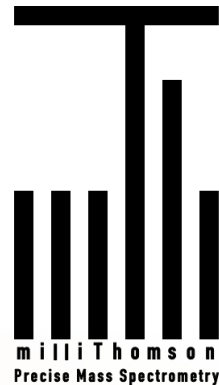


RAPID AND COMPLETE BIOPHARMACEUTICAL SEQUENCING USING A SINGLE TUNABLE PROTEASE

Johannes V. Janssens, Steven M. Yannone, Jennifer E. Van Eyk, **Simion Kreimer**



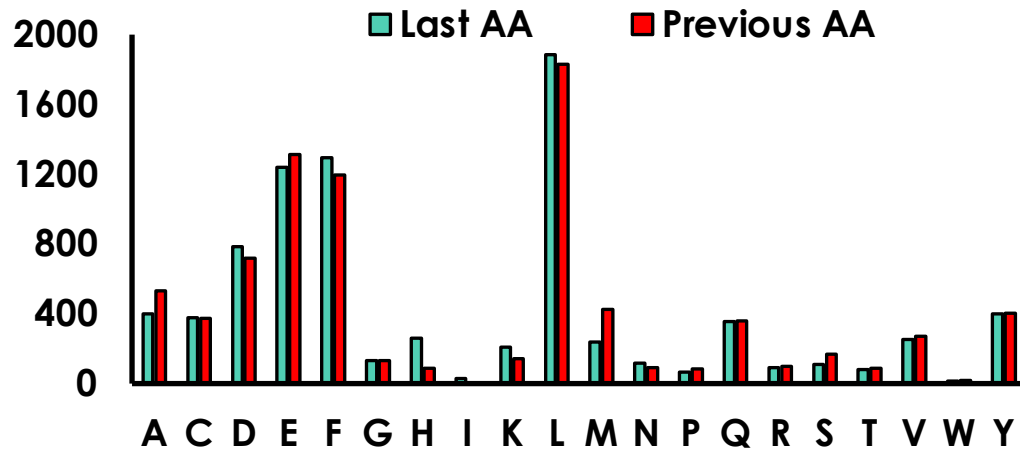


HYPERTHERMOACIDIC ARCHAEAL PROTEASES

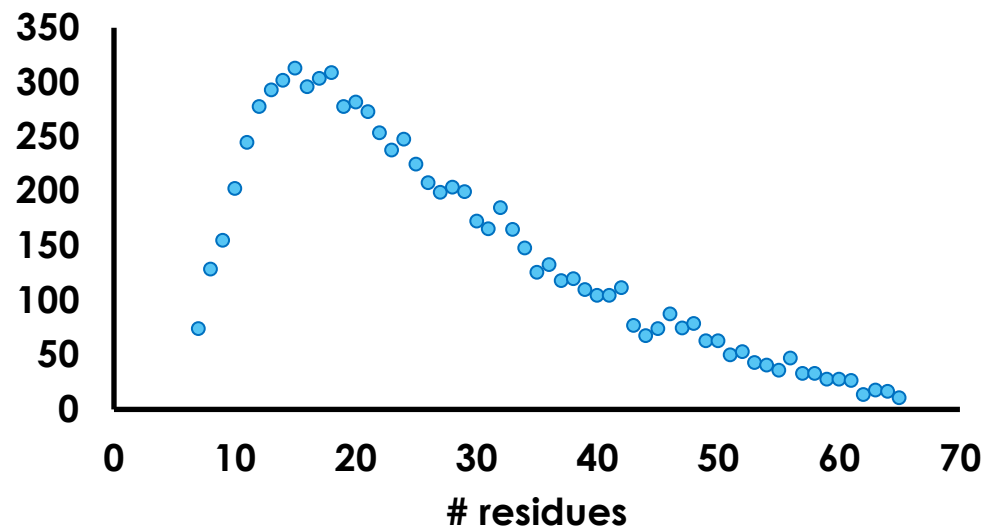
- Enzymes that break down proteins derived from extremophile single-celled Archaea
- Optimal function at high temperature and low pH; conditions that denature proteins we study
- High reaction speed and convenient 1-step sample preparation
- Cleavage specificity is lower than trypsin (actually helps with sequencing)

HTA CLEAVAGE MOTIFS ARE COMPLEX

Krakatoa Blood Digestion

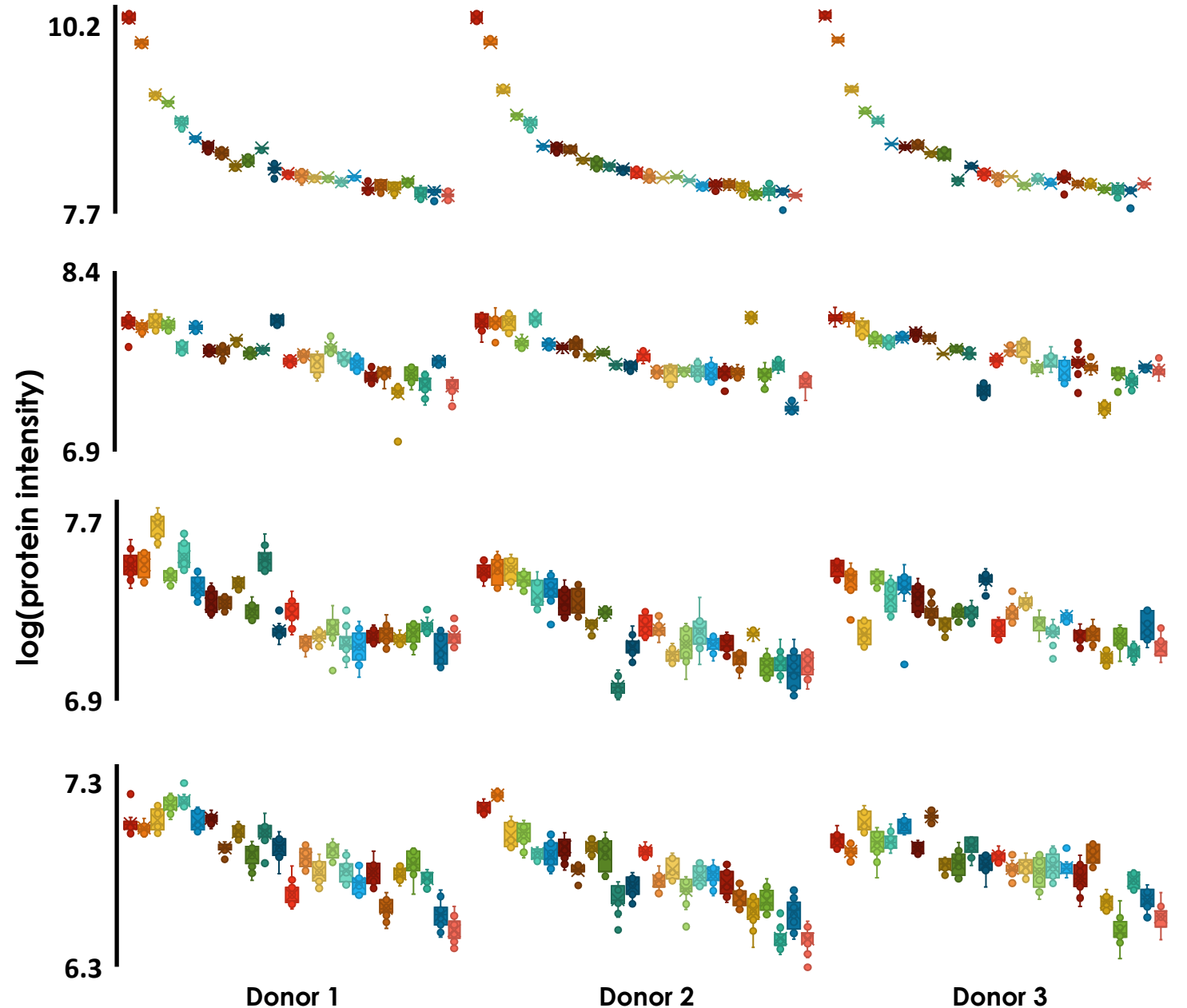
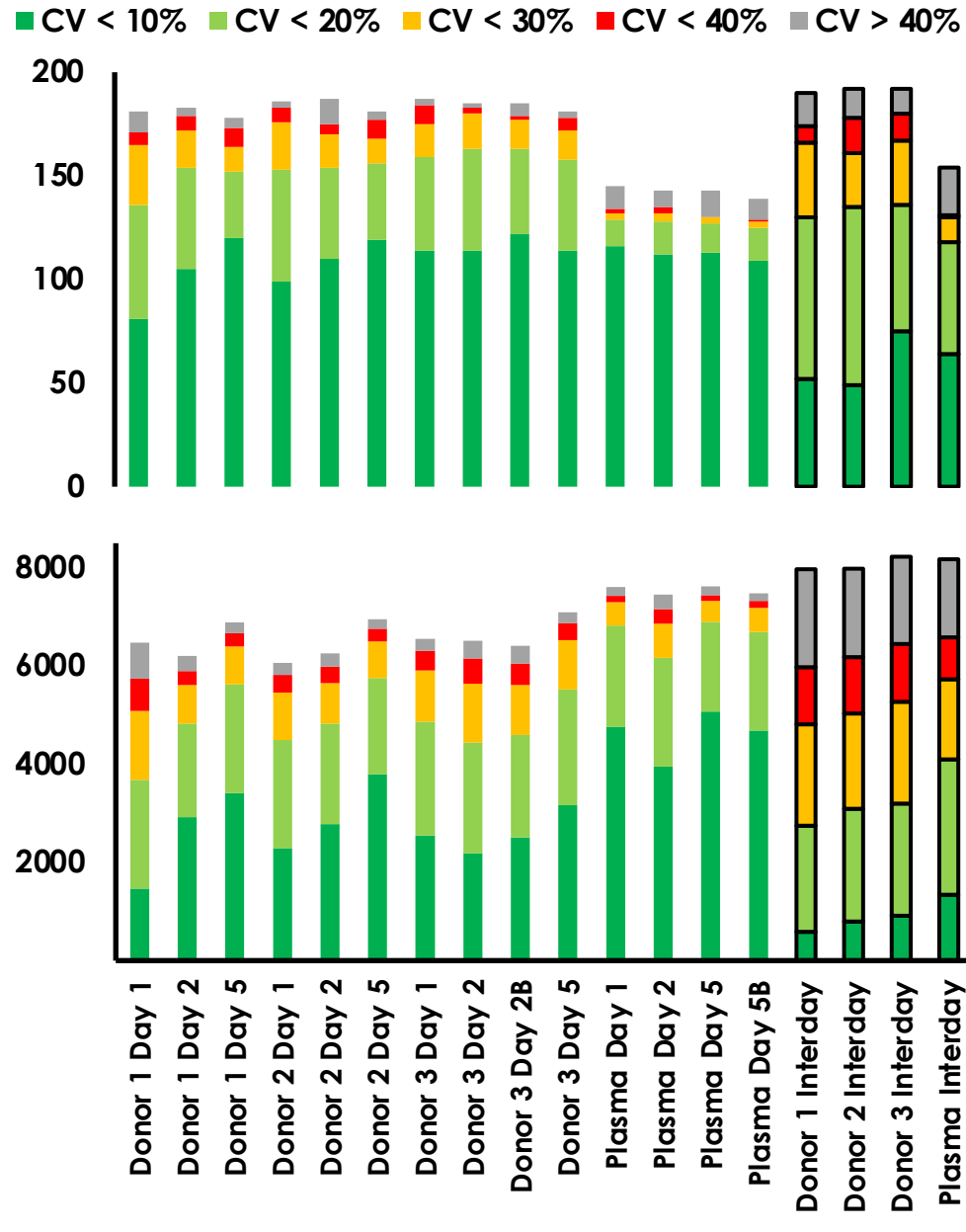


Peptide Length Distribution



AA	Previous	1st	2nd	3rd	4th	5th	Last	Next
A	532	811	673	732	605	826	400	715
C	374	99	237	130	193	199	377	357
D	719	450	491	396	463	571	784	406
E	1313	595	499	498	508	445	1241	565
F	1194	281	351	389	338	286	1296	259
G	133	470	223	325	481	445	130	460
H	86	205	283	425	438	306	262	138
I	0	146	172	148	114	194	29	0
K	141	630	593	752	680	723	210	688
L	1829	949	1081	957	982	749	1886	1016
M	424	125	142	108	93	106	239	120
N	92	316	201	435	374	320	118	290
P	83	347	1546	174	385	509	64	215
Q	359	203	120	213	290	238	357	212
R	98	471	168	444	340	420	92	482
S	169	746	428	686	670	483	110	754
T	89	494	305	429	456	545	80	447
V	272	769	656	873	707	717	253	596
W	19	66	34	60	63	115	15	21
Y	402	169	139	168	162	145	399	165

HTA PROTEOLYSIS IS REPRODUCIBLE

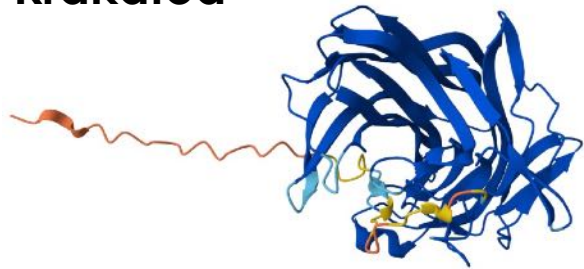


3 HTA PROTEASES

AMP



Krakatoa



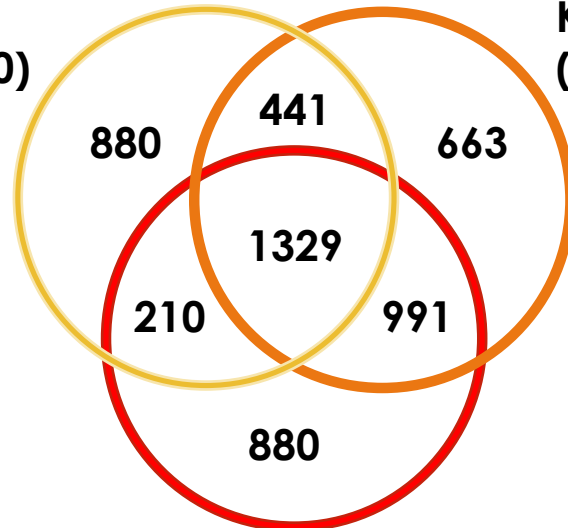
Vesuvius



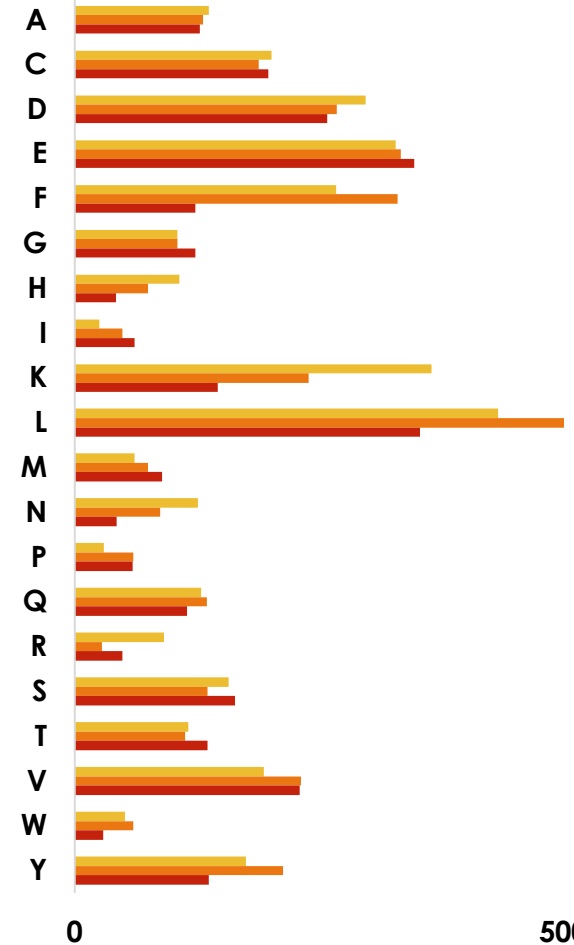
AMP
(3410)

Kra
(3394)

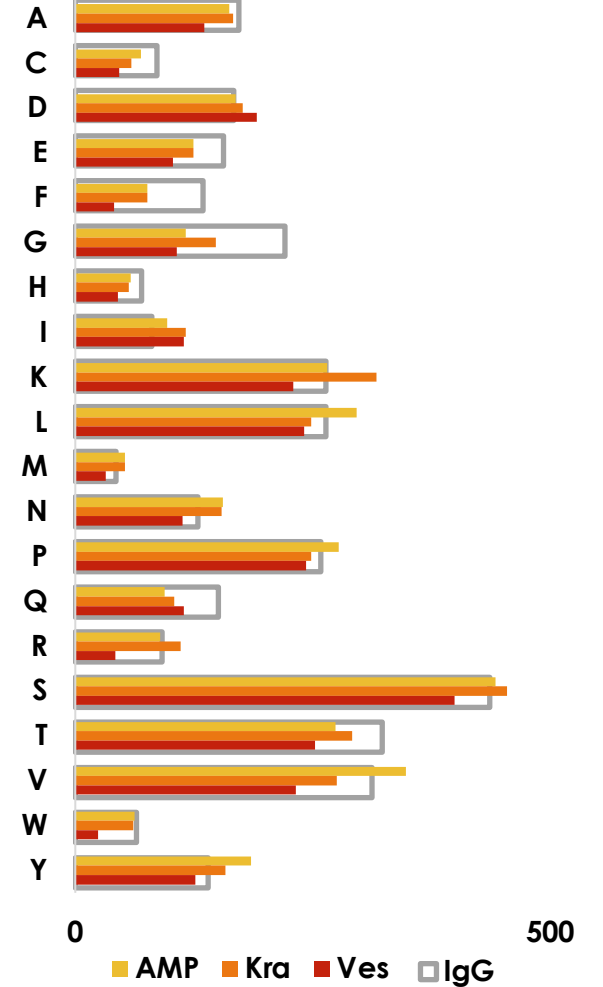
Ves
(2830)



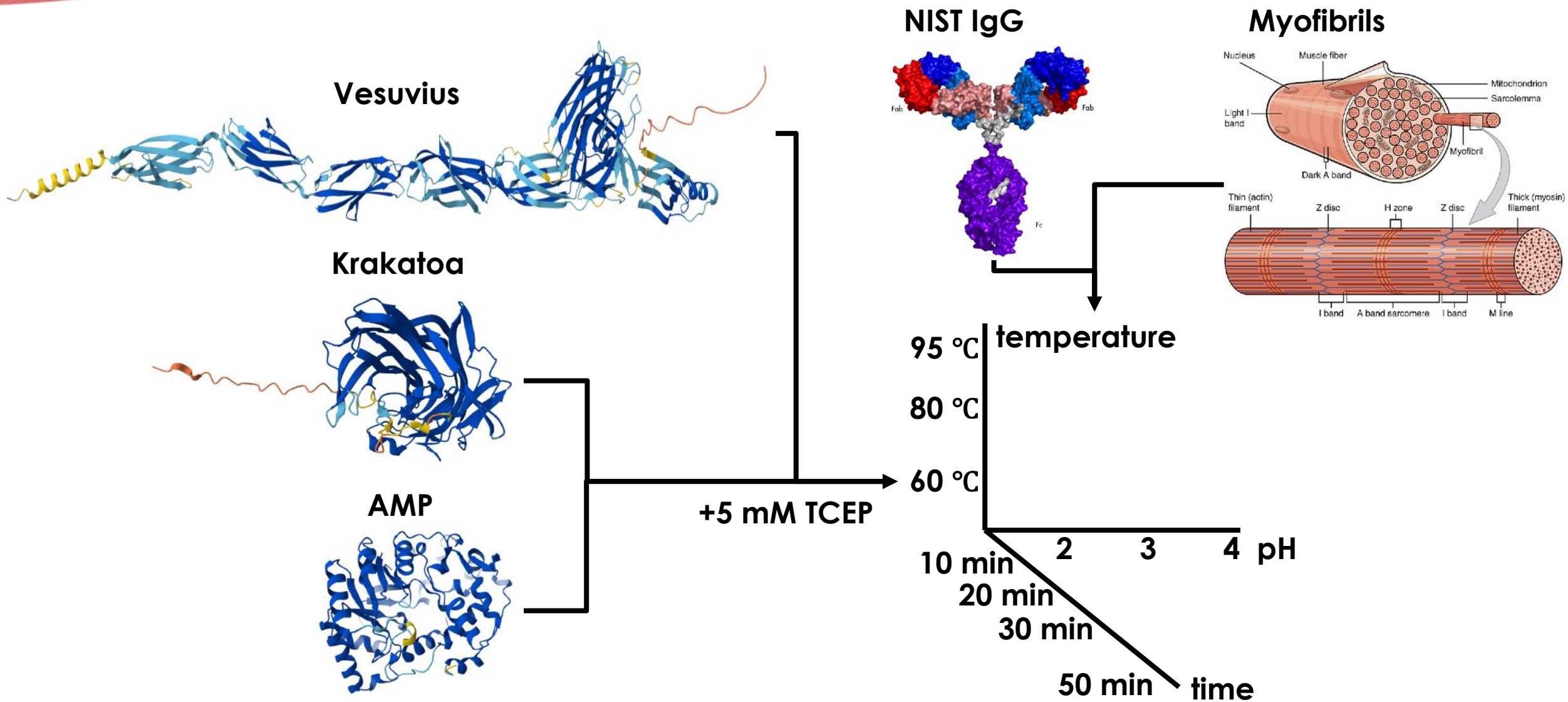
Last Residue Count
(C-terminal cleavage)



First Residue Count
(Follows AA distribution)



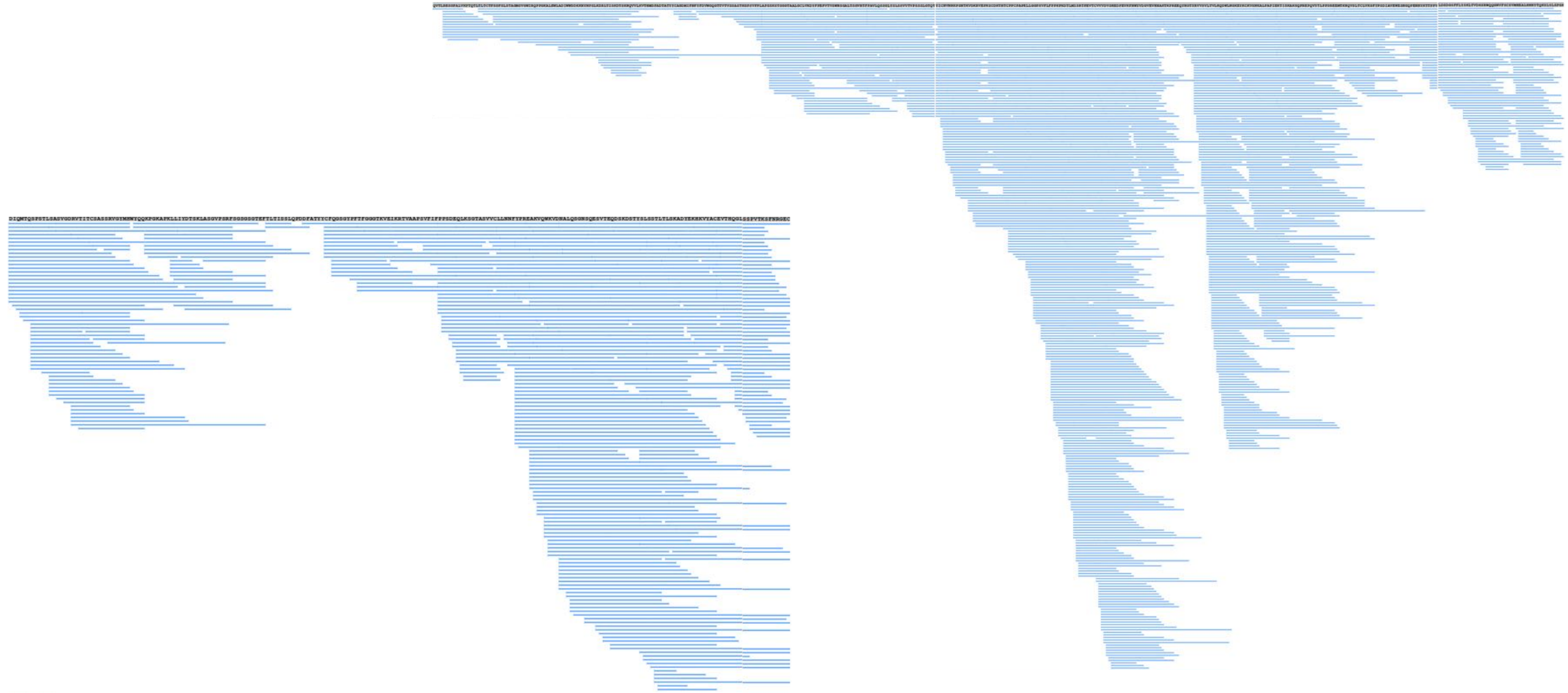
TUNING PROTEOLYSIS PARAMETERS



LC-MS ANALYSIS

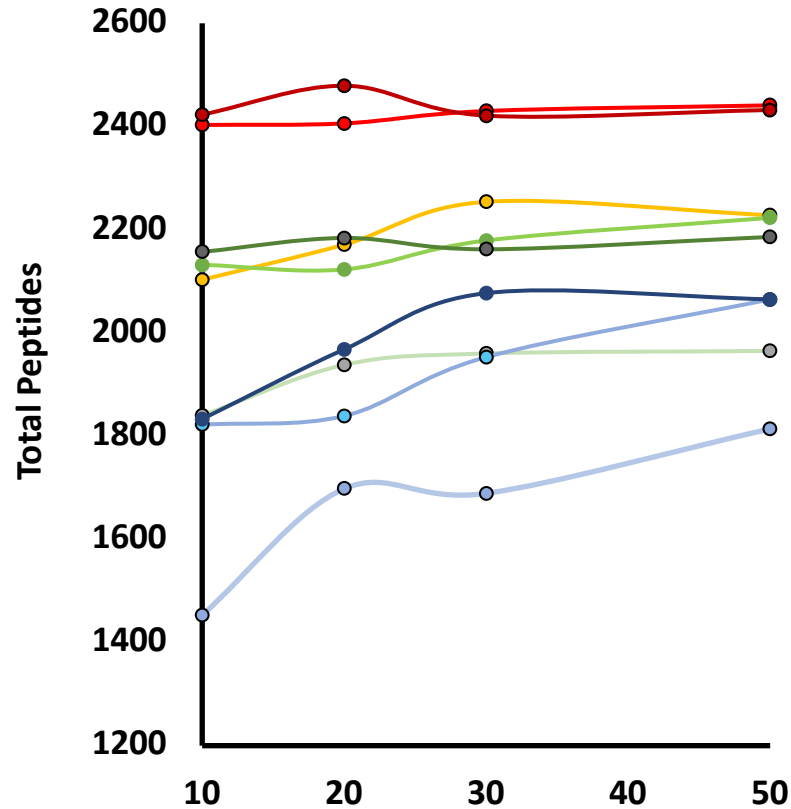
- NeoVanquish backflush trap and elute (1200 nL/min) and Exploris 480
- 25 min DDA and DIA run for each condition
- DDA data analyzed as “no specificity” proteolysis in PEAKS 12.5 to create libraries
- DIA data probed with corresponding library (protease x substrate)
- DIA results are used to compare proteolysis

100% SEQUENCE COVERAGE

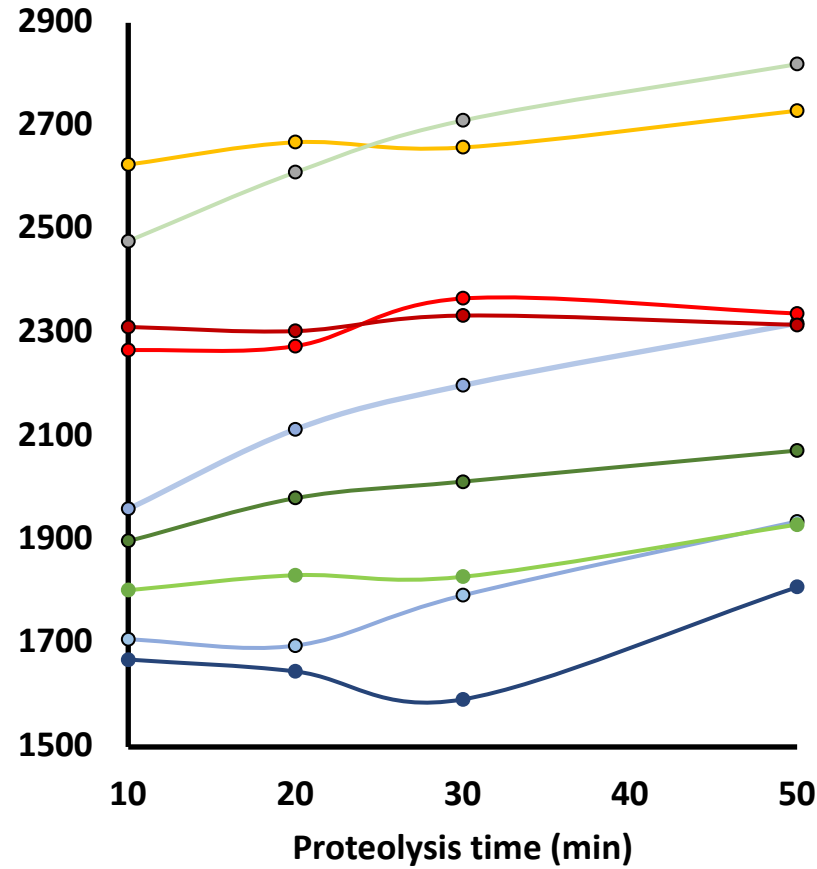


PEPTIDE COUNTS

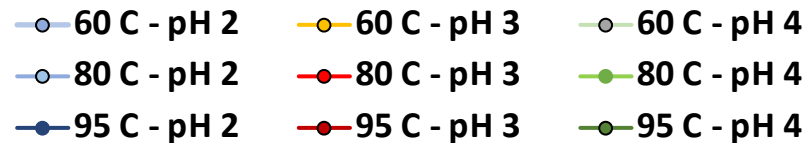
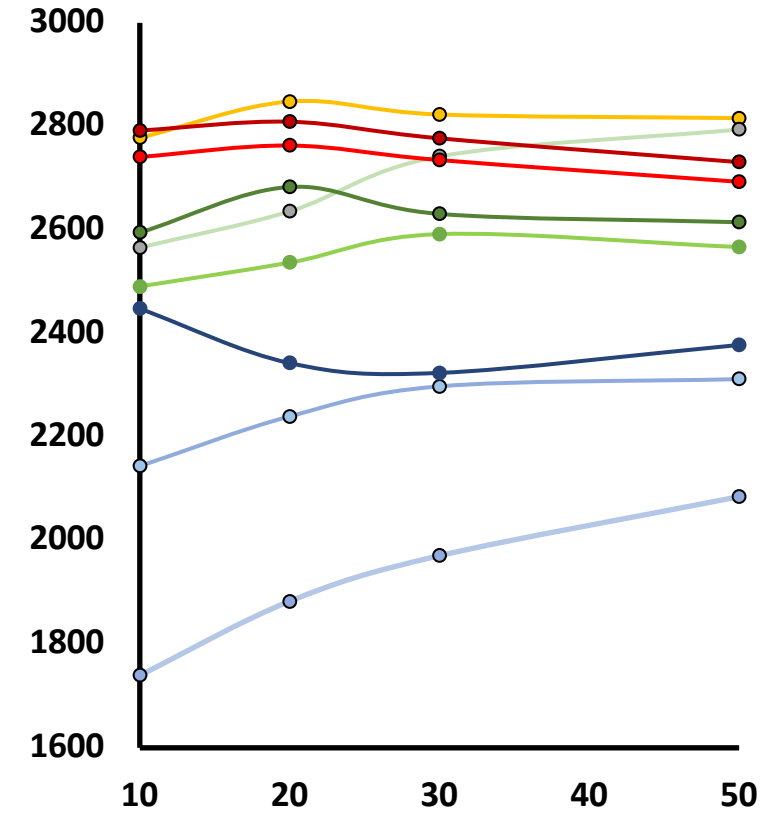
Vesuvius



AMP

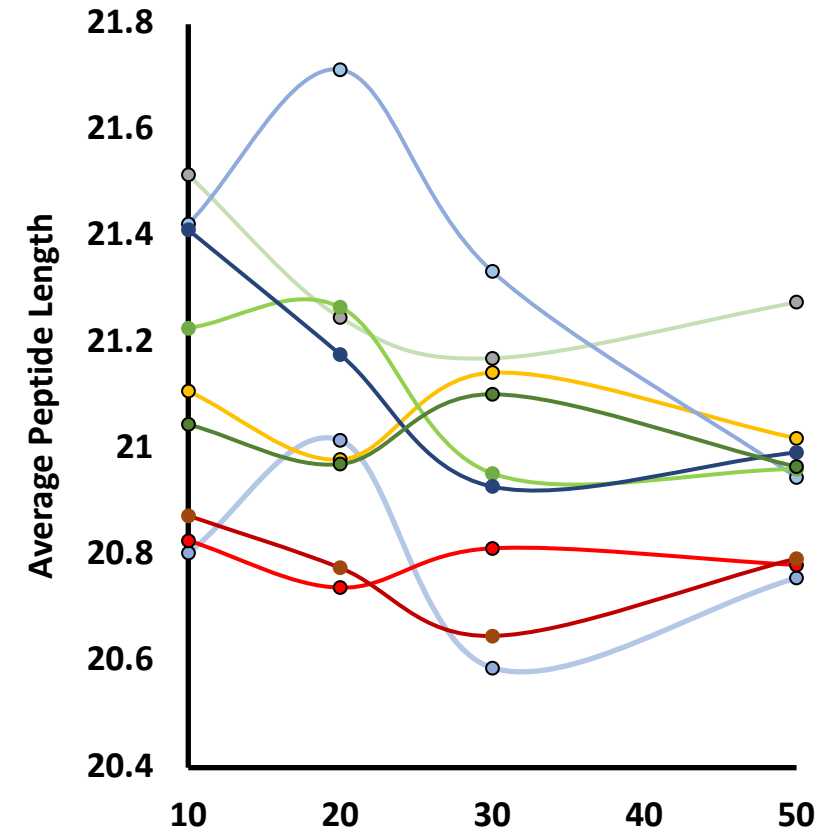


Krakatoa

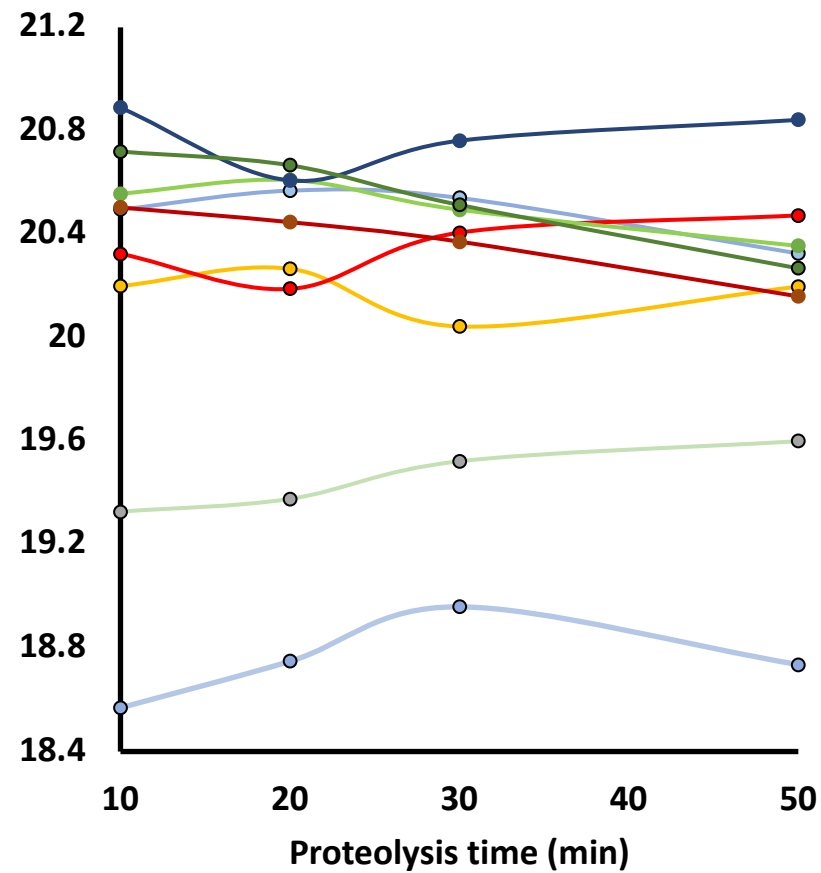


PEPTIDE LENGTH

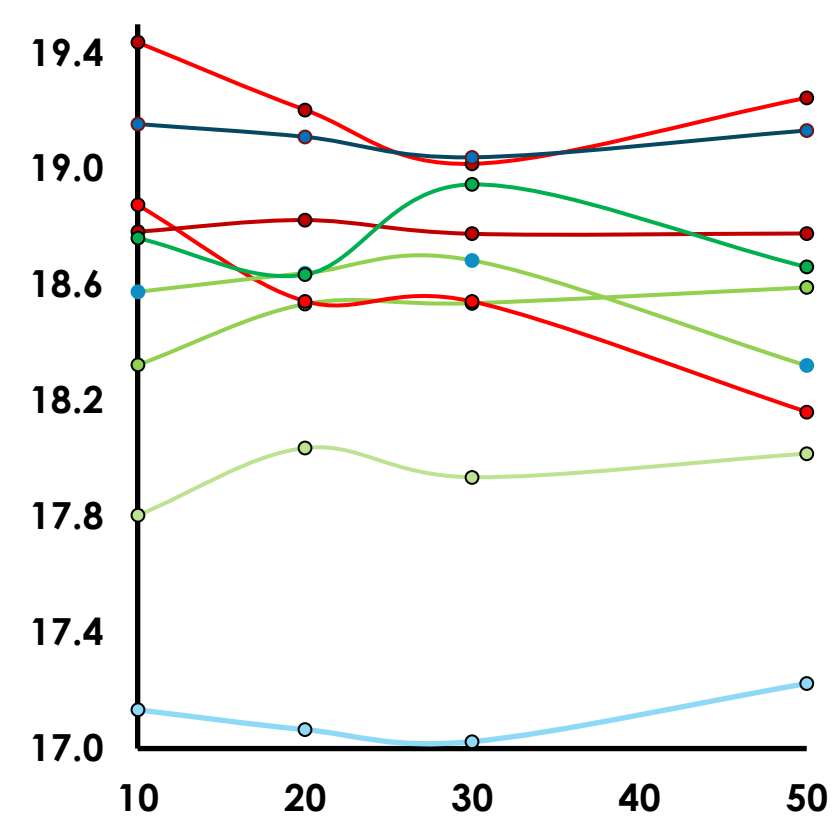
Vesuvius



AMP



Krakatoa



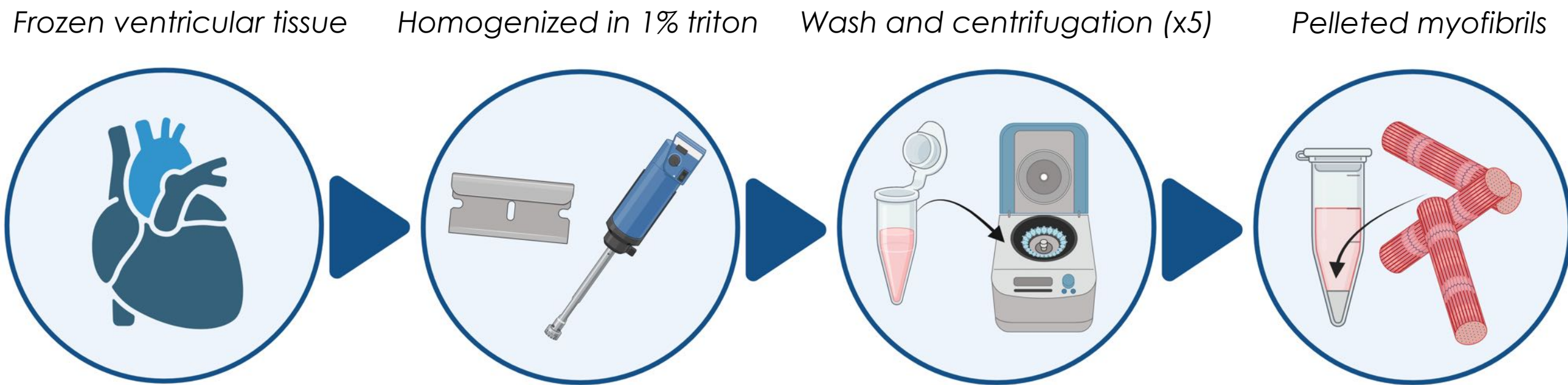
- 60 C - pH 2
- 80 C - pH 2
- 95 C - pH 2
- 60 C - pH 3
- 80 C - pH 3
- 95 C - pH 3
- 60 C - pH 4
- 80 C - pH 4
- 95 C - pH 4

PROTEIN INTENSITY

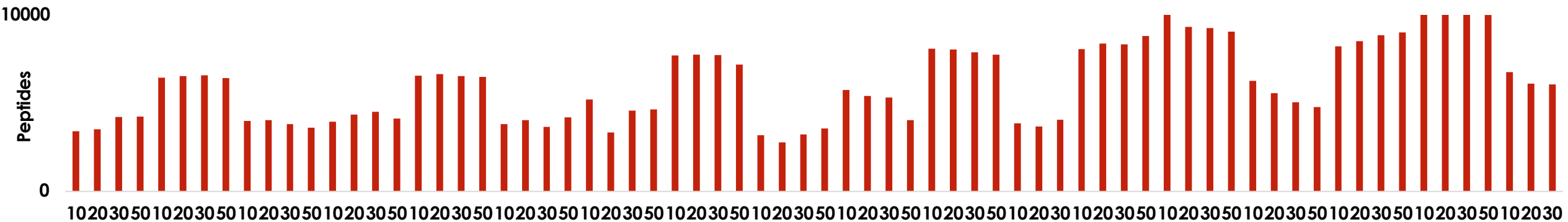
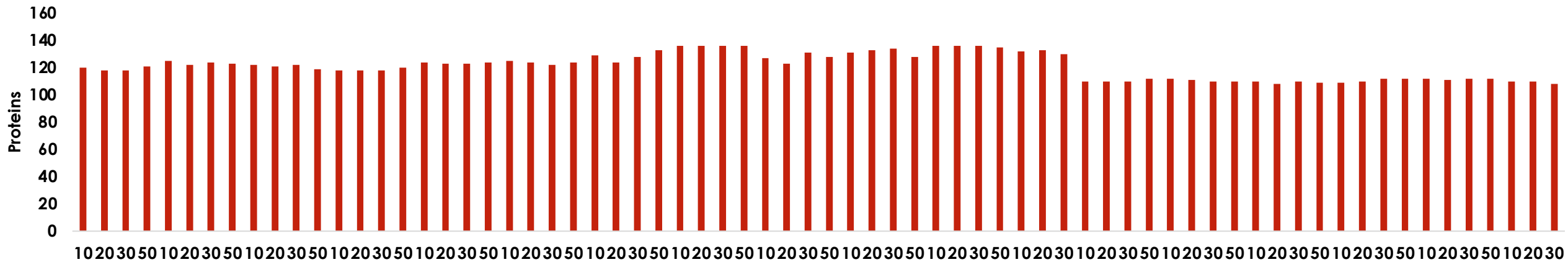
Temperature		60 C												80 C												95 C											
pH		2				3				4				2				3				4				2				3				4			
Duration		10	20	30	50	10	20	30	50	10	20	30	50	10	20	30	50	10	20	30	50	10	20	30	50	10	20	30	50	10	20	30	50	10	20	30	50
AMP	Light	7.9E +08	5.7E +08	5.9E +08	5.8E +08	1.1E +09	1.1E +09	1.2E +09	1.3E +09	7.1E +08	9.9E +08	1.1E +09	1.5E +09	1.2E +09	1.2E +09	1.2E +09	1.5E +09	1.2E +09	1.2E +09	1.2E +09	1.2E +09	1.7E +09	2.0E +09	2.1E +09	2.3E +09	1.2E +09	1.4E +09	1.4E +09	1.5E +09	1.0E +09	1.0E +09	1.0E +09	1.1E +09	1.9E +09	1.9E +09	1.9E +09	2.0E +09
	Heavy	2.0E +09	2.1E +09	2.0E +09	1.8E +09	1.1E +09	1.1E +09	1.1E +09	9.8E +08	1.5E +09	1.4E +09	1.2E +09	1.1E +09	8.5E +08	8.3E +08	7.7E +08	7.8E +08	1.1E +09	1.1E +09	1.0E +09	1.1E +09	5.6E +08	5.1E +08	5.3E +08	5.4E +08	8.2E +08	8.0E +08	8.1E +08	7.9E +08	1.1E +09	1.1E +09	1.2E +09	1.2E +09	6.1E +08	6.7E +08	6.4E +08	6.9E +08
	Combined	2.8E +09	2.7E +09	2.6E +09	2.4E +09	2.1E +09	2.2E +09	2.3E +09	2.2E +09	2.2E +09	2.4E +09	2.3E +09	2.6E +09	2.0E +09	2.0E +09	2.0E +09	2.3E +09	2.3E +09	2.3E +09	2.2E +09	2.2E +09	2.3E +09	2.5E +09	2.6E +09	2.8E +09	2.0E +09	2.2E +09	2.2E +09	2.3E +09	2.1E +09	2.2E +09	2.2E +09	2.3E +09	2.5E +09	2.6E +09	2.6E +09	2.7E +09
Kra	Light	5.2E +08	5.5E +08	5.9E +08	6.8E +08	1.4E +09	1.6E +09	1.7E +09	1.8E +09	1.0E +09	1.4E +09	1.6E +09	2.0E +09	1.7E +09	1.9E +09	1.9E +09	1.9E +09	1.7E +09	1.7E +09	1.8E +09	1.8E +09	3.2E +09	3.3E +09	3.3E +09	3.3E +09	2.1E +09	2.0E +09	2.1E +09	2.0E +09	1.6E +09	1.7E +09	1.7E +09	1.8E +09	3.2E +09	3.5E +09	3.2E +09	3.5E +09
	Heavy	3.1E +09	3.1E +09	3.1E +09	3.1E +09	2.8E +09	2.6E +09	2.5E +09	2.4E +09	2.8E +09	2.7E +09	2.6E +09	2.4E +09	2.1E +09	2.0E +09	2.1E +09	2.1E +09	2.5E +09	2.4E +09	2.4E +09	2.4E +09	2.0E +09	1.9E +09	1.9E +09	1.9E +09	2.1E +09	2.0E +09	2.0E +09	2.0E +09	2.4E +09	2.4E +09	2.3E +09	2.3E +09	1.9E +09	1.8E +09	1.8E +09	1.8E +09
	Combined	3.6E +09	3.6E +09	3.6E +09	3.7E +09	4.2E +09	4.2E +09	4.2E +09	4.2E +09	3.8E +09	4.0E +09	4.2E +09	4.4E +09	3.8E +09	3.9E +09	4.0E +09	4.0E +09	4.2E +09	4.1E +09	4.2E +09	4.1E +09	5.2E +09	5.2E +09	5.2E +09	5.3E +09	4.3E +09	4.1E +09	4.0E +09	4.0E +09	4.0E +09	4.0E +09	4.1E +09	4.1E +09	5.1E +09	5.2E +09	5.1E +09	5.3E +09
Ves	Light	1.6E +09	1.3E +09	1.2E +09	1.2E +09	1.9E +09	2.1E +09	2.1E +09	2.1E +09	2.7E +09	2.8E +09	2.9E +09	2.8E +09	1.7E +09	1.8E +09	1.9E +09	2.1E +09	1.9E +09	2.0E +09	2.0E +09	2.0E +09	3.3E +09	3.4E +09	3.4E +09	3.5E +09	2.0E +09	2.1E +09	2.3E +09	2.2E +09	1.9E +09	2.0E +09	2.0E +09	2.2E +09	3.4E +09	3.3E +09	3.2E +09	3.4E +09
	Heavy	2.6E +09	2.4E +09	2.4E +09	2.4E +09	2.0E +09	2.0E +09	1.9E +09	1.9E +09	2.0E +09	1.9E +09	1.9E +09	1.9E +09	2.2E +09	2.2E +09	2.3E +09	2.1E +09	2.0E +09	2.0E +09	2.0E +09	2.1E +09	1.8E +09	1.7E +09	1.7E +09	1.6E +09	2.2E +09	2.2E +09	2.0E +09	1.9E +09	2.1E +09	1.9E +09	1.9E +09	1.9E +09	1.7E +09	1.6E +09	1.6E +09	1.5E +09
	Combined	4.2E +09	3.7E +09	3.6E +09	3.5E +09	3.9E +09	4.1E +09	4.1E +09	4.0E +09	4.7E +09	4.7E +09	4.8E +09	4.7E +09	3.9E +09	4.0E +09	4.2E +09	4.3E +09	3.9E +09	4.0E +09	4.0E +09	4.1E +09	5.2E +09	5.1E +09	5.1E +09	5.1E +09	4.3E +09	4.3E +09	4.3E +09	4.2E +09	3.9E +09	3.9E +09	3.9E +09	4.0E +09	5.2E +09	5.0E +09	4.8E +09	4.9E +09

MYOFIBRIL PREPARATION

- Rod-like structures which can be extracted from heart muscle cells
- Made up of the contractile units of the heart (sarcomeres)
- Highly enriched in Myosin-6/7, cardiac actin, titin, tropomyosin and troponin subunits



MF IDENTIFICATIONS



2	3	4	2	3	4	2	3	4	2	3	4	2	3	4	2	3	4
80 C			95 C			80 C			95 C			80 C			95 C		
Vesuvius						AMP						Krakatoa					

CONCLUSION AND FURTHER DIRECTIONS

- The tested HTA proteases achieved 100% sequence coverage of NIST IgG and high sequence coverage of top myofilament proteins across a range of temperatures, pHs, and reaction durations.
- The profile of generated peptides was tuned by adjusting reaction parameters.

Future directions

- Establishing reproducibility for a subset of parameters.
- PTMs, induced modifications
- Quantitation of PTMs

THANKS! QUESTIONS?

- Van Eyk ACBRI Lab
- CinderBio
- US HUPO

