Method Development and Validation for the Determination of Two Protein Biomarkers by Multiplexed Online and Offline Hybrid LC-MS/MS in Human Heart Tissue

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Summary

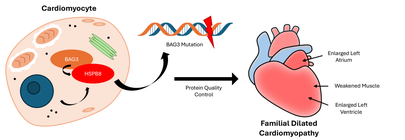
This study describes the development and validation of a multiplex LC-MS/MS assay to detect the protein biomarker BAG3 and its binding partner HSPB8 in heart tissue. BAG3 mutations are associated with familial dilated cardiomyopathy, making it an important biomarker for heart disease research. To overcome common challenges in multiplex protein assays, such as limited sensitivity, variable dynamic ranges, and interference from binding proteins, a hybrid workflow that analyzes a single digested tissue sample using both online antibody column anti-peptide enrichment and offline bead-based anti-peptide enrichment prior to LC-MS/MS analysis was developed. Earlier strategies using only one enrichment method showed limitations, including analyte carryover and low recovery, but combining online and offline approaches enabled reliable detection of both proteins. The final method was fully validated according to fit-for-purpose ICH M10 guidelines, demonstrating acceptable selectivity, accuracy, precision, sensitivity, and stability across a concentration range of 5 - 5,120 ng/mL in human heart tissue. Additionally, the assay was successfully translated to other species and matrices, including mouse and non-human primate tissues. Overall, this novel hybrid LC-MS/MS approach enables sensitive and selective quantification of multiple protein biomarkers from a single tissue sample with minimal processing.

Novelty

- Development and validation of a multiplexed protein biomarker in tissue using **simultaneous online and offline hybrid (anti-peptide immunoprecipitation)** LC-MS/MS approaches
- Tissue assay based on fit-for-purpose (CoU) application of ICH M10 guidelines
- Single sample processing (post-digest split) into online and offline hybrid LC-MS/MS approaches
- Translation to multiple species and matrices (mouse, NHP, human)
- Application of custom packed anti-peptide antibody column as well as offline bead-based anti-peptide enrichment post digestion of a single sample to detect a protein and its binding partner

Biological Background

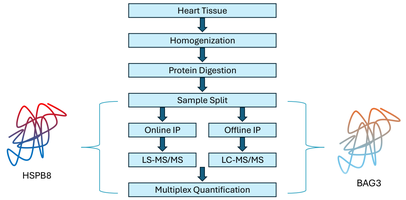
Multiplex protein biomarker assays in tissue present several analytical challenges, including limited sensitivity, wide dynamic ranges, the need for multiple immunocapture reagents, and potential interference when target proteins are bound to binding partners. BAG3 is a protein associated with familial dilated cardiomyopathy when mutated and interacts with the binding protein HSPB8, making both proteins important biomarkers for studying heart disease. However, accurately quantifying both proteins simultaneously in tissue can be difficult due to these methodological limitations, highlighting the need for improved analytical approaches capable of sensitive and selective multiplex detection.



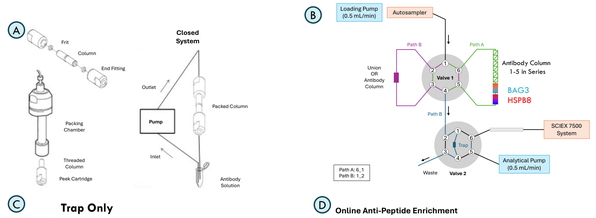
Methods

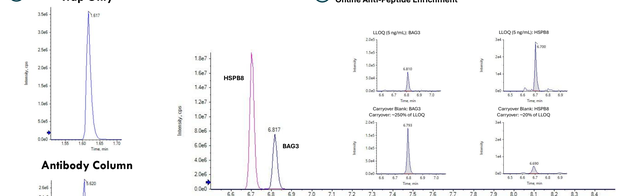
Method development included the assessment of various strategies to assay both proteins from a single sample. Online Anti-Peptide Enrichment - Strategies utilizing a custom packed online anti-peptide antibody column were initially investigated with variable success due to analyte carryover observed for BAG3 arising from the antibody column trap-and-elute configuration. Offline Anti-Peptide Enrichment - Next, a 2-plex approach utilizing offline bead-based anti-peptide antibody enrichment was tried again with limited success due to low recovery and sensitivity for HSPB8. The reasons for the various failures are shown in this poster which led to the ultimate approach. Multiplexed Online and Offline Anti-Peptide Enrichment - The final method utilized a combination of online and offline immunoprecipitation approaches which successfully allowed the assay to be developed and fully validated for both proteins according to fit-for-purpose (CoU) ICH M10 guidelines.

Approximately 10 mg of human heart tissue was homogenized in RIPA buffer using the Precellys Evolution Touch Homogenizer. The supernatant of the sample was isolated and subjected to heat denaturation for 10 minutes at 95°C. The sample was then reduced and alkylated and digested with RapiZyme Trypsin for 60 minutes at 70°C. After digestion, the sample was quenched for 15 minutes at 95°C and split into 2 different immunoaffinity workflows, one utilizing an online antibody column hybrid LC-MS/MS method and one utilizing an offline hybrid (anti-peptide) LC-MS/MS bead-based enrichment with Magne™ Protein G Beads using a KingFisher Flex system. The online sample workflow was injected directly onto the multidimensional LC-MS/MS system (schematic depicted in Figure A). The offline sample workflow was injected onto a direct inject LC-MS/MS system (schematic depicted in Figure E).



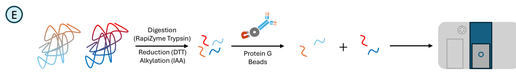
Online Anti-Peptide Enrichment

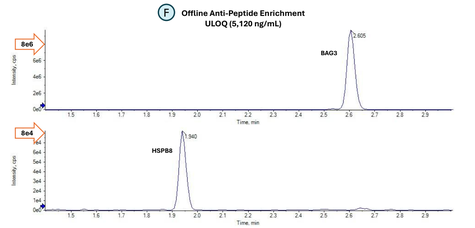




- **A** – Schematic illustrating the techniques used to custom pack an online antibody column
- **B** – Valve configuration of online antibody column setup (multiple pumps and multiple valves allowing assessment of the antibody column without altering any physical plumbing)
- **C** – Antibody column performance assessment comparing trap-and-elute and antibody column conditions; ~74% recovery was observed with the antibody column relative to trap-and-elute as shown in Figure C.
- **D** – During method development, online anti-peptide enrichment was evaluated for BAG3 and HSPB8. While both showed acceptable recovery, BAG3 exhibited significant carryover of ~250% of the LLOQ as shown in Figure D.

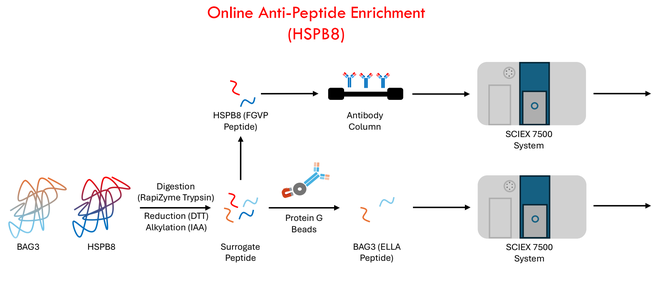
Offline Anti-Peptide Enrichment





- **E** – Figure E depicts a schematic of the offline bead-based anti-peptide enrichment workflow. During method development, offline enrichment was evaluated for both BAG3 and HSPB8.
- **F** – HSPB8 showed significantly lower recovery with the approach. At the ULOQ (5,120 ng/mL), HSPB8 signal (8e4) indicated insufficient sensitivity suggesting that this approach was not suitable to achieve an LLOQ of 5 ng/mL.

Ⓖ Final Method: Multiplexed Online and Offline Anti-Peptide Enrichment



- **G** – Schematic of hybrid workflow combining online and offline anti-peptide enrichment
  - By using this approach, we were able to significantly reduce the BAG3 carryover observed with online enrichment.
  - This method exhibited sufficient recovery of both BAG3 and HSPB8 enabling an LLOQ of 5 ng/mL.
- Skyline analysis was used to select optimal peptides and fragment ions for sensitivity
  - HSPB8 (FGVP peptide) quantified using online IP
  - BAG3 (ELLA peptide) quantified using offline IP

Results

The method was developed and fully validated for a 5 - 5,120 ng/mL curve range in human heart tissue meeting fit-for-purpose acceptance criteria of 25%. According to the ICH M10 guidelines, selectivity, accuracy, precision, linearity, sensitivity, matrix effects, stability, dilution integrity, parallelism, and reference standards were all validated for the context of use of this protein biomarker multiplex. It should be noted that the validation data did meet the more stringent acceptance criteria of ± 20% from nominal (± 25% at LLOQ) for both proteins but we adhered to the CoU acceptance set to 25% for future sample analysis.

Summary of Standard Curves - BAG3

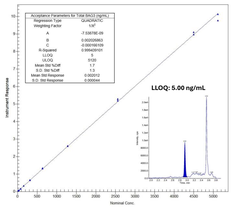
Calibration Standards:	Cal 5	Cal 10	Cal 20	Cal 40	Cal 80	Cal 160	Cal 320	Cal 640	Cal 1280	Cal 2560	Cal 4500	Cal 5120
	5.00 ng/mL	10.0 ng/mL	20.0 ng/mL	40.0 ng/mL	80.0 ng/mL	160 ng/mL	320 ng/mL	640 ng/mL	1280 ng/mL	2560 ng/mL	4500 ng/mL	5120 ng/mL
Mean	5.14	9.62	19.6	39.2	79.8	159	323	658	1310	2620	4450	5060
S.D.	0.236	0.342	0.62	0.957	1.47	3.85	4.29	10.9	16.3	69.2	108	99.7
%CV	4.6	3.6	3.2	2.4	1.8	2.4	1.3	1.7	1.2	2.6	2.4	2
%Bias	2.8	-3.8	-2	-2	-0.3	-0.6	0.9	2.8	2.3	2.3	-1.1	-1.2
n	6	6	6	6	6	6	6	6	6	6	6	6

Summary of Standard Curves - HSPB8

Calibration Standards:	Cal 5	Cal 10	Cal 20	Cal 40	Cal 80	Cal 160	Cal 320	Cal 640	Cal 1280	Cal 2560	Cal 4500	Cal 5120
	5.00 ng/mL	10.0 ng/mL	20.0 ng/mL	40.0 ng/mL	80.0 ng/mL	160 ng/mL	320 ng/mL	640 ng/mL	1280 ng/mL	2560 ng/mL	4500 ng/mL	5120 ng/mL
Mean	5.05	9.95	19.7	39.3	79.7	159	321	648	1310	2600	4490	5060
S.D.	0.16	0.239	0.378	0.935	1.14	3.06	5.34	8.07	33.5	51	113	167
%CV	3.2	2.4	1.9	2.4	1.4	1.9	1.7	1.2	2.6	2	2.5	3.3
%Bias	1	-0.5	-1.5	-1.8	-0.4	-0.6	0.3	1.3	2.3	1.6	-0.2	-1.2
n	6	6	6	6	6	6	6	6	6	6	6	6

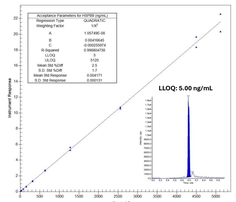
Offline IP

Calibration Curve for BAG3



Online IP

Calibration Curve for HSPB8



Accuracy and Precision (Inter-Assay) - BAG3

Surrogate Matrix:	Surr QC 5	Surr QC 15	Surr QC 200	Surr QC 2000	Surr QC 4000
	5.00 ng/mL	15.0 ng/mL	200 ng/mL	2000 ng/mL	4000 ng/mL
Mean Concentration Found (ng/mL)	5.36	14.7	199	2050	3960
Inter-run SD	0.215	0.536	5	45.8	115
Inter-run %CV	4	3.6	2.5	2.2	2.9
Inter-run %Bias	7.2	-2	-0.5	2.5	-1
n	15	15	15	15	15

Accuracy and Precision (Inter-Assay) - HSPB8

Surrogate Matrix:	Surr QC 5	Surr QC 15	Surr QC 200	Surr QC 2000	Surr QC 4000
	5.00 ng/mL	15.0 ng/mL	200 ng/mL	2000 ng/mL	4000 ng/mL
Mean Concentration Found (ng/mL)	5.54	14.9	203	2050	3950
Inter-run SD	0.275	0.439	3.29	40.1	95.5
Inter-run %CV	5	2.9	1.6	2	2.4
Inter-run %Bias	10.8	-0.7	1.5	2.5	-1.3
n	15	15	15	15	15

Accuracy and Precision (Inter-Assay) - BAG3

True Matrix:	True QC 0	True QC 15	True QC 200	True QC 2000	True QC 4000
	26.6 ng/mL	41.6 ng/mL	226 ng/mL	2030 ng/mL	4020 ng/mL
Mean Concentration Found (ng/mL)	27.3	41.1	224	2010	3940
Inter-run SD	1.38	0.556	4.19	42.2	109
Inter-run %CV	5.1	1.4	1.9	2.1	2.8
Inter-run %Bias	2.6	-1.2	-0.9	-1	-2
n	15	15	15	15	15

Accuracy and Precision (Inter-Assay) - HSPB8

True Matrix:	True QC 0	True QC 15	True QC 200	True QC 2000	True QC 4000
	13.2 ng/mL	28.2 ng/mL	213 ng/mL	2010 ng/mL	4010 ng/mL
Mean Concentration Found (ng/mL)	14.4	28.4	211	2000	3930
Inter-run SD	1.45	1.32	4.52	49.2	96
Inter-run %CV	10.1	4.6	2.1	2.5	2.4
Inter-run %Bias	9.1	0.7	-0.9	-0.5	-2
n	15	15	15	15	15
# > 25%Bias					

Conclusions

This study successfully developed and validated a multiplex LC-MS/MS assay capable of quantifying the protein biomarkers BAG3 and HSPB8 in human heart tissue using a hybrid workflow that combines online antibody column anti-peptide enrichment and offline bead-based anti-peptide enrichment. The method demonstrated sensitive, selective, and reliable performance across a wide concentration range and met fit-for-purpose ICH M10 validation criteria. Additionally, the assay was qualified for use in multiple species and tissue matrices, including human, mouse, and non-human primate samples. This approach enables efficient multiplex protein biomarker analysis from a single tissue digest with minimal sample handling.