.

6. Overlay each Immobiline DryStrip with PlusOne Immobiline DryStrip Cover Fluid (1.5-2 ml) to prevent evaporation and urea crystallization.

7. Slide the lid onto the Immobiline DryStrip Reswelling Tray and allow the Immobiline DryStrips to rehydrate at room temperature. A minimum of 10 h is required for rehydration; overnight is recommended, up to a maximum of 24 h.

Sample application using the cup-loading approach (analytical gels)

1. Pre-prepare electrode pads by cutting 5 mm x 15 mm pieces from the IEF Electrode Strips. Place on a clean dry surface such as a glass plate and soak with distilled water. Remove excess water by blotting with a paper towel, or filter paper.

Note: It is important that the pads are damp and not wet. Excess water may cause streaking.

2. Combine the required amount of each labelled protein extract (e.g. 5 µg Cy3 pooled internal standard, 5 µg Cy5 experimental sample).

3. Mix thoroughly by pipetting and leave on ice until use.

4. Place the Ettan IPGphor Cup Loading Strip Holders in the correct position on the Ettan IPGphor platform.

5. With a pair of forceps carefully remove the Immobiline DryStrip from the Immobiline DryStrip Reswelling Tray, taking care not to

damage the gel.

6. Allow the excess PlusOne Immobiline DryStrip Cover Fluid to run off the Immobiline DryStrip onto a piece of tissue. Do not allow the gel side to touch the tissue as it may stick to it.

7. Place the Immobiline DryStrip gel side up with the basic end (flat end of Immobiline DryStrip) flush with the flat end of the Ettan IPGphor Cup Loading Strip Holder.

8. Place a pre-prepared damp electrode pad (5 mm x 15 mm) onto the acidic and basic ends of the gel.

9. Clip down the electrodes firmly onto the electrode pads. Ensure that there is good contact with the Immobiline DryStrip and the metal on the outside of the strip holder.

10. Clip a cup loader onto the strip next to one of the electrodes. It should be positioned either at the acidic or basic end (see recommendations for IEF conditions on page 46), in between the two electrodes.

11. To check for a good seal fill the cup to the top with PlusOne Immobiline DryStrip Cover Fluid. Observe the level of the fluid to check if it is decreasing. If a leak is detected remove the PlusOne Immobiline DryStrip Cover Fluid and reposition the sample cup.

12. Apply at least 4 ml of PlusOne Immobiline DryStrip Cover Fluid to the Immobiline DryStrip holder allowing the oil to spread so it completely covers the Immobiline DryStrip.

13. Up to 100 µl of protein sample can now be loaded into the bottom of the sample cup.

14. Put the clear plastic strip cover onto the strip holder.

15. Cover apparatus to exclude light taking care not to cover the air vents. Metallic covers must not be used under any circumstances.

Your strips are now ready for isoelectric focusing.

Immobiline DryStrip preparation using the in-gel rehydration approach (high protein loads, e.g. preparative gels)

1. Deliver 500 µg labelled protein in a volume of 450 µl (for a 24 cm Immobiline DryStrip), slowly down the centre of the slot in the Immobiline DryStrip Reswelling Tray. The volume should not exceed the maximum volume determined for each Immobiline DryStrip size, shown in Table 5. Remove any large bubbles.

2. Remove the protective cover from the Immobiline DryStrip.

3. Position the Immobiline DryStrip with the gel side down and the pointed (acidic) end of the strip against the end of the slot nearest the spirit level.

4. Lower the Immobiline DryStrip onto the buffer. To help coat the entire Immobiline DryStrip and avoid air bubbles, gently lift and lower the strip along the surface of the buffer.

5. Overlay each Immobiline DryStrip with PlusOne Immobiline DryStrip Cover Fluid (1.5-2 ml) to prevent evaporation and urea crystallization.

6. Slide the lid onto the Immobiline DryStrip Reswelling Tray and allow the Immobiline DryStrips to rehydrate at room temperature. A minimum of 10 h is required for rehydration; overnight is recommended, up to a maximum of 24 h.

7. Pre-prepare electrode pads by cutting 5 mm x 15 mm pieces from the IEF Electrode Strips. Place on a clean dry surface such as a glass plate and soak with distilled water. Remove excess water by blotting with a paper towel, or filter paper.

Note: It is important that the pads are damp and not wet. Excess water may cause streaking.

8. Place the Ettan IPGphor Cup Loading Strip Holders in the correct position on the Ettan IPGphor platform.

9. With a pair of forceps carefully remove the Immobiline DryStrip from the Immobiline DryStrip Reswelling Tray, taking care not to damage the gel.

10. Allow the excess PlusOne Immobiline DryStrip Cover Fluid to run off the Immobiline DryStrip onto a piece of tissue. Do not allow the gel side to touch the tissue as it may stick to it.

11. Place the Immobiline DryStrip gel side up with the basic end (flat end of Immobiline DryStrip) flush with the flat end of the Ettan IPGphor Cup Loading Strip Holder.

12. Place a pre-prepared damp electrode pad (5 mm x 15 mm) onto the acidic and basic ends of the gel.

13. Clip down the electrodes firmly onto the electrode pads. Ensure that there is good contact with the Immobiline DryStrip and the metal on the outside of the strip holder.

14. Apply at least 4 ml of PlusOne Immobiline DryStrip Cover Fluid to the strip holder allowing the oil to spread so it completely covers the Immobiline DryStrip.

15. Put the clear plastic strip cover onto the strip holder.

16. Cover apparatus to exclude light taking care not to cover the air vents. Metallic covers must not be used under any circumstances.

Your strips are now ready for isoelectric focusing

Isoelectric focusing using the IPGphor IEF system

Focus the proteins overnight. A typical program used for analytical protein loads with 24 cm pH 3-10 strips, is shown in Table 6.

	Power	Ramp	Duration
1	300 V	Step-and-hold	3 h
2	600 V	Gradient	3 h
3	1000 V	Gradient	3 h
4	8000 V	Gradient	3 h
5	8000 V	Step-and-hold	4 h
6	500 V	Step-and-hold	48 h
50 µA per strip, 25 °C			

Table 6. Suggested focusing program for use with the Ettan IPGphor IEF system.

Strips should be removed as soon as possible after step 5 is completed. If they are left for more than 2 h at 500 V, strips should be ramped up to 8000 V over 30 min to refocus proteins before strips are removed.

Cathodic cup-loading may give better results for acidic IPG strips and DeStreak Rehydration Solution (code 17-6003-19) is recommended for use with IPG strips containing basic regions. Higher protein loads (e.g. for preparative gels) may require longer focusing times. More detailed guidelines for first dimension conditions and focusing parameters are available in the Ettan DIGE System User Manual (code 18-1173-17).

If the Immobiline DryStrip is not run immediately on the second dimension gel, it can be stored at –70 °C in a sealed container (e.g. equilibration tube or petri dish). The container has to be rigid because a frozen Immobiline DryStrip is very brittle and can easily be damaged. Do not equilibrate Immobiline DryStrip prior to storage, this must be carried out immediately before the second dimension separation.

❼ Second dimension SDS-PAGE electrophoresis

Important:
Low fluorescence glass plates must be used for 2–D DIGE fluorescent gels. See related products list, page 71 for recommended plates.
To prepare a gel for spot picking, attach reference markers to the glass plate treated with Bind-Silane (refer to page 50, "Preparing Ettan DALT preparative gels").

For detailed information consult the Ettan DIGE System User Manual (code 18-1173-17), the Ettan DALT*twelve* System User Manual (code 80-6476-53) or the Ettan DALT*six* System User Manual (code 80-6492-49)

Casting isocratic 2–D gels

1. For a full set of 14 gels, make up 900 ml acrylamide gel stock solution without adding the 10% (w/v) ammonium persulfate (APS) or 10% (v/v) TEMED, refer to page 5, "Other materials required". For best results filter the solution through a 0.2 µm filter to remove dust and insoluble matter.
2. Assemble the caster on a level surface, as described in the Ettan DIGE System User Manual (code 18-1173-17).
3. Connect one end of the feed tube to either a funnel or a peristaltic pump. Insert the opposite end into the grommet in the bottom of the balance chamber.
4. Pour 100 ml of displacing solution into the balance chamber.
5. When ready to pour the gels, add the appropriate volume of freshly prepared APS and TEMED to the acrylamide gel stock solution and mix.
6. Introduce the gel solution into the funnel or peristaltic pump taking care not to admit any air bubbles into the feed tube.
7. Allow the solution to enter the caster until it is 1–2 cm below the final desired height. Stop the flow of acrylamide and remove the feed tube from the balance chamber grommet. Once the feed tube is

removed, the dense displacing solution will enter the caster and force the remaining acrylamide solution into the gel cassettes to the desired height.
8. Immediately pipette 1-2 ml of water-saturated butanol onto each gel to create a level interface.
9. Allow the gels to polymerize for at least 3 h at room temperature before disassembling the caster. The gels can be stored in an airtight container at 2–8 °C covered with 1x SDS electrophoresis running buffer for up to 2 weeks.

Equilibration of focused Immobiline DryStrips

Note: Prior to the second dimension separation, strips loaded with saturation labelled samples should be equilibrated using DTT only. The iodoacetamide equilibration step used with CyDye DIGE Fluor minimal dyes must not be performed with CyDye DIGE Fluor saturation dyes.

1. Remove focused Immobiline DryStrips from the first dimension apparatus or if the strips have been frozen, allow them to warm to room temperature.
2. Incubate each strip in 10 ml equilibration solution containing DTT for 10 min with gentle agitation.
3. Meanwhile, prepare fresh agarose sealing solution and allow to cool slightly. Immediately before applying the Immobiline DryStrip to the second dimension gel, slowly pipette the molten agarose sealing solution between the glass plates at the top of the second dimension gel, taking care not to introduce bubbles. Do not allow the agarose to cool or solidify.
4. Briefly rinse the Immobiline DryStrip by submerging in a measuring cylinder of 1x SDS electrophoresis running buffer.
5. With forceps, carefully place the Immobiline DryStrip between the two glass plates of the gel. By convention, the acidic, or pointed end of the Immobiline DryStrip is on the left.

6. Gently position the strip so that it rests on the surface of the polyacrylamide gel. Avoid trapping air bubbles between the strip and gel. Handle the strips carefully to avoid damage to the first and second dimension gels. Allow the agarose sealing solution to solidify.
7. Load the gel plates into the Ettan DALT electrophoresis tank filled with 1x SDS electrophoresis running buffer (note: this running buffer contains 0.2% SDS) refer to page 5, "Other materials required".
8. Program the desired run parameters into the control unit. Running condition guidelines for 12 gels are given in Table 7.

Electrophoresis run time	Wattage per gel
16 h	2 W
8 h	4 W
4 h	8 W

Table 7. Recommended wattage settings for different gel running times for 12 gels.

Always set the gels to run at 15 °C and run until the bromophenol blue dye front reaches the bottom of the gel.
9. Once the gels have run, they can be scanned immediately. Ideally scan as soon as possible, e.g. on the same day. If the gels are to be scanned later the same day they can be stored in 1x SDS electrophoresis running buffer at ambient temperature in the dark. Overnight storage should be at 2–8 °C in the dark. Gels scanned more than a day after running will show spot diffusion. If 2–8 °C storage is employed, gel plates must be allowed to warm to ambient temperature prior to scanning

❽ Preparing Ettan DALT preparative gels

Protein spots can be picked directly from separated proteins labelled with CyDye DIGE Fluor saturation dyes. Reference markers (code 18-1143-34) are used by the picking software to determine the spot co-ordinates. Gels for spot picking must therefore be cast with two reference markers under the gel and the gel has to be bound to the glass plate to ensure that it does not deform during the picking process. If using DeCyder Differential Analysis Software to autodetect the white reference markers, please check to confirm correct detection of each marker.

1. Treat the larger Ettan DALT low fluorescence glass plate with Bind-Silane solution (refer to page 5, "Other materials required" for recipe). Pipette 4 ml of Bind-Silane solution over the surface of the plate and wipe with a lint free tissue until dry. Cover the plate with a lint-free tissue and leave on the bench for 1.5 h for the excess Bind-Silane to evaporate.

2. Once dry, place a reference marker halfway down the left side of the Bind-Silane treated plate, close to the spacer (without touching the spacer) where it will not interfere with the protein spot pattern. With a second marker, repeat this on the right side of the plate.

3. When ready to pour the gel, sandwich the Bind-Silane treated plate against an untreated glass plate. Place the glass plate cassette in the gel caster. Silanized plates should only be assembled immediately prior to gel pouring to avoid transfer of Bind-Silane between glass surfaces within the cassette.

❾ Scanning CyDye DIGE Fluor saturation dye gels using Typhoon Variable Mode Imager

Typhoon 9000 series Variable Mode Imager will optimally detect signal from the CyDye DIGE Fluor saturation dyes. It provides the sensitivity required for accurate quantitation of low-level signals. Gels can be scanned between glass plates, preventing drying and shrinkage, and allowing further rescanning if required.

If spots on the 2–D gel image show saturated signals (i.e. pixel value exceeds 100 000) then quantitation may not be accurate. When optimizing scan conditions, the maximum pixel value detected inside the region of interest on the gel should be in the range 50 000-80 000. To achieve this, it is recommended that a low resolution pre-scan is run and the PMT adjusted accordingly until the maximum pixel value falls within this range.

A few proteins may have high cysteine content and therefore be labelled to a much greater extent than the general protein population. These proteins will give much more intense spots than the other proteins on the gel. If this is the case, it is possible to scan at a higher PMT to saturate these few spots. This will enhance the detection of the low abundance protein spots but it must be recognized that the data from the saturated protein spots will not be quantitative and should therefore be disregarded.

1. After switching on Typhoon Variable Mode Imager, leave to warm up for 30 min before scanning.
2. Place the gel on the platen. Use the Gel Alignment Guides if scanning assembled gels.
3. Select Fluorescence Acquisition Mode and select the appropriate

Setup scan parameters.

4. Select Tray setting.

5. Select scan Orientation using the Gel Orientation Guide to ensure the correct option is chosen.

6. Select Press sample if scanning assembled gels.

7. Choose pixel size. Use 500 or 1000 µm for pre-scans and 100 µm for quantitative analytical scans.

8. Select Focal Plane, use +3mm if imaging assembled gels.

9. Select DIGE File Naming Format to ensure that unique filenames can be generated for each scan channel.

10. Press SCAN to start.

For further details please refer to Ettan DIGE System User Manual (code 18-1173-17) or Typhoon User Guide (code 63-0028-31)

⑩ Image analysis using DeCyder Differential Analysis Software

DeCyder Differential Analysis Software is a fully automated software suite developed for detection, quantitation, positional matching and differential protein expression analysis on images generated using Ettan DIGE system.

For a detailed guide to using DeCyder Differential Analysis Software, refer to DeCyder Differential Analysis Software User Manual (code 18-1173-16). A rapid understanding of the software and its capabilities can be obtained by working through the tutorials provided with software.

The software comprises four modules shown in Table 8.

DeCyder module	Function
DIA (Differential In-gel Analysis)	Protein spot detection Background subtraction In-gel normalization Gel artefact removal Quantitation All performed on a pair of images, from the same gel.
BVA (Biological Variation Analysis)	Matching of multiple images from different gels to provide statistical data on differential protein abundance levels between multiple groups. Fold-change, Student's T-test and ANOVA values can all be obtained.
Batch Processor	Fully automated image detection and matching of multiple gels without user interaction.
XML Toolbox	Extracts user specific data facilitating automatic report generation.

Table 8. The four modules of DeCyder Differential Analysis Software

Image analysis is performed using a number of complex algorithms, which have been designed specifically for use with multiplexed 2–D gel images. These algorithms form part of the built-in functionality of the software, and are performed automatically with minimum user intervention.

DeCyder Differential Analysis Software is compatible with Microsoft™ Windows™ XP Professional operating system. It incorporates operator-friendly graphical user interface (GUI), with pull-down menus and toolbars. The combination of text and XML output files means that all the data generated within DeCyder Differential Analysis Software can be easily stored and accessed for further investigation.

DeCyder Differential Analysis Software was developed in parallel with the 2–D DIGE methodology and therefore all the advantages of the technique are utilized in the software.

- The novel co-detection algorithm in the DeCyder Differential Analysis Software takes advantage of the identical spot patterns generated when multiple samples are resolved on the same gel. The algorithm generates identical spot boundaries for spots on images derived from the same gel.
- Conventional 2–D image analysis packages allow extensive user intervention during spot detection and editing. This can lead to subjective data analysis and may result in biased conclusions. DeCyder Differential Analysis Software is designed to provide automated spot detection, normalization, background subtraction, matching and spot statistics. The spot detection algorithms have been highly optimized to work with multiplexed fluorescently labelled proteins and this allows a high degree of automation. This minimizes user intervention, providing a more objective analysis of the data.
- DeCyder Differential Analysis Software utilizes the pooled internal standard experimental design. This allows unparalleled accuracy for

relative protein abundance quantitation and high confidence in experimental conclusions. 2–D DIGE is the only 2–D technique capable of multiplexing and therefore the only 2–D approach which enables the use of an internal standard.

• Use of the internal standard experimental design also allows the software to carry out gel-to-gel matching on the pooled internal standard samples only. Thus, very similar images are matched, increasing the user's confidence in inter-gel matching. As matching across internal standards is completed, the individual sample images co-detected with each internal standard are simultaneously matched into the dataset.

• DeCyder Differential Analysis Software in conjunction with CyDye DIGE Fluor dyes allows the analysis of results from experimental designs with various degrees of complexity. Studies ranging from a simple control/treated experiment through to a multi-condition experiment addressing multiple factors (e.g. dose and time) can be performed in a single analysis.

Additional information

Requirements for cell lysis buffer

It is essential that the pH of the protein solution used with CyDye DIGE Fluor saturation dyes is as close to pH 8.0 as possible. Below pH 8.0, the labelling efficiency is reduced. Above pH 8.2, non-specific labelling of lysine residues can occur, which can result in pI shifts on the gel. To ensure that the pH is maintained at pH 8.0, a buffer such as Tris should be included in the protein solution at 30 mM (HEPES, MOPS, CHES, MES and tricine are also suitable). Failure to include a suitable buffer will mean that the pH of the solution may deviate significantly from pH 8.0 leading to the labelling problems described above.

BEFORE sample labelling it is preferable to avoid the addition of compounds with primary amine or thiol groups. Reagents such as DTT and IPG buffer (thiols) or ampholytes (primary amines) may compete with the proteins for the maleimide dye. These are normally added in the sample buffer after the labelling step. If thiol or primary amine containing compounds are essential, then their effect on sample labelling should be investigated during the labelling optimization experiments.

For samples with high levels of DNA and/or RNA we recommend including 5 mM magnesium acetate in the cell lysis buffer to aid solubilization of nucleic acids.

Protein lysate sonication

Sonication with a small (micro) probe sonicator provides the best and most consistent method for disrupting cells for use in analyses using Ettan DIGE system. Sonication will completely disrupt the cells and will also shear the DNA and RNA in the cell, resulting in higher quality 2–D gels. The presence of large amounts of unsheared nucleic acids can cause vertical streaking in a 2–D gel. DNase and RNase can

be added but these may appear as protein spots on the 2–D gel. This protocol has been used to disrupt a range of cell and tissue types.

1. Clean the probe of the sonicator with 70% (v/v) ethanol and dry thoroughly with a clean tissue.
2. Place the sample tube in a beaker of ice water to keep it cold during sonication.
Note: If the sample is allowed to heat up in the presence of urea, some proteins may be carbamylated which will alter the charge (pI) of the protein, producing charge trains of protein across the gel.
3. Ensure that the sonicator microtip is suspended with its tip well below the surface of the liquid in the sample tube, but not touching the sides.
4. Start with the sonicator set initially at a low setting, such as 25% power or 5 μm amplitude. Increase the sonication gradually so that small white bubbles appear around the tip of the probe. This is the ideal sonication level. When the bubbles appear, do not increase power further as this will cause the protein sample to froth. If the samples do froth, briefly microfuge them and then continue sonicating.
5. When the sonication level has been optimized, sonicate for 20 sec bursts followed by a 1 minute cooling period. Repeat this process five times. Alternatively some sonicators have a pulse facility which can be used to achieve the equivalent sonication time. This process is completed when the sonicated solution is less cloudy than the original solution.
6. After sonication, centrifuge the samples at 12 000 x g for 5 min at 4 °C. Transfer the supernatant to a new tube and discard the pellet. The samples are now ready for protein quantitation or storage at -70 °C.

Adjustment of protein sample pH

If the pH of the protein sample is below pH 7.8 or above pH 8.2, do not proceed with the labelling. First adjust the pH of the sample before labelling.

In the following example the lysate pH is too low at pH 7.5 in a solution containing 7 M Urea, 2 M Thiourea, 4% CHAPS and 30 mM Tris.

1. Make an identical lysis solution at pH 9.5, without the protein (7 M Urea, 2 M Thiourea, 4% CHAPS, 30 mM Tris).
2. Mix increasing volumes of the new lysis solution to the protein sample. This will increase the pH of the protein sample as more cell lysis buffer is added. Stop when the pH of the protein sample is at pH 8.0. Alternatively, the pH of the lysate can be increased to pH 8.0 by the careful addition of 50 mM NaOH.

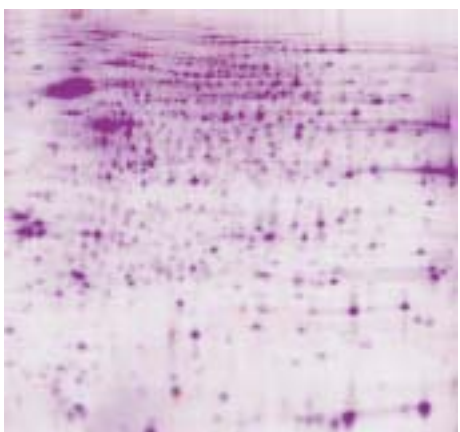
Cell and tissue types tested with CyDye DIGE Fluor saturation labelling

The following set of tables show protocols that have been used for a range of sample types, alongside examples of the 2–D images obtained. Standard recommended protocols and reagents were used unless otherwise stated. The protocols used here are not necessarily optimal methods for these sample types but do present a useful methodology along with an illustration of the image quality that can be obtained in each case.

All IEF programs used finished with a low voltage (500 V) step for 48 h. This step was intended to maintain the focusing of the proteins after the IEF program had completed. The number of hours spent at this voltage varied for each sample type but was generally significantly lower than the full 48 h programmed into the IEF unit. Strips were removed immediately upon completion of the IEF program. Where this was not possible and samples were left at 500 V for more than 2 h they were then refocused by ramping to 8000 V over a period of 30 min.

Extraction and Labelling Protocols	First and Second Dimension Conditions
Extraction Tissue washed with saline (0.9%), mechanically homogenized in cell lysis buffer (7 M urea, 2 M thiourea, 4% CHAPS, 40 mM Tris, pH 8.0, 1 ml per 0.1 g tissue) and centrifuged (13 000 rpm, 10 min, 4 °C). Pellet discarded and supernatant used for labelling. **Labelling** 5 µg protein labelled with 4 nmol TCEP and 8 nmol dye.	**First dimension** pH 3-10 NL, 24 cm Immobiline DryStrips. Ettan IPGphor IEF unit, anodic cup loading. 50 µA per strip 1. 300 V, 3 h, step 2. 600 V, 3 h, gradient 3. 1000 V, 3 h, gradient 4. 8000 V, 3 h, gradient 5. 8000 V, 4 h, step **Second dimension** 12.5% Ettan DALT gel, 2 W per gel overnight, 15 °C.

Gel Image

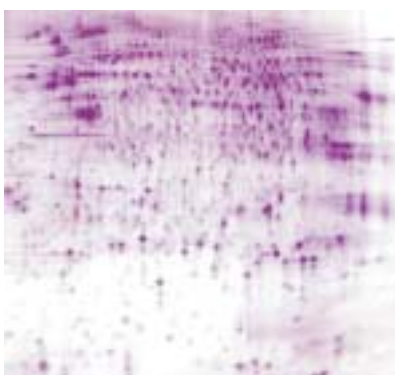


Cy3/Cy5 overlay for 5 µg protein labelled with CyDye DIGE Fluor Cy3 saturation dye (red) and 5 µg protein labelled with CyDye DIGE Fluor Cy5 saturation dye (blue).

Table 9. Mouse Brain

Extraction and Labelling Protocols	First and Second Dimension Conditions
Extraction Serum-free medium was poured off and cells washed twice with PBS in the flask. Without trypsinization, cell lysis buffer (2 M thiourea, 7 M urea, 4% CHAPS, 40 mM Tris, pH 8.0) was added to the flask. Cell lysate was pipetted out and sonicated on wet ice, with low-intensity 30 s pulses until the lysate turned clear. The sample was centrifuged (13 000 rpm, 10 min, 4 °C), the pellet discarded and the supernatant used for labelling. **Labelling** 5 µg protein labelled with 2 nmol TCEP and 4 nmol dye.	**First dimension** pH 3-10 NL, 24 cm Immobiline DryStrips. Ettan IPGphor IEF unit, anodic cup loading. 50 µA per strip 1. 300 V, 3 h, step 2. 600 V, 3 h, gradient 3. 1000 V, 3 h, gradient 4. 8000 V, 3 h, gradient 5. 8000 V, 4 h, step **Second dimension** 12.5% Ettan DALT gel, 2 W per gel overnight, 15 °C.

Gel Image

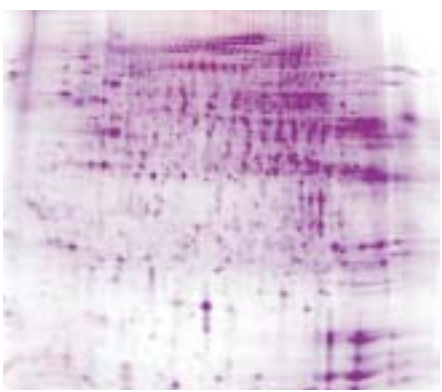


Cy3/Cy5 overlay for 5 µg protein labelled with CyDye DIGE Fluor Cy3 saturation dye (red) and 5 µg protein labelled with CyDye DIGE Fluor Cy5 saturation dye (blue).

Table 10. HEP G2 cultured cell line

Extraction and Labelling Protocols	First and Second Dimension Conditions
Extraction Tissue washed 4x with saline (0.9%) and mechanically homogenized in cell lysis buffer (8 M urea, 4% CHAPS, 30 mM Tris, pH 8.0, 1 ml per 0.1 g tissue). The supernatant was extracted and sonicated on wet ice, with low-intensity 30 s pulses until the lysate turned clear. The sample was centrifuged (13 000 rpm, 10 min, 4 °C), the pellet discarded and the supernatant used for labelling. **Labelling** 5 µg protein labelled with 2 nmol TCEP and 4 nmol dye.	**First dimension** pH 3-10 NL, 24 cm Immobiline DryStrips. Ettan IPGphor IEF unit, anodic cup loading. 50 µA per strip 1. 300 V, 3 h, step 2. 600 V, 3 h, gradient 3. 1000 V, 3 h, gradient 4. 8000 V, 3 h, gradient 5. 8000 V, 4 h, step **Second dimension** 12.5% Ettan DALT gel, 2 W per gel overnight, 15 °C.

Gel Image

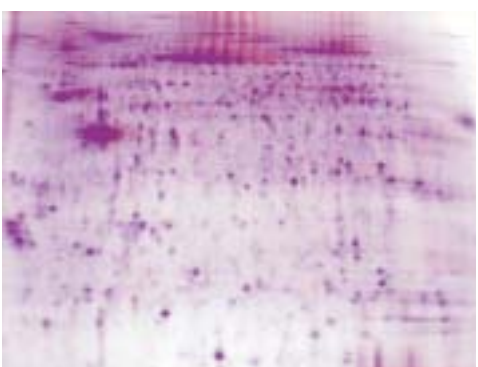


Cy3/Cy5 overlay for 5 µg protein labelled with CyDye DIGE Fluor Cy3 saturation dye (red) and 5 µg protein labelled with CyDye DIGE Fluor Cy5 saturation dye (blue).

Table 11. Rat liver

Extraction and Labelling Protocols	First and Second Dimension Conditions
Extraction Tissue washed 4x with saline (0.9%) and mechanically homogenized in cell lysis buffer (8 M urea, 4% CHAPS, 30 mM Tris, pH 8.0, 1 ml per 0.1 g tissue). The supernatant was extracted and sonicated on wet ice, with low-intensity 30 s pulses until the lysate turned clear. The sample was centrifuged (13 000 rpm, 10 min, 4 °C), the pellet discarded and the supernatant used for labelling. **Labelling** 5 μg protein labelled with 2 nmol TCEP and 4 nmol dye.	**First dimension** pH 3-10 NL, 24 cm Immobiline DryStrips. Ettan IPGphor IEF unit, anodic cup loading. 50 μA per strip 1. 300 V, 3 h, step 2. 600 V, 3 h, gradient 3. 1000 V, 3 h, gradient 4. 8000 V, 3 h, gradient 5. 8000 V, 4 h, step **Second dimension** 12.5% Ettan DALT gel, 2 W per gel overnight, 15 °C.

Gel Image



Cy3/Cy5 overlay for 5 μg protein labelled with CyDye DIGE Fluor Cy3 saturation dye (red) and 5 μg protein labelled with CyDye DIGE Fluor Cy5 saturation dye (blue).

Table 12. Rat lung.

Reagents tested for compatibility with CyDye DIGE Fluor saturation labelling

Labelling efficiency should be tested using the labelling optimization experiment (see page 27) in all the cases listed below:

- a new sample type is being used;
- the cell lysis buffer contains a reagent which hasn't been tested for compatibility with CyDye DIGE Fluor saturation labelling;
- the cell lysis buffer contains a reagent which has been tested for compatibility with CyDye DIGE Fluor saturation labelling, but is being used in a range known to affect labelling efficiency or is being used outside the recommended concentration range;
- the cell lysis buffer contains a combination of reagents that may or may not have been tested for compatibility with CyDye DIGE Fluor saturation labelling. The effect of different reagents on labelling efficiency is additive and may lead to unexpectedly poor labelling when one or more interfering reagents are used together.

Reagent	Effect on CyDye DIGE Fluor saturation labelling
Reducing agents TCEP, Tris-(2-carboxyethyl) phosphine	TCEP is used to reduce proteins before labelling with CyDye DIGE Fluor saturation dyes. The amount of TCEP (and dye) that are required to label a particular sample, are determined in the labelling optimization experiment. If TCEP is used in the cell lysis buffer, the optimum amount of TCEP added for the reduction step prior to labelling may be lower.

Reagent	Effect on CyDye DIGE Fluor saturation labelling
DTT, β-mercaptoethanol	Thiol-containing reagents react with saturation dyes. Therefore, the amount of TCEP (and dye) may need to be increased according to the amounts of these compounds present in the cell lysis buffer. The amount of TCEP (and dye) that are required to label a particular sample, are determined in the labelling optimization experiment.
Detergents	
CHAPS	4% recommended for use in the standard cell lysis buffer. This can be substituted with other detergents (see below). It is essential when using strong detergents (SDS, ASB14) that labelling is re-optimized as quantification of protein concentration may be affected.
Triton™ X-100	Use up to 4% .
NP40	Use up to 4%
SDS	Use up to 0.2% - No effect on labelling.
ASB14	Use up to 1% - No effect on labelling.
Buffers	
Tris	30-40 mM, pH 8.0 recommended. The pH during labelling is critical. pH 7.8-8.0 is optimal. pH >8.2 can cause non-specific labelling.
HEPES	Can cause focusing problems at high concentrations.

Reagent	Effect on CyDye DIGE Fluor saturation labelling
Protease inhibitors	
Protease Inhibitor Cocktail (Complete™), (contains AEBSF, 4-[2-aminoethyl]-benzolsulphonyl fluoride)	Compatible at manufacturer's recommended concentrations. To prevent charge trains forming, a protector reagent must be used. We recommend Pefabloc® SC^{PLUS}, AEBSF (Roche, code 1873601).
Other chemicals	
Amines	30 mM - 10% reduction in labelling
Ampholytes	0.5% - No effect on labelling 1% - 10% reduction in labelling 2% - 20% reduction in labelling
DNAse	No effect on labelling but extra spots may be visible in 2-D gel image.

Troubleshooting guide

Problems	Possible causes and remedies
❶ **The fluorescent signal is weak when scanned on a 2–D gel.**	**Possible cause 1.1:** The CyDye DIGE Fluor saturation dyes exceeded their expiry date prior to reconstitution, resulting in poor protein labelling. **Remedy 1.1:** Check the expiry date on the dye pack label. **Possible cause 1.2:** The reconstituted dye has been stored for too long, resulting in poor protein labelling. After reconstitution CyDye DIGE Fluor saturation dyes are only stable and useable until the expiry date detailed on the tube, or for 8 weeks, whichever is sooner. **Remedy 1.2:** Check the expiry date on the dye pack label and do not use dye that has been reconstituted for 8 weeks or more. **Possible cause 1.3:** The DMF used to reconstitute CyDye DIGE Fluor saturation dyes, was of poor quality or has been opened for longer than 3 months, resulting in poor protein labelling. **Remedy 1.3:** Always use 99.8% anhydrous DMF to reconstitute CyDye DIGE Fluor saturation dyes. Breakdown products of DMF include amines that compete with the protein for dye during the labelling step or cause dye degradation. **Possible cause 1.4:** The dyes have been exposed to light for long periods of time, resulting in loss of fluorescent signal. **Remedy 1.4:** Always store CyDye DIGE Fluor saturation dyes, in the dark. **Possible cause 1.5:** The dyes have been left out of the -15 °C to –30 °C freezer for a long period of time, resulting in poor protein labelling.

Remedy 1.5: Always store CyDye DIGE Fluor saturation dyes at -15 °C to -30 °C and only remove them for short periods to remove a small aliquot.

Possible cause 1.6: The wrong focal plane has been set on the Typhoon Variable Mode Imager.

Remedy 1.6: Set the focal plane to "+3 mm" for gels assembled between standard glass plates or "platen" for gels placed directly on the platen.

Possible cause 1.7: The Typhoon Variable Mode Imager settings are inappropriate.

Remedy 1.7: Ensure all parameters comply with recommended instrument settings.

Possible cause 1.8: The pH of the protein lysate is less than pH 7.8, resulting in poor protein labelling. This may be due to cell lysis causing a drop in pH or incomplete removal of the cell wash buffer prior to addition of the cell lysis buffer.

Remedy 1.8: Ensure the Tris buffer is present at 30 mM. Increase the pH of the cell lysis buffer by the addition of a small volume of 50 mM NaOH or cell lysis buffer at pH 9.5.

Possible cause 1.9: The pH of the protein lysate is more than pH 8.2, resulting in poor protein labelling.

Remedy 1.9: Decrease the pH of the cell lysis buffer by the addition of a small volume of 50 mM HCl.

Possible cause 1.10: Primary amines (e.g. pharmalytes or ampholytes) or thiols (e.g. DTT) are present in the labelling reaction competing with the protein for dye.

Remedy 1.10: Omit all exogenous primary amines and thiols from the labelling reaction.

Problems	Possible causes and remedies
	Possible cause 1.11: Interfering components are present in the labelling reaction at too high a concentration, resulting in poor protein labelling. **Remedy 1.11:** Remove the compounds from the labelling reaction if not essential. If they are essential test if the reduction in labelling efficiency can be counterbalanced by increasing TCEP/dye concentration. Investigate this using the method described in "Determining the optimum amount of TCEP/dye required to label a protein lysate", page 27. **Possible cause 1.12:** There is little or no protein in the protein lysate, or less lysate was loaded on the gel. **Remedy 1.12:** Test this by checking the protein lysate concentration using Protein Determination Assay (USB, code 30098). **Possible cause 1.13:** The protein lysate concentration is too low i.e. less than 0.55 mg/ml. **Remedy 1.13:** Make a new batch of protein lysate reducing the volume of cell lysis buffer to increase the protein concentration. Alternatively, precipitate the proteins and resuspend them in a smaller volume of cell lysis buffer. **Always check the pH and concentration of the resuspended sample before labelling.**
❷ **Protein spots on the 2–D gel show MW trains and/or streaking in the vertical direction.**	**Possible cause 2.1:** The amount of TCEP/dye used is too low. **Remedy 2.1:** Refer back to gels from the labelling optimization experiment to determine the correct amount. Repeat labelling optimization if necessary.

Problems	Possible causes and remedies
	Possible cause 2.2: There is insufficient SDS in the running buffer. **Remedy 2.2:** Prepare a fresh batch of running buffer ensuring that it contains 0.2% SDS. **Possible cause 2.3:** The concentration of Pharmalytes is too high. Pharmalytes complex with proteins at their isoelectric point. If too much Pharmalyte is present it may be difficult for proteins to resolubilize for transfer into the second dimension. **Remedy 2.3:** Ensure that the recommended concentration of Pharmalytes is used (no greater than 1% during rehydration).
3 **Cy3 and Cy5 labelled spots for the same protein show differential migration on the 2–D gel (i.e. some Cy3 and Cy5 labelled spots do not overlay).**	**Possible cause 3.1:** The amount of TCEP/dye used is too low. **Remedy 3.1:** Refer back to gels from the labelling optimization experiment to determine the correct amount. Repeat labelling optimisation if necessary. **Possible cause 3.2:** The sample press option has not been selected when scanning. **Remedy 3.2:** Ensure that the sample press option is used with assembled gels. **Possible cause 3.3:** Poor mixing during labelling, causing non-uniform labelling. **Remedy 3.3:** Mix vigorously by pipetting following each reagent addition during the labelling protocol.

Problems	Possible causes and remedies
4 **Protein spots on the 2–D gel show pI charge trains and/or streaking in the horizontal direction.**	**Possible cause 4.1:** The amount of TCEP/dye used is too high. **Remedy 4.1:** Refer back to gels from the labelling optimization experiment to determine the correct amount. Repeat labelling optimization if necessary. **Possible cause 4.2:** Poor mixing during labelling, causing non-uniform labelling. **Remedy 4.2:** Mix vigorously by pipetting following each reagent addition during the labelling protocol. **Possible cause 4.3:** The pH of the protein lysate is above pH 8.2, resulting in non-specific labelling of lysine residues. **Remedy 4.3:** Check the pH of the cell lysis buffer and reduce if necessary by adding a small volume of 50 mM HCl.

For further details of general 2–D electrophoresis troubleshooting, please refer to 2–D Electrophoresis, Principles and Methods Handbook

References

1 Ünlü, M. *et al.*, Difference gel electrophoresis: A single gel method for detecting changes in protein extracts. *Electrophoresis* **18**, 2071–2077 (1997).
2. Alban, A. *et al.*, A novel experimental design for comparative two dimensional gel analysis: two-dimensional difference gel electrophoresis incorporating an internal standard. *Proteomics* **3**(1), 36–44 (2003).
3. Gruber H. J. *et al.*, Anomalous fluorescence enhancement of Cy3 and Cy3.5 versus anomalous fluorescence loss of Cy5 and Cy7 upon covalent linking to IgG and noncovalent binding to avidin *Bioconjug. Chem.* **11** (5), 696–704 (2000).

Related products

CyDye DIGE Fluor Cy2 minimal dye (25 nmol)	RPK0272
CyDye DIGE Fluor Cy3 minimal dye (25 nmol)	RPK0273
CyDye DIGE Fluor Cy5 minimal dye (25 nmol)	RPK0275
CyDye DIGE Fluor Cy2 minimal dye (10 nmol)	25-8008-60
CyDye DIGE Fluor Cy3 minimal dye (10 nmol)	25-8008-61
CyDye DIGE Fluor Cy5 minimal dye (10 nmol)	25-8008-62
Immobiline DryStrip Reswelling Tray, 7-24 cm	80-6465-32
Ettan IPGphor IEF system	80-6414-02
Multiphor II IEF system	18-1018-06
DeStreak Rehydration Solution	17-6003-19
Ettan DALT*twelve* Large Vertical System	80-6466-27
Ettan DALT*six* Large Vertical System	80-6485-27
Low fluorescence glass plates for Ettan DALT	80-6442-14
Reference markers	18-1143-34
Ettan DIGE Gel Alignment Guides for Ettan DALT	80-6496-10
Gel Orientation Guide	80-6496-67
DeCyder Differential Analysis Software	18-1156-17
Ettan DIGE System User Manual	18-1173-17
Ettan Spot Picker	18-1145-28
Ettan Digester 100/120 VAC	18-1152-59
Ettan Digester 220/240 VAC	18-1142-68
Ettan MALDI-ToF Pro 120 V	18-1156-54
Ettan MALDI-ToF Pro 240 V	18-1156-53

For Immobiline DryStrips please refer to the catalogue.
For product information on Typhoon Variable Mode Imager, please inquire with your local Amersham Biosciences sales office.
For more details see Ettan DIGE System User Manual, catalogue and website (www.amershambiosciences.com/DIGE).

Legal

Product information

Product name	code
CyDye DIGE Fluor Labelling Kit for Scarce Samples	25-8009-83
CyDye DIGE Fluor Labelling Kit for Scarce Samples plus Preparative Gel Labelling	25-8009-84

Related products

see page 71

2–D Fluorescence Difference Gel Electrophoresis (Ettan DIGE) technology is covered by US Patent Numbers 6,043,025, 6,127,134, 6,426,190 and foreign equivalents and exclusively licensed from Carnegie Mellon University.
CyDye DIGE Fluor saturation dyes are covered by US Patent Number 6,048,982 and foreign equivalents and exclusively licensed from Carnegie Mellon University.
The purchase of CyDye fluors includes a limited license to use the CyDye fluors for internal research and development, but not for any commercial purposes. A license to use the CyDye fluors for commercial purposes is subject to a separate license agreement with Amersham Biosciences.

Amersham Biosciences has patent applications pending relating to its DeCyder software technology, including European patent application number 1,234,280.

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