Biochemistry Lab

SDS PAGE and Western blot

General Instructions

Background

When an electrical field is applied across a solution, the movement of the charged particles (proteins) is influenced not only by the charge but also the voltage, distance between electrodes, the size and shape of the molecule, temperature, and time. Polyacrylamide gels are polymerized products of acrylamide and bisacrylamide (n,n'-methylene bisacrylamide). When ammonium persulfate (APS) [$^{-}0_3$S-0-0-S0_3^{-}] is added to water it breaks down, forming unstable $O_4S^{-\cdot}$ free radicals, which can then initiate the polymerization reaction. TEMED (tetra methyl ethylene diamine) is a tertiary amine that reacts with these radicals to form TEMED free radicals, which in turn react with acrylamide to induce polymerization. The addition of bisacrylamide forms crosslinking of the long acrylamide molecules creating an average size pore. The size of the pore can be regulated by the concentration of acrylamide and bisacrylamide. Relatively small proteins will migrate faster through SDS-PAGE gels than larger proteins. Gels with lower percent bisacrylamide are more useful for larger proteins. In this case the smaller proteins will not be retarded by the size of the pores and will have a similar mobility. That is, they will run very close to each other at the bottom of the gel, separating the larger proteins more distinctly.

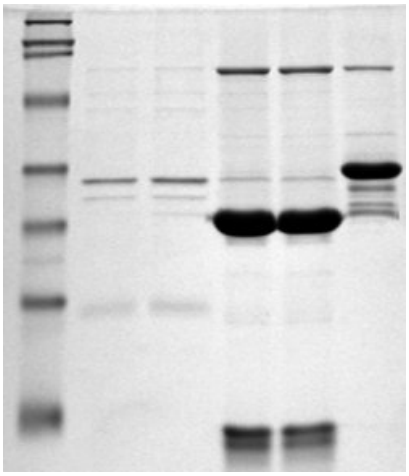

Fig 1: Coomassie Stained SDS PAGE

Traditional SDS PAGE - The gels often used in labs are discontinuous gels. These are gels that contain both a stacking gel and a resolving gel. When glycine from the upper reservoir enters the low pH of the stacking gel, it will principally be in the neutral form. This prevents glycine from being an effective carrier of electrical current. The Cl^- ions now carry the current and migrate toward the anode. During this step the Cl^- ion concentration becomes lower at the top of the gel and higher at the bottom of the stacking gel. As electrophoresis continues, protein molecules (which are negatively charged due to the SDS) will become greatly retarded, allowing the trailing protein molecules to catch up. The stacking gel is very low in percent to ensure there is little separation based on the pore size. This will ensure all of the proteins enter the resolving gel at the same time to get small tight bands.

After running a 10-12% SDS-PAGE gel, the gel is stained with Coomassie blue. Coomassie blue is a dye that will bind to the acidic amino acids in proteins. This method of staining will stain most proteins in the gel with as little as 0.1 µg of protein in a single band. Washing of the gel with destain solution will remove the unbound dye from the gel but not the protein, leaving behind several nice blue bands that show each protein.

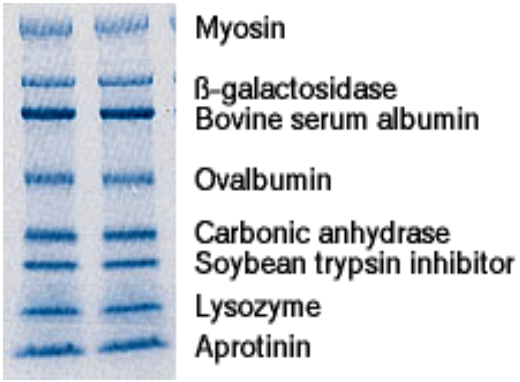

Fig 2 Prestained Molecular Weight Standards. Prestained standards are used in SDS-PAGE and Western Blotting applications. They provide a quick and easy way to assess blotting efficiency and allow continuous monitoring of protein separations during electrophoresis. Pre-stained standards can be used as a control for repetitive blotting experiments or in locating proteins for excision from unstained preparative gels.

Once the gel is destained, we can measure the relative mobility (Rf) of the proteins and use molecular weight standards to determine the molecular weight of an unknown protein. The Rf is calculated by dividing the distance the protein migrates by the distance the tracking dye migrates. *See Table I for molecular weights of some standards.*

The tracking dye (bromophenol blue) is a small colored molecule that runs much faster than proteins and approximates the movements of small ions in the electric field. Therefore, it is important not to run the dye off the bottom of the gel. A plot of the relative mobility for each standard protein vs. the log of the molecular

weight of the standard proteins will be linear, and the molecular weight of the unknown protein can be determined from its position on the plot. To determine the Rf, measure the distance the protein has moved from the top of the resolving gel to the center mass of the band of protein. Divide this value by the distance the dye traveled. This is the relative distance.

NextGel – NextGels are a novel mixture of acylamide, bisacrylamide, buffer, and SDS with a unique formulation that does NOT require a stacking gel for separation of proteins. However, if using this type of SDS PAGE, you must use the buffer solution supplied with the NextGel solution.

NextGel 10% 10 – 200 kDa

NextGel 12% 3.5 – 100 kDa

Pre-Cast Gels – There are many options to purchase pre-cast gels. While they are convenient, the cost is 15 times that of a "homemade" gel. Follow specific manufacturer's instructions for buffers and preparation of pre-cast gels.

Western blot / Immunoblotting use specific interactions of antibodies to detect a protein of interest. Western blotting can be divided into two steps: the transfer of the protein from the gel to the matrix (paper) and the detection of the antibody's epitope. We will be using the wet method to transfer the proteins, in which the gel and the immobilizing paper are sandwiched between buffer wetted filter paper through which a current is applied for 60 min. or overnight.

Safety: Coomassie stain is not harmful but care should be taken so as not to stain your clothing. Take it from me; it is very easy to spill a little bit onto yourself. I have a few shirts that are stained blue.

The SDS-PAGE Sample Buffer contains β-mercaptoethanol. This is harmful to breathe in large quantities. Unless the bottle of βME is spilled, there is little to no risk other than the stink. Try not to get any on your lab coat. It will make it smell like rotten eggs for quite some time!

The NextGel solution contains un-polymerized acrylamide. The monomer ***is a neurotoxin*** until it reacts with the APS and TEMED to form a cross-linked polymer, so **DO NOT GET ANY ON YOUR HANDS.** If you do get any on you, wash the area with soap and water. The TEMED stinks and is a potential teratogen. Once these chemicals are mixed together, there is no safety concern; however, treat them carefully prior to mixing.

References:

1. Hjelmeland, L.M. and Chrambach, A., Electrophoresis 1981, 2, 1-11

2. Sallantin, M., Huet, J., Demarteau, and Pernollet, J., Electrophoresis 1990, 11, 34-36

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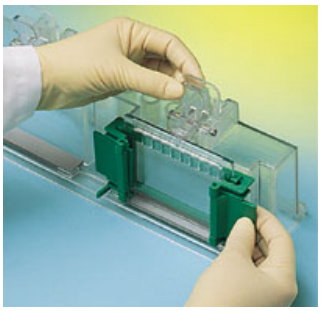
General Instructions

Instructions for NextGel preparation: See the video of how to prepare and run a NextGel.

Be certain not to add the APS and TEMED until you are ready to start the gel. The gel will start to polymerize within 3 or so minutes so be certain everything else is in hand and ready to start.



1. Ensure that your apparatus is clean and dry.
2. Pour 10 ml of NextGel solution to a 10 ml conical tube.
3. Add 100 µl 10% APS and 6 µl TEMED to the NextGel solution, place the cap onto the tube and mix by inversion.
4. Immediately pour the solution between your glass plates. Use a disposable transfer pipet. Fill until the solution to the top of the short glass plate.
5. IMMEDIATELY insert comb by sliding into the glass plates at an angle.
   - It will take 10-20 min for the gel to polymerize completely.
6. When the gel is polymerized, **the comb may be removed** gently, and the gel sandwich can be loaded into the electrophoresis apparatus.
7. **Prepare 500 ml of 1X running buffer** from the 10 X running buffer solution. *Record your calculations in your laboratory book.*

Preparing, Loading and Running the Samples: A typical protein load for a crude sample of protein for SDS PAGE is between 5 and 20 µg per lane. Too much protein will distort the bands, too little protein load will be difficult to detect by Coomassie staining.

1. Add the following mixtures in separate microfuge tubes:
   - For the bacterial lysate, mix 30 µl of the sample, 20 µl of water, and 20 µl of 5X sample buffer.
   - For the fraction samples, add 80 µl of each fraction and 20 µl of 5X sample buffer.
2. Boil the samples in a heat block at 100°C for 5 min.
3. Put the gel apparatus together and **fill the inner reservoir** to the top and pour the remaining buffer in the bottom.

4. **Typical Loading Order –** actual samples will vary, depending on what you saved at each step.
   - Lane 1: SDS PAGE Weight Standards (load 10 µl)
     - These are NOT a “ladder”; these are a standard mixture of purified proteins with a known mass. Do not call them a ladder, you will look like you don’t know the difference between a DNA gel and a protein gel!
   - Lane 2: Pre-induced cells
   - Lane 3: Induced cells or lysate supernatant
   - Lane 4: Resuspended insoluble sample (inclusion bodies and membranes)
   - Remaining Lanes: Fractions – typically load maximum until you know how the purification went.
   - ANY EMPTY LANES - Load 10 µl of 1X Sample buffer – use water to dilute the 5X sample buffer

5. Apply the power source to your gel box and turn on to 200 V until the dye is just at the bottom. **DO NOT STOP THE GEL TOO EARLY.** The results will be difficult to obtain because the proteins will be best resolved when the gel has run all of the way.

6. If you are proceeding to Western blotting, use the glass plate to carefully cut the gel in half vertically. *It helps if the glass plate is wet*. Lanes 1-4 will be stained in the next step, lanes 6-10 will be transferred in Part II in the Western blot.

7. Place the gel to be stained in the Coomassie stain and rock for 30 min. or overnight. Then rinse with water once. Add about 1/2 inch of destain solution. Leave it in destain overnight or up to one week. *LABEL YOUR SAMPLE.*

WESTERN BLOTTING:

Protein Transfer:

1. **Prepare 300 ml transfer buffer.** 30 ml of methanol plus 10X transfer buffer. QS to 300 ml.

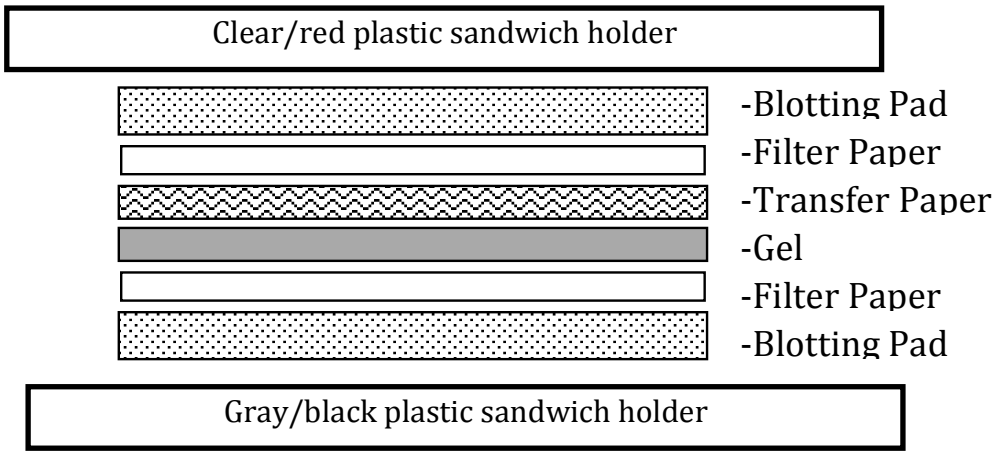
2. **Prepare the transfer membrane and filter paper.**
   If using immobilon (PVDF) transfer paper, pre-wet the paper for 30 seconds in methanol, rinse with distilled water and place in a shallow dish with transfer buffer.

3. If you are using nitrocellulose just rinse the paper in TTBS (Tween Tris-buffered saline) for 1 min.

4. **Prepare the polyacrylamide gel for transfer.** Soak the gel in transfer buffer for 10 minutes to remove salts that may result in poor transfers.

5. **Prepare the blotting pads.** Soak the pads in transfer buffer until they are saturated. Remove air bubbles by squeezing the pads while immersed in the buffer. Any remaining bubbles will block the transfer of the proteins.

6. **Assemble Blot Apparatus.** Place 1 blotting pad on the black part of the sandwich holder (***build on black***). Place the filter paper on the gel. Bubbles can be displaced with your fingers. On top of the filter paper, place the gel, followed by the transfer paper, another filter paper, and the final blotting pad, as shown below.



7. Transfer Protein: **Close the sandwich holder and place assembly into the gel box. Be certain to align the black with black and clear side with the red side of the transfer apparatus. Fill the inner portion with transfer buffer, place the stir bar into the box and add the ice cooler container. Transfer the protein at 100 V (constant) for 1 hour.**

Rule of thumb: **Smaller proteins < 50 kDa will be transferred after 0.8 hours; larger proteins > 100 kDa will take 1.5 hours. Alternatively, the blot can be transferred overnight at 30V in the cold room.**

Blotting and Detection:

1. **Wash the blot** by rinsing the blot for 5 min in TBS (Tris-buffered saline) in order to reduce spotting in the developed blot.
2. **Block the blot by soaking** for 30 min to one hour with 10 ml of 5% blocking buffer (TTBS and 5% dry milk [0.5 g dry milk per 10 ml TTBS]) on a rocking platform. If needed, this is a good stopping point. Seal it and leave the blot overnight in the cold room. **Below is a good example of a figure with both chromatograph and the gel results.** You will not combine the data as these authors did, but you can get a good idea of how the gel and chromatograph should be displayed and what a proper figure legend should look like.

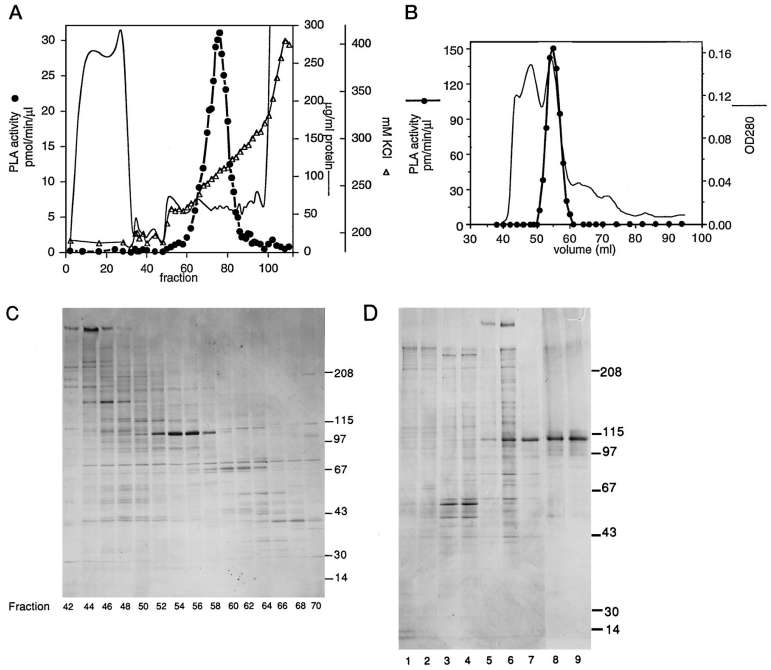


Figure 3: Chromatographic elution profiles and SDS-PAGE evaluation of PA-PLA purification. Elution profiles from Mono Q (A) and Superdex 200 (B) chromatography are shown. Elution volumes for markers on Superdex 200 (molecular mass in kDa in parentheses) were as follows: blue dextran 2000 (2,000,000), 43 ml; thyroglobulin (669), 47 ml; ferritin (440), 55 ml; catalase (232), 65 ml; aldolase (158), 67 ml; phosphorylase B (97), 68 ml; bovine serum albumin (67), 75 ml; ovalbumin (43), 83 ml; chymotrypsinogen (25), 91 ml. C, silver-stained gradient gel SDS-PAGE of the Superdex 200 fractions (2 µl each). D, silver-stained gradient gel SDS-PAGE of the pools from each of the stages of purification. In lanes 1-7, 1 µg of protein was loaded. In lanes 8 and 9, 50 ng of protein was loaded. Lanes 8 and 9 were stained separately in order to bring out minor bands. Lane 1, ammonium sulfate precipitate; lane 2, PEG precipitate; lane 3, SP pool; lane 4, concentrated SP pool; lane 5, Mono Q pool; lane 6, concentrated Mono Q pool; lane 7, Superdex 200 pool; lane 8, phenyl-Superose pool; lane 9, phenyl-CL-4B pool.

Table I: Calibrated BioRad Pre-Stained Low Range Molecular Weights for Pre-stained SDS-PAGE Standards

Protein	MW (Da)
Phosphorylase B	107,000
BSA	76,000
Ovalbumin	52,000
Carbonic anhydrase	36,800
Soybean trypsin inhibitor	27,000
Lysozyme	19,000

Molecular Weight Estimations and SDS PAGE: Often questions are posed regarding apparent discrepancies between protein size as determined by gels vs. other methods, such as sequence analysis. Two factors explain most of the observed variation.

The first factor is the amount of SDS bound to the protein. SDS is employed to disrupt secondary structure and give all proteins a constant charge/mass ratio, which is assumed to be 1.2g SDS/g protein. However, as stated in a review by Hjelmeland and Chrambach (1); “this assumption fails more frequently than is generally known.” The most common deviation from this assumption is probably a lower than normal amount of bound SDS. All else being equal, mobility would decrease, since the protein would have less of a negative charge.

A second source of error in molecular weight estimates is that protein mobility in the gel is more a function of molecular size (which is a function of both weight and length) than of molecular mass. It's generally assumed that with SDS proteins all exist in a random coil form, so the relationship between length and mass should be constant. Even assuming constant charge, if a protein has unreduced disulfide bonds or areas of incompletely disrupted secondary structure, it cannot unfold to full length and it would tend to run faster than expected in a typical SDS gel.

These deviations from the ideal can combine in every conceivable way, making it difficult to predict a net effect on migration rate. Nevertheless, the effects can be large. Unreduced BSA will run with an apparent size of about 55 kDa instead of 67 kDa. Furthermore, in smaller proteins a non-ideal region will have a larger proportional effect than the same region in a large protein. For example, polypeptides of approximately 2 kDa can give estimates which are off by a factor of 2 or more from actual size.

This is not to say that SDS-derived molecular weights are invalid, just that they have limitations. Most proteins will give estimates within a few percent of their actual weight by comparing them to appropriate calibration markers. And possible deviations from 'true' molecular weight do not affect the utility of SDS gels in identification, because even 'unusual' proteins, if prepared in the same way each time should run reproducibly on a given gel type.

Finally, care should also be taken in inferring precise size based on published weights for calibration markers. Even common proteins may have several slightly different size estimates reported in the literature, depending upon the methods of molecular weight measurement. *For a recent example see reference 2.*

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General Instructions

SDS-PAGE Gel Instructions:

The following recipes are more than enough for two 1.5 mm thick gels. The buffers/solutions do not need to be degassed if using a minigel.

Gel well capacity:

- At 1.0 mm gel thickness, 10 wells = 32 µl and 15 well = 18 µl
- At 1.5 mm gel thickness, 10 wells = 48 µl and 15 well = 27 µl

10% APS (stable frozen)

0.5 g Ammonium persulfate

5.0 ml H_2O

Coomassie Gel Stain, 1 liter

1.0 g Coomasie Blue R-120

450 ml methanol

450 ml H_2O

100 ml Glacial Acetic Acid

Coomassie Gel Destain, 1 liter

100 ml Methanol

100 ml Glacial Acetic Acid

800 ml H_2O

Pour the gel. Add APS and TEMED to the NextGel Solution, carefully pipet the solution into the glass sandwich until just below the top of the glass plate. Add the comb. Wait until the gel polymerizes. Tilt the apparatus to see if the gel has set.

Prepare the assembly.

1. When the gel is done setting, remove the gel cassette sandwich from the casting frame.
2. Place the gel cassette sandwich into the electrode assembly with the short plate facing inward.
3. Slide gel cassette sandwiches and electrode assembly into the clamping frame.
4. Press down the electrode assembly while closing the two cam levers of the clamping frame.
5. Lower the inner chamber into the mini tank. Use the spacer guide to load your samples.