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Simultaneous quantitation and discovery (SQUAD) of immunopeptidome with Orbitrap Ascend MultiOmics Tribrid mass spectrometer

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Keywords

Immunopeptidomics, immunopeptide, SQUAD, major histocompatibility complex, MHC, MHC Class I, Orbitrap Ascend MultiOmics Tribrid MS, tumor-specific antigens, neoantigens, Vanquish Neo, FAIMS Pro Duo

Goal

To assess the performance of a method for simultaneous targeted quantitation and data-dependent acquisition (DDA) discovery of histocompatibility complex (MHC) peptides on the Thermo Scientific™ Orbitrap™ Ascend MultiOmics Tribrid™ mass spectrometer.

Introduction

Immunopeptidomics is the study of the peptides presented by major MHC molecules on the surface of cells. These MHC peptides have major implications for many areas of research, including immunotherapy and personalized medicine. For example, many studies in this field aim to identify low-level, tumor-specific antigens (TSAs) with the goal of developing personalized immunotherapies to target cancerous cells with a high degree of specificity. Recently, non-canonical TSAs have been targeted as promising candidates for cancer treatment, as these peptides may be shared between many patients with the same subtype of cancer. In this and many other cases, it is critical to determine which TSAs a patient expresses and the quantity of these TSAs to understand the effectiveness of targeted immunotherapy. Success in recent clinical trials has accelerated interest in the field, and there is a clear need for analytical methods that push the limits of sensitivity in both depth of coverage for discovery and quantitative accuracy for validation and clinical usage.

Mass spectrometry (MS) is frequently used for immunopeptidomics as it is the only analytical technique that can directly measure and quantify MHC peptide presentation across a large number (tens of thousands) of targets. Therefore, large-scale untargeted profiling of the immunopeptidome is crucial for the discovery of novel targets and patient-specific TSAs. Due to the complexity of the data processing, DDA is often favored for this stage in the process. DDA enables discovery from databases and de novo peptide sequencing searches, producing interpretable spectra that can be manually validated. However, DDA is inherently stochastic and generally limited to MS[1] quantitation. Therefore, once putative target peptides are identified, follow-up experiments are often performed using targeted acquisition to help ensure accurate, sensitive and reproducible quantitation of a handful of targets. These targeted experiments are difficult to perform as they require high sensitivity and quantitative accuracy to be able to determine the quantity of very low-level antigen. In many cases, it is necessary to prepare double the amount of sample if both discovery and quantitation are required for a single sample. Doing so increases the experimental run time, doubles the cost of reagents and may not be possible with very limited clinical sample amounts.

In early-stage discovery, it may be necessary to perform unbiased MHC peptide profiling in a pilot group of samples, identify TSAs or other interesting peptide targets and then quantify these targets in a separate run. However, once a list of targets is developed, it is often necessary to obtain both targeted quantitation and discovery immunopeptidome profiles on a larger sample cohort. In this phase of research, the ability to perform deep immunopeptidome profiling while simultaneously obtaining rigorous targeted quantitation on known targets has the potential to save a significant amount of time and resources. Most mass spectrometers have a single mass analyzer, which means switching between targeted and discovery modes will negatively impact the performance of one or both data modalities. However, the Thermo Scientific™ Ascend Editions Tribrid™ instruments consist of both Orbitrap and linear ion trap detectors, which may be operated in parallel, enabling simultaneous quantitation and discovery (SQUAD).[1] In particular, advances in the ion parallelization on the Orbitrap Ascend MultiOmics MS minimize the impact of the targeted acquisition on the discovery data. The high-resolution Orbitrap Ascend MultiOmics MS is well suited for discovery, while the exceptional sensitivity of the linear ion trap is ideal for targeted quantitation (Figure 1). The linear ion trap is fast, sensitive and, unlike a triple quadrupole instrument, acquires a full MS/MS spectrum, which helps ensure unambiguous sequence annotation. We demonstrated the ability of SQUAD to profile the MHC peptidome (Figure 2) while obtaining absolute quantitation on 39 endogenous peptides and their heavy synthetic counterparts.

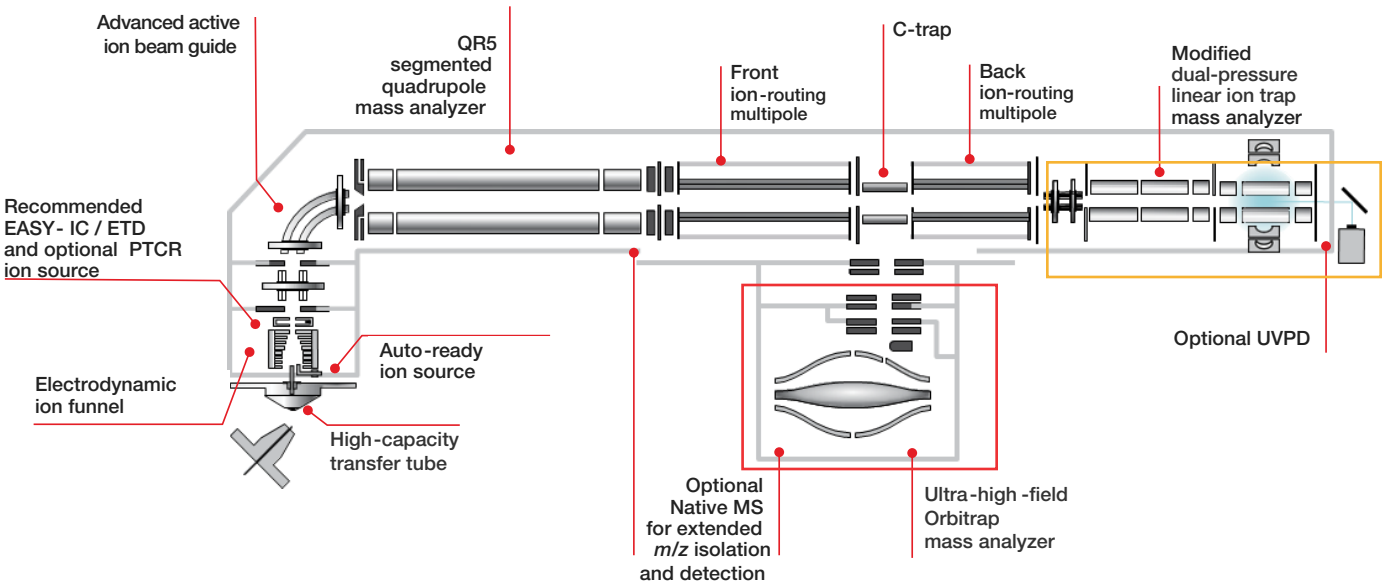


Figure 1. Schematic of Orbitrap Ascend MultiOmics MS. In SQUAD, discovery is performed in the Orbitrap (red box), while targeted quantitation is performed in the linear ion trap (yellow box) in a single injection.

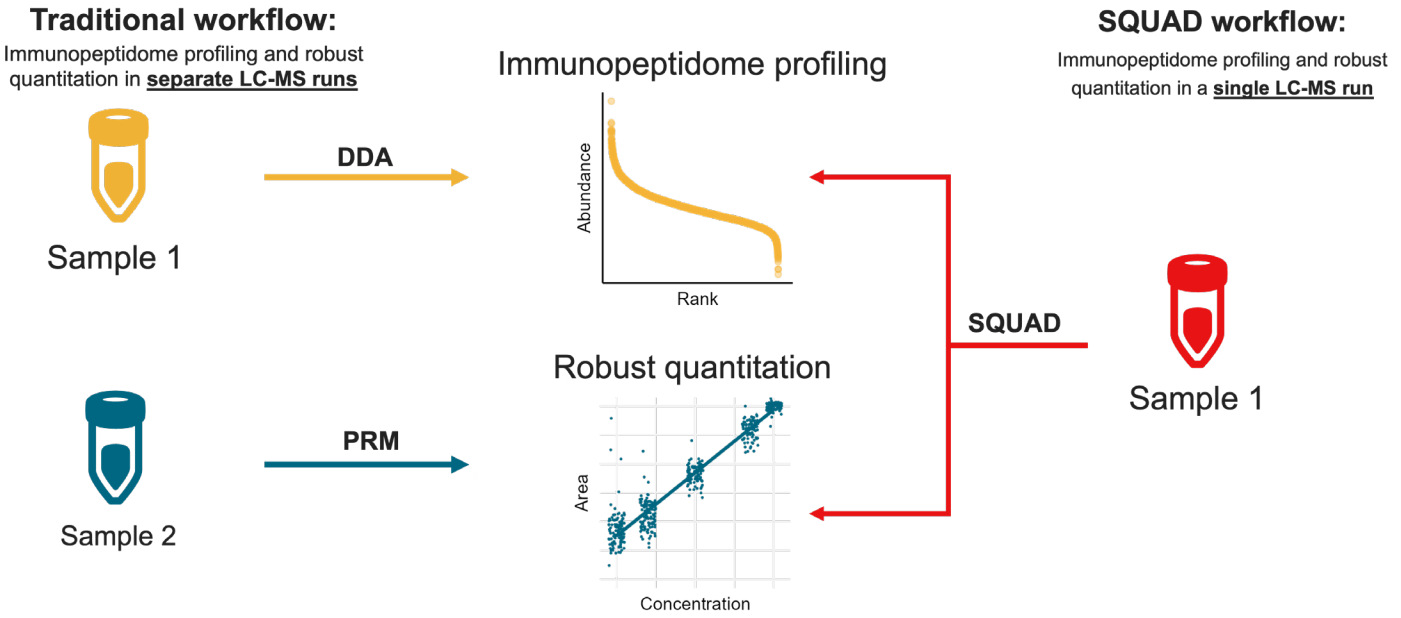


Figure 2. Comparison of traditional and SQUAD workflows

Experimental

Recommended consumables

- Thermo Scientific™ LC-MS Grade Water with 0.1% Formic Acid (FA) (Cat. no. LS118-500)
- Fisher Scientific LC-MS grade 80% Acetonitrile (ACN) with 0.1% Formic Acid (Cat. no. LS122500)
- Fisher Chemical™ Optima™ LC-MS Grade Formic Acid (Cat. no. A117-50)
- Fisher Chemical™ Optima™ LC-MS Grade Water (Cat. no. 10505904)
- Fisher Chemical™ Optima™ LC-MS Acetonitrile (Cat. No. A955-1)
- Thermo Scientific™ Pierce™ C18 Stage Tips (Cat. no. 87782)

Samples

- Cayman Chemical™ HCT 116 HLA Enriched Sample—contracted from Cayman Chemical
- Thermo Scientific™ Pierce™ Heavy Synthetic AQUA Peptides

LC columns

- IonOpticks Aurora Ultimate TS 25 cm (IonOpticks cat. no. AUR3-25075C18-TS)
- IonOpticks Column Heater (IonOpticks cat. no. HTS 902720000)
- Thermo Scientific™ PepMap™ Neo Trap Cartridge Holder (Cat. no.174502)

HPLC system

- Thermo Scientific™ Vanquish™ Neo UHPLC System (Cat. no. VN-S10-A-01), including:
  - Thermo Scientific™ Vanquish™ Neo Binary Pump/Split Sampler NT
  - Thermo Scientific™ Vanquish™ Column Compartment (Cat. no. VN-C10-A-01)

Mass spectrometer

- Orbitrap Ascend MultiOmics Tribrid Mass Spectrometer (Cat. no. FSN06-10000)
- Thermo Scientific™ FAIMS Pro Duo interface (Cat. no. OPTON-20068)
- Thermo Scientific™ Easy-Spray™ Source (Cat. no. ES081)

Data analysis software

- MacCoss Lab Software Skyline V23.1
- Bioinformatics Solutions Inc. PEAKS Studio 11
- FragPipe V22.0

MHC standard preparation

Class I MHC peptides were obtained by immunocapture with W6/32-conjugated resin on 100 million HCT-116 cells. After cleanup using Affinisep Stagetips, the starting material was diluted 100x with 0.1% formic acid. A dilution series was prepared by spiking 39 synthetic heavy labeled AQUA peptide standards into the MHC peptide sample at concentrations ranging from 100 amol to 10 fmol.

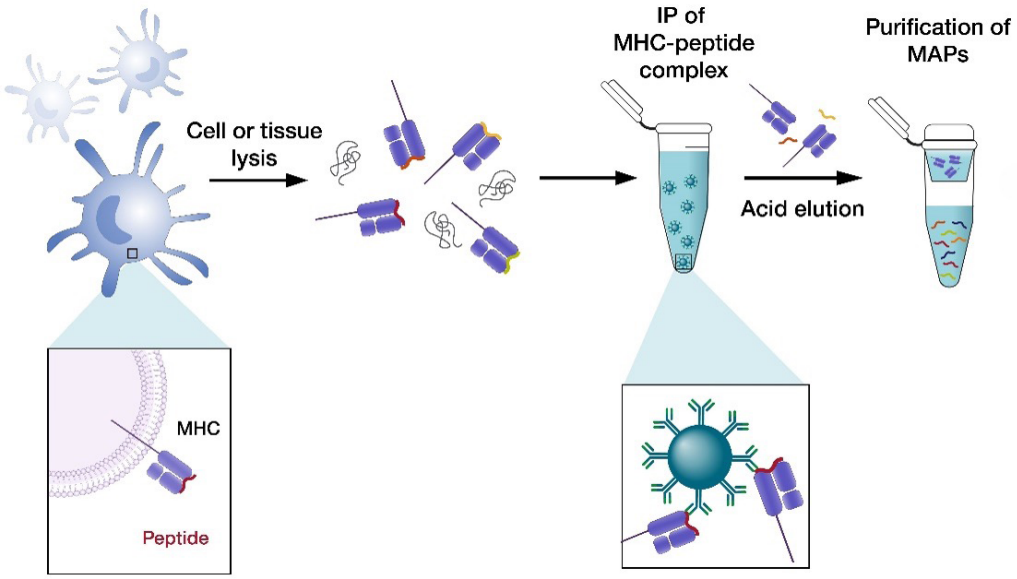


Figure 3. General sample preparation workflow for immunopeptidomics

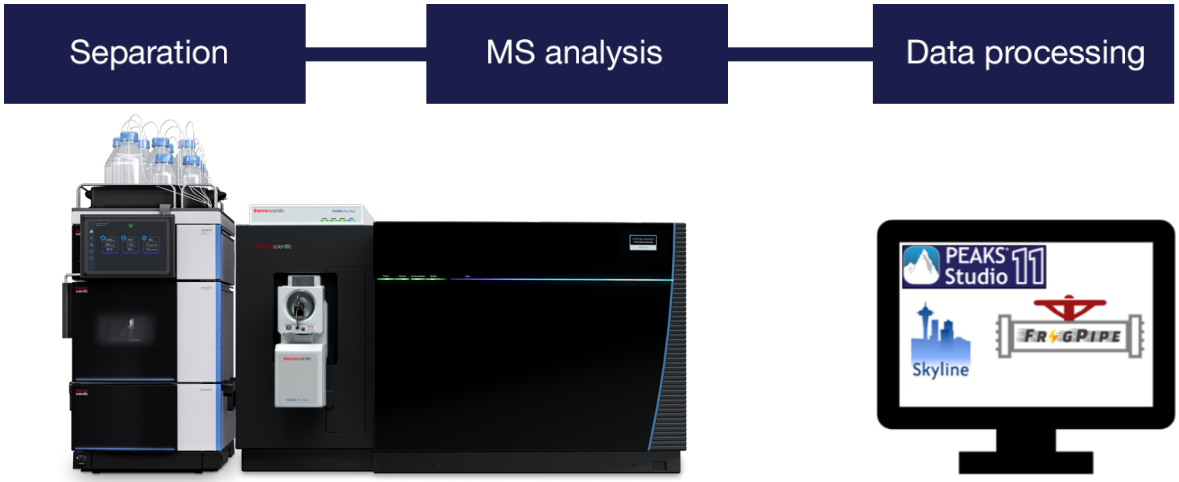


Figure 4. Workflow overview for SQUAD

LC conditions

Samples were analyzed using an IonOpticks Aurora Ultimate TS 25 cm column connected to a Vanquish Neo UHPLC system and an Orbitrap Ascend MultiOmics MS with a FAIMS Pro Duo Interface. A gradient length of 72 minutes was used. LC was operated in trap-and-elute mode to help ensure robust performance over time.

Table 1. HPLC conditions

Mobile phase A	0.1% FA in water
Mobile phase B	0.1% FA in 80% ACN
Flow rate	0.2 uL/min
Trap column	PepMap Neo Trap Cartridge
Trap loading volume	50 uL
Trap wash volume	4.882 uL
Trap equilibration volume	10.201 uL
Analytical column	Aurora Ultimate TS 25 cm
Column temperature	50°C
Autosampler temperature	7°C
Injection wash solvents	Strong wash: 0.1% FA in 80% ACN Weak wash: 0.1% FA in water
Needle wash	Enabled after-draw

Table 2. HPLC gradient

Time (min.)	Duration (min.)	%B	Flow rate (uL/min.)
0	0	2	0.5
1	1	5	0.5
1.1	0.1	5	0.2
61.1	60	35	0.2
63.1	2	70	0.2
67.1	4	99	0.2
67.2	0.1	99	0.5
72	4.8	99	0.5

Table 3. Global MS parameters

Source parameters	
Spray voltage (positive)	1,800
Capillary temperature	280
FAIMS mode	Standard resolution
Total carrier gas flow (L/min.)	3.5
MS global settings	
Expected LC peak width(s)	10
Advanced peak determination	True
Default charge state	2

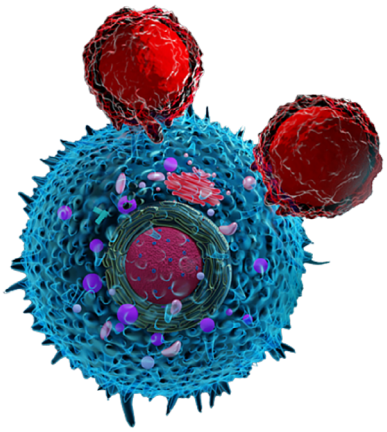
Table 4. DDA experiment parameters

Master scan (MS^1)	
Detector type	Orbitrap
Orbitrap resolution	120,000
Scan range (*m/z*)	300–800
RF lens (%)	30
Normalized AGC target	Standard
Maximum injection time (ms)	100
FAIMS voltages	–40 V or –60 V
Filters	
Dynamic exclusion	
Share dynamic exclusion list with other selected dynamic exclusion filters	False
Exclude after n times	1
Exclusion duration(s)	30
Mass tolerance	+/–10 ppm
Exclude isotopes	True
Perform dependent scan on single charge state per precursor only	True
Exclude within cycle	True
Charge state	
Include charge states	2–4
Include undetermined charge states	False
MIPS	
Monoisotopic peak determination	Peptide
Isolation window center	Most abundant peak
Data dependent	
Data dependent mode	Cycle time
Time between master scans (s)	0.6
ddMS2 OT HCD	
Isolation mode	Quadrupole
Isolation window	1.6
Activation type	HCD
HCD collision energy type	Normalized
HCD collision energy (%)	30
Detector type	Orbitrap
Orbitrap resolution	15,000
Scan range mode	Auto
AGC Target (%)	145
Maximum injection time (ms)	150

For both SQUAD and DDA-only experiments, the following parameters were used for two DDA experiments. One experiment used FAIMS CV of –40 V, and the other used FAIMS CV of –60 V (Figure 5).

Table 5. Parallel reaction monitoring (PRM) MS/MS parameters

Targeted MS^n Scan properties	
MS^n level	2
Isolation mode	Quadrupole
Isolation window (*m/z*)	1.6
Activation type	HCD
HCD collision energy type	Normalized
HCD collision energy (%)	30
Detector type	Ion trap
Ion trap scan rate	Rapid
Mass range	Normal
RF lens (%)	30
Normalized AGC target	Standard
Maximum injection time mode	Dynamic
Desired minimum points across the peak	6
FAIMS voltages	Defined in table
Time mode	Start/end time (1.5 min.)



A third experiment was added to the method in the SQUAD workflow (Figure 5). The target list consisted of 39 endogenous peptides with their heavy-labeled AQUA counterparts. Based on an unscheduled survey run, static scheduling windows of 1.5 minutes were determined. Optimal FAIMS CVs were determined based on survey runs of neat standards.

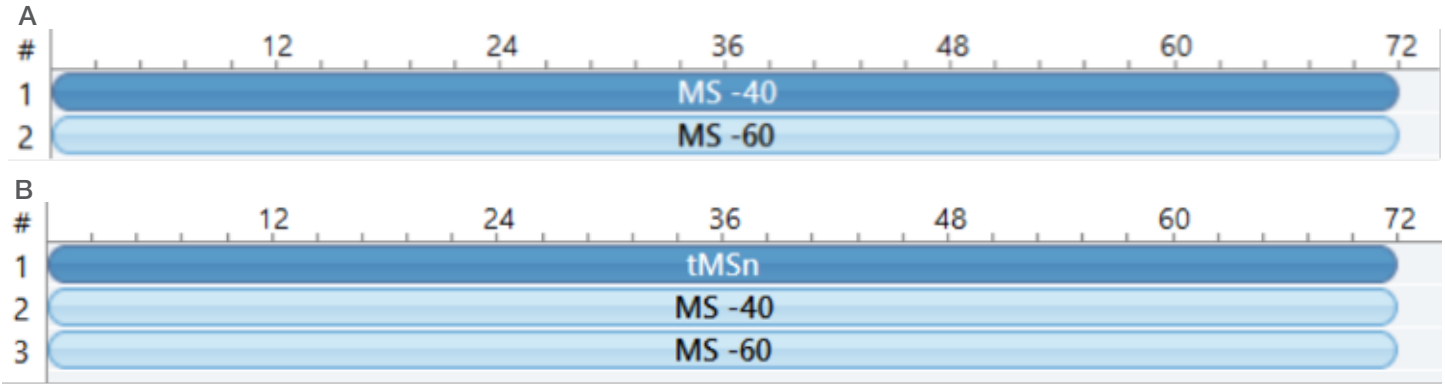


Figure 5. DDA-only (A) and SQUAD (B) method timelines

Data processing parameters

Conventional DDA and SQUAD DDA data were searched with PEAKS Studio 11 using the PEAKS DB workflow. Default Orbitrap HCD parameters and the human UniProt database were used (Taxon ID 9606, 20607 entries).

DDA data were also searched with MSFragger in FragPipe V22.0 using the standard MHC peptide workflow, filtered for peptides of length 7–15 amino acids. The AlphaPept model was used for spectral prediction and MSBooster rescoring.

PRM data were processed in Skyline V23.1 using scan filters to help ensure only linear ion trap scans of the appropriate CV were integrated. A tolerance of 0.5 Th was used for integration, and raw fragment area was used for quantitation.

Results

Immunopeptidome coverage with standard DDA

Deep coverage from low starting material is critical for immunopeptidomics experiments. We first demonstrate coverage with a conventional DDA approach. To test performance at low loads, we injected 1% of a sample generated from 1e8 HCT 116 cells. This is equivalent to material from approximately 1e6 cells. We searched the data with both PEAKS DB and MSFragger. Overall, both searches demonstrate deep immunopeptidome coverage (5,738 and 5,415 unique peptides from PEAKS and MSFragger, respectively), and the peptides fell in the expected range of 7–15mers (Figure 6).

Immunopeptidome coverage with SQUAD

We analyzed the same sample with conventional DDA and SQUAD, targeting 39 light/heavy peptide pairs. We expected some drop in coverage with SQUAD because some of the instrument time is now allocated to accumulate ions for PRM instead of DDA. The architecture of the Orbitrap Ascend MultiOmics MS minimized this loss in coverage, and we maintained 90% of the depth with SQUAD as we obtained with conventional DDA without losing dynamic range (Figure 7).

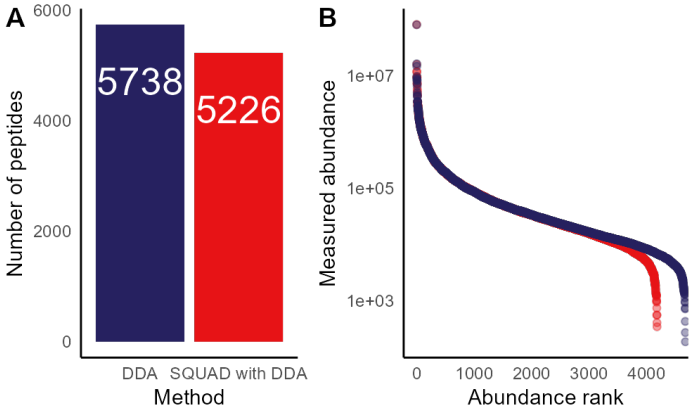


Figure 7. Comparison of 3 replicates (A) coverage and (B) dynamic range for traditional DDA and DDA with SQUAD

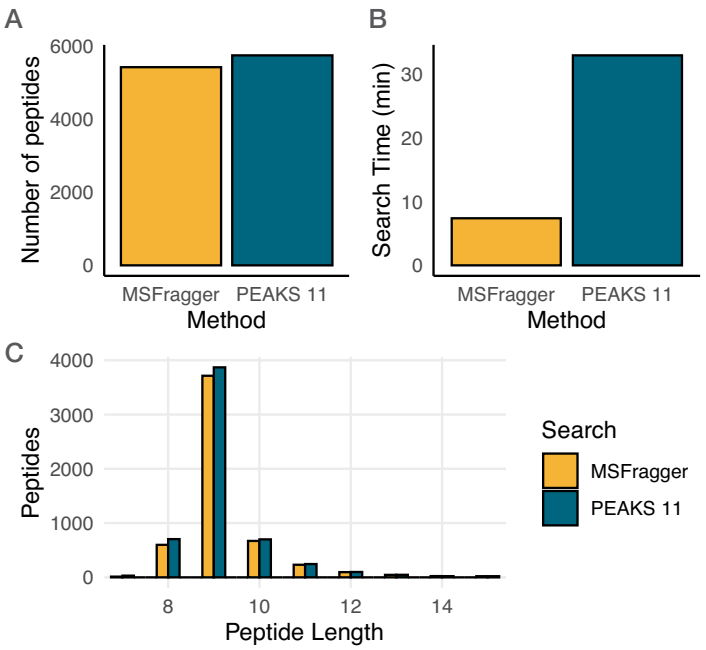


Figure 6. Comparison of search methods. Number of peptide IDs across three replicates (A), total search time (B) and peptide length distribution (C) for DDA data searched in both MSFragger and PEAKS 11.

Absolute quantitation with SQUAD

To evaluate the quantitative performance of SQUAD, we performed a dilution series of 39 heavy AQUA peptides in the same sample background. Heavy peptides were spiked in at concentrations ranging from 100 amol to 10 fmol, and the curve was acquired in triplicate. Overall, the quantitative data was of high quality for both light and heavy peptides across the dilution series (Figure 8). High-quality measurements were obtained for all 39-heavy peptides across a wide dynamic range (Figure 9), demonstrating the power of PRM in the linear ion trap for low-level MHC peptides.

PRM-based quantification dramatically increases sensitivity over MS^1-only quantitation that would normally be used in immunopeptidome profiling. On average, PRM run in a SQUAD experiment is 2x more sensitive than MS^1 quantitation for the heavy peptide standards, and increased sensitivity by 10–30x for some peptides (Figure 10). This increase in sensitivity and ability to reliably quantify known targets across all samples is a key advantage of PRM.

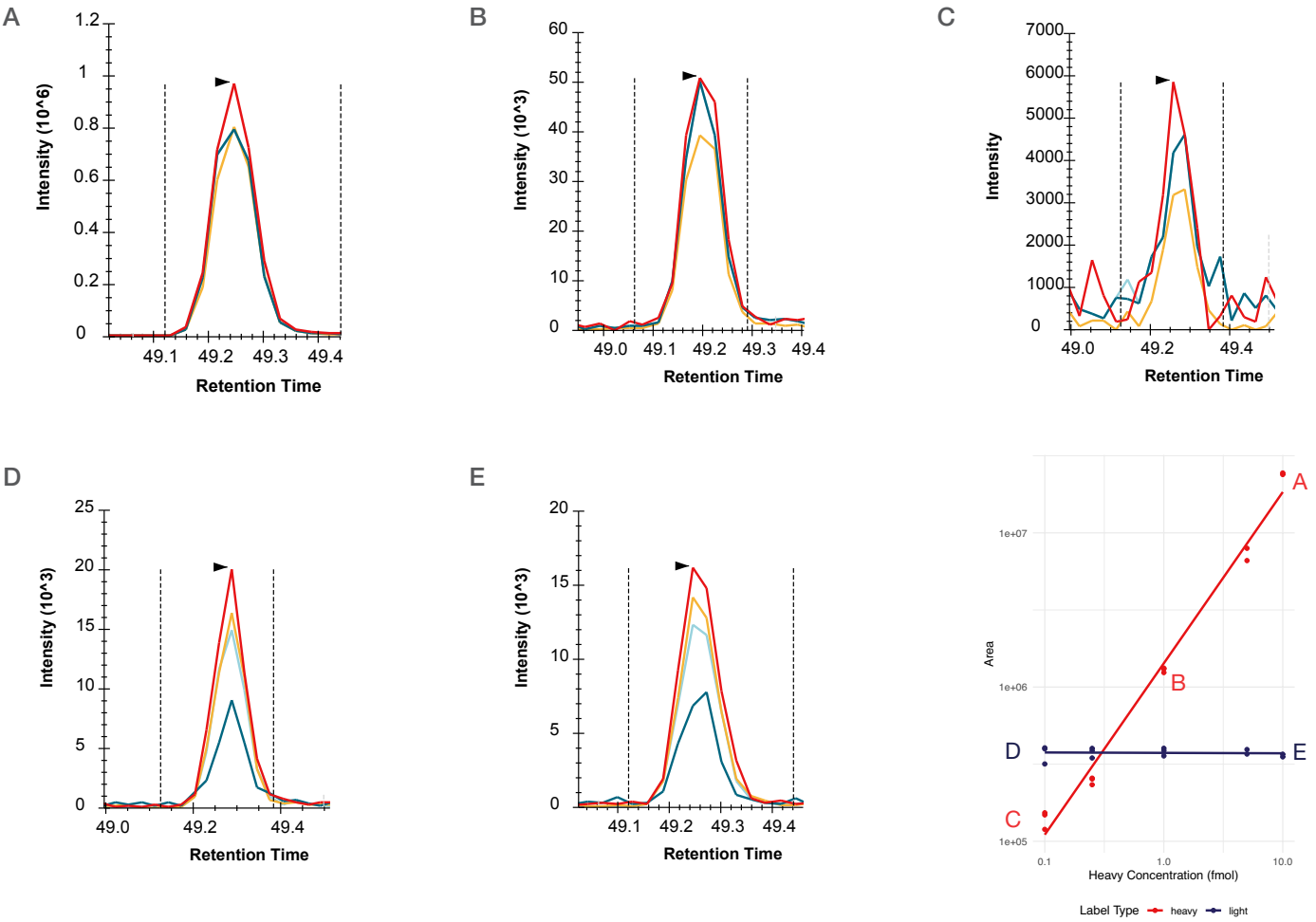


Figure 8. Example dilution curve obtained from targeted portion of SQUAD. Light (D and E) and heavy peptides (A, B and C) are shown across heavy peptide dilution series.

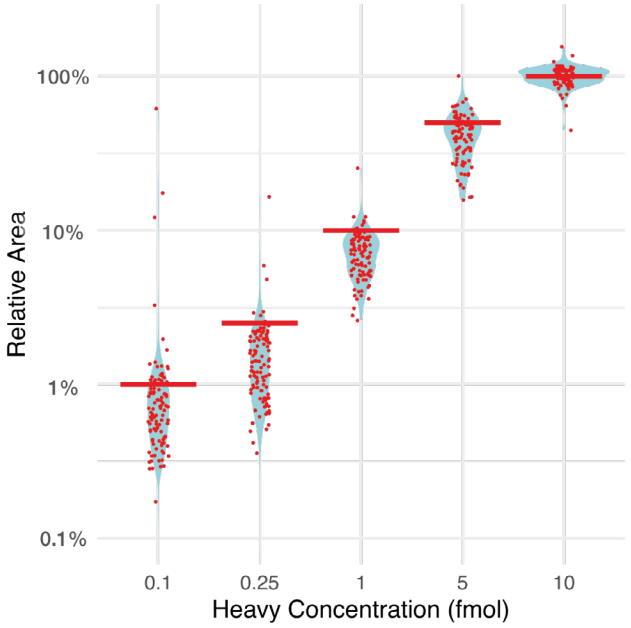


Figure 9. Dilution ratio for all 39 heavy peptides measured with SQUAD. Horizontal red lines indicate expected ratio.

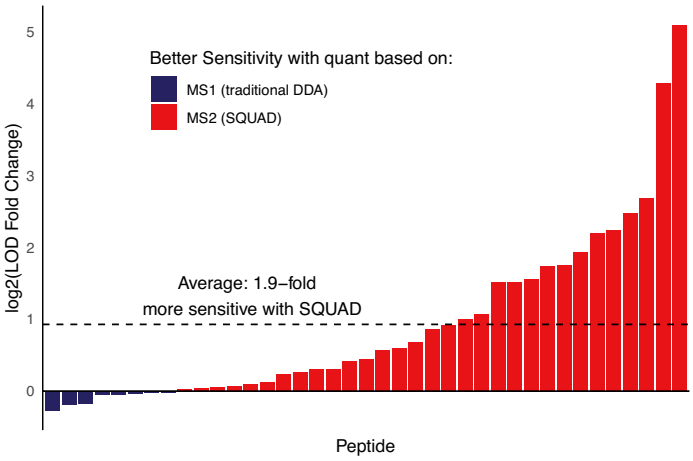


Figure 10. Pairwise comparison of lower limits of detection with SQUAD compared to conventional MS¹ area under the curve used for conventional DDA

Summary

- The Orbitrap Ascend MultiOmics Tribrid MS enables deep immunopeptidome profiling from 1e6 cell equivalents
- Combining PRM and DDA into one experiment increases sensitivity for known targets by 2x or more, while maintaining 90% depth of coverage
- The SQUAD workflow enables analytically rigorous quantitation and deep coverage from a single injection

References

1. Amer, B, *et al*. (2023, May). Simultaneous Quantitation and Discovery (SQUAD) Analysis: Combining the Best of Targeted and Untargeted Mass Spectrometry-Based Metabolomics. *Metabolites*. doi: 10.3390/metabo13050648.

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