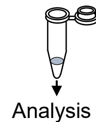
# CINDERBIO STANDARD PROTEOMICS DIGESTION PROTOCOL

## 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.**

**5-min/one-step HTA protocol**




Reconstitute sample in reducing HTA-buffer, add enzyme, incubate 80°C, pH=3.0, 5-60 minutes

## REQUIRED MATERIALS

| | |
|---|---|
| **Proteomics Grade Vesuvius:** CB14057– 2 Units/µL (16 nanograms/µL) | (200 Units provided) |
| and/or | |
| **Proteomics Grade Krakatoa:** CB23726– 2 Units/µL (16 nanograms/µL) | (200 Units provided) |
| **10x Protease Buffer:** 200mM $K_2HPO_4$, 400mM citric acid, pH 3.0 | (1000µL provided) |
| **Reductant:** 1mM reaction-compatible (pH 3.0) reductant (i.e. DTT, TCEP, BME) | (not provided) |
| **Low protein-binding reaction tubes:** (HTA-Proteases will adhere to tubes) | (not provided) |
| **Nano-pure water** | (not provided) |
| **Alkylating agent:** (NOT NEEDED/optional, IAA does not function at pH 3.0) | (not provided) |

## IN SOLUTION SAMPLE DIGESTION PROTOCOL

- Re-constitute protein sample in 1x Protease Buffer with 1mM reductant.
- Add protease at a dosage of 1.0 Unit enzyme/5µg substrate.
- Incubate reaction at 80°C for 5-60 minutes in a pre-heated block or PCR thermal cycler.
- Quench reaction on ice and analyze immediately or store cryogenically until analysis.

## NOTES:

Proteomics Grade Krakatoa-CB23726 and Vesuvius-CB14057 are compatible with simultaneous co-digestion of samples as useful. 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.

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. Digests over two hours will begin to show chemical cleavage at Aspartic acid (**D**) under these reaction conditions.

## 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 pipet 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 < 1 hour.** Reactions over 1 hour will begin to show increased chemical cleavage at aspartic acid residues. No advantage has been identified for longer reaction times. Adjust enzyme dosage and digestion times to achieve the desired results of your analyses and the desired timing within the range of 5 min-1 hour.

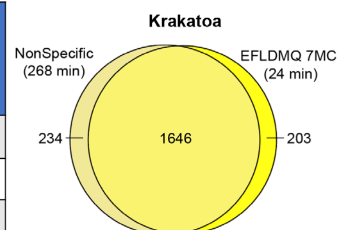
5) **Reduction and alkylation.** Note that IAA will not alkylate cysteines at pH 3.0 and the acidic conditions prevent disulfide reformation. An acid-compatible reductant should be used if the sample pH is set to 3.0 before reduction/alkylation (e.g. TCEP). Alternatively, reduction/alkylation can be 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.

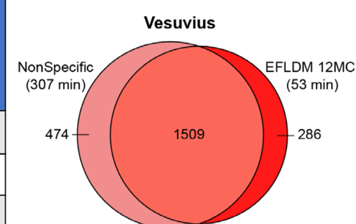
7) **HTA-Protease additive compatibilities.** HTA-Proteases are shipped in simple 1x reaction buffer (20mM $K_2HPO_4$, 40mM citric acid, pH 3.0). While our studies are not exhaustive, we find HTA-Proteases function well in citric, acetic, nitric, phosphoric, peracetic, and formic acids if the target 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.

8) **Data searches.** 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.

| KRAKATOA CB23726 | NonSpecific Search<br>Value (%max) | SemiSpecific EFLDMQ<br>+3 missed cleavages<br>Value (%max) | SemiSpecific EFLDMQ<br>+5 missed cleavages<br>Value (%max) | SemiSpecific EFLDMQ<br>+7 missed cleavages<br>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<br>Value (%max) | SemiSpecific EFLDM<br>+6 missed cleavages<br>Value (%max) | SemiSpecific EFLDM<br>+9 missed cleavages<br>Value (%max) | SemiSpecific EFLDM<br>+12 missed cleavages<br>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 (McCabe *et al.*) 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.

M.C. McCabe, V. Gejji, A. Barnebey, G. Suizdak, L.T. Hoang, T. Pham, K.Y. Larson, A.J. Saviola, S.M. Yannone, K.C. Hansen, From volcanoes to the bench: Advantages of novel hyperthermoacidic archaeal proteases for proteomics workflows, J Proteomics (2023) 104992.