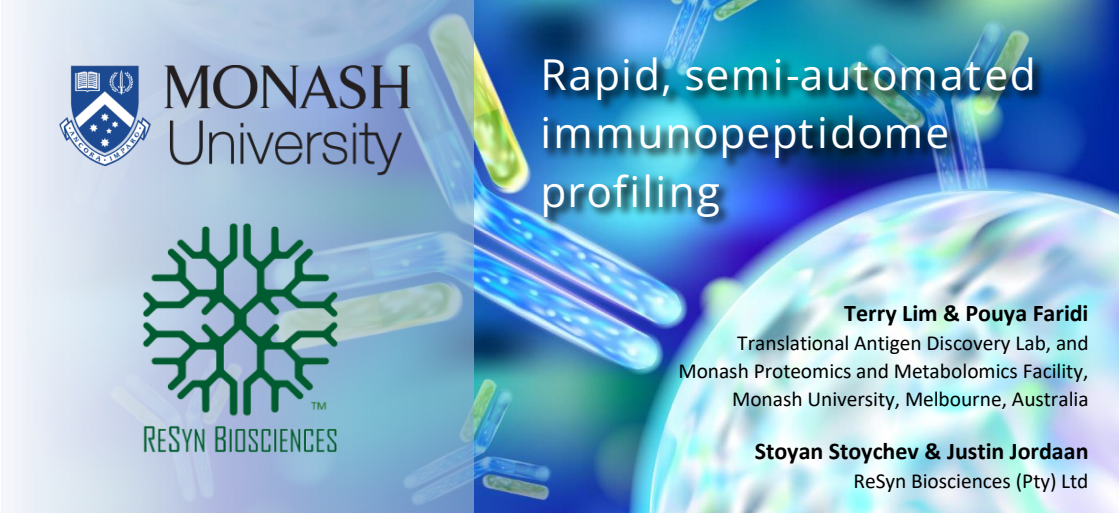

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INTRODUCTION

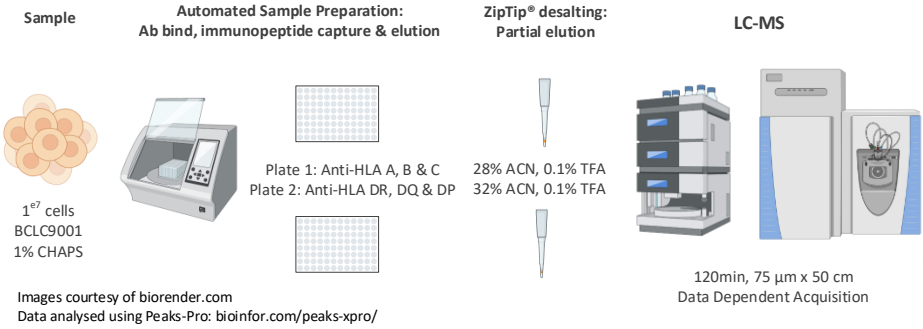
Immunopeptidome profiling refers to the global analysis of the **M**ajor **H**istocompatibility **C**omplex class I (MHC-I) and class II (MHC-II) associated peptides. The MHC system known as the human leukocyte antigen (HLA) in humans is located on the short arm of chromosome 6. HLA bound peptides play a crucial role in the development of adaptive immune response in vertebrates. Comprehensive immunopeptidome profiling can enable novel solutions in personalized antitumor, antiviral, antibacterial and autoimmune disease immunotherapies. There are however several analytical challenges associated with immunopeptidome profiling, as highlighted by the Human Immunopeptidome Project. These include peptide isolation uncertainty, the inability to analyze small amounts of biological material, low throughput, lack of sensitivity and reproducibility of mass spectrometry technologies, suboptimal identification rates, lack of experimental and computational standards, and the current lack of accessibility to large-scale community generated datasets.

MagReSyn® is a proprietary hyper-porous polymer backbone, allowing for a high-capacity to capture protein and peptide targets, while multi-point covalent immobilization of Protein A reduces leaching. In combination with the strong magnetic moment, for rapid microparticle clearance and ease of automation, we could tackle the current sample preparation challenges associated with immunopeptidome profiling including working with low input material, increasing throughput and reproducibility.

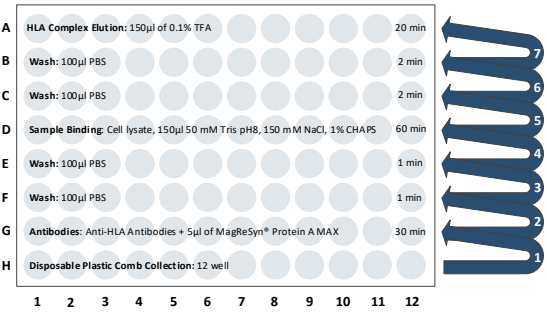
We describe a semi-automated method for MHC molecule immunoprecipitation, covering all the steps from anti-MHC antibody cocktail loading on magnetic microparticles, to the elution of purified MHC Class I and II complexes.

The method describes processing of up-to 12 samples in parallel but can be scaled to 96 samples without requirement for method re-optimization.

METHODS



Automated Sample Processing:



- **Plate 1**: For capture of HLA-Class-I peptides, 200 µg of Anti-HLA A, B & C (Leinco Technologies) in 150 µl PBS were aliquoted into row G, wells G1-G12.
- **Plate 2:** For capture of HLA-Class-II peptides, 100 µg of Anti-HLA DR, 50 µg of Anti-HLA DQ, 50 µg of Anti-HLA DP (Leinco Technologies) in 150 µl PBS were aliquoted into row G, wells G1-G12.
- MagReSyn® Protein A MAX beads (5 µl, 80 µg), pre-equilibrated in PBS, were added to the HLA antibody cocktail in both plates, row G, wells G1-G12.
- Wash buffer, 100 µl PBS, was added to both plates, rows F, E, C and B.
- Approximately 1^{e7} cells (BLCL9001, HLA-Class I: A*24:02, B*07:02, C*07:02, HLA-Class-II: DRB1*01:01, DQB1*05:01, DPB1*04:02) were re-suspended in 150 µl lysis buffer (1% CHAPS, 50mM Tris pH 8, 150mM NaCl) supplemented with protease and phosphatase inhibitor cocktail, loaded into both plates, row D, wells D1-D12.
- TFA (150 µl of 0.1%) was used as HLA complex eluate and loaded in both plates, row A, wells A1-A12.

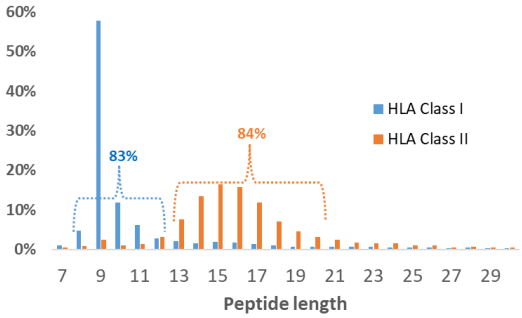
Desalting: C18 ZipTip® with elution of HLA-Class-I peptides using 28% ACN, 0.1% TFA or 32% ACN, 0.1% TFA for HLA-Class-II.

Data acquisition: Peptides solubilized in 2% ACN, 0.1% TFA, were loaded on a 100 µm, 2 cm nanoviper PepMap™ 100 trap column. This was followed by peptide elution at 250 nl/min over a 120 min gradient of 10 to 45% ACN, 0.2% formic acid (FA), on a 75 µm x 50 cm, PepMap™100 C18 analytical column (Thermo Fisher Scientific) connected to an Orbitrap HF (MS1: 60,000 resolution, AGC of 3^{e6}, 54 ms injection time; MS2: HCD at a collision energy of 32%, 30,000 resolution, an AGC of 2^{e5}, 120 ms injection time).

Data Processing: PEAKS X-Pro (Bioinformatics Solutions, Waterloo, Canada) was used with human database UniProtKB/Swiss-Prot. 'Unspecified enzyme digestion' was selected for the enzyme parameter and mass tolerances on MS1 and MS2 ions were 10 ppm and 0.01 Da respectively. Variable modifications were deamidation (NQ) and oxidation (M). A false discovery rate (FDR) of 1 % was applied.

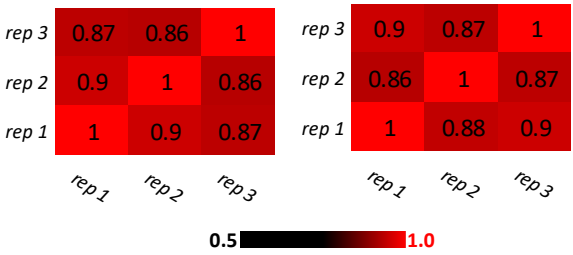
RESULTS

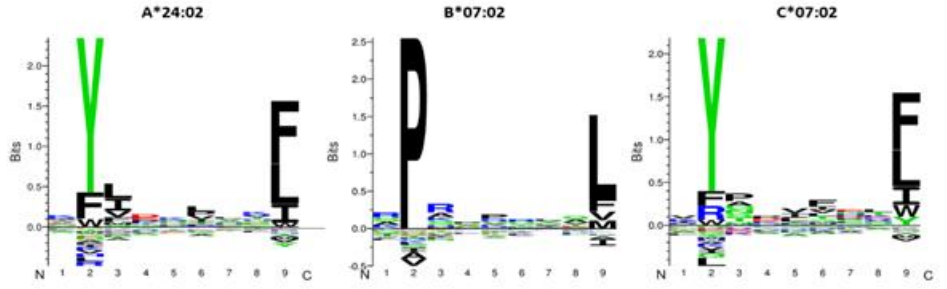
The KingFisher™ Duo method initially allows for anti-HLA antibody binding to the MagReSyn® Protein A MAX in row G, removal of unbound anti-HLA antibodies in rows F and E, and binding of HLA complexes in row D. Non-specifically bound species are removed in rows C and B, with a final double HLA elution in row A at room and elevated temperatures (70°C).



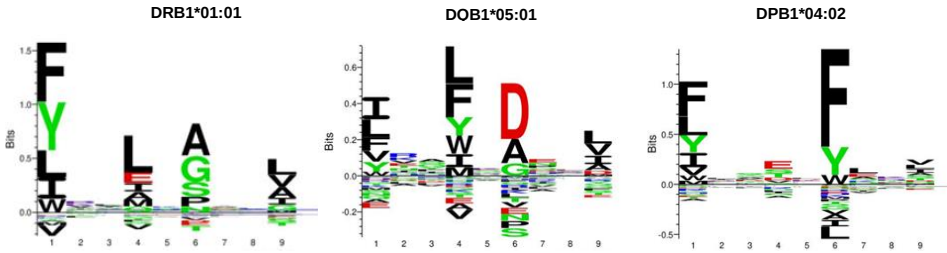
A total of 4,242 HLA I and 6,738 HLA II immunopeptides were identified across the three technical replicates. The HLA peptides were of expected size with over 83% of the Class I peptides containing 8 – 12 amino acids and 84% of HLA Class II peptides were 13-20 amino acids in length (**left**).

A high correlation across replicates was obtained for both Class I (**right**) and Class II (**far right**) peptides, illustrating the robustness of the workflow.





The predominant amino acid positions for peptides belonging to Class I A, B and C alleles (**above**) and Class II DR, DQ and DP alleles, (**below**) were as expected.



CONCLUSIONS

- ✓ We describe a high-throughput method for immunopeptide enrichment.
- ✓ The automated workflow has a total run time of approximately 120 min and allows for (i) loading anti-HLA antibodies on MagReSyn® Protein A MAX microparticles, (ii) removal of non-specifically captured anti-HLA antibodies, (iii) enrichment of HLA Class II and II peptides from a cell lysate and (iv) their elution.
- ✓ High reproducibility and enrichment efficiency were achieved whilst processing 12 samples in parallel on a KingFisher™ Duo magnetic handling station.
- ✓ The throughput can be further scaled up to 96 samples in parallel, processed in approximately 120 min, by utilising a KingFisher™ Flex or Apex magnetic bead handling stations, without the need for additional method re-optimization.

KingFisher protocols available on request.