TECHNICAL BULLETIN

ProteaseMAX™ Surfactant, Trypsin Enhancer

Instructions for Use of Products
V2071 and V2072

Revised 2/15
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ProteaseMAX™ Surfactant, Trypsin Enhancer

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1. Description

ProteaseMAX™ Surfactant[a] is designed to improve in-gel and in-solution protein digestion. ProteaseMAX™ Surfactant solubilizes proteins including difficult proteins (i.e., membrane proteins) and enhances protein digestion by providing a denaturing environment prior to protease addition. ProteaseMAX™ Surfactant ensures fast and efficient protein digestion with proteases such as trypsin (see Figure 1 for an example), chymotrypsin and Lys-C. For in-gel protein digestion, ProteaseMAX™ Surfactant offers time and labor savings. The digestion step is complete in 1 hour, and the surfactant provides concurrent extraction of peptides from gels, eliminating the need for post-digestion peptide extraction. The surfactant also improves recovery of longer peptides that are typically retained in the gel under a standard extraction protocol. For low nanogram protein levels, where protein identification is unreliable, ProteaseMAX™ Surfactant can improve probability of protein identification. Use of the surfactant for in-gel digestion of low protein amounts can be optimized by incorporation of easily adaptable modifications to the digestion protocol (see Section 5 for the protocol).

1. Description (continued)

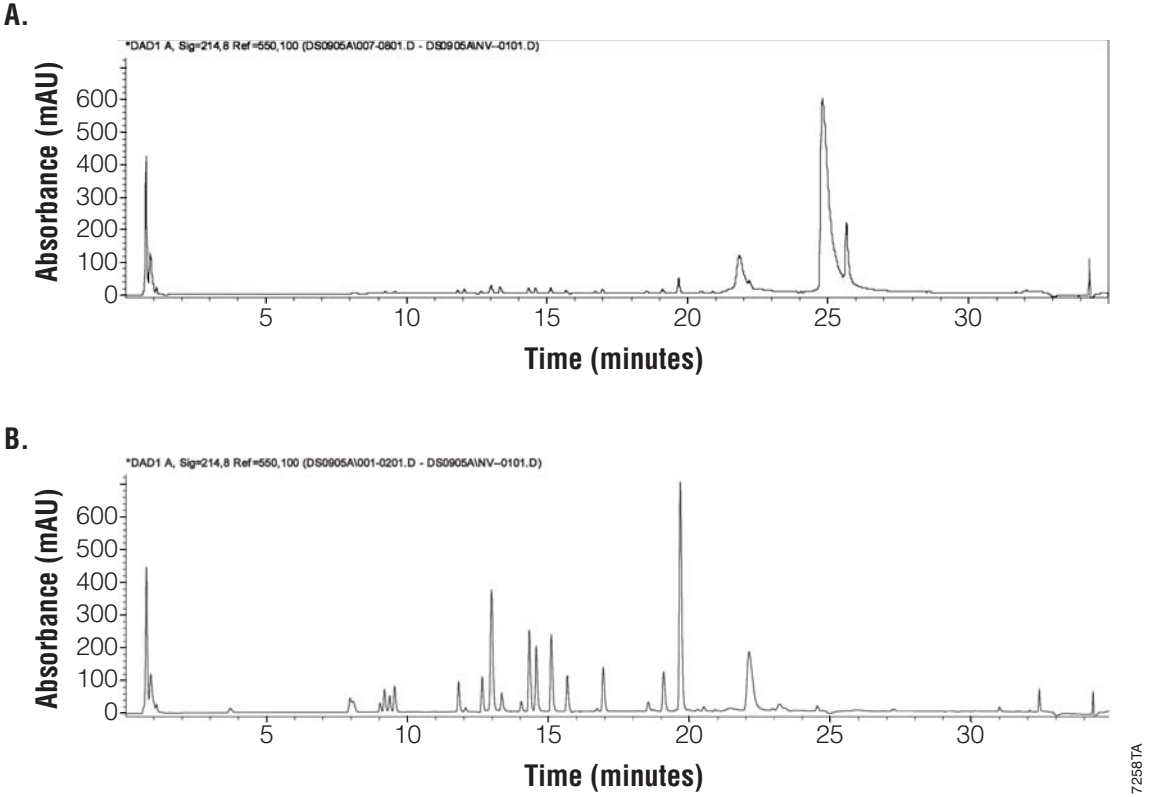


Figure 1. HPLC chromatograms of myoglobin digested with or without ProteaseMAX™ Surfactant. Myoglobin from horse heart was digested with trypsin at 50:1 ratio for 30 minutes at 37°C. Particulate material and the degraded surfactant were removed with centrifugation at 16,000 × *g* for 10 minutes, and the digests were resolved with a reverse-phase HPLC column. **Panel A.** Myoglobin was digested under standard conditions. The large peak at 25 minutes is an intact myoglobin. **Panel B.** Myoglobin was digested in the presence of 0.025% ProteaseMAX™ Surfactant. The peaks between 7 and 23 minutes are peptides. The arrow indicates degraded ProteaseMAX™ Surfactant, a small amount of which remains in the digest after centrifugation. Note that the surfactant elutes in the end of the gradient and does not interfere with peptide analysis.

ProteaseMAX™ Surfactant degrades over the course of a digestion reaction, yielding products that are compatible with downstream methods such as mass spectrometry (MS) and liquid chromatography (LC). No signal decrease or deterioration in chromatographic profiles in LC-MS have been observed after over one hundred runs with ProteaseMAX™ Surfactant-containing samples.

Advantages of ProteaseMAX™ Surfactant for In-Gel Protein Digestion

In-gel protein digestion normally requires overnight incubation followed by laborious peptide extraction. In the presence of the ProteaseMAX™ surfactant, protein digestion and peptide recovery are complete in a single step that can be as short as 1 hour (Figure 2). The surfactant enhances peptide extraction, eliminating the need for post-digestion extraction. In-gel digestion data quality is improved by the recovery of longer peptides that typically remain trapped in the gel under conventional conditions (Figure 3). In many cases, peptides in the range of 2,500–4,000Da are recovered in quantities sufficient for MS/MS analysis. The magnitude of the increase in peptide yield varies for different proteins. ProteaseMAX™ Surfactant degrades over the course of the in-gel digestion reaction allowing MS or LC analysis of the peptides directly after digestion. An investigation of an assortment of proteins of mouse or human origin showed that a ProteaseMAX™ Surfactant-assisted single-step in-gel digestion protocol provided reliable and robust protein identification and, in many cases, increased sequence coverage. The level of the degraded ProteaseMAX™ Surfactant can be minimized with solid phase extraction (SPE) or centrifugation if needed.

In-gel digestion of proteins at low nanogram amounts represents a challenging application. Probability of successful protein identification in gel decreases if protein amount drops below detection sensitivity of colloidal Coomassie® stain (15–20ng and less). In the presence of ProteaseMAX™ Surfactant, this probability substantially improves. A few optimization steps intended for maximizing peptide yield and minimizing interference from autoproteolytic tryptic peptides (see Section 5) help to achieve the best results. Study of a panel of mouse and human proteins digested in gel at low nanogram level indicated that ProteaseMAX™ Surfactant significantly improved probability of protein identification (Figure 5).

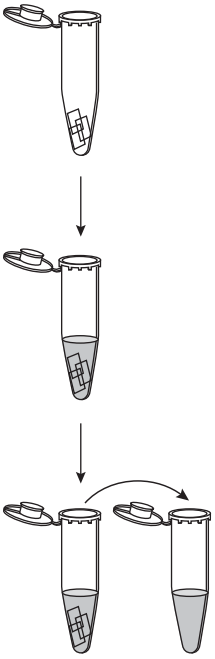
Advantages of ProteaseMAX™ Surfactant for In-Solution Protein Digestion

ProteaseMAX™ Surfactant solubilizes and denatures hydrophobic proteins (i.e., membrane proteins) at room temperature. ProteaseMAX™ Surfactant can be used as a single protein-solubilizing agent or as an additive with urea. As an additive with urea, ProteaseMAX™ Surfactant improves the solubilizing effect of urea.

Benefits of ProteaseMAX™ Surfactant

- Reduce protein digestion time.
- Eliminate the peptide extraction step following the in-gel digestion protocol.
- Improve recovery of longer peptides from gels.
- Improve MS identification of proteins present in gel at low amounts.
- Solubilize hydrophobic proteins within 1 hour or less at room temperature.
- Use ProteaseMAX™ Surfactant as an additive with urea. ProteaseMAX™ Surfactant improves urea solubilization efficiency.
- Perform downstream analyses (MS, HPLC) without interference from the surfactant.

1. Description (continued)



Prepare gel for digestion.
Destain and treat with reducing agent if needed.

Add trypsin and ProteaseMAX™ Surfactant.
Digest for 1 hour at 50°C or 2–4 hours at 37°C.
Protein is digested, peptides are extracted, and ProteaseMAX™ Surfactant is degraded over the course of digestion reaction.

Collect the digestion buffer (peptide solution), clean up peptides with SPE or centrifigation, and analyze with MS.

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Figure 2. In-gel protein digestion protocol. For the detailed protocol see Section 4.A (for in gel-digestion of Coomassie®-detectable protein amounts) or Section 5 (for in-gel digestion of low protein amounts).

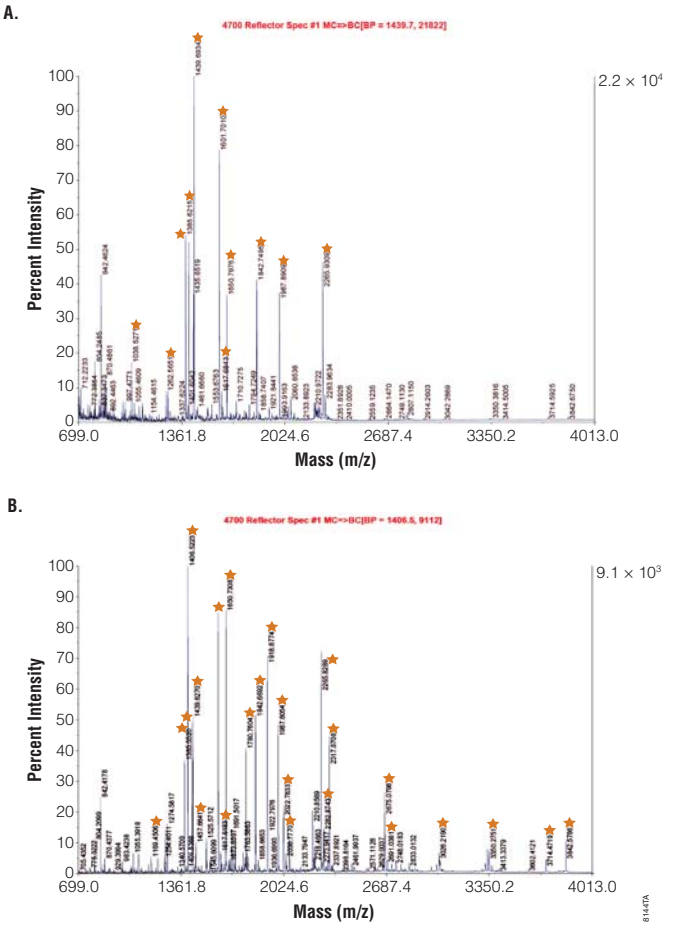

Figure 3. Efficient in-gel protein digestion with ProteaseMAX™ Surfactant (MALDI-TOF analysis). MALDI-TOF mass spectra of the protein in-gel digests performed with or without ProteaseMAX™ Surfactant. Membrane protein extract from mouse heart was resolved in 4–20% SDS-PAGE, and the gel was stained with Coomassie® R-250. A protein band with an apparent mass of 56kDa was excised from the gel, and protein was digested with or without ProteaseMAX™ Surfactant. **Panel A.** Conventional overnight digestion followed by peptide extraction with TFA/acetonitrile. **Panel B.** ProteaseMAX™ Surfactant-assisted 1-hour digestion as described in Section 4.A. Peptides obtained in either protocol were cleaned up with 10µl C18 ZipTip® pipette tips (Millipore) and analyzed with a 4800 MALDI-TOF/TOF Mass spectrometer with an OptiBeam™ on-axis laser (Applied Biosystems). The major protein in the band was identified as the β subunit of ATP synthase from H+-transporting mitochondrial F1 complex (assigned peptides are indicated by asterisks). Sequence coverage, MASCOT score and probability of a random match for the protein were 50%, 828 and 1×10^{-76} in the conventional protocol and 75%, 920 and 3.2×10^{-87} in the ProteaseMAX™ Surfactant-assisted protocol, respectively. Note the efficient recovery of long peptides (2,300–4,000Da long) in the presence of ProteaseMAX™ Surfactant.

1. Description (continued)

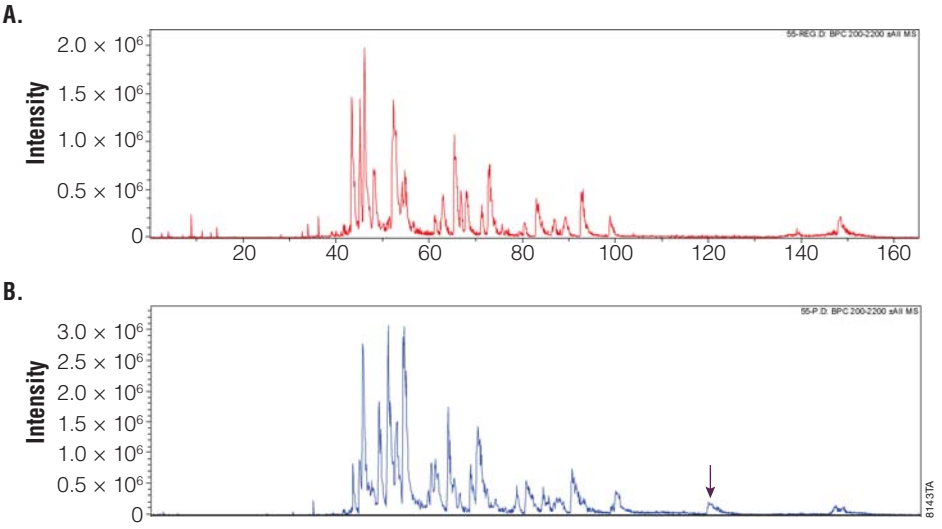

Figure 4. Efficient in-gel protein digestion with ProteaseMAX™ Surfactant (LC-MS analysis). Base peak chromatograms of protein in-gel digests performed with or without ProteaseMAX™ Surfactant. The same 56kDa protein band (see Figure 3 for more information regarding the protein) was analyzed. The protein band was excised from the gel, and protein was digested with or without ProteaseMAX™ Surfactant. **Panel A.** Conventional overnight digestion followed by peptide extraction with TFA/acetonitrile. **Panel B.** ProteaseMAX™ Surfactant-assisted 1-hour digestion as described in Section 4.A. Particulate material and the degraded ProteaseMAX™ Surfactant were removed with a 10-minute centrifugation at 16,000 × *g*. The digests were analyzed with Agilent 1100 series nano LC/MSD Ion Trap SL mass spectrometer. A small amount of degraded ProteaseMAX™ Surfactant remaining in the solution after centrifugation generated a detectable peak (indicated by an arrow on Panel B). Intensity of the peak of the residual degraded ProteaseMAX™ Surfactant varies depending on type of mass spectrometer and amount of injected sample (see Supplementary Figure, Section 6.C). Note that the degraded surfactant elutes at the end of the gradient. The degraded surfactant did not affect LC-MS analysis as measured by peptide peak retention, signal intensity or level of nonspecific noise. Two major proteins in the band were α and β subunits of ATP synthase from H+-transporting mitochondrial F1 complex. Coverage and MASCOT score for α subunit were 19% and 319 in the conventional protocol and 37% and 635 in the ProteaseMAX™ Surfactant-assisted protocol, respectively. The values for β subunit were similar in both protocols (data not shown).

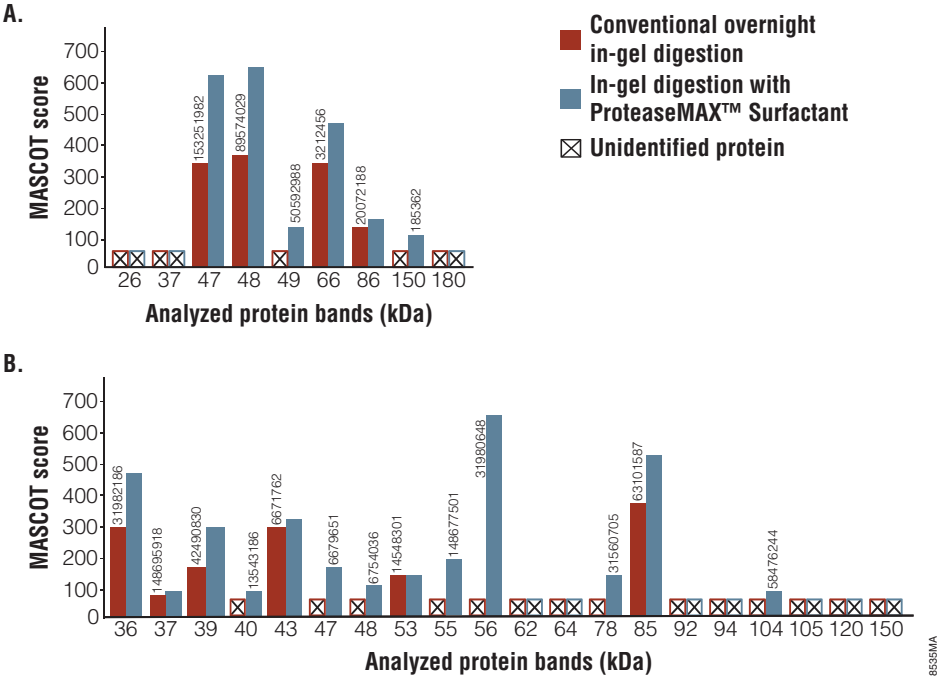

Figure 5. Improved rate of identification for in-gel digested proteins at low nanogram level. Human (**Panel A**) and mouse protein (**Panel B**) cell extracts were resolved in 4–20% SDS-PAGE, and the gel was stained with silver. Protein bands (amount of protein per band varied in the range of ~5–20ng) were excised from the gel and proteins were digested with or without ProteaseMAX™ Surfactant. Peptides obtained in either protocol were cleaned up with 10µl C18 ZipTip® pipette tips (Millipore) and analyzed with a 4800 MALDI-TOF/TOF mass spectrometer with an OptiBeam™ on-axis laser (Applied Biosystems). Red bars show MASCOT score for proteins digested with a conventional overnight in-gel digestion protocol followed by peptide extraction with TFA/acetonitrile. Blue bars show the score for the same proteins digested in the presence of ProteaseMAX™ Surfactant according to In-Gel Digestion Protocol for Low Protein Amounts. gi numbers for the identified proteins are indicated above the bars. Crossed bars indicate that no protein was identified in a band. From the total 29 analyzed protein bands, conventional protocol identified 10 proteins. ProteaseMAX™ Surfactant-assisted protocol identified 19 proteins. Notice overall increase in MASCOT score for proteins identified with ProteaseMAX™ Surfactant-assisted digestion protocol versus the same proteins identified with a conventional digestion protocol. The data demonstrate improved probability of identification of proteins present in gel at low nanogram amounts with the surfactant.

2. Product Components and Storage Conditions

PRODUCT	SIZE	CAT.#
ProteaseMAX™ Surfactant, Trypsin Enhancer	**1mg**	**V2071**

Includes:

- 1mg ProteaseMAX™ Surfactant, lyophilized

PRODUCT	SIZE	CAT.#
ProteaseMAX™ Surfactant, Trypsin Enhancer	**5 × 1mg**	**V2072**

Includes:

- 5 × 1mg ProteaseMAX™ Surfactant, lyophilized

Storage Conditions: Store lyophilized ProteaseMAX™ Surfactant at –20°C. See the product information sheet for expiration date. Store the 1% solution in single-use aliquots for up to 12 months at –20°C.

3. Before You Begin

3.A. General Considerations

ProteaseMAX™ Surfactant is acid-labile and should be dissolved in freshly prepared ammonium bicarbonate buffer (pH~7.8). Dissolving the surfactant in a buffer of lower pH will degrade the surfactant. Using ProteaseMAX™ Surfactant at concentrations higher than those suggested may lead to loss of peptide signal due to precipitation of the peptides.

ProteaseMAX™ Surfactant is a hydrophobic anionic sulfonate (sodium 3-((1-(furan-2-yl)undecyloxy) carbonylamino) propane-1-sulfonate) with a molecular weight of 425.51Da. It is designed to degrade over the course of a digestion reaction, eliminating the need for degradation after digestion. However, if degradation is still required (i.e., if digestion was carried out for a shorter period than recommended or ProteaseMAX™ Surfactant concentration was higher than recommended), ProteaseMAX™ Surfactant can be degraded either in 0.5% TFA for 15 minutes at 37°C, or at neutral pH by heat treatment at 95°C for 5 minutes. Degradation of the surfactant produces a hydrophilic zwitterionic species (M.W. = 139.17Da) and a neutral hydrophobic species (M.W. = 238.36Da), which do not interfere with mass spectrometry analysis when present in recommended amounts.

3.B. Preparation and Storage of ProteaseMAX™ Surfactant

Add 100µl of freshly prepared 50mM ammonium bicarbonate to a vial of ProteaseMAX™ Surfactant to give a 1% solution. The fine crystalline-like structure of ProteaseMAX™ Surfactant allows it to dissolve completely as soon as buffer is added. We recommend swirling the vial a few times to ensure proper mixing. Avoid rigorous shaking to prevent foaming. Keep the solution on ice until ready for use. For storage of ProteaseMAX™ Surfactant solution, we recommend dividing the stock solution into aliquots of desired size and snap-freezing on dry ice or in liquid nitrogen prior to storage at –20°C. As needed, thaw an aliquot, on ice if possible, or by hand-warming (use gloves to prevent contamination with keratin) for a few minutes. Keep the aliquot on ice after thawing. Although we recommend making single-use aliquots, the ProteaseMAX™ Surfactant can be refrozen up to five times without detectable loss of activity.

4. ProteaseMAX™ Surfactant Digestion Protocols

Notes:

1. ProteaseMAX™ Surfactant is designed to degrade over the course of a digestion reaction, eliminating the need for degradation after digestion. The degradation products generally do not interfere with liquid chromatography and mass spectral acquisition. However, at low protein or peptide concentrations it may be desirable to reduce the level of degraded surfactant to improve signal and minimize introduction of nonpeptide material to the system. The level of degraded ProteaseMAX™ Surfactant can be reduced with SPE or centrifugation. It is important to use C18 SPE tips of sufficient binding capacity (see the notes for in-gel and in-solution digestion protocols [Sections 4.A and 4.B] for recommendations). Since the hydrophobic degradation product has limited solubility, centrifugation can be used to reduce its level. A 10-minute centrifugation on a benchtop micro-centrifuge (14,000–16,000 × *g*) will remove 90–95% of the degraded surfactant.
2. To further minimize the introduction of degradation product into the mass spectrometer, we recommend diverting the trapping column flow away from the mass spectrometer after the peptides have been eluted. Consider that the degradation surfactant will bind to C18 solid phase and will elute at the end of the gradient after the peptides have eluted (Figure 4). Typically, switching at 85–95% acetonitrile should be sufficient, but this will vary by column. The presence of the degraded surfactant can be detected by UV absorbance at 220nm.
3. The long-term effect of residual ProteaseMAX™ Surfactant on the ion optics and capillary of mass spectrometers has been evaluated by analyzing signal intensities of standard digests before and after running injections of ProteaseMAX™ Surfactant-containing samples on an Agilent 1100 series nano LC/MSD Ion Trap SL mass spectrometer. No detectable signal decrease or deterioration in chromatographic profiles has been observed after over one hundred runs with ProteaseMAX™ Surfactant-containing samples.

Materials to Be Supplied by the User
(Solution compositions are provided in Section 6.A.)

Note: Mass spectrometry analysis is highly sensitive to contaminants. Use only the highest quality reagents and solvents (e.g., "mass spectrometry grade").

- NANOpure® (or equivalent grade) water
- methanol
- ethanol
- 50mM NH_4HCO_3
- acetonitrile (ACN)
- 1M dithiothreitol (DTT)
- 0.55M iodoacetamide (IAA)
- trifluoroacetic acid (TFA)
- trypsin (We recommend Trypsin Gold, Mass Spectrometry Grade, Cat.# V5280.)
- 50mM acetic acid
- ZipTip® (Millipore Cat.# ZTC18S096) or OMIX® pipette tips (Varian, Inc., Cat.# A5700310)

The following reagents are also required for in-solution digestions.

- –20°C acetone
- 8M urea

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4.A. In-Gel Digestion Protocol

Notes:

1. Steps 12 through 15 are critical, and it is important that they are performed as recommended. Be sure to use 0.01% ProteaseMAX™ Surfactant in the digestion solution (100X dilution of the stock solution). Higher surfactant concentrations may result in peptide loss. Prepare a 0.01% solution of the surfactant in the volume needed for digestion, and keep it on ice. The 0.01% solution cannot be stored for more than several hours due to limited stability. Any unused 0.01% ProteaseMAX™ Surfactant solution should be discarded.
2. Perform protein digestion at 50°C. We recommend using Trypsin Gold (Cat.# V5280) or Sequencing Grade Trypsin (Cat.# V5111), as these enzymes demonstrate high thermostability. Trypsin from other vendors may not be suitable for the protocol due to insufficient thermostability.
3. The volume of the digestion buffer is optimized for gel slices with dimensions of 1–2 × 5–6 × 1mm^3. Divide larger gel slices accordingly.

MALDI-TOF users: The protocol provides a reliable MS identification for protein amounts of about 50ng and up (Coomassie® R-250-detectable limit of detection for an average size [30–70kDa] protein) with MALDI-TOF analysis. For lower protein amounts we recommend using the in-gel digestion protocol for low protein amounts (Section 5). The 4800 MALDI-TOF/TOF Mass spectrometer with an OptiBeam™ on-axis laser irradiation (Applied Biosystems) was used for testing.

LC-MS users: The use of ProteaseMAX™ Surfactant should not affect the amount of protein detectable by LC-MS, assuming that the sample is injected without prior cleanup with SPE.

The protocol was tested with regular (R-250) and colloidal (G-250) Coomassie®- and silver-stained gels.

Washing

1. Resolve proteins by gel electrophoresis, and stain the gel. Wash with an appropriate destaining solution to remove nonspecifically bound stain.
2. Excise the protein band or spot of interest. Cut the gel slice onto 1mm^3 pieces, and transfer them to a 0.5 or 1.5ml microcentrifuge tube.

   Note: The protocol is optimized for gel slices of 1–2 × 5–6 × 1mm^3 dimension. If a gel slice is larger, divide it accordingly.
3. Wash with 200µl of NANOpure® water by vortex mixing for 30 seconds. After washing discard the water.

   Note: If many protein bands are being processed, put tubes on an orbital shaker (e.g., LabNet Orbit M60) and mix at 100–140 rpm.
4. Destain with 200µl of methanol:50mM NH_4HCO_3 (1:1 v/v) for 1 minute with intermittent vortex mixing. Discard supernatant. Repeat once.
5. Dehydrate for 5 minutes in 200µl of acetonitrile:50mM NH_4HCO_3 (1:1 v/v) with intermittent vortex mixing. Discard supernatant. Add 200µl of 100% acetonitrile, mix and incubate for 30 seconds. Discard the supernatant.
6. Dry in a Speed Vac® vacuum centrifuge for 5 minutes or until sample is dry.

Reduction/Alkylation

7. Rehydrate in 100µl of freshly prepared 25mM DTT in 50mM NH_4HCO_3, and incubate for 20 minutes at 56°C.
8. Discard supernatant. Add 100µl of freshly prepared 55mM iodoacetamide in 50mM NH_4HCO_3, and incubate in the dark for 20 minutes at room temperature.

   Note: Alkylation with iodoacetamide increases peptide mass by 57.02 for each cysteine.
9. Discard supernatant, and wash with 400µl of NANOpure® water by vortex mixing briefly. Discard supernatant. Repeat once.
10. Dehydrate for 5 minutes in 200µl of acetonitrile:50mM NH_4HCO_3 (1:1 v/v) with intermittent vortex mixing. Discard supernatant. Add 200µl of 100% acetonitrile, mix and incubate for 30 seconds. Discard the supernatant.
11. Dry in a Speed Vac® vacuum centrifuge for 5 minutes or until sample is dry.

Digestion

12. Rehydrate in 20µl of 12ng/µl trypsin in **0.01% ProteaseMAX™ Surfactant**:50mM NH_4HCO_3 for 10 minutes. Overlay with 30µl of **0.01% ProteaseMAX™ Surfactant**:50mM NH_4HCO_3 and gently mix for several seconds.

    Notes:

    1. We recommend using Trypsin Gold (Cat.# V5280) or Sequencing Grade Trypsin (Cat.# V5111), as these enzymes demonstrate high thermostability. Trypsin from other vendors may not be suitable for the protocol due to insufficient thermostability.
    2. The 0.01% ProteaseMAX™ Surfactant solution is made by diluting the 1% ProteaseMAX™ Surfactant solution 100X (see Section 3.B for preparation of the 1% solution) with 50mM NH_4HCO_3. Keep the 0.01% solution on ice until use to preserve its stability. The 0.01% solution should be used within several hours.
13. Incubate for 1 hour at 50°C. The digestion can also be performed at 37°C. A 2-hour incubation at 37°C is typically sufficient for digestion. We do not recommend continuing digestion for more than 1 hour at 50°C or 4 hours at 37°C. Incubating overnight might cause some decrease in peptide recovery.

    Notes:

    1. Protein digestion and peptide extraction are both completed within this step. Additional peptide extraction step (e.g., with acetonitrile) is not recommended, as it may cause peptide loss.
    2. ProteaseMAX™ Surfactant degrades over the course of the digestion reaction.
14. Collect the condensate from tube walls by centrifuging at 12,000–16,000 × *g* for 10 seconds.
15. Mix for a few seconds, and transfer the digestion reaction with extracted peptides into a new tube. Add TFA to a final concentration of 0.5% to inactivate trypsin. Mix and keep the tube on ice. If the digest is not going to be analyzed within several hours, snap-freeze it in dry ice and store at –20°C.

4.A. In-Gel Digestion Protocol (continued)

Peptide Handling

If the level of digested protein is sufficiently high the digest can be analyzed directly with MALDI-TOF. If the peptide signal is low, the peptides can be concentrated with a 10µl (0.6µl bed resin volume) C18 ZipTip® (Millipore; aspirate the digest into the tip to the maximal capacity. A 10µl Millipore tip can accommodate 20µl. Bind peptides with ten cycles of aspirating-dispensing). Avoid micro-C18 (0.2µl bed volume) tips for peptide cleanup. The use of micro-C18 tips can result in a decrease in peptide recovery due to competition of the degraded surfactant and peptides for binding sites.

For LC-MS, we recommend centrifuging the digest at 12,000–16,000 × *g* for 10 minutes to minimize introduction of nonpeptide material to a system. Centrifugation will remove particulate material and reduce level of the degraded ProteaseMAX™ Surfactant.

4.B. In-Solution Digestion Protocol (sample protocol for membrane protein)

Be sure to use the correct concentration of ProteaseMAX™ Surfactant in the digestion step (0.03% for cytoplasmic proteins or 0.05% for membrane proteins). Higher concentrations of surfactant might cause peptide loss or interfere with cleanup.

This protocol was developed using 50µg of membrane protein extract from mouse heart. Adjust volumes and quantity of reagents accordingly for larger amounts of protein. If urea is needed as a solubilizer, it can be used along with ProteaseMAX™ Surfactant. Use of a urea/ProteaseMAX™ Surfactant mix does not require any changes to the protocol.

Summary tables of volumes used are provided in Tables 1 and 2 at the end of the protocol. ProteaseMAX™ Surfactant concentrations given here refer to the concentration in the digestion reaction. You may use higher concentrations to solubilize protein.

Note: If you are starting with **protein that is in solution**, add 2.0µl of 1% ProteaseMAX™ Surfactant:50mM NH_4HCO_3, and proceed to Step 3 of this protocol. The final total digestion reaction volume will be 100µl (Table 2).

1. Precipitate the protein (50µg) from the extract or fraction with four volumes of –20°C acetone at –80°C for 20 minutes. Collect the protein by centrifugation at 12,000–16,000 × *g* for 10 minutes at 4°C. Discard the supernatant, and rinse the pellet with 300µl of (–20°C) acetone. Air-dry the pellet for 3–5 minutes.
2. Solubilize protein as follows:

   Solubilization with ProteaseMAX™ Surfactant alone: Add 20µl of 0.2% ProteaseMAX™ Surfactant:50mM NH_4HCO_3 to the pellet. Mix by vortexing for 2 minutes. Continue mixing by vortexing at medium speed, or if you are processing multiple samples, transfer the tubes onto an orbital shaker set at 100–140rpm. Avoid extensive foaming. Proteins will normally become soluble within 10–60 minutes, depending on the size of the protein pellet.

   Solubilization with ProteaseMAX™ Surfactant/urea mix: Add 15µl of 8M urea (dissolved in NANOpure® water), then add 20µl of 0.2% ProteaseMAX™ Surfactant/50mM NH_4HCO_3. Mix by vortexing or shaking on an orbital shaker as above.

   Note: Cytoplasmic proteins will typically solubilize in 0.1% ProteaseMAX™ Surfactant.

3. Add 50mM NH_4HCO_3 to a final volume of 93.5µl.
4. Add 1µl of 0.5M DTT. Incubate at 56°C for 20 minutes.
5. Add 2.7µl of 0.55M iodoacetamide. Incubate at room temperature in the dark for 15 minutes.
6. While performing Steps 4 and 5, dissolve trypsin to 1µg/µl with 50mM acetic acid and store on ice.
7. Add 1µl of 1% ProteaseMAX™ Surfactant and 1.8µl of 1µg/µl trypsin. Incubate at 37°C for 3 hours.

   Note: In our experiments, overnight digestion does not improve proteome coverage.
8. Collect the condensate from tube walls by centrifugation at 12,000–16,000 × *g* for 10 seconds. Add TFA to a final concentration of 0.5% to inactivate trypsin. Mix, and incubate at room temperature for 5 minutes. Mix, and keep the tube on ice, or proceed to peptide concentration and cleanup steps. If the digest is not going to be analyzed within several hours, snap-freeze it in dry ice and store at –20°C.

Peptide Handling

For LC-MS, we recommend centrifuging the digest at 12,000–16,000 × *g* for 10 minutes to minimize introduction of nonpeptide material to a system. Centrifugation will remove particulate material and reduce level of the degraded ProteaseMAX™ Surfactant.

If peptides are cleaned up with SPE, be sure to use a tip with sufficient binding capacity (e.g., 100µl OMIX® tips, Varian Cat.# A5700310). If the volume of the mixture exceeds 100µl or the amount of the protein exceeds 50µg, additional tips will be required. If you have not already done so in Step 8, add TFA to the final concentration of 0.5% before cleanup to ensure sufficiently low pH in the digest.

Table 1. Summary of In-Solution Solubilization/Digestion Reaction Volumes for Membrane Proteins.

Component	Membrane Protein (ProteaseMAX™ Surfactant only for solubilization) (µl)	Membrane Protein (ProteaseMAX™ Surfactant and urea for solubilization) (µl)
0.2% ProteaseMAX™ Surfactant: 50mM NH_4HCO_3 (for solubilization)	20	20
8M urea	—	15
50mM NH_4HCO_3	73.5	58.5
0.5M DTT	1.0	1.0
0.55M iodoacetamide	2.7	2.7
trypsin (1µg/µl)	1.8	1.8
1% ProteaseMAX™ Surfactant (for digestion)	1.0	1.0
Final Volume	**100**	**100**

4.B. In-Solution Digestion Protocol (sample protocol for membrane protein; continued)

Table 2. Summary of In-Solution Solubilization/Digestion Reaction Volumes for Cytoplasmic Proteins.

Component	Cytoplasmic Protein (from pellet) (µl)	Cytoplasmic Protein (from solution) (µl)
0.1% ProteaseMAX™ Surfactant: 50mM NH_4HCO_3 (for solubilization)	20	NA
1.0% ProteaseMAX™ Surfactant: 50mM NH_4HCO_3	—	2.0
50mM NH_4HCO_3	73.5	X
0.5M DTT	1.0	1.0
0.55M iodoacetamide	2.7	2.7
trypsin (1µg/µl)	1.8	1.8
1% ProteaseMAX™ Surfactant (for digestion)	1.0	1.0
Final Volume	**100**	**100**

5. In-Gel Digestion Protocol for Low Protein Amounts

Notes:

1. This protocol is designed to improve mass spectrometer identification of proteins present in gel below detection sensitivity of Coomassie® R-250 (down to about 5–10ng amounts for proteins of average size [30–70kDa]). For higher protein amounts we recommend the in-gel digestion protocol described in Section 4.A.
2. Probability of successful protein identification will vary depending on nature, amount and size of a protein and type of mass spectrometer used. See Figure 5 for an example.
3. This protocol was tested with silver- and Sypro Ruby-stained gels. It is optimized for gel slices of dimension 1–2 × 5–7 × 1mm^3. The protocol was validated with MALDI using an AB 4800 MALDI-TOF/TOF mass spectrometer and LC-MS on Agilent 1100 series nano LC/MSD Ion Trap SL mass spectrometer.

Washing

1. Resolve proteins by gel electrophoresis, and stain the gel. Wash with an appropriate destaining solution to remove nonspecifically bound stain.
2. Excise the protein band or spot of interest. Cut the gel slice onto 1mm^3 pieces, and transfer them to a 0.5 or 1.5ml microcentrifuge tube.

   Note: The protocol is optimized for gel slices of 1–2 × 5–6 × 1mm^3 dimension. If a gel slice is larger, divide it accordingly.

3. Wash with 200µl of NANOpure® water by vortex mixing for 30 seconds. After washing discard the water.

   Note: If many protein bands are being processed, put tubes on an orbital shaker (e.g., LabNet Orbit M60) and mix at 100–140 rpm.

4. Destain with 200µl of methanol:50mM NH_4HCO_3 (1:1 v/v) for 1 minute with intermittent vortex mixing. Discard supernatant. Repeat once.

5. Dehydrate for 5 minutes in 200µl of acetonitrile:50mM NH_4HCO_3 (1:1 v/v) with intermittent vortex mixing. Discard supernatant. Add 200µl of 100% acetonitrile, mix and incubate for 30 seconds. Discard the supernatant.

6. Dry in a Speed Vac® vacuum centrifuge for 5 minutes or until sample is dry.

Reduction/Alkylation

7. Rehydrate in 100µl of freshly prepared 25mM DTT in 50mM NH_4HCO_3 and incubate for 20 minutes at 56°C.

8. Discard supernatant. Add 100µl of freshly prepared 55mM iodoacetamide in 50mM NH_4HCO_3 and incubate in the dark for 20 minutes at room temperature.

   Note: Alkylation with iodoacetamide increases peptide mass by 57.02 for each cysteine.

9. Discard supernatant, and wash with 400µl of NANOpure® water by vortex mixing briefly. Discard supernatant. Repeat once.

10. Dehydrate for 5 minutes in 200µl of acetonitrile:50mM NH_4HCO_3 (1:1 v/v) with intermittent vortex mixing. Discard supernatant. Add 200µl of 100% acetonitrile, mix and incubate for 30 seconds. Discard the supernatant.

11. Dry in a Speed Vac® vacuum centrifuge for 5 minutes or until sample is dry.

Digestion

12. Rehydrate gel pieces in 20µl of 2ng/µl trypsin in 0.01% ProteaseMAX™ Surfactant:50mM NH_4HCO_3 for 10 minutes. Overlay with 10–15µl of 0.01% ProteaseMAX™ Surfactant:50mM NH_4HCO_3 (enough to cover gel pieces) and gently vortex for several seconds.

Notes:

1. The 0.01% ProteaseMAX™ Surfactant solution is made by diluting the 1% ProteaseMAX™ Surfactant solution 100X (see Section 3.B for preparation of the 1% solution) with 50mM NH_4HCO_3. Keep the 0.01% solution on ice until use. The 0.01% solution should be used within several hours.

2. Trypsin concentration is reduced to 2ng/µl (from a typical 10–12ng/µl concentration) to minimize interference from autocatalytic tryptic peptides. This reduction of trypsin concentration does not negatively affect protein digestion in the presence of ProteaseMAX™ Surfactant (the data will be sent upon request). A 2ng/µl trypsin solution is made by dilution of concentrated trypsin solution (for example, 500ng/µl solution) with 0.01% ProteaseMAX™ Surfactant:50mM NH_4HCO_3.

Do not prepare 2ng/µl solution using 50mM NH_4HCO_3 only (without ProteaseMAX™ Surfactant), as trypsin at such a low concentration will be largely absorbed by plasticware in the absence of ProteaseMAX™ Surfactant.

5. In-Gel Digestion Protocol for Low Protein Amounts (continued)

13. Incubate for 3 hours at 37°C. Incubating overnight might cause some decrease in peptide recovery.

    Note: Protein digestion is complete and ProteaseMAX™ Surfactant degrades over the course of the digestion reaction.

14. Collect the condensate from tube walls by centrifuging for 10 seconds at 12,000–16,000 × *g* in a desktop microcentrifuge.

15. Vortex for a few seconds, and transfer the digestion reaction into a new tube.

16. Add 20µl of 2.5% TFA to gel pieces and mix an orbital shaker (e.g., LabNet Orbit M60) at 100 rpm for 15 minutes. If the orbital shaker is unavailable, vortex for 15 minutes.

17. Combine the extract with the digest. Discard gel pieces.

18. Centrifuge the combined solution for 10 minutes at 12,000–16,000 × *g* in a desktop microcentrifuge. Transfer the supernatant into a new tube. Volume of the supernatant is typically between 30 to 40µl. Keep the tube on ice. If the digest is not going to be analyzed within several hours, snap-freeze it in dry ice and store at –20°C.

Peptide Handling

MALDI users: Concentrate peptides with a 10µl (0.6µl bed resin volume) C18 ZipTip® tip (Millipore; aspirate the digest into the tip to the maximum capacity. A 10µl Millipore tip can accommodate 20µl. Bind peptides with ten cycles of aspirating-dispensing).

LC-MS users: Directly inject the supernatant.

6. Appendix

6.A. Composition of Buffers and Solutions

50mM ammonium bicarbonate buffer (pH~7.8)

Dissolve 98.8mg of NH_4HCO_3 in 25ml of NANOpure® water. Prepare the buffer immediately before use. Keep it at room temperature until use.

1M DTT

Dissolve 154.25mg of DTT in a final volume of 1ml of NANOpure® water. Store on ice.

25mM DTT

Dilute the 1M DTT stock solution 40-fold with 50mM NH_4HCO_3 immediately before use.

0.55M iodoacetamide

Dissolve 40.7mg of iodoacetamide in 400µl of 50mM NH_4HCO_3. Prepare immediately before use and store in the dark.

iodoacetamide solution

Dilute 0.55M iodoacetamide solution 10-fold with 50mM NH_4HCO_3 immediately before use and store in the dark.

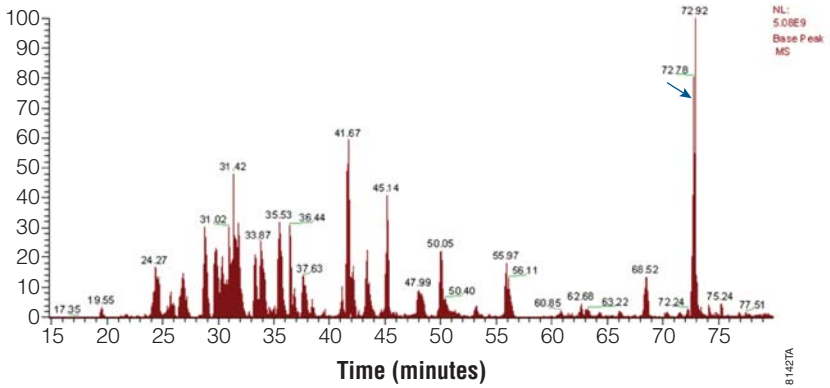
8M urea

Dissolve 480.5mg of mass spectrometry-grade urea crystals in a final volume of 1ml NANOpure® water.

6.B. Related Products

Product	Size	Cat.#
Trypsin Gold, Mass Spectrometry Grade	100µg	V5280
Sequencing Grade Modified Trypsin	100µg	V5111
Sequencing Grade Modified Trypsin, Frozen	100µg	V5113

6.C. Supplementary Figure



Supplementary Figure. LC-MS analysis of ProteaseMAX™ Surfactant-assisted in-gel protein digestion with LCQ Deca XP+/Eksigent 2D nano LC. Base peak chromatogram of in-gel-digested 56kDa protein from mouse membrane protein extract. The protein band was digested with the ProteaseMAX™ Surfactant-assisted protocol (Section 4.A). Particulate material and most of the degraded ProteaseMAX™ Surfactant were removed with a 10-minute centrifugation at 16,000 × *g*, and the digest was analyzed with LCQ Deca XP+/Eksigent 2D nano LC. The arrow indicates the degraded ProteaseMAX™ Surfactant remaining in the digest after centrifugation. The degraded surfactant was washed off the column after one blank run with an acetonitrile/formic acid gradient (data not shown).

Data provided courtesy of Dr. Chris Adams (Stanford University Mass Spec Facility).

7. Summary of Changes

The following changes were made to the 2/15 revision of this document:

1. The instructions for adding TFA were made consistent between Section 4.A, Step 15, and Section 4.B, Step 8.
2. The document design was updated.

[a]Patent Pending.