High sensitivity immunopeptidomics on the timsTOF SCP mass spectrometer

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Introduction

Mass spectrometric characterization of peptide antigens presented by the major histocompatibility complex is essential to understanding infection, cancer and autoimmunity (Figure 1).

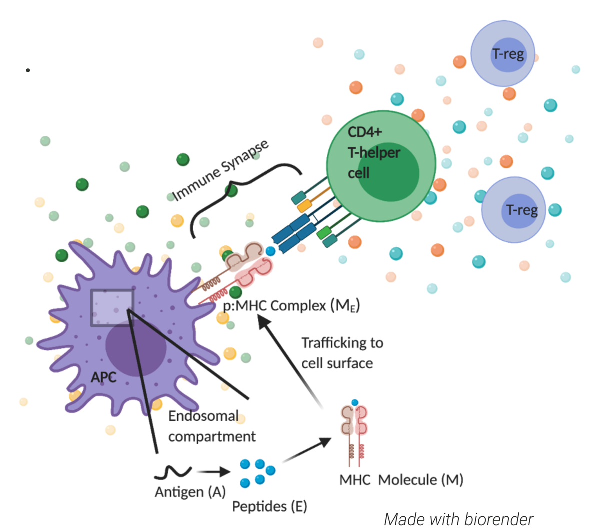


Figure 1

Due to their low abundance, non-tryptic nature and high-complexity, comprehensive LC-MS analysis remains challenging. Many peptides ionize as singly charged species hampering their identification.

In trapped ion mobility spectrometry (TIMS), these peptides are readily discriminated from multiply charged ion signals by their Collisional Cross Section, reducing the interference from co-isolations that usually occurs in pure m/z analyses.

Here, we use the timsTOF SCP system as a highly sensitive instrument with PASEF® for in depth analysis and show unprecedented coverage on low sample input of immunopeptide-like standard samples and real immunopeptidome.

Methods

Immunopeptidomic standards

1. HeLa protein lysate was reduced, alkylated and digested with protease elastase (cleaves at C-terminal of Ala, Val, Ser, Gly, Leu and Ile) at 37 °C for 3 h. Peptides were purified by SEP-PAK reversed phase SPE.
2. 2000 synthetic peptides were produced by solid-phase synthesis on cellulose membranes. Peptides were cleaved from the membrane into HLA specific pools of 250 peptides each, pools were combined at equimolar amounts.
3. Jurkat immunopeptidome was prepared from 2.5x10⁹ cells lysed and Immunoprecipitated with W632-Protein A beads, washed, eluted in acetic acid and purified by 5 Kda MWCO filtration and C18 stage-tip.

Mass Spectrometric Analysis

Peptides were dissolved in loading buffer and chromatographic separation performed using a nanoElute (gradient times of 36 min and 66 min) coupled to a captive spray ionization source on a TIMS-TOF SCP mass spectrometer. Data-dependent analysis was done primarily in ddaPASEF with 1 MS1 survey TIMS-MS and 10 PASEF MS/MS scans per cycle. Instrument settings (Charge state selection, Ion transfer and collision energy were modified to optimize data acquisition for identification of HLA peptide standards.

Raw data processing

Raw data files were analyzed in PEAKS Studio Xpro (Bioinformatic Solutions) using 20,606 reviewed human Uniprot entries. Enzyme specificity was set to nine, mass error windows were set at +/- 10 ppm for MS1 and 0.05 Da for MS2 with fixed modifications of Carbamidomethylation (C).

Results

Elastase digestion provides an HLA class I peptide standard

Class 1 HLA associated peptides are often shorter and found at lower charge states as they do not always possess R/K residues unlike tryptic peptides. To create a readily available standard that's simulates class I peptides we digested Hela lysates with elastase.

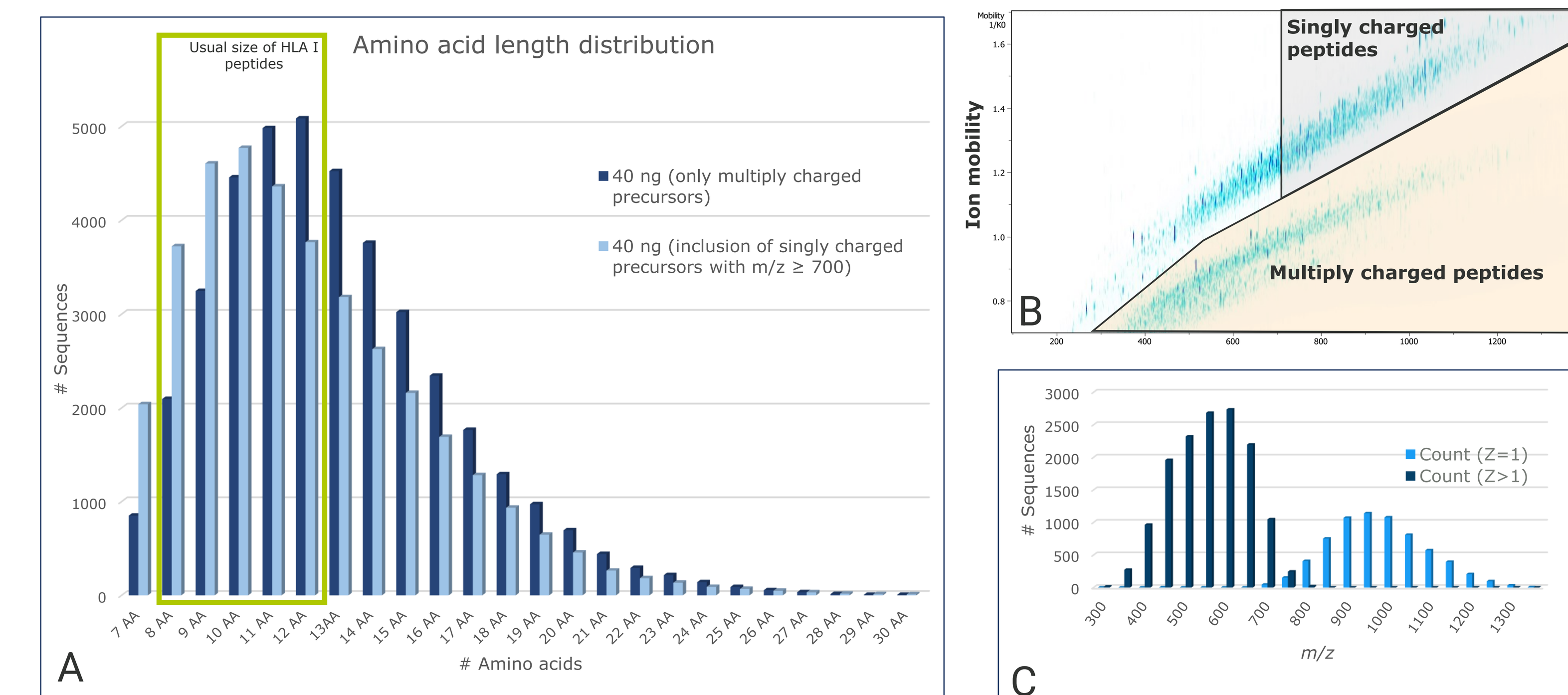
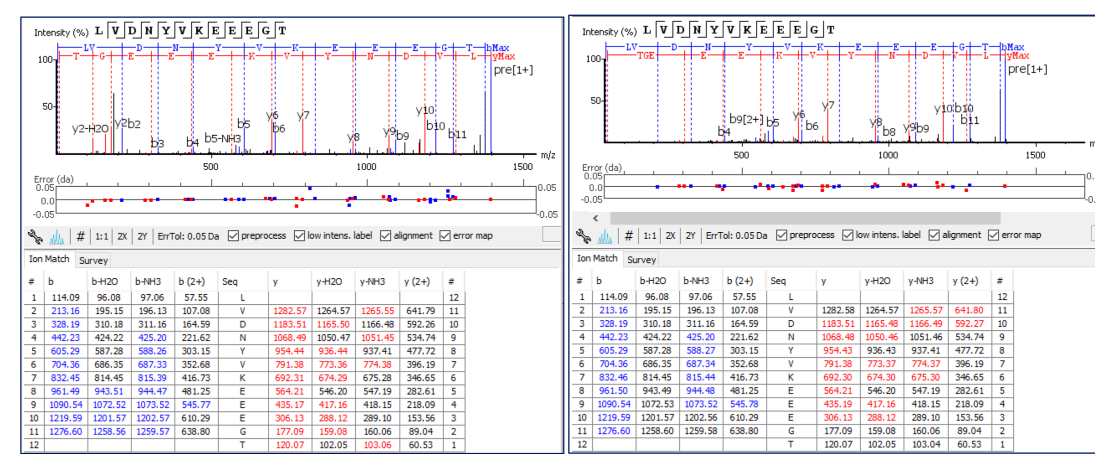


Figure 2

Figure 2A shows the number of peptide sequences identified of a given amino acid length (7 to 30) from a 40-ng injection of the Hela elastase digest. The length distribution is comparable to HLA class I peptides, with most peptides 8-12 amino acids in length. In addition, when singly charged species were selected, additional shorter peptides (7-10 amino acids) could be identified. Figure 2B-C shows the m/z distribution and identified sequences (8-12 amino acids) colored by charge state.

TIMS stepping enriches spectra but reduces ID rate



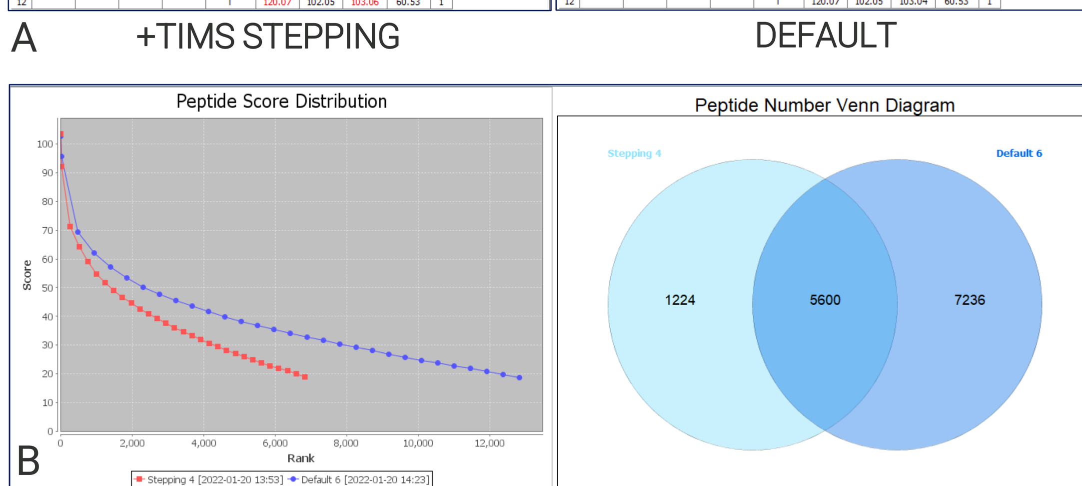


Figure 3

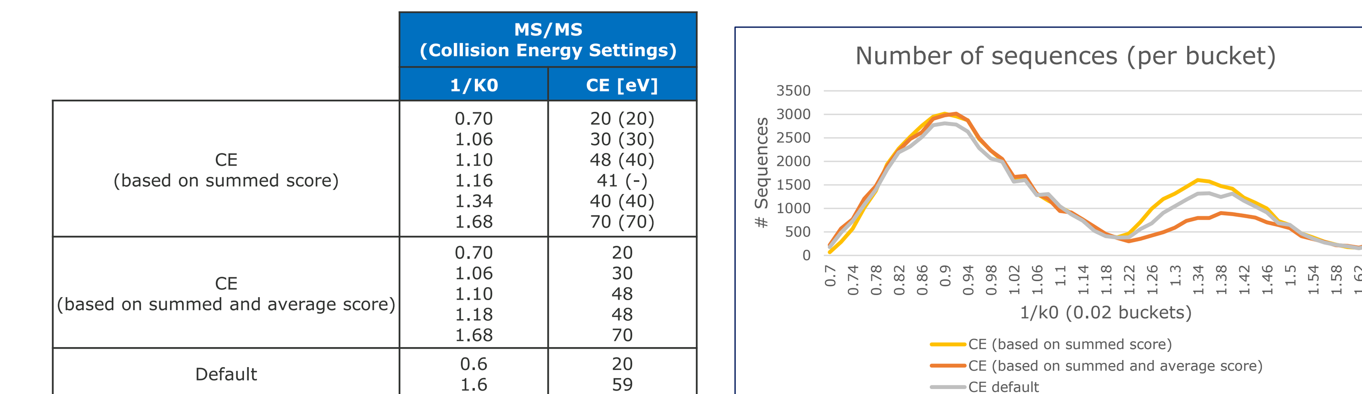
HLA peptides often fragment poorly. To investigate this, we implemented a dual TIMS ramp methodology (TIMS Stepping) able to enrich signal at low m/z.

Figure 3 A-B shows that TIMS stepping was able to enrich the low mass (<200 m/z) spectra, aiding with assignment of terminal amino acids.

To maintain the cycle time in TIMS stepping the number of precursors selected was reduced, this is detrimental to the number of peptide identifications in complex samples and the score profiles obtained in the standard (Figure 3 C-D).

CE Optimization leads to an improvement in peptide identification rates

In PASEF collision energy (CE) is set according to ion mobility (IM) values of precursors ions. By using set CE values (20,30,40,50,60,70,80 eV) and multiple injections with short gradients we determined the effect of CE on peptide identification rates and Peaks score at different IM buckets (data not shown). Two strategies were developed one based on **best summed score** and another **based on summed and average score** (Table 1).



	MS/MS (Collision Energy Settings)	
	1/K0	CE [eV]
CE (based on summed score)	0.70 1.06 1.10 1.16 1.34 1.68	20 (20) 30 (30) 48 (40) 41 (-) 40 (40) 70 (70)
CE (based on summed and average score)	0.70 1.06 1.10 1.18 1.68	20 30 48 48 70
Default	0.6 1.6	20 59

Table 1

Figure 4

Using standard injection of 40 ng over 66-minute gradient **best summed score** strategy gave the highest overall number of peptide identifications, this effect was pronounced at higher IM points where singly charged species are found. Overall CE optimization resulted in a modest 8% improvement over default strategy developed for proteomics (Figure 4).

Sensitivity for immunopeptidomic samples

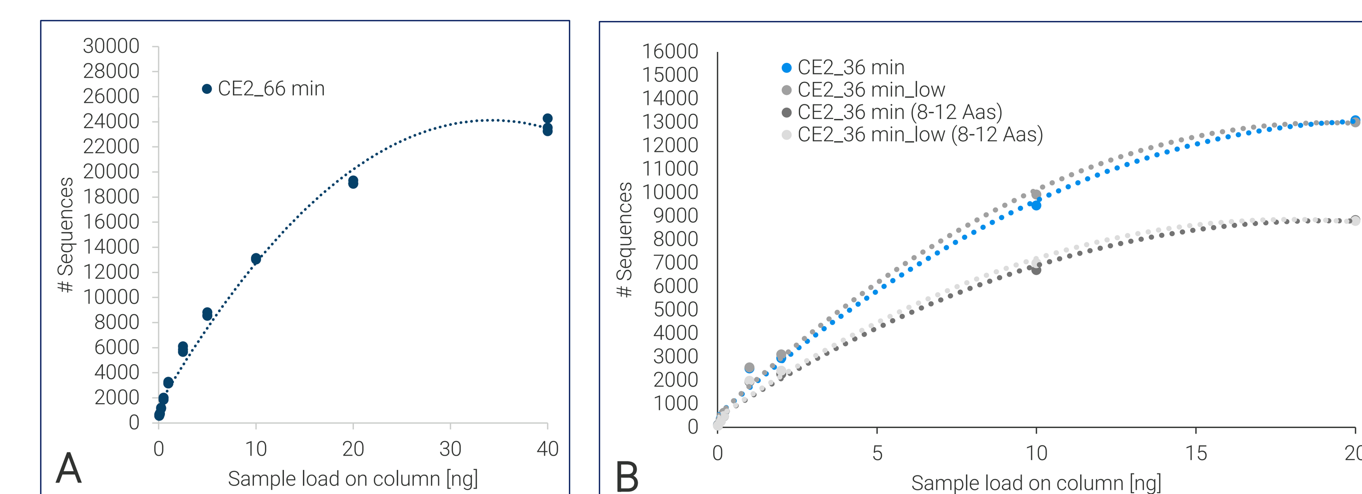


Figure 5

We assessed sensitivity of the SCP with a serial dilution of the elastase Hela digest (40 ng to 0.0625 ng) on 66-min gradient. using optimal CE and default intensity threshold (IT) of 500 and a target intensity (TI) of 20,000. We could readily detect 600 peptides from as little as 62.5 pg of peptides (Figure 5A).

We assessed combinations of IT (100, 250, 500) with TI (20,000 and 40,000). At 250/500 (IT) increasing to TI 40000 reduced the number sequences identified by 6%, and 20% at IT of 100 (data not shown).

Finally, we tested the default (IT=500 and TI=20000) and a low (IT=250 and TI=14000) ratio to see if lower IT could improve sensitivity with a lower TI to maintain the number MS/MS repetitions (Figure 5B). A modest improvement in peptide identifications was observed for 2 ng (2277 → 2415) and 10 ng (6704 → 6998) with no effect seen at higher or lower loads whilst Peaks scores were unaffected by these adjustments.

Validation and assessment of sequencing bias in synthetic HLA peptide standard

Using the optimal CE strategy developed we analyzed a pool of 1750 synthetic HLA peptides (20 fmol/peptide). In total 82% of target sequences could be identified and 87% of all sequence identified were expected (Figure 6A). We tested including 1+ ions (Pol) and found that this gave the highest number of overall peptides including 248 unique sequences (Figure 6B). Allele analysis indicated that this method was more effective in identifying hydrophobic sequences that bind A02:01 HLA compared to other methods (Figure 6 C,D).

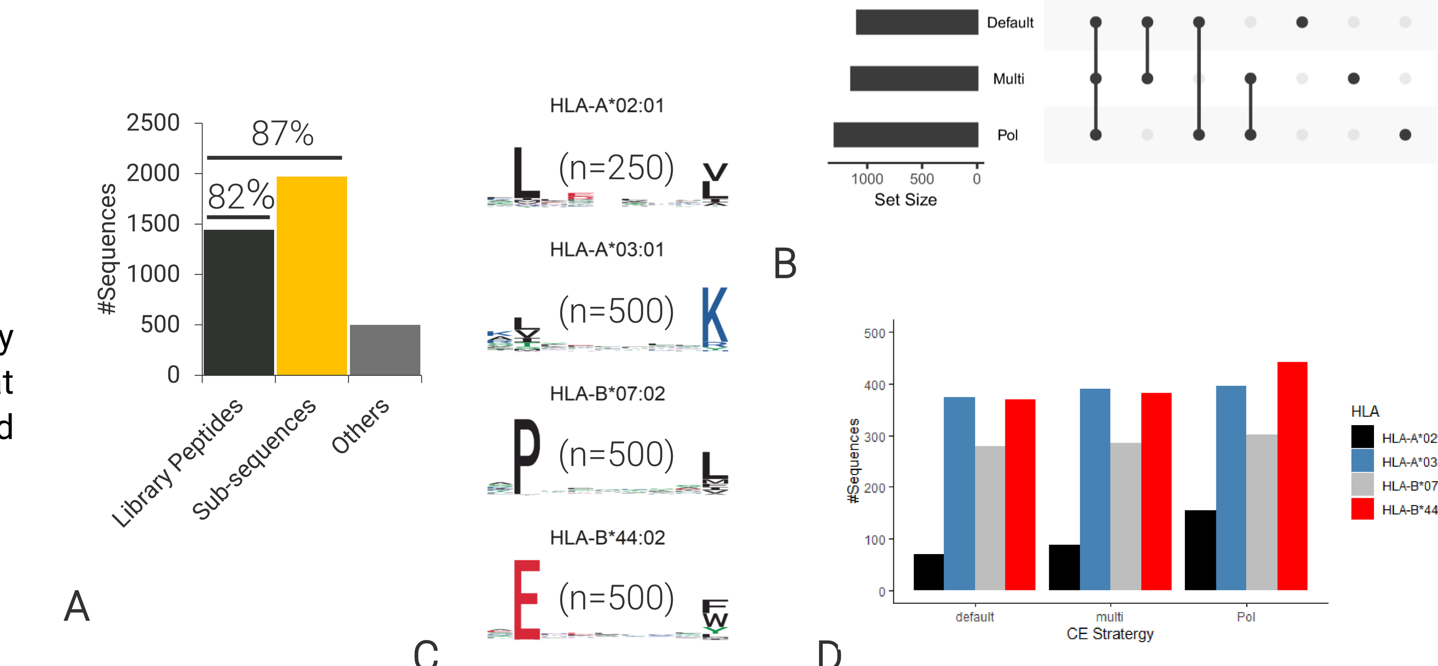


Figure 6

How many cells do you need if you buy a SCP?

Finally, to translate these results we analyzed a serial dilution of a **real immunopeptidomic sample** prepared from the Jurkat cell-line.

HLA-peptides could be detected with a peptide load of 0.00936 ng that was equivalent to 20,000 cells, with an exponential increase that appeared to reach saturation at 9.36 ng equivalent to 20,000,000 cells (Figure 7).

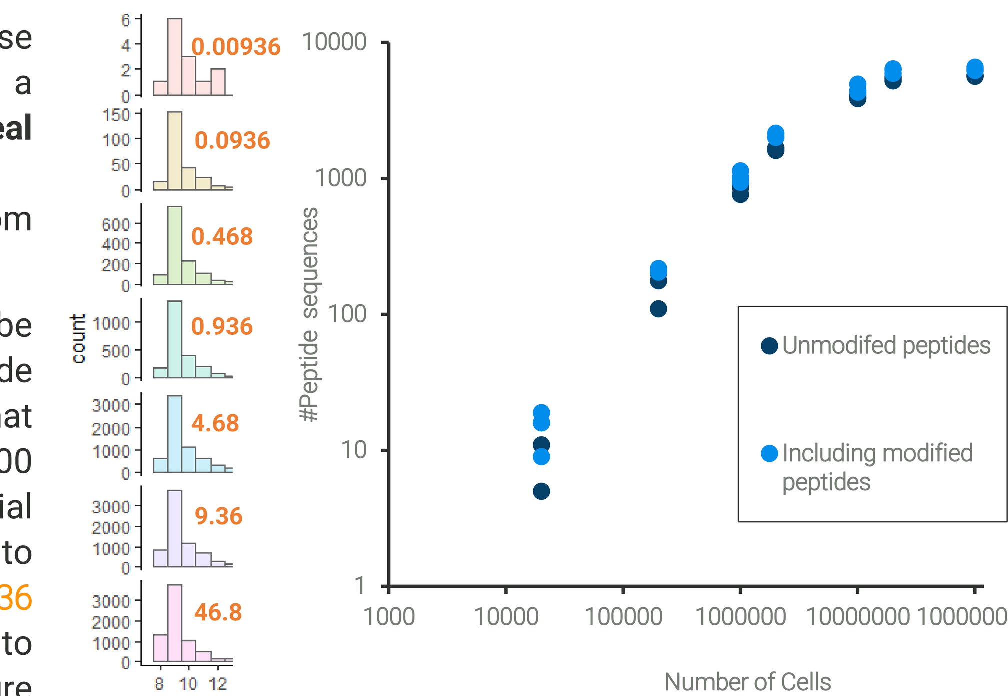


Figure 7

- We optimised the TIMS-TOF SCP for immunopeptidomics
- Isolation of singly charge species enhances peptide identification
- SCP results in deep immunopeptidome with lower starting material

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