Preparation, labelling, and detection of different protein lysate samples using CyDye DIGE Fluor dyes and Ettan DIGE system

key words:
- 2-D DIGE
- Difference Analysis
- Sample preparation
- 2-D electrophoresis
- Proteomics
- Minimal labelling

Two-dimensional fluorescence difference gel electrophoresis (2-D DIGE), a generic technique based on 2-D electrophoresis, can be applied to many different sample types. The methods described in this application note involve some different approaches for sample preparation that are compatible with minimal labelling, separation, and detection of proteins from different origins. The methods and examples provided here are intended as a starting point for more comprehensive protocol optimization.

Introduction

Two-dimensional fluorescence difference gel electrophoresis (2-D DIGE) allows detection and quantitation of real biological differences in protein abundance between different samples using cyanine dye labelling with spectrally resolvable CyDye™ DIGE Fluor minimal dyes (Fig 1). CyDye DIGE Fluor minimal dyes are NHS-esters that label lysine residues in proteins and are size- and charge-matched to enable multiplexing on a single gel.

Two-dimensional electrophoresis is a key technology for the separation of complex protein mixtures from biological samples in proteomics, and the sample preparation methods used are crucial to success. Comparative analysis of a sample using 2-D DIGE is dependent on many of the same parameters as normal 2-D analysis, such as the presence of chaotrope and detergent for protein solubilization.

Ettan™ DIGE system for 2-D DIGE is a powerful tool for separating complex mixtures of proteins by charge and size. The system includes Typhoon™ 9400 Variable Mode Imager, DeCyder™ Differential Analysis Software, and CyDye DIGE Fluor minimal dyes. Up to three samples can be co-electrophoresed on the same 2-D gel, which is then scanned with the Typhoon 9400 Variable Mode Imager. Differences are accurately quantitated using DeCyder Differential Analysis Software.

The recommended concentration of dye present in a protein-labelling reaction ensures that the amount of dye is limiting, leading to the labelling of approximately 1–2% of lysine residues. CyDye DIGE Fluor minimal dyes will therefore only label a small proportion of the total protein in a sample, hence the expression “minimal labelling”.

There are further considerations that specifically relate to the conditions for fluorescent labelling using CyDye DIGE Fluor minimal dyes, such as buffer concentration, pH, and the absence of high concentrations of interfering compounds. Using the correct conditions for minimal labelling enables good images to be obtained, which can then be used in DeCyder software for multi-gel 2-D difference analysis.

This study demonstrates the ability of the Ettan DIGE system to label, separate, and detect the proteins from a wide range of samples prepared using different lysis buffers, conditions, and methods. The similarities between the lysis buffers for different samples highlight the important parameters that need to be considered to obtain good results.
Products used

Amersham Biosciences products used in this application note:
- Ettan DALTsix Electrophoresis System 80-6485-27
- Ettan DALTtwelve Electrophoresis System 80-6466-27
- Multiphor II Electrophoresis System 18-1018-06
- MultiTemp III Thermostatic Circulator 18-1102-78
- Ettan IPIphor Isoelectric Focusing Unit 80-6414-02
- Typhoon 9400 Variable Mode Imager 63-0038-54
- Ettan DALT Gel 12.5, 26 × 20 cm 17-6002-36
- CyDye DIGE Fluor, Cy2 minimal dye RPK0272
- CyDye DIGE Fluor, Cy3 minimal dye RPK0273
- CyDye DIGE Fluor, Cy5 minimal dye RPK0275
- Pharmalyte, pH 3–10 17-0456-01
- Protein Determination Reagent US30098
- Ettan sample preparation kits and reagents
  - 2-D Quant Kit 80-6483-56
  - Urea 17-1319-01
  - CHAPS 17-1314-01
  - Dithiothreitol 17-1318-02
  - Tris 17-1321-01
  - PlusOne Sodium Dodecylsulfate 17-1313-01
  - PlusOne Glycerol 17-1325-01

Methods

For full method details, please refer to the respective manuals in references 1–9.

The recommended sample lysis buffer for Ettan DIGE system analysis contains 7 M urea, 2 M thiourea, 30 mM Tris, and 4% CHAPS. Other reagent combinations may be used to improve protein solubilization (9). However, this will need to be optimized for each sample to ensure compatibility with the minimal labelling step.

When starting work on a new sample type, or sample preparation method, it is important to perform a control experiment where the same sample is labelled, separated, and detected with all three dyes, and analyzed using DeCyder Differential Analysis Software. This enables an understanding of the underlying experimental variation for the new sample to be gained, and to determine the optimum sample preparation conditions.

Homogenization

Tissue (Table 1) was cut into small pieces 1–2 mm in size and placed into a tube with lysis buffer. Soft tissue such as mouse cerebellum was homogenized (X120 tissue homogenizer, Ingenieurbüro CAT, www.cat-ing.de) using five bursts of about 5-s duration each until a smooth paste was obtained. Hard tissue such as rat heart, liver, kidney, or mouse skeletal muscle was homogenized using up to 10 bursts of 30-s to 1-min duration.

Table 1. Preparation of protein lysates

<table>
<thead>
<tr>
<th>Cell or tissue type</th>
<th>Lysis buffer</th>
<th>Method of cell or tissue disruption*</th>
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</thead>
<tbody>
<tr>
<td>Caenorhabditis elegans (Fig 2)†</td>
<td>7 M urea, 2 M thiourea, 30 mM Tris, 4% CHAPS, pH to 8.5</td>
<td>3 mg/ml sample in water. Sample precipitated using acetone on ice for 1 h, centrifuged at +4 °C at 12 000 × g for 10 min. Supernatant discarded and pellet resuspended in lysis buffer.</td>
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<tr>
<td>Drosophila melanogaster (Fig 3)‡</td>
<td>7 M urea, 2 M thiourea, 25 mM Tris, 4% CHAPS, pH 8.0–8.5</td>
<td>Whole flies directly homogenized in lysis buffer. Incubated on ice for 1 h. Centrifuged at +4 °C in bench-top centrifuge for 20 min. Pellet discarded and protein concentration of supernatant determined.</td>
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<tr>
<td>Escherichia coli (Fig 4)</td>
<td>7 M urea, 2 M thiourea, 30 mM Tris, 4% CHAPS, pH to 8.5</td>
<td>Cells in lysis buffer sonicated on wet ice using 30-s pulses at pH to 8.5 7 microns amplitude with 1 min cooling period until clear. Centrifuged at +4 °C at 12 000 × g for 10 min. Pellet discarded and protein concentration of supernatant determined.</td>
</tr>
<tr>
<td>Human serum (Fig 5)</td>
<td>8 M urea, 40 mM Tris, 4% CHAPS, pH to 8.0</td>
<td>Protein concentration determined, sample diluted 1 to 4 in lysis buffer to give a concentration of 10 mg/ml.</td>
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<td>Mouse cerebellum (Fig 6)</td>
<td>8 M urea, 30 mM Tris, 4% CHAPS, pH to 8.5</td>
<td>Tissue sample rinsed four times with 10 ml 0.9% saline, drained, cut into small pieces, and homogenized at room temperature. Centrifuged at +4 °C at 12 000 × g for 10 min. Pellet discarded and protein concentration of supernatant determined.</td>
</tr>
<tr>
<td>Mouse skeletal muscle (Fig 7)</td>
<td>7 M urea, 2 M thiourea, 30 mM Tris, 4% CHAPS, pH to 8.5</td>
<td>Tissue sample rinsed four times with 10 ml 0.9% saline, drained, cut into small pieces, homogenized at room temperature. Centrifuged at +4 °C at 12 000 × g for 10 min. Pellet discarded and protein concentration of supernatant determined.</td>
</tr>
</tbody>
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Table 1. (continued)

<table>
<thead>
<tr>
<th>Cell or tissue type</th>
<th>Lysis buffer</th>
<th>Method of cell or tissue disruption</th>
</tr>
</thead>
<tbody>
<tr>
<td>NIH 3T3 fibroblasts, adherent cell line</td>
<td>7 M urea, 2 M thio urea, 10 mM Tris, 4% CHAPS, 5 mM magnesium acetate, pH to 8.0</td>
<td>Cells harvested by trypsinization, diluted with nine volumes of lysis buffer, incubated on ice for 30 min, and sonicated on wet ice using 25-s pulses at 5–6 microns amplitude with 1 min cooling period until clear. Centrifuged at +4 °C at 12,000 × g for 10 min. Pellet discarded and protein concentration of supernatant determined.</td>
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<tr>
<td>Rat heart (Fig 11)</td>
<td>7 M urea, 2 M thio urea, 10 mM Tris, 5 mM magnesium acetate, 4% CHAPS, pH to 8.0</td>
<td>1 g tissue homogenized in 10 ml lysis buffer. Lysate centrifuged at +10 °C at 12,000 × g for 1 h. Pellet discarded and protein concentration of supernatant determined.</td>
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<tr>
<td>Rat liver (Fig 12)</td>
<td>7 M urea, 2 M thio urea, 10 mM Tris, 5 mM magnesium acetate, 4% CHAPS, pH to 8.0</td>
<td>8 ml plasma mixed with 10 ml lysis buffer. Lysate centrifuged at +10 °C at 12,000 × g for 1 h. Pellet discarded and protein concentration of supernatant determined.</td>
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<td>Rat plasma (Fig 14)</td>
<td>7 M urea, 2 M thio urea, 10 mM Tris, 5 mM magnesium acetate, 4% CHAPS, pH to 8.0</td>
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<tr>
<td>Saccharomyces cerevisiae (Fig 15)</td>
<td>7 M urea, 2 M thio urea, 30 mM Tris, 4% CHAPS, pH to 8.5</td>
<td>Dried cell preparation resuspended in lysis buffer. Cells sonicated on wet ice using 30-s pulses at 7 microns amplitude with 1 min cooling period until clear. Centrifuged in a microcentrifuge for 5 min at 12,000 × g. Pellet discarded and protein concentration of supernatant determined.</td>
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<td>T24 bladder carcinoma cell line (Fig 16)</td>
<td>7 M urea, 2 M thio urea, 10 mM Tris, 5 mM magnesium acetate, 4% CHAPS, pH to 8.0</td>
<td>Cell culture medium removed. Cells were washed twice in PBS, scraped from the flasks, centrifuged, and cell pellets washed two times in 10 mM Tris pH 8, 5 mM magnesium acetate wash buffer. Pellets were resuspended in lysis buffer and stored at -70 °C. Protein concentration was determined.</td>
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Minimal labelling with CyDye DIGE Fluor minimal dyes

The following labelling protocol was used for samples separated on 24 cm Immobiline™ DryStrip gels and Ettan DALTsix/twelve format gels.

Dye reconstitution
A volume of 25 µl DMF (99.8% anhydrous, Aldrich 22,705–6) was added to each tube of dye (25 nmol) to give a 1 nmol/µl stock. An aliquot of each stock dye was further diluted 1:5 to give 200 pmol/µl dye in DMF, i.e. 2 µl stock dye + 8 µl DMF.

Sample labelling
Sample (50 µg) was labelled with 400 pmol of dye, i.e. by addition of 2 µl of the diluted dye at 200 pmol/µl (see above). After the dye was added, the samples were mixed thoroughly by pipetting vigorously.

The labelling reactions were carried out on ice for 30 min, followed by the addition of 2 µl of 10 mM l-lysine free base, (L-5501 Sigma), followed by a further 10-min incubation on ice.

The separate dye labelling reactions were combined and an equal volume of 2 × sample buffer (2 × sample buffer stock [7 M urea/2 M thio urea, 4% (w/v) CHAPS] was thawed and added to 2% [w/v] DTT and 2% [v/v] Pharmalyte™ 3–10) was added prior to cup loading onto rehydrated 24 cm Immobiline DryStrip gels, pH 3–10 NL.
First-dimension separation (IEF)

First-dimension separations were performed on Ettan IPGphor™ Electrophoresis Unit using anodic-cup loading with universal IPG strip holders. Alternatively, Multiphor™ II Electrophoresis Unit was used with cup loading. Fifty micrograms of protein labelled with each dye was loaded per strip.

Ettan IPGphor

Equipment: Ettan IPGphor isoelectric focusing unit, Cup Loading Strip Holder set.

Running conditions: 300 V, 3 h, step; 600 V, 3 h, gradient; 1000 V, 3 h, gradient; 8000 V, 3 h, gradient; 8000 V, 4 h, step; 500 V, 48 h, step.

Multiphor II

Equipment: Multiphor II horizontal electrophoresis unit. Cooling by MultiTemp™ III Thermostatic Circulator.

Running conditions: 5 W, 2 mA for all steps; 500 V, 1 Vh, gradient; 500 V, 1000 Vh, gradient; 3500 V, 3000 Vh, gradient; 3500 V, 50 000 Vh, gradient; 500 V, 10 000 Vh, step.

Equilibration

About 10–15 ml solution was used per strip. Each strip was equilibrated for 10 min in Equilibration solution 1 (50 mM TrisCl pH 8.8, 6 M urea, 30% [v/v] glycerol, 2% SDS, 0.5% DTT), and 10 min in Equilibration solution 2 (50 mM TrisCl pH 8.8, 6 M urea, 30% [v/v] glycerol, 2% SDS, 4.5% iodoacetamide) with gentle shaking/rolling.

Second-dimension separation

Ettan DALT Gel 12.5% SDS-polyacrylamide gels using low-fluorescence glass plates were prepared. Gels were run with 1× running buffer (25 mM Tris, 192 mM glycine, 0.2% SDS) in both upper and lower tank or with the top tank containing 2× buffer (50 mM Tris, 384 mM glycine, 0.2% SDS) and the bottom tank containing a 1× buffer (25 mM Tris, 192 mM glycine, 0.1% SDS). Gels were run overnight at a constant power of 5 W.

Detection

All gels were scanned using a Typhoon 9400 Variable Mode Imager at 100 µm resolution, with PMTs set in the 490–550 V range.

Table 2. Laser wavelength and emission filters used for scanning of CyDye DIGE Fluor minimal dyes with Typhoon 9400 Variable Mode Imager

<table>
<thead>
<tr>
<th>Dye</th>
<th>Laser (nm)</th>
<th>Emission filter (nm)</th>
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<tbody>
<tr>
<td>Cy2</td>
<td>Blue 2 (488)</td>
<td>520BP40</td>
</tr>
<tr>
<td>Cy3</td>
<td>Green (532)</td>
<td>580BP30</td>
</tr>
<tr>
<td>Cy5</td>
<td>Red (633)</td>
<td>670BP30</td>
</tr>
</tbody>
</table>

Results

Figures 4–13 and 16 are pseudocolored overlays of images from each of the three dyes. The different images are colored as follows before being combined: Cy™2 (green), Cy3 (red), and Cy5 (blue). Any protein spot that was preferentially detected or labelled will show up with one color being more intense. Figures 2, 3, 14, and 15 are Cy5 images only, pseudocolored black. For clarity, gel images show the pH 4–7 region, where most proteins are present.

Figures 2, 4, 8, 9, and 15 show different samples prepared in the recommended lysis buffer (7 M urea, 2 M thiourea, 4% CHAPS, 30 mM Tris, pH 8.5).

Figures 5, 6, and 7 show results from lysis buffers containing only 8 M urea as the chaotrope. Comparing Figures 6 and 7 with Figures 8 and 9 respectively shows the benefits of using a combination of 7 M urea/2 M thiourea as the chaotrope mix. An increased number of protein spots are seen when the latter chaotrope mix is used. For samples only containing soluble proteins such as serum and plasma, 8 M urea is usually sufficient.

Figures 10, 11, 12, 13, and 16 show the use of lysis buffers containing 10 mM Tris at pH 8.0 when labelling. There is some tolerance in the system for slightly suboptimal labelling conditions, however, 30 mM Tris at pH 8.5 is recommended as it gives the most reproducible results.

Different lysis methods were investigated, including sonication (Fig 4, 10, and 15), homogenization (Fig 3, 6, 7, 8, 9, 11, 12, and 13), and simply diluting the sample in lysis buffer (Fig 5, 14, and 16). One sample, Caenorhabditis elegans (Fig 2), was originally too dilute and was precipitated using acetone before being resuspended in lysis buffer.

Magnesium acetate was added to several samples to prevent nucleic acids causing problems during IEF (Fig 10, 11, 12, 13, 14, and 16). Sonication has also been found to be effective in mitigating the effects of nucleic acids.
Fig 2. 2-D analysis of Caenorhabditis elegans proteins on Ettan DALT 12.5% (26 x 20 cm) gel after focusing with 24 cm Immobiline DryStrip gel, pH 3–10 NL. Lysis buffer 7 M urea, 2 M thiourea, 30 mM Tris, 4% CHAPS (pH 8.5). Sample load was 50 µg of Cy5 dye-labelled protein only.

Fig 3. 2-D analysis of Drosophila melanogaster proteins on Ettan DALT 12.5% (26 x 20 cm) gel after focusing with 24 cm Immobiline DryStrip gel, pH 3–10 NL. Lysis buffer 7 M urea, 2 M thiourea, 25 mM Tris, 4% CHAPS (pH 8.0–8.5). Sample load was 50 µg of Cy5 dye-labelled protein only.

Fig 4. 2-D analysis of Escherichia coli proteins on Ettan DALT 12.5% (26 x 20 cm) gel after focusing with 24 cm Immobiline DryStrip gel, pH 3–10 NL. Lysis buffer 7 M urea, 2 M thiourea, 30 mM Tris, 4% CHAPS (pH 8.5). Sample load was 50 µg protein for each of the three dyes (150 µg total load).

Fig 5. 2-D analysis of human serum proteins on Ettan DALT 12.5% (26 x 20 cm) gel after focusing with 24 cm Immobiline DryStrip gel, pH 3–10 NL. Lysis buffer 8 M urea, 40 mM Tris, 4% CHAPS (pH 8.0). Sample load was 50 µg protein for each of the three dyes (150 µg total load).

Fig 6. 2-D analysis of mouse cerebellum proteins on Ettan DALT 12.5% (26 x 20 cm) gel after focusing with 24 cm Immobiline DryStrip gel, pH 3–10 NL. Lysis buffer 8 M urea, 30 mM Tris, 4% CHAPS (pH 8.5). Sample load was 50 µg protein for each of the three dyes (150 µg total load).

Fig 7. 2-D analysis of mouse skeletal muscle proteins on Ettan DALT 12.5% (26 x 20 cm) gel after focusing with 24 cm Immobiline DryStrip gel, pH 3–10 NL. Lysis buffer 8 M urea, 30 mM Tris, 4% CHAPS (pH 8.5). Sample load was 50 µg protein for each of the three dyes (150 µg total load).
2-D DIGE

Fig 8. 2-D analysis of mouse cerebellum proteins on Ettan DALT 12.5% (26 x 20 cm) gel after focusing with 24 cm Immobiline DryStrip gel, pH 3–10 NL. Lysis buffer 7 M urea, 2 M thiourea, 30 mM Tris, 4% CHAPS (pH 8.5). Sample load was 50 µg protein for each of the three dyes (150 µg total load).

Fig 9. 2-D analysis of mouse skeletal muscle proteins on Ettan DALT 12.5% (26 x 20 cm) gel after focusing with 24 cm Immobiline DryStrip gel, pH 3–10 NL. Lysis buffer 7 M urea, 2 M thiourea, 30 mM Tris, 4% CHAPS (pH 8.5). Sample load was 50 µg protein for each of the three dyes (150 µg total load).

Fig 10. 2-D analysis of NIH 3T3 fibroblast proteins on Ettan DALT 12.5% (26 x 20 cm) gel after focusing with 24 cm Immobiline DryStrip gel, pH 3–10 NL. Lysis buffer 7 M urea, 2 M thiourea, 10 mM Tris, 4% CHAPS (pH 8.0). Sample load was 50 µg protein for each of the three dyes (150 µg total load).

Fig 11. 2-D analysis of rat heart proteins on Ettan DALT 12.5% (26 x 20 cm) gel after focusing with 24 cm Immobiline DryStrip gel, pH 3–10 NL. Lysis buffer 7 M urea, 2 M thiourea, 10 mM Tris, 5 mM magnesium acetate, 4% CHAPS (pH 8.0). Sample load was 50 µg protein for each of the three dyes (150 µg total load).

Fig 12. 2-D analysis of rat liver proteins on Ettan DALT 12.5% (26 x 20 cm) gel after focusing with 24 cm Immobiline DryStrip gel, pH 3–10 NL. Lysis buffer 7 M urea, 2 M thiourea, 10 mM Tris, 5 mM magnesium acetate, 4% CHAPS (pH 8.0). Sample load was 50 µg protein for each of the three dyes (150 µg total load).

Fig 13. 2-D analysis of rat kidney proteins on Ettan DALT 12.5% (26 x 20 cm) gel after focusing with 24 cm Immobiline DryStrip gel, pH 3–10 NL. Lysis buffer 7 M urea, 2 M thiourea, 10 mM Tris, 5 mM magnesium acetate, 4% CHAPS (pH 8.0). Sample load was 50 µg protein for each of the three dyes (150 µg total load).
The overlaid images from all three dyes shown in the figures demonstrate the consistency of labelling and detection between the different dyes for many different sample types. When starting work on a new sample type, it is important to perform a control experiment where the same sample is labelled and detected with all three dyes, and analyzed using DeCyder Differential Analysis Software. This enables an understanding of the underlying experimental variation for the new sample type to be gained. An appropriate experimental design regime can then be selected.

The absence of color in the overlaid images from all three CyDye DIGE Fluor minimal dyes (Fig 3–12, 15) demonstrates the consistency of labelling and detection between the different dyes for many different sample types. The dyes have been designed to be matched for mass and charge. This ensures that a protein labelled with any of the CyDye DIGE Fluor minimal dyes will migrate to the same position on the 2-D gel, enabling multiplexing of up to three samples in the same 2-D gel.

Conclusions

2-D DIGE is a generic technique suitable for many different sample types and sample preparation methods. It offers high-resolution separation, and when lysis buffers compatible with the NHS-ester labelling chemistry are used, good 2-D DIGE results using Ettan DIGE system can easily be achieved.

Summary of critical parameters for efficient minimal labelling with CyDye DIGE Fluor minimal dyes:

- Recommended lysis buffer: 7 M urea, 2 M thiourea, 30 mM Tris, 4% CHAPS. Labelling problems may be experienced if the Tris concentration is reduced to 10 mM.

- Optimal pH for labelling is pH 8.5, however successful labelling can be achieved with a pH as low as 8.0.

- Avoid potential interfering compounds that react with NHS-esters, e.g. primary amines.
References

1. 2-D Electrophoresis
   Amersham Biosciences, code number 80-6429-60
2. Ettan DIGE User Manual
   Amersham Biosciences, code number 18-1164-40
3. Ettan IPGphor Isoelectric Focusing System Manual
   Amersham Biosciences, code number 80-6415-35
4. IPGphor Cup Loading Strip Holder Manual
   Amersham Biosciences, code number 80-6465-70
   Amersham Biosciences, code number 18-1103-43
   Amersham Biosciences, code number 18-1106-33
7. Ettan DALT System Manual
   Amersham Biosciences, code number 80-6476-53
   Amersham Biosciences, code number 63-0028-31
9. Optimizing sample preparation for 2-D electrophoresis

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

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