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This protocol is written to apply to an Amersham/Pharmacia Biotech style gel box. The procedure can be applied to any other manufacturers' apparatus except that the assembly of the gel sandwich and placement into a pouring stand may differ. Please refer to the individual manufacturers' instruction manuals.

- [ ] Assemble the glass plate sandwich of the electrophoresis apparatus according to manufacturer's instructions by stacking, in order, the notched aluminum plate, two 0.75 mm spacers, and the larger rectangular glass plate. Be sure to align the spacers properly with the ends flush with the top and bottom edge of the two plates when positioning the sandwiches. The gel(s) can be assembled and locked into the gel casting stand using the clamps and eccentric cams. In either case, **it is critical to align the spacers properly to prevent leaks during gel pouring.**

  We often will place a piece of Parafilm, folded in half, beneath the gel sandwich, to help form a tight seal. Additionally, a small dab of Vaseline can be placed at the corner of the gel near the spacers to help prevent leaks due to uneveness of the spacers.

- [ ] Prepare the appropriate concentration separating gel solution as needed using the accompanying table as a guideline. Preparing 25 ml of solution is sufficient for two mini gels with 0.75 mm spacers. Any excess gel solution can be used as a monitor of the polymerization process. **ADD AMMONIUM PERSULFATE AND TEMED LAST**.

- [ ] Immediately after adding the ammonium persulfate and TEMED, pipet the solution into the gel plate sandwich to the appropriate height. This can be determined by measuring using a sample comb prior to loading the gel solution. Remember to leave enough room to accommodate a stacking gel that is ~1cm high.

- [ ] Overlay the gel solution with water or water-saturated iso-butanol. Monitor for leaks at this point and allow the gel to polymerize for at least one hour. **If the gel is to be used to isolate a protein for the purpose of chemical sequencing we recommend that the gel be allowed to polymerize overnight to ensure maximum polymerization**.

  Unpolymerized acrylamide can react with primary amino groups and thiols and artificially block the amino terminus of the protein protein. In addition, free radicals left in the gel can catalyze other side-chain modifications that may be deleterious to proper sequencing.

- [ ] After the gel is polymerized, remove the iso-butanol or water and rinse the top of the gel with 1 x Tris HCl/SDS pH8.8. This can be left in place overnight if appropriate. Otherwise, rinse the surface of the gel with MilliQ water and pour a stacking gel.

  Polymerized gels can be stored for up to one week at 4°C wrapped in SaranWrap.

- [ ] To pour the stacking gel, remove any overlay buffer (or water) and partially insert the Teflon sample comb in the gel sandwich at an angle. Prepare the stacking gel solution (5 ml is enough for two gels) and pipet into the space above the separating gel so that it begins to fill in around the "teeth" of the sample comb. Push the comb down into the gel solution in a way so **that no air bubbles are trapped**. Add additional acrylamide solution as necessary to fill the spaces in the comb completely. Allow the gel to polymerize (~30 minutes).

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An optical discontinuity will be visible around the Teflon "teeth" upon polymerization. Alternatively, any unused acrylamide solution will polymerize in the beaker and act as a monitor of the polymerization process.

- ❑ After polymerization, carefully remove the comb and rinse the wells with water to remove any unpolymerized acrylamide. Clamp the gel onto the gel apparatus and place a sample well-template over the gel to identify the bottom of the sample wells. Fill the lower reservoir with 1 x electrode buffer so that it covers the bottom of the gel. Fill the upper reservoir with electrode buffer so that the wells are filled with buffer.

THE GEL SHOULD BE PRE-ELECTROPHORESED IF THE GEL IS TO BE USED FOR PROTEIN PURIFICATION PRIOR TO SEQUENCING. This is accomplished by including 1 mM sodium thioglycolate in the upper buffer during this step. The thioglycolate acts as a scavenger and reacts with unpolymerized acrylamide as it moves with the ion front thereby removing harmful acrylamide from the path of the migrating proteins. If the gel will be used for simple analysis this step may be omitted.

- ❑ Turn the power off and load the samples using a Hamilton syringe or gel loading tips.

  We recommend that samples destined for sequencing NOT be boiled but rather heated in sample buffer to 50-60°C for 15 minutes. This is usually sufficient for total denaturation.

  Preparing samples at approximately the same concentration and loading an equal volume to each well will ensure that all lanes are the same width and that the proteins run evenly. If unequal volumes of sample buffer are added to the wells, the lane with the larger volume will spread during electrophoresis and constrict adjacent lanes, causing distortions.

- ❑ Connect the power supply to the gel apparatus and run at 25 milliamps of constant current per gel. For a gel of 0.75 mm thickness, the gel will take about 1.5 hours to run. Cooling will be required if you run at higher amperages to prevent "smiling" (curvature of the migratory band).

- ❑ Disconnect the power supply after the bromophenol blue tracking dye has reached the bottom of the gel cassette and prepare for staining or electroblotting the gel.

TO REVIEW THE MAJOR PRECAUTIONARY STEPS IN PREPARING A GEL USED FOR PROTEIN SEQUENCE ANALYSIS.

1. **OVERNIGHT POLYMERIZATION TO REDUCE UNPOLYMERIZED ACRYLAMIDE**
2. **PRE-ELECTROPHORESIS WITH SODIUM THIOGLYCOLATE TO REMOVE ACRYLAMIDE**
3. **DO NOT BOIL SAMPLES TO REDUCE HEAT-INDUCED CLEAVAGE**
4. **REDUCE EXPOSURE OF GEL TO ACIDIC AND FIXING CONDITIONS, ie. stains (This reduces cleavage of internal ASP-PRO peptides bonds and reduced extraction efficiency due to protein fixation).**