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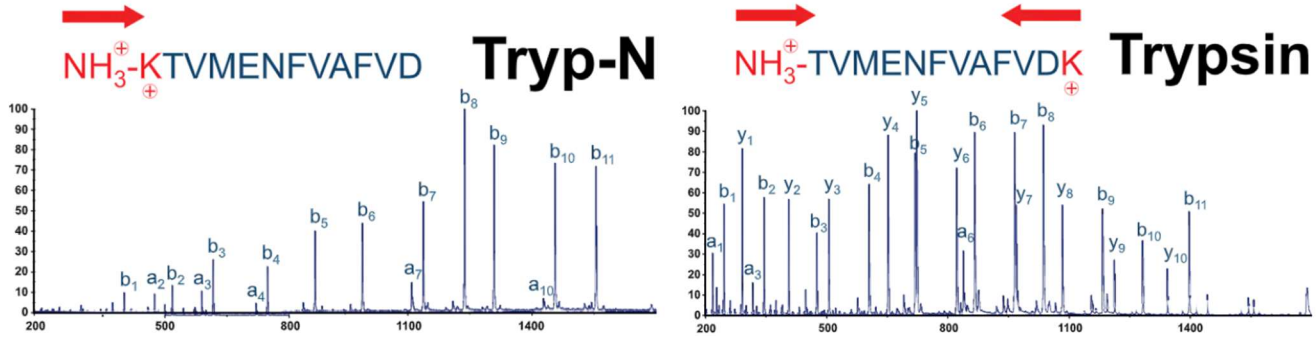
Tryp-N™ Sequencing Grade Kit



Patented Tryp-N™ is a high-performance protease engineered to accelerate protein digestion and enhance sensitivity in LC-MS analyses. Perfect for both routine and challenging samples, it works at higher temperatures (55 – 65 °C) to complete digestion in minutes to not many hours. This breaks down tough proteins while deactivating unwanted enzymes. Its activity can be precisely controlled with EDTA: limited proteolysis is fully reproducible.

Tryp-N™ cuts N-terminally to lysine and arginine residues, generating spectra dominated by b-ions. With up to a 35.4x increase in sensitivity over trypsin, you get cleaner, more accurate data; it's ideal for use in Multiple Reaction Monitoring (MRM) experiments. It produces predominant b-ion fragments, simplifying analysis for deeper protein coverage and post-translational modification studies.

Additional Reagents/Solutions (Not Included)

Liquid Chromatography-Mass Spectrometry (LC-MS) grade is recommended, where applicable

- Protein sample **(1 µg - 100 µg of total protein)**
- LC-MS grade Water
- LC-MS grade Isopropyl Alcohol (IPA)**
- LC-MS grade Methanol (MeOH)
- LC-MS grade Acetonitrile (ACN)
- LC-MS grade Formic Acid (FA)
- (Tris(2-carboxyethyl)phosphine) (TCEP)**
- Methyl Methanethiosulfonate (MMTS)**
- Benzonase® (optional)*
- S-Trap™ Micro Kit Protocol (optional)
- Rapigest™ (Waters, Catalog # 186001861)**
- Chloroform**

*** Benzonase® is susceptible to freeze-thaw cycles.**
**** Need with In-Solution Digestion Processing**

Equipment/Materials (Not Included)

- Single channel pipettes (P2 through P1000) and corresponding tips
- 1.7 mL sample tubes
- Benchtop centrifuge
- Vortex mixer
- Heat block (adjusted to 55 °C)
- pH paper (pH < 1, optional)
- Water bath or humidified incubator (adjusted to 55 °C)
- Lyophilizer or SpeedVac
- Positive pressure apparatus or vacuum manifold (optional)
- Sonicator (optional, recommended)

Contents:

Tryp-N™ Sequencing Grade Enzyme	1 x 10.0 µg 1 x 20.0 µg	50 mM Trimethylammonium Acetate, 2 mM $CaCl_2$, 100 µM $MnCl_2$ in LC-MS grade Water
Tryp-N™ Digestion Solution	1 x 250 µL 1 x 500 µL	100 mM Trimethylammonium Acetate pH 7.4 @ 55 °C, 4 mM $CaCl_2$ and 200 µM $MnCl_2$

RT = room temperature (20-25 °C), v/v = volume-to-volume ratio, w/w = weight-to-weight ratio

Protocol with S-Trap™ Micro Kit Processing:

Refer to Troubleshooting Tips.

1. **Denature Protein.** To 11.5 µL of liquid sample, add 11.5 µL of **Solution 1 (2x Lysis Solution)**. For solid samples, dilute **Solution 1 (2x Lysis Solution)** to 1x by adding an equal volume of LC-MS grade water. Add 23.0 µL of 1x Solution 1 to a solid sample. Sonicate or vortex to fully solubilize and denature proteins
2. If sample is viscous due to the presence of DNA, sheer it thoroughly by probe sonication or enzymatically with a nuclease such as Benzonase™ (see Appendix D). Unsheared DNA will clog the protein trap.
3. Clarify sample as desired by centrifugation (e.g. 13,000 x g for 8 minutes). Transfer clarified lysate to tubes. Pellet can be analyzed separately (see Appendix D).
4. **Reduce.** Add 1.0 µL of **Solution 2 (Reductant)**. Vortex briefly and incubate at 55 °C for 15 minutes.
5. **Alkylate.** Add 1.0 µL of **Solution 3 (Alkylator)**. Vortex briefly and incubate at RT for 10 minutes.
6. While the sample is incubating, add 8.1 mL of LC-MS grade MeOH to a bottle of **Solution 5 (Binding/Wash Solution)**. Mix thoroughly.
7. **Acidify.** Add 2.5 µL of **Solution 4 (Acidifier)** to the sample. Vortex briefly. pH paper can be used to ensure pH ≤ 1. Proceed to the next step immediately.
8. Add 165 µL of **Solution 5 (Binding/Wash Solution) with MeOH added** to the sample. Mix thoroughly.
   Note: *The sample may appear translucent at this step due to colloidal protein formation. Do NOT centrifuge.*
9. Place an **S-Trap™ Micro Spin Column** in a 1.7 mL sample tube for waste flow-through. Transfer the entire sample, including any insoluble material, to the top of the column.
   Note: *The column reservoir can hold ~275 µL of solution. For larger volumes, the column can be loaded multiple times with the solution from Step 8. When transferring into the column, do not disturb the matrix.*
10. **Trap Protein.** Centrifuge the **S-Trap™ Micro Spin Column** and waste flow-through tube at 10,000 x g for 30 seconds. Visually confirm that all solution passed through the column. If not, centrifuge again until no liquid remains. If the solution still hasn't passed through, the column may be centrifuged as high as 15,000 x g until all solution passed through. Discard flow-through and return the column to the 1.7 mL sample tube.
    Note: *The S-Trap™ Micro Spin Columns contain end in a Luer taper. A vacuum manifold or positive pressure can be used to draw solution through the column.*
11. **Clean Protein.** Add 150 µL of **Solution 5 (Binding/Wash Solution) with MeOH added** to the **S-Trap™ Micro Spin Column** and centrifuge at 10,000 x g for 30 seconds. Visually confirm that all solution passed through the column. Repeat washes 3 times; discard flow-through as necessary. Return the column to the 1.7 mL sample tube.
    Note: *For best results, rotate the column 180° between centrifugations. Marking the outside edge of the column makes it easier to track rotations.*
12. Centrifuge the **S-Trap™ Micro Spin Column** at 10,000 x g for 1 minute to fully remove all Binding/Wash Solution.
13. Transfer the protein-containing **S-Trap™ Micro Spin Column** to a clean 1.7 mL sample tube for digestion.
14. **Tryp-N™ Enzyme Solution for 100 µg Protein.** In a clean 1.7 mL sample tube, add 10.0 µL **Tryp-N™ Digestion Solution** and 6.0 µL LC-MS Grade Water followed by 4.0 µL **Tryp-N™ Sequencing Grade Enzyme** for a final volume of 20.0 µL, ensuring that the amount of Tryp-N™ in this solution is at a 1:50 (w/w) ratio with the total amount of protein in the sample (e.g. 2 µg per 100 µg of sample). The amount of Tryp-N™ will need to scaled for samples other than 100 µg. Tryp-N™ is supplied at a concentration of 0.5 mg/mL.
15. Transfer the entire 20.0 µL of Tryp-N™ Enzyme Solution to the **S-Trap™ Micro Spin Column**. The matrix is hydrophilic and will absorb the solution; no centrifugation is necessary.
    Note: *If bubbles are present, flick the tube gently to remove them and/or spin the column very briefly using a benchtop centrifuge. If any solution flows through, pipette it back on top of the column.*
16. **Incubate & Digest.** Loosely screw the cap on the **S-Trap™ Micro Spin Column** to limit evaporative loss so that the Tryp-N™ Enzyme Solution is not pushed through the column due to thermal expansion; allow air to escape. Pipette any solution that passes through back on top of the column. Place the column and sample tube in a 55 °C water bath or humidified incubator for 3 hours. If using a water bath, make sure the column matrix sits below the water level. Do NOT shake.
17. **Elute 1.** Remove the **S-Trap™ Micro Spin Column** with sample tube from the incubator and add 40.0 µL of **Solution 6 (Digestion Solution)** to the top of the column. Centrifuge at 10,000 x g for 1 minute.

18. **Elute 2.** Add 40.0 µL of **Solution 7 (Elution Solution)** to the top of the **S-Trap™ Micro Spin Column**. Centrifuge at 10,000 x g for 1 minute.
19. **Elute 3.** If hydrophobic peptides are of interest, add 40.0 µL of 50% (v/v) LC-MS grade ACN in 0.2% (v/v) LC-MS grade FA (solution not provided) to the top of the **S-Trap™ Micro Spin Column**. Centrifuge at 10,000 x g for 1 minute.
20. Lyophilize or SpeedVac eluted peptides collected from Steps 17-19 in the sample tube. Resuspend as needed for subsequent analysis (e.g. Aqueous Buffer A such as 5% ACN, 0.1% FA).

Protocol with In-Solution Digest Processing

Refer to Troubleshooting Tips.

1. Prepare a sample containing 1-100 µg of protein in 48.0 µL buffer at a pH between 6.5 and 8.5; LC-MS grade Water can be added to bring up the volume.
2. **Reduce.** Add 1.0 µL of 120 mM TCEP in LC-MS grade Water. Vortex briefly and incubate at 55 °C for 15 minutes.
3. **Alkylate.** Add 1.0 µL of 500 mM MMTS in LC-MS grade IPA. Vortex briefly and incubate at RT for 10 minutes.
4. **Precipitate Protein.**
    i. Add 400 µL of LC-MS grade MeOH and vortex thoroughly.
    ii. Add 100 µL of chloroform and vortex.
    iii. Add 350 µL of LC-MS grade Water. The mixture will become cloudy with precipitate. Vortex.
    iv. Centrifuge at 14,000 x g for 5 minutes. Three layers will result: an aqueous layer on top, a circular flake of protein in the interphase, and a chloroform-rich layer at the bottom.
    v. Remove and discard top aqueous layer carefully, avoiding disruption of the delicate protein interface.
    vi. Add 400 µL LC-MS grade MeOH and gently rock back and forth to mix the phases. Avoid extensive vortexing as this will break apart the protein interphase, making recovery more difficult.
    vii. Centrifuge at 20,000 x g for 5 minutes to force the flocculent precipitate into a pellet in the bottom of the tube.
    viii. Remove as much MeOH as possible; exercise care and caution as the pellet is delicate and easily disturbed. You should be able to remove all but a few µL of MeOH with care, which will speed drying.
    ix. Air dry until the pellet just starts to "shrink" at the edges. The ideal dryness is reached when the MeOH is almost fully evaporated, however the pellet is still translucent/white, and NOT clear, which indicates over-drying.
5. **Tryp-N™ Enzyme Solution for 100 µg Protein.** Add 10.0 µL **Tryp-N™ Digestion Solution** and 1.0 µL of 1% Rapigest™ (the final concentration will be 0.05% during the digestion). Sonicate and vortex as needed to fully solubilize the pellet. Once in solution, 5.0 µL LC-MS Grade Water followed by 4.0 µL **Tryp-N™ Sequencing Grade Enzyme** for a final volume of 20.0 µL, ensuring that the amount of Tryp-N™ in this solution is at a 1:50 (w/w) ratio with the total amount of protein in the sample (e.g. 2 µg per 100 µg of sample). The amount of Tryp-N™ will need to scaled for samples other than 100 µg. Tryp-N™ is supplied at a concentration of 0.5 mg/mL.
6. **Incubate & Digest.** Cap the tube and place in a 55 °C water bath or humidified incubator for 3 hours. Do NOT shake.
7. Optional: Stop digestion with the addition of 2.5 – 5 mM EDTA (final concentration).
8. As needed, cleave the MS-compatible detergent. For example, for an acid-cleavable detergent like Rapigest™, reduce the pH to ~2 – 3 by the addition of TFA or FA and incubate for at least 30 minutes at 37 °C.
9. Dry down sample to remove volatile buffer.
10. Resuspend sample as appropriate (e.g. in HPLC buffer A or in MALDI matrix solution) ideally with sonication, centrifuge out any insoluble matter, and take the supernatant for analysis.

Troubleshooting Tips

Problem	Possible Causes and Solutions
Protein precipitation during reduction and alkylation	Especially disulfide rich proteins can precipitate during reduction. Add chaotropes or MS-compatible detergents to keep proteins in solution.
Protein precipitation during digestion	**1.** The proteins of mesophilic organisms (those that grow in moderate temperatures) can undergo thermal precipitation at elevated temperatures. Solution 1: perform the digest at a lower temperature. Although the T_{opt} of Tryp-N™ is 65 °C, it performs well at 55 °C, a common temperature for reduction where thermal precipitation is not typically observed. Solution 2: add a MS-compatible, pH cleavable detergent like Rapigest™. **2.** Some proteins will precipitate in the presence of metals. Add a MS-compatible detergent such as Rapigest™.
Incomplete digestion	**1.** Metals must be present in the protein to digest before Tryp-N™ is added. Add digestion buffer or metals before adding Tryp-N™. **2.** Chelators (such as from EDTA containing protease inhibitors or also metal binding proteins) can inhibit Tryp-N™ activity. We recommend buffer exchange with, for example, a Pierce Zeba column or by precipitation in such cases. EDTA can also be overcome by adding sufficient additional calcium. **3.** Proteins may be poorly soluble or very compact. To remedy this, add an MS-compatible acid-cleavable detergent. pH-sensitive detergents are recommended over those that auto-hydrolyze (e.g. Pmax) due to the increased digestion temperature. **4.** At higher temperatures especially with shaking, the protein solution has a tendency to dry on the walls of sample tubes where it will remain undigested. Water baths are recommended, especially for small sample volumes.
Poor digestion specificity	**1.** Specificity can degrade during long digestions even at reduced temperatures. Reduce digestion time and/or amount of added Tryp-N™. **2.** The pH drift of buffers between room temperature and elevated digestion temperature can be significant and adversely affect specificity. pH buffers at the temperature of digestion.