HIGH THROUGHPUT AUTOMATED WORKFLOW FOR THE ANALYSIS OF MHC-I AND MHC-II SAMPLES BY LC-MS/MS

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BACKGROUND

- The process of **antigen presentation** is used by the body to monitor for malignancy, infection, or other abnormalities. Antigen-derived peptides are bound to MHC molecules for 'presentation' to patrolling T-cells and the specific immune-mediated removal of diseased/abnormal cells.
- The **immunopeptidome** is the repertoire of peptide antigens bound to MHC for presentation to T-cells. Typically, MHC-I bound peptides are ~8 to 11 AA in length, while MHC-II peptides are ~8 to 25 AA. The immunopeptidome is of paramount importance to understanding the immune system and is a logical source of targets for designing next-generation therapeutics and vaccines in **personalized medicine** for cancer, autoimmunity, and pathogenic infections.
- Currently the only method for comprehensive characterization of naturally presented MHC-bound peptides is **mass spectrometry**. However, the use of immunopeptidomics in basic and clinical immunology have been limited due to technical limitations, including low throughput and limited amounts of sample for analysis.

PURPOSE

- To reduce the material and time needed for immunopeptidome analysis, improve sample throughput and to demonstrate data quality and robustness of the optimized workflow

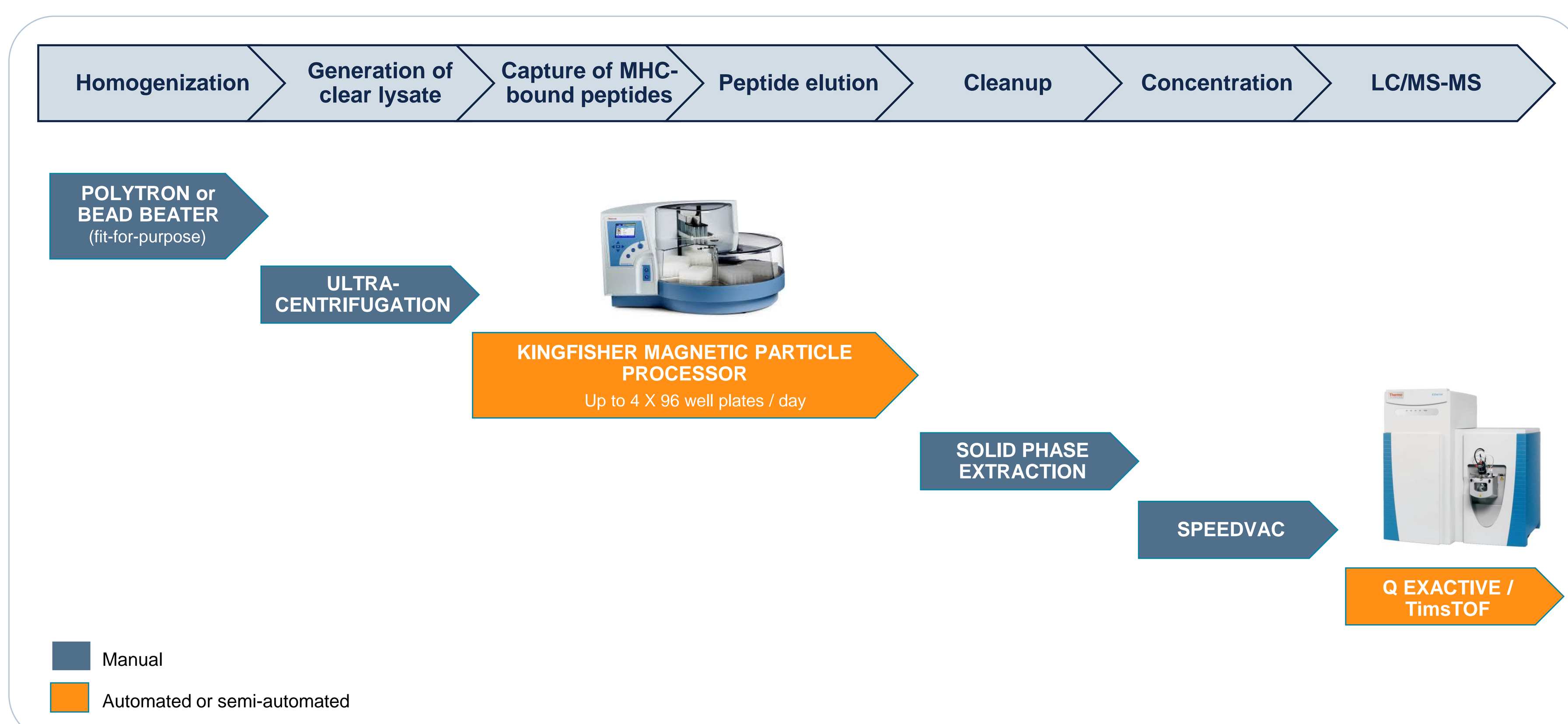
SIGNIFICANCE

- Automation will enable higher reproducibility and minimize potential processing error
- Smaller sample requirements and higher processing throughput will enable studies on larger cohorts with shorter turnaround times and faster results to clients

METHODS

- **Parameters tested for development of the high-throughput workflow:**
  - Tissue homogenization methods and lysis buffers
  - Capacity and non-specific binding of different magnetic beads
  - Stability of capture antibodies, reagents and use of protease inhibitor
  - Ratio of starting sample amount to antibody to beads
  - Minimum/optimal starting amounts for a range of matrix types
  - Preclearing of lysate with isotype control antibody
  - Pulldown incubation time
  - Washing and elution conditions
  - Detergent removal methods and peptide cleanup
  - Peptide reconstitution methods
  - Automation and overall optimization in well-plate format
- **Test samples**: Multiple matrices (cell lines, plasma and snap-frozen tissue) were tested during development. To verify the final method performance, snap-frozen tissues (lung, stomach, kidney, spleen, breast, uterus, intestine) were processed in lysis buffer using either a polytron or a high throughput bead beater, and ultracentrifugation was used to generate cleared lysate for immunoaffinity enrichment.
- **Sample processing**: Different magnetic beads from various suppliers were evaluated (non-specific binding, peptide recovery) and a final stationary phase selected. To capture the naturally presented MHC-bound peptides, a Deepwell format plate was used to incubate the cleared lysates with the appropriate antibody (anti-MHC-I or anti-MHC-II) in the presence of protease inhibitors. Immunocomplexes were purified using a KingFisher magnetic particle processor (Thermo) and eluates were cleaned up using SPE and dried down in a SpeedVac.
- **Mass spectrometry and data analysis**: Peptides were reconstituted and analyzed in DDA mode using a nanoAcquity UPLC (Waters) coupled to a high-resolution accurate mass Q Exactive mass spectrometer (Thermo) or to a TimsTOF (Bruker). Mass spectra was searched against the Uniprot human database reference proteome using the PEAKS (Bioinformatics Solutions) search engine, with deamidation and oxidation as variable modifications.

FINAL HIGH-THROUGHPUT WORKFLOW



HIGH-THROUGHPUT WORKFLOW RESULTS

FIGURE 1 A control test sample (pooled tissue lysates) was analyzed for MHC-I bound peptides using a range of starting amounts. **[A]** The number of identified peptides increases with increased sample amount. **[B]** Peak intensities of the 12 most abundant peptides increase with increased sample amount. **[C]** The identified peptides are in the expected size range of ~8-11 AA, with a consistent size distribution across the range of sample amounts tested.

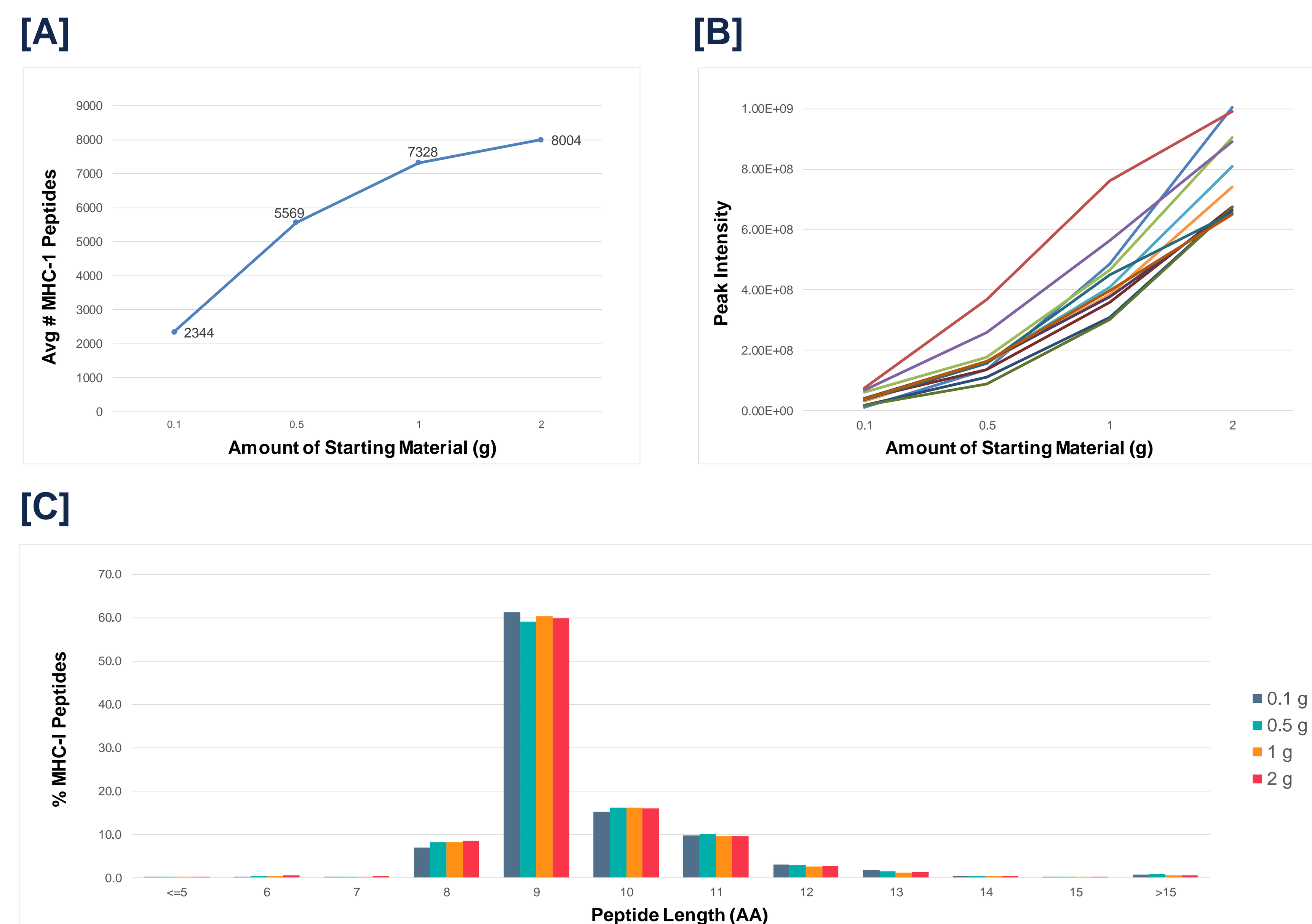


FIGURE 2 Reproducibility of the MHC-I workflow. Replicate aliquots of a control test sample (pooled tissue lysates) were processed on different days and/or plates, by two different operators, using 0.1 g or 0.5 g of starting material.

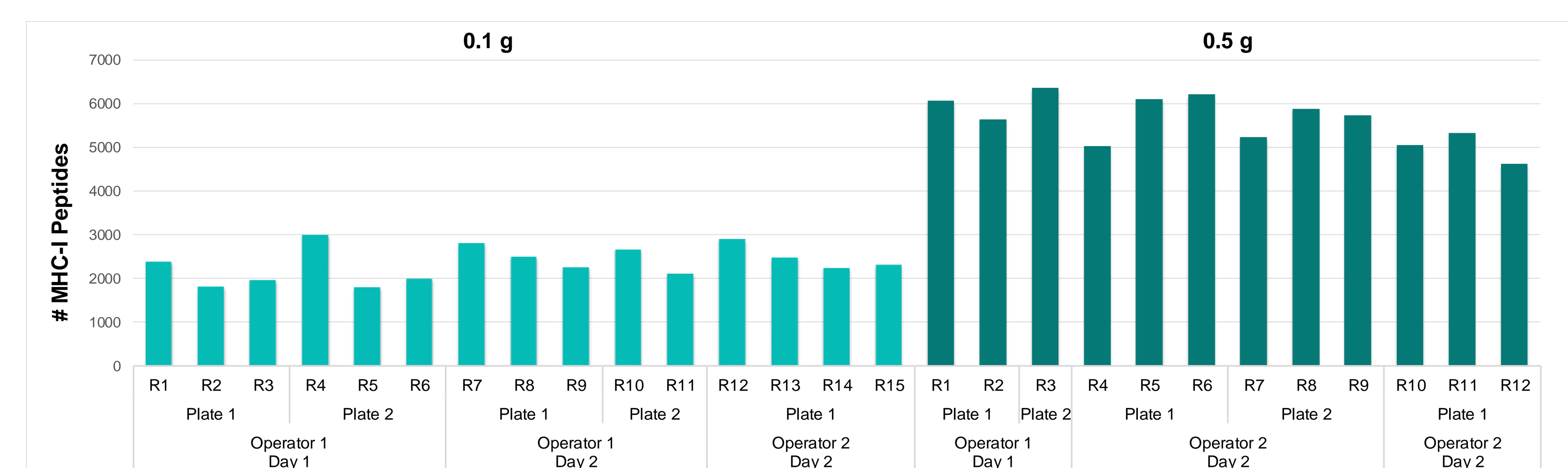
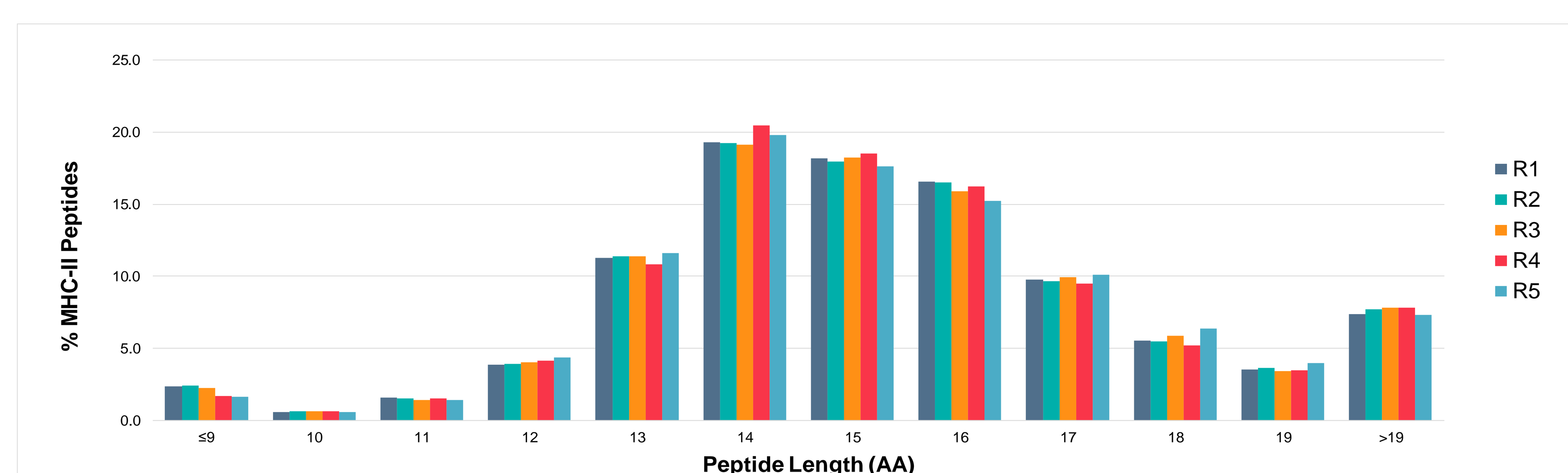


FIGURE 3 Reproducibility of the MHC-II workflow. Replicate aliquots of spleen tissue lysate were analyzed using different capture antibody lots. Approximately 800 to 1000 peptides were identified per 0.1 g starting amount of spleen tissue (data not shown), with the expected size range of ~8 to 25 AA and a consistent size distribution across the replicates.



SUMMARY

- We have developed a high-throughput workflow for the identification of naturally presented MHC-bound peptides from various biological matrices, with a ~120 sample / week per operator turnaround time
- Our data shows the identification of ~2000 to 8000 MHC-I bound peptides using 0.1 g to 2 g of a pooled tissue lysate. Data from MHC-II analysis of spleen tissue is also presented. The identified peptides match the respective expected size ranges for MHC-I and MHC-II, and their consistent size distributions over different starting amounts and replicates are indicative of the specificity of the workflow.
- Technical replicates by different operators on different days show good reproducibility
- The low sample requirements and higher throughput will enable more efficient, reliable analysis of sample-limited, large cohorts with shorter turnaround times, for discovery studies or targeted analysis of the immunopeptidome