Invitrosol™ LC/MS Protein Solubilizer

Cat. no. MS10007 **Store at –20°C**

Contents and Storage

The Invitrosol™ LC/MS Protein Solubilizer includes 5 ml of 5X Invitrosol™ LC/MS Protein Solubilizer and 50 µl of BSA standard tryptic digest (0.5 mg/ml in 1X Invitrosol™ solubilizer). Upon receipt, store the Invitrosol™ solubilizer at 4°C, and divide the BSA digest into smaller aliquots and store them at –20°C.

Description

Invitrosol™ LC/MS Protein Solubilizer is a proprietary surfactant blend that has unique binding and elution properties. This surfactant blend keeps a variety of hydrophobic proteins in solution, does not interfere with protease activity, and is also compatible with reverse-phase high-pressure liquid chromatography (RP-HPLC) separations of digested peptides and analysis via LC-coupled electrospray ionization/mass spectrometry (ESI/MS). The formulation includes surfactants that have been individually tested for suppression effects and factors that affect the resolution of peptide peaks eluted from reverse-phase chromatography. The Invitrosol™ LC/MS Protein Solubilizer is formulated to be directly compatible with LC/MS analysis at 1X concentration. The BSA tryptic digest in 1X Invitrosol™ solubilizer is provided for use as a standard to set up and optimize LC/MS methods before analyzing your own samples.

General Recommendations

- Always use ultrapure chemicals and deionized, sodium-free water in all procedures.
- All of these protocols are based on the use of trypsin as the protease for digestion. Other proteases will require buffers and reaction conditions other than those given here. Use empirical knowledge about the characteristics of your protein of interest to optimize procedures and yield.
- We recommend that you check your HPLC column performance and consistency prior to analyzing protein samples. Always condition and equilibrate the column well, especially if it is a new column. Perform a pilot run using the Invitrosol™ LC/MS Protein Solubilizer alone and/or with the BSA tryptic digest as a positive control.
- We strongly recommend the use of formic acid rather than trifluoroacetic acid (TFA) for all procedures. Formic acid is also recommended for the preparation of mobile phases for RP-HPLC (see Analysis of Digested Peptides), especially when directly coupled with ESI/MS instrumentation, as TFA has detrimental effects on ESI/MS.

Materials Needed

Several protocols are provided for the removal of incompatible buffer components and for digestion with trypsin. Since each protocol requires different materials, please read each protocol to ascertain the materials that you will need to have available before starting.

Removal of Incompatible Buffer Components (for Proteolysis in Solution)

These protocols are designed to remove buffer components that are often used for protein solubilization (especially hydrophobic proteins) but are incompatible with RP-HPLC and ESI/MS. These components, such as CHAPS, PEG, glycerol, SDS, and high salt concentrations, also may interfere with protease activity. If MS-incompatible reagents are present in your sample buffer, we suggest one of the following protocols to remove them. As noted in General Recommendations, these protocols are provided as a starting point. Use empirical knowledge about the characteristics of your protein of interest to optimize procedures and yield.

Acetone Precipitation

1. Add cold 80% (v/v) acetone to the protein sample and incubate on dry ice for at least 3 hours.
2. Centrifuge the tube at 14,000 x g at 4°C for 10 minutes.
3. Carefully remove the supernatant. Wash the pellet twice with cold acetone 100%, then air-dry the pellet.
4. Resuspend the pellet in 5X Invitrosol™ LC/MS Protein Solubilizer, using a volume equal to 1/5 of the required final volume for LC column loading. Note: The final volume will be dependent on your LC column's loading capacity. You may add 20% (w/v) freshly deionized urea (up to 10% w/v of the desired final volume in step 7) to facilitate dissolving the pellet. Urea will not interfere with any protease activity.
5. Vortex for 1–2 minutes, then incubate at 60°C for 5 minutes.
6. Vortex for 1–2 minutes, then incubate at 60°C for another 10 minutes or until the pellet is completely dissolved. **If necessary, sonicate for 30 minutes to ensure complete dissolution.**
7. Add 25 mM ammonium bicarbonate to obtain the desired final volume and an Invitrosol™ concentration of 1X.
8. Proceed to In-Solution Digestion with Trypsin.

Removal of Incompatible Buffer Components (for Proteolysis in Solution)—Continued

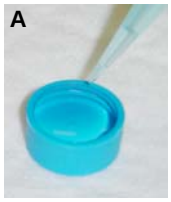
Ultrafiltration Buffer Exchange

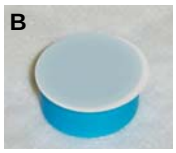
1. Mix your intact protein sample 1:1 (v/v) with 5X Invitrosol™ LC/MS Protein Solubilizer, and incubate at 37°C for 10 minutes.
2. Prepare and wash a Centricon® or similar centrifugal ultrafiltration device, following the manufacturer's directions.
3. Transfer the mixture of protein sample and Invitrosol™ solubilizer to the ultrafiltration device, being careful not to touch the membrane.
4. Centrifuge for 20 minutes at the recommended speed for the ultrafiltration device. Check the volume of the sample (retentate) every 5 minutes to prevent drying out and loss of the retentate.
5. Add 1X Invitrosol™ solubilizer (dilute the 5X Invitrosol™ with 25 mM ammonium bicarbonate) to the concentrated retentate to restore the original volume, and centrifuge again for 20 minutes.
6. Repeat step 5 twice, then continue centrifuging the sample until the retentate (containing the protein) is of the desired volume.
7. Proceed to In-Solution Digestion with Trypsin.

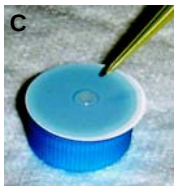
Drop Dialysis Buffer Exchange

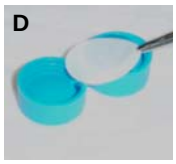
1. Prepare 8 ml of 1X Invitrosol™ solubilizer (1.6 ml of 5X Invitrosol™ LC/MS Protein Solubilizer + 6.4 ml of 25 mM ammonium bicarbonate). This will be the dialysis solution.
2. Mix 10 µl of intact protein sample with 10 µl of 5X Invitrosol™ Protein Solubilizer. Incubate at 37°C for 10 minutes.
3. Place an inverted 15 ml conical tube cap in a clean empty pipette tip box. Fill the cap to the brim with the dialysis solution (~3 ml; Figure A).
4. Using forceps, lay a nitrocellulose dialysis membrane (Millipore cat. no. VSP02500) over the filled cap (the membrane will not fit into the cap; Figure B). Most of the membrane should become wet immediately. If the membrane is not in contact with the dialysis solution, remove the membrane and add more solution to the cap.
5. Pipet the protein/ Invitrosol™ mixture from step 2 onto the center of the membrane (Figure C). Close the lid of the box. Allow buffer exchange at room temperature for 1 hour.
6. After 1 hour, place another inverted tube cap next to the first cap, and fill the second cap with fresh dialysis solution. Using forceps, VERY CAREFULLY slide the membrane over to the second cap (Figure D) so that the sample upon the membrane is not disturbed. Allow buffer exchange at room temperature for 1 hour.

   Note: The final volume (increased by swelling) of the protein sample drop after buffer exchange may vary and is dependent on the solutes present. For example, SDS increases the sample volume 2-3 fold, while glycerol causes a 2-fold increase. To concentrate the protein sample to the original volume, use vacuum drying. Ensure that the dryer trap is free of contamination. Avoid concentrating the sample to less than the original drop volume (20 µl from step 2) or to dryness.
7. Proceed to In-Solution Digestion with Trypsin.









In-Solution Digestion with Trypsin

This protocol may be used to digest an intact protein with sequencing-grade trypsin. The protein sample should have already undergone buffer exchange as described above. We recommend the use of modified trypsin (available from Promega or Roche Applied Science).

1. Reconstitute lyophilized trypsin with 25 mM ammonium bicarbonate, pH 8.0.
2. Add sufficient trypsin to the buffer-exchanged protein for an enzyme-to-substrate ratio between 1:25 and 1:100 (w/w).
3. Incubate the mixture for 12–18 hours at 37°C.
4. Proceed with RP-LC/MS analysis.

In-Gel Digestion with Trypsin

Use gloves, clean or prewashed microcentrifuge tubes, and a laminar flow hood to avoid keratin contamination of your samples.

1. Excise the desired gel band (from a 1D gel) or spot (from a 2D gel), cutting as close as possible to the band/spot to avoid background. Cut the excised gel into small pieces (no smaller than ~1 mm cubes), and transfer them to a clean microcentifuge tube.
2. Add 500 µl of 50% acetonitrile, 25 mM ammonium bicarbonate, pH 8.0. Incubate at room temperature for 15 minutes, then discard the supernatant carefully without removing the gel pieces.
3. Repeat step 2 until the gel pieces are sufficiently destained.
4. Add 200 µl of 100% acetonitrile to dehydrate the gel pieces.
5. Incubate for 5–10 minutes at room temperature, then discard the supernatant carefully without removing the gel pieces.
6. Dry the gel pieces in a clean centrifugal vacuum concentrator (e.g., SpeedVac® centrifuge).
7. Add enough trypsin solution (10 ng/µl dissolved in 25 mM ammonium bicarbonate, pH 8.0, 1X Invitrosol™ solubilizer; stored at 4°C) to cover the gel pieces.
8. Incubate on ice for at least one hour to allow the trypsin to penetrate the gel pieces. The cold temperature will help to prevent autolysis of the trypsin during this time.
9. Incubate overnight at 37°C.
10. Add 25 µl of 5% formic acid (FA), and incubate for 30 minutes at room temperature.
11. Vortex for 30 seconds, centrifuge at 14,000 x g for 1 minute, and collect the supernatant.
12. Add 25 µl of 5% FA, 50% acetonitrile to the pellet, and incubate for 30 minutes at room temperature.
13. Vortex for 30 seconds, centrifuge at 14,000 x g for 1 minute, and collect the supernatant, pooling it with the supernatant from step 11.
14. Concentrate the digested peptides using a SpeedVac® centrifuge.
15. Proceed with RP-LC/MS analysis.

Analysis of Digested Peptides

The digested peptides can be analyzed by reverse-phase high-pressure liquid chromatography/mass spectrometry (RP-HPLC/MS). We recommend 0.1% formic acid (FA) in ultrapure water as mobile phase A, and 0.1% formic acid in acetonitrile as mobile phase B.

1. Inject an appropriate bolus of sample for the column size. For a 4.6 mm ID column, load 10–15 µg of protein digest; for a 100–300 µm ID column, load 0.5–2 pmol.
2. Monitor the absorbance at 254 nm and, if using a multivariable wavelength detector, at 280 nm.
3. Run the appropriate gradient protocol—this must be determined empirically for each protein preparation, but a generic gradient should include a ramp from 5% to 70% acetonitrile in water with 0.1% FA over 50 minutes. The initial offline wash period (if analyzing by ESI-MS) should be at least 10 minutes to allow components of the Invitrosol™ solubilizer that elute in the void volume to pass undetected.

Expected Results

Figures 1-3 show the quality of protein samples and tryptic digests that have been treated with Invitrosol™ LC/MS Protein Solubilizer.

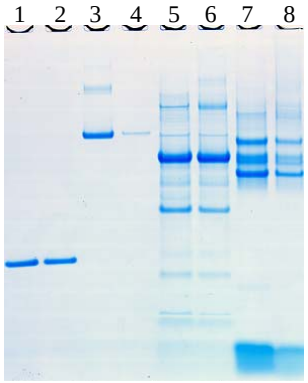


Figure 1. Separation of four protein samples on a 4-12% Bis-Tris NuPAGE® Gel before acetone precipitation, and after reconstituting the precipitated proteins in 1X Invitrosol™ LC/MS Protein Solubilizer. Lane 1: myoglobin; lane 2: myoglobin/Invitrosol™; lane 3: BSA; lane 4: BSA/Invitrosol™; lane 5: P450-2D6; lane 6: P450-2D6/Invitrosol™; lane 7: AChR; lane 8: AchR/Invitrosol™.

Note: Acetone precipitation is not a suitable method for some proteins, such as BSA (see low recovery in lane 4). In such cases, use an alternative method such as drop dialysis.

Expected Results—Continued

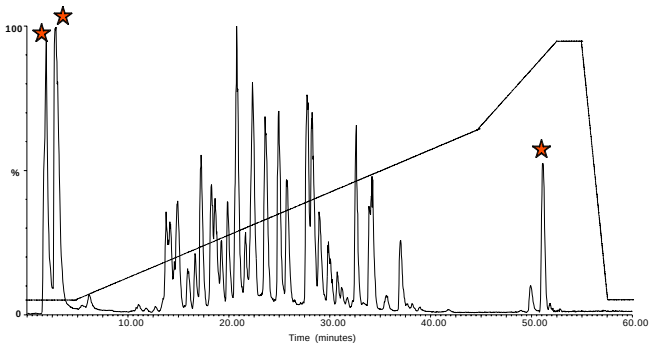


Figure 2. Total ion chromatogram (TIC) of tryptic digest of BSA in the presence of 1X Invitrosol™ solubilizer. Peaks indicated by stars correspond to components of the Invitrosol™ solubilizer. These peaks do not interfere with the peptide peaks.

Column:	Jupiter™ 005_C18 300Å, 250 x 4.6 mm
Flow rate:	0.3 ml/min
Mobile phase:	A: 01% FA in ultrapure water B: 0.1% FA in acetonitrile
Gradient:	A/B (95:5) to (35:65) in 45 minutes A/B (35:65) to (5:95) in 7.5 minutes
Column temp.:	24°C

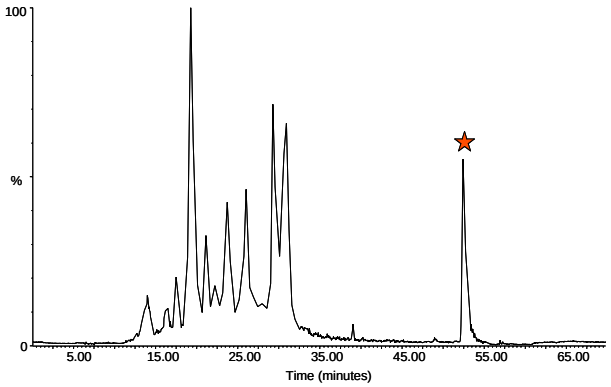


Figure 3. Total ion chromatogram (TIC) of tryptic digest of P450, buffer exchanged with 1X Invitrosol™ solubilizer. The peak indicated by the star corresponds to a component of the Invitrosol™ solubilizer. Components of the Invitrosol™ solubilizer that elute early were removed during a wash cycle applied to a C18 trap cartridge used in this configuration (compare to Invitrosol™ peaks shown in Figure 2).

Troubleshooting

You may find the information below helpful in troubleshooting your experiments. Most problems arise from the column or the gradient. Be sure that your column is well-conditioned and equilibrated.

Problem	Solution
No peaks corresponding to proteins in chromatogram.	• Proteins may have been lost during precipitation or buffer exchange. When performing acetone precipitation, be sure that you do not lose the protein pellet. When performing ultrafiltration or drop dialysis, do not tear the membrane or spill the sample. • Check protein recovery using 1D SDS-PAGE before and after the buffer exchange procedure.
Extra peaks in chromatogram.	• Some proteins are expressed or isolated with additional folding factors or chaperone proteins that improve solubility. Check for protein purity prior to buffer exchange using a simple 2D gel apparatus such as the ZOOM® IPGRunner™ system. • Follow the manufacturer's recommendations for sequencing-grade protease digestion exactly. Not using sequencing-grade protease, digestion for a very long time, or addition of excess protease will cause autolysis. The peptides resulting from autolysis may appear in the LC/MS analysis. • Hydrophobic peptides may have been carried over from a previous run. If the column was previously used for hydrophobic proteins, run one or more blanks until these contaminants are eliminated.
Poorly resolved MS spectra.	• Always check the instrument and method parameters by analyzing the provided BSA digest. • Be sure to remove MS-incompatible buffer components completely. The buffer exchange process may need to be repeated, or an alternative method selected.
Detergent peaks observed near peptide peaks rather than in the void volume.	• Check the HPLC instrument and method for correct setup, including flow and pressure. • Plumb a C18 trap cartridge in-line. • Check the column frits, sample loop, and plumbing for leaks or debris.

Product Qualification

Invitrosol™ LC/MS Protein Solubilizer is qualified at 1X concentration (1:4 (v/v) with ultrapure water) by RP-HPLC and monitoring of the absorbance at 214 nm. After analysis the following criteria are tested and must meet specified values: 1) the relative ratios of the surfactant components; 2) the presence of breakdown products; 3) the presence of sodium-induced clusters.

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