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Differential In-Gel Electrophoresis in a High-Throughput Environment

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1. Introduction

Proteomics deals with the large-scale analysis of proteins expressed by the genome of an organism. In general, the aim is to search for qualitative and quantitative changes in protein expression that occur as a function of development, disease, environmental insults, or treatment. Because of the complexity of the proteome of most organisms, the complete and rapid mapping of protein expression changes and characterization of posttranslational modifications is challenging and will require an analytical effort that exceeds the technology and throughput that exist in most standard biochemistry laboratories ***(1)***. While novel automated methods for rapid separation and analysis of proteins are being evaluated in many laboratories, it is widely accepted that two-dimensional gel electrophoresis (2-DE) is still the benchmark for large-scale separation of complex protein mixtures. The availability of standard reagents and equipment, and development of many modifications of the 2-DE process, have led to remarkable improvements in reproducibility ***(2,3)***. However, it is still difficult to fully duplicate the pattern of protein expression using conventional 2-DE methods ***(4,5)***, particularly when conducting multiple comparative and quantitative proteomics experiments in a high-throughput research environment where reproducibility and speed are critically important parameters.

The relatively new variant of 2-DE, differential in-gel electrophoresis (DIGE), promises to significantly improve the speed, reproducibility, and sensitivity of 2-DE-based experiments and performance of large-scale comparative proteomics studies using automated and semi-automated platforms. The DIGE concept involves the use of spectrally matched and resolvable fluorescent dyes to differentially label two to three protein samples prior to two-dimensional polyacrylamide gel electrophoresis (PAGE) on a single gel. The 2-DE gel pattern is then visualized by sequential imaging of the gel with a fluorescent scanner. Quantitative analysis of differential protein expression using the 2-D DIGE technology and appropriate software is fast and relatively more accurate than conventional 2-DE because it is based on the relative fluorescent intensities captured from a single gel, and errors attributable to gel-gel variations are nonexistent or minimal.

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Two DIGE procedures, "minimal" and "saturation" labeling, will be described in this chapter. In the minimal labeling procedure, two to three protein samples are separately labeled covalently with Cy2, Cy3, or Cy5 fluorescent dyes. The minimal labeling dyes contain an NHS ester active group that binds covalently to the lysine residue in proteins via an amide linkage. The protein-to-dye ratio is deliberately kept high (>95%) to ensure that only a single lysine residue on each protein is labeled in the minimal labeling protocol. The saturation labeling procedure is specifically developed for the analysis of scarce protein samples. The saturation labeling dyes (CyDye DIGE Fluor Cy3 and CyDye DIGE Fluor Cy5) contain maleimide reactive groups that covalently bind to the cysteine residues on proteins via a thioether linkage. The aim is to label all available cysteine residues.

In a high-throughput proteomics research environment, it is important to have a robust tracking system to permit the tracking of each sample, all proteins of interest, and all proteins that will be identified by mass spectrometry. Before proteins quantified by 2-D DIGE are analyzed by mass spectrometry, they must be picked from the gels, destained, digested, and extracted from the gel ***(6)***. Many reports have demonstrated that the DIGE technology affords great sensitivity and reproducibility ***(7–9)***, and because it is also amenable to automation ***(1)***, it is the variant of 2-D electrophoresis that we have found to be the most suitable for large-scale comparative and quantitative proteomics in a high-throughput biomedical research environment. This chapter covers the standard 2-D DIGE process and provides a brief introduction to the analysis of DIGE gels, and preparation of 2-D DIGE gels for automated picking of protein spots using a spot picker.

2. Materials

The materials listed below are required to successfully perform the GE Healthcare Biosciences minimal and saturation labeling 2-D DIGE procedure using matched and spectrally resolved cyanine dyes. Although there are a few reports indicating that other fluorescent dyes—e.g., Alexa™ dyes (Molecular Probes, Inc.)—can be used ***(5)***, the GE Healthcare DIGE package represents the most advanced and well-integrated DIGE package that is currently available commercially and that has been evaluated in many laboratories ***(1,4,7–9)***.

2.1. Minimal Labeling

1. Reagent-grade water.
2. Standard cell wash buffer: 5 m*M* magnesium acetate, 10m*M* Tris-HCl (pH 8.0). Store at 2–8°C and discard after 1 mo storage (*see* **Note 1**).
3. Lysis buffer: 7 *M* urea, 2 *M* thiourea, 4% CHAPS, 1% NP-40, 5 m*M* magnesium acetate, 30 m*M* Tris-HCl (pH 8.5), at 4°C. Store in aliquots at –20°C.
4. Mechanical homogenizer.
5. Microcentrifuge pestle with motor tool (Kimble/Kontes, Vineland, NJ).
6. pH indicator strips pH 2.0–9.0 (EM Science, Gibbstown, NJ).
7. Sodium hydroxide (NaOH), 50 m*M* solution.
8. Coomassie Plus protein assay reagent kit (Pierce Chemical Co., Rockford, IL).
9. Albumin Standard concentrate (Pierce Chemical Co., Rockford, IL).
10. Clear bottom 96-well microtiter plates.
11. Microtiter plate reader capable of reading absorbance at 595 nm.

12. 99.8% anhydrous dimethylformamide (DMF). Must be less than 3 mo old from date of opening.
13. Cy2, Cy3, and Cy5 minimal label dyes (GE Healthcare Biosciences, Piscataway, NJ).
14. Lysine (Aldrich Chemical Co., Milwaukee, WI).
15. DeStreak reagent (GE Healthcare Biosciences).
16. Immobilized pH gradient (IPG) buffer (GE Healthcare Biosciences).
17. Rehydration buffer: 7 *M* urea, 2 *M* thiourea, 4% CHAPS, 1% NP-40, 10% isopropanol, 5% glycerol, 1.2% DeStreak reagent, 0.5% IPG buffer, trace amount of bromophenol blue.
18. 2X rehydration buffer: 7 *M* urea, 2 *M* thiourea, 4% CHAPS, 1% NP-40, 10% isopropanol, 5% glycerol, 2.4% DeStreak reagent, 1.0% IPG buffer.
19. Bromophenol blue.
20. Immobiline DryStrips isoelectric focusing (IEF) gel strips (GE Healthcare Biosciences).
21. DryStrip cover fluid (GE Healthcare Biosciences).
22. Strip holders (GE Healthcare Biosciences).
23. IPGphor IEF unit (GE Healthcare Biosciences).
24. Sodium dodecyl sulfate (SDS) equilibration buffer: 6 *M* urea, 30% glycerol, 2% SDS, 50m*M* Tris-HCl (pH 8.8).
25. Equilibration buffer A: 1X SDS equilibration buffer, 0.5% dithiothreitol.
26. Equilibration buffer B: 1X SDS equilibration buffer, 4.5% iodoacetamide.
27. SDS-PAGE running buffer: 25 m*M* Tris, 192 m*M* glycine, 0.2% SDS.
28. Agarose sealing solution: 1X SDS-PAGE running buffer, 0.5% low melting point agarose, small amount of bromophenol blue.
29. 40% Acrylamide/bis solution (GE Healthcare Biosciences).
30. Bind-Silane (GE Healthcare Biosciences).
31. Low-fluorescence glass gel plates (GE Healthcare Biosciences).
32. Gel casting equipment.
33. TEMED (GE Healthcare Biosciences).
34. Ammonium persulfate (GE Healthcare Biosciences).
35. 1.5 *M* Tris-HCl, pH 8.8.
36. SDS-PAGE equipment (GE Healthcare Biosciences).
37. Gel fix solution: 10% methanol, 7% acetic acid.
38. Deep Purple Total Protein Stain (GE Healthcare Biosciences).
39. Development solution: 0.1% ammonium hydroxide.
40. Stabilization solution: 0 .75% acetic acid.
41. Decon™ (branded Contrad™ in the US).

2.2. Saturation Labeling

1. Reagent-grade water.
2. Cell lysis buffer: 7 *M* urea, 2 *M* thiourea, 4% (w/v) CHAPS, 30 m*M* Tris-HCl (pH to 8.0 with 1.0 *M* HCl). Aliquot and store at –15°C to –30°C. Discard after 3 mo.
3. Tris-(2-carboxyethyl) phosphine hydrochloride (TCEP) (Pierce Chemical Co., Rockland, IL).
4. Cy3 and Cy5 maleimide dye (GE Healthcare Biosciences).
5. IPG buffer (GE Healthcare Biosciences).
6. 1X rehydration buffer: 7 *M* urea, 2 *M* thiourea, 4% CHAPS, 1% NP-40, 10% isopropanol, 5% glycerol, 130 m*M* dithiothreitol (DTT), 1.0% IPG buffer, trace amount of bromophenol blue. Add IPG buffer and DTT immediately before use.
7. 2X rehydration buffer: 7 *M* urea, 2 *M* thiourea, 4% CHAPS, 1% NP-40, 10% isopropanol, 5% glycerol, 130 m*M* DTT, 2.0% IPG buffer.
8. Bromophenol blue.

9. Immobiline DryStrip IEF gel strips (GE Healthcare Biosciences).
10. DryStrip cover fluid (GE Healthcare Biosciences).
11. Strip holders (GE Healthcare Biosciences).
12. IPGphor IEF unit (GE Healthcare Biosciences).
13. SDS equilibration buffer: 6 *M* urea, 30% glycerol, 2% SDS, 50 m*M* Tris-HCl (pH 8.8).
14. Equilibration buffer A: 1X SDS equilibration buffer, 0.5% DTT.
15. SDS-PAGE running buffer: 25 m*M* Tris, 192 m*M* glycine, 0.2% SDS.
16. Agarose sealing solution: 1X SDS-PAGE running buffer, 0.5% low-melting-point agarose, small amount of bromophenol blue.
17. 40% Acrylamide/bis solution (GE Healthcare Biosciences).
18. Bind-Silane (GE Healthcare Biosciences).
19. Low-fluorescence glass gel plates (GE Healthcare Biosciences).
20. Gel casting equipment.
21. TEMED.
22. 10% Solution of ammonium persulfate.
23. 1.5 *M* Tris-HCl, pH 8.8.
24. SDS-PAGE equipment.
25. Gel fix solution: 10% methanol, 7% acetic acid.
26. Deep Purple Total Protein Stain (GE Healthcare Biosciences).
27. Development solution: 0.1% ammonium hydroxide.
28. Stabilization solution: 0.75% acetic acid.
29. Decon.

2.3. Quantitation of Protein Concentration

30. Coomassie Plus protein assay reagent or equivalent.
31. Spectrophotometer.

2.4. Isoelectric Focusing

1. Rehydration buffer: 7 *M* urea, 2 *M* thiourea, 4% (w/v) CHAPS, 1% (w/v) Pharmalyte™, broad range pH 3.0–10.0, 13 m*M* DTT. Prepare fresh by adding DTT (1 mg/mL) and Pharmalytes to 1X sample buffer. Use immediately and discard any unused material.
2. IPG strips (Immobiline™ DryStrip IPG strips, GE Healthcare Biosciences).
3. Reswelling tray (Immobiline™ DryStrip reswelling tray, GE Healthcare Biosciences).
4. Cup loading strip holders (Ettan™ IPGphor™ cup loading strip holder, GE Healthcare Biosciences).
5. DryStrip cover fluid (GE Healthcare Biosciences).
6. IEF and electrode strips (Ettan IPGphor IEF system, GE Healthcare Biosciences).
7. IEF electrode strips (GE Healthcare Biosciences).

2.5. SDS-PAGE Separation

1. Equilibration tubes or 9-cm diameter Petri dishes.
2. 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 stored at room temperature for 6 mo. DTT is then added immediately before use and any unused material discarded.
3. 12.5% acrylamide gel: 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 to prepare 14 24 cm × 24 cm gels.
4. Low fluorescence glass plates (GE Healthcare Biosciences).

5. Gel caster (Ettan DALT gel caster, GE Healthcare Biosciences).
6. Displacement solution: 375 m*M* Tris-HCl (pH 8.8), 50% glycerol, bromophenol blue (2 mg/100 mL). Prepare fresh and use immediately. Discard unused portion.
7. Agarose overlay solution: 0.5% low-melting-point (LMP) agarose preparation, 0.1% (w/v) bromophenol blue in 1X SDS electrophoresis running buffer (*see* **item 9**). Stable for 1 mo at room temperature.
8. Water-saturated butanol: Add 50 mL water to 50 mL butan-2-ol until two layers are visible. Stable for 6 mo at room temperature.
9. 1X SDS electrophoresis running buffer: 25 m*M* Tris, 192 m*M* glycine, 0.2% (w/v) SDS. Store at room temperature.
10. Electrophoresis system: e.g., Hoefer™ SE600 Ruby Gel system, Ettan DALTtwelve gel system, Ettan DALTsix gel system, or equivalent system.
11. Bind-Silane solution: 100 µL PlusOne Bind-Silane (code 17-1330-01). Add to 80 mL ethanol, 2 mL glacial acetic acid, and 18 mL water (GE Healthcare Biosciences).

2.6. Imaging and Image Analysis

1. Fluorescent gel imager (e.g., Typhoon™ 9000 series Variable Mode Imager, GE Healthcare Biosciences, or equivalent).
2. Image analysis software (DeCyder™ Differential Analysis Software, GE Healthcare Biosciences).

2.7. Post-DIGE Staining for Spot Picking and Mass Spectrometric Analysis

1. SYPRO® Ruby stain (Molecular Probes, Inc.).
2. SYPRO Ruby gel fixation solution: 30% (v/v) methanol and 7.5% (v/v) acetic acid in distilled water. Store at room temperature.
3. SYPRO Ruby gel destain solution: 10% (v/v) methanol and 6% (v/v) acetic acid in distilled water. Store at room temperature.

3. Methods

The methods described below outline the 2-D DIGE protocols for (1) the minimal labeling, (2) saturation labeling, (3) DIGE image analysis, and (4) protein spot processing; these have been successfully used in the authors' laboratory for 2-D DIGE analysis of protein isolated from human tissue, human cell lines, and cells procured by laser microdissection. Pay particular attention to the protocol because some of the methods used in preparation of protein samples for classical 2-D electrophoresis may not be compatible with DIGE. Also, note that there are some differences between the minimal and saturation labeling procedures.

3.1. Experimental Design

An experimental design needs to be carefully developed before carrying out 2-D DIGE experiments. The power of 2-D DIGE is fully realized when an in-gel internal standard is included. The in-gel standard is created by combining aliquots of all the biological samples in the experiment and labeling this mixture with one of the CyDye fluors (usually Cy2). This in-gel standard is then run on every single gel along with each individual sample. An example of the experimental design we have used successfully for analysis of human breast biopsies is shown in **Table 1**. The six-gel and three-gel experimental designs shown in **Table 1** will permit the comparison of protein spot

Table 1
Experimental Design for Two-Color (A) and Three-Color (B) Two-Dimensional Differential In-Gel Electrophoresis

A. Two-color analysis of protein from six samples (6-gel experiment)

Gel	CyDyes	
Number	Cy3 (test)	Cy2 (Internal standard)
1	50 µg Sample 1	50 µg (8.3 µg each of Sample 1–6)
2	50 µg Sample 2	50 µg (8.3 µg each of Sample 1–6)
3	50 µg Sample 3	50 µg (8.3 µg each of Sample 1–6)
4	50 µg Sample 4	50 µg (8.3 µg each of Sample 1–6)
5	50 µg Sample 5	50 µg (8.3 µg each of Sample 1–6)
6	50 µg Sample 6	50 µg (8.3 µg each of Sample 1–6)

B. Three-color analysis of protein from six samples (3-gel experiment)*

Gel		CyDyes	
Number	Cy3	Cy5	Cy2
1	50 µg Sample 1	50 µg Sample 2	50 µg (8.3 µg each of Sample 1–6)
2	50 µg Sample 3	50 µg Sample 4	50 µg (8.3 µg each of Sample 1–6)
3	50 µg Sample 5	50 µg Sample 6	50 µg (8.3 µg each of Sample 1–6)

*Using three colors reduces the number of gels required by 50%.

volumes across a range of samples and gels. The example given will allow (1) intra-gel co-detection of sample and internal standard protein spots and (2) inter-gel matching of internal standard samples across all the gels in the experiment.

3.2. Sample Preparation for 2-D DIGE

3.2.1. Cells

1. Harvest cells by centrifugation at 4°C in a suitable centrifuge.
2. Pour off growth medium without disturbing the cell pellet.
3. Wash cells by re-suspending the pellet in 1 mL of standard cell wash buffer in a microfuge.
4. Pellet in a benchtop centrifuge at 12,000*g* for 4 min at 4°C.
5. Carefully remove and discard the supernatant.
6. Re-suspend cell pellet in 1 mL of standard cell wash buffer.
7. Repeat **steps 4–6** three times.
8. Make sure that all standard cell wash buffer is removed after washing of cells.
9. Re-suspend the washed cell pellet in 500 µL of standard lysis buffer.
10. Homogenize with a mechanical homogenizer and leave on ice for 30 min, vortexing three to four times during this incubation. Take care not to heat the sample during homogenization.
11. Centrifuge the cell homogenate at 15,000*g* for 5 min at 4°C.
12. Re-homogenize the pellet using a microcentrifuge tube pestle with motor.
13. Centrifuge the cell homogenate at 15,000*g* for 10 min at 4°C.
14. Carefully draw off the supernatant with a Pasteur or adjustable pipet and place in a clean tube.

15. Check to insure the pH of the lysate is between 8.0 and 9.0 by spotting 1 µL of lysate on the indicator pads of the pH indicator strips (there are three pads). If the pH is too low, it can be raised by careful addition of 50 m*M* NaOH.
16. Place protein extract on ice and use immediately or store at –20°C to –80°C until needed.

3.2.2. Tissue

1. Place frozen tissue in a container big enough for the tissue and suitable for homogenization using a mechanical homogenizer.
2. Add approx 3 vols of ice-cold lysis buffer.
3. Rapidly homogenize for 3–5 s using a suitable laboratory homogenizer, set on high speed (*see* **Note 2**).
4. Place homogenate on ice immediately after homogenization. **Step 2** can be repeated up to three times. The generator on the homogenizer should be of sufficient size to handle the piece(s) of tissue.
5. Transfer the tissue homogenate to 1.5-mL microcentrifuge tube(s) and incubate on ice for 30 min. Vortex the homogenate three to four times during this incubation period.
6. Centrifuge the tissue homogenate at 15,000*g* for 5 min at 4°C.
7. Re-homogenize the pellet using a motorized microcentrifuge tube pestle.
8. Centrifuge the tissue homogenate at 15,000*g* for 10 min at 4°C.
9. Carefully draw off the supernatant and place in a clean tube.
10. Check to insure the pH of the lysate is between 8.0 and 9.0 by spotting 1 µL of lysate on the indicator pads pH indicator strips (there are three pads). If the pH is too low, it can be raised by careful additions of 50 m*M* NaOH.

3.3. Quantitation of Total Protein Concentration

It is absolutely essential that equal total protein concentrations be used in DIGE experiments. The method chosen for the determination of total protein concentration must be sensitive, reproducible, and compatible with 2-D DIGE buffer systems. The method must be compatible with detergents and thiourea, if these are present. The method described below is used routinely in the authors' laboratory for determining the total protein concentration of samples before 2-D DIGE.

1. Bring the Coomassie Plus dye reagent to room temperature and mix by inverting the tube several times.
2. Make dilutions of human serum albumin standard to obtain known concentrations (e.g., 0.125, 0.25, 0.5, 1.0, and 1.5 mg/mL). Include lysis buffer in the standards so the final concentration of lysis buffer in each assay well is identical.
3. Pipet 10 µL of the standards into microtiter plate wells, in duplicate.
4. For blank wells, pipet water with identical concentration of lysis buffer included. Prepare duplicate wells for the blank.
5. For sample wells add an amount of the lysate to duplicate wells; 1–2 µL should be enough to be in the range of the standard curve. Add enough water to the sample wells to bring the volume up to 10 µL.
6. Pipet into all wells 300 µL of the Coomassie Plus dye reagent and incubate at room temperature for 5 min.
7. Read the absorbance at 595 nm in a plate reader and calculate the total protein concentration based on the albumin standard curve.

Table 2
Examples of Dilutions of CyDye Used for Differential In-Gel Electrophoresis

Vol. of stock solution (µL)	Vol. of DMF (µL)	Total vol. (µL)	Conc. CyDye (pmol/µL)
1	4	5	200
2	3	5	400
2	2	4	500
1	–	1	1000

3.4. Differential Labeling of Protein Samples With CyDye DIGE Fluor

3.4.1. Minimal Labeling Protocol

The appropriate amount of CyDye fluor required for optimal labeling of specific protein samples will have to be determined individually. In general, the recommended ratio of fluor Cydye to protein is 400 pmol/50 µg. Examples of the Cydye concentrations that have been used are shown in **Table 2**.

The protocol described below has been found adequate for proteins isolated from human cell lines, biopsy specimens, and whole tissue. It has also been successfully used to study proteins isolated from cells procured by laser-assisted microdissection.

1. Re-suspend the Cydye as recommended by the manufacturer and dilute the stock Cydye 2.5 times with DMF. This makes a 400pmole/µL concentration of Cydye. Cydye fluor working solution is stable for only 1 wk at –20°C.
2. Transfer 50 µg of protein lysate to a clean microcentrifuge tube, and add 1 µL of diluted Cydye (400 pmol) (*see* **Note 3**).
3. Place tube on ice in an ice bucket, cover from light, and incubate for 30 min in the dark.
4. Add 10 m*M* lysine to stop the reaction. For every 1 µL of dye used, add 1 µL of 10 m*M* lysine.
5. Vortex, briefly centrifuge, and incubate the tube on ice, protected from light, for 10 min.
6. Determine the total volume in the tube and add an equal volume of 2X rehydration buffer. Vortex to mix, and centrifuge briefly.
7. Use immediately or store at –70°C, in the dark. Samples can be stored for 3 mo.

3.4.2. Saturation Labeling Protocol

The appropriate amount of CyDye fluor required for optimal labeling of specific protein samples must be determined for each batch of proteins. The protocol described below has been found adequate for proteins isolated from human cell lines, biopsy specimens, and whole-tissue samples. It has also been successfully used to study proteins isolated from cells procured by laser-assisted microdissection. As little as 5 µg of total protein can be labeled and analyzed.

1. Re-suspend the Cydye for saturation labeling as recommended by the manufacturer (GE Healthcare Inc.).
2. Transfer as little as 5 µg of protein lysate to a clean microcentrifuge tube, and add 1 µL of 2 m*M* TCEP (*see* **Note 4**).
3. Mix well using a pipet.
4. Briefly centrifuge tube and incubate at 37°C for 1 h. Protect the tube from light.
5. Add the Cydye to the tube. The amount of dye must be added at a 1:2 ratio to the TCEP (e.g., use 2 µL of Cydye when 1 µL of TCEP is used). Mix well using a pipet.

6. Centrifuge the tube briefly and incubate at 37°C. Protect the tube from light.
7. Determine the volume in the tube and stop the reaction by adding an equal volume of 2X rehydration buffer; mix well with a pipet.
8. The labeled samples can be used immediately or stored at –70°C, in the dark for up to 3 mo.

3.5. 2-D DIGE

3.5.1. IEF of CyDye Labeled Proteins—First Dimension

2-D DIGE is different from the standard 2D-PAGE because you can mix up to three differentially labeled protein samples before running on a single first- and second-dimension gel. The method described is for performing IEF with IPG strips using the Ettan IPGphor IEF system. Rehydration of the IPG strip can be done in the presence or absence of the labeled protein.

1. Choose an IEF strip length and range that is appropriate for the sample to be analyzed (*see* **Note 5**).
2. Combine the labeled samples in one tube. Samples that are to be combined must be labeled with different CyDye fluors.
3. Perform IEF using the recommended protocol for the chosen IEF strip.

3.5.2. PAGE of Cy Dye Labeled Proteins—Second Dimension

To identify and pick proteins of interest from 2-D DIGE gels using automatic spot pickers, e.g., Ettan Spot Picker (GE Healthcare Biosciences), the gels must be cast with two reference markers attached to the low-fluorescent glass plate, and the gel must be bound to the glass plate with Bind-Silane to prevent shifting and deformation during picking. Following the steps below carefully will enhance reproducibility. Focused strip must be equilibrated prior to running in the second dimension.

3.5.2.1. Preparation of DIGE Second-Dimension Gels for Automated Spot Picking

1. Clean low-fluorescence glass plates thoroughly and wash each plate in 1% Decon with a sponge to completely remove residual gel.
2. Soak plates in 1% Decon overnight and wash with a sponge.
3. Rinse plate with distilled water and leave to soak in 1% HCl (v/v) for 1 h.
4. Wash plate in 1% Decon and rinse with Milli-Q water.
5. Dry plates with lint-free tissue and protect from dust.
6. Coat the surface of the plate with 2–4 mL of Bind-Silane working solution and wipe with a lint-free tissue until dry.
7. Cover plate with a lint-free tissue and leave on bench for 1–1.5 h to dry (not leaving Bind Silane to dry before casting could create problems, because BindSilane will evaporate off the treated plate, coat the facing plate, and cause the gel to stick to the coating plate when it sets).
8. Attach reference markers on the treated glass plates. The markers should be placed where they will not interfere with the resolved proteins in the gel, and the spacers should not cover the markers.
9. Pour gels and allow to solidify before use.

3.5.2.2. Performing Second-Dimension DIGE

1. Equilibrate strips in equilibration buffer A for 15 min and in equilibration buffer B for 15 min, with gentle agitation during both steps. Note: If the focused strip contains samples that were labeled with saturation dye, they should be equilibrated for 15 min in equilibration buffer A only.

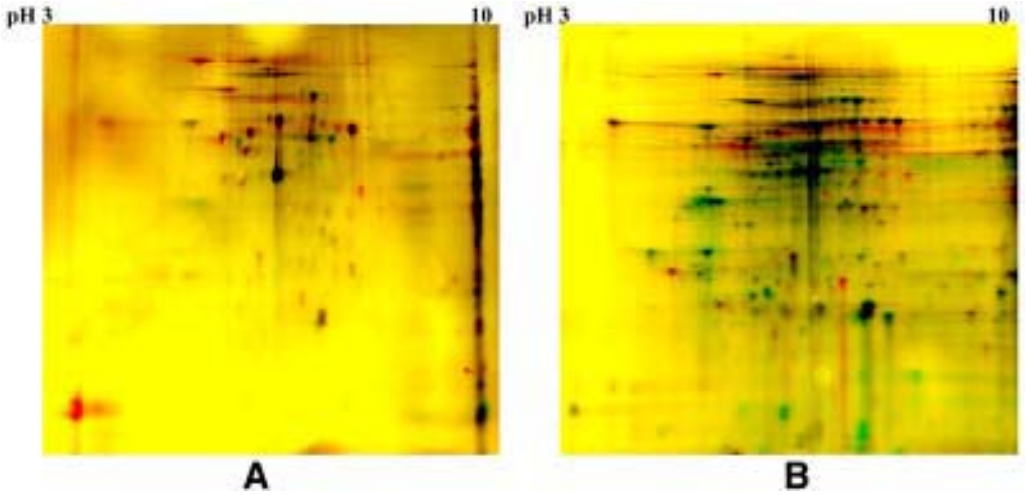

Fig. 1. Representative two-dimensional difference gel electrophoresis (2D-DIGE) images captured with the Typhoon 9400 Variable Mode Imager. The gel images show the signals generated following the labeling and analysis of 100 µg of total protein isolated from breast biopsies using **(A)** the 2D-DIGE "minimal" labeling protocol or **(B)** the 2D-DIGE "saturation" labeling protocol. Note that more candidate protein spots are visible on gel "B."

2. Carefully place equilibrated strips on top of the second-dimension gel and cover strip with melted agarose sealing solution.
3. Perform electrophoresis overnight (approx 16 h at 15°C and 2 W/gel). The best resolution in the second dimension is achieved if electrophoresis is performed overnight.

3.6. Image Capture, Spot Matching, and Spot Processing

The separated proteins are visualized by imaging on a fluorescence imager. In our high-throughput proteomics laboratory, we use the Typhoon 9400 Variable Mode digital imager for capturing of gel images after 2-D DIGE (**Fig. 1**). The protein spots are matched with the DIA and BVA modules of DeCyder, the gels are stained with SYPRO Ruby, a pick list is generated, and the protein spots are picked and processed using spot pickers and digesters or a fully automated spot-handling workstation ***(1)***. The use of DeCyder software, the Ettan spot picker, Ettan Digester (GE Healthcare Biosciences), or Ettan automated spot-handling workstation are beyond the scope of this protocol. Only brief overviews are presented. Detailed protocols can be found in the manuals accompanying each product.

3.6.1. Scanning of 2-D DIGE Gels

1. Carefully transfer gel to a suitable fluorescent scanner, e.g., the Typhoon Variable Mode Imager (GE Healthcare Inc.). Protect from light to avoid bleaching of dyes.
2. Obtain two scans of each gel (e.g., Cy2 and Cy3) if a two-color experiment was performed, and three scans of each gel (Cy2, Cy3, and Cy5) if a three-color experiment was performed. The excitation/emission wavelengths for Cy2, Cy3, and Cy5 are 488 nm/520 nm, 532 nm/580 nm, and 633 nm/670 nm, respectively. Use the protocol recommended for the scanner to position, select resolution (pixel size), and set focal plane.
3. Process the acquired image with image analysis software, e.g., DeCyder.

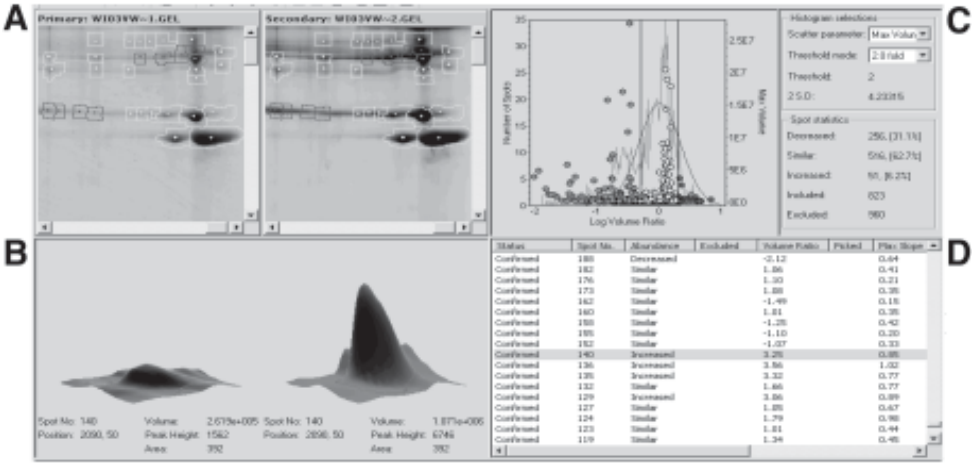

Fig. 2. Screen shot showing the DeCyder Difference In gel Analysis (DIA) workspace divided into four windows. **(A)** Image view: Cy3 (*primary window*) and Cy5 (*secondary window*) images generated by scanning a single DIGE gel at two wavelengths, **(B)** 3-D view: Simulated 3-D representation of the gel region shown in panel "A." **(C)** Histogram view: Shows the graphical representation of the data associated with the spot population, parameters used for generation of histogram and spots statistics window. In this example, the spots within the window defined by the two vertical lines sandwiching the peak (*twofold difference*) occur at similar levels between sample 1 (*primary*) and sample 2 (*secondary*), spots on the left of the window are more abundant in sample 1 and spots on the right of the window are more abundant in sample 2. Out of the 823-candidate proteins considered 516 (62.7%) were similar and 51 (6.2%) occurred at higher levels in sample 2. **(D)** Table view: Shows the tabulated data associated with the selected spot shown in panel "A" and "B."

3.6.2. Spot Matching

1. Use software capable of processing multiple 2-D images from a single and multiple gels. The most robust software currently available for DIGE is DeCyder. The co-detection algorithms in DeCyder permit comparison of the protein abundance of each sample to the reference, and generation of a 3-D image (**Fig. 2**).
2. Using the software, generate the ratio of (Cy3:Cy2) and (Cy5:Cy2) from all the gels, so that cross-sample comparison of protein abundance between samples on different gels can be performed.
3. Using the BVA module in DeCyder, automatically match the position of each protein spot on all the gels to a master gel and plot the relative abundance of each protein against the normalized internal standard (**Fig. 3**).

3.6.3. Staining With SYPRO Ruby, Generating Picking List, Picking and Processing Protein Spots

The picking and identification of protein spots by mass spectrometry after 2-D DIGE requires assigning of proteins for picking on the analyzed DIGE gels, spot detection on the preparative gel (gel prepared for picking and stained with SYPRO Ruby), identification of reference markers on gels, and exporting the picking list to the spot picker. All spots detected can be assigned for picking, or spots can be selected for picking based on the experimental design or specific properties of each spot.

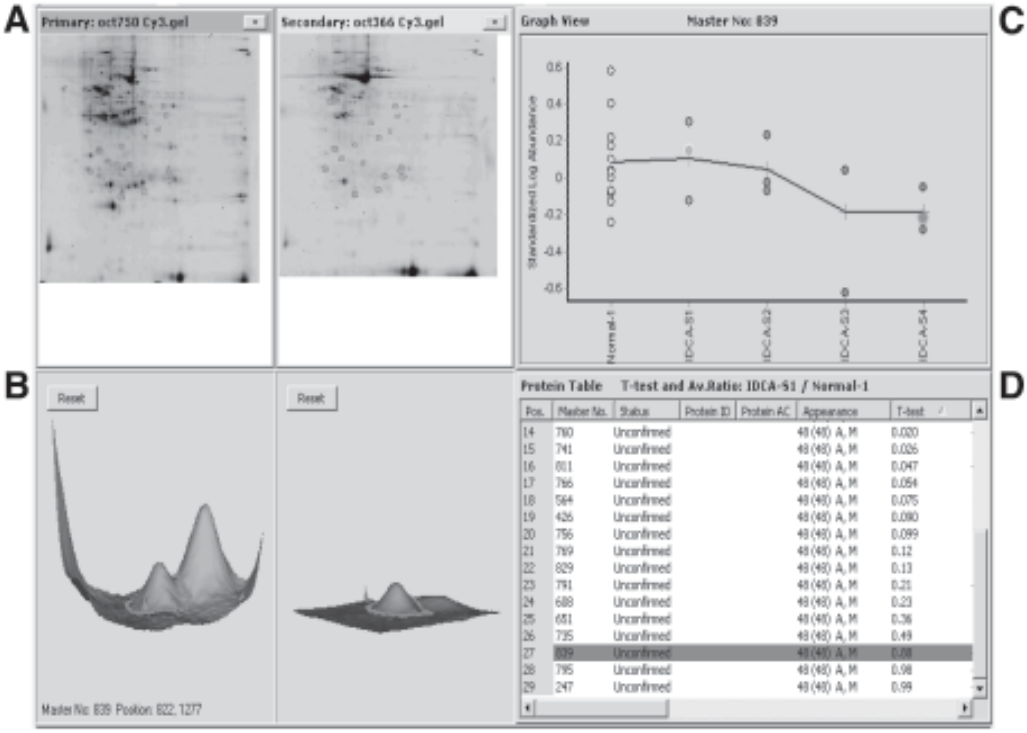

Fig. 3. Screen shot showing the DeCyder Biological Variation Analysis (BVA) workspace, divided into four windows. **(A)** Image view: Cy3 (*primary window*) and Cy5 (*secondary window*) images generated by scanning a single DIGE gel at two wavelengths, **(B)** 3-D view: Simulated 3-D representation of the gel region shown in panel "A." **(C)** Graphical view: Shows the graphical representation of the data associated with spot population. In this example, the relative abundance of protein number 839 in "normal" breast tissue and stage 1 to stage 4 IDCA of the breast is shown. In this examlple, there were twelve normal samples and three each for stage 1–4 IDCA. The line trace is the mean. **(D)** Table view: Shows the tabulated data associated with the selected spot shown in panel "A" and "B."

3.6.3.1. SYPRO Ruby Staining of 2-D DIGE Gels

To pick and identify proteins after 2-D DIGE, the gels must be stained with a fluorescent dye like SYPRO Ruby to ascertain that the majority of the unlabeled spots are detected and picked. All staining should be done in the containers suitable for SYPRO Ruby. Gels must be protected from light during the entire process to prevent bleaching of the dye.

1. Remove the top glass plate. The top is the plate that was not treated with Bind-Silane.
2. Place the DIGE gel, still attached to the bottom glass plate, into a suitable container (e.g., polypropylene, polycarbonate, or polyvinyl chloride tray).
3. Add SYPRO Ruby gel fix solution and incubate for at least 2 h on a shaking platform.
4. Pour off gel fixing solution.
5. Add SYPRO Ruby stain. Add enough stain to completely cover gel, protect from light, and incubate for at least 5 h or overnight with gentle shaking.
6. Pour off stain and wash gel with SYPRO Ruby gel destain solution for 1–2 h.

7. Briefly rinse gel with reagent-grade water.
8. Dry the back of the glass plate.
9. Place gel, glass side down, onto a clean, dust-free surface.
10. Wet the bottom edge of the gel with distilled water.
11. Place a clean, low-fluorescence glass plate on the gel, taking care not to damage gel or form bubbles.
12. Pick the gel up and drain excess water carefully.
13. Dry the outside of the glass plates.
14. Image the gel with the appropriate filter set and exposure times.
15. After scanning, remove the top plate and store the gel in the gel fixing solution.

3.6.3.2. Generating Picking List

Suitable software is needed for assigning of spots for picking. In our laboratory, we use the DIA and BVA modules of DeCyder for 2-D DIGE gel analysis and generation of the pick list. The DIA module is used for assigning spots for picking in small-scale experiments utilizing two samples. All spots can be picked by selecting a "Pick All" check box in DeCyder DIA workspace. The BVA module of DeCyder is used for assigning protein spots for picking based on the statistical information generated from the BVA workspace.

1. Upload a pair of analysis maps into the DIA module of DeCyder.
2. Identify the reference markers on the preparative gel.
3. Select the "Pick All" check box to pick all protein spots detected, or select spots for picking using the "Pick Filter" function.
4. The protein spots showing differences in expression can be picked on the basis of their physical properties—e.g., area, volume, and peak height.
5. Assign the analysis gel as the master gel, and then assign this master gel as the pick spot map.
6. From the spot map table select the preparative gel as the primary image and generate the picking list.
7. Transfer the picking list to the preparative gel.
8. Transfer the list to the spot picker and pick spots into microtiter plates. Use the protocol recommended for the spot picker and spot digester.

3.6.3.3. Processing Protein Spots

After identification of all protein spots of interest, the spots have to be excised from the gel manually or automatically with robotic spot pickers. In our laboratory, we use the Ettan spot picker for spot picking and the Ettan Digester for spot digestion, or the Spot Handling Workstation for fully automated spot picking, digestion, and matrix-assisted laser desorption/ionization (MALDI) target slide preparation ***(1)***. The procedure must be performed in a clean environment to prevent the entry of contaminating proteins, particularly keratin, into the protein identification workflow process at this stage (*see* **Note 6**).

4. Notes

1. A cell wash buffer should not contain primary amines, because primary amines such as ampholytes will compete with the proteins for fluors, thereby reducing the total number of proteins labeled.
2. Protein breakdown must be prevented. Homogenize the tissue immediately in lysis buffer and keep tube on ice all the time. When using a motorized homogenizer, make sure that the sample is not heated during the process by placing on ice intermittently.

Table 3
Recommended Amounts of TCEP and Dye Required for Labeling Optimization*

Gel	2 m*M* TCEP (μL)	TCEP (nmol)	2 m*M* 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

*Reproduced from the GE Healthcare saturation labeling protocol handbook.

3. The recommended protein concentration of the sample is between 5 and 10 mg/mL; however, samples containing 1mg/mL have been successfully labeled and used for 2-D DIGE.
4. For the best results, the amount of TCEP and CyDye DIGE used needs to be determined and optimized for each protein type being analyzed, or when a nonstandard cell lysis buffer is to be used. The recommended amounts are shown in **Table 3**.
5. Note that the IEF parameters may be different for different sample types and different batches of the same sample type.
6. Although keratins are a major contaminant, and attempts should be made to prevent their introduction into the workflow process, note that cytoskeletal keratins (especially the Type 1, e.g., the 40 kDa KRT19, also known as CK19, an epithelial biomarker) show differential expression as a function of disease. Many other keratins are also within the resolving limits of 2-D PAGE, and so when detected may not be a contaminant. Caution should, however, be used when interpreting results if it is observed that a batch of test and reference samples processed in the same way and separated in the same run all show elevated and/or comparable levels of keratin.

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