KERATIN CONTAMINATION
Keratin contamination is almost always observed as a background protein. Wear only nitrile gloves and rinse with HPLC grade water all trays, containers and surfaces that contact the gel (including gloves, staining trays, scalpels, razor blades, light box, and cutting area). It is strongly suggested to use freshly prepared solutions/buffers for the samples to be submitted to mass spectrometric analysis.

OTHER CONTAMINATIONS
Other commonly observed contaminating proteins are caseins and BSA from dry milk, media and/or sera commonly used in biology labs. These protein contaminations can be greatly minimized by extensive washing of cell pellets, glass ware and trays. Other contamination can be caused polymers from detergents, PPG and/or PEG. Thus, no detergents should be present in samples to be digested for mass spectrometric analysis. It should be noted, that many soaps and hand creams contain PEG's such that touching tubes, trays or gels with freshly washed or creamed hands can give rise to polymer contaminations.

SDS-PAGE HANDLING
1. Gloves and clean lab coats should be worn at all times when working with gels and associated reagents, tubes, tips and apparatus.
2. Whilst not essential if sufficient care is taken, the use of pre-cast gels can help reduce keratin contamination.
3. Fresh staining reagents should be used to reduce the risk of contamination and prevent accumulation of contaminants.
4. Use clean gel apparatus, staining trays and plastics (no dust!). Use new unopened boxes of tubes, tips etc. where possible, or make sure they have not been left open to air and/or plastics removed by ungloved hands.
5. After staining, gels should be washed thoroughly in high-purity water prior to band excision. 2 x 15 minutes should be sufficient. Longer washes are suggested for thick gels (more than 1.5mm).
6. For specific bands of interest, these should be cut from the gel with a clean, sharp razor blade. The band should be cut directly on the edge of the staining region; no borders of clear acrylamide should be left around the band.
7. Cut bands should be carefully placed into clean microcentrifuge tubes.
8. The microcentrifuge tubes can be stored at -20 °C until they are ready for further processing.
SDS-PAGE PROTOCOL

REAGENTS

TABLE 1

<table>
<thead>
<tr>
<th></th>
<th>SDS-PAGE 12%</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Separating</td>
</tr>
<tr>
<td>2DE-SDS</td>
<td></td>
</tr>
<tr>
<td>dH2O</td>
<td>13.4ml</td>
</tr>
<tr>
<td>1.5M Tris/HCl pH 8.8</td>
<td>10ml</td>
</tr>
<tr>
<td>0.5M Tris/Hcl pH 6.6</td>
<td>-</td>
</tr>
<tr>
<td>Acrylamide 30%</td>
<td>16ml</td>
</tr>
<tr>
<td>SDS 10%</td>
<td>400μl</td>
</tr>
<tr>
<td>APS 10%</td>
<td>200μl</td>
</tr>
<tr>
<td>TEMED</td>
<td>40μl</td>
</tr>
</tbody>
</table>

- Load in each lane about 100-150 μg total protein sample.
- For 2DE you will need:
  - Rehydration buffer: 8M Urea, 2% Triton X-100, 2% IPG Buffer, 40mM DTT, 1% Bromophenol Blue
  - SDS Equilibration Buffer: 6M Urea, 75mM Tris/HCl pH 8.8, 29,3% Glycerol (87%), 2%SDS, Bromophenol Blue. Prior to use add 100mg DTT per 10ml of Buffer

METHOD

PREPARATION OF GEL
1. Clean the glass plates with Ethanol solution. Let them dry in the air.
2. Assemble the gel-casting unit.
   - Form the gel sandwich by assembling the spacers and two glass plates in the clamps.
   - Align the bottom part of the spacers and two glass plates at the same level and then tighten the clamp.
- Place the gel sandwich onto the casting stand and insert the sample application comb. Mark the glass plate at a level ~1.5 cm below the bottom of the comb teeth.
3. Prepare the resolving gel according to Table 1. Mix the solution gently with TEMED and APS 10%. Pour the resolving gel. Carefully overlay the gel with an ~ 2-mm-deep layer of isopropanol solution. After polymerization is complete (~30 minutes), pour off the overlaying isopropanol and carefully remove any remaining liquid with filter paper without damaging the gel surface. Wash the gel surface with water.
4. Pour the stacking gel that you have prepared according to Table 1. Make sure that the solution is well mixed.
- Carefully overlay the resolving gel with the stacking gel solution until the height of the stacking gel is ~3 cm.
- Insert the Teflon comb into this solution leaving ~1-1.5 cm between the top of the resolving gel and the bottom of the comb. Make sure that no air bubbles are trapped beneath the teeth of the comb.
- Allow the stacking gel mixture to polymerize for ~2 hours. It is useful at this stage to mark the positions of the bottoms of the sample wells on the glass plates with a marker pen.
5. Carefully remove the sample comb from the stacking gel and assemble the cassette in the electrophoresis apparatus.
6. Fill the top reservoir with running buffer ensuring that the buffer fully fills the sample loading wells and check for any leaks from the top tank. If there are no leaks, fill the bottom tank with running buffer. Tilt the apparatus to dispel any bubbles caught under the gel.

**PREPARATION OF SAMPLES**

**PREPARE PROTEIN SAMPLE**
1. Mix the protein solution with 2X SDS-PAGE sample buffer in a 1:1 ratio. To ensure enough SDS is present (binding ratio for SDS and polypeptide is 1.4 gr SDS per gr of polypeptide), the concentration of protein in the final solution should not be higher than 10 µg/µl.
2. Heat the samples in a heat block or water bath for 2 min at 95 oC to denature the proteins and ensure the maximum amount of SDS binding to the proteins. Allow the samples to cool at room temperature. Remove any insoluble materials by centrifugation.

**LOADING AND RUNNING THE SAMPLES**
1. Use a pipette and gel-loading pipette tips to load the samples into the sample well.
2. Connect the power supply to the electrophoresis apparatus.
3. Pass a constant voltage through the gel until the bromophenol blue dye front reaches the bottom of the gel.
4. Turn off the power supply and disconnect the electrodes. Remove the gel plates from the apparatus and carefully remove a spacer. Use the spacer to gently pry the gel plates apart, leaving the gel stuck to one plate.

**PROTEIN VISUALIZATION**

**“BLUE SILVER” COOMASSIE COLLOIDAL BLUE STAIN**

Before staining, **Fixation with:**
- 30% Methanol
- 10% Acetic Acid

Incubate the gels in fixation solution for 30 minutes to 1 hour.
After fixation wash the gels 4 times with distilled water (15 minutes for each wash).

The final concentrations adopted in the working colloidal “blue silver” solution are:
- 0.12% dye
- 10% ammonium sulfate
- 10% phosphoric acid
- 20% methanol

This produces a dark green dye solution, which turns to a deep blue when adsorbed onto the polypeptide chains fixed in the polyacrylamide gel, or blotted onto membranes. The dye solution is prepared as follows, by sequentially adding the various ingredients as here indicated:

To a water solution (1/10 of the final volume) the desired amount of phosphoric acid is added, so that, in the final volume, its concentration will be 10%; to this, add the required amount of ammonium sulfate (in powder), calculated to obtain a final concentration of 10%. When the ammonium sulfate has dissolved, add enough Coomassie Blue G-250 (in powder) to obtain a final concentration of 0.12%. When all solids have dissolved, add water to 80% of the final volume. To this solution, under stirring, add anhydrous methanol to reach a 20% final concentration. This stock dye solution should be kept in a brown bottle and is stable at room temperature for >6 months.

*** For staining use 5 times the volume of the gel. For instance for a maxi gel (~50 mL of SDS-PAGE solution) you have to use about 200-250mL of this blue silver coomassie colloidal stain.
Keep the gels in staining solution for at least 3 hours, better overnight.

Destain with distilled water, change water 3-4 times.
NATIVE-PAGE PROTOCOL

REAGENTS

TABLE 2

<table>
<thead>
<tr>
<th></th>
<th>NATIVE PAGE 7%</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Separating</td>
</tr>
<tr>
<td>40 ml total</td>
<td>10ml total</td>
</tr>
<tr>
<td>dH$_2$O</td>
<td>20ml</td>
</tr>
<tr>
<td>1.5M Tris/HCl pH 8.8</td>
<td>10ml</td>
</tr>
<tr>
<td>0.5M Tris/HCl pH 6.6</td>
<td>-</td>
</tr>
<tr>
<td>Acrylamide 30%</td>
<td>9.32ml</td>
</tr>
<tr>
<td>APS 10%</td>
<td>200 μl</td>
</tr>
<tr>
<td>TEMED</td>
<td>20μl</td>
</tr>
</tbody>
</table>
Buffers for Native PAGE gels

10x Native Running Buffer

29 gr Tris
144 gr Glycine
Water until 1 litre
pH should be around 8.3

Use 1x Native running Buffer

5x Native Loading Dye

50% glycerol
0.5 M Tris pH 8.6
0.0125% B-bromophenol Blue

Use ½ sample amount of this Dye, for instance for 50ul sample, use 25ul Dye

To make 2x Native Loading Dye for empty lanes, just dilute 5x Dye in water