

instructions

product code

As listed below right

CyDye DIGE Fluors (minimal dyes) for Ettan DIGE

Reagents for labelling protein with Cy™2, Cy3 and Cy5 Fluors, before 2-Dimensional separation.

25-1900-27

25-1900-28

25-1900-30

25-8008-60

25-8008-61

25-8008-62.

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.



① 25190027 Rev-A, 2002



Handling

Storage

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

Expiry

For expiry date see outer packaging.

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Components

25-1900-27: 25 nmol of CyDye DIGE Fluor Cy2 minimal dye

25-1900-28: 25 nmol of CyDye DIGE Fluor Cy3 minimal dye

25-1900-30: 25 nmol of CyDye DIGE Fluor Cy5 minimal dye

25-8008-60: 10 nmol of CyDye DIGE Fluor Cy2 minimal dye

25-8008-61: 10 nmol of CyDye DIGE Fluor Cy3 minimal dye

25-8008-62: 10 nmol of CyDye DIGE Fluor Cy5 minimal dye

Safety warnings and precautions

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

Warning: 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: This dye is intensely coloured and very reactive. Care should be exercised when handling the dye to avoid staining clothing, skin, and other items. The toxicity of Cy2, Cy3 and Cy5 NHS Esters has not yet been evaluated.

Other materials required

Reconstitution of CyDye

99.8% anhydrous
Dimethylformamide (DMF)
less than 3 months old
from day of opening
(Aldrich 22,705-6)

Labelling

- Microfuge tubes 1.5 ml
- Standard Cell wash buffer, 10 mM Tris (pH 8.0), 5 mM Magnesium Acetate. Store in aliquots at -15 °C to -30 °C.
- Lysis buffer
30 mM Tris, 7 M Urea, 2 M Thiourea, 4% (w/v) CHAPS. Adjust to pH 8.5 with dilute HCl. Aliquots can be stored at -15 °C to -30 °C.
- Lysine
10 mM L-Lysine (Sigma™ L-5626)
- pH indicator strips (Sigma pH test strips pH 4.5–10.0 P4536)

For additional protocols

- 2 × Sample buffer
8 M Urea, 130 mM DTT,

- 4

Description

2-D Fluorescence Difference Gel Electrophoresis (Ettan™ DIGE) is a method to label proteins with CyDye Fluors that are subsequently separated using 2-Dimensional gel electrophoresis. This protocol is specifically designed to work using CyDye DIGE Fluors.

The Ettan DIGE technology is designed to simplify the process of detecting and identifying proteins using the 2-D electrophoresis technique by allowing the separation of up to three different protein samples in the same 2-D gel. Each of the three protein samples are labelled with one CyDye. After labelling the three samples are mixed together and run on the same Isoelectric focussing (IEF) and SDS PAGE gel. The ability to multiplex different samples on the same gel means that the different samples will be subject to exactly the same 1st and 2nd dimension running conditions so the same protein labelled with a CyDye will migrate to the same position on the 2-D gel, this helps limit experimental variation.

Each of the individual samples can then be visualised independently by selecting the individual excitation and emission wavelengths for each CyDye when fluorescence scanning.

4% (w/v) CHAPS, 2% (v/v) Pharmalyte™ 3-10 for IEF. Aliquots can be stored at -15 °C to -30 °C.

- Rehydration buffer
8 M Urea, 4% (w/v) CHAPS, 1% (v/v) Pharmalyte 3-10 for IEF
13 mM DTT.
- Sodium Hydroxide
50 mM NaOH
- 2 × Gel loading buffer
120 mM Tris (pH 6.8), 20% (v/v) (87% [v/v] Glycerol), 4% (w/v) SDS, 200 mM DTT and a few grains of Bromophenol Blue.
- 12.5% Acrylamide gel (for SE600)
32 ml Acrylamide/Bis 40% (v/v), 25 ml Tris (1.5 M pH 8.8), 1 ml SDS 10% (v/v), 1 ml Ammonium Persulfate 10% (w/v), 40 µl TEMED. Make up to 100 ml with distilled water.
- 1 × SDS electrophoresis running buffer
25 mM Tris, 192 mM Glycine, 0.2% SDS.
Store at room temperature.

Experimental design

The experimental design recommended is based on evidence that the experimental variation in a 2-D gel electrophoresis experiment is mostly due to gel to gel variation. Running multiple samples on a single gel reduces the number of gels required to produce the same number of data sets. The recommended protocol suggests that an internal standard sample be run on all gels within an experiment. The standard sample is generated by mixing together an aliquot of all the different samples in an experiment. This means that every protein from every sample will be represented in the standard that is present on all the gels. The standard sample will increase the confidence in matching between gels and will also allow the generation of accurate spot statistics between gels.

When using DeCyder™ software, use the experimental design outlined below. The example given is for protein samples, one derived from a control tissue and one a diseased tissue:

Mix 1/3 of each of the control and diseased samples together to create a standard sample. Label the standard sample with Cy2.

Label the remaining 2/3 of the control sample

- SYPRO Ruby protein gel stain 1 L, (Molecular Probes S-12000).
- SYPRO Ruby gel fixing solution 30% (v/v) Ethanol, 7.5% (v/v) Acetic acid
- Typhoon™ 9000 series (9400 series to scan Cy2 wavelengths)
- Hoefer™ SE600 or equivalent electrophoresis system
- Low Fluorescence glass plates (See Ettan™ DIGE user manual for more details)
- Immobililine™ DryStrip
- IPGphor™
- IPGphor cup loading strip holders
- Ettan DALT *twelve* gel system or equivalent electrophoresis system
- Ettan DALT *six* gel system

with Cy3.

Label the remaining 2/3 of the diseased sample with Cy5.

More complex experimental designs can be generated using the standard sample on all gels.

Minimal labelling

CyDye DIGE Fluors have an NHS ester reactive group, and are designed to covalently attach to the epsilon amino group of lysine of proteins via an amide linkage. The quantity of dye added to the sample is limiting in the reaction hence this method is referred to as 'minimal' labelling. This ensures that the dyes label approximately 1-2% of the available lysines and then only a single lysine per protein molecule.

The three dyes are matched for size and charge such that the three labelled protein samples are all run on the same 2-D gel and the same protein labelled with each CyDye will overlay.

The lysine amino acid in proteins carries a +1 charge at neutral or acidic pH. CyDye DIGE Fluors also carry an intrinsic +1 charge which, when coupled to the lysine, replaces the lysine's +1 charge with its own, ensuring that the pI of the protein does not significantly alter.

CyDye DIGE Fluors when coupled to the

protein add approximately 500 Da to the protein's mass in a uniform manner, giving an image comparable to equivalent silver stained gels in existing databases.

Spot picking

To spot pick from a gel containing CyDye labelled samples, post stain the gel with SYPRO Ruby to ensure that the majority of unlabelled protein is picked to give sufficient protein for MS identification. The migration difference between the unlabelled and labelled protein is due to the addition of a single CyDye molecule to the protein. This is more significant for low molecular weight proteins.

Protein identification

CyDye labelling of proteins does not affect the mass spectrometry data used to identify proteins as only 1-2% of lysine residues are labelled on a single protein. CyDye labelling of the lysine will only result in the loss of a single trypsin digest site per labelled protein.

Protocol

1 Introduction

This protocol provides all the information required to use CyDye DIGE Fluors to label proteins for 2-D electrophoresis experiments. It is recommended that the protocol is read thoroughly before using the system and that it is followed precisely. Reagents tested with Ettan DIGE are listed on page 21.

In the standard labelling protocol, proteins are first solubilized in a lysis buffer. The protein concentration should then be determined using a standard protein quantitation method. CyDye DIGE Fluors are then added to the protein lysate so that 50 µg of protein is labelled with 400 pmol of fluor. The reaction is incubated on ice in the dark for thirty minutes.

When handling proteins it is important to keep them on ice at all times to reduce the effect of proteases, and use plastic tubes as many proteins will adhere to glassware.

The fluorescent properties of Cy2, Cy3 and Cy5 can be adversely affected by exposure to light, so it is recommended that all labelling reactions are done in the dark, in microfuge tubes, and the exposure of protein labelled with CyDye to all light sources is kept to a minimum.

2 Preparation of a cell lysate compatible with CyDye DIGE minimal labelling

The example given here is that used with an *Escherichia coli* model system (See Ettan DIGE user manual for more details).

Recommendations for different cell types are listed on pages 19–20. Approximately 4×10^{10} *E. coli* cells will result in a 5 to 10 mg/ml of protein in 1 ml of lysis buffer.

1. Pellet the cells in a suitable centrifuge at 4 °C.

2. Pour off all growth media, taking care not to disturb the cell pellet.
3. Resuspend cell pellet in 1 ml of standard cell wash buffer in a microfuge tube.
4. Pellet the cells in a microcentrifuge at $12\,000 \times g$ for 4 min at $4\text{ }^{\circ}\text{C}$.
5. Remove and discard the supernatant.
6. Resuspend cell pellet in 1 ml of standard cell wash buffer in a microfuge tube.
7. Repeat steps 4 to 6 at least three times.
8. Ensure all the wash buffer has been removed with a fine pipette.
9. Resuspend the washed cell pellet in 1 ml of lysis buffer (30 mM Tris, 7 M Urea, 2 M Thiourea, 4% (w/v) CHAPS, pH 8.5) and leave on ice for 10 min.

Note: if the protein concentration is less than 5 mg/ml after protein quantitation, resuspend cells in a correspondingly smaller volume of lysis buffer in subsequent experiments.

10. Keep the cells on ice and sonicate intermittently until the cells are lysed. See 'Cell sonication', page 14.
11. Centrifuge the cell lysate at $4\text{ }^{\circ}\text{C}$ for 10 min at $12\,000 \times g$ in a microcentrifuge.
12. Transfer supernatant to a labelled tube. This is the cell lysate to be used for CyDye labelling. Discard the pellet.

Check that the pH of the cell lysate is still at pH 8.5 by spotting $1\ \mu\text{l}$ 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 'Adjustment' of protein sample pH', page 16.

Store cell lysates in aliquots at $-70\text{ }^{\circ}\text{C}$ until protein concentration is to be determined.

3 Reconstitution of CyDye in Dimethylformamide (DMF)

The dry CyDye must be reconstituted in DMF. Each vial of CyDye must be reconstituted in high quality anhydrous DMF (specification: $\leq 0.005\% \text{H}_2\text{O}$, $\geq 99.8\%$ pure, open for less than 3 months). On reconstitution in DMF the CyDye will give a deep colour; Cy2-yellow, Cy3-red, Cy5-blue.

Displacement of CyDye during manufacture or shipment of the fluors can be recovered to the bottom of the tube by pipetting the DMF down the side of the tube, vortexing vigously, and centrifuging.

The quality of the DMF used in all experiments is critical to ensure that the protein labelling is successful. The DMF must be anhydrous and every effort should be used 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 NHS ester CyDye reducing the concentration of fluor available for protein labelling. If in doubt use an unopened batch of DMF for reconstituting the fluor.

1. Take a small volume of DMF from its original container and dispense into a fresh microfuge tube.
2. Remove the CyDye from the $-15\text{ }^\circ\text{C}$ to $-30\text{ }^\circ\text{C}$ freezer and leave to warm for 5 minutes at room temperature.
3. After 5 minutes add the specified volume of DMF to each new vial of CyDye (see specification sheet supplied with the dye). This gives a CyDye stock solution of 1 mM.
4. Replace the cap on the dye microfuge tube and vortex vigorously for 30 seconds.
5. Centrifuge the microfuge tube for 30 seconds at $12\ 000 \times g$ in a microcentrifuge.

6. The fluor can now be used.

Unused CyDye 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 is only stable and useable until the expiry date detailed on the tube, or for 2 months, whichever is sooner.

4 Preparation of CyDye solution used to label proteins

1. Briefly spin down CyDye stock solution prepared in protocol 3, in a microcentrifuge.
2. Add one volume of CyDye stock solution to 1.5 volumes of high-grade DMF, to make 400 μ M CyDye solution. For example, take 2 μ l CyDye stock solution and add 3 μ l DMF to give 400 pmol CyDye in 1 μ l.

Note: Add the DMF first to the microfuge tube, followed by CyDye. 1 μ l of the working fluor solution now contains 400 pmol.

Quantity of CyDye to be used to label a protein lysate

It is recommended that 50 μ g of protein is labelled with 400 pmol of CyDye.

In each tube of CyDye there will be a 1 mM CyDye stock solution.

It is recommended that 400 pmol of CyDye per 50 μ g of protein to be labelled, between 100 pmol and 1000 pmol per 50 μ g of protein can be used. If labelling more than 50 μ g of protein then the same fluor to protein ratio must be used for all samples on the same gel.

Examples of CyDye dilutions that are used (recommended example is highlighted) are shown in Table 1, overleaf.

Table 1 – Examples of some widely used CyDye dilutions

Volume of stock CyDye (μl)	Volume of added DMF (μl)	Total volume (μl)	Concentration of CyDye (pmoles/ μl)
1	4	5	200
2	3	5	400
2	2	4	500
1	-	1	1000

The CyDye working solution is only stable for 1 week at $-15\text{ }^{\circ}\text{C}$ to $-30\text{ }^{\circ}\text{C}$.

5 Minimal labelling of a protein sample

The recommended concentration of the protein lysate is between 5 and 10 mg/ml. Samples containing from 1 mg/ml to 20 mg/ml have been successfully labelled using the protocol below.

The amount of CyDye used in the labelling reaction will have to be determined individually for the type of protein sample being analyzed.

1. Add a volume of protein sample equivalent to 50 μg to a microfuge tube. Bulk labelling reactions can also be done using more protein and fluor.
2. Add 1 μl of diluted CyDye to the microfuge tube containing the protein sample (i.e. 50 μg of protein is labelled with 400 pmol of fluor for the labelling reaction).
3. Mix and centrifuge briefly in a microcentrifuge. Leave on ice for 30 min in the dark.
4. Add 1 μl of 10 mM lysine to stop the reaction. Mix by pipetting and spin briefly in a microcentrifuge.
5. Leave for 10 min on ice in the dark.
6. Samples can now be stored for at least three months at $-70\text{ }^{\circ}\text{C}$ in the dark.

⑥ Loading samples onto IPG strips.

1. After the protein samples have been CyDye labelled, add an equal volume of 2 × sample buffer and leave on ice for 10 minutes.
2. Pool the protein samples that are going to be separated on the same 1st and 2nd dimension gel.

There are now two options

Option 1 - The samples can be loaded onto a rehydrated IPG strip via cup loading on the Multiphor II or IPGphor.

Option 2 - Follow the protocol outlined below if the IPG strips are to be rehydrated in the presence of the protein sample.

The total volume of labelled protein needs to be made up to the volume required for each IPG strip using the rehydration buffer.

One protein sample labelled with one CyDye: 20 µl

Add an equal volume 2× sample buffer: 20 µl + 20 µl = 40 µl

Add three samples together: 40 µl × 3

Total volume: 120 µl

A 24 cm IPG strip needs a total volume of 450 µl so add (450 µl – 120 µl) = 330 µl of rehydration buffer

Samples can now be run on an IPGphor, followed by the 2nd dimension on an Ettan Dalt *twelve* or *six* . For instructions on running 1st dimension IEF and 2nd dimension SDS PAGE gels, refer to the Ettan DIGE manual or the manuals supplied with the electrophoresis equipment.

Additional information

Requirements for Ettan DIGE protein lysis buffer

It is essential that the pH of the protein solution used with a CyDye DIGE Fluor is between pH 8.0–9.0.

To ensure that the pH remains between pH 8.0–9.0 a buffer such as Tris, Hepes or Bicarbonate should be included in the protein solution at a concentration of approximately 30 mM. Failure to include a suitable buffer will mean that the pH of the solution will fall below pH 8.0 resulting in little or no protein labelling. The Lysis buffer is required to work at 4 °C so the pH should be checked when the solution is chilled.

The protein solution should not contain any added primary amine compounds BEFORE labelling.

Primary amines, such as ampholytes, will compete with the proteins for CyDye. The result will be fewer CyDye labelled proteins, which might affect the data after scanning and spot detection.

See the section 'Reagents tested with Ettan DIGE', page 21.

Requirements for a cell wash buffer

A cell wash buffer should not lyse the cells, but it should dilute and remove any growth media or reagents that might affect the CyDye labelling process.

The cell wash buffer should not contain any primary amines.

A range of cell wash solutions such as 75 mM phosphate buffered saline (PBS) can be used in conjunction with the DIGE technology as long as their compatibility with the Ettan DIGE labelling is evaluated in controlled experiments (see 'Testing a new protein lysate for successful labelling', page 16).

Cell sonication

Sonication with a small (micro) probe sonicator provides the best and most consistent method for disrupting cells for use in Ettan DIGE.

Sonication will completely disrupt the cells and will also shear the DNA and RNA in the cell, resulting in higher quality 2-D gels. Presence of large amounts of unsheared nucleic acids can cause vertical streaking in a 2-D gel. DNases and RNases can be added but these may appear as protein spots on the 2-D gel. Sonication can be used on many different cell types including bacteria and mamalian cells.

1. Clean the probe of the sonicator with 70% (v/v) Ethanol and dry thoroughly with a clean tissue.
2. Place a beaker of ice water around the sample tube to keep it cold during sonication.

If the sample is allowed to heat up in the presence of urea, some proteins will 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 μ m amplitude. Increase the sonication gradually so that small white bubbles appear around the tip of the probe, this is now at an ideal sonication level. When the bubbles appear, do not increase the power any further as this will cause the protein sample to froth. If the samples do froth, briefly microfuge them and then continue sonicating with a reduced power level.
5. When the sonication is at the ideal level, sonicate for 20 second bursts followed by a 1 min cooling period. Repeat this process five times. Alternatively, some sonicators have a pulse facility which can be used to achieve the equivalent sonication time. Some samples may need further sonication cycles.
6. Sonication is complete when the solution appears significantly less cloudy than the starting solution.

7. After sonication, centrifuge the samples at $12\,000 \times g$ for 5 minutes at 4 °C. Remove the supernatant to a new tube and discard any pellet.

8. Samples are now ready for labelling with CyDye.

Adjustment of protein sample pH

If the pH of the protein sample is below pH 8.0 do not proceed with labelling with CyDye, first, increase the pH of the sample.

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, (7 M Urea, 2 M Thiourea, 4% CHAPS, 30 mM Tris [without the protein]) at pH 9.5.
2. Mix increasing volumes of the new lysis solution to the protein sample. This will increase the pH of the protein sample as more lysis buffer is added. Stop when the pH of the protein sample is at pH 8.5.

Alternatively, the pH of the lysate can be increased to pH 8.5 by the careful addition of dilute Sodium Hydroxide (50 mM).

Testing a new protein lysate for successful labelling

It is important to check that the labelling of the proteins has worked before the sample is used in Ettan DIGE.

The method involves running a small sample of your newly-labelled lysate on 1-D SDS-PAGE gel along with a control lysate that has already been labelled successfully. This gel is then scanned for CyDye, and the total fluorescence of each labelled sample is compared.

1. Label the new protein samples with Cy5 following the instructions in 'Protein sample labelling with CyDye', page 12. Cy5 labelled lysates have negligible cross talk with the SYPRO™ Ruby dye that might be used later in the experiment.
2. Add a volume of each CyDye labelled protein lysate equivalent to 50 µg into a microfuge tube.

3. Add an equal volume of 2 × gel loading buffer to the labelled protein lysate.
4. Heat the samples at 95 °C for 5 min to ensure full reduction of the proteins.
5. Make serial dilutions of each of the lysates in the 2 × gel loading buffer such as 25 µg, 12.5 µg and 6.25 µg. Make a 12.5% SDS-PAGE gel between low fluorescence glass plates (see Ettan DIGE User manual for glass plate recommendations). The gel should be made with wells at the top of the gel where the samples will be loaded. The SE600 gel system is recommend for this verification.
6. Load each protein serial dilution in successive lanes on the gel.
7. Run the samples until the Bromophenol Blue dye front has nearly reached the bottom of the gel.
8. Scan the gel at the Cy5 wavelength with Typhoon and carry out the statistics in ImageQuant™ software. If the labelling efficiency is comparable to the control, CyDye labelled samples can be subjected to 1st and 2nd dimension electrophoresis.
9. If further labelling is required to test a chemical component then the whole process should be repeated starting at point 1, or the sample can be resuspended in the recommended lysis buffer after using the PlusOne™ 2-D Clean Up Kit from Amersham Biosciences.

If labelling of the new lysate appears poor compared to the control sample the reason for this must be determined. The pH of the sample should be satisfactory, as this has previously been checked with pH test strips. The simplest explanation is that less of the new lysate was loaded on the gel than the control lysate. This can be tested for by post-staining the gel for total protein using SYPRO Ruby, or a general post stain such as silver staining. If an equivalent amount of the new sample and control sample were loaded on the gel this would suggest that there is a chemical component in the lysate that is affecting the

labelling reaction.

SYPRO Ruby staining the 1-D gel

1. Take the gel assembly apart after the electrophoresis and put the 1D gel directly into a polypropylene, polycarbonate or polyvinyl chloride tray. Add the SYPRO Ruby gel fixation solution (30% Ethanol, 7.5% Acetic acid) and incubate for at least 2 h on a shaking platform.
2. Pour off the fixing solution. Cover the gel with SYPRO Ruby stain.
3. Incubate the gel for at least two hours with gentle shaking, protected from the light.
4. Pour off the SYPRO Ruby.
5. Wash the gel in distilled water for 30 minutes. Repeat a further 3 times.
6. When the staining protocol is finished, clean, and dry two low fluorescence glass plates.
7. Place the gel onto one of the clean, dust-free glass plates, with the bottom edge of the gel furthest away.
8. Wet the bottom edge of the gel with distilled water.
9. Take a clean low-fluorescence glass plate, held so it is angled down and away and place one edge along the bottom of the gel.
10. Slowly lower the new glass plate onto the gel, taking care not to form bubbles. Bubbles can be avoided by ensuring that there is plenty of water on the gel, and by tapping the upper glass plate as it is lowered onto the gel.
11. When the new plate is flat on top of the gel, pick the gel up and let any excess water drain away. Dry any water off the outside of the plates. Be careful at this point as the gel and glass plates can easily slip apart.
12. Place the gel into the gel alignment guide appropriate for the gel

system and scan at the appropriate wavelength on Typhoon Imager (see Ettan DIGE User manual). NOTE: If preferred, the gel may be placed directly on the platen. If this method is used, wet the edge of the platen with Milli Q water and gently lower the gel making sure that air bubbles are excluded. Do not use the "Press Sample" function with bare wet gels.

Table 1. Typical cells and tissue types tested with Ettan DIGE.

Cell or tissue type	Lysis buffer	Method of cell or tissue disruption	Quantity of dye required to label 50 µg protein
<i>Saccharomyces cerevisiae</i>	2 M Thiourea 7 M Urea 30 mM Tris 4% CHAPS (pH to 8.5)	Pellet washed with PBS. Cells sonicated on wet ice and spun in a microfuge for 5 min at 12 000 × g. Pellet discarded and protein concentration determined.	400 pmol /50 µg
<i>Escherichia coli</i>	7 M Urea 2 M Thiourea 30 mM Tris 4% CHAPS (pH to 8.5)	Pellet sonicated on wet ice at amplitude 7 microns, for 30 s pulses. Spun in a centrifuge at 4 °C at 12 000 × g for 10 min. Pellet discarded and protein concentration determined.	400 pmol /50 µg

Cell or tissue type	Lysis buffer	Method of cell or tissue disruption	Quantity of dye required to label 50 µg protein
Rat liver	7 M Urea 2 M Thiourea 10 mM Tris 5 mM Magnesium Acetate 4% CHAPS (pH to 8.0)	1 g of tissue placed in 10 ml of lysis buffer. Tissue then homogenized and sonicated and lysate centrifuged at 10 °C, at 12 000 × g for 1 h. Pellet then discarded and protein concentration determined.	400 pmol /50 µg
Rat heart	7 M Urea 2 M Thiourea 10 mM Tris pH 8 5 mM Magnesium Acetate 4% CHAPS (pH to 8.5)	1 g of tissue placed in 10 ml of lysis buffer. Tissue then homogenized and sonicated and lysate centrifuged at 10 °C, at 12 000 × g for 1 h. Pellet then discarded and protein concentration determined.	400 pmol /50 µg
Tomato fruit wall	7 M Urea 2 M Thiourea 4% CHAPS 10 mM Tris (pH to 8.5)	Phenol based total protein extraction, including homogenizing step.	200 pmol /50 µg

For further examples of cell and tissue types tested with Ettan DIGE please refer to the Ettan DIGE User Manual which can be accessed on the web ([www.amershambiosciences.com/apatrix/upp00919.nsf/content/proteomics + DIGE protocols](http://www.amershambiosciences.com/apatrix/upp00919.nsf/content/proteomics+DIGE+protocols)).

Reagents tested with Ettan DIGE

Reducing agents

DTT 2 mg/ml – slight reduction in labelling
 5 mg/ml – 2× reduction in labelling
 10 mg/ml – 10× reduction in labelling
 CyDye will react with thiols at increased concentrations.

TCEP 0.5 M to 1 mM – slight reduction in labelling
 tris-(2-carboxyethyl) phosphine 2 mM – significant reduction in labelling
 β -mercaptoethanol significantly reduces labelling

Detergents

Triton™ X-100	use at 1%	17% reduction in labelling
NP40	up to 1%	No effect on labelling
SDS	up to 1%	No effect on labelling

Salts

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

Buffers

Tris Recommend 10–40 mM pH 8.5
 pH is very important. pH 8 to 9 is optimal.

HEPES Can cause focussing problems at high concentrations

Bicarbonate 5 mM pH 8.5 Acceptable

CHES	5 mM pH 9–9.5	Acceptable
PPA (3-piperidino- propionamide)	5 mM pH 8	Drop in sensitivity as PPA is a primary amine

Protease inhibitors

AEBSF (Pefabloc™) (4-(2-aminoethyl)- benzolsulphonyl fluoride)	causes charge trains unless protector primary amine
-------------------------------------------------------------------------	--------------------------------------------------------

Protease Inhibitor Cocktail (Complete™)	as above as contains AEBSF
--------------------------------------------	----------------------------

Aprotinin	compatible at recommended concentrations
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APMSF (4-amidino-phenyl) methane-sulphonyl fluoride)	compatible at manufacturer's recommended concentrations
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EDTA (ethylene diaminetetra- acetic acid)	compatible between 0.5 mM and 10 mM
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PMSF (phenylmethylsulphonyl fluoride)	compatible at manufacturer's recommended concentrations
---------------------------------------------	------------------------------------------------------------

Pepstatin A	compatible at manufacturer's recommended concentrations
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Troubleshooting guide

Problems

Possible causes and remedies

1

The pH of the protein lysate is less than pH 8 prior to labelling.

Possible cause: The lysis of the cells has caused a drop in the pH.

Remedy: Increase the buffering capacity of the Lysis buffer to 40 mM Tris (50 mM is the recommended maximum for Tris).

Possible cause: The cell wash buffer was not completely removed prior to addition of the lysis buffer.

Remedy: Increase the pH of the lysis buffer by the addition of a small volume of 50 mM NaOH. Or add an equal volume of the lysis buffer that is at pH 9.5.

2

The fluorescent signal is weak when scanned on a 2-D gel.

Possible cause: The fluors after reconstitution have a fixed lifetime in DMF that may have been exceeded.

Remedy: Check the expiry date on CyDye.

Possible cause: The DMF used to reconstitute CyDye was of poor quality or has been opened for longer than 3 months.

Remedy: Always use the 99.8% anhydrous DMF to reconstitute CyDye DIGE Fluors. Breakdown products of DMF include amines which compete with the protein for the CyDye labelling.

Possible cause: CyDye has been exposed to light for long periods of time.

Remedy: Always store CyDye in the dark.

Possible cause: CyDye has been left out of the -20 °C freezer for a long period of time.

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

Problems

Possible causes

Possible cause: The wrong focal plane has been set on the Typhoon.

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

Possible cause: The pH of the protein lysate is less than pH8.

Remedy: Increase the pH of the lysis buffer by the addition of a small volume of 50 mM NaOH. Or add an equal volume of the lysis buffer that is at pH 9.5.

Possible cause: Primary amines such as Pharmalyte or ampholytes are present in the labelling reaction competing with the protein for CyDye.

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

Possible cause: DTT or other substances such as SDS are present in the labelling reaction at too high a concentration.

Remedy: Remove the substances from the labelling reaction if not essential. If they are essential test if the reduction in labelling efficiency can be counterbalanced by increasing CyDye concentration. Investigate this using the 'Testing a new protein lysate for successful labelling', page 16

Possible cause: There is little or no protein in the protein lysate, or less lysate was loaded on the gel.

Remedy: Test this using the 'Testing a new protein lysate for successful labelling' section and 'SYPRO Ruby staining of a 1-D gel', page 18.

Possible cause: The protein lysate concentration is too low i.e. less than 1 mg/ml.

Remedy:

1. Make a new batch of protein lysate reducing the volume of lysis buffer to increase the protein

Problems

Possible causes

concentration.
2. Increase the ratio of CyDye to protein.
3. Precipitate the proteins and resuspend them in a smaller volume of lysis buffer.
Always check the pH and concentration of the new sample before labelling.

Possible cause: Incorrect fluor to protein ratio.

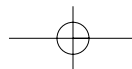
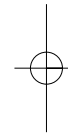
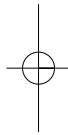
Remedy: 400 pmol of fluor per 50 µg of protein is recommended. If there is a large concentration of other components which can react with the fluor, then more fluor (up to 2 nmol per 50 µg of protein) can be used.

Related products

IPGphor	80-6414-02
Multiphor II	18-1018-06
Ettan DALT <i>twelve</i>	80-6466-27
Ettan DALT <i>six</i>	80-6485-27
SE600	80-6171-58
Low Fluorescence glass plates	
Ettan Dalt	80-6475-58
SE600	80-6179-94
Typhoon Imager	60-0038-54
DeCyder differential analysis software	56-3202-70

For Immobiline DryStrips please refer to the catalogue

For more details see Ettan DIGE user manual, catalogue and website ([www.amershambiosciences.com/optrix/upp00919.nsf/content/proteomics + DIGE + protocols](http://www.amershambiosciences.com/optrix/upp00919.nsf/content/proteomics%20+DIGE%20+protocols)).



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Product information

Product name	code
CyDye DIGE Fluors (minimal dyes) for Ettan DIGE	
Cy2 minimal dye 25 nmol	25-1900-27
Cy3 minimal dye 25 nmol	25-1900-28
Cy5 minimal dye 25 nmol	25-1900-30
Cy2 minimal dye 10 nmol	25-8008-60
Cy3 minimal dye 10 nmol	25-8008-61
Cy5 minimal dye 10 nmol	25-8008-62

Related products see page 26

2-D Fluorescence Difference Gel Electrophoresis (Ettan DIGE) technology is covered by US patent numbers US6,043,025 and US6,127,134 and foreign equivalents and exclusively licensed from Carnegie Mellon University under US patent number US5,268,486 and other patents pending.

Some of these products may only be available to collaborators and customers within certain of our technology access programmes.

The purchase of CyDye DIGE 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.

<http://www.amershambiosciences.com>

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