Suggestions for Sample Preparation for 2D Electrophoresis (2D SDS PAGE) at Kendrick Labs

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For advice: call 800-462-3417 (locally 608-258-1565) or email 2d@kendricklabs.com

I. Introduction

This booklet contains suggestions for sample preparation for 2D sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS PAGE) performed at Kendrick Labs under standardized conditions. (1, 2) Isoelectric focusing, the first dimension, is carried out in SDS-compatible tube gels, not immobilized by pH gradient strips, so both dimensions are SDS-compatible.

The quality of 2D results returned to you is highly dependent upon sample preparation. Generally sample preparation is as simple as sending us samples in SDS or urea buffer dissolved to a known protein concentration. Problems that arise because of high salt, protein insolubility or dilute concentration can usually be remedied. Following these suggestions will help you stay away from unfixable problems such as protease degradation, and produce the best possible results.

Changing sample preparation may alter 2D patterns so once you decide on a protocol, stick with it. If you are uncertain, try your protocol on one or two samples before proceeding to larger numbers.

If you have questions please email or call. We know your samples are precious and will do our very best to make your project successful.

General Instructions

Start by filling out the sample identification form from our web site (Submit Samples link). You'll have to decide how much sample to load, the gel staining method and method of analysis. Call or email for advice, or you can leave undecided lines blank and let our Lab Manager figure it out (he'll call you with any questions.)

A. How much protein should be loaded?

1.**Complex samples like whole cell lysates and cytosols** are usually run on large format 2D gels (LF, 20 x 22 cm). For LF, a 100 μg load is recommended for silver and a 500 μg load for Coomassie blue staining. The maximum volume that can be loaded is 150 μl. Cytosolic protein mixtures give cleaner patterns than whole cell lysates. For standard format 2D gels (SF, 13 x 15 cm) load 50 μg for silver and 200 μg for Coomassie staining.

2. **Purified proteins** are run on SF 2D gels. For Coomassie blue we suggest a load of about 2 μg/spot and for silver staining about 100 ng/spot. If in doubt triple the load. Overloading does not severely affect IEF of semi-purified proteins. Glycoproteins tend to focus over a broad pH range and should be

loaded at 10–20 μg protein for Coomassie and 1–2 μg for silver.

3. **Membrane fractions** may be run on either LF or SF gels depending on the sample. Lipids, polysaccharides, and polynucleotides in membrane samples cause streaks and decrease resolution and are the limiting factor. Nucleic acid fragments stain strongly with silver and may cause high background. Glycosylated proteins will appear fuzzy because of charge heterogeneity. Call for consultation about which gel size to use. In either case it is best to use silver stain and load 75 μg for SF gels, 150 μg for LF. If Coomassie blue staining is necessary for mass spectrometry we suggest 100 μg maximum load for SF and 400 μg for LF. When in doubt it is best to titrate the load up rather than going for broke when trying to load maximally.

4**. Other subcellular fractions, nuclear matrix for example** (3)**,** can be isolated as described in the literature and run on SF 2D gels. If enough material is available we will optimize gel conditions at no extra charge for such samples.

5. **Samples for Western blotting**: For research samples it is best to load the maximum amount possible on SF 2D gels. For complex samples such as whole cell lysates, the optimal load is 200 μg for both transblot-ting and duplicate 2D gels for mass spectrometry. Since 50 μl is the maximum loading volume, the sample must be ≥4 mg/ml protein. For HCP projects, LF gels are best. The Lab and Western Blot Managers will help you decide on load via a conference call.

B. Total amount required

If possible we would like to receive four times the recommended load to allow for free repeats in case initial problems arise. For purified proteins, please include enough for two to four runs (call or email to discuss). If your samples are too dilute, you may concentrate by lyophilization (but be careful of concentrating salts) or by protein precipitation.

C. What sample buffer should be used?

We use two buffers to dissolve samples: Sodium Dodecyl Sulfate (SDS) Boiling Buffer and Urea Sample Buffer. Recipes are given on the next page. About 95% of samples run at Kendrick Labs are dissolved in SDS buffer or in SDS: Urea buffer 1:1.

A huge advantage to using carrier ampholines with IEF tube gels instead of IPG strips is that SDS can be used in sample preparation. Although SDS is negatively charged it is stripped from the proteins during isoelectric focusing (IEF) to form micelles with the nonionic detergent IGEPAL, formally known as Nonidet P-40. The micelles migrate to the acidic end of the tube gel and form a bulb. (4)

Note: if your proteins of interest are basic, with pIs > 9.0, they will require non-equilibrium pH gradient 2D electrophoresis (NEPHGE), which is incompatible with SDS. Samples for NEPHGE must be dissolved in Urea Sample Buffer. (5)

Advantages of SDS: Heating biological samples in the presence of SDS dissolves proteins more completely than any other method. This is especially true for membrane proteins.(6) SDS sharpens 2D spots and increases the recoveries of most proteins in the gel. 2D gel patterns obtained in the presence of SDS are similar but not identical to those obtained with Urea Sample Buffer without SDS. However, they are quite reproducible. Sample preparation with SDS Buffer is *much easier* than with Urea Buffer. There are no undissolved pellets to discard.

Disadvantages of SDS: The acidic end of the tube gel (as much as 1.5 cm) becomes distorted due to formation of the SDS/IGEPAL bulb. To correct for this we run longer IEF tube gels for SDS-containing samples and remove the bulb, which contains no resolvable proteins. The remaining tube gel is the normal length.

Isoelectric point measurements may be unreliable in the presence of SDS because residual detergent may remain on some of the proteins. A few proteins become streaky in the presence of SDS.

II. Sample Buffers

The following gives formulas and references for various sample buffers. SDS and urea from different suppliers may give different 2D patterns so don't switch reagents in the middle of a project.
We recommend SDS from IBI Scientific (Peosta, IA cat # IB07062) and ultrapure urea from MP Biomedicals (Aurora, OH cat # 821519).

A. Urea Sample Buffer contains 9.5 M urea, and 4% Pharmalytes pH 3-10 (GE Healthcare, Piscataway, NJ). 2% w/v IGEPAL CA-630 (a non-ionic detergent, or Nonidet P-40), 5% beta-mercaptoethanol (BME), Ten 1 ml aliquots of this buffer are supplied in the mailing kit.

B. SDS Boiling Buffer contains 5% SDS, 5% BME, 10% glycerol and 60 mM Tris, pH 6.8. Protein

in solution at a final concentration of 35 mg/ml or less may be heated to boiling in this buffer to aid dissolution. Ten1 ml aliquots of this buffer are supplied in the mailing kit.

C. SDS Boiling Buffer Minus BME works best to dissolve protein pellets from cultured cells and other hard-to-dissolve samples prior to protein determinations using the BCA assay. (7) Note: SDS inhibits nuclease activity so polynucleotide digestion must be carried out prior to its addition. Seventeen 1 ml aliquots are supplied in the mailing kit.

D. Osmotic Lysis Buffer containing 10 mM Tris, pH 7.4, and 0.3% SDS may be used for cell lysis, for example in studying heat shock proteins.(8) Proteins in solution at a final concentration of 2.0 mg/ml or less may be heated to boiling in this buffer. Note: there are no sulfhydryl reducing agents (BME, DTT) in osmotic lysis buffer so the BCA protein assay may be performed. Seventeen 1 ml aliquots are supplied in the mailing kit.

E. 10× Nuclease Stock Solution(9) contains 50 mM $MgCl_2$, 100 mM Tris, pH 7.0, 50 units RNase (Ribonuclease A from bovine pancreas Type IIIA, Sigma cat# R5125) and 2500 units DNase (Deoxyribonuclease I, Type II from bovine pancreas, Sigma cat# D4527). Final concentrations for these enzymes should be 5 units/ml RNase and 250 units DNase in 5 mM $MgCl_2$ and 10 mM Tris-Cl, pH 7.0. Seventeen 100 µl aliquots are supplied in the mailing kit.

F. 100× Protease Inhibitor (PI) Stock Solution contains 20 mM AEBSF (Calbio-chem 101500), 1 mg/ml leupeptin (Sigma cat# L2884), 0.36 mg/ml E-64 (Sigma cat# E3132), 500 mM EDTA (Calbiochem cat# 34103), and 5.6 mg/ml benzamidine (Sigma cat# B6506). This stock solution should be added to samples at a final concentration of 1%. Four 100 µl aliquots are supplied in the mailing kit.

G. Phosphatase Inhibitors should be added for experiments involving protein phosphorylation. Phosphatase Inhibitor cocktails may be purchased from EMD Biosciences :Novagen cat# OC7850K (inhibits protein serine/theonine phosphatases) and cat #524625 (inhibits protein tyrosine phosphatases)]. Three 100 µ aliquots of each are supplied in the mailing kit.

H. OmniCleave™ Endonuclease from Lucigen cat# OC7810K) is a purified endonuclease that works in the presence of SDS. It reduces sample viscosity by degrading native DNA and RNA to di-, tri - and tetranucleotides. Dilute to 20 unit/µl with the dilution buffer supplied, i.e. add 0.5 ml to the tube of 10 kU, and use as needed.

III. 2D Pattern Visualization

A. Coomassie blue versus silver stain

Coomassie blue staining can detect as little as 0.05 µg/polypeptide spot. It is a quantitative stain (1) and fully compatible with mass spectrometry. Silver staining is much more sensitive and can detect 5 ng/ poly-peptide spot but is semiquantitative because different proteins saturate at different levels (10,11). A “special” silver stain is also offered for mass spectrometry of bacterial samples with a detection limit of ~10 ng/spot. (12). Note that proteins often stain negatively with special silver so quantification is not possible.

B. Sypro Ruby, Other fluorescent stains

We’ll stain your gels with fluorescent stains such as Sypro ruby, Cy dyes for DIGE etc., digitize the images with a Typhoon 9000 and analyze the patterns with Progenesis software. The stained gels can be returned to you either wet or dry.

IV. Preparing Samples

High concentrations (> 150 mM) of NaCl, KCl and other salts cause serious streaking problems as do lower concentrations of phosphate and buffers. Keep **salt concentrations as low as possible**. Dialyzing samples to remove salts and other low molecular weight substances is recommended. Lyophilization of dilute samples prior to adding sample buffer is ideal, **do not concentrate salts greater than 150 mM**. Beware of high viscosity due to concentration of non-ionic substances such as sucrose.

If there is a precipitant in the dissolved sample, pellet it by centrifugation. Precipitants tend to impede isoelectric focusing by plugging the top of the IEF tube gel.

A. In Urea Sample Buffer

Urea (H_2NCONH_2) is commonly used as a denaturant to solubilize proteins for isoelectric focusing, especially for purified and semi-purified proteins. Although urea does not dissolve proteins as well as SDS, there is no danger of it changing the apparent isoelectric point. It is often convenient to dilute a concentrated protein solution 1:1 or 2:1 with Urea Sample Buffer or simply to dissolve the lyophilized protein powder in the buffer. If the

sample does not dissolve well try heating it to 50°C for 10 min or freeze/thawing several times. **NEVER BOIL SAMPLES IN UREA**, isocyanates from the urea will react with the proteins to form charge isomer artifacts . (13)

B. In SDS Boiling Buffer

Add SDS Boiling Buffer to the sample, vortex thoroughly, and place the tube in a boiling water bath for 5 minutes. For example, if you have 320 µg protein in a microcentrifuge tube after lyophilization and want to load 150 µg in 30 µl, add 64 µl SDS Boiling Buffer, tap lightly to dissolve the powder, place in a boiling water bath for 2-5 min, and freeze. If samples are concentrated enough, dilute them 1:1 or 2:1 with SDS Boiling Buffer prior to heating. The 5% SDS in the buffer suffices to solubilize a protein concentration of 35 mg/ml based on the rule of 1.4 g SDS per gram of protein. (14)

C. Cultured cells

Remember to work quickly on ice to avoid proteolysis. Have Osmotic Lysis Buffer, 10× Nuclease Stock solution, 100x Protease Inhibitor (PI) Stock solution, phosphatase inhibitor cocktails (when necessary), SDS Boiling Buffer + BME (or Urea Sample Buffer), prelabeled microcentrifuge tubes, and dry ice ready. Prepare Osmotic Lysis Buffer by adding 100 µl of Nuclease Stock solution and 10 µl of PI stock/ml plus phosphatase inhibitor cocktails if required. Rinse the cells 3 times with cold buffered saline. Aspirate the excess liquid.

For plated cells, add prepared Osmotic Lysis Buffer directly to the cells on the cold dish. Try to add a volume to give 2–4 µg/µl of protein. A 35 mm diameter dish containing 40,000 cells/cm^2 (approximately 380,000 cells) would receive 50 µl of Osmotic Lysis Buffer. If the dish contains 400,000 cells/cm^2, then add 150 µl of Osmotic Lysis Buffer. While keeping the dish on ice, scrape the cells from the dish using a rubber policeman and mix them with the buffer. Transfer to a 1.5 ml microcentrifuge tube, vortex and incubate on ice for 5–30 min. The viscosity due to DNA and RNA should quickly disappear. If it doesn't, add 2 µl of OmniCleave™ or sonicate the cells for 5 min. Repeatedly draw the samples through a small-bore needle until the samples can be pipetted.

For suspended cells, pellet the cells by centrifugation and rinse them twice in cold, buffered saline by resuspending and centrifuging to remove serum proteins and/or unbound radiolabel. The final rinse should be in a 1.5 ml microcentrifuge tube. Estimate the amount of Osmotic Lysis Buffer to be added to give 2–4 µg/µ of protein. Use 40–50 ul per 2–4 million cells. Add prepared Osmotic Lysis Buffer to the pellet and vortex thoroughly. Use a pipette to resuspend if necessary. Vortex and incubate on ice for 5–30 min. If the mixture remains viscous, add 2 ul OmniCleave and or sonicate the cells for 5 min.

Remember to reserve an aliquot at this stage if you wish to determine protein concentration using the BCA method. The 0.3% SDS in the Osmotic Lysis Buffer, which helps to solubilize proteins and inhibit proteases, will not interfere with the BCA method. If the cells or pellets aren't dissolving, add more SDS Boiling Buffer minus BME and place the tubes in a boiling water bath for 5 min. You may still do protein determinations on < 20 µL aliquots using the BCA method.

At this point, add an equal amount of SDS Boiling Buffer containing BME or just add BME depending on previous steps and boil for 5 min. Alternatively, add an equal amount of Urea Sample Buffer and heat to 50°C for 1 min. **Do not boil samples in Urea Sample Buffer; do not add SDS Buffer to samples requiring NEPHGE.** Store the samples at -70°C until mailing or pickup.

D. Yeast, *S. aureus* & difficult-to-lyse cells

To each sample of gently pelleted cells, add 500 ul of osmotic lysis buffer containing PI Stock, nuclease stock, phosphatase inhibitor stock if needed, and 100 µg of washed glass beads (Sigma G9268, mesh size 425–6000 microns) per 50–100 ul washed cell pellet as described by Jazwinski. (15) Vortex sample thoroughly, freeze, centrifuge, and repeat until the pellet size has been substantially reduced. Add 400 µl of SDS Boiling Buffer minus BME, vortex, and freeze again. Place the tube in a boiling water bath for 5 min then centrifuge. Lyophilize the supernatant; remember to reserve an aliquot for protein determination. Dissolve the resulting residue in 1:1 diluted SDS Boiling Buffer to at least 5.0 mg/ml for Coomassie blue-stained gels or 1.0 mg/ml for silver-stained gels.

E. Immunoprecipitates e.g. see Brown et. al. (16) The amount of protein is small for IPs and there is no danger of overloading the gel. If possible, the amount of protein stripped off affinity beads for 2D should be 3-4 times that required for a 1D gel, since bands often are resolved into a string of spot isoforms. Duplicate Coomassie blue stained gels

may be run in tandem and matched to the Western blot film for mass spectrometry. Generally as much sample as possible is loaded on the Coomassie blue gel. *To ship IP's on beads, remove the supernatant and send the bead pellet on dry ice by express mail. Our Lab Manager will consult with you before eluting proteins from the beads at no charge.*

F. Animal Tissue e.g see Chen et.al. (17)
Weigh the tissue, freeze to -80°C, crush with mortar and pestle then place in a tissue homogenizer on ice. Add about 0.25 ml of Osmotic Lysis Buffer containing PI Stock, Nuclease Stock and Phosphatase inhibitor stock/100 mg tissue and homogenize on ice. Freeze/thaw twice. Allow the nucleases to react for 10–15 min on ice, add an equal amount of SDS Boiling Buffer - BME and place in a boiling water bath for 5–30 min. Cool the tissue on ice, centrifuge to pellet solids, determine protein concentration, and store at -70°C.

V. Determining Protein Concentration

There are four common spectrophotometric methods of determining protein concentration: BCA method (7) Lowry method (18), Bradford method (19), and absorbance at A_{280}/A_{260} (14). Reagents and protocols for the first three can be obtained respectively from ThermoFisher (Pierce) Sigma Chemical Co., and Bio-Rad Labs. We will accept protein determinations from any of these methods but would like to know which method was used so we can adjust the loads. The Bradford method tends to give higher values for mixtures than the values obtained from the BCA and Lowry methods.

We recommend the BCA method because it has good standard curve stability and relatively few compounds interfere (Pierce provides a list including 1% SDS and 3.0 M urea). Note, however, **BME and DTT strongly interfere with the BCA assay.** Remember to take aliquots for protein determination before the addition of BME- or DTT-containing buffers. Once samples are dissolved in either Urea Sample Buffer or SDS Boiling Buffer containing BME or DTT their protein concentrations cannot be measured. If samples are cloudy, dissolve them completely by boiling with SDS minus BME before performing the BCA assay. Please be sure to let us know if BME or DTT have been added to the samples when mailing. Note: if you are using the Bradford method SDS can't be used because > 0.1% SDS interferes with this assay.

VI. Protein Precipitation Methods

Precipitation techniques are commonly employed to separate sample proteins from interfering contaminants such as salts, detergents, nucleic acids, lipids and charged sugars, and to concentrate proteins without concentrating salt. Proteins in samples containing high salt require either dialysis or precipitation, for example those scheduled for silver staining with a protein concentration <1 mg/ml and salt concentration > 200 mM, and samples scheduled for Coomassie blue staining which have a protein concentration < 4 mg/ml and salt >200 mM.

Depending on composition one technique may be more suitable for your sample than another. Note that precipitation can alter the protein profile of a sample and should be avoided if possible.

A. Ethanol Precipitation is a simple method for removing sample contaminants. Suspend one part lysed or disrupted sample in nine parts absolute ethanol in an Eppendorf tube and vortex. Allow the suspension to sit at –80°C for at least 2 hours. Microfuge for 30 minutes at 12-14,000 rpm in a centrifuge cooled to 15-20°C (colder will bring down large SDS pellet). Carefully remove the ethanol from the samples and air-dry them for a short time to remove residual ethanol. See EtOH ppt white paper for details.

B. Trichloroacetic acid (TCA) in acetone is another common method used to precipitate proteins during sample preparation for 2-D electrophoresis and is more effective than either TCA or acetone alone at removing starch and sugars.

Suspend lysed or disrupted sample in 10% TCA in acetone with either 0.07% 2-mercaptoethanol or 20 mM DTT. Precipitate proteins for at least 45 minutes at -20°C. Pellet proteins by centrifugation at maximum rpm, 4°C, for 20 minutes. Wash the pellet twice with cold acetone containing either 0.07% 2-mercaptoethanol or 20 mM DTT by centrifuging at maximum rpm, 4°C, for 5 minutes. Remove residual acetone by air-drying.

C. Precipitation with ammonium acetate in methanol following phenol extraction (20) is useful with plant samples containing high levels of interfering substances. Begin by combining 100 ul sample with 150 ul liquid phenol (best quality by adding water to crystalline phenol). Add 10 ul of 10% SDS and 10 ul BME, vortex. Centrifuge for one min at maximum rpm's. Following centrifugation

remove upper phenol phase. Proteins are precipitated from the phenol phase with 0.1 M ammonium acetate in methanol. Pellet the precipitated proteins with centrifugation. The pellet is then washed several times with ammonium acetate in methanol. Remove ammonium acetate and allow any residual to evaporate.

D. Removal of lipid contaminants via protein precipitation with chloroform/methanol (21)

Transfer the sample to a 1.5 ml microcentrifuge tube and adjust the volume to 100 µl by lyophilization or adding water. Add 400 ul of methanol and vortex. Centrifuge the tube for 10 seconds at 9000xg, add 100 µl of chloroform and vortex. Centrifuge the sample for ten seconds, add 300 µl water and vortex vigorously to mix. Centrifuge the sample at 9000xg for three minutes at 4°C. At this point the liquid should have separated into two phases. If not, add an additional 100 µl of chloroform and centrifuge again.

Aspirate the upper phase with a narrow gauge hypodermic needle or a Pipetteman with a narrow tip. Transfer sample to a fresh microcentrifuge tube. Be careful not to disturb the interphase, the protein is present in the interphase at this point. It is advisable to leave 10-20 µl of upper phase behind. Add 300 µl of methanol to the lower phase in the original tube and vortex. Centrifuge at 14,000xg for ten minutes at 4°C. The protein should have formed a pellet. With small amounts of protein the pellet may be rough and smeared along the side of the tube. Transfer the supernatant and air-dry or redissolved the pellet.

VII. Mailing Samples

Fill out the sample ID form; label 1 ml Eppendorf or other small tubes with indelible ink. Aliquot samples into tubes and store frozen at -70°C until mailing. Mail samples on at least 6 pounds of dry ice along with sample ID form by overnight mail. Call 800-462-3417 or email 2d@kendricklabs.com with questions.

Pickup and delivery in the Madison, WI area is free. Call 608-258-1565 to arrange a time.

If buffers are unavailable, send the proper amount of protein aliquoted or lyophilized in a 1.5 ml Eppendorf tube. We will add the appropriate buffer as indicated on the sample ID form. There is no charge for this as long as the amount of standard buffer to be added is clear.

VIII. Reference List

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