Preparation of protein samples for SDS-polyacrylamide gel electrophoresis: procedures and tips

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Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) is the most widely used analytical method to resolve separate components of a protein mixture. It is almost obligatory to assess the purity of a protein through an electrophoretic method. SDS-PAGE simultaneously exploits differences in molecular size to resolve proteins differing by as little as 1% in their electrophoretic mobility through the gel matrix (1). The technique is also a powerful tool for estimating the molecular weights of proteins (2, 3). The success of SDS-PAGE as an indispensable tool in protein analysis has been attributed to three innovations that permitted the correlation of electrophoretic mobility with a protein's molecular mass (4). First was the introduction of discontinuous buffer systems where the sample and gel running buffers differ in both composition, Tris-HCl/Tris-glycine, and pH, 6.8/8.3, respectively (5, 6). Discontinuous buffer systems allow larger sample volumes to be loaded while maintaining good resolution of sample components because the proteins are focused, or "stacked," as thin bands prior to entering the resolving gel. Second was the use of the detergent sodium dodecyl sulfate (SDS) and reducing agents to denature proteins (7). SDS binds strongly to proteins at an approximate ratio of 1 dodecyl sulfate molecule per 2 amino acid residues (8). Therefore, the negative charge/unit mass ratio when SDS is bound to the polypeptide chain is similar for all proteins. Third was the combination of the first two discoveries employing a simple Tris-glycine buffer system (9). More recently, buffer combinations such as Tris-borate (10) and Tris-tricine (11) have improved the resolving power of the original methods. Modern SDS-PAGE has evolved to use microslab precast gels (12). Precast and packaged gels in a wide variety of gel formulations, acrylamide percentages, thicknesses, well formats, and buffer systems are now commercially available from several manufacturers. Therefore, successful SDS-PAGE analysis of protein samples no longer depends on tedious gel casting, buffer preparation and apparatus set-up, but on careful sample preparation and treatment prior to loading the gel. This article describes techniques and procedures as a guide for preparation of protein samples for SDS-PAGE analysis.

Sample buffer preparation

To ensure consistent and successful PAGE analysis, the highest purity reagents should be used to prepare sample buffer stock solutions. After a reliable source of electrophoresis reagents has been identified, the vendor and buffer component chemicals should be maintained. High purity electrophoresis, Ultrol® grade, and molecular biology grade reagents are available through Novagen's partner brand, Calbiochem. Solutions must be carefully and safely prepared, dated, and chemical lot numbers recorded. Concentrated stock solutions should not be stored for long periods of time. Tris base, rather than Tris-Cl, should be used for buffer preparation and pH adjustment made with HCl. Use of Tris-Cl

will result in a higher ionic strength, poor migration and diffuse protein bands (13). A respirator or dust mask should be worn when handling powdered SDS. Sulfhydryl reagents, dithiothreitol (DTT) and 2-mercaptoethanol (2-ME) can be unstable in solution and are toxic. These chemicals should be measured in a fume hood while wearing gloves and safety glasses. Although 2-ME is historically the chemical of choice for reduction of protein disulfide bonds in SDS-PAGE (7, 9), DTT is also a very effective alternative (14). Glycerol is added to increase the sample density, facilitating gel loading and preventing convective migration out of the sample wells. A small amount of bromphenol blue is added as a visual aid during sample loading and as a tracking dye, allowing easy monitoring of electrophoretic progress.

The sample buffer recipes listed in Table 1 are commonly used for Tris-glycine SDS-PAGE analysis of protein samples under denaturing, reduced conditions (7, 9, 13). When preparing these buffers, wear gloves to avoid keratin (skin protein) contamination. A heterogeneous cluster of bands around 55 kDa can be seen when a keratin-contaminated sample or sample buffer is used. This is particularly obvious with high sensitivity silver staining methods. The sample buffer should be divided into 1-ml aliquots and can be stored frozen (–70°C) for several months. Prior to use, warm (37°C) and mix the solution briefly to completely dissolve the SDS.

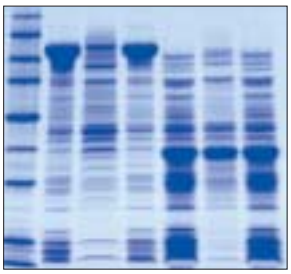
Table 1. SDS-PAGE sample buffer recipes

Component	Concentration	
	2X	4X
Tris-HCl, pH 6.8[1]	0.125 M	0.25 M
SDS	4%	8%
2-ME[2]	5%	10%
DTT[3]	0.15 M	0.3 M
Glycerol	20%	30%
Bromphenol blue	.01%	.02%

1. Prepared using Tris base, pH adjusted with HCl.
2. If 2-ME is used, omit DTT.
3. If DTT is used, omit 2-ME.

Protein sample preparation

Sample preparation is critical for clear and accurate resolution of protein bands. Photographic quality results are routinely possible if samples are carefully prepared. Common mistakes during sample preparation include using an incorrect protein-to-sample buffer ratio, delayed heating, overheating, failure to remove insoluble material, and overloading and underloading of protein. To prevent inadequate sample buffer-to-protein ratios, overloading, and underloading of samples, the protein concentration of the sample should be determined using a standard protein assay such as the CB-Protein Assay™, Non-Interfering Protein Assay™, or bicinchoninic acid (BCA) assay prior to sample buffer addition. Loading too much protein will result in distorted, poorly resolved bands in the overloaded lane and distorted electrophoretic patterns in adjacent lanes. Underloading simply prevents detection of minor components while even major bands will be too faint for photographic reproduction of the gel. Depending on the well size and gel thickness, the amount of protein loaded should range from 0.5–4.0 µg for purified samples and from 40–60 µg for crude samples if a Coomassie blue stain (e.g., RAPIDstain™) is used. Silver staining methods (such as the FASTsilver™ Kit) are approximately 100-fold more sensitive, and therefore require less protein per sample.



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SDS-PAGE sample buffer treatment is designed to completely dissociate all proteins into their subunit polypeptides. Proteins heated in the presence of SDS are denatured and imparted with a strong negative charge. Thiol reagents in the sample buffer reduce disulfide bonds. It is important to use enough sample buffer in order to maintain an excess of SDS. Most polypeptides bind SDS in a constant mass ratio of 1.4 µg SDS per 1.0 µg polypeptide, but a ratio of 3:1 is recommended (15). The 2X sample buffer prepared as shown in Table 1 contains 40 µg/µl SDS. Maintained reduction of protein sulfhydryls is essential in order to prevent intramolecular disulfide bond formation through oxidized cysteines. If artifactual band heterogeneity or unusual doublets are noted in SDS-PAGE results from samples containing sulfhydryls, insufficient reducing agent was present during sample treatment or the oxidation of cysteines may have occurred during the stacking phase of electrophoresis. These artifacts may be prevented if samples are treated with iodoacetamide (IAA) after heating in the appropriate concentration of sample buffer. The IAA treatment irreversibly blocks sulfhydryls and destroys excess reducing agent (16). Therefore, the sample buffer recipes in Table 1 do not necessarily indicate functional dilution factors, but rather they are convenient stock concentrations permitting correct addition of reagents to samples of high and low protein concentration.

Delayed heating of samples after sample buffer addition or excessive heating can cause electrophoretic artifacts due to protein degradation and peptide bond cleavage, respectively. Upon addition of SDS sample buffer, samples should be immediately mixed and heated to 85°C for three minutes. This treatment is usually sufficient to reduce disulfides, solubilize and dissociate proteins without peptide bond cleavage. Addition of SDS sample buffer will begin to denature most proteins. However, proteases are known to be resistant to SDS denaturation alone (15, 17). Partially denatured samples (particularly crude extracts) are therefore extremely sensitive to proteolytic degradation as protease active sites within the polypeptides become exposed by SDS treatment. Immediate heating limits degradation by completely denaturing all proteins including resistant proteases through the combination of heat, SDS, and reductant. Protease inhibitors may also be used during sample preparation to limit proteolysis. Excessive heating, e.g., 100°C for prolonged periods, may break peptide bonds or cause selective aggregation and band smearing (18). Asp-Pro bonds have been demonstrated to be sensitive to thermal cleavage.

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In some cases more extreme heating may be necessary to completely denature the protein (19, 20). Therefore, if prolonged heating at 100°C is necessary for complete dissociation of a thermally stable protein, the effects of such treatment upon peptide bond cleavage must be considered (21). Some proteins such as histones and membrane proteins may not completely dissolve by heating in SDS sample buffer alone and may require addition of 6–8 M urea or a nonionic detergent such as Triton X-100 (22, 23). After heat treatment in SDS sample buffer, insoluble material must be removed by brief centrifugation. This is easily accomplished by a two-minute spin in a microcentrifuge at 17,000 × g. Failure to remove precipitated insoluble material from the sample will cause streaking within the gel. The supernatant of the treated sample is now ready to load. The sample may be stored at 4°C overnight or frozen at –20°C for longer periods. Warm stored samples briefly at 37°C to redissolve the SDS and recentrifuge to remove insoluble material prior to loading.

Preparation of difficult samples

Samples that are dilute, acidic, very viscous, or that contain interfering compounds pose unique challenges to the SDS-PAGE analysis method. However, these difficult samples can be analyzed by SDS-PAGE through the application of one or more of the following pre-treatment techniques. Samples too dilute for analysis can be concentrated by several methods including lyophilization, spin concentrators, dialysis against concentrated polyethylene glycol (PEG), and absorption of excess solvent by exposure of the dialysis bag containing sample to dry PEG, Aquacide or gel filtration media such as Sephadex®. Samples concentrated through these methods may be dialyzed against 50 mM Tris-HCl, pH 6.8 to remove low molecular weight impurities prior to addition of SDS sample buffer. Dilute samples, acidic samples and samples containing interfering compounds such as potassium, guanidine hydrochloride, or ionic detergents can be precipitated by trichloroacetic acid or acetone to concentrate the proteins and remove contaminants. Protocols for TCA, acetone/methanol, and ethanol precipitation are described in references 4, 15, 17 and 24. A modified acetone precipitation method is also described in Novagen Technical Bulletin 012, available at www.novagen.com. Crude cell extracts are often extremely viscous due to the high concentration of unsheared nucleic acids. The high viscosity is problematic during gel loading, because samples are difficult to pipet and will not be evenly distributed in the sample well. Viscosity can be eliminated by treatment of samples with Benzonase® Nuclease prior to addition of sample buffer. This recombinant endonuclease completely degrades all forms of DNA and RNA and is free from proteolytic activity. Viscosity can also be reduced by physical shearing of the nucleic acids through sonication or by vigorous vortex mixing of the heated sample. Employing these pre-treatment protocols will allow successful SDS-PAGE analysis of samples that are very dilute, viscous or contaminated with interfering compounds.

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