HANDOUT

LECTURE-14

GEL-BASED ELECTROPHORESIS

TWO-DIMENSIONAL ELECTROPHORESIS WORKFLOW: STAINING AND IMAGE ANALYSIS

Slide 1:
Continuing from the previous lecture on gel based proteomics, that covered SDS-PAGE BN-PAGE, 2-DE and various advanced forms of gel-based proteomics, today's lecture talks about the workflow of 2-DE.

Slide 2:
Today's lecture will continue to cover the 2DE workflow, which, as a quick recap, has already covered the sequential steps of 1)isoelectric focusing, 2)equilibration of the IPG strip to make it compatible for the second dimension separation in SDS-PAGE, 3) SDS-PAGE to separate proteins based upon molecular weight, and 4) visualization of protein spots on the gel.

Slide 3:
The last lecture discussed two staining methods, of coomassie brlliant blue and silver staining.

Continuing with the same workflow, this discussion will continue to discuss other types of staining methods, including more sensitive stains such as SYPRO Ruby, Pro-Q Diamond and the Cyanine dyes.

Each one of these are used for various applications. For example, the Cyanine(Cy) dyes are used in one of the advanced forms of gel electrophoresis, known as 'Difference in gel electrophoresis (DIGE)'. In this method the Cy-dyes are mixed

together to facilitate seperation of proteins from 2 different test conditions on a single gel.

SYPRO ruby is one of the sensitive dyes and this stain is also used for looking at different types of protein patterns, and can be used for for dual staining in combination with another stain.

Post-translational modifications can be examined using a few stains such as Pro-Q diamond.

Slide 4:
SYPRO Ruby staining: This is one of the flourescent stains which is usually visualized in the UV or Blue light transluminescence. This stain is very sensitive and is an endpoint stain and generates very little background unlike the silver stain. Owing to high sensitivity, it can be useful to study low molecular weight, low abundance proteins and it can also be used for studying different types of post-translation modifications and hence is useful to study glycoproteins. SYPRO Ruby is also very useful for protein charectarization as it is very compatible for further processing by mass spectrometry.

Slide 5:
The following animation details the steps involved in performing SYPRO Ruby staining.

ANIMATION 1
SYPRO Ruby red:

Fixing the protein bands in the gel

The completed gel is first placed in a fixing solution of methanol and acetic acid that fixes the protein bands in the gel and minimizes any diffusion. This must be subjected to gentle shaking for around 30 min.

Staining the gel

After the fixation step, the gel is soaked in the flourescent SYPRO Ruby Red stain solution. The gel should be kept on the mechanical rocker for uniform shaking, resulting in uniform staining with the ruthenium-based chelate dye.

After the overnight step of staining, the gel can be washed with methanol and an acetic acid solution, on a rocker, to perform uniformone destaining. The MS compatible gel is then viewed using a UV or laser scanning instrument with an excitation wave length near 280 or 450 nm. This stain has 2 excitation maxima and an emission maxima of 610 nm. This animation demonstrates the staining obtained after after SYPRO Ruby Red staining of the 2-DE gel, showing seperation in the 2 dimensions of molecular weight and charge.

Slide 6:
Cyanine Dyes: These are very sensitive dyes and are water soluble derivatives of N-hydroxysuccinimide which can be covalently bound to the epsilon-amino groups of a protein's lysine residue. The quantity of dye added to the sample is limiting in the reaction and hence this reaction is refered to as 'minimal labelling', which ensures that the dyes label approximately 1-2 % of the available lysines and then only a single lysine per protein molecule. Multiple protein samples can be labeled with these dyes and then mixed together to separate the proteins on one gel. Now, unlike in traditional 2-DE, wherein the test and control samples are run on different gels and then compared, Cy-Dye labelling allows comparison of 2 or more samples on the same gel, by using multiple flourescence scanning parameters. This method is employed in DIGE, which represents one the very advanced forms of 2-DE and eliminates many gel-to-gel variation issues.

Slide 7:

While this topic will be covered in greater detail in the latter part of the course, a a brief animation for Cyanine dye staining is now described.

ANIMATION 2

Cyanine Dyes:
In this detection technique, the dyes are mixed with the samples prior to electrophoresis. The first sample is labeled with Cy3 dye and the second sample is labeled with the Cy5 dye. Now, an internal pool made from both samples 1 & 2 is labeled with 3rd dye, Cy2. Each protein sample as well as the standard internal pool is labeled with a differently fluorescent Cy dye which allows all the protein samples to be simultaneously run on a single 2-DE gel.

2D seperation

After dye labeling and mixing all the samples together, isoelectric focusing is performed, followed by the second dimension separation in SDS-PAGE.

Scanning the processed gels

The gel is then further scanned and viewed by illuminating it alternately with excitation wavelengths corresponding to the various Cy-dyes. This technique minimizes the gel-to-gel variations and allows a large number of samples to be run by using a fewer number of gels. Therefore, it provides very useful solution to various proteomics applications.

Slide 8:
Pro-Q diamond: This stain is a flourescent dye which is capable of detecting phosphorylation and is a sensitive method to study phosphorylation patterns in post-translationally modified proteins. This is very suitable for use in electrophoresis and it offers sensitivity at the nanogram level. However, exclusive use of the ProQ diamond

stain only would allow visualization of phosphorylated proteins in that gel. In order to further visualize (and charectarize by MS) the other proteins in the gel, dual staining is performed wherein the same gel is next stained with SYPRO Ruby. Visual comparison of the two images generated from a single gel sequentially stained by Pro-Q Diamond in Step 1 and then stained with SYPRO Ruby in Step 2 provides information about protein phosphorylation as well as global protein expression, allowing a deduction of the percentage of total proteins in any given sample that are phosphorylated. Combination of this methodology with MS provides a powerful tool for protein charectarization.

Slide 9:
Following is the animation demonstrating Pro-Q Diamond staining followed by dual staining.

ANIMATION 3

Pro-Q Diamond:
This is very useful staining technique for detection of phospho-proteins and when used in combination with SYPRO Ruby red it provides a comparative profile of total protein and phospho-protein contents.

Fixing the protein bands in the gel

The first step involves protein fixation followed by addition of Pro-Q diamond stain. This flourescent dye offers more sensitivity down to nanogram level and specifically detects phosphorylation at serine, thionine or tyrosine residues. For uniform staining, 8-12 hours of incubation with staining solution is required.

Staining the processed gel with Pro-Q Diamond

The staining step is followed by a wash with methanol and acetic acid. For uniform destaining, gentle shaking with the wash solution for atleast 6 hours is required. The gel can be further scanned at anemission maxima of 580 nm Often people combine this phospho stain along with SYPRO Ruby stain to obtain dual staining information which was earlier described in the lecture.

Scanning of the destained gel

Slide 10:

The previous animation demonstrates how the Pro-Q diamond stain can be used to detect PTM or phosphorylated forms of the proteins, and then deduce relative expression of these forms by using a dual staining technique wherein the second stain is SYPRO Ruby. This application has merit in that it also allows more than one detection protocol to be used on the same gel. It should be emphasized that the stain selection should be selected based upon the application.Samples with high protein oncentration can be stained with Coomasie Blue while those with low protein concentrations can be stained with more sensitive stains such as Silver or SYPRO Ruby. Studies examining PTM forms can be conducted using Pro-Q Diamond. while Cyanine dyes can be used to eliminate one a lot of gel artefacts. Following gel staining, the next step in visualization is image scanning.

Slide 11:

The slide lists comparisons of different stains available. Note the sensitivity is only approximate.

- Coomassie blue is one of the most commonly used stains which is employed in most of the laboratories worldwide. It is compatible with mass spectrometry. Sensitivity is in the range of 40-100 ng. Biosafe coomassie (an advanced form of the coomassie stain which is environmental friendly, nonhazardous and offers sensitivity in the range of 10 ng) is also MS compatible.
- Silver Or SYPRO Ruby stains are suitable when the protein extraction yields low protein quantities. having sensitivities in the range of 1 ng. While the original Silver stain has issues with MS compatibility, certain modifications, resulting in the development of ‘Silver Stain Plus’ have solved this issue. The SYPRO Ruby stain is MS compatible and is advantageous compared to the Silver stain in that it yields very low background staining on the gel. Infact, the linear dynamic range of SYPRO Ruby stain extends over three orders of magnitude, making it vastly

superior to Silver and Coomasie Blue stain, and helps in providing a very low background during staining.

Slide 12:

The following video will cover a laboratory demonstration of staining 2 dimensional gels using the coomassie or silver stains, followed by subsequent image acquisition and analysis.

Video demonstration

Gel staining and de-staining:

This process involves removal of the gel from the electrophoresis assembly followed by the treatment with the fixing ,staining and finally the de-staining solutions.

Gel removal and protein fixation:

- Soak the gel present the between the glass plates in fresh distilled water to remove any bound SDS.
- Pour the fixing solution containing methanol and acetic acid into another tray.
- Open the glass plates and carefully remove the IPG strip from the gel.
- Then transfer the gel into the fixing solution by gently sliding off from the glass plate.
- Place the tray on a mechanical shaker for an hour to ensure that protein spots get fixed on the gel, thereby minimising any diffusion.

Gel staining:

- Transfer the gel carefully into another tray containing the coomassie blue staining solution and place it on the shaker to ensure uniform contact of the gel with the solution.
- The negatively charged coomassie dye interacts with proteins through ionic and other non-covalent interactions thereby staining them with a blue colour.

- Add some more staining solution such that the gel is completely immersed and leave it overnight for a around 8-10 hours.

De-staining:

- Drain out the staining solution from the tray and pour the de-staining solution consisting of methanol and acetic acid into the tray.
- Place the tray on the shaker for around 6-8 hours until the background stain of the gel is completely removed and spots are clearly visible.

De-staining the gel

Another commonly employed technique for visualization of protein bands is the use of the silver staining solution. In this procedure the gels are first treated with the fixing solution followed by the silver stain and finally with the developing solution where the silver ions get reduced to metallic silver by an alkaline solution of formaldehyde and sodium carbonate. The dark protein bands can be viewed well against a light background. Shown below are gels containing separated proteins from serum, bacteria and plant samples that have stained with coomassie blue dye(bottom) as well as plant protein sample that has been stained with silver dye(top).

Slide 13:

The image is one2-DE gel image obtained from human serum, wherein one can see the molecular weight and isoelectric point of proteins separated in 2 dimensions. The first dimension is the isoelectric point (determined by the charge of the protein) and the second dimension is the molecular weight. For each of the protein spots one can obtain both informations for isoelectric point and molecular weight. This is a gel stained with coomassie stain.

Slide 14:

The next portion deals with with image aqquisition and scanning. Image analysis is another very important aspect of the 2-DE workflow.

Slide 15:
The different types of image scanners available from different vendors include the molecular imager densitometer and Typhoon variable mode imager. There are only a couple of examples from many staining image scanners available.

Slide 16:
Manual Image Analysis involves visual examination and sizing of every single spot on both control and test sample gels manually. This, as one can imagine, is an extremely tedious and inefficient process making statistical analysis to yeild interpretable results difficult. Automation of this process, using scanners and image analysis software is thus necessary.

Slide 17:
There are many commercially available software programs designed for 2-DE gel analysis. Almost all of these programs can:

- Enable spot identification
- Compare of test/treatment and control sample gel images.
- Overlay and crop these images
- Perform statistical analyses.

Slide 18:
- **The crop tool:** Thisis usually the first command used in these programs to help eliminate any area devoid of spots of interest, taking care to crop both the control and treatment gels uniformly. This tool allows for a very specific defined region of the gel to be cut from the entire gel, allowing selection of a region with a high spot density that can be used for further analysis.
- **Zoom Tool:** This is used to expand a specific area of the gel for futher analysis.

Slide 19:
Imaging overlaying: This is a very important aspect because if comparing 2 gels; one control and other treatment. Overlay of these images is required to oneto compare the spot pattern present on the two different gels.**Spot analysis:** This is the step following image overlay, wherein it is possible to obtain physical and statistical

parameters for each spot which is present on the gel. 3 dimensional views of each of the spots can be generated and how they differ from the control to the treatment samples can be determined, This involves comparison of the gels on a spot-by-spot basis. This process is a far more tedious and time consuming one than gel running.

Slide 20:
The images are obtained from from test (disease) and control (healthy) samples. Each of the spots is compared between the control and the test samples, and furthermore, different healthy controls andtest samples can be compared to see if overall change for any particular spot of interest reaches statistical significance between the 2 groups. It allows one to ask : If any particular spot (ie protein) is upregulated or downregulated, is this change uniform (ie identifiable in a majority of samples from either group)_and does it reach statistical significance?

Slide 21:
As seen in the slide, one spot of interest is compared across 16 gels. After using the software to zoom into that particular region, a 3D representation of each spot is obtained, which is then compared between the control (shown in yellow) and the test/disease (shown in blue) In this case, the 3D views of this particular protein shows a clear difference between the two groups, being higher (ie, upregulated) in the test/disease sample. Additionally, one can look at each of these spot's intensity in great detail and plot different types of parameters for percentage volume or spot intensity, allowing further analysis of that particular protein between the two groups

Slide 22:
The slide is a listing of various commercial softwares that are available for comparing the 2-DE gels. These are just a few examples of many different commercial applications designed for this purpose.

Slide 23:
Shown in the following animation is a step-by step procedure to perform 2-D gel analysis, using a generic software layout.

ANIMATION 4

2-D gel analysis software:

The first step is loading the gel image. It is possible to load either a single or multiple gel images simultaneously. This can be done by means of the load option in the file banner. One can save the gel images and then crop the area of interest.

Loading gel image

It is possible to crop the gels by selecting a specific region that is to be studied and then selecting the "crop gel" function. Cropping the gel helps in the selection of region with high spot density and/or to reduce the regions which contain high background with no spots.

Cropping of the gel images

Zooming into a selected region: if one wishes to expand a specific region the zoom tool can be used. A specific selected region of the gel can be zoomed into, allowing closer viewing of the spot and comparison of the spots between the two gels. This is particularly useful for gels with large number of spots. Once a suitable area is selected, cropped images from this area are then overlaid on top of each other. Overlaying of images is a particularly useful tool for the comparison of two gels. The gels are overlayed such that they appear merged and the spots that coincide will overlap with each other. This is extremely helpful while comparing the large clinical samples of controls and treatments so that one can obtain a clear indication of the proteins which are differentially expressed. Now, after the analysis one can look at the graphical representations of the 3D view of the selected spot(s). wherein the spots on the gels can be displayed as 3D graphs. Either the entire gel can be chosen or particular region can be selected for this representation. The peaks obtained in the graphical representation are directly correlated to the spot intensity.

3D view of the spots

Spot analysis: Every spot on the gel can be analysed by selecting the "detect spot" option. Various parameters such as smoothness, saliency and minimum area must be suitably adjusted for maximum clarity. Once this done, each spot will either be encircled or marked with a cross depending upon the setting along with the spot numbers.

Gel matching: This software facilitates interpretation of gel images by matching two different gel images which were obtained in the experiment. The matching spots are marked and after the matching is done any variation in the spot intensity and position can be indicated by the blue line as shown in the animation. This provides an understanding about the reproducibilty across the gels.

Detection of the spots

Spot matching

Once one have analysed the gel, a 'spot table' is generated which provides detailed information about any given spot including spot number, intensity, area and volume as well as the saliency. These parameters help to judge the quality of a gel.

Spot table

In addition to the physical parameters various statistical parameters can also be computed for each gel and for each spot on the gel such as central tendency, mean, median, dispersion, coefficient of variation, standard deviation or other statistical parameters. The scatterplots and histograms can also be plotted for clear data analysis. These can provide information regarding inter- and intra-gel variations.

Scatterplot

The Spot comparison: It is possible to specifically compare a particularly selected spot across the gels. When the gels are run with molecular weight markers with molecular weight of an unknown protein can be estimated from this information. For example, as

one can see in the animation, on the left hand side the molecular weight marker is loaded and now from that information one can compute the information for the unknown protein to calculate its molecular weight. These parameters in addition to the other physical and statistical parameters can be obtained for each spot.

Spot comparison

An advanced form of 2DE technique, which is DIGE (done using Cyanine dyes) is now discussed,While both DIGE and Cy dyes will be discussed at length later, the concept that DIGE analysis can be performed with specialized software which can overlay the images obtained from the scanners for Cy3, Cy5 and Cy2 labeled samples is introduced here. It allows comparison of 3 gel images simultaneously, out of which one is typically the pooled internal standard containing all proteins labeled with Cy2 while the other 2 are control and treatment samples labeled with either Cy3 or Cy5. Any changes implemented on one gel image such as cropping, spot selection, etc. will be implemented across all the three gel images in DIGE. Other features and tools for DIGE analysis are similar to those used for 2-DE analsis. The physical and statistical parameters of all the spots on the gels can be determined through their corresponding reports.

Slide 24:
The next step in this workflow is the spot picking which details how to excise the spot of interest from the gel. Now, most of the time, in abundance based proteomics where only the change in few proteins because of a given treatment, disease or condition is being examined, people use the comparison of their control and treatment gels. This (manual excision) is easily done when the number of protein changes occuring will not be too high—in the range of a 50-100 proteins. If one prints the spot map or the 2D gel image and keeps it side by side along with the gel, it is easy and possible to locate those spots on the given gel and then perform manual excision, along with various quality control checks to avoid any contamination. The other way of doing is to use the robot picker and excise the spot of interest. That is more precise but more costly. This would be

necessary if global expression of all the proteins (for eg., for a given sample or for a given organism if the proteome is unknown) was being studied.

Slide 25:
Two methods for the spot picking are shown. One is the manual excision of the spot where the user can take a scapel and make a cut on a particular gel for the given spot of interest and then transfer that into an eppendorf tube, label that and transfer that to a 96 well plate along with water. Ultrapure (millicure water or autoclaved water) needs to be used, , along with sterile scalpels and clean glassware, taking care to avoid contamination from the air or from gloves used. This procedure is preferably carried out in a laminar hood. This procedure is then followed by gel digestion and and mass spectrometry.

Slide 26:
The altervative method, robotic spot picking, is more accurate, more precise but also more costly. It is applicable when doing global protein profiling or while using flourescent dyes such as Cy dyes. For each selected spot, the robotic arm moves and picks up the individual spot.

Slide 27:
Following the completeion of the 2-DE workflow, other gel based methods to study the proteome will be briefly discussed.

Slide 28:
Other gel based methods (listed in the slide) have already been discussed. OFFGEL electrophoresis is an another new technology introduced from Agilent that will be covered briefly here.

Slide 29:
OFFGEL electrophoresis: As the name suggests in this method avoids separating proteins by a gel-based methodology.So OFFGEL electrophoresis can be performed on the IPG strip but subsequent gel approaches for protein seperation are not required and this method is mostly used for liquid chromatography-based mass spectrometry

applications wherein an avoidance of the artefacts introduced by gel-based methods is desired.-

Slide 30:

OFFGEL electrophoresis seperates proteins based on the isoelectric points of the proteins and the peptides. Following isoelectric focusing ,the components which are separated based on the pI values can be recovered in the liquid phase itself.

In this method, by the time the IEF has completed. The protein samples are already present in the liquid phase and one can remove aliquots from different pH ranges.This method is very compatible for further down stream processing in LC/MS or by immunodepletion.

Slide 31:
Briefly, the fractionation principle involved in OFFGEL electrophoresis method is covered here.

The IPG strip can be re-hydrated and tightly sealed against the frame of the well in the OFFGEL instrument. An example of seperation of 3 proteins(Proteins A, B and C) is provided here. Each of these proteins has a different isoelectric point as shown in the figure. Now in the IEF instrument, following application of the IPG strip, the proteins (in a liquid phase) are added, causing tightening of the wells which in turn causes equal distribution of proteins in the wells throughout the strip. This is then followed by addition of a coverslip to avoid evaporation.

Slide 32:
Following application of a high voltage, the proteins and peptides in the electric field will migrate because of the high voltage applied and proteins will start moving based on their charge(which is determined by the pH of their surroundings).

Slide 33:
When they reach a point where pH is equal to the isoelectric point then at this point, they become neutral and hence there will be no protein migration past this position.

The proteins A B and C had unique, well seperated pIs. Collecting fractions in different pH ranges thus ensures their efficient seperation in different fractions. While the supplied eaxmple is a that of a simple mixture of 3 proteins of different pH,However, ideally this method can be used for seperation of very complex protein mixtures to reduce the complexity of overall proteome and to separate or fractionate the overall proteome based on the isoelectric point and collect each of the fractions which can be separately further analysed using LC/MS approaches.

Slide 34:
Now, OFFGEL itself is sufficient and used widely for various LC/MS applications but the same manufacturer has introduced another technology allowing separation in the 2 dimensions of isoelectric point(ie, charge) and the molecular weight. So OFFGEL and on-chip electrophoresis methods can act as a substitute for gel-based protein separation by isoelectric point and the molecular weight. Now, the OFFGEL fractionation can be combined with high sensitive on-chip electrophoresis bio-analyser which enables 2-DE type of analysis with very high resolution and high sensitivity. This method is very suitable for studying differential protein expression for different applications.

Slide 35:
In summary, today's lecture has covered different types of staining techniques available for different applications, the comparison and analysis of 2-D images obtained from different sample groups and finally spot excision methods. It has also briefly looked at some variation to the 2DE process, such as OFFGEL fractionation. The next lecture will cover advanced electrophoresis technologies such as 2-Dimensional Difference in Gel Electrophoresis.