Removal of Interfering Substances in Samples
Prepared for 2-D electrophoresis

Introduction
Impurities such as ionic detergents, lipids, nucleic acids, salts and other ionic compounds, and even high-abundance proteins can impact a 2-D electrophoresis experiment by interfering with protein separation or by obscuring proteins of interest. These interfering substances can be endogenous (for example, phenolics, lipids, and nucleic acids) or exogenous (added during sample preparation; for example, salts and detergents). Either way, removing these impurities prior to analysis or mitigating their effect is often essential for good results.

General Considerations
Though removal or mitigation of interfering substances often yields clearer 2-D patterns and improves resolution of protein spots, any treatment of the sample can reduce yield and alter the relative abundance of sample proteins. Procedures for the removal of interfering substances represent a compromise between removal of non-protein contaminants and minimal interference with the integrity and relative abundance of the sample proteins. Since proteomics aims to study the relationship among proteins in their natural state, it is important to remove an interfering substance only when necessary and by using techniques appropriate for the sample.

Protein precipitation is a common general method for contaminant removal. Conditions are chosen under which sample proteins are selectively precipitated while leaving soluble the major nonprotein contaminants. Following centrifugation, the precipitated proteins are resuspended in a solution suitable for IEF.

Methods used in sample preparation for 2-D electrophoresis include precipitation with TCA and acetone (Damerval et al. 1986, Görg et al. 1988) and precipitation with methanol and chloroform (Wessel and Flügge 1984). Precipitation procedures also have the benefit of concentrating sample protein, which is often necessary for effective sample application.

Individual types of interfering contaminants cause specific problems and can be removed or mitigated in different ways. The most prevalent interfering contaminants and their removal methods are discussed next.

Nucleic Acids (DNA and RNA)
Nucleic acids, particularly DNA, can interfere with IEF (for example by clogging gel pores) and increase sample viscosity, thus limiting the effectiveness of cell lysis and sample application. Because smaller nucleic acids are generally tolerated better, strategies to reduce nucleic acid interference involve either shearing or enzymatic digestion: sonication shears DNA and renders the sample less viscous, and addition of nuclease digests nucleic acids to oligo- or mononucleotides.

Nucleases are often employed during sample preparation, particularly with bacterial lysates in which nucleic acid:protein ratios are high. Successful application of nuclease treatment requires attention to three factors:

- Nuclease may be inactive under the strongly denaturing conditions often used to prepare protein samples for 2-D electrophoresis.
DNase requires magnesium ions for activity

Nucleases are proteins and can appear in the 2-D pattern as extra spots

Benzonase is a nuclease with properties that make it particularly useful in sample preparation for 2-D electrophoresis (Chan et al. 2002). It is active in the presence of urea, and the amount required for treatment is usually not visible in a 2-D gel. It is applied in the presence of 1 mM MgSO4 or MgCl2. The magnesium ions are subsequently sequestered with EDTA in order to inhibit proteases that may require metal ions for activity.

Polysaccharides
Polysaccharides can interfere with electrophoresis by clogging gel pores and by forming complexes with proteins. Like nucleic acids, they can also cause a sample to be viscous, making it difficult to work with. Polysaccharides are a particularly prominent problem with plant-derived samples. Centrifugation may be used to remove high molecular weight polysaccharides. Phenol extraction, followed by precipitation with ammonium acetate in methanol, is a commonly used method that is very effective at removing polysaccharides in plant samples (Hurkman and Tanaka 1986, Wang et al. 2008).

Phenolic Compounds
Phenolic compounds are found in all plants and in some microorganisms and they can modify proteins in an enzyme-catalyzed oxidative reaction. The modification can cross-link proteins together or render them insoluble. The reaction can be prevented with reductants such as DTT, β-mercaptoethanol, or ascorbic acid, and the enzyme is inactivated by thiourea. Phenolic compounds may also be removed from the extract using the ReadyPrep 2-D cleanup kit (see the Products for Contaminant Removal sidebar) or by including polyvinylpyrrolidone (PVP) or polyvinylpolypyrrolidone (PVPP) in the extraction solution. These compounds bind phenolic compounds, and the precipitated complex can be removed from the extract by centrifugation (Toth and Pavia 2001). The phenol extraction procedure described above (see Polysaccharides) is also effective at removing phenolic contaminants (Hurkman and Tanaka 1986, Wang et al. 2008).

Lipids
Lipids can form insoluble complexes with proteins, but lipids can also complex with detergents, thereby reducing the detergents’ effectiveness at solubilizing protein. The effect of lipids can be minimized by using excess detergent (for example, 4% CHAPS in the lysis solution when preparing lipid-rich tissues such as brain). Precipitation methods that employ organic solvents (Damerval et al. 1986, Görg et al. 1988, Wessel and Flügge 1984) or the ReadyPrep 2-D cleanup kit can also be used to remove lipids.

Salt and Other Small Ionic Compounds
IEF requires samples that are free of salts and other small ionic compounds that may interfere with pH gradient formation. Salts formed from strong acids and strong bases (for example, NaCl) dissociate into their component base and acid, which is eventually drawn to either end of the IPG strip. Until this occurs, the conductivity of the IPG strip remains high and the voltage attained is low. The flow of ions from the IPG strip is accompanied by water flow, and one end of the strip may dry out, breaking electrical contact.

Weak acids and weak bases (for example, acetate, Tris, or ammonium ions) may not completely leave the IPG strip during focusing. These compounds interfere with the pH gradient, resulting in streaking and loss of resolution at one end of the pH range or the other (Figure 2.3). Amphoteric buffers such as HEPES can focus within the pH gradient, resulting in a portion of the pH gradient where proteins focus poorly.
Fig. 2.3. Effect of salt removal. E. coli extracts containing 1 M NaCl were separated by 2-D electrophoresis before and after treatment with the ReadyPrep 2-D cleanup kit. The samples were focused using 11 cm ReadyStrip pH 3–10 IPG strips and then separated on Criterion 8–16% Tris-HCl precast gels.

Samples of low ionic strength are desired, yet many samples contain salts and small ionic compounds that are either intrinsic to the sample type or have been introduced during sample preparation. Precipitation and dialysis methods are very effective at removing ionic contaminants, as is treatment with a desalting column (Chan et al. 2002).

Prevention of Keratin Contamination
Skin keratin is a common contaminant of 2-D gels and mass spectra. It may appear in silver-stained and fluorescently stained 2-D gels as an artifact focusing near pH 5 in the 50–70 kD region, or as an irregular but distinctive vertical streaking parallel to the SDS-PAGE direction of migration.

The best remedy for this keratin artifact is to avoid introducing it into the sample in the first place. Filter all monomer solutions, stock sample buffers, gel buffers, and electrode buffers through nitrocellulose and store them in sealed containers; then, clean the electrophoresis cell thoroughly with detergent. Above all, careful sample handling is important when sensitive detection methods are used, and gloves should be worn while handling samples, solution, or equipment.

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