

## Technical tips

### Session 7

#### NuPAGE Bis-Tris electrophoresis

This system consists of gels made at pH 6.4 (as opposed to the more typical pH 8.7 of Tris-Cl polyacrylamide gels). The lower pH offers several advantages over traditional gels, such as increased gel stability. Gels can be stored at room temperature for up to one year with no loss in resolution, a result of reduced polyacrylamide hydrolysis. The lower pH is also supposed to increase protein stability during electrophoresis. Because the protein separation region is at pH 7, as compared to 9.5 of traditional SDS-PAGE, the proteins are less susceptible to modifications such as deamination, alkylation and oxidation.

#### 2-D Gel electrophoresis and isoelectric focusing (IEF):

The technique of two-dimensional electrophoresis involves separating proteins in the **first dimension** according to **charge** (isoelectric focusing, IEF), followed by separating the focused proteins in the **second dimension** according to **molecular weight** by sodium dodecyl polyacrylamide gel electrophoresis (SDS-PAGE).

**Isoelectric focusing** gel electrophoresis (**first dimension**) separates proteins according to their **net charge**. At a given pH, a protein's net charge will depend on its relative number of positive and negative charges (which depend both on the amino-, carboxy- terminals and the net charge on the side chains of its amino acid residues). During isoelectric focusing, separation is accomplished by placing the protein in a pH gradient generated by an electric field. Under these conditions the protein migrates until it reaches the position in the pH gradient at which its net charge, or **isoelectric point**, is zero.

The **second dimension** of two-dimensional gel electrophoresis simply consists of SDS-PAGE in which either a strip or a tube gel from isoelectric focusing is fitted over an SDS-polyacrylamide gel and the proteins are separated according to molecular weight by electrophoresis.

The proteins may then be visualized by staining with Coomassie Brilliant Blue R250, silver stain, fluorescent dyes or through radioisotope detection (phosphorescence) after the proteins are metabolically labeled with  $^3\text{H}$ ,  $^{14}\text{C}$ , or  $^{35}\text{S}$  labeled amino acids. This two-dimensional array will produce spots that correspond to a single protein species in the sample. Using this technique combined with the 2D gel analysis system, thousands of different protein can be separated and information such as pI, molecular weight and protein amount can be determined. These spots can be excised for further analysis or the 2-D array can be analyzed for differences in protein quantity or in proteins present in the gel.

2-D gels can also be electroblotted to PVDF or nitrocellulose membranes for further analysis.

(Image removed due to copyright reasons.)

### **Mass spectrometry for protein identification**

In mass spectrometry, a substance is bombarded with an electron beam having sufficient energy to fragment the molecule. The positive fragments produced (cations and radical cations) are accelerated in a vacuum through a magnetic field and are sorted on the basis of mass-to-charge ratio. Since the bulk of the ions produced in the mass spectrometer carry a unit positive charge, the value  $m/e$  is equivalent to the molecular weight of the fragment. The analysis of mass spectroscopy information involves the re-assembling of fragments, working backwards to generate the original molecule. A schematic representation of a mass spectrometer is shown below:

(Image removed due to copyright reasons.)

A very low concentration of sample molecules is allowed to leak into the ionization chamber (which is under a very high vacuum) where they are bombarded by a high-energy electron beam. The molecules fragment and the positive ions produced are accelerated through a charged array into an analyzing tube. The path of the charged molecules is bent by an applied magnetic field. Ions having low mass (low momentum) will be deflected most by this field and will collide with the walls of the analyzer. Likewise, high momentum ions will not be deflected enough and will also collide with the analyzer wall. Ions having the proper mass-to-charge ratio, however, will follow the path of the analyzer, exit through the slit and collide with the Collector. This generates an electric current, which is then amplified and

detected. By varying the strength of the magnetic field, the mass-to-charge can be continuously varied.

The output of the mass spectrometer shows a plot of relative intensity vs the mass-to-charge ratio ( $m/e$ ). The most intense peak in the spectrum is termed the **base peak** and all others are reported relative to this peak's intensity. The peaks themselves are typically very sharp, and are often simply represented as vertical lines.

(Image removed due to copyright reasons.)

**Mass Spectrometry of Intact Proteins and Protein Fragments:** The mass of intact proteins can be measured with an accuracy of  $10,000 \pm 2$  Da. This ability provides a wealth of chemical information about protein chemical structure, including confirmation of amino acid sequence, detection and identification of chemical groups added post-translationally, and localization of proteolytic cleavage sites. The mass spectrum is like a *fingerprint* which reveals the structure of a compound either by skilled interpretation of the masses and abundances of *molecular ions* and their *fragment ions* or by comparison with records in a database (typically containing data for hundreds of thousands of prerecorded spectra).

This way, when you get the predicted sequence of small peptide fragments analyzed by mass spec you can later compare with full-length protein sequences in databases in order to identify your protein of interest. This is a very common method nowadays to determine proteins involved in the formation of complexes.

(A variant of mass spec is Matrix-Assisted Laser Desorption Ionization (MALDI): **MALDI is an ionization technique for large and/or labile molecules such as peptides, proteins, polymers, dendrimers, and fullerenes. This technique involves embedding analytes in a matrix which absorbs energy at the wavelength of the laser. The mechanism of the MALDI process, a combination of desorption and ionization, produces both positive and negative ions, usually in the forms of  $(M+H)^+$ ,  $(M+Na)^+$  and  $(M-H)^-$ . The technique also produces multiply charged ions, usually up to +3, as well as dimers, trimers, etc.)**