Compatibility of Deep Purple Total Protein Stain with Ettan DIGE system and MALDI-ToF mass spectrometry

Introduction

Two-dimensional fluorescence difference gel electrophoresis (2-D DIGE) using Ettan™ DIGE (Fluorescence Difference Gel Electrophoresis [1–4]) system is an established method for prelabelling protein samples with CyDye™ DIGE fluors prior to 2-D electrophoresis for differential analysis. Ettan DIGE system includes CyDye DIGE fluor dyes, Typhoon™ 9400 Variable Mode Imager, and DeCyder™ Differential Analysis Software.

The three currently available CyDye DIGE fluors used in Ettan DIGE system are each spectrally resolvable and are size- and charge-matched, which allows multiplexing of up to three separate protein mixtures on the same 2-D gel. This system is generally applied to analytical gels where it enables the incorporation of an internal standard on every 2-D gel yielding a more accurate statistical analysis of protein abundance. Each sample is compared with the same in-gel standard, so that when samples are compared across gels, each is measured relative to the standard, reducing the effects of gel-to-gel variation and increasing statistical confidence. Preparative gels poststained with a total protein stain are then matched to this data set to create a picklist of proteins of interest for further analysis by mass spectrometry.

Here we have evaluated the compatibility of Deep Purple™ Total Protein Stain (5,6) with Ettan DIGE system and mass spectrometry using Ettan MALDI ToF Pro in a model system investigating the differences between the human mammary gland cell line HBL-100 and the human ductal breast carcinoma BT-474.

Products used

Amersham Biosciences products used:

Ettan IPGphor IEF system	80-6414-02
Ettan DALTtwelve Large Vertical Electrophoresis System	80-6466-27
DALT Gel Caster	80-6467-22
Typhoon 9410 Variable Mode Imager & ImageQuant™ Image Analysis Software	63-0038-55
Deep Purple Total Protein Stain	RPN 6305
Pharmalyte 3–10 for IEF	17-0456-01
PlusOne Bind-Silane	17-1330-01
Immobiline DryStrip pH 3–10 NL, 24 cm	17-6002-45
Immobiline DryStrip Cover Fluid	17-1335-01
CyDye DIGE Fluor, Cy2 minimal dye	RPK 0272
CyDye DIGE Fluor, Cy3 minimal dye	RPK 0273
CyDye DIGE Fluor, Cy5 minimal dye	RPK 0275

Other products used

HBL-100 Human mammary gland cell line	ATCC No. HTB-124	ATCC, Manassas, Va., USA
BT-474 Human ductal breast carcinoma	ATCC No. HTB-20	ATCC, Manassas, Va., USA
DMF 99.8% anhydrous	22,705-6	Aldrich
Crew™ wipers	Z23681-0	Aldrich
Decon™ 90 (Scientific) Ltd	cln 010 010	M.J. Patterson
Methanol	101586	BDH
Glacial Acetic acid	10001	BDH
Ammonia Solution (sp. gr. 0.88–0.89, AnalaR)	100126	BDH
ZipTip$_{C18}$ P10	ZTC18S960	Millipore
Acetonitrile	152855	BDH
Ammonium bicarbonate	A-6141	Sigma
Trifluoroacetic acid	15311 2E	BDH

Amersham Biosciences

Methods

Sample labelling of analytical gels

Cell lysates were prepared by sonication, according to standard methods (7) in lysis buffer consisting of 5 mM magnesium acetate, 7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 30 mM Tris, pH 8. To derive statistically valid data on differences between the normal breast cells and breast cancer cells, equal amounts (25 µg) of each of the normal breast and breast cancer cell protein lysates were mixed and designated as the 'pooled internal standard'. A sufficient quantity of the standard sample was bulk labelled with CyDye DIGE Fluor Cy™2 minimal dye, to include a standard on every gel. Proteins (50 µg) from each of the normal and cancer protein cell lysates were labelled separately with CyDye DIGE Fluor Cy3 or Cy5 dye. These were bulk labelled in sufficient quantity for the number of gels required as shown on the experimental design in Table 1.

Table 1. 2-D DIGE experimental design for biological variation analysis

Gel number	Internal standard	Group 1	Group 2
Gel 1	Pooled Standard Cy2	Normal Cy3	Diseased Cy5
Gel 2	Pooled Standard Cy2	Normal Cy3	Diseased Cy5
Gel 3	Pooled Standard Cy2	Normal Cy3	Diseased Cy5
Gel 4	Pooled Standard Cy2	Normal Cy3	Diseased Cy5
Gel 5	Pooled Standard Cy2	Diseased Cy3	Normal Cy5
Gel 6	Pooled Standard Cy2	Diseased Cy3	Normal Cy5
Gel 7	Pooled Standard Cy2	Diseased Cy3	Normal Cy5
Gel 8	Pooled Standard Cy2	Diseased Cy3	Normal Cy5

The protein lysates were labelled following the standard protocol for minimal labelling with CyDye DIGE Fluor minimal dyes (7). A 50 µg aliquot of protein lysate in 7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 30 mM Tris, pH 8.5 was minimally labelled with 400 pmol CyDye DIGE Fluor minimal dyes for 30 min on ice in the dark. Lysine (10 mM) was added to the reaction in the same volume as the dye and incubated for a further 10 min on ice in the dark. The labelling reaction was stopped with the addition of equal volumes of 2× sample buffer containing 7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 2 mg/ml DTT, 2% (v/v) Pharmalyte™ 3–10 to each of the labelled protein samples.

Electrophoretic separation

Prior to isoelectric focussing (IEF), labelled samples were mixed as described in Table 1. Using this protocol, each gel contained Cy2-labelled standard and an individual sample labelled with Cy3 and Cy5.

Immobiline™ DryStrip gels pH 3–10 NL, 24 cm were rehydrated in 7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 2 mg/ml DTT, 1% Pharmalyte pH 3–10 in an Immobiline DryStrip Reswelling Tray prior to first-dimension separation using the cup-loading technique. Isoelectric focusing was carried out on an Ettan IPGphor™ electrophoresis system with Ettan IPGphor Manifold. The separation protocol consisted of five phases of graduated voltages from 300 to 8000 V with a total focusing of 64 000 Vh. Prior to the second dimension, strips were equilibrated for 10 min in a reducing buffer containing 6 M urea, 2% (w/v) SDS, 30% (v/v) glycerol, 32 mM DTT, 100 mM Tris, pH 8. This was followed by a 10 min alkylation in a buffer containing 6 M urea, 2% (w/v) SDS, 30% (v/v) glycerol, 240 mM iodoacetamide, 100mM Tris, pH 8. The strips were immediately applied to a 12.5% SDS-PAGE gel cast on DALT Gel Caster with PlusOne™ Bind-Silane coated low-fluorescence glass plates (26 × 20 cm) and 1-mm spacers. An overlay of 0.5% (w/v) agarose in running buffer containing 0.001% bromophenol blue was applied. Second-dimension electrophoresis was performed using Ettan DALT*twelve* electrophoresis system. Initially, gels were run at 2 W/gel overnight and the following morning the power was increased to 10 W/gel until the bromophenol blue dye front migrated off the bottom of the gel.

Imaging and analysis of analytical gels

Gel images for analysis were obtained using a Typhoon 9400 Variable Mode Imager, which is designed to optimally detect CyDye DIGE Fluor minimal dyes using the following settings: Cy2 (488 nm excitation laser and 520BP40 emission filter); Cy3 (532 nm excitation laser and 580BP30 emission filter); and Cy5 (633 nm excitation laser and 670BP30 emission filter).

The resulting images were processed using DeCyder Differential Analysis Software v 5.0. The spots on the gel were co-detected automatically as 2-D DIGE image pairs, which intrinsically links a sample to its in-gel standard. Matching between gels was performed using the in-gel standard from each image pair. The experimental set up and relationship between samples was assigned in DeCyder software. Each Cy3 or Cy5 gel image was assigned an experimental condition as Group 1 (normal) and Group 2 (diseased). All Cy2 images were assigned as the standard. The gel with the highest spot count was automatically assigned as the master gel. Student's T-test was performed for every matched spot set, comparing the average and standard deviation of protein abundance for a given spot between the normal and diseased groups. The protein spots were software filtered to only include proteins in the pick list

that demonstrated a significant change ($p = 0.0005$) in abundance.

Deep Purple protein staining

Staining gels with Deep Purple Total Protein Stain was performed according to the standard protocol. All steps were performed at room temperature on a shaking platform. Gels were first placed in fix solution (7.5% [v/v] acetic acid, 10% [v/v] methanol) overnight. Following 4 × 10 min washes in water, gels were incubated with gentle agitation for 1 h in Deep Purple stain (made up as a 1:200 dilution in water). Development of the stain was performed with 3 × 10 min incubations in a 1:1000 dilution of concentrated ammonia. Gels were then washed in signal stabilization solution (0.75% [v/v] glacial acetic acid) for a minimum of 10 min prior to imaging on a glass plate. A Typhoon 9410 Variable Mode Imager was used to collect the Deep Purple stained image. For standard 1-D and 2-D gels, the 532 nm laser was used for excitation with a 560LP emission filter. In the scanning of CyDye prelabelled analytical gels poststained with Deep Purple, the scanning parameters used were excitation with the 457 nm laser combined with a 610BP emission filter to avoid cross-talk with the Cy2 and Cy3 signal. Gels were stored at 4 °C in the signal stabilization solution.

Mass spectrometry and protein identification

For mass spectrometry analysis, preparative gels were run using a pool containing 250 µg each of unlabelled HBL-100 and BT-474 lysates (500 µg total). Following 2-D electrophoresis, the preparative gel was stained with Deep Purple following the standard protocol. The image was automatically matched to the master gel image in the analytical gel match-set using DeCyder software. A spot picking list was generated from DeCyder software and exported to Ettan Spot Picker. Gel spots were excised as 2-mm diameter plugs and delivered into 96-well microplates.

In-gel digestion was performed after washing the gel plugs twice with 50 mM ammonium bicarbonate, then 50% (v/v) acetonitrile in 50 mM ammonium bicarbonate followed by 100% (v/v) acetonitrile for dehydration. Following overnight digestion with 500 ng trypsin (Promega, USA) in 50 mM ammonium bicarbonate, pH 8 at room temperature, peptides were extracted using sequential steps of 1% (v/v) aqueous trifluoroacetic acid (TFA), followed by 50%(v/v) acetonitrile in 0.2% TFA. After drying out, the peptides were then resuspended in 10 µl of 0.1% TFA before being cleaned with ZipTip™$_{C18}$. The tips were rehydrated with three washes in 50% acetonitrile, equilibrated with three washes in 0.1% TFA before aspirating the peptides 10 times to ensure binding. The tips containing the peptides were washed three times in 0.1% TFA before being eluted in 3 µl of 50% acetonitrile/0.2% TFA.

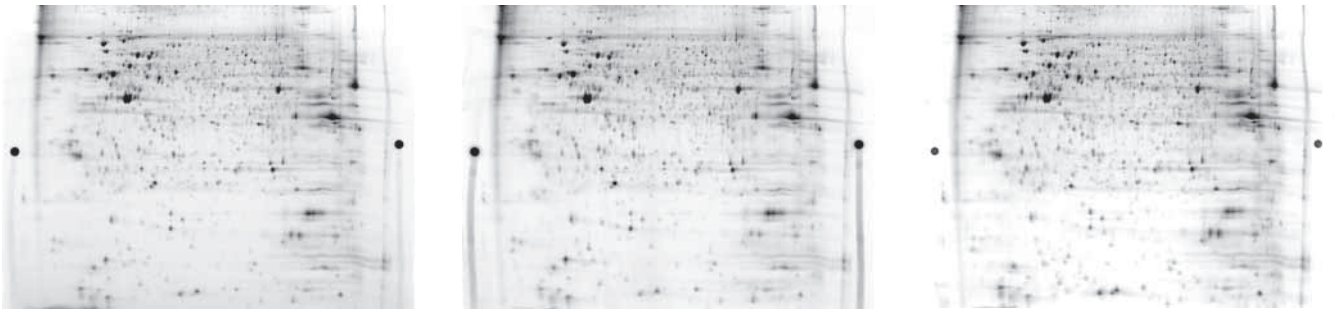
Peptide-mass fingerprinting was performed using Ettan MALDI-ToF Pro following the manufacturer's recommended protocol (8). MALDI-generated mass spectra were internally calibrated using trypsin peaks. The peptide masses were searched against the National Center for Biotechnology Information nonredundant mammalian database (9) using ProFound™ (10) and confirmed using a Mascot search from Matrixscience (11) and the SwissProt database from MS-Fit (12). One missed cleavage per peptide was allowed and an initial mass tolerance of 100 ppm was used in all searches. Partial oxidation for methionine was assumed.

Results and discussion

Use of Deep Purple in the analysis of preparative gels

A typical analytical 2-D DIGE experiment was performed and the CyDye labelled protein samples imaged according to their relevant spectra (Fig 1).

A preparative gel was also run and stained with Deep Purple (Fig 2a) and all the gel images were matched using DeCyder software, which assigns an individual spot number to all the validated spots. A number of proteins showed significant variation between the 'normal' and 'diseased' samples and a selection of these were picked (Fig 2b).



a. Cy2-labelled b. Cy3-labelled c. Cy5-labelled

Fig 1. Two-dimensional electrophoresis analytical gels: (a) 50 µg of Cy2-labelled pooled standard (normal and disease lysates); (b) 50 µg of Cy3-labelled HBL-100 normal breast cell lysate; and (c) 50 µg of Cy5-labelled BT-474 breast carcinoma.

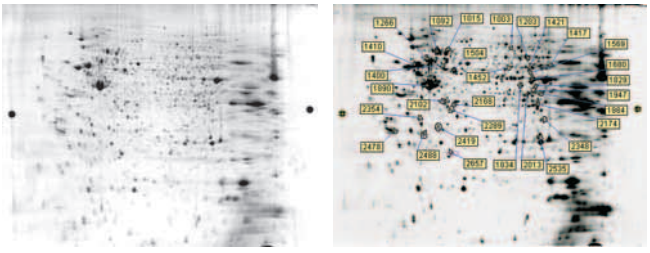


Fig 2. Deep Purple staining of a preparative 2-D gel: (a) 250 µg each of unlabelled HBL-100 human breast and BT-474 human breast carcinoma cell lysates stained with Deep Purple total protein stain; (b) image of the gel indicating the 30 differential spots that were picked.

A representative sample consisting of 10 of the 30 spots excised was processed to evaluate the compatibility of Deep Purple Total Protein Stain with mass spectrometry. Eight of these 10 gel plugs were positively identified by peptide-mass fingerprinting on Ettan MALDI-ToF Pro. It is clear that Deep Purple Total Protein Stain does not interfere with mass spectrometry determination. In the two spectra illustrated (Fig 3), a generally low background signal across the spectra is observed, and this was a feature noted in all the samples processed. This aided the protein identification process, resulting in a good score for expectation and sequence coverage, which provided greater confidence for the identification from peptide-mass searches.

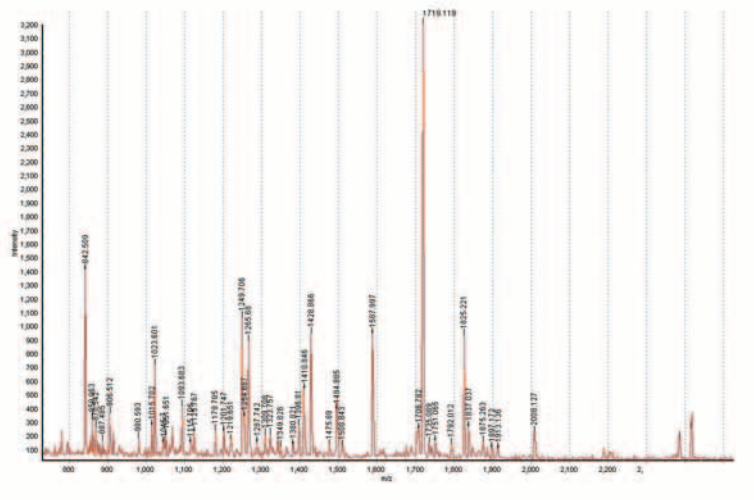
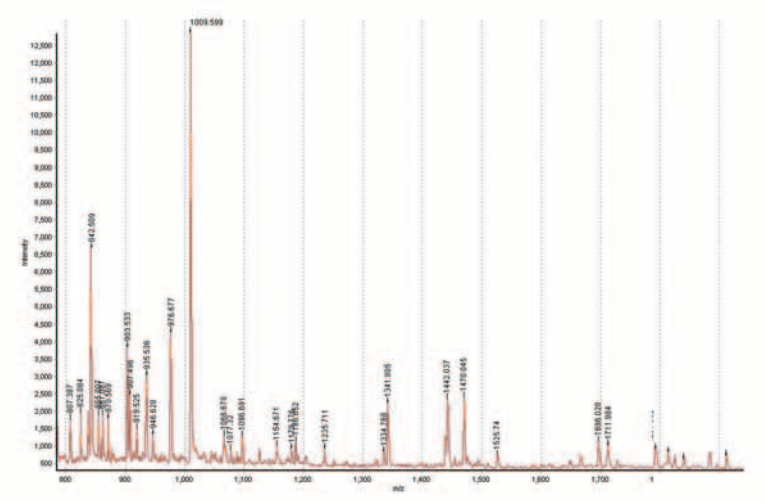
Spot No.	ID	MS Spectra
G1 1410	Vimentin Expectation 0.021 Coverage 25.5% pI 5.0 M_r 53 700	
F2 1829	Isocitrate dehydrogenase Expectation 0.010 Coverage 29.2% pI 6.2 M_r 46 600	

Fig 3. Mass spectra from Deep Purple stained proteins. Two proteins highlighted in Figure 2 were analyzed and identified using Ettan MALDI-ToF Pro; (above) spot 1410, identified as Vimentin; (below) spot 1829, identified as Isocitrate dehydrogenase.

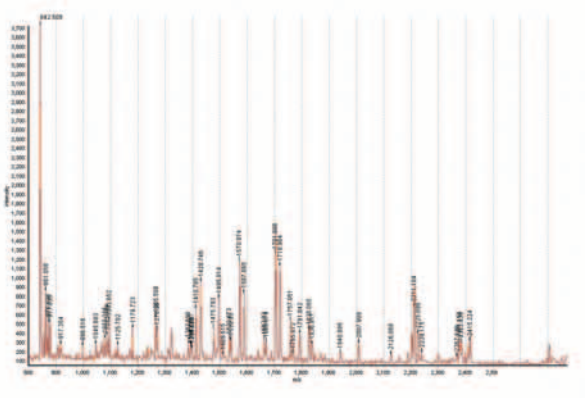
Use of Deep Purple in the analysis of analytical gels

The standard recommended workflow for the Ettan DIGE system is to run labelled analytical gels and match back to these with a stained preparative gel for spot picking and MS analysis. However, in some cases poststaining of the analytical gel may be required and MS analysis directly from a CyDye labelled sample may even be considered.

Deep Purple can be used to stain CyDye labelled analytical gels and the images can be processed successfully with the DeCyder Differential Analysis Software. Some carryover of signal from the CyDye label (depending on which label is used) may be observed in the Deep Purple image but this can be minimized on the Typhoon imager by use of the 457 nm laser for Deep Purple excitation combined with a 610BP emission filter. To illustrate the compatibility of the CyDye images and the Deep Purple stain, a number of the same spots as for the preparative gels were picked directly from the analytical gel (Fig 4a) and processed for MS analysis. Despite the low level of protein available on an analytical gel some of the highly abundant proteins were identified (see Fig 4b for one such example).

Vimentin

Expectation 0.00C
Coverage 43.8%
pI 5.1
M_r 53 600



a **b**

Fig 4. Deep Purple staining of an analytical gel for MS analysis. (a) image of the Deep Purple stain of the same gel as shown in Fig 1 after subsequent spot picking. (b) spot 1410 analyzed using Ettan MALDI-ToF Pro and identified as Vimentin.

Conclusions

Deep Purple Total Protein Stain, a new ultrasensitive fluorescent protein stain, has been applied to 2-D DIGE using Ettan DIGE system and MALDI mass spectrometry using Ettan MALDI-ToF. Deep Purple Total Protein Stain provides a high success rate of protein identification by peptide-mass fingerprinting with excellent scores for expectation and coverage leading to a high level of confidence in the identification from peptide mass searches.

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for more information

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