

PREOMICS

iST Technology

Enhanced Proteome Coverage Using iST with Orthogonal Proteases

This Technical Note demonstrates the compatibility of the *iST* workflow with orthogonal proteases, enabling expanded proteome coverage for applications such as peptide mapping and dark proteome exploration.

Key Takeaways

› **Easy-to-use, one-day protocol**

Fast and streamlined proteomics sample preparation in a single day.

› **Compatibility with a wide range of proteases**

Supports Try/Lys-C, Arg-C, Asp-N, Glu-C, and ProAlanase with minor workflow modifications.

› **Versatile workflow for enhanced coverage**

Seamlessly integrates alternative proteases to enhance sequence coverage and proteome insights.

Enhanced Proteome Coverage Using iST with Orthogonal Proteases

Chandni Natalia Kumar^{1,2}, Zehan Hu³, Chloé Moritz³

¹ Biomedical Center Munich, Division of Molecular Biology, Faculty of Medicine, LMU Munich, Planegg/Martinsried, Germany

² Biomedical Center (BMC), Protein Analytics Core Facility, Faculty of Medicine, LMU Munich, Planegg/Martinsried, Germany

³ PreOmics GmbH (Part of Biognosys Group), Planegg/Martinsried, Germany

Keywords

iST
Endoproteases
Sample Preparation
Proteomics
Peptide Mapping
Dark Proteome

Since its release in 2016, the *iST* kit has helped standardize and simplify proteomics sample preparation workflows. Trypsin remains the most widely used protease in bottom-up proteomics, enabling robust and reproducible protein identification across diverse sample types. However, certain biological questions require complementary peptide information that cannot be obtained through Trypsin/LysC (Try/Lys-C) digestion alone.

In this Technical Note, we demonstrate how the *iST* workflow can be combined with orthogonal proteases to expand the range of applications that can be addressed, including peptide mapping and dark proteome exploration.

Technical Details

	Product Specifications	Description / Values
<i>Kit</i>	iST 8x	1–8 samples
<i>Input</i>	HeLa Cell Pellet	1x10 ⁶ cells
<i>Alternative Enzymes</i>	Glu-C	Promega (Ref V1651)
	Arg-C	Promega (Ref V1881)
	Asp-N	Promega (Ref V1621)
	ProAlanase	Promega (Ref VA2171)

Table 1. Endoproteases experimental parameters used to digest HeLa cell pellet samples

Enzyme	Final Concentration	Resuspension Buffer	Activation Buffer
Glu-C	0.01 µg/µL	50 mM AMBIC, pH 7.5	NA
Arg-C	0.01 µg/µL	20 mM AMBIC, pH 7.5, 50 mM Tris-HCl, pH 7.5, 5 mM CaCl ₂ , 2 mM EGTA	50 mM AMBIC, 50 mM DTT, 2 mM EGTA (10X Stock)
Asp-N	0.002 µg/µL	10 mM Tris-HCl, pH 8.0, 50 mM AMBIC	NA
ProAlanase	0.01 µg/µL	Provided by manufacturer	NA
Try/Lys-C mix from the Kit (DIGEST)	NA	RESUSPEND buffer	NA

Materials and Methods

Four orthogonal proteases were compared with the classical Try/Lys-C mix provided in the *iST* kit. The alternative enzymes were prepared and used according to the provider recommendations (Table 1). The *iST* protocol was followed without adaptations unless otherwise specified.

HeLa cells (1×10^6) were lysed, and proteins were denatured, reduced, and alkylated in *iST* LYSE buffer. For each protease, a digestion reaction was performed by adding 50 µL of the corresponding resuspended enzyme solution, followed by incubation for 3 h at 37 °C. Enzymes were activated where required, as indicated in Table 1. Enzymatic activity was stopped by addition of STOP solution. For ProAlanase, enzymatic activity was terminated by heat inactivation (10 min at 95 °C) before adding STOP solution.

Peptides were then loaded onto *iST* cartridges, cleaned, and eluted following the standard *iST* protocol. After peptide drying and resuspension, one fifth of each sample corresponding to an injection amount of 150–200 ng of total peptide was analyzed on an Ultimate 3000 Dionex (ThermoFisher Scientific)–timsTOF Pro (Bruker) using a 50 min gradient at 300 nL/min on a *PepSep Classics* column (25 cm × 75 µm, 1.5 µm) in diaPASEF mode. Data were processed in *Spectronaut* (v20) using directDIA for diaPASEF.

Results and Discussion

To evaluate whether the *iST* workflow can be extended beyond standard Try/Lys-C digestion, orthogonal proteases were compared with respect to protein identifications and missed-cleavage rates. Identification depth varied considerably among proteases, whereas the sharing-degree analysis showed that most protein IDs were detected by multiple proteases (Figure 1A). Try/Lys-C generated the largest protein-ID set, yielding 7,553 distinct canonical protein accessions, of which 5,109 were identified in at least three protease datasets. Asp-N and Arg-C identified 6,621 and 5,174 proteins, respectively, followed by Glu-C with 3,951 and ProAlanase with 1,679.

Protein-ID overlap analysis showed that a substantial proportion of proteins was identified across multiple proteases, while additional protein groups were contributed by specific protease combinations and individual enzymes (Figure 1B). Across all five proteases, a total of 8,518 protein groups were detected. The UpSet analysis revealed that the largest intersection contained 1,759 protein IDs shared by Arg-C, Asp-N, Glu-C, and Try/Lys-C. Additional intersections were specific to smaller protease combinations or individual proteases, indicating that each digestion strategy sampled a distinct subset of the detectable proteome.

Missed-cleavage profiling provided an additional measure of digestion efficiency (Figure 1C). Try/Lys-C showed the most complete digestion, with 89.8% of distinct peptide sequences containing no missed cleavage. Asp-N and Arg-C also performed well, with 75.1% and 71.9% zero-missed-cleavage peptides, respectively.

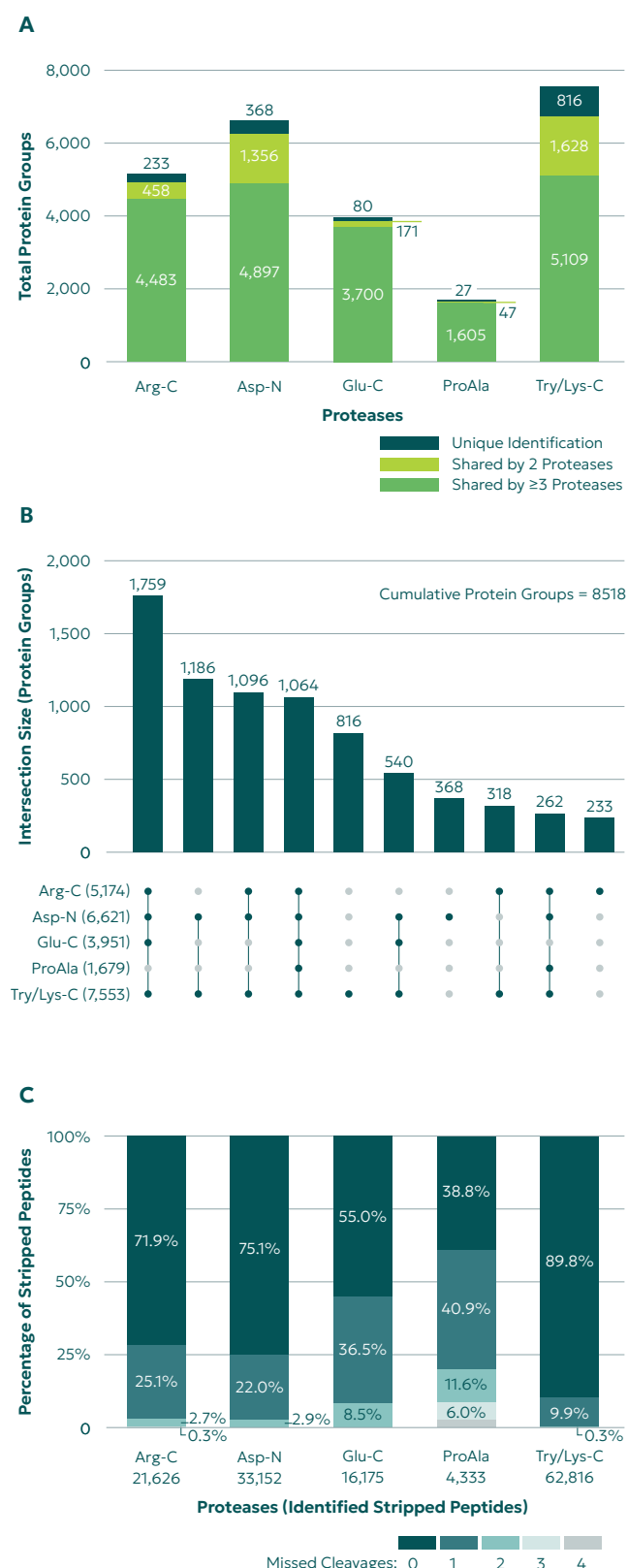


Figure 1. Protein identifications, protein-ID overlap, and digestion quality across proteases. (A) Per-protease protein IDs partitioned by sharing degree. (B) Protein-ID overlap shown as an UpSet plot; the 10 largest intersections are displayed. (C) Missed-cleavage composition by protease.

In contrast, Glu-C exhibited a larger fraction of peptides containing one missed cleavage, while ProAlanase showed the highest missed-cleavage burden, with only 38.8% of peptides free of missed cleavages and a substantial proportion containing two to four missed cleavages.

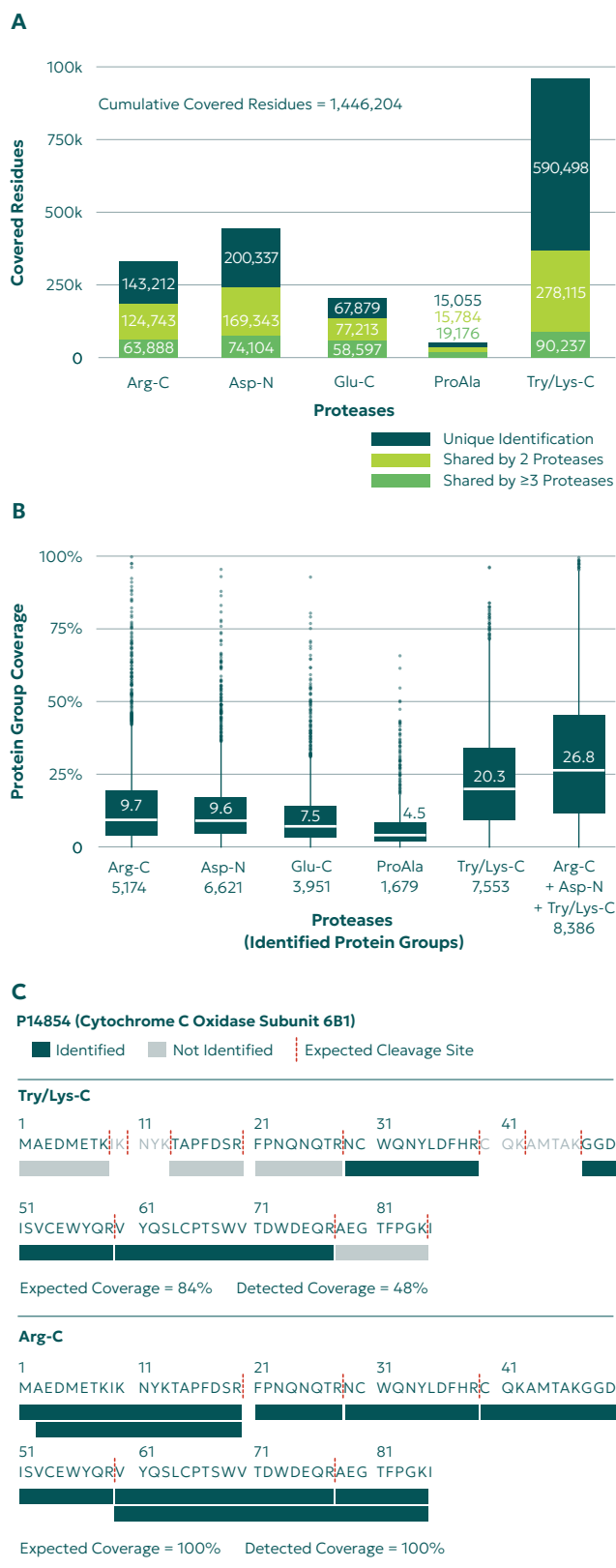
The elevated missed-cleavage rate observed for ProAlanase is consistent with previous reports and therefore does not appear to be specific to the *iST* workflow. Samodova et al. similarly reported substantial missed-cleavage frequencies in ProAlanase-digested proteome samples.¹ While this characteristic makes ProAlanase less suitable for global discovery proteomics, the enzyme may still offer value for targeting specific protein classes or for applications focused on maximizing sequence coverage through complementary protease strategies.

To assess protease complementarity at the residue level, covered amino acid positions were partitioned according to their degree of sharing among proteases (Figure 2A). Arg-C, Asp-N, and Try/Lys-C contributed the largest covered-residue sets, covering 331,843, 443,784, and 958,850 amino acid positions, respectively.

Importantly, each protease contributed a substantial number of unique residues that were not identified by the other proteases, highlighting their complementary cleavage specificities. Because Arg-C, Asp-N, and Try/Lys-C provided both the largest residue sets and the highest numbers of unique covered positions, their peptide evidence was combined to estimate cumulative sequence coverage.

The union of residues identified by these three proteases increased the median protein sequence coverage to 26.8% across 8,386 protein groups, compared with 20.3% for Try/Lys-C (7,553 PGs) alone and 4.5% for ProAlanase (1,679 PGs) (Figure 2B). Together, these results demonstrate that the compatibility of the *iST* workflow with different proteases enables expanded protein sequence coverage.

At the individual-protein level, P14854 (Cytochrome c oxidase subunit 6B1) illustrates how orthogonal cleavage specificity can improve sequence coverage for selected targets. In this example, Try/Lys-C leaves gaps because several predicted peptides are too short (<7 amino acids) for reliable recovery or identification, whereas Arg-C generates longer peptides that span these regions, enabling 100% sequence coverage.



Conclusions

The *iST* kit provides a standardized, rapid, and easy-to-use solution for LC-MS-based proteomics sample preparation. Here, we show that the workflow can be adapted to proteases with different cleavage specificities under the conditions tested. Although identification depth and sequence coverage differed by enzyme, Arg-C and Asp-N added complementary residue evidence to Try/Lys-C in the cumulative residue-union analysis. Overall, these findings expand the range of applications for the *iST* kit and support its use in peptide mapping, dark proteome exploration, and workflows where specific sequence coverage is beneficial.

References

- 1 Samodova, D. et al. Mol. Cell. Proteomics. 2020, 19 (12), 2139-2157.

Figure 2. Residue-level and protein-level sequence coverage. (A) Per-protease covered amino-acid positions partitioned by sharing degree. (B) PG.Coverage (Global) distribution for individual proteases and the cumulative Arg-C + Asp-N + Try/Lys-C combination. (C) Try/Lys-C and Arg-C cleavage-site coverage for P14854 Cytochrome c oxidase subunit 6B1.

Product	Manufacturer	Product Code
<i>iST 96x HT DV</i>	PreOmics GmbH	P.O.00198
<i>PepSep Classics (25 cm × 75 μm × 1.5 μm)</i>	Bruker Switzerland AG	Art.-Nr. 1893484
<i>Spectronaut</i>	Biognosys AG	support@biognosys.com

Ordering information

www.preomics.com/quote
order@preomics.com

Spectronaut

biognosys.com/software/spectronaut

Biognosys Group Technical Note — Enhanced Proteome Coverage Using *iST* with Orthogonal Proteases (V.1)
For research use only | Not for use in diagnostic procedures

biognosysgroup.com