A high-throughput and reproducible workflow for MRM analysis of biological samples

Qin Fu[1]*, Michael P Kowalski[2]*, Xiaoqian Liu[1], Jie Zhu[1], Graham J Threadgill[3], Christie L. Hunter[4] and Jennifer E. Van Eyk[1]
[1]Johns Hopkins Bayview Proteomics Center, Division of Cardiology, Department of Medicine, School of Medicine, Johns Hopkins University, Baltimore, MD, USA;
[2]Beckman Coulter Life Sciences, Indianapolis, IN, USA, [3]Beckman Coulter, Inc., Brea, CA, USA, [4]AB SCIEX, Foster City, CA, USA
*equal contribution

INTRODUCTION

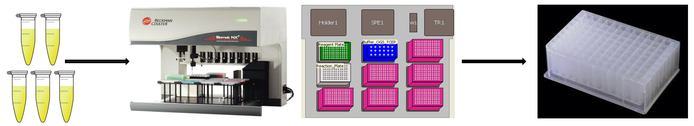
Multiple Reaction Monitoring (MRM) is a quantitative mass spectrometry-based method useful for quantifying peptides/proteins in biological samples. In contrast to immunoassays, MRM methods can be developed quickly and inexpensively in a multiplex format and are evolving into the method of choice for verification/validation of biomarkers. The challenge is to quickly and accurately process and analyze 100s to 1000s of samples.

Numerous steps in sample preparation for MS protein analysis can introduce analytical error beyond the requirements for bioanalytical methods. In addition, the throughput of MRM assays is limited by the LC-MS/MS step. Here we describe our implementation of automated sample processing in combination with an online desalt LCMS analysis strategy to improve the reproducibility and throughput of MRM assays. In addition, a multiplexed LCMS analysis further increased throughput of MRM analysis.

METHODS

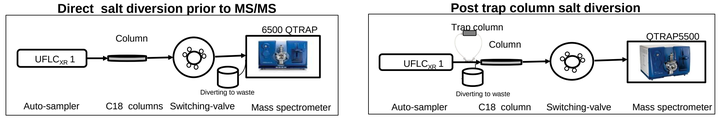
Pooled serum from healthy individuals were processed for MRM in a 96-well format using a Biomek NX^P automated liquid handling system. The conditions for each sample-preparation step (denaturation, reduction, alkylation, and trypsin digestion) were optimized to establish an automation-friendly procedure with a Protein Preparation Kit (AB SCIEX). Two online "desalting" strategies were achieved by either adding a trap column (linked to a QTRAP® 5500 system) or directly diverting salt fraction (from QTRAP® 6500 system) into waste. Two parallel reverse-phase chromatography systems (MPX™-2 System, AB SCIEX) were linked to either a QTRAP 5500 or 6500 system. Peptides from one column were eluted with an acetonitrile gradient into the mass spectrometer while the other column was washed, regenerated and loaded. A mixture of heavy isotope-labeled peptides was added to each sample as an internal standard.

Trypsin digestion with a Biomek NX^P Workstation



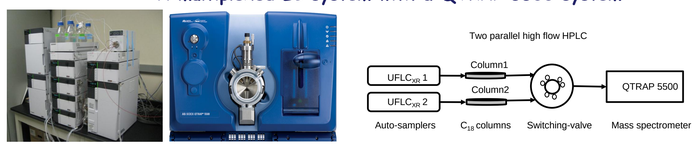
A scheme of serum protein denaturation, reduction, alkylation and trypsin digestion with a Biomek NX^P Workstation (Beckman Coulter, Inc.) and a Protein Preparation Kit (AB SCIEX)

"Online Desalting": two alternative approaches



A scheme of two alternative online "desalting" methods: 1). salt fraction was diverted off to waste prior to MS/MS (left); 2). salt fraction was diverted into waste post C18 trap column prior to C18 LC column (right).

A multiplexed LC system with a QTRAP 5500 system



MPX-2 System QTRAP 5500

Two parallel ultra flow and high pressure LC systems are configured into the MPX-2 system (Prominence UFLCXR components from Shimadzu) are configured with staggered sampling and injection into a single QTRAP 5500 mass spectrometer

Summary

1. The accuracy of a manual based quantitative MRM workflow was established by spiking exogenous beta-galactosidase (b-gal) protein. The coefficient of variation (CV) is calculated with isotopically labeled peptide as internal standard and b-gal protein spiked 171 individual plasma samples.
CV: 20-25%
2. A liquid handler (Biomek NX^P Workstation) based serum sample process combining with direct online "desalting" (diverting prior to MS) MRM protocol was established using exogenous b-gal and endogenous HSA and isotopically-labeled peptides as internal standards.
CV: <10%
3. The analytical variability of quantitative MRM was accessed by using exogenous b-gal and endogenous HSA and isotopically-labeled peptides as internal standards.

LC MS/MS	CV~ 4% for desalting/diverting prior to MS.
	CV~ 9% for online trap column desalting.
Offline SPE desalting + LC MS/MS:	CV~ 31%
Biomek NX^P + offline SPE + LC MS/MS	CV` 26%

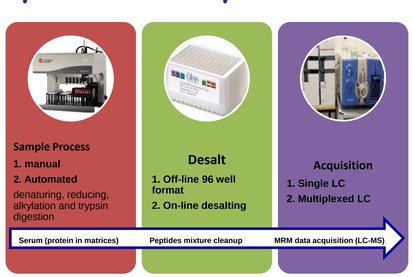
4. A multiplexed LC MS/MS MRM assay was established by using MPX-2 system (two parallel LC systems). The robust methods optimized in this study will facilitate the development of MRM assays for novel biomarker candidates.

The source of analytical variability of MRM analysis

Serum protein digestion
- Biomek-NX^P Workstation
- Manual

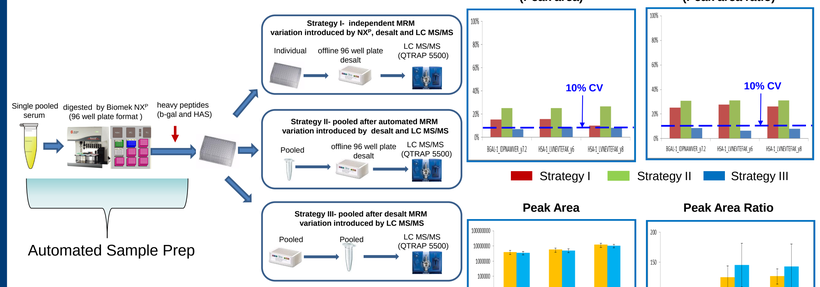
Sample clean up
- HLB 96 well plate
- On-line trap Elute

LC MS/MS
- Single LC
- Multiplexed LC



RESULT

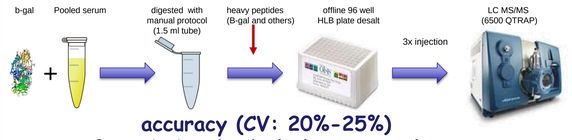
Source of MRM inaccuracy evaluation



Pooled human serum with exogenous b-gal digested with Biomek NX^P Workstation, then N_{15} peptide for b-gal and HSA were added. For strategy I, 12 individual digestion reactions processed through offline SPE in a 96 well format, 12 LC MS/MS was performed for individual digestion in triplicate injection. For Strategy II, 10 digestions were pooled then re-aliquoted into 10 offline SPE wells , LC MS/MS was performed for 10 individual offline SPE in triplicate injection. For strategy III, LC MS/MS was performance (14 consecutive injection) with a single sample post digestion and offline SPE

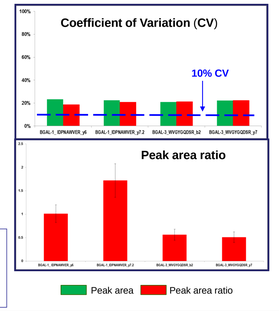
RESULT

A manual MRM workflow



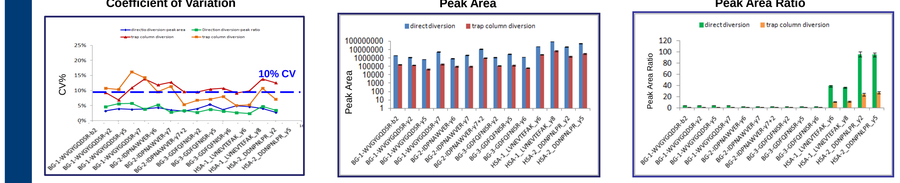
accuracy (CV: 20%-25%)
from 171 individual plasma samples

Beta-gal spiked into 171 individual plasma samples were denatured, reduced, alkylated and digested with Trypsin. The heavy peptides were then added as internal standards prior to SPE desalting via 96 well HLB plate format. Eluents were speed vacuum down to dryness and digested peptides were suspend to a 20% Acetonitrile/ 0.1% formic acid/H2O solution. MRM data was acquired by triplicate injection using 6500 QTRAP.



RESULT

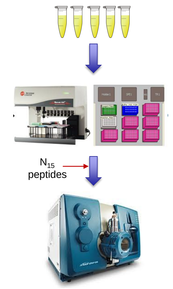
Coefficient variation of two "Online Desalting" approaches

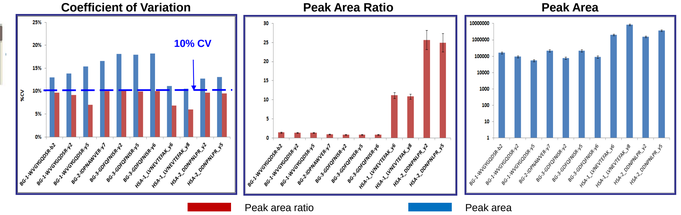


The variations of two online desalting approaches were evaluated. Heavy peptides of b-gal and HSA were added to b-gal spiked, then digested, serum. Formic acid was added to a final concentration of 0.1%. The mixture was spun at 16,000 g for 10 minutes. Then divided into vials for "online" diverting desalting with a QTRAP 6500 or online trap-elute with a QTRAP 5500.

RESULT

Precision of an automated MRM workflow

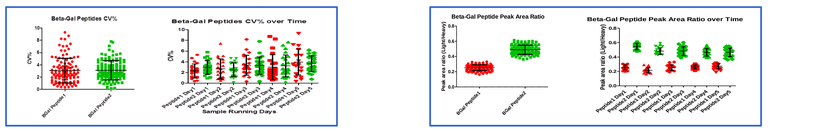




The precision of an automated MRM work flow which combined sample processing with a Biomek NX^P Workstation and online desalting LC MS/MS with a QTRAP 6500.

RESULT

Biomek NX^P Workstation workflow performance of 120 individual human plasma samples



Plasma from 120 individuals were processed through an automated MRM work flow and online desalting LC MS/MS with a QTRAP 6500.