LECTURE-6

2D Difference In Gel Electrophoresis (2D-DIGE)

HANDOUT

PREAMBLE

Proteome studies carried out by the traditional 2DE technique have limitations with respect to reproducibility, which can be attributed to gel-to-gel variations. These variations affect the quantitative comparison of protein expression levels. To address these issues and enhance the reproducibility, advanced gel-based technique, namely, Difference in Gel Electrophoresis was reported by Unlu et al. in 1997. This technique exploits the fact that two different proteins when labeled with two different fluorescent dyes (Cy3 and Cy5) can be visualized individually on one single gel. These fluorescent dyes are pH insensitive, photo-stable and spectrally distinct. Moreover, the use of an internal control, which is a pool of control and test sample, is labeled with a third fluorescent dye (Cy2), to facilitate co-detection, normalization and accurate quantification of protein samples.

OUTLINE OF LECTURE

1. Workflow for DIGE

2. Brief description of individual steps

3. Applications of DIGE

4. Advantages and limitations of DIGE

5. Conclusions

BOX-1: ABBREVIATIONS & TERMINOLOGIES

- DIGE: Difference In Gel Electrophoresis
- Cy Dyes: Cyanine dyes used for labeling of protein samples from different sources, which can be mixed and run together using electrophoresis process.
- Internal Control: A pool of equal amount of the two samples under study, which is labeled with a third fluorescent dye (usually Cy2).
- Co-detection: Simultaneous detection of protein spots from two different spots, which is enabled due to the multiplexing ability of DIGE.
- DeCyder™: DIGE Image Analysis software
- DIA: Differential In-gel Analysis module
- BVA: Biological Variation Analysis module

2. Workflow for DIGE

- Sample Preparation & Labeling
- IPG Strip Rehydration
- First dimension (IEF)
- Second dimension (SDS-PAGE)
- Scanning
- Image analysis and Data interpretation
- Protein identification of significant spots

Illustration: Difference Gel Electrophoresis (DIGE)

- *The pooled internal standard for DIGE is prepared by mixing equal amounts of all samples that are being run in the experiment. This prevents problems of gel-to-gel variations. Each protein sample as well as the internal standard is labeled with a differently fluorescing cyanine dye (Cy2, Cy3 and Cy5), which allows all protein samples to be simultaneously run on a single gel. The dye binds covalently to the e-amino group of lysine residues in proteins.*
- *The labeled protein samples are mixed and run on a single 2-DE gel. Separation takes place on the basis of isoelectric points of the proteins in one dimension and based on molecular weight of the proteins in the second dimension with the smaller proteins migrating further along the gel. The gel containing all the protein samples can be viewed by illuminating it alternately with excitation wavelengths corresponding to the various Cyanine dyes. Information on molecular weight and pI of proteins as well as differential expression can be obtained from these spots.*

A. Sample Preparation and Labeling:

The protein pellet dissolved in appropriate buffer is taken as the starting sample for labeling. The sample pH should be 8.5, and can be adjusted using 100mM NaOH. Each of the samples is labeled with one of the fluorescent dyes (for e.g. the control sample can be labeled with Cy3 and the test sample can be labeled with Cy5, or vice versa). An internal pool containing equal amount of the control and treated samples is mixed together and is labeled with a different fluorescent dye, usually Cy2. These dyes bind to the ε-amine groups of the protein's lysine residues. The dye swapping should be done to check reproducibility and efficiency of the working protocol. The reaction is quenched using 10 mM lysine, which combines with unbound dye molecules and stops the reaction. The labeled samples are then stored at 4°C, until they are further rehydrated.

Cyanine Dyes:

- N-hydroxy succinimidyl ester derivatives of cyanine.
- Bind to ε-amine groups of the protein's lysine residues.
- Lysine is targeted for cyanine labeling.
- Spectrally resolvable fluors that are matched for mass and charge.
- There is no change in signal over wide pH range used during first-dimension (IEF) separation.
- Discrete signal from each fluor with minimal cross talk contributes to high accuracy.
- Absorbance max for Cy3 dye is 550 nm and emission max 570 nm; and absorbance max for Cy5 dye is 649 nm and emission max 670 nm.

Illustration: Methodology for the labeling of proteins with cyanine dyes

- *Cyanine labeling enables accurate analysis of differential expression of proteins between the samples. It is possible to label three different samples within the same 2D gel, enabling accurate analysis of differences in protein abundance between samples by preventing gel-to-gel variation. Cyanine dyes are used for the differential labeling of the samples. Cy dyes have been designed to be both mass- and charge-matched. Spectrally resolvable with different excitation and emission wavelength. A representative protocol is demonstrated in following animation.*
- *Prepare stock solution and working solution as per the desired concentration of the dye. DMF used to reconstitute the fluors should be of high-quality (anhydrous, >99.8% pure). It must not become contaminated with water, which will start to degrade the DMF to amine compounds. The DMF stock solution should be replaced at least every 3 months. Vortex the content well. The final concentration of Dye should be 400pmol/μl.*
- *Cyanine Dye labeling - For labeling take protein sample equivalent to 50μg from test and control. For internal standard for a single gel take mixture of test and control (1:1) equivalent to 50μg. The pH of the sample should be 8.8 for the accurate labeling of the dye. Add 1μl each of cy2 to internal standard sample; Cy3 to test samples and Cy5 to control. Cy2 must always be labeled to internal standard.*
- *Mix the content well, vortex it and incubate in ice for 30 min in dark place. Avoid exposure to light, as dyes are light sensitive. The cyanine dyes label the e-amino group of the lysine in the protein. When a CyDye is coupled to the lysine, it replaces the lysine's single positive charge with its own, ensuring that the pI of the protein does not change. Only 3% of total protein is labeled. Add 1μl of 10mmol/L of lysine to the samples and incubate for 10 minutes in ice. Free lysine helps to quench the reaction, it goes and bind to free dyes. The labeled samples can be stored at -20°C or one can proceed for rehydration and IEF steps.*

B. Rehydration of IPG strips and IEF

The IPG strips need to be rehydrated with the labeled sample, which is mixed with the respective IPG buffer. The sample is then placed over the IPG strip and rehydration is allowed to occur by incubating the strips at RT for 16 hrs. After 1 hr, the samples are overlaid with mineral oil to prevent sample evaporation. The rehydrated strips are further used for isoelectric focusing (IEF). In IEF the rehydrated strips are first placed in the IEF tray and program for IEF run is selected as per the strip length and pH range. In IEF the applied voltage gradient brings about separation of proteins on the basis of their isoelectric point (pI), at which the net charge on a given protein is zero.

C. Second Dimension (SDS-PAGE)

The separation of proteins on the basis of their molecular weight is brought about in the Second dimension by SDS-PAGE. The focused strip is run on the gel in a direction, which is orthogonal to the IEF run. Follow previous lecture for more details of this step.

D. Scanning and Image Acquisition

After the second dimension run is completed, the gels are scanned at 3 different wavelengths, which are the excitation wavelengths of each of the 3 dyes, namely Cy3, Cy5 and Cy2. The resulting images are stored for further analysis.

E. Image Analysis and Data Interpretation

The saved images are further analyzed by using software especially designed for 2D-DIGE, such as DeCyder. The pair-wise analysis between test and control samples can

be performed using Differential In-gel Analysis (DIA) module, whereas the analysis between multiple samples belonging to two different groups can be performed using the Biological Variation Analysis (BVA) module of the software. Details of the data analysis will be described in next lecture.

3 Applications of DIGE

The 2D-DIGE technique has been used for several proteomic applications. Few representative studies are described here. Details of applications will be discussed in next lecture.

DIGE for detection of markers for cancer:

- Human tissue biopsies are obtained and protein is extracted. The 2D-DIGE is carried out as per the described protocol. The control samples of healthy control labeled with Cy3, whereas the treated samples (diseased patient for cancers) labeled with Cy5 or vice versa. An internal pool is labeled with Cy2.

APPLICATION	**REFERENCE**
Proteome analysis of human colorectal cancer tissue using DIGE	*Xui bai et al. 2010*
Biomarker discovery for esophageal cancer	*Norihisa Uemura et al. 2009*
Whole proteome analysis of drug treated rat muscle	*Kenyani et al. 2011*
Post-translational modifications	*DeKroon RM et al. 2012*

4. Advantages and Limitations of DIGE

Advantages:

- The amount of protein starting material required is very small as compared to the requirement for 2DE. DIGE is extremely sensitive, i.e. <1 fmol of protein can be detected and it can also enable the linear detection over a >10,000-fold protein abundance range.
- Higher reproducibility as two different samples can be analyzed on the same gel; therefore, differences in protein expression levels are purely attributed to biological variations.
- Accurate quantitative comparison, which can be attributed to the use of internal control, which allows normalization of spot intensities across different, gels and increases accuracy of protein expression differences.
- No post-electrophoretic processing (fixation or destaining) is necessary and thereby there is a reduction in protein loss particularly in the low molecular weight range.
- User bias can be eliminated by the co-detection method.

Limitations:

- Only two different samples can be analyzed on a single gel. For more than two samples, a large number of pair-wise comparisons would be required. Mass spectrometry based techniques such as iTRAQ could be used for 4 or 8-plexing.

- Spot excision is a problem since spots are not visible to the naked eye and hence require aids such as Robotic Spot picking. Alternatively, a normal 2-DE gel has to be run for these samples, which is then stained with Coomassie dye and spots of interest are excised.
- Fluorescence detection comes with several inherent problems such as high background, the detection of signals from non-protein sources (e.g. dust and residue on plates) and overlap of signals from different fluorophores.

CONCLUSION

DIGE technique has gained wide acceptance in proteomic studies because of its sensitive quantification abilities and reduced analytical variability. It also offers an advantage over 2-DE with respect to the reproducibility and accuracy in quantification. DIGE is an increasingly employed proteomic technique, which can be used to address several biological questions.

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