Development of a Robust and High-Throughput Proteomics Preparation Workflow for Bottom-Up Biomarker Analysis in Human Heart Tissue

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INTRODUCTION

Conducting bioanalysis of protein biomarkers in tissues often involves homogenization coupled with extensive purification steps followed by enzymatic digestions. The process is often labor intensive and time-consuming, which is not conducive to a high-throughput analysis for large sample sets. We sought out to develop an optimized method to generate reproducible results with reduced sample preparation time compared to current industry workflows. The optimized protocol is being applied to monitor multiple biomarkers with a data dependent (DDA) untargeted analysis to allow for post-acquisition data processing.

METHODS

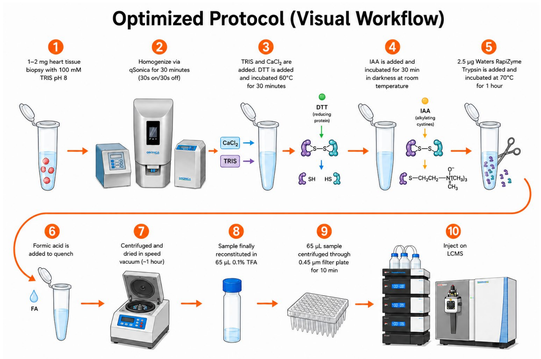
Original method parameters utilized for heart tissue sample digestions were cumbersome, tedious, and required manual intervention for multiple steps, occasionally requiring multiple analysts to complete digestions due to the length of the assay. The need for reduced sample preparation time was necessary for industry practices. One of the easiest ways to reduce assay length is to try another rapid enzyme for digestion, such as Waters RapiZyme Trypsin rather than Promega Trypsin Gold. In addition, sonication for tissue homogenization adds the ability for automation which increases robustness. Homogenization buffer, digestion length, and enzyme concentration were optimized for an untargeted protein analysis of heart tissues. In addition, sample analysis could be performed or transferred to a 96-well plate format for higher throughput.

Original vs. Optimized Protocol Comparison

ORIGINAL PROTOCOL		OPTIMIZED PROTOCOL	
Step	Duration	Step	Duration
1 1–2 mg heart tissue biopsy with RapiGest in ammonium bicarbonate	–	1 1–2 mg heart tissue biopsy with 100 mM TRIS pH 8	–
2 Incubate 50°C for 45 min	45 min	2 Homogenize via qSonica for 30 minutes (30s on/30s off)	30 min
3 DTT is added and incubated 100°C for 10 min	10 min	3 TRIS and $CaCl_2$ are added. DTT is added and incubated 60°C for 30 minutes	30 min
4 IAA is added and incubated for 30 min in darkness at room temperature	30 min	4 IAA is added and incubated for 30 min in darkness at room temperature	30 min
5 5 µg Promega Trypsin Gold is added and incubated at 37°C overnight (~16–24 hours)	16–24 h	5 2.5 µg Waters RapiZyme Trypsin is added and incubated at 70°C for 1 hour	1 h
6 Centrifuged and dried in speed vacuum (~1 hour)	1 h	6 Formic acid is added to quench	–
7 Formic acid is added and incubated at 37°C for 1 hour	1 h	7 Centrifuged and dried in speed vacuum (~1 hour)	1 h
8 Centrifuged and dried in speed vacuum (~1 hour)	1 h	8 Sample finally reconstituted in 65 µL 0.1% TFA	–
9 TFA is added and incubated at 37°C for 1 hour	1 h	9 65 µL sample centrifuged through 0.45 µm filter plate for 10 min	10 min
10 Centrifuged and dried in speed vacuum (~1 hour)	1 h	10 Grab a coffee and enjoy prior to injection ~20 minutes	~20 min
11 Sample finally reconstituted in 65 µL 0.1% TFA	–		
12 Centrifuge and transfer 55 µL aliquot to vial for analysis	10 min		
TOTAL DURATION: ~22.5 hours benchwork		TOTAL DURATION: ~4 hours benchwork	

TIME SAVINGS	STEPS REDUCED	SIMPLIFIED WORKFLOW	FASTER TO RESULTS
~82%	12 steps → 10 steps	Fewer incubations, drying steps, and reagent additions	From overnight/next-day workflow to same-day analysis
22.5 h → 4 h	(2 steps saved)		

Optimized Protocol (Visual Workflow)



RESULTS

Generation of a reliable, robust high throughput method involved several optimization parameters including sample homogenization and digestion procedures. Sample preparation time, replicate precision, and recovery of abundant proteins were utilized as markers for optimization experiments. Sonication of samples improved abundance levels and reduced variability compared to manual homogenization. Digestion buffer contents were tested for increased peptide abundances and protein sequence coverage. Enzyme choices and time of digestion played a key role in gaining greater sequence coverage of several ubiquitous and relevant proteins including albumin and collagen, which are ubiquitously present, and myosin which is one of the abundant proteins in heart tissue. For example, upon optimization, recovery for one of the abundant protein markers- Albumin, recovered 79 unique peptides with 88% sequence coverage, and 916 peptide spectral matches (PSMs) using RapiZyme trypsin enzyme while Trypsin Gold only yielded 55 unique peptides, 79% sequence coverage, and 198 PSMs. By benchmarking the recovery and consistency with abundant markers in the sample, we successfully developed a procedure to identify and semi-quantify multiple biomarkers specific to human heart.

Homogenization Optimization

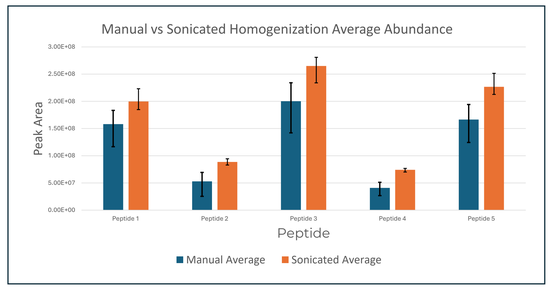


Figure 1: Average peptide abundances for N=3 replicates of heart tissue monitoring Myosin comparing manual vs sonication

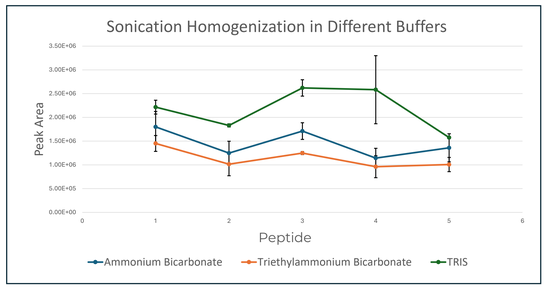


Figure 2: Heart tissues (N=2) were homogenized using qSonica with ammonium bicarbonate (ABC), triethylammonium bicarbonate (TEAB), or tris(hydroxymethyl)aminomethane (TRIS)

Digestion Optimization

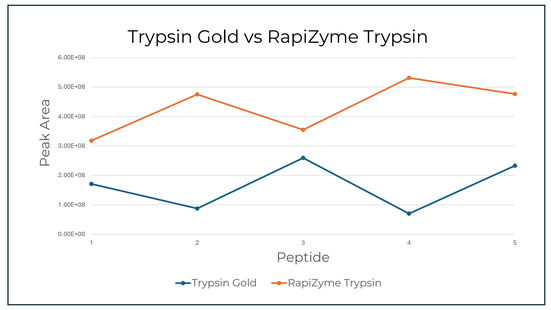


Figure 3: Heart tissues were digested with 5 µg per well of Trypsin Gold and RapiZyme Trypsin. Representative N=1

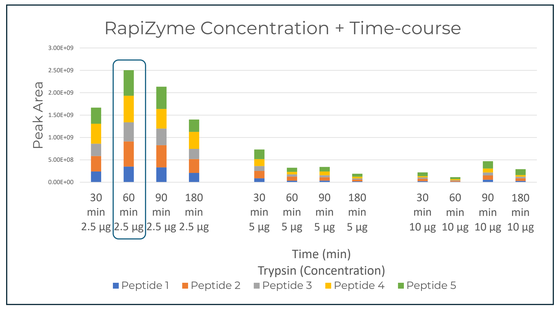


Figure 4: Comparison of RapiZyme Trypsin at 2.5, 5, and 10 µg per well for 30, 60, 90, or 180 minutes digestion time.
2.5 µg Trypsin with 60 minutes of digestion time was chosen as the best iteration.

Proteome Discoverer Results

Trypsin Gold

Accession	Description	Sum PEP Score	Coverage [%]	# Peptides	# PSMs	# Unique Peptides	Score Sequest HT
P12883	Myosin-7 [OS=Homo sapiens]	1682.549	74%	217	998	83	3059.87
P13533	Myosin-6 [OS=Homo sapiens]	1087.267	60%	153	538	24	1653.42
P02452	Collagen alpha-1(I) chain [OS=Homo sapiens]	398.245	45%	40	99	39	270.93
P02768	Albumin [OS=Homo sapiens]	372.656	79%	55	198	55	585.10

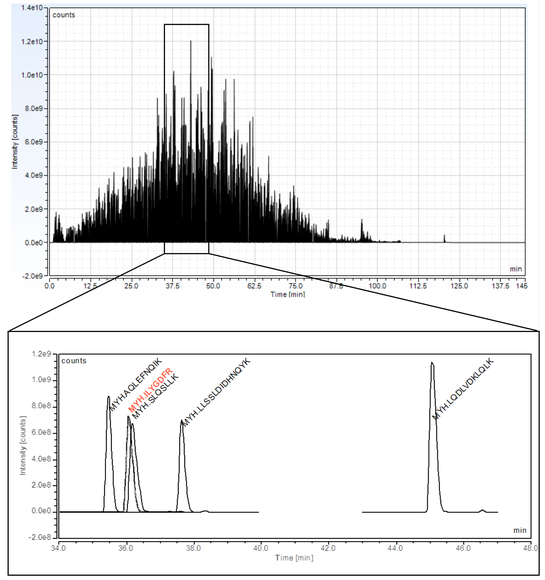
RapiZyme Trypsin

Accession	Description	Sum PEP Score	Coverage [%]	# Peptides	# PSMs	# Unique Peptides	Score Sequest HT
P12883	Myosin-7 [OS=Homo sapiens]	2645.712	84%	313	3642	116	12598.61
P13533	Myosin-6 [OS=Homo sapiens]	1676.126	71%	224	2065	36	6835.36
P02452	Collagen alpha-1(I) chain [OS=Homo sapiens]	672.079	61%	79	618	77	1670.26
P02768	Albumin [OS=Homo sapiens]	569.626	88%	79	916	79	2840.15

Figure 5: Processed results of human heart tissue using Proteome Discoverer. RapiZyme Trypsin has higher overall Sequest HT Scores, unique peptides, and coverage compared to Trypsin Gold

DATA PROCESSING

Samples were injected using a Thermo Vanquish Horizon liquid chromatograph coupled with a Thermo Exploris 240 Orbitrap mass spectrometer. A Waters Acquity Peptide HSS T3, 150x1.0-mm, 1.8µm column was utilized at a flow rate of 100 µL/min.



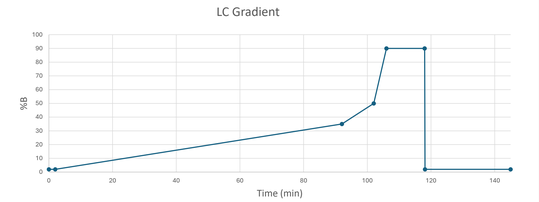


Figure 6: (Top) Total Ion Chromatogram (TIC) of a representative human heart tissue digest. (Middle) Extracted Ion Chromatogram (XIC) of 5 Myosin peptides with 5 ppm mass window. (Bottom) LC Gradient utilized.

Post processing was performed using Thermo Proteome Discoverer version 3.3. The Sequest(HT) algorithm was utilized with a 10-ppm precursor tolerance and 0.02-Da fragment mass tolerance with the Homo Sapiens (sp_canonical) protein database.

CONCLUSION

A successful high-throughput sample preparation method was developed to analyze biomarkers in human heart tissue by bottom-up proteomics. Benefits of the optimized method include decreased bench-time due to the rapid digestion duration, reduced variability between sample homogenizations due to sonication, increased sensitivity on the mass spectrometer, higher protein coverage, more peptide spectral matches, and increased sample-throughput based on the 96-well plate format.

The authors declare no competing financial interest.