Ultrahigh-throughput peptide quantitation of an acute phase protein panel using Acoustic Ejection Mass Spectrometry (AEMS) and peptide enrichment

Christie L Hunter[1], Bart Van Puyvelde[2,3], Oliver Wang[3], Maxim Zhgamadze[3], Qin Fu[3], Esthelle Hoedt[3], Maarten Dhaenens[2], Dieter Deforce[2], Jennifer E Van Eyk[3]

[1]SCIEX, Redwood City, CA, USA; [2]ProGenTomics, Laboratory of Pharmaceutical Biotechnology, Ghent University, Belgium; [3]Advanced Clinical Biosystems Research Institute, Cedars-Sinai Medical Center, Los Angeles, CA, USA

INTRODUCTION

For epidemiological and population studies that require the study of large cohorts (tens to hundreds of thousands of samples), strategies for higher throughput analysis while maintaining quantitative accuracy are needed. Ten acute phase plasma protein biomarkers representing different pathological pathways were incorporated into an automated immuno-enrichment assay (SISCAPA Assay Technologies), where target peptides representing each protein are enriched from plasma in a 96-well format. We've shown that Acoustic Ejection Mass Spectrometry (AEMS, Figure 1) using the Echo® MS system (SCIEX) can provide reproducible peptide quantitation at 1-3 seconds per sample on simplified peptide samples (minimal complexity matrix).

Here, a pilot cohort consisting of unhealthy and COVID-infected samples was analyzed using the combined peptide enrichment/AEMS workflow to test the feasibility of this ultra-high-throughput approach.

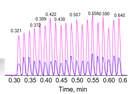
MATERIALS AND METHODS

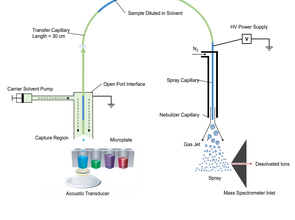
Sample preparation: A standard mixture of 20 stable isotope labeled peptides (PepCalMix, SCIEX), Beta-galactosidase digest (SCIEX) and other synthetic peptides were used for testing, as it provides a broad range of peptide properties. Peptides were diluted to various concentrations in small amounts of CHAPS/formic acid.

Automated plasma sample preparation: Plasma samples (50 µL) underwent denaturation, reduction/alkylation, trypsin digestion, heavy SIL addition, antibody capture and elution on the Beckman i7 Automation Workstation. The standard capture protocol for the acute phase 10-protein MRM assay was utilized, but with reduced CHAPS concentration (0.00025%) and modified washes to minimize salt.

AEMS conditions: Samples were analyzed at 1-3 seconds per sample using MRM analysis on an Echo® MS system. Carrier solvent used was 80% acetonitrile and 200 nM medronic acid with a flow rate of 500 µL/min. Droplet was optimized for best sensitivity. Each sample was run in triplicate.
Next, the sensitivity, reproducibility and repeatability were evaluated. In parallel, samples were analyzed using LC-MS on the SCIEX 6500 system with a 5-min gradient.

Data processing: Data were processed using the Analytics module in SCIEX OS software.





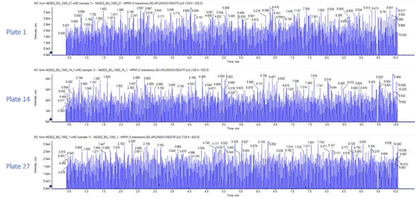
TECHNOLOGY

Acoustic Droplet Ejection: Acoustic energy is used to create, and then amplify, a standing wave in liquid in a sample well. As the amplitude of the standing wave increases, a point is reached when a single droplet is ejected from the wave at its central apex. Very consistent volume droplets are produced from the liquid in a plate well on a microsecond timescale. The sample plate is moved very rapidly and precisely over the fixed-position acoustic module by a pair of XY stepper motors.

Open Port Interface: The OPI is a pair of concentrically positioned tubes that are both open at one end. The outer tube has liquid (carrier solvent) delivered to it by a low-pressure liquid pump. The inner tube is connected, via a transfer line, directly to the capillary of the ESI source. The nebulizer gas (GS1) provides an aspirating "pull" that draws the solvent delivered from the outer tube into the inner tube. A vortex results where the carrier solvent makes the turn from the outer tube to the inner tube. The droplet from the acoustic ejection is captured in this vortex and carried to the MS system.

These 2 technologies form the Echo® MS system acoustic sampling module.

Ultrahigh-throughput quantitation
2 peptides – 10,260 samples – 4.75 hours



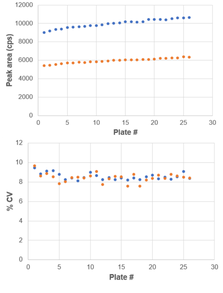


Figure 1. Ultrahigh-throughput peptide quantitation. (Left) Chromatograms of full 384-well plate measurement of two beta-galactosidase peptides. A total of 10,260 ejections of samples (2 MRM) were loaded from 27 plates. Time used was 2 seconds per sample.
(Right) High reproducibility of peptide quantitation on 27 runs of a 384-well plate.
VDEDQPFPAVPK - %CV <10% for each plate – 27 plate runs
DWENPGVTQLNR – %CV <10% for each plate – 27 plate runs

Plasma samples – SISCAPA

A total of 272 plasma samples from a cohort of samples collected from individuals presenting at the hospital with potential COVID infection. These samples were used to test the feasibility of combining SISCAPA sample preparation with Echo® MS system analysis for studying the changes in inflammation-relevant proteins with disease. An acute phase response protein panel (SISCAPA technologies) was used that consisted of 10 protein markers that cover the various aspects of inflammation (CRP, LPSBP, MBL, SAA, IgM, MPO, Hx, Alb, A1AG, C3). Three 96-well plates were prepared using the standard protocol optimized for the Beckman i7 Automation Station.

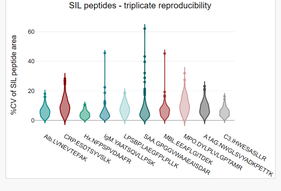


Figure 5. High reproducibility. Triplicate analysis was performed on each of the prepared samples. Reproducibility on the added SIL peptides was better than 10% CV for most peptides. Strong dependence of %CV on absolute area of peptide was observed in eluted sample.

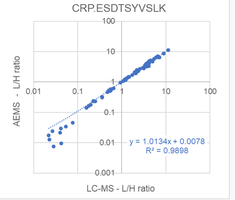


Figure 6. Accurate results. A subset of samples was also analyzed by LC-MS on the SCIEX 6500 system using MRM analysis. Ratios were compared to the ratios obtained from the Echo® MS system. Good correlation was obtained for all detectable peptides. The correlation of the peptide are ratios for CRP peptide between AEMS and LC-MS is shown.

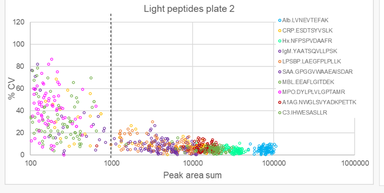


Figure 7. Detection of endogenous peptides. The peak areas for the captured endogenous peptides are plotted vs. the measured reproducibility across 3 repeat analyses by AEMS. Reproducibility strongly correlates with signal intensity. Most endogenous peptides are detectable with the current assay in the disease samples. The endogenous peptide signal for MBL and MPO are below the current detection level (gray dotted line). In healthy samples, CRP level drops to near the detection level.

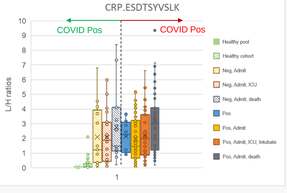


Figure 8. Biological interpretation. Using the clinical results provided based on accepted clinical tests, the samples were separated into biological groups based on an adapted ordinal scale for COVID disease status.* Measured ratios relative to the heavy peptide level for each protein were plotted by disease group to determine if a distinct pattern in the inflammation markers could be measured.

*SCIENCE IMMUNOLOGY, 10 Aug 2021, Vol 6, Issue 62.

Sensitivity

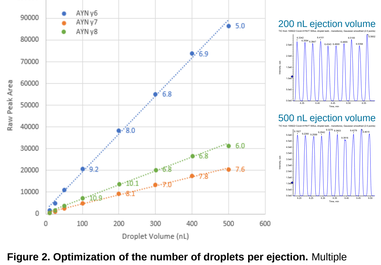


Figure 2. Optimization of the number of droplets per ejection. Multiple peptides were tested across a broad range of ejection volumes. Data is shown here for the SARS-CoV-2 NCAP peptide AYNVTQAFGR. Replicate ejections were performed to evaluate reproducibility (%CV shown as numbers on plot). Very good linearity was achieved up to 500 nL with excellent reproducibility.

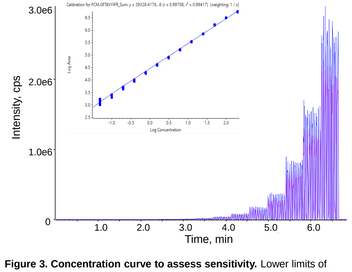


Figure 3. Concentration curve to assess sensitivity. Lower limits of quantitation were determined using standard bioanalytical cutoffs of <20% CV for precision and 80%-120% accuracy. Example data are shown for PCM.GFTAYYIPR, ejection raw data and linear calibration curve (300 nL ejection volume, 500 µL/min flow, 4 MRMs, dwell time 15ms). LLOQ of 0.05 fmol/µL was obtained for this peptide, with %CV of 14.4.

Peptide	Q1 m/z	LLOQ (fmol/µL)	%CV	Accuracy
PepCalMix.GAYVEVTAK	473.26	0.2	13.9	88.2
PepCalMix.LVGTPAEER	491.27	0.2	11.8	90.1
PepCalMix.LDSTSIPVAK	519.8	0.1	12.7	80.4
PepCalMix.AGLIVAEGVTK	533.32	0.02	11.8	111.8
PepCalMix.GFTAYYIPR	549.29	0.05	14.4	114
PepCalMix.AVGANPEQLTR	583.31	0.05	16.2	80.5
PepCalMix.VFTPLEVDVAK	613.35	0.2	7.5	111.8
PepCalMix.DGTFAVDGPGVIAK	677.86	0.05	14.5	110.3
PepCalMix.ALENDIGVPSDATVK	768.9	0.1	5.67	117.4
PepCalMix.TVESLFPEEAETPGSAVR	964.98	0.39	5.78	110.2
Average of all 20		**0.24**	**10.6**	**102.8**

Figure 4. Table of LLOQ values. LLOQs were determined for the 20 PepCalMix peptides. A selected subset is shown here along with the averages of all 20 peptides. The average sensitivity across the suite of peptides was 0.24 fmol/µL.

CONCLUSIONS

- The current best acquisition strategy uses a carrier solvent of acetonitrile with medronic acid, samples diluted in water/formic acid with a low amount of CHAPS (<0.0003%), and flow rates of ~450-500 µL/min
- Using these conditions, calibration curves were generated to assess the average lower limits of quantitation (typically 0.24 fmol/µL in plate)
- A large-scale reproducibility study was performed, with two light peptides in 384-well plates, across 27 plates, where 10,2600 samples were analyzed in 5 hours of acquisition time, providing intra-plate CVs of <10%
- Equivalent LC-MS time for same # of samples would take 45 days (up to 230x faster with AEMS)

- The first SISCAPA sample set (288 samples) was analyzed by AEMS, and very good reproducibility was observed for triplicate analysis
- Very good correlation between AEMS and LC-MS was observed for all peptides (note heavy peptide signal for CRP and SAA was on the edge of detection and the workflow has been adapted to fix this for round 2 of analysis)
- Biological interpretation using the NIAID COVID disease status classification is underway.

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