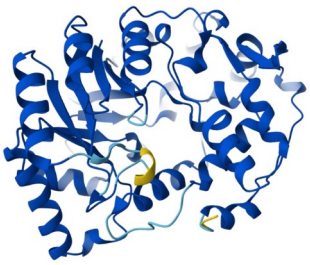
CinderBio

Version 1.4 – April 2026

PROTOCOLS FOR HYPER THERMOACIDIC ARCHAEAL PROTEASES (HTA-PROTEASES©)



Next Generation Proteases

Before using our enzymes, please read the entire protocol.

For effective HTA-protease proteomics performance:

DO NOT freeze HTA-Proteases
DO NOT use UREA or other reactive additives
DO NOT deviate from reaction pH of 3.0 (+/- 0.5 pH units)
DO NOT allow microbial contamination of enzyme aliquots

INTRODUCTION

The enzymes provided are ready-to-use next generation proteases and do not require any preparation. The reaction conditions of 80-90°C and pH 3.0 are sufficient to denature target proteins and prevent disulfide bond reformation allowing for simple, rapid, and effective proteomic analyses. These enzyme solutions should be stored aseptically at ambient laboratory conditions for up to 2 years without loss of activity and used according to guidelines herein.

SAMPLE PREPARATION

DO NOT USE UREA in your sample preparation as peptide/urea adducts form at pH 3 and 80°C and these adducts will impede data analyses. Samples do not require treatment with urea, guanidinium chloride, or other chaotropes or alkylating agents. The reaction conditions of 80-90°C and pH 3.0 are sufficient to denature target proteins and prevent disulfide reformation. This allows for simple, rapid, and effective sample preparation for proteomic analyses.

HTA-Proteases function optimally at pH 3.0 in 1x to 5x (60-300mM) phosphate/citrate buffer provided as a 10x stock (600mM). If your sample is buffered to a pH other than 3.0 **it is critical to establish a reaction pH of 3.0**. We recommend overwhelming the resident sample buffer with the provided phosphate/citrate pH 3.0 buffer to ensure a final reaction pH of 3.0 (+/- 0.5 pH units). **HTA-Proteases will function optimally at pH 3.0 in the phosphate/citrate buffer provided.** Additionally, our enzymes are tolerant of typical surfactants/detergents, including SDS and DDM, up to a 0.5% (w/v) concentration without observable diminishment in performance in most cases.

ENZYME DOSAGE

The completeness of proteolytic digestion for bottoms-up proteomics samples of varied origins is a function of 1) enzyme/substrate ratio (dose), 2) time of digestion, and 3) maintaining optimal digestion conditions (pH 3.0, 80-90°C, 5mM TCEP). Depending on your sample and your experimental goals these parameters can be varied to suit diverse workflows.

1 Unit of activity is defined as the amount of enzyme that will release 1 micro-mole of tyrosine equivalents at 80°C, pH 3.0, in 15-minutes with a hemoglobin substrate.

Krakatoa Dosage vs. Vesuvius Dosage: Approximately **3 times more Vesuvius activity is required as compared to Krakatoa** per unit-mass sample for comparable results. The enzyme concentration as packaged already reflects this difference in activity, so 1 μL of Vesuvius has 3 times the activity of 1 μL of Krakatoa. We have found that a time range of 5-60 minutes and an

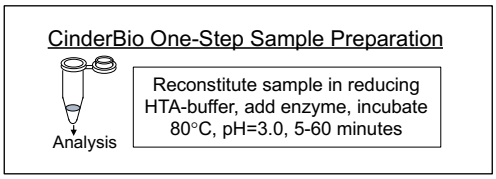
enzyme dose of **0.2-2.0 Units of Krakatoa** or **0.6-10 Units of Vesuvius** per microgram of substrate yields excellent results across a variety of protein samples. Digests over 60 minutes consistently show more chemical cleavage at aspartic acids (D) and can reduce protein identifications in some cases. To decrease incubation time, we suggest increasing your enzyme dose. See the Table below for recommended starting dosage based on experimental data:

Sample Type	Krakatoa Dose	Vesuvius Dose	igestion Time
Whole Blood, Serum, and Plasma[2]	2U/ug	6U/ug	15-60 minutes
Whole Cell Extracts (K562)[1]	0.2U/ug	0.6U/ug	15-60 minutes
Whole Cell Pellets[1]	0.2U/ug	0.6U/ug	15-60 minutes
Histones[1]	0.2U/ug	0.6U/ug	15-60 minutes
Antibody (De Novo)[3]	2U/ug	10U/ug	10-30 minutes

Note: We recommend starting with a simple digest of your samples according to the guidelines herein and optimize dose and time to suit your experiments and optimize your results. The recommended digestion times were established with reactions at 80°C for the noted time intervals and temperature ramping is not considered. Thermocyclers, preheated heat-blocks, water baths, or preheated ovens should be used to maintain a constant 80°C. While very simple, HTA-Protease methods and enzymes are unlike traditional methods and chemical reactivity must be considered at these temperatures and acidic pH. If you have technical questions or encounter any issues, we are happy to offer scientific support at info@cinderbio.com with any questions.

QUICK-START HTA-PROTEASE IN SOLUTION SAMPLE DIGESTION PROTOCOL

1) Reconstitute protein sample in 1-5x provided pH3 Protease Buffer and add 5mM TCEP.
2) Add Krakatoa at 0.2-2.0 Units/1µg substrate or Vesuvius at 0.6-6.0 Units/1µg substrate and mix.
3) Incubate reaction at 80°C for 30-minutes in a pre-heated block or PCR thermal cycler.
4) Quench reaction on ice and analyze immediately or store cryogenically until analysis.



NOTES: Reaction pH of 3.0 is critical for activity. Chaotropes are not needed and will chemically react with peptides diminishing PSMs. Standard cleanup methods are compatible with HTA digests but often not needed. Avoid water condensation on tube lid by heating entire tube.

MATERIALS REQUIRED

Proteomics Grade HTA-Protease (Krakatoa and/or Vesuvius) (provided)
10x HTA-Protease Buffer: 200mM K_2HPO_4, 400mM citric acid, pH 3.0 (provided)
Reductant: 5mM reaction-compatible (pH 3.0) reductant (i.e. TCEP) (not provided)
Low protein-binding tubes: (HTA-Proteases may adhere to some tubes) (not provided)
Alkylating agent: (**NOT NEEDED**, pH 3.0 inhibits disulfide bond reformation) (not needed)
Chaotropes: (**DO NOT USE**, Urea will adduct peptides and can severely impede data analysis)

ADDITIONALTECHNICAL RESOURCES

The 80°C temperature and pH 3.0 of HTA-Protease reaction conditions denature sample proteins and disrupt cellular assemblies and structures. Whole blood, whole cells, as well as extracts have all shown excellent performance with this simple and rapid protocol. The reaction pH of 3.0 severely inhibits disulfide re-formation and therefore blocking cysteines is not necessary.

HTA-Proteases have shown compatibility with the use of various Protein Aggregation Capture (PAC) methods. Our enzymes have demonstrated compatibility with S-Trap as well as acid-compatible beads from ReSyn Biosciences (MR-HYP-MagReSyn Hydroxyl Acid Stable) for SP3 methodologies.

Please refer to our publications for detailed experimental methods and established HTA-protease characteristics and applications (New advances available at cinderbio.com/publications).

HTA-Proteomics Introduction: Various MS systems, samples, and analytical software tools. PSM and ID comparisons of early HTA protocols to mature tryptic protocols.

Ref-1 McCabe MC, Gejji V, Barnebey A, Siuzdak G, Hoang LT, Pham T, Larson KY, Saviola AJ, Yannone SM, Hansen KC. "From volcanoes to the bench: Advantages of novel hyperthermoacidic archaeal proteases for proteomics workflows" J Proteomics. 2023;289:104992. Epub 2023/08/28. doi: 10.1016/j.jprot.2023.104992. PubMed PMID: 37634627. (https://pubmed.ncbi.nlm.nih.gov/37634627/)

HTA Clinical Proteomics: Novel biomarkers quantified in less than one hour from raw blood, serum, and plasma. Eight-minute sample intervals and reproducible 1-hour analyses of biofluids.

Ref-2 Yannone SM, Tuteja V, Goleva O, Leung DYM, Stotland A, Keoseyan AJ, Hendricks NG, Parker S, Van Eyk JE, Kreimer S. "Toward Real-Time Proteomics: Blood to Biomarker Quantitation in under One Hour" Anal Chem. 2025;97(12):6418-26. Epub 2025/03/21. doi: 10.1021/acs.analchem.4c05172. PubMed PMID: 40113440. (https://pubmed.ncbi.nlm.nih.gov/40113440/)

HTA *De Novo* sequencing: Multiple IgGs sequenced in single reactions in about an hour yielding 100% coverage and unmatched sequence redundancy/confidence at all CDRs.

Ref-3 Shamorkina TM, Paneda LP, Kadava T, Schulte D, Pribil P, Heidelberger S, Narlock-Brand AM, Yannone SM, Snijder J, Heck AJR. "Deep Coverage and Extended Sequence Reads Obtained with a Single Archaeal Protease Expedite de novo Protein Sequencing by Mass Spectrometry" bioRxiv. 2025. Epub 2025/06/12. doi: 10.1101/2025.05.26.656138. PubMed PMID: 40501842; PMCID: PMC12154832. (https://pubmed.ncbi.nlm.nih.gov/40501842/)

Difficult Protein sequencing: Project rescued by identifying specific protein sequence undetected by all previouly available enzymes and approaches.

Ref-4 Poole LG, Schmitt LR, Schulte A, Groeneveld DJ, Cline HM, Sang Y, Hur WS, Wolberg AS, Flick MJ, Hansen KC, Luyendyk JP. "Altered fibrinogen gamma-chain cross-linking in mutant fibrinogen-gamma(Delta5) mice drives acute liver injury" J Thromb Haemost. 2023;21(8):2175-88. Epub 2023/04/17. doi: 10.1016/j.jtha.2023.04.003. PubMed PMID: 37062522; PMCID: PMC10524487. (https://pubmed.ncbi.nlm.nih.gov/37062522/)

TIPS & TROUBLESHOOTING

1) **Reaction pH of 3.0 is critical**. Ensuring pH = 3 is critical for optimal HTA-Protease function. If your sample is already buffered to a neutral pH, you must overwhelm that buffer with the pH 3.0 buffer provided or otherwise adjust pH to 3.0. We recommend 2-5x molar amounts of acidic buffer to ensure resident buffers are overwhelmed and a final reaction pH of 3.0 (+/- 0.5 pH units) is established and maintained.

2) **DO NOT USE UREA.** Urea will chemically react with peptides under HTA reaction conditions to adduct peptides, thereby severely diminishing PSMs and protein IDs. Avoid even trace amounts of urea or other reactive additives in heated/acid reactions.

3) **Use low-binding tubes and pipette tips.** HTA-Proteases have high specific activity; in working concentrations, HTA-Proteases have a low total protein content. We recommend avoiding serial dilutions where possible and using low protein binding tubes for all reactions. Many customers have had success with Eppendorf Protein LoBind tubes.

4) **Some samples may form precipitates at pH 3.0.** Take precautions to ensure your devices will not be damaged by loading samples including solids. The conditions for HTA-Proteases can lead to precipitation of high protein-concentration samples such as blood or milk. You can avoid precipitates by reducing the protein concentration of your sample by dilution with pure water (prior to acidification) as useful; proteolysis will occur regardless.

5) **Limit reaction times to < 60 minutes.** Reactions over 60 minutes will begin to show increased chemical hydrolysis at aspartic acid residues, altering identifications and precursors. If additional cleavage at aspartic acid residues is desirable, reactions can be run >60 minutes or pretreated in reaction mix at 80°C/pH3.

6) **Reduction and alkylation.** Note that IAA is not needed to block disulfide reformation or scrambling as the low pH protonates cysteines and blocks disulfide reformation. Additionally, DTT is not as effective as a reductant at pH 3.0, we recommend using the acid-tolerant TCEP as a reductant for your HTA-Protease reactions at 1-5 mM concentration. The citrate component of the provided buffer is a weak reductant and reactions without additional reductants can be executed as desirable.

7) **Evaporation and Condensation.** HTA-Protease reaction conditions at 80°C will cause aqueous solutions to evaporate and possibly condense on tube caps. We recommend performing reactions in a PCR Thermal Cycler or incubator set to 80°C such that there is not a thermal gradient across the reaction tube.

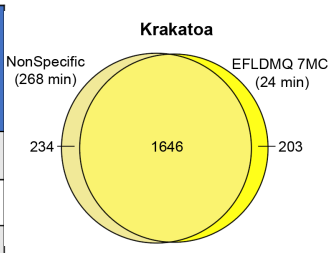
8) **Maintain HTA-Proteases aseptically.** The pH of HTA-Protease preparations is 3.0 and eliminates the growth of bacteria but some common fungi will grow at pH 3.0 if introduced to the sterile enzyme portions. Because these enzymes can store for up to 2 years at ambient conditions, care should be taken to avoid contamination with microbes/spores.

9) **HTA-Protease additive compatibilities.** HTA-Proteases are shipped in simple 1x reaction buffer (20mM K_2HPO_4, 40mM citric acid, pH 3.0). We find that typical surfactants/detergents (including SDS and DDM) are tolerated by HTA-Proteases up to approximately 0.5% (w/v). NaCl and KCl salt concentrations up to 250 mM are also tolerated. Forethought about possible chemical reactivity at pH 3.0 and 80°C with reaction additives is strongly encouraged.

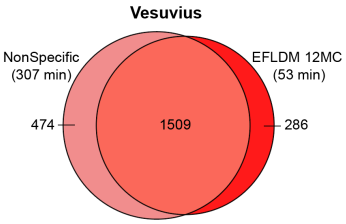
DATA SEARCHING HTA-PROTEASES

HTA-Protease data is compatible with all commonly used software packages given the settings are appropriate. PEAKS (Studio and AB; App Notes available online at cinderbio.com/publications), FragPipe, Spectronaut, DIA-NN, Protein Metrics, BioPharma Finder, and others have yielded excellent results. If maximal PSMs and IDs are desirable, nonspecific searches will yield maximal peptides and identifications. Alternatively, semi-specific searches are much more rapid with relatively minor losses in total identifications. An exemplary analysis of the impact of search settings on search time, protein IDs, and peptide IDs is shown below for both Krakatoa and Vesuvius.

KRAKATOA CB23726	NonSpecific Search Value (%max)	SemiSpecific EFLDMQ +3 missed cleavages Value (%max)	SemiSpecific EFLDMQ +5 missed cleavages Value (%max)	SemiSpecific EFLDMQ +7 missed cleavages Value (%max)
Search Time	268 min (100%)	12 min (4%)	20 min (7%)	24 min (9%)
Peptide ID's	15825 (100%)	7537 (48%)	12819 (81%)	14579 (92%)
Protein ID's	1880 (100%)	1466 (78%)	1795 (95%)	1849 (98%)



VESUVIUS CB14057	NonSpecific Search Value (%max)	SemiSpecific EFLDM +6 missed cleavages Value (%max)	SemiSpecific EFLDM +9 missed cleavages Value (%max)	SemiSpecific EFLDM +12 missed cleavages Value (%max)
Search Time	307 min (100%)	32 min (10%)	46 min (15%)	53 min (17%)
Peptide ID's	13159 (100%)	9562 (73%)	11010 (84%)	11456 (87%)
Protein ID's	1983 (100%)	1685 (85%)	1766 (89%)	1795 (91%)



Tables were generated by searching triplicate datasets of historical 90-minute digests of K562 cell extracts with the noted HTA-Protease using FragPipe version 20.0. Actual search times will vary depending on the computer used. Venn diagrams show the protein identification distributions for nonspecific and the noted semi-specific searches.

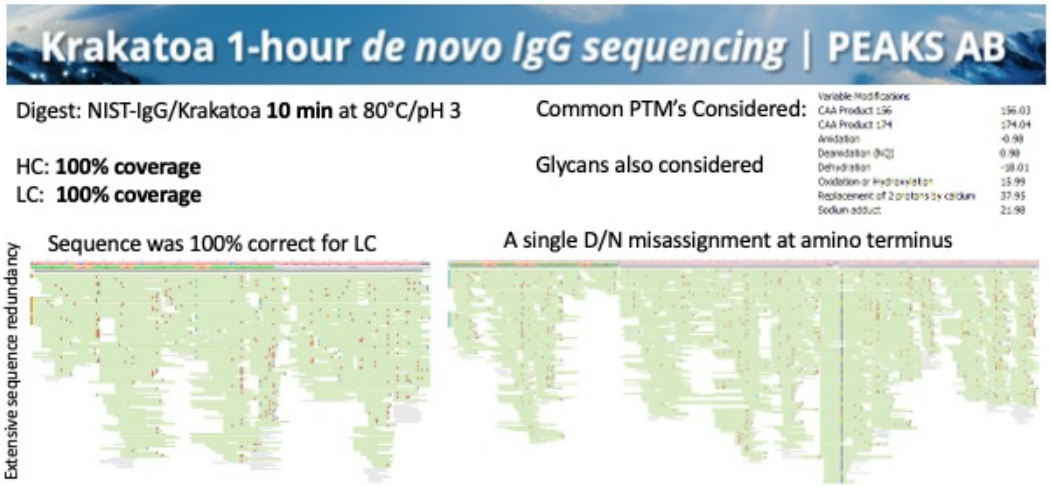
HTA-PTMs are substantively different than tryptic reactions. For optimal data analysis select HTA-appropriate modifications. (i.e. do not add carbamidomethylation w/out alkylation).

Trypsin	# Sites Modified	Vesuvius	# Sites Modified	Krakatoa	# Sites Modified
Deamidation (NQ)	2851	Deamidation (NQ)	370	Deamidation (NQ)	227
Carbamidomethylation (DHKE, X@N-term)	2200	Acetylation (Protein N-term)	158	Acetylation (Protein N-term)	149
Sodium adduct	960	Dehydration	150	Pyro-glu from Q	137
Acetylation (Protein N-term)	416	Pyro-glu from Q	144	Sodium adduct	119
Carbamylation	265	Sodium adduct	103	Dehydration	116
2-amino-3-oxo-butanoic_acid	248	Lysine oxidation to aminoadipic semialdehyde	43	Amidation	36
Phosphorylation (STY)	190	Pyro-glu from E	42	Pyro-glu from E	22
Oxidation (M)	187	Acetylation (K)	35	Acetylation (K)	21
Acetylation (N-term)	181	Deamidation (R)	32	Dimethylation(KR)	20
Methylation(KR)	122	Amidation	31	Deamidation (R)	14
Dehydration	119	Dimethylation(KR)	31	Phosphorylation (STY)	14
Lysine oxidation to aminoadipic semialdehyde	116	Methylation(KR)	31	Guanidination	13
Dihydroxy	104	Phosphorylation (STY)	28	Oxidation (M)	13
Deamidation (R)	104	Ammonia-loss (N)	21	Methylation(KR)	11
Dethiomethyl	93	Oxidation (M)	17	Lysine oxidation to aminoadipic semialdehyde	11
Biotinylation	72	Biotinylation	14	Biotinylation	9
Acetylation (K)	66	Carbamylation	14	Ammonia-loss (N)	9
Dimethylation(KR)	61	Guanidination	13	Carbamidomethylation (DHKE, X@N-term)	7
Double Carbamidomethylation (DHKE, X@N-term)	59	Acetylation (N-term)	12	Acetylation (N-term)	6
Pyro-glu from Q	56	Carbamidomethylation (DHKE, X@N-term)	11	Carboxymethyl (KW, X@N-term)	6
Replacement of proton by potassium	55	2-amino-3-oxo-butanoic_acid	10	Carbamylation	6

The data used to generate this table and figure are available at the Proteome-Xchange Consortium via the PRIDE partner repository with the data set identifier PXD041226. McCabe, M. C. *et al. J Proteomics* **289**, 104992, doi:10.1016/j.jprot.2023.104992 (2023).

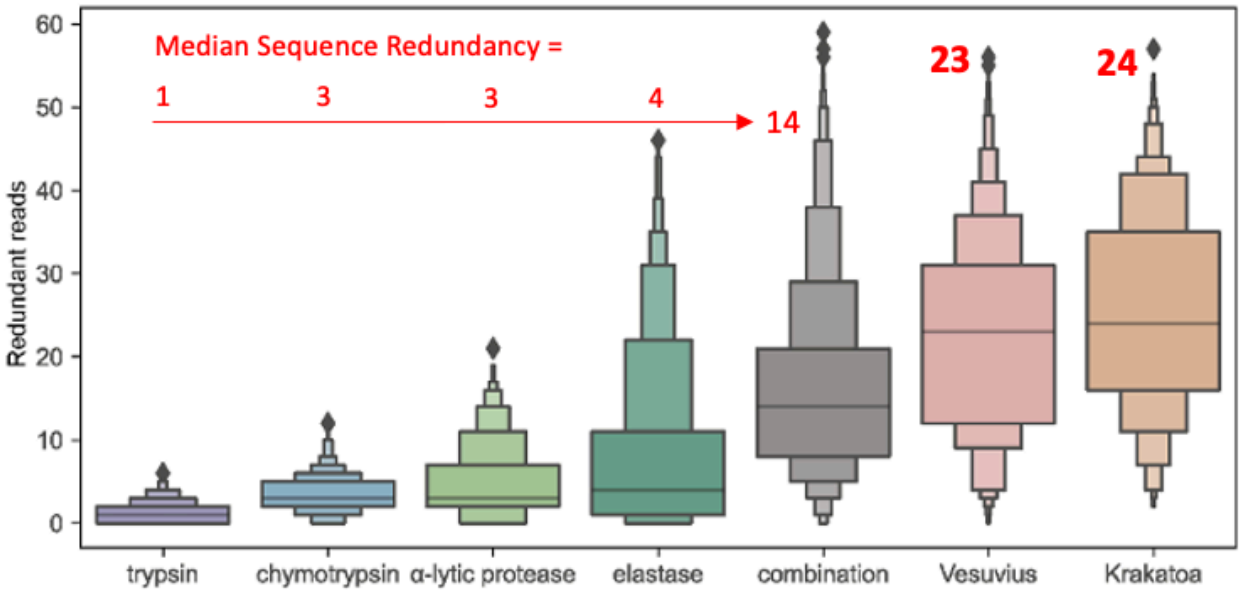
VALIDATED HTA-PROTEASE APPLICATIONS (JANUARY 2026)

1) ***De Novo* Sequencing.** The fastest and most effective *de* novo sequencing method available. HTA-Proteases reduce complex multi-enzyme *de novo* sequencing of antibodies to a single run and about one-hour in one run with 100% coverage and much higher sequence redundancy/confidence.



NOTE: Ile/Leu ambiguity is resolved by considering HTA-cleavage specificity for Leucine

A single HTA-Protease reaction/run outperforms multi-Enzyme approaches for *de novo* IgG sequencing in speed, simplicity, throughput, and coverage and depth/confidence.



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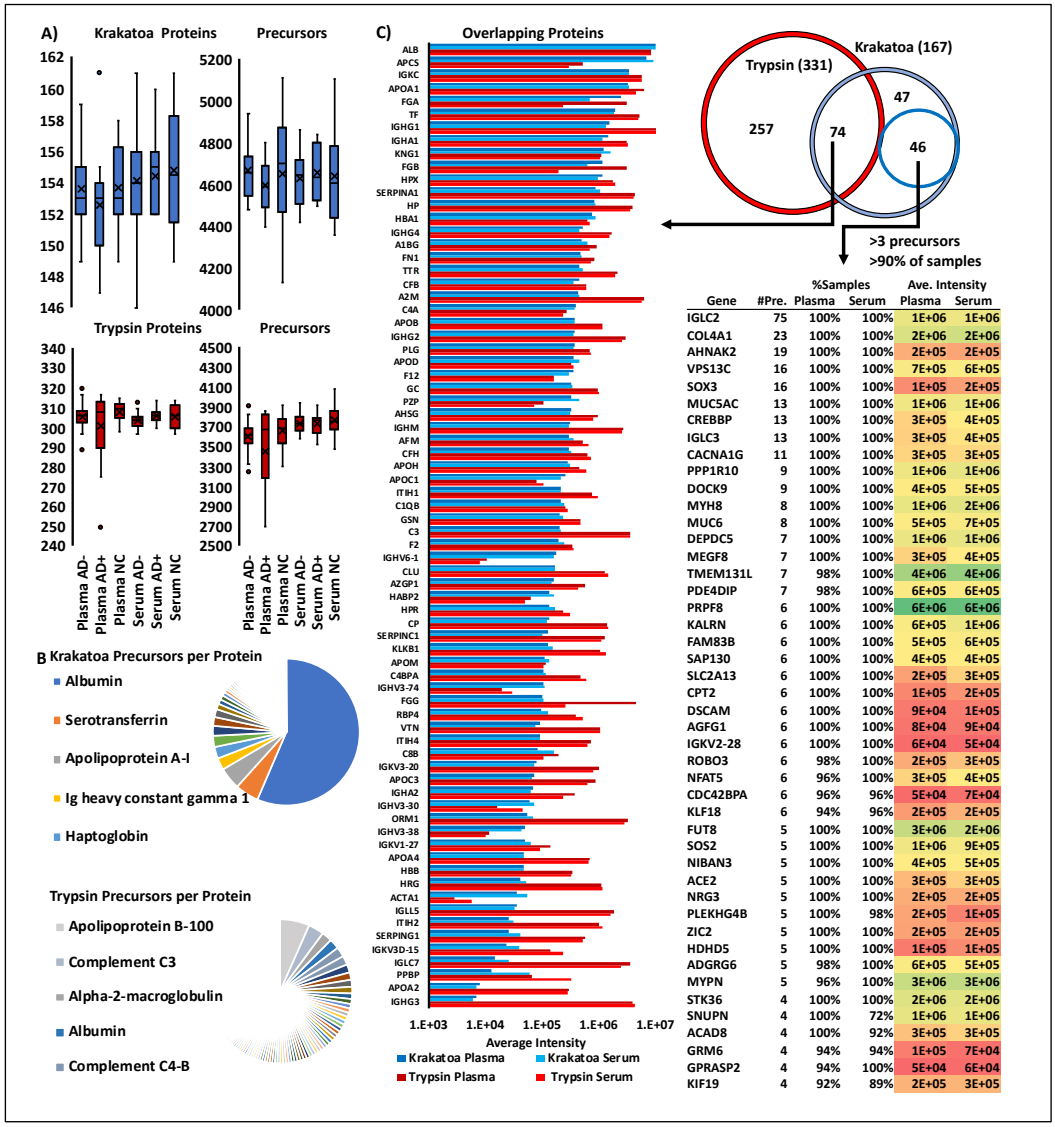
2) **Rapid Clinical Proteomics.** One-hour process for novel biofluid biomarker detection, monitoring, and discovery. HTA-digests of whole blood, serum, and plasma with a rapid one-step sample prep protocol reveal a protein set and labile peptide hormones not seen with trypsin with a 40-minute turnaround and 8-minute sample interval.

Rapid proteomics with biofluids identifies labile peptide hormones and protein identifications not seen with tryptic approaches. A direct digestion of whole blood, serum, or plasma for 20 minutes reveal quantitative and reproducible identifications of over 150 blood proteins including dozens of IDs unique to HTA-Protease methods, several PTMs, and additional sequence for identifications common to HTA-Proteases and trypsin.

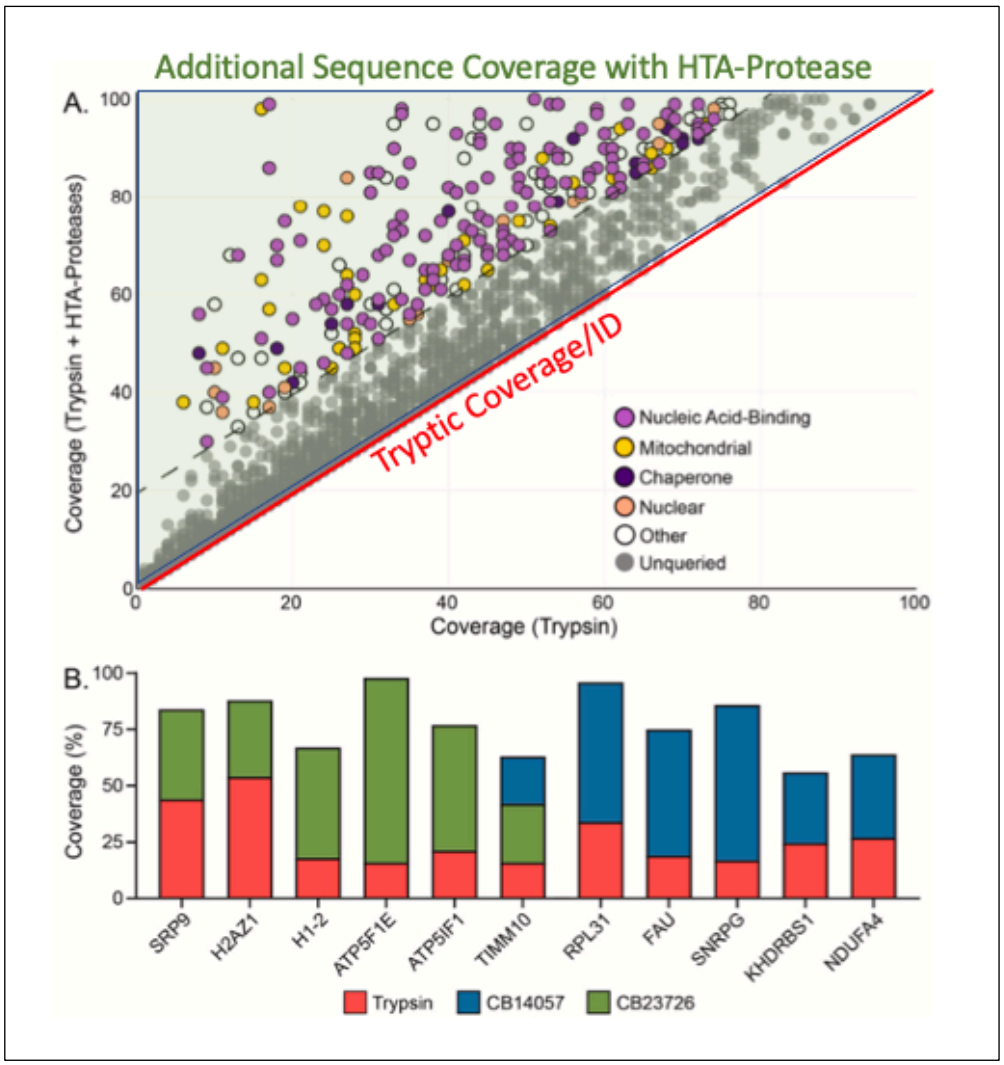


Gene	#Pre.	%Samples Plasma	%Samples Serum	Ave. Intensity Plasma	Ave. Intensity Serum
IGLC2	75	100%	100%	1E+06	1E+06
COL4A1	23	100%	100%	2E+06	2E+06
AHNAK2	19	100%	100%	2E+05	2E+05
VPS13C	16	100%	100%	7E+05	6E+05
SOX3	16	100%	100%	1E+05	2E+05
MUC5AC	13	100%	100%	1E+06	1E+06
CREBBP	13	100%	100%	3E+05	4E+05
IGLC3	13	100%	100%	3E+05	4E+05
CACNA1G	11	100%	100%	3E+05	3E+05
PPP1R10	9	100%	100%	1E+06	1E+06
DOCK9	9	100%	100%	4E+05	5E+05
MYH8	8	100%	100%	1E+06	2E+06
MUC6	8	100%	100%	5E+05	7E+05
DEPDC5	7	100%	100%	1E+06	1E+06
MEGF8	7	100%	100%	3E+05	4E+05
TMEM131L	7	98%	100%	4E+06	4E+06
PDE4DIP	7	98%	100%	6E+05	6E+05
PRPF8	6	100%	100%	6E+06	6E+06
KALRN	6	100%	100%	6E+05	1E+06
FAM83B	6	100%	100%	5E+05	6E+05
SAP130	6	100%	100%	4E+05	4E+05
SLC2A13	6	100%	100%	2E+05	3E+05
CPT2	6	100%	100%	1E+05	2E+05
DSCAM	6	100%	100%	9E+04	1E+05
AGFG1	6	100%	100%	8E+04	9E+04
IGKV2-28	6	100%	100%	6E+04	5E+04
ROBO3	6	98%	100%	2E+05	3E+05
NFAT5	6	96%	100%	3E+05	4E+05
CDC42BPA	6	96%	96%	5E+04	7E+04
KLF18	6	94%	96%	2E+05	2E+05
FUT8	5	100%	100%	3E+06	2E+06
SOS2	5	100%	100%	1E+06	9E+05
NIBAN3	5	100%	100%	4E+05	5E+05
ACE2	5	100%	100%	3E+05	3E+05
NRG3	5	100%	100%	2E+05	2E+05
PLEKHG4B	5	100%	98%	2E+05	1E+05
ZIC2	5	100%	100%	2E+05	2E+05
HDHD5	5	100%	100%	1E+05	1E+05
ADGRG6	5	98%	100%	6E+05	5E+05
MYPN	5	96%	100%	3E+06	3E+06
STK36	4	100%	100%	2E+06	2E+06
SNUPN	4	100%	72%	1E+06	1E+06
ACAD8	4	100%	92%	3E+05	3E+05
GRM6	4	94%	94%	1E+05	7E+04
GPRASP2	4	94%	100%	5E+04	6E+04
KIF19	4	92%	89%	2E+05	3E+05

Yannone SM, Tuteja V, Goleva O, Leung DYM, Stotland A, Keoseyan AJ, Hendricks NG, Parker S, Van Eyk JE, Kreimer S. "Toward Real-Time Proteomics: Blood to Biomarker Quantitation in under One Hour" Anal Chem. 2025;97(12):6418-26. Epub 2025/03/21. doi: 10.1021/acs.analchem.4c05172. PubMed PMID: 40113440. (https://pubmed.ncbi.nlm.nih.gov/40113440/)

3) **Additional Protein IDs and Sequence.** HTA-Proteases increase protein IDs, protein coverage, identify new PTMs and splice variants, and make many 'difficult' proteins tractable with MS/MS with data orthogonal to tryptic approaches.

HTA-Protease digests generate peptide sets that are non-overlapping with tryptic peptides and offer additional coverage for the majority of identified proteins as compared to trypsin alone. Identifications of non-tryptic regions of proteins allow identification of new PTMs and identification of regions of proteins that are refractory to detection and quantitation with common proteomics enzymes. The largest gains in coverage from digested whole cell lysate of K562 cells were biased for nucleic acid binding proteins (including histones), membrane and mitochondrial proteins, and other nuclear proteins. HTA-Protease data contributed additional sequence coverage for over 50 % of the tryptic identifications in addition to approximately 100 identifications that were only identified in the HTA-Protease data sets. (Data publicly available at ProteomeXchange Consortium via the PRIDE partner repository with the data set identifier PXD041226).



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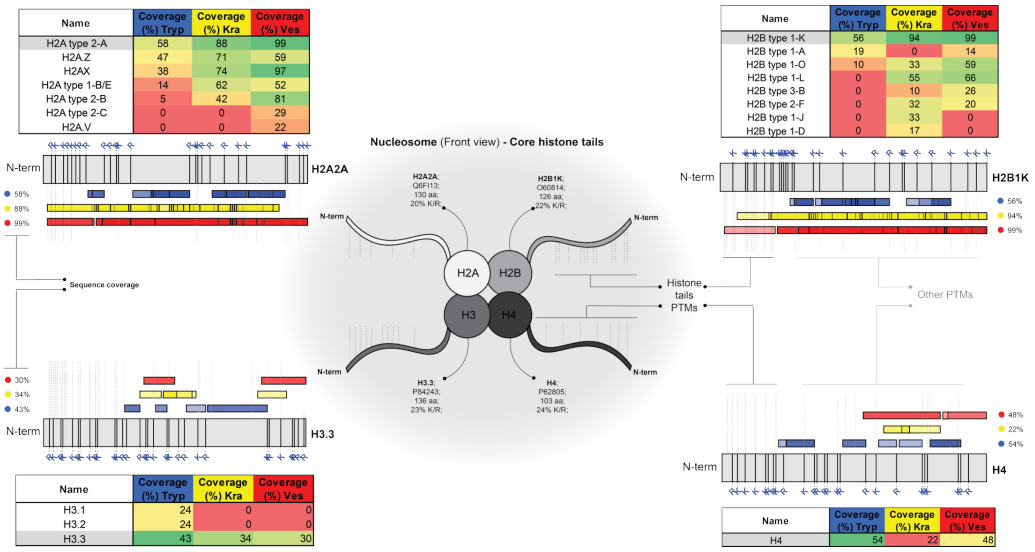
4) **Access to Histones and Nucleic Binding Proteins.** Histone coverage directly from whole cells or extracts without chemical manipulation using brief one-step sample preparation protocols.

Due to high abundance of K and R residues, Histones and other nucleic binding proteins are difficult to sequence with trypsin. HTA proteases prefer E, L, and F as cleavage sites and a large fraction of histone proteins can be mapped from whole cell lysates. K562 whole cell lysate data from McCabe, M. C. *et al.* show significant coverage for many histone variants even with an unoptimized one-step digestion that requires no additional blocking chemistries and no additional steps.

Direct Histone Data: Acquired from cell extract without any treatments or additives.

Histone coverage from K562 extract for HTA-Proteases and trypsin.



The proteomics data used to generate this figure are available at the Proteome-Xchange Consortium via the PRIDE partner repository with the data set identifier PXD041226.