



Mass Spectrometry Sample Preparation Guidelines

Keratin contamination is a significant problem when analyzing samples by MS. Gloves should be worn and all care should be taken to ensure keratin is not introduced while handling samples.

For protein: Samples should be submitted in Solid form with

1. **Samples for PMF must be tryptic or peptic digested and should be of no less than 0.1 nanomole in quantity.** A sample concentration of 1 mg/ml is ideal.
2. **Samples for intact protein mass analysis must be of high purity (>90%) and should be of no less than 100 microgram in quantity.**
3. **Salt and detergent free and should not be radioactive**
4. **Sample vials should be clearly labeled and sealed.**
5. **Solid samples can be posted in room temperature.**

Pathogenicity

Biological samples should be accompanied by documentation of potential pathogenicity or pathogen free status.

Protein Identification by Mass Spectrometry

Tryptic or peptic Digestion

For protein identification, you should cut out a gel spot or band and do an in-gel tryptic digestion or peptic digestion. The gel must be stained with a **mass spectrometry-compatible stain**, for example colloidal Coomassie Brilliant Blue R-250 or silver stain. Then the digested sample is dried and could be submitted for MALDI-MS and MS/MS analysis.

Pitfalls

If a band is not visible on a Coomassie-stained gel, the chances of obtaining identification are greatly reduced. At least 0.1 µg is preferable. The most common reason for failing to identify a protein, other than insufficient material, is contamination with keratin. Keratin can contaminate an entire gel, ruining any chances of identifying even an intensely-stained band. Peaks in the mass spectrum resulting from keratin peptides will reduce the intensity of those from the protein of interest, especially if the ratio of keratin to sample is high.

Peptide Mass Fingerprint (PMF)

The resulting mass spectrum of peptides or the peptide mass fingerprint (PMF), is searched against sequence databases. The PMF is compared to the masses of all theoretical tryptic peptides generated in silico by Mascot. Mascot produces a statistically-based Mowse score that indicates if any matches are significant or not.

MS/MS

MS/MS is used to increase the likelihood of getting a database match. The PMF only contains the masses of the peptides. MS/MS of peptides contains a spectrum of fragment ions that contain information about the amino-acid sequence. Adding up this information to the PMF allows Mascot to increase the statistical significance of a match.

Preparing gel slices for PMF or MS/MS

All gels from which bands or spots are to be excised for mass spectrometric analysis must be prepared so as to minimize keratin contamination. Samples can be electrophoresed on normal SDS-Polyacrylamide gels and the gel must be stained with a mass spectrometry-compatible stain, using Coomassie, Sypro or silver stains. Please note when using silver staining methods do not include glutaraldehyde in any of the steps. Coomassie stainable levels are required for PMF.

Manual in-gel digestion protocol

The digestion could follow Trypsin Gold, Mass Spectrometry Grade Protocol or these following protocols.

Protocol 1.

The digestion procedure is recommended to perform under positive pressure or laminar hood.

1. Cut the protein bands from the gel with sharp scalpel with total vol. < 20 μ l
2. Place the gel pieces into a 1.5 ml siliconized microcentrifuge tube. Add 200 μ l of washing solution (50% v/v Methanol, 5% v/v Acetic acid) and incubated at RT (\sim 30°C); overnight (until the background is nearly clear)
3. Carefully remove the washing solution and discard
4. Add 200 μ l of washing solution and incubated at RT (\sim 30°C); 2-3 hr.
5. Carefully remove the washing solution and discard
6. Add 200 μ l of Acetonitrile solution (for protein dehydration) (the gel pieces will have an opaque white color and smaller in size) and incubated at RT (\sim 30°C); 5 min
7. Carefully remove the acetonitrile solution and discard
8. Dry the gel at ambient temperature in a vacuum centrifuge (2-3 min)
9. Add 30 μ l of 10 mM DTT solution (for protein reduction) and incubated at \sim 25°C; 30 min
10. Carefully remove the DTT solution and discard
11. Add 30 μ l of 100 mM Iodoacetamide (IAA) solution (for protein alkylation) and incubated at \sim 25°C; 30 min; in dark condition
12. Carefully remove the IAA solution and discard
13. Add 200 μ l of Acetonitrile solution (Dehydration) and incubated at \sim 25°C; 5 min
14. Carefully remove the acetonitrile solution and discard
15. Add 200 μ l of 100 mM Ammonium bicarbonate solution (Rehydration) and incubated at \sim 25°C; 10 min
16. Carefully remove the ammonium bicarbonate solution and discard
17. Add 200 μ l of Acetonitrile solution (Dehydration) and incubated at \sim 25°C; 5 min
18. Carefully remove the acetonitrile solution and discard
19. Dry the gel at ambient temperature in a vacuum centrifuge (2-3 min)
20. Add 30 μ l of 20 ng/ μ l Trypsin solution (molecular grade is recommended) and incubated on ice 10 min (Rehydration); occasional vortex mixing
21. Centrifuge 30 sec. then carefully remove the excess trypsin solution from the sample
22. Add 5 μ l of 50 mM Ammonium bicarbonate (cold) and vortex & briefly centrifuge. Then incubate 37°C overnight (\sim 14-16 h)
23. Three extraction steps were perform by adding 30 μ l of 50 mM Ammonium bicarbonate and incubated at \sim 25°C; 10 min with occasional vortex mixing
24. Centrifuge 30 sec. and carefully collect supernatant and transfer to new microcentrifuge tube
25. Add 30 μ l of Extraction buffer (50% v/v Acetonitrile, 5% v/v Formic acid) and incubated at \sim 25°C; 10 min with occasional vortex mixing
26. Centrifuge 30 sec. and carefully collect the supernatant and combine the extract in the same tube
27. Add 30 μ l of Extraction buffer and incubated at \sim 25°C; 10 min with occasional vortex mixing
28. Centrifuge 30 sec. and carefully collect the supernatant and combine the extract in the same tube
29. Dry the extracted sample at ambient temperature in a vacuum evaporation centrifuge. Store the dry-samples at -20°C until analysis.

Protocol 2.

This protocol is based on Shevchenko A, Wilm M, Vorm O, & Mann M. Mass spectrometric sequencing of proteins from silver-stained polyacrylamide gels. *Anal Chem* 1996, 68:850-8. It could be used both Coomassie and silver-stained gel bands. The procedure includes reduction and acetamidation steps that may be skipped if desired. For heavily stained Coomassie bands, it is helpful to wash gel pieces for 1 hr in 100 mM NH_4HCO_3 prior to dehydrating with acetonitrile (step 2).

1. Excise band from Coomassie or silver stained gel. Cut gel band into 1 mm cubes using clean razor blade on a clean glass surface. Transfer to an Eppendorf tube.
2. Remove excess water with pipette. Add 25-35 μL acetonitrile to tube to cover gel pieces. Incubate 10 minutes at RT to dehydrate and shrink gel pieces.
3. Remove acetonitrile with pipette and dry in a vacuum evaporation centrifuge for 10 minutes.
4. Swell gel particles in 150 μL 10 mM DTT in 100 mM NH_4HCO_3 . Incubate 1 hr at 56°C.
5. Cool to RT. Replace DTT solution with 150 μL 55 mM iodoacetamide in 100 mM NH_4HCO_3 . Incubate 45 minutes at RT in the dark with occasional vortexing.
6. Remove solution and wash gel pieces with 150 μL 100 mM NH_4HCO_3 . Incubate 10 minutes at RT.
7. Remove NH_4HCO_3 solution with pipette. Add 150 μL acetonitrile to dehydrate gel pieces. Incubate 10 minutes at RT.
8. Repeat wash steps 6 through 7. Remove acetonitrile and speed-vac to dryness for 10 minutes.
9. Place tubes in ice water bath and swell gel particles in 25-35 μL digestion buffer. Incubate 45 minutes in ice water bath. Digestion buffer consists of 12.5 ng/ μL trypsin (Promega sequence-grade modified porcine trypsin, Cat. #V511A) in 50 mM NH_4HCO_3 . To make the digestion buffer, dissolve 20 μg Promega trypsin in 80 μL Promega trypsin buffer solution (50 mM acetic acid), and dilute with 50 mM NH_4HCO_3 to 12.5 ng/ μL .
10. Remove trypsin-containing buffer. Add 5-10 μL 50 mM NH_4HCO_3 without trypsin to keep pieces wet during cleavage. Incubate o/n at 37°C.
11. Spin 1 min at 14,000 rpm to spin down gel pieces. Save supernatant in a new eppendroff tube.
12. Add 20 μL 20 mM NH_4HCO_3 to cover gel pieces. Incubate 10 minutes at RT. Collect supernatant in the same eppendroff tube from step 11.
13. Add 25 μL 5% formic acid, 50% acetonitrile to the gel pieces. Incubate 20 minutes at RT.
14. Spin 1 min at 14,000 rpm. Remove formic acid/acetonitrile solution and save in the same eppendroff tube from step 11.
15. Repeat formic acid extraction (steps 13 through 14) twice more.
16. Dry extracted samples in a vacuum evaporation centrifuge to complete dryness. Store the dry-samples at -20°C until analysis.

Please note that MALDI samples should be free of SDS, and should not be radioactive. It is best to remove buffer salts and detergents (e.g. by dialysis) prior to analysis and to dissolve the sample in a suitable solvent (e.g. 0.1% TFA/water) which will not degrade the spectrum. If there is too much salt in a sample, the salt signal intensity is so large that it effectively suppresses out the sample signal, giving no sample spectrum. In cases where it is not possible to remove these contaminants the sample should be in a higher concentration. It may then be possible to dilute the sample to the point where the contaminants will have little effect on the spectrum.

Levels of buffers and detergents which exceed the following limits will probably cause noticeable degradation of the spectrum:

Phosphate buffer	>50mM	Ammonium bicarbonate	>30mM
Tris buffer	>100mM	Guanidine	>1M
Detergents (e.g. Triton-X)	>0.1%	SDS	>0.01%
Alkali metal salts	>1M	Glycerol	>1%
Sodium Azide	>1mM		

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