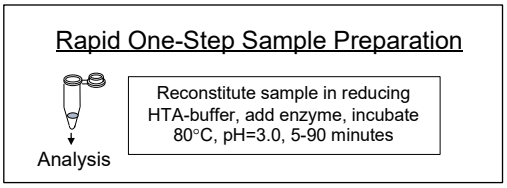
CINDERBIO STANDARD PROTEOMICS DIGESTION PROTOCOL

INTRODUCTION



Here we provide you with a proprietary HyperThermoacidic Archaeal protease (HTA-Protease©) derived from organisms that thrive in hot/acidic volcanic springs around the globe. The enzymes are provided as ready-to-use and do not require any preparation. Samples do not require treatment with urea, guanidinium chloride, other chaotropes or alkylating agents. The reaction conditions of 80°C and pH 3.0 are sufficient to denature target proteins for simple, rapid, and effective proteomic analyses. These enzyme solutions should be stored aseptically at ambient laboratory conditions and used according to instructions. **DO NOT FREEZE.**

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

Krakatoa Dosage vs. Vesuvius Dosage: Approximately **3-5 times more Vesuvius activity is required compared to Krakatoa** per unit mass sample for comparable results. We have found that a time range of 5-90 minutes and an enzyme dose of **0.2-2.0 Units of Krakatoa** or **0.6-10 Units of Vesuvius** per microgram of substrate protein has given good results across a variety of protein samples. We recommend starting at a Krakatoa dose of 1U/ug or a Vesuvius dose of 3U/ug for a 60 minute digestion to get your bearings with novel samples. 5mM reductant is important and pH 3.0 is critical for proper function of HTA-Proteases. Chaotropes are not needed and have proven to be counterproductive. Digests over 90 minutes have frequently shown fewer precursors and fewer identifications and consistently more chemical cleavage at aspartic acid (D).

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.

MATERIALS REQUIRED

Proteomics Grade Vesuvius: CB14057 (2U/ul or 10U/ul)	(provided)
Proteomics Grade Krakatoa: CB23726 (2U/ul or 10U/ul)	(provided)
10x Protease Buffer: 200mM K_2HPO_4, 400mM citric acid, pH 3.0	(provided)
Reductant: 5mM reaction-compatible (pH 3.0) reductant (i.e. DTT, TCEP)	(not provided)
Low protein-binding reaction tubes: (HTA-Proteases will adhere to tubes)	(not provided)
Alkylating agent: (NOT NEEDED, note IAA does not function at pH 3.0)	(not provided)

IN SOLUTION SAMPLE DIGESTION PROTOCOL

1) Re-constitute protein sample in 1x Protease Buffer with 5mM reductant.
2) Add protease at a dosage of 0.2-2.0 Unit enzyme / 1µg substrate.
3) Incubate reaction at 80°C for 5-90 minutes in a pre-heated block or PCR thermal cycler.
4) Quench reaction on ice and analyze immediately or store cryogenically until analysis.

NOTES:

The extreme heat and pH of HTA-Protease digestion conditions denatures sample proteins and disrupt cellular assemblies and structures. Whole blood, whole cells, as well as extracts have all shown good performance with this simple and rapid protocol.The reaction conditions at pH 3.0 prevent disulfide re-formation and therefore blocking of reduced cysteines is not necessary. HTA-Proteases function in dilute formic acid pH 3.0 without any additional additives and digests can be loaded directly onto the mass spectrometer if desirable.

Examples of Previous Experimentally Determined Krakatoa Reaction Conditions:

1) **Whole blood, serum, and plasma** are effectively digested with a 2U/ug dose for 20 minutes at pH 3.0, 80°C, with 5mM TCEP. The higher dose and the short digestion time was chosen to reduce the turnaround time and sampling intervals.
2) **K562 whole cell extracts** were efficiently digested with a 0.2U/ug dose for 90 minutes at pH 3.0, 80°C, with 5mM TCEP.
3) **Whole HAP1 cell pellets** showed effective digestion with 0.2U/ug incubated at 80°C for 60 minutes with shaking, then quenched on ice. Samples were desalted with Strata X33 RP column and dried down in a speed vac and frozen at -80C until injection.
4) **Histone Preparations** showed effective digestion without reductant or IAA when incubated for 60 minutes at 80°C, pH 3.0, with a dose of 0.2U/ug.
5) **De Novo Sequencing (Antibodies)** IgG's are effectively digested with a 1U/ug dose for 60 minutes at pH 3.0, 80°C, with 5mM TCEP.
6) **Proteins in solution** are effectively digested across the suggested range of doses and times and optimal reaction conditions should be empirically determined according to desired outcomes.

Importantly, HTA-Proteases are an entirely new class of proteases and we are optimizing and learning along with our collaborators and customers. We recommend starting with a simple digest of your samples according to the guidelines herein and optimize dose and time to suit your experiments.

Our publications are also a source of very detailed experimental methods.

1 Yannone, S. M. *et al.* Blood to Biomarker Quantitation in Under One Hour with Rapid Proteomics using a Hyperthermoacidic Protease. *bioRxiv*, doi:10.1101/2024.06.01.596979 (2024).

2 McCabe, M. C. *et al.* From volcanoes to the bench: Advantages of novel hyperthermoacidic archaeal proteases for proteomics workflows. *J Proteomics* **289**, 104992, doi:10.1016/j.jprot.2023.104992 (2023).

TIPS & TROUBLESHOOTING (PLEASE READ)

1) **Assure sample pH is 3.0**. Proper pH is critical for HTA-Protease function. If your sample is already buffered to a neutral pH you must overwhelm that buffer with pH 3.0 buffer, formic acid, or other common acid. We recommend 2-5x stochiometric amounts of acidic buffer to ensure a final reaction pH of 3.0 (+/- 0.5 pH units). Proper pH of 3.0 is CRITICAL for optimal enzyme function.

2) **Use low-binding tubes and pipette tips.** Because of the extremely high specific activity of HTA-Proteases the total protein concentration in HTA-Protease preparations is extraordinarily low. Aliquoting or serial transfer of enzymes in standard plasticware can deplete the liquid of enzyme. We recommend adding samples to reaction tubes first, to block plasticware with sample proteins prior to introducing HTA-Proteases.

3) **Some samples may form precipitates at pH 3.0.** Take precautions to ensure your devices will not be damaged by loading samples including some solids. We have found that proteolysis proceeds efficiently even in cases where precipitates form (e.g. milk casein). You can avoid precipitates by reducing the protein concentration of your sample by dilution (prior to acidification) as useful, but this is not required for proteolysis.

4) **Limit reaction times to < 90 mins.** Reactions over 90 mins will begin to show increased chemical cleavage at aspartic acid residues and diminishing identifications and precursors. Adjust enzyme dosage and digestion times to achieve the desired results of your analyses and the desired timing within the range of 5- 90 minutes.

5) **Reduction and alkylation.** Note that IAA will not alkylate cysteines at pH 3.0 and the acidic conditions prevent disulfide reformation. An acid-compatible reductant should be used if the sample pH is set to 3.0 before reduction/alkylation (e.g. TCEP). Alternatively, reduction/alkylation can be carried out at neutral pH with DTT/IAA and the sample pH then shifted to 3.0 after these chemical reactions are complete. Because disulfide reformation after reduction is severely inhibited by the pH 3.0 acidic reaction conditions, inclusion of TCEP in pH 3.0 buffers without subsequent alkylation may be the most efficient reaction conditions for many applications.

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

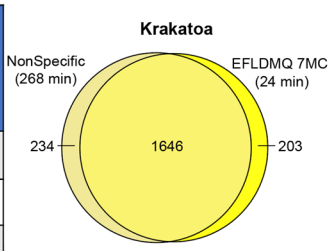
7) **HTA-Protease additive compatibilities.** HTA-Proteases are shipped in simple 1x reaction buffer (20mM K_2HPO_4, 40mM citric acid, pH 3.0). While our studies are not exhaustive, we find HTA-Proteases function well in citric, acetic, nitric, phosphoric, peracetic, and formic acids if the target pH of 3.0 is maintained. We also find that all tested surfactants/detergents are tolerated by HTA-Proteases up to approximately 0.5% (w/v) and NaCl and KCl salt concentrations up to 250 mM are tolerated. Forethought about possible chemical reactivity at pH 3.0 and 80°C with reaction additives is recommended.

DATA SEARCHING HTA-PROTEASE RESULTS

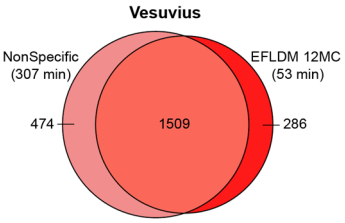
If maximal coverage is desirable, nonspecific searches will yield maximal identifications. Alternatively, semi-specific searches are much more rapid with relatively minor losses in total identifications. An analysis of the impact of search settings on search time, protein IDs, and peptide IDs is shown below for both Krakatoa and Vesuvius as a reference for preferred search settings and time required. HTA-Protease data is compatible with all commonly used software packages given the settings are appropriate.

NOTE: During data analysis do <u>not</u> add Carbamidomethylation as a fixed or variable modification if you did not alkylate.

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 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.

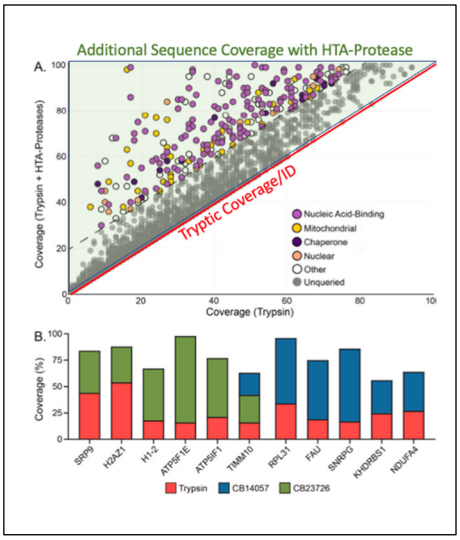
The mass spectrometry proteomics data used to generate this table and figure are available at the Proteome-Xchange Consortium via the PRIDE partner repository with the data set identifier PXD041226.

McCabe, M. C. *et al.* From volcanoes to the bench: Advantages of novel hyperthermoacidic archaeal proteases for proteomics workflows. *J Proteomics* **289**, 104992, doi:10.1016/j.jprot.2023.104992 (2023).

VALIDATED HTA-PROTEASE APPLICATIONS (AUGUST 2024)

1) Increased protein coverage, solve 'difficult' proteins, and identify new PTMS.

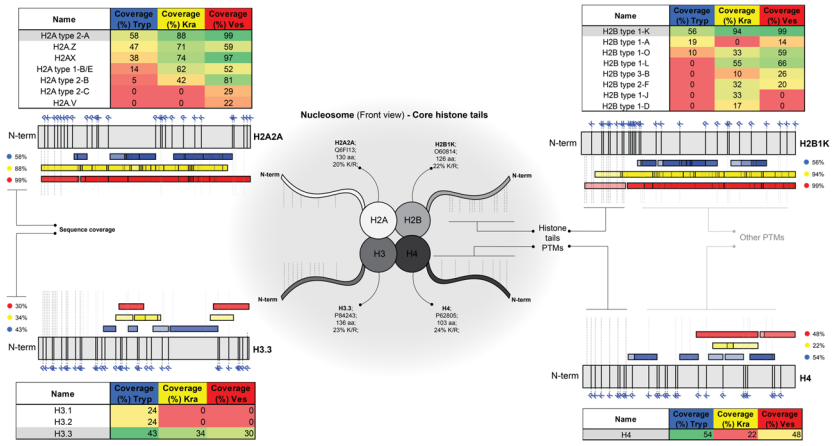
HTA-Protease digests generate peptide sets that are non-overlapping with tryptic peptides and offer additional coverage for the majority of identified proteins as compared to trypsin alone. Identifications of non-tryptic regions of proteins allow identification of new PTM's and identification of regions 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.



2) Histone coverage directly from whole cells or extracts without chemical intervention using brief one-step sample preparation protocols.

Histones and other nucleic binding proteins cause particular barriers for trypsin, often due to the high abundance of K and R residues. 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.

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



Name	Coverage (%) Tryp	Coverage (%) Kra	Coverage (%) Ves
H2A type 2-A	58	88	99
H2A.Z	47	71	59
H2AX	38	74	97
H2A type 1-B/E	14	62	52
H2A type 2-B	5	42	81
H2A type 2-C	0	0	29
H2A.V	0	0	22

Name	Coverage (%) Tryp	Coverage (%) Kra	Coverage (%) Ves
H2B type 1-K	56	94	99
H2B type 1-A	19	0	14
H2B type 1-O	10	33	59
H2B type 1-L	0	55	66
H2B type 3-B	0	10	26
H2B type 2-F	0	32	20
H2B type 1-J	0	33	0
H2B type 1-D	0	17	0

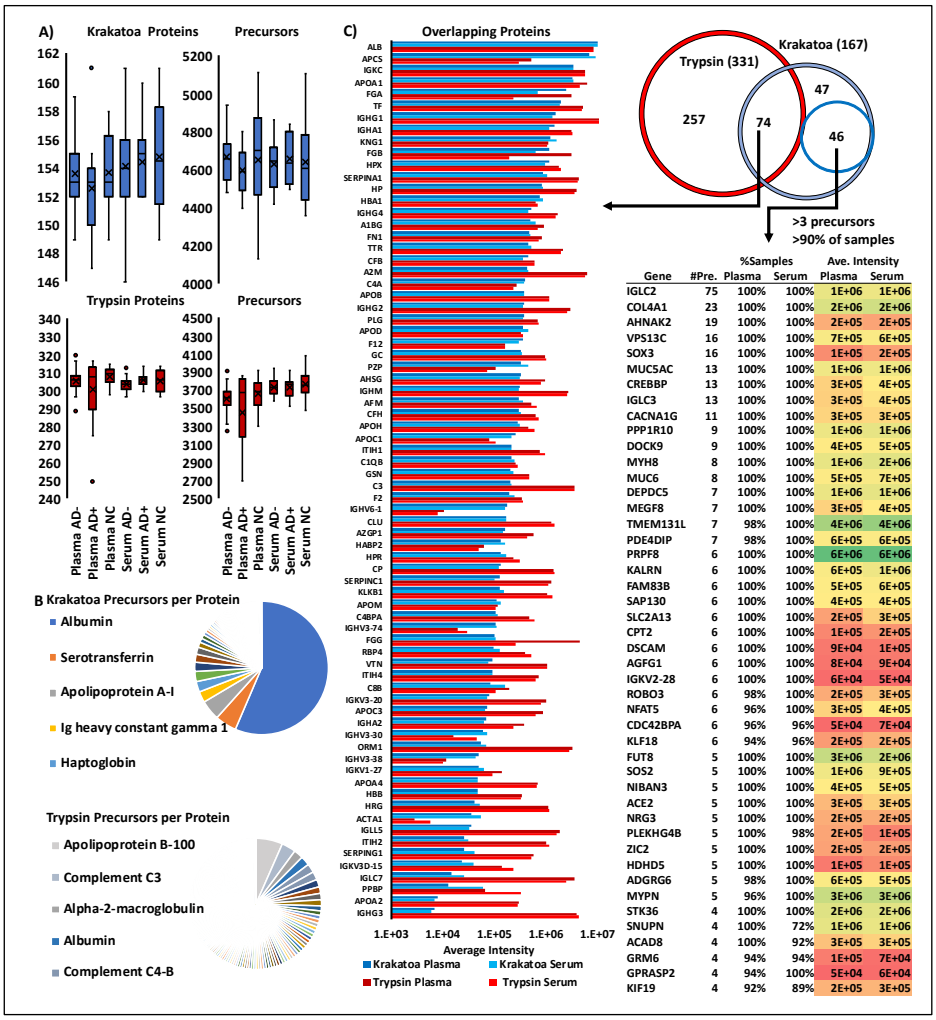
Name	Coverage (%) Tryp	Coverage (%) Kra	Coverage (%) Ves
H3.1	24	0	0
H3.2	24	0	0
H3.3	43	34	30

Name	Coverage (%) Tryp	Coverage (%) Kra	Coverage (%) Ves
H4	54	22	48

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.

3) **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 ID's unique to HTA-Protease methods, several PTM's, 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, S. M. *et al.* Blood to Biomarker Quantitation in Under One Hour with Rapid Proteomics using a Hyperthermoacidic Protease. *bioRxiv*, doi:10.1101/2024.06.01.596979 (2024).

4) **De-Novo Protein sequencing – HTA-Proteases provide a large number of overlapping peptides giving excellent sequence coverage and depth with simple and rapid digestion protocols. HTA-Proteases do not produce interfering peptides due to the low mass/mass dose and HTA-Proteases are not susceptible to autolysis.**

Exemplary mAb data kindly provided by an early-adopting customer. Digests were run according to the suggested protocols for 60 minutes.