Recommended use of Tryp-N™

The recommended **digestion buffer** is:
- 25 – 50 mM MS compatible acetate buffer, pH 7.4 at digestion temperature
- 2 mM $CaCl_2$
- 100 µM $MnCl_2$ ($ZnCl_2$ can also be used)
- 0.05% – 0.1% MS compatible pH cleavable detergent (not included; Waters RapiGest™ is recommended).

The recommended **digestion temperature** is 55 ºC. This can be optimized.

The recommended **digestion time** is 3 hrs. Significantly longer digestion times can result in loss of cleavage specificity. Depending on sample, shorter incubations times may be advantageous. This can be optimized.

The recommended **amount of protease to add** is 1:50 wt/wt (2% wt/wt or 1 µg Tryp-N per 50 µg protein to digest. This can be optimized.

Recommended digestion protocol

1) Reduce and alkylate your protein using standard protocols. Ensure all reducing reagent is removed prior to using Tryp-N (e.g. precipitate or use S-Trap™ sample processing see www.protifi.com/s-trap): reducing agents change the redox state of metals necessary for the catalytic activity of Tryp-N™ and can result in loss of specificity.

2A) If protein has been precipitated, resuspend the protein pellet in digestion buffer at around 1 mg/mL (sonicate and add detergent as needed). Verify buffer pH at digestion temperature before proceeding with digestion.

2B) If the protein is in solution, concentrate as needed, add detergent, then add 2x digestion buffer to 1x.

> **IMPORTANT NOTE 1:** reliable Tryp-N digestion requires a mass-spec compatible detergent especially Rapigest™.

3) Add **2% wt/wt (1:50)** of Tryp-N to the protein solution.

> **IMPORTANT NOTE 2**: the order of addition is important. Metals must be present in the protein to digest before protease is added. Add detergent first, then any additional buffer and metals, then Tryp-N.

4) Incubate for **3 hr at 55 ºC** without shaking (to prevent sample from drying on tube walls) optimally in a water bath.

5) Stop digestion with the addition of 2.5 – 5 mM EDTA (final concentration).

6) As needed, cleave the mass spec compatible detergent. For example for an acid cleavable detergent like Rapigest, reduce the pH to ~2 – 3 by the addition of TFA or formic acid and incubate at least 30 min at 37 ºC to cleave detergent.

6) Dry down sample to remove volatile buffer.

7) 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.

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Notes

Note 1: Optimize temperature, amount of added protease, detergent concentration and time for your protein of interest. Monitor by mass spectrometric and SDS-PAGE analysis.

Note 2 (IMPORTANT): The order of addition is important. Metals must be present, either added through the included digestion buffer or otherwise, before Tryp-N is added. Deviation from this order can result in failure to digest.

Note 3 (IMPORTANT): Detergent should be the first addition to protein to digest to prevent precipitation by metal chelation or thermal denaturation. Reliable Tryp-N digestion requires a mass-spec compatible detergent like Rapigest™ (even at 0.01%) or the addition of another denaturant such as 20% acetonitrile or isopropanol.

Note 4: The pH drift of digestion buffers when the temperature is raised from room temperature to the temperature of digestion is usually substantial and can adversely affect enzyme specificity. If you make your own buffer, make sure to pH the digestion buffer at the reaction temperature.

Note 5: Ammonium, trimethylammonium or triethylammonium acetate are all preferred buffers as they are volatile and mass spec compatible. Ammonium buffers are of course incompatible with amine labeling reagents such as iTRAQ or TMT. We recommend ammonium acetate when labeling will not be used and trimethyl- or triethylammonium acetate when labeling will be used.

Troubleshooting

Problem	Possible cause and solution
Protein precipitation during reduction and alkylation	**1.** 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 ºC, it performs well at 55 ºC, a common temperature for reduction where thermal precipitation is not typically observed. Solution 2: add a mass spec compatible, pH cleavable detergent like Rapigest. **2.** Some proteins will precipitate in the presence of metals. Add a mass spec 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 the protease. **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 protease. **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.