Translating peptide evidence into immunotherapy targets: Orbitrap Tribrid Apex MultiOmics MS delivers high-confidence spectral annotation

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Abstract

Purpose: To improve confidence in immunopeptidomic analyses for immunotherapy target discovery by enhancing peptide sequence coverage using the Thermo Scientific™ Orbitrap ™ Tribrid ™ Apex MultiOmics mass spectrometer (MS).

Methods: EThcD fragmentation was implemented and compared to HCD using a data-dependent acquisition (DDA) workflow. Subsequently, EThcD was incorporated into a combined DDA–PRM workflow to support concurrent discovery and targeted analysis in a single injection, improving sequence annotation of relevant targets. We called this method Neo-SCOAD: *Neoantigen Simultaneous Confirmation and Discovery analysis*.

Results: EThcD fragmentation on the Orbitrap Tribrid Apex MultiOmics MS enhances sequence annotation of HLA peptides. We demonstrate Neo-SCOAD leveraging EThcD to improve sequence confidence in known neoantigens or sequence variants while profiling the immunopeptidome.

Introduction

Immunopeptidomics systematically profiles HLA-presented peptides to reveal the functional antigen landscape that drives T cell recognition in health and disease. Because therapeutic decisions—such as neoantigen vaccine selection, adoptive T cell therapy design, and target de-risking—depend on precise epitope identity and context, the field critically requires high-confidence peptide-spectrum matches and rigorous verification of sequence and modification assignments. Ambiguities arising from low-abundance peptides, isobaric residues, PTMs, and noncanonical sequences can propagate false positives, undermining biological interpretation and clinical translation.

Materials and methods

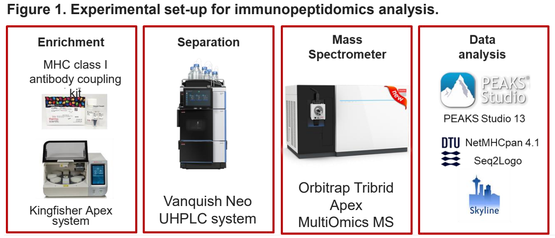
Sample preparation

HCT116 cells (ATCC) were cultured with or without 10 ng/mL interferon-γ (IFN-γ) treatment overnight prior to harvest and storage at −80 ° C. Sample preparation followed the Thermo Scientific™ Pierce™ MHC Class I Antibody Coupling Kit protocol and was performed using the Thermo Scientific™ KingFisher™ Apex Purification System. Cell pellets (10 million cells) were lysed in Thermo Scientific™ Mem-PER™ Membrane Solubilization Buffer. The W6/32 antibody was coupled to Thermo Scientific™ Pierce™ Protein A/G Magnetic Agarose Beads at room temperature for 1 hour. Lysates were incubated with the antibody-coupled beads for 2.5 hours at 4 ° C. Beads were then collected and washed with PBS and water, and bound peptides were eluted using 1% TFA for 2 minutes at room temperature. Eluted peptides were dried and resuspended in 0.1% formic acid prior to nanoLC–MS/MS analysis.

LC-MS/MS method

Peptides were separated on a Thermo Scientific™Vanquish™ Neo UHPLC System using IonOpticks Aurora® Ultimate™ 25 × 75 XT C18 UHPLC Column. Peptide loading was the equivalent to one million cells. Total run time was 72 min and Thermo Scientific™ EASY-Spray™ Ion Source was used. Peptides were analyzed by the Orbitrap Tribrid Apex MultiOmics MS.

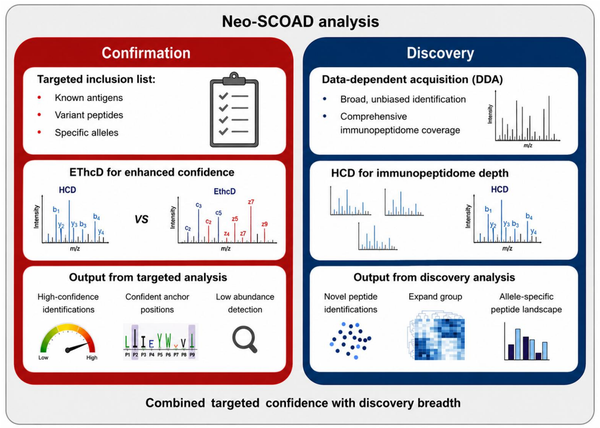
Figure 1. Experimental set-up for immunopeptidomics analysis.



Data processing

The data analysis was performed using PEAKS® Studio 13 with PEAKS® DeepNovo Peptidome workflow for database search and *de novo* peptide identification. Spectra were searched against UniProt human database (20,607 sequences) with no-enzyme option. The sequence motif and binding properties of 9-mer peptides were analyzed using Seq2Logo (ref.1)and NetMHCpan 4.1 (ref.2). Targeted scans (PRM) were analyzed with Skyline.

Figure 2. Neoantigen Simultaneous Confirmation and Discovery analysis (Neo-SCOAD).

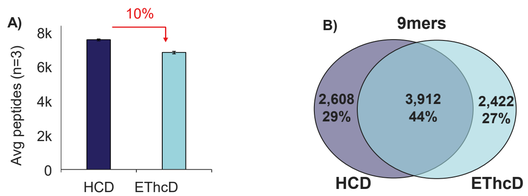


Results

EThcD fragmentation improves peptide sequence coverage compared to HCD fragmentation

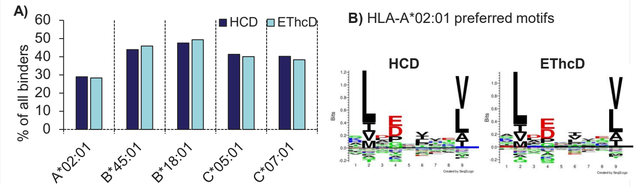
First, we evaluated the performance of the Orbitrap Tribrid Apex MultiOmics MS using EThcD *versus* HCD for the analysis of HLA Class-I peptides. Peptides identified analyzed in DDA mode (n = 3) using EThcD and HCD fragmentation were compared

Figure 4. HCD vs EThcD comparisons in DDA methods. A) Number of peptides identified using each DDA method with different fragmentation approach (HCD x EThcD). B) Distribution of CAA (%) from PEAKS, which corresponds to sequence coverage distribution.



Peptide identification indicate **complementary performance**, as each strategy uniquely contributes to overall identification rates.

Figure 5. Allele mapping in both datasets. No differences were observed in allele-designation between HCD and EThcD (A) nor were differences in the binding motifs of the peptides (B).



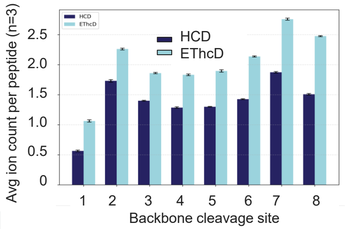
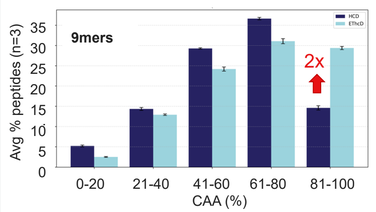


Figure 6. Fragment ions detected from HCD and EThcD runs.

EThcD provides more fragment ions per backbone cleavage site compared to HCD.

Figure 7. Peptide coverage distribution observed in the HCD and EThcD datasets for HLA Class-I 9mers. CAA (%) is the percent of number of confident amino acids in the sequence calculated by PEAKS.



The proportion of peptides with high sequence coverage (>80%) increases by 2-fold when using EThcD compared to HCD fragmentation.

Leverage EThcD on a SCOAD method for improved confidence of targets

The Neo-SCOAD method is a combination of targeted (tMS2) and discovery (DDA) experiments. 26 HLA Class-I peptides were targeted for improved sequence coverage

Figure 9. Peptide discovery rate comparing regular DDA method and Neo-SCOAD (26 targets)method.

Both regular DDA and Neo-SCOAD (discovery part) used HCD fragmentation with Orbitrap detection.

Peptide identification rates were higher with standard DDA, likely because it does not share cycle time with targeted acquisition, unlike Neo-SCOAD.

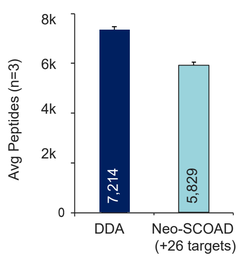
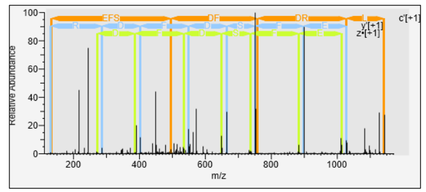
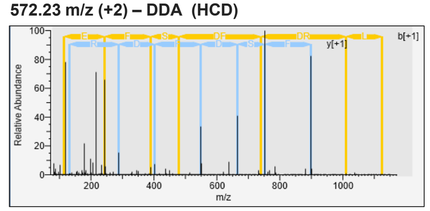


Figure 8. Peptide coverage distribution observed in the HCD and EThcD datasets for HLA Class-I 9mers. CAA (%) is the percent of number of confident amino acids in the sequence calculated by PEAKS.

572.23 m/z (+2) – Neo-SCOAD (EThcD)



572.23 m/z (+2) – DDA (HCD)



Ion series:
b/y
c/z

Conclusions

- The Orbitrap Tribrid Apex MultiOmics MS offers high versatility by combining three mass analyzers (Quadrupole, Ion trap, and Orbitrap), enabling both targeted and discovery experiments within a single injection
- EThcD fragmentation improves sequence coverage, leading to higher-confidence peptide annotation
- The Neo-SCOAD approach integrates targeted and discovery workflows, increasing annotation confidence of specific neoantigens while expanding immunopeptidome breath.

References

1. Thomsen, MCF and Nielsen, M. Nucleic Acids Research 2012; 40 (W1): W281-W287
2. Reynisson et al. Nucleic Acids Res. 2020 Jul 2;48(W1):W449-W454.

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