Reproducibility and Robustness of Large Scale Nanoflow Immunopeptidomics Study

Using the OptiFlow® Turbo V Ion Source on the TripleTOF® 6600+ LC-MS/MS System

Thomas Vander Schans, Saghar Kaabinejadian, Ken Jackson, Curtis McMurtrey
PureMHC, LLC

The human leukocyte antigen (HLA) is a polymorphic set of cell surface protein complexes that are used by the immune system to recognize foreign entities in the body. Class I HLA complexes bind to small (8-11 amino acids) peptide antigens derived from protein degradation products from within the cell and display them on the surface of the cell for cytotoxic T-cells to bind. The recognition of pathogen-derived or aberrant self-derived peptides leads to the destruction of the diseased cell but not the cytotoxic T-cell.

The study of the peptide complement that binds to these various HLA molecules, or immunopeptidomics, is an increasingly important area of research, however it is difficult to obtain large amounts of these HLA bound peptides. Therefore LC-MS

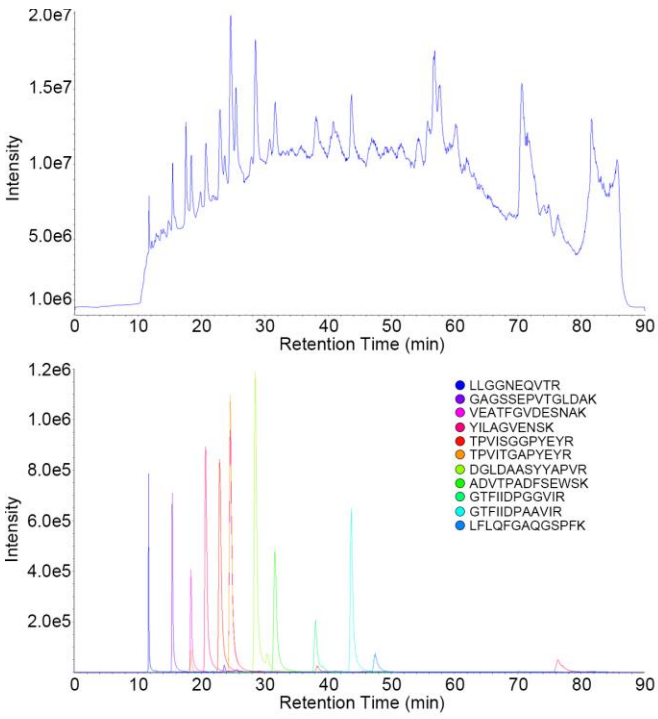

Figure 1. Chromatography Used in Study. (Top) Total ion chromatogram (TIC) of the HLA peptide sample (Injection 270). (Bottom) Extracted ion chromatograms (XICs) from the MS1 scan of the iRT peptides dosed into the same sample.

experiments require very high sensitivity and are typically done using nanoflow chromatography. To study the large number of samples now being analyzed in these studies, a robust and reproducible nanoflow chromatography system and source is increasingly important.

Here, the use of the OptiFlow Turbo V Ion Source and OptiFlow™ Interface were introduced into the nanoflow LC-MS platform being used by PureMHC in order to assess the impact on study reproducibility. SWATH® Acquisition is used to obtain a comprehensive analysis of the peptides in the samples for identification and specific quantification. The sample set consisted of 516 sample injections, performed over the course of 57 days with continuous operation, plus additional blank runs, QC runs, and calibration runs. No cleaning or instrument tuning was required during study.

Key Features of Nanoflow SWATH Acquisition for HLA Peptide Studies

- SWATH Acquisition on the TripleTOF 6600+ System using 24 variable Q1 windows provides a reproducible digital map of all detectable peptides in the sample that can be mined for quantitative peptide data
- The OptiFlow Source with associated probes and electrodes is an easy to use source for nanoflow LC with high long-term robustness and reproducibility

Methods

Sample Preparation: HLA complexes were purified from several primary human tissue samples using affinity chromatography with whole cell lysis. Purified HLA complexes were denatured, and peptide ligands were separated with off-line reverse phase HPLC into either 19 or 20 peptide containing fractions. Each fraction was dried and resuspended in a solution containing a 1:50 dilution of iRT peptides (Biognosys).

Chromatography: Fractions from samples were injected on a ChromXP C18, 3µm, 150mm x 0.075 mm nano column using trap elute mode on the NanoLC™ 425 HPLC System (SCIEX). A 70-minute gradient from 10-40% acetonitrile in 0.1% formic acid was used to separate the peptides. Column was heated to 40°C using the integrated OptiFlow column heater.

Mass Spectrometry: SWATH Acquisition was performed using the TripleTOF 6600+ System using an optimized 24 variable Q1 window method. The mass spectrometer was equipped with the OptiFlow Interface and OptiFlow Source, plumbed with the nanoflow probe and electrode.

Data Processing: SWATH Acquisition data was processed using the Skyline software. The top 4 MS/MS fragment XICs for each iRT peptide were summed and initial peak areas were reintegrated using mProphet. Confident iRT peptide assignments were made at a q-value <0.01. The first and last eluting iRT peptide were not extracted and are not shown due to column binding and elution inefficiencies.

Table 1. Reproducibility of iRT Peptides Across 516 Injections. Raw peak areas for the standard peptides were obtained and used to compute the variance across all injections. In addition, an average of the first 30 runs was compared to the last 40 runs to compute the # drop across the dataset.

Peptide	% CV Raw Peak Area	% Peak Area Drop Across Runs
GAGSSEPVTGLDAK	*21.5*	*24.8*
VEATFGVDESNAK	*28.4*	*40.3*
YILAGVENSK	*23.9*	*30.0*
TPVISGGPYEYR	*16.9*	*16.0*
TPVITGAPYEYR	*14.7*	*14.2*
DGLDAASYYAPVR	*16.7*	*28.1*
ADVTPADFSEWSK	*15.1*	*23.3*
GTFIIDPGGVIR	*23.6*	*26.1*
GTFIIDPAAVIR	*32.7*	*12.9*

Peak Area Reproducibility

The standard peptides were spiked into each separate complex peptide sample before LC-MS analysis. The peak areas of these standard peptides could then be monitored across the sample set to understand the system reproducibility. Figure 2 shows the raw peak area for each peptide in every run, visually showing the variance of each measurement. The peak area reproducibility for each was computed and shown in Table 1. Note this indicates the reproducibility of the full experiment with complex immunopeptide pools from human tissue samples, including

Figure 2. Peak Area Reproducibility of iRT Peptides. iRT peptides were spiked into 516 fractions from tissue-derived complex immunopeptide pool samples and monitored across the batch. Using a single column and single electrode across the full project. Excellent raw peak area reproducibility was observed for the peptides. Some run to run variance of the standard peptides is observed due to the different sample matrices, peptide pipetting and the normal LC-MS sources of variance. However, in general across the 516 injections, the peak area stayed very stable.

matrix effects created by each separate sample, the error introduced during the spiking of the standard peptides, as well as the normal LC-MS variance. Together, the CV% for each iRT peptide ranged from 14.7% - 28.4% with an average CV% of 21.5% across all iRT peptides (Table 1).

As expected with complex biological samples, there was a steady drop in the raw signal intensity for all iRT peptides. All peptides showed a linear decrease in signal intensity (average slope -5.5e3) except GTFIIDPGGVIR and GTFIIDPAAVIR both of which had linear slopes that were not significantly different from zero (F-test, p-value >0.01). The % signal drop for each peptide was also computed, by comparing the average value for the first 40 runs to the average value of the last 40 runs (Table 1). Overall the % signal drop was low, averaging only 24.0%.

Electrode Robustness

A large part of system reproducibility relies on electrode robustness, and remarkably, only a single electrode was used for the full study. The electrode was viewed under the microscope after all 516 injections and, although there was residue buildup, there was no sign of occlusion or degradation (Figure 3, top). Another good test is to monitor the column pressure across the sample set, as any occlusion in the electrode would result in an increase in pressure. Minimal pressure increases were observed (Figure 3, bottom).

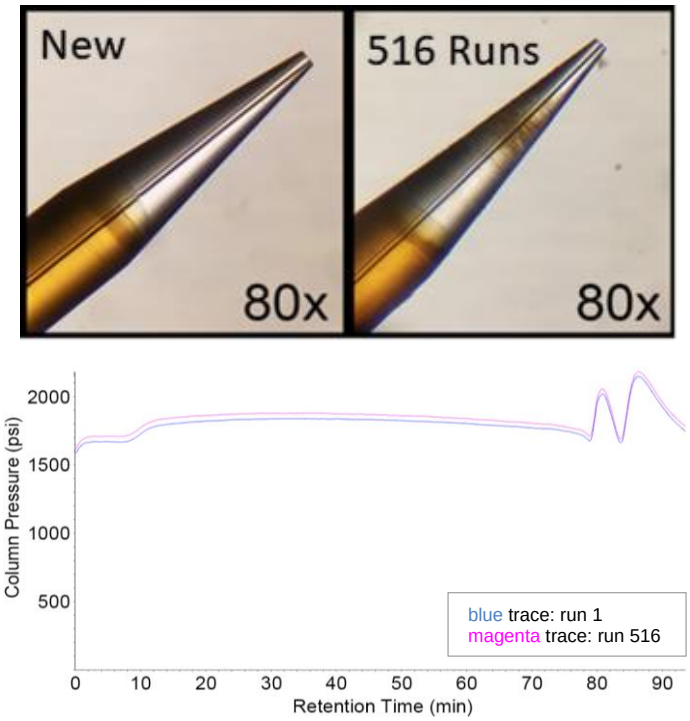

Figure 3. Electrode Robustness. (Top) A single electrode was used for the full study. After the study, there was no sign of any occlusion in the electrode tip under the microscope (top right) compared to a new electrode (top left). (Bottom) Very little change in the LC system pressure traces across the data set was observed (run 1 – blue trace vs run 516 – magenta trace), highlighting the robustness of the electrode.

Conclusions

Using the OptiFlow Source with the NanoLC 425 System, a complete nanoflow study of over 500 injections of complex samples was run with a single column and electrode. Very good raw peak area reproducibility was observed across the dataset when monitoring the signals from the standard peptides spiked into each sample. The signal intensity stayed relatively constant over the runs with only a small decrease in signal across the study.

This data highlights the reproducibility of the electrode design with the OptiFlow Source, providing long-lasting spray tip lifetime and good reproducibility enabling much larger studies to be run using nanoflow chromatography.

Much larger studies can now be run using nanoflow chromatography with the OptiFlow Source and TripleTOF 6600+ System. With the novel and reliable electrode design, this study highlights the reproducibility of the electrode as well as long-lasting spray tip.

SCIEX

Headquarters
500 Old Connecticut Path | Framingham, MA 01701 USA
Phone 508-383-7700
sciex.com

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