NIH-PA Author Manuscript
NIH-PA Author Manuscript
NIH-PA Author Manuscript

NIH Public Access
Author Manuscript
Nat Protoc. Author manuscript; available in PMC 2007 October 8.

Published in final edited form as:
Nat Protoc. 2006 ; 1(4): 1732–1742.

Two-dimensional fluorescence difference gel electrophoresis for comparative proteomics profiling

Nilesh S Tannu and **Scott E Hemby**
Department of Physiology and Pharmacology, Wake Forest University School of Medicine, Winston-Salem, North Carolina 27157, USA.

Abstract

Quantitative proteomics is the workhorse of the modern proteomics initiative. The gel-based and MuDPIT approaches have facilitated vital advances in the measurement of protein expression alterations in normal and disease phenotypic states. The methodological advance in two-dimensional gel electrophoresis (2DGE) has been the multiplexing fluorescent two-dimensional fluorescence difference gel electrophoresis (2D-DIGE). 2D-DIGE is based on direct labeling of lysine groups on proteins with cyanine CyDye DIGE Fluor minimal dyes before isoelectric focusing, enabling the labeling of 2–3 samples with different dyes and electrophoresis of all the samples on the same 2D gel. This capability minimizes spot pattern variability and the number of gels in an experiment while providing simple, accurate and reproducible spot matching. This protocol can be completed in 3–5 weeks depending on the sample size of the experiment and the level of expertise of the investigator.

INTRODUCTION

The comprehensive sequencing of human and other important genomes has immensely enhanced our insight into the cellular machinery of higher organisms[1]. This has largely been accomplished by the innovations in large-scale analysis of mRNA expression, through the advent of high-throughput technologies such as microarrays, serial analysis of gene expression and differential display[2]. Similar methods for the analysis of global protein expression using high-throughput gel- and non-gel-based protein fractionation techniques coupled to protein identification by high-throughput tandem mass spectrometry (MS/MS)-based automated software algorithms have evolved gradually over the last few decades to their present state[3–14]. These techniques are essential for expanding our understanding of the intricate orchestration of cellular events and for enabling synthesis of the genome with proteome data in health and disease. The necessity to evaluate the proteome is based on the knowledge that higher organisms have several means for controlling cellular function and that proteins mediate the greater part of biological events in the cell, even though certain RNAs can act as effector molecules. However, there are several factors that prevent the linear association of RNA and protein levels (transcription to translation)[15,16], including single genes that can be translated in several protein products, differential responsivity of mRNA and proteins to intra- and extracellular stimuli, post-transcriptional changes in mRNA (alternative splicing, RNA editing), post-translational modifications of proteins that affect functionality and trafficking (phosphorylation, glycosylation, methylation, palmitoylation)[17] and, most recently, the discovery of microRNAs, which regulate the conversion of mRNAs to proteins[18–20]. To evaluate comprehensively the cellular alterations in health and disease, it is essential to determine the alternations in proteins in as complete a manner as possible[21]. To harness the vast amount of proteomic information available in the cell, there has been an expansion and

Correspondence should be addressed to S.E.H. (shemby@wfubmc.edu).

COMPETING INTERESTS STATEMENT The authors declare that they have no competing financial interests.

NIH-PA Author Manuscript NIH-PA Author Manuscript NIH-PA Author Manuscript

development of various separation chemistries, mass spectrometry (MS) and bioinformatics[22–24]. Rather than traditional approaches such as Western blot analysis or immunocytochemical techniques, which examine one or a few proteins at a time, the development of proteomic technologies has enabled the concurrent examination of large numbers (thousands) of proteins in multiple samples. Technologies that allow simultaneous assessment of multiple samples in a quantitative manner (i.e., expression proteomics) have become the preferred approaches[25]. Gel-based and MuDPIT approaches have enabled important advances in the measurement of protein expression alterations in normal and disease states and provide complementary analysis of proteins within samples[22,26].

2DGE is a widely used separation method in which proteins are first separated by isoelectric focusing (IEF) and then by molecular mass in the second dimension on application of an electrical potential across a solid-based gel. In general, approximately 1,000–2,000 protein spots can be visualized on a gel, depending on the visualization technique, the p*I* range of the first dimension and the size of the 2DGE. Some of the inherent caveats/disadvantages associated with 2DGE are as follows: (i) many protein spots are likely comprised of multiple proteins; (ii) individual proteins may migrate as multiple spots based on differential digestion; (iii) labor-intensive image analysis requires gel matching and manual removal of artifacts, (iv) poor spot resolution at higher p*I* values, (v) difficulty in electrophoresing large and hydrophobic proteins in the first dimension separation; and (vi) extreme acidic and basic proteins are not well represented[27]. The technical variability seen with 2DGE is due to sample preparation, sources of reagent, staining methods, image analysis software and individual experimenter variability, and contributes a coefficient of variation of approximately 20–30%[28].

One of the recent technical advances in 2DGE has been the development of the multiplexing fluorescent 2D-DIGE method[11], which relies on direct labeling of the lysine groups on proteins with cyanine (Cy) dyes before IEF. A critical aspect of the use of 2D-DIGE technology is the ability to label 2–3 samples with different dyes and electrophorese all the samples on the same 2D gel, thus reducing spot pattern variability and the number of gels in an experiment and yielding simple and accurate spot matching. The CyDye DIGE Fluor minimal dye has an *N*-hydroxysuccinimidyl ester reactive group (Fig. 1). It is designed to form a covalent bond with the epsilon amino group of lysine in proteins via an amide linkage. The single positive charge of the CyDye replaces the single positive charge of the lysine at neutral and acidic pH, keeping the p*I* of the protein relatively unchanged. The labeling reaction is dye limiting and the ratio of CyDye and the sample (100–300 pmol:50 μg) ensures that the dyes label approximately 1–2% of lysine residues. Therefore, each labeled protein carries only one dye label and is visualized as a single protein spot. It should be noted that CyDye labeling adds a mass of approximately 500 Da to labeled proteins; however, this addition does not affect the 2D gel pattern, as all visualized proteins are labeled. “Minimal” labeling is performed to tag only one lysine residue in each protein to prevent the vertical train of spots due to the added mass of each fluorophore and to prevent protein precipitation due to increased hydrophobicity. The ratio has been optimized to label the less abundant proteins, at the same time keeping the highly abundant proteins in the linear dynamic range for quantitative image analysis. The labeling reaction is quenched by the addition of lysine.

The more popular experimental design for 2DIGE is the use of a pooled internal standard (sample composed of equal aliquots of each sample in the experiment) labeled with the Cy2 dye and labeling the control and the diseased/treatment groups with either Cy3 or Cy5 dyes counterbalanced across the samples (Fig. 1). The internal standard is essential for assessing biological and experimental (between gels) variations and increasing the robustness of statistical analysis. The individual protein data from the control and diseased/treatment (Cy5 or Cy3) samples are normalized against the Cy2 dye-labeled sample, Cy5:Cy2 and Cy3:Cy2. The CyDye-labeled proteins, fractionated by 2D-DIGE, are scanned by a Typhoon variable

NIH-PA Author Manuscript NIH-PA Author Manuscript NIH-PA Author Manuscript

mode imager. Sequential scanning of Cy2-, Cy3- and Cy5-labeled proteins is achieved by the following laser/emission filters: 488/520, 532/580 and 633/670nm, respectively. Scanned images of fluorescently labeled proteins are sequentially analyzed by differential in-gel analysis (DIA; performs Cy5/Cy3:Cy2 normalization; Fig. 2) followed by biological variation analysis (BVA; performs inter-gel statistical analysis to provide relative abundance in various groups; Fig. 3). Log abundance ratios are then compared between control and diseased/treatment samples from all gels using the chosen statistical analysis (e.g., *t*-test and ANOVA) from software packages such as DeCyder (GE HealthCare) or Progenesis (Nonlinear)[29–31]. 2D-DIGE offers the most reliable quantitation of any 2DGE method, is comparable in sensitivity to silver staining method and is compatible with the downstream MS protein characterization (as most lysine residues remain untagged and are accessible for tryptic digestion). This method has the advantage of being able to quantify the protein spots that are uniquely present in one group owing to the presence of an internal standard. The accuracy of quantitation as well as the statistical confidence obtained for the differentially regulated gene products is significantly greater with 2D-DIGE than with 2DGE, although one caveat is ambiguity for lower abundance protein spots[3,31,32]. A significant caveat of the approach is that proteins with a high percentage of lysine residues are possibly labeled more efficiently than proteins with few or no lysines. Therefore, the possibility remains that a high-abundance protein spot in the conventional 2DGE can be a medium- or even low-abundance protein in 2D-DIGE owing to low lysine content. The lesser abundance proteins detected by 2D-DIGE are mostly unidentifiable, owing to the limitations of the current in-gel digestion as well as the detection limits of most of the currently available mass spectrometers. Another potential drawback of 2D-DIGE is that the fluorophores, equipment and software are currently proprietary to GE Healthcare and therefore may be financially limiting for academic labs. Nonetheless, the 2D-DIGE approach offers tremendous promise and has been used increasingly by researchers to address a wide range of biological questions, including substance abuse[3] and other psychiatric illnesses[33–35], hematology/oncology[36–49], toxicity[50–53], as well as proteomic profiling in eukaryotic[54–57] and prokaryotic cells[58–67].

MATERIALS

REAGENTS

- 2D clean-up kit (cat. no. 80-6484-51; GE HealthCare); alternative sources are ReadyPrep 2-D Cleanup Kit (Bio-Rad) and 2-D Sample Prep Kits (Pierce Biotechnology)
- Sample buffer (30 mM Tris-HCl, 2 M thiourea, 7 M urea and 4% CHAPS, pH 8.5)
- 0.1 NaOH
- 2D-Quant kit (cat. no. 80-6484-51; GE HealthCare)
- CyDye DIGE Fluor minimal dyes 2, 3, and 5 (cat. no. 80-6484-51; GE HealthCare)
- >99.5% pure dimethylformamide (DMF: USB Corporation)
- 10 mM lysine
- Rehydration buffer (2 M thiourea, 7 M urea, 2% dithiothreitol (DTT), 4% CHAPS and 2% Pharmalyte (GE HealthCare))
- Destreak rehydration buffer (GE HealthCare)
- Immobiline DryStrips (240×3×0.5 mm, linear 4–7/6–9/3–10 pH; GE Healthcare); alternative sources are Bio-Rad, Sigma-Aldrich and Isogen Lifesciences

NIH-PA Author Manuscript NIH-PA Author Manuscript NIH-PA Author Manuscript

- Reducing buffer (6 M urea, 1.5 M Tris-HCl, pH 8.8, 30% (vol/vol) glycerol, 2% (wt/vol) SDS and 2% (wt/vol) DTT). ▲ **CRITICAL** Ensure that all the solutions containing urea are prepared freshly and also not heated above 37°C to prevent protein carbamylation and subsequent formation of charge trains on the 2D gel.
- Alkylating buffer (6 M urea, 1.5 M Tris-HCl, pH 8.8, 30% (vol/vol) glycerol, 2% (wt/vol) SDS and 2.5% (wt/vol) iodoacetamide)
- Pre-cast 8–15% gradient SDS-PAGE (2,400×2,000×1 mm; Jule Inc.)
- Fixing solution (30% (vol/vol) methanol and 7.5% (vol/vol) acetic acid)
- SyproRuby (Molecular Probes)
- Destain solution (10% methanol and 6% glacial acetic acid)
- Ammonium bicarbonate
- Acetonitrile
- Methanol
- Trifluoroacetic acid (TFA)
- Sequencing grade lyophilized trypsin (Promega)
- α-Cyano-4-hydroxy-cinnamic acid (CHCA; Sigma-Aldrich)

EQUIPMENT

- Ettan™ IPGphor™ apparatus (GE HealthCare); alternative equipment is PROTEAN IEF System (Bio-Rad), Multiphor II Horizontal Electrophoresis Unit (PerkinElmer) or UniPhor Horizontal Electrophoresis Unit (Sigma-Aldrich)
- Ettan Dalt II System (GE HealthCare); alternative equipment is PROTEAN Plus Dodeca™ Cell (Bio-Rad).
- Typhoon 9400™ scanner (GE Healthcare); alternative equipment is FLA 5100 Imaging System (FUJIFILM) or Ettan DIGE imager (GE HealthCare)
- Ettan™ Spot Handling Workstation (GE HealthCare)
- Mass spectrometer: Applied Biosystems 4700 Proteomics Analyzer (MALDI-TOF/TOF); most other mass spectrometers are capable of analysis; however, MALDI-TOF-TOF has an advantage of higher throughput and provides detailed peptide sequence analysis
- DeCyder™ (GE HealthCare); alternative software is Progenesis (Nonlinear Dynamics)—the most automated of the softwares available for DIGE analysis—or Delta2D (DECODON)
- ImageQuant™ (GE HealthCare); alternative software is MultiGauge (FUJIFILM)
- PS Explorer (Applied Biosystems)

PROCEDURE

Preparation

1| Precipitate the protein sample using the 2D clean-up kit according to the manufacturer's recommendations at −20 °C overnight.

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

2| The next day, pellet the sample by centrifugation at 13,400*g* for 5 min at 4 °C and air-dry the pellet for 2 min.

▲ **CRITICAL STEP** The pellet will not dissolve in sample buffer if over-dried.

3| Resuspend the pellet in adequate rehydration buffer. Start by adding 50 μl of sample buffer and add additional 10 μl volumes until the pellet dissolves.

▲ **CRITICAL STEP** Add DTT and IPG buffer fresh to the rehydration buffer.

4| Determine the protein concentration using the 2D-Quant kit. It is compatible with the reagent concentration in the sample buffer.

5| Make sure that the protein concentration is in the range of 5–10 mg ml^{-1} by adequately concentrating or diluting the samples.

6| Prepare a normalization pool (standard pool) comprising equal amounts from all the samples present in the two conditions to be compared.

▲ **CRITICAL STEP** Make sure that each sample from both the conditions is used in the exact same amount to make up the internal standard. This is crucial, so that every protein in the experiment has an internal standard and for the following to be true, "This method has the advantage of being able to quantify the protein spots which are uniquely present in one group due to the presence of internal standard".

7| Check the pH of all the samples by adding a very small amount of sample (3 μl) to a pH indicator strip. Make sure that the sample pH is between 8.0 and 9.0. Usually you have to increase the pH using 0.1 NaOH or 30 mM Tris-HCl.

8| Reconstitute the CyDye DIGE Fluor minimal dye in 10 μl of DMF by centrifuging at 12,000*g* for 30 s to make a stock of 1,000 pmol μl^{-1}. Ensure that the dye has a deep color at this point (Cy2–yellow, Cy3–red and Cy5–blue). Wrap the tubes in aluminum foil and return to −20 °C until needed for use.

▲ **CRITICAL STEP** Ensure that the DMF is water free as the reactive groups of the dyes are very water-sensitive. Once reconstituted, the Cy dyes should be used within 3–4 weeks, ensuring water-free storage conditions (e.g., use silica gel).

9| Create a working solution of 200 pmol μl^{-1} of CyDye by adding 4 μl of DMF to 1 μl of stock solution. Based on the protein quantitation, label 50 μg of each sample from conditions 1 and 2 with 1 μl of working solution of either Cy3 or Cy5 dye in such a way that Cy3 and Cy5 are swapped equally among the samples from the two conditions (Fig. 1). At the same time, label an aliquot of 50 μg of internal standard sample with 1 μl of working solution of Cy2 label to be mixed with corresponding aliquots of Cy3- and Cy5-labeled samples for each gel. Mix the sample and dye by vortexing vigorously and keep the sample on ice for 30 min in the dark.

▲ **CRITICAL STEP** A preliminary study has to be performed to ensure optimum labeling of the comparatively less abundant protein spots while still maintaining the most abundant protein spots in the dynamic linear range for quantitative image analyses. A range of ratios for protein concentration:CyDye amount (50 μg: 100 pmol to 50 μg: 400 pmol) should be tested to ensure the optimal ratio for the sample of interest.

10| Now add 10 μl of 10 mM lysine to each sample to quench the labeling reaction. Vortex, centrifuge at 13,400*g* for 10 s and keep on ice for 10 min.

■ **PAUSE POINT** The labeled samples can be stored at −70 °C for up to 3 months.

11| At this stage, matched samples from conditions 1 and 2 (labeled with Cy3 or Cy5) along with an aliquot of internal standard (labeled with Cy2) should be pooled together.

NIH-PA Author Manuscript NIH-PA Author Manuscript NIH-PA Author Manuscript

Thus, each 2D gel will have three labeled samples: one each from internal standard, and conditions 1 and 2. This is unique to 2D-DIGE when compared with traditional 2DGE.

12| Create a sample for the 'pick gel', with equal amounts of all the samples (conditions 1 and 2) mixed together to make up 750–950 µg, combined with 50 µg of Cy2-labeled internal standard.

13| Add adequate rehydration buffer to make the volume of the samples prepared in Steps 11 and 12 up to 450 µl and add 50 µl of Destreak rehydration buffer. The samples are ready for IEF.

14| Perform the IEF according to the setup shown below using the 24-cm pH 4–7 NL Immobiline DryStrips on an Ettan™ IPGphor™ apparatus. First, an appropriate sample volume should be loaded into the IPG cup loading tray holder. With utmost care, remove the protective cover from the IPG DryStrip and position it with the gel side down into the IPG tray. Ensure that the acidic end approximates the positive electrode of the IPGphor apparatus and that no bubbles are trapped under the IPG DryStrip. Finally, overlay PlusOne DryStrip Cover Fluid over the DryStrip to prevent evaporation and urea crystallization. The methodology should be optimized for the tissue in context by varying the voltage and duration of different steps, ensuring that the rehydration is always ≥ 10 h for adequate current flow during IEF.

Method	Volts	Volt-hours	Duration (h)
Step-and-hold	0	0	10
Step-and-hold	100	100	1
Step-and-hold	500	500	1
Step-and-hold	1,000	1,000	1
Gradient	8,000	13,500	1.7
Step-and-hold	8,000	60,000	7.5
Step-and-hold	500		24

▲ **CRITICAL STEP** The length of the pH strip, its pH range as well as the IEF setup should be empirically determined to provide the best possible resolution for your sample of interest.

■ **PAUSE POINT** The Immobiline DryStrips can be stored at −70 °C for up to 3 months. Ensure that the strips are kept in a rigid container, as the frozen strips are very brittle.

15| Equilibrate the IEF DryStrips to reduce the disulfide bonds by gently rocking them in 10 ml of reducing buffer/strip for 10 min. Immediately after this, alkylate the -SH groups of proteins by gently rocking the proteins in 10 ml of alkylating buffer/strip for 10 min. The SDS in the buffers also helps the proteins to acquire a negative charge, which drives their migration under the electrical current. Before proceeding to the next step, rinse the IEF strip in the SDS electrophoresis running buffer.

▲ **CRITICAL STEP** Use fresh DDT and iodoacetamide in the reducing and alkylating buffers, respectively. Do not exceed the stipulated times of alkylation and reduction, as there is a possibility of protein loss. Perform this step as close as possible to running the second dimension.

16| Prepare a 0.6% agarose overlay solution by heating. Slowly pipette the agarose solution, making sure that no bubbles are introduced, up to the top of the glass plate of a pre-cast 8–15% gradient SDS-PAGE. Place the IEF strip between the glass plates and push it with a thin plastic spacer ensuring that the IEF strip rests on the SDS-PAGE.

▲ **CRITICAL STEP** Avoid the cooling and solidifying of agarose before inserting the IEF strip. Avoid inserting any bubbles between the IEF strip and the SDS-PAGE. Ensure

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

that low-fluorescence glass plates with a reference marker are used. This is critical for the background pixel values of the scanned images to be at the lowest possible. Also ensure that all the pre-cast gels are from the same batch to avoid variability in the second dimensional fractionation of proteins. Ensure that the agarose has solidified before starting the second dimension run.

17| Fill the buffer tank of the Ettan Dalt II System with SDS electrophoresis running buffer and cool it to 15 °C. At 15 °C, load wet gel cassettes (with IEF strips) along with blank cassettes (depending on the number of gels to be run on a unit that is capable of running 12 gels at a time). Once all the cassettes are in place, pour running buffer to attain a level in the tank that is mid-way between the minimum and maximum permissible levels. The proteins are then separated on the basis of their molecular weight at 4 W overnight until the bromophenol blue dye front reaches the bottom of the gel.

18| Fix the slab gels, 2D-DIGE (if 'n' ≥ 10 gels) and the 'pick gel', overnight in fixing solution followed by washing the gels twice with distilled water (DW; 500 ml) for 10 min per wash. After fixation, stain the 'pick gel' overnight with 500 ml of SyproRuby™ stain. The next day, wash away excess stain by washing two times for 10 min each with destaining solution[29]. Alternatively, DeepPurple™ can be used as a post-stain for the 'pick gel'. This stain has some advantages over SyproRuby™ with regard to the success of protein identification[68].

19| Scan the gels using a Typhoon 9400 scanner at 100 μm resolution, as elaborated in the equipment setup. The first step is to perform a quick pre-scan at 500 μm resolution to figure out an optimal photomultiplier tube (PMT) value. Once a PMT value is noted for each channel, which gives the desired pixel intensity, measured by ImageQuant, the gels should be scanned at 100 μm resolution.

CyDye	Laser	Excitation filter (nm)	Emission filter (nm)	PMT	Maximum pixel intensity
Cy2	Blue	488	520 BP	Variable	80,000–95,000
Cy3	Green	532	580 BP	Variable	80,000–95,000
Cy5	Red	633	670 BP	Variable	80,000–95,000

▲ **CRITICAL STEP** It is essential that the maximum pixel intensities of all the three images do not differ from each other by more than 10,000–15,000. If the most intense protein spot is saturated (> 100,000), the gels should be re-scanned using a lower PMT value to bring all the protein spots in the linear dynamic range. This is crucial to obtain any meaningful quantitative comparison between the gel images.

20| Analyze the image using the DeCyder™ software (Fig. 2). The DIA should be used for intra-gel analysis for protein spot detection as well as for normalization of Cy3 and Cy5 gel images with respect to the Cy2 image. After spot detection, the abundance changes are represented by the normalized volume ratio (Cy3:Cy2 and Cy5:Cy2).

▲ **CRITICAL STEP** Make sure that extraneous protein spots are removed and that all true protein spots are included by manually examining all the protein spots detected. The eventual quantitative data are very robust if this is adhered to strictly, even though it is a time-consuming process. On the basis of experience for our system, the following spot filtering parameters help us significantly in reducing the time required to achieve the above: slope >1.0, area <350, peak height <350 and volume <100,000. These can be optimized for a particular system based on the distribution of protein spots and their intensities.

21| Use the BVA for inter-gel analysis (Fig. 3). After manually landmarking all the gels, the remaining protein spots should be matched in the automatic mode. The protein spot

NIH-PA Author Manuscript NIH-PA Author Manuscript NIH-PA Author Manuscript

matches should then be confirmed manually for all the gels. A Student's *t*-test should be applied to generate a list of protein spots that were differentially regulated between conditions 1 and 2. The Protein Statistics section included in the BVA can also be used for two-way ANOVA between conditions 1 and 2 as well as for one-way ANOVA between different groups, if more than two conditions have been included in the study. A significant caveat is that for lower abundance protein spots, the statistical significance of the data will not always be true and should be validated by other confirmatory techniques such as western blotting.

22| The protein spots of interest, differentially as well as constitutively regulated, should be picked by a Spot Picking Ettan™ Spot Handling Workstation. Differentially expressed spots provide information about proteins that may be regulated in the experimental or disease state. Constitutively expressed spots provide a more complete assessment of the proteome of interest, to account for normal variation in proteomic assessment between samples and build the sequence databases for the organism and tissues of interest.

▲ **CRITICAL STEP** The calibration of the *Z*-dimension is very crucial, as the head can be easily damaged if the proper configuration is not achieved. It is a good idea to program a critical parameter to avoid the head from being critically damaged. Also, one has to be careful to place the 2D gel holders and gel plug well plates in the correct location according to the particular programmed run. Otherwise, the robotics will not function properly and will require tedious and time-consuming efforts to re-baseline the equipment.

Processing for mass spectrometric analysis

23| Wash the gel plugs twice for 20 min using 50 mM ammonium bicarbonate/50% (vol/vol) methanol in water. Follow it with one more wash with 75% (vol/vol) ACN in DW for 30 min or until the gel plugs turn opaque. Dry the gel plugs in a SpeedVac at 40 °C for 10 min and cool the samples to room temperature (25 °C) before proceeding.

24| If reliable mass spectral data are not obtained, first reduce the samples by incubating at 56 °C for 45 min in 40 µl of 50 mM DTT/100 mM ammonium bicarbonate. Discard the solution and cool the samples. Then, immediately alkylate the samples by adding 40 µl of 100 mM iodoacetamide and incubating them at room temperature for 30 min in the dark. Discard the solution.

25| Give a final wash by adding 100 µl of 50 mM ammonium bicarbonate/50% (vol/vol) methanol in water for 10–15 min at room temperature. Dry the gel plugs by vacuum centrifugation.

26| Incubate the gel plugs overnight (12–16 h) at 37 °C in 140 ng of sequencing grade trypsin and transfer the supernatant to a low-retention 96-well plate.

27| Extract the peptides from the gel pieces twice sequentially using 100 µl of extraction buffer (50% (vol/vol) acetonitrile and 0.1% (vol/vol) TFA in DW) and pool it with the above supernatant. Dry the combined supernatants by vacuum centrifugation.

28| Dissolve the dried peptides from each gel plug in 5 µl of 50% (vol/vol) acetonitrile and 0.1% TFA in DW and deposit 0.5 µl on the stainless-steel MALDI target plate. After air-drying the protein spot, add to each spot 0.5 µl of 6 mg ml^{-1} of α-cyano-4-hydroxy-cinnamic acid in 50% (vol/vol) acetonitrile and 0.1% TFA in DW.

Mass spectrometry using Applied Biosystems 4700 Proteomics Analyzer

29| Perform MS on each protein spot as elaborated. The mass spectrometer should be used in reflector mode for positive ion detection. The MS spectra should be accumulated by at least 2,000 laser shots. The laser wavelength and the repetition rate should be 355 nm and

NIH-PA Author Manuscript NIH-PA Author Manuscript NIH-PA Author Manuscript

200 Hz, respectively. The peak detection criteria should be the following: minimum *S/N* of 8; a local noise window width (mass/charge (*m/z*)) of 200; and a minimum peak width at full-width half-maximum (bins) of 2.9. The mass spectra should be calibrated using at least two of the tryptic auto-digest products: fragment 100–107 ($[M+H]^+$=842.51 Da), fragment 98–107 ($[M+H]^+$=1,045.556 Da) and fragment 50–69 ($[M+H]^+$=2,211.105 Da). After acquiring the mass spectra for peptide fragments, the ten most intense precursor ions should be selected for acquiring MS/MS spectra[14]. The criteria used to filter the monoisotopic precursor for the MS/MS should be the following: a minimum *S/N* of 25; exclusion of the most commonly observed peptide peaks for trypsin and keratin; and exclusion of the precursors within 150 resolutions. In the TOF1 stage, all ions are accelerated to 1 kV under conditions promoting metastable fragmentation. The peak detection criteria that should be used are the following: *S/N* of 8 and local noise window width of 250 (*m/z*).

30| Submit the mass lists from MS and MS/MS to GPS Explorer, which primarily uses a MASCOT search engine to search against the appropriate species database from NCBI. The parameters that should be used are that one allowed missed cleavage, ±50 ppm for *m/z* error for MS and 0.1 Da *m/z* error for MS/MS, partial modification of cysteine (carbamidomethyl-cysteine) and methionine (oxidized). The identification of protein is confirmed when the MASCOT confidence interval is greater than 95%. The protein mass and p*I* accuracy on the 2D gel can also be used as a good guide to confirm protein identification. Alternatively, one of the following protein identification search engines can be used: MASCOT: http://www.matrixscience.com/search_form_select.html; ProFound: http://prowl.rockefeller.edu; MS-Fit: http://prospector.ucsf.edu/ or Aldente: http://www.expasy.org/tools/aldente/.

● **TIMING—**Day 1: Step 1, 2 h.

Day 2: Step 2, 15 min; Step 3, 30–60 min; Step 4, 2 h; Step 5, 30–60 min; Step 6, 30–60 min; Step 7, 30–60 min.

Day 3: Step 8, 15–30 min; Step 9, 1–2 h; Step 10, 20–30 min; Step 11, 30–60 min; Step 12, 30–60 min; Step 13, 1–2 h.

Days 4–5: Step 14.

Day 5: Step 15, 30–60 min; Step 16, 1–2 h; Step 17, 30–60 min.

Day 6: Step 18, 30–60 min.

Days 7–10: Step 19, depends on *'n'*, 2–3 gels can be scanned per day.

Days 11–13: Step 20, depends on the level of expertise.

Days 14–16: Step 21, depends on the level of expertise.

Day 17: Step 22.

Day 18: Step 23, 30–60 min; Step 24, 90 min; Step 25, 1–2 h; Step 26, overnight.

Day 19: Step 27, 1–2 h; Step 28, 2–5 h (time is dependent on the number of protein spots spotted onto the MALDI plate).

Day 20: Step 29, 2–8 h (depends on the number of protein spots processed and the level of expertise).

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

Days 21–22: Step 30, depends on the level of expertise.

? TROUBLESHOOTING—Troubleshooting advice can be found in Table 1.

ANTICIPATED RESULTS

Using as little as 75 μg of protein per sample, the 2D-DIGE is can compare quantitative expression levels of 1,000–2,000 unique proteins between two different phenotypic cells, and this is dependent on the cellular fraction, the p*I* range and the size of the 2DGE used for the experiment (Fig. 1). For identification of proteins of interest, however, additional protein (at least ≈50–150 μg per sample) is needed for running a 'pick gel'. Post-staining the 'pick gel' with SyproRuby™/DeepPurple™ dyes is necessary to decide which protein spots should be processed for mass spectrometric analysis. A protein spot with staining intensity more than 9.80×10^5 gives reliable identification of protein by MS.

References

1. Tannu, N.; Hemby, SE. Progress in Brain Research: Functional Genomics and Proteomics in the Clinical Neurosciences. Hemby, SE.; Bahn, S., editors. 158. Elsevier; Amsterdam: 2006.
2. Venter JC, et al. The sequence of the human genome. Science 2001;291:1304–1351. [PubMed: 11181995]
3. Tannu N, Mash DC, Hemby SE. Cytosolic proteomic alterations in the nucleus accumbens of cocaine overdose victims. Mol Psychiatry. 200610.1038/sj.mp.4001914
4. O'Farrell PH. High resolution two-dimensional electrophoresis of proteins. J Biol Chem 1975;250:4007–4021. [PubMed: 236308]
5. Bjellqvist B, et al. Isoelectric focusing in immobilized pH gradients: principle, methodology and some applications. J Biochem Biophys Methods 1982;6:317–339. [PubMed: 7142660]
6. Shomo RE II, Chandrasekaran A, Marshall AG, Reuning RH, Robertson LW. Laser desorption/fourier transform ion cyclotron resonance mass spectrometry: digoxin, digitoxin, and their reduced and sugar-hydrolyzed metabolites. Biomed EnvironMass Spectrom 1988;15:295–302.
7. Wildenauer DB, et al. Analysis of cerebrospinal fluid from patients with psychiatric and neurological disorders by two-dimensional electrophoresis: identification of disease-associated polypeptides as fibrin fragments. Electrophoresis 1991;12:487–492. [PubMed: 1915241]
8. Henzel WJ, et al. Identifying proteins from two-dimensional gels by molecular mass searching of peptide fragments in protein sequence databases. Proc Natl Acad Sci USA 1993;90:5011–5015. [PubMed: 8506346]
9. Pappin DJ, Hojrup P, Bleasby AJ. Rapid identification of proteins by peptide-mass fingerprinting. Curr Biol 1993;3:327–332. [PubMed: 15335725]
10. Jonscher KR, Yates JR III. The quadrupole ion trap mass spectrometer —a small solution to a big challenge. Anal Biochem 1997;244:1–15. [PubMed: 9025900]
11. Unlu M, Morgan ME, Minden JS. Difference gel electrophoresis: a single gel method for detecting changes in protein extracts. Electrophoresis 1997;18:2071–2077. [PubMed: 9420172]
12. Yates JR III. Database searching using mass spectrometry data. Electrophoresis 1998;19:893–900. [PubMed: 9638935]
13. Gygi SP, et al. Quantitative analysis of complex protein mixtures using isotope-coded affinity tags. Nat Biotechnol 1999;17:994–999. [PubMed: 10504701]
14. Tannu NS, et al. Paraffin-wax-coated plates as matrix-assisted laser desorption/ionization sample support for high-throughput identification of proteins by peptide mass fingerprinting. Anal Biochem 2004;327:222–232. [PubMed: 15051539]
15. Anderson L, Seilhamer J. A comparison of selected mRNA and protein abundances in human liver. Electrophoresis 1997;18:533–537. [PubMed: 9150937]
16. Gygi SP, Rochon Y, Franza BR, Aebersold R. Correlation between protein and mRNA abundance in yeast. Mol Cell Biol 1999;19:1720–1730. [PubMed: 10022859]

NIH-PA Author Manuscript NIH-PA Author Manuscript NIH-PA Author Manuscript

17. Roberts GC, Smith CW. Alternative splicing: combinatorial output from the genome. Curr Opin Chem Biol 2002;6:375–383. [PubMed: 12023119]
18. Lagos-Quintana M, Rauhut R, Lendeckel W, Tuschl T. Identification of novel genes coding for small expressed RNAs. Science 2001;294:853–858. [PubMed: 11679670]
19. Lee Y, Jeon K, Lee JT, Kim S, Kim VN. MicroRNA maturation: stepwise processing and subcellular localization. EMBO J 2002;21:4663–4670. [PubMed: 12198168]
20. Sontheimer EJ, Carthew RW. Silence from within: endogenous siRNAs and miRNAs. Cell 2005;122:9–12. [PubMed: 16009127]
21. Freeman WM, Hemby SE. Proteomics for protein expression profiling in neuroscience. Neurochem Res 2004;29:1065–1081. [PubMed: 15176464]
22. Aebersold R, Mann M. Mass spectrometry-based proteomics. Nature 2003;422:198–207. [PubMed: 12634793]
23. Schrader W, Klein HW. Liquid chromatography/Fourier transform ion cyclotron resonance mass spectrometry (LC-FTICR MS): an early overview. Anal Bioanal Chem 2004;379:1013–1024. [PubMed: 15221183]
24. Douglas DJ, Frank AJ, Mao D. Linear ion traps in mass spectrometry. Mass Spectrom Rev 2005;24:1–29. [PubMed: 15389865]
25. Nilesh, S.; Tannu, SEH. Progress in Brain Research. Bahn, SEHaS, editor. 45. Elsevier; 2006.
26. Yates JR III, Gilchrist A, Howell KE, Bergeron JJ. Proteomics of organelles and large cellular structures. Nat Rev Mol Cell Biol 2005;6:702–714. [PubMed: 16231421]
27. Van den Bergh G, Clerens S, Vandesande F, Arckens L. Reversed-phase high-performance liquid chromatography prefractionation prior to two-dimensional difference gel electrophoresis and mass spectrometry identifies new differentially expressed proteins between striate cortex of kitten and adult cat. Electrophoresis 2003;24:1471–1481. [PubMed: 12731035]
28. Molloy MP, Brzezinski EE, Hang J, McDowell MT, VanBogelen RA. Overcoming technical variation and biological variation in quantitative proteomics. Proteomics 2003;3:1912–1919. [PubMed: 14625853]
29. Tannu N, Hemby SE. Quantitation in two-dimensional fluorescence difference gel electrophoresis: effect of protein fixation. Electrophoresis 2006;27:2011–2015. [PubMed: 16607608]
30. Tonge R, et al. Validation and development of fluorescence two-dimensional differential gel electrophoresis proteomics technology. Proteomics 2001;1:377–396. [PubMed: 11680884]
31. Alban A, et al. A novel experimental design for comparative two-dimensional gel analysis: two-dimensional difference gel electrophoresis incorporating a pooled internal standard. Proteomics 2003;3:36–44. [PubMed: 12548632]
32. Knowles MR, et al. Multiplex proteomic analysis by two-dimensional differential in-gel electrophoresis. Proteomics 2003;3:1162–1171. [PubMed: 12872217]
33. Hu Y, Malone JP, Fagan AM, Townsend RR, Holtzman DM. Comparative proteomic analysis of intra- and interindividual variation in human cerebrospinal fluid. Mol Cell Proteomics 2005;4:2000–2009. [PubMed: 16199891]
34. Karp NA, Griffin JL, Lilley KS. Application of partial least squares discriminant analysis to two-dimensional difference gel studies in expression proteomics. Proteomics 2005;5:81–90. [PubMed: 15744836]
35. Swatton JE, Prabakaran S, Karp NA, Lilley KS, Bahn S. Protein profiling of human postmortem brain using 2-dimensional fluorescence difference gel electrophoresis (2-D DIGE). Mol Psychiatry 2004;9:128–143. [PubMed: 14708031]
36. Lee IN, et al. Identification of human hepatocellular carcinoma-related biomarkers by two-dimensional difference gel electrophoresis and mass spectrometry. J Proteome Res 2005;4:2062–2069. [PubMed: 16335951]
37. Zhou G, et al. 2D differential in-gel electrophoresis for the identification of esophageal scans cell cancer-specific protein markers. Mol Cell Proteomics 2002;1:117–124. [PubMed: 12096129]
38. Brown LM, et al. Quantitative and qualitative differences in protein expression between papillary thyroid carcinoma and normal thyroid tissue. Mol Carcinog 2006;45:613–626. [PubMed: 16788983]

NIH-PA Author Manuscript NIH-PA Author Manuscript NIH-PA Author Manuscript

39. Nakashima D, et al. Protein expression profiling identifies maspin and stathmin as potential biomarkers of adenoid cystic carcinoma of the salivary glands. Int J Cancer 2006;118:704–713. [PubMed: 16094606]
40. Nishimori T, et al. Proteomic analysis of primary esophageal squamous cell carcinoma reveals downregulation of a cell adhesion protein, periplakin. Proteomics 2006;6:1011–1018. [PubMed: 16400690]
41. Seike M, et al. Proteomic signatures for histological types of lung cancer. Proteomics 2005;5:2939–2948. [PubMed: 15996008]
42. Somiari RI, Somiari S, Russell S, Shriver CD. Proteomics of breast carcinoma. J Chromatogr B Anal Technol Biomed Life Sci 2005;815:215–225.
43. Seike M, et al. Proteomic signature of human cancer cells. Proteomics 2004;4:2776–2788. [PubMed: 15352251]
44. Somiari RI, et al. High-throughput proteomic analysis of human infiltrating ductal carcinoma of the breast. Proteomics 2003;3:1863–1873. [PubMed: 14625848]
45. Chan HL, et al. Proteomic analysis of redox- and ErbB2-dependent changes in mammary luminal epithelial cells using cysteine- and lysine-labelling two-dimensional difference gel electrophoresis. Proteomics 2005;5:2908–2926. [PubMed: 15954156]
46. Alfonso P, et al. Proteomic expression analysis of colorectal cancer by two-dimensional differential gel electrophoresis. Proteomics 2005;5:2602–2611. [PubMed: 15924290]
47. Canals F, Colome N, Ferrer C, Plaza-Calonge Mdel C, Rodriguez-Manzaneque JC. Identification of substrates of the extracellular protease ADAMTS1 by DIGE proteomic analysis. Proteomics 2006;6 (suppl 1):S28–S35. [PubMed: 16511810]
48. Yu KH, Rustgi AK, Blair IA. Characterization of proteins in human pancreatic cancer serum using differential gel electrophoresis and tandem mass spectrometry. J Proteome Res 2005;4:1742–1751. [PubMed: 16212428]
49. Ornstein DK, Petricoin EF III. Proteomics to diagnose human tumors and provide prognostic information. Oncology (Williston Park) 2004;18:521–529. [PubMed: 15134357]discussion 529–532
50. Alm H, et al. Proteomic evaluation of neonatal exposure to 2,2,4,4,5-pentabromodiphenyl ether. Environ Health Perspect 2006;114:254–259. [PubMed: 16451863]
51. Roelens SA, et al. Neurotoxicity of polychlorinated biphenyls (PCBs) by disturbance of thyroid hormone-regulated genes. Ann NY Acad Sci 2005;1040:454–456. [PubMed: 15891087]
52. Kleno TG, et al. Mechanisms of hydrazine toxicity in rat liver investigated by proteomics and multivariate data analysis. Proteomics 2004;4:868–880. [PubMed: 14997507]
53. Ruepp SU, Tonge RP, Shaw J, Wallis N, Pognan F. Genomics and proteomics analysis of acetaminophen toxicity in mouse liver. Toxicol Sci 2002;65:135–150. [PubMed: 11752693]
54. Tian Q, et al. Integrated genomic and proteomic analyses of gene expression in mammalian cells. Mol Cell Proteomics 2004;3:960–969. [PubMed: 15238602]
55. Dowsey AW, et al. Examination of 2-DE in the Human Proteome Organisation Brain Proteome Project pilot studies with the new RAIN gel matching technique. Proteomics 2006;6:5030–5047. [PubMed: 16927431]
56. Frohlich T, et al. Analysis of the HUPO Brain proteome reference samples using 2-D DIGE and 2-D LC-MS/MS. Proteomics 2006;6:4950–4966. [PubMed: 16927427]
57. Focking M, et al. 2-D DIGE as a quantitative tool for investigating the HUPO Brain Proteome Project mouse series. Proteomics 2006;6:4914–4931. [PubMed: 16927420]
58. Yan JX, et al. Fluorescence two-dimensional difference gel electrophoresis and mass spectrometry based proteomic analysis of Escherichia coli. Proteomics 2002;2:1682–1698. [PubMed: 12469338]
59. Gade D, Thiermann J, Markowsky D, Rabus R. Evaluation of two-dimensional difference gel electrophoresis for protein profiling. Soluble proteins of the marine bacterium Pirellula sp strain 1. J Mol Microbiol Biotechnol 2003;5:240–251. [PubMed: 12867748]
60. Corbett M, et al. Identification of a new quorum-sensing-controlled virulence factor in Erwinia carotovora subsp atroseptica secreted via the type II targeting pathway. Mol Plant Microbe Interact 2005;18:334–342. [PubMed: 15828685]

NIH-PA Author Manuscript NIH-PA Author Manuscript NIH-PA Author Manuscript

61. Lopez-Campistrous A, et al. Localization, annotation, and comparison of the Escherichia coli K-12 proteome under two states of growth. Mol Cell Proteomics 2005;4:1205–1209. [PubMed: 15911532]
62. Gade D, Gobom J, Rabus R. Proteomic analysis of carbohydrate catabolism and regulation in the marine bacterium *Rhodopirellula baltica.* Proteomics 2005;5:3672–3683. [PubMed: 16127727]
63. Rathsam C, et al. Two-dimensional fluorescence difference gel electrophoretic analysis of *Streptococcus mutans biofilms.* J Proteome Res 2005;4:2161–2173. [PubMed: 16335963]
64. VerBerkmoes NC, et al. Integrating "top-down" and "bottom-up" mass spectrometric approaches for proteomic analysis of *Shewanella oneidensis.* J Proteome Res 2002;1:239–252. [PubMed: 12645901]
65. Busler VJ, et al. Protein–protein interactions among *Helicobacter pylori* cag proteins. J Bacteriol 2006;188:4787–4800. [PubMed: 16788188]
66. Vipond C, et al. Proteomic analysis of a meningococcal outer membrane vesicle vaccine prepared from the group B strain NZ98/254. Proteomics 2006;6:3400–3413. [PubMed: 16645985]
67. Slabas AR, Suzuki I, Murata N, Simon WJ, Hall JJ. Proteomic analysis of the heat shock response in Synechocystis PCC6803 and a thermally tolerant knockout strain lacking the histidine kinase 34 gene. Proteomics 2006;6:845–864. [PubMed: 16400687]
68. Tannu NS, Sanchez-Brambila G, Kirby P, Andacht TM. Effect of staining reagent on peptide mass fingerprinting from in-gel trypsin digestions: a comparison of SyproRubytrade mark and DeepPurpletrade mark. Electrophoresis 2006;27:3136–3143. [PubMed: 16800026]

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

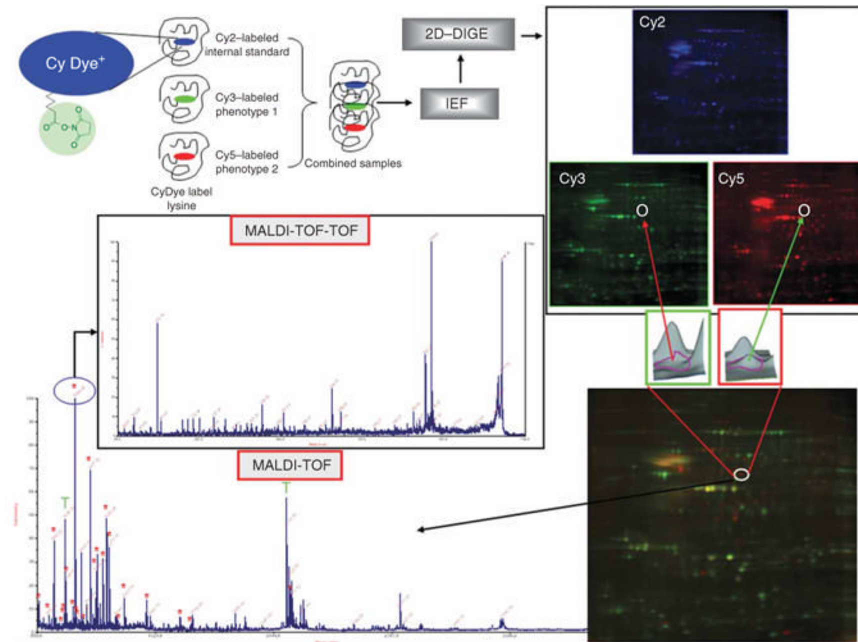

Figure 1.
Schematic of 2D-DIGE experiment. A normalization pool comprising all the samples from condition 1 (phenotype 1) and condition 2 (phenotype 2) is labeled with Cy2 dye. The condition 1 and condition 2 samples are labeled such that half of the samples from each group are labeled with Cy3 dye and the remaining half with Cy5 dye. Each 2D-DIGE gel comprises normalization pool (Cy2), condition 1 (Cy3/Cy5) and condition 2 (Cy3/Cy5) samples. The three scanned images from each 2D-DIGE are further analyzed by DeCyder Image analysis software to generate the differentially regulated protein spots (elaborated in Figs. 2 and 3). The differentially regulated protein spots, as depicted in the 2D and 3D images, are further processed by MS (elaborated in the text). The mass list from the mass spectra (MALDI-TOF) is used for peptide mass fingerprinting for protein identification. Some of the precursor ions from MS are further processed by MS/MS (MALDI-TOF-TOF) to generate confirmatory sequence information.

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

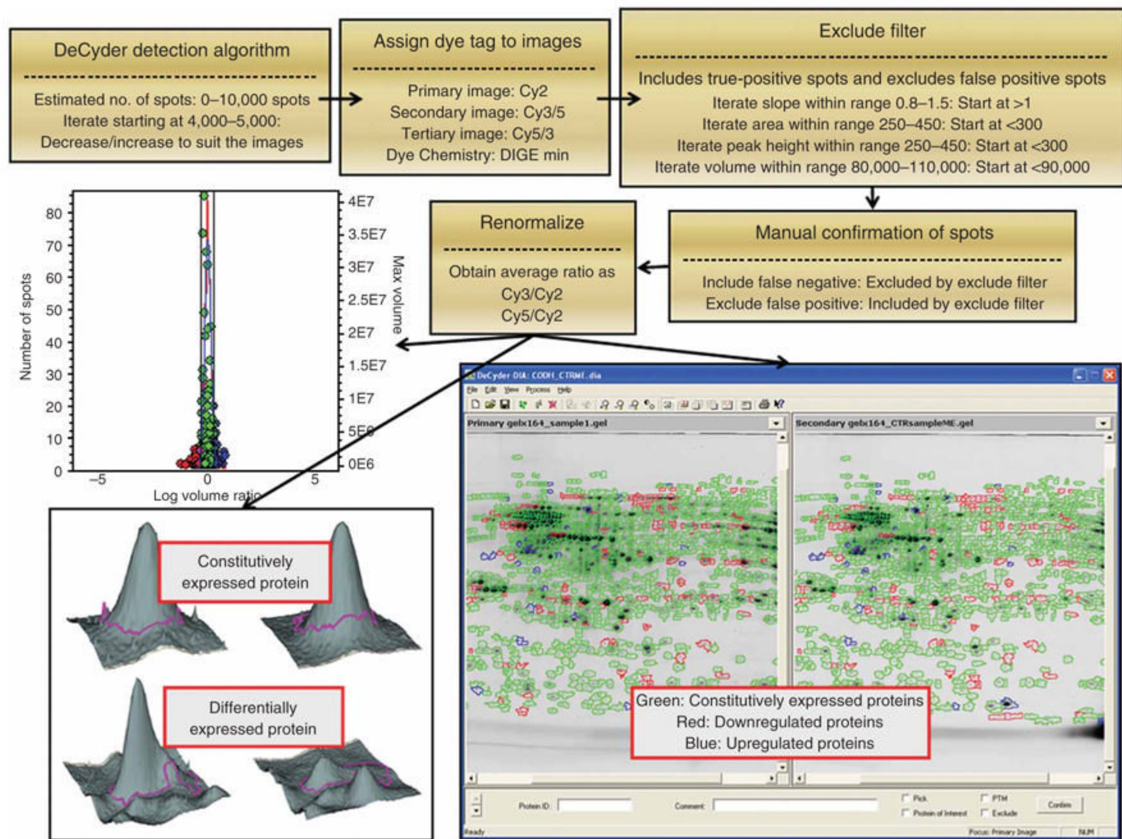

Figure 2.

Schematic of DeCyder DIA. The DeCyder image analysis starts with the DIA module. A basic protocol is depicted step by step in the figure. The image analysis has to be iterated at some steps for optimal generation of quantitative expression data based on the images generated by particular samples. In this stage of image analysis, a single gel consisting of three images is processed for spot co-detection. In this module, the spot volumes are normalized to the normalization pool: Cy3/Cy2 and Cy5/Cy2. After normalization, the volume ratios are generated as Cy3/Cy5. It can be appreciated in the figure that the vast majority of protein spots are constitutively expressed (≈90% protein spots: green boundaries) as opposed to the differentially regulated protein spots, in accordance with normal distribution.

NIH-PA Author Manuscript NIH-PA Author Manuscript NIH-PA Author Manuscript

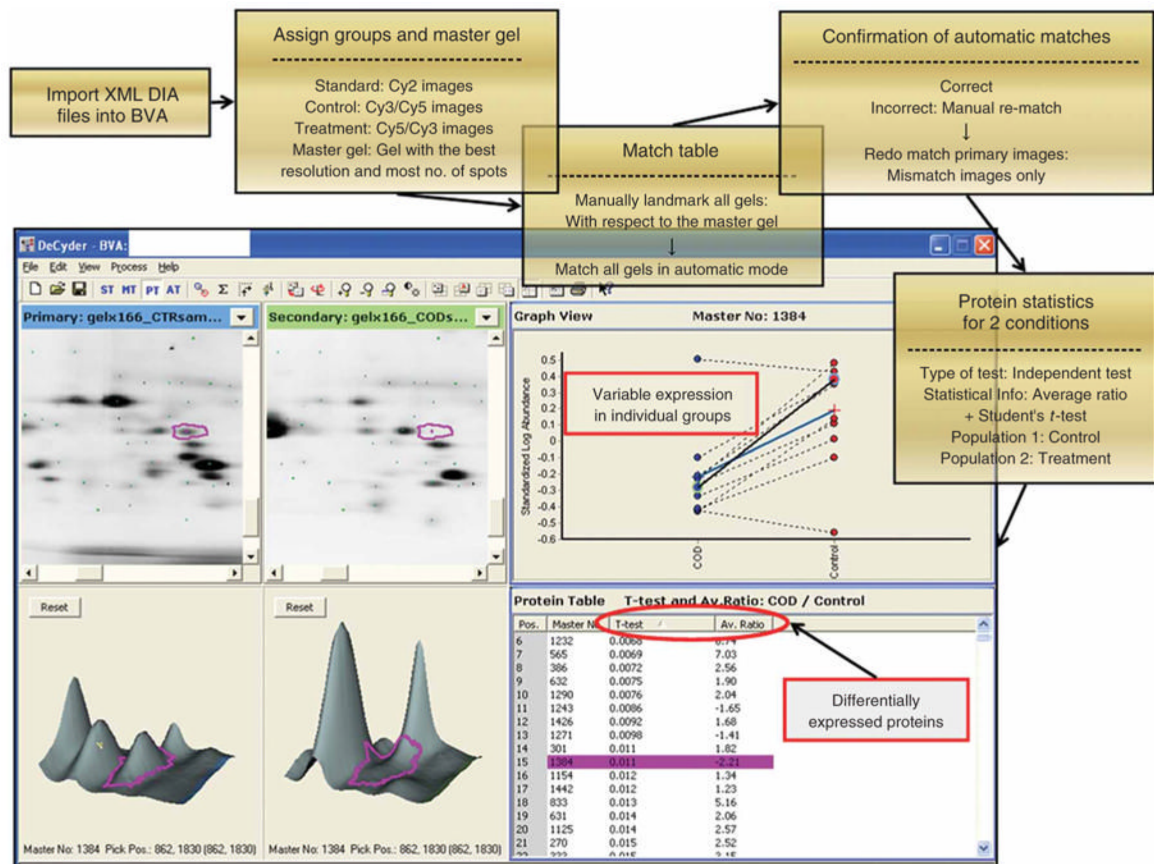

Figure 3.

Schematic of DeCyder BVA. The figure shows a schematized step-by-step protocol that can be adapted for BVA. This module primarily allows comparison of multiple gels. The most important processing done BVA is to match the protein spots across all the gels and images. After image matching, the volume information from DIA is used to generate average ratios for each protein spot for subsequent statistical analysis (*t*-test or ANOVA) to generate a list of differentially regulated protein spots with statistical significance in their expression. This module allows the user to visualize the 2D and 3D images of individual protein spots with differential expression as well as their within-group variability.

NIH-PA Author Manuscript
NIH-PA Author Manuscript
NIH-PA Author Manuscript

TABLE 1

Troubleshooting table.

Problem	Cause	Solution
	Sample preparation	
Strong signal for protein spots with only one CyDye.	Inappropriate protein quantitation.	Repeat the protein quantitation and make sure that the various detergents and ingredients of the buffers are compatible with the methodology of protein quantitation.
	Variable denaturation/solubilization.	Ensure the use of the same buffer from a single tube for all the samples rather than different aliquots of buffer for different samples.
Unexpected protein spots in some samples.	Sample contamination before labeling.	Ensure clean conditions for all the equipment used, also wear gloves and a full-sleeve gown during all steps of sample handling.
Weak fluorescent signal.	Inappropriate dye:protein ratio.	Run a preliminary 1D gel with various dye:protein ratios to find the ratio suitable to give adequate signal.
	Low sample pH before labeling.	Check sample pH immediately before labeling and ensure it is 8.5.
	Less than adequate quality of DMF.	The DMF used should be >99.5% pure and anhydrous. Use a bottle that has been opened in the last 3 months.
	DTT (thiol agents) and ampholytes(primary amines) compete with the CyDye labeling.	Ensure that DTT and the ampholytes do not exceed the recommended concentrations. Alternatively, the amount of dye used can also be increased.
No fluorescent signal for one CyDye.	Probably no dye was present in the vial.	A preliminary scan of an extremely small drop of CyDye directly on a glass plate is warranted to ensure the presence of dye.
	Isoelectric focusing	
Desired voltage/current is not attained.	The electrical circuit is incomplete.	Ensure that the external electrodes of the gel-holding trays are in contact with the appropriate electrodes of the IPGphor. Ensure that the gel is in contact with the internal electrodes of the gel-holding tray.
	The IPG DryStrip is not dehydrated for the appropriate time.	Add more rehydration buffer and increase the rehydration time.
	Inappropriate concentration of ampholytes.	Use a concentration of 1% (vol/vol) ampholytes.
Inadequate protein focusing.	High concentration of salts in the lysis buffer.	Perform a sample clean-up by using the 2D clean-up kit or acetone precipitation.
	SDS-PAGE	
Charge trains on the second dimension.	Carbamylation of proteins.	All solutions containing urea should be freshly prepared and not heated above 30 °C. Try not to store the sample in a urea-containing buffer for a longer period.
+	Suboptimal IEF.	Perform a sample clean-up by using the 2D clean-up kit or acetone precipitation. Increase the volt-hours for IEF.
	High sample load.	Adjust the total sample loaded on one gel by appropriate adjustments in all the three CyDye-labeled samples. The loading amount will be dictated by the size of the second dimension.
Vertical streaks.	Inappropriate SDS.	Use appropriate SDS during gel preparation and in running buffer.
	Gel imaging	
Spots are not seen after scan.	Inappropriate use of laser and emission filters.	Select the appropriate laser and emission filter according to the guidelines for gel imaging.
	Too low PMT.	Increase the scanning PMT voltage until you obtain a desirable signal.
Image is black.	Too high PMT.	Decrease the scanning PMT voltage until you obtain a desirable signal.
Nonspecific speckling.	Dirt on gel plates, platen or the gel itself.	Clean the gel plates and the scanner platform.
	Image analysis	
All the spots are not detected during spot detection of DIA.	The areas outside the gel are detected as spots rather than the gel.	Crop the gel image so as to remove areas extraneous to the gel image.
	Less number of spots specified for detection.	Increase the number of spots to be detected during spot detection.
Spot matching not possible across gels in an experiment.	Similar conditions were not maintained during sample preparation and 2-D separation of proteins across samples.	The most common culprit is the use of IEF DryStrips and second-dimension gel from different batches. Ensure the use of gels from the same batch.
	Sample preparation and MS	
Inappropriate picking of gel spots.	Reference markers not marked in the correct order.	Always select the reference marker on the left first, followed by the one on the right.
Low signal on MS.	Low starting material of protein spot.	Run multiple pick gels, combine similar protein spots from multiple gels and process them as a pool.
	Incomplete trypsin digestion.	Possibly due to incomplete unfolding of proteins in gel. Repeat reduction-alkylation before trypsin digestion.

NIH-PA Author Manuscript NIH-PA Author Manuscript NIH-PA Author Manuscript

Problem	Cause	Solution
No protein identification after database search.	The ammonium bicarbonate used for trypsin reconstitution interferes with ionization.	Perform a ZipTip C18 clean-up. Alternatively, the sample can be washed on-plate with ice-cold 0.1% TFA.
	Matrix suppression of signal.	Reduce the amount of matrix used for spotting of sample on the MALDI plate.
	Mass spectrum not calibrated.	Calibrate the mass spectrum using the tryptic auto-digest fragments as described in PROCEDURE.
	Incorrect use of species.	Use the right species corresponding to the one used for the study in question.
	Database of the species incomplete.	Perform *de novo* sequencing. Another option is to perform a broader database search.
	Too stringent conditions for database search.	Use less stringent conditions for database search: allow for1–2 trypsin cleavages, relax the mass error for MS to 100–200 ppm and MS/MS to 0.5 Da.