Protein Sample Preparation for nanoLC-MS
Hongwei Zhao - August/2013

✓ This protocol is suitable for our maXis impact-qTOF-MS and amaZon speed ETD ion trap MS systems.
✓ To avoid human keratin contamination from skin and hair, always take powder-free gloves, while taking a mask also recommended.
✓ Always use Milli-Q ultrapure water (the Milli-Q water system must be maintained well, if not, please use proteomics grade or HPLC grade water) for preparing all of buffers and solutions.
 Note: Always use LC-MS grade water to prepare solution for ZipTip C18 desalt procedure.
✓ Always use ultrapure chemicals and solvents
✓ Always use high quality microcentrifuge tubes. USA scientific 1.5 ml Seal-Rite tubes (Catalog #1615-5500) recommended.
✓ Clean and autoclaved tubes and pipette tips recommended.
✓ Desalt using Millipore ZipTip C18 is required before running your samples in our maXis impact qTOF MS system and amaZon ion trap MS system.

1D gel

<table>
<thead>
<tr>
<th>Running gel</th>
<th>7% (mL)</th>
<th>10% (mL)</th>
<th>12% (mL)</th>
<th>15% (mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H₂O</td>
<td>5.1</td>
<td>4.1</td>
<td>3.4</td>
<td>2.4</td>
</tr>
<tr>
<td>1.5 M Tris-HCl, pH 8.8</td>
<td>2.5</td>
<td>2.5</td>
<td>2.5</td>
<td>2.5</td>
</tr>
<tr>
<td>10% (w/v) SDS</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>Acrylamide/Bisacrylamide</td>
<td>2.3</td>
<td>3.3</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>30%/0.8% w/v (Bio-Rad)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10% ammonium persulfate</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>TEMED</td>
<td>0.006</td>
<td>0.006</td>
<td>0.006</td>
<td>0.006</td>
</tr>
</tbody>
</table>

For one 1 mm minigel, add 4.5 mL running gel solution

<table>
<thead>
<tr>
<th>Stacking gel</th>
<th>3.75% for 2 gels, (mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H₂O</td>
<td>1.875</td>
</tr>
<tr>
<td>0.5 M Tris-HCl, pH 6.8</td>
<td>0.75</td>
</tr>
<tr>
<td>10% (w/v) SDS</td>
<td>0.03</td>
</tr>
<tr>
<td>Acrylamide/Bisacrylamide</td>
<td>0.375</td>
</tr>
<tr>
<td>30%/0.8% w/v (Bio-Rad)</td>
<td></td>
</tr>
<tr>
<td>10% ammonium persulfate</td>
<td>0.03</td>
</tr>
<tr>
<td>TEMED</td>
<td>0.003</td>
</tr>
</tbody>
</table>

For one 1 mm minigel, add 1.5 mL stacking gel solution, insert comb to form wells, polymerize for at least 30 min.

<table>
<thead>
<tr>
<th>SDS-PAGE running buffer</th>
<th>1 X</th>
<th>2 X</th>
<th>5 X</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycine (g)</td>
<td>14.4</td>
<td>28.8</td>
<td>72</td>
</tr>
<tr>
<td>Tris-base</td>
<td>3.02</td>
<td>6.04</td>
<td>15.1</td>
</tr>
<tr>
<td>SDS</td>
<td>1</td>
<td>2</td>
<td>5</td>
</tr>
</tbody>
</table>

For 12% minigel, we usually run it at 120 v constant for 20 min, followed by 80 v for 2 h.
For a pure protein, 3-5 ug per loading recommended; for a protein mixture extracted from bacteria, 50 ug per loading recommended.

<table>
<thead>
<tr>
<th>5X sample loading buffer (1 volume sample and 4 volume buffer)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris-HCl, pH 6.8, (0.605g Tris-base in 10 mL H₂O)</td>
<td>5 mL</td>
</tr>
<tr>
<td>SDS</td>
<td>0.5 g</td>
</tr>
<tr>
<td>Glycerol</td>
<td>5 mL</td>
</tr>
<tr>
<td>Bromophenol blue R-250</td>
<td>0.005g</td>
</tr>
<tr>
<td>2-mercaptoethanol</td>
<td>1 mL</td>
</tr>
<tr>
<td>Aliquot to 0.5 mL, store at -20 °C</td>
<td></td>
</tr>
</tbody>
</table>

**Staining and destaining**

**Staining**

Stain gel for 0.5-1h by shaking in staining solution (270 mL ethanol, 100 mL acetic acid, 5 g CuSO₄·5H₂O and 0.4 g Coomassie Brilliant Blue R-250, add water to make 1 L solution)

**Destaining**

Destain gel by shaking in destaining solution (120 mL ethanol, 70 mL acetic acid and 810 mL water) for 3~5 x 30 min. Wash gel with Milli-Q water 2~3 x 5 min after destaining.

**In gel digestion**

- Ammonium bicarbonate (NH₄HCO₃), dithiothreitol (DTT) and iodoacetamide (IAA) solutions should be freshly prepared every day.
- Prepare 100 mM NH₄HCO₃ first (237 mg NH₄HCO₃ in 30 mL milli-Q water).

1. cut the target protein band from gel as closely as possible and chop gel pieces into 1 mm³ and transfer to a microcentrifuge tube, do not cut off too big gel piece.

2. add 200 ul destaining solution (25 mM NH₄HCO₃, 50% ACN), incubate at RT for 30 min, discard the solution, repeat until the gel becomes white.

3. remove destaining solution and dry completely in a speed-vacuum for 15 min.

4. add 50 ul of freshly prepared 10 mM DTT (15.4 mg DTT in 10 mL of 100 mM NH₄HCO₃), incubate at 56 °C for 30 min.

5. discard DTT solution, add 50 ul 100 ACN for 5 min (repeat 2X)

6. dry in a speed-vacuum for 15 min

7. add 50 ul of 55 mM iodoacetamide (101.75 mg in 10 mL of 100 mM NH₄HCO₃) to alkylate cysteines, incubate for 20 min in dark at RT.
8, centrifuge at 10000 rpm for 2 min and discard the solution, wash the gel with 200 ul of 100 mM NH₄HCO₃, vortex for 10 min, then remove washing solution.

9, dehydrate with 200 ul of ACN and incubate for 10 min at RT. Gel pieces should shrink and become an opaque-white color within 5 min, if they don’t, then repeat this step.

10, remove ACN and dry completely in a speed-vacuum (15 min).

11, add 40 ul of trypsin solution (Promega sequencing grade, 20 ng/ul in 25 mM NH₄HCO₃) and incubate at 4 °C for 15 min

12, remove excess trypsin solution and overlay the gel pieces with 30-40 ul of 25 mM NH₄HCO₃, seal the tubes using parafilm.

13, incubate overnight (12-16h) at 37 °C waterbath, Note: check if the gel pieces is still overlayed by NH₄HCO₃ after 1 h incubation, if not, add more NH₄HCO₃.

**Peptides Extraction**

14, carefully remove the parafilm from the microcentrifuge tube, spin the gel pieces down at 10000 rpm for 2 min and collect solution into a fresh and clean tube.

15, add 30 ul of 60% ACN/1% TFA (or 5% FA) to the gel pieces, sonicate for 10 min, collect solution and combine it with the solution above, repeat this step again.

16, dry the solution in speed-vacuum, use immediately otherwise store at -20 °C.

Note: Sample desalt is required before LC-MS analysis, because ESI/CSI source is very sensitive to salt.

**Sample desalt by ZipTip**

- If the sample is dry, rehydrate it in 20-30 ul 1% formic acid (FA) / 5% ACN, sonicate for 10 min and spin sample solution down.
- **Avoid introducing air** through the tip during the procedure, pipetting up and down gently.
- Always use LC-MS grade ACN and water in this procedure.
- Before equilibrate the ZipTip, add 100 ul of 1% FA into a clean tube and 10 ul elution solution (60% ACN / 1% FA) in another clean tube.

1, set pipette to 10 ul and attach a Milipore C18 ZipTip.

2, equilibrate the ZipTip: pipette up 100% ACN and discard ACN to waste, repeat 3 times, followed by pipetting up 1% FA and discard solution to waste, repeat 3 times.

3, load the digested sample by pipetting up and down for 10 times.

4, wash the ZipTip by pipetting up 1% FA and discard it to waste, repeat 6 times.
5, pipette the ZipTip up and down in the elution solution for 10 times. (C18 ZipTip can be reused for the same sample from step 3 to 5 to best recover peptides from the solution)

6, dry the ZipTip recovered sample in a speed-vacuum for 10 min, add 20-100 ul (based on your protein quantity) of 0.1% FA (not 1% FA here) / 5% ACN and sonicate for 10 min, then spin down the solution, store at -20 °C for a few days if not do LC-MS analysis today.