

Development of a Multiplexed SRM Assay for Osteoarthritis Biomarkers

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Overview

Purpose: Develop an SRM assay for osteoarthritis biomarkers.

Methods: A panel of proteotypic peptides derived from differentially expressed proteins in synovial fluid samples from osteoarthritis patients were developed into an SRM assay. Synthetic heavy labeled peptides were used as quantitative standards.

Results: The SRM assay panel of synovial fluid peptides was successfully applied to clinical synovial fluid samples.

Introduction

Osteoarthritis (OA) is a prevalent, poorly understood disease whose management would be significantly advanced by the development of assays for early diagnosis and/or disease prognosis. Previous discovery work has identified candidate protein biomarkers for OA (1) including afamin and proteoglycan 4. Recently, emphasis has been placed on advancing proteomics studies from discovery and/or relative quantification to validated quantitative methods in an effort to establish clinical assays. Targeted selective ion monitoring (SRM) assays provide a vehicle for cost effective, high-throughput quantification and monitoring of specific disease biomarkers. In this study, we applied novel software to integrate information from MS/MS discovery spectra generated on a Thermo Scientific LTQ Orbitrap™ platform to facilitate the development of osteoarthritis specific SRM assays on a TSQ Quantum Ultra™ triple quadrupole mass spectrometer. The SRM assays were used to interrogate synovial fluid patient samples and demonstrate the applicability of this approach for potential OA screening.

Methods

Clinical samples

Synovial fluid samples were collected from participants with full consent and approval under IRB protocols. The study set consisted of 4 normal and 12 late stage Osteoarthritis patient synovial fluid samples.

Sample preparation

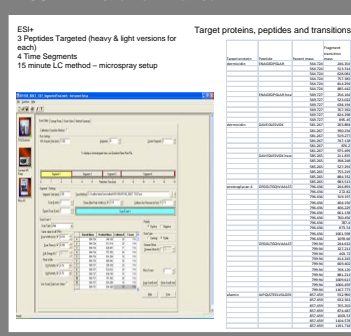
Add 25 μ L of synovial fluid (protein concentration: 40mg per mL) using a wide bore tip to 100 μ L 8 M GuHCl/150 mM Tris/10 mM DTT pH 8.5 and vortex aggressively. Incubate at 37 °C for 60 minutes. Remove and cool to room temperature. Add 11 μ L of 500 mM iodoacetic acid/1 M Tris pH 8.5 to each sample. Final concentration = 45 mM. Alkylate in the dark for 60 minutes. Quench reaction with the addition of 2 μ L of 2 M DTT. Final concentration = 5 mM. Dilute samples to 3 mL with the addition of 50 mM Tris/5 mM CaCl₂ pH 8.0. Add 20 μ g of sequencing grade trypsin (Promega). Incubate at 37 °C for 24 hours. Quench reaction with the addition of TFA to 1%. Using a Thermo Scientific Hypersil Gold™ C18 SPE 100 mg column. Clean up each digest sample by equilibrating the column with 1 x ACN followed by 3 x 0.25% TFA/water. Load sample, rinse column with 5 x 0.25%TFA/water. Elute using 2 x 100 μ L Final protein concentration: 5 μ g per 1 μ L

SRM assays

SRM assays were developed on a Thermo Scientific TSQ Quantum Ultra triple quadrupole mass spectrometer, Surveyor MS pump, Micro Autosampler and an IonMax Source equipped with a low flow metal needle. Reverse phase separations were carried out on a 1mm X 50mm Hypersil Gold 1.9 μ m C18 particle. Solvent A was LC-MS grade water with 0.2% (v/v) formic acid, and solvent B was LC-MS grade 30% (v/v) acetonitrile with 0.2% (v/v) formic acid (Optima grade reagents, Fisher Scientific).

Thermo Scientific SRM Workflow prototype software (available upon request) was used for targeted protein quantification. This software algorithm is used to predict candidate peptides and for choosing multiple fragment ions for SRM assay design, building an instrument method and a sequence file, and also for automatic peptide identity confirmation and quantitative data processing. For the workflow described herein, the differentially expressed peptides that were identified in the LC-MS/MS discovery MS data were imported directly into SRM builder software. Transitions were chosen based on the predominant fragments observed in the discovery data (>5 transitions per peptide). Peptides were identified by co-eluting light and heavy transitions derived from synthetic peptide standards.

FIGURE 1. Instrument method



Results

FIGURE 2. LC methods

A: Water 0.2% FA
B: ACN 0.2% FA

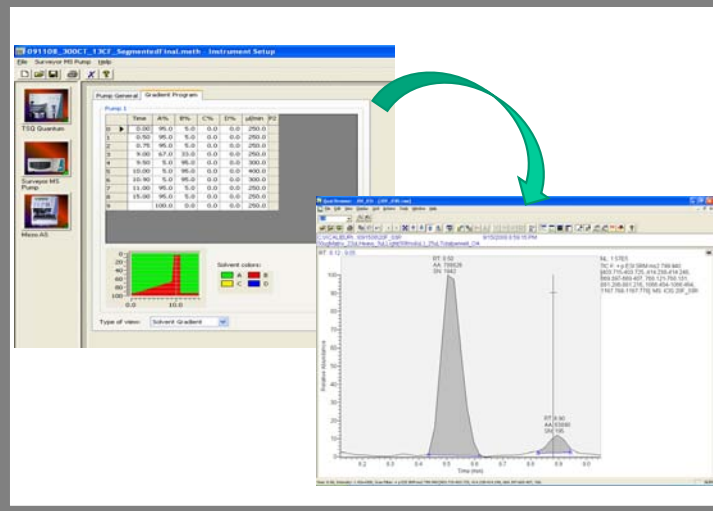
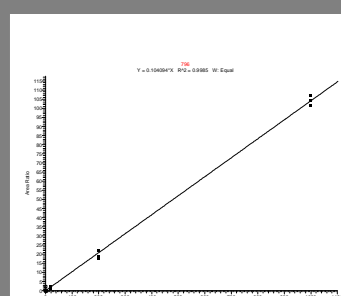


FIGURE 3. Peak width

Peak width 10-13 seconds
No smoothing
9 scans across the peak
0.100s scan time per transition
All transitions summed per peptide

FIGURE 4. Calibration curves for target peptide

A. Neat peptide 769
LOD 200 attomole on column
LOQ 500 attomole on column



B. Peptide 769 in matrix
Heavy Constant/Light Varied
in 50 μ g Matrix

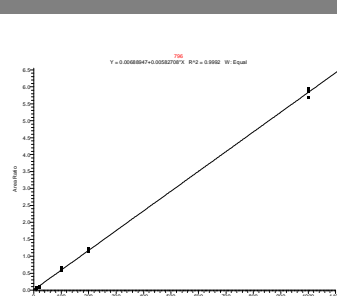


FIGURE 5. Internal standard reproducibility

CVs less than 15%

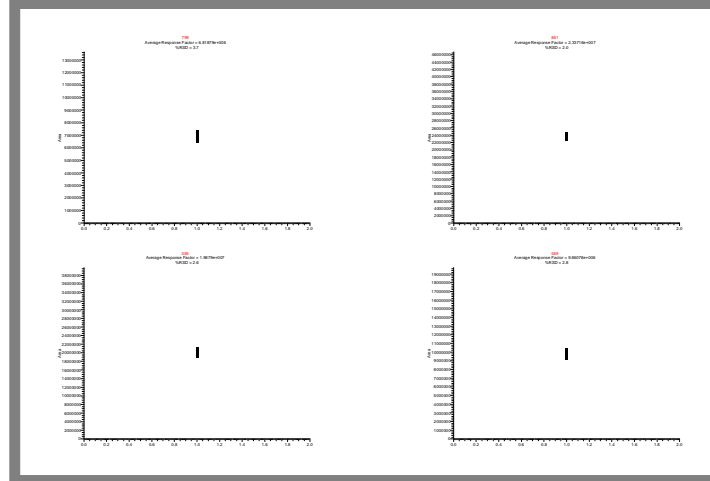


FIGURE 6. Overlaid transitions for peptide at 796.436 m/z

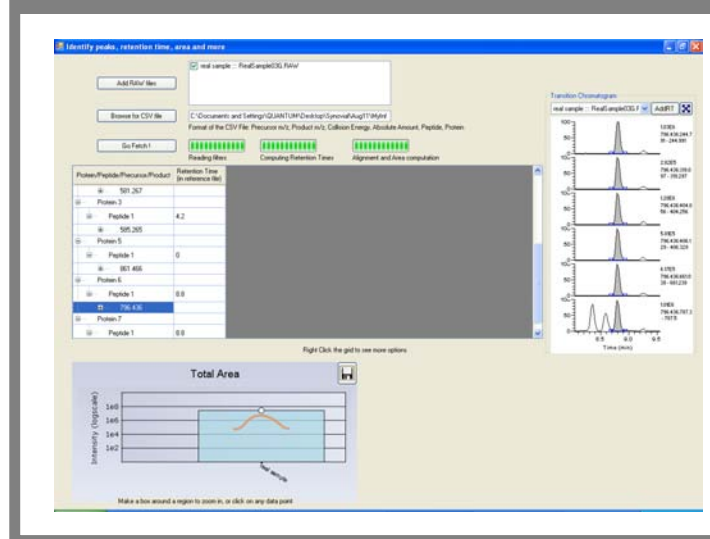


FIGURE 7. Reproducibility of the assay

Samples	Concentration fmol on column	%CV
Realt01-1	19.67705012	15.04550004
Realt01-2	22.99322395	
Realt01-3	16.93172492	
Realt02-1	20.46779713	13.52160444
Realt02-2	15.9790745	
Realt02-3	18.39680434	
Realt03-1	14.50582975	8.593368411
Realt03-2	17.17938933	
Realt03-3	16.42658485	
Realt04-1	20.85621553	27.37228961
Realt04-2	14.96462362	
Realt04-3	24.85198403	
Realt05-1	31.80237169	19.9947663
Realt05-2	21.87363497	
Realt05-3	31.64143519	
Realt06-1	47.24274124	2.27405372
Realt06-2	45.16403949	
Realt06-3	48.49501995	
Realt07-1	27.35625462	24.88928564
Realt07-2	20.87544162	
Realt07-3	34.61469316	
Realt08-1	18.6914715	13.21588261
Realt08-2	14.65633161	
Realt08-3	18.53381733	
Realt09-1	35.91239532	5.126161015
Realt09-2	32.99491879	
Realt09-3	32.1752218	
Realt10-1	41.9793492	9.558992755
Realt10-2	35.24188886	
Realt10-3	41.61049422	
Realt11-1	18.44718564	26.68752716
Realt11-2	12.49601233	
Realt11-3	21.74377134	
Realt12-1	33.42947072	15.94150895
Realt12-2	37.61482235	
Realt12-3	27.23468955	
Realt13-1	19.1254248	17.34612579
Realt13-2	22.11032119	
Realt13-3	26.9363266	
Realt14-1	20.21800715	20.26723433
Realt14-2	27.470025	
Realt14-3	19.1776247	
Realt15-1	19.0529449	6.604498235
Realt15-2	18.81519717	
Realt15-3	16.85769944	
Realt16-1	22.80569601	16.30208948
Realt16-2	23.4298123	
Realt16-3	29.53434864	

Sample SRM data from peptide DAVEDESIVG

- Triplicate samples
- % CV ranged from 2-26%
- Average concentration in controls = 18.4 +/- 3.0
- Average concentration in late-stage OA samples = 27.5 +/- 4.0

Conclusions

- LC-MS/MS discovery experiments identified target proteins for osteoarthritis
- Discovery LC-MS/MS data were used to develop SRM target peptides and methods
- Multiplexed SRM assay was developed for 4 proteotypic peptides from 3 proteins
- At least 5-8 transitions (fragment ions) were measured for each peptide
- Synthetic heavy peptides were used as internal quantitative standards
- The developed method is rapid, (15 minutes), robust (microspray) and sensitive (200 amoles on column)
- CV's for most peptides ranged from 2-25%
- Proof-of-concept was demonstrated using normal and late stage osteoarthritis synovial fluid patient samples

References

1. Gobezie R, Kho A, Krastins B, Sarracino DA, Thornhill TS, Chase M, et al. High abundance synovial fluid proteome: distinct profiles in health and osteoarthritis. Arthritis Res Ther 2007;9(2):R36.