Development Of A Quantitative Hybrid LC-MS/MS Method For Multiple High Abundant Protein Biomarkers In Human Heart Tissue

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INTRODUCTION

Biomarker assay development in tissues can pose multiple challenges including complete extraction of the protein from the tissue, wide endogenous ranges, immunocapture (IP) reagent availability, co-interactions with other proteins limiting successful IP extraction. BAG3 is a clinically relevant cardiac biomarker, with mutations linked to familial dilated cardiomyopathy, and exists in complex with its binding partner HSPB8 protein. Simultaneous quantitation of both proteins from limited heart tissue is analytically challenging. Current work showcases the challenges in the development of a multiplexed hybrid LC-MS/MS assay to quantify levels of both BAG3 and HSPB8 from a single human heart tissue sample using complementary offline and online immunoaffinity enrichment strategies. Method development included optimization of tissue homogenization process, identifying the right surrogate matrix, and evaluating multiple enrichment strategies. Upon method optimization, a combined offline and online approach was adopted from a single post-digest sample split, minimizing sample consumption while maintaining assay performance.

CONSTRAINTS AND CONSIDERATIONS

Key Considerations for LC–MS/MS Protein Biomarker Assay Development

When developing an assay for the quantification of protein biomarkers using LC–MS/MS, several factors play a key role in ensuring the assay is robust, reproducible, and fit for its intended context of use. Below is a checklist to consider during **method development**.

1. MATRIX

- **Availability of Matrix:** Rare vs readily available
- **Sourcing Healthy vs Diseased Matrix:** Consider biological relevance and consistency of source
- **If Tissue Matrix:** Homogenization buffer and ratio for consistent recovery

2. ANALYTE

- **Endogenous:** Identification of right surrogate matrix
- **Abundance:** Low abundant or high abundant? Need for parallelism and identifying the right diluent
- **Availability of Protein Standard:** Recombinant vs cell lysate vs peptide standard
- **Matrix Effect:** "Normal levels" vs Disease state levels

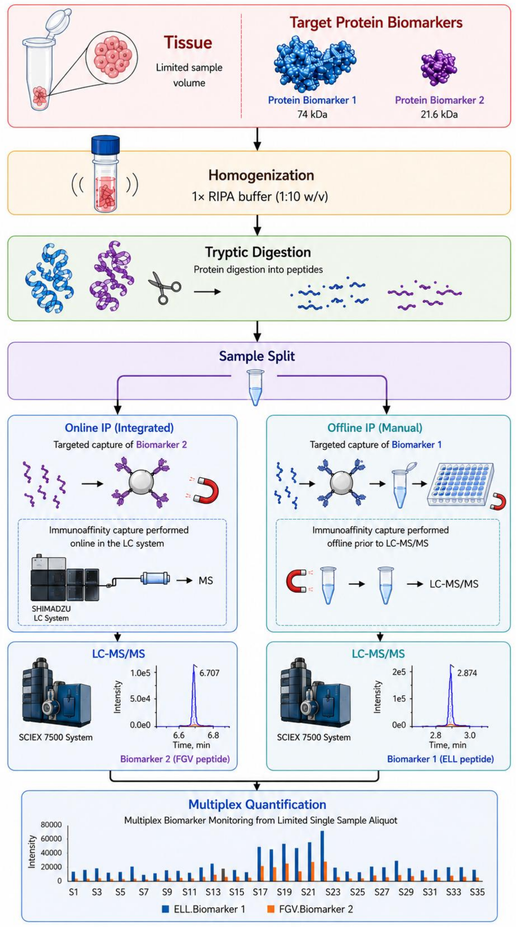
3. ASSAY CONSIDERATIONS

- **Availability of capture reagents:** Antibody or affinity reagent availability, specificity, and performance
- **Multiplexing:** Basal levels of each biomarker and range identification
- **Establishing Fit–for–Purpose Quality Controls:** Consideration to see if 1 set can work for both biomarkers
- **Need for unique peptide to monitor** vs any peptide from the biomarker that ionizes well
- **Other assay parameters:** Selectivity, Carryover, Dilution integrity, Stability

Careful consideration of these factors during assay development will help ensure a **robust, reproducible**, and **fit–for–purpose** LC–MS/MS assay for protein biomarker quantification.

Human Heart Tissue		
Biomarker 1		**Biomarker 2**
460 - 5000 ng/mL	"Normal" Level Ranges	280 - 3000 ng/mL
5-5120 ng/mL	Std Curve Range	5-5120 ng/mL
50x diluted	Quality control basal sample	50x diluted
1x RIPA Buffer	Diluent	1xRIPA Buffer
non-dilute to 200x dilute	Parallelism	non-dilute to 200x dilute
Direct digest with anti-peptide Ab IP	Extraction	Direct digest with anti-peptide Ab IP
Offline anti-peptide Ab IP to minimize Carryover issues	Approach Utilized	On-line Ab column to maximize Recovery

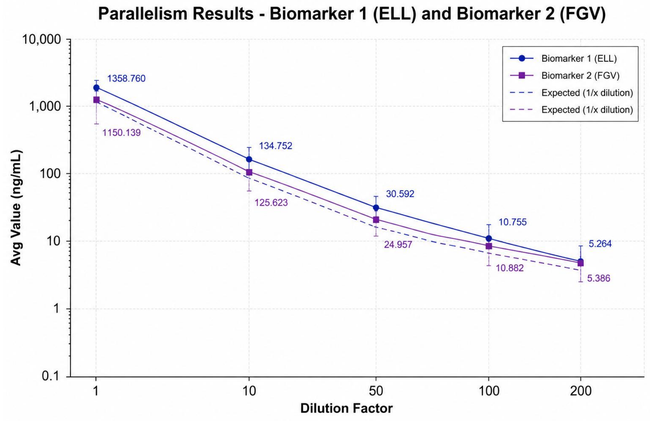
HYBRID LC-MS/MS WORKFLOW WITH PEPTIDE-LEVEL CLEAN-UP



METHOD OPTIMIZATION DATA

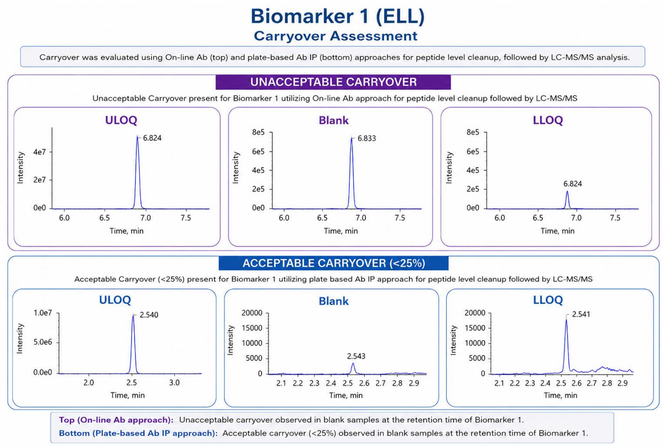
Parallelism

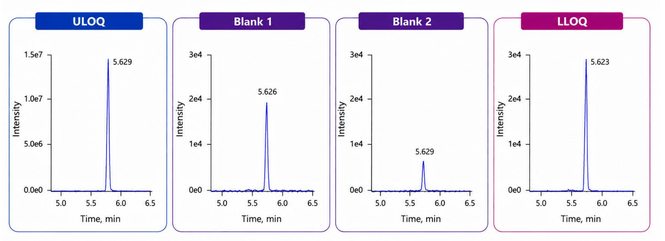
Parallelism was successfully established up to 200-fold for both biomarkers



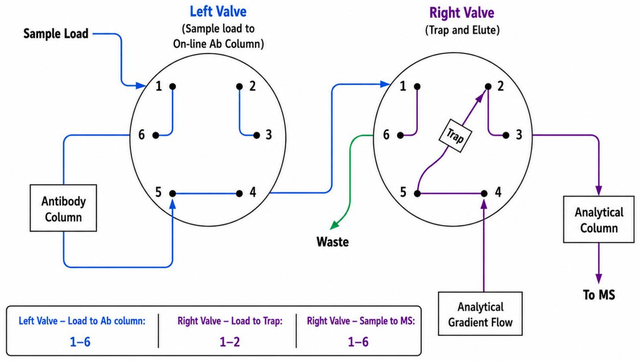
Carryover

Single sample was digested and split assay was developed to tackle carryover issue with biomarker 1. A plate-based anti-peptide IP eliminated the need for on-line Ab column and trap before MS analysis. Cause for carryover was identified to be with Ab column and trap for ELL peptide





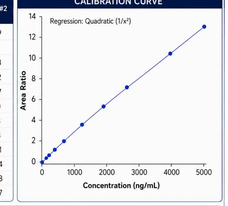
Online Ab column, Trap and Elute Configuration



RESULTS

BIOMARKER 1 PRECISION AND ACCURACY

Actual Concentration (ng/mL)	Num. Values	Mean	%CV	% Accuracy	Value #1	Value #2
5	2 of 2	5.14	4.1	102.8	4.99	5.29
10	2 of 2	9.69	0.3	96.9	9.67	9.71
20	2 of 2	19.3	3.6	96.7	18.8	19.8
40	2 of 2	38.4	4.5	96.0	39.6	37.2
80	2 of 2	81.2	4.3	101.5	78.8	83.7
160	2 of 2	160	0.0	99.8	160	160
320	2 of 2	320	1.4	99.9	316	323
640	2 of 2	663	1.0	103.6	658	668
1280	2 of 2	1326	3.7	103.6	1360	1291
2560	2 of 2	2609	4.1	101.9	2684	2534
4500	2 of 2	4352	3.7	96.7	4467	4238
5120	2 of 2	5152	0.2	100.6	5146	5157



BIOMARKER 1 QUALITY CONTROLS IN 50x DILUTED HUMAN HEART HOMOGENATE

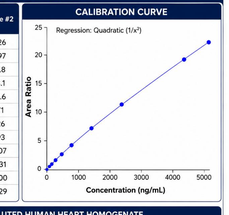
QC Level	Actual Concentration (ng/mL)	Adjusted Concentration (ng/mL)	Mean	% CV	% Accuracy	Value #1	Value #2	Value #3
Basal	20.8	20.8	20.8	2.2	100.0	20.3	21.2	21.0
Low QC	15.0	35.8	35.0	4.6	97.7	35.4	33.2	36.4
Mid QC	2000	2021	2007	3.1	99.3	2052	1936	2033
High QC	4000	4021	3884	5.2	96.6	3905	4073	3674

Surrogate Curve and Matrix Quality Controls

1x RIPA buffer was used as a surrogate matrix for a 2-plex assay. Based on the endogenous values detected, standard curve range was set from 5-5,120 ng/mL. Due to limited tissue availability, single sample volume of 25 µL was utilized to quantitate levels of both biomarkers implementing a split assay format. A 50x dilution was employed to prepare matrix QCs to bring the quantitation levels within the range of the curve. This decision was supported with the parallelism data obtained.

BIOMARKER 2 PRECISION AND ACCURACY

Actual Concentration (ng/mL)	Num. Values	Mean	%CV	% Accuracy	Value #1	Value #2
5	2 of 2	5.17	2.4	103.4	5.09	5.26
10	2 of 2	9.52	8.2	95.2	10.1	8.97
20	2 of 2	19.3	3.6	96.3	18.8	19.8
40	2 of 2	39.0	3.3	97.5	39.9	38.1
80	2 of 2	80.9	3.0	101.1	79.2	82.6
160	2 of 2	165	4.9	103.3	159	171
320	2 of 2	318	3.7	99.4	310	326
640	2 of 2	667	5.5	104.2	641	693
1280	2 of 2	1289	1.9	100.7	1271	1307
2560	2 of 2	2556	1.4	99.9	2581	2531
4500	2 of 2	4367	2.2	97.1	4435	4300
5120	2 of 2	5217	3.0	101.9	5105	5329



BIOMARKER 2 QUALITY CONTROLS IN 50x DILUTED HUMAN HEART HOMOGENATE

QC Level	Actual Concentration (ng/mL)	Adjusted Concentration (ng/mL)	Mean	% CV	% Accuracy	Value #1	Value #2	Value #3
Basal	9.78	9.78	9.78	2.1	100.0	9.73	9.60	10.0
Low QC	15.0	24.8	23.6	4.4	95.1	23.6	22.5	24.6
Mid QC	2000	2010	2042	2.8	101.6	2067	1978	2082
High QC	4000	4010	3955	2.9	98.6	3852	4076	3937

CONCLUSION

A multiplexed hybrid LC-MS/MS method was successfully developed and fully validated for a fit for purpose context of use (CoU) format for the simultaneous quantitation of BAG3 and HSPB8 in human heart tissue over a concentration range of 5–5,120 ng/mL with an acceptance criteria set at ±25%. During method development, parameters evaluated included optimal homogenization ratio and buffer selection, surrogate matrix, quality control concentrations, parallelism, selectivity, accuracy and precision, sensitivity, matrix effects, stability, dilution integrity, and reference standard performance. The validated multiplex assay was further qualified in non-human primate and mouse heart and liver tissues, demonstrating translatability across species and matrices. A single-sample, split-workflow multiplex biomarker quantitative hybrid LC-MS/MS method in tissue samples using online and offline IP in tandem was successfully developed and validated under ICH M10 guidance.

The authors declare no competing financial interest.