

## Sample Preparation Guidelines

### **I. Guidelines for maximum concentrations of salts, etc. before sample cleanup.**

Note that higher concentrations usually result in excessive loss of target material or extremely poor digestion. Volatile buffers (i.e., ammonium salts, formate) are recommended. Call or e-mail for detailed information.

Detergents: < 0.1%

SDS: < 0.01%

Glycerol: < 1%

Tris buffer: < 50mM

Phosphate buffers: avoid

Sodium Azide: < 1mM

Alkali metal salts: < 1M

Ammonium bicarbonate: < 30mM

Guanidine: < 1M

### **II. Recommended concentration of samples**

Peptide: 0.1 - 1 pmol/ □

Protein (6-60 kDa): 1 - 10 pmol/ □

Protein (60-100 kDa): 2- 20 pmol/ □

Protein (>100 kDa): 4 - 40 pmol/ □

### **III. Sample preparation techniques for MS Analysis**

#### **1. Sample preparation for MS of proteins and peptides in solution.**

A protein that is at least a major component in a mixture can be analyzed using MS without SDS-PAGE. Mixtures of peptides can readily be analyzed, however, poorly ionizing peptides may be suppressed and may require additional separation techniques.

#### **A. Use of volatile salts.**

The easiest way to rid samples of contaminants is to not use anything that is a potential problem. Avoidance of detergents, PEGs, and salts always lessens time and work required for sample cleanup. Ammonium salts can often be substituted in dialysis, sizing, and ion exchange chromatography.

#### **B. Dialysis**

An effective method for exchange of high concentrations of unfriendly salts, etc. with low concentrations of more MS friendly solutions.

This method can be applied to large volumes (dialysis tubing - RINSE WELL) as well as small volumes (Drop dialysis - FOR PROTEINS, NOT PEPTIDES).

*REMEMBER - always dialyze against low (<10 mM) concentrations of ammonium salts rather than pure water.*

#### **C. Micro-gel filtration**

Home made or commercially available micro-devices for desalting can be used to remove excess salt.

#### **D. Micro reversed-phase cartridges.**

Several different types of commercially available products (e.g., ZipTips<sup>®</sup>) are available including high-throughput devices.

#### **E. Precipitation**

Precipitation methods vary for different classes, but may work well for desalting or removal of detergents. (i.e., for proteins > 15000 Da)

**2. Sample prep for very hydrophobic/membrane proteins and peptides. Analysis of hydrophobic proteins is often challenging and may require repeated efforts before satisfactory results have been obtained. Some guidelines include:**

##### **A. Run a 1-D SDS-PAGE from a relatively purified fraction.**

Eliminating as many unwanted proteins as possible will increase chances of successful analysis.

##### **B. Use acid labile detergents if possible.**

Commercially available acid-labile detergents from Millipore are recommended. Octyl-glucoside at modest concentrations (Final conc. < 20 mM) is relatively MS friendly.

##### **C. Solubilize in Acid.**

Formic acid (~ 70%) is an MS friendly acid for solubilizing proteins.

##### **D. Solubilize in Urea and/or Guanadinium/HCl.**

Trypsin is still quite active in 2M urea or Guanadinium/HCl.

##### **E. Use more polar columns with non-polar solvents for chromatography.**

A variety of groups have reported using different chromatography methods to separate hydrophobic peptides. Some include diphenyl columns, isopropanol in the mobile phase, and high acid.

### **3. In-gel digestion procedures for preparation of MS samples**

#### **A. General Guidelines.**

1. Wear appropriate gloves during all steps.
2. Use Eppendorf brand plastic ware if possible (or other tested plasticware).
3. Avoid using high percentage acrylamide gels (>12%) in your preparation.
4. Ammonium Acetate (pH 7) can be substituted for Ammonium bicarbonate when appropriate.
5. More gel pieces are not necessarily better. Overall efficiency is much higher with a single concentrated gel rather than several dilute.
6. Pool samples after extraction.

#### **B. Staining Techniques.**

##### **1. Zinc.**

Zinc stain is the preferred method for in gel digestions because this is a negative stain. A kit is available from BioRad (cat. #161-0440) consisting of an imidazole solution and a zinc sulfate solution.

**a. Stain.** Immediately after electrophoresis, the gel is soaked in imidazole solution (0.2 M) for 10 min and then transferred to zinc sulfate solution (0.2 M). The background becomes opaque while the protein bands remain clear. When the desired degree of opacity is achieved (1-2 min), the gel is transferred to water. The gel can then be scanned and handled for sample preparation.

**b. Destain.** No destain is needed with zinc stain.

## 2. Coomassie blue staining.

**a. Stain.** It is important to stain the gel for the minimum amount of time necessary to see the protein bands. This minimizes the degree to which proteins are irreversibly fixed in the gel. The Pierce Gelcode blue stain reagent works well and requires a very short staining step.

**b. Destain.** Procedures vary dependent upon the type of Coomassie stain used. If Coomassie Brilliant Blue R-250 is used, destain with 20% methanol, 0.5% acetic acid.

## 3. Silver staining.

This is the least preferable staining technique. If silver staining must be used, modified techniques are available in the literature with a milder fixing step. A mass spectrometry friendly silver stain kit is now offered by Invitrogen and other commercial sources.

## C. Gel preparation for digestion.

1. Place the gel on a clean transparency sheet on a light box. Excise the protein band and chop it into small pieces with a cleaned scalpel or razor blade. It is important to prevent keratin contamination at this step. For best results excise the gel bands in a HEPA filtered laminar flow hood. Place gel pieces into a microcentrifuge tube (Eppendorf brand is recommended).

2. Wash the gel with a suitable volume of 100 mM ammonium bicarbonate (pH 8.5) in 50% acetonitrile (ACN) three times for 20 minutes. Wash with 100% ACN for 10 min. The gel pieces should be shrunken and opaque white with a clear, colorless supernatant. For Coomassie gels, if blue stain remains, rehydrate with 100 mM ammonium bicarbonate (pH 8.5) and repeat step 3b.

3. Dry the gel pieces using a centrifugal evaporator for 15 min.

## D. Reduction and Alkylation.

1. Reduce the gel pieces using 30  $\mu$ l of 20 mM dithiothreitol (DTT) in 100 mM ammonium bicarbonate, 5% ACN for 1 hr at 55°C.

2. Remove the supernatant and wash the gel pieces with 100  $\mu$ l 100 mM ammonium bicarbonate for 10 min.

3. Remove the supernatant and dehydrate the gel pieces with 100  $\mu$ l 100% ACN for 20 min.

4. Add 30  $\mu$ l 100 mM iodoacetamide, 100 mM ammonium bicarbonate. Incubate for 30 min in the dark at room temperature.

5. Remove the supernatant and wash the gel pieces with 100  $\mu$ l 100 mM ammonium bicarbonate for 10 min.

6. Remove the supernatant and dehydrate the gel pieces with 100  $\mu$ l 100% ACN for 20 min.

7. Wash and swell the gel pieces with 100  $\mu$ l 100 mM ammonium bicarbonate for 10 min.

8. Remove the supernatant and dry the gel pieces in 100  $\mu$ l 100% ACN for 20 min.

9. Remove the supernatant and dry the gel pieces in a centrifugal evaporator for 15 min.

## E. Digestion.

1. Swell the gel pieces in digestion buffer (50 mM ammonium bicarbonate, enzyme (12.5 ng/  $\mu$ l) trypsin, sequencing grade) on ice for 45 min. The minimum volume (20-30  $\mu$ l for most gel pieces) of buffer necessary to swell the gel should be used. For best results, the enzyme:substrate ratio for trypsin should be about 1:50-1:100 (similar for Asp-N and Lys-C), so the concentration of enzyme can be adjusted accordingly.
2. Add 5-25  $\mu$ l 50 mM ammonium bicarbonate if necessary to keep the pieces wet.
3. Incubate at 37°C for 6-15 hr.

#### **F. Extraction.**

1. Collect the supernatant of the digestion solution.
2. Extract 1X with 100  $\mu$ l of 20 mM ammonium bicarbonate for 20 min and collect the supernatant (all supernatants are pooled).
3. Extract 2X with 100  $\mu$ l 1% TFA in 50% ACN for 20 min.
4. Extract 1X with 100  $\mu$ l 100% ACN for 20 min.
5. Dry the combined supernatants in a centrifugal evaporator.  
Store sample at -20°C for later analysis.