Retrospective Alignment and Chromatogram Libraries Enable High-Throughput Targeted Assays

Philip M. Remes[1], Cristina Jacob[1], Jesse Canterbury[1], Ping Yip[1], Will Barshop[1], Lilian Heil[2], Eric Huang[2], Michael J. MacCoss[2]
[1]Thermo Fisher Scientific, San Jose CA; [2]University of Washington, Genome Sciences Department, Seattle WA

ABSTRACT

Purpose: Demonstrate improvements of real-time alignment on targeted quantitation.

Methods: A chromatogram library was used to detect and refine targets. LOQ's for 1- and 2-minute acquisition segments were compared.

Results: LOQ's generally improved by $\sqrt{Extra\ Time} = \sqrt{F}$. Alternatively F more targets could be included without worsening LOQ.

INTRODUCTION

Parallel and Selected Reaction Monitoring (PRM/SRM) represent the gold standard in data quality, but traditionally are limited to 10's or at most a few 100 analytes because of instrument throughput limitations and the difficulty in developing and maintaining such assays, especially as retention times drift. Real-time alignment technology improves both the throughput and maintenance issues, enabling many 1000's of targets per assay[1], but doesn't directly address the method development step. Here we describe a method creation workflow based on a chromatogram library[2] and additionally demonstrate how analytical performance is improved by using the narrower scheduling windows afforded by real-time alignment.

MATERIALS AND METHODS

Sample Preparation

Human and chicken plasma were digested and prepared at 0.2 ug/ul. They were mixed 50:50 by volume and diluted 3x for 5 steps with the Chicken plasma to give 7 levels, given in % human: 50, 16.6, 5.5, 1.9, 0.6, 0.2, 0.0. Samples were injected 3 times at each level for each experimental condition.

Liquid Chromatography

A Thermo Scientific™ UltiMate™ 3000 RSLCnano LC system was used with a 150 x 0.15 mm column (ES906A) at 1 ul/min with a 30 minute gradient.

Chromatogram Library

4 Gas-Phase fractionation experiments were performed to detect peptides and refine a set of high-quality targets. These experiments included 15 very fast alignments acquisitions with 40 Th isolation widths, and 150 slower acquisitions with 1 Th isolation width. Quadrupole mass filter isolation and ion trap analysis was used for all acquisitions.

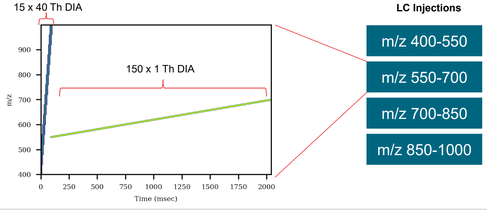
Data Analysis

The 1 Th acquisitions were searched with Thermo Scientific™ Proteome Discoverer™ 3.0. The results were imported to Skyline, where custom reports were generated and analyzed by Python scripts to perform precursor filtering and generate instrument methods.

Dilution Curve Experiments

LOQ's were determined using Pino's method[3] for 3 Conditions: 790 precursors with 1 min and 2 min PRM acquisition segments, and 1878 precursors with 1 min acquisition segments.

Figure 1. The chromatogram library is built from 4x LC experiments. Each experiment cycle had two parts: a set of 15 alignment scans used later for real-time alignment, and 150 analytical scans used to identify and refine peptide targets.



DATA ANALYSIS AND PRECURSOR REFINEMENT

Chromatogram Library Search Results

4435 unique human and chicken peptides from 581 protein groups were detected in the library runs. Figure 2 gives the breakdown of these peptides by organism. Figure 2b gives the distribution of peptide m/z.

Figure 2a. Distribution of identified peptides. 2218 human, 1993 chicken, and 224 homologues.

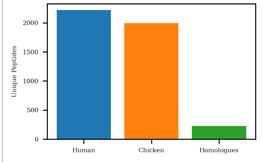
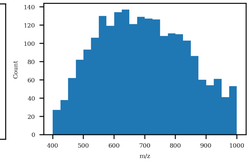


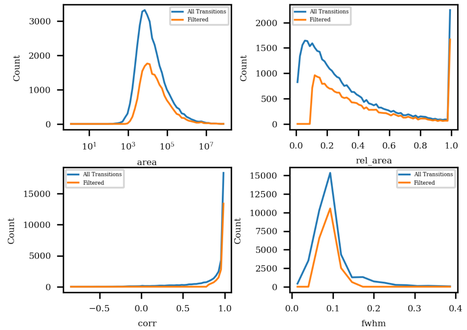
Figure 2b. Distribution of identified peptides by m/z.



Precursor Refinement

The search results were imported to Skyline using data only from a 0.25 min window around the search id times for all precursors with >= 3 transitions and for up to 20 transitions. The chromatograms were exported with a custom report to a text file for offline analysis. A set of filters were applied to the transitions, requiring absolute area > 1e3, relative area > 0.1, and full width half maximum width between 0.06 and 0.15 minutes. Searle filtering[2] was applied using a correlation coefficient of 0.8 of the transitions to their normalized median. Finally, precursors were required to have at least 3 such quality transitions. A total of 1878 precursors met these requirements, called the Big set. Figure 3 shows distributions of the transition metrics. 790 of these precursors (the Small set) were selected with peak width < 11 seconds and that had the best CV in 2 more targeted injections.

Figure 3. Filters applied to the transitions of the identified peptides: Absolute area, relative area, Searle correlation, time width.



METHOD CREATION

Target Scheduling

Methods were created for the 3 conditions: 790 precursors (Small) with 1- or 2-min acquisition segments, and 1878 precursors (Big) with 1 min acquisition segments. Small 1 min has ~2x fewer active precursors at any time than the other conditions, and therefore is able to inject for ~2x longer.

Figure 4a. Estimated instrument time needed for acquisitions compared to 8 points per peak threshold.

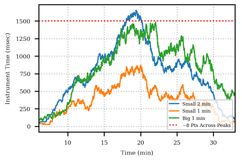
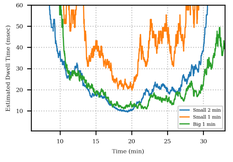


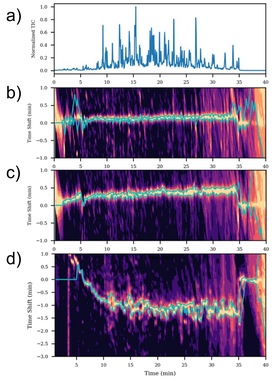
Figure 4b. Estimated injection time available for acquisitions.



Retrospective Real-Time Alignment

We call the alignment process "retrospective" because during the targeted experiment, alignment scans are acquired and compared to data collected earlier to adjust the triggering windows in real time. The last library fraction was used for alignment. The precursor times from each of the other library fractions were adjusted relative to this fraction. Despite that the library samples were 50/50 human/chicken, the alignment process worked even at 0/100 human/chicken (Figure 4b). The alignment worked for the life of the first column, 1 month, and continued to work with the new replacement column (Figure 4d).

Figure 4. Cross correlation alignment plots to 50/50 human/chicken control data from chromatogram library. Light colors are areas of high correlation. Cyan lines are left-right boundaries of apex, representing width of real-time triggering extra buffer. a) Representative LC chromatogram. b) Alignment of 100% chicken sample. c) Alignment of 50/50 human/chicken sample. d) Alignment of 50/50 human/chicken sample with new column after 1 month.

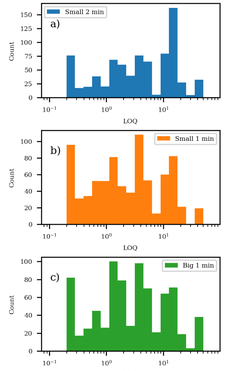


RAW RESULTS

Limits of Quantitation

The data were analyzed to determine the limits of quantitation (LOQ) using the last concentration of human to chicken plasma at which the coefficients of variation were below 20%. The summary of the results is shown in Figure 5. Overall, the results for Small 1 min and Big 1 min are better than for Small 2 min. The Big 1 min data were unfortunately acquired with a different column where the intensities were slightly higher than previously. The experiment needs to be repeated using random injection orders on the same column.

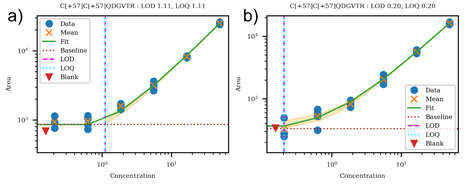
Figure 5. Histograms of LOQ, given in percent human to chicken plasma dilution. a) 790 precursors with 2 min acquisition segments. b) 790 precursors with 1 min acquisition segments. c) 1878 precursors with 1 min acquisition segments



Typical Quantitative Measurements

Using Pino's method we can estimate the precision of the measurements between the actual sample levels, using a statistical technique called "boot-strapping"[3]. In Figure 6 the estimated uncertainty bounds are represented with tan shading. The typical result is that with more injection time the LOQ's improve, as expected.

Figure 6. Representative quantitation curves, here for the peptide CCQDGVTR for the 790 precursor assays with acquisition segments of a) 2 min and b) 1 min.

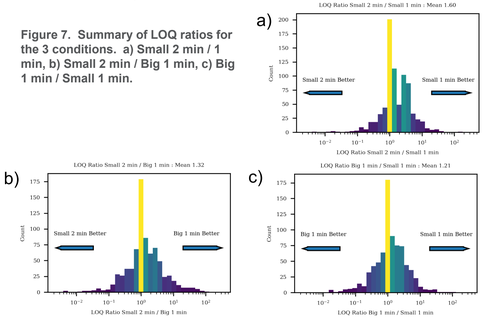


RESULT SUMMARY

Limit of Quantitation Ratios

We are principally interested in observing the effect of additional injection time on quantitative figures of merit. The standard deviation of a random Poisson process decreases with the square root of increasing the mean number of events. We observe an average LOQ improvement of 1.6x for the 2 versus 1-minute conditions (Figure 7a), in line with expectations. We expected the Big assay results to be worse than the Small 1 min results by a similar factor (1.6 instead of actual 1.2), and to be equivalent to the Small 2 min results (1.0 instead of actual 1.3). We attribute the good performance of the Big assay to the different LC column used, that had somewhat higher intensities, even for the QC samples. We will repeat all conditions on the same column with random experiment order in the future.

Figure 7. Summary of LOQ ratios for the 3 conditions. a) Small 2 min / 1 min, b) Small 2 min / Big 1 min, c) Big 1 min / Small 1 min.



CONCLUSIONS

Chromatogram libraries enable easy PRM assay development. Retrospective real-time alignment allows to perform high throughput targeted assays with narrower acquisition segments, typically by a factor $F = 2 - 4$, resulting in higher quality data:

- For the same number of targets, LOQ's improve by $\sim\sqrt{F}$, or
- The number of targets can be increased by $\sim F$ while achieving the same LOQ's.

REFERENCES

1. Remes et. al. Analytical Chemistry v92, i17, 11809, 2020.
2. Searle et. al. Nat. Comm. V9, i1, 5128, 2018.
3. Pino et. al. J. Prot. Research, v19, i3, 1147, 2020.

TRADEMARKS/LICENSING

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