

# Chapter 2

## The Basics of 2D DIGE

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### Abstract

The technique of two-dimensional (2D) gel electrophoresis is a powerful tool for separating complex mixtures of proteins, but since its inception in the mid 1970s, it acquired the stigma of being a very difficult application to master and was generally used to its best effect by experts. The introduction of commercially available immobilized pH gradients in the early 1990s provided enhanced reproducibility and easier protocols, leading to a pronounced increase in popularity of the technique. However gel-to-gel variation was still difficult to control without the use of technical replicates. In the mid 1990s (at the same time as the birth of “proteomics”), the concept of multiplexing fluorescently labeled proteins for 2D gel separation was realized by Jon Minden’s group and has led to the ability to design experiments to virtually eliminate gel-to-gel variation, resulting in biological replicates being used for statistical analysis with the ability to detect very small changes in relative protein abundance. This technology is referred to as 2D difference gel electrophoresis (2D DIGE).

**Key words:** Two-dimensional gel electrophoresis, 2D DIGE, CyDye, Multiplexing, Difference gel electrophoresis

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### 1. Introduction

Two-dimensional (2D) gel electrophoresis allows for the simultaneous separation of thousands of proteins and was pioneered by O’Farrell (1), utilizing a denaturing environment. The technique separates the proteins based on their charge in the first dimension using isoelectric focusing (IEF), where the proteins will migrate to their isoelectric point (pI). In the second dimension, the proteins are separated based on molecular weight by the use of classical SDS-PAGE (2). The traditional use of carrier ampholytes to establish the pH gradient in the first dimension led to a number of issues, most notably the lack of reproducibility. This resulted in the development of immobilized pH gradients (3), leading to commercially available precast gels for the first dimension that allowed for enhanced reproducibility (since they do not rely on carrier

ampholytes to establish the pH gradient). Despite this increase in reproducibility, there were still problems with gel-to-gel reproducibility requiring the use of technical replicates to remove artifacts of experimental variation. This can result in a prohibitive number of gels, especially for complex experimental designs.

A breakthrough in 2D gel electrophoresis arrived with the introduction of the ability to multiplex fluorescently labeled proteins on the same gel (4). This technique is referred to as 2D difference gel electrophoresis (2D DIGE) (see reviews (refs. 5–7)). The fluorescent dyes used are specially modified cyanine dyes (CyDye™ DIGE fluors) which are matched for molecular weight and charge and provide a useable dynamic range of up to 4 orders of magnitude. There are two approaches to the labeling; the most common approach is termed minimal labeling, where the dye binds to a restricted number of lysine residues. For certain samples an alternative approach termed saturation labeling is used, where the dyes bind to all of the accessible cysteine residues.

The minimal dyes (Cy™2, Cy3, and Cy5) all have an approximate molecular weight of 450 Da and carry a +1 charge (this replaces the +1 charge of the lysine resulting in no overall change to the pI). The dye-to-protein ratio is controlled such that only a small percentage of the total available lysine population is labeled with the CyDye to avoid multiple labels per protein. By utilizing size- and charge-matched dyes, the labeled proteins will comigrate on the 2D gel and allow precise image overlay from each sample.

For the saturation dyes (Cy3 and Cy5) the opposite strategy is used for labeling. The dye and reductant concentrations are optimized to ensure that all the reduced cysteine residues are labeled with the CyDye, resulting in an increase in signal. These dyes have a molecular weight of 680 Da and are neutrally charged. Samples labeled with saturation dyes will exhibit altered spot migrations due to the number of cysteines present and will thus display a different spot pattern compared to the minimal labeling approach. However, the samples within the same gel will comigrate such that differential analysis can still be performed.

Running differently labeled samples in a single gel and analyzing the resulting images can provide possible proteins of interest. However, to allow for biological variation the use of biological replicates for statistical confidence is necessary, so multiple gels still need to be run. To overcome the problems of gel-to-gel variation, one of the dyes is used to label a pooled internal standard (sometimes referred to as a pooled internal reference) (see Fig. 1). The pooled internal standard is comprised of all the potential detectable proteins in the experiment such that it is a combination of equal aliquots of each of the samples to be analyzed (see Table 1). The virtual elimination of gel-to-gel variation (coupled with multiplexing) now allows for the running of biological replicates such that the number of gels to be run is dramatically reduced

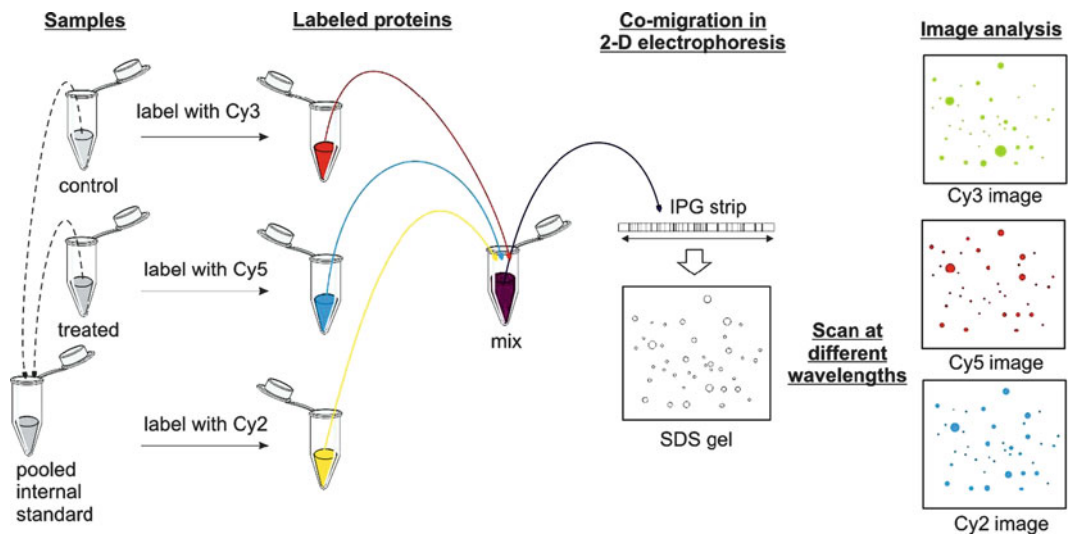


Fig. 1. Workflow for a minimal CyDye labeling experiment. After CyDye labeling, the three samples are separated on the same 2D gel and then imaged for the resulting fluorescence associated with each CyDye. Permission to reproduce from Westermeier and Scheibe (8).

**Table 1**  
**Experimental design for a minimal labeling experiment, incorporating a dye swap and including a pooled internal standard (standard). This scenario allows for looking at two different conditions: 1 and 2**

	Cy2	Cy3	Cy5
Gel 1	Standard	Sample 1a	Sample 2d
Gel 2	Standard	Sample 2c	Sample 1b
Gel 3	Standard	Sample 1c	Sample 2b
Gel 4	Standard	Sample 2a	Sample 1d
Standard = s1a + s1b + s1c + s1d + s2a + s2b + s2c + s2d			

The letters *a*, *b*, *c*, and *d* denote biological replicates

compared to classical detection techniques (9) (see Fig. 2). The 2D DIGE system benefits from the pooled internal standard in several ways; it is used to help normalize the signal between and within each gel by comparing the ratio of each labeled protein spot to the internal standard and then to the same protein spot in the other gels. In addition, the pooled internal standard is used as a standard map to match protein spots across multiple gels since all of the spots in the internal standard should be present across all of the gels.

### Traditional 2D gel electrophoresis (Silver, Coomassie, SYPRO Ruby, Deep Purple)

Need gel replicates and biological replicates.

Minimum needed for statistics is 3 of each.

No. of gels (N) = no. of samples (y) x 3 (biological reps.) x 3 (gel reps.)

$N = y \times 3 \times 3$

Example 1: control versus treated (y = 2);

$N = 2 \times 3 \times 3 = 18$  gels.

Example 2: control versus treated versus treated+drug (y=3);

$N = 3 \times 3 \times 3 = 27$  gels.

### 2D DIGE (Minimal Labeling)

Only need biological replicates (since gel-to-gel variation is virtually eliminated with this technique).

Minimum needed for statistics is 3 - but to properly enable reverse labeling 4 is recommended. The multiplexing capability allows three samples per gel (one of these is the internal standard so two real samples are run per gel).

No. of gels (N) =  $\frac{\text{no. of samples (y)} \times 4 \text{ (biological reps.)}}{2 \text{ (since two samples on each gel)}}$   $N = \frac{y \times 4}{2}$

Example 1: control versus treated (y = 2);

$N = \frac{2 \times 4}{2} = 4$  gels.

Example 2: control versus treated versus treated+drug (y=3);

$N = \frac{3 \times 4}{2} = 6$  gels.

Fig. 2. Calculation for the minimum number of gels to be run for a traditional 2D experiment compared to a 2D DIGE experiment.

It is important with this technique that the three fluorescent dyes (Cy2, Cy3, and Cy5) are imaged with an appropriate device that can not only independently excite these fluorors but is also able to distinguish between the three resulting spectra and avoid any cross talk issues, which would interfere with quantification. A laser scanner capable of blue, green, and red excitation and equipped with the appropriate band pass filters for the corresponding emission is highly recommended. Also, a suitably designed image analysis software package should be used to perform the required calculations (7). In particular, the software's ability to properly handle the codetection of the images within each gel, and the normalization against the internal standard, will influence the accuracy and reliability of the quantification.

Since the inception of 2D DIGE in 1997, there are now over 2,500 papers (as of May 2011, Ishida Y, GE Healthcare, personal communication). Many types of samples have been investigated using this technique, including a wide range of plant and animal species (7). Recently, advances have been made in furthering the utility of the technique by exploring niche applications. Such examples include cell surface labeling (10–12), reduced vs. nonreduced states (13), host cell protein monitoring (14), and samples from laser microdissection (15), to name but a few.

## 2. Materials

The use of high-quality electrophoresis/proteomic-grade chemicals is paramount to achieving successful experiments with resulting identifications—this is especially true of the quality of the water used in all buffers and solutions and should be of 18 M $\Omega$  or less.

### **2.1. First Dimension of 2D Electrophoresis**

1. Rehydration buffer: 7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 40 mM dithiothreitol (DTT), 0.5% (v/v) carrier ampholyte.
2. Immobilized pH gradient (IPG) strips (see Note 1).

### **2.2. Second Dimension of 2D Electrophoresis**

1. SDS equilibration buffer: 6 M urea, 30% glycerol, 2% sodium dodecyl sulfate (SDS), 0.002% bromophenol blue, 75 mM Tris-HCl, pH 8.8 (step 1: DTT followed by step 2: iodoacetamide).
2. SDS gel: acrylamide (10%), bisacrylamide (3%), SDS (0.1%), ammonium persulfate, TEMED, 0.37 M Tris-HCl, pH 8.8 (see Note 1).
3. SDS running buffer: 25 mM Tris-base, 192 mM glycine, 0.2% SDS.
4. Bind-Silane (see Note 2).

### **2.3. Labeling (Minimal Dye Approach)**

1. CyDye DIGE fluors (Cy2, Cy3, and Cy5).
2. Dimethyl formamide (DMF) (see Note 3).
3. Labeling buffer: 7 M urea, 2 M thiourea, 4% CHAPS, 30 mM Tris-HCl, pH 8.5.
4. 10 mM lysine.
5. pH test paper 7.5–9.5 (see Note 4).
6. 50 mM NaOH.

### **2.4. General Reagents**

1. Ethanol.
2. Glacial acetic acid.
3. Deionized water.
4. Fluorescent stain (e.g., Deep Purple™, SYPRO® Ruby).

### **2.5. General Apparatus**

1. First dimension electrophoresis unit.
2. Second dimension electrophoresis unit.
3. Power supply.
4. Temperature controlled recirculating water bath.
5. Ice bucket/ice.
6. Imaging device (laser scanner).
7. Analysis software.

### 3. Methods

This protocol describes minimal labeling. Full details for performing a saturation labeling experiment can be found in the associated product booklet (16).

#### 3.1. Sample Preparation

The sample is prepared as for classical 2D gel electrophoresis (17), except that primary amines, carrier ampholytes, and thiols are omitted from the buffers. It is then usual to concentrate the resulting sample (e.g., by precipitation) and resuspend it in labeling buffer (7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 30 mM Tris-HCl, pH 8.5) to a concentration of between 5 mg/mL and 10 mg/mL (though 1 mg/mL to 20 mg/mL have been successfully used) (see Note 5). The pH of this resulting sample is then checked with pH test paper such that the pH is between 8 and 9 (and adjusted with 50 mM NaOH if necessary). If the pH is below 8.0, then the dye will not bind, and if the pH is over 9.0, then multiple dyes can bind to the protein or to different amino acids.

The internal standard is prepared by pooling together equal aliquots of all the biological replicates in the experiment (see Table 1).

#### 3.2. Sample Labeling

The labeling protocol (18) involves the resuspension of each lyophilized CyDye in DMF to create a stock solution of 1 mM. To limit any effects of photobleaching on the fluors, all subsequent steps are performed in the dark.

The dye-to-protein ratio is controlled at 400 pmol of dye to 50  $\mu$ g of protein (though 100–1,000 pmol have been successfully used)—bulk labeling can also be performed by keeping this ratio constant. It is recommended to label the pooled internal standard with the Cy2 dye and then to perform a dye swap with each of the sample types in the experiment such that an equal number are labeled with Cy3 as with Cy5 (see Table 1; Notes 6 and 7). The labeling reaction is performed on ice for 30 min, and then, the labeling reaction is terminated by the addition of lysine to quench any unreacted dye (for 10 min on ice).

#### 3.3. 2D Gel Electrophoresis

The labeled samples are mixed appropriately for loading onto the first dimension IPG strips, either by in-gel rehydration, cup loading, or paper-bridge loading (17). The samples are made up to the correct volume for sample loading ensuring that the final buffer concentrations are 7 M urea, 2 M thiourea, 4% CHAPS, 0.5% carrier ampholyte, and 40 mM DTT.

Standard IEF separation protocols are subsequently followed (17). After the first dimension a two-step equilibration procedure is performed. This procedure saturates the IPG strip with the SDS buffer system required for the second dimension separation.

The equilibration solution contains urea, glycerol, reductant, SDS, and tracking dye. The second equilibration step replaces the reductant with iodoacetamide to alkylate the reduced cysteine residues. The strips are then sealed with agarose on to the top of the second dimension gel and then separated for molecular weight by classical SDS-PAGE.

### **3.4. Gel Imaging**

If the gel is to be imaged while still between the glass plates or attached to a plastic backing, then the glass or plastic must have low fluorescent properties to minimize any background issues (autofluorescence) that could compromise quantification.

The gels are imaged with a suitable fluorescent imager that is capable of exciting the three dyes independently and has the necessary band pass filters to avoid cross talk (see Note 8).

The image capture is then performed as described in the instrument manual, with the following guidelines:

- (a) The final image is scanned at 100- $\mu$ m resolution.
- (b) The file format should be a 16 bit .tif (or similar).
- (c) Steps should be taken to avoid introducing any fluorescent particles (dust, lint, etc.).
- (d) The gel images should first be prescanned using a short-exposure or low-resolution setting so that the final image capture settings can be optimized to avoid saturation while taking advantage of the full dynamic range.

### **3.5. Image Analysis**

Each gel set (three images) should be cropped to remove any areas that are redundant from the analysis (such as the dye front and IPG strip) that may interfere with spot detection and normalization. The cropping should be performed to keep similar spot patterns the same rather than using similar sized crop areas. The analysis software should allow for the use of the pooled internal standard to facilitate the normalization and spot matching procedures. It is usual for the software to incorporate some statistical tools to allow for the assignment of spots of interest that can then be exported as a pick list. These protein spots can then be excised for further analysis, such as by mass spectrometry to identify the protein.

### **3.6. Gel Processing for Spot Picking**

For spot picking, it is necessary to poststain a designated gel with a total protein detection system (e.g., silver, Coomassie®, or ideally a fluorescent stain such as Deep Purple or SYPRO Ruby) (see Note 9).

The reason for this procedure is that if the original CyDye spot coordinates were used, then there is the possibility that only the protein with the dye attached will be picked (a small percentage of the total protein) as this has an approximate 450-Da molecular weight shift to a higher position in the gel (this is the same for all three dyes). The bulk of the protein lies at a slightly lower molecular

weight and will be more of an issue for the lower molecular weight proteins, but this procedure should be performed as standard practice. Spots of interest can now be matched to the pick gel image by using the analysis software. The pick gel can be run as a separate preparative gel on its own, or extra unlabeled protein (made up as for the internal standard) can be added equally to all of the analytical gels such that each gel is then a potential pick gel.

### **3.7. Spot Processing and Identification**

The excised protein spot can be enzymatically digested (usually with trypsin), and the resulting peptides can be analyzed with a mass spectrometer. The most commonly used techniques include matrix-assisted laser desorption/ionization (MALDI) or electrospray ionization (ESI) mass spectrometry (MS). Once a protein has been identified by MS, it can be very useful to verify its identity. This can be achieved by Western blotting if an antibody is available against the target of interest. Western blotting combined with a 2D gel can be a very powerful approach since an SDS-PAGE gel alone will not detect the posttranslational modifications that result in different charge forms of the same protein being present. If blotting an actual 2D DIGE gel from an experiment, it is important that the reporter molecule should not interfere with the signal from the CyDyes such that the antibody can be linked to an enzyme (such as horse radish peroxidase, HRP) for chemiluminescent detection or the antibody can be linked to an infrared reporter molecule. It must be remembered that the total protein from control, treated, and internal standard will now be detected, so this approach is more useful for confirmation of location and identification. However, if using a two-dye system (see Note 6) for the DIGE experiment (e.g., Cy 3 and Cy5), then the third dye (Cy2) could be used as a reporter molecule on the primary or secondary antibody.

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## **4. Notes**

1. Reproducibility of spot patterns can be facilitated by the use of precast gels for both the first and second dimension.
2. To facilitate accurate spot picking, it is strongly recommended to immobilize the gel to prevent swelling or shrinking during the staining procedure. This can be achieved by treating one of the two low fluorescent glass plates (one pair of plates is used per gel) with Bind-Silane. Another approach is to use a low fluorescent plastic-backed gel.

Reference markers can be attached to the support surface prior to gel casting or imaging to enable more accurate spot picking with robotic instrumentation—these markers will serve



as “anchor points” such that the pixel coordinates from the software can be accurately converted to picking coordinates.

3. DMF is used to reconstitute the CyDye and should be anhydrous. Poor-quality DMF will result in reduced labeling efficiency and reduced shelf life for the dyes. Water accumulation and amine-containing byproducts can be avoided by the addition of a 4 Å molecular sieve (cat. no. M2635, Sigma-Aldrich®) to absorb these impurities and the water.
4. Wider pH range test papers are not accurate enough.
5. The initial determination of protein concentration should be verified using an assay that is compatible with the reagents that are used in classical 2D gel electrophoresis. Chemicals such as urea and DTT can interfere with standard protein assays. Labeling should be performed at the same protein concentration across all the samples in the experiment.
6. The incorporation of a dye swap in a 3-dye approach negates any chance for dye bias. Utilizing a 2-dye approach will also negate this dye bias (19).
7. Please see the paper by Karp et al. (19) for a discussion on how many replicates should be run in an experiment. Another paper by Karp et al. discusses when pooling or subpooling of samples can be employed (20).
8. For the minimal labeling CyDyes, see also Table 2.
 

Cy2 has an excitation maximum at 491 nm and emission maximum at 509 nm.

Cy3 has an excitation maximum at 553 nm and emission maximum at 569 nm.

Cy5 has an excitation maximum at 645 nm and emission maximum at 664 nm.
9. The gel should only be fixed (usually in a combination of acid and alcohol) after the gel has been imaged since the use of ethanol can interfere with the fluorescent properties of the CyDyes. The gel should not be fixed if Western blotting will be

**Table 2**  
**The colors related to the different CyDyes used in a minimal labeling experiment**

CyDye	Reagent color	Laser excitation	Emission fluorescence
Cy2	Yellow	Blue	Green
Cy3	Red	Green	Orange
Cy5	Blue	Red	Red

performed. If the gels need to be stored prior to scanning, then they can be kept under SDS running buffer in a light-tight container at 4°C. It is recommended to allow the gels to warm up to room temperature before imaging, as fluorescent intensity is temperature dependent. The ethanol used should be free of hexanes or other nonalcohol organic solvent impurities that can contribute to background fluorescence.

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### Trademarks

CyDye™, Cy™, and Deep Purple™ are trademarks of GE Healthcare Ltd., a General Electric company.

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SYPRO® is registered trademark of Molecular Probes, Inc.

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## References

1. O'Farrell PH. (1975) High resolution two-dimensional electrophoresis of proteins. *J. Biol. Chem.* 250: 4007–4021.
2. Friedman, D, Hoving, S, Westermeier, R. (2009) Isoelectric focusing and two-dimensional gel electrophoresis. *Methods Enzymol.* 463: 515–540.
3. Gorg A, Drews O, Luck C, Weiland F, Weiss W (2009) 2DE with IPGs. *Electrophoresis* 30: 122–132.
4. Ünlü, M, Morgan, ME, Minden JS. (1997) Difference gel electrophoresis: A single gel method for detecting changes in protein extracts. *Electrophoresis.* 18: 2071–2077.
5. Lilley KS, Friedman DB (2004) All about DIGE: quantification technology for differential-display 2D-gel proteomics. *Expert Rev. Proteomics* 1: 401–409.
6. Marouga, R, David, S., Hawkins, E (2005) The development of the DIGE system: 2D fluorescence difference gel analysis technology. *Anal. Bioanal. Chem.* 382: 669–678.
7. Loyland, SM, Rozanas, CR (2008) Capabilities using 2-D DIGE in Proteomics Research: The New Gold Standard for Two-dimensional Gel Electrophoresis. *Methods Mol. Biol.*, 441: 1–18.
8. Westermeier, R., and Scheibe, B. (2008) Difference Gel Electrophoresis Based on Lys/Cys Tagging In: Posch, A. Ed. 2D PAGE: Sample preparation and Fractionation. *Methods in Molecular Biology* 424, Humana Press, Totowa, NJ., p. 75.
9. Alban, A., David, S., Björkstén, L., Andersson, C., Sloge, E., Lewis, S., Currie, I. (2003) A novel experimental design for comparative two-dimensional gel analysis: Two-dimensional difference gel electrophoresis incorporating a pooled internal standard. *Proteomics* 3: 36–44.
10. Mayrhofer, C., Krieger, S., Allmaier, G., Kerjaschki, D. (2006) DIGE compatible labeling of surface proteins on vital cells in vitro and in vivo. *Proteomics* 6: 579–585.

11. Sidibe, A., Yin, X., Tarelli, E., Xiao, Q., Zampetaki, A., Xu, Q., Mayr, M. (2007) Integrated Membrane Protein Analysis of Mature and Embryonic Stem Cell-derived Smooth Muscle Cells using a Novel Combination of Cy-dye/Biotin Labeling. *Mol. Cell Proteomics* 6: 1788–1797.
12. Selective labeling of cell-surface proteins using CyDye DIGE Fluor minimal dyes. Application Note 11-0033-92 AB, GE Healthcare.
13. Hurd, TR, Prime, T, Harbour, ME, Lilley, KS, Murphy, MP (2007) Detection of Reactive Oxygen Species-sensitive Thiol Proteins by Redox Difference Gel Electrophoresis. Implications for mitochondrial redox signaling. *Biol. Chem.* 282: 22040–22051.
14. Jin, M., Szapiel, N., Zhang, J., Hickey, J., Ghose, S. (2010) Profiling of host cell proteins by two-dimensional difference gel electrophoresis (2D-DIGE): Implications for downstream process development. *Biotechnol. Bioeng.* 105: 306–316.
15. Kondo, T. and Hirohashi, S. (2006) Application of highly sensitive fluorescent dyes (CyDye DIGE Fluor saturation dyes) to laser microdissection and two-dimensional difference gel electrophoresis (2D-DIGE) for cancer proteomics. *Nature Protocols* 1(6): 2940–2956.
16. Amersham CyDye DIGE Fluor Labeling Kit for Scarce Samples 25-8009-83 AD. Instruction manual, GE Healthcare.
17. 2D Electrophoresis, Principles and Methods. 80-6429-60 AD. Handbook, GE Healthcare.
18. Amersham CyDye DIGE Fluors (minimal dyes) for Ettan DIGE 28-9531-63 AF. Instruction manual, GE Healthcare.
19. Karp, NA, McCormick, PS, Russell, MR, Lilley KS (2007) Experimental and statistical considerations to avoid false conclusions in proteomics studies using differential in-gel electrophoresis. *Mol. cell. Proteomics* 6: 1354–1364.
20. Karp, NA, Lilley KS (2009) Investigating sample pooling strategies for DIGE experiments to address biological variability. *Proteomics* 9: 388–397.

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